Synthesis and fluorescence studies of fluorescently labelled phosphoramidites: synthons for multiple labelled oligonucleotides

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Two highly fluorescent compounds, viz. 6-[6-isobutyryl-amino-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 have been synthesized and attached to uridine and 2′-deoxyuridine at the C-5 position using a suitable linker, 5-aminopentanol-1, and to 2′-deoxycytidine at the C-4 position using spermine as a linker molecule. These nucleosides have been converted to their respective phosphoramidites and thus used to synthesize labelled oligonucleotides, showing appreciable fluorescence, on solid support.

Keywords: Fluorophores, labelled oligonucleotides, linkers, phosphoramidites.

FLUORESCENTLY labelled probes are widely used in molecular biology and have attracted attention in the detection of nucleic acids hybridization[1], DNA sequencing[2,3], nucleic acid–protein interaction[4] and applications in medical diagnostics[5]. Fluorophores represent an attractive class of reporter molecules since they are stable, directly detectable and offer an opportunity for the simultaneous detection of multiple probes[6]. Fluorescent molecules are replacing radioisotopes, as these molecules on excitation emit light in the UV–visible region and can be detected directly without requiring any procedural work-up[7].

Fluorescence is dependent on solvent, pH, temperature and concentrations. Hence fluorescent probes can be used for studies of molecular interactions[8], cellular functions, biochemical processes[9], etc.

Fluorescent labels are chemically incorporated directly during oligonucleotide synthesis to control the number of labels in each DNA molecule. Fluorescent molecules can be covalently attached by various enzymatic[10] or chemical methods[11–13] through various active sites on bases, sugars (3′, 5′) or phosphate units of oligonucleotides[14–16]. These molecules are attached at the end of spacer arms or side chains of nucleobases and sugars. Nucleosides bearing side arms terminating in groups like $-NH_2$, have often been used as linkers for post-synthesis modifications[17].

We have synthesized two highly fluorescent molecules, viz. 6-[6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl]-hexanoic acid (fluorophore **1**) and 6-(6-dimethylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (fluorophore **2**) and attached them to nucleosides – uridine and 2′-deoxyuridine at the C-5 position and finally converted them to their respective phosphoramidites (Figure 1 *a–c*). In the present work, we have introduced five-carbon chain at C-5 of 5-bromouracil and 5-iodo-2′-deoxyuridine. Nucleoside of 5-bromouracil has been synthesized by first attaching 5-aminopentanol-1 at the C-5 position and then coupling with ABR (β-D-ribofuranose-1-acetate-2,3,5-tribenzoate)[18].

The linker arm at the C-5 position of 5-bromouracil has been attached using Williamson's synthetic procedure for ethers. FMOC-protected 5-aminopentanol-1 is used as the linker arm. FMOC-protected aminopentanol (1.456 g, 5.14 mmol) dissolved in DMSO (20 ml) was added dropwise (8 ml) 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 the 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 150 ml water saturated with NaCl and then extracted with ethylacetate (40 ml × 3) and reduced to gum at low temperature, around 40°C.

Nucleoside of uracil was synthesized using ABR (1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose). Uracil bearing side chain at the 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. Then 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. 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), *p*-toluenesulfonyl chloride (1.9 g, 10 mmol) added 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. The reaction mixture was concentrated, the crude 4-*N*-*p*-toluenesulfonyl-2′-deoxycytidine was redissolved in anhydrous pyridine (20 ml), DMTrCl (1.7 g, 5 mmol) added 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, spermine (10 equivalents) added 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,

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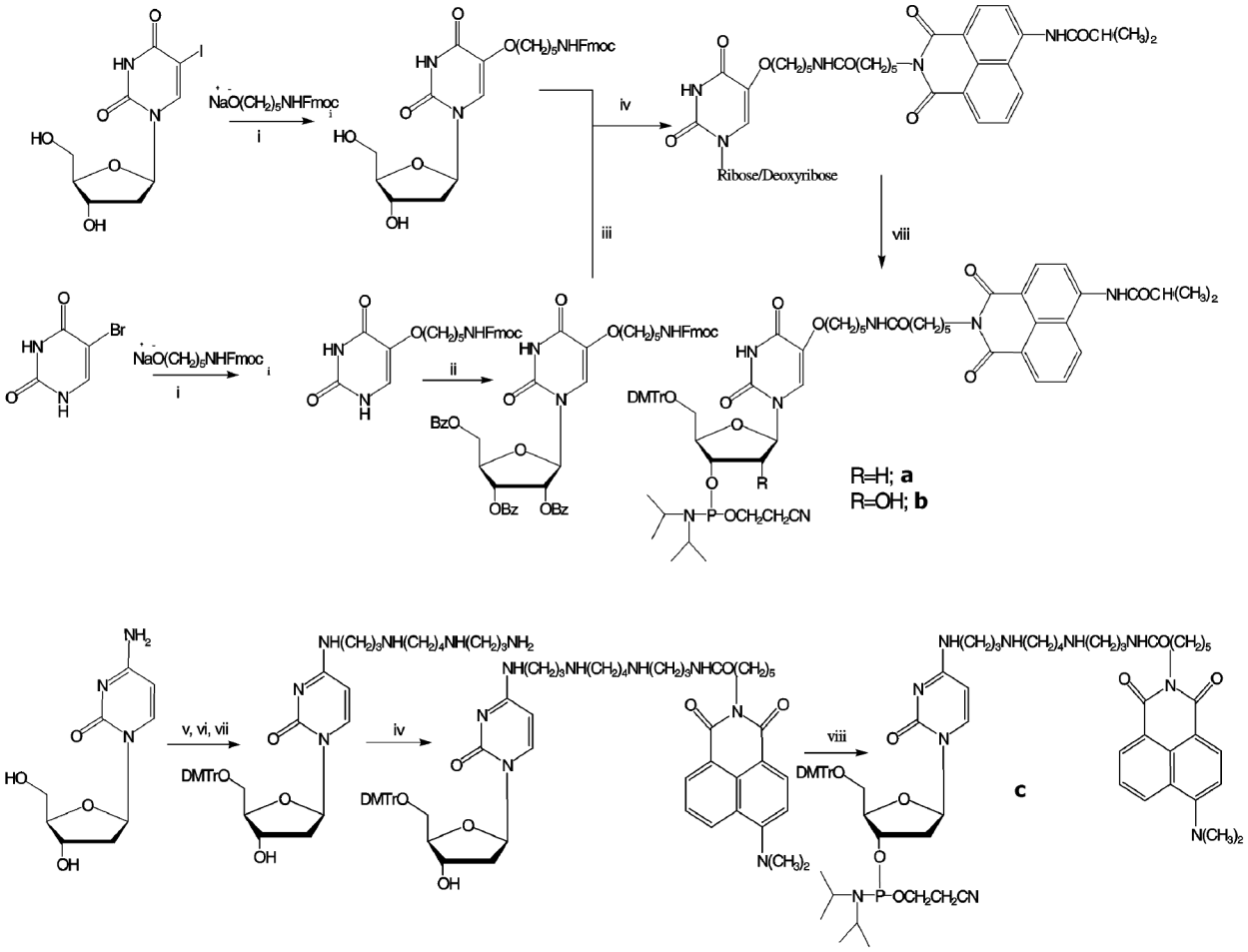

Figure 1. Synthesis of fluorescently labelled phosphoramidites. (i) DMSO, 18 h stirring at rt; (ii) ABR, HMDS, TCS, $SnCl_4$, CH_3CN, 20 h stirring; (iii) MeOH, NaOMe, 1.5 h; (iv) 6-[6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl]-hexanoic acid or 6-(6-dimethylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic 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.

Spectral data for compound ***a*** (in Figure 1): ^{1}HNMR ($CDCl_3$); 1.05 (d, 12H), 1.24 (s, 9H), 1.29 (m, 4H), 1.57 (m, 4H), 1.66 (t, 2H), 2.18 (t, 2H), 4.28 (s, 1H), 2.97 (m, 2H), 3.2 (t, 4H), 3.57 (s, 1H), 3.96 (s, 1H), 5.93 (s, 1H), 6.8–7.97 (m, 18H); UV (MeOH) λ_{max} 310; ^{31}PNMR ($CDCl_3$) 146.

Spectral data for compound ***b*** (in Figure 1): ^{1}HNMR ($CDCl_3$); 1.05 (d, 12H), 1.24 (s, 9H), 1.29 (m, 4H), 1.57 (m, 4H), 1.66 (t, 2H), 2.18 (t, 2H), 2.22 (d, 2H), 2.97 (m, 2H), 3.2 (t, 4H), 3.57 (s, 1H), 3.66 (d, 2H), 3.9 (t, 2H), 3.96 (s, 1H), 5.85 (s, 1H), 6.8–7.97 (m, 18H); UV (MeOH) λ_{max} 310; ^{31}PNMR ($CDCl_3$) 146.

Spectral data for compound ***c*** (in Figure 1): ^{1}HNMR ($CDCl_3$); 1.05 (d, 12H), 1.29 (t, 2H), 1.41 (t, 2H), 1.59 (t, 2H), 1.67 (m, 4H), 2.22 (d, 2H), 2.55 (t, 8H), 2.65(t, 2H), 2.85 (s, 6H), 2.97 (m, 2H), 3.2 (t, 4H), 3.57 (s, 1H), 3.91 (t, 1H), 6.8–7.9 (m, 18H); UV (MeOH) λ_{max} 322; ^{31}PNMR ($CDCl_3$) 146.3.

the organic phase was concentrated under reduced pressure and the product was purified by silica gel column chromatography with DCM and methanol.

The FMOC group at the linker arm in uridine and the 2′-deoxyuridine and benzoyl groups at the sugar in uridine were removed by treating the nucleosides with sodium methoxide at room temperature for 1.5 h. At last the fluorophore is attached to the nucleosides bearing the 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) suspended in DMF (5 ml) was added to the supernatant. To this reaction mixture, TEA (1 ml) was added, stirred and left overnight. The reaction mixture was filtered and dried *in vacuo*. The products formed were characterized by TLC due to the presence of fluorescence and purified chromatographically.

The fluorescently labelled nucleosides were finally converted to their respective phosphoramidites[21] (Figure 1 *a–c*). Labelled nucleosides were dried under reduced pressure for several hours and dissolved in anhydrous acetonitrile (7.5 ml) separately. Diisopropylethylamine (1.2 ml, 6 mmol) was added and 2-cyanoethyldiisopropyl chlorophosphoramidite (0.71 ml, 3 mmol) was added drop-

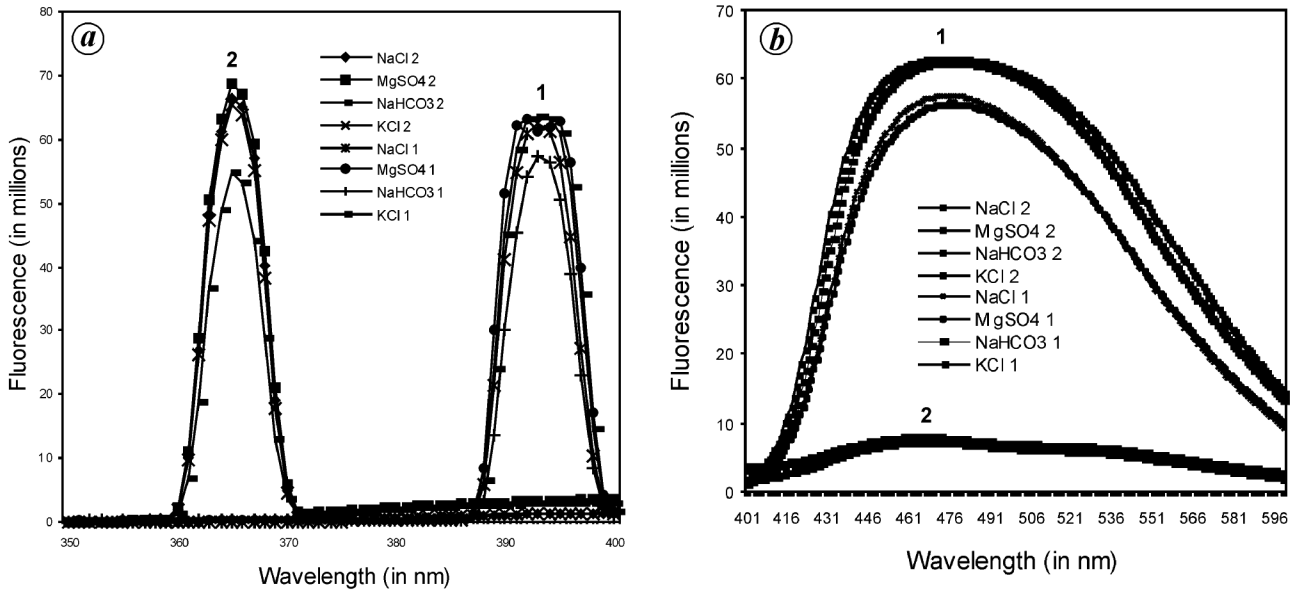

Figure 2. Excitation spectra (*a*) and emission spectra (*b*) of fluorophores **1** and **2** at 397 and 368 nm respectively, in inorganic media.

wise to the solution and left for 1.5 h at room temperature. 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. Fractions containing 3′-*O*-phosphoramidite were evaporated and lyophilized. The synthetic procedure has been outlined in Figure 1.

The oligonucleotides have been synthesized on Pharmacia LKB Gene Assembler Plus on 0.2 μmol scale using standard protocols. The DMT group from LCAA-CPG attached 11-mer, DMT-d(GTGGGTTAAGA) was removed using dichloroacetic acid in dichloromethane. 2′-Deoxyuridine-3′-*O*-phosphoramidite activated by tetrazole to form 2′-deoxyuridine-3′-*O*-phosphomonotetrazolide was added onto the column, as it undergoes rapid nucleophilic substitution reaction with hydroxyl group of CPG-bound oligonucleotide and forms an internucleotide phosphite-triester linkage. The coupling yield of this step was 98.9% as detected by trityl analysis[20]. This phosphite-triester was oxidized to phosphotriester using iodine in THF, 2,6-lutidine and water. This fluorescently labelled oligonucleotide was delinked from the LCAA-CPG support by ammonia treatment and purified on reversed phase HPLC, after removal of ammonia and phosphate protecting groups, using 0.1 M triethylammonium acetate and acetonitrile.

The above procedure was repeated with support-bound DMT-d(CTTAACCCACT) and fluorescently labelled 2′-deoxycytidine-3′-*O*-phosphoramidite to synthesize fluorescently labelled oligonucleotide, d(C*CTTAACCCACT).

We have studied the fluorescence of fluorophores, phosphoramidites at 10 μmol/l and oligonucleotides at 0.03 OD concentration levels. The fluorescence of fluorophores was recorded in 1 M solution of NaCl, $MgSO_4$, $NaHCO_3$ and KCl and scanned in the range 350–600 nm. Fluorophores **1** and **2** were excited at wavelengths 397 and 368 nm, respectively and their excitation spectra have been shown in Figure 2 *a*.

Fluorophore **1** showed higher degree of fluorescence than fluorophore **2**. Fluorescence of these fluorophores has been studied in inorganic medium. The results showed that inorganic ions enhanced fluorescence of fluorophore **2** to a lesser extent compared to fluorophore **1** (Figure 2 *b*). Hence good fluorescence signals can be obtained when these fluorophores are used in biological media having inorganic ions.

The fluorescence of phosphoramidites of these labelled nucleosides was recorded in dichloroethane and both were scanned in the range 400–700 nm. Excitation wavelengths were fixed at 397 nm for uridine and 2′-deoxyuridine, and 368 nm for 2′-deoxycytidine, respectively. The emissions were recorded at 465 nm. Phosphoramidites of labelled 2′-deoxycytidine showed higher fluorescence than phosphoramidites of labelled uridine (Figure 3 *a*). In general, phosphoramidites show higher sensitivity than labelled nucleosides.

Fluorescence of both the labelled oligonucleotides at 0.03 OD each, was studied in phosphate buffer (0.01 M; pH 7.1). d(U*GTGGGTTAAGA) and d(C*CTTAACCCACT) were excited at 397 and 368 nm, respectively, and scanned in the range 400–650 nm. These labelled oligonucleotides show appreciable degree of fluorescence (Figure 3 *b*).

Thus, we have evolved a short and efficient way of synthesizing labelled phosphoramidites bearing indigenously developed fluorophores with high degree of fluorescence, which have been used for synthesizing fluorescently labelled oligonucleotides. Further, these nucleoside

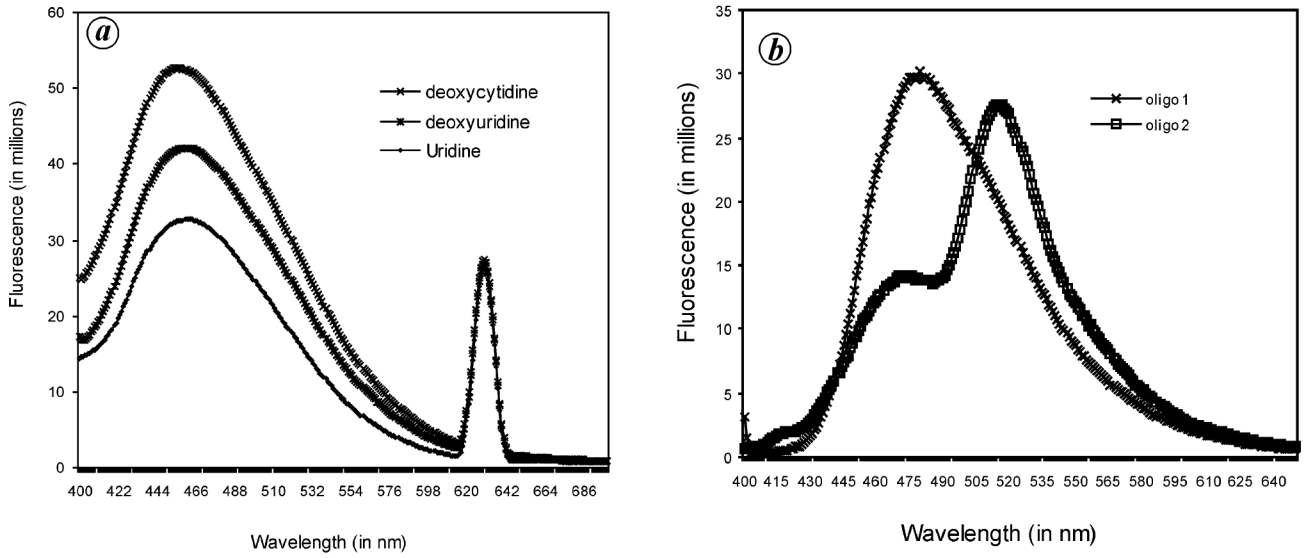

Figure 3. Emission spectra of labelled phosphoramidites (*a*) and oligonucleotides (*b*).

phosphoramidites can be used for multiple labelling of oligonucleotides (DNA/RNA) during their solid phase synthesis and the respective nucleoside triphosphates can be utilized for synthesizing multiple-labelled oligonucleotides using the enzymatic procedure – a method comparable to the template-directed synthesis of digoxigenin labelled oligonucleotides. Work is in progress in our laboratory.

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