Supplementary information for:

Recognition-Controlled Membrane Translocation for Signal Transduction across Lipid Bilayers

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Experimental procedures

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 400-MHz Bruker spectrometer. Chemical shifts are reported as δ values in *ppm*. Flash chromatography was carried out on an automated system (Combiflash Companion) using pre-packed cartridges of silica (50 μm PuriFlash® Column). GPC purification of vesicles was carried out using GE Healthcare PD-10 desalting columns prepacked with Sephadex G-25 medium. Fluorescence spectroscopic data were recorded using a Cary Eclipse fluorescence spectrophotometer (Agilent). pH measurements were conducted using a Mettler-Toledo "*Seven Compact"* pH meter equipped with an "*In-lab Micro"* electrode. Vesicles were prepared as described below using Avestin "*LiposoFast"* extruder apparatus, equipped with polycarbonate membranes with 200 nm pores.

Materials

All reagents and solvents were used without further purification. Lipids were purchased from Sigma Aldrich and used without further purification.

Fluorescence experiments

Fluorescence excitation experiments were recorded using the following parameters: emission wavelength = 510 nm, excitation range 380−480 nm, recorded at 2 minute intervals, with the excitation and emission slits set at 5 nm.

Vesicle signal transduction experiments were repeated a minimum of two times, and the kinetic constants reported are averaged values. All experiments were repeated on independent samples to confirm reproducibility.

Dynamic Light Scattering (DLS) Experiments

DLS experiments were undertaken using a Malvern Zetasizer Nano S90 apparatus, equipped with a 4 mW HeNe laser source (633 nm). The sample was placed in a thermostat-controlled cell-holder maintained at 298 K and the scattered light collected at an angle of 173°. Unimodal intensity weighted distributions of the hydrodynamic diameters were obtained and the averaged values of three measurements reported.

General procedure for vesicles preparation

Vesicles suspensions were prepared as follows:

To a round bottom flask was added chloroform/ethanol solutions of the lipids (1,2-Dioleoyl-*sn*-glycero-3-phosphocholine DOPC and 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine DOPE, in a 3:2 ratio), a chloroform solution of **1**, and aqueous $CuCl_2$, obtain final concentrations of 1 mM lipids, and 10 μM **1**·Cu^{2+}. The solvent was removed *in vacuo* and dried under high vacuum for 2 h. A 100 mM solution of HEPES buffer at pH 7 (2.5 mL) and aqueous solutions of ester **2** and $ZnCl_2$ (to obtain final concentrations of 250 μM) were added to the flask containing the lipids, and sonicated for 1 min. The suspension was subjected to 5 cycles of freeze-thaw using liquid nitrogen. The suspension was extruded 19 times through a polycarbonate filter with 200 nm pores in an Avestin Lipofast apparatus,[1] and then the vesicles were separated by the bulk solution using prepacked GPC columns eluting with a 100 mM HEPES buffer solution at pH 7. The average size of the vesicles in the sample was determined by DLS measurements (see Table S1) to be approximately 200 nm.

Synthetic procedures

Compounds **2**[2] and **8**[3] were prepared as previously described.

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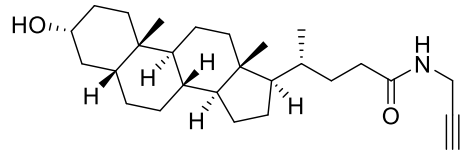

Lithocholic acid (1.00 g, 2.65 mmol) and *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC·HCl, 760 mg, 3.98 mmol) were dissolved in dry DCM (150 mL) in the presence of dry triethylamine (1.8 mL, 13.3 mmol) and catalytic DMAP (~2 mg). To the solution was added propargyl amine (220 mg, 3.97 mmol) and the reaction stirred at rt for 16 h, before washing with 1 M HCl (2 × 20 mL) and brine (2 × 20 mL). The organic layer was dried over $MgSO_4$, filtered, and the solvents removed *in vacuo*. The crude was purified by flash chromatography (SiO_2, 0-10% gradient of methanol in dichloromethane) to afford the product as a white solid (410 mg, 40%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.58 (br. s, 1H), 4.06 (m, 2H), 3.63 (m, 1H), 2.30-2.23 (m, 2H), 2.10-2.05 (m, 1H), 1.97-0.96 (m, 26H), 1.58 (s, 1H) 0.93-0.91 (m, 6H), 0.64 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 173.1, 79.7, 71.9, 71.6, 56.5, 56.0, 42.8, 42.1, 40.4, 40.2, 36.5, 35.9, 35.4, 35.3, 34.6, 33.3, 31.6, 30.6, 29.2, 28.3, 27.2, 26.4, 24.2, 23.4, 20.8, 18.4, 12.1. HRMS calc. for $C_{27}H_{43}NO_2H^+$: 414.3372; found: 414.3372.

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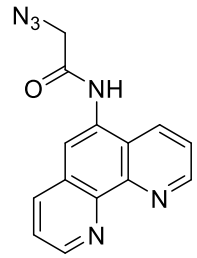

1,10-Phenanthrolin-5-amine (220 mg, 1.13 mmol) was suspended in a mixture of dry triethylamine (0.19 mL) and dry THF (40 mL). The mixture was cooled to 0 °C and chloroacetyl chloride (0.2 mL, 2.8 mmol) in THF (5 mL) was added dropwise. The reaction mixture was stirred at r.t. for 16 hrs before the solvents were removed *in vacuo*. The residue was suspended in DMF (10 mL) before sodium azide (220 mg, 3.4 mmol) was added, which immediately resulted in all solids going into solution. The solution was stirred at 60 °C for 16 h, before cooling to rt. The mixture was diluted with water (10 mL) and extracted with DCM (100 mL). The solvents were removed *in vacuo* and the crude purified by flash chromatography (SiO_2, 5-20% gradient of methanol in dichloromethane) to afford the product as a yellow solid (130 mg, 40%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 9.14 (br. s, 1H), 9.13 (dd, ^3J = 4.3 Hz, ^4J = 1.5 Hz, 1H), 9.08 (dd, ^3J = 4.3 Hz, ^4J = 1.5 Hz, 1H), 8.35 (dd, ^3J = 8.4 Hz, ^4J = 1.5 Hz, 1H), 8.31 (s, 1H), 8.19 (dd, ^3J = 8.1 Hz, ^4J = 1.5 Hz, 1H), 7.60 (m, 2H), 4.34 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 165.9, 150.2, 150.0, 146.4, 144.4, 136.1, 129.6, 128.2, 123.7, 123.6, 122.9, 119.5, 53.0, 50.8. HRMS calc. for $C_{14}H_{10}N_6OH^+$: 279.0994; found: 279.0993.

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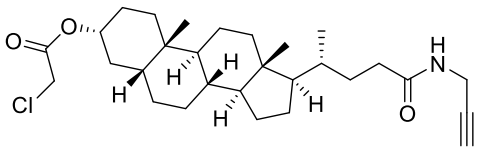

To a solution of **4** (165 mg, 0.242 mmol), 4-dimethylaminopyridine (1.0 mg, 0.01 mmol) and triethylamine (35 mg, 0.290 mmol) in DCM (30 mL) at 0°C was added chloroacetylchloride (29 mg, 0.29 mmol) in DCM (5 mL) dropwise. The reaction was allowed to warm to rt and stirred for a further 16 h. The reaction was washed with water (2 × 10 mL), dried over $MgSO_4$, filtered and the solvents removed *in vacuo*. The crude was purified by column chromatography (SiO_2, 0-15% gradient of MeOH in DCM) to afford the product as a white solid (110 mg, 90%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.60 (br. s, 1H), 4.84 (m, 1H), 4.04 (s, 2H), 4.00 (s, 2H), 2.36-2.26 (m, 2H), 2.25 (s, 1H), 2.10-0.96 (m, 25H), 1.58 (s, 1H) 0.93-0.91 (m, 6H), 0.66 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 173.2, 166.7, 71.6, 56.4, 56.0, 42.9, 41.9, 41.0, 40.3, 35.8, 35.1, 34.3, 33.3, 32.2, 31.5, 29.6, 28.9, 28.1, 27.0, 26.5, 26.2, 24.2, 23.3, 20.9, 18.7, 11.9. HRMS calc. for $C_{29}H_{44}NO_3ClH^+$: 490.3088; found: 490.3089.

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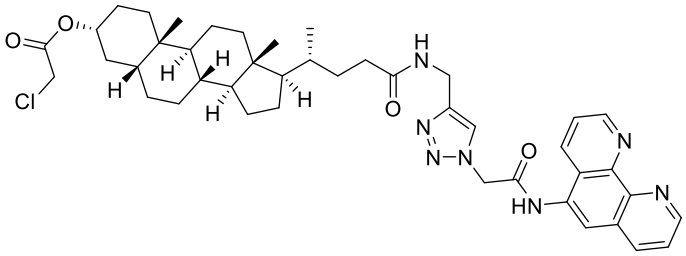

To a solution of **6** (90 mg, 0.180 mmol) and **5** (50 mg, 0.180 mmol) in DCM (25 mL) was added tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 16 mg, 0.03 mmol) and $Cu(CH_3CN)_4PF_6$ (11 mg, 0.03 mmol) and the reaction mixture stirred for 16 hr at r.t. The reaction mixture was diluted with DCM (50 mL), then washed with basic EDTA solution (0.1 M, 2 × 5 mL), water (2 × 10 mL), dried over $MgSO_4$, filtered and the solvents removed *in vacuo*. The crude was purified by column chromatography (SiO_2, 0-15% gradient of MeOH in DCM) to afford the product as a pale yellow solid (76 mg, 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 9.00 (d, ^3J = 4.0 Hz, 1H), 8.92 (d, ^3J = 4.0 Hz, 1H), 8.38 (d, ^3J = 7.8 Hz, 1H), 8.10 (d, ^3J = 7.8 Hz, 1H), 8.05 (s, 1H), 7.74 (s, 1H), 7.60 (m, 2H), 7.51 (m, 1H), 5.29 (br. s, 2H), 5.17 (s, 2H), 4.64 (m, 1H), 4.31 (m, 2H), 4.89 (s, 2H), 2.78 (s, 3H), 2.09 (m, 1H), 1.90 (m, 1H), 1.80-0.82 (m, 21H), 0.76 (m, 6H), 0.50 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 209.3, 175.2, 167.5, 165.7, 149.8, 149.6, 145.8, 145.2, 144.1, 136.4, 131.2, 130.5, 128.2, 124.7, 123.6, 123.1, 120.8, 104.0, 76.9, 56.4, 55.9, 52.5, 42.7, 41.8, 41.1, 40.3, 40.1, 35.6, 34.8, 34.5, 34.1, 32.9, 31.9, 31.6, 28.0, 27.0, 26.3, 26.1, 24.1, 23.0, 20.8, 18.1, 11.5. HRMS calc. for $C_{43}H_{54}N_7O_4ClH^+$:768.4004; found: 768.4016.

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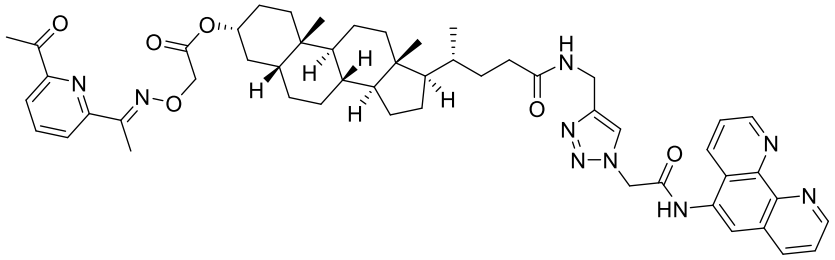

To a solution of mono-oxime **8** (13 mg, 0.050 mmol) in DMF (5 mL) at 0°C was added potassium carbonate (20 mg, 0.150 mmol). After warming to rt the mixture was stirred for 1h, before cooling to 0°C and adding a solution of **7** (38 mg, 0.050 mmol) in DMF (5 mL) dropwise. The reaction was warmed to rt and stirred for a further 16 h, before diluted with ethyl acetate (50 mL), washed with 5% LiCl $_{(aq)}$ (3 × 5 mL), dried over $MgSO_4$, filtered and the solvents removed *in vacuo*. The crude was purified by column chromatography (SiO_2, 0-15% gradient of MeOH in DCM) to afford the product as a pale yellow solid (11 mg, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 9.40 (s, 1H), 9.21 (d, ^3J = 4.0 Hz, 1H), 9.15 (d, ^3J = 4.0 Hz, 1H), 8.38 (d, ^3J = 7.7 Hz, 1H), 8.30 (d, ^3J = 7.7 Hz, 1H), 8.09 (d, ^3J = 8.0 Hz, 1H), 8.00 (d, ^3J = 8.0 Hz, 1H), 7.89 (s, 1H), 7.80 (t, ^3J = 7.8 Hz 1H), 7.65 (m, 2H), 6.33 (br. t, ^3J = 5.7 Hz, 1H, N*H*), 5.37 (s, 2H), 4.85 (m, 1H), 4.77 (s, 2H), 4.66 (d, ^3J = 5.7 Hz, 2H), 2.77 (s, 3H), 2.49 (s, 3H), 2.30-2.06 (m, 2H), 1.90-0.93 (m, 26 H), 0.91 (s, 3H), 0.86 (m, 3H), 0.57 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 200.9, 173.6, 166.8, 164.2, 157.0, 153.6, 152.5, 144.7, 137.0, 134.4, 129.8, 129.0, 128.2, 124.2, 121.4, 118.4, 71.5, 56.6, 55.8, 54.4, 47.3, 42.7, 41.9, 41.3, 40.7, 40.2, 35.8, 35.5, 34.9, 34.6, 33.3, 32.1, 31.5, 29.3, 28.2, 27.0, 26.5, 26.3, 25.7, 24.1, 23.7, 20.8, 18.3, 12.1, 10.2. HRMS calc. for $C_{56}H_{63}N_9O_6H^+$: 910.4966; found: 910.4960.

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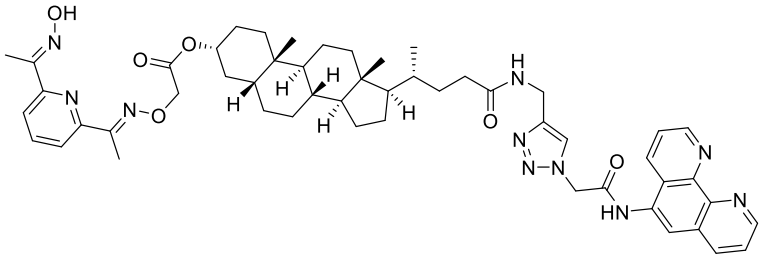

To a solution of **9** (8.0 mg, 0.0088 mmol) in 1:1 $CHCl_3$/ethanol (5 mL) was added a solution of hydroxylamine hydrochloride (0.7 mg, 0.011 mmol) and sodium acetate (0.9 mg, 0.011 mmol) in H_2O (0.5 mL) and the reaction heated at 60°C for 16 h. The reaction was diluted with $CHCl_3$ (10 mL), and washed with water (2 × 5 mL). The organic layer was dried over $MgSO_4$, filtered and the solvents removed *in vacuo* to yield the product as a pale yellow solid (7.5 mg, 93%). ^{1}H NMR (400 MHz, 1:1 $CDCl_3$/CD_3OD) δ (ppm): 9.14 (app s., 1H), 9.06 (app s., 1H), 8.58 (d, ^3J = 7.9 Hz, 1H), 8.30 (m, 1H), 8.19 (m, 1H), 7.93 (s, 1H), 7.86-7.76 (m, 3H), 7.72-7.60 (m, 2H), 4.79 (m, 1H), 4.73 (s, 2H), 4.48 (s, 2H), 4.21 (m, 2H), 2.40 (s, 3H), 2.31 (s, 3H), 2.30-1.60 (m, 11H), 1.55-0.82 (m, 26H), 0.59 (s, 3H). ^{13}C NMR (100 MHz, 1:1 $CDCl_3$/CD_3OD) δ (ppm): 175.5, 170.3, 168.3, 165.8, 157.8, 156.0, 153.8, 152.7, 149.8, 149.6, 145.9, 145.0, 144.2, 136.4, 132.2, 131.4, 131.0, 128.8, 128.3, 124.7, 123.8, 123.1, 120.2, 120.0, 75.5, 71.1, 68.1, 56.3, 56.1, 42.5, 41.9, 40.4, 40.0, 38.8, 35.7, 35.5, 34.8, 34.5, 32.9, 32.1, 31.6, 29.5, 28.1, 26.7, 26.5, 26.3, 24.1, 23.6, 22.8, 20.6, 17.9, 13.5. HRMS calc. for $C_{52}H_{64}N_{10}O_6H^+$: 925.5089; found: 925.5087.

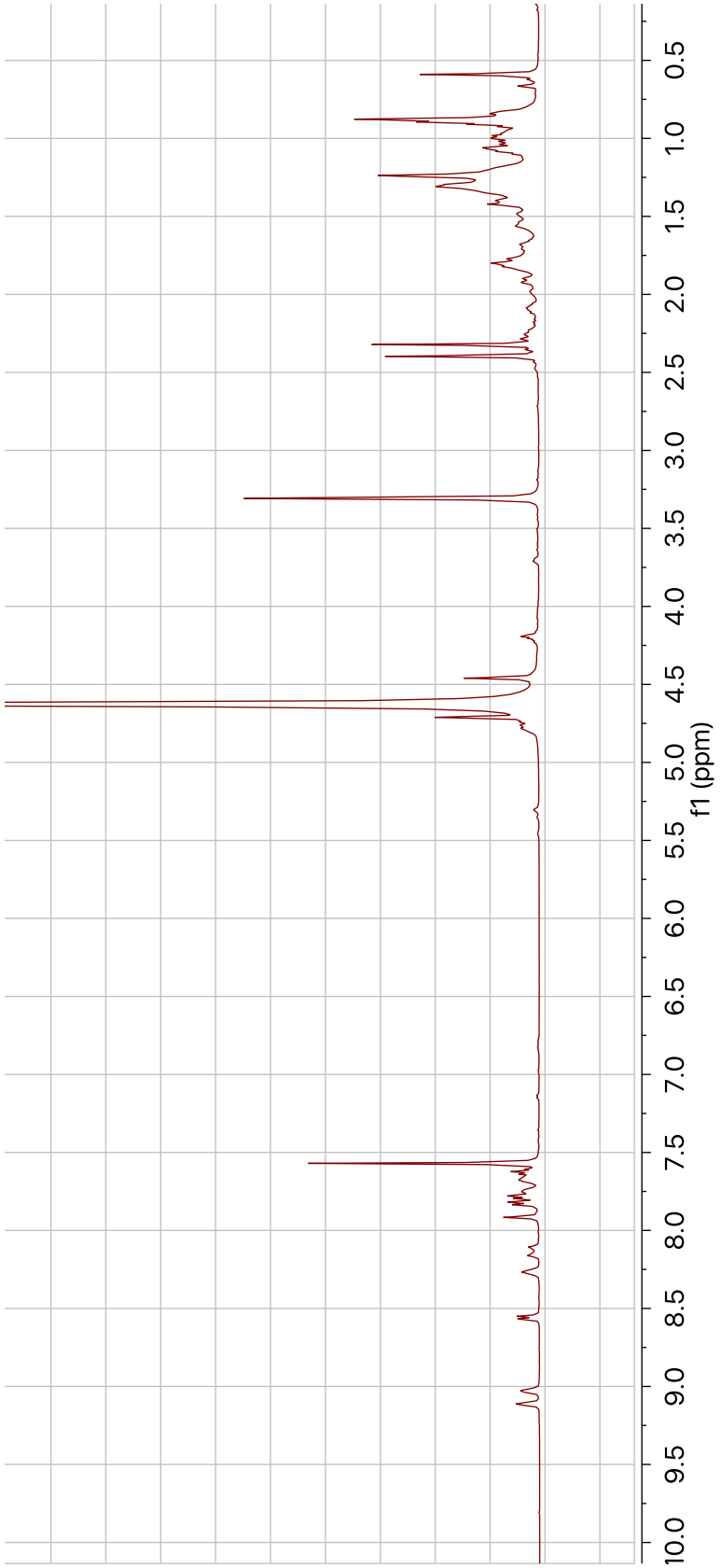

Figure S1. ^{1}H NMR spectrum of **1** (400 MHz, 1:1 $CDCl_3/CD_3OD$, 298 K)

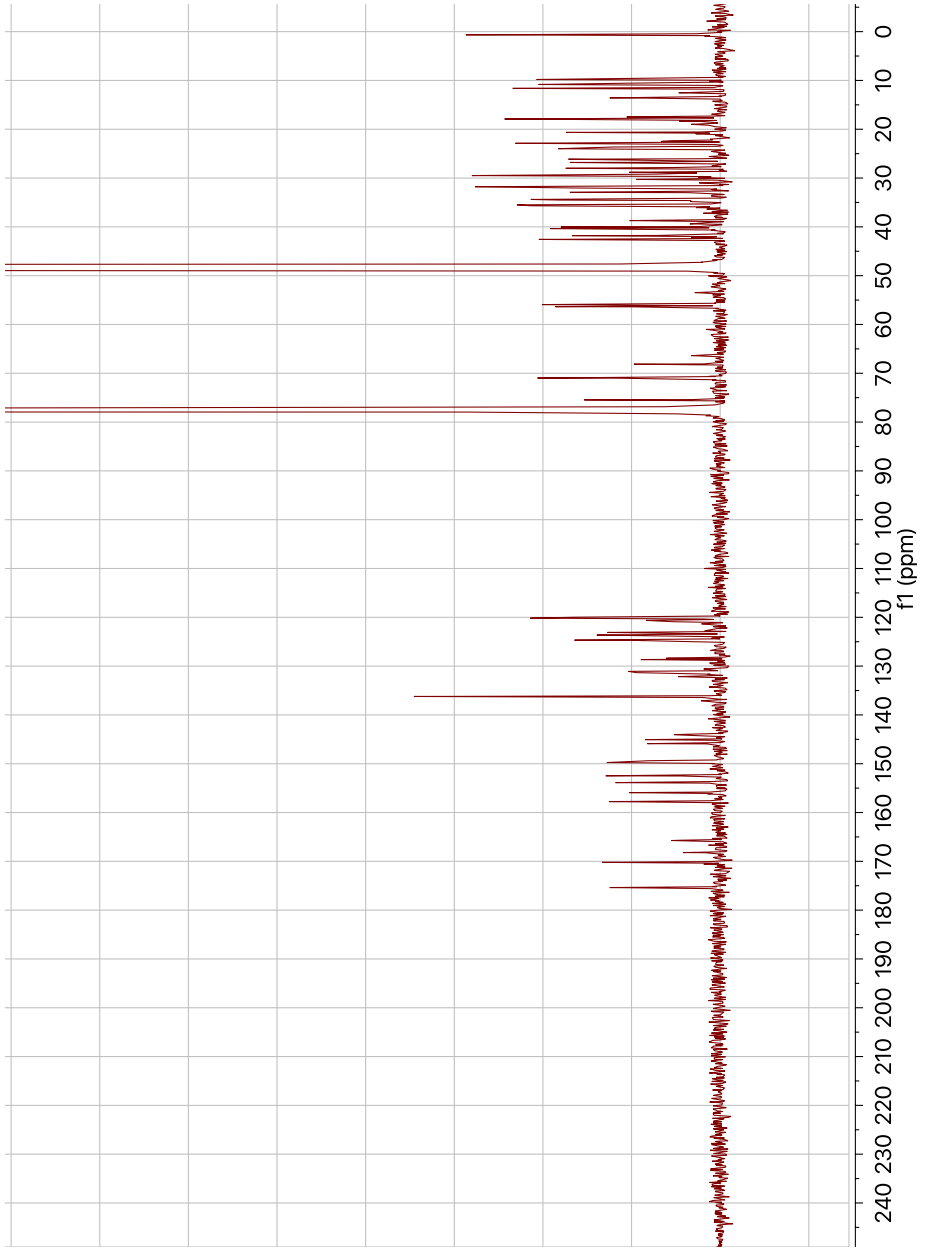

Figure S2. ^{13}C NMR spectrum of **1** (100 MHz, 1:1 $CDCl_3/CD_3OD$, 298 K)

2. Additional data

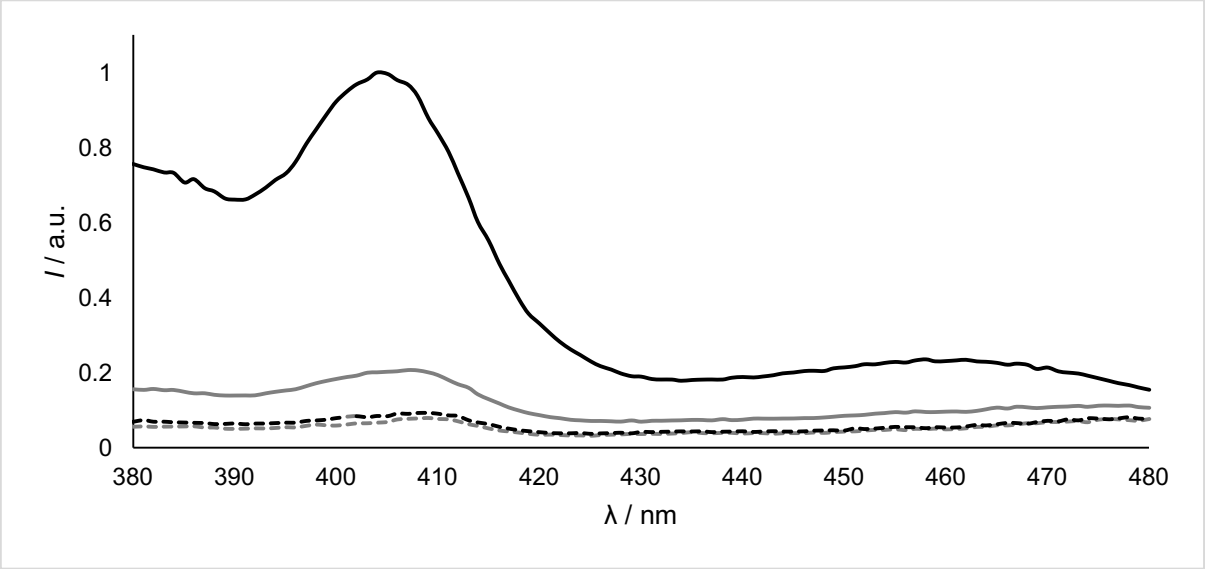

Figure S1. Normalised fluorescence excitation spectrum of vesicles composed of DOPC/DOPE lipids + 1% 1·Cu, in the presence and absence of encapsulate substrate 2. λ_{em} = 510 nm. Solid lines: vesicles composed of lipids with 1 mol% **1·Cu** containing 250 μM **2**, before (grey) and after (60 mins, black) addition of 1 eq. EDTA to the vesicle suspension. Dashed lines: vesicles composed of lipids with 1 mol% **1·Cu** in the absence of **2**, before (grey) and after (black) addition of 1 eq. EDTA.

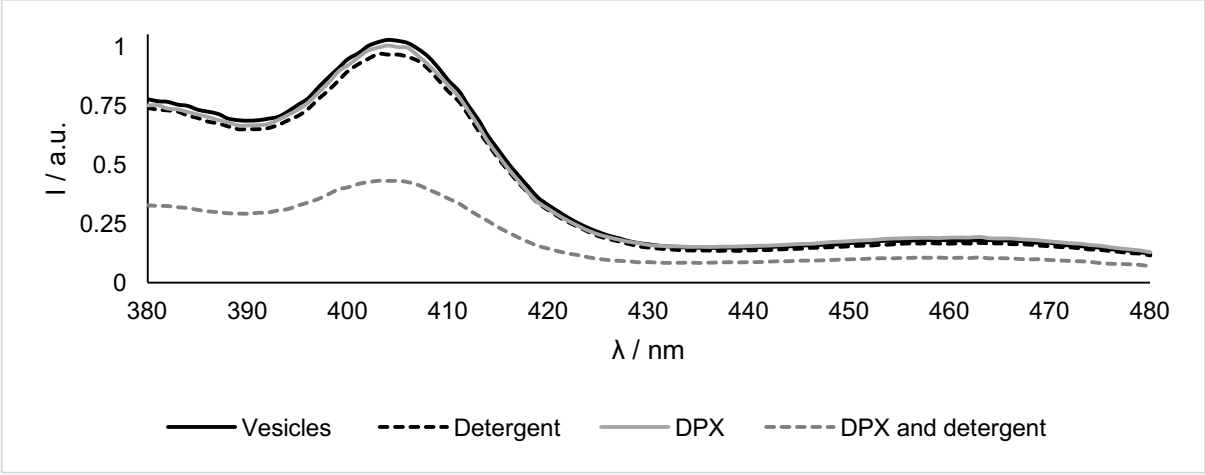

Figure S2. Vesicle integrity determination experiment using *p*-xylene-bis-pyridinium bromide (DPX) quencher. Normalised fluorescence excitation spectrum (emission at 510 nm) of vesicles containing 1 mol% **1·Cu** and encapsulated **3** (250 μM), generated by the hydrolysis of ester **2** in a signalling experiment. Spectra were recorded in the presence and absence of external DPX (2.5 mM), and following detergent addition (Triton X-100, 1 mM), and demonstrate that the substrate ester remains encapsulated inside the vesicles during the experiment.

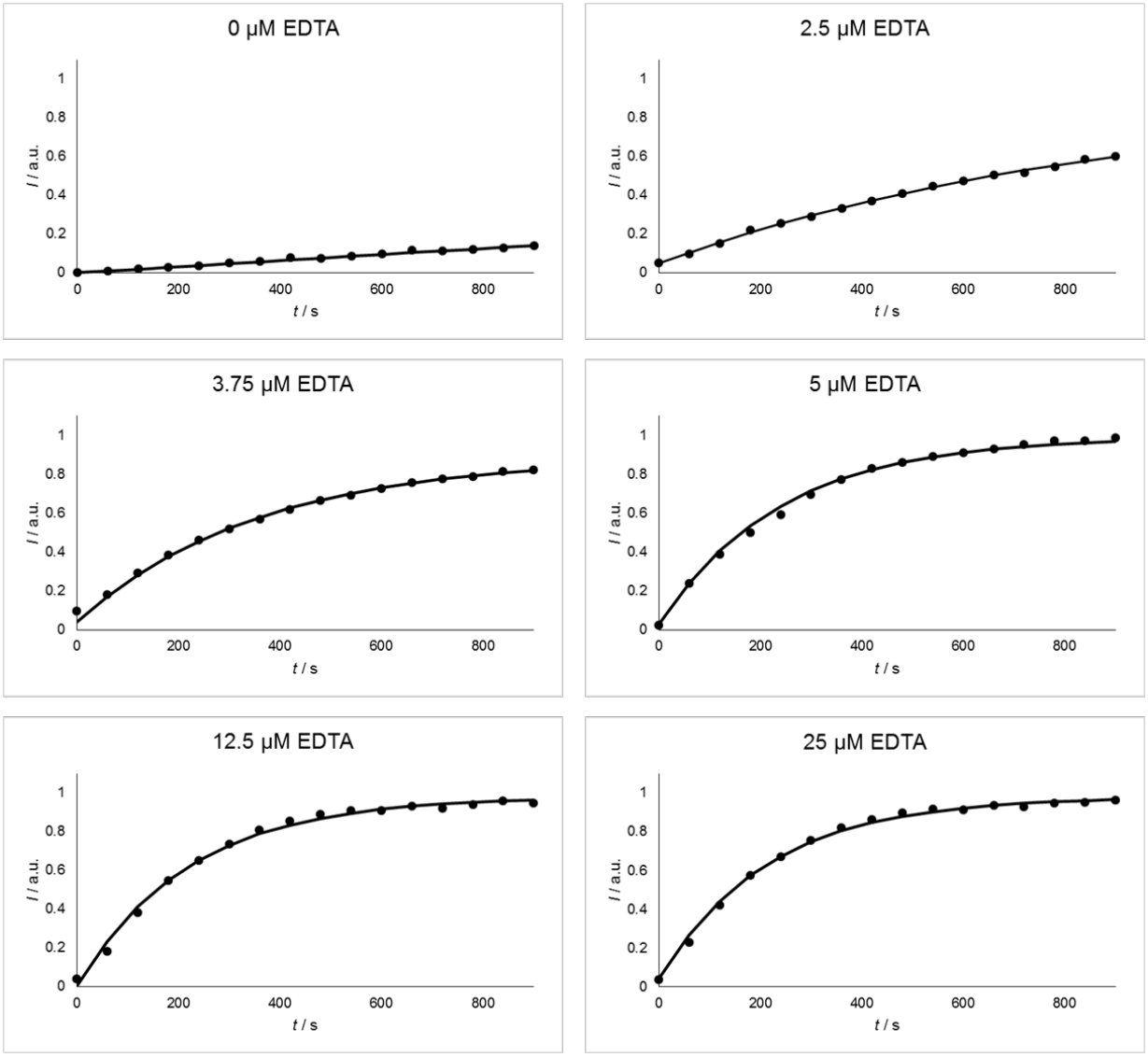

Figure S3. EDTA dependence of the observed rate constant. Fluorescence emission intensity at 510 nm, exciting at 415 nm (points), fitted to a pseudo-first order rate equation (black line). Experiment conducted with 200 nm DOPC/DOPE vesicles (1 mM lipids, 1 mol% **1·Cu**) containing 100 mM HEPES buffer at pH 7, 250 μM $ZnCl_2$ and 250 μM substrate **2**. Aliquots of EDTA solution were added to the vesicle suspension to reach the stated concentration.

Table S1: Dynamic Light Scattering (DLS).

Sample	*d* / nm	PdI
Vesicles	230.3	0.31
Vesicles + 5 μM EDTA	233.6	0.33

Average vesicles dimensions determined by DLS and the corresponding poly-dispersity index. Data averaged over 3 measurements, for DOPC/DOPE vesicles prepared by passing through 200 nm membranes as described above, and recorded in the presence and absence of 5 μM EDTA at 298 K.

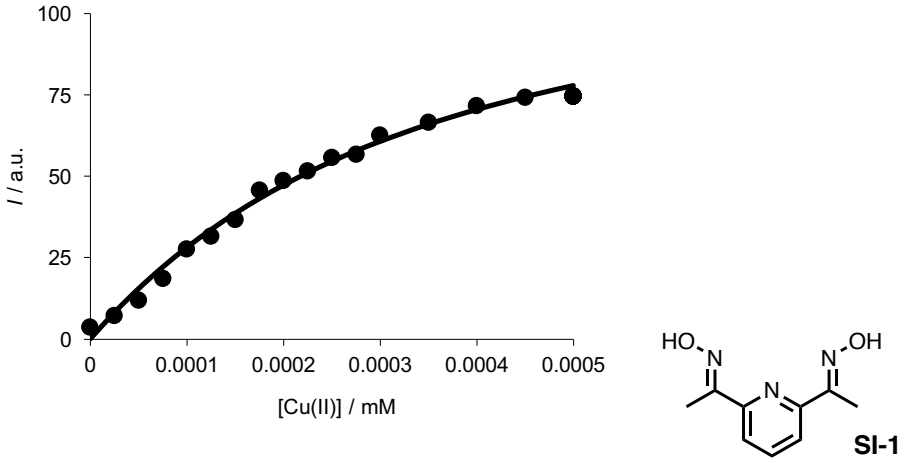

Figure S4. Emission intensity as a function of Cu(II) concentration for model compound SI-1. Fluorescence Cu(II) binding titration with bis-oxime **SI-1** (10^{-7} M) in water. Solid points: emission at 340 nm ($\lambda_{excitation}$ = 230 nm). $CuCl_2$ added as a 10 μM aqueous solution to a 0.1 μM aqueous solution of **SI-1**. T = 298 K.

Data fitted to a 1:1 binding isotherm (solid line), to obtain log K = 6.8 ± 0.4 (average of two titrations). For comparison, the literature value for the related bis-amido oxime is log K_1 = 6.6.[4] Compound **SI-1** was prepared according to literature procedures.[5]

References

(1) MacDonald, R. C.; MacDonald, R. I.; Menco, B. P.; Takeshita, K.; Subbarao, N. K.; Hu, L. R. *Biochim. Biophys. Acta* **1991**, *1061*, 297
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