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

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Abstract: Fluorescent benzo[*a*]phenoxazinium chlorides possessing undecylamino chains with functionalised ending-groups (hydroxyl, carboxylic acid and the ester group) as substituents at the 5-position of the heterocycles were successfully synthesised and characterised. These compounds were used in photophysical studies with DNA, and compared to the corresponding analogue with a non-functionalised terminal (methyl group). It was found that the functionalised terminal exerts a dramatic influence on the type of interaction with the hydroxyl group promoting intercalation, while the ester group promotes groove binding.

Keywords: Benzo[*a*]phenoxazinium dyes; Nile Blue; DNA probes; Near-Infrared fluorophores; Functionalised probes.

In recent years, the development of fluorescent probes, which offer a wealth of information in various fields, has attracted the interest of researchers.[1-3] The strong influence of the surrounding medium on fluorescence emission, has led to fluorescent molecules being used as probes for the investigation of physicochemical, biochemical and biological systems. The solubility of the probes and the resulting specific interactions that can be established with the system to be probed are governed by their chemical nature; the hydrophobic, hydrophilic or amphiphilic character of the probe is essential in this regard. The presence of a long hydrocarbon chain in the fluorescence probe allows it to bind easily with the hydrophobic parts of biomolecules, enabling the fluorophore moiety to probe its environment.[4]

Studies on the interaction between DNA and ligands are particularly important for therapeutic[5] and scientific reasons.[6,7] Among other molecules, Nile Blue, a benzo[*a*]phenoxazinium dye with a planar and rigid structure, has been reported as a DNA probe,[8] and was considered to be a good intercalator of the DNA double helix.[9] Mitra and collaborators clearly identified non-specific

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electrostatic and intercalative modes of interaction of the label with DNA at lower and higher DNA concentrations, respectively.[10] The minor or major groove DNA binding of molecules is another possibility of interaction with nucleic acids.[11,12]

Bearing in mind earlier observations, combined with our current research interest on benzo[*a*]phenoxazinium dyes,[13] and following on from our previous evaluation of the potential of this family of fluorophores as DNA labels,[13e] it was decided to synthesise fluorescent benzo[*a*]phenoxazinium chlorides bearing undecylamino side-chains with functionalised ending-groups. The main purpose of the work described was to study the effect of these terminal groups, which would function as an anchor in the DNA chain, thus facilitating and stabilizing the interaction of the fluorochrome moiety with DNA bases.

Benzo[*a*]phenoxazinium chlorides **1a-c** were synthesised by condensation of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **2** with *N*-substituted-naphthylamines **3a-c**, in an acidic medium (Scheme 1). The required 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **2** was synthesised, using the usual procedure[14] involving treatment of the corresponding 3-ethylamino-4-methylphenol with sodium nitrite in an acid solution. Intermediates **3a**,**b** were prepared by alkylation, in ethanol, of 1-naphthylamine with 11-bromoundecan-1-ol and 12-bromododecanoic acid, respectively.[15] Hydrolysis of the ester group of the intermediate **3b** (1 M NaOH/1,4-dioxane) yielded the corresponding 12-(naphthalen-1-ylamino)dodecanoic acid **3c**. After column chromatography purification or isolation by extraction (**3c**), these compounds were obtained as oils (**3a**, 73%; **3b**, 70%, together with compound **3c** in 17%) or an oily solid (**3c**, 90%), and were characterised by high resolution mass spectrometry, IR and NMR (^{1}H and ^{13}C) spectroscopy.

The reaction of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **2** with functionalised precursors 11-(naphthalen-1-ylamino)undecan-1-ol **3a** and ethyl 12-(naphthalen-1-ylamino)dodecanoate **3b**, in the presence of hydrochloric acid, refluxed in ethanol, produced the benzo[*a*]phenoxazinium chlorides **1a**,**b**.[16] In the preparation of compound **1c**, the nitroso intermediate **2** reacted with 12-(naphthalen-1-ylamino)dodecanoic acid **3c**, in an acidic medium, using DMF as a solvent and heating at 80 ºC. Compound **1d** was synthesised by condensation of nitrosophenol **2** with *N*-dodecylnaphthalen-1-amine **2d**, in the presence of hydrochloric acid, refluxed in ethanol, as previously described.[13e]

After purification by column chromatography, cationic dyes **1a-c** were isolated as solid materials in moderate to high yields (Table 1) and were fully characterised by the usual analytical techniques.

<Scheme 1>

<Table 1>

Electronic absorption and emission spectra of 10^{-6} M solutions of benzo[*a*]phenoxazinium chlorides **1a-c**, in degassed absolute ethanol, were measured and the summarised data are presented in Table 1, in comparison with compound **1d**.[13e]

The longest wavelength of maximum absorption (λ_{max}) of all compounds was located between 616 and 629 nm, with molar absorptivities ranging from 58 327 to 63 128 $M^{-1}cm^{-1}$. Regarding fluorescence properties, the quantum yields (Φ_F) were calculated using Oxazine 1 as a standard (Φ_F = 0.11 in ethanol),[17] which was excited at 590 nm, the excitation wavelength used for each one of the compounds to be tested. Emission maxima (λ_{em}) for all compounds in ethanol was at about 655 nm, the Stokes' shifts were from 26 to 38 nm. All compounds exhibited similar levels of fluorescence, with Φ_F 0.24-0.29.

As a preliminary photophysical study for the use of benzo[*a*]phenoxazinium derivatives **1a-d** as DNA non-covalent markers, absorption and emission spectra were measured as a function of DNA content, keeping the concentration of fluorophore at 2×10^{-6} M.[18] Compounds **1a-d** behave differently depending on the side-chain terminating group. In Figures 1 to 4, normalised emission and absorption spectra are shown for fluorophores with hydroxyl (**1a**), ethyl ester (**1b**), carboxylic acid (**1c**) and methyl (**1d**) terminations, for various P/D values, which represents the concentration ratio between DNA phosphate groups and fluorophore molecules. It can be concluded that the presence of the hydroxyl group (compound **1a**) promotes a greater interaction of the benzo[*a*]phenoxazinium unit with the nucleotide bases. In this case, a 20 nm shifted emission appears above P/D = 10; this can safely be attributed to an intercalation complex of the benzo[*a*]phenoxazinium moiety with the nucleotide bases (Figure 1). The fact that the hydroxyl termination facilitates intercalation, indicates a more favourable fitting of the side chain in the DNA backbone. Absorption spectra confirms the formation of a nucleotide/benzo[*a*]phenoxazinium complex for compound **1a** due to the appearance of new absorption bands at P/D>10. The observed broad absorption spectra in water was previously reported by us for similar compounds[13d,e] and interpreted by the presence of H-aggregates (580

nm), the neutral basic form stabilized by the ethanol enriched solvation shell (~500 nm) and its H-aggregate (~450 nm). In the case of compound **1a**, the absorption spectra shows H-aggregates and the cationic acid form (~620 nm). Upon DNA addition, up to P/D = 5, the fraction of H-aggregates increases with a concomitant decrease of the fluorescence quantum yield (see inset of right panel in Figure 1). This is explained by electrostatic binding, which favours aggregate formation. The intercalation of compound **1a** in double stranded DNA is further confirmed by DNA melting studies. Above 80ºC, the double strand separates into two complementary single-strand (ss) DNA chains. This process is partially reversible due to chain dynamics, which hinders the exact recombination of the complementary ss-DNA strains. Compound **1a** reports this process as seen in Figure 1: upon heating, the spectrum of the DNA solution at P/D = 100 becomes similar to that obtained in the absence of DNA; after cooling to room temperature, the absortion spectrum regains its form but with less intensity.

< Figure 1>

Compound **1b** with an ethyl ester termination shows a different behaviour. At low P/D a huge enlargement of the blue side of the spectrum was observed, corresponding to an emission band at around 600 nm (Figure 2). This emission corresponds to the basic neutral form of the compound[13] and is already observable in the absence of DNA. At P/D = 1, a sudden increase of the basic form is observed, followed by a gradual decrease and enlargement of the red side of the spectrum for higher P/D values. These results can be interpreted by initial groove binding in such a way that the 5-amino group is protected from H-bond interaction. This interaction precludes the appearance of base form emission in an aqueous environment.[13e] The fact that the quantum yield of the basic form is ~5 times lower[13a], explains the observed initial decrease of the fluorescence quantum yield with a P/D value. The appearance of the basic form is correspondingly observable in UV-Vis absorption measurements. As the amount of DNA increases, some of the molecules of the fluorophore intercalate and, consequently, the red shifted emission starts to appear. When the termination is a carboxylic acid group (compound **1c**), the interaction with DNA is very low with no observable red shifted emission within the range of the P/D values studied (Figure 3). This fact is probably due to an electrostatic repulsion between the deprotonated carboxylic group (pK_a between 4 and 5) and the phosphate groups of DNA. Yet, it is possible to observe a marked change in the absorption spectra for the higher P/D values although the fluorescence properties remain more or less constant.

< Figure 2>

< Figure 3>

For compound **1d,** only a very small enlargement of the red side of the emission spectrum was observed for the higher P/D values. This confirms our previous observations[13e] that apolar/non-functionalised long side chains hinder the intercalation of the benzo[*a*]phenoxazinium unit (Figure 4). The fluorescence quantum yield shows the same initial decrease as for compound **1a** and **1b**. As the absorption spectra are dominated by H-aggregates, the experimental results suggest that electrostatic binding is the main form of DNA interaction for compound **1d**.

< Figure 4>

Conclusion

5,9-Diaminobenzo[*a*]phenoxazinium dyes **1a-c** possessing a C_{11} side chain, at the 5-amino position, with functionalised terminating groups (hydroxyl, ester and carboxylic acid) were successfully synthesised. Considering their longer wavelength of absorption and emission maxima in connection with high fluorescence, these cationic dyes were photophysically evaluated in terms of their ability to interact with DNA, in comparison with the corresponding analogue with a non-functionalised ending group. It was concluded that the hydroxyl termination enhances intercalation binding, while the ester group seems to favour association into DNA grooves. The carboxylic acid fluorophore, which exists in its carboxylate form at pH = 7, showed very little interaction with DNA, due to an electrostatic repulsion with DNA phosphate groups.

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15. Typical procedure for the synthesis of **3a-c** (described for **3a**): To a solution of 1-naphthylamine (1.0 g; 6.98×10^{-3} mol) in ethanol (2 mL), 11-bromoundecan-1-ol (2.15 g, 8.56×10^{-3} mol) was added and the resulting mixture was refluxed for 25 hours, and monitored by TLC (silica: dichloromethane/*n*-hexane, 4:6). The solvent was removed under reduced pressure and the crude mixture was purified by column chromatography on silica gel using dichloromethane/*n*-hexane, mixtures of increasing polarity, as the eluent. 11-(Naphthalen-1-ylamino)undecan-1-ol **3a** was obtained as a violet oil (1.80 g, 73%). R_f = 0.32 (silica:

dichloromethane/*n*-hexane, 4:6). FTIR (neat): ν_{max} 3429, 2954, 2925, 2853, 1644, 1585, 1527, 1482, 1463, 1407, 1378, 1345, 1254, 1152, 1120, 1074, 1028, 989, 935, 886, 784, 766, 741, 666 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.20-1.48 (14 H, 2×m, 7×CH_2), 1.50-1.65 (2 H, m, NHCH$_2$*CH$_2$*), 1.70-1.80 (2 H, m, *CH$_2$*CH$_2$OH), 3.29 (2 H, t *J* 6.8 Hz, *CH$_2$*OH), 3.65 (2 H, t *J* 6.8 Hz, NH*CH$_2$*CH$_2$), 6.66 (1 H, d *J* 7.2 Hz, 4-H), 7.27 (1 H, d *J* 8.4 Hz, 2-H), 7.39 (1 H, t *J* 7.6 Hz, 3-H), 7.43-7.51 (2 H, m, 6-H and 7-H), 7.80-7.86 (2 H, m, 8-H and 5-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 25.66 (CH_2), 27.27 (CH_2), 29.30 (CH_2), 29.35 (CH_2), 29.40 (*CH$_2$*CH$_2$OH and CH_2), 29.46 (CH_2), 29.51 (CH_2), 32.69 (NHCH$_2$*CH$_2$*), 44.27 (*CH$_2$*OH), 62.91 (NH*CH$_2$*CH$_2$), 104.36 (C-4), 117.13 (C-2), 119.74 (C-5), 123.30 (C-4a), 124.52 (C-7), 125.57 (C-6), 126.8 (C-3), 128.58 (C-8), 134.24 (C-8a), 143.40 (C-1) ppm. HRMS: *m/z* (EI): calcd. for $C_{21}H_{31}NO$ [M^+] 313.2406; found 313.2408.

16. Typical procedure for the synthesis of **1a-c** (described for **1a**): To a cold solution (ice bath) of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **2** (0.175 g; 9.71×10^{-4} mol), in ethanol (3 mL), 11-(naphthalen-1-ylamino)undecan-1-ol **3a** (0.290 g; 9.71×10^{-4} mol) and concentrated hydrochloride acid (5.0×10^{-2} mL) were added. The mixture was refluxed for 7 hours and monitored by TLC (silica: dichloromethane/methanol, 9:1). The solvent was removed under reduced pressure and the crude mixture was purified by colunm chromatography on silica gel using dichloromethane/methanol 92:8. *N*-(5-(11-Hydroxyundecylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethaneminium chloride **1a** was obtained as a blue solid (0.19 g, 64%). Mp 172.4-175.6 ºC. R_f = 0.32 (silica: dichloromethane/methanol, 9:1). FTIR (KBr): ν_{max} 3424, 2953, 2925, 2854, 1642, 1610, 1592, 1561, 1545, 1521, 1452, 1376, 1315, 1261, 1185, 1163, 1131, 1086, 1011, 666 cm^{-1}.^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.20-1.60 (19 H, 3×m, 8×CH_2 and NHCH$_2$*CH$_3$*), 1.79 (2 H, br s, NHCH$_2$*CH$_2$*), 2.44 (3 H, s, CH_3), 3.20-3.35 (2 H, m, NH*CH$_2$*CH$_2$), 3.40-3.60 (2 H, m, NH*CH$_2$*CH$_3$), 3.64 (2 H, t *J* 4.8 Hz, *CH$_2$*OH), 6.19 (1 H, s, 8-H), 6.27 (1 H, s, 6-H), 6.62 (1 H, br s, NH), 7.45 (1 H, s, 11-H), 7.84 (2 H, br s, 2-H and 3-H), 8.40 (1 H, br s, NH or OH), 8.80 (1 H, br s, 1-H), 9.17 (1 H, br s, 4-H), 11.17 (1 H, br s, NH or OH) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 13.90 (NHCH$_2$*CH$_3$*), 18.11 (CH_3), 25.65 (CH_2), 27.07 (CH_2), 28.66 (NHCH$_2$*CH$_2$*), 29.20 (CH_2), 29.24 (CH_2), 29.27 (CH_2), 29.32 (CH_2), 29.37 (CH_2), 32.73 (CH_2), 38.67 (N*CH$_2$*CH$_2$), 44.64 (NH*CH$_2$*CH$_3$), 62.90 (CH_2OH), 92.57 (C-6), 93.30 (C-8), 123.94 (Ar-C), 124.05 (C-1), 126.0 (C-4), 126.52 (C-10), 129.35 (Ar-C), 130.28 (C-3), 130.69 (Ar-C), 131.05 (C-11), 131.74 (C-2), 134.27 (Ar-C), 146.69 (Ar-C), 150.80 (Ar-C), 153.81 (C-9), 156.37 (C-5) ppm. HRMS: *m/z* (EI): calcd. for $C_{30}H_{40}N_3O_2$ [M^+] 474.31341; found 474.31150.

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18. Natural double-stranded salmon sperm DNA was obtained from Invitrogen. 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, A260/A280 = 1.95 (good-quality DNA has an A260/A280 ratio higher than 1.8).[19] The DNA concentration in number of bases (or phosphate groups) was determined from the molar extinction coefficient ε = 6600 M^{-1} cm^{-1} at 260 nm.[20] 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 hours to stabilize.

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CAPTIONS

Table 1. Synthesis, UV-Vis and fluorescence data for compounds **1a-d** in ethanol.

Figure 1. Absorption spectra (left panel) and normalised fluorescence intensity (right panel) of compound **1a** in buffered (pH = 7) aqueous solutions of DNA. The full line curves are identified by the corresponding P/D value. The dashed and dotted lines correspond, respectively, to the absorption of a solution with P/D = 100 at 80ºC and after cooling back to room temperature. The inset shows the maximum fluorescence intensity (●) and this quantity divided by the absorbance at the excitation wavelength (570 nm) (○) as a function of P/D.

Figure 2. Absorption spectra (left panel) and normalised fluorescence intensity (right panel) of compound **1b** in buffered (pH = 7) aqueous solutions of DNA. The curves are identified by the corresponding P/D value. The inset shows the maximum fluorescence intensity (●) and this quantity divided by the absorbance at the excitation wavelength (570 nm) (○) as a function of P/D.

Figure 3. Absorption spectra (left panel) and normalised fluorescence intensity (right panel) of compound **1c** in buffered (pH = 7) aqueous solutions of DNA. The curves are identified by the corresponding P/D value. The inset shows the maximum fluorescence intensity (●) and this quantity divided by the absorbance at the excitation wavelength (570 nm) (○) as a function of P/D.

Figure 4. Absorption spectra (left panel) and normalised fluorescence intensity (right panel) of compound **1d** in buffered (pH = 7) aqueous solutions of DNA. The curves are identified by the corresponding P/D value. The inset shows the maximum fluorescence intensity (●) and this quantity divided by the absorbance at the excitation wavelength (570 nm) (○) as a function of P/D.

TABLES

Table 1

Compound	Yield [%]	λ_{max} [nm] (ε, $M^{-1}cm^{-1}$)	λ_{em} [nm]	Φ_F	Stokes' shift [nm]
1a	64	616 (59 749)	654	0.29	38
1b	49	629 (62 394)	655	0.28	26
1c	33	625 (58 327)	655	0.24	30
1d[13e]	83	627 (63 128)	655	0.27	28

FIGURES

Figure 1

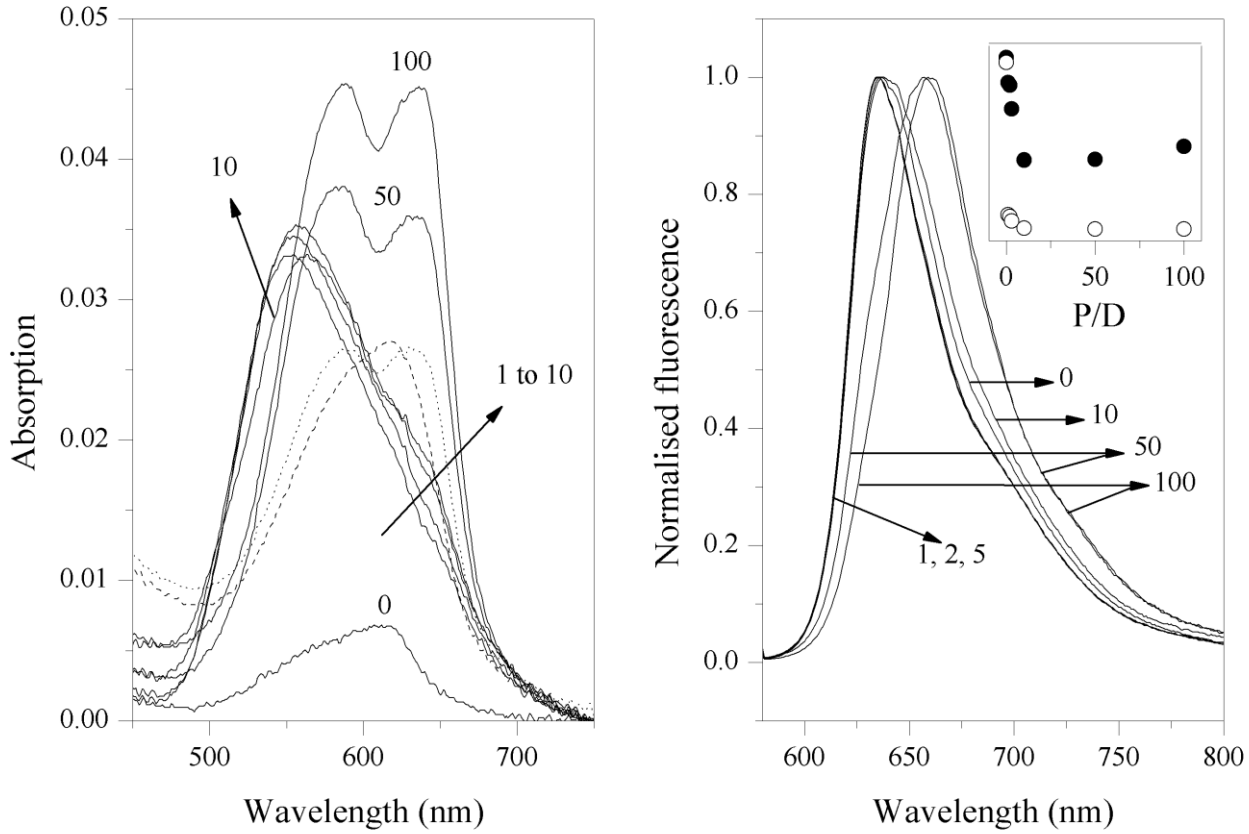
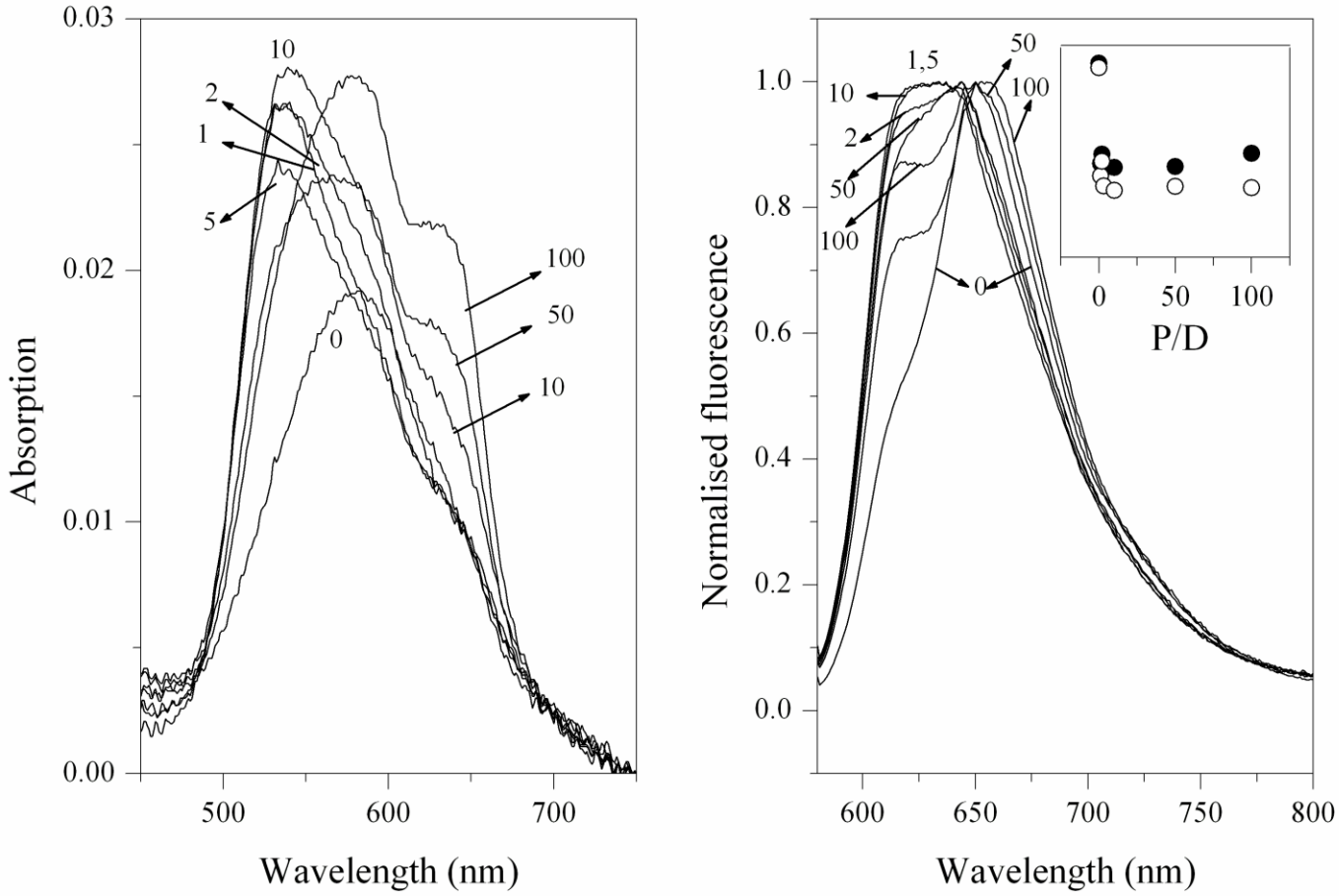


Figure 2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0.02
0.01
0.00
500
600
700
10
2
1
5
100
50
10
0
Normalised fluorescence
Wavelength (nm)
1.0
0.8
0.6
0.4
0.2
0.0
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650
700
750
800
1,5
50
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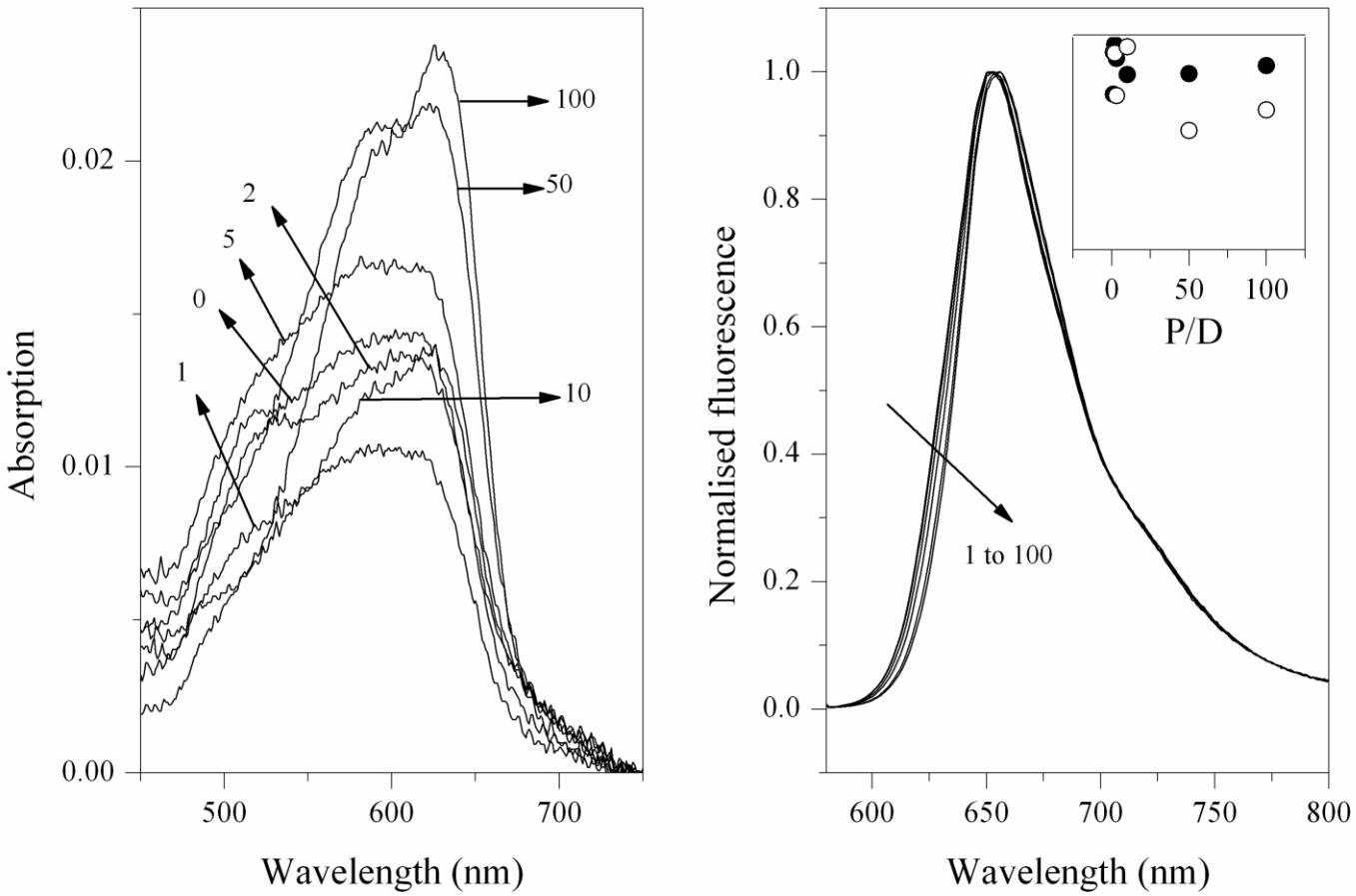
2
50
100
0
P/D
0
50
100

Figure 3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Wavelength (nm)
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2
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Normalised fluorescence
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0.2
0.4
0.6
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650
700
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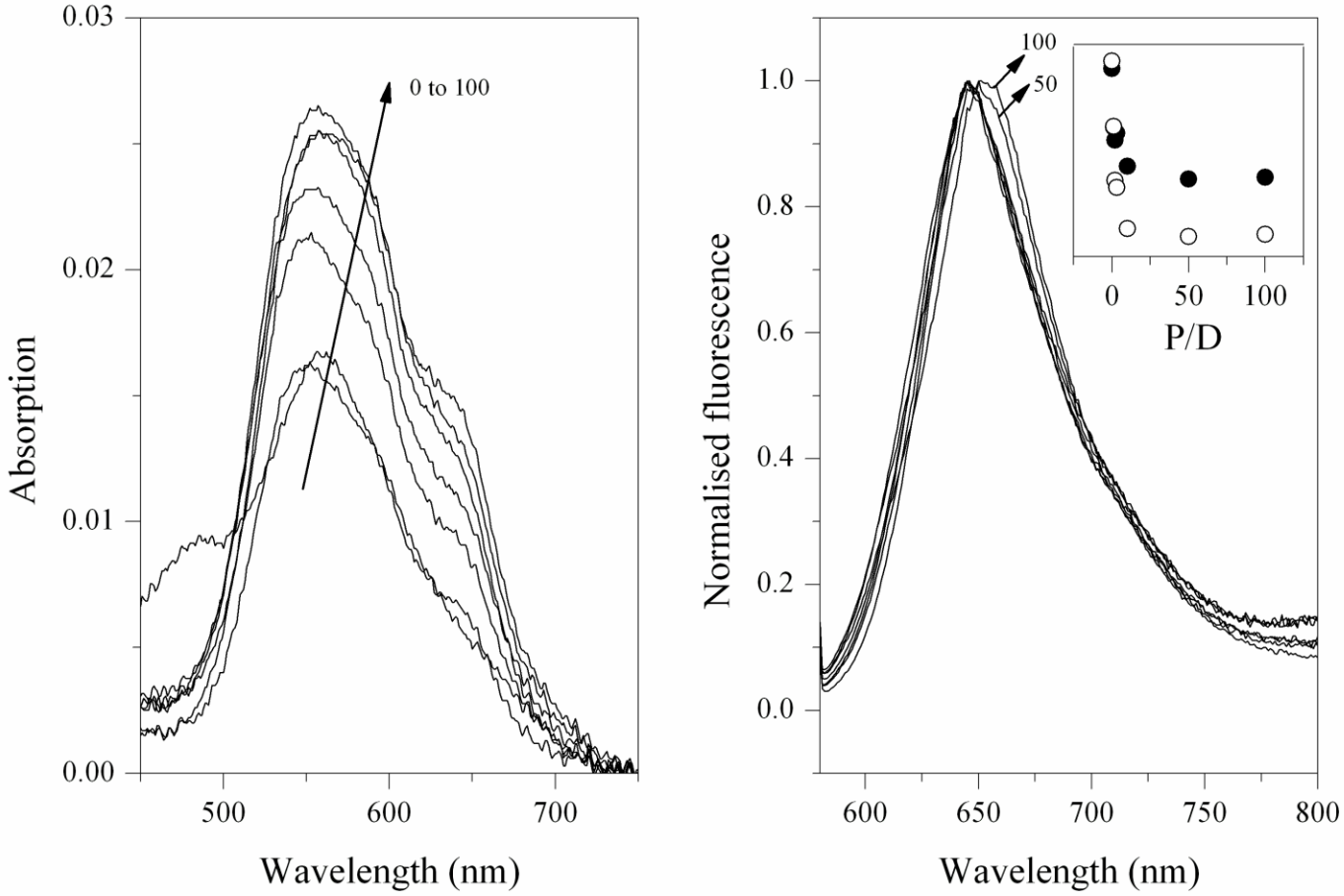
800
Wavelength (nm)
1 to 100
0
50
100
P/D

Figure 4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0.02
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Absorption
0 to 100
500
600
700
Wavelength (nm)
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0.4
0.2
0.0
Normalised fluorescence
100
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100
P/D
600
650
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750
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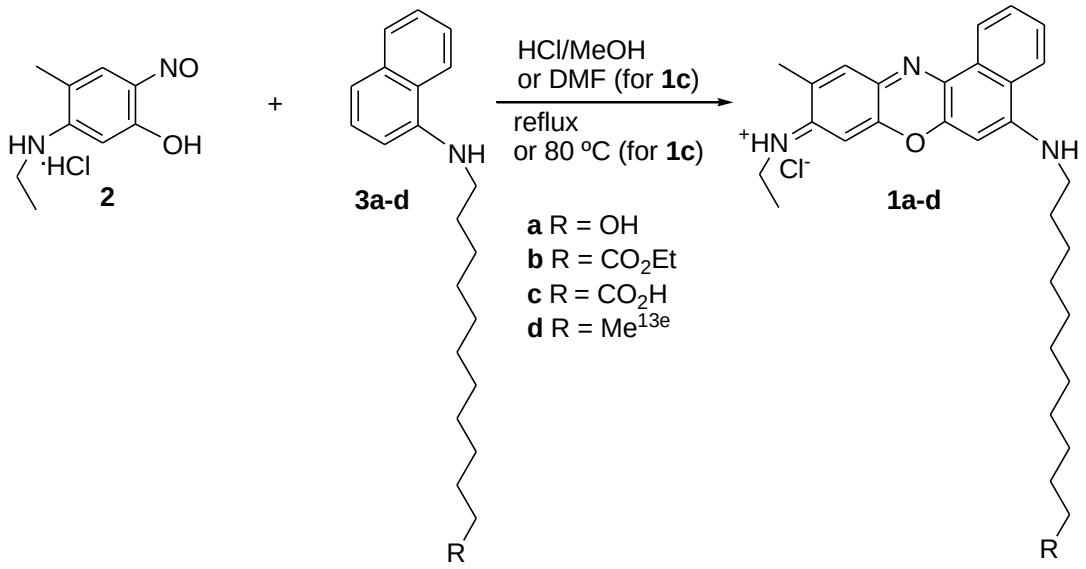
Wavelength (nm)

SCHEMES

Scheme 1



GRAPHICAL ABSTRACT

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

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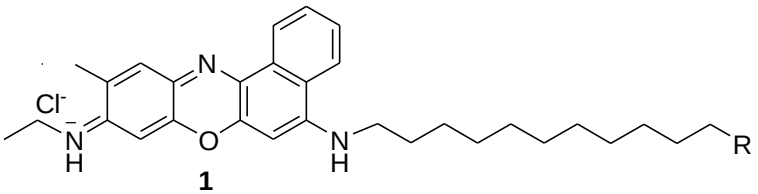

1

a R = OH
b R = CO_2Et
c R = CO_2H
d R = Me