

# **Caged AG10: New tools for spatially predefined mitochondrial uncoupling**

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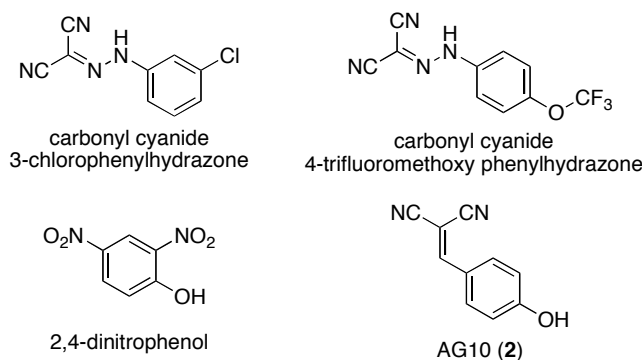
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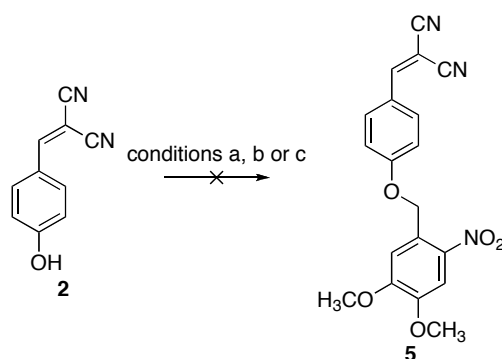
## **Electronic Supplementary Information**

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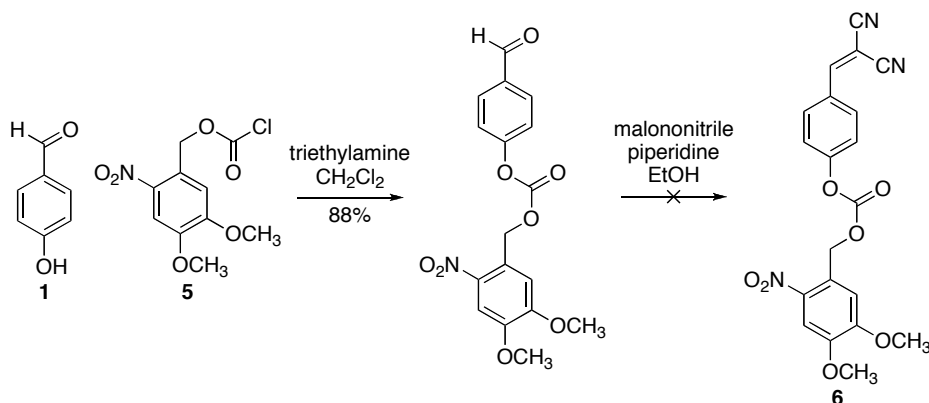


**Fig. S1** The structures of compounds known to act as protonophores. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), 2,4-dinitrophenol (DNP) and AG10 (2).

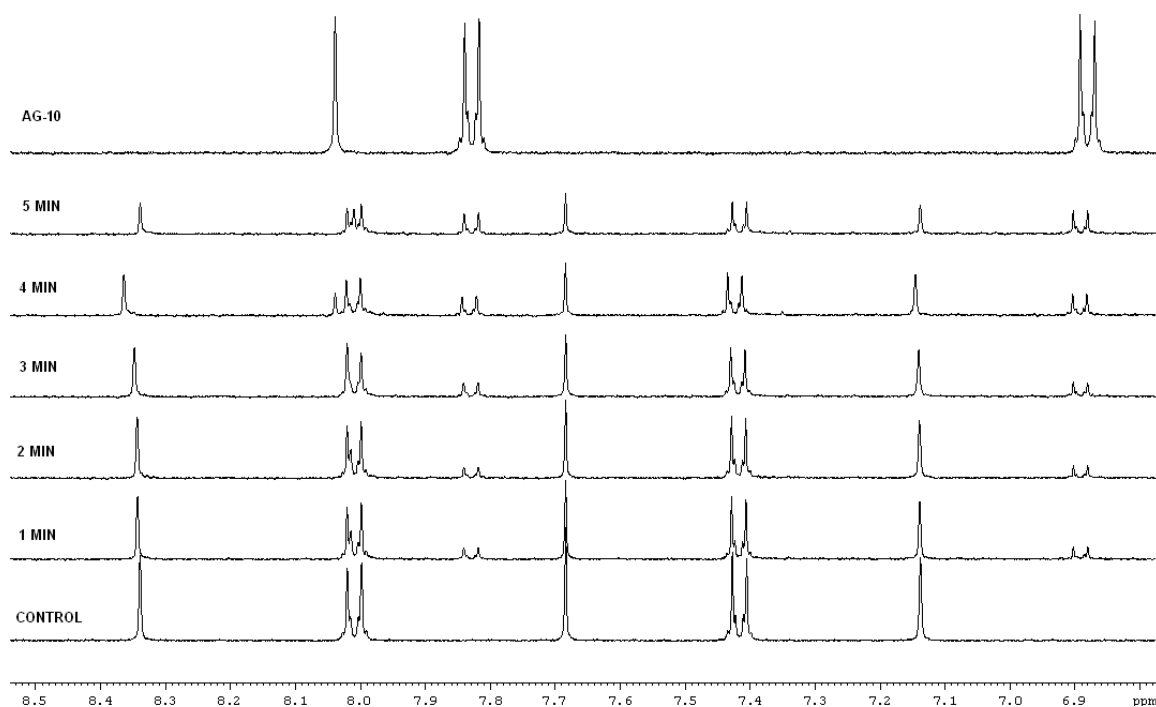


**Fig. S2** The attempted synthesis DMNB-caged AG10 from AG10. *Reagents and conditions:* a. <sup>t</sup>BuOK, THF, rt; b. K<sub>2</sub>CO<sub>3</sub>, DMF, 40 °C; c. NaH, DMF, rt.

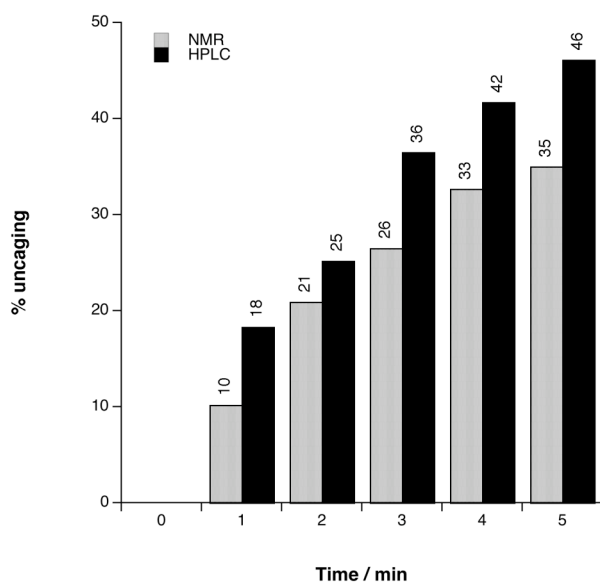
It was envisaged that deprotonation of AG10 and reaction with 4,5-dimethoxy-2-nitrobenzyl (DMNB) bromide would furnish the desired DMNB-caged AG10 (5). However, treatment of 2 with <sup>t</sup>BuOK in DMF at room temperature failed to produce any of the desired product. Subsequent reactions employing K<sub>2</sub>CO<sub>3</sub> in DMF at 40 °C or using NaH in DMF at room temperature also failed to give the caged AG10 derivative 5. It was hypothesised that AG10 failed to react with DMNB bromide because of its very weak nucleophilic nature. The phenolate anion of 2 is likely to be a poor nucleophile due to delocalisation of the negative charge across the extended conjugated system, which includes the nitrile groups that are linked to the aromatic ring by the C-C double bond. To overcome this problem, 4-hydroxybenzaldehyde was first reacted with DMNB bromide and the condensation reaction then performed. As 4-hydroxybenzaldehyde has a less extensive conjugated  $\pi$ -system than AG10, it was envisaged that this compound would be nucleophilic enough to react with DMNB bromide. This proved to be the case and the synthesis of the DMNB-caged aldehyde 4 and subsequent condensation with malononitrile, to give the desired DMNB-caged AG10 derivative 5, proceeded smoothly.



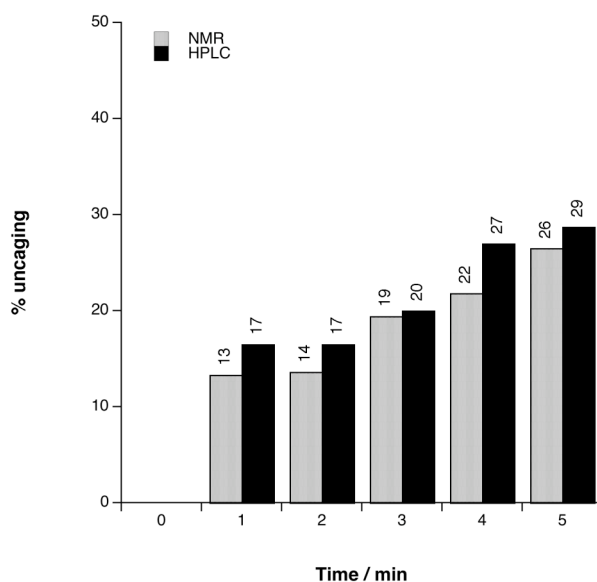
**Fig. S3** The attempted alternative synthesis of CDMNB-caged AG10.



**Fig. S4** The photolysis of **5** using a 95 mJ (pulse energy) laser with a wavelength of 355 nm. The partial  $^1\text{H}$  NMR spectrum labelled control shows DMNB-caged AG10 before photolysis. The following partial  $^1\text{H}$  NMR spectra show a sample of DMNB-caged AG10 after being photolysed for the time shown. The partial  $^1\text{H}$  NMR spectrum labelled AG10 shows a synthetic sample of AG10. It can be seen from these spectra that photolysis of DMNB-caged AG10 leads to the loss of DMNB-caged AG10 and the appearance of AG10.



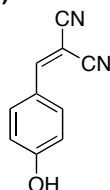
**Fig. S5A** The percentage of AG10 present after photolysis of DMNB-caged AG10 (**5**) for the time shown. Analysed by  $^1\text{H}$  NMR (grey bars) and HPLC (black bars).



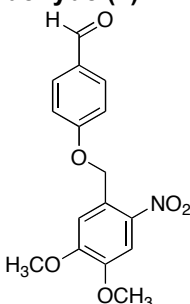
**Fig. S5B** The percentage of AG10 present after photolysis of CDMNB-caged AG10 (**3**) for the time shown. Analysed by  $^1\text{H}$  NMR (grey bars) and HPLC (black bars).

**General experimental methods**

<sup>1</sup>H NMR spectra were recorded at 300.1 MHz using deuteriochloroform (or other indicated solvent) as reference and internal deuterium lock. The chemical shift data for each signal are given as  $\delta$  in units of parts per million (ppm) relative to tetramethylsilane (TMS) where  $\delta_{\text{TMS}} = 0.00$  ppm. <sup>13</sup>C NMR spectra were recorded at 125 MHz, 100 MHz or 75.5 MHz using the PENDANT sequence and internal deuterium lock with broadband proton decoupling and internal deuterium lock. The chemical shift data for each signal are given as  $\delta$  in units of ppm relative to TMS where  $\delta_{\text{TMS}} = 0.00$  ppm. IR spectra were recorded as nujol mulls or thin films between NaCl disks, as indicated. Absorption maxima are reported in wavenumbers (cm<sup>-1</sup>). Melting points are uncorrected. Analytical thin layer chromatography (TLC) was carried out on pre-coated 0.25 mm F<sub>254</sub> silica gel plates. Visualisation was by absorption of UV light, or thermal development after dipping in either an aqueous solution of potassium permanganate, potassium carbonate and sodium hydroxide or a solution of ninhydrin in butan-2-ol. Flash Column chromatography was carried out on silica gel or on activated aluminium oxide as indicated, under a positive pressure of compressed air. HPLC was conducted using Gilson equipment detecting at 255 nm, employing a Phenomenex Gemini 5  $\mu$  C18 110A column with a gradient elution of H<sub>2</sub>O and MeCN with 0.05 % TFA. Dichloromethane was distilled from calcium hydride in a recycling still, or by passage through two columns of alumina using an MBRAUN (SPS-800) solvent purification system. Diethyl ether was distilled from sodium in a recycling still using benzophenone ketyl as an indicator. Anhydrous *N,N*-dimethyl formamide was dried by distillation from 4 Å molecular sieves onto 4 Å molecular sieves under an atmosphere of nitrogen. Reagents and solvents were purchased from commercial sources and were purified and dried, where necessary, by standard techniques.<sup>1</sup> Where appropriate, and if not stated otherwise, all non aqueous reactions were performed under an inert atmosphere of nitrogen or argon, using a vacuum manifold with nitrogen passed through 4 Å molecular sieves and self-indicating silica gel. *In vacuo* refers to the use of a rotary evaporator attached to a diaphragm pump. Hexane refers to a mixture of hexanes and petroleum ether to the fraction boiling between 40-60 °C. Room temperature (rt) refers to the temperature of 25 °C.

**4-(Hydroxybenzylidene)malononitrile (AG10, 2)**

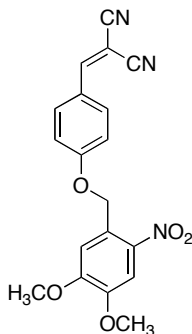
To a solution of 4-hydroxybenzaldehyde (**1**, 10.0 g, 82 mmol) and malononitrile (5.6 g, 85 mmol) in ethanol (50 mL) was added of piperidine (10 drops) and the reaction solution heated under reflux for 2 h. After this time, water was added and the resulting solid was removed by filtration. The solid was crystallised from ethanol/hexane to afford **2** as a yellow solid (12.56 g, 90%). *R<sub>f</sub>* 0.42 (hexane/ethyl acetate 6:4); mp 180-182 °C (from acetone/hexane, lit.<sup>2</sup> 180 °C); <sup>1</sup>H NMR (300 MHz; acetone-D<sub>6</sub>):  $\delta$  9.69 (bs, 1H, OH), 7.98 (s, 1H, CH), 7.85 (d, *J* = 8.9 Hz, 2H, Ar), 6.93 (d, *J* = 8.9 Hz, 2H, Ar); <sup>13</sup>C NMR (75 MHz; D<sub>6</sub>-acetone):  $\delta$  164.4, 160.7, 134.7, 124.5, 117.5, 115.6, 114.7, 77.6; HRMS *m/z* (ES<sup>-</sup>) [found (M - H)<sup>-</sup> 169.0395, C<sub>10</sub>H<sub>5</sub>N<sub>2</sub>O requires M<sup>-</sup>, 169.0402]; *m/z* (ES<sup>-</sup>), 169 ([M - H]<sup>-</sup>, 100); Anal. Calcd for C<sub>10</sub>H<sub>6</sub>N<sub>2</sub>O: C, 70.6; H, 3.6; N, 16.5. Found: C, 70.4; H, 3.2; N, 16.4.

**4-(4',5'-Dimethoxy-2'-nitrobenzyloxy)benzaldehyde (4)**

To a solution of 4-hydroxybenzaldehyde (**1**, 300 mg, 2.45 mmol) in DMF (10 mL) was added potassium carbonate (678 mg, 4.90 mmol) and 4,5-dimethoxy-2-nitrobenzyl bromide (677 mg, 2.45 mmol) and the reaction mixture was stirred at 40 °C overnight. The reaction mixture was cooled and water was added. After stirring for 10 min the precipitated solid was filtered and washed with water. The solid was dissolved in dichloromethane and the solution was dried over sodium sulfate, filtered and concentrated *in vacuo*. Purification with silica gel column chromatography eluting with petroleum ether and ethyl acetate (8:2)

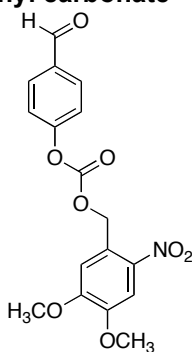
afforded **4** (400 mg, 51%) as a colourless solid.  $R_f$  0.37 (hexane/ethyl acetate 6:4); mp 166-168 °C (from hexane);  $\nu_{\max}$  (nujol)/ $\text{cm}^{-1}$  2924 (w), 1691 (s), 1690 (m), 1604 (m), 1521 (s), 1279 (s);  $^1\text{H}$  NMR (300 MHz;  $\text{CDCl}_3$ ):  $\delta$  9.85 (s, 1H, CHO), 7.81 (d,  $J$  = 8.8 Hz, 2H, Ar), 7.72 (s, 1H, Ar), 7.21 (s, 1H, Ar), 7.08 (d,  $J$  = 8.8 Hz, 2H, Ar), 5.52 (s, 2H,  $\text{CH}_2$ ), 3.91 (s, 3H,  $\text{OCH}_3$ ), 3.89 (s, 3H,  $\text{OCH}_3$ );  $^{13}\text{C}$  NMR (100 MHz;  $\text{CDCl}_3$ ):  $\delta$  190.7, 162.9, 154.0, 148.2, 139.2, 132.1, 130.7, 128.1, 115.3, 109.3, 108.2, 67.3, 56.5; HRMS  $m/z$  ( $\text{ES}^+$ ) [found ( $\text{M} + \text{Na}$ ) $^+$  340.0791,  $\text{C}_{16}\text{H}_{15}\text{NO}_6\text{Na}$  requires  $\text{M}^+$ , 340.0797];  $m/z$  ( $\text{ES}^+$ ), 340 ( $[\text{M} + \text{Na}]^+$ , 100); Anal. Calcd for  $\text{C}_{16}\text{H}_{15}\text{NO}_6$ : C, 60.6; H, 4.8; N, 4.4. Found: C, 61.0; H, 4.7; N, 4.3.

#### 4-(4',5'-Dimethoxy-2'-nitrobenzyloxy)benzylidenemalononitrile (**5**)

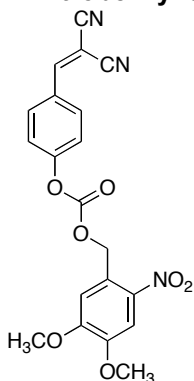


To a solution of 4-(4',5'-dimethoxy-2'-nitrobenzyloxy)benzaldehyde **4** (10.0 g, 82 mmol) and malononitrile (5.6 g, 85 mmol) in ethanol (20 mL) was added piperidine (5 drops) and the reaction solution was heated under reflux for 2 h. After this time, water was added and the resulting solid removed by filtration to afford **5** (160 mg, 70%) as a yellow solid.  $R_f$  0.48 (hexane/ethyl acetate 6:4); mp 226-228 °C (from ethanol);  $\nu_{\max}$  (nujol)/ $\text{cm}^{-1}$  2924 (m), 2854 (m), 2222 (w), 1601 (m), 1584 (m), 1267 (s);  $^1\text{H}$  NMR (300 MHz;  $\text{D}_6\text{-DMSO}$ ):  $\delta$  8.48 (s, 1H, CH), 8.06 (d,  $J$  = 9.0 Hz, 2H, Ar), 7.80 (s, 1H, Ar), 7.39 (s, 1H, Ar), 7.36 (d,  $J$  = 9.0 Hz, 2H, Ar), 5.60 (s, 2H,  $\text{CH}_2$ ), 3.94 (s, 3H,  $\text{OCH}_3$ ), 3.93 (s, 3H,  $\text{OCH}_3$ );  $^{13}\text{C}$  NMR (125 MHz;  $\text{D}_6\text{-DMSO}$ ):  $\delta$  162.9, 160.3, 153.1, 148.1, 139.9, 133.2, 125.8, 124.5, 115.8, 114.5, 113.6, 111.9, 108.5, 77.4, 67.1, 56.2, 56.1; HRMS  $m/z$  ( $\text{EI}^+$ ) [found  $\text{M}^+$  365.1008,  $\text{C}_{19}\text{H}_{15}\text{N}_3\text{O}_5$  requires  $\text{M}^+$ , 365.1006];  $m/z$  ( $\text{CI}^+$ ) 383 ( $[\text{M} + \text{NH}_4]^+$ , 20), 196 ( $[\text{C}_9\text{H}_{10}\text{NO}_4]^+$ , 10), 182 (30), 168 ( $[\text{C}_{10}\text{H}_5\text{N}_2\text{O}]^+$ , 100).

#### 4',5'-Dimethoxy-2'-nitrobenzyl 4-formylphenyl carbonate

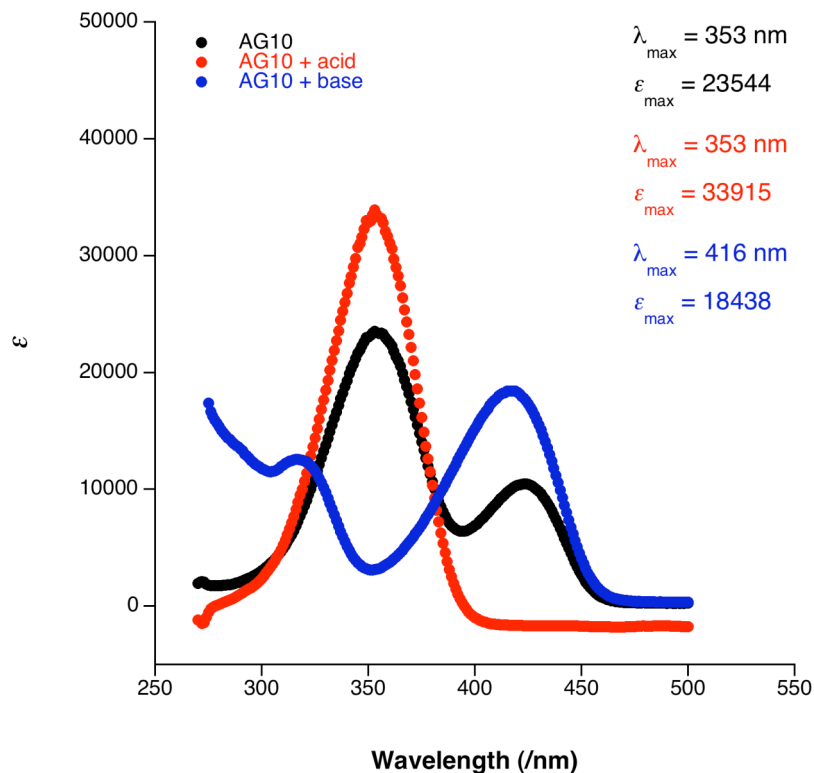


A stirred solution of 4-hydroxybenzaldehyde (**1**, 100 mg, 0.82 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (10 mL) was cooled to 0 °C. 4,5-Dimethoxy-2-nitrobenzyl chloroformate (678 mg, 2.46 mmol) and triethylamine (247 mg, 0.34 mL, 2.45 mmol) were then added and the reaction solution was stirred for 2 h. After this time, TLC analysis indicated complete consumption of the starting materials. The solvents were removed *in vacuo* and the crude material was purified by silica gel column chromatography eluting with hexane and ethyl acetate (7:3) to afford the title compound (260 mg, 88%) as yellow solid.  $R_f$  0.33 (hexane/ethyl acetate 6:4); mp 136-138 °C (from hexane);  $\nu_{\max}$  (thin film)/ $\text{cm}^{-1}$  2949 (w), 2850 (w), 1768 (s), 1700 (s), 1525 (s), 1279 (s);  $^1\text{H}$  NMR (300 MHz;  $\text{CDCl}_3$ ):  $\delta$  10.03 (s, 1H, CHO), 7.97 (d,  $J$  = 8.7 Hz, 2H, Ar), 7.80 (s, 1H, Ar), 7.43 (d,  $J$  = 8.7 Hz, 2H, Ar), 7.14 (s, 1H, Ar), 5.74 (s, 2H,  $\text{CH}_2$ ), 4.05 (s, 3H,  $\text{OCH}_3$ ), 4.01 (s, 3H,  $\text{OCH}_3$ );  $^{13}\text{C}$  NMR (75 MHz;  $\text{CDCl}_3$ ):  $\delta$  190.7, 155.4, 153.8, 152.4, 148.7, 139.9, 134.3, 131.4, 125.5, 121.7, 110.4, 108.4, 67.5, 56.6, 56.5; HRMS  $m/z$  ( $\text{ES}^+$ ) [found ( $\text{M} + \text{Na}$ ) $^+$  384.0710,  $\text{C}_{17}\text{H}_{15}\text{NO}_8\text{Na}$  requires  $\text{M}^+$ , 384.0719];  $m/z$  ( $\text{ES}^+$ ), 384 ( $[\text{M} + \text{Na}]^+$ , 80); Anal. Calcd for  $\text{C}_{17}\text{H}_{15}\text{NO}_8$ : C, 56.5; H, 4.2; N, 3.8. Found: C, 56.8; H, 4.2; N, 3.9.

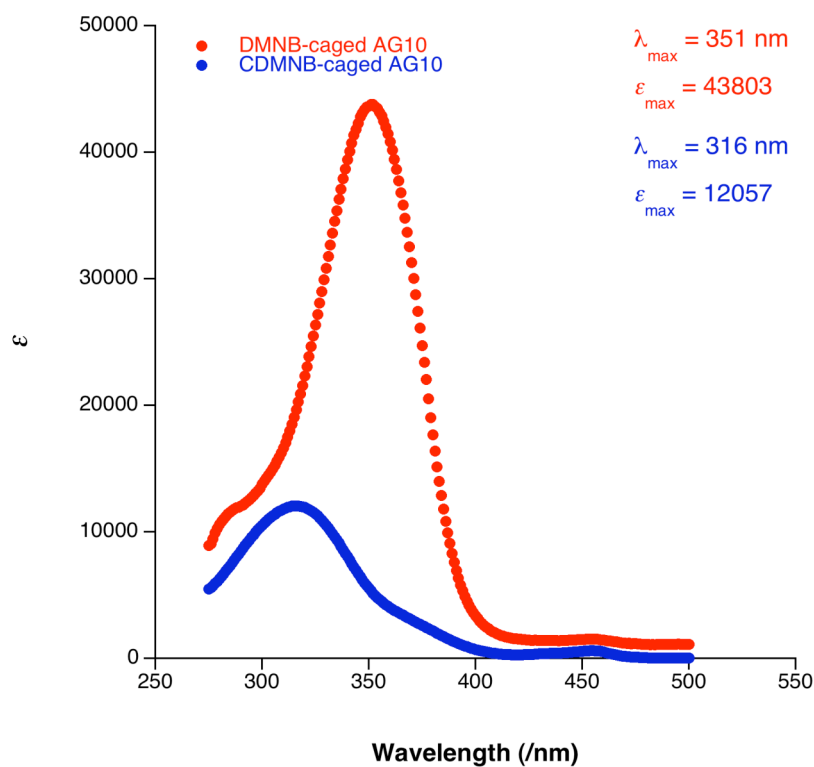
**4-(2,2-Dicyanovinyl)phenyl 4',5'-dimethoxy-2'-nitrobenzyl carbonate (3)**

A stirred solution of 4-(hydroxybenzylidene)malononitrile (**2**, 100 mg, 0.59 mmol) in THF (5 mL) was cooled to 0 °C. 4,5-Dimethoxy-2-nitrobenzyl chloroformate (488 mg, 1.77 mmol) and triethylamine (181 mg, 0.25 mL, 1.79 mmol) were added and the reaction solution was stirred for 3 h at room temperature. After this time the solvent was removed *in vacuo* and the residue was purified using silica gel column chromatography eluting with hexane and ethyl acetate (3:2) to afford **3** (96 mg, 40%) as a yellow solid.  $R_f$  0.44 (hexane/ethyl acetate 6:4); mp 192-194 °C (from chloroform);  $\nu_{\max}$  (nujol)/ $\text{cm}^{-1}$  2924 (s), 2854 (s), 2229 (w), 1771 (w), 1463 (s);  $^1\text{H}$  NMR (300 MHz;  $\text{D}_6$ -DMSO):  $\delta$  8.55 (s, 1H, CH), 8.03 (d,  $J$  = 8.8 Hz, 2H, Ar), 7.73 (s, 1H, Ar), 7.56 (d,  $J$  = 8.8 Hz, 2H, Ar), 7.24 (s, 1H, Ar), 5.59 (s, 2H,  $\text{CH}_2$ ), 3.90 (s, 3H,  $\text{OCH}_3$ ), 3.88 (s, 3H,  $\text{OCH}_3$ );  $^{13}\text{C}$  NMR (100 MHz; DMSO- $\text{D}_6$ ):  $\delta$  158.7, 155.0, 153.6, 152.1, 148.7, 139.9, 132.4, 128.7, 125.1, 122.1, 113.4, 112.3, 110.6, 108.3, 82.7, 67.5, 56.5, 56.4; HRMS  $m/z$  ( $\text{ES}^+$ ) [found ( $\text{M} + \text{Na}$ ) $^+$  432.0810  $\text{C}_{20}\text{H}_{15}\text{N}_3\text{O}_7\text{Na}$  requires  $\text{M}^+$ , 432.0808];  $m/z$  ( $\text{ES}^+$ ), 432 ( $[\text{M} + \text{Na}]^+$ , 100); Anal. Calcd for  $\text{C}_{20}\text{H}_{15}\text{N}_3\text{O}_7$ : C, 58.7; H, 3.7; N, 10.3. Found: C, 58.3; H, 3.6; N, 10.0.

## UV/Vis Spectra



**Fig. S6** AG10 UV/Vis spectrum (0.1 mM in MeOH) (black), AG10 UV/Vis spectrum (0.1 mM in MeOH, HCl added to attain pH 2) (red), AG10 UV/Vis spectrum (0.1 mM in MeOH, DBU added to attain pH 12) (blue).



**Fig. S7** DMNB-caged AG10 UV/Vis spectrum (0.1 mM in DMSO) (red); CDMNB-caged AG10 UV/Vis spectrum (0.1 mM in DMSO) (blue).

## References

1. W. L. F. Armarego and C. L. L. Chai, *Purification of laboratory chemicals*, 5th edn., Butterworth Heinemann, 2003.
2. A. Gazit, P. Yaish, C. Gilon and A. Levitzki, *J. Med. Chem.*, 1989, **32**, 2344-2352.