Supplementary Material

Discovery of Novel Phenaleno Isoquinolinium-Based Fluorescence Imaging Agents for Sentinel Lymph Node Mapping

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Experimental section

General

Flash column chromatography was performed using E. Merck 230-400 mesh silica gel. Column Chromatography were monitored using analytical thin-layer chromatography (TLC) carried out on 0.25 Merck silica gel plates (60 F-254) using UV light as a visualizing. ^{1}HNMR and ^{13}C NMR spectra were recorded on a Bruker Advance II/DPX 400 (400 MHz ^{1}H,100 MHz ^{13}C) spectrometer with chemical shifts reported relative to residual deuterated solvent peaks. ^{1}H NMR spectra are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet). ^{13}C NMR spectra were referenced to the residual $CDCl_3$ (77.26 ppm), DMSO (39.5 ppm). Fluorescence data were recorded on a Fluoromax plus spectrofluorometer of Horiba scientific at ibs center for NanoMedicine. High resolution mass spectra (HRMS) were obtained using a Shimadzu Nexera XR system at Daegu-Gyeongbuk Medical Innovation Foundation. The absolute quantum yields were measured by Fluoromax plus spectrofluorometer of Horiba scientific

Synthesis

Preparation of N-(pyren-1-ylmethyl)butan-1-amine (CAS No. 94964-63-3, 1a): To a 25 mL rbf were added 1-pyrene aldehyde (2.17 mmol, 1.5 g), *n*-butylamine (2.17 mmol, 102 mL) and methanol (4 mL). The resulting solution was stirred and refluxed for 24 h. After cooling to 0 ^{o}C, sodium borohydride (6.51 mmol, 0.25 g) was added to this solution and stirred for 24 h. Water was slowly added to the resulting mixture and then washed with

CH_2Cl_2 for three times. The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure giving a residue that was subjected to column chromatography (*n*-hexane:ethyl acetate=5:1) to give the desired product in 78% yield (white solid, 0.49 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (d, *J* = 9.24 Hz, 1H), 8.19-8.12 (m, 4H), 8.04 (s, 2H), 8.03-7.98 (m, 2H), 5.30 (s, 1H), 4.50 (s, 2H), 2.80 (t, *J* = 7.16 Hz, 2H), 1.43-1.35 (m, 2H), 0.92 (t, *J* = 7.36 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 134.5 131.6, 131.1, 130.8, 129.3, 127.8, 127.7, 127.26, 127.22, 126.1, 125.3, 125.29, 125.21, 124.9, 123.4, 52.2, 50.0, 32.6, 20.8, 14.2.

General Procedure: Preparation of Isoquinolinium Salts (MF37-MF40)

Preparation of 9-butyl-7,8-diphenylphenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (MF37): To a 5 mL reaction vial were added *N*-(pyren-1-ylmethyl)butan-1-amine (0.3 mmol, 86.8 mg), diphenylacetylene (0.45 mmol, 80 mg), copper acetate (0.6 mmol, 108 mg), $[Cp^*RhCl_2]_2$ (5 mol%), sodium tetrafluoroborate (0.45 mmol, 50 mg) and methanol (1 mL). The resulting solution was stirred at 100 ^{o}C for overnight, filtered, and the filtrate was concentrated under reduced pressure giving a residue that was subjected to column chromatography (CH_2Cl_2:MeOH=5:1) to give **MF37** in 60% yield (red solid, 0.1 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.03 (s, 1H), 9.53 (d, *J* = 9.16 Hz, 1H), 8.62 (d, *J* = 9.12 Hz, 1H), 8.40 (d, *J* = 7.64 Hz, 1H), 8.25 (d, *J* = 7.2 Hz, 1H), 8.14 (d, *J* = 8.12 Hz, 2H), 8.03 (s, 1H), 7.84 (d, *J* = 9.12 Hz, 1H), 7.43-7.37 (m, 8H), 7.29-7.27 (m, 2H), 4.90 (t, *J* = 8.04 Hz, 2H), 1.97-1.91 (m, 2H), 1.34 (quint, *J* = 7.44 Hz, 2H), 0.77 (t, *J* = 7.32 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 146.1, 143.3, 138.7, 138.5, 135.3, 134.2, 133.7, 132.6, 131.5, 131.2, 130.56, 130.51, 130.3, 130.1, 129.0, 128.75, 128.70, 128.6, 128.2, 127.9, 127.4, 124.3, 123.2, 122.2, 121.5, 120.3, 59.7, 34.3, 19.4,

13.2; HRMS (ESI) calcd for $C_{35}H_{28}N^+$ 462.2216, found 462.2214.

***9-butyl-7,8-bis(4-methoxyphenyl)phenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (MF38)*:** General procedure was used employing *N*-(pyren-1-ylmethyl)butan-1-amine and 1,2-bis(4-methoxyphenyl)ethyne to give the desired product **MF38** (red solid, 82% yield, 0.1 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.99 (s, 1H), 9.56 (d, *J* = 9.12 Hz, 1H), 8.68 (d, *J* = 9.12 Hz, 1H), 8.44 (d, *J* = 7.6 Hz, 1H), 8.27 (d, *J* = 7.2 Hz, 1H), 8.17-8.13 (m, 2H), 8.08 (s, 1H), 7.86 (d, *J* = 9.08 Hz, 1H), 7.27-7.25 (m, 2H), 7.18-7.16 (m, 2H), 6.96-6.91 (m, 4H), 4.89 (t, *J* = 7.88 Hz, 2H), 3.88 (s, 3H), 3.85 (s, 3H), 1.97-1.89 (m, 2H), 1.41-1.32 (m, 2H), 0.81 (t, *J* = 7.32 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.7, 159.8,146.0, 143.7, 139.0, 138.9, 136.0, 133.8, 132.8, 132.1, 131.9, 131.8, 130.8, 130.5, 129.2, 128.4, 128.1, 127.7, 126.6, 124.6, 123.6, 122.5, 121.7, 120.6, 114.4, 114.3, 59.9, 55.6, 55.5, 34.5, 19.7, 13.5; HRMS (ESI) calcd for $C_{37}H_{32}NO_2^+$ 522.2428, found 522.2428.

***9-butyl-7,8-bis(4-fluorophenyl)phenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (MF39)*:** General procedure was used employing *N*-(pyren-1-ylmethyl)butan-1-amine and 1,2-bis(4-fluorophenyl)ethyne to give the desired product **MF39** (orange solid, 67% yield, 78 mg). ^{1}H NMR (400 MHz, DMSO) δ 11.31 (s, 1H), 9.66 (d, *J* = 9.24 Hz, 1H), 8.97 (d, *J* = 9.16 Hz, 1H), 8.72 (d, *J* = 7.8 Hz, 1H), 8.57 (d, *J* = 7.36 Hz, 1H), 8.45 (d, *J* = 9.2 Hz, 1H), 8.35 (t, *J* = 7.68 Hz, 1H), 8.29 (d, *J* = 9.2 Hz, 1H), 8.20 (s, 1H), 7.66 (dd, *J* = 8.58, 5.48 Hz, 2H), 7.44 (dd, *J* = 8.56, 5.6 Hz, 2H), 7.33 (dd, *J* = 18.74, 9.04 Hz, 4H), 4.66 (t, *J* = 7.76 Hz, 2H), 1.94-1.90 (m, 2H), 1.32-1.24 (m, 2H), 0.76 (t, *J* = 7.32 Hz, 3H); ^{13}C NMR (100 MHz, DMSO) δ 163.7, 163.0, 161.2, 160.6, 146.2, 142.8, 137.9, 137.4, 135.0, 133.5, 133.19, 133.10, 132.6, 132.5, 131.9, 131.0, 130.5 (d, J_{C-F} = 2.54 Hz), 130.3, 129.4, 128.8,

128.7, 128.4, 127.8, 127.6 (d, J_{C-F} = 3.42 Hz), 123.5, 122.6, 121.9, 120.8, 120.3, 115.6, 115.4, 58.8, 32.7, 18.9, 13.0; HRMS (ESI) calcd for $C_{35}H_{26}F_2N^+$ 498.2028, found 498.2027.

***9-butyl-7,8-dipropylphenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (MF40)*:** General procedure was used employing *N*-(pyren-1-ylmethyl)butan-1-amine and 4-octyne to give the desired product **MF40** (yellow solid, 74% yield, 50 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.19 (s, 1H), 9.52 (d, *J* = 9.0 Hz, 1H), 8.67 (d, *J* = 8.96 Hz, 1H), 8.53 (s, 1H), 8.49 (d, *J* = 7.76 Hz, 1H), 8.32 (d, *J* = 7.16 Hz, 1H), 8.27 (d, *J* = 9.12 Hz, 1H), 8.20-8.11 (m, 2H), 5.03 (t, *J* = 7.84 Hz, 2H), 3.39-3.35 (m, 2H), 3.21-3.17 (m, 2H), 2.10-2.06 (m, 2H), 1.95-1.83 (m, 4H), 1.64-1.58 (m, 2H), 1.31-1.23 (m, 6H), 1.05 (t, *J* = 7.28 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 146.6, 143.0, 138.9, 135.9, 135.1, 134.0, 132.6, 131.8, 131.1, 130.8, 129.3, 128.4, 128.1, 127.7, 124.4, 123.7, 122.5, 121.3, 117.7, 58.8, 35.0, 31.5, 31.3, 24.4, 23.7, 20.0, 14.9, 14.7, 13.7; HRMS (ESI) calcd for $C_{29}H_{32}N^+$ 394.2529, found 394.2525.

***9-butyl-7,8-bis(4-(dimethylamino)phenyl)phenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (MF41)*:** General procedure was used employing *N*-(pyren-1-ylmethyl)butan-1-amine and 4,4'-(ethyne-1,2-diyl)bis(N,N-dimethylaniline) to give the desired product **MF41** (dark brown solid, 67% yield, 43 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.85 (s, 1H), 9.92 (d, *J* = 9.2 Hz, 1H), 8.73 (d, *J* = 9.16 Hz, 1H), 8.48 (d, *J* = 7.64 Hz, 1H), 8.27 (d, *J* = 7.16 Hz, 1H), 8.19-8.13 (m, 3H), 7.91 (d, *J* = 9.12 Hz, 1H), 7.10-7.06 (m, 4H), 6.72 (dd, *J* = 6.84, 2.0 Hz, 2H), 6.65 (d, *J* = 8.88 Hz, 2H), 5.01 (t, *J* = 7.92 Hz, 2H), 3.03 (s, 6H), 3.00 (s, 6H), 1.95-1.91 (m, 2H), 1.37-1.30 (m, 2H), 0.80 (t, *J* = 7.32 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.8, 150.1, 147.4, 144.6, 139.3, 138.4, 136.3, 133.3, 132.4, 132.0, 131.7, 131.5,

131.0, 130.9, 129.0, 127.9, 127.8, 124.6, 123.8, 123.6, 122.2, 122.0 ,121.0 ,118.7, 112.1, 111.7, 59.4, 40.5, 40.3, 34.7, 29.97, 29.94, 19.8, 13.6; HRMS (ESI) calcd for $C_{39}H_{38}N_3$+ 548.3060, found 548.3061.

Animals: Specific pathogen-free six-week-old, female Balb/c nude mice were obtained from SLC, Inc. (Shizuoka, Japan). All animal experimental procedures were conducted in strict accordance with the appropriate institutional guidelines for animal research. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the Laboratory Animal Center of Daegu-Gyeongbuk Medical Innovation Foundation (approval no. DGMIF-20032403-00).

Cells: Chinese hamster ovary (CHO) and L929 cells were grown in RPMI 1640 medium (Hyclone, Logen UT, USA) supplemented with 10% fetal bovine serum (FBS: Hyclone) and 1% penicillin-streptomycine (Gibco, Grand Island, NY, USA). Murine macrophage cells (Raw 264.7) were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. (Sigma).

Cell proliferation assay: Cell proliferation assay was performed using a Cell Counting Kit (CCK-8, Dojindo Laboratories, Tokyo, Japan). CHO, L929, and RAW 264.7 cells were seeded at 1×10^4 cells per well in 96-well plates, followed by incubation of various concentration of MF37, 38, 39, and 40 for 24h. Ten microliters of CCK-8 solution were added to each well at 24 h after incubation with various concentration of MF37, 38, 39, and 40 and then the plate was incubated at 37 ^{o}C for 1 h. Absorbance at 450 nm was measured using a microplate reader (BMG Labtech, Offenburg, Germany).

Immunofluorescence microscopy imaging: L929 of normal fibroblast cell lines and Raw264.7 of macrophage cell lines were treated with MF compounds for 0.5 h and washed three times with phosphate-buffered saline (PBS) and visualized with a confocal laser scanning microscope (LSM 800; Carl Zeiss, Oberkochen, Germany).

Animal studies: 1 mg/kg MF compounds was subcutaneously injected into the footpad of mice (N=5) and FLI images were acquired at indicated times post-injection. Upon imaging acquisition, mice were sacrificed and all organs, including lymph nodes, excised and imaged using an IVIS Spectrum CT.

***In-vivo* fluorescence imaging:** *In-vivo* fluorescence images were acquired using an IVIS Spectrum CT (PerkinElmer). The scan times ranged from 1 s to 5 min depending on the intensity of the emitted fluorescence signal. All *in-vivo* FLI were obtained with the following settings: Ex/Em, 465 nm/720 nm; exposure time, 1-2 s; f/stop, 2; binning, 8; and field of view, 21.8. The image was thresholder to maximize visualization of the region of interest and minimize background fluorescence. Grayscale and fluorescence color images were superimposed using LIVINGIMAGE v 2.12 (Perkin Elmer) and IGOR Image Analysis FX (Wave Metrics, Lake Oswego, OR, USA) software. Signal intensity is expressed in units of Total Radiant Efficiency [p/s] / [μW/cm²].

Fluorescence Microscopy: After killing, sentinel lymph nodes and control lymph nodes were removed and frozen at -80°C. Frozen section (30 μm) of all the lymph nodes were prepared after embedding in Tissue Tek ® O.C.T. compound (Sakura Finetek, Torrance, CA, USA). Fluorescence was observed under an upright epifluorescence microscope (IX-71 Provis, Olympus, Rungis, France) equipped with a 100-W mercury vapor lamp and a Peltier cooled CCD camera (DP71, Olympus). The filter set used consisted of a 400- to 440-nm band-pass

excitation filter, a 570-nm dichromic mirror, and a 590-nm long-pass filter. Fluorescence images were recorded at a magnification of ×40.

Measuring of absolute quantum yield: 20 μL of MF compounds (10 mM in DMSO) was diluted by 2 mL of dichloromethane. The quantum yields of Corresponding solution was analyzed with Fluoromax plus spectrofluorometer.

Toxicity study: To determine the acute toxicity of M37-MF40 *in-vivo*, six ICR mice of each group were intravenously injected with 1 mg/kg of respective MF compounds in a total volume of 100 uL with DMSO and saline (MF compounds: DMSO: saline=25%:5%:70%). The control mice (n =6) were not treated any circumstances. The body weight and physical activities were observed within 7 days. All mice were sacrificed and collected blood samples were examined for serum biochemistry analysis according to the manufacturer's instructions (TBA 120-FR; Toshiba, JP). All tissues were fixed in 10% neutral buffered formalin (BBC Biochemicals, Mount Vernon, WA, USA) for histopathological evaluation. A tissue processor (Thermo Fisher Scientific, Inc., Runcorn, UK) was used to prepare tissue samples from the formalin-fixed samples for analysis by fixing, staining and dehydrating. The paraffin-embedded tissue blocks were cut at a 4-μm thickness and mounted onto glass slides. Staining was performed with hematoxylin and eosin using an autostainer (Dako Coverstainner; Agilent, Santa Clara, CA, USA). Histopathological evaluation on the samples was performed in a blind manner.

Statistical analysis: All data are expressed as the mean ± standard deviation (SD) from at least three independent experiments, and statistical significance of the difference was determined by unpaired Student's tests using GraphPad Prism 8 (GraphPad. Software Inc). Differences with p-values less than 0.05 were considered statistically significant.

Figures of experimental section

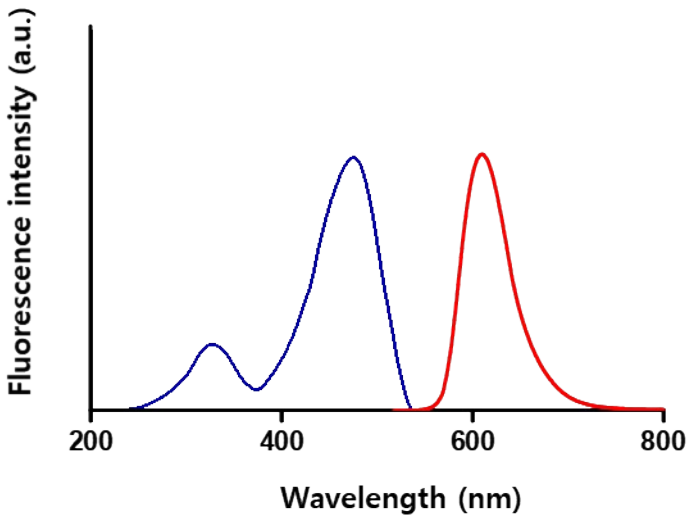


Figure S1. Fluorescent spectrum of **MF 37**

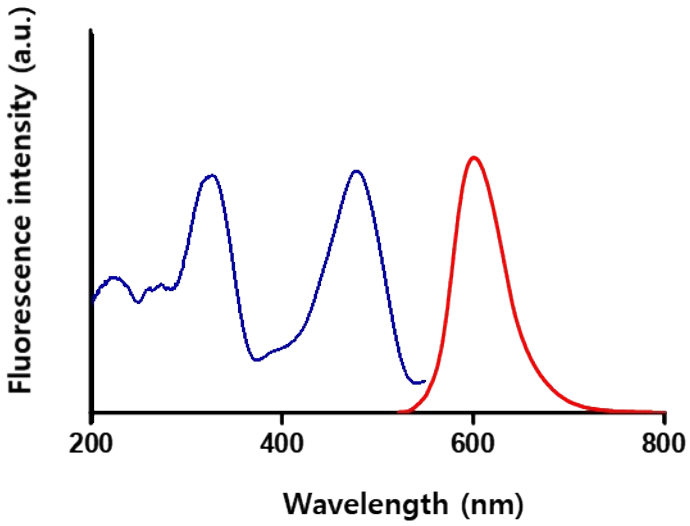


Figure S2. Fluorescent spectrum of **MF 38**

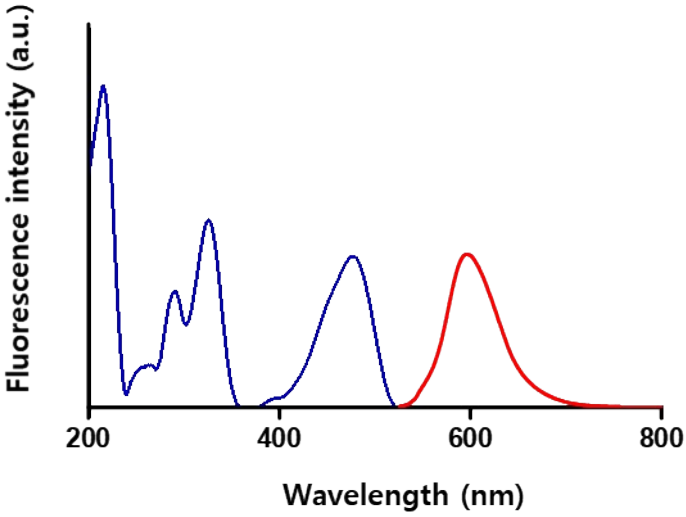


Figure S3. Fluorescent spectrum of **MF 39**

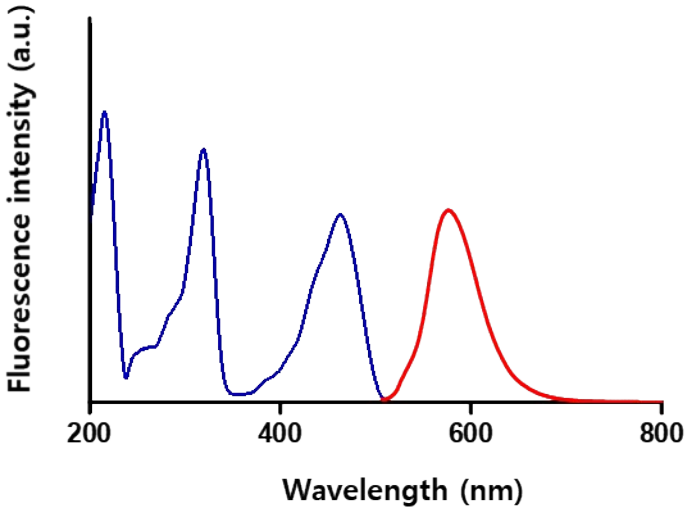


Figure S4. Fluorescent spectrum of **MF 40**

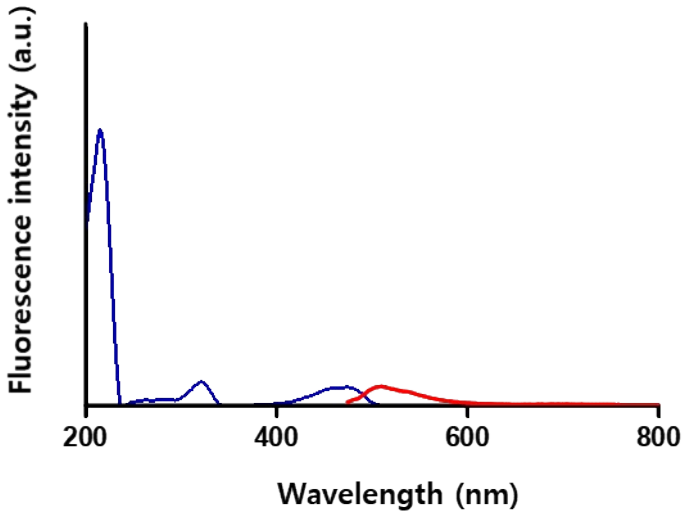


Figure S5. Fluorescent spectrum of **MF 41**

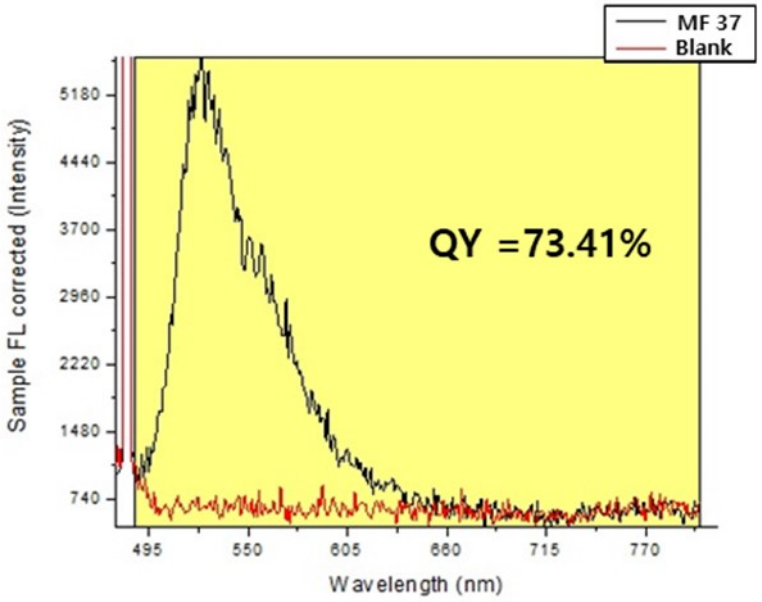


Figure S6. Absolute PL QY measurements of **MF37**

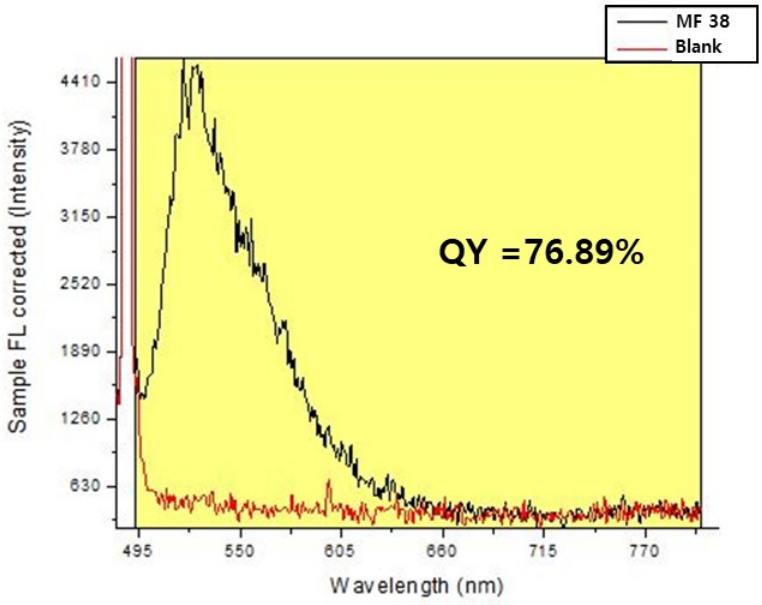


Figure S7. Absolute PL QY measurements of **MF38**

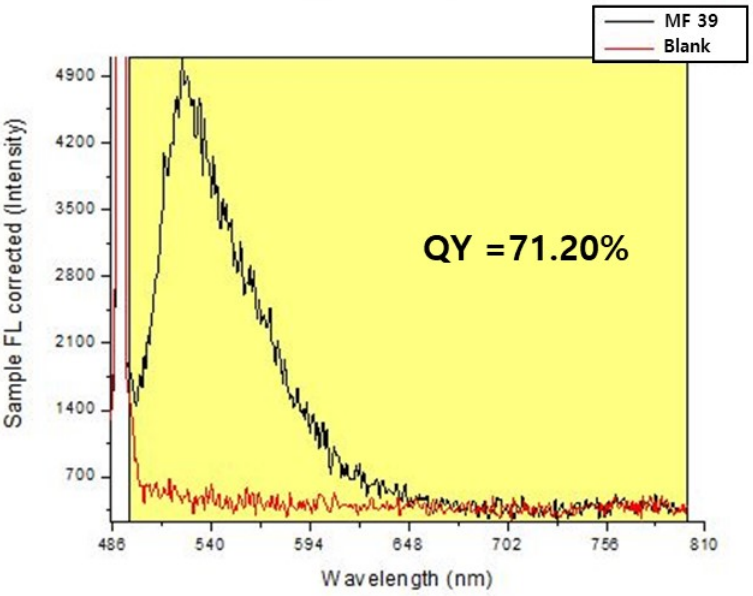


Figure S8. Absolute PL QY measurements of **MF39**

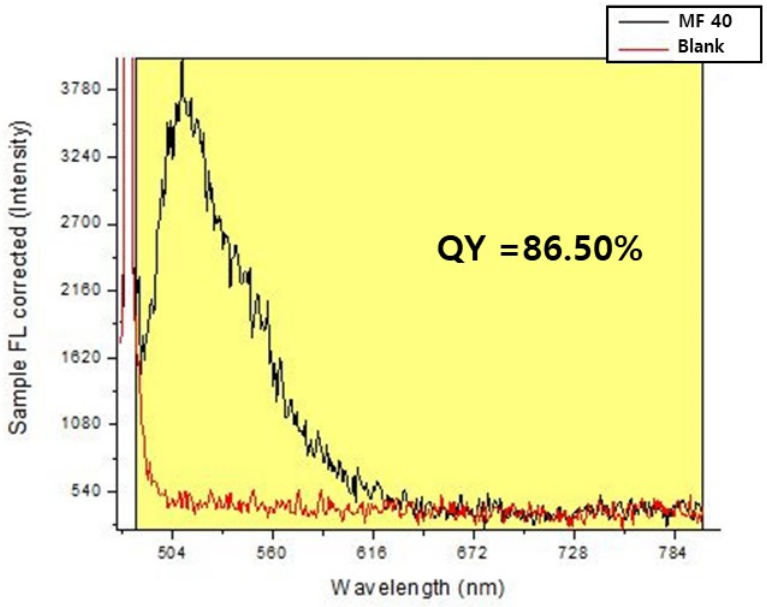


Figure S9. Absolute PL QY measurements of **MF40**

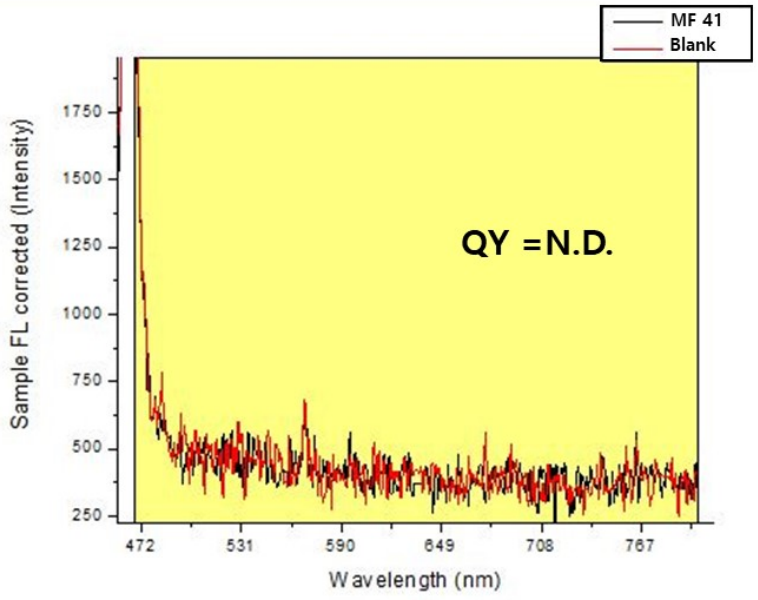


Figure S10. Absolute PL QY measurements of **MF41**

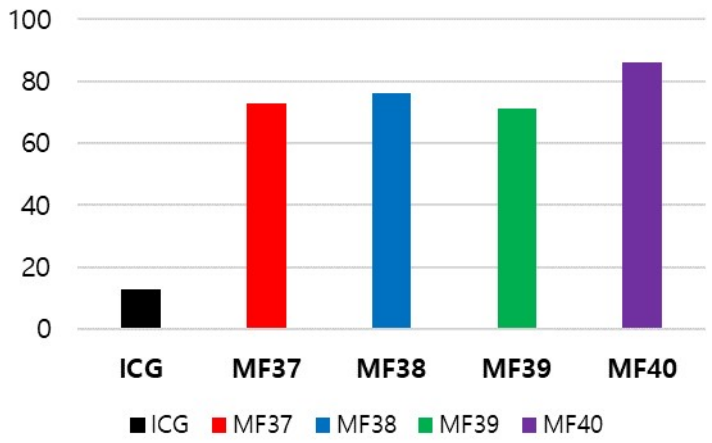


Figure S11. Comparison of PL QY among ICG and MF compounds (**MF37-40**)[1]

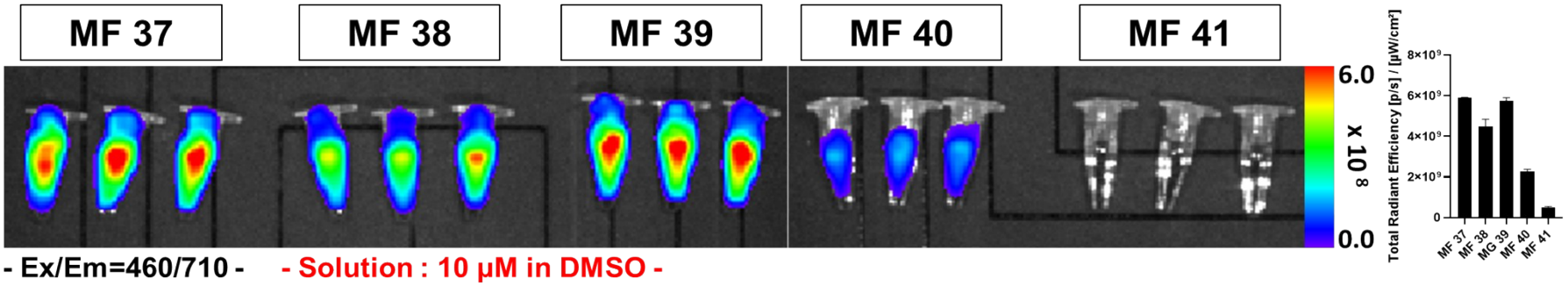


Figure S12. *In-vitro* fluorescent imaging of tube containing **MF37**, **MF38**, **MF39**, **MF40**, and **MF41** solutions

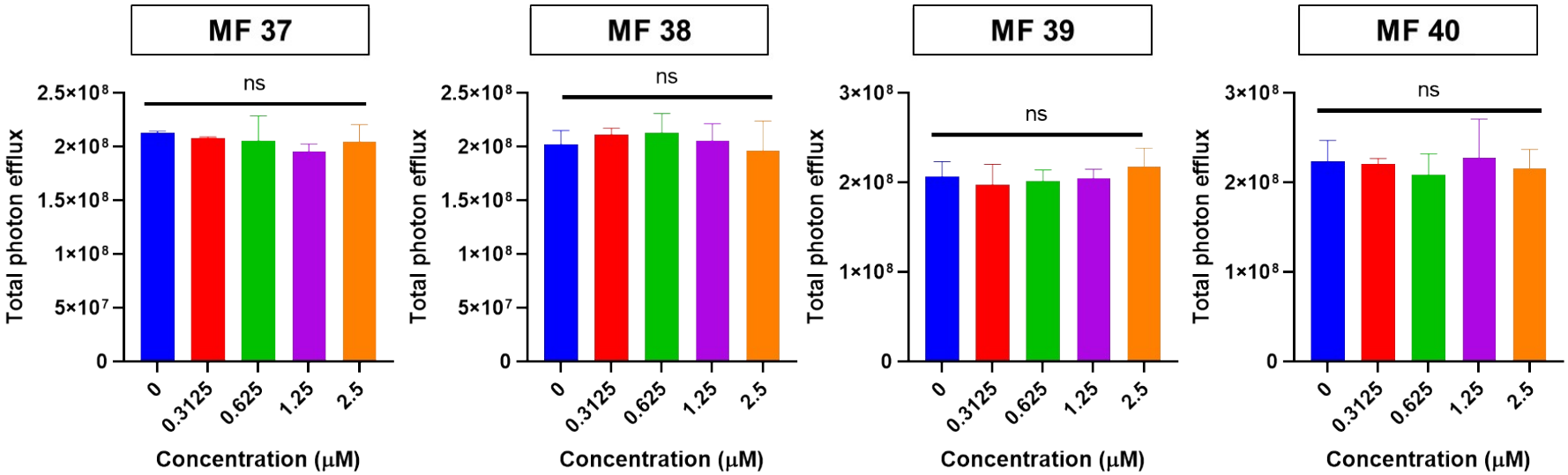


Figure S13. Caspase 3/7 activity in MF compounds-treated Raw264.7 cells

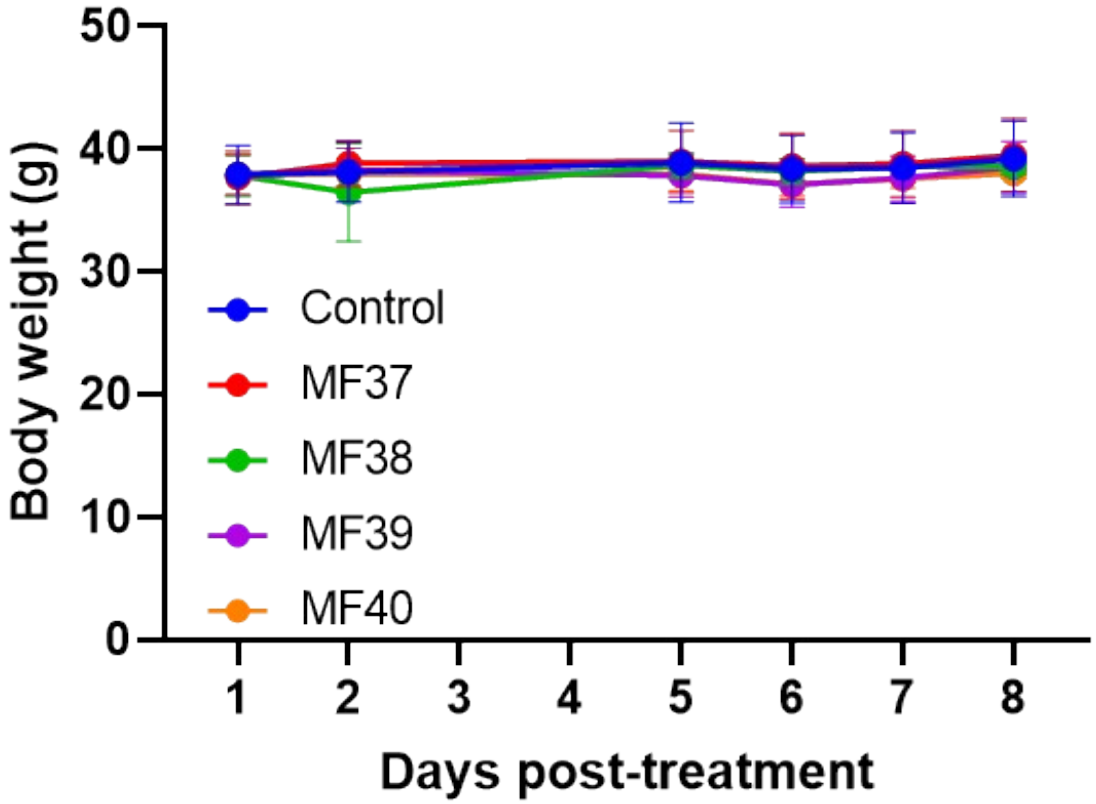


Figure S14. Body weight measurement

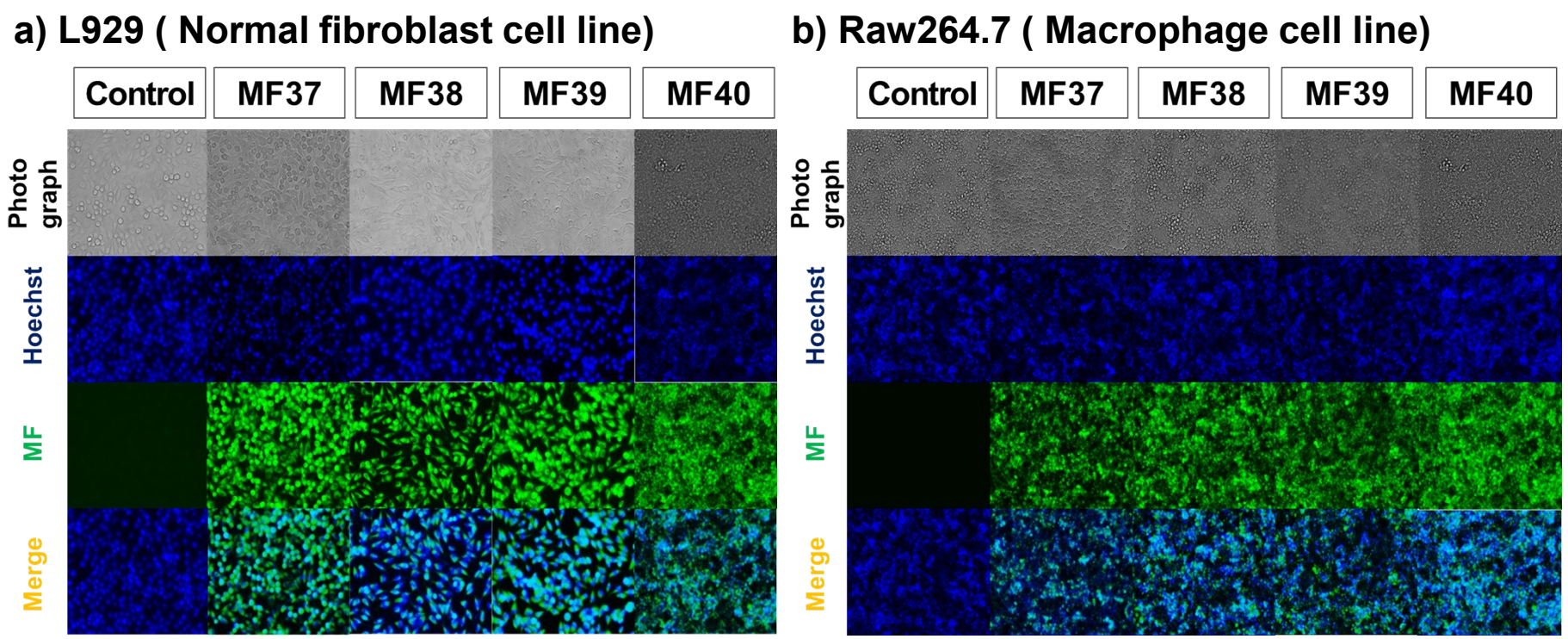
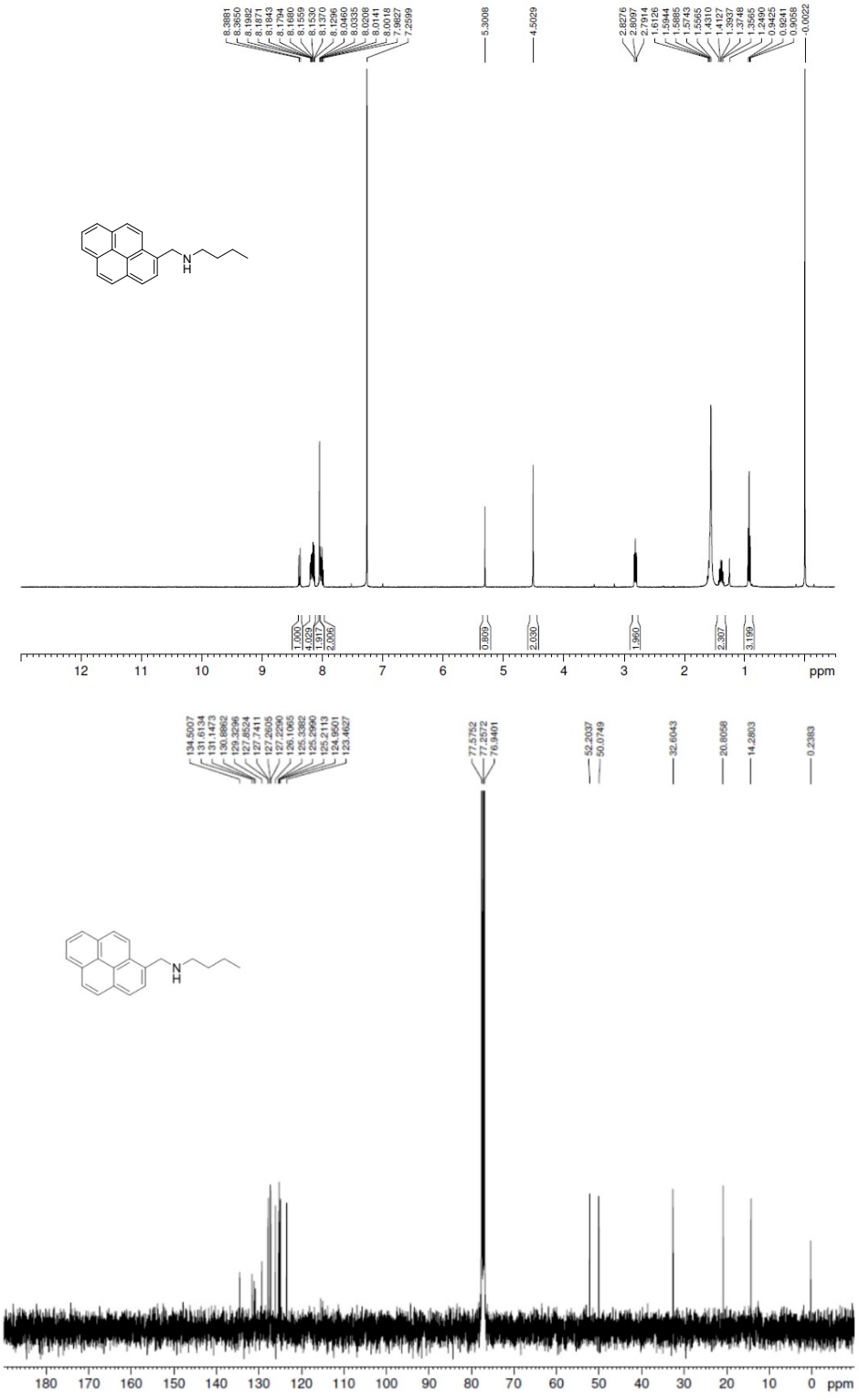


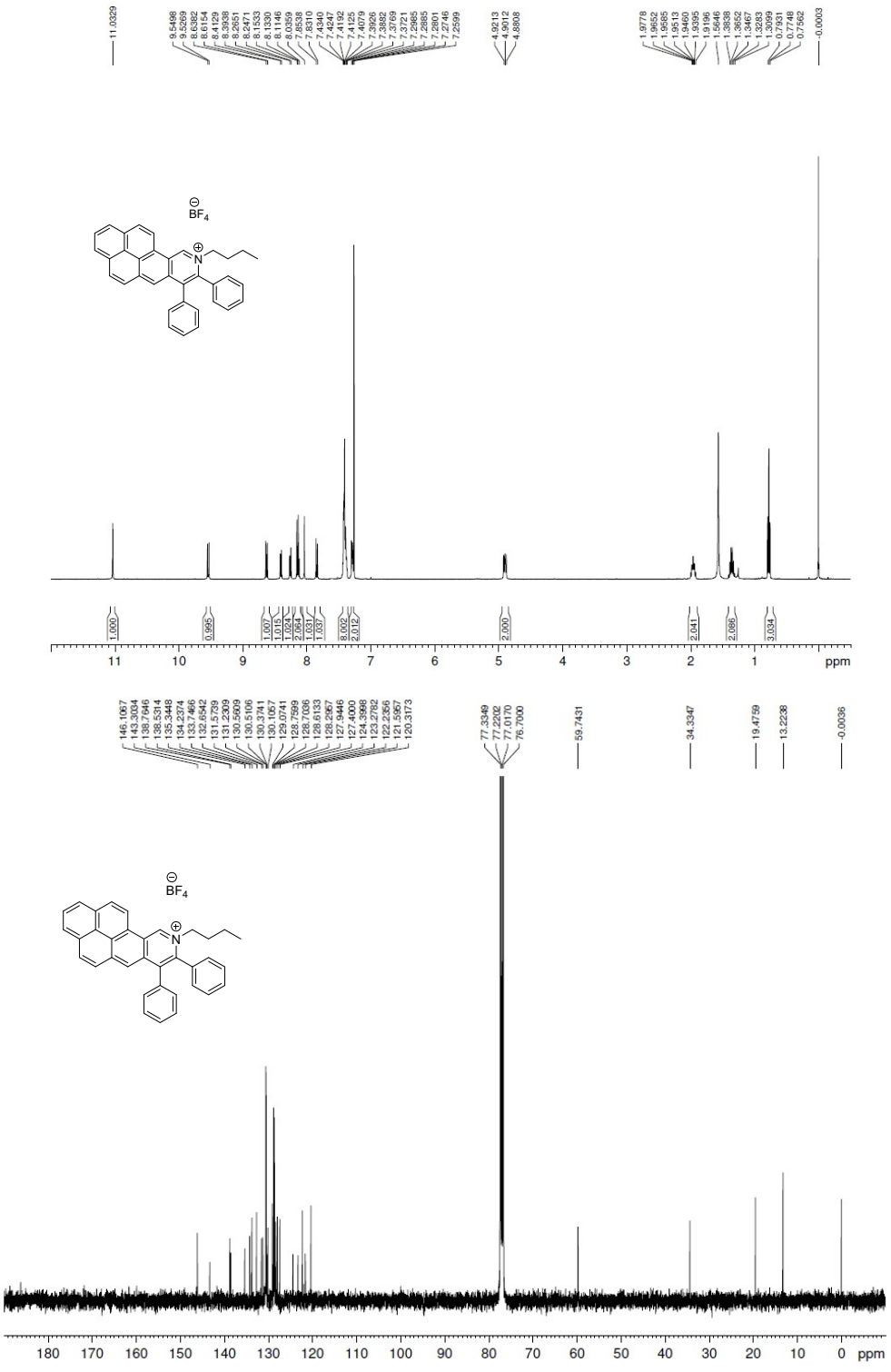
Figure S15. Cellular uptake of MF compounds in normal fibroblast L929 and immune Raw264.7 cells

^{1}H and ^{13}C NMR spectra

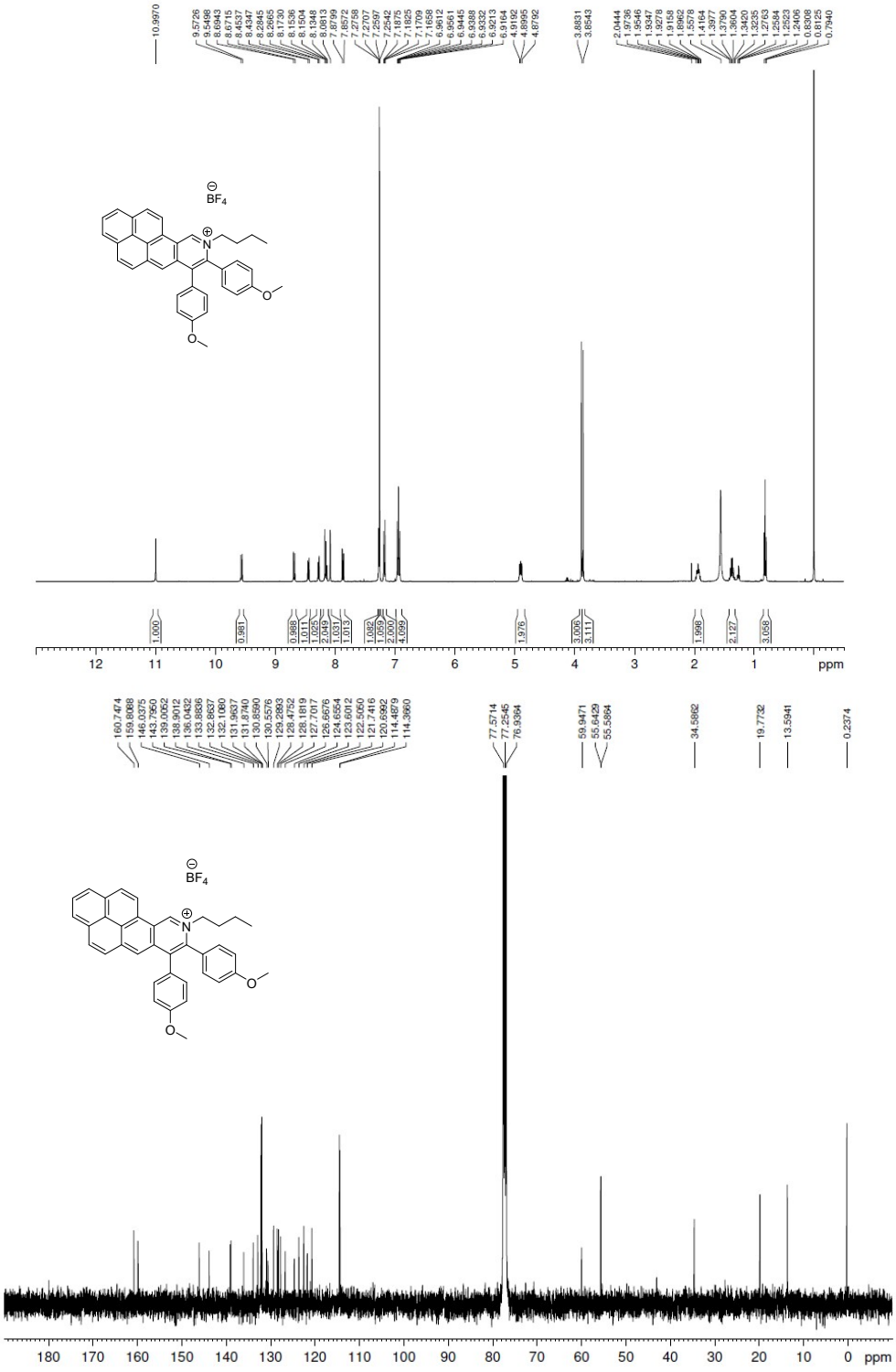
- *N*-(pyren-1-ylmethyl)butan-1-amine (CAS No. 94964-63-3, **1a**)



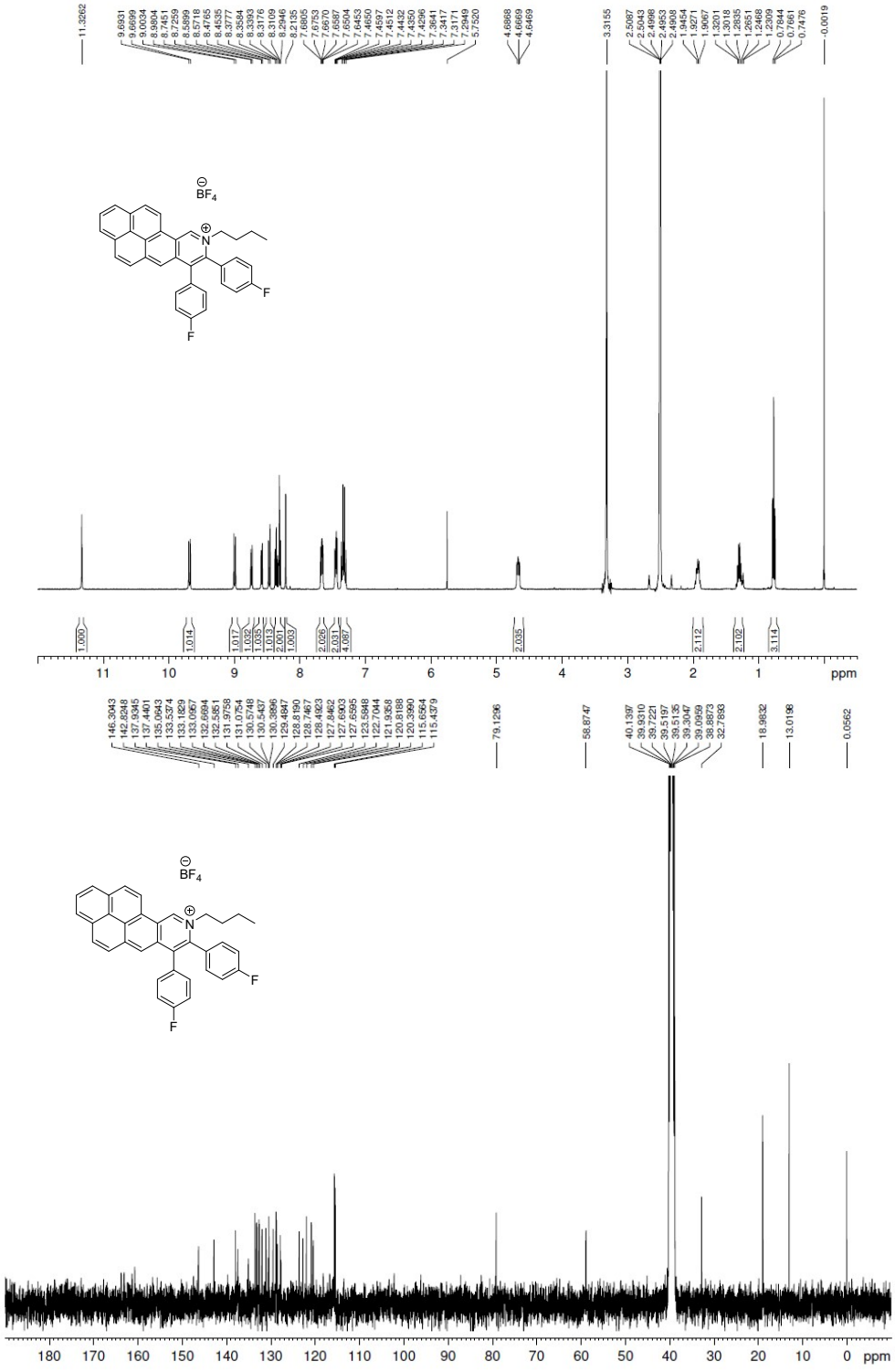
- 9-butyl-7,8-diphenylphenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (**MF37**)



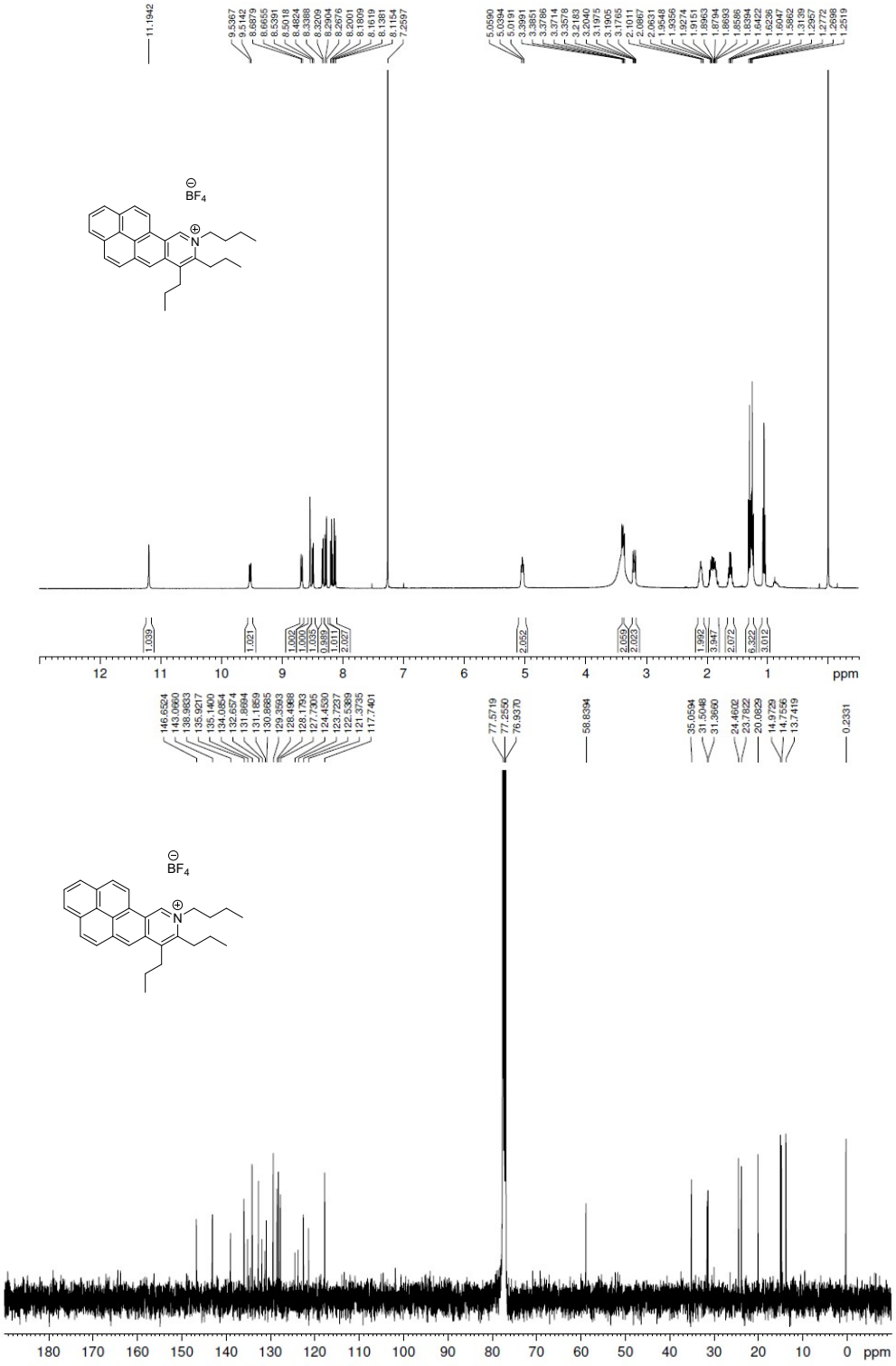
- 9-butyl-7,8-bis(4-methoxyphenyl)phenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (**MF38**)



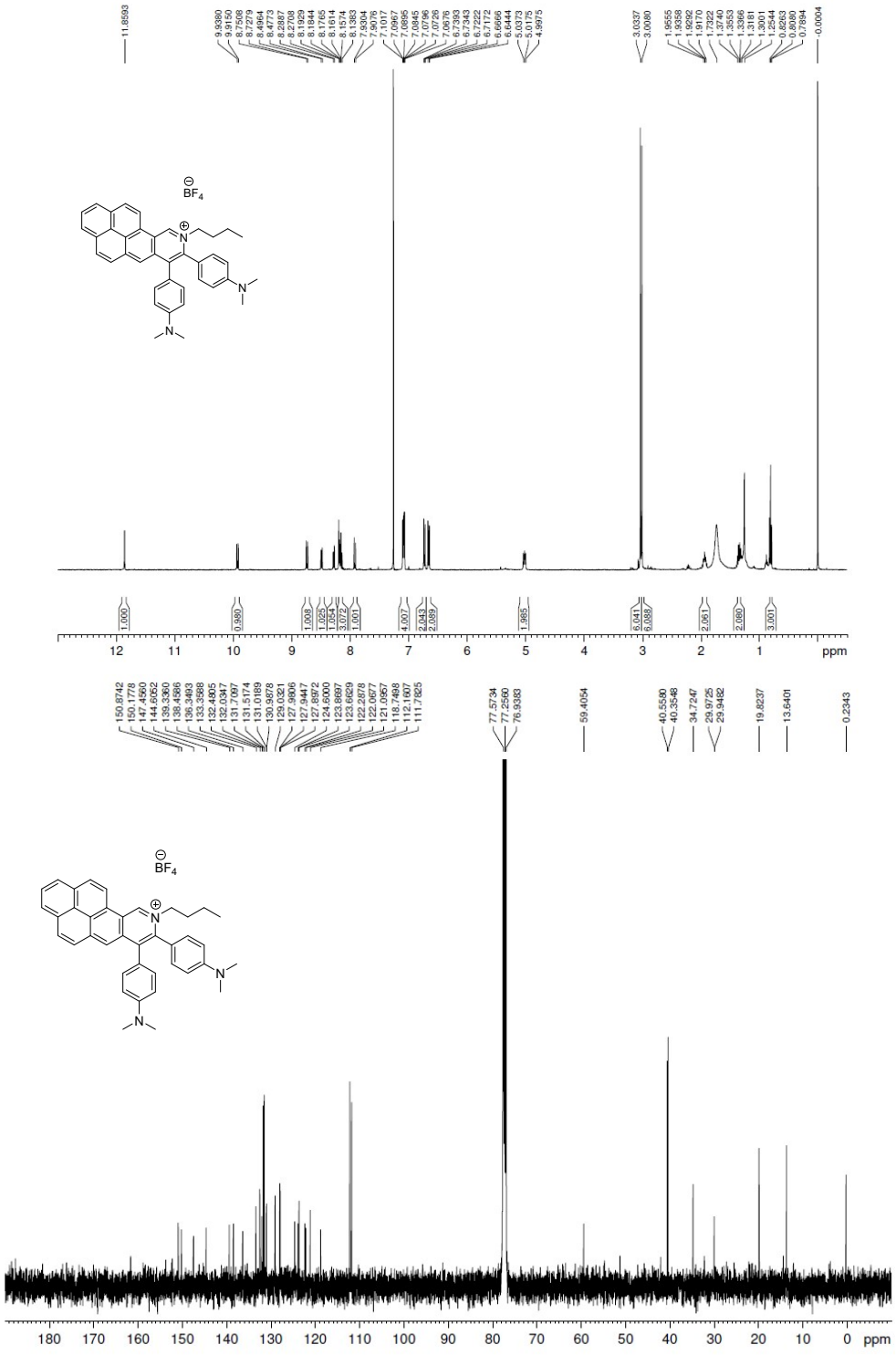
- 9-butyl-7,8-bis(4-fluorophenyl)phenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (**MF39**)



- 9-butyl-7,8-dipropylphenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (**MF40**)



- 9-butyl-7,8-bis(4-(dimethylamino)phenyl)phenaleno[1,9-gh]isoquinolin-9-ium tetrafluoroborate (**MF41**)



References

[1] R. C. Benson and H. A. Kues, *J. Chem. Eng. Data*, 1977, **22**, 379.