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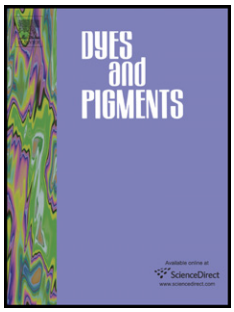

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Novel Nile Blue derivatives as fluorescent probes for DNA

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Highlights

- New NIR cationic dyes based on benzo[*a*]phenoxazinium chlorides were synthesised.
- The dyes showed intercalative behaviour, aggregation or groove binding in DNA.
- They display promising capabilities as DNA colored markers in electrophoresis assays.

GRAPHICAL ABSTRACT

Novel Nile Blue derivatives as fluorescent probes for DNA

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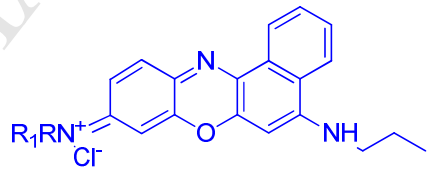
A new set of NIR fluorescent benzo[*a*]phenoxazinium chlorides was synthesised. Photophysical studies and agarose gel electrophoresis experiments with these Nile Blue derivatives revealed their potential as DNA markers and probes.



R and / or $R_1 = (CH_2)_nCH_3$, $n = 1, 7, 11$

Novel Nile Blue derivatives as fluorescent probes for DNA

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Abstract: Cationic dyes based on benzo[*a*]phenoxazinium chlorides either mono- or disubstituted with ethyl, octyl and dodecyl groups at the amino side chain at the 9-position of the tetracyclic ring, and possessing a propyl group at the 5-amino position were synthesised, characterised and used in photophysical studies with DNA and in agarose gel electrophoresis assays. Photophysical results showed that compounds with diethyl and octyl substituents intercalated DNA above a phosphate dye ratio of 10. All compounds associated with DNA as evidenced by their use as colored markers of migrating DNA bands in agarose gel electrophoresis experiments.

Keywords: Nile Blue; Benzo[*a*]phenoxazinium dyes; DNA probes; Near-infrared probes; Cationic dyes

1. Introduction

In the recent years, labeling of biological matter such as amino acids, proteins and cells with synthetic fluorescent reagents has become an indispensible tool in bioanalytical sciences [1,2]. Among the fluorescent reagents, phenoxazine core based molecules are extensively studied due to their profound applications in terms of extra stability and brightness [3-5]. Nile Blue (NB), a phenoxazine dye, contains a planar rigid aromatic system which enables the intercalation into the non-planar interior of the DNA helix [6]. Due to this intercalation binding [7], NB and derivatives were used as fluorescent labels in therapeutic [8-10] and scientific areas [11-14].

NB in comparison with other conventional intercalators proved to be superior mainly due to low toxicity and good sensitivity for DNA quantification purposes [6]. In addition, NB was used as marker for the detection of DNA in electrophoresis gels [15]. The intercalation of

small organic molecules or ligands with nucleic acids is mainly associated with π–stacking [16]. Despite the importance and application of NB as molecular fluorescent probe, limited research has been done with new derivatives of the benzo[*a*]phenoxazinium dye family. In continuation of our earlier observations, combined with synthetic conciseness [17-20] and evaluation of the potential of this family of fluorophores as DNA labels [21,22], we synthesized new benzo[*a*]phenoxazinium chlorides either mono- or disubstituted with different alkyl groups at the amino side chain at the 9-position of the tetracyclic ring, and having the propyl group at the 5-amino position. The photophysical behavior of these new Nile Blue derivatives in the presence of DNA was evaluated and also electrophoresis experiments in agarose gel were performed in order to assess their potential as DNA markers.

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. 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 analyses were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. Absorption spectra (200-800 nm) were obtained using Shimadzu UV/3101PC spectrophotometer and fluorescence spectra with a Spex Fluorolog spectrofluorometer. Commercially available reagents and solvents were used without further purification.

*2.2. Synthetic methods for the preparation of precursors **2-4***

2.2.1. 5-(Dioctylamino)-2-nitrosophenol hydrochloride **2**.

To an ice-cold solution of 3-(dioctylamino)phenol (0.333 g, 1 mmol) in ethanol (1 mL), concentrated hydrochloric acid (0.21 mL) was added and stirred during 5 min. The solution of sodium nitrite (0.076 g, 1.1 mmol) in water (0.1 mL) was then added drop-wise within an interval of 20-25 min. The resulting mixture was stirred for 3 h and monitored by TLC (dichloromethane/methanol, 9.5:0.5 and 9:1 vol/vol). After evaporation of the reaction, compound **1** was obtained as an orange oily solid (0.399 g) and was used in the following step without any purification.

3-(Octylamino)phenol and 3-(dioctylamino)phenol

To a solution of 3-aminophenol (1.0 g, 9.10 mmol) in ethanol (6 mL), 1-bromooctane (1.86 mL, 10.8 mmol) was added and the reaction mixture was refluxed for 18.5 h. The progress of the reaction was monitored by TLC which showed the presence of two products. After purification by column chromatography on silica gel with dichloromethane and dichloromethane/methanol (mixtures of increasing polarity), as the eluent, 3-(octylamino)phenol was obtained as a brown oil (1.018 g, 51%). TLC (dichloromethane): R_f = 0.21. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.84 (t, J = 7.2 Hz, 3 H, NH$(CH_2)_7CH_3$), 1.10-1.22 (s, 10 H, NHCH$_2$CH$_2$*(CH$_2$)$_5$*CH$_3$), 1.72-1.85 (m, 2 H, NHCH$_2$*CH$_2$*(CH$_2$)$_5$CH$_3$), 3.26 (t, J = 8.4 Hz, 2 H, NH*CH$_2$*CH$_2$(CH$_2$)$_5$CH$_3$), 6.84 (dd, J = 8.2 and 2.0 Hz, 1 H, 4-H), 6.99 (dd, J = 8.2 and 1.2 Hz, 1 H, 6-H), 7.14 (t, J = 8.4 Hz, 1 H, 5-H), 7.20 (t, J = 2.0 Hz, 1 H, 2-H), 7.83 (br s, 2 H, NH, OH). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 14.00 (NH$(CH_2)_7CH_3$), 22.50 (CH_2), 25.46 (NHCH$_2$*CH$_2$*(CH$_2$)$_5$CH$_3$), 26.50 (CH_2), 28.88 (CH_2), 29.00 (CH_2), 31.60 (CH_2), 52.60 (NH*CH$_2$*CH$_2$(CH$_2$)$_5$CH$_3$), 109.77 (C-2), 113.79 (C-6), 116.99 (C-4), 130.98 (C-5), 135.54 (C-3), 157.61 (C-1). IR (Neat): ν_{max} = 3261, 2956, 2927, 2857, 2778, 2467, 1621, 1601, 1576, 1503, 1485, 1468, 1427, 1378, 1316, 1289, 1223, 1152, 1082, 1000, 959, 861, 782, 737, 688, 666 cm^{-1}. HRMS: m/z (TOF EI): calcd. for $C_{14}H_{23}NO$ [M^+]: 221.1780; found: 221.1784.

In addition to the 3-(octylamino)phenol, 3-(dioctylamino)phenol was also obtained as a brown oil (0.712 g, 24%). TLC (dichloromethane): R_f = 0.56. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.90 (t, J = 6.9 Hz, 6 H, (N$((CH_2)_7CH_3)_2$), 1.28 (br s, 20 H, N(CH$_2$CH$_2$(*CH$_2$*)$_5$CH$_3$)$_2$), 1.58 (br s, 4 H, N(CH$_2$*CH$_2$*(CH$_2$)$_5$CH$_3$)$_2$), 3.24 (t, J = 7.8 Hz, 4 H, N(*CH$_2$*CH$_2$(CH$_2$)$_5$CH$_3$)$_2$), 6.38 (br s, 3 H, 2-H, 4-H, 6-H), 7.09 (t, J = 7.8 Hz, 1 H, 5-H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 14.04 (2×CH_3), 22.58 (2×CH_2), 26.75 N(CH$_2$*CH$_2$*(CH$_2$)$_5$CH$_3$)$_2$), 27.00 (2×CH_2), 29.24 (2×CH_2), 29.35 (2×CH_2), 31.75 (2×CH_2), 52.30 (N(*CH$_2$*CH$_2$(CH$_2$)$_5$CH$_3$)$_2$), 100.82 (C-2), 105.93 (C-6, C-4), 130.15 (C-5), 148.18 (C-3), 157.01 (C-1). IR (Neat): ν_{max} = 3299, 2955, 2926, 2855, 2726,

1619, 1579, 1504, 1467, 1400, 1370, 1276, 1241, 1221, 1167, 1142, 1114, 1013, 999, 832, 821, 753, 723, 688 cm^{-1}. HRMS: m/z (TOF EI): calcd. for $C_{22}H_{39}NO$ [M^+]: 333.3032; found 333.3034.

*2.2.2. 2-Nitroso-5-(octylamino)phenol hydrochloride **3***

Starting from 3-(octylamino)phenol (0.336 g, 1.52 mmol) in ethanol (2 mL) and concentrated hydrochloric acid (0.40 mL), using sodium nitrite (0.115 g, 1.67 mmol) in water (0.1 mL), and following the same procedure as described before for the preparation of **1**, compound **2** was obtained as an orange oily solid (0.436 g) and was used in the following step without any purification.

*2.2.3. 5-(Dodecylamino)-2-nitrosophenol hydrochloride **4***

Starting from 3-(dodecylamino)phenol (0.416 g, 1.5 mmol) in ethanol (2 mL) and concentrated hydrochloric acid (0.40 mL), using sodium nitrite (0.114 g, 1.65 mmol) in water (0.1 mL), and following the same procedure as described before for the preparation of **1**, compound **2** was obtained as an orange oily solid (0.515 g) and was used in the following step without any purification.

3-(Dodecylamino)phenol

To a solution of 3-aminophenol (2.0 g, 0.183 mmol) in ethanol (6 mL), 1-bromododecane (6.5 mL, 0.273 mmol) was added and the reaction mixture was refluxed for 10 h. After purification by column chromatography on silica gel with dichloromethane and dichloromethane/methanol, mixtures of increasing polarity, as the eluent, 3-(dodecylamino)phenol was obtained as a brown solid (2.931g, 58%). TLC (dichloromethane): R_f = 0.17. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.87 (t, J = 7.2 Hz, 3 H, $NH(CH_2)_{11}\mathit{CH_3}$), 1.10-1.35 (m, 18 H, $NHCH_2CH_2(\mathit{CH_2})_9CH_3$), 1.70-1.90 (m, 2 H $NHCH_2\mathit{CH_2}(CH_2)_9CH_3$), 3.26 (t, J = 8.4 Hz, 2 H, $NH\mathit{CH_2}CH_2(CH_2)_9CH_3$), 6.82 (dd, J = 8.4 and 1.6 Hz, 1 H, 4-H), 6.98 (dd, J = 8.0 and 1.2 Hz, 1 H, 6-H), 7.14 (t, J = 8.0 Hz, 1H, 5-H), 7.22 (br s, 1 H, 2-H), 8.4 (br s, 2 H, NH, OH). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 14.07 ($NH(CH_2)_{11}\mathit{CH_3}$), 22.64 ($CH_2$), 25.55 ($NHCH_2\mathit{CH_2}(CH_2)_9CH_3$), 26.57 ($CH_2$), 29.00 ($CH_2$), 29.31 ($CH_2$), 29.43 ($CH_2$), 29.49 ($CH_2$), 29.58 ($CH_2$), 29.59 ($CH_2$), 31.87 ($CH_2$), 52.54 ($NH\mathit{CH_2}CH_2(CH_2)_9CH_3$), 109.69 (C-2), 113.72 (C-6), 116.92 (C-4), 130.97 (C-5), 135.67 (C-3), 157.65 (C-1). FTIR (KBr 1%): ν_{max} 3282, 2918, 2851, 2730, 2630, 2600, 2414, 1619, 1571, 1499, 1484, 1468, 1417, 1382, 1338, 1318, 1288, 1250, 1237, 1216, 1176, 1153, 1133, 1087 cm^{-1}. HRMS: m/z (EI): calcd. for $C_{18}H_{31}NO$ [M^+]: 277.2406; found: 277.2410.

In addition to the 3-(dodecylamino)phenol, 3-(didodecylamino)phenol was also obtained as a purple solid (1.598g, 20%). TLC (dichloromethane): R_f = 0.53. ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 0.85 (t, J = 7.2 Hz, 6 H, N((CH$_2$)$_{11}$*CH$_3$*)$_2$), 1.10-1.40 (m, 36 H, N(CH$_2$CH$_2$*(CH$_2$)*$_9$CH$_3$)$_2$), 1.92 (br s, 4 H, N(CH$_2$*CH$_2$*(CH$_2$)$_9$CH$_3$)$_2$), 3.29 (br s, 2 H, N*CH$_2$*CH$_2$(CH$_2$)$_9$CH$_3$), 3.47 (br s, 2 H, N*CH$_2$*CH$_2$(CH$_2$)$_9$CH$_3$), 7.16 (br s, 2 H, 4-H, 6-H), 7.28 (t, J = 8.0 Hz, 1 H, 5-H), 7.45 (br s, 1 H, 2-H), 8.68 (br s, 1 H, OH). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ= 14.03 ((N(CH$_2$)$_{11}$*CH$_3$*)$_2$), 22.59 (2×CH$_2$), 24.77 (N(CH$_2$*CH$_2$*(CH$_2$)$_9$CH$_3$)$_2$), 26.46 (2×CH$_2$), 28.93 (2×CH$_2$), 29.24 (2×CH$_2$), 29.34 (2×CH$_2$), 29.38 (2×CH$_2$), 29.49 (2×CH$_2$), 29.52 (2×CH$_2$), 31.82 (2×CH$_2$), 59.11 (N(*CH$_2$*CH$_2$(CH$_2$)$_9$CH$_3$)$_2$), 108.91 (C-2), 113.21 (C-6), 118.19 (C-4), 130.96 (C-5), 138.05 (C-3), 158.89 (C-1). FTIR (KBr 1%): ν_{max} 3175, 3046, 2916, 2850, 2636, 2605, 2537, 1613, 1511, 1490, 1471, 1446, 1418, 1406, 1373, 1355, 1318, 1284, 1226, 1212, 1204, 1181, 1141, 1126, 1091, 1044 cm^{-1}. HRMS: m/z (TOF EI): calcd. for $C_{30}H_{55}NO$ [M^+]: 445.4284; found: 445.4283.

*2.3. General procedure for the synthesis of benzo[a]phenoxazines **6-9***

To a cold solution (ice bath) of 2-nitrosophenol hydrochloride (1.5 mmol) in methanol (3 mL), *N*-propylnaphthalen-1-amine **5** (1 mmol) and concentrated hydrochloride acid (40 µL) were added. The mixture was refluxed until the completion of the reaction and was monitored by TLC with chloroform and mixtures of chloroform/methanol, as the eluent. The solvent was evaporated and the crude mixture was purified by column chromatography on silica gel with chloroform and chloroform/methanol, mixtures of increasing polarity, as the eluent. The expected benzo[*a*]phenoxazines **6**-**9** were isolated as blue solids in good yields.

*2.3.1. N-Octyl-N-(5-(propylamino)-9H-benzo[a]phenoxazin-9-ylidene)octan-1-aminium chloride **7***

The product of the reaction of 5-(dioctylamino)-2-nitrosophenol hydrochloride **2** (0.399 g, 1.0 mmol) with *N*-propylnaphthalen-1-amine **5** (0.124 g, 0.67 mmol) (reflux time 14 h) was chromatographed with chloroform and chloroform/methanol (mixtures of increasing polarity), as the eluent, to give compound **7** (0.439 g, 78%). Mp = 150-152°C. TLC (chloroform/methanol, 9.4:0.6): R_f = 0.20. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.80-1.0 (m, 6 H, NHCH$_2$CH$_2$*CH$_3$*, N(CH$_2$)$_7$*CH$_3$*), 1.11 (br s, 3 H, N(CH$_2$)$_7$*CH$_3$*), 1.10-1.50 (br s, 20 H, N(CH$_2$CH$_2$(*CH$_2$*)$_5$CH$_3$)$_2$), 1.72 (br s, 4 H, N(CH$_2$*CH$_2$*(CH$_2$)$_5$CH$_3$)$_2$), 1.98 (br s, 2 H, NHCH$_2$*CH$_2$*CH$_3$), 3.50 (br s, 4 H, N(*CH$_2$*CH$_2$(CH$_2$)$_5$CH$_3$)$_2$), 3.86 (br s, 2 H, NH*CH$_2$*CH$_2$CH$_3$), 6.61 (br s, 2 H, 6-H, 8-H), 6.95 (br s, 1 H, 10-H), 7.70-7.90 (m, 3 H, 2-H, 11-H, 3-H), 8.86 (br

s, 1 H, 4-H), 9.45 (br s, 1 H, 1-H), 11.90 (br s, 1 H, NH). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 11.58 ($N(CH_2)_7\mathit{CH_3}$), 14.00 ($NHCH_2CH_2\mathit{CH_3}$, $N(CH_2)_7\mathit{CH_3}$), 22.37 ($2\times CH_2$), 22.54 ($NHCH_2\mathit{CH_2}CH_3$), 26.97 ($2\times CH_2$), 27.36 ($N(CH_2\mathit{CH_2}(CH_2)_5CH_3)_2$), 29.15 ($2\times CH_2$), 29.29 ($CH_2$), 29.60 ($CH_2$), 31.68 ($2\times CH_2$), 46.53 ($NH\mathit{CH_2}CH_2CH_3$), 52.01 ($N(\mathit{CH_2}CH_2(CH_2)_5CH_3)_2$), 93.62 (C-6), 95.98 (C-8), 113.46 (C-10), 124.07 (C-4), 124.32 (Ar-C), 126.65 (C-1), 128.27 (Ar-C), 130.74 (Ar-C), 130.94 (C-3), 132.12 (C-2), 132.40 (C-11), 135.97 (Ar-C), 147.34 (Ar-C), 151.63 (Ar-C), 153.00 (C-9), 158.92 (C-5). IR (KBr 1%) ν_{max} = 3446, 3154, 3012, 2954, 2924, 2853, 1641, 1586, 1545, 1494, 1480, 1455, 1433, 1330, 1289, 1273, 1236, 1213, 1180, 1162, 1146, 1120, 1053, 1012, 1001, 972, 923, 915, 900, 886, 870, 855 cm^{-1}. HRMS: m/z (EI): calcd. for $C_{35}H_{50}N_3O$ [M^++1]: 528.39426; found: 528.39484.

*2.3.2. N-(5-(Propylamino)-9H-benzo[a]phenoxazin-9-ylidene)octan-1-aminium chloride **8***

The product of the reaction of 2-nitroso-5-(octylamino)phenol hydrochloride **3** (0.436 g, 1.52 mmol) with *N*-propylnaphthalen-1-amine **5** (0.140 g; 0.75 mmol) (reflux time 8 h) was chromatographed with chloroform and chloroform/methanol (mixtures of increasing polarity), as the eluent, to give compound **8** (0.240 g, 35%). Mp = 165-168°C. TLC (chloroform/methanol, 9:1): R_f = 0.45; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.86 (t, J = 6.8 Hz, 3 H, $NHCH_2CH_2\mathit{CH_3}$), 1.07 (t, J = 6.8 Hz, 3 H, $NH(CH_2)_7\mathit{CH_3}$), 1.20-1.43 (m, 10 H, $NHCH_2CH_2(\mathit{CH_2})_5CH_3$), 1.74 (br s, 2 H, $NHCH_2\mathit{CH_2}(CH_2)_5CH_3$), 1.83-2.0 (m, 2 H, $NHCH_2\mathit{CH_2}CH_3$), 3.11 (br s, 2 H, $NH\mathit{CH_2}CH_2(CH_2)_5CH_3$), 3.53 (br s, 2 H, $NH\mathit{CH_2}CH_2CH_3$), 6.22 (s, 2 H, 6-H, 8-H), 7.15 (br s, 1 H, 10-H), 7.35 (d, J = 8.4 Hz, 1 H, 11-H), 7.68-7.86 (m, 2 H, 2-H, 3-H), 8.43 (br s, 1 H, NH), 8.59 (d, J = 7.6 Hz, 1 H, 4-H), 8.83 (br s, 1 H, 1-H), 10.35 (br s, 1 H, NH). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 11.57 ($NH(CH_2)_7\mathit{CH_3}$), 14.04 ($NHCH_2CH_2\mathit{CH_3}$), 21.98 ($NHCH_2\mathit{CH_2}CH_3$), 22.58 (CH_2), 27.15 (CH_2), 28.51 (CH_2), 29.19 ($NHCH_2\mathit{CH_2}(CH_2)_5CH_3$), 29.25 ($CH_2$), 31.76 ($CH_2$), 43.90 ($NH\mathit{CH_2}CH_2(CH_2)_5CH_3$), 46.24 ($NH\mathit{CH_2}CH_2CH_3$), 92.34 (C-6), 94.13 (C-8), 118.34 (C-10), 123.35 (C-4), 123.79 (C-1), 125.04 (C-3), 129.51 (Ar-C), 130.22 (Ar-C), 130.67 (C-2), 131.35 (C-11), 131.82 (Ar-C), 132.42 (Ar-C), 147.87 (Ar-C), 150.52 (Ar-C), 156.11 (C-9), 157.14 (C-5). IR (KBr 1%) ν_{max} = 3431, 3173, 2927, 2853, 1640, 1588, 1547, 1494, 1454, 1432, 1384, 1322, 1272, 1254, 1234, 1181, 1160, 1147, 1118, 1000, 948, 854, 830 cm^{-1}. HRMS (FAB) m/z: calcd for $C_{27}H_{34}N_3O$ [M^+]: 416.26964; found: 416.26896.

*2.3.3. N-(5-(Propylamino)-9H-benzo[a]phenoxazin-9-ylidene)dodecan-1-aminium chloride **9***

The product of the reaction of 5-(dodecylamino)-2-nitrosophenol hydrochloride **4** (0.515 g, 1.5 mmol) with *N*-propylnaphthalen-1-amine **5** (0.139 g; 0.75 mmol) (reflux time 10 h) was

chromatographed with chloroform and chloroform/methanol (mixtures of increasing polarity), as the eluent, to give compound **9** (0.229 g, 30%). Mp = 151-153 °C. R_f = 0.46 (chloroform/methanol, 9:1). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.88 (t, J = 7.2 Hz, 3 H, $NH(CH_2)_{11}\mathit{CH_3}$), 1.10 (br s, 3 H, $NHCH_2CH_2\mathit{CH_3}$), 1.26-1.43 (m, 18 H, $NHCH_2CH_2(\mathit{CH_2})_9CH_3$), 1.76 (br s, 2 H, $NHCH_2\mathit{CH_2}(CH_2)_9CH_3$), 1.95 (br s, 2 H, $NHCH_2\mathit{CH_2}CH_3$), 3.06 (br s, 2 H, $NH\mathit{CH_2}CH_2(CH_2)_9CH_3$), 3.54 (br s, 2 H, $NH\mathit{CH_2}CH_2CH_3$), 6.21 (s, 2 H, 8-H, 6-H), 7.13 (br s, 1 H, 10-H), 7.36 (br s, 1 H, 11-H), 7.77 (br s, 2 H, 3-H, 2-H), 8.23 (br s, 1 H, NH), 8.67 (br s, 1 H, 4-H), 8.86 (br s, 1 H, 1-H), 10.37 (br s, 1 H, NH). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 11.67 ($NHCH_2CH_2\mathit{CH_3}$), 14.09 ($NH(CH_2)_{11}\mathit{CH_3}$), 22.07 ($2\times CH_2$), 22.65 ($NHCH_2\mathit{CH_2}CH_3$), 27.21 ($2\times CH_2$), 28.53 ($NHCH_2\mathit{CH_2}(CH_2)_9CH_3$), 29.32 ($CH_2$), 29.57 ($CH_2$), 29.61 ($2\times CH_2$), 29.63 ($CH_2$), 31.88 ($CH_2$), 44.00 ($NH\mathit{CH_2}CH_2CH_3$), 52.01 ($NH\mathit{CH_2}CH_2(CH_2)_9CH_3$), 92.60 (C-6), 94.32 (C-8), 123.39 (C-10), 123.88 (C-4), 125.42 (Ar-C), 129.62 (C-1), 130.22 ($2\times$Ar-C), 130.72 (C-3), 131.47 (C-2), 131.75 (C-11), 132.52 (Ar-C), 147.81 (Ar-C), 150.50 (Ar-C), 155.93 (C-9), 157.17 (C-5). IR (KBr 1%): ν_{max} 3159, 2923, 2850, 1643, 1585, 1548, 1519, 1494, 1450, 1434, 1416, 1384, 1317, 1303, 1286, 1271, 1254, 1235, 1186, 1171, 1160, 1145, 1119, 1097, 1056, 1014, 999, 975, 951, 855, 828 cm^{-1}. HRMS: m/z (FAB): calcd. for $C_{31}H_{42}N_3O$ [M^+]: 472.33224; found: 472.33105.

2.4. Preparation of DNA mother solution

Natural double-stranded salmon sperm DNA was obtained from Invitrogen as a 10 mg/mL aqueous solution with an average size of 2000 bp. Mother solutions of salmon sperm DNA were made in 10 mM Tris-HCl buffer (pH = 7.4), with 1 mM EDTA. The purity of DNA was checked by monitoring the absorption spectrum and the ratio of the absorbance at 260 and 280 nm, A_{260}/A_{280} = 1.95 (good-quality DNA has an A_{260}/A_{280} ratio higher than 1.8) [24]. The DNA concentration in number of bases (or phosphate groups, [P]) were determined from the molar extinction coefficient ε = 6600 M^{-1}cm^{-1} at 260 nm [25]. Appropriate amounts of 10^{-3} M ethanolic solutions of the compounds were added to DNA solutions at desired concentrations. The solutions were left at least 24 h to stabilize.

2.5. Electrophoresis

The electrophoretic apparatus was a BIO RAD Power Pac 300 run at an electric potential difference of 100V. The distance between the electrodes in the electrophoretic chamber was 20 cm such that the applied electrical field was 5V/cm.

2.5.1. Typical procedure for electrophoresis experiments

Solutions of benzo[*a*]phenoxazinium chlorides **6**-**9** (D) in DNA with molar ratio P/D = 5 were prepared using 12 µL of salmon sperm DNA in Tris-buffer solution, [P] = 0.0025 M, 6 µL of 1 mM solution of compounds **6**-**9** in ethanol, 10 µL of glycerine/water 1:1 as sample loading buffer and 32 µL of TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). A volume of 40 µL of the resulting solution was loaded in wells obtained after gelification of a 0.3% solution of agarose gel in TAE buffer. The amount of DNA per well was 6 µg. In regular time intervals the electrophoresis run was interrupted and images captured with a digital camera.

3. Results and discussion

3.1. Synthetic methods

Condensation reaction of 5-(diethylamino-2-nitroso)phenol hydrochloride **1** [18], 5-(dioctylamino)-2-nitrosophenol hydrochloride **2**, 2-nitroso-5-(octylamino)phenol hydrochloride **3** and 5-(dodecylamino)-2-nitrosophenol hydrochloride **4** with *N*-propylnaphthalen-1-amine **5** [22], in acid medium under reflux in methanol followed by purification through column chromatography on silica gel, gave benzo[*a*]phenoxazinium chlorides **6** (49%), **7** (35%), **8** (78%) and **9** (30%), respectively (Scheme 1). 2-Nitrosophenol hydrochlorides **1**-**4** were obtained by nitrosation of 3-(diethylamino)phenol, 3-(dioctylamino)phenol, 3-(octylamino)phenol and 3-(dodecylamino)phenol, with sodium nitrite and hydrochloric acid, in a mixture of ethanol-water as the solvent [23]. The latter precursors resulted from the alkylation of 3-aminophenol with the appropriate bromoalkane, namely bromoethane, 1-bromooctane and 1-bromododecane.

<Scheme 1>

3.2. Physical studies

Electronic absorption and emission spectra of 2×10^{-6} M solutions in degassed absolute ethanol and in physiological simulated conditions (pH 7.4, Tris-HCl buffer solutions) were measured for the synthesised benzo[*a*]phenoxazinium chlorides **6**-**9** (Table 1). The fluorescence quantum yields (Φ_F) were determined using Oxazine 1 as a standard (Φ_F = 0.11 in

ethanol) [3] at 570 nm excitation. As in previous studies with heterocycles of the same family [17], the photophysical behaviour is determined by the coexistence of an acidic cationic form of benzo[*a*]phenoxazinium chlorides (BzH^+) and a neutral basic form of benzo[*a*]phenoxazinium chlorides (Bz). The acidic form shows maximum absorption wavelength in the 627-647 nm range and an emission in the 632-683 nm spectral region whereas the neutral form has maximum absorption near 500 nm and a broad emission centred at ~600 nm with a ~10 times lower fluorescence quantum yield. In water it was previously reported that Bz neutral form is absent and non-emissive H-aggregates appeared with an approximately 50 nm blue shifted absorption relative to the observed for the acidic form. This aggregation resulted in a decrease of the fluorescence quantum yield.

In the case of the studied compounds **6**-**9** it is observed that only compound **6** maintains a high fluorescence quantum yield in physiological pH indicating that the fraction of aggregation is very small. The red shift of absorption maxima, when changing from ethanol to physiological pH, is indicative of a π-π^* electronic transition.

<Table 1>

In compounds **7**-**9**, significant aggregation occurs with broad bands appearing near 580 nm and a huge decrease in fluorescence quantum yields. Comparing compounds **8** and **9**, it is seen that the increase of side-chain length on the 9-amino moiety results in an increased fraction of aggregation and blue shift of the emission from 650 to 632 nm.

Compound **7** with a dioctylated amine shows the highest level of aggregation and evidence for the presence of the neutral Bz form probably stabilized by an ethanol enriched solvation shell (the preparation of all aqueous buffered solutions proceeded by addition of a 1 mM mother solution of the compounds in ethanol) .

As a continuation of our previous studies [21,22] concerning the use of benzo[*a*]phenoxazinium derivatives as DNA non-covalent markers, absorption and emission spectra were obtained as a function of DNA phosphate group to compound molar ration (P/D). In Figures 1 to 4, area normalized absorption spectra, as well as normalised fluorescence spectra, are shown for compounds **6**-**9**.

The behaviour of compound **6**, which is similar to Nile Blue, was already published in a previous study [21] and, for completeness, its results are replotted in this work (Figure 1). Briefly, for low P/D values a decrease in absorption is observed with an appearance of a blue shifted shoulder and a corresponding decrease in fluorescence quantum yield maintaining the spectral shape and position. These results were interpreted as electrostatic binding that

promotes formation of H-aggregates. For higher P/D values (>50), distinct photophysical behaviour occurs: a red shift of both absorption and emission spectra with a slight increase in the fluorescence quantum yield. This behaviour was interpreted [21] and proved [22] to indicate dye intercalation into the DNA double strand.

< Figure 1>

For dyes **7-9** very different behaviour (Figures 2-4) were observed. The dialkylated derivative **7** mainly showed variation in the fraction of basic neutral form and a decrease in aggregation. The corresponding fluorescence intensity decreases as the quantum yield of the neutral form is much lower than that of the cationic form. This could result from groove binding [22,23]. For P/D>50, a red shift is observed in fluorescence spectra with an increase in quantum yield (Figure 3). This behaviour could indicate intercalation but the noise level of the experimental spectra does not allow a definite conclusion.

< Figure 2>

In absorption, dye **8** shows a similar behaviour to dye **6** (Figure 3). The only difference is that aggregates exist in the absence of DNA and consequently the absorption is lower, as well as the fluorescence quantum yield. However, in the emission behaviour, while there is a decrease in fluorescence quantum yield, a red shift is also observed (Figure 3B). As a result either the formed aggregates are distorted originating a red J-aggregate type emission, or another type of DNA binding is operative. In this last case, it should be groove binding as, similarly to compound **6**, for higher P/D values (>50) the quantum yield rises and both absorption and emission spectra shifts to the red. These last observations correspond to intercalative binding.

In a previous study [21], a similar compound possessing the octyl group on the 5-amino unit instead of in the 9-position showed a similar behaviour, although the red shift in emission spectra was only observed when the dye intercalates (high P/D values). From this fact, it can be concluded that these type of compounds can either intercalate DNA through the fused benzene ring into the phenoxazine moiety or enter the DNA double strand using the molecular side that is less constrained.

< Figure 3>

Compound **9** is similar to **8** but possesses a dodecyl group on the 9-amino group, yet a different photophysical behaviour is observed as a result of DNA interaction (Figure 4). It was previously verified [22] that apolar/non-functionalised long side chains hinder the intercalation of the benzo[*a*]phenoxazinium unit. For compound **9**, no red shift in absorption is observed. Instead, a blue shift occurs for P/D=1 after which the spectral shape remains constant with an initial fast increase in absorption intensity followed by a slow one after P/D≥10. In emission the spectral position and shape remains approximately constant with a decrease in quantum yield up to P/D=5, above which there is a red shift of the spectrum with no appreciable change in quantum yield. Up to P/D=5, the behaviour is compatible with H-aggregation, but in a local environment different from what is experienced in the case of compound **8**. Above P/D=10, the gradual red shift in emission spectra is similar to that observed for compound **8**, but the interpretation of the emission spectra is not straightforward because, as the quantum yield is very low, the noise level is high and increases with wavelength, as the photomultiplier gradually loses efficiency as its detection limit of 800 nm approaches.

< Figure 4>

In order to access the potential of dyes **6**-**9** as DNA markers in agarose gel electrophoresis assays and to further assess the strength of the binding of the compounds with DNA, Figures 5 and 6 show results obtained from electrophoresis runs performed at a P/D ratio of 5 for which the interaction with DNA is dominated by electrostatic and groove binding. The standard procedure used in this type of assay is ethidium bromide dispersed in the agarose gel and interacting with DNA via intercalation. This compound is very carcinogenic and suitable alternatives are of interest. In the present experimental setup, only DNA is stained resulting in much lesser use of the synthetic compound. The curves shown in Figures 5 and 6 were obtained by transforming the image, taken with a digital camera after fixed periods of time, into a gray scale and averaging the pixel intensity (0-dark; 255-white) in each lane as a function of the distance from the wells where the DNA solutions were initially placed. The baseline was subtracted from each curve to allow a better visualization.

< Figure 5>

The migration of DNA to the positive pole was seen as either blue (compounds **6**, **8**, **9**) or orange (compound **7**) travelling bands. This confirms the binding of the compounds to DNA, as migration of free compounds would have occurred in the opposite direction towards

the negative pole. The bands faded after 30 min, but during this period they were clearly seen by the unaided eye.

DNA lanes with compounds **6**, **8** and **9** showed similar migrating velocities whereas DNA marked with compound **7** migrated more slowly. As the bands are migrating a tail is left on the gel. This tailing implies that some fraction of the dye leaves DNA and either starts migrating to the opposite side or spreads within the gel. Both of these processes account for the fading of the DNA bands. The released molecules are expected to be those that were electrostatically binded as the applied electric field weakens this interaction. Compound **6** shows a lower amount of this tracing tail, as can be seen in the curves in Figure 5, by a lower intensity before the peak corresponding to the DNA band. This tail can be a hindrance when trying to separate mixtures of DNA chains with different sizes according to different migration rates. Still, we expect that these compounds, especially **6**, to be useful in such assays, as the DNA band remains more intense than the background and also because the released dye from the faster moving DNA chains could rebind larger DNA sequences that migrate more slowly. Nile blue, which is similar to compound **6**, has already been shown to be useful in DNA separations [26] although the experimental setup involved marking all the gel, use of higher electrical fields (17V/cm compared with 5V/cm used in this work) and an induction time of 15 min, that probably is necessary in order to concentrate the dye, travelling in opposite direction to DNA, around the moving DNA spots. As a preliminary experiment to show whether there is sensitivity to the size of the DNA strand, an electrophoresis assay was performed with the same type of DNA but downsized using ultrasonication (QSonica Misonix S-4000 with 80W irradiation during 1 h). Figure 6 shows the obtained results for compounds **6**, **8** and **9**. It can be seen that the migration velocities are higher as expected for a smaller sized DNA chain. Compound **6** migrates slightly faster than the other compounds and almost without a tail. For compounds **8** and **9** the tail is very prominent and inhomogeneous.

The results for compounds **7**, **8** and **9** indicates that the free compound might locally change the agarose-gel structure due to its hydrophobic moieties leading to changes in migration velocity of DNA and in gel homogeneity. These electrophoresis experiments also showed that compound **7** which, from spectroscopy data, seemed to have a weak interaction with DNA, also binds to DNA although not so strongly as the other compounds.

< Figure 6>

4. Conclusion

Benzo[*a*]phenoxazinium chlorides either mono- (octyl and dodecyl groups) or disubstituted (ethyl and octyl groups) at the 9-position of the tetracyclic ring, and having the propyl group at the 5-amino position were synthesized. Through photophysical behavior at varying P/D molar ratio and agarose gel electrophoresis experiments it was concluded that all compounds interact with DNA. While compounds **6** and **8** clearly showed intercalative behavior, the more hydrophobic derivatives **7** and **9** mainly showed either aggregation on the surface of the DNA or groove binding. Combining the results of this contribution with previous studies [22] it is possible to conclude that the benzo[*a*]phenoxazinium intercalates DNA or from the phenoxazinium side that is less constrained or in a slating orientation through the fused benzene ring. The compounds, especially compounds **6** and **8**, also showed promising capabilities as DNA coloured markers in electrophoresis assays without the need for staining the gel.

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References

[1] Gonçalves MST. Fluorescent labelling of biomolecules with organic probes. Chem Rev 2009;109:190-212.

[2] Link M, Kele P, Achatz DE, Wolfbeis OS. Brightly fluorescent purple and blue labels for amines and proteins. Bioorg Med Chem Lett 2011;21:5538-42.

[3] Sens R, Drexhage KH. A new synthesis of symmetric boraindacene (BODIPY) dyes. J Lumin 1981;24:709-12.

[4] Hartmann H. Molekiildesign bei FIuoreszenzfarbstoffen. Chimia 1994;48:512-14.

[5] Tung CH. Fluorescent peptide probes for in vivo diagnostic imaging. Peptide Science 2004;76:391-403.

[6] Chen QY, Li DH, Yang, HH, Zhu QZ, Xu JG, Zhao Y. Interaction of a novel red-region fluorescent probe Nile Blue with DNA and its application to nucleic acids assay. Analyst 1999;124:901-06.

[7] Lerman LS. Structural considerations in the interaction of DNA and acridines. J Mol Biol 1961;3:18-30.

[8] Rosenberg B, Camp LV, Trosko JE, Mansour VH. Platinum compounds: a new class of potent antitumour agents. Nature 1969;222:385-86.

[9] Lippard SJ. Platinum complexes: probes of polynucleotide structure and antitumor drugs. Acc Chem Res 1978;11:211-17.

[10] Shahabadi N, Moghadam NH. Study on the interaction of the antiviral drug, zidovudine with DNA using neutral red (NR) and methylene blue (MB) dyes. J Luminesc 2013;134:629-34.

[11] Johnston DH, Glasgow KC, Thorp HH. Electrochemical measurement of the solvent accessibility of nucleobases using electron transfer between DNA and metal complexes. J Am Chem Soc 1995;117:8933-38.

[12] Blackburn GM, Gait MJ. Nucleic acids in chemistry and biology. IRL Press New York 1990.

[13] Deligeorgieva TG, Kaloyanovaa S, Vaquero JJ. Intercalating cyanine dyes for nucleic acid detection. Recent Pat Mater Sci 2009;2:01-26.

[14] Yarmoluk SM, Kovalska VB, Losytskyy MY. Symmetric cyanine dyes for detecting nucleic acids. Biotech Histochem 2008;83:131-45.

[15] Yang YI, Hong HY, Lee IS, Bai DG, Yoo GS, Choi JK. Detection of DNA using a visible dye Nile Blue in electrophoresed gels. Anal Biochem 2000:280;322-24.

[16] Mahadevan S, Palaniandavar M. Spectroscopic and voltametric studies of copper(II) complexes of bis(pyrid-2-yl)-di/trithia ligands bound to calf thymus DNA. Inorg Chim Acta 1997:254;291-302.

[17] Frade VHJ, Gonçalves MST, Coutinho PJG, Moura JCVP. Synthesis and spectral properties of long-wavelength fluorescent dyes. J Photochem Photobiol A: Chem 2007:185;220-30.

[18] Alves CMA, Naik S, Coutinho PJG, Gonçalves MST. New long alkyl side-chain benzo[*a*]phenoxazines as micellisation probes. Tetrahedron Lett 2009:50;4470-74.

[19] Naik S, Alves CMA, Coutinho PJG, Gonçalves MST. *N*-(Di)icosyl-substituted benzo[*a*]phenoxazinium chlorides: synthesis and evaluation as near-infrared membrane probes. Eur J Org Chem 2011:2491-97.

[20] Firmino ADG, Gonçalves MST. Bifunctionalised long-wavelength fluorescent probes for biological applications. Tetrahedron Lett 2012:53;4946-50.

[21] Alves CMA, Naik S. Coutinho PJG, Gonçalves MST. Novel long alkyl side chain benzo[*a*]phenoxazinium chlorides: synthesis, photophysical behaviour and DNA interaction. Tetrahedron 2009:65;10441-52.

[22] Alves CMA, Naik S, Coutinho PJG, Gonçalves MST. Novel DNA fluorescence probes based on *N*-[5-(11-functionalised-undecylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene]propan-1-aminium chlorides: synthesis and photophysical studies. Tetrahedron Lett 2011:52;112-16.

[23] Crossley ML, Turner RJ, Hofmann CM, Dreisbach PF, Parker RP. Chemotherapeutic dyes. II. 5-Arylamino-9-dialkylaminobenzo[*a*]phenoxazines. J Am Chem Soc 1952:74;578-84.

[24] Cao Y, He XW. Studies of interaction between Safranine T and double helix DNA by spectral methods. Spectrochim Acta A: Mol Biomol Spectrosc 1998:54;883-92.

[25] Renault E, Fontaine-Aupart MP, Tfibel F, Gardes-Albert M, Bisagni E. Spectroscopic study of the interaction of pazelliptine with nucleic acids. J Photochem Photobiol B: Biol 1997:40; 218-27.

[26] Adkins S, Burmesiter M, Visualization of DNA in agarose gels as migrating colored bands: applications for preparative gels and educational demonstrations. Anal Biochem 1996:240;17-23.

CAPTIONS

Table 1. UV-Vis-NIR absorption and fluorescence data for 2×10^{-6} M solutions of compounds **6**-**9** in ethanol and buffered (pH = 7.4) aqueous solutions.

Scheme 1. Synthesis of benzo[*a*]phenoxazinium chlorides **6**-**9**.

Figure 1. Area normalised absorption spectra (left panel) and normalised fluorescence intensity at 570 nm excitation (right panel) of compound **6** in buffered (pH = 7.4) aqueous solutions of DNA. The curves are identified by the corresponding P/D value. The insets show either the area of the absorption spectra (left panel) or the fluorescence quantum yield as a function of P/D (right panel).

Figure 2. Area normalised absorption spectra (left panel) and normalised fluorescence intensity at 570 nm excitation (right panel) of compound **7** in buffered (pH = 7.4) aqueous solutions of DNA. The curves are identified by the corresponding P/D value. The insets show either the area of the absorption spectra (left panel) or the fluorescence quantum yield as a function of P/D (right panel).

Figure 3. Area normalised absorption spectra (left panel) and normalised fluorescence intensity at 570 nm excitation (right panel) of compound **8** in buffered (pH = 7.4) aqueous solutions of DNA. The curves are identified by the corresponding P/D value. The insets show either the area of the absorption spectra (left panel) or the fluorescence quantum yield as a function of P/D (right panel).

Figure 4. Area normalised absorption spectra (left panel) and normalised fluorescence intensity at 570 nm excitation (right panel) of compound **9** in buffered (pH = 7.4) aqueous solutions of DNA. The curves are identified by the corresponding P/D value. The insets show either the area of the absorption spectra (left panel) or the fluorescence quantum yield as a function of P/D (right panel).

Figure 5. Agarose gel electrophoresis assay of salmon sperm DNA marked with compounds **6**-**9**. In each picture, two white lines mark the position of the wells, the order of the compounds in the lanes are, from top to bottom, in same order as in the legend of the graph. Top figure after 10 min run. Bottom figure after 20 min run. The graph plots the pixel intensity in gray scale (0-black; 255-white) for each lane as a function of distance from the well.

Figure 6. Agarose gel electrophoresis assay of ultrasonicated Salmon sperm DNA marked with compounds **6**, **8** and **9**. In each picture, two white lines mark the position of the wells, the order of the compounds in the lanes are, from top to bottom, in same order as in the legend of the graph. Top figure after 5 min run. Bottom figure after 20 min run. The graph plots the pixel intensity in gray scale (0-black; 255-white) for each lane as a function of distance from the well.

TABLE

Table 1

Compound	Ethanol		Tris-HCl buffer solution (pH = 7.4)	
	λ_{abs} (nm)	λ_{em} (nm)	λ_{abs} (nm) [(a)]	λ_{em} (nm)
	ε (10^4 $M^{-1}cm^{-1}$)	Φ_F	ε (10^4 $M^{-1}cm^{-1}$)	Φ_F
6	507; 637	669	642	683
	1.69; 2.82	0.26	3.1	0.25
7	514; 641	676	521; 610 *sh*; 644	646
	1.80; 2.91	0.16	0.46; 0.34; 0.40	0.0063
8	506/627	645	530 *sh*; 588; 647 *sh*	650
	1.60; 3.47	0.38	0.7; 1.0; 0.8	0.052
9	510; 628	645	583; 644 *sh*	632
	1.65; 4.27	0.35	1.0; 0.68	0.018

(a) *sh*: shoulder

Scheme 1

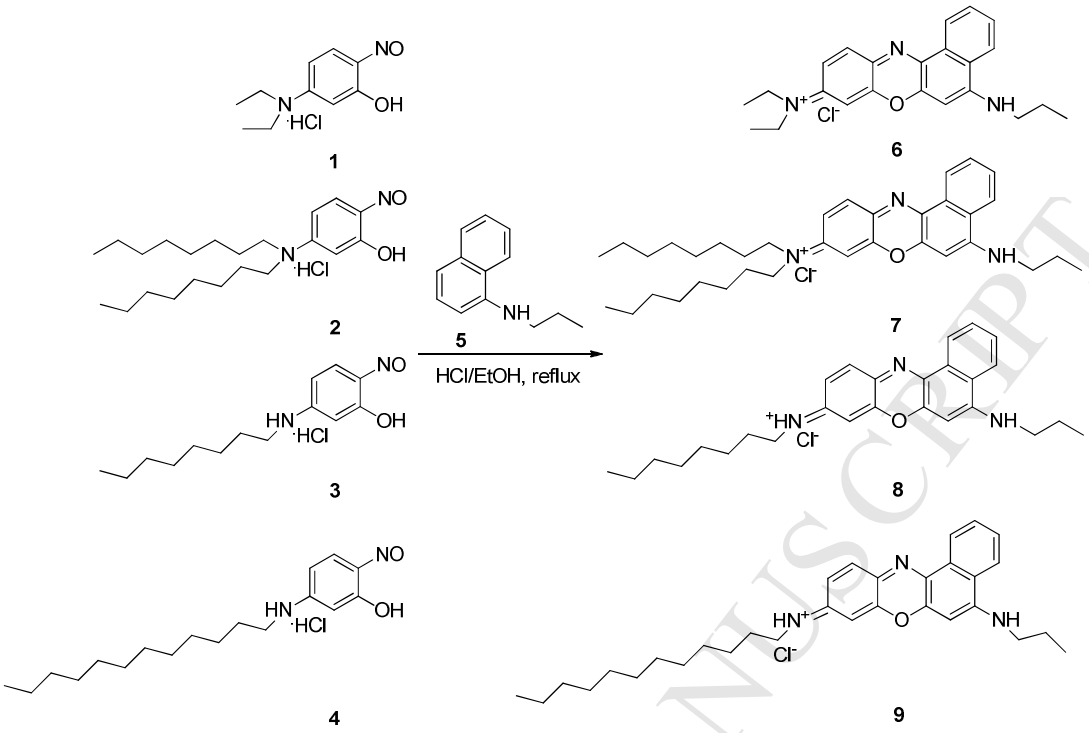

Figure 1

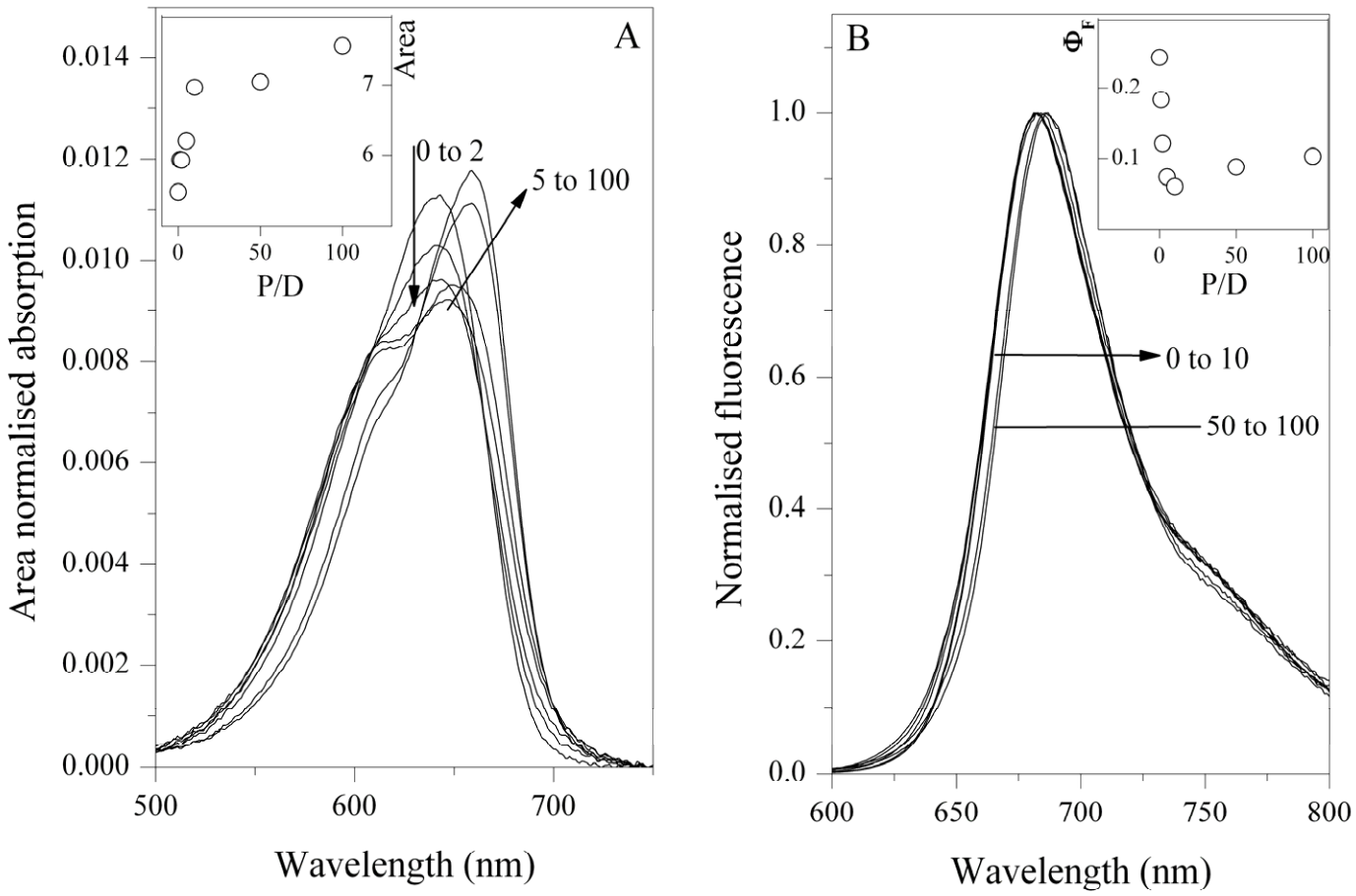

Figure 2

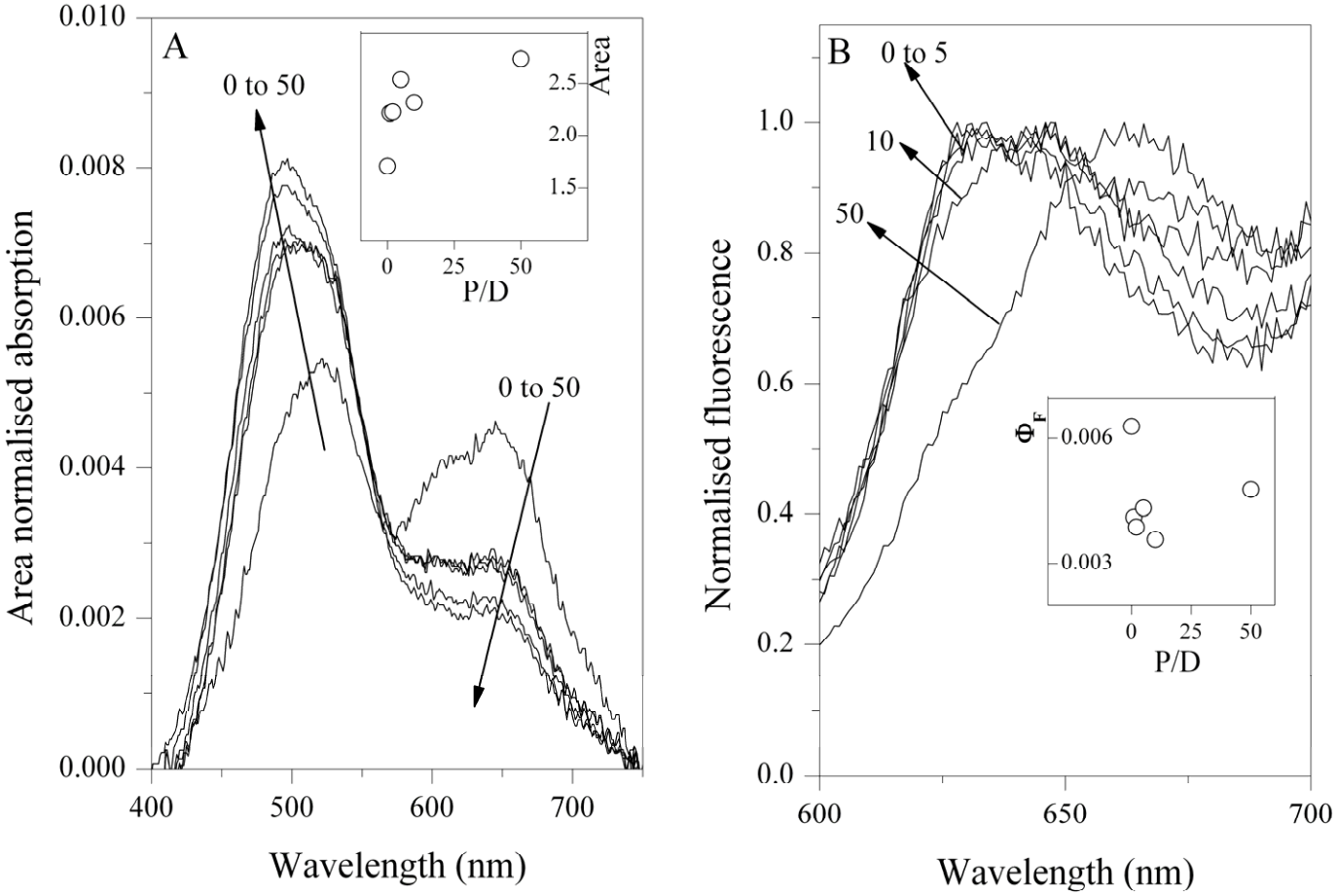

Figure 3

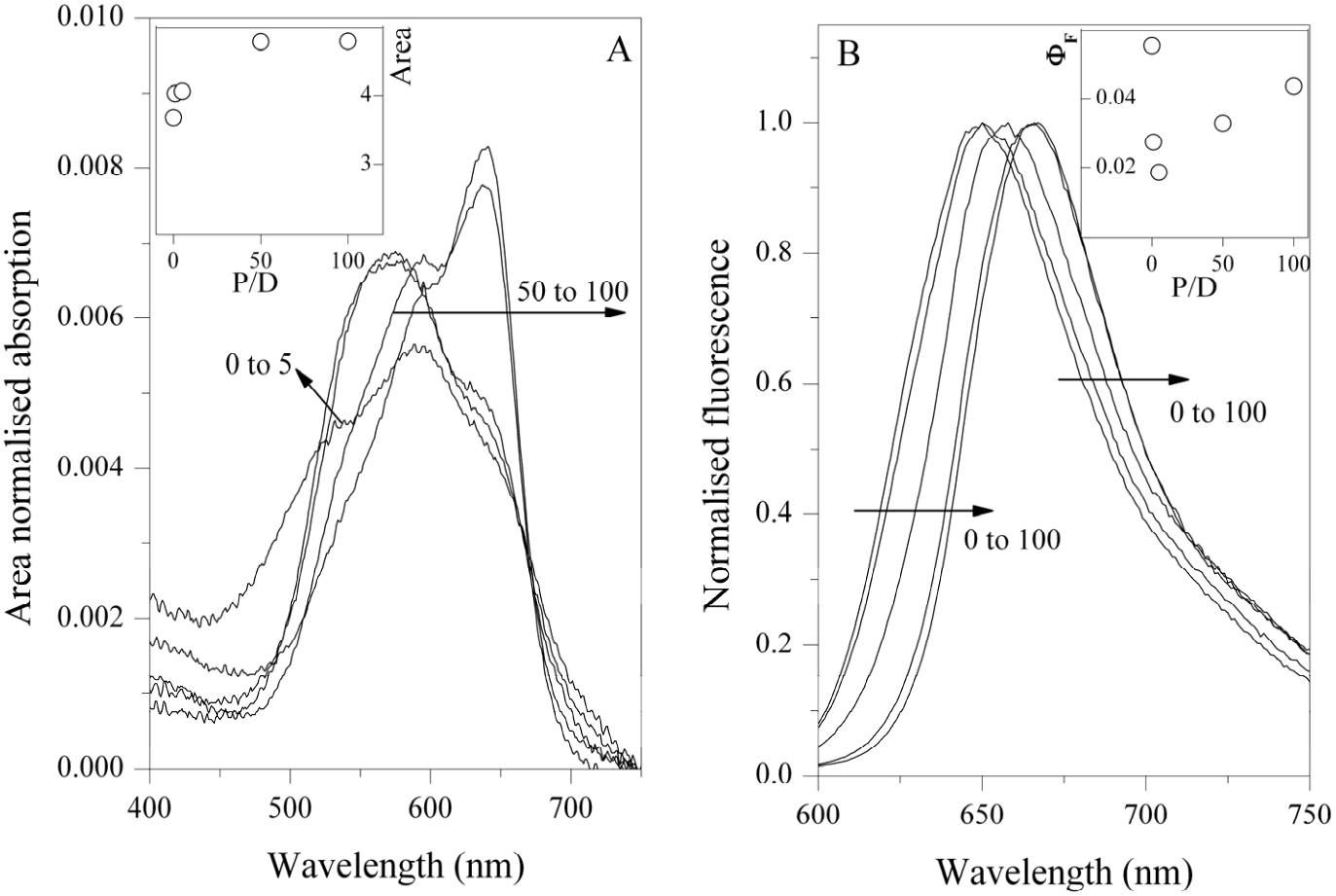

Figure 4

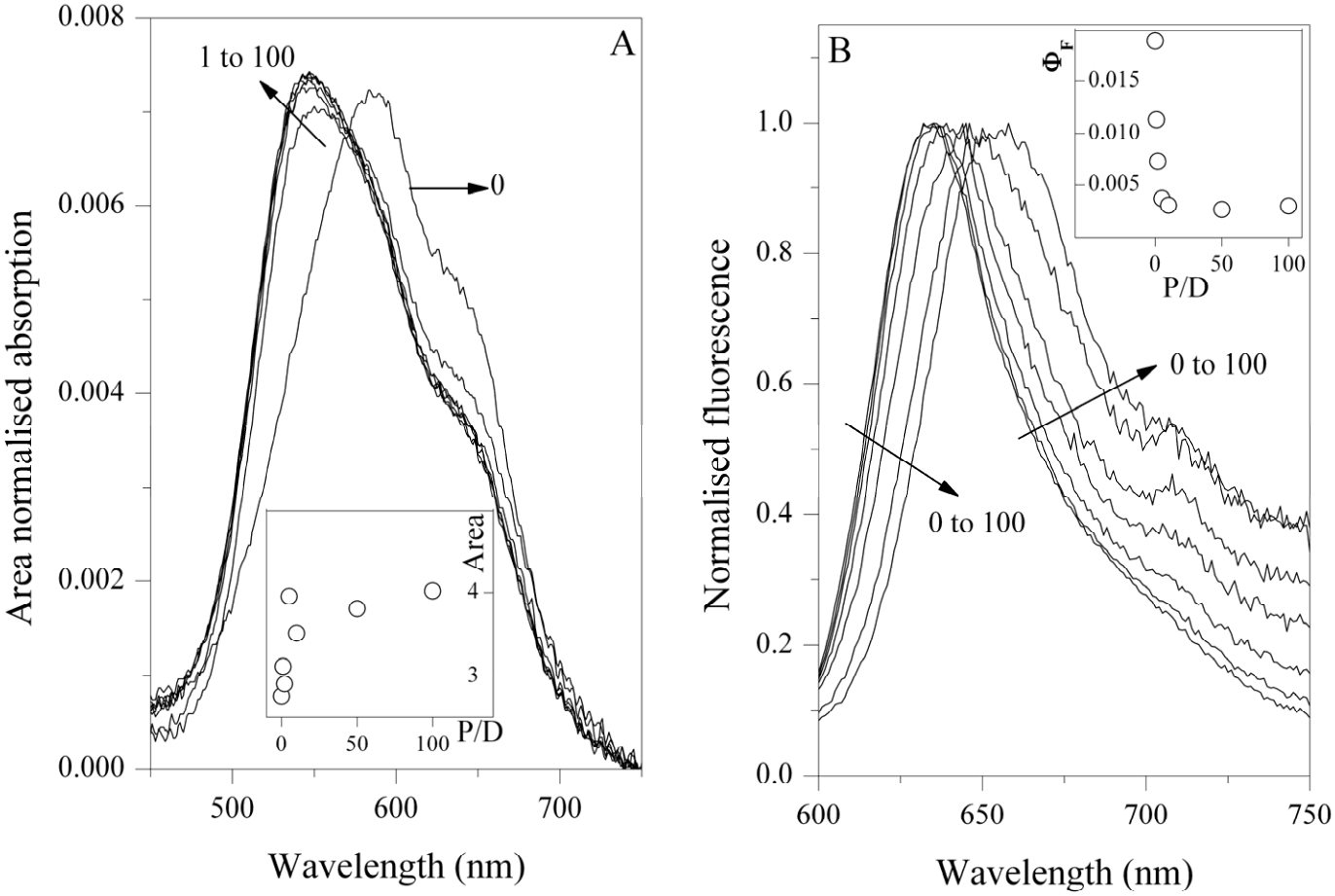

Figure 5

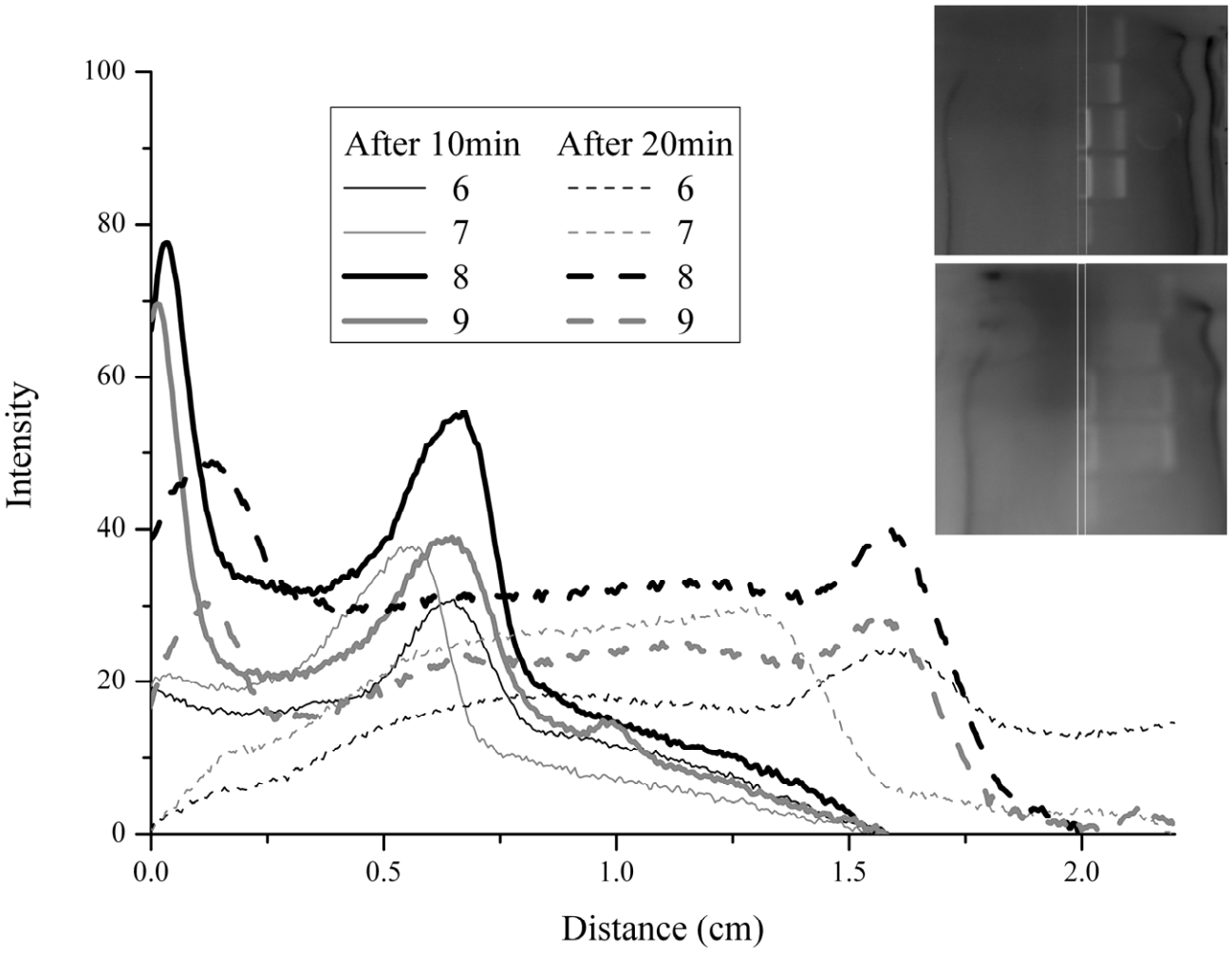

Figure 6