Supporting Information

for

BODIPY-based fluorescent liposomes with sesquiterpene lactone trilobolide

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Additional information, characterization methods, experimental, analytical data, and supporting images from live-cell fluorescence microscopy

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1. Chemistry

1.1. Methods and apparatus

Cholesterol was purchased from Sigma-Aldrich (Corp., St. Louis, USA), acetylene-PEG₃amine from Click chemistry tools (Scottsdale, USA). For thin-layer chromatography (TLC), aluminum silica gel sheets were used for detection in UV light (TLC Silica gel 60 F_{254} , Merck). A diluted solution of sulfuric acid in methanol was used and plates were successively heated for TLC visualization. Silica gel (30-60 µm, SiliTech, MP Biomedicals) was used for column chromatography. NMR spectra (¹H 300 MHz and ¹³C 75 MHz) were recorded with a Varian Gemini 300 (Varian, Palo Alto, USA). Chemical shifts are given in δ (ppm). FTIR spectra were measured by Nicolet iS10 (Thermo Scientific, Waltham, USA) using ATR (KBr crystal) technique (symbolism; *s* strong, *vs* very strong, *m* medium, *w* weak, *vw* very weak). HRMS were measured by LTQ ORBITRAP VELOS with HESI⁺/HESI⁻ ionization (Thermo Scientific, Waltham, USA). Optical rotations were measured by an Autopol VI polarimeter (Rudolph Research Analytical, Hackettstown, USA). Methyl 4-iodo-L-phenylalaninate, propargyl cholesteryl ether [1], Tb-N₃VA [2], and BODIPY-BA [3] were synthesized according to previously described protocols.

1.2. Synthesis

Methyl N-(5-azidopentanoyl)-4-iodo-L-phenylalaninate (1)

Pyridine (15 mL) and T3P (6 mL, 0.02 mol, 50% solution) were added to a solution of methyl 4-iodo-L-phenylalaninate hydrochloride (1.5 g, 4.9 mmol) and azidovaleric acid (0.85 g, 5.9 mmol) in AcOEt (10 mL) at 0 °C. Then, the mixture was stirred at rt for 24 h. After the completition of the reaction, the mixture was dilluted with AcOEt (50 mL), the organic layer was washed with brine (3×50 mL), dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The product was purified by column chromatography

on silica gel using a gradient mobile phase (DCM/MeOH 100:0 to 98:2). Evaporation of the solvent yielded a white-yellow product (1.6 g, 3.7 mmol) in 70% yield. ¹H NMR (300 MHz, CDCl₃) δ ppm: 7.54 (d, *J* = 8.2 Hz, 2 H), 6.80 (d, *J* = 8.2 Hz, 2 H), 6.29 (d, *J* = 7.9 Hz, 1 H), 4.87 - 4.73 (m, 1 H), 3.66 (s, 3 H), 3.19 (t, *J* = 6.6 Hz, 2 H), 3.08 - 2.98 (m, 1 H), 2.95 - 2.84 (m, 1 H), 2.13 (t, *J* = 7.0 Hz, 2 H), 1.65 - 1.53 (m, 2 H), 1.53 - 1.41 (m, 2 H). ¹³C NMR (75MHz, CDCl₃) δ ppm: 172.4, 172.2, 137.8, 135.9, 131.5, 92.8, 53.0, 52.7, 51.3, 37.6, 35.7, 28.4, 22.8. LRMS-ESI: found 306.0 [M+H]⁺ and 324.0 [M+NH₄]⁺.

4-(BODIPY)-N-(5-azidopentanoyl)phenylalanine (3)

A solution of LiOH (166 mg) in water (8 mL) was added to a solution of methyl ester (1, 1 g, 2.3 mmol) in THF (16 mL), and the mixture was stirred for 3 h. Then, the pH value was changed to acidic by HCl, and the product was extracted by AcOEt (5×25 mL). The organic layers were combined, washed with brine $(2 \times 100 \text{ mL})$ and dried over MgSO₄. The solvents were evaporated under reduced pressure. The product 2 was used in the following reaction without purification (LRMS-ESI: 415.0 [M-H]⁻). The acid 2 (388 mg, 0.93 mmol) and BODIPY-BA [3] (480 mg, 1.3 mmol) were dissolved in a mixture of toluene/methanol/water (10:3:2; 15 mL). Potassium carbonate (1.28 g, 9.3 mmol) and Pd(PPh₃)₄ (108 mg, 0.1 mmol) were added, and the mixture was heated at 60 °C for 24 h. Chloroform (30 mL) was added, and the mixture was filtered. The organic layer was washed with diluted HCl (50 mL), water (50 mL) and a 1 M solution of ammonium chloride (50 mL), then dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The product was purified by column chromatography on silica gel using a gradient mobile phase (DCM/MeOH 100:0 to 98:2). Evaporation of the solvent yielded a product as brick solids (500 mg, 0.82 mmol) in 88% yield. ¹H NMR (600 MHz, CDCl₃+CD₃OD 2 drops): δ ppm 7.74 (m, 2H), 7.62 (m, 2H), 7.35 (m, 2H), 7.30 (m, 2H), 6.01 (br s, 2H), 4.85 (dd, 1H, J = 7.0, 5.4 Hz), 3.30 (dd, 1H, J = 14.0, 5.4 Hz), 3.23 (m, 2H), 3.11 (dd, 1H, J = 14.0, 7.0 Hz), 2.56 (s, 6H), 2.23 (m, 2H), 1.66 (m, 2H), 1.54 (m, 2H), 1.45 (s, 6H). ¹³C NMR (150.9 MHz), CDCl₃+CD₃OD 2 drops): δ ppm 173.50, 173.07, 155.50, 143.32, 141.61, 141.30, 138.60, 136.21, 133.84, 131.47, 129.94, 128.51, 127.47, 127.05, 121.33, 53.05, 51.08, 37.13, 35.50, 28.18, 22.70, 14.48. For graphical assignment of the ¹H and ¹³C NMR, see section 5.1 - Figure S1. IR (CHCl₃, neat): 3285 (*s*), 3071 (*w*), 2952 (*m*), 2862 (*w*), 2095 (*s*), 1736 (*vs*), 1648 (*s*), 1541 (*s*), 1485 (*w-m*), 1436 (*m*), 1351 (*w-m*), 1272 (*m*), 1260 (*m-s*), 1219 (*m*), 1173 (*w*), 1009 (*w*), 817 (*w*). HRMS-ESI: *monoisotopic mass* 612.2832 Da, found *m/z* 611.2827 [M-H]⁻; section 5.2 - Figure S11. $\varepsilon = 47$ 000 dm³·mol⁻¹·cm⁻¹. [α]_D²⁰+11 (*c* = 1; CHCl₃).

ChL-BODIPY-acid (4)

Procedure for CuAAC: To a solution of 3-*O*-propargylcholesterylether (83 mg, 0.2 mmol) and BODIPY derivative **3** (100 mg, 0.16 mmol) in DMF (2 mL) were added CuSO₄·5H₂O (4 mg, 0.02 mmol), sodium ascorbate (6.5 mg, 0.03 mmol) and TBTA (17 mg, 0.03 mmol). The mixture was placed onto a microwave reactor (MW) and stirred at 60 °C for 90 min. The solvent was evaporated and the product was purified by column chromatography on silica gel (DCM/MeOH 100:0 to 98:2) to obtain product **4** as a brick coloured gel (82 mg, 0.08 mmol) in 49% yield. ¹H NMR (600 MHz, CDCl₃): *δ* ppm: 7.69 (m, 2H), 7.57 (m, 2H), 7.32 (m, 2H), 7.26 (m, 2H), 7.25 (s, 1H), 6.38 (br s, 1H), 5.98 (br s, 2H), 5.34 (m, 1H), 4.91 (m, 1H), 4.68 (br s, 2H), 4.33 (m, 2H), 3.35 (tt, 1H, *J* = 11.0, 11.0, 4.5, 4.5 Hz), 3.24 (dd, 1H, *J* = 14.0, 5.0 Hz), 3.06 (dd, 1H, J = 14.0, 7.0 Hz), 2.56 (s, 6H), 2.41 (m, 1H), 2.26 (m, 1H), 2.22 (m, 2H), 2.00 (dt, 1H, *J* = 13.0, 3.5, 3.5 Hz), 1.96 (m, 2H), 1.88 (m, 2H), 1.85 (m, 1H), 1.82 (m, 1H), 1.63 (m, 1H), 1.56 (m, 1H), 1.52 (m, 2H), 1.50 (m, 1H), 1.13 (m, 4H), 1.08 (m, 1H), 1.06 (m, 1H), 1.04 (m, 1H), 0.99 (m, 1H), 0.988 (s, 3H), 0.97 (m, 1H), 0.91 (m, 1H),

0.908 (d, 3H, J = 6.5 Hz), 0.865 (d, 3H, J = 6.5 Hz), 0.86 (d, 3H, J = 6.5 Hz), 0.664 (s, 3H); Fig. S5. ¹³C NMR (150.9 MHz, CDCl₃) δ ppm: 173.33, 172.56, 155.52, 142.99, 141.35, 141.03, 140.43, 138.55, 135.92, 133.96, 131.40, 130.00, 128.52, 127.35, 127.06, 122.02, 121.24, 79.41, 61.46, 56.71, 56.14, 53.07, 50.10, 49.81, 42.29, 39.73, 39.49, 38.92, 37.10, 36.81, 36.16, 35.76, 35.14, 31.91, 31.85, 29.15, 28.20, 28.00, 24.26, 23.83, 22.80, 22.54, 22.57, 21.04, 19.33, 18.69, 14.59, 14.54, 11.84; Fig. S6. For graphical assignment of the ¹H and ¹³C NMR, see section 5.1 - Figure S2. IR (CHCl₃, neat): 3287 (*br*), 3028 (*w*), 2931 (*m*), 2866 (*m*), 2360 (*m*), 2340 (*m*), 1958 (*w*), 1919 (*w*), 1844 (*w*), 1771 (*w*), 1734 (*m*), 1669 (*m*), 1636 (*m*), 1544 (*s*), 1510 (*s*), 1468 (*m*), 1308 (*m*), 1194 (*s*), 1157 (*m*), 1085 (*m*), 981 (*m*), 817 (*w*). HRMS-ESI: *monoisotopic mass* 1036.6537 Da, 1059.64465 [M+Na]⁺; section 5.2 -Figure S12. ε = 53 000 dm³·mol⁻¹·cm⁻¹. [α]_D²⁰ + 5.33 (*c* = 1; CHCl₃).

ChL-BODIPY alkyne (5)

EDCI (16.6 mg, 0.09 mmol), 4-DMAP (10.6 mg, 0.09 mmol) and HOBt (3.9 mg, 0.03 mmol) were added sequentially to a solution of **4** (60 mg, 0.06 mmol) and amino-PEG₄-acetylene (19 mg, 0.08 mmol) in DMF (5 mL) at 0 °C. The cooling bath was removed and the mixture was stirred for 24 h. Additional EDCI (5.5 mg, 0.03 mmol) was added, and stirring at rt continued for the next 24 h. The solvent was evaporated under reduced pressure, and the product was purified by column chromatography on a silica gel (DCM/MeOH 100:0 to 98:2) to obtain product **5** as a brick like gel (66 mg, 0.05 mmol) in 92% yield. ¹H NMR (600 MHz, CDCl₃) δ ppm: 7.73 (m, 2H), 7.61 (m, 2H), 7.54 (s, 1H), 7.345 (m, 2H), 7.31 (m, 2H), 6.62 (t, 1H, *J* = 5.2 Hz), 6.42 (d, 1H, *J* = 8.0 Hz), 5.99 (br s, 2H), 5.35 (m, 1H), 4.70 (ddd, 1H; *J* = 8.0, 7.0, 6.2 Hz), 4.67 (br s, 2H), 4.31 (m, 2H), 4.20 (d, 1H, *J* = 2.2 Hz), 3.69 (m, 2H), 3.68–3.44 (m, 12H), 3.42 (m, 2H), 3.32 (tt, 1H, *J* = 11.2, 11.2, 4.5, 4.5 Hz), 3.14 (dd, 1H, *J* = 13.6, 7.0 Hz), 3.09 (dd, 1H, *J* = 13.6, 6.2 Hz), 2.56 (s, 6H), 2.44 (t, 1H, *J* = 2.2 Hz), 2.41 (ddd, 1H,

J = 13.0, 4.5, 2.2 Hz), 2.25 (m, 1H), 2.23 (m, 2H), 2.00 (m, 1H), 1.965 (m, 1H), 1.95 (m, 1H), 1.90 (m, 2H), 1.85 (m, 1H), 1.825 (m, 1H), 1.65 (m, 2H), 1.57 (m, 1H), 1.515 (m, 2H), 1.495 (m, 1H), 1.49 (m, 1H), 1.44 (m, 1H), 1.43 (m, 1H), 1.43 (s, 6H), 1.37 (m, 1H), 1.33 (m, 2H), 1.255 (m, 1H), 1.15 (m, 1H), 1.14 (m, 1H), 1.12 (m, 2H), 1.085 (m, 1H), 1.06 (m, 1H), 1.04 (m, 1H), 0.994 (s, 3H), 0.99 (m, 1H), 0.98 (m, 1H), 0.915 (m, 1H), 0.912 (d, 3H, J = 6.6 Hz), 0.866 (d, 3H, J = 6.6 Hz), 0.862 (d, 3H, J = 6.6 Hz), 0.672 (s, 3H); Fig. 7. ¹³C NMR (150.9 MHz, CDCl₃) δ ppm: 171.70, 170.60, 155.48, 145.92, 143.00, 141.37, 141.07, 140.61, 138.40, 136.44, 133.91, 131.40, 129.98, 128.50, 127.35, 127.01, 122.22, 121.80, 121.21, 79.60, 78.94, 74.72, 70.51, 70.49, 70.44, 70.29, 70.15, 69.51, 69.02, 61.62, 58.36, 56.70, 56.10, 54.22, 50.10, 49.86, 42.27, 39.72, 39.47, 39.30, 38.99, 38.46, 37.11, 36.80, 36.14, 35.74, 35.31, 31.89, 31.84, 29.52, 28.27, 28.19, 27.97, 24.24, 23.78, 22.78, 22.52, 22.23, 21.02, 19.32, 18.67, 14.56, 14.52, 11.82; Fig. S8. For graphical assignment of the ¹H and ¹³C NMR, see section 5.1 - Figure S3. IR (CHCl₃, neat): 3284 (br), 3072 (w), 2932 (m), 2867 (m), 2360 (w), 2113 (w), 1958 (w), 1639 (m), 1544 (s), 1511 (m), 1468 (m), 1366 (m), 1307 (m), 1261 (s), 1195 (s), 1089 (s), 981 (m), 836 (w), 711 (w). HRMS-ESI: monoisotopic mass 1249.7902 Da, found m/z 1250.79895 $[M+H]^+$, 1272.78075 $[M+Na]^+$ and 1288.75415 $[M+K]^+$; section 5.2 -Figure S13. $\varepsilon = 58\ 000\ \text{dm}^3 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. $[\alpha]_D^{20} - 10.3\ (c = 1, \text{CHCl}_3)$.

Tb-BODIPY-ChL construct (6)

This compound was synthesized by following the click protocol described for compound **4**. In reaction: Tb-N₃VA (15.5 mg, 0.03 mmol), ChL-BODIPY alkyne **5** (35 mg, 0.03 mmol), CuSO₄·5H₂O (0.2 equiv), sodium ascorbate (0.3 equiv) and TBTA (0.2 equiv), DMF (2 mL), the mixture was placed onto MW and stirred at 60 °C for 5 h. Chromatography on silica gel (2% of MeOH in DCM) gave product **6** (42 mg, 0.023 mmol) in the yield of 84% as a brick coloured gel which solidified upon storage. ¹H NMR (600 MHz), CDCl₃) δ ppm:

7.72 (m, 2H), 7.64 (s, 1H), 7.575 (m, 2H), 7.54 (s, 1H), 7.34 (m, 2H), 7.29 (br, 1H), 7.28 (m, 2H), 6.92 (d, 1H, J = 8.0 Hz), 6.11 (qq, 1H, J = 7.2, 1.5 Hz), 5.99 (br s, 2H), 5.80 (t, 1H, J =3.8 Hz), 5.75 (q, 1H, J = 1.8 Hz), 5.60 (m, 1H), 5.34 (m, 1H), 4.71 (dt, 1H; J = 8.0, 7.0, 7.0Hz), 4.685 (d, 1H, J = 12.0 Hz), 4.65 (d, 1H, J = 12.0 Hz), 4.64 (br s, 2H), 4.34 (m, 1H), 4.32 (m, 3H), 4.27 (m, 1H), 3.71 (m, 2H), 3.66–3.59 (m, 8H), 3.52 (m, 1H), 3.47 (m, 1H), 3.46 (m, 1H), 3.38 (m, 1H), 3.30 (tt, 1H, J = 11.2, 11.2, 4.5, 4.5 Hz), 3.28 (m, 2H), 3.055 (dd, 1H, J = 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 11.2, 1 13.5, 7.0 Hz), 3.00 (dd, 1H, J = 13.5, 7.0 Hz), 2.98 (dd, 1H, J = 14.6, 3.8 Hz), 2.59 (m, 1H), 2.56 (s, 6H), 2.39 (m, 1H), 2.36 (dd, 1H, J = 14.6, 3.8 Hz), 2.34 (m, 2H), 2.27 (m, 1H), 2.23 (m, 1H), 2.19 (m, 1H), 2.02 (dq, 3H, J = 7.2, 1.5 Hz), 2.00 (m, 1H), 1.96 (m, 1H), 1.94 (m, 1H), 1.94 (s, 3H), 1.93 (m, 2H), 1.92 (p, 3H, $J = 4 \times 1.5$ Hz), 1.92 (m, 3H), 1.88 (m, 2H), 1.85 (m, 1H), 1.82 (m, 1H), 1.66 (m, 1H), 1.61 (m, 2H), 1.58 (m, 2H), 1.56 (m, 1H), 1.51 (m, 2H), 1.49 (m, 1H), 1.48 (m, 1H), 1.44 (m, 1H), 1.425 (m, 1H), 1.425 (s, 6H), 1.405 (s, 3H), 1.35 (s, 3H), 1.335 (m, 1H), 1.33 (m, 1H), 1.25 (m, 1H), 1.15 (m, 1H), 1.135 (m, 1H), 1.12 (m, 2H), 1.08 (m, 1H), 1.06 (m, 1H), 1.04 (m, 1H), 0.99 (s, 3H), 0.99 (m, 1H), 0.975 (m, 1H), 0.92 (m, 1H), 0.92 (s, 3H), 0.866 (d, 3H, J = 6.6 Hz), 0.861 (d, 3H, J = 6.6 Hz), 0.67 (s, 3H); Fig. S9. ¹³C NMR (150.9 MHz, CDCl₃) δ ppm: 176.42, 172.46, 171.85, 171.01, 170.33, 167.70, 155.49, 145.90, 144.98, 143.00, 142.74, 141.39, 141.12, 140.52, 138.31, 138.23, 136.49, 133.88, 132.05, 131.40, 130.02, 128.52, 127.83, 127.33, 126.88, 122.88, 122.48, 121.88, 121.22, 85.70, 79.69, 79.13, 78.65, 78.58, 77.63, 70.44, 70.41, 70.04, 69.79, 69.33, 66.69, 64.66, 61.47, 56.70, 56.11, 54.28, 51.68, 50.08, 49.81, 42.28, 39.72, 39.48, 39.32, 38.99, 38.58, 38.28, 37.09, 36.79, 36.15, 35.75, 35.39, 33.46, 32.37, 31.89, 31.84, 29.35, 29.29, 28.25, 28.20, 27.98, 24.25, 23.79, 22.79, 22.53, 22.37, 21.38, 21.16, 21.02, 20.70, 19.32, 18.68, 15.98, 14.57, 14.53, 13.13, 11.82; Fig. S10. For graphical assignment of the ¹H and ¹³C NMR, see section 5.1 - Figure S4. IR (CHCl₃, neat): 3289 (br), 2927 (s), 2857 (m), 2367 (w), 2247 (w), 1958 (w), 1784 (m), 1731 (m), 1545 (m), 1465 (m), 1368 (m), 1247 (m), 1195 (m-s),

1156 (*s*), 1086 (*m*-*s*), 1050 (*m*), 982 (*m*), 838 (*w*), 732 (*w*), 609 (*w*). HRMS-ESI: *monoisotopic* mass 1814.0414 Da, found m/z 1815.05198 [M+H]⁺, 1837.03136 [M+Na]⁺ and 1853.00736 [M+K]⁺; section 5.2 - Figure S14. $\varepsilon = 45\ 000\ dm^3 \cdot mol^{-1} \cdot cm^{-1}$. $[\alpha]_D^{20} - 99\ (c = 1, CHCl_3)$.

2. Liposome preparation

Liposomes were prepared by mixing stock solutions of construct 6 ($1 \text{ mg} \cdot \text{mL}^{-1}$), 1,2dipalmitoyl-3-trimethylammoniumpropane (DPTAP; 5 mg·mL⁻¹), 1,2-dioleoyl-sn-glycero-3phosphoethanolamine (DOPE; $2 \text{ mg} \cdot \text{mL}^{-1}$) and cholesterol ($1 \text{ mg} \cdot \text{mL}^{-1}$) to yield a final concentration of compound **6** of 0.1 mg·mL⁻¹ (55.13 μ M). The appropriate mixture ratios of the stock solutions in chloroform were 10:10:40:40 (wt %) of construct 6/DPTAP/DOPE/cholesterol. The resulting mixture was evaporated to dryness under a stream of pressurized air, and solvent residues were removed by evacuation under reduced pressure (8-100 mbar) for at least 16 h. The lipid residues were hydrated in physiological solution at 60 °C for 60 min with shaking to yield multilamellar vesicles. The resulting dispersion was processed to give large unilamellar vesicles by extruding the mixture 19 times through Millipore (Bedford, MA, USA) 100 nm pore size polycarbonate filters using a Liposo-Fast extruder. The liposome composition is depicted in Table S1.

Component	wt [%]	$c [\mu g \cdot m L^{-1}]$
Construct 6	10	100
Cholesterol	10	100
1,2-Dipalmitoyl-3-trimethylammonium-		
	40	400
propane		
1,2-Dioleoyl-sn-Glycero-3-		
	40	400
phosphoethanolamine		

Table S1. Composition of liposomes with incorporated construct 6

3. Atomic force microscopy

Atomic force microscopy (AFM) (instrument VEECO CP II, tapping mode, Si probe RTESPA-CP with the spring constant 20–80 N·m⁻¹) was used as the main method for characterization of the prepared liposomes by evaluating their surface morphology and roughness. For this purpose, the liposome samples (1 mg·mL⁻¹) with construct **6** were loaded (4 and 8 μ L) on a microscopic glass coverslip, let semi-dry overnight and subjected to AFM analysis. The mean roughness value (R_a) represents the average of the deviations from the center plane of a sample. The R_a determination is calculated based on the following equation:

$$R_a = \frac{\sum_{i=1}^{N} \left| Z_i - Z_{cp} \right|}{N}$$

where Z_{cp} is the value of the central plane, Z_i is the actual Z value, and N is the number of points, where the Z_i is evaluated.

4. Cell assays

4.1. Cell uptake of Tb-BODIPY-ChL construct 6

In our study, the following human cancer cell lines were used: U-2 OS (cells from osteosarcoma) and HeLa (cells from cervical carcinoma). Cells were cultivated in DMEM medium (Sigma-Aldrich, USA) with stable L-glutamine, supplemented with 10% fetal bovine serum (Thermo Scientific, USA), and maintained at 37 °C in humidified atmosphere with 5% of CO₂.

Cells $(1 \cdot 10^5 \text{ per dish})$ were seeded into 35 mm cultivation dishes with a glass bottom for live-cell imaging (Ibidi, Germany) and left to adhere for 16 h. The attached cells were washed with phosphate buffered saline (PBS, pH 7.4, 37 °C) and incubated with construct **6** (13–500 nM) and liposomes with encapsulated construct **6** at a concentration range of 13 nM–1.25 μ M, in complete medium without phenol red (FluoroBrite DMEM; Thermo Scientific) at 37 °C for 1, 2, 7, 16 h, and 48 h, respectively. After the incubation period, the cells were washed with PBS and fed with fresh medium without phenol red and subjected to fluorescence microscopy.

4.2.Fluorescence microscopy

Cell uptake and the intracellular localization of Tb-BODIPY-ChL construct **6** and the liposome formulation were studied by real-time live-cell fluorescence microscopy in the subjected cell lines at 37 °C and in 5% CO₂ atmosphere. The images were acquired by an inverse fluorescence microscope Olympus IX-81, xCellence System using high-stability 150 W xenon arc burner, FITC filter (Olympus, Japan) and EM-CCD camera C9100-02 (Hamamatsu, Germany). Living cells were analyzed by a $60 \times$ oil immersion objective (NA 1.4, Olympus, Japan). All images were deconvolved using xCellence software.

4.3. Nitric oxide release in primary macrophages

The resident peritoneal macrophages collected from individual female Wistar rats weighing 175-185 g (VELAZ, Czech Republic; approved institutional protocol MSMT-15894/2013-310) were washed, resuspended and seeded into 96-well round-bottom microplates (2.10⁵ cells per well) in complete RPMI-1640 medium. The complete RPMI-1640 culture medium contained 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, $50 \,\mu\text{g}\cdot\text{mL}^{-1}$ gentamicin, and $5 \cdot 10^{-5}$ M 2-mercaptoethanol (all Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured for 24 h in the presence of the tested compounds (stock solutions of **Tb** and compounds 4, 5, 6 were prepared 100 mM in DMSO). Untreated cells were incubated only with the vehicle (DMSO). For comparative purpose of immunomodulatory activity, 100 pg·mL⁻¹ lipopolysaccharide (LPS) was used. Peritoneal macrophages were maintained at 37 °C, 5% CO₂ in humidified Heraeus incubator. All experimental variants were run in duplicates. The concentration of nitrites was detected in individual, cell-free samples (50 µL) incubated 10 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanilamide/0.1% naphtylendiamine/2.5% H₃PO₄). The absorbance was recorded at 540 nm using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to concentration (µM) of nitrite.

4.4. Statistical analysis

Analysis of variance (ANOVA) and graphical presentation of data were done using GraphPad Prism 6, San Diego, CA.

5. Analytical supplement

5.1. Graphical assignment of the ¹H and ¹³C NMR spectra of the key compounds



Figure S1. Interpretation of the NMR spectra of compound 3.



Figure S2. Interpretation of the NMR spectra of compound 4.



Figure S3. Interpretation of the NMR spectra of compound 5.



Figure S4. Interpretation of the NMR spectra of construct 6.



Figure S5. ¹H NMR spectrum (600 MHz) of compound 4 CDCl₃.



Figure S6. ¹³C NMR spectrum (150.9 MHz) of compound 4 in CDCl₃.





Figure S8. ¹³C NMR spectrum (150.9 MHz) of compound 5 in CDCl₃.





Figure S10. ¹³C NMR spectrum (150.9 MHz) of construct 6 in CDCl₃.

5.2. High-resolution mass spectra



Figure S11. HRMS-ESI spectra of compound 3.







Figure S13. HRMS-ESI spectra of compound 5.



Figure S14. HRMS-ESI spectra of construct 6

5.3. HPLC chromatogram of the target construct 6



Figure S15. HPLC-MS analysis of the final construct 6 (column RP C-18 Cromasil, flow 0.4 mL/min,

50% MeOH / MeOH gradient (time (min) MeOH (%)); 1/50; 2/60; 3/70; 4/90; 5/100; 15/100).

6. Live-cell imaging



Figure S16. A panel of images from live-cell fluorescence microscopy: intracellular localization of liposomes with construct **6** (43 nM) in U-2 OS cells after 1 h of incubation: A) bright field; B) construct **6** in liposomes; C) merge of images A and B.



Figure S17. A panel of images from live-cell fluorescence microscopy: intracellular localization of liposomes with construct **6** (125 nM) in HeLa cells after 2 h of incubation: A, D) bright field; B, E) construct **6** in liposomes; C, F) merges of images A and B and D and E.



Figure S18. A panel of images from live-cell fluorescence microscopy: intracellular localization of liposomes with construct **6** (1.25 μ M) in U-2 OS cells after 2 and 7 h of incubation: A, D) bright field; B, E) construct **6** in liposomes; C, F) merges of images A and D and B and E.

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