Electronic Supplementary Information

Cationic octahedral molybdenum cluster complexes functionalized with mitochondria-targeting ligands: photodynamic anticancer and antibacterial activity

Kaplan Kirakci,*[a] Jaroslav Zelenka,[b] Michaela Rumlová,[c] Josef Cvačka,[d] Tomáš Ruml,[b] Kamil Lang*[a]

[a]Institute of Inorganic Chemistry of the Czech Academy of Sciences, 250 68 Řež, Czech Republic

[b]Department of Biochemistry and Microbiology, University of Chemistry and Technology Prague, Technická 5, 166 28 Praha 6, Czech Republic

[c]Department of Biotechnology, University of Chemistry and Technology Prague, Technická 5, 166 28 Praha 6, Czech Republic

[d]Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Flemingovo nám. 2, 166 10 Praha 6, Czech Republic

* Corresponding authors: kaplan@iic.cas.cz (Kaplan Kirakci), lang@iic.cas.cz (Kamil Lang)

Content

Experimental

Reagents and general procedures. Starting compound $Na_2[Mo_6I_8(OMe)_6]$ and compounds **1** and **2** were prepared according to previously published procedures [K. Kirakci, K. Fejfarová, M. Kučeráková and K. Lang, *Eur. J. Inorg. Chem.,* 2014, **14**, 2331-2336]. Molybdenum, iodine, sodium methoxide, (4-carboxybutyl)triphenylphosphonium bromide, and 4-carboxy-1-methylpyridinium chloride were obtained from Sigma Aldrich and used as received. Solvents for synthesis were purchased from Penta (Czech Republic) and dried over molecular sieves (3 Å).

Instrumental methods. ^{1}H NMR spectra were measured using a Varian Mercury 400^{Plus} instrument (Agilent Technologies, Santa Clara, CA, USA). The chemical shifts are referred to the residual ^{1}H signal of a deuterated solvent. High-resolution mass spectrometry measurements were performed using an LTQ Orbitrap XL hybrid instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. C, H, N elemental analysis was performed using a Vario EL Cube analyser (Elementar GmbH, Langenselbold, Germany).

UV-vis absorption spectra were recorded on a Perkin Elmer Lambda 35 spectrometer. Luminescence properties were monitored on a Fluorolog 3 spectrometer equipped with a cooled TBX-05-C photon detection module (Horiba Jobin Yvon) or a Hamamatsu H10330-45 photomultiplier. The latter set-up was used for the estimation of the quantum yields of singlet oxygen formation, Φ_Δ, using the comparative method with perinaphthenone as a standard (Φ_Δ = 0.98 ± 0.08 in oxygen-saturated water) [R. Schmidt, C. Tanielian, R. Dunsbach and C. Wolff, *J. Photochem. Photobiol*. 1994, **79**, 11–17]. The measurement was carried out in oxygen-saturated water solutions with matched absorbances at the excitation wavelength of 400 nm. The intensities of the $O_2(^1\Delta_g)$ phosphorescence bands for the standard and optically matched water solutions of **1** and **2** were compared. The same instrument was also used for luminescence lifetime experiments

using an excitation at 390 nm (SpectraLED-390, Horiba Scientific). The decay curves were fitted to exponential functions by the iterative reconvolution procedure of the DAS6 software (v. 6.8, Horiba Jobin Yvon). Absolute photoluminescence quantum yields were measured using a Quantaurus QY C11347-1 spectrometer (Hamamatsu). To perform measurements under different concentrations of dissolved oxygen, water solutions were saturated with oxygen, air, or argon.

The stock solutions of **1** and **2** were prepared in DMSO. To prepare water solutions, the aliquots were taken and diluted with water. The resulting DMSO concentration in solutions was less than 3 v/v%.

Preparation of $[Mo_6I_8(OCOC_4H_8PPh_3)_6]Br_4$ ***(1)***. A mixture of $Na_2[Mo_6I_8(OMe)_6]$ (200 mg, 116 μmol) and of (4-carboxybutyl)triphenylphosphonium bromide (292 mg, 696 μmol) in 5 mL DMSO was heated at 110ºC for 18 hours under Ar atmosphere. Upon cooling, 40 mL of diethyl ether were added, and the upper phase was discarded. This operation was repeated twice, yielding an orange solid that was further dissolved in 5 mL of chloroform and filtrated. Then, 40 mL of acetone were added to the resulting red solution and the precipitate was washed two times with 40 mL of diethyl ether to yield 320 mg of an orange powder after drying under reduced pressure (yield 71%).

^{1}H NMR (400 MHz, DMSO-d6, δ ppm) 1.52 (br s, 2H, CH_2), 1.63 (br s, 2H, CH_2), 1.96 (br s, 2H, CH_2), 3.59 (br s, 2H, CH_2), 7.78 (m, 15H, ar.) (Fig. S1); HRMS (ESI+) calcd. for $C_{138}H_{138}O_{12}I_8Mo_6P_6$ ($[Mo_6I_8(OCOC_4H_8PPh_3)_6]^{4+}$) *m/z* 943.8818, found 943.8846 (Fig. S2); Elemental analysis calcd (%) for $Br_4C_{138}H_{138}I_8Mo_6O_{12}P_6$: C 40.57, H 3.41; found: C 40.20, H 3.50; UV-vis (DMSO): λ/nm (ε/$M^{-1}cm^{-1}$) = 355 (7600), 400 (6100).

Preparation of $[Mo_6I_8(OCOC_5H_4NMe)_6]Cl_4$ ***(2)***. A mixture of $Na_2[Mo_6I_8(OMe)_6]$ (200 mg, 116 μmol) and of 4-carboxy-1-methylpyridinium chloride (121 mg, 696 μmol) in 5 mL DMSO was heated at 110ºC for 18 hours under Ar atmosphere. Upon cooling, 40 mL of diethyl ether were added and the upper phase was discarded after phase separation. This operation was repeated two times yielding an orange solid that was further dissolved in 5 mL of DMF and filtrated. Then, 40 mL of diethyl ether were added to the resulting orange solution and the precipitate was washed one time with 40 mL of absolute ethanol and two times with 40 mL of diethyl ether to yield 153 mg of an orange powder after drying under reduced pressure (yield 55%).

^{1}H NMR (400 MHz, DMSO-d6, δ ppm) 4.32 (s, 3H, CH_3), 8.18 (d, 1H, ar.), 8.89 (d, 1H, ar.) (Fig. S1); HRMS (ESI−) calcd. for $C_{42}H_{42}O_{12}N_6Cl_6I_8Mo_6$ ($\{[Mo_6I_8(OCOC_5H_4NMe)_6]Cl_6\}^{2-}$) *m/z* 1317.3842, found 1317.3861 (Fig. S3); Elemental analysis calcd (%) for $C_{42}Cl_4H_{42}I_8Mo_6N_6O_{12}$: C 19.74, H 1.66, N 3.29; found: C 20.33, H 2.24, N 3.51; UV-vis (DMSO): λ/nm (ε/$M^{-1}cm^{-1}$) = 360 (6100), 400 (4200).

Cell culture. Human cervix carcinoma HeLa cells were cultured in the EMEM medium (Sigma-Aldrich) supplemented with 0.5 mM glutamine and 5% fetal bovine serum at 37°C in atmosphere containing 5% CO_2. Uptake, toxicity, and phototoxicity experiments were performed 24 - 48 h after seeding. Fixed volumes of DMSO with various concentrations of **1** and **2** were added to the culture media to ensure less than 2 v/v % of DMSO in the cellular environment and incubated for the indicated time (typically 2 h). Then, the medium was changed and the cells were further processed.

Flow cytometry analysis.

A) Internalization of compounds: HeLa cells loaded with **1** or **2** were washed with phosphate-buffered saline (PBS) and trypsinized. The cells were subjected to flow cytometry analysis (excitation at 405 nm, emission recorded at 655 - 685 nm). Alternatively, bacterial cultures loaded with **1** or **2** were measured without further processing. Data are expressed as medians of cellular fluorescence intensity with subtracted medians of control (unloaded) cells/bacteria (see Fig. 3A, B; Fig. 6).

B) Apoptosis and necrosis analysis: Irradiated HeLa cells growing in a six-well plate were harvested by the standard trypsinization procedure. The cells were gently washed with cold PBS, resuspended in the annexin-binding buffer, and labeled with FITC Alexa Fluor® 488 annexin V and propidium iodide according to manufacturer's protocol for the Dead Cell Apoptosis Kit (Invitrogen). The stained cells (ten thousand events) were analyzed using a BD FACSAria III flow cytometer and the results were processed with the BD FACSDiva software 8.

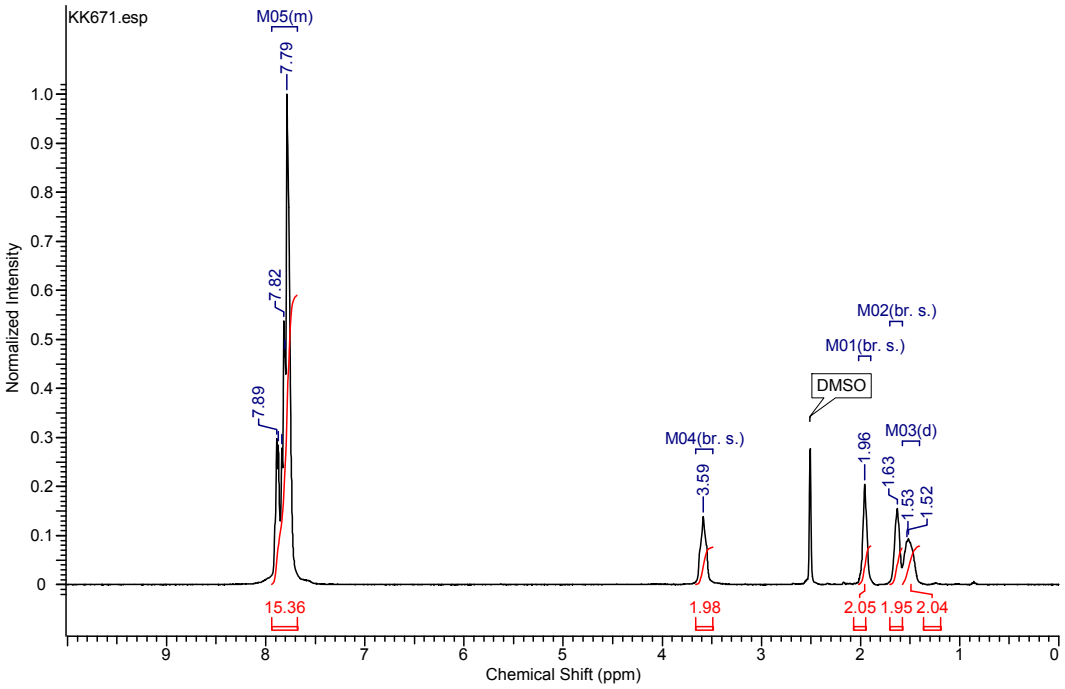
Confocal microscopy was performed with an Andor xD spinning disc confocal microscope on an Olympus platform with HeLa cells grown on Petri dishes with a coverslip bottom (MatTek) cultured under standard conditions. Luminescence of **1** was recorded using a set-up containing a 405 nm excitation laser and 600/700 nm emission filter. MitoTracker Green and LysoTracker Green (Thermo Fisher) were excited at 488 nm and corresponding fluorescence was recorded at 525 nm. The image reconstruction was performed with the iQ3 software (Andor).

Toxicity and phototototoxicity. HeLa cells were incubated with **1**, **2** or hydrolyzed **1** (11 days in water solution prior to use) in the EMEM medium for 2 h, washed with the fresh EMEM

medium, and subsequently irradiated or kept in the dark. In some cases, HeLa cells were incubated with **1** for 2 h or 24 h in the EMEM medium supplemented with 0.5 mM glutamine and 5% fetal bovine serum (comparative experiments presented in Figure S8 and S11). Irradiation was performed with a 12x10 W LED light source (Cameo) at 460 nm (15 min, 20 mW cm^{-2}). The cell viability was assayed with the resazurin assay (Sigma-Aldrich) according to manufacturer's protocol after 24 h from incubation / irradiation.

Bactericidal testing. The bacterial strains from the collections of the University of Chemistry and Technology Prague were cultured at plate count agars at 37°C overnight and used immediately or after 1 month storage at 4°C. For the toxicity determination, a suspension of bacteria with density 0.5 McFarland in phosphate-buffered saline was prepared, mixed with the compounds (**1**, **2** or hydrolyzed **1** for 11 days in water solution prior to use) dissolved in DMSO to reach maximally 5% (v/v) DMSO, and incubated 30 min in dark. Then, the sample was subjected to flow cytometry, incubated in dark, or irradiated with a blue LED light (460 nm, 20 mW cm^{-2}) for 30 min. Next, the bacterial suspensions were serially diluted and spread on plates with plate count agar. The plates were incubated overnight at 37°C. Then, the number of colony-forming units (CFU) per mL of initial inoculum was calculated.

Figure S1. ^{1}H NMR (top) spectra of **1** (top) and **2** (bottom) in DMSO-d_6.



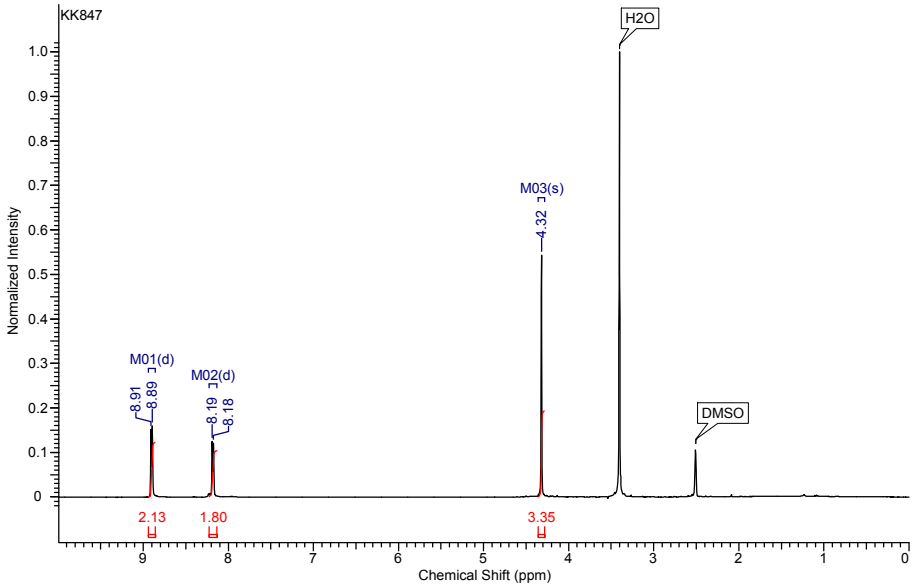


Figure S2. High resolution electrospray ionization-mass spectrum of **1** in the positive mode (top) and mass spectrum of **1** compared with the corresponding calculated pattern (bottom).

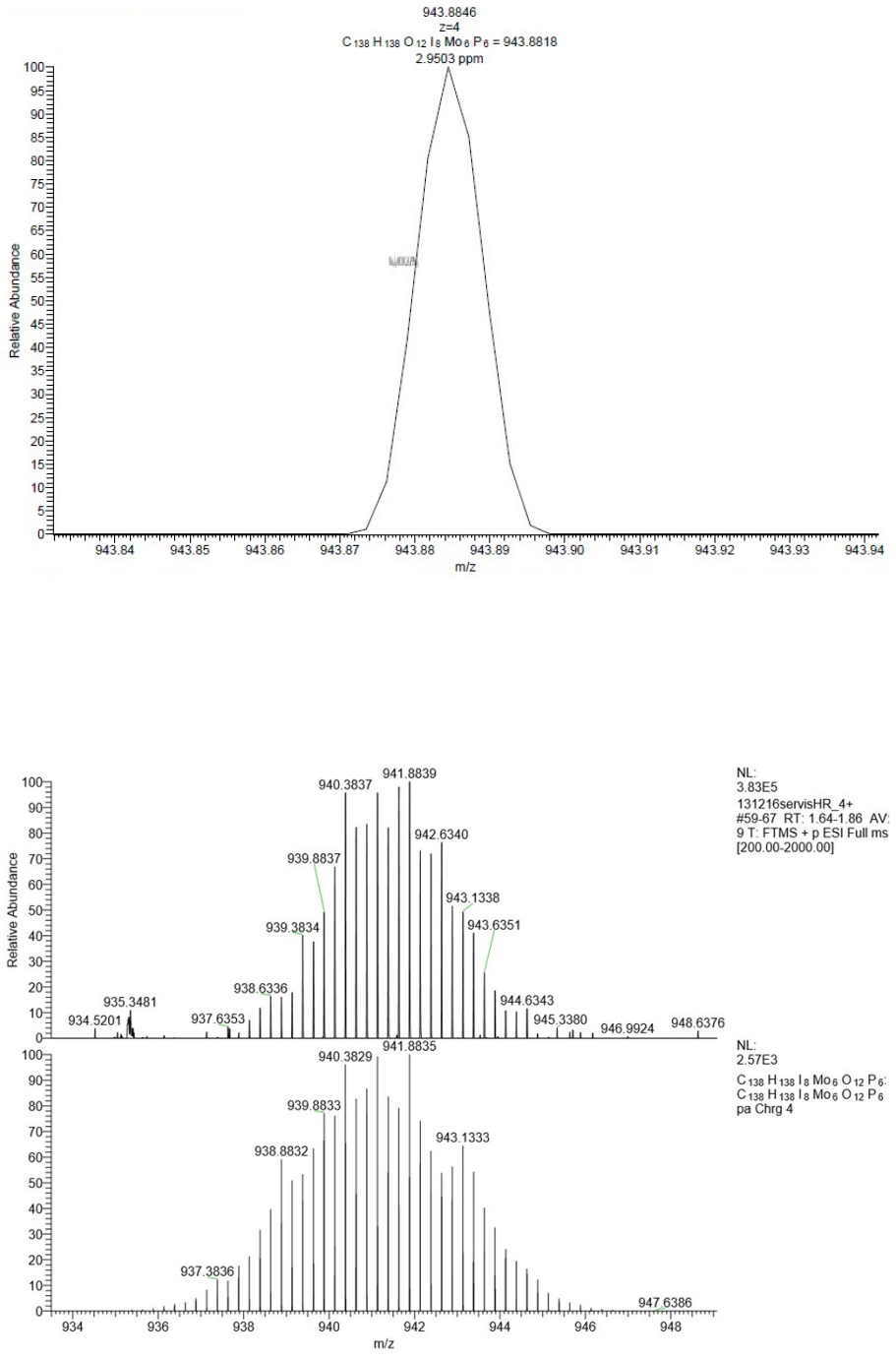


Figure S3. High resolution electrospray ionization-mass spectrum of **2** in the negative mode (top) and mass spectrum of **2** compared with the corresponding calculated pattern (bottom).

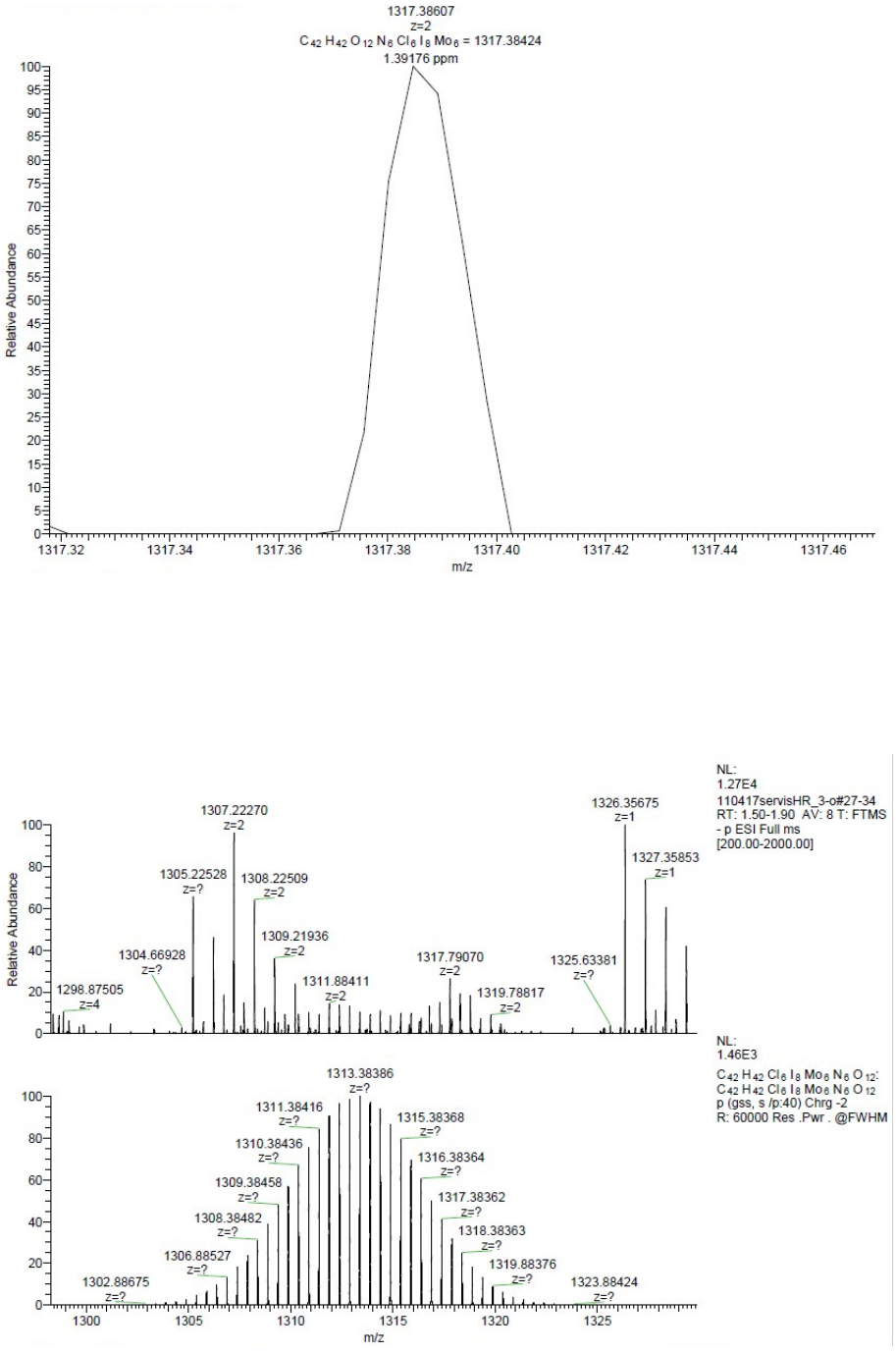
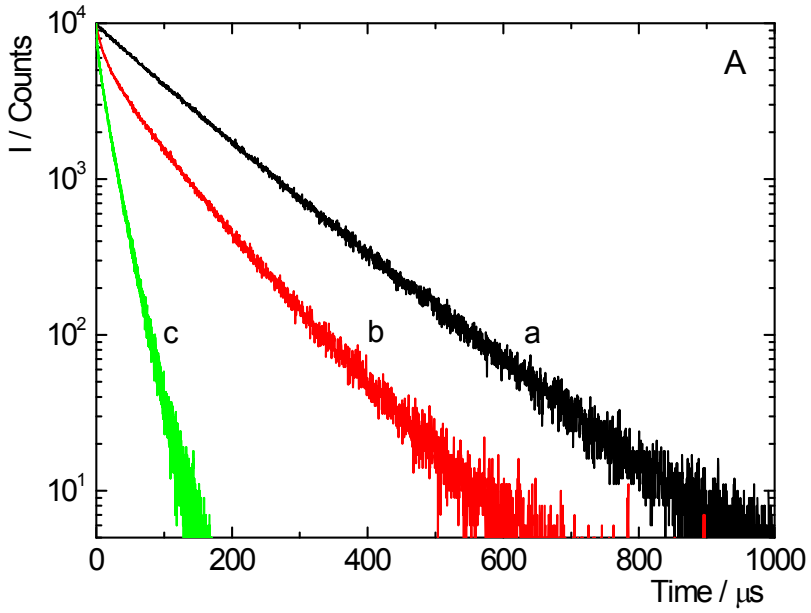


Figure S4. Luminescence decay curves of fresh solutions of **1** (A) and **2** (B) in oxygen-free (a), air- (b) and oxygen-saturated (c) water.

The samples were excited at 390 nm and luminescence was recorded at 700 nm (A) 690 nm (B).



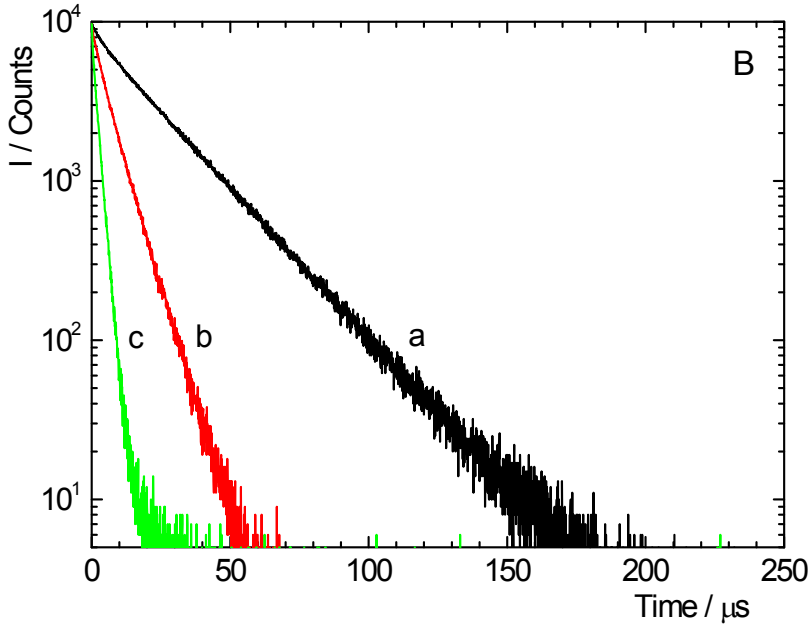
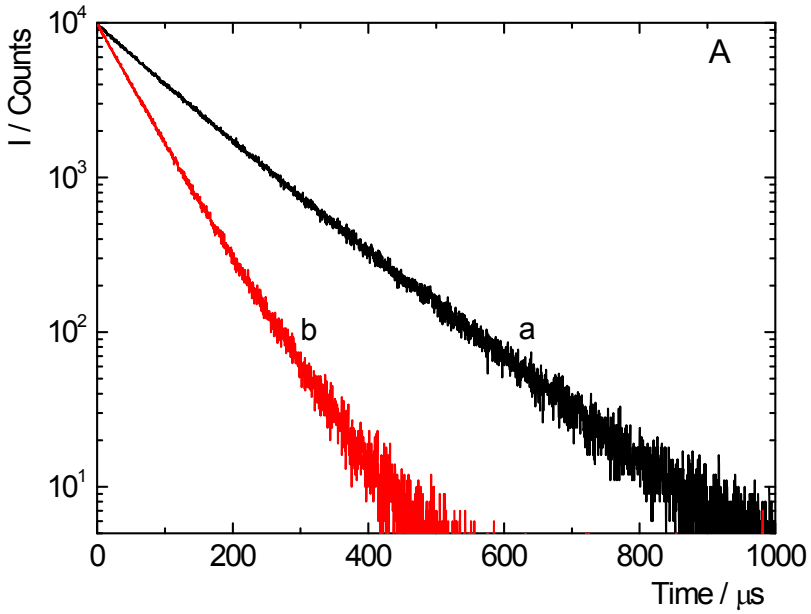


Figure S5. Fluorescence decay curves of **1** (A) and **2** (B) in oxygen-free water: fresh (a) and 11 days old solution (b).

The samples were excited at 390 nm and luminescence was recorded at 700 nm (A) 690 nm (B).



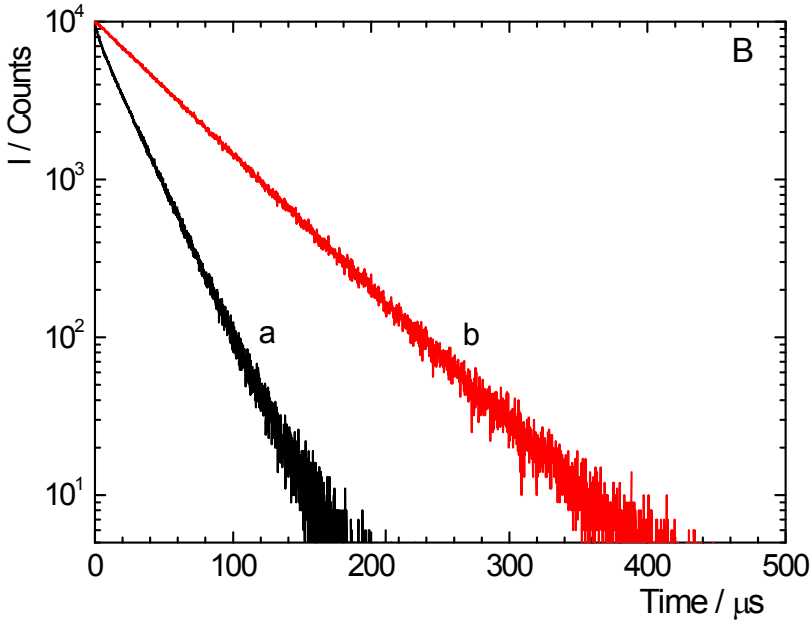


Figure S6. Kinetics of the hydrolysis of **1** and **2** in water as obtained from the changes in luminescence quantum yields over time.

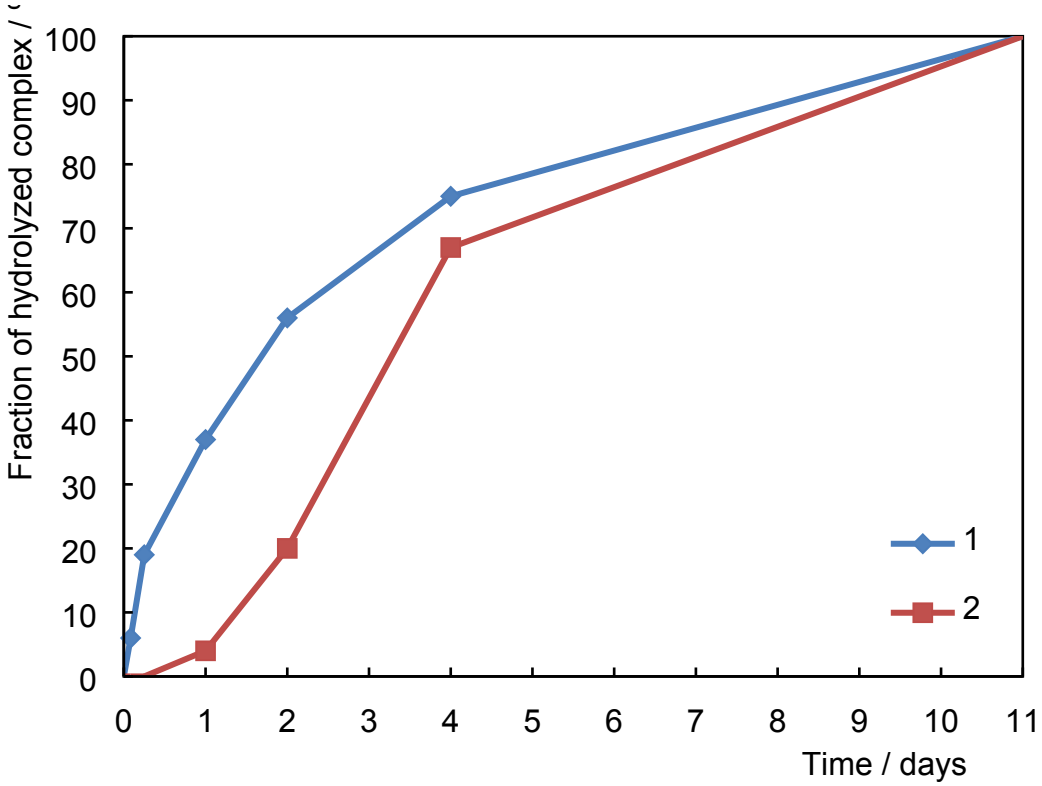


Figure S7. Flow cytometry histograms.

(A) HeLa cells incubated with 0.5, 1, 2, 5, 10 and 20 μM of **1** (from light to dark tone of green, respectively).

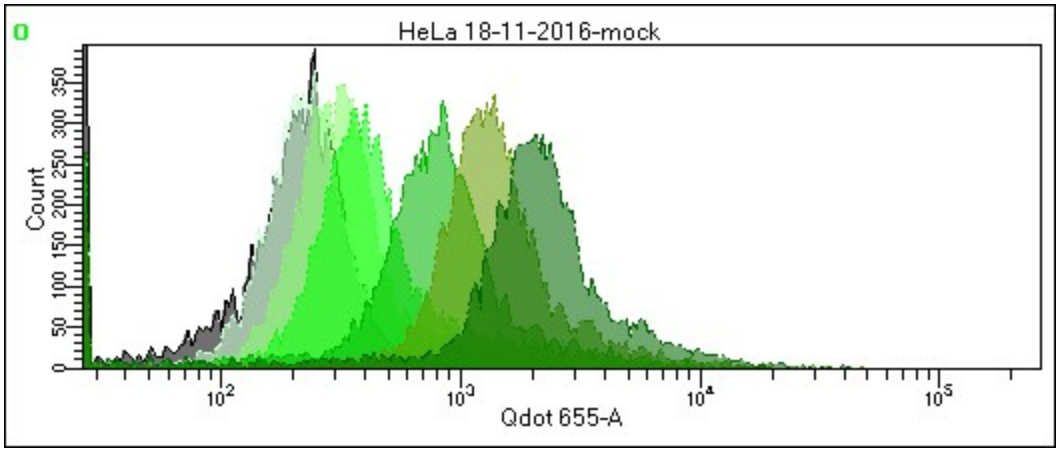
(B) HeLa cells incubated with 10 μM of **1** for 30, 60, 90, 120 min (from light to dark tone of purple, respectively).

(C) HeLa cells incubated with 10 μM **2**.

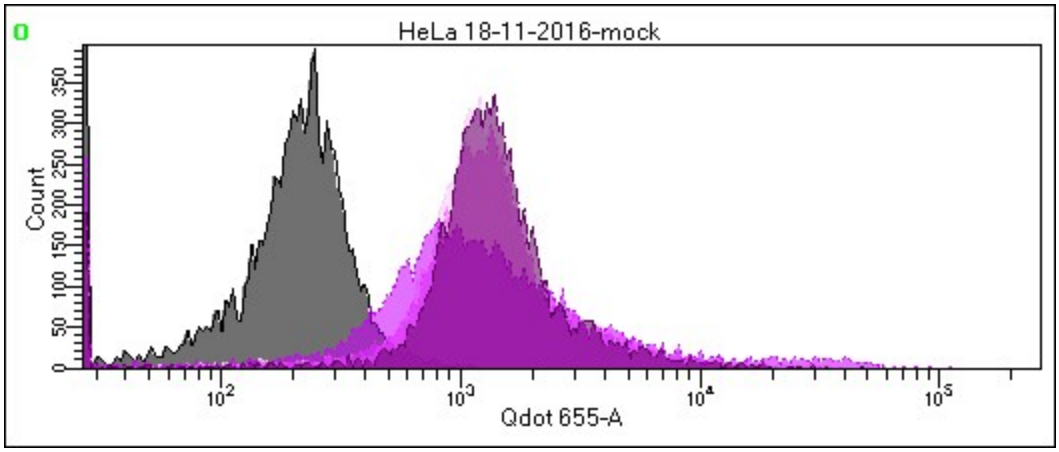
(D) HeLa cells incubated with hydrolyzed **1** (10 μM, 11 days in water solution prior to use).

Dark grey color is the control.

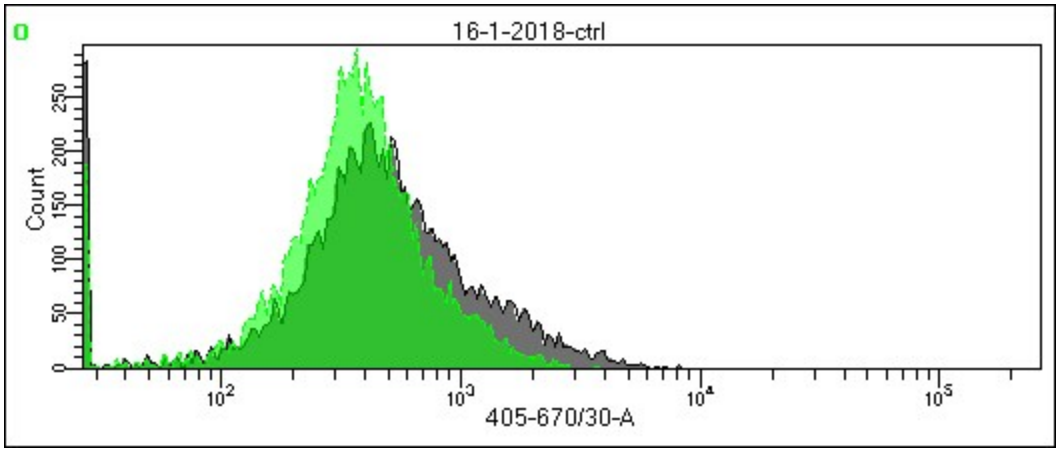
A



B



C


16-1-2018-ctrl
Count
0
50
100
150
200
250
10^2
10^3
10^4
10^5
405-670/30-A

D

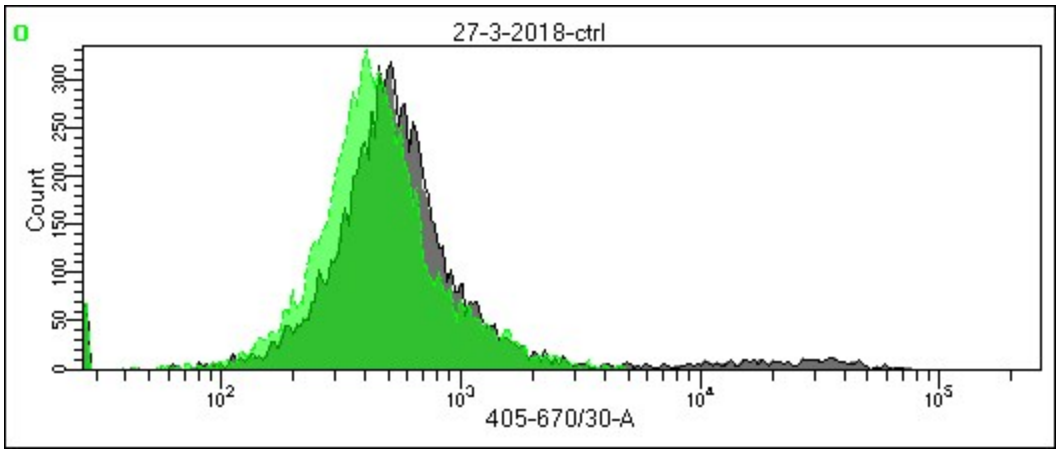

27-3-2018-ctrl
Count
0
50
100
150
200
250
300
10^2
10^3
10^4
10^5
405-670/30-A

Figure S8. A) Uptake (0.5 μM **1**) and B) phototoxicity of **1** under specified conditions:

"2h + 22 h" - incubation with **1** in the EMEM medium for 2 h, followed by 22 hours of washout in the fresh EMEM medium supplemented with 0.5 mM glutamine and 5% fetal bovine serum, followed by flow cytometry analysis / irradiation.

"2 h" - incubation with **1** for 2 h in the EMEM medium, followed by flow cytometry analysis / irradiation.

Irradiation was performed with a 12x10 W LED light source (Cameo) at 460 nm (15 min, 20 mW cm^{-2}). The cell viability was assayed with the resazurin assay according to manufacturer's protocol after next 24 h.

Cell viability values are presented as relative ones to the cell viability measured under the same experimental conditions, however in the absence of **1**.

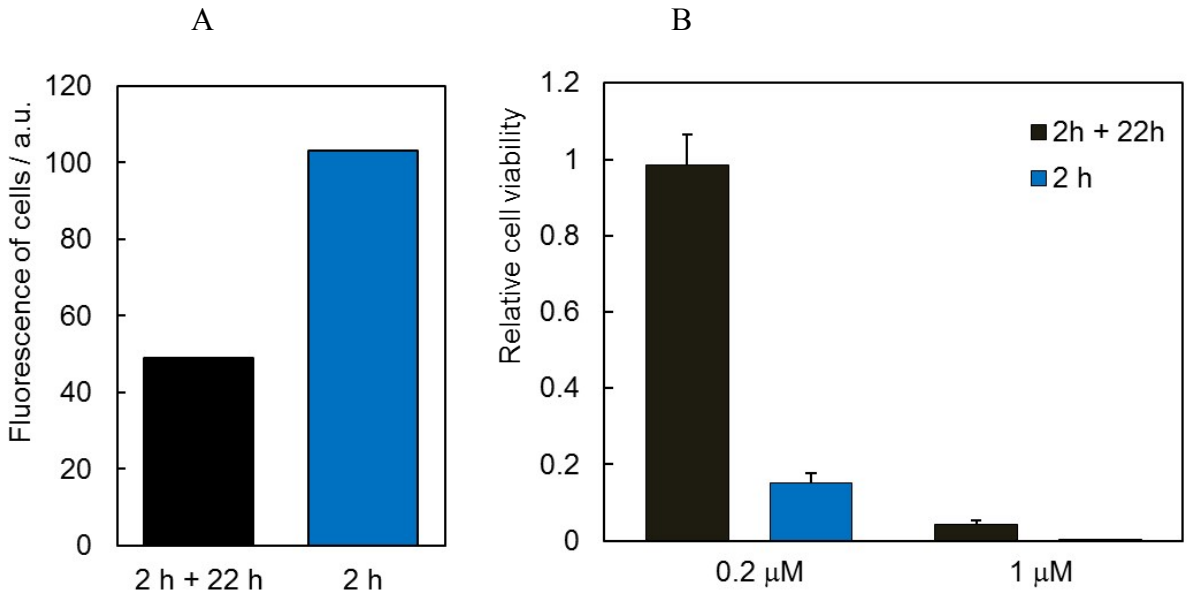


Figure S9. A) Uptake (0.5 μM **1**) and B) phototoxicity of **1** under specified conditions:

"2 h + FBS" - incubation with **1** for 2 h in the EMEM medium supplemented with 0.5 mM glutamine and 5% fetal bovine serum, followed by flow cytometry analysis / irradiation.
"2 h" - incubation with **1** for 2 h in the EMEM medium, followed by flow cytometry analysis / irradiation.

Irradiation was performed with a 12x10 W LED light source (Cameo) at 460 nm (15 min, 20 mW cm^{-2}). The cell viability was assayed with the resazurin assay according to manufacturer's protocol after next 24 h.
Cell viability values are presented as relative ones to the cell viability measured under the same experimental conditions, however in the absence of **1**.

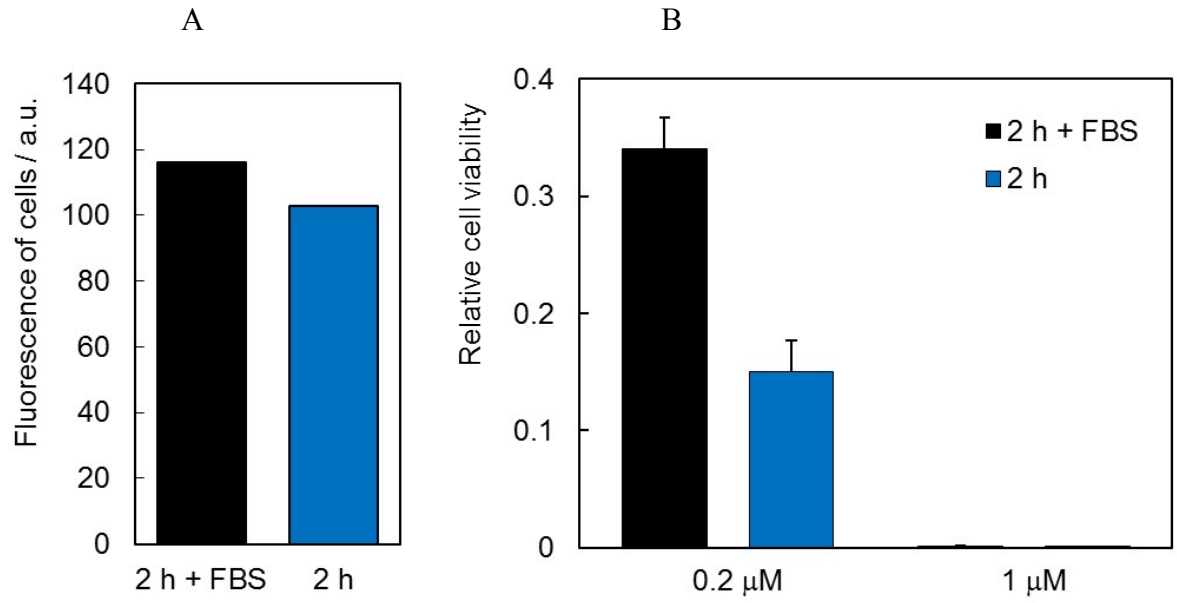


Figure S10. Cell viability of HeLa cells incubated with varying concentrations of **2**.
Dark toxicity: 2 h of incubation with specified concentrations in the dark, measured after 24 h. Phototoxicity: 2 h incubation with specified concentrations in the dark followed by irradiation with 460 nm light (20 mW cm^{-2}, 15 min), measured after 24 h. The result labeled 0 μM belongs to the control experiment where cells were irradiated in the absence of **2**.

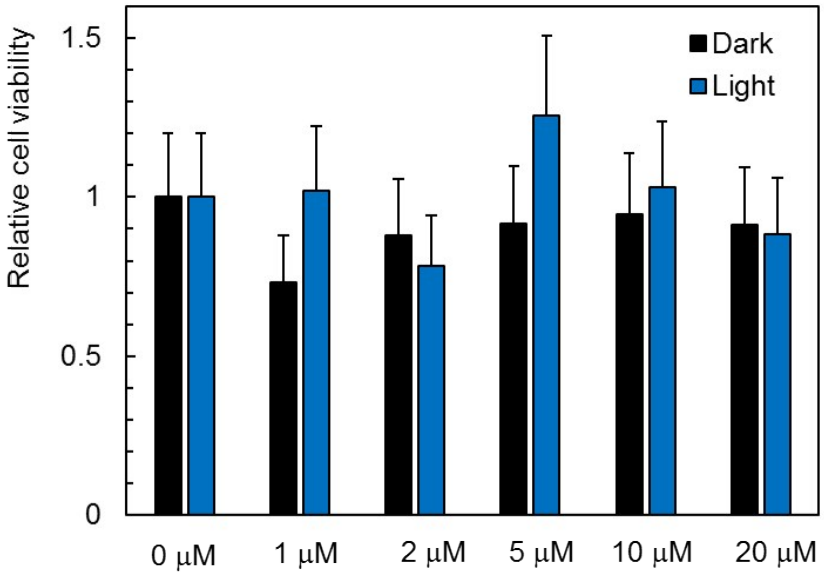


Figure S11. Cell viability of HeLa cells incubated with varying concentrations of hydrolyzed **1** (11 days in water solution prior to use) for 2 h followed by irradiation with 460 nm light (20 mW cm^{-2}, 15 min), measured after 24 h. The result labeled 0 μM belongs to the control experiment where cells were irradiated in the absence of hydrolyzed **1**.

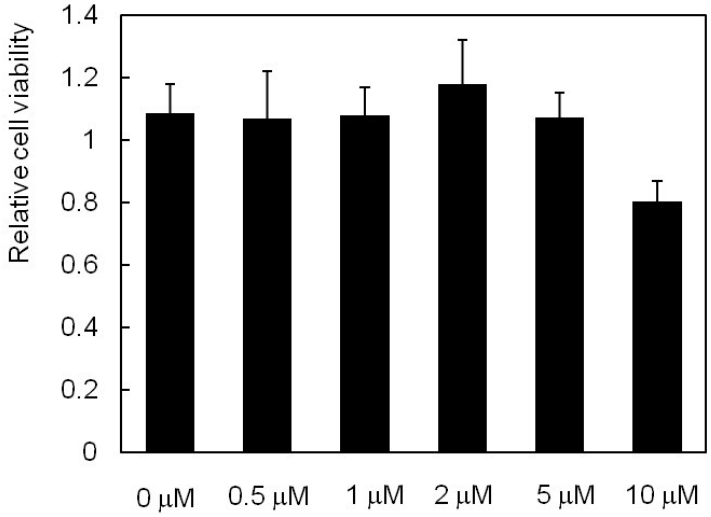


Figure S12. A) Uptake (0.5 μM **1**) and B) phototoxicity of **1** under specified conditions:

"24 h + FBS" - 24 h incubation with **1** in the EMEM medium supplemented with 0.5 mM glutamine and 5% fetal bovine serum, followed by flow cytometry analysis / irradiation.
"2 h + FBS" incubation with **1** for 2 h in the EMEM medium supplemented with 0.5 mM glutamine and 5% fetal bovine serum, followed by flow cytometry analysis / irradiation.

Irradiation was performed with a 12x10 W LED light source (Cameo) at 460 nm (15 min, 20 mW cm^{-2}). The cell viability was assayed with the resazurin assay according to manufacturer's protocol after next 24 h.
Cell viability values are presented as relative ones to the cell viability measured under the same experimental conditions, however in the absence of **1**.

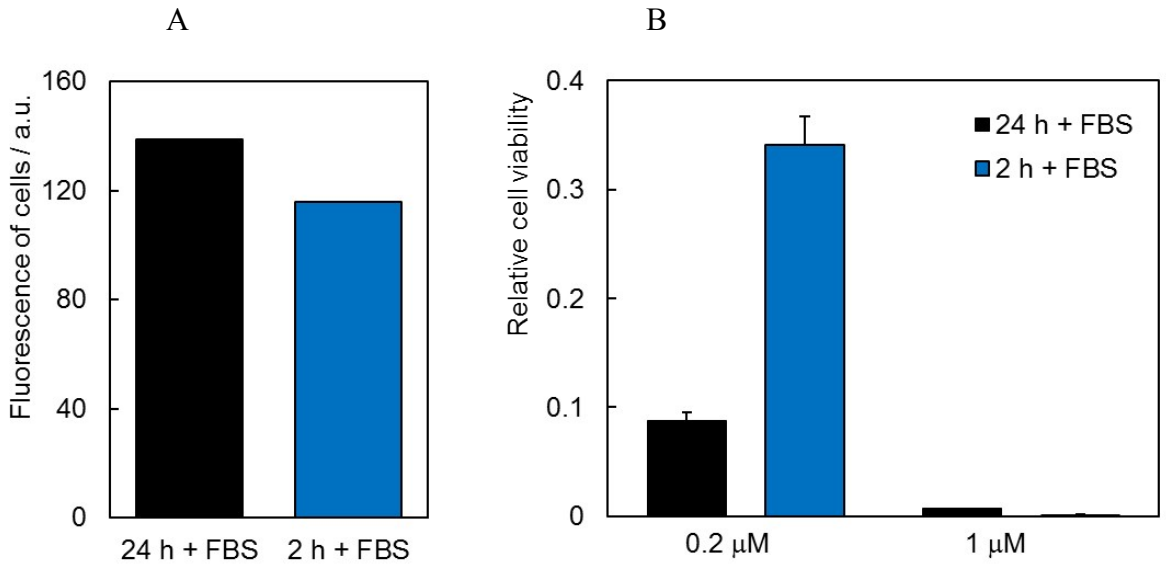


Figure S13. Photoinactivation of Gram-positive *Staphylococcus aureus* (top) and *Enterococcus faecalis* (bottom) incubated with 50 μM **2** and hydrolyzed **1** (11 days in water solution prior to use). Bacteria were incubated 30 min with the complexes and irradiated 30 min by the 460 nm light source (20 mW cm^{-2}). The control experiment was performed in the dark.

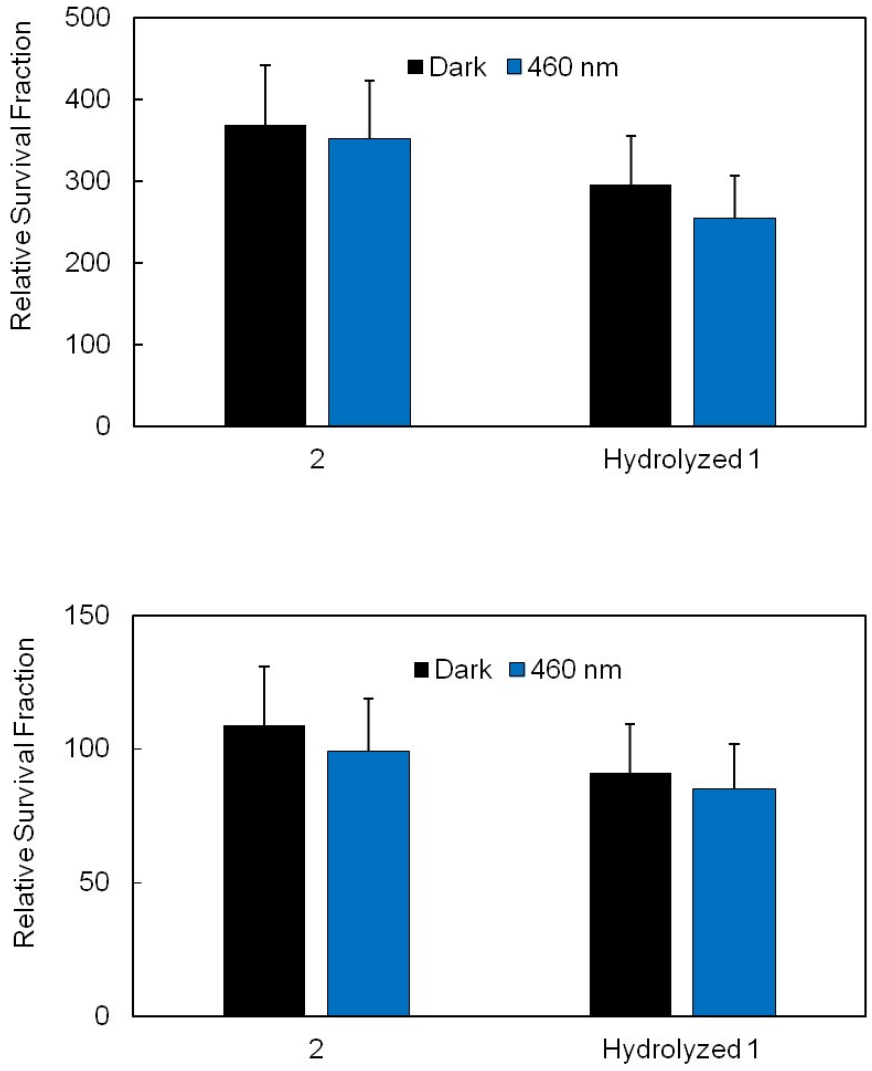
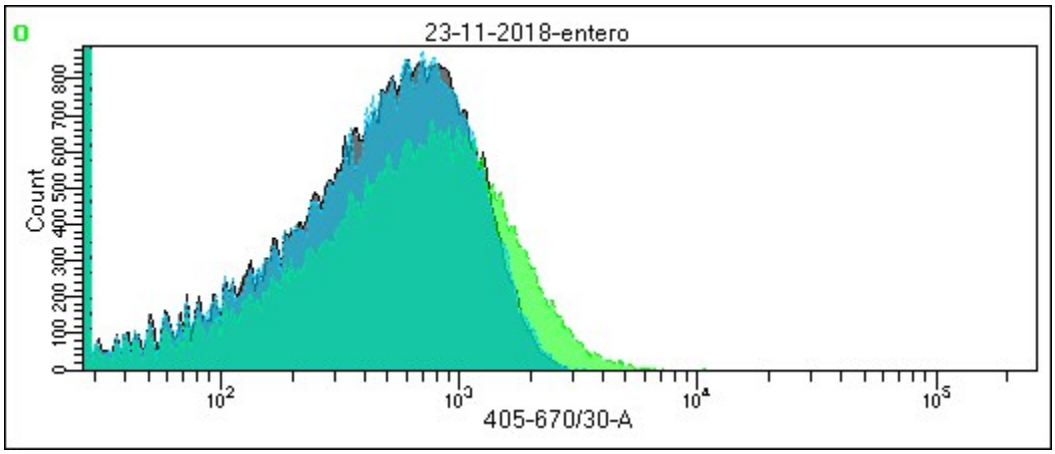


Figure S14. Uptake of 10 µM **1** (green) or **2** (blue) by Gram-positive *Enterococcus faecalis* (A) and Gram-negative *Escherichia coli* (B) measured using flow cytometry after 1 h incubation. Control cells are given in black.

A



B