Syntheses, characterisation and fluorescence study of some novel naphthalimide derivatives

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Three fluorescent derivatives viz. 4-tosylamido-1,8-naphthalic anhydride, 4-dansylamido-1,8-naphthalimido-N-pentanol and 4-dabsylamido-1,8-naphthalimido-N-pentanol have been prepared and characterised. The comparative fluorescence studies and quantum yield estimation has been carried out in different solvents such as methanol, dioxane, water, water-methanol gradient, normal aqueous solutions of sodium carbonate, hydrochloric acid and buffers like ammonium acetate, 1X TRIS-EDTA and the effect of inorganic salts like sodium chloride, potassium chloride, magnesium sulphate has also been studied. Thus, optimal conditions for maximum fluorescence yield has been worked out. The fluorescent derivatives reported herein have the potential to be used for covalent as well as non-covalent labelling of various biomolecules, specially to synthetic oligonucleotides. The labelled oligonucleotides have been proved to be of immense value as probes in clinical diagnostics.

Fluorescent molecules represent a class of reporter groups, which are presently being used for labelling of oligonucleotides and/or nucleic acids. Fluorescent probes are being explored as a potential alternative to radioisotopic labelling since the latter is beset with certain problems like short shelf life of isotopes, associated health hazards, storage and disposal. Being dependent on local environment fluorescence has been proved as an indispensable tool for the study of molecular interactions and miscellaneous cellular functions. Recently, the fluorescently labelled oligonucleotides are being used in nucleic acids sequencing, techniques of fluorescence in situ hybridisation and chip based DNA arrays prepared using combinatorial methods and also the advent of fluorescence resonance energy transfer (FRET) has made possible real time monitoring of oligonucleotide hybridisation.

A number of fluorescent molecules are known which have been used for covalent labelling of oligonucleotides such as biotin, fluorescein, dansyl chloride, rhodamine, pyrene and others. Many naphthalic anhydride/naphthalimido derivatives have been used to investigate various non-covalent and/or covalent interactions of oligonucleotides. In our laboratory fluorescent naphthalimido derivatives have been used to study photoinduced DNA strand scission, intercalation and sequence specific chemical recognition of nucleic acids.

4-Acetylamino-1, 8-naphthalimido-N-caproic acid has been used as a reporter group for some antisense oligonucleotides. However, for their successful application the need was to enhance their quantum yields, so that the minimum detectable level could be comparable to radioactive labels.

In the present work we report the preparation and characterisation of three new fluorescent naphthalic anhydride/naphthalimido derivatives viz. 4-tosylamido-1,8-naphthalic anhydride, 4-dansylamido-1,8-naphthalimido-N-pentanol and 4-dabsylamido-1,8-naphthalimido-N-pentanol. Their preliminary fluorescence studies have been carried out by preparing solutions in methanol, dioxane, water-methanol gradient, water, sodium carbonate, hydrochloric acid, buffers like ammonium acetate and 1X TRIS-EDTA and in the presence of salts like sodium chloride, potassium chloride and magnesium sulphate. The respective quantum yields have been determined using quinine sulphate as emission standard. Their quantum yield is found to be higher than the derivatives reported earlier. Their minimum detectable concentrations makes them more useful labels for use as reporter groups.

Results and Discussion

4-Amino-1,8-naphthalic anhydride, the basic unit was condensed with fluorescent ligands viz. toluene-p-sulphonyl chloride (not fluorescent), dansyl...
chloride and dabsyl chloride in order to get flat, planar polycyclic system with greater degree of conjugation and favourable solubility behaviour due to the sulphonamide linkage and therefore, expected to produce higher emission and quantum yield. The flat polynuclear system is expected to intercalate better in case of its covalent/noncovalent interaction with an oligonucleotide sequence.

The synthesis of the three naphthalimide derivatives viz. 4-tosylamido-1,8-naphthalic anhydride 4, 4-dansylamido-1,8-naphthalimido-N-pentanol 7 and 4-dabsylamido-1,8-naphthalimido-N-pentanol 8 was carried out starting from acenaphthene, through a series of chemical reactions (Scheme I). The 5-aminopentanol acts as spacer which plays a significant role in avoiding spatial interaction of the fluorophore with the oligonucleotide strand while investigating various covalent interactions of oligonucleotide strand. The 5-aminopentanol spacer is significant in the sense that its length can be varied as necessitated...
Table 1 — Wavelength of maximum absorption (λ, in nm) and molar extinction coefficient (ε in x 10^7 M⁻¹ dm³) for 4, 7 and 8 in different solutions of concentration 10 μM/L.

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Solvents</th>
<th>4-Tosylamido-1,8-naphthalic anhydride 4</th>
<th>4-Dansylamido-1,8-naphthalimido-N-pentanol 7</th>
<th>4-Dubyslamido-1,8-naphthalimido-N-pentanol 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>aq. TRIS-EDTA</td>
<td>λ: 314, ε: 46</td>
<td>λ: 316, ε: 56</td>
<td>λ: 410, ε: 38</td>
</tr>
</tbody>
</table>

Figure 1 — Relative fluorescence intensity of 4, 7 and 8 in various solutions of concentration 10 μM/L. Methanol; 2. Dioxane; 3. TRIS-EDTA; 4. aq. ammonium acetate; 5. aq. sodium chloride; 6. aq. potassium chloride; 7. aq. magnesium sulphate; 8. Water; 9. aq. sodium carbonate; 10 aq. hydrochloric acid.

and the free hydroxy function can be readily phosphitylated and then coupled with appropriately derivatised oligonucleotide using phosphoramidite approach of oligonucleotide synthesis. Alternatively, it can be coupled directly by generating appropriate functionality on the desired site of oligonucleotides. The compounds prepared in the present work were characterised by m.p., UV, ¹H NMR, elemental data and were purified by HPLC.

Fluorescence measurement of compounds 4, 7 and 8 was done by preparing the stock solutions in concentration of 10 mM / L in various solvents and diluting these sequentially 10 μM / L, 7.5 μM / L, 5.0 μM / L and 2.5 μM / L, respectively. The absorption spectra was recorded by using 10 μM / L solutions of the three compounds. The wavelength of maximum absorption (λ_max) and corresponding molar extinction coefficients (ε) of the three derivatives are summarised in Table 1. It could be easily followed from the data that there is a significant downward shift in absorption wavelength with increase in the polarity or ionisation of the solvents. However, with variation in the chemical structure of the three derivatives and also the change of solvents and/or solvent systems the excitation wavelength was not effected much but the change in emission wavelength was significant.

There are number of matters to be taken into account while interpreting these data. Most important are the effects of solvent caused by the difference in hydrophobicity, protogenic property and permittivity (dielectric constant). Of the solvents used for studying fluorescence characteristics of compounds 4, 7 and 8, more pronounced emission was observed in organic phase than aqueous phase, protic solvents (methanol) produced higher emission intensities than non-protic solvent like dioxane (Figure 1), the fluorescence intensity of these compounds was found to decrease in the order; methanol>dioxane>TRIS-EDTA>normal aqueous solutions of ammonium acetate>sodium chloride> potassium chloride > magnesium sulphate > water > sodium carbonate > hydrochloric acid. A comparison of the fluorescence characteristics of the three compounds reveals that under similar conditions compound 7 invariably produces greater emission than compound 4 followed by compound 8. This is due to the structural characteristics of these
compounds as 7 has higher degree of conjugation as compared to 4 whereas in 8 azo group may act as electron sink (Figure 1; Scheme I).

A detailed fluorescence study was undertaken for compound 7 since it shows maximum quantum yield and highest wavelength. It was observed that on dilution to various micromolar concentration viz. 7.5 μM/L, 5.0 μM/L and 2.5 μM/L there was a relative decrease in fluorescence intensity of the compound as expected (Figure 2). A comparative fluorescence spectra of the compound 7 was recorded in water - methanol gradient and it was found that 25:75; water : methanol produced highest emission intensity. This may be due to the peculiar solubility characteristics of the compound (Figure 3).

The presence of ions invariably increased the fluorescence intensity as compared to pure aqueous phase in case of all three derivatives viz. compounds 4, 7 and 8. It was found that the ions exert variable effects, maximum increase in intensity was observed in the presence of Na⁺ followed by K⁺ (monovalent) and divalent Mg²⁺ (Figure 4). Similarly pH was also found to influence the emission characteristics of these compounds. Almost negligible emission was obtained in normal aqueous solutions of hydrochloric acid, even though emission in sodium carbonate solution was satisfactory. This may be due to the protonation of amino functionality present in 7 and 8. Same was the case with buffers, where 1 X TRIS-EDTA buffer (pH 8.0) produced more pronounced emission than ammonium acetate buffer (pH 7.0) thereby indicating that alkaline pH is more favourable (Figure 5).

The quantum yield of the three derivative was estimated in all solutions mentioned herein. Secondary method of quantum yield estimation developed by Parker-Rees¹⁸ was used employing 1.26 × 10⁻⁶ M quinine sulphate in 0.1 N sulphuric acid (Φₛ = 0.55) as quantum yield reference. The expression as shown in Equation (1) was used¹⁹.

$$\Phi_α = \Phi_\alpha = A_α I_α n_α^2 / A_u I_u n_u^2 \quad \ldots (1)$$

where \(\Phi\) is the quantum yield, \(A\) represents the absorption at the wavelength of excitation, \(I\) is the integrated fluorescence intensity when the sample and the reference were excited at the same wavelength under similar instrument settings, \(n\) is the refractive index, subscripts \(s\) and \(u\) indicate the standard (quinine sulphate) and unknown fluorophore, respectively. The respective Φ values of the three derivatives in different solutions are summarised in Table II. It can be seen that Φ values are more/less in conformity with our interpretation of emission spectra in preceding section.

Thus, the covalent attachment of two separate fluorescent molecules viz. 4-amino-1, 8-naphthalic anhydride and dansyl and dabsyl chloride via a sulphonamide linkage and subsequent attachment of a
Figure 3 — Relative fluorescence intensity of 7 in water methanol gradient. 1. 100 % water; 2. 25 : 75; 3. 50 : 50; 4. 75 : 25; 7. 100 % methanol.

Figure 4 — Relative fluorescence intensity of 7 in normal aqueous solutions of inorganic salts. 1. NaCl; 2. KCl; 3. MgSO₄.
5-carbon spacer via an imide linkage has resulted in highly potential reporter groups viz. 4-dansylamido-1,8-naphthalimido-N-pentanol and 4-dabsylamido-1,8-naphthalimido-N-pentanol. From the optimization of experimental conditions i.e. solvents and/or solvent systems, ions, pH etc. has indicated that these molecules when attached (covalently/non-covalently) to biomolecules specially normal or modified oligonucleotides can prove to be efficient probes.

Experimental Section

Acenaphthene, 5-aminopentanol-1, dimethylaminonaphthalene sulphonyl (dansyl) chloride, toluene-p-sulphonyl (tosyl) chloride and 4-dimethylamino-4'-diazenobenzene sulphonyl (dabsyl) chloride were purchased from Merck-Schuchardt, Germany. All the solvents used were obtained from E. Merck, Co., Mumbai and were further purified and distilled prior to use. TLC was carried out on silica gel G. Melting points reported herein are uncorrected. \(^{1}H\) NMR spectra were obtained with Brukers DMX 500, elemental data with Shimadzu 34-408 analyser. Purity of compound was checked by HPLC (Pharmacia LKB-DBF) using reverse phase C\(_{18}\) columns employing UV-VIS detector. Absorption spectra was recorded in Hitachi 220S spectrophotometer whereas fluorescence spectra was recorded on Kontron SFM 25 spectrofluorometer. All the solvents employed for measuring fluorescence were degassed prior to use.

5-Nitroacenaphthanene 1. Acenaphthene (24 g / 0.16 M) was dissolved in hot glacial acetic acid (192.0 mL) and cooled gradually to 10\(^{\circ}\C\) to form a crystalline magma. Nitric acid (12.0 mL) was added dropwise to this magma for 30 min keeping the temperature at 15\(^{\circ}\C\). Now the temperature was allowed to rise at 30-35\(^{\circ}\C\) and stirred the reaction mixture for another 30 min. The mixture was then poured into crushed ice to precipitate 5-nitroacenaphthene. It was washed and crystallised from glacial acetic acid as yellow needles, yield 68% (16.32 g), m.p. 106\(^{\circ}\C\), \(R_f\) 0.4 (Hexane : Benzene; 7:3 v/v); UV (MeOH): 370 nm.

4-Nitro-1,8-naphthalic anhydride 2. Compound 1 (10.0 g / 50.0 mM) was taken in glacial acetic acid (150.0 mL). Sodium dichromate (60.0 g / 0.2M) was gradually added to it with continuous stirring. The reaction mixture was incubated on a water-bath for 3-4 hr. This was cooled and transferred to cold water and filtered to separate the orange precipitate. The precipitate was crystallised from hot glacial acetic acid as colourless needles, yield 62% (6.2 g); m.p. 229\(^{\circ}\C\); \(R_f\) 0.5 (Benzene), UV (MeOH): 340 nm; \(^{1}H\) NMR (\(\delta\), ppm): 6.37-7.02 (m, 5H, ArH). Elemental analysis; Found: C,58.99; H,2.55; N,5.59. Calculated for C\(_{12}\)H\(_{15}\)NO\(_{3}\): C,59.25; H,2.05; N,5.76%.
4-Amino-1,8-naphthalic anhydride 3. Compound 2 (1.95 g / 8.0 mM) was taken in glacial acetic acid and iron was added to it. The reaction mixture was gently warmed over water-bath for 3-4 hr. After ensuring complete reaction, the reaction mixture was filtered hot. The amino derivative present in the filtrate, was converted to hydrochloride salt, which on neutralisation with ammonia gave the reddish orange crystals of 3, yield 52% (3.0 g); m.p. >320°C; Rf 0.2 (Benzene : MeOH; 9:1 v/v), UV (MeOH): 345 nm; 1H NMR (δ ppm): 6.32-7.29 (m, 5H, Ar-H). Elemental analysis; Found: C, 62.65; H, 4.17; N, 6.35. Calculated for C₂₇H₂₀N₄O₃S: C, 62.79; H, 4.27; N, 6.29.

4-Tosylamido-1,8-naphthalic anhydride 4. Compound 3 (0.84 g / 4.0 mM) was dissolved in dry pyridine (10.0 mL) and toluene-p-sulphonyl chloride (1.08 g / 6.0 mM) was added to it followed by triethyl amine (2.0 mL) as catalyst. The reaction mixture was gently refluxed for 3 hr. After cooling to room temperature, the reaction mixture was transferred to water and the product was extracted with dichloromethane. The organic layer was kept over sodium sulphate, which was filtered and evaporated in vacuo. The product was purified by column chromatography using solvents dichloromethane and methanol in increasing gradient, yield 79% (0.67 g), m.p. 232°C; Rf 0.6 (Benzene : MeOH; 7:3, v/v); UV (MeOH): 323 nm; 1H NMR (δ, ppm): 3.11 (s, 6H, -CH₃), 6.43-7.89 (m, 13H, Ar-H). Elemental analysis; Found: C, 62.65; H, 4.17; N, 6.35. Calculated for C₃₂H₂₇N₂O₅S: C, 64.57; H, 4.03; N, 6.27.

4-Dabsylamido-1,8-naphthalic anhydride 6. Compound 3 (1.08 g / 4.0 mM), was dissolved in pyridine (5.0 mL) and dabsyl chloride (1.28 g / 10.0 mM) and triethyl amine (2.0 mL) were added. Procedure adopted was same as for compounds 4 and 5, yield 70% (0.588 g); m.p. 220°C; Rf 0.6 (Benzene : MeOH; 7:3, v/v); UV(MeOH): 424 nm; 1H NMR (δ ppm): 3.14 (s, 6H, -CH₃), 6.66-7.96 (m, 13H, Ar-H). Elemental analysis; Found: C, 63.53; H, 4.10; N, 9.62. Calculated for C₃₅H₃₀N₂O₅S: C, 63.67; H, 4.08; N, 9.38.

4-Dansylamido-1,8-naphthalimido-N-pentanol 7. Compound 5 (0.44 g / 1.0 mM) was dissolved in ethanol (5.0 mL). 5-Aminopentanol (0.12 g / 1.2 mM) was added to it and reaction mixture was refluxed gently on a water-bath for 1.5 hr. This was cooled to room temperature and evaporated in vacuo to dryness. The product was purified by column chromatography using dichloromethane and methanol in linear gradi-
ent, yield 82% (0.361 g); m.p. 212°C; Rf 0.7 (Benzene: MeOH; 7:3, v/v), UV (MeOH): 324 nm; 1H NMR (δ, ppm): 2.40 (m, 2H, C-4), 2.43-2.46 (m, 2H, C-3), 2.49 (m, 2H, C-2), 3.21 (s, 6H, -CH3), 3.76 (m, 2H, C-5), 6.50-7.15 (m, 11H, Ar-H). Elemental analysis; Found: C,66.67; H,5.62; N,5.97. Calculated for C29H29N3O3S: C,66.79; H,5.56; N,6.14%.

4-Dabsylamido-1,8-naphthalimido-N-pentanol 8.

This was synthesised by a method parallel to reported above for 7 using compound 6 (0.51 g / 1.0 mM), 5-aminopentanol (0.12 g / 1.0 mM) and ethanol (5.0 mL), yield 74 %; Rr 0.8 (Benzene: MeOH - 7:3, v/v); m.p. > 220°C; UV(MeOH): 426 nm; 1HNMR (δ, ppm): 3.19 (s, 6H, -CH3), 6.63-7.32 (m, 13H, Ar-H). Elemental analysis; Found: C, 63.79; H, 5.15; N, 11.68. Calculated for C31H31N3O3S: C, 63.58; H, 5.29; N, 11.96%.

Fluorescence measurements

Stock solution of compounds was prepared in concentration of 10.0 mM / L in methanol, dioxane, water and water-methanol gradients (75:25, 50:50, 25:75), normal aqueous solutions of sodium carbonate, hydrochloric acid, sodium chloride, potassium chloride, magnesium sulphate, ammonium acetate buffer (pH adjusted to 7.0) and 1X TRIS-EDTA buffer (pH adjusted to 8.0). These were sequentially diluted to make solutions of concentration 10 μM / L, 7.5 μM / L, 5.0 μM / L, 2.5 μM / L. The absorption spectra was recorded with solutions of concentration 10 μM / L to access the wavelength of maximum absorption.

Fluorescence spectra for derivatives reported herein were recorded at different micromolar concentrations on Kontron SFM 25 spectrofluorometer and solvents employed for this study were degassed prior to use. The compounds were excited at their respective wavelength of maximum absorption and emission was scanned in the range of 200-600nm. Quantum yields were estimated using 1.26 × 10^-6 M quinine sulphate in 0.1 N sulphuric acid (Table II). All spectral studies were carried simultaneously (unknown / standard) and at room temperature.

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References