Electronic Supplementary Material (ESI) for ChemComm.

Supporting information for

Microscope laser assisted photooxidative activation of bioorthogonal ClickOx probes

Attila Kormos, Dóra Kern, Alexandra Egyed, Bianka Söveges, Krisztina Németh, Péter Kele*

Chemical Biology Research Group, Institute of Organic Chemistry, Research Centre for Natural Sciences, Magyar tudósok krt. 2, H-1117, Budapest, Hungary

CONTACT

Péter Kele: kele.peter@ttk.hu

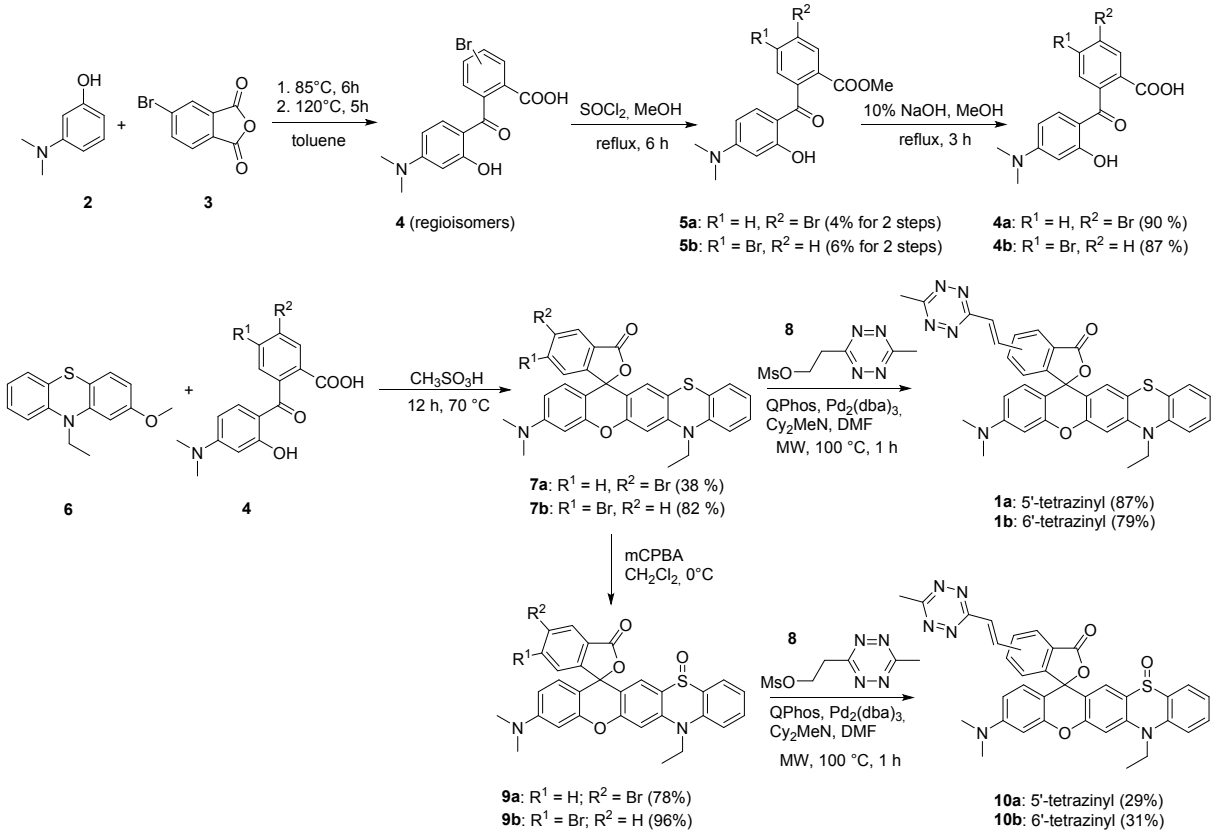
Table of Contents

General

All starting materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck, Alfa Aesar, Reanal, Molar Chemicals, Fluorochem) and used without further purification.
Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} precoated aluminum TLC plates from Merck. Flash column chromatography was performed on Teledyne Isco CombiFlash® Rf^{+} automated flash chromatographer with silica gel (25-40 μm) from Zeochem. Microwave experiments were performed on an AntonPaar Monowave 400 microwave reactor using 10 mL sealed glass tubes and for each experiment, fast heating to 100°C and maintaining constant temperature for one hour.
NMR spectra were recorded on a Varian Inova 500 MHz spectrometer. Chemical shifts (δ) are given in parts per million (ppm) using solvent signals as the reference. Coupling constants (*J*) are reported in Hertz (Hz).
Analytical RP-HPLC-UV/Vis-MS experiments were performed on a SHIMADZU LCMS-2020 system by using a Gemini C18 column (100×2.00 mm I.D.) with 5 μm silica (110 Å pore size) as a stationary phase with a photodiode array UV/Vis (l=220-800 nm) and an ESI-MS detector. Linear gradient elution (0 min 0% B; 2.0 min 100% B; 3.5 min 100% B; 4.5 min 0% B; 5.0 min 0% B) with eluents A (2% NH_4HCO_3, 5% MeCN, and 93% H_2O) and B (2% NH_4HCO_3, 80% MeCN, and 18% H_2O) was used at a flow rate of 1.0 mL min^{-1} at 30°C. The samples were dissolved in MeCN - H_2O mixture.
Semipreparative HPLC was performed on a Hanbon Semiprep NP7010C system using a Gemini C18 column (150 × 21 mm I.D.) with 5 μm silica (110 pore size) as a stationary phase.

Spectroscopic measurements were performed on a Jasco FP-8300 spectrofluorometer and a JASCO V-750 spectrophotometer in all-sodium PBS (pH=7.4, containing 0.1% SDS) at r. t. Quartz cuvettes with path length of 1 cm were used.
The exact masses were determined with an Agilent 6230 time-of-flight mass spectrometer.

Syntheses and characterization

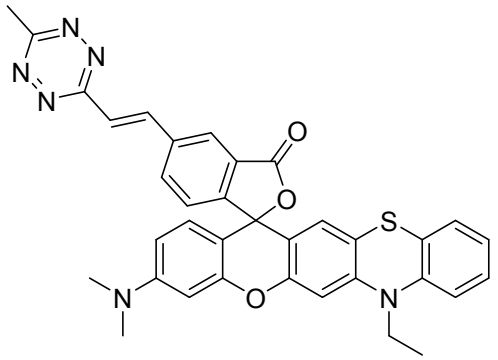


Scheme S1. Synthesis of Rhodaphenothiazines **1a** and **1b**

General procedure for 1a, 1b, 10a and 10b:

A mixture of bromo-rhodamine derivative (**7a, 7b, 9a** or **9b**) (0.1 mmol), tetrazine (**8**) (21.7 mg, 0.11 mmol, 1.0 eq), *N,N*-dicyclohexylmethylamine (0.4 mmol, 86 µl, 4 eq), tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$, 0.01 mmol, 9.2 mg, 0.01 eq) and 1,2,3,4,5-pentaphenyl-1′-(di-*tert*-butylphosphino)ferrocene (QPhos, 0.01 mmol, 7.1 mg, 0.01 eq) in 3 mL absolute dimethylformamide in a 10 mL sealed microwave pressure tube with magnetic stir bar was heated in a microwave reactor at 100 °C for 60 min. The solvent was evaporated in vacuo and the crude products were purified by silica flash chromatography (CH_2Cl_2: MeOH starting from 100 : 1 to 9 : 1), then with preparative-HPLC (H_2O : MeCN starting from 95 : 5 to 0 : 100 containing 1% HCOOH).

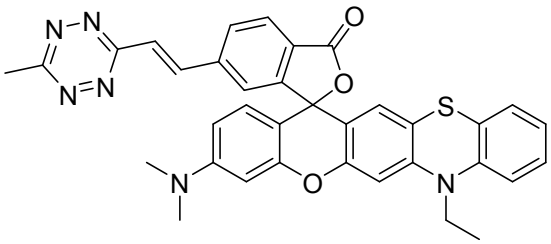
(*E*)-3-(dimethylamino)-7-ethyl-5'-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-3'*H*,7*H*-spiro[chromeno[2,3-*b*]phenothiazine-14,1'-isobenzofuran]-3'-one (1a)



Pale blue powder (53 mg, 0,087 mmol; 87 %)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.42 (d, *J* = 16.3 Hz, 1H), 8.29 (s, 1H), 7.93 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.60 (d, *J* = 16.3 Hz, 1H), 7.23 (d, *J* = 8.0 Hz, 1H), 7.14 (t, *J* = 7.5 Hz, 1H), 7.03 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.93 – 6.83 (m, 2H), 6.74 (s, 1H), 6.63 (d, *J* = 8.8 Hz, 1H), 6.51 – 6.45 (m, 2H), 6.42 (dd, *J* = 8.8, 2.1 Hz, 1H), 3.97 (q, *J* = 6.8 Hz, 2H), 3.09 (s, 3H), 2.99 (s, 6H), 1.48 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.0, 166.9, 164.6, 154.2, 152.7, 152.4, 151.9, 147.3, 143.8, 139.0, 137.2, 134.4, 128.8, 128.4, 127.53, 127.51, 125.79, 124.9, 124.3, 123.8, 123.2, 123.0, 119.4, 115.5, 112.7, 109.2, 105.8, 103.3, 98.6, 84.3, 42.4, 40.4, 21.4, 12.9. HRMS: m/z calcd. for $[C_{35}H_{29}N_6O_3S]^+$: 613.2016, found: 613.2012 $[M+H]^+$.

f(*E*)-3-(dimethylamino)-7-ethyl-6'-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-3'*H*,7*H*-spiro[chromeno[2,3-*b*]phenothiazine-14,1'-isobenzofuran]-3'-one (1b)

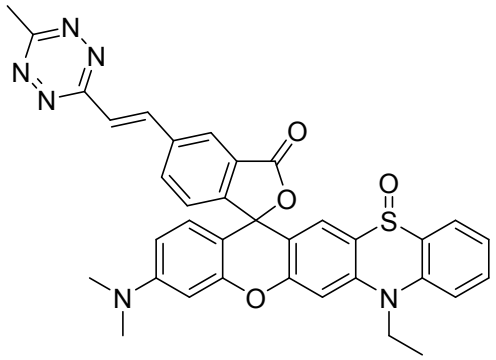


Pink-grey powder (47 mg, 0,079 mmol; 79 %)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, *J* = 16.3 Hz, 1H), 8.07 (d, *J* = 8.0 Hz, 1H), 7.87 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.47 (d, *J* = 16.3 Hz, 1H), 7.42 (s, 1H), 7.16 – 7.10 (m, 1H), 7.02 (dd, *J* = 7.9, 1.4 Hz, 1H), 6.91 – 6.85 (m, 2H), 6.75 (s, 1H), 6.65 (d, *J* = 8.8 Hz, 1H), 6.51 (s, 1H), 6.50 (d, *J* = 2.5 Hz, 1H), 6.43 (dd, *J* = 9.0, 2.5 Hz, 1H), 3.97 (q, *J* = 6.9 Hz, 2H), 3.04 (s, 3H), 2.99 (s, 6H), 1.48 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.9, 166.8, 164.4, 154.2, 152.7, 152.4, 151.9, 147.3, 143.8, 141.8, 139.0, 129.6, 128.9, 128.1, 127.53, 127.49, 125.82, 125.79, 124.5, 123.8, 123.1, 123.0, 119.4, 115.5, 112.8, 109.3, 105.9, 103.3, 98.7, 84.6, 42.4, 40.4, 21.4, 12.9.

HRMS: m/z calcd. for $[C_{35}H_{29}N_6O_3S]^+$: 613.2016, found: 613.2013 $[M+H]^+$.

(*E*)-3-(dimethylamino)-7-ethyl-5'-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-3'*H*,7*H*-spiro[chromeno[2,3-*b*]phenothiazine-14,1'-isobenzofuran]-3'-one 12-oxide (10a)



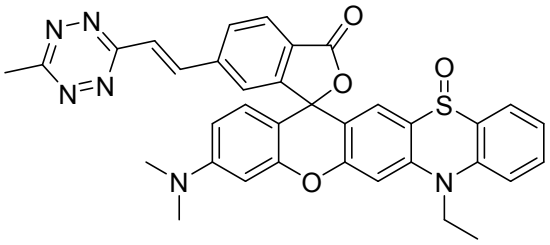
Pink powder (18 mg, 0,029 mmol; 29 %)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.43 (d, *J* = 16.3 Hz, 1H), 8.35 (s, 1H), 7.92 (dd, *J* = 7.9, 1.3 Hz, 1H), 7.84 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.65 – 7.58 (m, 2H), 7.48 – 7.43 (m, 2H), 7.29 (s, 1H), 7.23 (t, *J* = 7.4 Hz, 1H), 7.19 (d, *J* = 8.1 Hz, 1H), 6.68 (d, *J* = 8.8 Hz, 1H), 6.56 (d, *J* = 2.0 Hz, 1H), 6.46 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.38 (qd, *J* = 7.0, 3.0 Hz, 2H), 3.09 (s, 3H), 3.02 (s, 6H), 1.65 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 168.5, 166.9, 164.5, 154.9, 152.6, 152.5, 140.1, 138.8, 137.8, 137.5, 136.2, 134.3, 133.2, 132.9, 131.9, 128.8, 128.4, 124.8, 124.7, 124.6, 123.4, 122.4, 120.8, 115.8, 114.1, 109.6, 103.2, 98.8, 81.6, 43.3, 40.4, 21.4, 12.1.

HRMS: m/z calcd. for $[C_{35}H_{29}N_6O_4S]^+$: 629.1965, found: 629.1939 $[M+H]^+$.

(*E*)-3-(dimethylamino)-7-ethyl-6'-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-3'*H*,7*H*-spiro[chromeno[2,3-*b*]phenothiazine-14,1'-isobenzofuran]-3'-one 12-oxide (10b)



Pink powder (20 mg, 0,031 mmol; 31 %)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (d, *J* = 15.9 Hz, 1H), 8.13 (d, *J* = 8.2 Hz, 1H), 7.90 (d, *J* = 8.4 Hz, 1H), 7.83 (d, *J* = 7.3 Hz, 1H), 7.61 (t, *J* = 8.1 Hz, 1H), 7.48 – 7.36 (m, 4H), 7.30 (s, 1H), 7.21 (t, *J* = 7.4 Hz, 1H), 6.70 (d, *J* = 9.2 Hz, 1H), 6.56 (d, *J* = 2.4 Hz, 1H), 6.46 (dd, *J* = 9.2, 1.5 Hz, 1H), 4.38 (q, *J* = 7.8 Hz, 2H), 3.02 (s, 3H), 3.02 (s, 6H), 1.65 (t, *J* = 6.9 Hz, 3H).

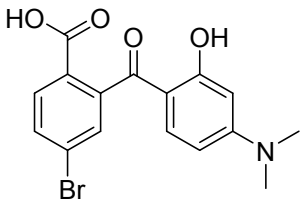
^{13}C NMR (126 MHz, $CDCl_3$) δ 168.5, 166.9, 164.3, 154.9, 154.1, 152.5, 152.5, 141.9, 140., 138.8, 137.8, 133.2, 132.9, 131.9, 129.9, 128.8, 128.0, 126.1, 124.7, 124.5, 122.8, 122.3, 120.8, 115.8, 114.2, 109.6, 105.9, 103.2, 98.8, 82.7, 43.3, 40.4, 21.4, 12.0.

HRMS: m/z calcd. for $[C_{35}H_{29}N_6O_4S]^+$: 629.1965, found: 629.1973 $[M+H]^+$.

General procedure for 4a and 4b:[1]

The esters (**5a** or **5b**, 1.13 g, 3.00 mmol) individually were dissolved in methanol and 11 eq 10 % NaOH was added. The mixtures were refluxed for 3 h. Then the solvent was evaporated and the residues were dissolved in 20 mL distilled water. The pH of the solutions was set to ~1.5 with 5 % H_2SO_4. The resulting precipitate was filtered, washed with water, and dried under vacuum.

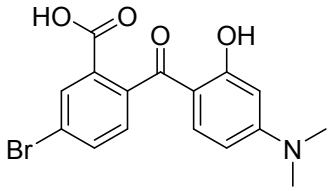
4-Bromo-2-(4-(dimethylamino)-2-hydroxybenzoyl)benzoic acid (4a)



Yellow crystals (986 mg, 2.71 mmol, 90 %).

^{1}H NMR (500 MHz, DMSO) δ 12.31 (s, 1H), 7.89 (d, *J* = 8.4 Hz, 1H), 7.82 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.61 (d, *J* = 1.8 Hz, 1H), 6.83 (d, *J* = 9.1 Hz, 1H), 6.23 (dd, *J* = 9.1, 2.4 Hz, 1H), 6.10 (d, *J* = 2.4 Hz, 1H), 3.01 (s, 6H). MS: m/z calcd. for $[C_{16}H_{15}BrNO_4]^+$: 364, found: 364 $[M+H]^+$.

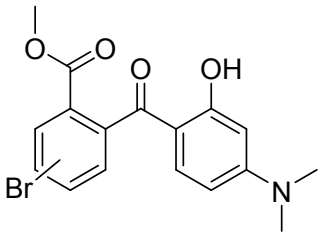
5-Bromo-2-(4-(dimethylamino)-2-hydroxybenzoyl)benzoic acid (4b)



Yellow crystals (946 mg, 2.60 mmol, 87 %).

^{1}H NMR (500 MHz, DMSO-d6) δ 12.37 (s, 1H), 8.06 (d, *J* = 1.9 Hz, 1H), 7.89 (dd, *J* = 8.1, 2.0 Hz, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 6.84 (d, *J* = 9.1 Hz, 1H), 6.22 (dd, *J* = 9.1, 2.4 Hz, 1H), 6.10 (d, *J* = 2.4 Hz, 1H), 3.01 (s, 6H). MS: m/z calcd. for $[C_{16}H_{15}BrNO_4]^+$: 364, found: 364 $[M+H]^+$.

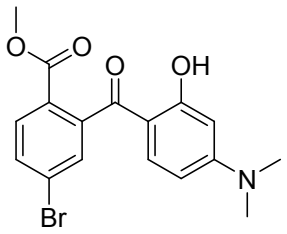
Methyl 4- and 5-bromo-2-(4-(dimethylamino)-2-hydroxybenzoyl)benzoate (5a, 5b)[1]



5-Bromophthalic anhydride (**3**) (12 g, 53 mmol, 1 eq) and 3-dimethylaminophenol (**2**) (8 g, 58 mmol, 1.1 eq) were dissolved in toluene (120 mL). The solution was stirred for 16 h at 85 °C, then 5 h at 120 °C. After the solvent was removed, the residue was dissolved in methanol (100 mL). Keeping the reaction mixture at 0 °C, 6 ml thionyl chloride was added dropwise through 30 min. The reaction was refluxed overnight, then the solvent was removed. The residue was extracted with CH_2Cl_2 and

water, the organic phase was dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure. The dark pink crude product was purified by silica flash chromatography (hexane : CH_2Cl_2 – 15 : 1) to give yellow crystals (5.8 g, 15.3 mmol, 29 %).

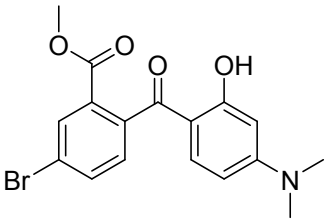
Methyl 4-bromo-2-(4-(dimethylamino)-2-hydroxybenzoyl)benzoate (5a)



In order to gain compound **5a**, the mixture of the two isomers was recrystallised from methyl *tert*-butyl ether twice to give the required isomer. Yellow crystals (2.34 mmol, 619 mg).

^{1}H NMR (500 MHz, DMSO-d6) δ 12.24 (s, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.86 (dd, J = 8.4, 1.9 Hz, 1H), 7.68 (d, J = 1.8 Hz, 1H), 6.85 (d, J = 9.1 Hz, 1H), 6.24 (dd, J = 9.2, 2.4 Hz, 1H), 6.12 (d, J = 2.4 Hz, 1H), 3.66 (s, 3H), 3.02 (s, 6H). MS: m/z calcd. for $[C_{17}H_{17}BrNO_4]^+$: 378, found: 378 $[M+H]^+$.

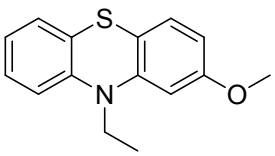
Methyl 5-bromo-2-(4-(dimethylamino)-2-hydroxybenzoyl)benzoate (5b)



The 1:1 ratio mixture of **5a** and **5b** was recrystallized from diisopropyl ether twice to give the required isomer (**5b**). Yellow crystals (3.22 mmol, 853 mg).

^{1}H NMR (500 MHz, DMSO-d6) δ 12.32 (s, 1H), 8.09 (d, J = 1.9 Hz, 1H), 7.94 (dd, J = 8.1, 2.0 Hz, 1H), 7.42 (d, J = 8.1 Hz, 1H), 6.87 (d, J = 9.1 Hz, 1H), 6.22 (dd, J = 9.2, 2.4 Hz, 1H), 6.12 (d, J = 2.3 Hz, 1H), 3.68 (s, 3H), 3.02 (s, 6H). MS: m/z calcd. for $[C_{17}H_{17}BrNO_4]^+$: 378, found: 378 $[M+H]^+$.

10-Ethyl-2-methoxy-10*H*-phenothiazine (6)



2-Methoxyphenothiazine (2.3 g, 10 mmol) was dissolved in dry DMF, then NaH (600 mg, 15 mmol) and iodoethane (2.34 g, 15 mmol) were added at 0 °C. The mixture was stirred for 14 h at rt. After the reaction was completed, the solvent was evaporated under reduced pressure. The residue was dissolved in water and diethyl-ether (100 cm^3–100 cm^3), then the aqueous phase was extracted two more times. The collected organic phase was washed with brine and dried over $MgSO_4$. After evaporation of the solvent

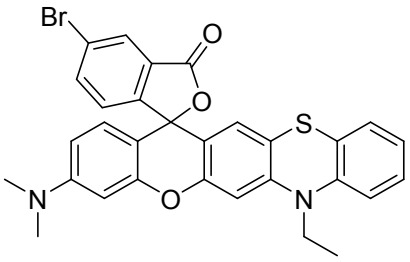
the crude product was used without further purification. White powder (2.40 g, 9.33 mmol, 93 %)

^{1}H NMR (500 MHz, DMSO-d6) δ 7.20 – 7.15 (m, 1H), 7.12 (dd, J = 7.6, 1.5 Hz, 1H), 7.04 – 6.98 (m, 2H), 6.92 (td, J = 7.5, 0.8 Hz, 1H), 6.57 – 6.52 (m, 2H), 3.91 (q, J = 6.9 Hz, 2H), 3.74 (s, 3H), 1.29 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d6) δ 159.5, 145.8, 144.2, 127.30, 127.29, 126.8, 123.7, 122.3, 115.5, 113.8, 107.2, 102.7, 55.2, 41.0, 12.7. MS: m/z calcd. for $[C_{15}H_{16}NOS]^+$: 258, found: 258 $[M+H]^+$.

General procedure for 7a and 7b:

4a or **4b** (360 mg, 1.0 mmol) were dissolved in methansulfonic acid (1mL), then phenothiazine **6** (257 mg, 1.0 mmol) was added. The mixture was stirred for 12 hours at 70 °C. After the reaction was completed, the solution was poured on ice and the pH was set to 7–8 with $NaHCO_3$ solution. The mixture was stirred for 20 minutes more, then it was extracted with CH_2Cl_2 three times. The collected organic phase was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was purified on silica flash cromatography (CH_2Cl_2 : methanol – 9:1).

5'-Bromo-3-(dimethylamino)-7-ethyl-3'*H*,7*H*-spiro[chromeno[2,3-b]phenothiazine-14,1'-isobenzofuran]-3'-one (7a)



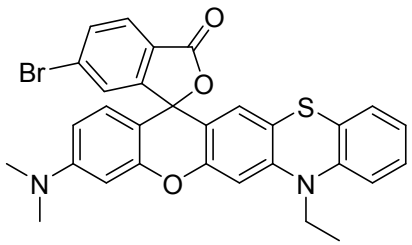
Blue powder (217 mg, 0,38 mmol; 38 %)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.14 (d, *J* = 0.9 Hz, 1H), 7.75 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.14 (t, *J* = 7.3 Hz, 1H), 7.04 (t, *J* = 6.5 Hz, 2H), 6.92 – 6.86 (m, 2H), 6.72 (s, 1H), 6.58 (d, *J* = 8.8 Hz, 1H), 6.48 – 6.43 (m, 2H), 6.41 (dd, *J* = 8.9, 2.3 Hz, 1H), 3.96 (q, *J* = 6.9 Hz, 2H), 2.98 (s, 6H), 1.47 (t, *J* = 6.9 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 168.0, 152.7, 152.4, 151.9, 151.7, 147.3, 143.8, 138.1, 129.5, 129.4, 128.8, 128.1, 127.53, 127.52, 125.8, 125.7, 123.8, 123.0, 119.4, 115.4, 112.4, 109.2, 105.5, 103.2, 98.6, 84.3, 42.4, 40.3, 12.9.

HRMS: m/z calcd. for $[C_{30}H_{24}BrN_2O_3S]^+$: 571.0685, found: 571.0681 $[M+H]^+$.

6'-Bromo-3-(dimethylamino)-7-ethyl-3'*H*,7*H*-spiro[chromeno[2,3-b]phenothiazine-14,1'-isobenzofuran]-3'-one (7b)



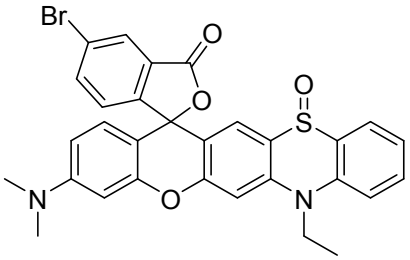
Blue powder (467 mg, 0,82 mmol; 82 %)

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.86 (d, *J* = 8.7 Hz, 1H), 7.72 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.29 (dd, *J* = 1.6, 0.5 Hz, 1H), 7.17 – 7.11 (m, 1H), 7.05 (dd, *J* = 7.6, 1.4 Hz, 1H), 6.92 – 6.86 (m, 2H), 6.72 (s, 1H), 6.60 (d, *J* = 8.8 Hz, 1H), 6.48 – 6.44 (m, 2H), 6.42 (dd, *J* = 8.9, 2.6 Hz, 1H), 3.96 (q, *J* = 6.9 Hz, 2H), 2.99 (s, 6H), 1.47 (t, *J* = 7.0 Hz, 3H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 168.7, 155.0, 152.6, 152.4, 151.8, 147.4, 143.8, 133.4, 130.3, 128.8, 127.6, 127.52, 127.46, 126.4, 126.1, 125.7, 123.8, 123.0, 119.4, 115.58, 112.4, 109.2, 105.4, 103.2, 98.6, 83.5, 42.4, 40.3, 12.9.
HRMS: m/z calcd. for $[C_{30}H_{24}BrN_2O_3S]^+$: 571.0685, found: 571.0681 $[M+H]^+$.

General procedure for 9a and 9b:

To a solution of **7a** or **7b** (570 mg, 1.0 mmol) in CH_2Cl_2 was added mCPBA (246 mg, 70% wet with water, 1.0 mmol) at 0°C and stirred for 1h at rt. After the reaction was complete, the mixture was washed 3 times with saturated $NaHCO_3$ solution, dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The crude product was purified on silica flash cromatography (CH_2Cl_2 : methanol – 9:1).

5'-Bromo-3-(dimethylamino)-7-ethyl-3'*H*,7*H*-spiro[chromeno[2,3-b]phenothiazine-14,1'-isobenzofuran]-3'-one 12-oxide (9a)

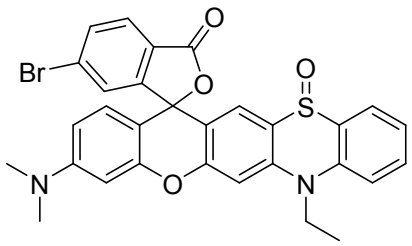


Pink powder (458 mg, 0.78 mmol, 78%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.20 (dd, *J* = 1.7, 0.5 Hz, 1H), 7.85 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.74 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.61 (ddd, *J* = 8.8, 7.2, 1.6 Hz, 1H), 7.44 (d, *J* = 8.5 Hz, 1H), 7.40 (s, 1H), 7.26 – 7.20 (m, 2H), 6.99 (dd, *J* = 8.1, 0.4 Hz, 1H), 6.63 (d, *J* = 8.8 Hz, 1H), 6.53 (d, *J* = 2.5 Hz, 1H), 6.44 (dd, *J* = 8.9, 2.6 Hz, 1H), 4.41 – 4.28 (m, 2H), 3.01 (s, 6H), 1.63 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 167.6, 154.9, 152.53, 152.47, 151.5, 140.0, 138.1, 137.8, 133.2, 132.8, 131.8, 129.4, 128.7, 128.4, 125.5, 124.8, 124.1, 122.4, 120.8, 115.8, 113.8, 109.5, 105.51, 103.2, 98.7, 83.0, 43.3, 40.3, 12.0.

HRMS: m/z calcd. for $[C_{30}H_{24}BrN_2O_4S]^+$: 587.0634, found: 587.0633 $[M+H]^+$.



6'-Bromo-3-(dimethylamino)-7-ethyl-3'*H*,7*H*-spiro[chromeno[2,3-b]phenothiazine-14,1'-isobenzofuran]-3'-one 12-oxide (9b)

Pink powder (564 mg, 0.96 mmol, 96%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.92 (d, *J* = 8.2 Hz, 1H), 7.85 (dd, *J* = 7.6, 1.5 Hz, 1H), 7.75 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.64 – 7.57 (m, 1H), 7.46 – 7.39 (m, 2H), 7.27 – 7.20 (m, 3H), 6.64 (d, *J* = 8.8 Hz, 1H), 6.52 (d, *J* = 2.5 Hz, 1H), 6.46 (dd, *J* = 8.9, 2.5 Hz, 1H), 4.40 – 4.27 (m, 2H), 3.00 (s, 6H), 1.62 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 168.3, 154.79, 154.75, 152.5, 152.3, 140.0, 137.8, 133.6, 133.2, 132.9, 131.9, 130.2, 128.7, 127.2, 126.7, 126.0, 124.7, 122.4, 120.8, 115.8, 113.7, 109.6, 105.4, 103.2, 98.7, 82.3, 43.2, 40.3, 12.0.

HRMS: m/z calcd. for $[C_{30}H_{24}BrN_2O_4S]^+$: 587.0634, found: 587.0638 $[M+H]^+$.

Spectral measurements

Photophysical measurements were performed on a JASCO V-750 Spectrophotometer and a JASCO FP-8300 spectrofluorometer. A stock solution in MeOH was prepared from the solid dyes (1 mM). To obtain the BCN conjugates, all of the dyes (1 mM) were reacted with BCN (1.1 mM) in MeOH at room temperature for 15 min. The completion of the reaction was monitored by LC-MS

The excitation and emission spectra were measured in all-sodium PBS (pH=7.4) with 0.1 m/V% SDS, in order to prevent aggregation in 2.5 μM solutions (Figure S3). To record the emission spectra dyes **1** and their BCN conjugates were excited at 600 nm, dyes **10** and their BCN conjugates were excited at 500 nm. For the excitation spectra emission was recorded at 750 nm for dyes **1** and their BCN conjugates, and at 650 nm for dyes **10** and their BCN conjugates.

Quantum yields were determined using cresyl violet in methanol (Φ=0.54)[2] and rhodamine B in basic ethanol (Φ=0.65)[3] as standard.

The photooxidation experiments were performed using commercial green or orange LED as light source (10 W High power COB-LED chips (8.0-10V, 840-1000mA, 600-800LM) operate 3 in series circuit connected to current generator: AC 100-260V in, DC 20-40V 900mA 30W out, for the emission spectrum, see Figure S1). Sample solutions were prepared in MeOH from the dyes (0.5 mM). To obtain the BCN conjugates, the dyes were reacted with 1.1 equiv BCN. The samples were irradiated for a given time while oxygen was bubbled through the solutions using continuous water cooling for the light source and a petri dish to prevent any heating issues. The emission spectra were measured in all-sodium PBS (pH=7.4) with 0.1 m/V% SDS in 2.5 μM solution. The emission intensity at 585 nm (characteristic emission of **10** and their BCN conjugates) were compared. Control experiments were performed with no oxygen bubbling during irradiation, with oxygen bubbling in the dark, and irradiating a sample containing 0.1 M NaN_3 as a singlet oxygen quencher while bubbling oxygen (see Figure S2).

The RNO method of singlet oxygen determination was carried out as follows.[4] A 100 mM oxygenated solution of the photosensitizer in PBS containing 0.01 M histidine and 50 mM *N,N*-dimethyl-4-nitrosoaniline (RNO) was illuminated using green LED and its absorbance was measured periodically without further dilution.

Polarity dependence was measured in solvents with different dielectric constants (dioxane:water mixtures from 100% to 10%, with 10% increments)[5] in the concentration of 1 μM.

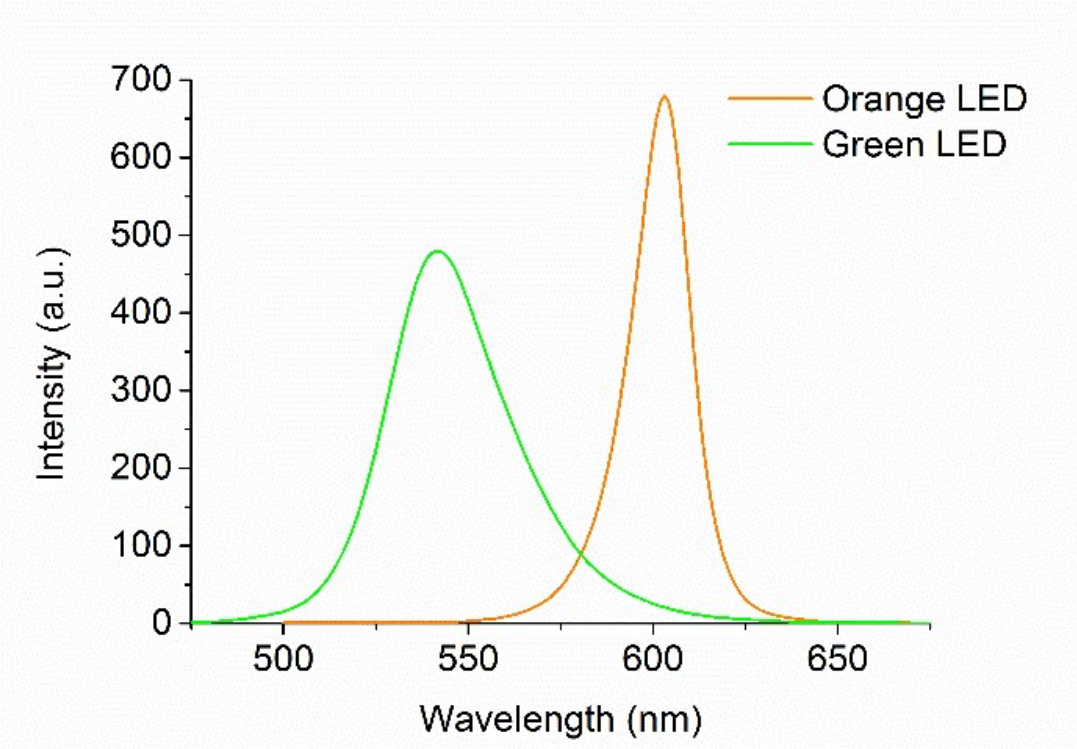


Figure S1. Emission spectra of the LEDs used for the photooxidation experiments

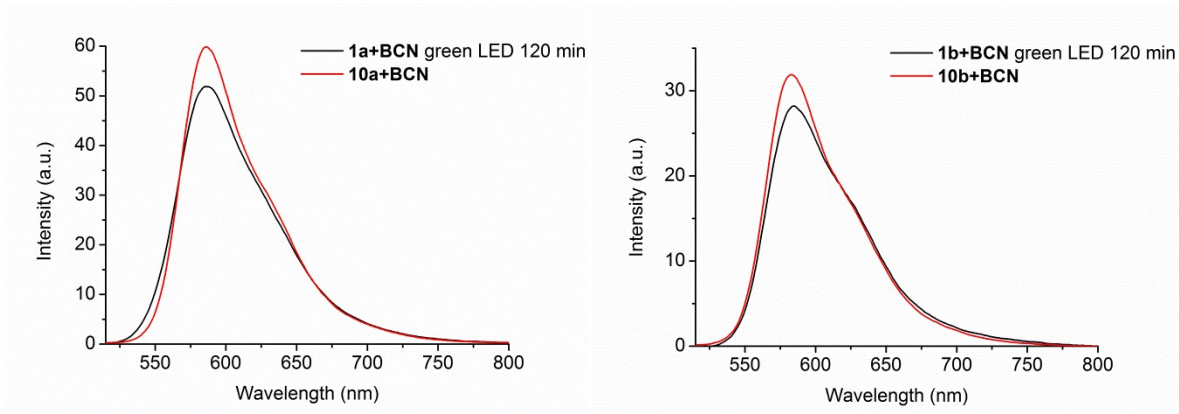


Figure S2. Emission spectra of probes **1.BCN** in PBS-SDS after illumination with green LED for 120 min in oxygenated methanolic solutions with spectra of probes **10.BCN**. in PBS-SDS for comparison

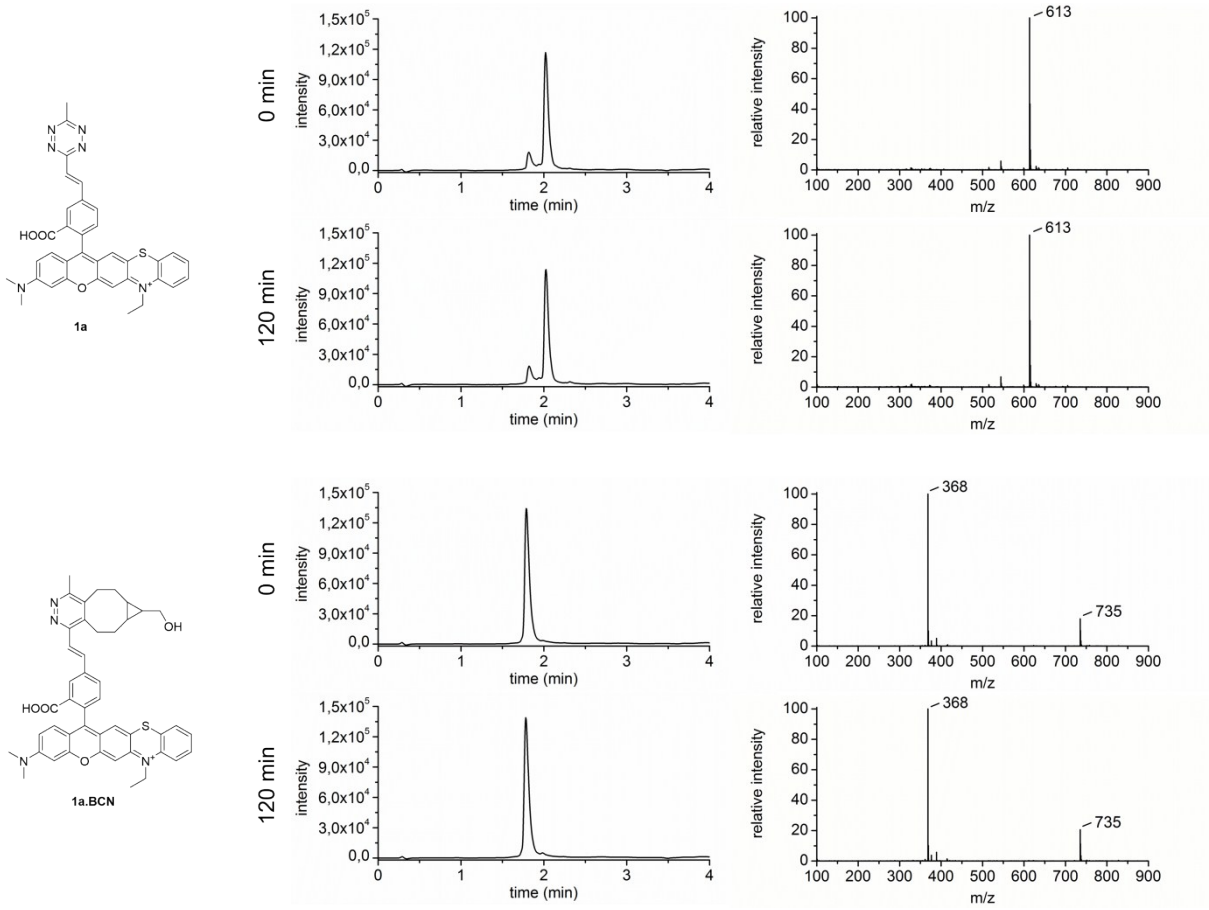


Figure S3. HPLC chromatograms of **1a** (top two rows) and **1a.BCN** (bottom two rows) in the presence of NaN_3 before and after illumination with green LED for 120 min, and the corresponding MS spectra

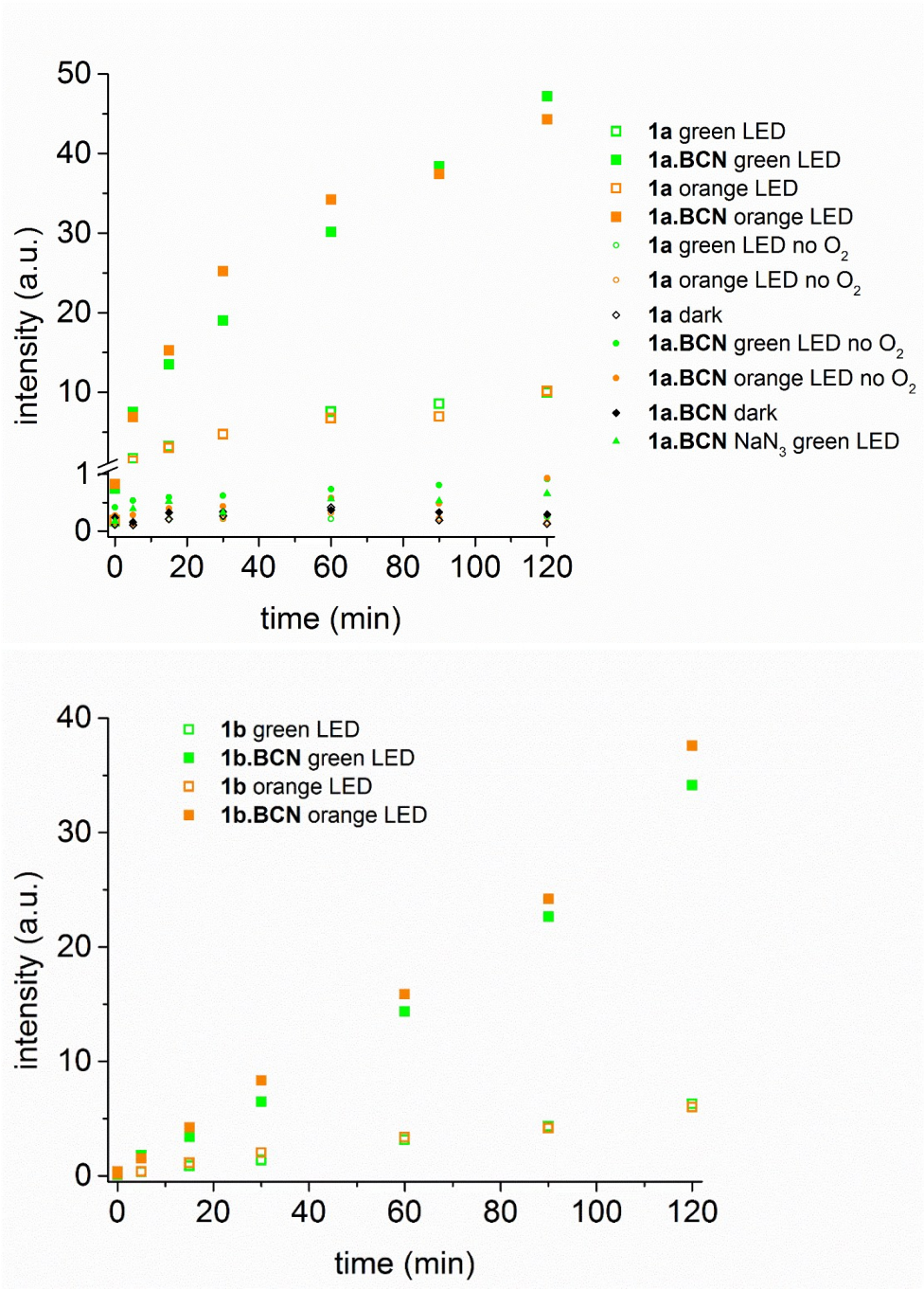


Figure S4. Oxidation of probes **1a** and **1b** and their BCN-conjugates in methanolic solutions upon green or orange LED illumination.

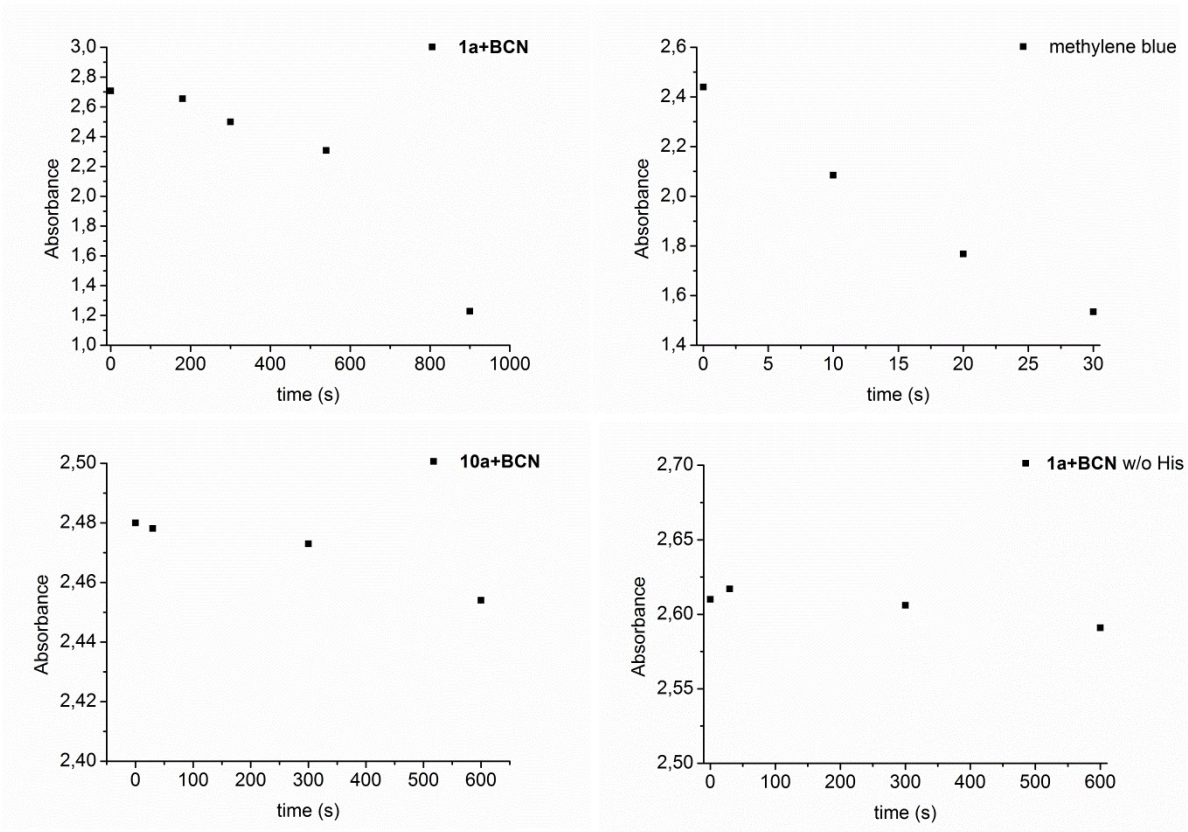


Figure S5. Changes in absorption of RNO (at 440 nm) upon irradiation with green LED using **1a.BCN** (top left), methylene blue (top right) or **10a.BCN** (bottom left) as photosensitizers. Control experiment using **1a.BCN** in the absence of histidine is shown bottom right.

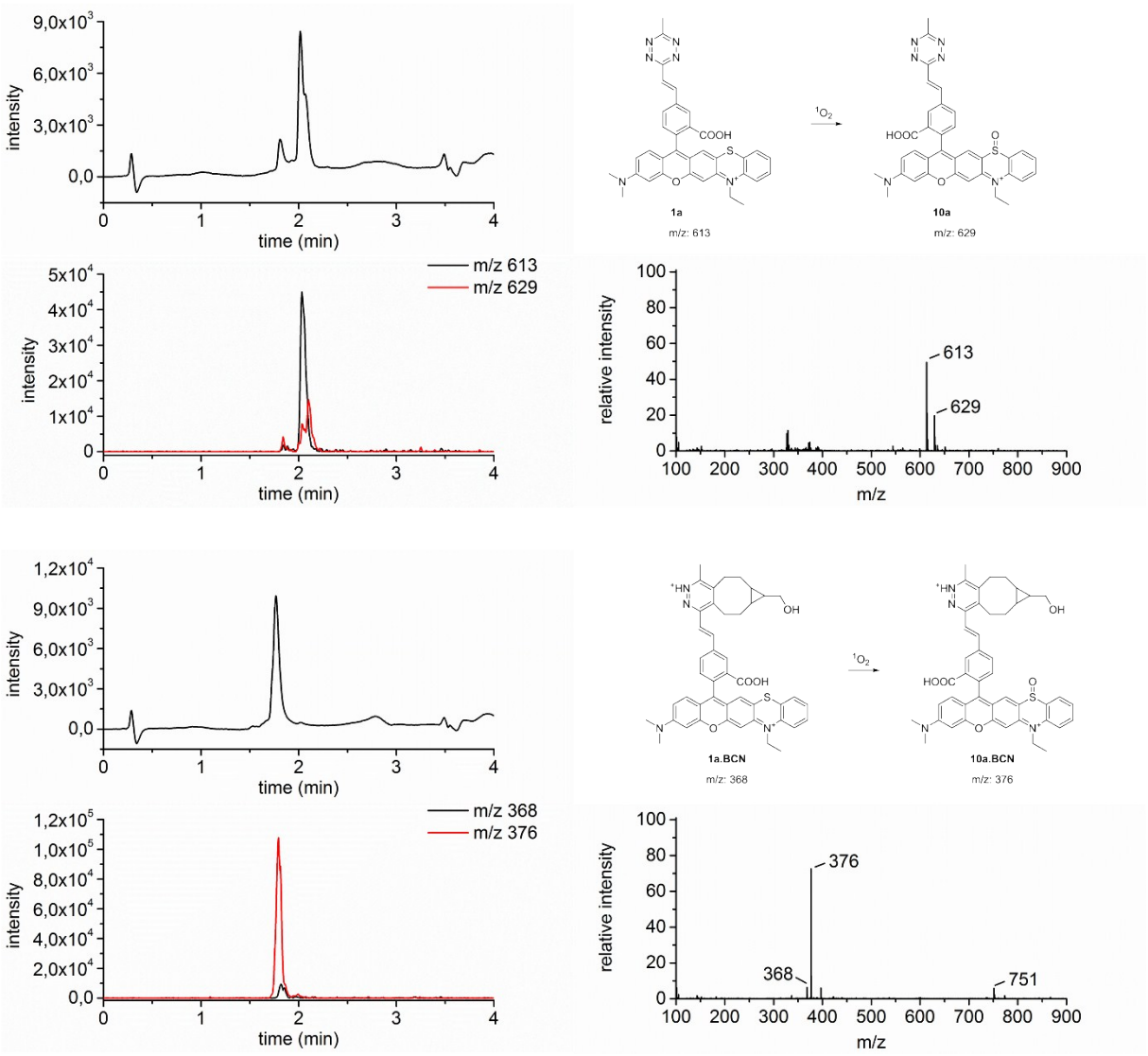


Figure S6. HPLC and ion chromatograms of **1a** (top two rows) and **1a.BCN** (bottom two rows) after illumination with green LED in oxygenated methanolic solutions for 120 min, and the corresponding MS spectra

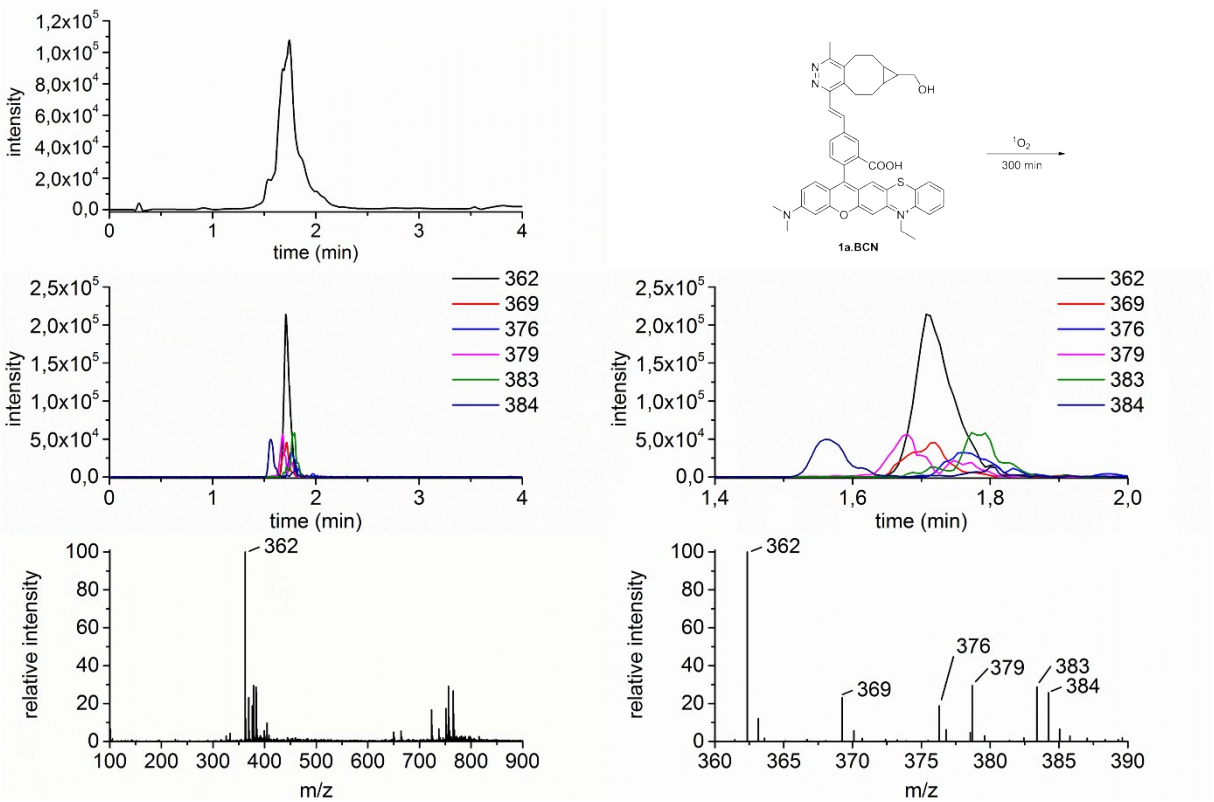
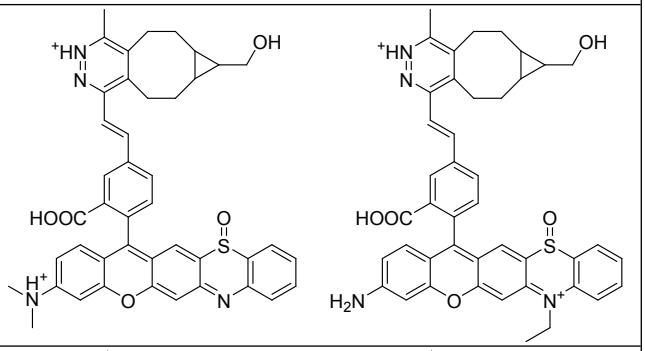
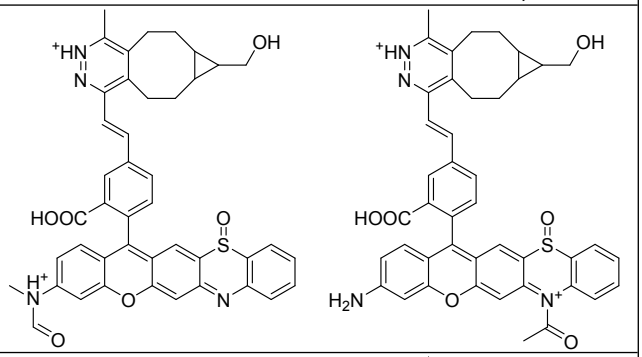
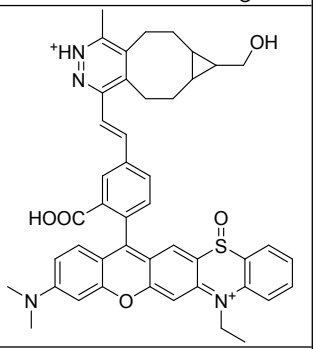
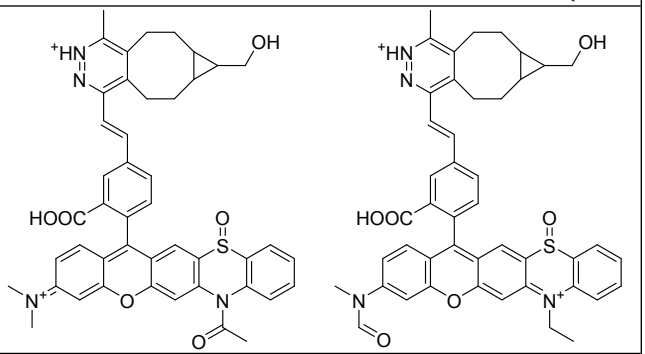
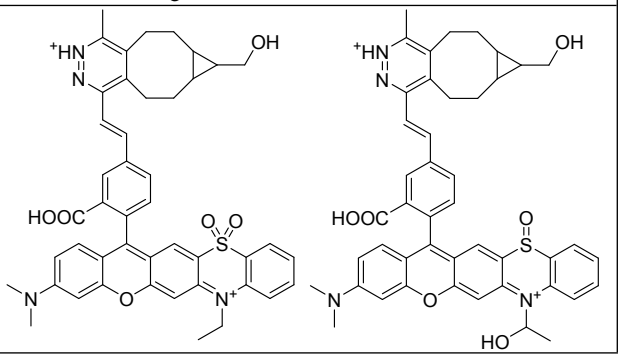


Figure S7. HPLC (top left) and ion chromatograms (middle row) of **1a.BCN** after illumination with green LED in oxygenated methanolic solutions for 300 min and the combined MS spectra taken between 1.5 and 2 min retention times (bottom row)

Table S1. Possible products after green LED illumination of **1a.BCN** for 300 min

m/z	Possible structures
362	
369	
376	
383	
384	

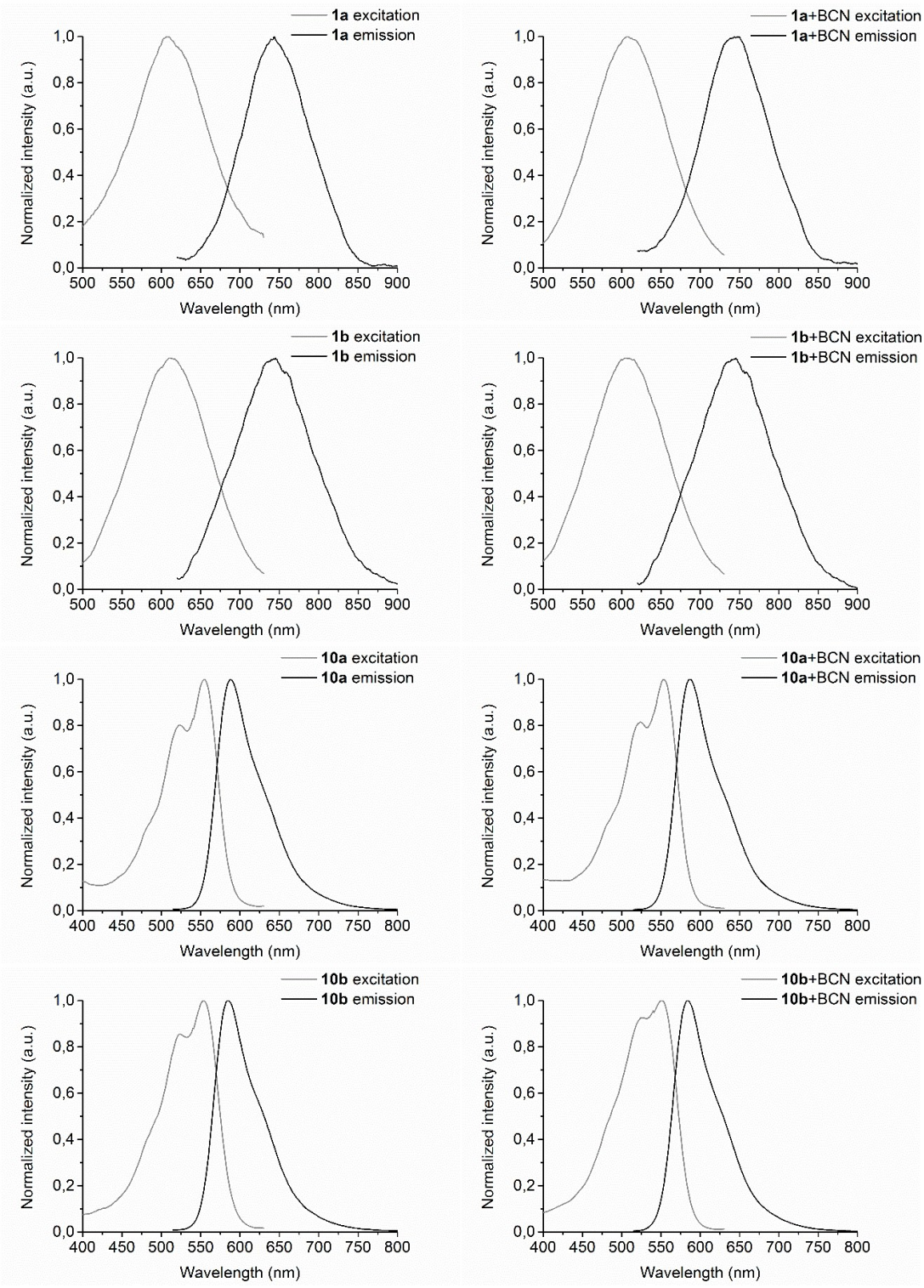


Figure S8. Excitation and emission spectra of probes **1** and **10** and their BCN conjugates in PBS-SDS

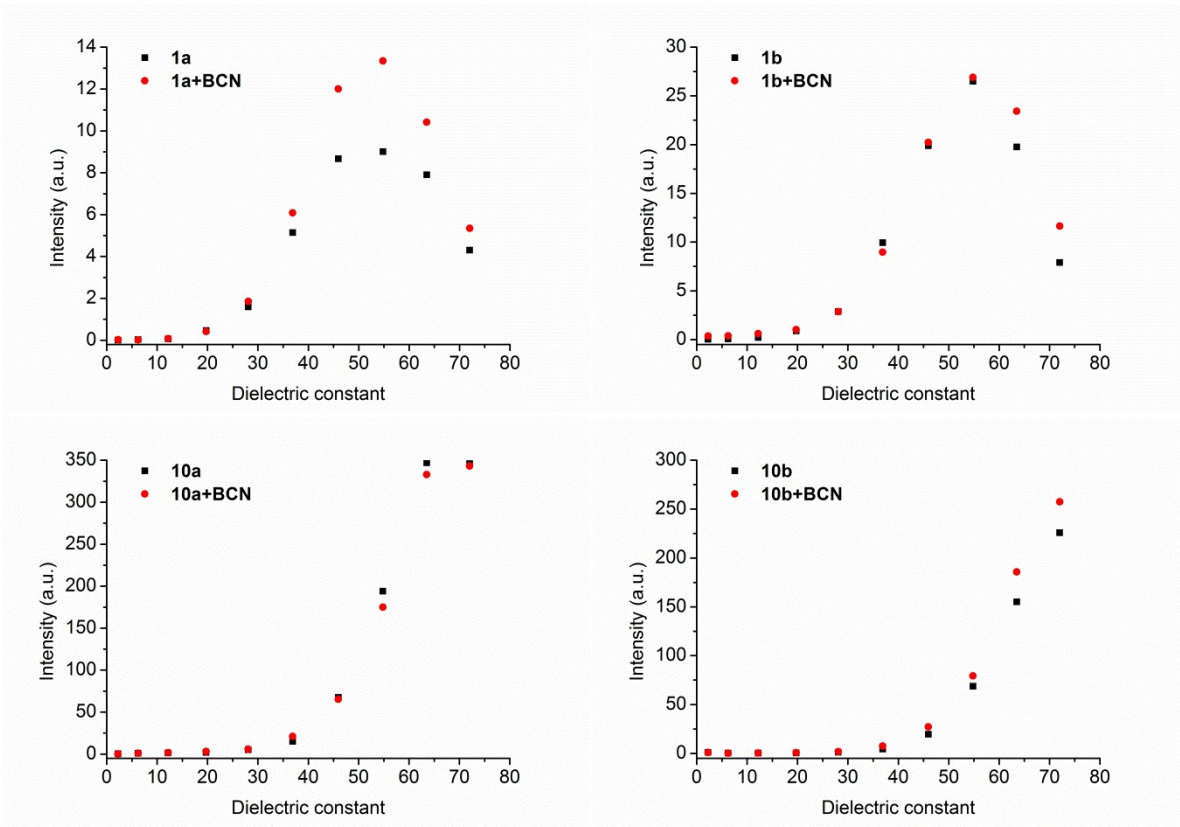


Figure S9. Polarity dependence of probes **1**, **10** and their BCN conjugates

Biological experiments

Cell culture

COS-7 cells (Sigma 87021302) were maintained in Dulbecco's modified Eagle's medium (Life Technologies 41965-039) supplemented with 1% penicillin-streptomycin (Sigma P0781), 1% L-Glutamine (Sigma G7513), 1% sodium pyruvate (Life Technologies 11360), and 10% FBS (Sigma F7524). Cells were cultured at 37°C in a 5% CO_2 atmosphere and passaged every 3–4 days up to 20 passages.

Actin labeling[6]

Actin labeling was performed based on the procedure published by Wieczorek et al.[7] and Meimetis et al.[8] COS-7 cells were transferred into ibidi µ-slide 8 well glass bottom plates (15,000 cell/well) and incubated for 20–24 h at 37°C in a 5% CO_2 atmosphere. Cells were washed with PBS then fixed (4% PFA for 10 min at 25°C) and permeabilized (0.1% Triton-X-100 for 5 min at 25°C). Phalloidin-BCN treatment was carried out in a final concentration of 1 µg/mL, (200 µg/mL stock concentration in MeOH) was freshly diluted in labelling buffer (10 mM TBS (pH 7.4), 0.1% Triton-X-100 and 2% BSA) for 40 min at 25°C. After a washing step cells were labeled with the fluorogenic dye (**1a**, **1b**, **10a** or **10b**) in 10 µM concentration (in PBS) for 30 min at 25 °C in dark). Cells were washed with PBS twice and were imaged. In the case of no-wash condition imaging was carried out without the last washing step.

Synthesis of phalloidin-BCN derivative

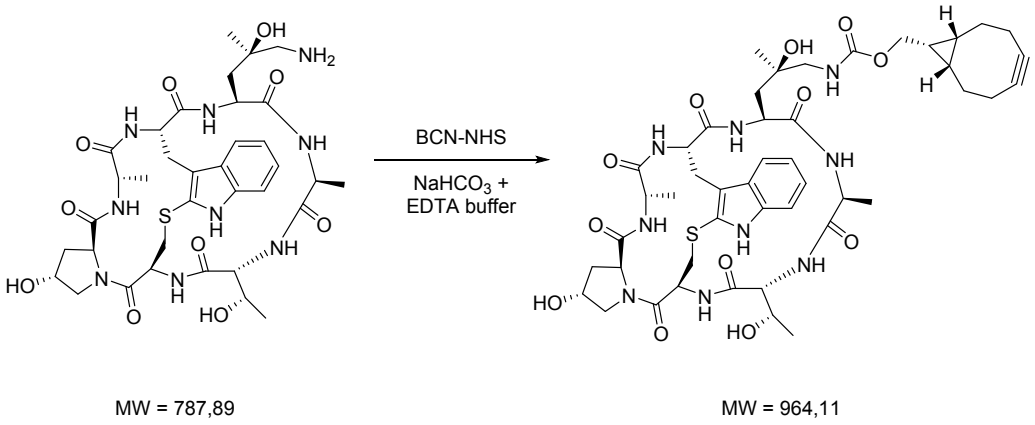


Figure S10. Conjugation of BCN and phalloidin

Synthesis of phalloidin-BCN was performed as Knorr et al.[6] with slight modifications. Briefly, aminophalloidin (Bachem H7634.0001; 120 µg, 140 nmol, 1.0 eq – *140 µL from the 1 mM stock*

solution) and BCN-NHS (Sigma 744867; 757 µg, 2.6 µmol, 19.0 eq – *26 µL from the 100 mM stock solution*) was mixed in 110 mM Na_2CO_3/$NaHCO_3$ + 2.2 mM EDTA buffer pH 9.0 (1250 µl). The reaction mixture was incubated in dark at 25°C for 2 h, then was purified with reversed phase HPLC on a Phenomenex Jupiter 10 µm C18 250 mm x 10 mm column (H_2O:MeCN both with 0.1% trifluoroacetic acid, gradient elution from 5% to 75% MeCN in 30 min) to give phalloidin-BCN (30 µg, 22%). HRMS $[M+H]^+$ calcd. for $[C_{46}H_{63}N_9O_{12}S]^+$ 964.4239; found 964.4280.

Confocal and STED imaging and analysis

Confocal and STED images were acquired on a Leica TCS SP8 STED 3X microscope using the 552 nm and 638 nm laser for excitation; 660 nm STED (1.5 W, continuous wave) laser for depletion. The images were taken using a Leica HC PL APO 100x/1.40 oil immersion objective along with Leica HyD detector. Using the Huygens STED Deconvolution Wizard (Huygens Software), only a moderate degree of deconvolution was applied to the recorded STED images to avoid deconvolution artifacts. The deconvolution was based on theoretical point spread function (PSF) values. Images were analyzed using Leica Application Suite X and ImageJ software (NIH). Gaussian non-linear fitting and full width at half maximum values (FWHM) were obtained with Origin Pro 9 software.

Actin labeling using 10a

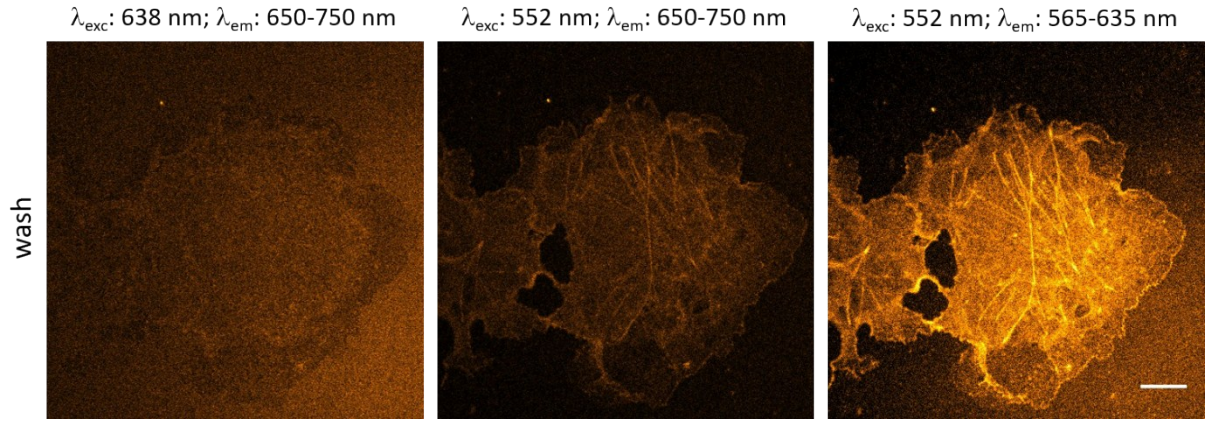


Figure S11. Actin filament staining. COS7 cells were tagged with cyclooctynylated phalloidin, then labeled with **10a** and washed (scale bar: 10 µm).

Supplementary references

1 H. Yu, Y. Xiao and H. Guo, *Org. Lett.* 2012, **14**, 2014–2017.

2 S. J. Isak and E. M. Eyring, *J. Phys. Chem.* 1992, **96**, 1738–1742.

3 R. F. Kubin and A. N. Fletcher, *J. Lumin.* 1982, **27**, 455–462.

4 I. Kraljić and S. El Mohsni, *Photochem. Photobiol.* 1978, **28**, 577–581.

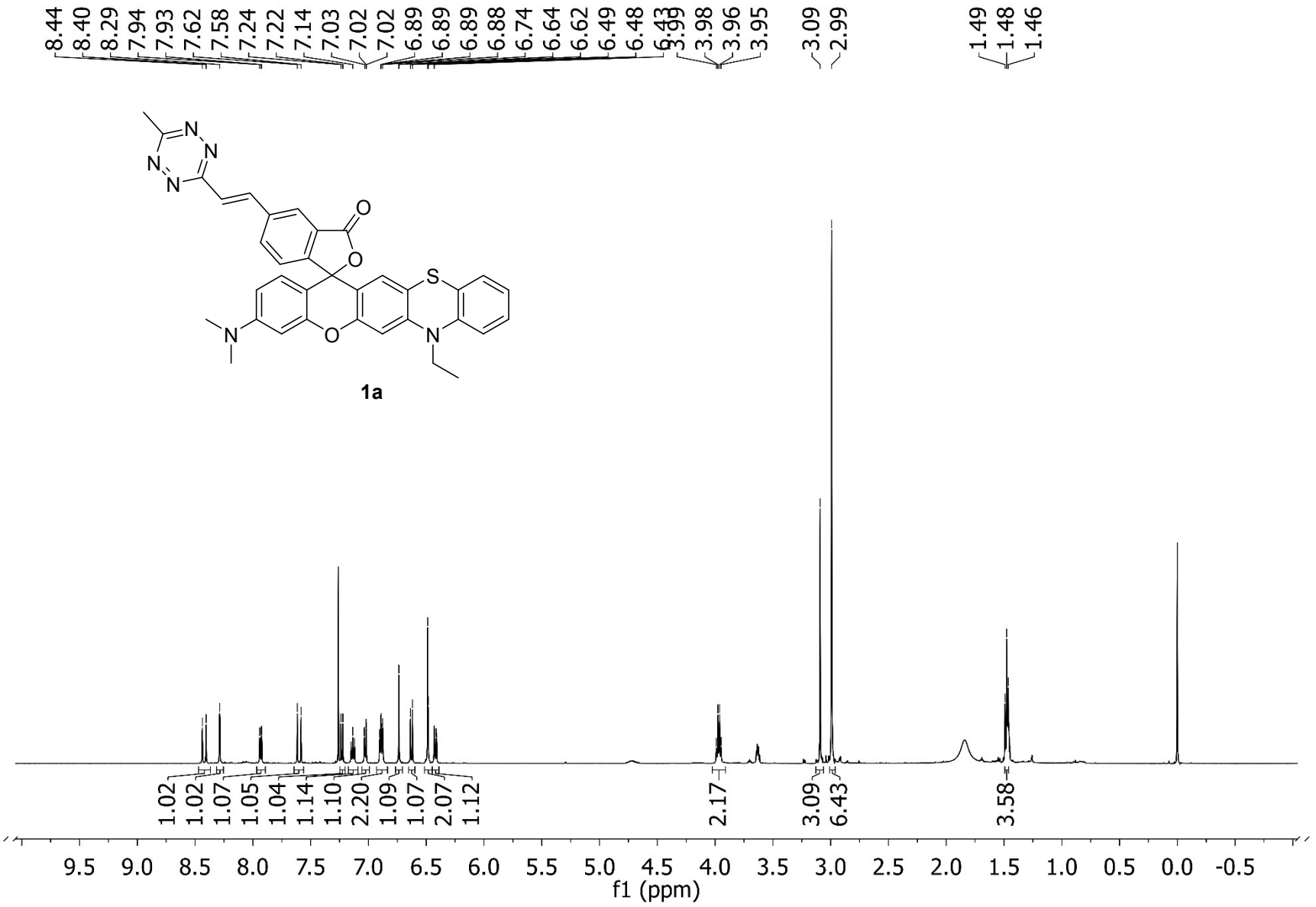
5 F. E. Critchfield, J. A. Gibson and J. L. Hall, *J. Am. Chem. Soc.* 1953, **75**, 1991–1992.

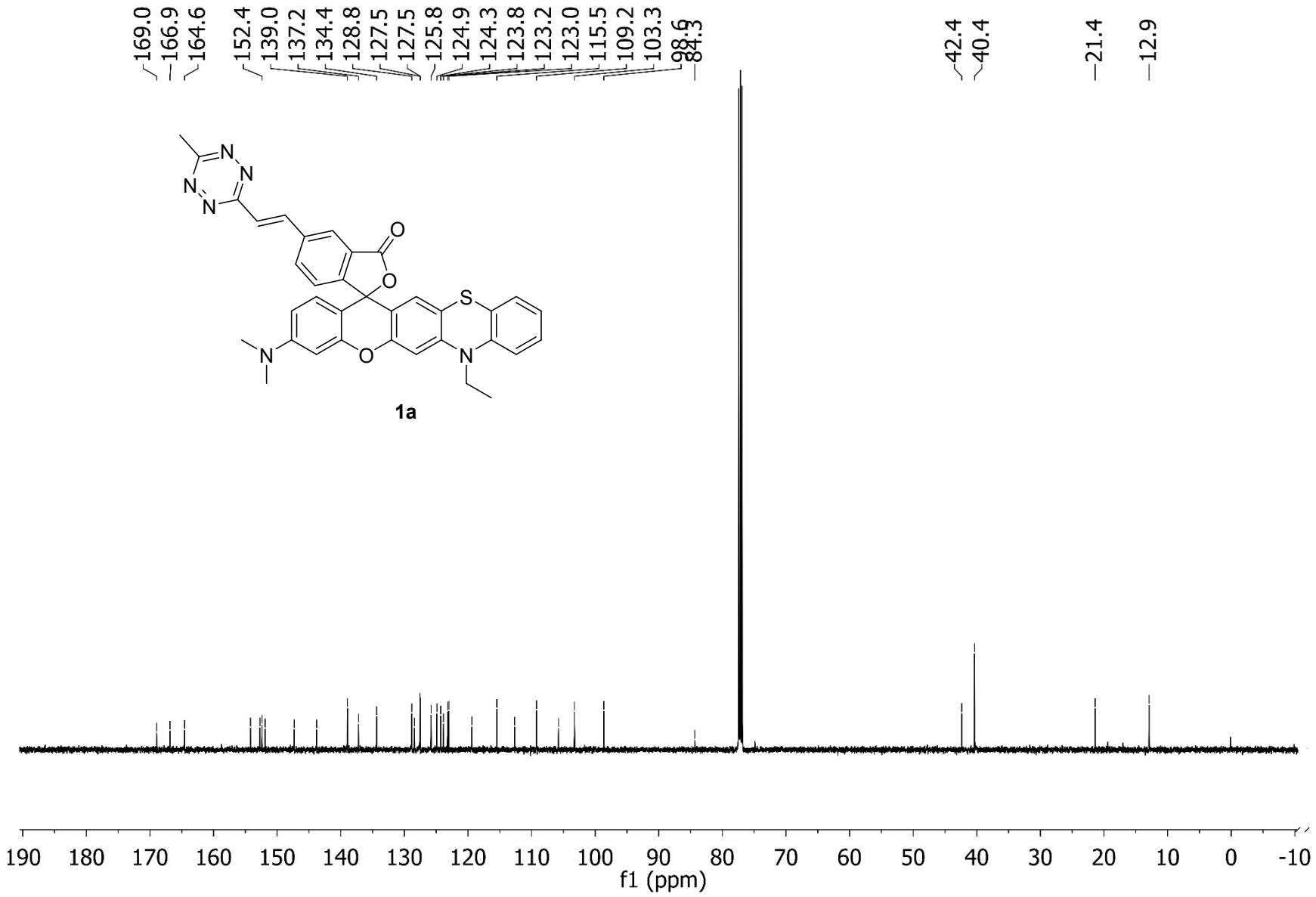
6 G. Knorr, E. Kozma, J. M. Schaart, K. Nemeth, G. Torok and P. Kele, *Bioconjugate Chem.* 2018, **29**, 1312–1318.

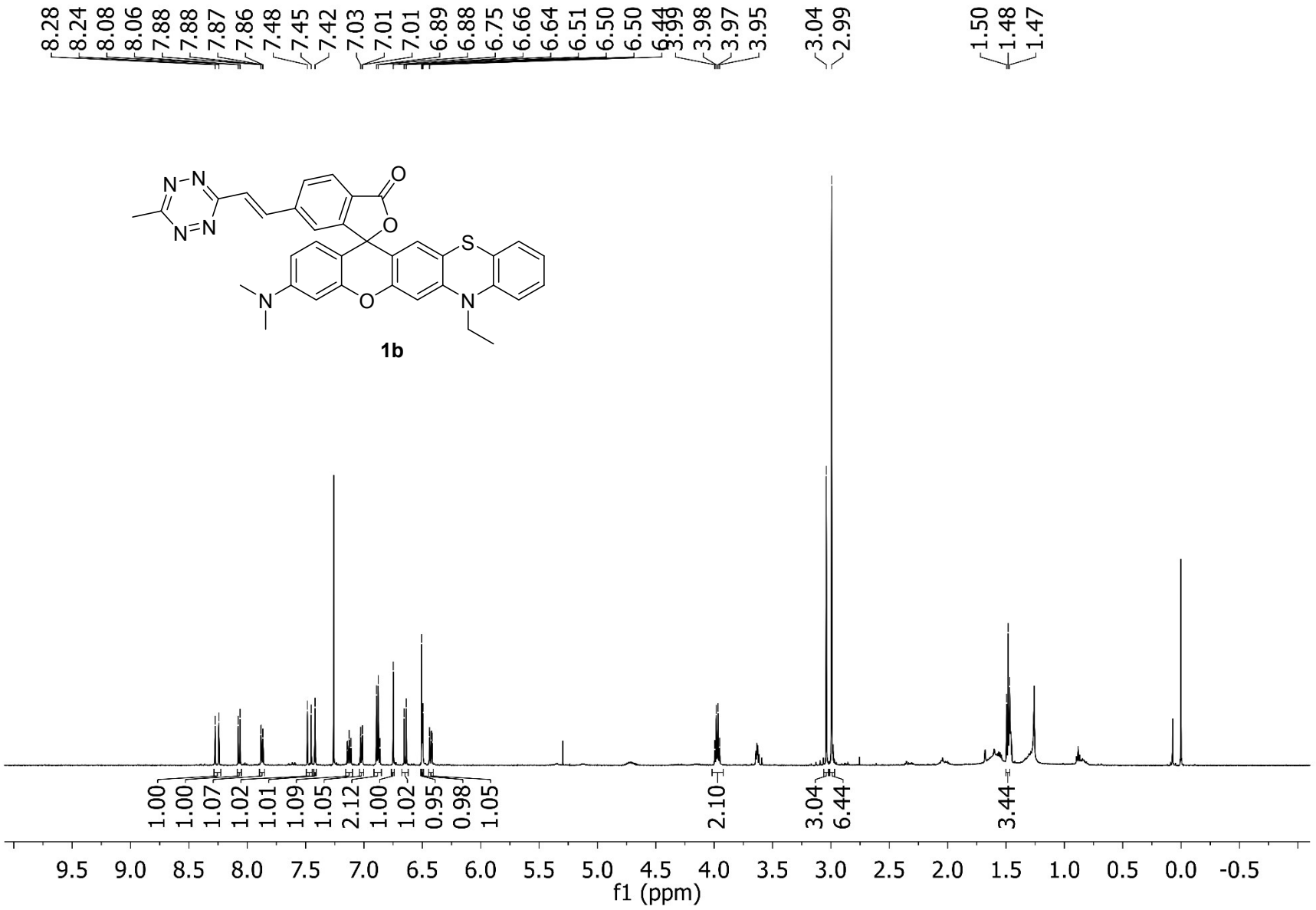
7 A. Wieczorek, P. Werther, J. Euchner and R. Wombacher, *Chem. Sci.* 2017, **8**, 1506–1510.

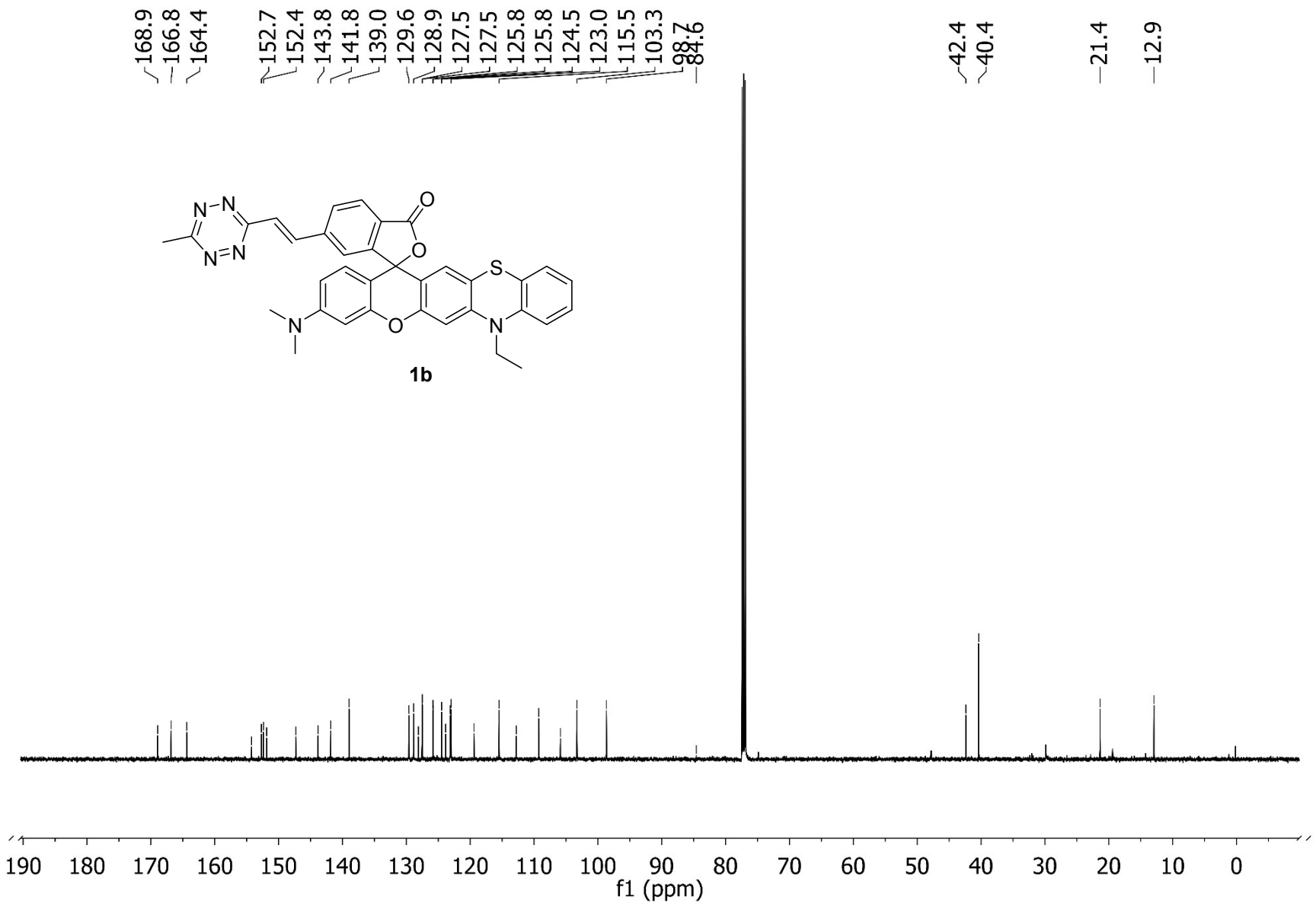
8 L. G. Meimetis, J. C. Carlson, R. J. Giedt, R. H. Kohler and R. Weissleder, *Angew. Chem. Int. Ed.* 2014, **53**, 7531–7534.

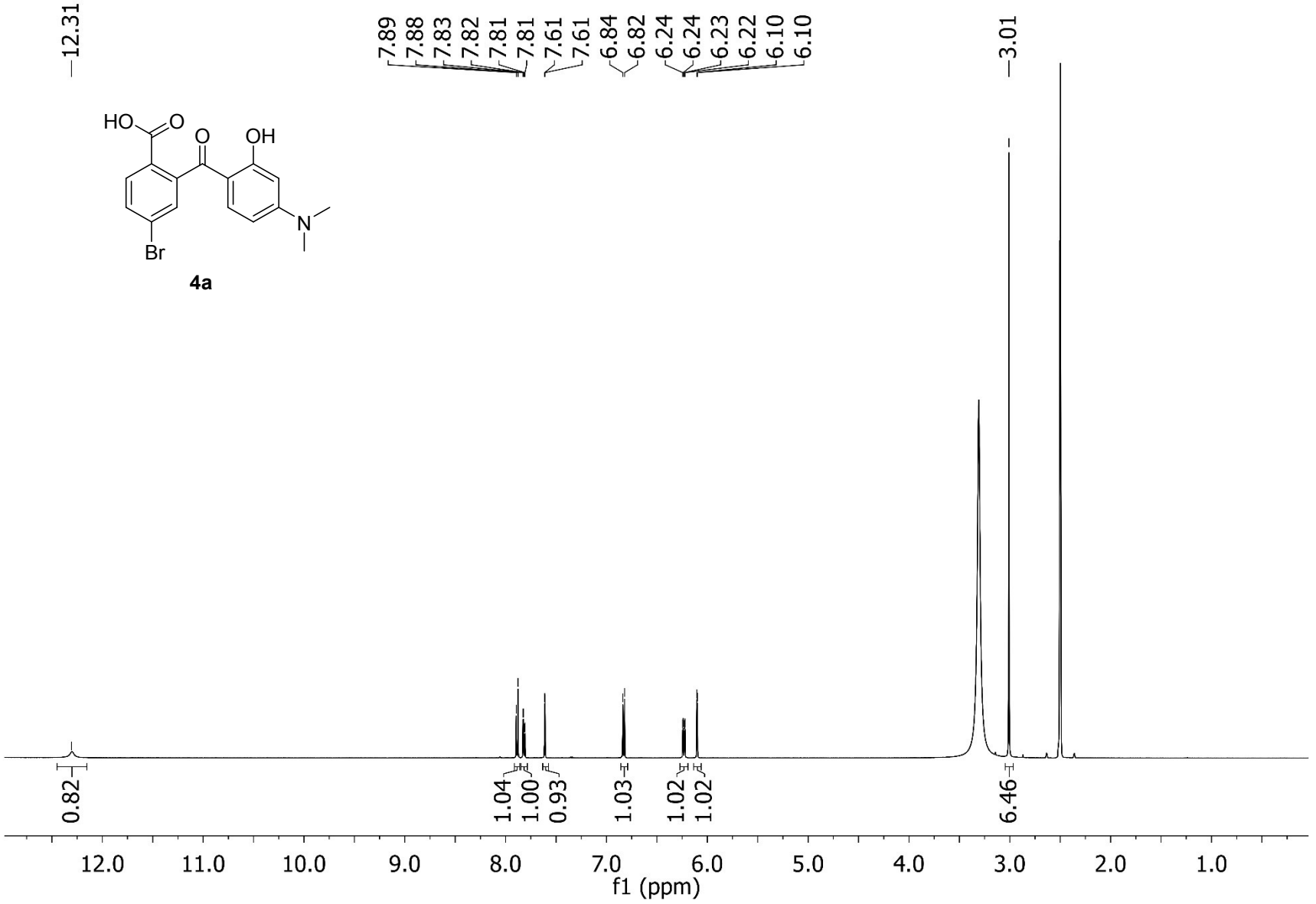
NMR spectra

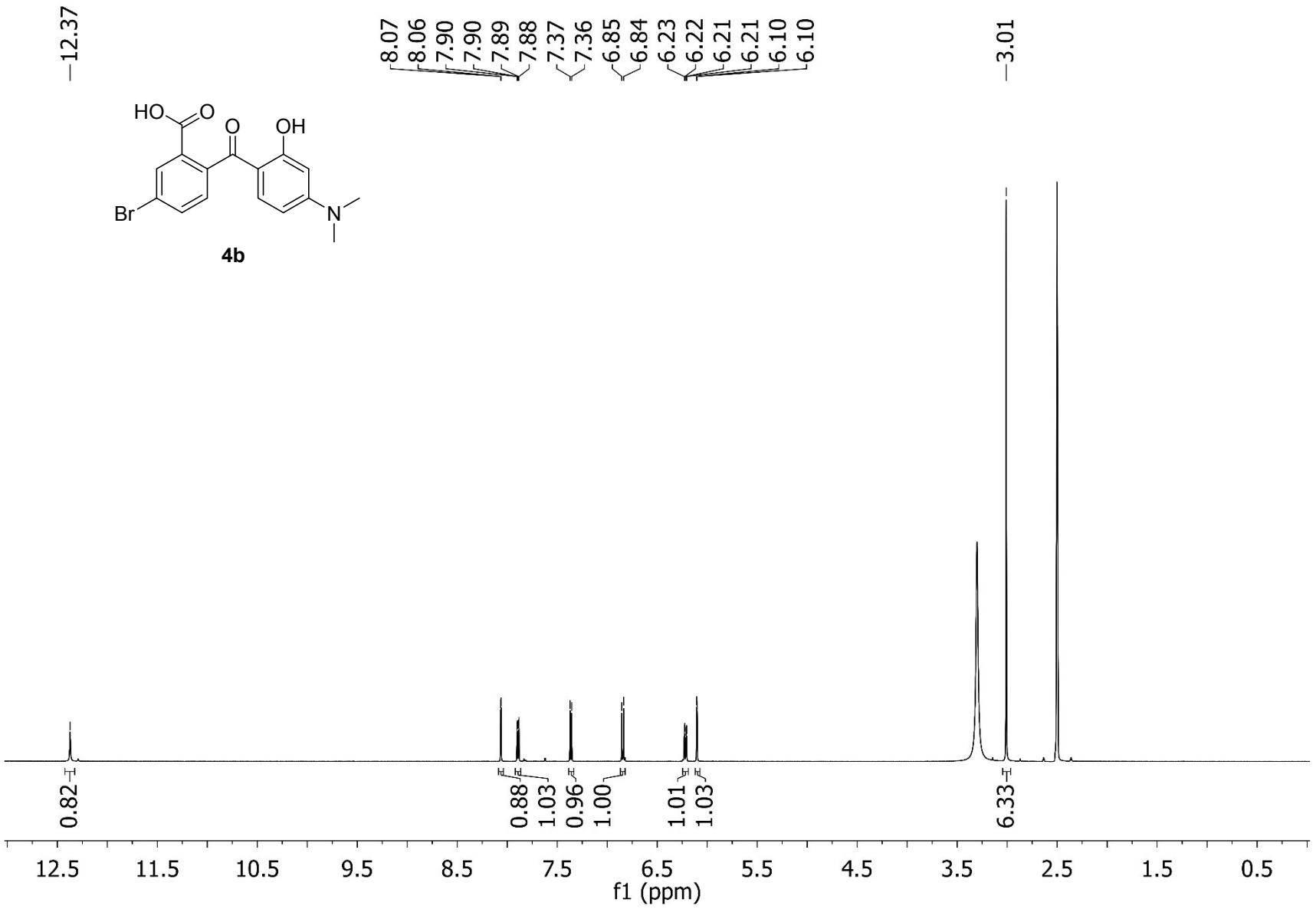

8.44
8.40
8.29
7.94
7.93
7.62
7.58
7.24
7.22
7.14
7.03
7.02
7.02
6.89
6.89
6.89
6.88
6.74
6.64
6.62
6.49
6.48
6.43
3.99
3.98
3.96
3.95
3.09
2.99
1.49
1.48
1.46
1a
1.02
1.02
1.07
1.05
1.04
1.14
1.10
2.20
1.09
1.07
2.07
1.12
2.17
3.09
6.43
3.58
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

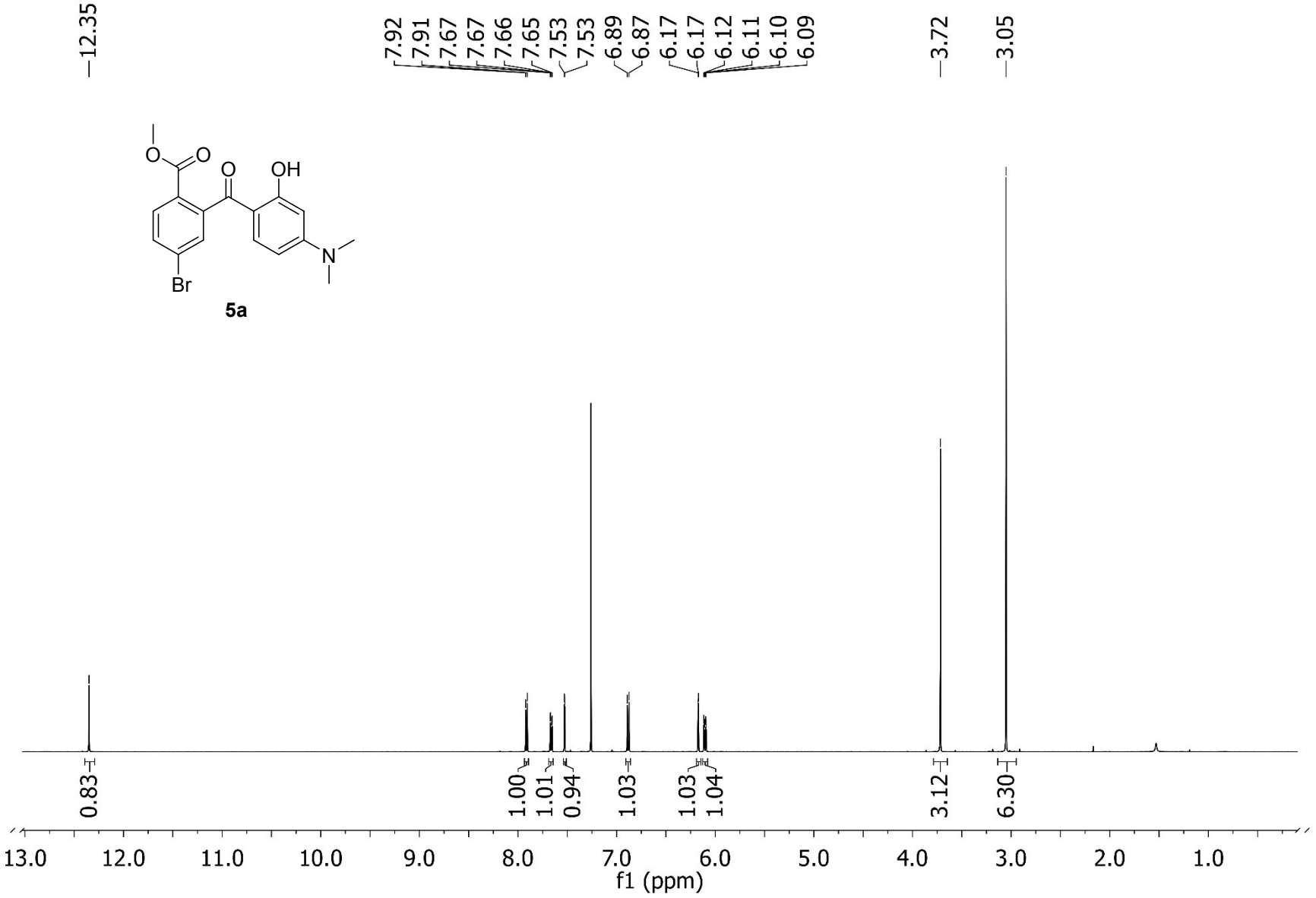

169.0
166.9
164.6
152.4
139.0
137.2
134.4
128.8
127.5
127.5
125.8
124.9
124.3
123.8
123.2
123.0
115.5
109.2
103.3
98.6
84.3
42.4
40.4
21.4
12.9
1a
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

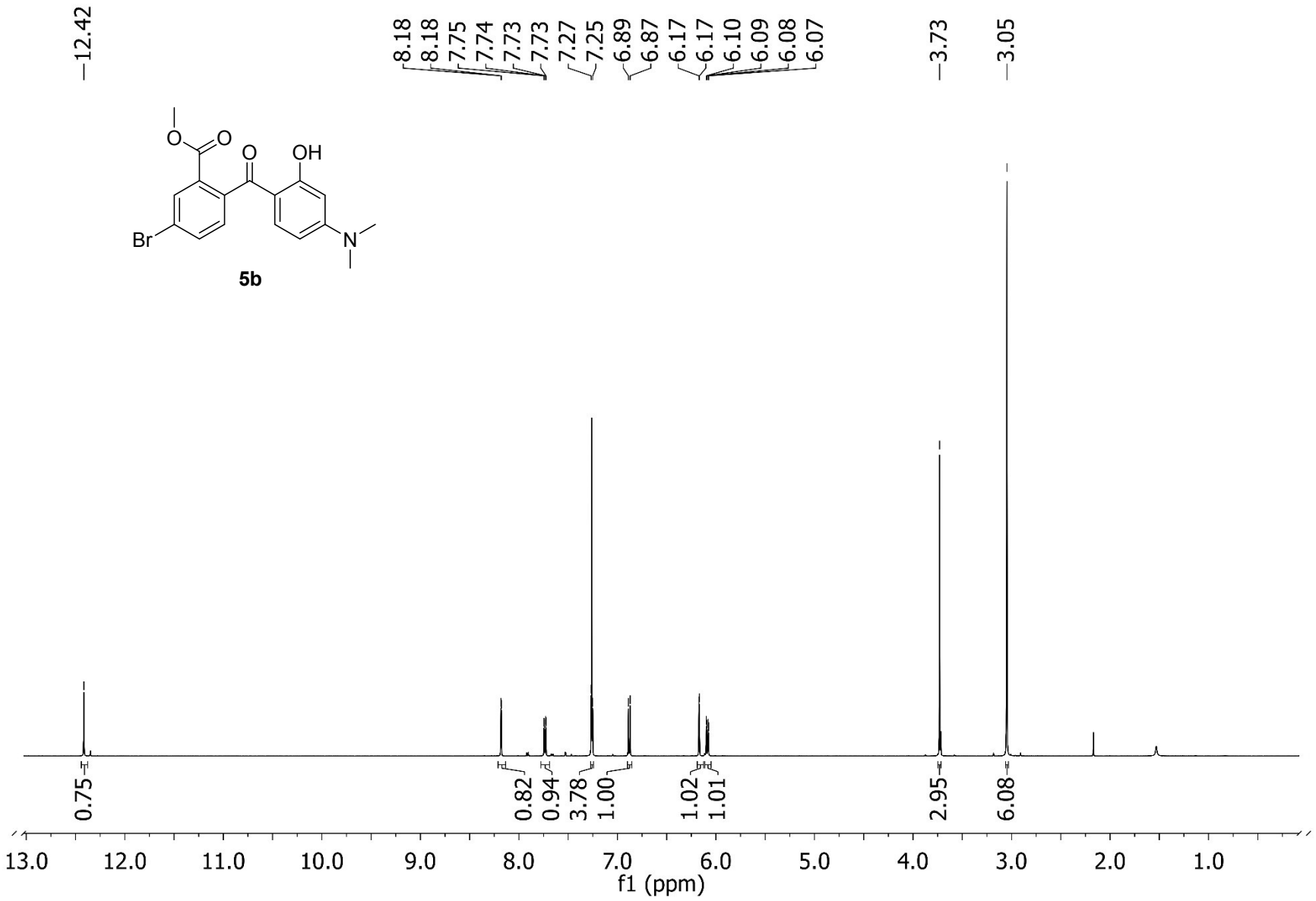

8.28
8.24
8.08
8.06
7.88
7.88
7.87
7.86
7.48
7.45
7.42
7.03
7.01
7.01
6.89
6.88
6.75
6.66
6.64
6.51
6.50
6.50
6.44
3.99
3.98
3.97
3.95
3.04
2.99
1.50
1.48
1.47
1b
1.00
1.00
1.07
1.02
1.01
1.09
1.05
2.12
1.00
1.02
0.95
0.98
1.05
2.10
3.04
6.44
3.44
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

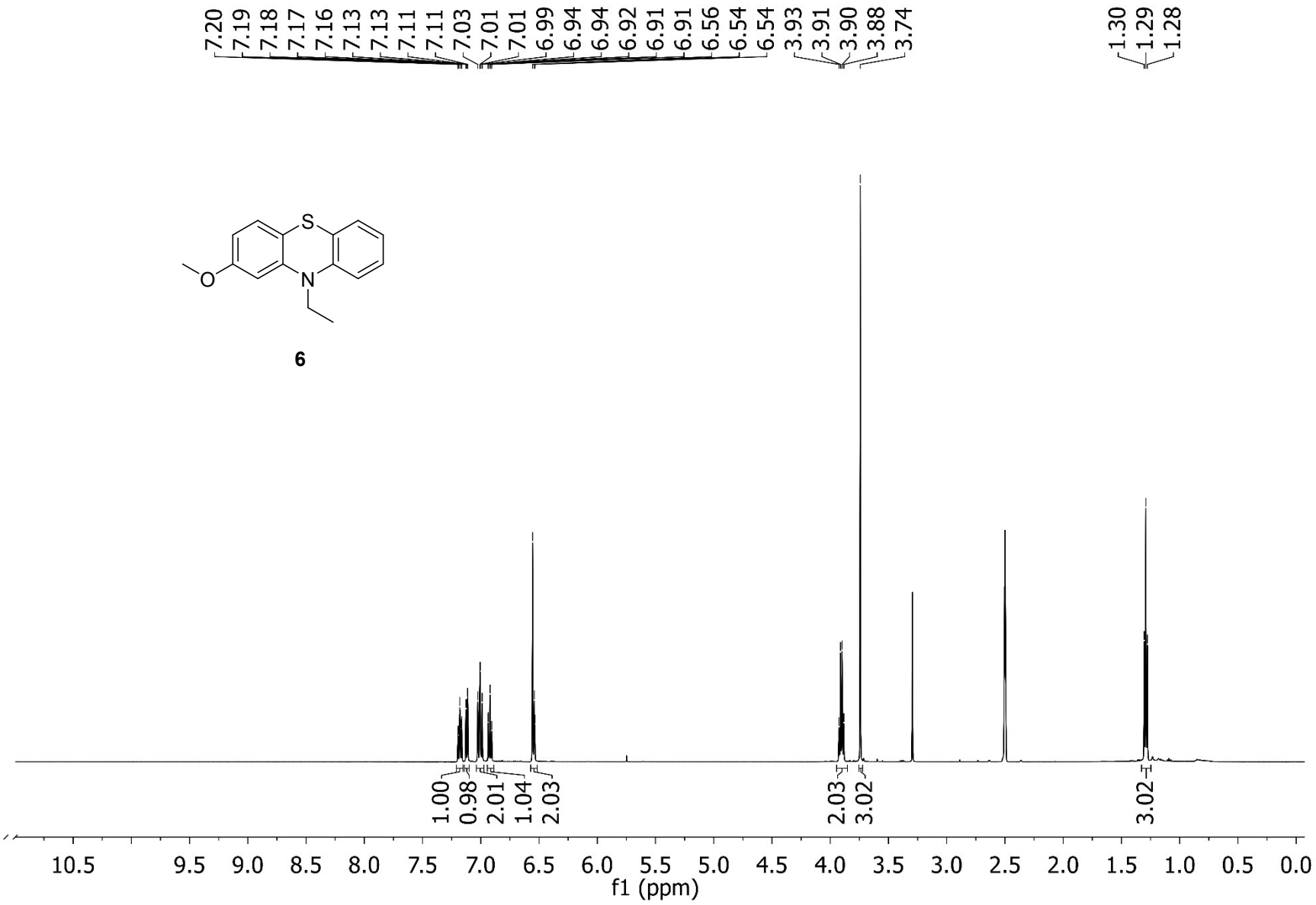

168.9
166.8
164.4
152.7
152.4
143.8
141.8
139.0
129.6
128.9
127.5
127.5
125.8
125.8
124.5
123.0
115.5
103.3
98.7
84.6
42.4
40.4
21.4
12.9
1b
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

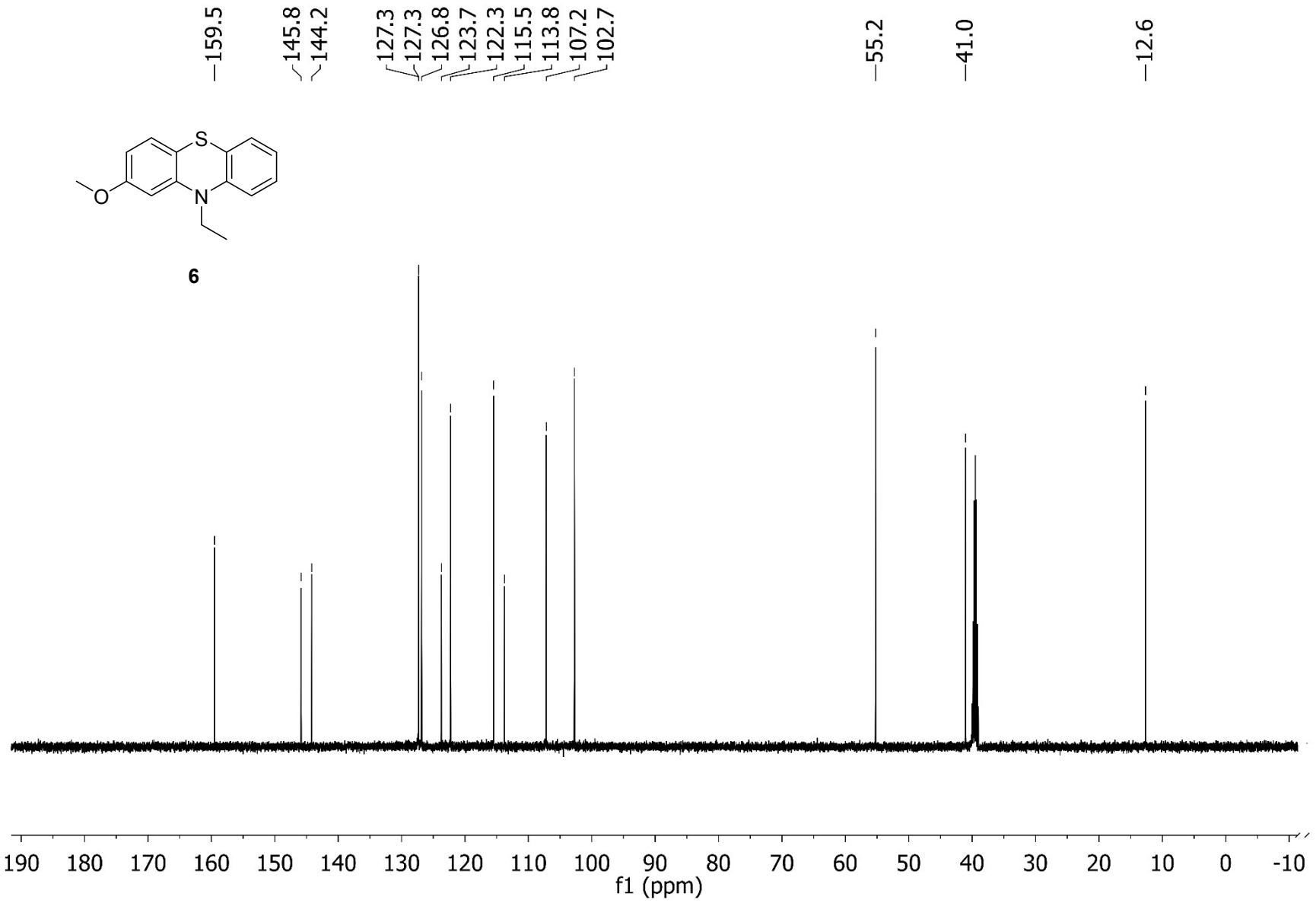

12.31
7.89
7.88
7.83
7.82
7.81
7.81
7.61
7.61
6.84
6.82
6.24
6.24
6.23
6.22
6.10
6.10
3.01
HO
O
O
OH
N
Br
4a
0.82
1.04
1.00
0.93
1.03
1.02
1.02
6.46
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
f1 (ppm)

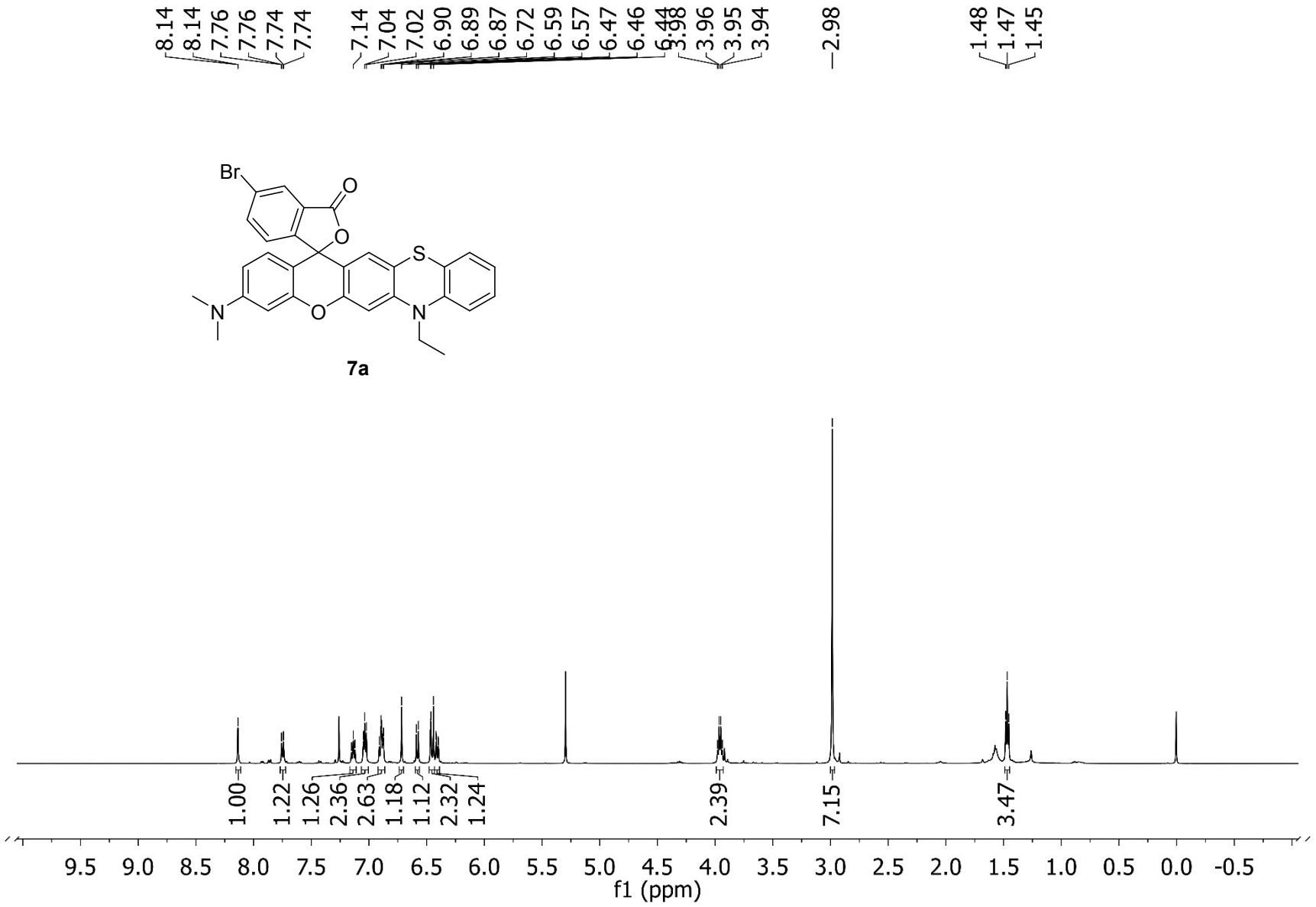

12.37
8.07
8.06
7.90
7.90
7.89
7.88
7.37
7.36
6.85
6.84
6.23
6.22
6.21
6.21
6.10
6.10
3.01
HO
O
O
OH
Br
N
4b
0.82
0.88
1.03
0.96
1.00
1.01
1.03
6.33
12.5
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
f1 (ppm)


12.35
7.92
7.91
7.67
7.67
7.66
7.65
7.53
7.53
6.89
6.87
6.17
6.17
6.12
6.11
6.10
6.09
3.72
3.05
O
O
O
OH
N
Br
5a
0.83
1.00
1.01
0.94
1.03
1.03
1.04
3.12
6.30
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
f1 (ppm)

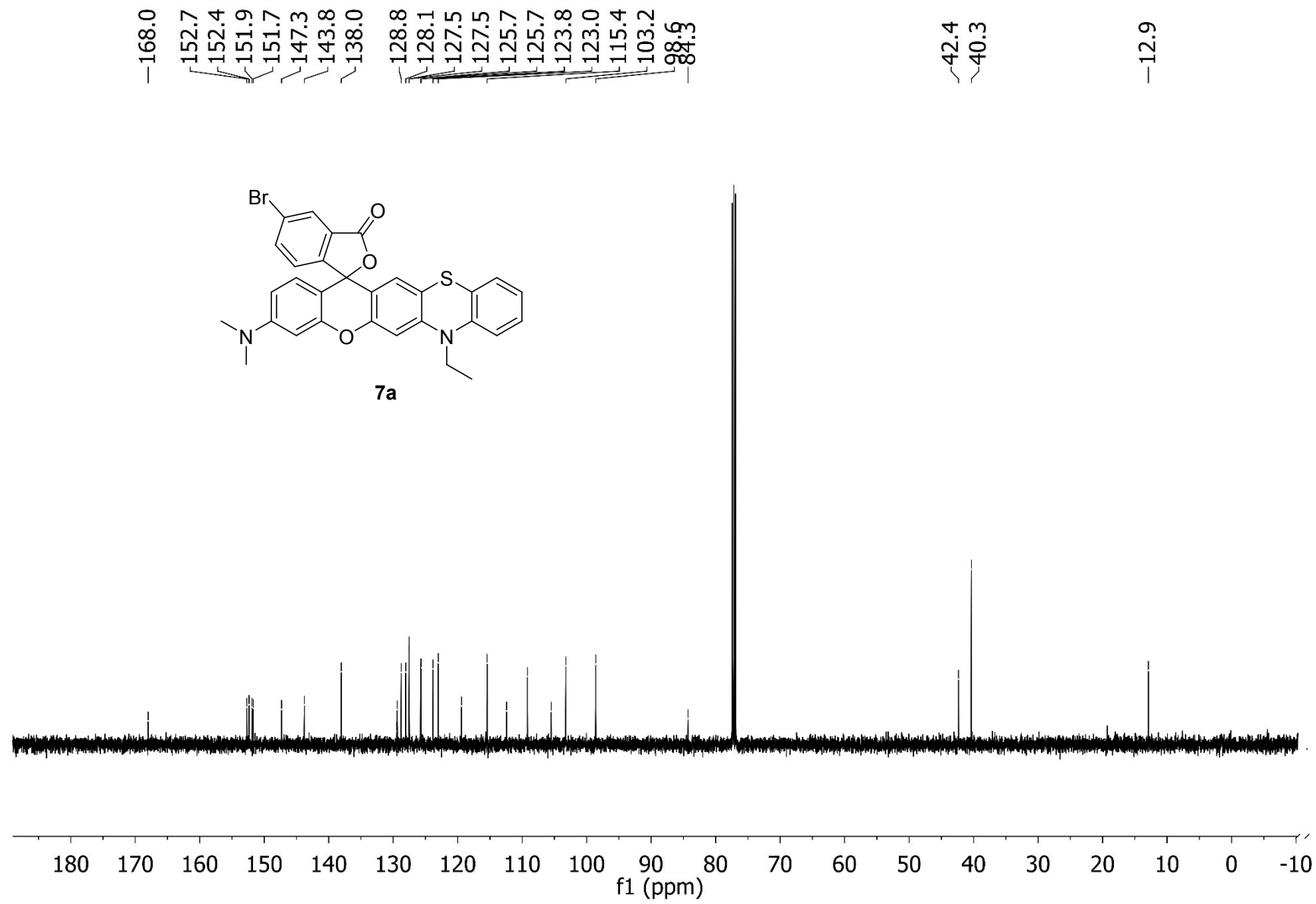

12.42
8.18
8.18
7.75
7.74
7.73
7.73
7.27
7.25
6.89
6.87
6.17
6.17
6.10
6.09
6.08
6.07
3.73
3.05
O
O
O
OH
Br
N
5b
0.75
0.82
0.94
3.78
1.00
1.02
1.01
2.95
6.08
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
f1 (ppm)

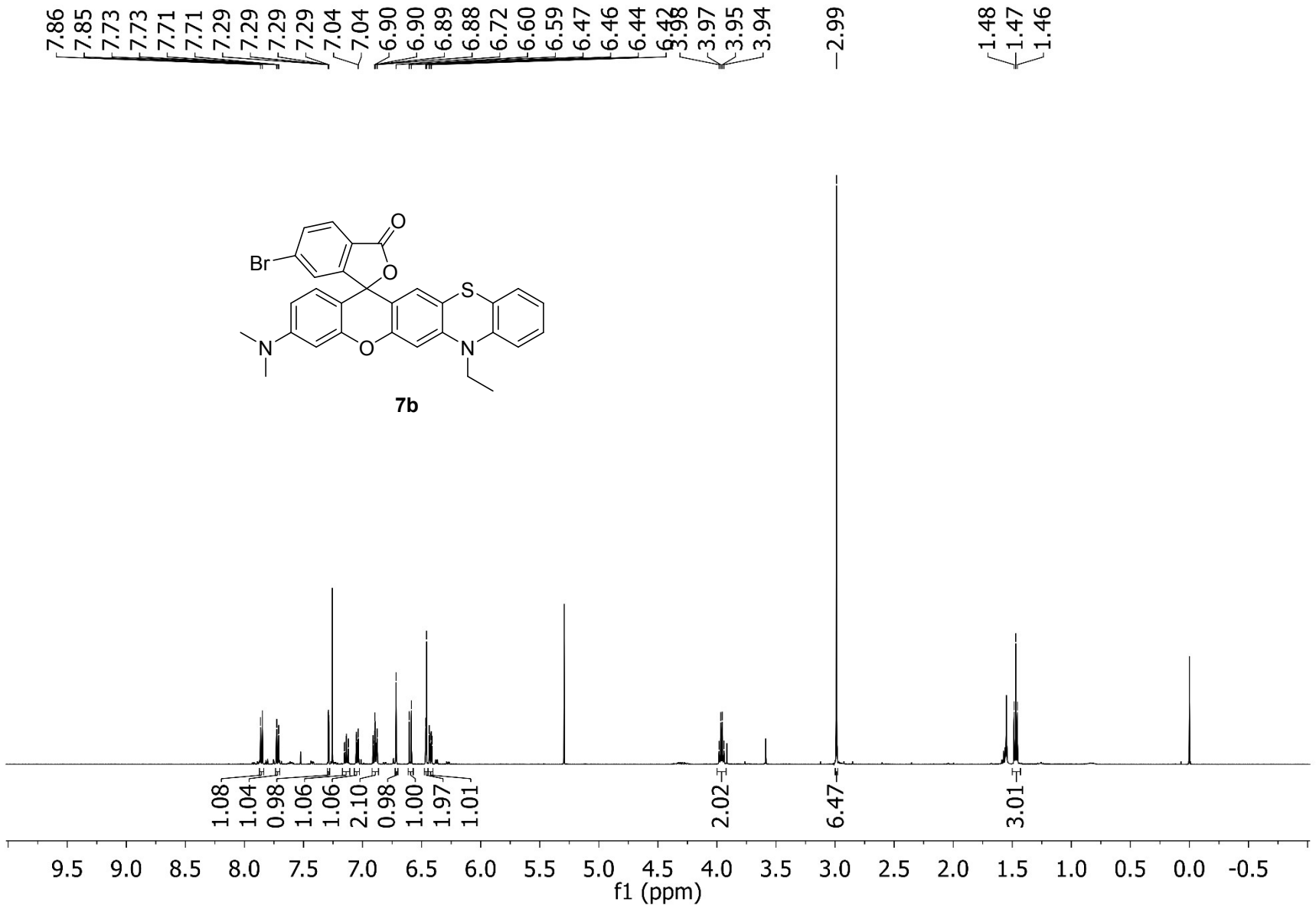

7.20
7.19
7.18
7.17
7.16
7.13
7.13
7.11
7.11
7.03
7.01
7.01
6.99
6.94
6.94
6.92
6.91
6.91
6.56
6.54
6.54
3.93
3.91
3.90
3.88
3.74
1.30
1.29
1.28
S
O
N
6
1.00
0.98
2.01
1.04
2.03
2.03
3.02
3.02
10.5
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

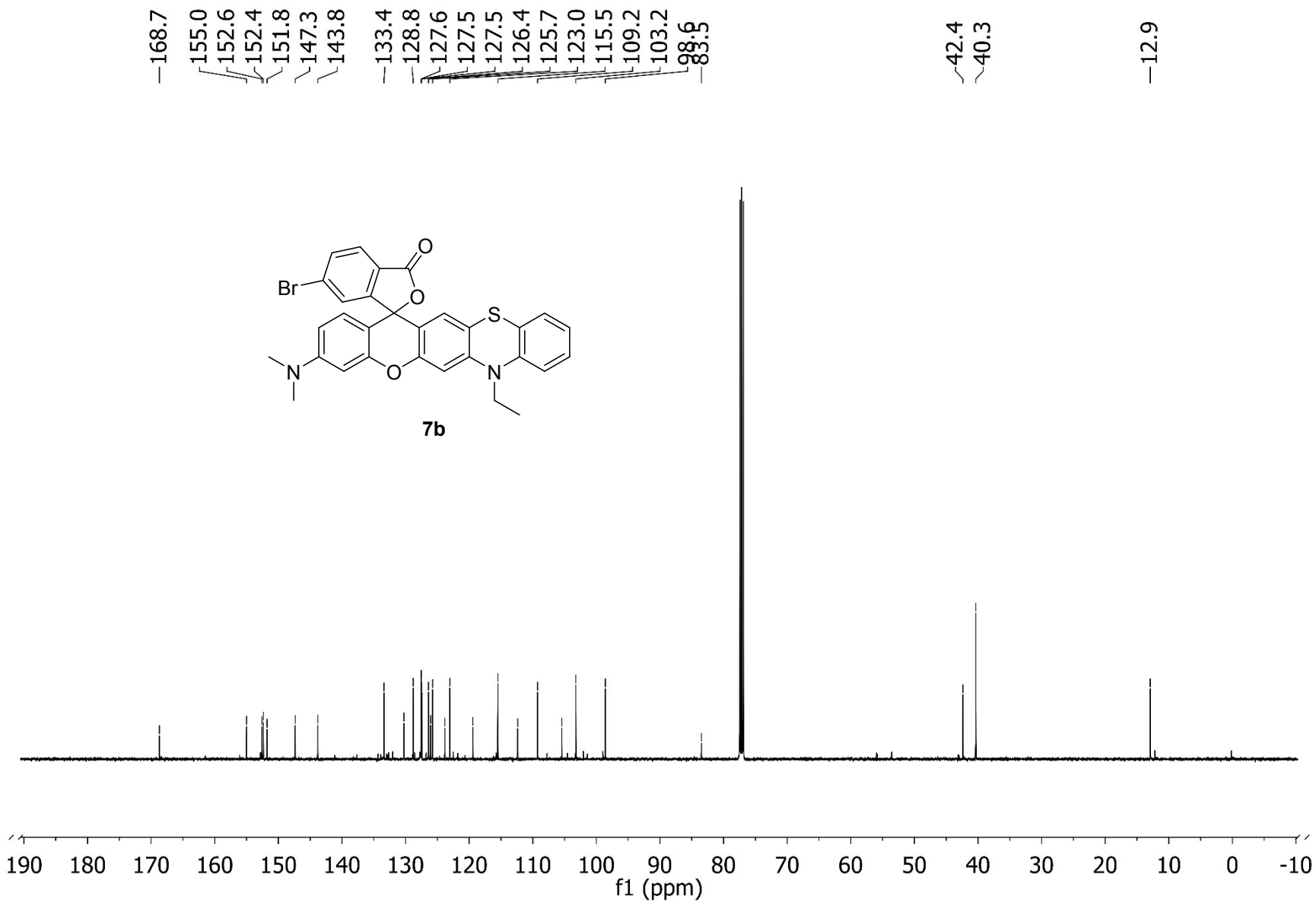

159.5
145.8
144.2
127.3
127.3
126.8
123.7
122.3
115.5
113.8
107.2
102.7
55.2
41.0
12.6
S
O
N
6
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

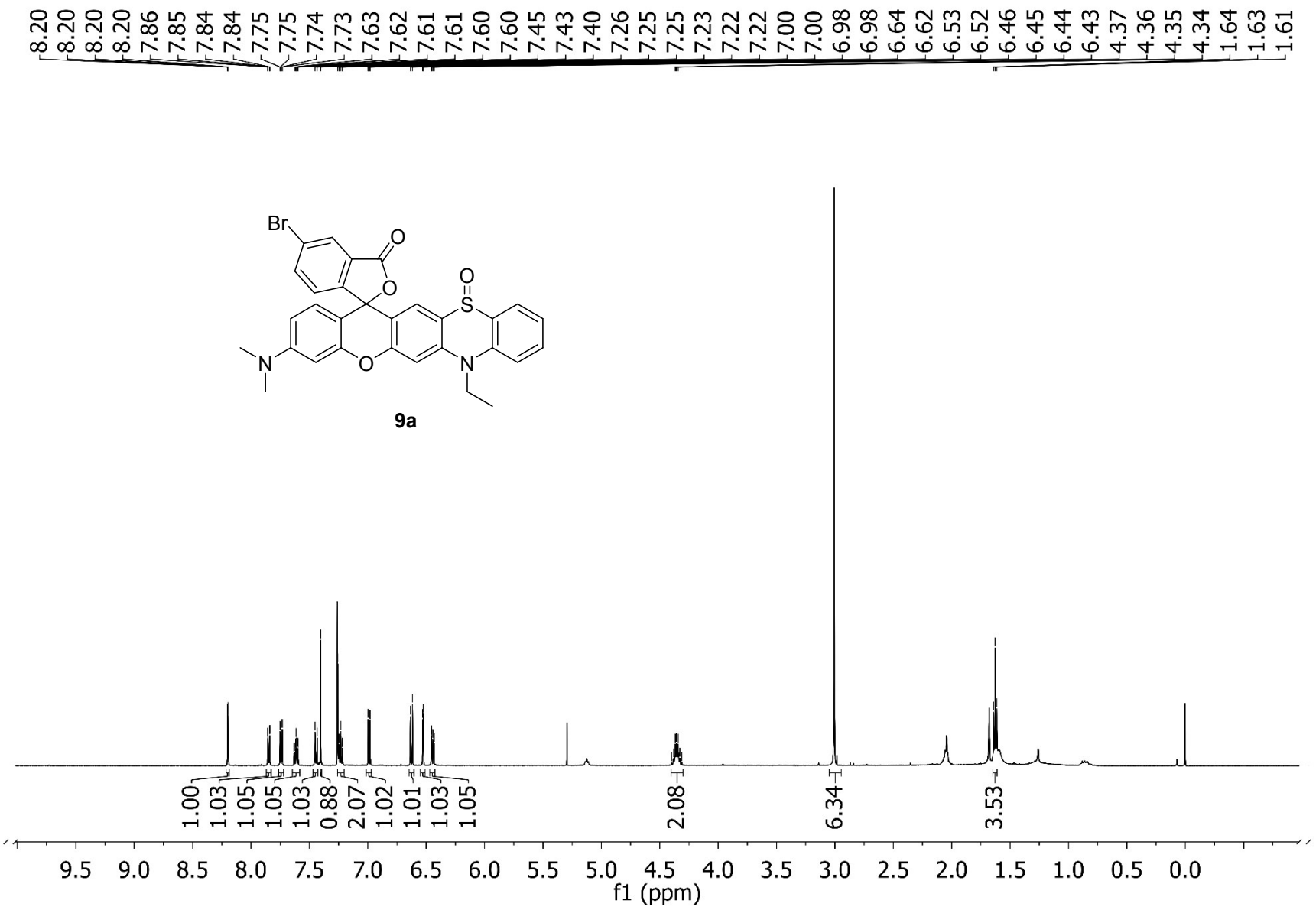

8.14
8.14
7.76
7.76
7.74
7.74
7.14
7.04
7.02
6.90
6.89
6.87
6.72
6.59
6.57
6.47
6.46
6.44
3.98
3.96
3.95
3.94
2.98
1.48
1.47
1.45
Br
O
O
S
N
O
N
1.00
1.22
1.26
2.36
2.63
1.18
1.12
2.32
1.24
2.39
7.15
3.47
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

7a

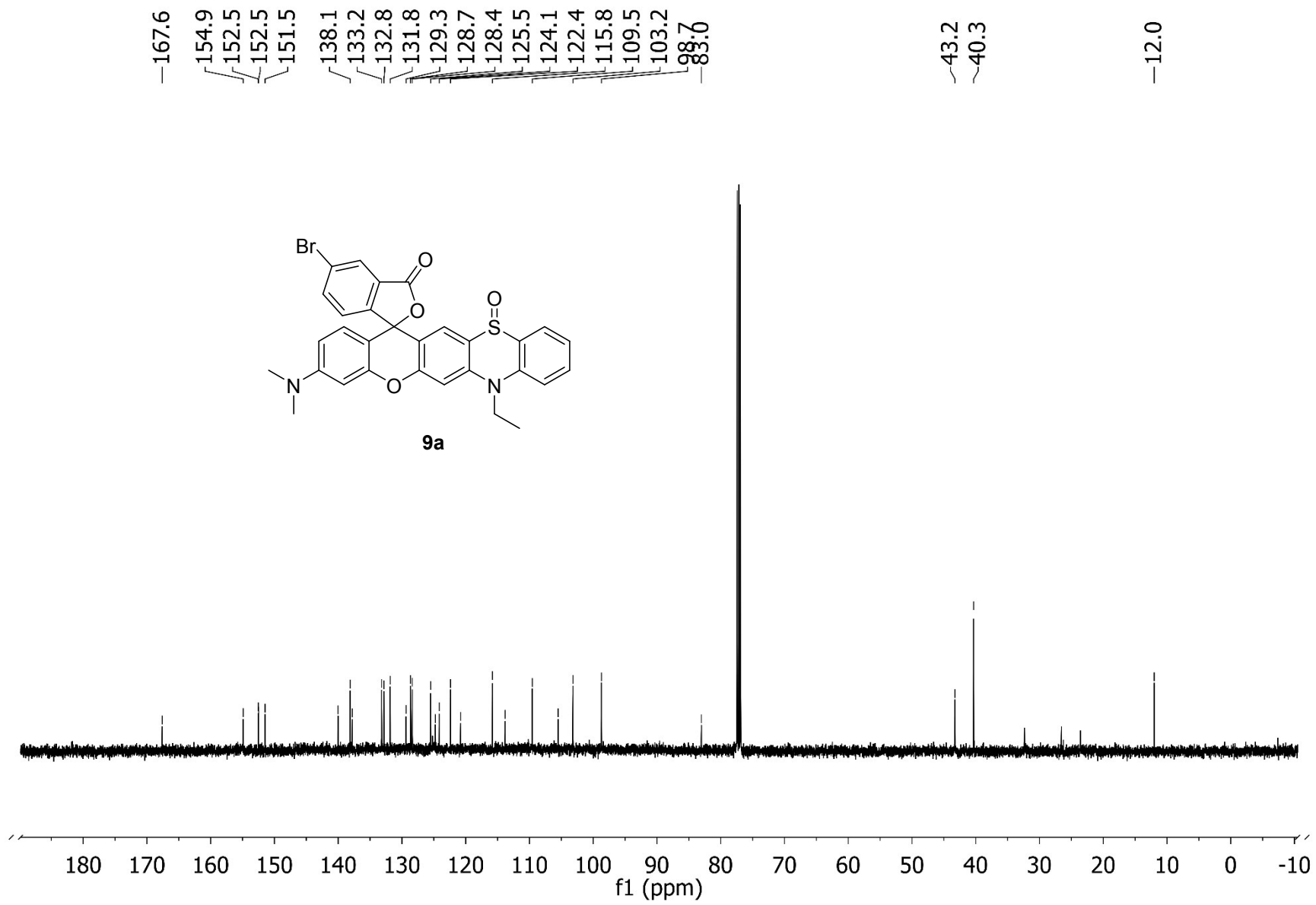

168.0
152.7
152.4
151.9
151.7
147.3
143.8
138.0
128.8
128.1
127.5
127.5
125.7
125.7
123.8
123.0
115.4
103.2
98.6
84.3
42.4
40.3
12.9
Br
O
O
S
N
O
N
7a
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

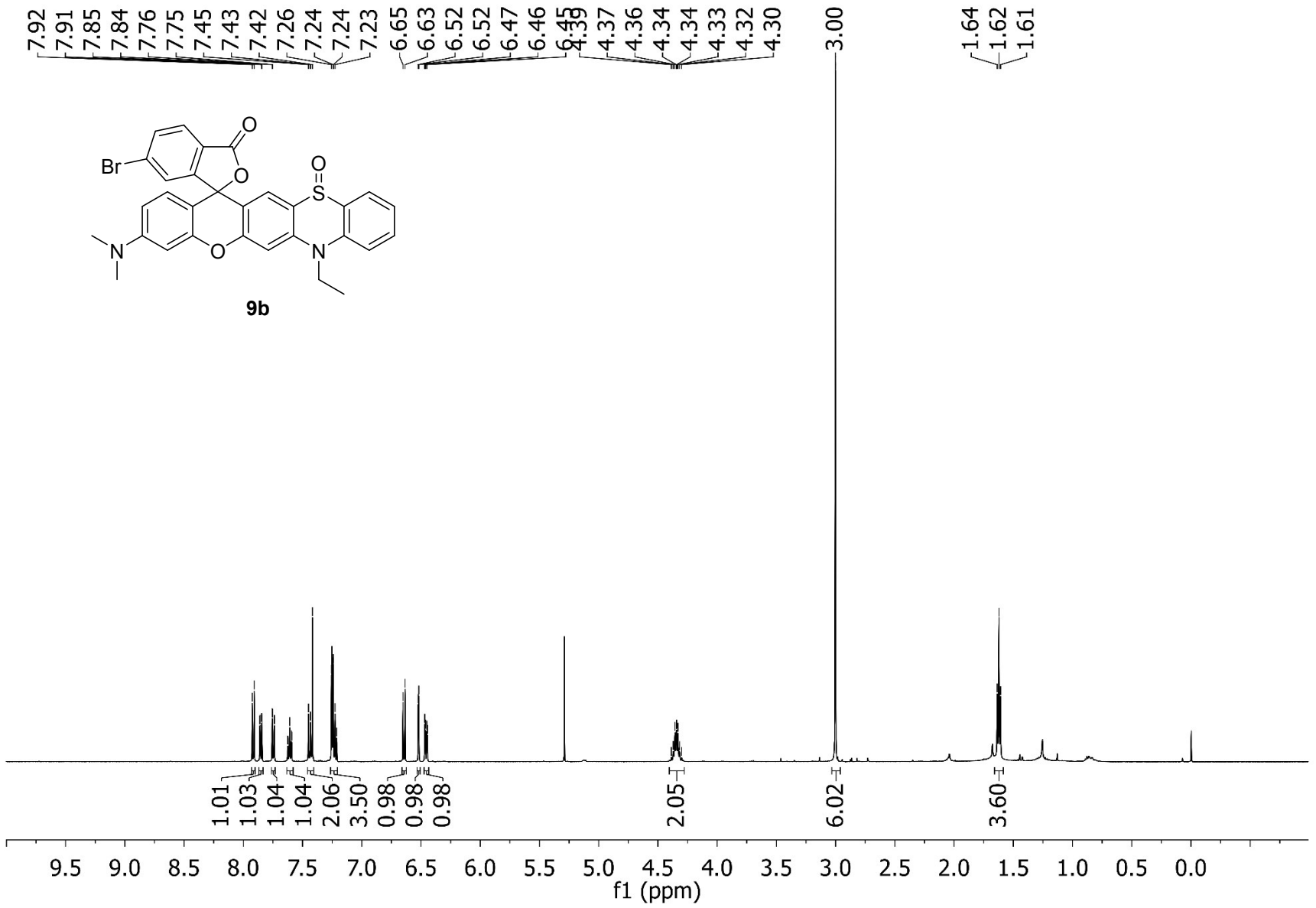

7.86
7.85
7.73
7.73
7.71
7.71
7.29
7.29
7.29
7.29
7.04
7.04
6.90
6.90
6.89
6.88
6.72
6.60
6.59
6.47
6.46
6.44
6.42
3.98
3.97
3.95
3.94
2.99
1.48
1.47
1.46
Br
O
O
S
N
O
N
7b
1.08
1.04
0.98
1.06
1.06
2.10
0.98
1.00
1.97
1.01
2.02
6.47
3.01
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)


168.7
155.0
152.6
152.4
151.8
147.3
143.8
133.4
128.8
127.6
127.5
127.5
126.4
125.7
123.0
115.5
109.2
103.2
98.6
83.5
42.4
40.3
12.9
Br
O
O
S
N
O
N
7b
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

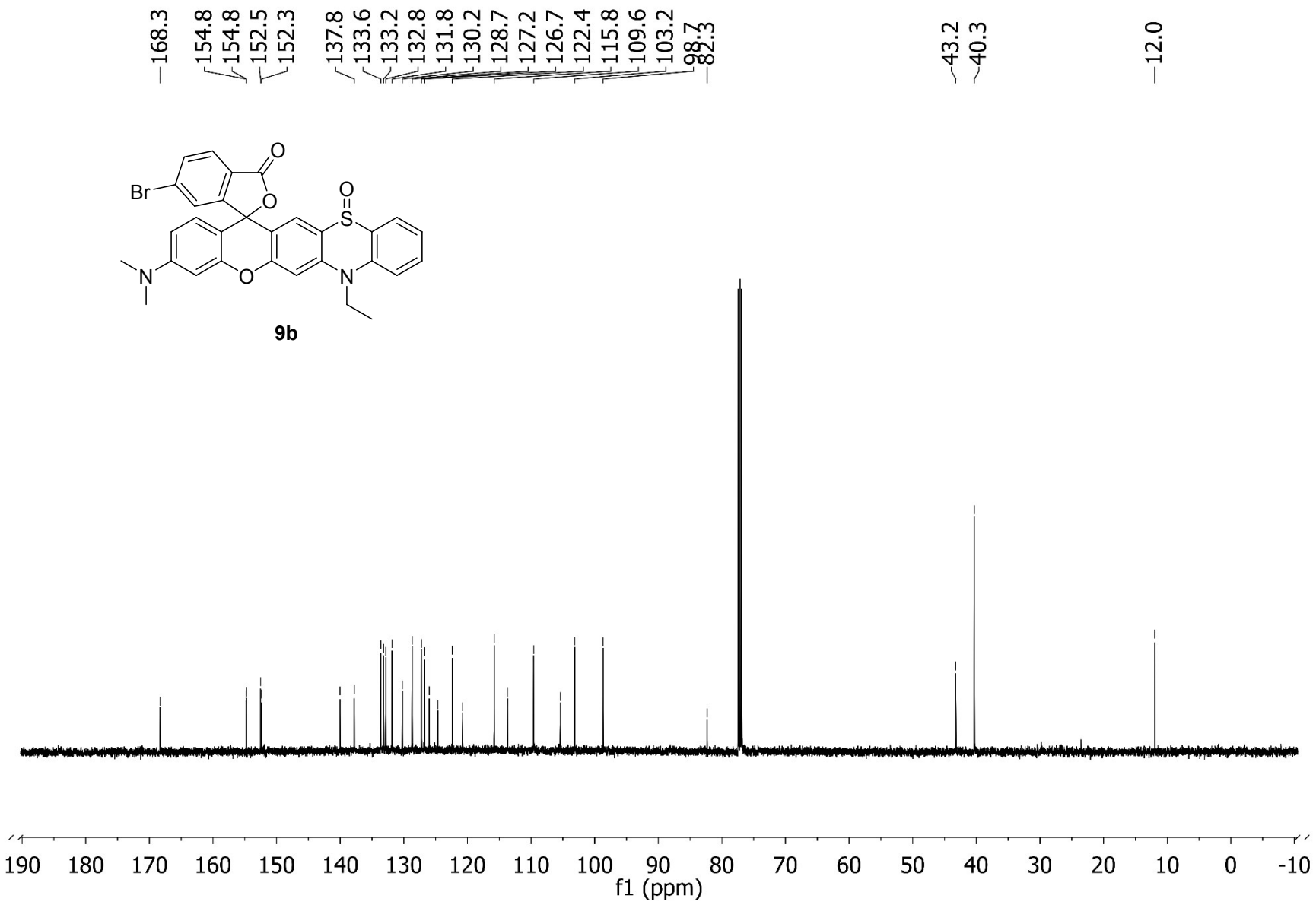

8.20 8.20 8.20 8.20 7.86 7.85 7.84 7.84 7.75 7.75 7.74 7.73 7.63 7.62 7.61 7.61 7.60 7.60 7.45 7.43 7.40 7.26 7.25 7.25 7.23 7.22 7.22 7.00 7.00 6.98 6.98 6.64 6.62 6.53 6.52 6.46 6.45 6.44 6.43 4.37 4.36 4.35 4.34 1.64 1.63 1.61
Br
O
O
S
O
N
O
N
1.00 1.03 1.05 1.05 1.03 0.88 2.07 1.02 1.01 1.03 1.05 2.08 6.34 3.53
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

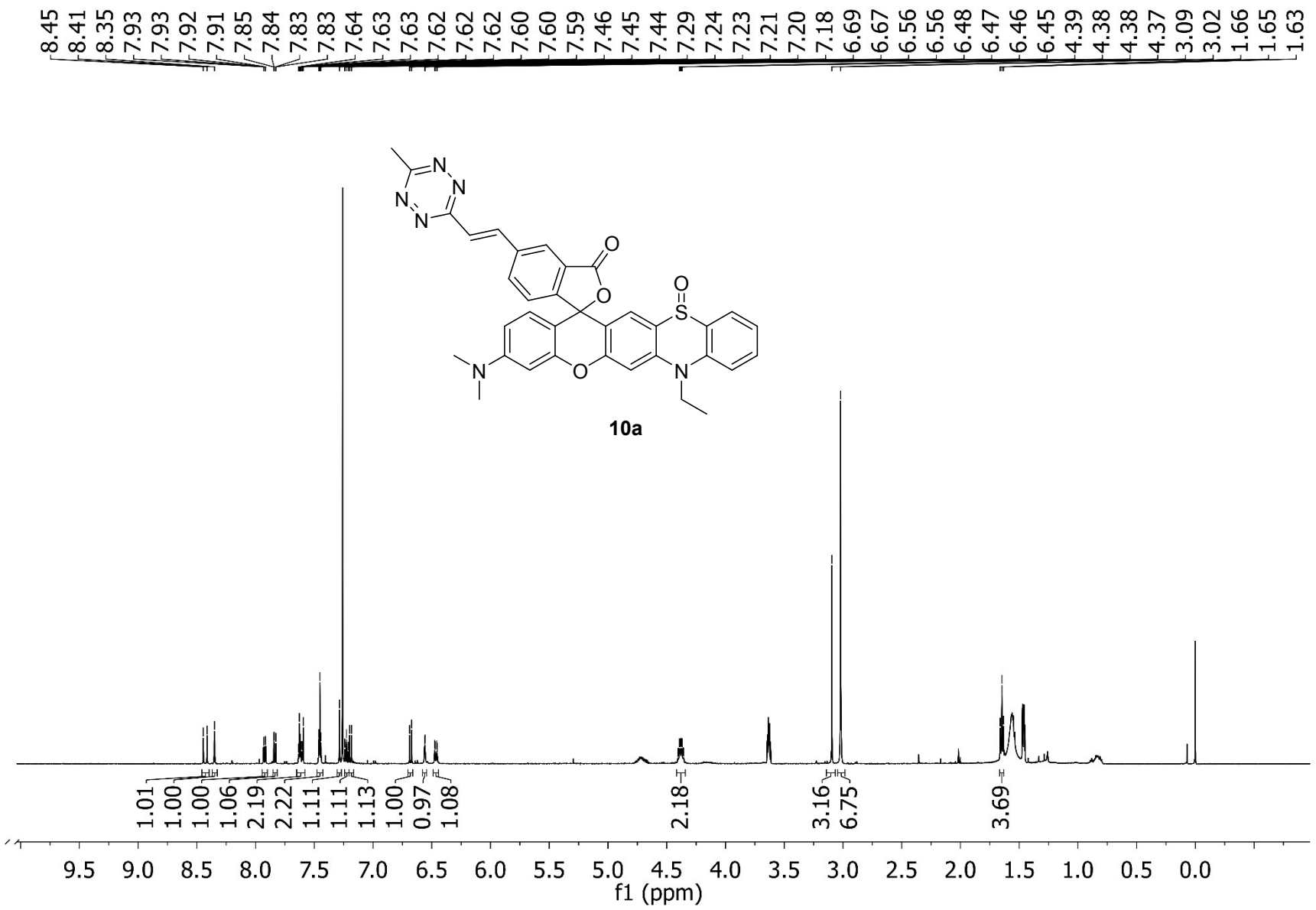
9a

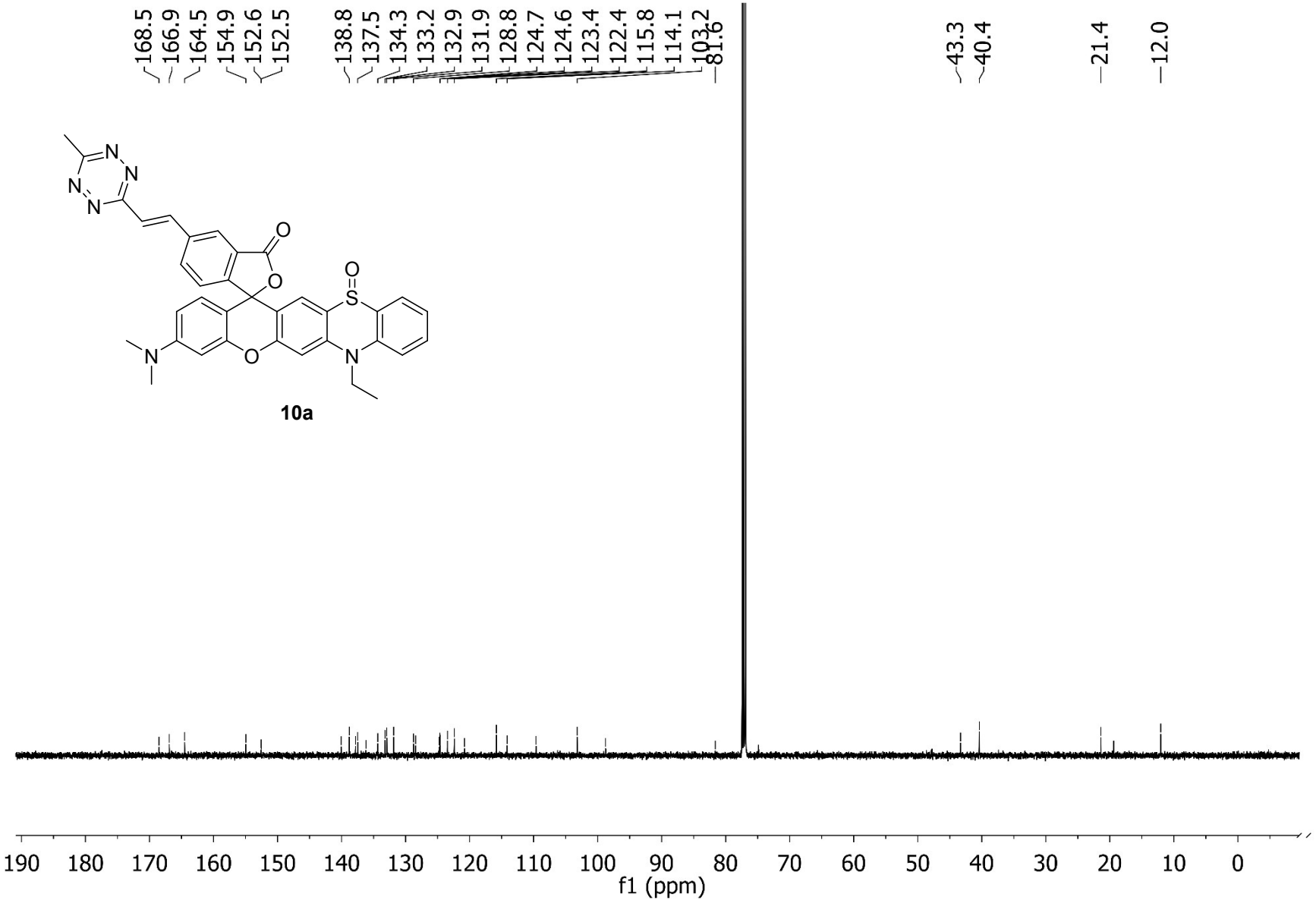

167.6
154.9
152.5
152.5
151.5
138.1
133.2
132.8
131.8
129.3
128.7
128.4
125.5
124.1
122.4
115.8
109.5
103.2
98.7
83.0
43.2
40.3
12.0
Br
O
O
S
O
N
O
N
9a
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

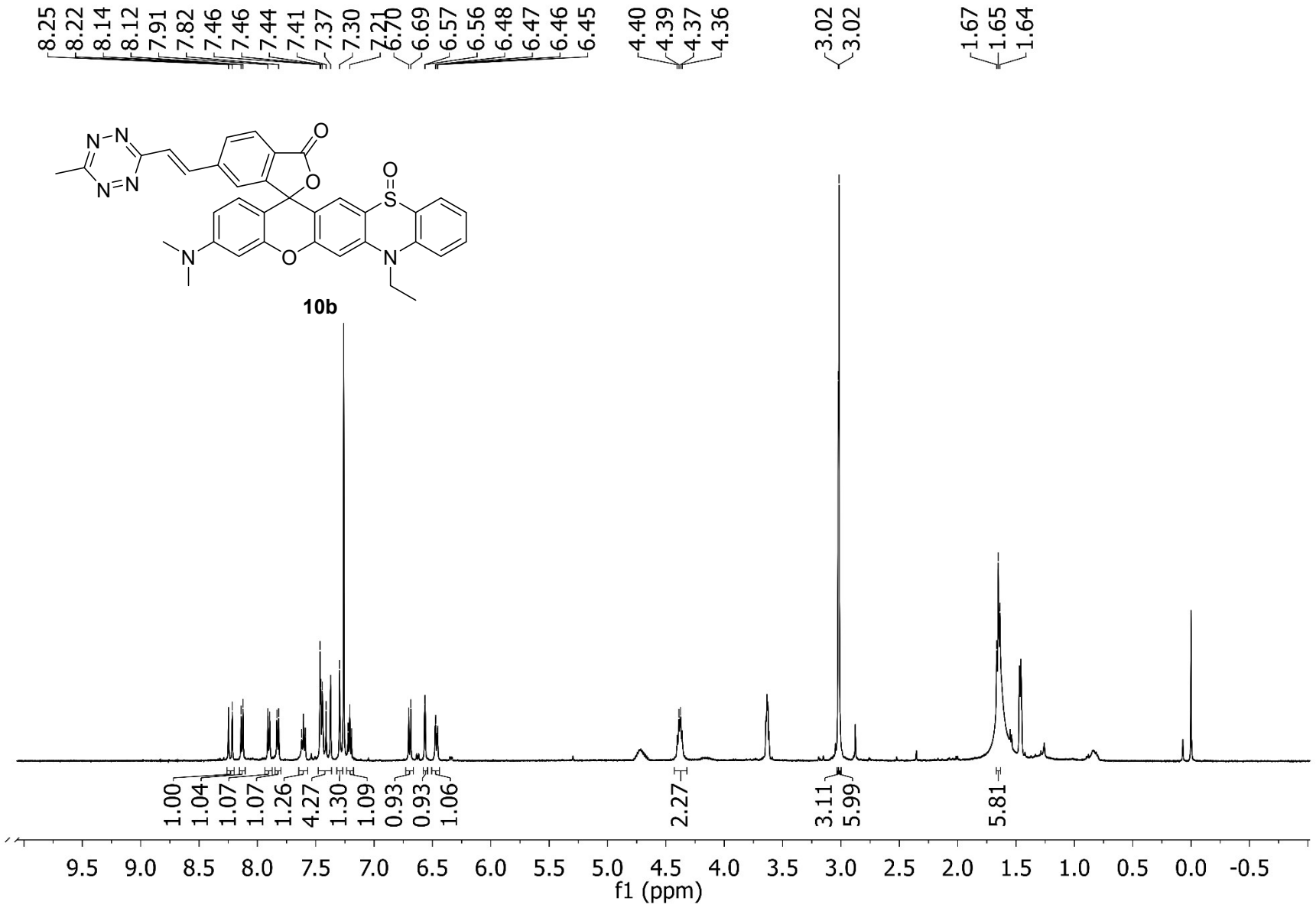

7.92
7.91
7.85
7.84
7.76
7.75
7.45
7.43
7.42
7.26
7.24
7.24
7.23
6.65
6.63
6.52
6.52
6.47
6.46
6.45
4.39
4.37
4.36
4.34
4.34
4.33
4.32
4.30
3.00
1.64
1.62
1.61
Br
O
O
O
S
N
O
N
1.01
1.03
1.04
1.04
2.06
3.50
0.98
0.98
0.98
2.05
6.02
3.60
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

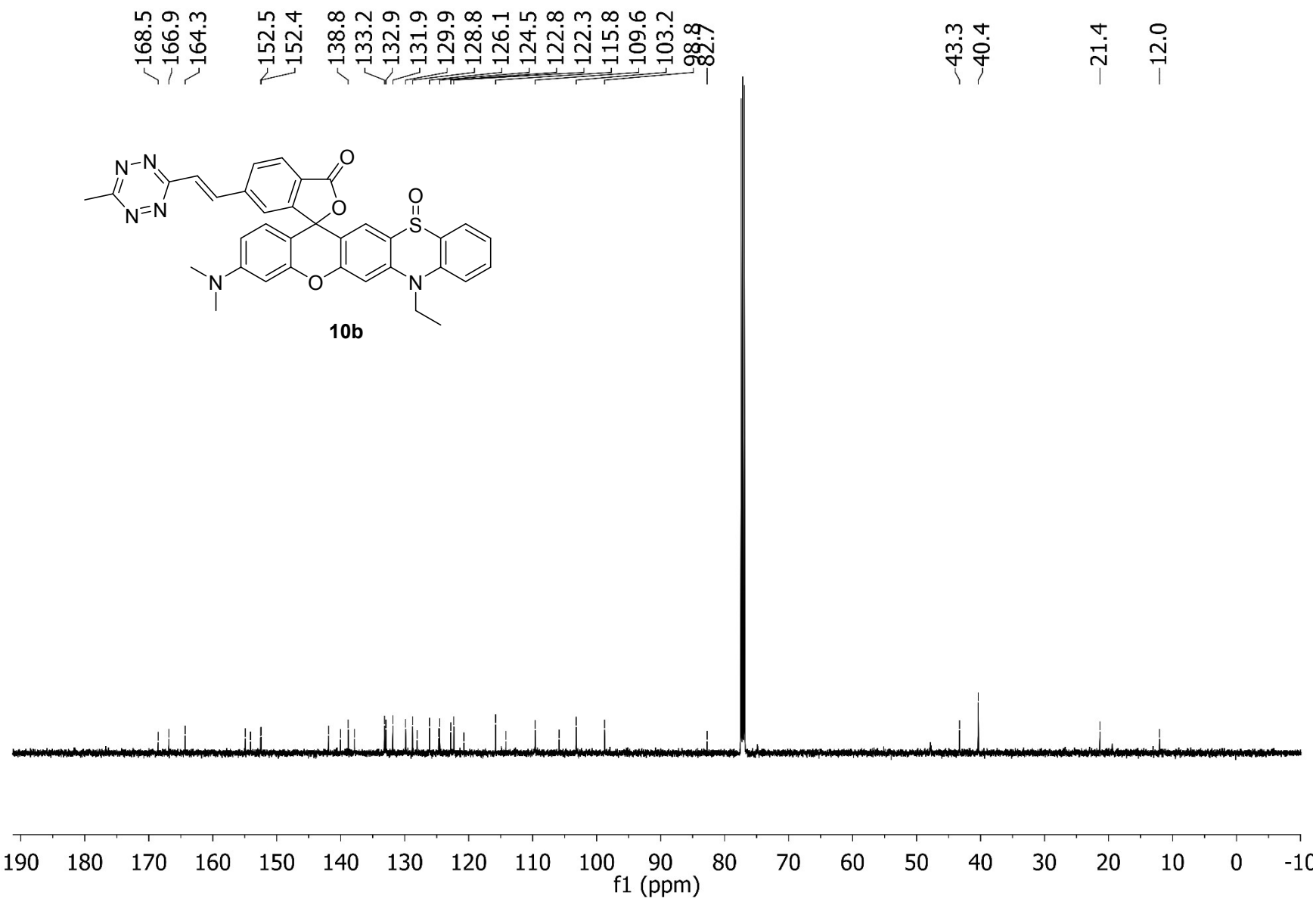
9b


168.3
154.8
154.8
152.5
152.3
137.8
133.6
133.2
132.8
131.8
130.2
128.7
127.2
126.7
122.4
115.8
109.6
103.2
98.7
82.3
43.2
40.3
12.0
Br
O
O
S
O
N
O
N
9b
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)


8.45 8.41 8.35 7.93 7.93 7.92 7.91 7.85 7.84 7.83 7.83 7.64 7.63 7.63 7.62 7.62 7.62 7.60 7.60 7.59 7.46 7.45 7.44 7.29 7.24 7.23 7.21 7.20 7.18 6.69 6.67 6.56 6.56 6.48 6.47 6.46 6.45 4.39 4.38 4.38 4.37 3.09 3.02 1.66 1.65 1.63
10a
1.01 1.00 1.00 1.06 2.19 2.22 1.11 1.11 1.13 1.00 0.97 1.08 2.18 3.16 6.75 3.69
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


168.5
166.9
164.5
154.9
152.6
152.5
138.8
137.5
134.3
133.2
132.9
131.9
128.8
124.7
124.6
123.4
122.4
115.8
114.1
103.2
81.6
43.3
40.4
21.4
12.0
10a
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


8.25
8.22
8.14
8.12
7.91
7.82
7.46
7.46
7.44
7.41
7.37
7.30
7.21
6.70
6.69
6.57
6.56
6.48
6.47
6.46
6.45
4.40
4.39
4.37
4.36
3.02
3.02
1.67
1.65
1.64
10b
1.00
1.04
1.07
1.07
1.26
4.27
1.30
1.09
0.93
0.93
1.06
2.27
3.11
5.99
5.81
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)


168.5
166.9
164.3
152.5
152.4
138.8
133.2
132.9
131.9
129.9
128.8
126.1
124.5
122.8
122.3
115.8
109.6
103.2
98.8
82.7
43.3
40.4
21.4
12.0
10b
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)