Functionalising the azobenzene motif delivers a light-responsive membrane-interactive compound with the potential for photodynamic therapy applications

Theodore J. Hester,[a] Sarah R. Dennison,[b] Matthew J. Baker,[c] Timothy J. Snape*[,b]

[a] School of Forensic and Investigative Sciences, University of Central Lancashire, Preston, PR1 2HE, UK.

[b] School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, PR1 2HE, UK. Tel: +44 (0)1772 895805; E-mail: tjsnape@uclan.ac.uk.

[c] WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, G1 1XL, UK. Tel : +44 (0)141 548 4700; E-mail: matthew.baker@strath.ac.uk.

Generic information

Commercially available reagents were used as received without purification. Analytical thin layer chromatography (TLC) was performed with plastic-backed TLC plates coated with silica G/UV_{254}, in 10% EtOAc in petroleum ether. The plates were visualised by UV light (254 nm) or *p*-anisaldehyde solution. Flash column chromatography was conducted with Davisil silica 60Å (40-63 μm) under bellows pressure. Low resolution mass spectra were recorded on a Thermo FinniganLCQ Advantage MAX using electron spray ionisation (ESI) and high resolution mass spectra were gratefully recorded by the EPSRC National Mass Spectrometry Service at Swansea University, UK. ^{1}H and ^{13}C NMR spectra were recorded on a BrukerAvanceDPX 300 (300 MHz) or a Bruker 400 (400 MHz) spectrometer. All chemical shifts (δ) are quoted in parts per million (ppm) relative to a calibration reference of the residual protic solvent; C*H*Cl_3 (δ_H 7.26, s) was used as the internal standard in ^{1}H NMR spectra, and ^{13}C NMR shifts were referenced using *C*DCl_3 (δ_C 77.16, t) with broad band decoupling and the *J* values are measured in Hertz. IR spectra were recorded on a NICOLET iS10. Petroleum ether refers to the fraction that boils between 40-60 °C.

Procedure for the synthesis *trans*-bis(4-octylphenyl)diazene, 4

4-Octylaniline (0.513 g, 2.5 mmol) was dissolved in acetonitrile (6 ml) and copper(I) bromide (0.179 g, 1.25 mmol) and TEMPO (0.195 g, 1.25 mmol) were added. The reaction mixture was stirred vigorously at 60 °C for 24 hrs. After completion (TLC) the reaction mixture was diluted with water (2 × 50 ml) and the product extracted using diethyl ether (2 × 50 ml). The organic layer was washed with water (50 ml) and brine (50 ml). The organic phase was dried ($MgSO_4$) and briefly purified by filtration through a short silica column (diethyl ether) to yield the title compound as a pure solid product (0.38 g, 73%). R_f (10% EtOAc in petroleum ether): 0.86. ^{1}H NMR ($CDCl_3$, 300MHz): δ_H 0.88 (t, 6H, *J*= 7.0Hz), 1.25-1.33 (m, 20H), 1.60-1.70 (m, 4H), 2.67 (t, 4H, *J*= 7.5Hz), 7.31 (d, 4H, *J*= 8.5Hz), 7.82 (d, 4H, *J*= 8.5Hz). ^{13}C NMR ($CDCl_3$, 75MHz): δ_C 14.3 (CH_3), 22.8 (CH_2), 29.40 (CH_2), 29.44 (CH_2), 29.6 (CH_2), 31.5 (CH_2), 32.0 (CH_2), 36.0 (CH_2), 122.8 (CH),

129.2 (CH), 146.4 (C), 151.1 (C); MS (GC-MS): 406 [M^+, (100%)]. HRMS $[M+H]^+$ 407.3421, $C_{28}H_{42}N_2+H^+$ requires 407.3421.

Representative UV experiment for the conversion of *trans*-4 into *cis*-5

A 0.125 mM stock solution of **4** in isooctane (or $CDCl_3$, or 50:50 *n*-propanol:water) was prepared. A 1.2 ml quartz glass cuvette with an optical path length of 1 cm was filled with 1 ml of the sample solution. The cuvette was arranged in a temperature controlled box with a sliding lid, allowing for irradiation of the sample with a 40 W UV 365nm lamp. A halogen lamp served as the light source and illuminated the sample with an optical fibre for recording the spectra. On the opposite side was a spectrometer (Shimadzu UV-3600) connected with another optical fibre. The slit was adjusted to meet a bandwidth of 20 nm. Calibration of the optical set-up was achieved by recording a baseline, after which spectra from 200 nm to 700 nm were taken every 10 seconds of UV 365 nm irradiation of the sample. Figure 1 displays the results.

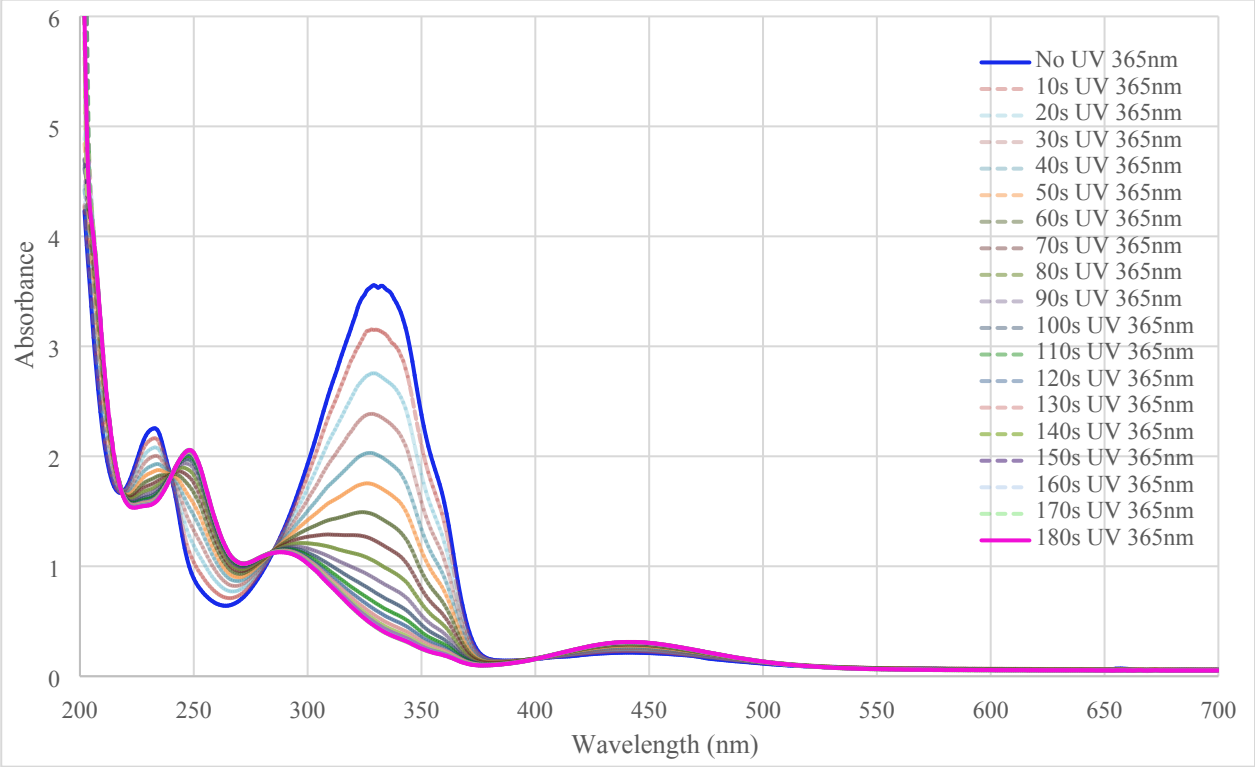


Figure 1. Plot displaying the UV-Vis absorbance pattern of a 0.125mM solution of **4** in isooctane before irradiation (blue line) with 365 nm UV light, and after every 10 seconds of irradiation with 365 nm UV light, up to 3 minutes of irradiation (pink line).

Representative UV experiment for the reverse conversion of *cis*-5 into *trans*-4

A relaxation experiment was carried out in a similar manner. Using a 532 nm laser as the light source and the now "excited" sample of *cis*-**5** (0.125mM solution), a similar plot was made for the absorbance pattern of the same "excited" sample after illumination with the 532 nm laser for 30 second intervals, up to 10 minutes of illumination. Figure 2 displays the results.

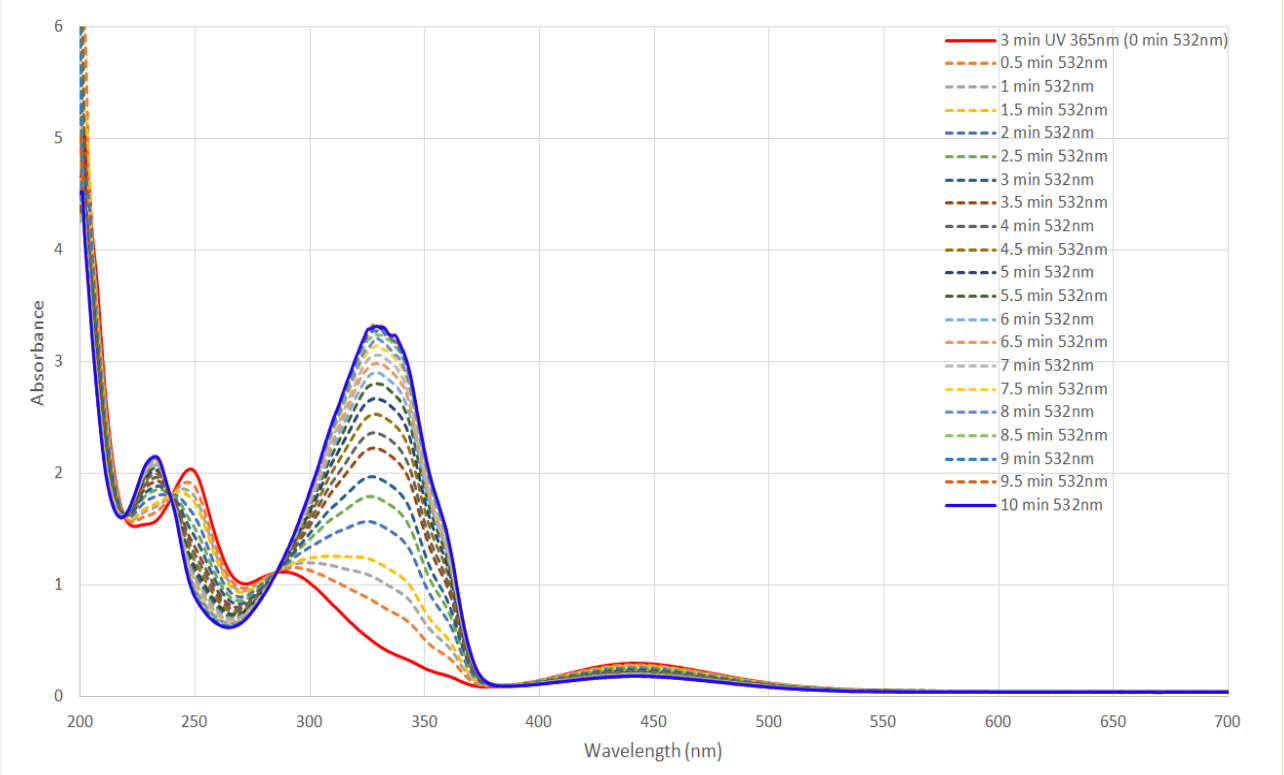


Figure 2. Plot displaying the UV-Vis absorbance pattern of a 0.125mM solution of **5** every 30 seconds with irradiation using a 532 nm laser, for up to 10 minutes of irradiation.

Conformational switching using 1H-NMR

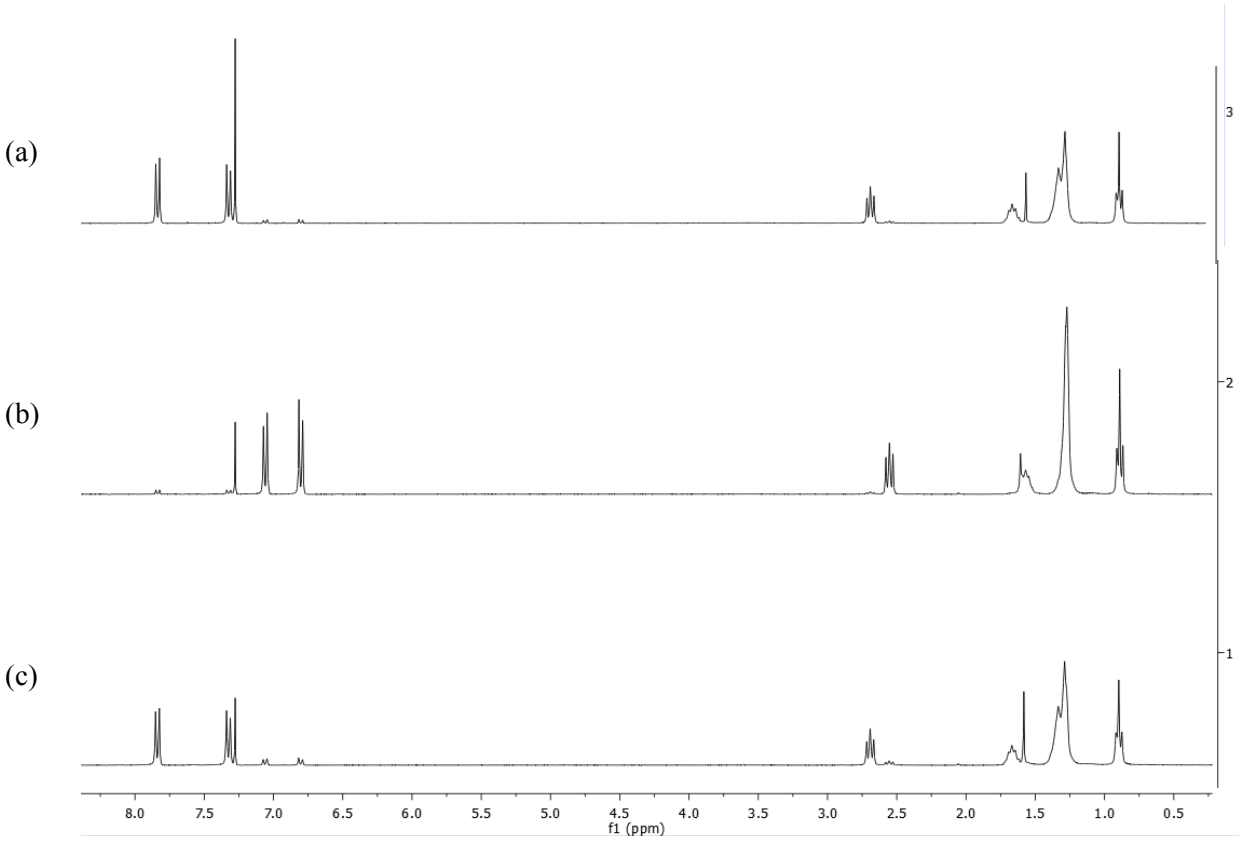


Figure 3. 300 MHz ^{1}H NMR spectra of **4** in $CDCl_3$ (a) prior to irradiation (i.e. in the *trans*-conformation), (b) after 21hrs of UV 365nm irradiation (in the *cis*-conformation), (c) after exposure of the irradiated sample to darkness and heat (back to *trans*).

Assay of calcein leakage

Phospholipids DMPG (dimyristoylphosphatidylglycerol), DMPE (dimyristoylphosphatidylethanolamine), DMPS (dimyristoylphosphatidylserine) and DMPC (dimyristoylphosphatidylcholine) (7.5 mg) were separately dissolved in chloroform and evaporated off under a stream of nitrogen before being further dried under vacuum for 12 hours to form a thin film. The lipid film was then hydrated with 5.0 mM HEPES (1 ml) containing 70 mM calcein. The suspension was vortexed for 5 min before being sonicated for 30 min. The solution then underwent 3 cycles of freeze-thawing. Liposomes were extruded 11 times through a 0.1 μm polycarbonate filter using an Avanti polar lipids min-extruder apparatus. Calcein entrapped vesicles were separated from free calcein by gel filtration using a Sephadex G75 column [Sigma-Aldrich, UK] which was rehydrated overnight in 20 mM HEPES, 150 mM NaCl and 1.0 mM EDTA. The column was eluted with 5 mM HEPES pH 7.5. The total lipid concentration used was 100μM.

The calcein release assay was performed (and repeated four times) by combining 2 mL of a solution containing HEPES (20 mM), NaCl (150 mM) and EDTA (pH 7.4, 1.0 mM), and 20 µl calcein vesicles, followed by the addition of the test compounds from a stock solution. The fluorescence intensity of calcein was measured using a FP-6500 spectrofluorometer [JASCO, Tokyo, Japan], with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. To measure maximum fluorescence, 20 µl of Triton X-100 was used to dissolve the vesicles. The percentage of dye leakage was then calculated using the following equation:

$$\text{Percentage leakage} = [(F - F_0)/(F_{\text{Triton}} - F_0)] \times 100$$

Where F_0 is the fluorescence intensity of the lipid vesicles, F is the maximum fluorescence intensity in the presence of **4** and **5**, and F_{triton} is the intensity after the addition of Triton X-100.

Assay of calcein leakage - raw data

*Concentration dependent calcein leakage for compounds **4** and **5***

For paper								
Concentration	PE **4**	PESD	PG **4**	PGSD	PE **5**	PESD	PG **5**	PGSD
0	0	0	0	0	0	0	0	0
3	1.454051	0.661157	4.455072	0.298218	8.477808	2.472953	21.32935	1.266341
6	8.188044	1.289	14.86628	1.040287	25.94753	1.148625	43.48272	0.170784
12	10.40827	0.302397	28.57849	0.210872	33.43999	0.223727	54.71537	0.307486
24	12.79217	1.526472	29.76554	0.892392	37.48279	2.78165	58.57625	0.550551
Concentration	PC **4**	PCSD	PS **4**	PSSD	PC **5**	PCSD	PS **5**	PSSD
0	0	0	0	0	0	0	0	0
3	6.33579	1.215444	6.017116	0.649266	36.63035	1.547194	31.59183	1.888012
6	16.30988	2.524707	25.55313	0.488715	43.0953	2.529172	44.24949	0.171525
12	19.01268	2.93474	28.67989	0.79234	51.25844	0.003179	63.91695	0.528449
24	26.28465	4.795057	32.85612	0.542417	52.73049	1.144758	62.85722	2.192758

PE **4** refers to the value for compound **4** against DMPE and PESD refers to the Standard Deviation for that result etc.

*Time course for **4***

Concentration	1 hour	SDPC	2 hours	SDPC	3 hours	SDPC	6 hours	SDPC	7 hours	SDPC	8 hours	SDPC	24 hours	SDPC	DMPC
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	5.018739	0.023488	21.11801	0.417579	17.99262	1.713292	20.22751	3.959465	20.84906	1.186005	21.8263	6.73431	20.15911	0.931214	
6	10.45687	0.283215	24.32451	0.335291	34.8897	2.46838	32.38035	0.674098	33.37836	2.397743	33.52116	2.824111	35.10132	0.704035	
12	29.86158	0.048306	28.1518	0.426934	44.40604	2.848096	44.83738	1.328885	46.72743	0.922806	45.86304	8.283912	45.97323	0.52316	
24	29.73163	0.079487	27.68747	0.518009	45.51702	1.474361	44.66034	1.251529	45.08364	0.397177	44.56104	8.41731	45.98249	0.429262	
Concentration	1 hour	SDPC	2 hours	SDPC	3 hours	SDPC	6 hours	SDPC	7 hours	SDPC	8 hours	SDPC	24 hours	SDPC	DMPE
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	7.970968	2.266309	7.206571	0.672669	4.218732	0.290789	5.624159	0.057321	4.998241	0.4751	5.599733	1.298899	5.960341	1.171581	
6	12.19124	0.841769	15.8264	0.760533	21.34657	0.610686	20.08843	0.056272	20.6269	0.496357	18.68053	1.951687	19.57746	1.221234	
12	12.49159	1.27127	27.24008	0.690267	29.42735	0.954668	27.45322	4.306728	28.70988	0.88087	29.4222	4.070261	27.39067	0.185133	
24	12.71269	0.659692	25.57317	0.393166	30.53504	0.895892	30.01772	0.062175	30.28176	0.824303	29.69374	5.17224	30.66705	0.578795	
Concentration	1 hour	SDPC	2 hours	SDPC	3 hours	SDPC	6 hours	SDPC	7 hours	SDPC	8 hours	SDPC	24 hours	SDPC	DMPG
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	5.553978	0.105409	11.09347	0.633543	6.729816	1.05942	6.673053	2.157294	6.749762	0.182165	5.792249	7.544169	5.885126	1.425191	
6	14.20937	0.442459	17.5273	0.090194	18.84486	2.090659	18.6768	1.017903	16.02927	0.522518	17.38253	3.038959	16.47979	1.492244	
12	30.90894	3.243638	27.84065	0.390078	25.81695	2.642466	26.6043	2.79465	30.2023	0.363425	31.69767	3.017937	33.79879	3.228643	
24	31.39451	4.18942	32.92712	0.273091	30.64467	2.782304	30.71553	0.858217	31.80094	0.412424	32.57746	0.954699	32.58161	1.524929	
Concentration	1 hour	SDPC	2 hours	SDPC	3 hours	SDPC	6 hours	SDPC	7 hours	SDPC	8 hours	SDPC	24 hours	SDPC	DMPS
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	9.755577	0.210558	8.835161	0.671986	8.472383	0.173749	10.22265	1.539636	12.09483	0.917973	12.49733	0.916059	11.68821	0.506969	
6	23.99086	0.356421	29.61932	0.484849	30.43301	0.314942	33.21985	4.638353	31.46631	0.592375	29.50034	2.780728	33.8848	1.26112	
12	28.34516	0.743918	35.90449	0.574226	32.95658	0.412942	33.8032	0.236761	36.14493	4.163879	35.5892	2.823519	36.70023	1.34215	
24	31.30967	1.620408	35.64558	0.443411	40.41876	0.407662	47.86428	0.330453	42.77165	2.057834	45.24802	0.977833	49.03838	1.909264	

SD = standard deviation

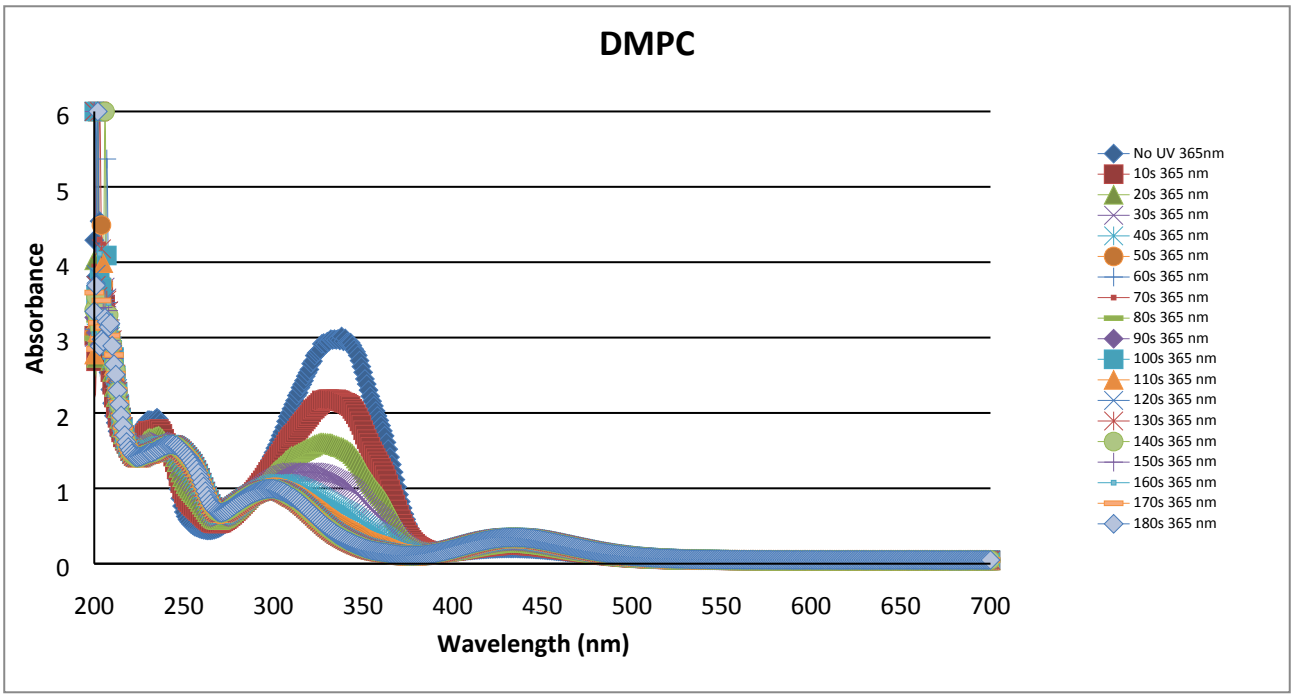
Time course for ***5***

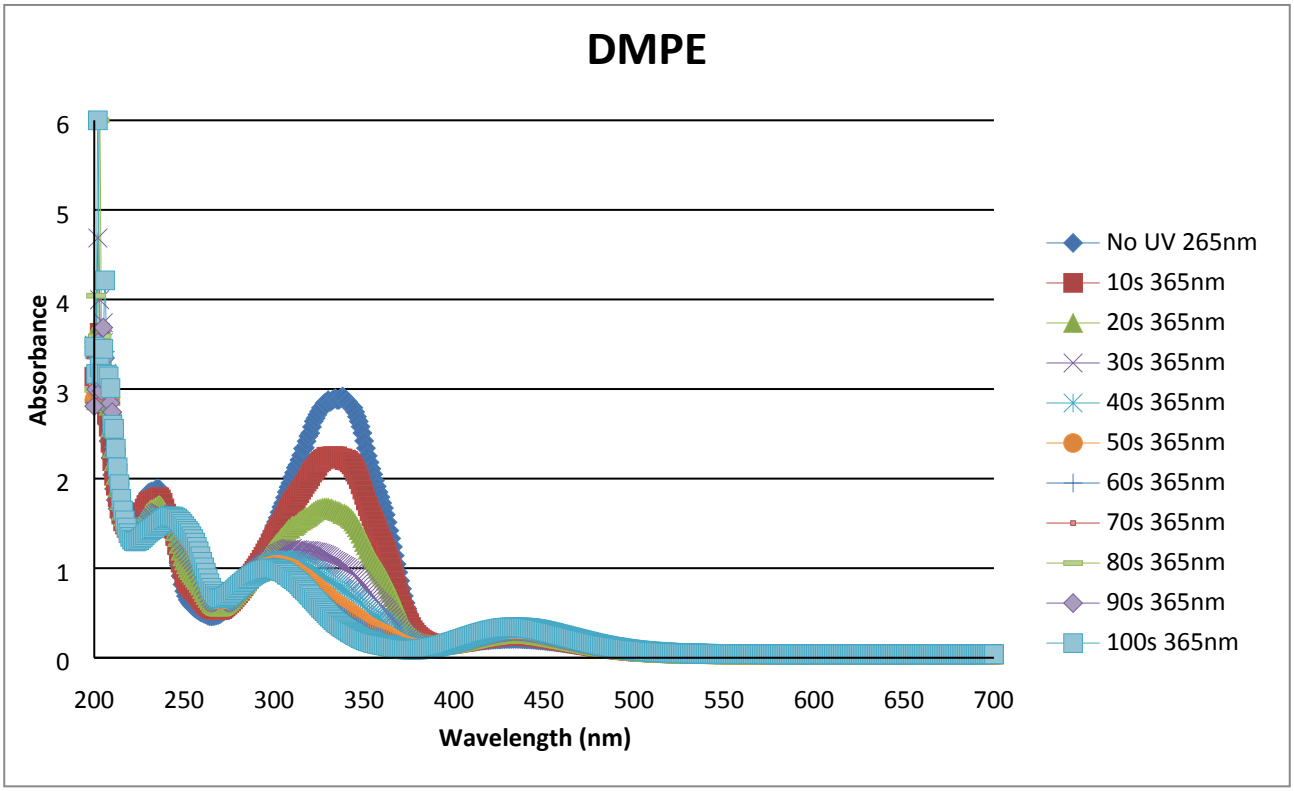
Concentration	1 hour	SDPC	2 hours	SDPC	3 hours	SDPC	6 hours	SDPC	7 hours	SDPC	8 hours	SDPC	24 hours	SDPC	DMPC
0	0		0	0	0	0	0	0	0	0	0	0	0	0	
3	17.93338	0.716649	16.74396	0.348902	19.13897	0.812536	17.70825	0.614096	21.80772	0.289735	21.80772	4.810218	19.57279	0.684009	
6	20.71582	0.168799	26.6636	0.496326	33.16092	1.366511	30.60278	0.699061	29.76916	0.336258	29.76916	6.015039	34.44949	0.432223	
12	44.2906	0.542846	45.95289	0.832539	44.11166	3.173547	44.32006	3.3852	45.22481	0.221268	45.22481	10.25369	45.63838	0.474647	
24	45.23099	0.327837	46.06655	0.563076	45.05948	2.845608	55.59701	1.163454	56.03464	7.102913	56.03464	12.41169	55.61248	0.452511	
Concentration	1 hour	SDPC	2 hours	SDPC	3 hours	SDPC	6 hours	SDPC	7 hours	SDPC	8 hours	SDPC	24 hours	SDPC	DMPE
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	14.97484	1.00713	18.51135	0.295646	37.95992	1.00022	35.26872	2.420112	37.32262	0.139091	36.91272	1.115753	36.17246	0.342522	
6	26.04455	0.389687	22.34141	0.249153	49.12148	0.84771	45.45817	2.643398	47.97191	0.192905	44.81429	2.851731	48.28786	0.479342	
12	37.07745	0.098917	34.83172	0.267046	51.45635	0.849291	53.37777	0.09025	52.92901	0.389225	51.82231	1.462547	52.83867	0.219981	
24	41.40471	0.147033	36.39238	0.204173	54.24827	7.648951	52.61365	0.082065	55.14469	0.503589	52.60731	1.665379	55.1725	0.640164	
Concentration	1 hour	SDPC	2 hours	SDPC	3 hours	SDPC	6 hours	SDPC	7 hours	SDPC	8 hours	SDPC	24 hours	SDPC	DMPG
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	11.41158	1.245486	18.20961	0.432786	22.67374	1.178371	25.53768	1.877078	24.46873	3.17906	23.58157	3.302796	22.6526	1.893696	
6	43.11942	0.206259	41.44116	0.588607	46.05021	2.338623	44.81428	1.55793	39.38854	3.206736	41.79498	1.358347	45.06644	2.392349	
12	51.21005	0.529225	59.8112	0.237337	63.84042	4.98852	59.75319	2.848684	62.15496	1.130414	64.1633	4.442131	63.62464	2.306395	
24	53.03237	0.667163	56.69497	0.759465	68.92871	3.644332	59.31676	0.322742	63.93774	0.41691	67.36215	1.066063	67.62704	2.219026	
Concentration	1 hour	SDPC	2 hours	SDPC	3 hours	SDPC	6 hours	SDPC	7 hours	SDPC	8 hours	SDPC	24 hours	SDPC	DMPS
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	20.48179	0.156664	27.15628	0.593473	31.73532	0.133561	33.46094	0.116429	30.63857	0.532186	31.36137	0.908542	33.01261	2.275782	
6	39.1723	0.119978	49.6975	3.459168	56.30448	0.194222	58.38562	0.037684	65.82152	2.544688	65.44829	1.914212	66.00703	2.564439	
12	63.6098	0.114807	65.92554	0.911167	68.71514	0.294448	76.09634	0.038598	78.45653	3.067228	77.81025	1.105945	77.16199	3.551942	
24	65.12044	0.254856	66.51803	3.545652	71.49974	0.771604	83.42133	0.110259	86.95928	6.629255	85.308	1.82009	89.89901	3.534515	

SD = standard deviation

Representative UV experiments for the conversion of *trans*-4 into *cis*-5 in the presences of liposomes composed of DMPC and DMPE lipids

Following the procedure above [A 0.25 mM stock solution of **4** in *n*-propanol was prepared and added to an equal volume of liposomes in aqueous buffer. A 1.2 ml quartz glass cuvette with an optical path length of 1 cm was filled with 1 ml of the sample solution.], the following identical spectral series were obtained with DMPC (top) and DMPE (bottom) to those produced in the absence of the liposomes.


DMPC
Absorbance
Wavelength (nm)
No UV 365nm
10s 365 nm
20s 365 nm
30s 365 nm
40s 365 nm
50s 365 nm
60s 365 nm
70s 365 nm
80s 365 nm
90s 365 nm
100s 365 nm
110s 365 nm
120s 365 nm
130s 365 nm
140s 365 nm
150s 365 nm
160s 365 nm
170s 365 nm
180s 365 nm


DMPE
Absorbance
Wavelength (nm)
No UV 265nm
10s 365nm
20s 365nm
30s 365nm
40s 365nm
50s 365nm
60s 365nm
70s 365nm
80s 365nm
90s 365nm
100s 365nm