Synthesis and kinetic studies of cyclisation-based self-immolative spacers

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The Supporting Information reports on:

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pKa measurements

All the reported self-immolative spacers are based on phenol cores. We determined the proton exchange constant for all of them by analysing the evolution of their UV-Vis absorption spectra on pH in CH₃CN/0.1 M Britton-Robinson buffer 1:1 (v:v) (figure 1 to 4).

Figure 1: pH dependence of the absorption spectrum of 2-hydroxyphenolcarbamate 2-HPC\(^{H}\) (10 µM; pH from 7.6 to 10.9).
Figure 2: pH dependence of the absorption spectrum of 2-hydroxy-4-methoxy-phenolcarbamate 2-HPC\textsubscript{CH3} (10 µM; pH from 6.3 to 10.9)

Figure 3: pH dependence of the absorption spectrum of 2-hydroxy-4-bromo-phenolcarbamate 2-HPC\textsubscript{Br} (10 µM; pH from 6.4 to 10.5)

Figure 4: pH dependence of the absorption spectrum of 2-hydroxy-4-nitro-phenolcarbamate 2-HPC\textsubscript{NO2} (10 µM; pH from 6.4 to 9.8)
The pH dependence of the absorption spectra of the phenols has been analysed with the SPECFIT/32\textsuperscript{TM} Global Analysis System (Version 3.0 for 32-bit Windows systems) in order to extract the pKa. The extracted values are given in table 1.

Table 1: pKa of the phenol cores contained in cyclising self-immolative spacers.

<table>
<thead>
<tr>
<th>Phenol</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Phenol Structure 1]</td>
<td>10.7 ± 0.1</td>
</tr>
<tr>
<td>![Phenol Structure 2]</td>
<td>10.8 ± 0.1</td>
</tr>
<tr>
<td>![Phenol Structure 3]</td>
<td>10.0 ± 0.1</td>
</tr>
<tr>
<td>![Phenol Structure 4]</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>![Phenol Structure 5]</td>
<td>10.2 (supplier data)</td>
</tr>
</tbody>
</table>
**Irradiation experiments**

The irradiations relying on one-photon excitation were performed in 54 μL homogeneously illuminated quartz fluorescence cuvettes (Hellma) without stirring at 293 K in CH₂CN/0.1 M Britton-Robinson pH 8 or pH 5 buffer 1/1 (v/v). Excitations were performed using a light-emitting diode (PE-2, CoolLED, Andover, United Kingdom; emission at 365±25 nm) and the resulting signals were observed at 660 nm.

**Kinetic measurements**

The self-immolation process is initiated by photocleavage of the 4,5-dimethoxy-2-nitrobenzyl moiety which involves a multi-step radical mechanism, but which can be considered as a single step for our kinetic measurement considering that the whole process occurs in the millisecond timescale (*Chem. Eur. J.*, 2006, 12, 6865-6879). Moreover, the 4,5-dimethoxy-2-nitrosobenzaldehyde formed in this reaction is not known to absorb at 365 nm or 660 nm (*J. Am. Chem. Soc.*, 1988, 110, 7170-7177), thus does not interfere with our measurements. DDAO emits a strong fluorescence in its free-phenol form, leading us to conclude that the contribution of the overall fluorescence could be due to the free fluorophore; however we cannot exclude a contribution of the intermediary uncaged compound. As in on our previous work (*Chem. Eur. J.*, 2013, 19, 11717-11724; supporting information, equation 13), the temporal evolution of the overall fluorescence signal could be fitted with the following equation:

\[
I_{DDAO}^F(t) = I_{DDAO}^F(\infty) \left( q_{DDAO}^{CP} \frac{c_{DDAO}(t)}{c_{DDAO}(0)} + \frac{c_{DDAO}(t)}{c_R(0)} \right)
\]

With:

- \( I_{DDAO}^F(t) \): Intensity of fluorescence (at 660 nm) at t time.
- \( I_{DDAO}^F(\infty) \): Maximal intensity of fluorescence (at 660 nm) at t = \( \infty \).
- \( q_{DDAO}^{CP} \): Brightness of the intermediary phenol formed after photocleavage.
- \( c_{DDAO}(t) \): Concentration of the intermediary phenol at t time
- \( c_{DDAO}(0) \): Initial concentration of the intermediary phenol
- \( c_{DDAO}(t) \): Concentration of DDAO at t time
- \( c_R(0) \): Initial concentration of the caged compound (2-HPC or TML derivatives)

**Experimental curves**

Figures 5-10 display the temporal evolution of the fluorescence emission from solutions initially containing the caged precursors and continuously submitted to illumination. They all exhibited the expected signal growth associated to quantitative DDAO liberation. Moreover the biexponential fit with equation (1) was found satisfactory, which enabled us to extract the \( k_2 \) values.
Figure 5: Temporal evolution of the fluorescence emission ($\lambda_{em}=660$ nm) by illuminating at $\lambda_{exc}=365 \pm 25$ nm a $10.0 \pm 0.3 \mu$M solution of compound 2-HPC$_{NO_2}$ at $1.4 \times 10^{-9}$ Einst$^{-1}$ intensity of light and at 293K. Experimental data in red, fits with equation (1) in black. From the signal calibration with the fluorophore, we extracted $10.0 \pm 0.5 \mu$M for the averaged final DDAO concentration, which demonstrated the DDAO liberation to be quantitative. From the fits, we retrieved $k_2 = 5.2 \pm 0.5 \times 10^{-2}$ min$^{-1}$; Solvent: CH$_3$CN/0.1 M Britton-Robinson pH 5 buffer 1/1 (v/v).
Figure 6: Temporal evolution of the fluorescence emission ($\lambda_{\text{em}}=660$ nm) by illuminating at $\lambda_{\text{exc}}=365 \pm 25$ nm a $10.0 \pm 0.3 \, \mu$M solution of compound 2-HPC$_{\text{NO2}}$ at $1.4 \times 10^{-9}$ Emt$^{-1}$ intensity of light and at 293K. Experimental data in red, fits with equation (1) in black. From the signal calibration with the fluorophore, we extracted $10.0 \pm 0.5 \, \mu$M for the averaged final DDAO concentration, which demonstrated the DDAO liberation to be quantitative. From the fits, we retrieved $k_2 = 6.6 \pm 0.5 \times 10^{-2}$ min$^{-1}$; Solvent: CH$_3$CN/0.1 M Britton-Robinson pH 8 buffer 1/1 (v/v).
Figure 7: Temporal evolution of the fluorescence emission ($\lambda_{em}=660$ nm) by illuminating at $\lambda_{exc}=365 \pm 25$ nm a 10.0 ± 0.3 μM solution of compound 2-HPC at 1.4 $10^9$ EIns$^2$ intensity of light and at 293K. Experimental data in red, fits with equation (1) in black. From the signal calibration with the fluorophore, we extracted 10.0 ±0.5 μM for the averaged final DDAO concentration, which demonstrated the DDAO liberation to be quantitative. From the fits, we retrieved $k_2 = 1.9 \pm 0.2 \times 10^{-3}$ min$^{-1}$; Solvent: CH$_3$CN/0.1 M Britton-Robinson pH 8 buffer 1/1 (v/v).
Figure 8: Temporal evolution of the fluorescence emission ($\lambda_{\text{em}}=660 \text{ nm}$) by illuminating at $\lambda_{\text{exc}}=365 \pm 25 \text{ nm}$ a $10.0 \pm 0.3 \text{ M}$ solution of compound 2-HPC$_{\text{HCl}}$ at $1.4 \times 10^{-9} \text{ Einst}^{-1}$ intensity of light and at 293K.

Experimental data in red, fits with equation (1) in black. From the signal calibration with the fluorophore, we extracted $10.0 \pm 0.5 \text{ M}$ for the averaged final DDAO concentration, which demonstrated the DDAO liberation to be quantitative. From the fits, we retrieved $k_2 = 2.9 \pm 0.3 \times 10^{-1} \text{ min}^{-1}$; Solvent: CH$_3$CN/0.1 M Britton-Robinson pH 8 buffer 1/1 (v/v).
Figure 9: Temporal evolution of the fluorescence emission ($\lambda_{\text{em}} = 660$ nm) by illuminating at $\lambda_{\text{exc}} = 365 \pm 25$ nm a $10.0 \pm 0.3 \mu$M solution of compound 2-HPC$^\text{Br}$ at $1.4 \times 10^{-9}$ Eins$^{-1}$ intensity of light and at 293K. Experimental data in red, fits with equation (1) in black. From the signal calibration with the fluorophore, we extracted $10.0 \pm 0.5 \mu$M for the averaged final DDAO concentration, which demonstrated the DDAO liberation to be quantitative. From the fits, we retrieved $k_2 = 1.1 \pm 0.3 \times 10^{-1}$ min$^{-1}$; Solvent: CH$_3$CN/0.1 M Britton-Robinson pH 8 buffer 1/1 (v/v).
Figure 10: Temporal evolution of the fluorescence emission ($\lambda_{em}=660$ nm) by illuminating at $\lambda_{exc}=365 \pm 25$ nm a $10.0 \pm 0.3$ $\mu$M solution of compound TML at $1.4 \times 10^9$ Eins$^{-1}$ intensity of light and at 293K. Experimental data in red, fits with equation (1) in black. From the signal calibration with the fluorophore, we extracted $10.0 \pm 0.5$ $\mu$M for the averaged final DDAO concentration, which demonstrated the DDAO liberation to be quantitative. From the fits, we retrieved $k_2 = 3.3 \pm 0.3 \times 10^{-1}$ min$^{-1}$; Solvent: $CH_3CN/0.1$ M Britton-Robinson pH 8 buffer 1/1 (v/v).
Figure 11: Fluorescence emission ($\lambda_{em}=660 \text{ nm}$) by illuminating at $\lambda_{exc}=365 \pm 25 \text{ nm}$ a $10.0 \pm 0.3 \mu\text{M}$ solution of the free DDAO at $1.4 \times 10^{-9} \text{ Eins}^{-1}$ intensity of light and at 293K. Solvent: CH$_3$CN/0.1 M Britton-Robinson pH 8 buffer 1/1 (v/v) corresponding to the maximum fluorescence observed after decaging.
Figure 12: $^1$H NMR spectrum of compound 1
Figure 13: $^{13}$C NMR spectrum of compound 1
Figure 14: HRMS spectrum of compound 1
Figure 15: $^1$H NMR spectrum of compound 2
Figure 16: $^{13}$C NMR spectrum of compound 2
Figure 17: HRMS spectrum of compound 2
Figure 18: $^1H$ NMR spectrum of compound 3
Figure 19: $^{13}$C NMR spectrum of compound 3
Figure 20: HRMS spectrum of compound 3
Figure 21: $^1$H spectrum of compound 4
Figure 22: $^{13}$C NMR spectrum of compound 4
Figure 23: HRMS spectrum of compound 4
Figure 24: $^1$H NMR spectrum of compound 5
Figure 25: $^{13}$C NMR spectrum of compound 5
Figure 26: HRMS spectrum of compound 5
Figure 27: $^1$H NMR spectrum of compound 6
Figure 28: $^{13}$C NMR spectrum of compound 6
Figure 29: HRMS spectrum of compound 6
Figure 30: $^1$H NMR spectrum of compound 7
Figure 31: $^{13}$C NMR spectrum of compound 7
Figure 32: HRMS spectrum of compound 7
Figure 33: $^1$H NMR spectrum of compound 8
Figure 34: $^{13}$C NMR spectrum of compound B
Figure 35: HRMS spectrum of compound 8
Figure 36: $^1$H NMR of compound 9
Figure 37: $^{13}$C NMR spectrum of compound 9
Figure 38: HRMS spectrum of compound 9
Figure 39: $^1$H NMR spectrum of compound 10
Figure 40: $^{13}$C NMR spectrum of compound 10
Figure 41: HRMS spectrum of compound 10
Figure 42: $^1$H NMR spectrum of compound 11
Figure 43: $^{13}$C NMR spectrum of compound 11
Figure 44: HRMS spectrum of compound 11
Figure 45: $^1$H NMR spectrum of compound 12
Figure 46: $^{13}$C NMR spectrum of compound 12
Figure 47: HRMS spectrum of compound 12
Figure 48: $^1$H NMR spectrum of compound 13
Figure 49: $^{13}$C NMR spectrum of compound 13
Figure 50: HRMS spectrum of compound 13
Figure 51: $^1$H NMR spectrum of compound 14
Figure S2: $^{13}$C NMR of compound 14
Figure 53: HRMS spectrum of compound 14
Figure 54: $^1H$ NMR spectrum of compound 15
Figure 55: $^{13}$C NMR spectrum of compound 15
Figure 56: HMRS spectrum of compound 15
Figure 57: $^1$H NMR spectrum of compound 16
Figure 58: $^{13}$C NMR spectrum of compound 16
Figure 59: HRMS spectrum of compound 16
Figure 60: $^1$H NMR spectrum of compound 2-HPC$_{NO_2}$
Figure 61: $^{13}$C NMR spectrum of compound 2-HPC$^{NO2}$
Figure 62: HRMS spectrum of compound 2-HPC\textsuperscript{NO2}
Figure 63: $^1$H NMR spectrum of compound 2-HPC$^{14}$
Figure 64: $^{13}$C NMR spectrum of compound 2-HPC$^h$
Figure 65: HRMS spectrum of compound 2-HPC ii
Figure 66: $^1$H NMR spectrum of compound 2-HPC$^\text{OCH}_3$
Figure 67: $^{13}$C NMR spectrum of compound 2-HPC$_{OCH_3}$
Figure 68: HRMS spectrum of compound $2\text{-HPC}^{\text{OCH}_3}$
Figure 69: $^1$H NMR spectrum of compound 2-HPC$^{br}$
Figure 70: $^{13}$C NMR spectrum of compound 2-HPC$^{Br}$
Figure 71: HRMS spectrum of compound 2-HPC\textsuperscript{Br}
Figure 72: $^1$H NMR spectrum of compound 17
Figure 73: $^{13}$C NMR spectrum of compound 17
Figure 74: HRMS spectrum of compound 17
Figure 75: $^1$H NMR spectrum of compound 18
Figure 76: $^{13}$C NMR spectrum of compound 18
Figure 77: HRMS spectrum of compound 18
Figure 78: $^1$H NMR spectrum of compound 19
Figure 79. $^{13}$C NMR spectrum of compound 19
Figure 80: HRMS spectrum of compound 19
Figure 81: $^1$H NMR spectrum of compound 20
Figure 82: $^{13}$C NMR spectrum of compound 20
Figure 83: HRMS spectrum of compound 20
Figure 84: $^1$H NMR of compound 21
Figure 85: $^{13}$C NMR spectrum of compound 21
Figure 86: HRMS spectrum of compound 21
Figure 87: $^1$H NMR spectrum of compound TML
Figure 88: $^{13}$C NMR spectrum of compound TML
Figure 89: HRMS spectrum of compound TML
Figure 90: UV absorbance of free DDAO (blue) and TML at 0 (black) and 1000s (red) in acetonitrile/buffer: no absorption at 640 nm corresponding to free DDAO appeared, which proves the stability of the TML ester during this time.
The purity of the compounds was evaluated by HPLC (Waters ACQUITY UPLC BEH C18 1.7µm VanGuard® Pre-Column 3/Pk 2.1 x 5mm Column). Conditions of HPLC analysis introduce 2 peaks when purified carbamate and ester linked DDAO compounds are analysed. Thus, the purity was calculated by the sum of these 2 peaks.

Figure 91: HPLC spectrum of 2-HPC\textsuperscript{NO2}, showing retention time of 1.51 min in 80 to 90% gradient of CH\textsubscript{3}CN/water/0.1% formic acid. The purity of the desired compound is 96.4%.
Figure 92: HPLC spectrum of 2-HPC\textsuperscript{4}, showing retention time of 1.61 min in 80 to 90% gradient of CH\textsubscript{3}CN/water/0.1% formic acid. The purity of the desired compound is 99.8%.
Figure 93: HPLC spectrum of 2-HPC<sub>O</sub>CH<sub>3</sub>, showing retention time of 1.59 min in 80 to 90% gradient of CH<sub>3</sub>CN/water/0.1% formic acid. The purity of the desired compound is 72.8%.
Figure 94: HPLC spectrum of 2-HPC<sub>Br</sub>, showing retention time of 1.96 min in 80 to 90% gradient of CH<sub>3</sub>CN/water/0.1% formic acid. The purity of the desired compound is 96.2%.
Figure 95: HPLC spectrum of TML, showing retention time of 2.34 min in 80 to 90% gradient of CH$_3$CN/water/0.1% formic acid. The purity of the desired compound is 94.7%.