Fluorescent light-up probe with "AIE + ESIPT" characteristics for specific detection of lysosomal esterase

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Experimental Section

Materials and method

2,4-Dihydroxybenzaldehyde, 1,4-dibromobutane, Caesium carbonate, hydrazine monohydrate, acetic anhydride, morpholine, ethanol, anhydrous DMF, esterase from porcine liver, lysozyme, and Cathepsin B were all purchased from Aldrich.

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). The time-dependence fluorescence scans of **AIE-Lyso-1** were conducted with microplate reader (TECAN). ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. The molecular mass was acquired using ion trap-time-of-flight mass spectrometry (MS-IT-TOF) (Shimadzu).

Cell culture and imaging

Cell Culture

MCF-7 cell lines were provided by American Type Culture Collection. MCF-7 breast cancer cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Scientific) and maintained in a humidified incubator at 37 ^{o}C with 5% CO_2. Before experiment, the cells were pre-cultured until confluence was reached.

Cell Imaging

MCF-7 cells were seeded at a density of 2 $\times 10^4$/well in the chamber (LAB-TEK, Chambered Coverglass System) and grown for 18 h at 37°C in a humidified 5% CO_2 incubator. The cells were washed twice with 1 × PBS buffer and 0.3ml fresh

serum-free medium containing 1.0 μM **AIE-Lyso-1** solution was then added to the chamber.

For real-time fluorescence imaging, 1.0 μM **AIE-Lyso-1** solution was incubated with MCF-7 cells, and the images were collected at different time points by CLSM (Zeiss LSM 410, Jena, Germany) with imaging software (Olympus Fluoview FV1000). The intensity images of **AIE-Lyso-1** were recorded with the emission in the range of 515-560nm. Excitation wavelength: 405 nm.

For co-staining between **AIE-Lyso-1** and LysoTracker Red, the MCF-7 cells were first incubated with 1.0 μM **AIE-Lyso-1** solution for 2 h, and then the cells were rinsed and incubated in the medium containing 100 nM Lyso-tracker Red for 1 h. Confocal images were collected with a confocal laser scanning microscopy: **AIE-Lyso-1** was excited at 405 nm (1% laser power) and the fluorescence was collected at 515−560 nm; LysoTracker Red was excited at 560 nm (18% laser power) and fluorescence was collected at 581−688 nm.

For Photostability study, MCF-7 cells were stained with 1.0 μM **AIE-Lyso-1** and Lyso-tracker Red for 1 h at 37 °C under 5% CO_2. The changes of fluorescence intensity with scan time were determined by CLSM (Zeiss LSM 410, Jena, Germany). Excitation wavelength: 405 nm (for **AIE-Lyso-1**, 1% laser power) and 560 nm (for LysoTracker Red, 18% laser power); emission filter: 515−560 nm (for **AIE-Lyso-1**) and 581−688 nm (for LysoTracker Red). The data were obtained from replicate experiments (n = 3).

Cell viability evaluated by MTT Assay

MCF-7 cancer cells were seeded in 96-well plates at a density of 4 $\times 10^4$ cells/mL. After 24 h incubation, the cells were exposed to a series of doses of the probe **AIE-Lyso-1** at 37 ^oC. After the designated time intervals, the sample wells were washed twice with 1 × PBS buffer and freshly prepared MTT solution (0.5 mg/mL, 100 μL) in culture medium was added into each sample well. The MTT medium solution was carefully removed after 3 h incubation in the incubator for the sample wells, whereas the control wells without addition of MTT solution were washed twice with 1 × PBS buffer. DMSO (100 μL) 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 the differentiation between the absorbance of the sample well and 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.

Synthesis and Characterization

Synthesis of 4-(4-bromobutoxy)-2-hydroxybenzaldehyde (4): 2,4-Dihydroxybenzaldehyde **3** (276 mg, 2.0 mmol) and 1,4-dibromobutane (214 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 ^oC 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 (177 mg, 65% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.47 (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), 4.05 (t, J = 5.2 Hz, 2H), 3.48 (t, J = 6.0 Hz, 2H), 2.06–1.97 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): 194.3, 166.0, 164.5, 135.2, 115.2, 108.6, 101.1, 67.4, 33.1, 29.2, 27.6; HRMS (ESI): m/z $[M]^+$ calcd for $C_{11}H_{13}BrO_3$: 272.0048; found: 272.2673.

Synthesis of 6,6'-((1*E*,1'*E*)-hydrazine-1,2-diylidenebis(methanylylidene))-bis(3-(4-bromobutoxy)phenol) (5):

4-(4-Bromobutoxy)-2-hydroxybenzaldehyde (272 mg, 1 mmol) **4** was dissolved in absolute ethanol (10 mL), followed by addition of hydrazine monohydrate (25 mg, 0.5 mmol), and 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 **5** was obtained as a yellow powder solid (216 mg, 80% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.48 (br s, 2H), 8.58 (s, 2H), 7.22 (d, J = 8.4 Hz, 2H), 8.51–8.50 (m, 4H), 4.04 (t, J = 5.6 Hz, 2H), 3.48 (t, J = 6.4 Hz, 2H), 2.09–1.95 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz): 163.2, 162.7, 161.8, 133.6, 111.0, 107.9, 101.7, 67.1, 33.3, 29.4, 27.7. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{22}H_{27}Br_2N_2O_4$: 541.0338; found: 541.2141.

Synthesis of 6,6'-((1*E*,1'*E*)-hydrazine-1,2-diylidenebis(methanylylidene))-bis(3-(4-morpholinobutoxy)-phenol) (2):

6,6'-((1*E*,1'*E*)-hydrazine-1,2-diylidenebis(methanylylidene))bis(3-(4-bromobutoxy)-phenol) **5** (270 mg, 0.5 mmol) was added into morpholine (4 mL), and the mixture was refluxed under nitrogen for 4 h, then the mixture was dried under vacuum and extracted with dichloromethane (40 mL × 3). The extracts were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was further recrystallized from ethanol/hexane to give a yellow solid (263 mg, 95% yield). ^{1}H NMR (DMSO–d_6, 400 MHz): δ 11.47 (br s, 2H), 8.85 (s, 2H), 7.52 (d, J = 8.4 Hz, 2H), 6.56 (d, J = 8.4 Hz, 2H), 6.51 (s, 2H), 4.03 (t, J = 6.4 Hz, 4H), 3.56 (br s, 8H), 2.33–2.29 (m, 12H), 1.75–1.54 (m, 8H); ^{13}C NMR (DMSO–d_6, 100 MHz): 162.7, 162.0, 160.6, 132.7, 111.3, 107.5, 101.4, 67.6, 66.2, 57.6, 53.3, 26.3, 22.2; HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{30}H_{43}N_4O_6$: 554.3104; found: 555.3006.

Synthesis of ((1*E*,1'*E*)-hydrazine-1,2-diylidenebis(methanylylidene))bis(3-(4-morpholinobutoxy)-6,1-phenylene) diacetate (AIE-Lyso-1):

6,6'-((1*E*,1'*E*)-hydrazine-1,2-diylidenebis(methanylylidene))bis(3-(4-morpholinobutoxy)phenol) **2** (277 mg, 0.5 mmol) was dissolved in DMF (5 mL), followed by addition of Cs_2CO_3 (326 mg, 1.0 mmol), acetic acid anhydride (204 mg, 2.0 mmol), and the mixture was stirred at 60^{o}C for 8 h. After cooling to room temperature, the solvent

was removed under vacuum, and the residue was extracted with dichloromethane (40 mL × 3). The extracts were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was further recrystallized from ethanol/hexane to give a yellow solid (258 mg, 81% yield). ^{1}H NMR (DMSO–d_6, 400 MHz): δ 8.56 (s, 2H), 7.94 (d, J = 8.8 Hz, 2H), 6.99 (d, J = 7.2 Hz, 2H), 6.88 (s, 2H), 4.08–3.44 (m, 20H), 1.77–1.76 (m, 8H); ^{13}C NMR (DMSO–d_6, 100 MHz): 169.1, 161.6, 156.6, 151.2, 130.0, 118.6, 113.2, 109.3, 67.4, 63.3, 55.6, 51.1, 25.6, 20.8, 19.8. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{34}H_{47}N_4O_8$: 638.3316; found: 639.3193.

Synthesis of ((1*E*,1'*E*)-hydrazine-1,2-diylidenebis(methanylylidene))bis(3-(4-bromobutoxy)-6, 1-phenylene) diacetate 6:

6,6'-((1*E*,1'*E*)-hydrazine-1,2-diylidenebis(methanylylidene))bis(3-(4-bromobutoxy)phenol) **5** (270 mg, 0.5 mmol) was dissolved in DMF (5 mL), followed by addition of Cs_2CO_3 (326 mg, 1 mmol), acetic acid anhydride (204 mg, 2.0 mmol), and the mixture was stirred at 60^{o}C for 8 h. After cooling to room temperature, the solvent was removed under vacuum, and the residue was extracted with dichloromethane (40 mL × 3). The extracts were washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was further recrystallized from ethanol/hexane to give a yellow solid (268 mg, 86% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.65 (s, 2H), 8.00 (d, J = 8.8 Hz, 2H), 6.85 (d, J = 8.8 Hz, 2H), 6.67 (s, 2H), 4.04 (t, J = 6.0 Hz, 4H), 3.49 (t, J = 6.0 Hz, 4H), 2.38 (s, 6H), 2.09–1.95 (m, 8H); ^{13}C NMR ($CDCl_3$, 100 MHz): 169.0, 162.0, 156.6, 151.5, 129.7, 118.9, 113.2, 108.7, 67.3, 33.3, 29.3, 27.7, 21.0. HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{26}H_{31}Br_2N_2O_6$: 625.0549; found: 625.3316.

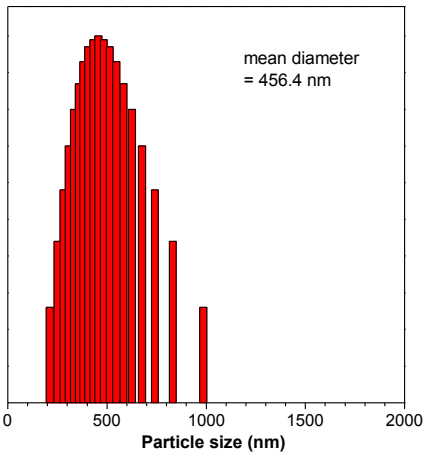


Fig. S1 (A) Size distribution of nanoparticles of **2** suspended in aqueous mixture, the polydiversity = 0.264.

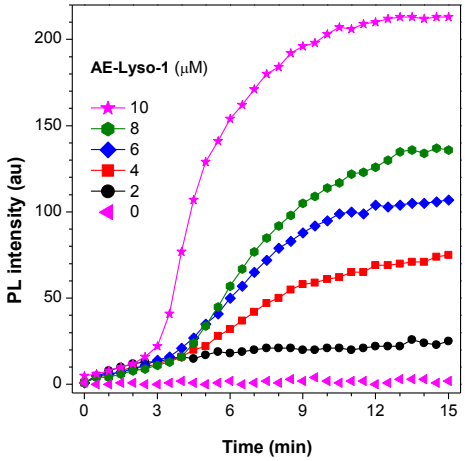


Fig. S2 Progress curves of hydrolysis of probe **AIE-Lyso-1** at a series of concentrations (0–10 μM) upon incubation with esterase (0.5 U mL^{-1}) in PBS buffer solution (pH = 7.4) with $\lambda_{ex/em}$ = 356/532 nm.

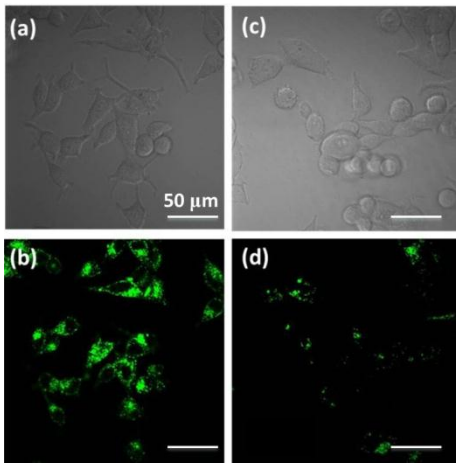


Fig. S3 (a, b) normal MCF-7 cells treated with 1.0 μM of **AIE-Lyso-1** only; (c, d) MCF-7 cells were pretreated with 0.4 mM AIEBSF for 30 min and then incubated with 1.0 μM of **AIE-Lyso-1** for 30 min. Scale bar = 50 μm.

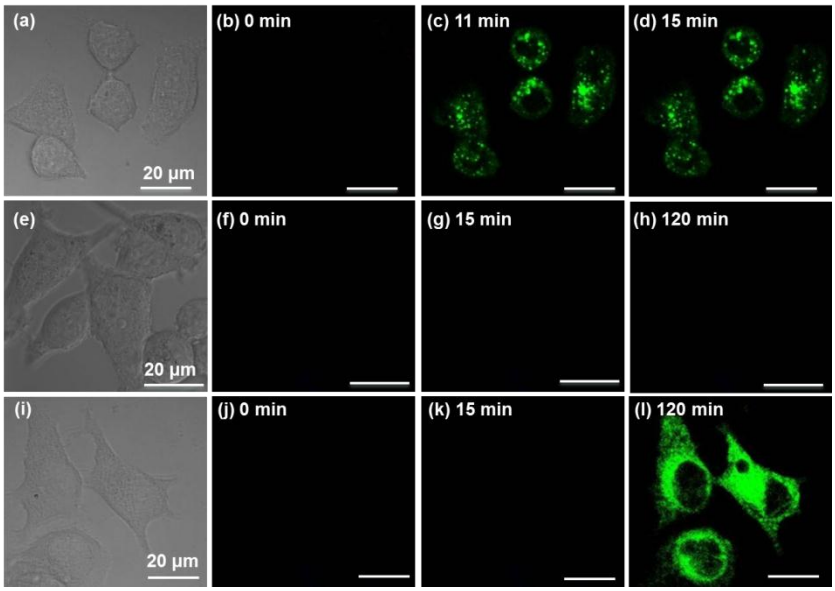


Fig. S4 Real-time fluorescence images showing the MCF-7 cells with (a, b, c, d) 1.0 μM of **AIE-Lyso-1**; (e, f, g, h) 1.0 μM of **6**; (i, j, k, l) 5.0 μM of **6**; Scale bar = 20 μm.

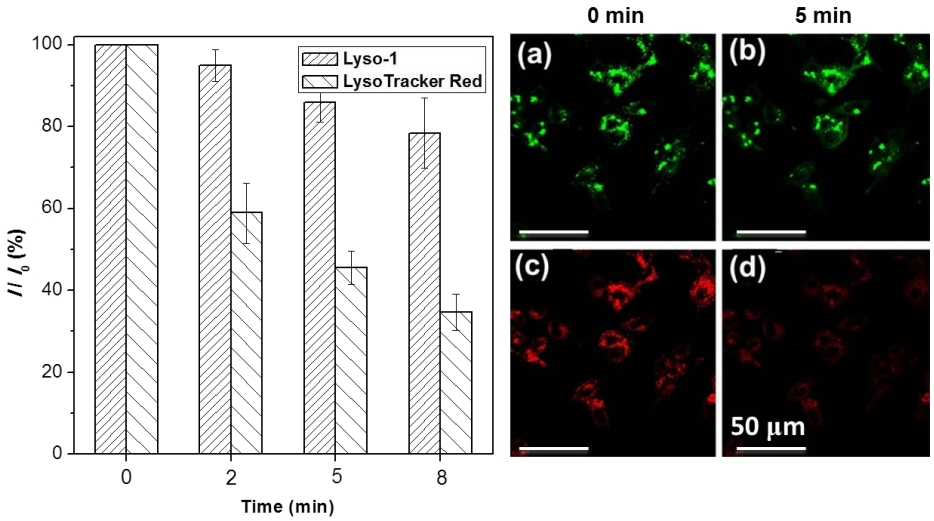


Fig. S5 (Left) I/I_0 (%) of fluorescent emission of **AIE-Lyso-1** (1.0 μM) and LysoTracker Red (50 nM) with increasing time. Excitation wavelength: 405 nm (for **AIE-Lyso-1**) and 560 nm (for LysoTracker Red); emission filter: 515−560 nm (for **AIE-Lyso-1**) and 581−688 nm (for LysoTracker Red); (Right) fluorescent images of living MCF-7 cells stained with **AIE-Lyso-1**(1.0 μM) and LysoTracker Red (50 nM) at 0 and 5 min. Scale bar = 50 μm.

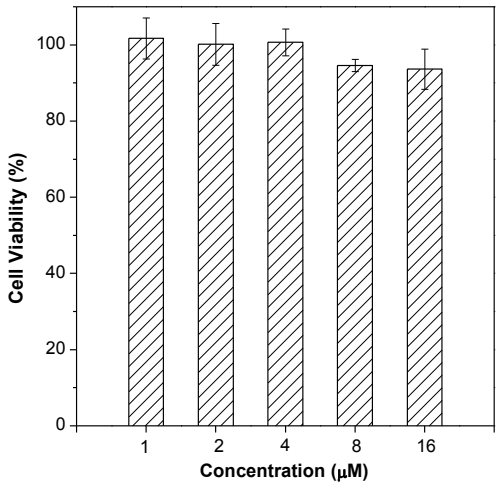
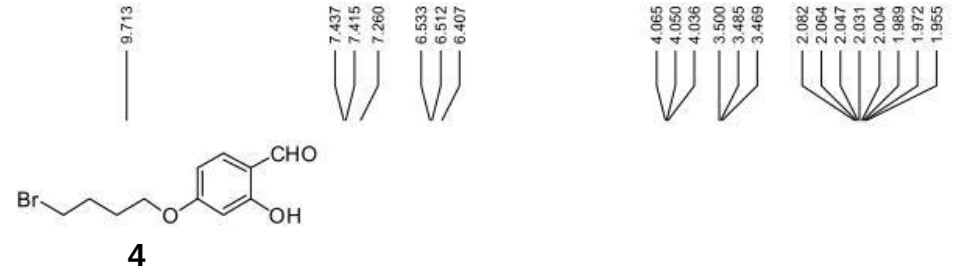


Fig. S6 Metabolic viability of MCF-7 breast cancer cells after incubation with **AIE-Lyso-1** at concentrations of 1, 2, 4, 8 and 16 μM for 24 h.



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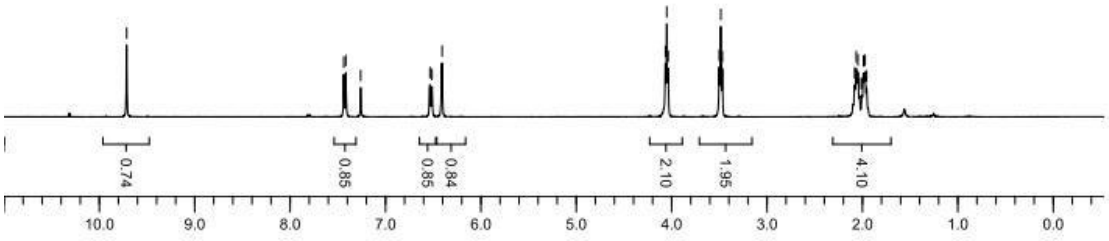

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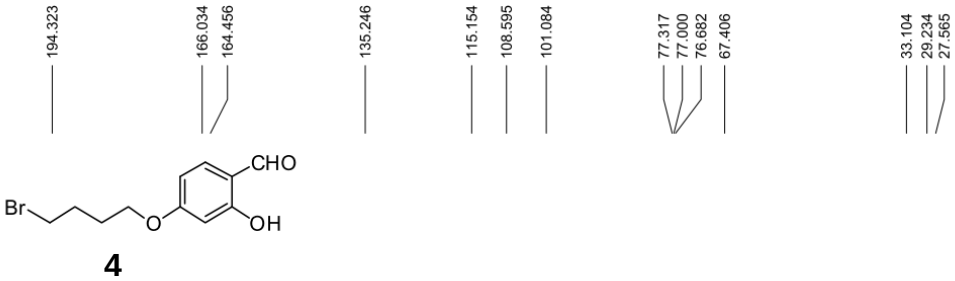

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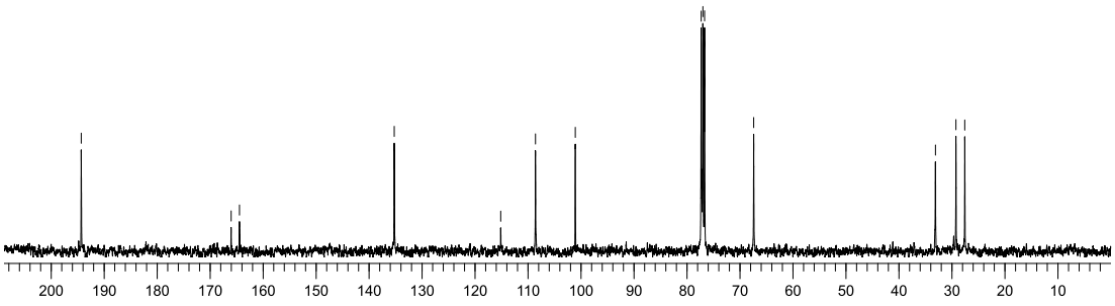

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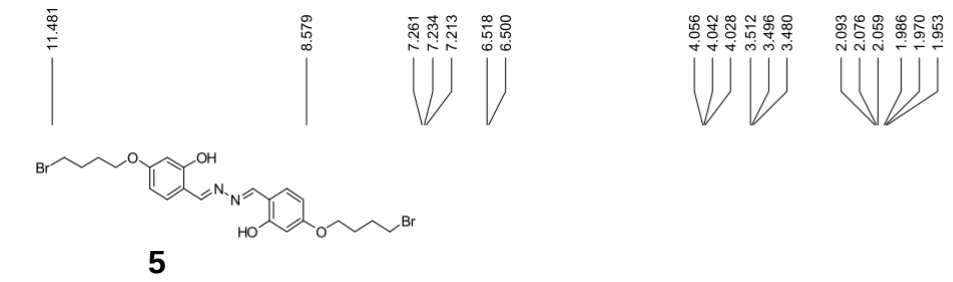

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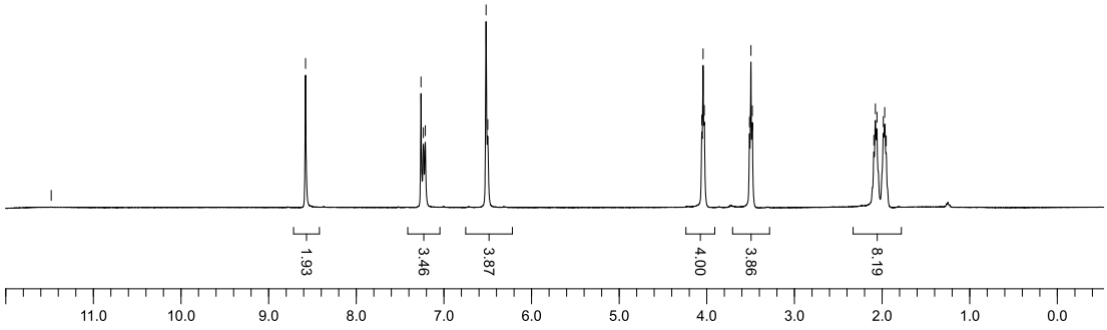

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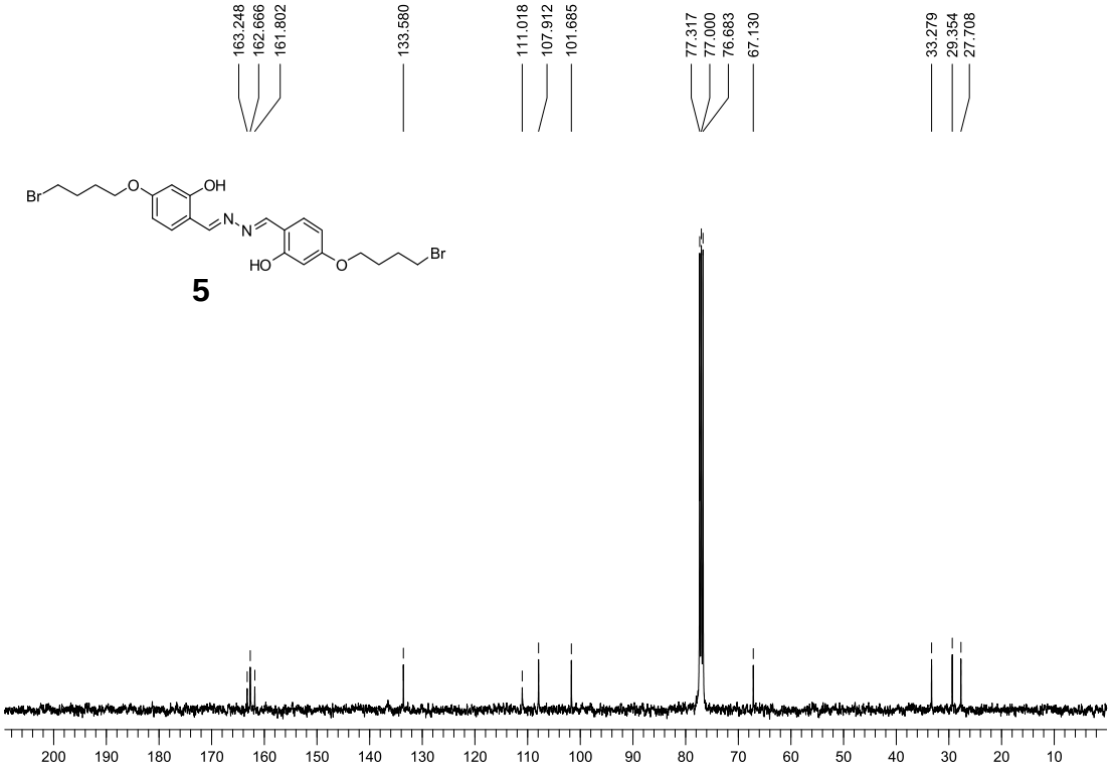

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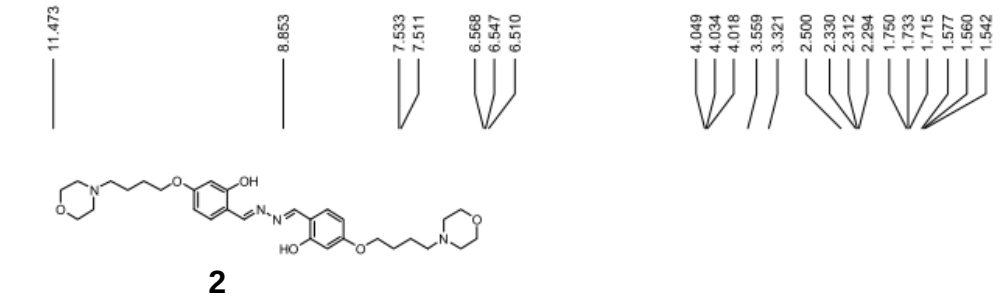

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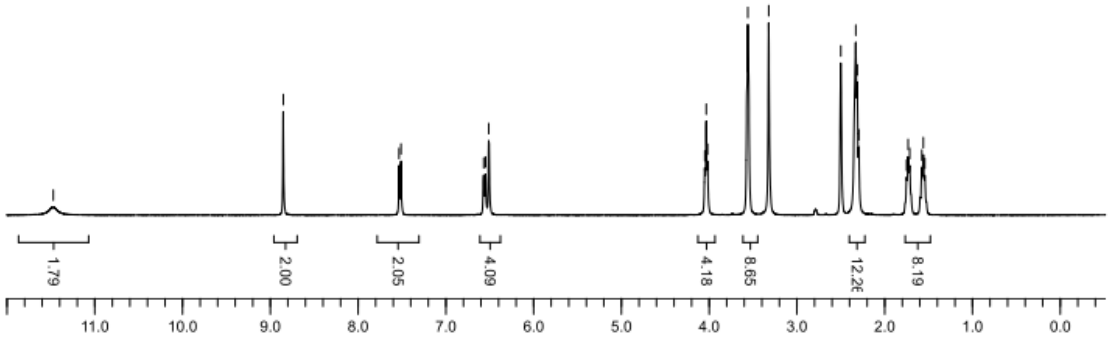

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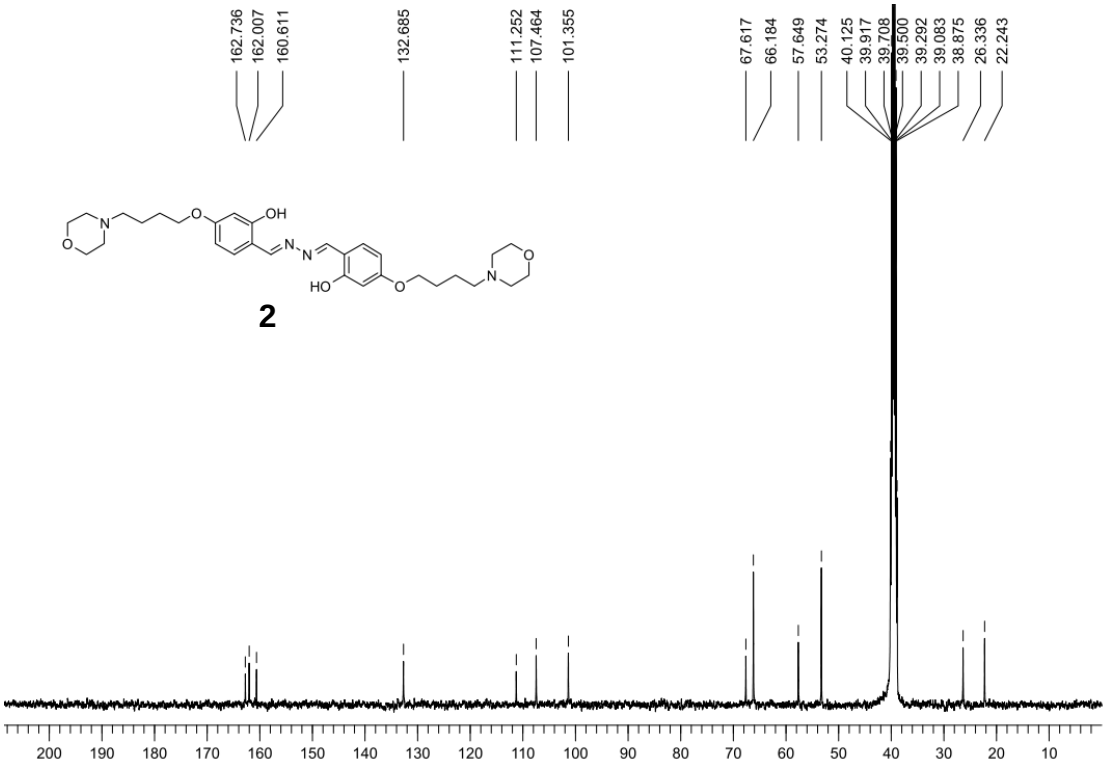

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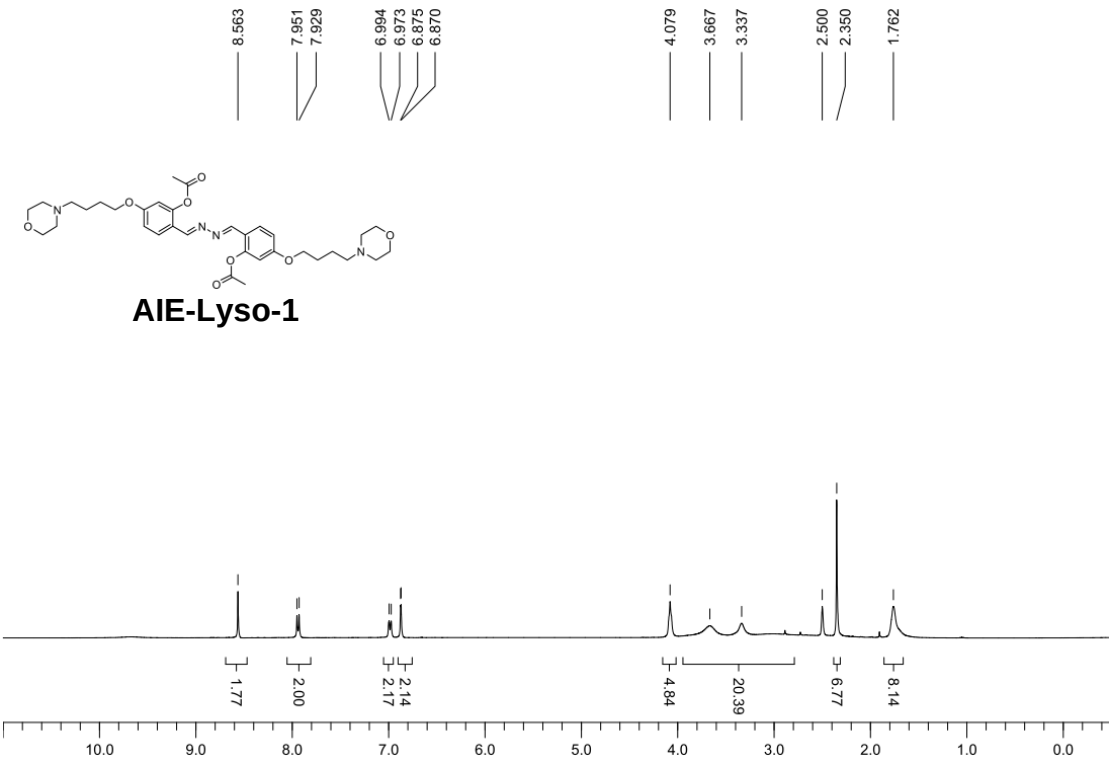
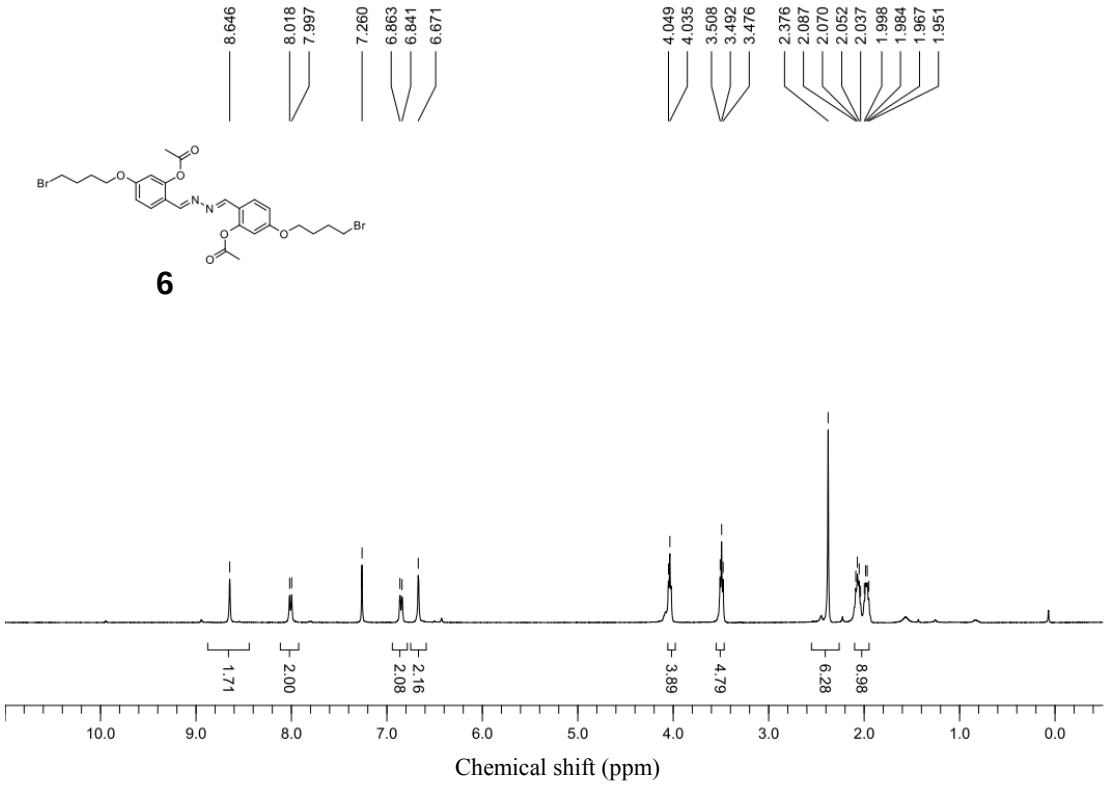


AIE-Lyso-1

AIE-Lyso-1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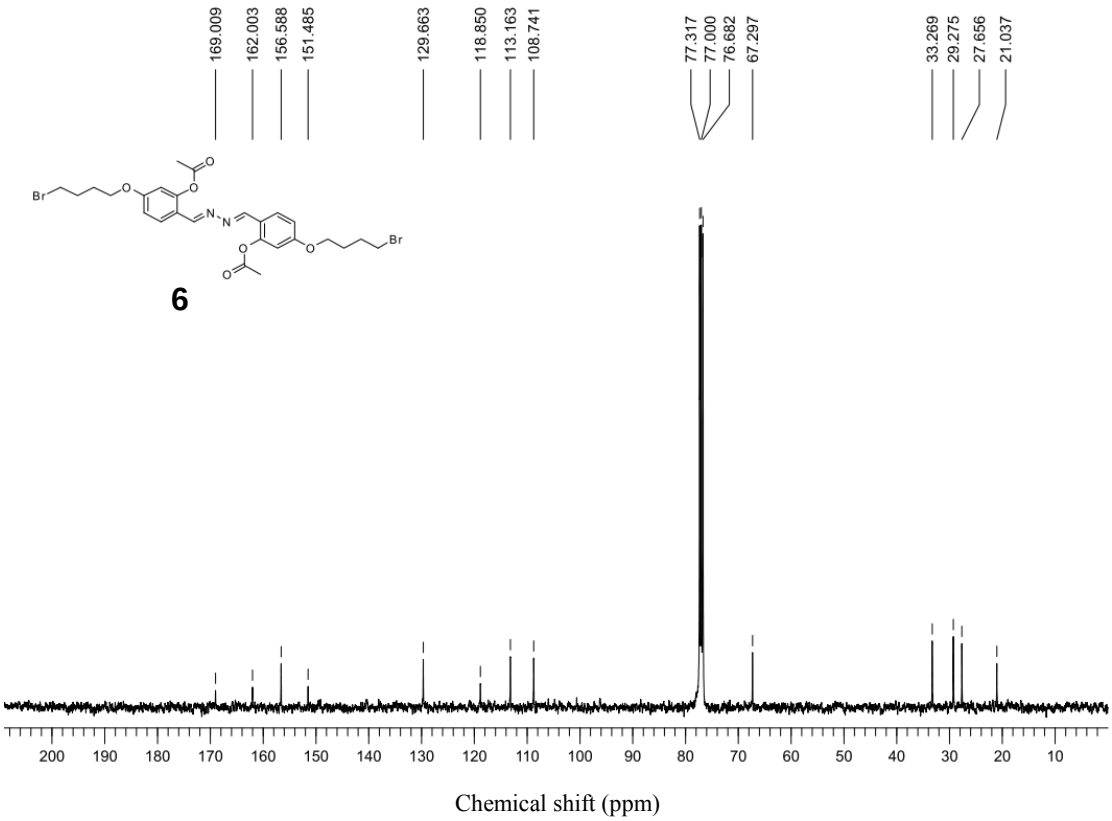
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Chemical shift (ppm)

6