Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Photostable Polymorphic Organic Cages for Targeted Live Cell Imaging

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1. Experimental Section

Materials. All reagents and solvents were purchased from commercial sources and used without further purification. ^{1}H-NMR spectra were performed at 298 K and 400 MHz with $CDCl_3$ solutions at 5mg/ml concentration with an Avance III Bruker Corporation instrument. The electrospray mass spectra were obtained using Bruker microOTOF-Q and apex ultra FT-ICR instruments. UV and fluorescence spectra were recorded on SHIMADZU UV-2600 UV-Vis spectrophotometer and CARY Eclipse fluorescence spectrophotometer. The solution were prepared for UV and fluorescence measurements by 1 h sonification of organic cages in DMSO followed by filtration. MCF-7 cells (ATCC HTB-22), Hela cells (ATCC CCL-2), HEK-293 cells (ATCC CRL-1573), and HDF cells (ATCC PCS-201-010) were purchased from ATCC. MTT cell viability kit was purchased from Bio vision incorporated. Cell viability was measured using a Bio-Rad xMark Microplate Absorbance Spectrophotometer. Confocal laser scanning microscopy (CLSM) cell imaging experiments were performed on a Leica TCS SP8 MP confocal fluorescence microscope.

Cell culture. MCF-7 cells (ATCC HTB-22) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/streptomycin (PS) at 37 °C under 5% CO_2. MCF-7 cells were then plated on MatTek's 35mm glass bottom dishes at 1×10^5 cells/dish and incubated at 37 °C under 5% CO_2 overnight before performing experiments.

Cytotoxicity Studies. MCF-7 cells were plated at 2×10^4 cells/well in plastic 96 well plates. After 24 h, the media was removed, and cells were rinsed once with FBS-free DMEM. Cells were incubated again in FBS free DMEM containing either **OC1**, **OC2**, or **OC3** for 24 h at 37 ºC in a 0.5% DMSO vehicle. After incubation, the media was removed and the cells were incubated for 3 h with MTT Reagent at 37 °C. After incubation, cells were treated with MTT Solvent for 15 min. at room temperature. Absorbance was measured at 590 nm.

CLSM imaging and colocalization studies. Fluorescence imaging was performed on Leica SP8 confocal microscope. Imaging was done using FluoroBrite DMEM (Gibco).

MCF-7 cells were plated in glass bottom dishes containing 2 ml of DMEM enriched with 10% FBS and 1% PS. Adherent cells are washed 24 h post incubation with FBS free DMEM and treated with either **OC1**, **OC2**, or **OC3** for over 3h then rinsed 2x with FBS free media followed by treatment with 150 nM MitoTracker Red (Invitrogen), or 60 nM LysoTracker Red (Invitrogen) for 15 minutes at 37 ºC. After this incubation, the media was aspirated, and cells were rinsed 2X with FluoroBrite and imaged on Leica Sp8.

Mechanism of Uptake Studies. MCF-7 cells were plated on glass-bottom imaging dishes and 6-well plate in 2.00 mL 10% FBS 1% PS DMEM. After 24 h, dishes were rinsed 2X with FBS-free DMEM and treated with either 1) 1 μg/mL chlorpromazine (clathrin-mediated endocytosis inhibitor), 2) 1 μg/mL filipin (FIL) (caveolae-mediated endocytosis inhibitor), 3) 40 μM amiloride (macropinocytosis inhibitor), or all three together for 30 minutes. After treatment, dishes were rinsed 2X with FBS-free DMEM and incubated with **OC1** for 1 h. Media was removed and dishes were rinsed 2X in FluoroBrite and imaged and quantified on FACS.

Temperature-Dependent Uptake Studies. MCF-7 cells were plated in glass-bottom dishes containing 2.00 mL of 10% FBS 1% PS DMEM. The next day, adherent cells were washed 2X with FBS-free DMEM. Cells were then incubated in 2 mL FBS-free DMEM containing **OC1** in either 37 ºC, 27 ºC, or 4 ºC for 3 h. The media was removed, and cells were rinsed 2X in FluoroBrite and imaged. Fluorescence was quantified on FACS.

Live Cell Imaging. MCF-7 cells were plated in glass bottom dishes containing 2 ml of DMEM enriched with 10% FBS and 1% PS. Adherent cells are washed 24 h post incubation with FBS free DMEM and treated with **OC1** and 150 nM MitoTracker Red (Invitrogen), and imaged at 37 ºC under 5% CO_2 on Leica Sp8.

Fixed cell imaging. MCF-7 cells were plated in glass bottom dishes containing 2 ml of DMEM enriched with 10% FBS and 1% PS. Adherent cells are washed 24 h post incubation with FBS free DMEM and treated with150 nM MitoTracker Red (Invitrogen)and washed after 15 mins, cells are then fixed using 4% paraformaldehyde in PBS. Cells are treated with **OC1** post fixation and imaged on Leica Sp8.

Synthesis of OC1.

1,3,5-triformylphloroglucinol has been prepared according to literature reports.[1,2] To 30 ml trifluoroacetic acid (TFA) was added hexamethylene tetraamine (5.032 g) and dried phloroglucinol (2.004 g). The solution was heated at 100 ºC for 6 h. Approximately 50 mL of 3 M HCl was added and the solution was heated at 100 ºC for 3 h. After cooling to room temperature, the solution was extracted with ca. 350 mL dichloromethane, dried over sodium sulfate, and filtered. Rotary evaporation of the solution afforded light yellow powder. Finally, washing with ethanol afforded the pure product.

42 mg (0.2 mmol) of 1,3,5-triformylglucinol and 34 mg (0.3mmol) of (1R, 2R)-1,2-diaminocyclohexane were refluxed for 48 h in 50 mL of chloroform. The mixture was cooled down, filtered and evaporated. The pure product **OC1** recrystallized from DMSO/chloroform with 48% yield. ^{1}H NMR (400MHz NMR, $CDCl_3$) δ 10.47-10.53 (dd, 6H, NH); 7.64-7.67 (d, 6H, C=CH-NH); 2.90 (m, 6H, cyclohexane H_c); 2.27 (m, 6H, cyclohexane H_d); 1.91 (m, 6H, cyclohexane $H_{d'}$); 1.54 (m, 6H, cyclohexane He), 1.42 (m, 6H, cyclohexane He'); ^{13}C NMR (100 MHz, $CDCl_3$): δ 184.80 (C=O), 156.24 (C=CH-NH), 107.0 (C=CH), 68.0 (cyclohexane C_c), 31.62 (cyclohexane C_d), 24.75(cyclohexane C_e). TOF MS m/z: $[M+H]^+$ Calcd for $C_{36}H_{43}N_6O_6$ 655.3239; Found 655.3394.

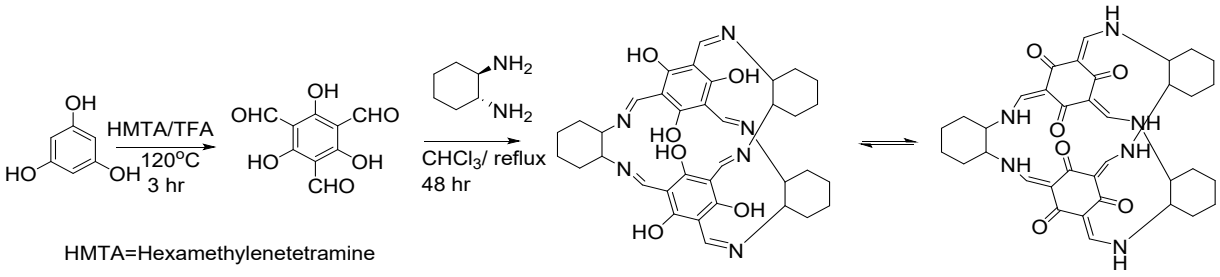


Figure S1. General synthetic scheme of **OC1** starting from 2,4,6-trihydroxy benzene.

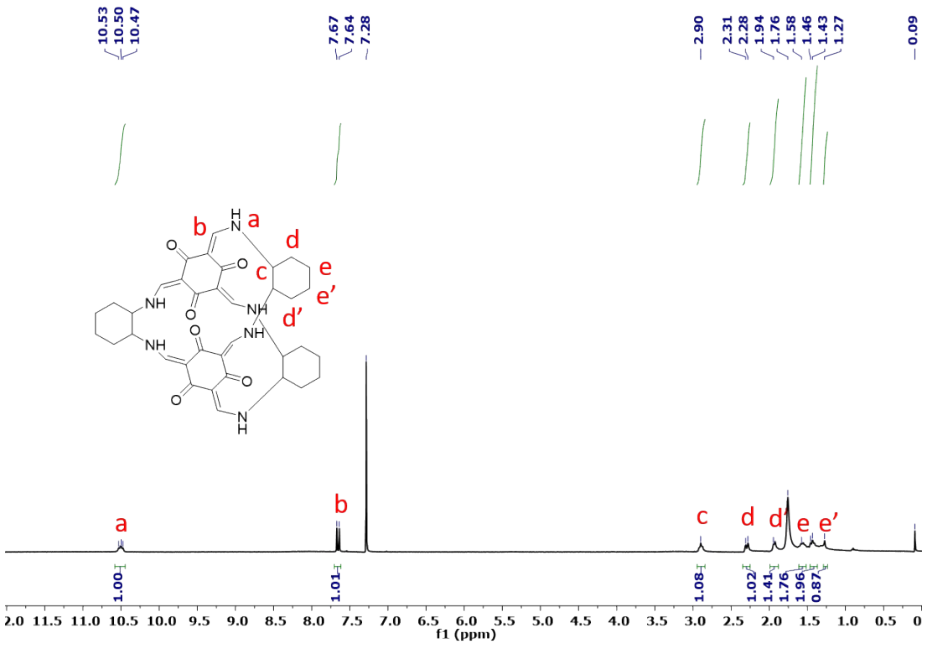


Figure S2. ^{1}H-NMR of **OC1** (400MHz NMR, $CDCl_3$).

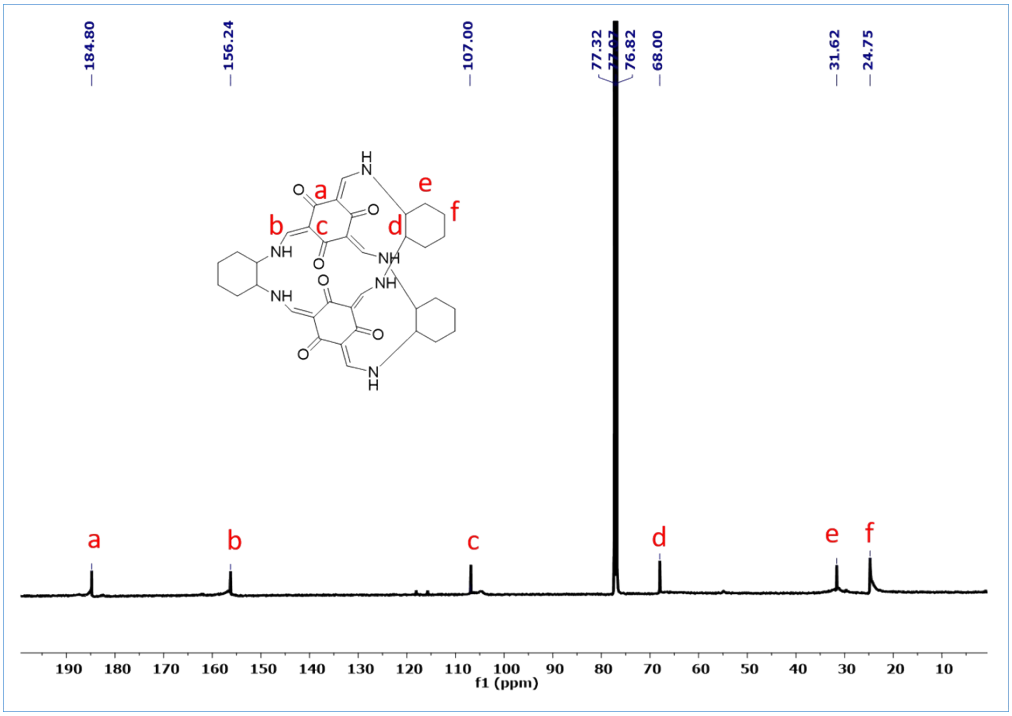


Figure S3. ^{13}C NMR of **OC1** (100 MHz NMR, $CDCl_3$).

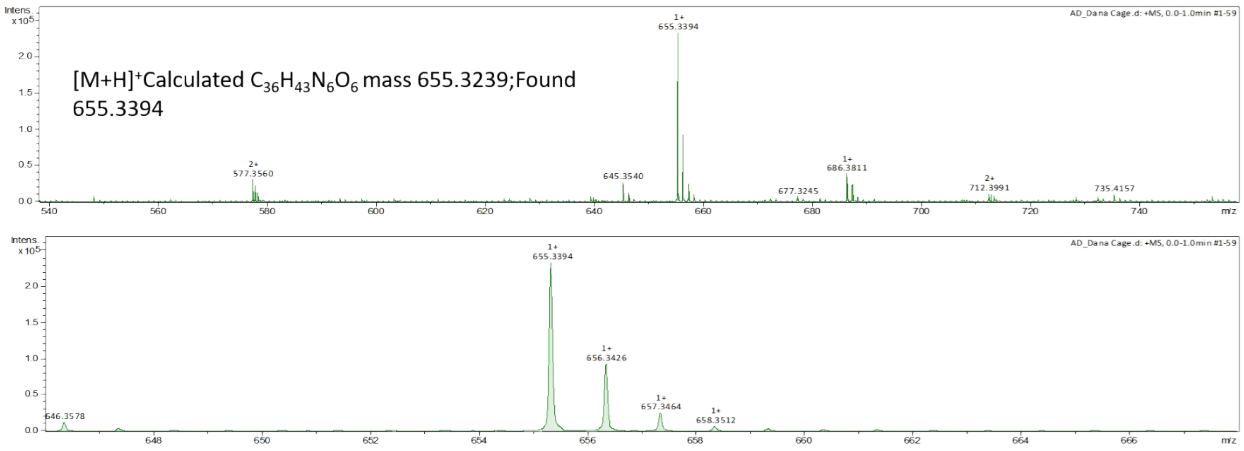


Figure S4. TOF-Mass of **OC1**.

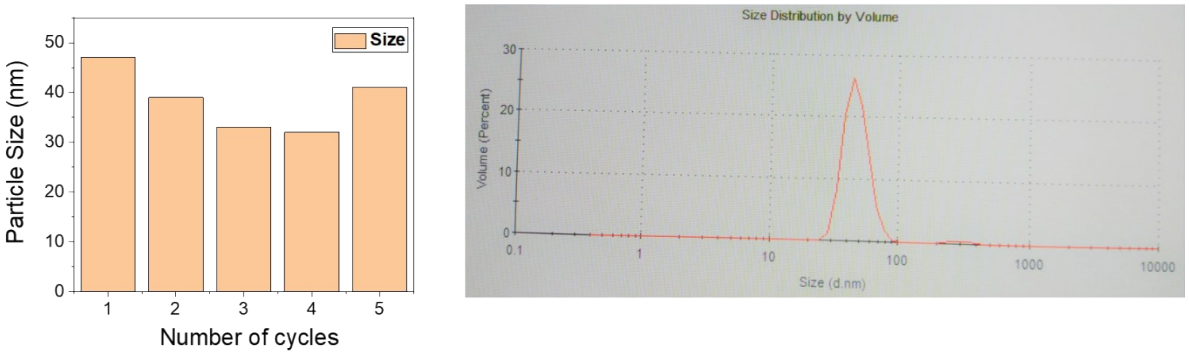


Figure S5. Particle size of **OC1** in DMSO-water.

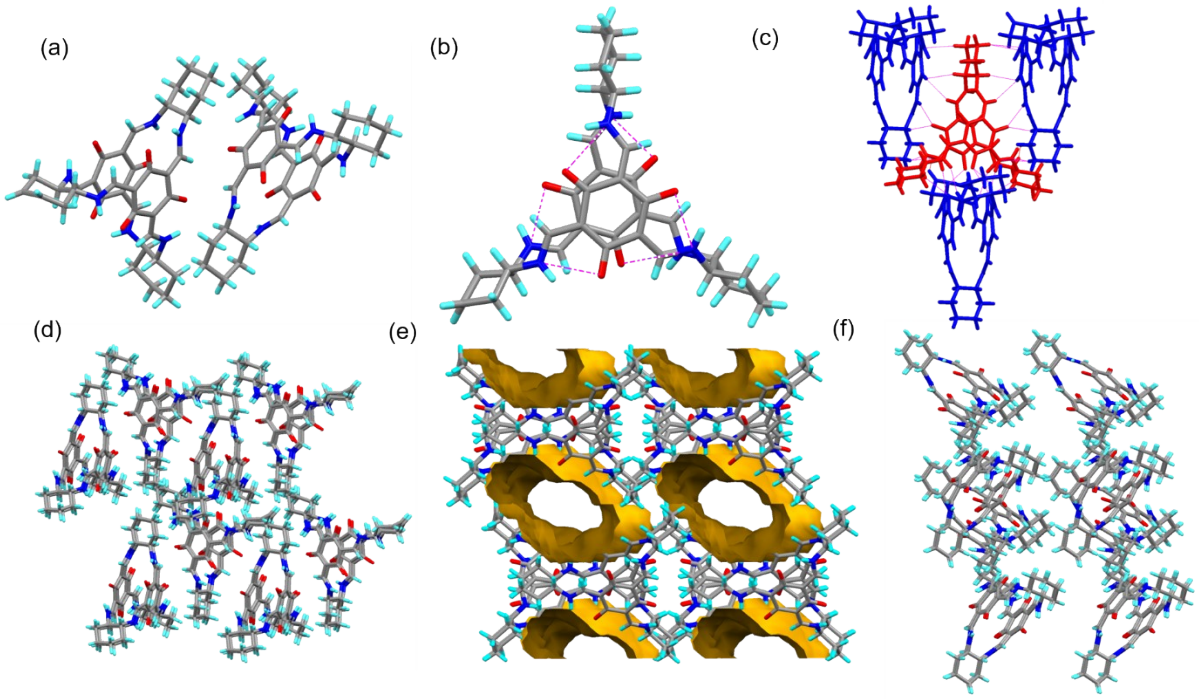


Figure S6. (a) Asymmetric unit of **OC1**. (b, c) Single crystal structure of **OC1**, which suggests keto-enol tautomeric form, is stabilized by hydrogen bonding. (d) Packing of **OC1** along *a*-axis. (e) Packing of **OC1** along *c*-axis. (f) Packing of **OC1** along *b*-axis.

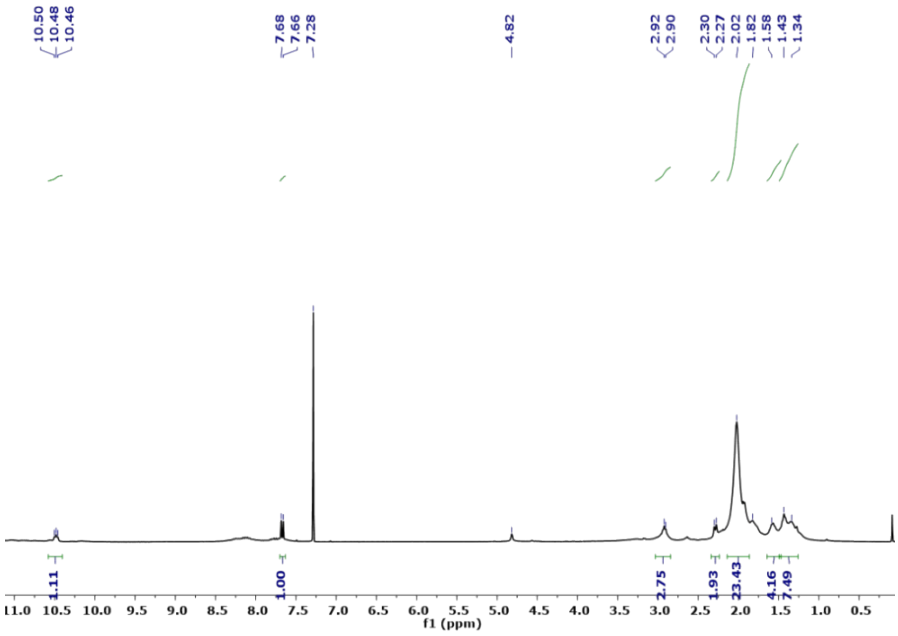


Figure S7. ^{1}H NMR of **OC1** (400MHz NMR, $CDCl_3$) indicating the stability of the cage in water.

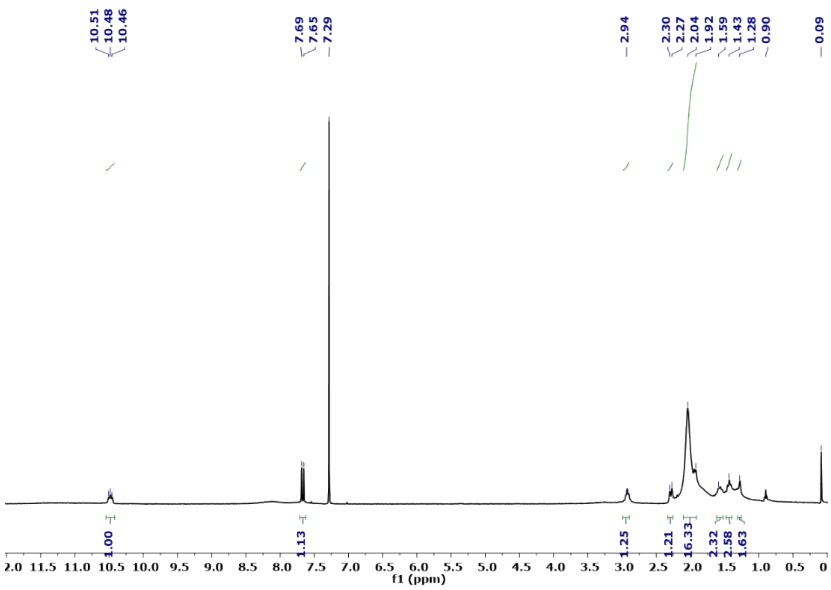


Figure S8. ^{1}H NMR of **OC1** (400MHz NMR, $CDCl_3$) indicating the stability of the cage in bovine serum-free Dulbecco's modified eagle medium (DMEM).

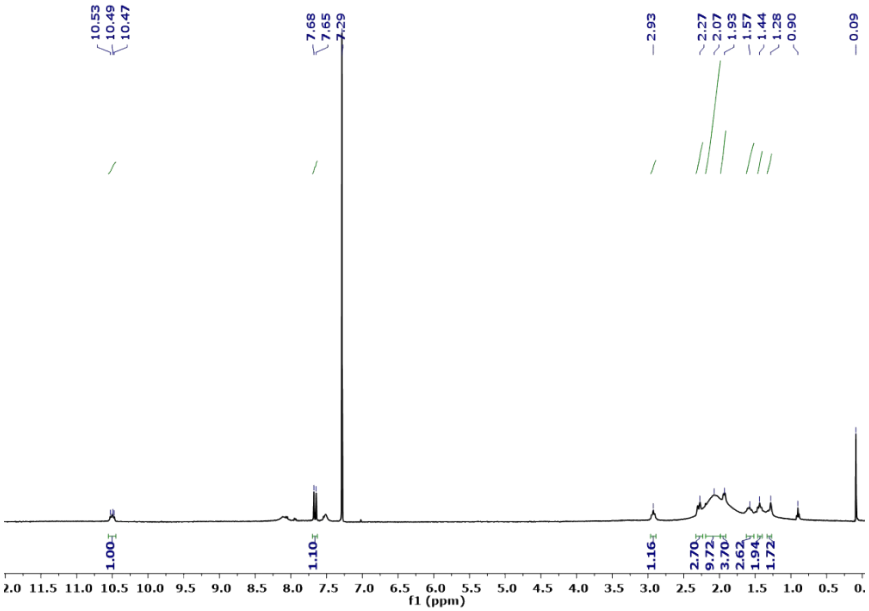


Figure S9. ^{1}H NMR of **OC1** (400MHz NMR, $CDCl_3$) indicating the stability of the imine cage in aqueous solution at pH-4.

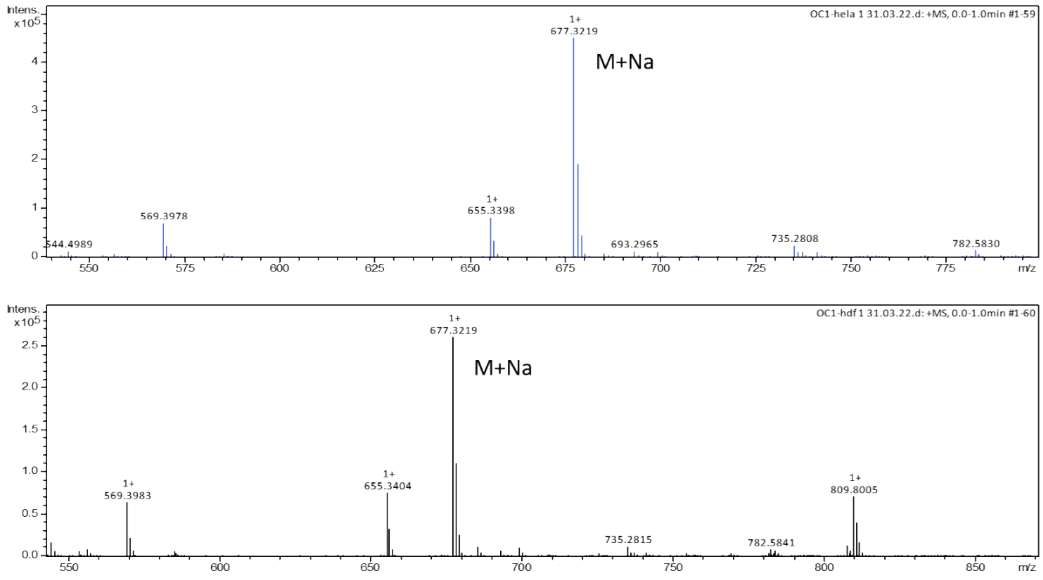


Figure S10. TOF-Mass of **OC1** indicating the stability of the cage inside the cell.

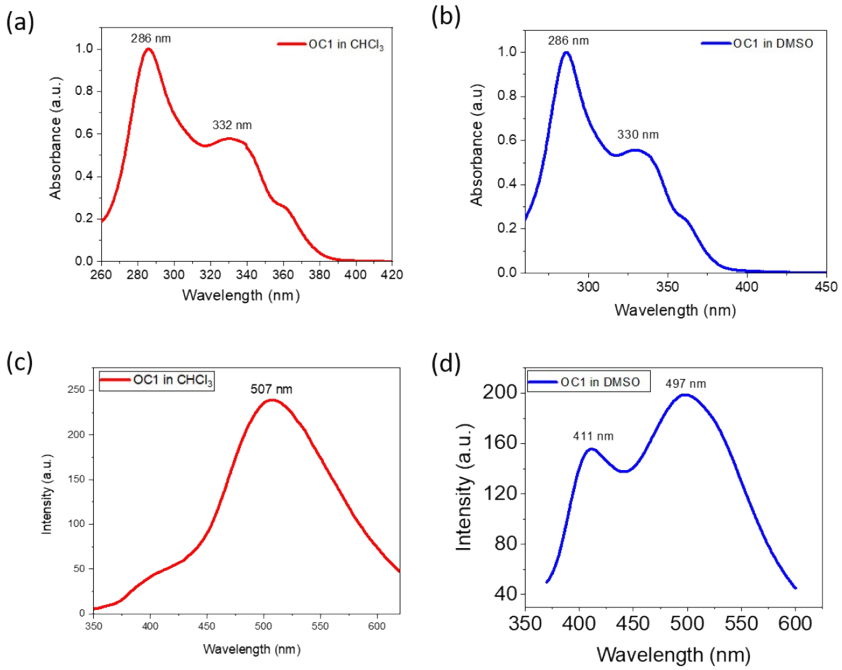


Figure S11. UV−visible absorption (a,b) and emission spectra (c,d) of **OC1** in chloroform and DMSO (without correction).

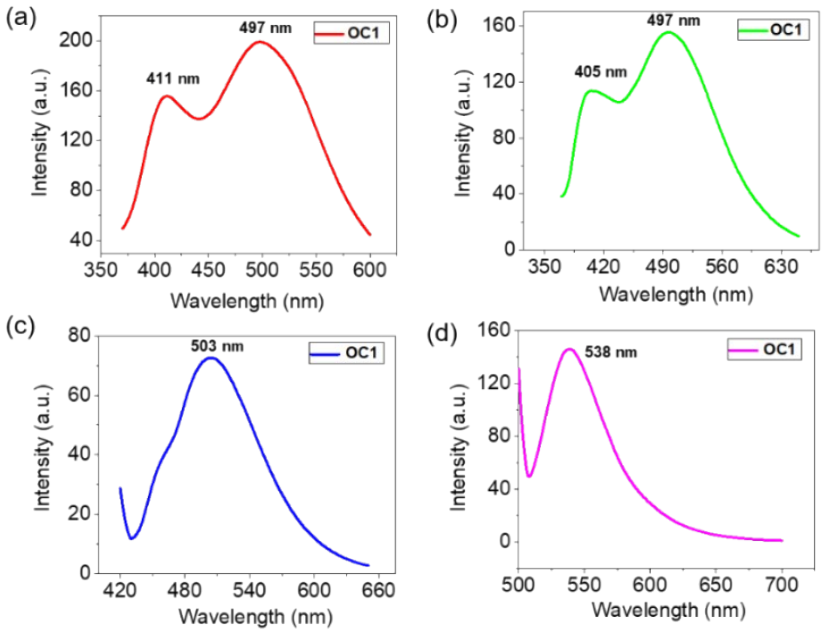


Figure S12. Fluorescence emission spectra of **OC1** in DMSO upon excitation at (a) 330 nm, (b) 350 nm, (c) 400 nm, (d) 480 nm (without correction).

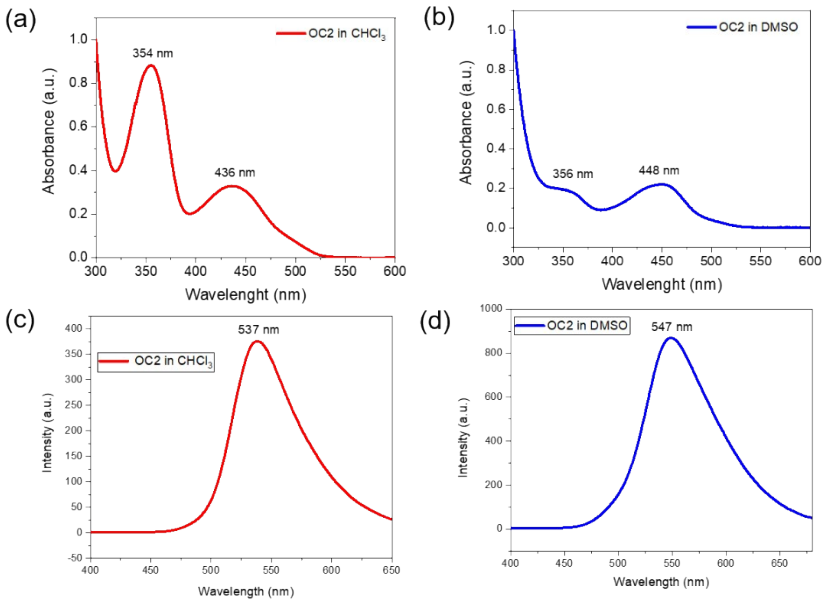


Figure S13. UV−visible absorption (a,b) and emission spectra of **OC2** in $CHCl_3$ and DMSO (without correction).

Quantum yield and Molar Extinction Coefficient of OC1, OC2 and OC3

Cages are not soluble in water. The molar extinction coefficient of organic Cage **OC1** in organic solvents was estimated to be: 41111 M^{-1} cm^{-1} in $CHCl_3$ and 46806 M-1 cm^{-1} in DMSO. Fluorescence quantum yield of Organic Cage **OC1** and **OC2** in organic solvents was measured with comparative method using Coumarin 1 dye in ethanol as a reference for OC1 (Φ = 73%) and DCM (4-(dicyanomethylene)-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran in $CHCl_3$ (Φ = 35%) as a reference for **OC2**. Fluorescence quantum yield of **OC1** was determined to be Φ = 0.2% in DMSO and Φ = 0.4% in $CHCl_3$. However, large difference in fluorescence quantum yields of reference and studied system may introduce a minor error. Due to a good overlap of both the absorption and fluorescence spectra of **OC2** and **OC1**, fluorescence quantum yield of **OC1** can also be determined using **OC2** as a reference (the fluorescence quantum yield of **OC2** was determined to be Φ = 11.7% in $CHCl_3$ and Φ = 6.1% in DMSO). Since **OC2** revealed unexpected excitation dependent fluorescence quantum yield, we added this effect in our calculation. The calculated fluorescence quantum yield of **OC1** in DMSO (Φ = 0.25%) is in good agreement with the value obtained with Coumarin 1 as reference. Fluorescence quantum yield of **OC3** was estimated by comparative method using Coumarin 1 dye in ethanol as a reference, giving the value of Φ=0.5%. Molar extinction coefficient of **OC3** in $CHCl_3$ at peak 320 nm was estimated to be 4339 M^{-1} cm^{-1}.

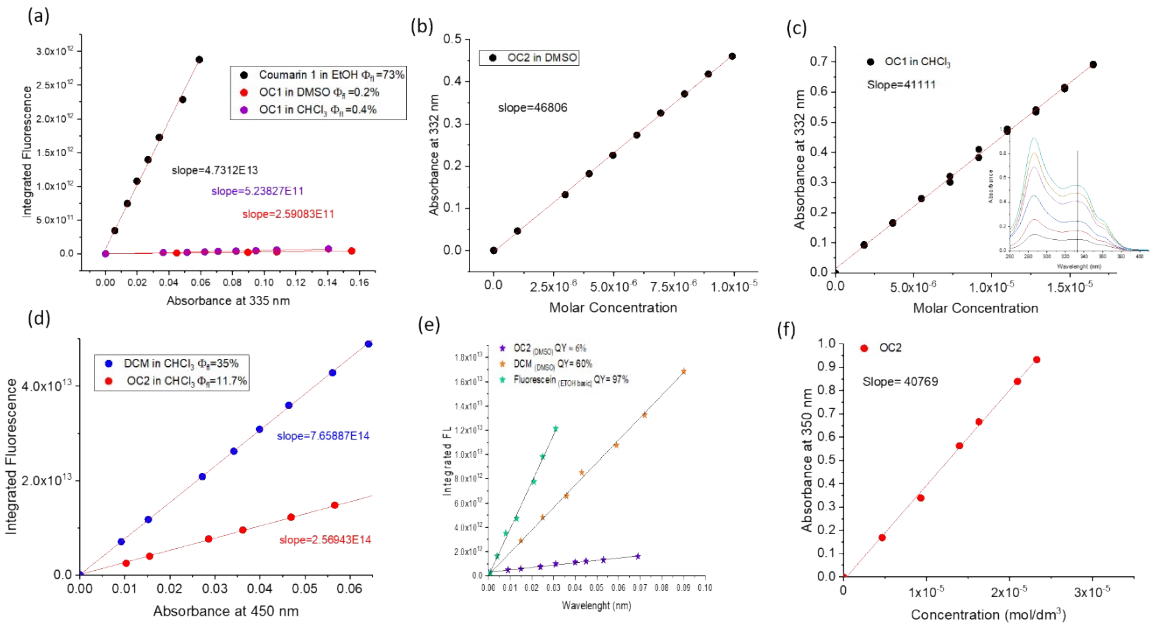


Figure S14. (a-c) Fluorescence quantum yield and molar extinction coefficient of **OC1**. (d-f) Fluorescence quantum yield and molar extinction coefficient of **OC2**.

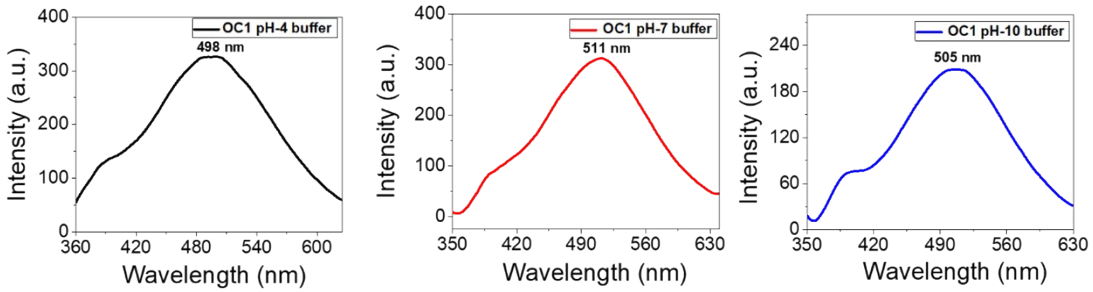


Figure S15. Fluorescence spectra of **OC1** at different pH showing emissive characteristic (without correction).

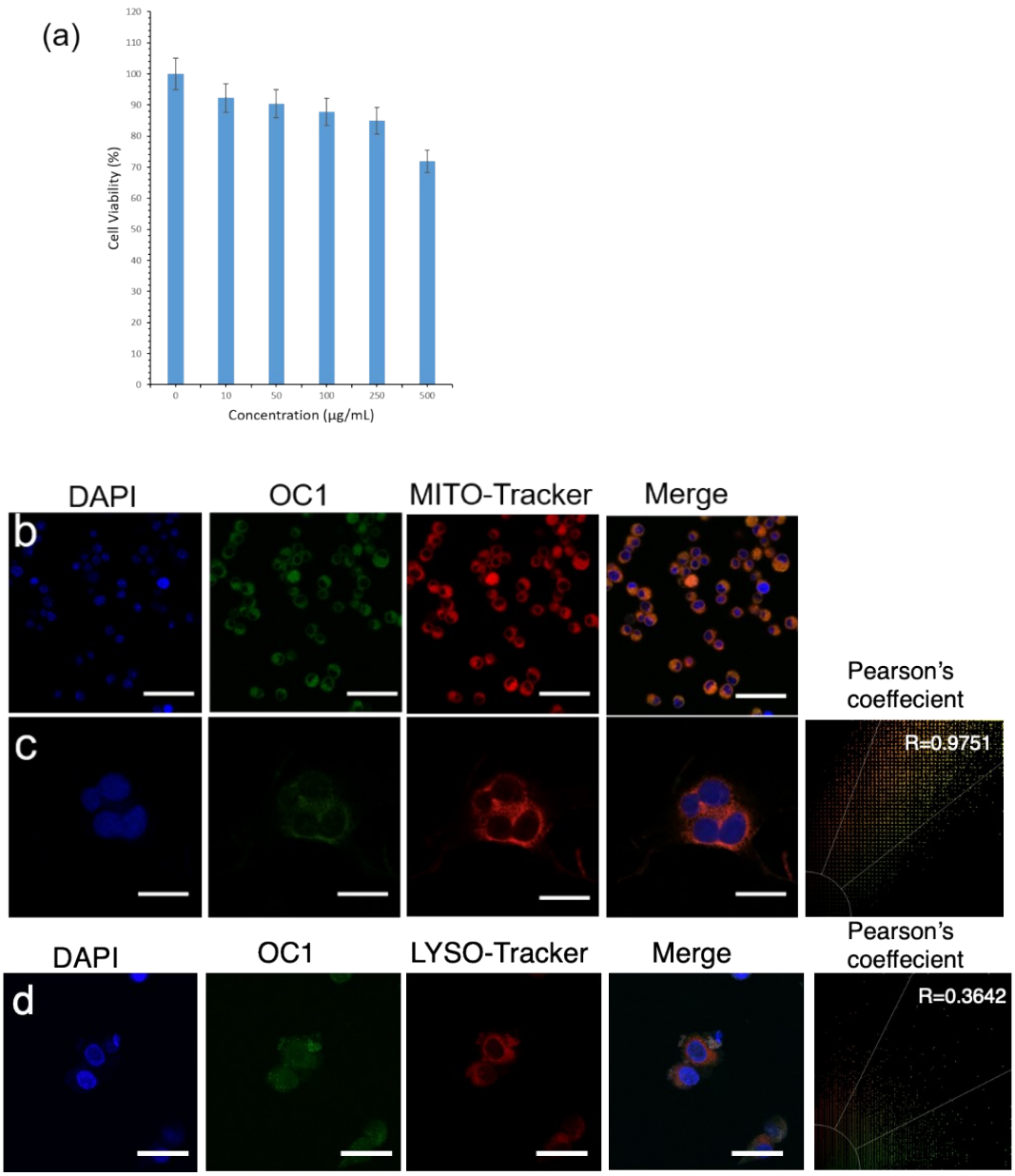
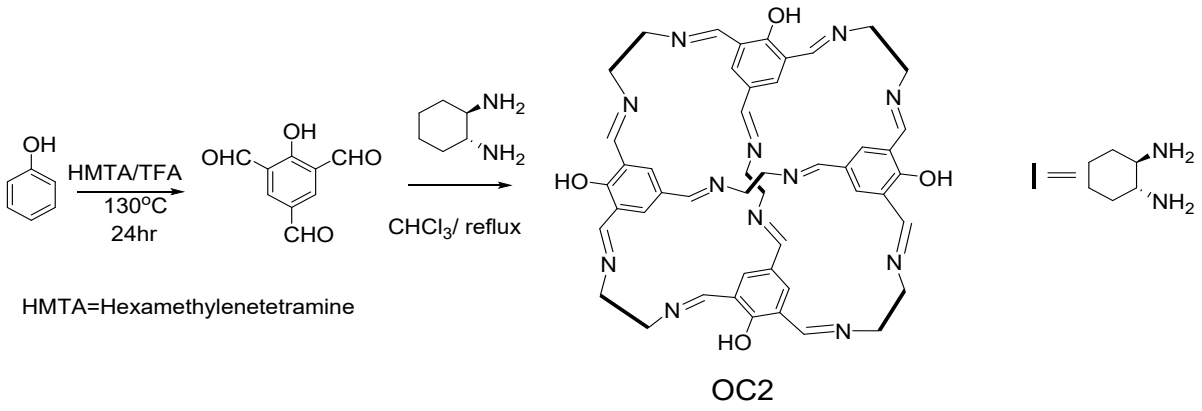


Figure S16. (a) Cytotoxicity studies of **OC1** in MCF-7 cells over 24 h using MTT assay. (b) CLSM colocalization of **OC1** (abs/em ~495/519nm) with commercial probes in Live MCF-7 cells. (Scale bar at 30 µm) (c) Colocalization of **OC1** with the commercial probe MitoTracker RedFM (abs/em ~581/644 nm) in MCF-7 cells with a Pearson's correlation coefficient of 0.9751. (Scale bar at 30 µm). (d) Colocalization of **OC1** with the commercial probe LysoTracker Red (abs/em ~577/590 nm) in MCF-7 cells with a Pearson's correlation coefficient of 0.3642. (Scale bar at 30 µm).

Synthesis of OC2.

2-hydroxy-1,3,5-triformylbenzene was prepared from phenol and hexamethylenetatraamine (HMTA).[3] Phenol (5.93 g) and HMTA (17.2 g) were placed in a round-bottomed flask, and 60 mL of trifluoroacetic acid was added. The resulting mixture was stirred at 130 °C for about 24 h. Then the reaction mixture was then heated to 150 °C and stirred for 3 h. The mixture was cooled to 100 °C, treated with 100 mL of 4M HCl, heated at 105 °C for 30 min and cooled to room temperature overnight. The precipitated yellowish solid was filtered off, washed with water, ethanol, and dichloromethane and dried in air.

Trans-(1R,2R)-diaminocyclohexane (0.171 g, 1.5 mmol) and 2-hydroxy-1,3,5-triformylbenzene (0.178 g, 1 mmol) were dissolved in 50 ml of chloroform and refluxed for 72 h. The solvent was evaporated under vacuum and the solid product was obtained quantitatively. The crystalline solid **OC2** was obtained from a DMSO/chloroform solution in 40% yield. ^{1}H NMR of **OC2** (400 MHz, $CDCl_3$). δ 14.69-14.55 (d, 4H), 8.65 – 8.62 (m, 4H), 8.35 – 8.33 (m, 4H), 8.12 (d, 4H), 7.99 (d, 8H), 3.43-8.33 (d, , 12H), 1.84 (m, 12H), 1.86-1.74 (s, 24H), 1.48 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.75 (C=N), 160.35 (C-OH), 155.41 (C-C=N,), 132.24 (C-C), 126.18- 123.40 (C-C=N),), 119.18 (m), 74.30 (cyclohexane C), 33.12 (cyclohexane C), 24.26 (cyclohexane C); TOF-MS Calcd for $C_{72}H_{85}N_{12}O_4$ $[M+H]^+$: 1181.6817; Found 1181.6695.



OC2

Figure S17. General synthetic scheme of **OC2** starting from phenol.

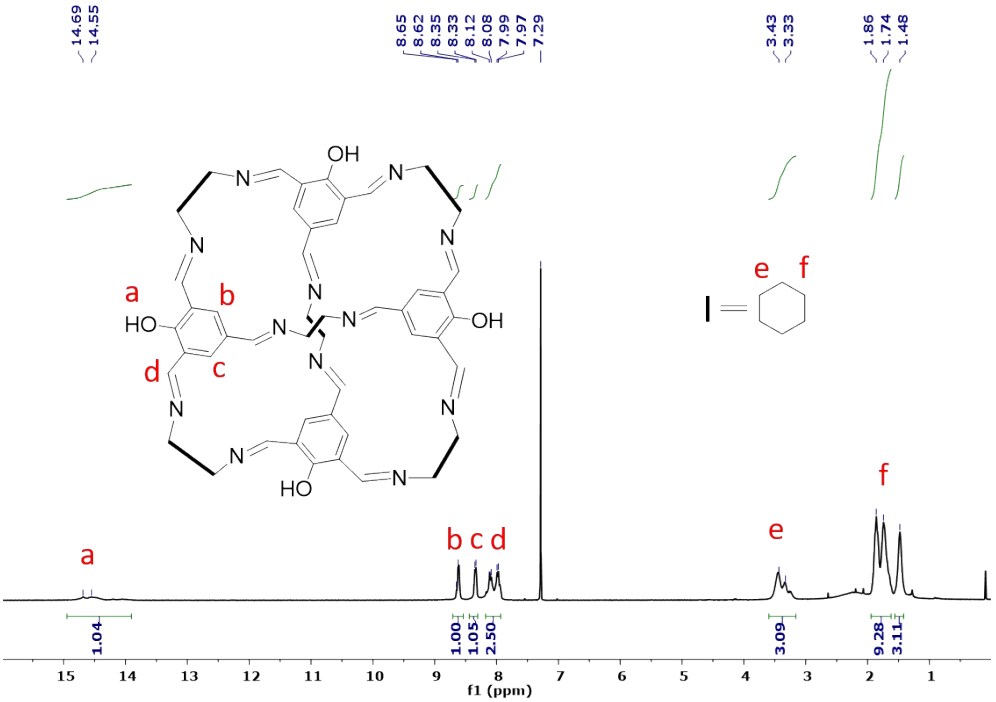


Figure S18. ^{1}H NMR of **OC2** (400 MHz, $CDCl_3$). δ 14.69-14.55 (d, 4H), 8.65 – 8.62 (m, 4H), 8.35 – 8.33 (m, 4H), 8.12 (d, 4H), 7.99 (d, 8H), 3.43-8.33 (d, , 12H), 1.84 (m, 12H), 1.86-1.74 (s, 24H), 1.48 (s, 12H).

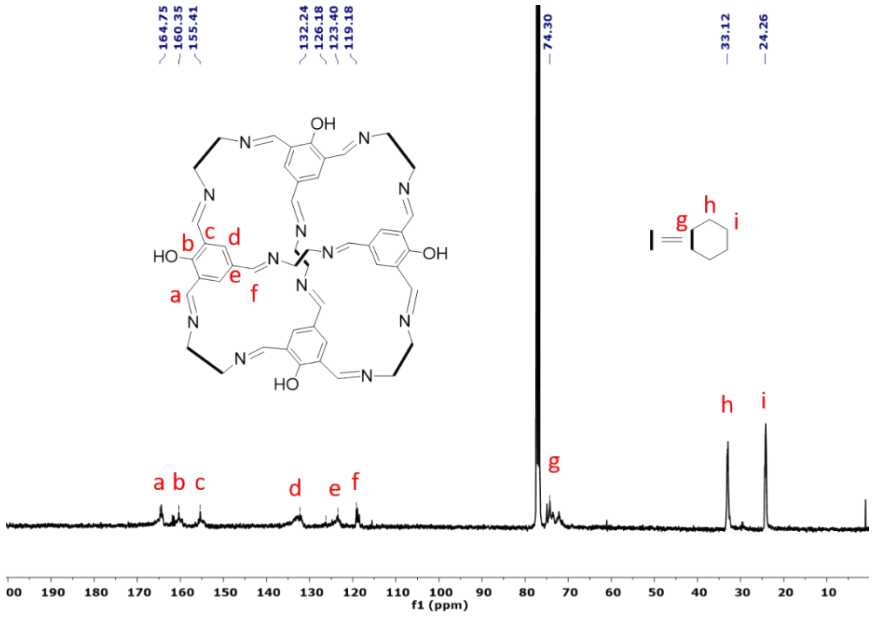


Figure S19. ^{13}C NMR of **OC2** (100MHz NMR, $CDCl_3$).

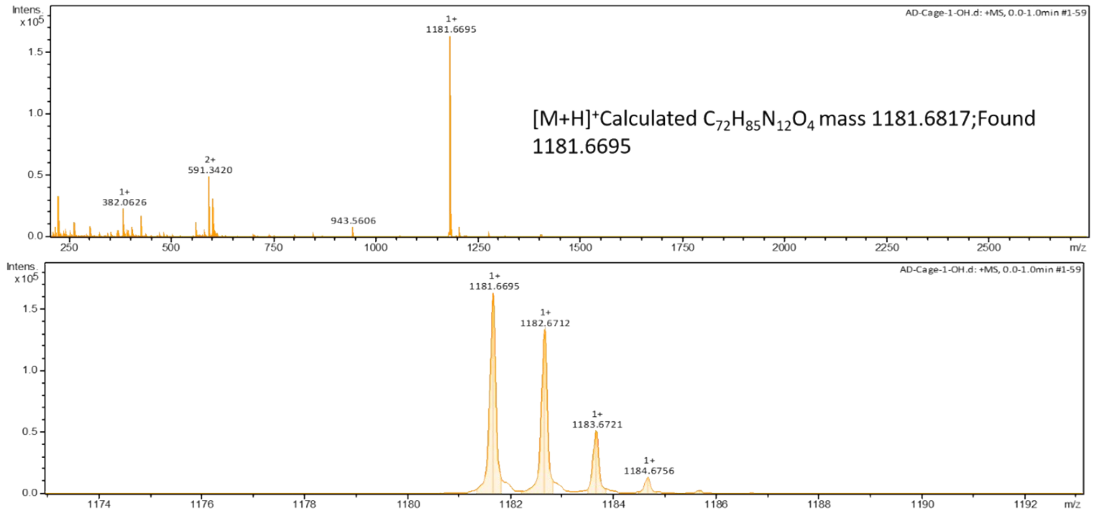


Figure S20. TOF-Mass of **OC2**.

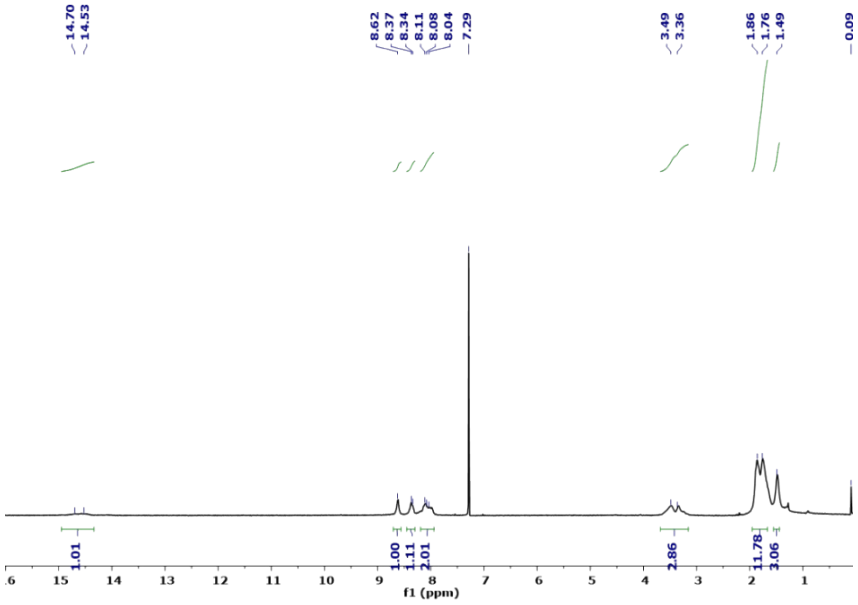
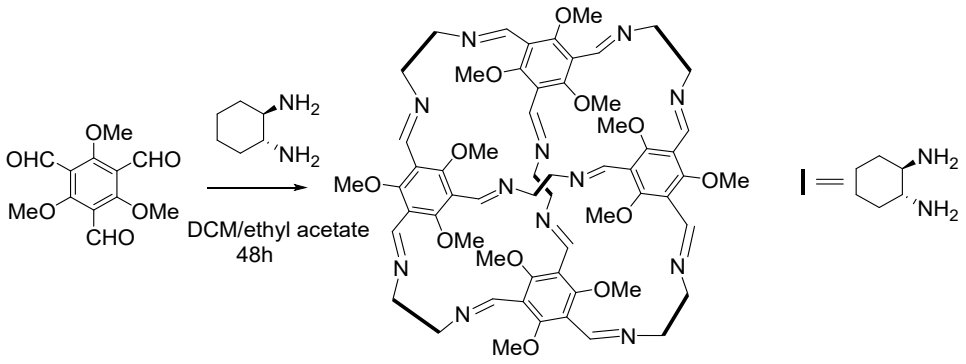


Figure S21. ^{1}H NMR of **OC2** (400MHz NMR, $CDCl_3$) indicating the stability of the imine cage in Serum.

Synthesis of OC3.

To 1,3,5 Trimethoxy benzene (252 mg, 2 mmol) in a round bottom flask was added 20 ml of ethyl acetate. The solution was placed in an ice bath followed by slow addition (~1ml/12min) of (1R, 2R) 1,2- diamino cyclohexane (3 mmol, 171 mg, 3mmol) in 20 ml of DCM. During the addition, temperature should be maintained at near to 0 °C. A catalytic amount (~5μL) of TFA was added after the complete addition. The reaction mixture was then allowed to stay overnight for slow evaporation. The microcrystalline product was isolated and recrystallized from 1:1 mixture of chloroform and acetonitrile.[4] ^{1}H NMR (400MHz NMR, $CDCl_3$); δ 8.55 (s, 12H), 3.55 (s, 36H), 3.43 (s, 12H), 1.51-1.86 (m, 48H) ppm. ^{13}C NMR (100MHz NMR, $CDCl_3$): δ 162.57 (C=N), 153.80 (C-OMe), 120.85 (C-C=N), 62.60 (O-Me), 33.70 (cyclohexane C), 24.61 (cyclohexane C) ppm. TOF MS m/z: $[M+H]^+$ Calcd for $C_{84}H_{108}N_{12}O_{12}$ 1477.8288; Found 1477.8490.



OC3

Figure S22. General synthetic scheme of **OC3** starting from 2,4,6-Trimethoxy-benzene-1,3,5-tricarbaldehyde.

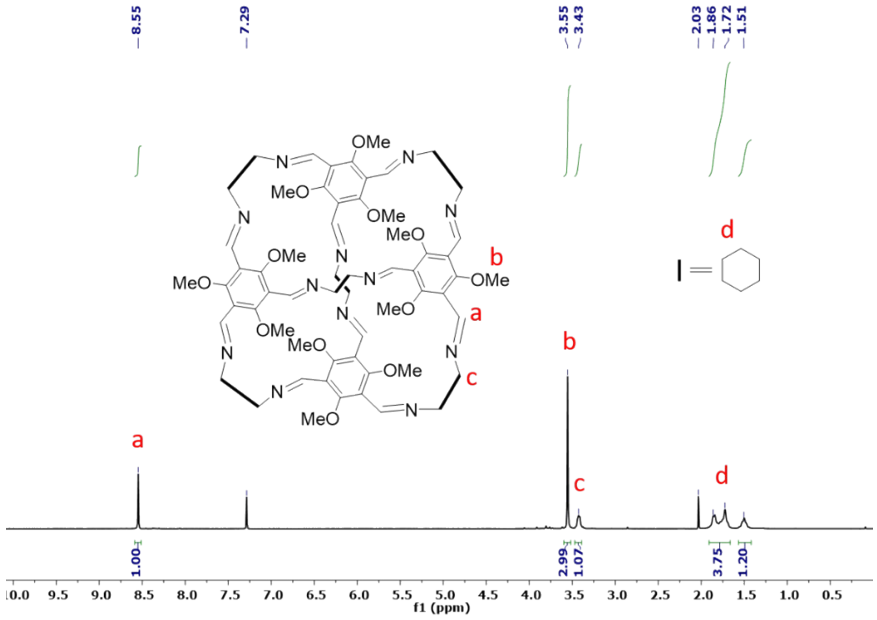


Figure S23. ^{1}H NMR of **OC3** (400MHz NMR, $CDCl_3$). δ 8.55 (s, 12H), 3.55 (s, 36H), 3.43 (s, 12H), 1.51-1.86 (m, 48H) ppm.

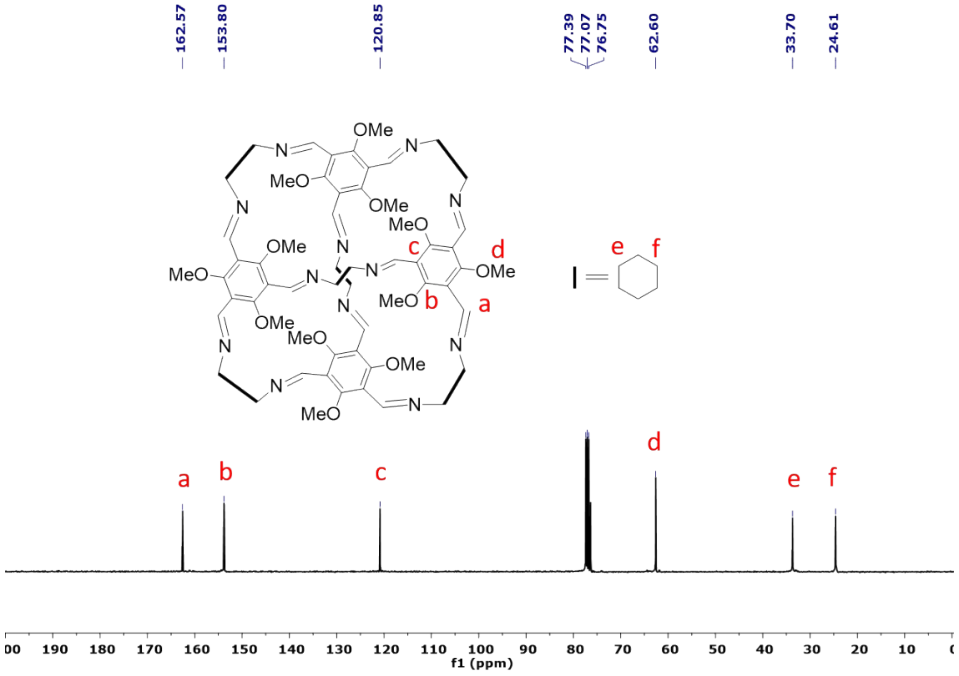


Figure S24. ^{13}C NMR of **OC3** (100MHz NMR, $CDCl_3$).

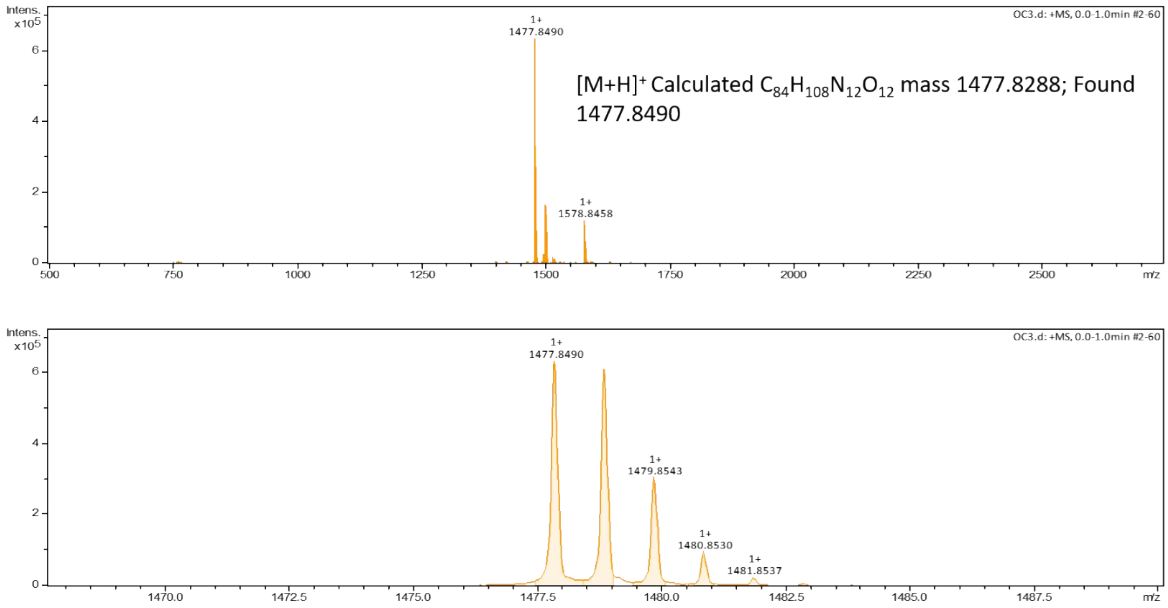


Figure S25. TOF-Mass of **OC3**.

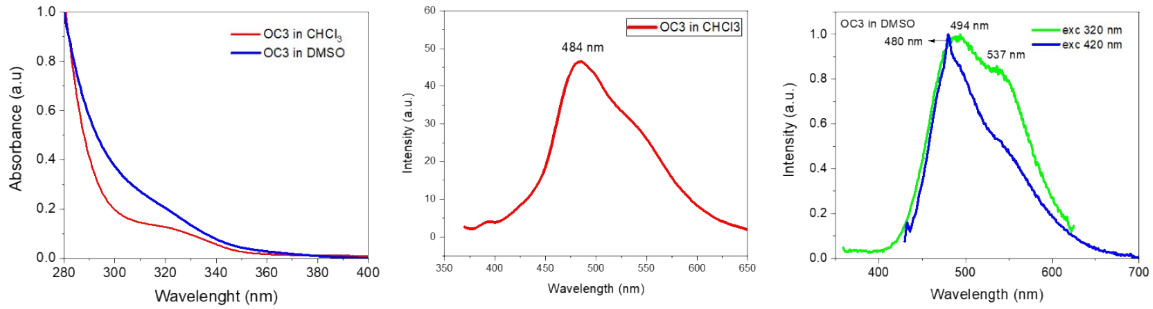


Figure S26. UV−visible absorption and emission spectra of **OC3** in $CHCl_3$ and DMSO (without correction).

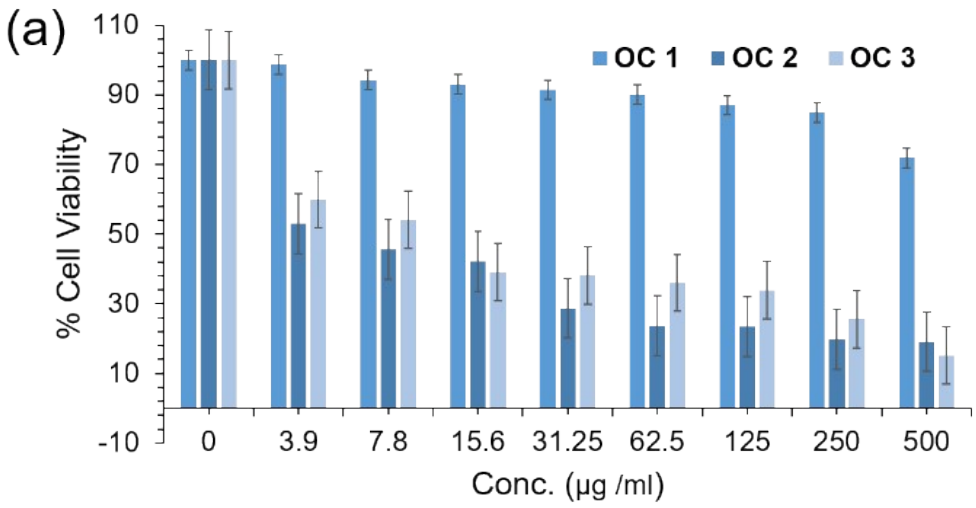


Figure S27. Cytotoxicity studies of **OC1**, **OC2**, and **OC3** in MCF-7 cells over 24 h.

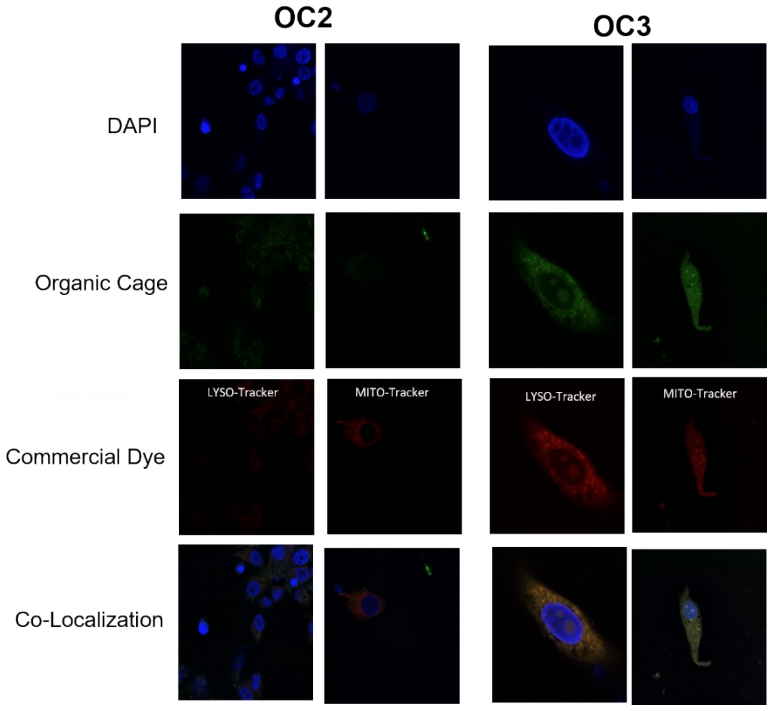


Figure 28. CLSM of MCF-7 cells incubated with either 7.8 µg/ml **OC2** or 3.9 µg/ml **OC3** for 3 hrs showing the colocalization oft he organic cages with the commercial probes. (Scale bar at 30– 1 µm).

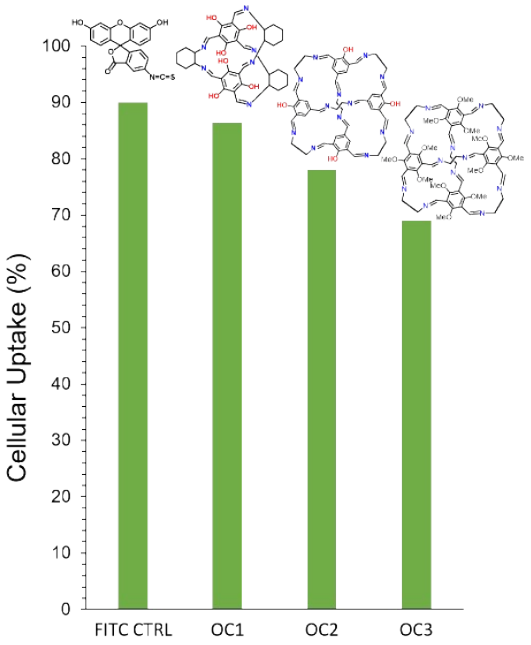


Figure 29. Cell uptake of 3.9μg/ml of **OC1**, **OC2**, and **OC3** measured by FACS.

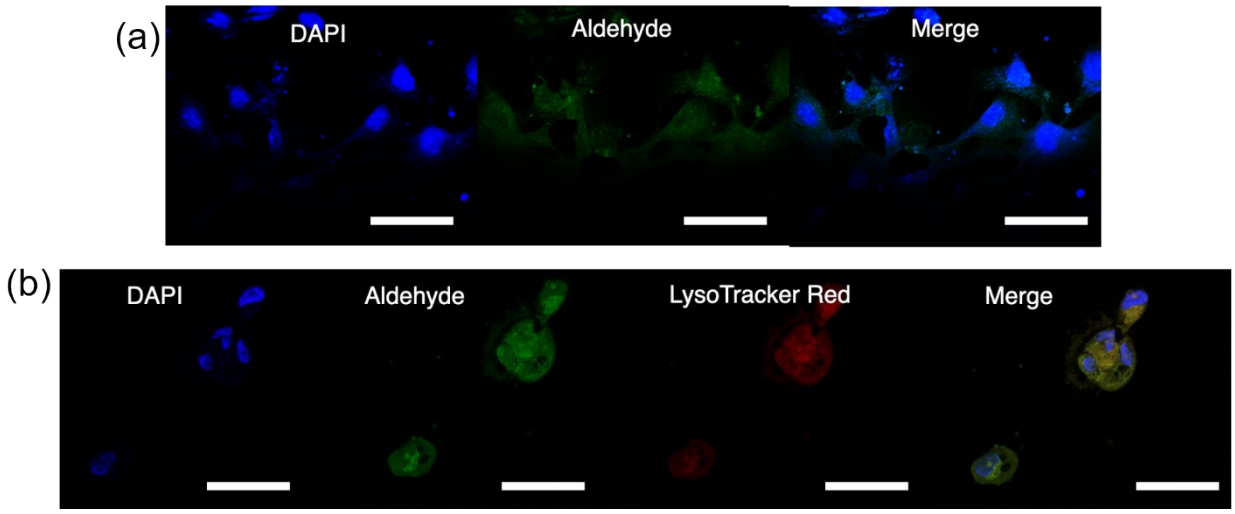


Fig S30. CLSM images of 1,3,5-triformylphloroglucinol aldehyde in MCF-7 cells. (a) Aldehyde distribution inside the cell. (b) Co-localization of the aldehyde in lysosomes. (Scale bar at 30μm).

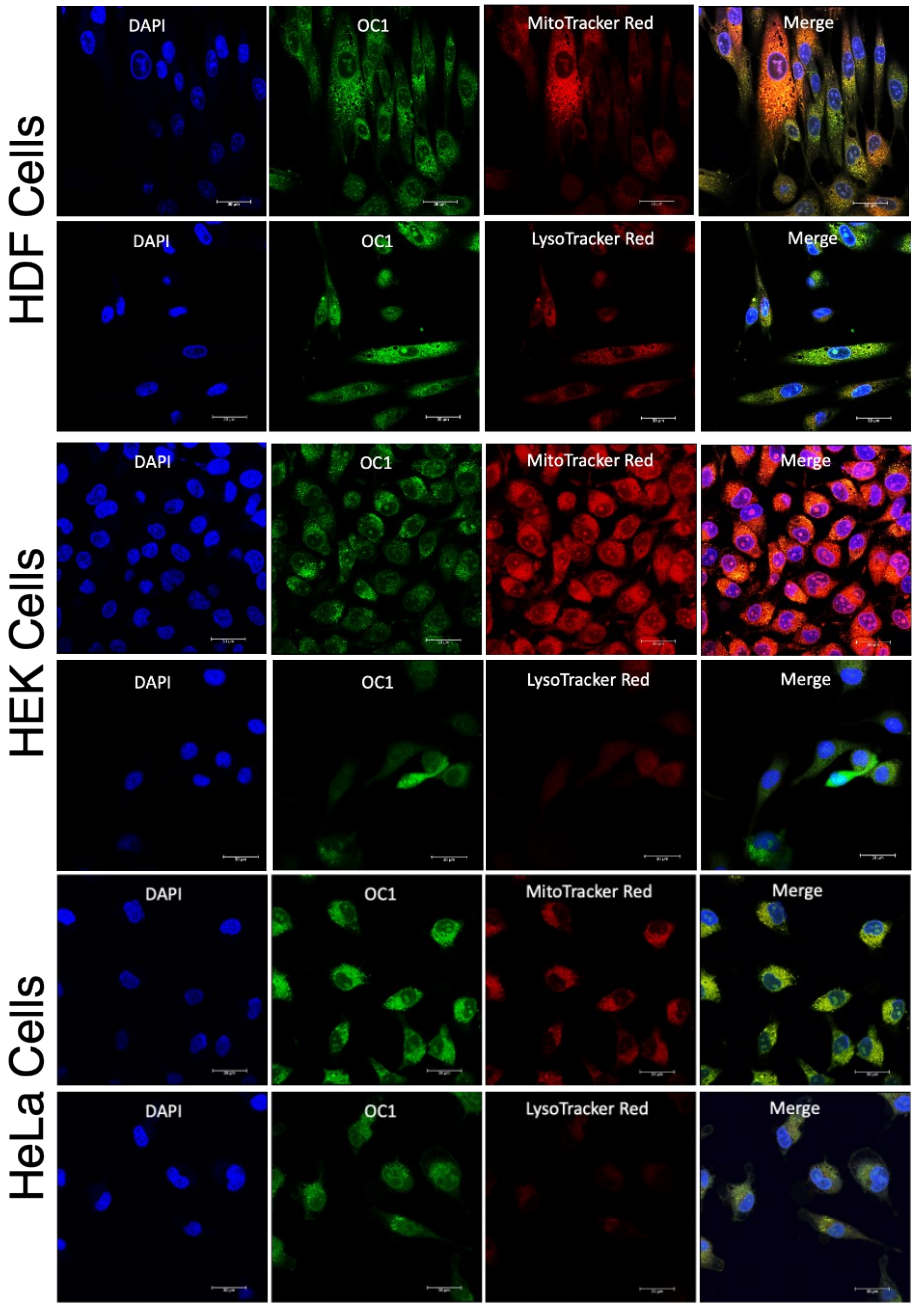


Figure S31. CLSM of Live HDF, HEK, and HeLa cells incubated with 250µg/ml **OC1** (abs/em ~495/519nm) for 3 hrs, showing the colocalization and affinity towards the mitochondria by overlapping with the commercial probe MitoTracker RedFM (abs/em ~581/644 nm). (Scale bar at 30 µm).

Single Crystal X-ray Diffraction. Single crystals of the **OC1**, was mounted in a Hampton cryoloop with *Paratone*® N oil cryoprotectant. In this case, a suitable crystal of appropriate size was selected from the mother liquor and immersed in *Paratone*® N oil and then it was mounted on the tip of a glass fiber and cemented using epoxy resin. Single crystal X-ray diffraction (SCXRD) was performed using a Bruker D8-Venture single crystal X-ray diffractometer equipped with a digital camera diffractometer using graphite-monochromated Mo-Kα radiation (0.71073 Å) at 120 K temperature. The linear absorption coefficients, scattering factors for the atoms and the anomalous dispersion corrections were taken from International Tables for X-ray Crystallography. Data integration and reduction were performed using SaintPlus 6.01[5] software. Absorption correction was performed by multi-scan method implemented in SADABS.[6] Space group was determined using XPREP implemented in APEX-III.[7] The structures were solved by direct methods using SHELXS-2008 and refined using SHELXL-2018.[8] X-Seed[9] was used as the graphical interface for the SHELX program suite. For all the cases, non-hydrogen atoms were refined anisotropically. All other hydrogen atoms are geometrically fixed using riding atom model. Attempts to identify the disordered solvent molecules have failed. Therefore, final refinement was done by SQUEEZE procedure implemented in PLATON.[10] Data collection, structure refinement parameters and crystallographic data for the crystals are given in Table S1.

Table S1. Crystal data and structure refinements for **OC1**.

Identification code	**OC1**	Reported **OC1**
Empirical formula	$C_{75.5}H_{85.5}Cl_{10.5}N_{12}O_{12}$	$C_{36}H_{42}N_6O_6.3(CHCl_3)$
Formula weight	1725.28	1012.86
Temperature (K)	120(2)	100(2)
Radiation	Mo-Kα	Mo-Kα
Wave length (λ)	0.71073	0.71073
Crystal system	Triclinic	Monoclinic
Space group	*P*1	*C2*
a [Å]	12.733(4)	19.477(2)
b [Å]	13.433(5)	24.208(2)
c [Å]	14.738(5)	13.2882(16)
α [°]	63.059(8)	90
β [°]	71.223(9)	131.418(6)

γ [°]	73.702(9)	90
Volume [Å^3]	2100.3(1)	4698.5(9)
Z	1	4
Density (calculated)[g cm^{-3}]	1.364	1.432
Absorption coefficient [mm^{-1}]	0.413	0.587
F(000)	897	-
Refl. used [$I > 2\sigma(I)$]	14326	7680
Independent reflections	53661	11328
R_{int}	0.1454	0.0268
Refinement method	F^2	F^2
GOF	0.971	1.034
Final R indices [$I>2\sigma$(I)]	$R_1 = 0.0873$, $wR_2 = 0.2043$	$R_1 = 0.0844$, $wR_2 = 0.1957$
CCDC Number	2144153	Ref 2

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