Supporting Information

Novel NIR-II organic fluorophores for bioimaging beyond 1550 nm

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Author contributions

Y. Xiao and X. Hong conceived and designed the experiments. Y. Li, Y. Liu, T. Tian, W. Zhou, Y. Cui, X. Cheng, X. Wang performed the experiments. Y. Li, Q. Li, Q. Ding, X. Zeng, X. Meng, X. Wang, Y. Li, H. Deng, J. Wu, Z. Deng, X. Hong and Y. Xiao analyzed the data. Y. Li, Q. Li, X. Hong wrote the manuscript. All authors discussed the results and commented on the manuscript.

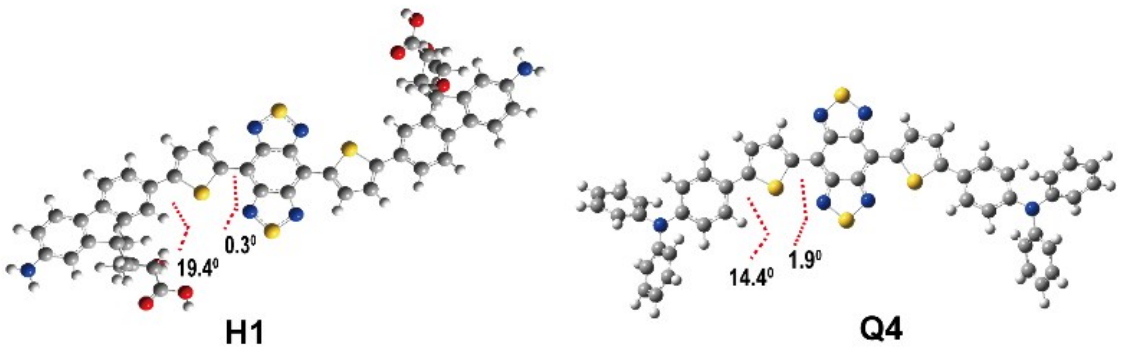


Figure S1. Optimized ground state geometries (S_0) of **H1** and **Q4** by using the method of the optimally B3LYP/6-31G(d) scrf with Gaussian 09 software.

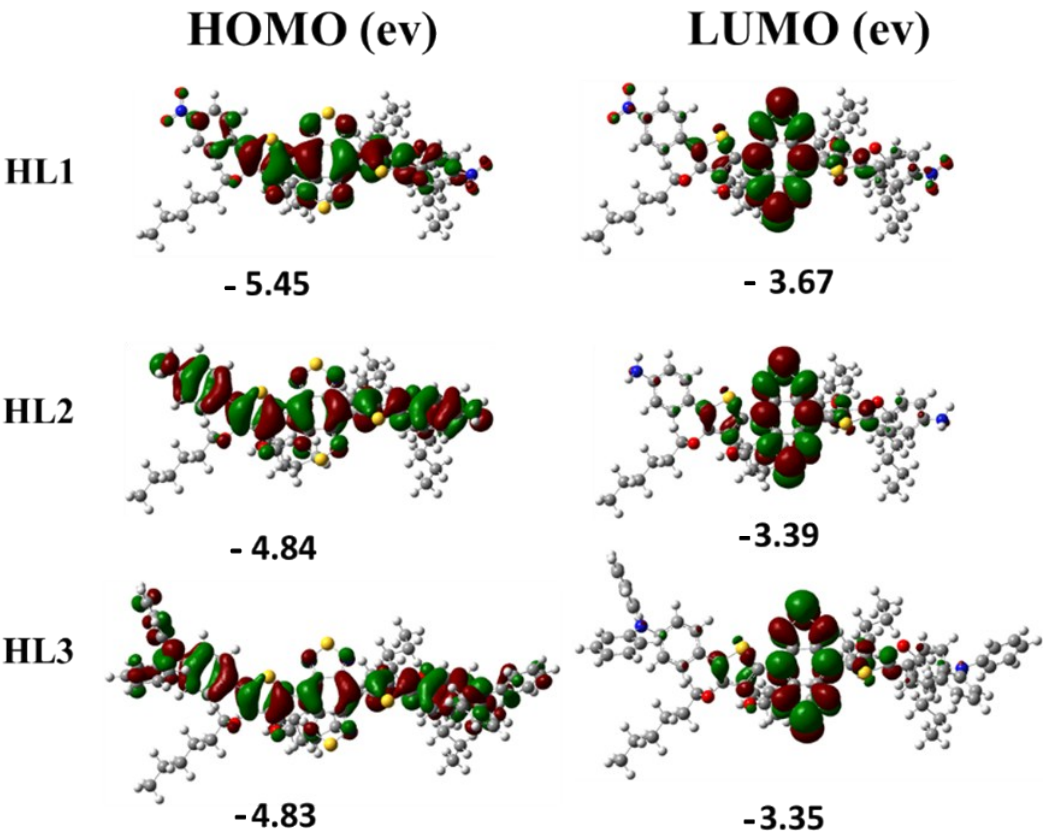


Figure S2. Highest occupied molecular orbitals (HOMOs) and lowest unoccupied molecular orbitals (LUMOs) of **HL1, HL2**, and **HL3** using Gaussian 09 time-dependent density functional theory (TD-DFT) calculations at B3LYP/6-31G(d) scrf = solvent = dichloromethane level.

Dyes	λ_{abs}(nm)	λ_{em}(nm)	Stokes Shifts	HOMO (eV)	LUMO (eV)	E_{gap} (eV)
H1	~850	~1100	~250	-4.71	-3.5	1.21
Q4	~880	~1100	~220	-4.58	-3.46	1.12
HL1	~643	~922	~279	-5.45	-3.67	1.78
HL2	~762	~1062	~300	-4.84	-3.39	1.45
HL3	~725	~1050	~325	-4.83	-3.35	1.48

Table S1. Calculated and experimental optical properties of **H1**, **Q4**, and **HL1-HL3.**

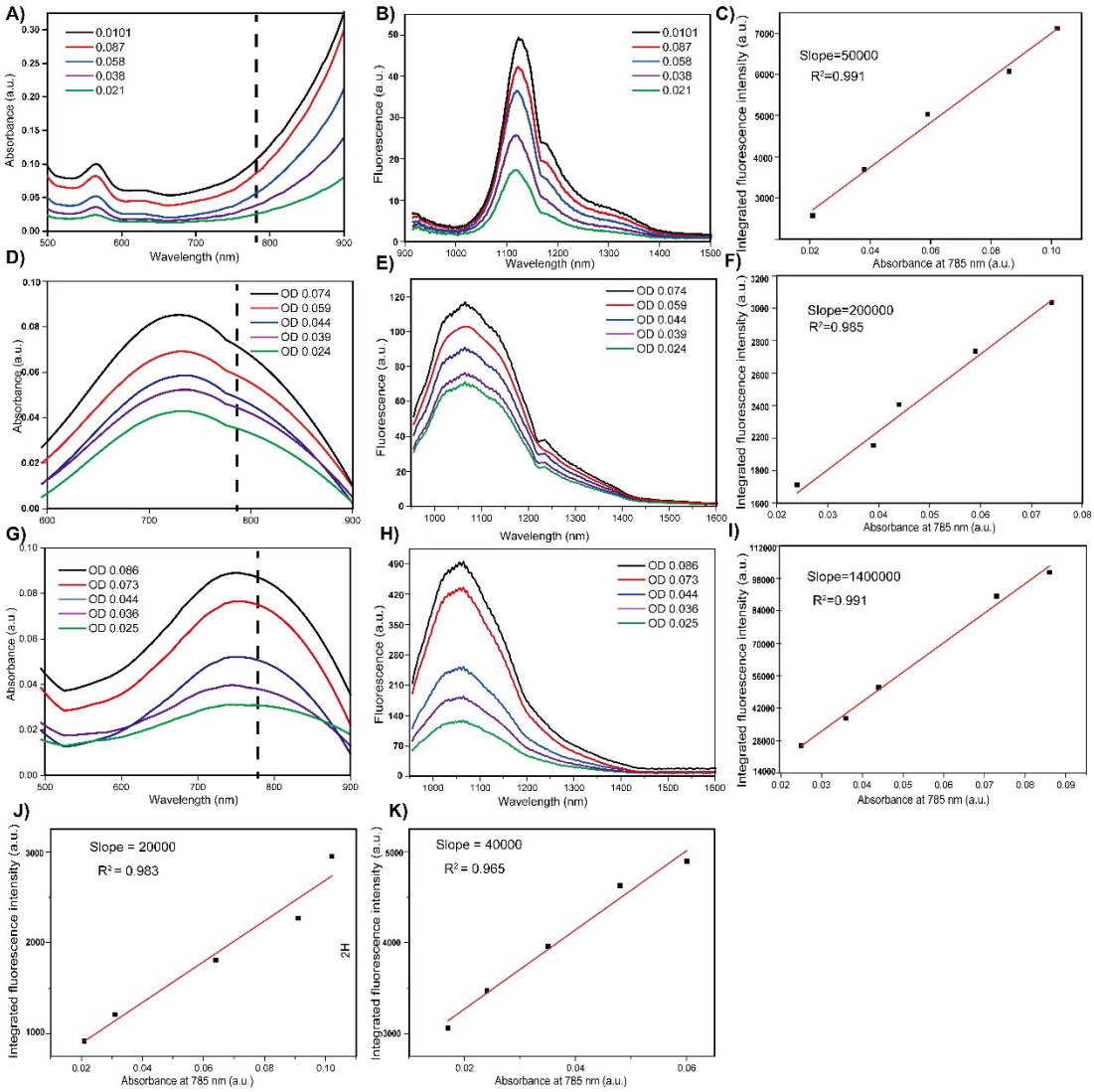


Figure S3. Fluorescence quantum yield measurements of **HL3** in THF. The method was reported in our previous work [1] and IR-26 is the reference with 0.5% quantum yield in DCE. Absorbance (A, D, G) and fluorescence (B, E, H) spectra of IR-26 in

DCE (A, B), **HL3** in THF (D, E), and **HL3** dots in water (G, H). The slope of IR-26 in DCE (C), **HL3** in THF (F), **HL1** in THF (J), **HL2** in THF (K) and **HL3** dots in water (I). The equation was below:

$$QY_{sam} = QY_{ref} \times \frac{S_{sam}}{S_{ref}} \times \left(\frac{n_{sam}}{n_{ref}}\right)^2$$

Where QY_{sam} is the QY of **HL3** dots, QY_{ref} is the quantum yield of IR-26 (~0.5%), S_{sam} and S_{ref} are the slopes obtained by linear fitting of the integrated fluorescence intensity of **HL3** dots (1000-1600 nm) or (1550-1600 nm) and IR-26 (1000-1600 nm) against the absorbance at 785 nm. n_{sam} and n_{ref} are the refractive indices of their respective solvents (water:1.333 and THF: 1.4)

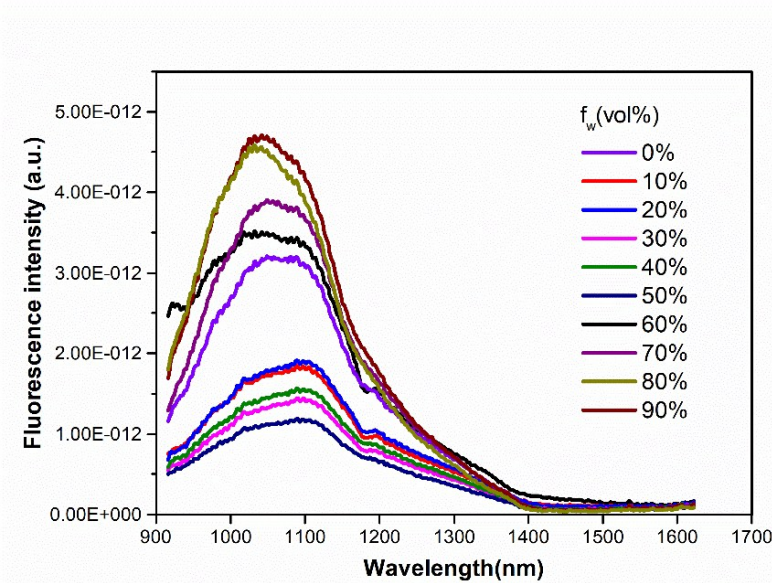


Figure S4. Fluorescence emission spectra of **HL2** in water /THF mixtures with different fw (0% to 90%). Excitation wavelength at 785 nm.

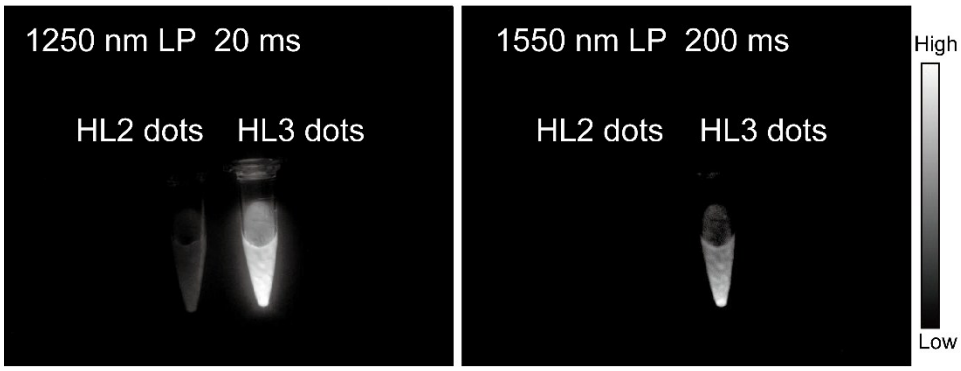


Figure S5. The NIR-II fluorescence images of **HL2** dots (0.4 mg/mL) and **HL3** dots (0.4 mg/mL) using 1250 nm and 1550 nm LP.

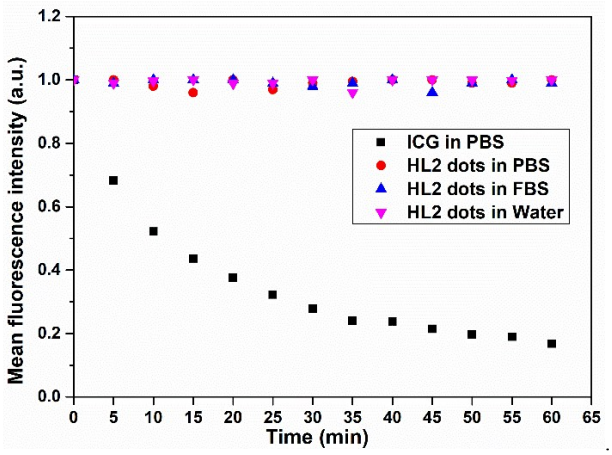


Figure S6. The photo-stability of **HL2** dots in water, FBS, PBS and ICG in PBS with continuous 808 nm laser irradiation (60 min, 90mW cm^{-2}).

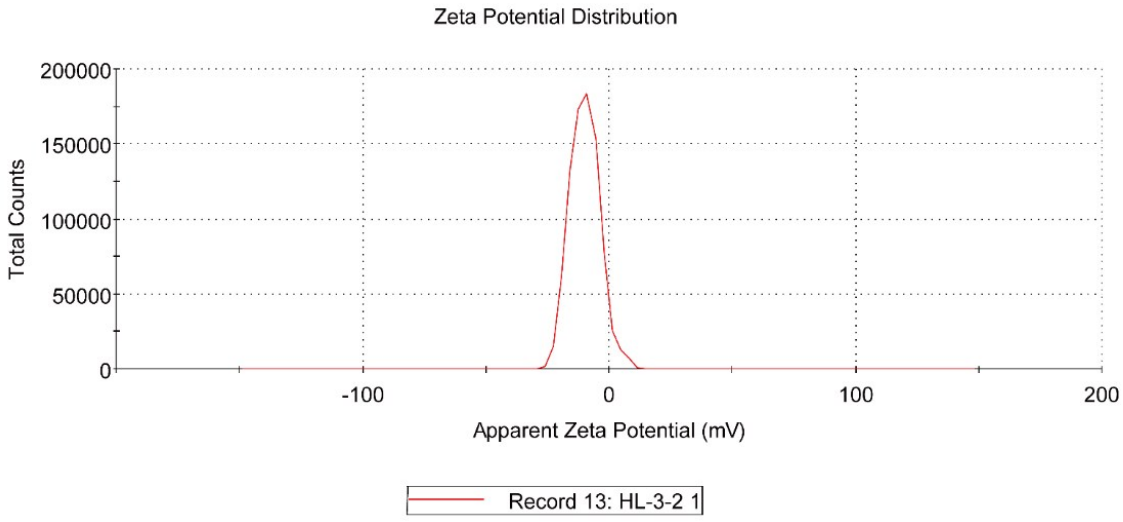


Figure S7. Zeta potential (-9.2 eV) of **HL3** dots in water.

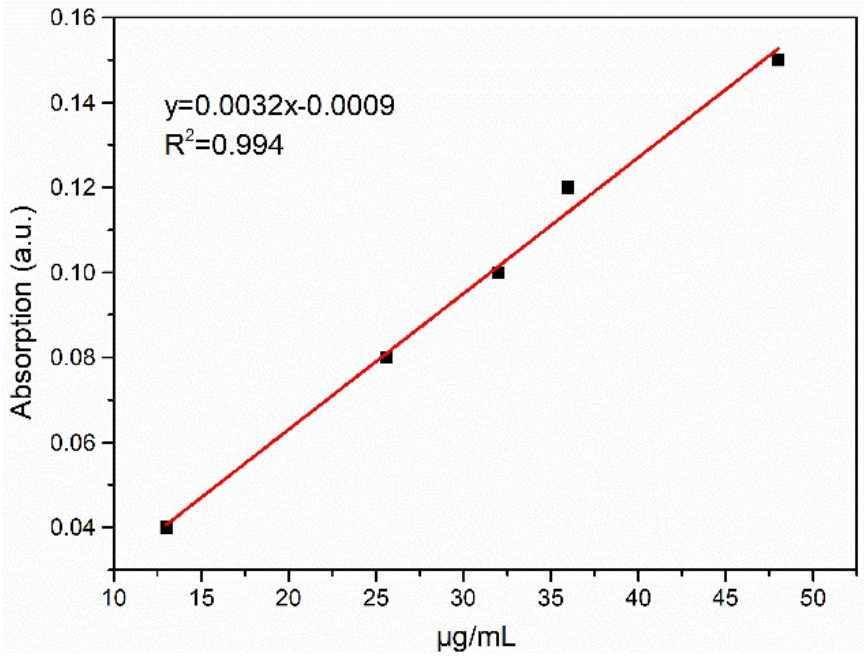


Figure S8. The encapsulation efficiency of **HL3** dots.

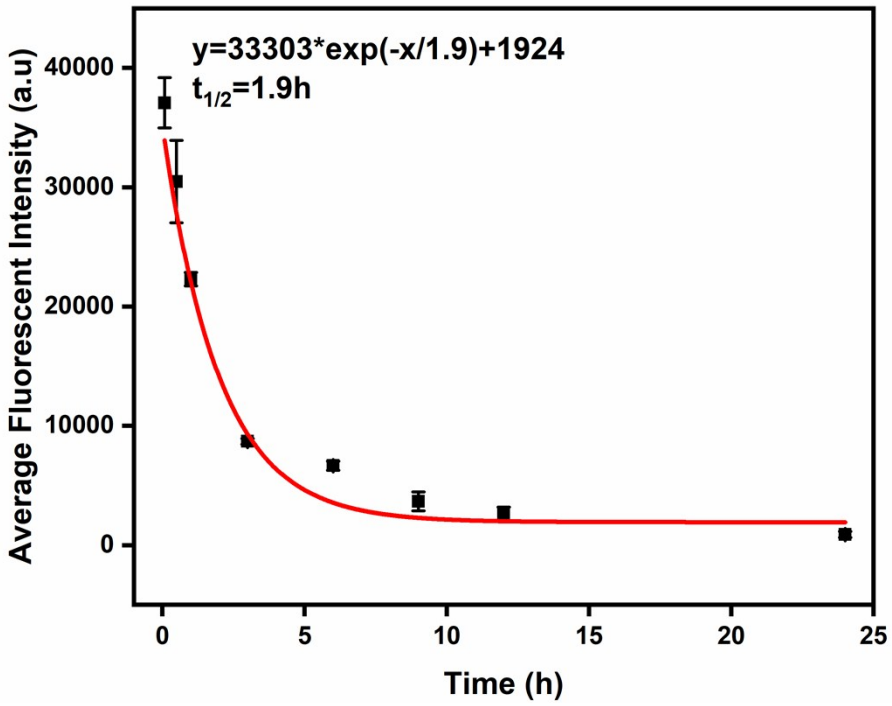


Figure S9. The blood half-life circulation curve of **HL3** dots in female KM mice was determined to be 24 hours by fitting the data from the mean fluorescence intensity of blood sample at particular time points after tail vein injection of probe using a first-order exponential decay (n=3).

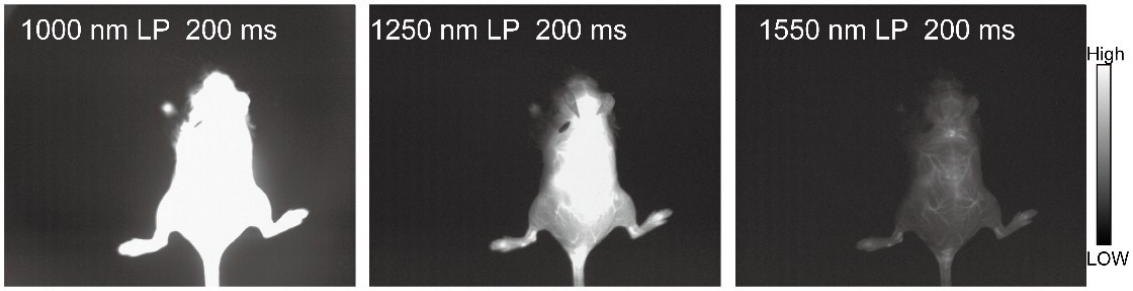


Figure 10. The NIR-II *in vivo* fluorescence images of **HL3** dots (0.8 mg/mL) using 1000 nm LP, 1250 nm and 1550 nm LP (200 ms).

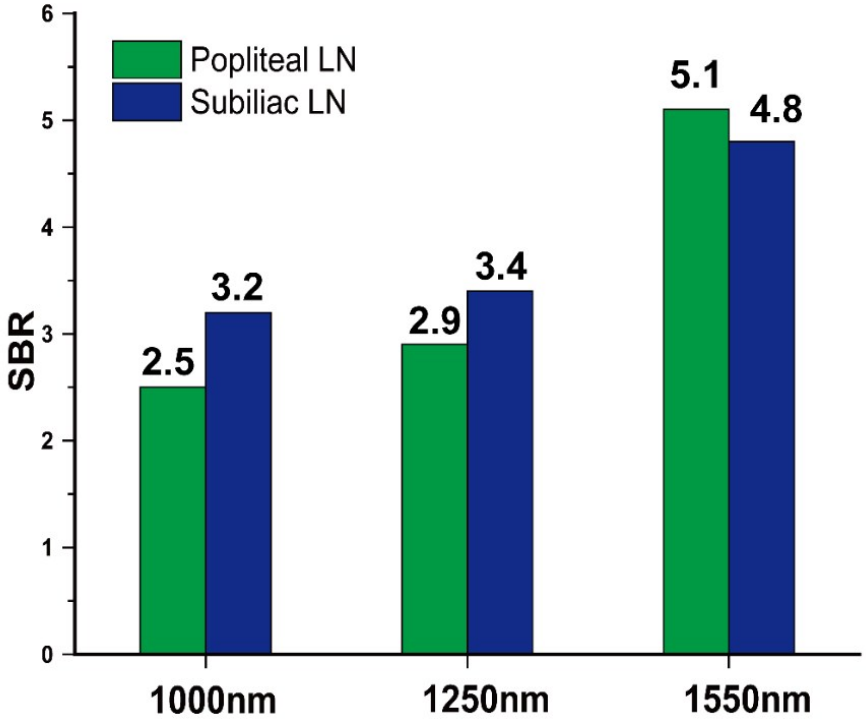


Figure S11. The different SBR of popliteal and subliliac LNs with different LP.

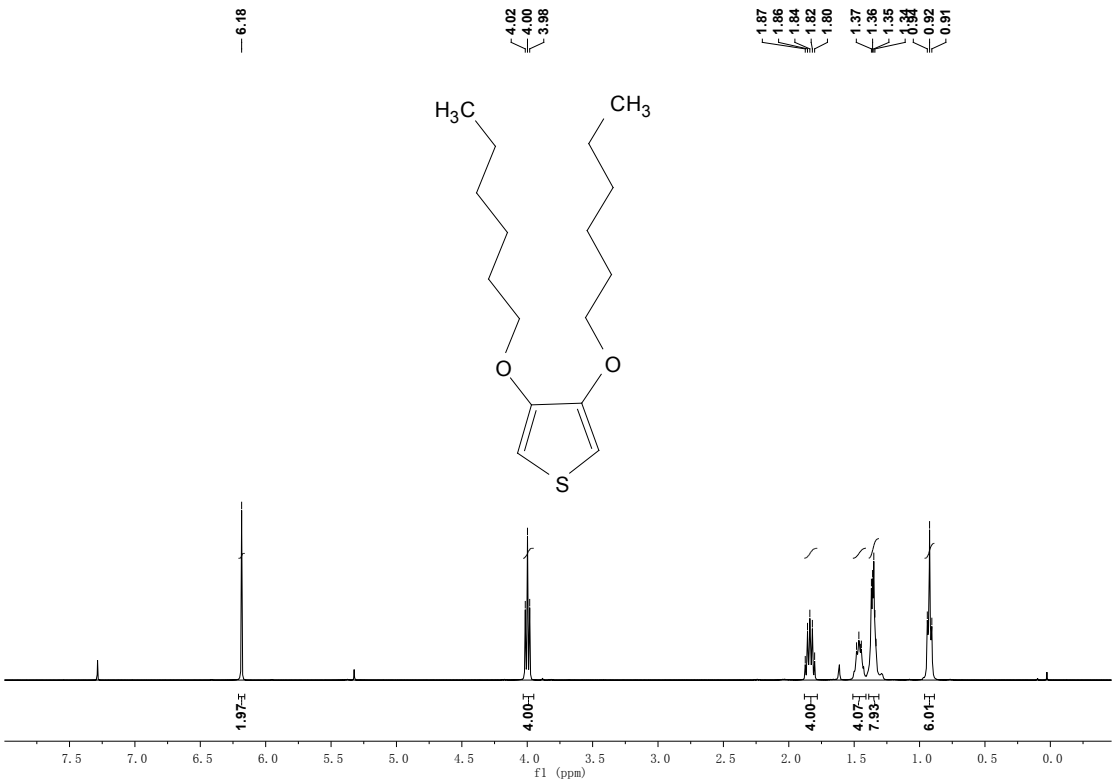


Figure S12. ^{1}H NMR of compound 3,4-bis(hexyloxy)thiophene

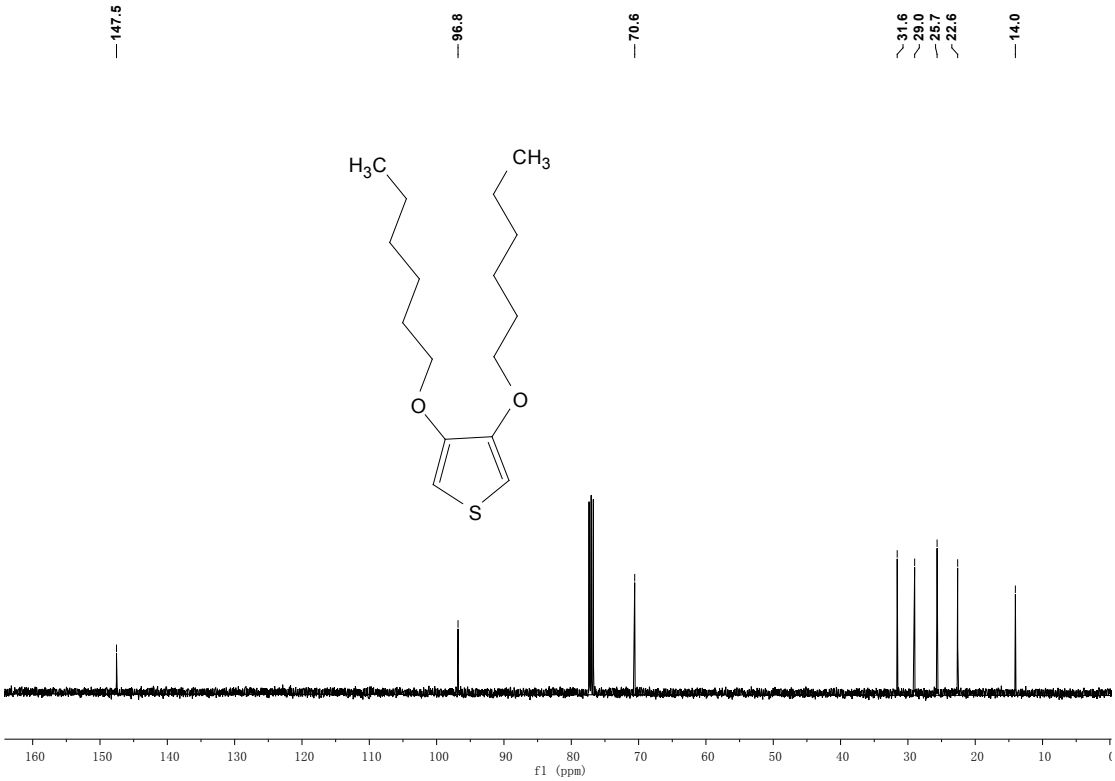


Figure S13. ^{13}C NMR of compound 3,4-bis(hexyloxy)thiophene.

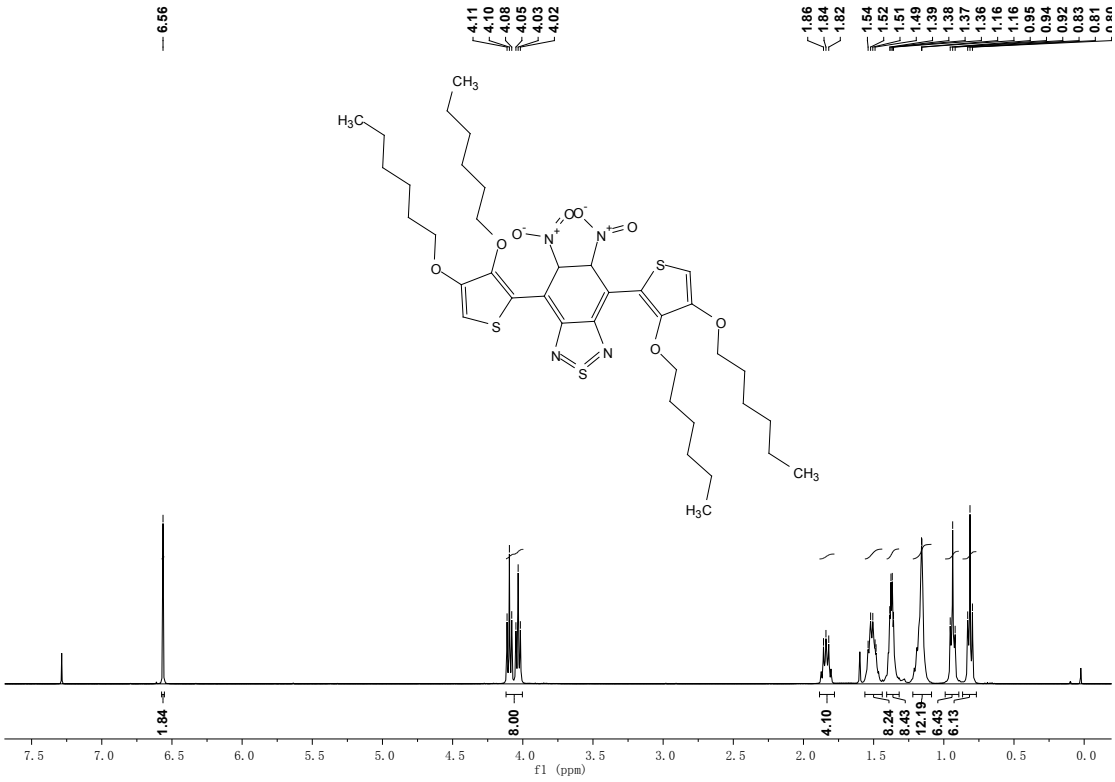


Figure S14. ^{1}H NMR of compound **2**

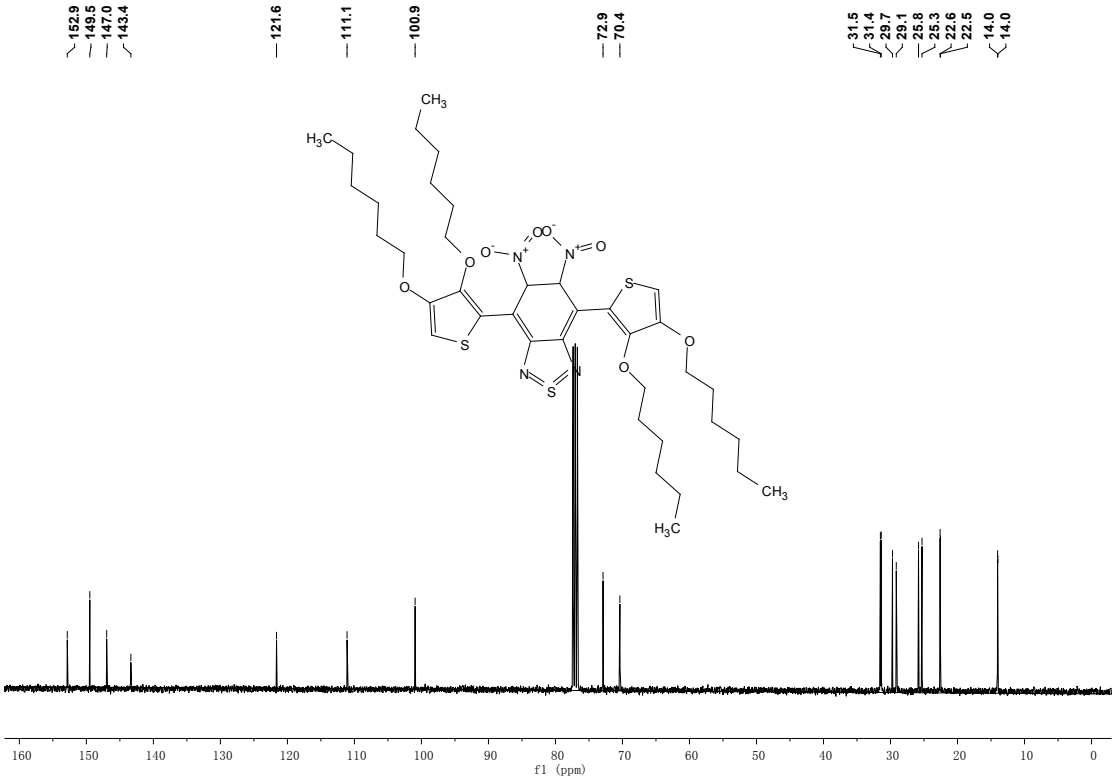


Figure S15. ^{13}C NMR of compound **2**

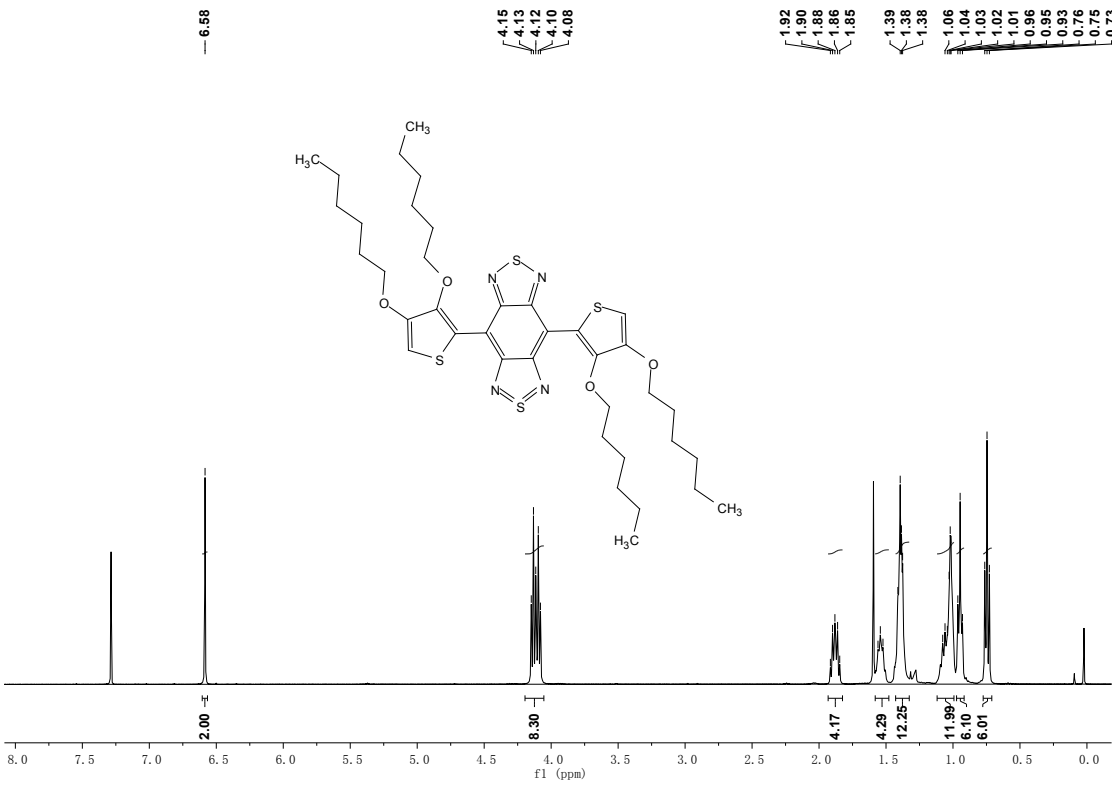


Figure S16. ^{1}H NMR of compound **3**

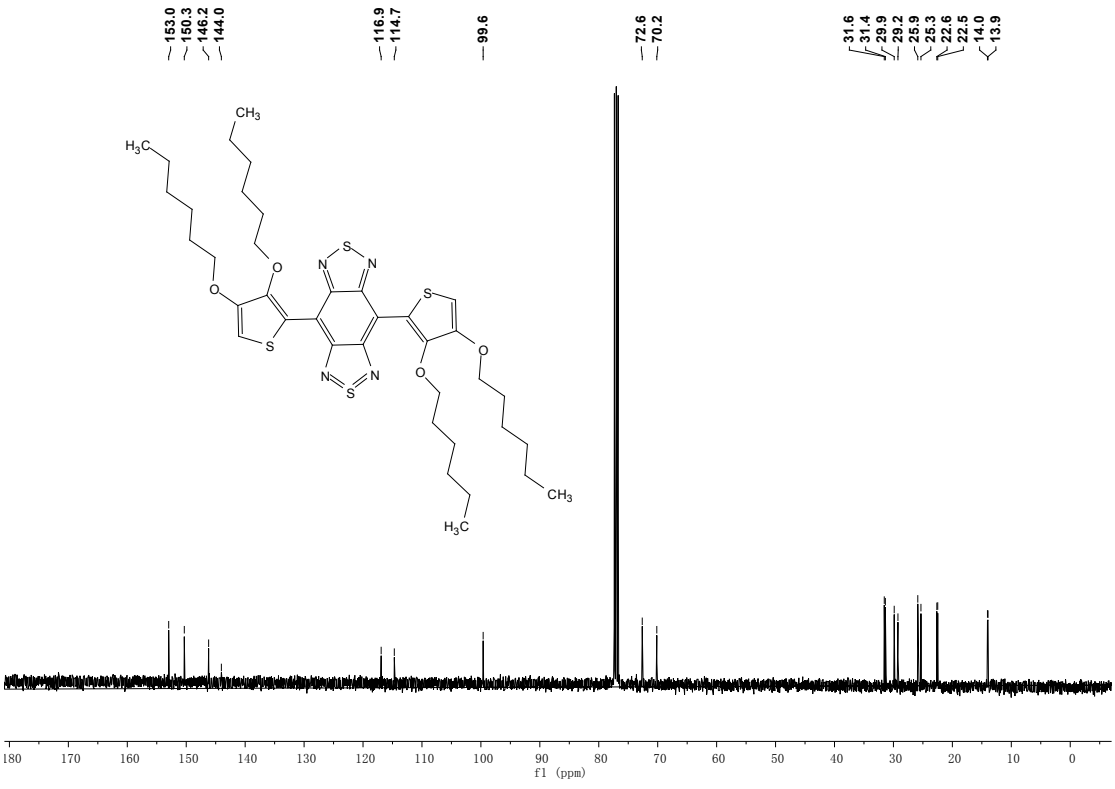


Figure S17. ^{13}C NMR of compound **3**

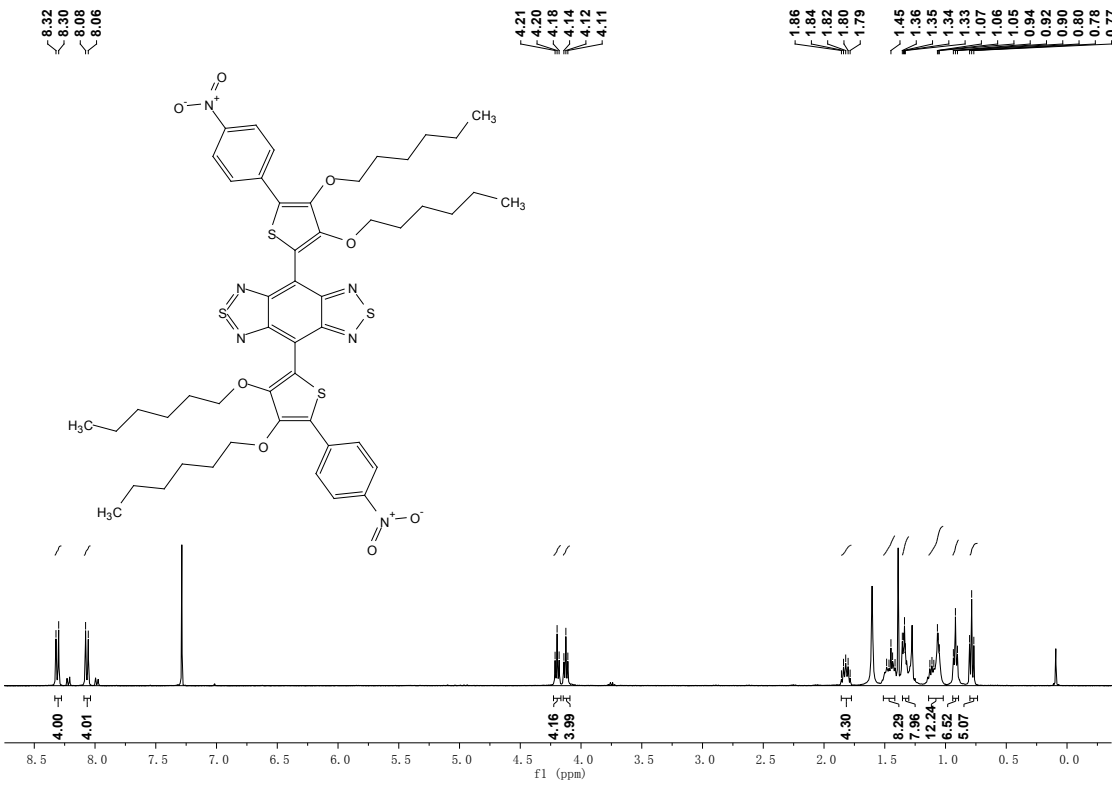


Figure S18. ^{1}H NMR of compound **HL1**

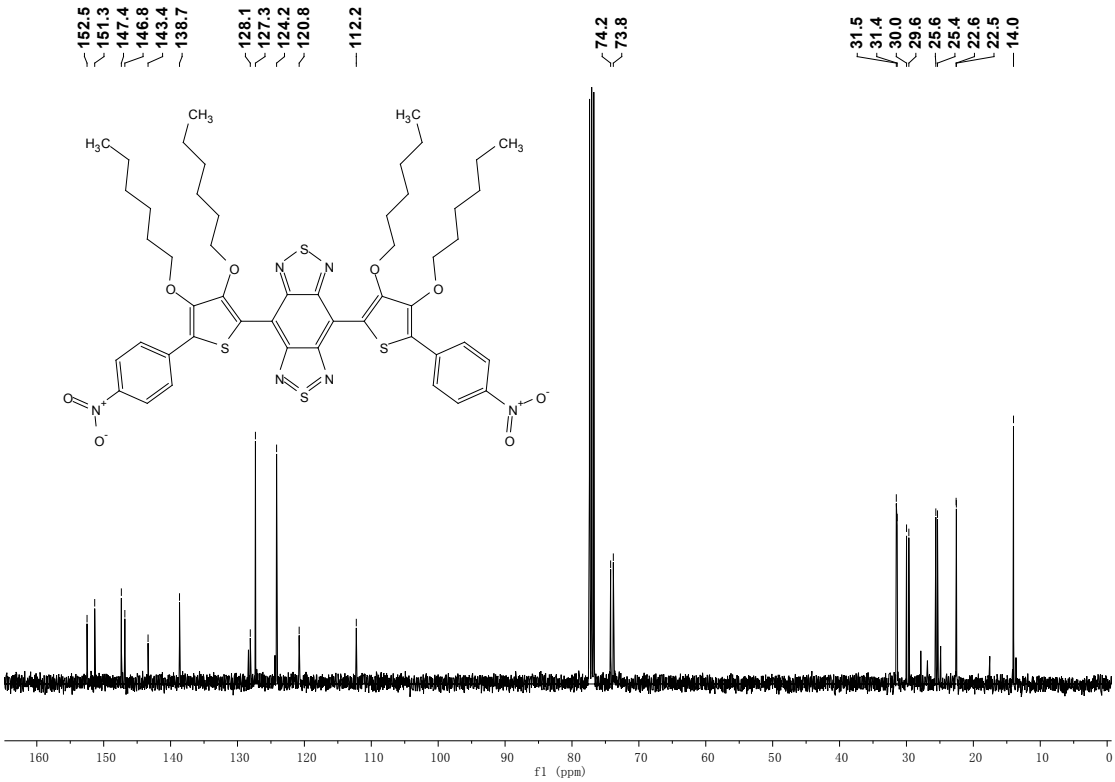


Figure S19. ^{13}C NMR of compound **HL1**

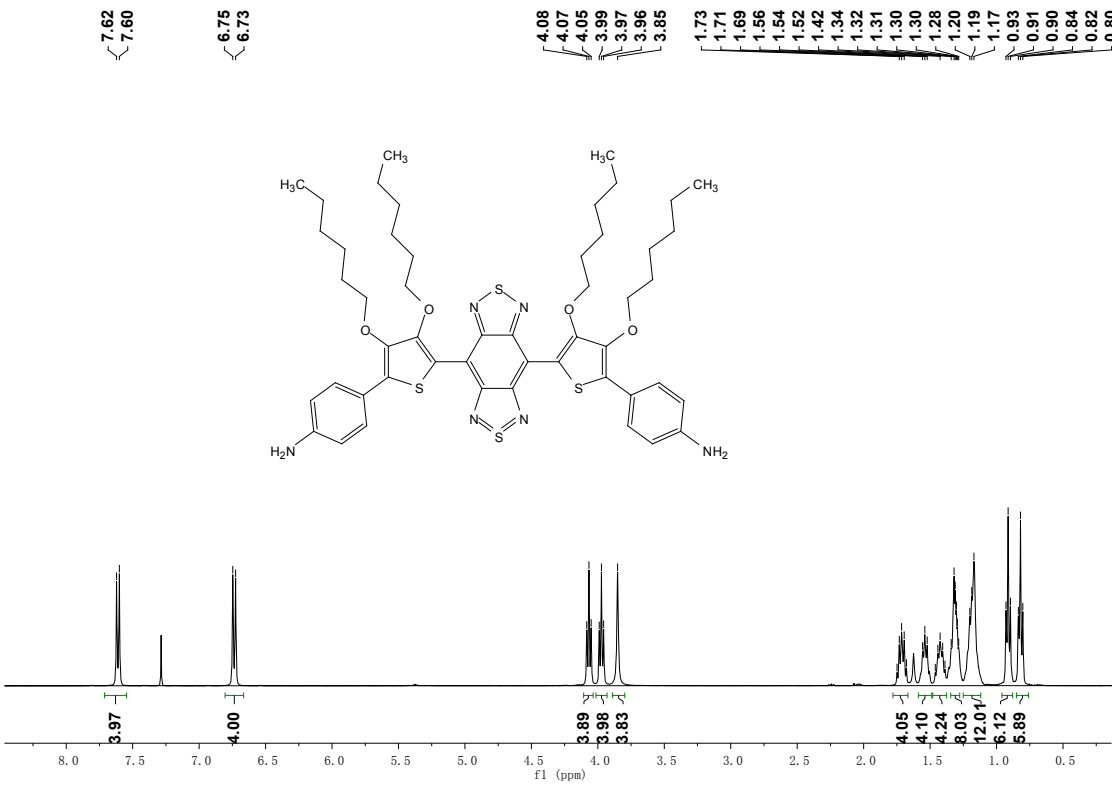


Figure S20. ^{1}H NMR of compound **HL2**

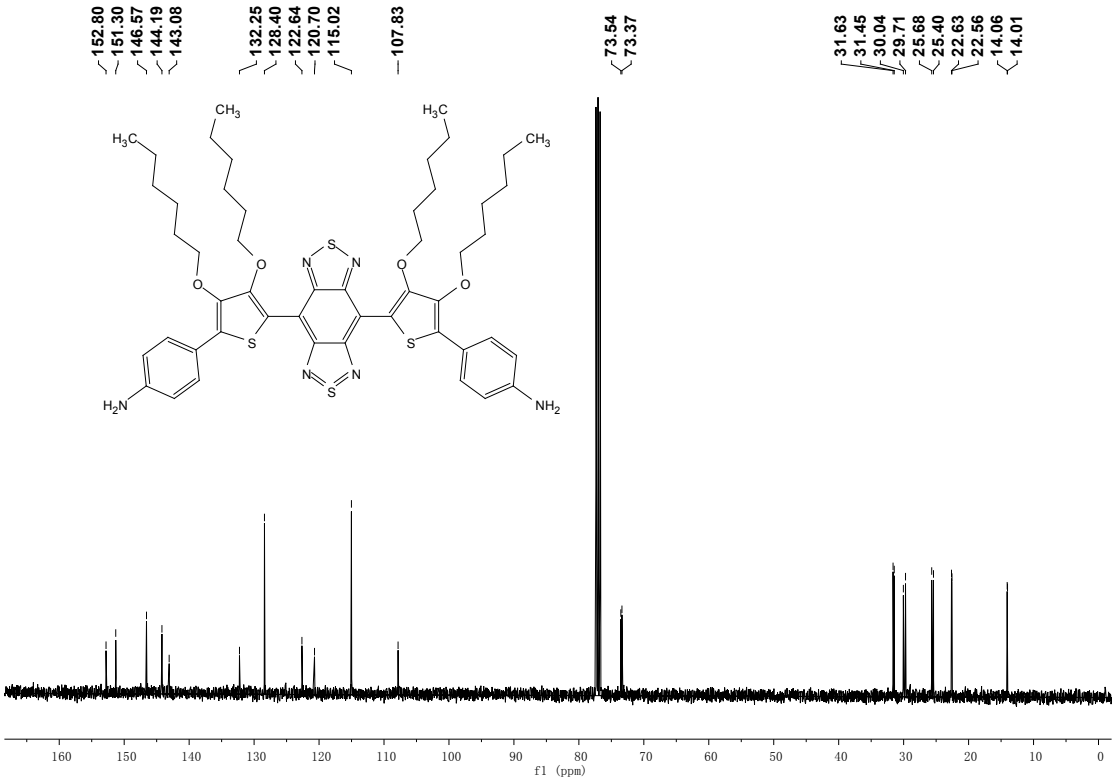


Figure S21. ^{13}C NMR of compound **HL2**

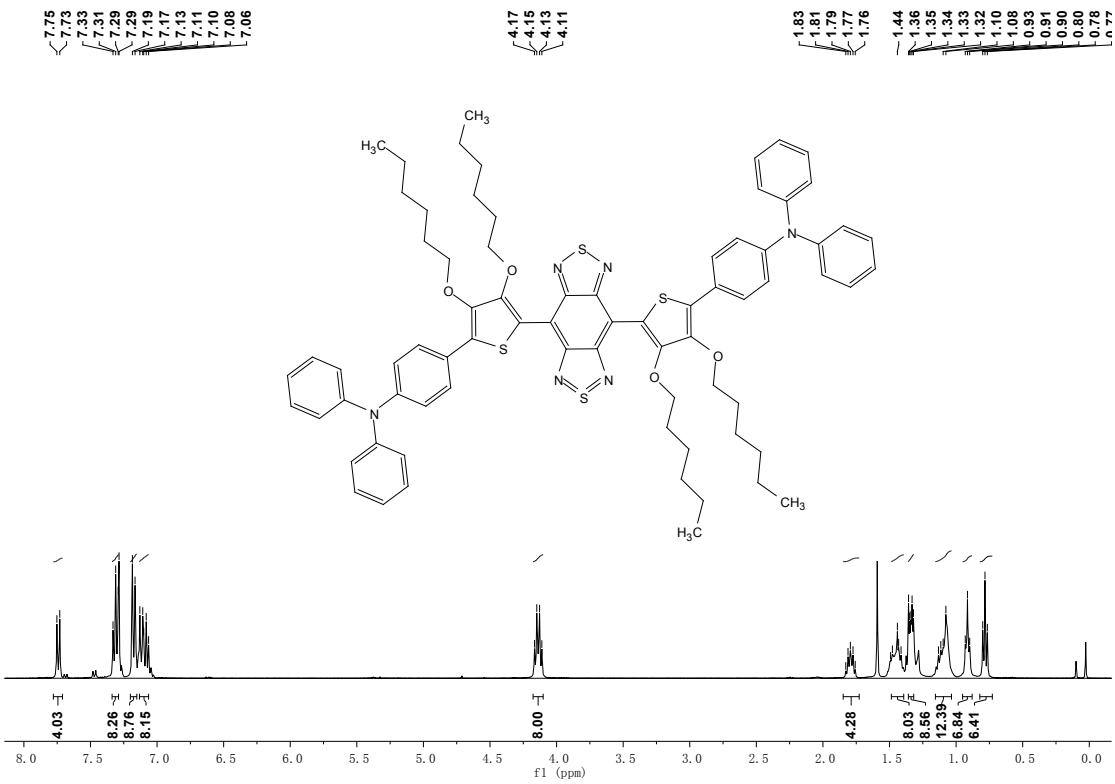


Figure S22. ^{1}H NMR of compound **HL3**

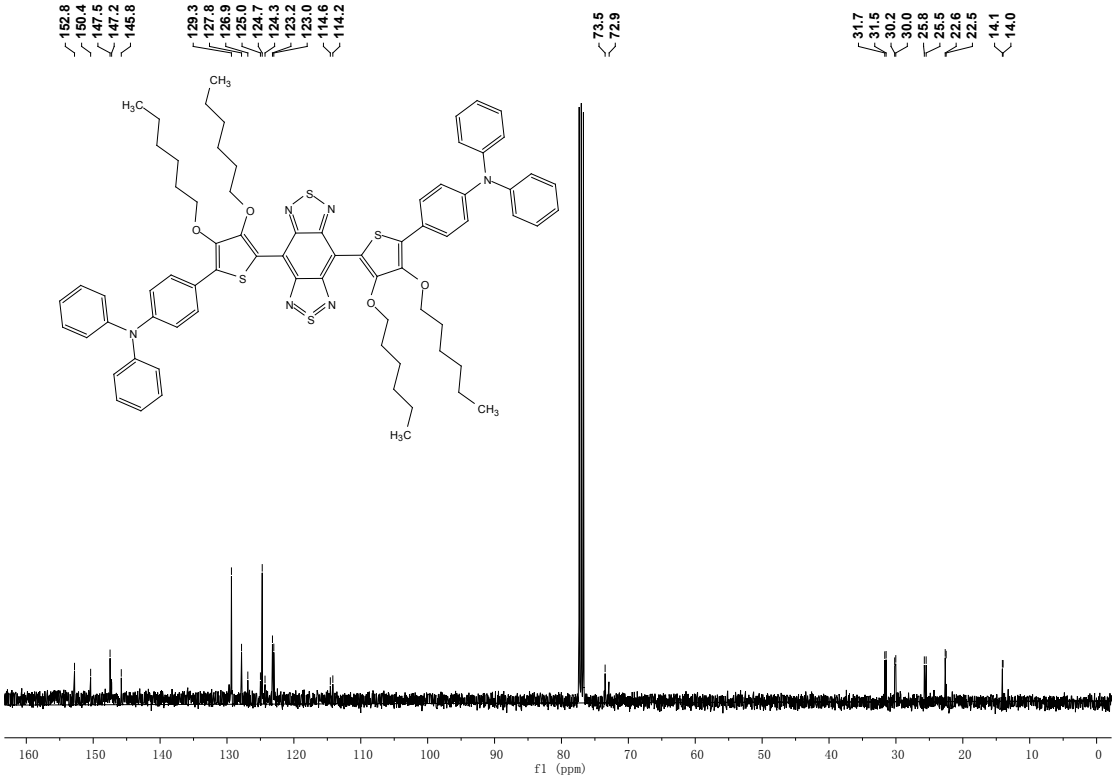


Figure S23. ^{13}C NMR of compound **HL3**

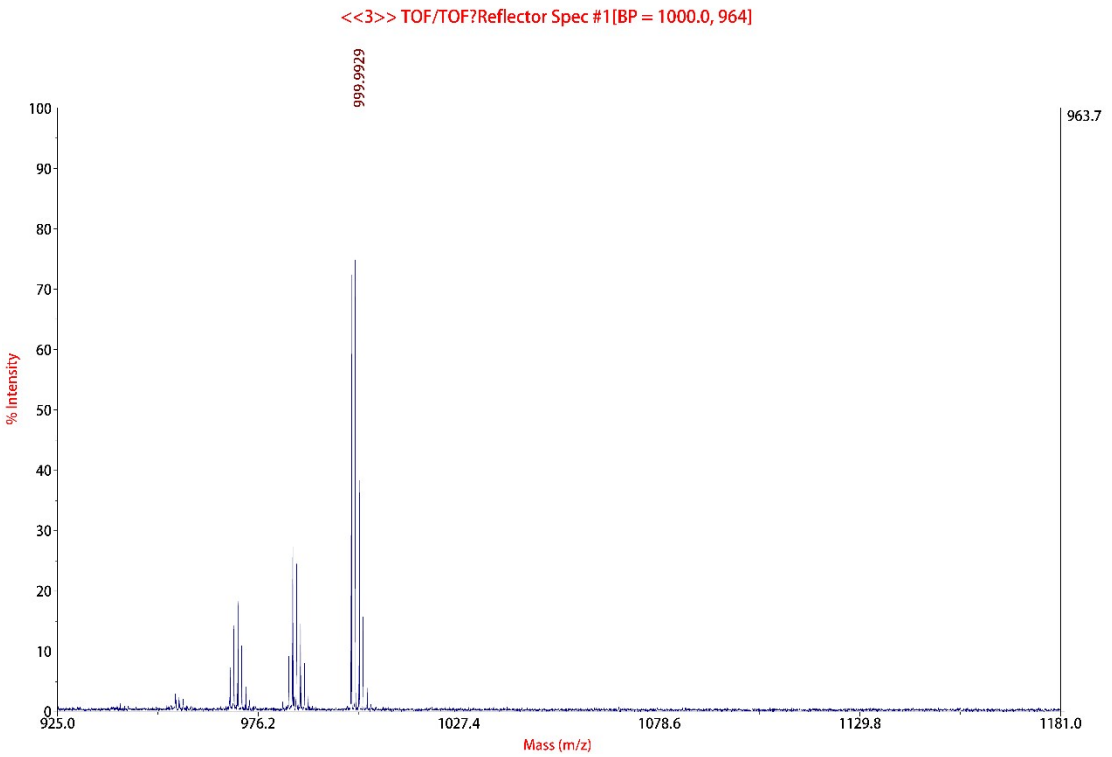


Figure S24. MOLDI-TOF-MS of **HL1**

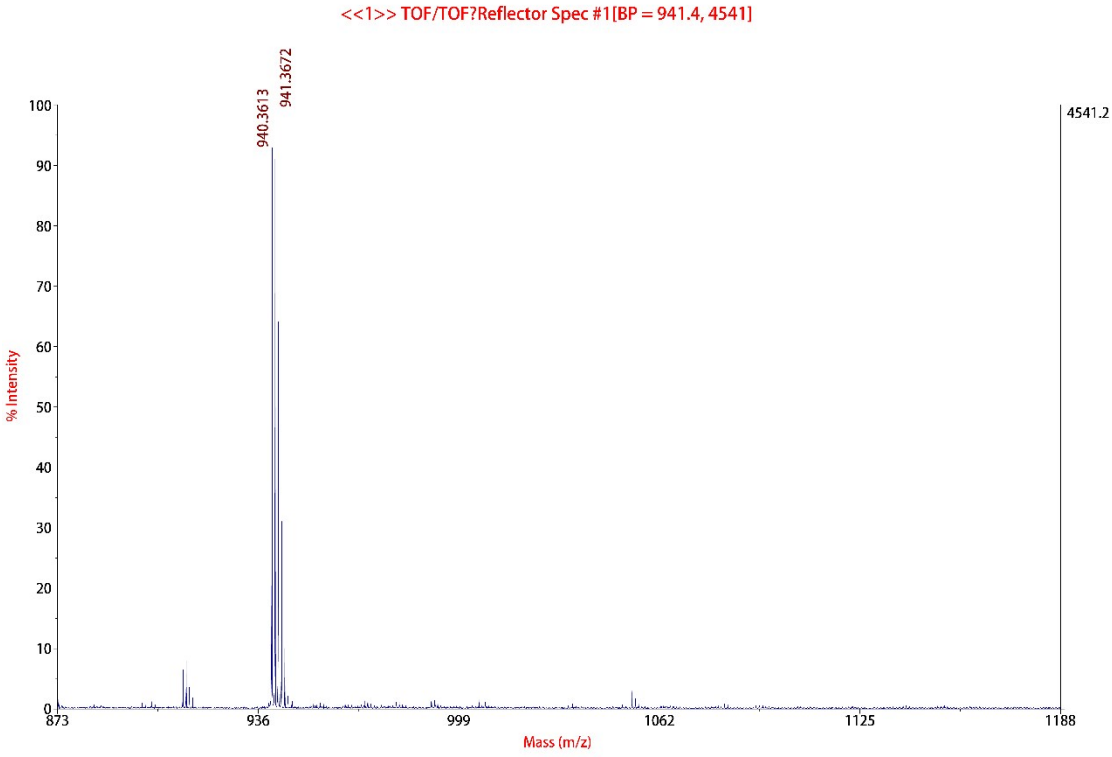


Figure S25. MOLDI-TOF-MS of **HL2**

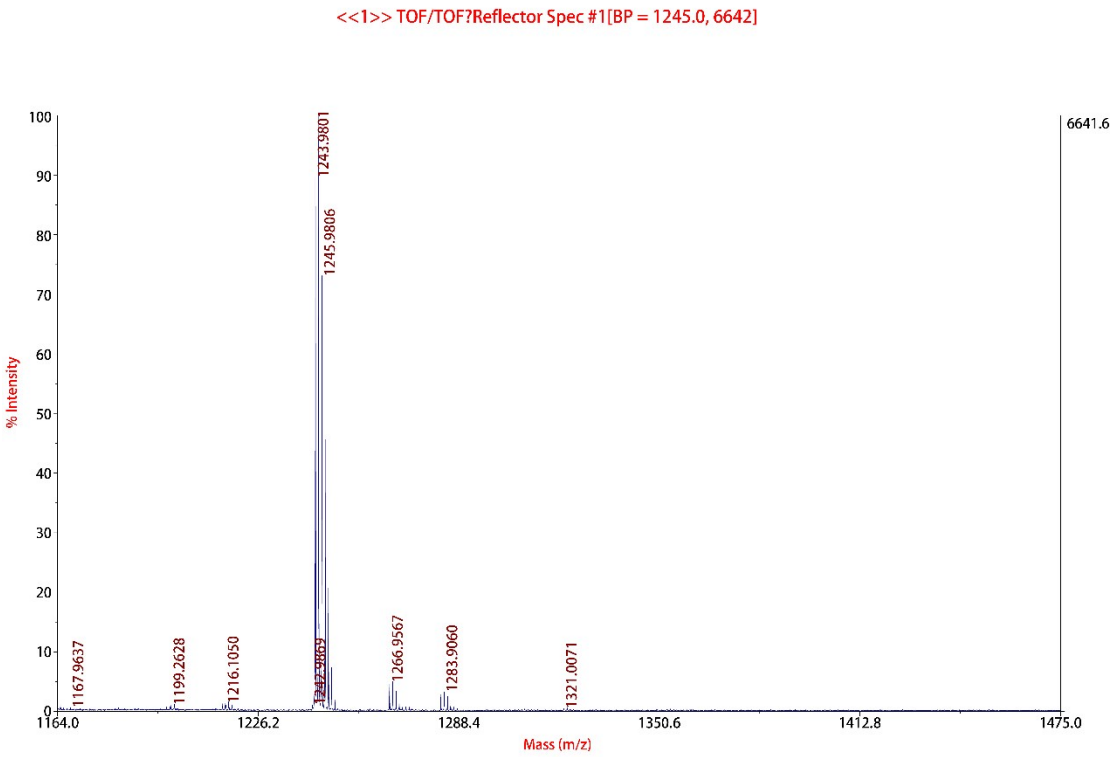
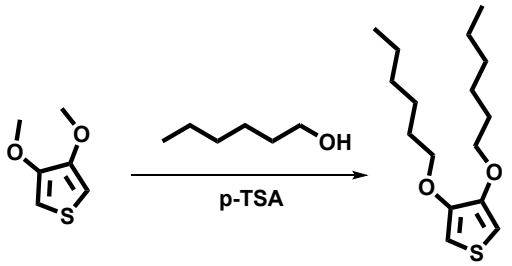


Figure S26. MOLDI-TOF-MS of **HL3**

Materials and General Procedure

The synthesis reagents were purchased from commercial suppliers (such as Aldrich, Adamas, Energy Chemical, Sinopharm Group Co., Ltd.) and used without further purification unless otherwise noted. N, N-Dimethylformamide (DMF) and dichloromethane (CH_2Cl_2) were distilled from calcium hydride. Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone. Anhydrous pyridine was freshly distilled using calcium hydride. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ at room temperature using a Bruker AV400 magnetic resonance spectrometer. ESI-MS were performed on Finnigan LCQ advantage mass spectrometer. MALDI-TOF-MS characteristics were performed on an AB SCIEX 5800 MALDI TOF mass spectrometer. Analytical and preparative TLC were performed on silica gel plates, and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals. UV-vis-NIR spectra were tested with a SHIMADZU UV-2600 or PerkinElmer Lambda 25 spectrophotometer. NIR fluorescence spectrum was performed on an Applied NanoFluorescence spectrometer at room temperature with an excitation laser source of 785 nm and 808 nm. The NIR-II in vivo imaging system was purchased from Suzhou NIR-Optics Technologies CO., Ltd.

Synthesis and characterization

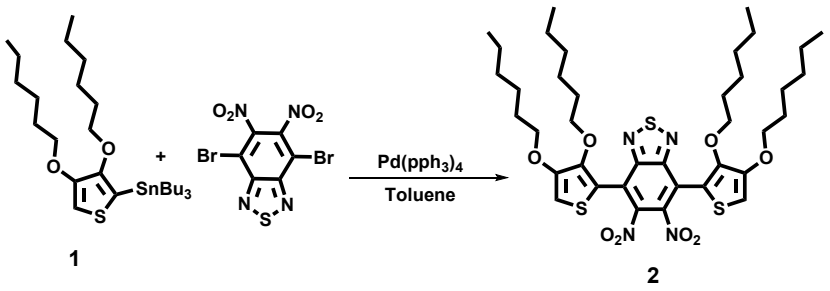


Synthesis of compound 3,4-bis(hexyloxy)thiophene

3,4-dimethoxythiophene (1.5 g, 10.40 mmol) and trifluoromethanesulfonic acid (50 μL, 0.52 mmol) were dropped into 1-Hexanol (6.5 mL). The reaction mixture stirred at 100℃ for 10 h under an argon atmosphere. Cooling down to room temperature, the reaction mixture was extracted with DCM. The organic phase was dried with anhydrous Na_2SO_4. And the solvent was removed under reduced pressure. The crude product was purified by column chromatography (PE) to afford compound **1** as colorless oil (1.7 g, 57.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 8.7 Hz, 4H), 7.31 (t, *J* = 7.9 Hz, 8H), 7.18 (d, *J* = 8.0 Hz, 8H), 7.13 – 7.06 (m, 8H), 4.14 (dd, *J* = 15.2, 6.7 Hz, 8H), 1.85 – 1.73 (m, 4H), 1.44 (dd, *J* = 14.8, 11.0 Hz, 8H), 1.34 (dd, *J* = 7.1, 3.4 Hz, 8H), 1.11 (dd, *J* = 14.6, 7.6 Hz, 12H), 0.91 (t, *J* = 6.6 Hz, 6H), 0.78 (t, *J* = 6.9 Hz, 6H).^{13}C NMR (101 MHz, $CDCl_3$) δ 147.5, 96.8, 70.5, 31.5, 29.0, 25.6, 22.6, 14.0. ESI-MS Calcd for: $C_{16}H_{28}O_2S^+$ ($[M+H]^+$): 284.5. Found: 284.9.

Synthesis of compound 1

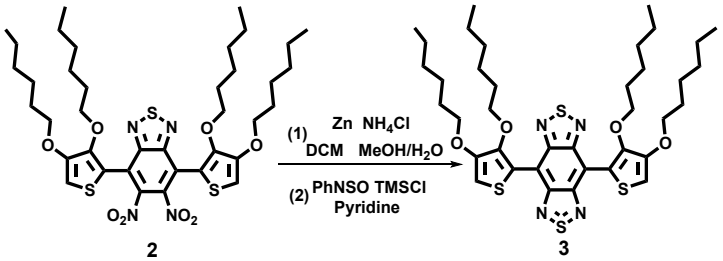
The solution of 3,4-bis(hexyloxy)thiophene (690 mg, 2.43 mmol) in dry THF (15 mL), *n*-butyllithium (2 mL, 2.91 mmol) was added dropwise into the reaction solution and stirred at -78 ℃. After the reaction mixture stirred for 2 h, chlorotributyltin (0.9 mL, 2.91 mmol) was added to the solution. The reaction mixture stirred over night at -78 ℃. After reaction, the mixture was quenched with potassium fluoride andextracted with EA (30 mL) and concentrated under reduced pressure to give compound **1**. The compound **1** was utilized to next step without further purification.



1 2

Synthesis of compound 2

A solution of 4,7-dibromo-5,6-dinitrobenzo[c][1,2,5]thiadiazole (275 mg, 0.72 mmol) and compound **1** (1 g, 1.8 mmol) in dry THF (15 mL), $Pd(PPh_3)_4$ (83 mg, 0.07 mmol) was added to the reaction mixture. The reaction mixture was heated in an oil bath at 80 ℃ for 18 h under an argon atmosphere. After cooling down to room temperature, the reaction mixture was poured into 100 mL water and extracted with EA (30 mL), the organic phase was dried with anhydrous Na_2SO_4 and concentrated in vacuo and the residue was purified by silica gel chromatography (PE:EA=70:1) to give compound **2** (163 mg, 28.7% yield) as an orange-red oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.56 (s, 2H), 4.06 (dt, *J* = 24.6, 6.5 Hz, 8H), 1.89 – 1.78 (m, 4H), 1.56 – 1.44 (m, 8H), 1.37 (dd, *J* = 7.2, 3.5 Hz, 8H), 1.16 (d, *J* = 1.2 Hz, 12H), 0.94 (t, *J* = 7.0 Hz, 6H), 0.81 (t, J = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.8, 149.4, 146.9, 143.3, 121.6, 111.1, 100.9, 72.9, 70.3, 31.5, 31.3, 29.7, 29.1, 25.8, 25.30, 22.6, 22.5, 14.0, 13.9. ESI-MS Calcd for: $C_{38}H_{54}N_4O_8S_3^+$ ([M+H]$^+$): 791.1. Found: 791.6.



2 3

Synthesis of compound 3

Compound **2** (80 mg, 0.10 mmol) was dissolved in DCM (8 mL) and 90% MeOH (4 mL). Zinc powder (394 mg, 6.05 mmol) and ammonium chlorid (171 mg, 3.63 mmol) were added to the solution. The reaction mixture stirred for 2 h at room temperature. The reaction solution was filtered to remove zinc powder and the resulting solution was

extracted with DCM and concentrated in vacuo. The crude product was used for the next step without further purification.

The chlorotrimethylsilane (0.7 mL, 6.05 mmol) and N-thionylaniline (0.7 mL, 8.07 mmol) were added to the solution of above product in pyridine (4 mL). The reaction mixture stirred over night at 85 ℃ under an argon atmosphere. The reaction solution was poured into 100 mL water and extracted with EA (50 mL) and dried with anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography (PE:EA=100:1) to afford compound **3** as a blue solid (52 mg, 56.2% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (s, 2H), 4.20 – 4.05 (m, 8H), 1.93 – 1.82 (m, 4H), 1.58 – 1.48 (m, 4H), 1.39 (dd, *J* = 8.9, 5.2 Hz, 12H), 1.12 – 0.99 (m, 12H), 0.95 (t, *J* = 6.9 Hz, 6H), 0.75 (t, *J* = 6.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.9, 150.3, 146.2, 144.0, 116.9, 114.6, 99.6, 72.6, 70.1, 31.5, 31.3, 29.8, 29.2, 25.8, 25.3, 22.6, 22.4, 14.0, 13.9. MALDI-TOF-MS Calcd for: $C_{38}H_{54}N_4O_4S_4$ ([M+H]+): 758.30, found: 759.20.

Synthesis of compound 4

To the solution of compound **3** (50 mg, 0.07mmol) in DMF (4 mL) and acetonitrile (2 mL), NBS (13 mg, 0.07 mmol) and HBr (0.05 mL) were added to the reaction mixture. NBS was added once an hour. The reaction mixture was heated to 65℃ for 3h in the dark under an argon atmosphere. After the reaction solution cooled down to room temperature, it was extracted with EA and dried with anhydrous Na_2SO_4. The organic layer evaporated to give compound 4 of blue solid. The compound 4 was utilized to next step without further purification.

Synthesis of compound HL1

Compound **4** (40 mg, 0.04 mmol) was dissolved in distilled THF (5 mL) and 4,4,5,5-tetramethyl-2-(4-nitrophenyl)-1,3,2-dioxaborolane.(26mg, 0.10 mmol), $PdCl_2(dppf)_2$DCM (7 mg, 0.01 mmol) and KOAC (9 mg, 0.09 mmol) in 2 mL distilled water were added to the reaction solution. The reaction mixture stirred for 3h at 75℃ under an argon atmosphere. Cooling down to the room temperature, the reaction mixture was extracted with DCM. The organic phase was dried with anhydrous Na_2SO_4

and removed solvent under reduced pressure. The crude product was purified by column chromatography (PE:EA=20:1) to afford **HL1** as a green solid (17 mg, 39.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (d, *J* = 8.9 Hz, 4H), 8.07 (d, *J* = 8.9 Hz, 4H), 4.20 (t, *J* = 6.6 Hz, 4H), 4.12 (t, *J* = 6.5 Hz, 4H), 1.86 – 1.77 (m, 4H), 1.46 (dd, *J* = 13.1, 6.6 Hz, 8H), 1.34 (t, *J* = 3.2 Hz, 8H), 1.14 – 1.02 (m, 12H), 0.92 (t, *J* = 6.7 Hz, 6H), 0.78 (d, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.5, 151.3, 147.4, 146.8, 143.4, 138.7, 128.1, 127.3, 124.2, 120.8, 112.2, 74.2, 73.8, 31.5, 31.4, 30.0, 29.6, 25.6, 25.4, 22.6, 22.5, 14.0. MALDI-TOF-MS Calcd for: $C_{50}H_{60}N_6O_8S_4$ ([M+H]+): 1000.34, found: 1000.964.

Synthesis of compound HL2

Compound **4** (60 mg, 0.07 mmol) was dissolved in dry THF (5 mL) and tert-butyl(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)carbamate (51 mg, 0.16 mmol), $PdCl_2(dppf)_2$DCM (15 mg, 0.01 mmol) and KOAC (10 mg, 0.1 mmol) in 2 mL distilled water were added to the reaction solution. The reaction mixture stirred for 3 h at 75℃ under an argon atmosphere. Cooling down to the room temperature, the reaction mixture was poured into 100 mL water and extracted with DCM (30 mL). The organic phase was dried with anhydrous Na_2SO_4 and removed solvent under reduced pressure. The crude product used directly into the next step. TFA (2 mL) was added to the solution of the mixture in DCM (3 mL). The reaction solution was evaporated. The crude product was purified by column chromatography (PE:EA=5:1) to afford **HL2** as a green solid. (8 mg, 12.1% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 8.5 Hz, 4H), 6.74 (d, *J* = 8.5 Hz, 4H), 4.07 (t, *J* = 6.6 Hz, 4H), 3.97 (t, *J* = 6.6 Hz, 4H), 3.85 (s, 4H), 1.78 – 1.67 (m, 4H), 1.53 (dd, *J* = 13.8, 6.8 Hz, 4H), 1.48 – 1.38 (m, 4H), 1.31 (dt, *J* = 6.9, 6.3 Hz, 8H), 1.25 – 1.12 (m, 12H), 0.91 (t, *J* = 6.7 Hz, 6H), 0.82 (t, *J* = 6.7 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.8, 151.3, 146.6, 144.2, 143.08, 132.2, 128.4, 122.6, 120.7, 115.0, 107.8, 73.5, 73.4, 31.6, 31.4, 30.0, 29.7, 25.7, 25.4, 22.6, 22.6, 14.1, 14.0. MALDI-TOF-MS Calcd for: $C_{50}H_{64}N_6O_4S_4$ ($[M+H]^+$): 940.39, found: 941.3672.

Synthesis of compound HL3

Compound **4** (45 mg, 0.05 mmol) was dissolved in distilled THF (5 mL) and N,N-

diphenyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline (44 mg, 0.12 mmol), $PdCl_2(dppf)_2DCM$ (8 mg, 0.01 mmol) and K_2CO_3 (14 mg, 0.10 mmol) in 2 mL distilled water were added to the reaction solution. The reaction mixture stirred for 3h at 75℃ under an argon atmosphere. Cooling down to the room temperature, the reaction mixture was extracted with DCM. The organic phase was dried with anhydrous Na2SO4 and removed solvent under reduced pressure. The crude product was purified by column chromatography (PE:EA=10:1) to afford **HL3** as a green solid (18 mg, 29.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 8.7 Hz, 4H), 7.31 (t, *J* = 7.9 Hz, 8H), 7.18 (d, *J* = 8.0 Hz, 8H), 7.13 – 7.06 (m, 8H), 4.14 (dd, *J* = 15.2, 6.7 Hz, 8H), 1.85 – 1.73 (m, 4H), 1.44 (dd, *J* = 14.8, 11.0 Hz, 8H), 1.34 (dd, *J* = 7.1, 3.4 Hz, 8H), 1.11 (dd, *J* = 14.6, 7.6 Hz, 12H), 0.91 (t, *J* = 6.6 Hz, 6H), 0.78 (t, *J* = 6.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.7, 150.3, 147.4, 147.2, 145.8, 129.3, 127.8, 126.8, 125.0, 124.7, 124.3, 123.2, 123.0, 114.5, 114.1, 73.4, 72.8, 31.6, 31.4, 30.1, 30.0, 25.7, 25.4, 22.6, 22.4, 14.0, 13.9. MALDI-TOF-MS Calcd for: $C_{74}H_{80}N_6O_4S_4$ ([M+H]+): 1244.51, found: 1245.9806.

Fabrication of HL2 and HL3 dots

HL2 and **HL3** dots were prepared through a nanoprecipitation method by employing DPPE-5KPEG as a encapsulation matrix.[2] Subsequently **HL2**/THF and **HL3**/THF mixture (1 mg/mL) were added dropwise into the DPPE-5KPEG (10 mg) in 10 mL deionized water solution under continuous sonication in an ice bath. Then, the leftover tetrahydrofuran in the mixture was eliminated completely under an inert airflow. The redundant DPPE-5KPEG was eliminated by ultraltration using 40 kDa centrifugal filter equipments to obtain **HL2** dots and **HL3** dots.

The molar extinction coefficients (ε)

The absorbance spectra of different concentrations of **HL1-HL3** in THF, **HL2** dots and **HL3** dots in water were investigated.

The molar extinction coefficients (ε) of **HL1-HL3** in THF were measured as 8.3 $\times 10^3$ $L.mol^{-1}.cm^{-1}$, 4.1 $\times 10^3$ $L.mol^{-1}$ and cm^{-1}, 7 $\times 10^3$ $L.mol^{-1}.cm^{-1}$ respectively. The molar absorption coefficient of **HL2** dots was measured as ~ 6 $\times 10^3$ $L.mol^{-1}.cm^{-1}$. The molar extinction coefficients (ε) of **HL3** dots in water was measured as 9.3 $L.mol^{-1}.cm^{-1}$.

Cell Culture and Animal Model

Human hepatocyte cells L929 were purchased from the China Center for Type Culture Collection (CCTCC). All cells were grown in a humidified atmosphere at 37 °C with 5% CO_2 atmosphere. L929 cells were maintained in Mimumum Essentiul Medium (MEM, Gibco), supplemented with 10% fetal bovine serum, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin. All animal experiments were performed according to the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University.

The cell viability assessment of HL3 dots**.**

The cytotoxicity of **HL3** dots was investigated by a standard MTT assay. The cell viability was measured using mouse fibroblast cells L929 for 24 h incubation. The L929 were seeded in a 96-well plate (around 5000 cells per well). After 12 h, the medium

was substituted with the fresh medium contained **HL3** dots with different concentrations. Followed by incubation for 24 h, then a standard MTT method was performed for measuring the cell viability.

***In Vivo* NIR-II Fluorescence Imaging.**

All NIR-II fluorescent images were collected using a NIR-II imaging system with the indium-gallium-arsenide (InGaAs) camera (Princeton Instruments). The excitation light source was an 808 nm diode laser. The laser power density was 90 mW cm^{-2} with 1000 nm, 1250 nm and 1550 nm long-pass filter during *in vivo* imaging. The C57BL/6 and KM mice (n=3 per group) were anesthesized by intraperitoneal injection of pentobarbital sodium solution (50 mg kg^{-1}) during the NIR-II imaging. For *in vivo* whole body and cerebral vasculature imaging, the athymic C57BL/6 mice (n=3 per group) were given **HL3** dots (200 μL, 1 mg/mL) via tail vein injection. For *in vivo* lymph nodes imaging, **HL3** dots (15 μL, 1 mg/mL) were injected intra-dermally at the left forefoot pad of KM mice (n = 3 per group). After injection, the mice were mounted in the prone position beneath the laser for imaging at various time points. And animals were mounted in the NIR-II imaging system at different time points.

Reference

[1] X. Zeng, Y. Xiao, J. Lin, S. Li, H. Zhou, J. Nong, G. Xu, H. Wang, F. Xu, J. Wu, Z. Deng, X. Hong, *Adv. Healthcare Mater.*, 2018, ***7***, 1800589.

[2] J. Lin, X. Zeng, Y. Xiao, L. Tang, J. Nong, Y. Liu, H. Zhou, B. Ding, F. Xu, H. Tong, Z. Deng and X. Hong, *Chem. Sci*., 2019, ***10***, 1219-1226.