GRAPHICAL ABSTRACT

Synthesis, photophysical characterisation and photostability studies of NIR probes with aliphatic, aromatic and chlorinated terminals in 5- and 9-amino positions of benzo[*a*]phenoxazines

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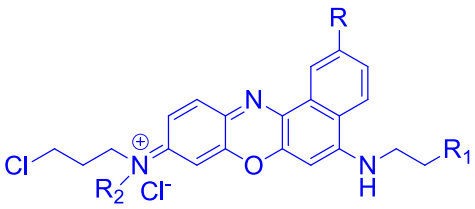
A new series of benzo[*a*]phenoxazinium chlorides possessing mono- or disubstituted amines with 3-chloropropyl groups at the 9-position, isopropyl, cyclohexyl and phenyl groups as terminals at 5-postion was synthesised. Photophysical studies were performed in dry ethanol and aqueous solutions. The photostability of these compounds in different media was also investigated.



R = OH, H
R_1 = $CH(CH_3)_2$, C_6H_{11}, C_6H_5
R_2 = $(CH_2)_3Cl$

4a-d

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Synthesis, photophysical characterisation and photostability studies of NIR probes with aliphatic, aromatic and chlorinated terminals in 5- and 9-amino positions of benzo[*a*]phenoxazines

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HIGHLIGHTS

- A new series of benzo[*a*]phenoxazinium chlorides was synthesised in moderate yields.
- They exhibit high fluorescence quantum yields in ethanol.
- The fluorochromophores displayed more photostability in biological media and membranes, than in aqueous solutions.

Synthesis, photophysical characterisation and photostability studies of NIR probes with aliphatic, aromatic and chlorinated terminals in 5- and 9-amino positions of benzo[*a*]phenoxazines

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Abstract: A new series of benzo[*a*]phenoxazinium chlorides possessing mono- or disubstituted amines with 3-chloropropyl groups at the 9-position, isopropyl, cyclohexyl and phenyl groups as terminals at 5-postion was synthesised. Photophysical studies were performed in dry ethanol and aqueous solutions. The terminals at the 5-amino position were found to influence the acid-base equilibrium. The presence of hydroxyl functionality at 2-position was found to introduce an additional basic form that is the one in equilibrium with the cationic acid form in dry ethanol solutions. The photostability of these compounds in different media was also investigated and a high resistance to photobleaching in model biological membranes was observed. In proteins a moderate of 20% photobleaching occurs in 1h 30min, while in water more than 60% of the compound molecules are photodegraded during the same time interval.

Keywords: Nile Blue; Benzo[*a*]phenoxazines; Near-infrared fluorescent probes; Photostability; Fluorochromophores

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1. Introduction

Synthetic fluorescent labels are indispensable tools for quantitative detection in various fields of modern sciences [1]. These are employed in several separation techniques, and are quite remarkable for their fluorescence detection even to the single molecular level as in case of laser-assisted fluorometry [2]. Fluorescence imaging in longer wavelength region (600-1000 nm) is particularly appealing as biomolecules display less background fluorescence and deeper penetration into the substances [3-6]. Several fluorescent dyes reported in the literature suffer from serious drawbacks such as their cumbersome synthesis, limited availability and biological interference [7]. Among the polycylic oxazines, benzo[*a*]phenoxazinium salts, such as Nile Blue (NB) derivatives are attractive fluorophores due to their sensitivity, photostable nature, high fluorescence quantum yield and in general favourable photophysical properties in the near-infrared spectral region [8]. These compounds are used as fungicide agents [9] and exhibit potent antiprotozoal activities, being even quite effective when attached with substituted phenyl rings to the phenoxazinium skeleton [10]. Benzo[*a*]phenoxazinium chlorides can also function as covalent probes for organic and biological molecules, namely amino acids, proteins, peptides and DNA, as well as in the non-covalent labelling of nucleic acids for use in monitoring changes in protein conformation [11,12], labelling of lysosomes during cell division and apoptosis [13] and imaging tumors that over-express cyclooxygenase-2 [14].

Considering the importance and as a continuation of our current research interest towards the design, synthesis and application of NB derivatives with different substituents [15-25], the present work is focused in the preparation of a new set of benzo[*a*]phenoxazinium chlorides with aliphatic, aromatic and chlorinated terminals in 5- and 9-amino positions of the heteroaromatic system. The photophysical behaviour of these compounds in anhydrous ethanol and in aqueous solutions was investigated along with photostability studies in water and in

biological medium. These measurements are important for evaluating the suitability of the synthesised compounds as biological markers in imaging applications.

2. Experimental section

2.1. Material and instruments

All melting points were measured on a Stuart SMP3 melting point apparatus. TLC analysis was carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer. UV-Vis absorption spectra (200 - 800 nm) were obtained using Shimadzu UV/3101PC spectrophotometer and fluorescence spectra with Spex Fluorolog spectrofluorometer. NMR spectra were obtained on a Varian Unity Plus Spectrometer at an operating frequency of 300 MHz for ^{1}H and 75.4 MHz for ^{13}C or a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C using the solvent peak as internal reference at 25 ºC. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Low and high resolution mass spectrometry analysis were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. Photostability studies were carried out using a xenon arc lamp (OSRAM HBO 200 W) equipped with a bandpass filter centered 600 ± 10 nm (ThorLabs, New Jersey, USA). Commercially available reagents were used as received.

*2.1. Synthetic method for the preparation of precursor **3a***

*2.1.1. 5-(Isopentylamino)naphthalen-2-ol **3a***

To a solution of 5-aminonaphthalen-2-ol (0.477 g, 3.0 mmol) in ethanol (2 mL), 1-bromo-3-methylbutane (0.644 g, 3.3 mmol) was added, and the resulting mixture was refluxed for 5 hours. The progress of reaction was monitored by TLC (dichloromethane/methanol, 9.5:0.5). After completion of the reaction, the solvent was evaporated and the mixture was purified by column chromatography on silica gel using mixture of dichloromethane and methanol (9.5:0.5) as the eluent. Compound **3a** was obtained as colourless solid (0.528 g, 77%). mp 111-113 °C. R_f = 0.55 (dichloromethane/methanol 9.5:0.5). IR (KBr 1%): ν_{max} = 3353, 3062, 2956, 2927, 2869, 1623, 1581, 1531, 1469, 1407, 1368, 1339, 1270, 1219, 1171, 1151, 1027, 996, 957, 862, 813, 795, 772, 743 cm^{-1}. ^1H NMR (CD_3OD, 400 MHz): δ_H 1.01 (d, J = 6.4 Hz, 6H, $NHCH_2CH_2CH(CH_3)_2$, 1.70 (m, 2H, $NHCH_2CH_2CH(CH_3)_2$), 1.76-1.91 (m, 1H, $NHCH_2CH_2CH(CH_3)_2$), 3.28 (t, J = 7.6 Hz, 2H, $NHCH_2CH_2CH(CH_3)_2$), 6.52 (d, J = 7.2 Hz, 1H, 6-H), 7.0-7.06 (m, 2H, 8-H, 3-H), 7.07 (d, J = 2.6 Hz, 1H, 1-H), 7.32 (t, J = 7.6 Hz, 1H, 7-H), 7.71 (d, J = 9.2 Hz, 1H, 4-H) ppm. ^{13}C NMR (CD_3OD, 101.6 MHz): δ_C 22.64 ($NHCH_2CH_2CH(CH_3)_2$), 26.19 ($NHCH_2CH_2CH(CH_3)_2$), 38.41 ($NHCH_2CH_2CH(CH_3)_2$), 42.47 ($NHCH_2CH_2CH(CH_3)_2$), 102.78 (C-6), 110.51 (C-1), 116.01 (C-8 and C-3), 118.74 (C-4a), 121.85 (C-4), 127.49 (C-7), 135.70 (C-8a), 143.73 (C-5), 153.32 (C-2) ppm.

*2.2. General procedure for the synthesis of benzo[a]phenoxazines **4a-d** and **5***

To a cold solution (ice bath) of nitrosophenols **1** or **2** (1.5 mmol) in ethanol (2 mL), 5-aminonaphthalen-2-ol or naphthalen-1-amine derivatives **3a-d** (1.0 mmol) and concentrated hydrochloride acid (40 μL) were added. The mixture was refluxed during the time mentioned below, and monitored by TLC. After evaporation of the solvent and column chromatography purification on silica gel with dichloromethane and dichloromethane/methanol, mixtures of

different polarity, as the eluent, the expected benzo[*a*]phenoxazines **4a-d** and **5** were isolated as blue solids in moderate yields.

2.2.1. 3-Chloro-N-(2-hydroxy-5-(isopentylamino)-9H-benzo[a]phenoxazin-9-ylidene)propan-1-aminium chloride **4a**

The product of the reaction of 5-((3-chloropropyl)amino)-2-nitrosophenol hydrochloride **1** (0.158 g, 0.629 mmol) in ethanol (1 mL) and concentrated hydrochloric acid (0.017 mL) with 5-(isopentylamino)naphthalen-2-ol **3a** (0.086 g; 0.377 mmol) (reflux time 3h) was chromatographed with dichloromethane and dichloromethane/methanol 9.0:1.0 to give compound **4a** as a blue solid (0.166 g, 49%). mp 200-202 °C. R_f = 0.50 (dichloromethane/methanol, 9:1). IR (KBr 1%): ν_{max} 3400, 3200, 2955, 2925, 2854, 1588, 1546, 1464, 1324, 1270, 1221, 1155, 1126, 1035, 817, 720 cm^{-1}. ^1H NMR (CD_3OD, 400 MHz): δ_H 1.09 (d, J = 6.4 Hz, 6 H, $NHCH_2CH_2CH(\mathit{CH_3})_2$, 1.70-1.85 (m, 4 H, $NHCH_2\mathit{CH_2}CH(CH_3)_2$ and $NHCH_2CH_2\mathit{CH}(CH_3)_2$), 2.18 (quint, J = 6.4 Hz, 2 H, $NHCH_2\mathit{CH_2}CH_2Cl$), 3.51 (t, J = 6.8 Hz, 2 H, $NH\mathit{CH_2}CH_2CH(CH_3)_2$), 3.60-3.72 (m, 2 H, $NH\mathit{CH_2}CH_2CH_2Cl$), 3.75 (t, J = 6.4 Hz, 2 H, $NHCH_2CH_2\mathit{CH_2}Cl$), 6.64 (s, 1 H, 6-H), 6.67 (d, J = 1.6 Hz, 1 H, 8-H), 6.99 (d, J = 9.2 Hz, 1 H, 10-H), 7.17 (dd, J = 8.8 and 2.0 Hz, 1 H, 3-H), 7.63 (d, J = 8.8 Hz, 1 H, 11-H), 8.0 (t, J = 3.2 Hz, 1 H, 1-H), 8.11 (d, J = 8.8 Hz, 1 H, 4-H) ppm. ^{13}C NMR (CD_3OD, 101.6 MHz): δ_C 22.89 ($NHCH_2CH_2CH(\mathit{CH_3})_2$), 23.12 ($NHCH_2CH_2CH(\mathit{CH_3})_2$), 27.45 ($NHCH_2\mathit{CH_2}CH(CH_3)_2$), 32.62 ($NHCH_2\mathit{CH_2}CH_2Cl$), 38.57 ($NHCH_2CH_2\mathit{CH}(CH_3)_2$), 41.60 ($NH\mathit{CH_2}CH_2CH(CH_3)_2$), 43.06 ($NH\mathit{CH_2}CH_2CH_2Cl$), 44.21 ($NHCH_2CH_2\mathit{CH_2}Cl$), 93.27 (C-6), 95.85 (C-8), 110.03 (C-1), 117.17 (C-10), 117.29 (Ar-C), 119.51 (Ar-C), 120.12 (C-3), 126.32 (C-4), 130.69 (C-11), 135.07 (2×Ar-C), 135.47 (Ar-C), 153.28 (C-9), 157.38 (Ar-C), 159.45 (C-5), 162.77 (Ar-C) ppm. HRMS: m/z (ESI): Found $[M]^+$: 424.17803; $C_{24}H_{27}ClN_3O_2$ requires $[M]^+$: 424.17863.

2.2.2. 3-Chloro-N-(5-(isopentylamino)-9H-benzo[a]phenoxazin-9-ylidene)propan-1-aminium chloride **4b**

The product of the reaction of 5-((3-chloropropyl)amino)-2-nitrosophenol hydrochloride **1** (0.114 g, 0.454 mmol) in ethanol (1 mL) and concentrated hydrochloric acid (0.012 mL) with *N*-isopentylnaphthalen-1-amine **3b** (0.058 g; 0.60 mmol) (reflux time 8 h) was chromatographed with dichloromethane and dichloromethane/methanol 9.0:1.0 to give compound **4b** as a blue solid (0.148 g, 66%). mp 168-170 °C. R_f = 0.58 (dichloromethane/methanol, 9.5:0.5). IR (KBr 1%): ν_{max} 3440, 2958, 1641, 1590, 1548, 1497, 1454, 1433, 1321, 1282, 1186, 1158, 1122, 999, 773 cm^{-1}. ^1H NMR (CD_3OD, 400 MHz): δ_H 1.08 (d, J = 6.4 Hz, 6 H, $NHCH_2CH_2CH(\mathit{CH_3})_2$), 1.76-1.89 (m, 3 H, $NHCH_2\mathit{CH_2}CH(CH_3)_2$ and $NHCH_2CH_2\mathit{CH}(CH_3)_2$), 2.17-2.26 (m, 2 H, $NHCH_2\mathit{CH_2}CH_2Cl$), 3.61 (t, J = 6.8 Hz, 2 H, $NH\mathit{CH_2}CH_2CH(CH_3)_2$), 3.74-3.83 (m, 4 H, $NHCH_2CH_2\mathit{CH_2}Cl$ and $NH\mathit{CH_2}CH_2CH_2Cl$), 6.90 (s, 1 H, 8-H), 7.03 (s, 1 H, 6-H), 7.15 (d, J = 8.4 Hz, 1 H, 10-H), 7.82-7.92 (m, 2 H, 11-H and 3-H), 7.97 (t, J = 7.6 Hz, 1 H, 2-H), 8.39 (d, J = 8.4 Hz, 1 H, 4-H), 8.98 (d, J = 7.6 Hz, 1 H, 1-H) ppm. ^{13}C NMR (CD_3OD, 100.6 MHz): δ_C 22.82 ($NHCH_2CH_2CH(\mathit{CH_3})_2$), 22.91 ($NHCH_2CH_2CH(\mathit{CH_3})_2$), 27.42 ($NHCH_2\mathit{CH_2}CH(CH_3)_2$), 32.63 ($NHCH_2\mathit{CH_2}CH_2Cl$), 38.35 ($NHCH_2CH_2\mathit{CH}(CH_3)_2$), 41.64 ($NH\mathit{CH_2}CH_2CH(CH_3)_2$), 42.95 ($NH\mathit{CH_2}CH_2CH_2Cl$), 44.30 ($NHCH_2CH_2\mathit{CH_2}Cl$), 92.39 (C-6), 95.86 (C-8), 123.84 (C-4), 125.03 (C-10), 125.76 (C-1), 131.11 (C-3), 132.02 (Ar-C), 132.79 (2×Ar-C), 133.09 (C-2), 134.02 (C-11), 135.39 (2×Ar-C), 153.53 (C-9), 158.0 (Ar-C), 159.63 (C-5) ppm. HRMS: m/z (ESI): Found $[M]^+$: 408.18308; $C_{24}H_{27}ClN_3O$ requires $[M]^+$: 408.18372.

2.2.3. 3-Chloro-N-(5-((2-cyclohexylethyl)amino)-9H-benzo[a]phenoxazin-9-ylidene)propan-1-aminium chloride **4c**

The product of the reaction of 5-((3-chloropropyl)amino)-2-nitrosophenol hydrochloride **1** (0.136 g, 0.541 mmol) in ethanol (1 mL) and concentrated hydrochloric acid

(0.013 mL) with *N*-(2-cyclohexylethyl)naphthalen-1-amine **3c** (0.082 g; 0.325 mmol) (reflux time 3h) was chromatographed with dichloromethane and dichloromethane/methanol 9.0:1.0 to give compound **4c** as a blue solid (0.118 g, 52%). mp 248-250 °C. R_f = 0.43 (dichloromethane/methanol, 9:1). IR (KBr 1%): ν_{max} 3444, 2926, 1640, 1588, 1548, 1493, 1448, 1431, 1317, 1268, 1202, 1157, 1122, 998, 819, 773 cm^{-1}. ^1H NMR δ_H (CD_3OD, 400 MHz), 1.0-1.60 (3×m, 9H, CH Cy), 1.62-1.80 (m, 2H, $NHCH_2\mathit{CH_2}C_6H_{11}$), 1.81-1.90 (m, 2H, CH Cy), 2.10-2.25 (m, 2H, $NHCH_2\mathit{CH_2}CH_2Cl$), 3.40-3.60 (m, 2H, $NHCH_2CH_2\mathit{CH_2}Cl$), 3.62-3.85 (m, 4H, $NH\mathit{CH_2}CH_2CH_2Cl$ and $NH\mathit{CH_2}CH_2C_6H_{11}$), 6.82 (d, *J* = 2.0 Hz, 1 H, 8-H), 6.92 (s, 1 H, 6-H), 7.11 (dd, *J* = 9.0 and 1.6 Hz, 1 H, 10-H), 7.77-7.86 (m, 2H, 11-H and 3-H), 7.93 (t, *J* = 8.0 Hz, 1 H, 2-H), 8.34 (d, *J* = 8.0 Hz, 1 H, 4-H), 8.90 (d, *J* = 8.0 Hz, 1 H, 1-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 27.33 (CH Cy), 27.41 (CH Cy), 27.55 (*CH₂* Cy), 32.61 (*CH₂* Cy), 33.08 ($NHCH_2\mathit{CH_2}CH_2Cl$), 34.31 (*CH₂* Cy), 36.98 ($NHCH_2\mathit{CH_2}C_6H_{11}$ and *CH* Cy), 41.68 ($NHCH_2CH_2\mathit{CH_2}Cl$), 43.00 ($NH\mathit{CH_2}CH_2CH_2Cl$), 43.88 ($NH\mathit{CH_2}CH_2C_6H_{11}$), 94.34 (C-6), 95.60 (C-8), 123.84 (Ar-C), 124.25 (Ar-C), 124.92 (C-4), 125.67 (C-1), 126.10 (Ar-C), 131.07 (C-3), 131.83 (Ar-C), 131.91 (Ar-C), 132.62 (C-2), 133.04 (Ar-C), 133.69 (Ar-C), 133.96 (C-11), 135.20 (Ar-C), 153.29 (Ar-C), 157.93 (C-9), 159.40 (C-5) ppm. HRMS: m/z (ESI): Found $[M]^+$: 448.21449; $C_{27}H_{31}ClN_3O$ requires $[M]^+$: 448.21502.

2.2.4. 3-Chloro-N-(5-(phenethylamino)-9H-benzo[a]phenoxazin-9-ylidene)propan-1-aminium **4d**

The product of the reaction of 5-((3-chloropropyl)amino)-2-nitrosophenol hydrochloride **1** (0.116 g, 0.462 mmol) in ethanol (1 mL) and concentrated hydrochloric acid (0.011 mL) with *N*-phenethylnaphthalen-1-amine **3d** (0.068 g; 0.277 mmol) (reflux time 3h) was chromatographed with dichloromethane and dichloromethane/methanol 9.0:1.0 to give compound **4c** as a blue solid (0.092 g, 40%). mp 220-222 °C. R_f = 0.52 (dichloromethane/methanol, 9:1). IR (KBr 1%): ν_{max} 3420, 2925, 2855, 1639, 1583, 1543,

1492, 1450, 1432, 1313, 1287, 1259, 1172, 1154, 1124, 994, 828, 773, 738 cm^{-1}. ^1H NMR δ_H (CD_3OD, 400 MHz), 1.36 (m, 2 H, $NHCH_2CH_2CH_2Cl$), 3.17 (t, *J* = 7.2 Hz, 2 H, $NHCH_2CH_2C_6H_5$), 3.58 (t, *J* = 6.8 Hz, 2 H, $NHCH_2CH_2C_6H_5$), 3.76 (t, *J* = 6.4 Hz, 2 H, $NHCH_2CH_2CH_2Cl$), 3.99 (t, *J* = 7.2 Hz, 2 H, $NHCH_2CH_2CH_2Cl$), 6.82 (s, 1 H, H-8), 6.90 (s, 1 H, 6-H), 7.12 (dd, *J* = 9.2 and 2.0 Hz, 1 H, 10-H), 7.20-7.24 (m, 1 H, Ar-H), 7.28-7.36 (m, 4 H, 4×Ar-H), 7.79-7.89 (m, 2 H, 11-H and 3-H), 7.92 (t, *J* = 7.6 Hz, 1 H, 2-H), 8.29 (d, *J* = 8,0 Hz, 1 H, 4-H), 8.90 (d, *J* = 7.2 Hz, 1 H, 1-H) ppm. ^{13}C NMR δ_C (CD_3OD, 100.6 MHz), 32.61 ($NHCH_2CH_2CH_2Cl$), 35.86 ($NHCH_2CH_2C_6H_5$), 41.68 ($NHCH_2CH_2C_6H_5$), 42.97 ($NH_2CH_2CH_2CH_2Cl$), 47.12 ($NHCH_2CH_2CH_2Cl$), 94.53 (C-6), 95.53 (C-8), 123.75 (C-4), 124.86 (C-10), 125.68 (C-1), 129.82 (Ar-C), 129.86 (Ar-C), 130.09 (2×Ar-C), 130.77 (Ar-C), 131.05 (C-3), 132.08 (Ar-C), 132.40 (1×Ar-C), 132.65 (Ar-C), 133.03 (C-2), 133.59 (Ar-C), 134.04 (C-11), 135.10 (Ar-C), 139.39 (Ar-C), 153.20 (C-9), 158.03 (Ar-C), 159.51 (C-5) ppm. HRMS: m/z (ESI): Found $[M]^+$: 442.16734; $C_{27}H_{25}ClN_3O$ requires $[M]^+$: 442.16807.

2.2.5. 3-Chloro-N-(3-chloropropyl)-N-(5-(isopentylamino)-9H-benzo[a]phenoxazin-9-ylidene) propan-1-aminium chloride **5**

The product of the reaction of 5-(bis(3-chloropropyl)amino)-2-nitrosophenol hydrochloride **2** (0.113 g, 0.345 mmol) in ethanol (1 mL) and concentrated hydrochloric acid (0.008 mL) with *N*-isopentylnaphthalen-1-amine **3b** (0.044 g, 0.60 mmol) (reflux time 3 h) was chromatographed with dichloromethane and dichloromethane/methanol 9.0:1.0 to give compound **5** as a blue solid (0.133 g, 58%). mp 221-223 °C. R_f = 0.14 (dichloromethane/methanol, 9.5:0.5). FTIR (KBr 1%): ν_{max} 2956, 1639, 1587, 1546, 1491, 1456, 1434, 1330, 1279, 1220, 1178, 1159, 1123, 999, 775 cm^{-1}. ^1H NMR (CD_3OD, 400 MHz): δ_H 1.08 (d, *J* = 6.0 Hz, 6 H, $NHCH_2CH_2CH(CH_3)_2$), 1.75-1.90 (m, 3 H, $NHCH_2CH_2CH(CH_3)_2$ and $NHCH_2CH_2CH(CH_3)_2$), 2.19-2.30 (m, 4 H, $N(CH_2CH_2CH_2Cl)_2$), 3.73-3.90 (m, 10 H, $NHCH_2CH_2CH(CH_3)_2$, $N(CH_2CH_2CH_2Cl)_2$) and $N(CH_2CH_2CH_2Cl)_2$), 7.04 (s, 2 H, 8-H and 6-

H), 7.34 (d, *J* = 8.8 Hz, 1 H, 10-H), 7.87 (t, *J* = 7.2 Hz, 1 H, 3-H), 7.92-8.02 (m, 2 H, 2-H and 11-H), 8.40 (d, *J* = 8.0 Hz, 1 H, 1-H), 8.96 (d, *J* = 8.0 Hz, 1 H, 4-H) ppm. ^{13}C NMR (CD_3OD, 100.6 MHz): δ_C 22.82 ($NHCH_2CH_2CH(\mathit{CH_3})_2$), 27.41 ($NHCH_2\mathit{CH_2}CH(CH_3)_2$), 31.36 ($N(CH_2\mathit{CH_2}CH_2Cl)_2$), 38.39 ($NHCH_2CH_2\mathit{CH}(CH_3)_2$), 43.15 ($NH\mathit{CH_2}CH_2CH(CH_3)_2$), 44.49 ($N(\mathit{CH_2}CH_2CH_2Cl)_2$), 44.30 ($N(CH_2CH_2\mathit{CH_2}Cl)_2$), 94.88 (C-6), 97.60 (C-8), 116.20 (C-10), 124.01 (C-1), 125.11 (Ar-C), 125.91 (C-4), 131.17 (Ar-C), 131.40 (C-3), 132.71 (Ar-C), 133.34 (C-2), 133.99 (C-11), 136.60 (Ar-C), 149.44 (Ar-C), 153.76 (C-9), 155.62 (Ar-C), 160.11 (C-5) ppm. HRMS: m/z (ESI): Found $[M]^+$: 484.19154; $C_{27}H_{32}Cl_2N_3O$ requires $[M]^+$: 484.19169.

*2.3. Typical procedure for the photostablity studies with benzo[a]phenoxazinium chlorides **4b** and **5**.*

Solutions of the compounds **4b** and **5** (C = 1×10^{-5} M) was prepared in ethanol, water, buffered solutions of BSA and membranes (soya lecithin) in a 5 mL flask. Then a volume of 2 mL was pipetted into a quartz cuvette and placed directly in the path of incident light with an irradiance of 10 $mWcm^{-2}$ (Xenon arc lamp, OSRAM HBO 200 W) equipped with a band pass filter centered on 600 ± 10 nm (ThorLabs, New Jersey, USA). The absorbance was recorded from the initial (t = 0 min) to the maximum (t = 110 min) irradiation with various time intervals in between.

3. Results and discussion

3.1. Synthetic methods

The synthesis of benzo[*a*]phenoxazinium chlorides **4a-d** and **5** was initiated with the preparation of *N*-alkylated derivatives of 3-aminophenol, 5-aminonaphthalen-2-ol and

naphthalen-1-amine. The reaction of 3-aminophenol with 1-bromo-3-chloropropane followed by silica gel column chromatography purification afforded 3-(3-chloropropylamino)phenol and 3-(bis(3-chloropropyl)amino)phenol, respectively. The nitrosation of these compounds was carried out with sodium nitrite in hydrochloric acidic solution to obtain 5-(3-chloropropylamino)-2-nitrosophenol hydrochloride **1** and 5-(bis(3-chloropropyl)amino)-2-nitrosophenol hydrochloride **2** [15].

The 5-(isopentylamino)naphthalen-2-ol **3a** was obtained by the *N*-alkylation reaction of 5-aminonaphthalen-2-ol with 1-bromo-3-methylbutane in ethanol under reflux conditions. Similarly, the precursors *N*-isopentyl-naphthalen-1-amine **3b**, *N*-(2-cyclohexylethyl)naphthalen-1-amine **3c** and *N*-(phenethyl)naphthalen-1-amine **3d** were synthesised by the reaction of naphthalen-1-amine with 1-bromo-3-methylbutane, (2-bromoethyl)cyclohexane and (2-bromoethyl)benzene in ethanol following the procedure described [16]. After purification by silica gel column chromatography, intermediates **3a-d** were isolated and spectroscopic data were in accordance with the expected structures.

The reaction of 3-(3-chloropropylamino)phenol hydrochloride **1** or 3-(bis(3-chloropropyl)amino)phenol hydrochloride **2** with *N*-alkylated derivatives of 5-aminonaphthalen-2-ol and naphthalen-1-amine **3a-d** in an acidic medium afforded the corresponding benzo[*a*]phenoxazinium chlorides **4a-d** and **5**, respectively (Scheme 1). Thus, reaction of nitrosophenol **1** with intermediates **3a-d** in ethanol, in the presence of concentrated hydrochloric acid, and after silica gel column chromatography purification gave the benzo[*a*]phenoxazinium chlorides **4a-d**, possessing the isopropyl, cyclohexyl and phenyl terminals at 5-position, monosubstituted with the 3-chloropropyl group at the amine of 9-position. Similarly, the nitrosophenol **2** reacted with precursors **3a**, producing the cationic dye **5**, but with the amino of 9-position of the polycyclic system disubstituted with 3-chloropropyl groups. All these compounds were obtained as blue solids in moderate yields and were fully

characterised by high resolution mass spectrometry, IR and NMR (^{1}H and ^{13}C) spectroscopy (Table 1).

<Scheme 1>

3.2. Physical studies

Electronic absorption and fluorescence spectra of solutions of fluorophores **4a-d** and **5** in absolute ethanol and water were measured at various concentrations. Summarised data of this study is presented in Table 1.

<Table 1>

The fluorescence quantum yields Φ_F were evaluated using oxazine 1 in ethanol as standard (Φ_F = 0.11) [26] at 575 nm or 470 nm excitation.

Figure 1 shows absorption and fluorescence spectra, at 470 nm excitation, of the compounds in water (panels A and B), ethanol (panels C and D) and basified (addition of a small amount of tetraethylammonium hydroxide) or acidified (addition of a small amount of trifluoroacetic acid) ethanol (panels E and F).

Previous studies of benzo[*a*]phenoxazinium chloride derivatives [27,28] showed that the absorption spectra in ethanol media is composed of two contributions. The acidic form (BzH^+) and other, that is the neutral form (Bz), which is ~100 nm blue shifted. This is clearly seen in Figure 1C and from the distinct behaviour in basified or acidified ethanol (Figure 1E).

<Figure 1>

In ethanol media, compound **5** which is di-alkylated at 9-aminoposition, shows a higher fraction of basic form. Globally, when comparing with similar compounds without a bulky group at the terminal of 5-amino position [15], it is possible to conclude that the bulky groups used in this study, significantly increase the fraction of basic form. The absorption spectra of compound **4a**, possessing OH functionality at position 2, shows a very distinct behaviour. It can be seen that in basified ethanol the usual neutral form appears (Figure 1E) with a slight blue shift whereas in normal ethanol what would be the contribution from that form appears deviated more than 50 nm in comparison with the other compounds (Figure 1C). This deviation can be understood by considering an additional form that corresponds to deprotonation of the OH group with possible interaction, by H-bond, with solvent molecules. In that basic form the π-conjugation system would not be significantly changed resulting in a less blue shifted absorption than when the deprotonation occurs at the 5-position. As in previous studies [15, 17], compound **5** shows that double alkylation at 9-position results in red shift of the acid form absorption.

In water media, as observed in our previous studies for similar compounds [15, 27, 28], non-emissive H-aggregates of the acidic form are observed as a blue shoulder around 600 nm or as a flat absorption band (Figure 1A). The aggregation fraction increases with compound concentration resulting in corresponding spectral changes. This has been seen in previous studies on this type of compounds and also occurs for these compounds **4a-d** and **5** (data not shown).

Variations in the absorption spectra of these compounds in anhydrous ethanol with its concentration corresponds to changing ratios of the acid and basic forms of the compounds as can be concluded from Figure 2. Typical variations are shown in Figure 2B being that, as stated previously, compound **4a** behaves differently (Figure 2A).

<Figure 2>

With the exception of compound **4a**, the experimental spectra were fitted to a weighted sum of the spectra obtained in acidified and basified ethanol. For compound **4a**, the spectrum of the basic form was defined by a sum of two Gaussian functions. Figures 2A and 2B shows the result of this fitting procedure together with the used spectra of acid and basic forms.

Considering that acid and basic forms are at chemical equilibrium, the following equation can be derived for the fraction of basic form, f_b:

$$\frac{(1-f_b)}{f_b}K_a - \frac{f_b C_T}{2} + \sqrt{\left(\frac{f_b C_T}{2}\right)^2 + K_{EtOH}} = 0 \qquad (1)$$

where C_T is the compound total concentration, K_a, is the dissociation constant and K_{EtOH} is ethanol self-dissociation constant. This model was previously and successfully applied in compounds of the same family [17, 28]. In Figure 2C it is shown that the fitting of the fraction of basic form, obtained from spectral deconvolution procedure, with eq. (1). In Table 2 the resulting equilibrium constants are reported for an ethanol self-dissociation constant of $7.97\times10^{-13}M^2$, obtained globally for all data in Figure 2C. As in a previous study [17] the value of the ionic product corresponding to the ethanol self-dissociation equilibrium is much higher than expected [29] and, as stated therein, can probably be accounted from specific interactions of the studied compound with ethanol solvating molecules. For compounds without substitution at the 2-position and monosubstitued at the 9-amino position, the obtained K_a values follows the order cyclohexyl < benzyl < isopropyl which seems related to decreasing order of steric hindrance in the terminal at the 5-amino position. Disubstitution at the 9-amino position has already been reported to increase the dissociation equilibrium constant [17].

<Table 2>

The fluorescence spectra in ethanol (Figures 1D and 1F) shows that, with the exception of compound **4a**, it is possible to completely displace the acid-base equilibrium into the basic form by the addition of TEAH. As already reported for similar compounds [27, 28], the basic form has a broad fluorescence band centered at ~610 nm. The calculated quantum yields are located between 0.019 and 0.027 (Table 1). For compound **4a** a very low and broad emission is observed indicating that the hydroxyl group at the 2-position has a profound effect on the emission of the neutral basic form of this family of compounds.

In order to fully displace the acid-base equilibrium into the acid form TFA was added to the ethanolic solution. However, two types of emission bands were observed (Figure 1F) upon excitation with 470 nm wavelength. From previous studies [15,27,28], the band centered between 644-666 nm with a high quantum yield corresponds to the acid form emission. The other, near 540 nm, has recently been attributed to a tautomeric form (proton displacement resulting in localization of the positive charge in one of the 5- or 9-amino positions) with the corresponding excitation spectrum showing absorption at ~510 nm and ~480 nm [15]. These bands are not seen in the absorption spectra in acidified ethanol (Figure 1E) so that the impact of the tautomerization process on the quantification of the acid-base equilibria (Table 2) should be very low. Only in compounds **4a** and **5** the tautomeric forms are absent so that the tabulated values of the quantum yields of these compounds are 38% and 44% respectively, which should be close to the actual values. For the other compounds, the calculated quantum yields with an excitation wavelength of 575 nm at which only the acid form emission is recorded, increases from ethanol to acidified ethanol. This is expected as the fraction of basic form which has low quantum yield reduces practically to zero upon acidification with TFA (Figure 1E). Those quantum yields lies between 45% and 47% and expected to be very close to the actual values as the tautomeric forms were not detected in the absorption spectra in acidified ethanol.

In water, only small amounts of tautomer emission was observed for compounds **4b** and **4a** (Figure 1B). Considering that no basic form is observed in the corresponding absorption

spectrum (Figure 1A) and also the existing H-aggregates are non-emissive, the obtained values of fluorescence quantum yield are the actual values for the acid form multiplied by its fractional contribution to the absorption at the excitation wavelength. Assuming similar fluorescence quantum yield values to those obtained in acidified ethanol it is possible to estimate the fraction of absorption due to aggregates at 575 nm as being 76%, 78%, 66%, 61% and 78%, respectively, for compound **4a-d** and **5**. As H-aggregates absorption occurs towards the blue of the monomer, these high aggregation levels explains the observed blue shift of the absorption spectra from ethanol to water whereas the opposite trend is observed in emission data (Table 1). The same reasoning explains the higher values of Stokes shifts observed in water when compared in ethanol.

In order to utilize the studied compounds either as dyes, fluorescent probes or biomarkers in fluorescence imaging it is important to evaluate their photostability in various conditions. Figure 3 shows the results of variations of absorption spectra of compounds **4b** and **5** in water, BSA protein and soybean lecithin lipid membranes, upon irradiation with light at 600 nm obtained from a 200 W Xe arc-lamp filtered by an interference filter with ~20% transmission at 600 nm and 10 nm fwhm.

<Figure 3>

The photostability depends on the medium where the compound resides and increases in the order membranes > BSA > water. Compound **5**, which is disubstituted at the 9-amino position, is marginally more photostable than compound **4b** (Figure 3E). In water, the compounds shows less photostability reaching 59% and 61% photodegradation for compounds **5** and **4b** respectively, after 90min irradiation. The H-aggregates are more photolabile as evident from the absorption spectra in water which show a red shift with an increase of the irradiation time (Figure 3A and 3B). In the studied models of biological media (Figure 3C and

3D), no significant spectral changes are observed. Interestingly, the fraction of aggregation is higher in BSA than in membranes and is much higher for compound **4b** than for compound **5**. In BSA medium, the basic form is clearly seen at ~510 nm only for compound **5** (Figure 3C). Both previous features and the slightly higher stability of compound **5** are probably a consequence of di-alkylation at 9-amino position. In BSA the photodegradation reaches only 19% for compounds **5** and **4b**, respectively, after 50 min of irradiation. In soybean lecithin membranes, the photostability is very high reaching only 5% for compound **4b** upon 110 min of irradiation, and no photodegradation was observed for compound **5** within 120 min of irradiation. Considering that light intensity used in the photostability experiment was 1.65 mW with a beam diameter of 8mm, 120 min exposure corresponds to 59.4s of irradiation with a 594 nm 2 mW HeNe laser with 0.8 mm beam diameter. Assuming 1 sec acquisition time per pixel it would be possible to image a 8 mm circular zone with 800 μm spatial resolution for more than 50 times, without significant photobleaching of compound **5** when incorporated in biological membranes. The spatial resolution can be improved by focusing the laser, but the field of view as well as the time per pixel needs to be reduced in order to not exceed the maximum number of photons per molecule during the acquisition of each picture. Considering the used experimental conditions in the photostability experiments, 120 min irradiation time corresponds to 5.95×10^{-5} mol of photons. Each molecule within the irradiated cylinder, with 8 mm diameter and 1 cm length, was thus exposed, in average, to 1.2×10^{4} photons per molecule without suffering appreciable photodegradation.

4. Conclusion

Four new benzo[*a*]phenoxazinium chlorides possessing mono- or disubstituted amines with 3-chloropropyl groups at the 9-position, isopropyl, cyclohexyl and phenyl groups as terminals at

5-aminoposition of the polycyclic system were efficiently synthesised. The photophysical behaviour of these compounds was evaluated in dry ethanol and water. Acid base equilibria in ethanolic medium were found to be influenced by the presence of bulky groups in the 5-amino position. A hydroxyl group at the 2-position introduced an additional deprotonation site giving different photophysical behaviour for that type of compounds. The dialkylation at the 9-amino position mainly induced a red shift on the absorption and emission spectra and originated a higher percentage of the neutral basic form. Photostability studies showed that dialkylation at the 9-amino position induces a slightly higher resistance to photobleaching, H-aggregates showed more lability than monomers in water. Overall, based on the results obtianed, namely the absorption and fluorescence emission above 600 nm (600-700 nm) and the good photostability particularly in biological media, the benzo[*a*]phenoxazinium chlorides described are potential interesting aleternative probes for bioapplications.

Acknowledgments

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CAPTIONS

Scheme 1. Synthesis of benzo[*a*]phenoxazinium chlorides **4a-d** and **5**.

Table 1. Yields and photophysical data of compounds **4a-d** and **5** in dry ethanol, acidified ethanol with TFA, basified ethanol with TEAH and in aqueous solution ($C = 1\times10^{-5}$ M).

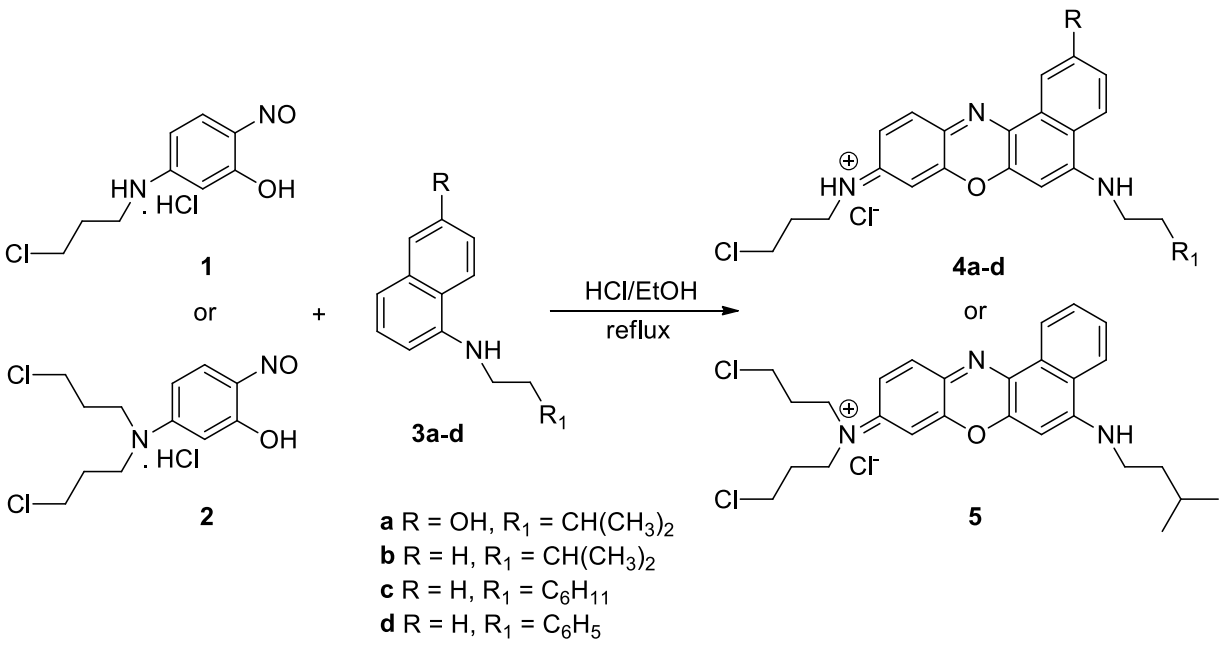
Table 2. Equilibrium dissociation constants of compounds **4a-d** and **5** in dried ethanol.

Figure 1. Normalised absorption/emission spectra of compounds **4a-d** and **5** ($C = 1\times10^{-5}$ M) in water (A/B), ethanol (C/D) and either acidified or basified ethanol with respectively TFA or TEAH (E/F).

Figure 2. Absorption spectra of compounds **4a** (A) and **5** (B) in dried ethanol media at concentrations from 7×10^{-6} M to 26×10^{-6} M (solid lines - experimental; grey lines - fitted spectrum); the dotted line is the spectrum of 1×10^{-5} M concentration in acidified ethanol; the dashed line is the spectrum correspondent to the basic form that, in the case of compound **4a** (A) an additional spectrum represented by dash-dot-dot line is the fitted spectrum of the basic form that is involved in the acid-base equilibria. Panel C shows the obtained fraction of basic form of compounds **4a-d** and **5** in anhydrous ethanol media.

Figure 3. Photostability experiments with 600nm irradiation light. Normalised absorption spectra of compounds **4b** and **5** in water (A, B), in BSA proteins (C) and in soybean lecithin membranes (D). Panel E shows the remaining fraction of molecules as a function of irradiation time with the inset representing the initial absorption of each studied system.

Scheme 1



a R = OH, R_1 = $CH(CH_3)_2$
b R = H, R_1 = $CH(CH_3)_2$
c R = H, R_1 = C_6H_{11}
d R = H, R_1 = C_6H_5

Table 1

Compound		**4a**	**4b**	**4c**	**4d**	**5**
Yield (%)		66	49	52	40	58
Solvent						
Ethanol	λ_{abs} (nm)	616	620	621	625	630
	ε (10^4 $M^{-1}cm^{-1}$)	2.5	0.8	1.1	1.6	0.4
	λ_{em}//fwhm(nm) [a]	644//55	645//50	645//50	645//52	666//56
	Φ_F [a]	0.11	0.36	0.38	0.45	0.40
	Δ (nm) [a]	28	25	24	20	36
Ethanol acidified with TFA	λ_{abs}//fwhm(nm)	617//75	619//72	620//69	623//66	628//74
	ε (10^4 $M^{-1}cm^{-1}$)	5.2	3.7	4.9	5.2	4.3
	λ_{em}//fwhm(nm) [a]	650//69	650//61	650//62	650//64	667//64
	Φ_F [a]	0.38	0.46	0.47	0.45	0.44
	Δ (nm) [a]	33	31	30	27	39
Ethanol basified with TEAH	λ_{abs}//fwhm(nm)	482//94	493//96	493//92	497//91	499//88
	ε (10^4 $M^{-1}cm^{-1}$)	2.6	1.7	2.1	2.5	1.9
	λ_{em}//fwhm(nm) [b]	622	611//97	610//98	611//100	611//110
	Φ_F [b]	0.0004	0.021	0.027	0.027	0.019
	Δ (nm) [b]	140	118	117	114	112
Water	λ_{abs}//fwhm(nm)	604//166	600//116	612//106	618//94	631//95
	ε (10^4 $M^{-1}cm^{-1}$)	1.03	1.48	1.43	1.99	1.64
	λ_{em}//fwhm(nm) [a]	650//57	653//56	653//58	656//60	677//62
	Φ_F [a]	0.089	0.10	0.16	0.18	0.098
	Δ (nm) [a]	46	53	41	38	46

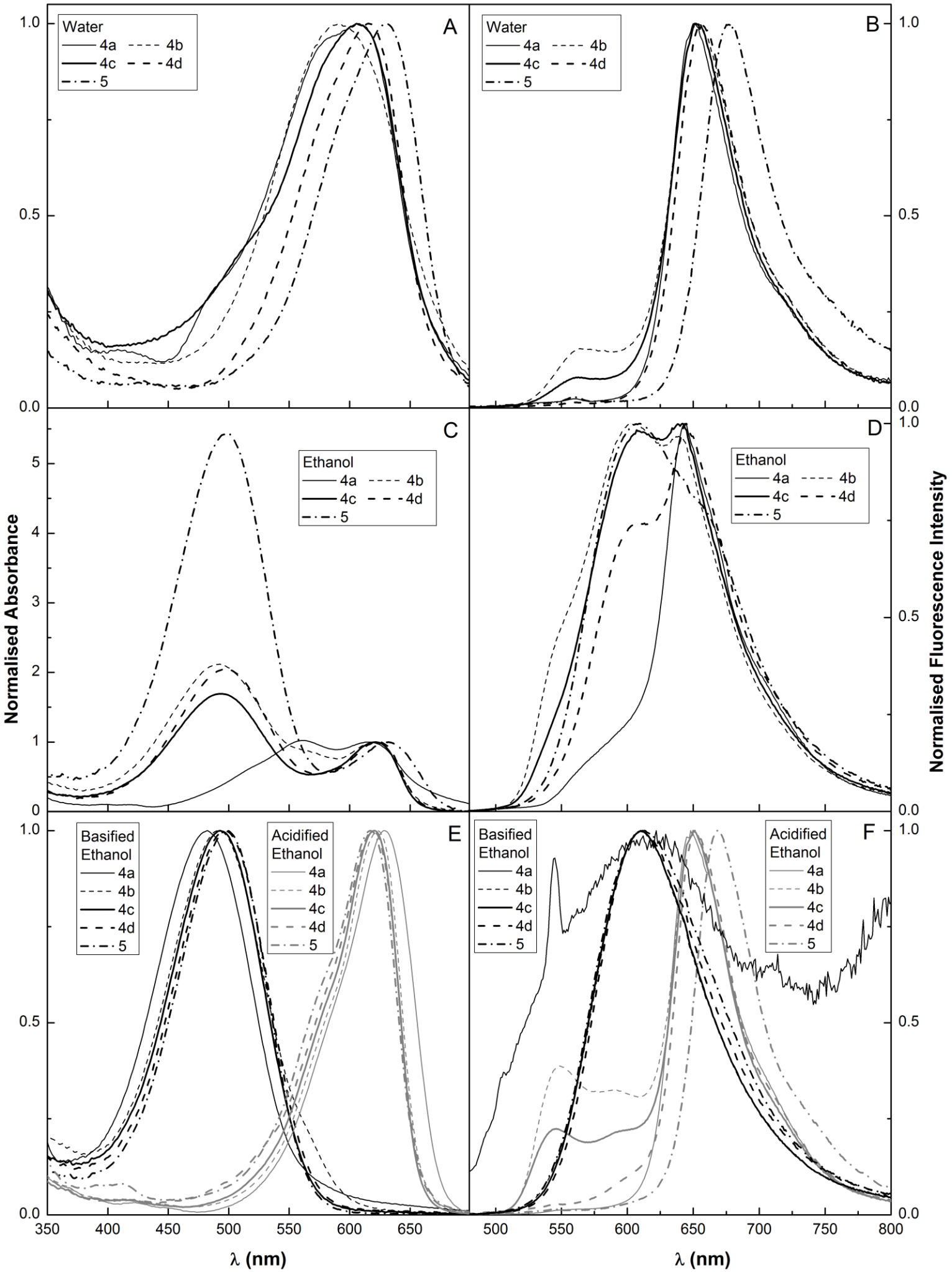
(a) Emission spectra obtained with excitation at 575 nm; (b) Emission spectra obtained with excitation at 470 nm

Table 2

Compound	**4a**[(*)]	**4b**	**4c**	**4d**	**5**
K_a (10^{-5} M)	1.1	4.5	2.7	3.2	7.2

(*) Obtained by fitting the absorption spectrum of the basic form

Figure 1


A
Water
4a
4b
4c
4d
5
B
Water
4a
4b
4c
4d
5
C
Ethanol
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4b
4c
4d
5
D
Ethanol
4a
4b
4c
4d
5
E
Basified Ethanol
4a
4b
4c
4d
5
Acidified Ethanol
4a
4b
4c
4d
5
F
Basified Ethanol
4a
4b
4c
4d
5
Acidified Ethanol
4a
4b
4c
4d
5
Normalised Absorbance
Normalised Fluorescence Intensity
λ (nm)
λ (nm)

Figure 2

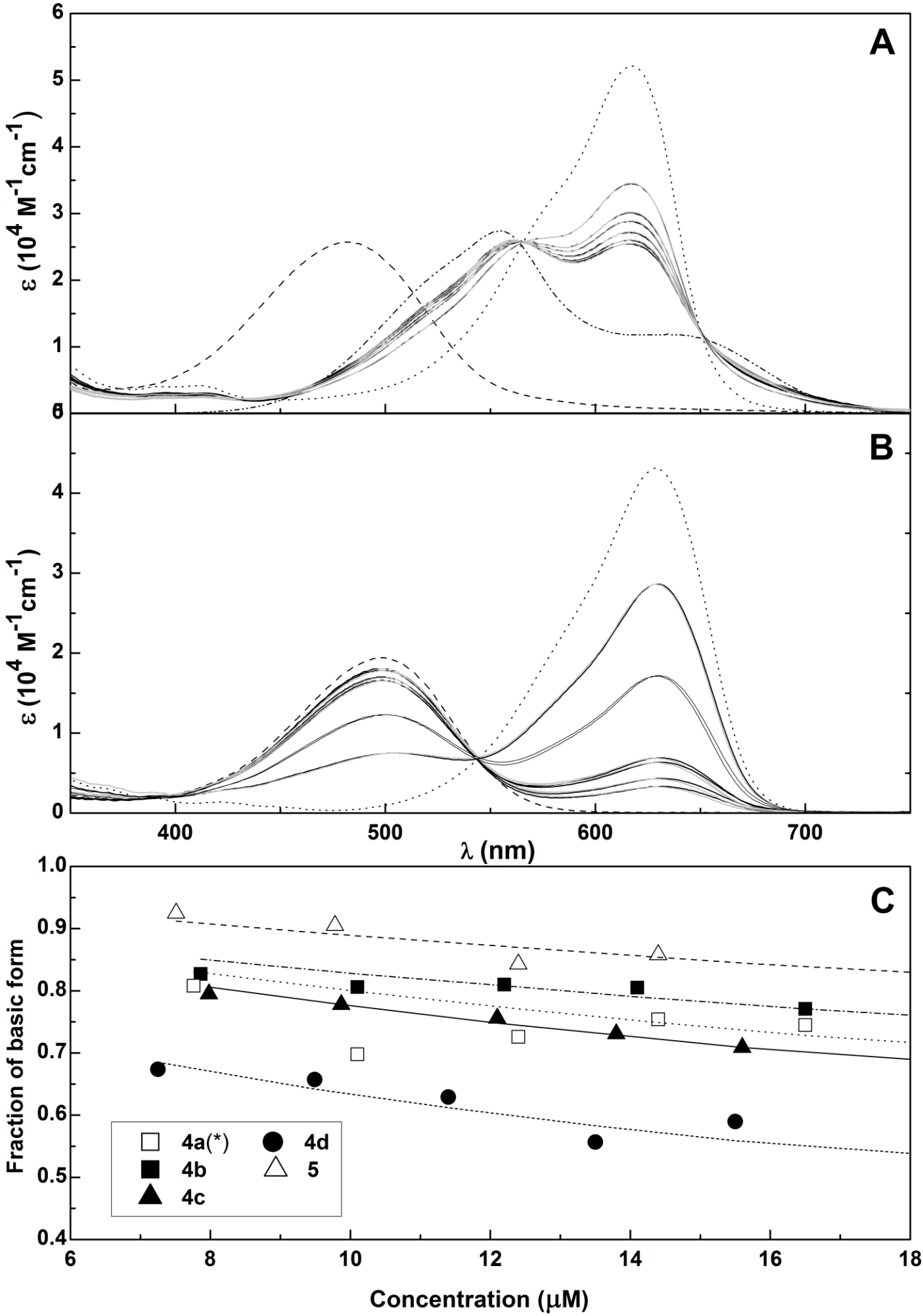
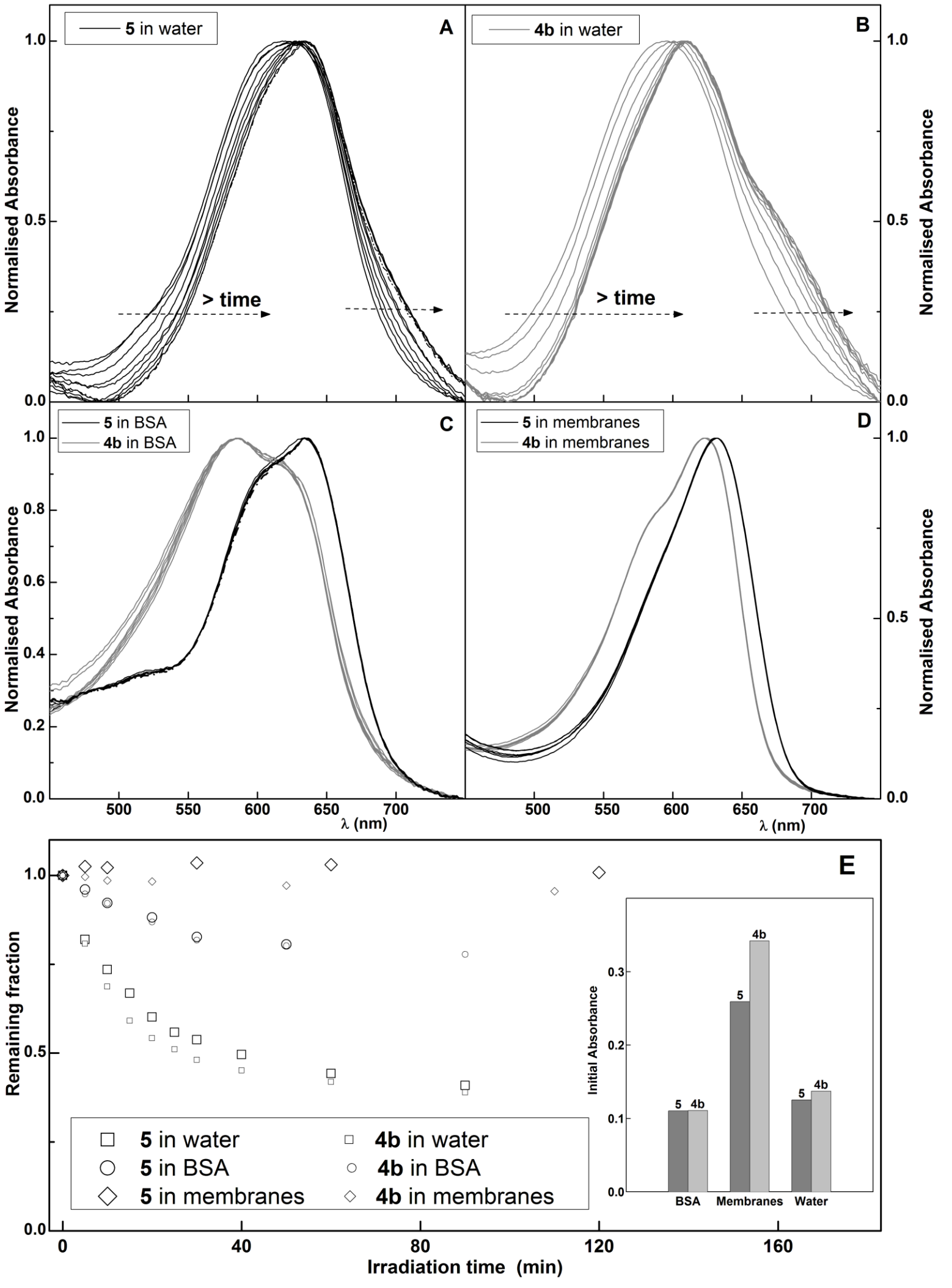

A
B
C
ε (10^4 M^-1 cm^-1)
λ (nm)
Fraction of basic form
Concentration (μM)
4a(*)
4b
4c
4d
5

Figure 3


A
5 in water
> time
Normalised Absorbance
B
4b in water
> time
Normalised Absorbance
C
5 in BSA
4b in BSA
Normalised Absorbance
λ (nm)
D
5 in membranes
4b in membranes
Normalised Absorbance
λ (nm)
E
Remaining fraction
Irradiation time (min)
5 in water
4b in water
5 in BSA
4b in BSA
5 in membranes
4b in membranes
Initial Absorbance
5
4b
BSA
Membranes
Water