Supporting Information

Novel small-molecule fluorophores for in vivo NIR-IIa and NIR-IIb imaging

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Author contributions

Y. Xiao conceived and designed the experiments. Q. Li, Q. Ding, Y. Li, X. Zeng, Y. Liu, S. Lu performed the experiments. Q. Li, X. Zeng, Q. Ding, Y. Li, Y. Liu, H. Zhou, X. Meng, X. Wang, J. Wu, Z. Deng and Y. Xiao analyzed the data. Y. Xiao and Q. Li wrote the manuscript. All authors discussed the results and commented on the manuscript.
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. Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone. Dimethylformamide (DMF) and Dichloromethane (CH$_2$Cl$_2$) were distilled from calcium hydride. Anhydrous pyridine was freshly distilled using calcium hydride. 4,7-bis(7-bromo-2,3-dihydrothieno[3,4-b][1,4]dioxin-5-yl)-5,6-dinitrobenzo[c][1,2,5]thiadiazole (intermediate_1) was synthesized according to our previous report.[1] $^1$H 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 Nano Fluorescence 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

The synthetic route of HQL2

Synthesis of tributyl(3-dodecyclohexa-1,3-dien-1-yl)stannane
Compound 1 (6 g, 0.02 mol) was dissolved in 35 mL of dry THF in a rounded-bottom flask under a N₂ atmosphere. 11.4 mL of n-butyllithium (2.5 M in hexane) was added dropwise at −78 °C. After stirring for 1 h, tributyltin chloride (9.28 g, 0.028 mol) was added in one portion. Then the reaction was warm to room temperature and stirred overnight. Finally, the solution was purged into cold water. The organic phase was separated, and the aqueous layer was twice extracted with 50 mL of ether. The organic layers were collected and dried over anhydrous MgSO₄. After removing the solvent, the crude product tributyl(3-dodecylcyclopenta-1,3-dien-1-yl)stannane 2 as light yellow oil (5.4 g) was obtained and used in the next step without further purification.

**Synthesis of compound 4**

A solution of compound 2 (3 g, 5.5 mmol) in Toluene (15 mL) was treated with 4-bromo-N,N-diphenylaniline (1.79 g, 5.5 mmol) and tetrakis(triphenylphosphine) palladium (0) (638 mg, 0.5 mmol), heated at 110° C under a N₂ atmosphere for 8 hours, cooled to ambient temperature, poured into ethyl acetate (10 mL), washed with saturated KF, and brine, dried with anhydrous Na₂SO₄, filtered, and concentrated. The concentrate was purified by column chromatography on silica gel to give the desired product 4 (1.5 g, yield 55.6%) as colorless oil.¹H NMR (400 MHz, CDCl₃) δ 7.56-7.54 (m, 2H), 7.35-7.32 (m, 4H), 7.22-7.11 (m, 8H), 6.88(s, 1H), 2.69-2.68 (m, 2H), 1.74 (s, 2H), 1.39(m, 18H), 1.01-1.00(m,3H).¹³C NMR (101 MHz, CDCl₃) δ 147.7, 147.2, 144.3, 144.0, 129.4, 129.1, 126.6, 124.5, 124.0, 123.8, 123.1, 118.8, 32.1, 30.8, 30.6, 29.9, 29.7, 29.6, 22.9, 14.3. ESI-MS Calcd for: C₃₄H₄₂NS⁺ ([M+H]+): 496.30. Found: 496.37.

**Synthesis of compound 5**
Compound 4 (1.5 g, 3 mmol) was dissolved in 20 mL of dry THF in a rounded-bottom flask under a N$_2$ atmosphere. 1.4 mL of $n$-butyllithium (2.5 M in hexane) was added dropwise at $-78$ °C. After stirring for 1 h, tributyltin chloride (1.18 g, 3.6 mmol) was added in one portion. Then the reaction was warm to room temperature and stirred overnight. Finally, the solution was purged into cold water. The organic phase was separated, and the aqueous layer was twice extracted with 50 mL of ether. The organic layers were collected and dried over anhydrous MgSO$_4$. After removing the solvent, the crude product 4-(4-dodecyl-5-(tributylstannyl)thiophen-2-yl)-N,N-diphenylaniline 5 as light yellow oil (1.1 g) was obtained and used in the next step without further purification.

**Synthesis of compound 6**

To a solution of compound 5 (1.1 g, 1.2 mmol) and intermediate_1 (4,7-dibromo-5,6-dinitrobenzo[c][1,2,5]thiadiazole) (370 mg, 0.9 mmol) in Toluene (20 mL) was added tetrakis(triphenylphosphine) palladium (0) (223 mg, 0.19 mmol) at a N$_2$ atmosphere and degassed for 3 times. Then the mixture was stirred at 110 °C for 10 hours. After reaction, the mixture was cooled to ambient temperature, and poured in to 100 mL water, and extracted with EA (50 mL x3), washed with brine (100 mL), dried with anhydrous Na$_2$SO$_4$, concentrated and purified with column chromatography on silica gel to give the desired product 6 (0.85 g, yield 51%) as purple solid.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.52 (d, $J$ = 8.6 Hz, 2H), 7.37-7.29 (m, 8H), 7.26(s,2H), 7.17 (d, $J$ = 7.9 Hz, 2H), 7.12-7.06 (m, 16H), 2.48-2.45 (m, 4H), 1.64-1.60 (m, 36H), 0.96 (t, $J$ = 7.3 Hz, 3H),

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 153.4, 148.1, 147.3, 146.5, 144.0, 129.4, 127.1, 126.8, 124.8, 124.0,123.8, 123.4, 123.2, 121.0, 31.9, 29.7, 27.9, 26.9, 22.7, 17.6, 14.2, 13.7. ESI-MS Calcd for:
C\textsubscript{74}H\textsubscript{81}N\textsubscript{6}O\textsubscript{4}S\textsubscript{3}\textsuperscript{3+} ([M+H]\textsuperscript{+}): 1213.54. Found: 1213.68.

**Synthesis of compound HQL2**

\[ \text{HQL2} \quad \text{R= n-Decyl} \]

Zinc dust (500 mg, 9.4 mmol) and NH\textsubscript{4}Cl (200 mg, 3.07 mmol) were added to a stirred solution of compound 6 (100 mg, 0.08 mmol) in dichloromethane (5 mL) and 90% methanol (5 mL) under an N\textsubscript{2} atmosphere. After being stirred at room temperature for 4 h, the solution was filtered through Celite pad, diluted with dichloromethane, and washed with water, saturated aqueous NaHCO\textsubscript{3}, and saturated aqueous brine. The organic phase was dried over anhydrous MgSO\textsubscript{4}, filtered and concentrated under vacuum to afford a yellow solid which was used into the next step without further purification.

To a dark yellow solution in anhydrous pyridine (1 mL) was added N-thionylaniline (0.45mL, 4.02 mmol, 560 mg) and chlorotrimethylsilane (0.45 mL, 5.1 mmol, 560 mg). The mixture was heated in an oil bath at 80 °C for 12 h. The reaction mixture was allowed to cool down to room temperature, poured into iced water and 1M HCl (aq.), extracted with ethyl acetate. The combined organic layer was washed saturated aqueous brine, dried over anhydrous MgSO\textsubscript{4}, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (15:1 petroleum ether: ethyl acetate) to yield the product HQL2 as light blue solid (15 mg, two step 45% yield).

\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) δ 7.61 (d, \textit{J} = 8.3 Hz, 4H), 7.41 (s, 2H)7.32 (t, \textit{J} = 8.3 Hz, 8H), 7.23-7.04 (m, 16H), 2.64 (t, \textit{J} = 7.8 Hz, 4H), 1.67-1.60 (m, 4H), 1.42-1.08 (m, 36H), 0.90 (t, \textit{J} = 6.6 Hz, 6H). \textsuperscript{13}C NMR (101 MHz, CDCl\textsubscript{3}) δ 153.3, 147.6, 147.5, 147.0, 145.7, 129.4, 128.2, 126.8, 124.7, 124.7, 123.4, 116.1, 32.0, 30.5, 30.4, 29.7, 29.4, 25.7, 22.7, 14.2. MALDI-TOF-MS Calcd for: C\textsubscript{74}H\textsubscript{81}N\textsubscript{6}O\textsubscript{4}S\textsubscript{3}\textsuperscript{3+} ([M+1]\textsuperscript{+}): 1181.53, found: 1181.2.(Fig. S7)

The synthesis route of HQL1
The compound HQL1 was obtained from 4-bromo-N,N-diphenylaniline by the method similar to that of HQL2.

Cell Culture and Animal Model

Human hepatocyte cells L02 were purchased from the China Center for Type Culture Collection (CCTCC). All cells were grown in a humidified atmosphere at 37 °C with 5% CO₂ atmosphere. L02 cells were maintained in Minimum Essential Medium (MEM, Gibco), supplemented with 10% fetal bovine serum, 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin. For U87 subcutaneous tumor model establishment, U87 cells (roughly 2 × 10⁶ in 75 μL of FBS-free DMEM medium) were subcutaneous injected into the right back leg of the 6-week-old female athymic nude mice (nu/nu) which were purchased from Charles River Laboratories (Beijing, China). The tumors were allowed to reach ~200 mm³ for small animal fluorescence imaging studies (tumor volume = Length*Width*Width/2). 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.

Preparation and Characterization of HQL2 dots

HQL2 (5 mg) and DSPE-PEG5K (40 mg) were completely dissolved in THF (1 mL). Then the mixture was added dropwise into one grade water (10 mL) under strong sonication. After 5 min sonication, the organic solvent was removed under N₂ flow, then the resulting mixture was washed several times using a 30 kDa centrifugal filter units and concentrated to yield HQL2 dots (~ 5 mg/mL). The resultant was stored in dark at 4 °C for further usage. The size and morphology were
characterized by Transmission electron microscopy (TEM) images on a JEM-2100 TEM system at an accelerating voltage of 200 kV. The hydrodynamic diameter and zeta potentials were measured using a Malvern Zetasizer Nano ZS. The UV-vis-NIR spectra of HQL2 dots was tested with a PerkinElmer Lambda 25 UV-Vis spectrophotometer. The corresponding NIR fluorescence spectrum was recorded on an Applied Nano Fluorescence spectrometer at room temperature with an excitation laser source of 808 nm.

**The cell viability assay of HQL2 dots**

The cytotoxicity of HQL2 dots was investigated by a standard MTT assay. The cell viability was measured using human hepatocyte cells L02 for 24 h incubation. The L02 cells were seeded in a 96-well plate (around 5000 cells per well). After 12 h, the medium was substituted with the fresh medium contained HQL2 dots with different concentrations. Followed by incubation for another 24 h, then a standard MTT method was performed for measuring the cell viability.

**In vivo vascular 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 different filter (1000 nm LP, 1250 nm LP, 1320 nm LP and 1550 nm LP) during *in vivo* imaging. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium solution (50 mg kg\(^{-1}\)) during the NIR-II imaging. For *in vivo* vascular imaging, the C57 Balb/c female mice and the athymic nude mice (nu/nu) bearing subcutaneous U87 tumor were given HQL2 dots (0.2 mL, 5 mg/mL) via tail vein injection. After injection, the mice were mounted in the prone position and supine position beneath the laser for vascular imaging.
Figure S1. $^1$H NMR of compound 4

Figure S2. $^{13}$C NMR of compound 4
Figure S3. $^1$H NMR of compound 6

Figure S4. $^{13}$C NMR of compound 6

Figure S5. $^1$H NMR of compound HQL2
Figure S6. $^{13}$C NMR of compound HQL2

Figure S7. The MALDI-TOF-MS spectrum of HQL2

Figure S8. $^1$H NMR of 4a
Figure S9. $^{13}$C NMR of 4a

Figure S10. $^1$H NMR of compound 6a

Figure S11. $^{13}$C NMR of compound 6a
Figure S12. $^1$H NMR of HQL1

Figure S13. $^{13}$C NMR of HQL1
Figure S14. The MALDI-TOF-MS spectrum of HQL1.

Figure S15. HOMO and LUMO orbital surfaces of HQL1, HQL2 and Q4 using DFT B3LYP/6-31G(d) scrf = (cpcm, solvent=dichloromethane) method. And the Optimized ground-state (S0) of these molecules and the relative dihedral angles.
**Figure S16.** The fluorescence intensity ratio ($I/I_0$) of HQL1 in different $f_w$, $I_0$ is the fluorescence intensity of HQL1 in pure THF and the fluorescence image of HQL1 in different $f_w$ with 808 nm excitation (5 ms exposure time, 90 mW cm$^{-2}$)

**Figure S17.** The Zeta potential of HQL2 dots
Figure S18. Absorbance (A, D, G) and fluorescence (B, E, H) spectra of HQL2 in DCM (A, B), HQL2 dots in water (D, E), and IR-26 in DCE (G, H). The slope of HQL2 in DCM (C), HQL2 dots in water (F), and IR-26 in DCE (I). Fluorescence quantum yield measurements of HQL2 in DCM and HQL2 dots in water were calculated according to a standard equation in the previous literature [2]. 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 HQL2 dots, \( QY_{ref} \) is the quantum yield of IR-26 (~0.05%), \( S_{sam} \) and \( S_{ref} \) are the slopes obtained by linear fitting of the integrated fluorescence intensity of HQL2 dots (1300-1600 nm) or (1500-1600 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 DCM: 1.42).
Figure S19. Absorbance (A, D, G) and fluorescence (B, E, H) spectra of HQL1 in DCM (A, B), HQL1 dots in water (D, E), and IR-26 in DCE (G, H). The slope of HQL1 in DCM (C), HQL1 dots in water (F), and IR-26 in DCE (I). The method of calculation fluorescence quantum yield of HQL1 in DCM and HQL1 dots in water were similar to the QY of HQL2.
Figure S20. The blood half-life circulation curve of HQL2 dots in female KM mice was determined to be ~226.5 min by fitting the data from the mean fluorescence intensity of blood samples at different time points after tail vein injection of HQL2 dots using a first-order exponential decay.
Fig. S21 (A) The acquired TEM image and (B) DLS results of a sample of the HQL2 dots. (C) The acquired UV-Vis-NIR absorption spectrum (black line) and fluorescence emission spectrum (red line) of the HQL2 dots in water. (D) Digital photograph of the HQL2 dots (left) and fluorescence signals of the dots (right) under different long-pass (LP) filters with an 808 nm-wavelength laser excitation (90 mW cm\(^{-2}\)). (E) The photostability levels of the HQL2 dots in water, PBS and FBS media. ICG in water was used as a reference. (These experiments were conducted with an 808 nm excitation, 180 mW cm\(^{-2}\).) (F) The viabilities of various samples of L02 human hepatocyte cells incubated with different concentrations of HQL2 dots for 24 h.

Reference
