Supporting Information for

Scalable and Concise Synthesis of Water-Soluble Dichlorofluorescein Derivatives Displaying Tissue Permeation in Live Zebrafish Embryos

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K.K. designed the synthesis. F.S., A.L.G, and V.D.M. performed synthesis. F.S. performed fluorescence spectroscopic studies. N.A.H. designed zebrafish experiments. E.D.G. performed the zebrafish experiments. L.A.D. assisted recording photos of dye-treated zebrafish. K.K., E.D.G., and N.A.H. wrote the manuscript.

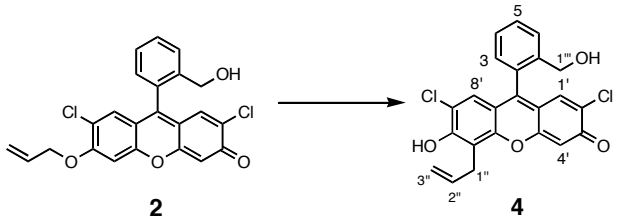
Abbreviations. See http://pubs.acs.org/paragonplus/submission/joceah/index.html

A. Preparation of Dichlorofluorescein Derivatives

General techniques. All reactions were carried out with dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogenous materials, unless otherwise stated.

All reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25-mm E. Merck silica gel plates (60F-254) using UV-light (254 nm), 2.4% phosphomolybdic acid/1.4% phosphoric acid/5% sulfuric acid in water, anisaldehyde in ethanol, or 0.2% ninhydrin in ethanol and heat as developing agents. TSI silica gel (230-400 mesh) was used for flash column chromatography.

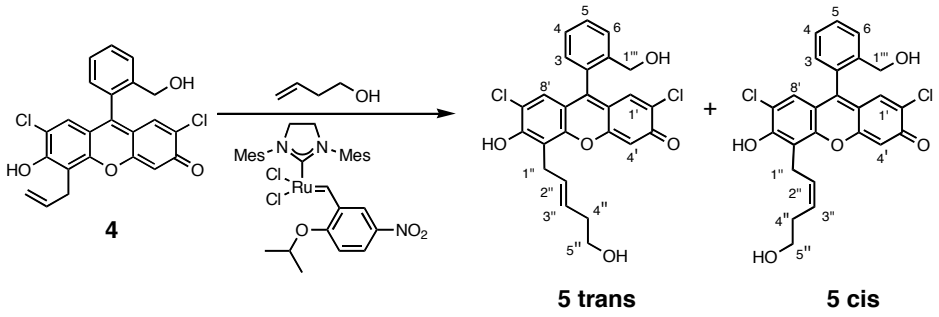
NMR spectra were recorded on AM300 (Bruker) instruments and calibrated using a solvent peak or tetramethylsilane as an internal reference. The following abbreviations are used to indicate the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. High-resolution mass spectra were obtained by using EBE geometry.



2 **4**

Preparation of 4. A solution of compound **2** (1.43 g, 3.35 mmol) in Ph_2O (5 mL) was stirred at 150 °C for 12 h. After cooled to 24 °C, the reaction mixture was transferred directly to a silica gel column. The column chromatography was performed with 10 % isopropyl alcohol in hexanes to afford compound **4** as a red-orange solid (1.15 g, 80%).

Data for **4**: mp = 186—188 °C; R_f = 0.59 (50% EtOAc in hexanes); IR (in CH_2Cl_2): 3305 (br, OH), 3074, 2923, 2855, 1635, 1598, 1482, 1446, 1356, 1278, 1213, 737 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 293K): δ 7.42–7.35 (m, 2H, Ar), 7.30–7.28 (m, 1H, Ar), 6.89–6.87 (m, 3H, Ar; overlap of two singlets and one doublet), 6.77 (s, 1H, Ar), 6.01 (ddt, *J* = 17.4, 9.9, 6.3 Hz, 1H, 2′′-H), 5.29 (br s, 2H, 1′′-H), 5.14 (dd, *J* = 17.4, 1.7 Hz, 1H, 3′′-H_{trans}), 5.05 (dd, *J* = 9.9, 1.5 Hz, 1H, 3′′-H_{cis}), 3.63 (br d, *J* = 6.3 Hz, 2H, 1′′-H); ^{13}C NMR (75 MHz, CD_3OD, 293K): δ 155.3, 152.5, 151.2, 149.2, 145.3, 140.1, 138.7, 130.4, 129.8 (two carbons), 127.6, 124.6, 122.2, 118.6, 118.1, 117.5, 117.3, 117.1, 115.6, 104.4, 85.0, 73.0, 29.0; HRMS (EI+) calcd for $C_{23}H_{16}Cl_2O_4$ $[M]^+$ 426.0426, found 426.0419.

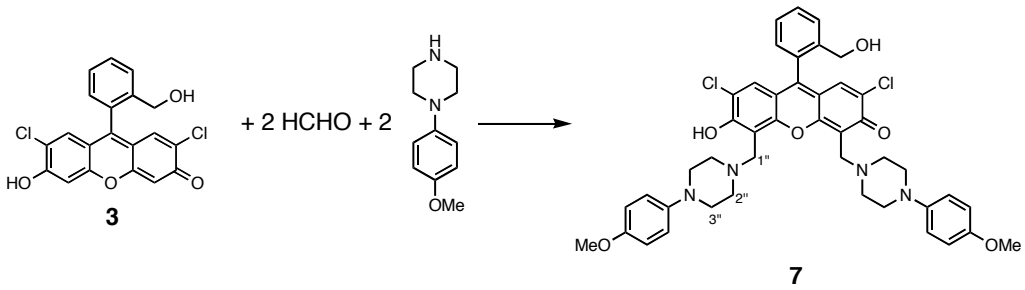


4 **5 trans** **5 cis**

Preparation of 5. To a solution of the ruthenium catalyst (7.5 mg, 0.011 mmol) in DCE (0.5 mL) was added compound **4** (34 mg, 0.075 mmol, in 0.8 mL DCE) under a nitrogen atmosphere at 24 °C. The resulting mixture was stirred for 5 min at the same temperature and 3-buten-1-ol (20 μL, 0.225 mmol) was added dropwise at 24 °C. The resulting mixture was heated at 45 °C for 3 h. Subsequently, the reaction mixture was cooled to 24 °C, and the solvent was removed in vacuo. The residue was purified by flash column chromatography (5 → 50 % EtOAc in hexanes containing 1% HOAc) to afford compound **5** as a red solid (15 mg, 42 %; 52% based on 9 mg of recovered **4**). For characterization, **5 trans** (major product) and **5 cis** (minor product) were separated by HPLC (20 → 100 % CH_3CN in H_2O; C18 column). The major product was fully characterized, and the minor product was partially characterized.

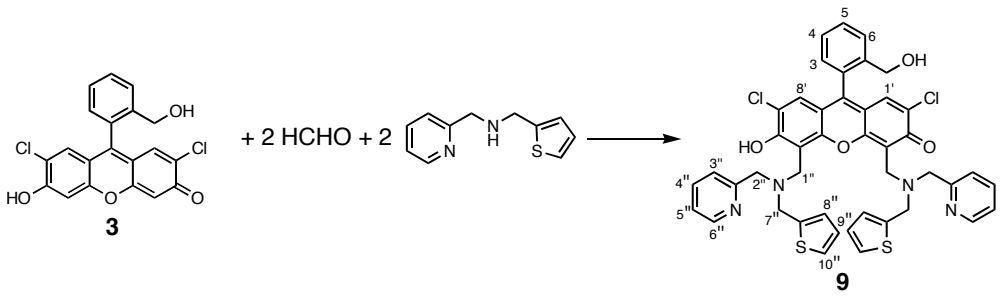
Data for **5 trans**: R_f = 0.15 (60% EtOAc in hexanes containing 1% HOAc); IR (in CH_2Cl_2): 3300 (br, OH), 3070, 2926, 2854, 1632, 1582, 1482, 1443, 1354, 1283, 1212, 1014, 736 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 293K): δ 7.40–7.35 (m, 2H, Ar), 7.31–7.26 (m, 1H, Ar), 6.89 (s, 1H, Ar), 6.88 (br d, 1H, *J* = 7.5 Hz), 6.86 (s, 1H, Ar), 6.76 (s, 1H, Ar), 5.79–5.68 (m, 1H, 2′′-H), 5.62–5.51 (m, 1H, 3′′-H), 5.28 (br s, 2H, 1′′′-H), 3.65–3.59 (m, 2H, 1′′-H and 5′′-H), 2.27 (app q, *J* = 12.3, 6.0 Hz, 2H, 4′′-H), 2.11 (s, 1H, OH). ^{13}C NMR (75 MHz, $CDCl_3$, 293K): δ 151.9, 150.3, 149.7, 147.9, 143.8, 138.7, 130.2, 128.7, 128.6, 128.4, 127.5, 125.8, 123.7, 121.0, 118.3, 118.0, 115.4, 115.3, 115.2, 103.8, 83.4, 72.3, 61.9, 35.9, 27.2; LRMS (ESI+) $C_{25}H_{20}Cl_2O_5$ $[M+H]^+$ 493 (18%), $(M+Na)^+$ 493 (35%), $(M+K)^+$ 493 (35%), (EI+) $(M-H)^+$ 469 (30%), $(M)^+$ 470 (22%), $[M+H]^+$ 471 (25%).

Data for **5 cis**: R_f = 0.19 (60% EtOAc in hexanes containing 1% HOAc); ^1H NMR (300 MHz, CD_3OD, 293K): δ 7.40–7.27 (m, 3H, Ar), 6.90 (s, 1H, Ar), 6.89–6.87 (m, 1H, Ar), 6.86 (s, 1H, Ar), 6.75 (s, 1H, Ar), 5.73–5.65 (m, 1H, 2′′-H), 5.53–5.48 (m 1H, 3′′-H), 5.28 (s, 2H, 1′′′-H), 3.76 (t, *J* = 6.3 Hz, 2H, 5′′-H), 3.69 (d, *J* = 6.6 Hz, 2H, 1′′-H), 2.60 (app q, *J* = 6.3 Hz, 2H, 1′′-H).



Compound 7. A solution of 1-(4-methoxy-phenyl)-piperazine (580 mg, 3.0 mmol) and paraformaldehyde (630 mg, 21.0 mmol) in MeCN (3 mL) was refluxed for 30 min under a nitrogen atmosphere. To this resulting solution was added a solution of **3** (387 mg, 1mmol) in H_2O/MeCN (1:1; 24 mL) at 24 °C. The resulting mixture was refluxed for 24 h in the dark under a nitrogen atmosphere. After the reaction mixture was cooled to 24 °C, most of the organic solvent was removed *in vacuo*. The aqueous residue was extracted with CH_2Cl_2 (30 mL × 3). The combined organic layers were dried over Na_2SO_4. After being filtered and concentrated, the crude product was purified by flash chromatography (80:1 CH_2Cl_2/MeOH) to afford compound **7** (510 mg, 64%) as a red-pink foam.

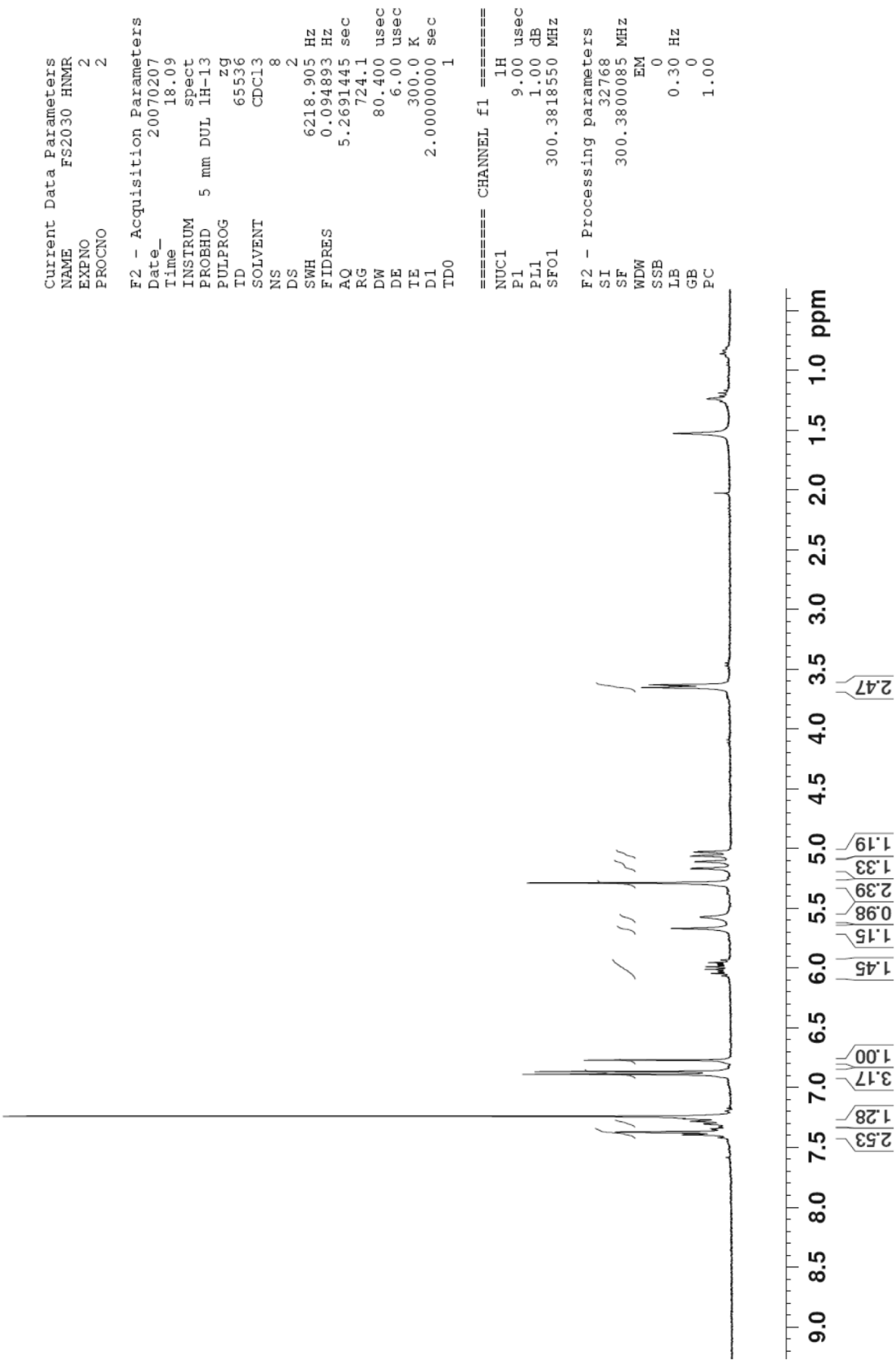
Data for **7**: R_f = 0.35 (50% EtOAc in hexanes); IR (in CH_2Cl_2): 3426 (br, O-H), 2925, 2850, 1624, 1511, 1458, 1398, 1346, 1246, 1035, 821 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 293K): δ 7.43–7.29 (m, 4H, Ar), 6.91–6.78 (m, 10H, Ar), 5.22 (s, 2H, C*H*$_2$OH), 4.06 (s, 4H, 1"-H), 3.74 (s, 6H, OCH_3), 3.16 (br s, 8H, 3"-H), 2.85 (br s, 8H, 2"-H); ^{13}C NMR (75 MHz, $CDCl_3$, 293K): δ 154.6, 154.4, 147.0, 145.0, 143.0, 139.4, 128.6, 128.4, 123.8, 121.0, 118.9, 116.5, 116.0, 115.8, 114.5, 108.3.5, 83.5, 71.8, 55.5, 54.6, 53.0, 50.7, 29.7; HRMS (ESI+) calcd for $C_{44}H_{45}Cl_2N_4O_6$ $[M+H]^+$ 795.2716, found 795.2739.



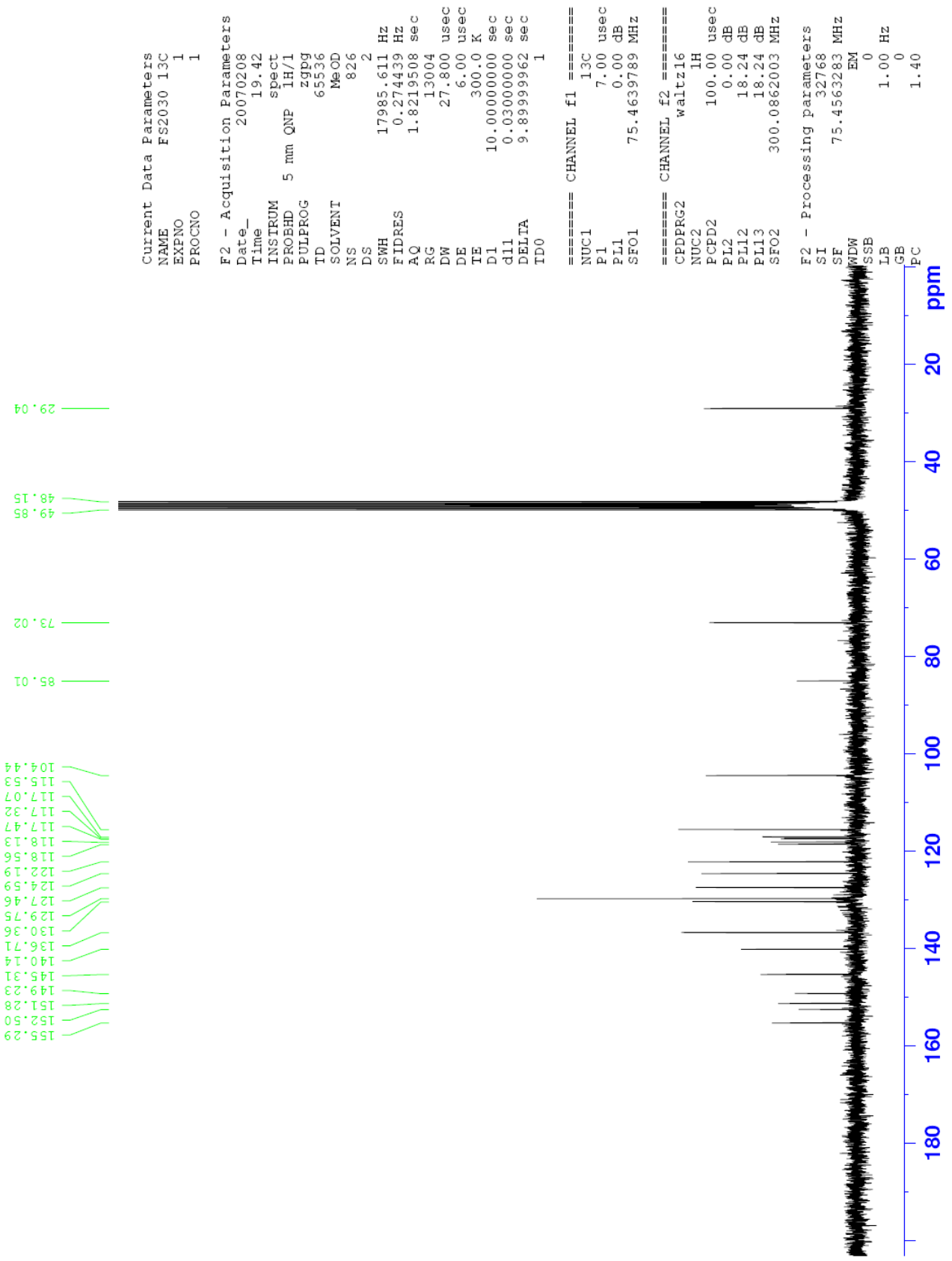
Compound 9. *N*-((Pyridin-2-yl)methyl)(thiophen-2-yl)methanamine was prepared according to the literature (Huisman, M.; Koval, I. A.; Gamez, P.; Reedijk, J. *Inorg. Chim. Acta.* **2006**, *359*, 1786-1794). A solution of this amine (150 mg, 0.73 mmol) and paraformaldehyde (38 mg, 1.26 mmol) in MeCN (4.5 mL) was refluxed for 1 h under a nitrogen atmosphere. A slurry of **3** (93 mg, 0.24 mmol) in H_2O/MeCN (1:1; 5 mL) was added to the solution at 24 °C. The resulting mixture was then refluxed for 22 h in the dark under a nitrogen atmosphere. After cooled to 24 °C, the reaction mixture was filtered and washed with Et_2O. The crude product was purified by silica gel flash chromatography (20:1 CH_2Cl_2/MeOH) to afford compound **9** (89 mg, 45%) as a pink solid.

Data for **9**: mp = 204–207 °C; R_f = 0.22 (50% EtOAc in hexanes); IR (in CH_2Cl_2): 3436 (br, OH), 3089, 3034, 2918, 2853, 1626, 1595, 1469, 1434, 1378, 1359, 1277, 1207, 1007, 755 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 293K): δ 8.61 (d, *J* = 3.3 Hz, 2H, 6"-H), 7.69 (apparent t, *J* = 7.5 Hz, 2H, 4"-H), 7.37–7.29 (m, 4H, 3"-H, 5"-H), 7.24–7.19 (m, 4H, 3, 4, 5, 6-H), 6.94–6.78 (m, 8H, Ar), 5.22 (s, 2H, C*H*$_2$OH), 4.05 (s, 4H, 2"-H), 3.98-3.85 (m, 8H, 1"-H, 7"-H); ^{13}C NMR (75 MHz, $CDCl_3$, 293K): δ 156.8, 154.4, 153.0, 149.2, 147.2, 143.6, 139.1, 138.3, 137.0, 128.5, 126.9, 125.8, 123.8, 123.5, 122.7, 120.9, 116.7, 116.1, 110.2, 109.7, 83.6, 71.9, 58.3, 51.5, 49.3; LRMS (EI+) calcd for $C_{44}H_{36}Cl_2N_4O_4S_2$ $[M]^+$ 818, found 823 (3%), 821 (6%), 819 (2%), 617 (64%), 616 (26%), 615 (72%), 509 (22%), 508 (5%), 507 (30%), 411 (100%), 413 (80%); HRMS (ESI+) calcd for $C_{44}H_{39}Cl_2N_4O_4S_2$ $[M+3H]^+$ 821.1790, found 821.1775.

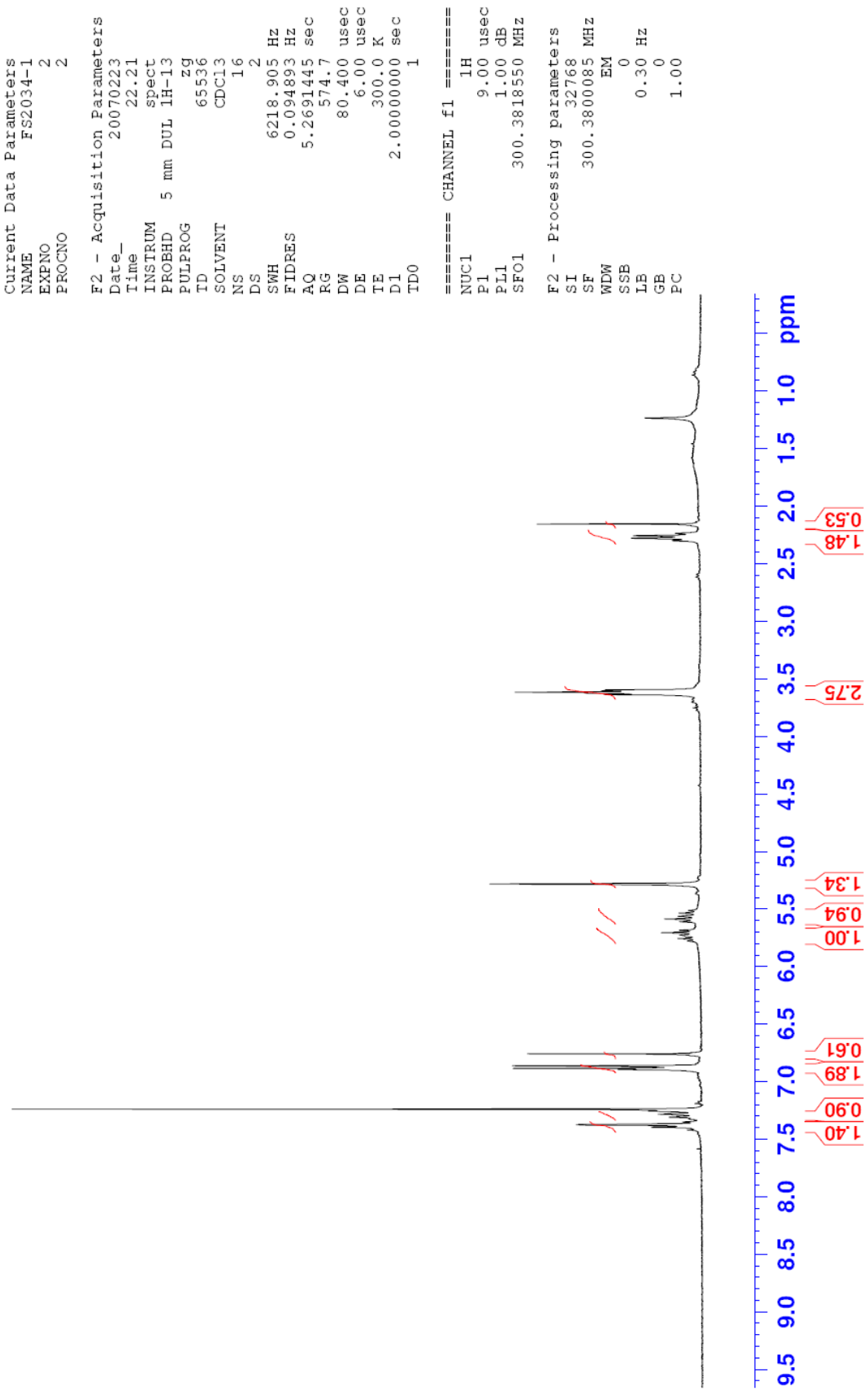
^{1}H NMR spectrum of compound **4**: $CDCl_3$, 293K, 300 MHz



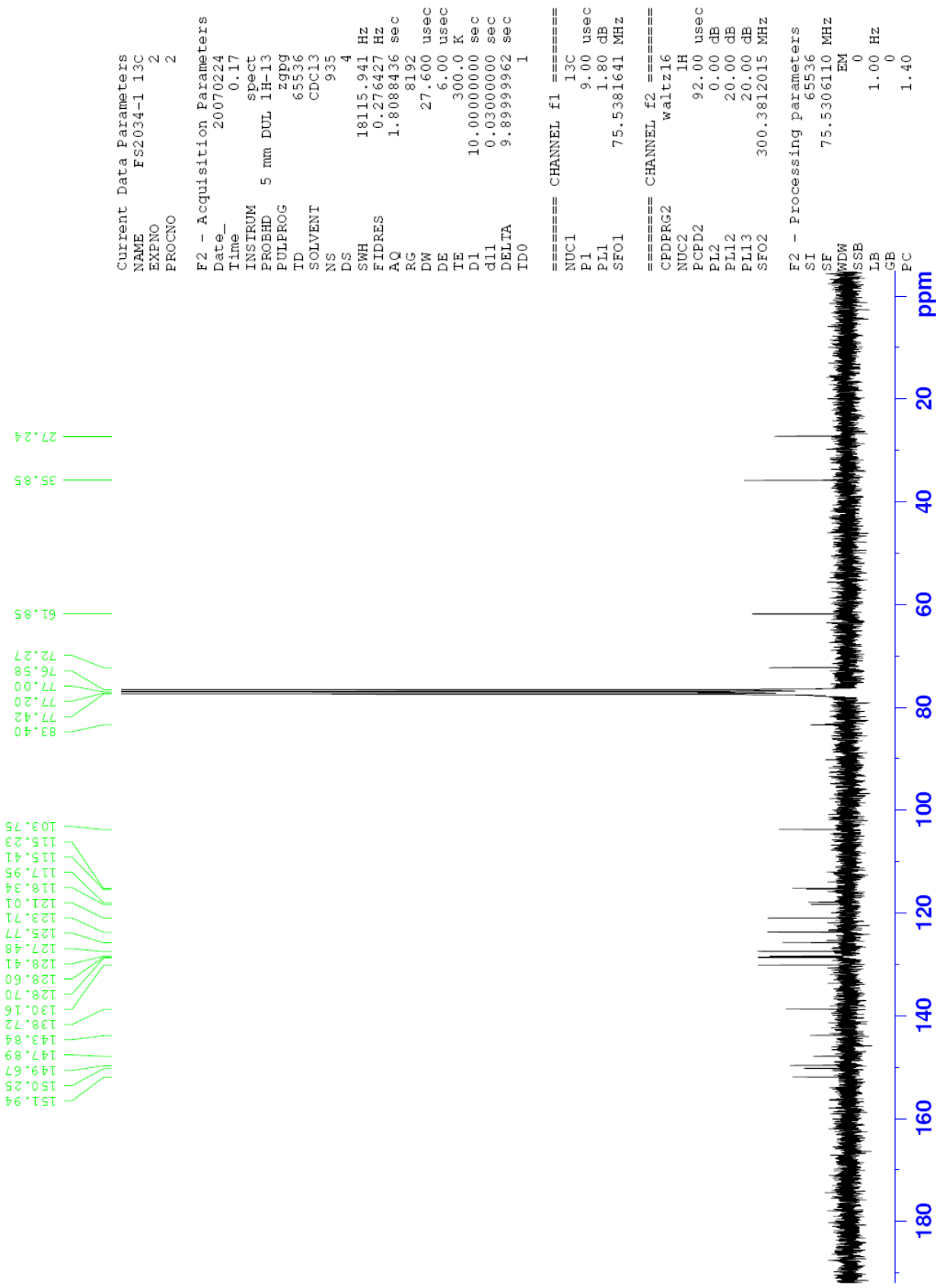
^{13}C NMR spectrum of compound **4**: CD_3OD, 293K, 75 MHz



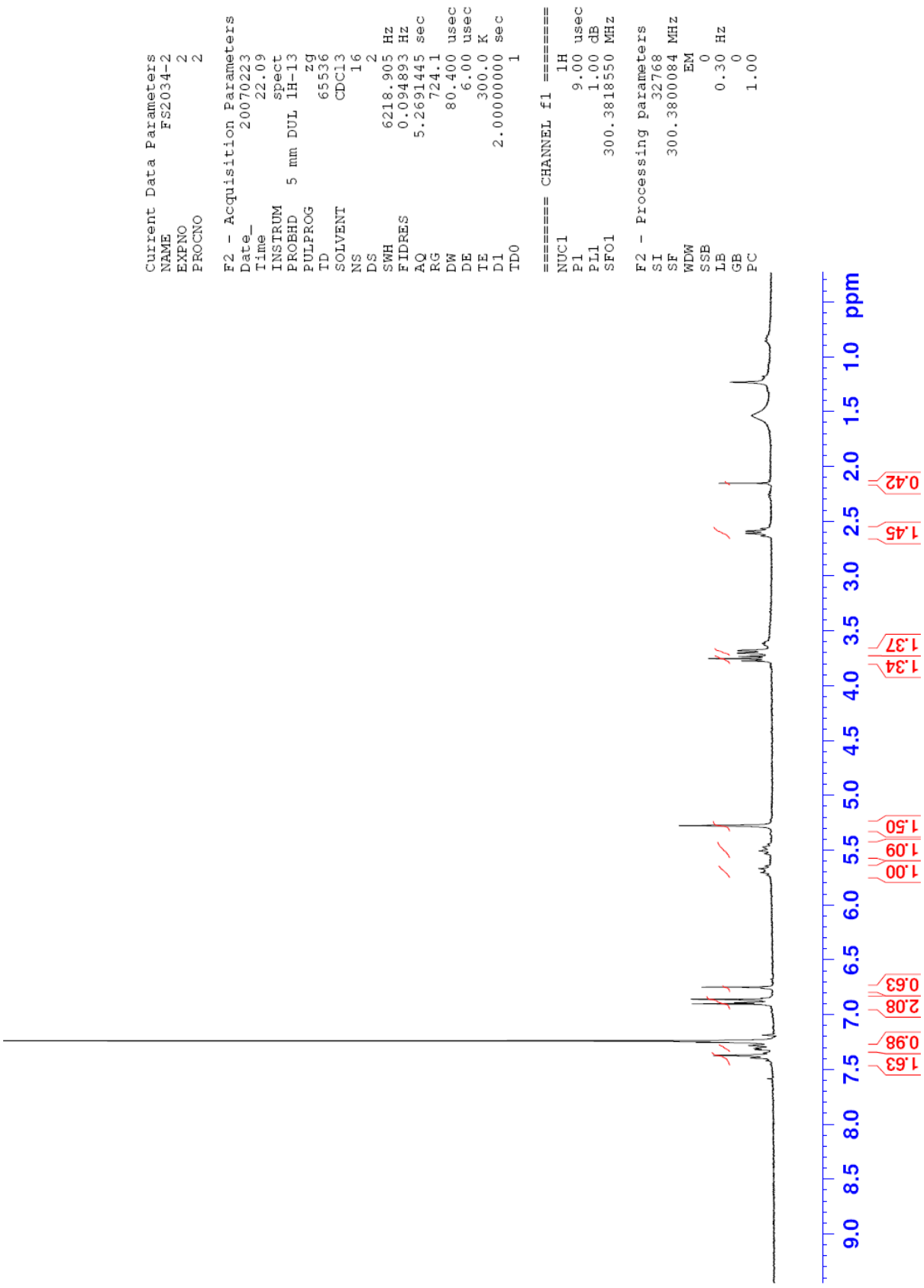
^{1}H NMR spectrum of compound **5 (trans)**: $CDCl_3$, 293K, 300 MHz



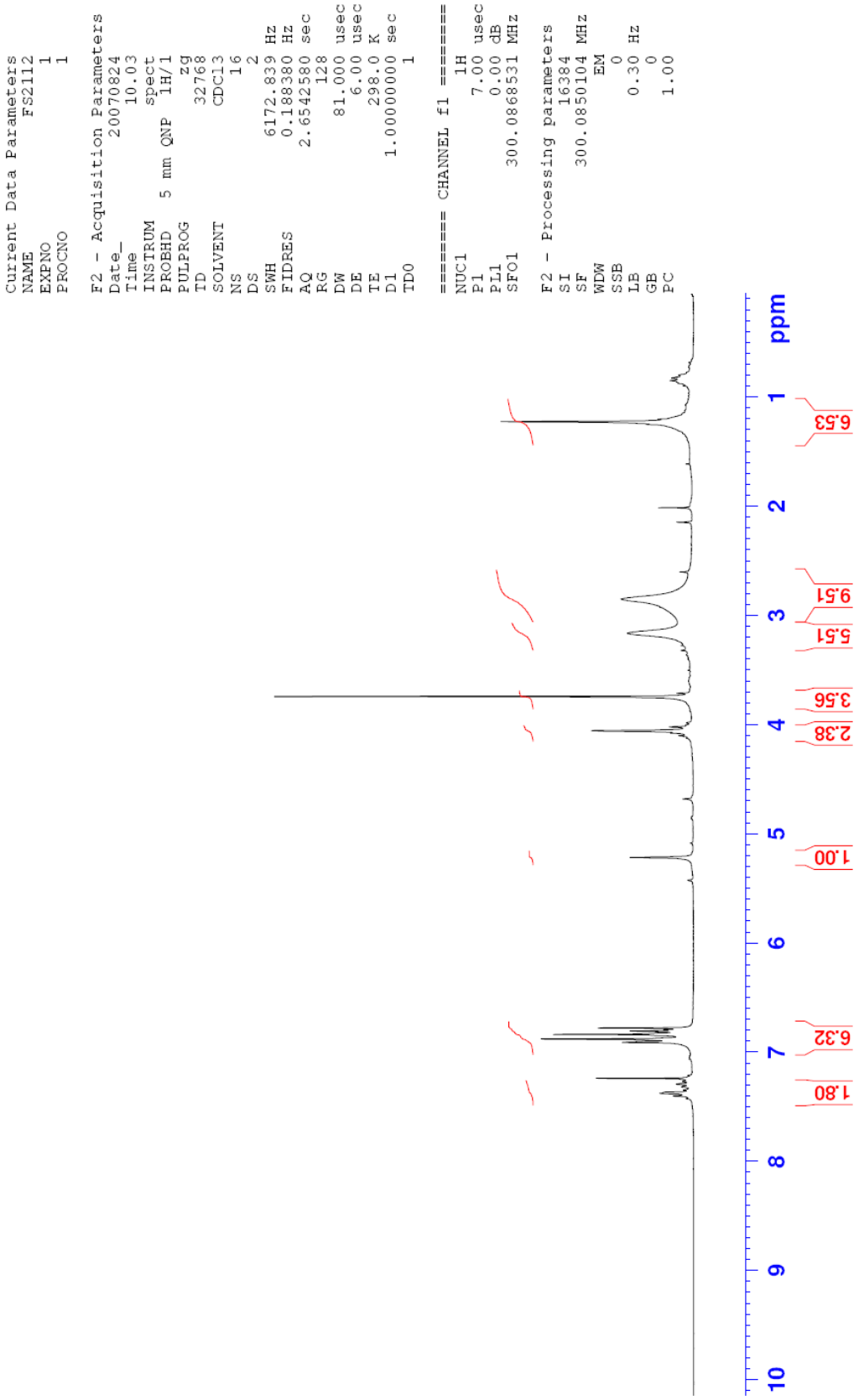
^{13}C NMR spectrum of compound **5 (trans)**: $CDCl_3$, 293K, 75 MHz



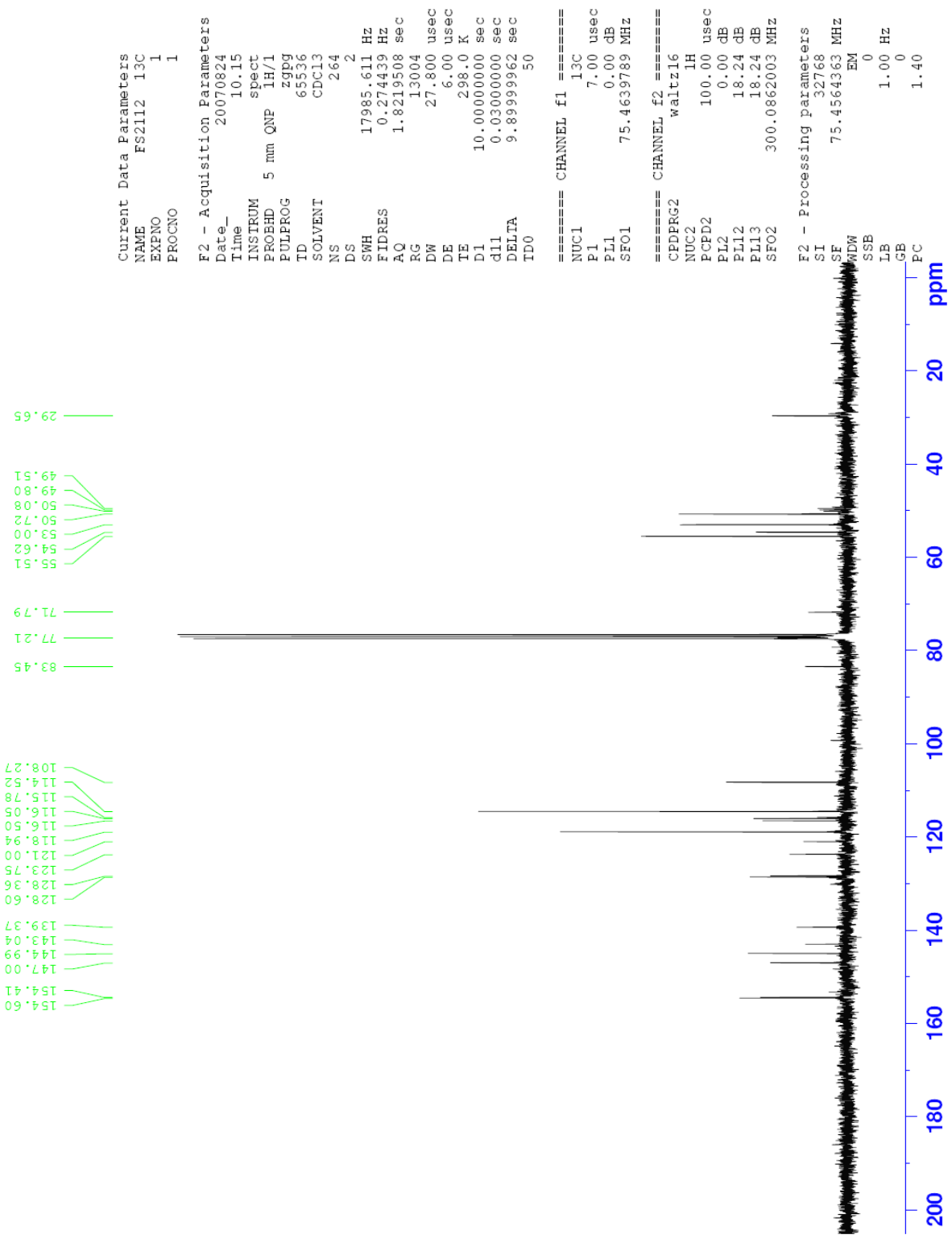
^{1}H NMR spectrum of compound **5 (cis)**: $CDCl_3$, 293K, 300 MHz



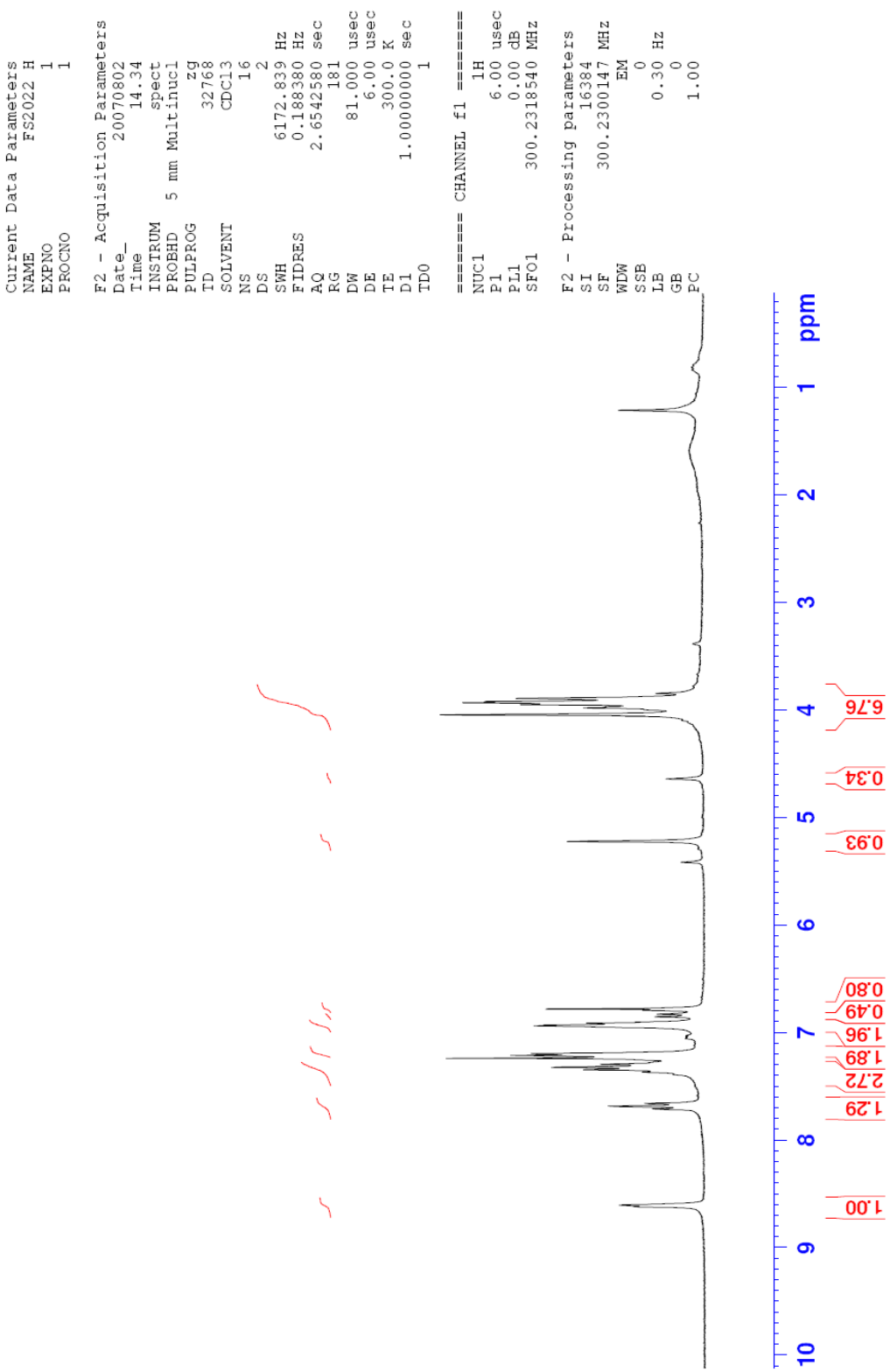
^{1}H NMR spectrum of compound **7**: $CDCl_3$, 293K, 300 MHz



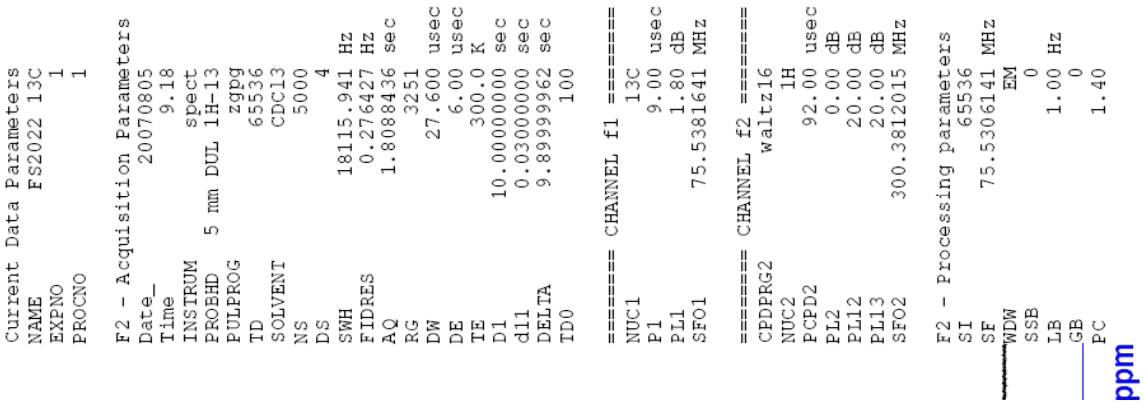
^{13}C NMR spectrum of compound **7**: CD_3OD, 293K, 75 MHz



^{1}H NMR spectrum of compound **9**: $CDCl_3$, 293K, 300 MHz



^{13}C NMR spectrum of compound **9**: $CDCl_3$, 293K, 75 MHz



B. UV and Fluorescent Spectroscopic Analyses

UV-Visible Spectroscopy. Absorption spectra were acquired on a Cary 50 UV-Vis spectrometer, under the control of Windows-based PC's running the manufacturer's supplied software.

Fluorescence Spectroscopy. All fluorescence measurements were acquired in a 1-cm x 1-cm quartz cell using a Spex Fluorolog fluorometer with 0.7-nm bandwidth slits, exciting at 490 nm and collecting emission from 496–620 nm. All spectra were corrected for emission intensity using the manufacturer's supplied photomultiplier curves.

Relative Quantum Yields. To determine quantum yields relative to fluorescein, stock solutions of **3** and **4** were prepared in DMSO (1 mM) and diluted in borate buffer (pH = 10) to OD_{490} = 0.12. The samples were excited at 490 nm and the integrated emission spectra were compared. The quantum yields of all compounds were referenced to fluorescein in 0.1 N NaOH (Φ = 0.95).[1]

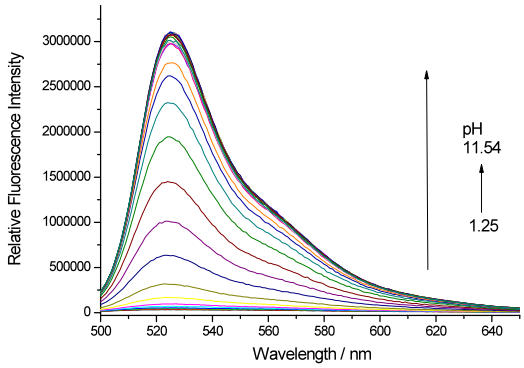
pH Titration of Compound 3

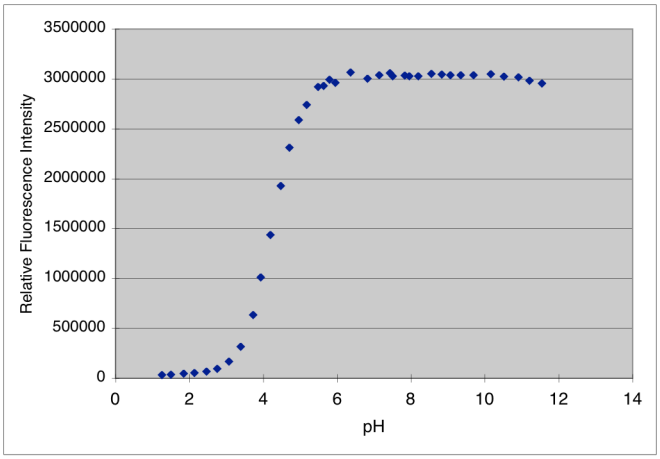
Method: An aqueous 100 mL solution containing compound **3** (5 μM) and NaCl (1.0 M) was prepared. To one half of this mixture solution (50 mL) was added a small magnetic stir bar in a vial with a pH electrode. The pH of the solution was changed by adding 0.1 N or 1.0 N HCl solution dropwise while stirring and the fluorescence spectrum was recorded at ~ 0.3 pH intervals. The other half of this mixture solution (50 mL) was titrated with 0.1 N or 1.0 N NaOH solution. λex = 497 nm. λem = 523 nm. Slit width: 1 nm for excitation and emission. In order to calculate the pKa, the pH dependence of fluorescence spectra were analyzed using the following equation (El-shishtawy, R. M.; Almeida, P. *Tetrahedron* **2006**, *62*, 7793-7798).

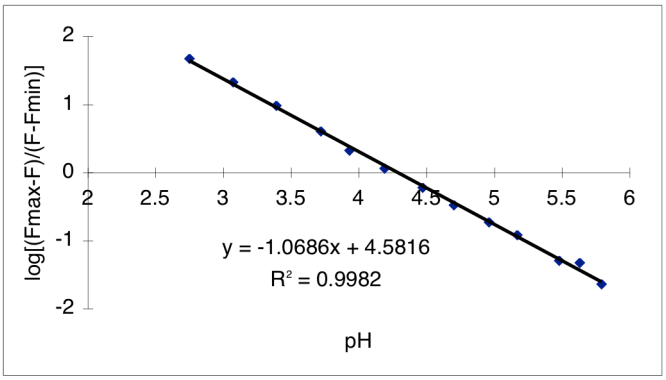
$$pH = pK_a - \log[(F_{max}-F)/(F-F_{min})]$$

Results

2',7'-dichlorofluorescein (used as a control)

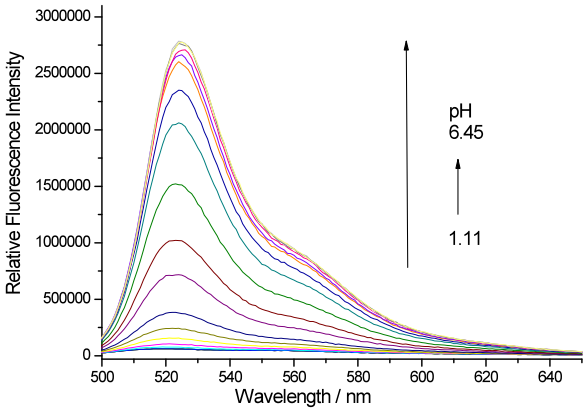


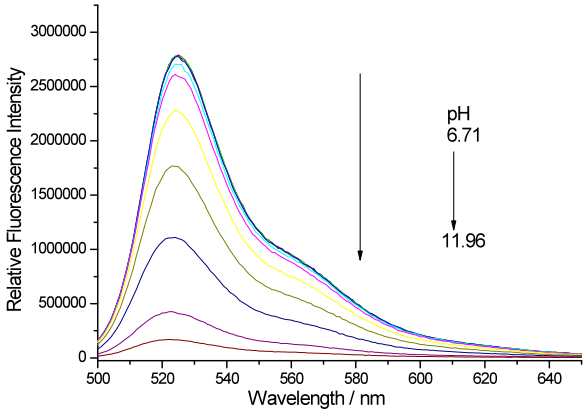


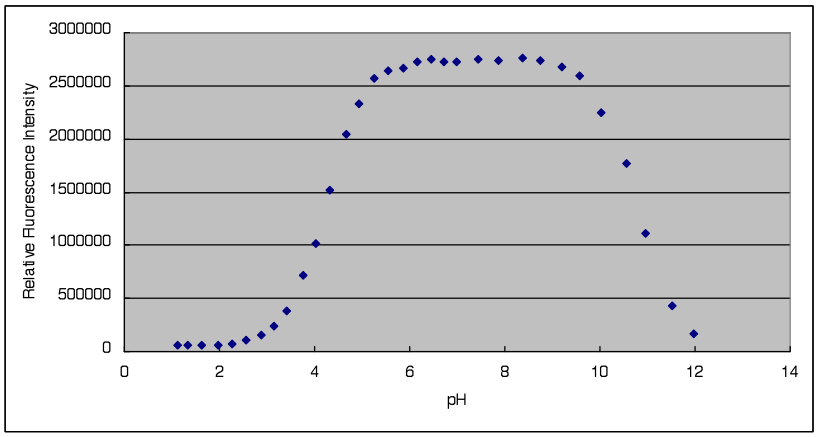


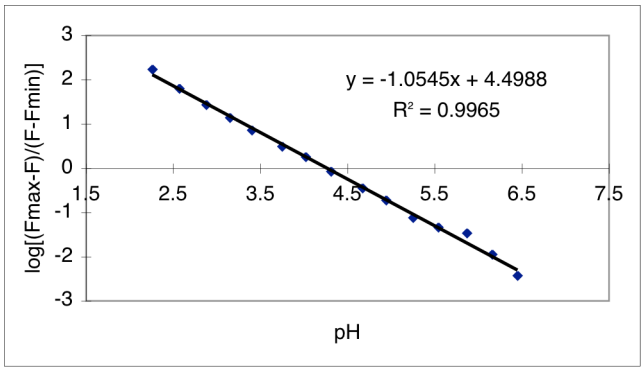
$pK_a = 4.29$

Compound **3**

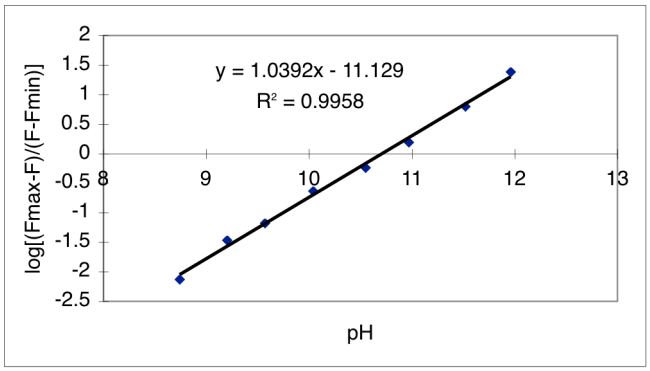








pKa = 4.27



pKa = 10.71

C. Zebrafish Studies

Table S1. Zebrafish embryo (48 hours post-fertilization) viability after 44.5 h of exposure to Pittsburgh Green (**3**) or Yellowgreen (**4**). Means of three independent experiments (*n* = 35) are presented.

Test Solution	Concentration (μM)	Mean Viable Embryos ± SD
Pittsburgh Green	25	29.3 ± 4.0 **
	8	33.7 ± 1.5
	2.5	35.0 ± 0.0
	0.8	34.0 ± 1.0
	0.25	34.7 ± 0.6
Pittsburgh Yellowgreen	25	0.0 + 0.0 **
	8	0.7 ± 1.2 **
	2.5	33.7 ± 1.5
	0.8	35.0 ± 0.0
	0.25	34.7 ± 0.6
BODIPY TR methyl ester	25	34.7 ± 0.6
Control solution	n/a	34.7 ± 0.6
HEPES-buffered embryo medium	n/a	34.3 ± 0.6

** significant at $p < 0.001$; SD = sample standard deviation

Zebrafish Strains and Maintenance. Zebrafish were maintained under standard conditions and staged as previously described.[2] Embryos were collected from group matings of wild-type AB adults, separated from unfertilized eggs, and pooled. Animal welfare was monitored by the University of Pittsburgh Division of Laboratory Animal Resources.

Vital Staining of Zebrafish Embryos. Embryos were stained as previously described with some modifications.[3] Stock solutions of Pittsburgh Green (**3**), Pittsburgh Yellowgreen (**4**), and 2',7'-dichlorofluorescein (DCF) were each prepared at 10 mM in DMSO. BODIPY TR methyl ester (Invitrogen, Carlsbad, CA) was supplied as a 5 mM stock in DMSO. All stocks were protected from light and stored in aliquots at -20 °C. Stock solutions were diluted in HEPES-buffered embryo medium (5 mM HEPES pH 7.2, 5 mM NaCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.17 mM KCl) to yield 25 μM staining solutions with normalized DMSO contents of 2% (v/v).

At 24 hours post-fertilization (hpf), embryos were manually dechorionated with fine forceps and added in groups of 20 to agarose-coated wells of a 12-well plate containing HEPES-buffered embryo medium. Excess medium was carefully removed from each well, avoiding exposure of the embryos to air, and replaced with 1 ml of staining solution or control solution (2% DMSO in HEPES-buffered E3). The plates were then covered in foil and incubated at room temperature with gentle shaking for 1 h. After staining, the embryos were transferred to agarose-coated 60 mm dishes and washed 3 times for 10 min each in HEPES-buffered embryo medium with gentle shaking.

Toxicity Screening. Stock solutions of Pittsburgh Green, Pittsburgh Yellowgreen, and BODIPY TR methyl ester were each diluted in HEPES-buffered embryo medium to produce 25 μM test solutions with normalized DMSO contents of 0.5% (v/v). The 25 μM Pittsburgh Green and Yellowgreen test solutions were then further diluted in HEPES-buffered embryo medium containing 0.5% DMSO to yield 8 mM, 2.5 mM, 0.8 mM, and 0.25 mM test solutions.

Groups of 35 chorionated, late-blastula (3.5 hpf) embryos were added to wells of 6-well plates containing HEPES-buffered embryo medium. The medium was removed and immediately replaced with 3 ml of test solution, HEPES-buffered embryo medium, or control solution (0.5% DMSO in HEPES-buffered embryo medium). Plates were wrapped in foil and incubated at 28.5 ^{o}C with gentle shaking for 44.5 h. During the incubation period, the embryos were checked periodically and visibly necrotic embryos were removed. Viability was assessed via cardiac movements.

Statistical Analysis. Single factor analyses of variance were performed to determine if any significant treatment effects were contained within each data set. If the null hypothesis was rejected, then the least significant difference between any two means was calculated at probabilities of 0.05, 0.01 and 0.001. The mean of each test group was compared to that of the control solution group at these *p* values to determine the degree of statistical significance.

Imaging of Zebrafish Embryos. Embryos used for imaging were anesthetized in HEPES-buffered embryo medium containing 0.64 mM Tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich, St. Louis, MO). Digital photographs were taken using a stereomicroscope (Leica Microsystems, Wetzlar, Germany) and CCD camera (QImaging, Burnaby, BC, Canada). For fluorescent imaging, filter sets (Chroma Technology, Rockingham, VT) were used to visualize Pittsburgh Green, Pittsburgh Yellowgreen, DCF (all at Ex 480/40, Em 535/50) or BODIPY TR methyl ester (Ex 575/50, Em 640/50).

Confocal optical sections were collected at 1 μm intervals with a 20x objective using a laser scanning confocal head (Leica Microsystems) mounted on an inverted microscope (Leica Microsystems). Excitation was provided by an argon laser at 488 nm (Pittsburgh Green, Pittsburgh Yellowgreen, and DCF) and a HeNe laser at

543 or 594 nm (BODIPY TR methyl ester). Image stacks were rendered and analyzed with image analysis software (ImageJ, version1.37v; Wayne Rasband, NIH/NIMH, http://rsb.info.nih.gov/ij/).

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