Electronic Supplementary Material (ESI) for Nanoscale.

Supporting information

Photoinduced electron transfer reaction in mitochondria for spatiotemporal selective photo-oxidation of lipids by donor/acceptor linked molecules

Yuta Takano,*[a,b] Erina Hanai,[c] and Hiroshi Imahori*[a,c]

[a]Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

[b]Research Institute for Electronic Science, Hokkaido University, Kita-20 Nishi-10, Kita-ku, Sapporo 001-0020, Japan

[c]Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Nishikyo-ku, Kyoto 615-8510, Japan

*Correspondence to (Y.T.) tak@es.hokudai.ac.jp and (H.I.) imahori@scl.kyoto-u.ac.jp

Materials and methods

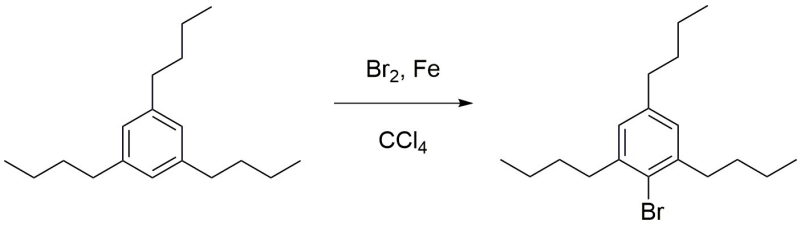
Materials:

9-Mesityl-10-methylacridinium perchlorate (**3**) was purchased from Tokyo Chemical Industry Co., Ltd. (Japan) COATSOME EL-11-A (Fig. S1) (Lipid composition: 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC):cholesterol:1-Palmitoyl-2-oleoylphosphatidyl glycerol (POPG) = 30:40:30 mol%. It is unilamellar vesicle with high monodispersity based on the DLS measurements) was obtained from NOF Co. (Japan). Cardiolipin (Fig. S2) (1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (sodium salt)) was purchased from Avanti Polar Lipids Inc. (AL, USA). All other solvents and chemicals were of reagent-grade quality, purchased commercially, and used without further purification unless otherwise noted. Thin layer chromatography (TLC) and column chromatography were performed with Silica gel 60 F254 (Japan Merck, Japan) and SiliaFlash F60 (230 – 400 mesh; SiliCycle Inc., Canada), respectively. Dulbecco's phosphate buffered saline (PBS) and Hank's Balanced Salt Solution (HBSS) were obtained from Thermo Fisher Scientific Inc. (MA, USA). Cell culture dishes and glass bottom dishes were purchased from Corning Inc. (MA, USA).

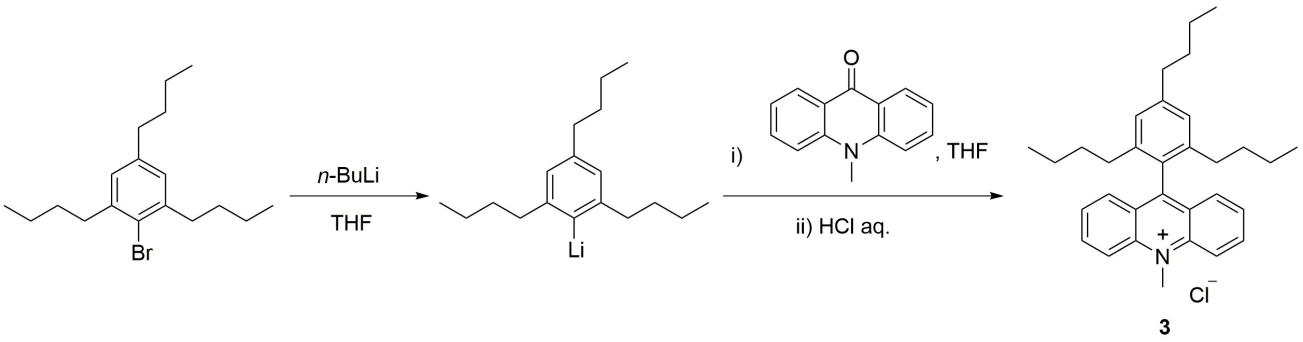
General Procedures:

^{1}H NMR spectra were measured by JEOL JNM-EX400 or JEOL-AL300 NMR spectrometer. Electro spray ionization (ESI) mass spectra were obtained using a Thermo Fischer Scientific EXACTIVE spectrometer. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectra were obtained using a Ultraflex Ⅲ−KE TOF/ TOF system with 9-aminoacridine as a matrix for negative mode. UV-vis spectra of solutions were measured with a Perkin-Elmer Lambda 900UV/vis/NIR spectrometer. Steady-state fluorescence spectra were obtained by a HORIBA SPEX Fluoromax-4 or HORIBA Nanolog spectrofluorometer.

Synthesis



To three neck round bottom flask was charged with 1,3,5-tributylbenzene (599 mg, 2.43 mmol) and iron powder (18.8 mg, 337 μmol) in dried CCl_4 (9 mL). Resulted solution was cooled to 0 ºC. Solution of Br_2 (0.07 mL, 2.72 mmol 1.1 eq.) in dried CCl_4 (3 mL) was added dropwise slowly. Then the mixture was stirred at r.t. for 3 hrs. The mixture was washed two times with water, NaOH aq. and then water. Organic layer was separated, dried over $MgSO_4$ and purified by silica-gel chromatography with hexane as an eluent. 1-bromo-2,4,6-Tributylbenzene was obtained as colorless oil (yield: 85%, 669 mg, 2.07 mmol).
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.86 (s, 2H), 2.72 (t, J = 7.8 Hz, 4H), 2.49 (t, J = 7.8 Hz, 2H), 1.58 (m, 6H), 1.35 (m, 6H), 0.95 (t, J = 3.9 Hz, 9H). HRMS (EI): m/z Calcd. for $C_{18}H_{39}Br$ (M^+) 324.15, Found 324.15. IR(neat): ν = 2957, 2930, 2860, 1458, 1019 cm^{-1}.



3

A solution of 1-bromo-2,4,6-tributylbenzene (0.661 g, 2.03 mmol, 3.0 eq.) in THF (12.5 mL) was stirred and cooled to -78 ºC. *n*-butyllithium (1.55 M in hexane (1.35 mL), 3.0 eq.) was added and the solution was stirred for 5.5 hrs at -78 ºC. Then 10-methyl-9(10H)-acridone (142 mg, 678 µmol) was added to the solution and stirred at r.t. for 15 hrs. 1 M HCl (15 mL) was added to the mixture and stirred for additional 2 hrs. The mixture was extracted with CH_2Cl_2 three times. The organic layer was washed with brine and dried over $CaCl_2$. The solution was condensed in a reduced pressure until 3 mL remained. Then hexane (30 mL) was added to the residue, filtered and the solid was collected. The solid was purified by alumina column chromatography. The eluent was changed from chloroform to 2% MeOH /chloroform. And the product was further purified by HPLC with using a COSMOSIL®C18-EB column (nacalai tesque. co., Ltd. Japan) and MeOH as the eluent. The elution was collected and solvent was removed in a reduced vacuum. **2** was obtained as dark green solid (yield 10%, 67.8 µmol, 32.1 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.15 (dd, 2H), 8.546 (dt, 2H), 7.85 (dd, 2H), 7.79 (dt, 2H), 7.19 (s, 2H), 5.40 (s, 3H), 2.76 (t, *J* = 7.82 Hz, 2H), 1.88 (t, *J* = 7.82 Hz, 4H), 1.74 (m, 2H), 1.50 (m, 2H), 1.19 (m, 4H), 1.03 (t, *J* = 7.32 Hz, 3H), 0.94 (m, 4H), 0.55 (t, *J* = 7.32 Hz, 6H). HRMS (ESI): m/z Calcd. for $C_{32}H_{40}N$ ([M^+]) 438.32, Found 438.31.). IR(film): ν = 2971, 2960, 2865, 1609, 1549, 1541, 1458, 765 cm^{-1}.

Ion Exchange of 3 to 1

3 (208 mg, 510 µmol) was dissolved in methanol: acetonitrile =1:1 (v/v) and slowly passed through an ion exchange column in Cl^- cycle (Amberlite-IRA 410 (JapanMerck), pre-rinsed with methanol: acetonitrile =1:1 (v/v) over night) for taking more than 2 hrs. The column was rinsed with another portion of methanol: acetonitrile =1:1 (over 2 hrs). After evaporation of solvent of the eluent, **1** (yellow solid) was obtained (yield: 80%, 408 µmol, 140 mg). m.p.: 238 ºC, ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.13 (dd, 2H, Acr), 8.48 (dt, 2H, Acr), 7.83 (s, 4H, Acr), 7.15 (s, 2H, Acr), 5.32 (s, 3H, NMe), 2.49 (s, 3H, Me), 1.72 (s, 6H, Me). HRMS (ESI): *m/z* calcd for $(C_{23}H_{22}N)_2Cl$ ([2M+Cl]$^-$), 659.32, Found 659.32.

cf. 3: ^{1}H NMR (300 MHz; $CDCl_3$): δ 8.78 (2H, s, Acr), 8.40 (2H, s, Acr), 7.83 (4H, d, *J* = 14 Hz, Acr), 7.16 (2H, s, Acr), 5.11 (3H, s, NMe), 2.49 (3H, s, Me), 1.74 (6H, s, Me).

Electrochemical Measurements

Electrochemical measurements were performed using a BAS ALS 630a electrochemical analyzer. Redox potentials were determined by second alternating current voltammetry (2nd ACV) in PBS. A glassy carbon (3 mm diameter) working electrode, Ag/AgCl (sat. KCl) reference electrode, and Pt wire counter electrode were employed. Ferrocene was used as an external standard for the 2nd ACV

measurements.

Incorporation into Liposome

Mixture of 1.5 mM COATSOME EL-11-A (Fig. S1) solution of PBS (971 μL) and 1.0 mM compound (**1** or **2**) (29 μL) were vortexed, and incubated at 37 °C for 1 h. Then, absorbance was measured. The resultant solution was passed into a NAP-10 column (GE Healthcare Life Sciences), fractionated to 1 mL (first: fra.1, second: fra.2…) and measured absorbance of each fractions. For a control condition, solution of 1.0 mM compounds (29 μL) in PBS (971 μL) were also conducted in the absence of COATSOME.

Nanosecond Transient Absorption (TA) Measurements

Nanosecond TA measurements were carried out using the laser system (UNISOKU Co., Ltd., Osaka, Japan). Solvents were deaerated by argon bubbling for 1 h before measurements. A solution containing a compound (30 μM) or a mixture of compound (30 μM) and COATSOME (molar ratio: 1/50) was excited by a Panther OPO pumped by a Nd:YAG laser (Continuum SLII-10, 4-6 ns FWHM), and the photodynamics were monitored by continuous exposure to a Xe lamp as a probe light and a photomultiplier tube (Hamamatsu 2949) as a detector. CS yields were obtained by the comparative method.[1,2]

Detection of Photoinduced Superoxide in a Cuvette

Detection of ROS with MitoSOX (Thermo Fisher Scientific Inc., MA, USA) was performed on the basis of the manufacture's protocol. Briefly, 1.0 μM solution of a compound (**1**, **2**, or reference water soluble free-base porphyrin[4,5]) and 5.0 μM MitoSOX in 50 μM COATSOME/H_2O solution were mixed and irradiated in a cuvette by Xe lamp (425-435 nm, 630 mJ cm^{-2}). The resulting solutions were immediately subjected to the spectrofluorometer with an excitation wavelength of 510 nm and monitored between 580-630 nm. The measurements were repeated for three times at least and results were statistically processed.

Detection of Photoinduced Singlet Oxygen

Detection of ROS by the Singlet Oxygen Sensor Green (SOSG) reagent (Thermo Fisher Scientific Inc., MA, USA) was performed on the basis of the manufacture's protocol. Briefly, 1.0 μM solution of a compound (**1**, **2**, or reference water soluble free-base porphyrin[3,4]) and 25.0 μM SOSG in 50 μM COATSOME/H_2O solution were mixed and irradiated in a cuvette by Xe lamp (425-435 nm, 630 mJ cm^{-2}). The resulting solutions were immediately subjected to the spectrofluorometer with an excitation wavelength of 504 nm and monitored between 520-530 nm. The measurements were repeated for three times at least and results were statistically processed.

HeLa Cell Culture

HeLa cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) with low glucose supplemented with 10% Fetal bovine serum (FBS) (Japan Bioserum, Japan) supplemented with 60 U mL^{-1} penicillin and 60 μg mL^{-1} streptomycin. The cells were cultured in 5% CO_2 and 95% air, and

were passaged every 3–4 days. HeLa cells were used for the following experiments with this culturing condition unless stated.

Confocal Laser Microscopy for Colocalization Determination

HeLa cells were cultured on a glass bottom dish with 1.0×10^5 cells mL^{-1} and cultured for 1 day. After 1 day, medium was removed, washed twice with PBS(−) and filled with serum free culture medium. Then, Mito-Tracker Red® (Thermo Fisher Scientific Inc., MA, USA) was added to the HeLa cells (final concentration: 25 nM) and incubated under an atmosphere of 5% CO_2/air at 37◦C. After a 12 min incubation, compound (final concentration: 10 μM) was added and incubation 3 min additionally and then observed by Zeiss LSM780 (Carl Zeiss Microscopy GmbH, Jena, Germany), which equips a 40 oil objective lens (NA 0.75), GaAsP (32x) array and fs–pumped pulsed laser. The cells were excited with a 561 nm light for detecting MitoTracker® Red and a 440 nm light for detecting compounds.

Calculation of Weighted Co-localization Coefficients

The Zeiss LSM780 Zen software was used to measure the weighted colocalization coefficients from three separate experiments. The weighted colocalization coefficients take into account the intensity value of the summed pixels. The weighted colocalization coefficients use the same equation as the colocalization coefficients,[5] but the value for each pixel is equal to its intensity value. Their values will range from 0 to 1. In this analysis, a pixel with bright intensity represents a higher number of fluorophores than a pixel with low intensity.

Detection of Photoinduced Apoptosis

Detection of cell apoptosis/necrosis was carried out by using Annexin V-FITC Apoptosis Kit (nacalai tesque co. Ltd., Japan), which contains Annexin V-FITC conjugate for early apoptosis detection and propidium iodide (PI) for late apoptosis and necrosis detection, according to the manufacture's protocol. Briefly, HeLa cells were cultured on a glass bottom dish with 1.7×10^5 cells mL^{-1} and cultured for 1 day. Then, the medium was removed, washed with HBSS(+) once and filled with serum free culture medium. Then, a compound (**1** or **2**) was added to the HeLa cells (final concentration of compound, 2.5 μM) and cells were incubated under 5% CO_2/air at 37°C. After 10 min incubation, the medium was replaced with fresh phenol red-free medium containing serum. Then, the cells were illuminated by the Xe lamp (4.4 mW cm^{-2}, 425-435 nm) for 255 sec (total 1124 mJ cm^{-2}) and incubated under an atmosphere of 5% CO_2/air at 37°C. After a 1 hr, the cells were treated with the Annexin V-FITC/PI solution, incubated for 10 min at room temperature. After 10 min, the medium was replaced with HBSS(+), and then observed by Fluoview-10i (Olympus Co., Japan) . Fluorescent cells were counted as cells in early apoptosis (green) or those in late apoptosis or necrosis (red or yellow).

Statistical Analyses

Statistical analysis was performed using *Igor* Pro v6.3 using a *one-way ANOVA* with a *Dunnett's* post hoc *test* for significance.

Measurements of Cardiolipin (CL) Oxidation (1) in a Glass Cuvette or (2) in Cells

(1) In a cuvette

Detection of cardiolipin (CL) oxidation was performed by Liperfluo reagent (Dojindo Co., Ltd., Kumamoto, Japan) on the basis of the manufacture's protocol. Briefly, 1.0 μM compound, 3.3 μM CL and 1.7 μM Liperfluo in PBS were immediately subjected to the spectrofluorometer with an excitation wavelength of 488 nm. Then the solutions were irradiated in a cuvette by Xe lamp (425-435 nm, 630 mJ cm^{-2}) and immediately subjected to the spectrofluorometer with an excitation wavelength of 488 nm. The measurements were repeated for three times at least and results were statistically processed.

(2) In cells

Detection of lipid oxidation in mitochondria was carried out by using Mito-PeDPP (Dojindo Co., Ltd., Kumamoto, Japan). Briefly, HeLa cells were cultured on a glass bottom dish with 1.0×10^5 cells mL^{-1} and cultured for 1 day. Then, the medium was removed, washed with PBS(−) twice and filled with serum free culture medium. Then, Mito-PeDPP was added to the HeLa cells (final concentration: 500 nM) and incubated under an atmosphere of 5% CO_2/air at 37◦C. After 10 min incubation, compound was added (final concentration: 1.0 μM) and incubated for 5 min additionally and then observed by Zeiss LSM780 (Carl Zeiss Microscopy GmbH, Jena, Germany), which equips a 40 x oil objective lens (NA 0.75), GaAsP (32x) array. Images were recorded with a spatial resolution by 500×500 nm^2 $pixel^{-1}$. The cells were excited with 488 nm light of diode laser for detecting Mito-PeDPP and 440 nm light for exciting compound. Data points were collected every 1 sec and one data point is integration of the signal for 1 sec. The measurements were repeated for three times at least and results were statistically processed.

Reference

(1) T. Tsudaka, H. Kotani, K. Ohkubo, T. Nakagawa, N. V. Tkachenko, H. Lemmetyinen and S. Fukuzumi, *Chem. Eur. J.*, 2017, **23**, 1306-1317.

(2) T. Higashino, T. Yamada, M. Yamamoto, A. Furube, N. V. Tkachenko, T. Miura, Y. Kobori, R. Jono, K. Yamashita and H. Imahori, *Angew. Chem. Int. Ed.*, 2016, **55**, 629-633.

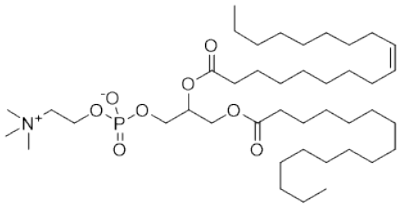
(3) T. Numata, T. Murakami, F. Kawashima, N. Morone, J. E. Heuser, Y. Takano, K. Ohkubo, S. Fukuzumi, Y. Mori and H. Imahori, *J. Am. Chem. Soc.*, 2012, **134**, 6092-6095.

(4) Y. Takano, T. Numata, K. Fujishima, K. Miyake, K. Nakao, W. D. Grove, R. Inoue, M. Kengaku, S. Sakaki, Y. Mori, T. Murakami and H. Imahori, *Chem. Sci.*, 2016, **7**, 3331-3337.

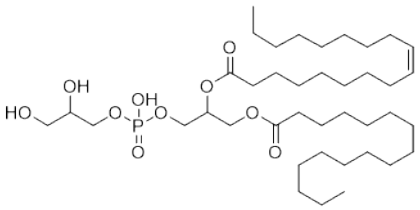
(5) E. M. M. Manders, F. J. Verbeek and J. A. Aten, *J Microsc-Oxford*, 1993, **169**, 375-382.

(6) Y. Y. Tyurina, V. Kini, V. A. Tyurin, I. I. Vlasova, J. F. Jiang, A. A. Kapralov, N. A. Belikova, J. C. Yalowich, I. V. Kurnikov and V. E. Kagan, *Mol. Pharmacol.*, 2006, **70**, 706-717.

POPC:



POPG:



Cholesterol:

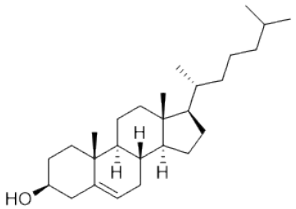


Fig. S1 Structures of the components of COATSOME EL-11-A.

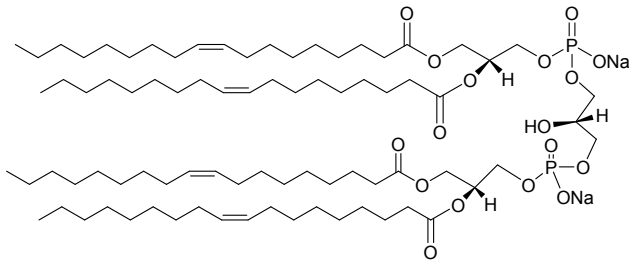


Fig. S2 Structure of a representative cardiolipin used in the present study.

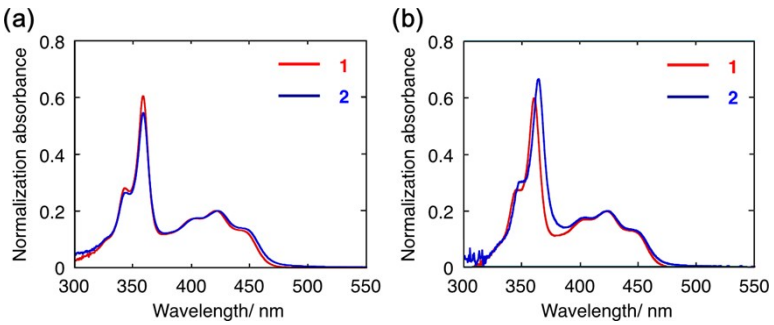


Fig. S3 UV-vis absorption spectra of **1** and **2** (a) in PBS and (b) in the COATSOME in H_2O. The absorbance was normalized to be 0.20 at the peak around 420 nm.

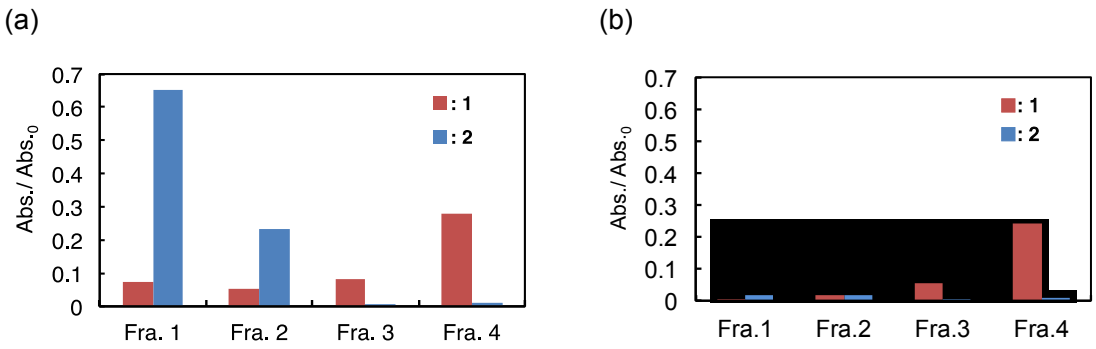


Fig. S4 (a) Incorporation ratio into the COATSOME after the elution from a size exclusion (NAP10) column. (b) Reference experiments of the elution in the absence of the COATSOME. The ratios (Abs./ $Abs._0$) were calculated based on the absorbance of the compounds at 425 nm before ($Abs._0$) and after (Abs.) the elution. Integrated ratios of Fra.1-4 were less than 1.0 respectively because there were still remained compounds in the column after the elution of Fra.4.

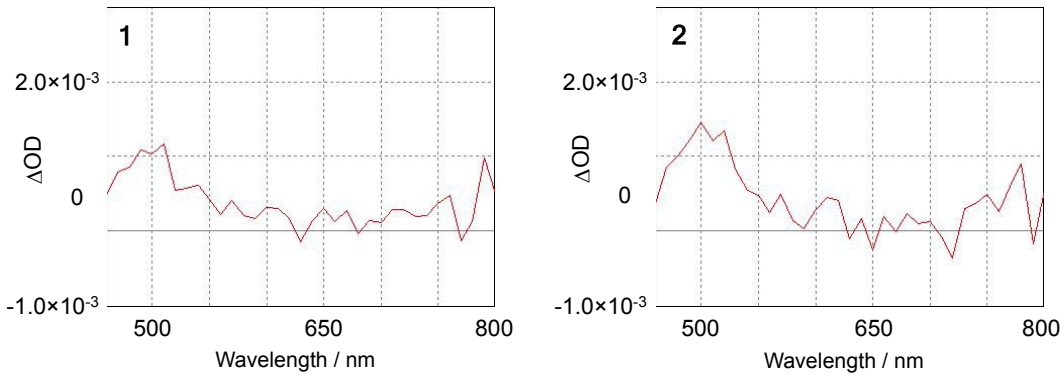


Fig. S5 Nanosecond time-resolved TA spectra of (a) **1** and (b) **2** in PBS taken at 1.0 μs after laser excitation of 5.0 mJ pulse^{-1} at 430 nm where the absorbance was adjusted to be identical (0.30).

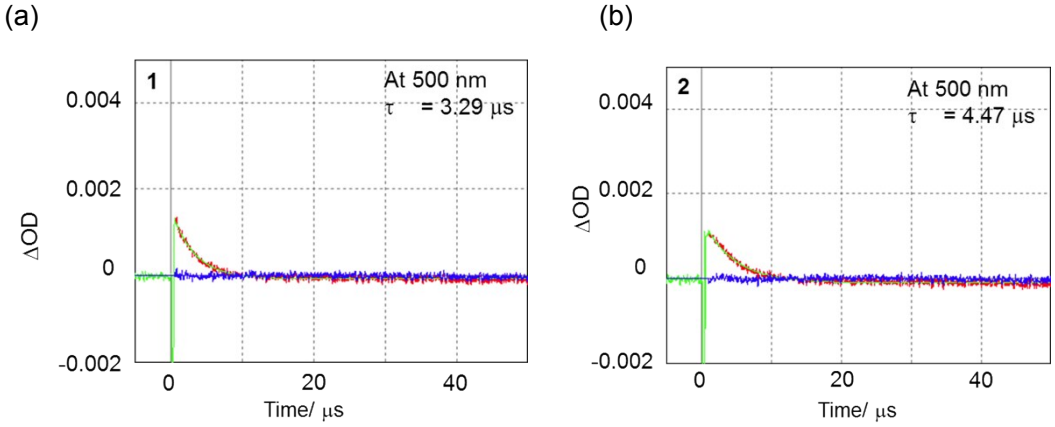


Fig. S6 Decay time profiles of (a) **1** and (b) **2** at 500 nm arising from $D^{\bullet+}$ and the $A^{\bullet}$ which were obtained by nanosecond time-resolved TA measurements in PBS after nanosecond laser excitation. The profiles were obtained by the excitation at 430 nm where the absorbance was adjusted to be identical (0.30). Red plots are the observed signals, green line is drawn by bi-exponential fitting, and blue line is the residual after the fitting.

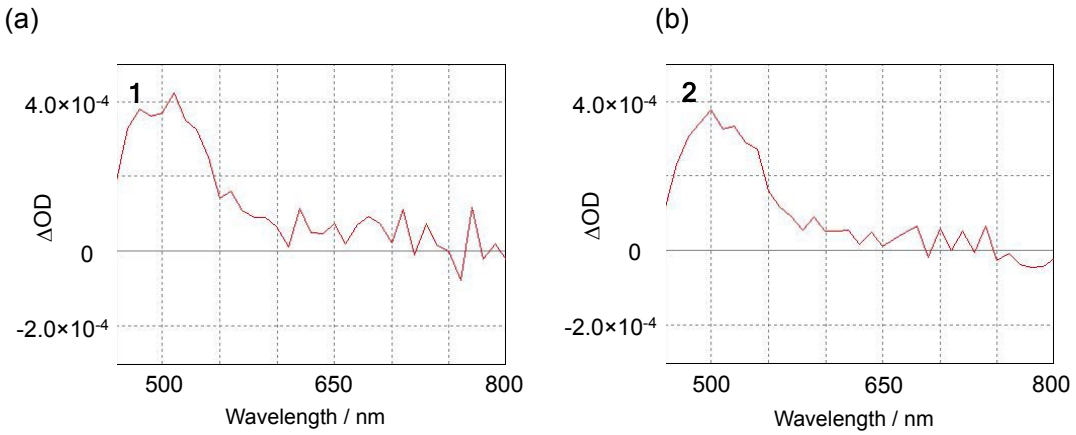


Fig. S7 Nanosecond time-resolved TA spectra of (a) **1** and (b) **2** in COATSOME/PBS taken at 50 μs after laser excitation of 5.0 mJ pulse^{-1} at 430 nm where the absorbance was adjusted to be identical (0.30).

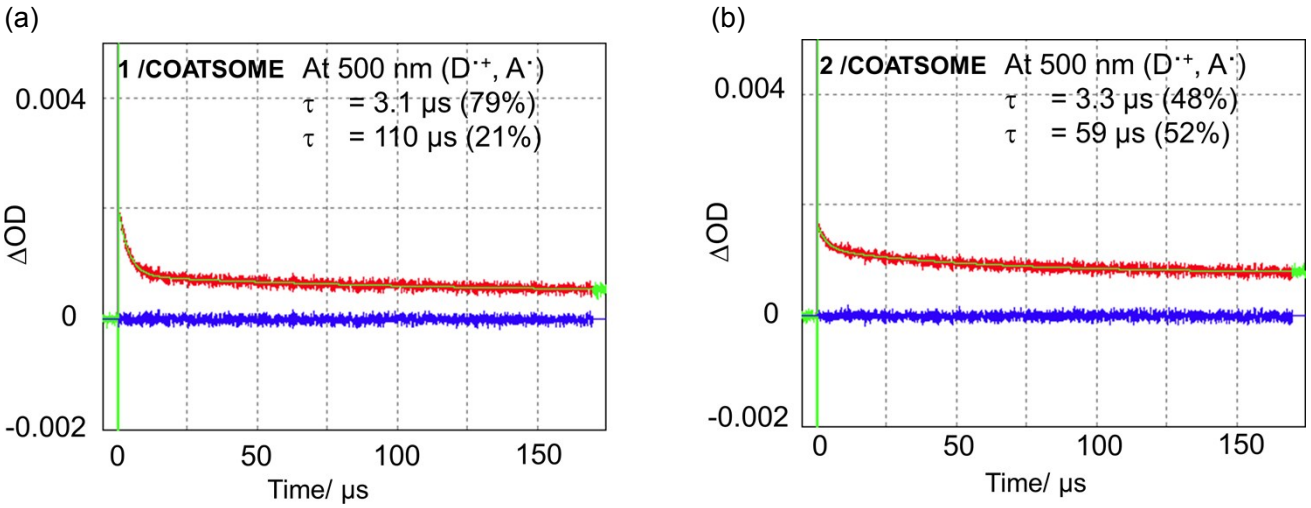
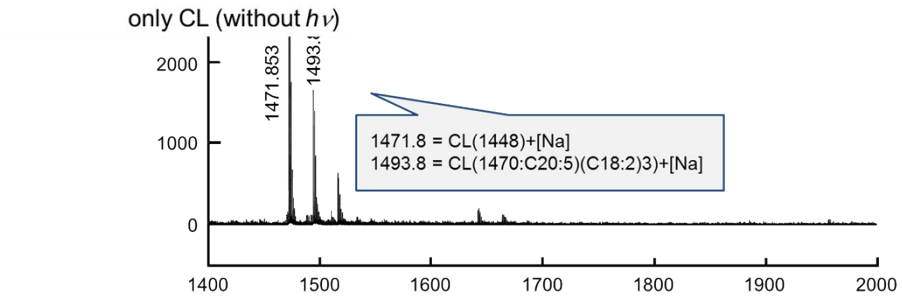
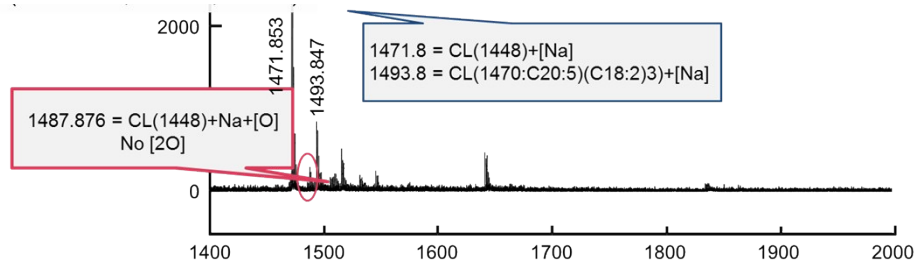


Fig. S8 Decay time profiles of (a) **1** and (b) **2** at 500 nm arising from Acr• and the aryl radical cation which were obtained by nanosecond time-resolved TA measurements in COATSOME/PBS after nanosecond laser excitation. The profiles were obtained by the excitation at 430 nm where the absorbances were adjusted to be identical (0.30). Red plots are the observed signals, green line is drawn by bi-exponential fitting, and blue line is the residual after the fitting.

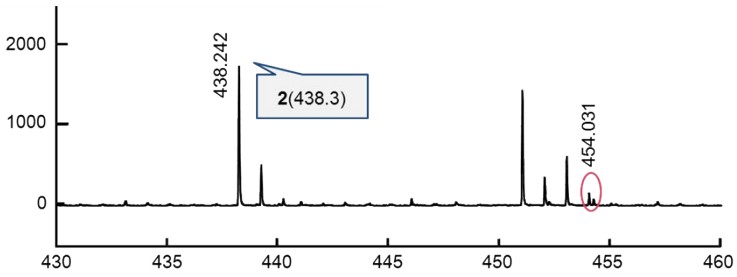
(a)



(b)



(c)



(d)

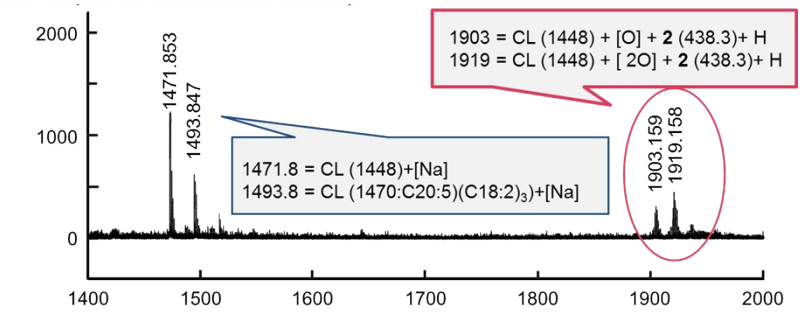
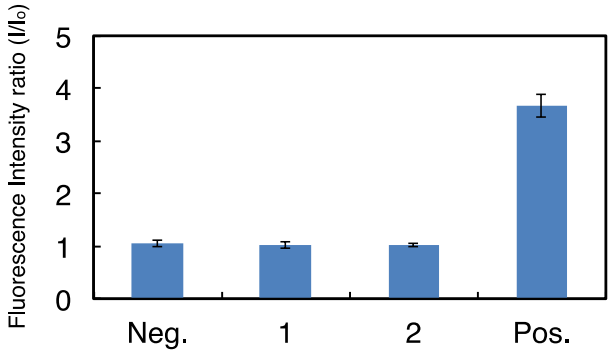
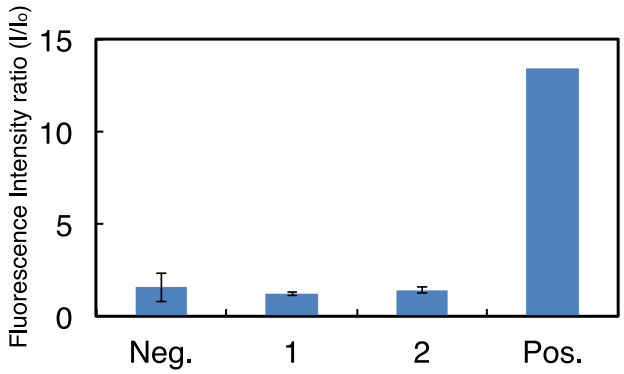


Fig. S9 MALDI-TOF mass spectra of (a) solely CL without illumination, (b) solely CL with illumination, (c) solely **2** with illumination and (d) CL and **2** with illumination. Conditions: Xe lamp, wavelength, 425-435 nm, 1000 mJ cm^{-2}. Results indicate that doubly-oxygenated CL conjugate with **2** was observed only in the condition using CL and **2** together with illumination as shown in (d).

(a)



(b)



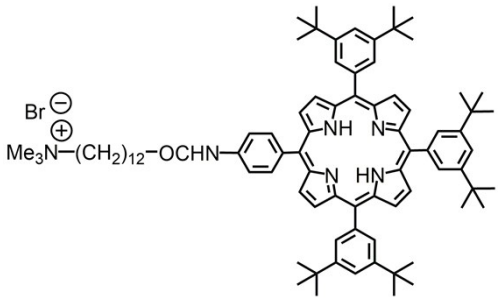


Fig. S10 Detection of (a) singlet oxygen ($^1O_2$) by Singlet Oxygen Sensor Green (SOSG) and (b) superoxide by Mito-SOX, in COATSOME/H_2O. Conditions: Xe lamp, wavelength, 425-435 nm, 630 mJ cm^{-2}. Error bars indicate S.D. (*n* = 3). Positive control: Water soluble free-base porphyrin shown in the figure.[4,5]

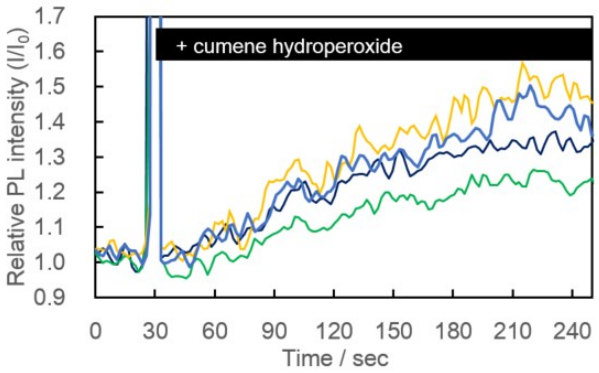


Fig. S11 CL oxidation in mitochondria of HeLa cells recorded by confocal laser microscope for longer term than that shown in Figure 3 in the presence of 1.0 mM cumene hydroperoxide without illumination. Large spike signal around 30 sec was caused by the room light which was used during the addition of cumene hydroperoxide.

Scheme S1. Energy diagram where energy levels were obtained from the second alternating current voltammetry as shown in Table S1. The oxidation potentials of cardiolipin (CL) were from Ref.6.

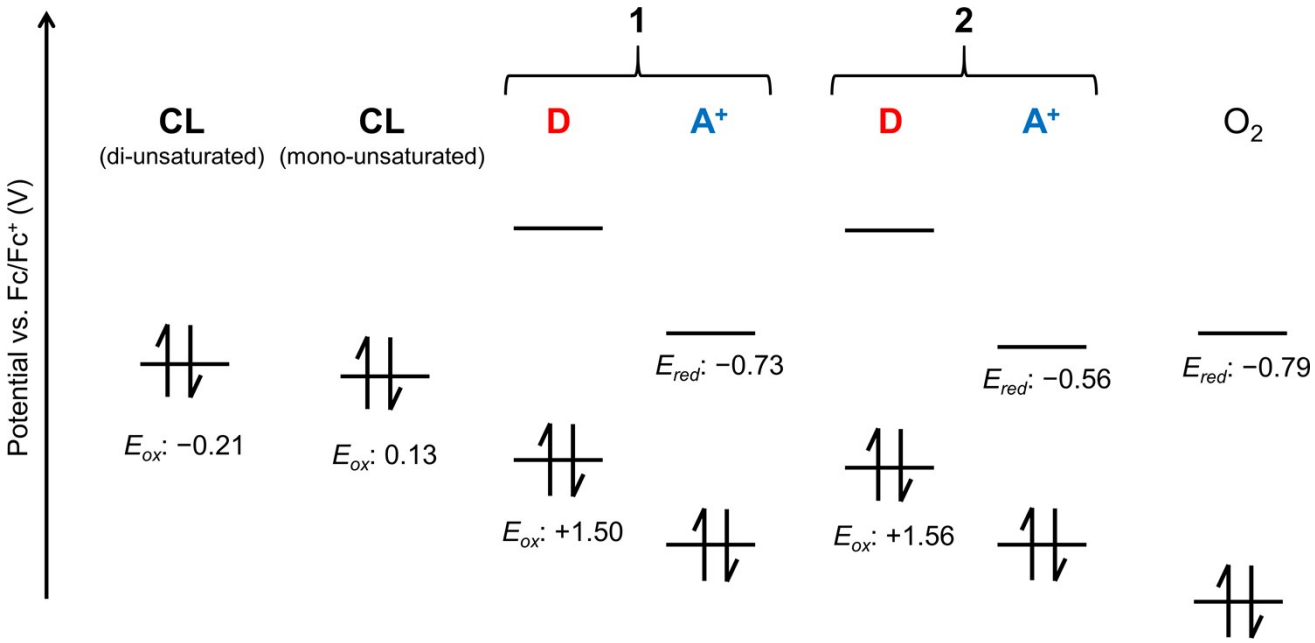


Table S1. Redox potentials of 1 and 2[a]

compound	E_{ox} / V ($D^{\bullet+}$/D)	E_{red} / V (A^{+}/$A^{\bullet}$)
1	1.50	–0.73
2	1.56	–0.56
O_2		–0.79

[a]Values were obtained by the second alternating current voltammetry. Condition; vs. Fc/Fc^{+}, solvent: PBS, working electrode: glassy carbon, counter electrode: platinum wire, reference electrode: Ag/AgCl (saturated KCl), amplitude, 25 mV.

Table S2. CS properties of **1** and **2**.

Medium	compound	k_{BET1} / s^{-1} [a]	k_{BET2} / s^{-1} [a]	$\Phi_{calcd.}$ [b]
PBS	**1**	3.0×10^{5}		0.08
	2	2.2×10^{5}		0.07
COATSOME/ PBS	**1**	3.2×10^{5} (79%)	9.0×10^{3} (21%)	0.16
	2	3.0×10^{5} (48%)	1.7×10^{4} (52%)	0.14

[a] Values in parenthesis in denote relative amplitude. [b] Φ_{calcd} were obtained by the comparative method.[1,2]