Anionic hexadeca-carboxylate tetrapyrazinoporphyrazine: synthesis and in vitro photodynamic studies of a water-soluble, non-aggregating photosensitizer

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Synthesis

Preparation of triethyl benzene-1,3,5-tricarboxylate (3).
A reported procedure has been adopted for synthesis of this compound. Trimesic acid (20 g, 95 mmol) was dissolved in hot absolute ethanol (145 mL) and sulfuric acid (3.2 mL) was added after dissolution. The mixture was refluxed for 1.5 h and then the condenser was replaced for descending one and about 80 mL of ethanol was distilled off. The reflux then continued for next 3.5 h with appearance of white solid after 1.5 h. The reaction was cooled down and neutralized by saturated NaHCO₃ solution. The white precipitate was collected by filtration and washed by water to yield white solid (23.9 g, 85%) of sufficient purity for next reactions. The analytical sample was crystallized from ethanol. m.p. 132.2-133.1 °C (lit. 136 °C); ¹H NMR (CDCl₃, 500 MHz): δ 8.84 (3H, s), 4.43 (6H, q, J = 7.1 Hz), 1.42 (9H, t, J = 7.1 Hz); ¹³C NMR (CDCl₃, 125 MHz): δ 165.0, 134.4, 131.4, 61.7, 14.3. Data corresponded well to those published by Kathiresan et al.¹

Preparation of 3,5-bis(ethoxycarbonyl)benzoic acid (4).
A reported procedure has been adopted for synthesis of this compound. The triester 3 (36.1 g, 123 mmol) was dissolved in hot THF (75 mL) and absolute ethanol (120 mL) was added together with finely ground KOH (8.9 g, 135 mmol, contains 85% of the material). The mixture was refluxed for 24 h, cooled down and the volatiles were removed under reduced pressure. Distilled water (450 mL) was added to the remaining solid and the mixture was washed three times with dichloromethane. The organic phase was dried with Na₂SO₄ and evaporated under reduced pressure to recover unreacted triester 3 (6.3 g, 17%). The water phase was acidified with HCl and the white precipitate that formed was collected by filtration and washed with water. The white solid (19.6 g, 60%) contained the product 4 with the traces of 5-(ethoxycarbonyl)isophthalic acid and was of sufficient purity for next reactions. The analytical sample was crystallized from ethanol. m.p. 150.0-150.9 °C (lit. 153-154 °C); ¹H NMR (CDCl₃, 300 MHz): δ 10.94 (1H, bs), 8.98-8.84 (3H, m), 4.45 (4H, q, J = 7.1 Hz), 1.44 (6H, t, J = 7.1 Hz); ¹³C NMR (CDCl₃, 75 MHz): δ 170.4, 164.8, 135.4, 135.0, 131.7, 130.2, 61.8, 14.3. Data corresponded well to those published by Leon et al.²

Preparation of diethyl 5-(hydroxymethyl)isophthalate (5).
A reported procedure has been adopted for synthesis of this compound. The monocarboxy derivate 4 (17.6 g, 66.1 mmol) was dissolved in anhydrous THF (100 mL) under argon. While cooling the flask by ice water, the 1M solution of BH₃·THF (100 mL, 100 mmol) was added by dropping funnel over a period of 1 h under intense stirring and the white precipitate formed. After all BH₃·THF was added, the reaction was stirred at rt for another 5 h. The reaction was quenched by THF/water 1:1 (150 mL) that was added in a dropwise manner while the reaction flask was cooled by ice water. THF was removed under reduced pressure and the residual water was washed three times with ethyl acetate. Organic layer was dried with Na₂SO₄ and evaporated under reduced pressure. The crude product was purified via column chromatography on silica with hexane/ethyl acetate (1:1) as the eluent to yield white solid (13.4 g, 79%). The analytical sample was crystallized from ethanol/water. m.p. 82.3-83.1 °C (lit. 82-83 °C); ¹H NMR (CD₃SOCD₂, 300 MHz): δ 8.36-8.27 (1H, m), 8.16-8.09 (2H, m), 5.49 (1H, t, J = 5.7 Hz), 4.62 (2H, d, J = 5.7 Hz), 4.33 (4H, q, J = 7.1 Hz), 1.33 (6H, t, J = 7.1 Hz); ¹³C NMR (CD₃SOCD₂, 75 MHz): δ 165.2, 144.5, 131.3, 130.5, 128.0, 62.0, 61.3, 14.3. Data corresponded well to those published by Kathiresan et al.¹

Preparation of diethyl 5-formylisophthalate (6).
A reported procedure has been adopted for synthesis of this compound. The alcohol 5 (13.4 g, 53.1 mmol) was dissolved in anhydrous chloroform stabilized by amylene (120 mL) under argon. The solution was stirred at rt and pyridinium chlorochromate (28.6 g, 132.8 mmol) was added stepwise. The brown-black suspension was stirred further at rt for 4 h. The solution was then gently decanted
from the black slurry and poured into diethylether. The black slurry in the flask was washed several times with diethylether, the solutions were combined and filtered through Celite. The crude product was purified via column chromatography on silica with hexane/ethyl acetate (1:1) as the eluent to yield white solid (11.8 g, 89%). m.p. 88.0-88.9 °C (lit.4 90 °C); 1H NMR (CDCl$_3$, 300 MHz): δ 10.12 (1H, s), 8.90 (1H, t, J = 1.7 Hz), 8.69 (2H, d, J = 1.8 Hz), 4.44 (4H, q, J = 7.1 Hz), 1.43 (6H, t, J = 7.1 Hz); 1H NMR (CDCl$_3$, 300 MHz): δ 190.5, 164.7, 136.7, 135.6, 134.1, 132.1, 61.8, 14.3. NMR data corresponded well to those published by Dy et al.3
Characterization

NMR spectra

Figure S1. $^1$H NMR spectrum (D$_2$O, 500 MHz) of compound 1. Asterisk indicates residual signal of non-deuterated solvent, triangle is acetone used to lock the signal.

Figure S2. $^{13}$C NMR spectrum (D$_2$O, 125 MHz) of compound 1. Triangle is acetone used to lock the signal.
**Figure S3.** $^1$H NMR spectrum (CDCl$_3$/C$_5$D$_5$N 3:1, 300 MHz) of compound 2Mg. Asterisks indicate residual signals of non-deuterated solvents, triangle indicates water.

**Figure S4.** $^{13}$C NMR spectrum (CDCl$_3$/C$_5$D$_5$N 3:1, 75 MHz) of compound 2Mg. Asterisks indicate signals of solvents.
**Figure S5.** $^1$H NMR spectrum (CDCl$_3$/C$_5$D$_5$N 3:1, 300 MHz) of compound 2H. Asterisks indicate residual signals of non-deuterated solvents, triangle indicates water.

**Figure S6.** $^{13}$C NMR spectrum (CDCl$_3$/C$_5$D$_5$N 3:1, 75 MHz) of compound 2H. Asterisks indicate signals of solvents.
Figure S7. $^1$H NMR spectrum (CDCl$_3$/C$_5$D$_5$N 3:1, 300 MHz) of compound 2Zn. Asterisks indicate residual signals of non-deuterated solvents, triangle indicates water.

Figure S8. $^{13}$C NMR spectrum (CDCl$_3$/C$_5$D$_5$N 3:1, 75 MHz) of compound 2Zn. Asterisks indicate signals of solvents.
Figure S9. $^1$H NMR spectrum (CDCl$_3$, 500 MHz) of compound 7. Asterisk indicates residual signal of non-deuterated solvent, triangle indicates water.

Figure S10. $^{13}$C NMR spectrum (CDCl$_3$, 125 MHz) of compound 7. Asterisk indicates signal of solvent.
Figure S11. $^1$H NMR spectrum (CDCl$_3$, 300 MHz) of compound 8. Asterisk indicates residual signal of non-deuterated solvent, triangle indicates water.

Figure S12. $^{13}$C NMR spectrum (CDCl$_3$, 75 MHz) of compound 8. Asterisk indicates signal of solvent.
Figure S13. $^1$H NMR spectrum (CD$_3$SOCD$_3$, 300 MHz) of compound 9. Asterisk indicates residual signal of non-deuterated solvent, triangle indicates water.

Figure S14. $^{13}$C NMR spectrum (CD$_3$SOCD$_3$, 75 MHz) of compound 9. Asterisk indicates signal of solvent.
Figure S15. $^1$H NMR spectrum (CD$_3$COCD$_3$, 300 MHz) of compound 10. Asterisk indicates residual signal of non-deuterated solvent.

Figure S16. $^{13}$C NMR spectrum (CD$_3$COCD$_3$, 75 MHz) of compound 10. Asterisk indicates signal of solvent.
Mass spectra

Figure S17. MALDI-TOF mass spectra of compound 1 (negative reflectron mode).

Figure S18. MALDI-TOF mass spectra of compound 2Mg (positive reflectron mode).

Figure S19. MALDI-TOF mass spectra of compound 2H (positive reflectron mode).
Figure S20. MALDI-TOF mass spectra of compound 2Zn (positive reflectron mode).

Chromatograms

Figure S21. Elution diagram of 1 from gel filtration on Sephadex G-25 monitored as its absorbance at 370 nm after 500× dilution of each fraction. Bed diameter: 10 mm. Bed volume: 21 mL. Flow rate 0.3 mL min⁻¹. Two independent experiments (two batches) are shown.

Figure S22. a) HPLC chromatogram of separation of compound 1 (Hypersil BDS C18 column (100 × 4.6 mm, particle size 2.4 μm), gradient elution: triethylamine acetate buffer (50 mM, pH 6.3, mobile phase A) and methanol (mobile phase B), λ = 647 nm). b) Enlarged part of the chromatogram. c) Absorption spectrum at t = 9.8 min.
Detection of singlet oxygen in water

**Figure S23.** a) Visible spectra of 1 (black) and methylene blue (MB, standard, blue) in D$_2$O (0.1 M phosphate buffer, pD = 7.1) with absorbances at excitation wavelength ($\lambda_{\text{exc}} = 650$ nm) for calculation of singlet oxygen quantum yield. b) Comparison of singlet oxygen phosphorescence generated from 1 (black) and MB (blue) in D$_2$O (0.1 M phosphate buffer, pD = 7.1). Excited at 650 nm.

**Figure S24.** Kinetics of the triplet states of 1 in oxygen-, air-, and argon saturated D$_2$O monitored at 500 nm. Transient absorption was measured using a LKS 20 laser kinetic spectrometer (Applied Photophysics, UK) equipped with a 150 W Xe lamp, pulse unit and R928 photomultiplier (Hamamatsu) upon laser excitation at 650 nm.
Absorption and fluorescence spectra

Figure S25. Absorption spectra of 2Zn (a), 2Mg (b) and 2H (c) in THF (red) and toluene (black) at concentrations approximately 1 µM.
Figure S26. Normalized absorption (orange), emission (blue) and excitation (black dashed) spectra of 2Zn (a), 2Mg (b) and 2H (c) in pyridine and 1 (d) in water.

Figure S27. Mutual quenching of the fluorescence of LysoTrackerBlue (a, $\lambda_{exc} = 354$ nm) and compound 1 (b, $\lambda_{exc} = 588$ nm) in buffer solution with pH = 5.1. Blue lines - the dye alone (1 $\mu$M) in buffer, red lines – after mixing the two dyes (final concentration of each component is 1 $\mu$M).
Figure S28. a) Absorption spectra of LysoTrackerBlue (black) and compound 1 (magenta) in buffer solution with pH = 5.1 at concentration of 1 µM. b) Sum (blue) of the absorption spectra of 1 and LysoTrackerBlue in buffer solution with pH = 5.1 at concentration of 1 µM and absorption spectrum of the mixture of these two compounds (red) after mixing the two dyes (final concentration of each component is 1 µM).

Figure S29. a) Changes in absorption spectra of 1 (c ~ 1 µM) in buffer of different pH. b) Dependence of ratio of absorbance at 650 nm and 359 nm of 1 on pH.
Literature data on photodynamic activity of phthalocyanines

**Table S1.** Photodynamic activity of water soluble anionic and cationic Pcs and aza-analogs.

<table>
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<tr>
<th>Compound</th>
<th>Type</th>
<th>Cells</th>
<th>Irradiation</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
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<td>HeLa</td>
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<td>This work</td>
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<td>J774</td>
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<td>~ 1</td>
<td>5</td>
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Fluorescence microscopy

**Figure S30.** Subcellular localization of compound 1 (red) in HeLa cells visualized by fluorescence microscopy after co-incubation with MitoTracker (green). No co-localization is observed.

**Figure S31.** Subcellular localization of Rab7a-GFP (A, green) and Lamp1-RFP (B, red) on endo-lysosomal vesicles in HeLa cells visualized by fluorescence microscopy.
Experiments with pH inside the cells

**Figure S32.** Determination of lysosomal pH using LysoSensor Yellow/Blue DND-160. Green dots are data for calibration curve determined in buffer (made in duplicate), black line is the best fit. The lysosomal pH in HeLa cells was subsequently determined to be 4.9 using the calibration curve.

**Figure S33.** Effect of alkalization of lysosomes in HeLa cells with 20 mM NH₄Cl on fluorescence intensity of 1 (λ_exc = 375 nm, λ_em = 671 nm) monitored by Tecan Infinite M 200 plate reader. Five independent experiments were performed. Cells were seeded on 96-well plates and incubated with 1 for 12 h. Ammonium chloride was added for 15 min prior to fluorescent measurement. Key: *, p < 0.05.
Interaction of BSA with 1

**Figure S34.** a) Determination of binding constant ($K_b$) between BSA and compound 1 in SFM. ($c_{(BSA)} = 2$ µM, $\lambda_{exc}=280$ nm, $\lambda_{em}=340$ nm). b) Dependence of normalized $\Phi_F$ of 1 (red) and Pc-Im$_{16}$ (blue) in SFM on the amount of added BSA. Dotted line indicates the typical amount of BSA in SCM.

**In vitro photodynamic activity**

**Figure S35.** Phototoxicity ($\lambda > 570$ nm, 12.4 mW cm$^{-2}$, 15 min, 11.2 J cm$^{-2}$) of 1 against the HeLa cells. Compound 1 was applied as a solution in SFM (green, triangle) or in SCM (red, dots). Typically four independent experiments, each in quadruplicate, were performed. Key: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

**References**