Supporting Information

For

Effective Two-photon Excited Photodynamic Therapy of Xenograft Tumors Sensitized by Water-Soluble Bis(arylidene)cycloalkanone Photosensitizers

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Singlet oxygen quantum yield (Φ_Δ): Φ_Δ was determined by using 1,3-diphenylisobenzofuran (DPBF) as the scavenger[1] and Rose Bengal as the reference (Φ_Δ = 0.47).[2] Photosensitizers and DPBF were mixed in DMF and the mixture was stirred vigorously to ensure the saturation of air during the experiment. The concentrations of all photosensitizers were adjusted to possess same absorbance (0.1) at 473 nm and the initial concentrate of DPBF is 6.4×10^{-5} M. The absorbance change of DPBF under the irradiation of 473 nm laser was detected by Uv-vis spectrometer. The slope of the photodegradation rate (percentage declines) of DPBF at 406 nm vs. irradiation time is an indication of a photosensitizer's efficiency to generate singlet oxygen (the larger the slope, the better the efficiency is). The experimental results are shown in Figure S1. The Φ_Δ values of **1-6** were calculated by the following equation:

$$\Phi_\Delta^S = \frac{k_S}{k_R} \times \Phi_\Delta^R \qquad (1)$$

Where, k is the slope of the photodegradation rate of DPBF; the superscripts/subscripts S and R stand for the sample and the reference, respectively.

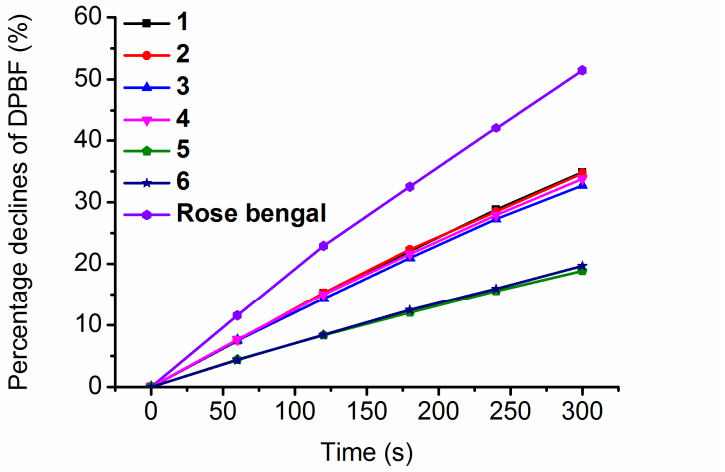

Figure S1 The percentage declines of DPBF vs. irradiation time under 473 nm laser in the presence of **1-6** or Rose Bengal.

Transient properties and triplet quantum yield (Φ_T): Transient properties were recorded on an Edinburgh LP920 laser flash photolysis system. A Nd:YAG laser (Continuum Surelite I 10, 355 nm, 4-6 ns) was used as the excitation source. The analyzing light was from a pulsed Xe920 xenon lamp. Samples were contained in a quartz cell with an optical path length of 1 cm. The signal was detected by a Hamamatsu R928B detector, and then was displayed and recorded on a Tektronix TDS 3012B oscilloscope and a computer. The concentrations of the photosensitizers were 2×10^{-4} M in toluene.

The transient spectra of **1-6** in nitrogen-saturated toluene and their decay profiles at 660 nm are shown in Figure S2. The transient spectra of **1-4** are very similar and exhibit a broad absorption band within 550-850 nm. The transient spectra of **5-6** exhibit a broad absorption band within 525-800 nm. Only one species is involved in the photolysis process of each photosensitizer. Based on the transient life times (τ_T) get from the decay profiles, the oxygen quenching rate constant (k_q) values were calculated as $2.4\text{-}3.0\times10^{9}$ S^{-1} M^{-1} (oxygen concentration in air-saturated toluene was used as 2×10^{-3} M)[3] (Table S1). These k_q values are close to the diffusion limit,[4] indicating that these transient absorptions are from triplet states.

The triplet quantum yields (Φ_T) of **1-6** were measured according to a literature procedure by checking the energy-transfer from photosensitizers to β-carotene using benzophenone as the reference.[5] Samples were prepared by mixing photosensitizers and β-carotene in toluene together. The concentrations of all photosensitizers were adjusted to possess same absorbance (0.5) at 355 nm and the concentration of β-carotene was 2×10^{-4} M. Due to the low triplet state yield of β-carotene, the direct excitation of β-carotene alone did not produce any significant transient signal. In the mixed solution, more β-carotene triplet could be generated by energy-transfer from the triplet states of **1-6** or benzophenone. With the growth transient absorbance (ΔA) of β-carotene between with and without photosensitizers at 540 nm, the Φ_T values of **1-6** can be estimated by the following equation:

$$\Phi_T^S = \Phi_T^R \frac{\Delta A^S}{\Delta A^R} \frac{k_{obs}^R - k_0^R}{k_{obs}^R} \frac{k_{obs}^S}{k_{obs}^S - k_0^S} \cdots \quad (2)$$

where superscripts S and R denote the sample and the reference, respectively, k_{obs} is the pseudo-first-order rate constant for the growth of the β-carotene triplet state, and k_0 is the rate constant for the decay of the triplet states of photosensitizers with same

absorbance (0.5) at 355 nm (in the absence of β-carotene). The Φ_T of benzophenone in toluene was taken as 1. The reliability of Φ_T data obtained in this method depends on the validity of the assumption that the energy-transfer efficiency from **1**-**6** or benzophenone to β-carotene is 100%. The error of this method was estimated about ±20%.[5]

Singlet oxygen quantum yield (Φ_Δ, determined by using DPBF as the scavenger and tetraphenylporphyrin as the reference with a yield of 0.68 in toluene[6]) and triplet state quantum yield (Φ_T) of **1**-**6** in toluene are listed in Table S1. For the singlet oxygen generates from the triplet state of a sensitizer, Φ_Δ can be expressed as the following equations:[7]

$$\emptyset_\Delta = \emptyset_T P_T^{O_2} f_\Delta^T \tag{3}$$

$$P_T^{O_2} = \frac{k_q[O_2]}{\frac{1}{\tau_T^{N_2}} + k_q[O_2]} \tag{4}$$

where Φ_T is the triplet quantum yield of the sensiziter, $P_T^{O_2}$ is the fraction of triplet-state quenched by ground state oxygen, f_Δ^T defined as sensitization efficiency is the fraction of quenching events that result in the production of singlet oxygen, k_q is the rate constant of the triplet photosensitizer reacting with the ground state oxygen, $[O_2]$ is the concentration of ground state oxygen, and $\tau_T^{N_2}$ is the lifetime of the triplet-state in nitrogen-saturated solution.

From the equations, values of f_Δ^T and $P_T^{O_2}$ of **1**-**6** were calculated and listed in Table S1. The $P_T^{O_2}$ values of **1**-**4** are close to 0.72, meaning that the triplet-states of **1**-**4** were partly quenched by ground state oxygen. The $P_T^{O_2}$ values of **5**-**6** are only 0.30. For **1**-**4** or **5**-**6**, their f_Δ^T and $P_T^{O_2}$ are almost equal, indicating that the photophysical process involved in the generation of singlet oxygen was not affected by the modification of PEG chains.

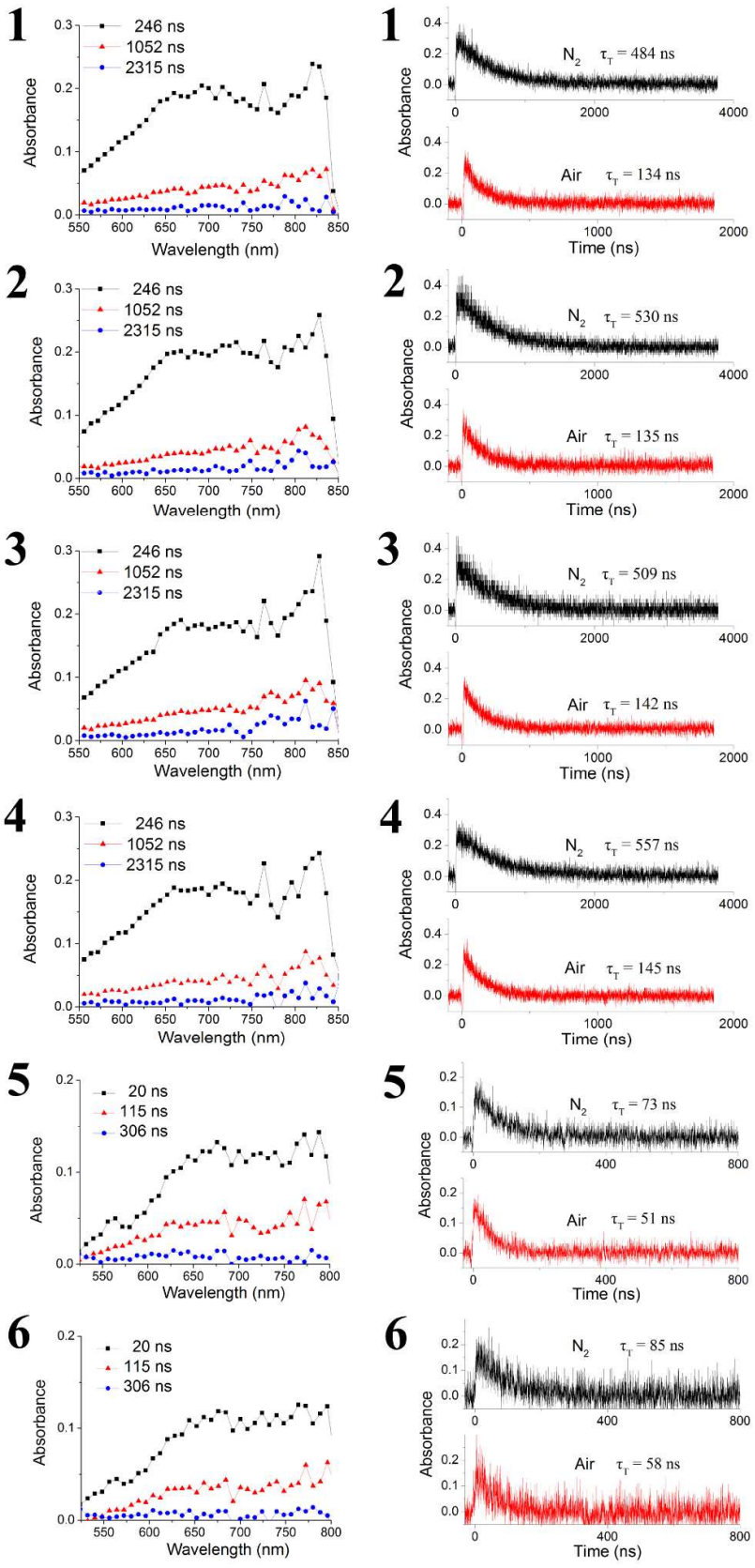

Figure S2 Left: The transient absorption spectra of **1**-**6** in nitrogen-saturated toluene. Right: The decay profiles of **1**-**6** at 660 nm in nitrogen-saturated (black lines) and air-saturated (red lines) solutions, respectively.

Table S1 Φ_Δ and the triplet state properties of **1-6** in toluene[a]

Comp.	Φ_T[b]	Φ_Δ[c]	τ_T^{N2} (ns)	τ_T^{Air} (ns)	k_q (10^9 S^{-1} M^{-1})	$P_T^{O_2}$	f_Δ^T
1	0.65	0.33	484	134	2.57	0.72	0.71
2	0.62	0.31	530	135	2.63	0.74	0.68
3	0.67	0.31	509	142	2.42	0.72	0.66
4	0.66	0.35	557	145	2.43	0.73	0.73
5	0.22	0.066	73	51	2.95	0.30	1.0
6	0.21	0.068	85	58	2.74	0.32	1.0

[a]Φ_T is the triplet-state quantum yield, the experimental uncertainty amounts to ± 20%; Φ_Δ is the singlet oxygen quantum yield, , the experimental uncertainty amounts to ± 10%; $\tau_T^{N_2}$ and τ_T^{air} are the lifetimes of the triplet-state in nitrogen-saturated and air-saturated solutions, respectively; k_q is oxygen quenching rate constant toward the triplet-state; $P_T^{O_2}$ is the fraction of triplet-states quenched by ground state oxygen; f_Δ^T is the fraction of quenching events that result in the production of singlet oxygen. [b]The experimental uncertainty amounts to ± 20%. [c]The experimental uncertainty amounts to ± 10%.

Two-photon excited fluorescence (2PEF) method: TPA cross-section (σ_2) values of compounds in DMF (2×10^{-4} M) were determined using 2PEF method as described by Xu and Webb.[8] The excitation light source was a mode-locked Tsunami Ti:sapphire laser (720–880 nm, 80 MHz, <130 fs). Up-converted fluorescence spectra were recorded in a direction perpendicular to the laser beam using a fiber spectrometer (Ocean Optics USB2000 CCD) as detector. Rhodamine 6G in methanol solution (10^{-4} M) was used as the reference.[9] The σ_2 values of **1**-**6** were calculated by the following equation:

$$\sigma_{2s} = \frac{F_s \Phi_r \eta_r c_r}{F_r \Phi_s \eta_s c_s} \sigma_{2r} \quad (5)$$

Where, the subscripts *r* and s stand for the reference and sample, respectively; *F* is the integral area of the up-converted fluorescence; Φ is the fluorescence quantum yield; η is the overall fluorescence collection efficiency of the experimental apparatus, and *c* is the concentration.

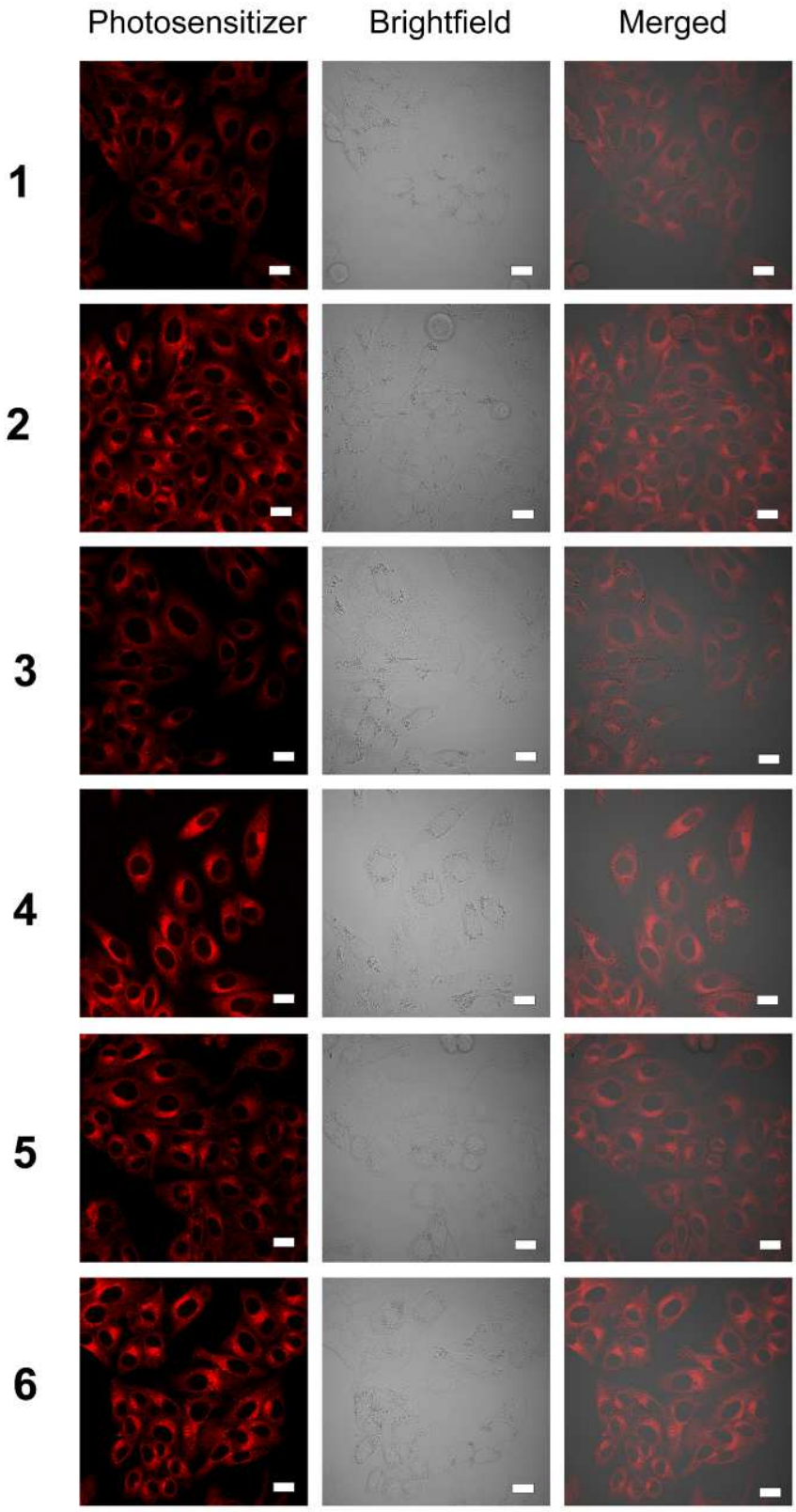

Figure S3 Confocal images obtained after incubation of HepG2 cells with 5 μM **1-6** for 4 h. Scale bars denote 20 μm.

Intracellular distribution: HepG2 cells were dispersed in 1 ml culture in a 35 mm coverglass chamber with a density of 1×10^5 cells/ml. After 24 h, the cells were incubated with photosensitizer-containing (5 μM) culture for 4 h, then washed with PBS for two times and further incubated with MitoLite™ NIR for 30 min. After removing the nonspecifically absorbed probe, the cells were observed on a CLSM (Olympus FV1000). Photosensitizers **1-6** were excited at 488 nm and monitored at 570-670 nm, while MitoLite™ NIR was excited at 635 nm and monitored at 650-750 nm. Control experiments demonstrated that such parameters were able to distinguish the fluorescence from **1-6** and MitoLite™ NIR due to the facts that photosensitizers **1-6** cannot be excited by 635 nm laser and MitoLite™ NIR has no absorption band at 488 nm.

Cellular uptake: Cellular uptake profiles were determined by lysis method plus with fluorescence spectrometer. Briefly, the cells in 12-well plates were incubated with 10 μM photosensitizers for certain time, washed three times with PBS, added with 200 μl lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100), put on ice for 10 min, then added with 1 ml PBS. The fluorescence signal of the final solution was measured. Standard curves were obtained by dissolving photosensitizers in lyzed solution of blank cells. The photosensitizer concentration was converted to intracellular concentration by assuming that the volume of each HepG2 cell was 2.83×10^{-9} ml and the photosensitizer was distributed equally within the cell.[10]

In vitro ROS test: HepG2 cells were seeded in a coverglass chamber (diameter 35 mm) with a density of 1×10^5 cells per well in culture media. After 24 h, the cells were divided into three groups: A, B, and C. Group A was only incubated with 10 μM DCFH-DA[11] for 0.5 h, while the groups of B and C were incubated with 5 μM photosensitizer **4** for 4 h and were further incubated with 10 μM DCFH-DA for 0.5 h. Thereafter, group A and group C were washed with PBS, and irradiated by 800 nm fs laser for 10 min (1.6 W cm^{-2}). After all groups were carefully washed with PBS, their fluorescence images were obtained by CLSM (excited at 488 nm and collected in 570-670 nm). The obtained images were analyzed by ImageJ software (http://rsbweb.nih.gov/ij/). The results of fluorescence intensity are provided in Figure S4.

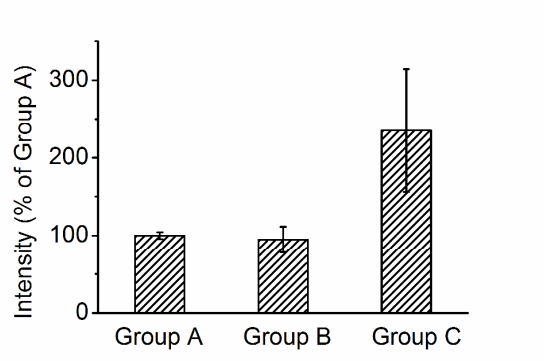

Figure S4 In vitro ROS test. Group A (control group, without photosensitizer): Cells were incubated with 10 μM DCFH-DA for 0.5 h and irradiated by 800 nm fs laser. Group B (control group, without laser): Cells were incubated with 5 μM photosensitizer **4** for 4 h and 10 μM DCFH-DA for 0.5 h. Group C (2PE-PDT group): Cells were incubated as Group B and then irradiated by 800 nm fs laser. The error bars denote standard deviation of three replicates.

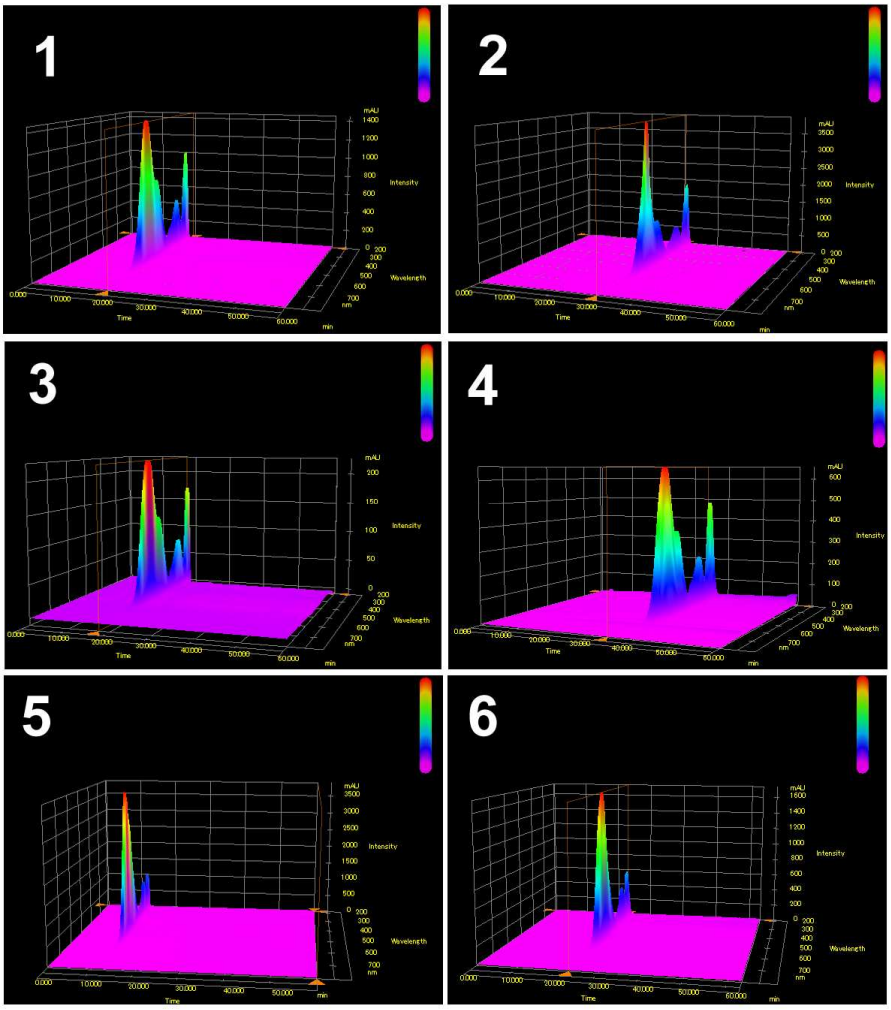

Figure S5 HPLC chromatogram of photosensitizers **1-6**.

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