A fluorescent light-up probe with AIE characteristics for specific mitochondrial imaging to identify differentiating brown adipose cells

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Experimental Section

Materials and method

All chemicals were purchased from Aldrich unless indicated.

The UV-vis absorption spectra were obtained using UV-vis spectrometer (Shimadzu, UV-1700, Japan). PL measurements were carried out on a Perkin-Elmer LS-55 equipped with a xenon lamp excitation source and a Hamamatsu (Japan) 928 PMT, using 90° angle detection for solution samples. The cells were imaged by fluorescence microscope (Nikon A1 Confocal microscope). ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. Chemical shifts were reported in parts per million (ppm) referenced with respect to residual solvent ($CDCl_3$ = 7.26 ppm, $(CD_3)_2SO$ = 2.50 ppm or tetramethylsilane $Si(CH_3)_4$ = 0 ppm). The molecular mass was acquired using ion trap-time-of-flight mass spectrometry (MS-IT-TOF) (Shimadzu). Confocal imaging was done using Nikon A1R microscope. Excitation and emission wavelength: 405 nm and 500–550 nm for **AIE-MitoGreen-1;** 560 nm and 581–688 nm for MT.

Cell culture and imaging

Cell Culture

A wild-type brown preadipose cell line WT-1 was obtained from Kai Ge lab. Cells were differentiated into mature adipocytes using a protocol described previously:[1] Brown preadipose cells were passaged in 10% fetal bovine serum(FBS) in DMEM. To prime cells for differentiation to mature adipocytes, cells were first incubated with 0.02 μM insulin and 1 nM T3 in 10% FBS/DMEM for three days. Next, on the day of differentiation (day 1), the medium was replaced by 0.02 μM insulin, 1 nM T3, 0.125 mM indomethacin, 2 μg/ml dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine for two days. On day 3, this medium was replaced by 0.02 μM insulin and 1 nM T3 in 10% FBS/DMEM. This change of medium was repeated on day 5. On day 7, the cells were harvested.

HeLa cells were cultured in the MEM containing 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) in a 5% CO_2 humidity incubator at 37°C.

Cell Imaging

Brown adipose cells were seeded on micro-slide, and incubated in DMEM medium containing 10% fetal bovine serum for overnight. Probes were then added at these final concentrations (**AIE-MitoGreen-1**, 5 μM; MitoTracker Red FM, 50 nM) unless otherwise stated. Incubation was done for 20 min at 37 ^{o}C, and the probe was washed off and replaced with fresh medium. In wash-free imaging experiments, the probe remained in the well. For imaging of mature adipose cells, preadipose cells were induced to differentiation as described previously.[1] For Oil-Red-O Staining, Oil-Red-O stock solution was prepared by dissolving its powder to isopropanol to give a final concentration of 5 mg/ml. Oil-Red-O working solution was prepared by mixing 3 parts of Oil-Red-O stock solution with 2 parts of distilled water, and filtering through a 0.2 μm microfilter to remove undissolved particulates. Cell samples were fixed with 4% formaldehyde for 20 min at room temperature. Oil-Red-O working solution was added for 1 hour at room temperature, rinsed with water and stored at 4□C in water.

HeLa cells were grown overnight on a 35 mm petri dish with a cover slip or a plasma-treated 25 mm round cover slip mounted to the bottom of a 35 mm petri dish with an observation window. The live cells were stained with 5 μM of **AIE-MitoGreen-1** for 20 min (by adding 2 μL of a 5 mM stock solution of **AIE-MitoGreen-1** in DMSO to 2 mL culture medium) or 50 nM MitoTracker® Red FM (MT) for 15 min (by adding 0.5 μL of a 200 μM stock solution of MT in DMSO to 2 mL culture medium). The cells were imaged under an FL microscope (BX41 Microscope) using different combination of excitation and emission filters for each dye: for **AIE-MitoGreen-1**, excitation filter = 330–385 nm, dichroic mirror = 400 nm, and emission filter = 420 nm long pass; for MT, excitation filter = 540–580 nm, dichroic mirror = 600 nm, and emission filter = 610 nm long pass.

For photostability test, after HeLa cells were incubated with 5.0 μM **AIE-MitoGreen-1** for 20 min at 37 °C, the changes of fluorescence intensity with scan time were determined by CLSM (Zeiss LSM 410, Jena, Germany). Excitation wavelength: 405 nm (1% laser power); emission filter: 500−550 nm. The data were obtained from replicate experiments (n = 3).

Cell viability evaluated by MTT Assay

Brown preadipose cells were seeded in 12-well plates at a density of 4 ×10^4 cells/mL. After 24 h incubation, the cells were exposed to a series of doses of the probe **AIE-MitoGreen-1** and MT at 37 ^{o}C. After 24 hours, MTT solution (Sigma) was added and kept 3 h in the incubator. MTT solubilization solution was then added into each well and the plate was gently shaken for 10 min at room temperature to dissolve all the precipitates formed. The absorbance of individual wells at 570 nm was then monitored by the microplate reader. The absorbance of MTT in the sample well was determined by subtracting the absorbance of the sample well from that of the corresponding control well. Cell viability was expressed by the ratio of the absorbance of MTT in the sample wells to that of the cells incubated with culture medium only.

References

1. J. Klein, M. Fasshauer, M. Ito, B. B. Lowell, M. Benito and C. R. Kahn, *J. Biol. Chem.*, 1999, **274**, 34795–34802.

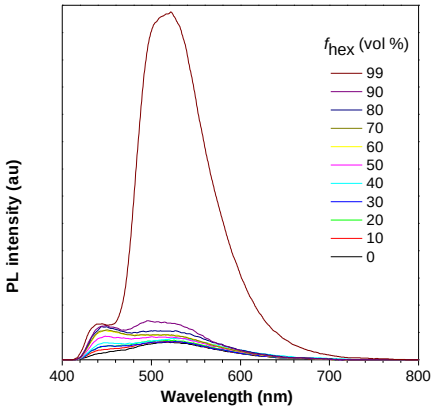


Fig. S1 (a) PL spectra of **AIE-MitoGreen-1** in ethanol and ethanol/hexane mixtures with different hexane fractions (f_{hex}); Concentration: 10 μM; excitation wavelength: 356 nm.

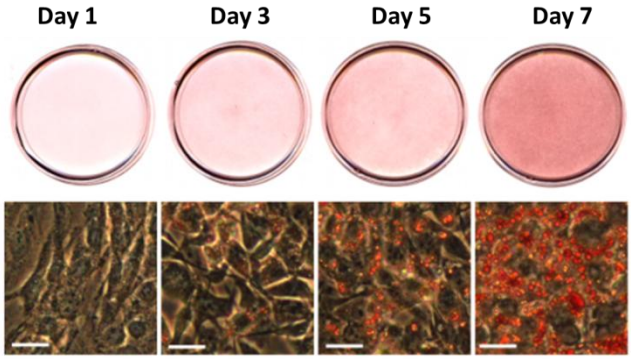


Fig. S2 Lipid droplets are stained using Oil-Red-O for WT-1 brown preadipose cells under differentiating medium for 1, 3, 5, 7 days. Scale bar = 50 μm.

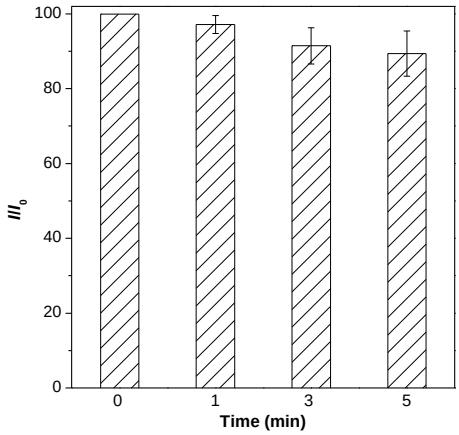


Fig. S3 I/I_0 of fluorescent emission of **AIE-MitoGreen-1** (5 μM) with increasing time. Excitation wavelength: 405 nm (for **AIE-MitoGreen-1**); emission filter: 515−560 nm.

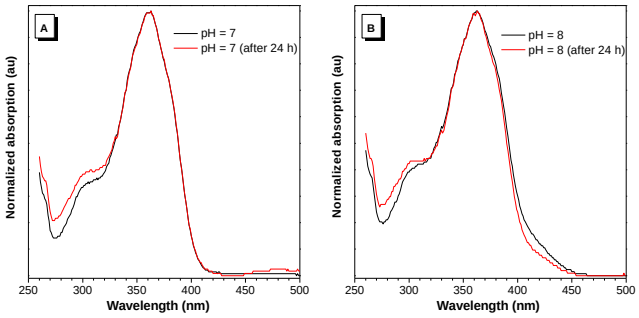


Fig. S4 Normalized UV spectra of **AIE-MitoGreen-1** in buffer solutions with pH of 7 and 8 at 0 and 24 h; Concentration: 10 μM.

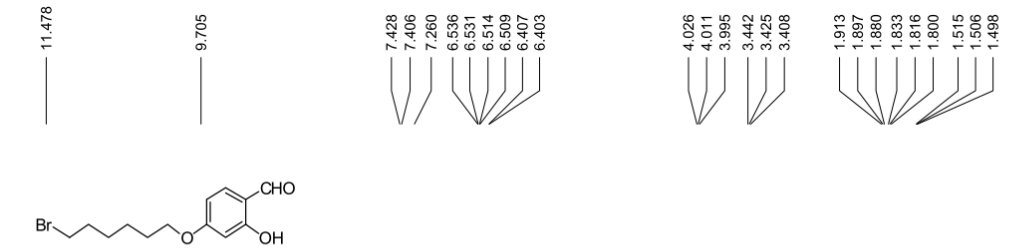
Synthesis and Characterization

Synthesis of (**2**): 2,4-dihydroxybenzaldehyde (276 mg, 2.0 mmol) and 1,6-dibromobutane (482 mg, 2.0 mmol) were first dissolved in DMF (10 mL), followed by addition of Cs_2CO_3 (652 mg, 2.0 mmol). The mixture was stirred at 60 ^{o}C under nitrogen for 12 h. After cooling to room temperature, the reaction mixture was extracted by dichloromethane (40 mL × 3), the combined dichloromethane fractions were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was further separated by column chromatography (silica, petroleum ether:ethyl acetate = 20:1) to give a colorless oil of **2** (402 mg, 67% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.48 (s, 1H), 9.71 (s, 1H), 7.42 (d, J = 8.4 Hz, 1H), 6.52 (d, J = 8.4 Hz, 2H), 6.40 (s, 1H), 4.01 (t, J = 6.0 Hz, 2H), 3.43 (t, J = 6.8 Hz, 2H), 1.91–1.80 (m, 4H), 1.52–1.50 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): 194.3, 166.3, 164.5, 135.2, 115.0, 108.7, 101.0, 68.3, 33.7, 32.6, 28.7, 27.8, 25.2; HRMS (ESI): m/z $[M + H]^+$ calculated for $C_{13}H_{18}BrO_3$: 302.1842; found: 302.2030.

Synthesis of (**3**): 4-((6-bromohexyl)oxy)-2-hydroxybenzaldehyde (300 mg, 1 mmol) was dissolved in absolute ethanol (10 mL), followed by addition of hydrazine monohydrate (25 mg, 0.5 mmol). The mixture was refluxed for 4 h. Precipitates were filtrated under vacuum and washed with absolute ethanol three times, then dried under vacuum. Pure product of **3** was obtained as a yellow powder solid (253 mg, 85% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.74 (br s, 2H), 8.57 (s, 2H), 7.21 (d, J = 8.4 Hz, 2H), 6.50 (d, J = 8.4 Hz, 2H), 4.00 (t, J = 6.4 Hz, 4H), 3.43 (t, J = 6.4 Hz, 4H), 1.92–1.51 (m, 16H); ^{13}C NMR ($CDCl_3$, 100 MHz): 163.4, 162.7, 161.7, 133.5, 111.0, 107.9, 101.7, 68.0, 33.6, 32.7, 28.9, 27.9, 25.2. HRMS (ESI): m/z $[M]^+$ calculated for $C_{26}H_{34}Br_2N_2O_4$: 596.0885; found: 596.3950.

Synthesis of (**AIE-MitoGreen-1**):

Compound **3** (298 mg, 0.5 mmol) was dissolved in pyridine (3 mL), and the mixture was stirred at reflux for 12 h. After completion of the reaction, the mixture was cooled to room temperature. The solvent was then removed by evaporation under pressure, the residue was recrystallized from a mixture of hexane/ethyl acetate, and the product **AIE-MitoGreen-1** was obtained as a yellow solid (351 mg, 93%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.02 (d, J = 5.6 Hz, 4H), 8.69 (s, 2H), 8.60 (t, J = 7.6 Hz, 2H), 8.12 (t, J = 6.4 Hz, 4H), 7.35 (d, J = 8.4 Hz, 2H), 6.53–6.47 (m, 4H), 4.67 (t, J = 7.2 Hz, 4H), 4.03 (t, J = 6.0 Hz, 4H), 2.11–1.48 (m, 16H); ^{13}C NMR (CD_3OD, 100 MHz): 165.0, 164.4, 162.8, 147.1, 146.1, 135.0, 129.7, 112.8, 108.7, 102.8, 69.2, 63.2, 32.5, 30.0, 27.0, 26.7; HRMS (ESI): m/z $[M – Br]^+$ calculated for $C_{36}H_{44}BrN_4O_4$: 675.2540; found: 675.2624.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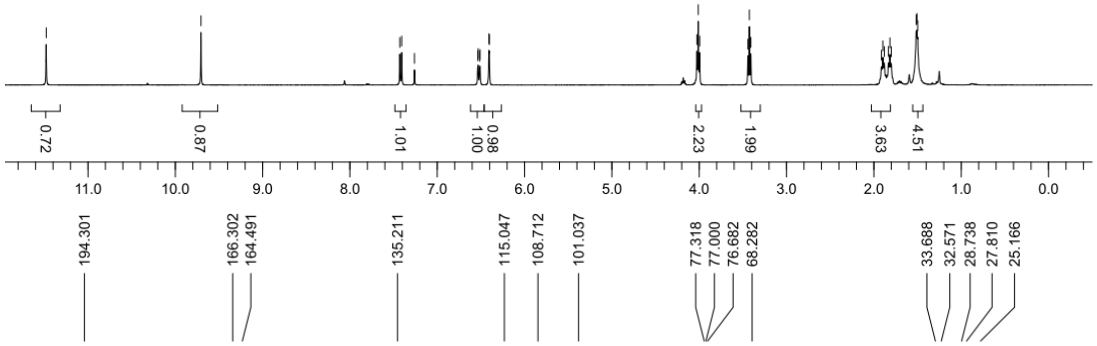
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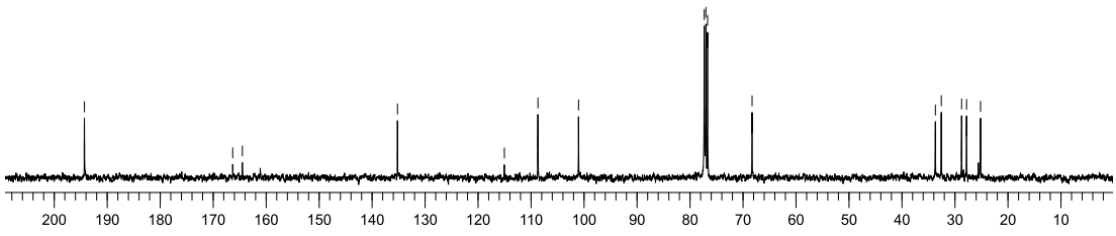
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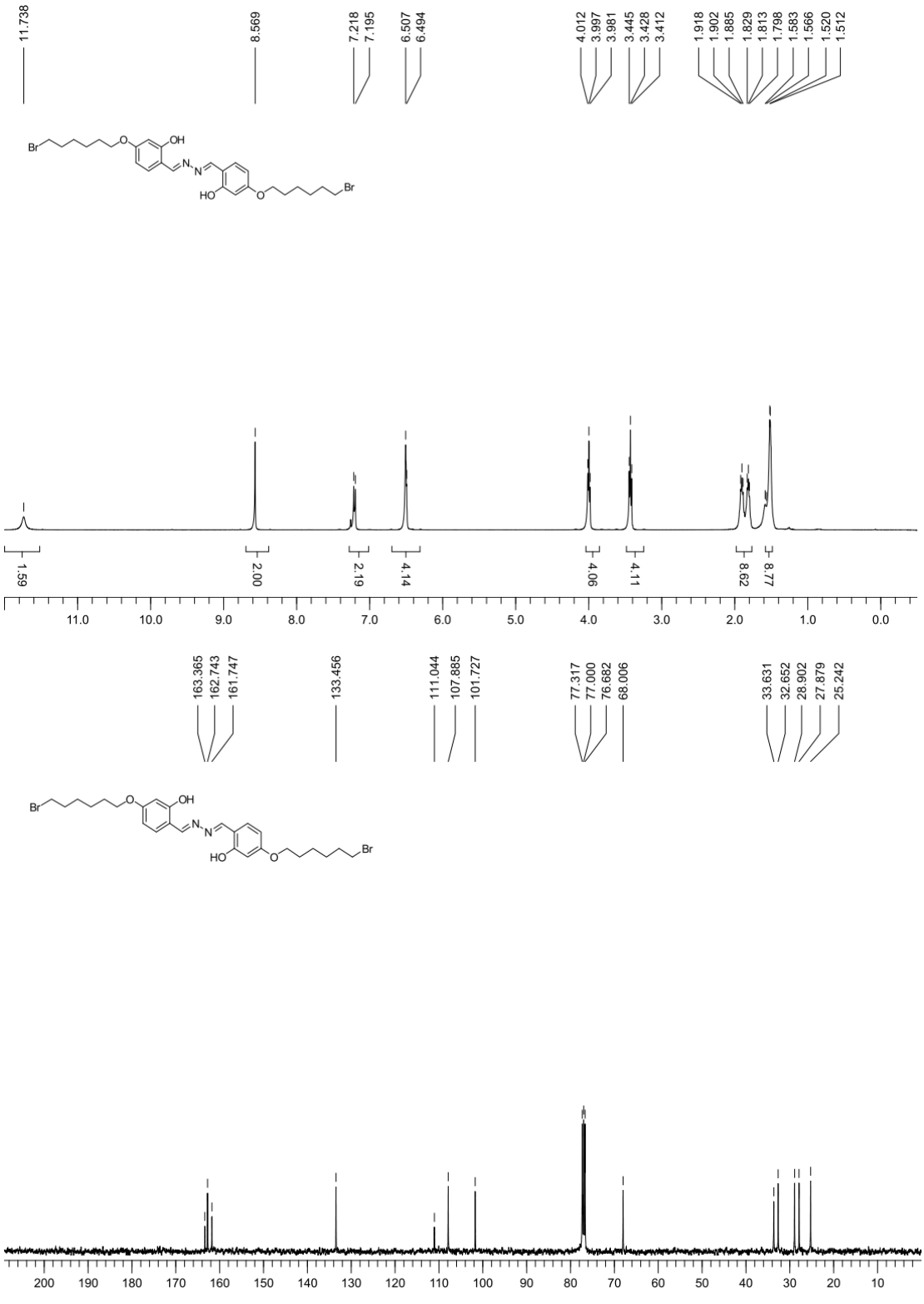
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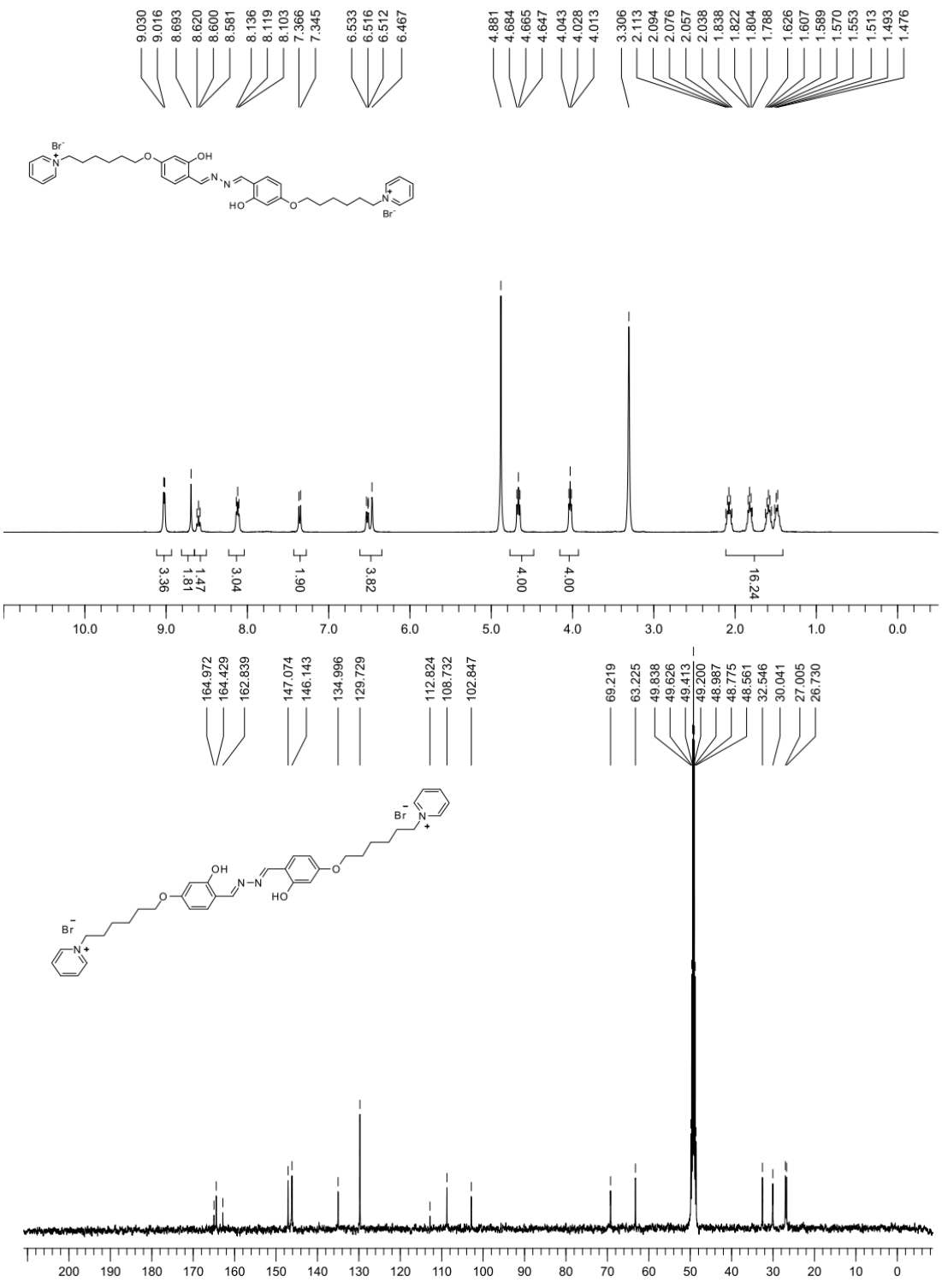
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