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ORIGINAL PAPER

Synthesis and Fluorescence Studies of Some New Fluorophores and Their Effect on Hybridization of Oligodeoxyribonucleotides

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Abstract Some novel fluorophores, viz. 6-(6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**1**), 6-(6-dimethylamino-1,3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**2**), 6-(6-benzoylamino-1, 3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**3**), 6-(6-amino-1-oxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**4**) and 6-(6-amino-1H,3H-benzo[de] isoquinolin-2-yl)-hexanoic acid (**5**) have been designed, synthesized and characterized. Their comparative fluorescence has been studied in different organic solvents and aqueous solutions containing inorganic ions. Out of these, two fluorophores, **1** and **2** have been used for labelling of nucleosides which were finally converted into their phosphoramidites, and used for labelling of oligodeoxyribonucleotides through covalent attachment. These fluorophores after attachment on oligodeoxyribonucleotides showed good fluorescence signals and higher hybridisation affinity than unlabelled oligodeoxyribonucleotides.

Keywords Fluorophores · Fluorescence · Oligodeoxyribonucleotides · Hybridization · Thermal denaturation

Introduction

Fluorescently labelled oligonucleotides have tremendous applications in biotechnology, molecular and clinical diagnostics [1], therapeutics [2] and molecular biology. Fluorescence methods are extremely widespread in chemistry and biology. They give useful information on structure [3], distance, orientation, complexation [4] and location of biomolecules. In addition, time-resolved methods are increasingly used in measurements of dynamics and kinetics [5]. As a result, many strategies for fluorescent labelling of biomolecules, like nucleic acids [6] have been developed.

Fluorescence techniques are used widely in nucleic acids research to study the structure of nucleic acids as well as their interaction with proteins. In addition, numerous methods for the detection and quantification of DNA and RNA based on fluorescence have been developed [7] and that is why, understanding the mechanism of possible interactions between the fluorophores and nucleic acids is important. With the advent of automated DNA synthesis and phosphoramidite chemistry, it has become possible to attach fluorophores covalently to an oligonucleotide of interest [8].

Radioisotopes are the common labels but these are now being replaced by fluorophores due to some disadvantages, like cumbersome nature of autoradiographic/scintillation counting method, isotope instability, health hazards and disposal problems [9].

Fluorescent molecules are not very common and those which do fluoresce are almost invariably fairly rigid aromatic rings or ring system. In biological systems, quenching is usually a result of either collisional processes (either a chemical reaction or simply collision with exchange of energy) or a long- range radiative process called resonance energy transfer. These factors are usually expressed in an experimental situation involving solutions as an effect of the solvent or dissolved compounds (called Quenchers), temperature, pH, neighbouring chemical groups, or the concentration of the fluorophore; so fluorescent probes can be used for studies of molecular interactions [10], cellular functions [11] and biochemical processes [12] etc.

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Fluorescent molecules can be covalently attached to oligonucleotides [13, 14] by various enzymatic [15] or chemical methods [16, 17] through various active sites on bases, sugars (3′, 5′) or phosphate units. These molecules are attached at the end of spacer arms or side chains of nucleobases [15], sugars and oligonucleotides utilizing $-NH_2$ or –SH functional groups [18]. We have labelled nucleosides and oligodeoxyribonucleotides, bearing primary aliphatic amino group at C-4/C-5 position of bases having aliphatic carbon chain and at 5′-terminus, respectively, with fluorophores of high sensitivity. Phosphoramidites of these labelled nucleosides have been synthesized successively.

The present work focuses on synthesis of some novel fluorophores, viz. 6-(6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**1**), 6-(6-dimethyl amino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**2**), 6-(6-benzoyl amino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**3**), 6-(6-amino-1-oxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**4**) and 6-(6-amino-1H,3H-benzo[de] isoquinolin-2-yl)-hexanoic acid (**5**). The comparative fluorescence studies of these fluorophores and 6-(6-amino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**6**), have been done in methanol. Fluorescence of **1** and **2** have been studied in different media including dioxane, water-methanol, aqueous solutions of NaCl, $MgSO_4$, $NaHCO_3$ and KCl at a micro molar concentration. The results showed that the fluorophores reported herein have sufficiently good emission quanta, sensitivity upto pico mole level and can be suitably used for labelling of oligodeoxyribonucleotides. Fluorescence of labelled nucleosides and phosphoramidites were recorded in methanol and that of oligodeoxyribonucleotides in phosphate buffer.

Materials and methods

Chemicals, oligonucleotides and buffers

All chemicals and solvents used were purchased from Sigma-Aldrich (India) and Merck (India) and were of analytical grade. Oligonucleotide synthesis was carried out on Pharmacia LKB-Gene Assembler plus. The following sequences were used: *oligo-1*: DMT-AGT GGG TTA AGA, *oligo-2*: DMT-TCT TAA CCC ACT CGG. The buffers used for purification using HPLC and hybridization studies were: CH_3COONH_4, 0.1 M, pH 7.1 and CH_3CN and 0.01 M $NaH_2PO_4 \cdot 2H_2O$, 0.01 M Na_2HPO_4 and 1.0 m NaCl, pH 7.2, respectively.

Spectroscopy

Absorption spectra were recorded on a Perkin-Elmer Lambda Bio 20 UV-visible spectrophotometer fitted with a PTP-6 (Peltier Temperature Programmer) device and fluorescence spectra on Fluoromax-3 (Jovin Yvon Horiba, Tokyo, Japan) spectrofluorometer. Purification of oligodeoxyribonucleotides was carried out on HPLC Agilent 1100 using an RP C18 Lichrosphere column employing a UV-visible detector.

Synthesis of fluorophores

6-(6-Amino-1,3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid was prepared according to published procedures [17].

*6-(6-Isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)- hexanoic acid (**1**)*

6-(6-Amino-1,3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (50 mg, 0.15 mmol) was dried using anhydrous pyridine (10 mL × 3) and dissolved in anhydrous pyridine (2 mL). Reaction mixture was cooled on ice bath. After 30 min, isobutyric anhydride (3 mL) was added and the reaction mixture was removed from ice bath. Reaction was left overnight for stirring. After 24 h, reaction mixture was chilled on ice bath and cold water (2 mL) was added. After 15 min, it was concentrated and poured into water and washed with ether and left for crystallization. Compound was obtained in 69% yield. R_f, 0.3, (DCM); UV(MeOH): λ_{max} 397 nm;^{1}H NMR ($CDCl_3$, TMS = 0.00): 7.97–6.79 (d, 5H, Ar-H), 3.2–1.29 (d, 8H); 2.78 (d, H), 2.23 (s, 2H); 1.19 (s, 6H);^{13}C NMR ($CDCl_3$): 18.0, 24.8, 26.8, 28.7, 35.8, 42.0, 111.5, 116.3, 120.4, 125.9, 126.7, 127.0, 130.3, 137.7, 138.3, 151.6, 165.9, 177.2; MS (EI) m/z: 396.17 (M^+); $C_{22}H_{24}N_2O_5$ C, 66.65; H, 6.10; N, 7.07; found: C, 66.25; H, 6.15; N, 6.9; ΔG°: 28.42 [kJ/mol]; ΔH°: −557.21 [kJ/mol].

*6-(6-Dimethylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**2**)*

6-(6-Amino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (3.26 g, 10 mmol) was dissolved in dried DMF (10 mL) and chilled on ice bath. Tributylamine (4.8 mL, 20 mmol) was added and the reaction mixture was stirred. After 15 min, when reaction mixture was chilled sufficiently, methyl iodide (1.8 mL, 28 mmol) was added dropwise and allowed to overnight stirring at room temperature. After 24 h, dried ether (100 mL) was added and the reaction mixture was placed in refrigerator for 2 h. It was then concentrated and extracted with DCM and water. Crystals were obtained in DCM fraction in 71% yield. R_f, 0.8, (DCM:MeOH::9:1, v/v); UV(MeOH): λ_{max} 268 nm;^{1}H

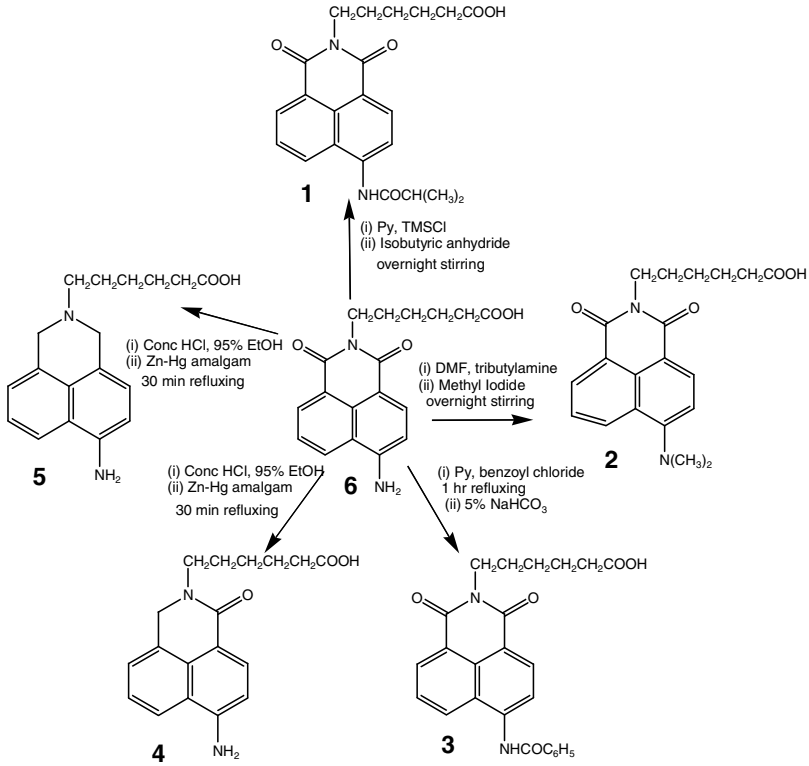

Fig. 1 Synthesis of fluorophores **1–5**

NMR ($CDCl_3$, TMS = 0.00):): 7.97–6.79 (d, 5H, Ar-H), 3.2–1.29 (t, 8H); 2.23 (s, 2H); 2.85 (s, 6H);^{13}C NMR ($CDCl_3$): 24.8, 26.8, 28.7, 35.8, 42.0, 44.0, 116.8, 121.0, 126.6, 127.7, 128.5, 134.4, 138.6, 161.3, 165.9, 177.0; MS (EI) m/z: 356.16 (M^+); Anal. calcd. for $C_{20}H_{22}N_2O_4$ C, 67.78; H, 6.26; N, 7.90; found C, 67.7; H, 6.19; N, 7.94; ΔG°: 183.88 [kJ/mol]; ΔH°: −330.91 [kJ/mol].

*6-(6-Benzoylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**3**)*

6-(6-Amino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (1.63 g, 5 mmol) was dried with anhydrous pyridine and dissolved in dry pyridine (25 mL). Benzoyl chloride (5.8 mL, 49.7 mmol) was added and the reaction mixture refluxed for 1 h. After heating, it was cooled gradually and ice was added. After 1 h, 5% $NaHCO_3$ (50 mL) solution was added and extracted with DCM and water. White crystals of the product were obtained in 83% yield. R_f, 0.7 (DCM:MeOH::9:1, v/v); UV(MeOH): λ_{max} 388 nm;^{1}H NMR ($CDCl_3$, TMS = 0.00): 7.97–6.79 (d, 5H, Ar-H), 7.95–7.44 (m, 5H Ar-H); 3.2–1.29 (t, 8H); 2.23 (s, 2H);^{13}C NMR ($CDCl_3$): 24.8, 26.8, 28.7, 35.8, 42.0, 111.5, 116.3, 120.4, 125.9, 126.7, 127.3, 128.6, 130.3, 131.9, 133.5, 137.7, 138.3, 151.6, 165.9, 177.0; MS (EI) m/z: 436.15 (M^+); Anal. calcd. for $C_{25}H_{22}N_2O_5$ C, 69.76; H, 5.15; N, 6.51; found C, 69.71; H, 5.0; N, 6.72; ΔG°: 168.53 [kJ/mol]; ΔH°: − 377.32 [kJ/mol] (Fig. 1).

*6-(6-Amino-1-oxo-1H,3H-benzo[de] isoquinolin-2-yl)-hexanoic acid (**4**)*

To a mixture of water and HCl (1.25 + 0.04 mL), added Zn (1.0 g) and $HgCl_2$ (40 mg) and stirred the mixture for 15 min. The liquid was decanted and this amalgamated zinc was used immediately. Amalgamated zinc (0.5 g) and conc HCl (0.3 mL) were gently refluxed for 15 min. 6-(6-Amino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (25 mg, 0.076 mmol), conc HCl (0.6 mL) and absolute ethanol (1.7 mL) were mixed in a flask and added dropwise to the above solution over 5 min. After addition, it was refluxed for 30 min and the liquid was decanted from Zn metal pieces. Reaction mixture was extracted with DCM and water. 6-(6-Amino-1-oxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid were obtained in 51% yield. R_f, 0.8, (DCM:MeOH::9:1, v/v); UV(MeOH): λ_{max} 352 nm;^{1}H NMR ($CDCl_3$, TMS = 0.00): 7.58–6.62 (m, 5H, Ar-H); 4.9 (s, 2H, Ar-H); 3.2–1.29 (t, 8H); 2.23 (s, 2H);^{13}C NMR ($CDCl_3$): 24.8, 27.1, 29.2, 35.8, 47.5, 53.7, 107.9, 119.3, 121.4, 123.6, 125.6, 127.1, 129.0, 134.4, 143.2, 166.4, 177.0; MS (EI) m/z: 314.15 (M^+); Anal. calcd. for $C_{18}H_{20}N_2O_3$ C, 69.21; H, 6.45; N, 8.97; found C, 68.9; H, 6.59; N, 9.0; ΔG°: 82.14 [kJ/mol]; ΔH°: − 309.01 [kJ/mol].

*6-(6-Amino-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (**5**)*

Compound **5** was prepared by repeating the above procedure and obtained in 48% yield. R_f, 0.6 (DCM:MeOH::9:1, v/v); UV(MeOH): λ_{max} 340 nm;^{1}H NMR ($CDCl_3$, TMS = 0.00): 7.47–6.39 (m, 5H, Ar-H); 4.06 (s, 4H, Ar-H); 2.36–1.29 (t, 8H); 2.23 (s, 2H);^{13}C NMR ($CDCl_3$): 24.8, 27.4, 29.8, 35.8, 53.9, 60.2, 109.3, 119.2, 120.6, 123.0, 124.5, 125.6, 126.2, 130.2, 132.0, 140.5, 177.0; MS (EI) m/z: 300.17 (M^+); Anal. calcd. for $C_{18}H_{22}N_2O_2$ C, 72.46; H, 7.43; N, 9.39; found C, 72.5; H, 7.3; N, 9.4; ΔG°: 204.73 [kJ/mol]; ΔH°: −171.31 [kJ/mol].

Synthesis of 5-[5-*N*-(9-fluorenylmethoxycarbonyl)-aminopentanoxy] uracil and 5-[5-*N*-(9-fluorenylmethoxycarbonyl)-aminopentanoxy]-2′-deoxyuridine

The linker arm at C-5 position of 5-bromouracil has been attached by using Williamson's synthetic procedure for ethers. FMOC protected 5-aminopentanol-1 has been used as linker arm. FMOC protected aminopentanol (1.456 g, 5.14 mmol) dissolved in DMSO (20 mL) was added dropwise to Na metal (140 mg) and stirred for 2.5 h and divided in two parts. 5-Bromouracil and 5-iodo-2′-deoxyuridine (0.8 g each) were added to first and second part, respectively, and the reaction mixtures were stirred for 18 h at room temperature. The dense violet coloured solutions were poured into water saturated with NaCl (150 mL) and then extracted with ethylacetate (40 mL × 3) and reduced to gum at low temperature, around 40°C. 5-[5-*N*-(9-fluorenylmethoxycarbonyl)-aminopentanoxy] uracil and 5-[5-*N*-(9-fluorenylmethoxycarbonyl) aminopentanoxy]-2′-deoxyuridine (7) were obtained and used without further purification.

*5-[5-N-(9-Fluorenylmethoxycarbonyl)-aminopentanoxy] uracil (2′, 3′, 5′-tri-O-benzoyl-β-D-ribo furanose) (**8**)*

Nucleoside of uracil was synthesized using ABR (1-*O*-acetyl-2, 3, 5-tri-*O*-benzoyl-*β*-D-ribofuranose). Uracil bearing side chain at C-5 position (675 mg, 1.37 mmol) was stirred with ABR (781.2 mg, 1.55 mmol) in acetonitrile (23.25 mL) and HMDS (0.26 mL, 1.24 mmol), TCS (0.158 mL, 1.24 mmol) and $SnCl_4$ catalyst (0.22 mL, 1.86 mmol) in CH_3CN (7.8 mL) were added and stirred for 20 h at room temperature. DCM (25 mL) was added and the reaction mixture was extracted twice with saturated solution of $NaHCO_3$ (aqueous). The organic layer was washed with saturated NaCl (aqueous), dried over anhydrous Na_2SO_4 and reduced to a small volume under vacuum.

*5′-O-Dimethoxytrityl-4-N-(tris-4, 9, 13-triazatridecane-1-yl)-2′-deoxycytidine (**9**)*

2′-Deoxycytidine hydrochloride (1.32 g, 5 mmol) was transiently *O*-protected with trimethylsilyl chloride (2.5 mL, 2 mmol) in anhydrous pyridine (20 mL). To this solution added *p*-toluenesulfonyl chloride (1.9 g, 10 mmol) and the reaction continued overnight [19] at 60°C. After workup in dichloromethane and aqueous saturated $NaHCO_3$, the organic phase was concentrated. The product was desilylated by treating with a mixture of pyridine and concentrated ammonia (15 mL each) for 4 h. Reaction mixture was concentrated. The crude 4-*N*-*p*-toluenesulfonyl-2′-deoxycytidine was redissolved in anhydrous pyridine (20 mL), added DMTrCl (1.7 g, 5 mmol) and the reaction was allowed to proceed for 3 h. After workup in DCM and aqueous 0.5 M $NaHCO_3$, the organic phase was dried and concentrated. The pure 5′-*O*-dimethoxytrityl-4-*N*-*p*-toluenesulfonyl-2′-deoxycytidine was obtained after silica gel column chromatography with DCM and methanol. This compound was again dissolved in anhydrous pyridine, added spermine (10 equivalents) and the reaction vessel was kept in an oven at 70°C for 19 h. After completion of the reaction, it was partitioned between water and DCM and the organic phase was concentrated under reduced pressure and the product was purified by silica gel column chromatography with DCM and methanol.

Attachment of fluorophore at linker arms of nucleosides

The FMOC group at linker arm in the uridine and 2′-deoxyuridine and benzoyl groups at sugar in uridine were removed by treating the protected nucleosides with sodium methoxide at room temperature for 1.5 h. At last, the fluorophore is attached to nucleosides bearing linker arm [20]. DCC (515 mg, 2.5 mol) was added separately to fluorophores 1 and 2 (311 mg, 1 mmol each) in dioxane (4 mL), dry pyridine (0.2 mL) and *p*-nitrophenol (140 mg, 1 mmol). The dicyclohexylurea precipitated out after 2.5 h. The precipitate was filtered out and each nucleoside (1 mmol) (**7**, **8**, **9**) suspended in DMF (5 mL) was added to the supernatant, separately. To these reaction mixtures, TEA (1 mL) was added, stirred and left overnight. The reaction mixtures were filtered and dried *in vacuo*. The products formed were identified by TLC due to the presence of fluorescence and purified chromatographically.

Synthesis of labelled phosphoramidites (**10**, **11** and **12**)

The fluorescently labelled nucleosides were finally converted to their respective phosphoramidites [21]. Labelled nucleosides were dried under reduced pressure for 5 h and dissolved in anhydrous acetonitrile (7.5 mL) separately.

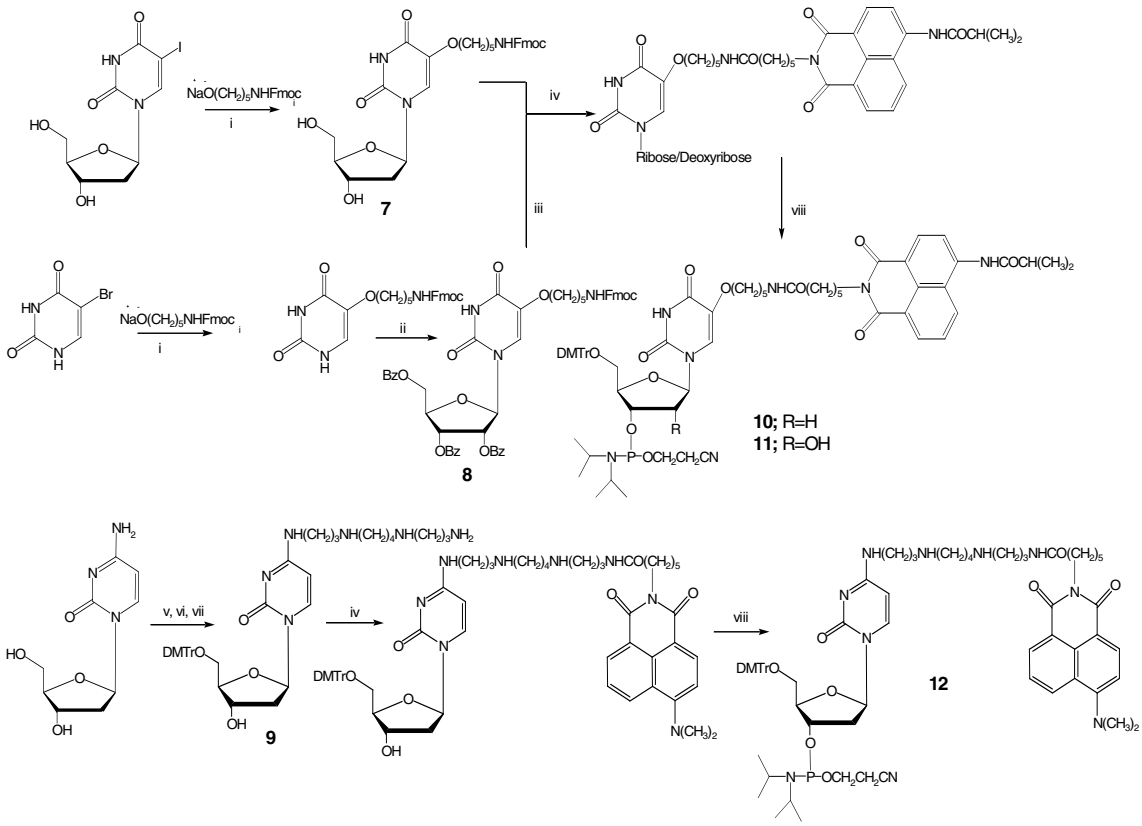

Fig. 2 Synthesis of fluorescently labelled phosphoramidites. (i) DMSO, 18 h stirring at rt; (ii) ABR, HMDS, TCS, $SnCl_4$, CH_3CN, 20 h stirring; (iii) MeOH, NaOMe, 1.5 h; (iv) 6-(6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid or 6-(6-dimethylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid/p-nitrophenol/DCC in dioxane pyridine.; (v) TMSCl in pyridine/p- TsCl, 10 h stirring, 60°C; (vi) pyridine-ammonia, 4 h stirring, DMTrCl in pyridine, 3 h stirring; (vii) spermine in pyridine, 19 h stirring at 70°C; (viii) acetonitrile, DIPEA, 2-cyanoethyldiisopropylchlorophosphoramidite, 1.5 h stirring

Diisopropylethylamine (1.2 mL, 6 mmol) was added and 2-cyanoethyldiisopropyl chlorophosphoramidite (0.71 mL, 3 mmol) was added dropwise to the solution and left for 1.5 h at rt. The reaction mixture was worked up with saturated aqueous solution of $NaHCO_3$. The reaction mixture was extracted three times with DCM containing 1% TEA. The combined layers were dried with anhydrous Na_2SO_4 and evaporated till dryness. The product was purified by silica gel column chromatography in ethylacetate and hexane containing 1% TEA. The fractions containing 3′-*O*-phosphoramidite were collected and lyophilized. The synthetic procedure has been outlined in Fig. 2.

Synthesis of labelled oligodeoxyribonucleotides

The oligodeoxyribonucleotides have been synthesized on Pharmacia LKB Gene Assembler Plus on 0.2 μmol scale using standard protocols. The CPG attached 12-mer, DMT-d(AGTGGGTTAAGA), was placed into a vial and the resin was washed with anhydrous HPLC grade dioxane (3 mL × 3). Carbonyldiimidazole (50 mg) dissolved in dioxane (1.0 mL) was added to it and the reaction was allowed to proceed for 30 min at room temperature with occasional shaking. After this, the solid support was washed well with dioxane (3 mL × 3). The 5′-activated oligodeoxyribonucleotide was then allowed to react with hexamethylenediamine (1.0 mL, 0.2 M) dissolved in dioxane:water (9:1) in the same reaction vessel. The reaction was allowed to proceed for 20 min at room temperature. Finally, the support was washed with dioxane and methanol. Similarly, the other oligodeoxyribonucleotide, DMT-d(TCTTAACCCACT), was also derivatised at its 5′-end to bear a primary amino group.

These derivatised oligodeoxyribonucleotides were cleaved from their support with concentrated ammonia (25%, 4 mL) for 1 h at rt. The support was filtered out and the aqueous ammonical solution was heated at 55°C for 10 h. Ammonia was removed under vacuum and the oligodeoxyribonucleotides were then purified by Reversed Phase HPLC using C_{18} Lichrosphere column and gradient buffer CH_3COONH_4, 0.1 M, pH 7.1 (A) and CH_3CN (B) having flow rate, 1 mL/min and a linear gradient from 0 to 25% B in 25 min. The absorption was monitored at 260 nm for peak detection. The retention time observed for d(AGTGGGTTAAGA) (10 OD, 280 μg) was 9 min and for d(TCTTAACCCACT) (11

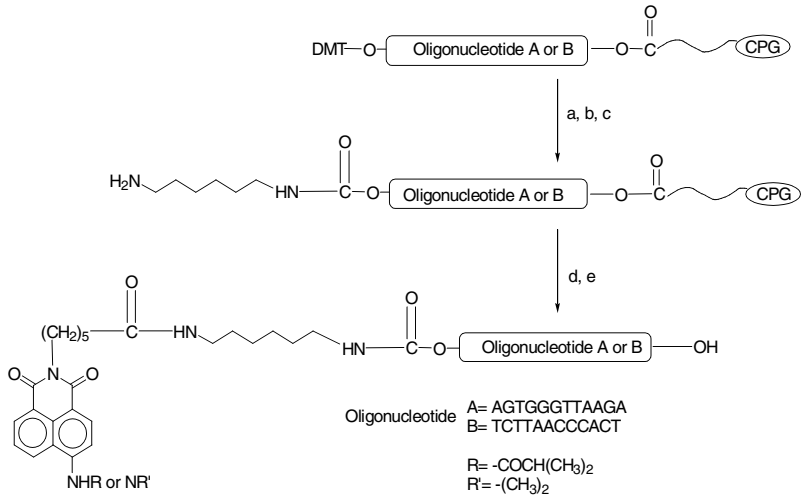

Fig. 3 Fluorescent labelling of oligodeoxyribonucleotides. (a) Dichloroacetic acid in dichloromethane (3%). (b) Carbonyldiimidazole dissolved in dioxane. (c) Hexamethylenediamine in dioxane:water. (d) Concentrated ammonia (25%) 10 h at 55°C. (e) *p*-Nitrophenyl ester of fluorophore dissolved in $Na_2CO_3/NaHCO_3$ buffer (pH 9.0):DMF:H_2O::5:2:3, v/v

OD, 363 μg) was 8.2 min. The desired fractions were pooled and lyophilized.

p-Nitrophenyl esters [17] of fluorophores were prepared by dissolving the fluorophores (0.054 mM) separately in dry dioxane (2.0 mL) containing dry pyridine (0.2 mL), TEA (0.2 mL) and *p*-nitrophenol (7.56 mg, 0.054 mM). Dicyclohexylcarbodiimide (27.8 mg, 0.135 mM) was added and the reaction mixture was left for 2.5 h with occasional shaking. Dicyclohexylurea was removed by filtration to obtain the *p*-nitrophenyl ester of 6-(6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid and 6-(6-dimethylamino-1,3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (yield 65%) (Fig. 4).

5′-Alkylamino oligodeoxyribonucleotides (6.5 OD and 8 OD for oligonucleotides A and B, respectively) were dissolved separately in 250 μL solutions of a mixture of 0.1 M $Na_2CO_3/NaHCO_3$ (pH 9.0). To these solutions, 500 μL solutions of *p*-nitrophenyl ester of fluorophores dissolved in a mixture of 1.0 M $Na_2CO_3/NaHCO_3$ buffer (pH 9.0):DMF:H_2O (5:2:3. v/v) were added and the reaction mixtures were vortexed and wrapped with aluminium foil to prevent light exposure. The synthetic procedure has been outlined in Fig. 3. The reaction mixture was stirred for 8 h at room temperature in dark. The labelled oligodeoxyribonucleotides were then passed through Sephadex G-25 column using ethanol-water (8:2, v/v) as eluent. The filtrates containing fluorescently labelled oligodeoxyribonucleotides were collected, concentrated and resuspended in water (1.0 mL) and purified by Reversed Phase HPLC using C_{18} Lichrosphere column and gradient buffer CH_3COONH_4, 0.1 M, pH 7.1 (A) and CH_3CN (B) having flow rate, 1 mL/min and a linear gradient from 0 to 25% B in 25 min. The absorption was monitored at 260 nm for peak detection. The retention time observed for labeled d(AGTGGGTTAAGA) was 16.5 min and for d(TCTTAACCCACT) was 15.8 min. The desired fractions were pooled and lyophilized. Finally, 150 μg (6 OD; 0.03 μmol) and 165 μg (7 OD; 0.035 μmol) labeled oligodeoxyribonucleotides were obtained. Analysis of ESI-MS of labeled d(AGTGGGTTAAGA) and labeled d(TCTTAACCCACT), m/z = 4285.53 and m/z = 4034.67, respectively.

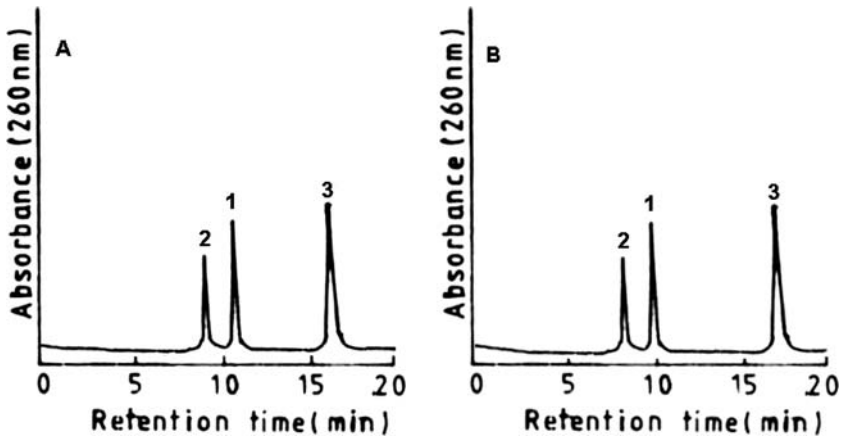

Fig. 4 RPHPLC Profile of oligodeoxyribonucleotides (**A**) d(AGTGGGTTAAGA) and (**B**) d(TCTTAACCCACT); **1**, oligodeoxyribonucleotide with 5′-DMT group; **2**, Oligodeoxyribonucleotide with linker arm; **3,** fluorescently labelled oligodeoxyribonucleotide. Purification was done using C_{18} Lichrosphere column and gradient buffer CH_3COONH_4, 0.1 M, pH 7.1 and CH_3CN

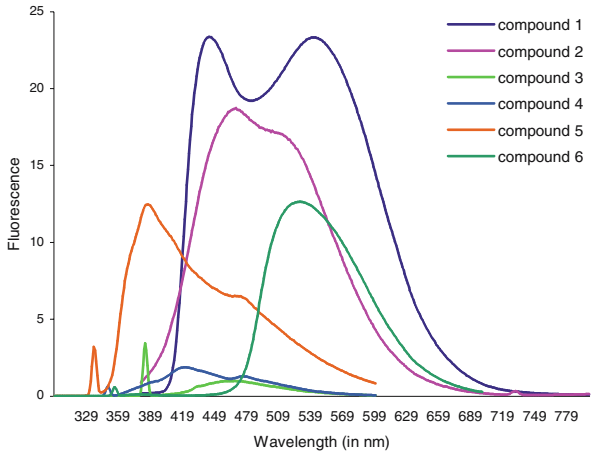

Fig. 5 Comparative fluorescence of all fluorophores in MeOH

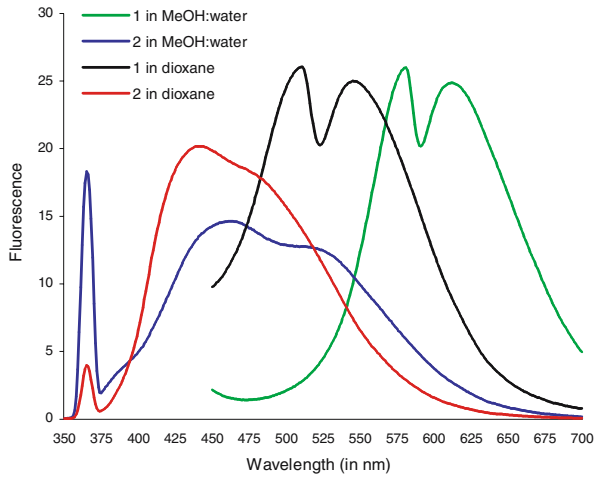

Fig. 6 Comparative fluorescence of compounds 1 & 2 in dioxane and MeOH:water

Results and discussion

The present work is an effort towards the development of highly sensitive fluorophores. Several such fluorophores have been synthesized and characterized. These fluorophores contain $–NH_2$, $–NHCOCH(CH_3)_2$ and $–N(CH_3)_2$ groups as chromophores which enhance the degree of fluorescence in the molecules whereas chromophore like $–COC_6H_5$ reduces the degree of fluorescence. Some of the fluorophores showed very good fluorescence and have been used for labelling of oligodeoxyribonucleotides. These fluorophores have detection limits upto 10^{-9} mol/L.

1,8-Naphthalimide derivatives have two sub-systems, viz. the naphthalene ring and a dicarboximide (–CO–N–CO–) group in a six membered ring. The photophysical properties and the colour characteristics of 1,8-naphthalimide depend basically on the polarization of the molecules. The irradiation induced polarization of 1,8-naphthalimide molecules causes an electron donor-acceptor interaction between the substituents at C-4 and the carbonyl groups from the imide structure of the chromophorous system.

The fluorescence of the molecules depends on their compositions and certain substituents have very appreciable effect. Electron releasing substituents have favourable effect [22] which explained the high fluorescence of fluorophores **1** and **2**, while in case of fluorophore **3**, fluorescence decreased as benzoyl substituent does not donate electron like isobutyryl and dimethyl groups. An interesting result was obtained when one of the oxo groups was reduced, the fluorescence decreased drastically whereas the fluorescence remained almost the same as that of original compound **6**, when both of the oxo groups were reduced *albeit* the colour changed from greenish yellow to greenish red. This behaviour may be due to the interaction between 1,8-naphthalimide and methanol molecules. The H-bonds between hydroxyl groups of the methanol and the carboxylic oxygen atom (C=O) from the naphthalimide system occur in the hydroxylated methanol solution. These H-bonds favour the radiation-less transition causing a decrease of the fluorescence quantum yield of 1,8-naphthalimides.

The fluorophores were dissolved separately in MeOH at 10 μM concentrations and their fluorescence was recorded (Fig. 5). The quantum yields of compounds were estimated on the basis of absorption and fluorescence spectra. The quantum yields were found in the range 0.52–0.12. Their photophysical properties of these fluorophores have been summarized in Table 1.

The decreasing order of fluorescence of these fluorophores was **1** > **2** > **6** ≈ **5** > **4** > **3**. We have chosen **1** and **2** for labelling of nucleosides and oligodeoxyribonucleotides and studying their effect on oligodeoxyribonucleotide hybridization.

The fluorescence of these two molecules was also recorded in organic and inorganic media, like dioxane, methanol-water (50:50) and NaCl, KCl, $MgSO_4$ and

Table 1 Photophysical properties of fluorophores **1–5**

Compounds	λ_{max} (nm)	ε (L/mol cm × 10^4)	λ_{ex} (nm)	λ_{em} (nm)	Φ
1	397	1.12	397	446	0.52
2	368	1.05	368	470	0.47
3	384	0.73	388	465	0.12
4	352	0.61	352	425	0.29
5	337	0.99	340	390	0.36

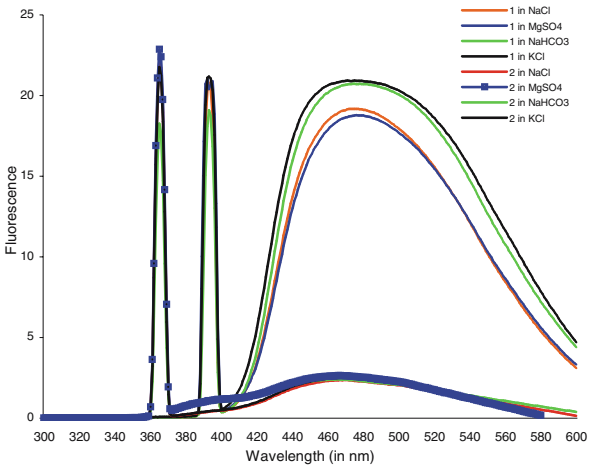

Fig. 7 Comparative fluorescence of compounds 1 and 2 in different inorganic media

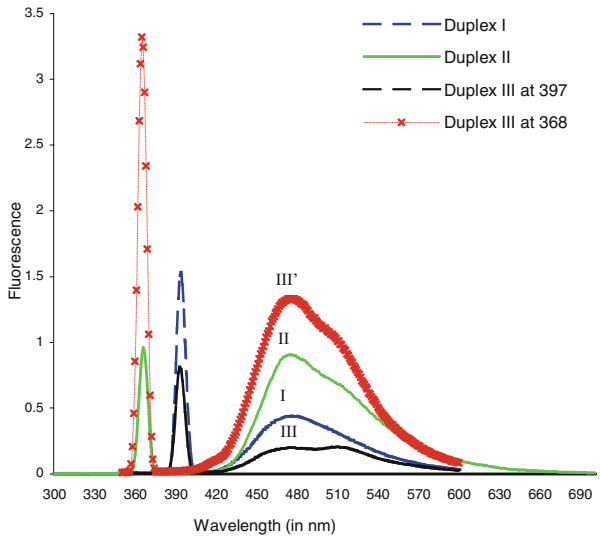

Fig. 8 Comparative fluorescence of oligodeoxyribonucleotides in phosphate buffer

$NaHCO_3$. Fluorescence of both the fluorophores increased to a little extent in dioxane than in methanol while in methanol-water, fluorescence of **1** increased a little but in the case of **2**, it decreased noticeably (Fig. 6). Fluorophore **1** showed higher degree of fluorescence in inorganic media than **2**, and hence it may be a better choice for biological systems (Fig. 7). However, the fluorescence of both the fluorophores was lower in inorganic media than in organic media.

The coupling of these fluorophores with oligodeoxyribonucleotides through its activated ester is efficient and fluorophores **1** and **2** have been covalently attached to oligodeoxyribonucleotides bearing 5′-NH_2 function generated by the reaction between oligodeoxyribonucleotides and hexamethylenediamine mediated by CDI. Such amino protected oligodeoxyribonucleotides can be stored for longer time also.

The DMT-oligodeoxyribonucleotides, amino linker bearing oligodeoxyribonucleotides and fluorescently labelled oligodeoxyribonucleotides were purified by HPLC on C_{18} column using gradient buffer CH_3COONH_4, 0.1 M, pH 7.1 and CH_3CN and absorption monitored at 260 nm for peak detection. The retention time observed for d(AGTGGGTTAAGA) were 9, 10.7 and 16.5 min (Fig. 4A) and for d(TCTTAACCCACT) were 8.2, 10 and 15.8 min (Fig. 4B). Fluorescently labelled oligodeoxyribonucleotides were also monitored through UV detector of HPLC (Fig. 8).

In order to assess the effect of these fluorophores on hybridisation we have taken the following four duplexes for fluorescence and thermal denaturation studies (Fig. 9).

The fluorescently labelled oligodeoxyribonucleotides (0.44 μM each) were dissolved separately in sodium phosphate buffer (0.01 M; pH 7.1), mixed and heated to 95°C in a water bath and allowed to hybridise. The fluorescence of these labelled duplexes was scanned in the range of 300–700 nm at room temperature. Excitation wavelengths were fixed at 397 and 368 nm for duplexes I and II, respectively and the duplex III was excited once at 397 nm (III) and then at 368 nm (III′). The emissions were recorded at 475 nm. The study showed that fluorescence of fluorophore **2** increased 1.5 times when used in combination with **1** as compared to its fluorescence observed in the case of duplex II at the same excitation wavelength, i.e., 368 nm. Whereas the fluorescence of the fluorophore **1** decreased to half when used in combination with fluorophore **2** at its excitation wavelength 397 nm in comparison to its fluorescence in duplex I (Fig. 8).

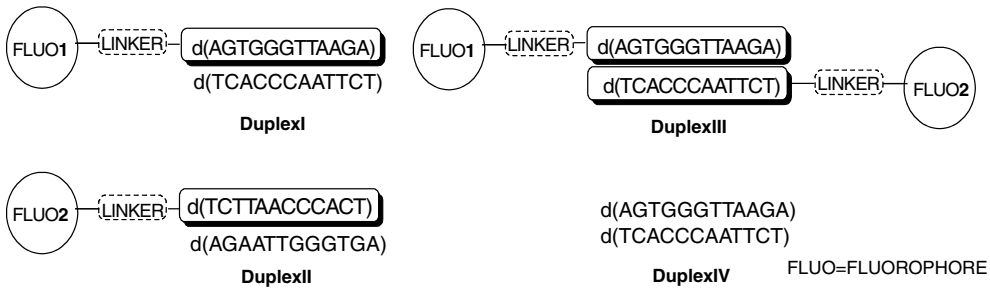

Fig. 9 Fluorescently labelled duplexes (I–III) and unlabelled duplex (IV)

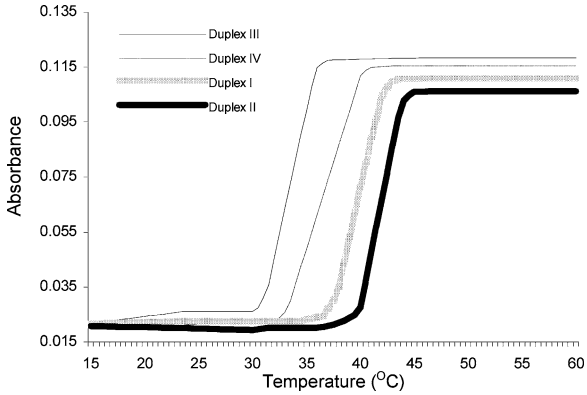

Fig. 10 Thermal denaturation graph of duplexes I–IV

The thermal denaturation studies (Fig. 10) showed that the singly labelled duplexes I and II melted at a higher temperature (T_m; 39 and 41°C, respectively) than the unlabelled duplex IV (T_m; 36°C), whereas the doubly labelled duplex III melted at slightly lower temperature (T_m; 34°C). The increase in Tm in the case of duplexes I and II derives support from an earlier literature where oxygen atoms enhance the stability of duplex by interacting in the major grooves of duplexes [23]. However, lowering of T_m in the case of duplex III remains unanswered.

An interesting observation was that the fluorescence of labelled oligodeoxyribonucleotide, d(TCTTAACCCACT)-Linker-Fluorophore-2, increased when it formed a duplex II with its complementary sequence. This clearly has been supported by fluorescence measurement at varying temperature where the fluorescence was drastically decreased beyond its melting point, i.e., 41°C. However, the fluorescence was almost unchanged in the case of duplexes I and III (Fig. 11).

The covalent attachment of these fluorophores to nucleosides, uridine and 2′-deoxyuridine, through linker arm has

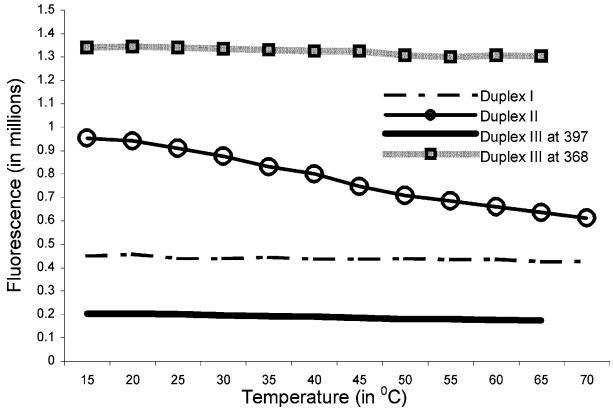

Fig. 11 Comparative fluorescence of duplexes I–III with respect to temperature

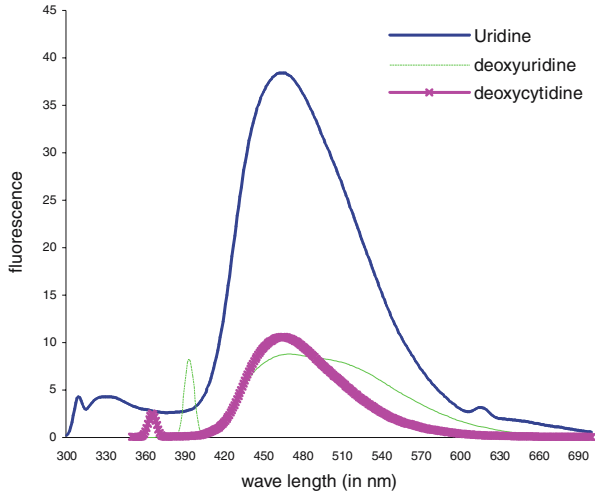

Fig. 12 Comparative fluorescence of labelled nucleosides uridine, deoxyuridine and deoxycytidine in MeOH

been done using a new method, i.e., Williamson's synthetic procedure used for synthesis of ethers. This procedure is simple and does not require costly chemicals as needed in Pd or Hg catalyzed olefination reactions. Further, their phosphoramidites were synthesized which can be used for synthesis of multiple labelled oligonucleotides.

Fluorescence of fluorescently labelled nucleosides and phosphoramidites was recorded in MeOH at 10 μM concentrations which showed that fluorescence in case of phosphoramidites of deoxycytidine and deoxyuridine, increased considerably while fluorescence of phosphoramidites of uridine increased slightly (Figs. 12 and 13).

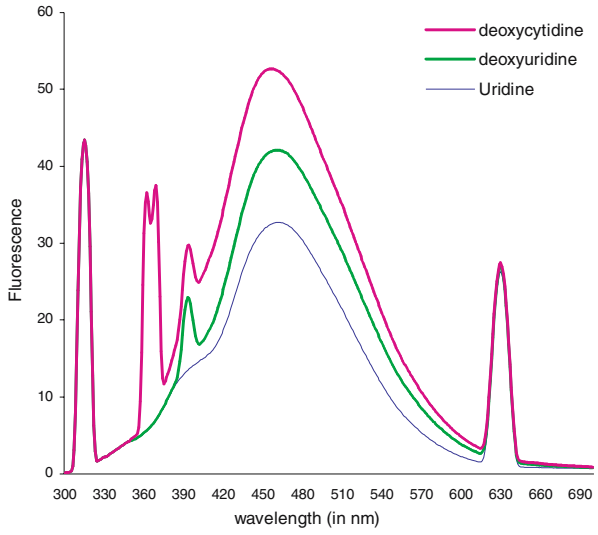

Fig. 13 Comparative fluorescence of phosphoramidites of labelled uridine, deoxyuridine and deoxycytidine in MeOH

Conclusion

We have synthesized highly sensitive fluorophores for labelling of oligonucleotides or other biological molecules and converting them into suitable probes. These molecules can also be used in multiple labelling of oligonucleotides for getting enhanced signals of fluorescence at very low concentrations. These molecules are supposed to have tremendous potential for applying to biological systems as these are very stable in inorganic media and give good fluorescence in the presence of ions like Na^+, K^+, etc. The synthesis of multiple labelled oligonucleotides is in progress in our laboratory.

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