

A simplified synthetic route for the photo-sensitizer TPCS 2a - validation against human (T24) and rat (AY27) bladder cancer cell models

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Research Article

Keywords:

Posted Date: August 31st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10728553/v1>

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Additional Declarations: No competing interests reported.

A simplified synthetic route for the photo-sensitizer TPCS_{2a} - validation against human (T24) and rat (AY27) bladder cancer cell models

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Abstract

The tetraphenyl chlorin disulfonate, TPCS_{2a}, is a synthetic and light-activated photosensitizer (PS) that is used for photodynamic therapy (PDT) or by releasing entrapped cytotoxic drugs from cancer cell endosomes and lysosomes through the generation of reactive oxygen species (ROS). In this study a new and simplified synthetic route for TPCS_{2a} and its pre-cursors TPPS_{2a} and TPPS_{2o}, was developed and evaluated. The laboratory-synthesized compounds exhibited photophysical properties (absorption/fluorescence) identical to those of commercially available counterparts and demonstrated efficient singlet oxygen generation from their triplet states. In addition, the compounds were benchmarked in cell viability assays using human (T24) and rat (AY27) bladder cancer cell lines. When used *in vitro* in combination with blue light (435 nm), TPCS_{2a} induced significant cell death, as confirmed by confocal microscopy, short-term metabolic assays (MTT), and long-term clonogenic assays. These effects were observed in both PDT and photochemical internalization (PCI) applications.

1. Introduction

Tetraphenyl chlorin disulfonate (TPCS_{2a}) is a synthetic photosensitizer (PS) derived from the macrocyclic aromatic chlorin core (Figure 1A). The PS can enter cancer cells and can be activated by light to produce reactive oxygen species (ROS), leading to the disruption of various intracellular compartments upon systemic administration [1]. TPCS_{2a} was specifically developed for deep tissue photo-dynamic applications by reducing one of the pyrrole double bonds in the porphyrin ring of the amphiphilic precursor TPPS_{2a}. This modification enhances its photophysical properties while preserving sulfonated side-chains [2-5]. The compound has been extensively employed in the patented photo-chemical internalization (PCI) technology for light-controlled intracellular drug delivery [6-9]. Photosensitizers of this class, including related chlorins, serve as versatile scaffolds in structure-activity relationship studies. By modifying the peripheral functional groups around the tetrapyrrole core, researchers can optimize physicochemical properties and therapeutic efficacy [10, 11].

The increased optical absorbance of TPCS_{2a} in the red region of the Q-bands compared to the corresponding porphyrin spectrum, enables deeper light activation down to the subcutaneous tissue level [12]. Although relatively new in the clinical treatment of human cancers, TPCS_{2a} has already demonstrated therapeutic potential in squamous cell carcinoma of the head and neck [13, 14]. In parallel, the compound is widely used *in vitro* as a PS for both PDT and PCI [4, 9, 15-20].

At the time this study was initiated, the commercial availability of sulfonated and disulfonated porphyrins and particularly TPCS_{2a}, was partially limited. It was therefore decided to synthesize the compounds through direct sulfonation of tetraphenyl porphyrin (TPP). Although sulfonation can be readily achieved using sulfur trioxide in sulfuric acid, the present work employed concentrated sulfuric acid, as this approach provided superior control over the reaction. However, it required the subsequent chromatographic separation of the two regioisomeric disulfonated products, TPPS_{2a} (adjacent) and TPPS_{2o} (opposite). Literature procedures for the preparation of TPCS_{2a} from TPPS_{2a} typically employ either photochemical or chemical reduction with diimide [2]. The selective reduction of one *meso*-double bond in the porphyrin macrocycle is well documented [21]. The most commonly used method for reduction is the procedure by using a diimide

as the reducing agent as published by Whitlock et al. (1969) [22]. The diimide is generated from the hydrazine group of *p*-toluenesulfonyl hydrazide by a mild base under heating in either 3-picoline or pyridine. A major limitation of this approach is the poor regio-selectivity and over-reduction, which can lead to substantial amounts of the corresponding bacteriochlorin TPBS_{2a}. These compounds are only differentiated by an extra double bond and can be hard to separate, and a *o*-chloranil oxidation step is needed to re-oxidize the formed bacteriochlorin to chlorin species. Typically, TPCS_{2a} is used as an ethanolamine salt. By using ethanolamine, under anhydrous conditions as dual solvent and base system, eliminates reaction side products, leaving only minor impurities of bacteriochlorin [23].

The present study reports a modified synthetic protocol that efficiently suppresses bacteriochlorin formation during the preparation of TPCS_{2a}, resulting in only minor impurities. The laboratory-synthesized compound was fully characterized with respect to its photophysical properties and subsequently evaluated in two bladder cancer cell lines (AY27 and T24). Cell viability was assessed following photochemical internalization (PCI) and photodynamic therapy (PDT) in combination with blue light illumination. The PCI technology significantly enhances the intracellular delivery and biological activity for molecules with poor plasma membrane permeability, such as the chemotherapeutic agent bleomycin (BLM) and other therapeutic macromolecules, [1, 15, 24]. Additionally, aluminum phthalocyanine disulfonate (AlPcS_{2a}), a well-established photosensitizer used in clinical PDT, was included as a reference compound. It was tested on the rat bladder cancer cell line (AY27) as a reference for assessing the efficacy of TPCS_{2a} [25, 26].

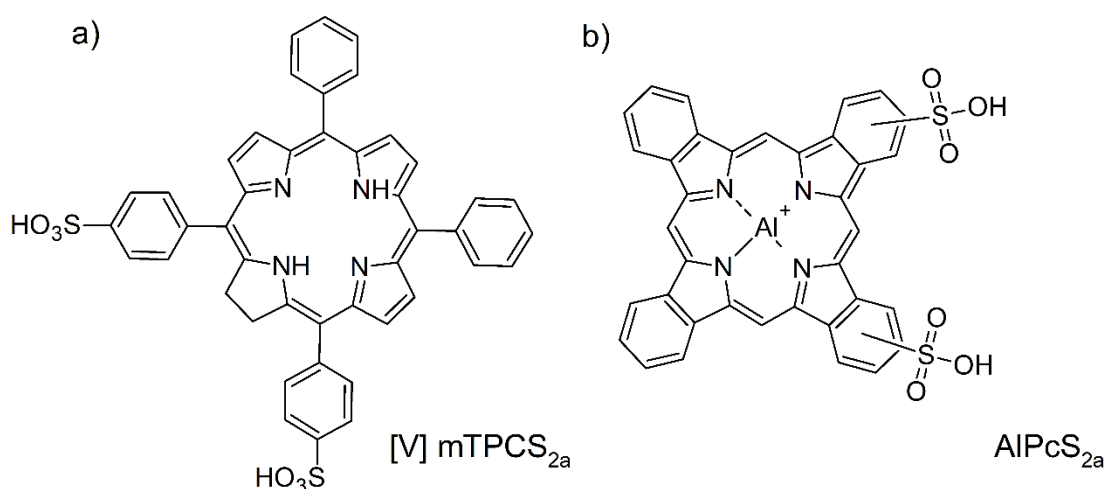


Figure 1. Structural formulas. a) of tetraphenyl chlorin disulfonate (TPCS_{2a}). And b) aluminum phthalocyanine disulfonate (AlPcS_{2a}).

2. Materials and Methods

2.1 Chemicals

RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), sodium pyruvate, nonessential aminoacids, trypsin and phosphate buffered saline (PBS) were from Gibco BRL, Life Technologies (Inchinnan, Scotland). Gentamicin sulphate was from Schering Corp (Kenilworth, NJ) and absolute ethanol from Arcus A/S (Oslo, Norway), the MTT solution; 3-(4, 5 dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) was from Sigma-Aldrich (St. Louis, MO). The AlamarBlue reagent (Catalog No. A50101, invitrogen alamarBlue™HS Cell Viability Reagent) was ordered from Thermo Fisher Scientific, Waltham, MA, US. Bleomycin powder (15000 IU, Baxter, Norway) was dissolved with 0.9% salt water as stock (1000 IU) and stored at -20 °C in aliquots.

Aluminum phthalocyanine disulfonate (AlPcS_{2a}), Product No. P40632, from Frontier Scientific®, Newark DE, US, sulphuric acid (H₂SO₄, Supelco®), chloroform (LiChromsolv), Methanol, ethanolamine, *p*-toluenesulfonyl hydrazide (*p*-TsNHNH₂) from Merck. The photosensitizer TPCS_{2a}, with singlet oxygen generation, was synthesized at the PET Senter, St. Olavs Hospital, Trondheim, Norway, and photo-physically examined at Department of Physics, NTNU Trondheim, Norway.

2.2 Synthesis of TPCS_{2a} from 5, 10, 15 20 -tetraphenylporphine (TPP)

The photosensitizer TPCS_{2a} was synthesized at the PET Centre, St. Olavs Hospital, Trondheim, Norway and the sulfonated intermediates were separated by flash chromatography (SupelTM Flash Cartridge, Supelco) according to the procedure described by Dahl et al. [26], with modifications. Initially, tetraphenylporphyrin sulfonic acid isomers were prepared from 5,10,15,20-tetraphenylporphyrin (TPP) according to Zhang et al. [27]. TPP (5.00 g, 6.5 mmol) was added to concentrated sulfuric acid (H₂SO₄, 4 mL) at 4 °C under a nitrogen atmosphere. The reaction mixture was stirred

thoroughly and then heated at 43 °C for 18 h. The reaction was quenched by pouring the mixture onto crushed ice, and the resulting precipitate was isolated by centrifugation at 3000g for 15 min. The solid material was washed sequentially with water (10 mL) and acetone (10 mL) at 18 °C.

The crude mixture of TPPS isomers was separated by flash chromatography using a Supelco Supel™ Flash Cartridge, product no. FCISI025. Prior to separation, the cartridge was conditioned with dichloromethane/methanol/ethanolamine (DCM/MeOH/MEA, 79:20:1, v/v/v). The disulfonated porphyrin regioisomers were eluted using a gradient of MeOH in DCM containing a constant amount of MEA (1%), starting at 23% MeOH and increasing to 30% MeOH. This fraction contained TPPS_{2a}, 5,10-bis(4-sulfonatophenyl)-15,20-diphenylporphyrin [I], in which the sulfonic acid groups are located on adjacent phenyl rings, and TPPS_{2o} [II], the corresponding opposite disulfonated regioisomer. Subsequently, the mobile phase was changed to 100% MeOH to elute the trisulfonated product, TPPS₃ [IV]. Fractions of 40 mL were collected in plastic tubes. Fractions containing TPPS_{2a} and TPPS_{2o} were evaporated to dryness and further purified by preparative HPLC using an Agilent InfinityLab LC Series 1260 Infinity II system. The purified TPPS_{2a} fractions were dried under vacuum and nitrogen and stored at 4 °C protected from light at all times.

The isolated disulfonated porphyrin TPPS_{2a} [I] was subsequently reduced to the corresponding chlorin, TPCS_{2a}, using a Whitlock diimide reduction procedure with ethanolamine as both base and solvent [22]. TPPS_{2a} (150 mg, 0.20 mmol) was dissolved in ethanolamine (3 mL), and the mixture was heated on a rotary evaporator at 80 °C for 4–5 h. A small amount of ethanolamine was distilled from the reaction mixture under reduced pressure to ensure complete removal of residual water. *p*-Toluenesulfonyl hydrazide (*p*-TsH, 188 mg, 1.0 mmol) was then added under a nitrogen atmosphere, and the reaction mixture was stirred in an oil bath at 70 °C for 19 h. An additional portion of *p*-TsH (205 mg, 1.1 mmol) was added under nitrogen, and the reaction was continued at 70 °C for 24 h. The mixture was then cooled under reduced pressure.

The crude reduction mixture was purified by flash chromatography using a preconditioned cartridge and a DCM/MeOH/MEA mobile phase, analogous to the procedure described above. A gradient of MeOH in DCM containing 1% MEA was applied, increasing the MeOH content from 20% to 26%. Final purification was performed by preparative HPLC to isolate TPCS_{2a} as a mixture of stereoisomers and to remove residual TPBS_{2a} [VI] and minor byproducts.

Characterization of TPCS_{2a}. The identity of the isolated TPCS_{2a} was supported by ¹H NMR spectroscopy (Bruker Avance NEO 600 MHz spectrometer), analytical HPLC, and UV–vis spectroscopy. The ¹H NMR spectrum was consistent with the reported spectrum of di(monoethanolammonium) TPCS_{2a} [Berg et al, 2011 [2] ¹H NMR (methanol-d₄, [600 MHz]) δ 8.65–8.75 (m, 2H), 8.25–8.40 (m, 2H), 8.05–8.25 (m, 8H), 8.00–8.10 (m, 2H), 7.70–7.95 (m, 2H), 7.80–7.85 (m, 2H), 7.41–7.80 (m, 6H), 4.05–4.20 (m, chlorin CH₂), 3.60 (m, monoethanolammonium CH₂), 2.77 (t, monoethanolammonium CH₂). The appearance of the overlapping chlorin methylene resonances around δ 4.17 (CH₂) supported reduction of the corresponding porphyrin precursor TPPS_{2a} to TPCS_{2a} isomers. The ¹H NMR spectrum was further confirmed by 2D HSQC to assign the CH/CH₃ and CH₂ in opposite phase (Attached in supp. Inf.). The chemical structures of the compounds included in the synthetic pathway to TPCS_{2a} are shown in Figure 2.

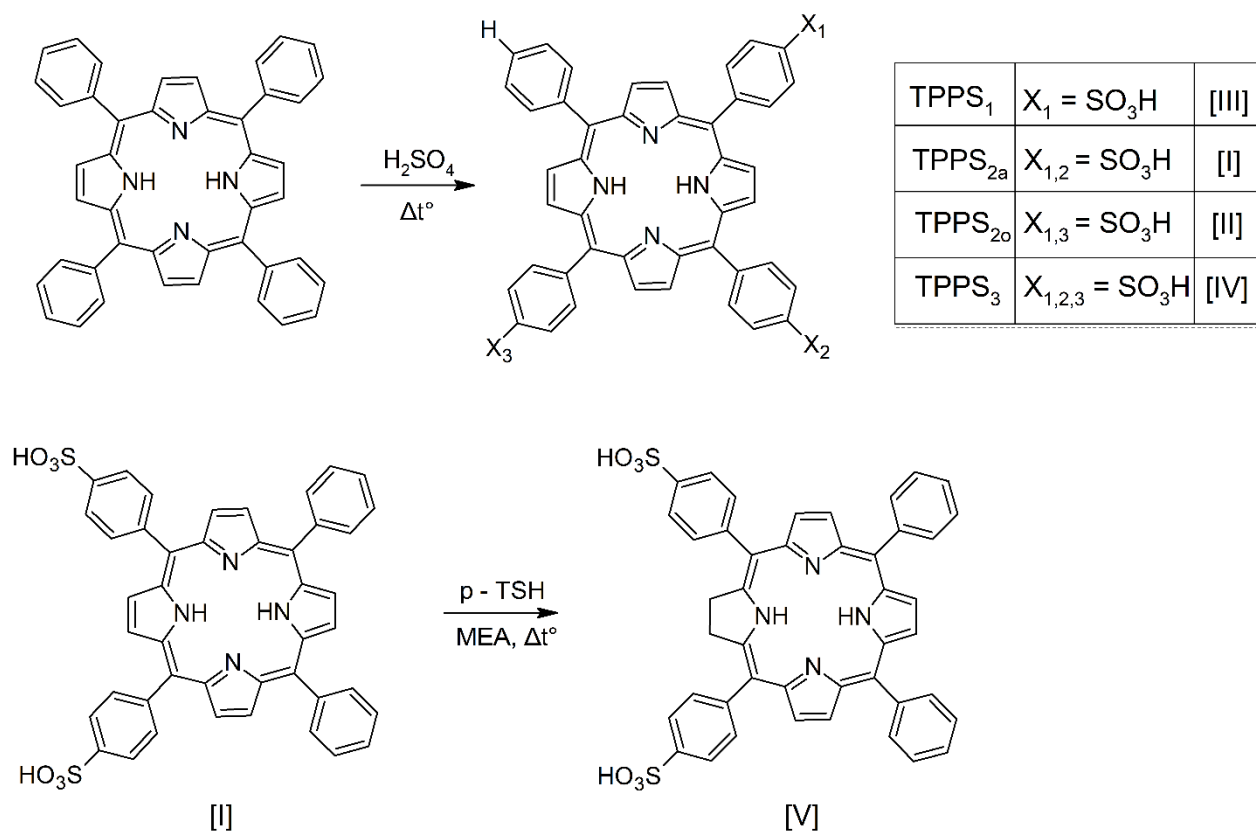


Figure 2. Structural overview of the synthetic route to TPCS_{2a}. Sulfonation of TPP by heating in H₂SO₄ affords a mixture of TPPS intermediates with 1-3 sulfonate groups. Isolated TPPS_{2a} [I] is subsequently reduced with *p*-toluenesulfonyl hydrazide (*p*-TSH) in monoethanolamine (MEA), giving TPCS_{2a} [V] as a mixture of regioisomers formed by reduction of one pyrrolic double bond.

2.5 Optical spectroscopy

The steady state absorption spectra of freshly produced TPCS_{2a} were recorded using a Shimadzu UV-1601PC spectrophotometer as earlier described by Callaghan S. et al. and Hageberg I. U. et al. [10, 28]. Measurements were performed with 10 mm quartz cuvettes (Hellma Precision) with a teflon cap allowing for gas-purging using syringe needles. Steady state photoluminescence measurements were carried out employing a PTI Quantamaster 8075-22 (Horiba Scientific) equipped with Double Mono 300 spectrometer chambers for both excitation and emission. A Hamamatsu R928 PMT was used for detection in the range 185 – 950 nm and one OB-75X (75W Xenon arc lamp) was used as the light source. Data acquisition and basic data-handling of steady state luminescence data were carried out with the Felix Data Analysis software and further processed and presented using Origin Pro.

Time-resolved fluorescence decays were recorded using an IBH time-correlated single photon counting (TCSPC) spectrometer system with 1 nm resolved emission monochromator (5000 M, Glasgow, UK). The system was equipped with a TBX-04D picosecond photon detection module, and the sample was excited using an IBH LED operating at 469 nm. The measured decay-trace was analyzed using deconvolution fitting with the IBH Data Station v 2.1 software and details how to process and interpret the TESA spectra, are earlier described [10, 28, 30].

2.6 Transient Singlet Oxygen Luminescence Measurements

The singlet oxygen luminescence lifetime and yield were measured from the transient luminescence of a solution containing the dye in a standard 90° configuration [31]. A tunable OPO laser, NT342A-SH-10-WW (Ekspla, Lithuania) was used for excitation. Typically, pulses of energy 40 – 50 mJ were included. For transient recording, a PMT (R5509, Hamamatsu) and a bandpass interference filter with maximum transmission at 1272.5 nm and a long pass filter transmitting above 780 nm were used in conjunction to avoid spurious signals and an Infiniium BDSU Oscilloscope (Keysight, United States) for collecting and to monitor data. Time-gated electronics controlled the time between the laser excitation and the recording of the luminescence transient. To obtain a reference signal (minimum amount of air/oxygen) the sample cell was bubbled with argon gas until the signal vanished. This typically took 10 min – 15 min.

2.7 Blue light source for *in vitro* cell experiments

Culture dishes were illuminated (from below, at room temperature) by using a LumiSource® blue light box (PCI Biotech, Norway), consisting of 4 Osram tubes (18 W, peak wavelength 435 nm, irradiance of 13 mW/cm²), measured with an Optometer UDT model 161, radiometer-photometer (United Detector Technology, Culver City, CA, USA), giving a total light dose of 7.8 J/cm² on the cell level during a 10 min illumination period. The light was detected near the bottom of the dish and before light treatment.

2.8 Cell cultivation

The AY27 cell line originates from a transitional cell carcinoma in a rat bladder and was supplied by Professor Steven H. Selman from the Medical College of Ohio, Toledo, USA in 2004. The AY27 cells were grown in RPMI-1640 medium (Catalogue No. R0883 Sigma Aldrich) and supplemented with fetal bovine serum (10%, Catalogue No. F-7524), 0.33% L- Glutamine (200 mM, Catalogue No. G-7513), and 1% penicillin/streptomycin (100 U/mL, 100U/mL, Catalogue No. P-0781). The T24 carcinoma cells (ATCC HTB-4) are transitional cell carcinoma from human urinary bladder and were grown in DMEM medium (Catalogue No. 11960044, Gibco) containing 10% fetal bovine serum, 1% penicillin/streptomycin, 0.33% L-glutamine and 1mM of sodium pyruvate (Catalogue No. S8636, Sigma Aldrich). Both cell lines were grown in sterile 75 cm² culture flasks and kept in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂ and sub cultivated approximately twice a week. Both light sensitive solutions and cells were covered with aluminum foil during all experiments.

2.9 Cell survival analyses

The cell survival studies were performed by three different protocols. the Alamar Blue assay and the mitochondrial dehydrogenase assay (MTT) and the clonogenic assay. The colorimetric assay MTT assesses the cell viability by measuring activity of enzymes that reduce MTT solution to formazan dyes, giving a purple color. The clonogenic assay documents the ability of a single cell to divide and produce a colony of identical daughter cells as (a clone, minimum 50 cells per clone).

2.10 Photodynamic experiments and viability assays

The cells were seeded in Petri dishes (D=6 cm, Nunc Denmark) at a density of 0.5×10^6 cells per dish the day before adding of growth medium containing TPCS_{2a} (0.2 µg/mL, 18 h, 37 °C) in the dark. The concentration of the PS was based on studies by group of Berg [32]. And the cells were grown overnight (24 h) in regular culture medium prior to viability assays (MTT) [33]. The following control samples were included and considered as “dark toxicity”: Two or three control dishes were exposed to light or TPCS_{2a} alone. The MTT solution (0.5 mg/ml) was freshly made in growth medium from a stock solution (5 mg/mL) and the cell cultures were washed (PBS, 2 mL) before adding the colouring reagents (3 mL) followed by incubation (1 h, 37 °C, 5% CO₂). The cells were detached by isopropanol (2 mL) and orbital shaking (80 rpm, 30 min) before measuring the absorbance (Shimadzu UV-1700 spectrophotometer, λ =595 nm). Isopropanol was used as a reference.

For Alamar Blue analyses (Thermo Fisher Scientific UK) the cell suspensions were diluted to make a concentration of 0.025×10^6 cells/mL and added (200 µL/well) to 96-well black plates (Costar® Corning incorporated, Sigma Aldrich) and the control rows and rows only receiving light were refilled with the same amount of normal growth medium. The cells were incubated (24h, 37 °C, 5% CO₂) before washing and incubation with TPCS_{2a} (0.2 µg/mL, 18 h, 37 °C) in the dark. The blue light illumination was performed (1 min, 13 mW/cm²) after washing (PBS x 3) and before incubation overnight (24 h). Then AlamarBlue™ reagent was diluted in growth medium (10%) and added to the cells and further incubated (3h, 37 °C, 5% CO₂). The fluorescence (excitation wavelength 560 nm, emission wavelength 590 nm) of each well was measured after 30 min of acclimatising using a plate reader (SpectraMax®i3x, Molecular Devices with SoftMax Pro 6.5.1 software).

In parallel with MTT, clonal cell survival was evaluated. A homogenous cell suspension (1000 cells) was seeded in dishes (D=6 cm) with growth medium (3 ml, 37 °C) and incubated for 6-7-days or 13-17 days (5% CO₂, 37 °C). Then colonies were washed (H₂O), fixed (glutaraldehyde, 6% in H₂O) and stained (crystal violet, Sigma-Aldrich) before manual counting. Duplicate plates were used for treatment and controls, and experiments were carried out in duplicates or triplicates. The average calibrated from the control wells ($I_{Control}$), for all viability assays were set to 100% cell viability. By using the equation below (Eq. 1) the cell viability for each groups measurement ($I_{Treated}$) were calculated.

$$\% \text{ Cell Viability} = \frac{I_{Treated}}{I_{Control}} \quad \text{Eq. 1}$$

2.12 Confocal microscopy of AY27 and T24 cell lines after bleomycin-based PCI

The AY27 and T24 cells were tyrosinated, centrifuged and counted as previously described and further seeded (8000 cells per dish and in 8-well chambered coverslips ($A_{\text{well}} = 1 \text{ cm}^2$) (μ -Slide 8 Well, Ibidi GmbH, Gräfelting, Germany) one day before washing (PBS) and incubation of TPCS_{2a} (1–3 $\mu\text{g/mL}$, 18 h, 37 °C, 5% CO₂). The next day the PCI treatment was performed after washing (PBS X 3) and before adding BLM (0.3 μM , 4 h). During the BLM incubation period the cells were illuminated (90 s) 1 h before end of the incubation and before confocal measurements at 5 and 60 min after light treatment. For investigating localization of the photosensitizer in cell compartments CellMask Yellow (1X, 5 min), Mitotracker Green (50 nM, 45 min) or LysoTracker Blue (150 nM, 1h 45min) were added before washing with PBS (x2). Images were recorded using a Leica SP8 confocal microscope using an HC PL APO CS2 63x/1.40W objective using distilled water as immersion liquid. All the emissions were detected through a 1 Airy Unit pinhole and the laser power, detector gain, and offset were chosen to minimize auto-fluorescence in the control samples where no photosensitizer was added. The laser wavelengths, emission bandpass, gain and zoom were set in the LAS X software.

3 Results

3.1 Chromatography of tetraphenylporphyrines

The sulfonation of tetraphenylporphyrin (TPP) afforded a mixture of sulfonated TPPS derivatives that were analyzed by HPLC and separated by flash chromatography. The analytical HPLC chromatogram showed one minor early-eluting impurity at approximately 4.8 min, followed by four assigned TPPS products (Fig. 3). The major peak was assigned to TPPS₃ [IV] ($t_R \sim 7.8 \text{ min}$). The two disulphonated regioisomers TPPS_{2o} [II] and TPPS_{2a} [I] eluted at ($t_R \sim 9.9 \text{ min}$) and ($t_R \sim 11.3 \text{ min}$), respectively, whereas the monosulfonated TPPS₁ [III] eluted later ($t_R \sim 13.4 \text{ min}$). The early-eluting impurity was not isolated or further characterized and was generally removed during the water/acetone washing step.

Because of the similarity between the TPPS_{2a} and TPPS_{2o} isomers, they were isolated as a mixture following the flash chromatography. The only difference is that TPPS_{2a} has two adjacent sulfonic side groups while TPPS_{2o} has the sulfonic groups opposite of each other and was most efficiently purified by preparative HPLC. TPPS₁ and TPPS₃ had a bigger difference in polarity from the disulfonated isomers on silica gel and were readily removed by flash chromatography. The analytical HPLC of the crud reaction mixture of TPPS reduction by p-TSH to TPCS_{2a} stereoisomers [V] and bacteriochlorin TPBS_{2a} [VI] after 18h, are present in Figure 4.

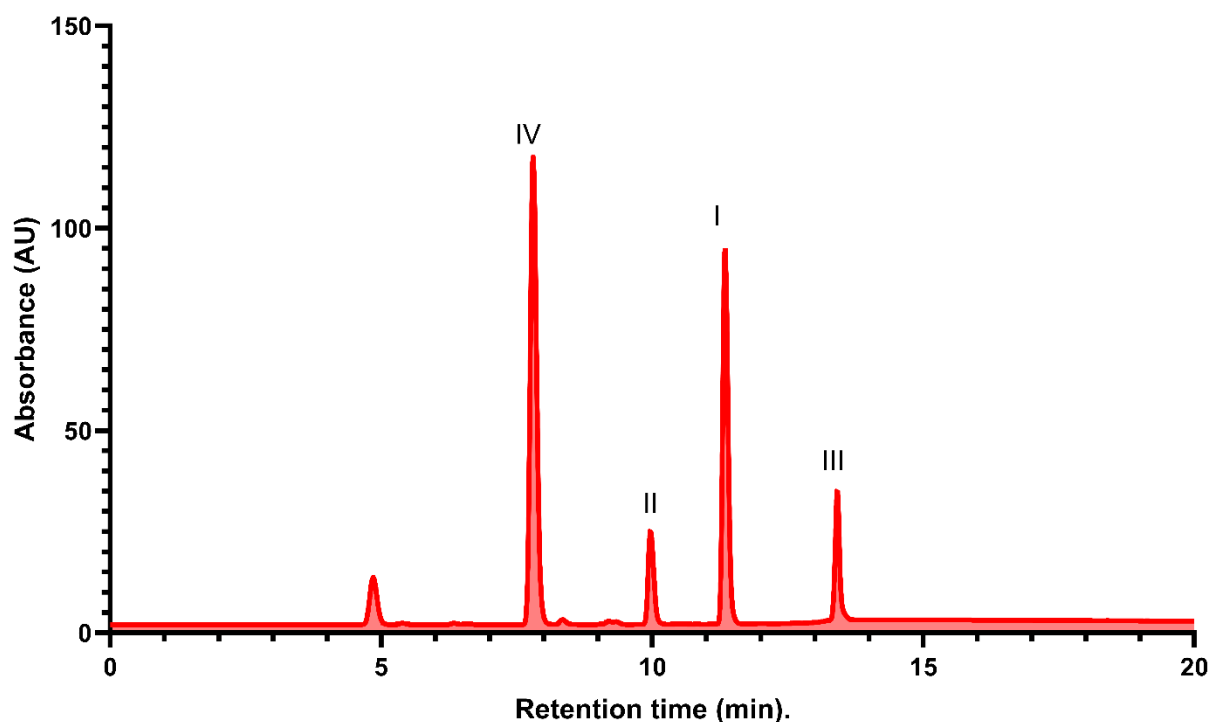


Figure 3. HPLC chromatogram of sulfonated products obtained from tetraphenylporphyrin (TPP). The assigned peaks correspond to TPPS₃ [IV] ($t_R \sim 7.8 \text{ min}$), TPPS_{2o} [II] ($t_R \sim 9.9 \text{ min}$), TPPS_{2a} [I] ($t_R \sim 11.3 \text{ min}$), and TPPS₁ [III] ($t_R \sim 13.4 \text{ min}$). The minor eluting peak at approximately 4.8 min was not isolated or characterized.

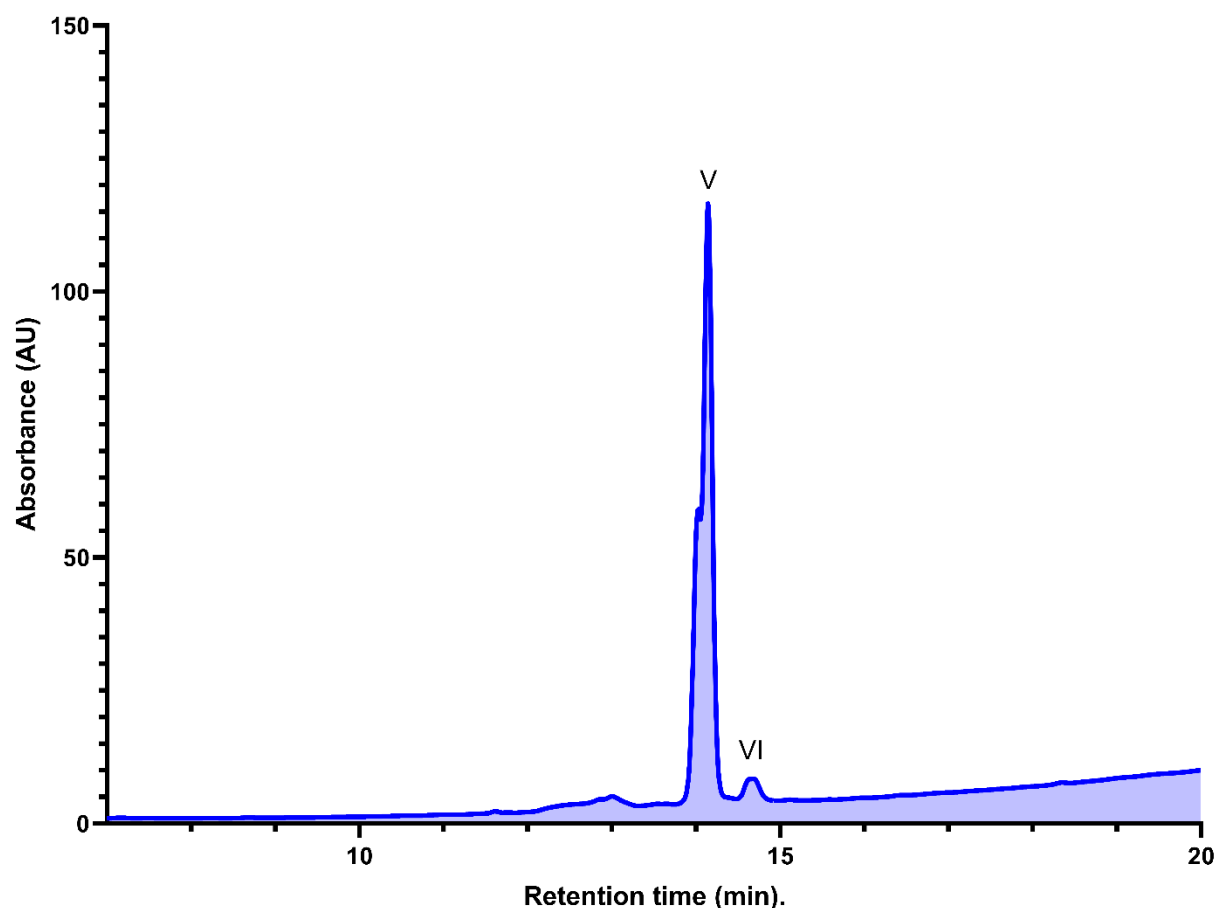


Figure 4. Analytical HPLC chromatogram of crud reaction mixture after p-TSH reduction of TPPS_{2a} to TPCS_{2a} stereoisomers [V] and bacteriochlorin TPBS_{2a} [VI].

3.3 NMR spectroscopy of tetrapyrroles

The ¹H NMR spectrum of the isolated material was consistent with di(monoethanolammonium salt) TPCS_{2a}. The aromatic and macrocycle region was observed as a series of overlapping multiplets between appr. δ 7.4 and 8.9 ppm, with resolved/maximal resonances at δ 8.87, 8.59, 8.29, 8.21, 7.80, and 7.41 ppm. These resonances correspond to the meso-phenyl, the meso sulfonated phenyl groups, the β-pyrrolic, imine, and alkene protons of the chlorin macrocycle. The complexity and overlapping regions of the aromatic part of the spectrum is expected because the isolated product is not a single isomer of TPCS_{2a}, but a mixture of three isomers as previously reported by Berg et.al. The most important confirmation from the NMR spectrum was the confirmation of the reduced double bond at the pyrrolidine CH₂ in the δ 4.1 ppm region, confirming the conversion of the TPPS_{2a} to the TPCS_{2a}. Additional aliphatic resonances at δ 3.70–3.60 and δ 2.78 ppm were assigned primarily to the monoethanolammonium counterions on the sulfonated phenyl groups. The 2D HSQC spectra further supported the assignment. The HSQC spectrum showed direct ¹H–¹³C correlations for the aromatic/macrocycle CH protons in the δ_H 7.4–8.9/δ_C 119–135 ppm region and aliphatic methylene correlations in the δ_H 4.2–2.8 ppm region (Figure S1). The methylene correlations appeared with opposite phase relative to CH correlations, supporting assignment of the δ 4.17 ppm resonances to CH₂ chlorin protons rather than aromatic CH protons.

3.4 Absorption and fluorescence emission of the tetrapyrroles TPPS_{2o}, TPPS_{2a}, and TPCS_{2a}

The absorption spectra show the characteristic strong Soret band around 421 nm, considerably broadened and stronger for the TPCS_{2a} variant (Figure 5). The vibrational splitting into 4 sub-bands is typical for a non-metalated central ring structure for both chlorins and the porphyrins [10, 34]. The Q-band region at approx. 500 – 675 nm is in comparison weaker for the porphyrins. All compounds show 2 distinct emission bands, also typical for the free-base form of the ring structure, with the one with highest photon energy coinciding with the lowest photon absorption at 650 nm. The low-energy sub-band is considerably weaker for the chlorin TPCS_{2a}. The fluorescence life-time of the emissions were characterized using time-correlated single photon counting found to be similar, in the range 10.6 – 12.3 ns (Figure S2).

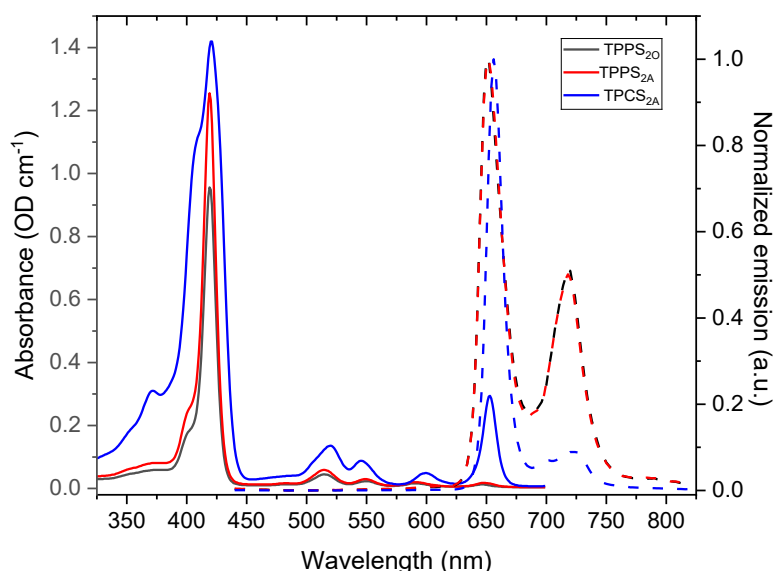


Figure 5: Absorption (solid lines) and fluorescence emission (dashed lines) of TPPS₂₀, TPPS_{2a}, and TPCS_{2a} in TRIS buffer (5 μ g/mL). The emission spectra were recorded upon excitation at $\lambda = 420$ nm.

3.5 Singlet Oxygen Production

Since the singlet oxygen quantum yield of the related porphyrins and chlorins have been reported extensively elsewhere [10, 35]; only representative results indicating singlet-oxygen activity from transient luminescence at 1270 nm for TPCS_{2a}, are reported here (see Materials and Methods). Firstly, the Soret-band corresponding to blue light (420 nm) was selected as excitation wavelength for the transient oxygen luminescence of TPCS_{2a} using ethanol and DMSO as solvents. As shown in Figure 6, these solvents give a very similar luminescence response with typical decays in the μ s time-scale. Secondly, the singlet oxygen yield of TPCS_{2a} in ethanol was estimated by comparison with an analogous chlorin based ring compound, ChloGA-proto (QE = 70%) with different substituents attached to the central ring [10] (Figure S3). The absorption spectrum in Figure S3 (A) shows a close-up of the Q-bands, and as can be seen, the absorption bands coincide very well in the 490 - 610 nm region. In Figure S3 (B) the transient single oxygen emission at 1270 nm in ethanol is shown upon excitation of the Q-band at 511 nm. Compensating for the difference in absorbance at 511 nm, the transient luminescence is of similar magnitude and decay time.

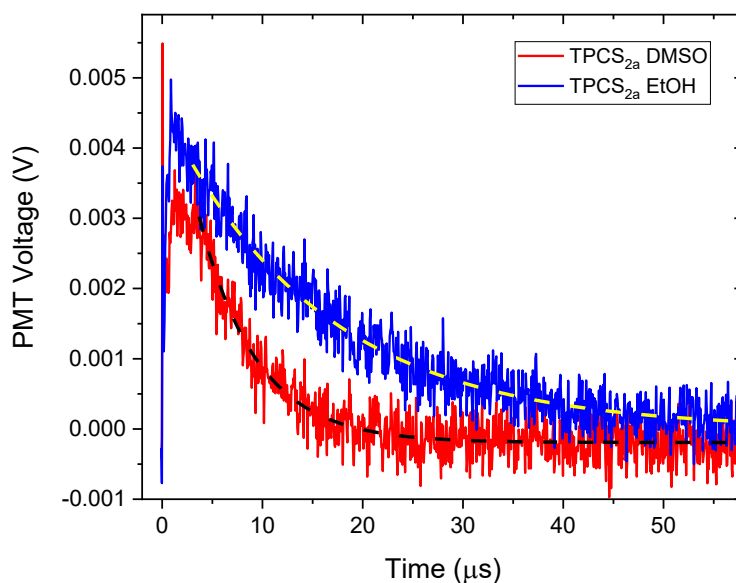


Figure 6. Transient singlet oxygen luminescence recorded at 1275 nm. TPCS_{2a} (1.3 μ M in ethanol (EtOH) and DMSO) was illuminated at 420 nm (absorption OD 0.2) with a Tunable pulsed (5 ns) OPO laser NT 342A-SH-10WW (Ekspla, Lithuania). Dashed curves are fits using 15.5 ± 0.4 μ s and 5.7 ± 0.2 μ s for ethanol and DMSO, respectively.

3.6 Photodynamic effects of tetraphenyl derivates on AY27 cells – MTT assay

The viability effects of tetraphenyl derivates were tested on the rat bladder cell line by short-time MTT assay (Figure 7) and resulted in a LD₅₀ value after 80s for TPCS_{2a}, but for TPPS_{2a} a LD₅₀ after about 160 s. For TPCS_{2o}, only a negligible effect (about 10%) was observed, and the control cells were weakly affected during the illumination period (blue light, 435 nm). These information documents that TPCS_{2a} were the most suitable PS and were further used in both standard PDT experiments and for PCI technology.

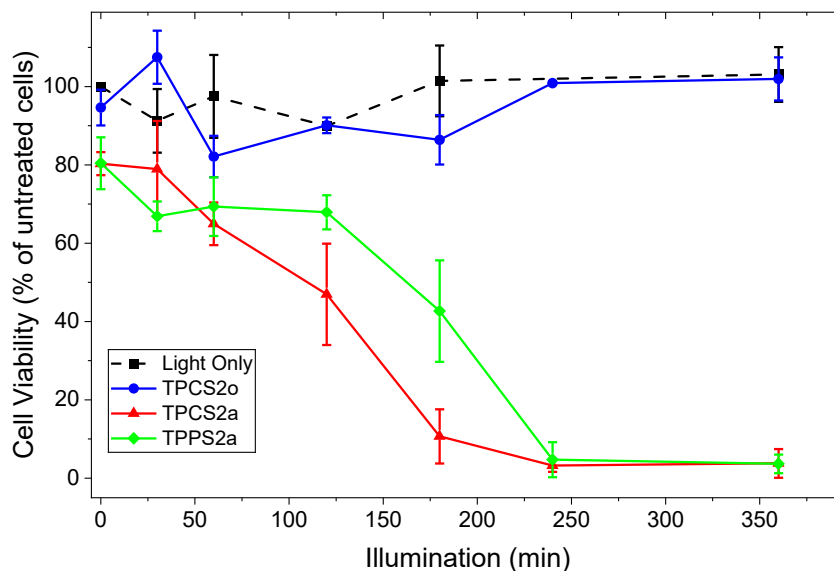


Figure 7. Effects of photochemical treatment (MTT assay) by using TPCS_{2o}, TPPS_{2a}, TPCS_{2a}; all incubated with 0.2 μ M (18 h, 37 °C) in the dark. The cell survival of AY27 rat bladder cancer cells was measured as a function of blue light exposure (435 nm, 13 mW/cm²), and the LD₅₀ doses correspond to 2.47 J cm⁻². Cell viability was performed by using MTT assay 24 h post light exposure and the results are present as mean values $\pm$ SD from five experiments.

3.7 Cytotoxic effects of TPCS_{2a} and AlPcS_{2a} on AY27 cells - AlamarBlue assay

A comparison was made according to cytotoxic effects between the synthesized photosensitizer TPCS_{2a}, and the commercial aluminum phthalocyanine disulfonate (AlPcS_{2a}, Frontier Scientific), the chemical compound with which PCI technology was introduced by group of Professor Berg [36]. This study was conducted to test the efficacy of the commercialized drug AlPcS_{2a} against our own synthesized compound (Figure 8, Figure S4). In the present experiments the incubation of the photosensitizers (24 h, 37°C, dark) with different concentrations of TPCS_{2a} (0-100 μ g/mL) and AlPcS_{2a} (0-10 μ g/mL), resulted in 50% cell death by 2 μ g/mL for AlPcS_{2a} and about 42 μ g/mL by TPCS_{2a} respectively analyzed by AlamarBlue assay from three separate assays. This indicates that AY27 cells are much more sensitive to AlPcS_{2a} compared to TPCS_{2a}, by reaching a complete cell death after an incubation with 7.5 μ g/mL compared to TPCS_{2a} which had no live cells by using a concentration of 100 μ g/mL.

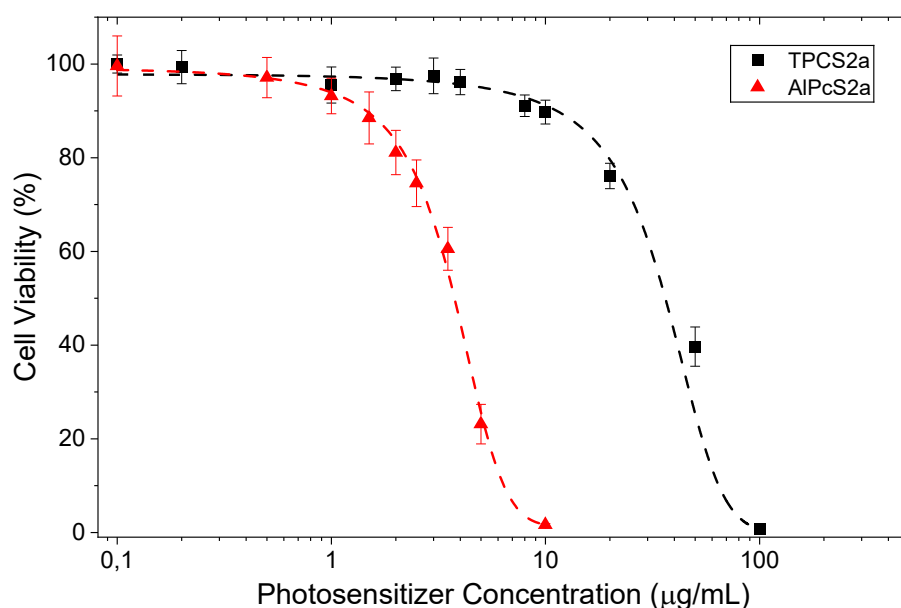


Figure 8. Comparison between cytotoxic effects of synthesized TPCS_{2a} (0.1 – 10 µg/mL) and AlPcS_{2a} (0.1-100 µg/mL) on rat bladder cancer cells (AY27) by employing the AlamarBlue assay as described in Material and Methods. Each datapoint in the plot represents metabolic activity relative to control cells incubated in a photosensitizer-free medium. The results are present as mean values $\pm$ SD from six parallel wells. The dashed curves are fits to the standard dose response yielding the IC₅₀ values of 3.66 ± 0.10 and 36.2 ± 0.30 µg/mL for AlPcS_{2a} and TPCS_{2a}, respectively.

3.8 Cytotoxic effects of TPCS_{2a} - Clonogenic assay

The long-time effect (6-7 days) of PDT and PCI was measured by clonogenic assay as described in Material and Methods. The results show almost similar effects for both controls and BLM treatment which never reach LD₅₀ (Figure 9), while the present result for LD₅₀ of both PDT and PCI were 90 s and 130 s respectively.

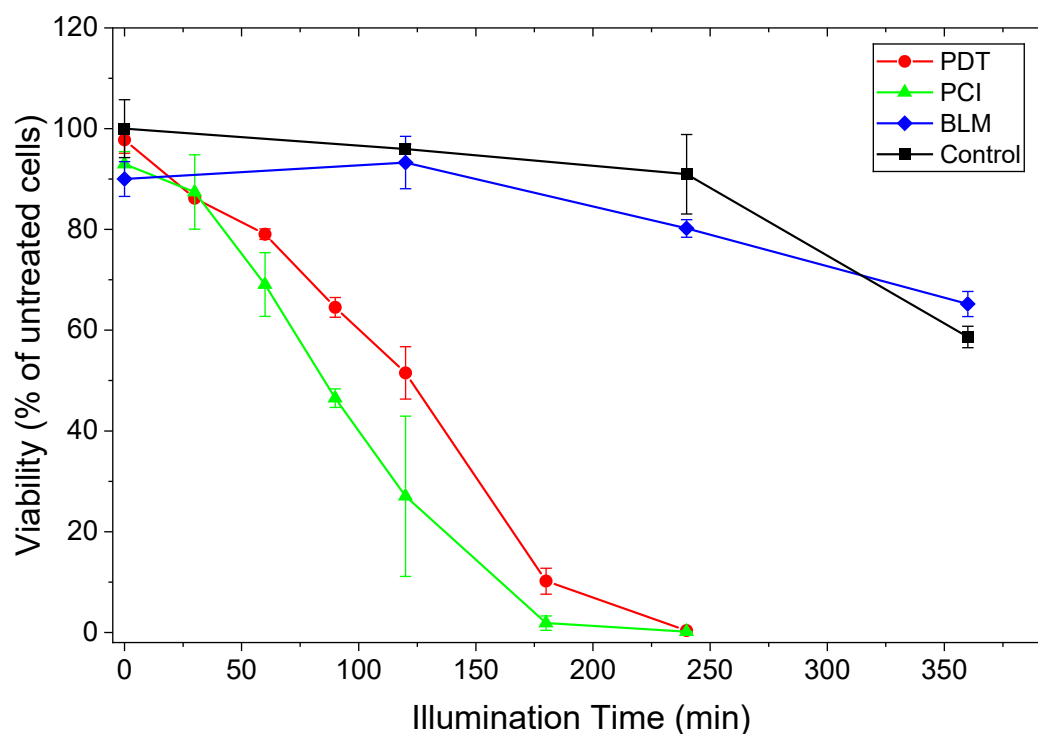


Figure 9. Survival of clonogenic rat bladder cancer cells (AY27) after incubation of BLM (0.1 µM, 4h) or TPCS_{2a} (0.2 µg/ml) alone or in combination. Data are presented as mean value $\pm$ SEM of duplicates or triplicates from four different experiments, relative to untreated cells.

3.9 Confocal microscopy on AY27 and T24 cells after incubation of TPCS_{2a}

To investigate the localization of synthesized TPCS_{2a} in the bladder cancer cells, co-staining experiments were performed with different organelle markers in both AY27 and T24 cells for additional information about the properties of the photosensitizer. First, the testing by confocal microscopy of two different concentrations of TPCS_{2a}; 1 $\mu\text{g/mL}$ and 3 $\mu\text{g/mL}$ was performed (Figure S5) giving the highest fluorescence by 3 $\mu\text{g/mL}$. Co-localization experiments in AY27 with LysoTracker Blue, Mitotracker Green or Cell Mask Yellow showed that TPCS_{2a} localized in separated well-defined clusters (Figure S6). Representative images of the incubation and PDT process are shown in Figure 10 (AY27) and Figure 11 (T24). Firstly, upon TPCS_{2a} incubation both cell lines appear slightly rounded up with PS in the cytosol (A). By coloring Cell Mask Yellow, the cell membrane is clearly visible in both cell lines, and for T24 it is showing a marked and broad shape. In the AY27 cells the nucleus is manifested as a circular and darker area (B).

Five minutes post BLM (0.3 μM , 4 hours)-PCI treatment including light (90s, 435 nm, 13 mW/cm²), the cells were swollen, and the cell membrane of AY27 cells appears to still be strongly stained. In contrast, for the T24 cells, the cell membrane is weaker, but at the same time clusters of PS are evident inside the cytoplasm (C). Cell division was also observed in T24 cells, indicating that the cells are alive (C), but after further post incubation (60 min, 37 °C) complete destruction occurred with membrane leakage and Cell Mask Yellow throughout the cytosol (D) in both cell lines.

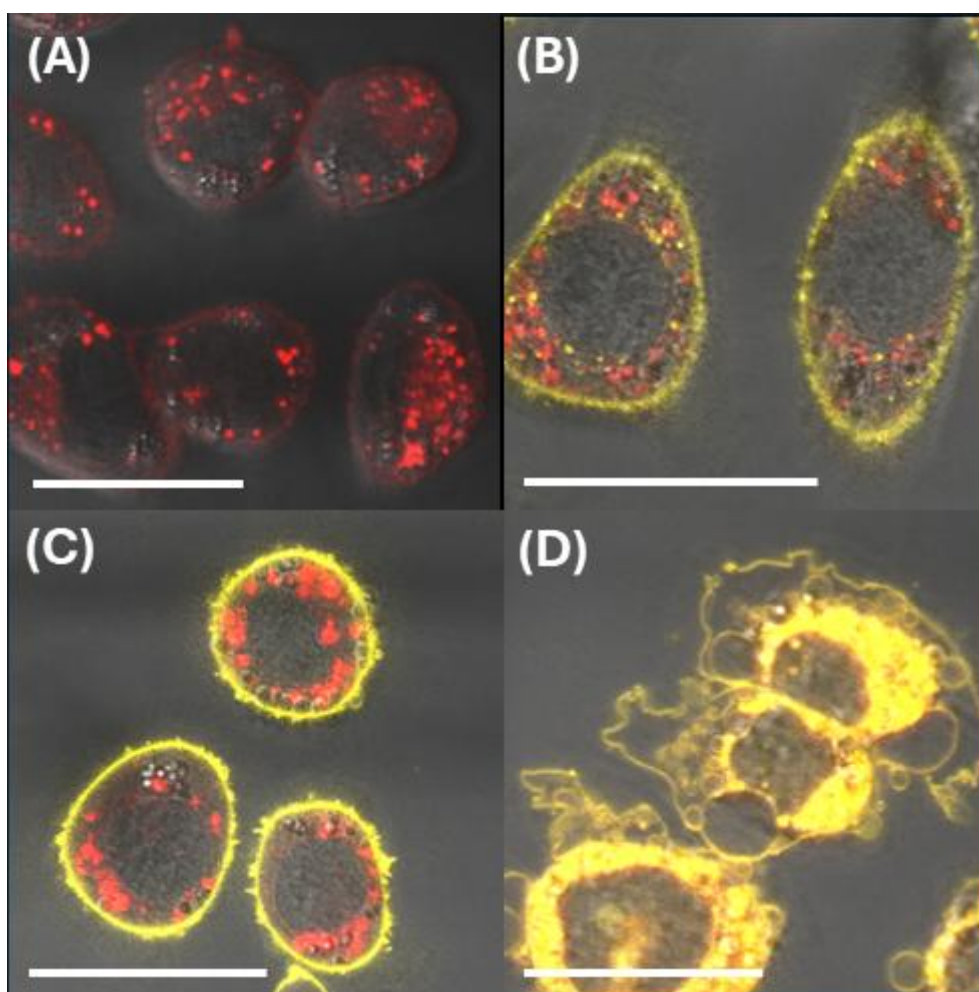


Figure 10: Representative images used for co-localization analysis on AY27 cells by coloring of Cell Mask Yellow: A) Incubation with TPCS_{2a} (3 $\mu\text{g/mL}$, 18 h, 37 °C), B) Incubation with TPCS_{2a} and colored with Cell Mask Yellow (5 μM , 5 min), C) Post 5 min after BLM-PCI including blue light (90 s, 435 nm, 13 mW/cm²). D) Post 60 min after BLM-PCI including blue light (90 s, 435 nm, 13 mW/cm²). Scale bar is 25 mm.

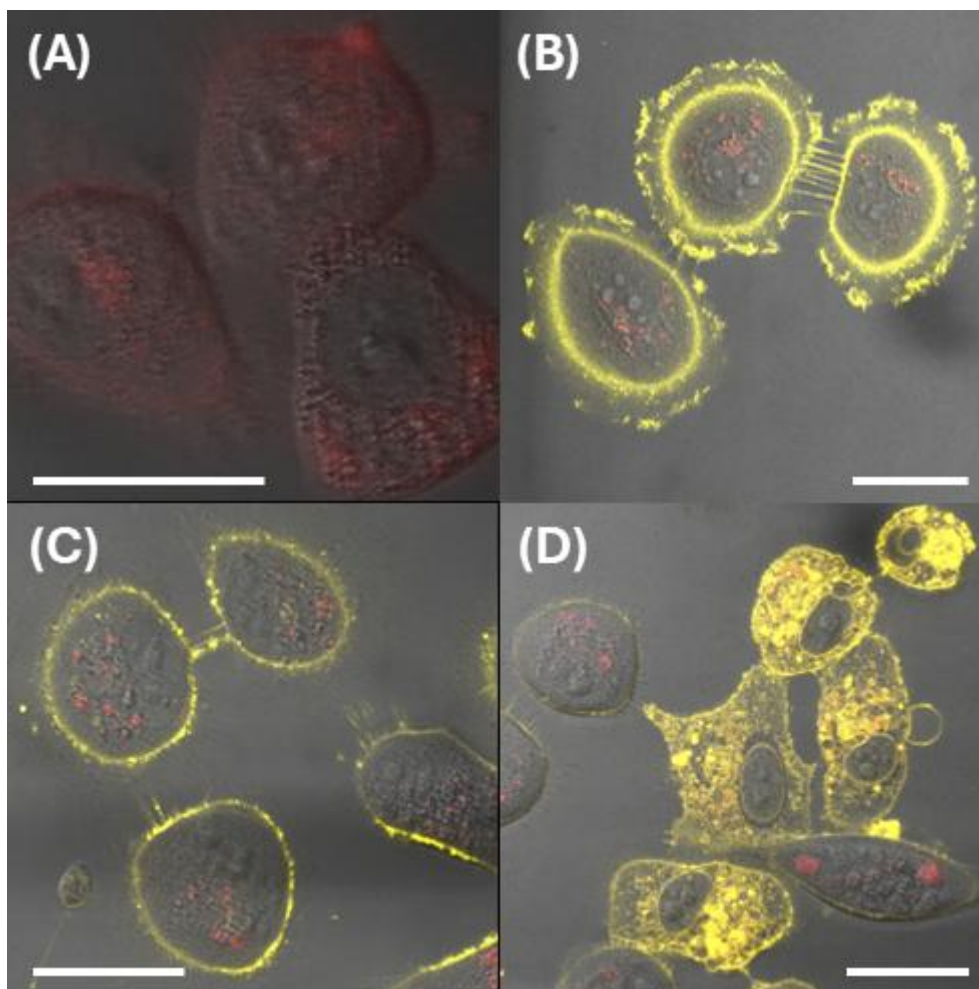


Figure 11: Representative images used for co-localization analysis on T24 cells by coloring of Cell Mask yellow: A) Incubation with TPCS_{2a} (3 ug/mL, 18 h, 37 °C), B) Incubation with TPCS_{2a} and colored with CellMask Yellow (5 uM, 5 min, 37 °C), C) Post 5 min after BLM-PCI including blue light (90 s, 435 nm, 13 mW/cm²). D) Post 60 min after PCI including blue (90 s, 435 nm, 13 mW/cm²). Scale bar is 25 mm.

3 Discussion

One of the aims of the project was to synthesize TPCS_{2a} from 5, 10, 15 20 -tetraphenylporphine (TPP) and to avoid high levels of bacteriochlorine. Several methods have earlier been described; the one with diimide reduction resulted in three isomers as described by Berg et al. in the ratio 1:2:1 [2]. Although PDT with bacteriochlorin has shown promising cytotoxic effects in myeloma cells by Mroz P. et al. [37], the influence of this byproduct after PS synthesis is not desirable in treatment of cancer cells. A possible and simpler method is to synthesis from TPPS_{2a} as described by Zhang et al. [27] and used in this project as present in Figure 2. It is well documented in literature that the use of ethanolamine can facilitate regioselective hydrogenation in the diimide reduction without causing double-bond migration or over-reduction, often in combination with 2-methoxyethanol as a solvent [23]. By using ethanolamine as a mild, polar organic base and cosolvent we achieved good solubility of the disulfonated porphyrins and the diimide precursor p-TSH without the need for 2-methoxyethanol cosolvent. The sulphonate groups at the one side of the aromatic macrocycle makes it hydrophilic and on the opposite side the hydrophobic and unsubstituted part of the molecule. Because of these chemical properties, the PS accumulates well in the lipid bilayer, and the physio-chemical properties of the PSs are of importance because the molecules must be able to penetrate the cellular membrane. By incubation of 18 h, using the synthesized PS, as described in the present study, the *in vitro* results correlate well with data on AY27 cells by Arentsen [15, 38] and also on squamous cell carcinoma (A431) by Baglo et al. [38].

Spectroscopic investigation confirmed the well-known absorption in the strong Soret-peak in the blue 420 nm region and four weaker but distinct Q-bands at 510 – 650 nm (Figure 5). Fluorescence can be observed in the red region (650 – 720 nm) useful for investigating PS localization in cell models [35]. Furthermore, by exciting Q-bands with a ns laser singlet oxygen singlet oxygen emission at 1275 nm can be observed (Figure 6) because of spin-transfer from excited triplet states to ambient oxygen dissolved in the samples [10].

The *in vitro* studies by incubation of the synthesized TPCS_{2a} document significant PDT-cell-killings effects in the two bladder cancer cell lines, but T24 cells have a lower uptake compared to the AY27 cells (Figure 10 and 11). In another study on AY27 cells with incubation of glycosylated chlorins and blue light (435 nm) illumination, PDT effects were observed with apoptosis and necrosis as early as 30s; and the results are consistent with our data [10]. Further, by using BLM-based PCI technology resulted in a clear decrease in cell viability and is well correlated with our previous data [38] on both cell lines. About long-time survival, non-illuminated AY27 and T24 cells treated with TPCS_{2a} were almost identical to the control cells and indicate a low dark toxicity, but for investigating the localization of the PSs in the cell, co-staining experiments were performed with different organelle markers (Figure S3).

A cytostatic drug like e.g. BLM, follows a similar route as the PS to penetrate the cellular membranes by endocytosis. When BLM molecules enter the cytosol after illumination, they bind to specific regions of DNA with complexation with Fe (II) and molecular oxygen and result in i. a. double-stranded and single-stranded DNA cleavage [1, 39]. The endocytic transport pathways for BLM and its translocation into nucleus are not fully understood, but the cell death effects by BLM-PCI on AY27 cells have been documented in a series of studies *in vitro* [1], and *in vivo* [13, 18, 38, 40].

After testing the viability of tetraphenyl derivatives on rat bladder cancer cells, which clearly showed that the synthesized TPCS_{2a} was the best candidate (Figure 7), further clonogenic assays were performed by using a PS concentration of 0.2 µg/mL based on previous studies on the same cell line [18, 38]. The clonogenic results are in accordance with data from another research group [18, 38] and document a reduced colony forming capacity by BLM-PCI (~4%) compared to the PDT treated ones (Figure 9). In contrast, the clonogenic effect by BLM or light alone was not more than 20% in decrease of cell viability during 250s of light.

By comparing the spectroscopic data (Figure 5) with results from Lilletvedt et al. [41, 42], they are convincingly similar. And in our case the light dose by 20s is most relevant for long-term clonogenicity assays as longer exposures result in dead cells with no clonal survival (Figure 9). The clonogenicity experiments indicate a clear reduced colony-forming capacity by PCI treated AY27 cells compared to the PDT treated ones.

The localization of the PS are assumed in the endocytic vesicles, and beneficial for PCI application [43]. By confocal microscopy studies additional information's were performed on properties of TPCS_{2a} and even more clearly by BLM-PCI (Figure 10 and 11) and the detailed colocalization were performed by using Cell Mask Yellow with focus on the cell membrane differences. A convincing and clear localization of the PS in the plasma membranes of both cell lines, and subsequent incubation of the cytostatic drug BLM in combination with blue light (90s), resulted in minor changes in T24 cells post 5 min, but a strong fluorescence in the plasma membrane of AY27 cells. Further, a complete cell destruction was observed post 60 min. In both cell lines, granulated clusters of the photosensitizers were observed inside the plasma membrane of both AY27 and T24 cells.

The main photosensitizers TPCS_{2a} and AlPcS_{2a}, who have been utilized in PCI experiments and documented to localize in endocytic vesicles both *in vitro* and *in vivo* [36, 44, 45], it was of interest to compare the toxicity of both PSs on the AY27 cells. These comparisons were interesting because PCI technology was established by using AlPcS_{2a} [4] and later replaced by TPCS_{2a} (brand name Amphinex®) from PCI Biotech AS, Oslo. The dose-response at LD₅₀ (about 3 µM) correlate well with the 4 µM-results from Didamson et al. [25] on esophageal CSC-cells and suggests that the synthesized photosensitizer is effective for treating on both AY27 and CSC cells at low concentrations. The present incubation with AlPcS_{2a} indicate a cell killing (LD₅₀) about 13 times more compared to our own produced TPCS_{2a}. That is in accordance with BLM studies on tumors of different organs in BALB/c (nu/nu) mice [46], and document that AlPcS_{2a}-PCI induced a more pronounced delay compared to TPCS_{2a}-PCI.

Conclusions

It is successfully demonstrated a convenient synthetic approach to produce tetraphenyl chlorine (TPCS_{2a}/TPCS_{2o}) by using a new and simpler route for synthesis of the PS and its pre-compounds TPPS_{2a} and TPPS_{2o}. The compounds were compared to properties of commercial variants along with capability to produce singlet oxygen from their triplet states. The synthesized TPCS_{2a} has shown to be an efficient PCI photosensitizer *in vitro*, tested by two bladder cancer cell lines (AY27 and T24), and demonstrate enhanced cytotoxic effects compared to PDT. Overall, the documented and evaluated novel synthesis of TPCS_{2a} may be actual for further development in photodynamic therapy and in synthesis of other chlorin compounds where over reduction to bacteriochlorin is an issue.

Conflict of interest: None

Acknowledgements

This work was financially supported by The Liaison Committee for education, research, and innovation in Central Norway and grants from the Cancer Research Foundation of St. Olav's University Hospital, Trondheim. Also, support by Gun Svendsen (Byggteknikk Invest AS), Trondheim, Norway and "Forskningsstiftelsen", arranged by Kristian Berg, the Norwegian Radium Hospital, Oslo, Norway. The authors want to thank the following persons from NTNU; Kristin Sæterbø and Johannes Bergvoll (Department of Physics), Markus Haug (Centre of Molecular Inflammation Research (CEMIR) and Ellen M. S. Flatby (University Library). Also, thanks for detail information's by Tengzhi Liu, PET Senter, St. Olav's Hospital, Trondheim/ARTBIO, Oslo, and the permanent employees at Department of Natural Sciences at the University of Agder, Kristiansand.

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Supplementary figures

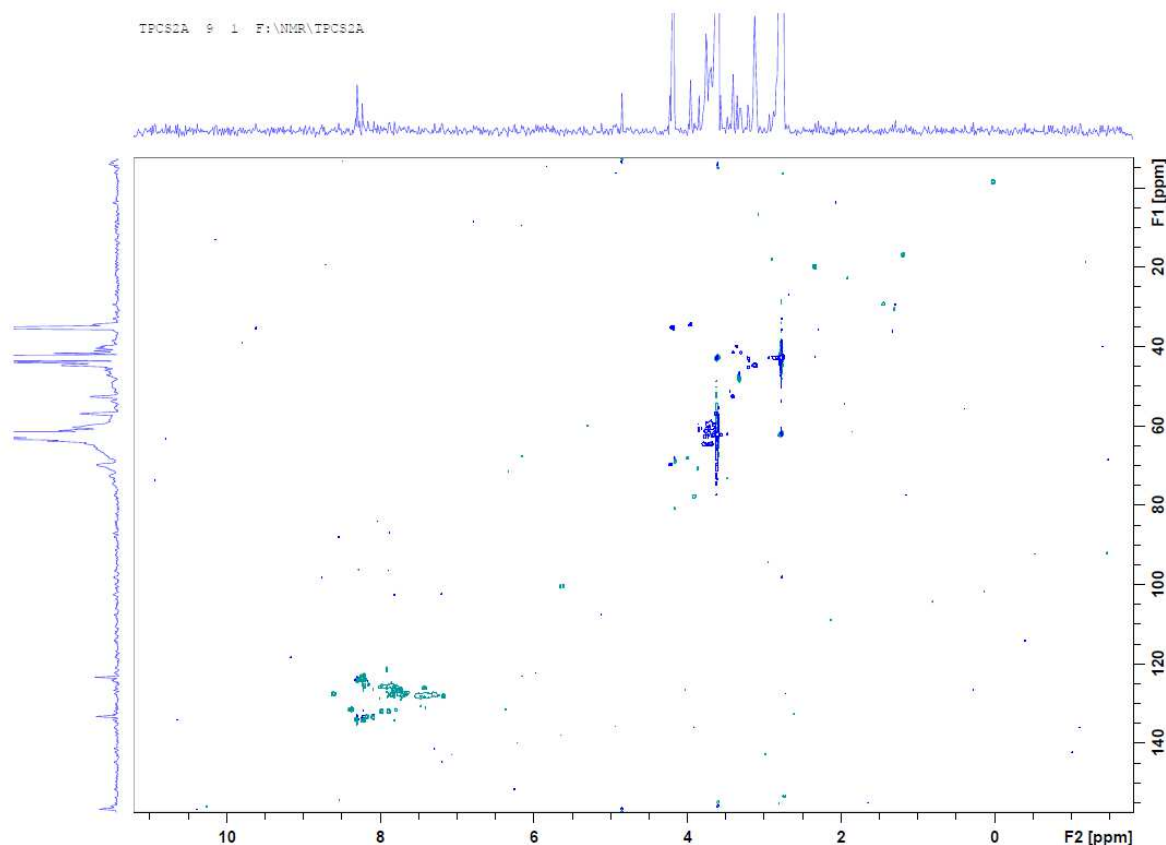


Figure S1. HSQC spectra of synthesized TPCS_{2a} documented the ¹H-¹³C correlation between the aromatic/macrocycle CH protons in the δ_H 7.4–8.9/δ_C 119–135 ppm region and aliphatic methylene correlations in the δ_H 4.2–2.8 ppm region. The methylene CH₂ correlations appear in opposite phase to the aromatic CH protons.

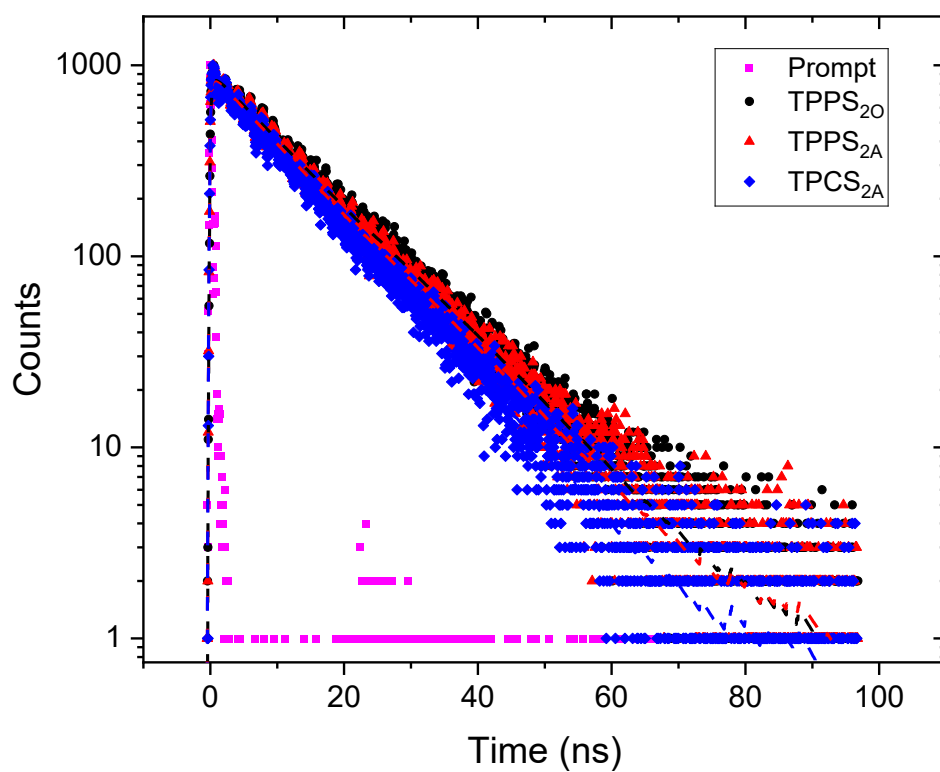


Figure S2. Time-correlated Single Photon Counting decay traces of the solutes in ethanol. The excitation wavelength was 403 nm and the emission in the range 660 – 730 nm was collected.

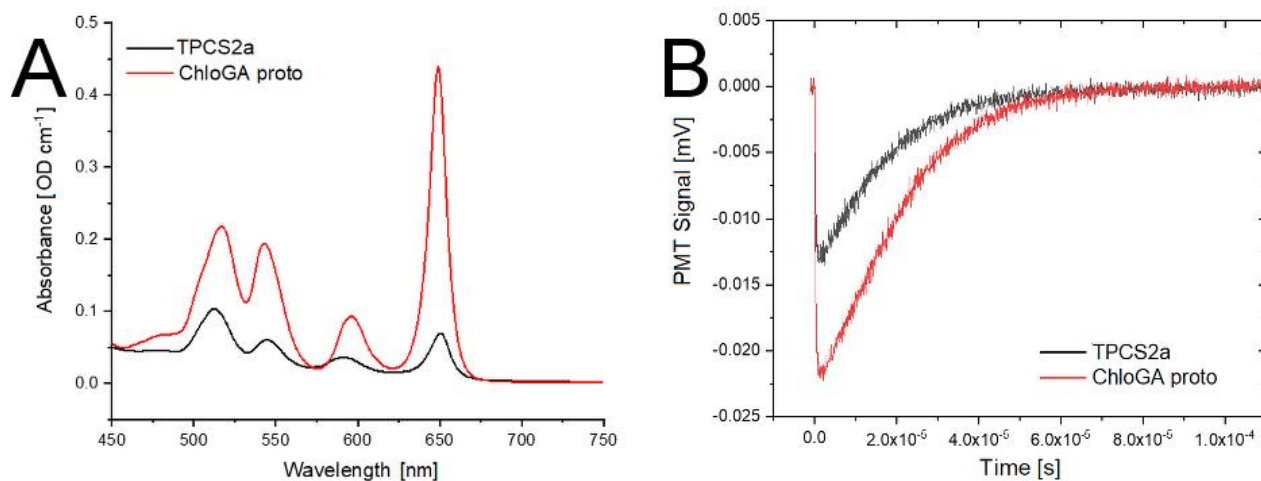


Figure S3. Time-correlated Single Photon Counting decay traces of the solutes in ethanol. The excitation wavelength was 403 nm and the emission in the range 660 – 730 nm was collected.

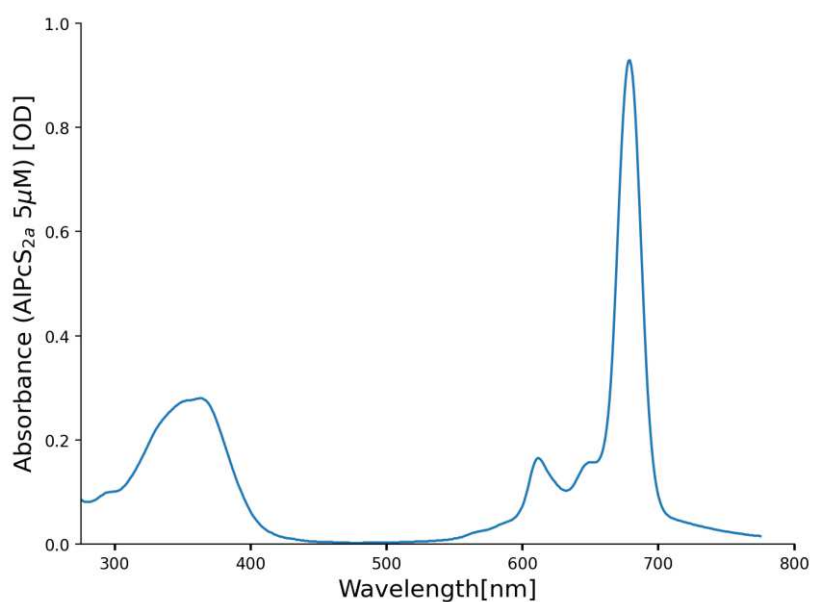


Figure S4. Absorbance spectrum of AlPcS_{2a} (5 μM) using a Shimadzu UV/VIS UV-1601 PC Spectrophotometer [47].

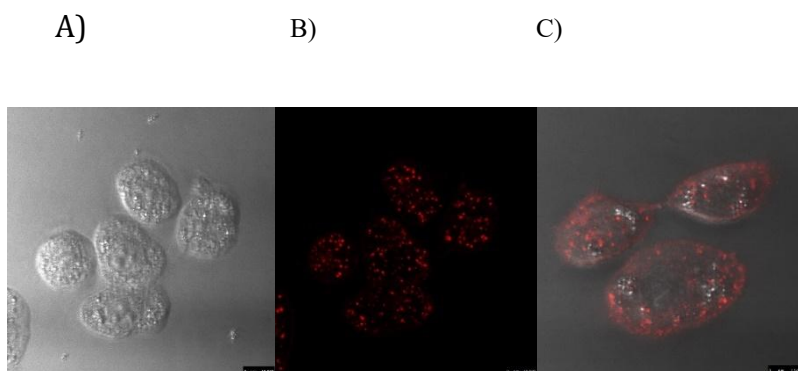


Figure S5. Confocal measurements of human bladder cancer cells (T24). Without any treatment in A), incubation with only synthesized TPCS_{2a} (1 μg/mL) in B) and TPCS_{2a} (3 μg/mL) in C).

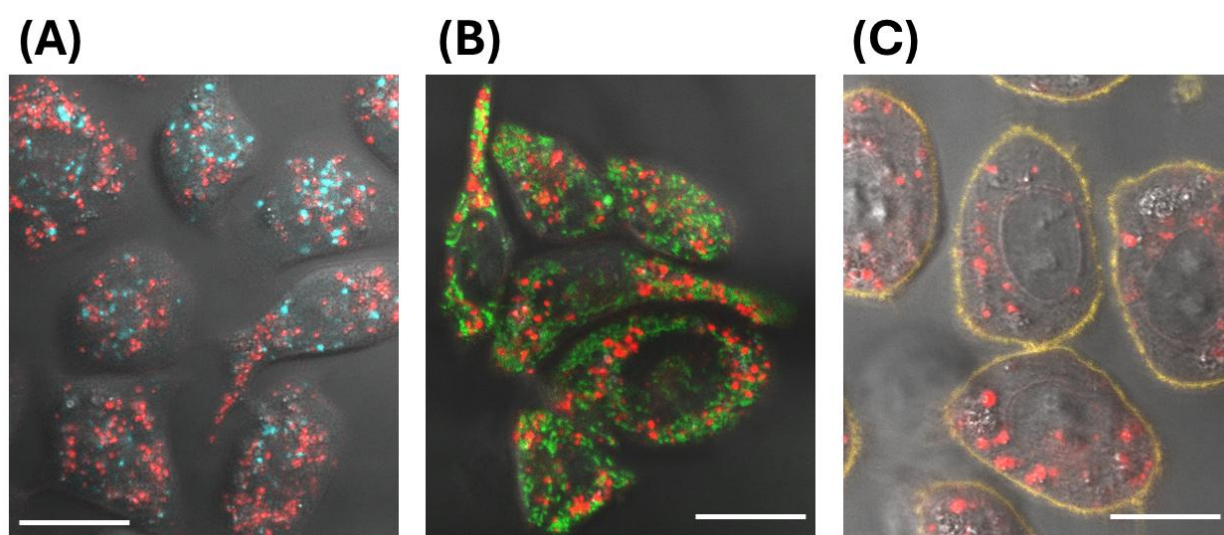


Figure S6. Imaging TPCS_{2a} (3 ug/mL) in AY27 together with (A) LysoTracker Blue (B) MitoTracker Green, and (C) CellMask Yellow. Scale bar is 25 μm.