Supporting Information

"Visualization of Multiple Localizations of GLUT4 by Fluorescent Probes of PYP-tag with Designed Unnatural Warhead"

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1. Experimental Procedures

Materials and instruments

General chemicals were of the best grade available and supplied by Tokyo Chemical Industries, FUJIFILM Wako Pure Chemical Corporation, Sigma-Aldrich Co. and Kishida Chemical Co., and used without further purification. Enzymes for molecular biological manipulations were purchased from Takara Bio and New England Biolabs. pQCXIN was purchased from Clontech. Synthetic oligonucleotides were purchased from Invitrogen. NMR spectra (Bruker Ascend 500 with 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR) were recorded by using tetramethylsilane as an internal standard.

High-resolution mass spectra (HRMS) were recorded using JEOL JMS-700. Normal-phase column chromatography was conducted using silica gel, BW-300 (Fuji Silysia Chemical Ltd.). Reversed-phase high-performance liquid chromatography (RP-HPLC) purification was conducted using an Inertsil ODS-3 column (4.6 or 10.0 mm × 250 mm, GL-Science, Inc.). Gel-filtration chromatography was performed using a Superdex 75 10/300 GL column (GE Healthcare Life Sciences) connected to an ÄKTA explorer system (GE Healthcare Life Sciences) or an NGC Chromatography System (Bio-Rad). Spectroscopic experiments were carried out using a fluorometer (Hitachi F7000 spectrometer) with a photomultiplier voltage of 700V.), and an absorption spectrophotometer (JASCO, V-650). Fluorescent bands in gels were detected using a variable mode laser scanner, Typhoon FLA 9500 (GE Healthcare Life Science). Fluorescence live-cell images were acquired using a confocal laser scanning microscope (Olympus FLUOVIEW FV10i) or a fluorescence microscope (KEYENCE, BZ-X700) with a 60× lens.

Syntheses of compounds

1-chloro-*N*-(4-methoxyphenyl)methanesulfonamide (1)

4-Methoxyaniline (100.78 mg, 0.82 mmol), chloromethanesulfonyl chloride (90 µl, 0.99 mmol) and triethyamine (TEA, 230 µl, 1.65 mmol) were dissolved in dichloromethane (DCM, 6.0 ml) on ice and stirred at room temperature (r.t.) under N_2 for 6 h. DCM was added to the mixture and washed sequentially with 5% (v v^{-1}) HCl aq., water, $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. The dried residue was collected by dissolving it in diethyl ether and dried *in vacuo* to give **1** (92.6 mg, 0.39 mmol, 48%). ^{1}H NMR (500 MHz, acetone): δ 7.52 (d, 2H, J = 9.0 Hz), 7.03 (d, 2H, J = 9.0 Hz), 5.39 (s, 2H), 3.87 (s, 3H). ^{13}C NMR (125 MHz, acetone): δ 162.14, 133.70, 126.15, 115.06, 58.52, 55.75. HRMS (FAB–) Calcd. for $[M–H]^-$ 234.0070, found 233.9995.

1-chloro-*N*-(4-fluorophenyl)methanesulfonamide (2)

4-Fluoroaniline (90 µl, 0.94 mmol), chloromethanesulfonyl chloride (100 µl, 1.10 mmol) and TEA (250 µl, 1.79 mmol) were dissolved in DCM (6.0 ml) on ice and stirred at r.t. under N_2 for 6 h. DCM was added to the mixture and washed sequentially with 5% (v v^{-1}) HCl aq., water, $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. The dried residue was collected by

dissolving it in diethyl ether and dried *in vacuo* to give **2** (201 mg, 0.90 mmol, 96%). ^{1}H NMR (500 MHz, acetone): δ 7.43 (m, 2H), 7.17 (m, 2H), 4.81 (s, 2H). ^{13}C NMR (125 MHz, acetone): δ 160.99, 134.25, 124.95, 116.53, 54.13. HRMS (FAB–) Calcd. for $[M–H]^-$ 221.9870, found 221.9792.

4-fluorophenyl chloromethanesulfonate (3)

4-Fluorophenol (100.27 mg, 0.89 mmol), chloromethanesulfonyl chloride (98 µl, 1.08 mmol) and TEA (250 µl, 1.79 mmol) were dissolved in DCM (6.0 ml) on ice and stirred at r.t. under N_2 overnight. DCM was added to the mixture and washed sequentially with 5% HCl aq., water, $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. The dried residue was purified by silica gel column chromatography (hexane/ethyl acetate = 85/15) to give **3** (114.3 mg, 0.51 mmol, 57%). ^{1}H NMR (500 MHz, acetone): δ 7.45 (d, 2H), 7.30 (d, 2H), 5.30 (s, 2H). ^{13}C NMR (125 MHz, acetone): δ 161.86, 146.04, 124.64, 117.35, 53.33. HRMS (FAB–) Calcd. for $[M–H]^-$ 222.9710, found 222.9635.

2-chloro-*N*-(4-fluorophenyl)acetamide (4)

4-Fluoroaniline (86 µl, 0.91 mmol), chloroacethyl chloride (80 µl, 1.01 mmol) and TEA (150 µl, 1.08 mmol) were dissolved in dimethylformamide (DMF 3.0 ml) on ice and stirred at r.t. under N_2 for 3h. After removing the solvent, DCM was added to the mixture and washed sequentially with 5% (v v^{-1}) HCl aq., water, $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. The dried residue was purified by silica gel column chromatography (hexane/ethyl acetate = 90/10) to give **4** (132.1 mg, 0.70 mmol, 77%). ^{1}H NMR (500 MHz, acetone): δ 9.47 (br. s, 1H), 7.70 (m, 2H), 7.11 (m, 2H), 4.23 (s, 2H). ^{13}C NMR (125 MHz, acetone): δ 165.00, 159.54, 135.41, 122.09, 115.81, 43.71. HRMS (FAB–) Calcd. for $[M–H]^-$ 186.0200, found 186.0124.

2-chloro-*N*-(4-(trifluoromethyl)phenyl)acetamide (5)

4-Aminobenzotrifluoride (80 µl, 0.65 mmol), chloroacethyl chloride (60 µl, 0.75 mmol) and TEA (174 µl, 1.08 mmol) were dissolved in DCM (6.0 ml) on ice and stirred at r.t. under N_2 overnight. DCM was added to the mixture and washed sequentially with 5% (v v^{-1}) HCl aq. and water and $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. After adding hexane, the precipitate was collected by washing with hexane and dried *in vacuo* to give **5** (141.6 mg, 0.60 mmol, 92%). ^{1}H NMR (500 MHz, acetone): δ 9.77 (br. s, 1H),

7.90 (d, 2H, *J* = 8.5 Hz), 7.69(d, 2H, *J* = 8.5 Hz), 4.29 (s, 2H). ^{13}C NMR (125 MHz, acetone): δ 165.61, 142.65, 126.62, 125.76, 123.94, 120.05, 43.75. HRMS (FAB–) Calcd. for $[M–H]^-$ 236.0168, found 236.0094.

2-chloro-*N*-(4-cyanophenyl)acetamide (8)

4-Aminobenzonitrile (100.52 mg, 0.85 mmol), chloroacethyl chloride (80 µl, 1.01 mmol) and TEA (240 µl, 1.72 mmol) were dissolved in DCM (6.0 ml) on ice and stirred at r.t. under N_2 overnight. DCM was added to the mixture and washed sequentially with 5% (v v^{-1}) HCl aq., water, $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated to give **8** (93.4 mg, 0.48 mmol, 56%). ^{1}H NMR (500 MHz, acetone): δ 9.83 (br. s, 1H), 7.89 (d, 2H, *J* = 8.5 Hz), 7.75 (d, 2H, *J* = 8.5 Hz), 4.29 (s, 2H). ^{13}C NMR (125 MHz, acetone): δ 165.77, 143.20, 133.72, 120.22, 119.01, 107.49, 43.77. HRMS (FAB–) Calcd. for $[M–H]^-$ 193.0247, found 193.0166.

2-chloro-*N*-(4-chlorophenyl)acetamide (9)

4-Chloroaniline (99.68 mg, 0.78 mmol) and chloroacethyl chloride (75 µl, 0.94 mmol) and TEA (220 µl, 1.58 mmol) were dissolved in DCM (6.0 ml) on the ice and stirred at r.t. under N_2 for 7h. DCM was added to the mixture and washed sequentially with 5% (v v^{-1}) HCl aq., water, $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. The dried residue was purified by silica gel column chromatography (hexane/ethyl acetate = 75/25) to give **9** (81.4 mg, 0.40 mmol, 51%). ^{1}H NMR (500 MHz, acetone): δ 9.56 (br. s, 1H), 7.70 (d, 2H, *J* = 9.0 Hz), 7.36 (d, 2H, *J* = 9.0 Hz), 4.24 (s, 2H). ^{13}C NMR (125 MHz, acetone): δ 165.18, 138.09, 129.28, 121.68, 43.74. HRMS (FAB–) Calcd. for $[M–H]^-$ 201.9905, found 201.9828.

***N*-(5-bromopyridin-2-yl)-2-chloroacetamide (10)**

2-Amino-5-bromopyridine (100.40 mg, 0.58 mmol), chloroacethyl chloride (56 µl, 0.70 mmol) and TEA (160 µl, 1.15 mmol) were dissolved in DCM (6.0 ml) on ice and stirred at r.t. under N_2 for 7 h. DCM was added to the mixture and washed sequentially with 5% (v v^{-1}) HCl aq., water, $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. The dried residue was purified by silica gel column chromatography (DCM/methanol = 98/2) to give **10** (57.3 mg, 0.23 mmol, 40%). ^{1}H NMR (500 MHz, acetone): δ 9.80 (br. s, 1H), 8.39 (dd, 1H, *J* = 2.5 Hz, *J* = 0.5 Hz), 8.16 (d, 1H, *J* = 9.0 Hz), 8.00 (dd, 1H, *J* = 9.0 Hz,

J = 2.5 Hz), 4.38 (s, 2H). ^{13}C NMR (125 MHz, acetone): δ 165.82, 151.13, 149.45, 141.30, 115.60, 114.87, 43.81. HRMS (FAB–) Calcd. for $[M–H]^-$ 248.9352, found 248.9429.

2-chloro-*N*-(5-cyanopyridin-2-yl)acetamide (11)

2-Amino-5-cyanopyridine (100.88 mg, 0.85 mmol), chloroacethyl chloride (80 µl, 1.01 mmol) and TEA (235 µl, 1.69 mmol) were dissolved in DCM (6.0 ml) on ice and stirred at r.t. under N_2 overnight. DCM was added to the mixture and washed sequentially with 5% (v v^{-1}) HCl aq., water, $NaHCO_3$ aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. The dried residue was purified by silica gel column chromatography (hexane/ethyl acetate = 80/20) to give **11** (38.5 mg, 0.20 mmol, 24%). ^{1}H NMR (500 MHz, acetone): δ 10.15 (br. s, 1H), 8.70 (dd, 1H, *J* = 2.0 Hz, *J* = 1.0 Hz), 8.35 (dd, 1H, *J* = 8.5 Hz, *J* = 1.0 Hz), 8.22 (dd, 1H, *J* = 8.5 Hz, *J* = 2.0 Hz), 4.44 (s, 2H). ^{13}C NMR (125 MHz, acetone): δ 166.41, 154.71, 152.53, 142.54, 117.13, 113.76, 105.65, 43.87. HRMS (FAB+) Calcd. for $[M–H]^+$ 196.0199, found 196.0276.

2-bromo-N-(4-hydroxyphenyl)acetamide (12)

4-nitrophenol (1.39 g, 10.02 mmol) and NaH (603.2 mg, 15.08 mmol) were dissolved in tetrahydrofuran (THF, 40 mL) and stirred at 0 ºC for 1 h. Chloromethyl methyl ether (1.2 ml, 15.80 mmol) was added and stirred at room temperature for 3 h. After quenching by water and removing the solvent, diethyl ether was added to the mixture and washed with water and brine. Then the organic layer was dried over with Na_2SO_4 and evaporated. The mixture (1.74 g, 9.50 mmol) and Pd/C (174 mg) were dissolved in methanol (50 ml) and stirred at room temperature under H_2 for 6h. After filtration by celite agent, the solvent was removed. The mixture (1.41 g, 9.20 mmol) and bromoacethyl bromide (880 µl, 10.16 mmol) and TEA (1.5 ml, 10.76 mmol) were dissolved in DMF (24 ml) on the ice and stirred at room temperature under N_2 for 5.5 h. After removing the solvent, DCM was added to the mixture and washed with 5% HCl aq. and water and $NaHCO_3$ aq. and brine. Then the organic layer was dried over with Na_2SO_4 and evaporated. The mixture (1.13 g, 4.12 mmol) and TFA (2.0 ml) were dissolved in DCM (9 ml) and stirred at room temperature for 4 h. After removing the solvent, the residue was purified by silica gel column chromatography (hexane/ethyl acetate = 65/35) to give compound 4 (219.3 mg, 0.95 mmol, 4 steps, 10%).^{1}H NMR (500 MHz, Acetone-d_6) δ 9.33 (s, 1H, a), 8.30 (s, 1H, d),

7.47 (d, 2H, J_{bc} = 9.0 Hz, b), 6.79 (d, 2H, J_{cb} = 9.0 Hz, c), 3.99 (s, 2H, e). ^{13}C NMR (125 MHz, acetone) δ 164.47, 154.56, 131.42, 121.65, 115.68, 29.62. HRMS (FAB+) Calcd. for $[M+H]^+$ 229.9738, found 229.9810.

tert-butyl(2-methyl-4-(trifluoromethyl)phenyl)carbamate (13)

2-Methyl-4-(trifluoromethyl)aniline (9.01 g, 51.46 mmol) and di-*tert*-butyl dicarbonate (Boc_2O, 13.47 g, 61.70 mmol) were dissolved in THF (60 ml) and stirred at 90 °C overnight. After removing the solvent, the mixture was dissolved in ethyl acetate (AcOEt) and washed sequentially with 10% (w v^{-1}) citric acid aq. and brine. The organic layer was dried with Na_2SO_4 and evaporated. The dried residue was washed with hexane, collected and dried *in vacuo* to give **13** (8.30 g, 30.14 mmol, 59%). ^{1}H NMR (500 MHz, acetone): δ 8.00 (d, 1H, *J* = 8.5 Hz), 7.86 (br. s, 1H), 7.51 (s, 1H), 7.50 (d, 1H, *J* = 8.5 Hz), 2.38 (s, 3H), 1.50 (s, 9H). ^{13}C NMR (125 MHz, acetone): δ 154.67, 142.69, 130.80, 128.83, 127.52, 126.01, 125.04, 122.95, 81.52, 29.33, 18.86. HRMS (FAB–) Calcd. for $[M–H]^–$ 274.1133, found 274.1052.

tert-butyl(2-(bromomethyl)-4-(trifluoromethyl)phenyl)carbamate (14)

13 (3.0 g, 0.00109 mol), *N*-bromosuccinimide (NBS, 2.1 g, 0.0120 mmol) and 2,2'-azobis(2-methylpropionitrile) (AIBN, 0.54 g, 0.00327 mmol) were dissolved in CCl_4 and refluxed at 100 °C for 2 h. Subsequently, the mixture was evaporated to give brown solid. The solid was dissolved in AcOEt and washed with MilliQ water and brine. The organic layer was dried with Na_2SO_4 and evaporated to give brown solid, which was dissolved in $CHCl_3$ at 60 °C and cooled to 20 °C for recrystallization. Thus, obtained crystals were filtrated and washed with cold $CHCl_3$ and diethyl ether to give **14** (1.80 g, 0.00508 mmol, y. 47%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.11 (d, 1H, *J* = 9.0 Hz), 7.58 (dd, *J* = 9.0, 2.0 Hz, 1H), 7.53 (d, *J* = 1.5 Hz, 1H), 6.86 (s, 1H), 4.49 (s, 2H), 1.56 (s, 9H), ^{13}C NMR (125 MHz, $CDCl_3$): δ 152.28, 127.08, 127.05, 127.02, 126.99, 126.00, 121.61, 81.79, 29.98, 28.27, 25.18. HRMS (FAB–) Calcd. for $[M–H]^–$ 353.0238, found 353.0163.

1,2-bis(2-bromoethoxy)ethane (15)

Triethyllene glycol (6.86 g, 0.0457 mmol) was dissolved in THF (60 ml) on ice. The solution was mixed with triphenyl phosphine (23.99 g, 0.0914 mmol) then slowly added with CBr_4 (30.30 g, 0.0914 mmol). The mixture was stirred at

r.t. for 2 h. The white precipitates were removed and evaporated. The residue was purified by silica gel column chromatography (ethanol: hexane) to give **15** (9.02 g, 0.0329 mmol, y. 55.6%).

2-(2-(2-(2-bromoethoxy)ethoxy)ethyl)isoindoline-1,3-dione (16)

15 (5.0 g,18.1 mmol) was dissolved in THF (50 ml) and added potassium phthalimide (3.4 g, 18.1 mmol) dissolved in DMF (20 ml). The solution was refluxed and stirred at 90 °C under N_2 for 3 h. White solid precipitated from the reaction mixture was filtered, and the cleared filtrate was evaporated. The dried residue was dissolved in AcOEt, washed with $NaHCO_3$ and brine, and dried with Na_2SO_4. It was then purified by silica gel column chromatography to give **16** (2.1 g, 0.006 mmol, 71.3%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.85 (q, *J* = 3.0 Hz, 2H), 7.72 (q, *J* = 3.0 Hz, 2H), 3.90 (t, *J* = 6.0 Hz, 2H), 3.77–3.61 (m, 8H), 3.38 (t, *J* = 6.5 Hz, 2H) ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.29, 133.96, 132.15, 123.27, 71.20, 70.50, 70.13, 68.01, 37.29, 30.27. HRMS (FAB+): Calcd. for $[M+Na]^+$ 341.0263, found 341.0342.

2-(2-(2-(2-mercaptoethoxy)ethoxy) ethyl)isoindoline-1,3-dione (17)

16 (1.0 g,3.0 mmol) and 70% NaSH (257 mg, 3.2 mmol) were dissolved in DMF (7 ml). The solution was added tetrabutylammonium bromide (TBAB, 283 mg, 0.87mmol) and stirred at r.t. under N_2 for 1.5 h. After addition of diethyl ether, the reaction mixture was evaporated to remove DMF. Then the residue was dissolved in methanol, and the product was purified by silica gel column chromatography to give **17** (528 mg, 1.78 mmol, 60%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.85 (q, *J* = 3.0 Hz, 2H), 7.72 (q, *J* = 3.0 Hz, 2H), 3.90 (t, *J* = 6.0 Hz, 2H), 3.77–3.61 (m, 8H), 3.38 (t, *J* = 6.5 Hz, 2H) ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.29, 133.96, 132.15, 123.27, 71.20, 70.50, 70.13, 68.01, 37.29, 30.27. HRMS (FAB+): Calcd. for $[M+H]^+$ 296.0951, found 296.0949.

***tert*-butyl(2-(((2-(2-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)ethoxy)ethyl)thio)methyl)-4-(trifluoromethyl)phenyl)carbamate (18)**

17 (473 mg, 1.601 mmol) was dissolved in THF (5 ml) and added **14** (623 mg, 1.761 mmol). The mixture was stirred at r.t. for a while before TEA (178,26 mg, 1.761 mmol) was added and stirred for 30 min. After removing white precipitate, the filtrate was evaporated to give a yellow oil. The product was purified by silica gel column chromatography to give **18** (540 mg, 0.950 mmol, y.

59%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.03 (d, J = 9.0 Hz, 1H), 7.78 (q, *J* = 3.0 Hz, 2H), 7.67 (q, *J* = 3.0 Hz, 2H), 7.59 (s, 1H), 7.47 (dd, J= 9.0 Hz, 2.0 Hz, 1H), 7.40 (d, J = 2.0 Hz, 1H), 3.90 (t, *J* = 5.5 Hz, 2H), 3.81 (s, 2H), 3.75 (t, *J* = 6.0 Hz, 2H), 3.67–3.59 (m, 6H), 2.49 (t, *J* = 6.0 Hz, 2H), 1.54 (s, 9H) ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.24, 152.75, 140.40, 133.93, 132.04, 127.64, 127.61, 127.57, 126.33, 125.29, 123.18, 121.25, 81.08, 72.01, 70.34, 70.15, 68.03, 37.34, 33.49, 30.25, 28.34. HRMS (FAB+): Calcd. for $[M+Na]^+$ 591.1747, found 591.1762.

tert-butyl(2-(((2-(2-(2-aminoethoxy)ethoxy)ethyl)thio)methyl)-4-(trifluoromethyl)phenyl)carbamate (19)

18 (36 mg, 0.0633 mmol) was dissolved in methanol (500 µl) and stirred at r.t. for a while. Then the solution was added 10% (v v^{-1}) hydrazine monohydrate (3.65 mg, 0.0730 mmol) dissolved in methanol (60 µl) and stirred at r.t. for 150 min. After removing white precipitate, the filtrate was evaporated to give colorless oil. The product was separated by silica gel column chromatography to give **19** as crude.

PCAFgreen (ATTO488): 6-amino-9-(6-((1-(2-((chlorocarbonyl)amino)-5-(trifluoromethyl)phenyl)-12-oxo-5,8-dioxa-2-thia-11-azapentadecan-15-yl)(methyl)carbamoyl)cyclohexa-2,4-dien-1-yl)-4,5-disulfo-6,7,8,8a-tetrahydro-3H-xanthen-3-iminium

19 (15 mg, 0.0342 mmol) and ATTO 488-NHS (25.88 mg, 0.0376 mmol) were dissolved in DMF (1 ml). The solution was added 10% (v v^{-1}) TEA in DMF (57.2 µl) and allowed to stand for 1 min. The solution was evaporated, and the product was purified by silica gel column chromatography (methanol/DCM). The product was dissolved in DCM, added TFA and stirred at r.t. for deprotecting the Boc group. The solution was evaporated, reacted with chloroacetyl chloride (3.37 µl, 0.030 mmol) and purified by HPLC after removal of the solvent to give PCAFgreen (25.9 mg, 0.0256 mmol, y. 75%).^{1}H NMR (500 MHz, DMSO-d_6): δ 9.82 (s, 1H), 8.72 (s, br, 3H), 7.83-7.49 (m, 7H), 7.15 (d, *J* = 9.0 Hz, 2H), 7.04 (d, *J* = 9.0 Hz, 2H), 4.39 (s, 2H), 3.93 (s, 3H), 3.47–3.07 (m, 12H), 2.85 (s, 2H), 2.63 (s, 1H), 2.53 (t, J = 7.0 Hz, 1H), 1.79 (t, J = 7.0 Hz, 1H), ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.86, 167.76, 165.82, 155.93, 154.97, 139.61, 136.57, 132.88, 132,80, 132.56, 130.82, 130.62, 130.26, 129.92. HRMS (FAB+): Calcd. for $[M+H]^+$1030.1423, found 1030.1437.

PCAForange (5-TAMRA):

5-(12-(2-((chlorocarbonyl)amino)-5-(trifluoromethyl)phenyl)-1-hydroxy-5,8-dioxa-11-thia-2-azadodecyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate

19 (22 mg, 0.0456 mmol) and 5-TAMRA-NHS (22 mg, 0.0411 mmol) were dissolved in DMF (1 ml). The solution was added 10% (v v^{-1}) TEA in DMF (70 µl) and allowed to stand for 1min.The solution was evaporated, and the product was purified by silica gel column chromatography (methanol:DCM) The product was dissolved in DCM, added TFA and stirred at r.t. for deprotecting the Boc group. The solution was evaporated, reacted with chloroacetyl chloride (3.37 µl, 0.030 mmol) and purified by HPLC after removal of the solvent to give PCAForange (27.7 mg, 0.526 mmol, y. 71%). ^{1}H NMR (500 MHz, MeOD): δ 8.62 (d, *J* = 1.5 Hz, 1H), 8.10 (dd, *J* = 8.0 Hz, 2.0 Hz, 1H), 7.83 (d, *J* = 8.0 Hz, 1H), 7.69 (d, *J* = 1.5 Hz), 7.54 (dd, *J* = 8.5 Hz, 1.5 Hz, 1H), 7.36 (d, *J* = 7.5 Hz, 1H), 7.18 (d, *J* = 9.5 Hz, 2H), 7.00 (dd, *J* = 9.5 Hz, 2.5 Hz), 6.94 (d, *J* = 2.5 Hz, 2H), 4.28 (s, 2H), 3.93 (s, 2H), 3.72 (t, *J* = 5.0 Hz, 2H), 3.69–3.63 (m, 8H), 3.28 (s, 12H), 2.60 (t, *J* = 6.5 Hz, 2H), ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.33, 166.39, 159.98, 157.64, 157.42, 138.76, 135.99, 135.90, 132.13, 130.92, 129.83, 129.25, 128.93, 128.90, 127.27, 127.24, 125.06, 125.03, 124.36, 124.33, 113.83, 113.36, 95.98, 70.92, 69.96, 69.93, 69.08, 42.48, 39.75, 39.46, 32.01, 30.38. HRMS (FAB+): Calcd. for $[M+H]^+$ 827.2488, found 827.2508.

PCAFred (SiR650):

***N*-(2-(2-(2-((2-(2-chloroacetamido)-5-(trifluoromethyl)benzyl)thio)ethoxy)ethoxy)ethyl)-3,7-bis(dimethylamino)-5,5-dimethyl-3'-oxo-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-6'-carboxamide**

SiR650 (9.22 mg, 0.0271 mmol) was dissolved in DMF (3 ml) and added hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU, 9.54 mg, 0.00254 mmol) and *N*-methylmorpholine (NMM, 9.19 µl, 0.0836 mmol) on ice. Subsequently, **19** (17 mg, 0.0388 mmol) dissolved in dry DMF (12.2 mg) was added, and the mixture was stirred on ice for 90 min. Then the mixture was evaporated to give a crude oil. The crude product was dissolved in DCM, added TFA for deprotecting Boc group and stirred at r.t. Then the mixture was evaporated, reacted with chloroacetyl chloride (3.37 µl, 0.030 mmol) and purified by HPLC after removal of the solvent to give PCAFred. (5.95 mg, 0.0666 mmol, y. 25%) ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.35 (s, 1H), 8.07 (d, *J* = 8.5 Hz 1H), 8.04 (d,

J = 8.0 Hz 1H), 7.90 (d, *J* = 8.0 Hz 1H), 7.75 (s, 1H), 7.55 (d, *J* = 8.5 Hz 1H), 7.48 (s, 1H), 7.42 (d, *J* = 2.5 Hz 1H), 7.07–6.94 (m, 5H) , 4.25 (s, 2H), 3.89 (s, 2H), 3.67–3.60 (m, 10H), 3.12 (s, 12H), 2.60 (t, *J* = 6.5 Hz, 2H), 0.62 (s, 3H), ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.04, 167.69, 166.44, 148.82, 141.51, 141.04, 140.03, 132.45, 130.54, 130.09, 129.25, 128.97, 128.94, 128.68, 128.42, 127.07, 127.04, 126.30, 126.16, 125.17, 124.15, 123.07, 118.74, 72.67, 71.67, 71.64, 71.00, 44.51, 41.62, 35.20, 32.29, 1.45, 1.22. HRMS (FAB+): Calced. for $[M+H]^+$ 915.2597, found 915.2622.

PCAFfar-red:
***N*-(2-(2-(2-((2-(2-chloroacetamido)-5-(trifluoromethyl)benzyl)thio)ethoxy)ethoxy)ethyl)-1',9',11',11'-tetramethyl-3-oxo-2',3',7',8',9',11'-hexahydro-1'H,3H-spiro[isobenzofuran-1,5'-silino[3,2-f:5,6-f']diindole]-6-carboxamide**

SiR700 (15.37 mg, 0.0271 mmol) was dissolved in DMF (4 ml), and added HATU (15.13 mg, 0.00304 mmol) and NMM (14.58 µl, 0.132 mmol) on ice. Subsequently, **19** (19.38 mg, 0.0442 mmol) dissolved in dry DMF (4 ml) was added, and the mixture was stirred on ice for 90 min. Then the mixture was evaporated to give a crude oil. The crude product was dissolved in DCM, added TFA and stirred at r.t. for deprotecting the Boc group. The mixture was evaporated, reacted with chloroacetyl chloride (3.37 µl, 0.030 mmol) and purified by HPLC after removal of the solvent to give PCAFfar-red. (5.40 mg, 0.00589 mmol, y. 22%)^{1}H NMR (500 MHz, $CDCl_3$): δ 9.40 (s, 1H), 8.18 (d, *J* = 8.0 Hz 1H), 8.07 (d, *J* = 8.5 Hz 1H), 7.91 (d, *J* = 7.5 Hz 1H), 7.67 (s, 1H), 7.55 (d, *J* = 8.5 Hz 1H), 7.51(s, 1H), 6.78 (s, 2H), 6.61 (s, 2H) , 4.25 (s, 2H), 3.88(s, 2H), 3.69–3.61 (m, 15H), 3.10 (s, 3H), 2.90 (t, *J* = 8Hz, 4H), 2.62 (t, *J* = 6 Hz, 2H), ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.60, 165.09, 155.65, 138.63, 137.91, 132.91, 132.12, 131.75, 130.41, 128.91, 128.31, 128.25, 127.49, 127.46, 127.22, 127.10, 126.96, 125.52, 125.48, 124.90, 123.78, 122.74, 113.67, 71.14, 70.20, 70.18, 69.47, 54.78, 43.08, 40.08, 34.16, 33.68, 32.96, 30.94, 26.66. HRMS (FAB+): Calced. for $[M+H]^+$ 827.2488, found 827.2508.

Preparation of Plasmid and recombinant protein

pQCXIN-PYP-GLUT4^{wt}

For construction of pQCXIN-PYP-GLUT4^{wt}, the DNA fragment encoding the PYP-GLUT4^{wt} fusion protein was prepared from pcDNA-PYP-GLUT4^{wt} by PCR using the primers 5'-GTTATAGCGGCCGCTCAGCCACCATGCCGTCG-3'

and 3'-TGACTTCGTACGTTACTTATCGTCGTCATCCTTGTAATCC-5'. The PYP-GLUT4^{wt} fragment and pQCXIN empty vector (Clontech) were digested with *Not*I and *Bsi*WI, and ligated to form pQCXIN-PYP-GLUT4^{wt}.

pQCXIN-PYP-GLUT4^{N57Q}

For construction of pQCXIN-PYP-GLUT^{N57Q}, the DNA fragment encoding the PYP-GLUT4^{N57Q} fusion protein was prepared from pcDNA-PYP-GLUT4^{N57Q} by PCR using the primers 5'-GTTATAGCGGCCGCTCAGCCACCATGCCGTCG-3' and 3'-TGACTTCGTACGTTACTTATCGTCGTCATCCTTGTAATCC-5'. The PYP-GLUT4 fragment and pQCXIN empty vector (Clontech) were digested with *Not*I and *Bsi*WI, and ligated to form pQCXIN-PYP-GLUT^{N57Q}.

pET21b-His-HA-PYP

For construction of pET-His-HA-PYP, the DNA fragment encoding the HA-PYP fusion protein was prepared from pcDNA-HA-PYP-NLS by PCR using the primers 5'-GATGTAGGATCCGATGACGATGACAAAATGGGATATCCATATGATGTTCCAG-3' and 3'-CTATTGGCGGCCGCTTAGACGCGCTTGACGAAGAC -5'. pET-His-PYP were digested with *Bam*HI and *Not*I, and ligated to form pET-His-HA- PYP.

pcDNA-HA- PYP

For construction of pcDNA-HA-PYP, the DNA fragment encoding the HA-PYP fusion protein was prepared from pcDNA -HA-PYP-NLS by PCR using primers, 5'-GCTGTA AAGCTTATGGAACACGTAGCCTTC-3' and 3'-CAGCTGGAATTCTCAGACGCGCTTGACGAAGAC-5'. The HA-PYP fragment and pcDNA-HA-PYP-NLS vector (Clontech) were digested with *Nhe*I and *Eco*RI, and ligated to form pcDNA-HA-PYP.

Preparation of recombinant proteins.

E. coli BL21 (DE3) (Novagen) transformed with pET-His-HA-PYP grown in LB to an optical density of 0.6 at 600 nm was induced with 100 µM IPTG at 20 °C overnight. Following cell lysis, supernatant was obtained by centrifugation at 15000 rpm for 20 min and transferred to a column packed with cOmplete His-Tag

purification resin (Roche). The resin was washed with 50 mM sodium phosphate buffer pH 8.0 containing 300 mM NaCl, 5 mM imidazole and 1 mM DTT. Proteins absorbed to the resin were eluted in 50 mM sodium phosphate buffer pH 8.0 containing 300 mM NaCl, 250 mM imidazole and 1mM DTT. The eluted fractions containing the target protein were further purified by gel filtration chromatography (Superdex^{TM} 75 10/300 GL, GE healthcare Life Science) using the assay buffer (20 mM HEPES buffer pH 7.4 containing 150 mM NaCl). Purity and size of the protein were confirmed by SDS–PAGE. The purified protein was flash-frozen in liquid nitrogen and stored in a –80 °C freezer.

Cell culture.

HeLa cells, HEK293T cells were purchased from RIKEN BRC and tested for mycoplasma contamination. HEK293T and HeLa cells were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin), unless otherwise specified.

Preparation of stable cell lines.

Platinum-A Retroviral Packaging Cells (Cell Biolabs, Inc.) were transfected with pQCXIN-PYP^{wt}-GLUT4 and pQCXIN-PYP^{wt}-GLUT-^{N57Q} using PEIMAX (Polysciences, Inc.) according to the manufacturer's protocol and cultured in Opti-MEM at 37 °C for 16 h. After replacing the medium with DMEM containing 10% FBS, the cells were further cultured at 37 °C for 24 h. The culture medium was filtrated using a 0.45 μm filter to obtain a virus solution, which was mixed with polybrene to the final concentration of 8 μg ml^{-1}. HeLa cells were mixed with the virus solution and incubated at 37 °C for 24 h in DMEM containing 10% FBS and 500 μg ml^{-1} geneticin. Further subculturing was conducted to isolate a single clone of the cells.

Comparative labeling efficiency of PYP-tag with PCAForange and TAMRA-DNB in live cells.

HEK293T cells were transfected with pcDNA-HA-PYP using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. After incubation of the cells at 37 °C for 24 h, the cells were washed with PBS three times. The cells were incubated with 5 μM PCAForange or TAMRA-DNB in DMEM for 1 h for labeling. After the incubation, the cells were washed with 1 ml PBS three times.

The washed cells were gathered with cell scraper and lysed in the lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v v^{-1}) NP40) on ice for 15 min. The cell lysate was centrifuged at 15000 rpm for 15 min at 4 °C. The HA-PYP fusion protein was isolated from the supernatant using the HA-tagged Protein Purification Kit (Medical & Biological Laboratories Co., Ltd.) according to the manufacturer's protocol. The sample was heated at 95 °C for 5 min and subsequently analyzed by SDS–PAGE. After the fluorescence image was taken by Typhoon FLA 9500 with excitation at 532 nm, the gel was stained with Coomassie Brilliant Blue.

Fluorescence measurements.

The fluorescence measurements were taken after each probe (5 μM) was incubated with or without PYP-tag (10 μM) in 20 mM HEPES at pH 7.4 containing 150 mM NaCl and 1% (v v^{-1}) DMSO at 37 °C for 1 h. The fluorescence spectra were recorded at the excitation wavelength of 501 nm for PCAFgreen, 554 nm for PCAForange, 652 nm for PCAFred and 698 nm for PCAFfar-red.

Fluorescence quantum yield measurements.

Fluorescence quantum yields of PCAFprobes were measured in 20 mM HEPES at pH 7.4 containing 150 mM NaCl and 1% (v v^{-1}) DMSO at 37 °C. For reference measurements, fluorescein in 0.1 N NaOH (aq.) (Φ_{ref} = 0.92)[3], rhodamine B in ethanol (Φ_{ref} = 0.65)[4] and Cye5 in PBS (Φ_{ref} = 0.27)[5] were used. Fluorescence quantum yields (Φ_F) were determined using the following equation:

$$\Phi_F = \Phi_{ref} (A_{ref} F_s n^2_s / A_s F_{ref} n^2_{ref})$$

where A is the absorbance at the excitation wavelength, F is the relative sectional quadrature of fluorescence intensity, and n is the average refractive index of the solvent used, and the subscripts s and ref stand for the sample and the reference, respectively.

Kinetic analysis of protein labeling reaction.

The second-order reaction rate constants, k_2, of the labeling reactions of PYP-tag with each probe were determined by reacting each probe with an excess amount of PYP-tag and analyzing the product by SDS–PAGE. Fluorescence images of the gels were captured using Typhoon FLA 9500

(Cytiva) with excitation at 473 nm (PCAFgreen), 532 nm (PCAForange) or 635 nm (PCAFred and PCAFfar-red). Quantification was performed by the intensity of fluorescence signal of the bands in the gel. The analyses were carried out by reacting PYP-tag (2 μM for PCAFgreen and 1 μM for PCAForange, PCAFred and PCAFfar-red) against a constant concentration of the probes (PCAFgreen:1 μM, PCAForange, PCAFred, PCAFfar-red: 500 nM). k_2 was obtained by fitting the fluorescence band data to the following equation.

$$\frac{F_t}{F_{\max}} = \frac{e^{\{k_2 t([A]_0 - [B]_0)\}} - 1}{e^{\{k_2 t([A]_0 - [B]_0)\}} - \frac{[B]_0}{[A]_0}}$$

$[A]_0$ = Initial concentration of Probe
$[B]_0$ = Initial concentration of Protein
F_t = Fluorescence intensity of SDS-PAGE gel bands.
F_{max} = Maximum fluorescence intensity of SDS-PAGE gel bands.

Labeling reaction of PYP-tag with Compounds 5-7.

PYP-tag (10 μM) was incubated with each of **5-7** (20 μM) at 37 °C for 10, 20, 30, 40, 50, 60 min in 20 mM HEPES buffer pH 7.4 containing 2% (v v^{-1}) DMSO. TMBDMA (8 μM) was then added to label unreacted PYP-tag and incubated for a further 30 minutes. And maleimide was added to stop the reaction, the reaction mixture was heated at 95 °C for 5 min and subjected to SDS–PAGE. After the fluorescence image was taken by Typhoon FLA 9500 with excitation at 473 nm, the gel was stained with Coomassie Brilliant Blue.
The bound fraction was calculated by comparing the measured signal intensity with the intensity expected when PYP-tag was bound fully by the ligand.

Reactivity of compounds 1–11 with PYP-tag.

PYP-tag (10 μM) and each of **1**–**11** (0, 10, 20, 50, 100 μM) were incubated in 20 mM HEPES buffer pH 7.4 containing 3% (v v^{-1}) DMSO for 60 min at 37 °C. Then, TMBDMA (8 μM) was added and incubated for 30 min, and the reaction was terminated by the addition of maleimide (100 mM). The reaction was then heat denatured and subjected to SDS–PAGE. The fluorescence image of the gel was taken by Typhoon FLA 9500 with excitation at 473 nm to quantify the

proportion of PYP-tag that was labeled by TMBDMA.

Reactivity of compounds 5-7 in the presence of glutathione.

5-7 (20 µM) were individually incubated with glutathione (10 mM) in 20 mM HEPES buffer pH 7.4 containing 2% (v v^{-1}) DMSO and 150 mM NaCl for 30 min at 37 °C. Subsequently, the reaction mixtures were analyzed by HPLC. Reversed-phase HPLC analysis was performed using an Inertsil® ODS-3 column with Buffer A comprised of H_2O supplemented with 0.1% (v v^{-1}) HCOOH and Buffer B comprised of CH_3CN supplemented with 0.1% (v v^{-1}) HCOOH. A linear gradient of 10% to 90% B over 0 to 30 min was applied at a flow rate of 1 ml min^{-1}. The eluate was monitored by absorption at 254 nm.

In vitro protein labeling.

PYP-tag (15 µM) was incubated with PCAForange (10 µM) at 37 °C for 30 min in 20 mM HEPES buffer (1% DMSO,150 mM NaCl, pH = 7.4). The reaction mixture was heated at 95 °C for 5 min and subjected to SDS–PAGE. After the fluorescence image was taken by Typhoon FLA 9500 with excitation at 532 nm, the gel was stained with Coomassie Brilliant Blue.

Protein labeling in cell lysate.

For protein labeling in cell lysate, HeLa cells were cultured in DMEM with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin at 37 °C under 5% CO_2. The cells suspend in the assay buffer were frozen and thawed three times to lyse the cells. The cell lysate was clarified by centrifugation at 15,000 rpm for 30 min to obtain the supernatant. Total protein final concentration of the cell lysate was quantified by a Bradford assay kit (Bio-Rad). PYP-tag (1 µM) and PCAFprobes (2 µM of a single probe per reaction) were reacted in the cell lysate containing cellular proteins at a final concentration of 1.2 mg ml^{-1} at 37 °C for 30 min. Subsequently, the samples were subjected to SDS–PAGE. After the fluorescence image was obtained by Typhoon FLA 9500 with excitation at 532 nm, the gels were stained with Coomassie Brilliant Blue.

Reactivity of PCAFgreen, PCAForange, PCAFred, PCAFfar-red in the presence of glutathione.

PCAFprobes (10 µM) were incubates with PYP-tag (20 µM) in the

presence or absence of glutathione (10 mM) in 20 mM HEPES buffer pH 7.4 containing 2% (v v^{-1}) DMSO and 150 mM NaCl for 1 h at 37 °C. Subsequently, the samples were subjected to SDS–PAGE. After the fluorescence image was obtained by Typhoon FLA 9500 (Cytiva) are shown. λ_{ex}(PCAFgreen) = 473 nm, λ_{ex}(PCAForange) = 532 nm, λ_{ex}(PCAFred) = 635 nm, λ_{ex}(PCAFfar-red) = 635 nm, the gels were stained with Coomassie Brilliant Blue.

Live-cell imaging.

Live-cell imaging of HEK293T cells transfected with pcDNA-PYP-NLS, pcDNA- MBP-PYP or pcDNA-PYP-EGFR was performed with the PCAFprobes. Transfection of HEK293T cells with pcDNA-PYP-NLS, pcDNA-MBP-PYP or pcDNA-PYP-EGFR was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. After incubation of the transfected cells at 37 °C for 24 h, the cells were washed with Hanks' Balanced Salt Solution (HBSS) three times. The transfected cells were incubated with each probe (500 nM) in DMEM at 37 °C for 60 min. After the labeling reaction, the unreacted probe was removed by washing the cells with HBSS three times. Subsequently, the medium was replaced with fresh DMEM. Microscopic images of the transfected cells were recorded using a confocal laser-scanning microscope with excitation at 473 nm for PCAFgreen, 559 nm for PCAForange or 635 nm for PCAFred and PCAFfar-red coupled with an emission filter of 490–590 nm for PCAFgreen, 570–670 nm for PCAForange and 660–760 nm for PCAFred and PCAFfar-red.

Comparative labeling of PYP-tag with PCAForange and TAMRA-DNB in live cells.

HEK293T cells were transfected with pcDNA-PYP-NLS by using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. After incubation of the transfected cells at 37 °C for 24 h, the cells were washed with HBSS three times. The transfected cells were incubated with PCAForange (1 µM) or TAMRA-DNB (1 µM) for 60 min in DMEM at 37 °C. Time-lapse imaging using a confocal laser-scanning microscope with excitation at 559 nm and a 570–670 nm emission filter was performed for the analysis.

Live-cell imaging of PYP-GLUT4^{WT} and PYP-GLUT4^{N57Q} labeled

with PCAFgreen and PCAForange.

Stable HeLa cell lines expressing PYP-GLUT4^{WT} and PYP-GLUT4^{N57Q} were maintained in DMEM supplemented with 10% FBS. For the imaging, the medium was removed, and the cells were incubated in Krebs-Ringer bicarbonate (KRB) buffer for 3 h. Subsequently, PCAFgreen (2 μM) and insulin (100 nM) were then added to the cells and incubated for 20 minutes. After washing cells with KRB containing insulin, then PCAForange (1 μM) was added and incubated for 30 minutes. After washing the cells with KRB containing insulin three times to remove the probes, the cells were observed by confocal microscopy. For samples with the insulin stimulation, insulin (100 nM) was also added to HBSS.

Immunostaining of PYP-GLUT4WT and PYP-GLUT4N57Q using PCAFgreen.

Stable cells were incubated in KRB buffer for three hours, and then incubated with PCAFgreen (2 μM) for 20 min. After washing, the cells were further incubated in KRB buffer for 40 min, washed three times with PBS, and then incubated with formaldehyde solution on ice for 15 min to fix the cells. The cells were washed three times with PBS, incubated with 0.1% Triton for 5 minutes at room temperature, and then washed with PBS. The cells were treated with 5% BSA for 30 minutes, and then probed with anti-FLAG primary antibody in the presence of 3% BSA for an hour at room temperature. The cells were then washed to remove excess primary antibody, and probed with secondary antibody (anti-mouse conjugated Cy3) for an hour at room temperature. After the antibody staining, microscopic images were taken.

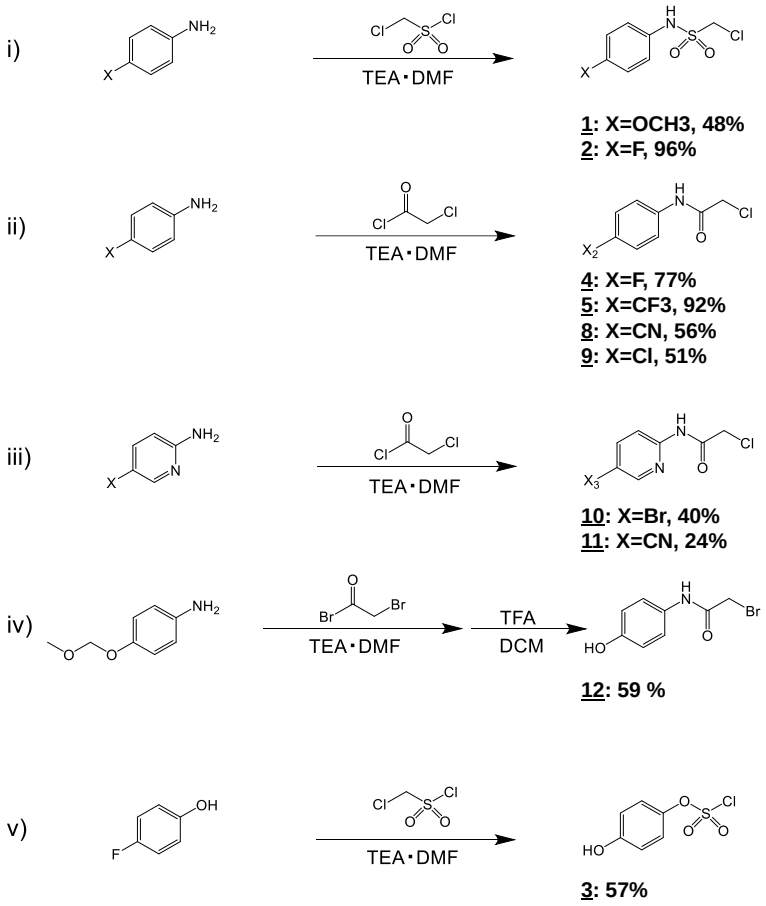
Co-staining of GLUT4 with lysosomes and golgi apparatus.

For co-staining of GLUT4 with lysosomes, the same live-cell imaging protocol described above was used except for the addition of a lysosome-labeling fluorescent dye (Invitrogen™ LysoTracker™ Deep Red) five minutes before the cell washing step prior to observation under the microscope. For visualization of the Golgi apparatus, the same protocol described above for live-cell imaging for locating PYP-GLUT was used except for the addition of BODIPY™ TR Ceramide complexed to BSA (Invitrogen) according to the manufacturer’s protocol for labeling the Golgi apparatus.

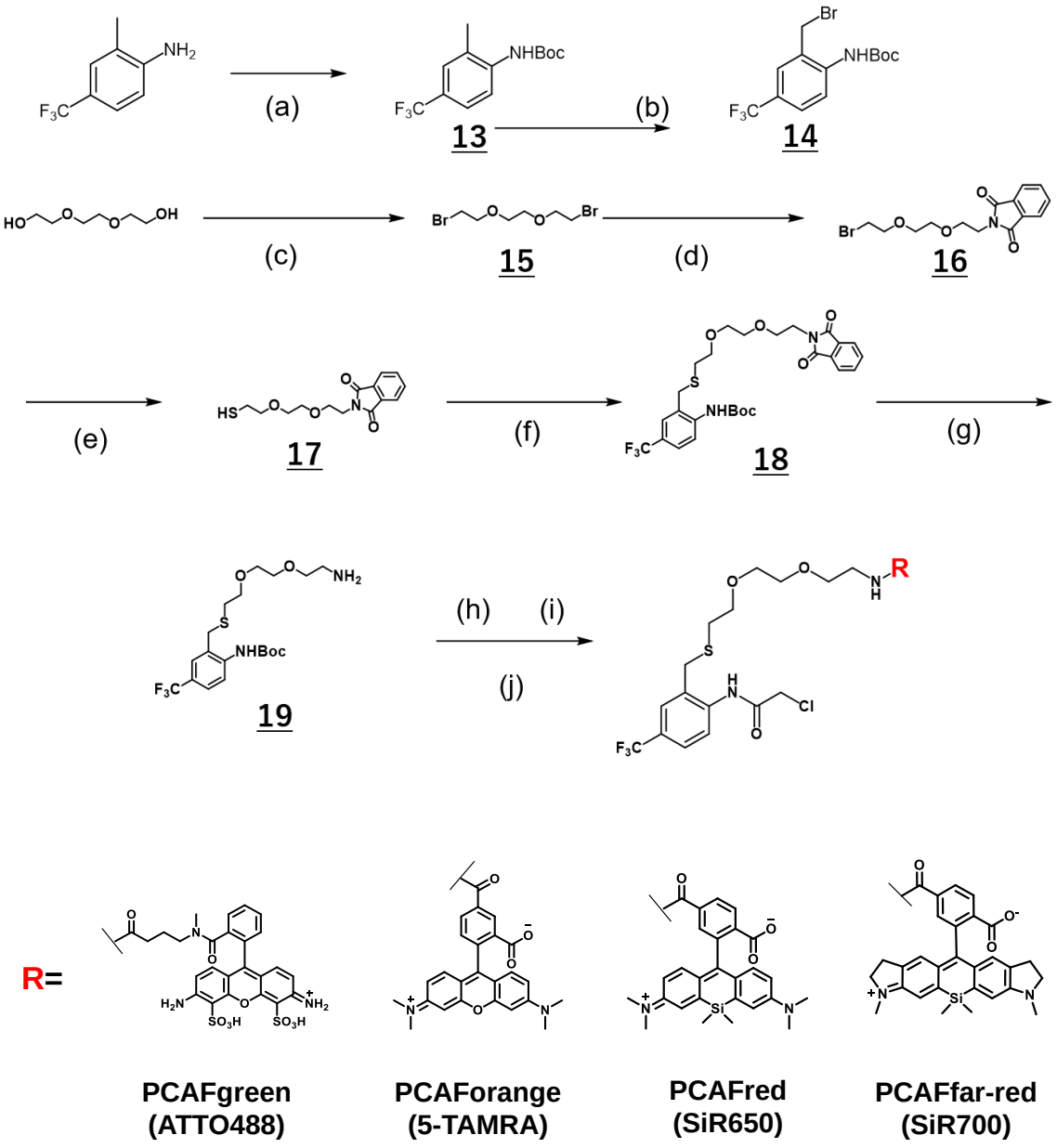
Time-lapse imaging of PYP-GLUT4 ^{WT} in a single cell labeled with PCAFgreen and PCAForange.

Time-lapse imaging was performed after incubating the PYP-GLUT4-stable cell lines in KRB for 3 h. PCAFgreen (2 μM) and PCAForange (1 μM) were added to the cells simultaneously and incubated for 20 min. Subsequently, the probes were washed off by rinsing the cells with HBSS three times, and the cells were incubated for another 20 minutes. After the incubation, insulin (100 nM) was added, and the image of the cells was captured every 10 minutes by confocal microscope.

2. Supplementary Schemes

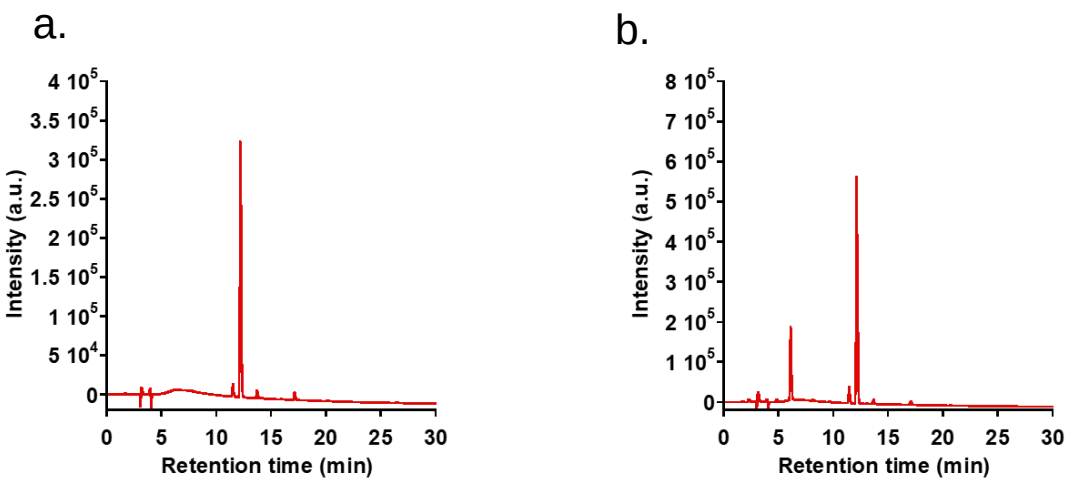


Supplementary Scheme 1. Synthesis of PYP-tag ligands.

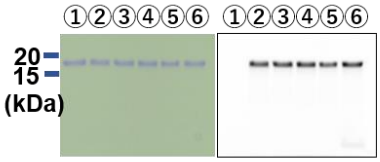


Supplementary Scheme 2. Synthesis of PCAFgreen, PCAForange, PCAFred and PCAFfar-red. (a) Boc_2/THF, (b) NBS, AIBN/CCl_4, (c) PH_3P, CBr_4/THF, (d) potassium phthalimide/THF, (e) NaSH, TBAB/DMF, (f) **13**, TEA/THF, (g) H_2NNH_2/MeOH, (h) ATTO488-NHS or 5-TAMRA-NHS or SiR650-NHS or Sir700-NHS/DMF, (i) 10% (v v^{-1}) TFA/CH_2Cl_2, (j) chloroacetyl chloride/DMF.

3. Supplementary Figures



Supplementary Figure 1. HPLC analysis of **a, 12** stored at –80 °C dry and **b**, **12** stored at –80 °C in DMSO for one week. For the HPLC analysis, an Inertsil® ODS-3 column, Buffer A comprised of H_2O with 0.1% (v v^{-1}) HCOOH, Buffer B comprised of CH_3CN with 0.1% (v v^{-1}) HCOOH, and a linear gradient of 10% to 90% B over 0 to 30 min were used. The eluate was monitored with λ_{abs} = 254 nm.



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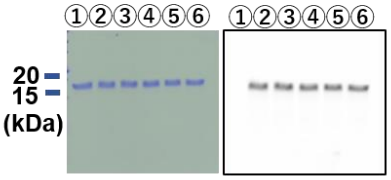

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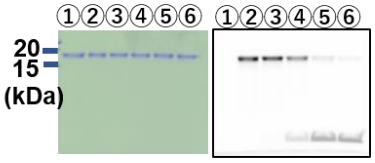

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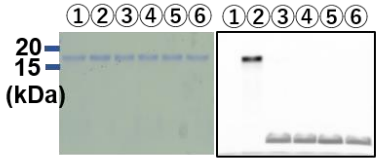

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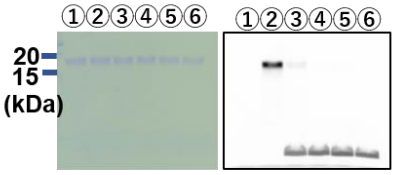

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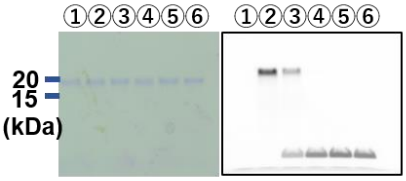

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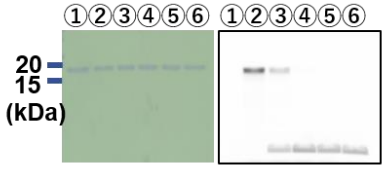
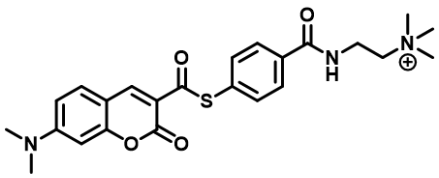


9

10

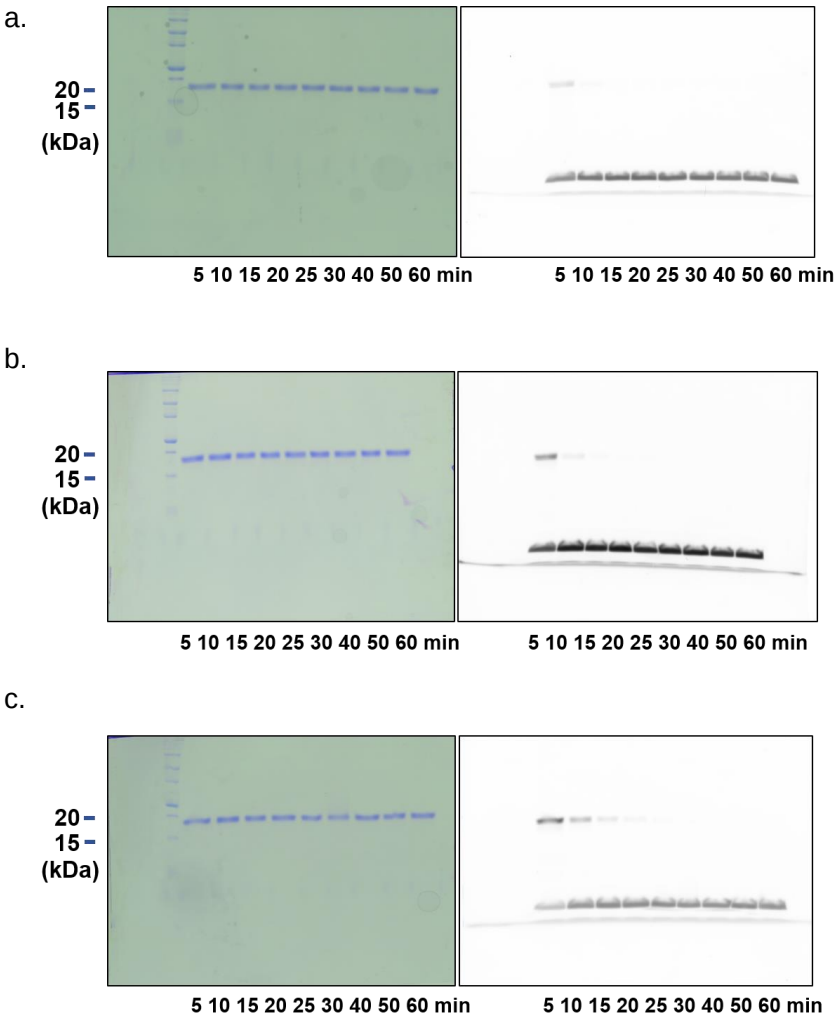
11

	①	②	③	④	⑤	⑥
PYP-tag (uM)	10	10	10	10	10	10
TMBDMA (uM)	0	8	8	8	8	8
Compound (uM)	0	0	10	20	50	100

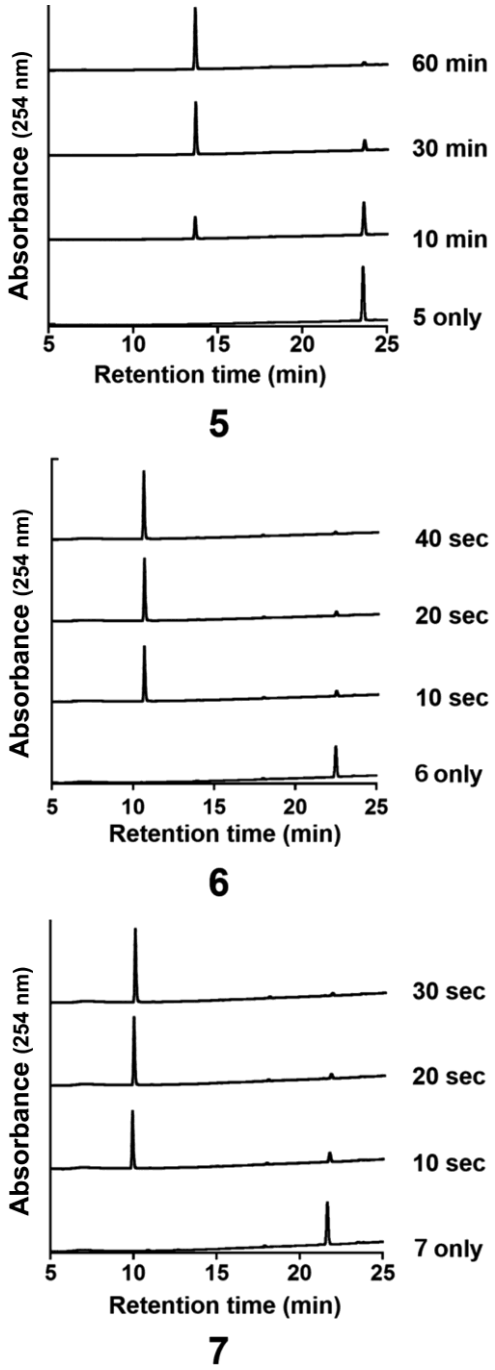


TMBDMA

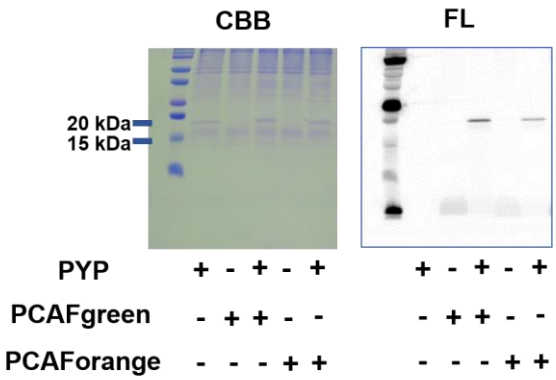
Supplementary Figure 2. SDS–PAGE analysis of the efficiency of labeling of PYP-tag by **1**–**11**. Each of **1**–**11** at the concentration of 0, 10, 20, 50 and 100 μM was mixed with 10 μM PYP-tag at 37 °C for 1 h, and unlabeled PYP-tag was fluorescently labeled by incubating the reaction mixture with 8 μM TMBDMA for 30 min. Buffer condition: 20 mM HEPES buffer pH 7.4 containing 3% (v v^{-1}) DMSO and150 mM NaCl. Images of Coomassie Brilliant Blue (CBB)-stained and fluorescence gel (λ_{ex} = 473 nm) are displayed on the left and right, respectively.



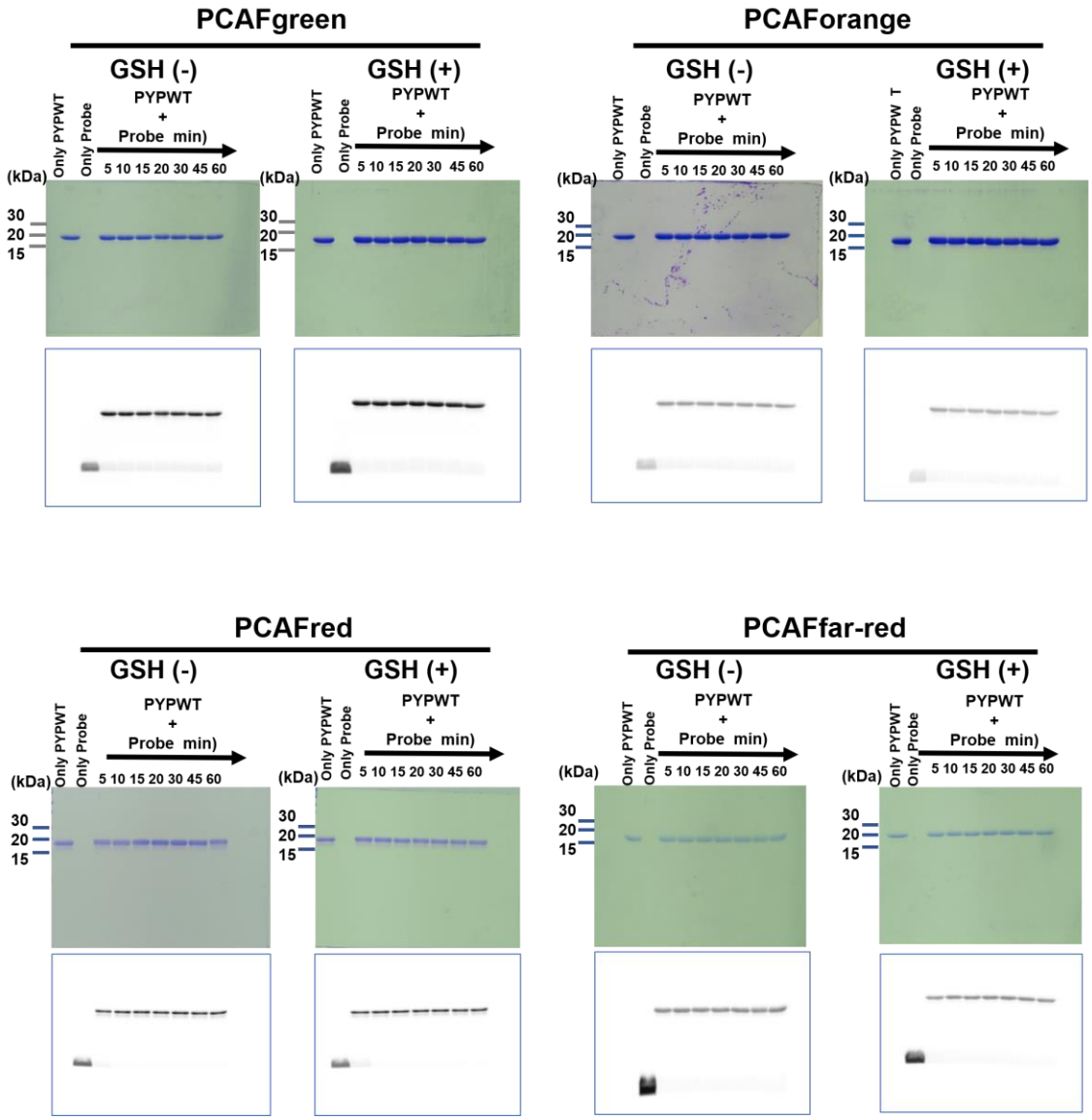
Supplementary Figure 3. SDS–PAGE analysis for determining the preferential binding of PYP-tag by the ligands **a. 5**, **b. 6** and **c. 7**. The labeling reaction was performed as described in the Methods section except 20 µM of the ligand and the HEPES buffer supplemented with 150 mM NaCl were used. After the reaction, all samples were immediately mixed with 8 µM TMBDMA and incubated for another 30 minutes before being analyzed by SDS-PAGE. The fluorescence signal of the band corresponding to the labelled PYP-tag was measured. The fluorescent signals of the bands corresponding to the labeled PYP-tag were measured at λ_{ex} = 473 nm. The bound fraction was calculated by comparing the measured signal intensity with the intensity expected when PYP-tag was bound fully by the ligand.



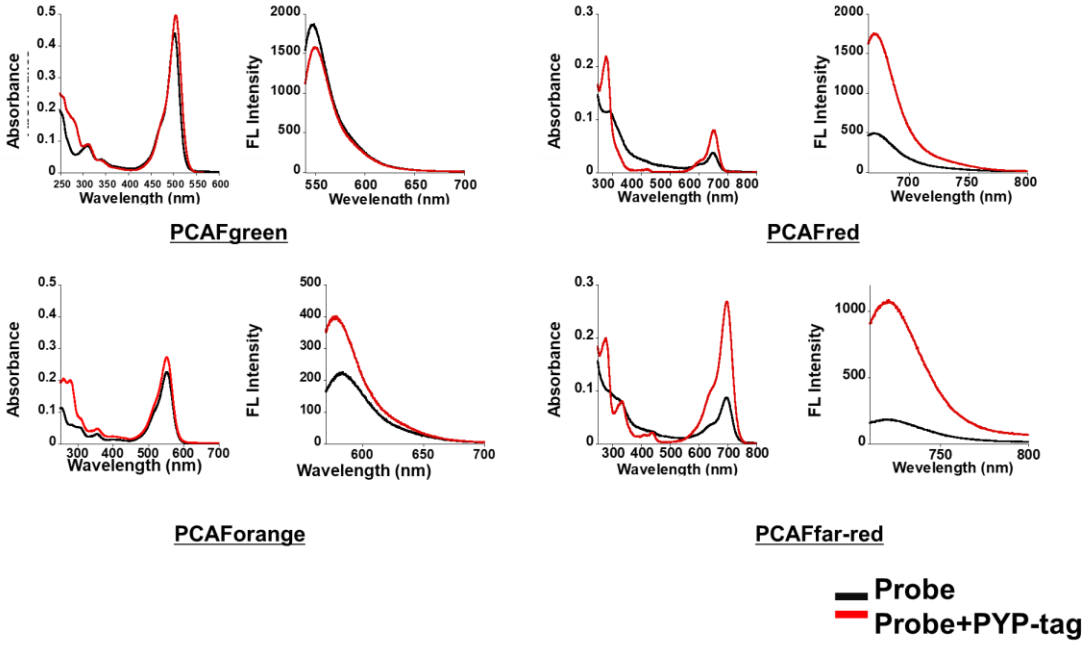
Supplementary Figure 4. HPLC analysis of the reaction between compound **5**, **6** or **7** (20 μM) and glutathione (10 mM). Buffer condition: 20 mM HEPES buffer at pH 7.4 with 2% (v v^{-1}) DMSO and 150 mM NaCl. For the HPLC analysis, an Inertsil® ODS-3 column, Buffer A comprised of H_2O with 0.1% (v v^{-1}) HCOOH, Buffer B comprised of CH_3CN with 0.1% (v v^{-1}) HCOOH and a linear gradient of 10% to 90% B over 0 to 30 min were used.



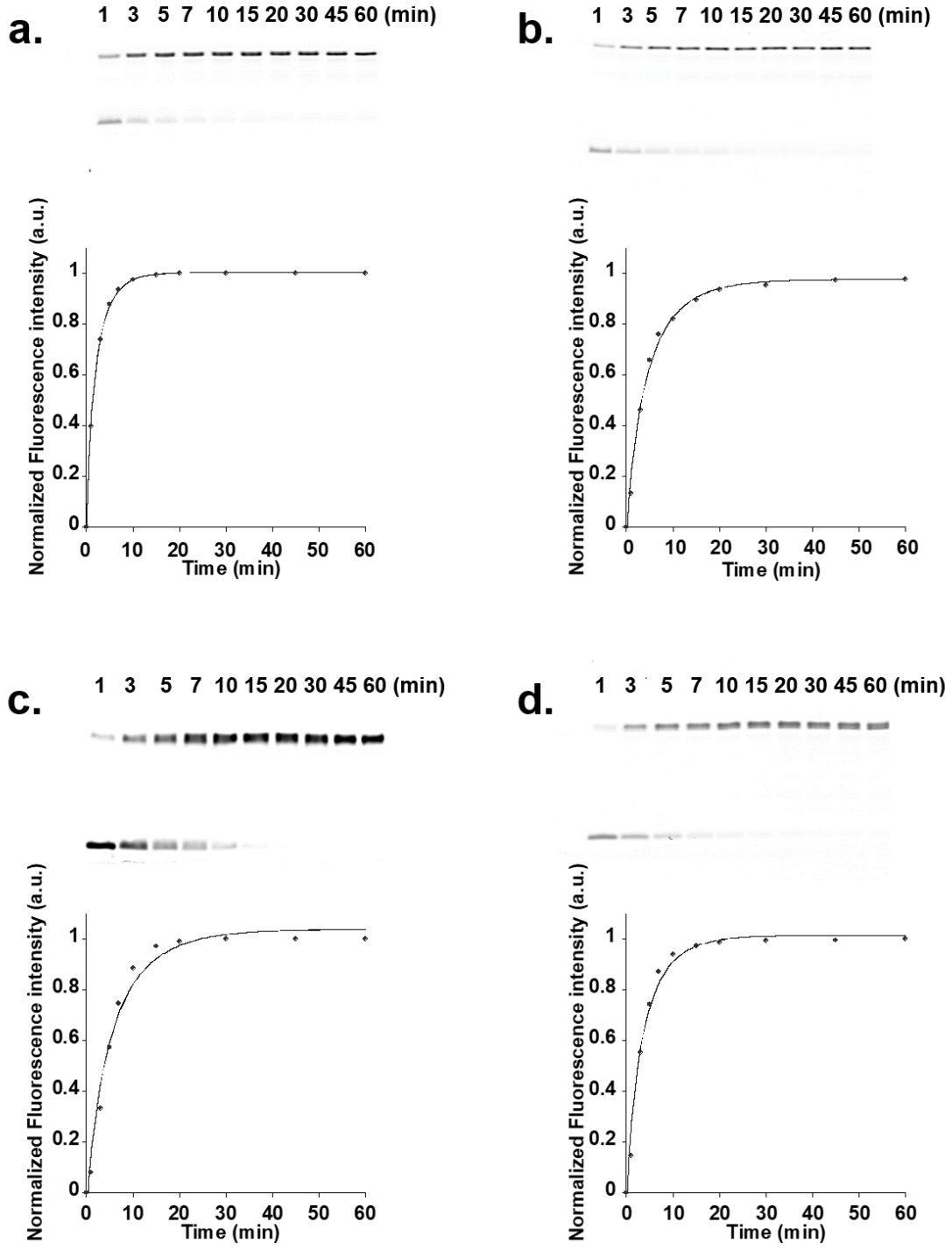
Supplementary Figure 5. Protein labeling experiments with PCAForange in lysate of NIH3T3 cells. PYP (1 μM) was reacted with PCAForange or PCAFgreen (500 nM) in NIH3T3 cell lysate (1.34 mg/mL).



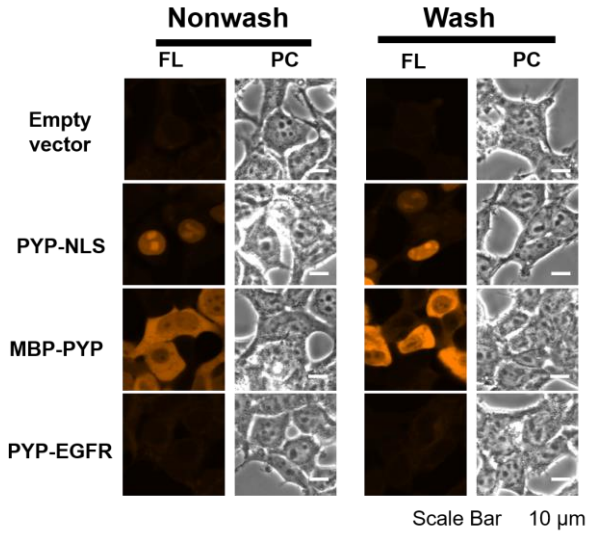
Supplementary Figure 6. SDS–PAGE analysis of the ability of PCAF probes to label PYP-tag in the presence of glutathione (GSH). Each of the PCAF probes at 10 μM was reacted with 20 μM PYP-tag in the presence or absence of 10 mM GSH at 37 °C for 1 h. Buffer condition: 20 mM HEPES buffer pH 7.4, 3% (v v^{-1}) DMSO and 150 mM NaCl. Images of CBB-stained (top) and fluorescence-visualized (bottom) gels captured by Typhoon FLA 9500 (Cytiva) are shown. λ_{ex}(PCAFgreen) = 473 nm, λ_{ex}(PCAForange) = 532 nm, λ_{ex}(PCAFred) = 635 nm, λ_{ex}(PCAFfar-red) = 635 nm.



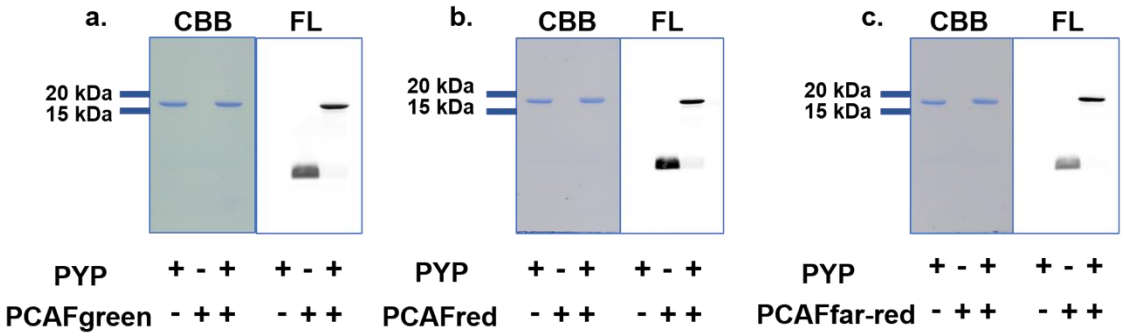
Supplementary Figure 7. Absorption (left) and fluorescence (right) spectra of PCAFgreen, PCAForange, PCAFred and PCAFfar-red alone (black) and after reacting with PYP-tag (red). The concentration of the probe and the protein were 5 μM and 10 μM, respectively.



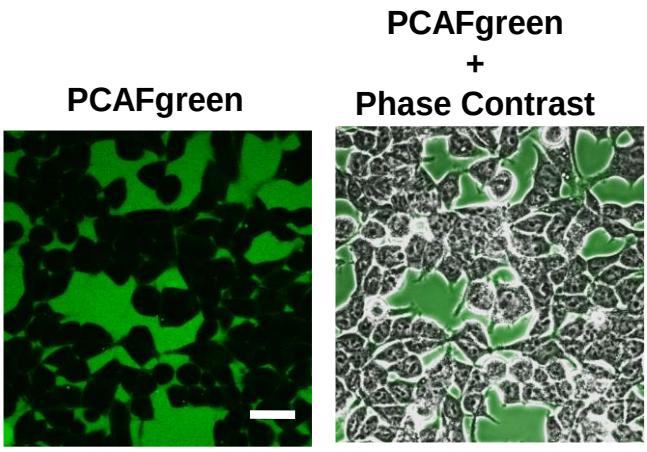
Supplementary Figure 8. SDS–PAGE analysis of the time-course of the reaction between PYP-tag and the PCAF probe **a.** PCAFgreen, **b.** PCAForange, **c.** PCAFred and **d.** PCAFfar-red.



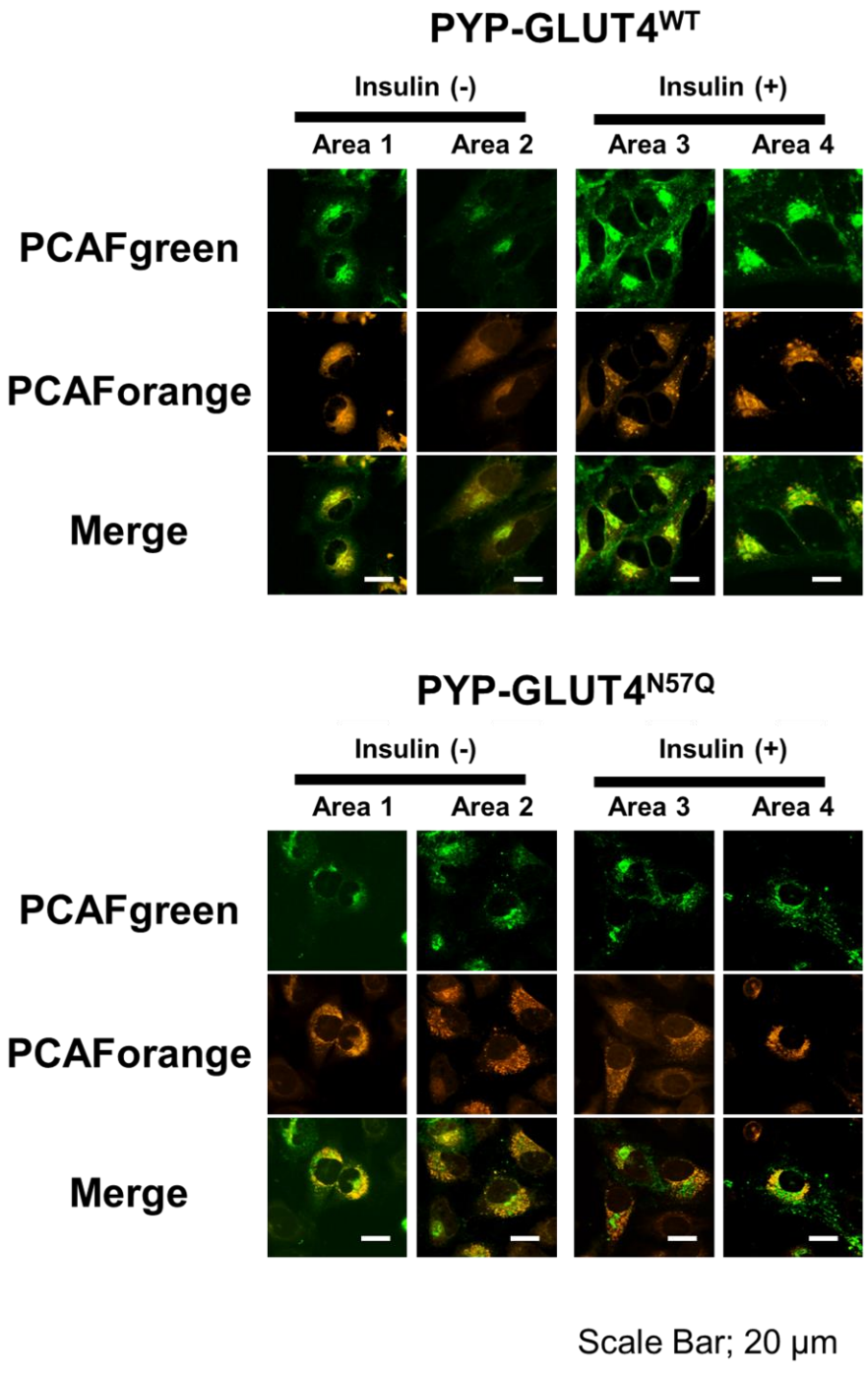
Supplementary Figure 9. Live-cell imaging of PYP-NLS, MBP-PYP and PYP-EGFR fusion proteins expressed in HEK293T cells labeled with PCAForange. The cells were incubated with 1 μM PCAForange for 60 min at 37 °C, washed or unwashed and imaged with a confocal fluorescence microscope. Fluorescence (FL, λ_{ex} = 559 nm) and phase contrast (PC) images are shown.



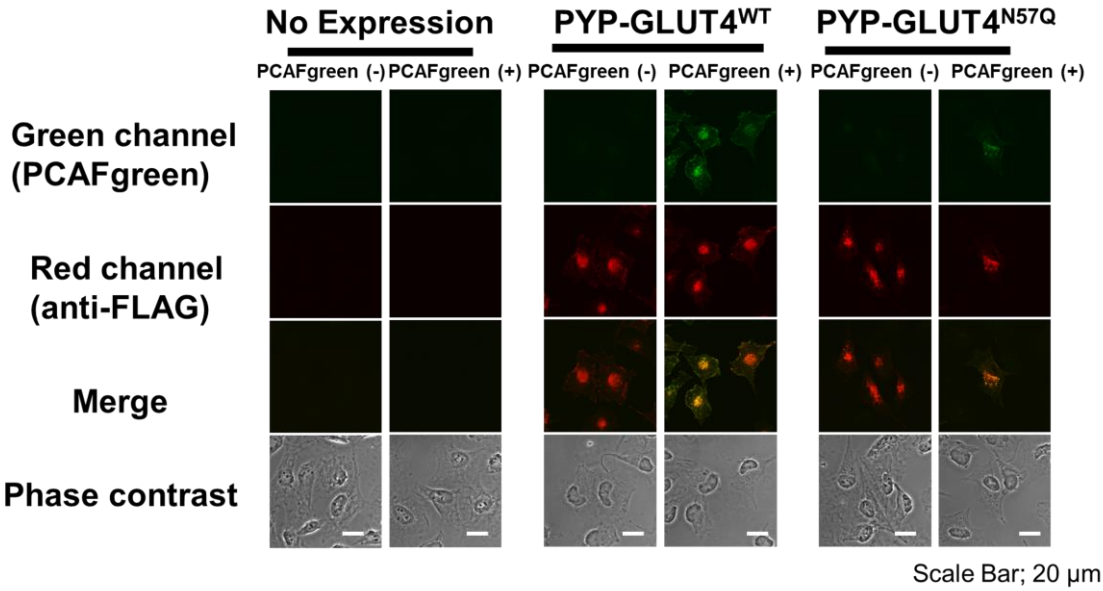
Supplementary Figure 10. SDS–PAGE analysis of labeling reactions of PYP-tag with **a.** PCAFgreen, **b.** PCAFred and **c.** PCAFfar-red. PYP-tag (15 μM) was reacted with the probes (10 μM) at 37 °C for 30 min. λ_{ex}(PCAFgreen) = 473 nm, λ_{ex}(PCAForange) = 532 nm and λ_{ex}(PCAFred and PCAFfar-red) = 635 nm.



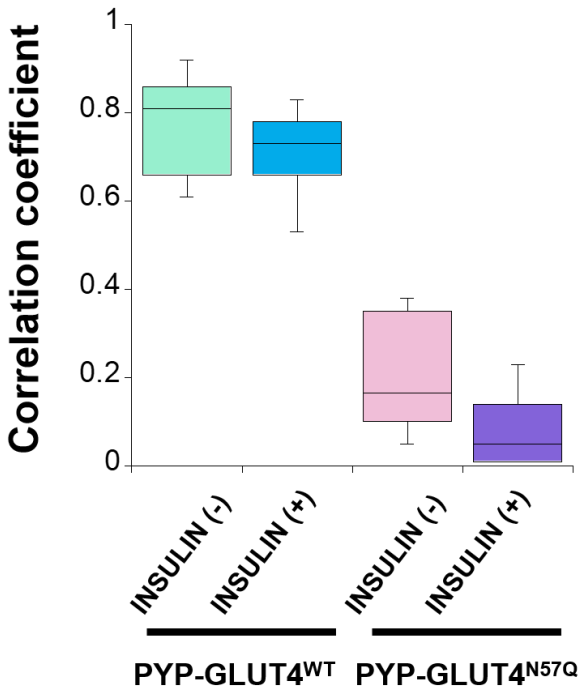
Supplementary Figure 11. Live-cell imaging of non-transfected HeLa cells incubated with PCAFgreen to confirm that the HeLa cell membrane is impermeable to the probe. The cells were incubated with 1 µM PCAFgreen for 30 min and imaged under a fluorescence microscope. λ_{ex}(PCAFgreen) = 473 nm. Scale Bar (white, left panel): 20 µm.



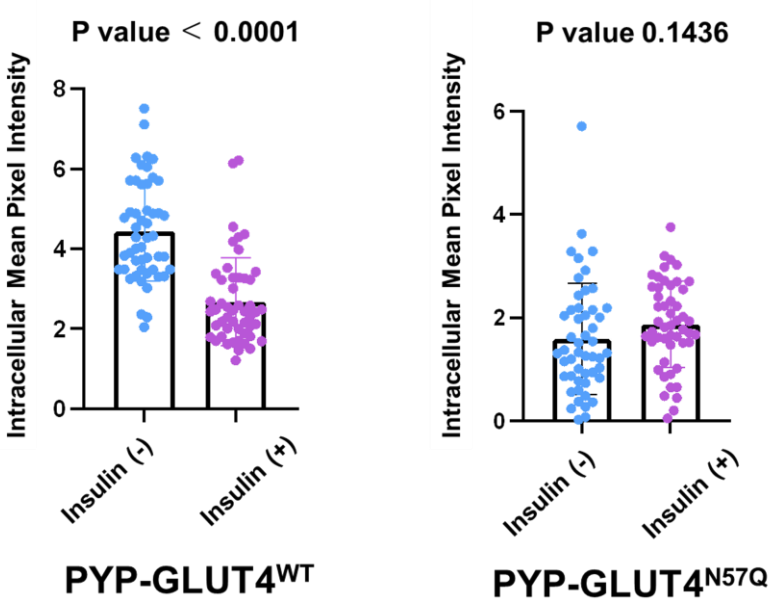
Supplementary Figure 12. Multicolor live-cell imaging with the PCAF color palette to examine the localization of PYP-GLUT4^{WT} and PYP-GLUT4^{N57Q} with or without insulin stimulation. Images in two areas different from Figure 6a are shown. HeLa cells stably expressing PYP-GLUT4^{WT} and PYP-GLUT4^{N57Q} were labeled with 2 μM PCAFgreen or 1 μM PCAForange with or without insulin stimulation and visualized under a confocal fluorescence microscope.



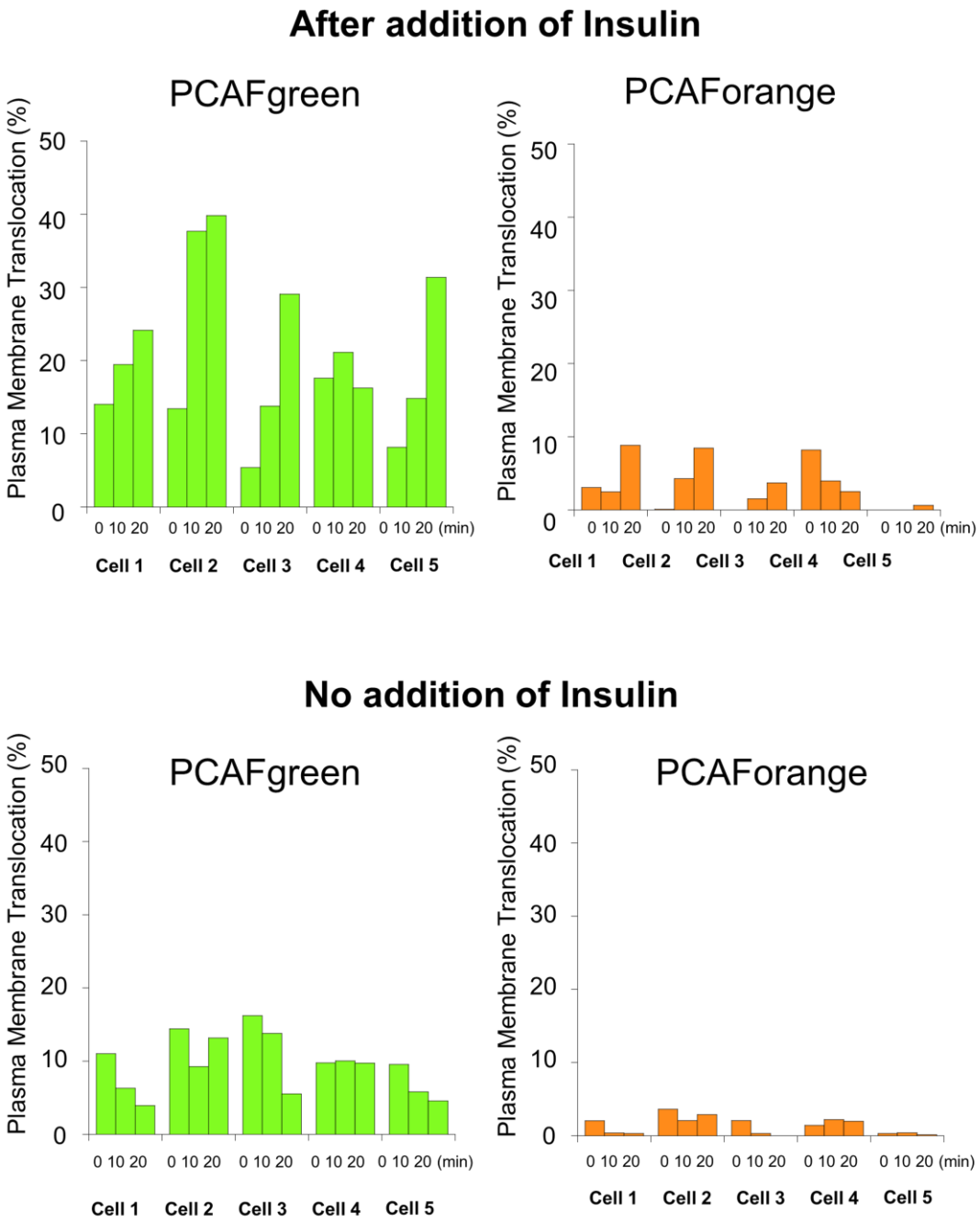
Supplementary Figure 13. PYP-GLUT4^{WT} and PYP-GLUT4^{N57Q} probed with PCAFgreen or anti-FLAG. HeLa cells stably expressing PYP-GLUT4^{WT} and PYP-GLUT4^{N57Q} along with untransfected HeLa cells (No Expression) were labeled with 2 μM PCAFgreen without insulin stimulation in living cells, followed by fixation and immunostaining with anti-FLAG, and visualized under a confocal fluorescence microscope. FLAG-tag is included in both PYP-GLUT4 constructs and is detected using anti-FLAG and its secondary antibody conjugated to Cy3. Laser: Ex: 473 nm (Em: 490-540 nm) for PCAFgreen, Ex: 559 nm (Em: 570-620 nm) for anti-FLAG.



Supplementary Figure 14. Degree of colocalization between PCAFgreen- and PCAForange-labeled proteins inside cells. Co-localization was quantified to examine the distribution of PCAFgreen and PCAForange signals within cells in the experiments performed in Figure 6a. The vertical axis is Pearson's correlation coefficient between PCAFgreen and PCAForange localization.



Supplementary Figure 15. Intracellular mean pixel intensity of individual cells that have internalized GLUT4 proteins labeled with PCAF probes. The intracellular mean pixel intensity is obtained by calculating intracellular fluorescence intensity divided by intracellular area (total pixels) using ImageJ. The distribution of the intracellular mean pixel intensity (fluorescence intensity / cell) of PCAFgreen-labeled cells (N = 50) in the presence (+) or absence (–) of 100 nM insulin was compared between the PYP-GLUT4^{WT} and PYP-GLUT4^{N57Q} cell lines. Stable HeLa cell lines expressing PYP-GLUT4^{WT} and PYP-GLUT4^{N57Q} were maintained in DMEM supplemented with 10% FBS. For the imaging, the medium was removed, and the cells were incubated in Krebs-Ringer bicarbonate (KRB) buffer for 3 h. Subsequently, PCAFgreen (2 μM) and insulin (100 nM) were added to the cells and incubated for 20 min. After washing the cells with HBSS three times to remove the probes, the cells were observed by confocal microscopy. For samples with the insulin stimulation, insulin (100 nM) was also added to HBSS. PCAFgreen: λ_{ex} = 473 nm; Statistical analyses of the fluorescence densities determined from images of cells labeled with 2 μM of PCAFgreen were performed. Median (bar) with interquartile range (error bar) is shown for each condition. Two-tailed unpaired Student's t-test was used to show that the difference in the intracellular mean pixel intensity in the presence vs. the absence of insulin was statistically significant. The plot was prepared using the GraphPad Prism software.



Supplementary Figure 16. Quantification of GLUT4 translocation to the plasma membrane shown in Figure 6e. The vertical axis of the graph represents ratio of the mean pixel intensity on the membrane to the mean intracellular pixel intensity.

4. Supplementary Tables

Probes	k^2 [a] [M^{-1} s^{-1}]
PCAFgreen	9.8x10 3
PCAForange	4.5x10^3
PCAFred	3.7x10^3
PCAFfar-red	5.8x10^3
TAMRA-DNB	7.9x10^2
TAMRA-DNB2	3.3x10^2
FCANB	1.3x10^2
FCDNB	5.4x10^2
AT-DNB	1.1x10^3
AT-DNB2	7.4x10^2
Cy3NB	2.6x10^2
Cy3DNB	4.2x10^2
Cy3DNB2	4.9x10^2
Cy5NB	4.1x10^2
Cy5DNB	5.8x10^2
Cy5DNB2	7.2x10^2

Supplementary Table 1. Second-order rate constants (k^2)[1,2] of various probes, including the PCAF probes developed in the current study, for binding of PYP-tag. All measurements were made in triplicate.

5. Supplementary References

1. Hori, Y., Hirayama, S. & Kikuchi, K. Development of cyanine probes with dinitrobenzene quencher for rapid fluorogenic protein labelling. *Philos. Trans A Math. Phys. Eng. Sci.* **375**, 20170018 (2017).
2. Hirayama, S., Hori, Y., Benedek, Z., Suzuki, T. & Kikuchi, K. Fluorogenic probes reveal a role of GLUT4 N-glycosylation in intracellular trafficking. *Nat. Chem. Biol.* **12**, 853–859 (2016).
3. Fries, F. & Reineke, S. Statistical treatment of photoluminescence quantum yield measurements. *Sci. Rep.* **9**, 15638 (2019).
4. R.F. Kubin, A.N. Fletcher, Fluorescence quantum yields of some rhodamine dyes. *J. Lumin.* **27**, 455–462 (1982)
5. R.B. Mujumdar, L.A. Ernst, S.R. Mujumdar, C.J. Lewis & A.S. Waggoner, Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. *Bioconjug. Chem.* **4**, 105–111(1993)

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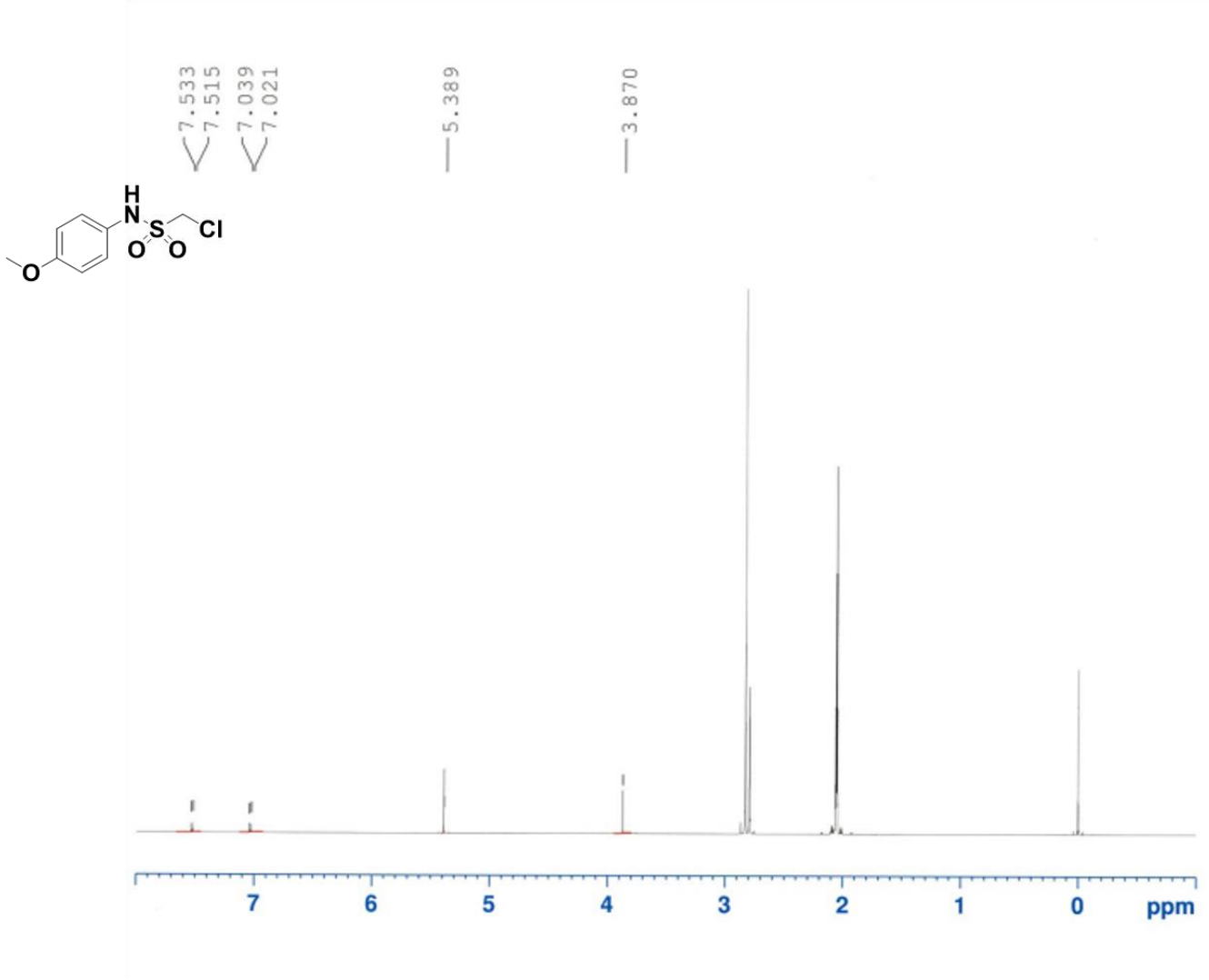

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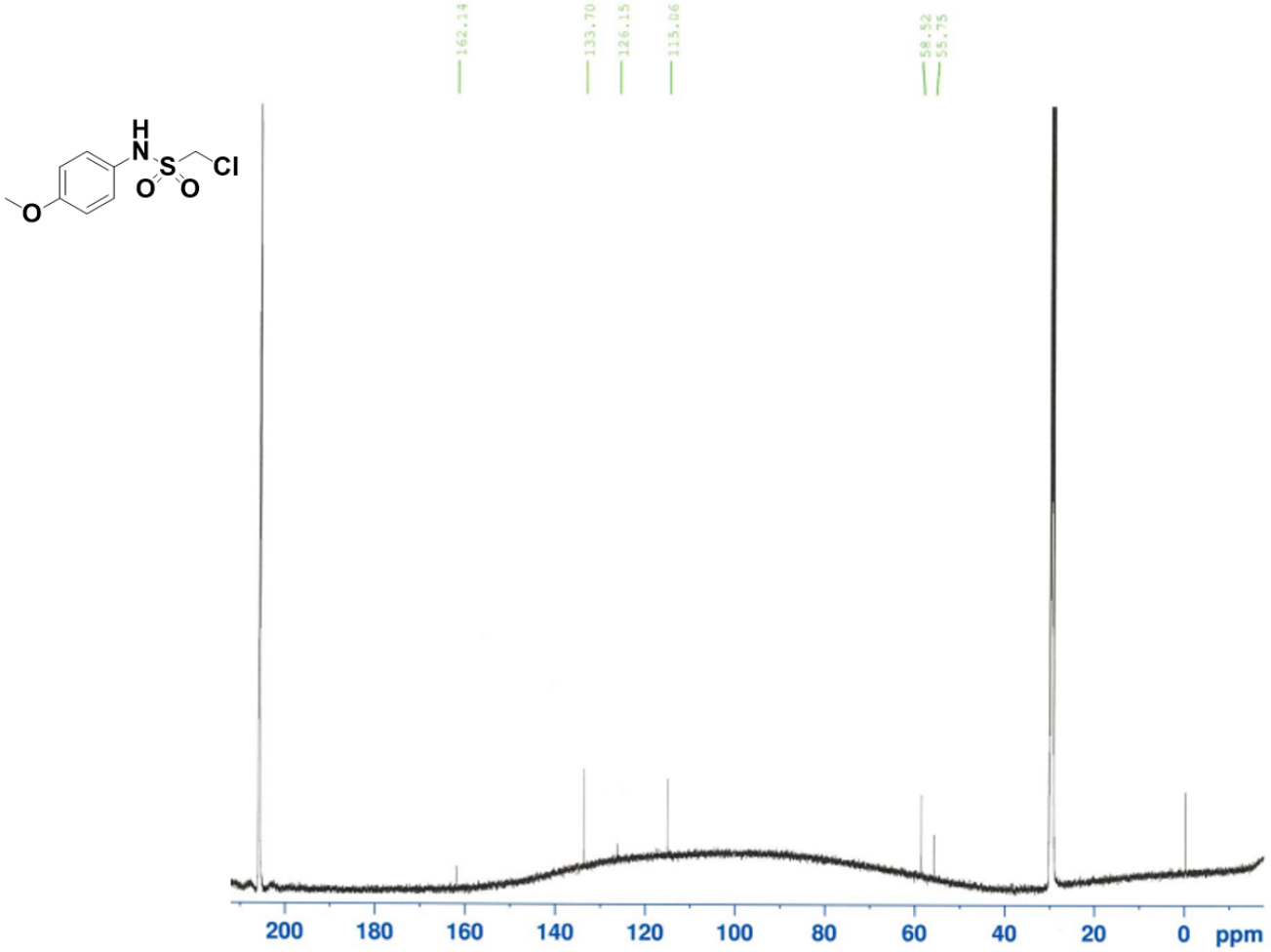

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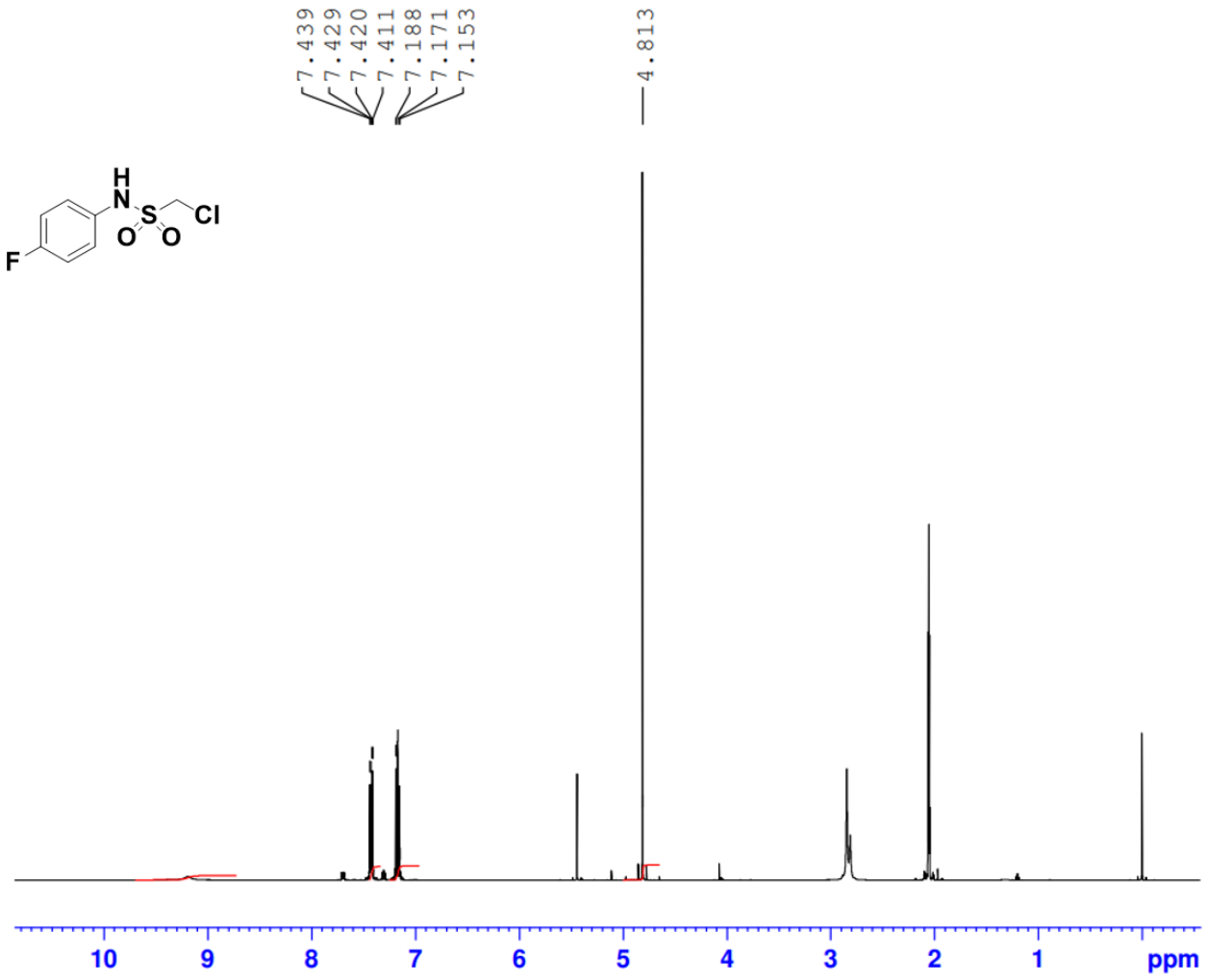

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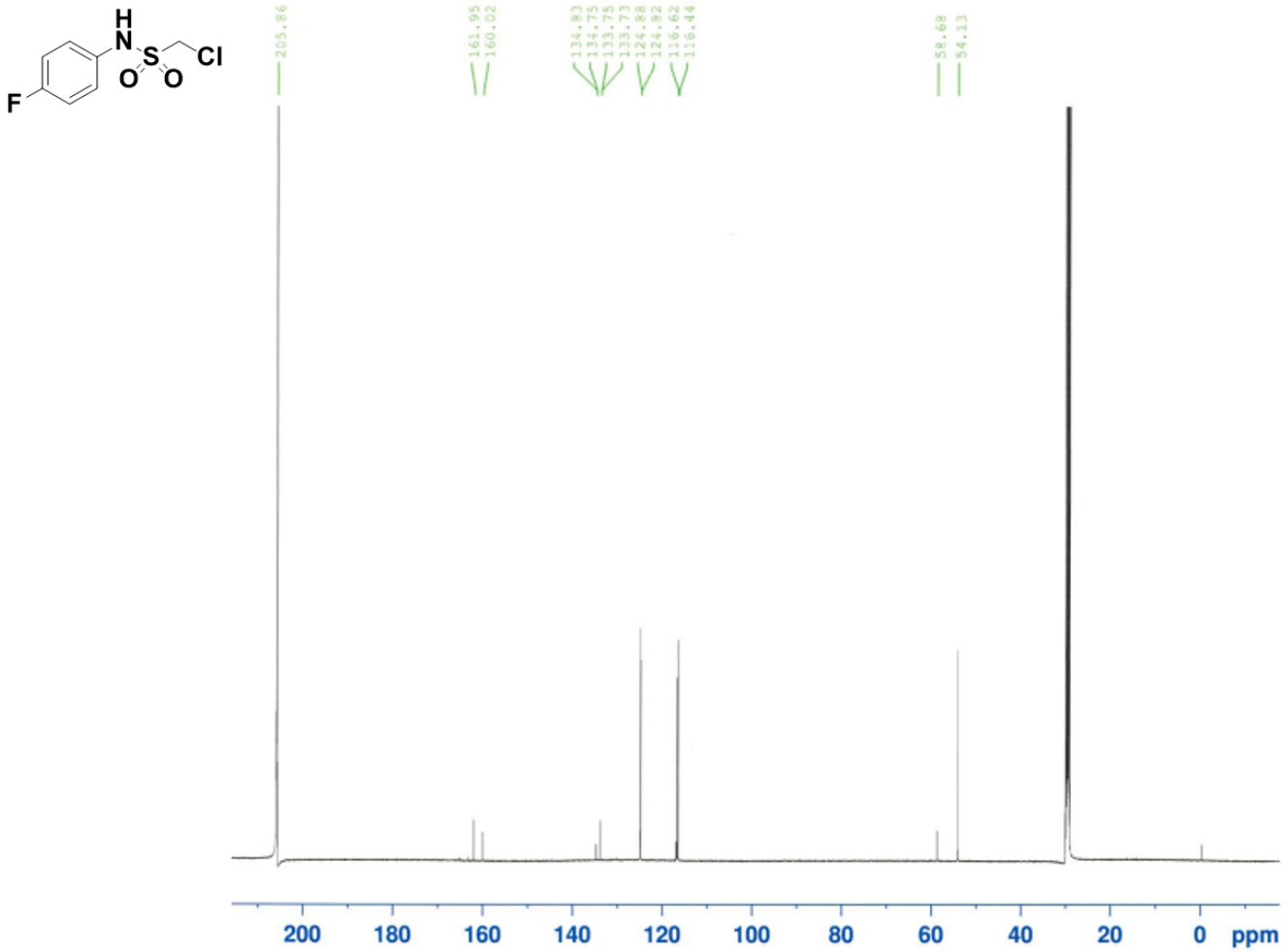

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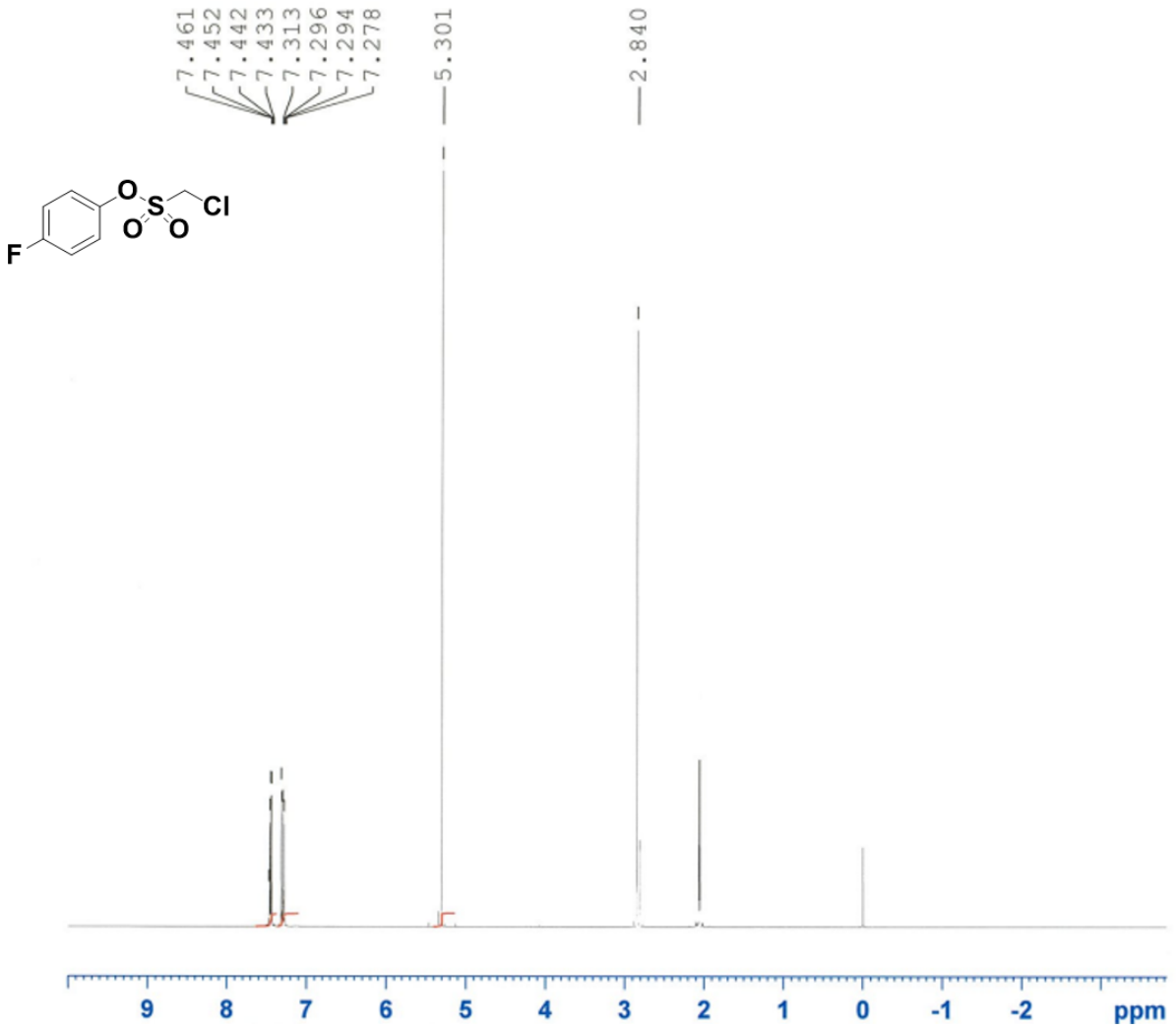

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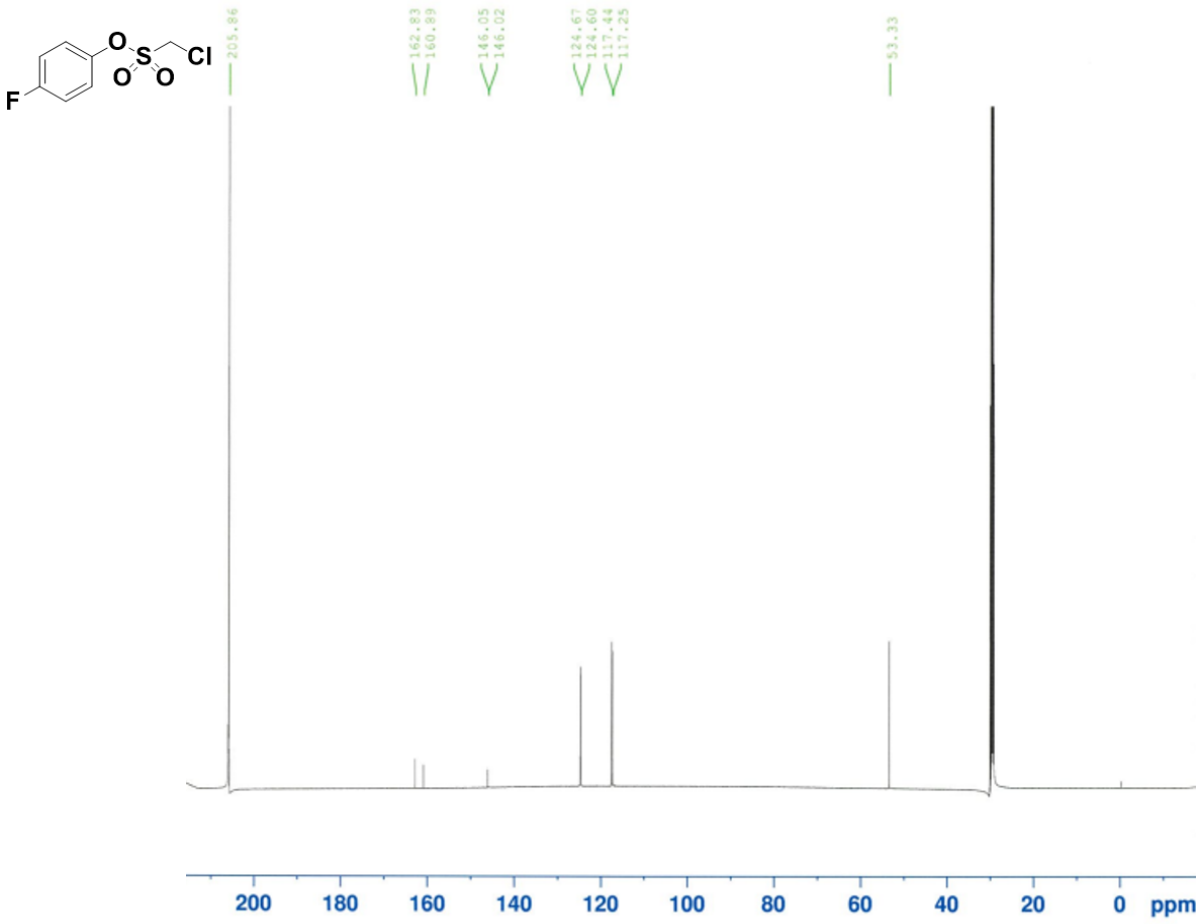

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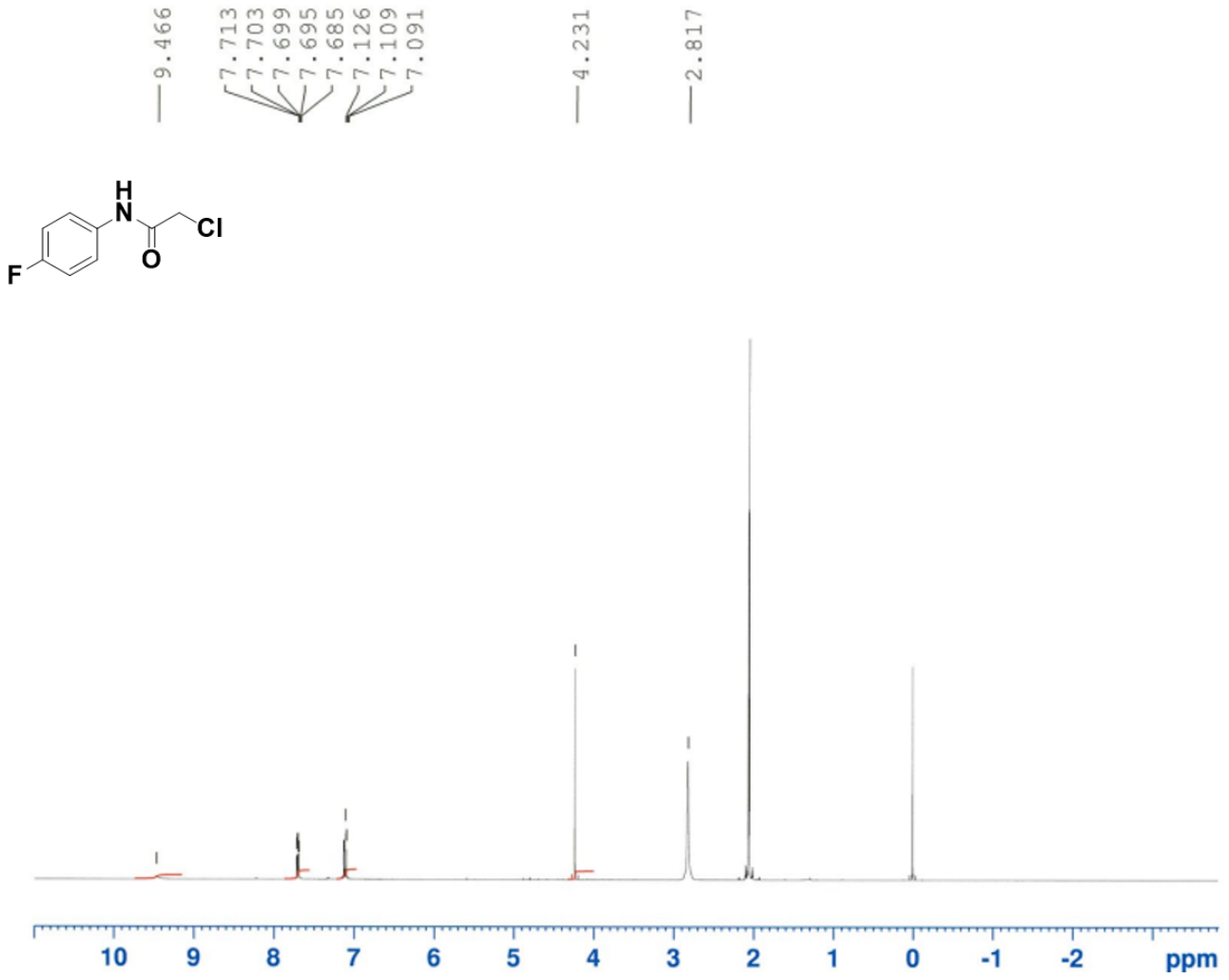

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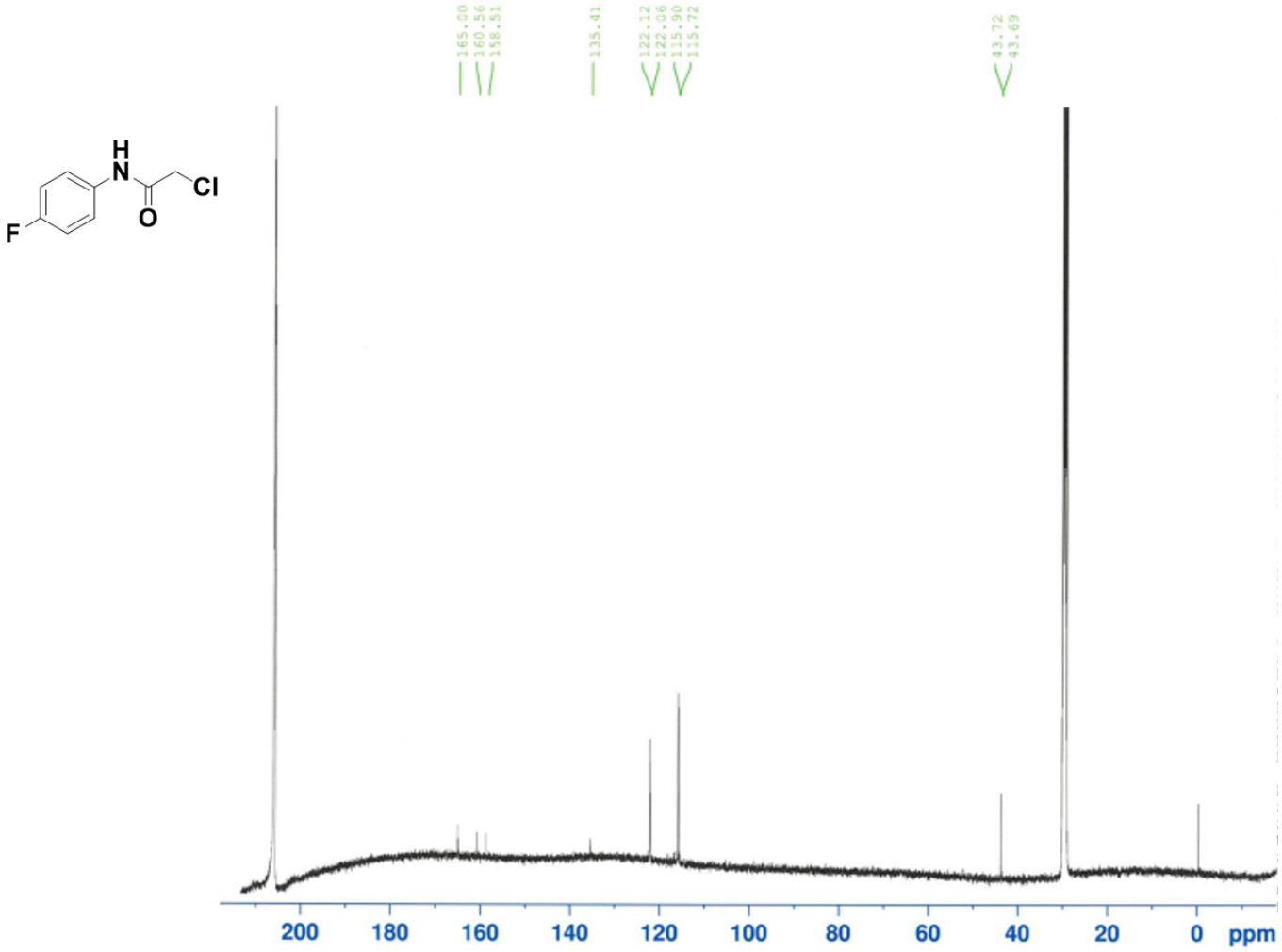

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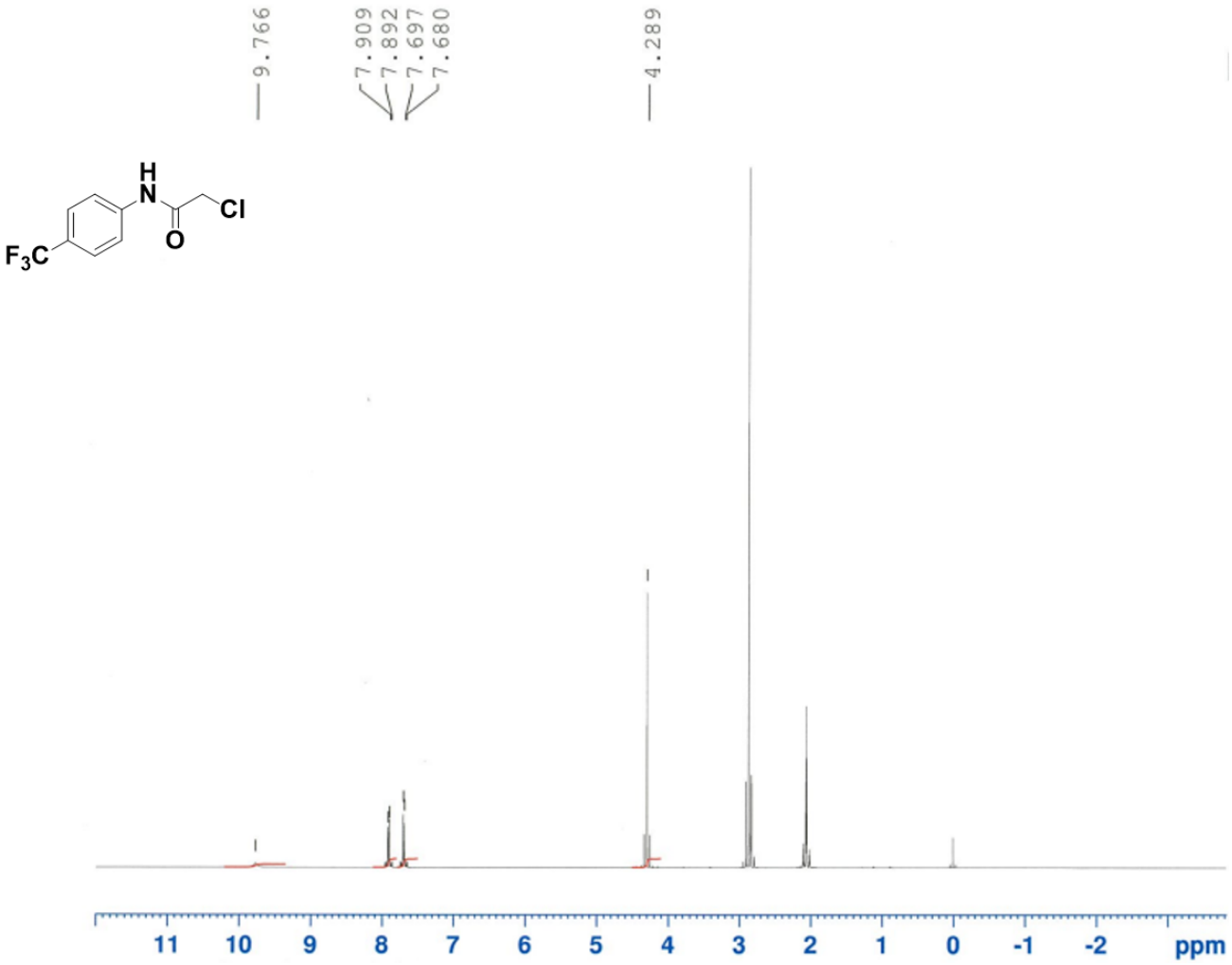

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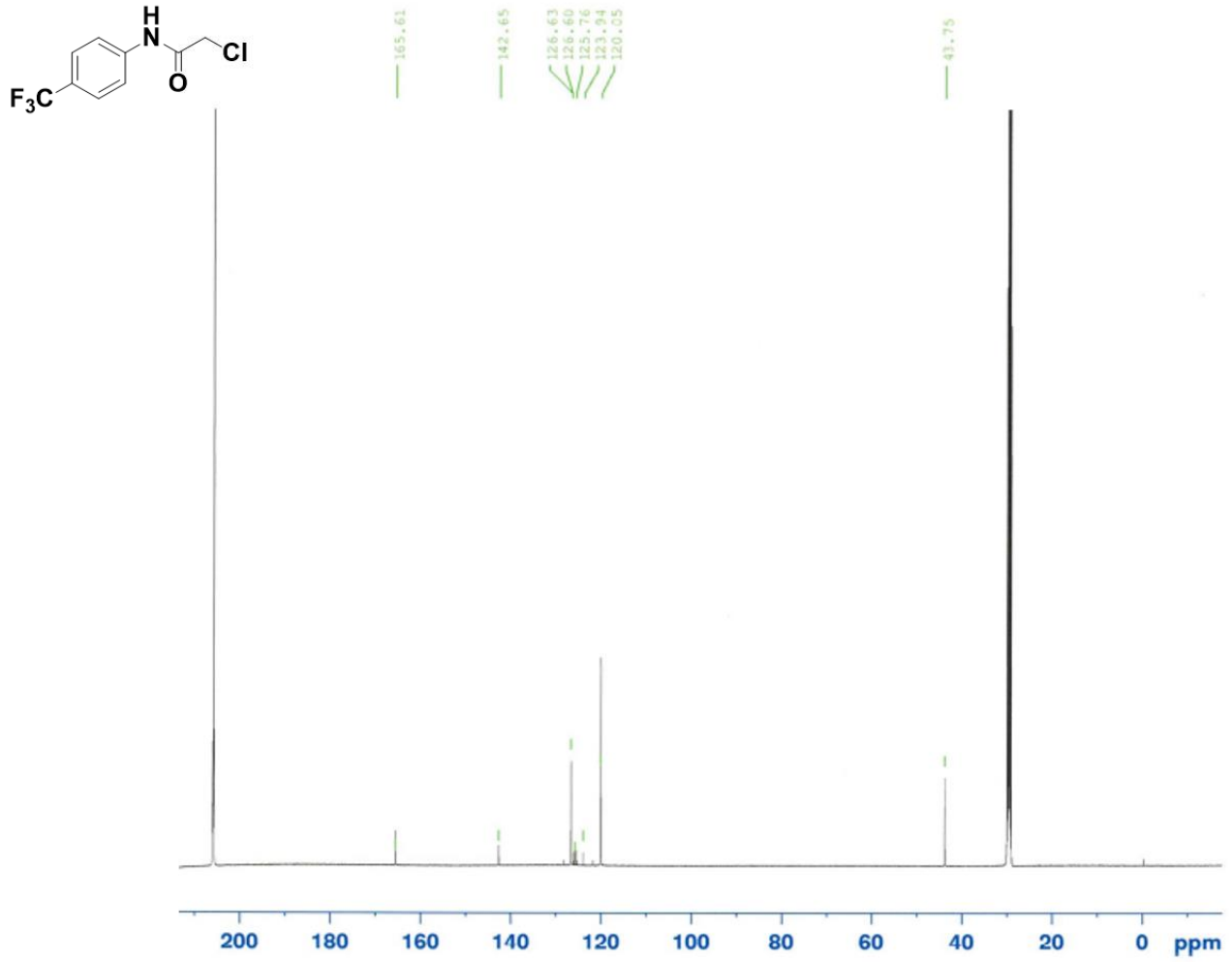

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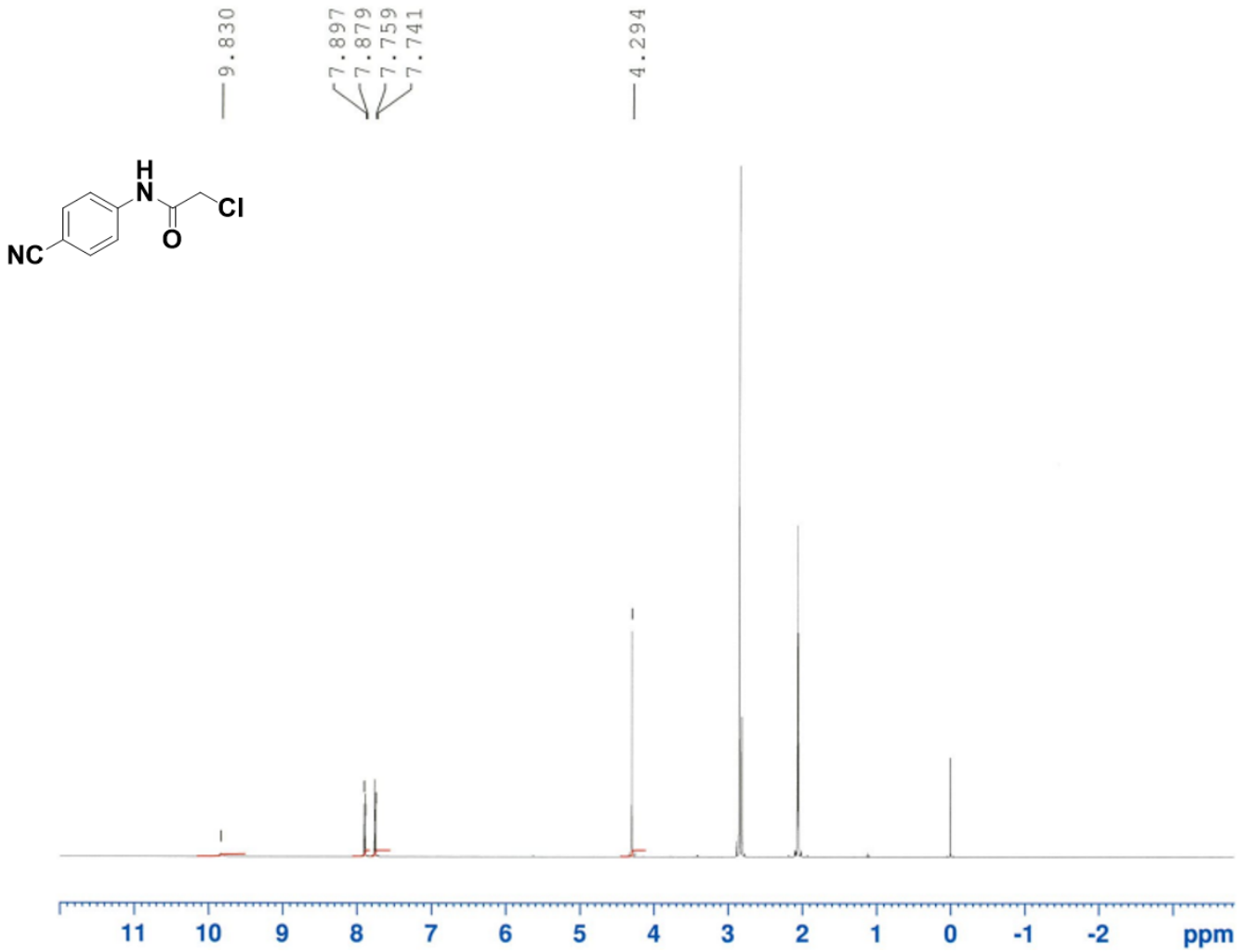

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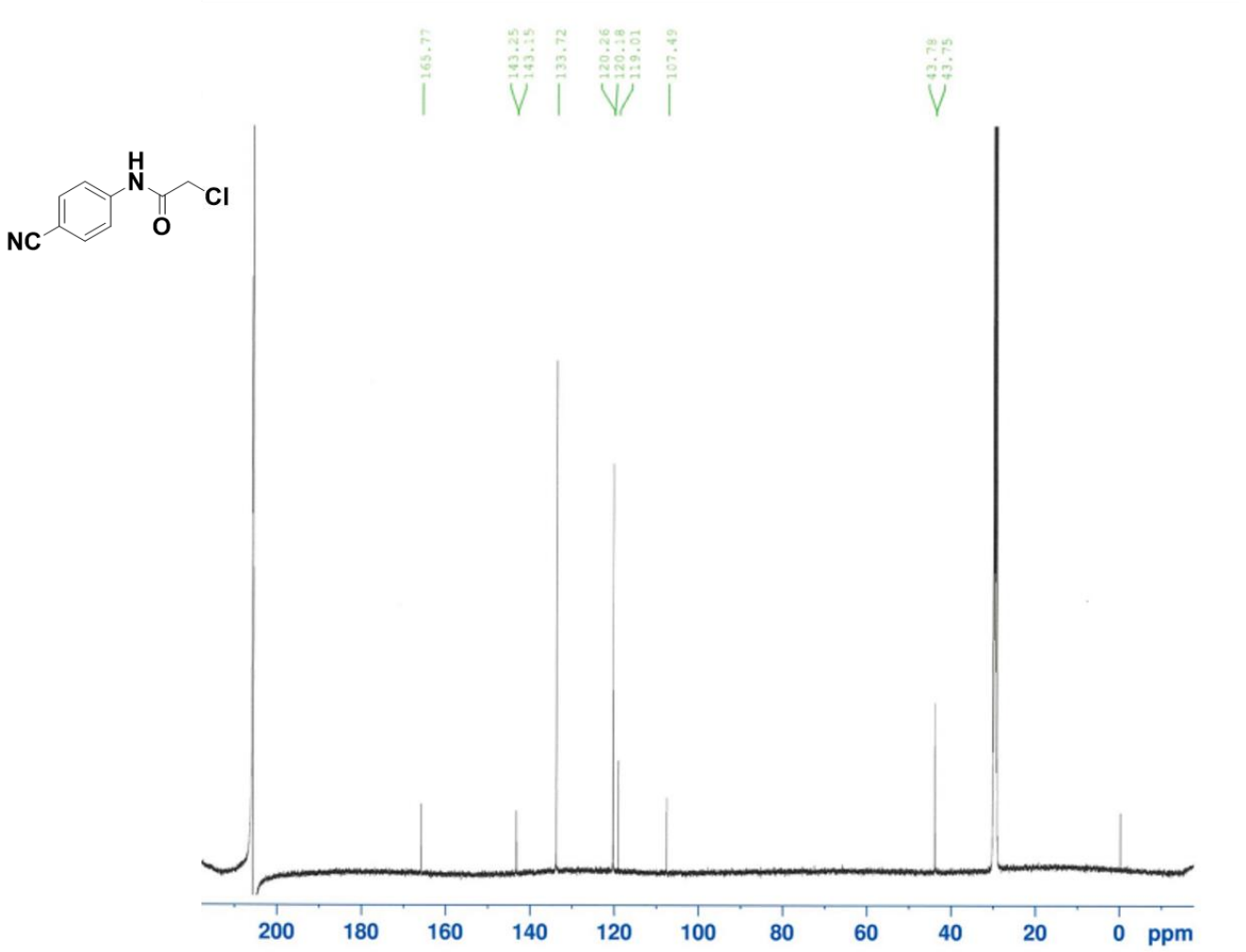

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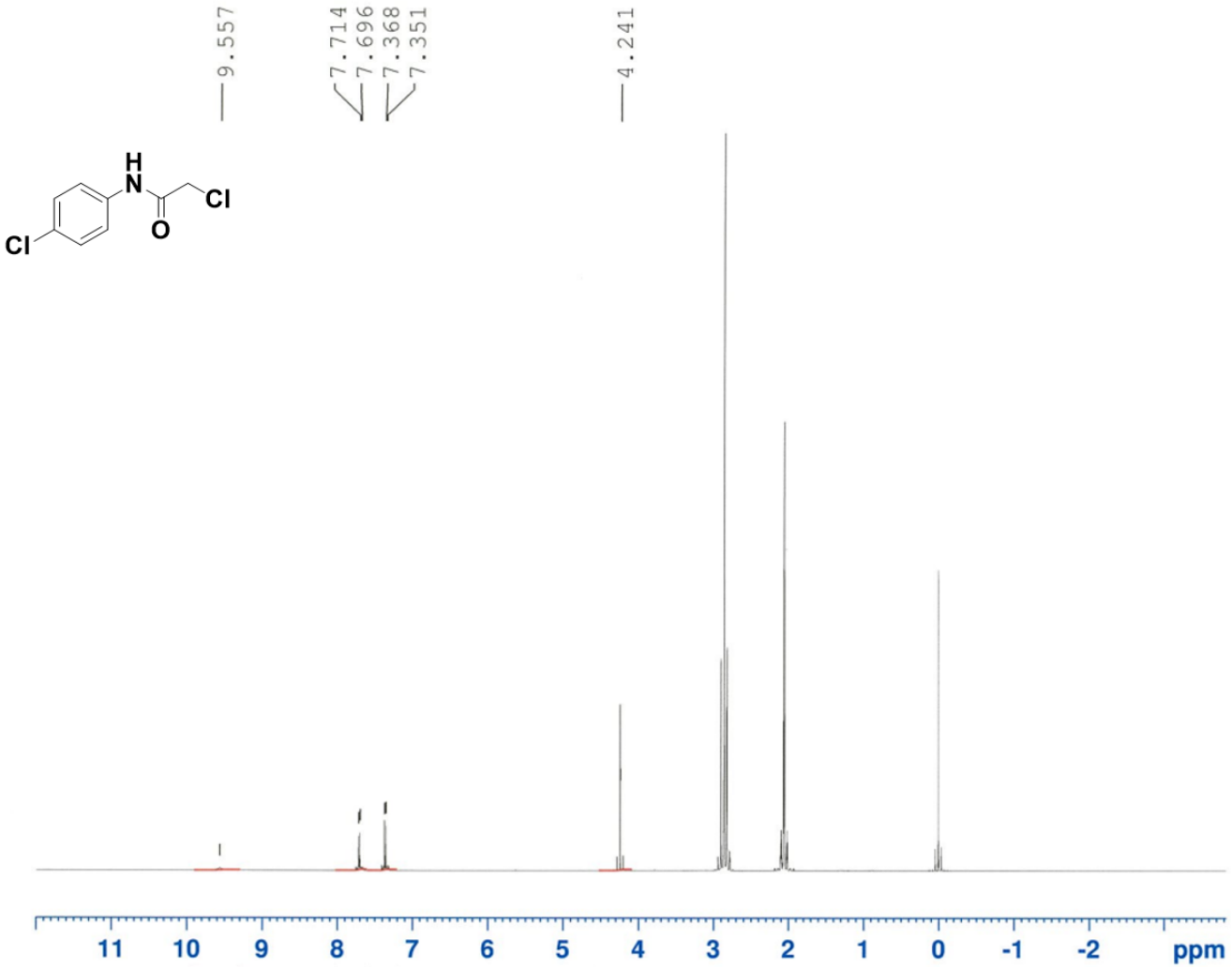

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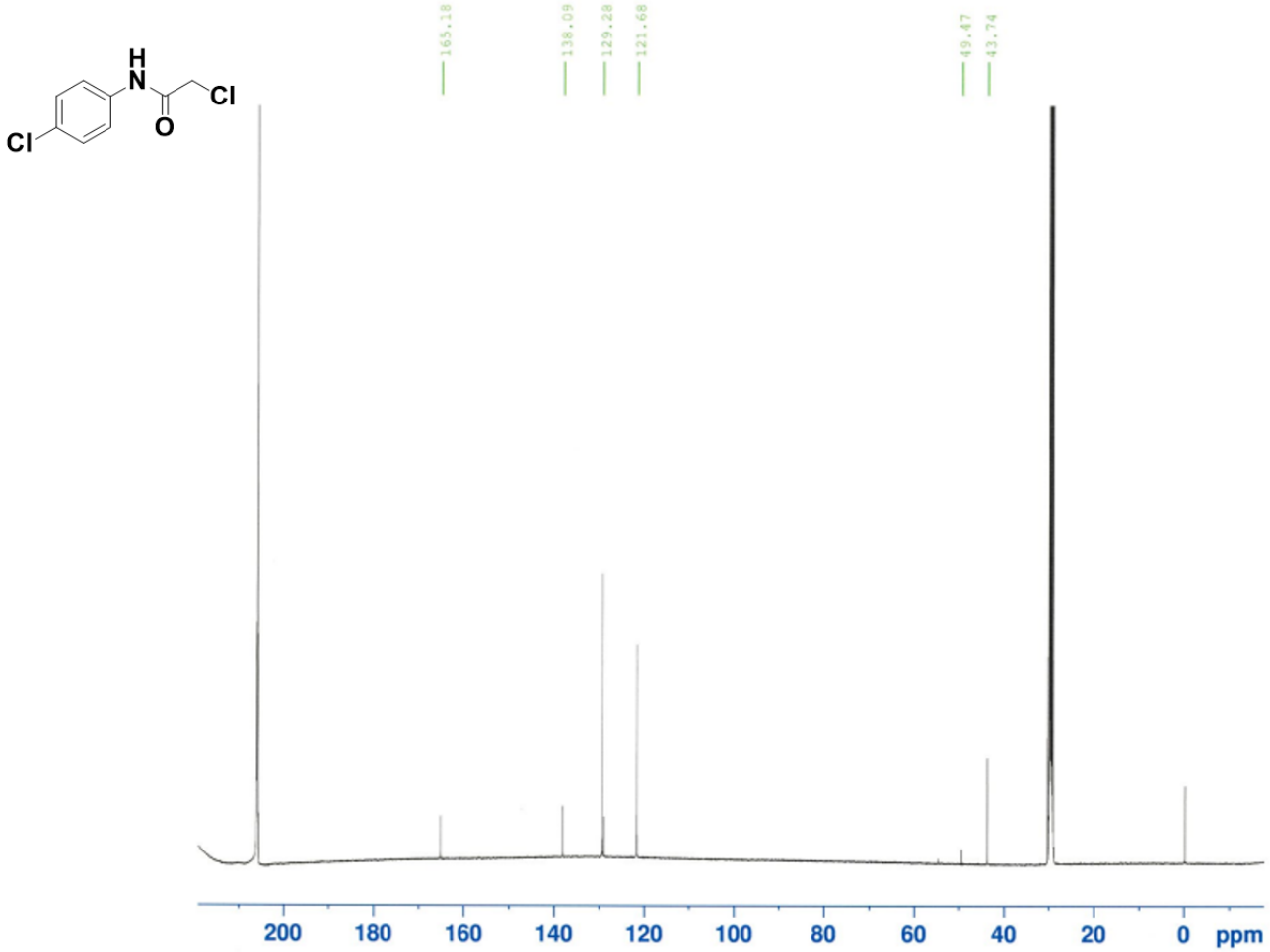

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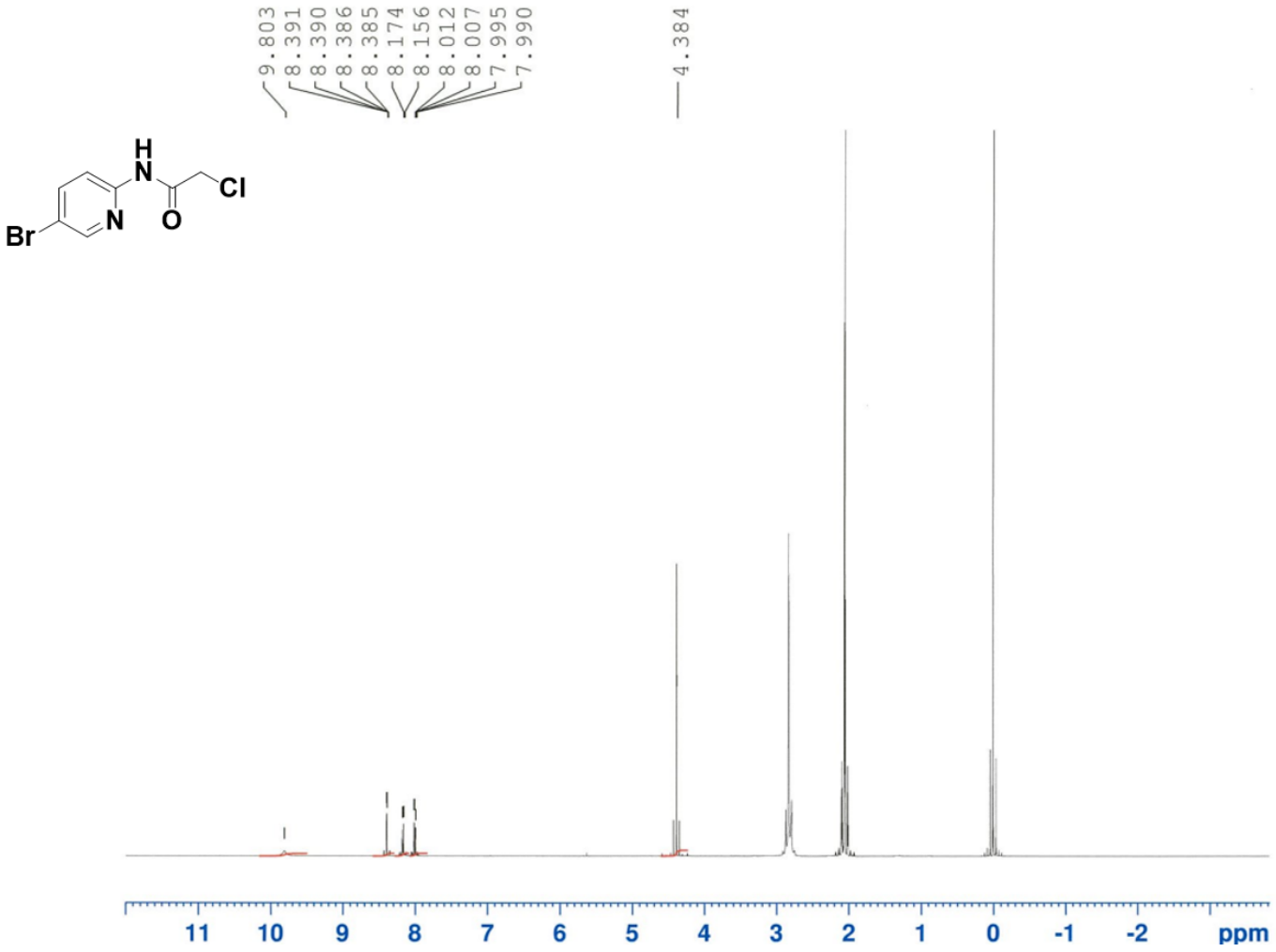

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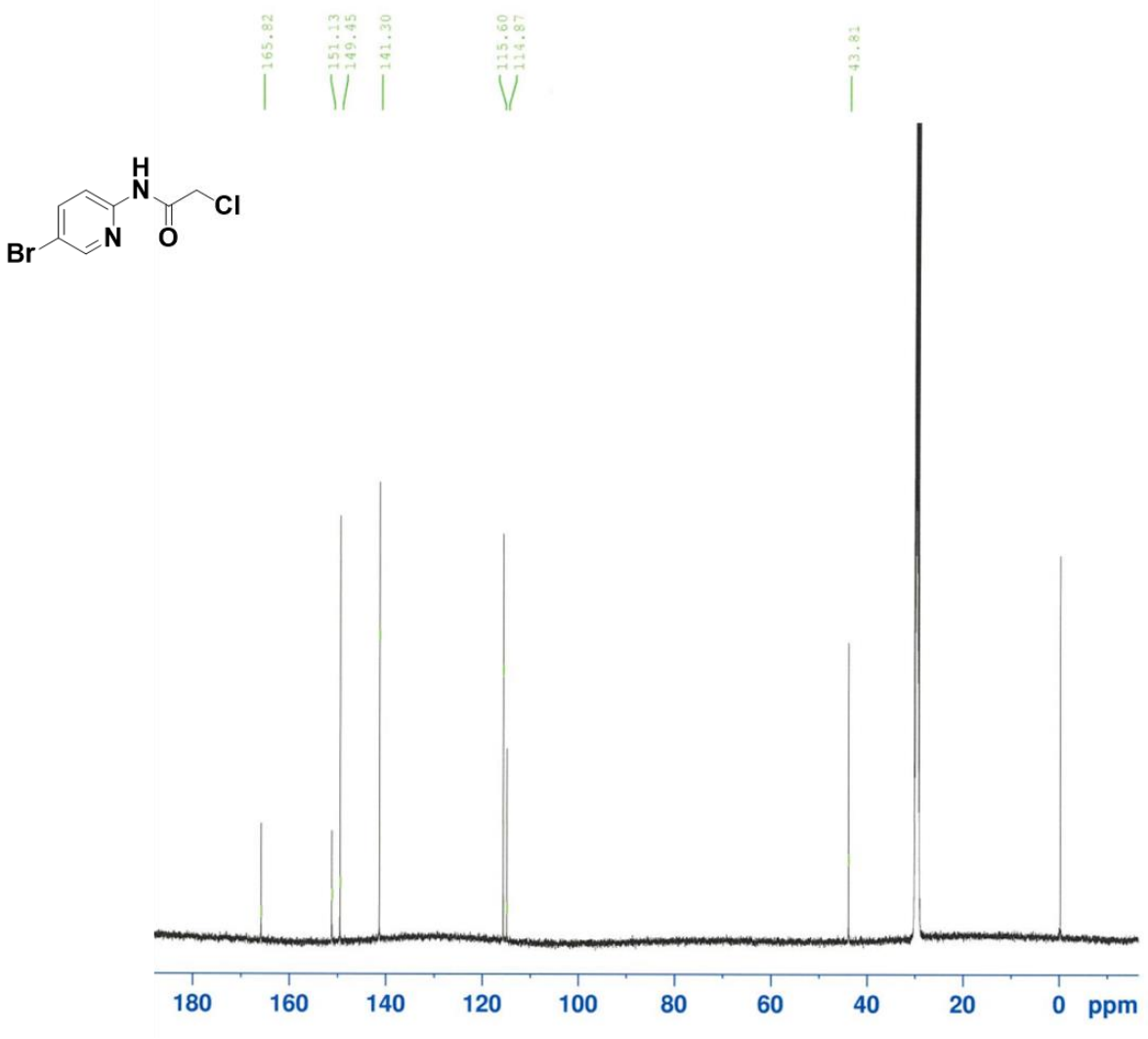

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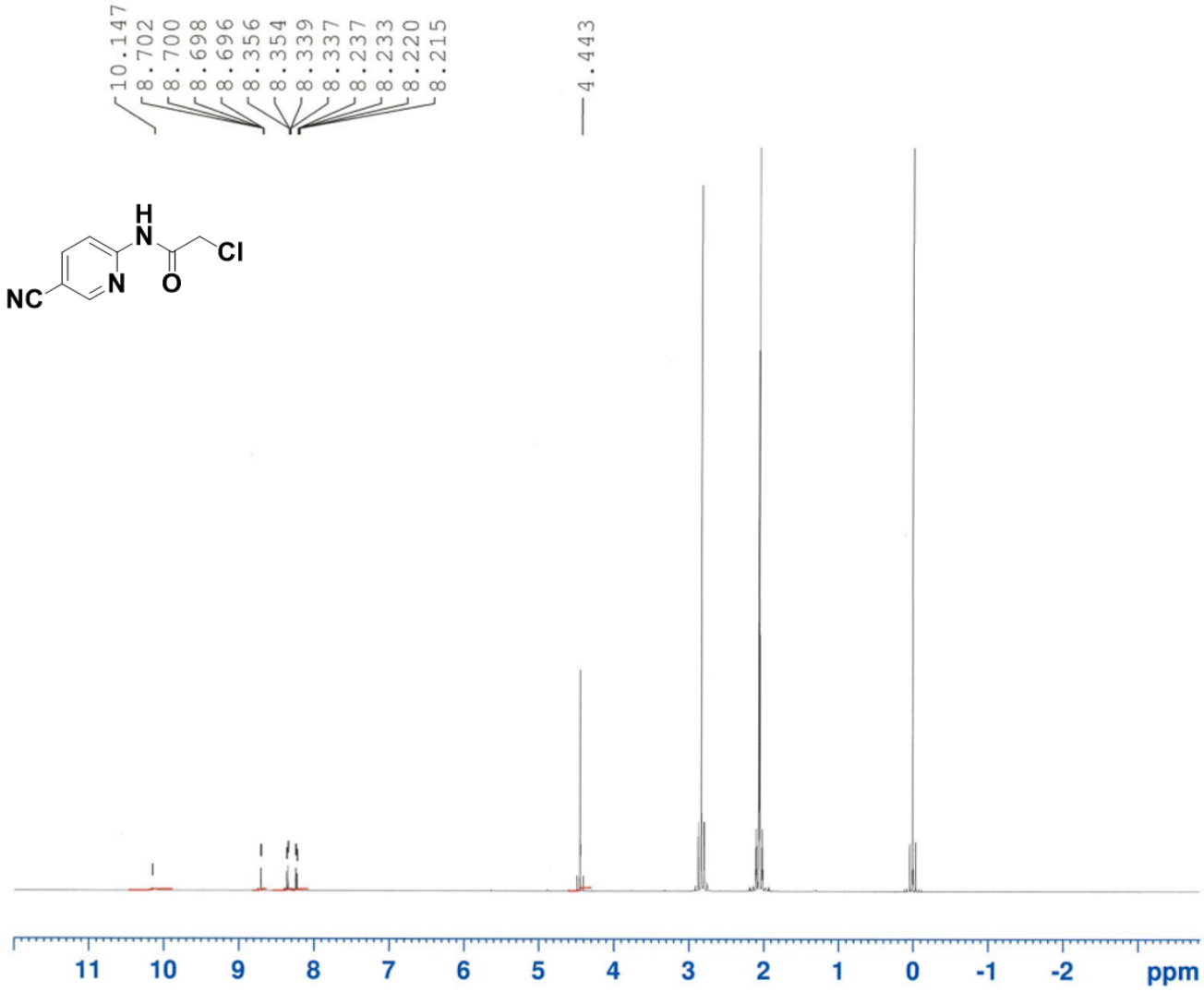

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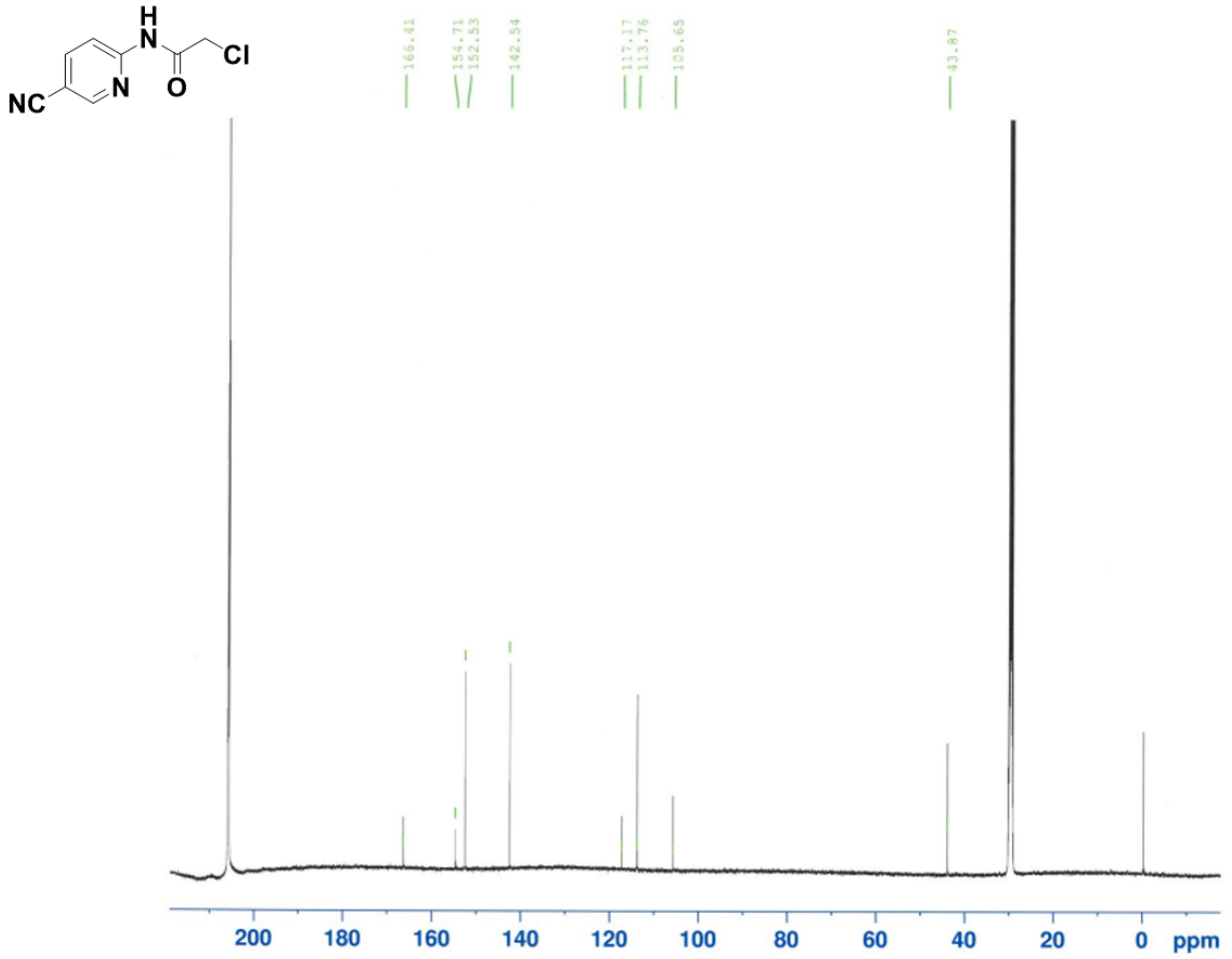

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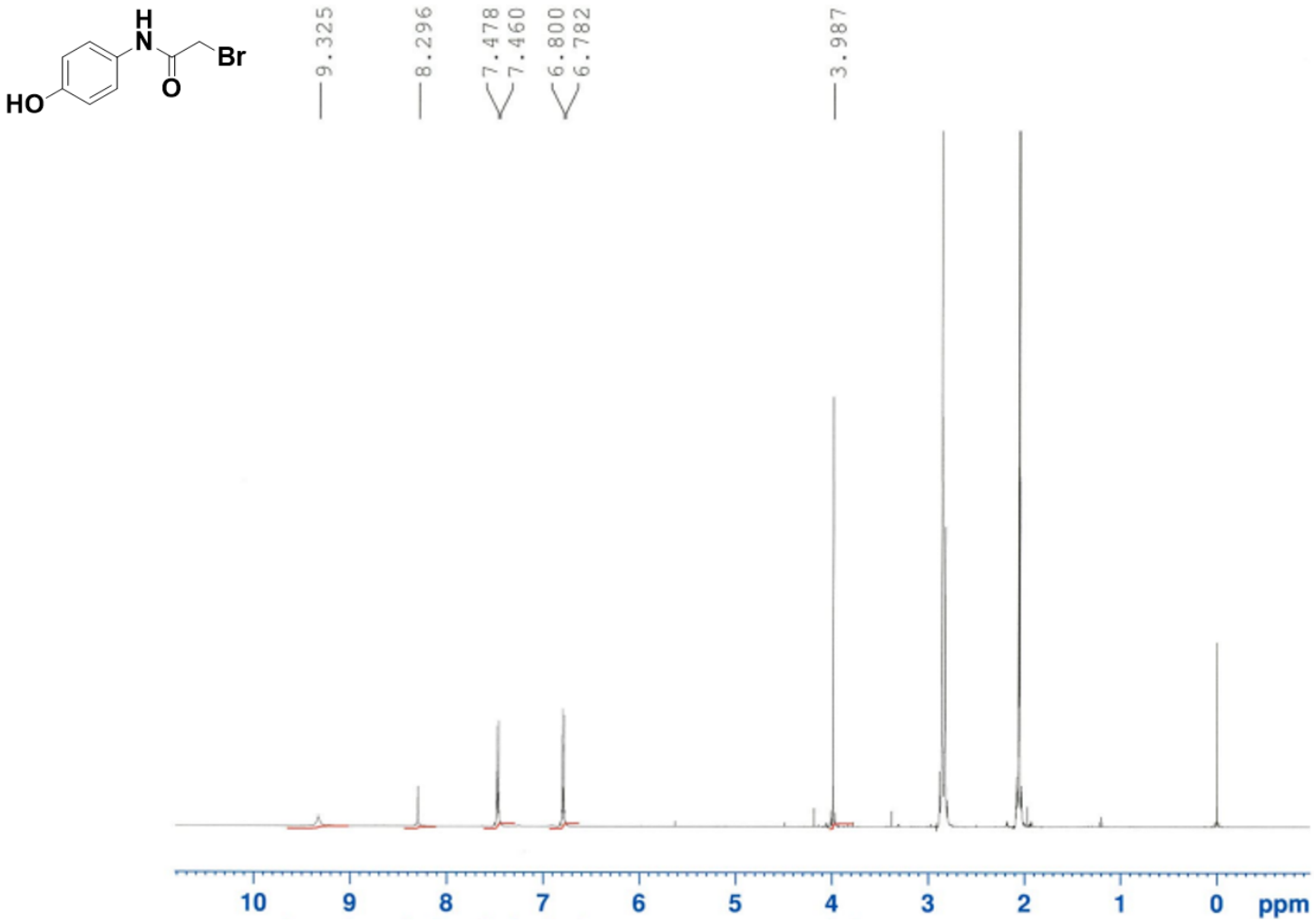

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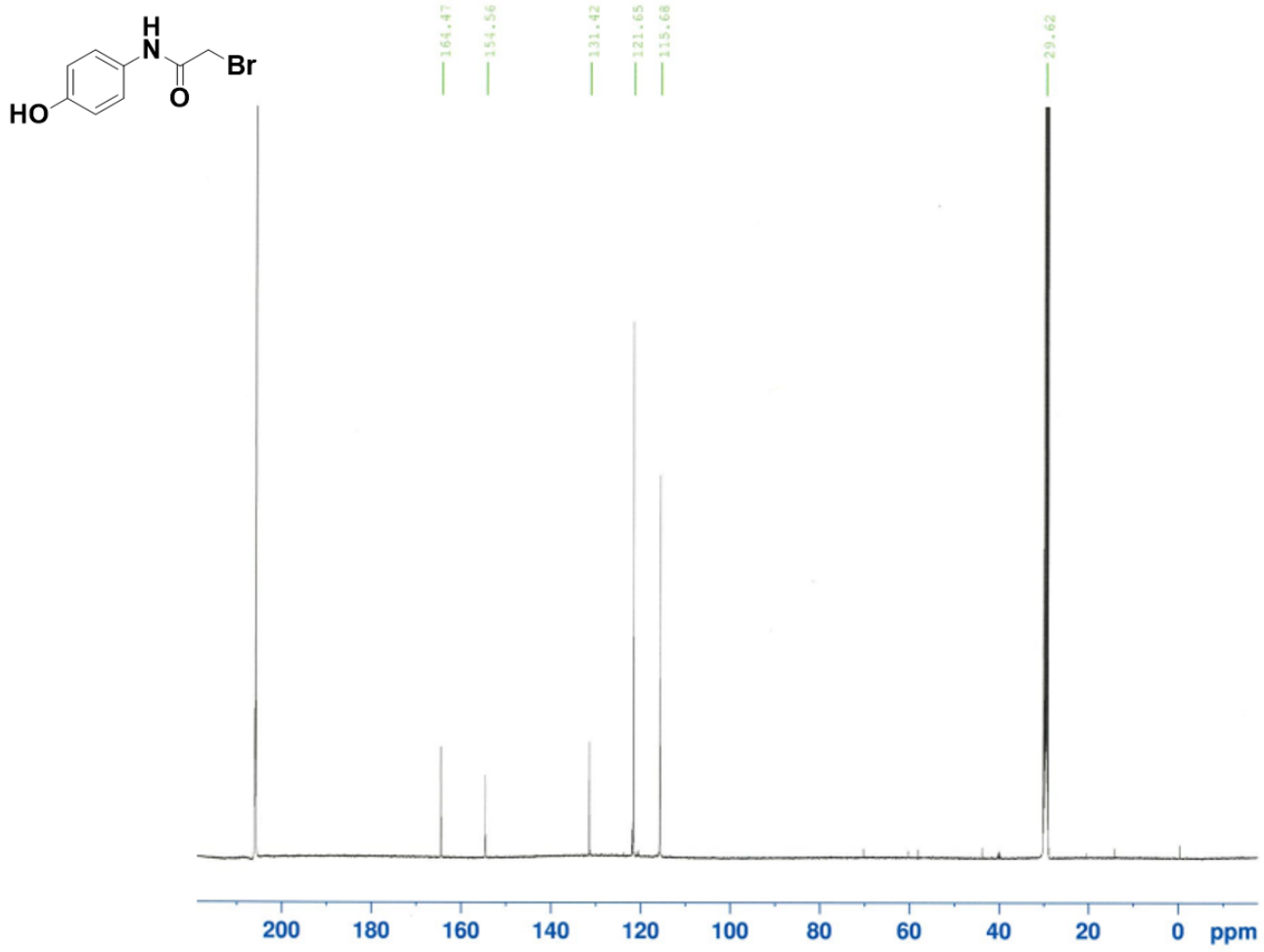

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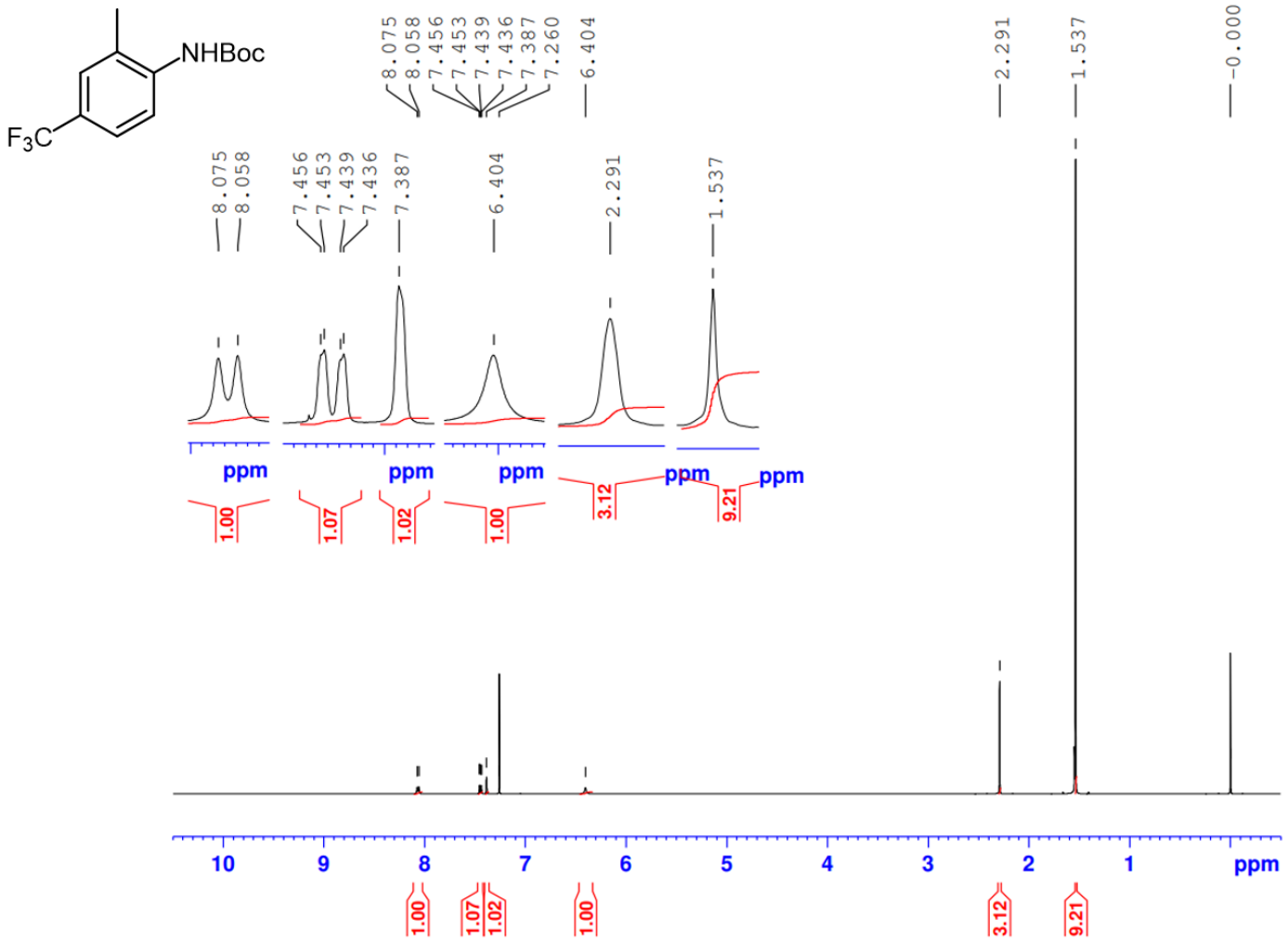

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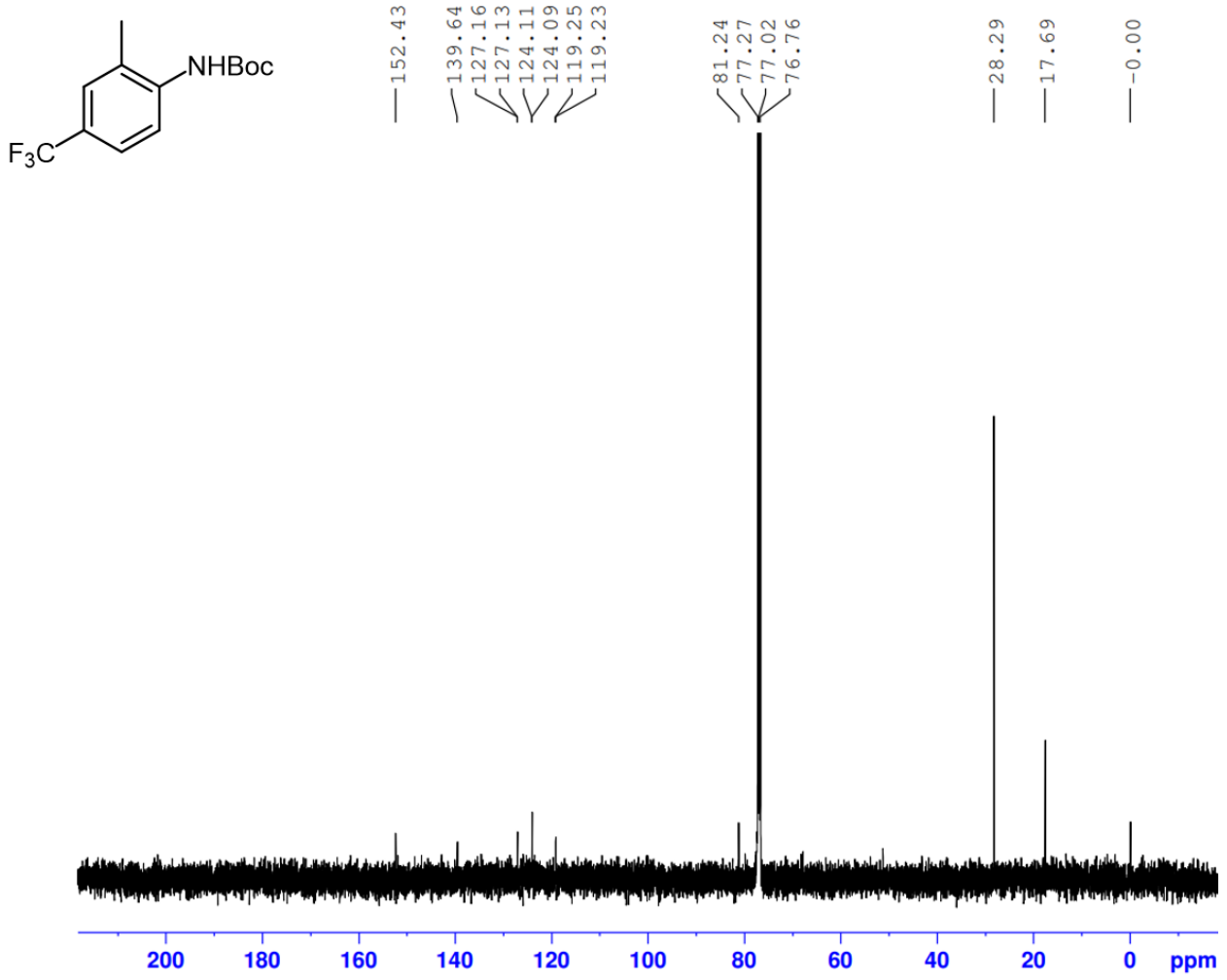

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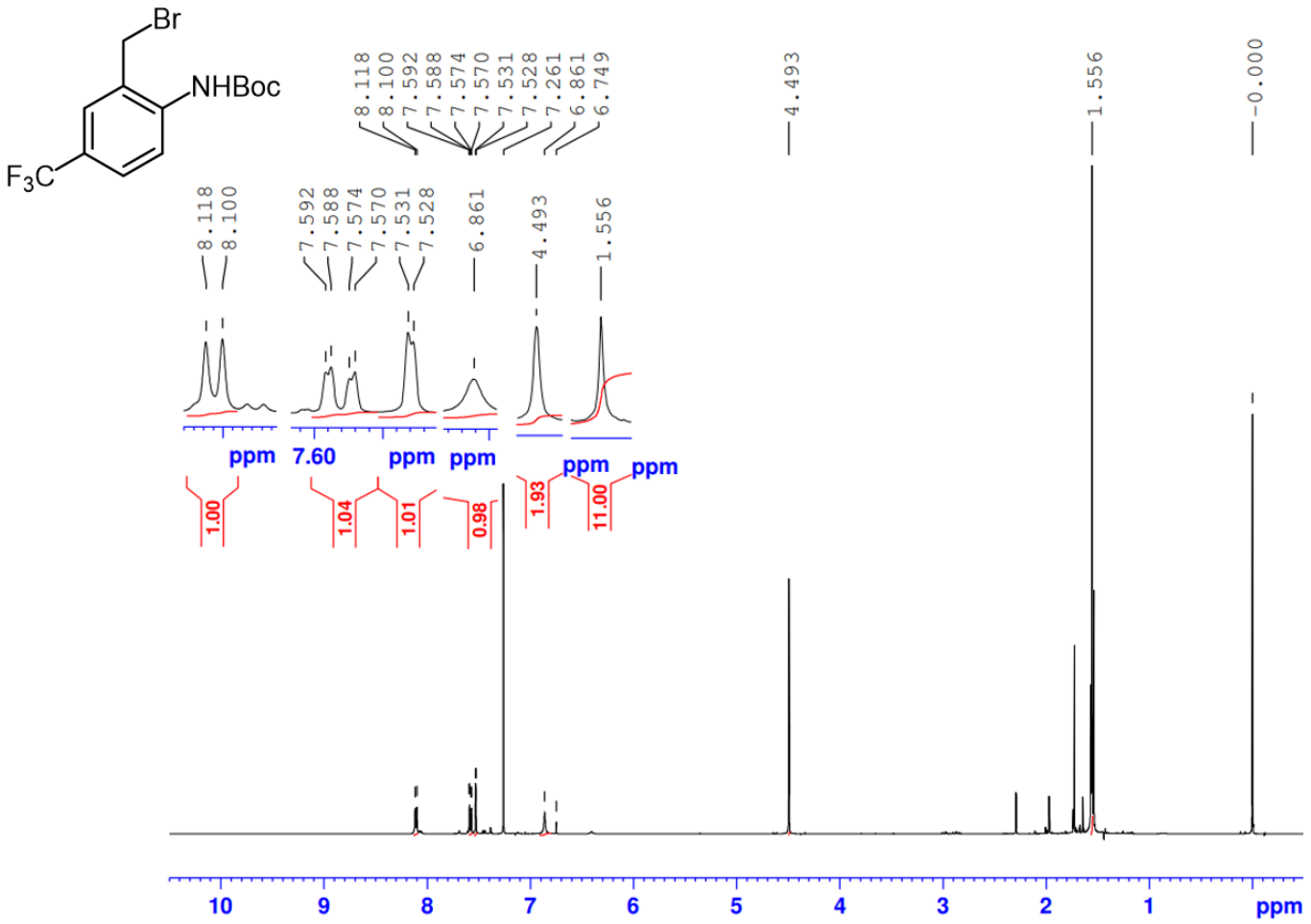

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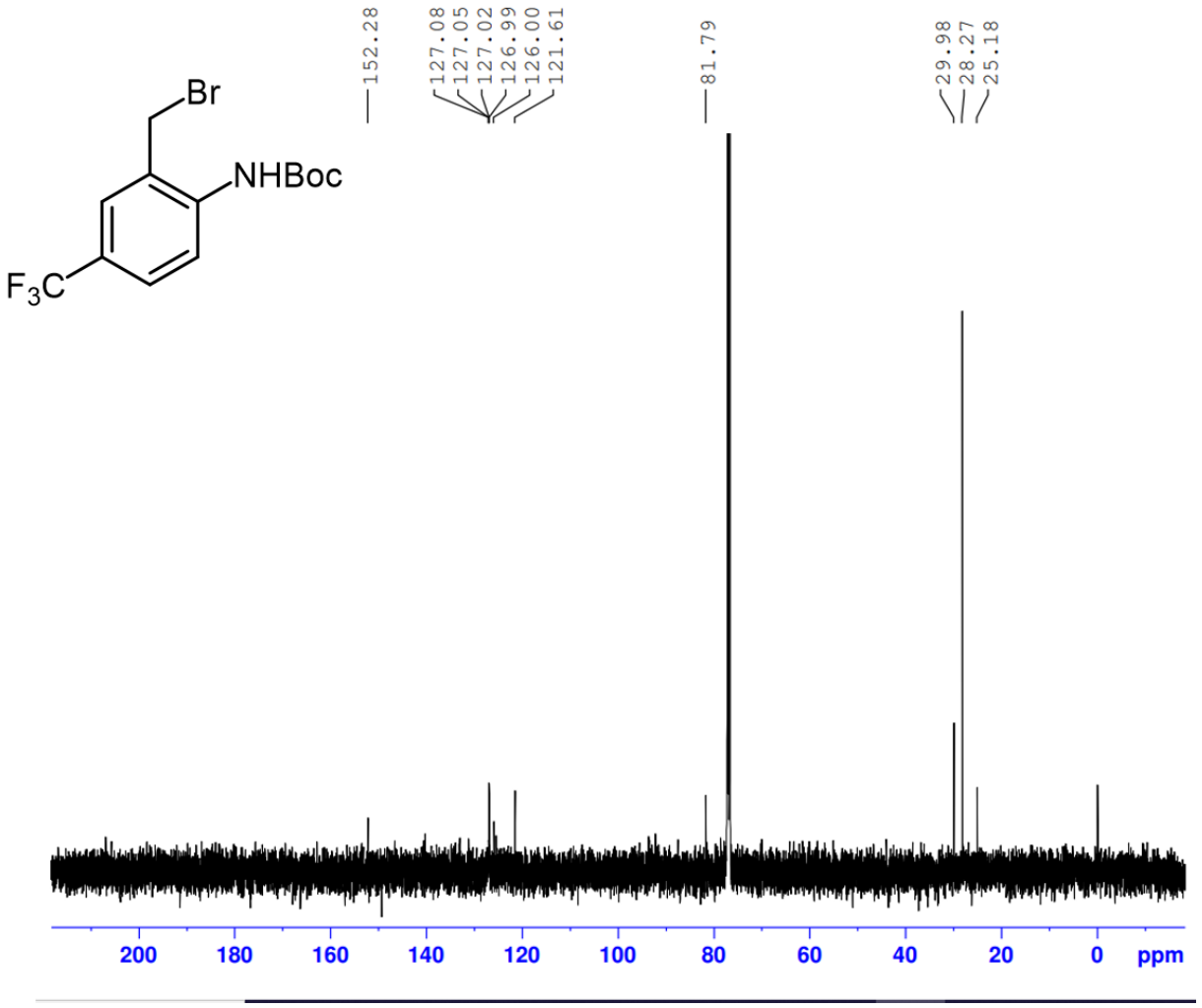

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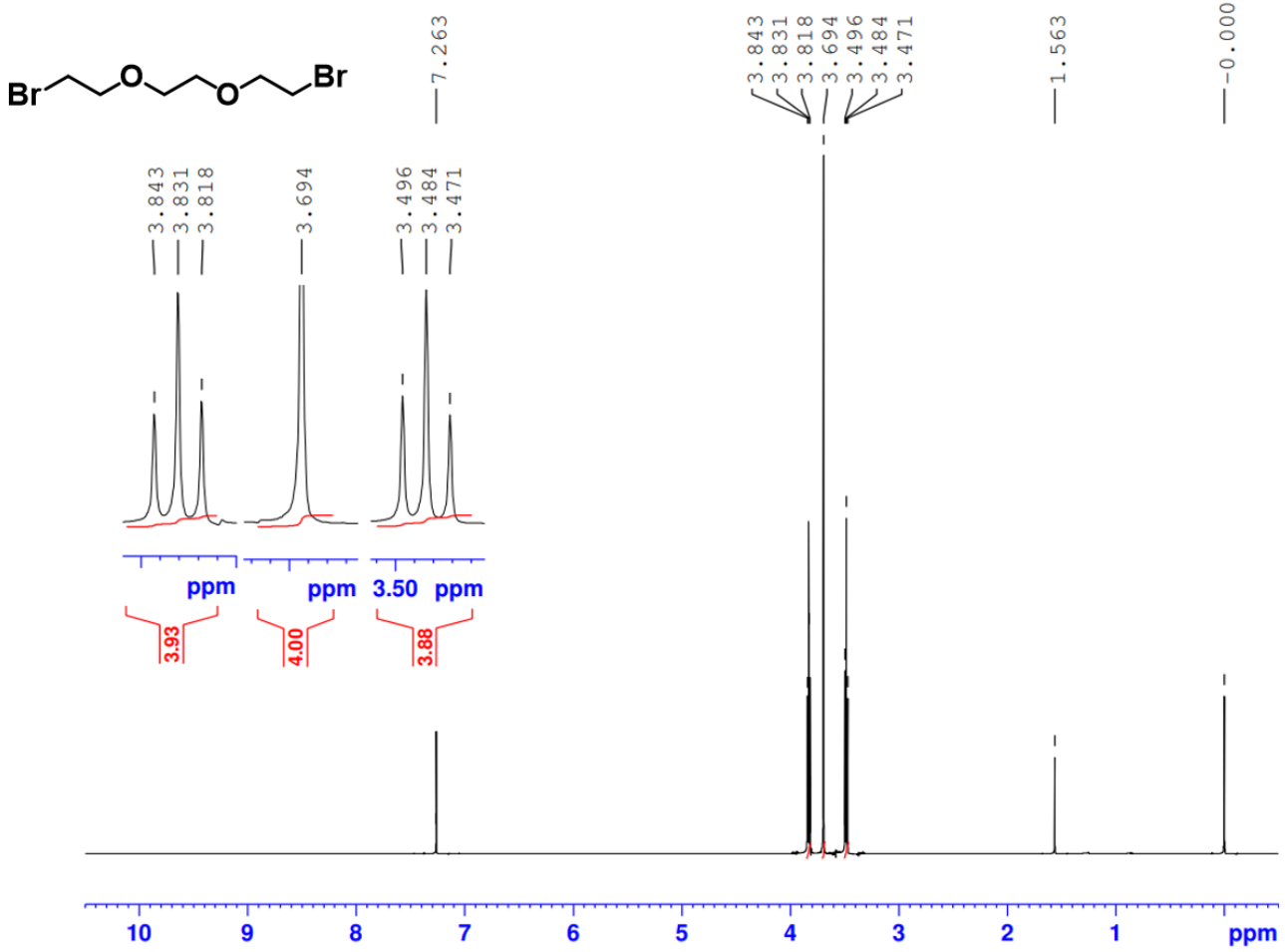

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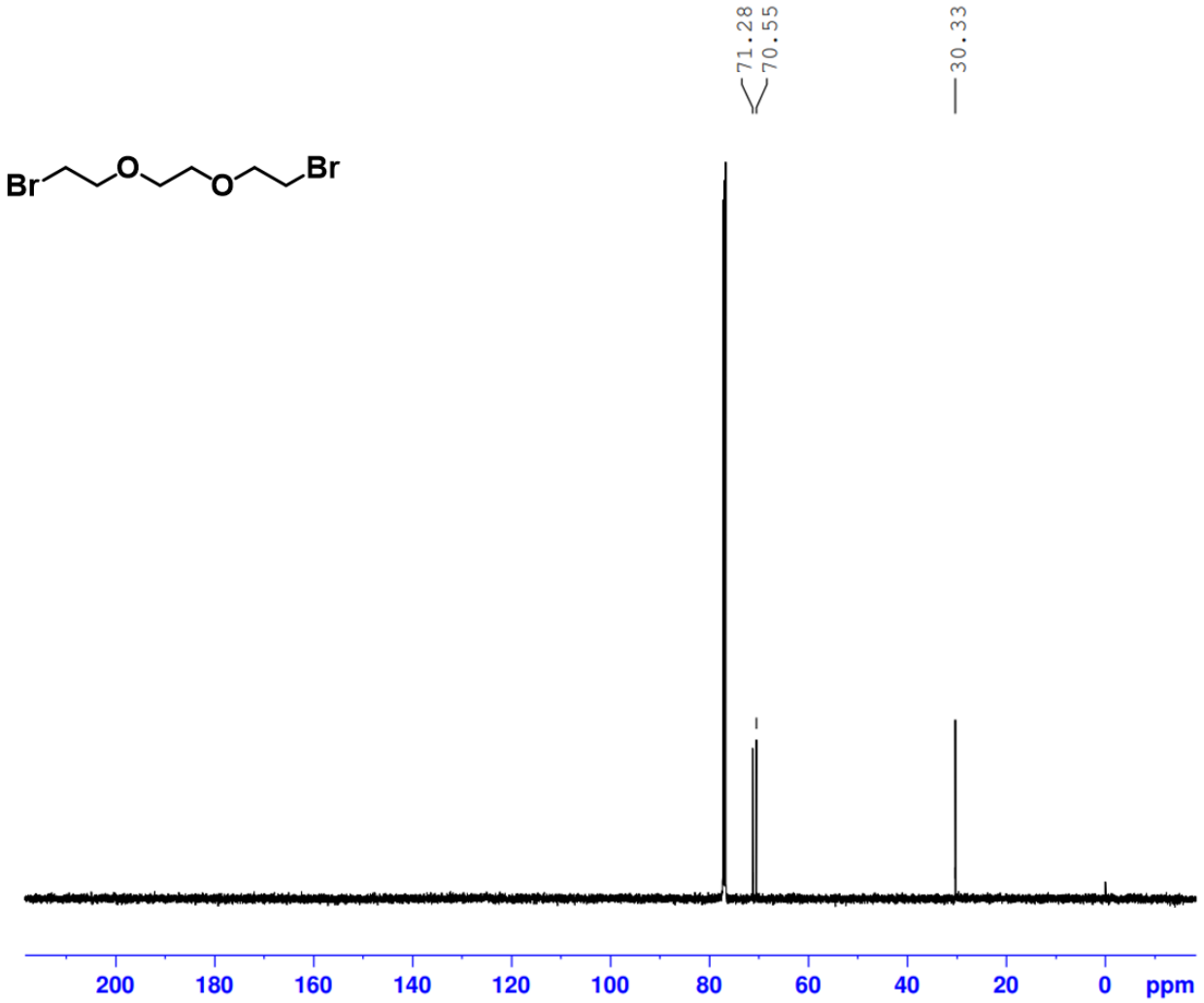

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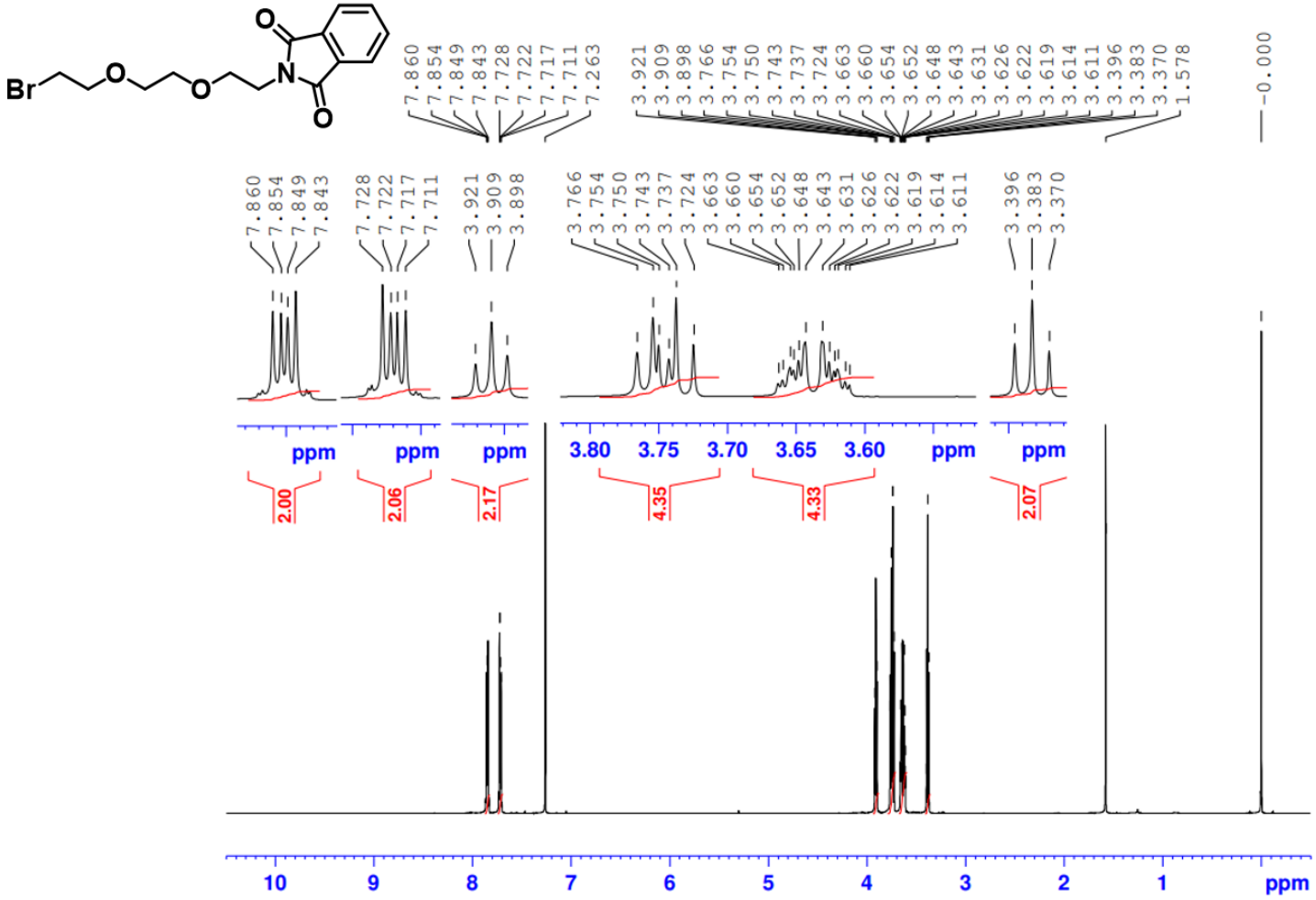
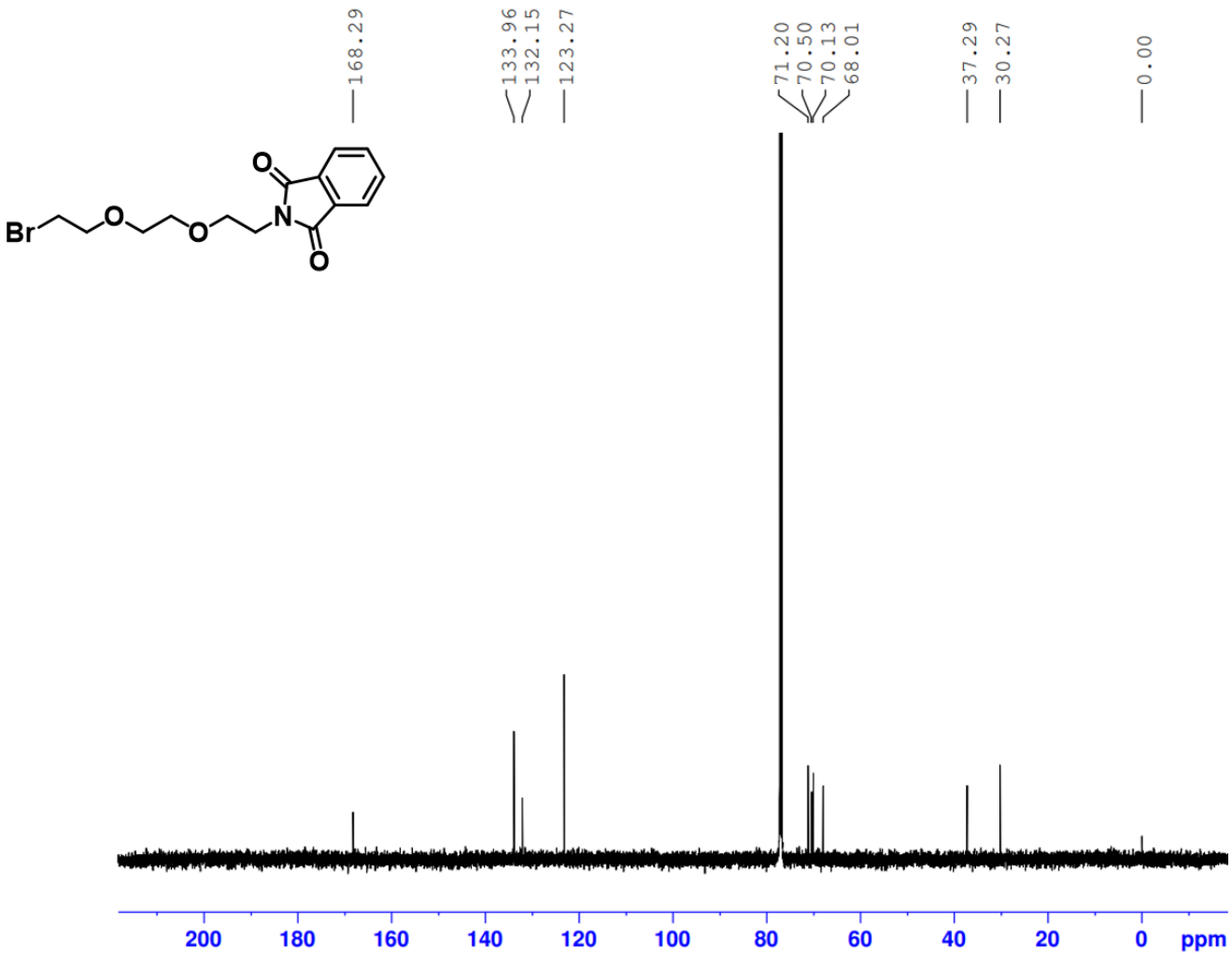
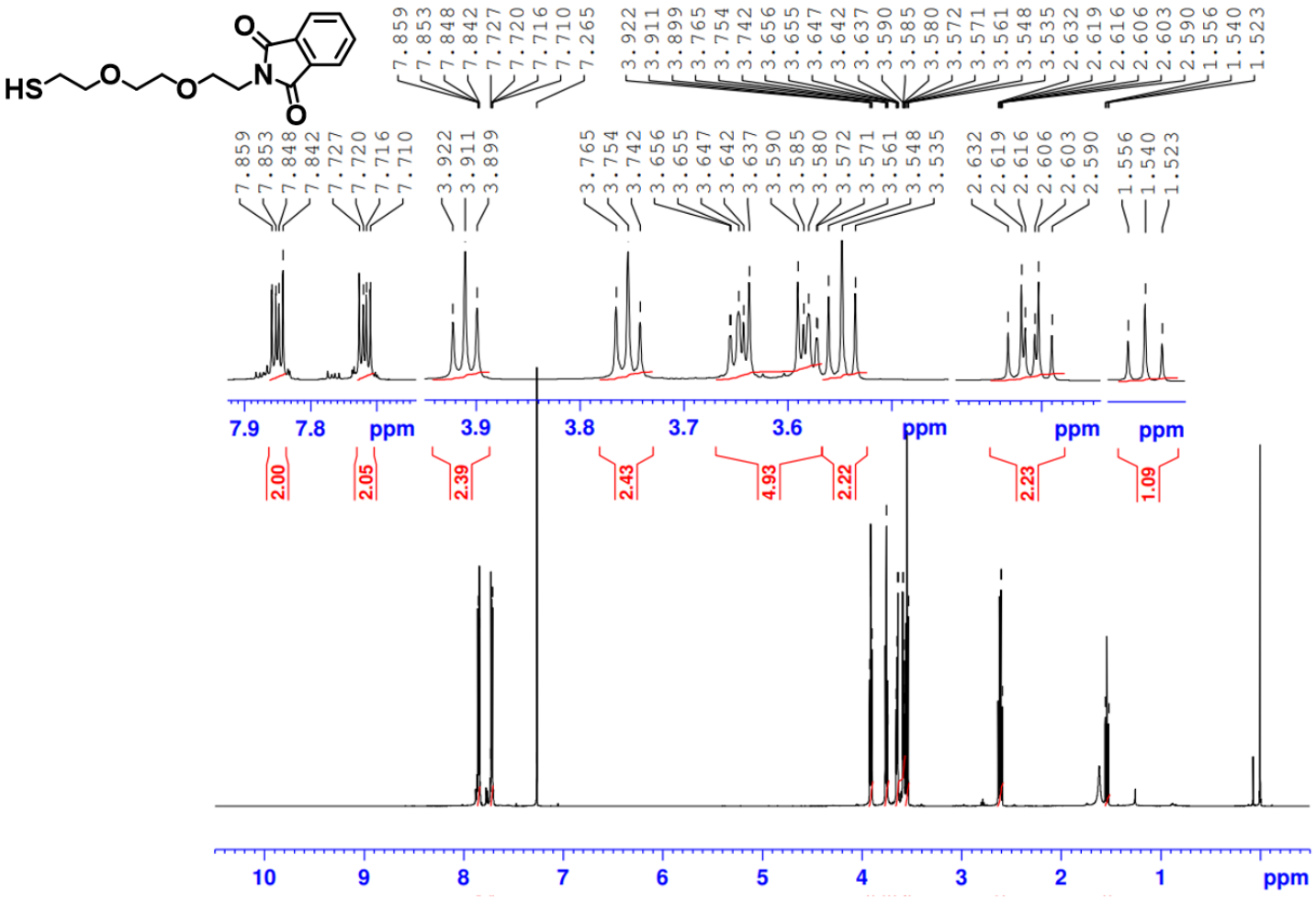


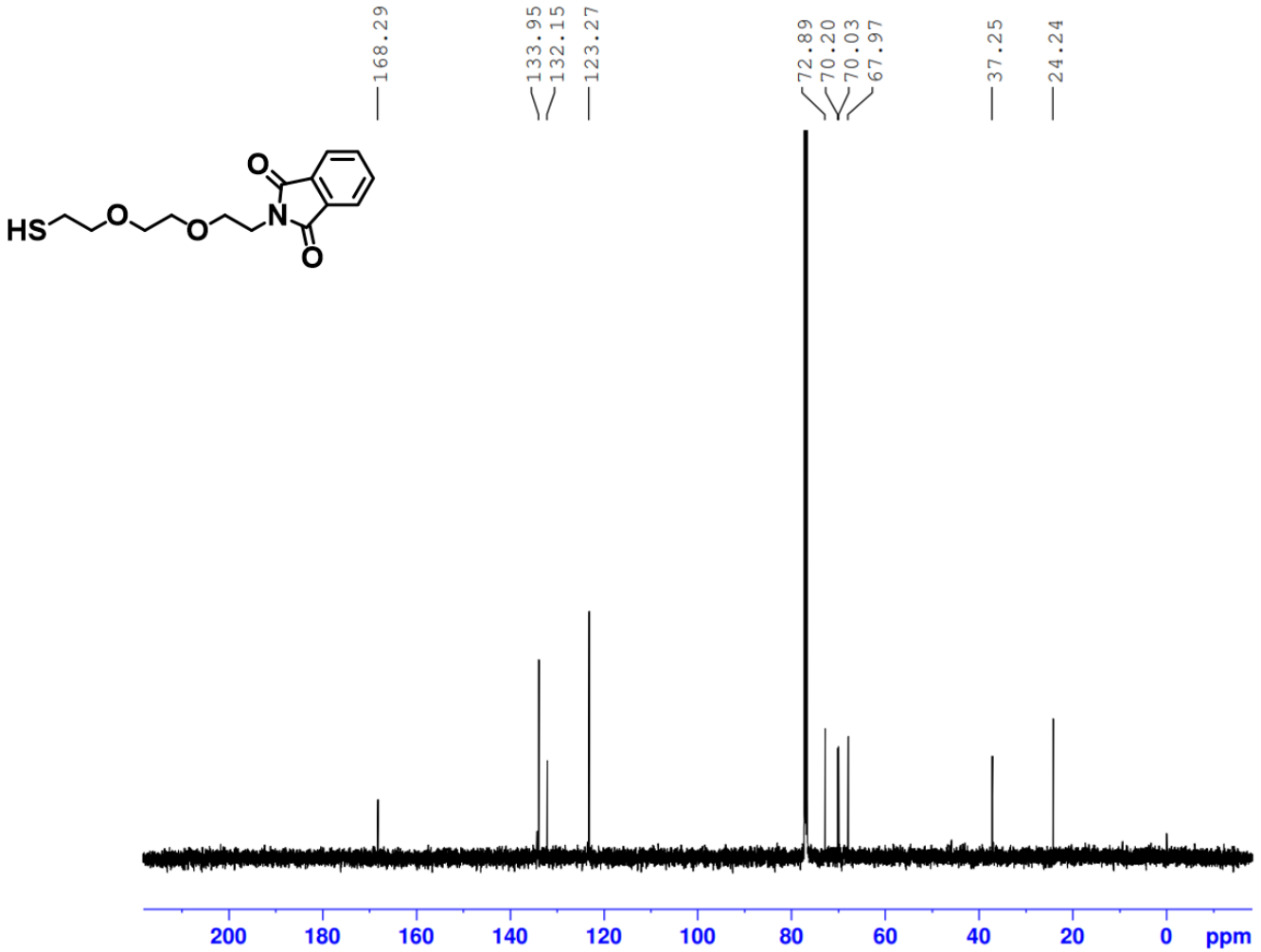
^{13}C NMR spectra of compound **16**



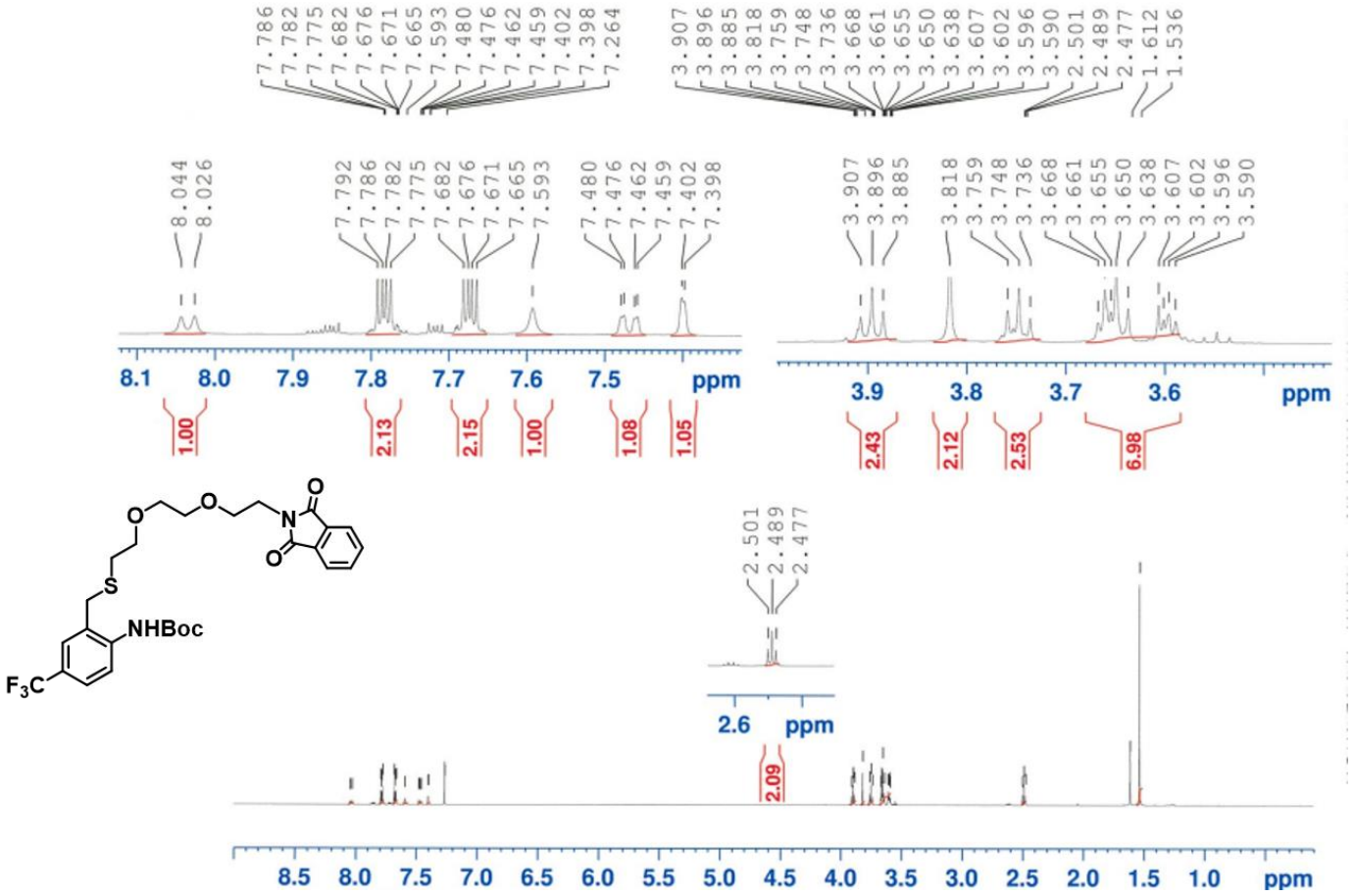
^{1}H NMR spectra of compound **17**



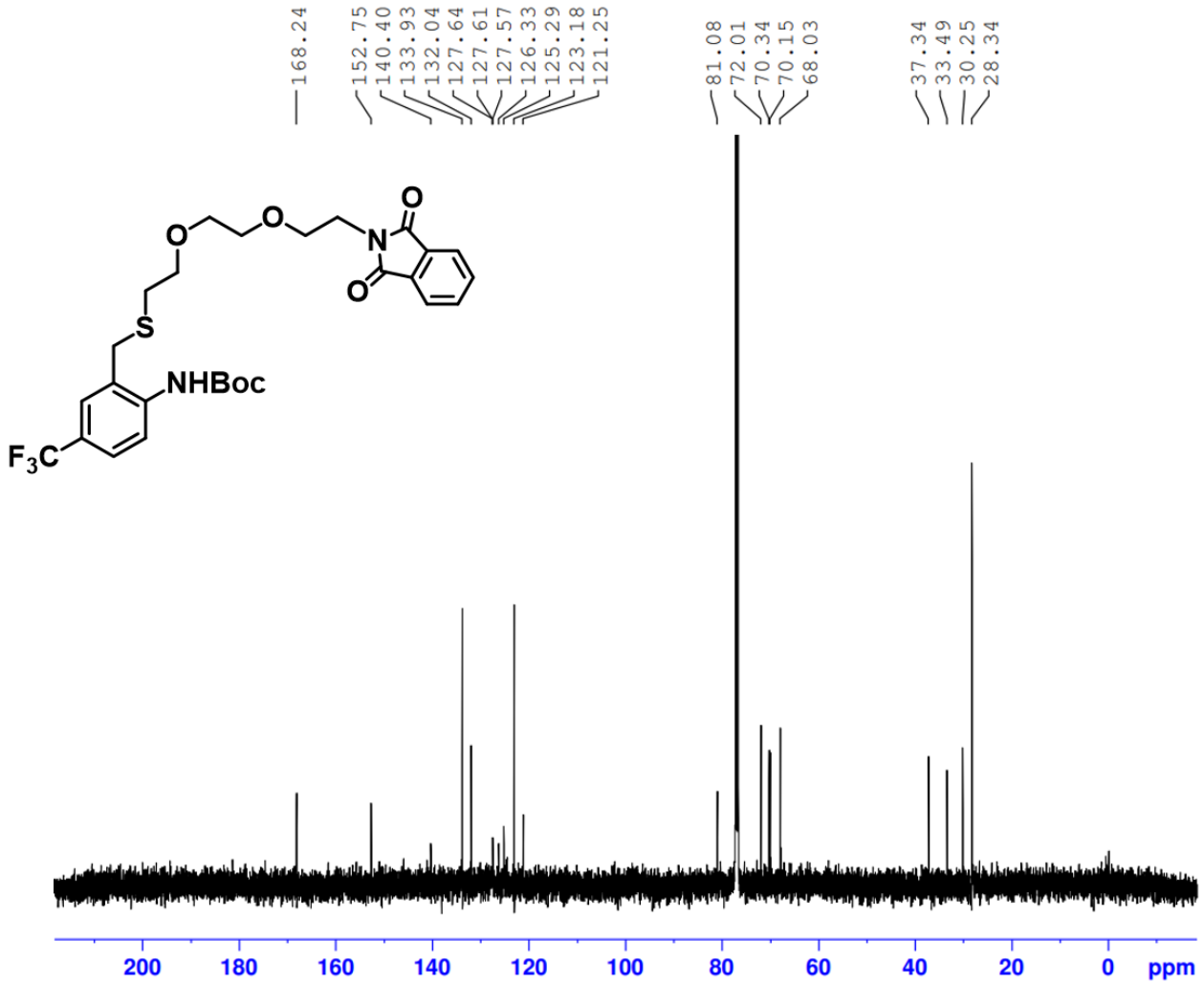
^{13}C NMR spectra of compound **17**



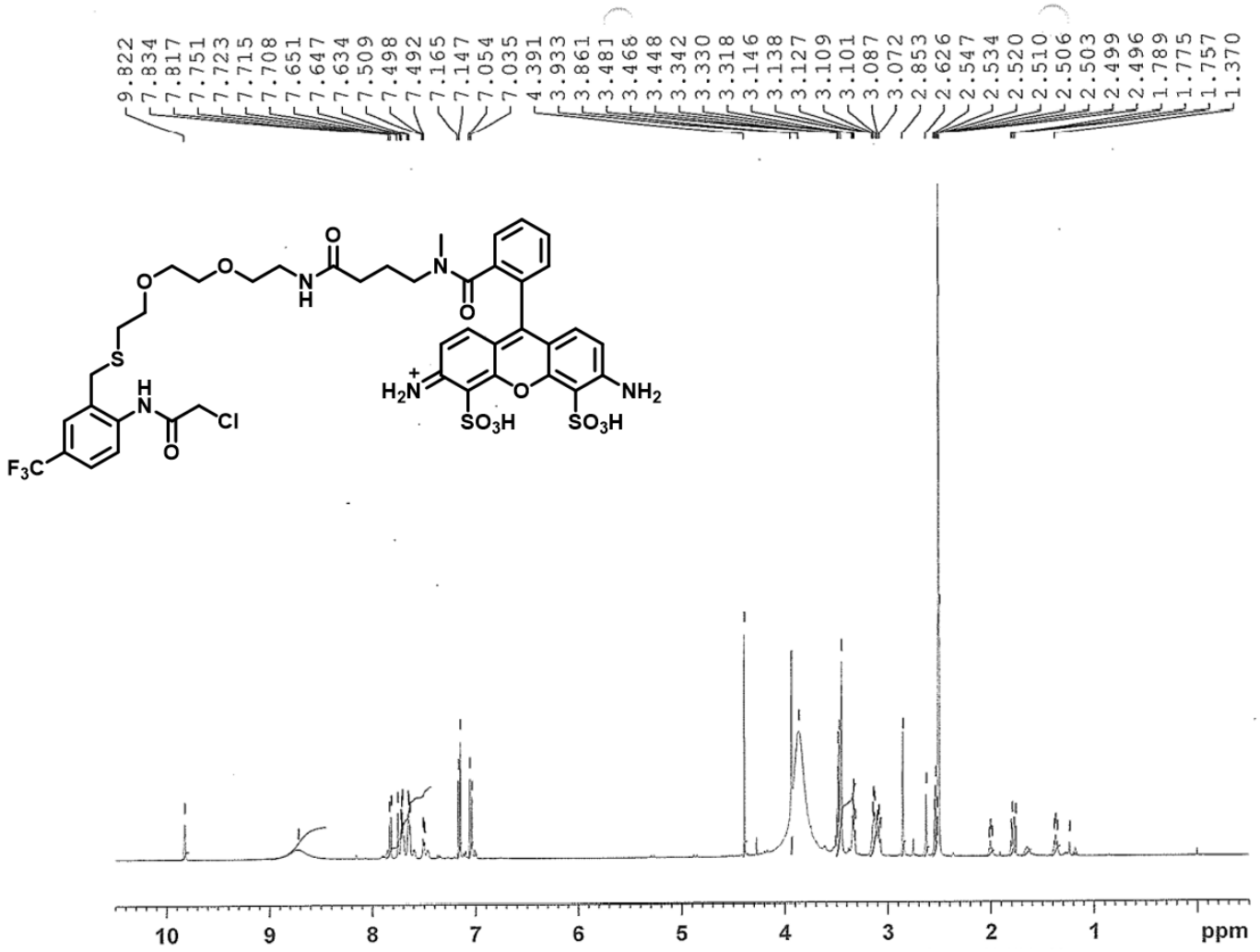
^{1}H NMR spectra of compound **18**



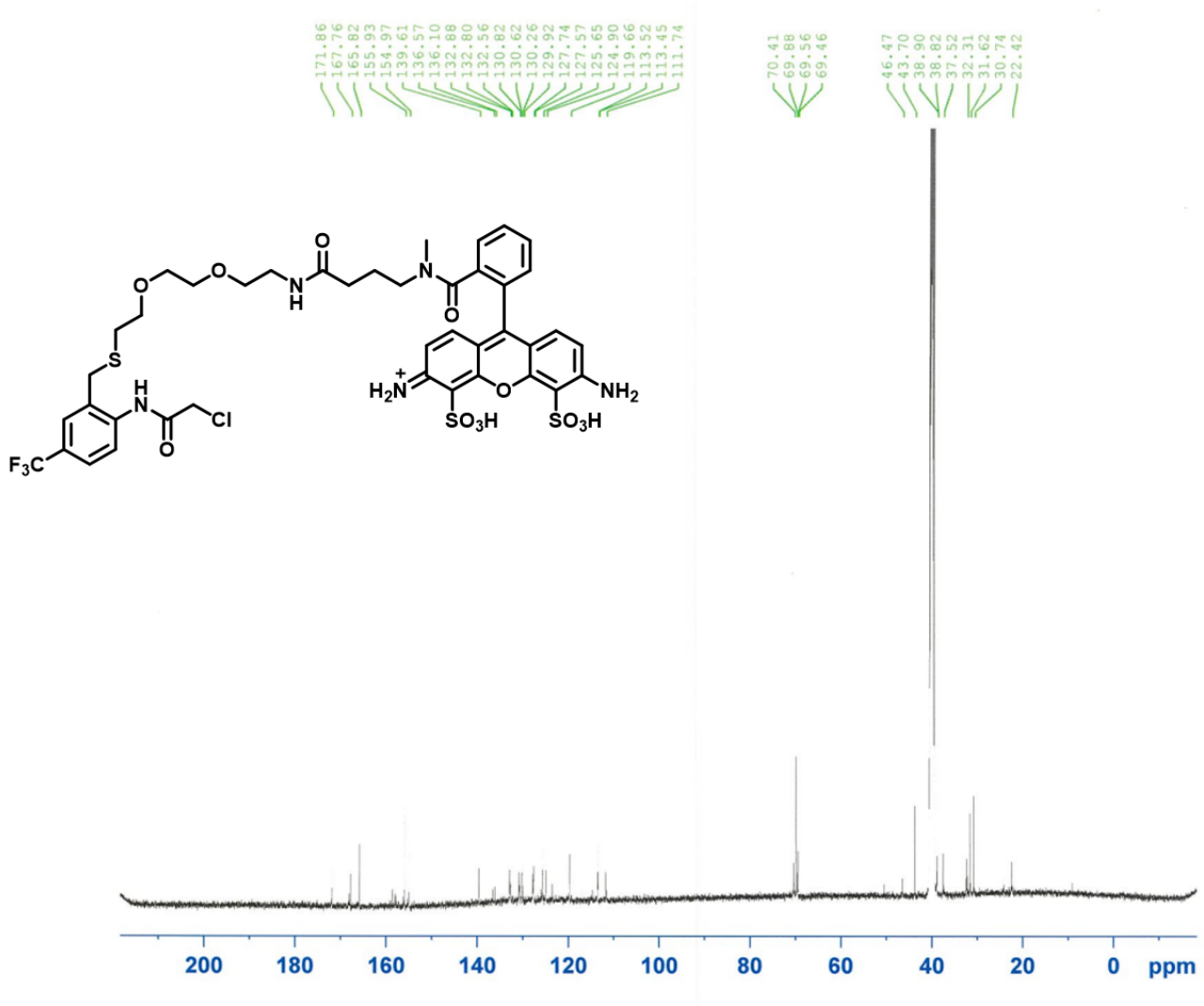
^{13}C NMR spectra of compound **18**



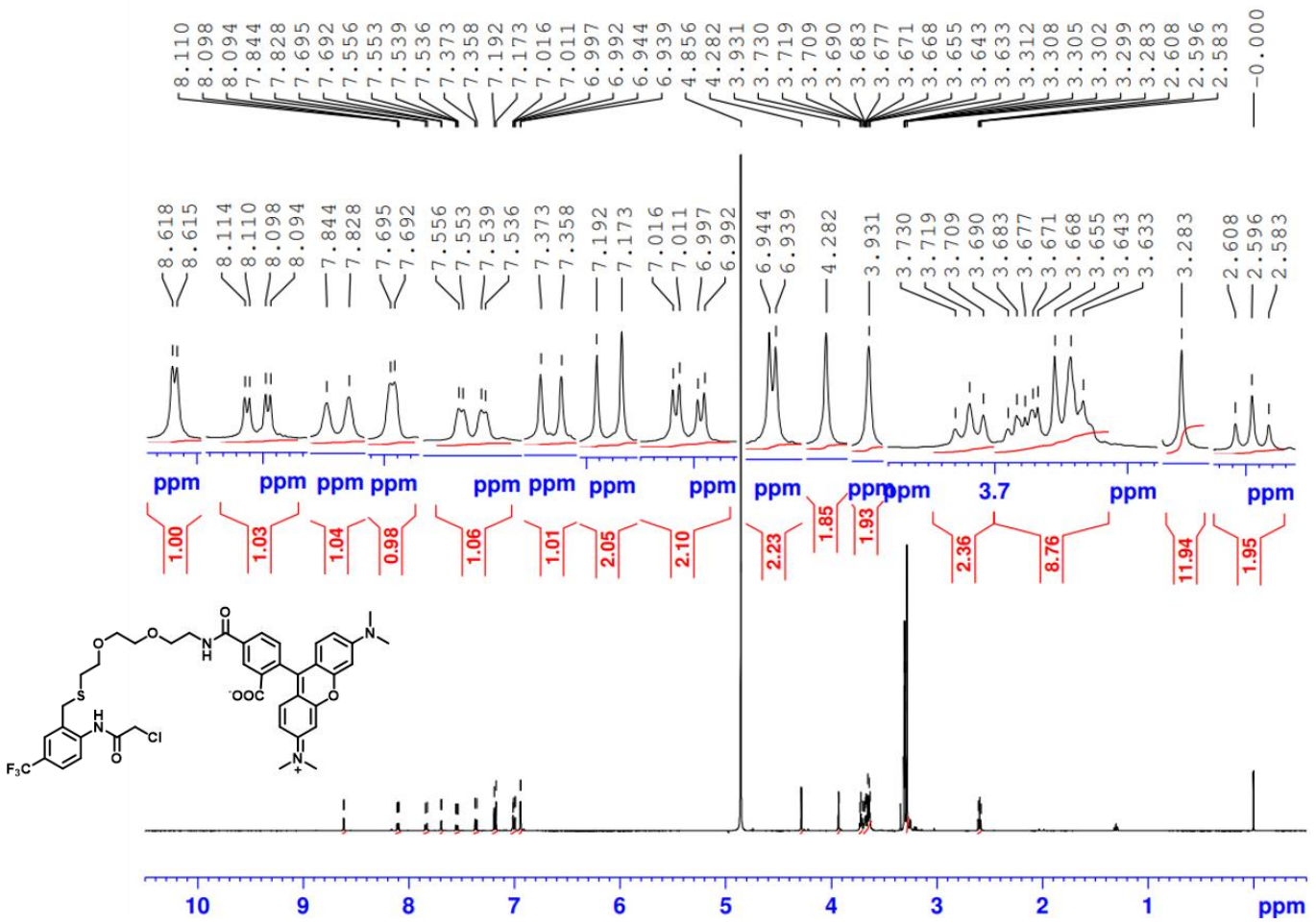
^{1}H NMR spectra of PCAFgreen



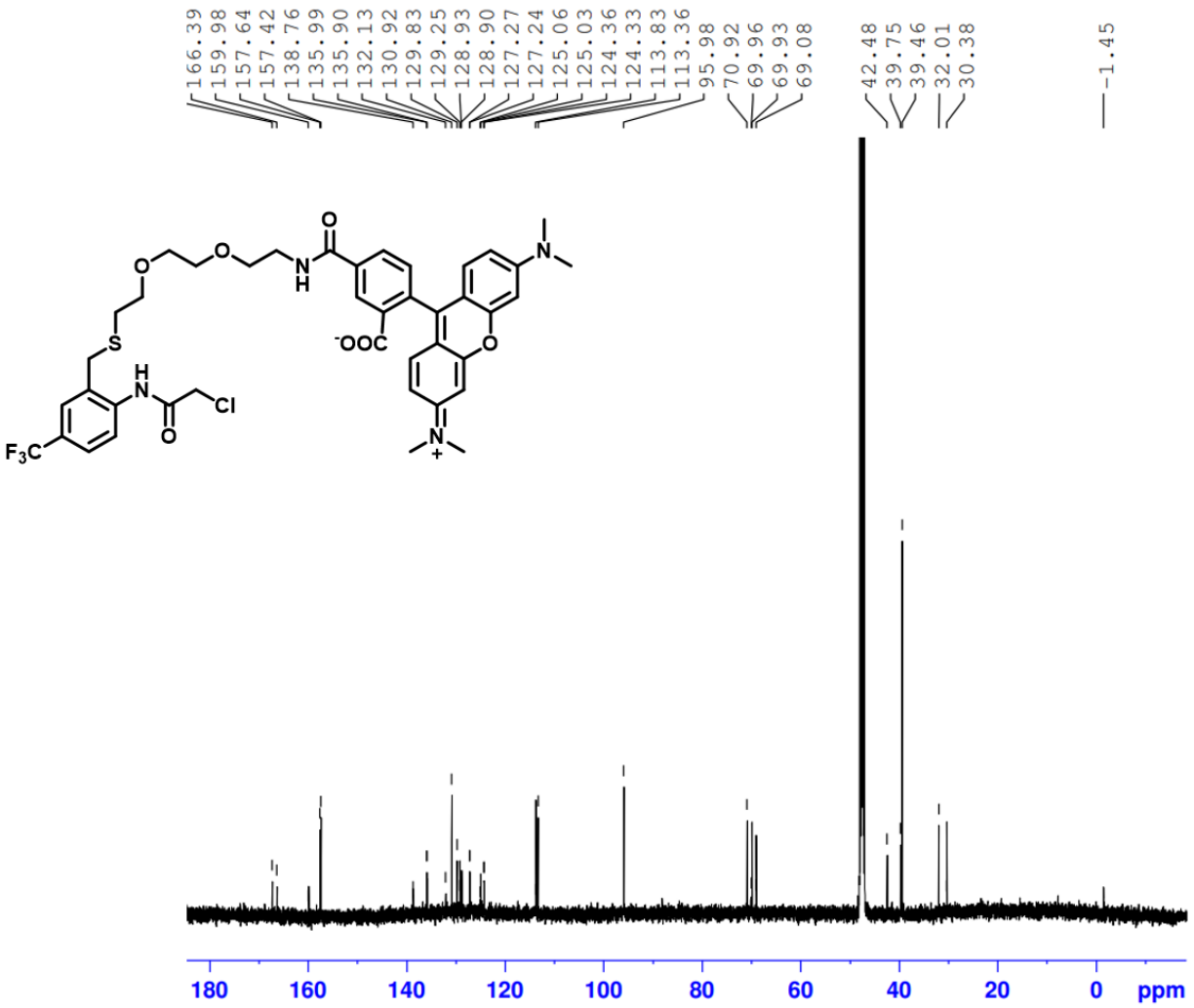
^{13}C NMR spectra of PCAFgreen



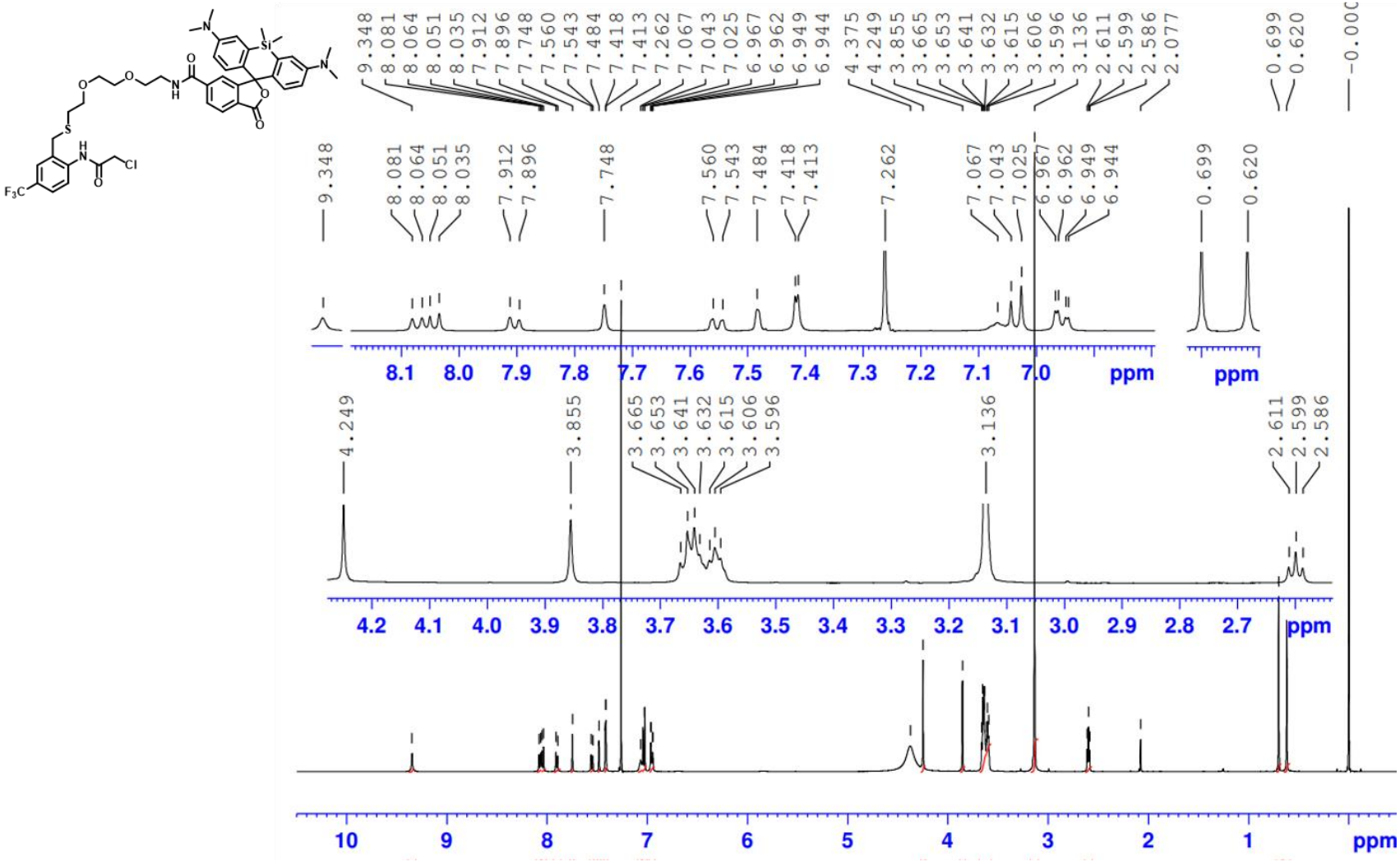
^{1}H NMR spectra of PCAForange



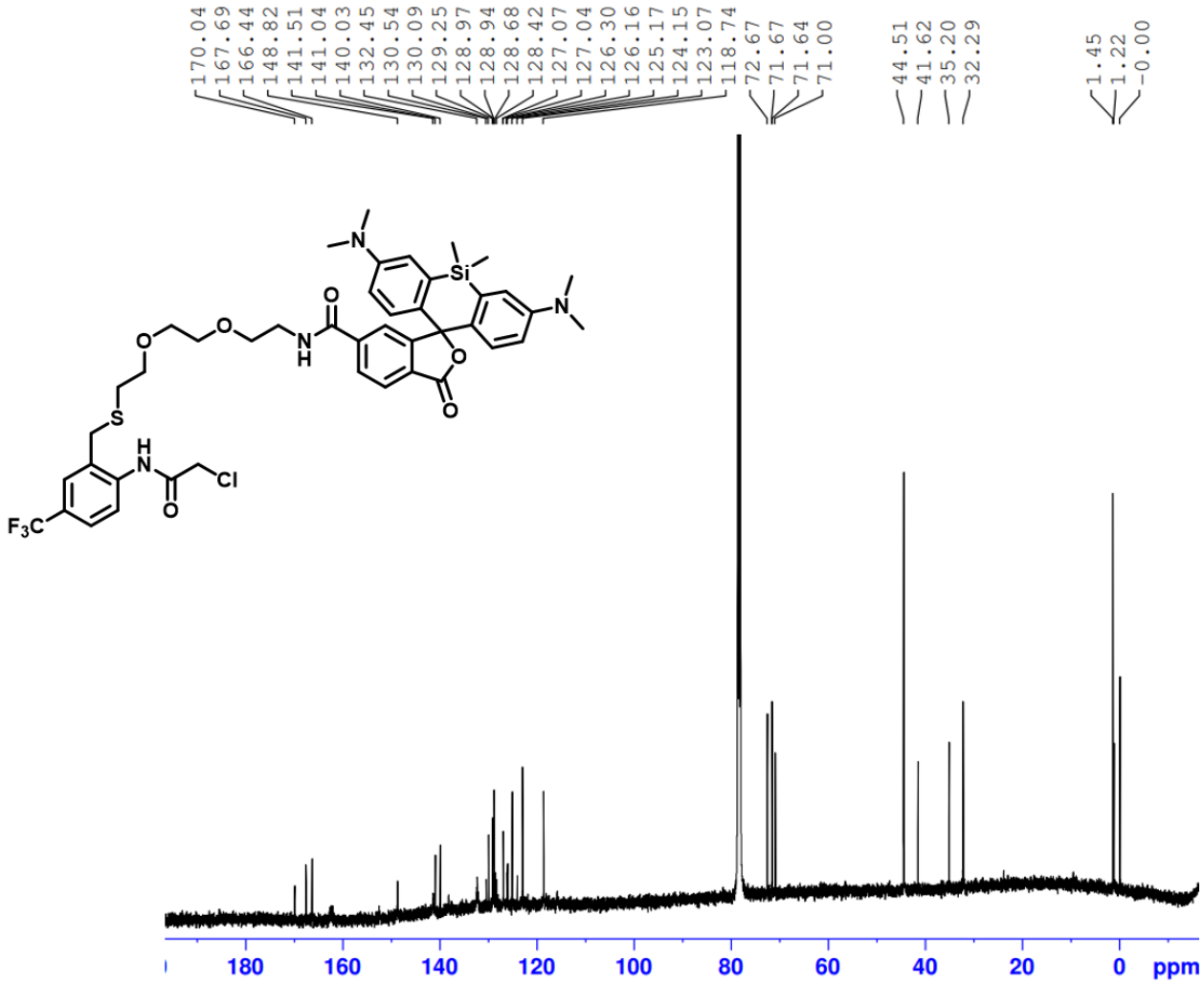
^{13}C NMR spectra of PCAForange



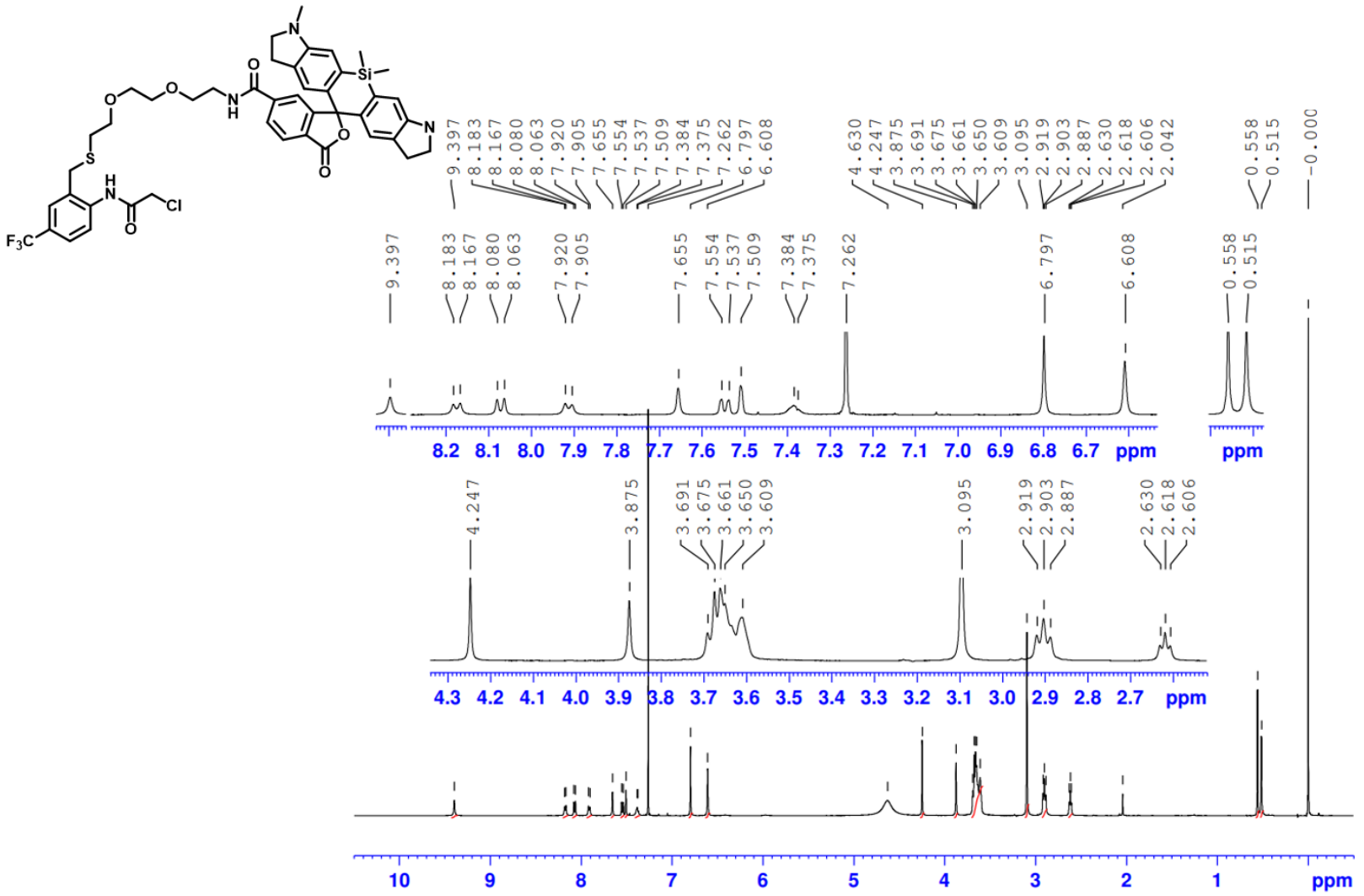
^{1}H NMR spectra of PCAFred



^{13}C NMR spectra of PCAFred



^{1}H NMR spectra of PCAFfar-red



^{13}C NMR spectra of PCAFred