Supporting Information

Aerobic Oxidation of in Situ Generated Cyanine Dyes Leading to DNA Damage

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1. General Information.

All commercially available materials were used as supplied without further purification, unless otherwise noted. Proton and carbon NMR were recorded on Bruker Avance 500 spectrometer equipped with cryoprobe. Chemical shifts are reported in parts-per-million (ppm). Deuterated solvent was used as a lock and residual protiated solvent peak or TMS was used as reference. FIDs are available upon request. UV–vis spectra were recorded in 1 cm Starna Cells cuvette on Agilent 8453 UV–vis spectrometer. Mass spectrometry experiments were recorded using ion cyclotron resonance Fourier Transform MS using electro spray ionization. Gel electrophoresis experiments were carried out in Bio-Rad Sub-Cell GT (15x20 cm gel tray) and/or Labnet ENDURO (10x10 cm gel tray) gel boxes. DNA optical melting curves were recorded on Cary-Bio UV–vis spectrophotometer (Varian Inc.) equipped with a thermo controlling cell. Deoxygenated samples were prepared in a LabMaster 130 glovebox in an atmosphere of nitrogen. Solutions were deoxygenated by 3 freeze-pump-thaw cycles under atmosphere of argon and stored in the glovebox under a nitrogen atmosphere.

2. Synthesis

2.1. Bis(N-methylpyridin-4-ium)methane Iodide (4)

To a solution of bis(pyridin-4-yl)methane (50 mg, 0.30 mmol, obtained according to\(^1\)) in 5 mL of acetonitrile was added an excess of methyl iodide (240 mg, 1.6 mmol) and the stirred reaction mixture was refluxed overnight. After cooling to room temperature, the precipitate was filtered, washed with 0.5 mL of acetonitrile, 2x5 mL of diethyl ether and dried in vacuo to yield 4 as a pale yellow solid (106 mg, 80%). Mp = > 300 °C (decomp.) \(^1\)H NMR (500 MHz, D\(_2\)O): δ 8.63 (d, 4H, J = 6.65 Hz), 7.81 (d, 4H, J = 5.87 Hz), 4.54 (s, 1H, fast exchange), 4.25 (s, 6H). \(^13\)C NMR (125 MHz, D\(_2\)O): δ 156.35, 145.12, 128.35, 125.01, 47.64. HRMS (ESI): calcd. for C\(_{13}\)H\(_{16}\)N\(_2\)\(^2+\) [M-2I]\(^2+\), 100.06513, found 100.06512.

2.2 Bis(N-methylpyridin-4-ium)methanone Iodide

To a solution of dipyridin-4-ylmethanone (Sigma-Aldrich, ≥ 95 %, 50 mg, 0.27 mmol) in 5 mL of acetone (freshly distilled from CaH\(_2\)) was added an excess of methyl iodide (240 mg, 1.6 mmol) and the reaction mixture was refluxed overnight under an argon atmosphere. After cooling to room temperature, the precipitate was filtered, washed with 0.5 mL of dry acetone, 2x5 mL of anhydrous diethyl ether and dried in vacuo to yield bis(N-methylpyridin-4-ium)methanone iodide as an orange solid (100 mg, 85 %). Mp = > 300 °C (decomp.) \(^1\)H NMR (500 MHz, CD\(_3\)CN): δ 8.96 (d, 4H, J = 6.56 Hz), 8.30 (d, 4H, J = 6.10 Hz), 4.43 (s, 6H). \(^13\)C NMR (125 MHz, CD\(_3\)CN): δ 188.15, 147.36, 147.06, 127.76, 49.07. HRMS (ESI): calcd. for C\(_{13}\)H\(_{14}\)N\(_2\)O\(^2+\) [M-2I]\(^2+\), 107.05476, found 107.05470.
2.3. Bis(N-methylpyridin-4-ium)methanediol Iodide (5)

Bis(N-methylpyridin-4-ium)methanone iodide was dissolved in D$_2$O and immediate formation of Bis(N-methylpyridin-4-ium)methanediol iodide (5) was observed by $^1$H NMR. $^1$H NMR (500 MHz, D$_2$O): δ 8.71 (d, 4H, J = 6.61 Hz), 8.19 (d, 4H, J = 6.37 Hz), 4.25 (s, 6H), $^{13}$C NMR (125 MHz, D$_2$O): δ 159.80, 145.90, 125.03, 93.30, 47.88. HRMS (ESI) of D$_2$O solution: calcd. for C$_{13}$H$_{14}$D$_2$N$_2$O$_2$$^{2+}$ [M-2I]$^{2+}$, 117.06632, found 117.06626.

2.4. Tetrakis(N-methylpyridin-4-ium)ethane derivatives

1,1,2,2-Tetrakis(N-methylpyridin-4-ium)ethane iodide (1), 1,1,2,2-Tetrakis(N-methylpyridin-4-ium)ethylene iodide (2) and 1,1,2,2-Tetrakis(N-methylpyridin-4-ium)ethylene oxide (3) were synthesized according the previously reported procedure.$^2$
3. Experimental Data.

3.1. $^1$H NMR of the Formation of 5 from 4 in D$_2$O.

A sample of 1 mg of 4 was dissolved in 0.5 mL of D$_2$O and the spontaneous reaction of the gem-diol 5 formation was monitored over the period of 2 weeks. Between the measurements, the opened NMR tube was stored under ambient light during daytime and kept in the dark during overnight periods. Figure S1 shows a fragment of the $^1$H NMR evolution data showing the evolution of 8.03 peak assigned to 4”.

![Figure S1. Fragment of $^1$H NMR monitoring of 4 -> 5 transition in air. Boxed sections show peaks assigned to 4”. 4.26 ppm signal can be observed at the initial stages of the reaction. Left and right parts are not scaled to aid in visualization.](image-url)
3.2. UV-vis Monitoring of the 480 nm Absorption.

To 3 mL of 0.43 mM aqueous solution of 4 were added few drops of saturated aqueous solution of NaHCO₃. UV-vis measurements in the opened cuvette were taken every 3-5 minutes. Solution was thoroughly mixed before each measurement. Time evolution of the 480 nm band in phosphate-buffered saline (PBS, pH=7.4) solution was followed in the same way. In PBS experiment, solution was stored in the dark during the night periods and under ambient light during daytime.

![Graph showing UV-vis monitoring of the 480 nm band of 4’ in PBS within 3 days.](image)

**Figure S2.** UV-vis monitoring of the 480 nm band of 4’ in PBS within 3 days.

Exergonicity of the oxidation of 4’ can be estimated from the reduction potentials of superoxide and oxidation potential of 4’.³

\[
\text{O}_2 + e^- \rightarrow \text{O}_2^- \quad \text{E}_{\text{red}} = -0.18 \ \text{V}
\]

Reduction potential of 4’ (‘dye’ in the following scheme) can be estimated from the empirical equation by Ferre and Larive.⁴

\[
dye \rightarrow \text{dye}^+ + e^- \quad \text{E}_{\text{ox}} = 0.77 \ \text{V}
\]

Positive cell potential (0.59 V) indicates that oxidation of 4’ should be spontaneous as observed under experimental conditions.
3.3. DNA Binding Experiments.

All measurements were performed in 1 mm FireflySci cuvette.

A 9.5 µM solution of 4’ obtained by treating a 0.2 mL sample of a slightly acidic solution of 4 (HCl, pH = 5) with 0.1 mL of 40 mM PBS and 0.1 mL of deionized water (DI) (C (4) = 3.7 mM) was titrated with incremental additions of CT-DNA solution (10 mg/ mL, UltraPure Calf Thymus DNA, Fisher) until reaching 2x excess of DNA base pairs with respect to the dye. Concentrations of 4, 4’ and CT-DNA were derived from the absorbance values and molar absorptivity values. Spectra were corrected for small dilution effect associated with titration.

ε<sub>258</sub> (4) = 6758 cm<sup>-1</sup> M<sup>-1</sup>, ε<sub>270</sub> (4) = 2184 cm<sup>-1</sup> M<sup>-1</sup>

ε<sub>480</sub> (4’) = 1.4·10<sup>5</sup> cm<sup>-1</sup> M<sup>-1</sup> (literature data)<sup>5</sup>

ε<sub>260</sub> (CT-DNA) = 13200 bp cm<sup>-1</sup> M<sup>-1</sup> (literature data)<sup>6</sup>

**Figure S3.** Titration of 4’ with CT-DNA in 10 mM PBS.

Due to air sensitivity of 4’ its absorption does not stay constant during the measurements both in the absence and presence of DNA. Therefore, normalized absorption spectra are shown in the inset of Figure S3.
A slightly acidic (HCl, pH = 5) 7.3 mM solution of 1 (0.4 mL) was split in 2 equal portions. To the first part of this solution, 0.1 mL of 40 mM PBS (pH = 7.4) and 0.1 mL of DI water were added, and the 520 nm absorption band was recorded. To the second half of the solution, 0.1 mL of 40 mM PBS (pH = 7.4) and 0.1 mL of CT-DNA solution were added. Due to the lack of the data for molar absorptivity for 1’ we used 1 equivalent of CT-DNA base pairs with respect to the total solute concentration. Concentrations of 1 and CT-DNA were derived from the absorbance values and molar absorptivity values.

\[ \varepsilon_{262}(1') = 11932 \text{ cm}^{-1} \text{ M}^{-1}, \varepsilon_{275}(1') = 4048 \text{ cm}^{-1} \text{ M}^{-1} \]

**Figure S4.** Normalized characteristic absorption bands of 1’ in the absence and presence of 1 equivalent of CT-DNA base pairs.
3.4. DNA Optical Melting Experiments.

Due to the instability of alkane salts and the oxidation intermediates and long duration of the optical melting experiments, we tested the DNA duplex stabilizing properties of a stable oxidation product 2.

To 3.6 mL PBS (10 mM + 100 mM NaCl) was added 4 µL of a 12-mer stock solution (12-mer, 33.3 % of GC content; 5’ – GTTAGTATATGG – 3’ (one strand shown), Integrated DNA Technologies). Using $\varepsilon_{260}$ (12-mer) = 244000 cm$^{-1}$ M$^{-1}$, concentration of the DNA base pairs was estimated as 29 µM. To that, 70 µL of 0.38 mM aqueous solution of 2 was added (1 equivalent with respect to the DNA base pairs). Melting curves were obtained in the absence and the presence of 1 equivalent of 2.

$\varepsilon_{274}$ (2) = 20839 cm$^{-1}$ M$^{-1}$

DNA absorbance at 260 nm was monitored on Cary-Bio UV–vis spectrophotometer (Varian Inc.) equipped with a thermo controlling cell. Solutions of DNA oligonucleotide in 1 cm quartz cuvette were thermostated at 20 ºC for 3 min. Stirred solutions were heated from 20 ºC to 60 ºC at 0.5 ºC/min. rate. Melting temperatures of pure DNA oligonucleotide, Tm (DNA), and in the presence of 2, were determined from the first derivative curves (dA260/dT vs T).

![Figure S5](image-url)

**Figure S5.** First derivatives of the 12-mer DNA thermal melting curves in the presence (red) and absence (black) of 2.
3.5. DNA Gel Electrophoresis.

A solution of 0.5 μL ΦX174 RF I DNA (1 mg/mL, 5386 base pairs length, New England Biolabs (NEB) was transferred in a 0.6 mL polypropylene tube (Fisher Scientific, 02-681-300). Into each tube was added 2 μL of 40 mM PBS. After that, tubes were filled with decreasing amounts of water (BioPerformance Certified, Sigma-Aldrich) according to the table S1. To these samples increasing amounts of acidic solution of 1 (HCl, pH = 5) were added according to the Table S1 so that the final volume in each tube was 10.5 μL. To achieve the desired molar ratios of 1/(DNA bp) an acidic solution of 1 with $A_{262} = 2.3$ was used. First control sample contained 0.5 μL ΦX174 RF I DNA, 2 μL of PBS and 8 μL of water. Second control contained 0.5 μL ΦX174 RF I DNA, 2 μL of PBS and 8 μL of aqueous pH = 5 HCl solution (blank). Every sample was vortexed after each addition described above. Final solutions were vortexed every 10 minutes for 2 hrs. Lids of polypropylene tubes were kept opened throughout the experiment.

After 2 hrs, to each solution was added 3.5 μL of water followed by the addition of 1.5 μL of Blue Juice Loading dye (ThermoFisher). Samples were loaded in 15x20 cm 1 % agarose gel (1X TAE buffer). A 10 μL of solution of 1kb DNA ladder (NEB) was loaded in the first well as a reference. Typically gel electrophoresis was run for approximately 14 hrs at 35 V. Gels were post-stained in GelRed Nucleic Acid Stain (Biotium) in 1X TAE buffer (10 μL of 10000X GelRed + 100 mL of 1X TAE buffer) for 6 hrs.

Solutions containing 4 were prepared in the similar fashion (Table S2). Since the oxidation was performed for 2 days solutions were periodically diluted with distilled water to compensate water loss due to the slow evaporation from the opened polypropylene tubes. Solutions were not vortexed during overnight storage.

DNA samples containing 2 and 3 were prepared in similar fashion and loaded in 10x10 cm 1 % agarose gels (GelRed pre-stain, 5 μL of 10000X GelRed + 50 mL of 1X TAE buffer). 10x10 cm agarose gels were run for approximately 7 hrs at 35 V. Gels were visualized using a 302 nm transilluminator (TR-302, Ultraviolet) and photographed by a digital camera through an ethidium bromide filter. Densitometric analysis of the DNA bands was performed in Image Studio Lite software.

Deoxygenated DNA samples were prepared in a glovebox in an atmosphere of nitrogen. All solutions were degassed before transferring to a glovebox.

Table S1. Preparation of DNA mixtures with 1 ($A_{262} (1) = 11932 \text{ cm}^{-1} \text{ M}^{-1}$).
Table S2. Preparation of DNA mixtures with 4 ($\varepsilon_{258}(4) = 6758$ cm$^{-1}$ M$^{-1}$).

|       | CONTROL | 1 per 25 | 1 per 12 | 1 per 6 | 1 per 3 | 1 per 2 | 1 per 1.56 | 1 per 1 | 1.25 per 1 | 1.5 per 1 | 1.75 per 1 | 2 per 1 |
|-------|---------|----------|----------|---------|---------|---------|-------------|---------|------------|-----------|------------|--------|
| 0     | 1.0     | 2.0      | 3.0      | 4.0     | 5.0     | 6.0     | 7.0         | 8.0     | 9.0        | 10.0      | 11.0       | 12.0   |
| φX174 | 0.5     | 0.5      | 0.5      | 0.5     | 0.5     | 0.5     | 0.5         | 0.5     | 0.5        | 0.5       | 0.5        | 0.5    |
| PBS 10x | 2.0 | 2.0      | 2.0      | 2.0     | 2.0     | 2.0     | 2.0          | 2.0     | 2.0        | 2.0       | 2.0        | 2.0    |
| DS_sol | 0.0    | 0.16     | 0.33     | 0.67    | 1.34    | 2.01    | 2.57         | 4.03    | 5.03       | 6.04      | 7.05       | 8.00   |
| water | 8.0    | 7.84     | 7.67     | 7.33    | 6.66    | 5.99    | 5.43         | 3.97    | 2.97       | 1.96      | 0.95       | 0.00   |
| V(total) | 10.5 | 10.5     | 10.5     | 10.5    | 10.5    | 10.5    | 10.5          | 10.5    | 10.5       | 10.5      | 10.5       | 10.5   |

A = 1.3 @ 258 nm

Figure S6. Mixtures of 2 (lanes 1-9) and 3 (lanes 1a-10a) with φX174 RF I DNA analyzed by gel electrophoresis. Experiments performed in the conditions described above. No DNA damage was observed. Lanes 1, 1a, 2, 2a – control samples (water and HCl blank, correspondingly). Lanes 3-9 – increasing amounts of 2 (from 1 molecule/50 bp to 1 molecule /1 bp: 1/50, 1/25, 1/12, 1/6, 1/3, 1/2, 1/1). Lanes 3a-10a – increasing amounts of 3 (from 1 molecule/50 bp to 1 molecule /1 bp: 1/50, 1/37, 1/25, 1/12, 1/6, 1/3, 1/2, 1/1). Solutions of 2 and 3 in water were used. ($\varepsilon_{274}(2) = 20840$ cm$^{-1}$ M$^{-1}$, $\varepsilon_{264}(3) = 11613$ cm$^{-1}$ M$^{-1}$).

Figure S7. Left – mixtures of 4 with φX174 RF I DNA prepared and kept in anaerobic conditions for 2 days analyzed by gel electrophoresis. Lane 1 – control sample. Lanes 2-12 – increasing amounts of 4 (from 1 molecule/25 bp to 2 molecule/1bp: 1/25, 1/12, 1/6, 1/3, 1/2, 1/1.56, 1/1, 1.25/1, 1.5/1, 1.75/1, 2/1). Right – results of the densitometric analysis of the DNA bands indicating the absence of DNA damage with respect to control.
Figure S8. Mixtures of 1 with ΦX174 RF I DNA prepared and kept in anaerobic conditions for 2 hrs analyzed by gel electrophoresis. Lane 1, 2 – control samples. Lanes 3-11 – increasing amounts of 1 (from 1 molecule/50 bp to 2 molecules/1bp: 1/50, 1/25, 1/12, 1/6, 1/3, 1/2, 1/1.56, 1/1, 2/1).

While in the glovebox, solutions permanently exhibited characteristic purple color of 1’. Oxidation of 1’ occurred during the transfer of the samples from the glovebox and while loading the samples in a gel due to much higher air sensitivity of 1’ compared to 4’.

Figure S9. Mixtures of 5 with ΦX174 RF I DNA analyzed by gel electrophoresis. Lane 1 – control sample. Lanes 2-6 – increasing amounts of 5 (from 1 molecule/12 bp to 2 molecules/1 bp: 1/12, 1/3, 1/1.56, 1/1, 2/1). No DNA damage was observed.
4. Qualitative Peroxide Test

To 1.5 mL of a solution of 2 mg of 4 in deionized water (DI) was added 30x excess of solid sodium iodide and solid starch (TEST). The first control sample did not have 4 and contained the same amounts sodium iodide and starch (Control#1). The second control sample (Control#2) contained starch, sodium iodide and aqueous hydrogen peroxide (0.3 mL of a solution obtained by diluting 3 mg of 30% H₂O₂ in 20 mL of water was further diluted in 1.2 mL of DI water). Small concentration of H₂O₂ in this control represents a similar concentration of hydroperoxide 4″ which can be achieved during the first hours of oxidation of 2 mg of 4.

We performed test in the absence of acid since compound 4 is stable in acidic conditions similar to 1.²

![Figure S10](image)

**Figure S10.** Left to right – Control#1, Control#2, TEST after 3 hrs of exposure.

Formation of the starch-iodide (I₃⁻) complex in Control#2 and TEST samples supports the formation of the hydroperoxide 4″.

To aid in the visualization Control#2 and TEST samples were decanted and diluted with deionized water (Figure S11).

![Figure S11](image)

**Figure S11.** Left to right – Control#1, Control#2, TEST 3 hrs of exposure (Control#2 and TEST decanted and diluted with DI water).
5. UV-vis Absorption of All Compounds.

Figure S12. UV-vis absorption of bis(N-methylpyridin-4-ium)methane iodide (4) in water.

Figure S13. UV-vis absorption of bis(N-methylpyridin-4-ium)methanediol iodide (5) in water.
Figure S14. UV-vis absorption of bis(N-methylpyridin-4-ium)methanone iodide in acetonitrile.
5. MS of All Compounds.

Figure S15. MS of bis(N-methylpyridin-4-ium)methane iodide (4).
Figure S16. MS of bis(N-methylpyridin-4-ium)methanone.
Figure S17. MS of bis(N-methylpyridin-4-iium)methanediol iodide (5) (D$_2$O solution).
6. NMR of All Compounds.

Figure S18. $^1\text{H}$ NMR (D$_2$O, 500 MHz) spectrum of bis(N-methylpyridin-4-ium)methane iodide (4).
Figure S19. $^{13}$C NMR (D$_2$O, 125 MHz) spectrum of bis(N-methylpyridin-4-ium)methane iodide (4).
Figure S20. $^1$H NMR (CD$_3$CN, 500 MHz) spectrum of bis(N-methylpyridin-4-ium)methanone.
Figure S21. $^{13}$C NMR (CD$_3$CN, 125 MHz) spectrum of bis(N-methylpyridin-4-ium)methanone.
Figure S22. $^1$H NMR (D$_2$O, 500 MHz) spectrum of bis(N-methylpyridin-4-ium)methanediol iodide (5).
Figure S23. $^{13}$C NMR (D$_2$O, 125 MHz) spectrum of bis(N-methylpyridin-4-i um)methanediol iodide (5).
7. References.

1. Gaus, P.L.; Haim, A.; Johnson, F. J. Org. Chem. 1977, 42, 564.

2. Tcyrulnikov, N.A.; Tikhomirova, A.A.; Tcyrulnikov, S.; Wilson, R.M. Org. Lett. 2018, 20, 1279.

3. Armstrong, D.A.; Huie, R.E; Koppenol, W.H.; Lymar, S.V; Merényi, G.; Neta, P.; Ruscic, B.; Stanbury, D.M.; Steenken, S.; Wardman, P. Pure Appl. Chem. 2015, 87, 1139.

4. Ferre, Y.; Larive, H.; Vincent, E.-J. Photogr. Sci. Eng. 1974, 18, 457.

5. Tolbert, L. M.; Zhao, X. J. Am. Chem. Soc. 1997, 119, 3253.

6. Lee, H.M.; D.S. Yang, C.F.; Lam, H.Y.; Yan, S.C.; Che, C.M.; Ma, D.L.; Leung, C.H. Chem. Commun. 2010, 46, 4680.