Supporting Information

Diazachlorin and Diazabacteriochlorin for One- and Two-Photon Photodynamic Therapy

Jean-François Longevial, Ayaka Yamaji, Dina Aggad, Gakhyun Kim, Wen Xi Chia, Tsubasa Nishimura, Yoshihiro Miyake, Sébastien Clément Juwon Oh, Morgane Daurat, Christophe Nguyen, Dongho Kim,* Magali Gary-Bobo,* Sébastien Richeter,* and Hiroshi Shinokubo*

Department of Molecular and Macromolecular Chemistry, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-8603, Japan.
Institut Charles Gerhardt de Montpellier, UMR 5253 CNRS-ENSCM-UM, 34296 Montpellier Cedex 05, France
Institut des Biomolécules Max Mousseron, UMR 5247 CNRS-UM, 34093 Montpellier Cedex 05, France
Spectroscopy Laboratory for Functional π-Electronic Systems and Department of Chemistry, Yonsei University, Seoul 03722, Korea

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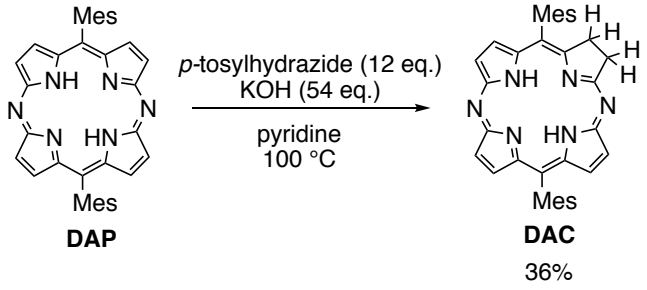
Instrumentation and Materials

^{1}H NMR (500 MHz) and ^{13}C{^{1}H} NMR (126 MHz) spectra were recorded on a Bruker AVANCE III HD spectrometer. Chemical shifts were reported as the delta scale in ppm relative to $CDCl_3$ (δ = 7.26 ppm) for ^{1}H NMR and $CDCl_3$ (δ = 77.16 ppm) for ^{13}C{^{1}H} NMR. UV/vis/NIR absorption spectra were recorded either on a Shimadzu UV-2550 or a JASCO V670 spectrometer. Emission spectra were measured in 1 cm quartz cell on a JASCO FP-6500 spectrometer and absolute fluorescence quantum yields were measured by photon-counting method using an integration sphere. Mass spectra were recorded on a Bruker microTOF using ESI-TOF method for 2-propanol solutions. X-ray data were taken on a Bruker D8 QUEST X-ray diffractometer equipped with PHOTON 100 CMOS active pixel sensor detector and IμS microfocus source using Mo-Kα radiation (λ = 0.71073 Å) or a Rigaku CCD diffractometer (Saturn 724 with MicroMax-007) with Varimax Mo optics using graphite monochromated Mo-Kα radiation (λ = 0.71075 Å). Unless otherwise noted, materials obtained from commercial suppliers were used without further purification.

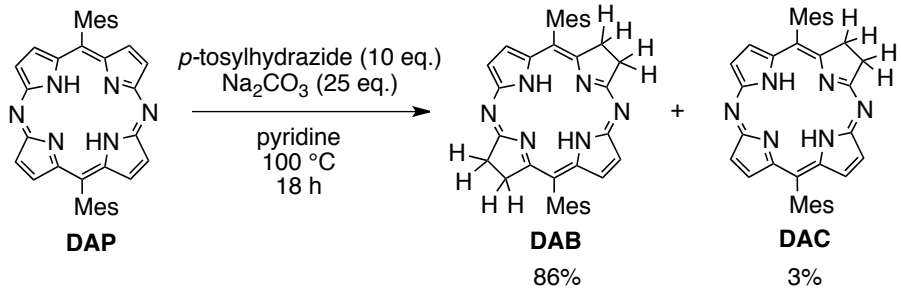
Synthesis

Diazachlorin (DAC)



A Schlenk tube containing **DAP** (13.7 mg, 0.025 mmol) was evacuated and refilled with argon gas. To the flask, *p*-tosylhydrazide (9.4 mg, 2 equiv), potassium hydroxide (75.6 mg, 54 equiv), and degassed pyridine (5 mL) were added. The reaction mixture was heated to 100 °C and stirred for 3 h in the dark. Then, additional *p*-tosylhydrazide (46.5 mg, 10 equiv) was added to the solution and the mixture was stirred for additional 20 h. The progress of the reaction was monitored by TLC analysis. After the reaction completed, pyridine was removed under reduced pressure. The residue was dissolved with EtOAc and washed with water several times. The organic layer was separated and dried over Na_2SO_4. The solvent was removed by rotary evaporator and the solid residue was subjected on silica gel column chromatography (hexane/EtOAc = 3/2 as an eluent) to yield **DAC** as a green solid (4.9 mg, 36%).

Diazabacteriochlorin (DAB)



A Schlenk tube containing **DAP** (13.9 mg, 0.025 mmol) was evacuated and refilled with argon gas. To the flask, *p*-tosylhydrazide (46.5 mg, 10 equiv), sodium carbonate (66.2 mg, 25 equiv) and degassed pyridine (10 mL) were added. The mixture was heated to 100 °C and stirred for 18 h in the dark. The progress of the reaction was monitored by TLC analysis. After the reaction completed, pyridine was removed under reduced pressure. The residue was dissolved with EtOAc and washed with water several times. The organic layer was separated and dried over Na_2SO_4. The solvent was removed by rotary evaporator and the solid residue was subjected to silica gel column chromatography (hexane/EtOAc = 4/1 as an eluent) to yield **DAB** as a green solid (12.0 mg, 86%) and **DAC** as a green solid (0.4 mg, 3%).

Compounds Data

DAC: ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.95 (d, J = 5.0 Hz, 1H, β-H), 8.70 (d, J = 4.0 Hz, 1H, β-H), 8.64 (d, J = 4.5 Hz, 1H, β-H), 8.53 (d, J = 4.5 Hz, 1H, β-H), 8.35 (d, J = 4.5 Hz, 1H, β-H), 8.32 (d, J = 5.0 Hz, 1H, β-H), 7.25 (s, 2H, Mes-*meta*), 7.23 (s, 2H, Mes-*meta*), 4.55–4.58 (m, 2H, sp^3-β), 4.01–4.04 (m, 2H, sp^3-β), 2.59 (s, 3H, Mes-*para*), 2.56 (s, 3H, Mes-*para*), 1.90 (s, 6H, Mes-*ortho*), 1.89 (s, 6H, Mes-*ortho*), -0.49 (br-s, 2H, inner-NH) ppm; ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$): δ 175.9, 173.5, 160.5, 151.5, 146.0, 145.3, 141.9, 138.9, 138.5, 138.2, 138.1, 136.0, 135.5, 133.8, 132.8, 132.1, 131.5, 129.1, 128.2, 128.2, 128.0, 126.5, 122.5, 113.0, 35.3, 35.1, 21.6, 21.5, 21.3, 21.1 ppm. UV/Vis (CH_2Cl_2): λ_{max} (ε [$M^{-1}cm^{-1}$]) = 389 (86 000), 491 (7 000), 522 (8 600), 610 (6 200), and 665 (67 000) nm; HR-MS (ESI-MS): m/z = 551.2926, calcd for $(C_{36}H_{34}N_6)^+$ = 551.2918 [$(M + H)^+$].

DAB: ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.52–8.53 (m, 2H, β-H), 8.08–8.09 (m, 2H, β-H), 7.22 (s, 4H, Mes-*meta*), 4.42–4.45 (m, 4H, sp^3-β), 3.85–3.88 (m, 4H, sp^3-β), 2.54 (s, 6H, Mes-*para*), 1.89 (s, 12H, Mes-*ortho*), –0.69 (br-s, 2H, inner-NH) ppm; ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$): δ=169.5, 166.2, 143.9, 138.0, 137.9, 136.3, 135.6, 128.9, 124.0, 123.4, 116.0, 35.3, 33.7, 21.5, 20.8 ppm; UV/Vis (CH_2Cl_2): λ_{max} (ε [$M^{-1}cm^{-1}$]) = 325 (92 000), 371 (86000), 431 (5 100), 448 (6 800), 482 (14 000), 666 (4 900), 731 (11 000), 772 (130 000) nm; HR-MS (ESI-MS): m/z = 553.3057, calcd for $(C_{36}H_{36}N_6)^+$ = 553.3074 [$(M + H)^+$]. Single crystals were obtained by vapor diffusion of methanol into a chlorobenzene solution. $C_{36}H_{36}N_6$, M_w = 552.71, monoclinic, space group $P2_1/c$, a = 13.0765(6), b = 13.7569(6), c = 8.1842(4) Å, β = 101.4200(10)°, V = 1443.13(12) $Å^3$, Z = 2, D_{calc} = 1.272 g/cm^3, T = 113.(2) K, R = 0.0405 ($I > 2.0\ \sigma(I)$), R_w = 0.1014 (all data), GOF = 1.061. Crystallographic data for **DAB** has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-1865986.

NMR Spectra of Compounds

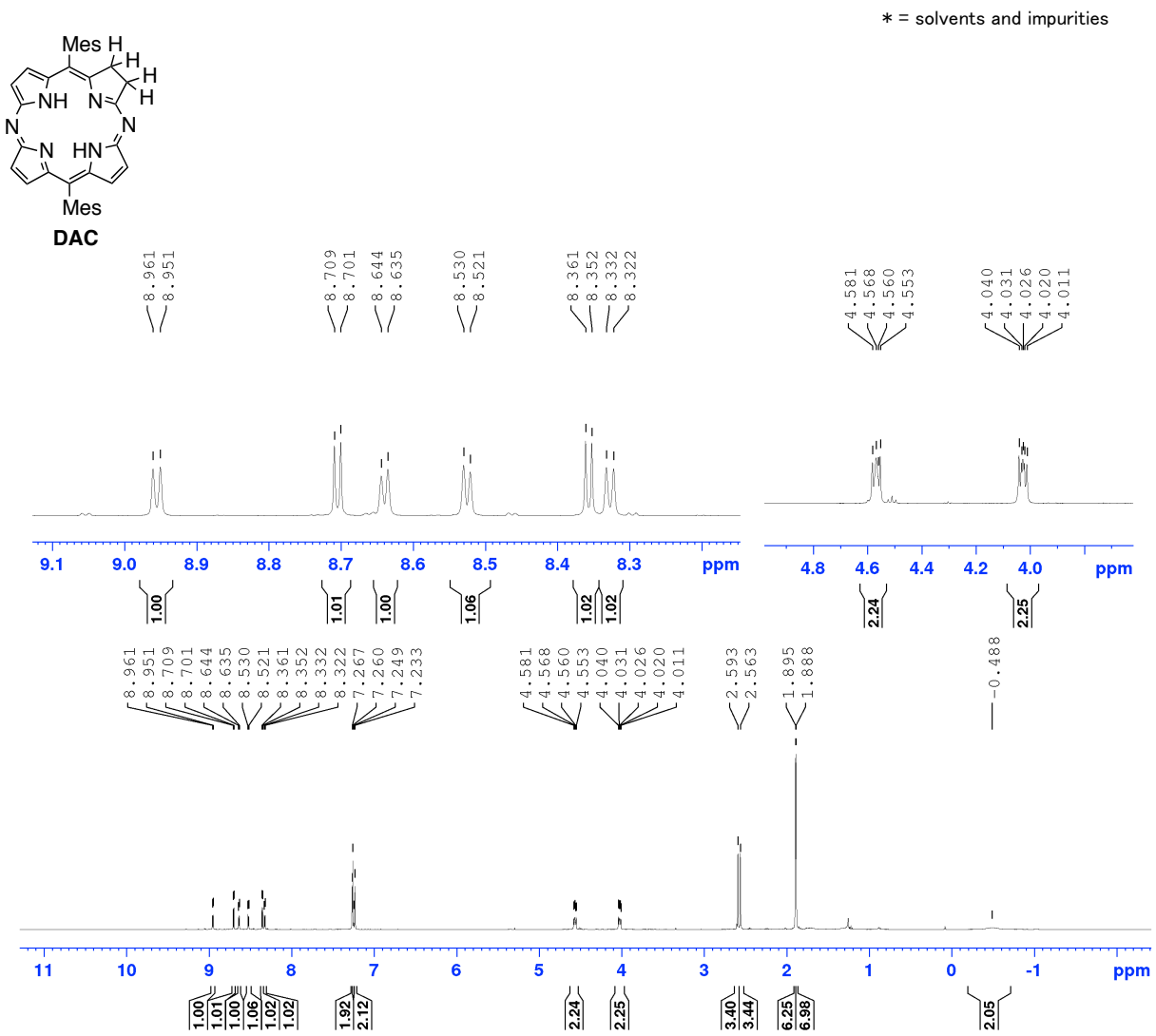


Figure S1. ^{1}H NMR spectrum of **DAC** in $CDCl_3$.

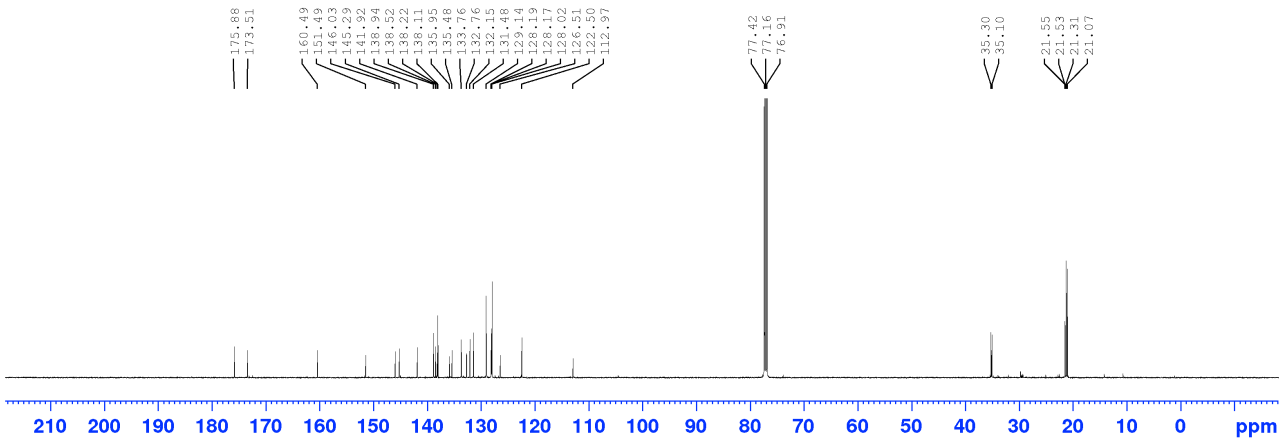
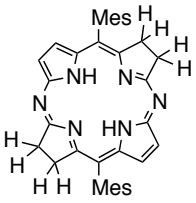


Figure S2. ^{13}C{^{1}H} NMR spectrum of **DAC** in $CDCl_3$.



DAB

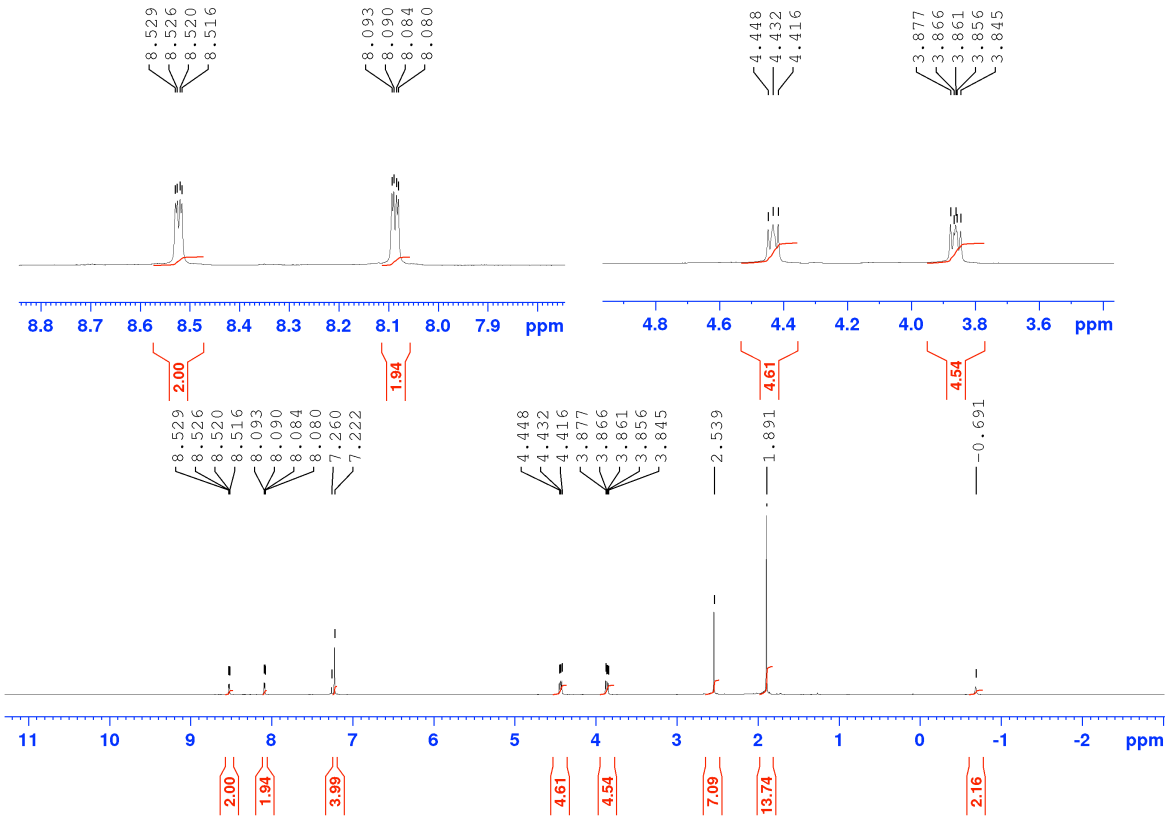


Figure S3. ^{1}H NMR spectrum of **DAB** in $CDCl_3$.

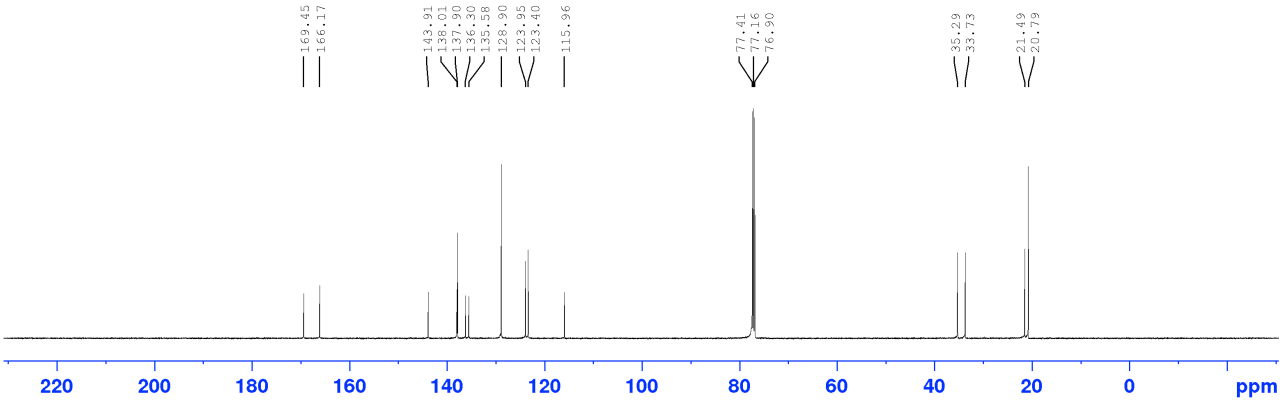


Figure S4. $^{13}C\{^{1}H\}$ NMR spectrum of **DAB** in $CDCl_3$.

Stability of DAC and DAB

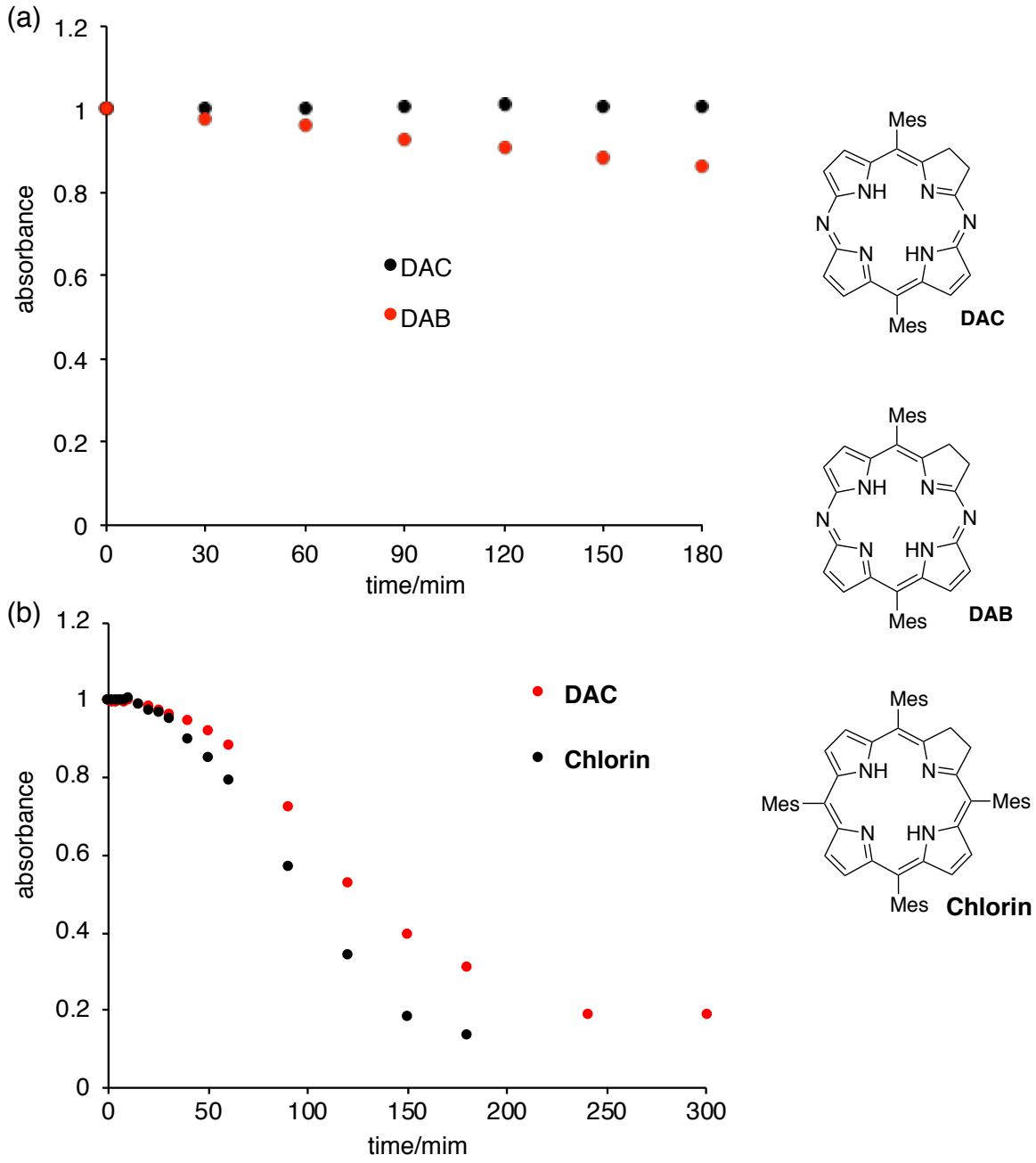


Figure S5. (a) Change of absorbance of **DAC** (665 nm) and **DAB** (772 nm) in CH_2Cl_2 (5.0 μM) under ambient conditions (room light). (b) Change of absorbance of **DAC** (665 nm) and **Chlorin** (652 nm) in CH_2Cl_2 (5.0 μM) at room temperature upon photoirradiation (>380 nm) with high-pressure mercury lamp (USHIO USH-250SC (250W)).

Singlet Oxygen Generation Quantum Yield

In order to evaluate the singlet oxygen generation quantum yield, photoluminescence (PL) measurements were carried out using a SPEX Nanolog 3–211 (Horriba) spectrofluorimeter. The PL signal was analyzed using an NIR spectrometer equipped with an InGaAs array detector (Symhony II). Tetraphenylporphyrin (TPP) in aerated toluene was chosen as a reference sensitizer (Φ_Δ = 0.70)[S3] to evaluate the singlet oxygen generation efficiency of diazaporphyrins. **DAP**, **DAC** and **DAB** display the PL bands of singlet oxygen over the range 1240-1320 nm with the peak maxima at 1272 nm. Applying equation 1, the singlet oxygen quantum yields for both samples can be determined.[S4, S5]

$$\Phi_\Delta^{\,s} = \Phi_\Delta^{\,r} \frac{(1-10^{Abs^r})}{(1-10^{Abs^s})} \frac{I_\Delta^{\,s}}{I_\Delta^{\,r}} \qquad (1)$$

While $\Phi_\Delta^{\,r}$ (0.70) is the singlet oxygen generation efficiency of TPP as a reference in aerated toluene, $I_\Delta^{\,s}$ and $I_\Delta^{\,r}$ stand for the singlet oxygen PL intensities at the maxima peaks for the samples and TPP, respectively, and $Abs^{s,r}$ refer to the absorbance of the samples and TPP at the excitation wavelength, respectively.

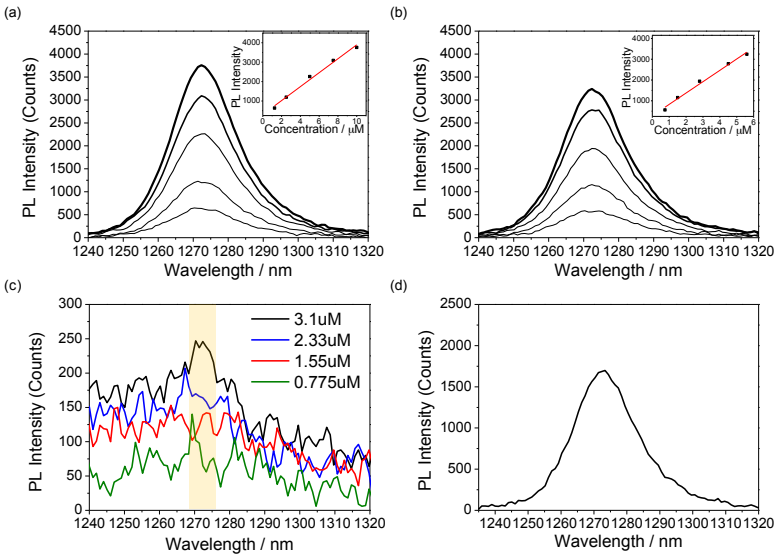


Figure S6. Photoluminescence from singlet oxygen in aerated toluene. The sensitizers are (a) **DAP**, (b) **DAC**, (c) **DAB**, and (d) **H_2TPP** (Reference, 32.6 μM) excited at 629, 667, 771 and 646 nm, respectively. Inset shows a linear fit according to the PL intensity maxima at 1272 nm versus concentration.

Time-Resolved Spectroscopy

Femtosecond Transient Absorption Measurements

The femtosecond time-resolved transient absorption (*fs*-TA) spectrometer consists of an optical parametric amplifier (OPA; Palitra, Quantronix) pumped by a Ti: sapphire regenerative amplifier system (Integra-C, Quantronix) operating at 1 kHz repetition rate and an optical detection system. The generated OPA pulses have a pulse width of ~ 100 fs and an average power of 1 mW in the range of 280-2700 nm, which are used as pump pulses. White light continuum (WLC) probe pulses were generated using a sapphire window (3 mm thick) by focusing a small portion of the fundamental 800 nm pulses, which was picked off by a quartz plate before entering the OPA. The time delay between pump and probe beams was carefully controlled by making the pump beam travel along a variable optical delay (ILS250, Newport). Intensities of the spectrally dispersed WLC probe pulses are monitored by a High Speed Spectrometer (Ultrafast Systems) for both visible and near-infrared measurements. To obtain the time-resolved transient absorption difference signal (ΔA) at a specific time, the pump pulses were chopped at 500 Hz and absorption spectra intensities were saved alternately with or without pump pulse. Typically, 4000 pulses excite the samples to obtain the *fs*-TA spectra at each delay time. The polarization angle between pump and probe beam was set at the magic angle (54.7°) using a Glan-laser polarizer with a half-wave retarder in order to prevent polarization-dependent signals. Cross-correlation *fwhm* in pump-probe experiments was less than 200 fs and chirp of WLC probe pulses was measured to be 800 fs in the 400-800 nm region. To minimize chirp, all reflection optics in the probe beam path and a quartz cell of 2 mm path length were used. After *fs*-TA experiments, the absorption spectra of all compounds were carefully examined to detect if there were artifacts due to degradation and photo-oxidation of samples. The three-dimensional data sets of ΔA versus time and wavelength were subjected to singular value decomposition and global fitting to obtain the kinetic time constants and their associated spectra using Surface Xplorer software (Ultrafast Systems).

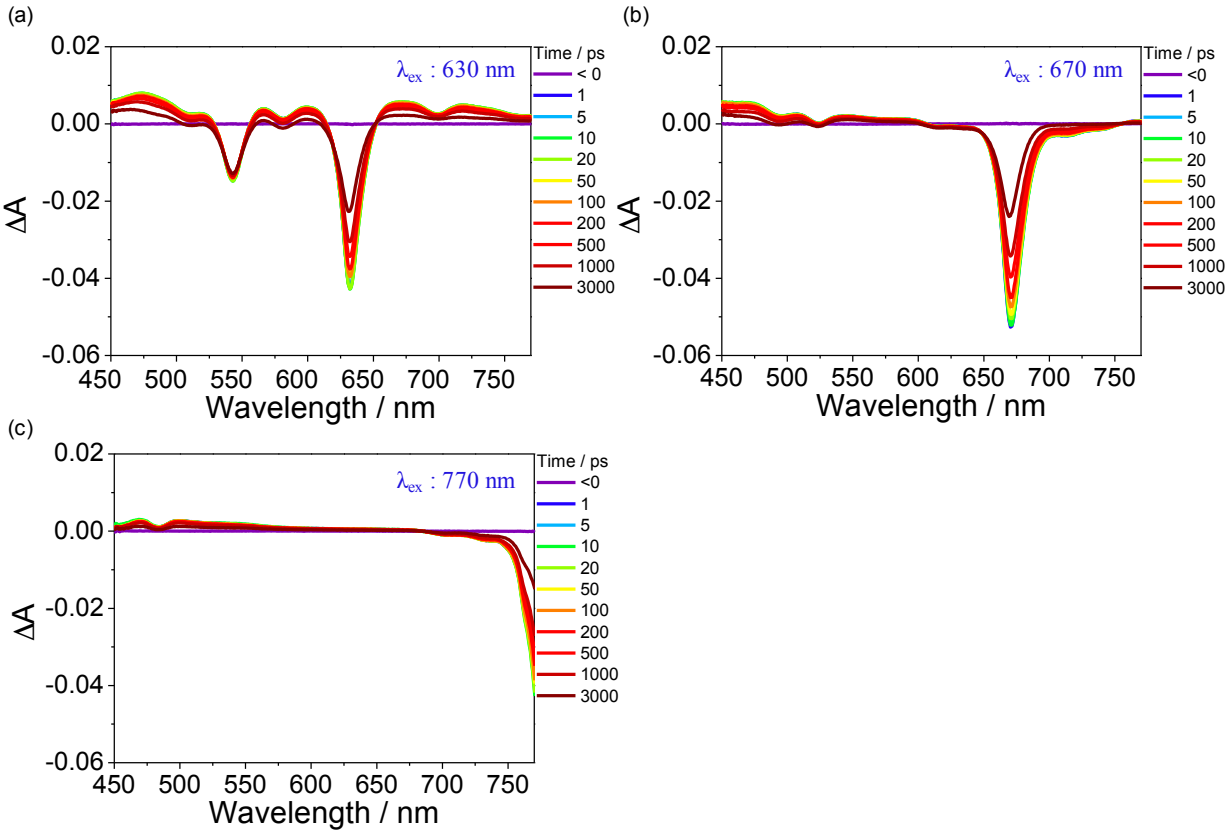


Figure S7. Femtosecond transient absorption spectra of (a) **DAP**, (b) **DAC**, and (c) **DAB** in argon purged toluene obtained by photoexcitation at 630 nm (for **DAP**), 670 nm (for **DAC**) and 770 nm (for **DAB**).

Sub-Nanosecond Transient Absorption Measurements

The sub-nanosecond time-resolved transient absorption spectrometer consists of Optical Parametric Amplifiers (Palitra, Quantronix) pumped by a Ti:sapphire regenerative amplifier system (Integra-C, Quantronix) operating at 1 kHz repetition rate for excitation pulse, Ti:sapphire laser (Maitai BB, SpectraPhysics) which provides a repetition rate of 80 MHz with ~100 fs pulses and an optical detection system. The generated OPA pulses, which were used as pump pulses, had a pulse width of ~100 fs and an average power of 100 mW in the range 280–2700 nm. 80 MHz pulse train in the range from 710–990 nm from Ti:sapphire laser generates supercontinuum pulses, which were used as probe pulses, by photonic crystal fiber (FemtoWHITE 800, NKT Photonics). To obtain the time-resolved transient absorption difference signal (ΔA) at a specific time, randomly-interleaved-pulse-train method[S6] is used with photodiodes (FPD510-V, Menlosystems) and oscilloscope. To minimize the triplet annihilation process, measurements were conducted under paraffin oil condition.

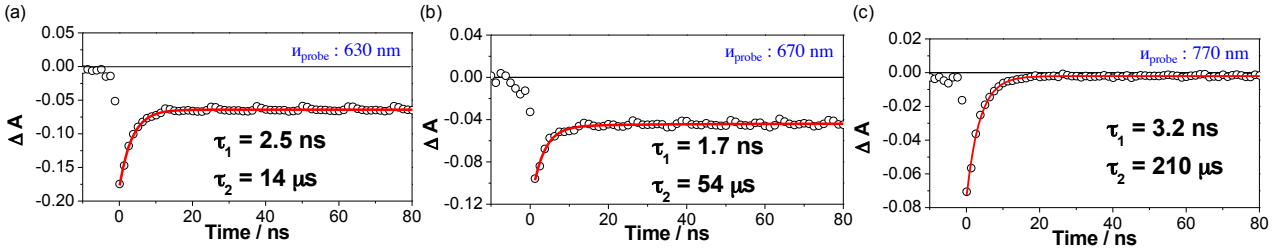


Figure S8. Sub-nanosecond TA kinetic profiles of (a) **DAP**, (b) **DAC**, and (c) **DAB** recorded in argon purged toluene.

Nanosecond Transient Absorption Measurements

The nanosecond transient absorption spectra were obtained using nanosecond flash photolysis techniques. Specifically, a tunable excitation pulse was generated using an Optical Parametric Oscillator system (Continuum, Surelite OPO), which was pumped by 355 nm from the third-harmonic output of a Q-switched Nd:YAG laser (Continuum, Surelite II-10). The time duration of the excitation pulse was ca. 6 ns, and the 11 pulse energy was ca. 2 mJ/pulse. A CW Xe lamp (150 W) was used as the probe light source for the transient absorption measurement. The probe light was collimated on the sample cell and was spectrally resolved using a 15 cm monochromator (Acton Research, SP150) equipped with a 600 grooves/mm grating after passing the sample. The spectral resolution was approximately 3 nm for the transient absorption experiment. The light signal was detected using an avalanche photodiode (APD). The output signal from the APD was recorded using a 500 MHz digital storage oscilloscope (Lecroy, WaveRunner 6050A) for the temporal profile measurement. Since the triplet-state dynamics of molecules in solution are strongly dependent on the concentration of oxygen molecules dissolved in solution, we attempted to remove oxygen by degassing with Ar gas for 15 min. To minimize the triplet annihilation process, measurements were conducted under paraffin oil condition.

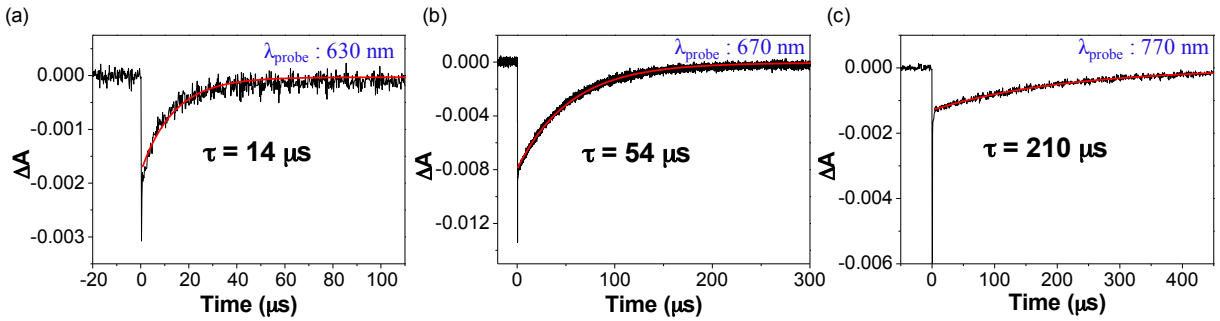


Figure S9. Nanosecond TA kinetic profiles of (a) **DAP**, (b) **DAC** and (c) **DAB** recorded in argon purged toluene.

Picosecond Time-Resolved Fluorescence Measurements

A time-correlated single-photon-counting (TCSPC) system was used for measurements of spontaneous fluorescence decay. As an excitation light source, we used a mode-locked Ti:sapphire laser (Spectra Physics, MaiTai BB) which provides ultrashort pulse (center wavelength of 800 nm with 80 fs at FWHM) with high repetition rate (80 MHz). This high repetition rate was reduced to 800 kHz by using homemade pulse-picker. The pulse-picked output was frequency doubled by a 1-mm-thick BBO crystal (type-I, θ = 29.2°, EKSMA). The fluorescence was collected by a microchannel plate photomultiplier (MCP-PMT, Hamamatsu, R3809U51) with a thermoelectric cooler (Hamamatsu, C4878) connected to a TCSPC board (Becker & Hickel SPC-130). The overall instrumental response function was about 25 ps (FWHM). A vertically polarized pump pulse by a Glan-laser polarizer was irradiated to samples, and a sheet polarizer set at an angle complementary to the magic angle (54.7°), was placed in the fluorescence collection path to obtain polarization-independent fluorescence decays.

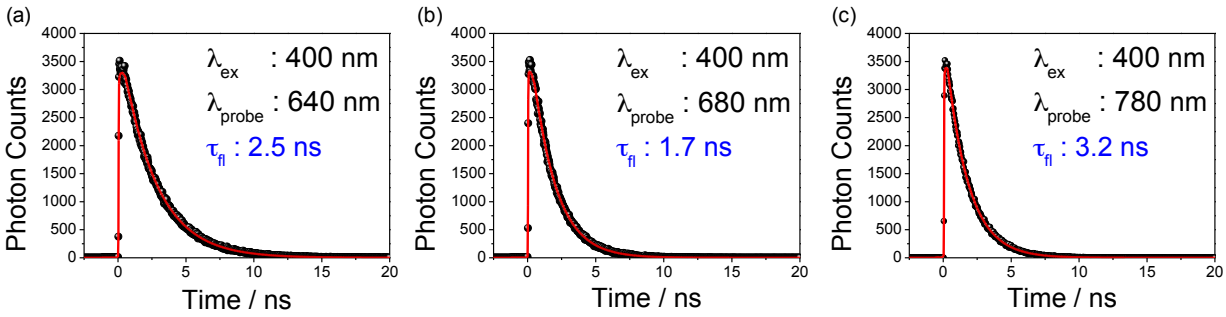


Figure S10. Time-resolved fluorescence decay profiles of (a) **DAP**, (b) **DAC** and (c) **DAB** recorded in toluene.

In Vitro One-Photon Excitation Photodynamic Therapy

Cell Culture

For cell culture, MCF-7 human breast cancer cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 50 μg mL^{-1} gentamycin. Cell types were then grown in humidified atmosphere at 37 °C under 5% CO_2.

Fluorescence Imaging on MCF-7 Cells

Cells were grown on histological slides in complete culture medium and exposed overnight to **DAP**, **DAC** or **DAB** (15 μM). Slides were washed with phosphate-buffered saline (PBS) and examined under a fluorescence microscope (Axioplan2, Carl Zeiss, Feldbach, Switzerland) with filters set at 365 nm excitation light (BP 365/12, FT 395, LP 397) for the green fluorescence of CellMask™ (cell membranes visualization) and 535 nm excitation light (BP 510–560, FT 580, LP 590) for the red fluorescence of diazaporphyrins.

Photodynamic Therapy

Cells were seeded into 96-well plates at 3×10^3 cells/well in 100 μL of culture medium and allowed to grow for 24 h. Cells were then incubated for 24 h with DMF solutions supplemented with **DAP**, **DAC**, or **DAB**. After incubation, cells were irradiated with laser (630–680 nm; 6 mW cm^{-2}). Two days after irradiation, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the phototoxicity of **DAP**, **DAC** or **DAB**. Cells were incubated in the presence of MTT (0.5 mg mL^{-1}) for 4 h to determine the mitochondrial enzyme activity. Then, MTT precipitates were dissolved in 150 μL of an ethanol/DMSO (1:1) solution and its absorbance at 540 nm was recorded.

Imaging and PDT Results on MCF-7 Cells

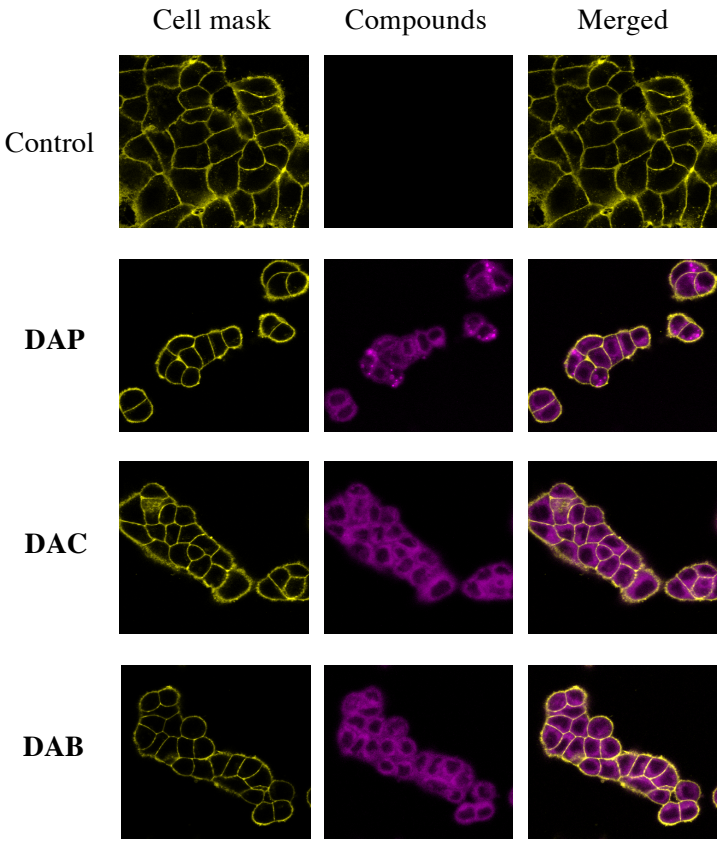


Figure S11. Fluorescence imaging of MCF-7 cells incubated for 24 h with 15 μM of **DAP**, **DAC** and **DAB**. The cell membranes appear in yellow and **DAP**, **DAC** and **DAB** in purple.

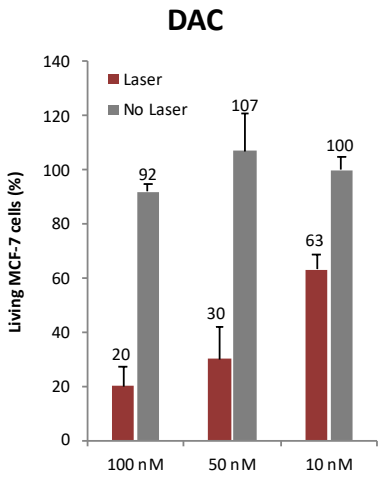


Figure S12. PDT evaluations of **DAC** at lower concentrations on MCF-7 cells under monophotonic irradiation (650 nm).

ROS Production Analysis

MCF-7 cells were seeded into 96-well plates at 10^4 cells/well in 200 μL of culture medium and allowed to grow for 24 h. Cells were then incubated for 24 h with DMF solutions of **DAP**, **DAC** or **DAB**. Then, cells were incubated 45 min with DCFDA solution and irradiated in the same conditions as for PDT experiments. After irradiation, cells were rinsed and imaged on a fluorescent microscope under a 490 nm excitation as described in the DCFDA-cellular Reactive Oxygen Species Detection Assay Kit (abcam).

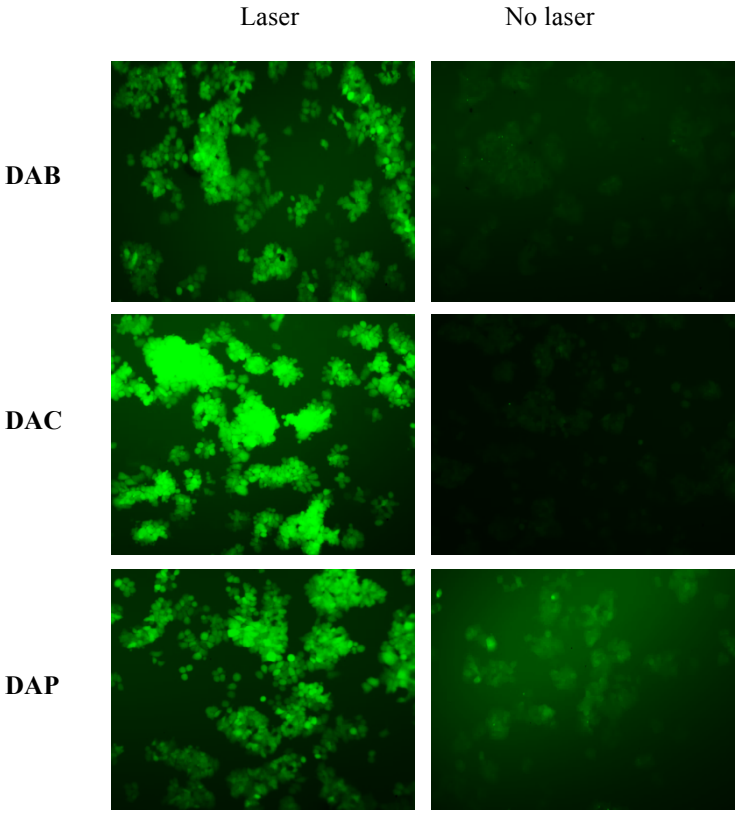


Figure S13. ROS visualization in MCF-7 cells using fluorescent microscope. Cells were incubated with **DAB** or **DAC** or **DAP** (5 μM) during 24 h. Then, cells were incubated with the DCFH2-DA reagent (for 45 minutes) which will be oxidized to DCF in the presence of ROS. Cells were excited with a monophotonic laser (630–680 nm; 6 mW cm^{-2}) and observed on a fluorescent microscope. Fluorescence intensity is proportional to the amount of generated ROS.

Two-Photon Absorption Measurements

The two-photon absorption spectrum was measured in the NIR region using the open-aperture Z-scan method with 176 fs pulses from an optical parametric amplifier (Light Conversion, ORPHEUS) operating at a repetition rate of 10 kHz generated from a Yb:KGW regenerative amplifier system (Light Conversion, PHAROS). After passing through a 10 cm focal length lens, the laser beam was focused and passed through a 1 mm quartz cell. Since the position of the sample cell could be controlled along the laser beam direction (z axis) using the motored controlled delay stage, the local power density within the sample cell could be simply controlled under constant laser intensity. The transmitted laser beam from the sample cell was then detected by the same photodiode as used for reference monitoring. The on-axis peak intensity of the incident pulses at the focal point, I_0, ranged from 60 to 130 GW cm^{-2}. For a Gaussian beam profile, the nonlinear absorption coefficient can be obtained by curve fitting of the observed open-aperture traces $T(z)$ with the following equation:

$$T(z)=1-\frac{\beta I_0(1-e^{-\alpha_0 l})}{2\alpha_0[1+(z/z_0)^2]}$$

where α_0 is the linear absorption coefficient, l is the sample length, and z_0 is the diffraction length of the incident beam. After the nonlinear absorption coefficient has been obtained, the TPA cross section $\sigma^{(2)}$ of one solute molecule (in units of GM, where 1 GM = 10^{-50} cm^4 s photon^{-1} molecule^{-1}) can be determined by using the following relationship:

$$\beta=\frac{10^{-3}\sigma^{(2)}N_A d}{h\nu}$$

Where N_A is the Avogadro constant, d is the concentration of the compound in solution, h is the Planck constant, and ν is the frequency of the incident laser beam.

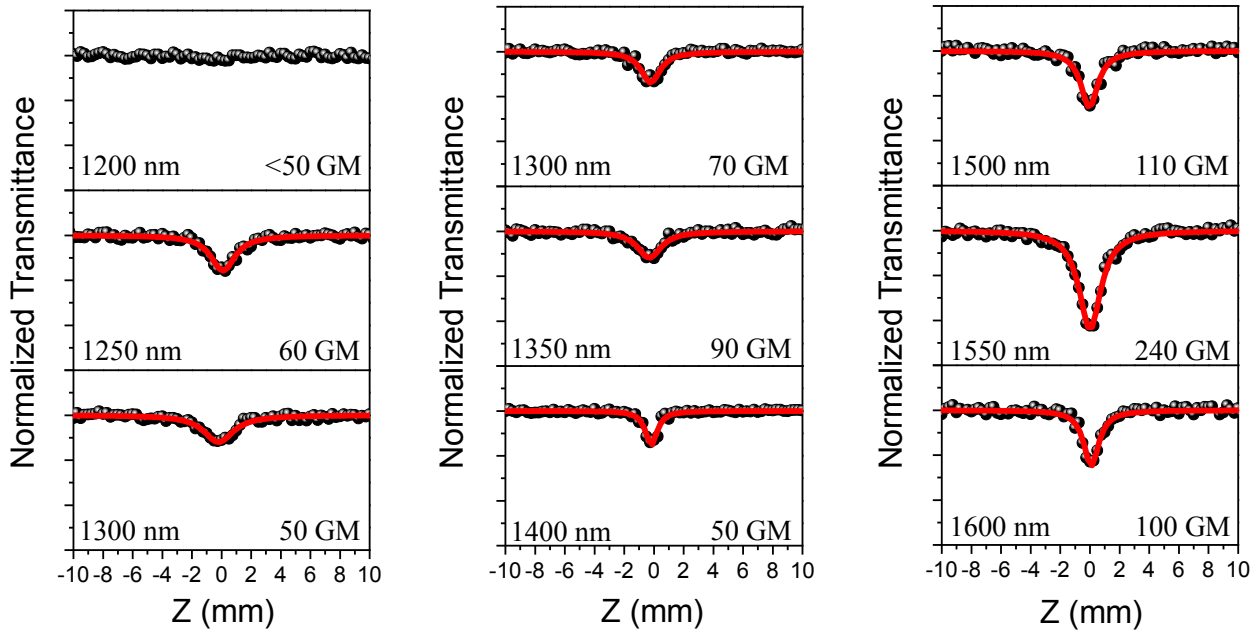


Figure S14. Z-scan curves of **DAP** (left), **DAC** (middle) and **DAB** (right) recorded in toluene. The fitted curves were probed by photoexcitation in the range from 1200 to 1600 nm.

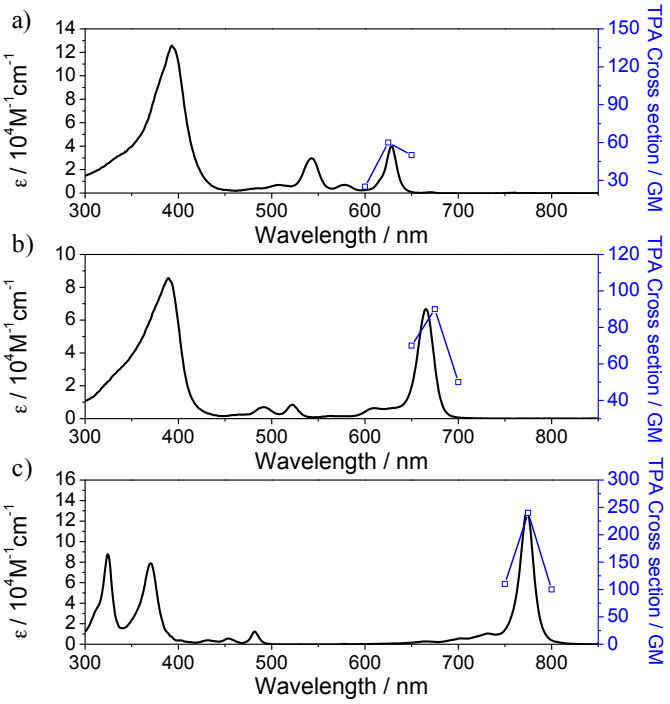


Figure S15. One-photon and two-photon absorption of (a) **DAP**, (b) **DAC** and (c) **DAB** recorded in toluene. The TPA spectra are displayed at $\lambda_{ex}/2$.

In Vitro Two-Photon Excitation Photodynamic Therapy

Cell Culture

Human breast adenocarcinoma cells MDA-MB-231 expressing the green fluorescence protein (GFP) (purchased from ATCC) were cultured in DMEM Media - GlutaMAX™-I (containing 4.5 g L^{-1} of D-glucose) supplemented with 10% fetal bovine serum and 50 μg mL^{-1} gentamycin and allowed to grow in humidified atmosphere at 37 °C under 5 % CO_2.

Cytotoxicity Measurement

For in vitro cytotoxicity analysis, MDA-MB-231 cells were seeded into a 96 well plate, 2000 cells per well in 200 μL of culture medium and allowed to grow for 24 h. Then cells were treated with increasing concentrations of **DAB**, and after 3 days, the MTT assay was performed as previously described.[1]

Two-Photon Fluorescence Imaging

The day prior to the experiment, MDA-MB-231 cells expressing GFP were seeded onto bottom glass dishes (World Precision Instrument, Stevenage, UK) at a density of 10^6 cells.cm^{-2}. Adherent cells were then washed once and incubated in 1 mL medium with or without **DAB** at a concentration of 5 μM for 24 h. Fifteen minutes before the end of incubation, cells were loaded with Cell Mask orange (Invitrogen, Cergy Pontoise, France) for membrane staining at a final concentration of 5 μg mL^{-1}. Before visualization, cells were gently washed with cell media. Cells were then scanned with an LSM 780 LIVE confocal microscope (Carl Zeiss, Le Pecq, France), at 760 or 800 nm for **DAB**, 488 nm for GFP and 561 nm for cell membranes. All images were performed with a high magnification (63 × /1.4 OIL DIC Plan-Apo).

Two-Photon Excitation Photodynamic Therapy

MDA-MB-231 cells were seeded into a 384 multi-well glass-bottomed plate (thickness 0.17 mm) with a black polystyrene frame at a concentration of 1000 cells per well in 50 μL of culture medium, and allowed to grow for 24 h. Then, cells were treated with **DAB** (5 μM) and cells were submitted or not to laser irradiation after 24 h with the Carl Zeiss Microscope (laser power input 3W). Half of the well was irradiated at 760, 800 or 980 nm by three scans of 1.57 s duration in 4 different areas of the well. The laser beam was focused by a microscope objective lens (Carl Zeiss 10 × /0.3 EC Plan-Neofluar). The scan size does not allow irradiating more areas without overlapping. After 2 days, the MTT assay was performed as previously described[1] and was corrected according to the following formula Abs control – 2 × (Abs control – Abs **DAB**).

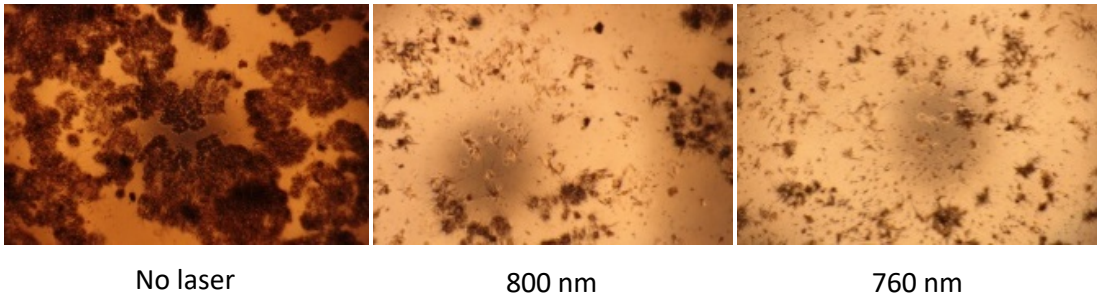


Figure S16. Photographs of MDA-MB-231 cells after TPE-PDT treatment with **DAB**.

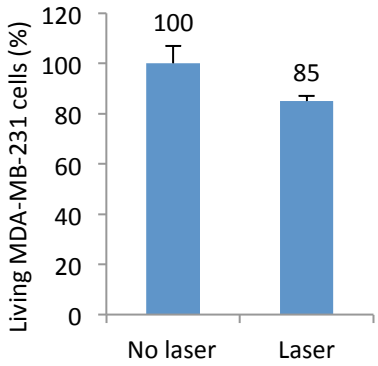


Figure S17. PDT activity of **DAB** under 980 nm biphotonic irradiation at 5 µM.

ROS Production Analysis

MDA-MB-231 cells were seeded into 384-well plates at 1000 cells per well in 50 µL of culture medium and allowed to grow for 24 h. Cells were then incubated for 24 h with DMF solutions of **DAB**. Then, cells were incubated 45 min with DCFDA solution. After incubation, cells were rinsed and imaged on a fluorescent microscope under a 490 nm excitation as described in the DCFDA-cellular Reactive Oxygen Species Detection Assay Kit (abcam). This imaging is performed before and after biphotonic irradiation at 760 nm (1 pulse of 1.57 secondes).

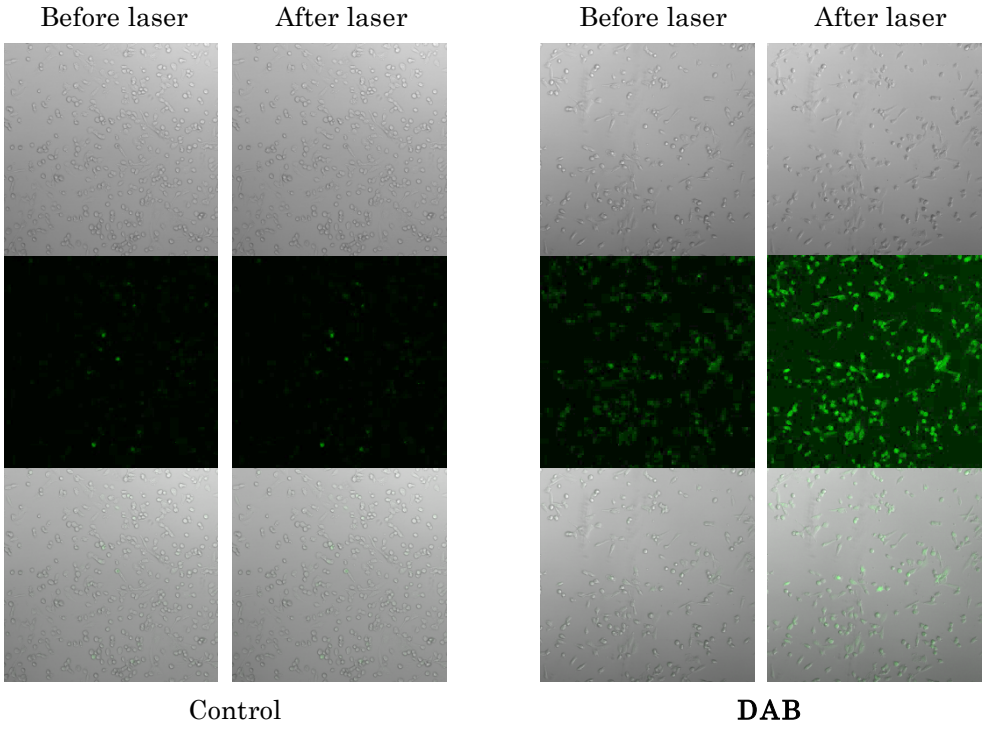


Figure S18. ROS visualization in MDA-MB-231 cells using confocal microscopy. ROS production of **DAB** (5 µM) under 760 nm biphotonic irradiation. Before biphotonic irradiation, the cells were incubated with the DCF-DA reagent (for 45 min), which is oxidized in DCF in presence of ROS. Fluorescence intensity is proportional to the amount of generated ROS (detection at 535 nm).

In Vivo Two-Photon Excitation Photodynamic Therapy

Cells Preparation for Injection into Zebrafish Larvae

The day of experiment, the MDA-MB-231 cells were washed twice, trypsined and then, stained with 2 µg mL^{-1} of Dil Stain (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Invitrogen™), for 15 min at 37 °C. In parallel, the cells were deposited on a counting chamber (MARIENFELD SUPERIOR), to determine the number of cells per liquid volume unit. The stained

cells were diluted to a final concentration of 10^8 cells mL^{-1} in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 10% fetal bovine serum. The cells were maintained in ice until injection, which must be done within 3 h after the preparation of the cells.

Zebrafish

All experiments with zebrafish embryos were done according to the guidelines of the European Community Council directive 2010/63/EU and to the accreditation number of the zebrafish facility A34-172-37 of the University of Montpellier. *Casper* line, a zebrafish pigmentation mutant, were purchased from the Zebrafish International Resource Center (ZIRC) as embryos and raised to adulthood in Verdier's lab facilities. Only fish directly from ZIRC or their F1 offspring were used as egg producers to avoid inbreeding effects. Embryos were obtained from pairs of adult fish by natural spawning and raised at 28.5 °C in tank water. Embryos and larvae were staged according to the literature.[2]

Injection of DAB

Zebrafish larvae were manually dechorionated and anesthetized. 100 cells.nL^{-1} were injected into the perivitelline space of larvae aged of 24 to 30 h post fertilization (hpf); between 5 and 10 nL were injected each time. The injected or non-injected larvae were allowed to develop at 32 °C and were observed at 24 h post injection (hpi) under an Olympus MVX10 epifluorescence microscope. Only the larvae with homogeneous and comparable xenografted tumor were kept. Then, **DAB** was diluted to 40 μM in water solution containing 1% phenol red and intravenously injected (5 nL) in xenografted tumor zebrafish larvae of 4 days post fertilization. For each condition, the **DAB** injected or non-injected larvae were divided into 2 set: one that will be irradiated (760 nm biphotonic laser excitation) and the other not. In the order to follow and compare the xenografted tumor evolution, the irradiated or non-irradiated larvae were allowed to develop at 32 °C until the 6th day.

Two-Photon Excitation Photodynamic Therapy with Zebrafish

For two-photon excited irradiation, zebrafish larvae were placed into multi-well glass bottom (thickness 0.17 mm) and submitted to laser irradiation. Two-photon irradiation was performed on a confocal microscope equipped with a mode-locked Ti:sapphire laser generating 100 fs wide pulses at 80 MHz rate. The laser beam was focused by a microscope objective lens (10×, NA 0.4). The

wells were irradiated at 800 nm by 3 scans of 1.25 s each and the surface of the scanned areas was 1.5 × 1.5 mm^2, with a slice depth of 0.67 μm.

Zebrafish Fluorescence Imaging

The injected or non-injected larvae were imaged before and 2 days after two-photon irradiation. Each larvae was placed into multi-well glass bottom (thickness 0.17 mm) and scanned with a LSM 780 LIVE confocal microscope (Carl Zeiss, Le Pecq, France), at 561 nm. All images were performed by a confocal microscope objective 20× (Plan-Apochromat 20×/0.8 M27).

Immunofluorescence Staining *in toto* Zebrafish

The larva were fixed at 6 days post fertilization with a solution of 4% paraformaldehyde in PBS + 0.5% Triton at room temperature for 3 h under hood. Larva were then washed with PBS at least 3 times. Fixed larvae were permeabilized using Triton in PBS for 2 h at room temperature. In order to saturate the potential unspecific sites antibodies, the larvae were then blocked with blocking solution containing: PBS 1× + BSA 1× + DMSO 1× + 10% Donkey serum (Sigma-Aldrich D9663) for 1 day at 4 °C. Primary cleaved caspase-3 (Asp175) antibodies (Cell Signaling Technology) were used to specific labeling the apoptosis signal. The recommended antibody dilution is 1:400 in blocking solution containing 2% of Donkey serum. The larvae were incubated over-week end at 4 °C and then washed during the day in PBS. At the end of the day, the larvae were labeled with the secondary antibody conjugated to Cy5 at the dilution of 1:500 and incubated over night at 4 °C. Then, the larvae were washed whole day in PBS. The immunostained larvae were mounted flat onto transparent slides with a coverslip (using Dako fluorescent mounting medium).

Zebrafish 3D Imaging

Confocal fluorescence microscopy was performed using a Leica DM2500CSQ upright microscope with a Leica TCS SPE confocal scan head, differential interference contrast (DIC) optics and a SuperZGalvo SPE z-step controller. The acquisition of 3 Dimensions stacks was performed with a 63× Leica Apo oil 1.15 NA. Final image analysis and reconstitution were performed using Imaris ×64 8.02 software.

TPE-PDT Results with Zebrafish

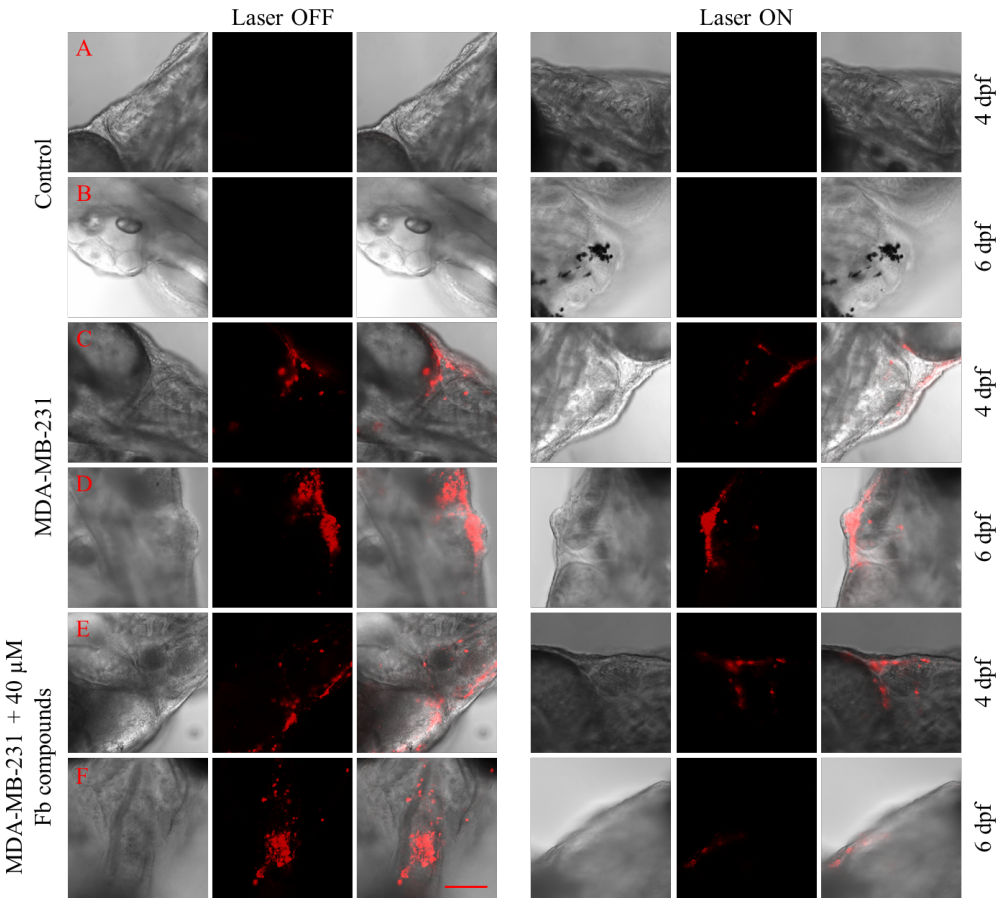


Figure S19. Confocal fluorescence microscopy of 4 and 6 days post-fertilization (dpf) live larvae. For each column (Laser OFF and Laser ON), the left panels represent the bright field channel, the middle the Dil staining (Red) and the right the overlays. All images were performed on the perivitelline space (the injection site) by the objective 20×. Scale bar represent 10 μm for all photographs. (A) 4 dpf lives control larvae. (B) 6 dpf lives control larvae. (C) 4 dpf lives larvae injected with MDA-MB-231 cells alone. (D) Xenografts evolution in 6 dpf lives larvae injected with MDA-MB-231 cells alone. (E) 4 dpf lives larvae injected with MDA-MB-231 cells and 40 μM **DAB**. (F) Xenografts evolution in 6 dpf lives larvae injected with MDA-MB-231 cells and 40 μM **DAB**.

Immunostained Zebrafish

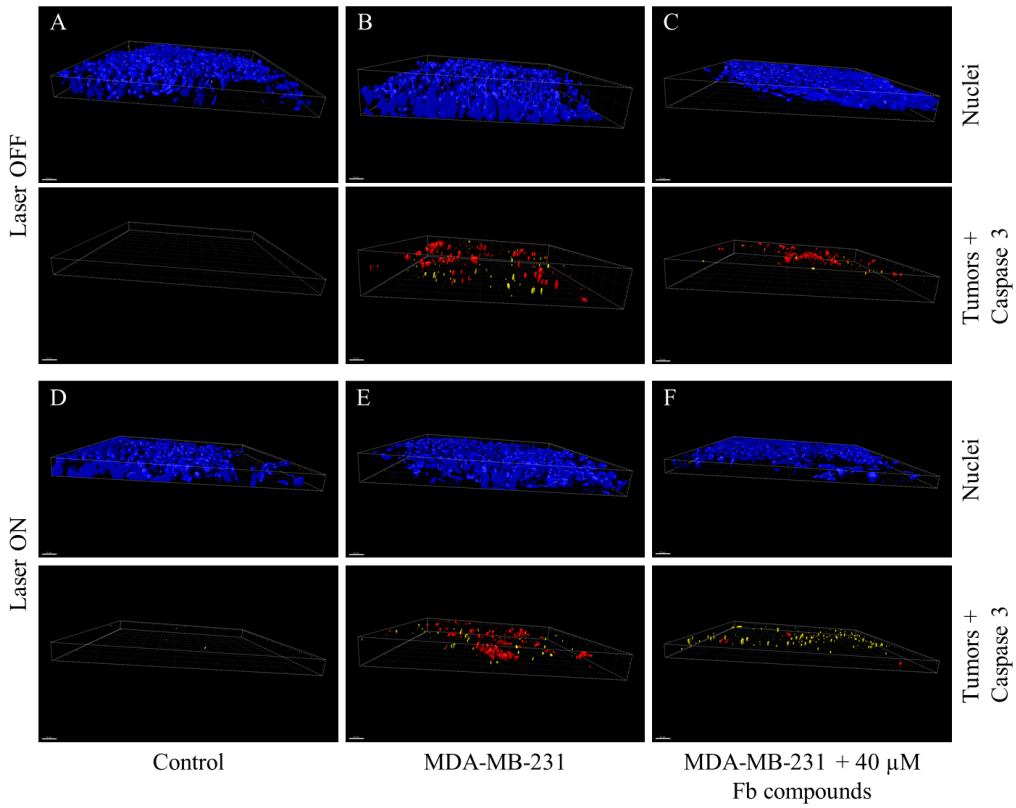


Figure S20. Xenografts apoptosis induced by **DAB**. Three dimensions reconstitution of three-dimensional stacks confocal microscopy imaging of immunostained fixed whole injected or non-injected zebrafish larvae at 6 days post fertilization. The three-dimensional stacks were performed on the perivitelline space. Each time, laser OFF and laser ON lines represent two different acquisitions: the nuclei on the top and the tumors with caspase 3 signal on the bottom. All images were performed with 63 × Leica Apo oil and the final reconstitution were performed using Imaris ×64 8.02 software. All panels represent the overlays of the three channels: blue nuclei, red MDA-MB-231 cells and yellow caspase 3. The top panels represent the zebrafish larvae without any irradiation. The bottom panels represent the zebrafish larvae submitted to two photons laser irradiation. (A) and (D) Nuclei of fixed control larvae without or with laser irradiation, respectively. (B) and (E) Red tumor extent among the blue nuclei of fixed larvae injected with MDA-MB-231 cells alone without or with laser irradiation, respectively. (C) Red tumor extent among the blue nuclei of fixed larvae injected with MDA-MB-231 cells and **DAB** (40 μM) without laser irradiation. (F) Red tumor restriction among the blue nuclei and punctuated with multiple yellow dots of fixed larvae injected with MDA-MB-231 cells and **DAB** (40 μM) after laser irradiation. Scale bar represents 10 μm for all photos.

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