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A Probe for Detection of G-Rich Target Strands through Fluorescence Quenching[1]

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Abstract—A modified fluorescent probe U^{F}AA AAT CTC CGC CGC was synthesized using the nucleoside analogue 3'-*O*-(*N,N'*-diisopropylamino-2-cyanoethoxyphosphinyl)-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(dansyl-1-sulfonamidohexylaminocarbonyl)uridine for hybridization studies with perfectly matched (U/A) complementary DNA and with a DNA strand having similar G-rich telomeric units at their 3'-ends. Data on the thermal stability and decrease in fluorescence intensity due to the presence of dG units clearly demonstrated the potential application of this approach in DNA diagnostics in homogeneous hybridization assays.

Key words: deoxyguanosine, quenching, telomere

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INTRODUCTION

Telomeres, the critical components of genomes, are specialized nucleoprotein structures that cap the end of almost every eukaryotic chromosome. Among eukaryotes, several characteristic features of telomeric DNAs are highly conserved and one of their important functions consists in the chromosome stabilization from the loss of DNA during cell division just like a "maintenance workshop". Telomerase, a cellular nucleoprotein reverse transcriptase, is responsible for the strand elongation. The dynamic telomeric nucleoprotein structure interacts with telomerase to prevent the gradual loss of DNA sequence from the chromosome end. The 3'-end of each strand of the duplex linear chromosomal DNA molecules is simply a tandemly repeated sequence of clusters of G residues as an overhang [1]. With limited variations, eukaryotic species has a characteristic telomeric repeat sequence and telomerase enzyme is responsible for the synthesis of G-rich strand. Telomerase first reported [2] in Tetrahymena is shown to have repeats of telomeric DNA sequences TTGGGG. Thus, utilizing the characteristic telomeric G-rich strands we have designed and synthesized a modified complementary fluorescence probe in which the microenvironment-sensitive dansyl fluorophore is covalently attached at 2'-hydroxyl of 5'-terminal uridine through a suitable spacer arm of six carbon chain. It is well known that the not planar dansyl molecule exhibits enhancement in fluorescence intensity in the hydrophobic environment [3, 4].

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RESULTS AND DISCUSSION

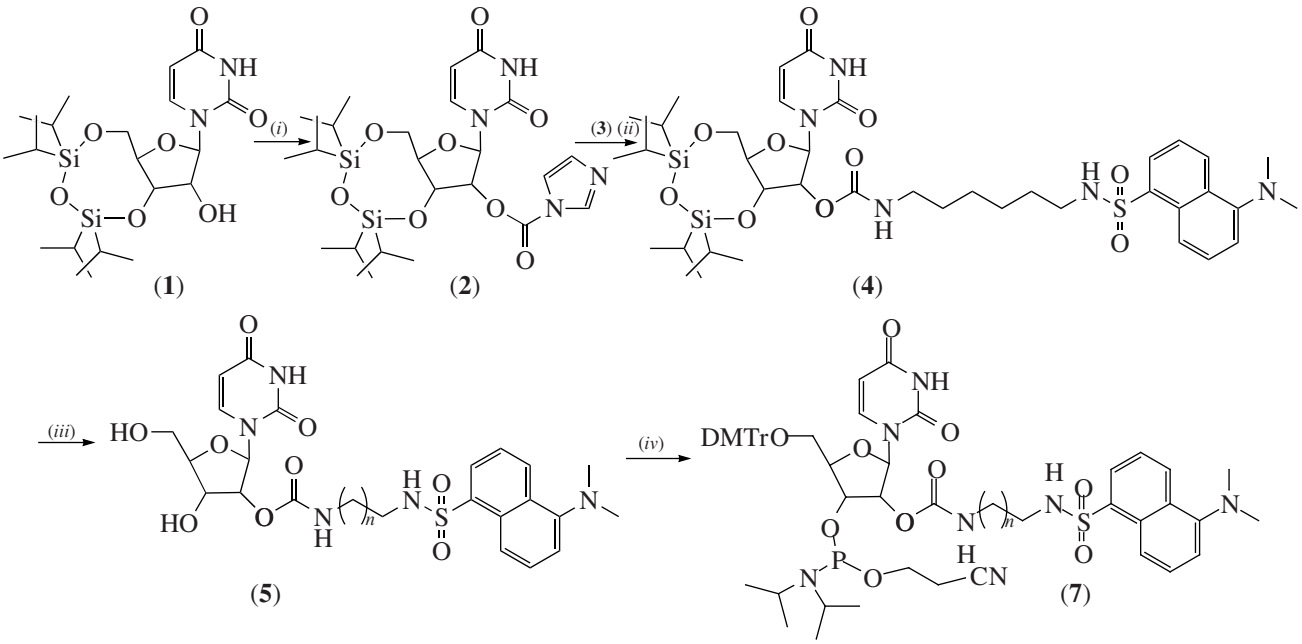
We carried out the chemical synthesis of a modified nucleoside analogue 3'-*O*-(*N,N'*-diisopropylamino-2-cyanoethoxyphosphinyl)-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(dansyl-1-sulfonamidohexylamino carbonyl) uridine (**7**) as shown in scheme 1. 3',5'-*O*-(Tetraisopropyldisiloxane-1,3-diyl)uridine (**1**) was obtained by preliminary protection of 3',5'-hydroxy groups of uridine with silylating reagent as previously described in [5] and then treatment with CDI in dry dichloromethane to get 2'-*O*-(imidazol-1-ylcarbonyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine (**2**) in quantitative yield. The imidazolyl derivative was subsequently condensed with 6-aminohexyldansylsulfonamide (**3**) by overnight stirring of the reaction mixture; the desired product was isolated by column chromatography on silica gel. For the synthesis of (**3**), hexamethylene-1,6-diamine was dissolved in 10% $NaHCO_3$ and treated with dansyl chloride. Monitoring by TLC showed the complete conversion of starting materials into a product, which was further confirmed through bright fluorescence observed by illumination of the TLC plates with UV lamp at 354 nm and also by the ninhydrin detection of blue spot. The silylating reagent was removed from the 2'-*O*-modified uridine carbamate (**4**) by tetrabutylammonium fluoride in THF for 20 min (scheme). The crude product after complete work up and purification was tritylated under anhydrous condition with 4,4'-dimethoxytrityl chloride (DMTr-Cl) in the presence of dimethylaminopyrimidine (DMAP) and catalytic amount of triethylamine (TEA) to obtain compound (**6**) in good yield [6]. Phosphitylation of compound (**6**) was done under the atmosphere of argon in dry dichloromethane using 2-cyanoethyl *N,N,N'*,*N'*-tet-

Properties of oligonucleotide

Entry	Oligonucleotide sequences (5' ⟶ 3')[a]	Yield (A_{260})	Retention time, min	T_m/°C		ΔT_m/°C	
				ODN1	ODN2	ODN1	ODN2
ODN1	U^FAA AAT CTC CGC CGC	23.6 O.D.	13.7				
ODN2	UAA AAT CTC CGC CGC	23.3 O.D	13.4				
ODN3	GCG GCG GAG ATT TTA	26.4 O.D	13.8	54.1	54.3		
ODN4	GCG GCG GAG ATT TT*G*	24.5 O.D	14.3	53.9	55.44	(–) 0.2	(+) 1.14
ODN5	GCG GCG GAG ATT TT*GG*	23.8 O.D	14.7	53.83	55.47	(–) 0.27	(+) 1.17
ODN6	GCG GCG GAG ATT TT*GGG*	22.9 O.D	14.8	53.79	55.49	(–) 0.31	(+) 1.19

[a] Aq. NH_4OH (30%), 60°C, 16 h; oligonucleotides were synthesized at 0.2 μM scale using standard phosphoramidite chemistry.

raisopropylphosphoramidite (Bis-reagent) and pyridiniumtrifluoroacetate (Py · TFA) as an activator. After completion of reaction, the crude product was immediately applied onto a short silica gel column and eluted with ethyl acetate and dichloromethane (1% TEA) to furnish the phosphoramidite of 2'-*O*-carbamate uridine (**7**). The title compound was stored under anhydrous condition at –20°C and used further for the synthesis of modified oligonucleotide fluorescent probe.



Scheme. (*i*) *N*,*N*'-carbonyldiimidazole/DCM; (*ii*) 5-dimethyl- aminonaphthalene-1-[*N*-(6-aminohexyl)] sulfonamide, (**3**), in DCM; (*iii*) TBAF/THF; (*iv*) a. DMTr-Cl/DMAP/TEA; b. 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropyl phosphoramidite (Bis-reagent)/Py, TFA, $n = 5$.

The modified nucleoside analogue (**7**) was incorporated in last coupling cycle of oligonucleotide synthesis to obtain fluorescent probe 5'-U^FAA AAT CTC CGC CGC-3'. The complementary oligonucleotides ODN3 to ODN6 (table) were synthesized keeping the 3'-end oligonucleotide nucleobase sequences similar to the chromosomal telomeric strands. To cleave oligomers from the solid support and to remove base labile-protecting groups, oligomers were treated with 30% aqueous ammonia at 60°C, for 16 h. The ammonia solution was evaporated in a SpeedVac, redissolved in water (200 μl), subjected to desalting (reversed phase C18 silica gel column), and eluted with 30% acetonitrile in water. For stringent purification, oligomers were first subjected to anion-exchange chromatography on FPLC using buffer A (0.1 M NaCl, pH 12.0) and buffer B (1 M NaCl, pH 12.0) with a gradient of 0 to 100% B for 35 min and then analyzed on RP HPLC using a gradient of ammonium acetate (0.1 M, pH 7.1) and acetonitrile (from 0 to 50% B for 30 min).

Hybridization studies, measurement of T_m-values and fluorescence studies of duplexes were carried

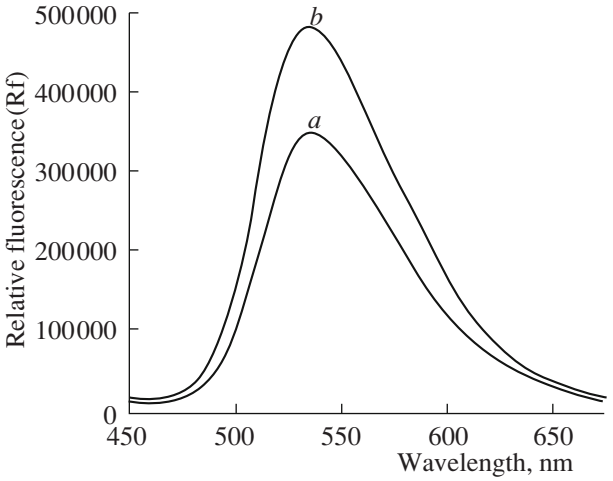


Fig. 1. Relative fluorescence intensity of oligonucleotide fluorescence probe (ODN1) of 2.5×10^{-5} M concentration (*a*) and of duplex (*b*) formed after hybridization with perfectly matched complementary oligonucleotide strand (ODN3) of 3.0×10^{-5} M concentration at 25°C in a quartz cuvette of 1-cm path length; λ_{ex} = 335 nm; power: 150 W xenon lamp; excitation and emission slit widths = 4 nm.

out in 0.1 M phosphate buffer containing 0.1 M NaCl and 5.0 mM $MgCl_2$ at pH 8.0. To facilitate complete hybridization, complementary oligonucleotide strands were taken in equimolar concentrations and subjected to annealing process by incubating complementary strands for 15 min at 90°C and allowed to cool down slowly below room temperature. For comparative thermal stabilities, both fluorescent probe (ODN1) and an unmodified probe ODN2 were hybridized with target strand ODN3 (wild-type), ODN4 (mismatch A/G), ODN5 mismatched with one extra G and ODN6 mismatched as well as two extra G moiety as an overhang. The melting points of duplexes were obtained by lowering the temperature slowly (0.5°C min^{-1}) from 75 to 15°C and measuring the change in absorbance at a single wavelength 254 nm at each temperature. T_m values represent the midpoint of the transition as obtained by fitting the melting profiles with a sigmoidal expression. T_m value data were recorded for each duplex by repeating the cycle four times and an average of that was taken with ±1% error. T_m value data obtained (table) has shown a significant degree of stabilization (1.14–1.19°C) for the duplexes formed with unmodified probe while destabilization in the range of 0.2–0.31°C with fluorescent probe. The more degree of enhancement in the T_m value with unmodified probe and complementary target strands having dG instead of dA at the 3'-end within the duplex loop as well as an overhang confirmed the affinity for such kind of duplexes thereby confirming the involvement of non-Watson–Crick type wobble-base pairing [7, 8] between the mismatch nucleobase pairs (U/G). Conversely, the destabilization observed in case of duplexes formed with modified fluorescent probe and target strands, appears to be due to the attachment of fluorophore at the 2'-position through the carbamate approach. Indubitably a carbamate linkage creates some sort of destabilization in duplex formation but seems to be a good strategy for the covalent incorporation of ligands for several biological studies [9]. The choice of spacer arm is crucial to make a suitable distance between the fluorophore and DNA strand. In the present study we placed a spacer arm of six-carbon chain to avoid any kind of perturbation in stacking arrangements of nucleobases due to fluorophore and its interaction vice-versa.

The fluorescence spectra of fluorescence probes were recorded at 2.5×10^{-5} M concentrations in phosphate buffer containing 0.1 M NaCl. UV-absorption spectra showing absorbance at 335–340 nm due to fluorophore and 255–265 nm due to either monomer or oligomers (Figure not given) and fluorescence emission spectra at 530 nm wavelength clearly confirmed the retention of the fluorophore in the modified oligonucleotides. Figure 1 is demonstrating the relative change in fluorescence intensity of the single-stranded dansyl labeled oligonucleotide fluorescence probe (ODN1) alone and of the duplex formed after complete hybridization with perfectly matched complementary oligonucleotide strand (ODN3). An increase in the fluorescence intensity of duplex with a small blue-shift of the emission maximum by 3 nm clearly demonstrates that the attached dansyl fluorophore with linker arm is moved towards the less polar environment. To observe the change in the relative fluorescence intensity of duplexes, of modified labeled probe with its complementary strands, first, a model experiment was performed in which to a perfectly matched target complementary strand (ODN3) of 2.5×10^{-5} M concentration in phosphate buffer, fluorescence probe (ODN1) was sequentially added in increasing order as: 0.125×10^{-5} M; 0.25×10^{-5} M; 0.5×10^{-5} M; 1.0×10^{-5} M; 1.5×10^{-5}M; 2.0×10^{-5} M; 2.5×10^{-5} M; 3.5×10^{-5} M and 4.0×10^{-5} M. From Fig. 2 (A and B) it has been observed that as the concentration of probe ODN1 increased to the target strand, there is gradual enhancement in the fluorescence signal and at 1 : 1 stoichiometry, enhancement in the fluorescence signal with blue-shift of around 3 nm in emission intensity, was reached to saturation as on further increasing the concentration of fluorescence probe ODN1 no further substantial enhancement in the fluorescence signal was observed. Thus, indicating further that on complete duplex formation, dansyl fluorophore moved towards more hydrophobic environment (towards minor groove region) henceforth, resulting higher fluorescence signal [3, 4].

Figure 3 shows the pattern of relative fluorescence intensity of different oligonucleotide duplexes formed after the complete hybridization with fluorescent probe ODN1. The synthetic G-rich target strands have mismatch of "dG" in place of "dA" corresponding to complementary uridine analogues of DNA strands and/or as overhang. There was gradual decrease in the fluorescence intensity signal without any significant shifts for

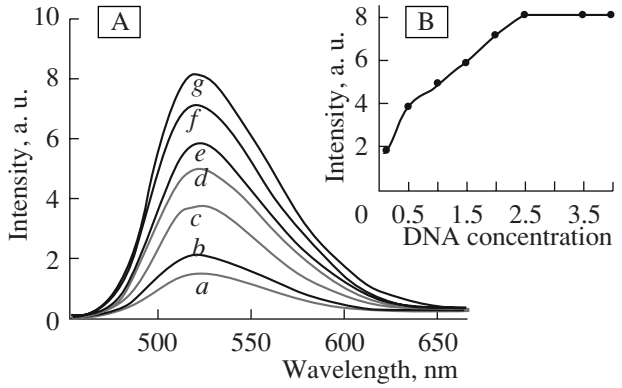


Fig. 2. (A) Increase in fluorescence intensity on increasing the concentration of monodansylated fluorescent oligonucleotide probe (ODN1) stepwise: 0.125×10^{-5} M (*a*), 0.25×10^{-5} M (*b*), 0.5×10^{-5} M (*c*), 1.0×10^{-5} M (*d*), 1.5×10^{-5} M (*e*), 2.0×10^{-5} M (*f*); 2.5×10^{-5} M (*g*). (B) Showing the trend for increase in the relative fluorescence intensity. At 25°C in a quartz cuvette of 1 cm path length. λ_{ex} = 335 nm. Power: 150 W Xenon lamp. Excitation and emission slit width at 4 nm.

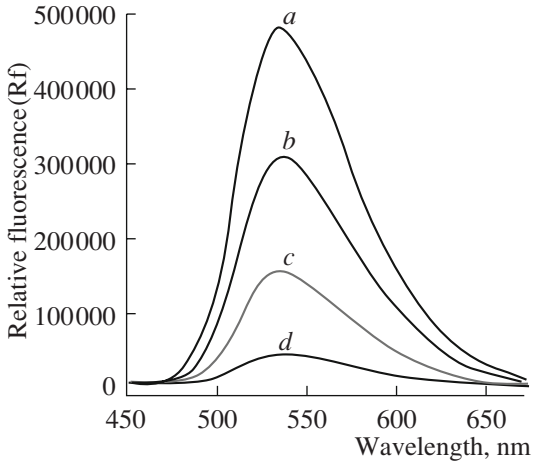


Fig. 3. Decrease in fluorescence intensity of duplexes formed between monodansylated fluorescent oligonucleotide probe (ODN1) of 2.5×10^{-5} M concentration on hybridization in phosphate buffer with target strands of 3.0×10^{-5} M concentrations; perfectly matched complementary strand ODN3 (*a*); strands having one "dG", ODN4 (*b*); two "dGs", ODN5 (*c*) and three "dGs", ODN6 (*d*). At 25°C in a quartz cuvette of 1 cm path length. λ_{ex} = 335 nm. Power: 150 W Xenon lamp. Excitation and emission slit width at 4 nm.

duplexes, formed with hybridization of fluorescent probe ODN1 and complementary oligonucleotides ODN4 (one dG, opposite to 2'-*O*-modified uridine unit), ODN5 (two dGs, one as overhang) and ODN6 (three dGs, two as overhang) respectively. The decrease in fluorescence intensity was observed in the order of 0.31×10^6, 0.15×10^6 and 0.05×10^6 respectively as compared to the perfectly matched duplex. This decrease in fluorescence signal was approximately ~10 times when three "dGs" were employed in the complementary target strand. It is very well established that guanosine moiety is capable for 'contact mediated' quenching of the fluorescence intensity of fluorophores due to low oxidation potential [10–12] and as shown in previous report in case of hairpin probe [13] we further, utilized this inherent quenching properties of guanosine moieties with complementary G-rich target strands which are very much close to the telomeric repeat strands of the chromosomes.

Thus the methodology employed with sufficient sensitivity is worth to use for in vitro studies and its potential application in biological research for detection of target molecules through quenched fluorescence signal in homogeneous hybridization assays. The introduction of deoxyguanosine units in the complementary strands at the terminal end, either within the strand, just opposite to the fluorophore or close to the fluorophore as overhang and relative decrease in fluorescence signal clearly demonstrates the quenching efficiency of the dG nucleobase which can be further exploited for the detection of deoxyguanosine rich target strands.

EXPERIMENTAL

General: Dansyl chloride, dimethylaminopyridine (DMAP), carbonyldiimidazole (CDI), tetrabutylammonium fluoride (TBAF)/THF, hexamethylene-1,6-diamine were from Aldrich. Py · TFA, LCAA-CPG loaded nucleosides of Perseptive Biosystem Gmbh (LCAA-CPG, 500 Å, 39.0 μmol/g). 2-Cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphoramidite (Bis-reagent), 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPDS-Cl), 4,4'-dimethoxytrityl chloride (DMTr-Cl) and different amidites were obtained from ChemGenes, USA. Oligonucleotide synthesis grade acetonitrile, 1*H*-tetrazole were purchased from Applied Biosystem, Perkin-Elmer, Switzerland. *UV*-visible absorbance spectra and thermal denaturation experiments (T_m-value) were performed on a Perkin-Elmer Lambda Bio 20 UV-visible spectrometer with PTP 1 (Peltier Temperature programmer). Fluorescence spectra were recorded on Perkin-Elmer spectrofluorimeter. All the fluorescence spectra were recorded at 25°C in a quartz cuvette of 1 cm path length by exciting the molecule at 335 nm wavelength and scanning the spectra between 450 to 700 nm wavelength with full power of 150 W xenon lamp and keeping the excitation and emission slit width at 4 nm throughout whole experiment. Purification of oligonucleotides was carried by anion-exchange chromatography on fast protein liquid chromatography (FPLC, Pharmacia) and reverse-phase high performance liquid chromatography (RP-HPLC, Shimadzu) using an RP C-18 Lichrosphere column employing a UV-visible detector. Synthetic grade solvents used were from Qualigens and were purified and dried prior to use. ^{1}H NMR and ^{31}P NMR were recorded on a JEOL AL300 FT-NMR spectrophotometer. ^{1}H NMR was recorded in DMSO-d_6 solvent. All the oligomers were assembled on the standard supports of labile nucleoside phosphoramidite at 0.2 μmol scale on ABI 392 DNA/RNA synthesizer using standard phosphoramidite

chemistry. The overall coupling yield was found more than 85% during the course of automated synthesis of oligonucleotides.

3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine (1). 3',5'-OH of uridine was first protected by treating with silylating reagent 1,1,3,3-tetraisopropyldisiloxane-1,3-dichloride in pyridine under anhydrous condition to give (**1**) (yield 75%) as described previously [5].

2'-*O*-(Imidazol-1-ylcarbonyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine (2). The compound (**1**) (5 mM, 2.434 g) was evaporated twice with anhydrous acetonitrile (2 × 20 ml) and dissolved in dry diclorometh ane (25 ml). *N*,*N*'-carbonyldiimidazole (CDI) (6.0 mM, 1 g) was added. The reaction mixture was stirred for 2 h. TLC showed the conversion of starting material into product of lower mobility. The solution was washed with water (2 × 30 ml), dried over sodium sulphate, filtered and evaporated in vacuum to get imidazolyl derivative as white foam. Yield 2.24 g (91.9%). R_f 0.41 (EtOAc). MALDI-TOF (2,4,6-THAP + citrate): $[M + Na]^+$ calcd. 603.77, found 604.58, $[M + K]^+$ calcd. 619.88, found 620.55. ^{1}H NMR (δ, ppm): 11.3 (1 H, s, H3), 8.3 (1 H, s, imidazole), 7.69 (1 H, s, imidazole), 7.93 (1 H, d, *J* 7.8 Hz, H6), 6.97 (1 H, s, imidazole), 5.75 (1 H, s, H1'), 5.67 (1 H, d, *J* 5.2 Hz, H2'), 5.65 (1 H, d, *J* 8.0 Hz, H5), 4.71 (1 H, m, H3'), 4.23–3.95 (3 H, m, H4', H5'), 1.12–0.9 (28 H, m, Pr^i).

6-Aminohexyldansylsulphonamide (3). Dansyl chloride (5.0 mM, 1.35 g) was taken in THF (25 ml) and added dropwise to a well stirred ice cold 10% $NaHCO_3$ aqueous solution of hexamethylene-1,6-diamine (20.0 mM) for 3 h. Completion of reaction was monitored on TLC. The crude product was poured in water and extracted with ethyl acetate (3 × 25 ml). The organic layer was pooled, concentrated in vacuum and eluted with EtOAc/hexane (2 : 8) on silica gel column to get 6-aminohexyldansylsulphonamide (**3**). Yield 2.33 g (78%). R_f 0.34 (8 : 2 : DCM/MeOH). UV λ_{max} (MeOH) : 251, 334, 385 nm. ^{1}H NMR (δ, ppm); 2.38–2.69 (12 H, m, $(-CH_2)_6NH_2$), 2.89 (6 H, s, $(CH_3)_2N$), 4.62 (3 H, br, NH), 8.21 (1 H, d, *J* 8.1 Hz, H2) 7.67–8.42 (3 H, m, naphthoyl) 6.44–6.50 (2 H, m, naphthoyl). λ_{max} 335 nm, λ_{em} 535 nm (in methanol).

2'-*O*-(Dansyl-6-sulphonamidohexylaminocarbonyl)-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine (4). Imidazolyl derivative of uridine (3.5 mM, 2 g) was taken in dry dichloromethane (30 ml) and (**3**) (4.5 mM, 1.58 g) was added dropwise for 1 h under argon atmosphere. After complete addition the reaction mixture was allowed to stir overnight. The reaction mixture was washed with water and 3% citric acid. The organic layer was kept over sodium sulphate and evaporated in vacuum. The residue was applied to silica column and product was isolated using gradient ethyl acetate in chloroform. Fractions containing the product were evaporated in vacuum to give compound (**4**) as white solid foam. Yield 2.82 g (94%). R_f 0.45 (DCM/MeOH: 8 : 2 v/v). MALDI-TOF (2,5-DHBA): $[M + Na]^+$ calcd. 884.3, found 885.2, $[M + K]^+$ calcd. 900.3, found 900.9. ^{1}H NMR (δ, ppm): 11.38 (1 H, s, H3), 7.11 (1 H, t, *J* 5.8 Hz, NHOCO), 3.28–3.34 (12 H, m, $-NHCH_2-CH_2-CH_2-NH-$), 2.85 (6 H, s, NCH_3), 4.57 (1 H, br, SO_2NH), 8.01 (1 H, t, naphthoyl), 8.52–7.87 (3 H, m, naphthoyl), 6.44–6.50 (2 H, m, H1', naphthoyl), 7.59 (1 H, d, *J* 7.9 Hz, H6), 5.16 (1 H, d, *J* 8.1 Hz, H5), 5.68 (1 H, d, H1'), 5.49 (1 H, m, H2'), 4.87 (1 H, m, H3'), 3.91 (1 H, m, H4'), 3.60 (2 H, m, H5'), 0.87–1.24 (28 H, m, Pr^i).

2'-*O*-(Dansyl-6-sulphonamidohexylaminocarbonyl)uridine (5). Compound (**4**) (3 mM, 2.58 g) was dissolved in THF (10 ml) and 1 M solution of TBAF in THF (5.0 ml) was added with stirring for 20 min. The reaction mixture was poured in 5% $NaHCO_3$ solution and extracted several times with ethyl acetate. The organic layer was concentrated, dried, filtered and again evaporated to afford the compound as a gummy mass. Yield 1.76 g (95%). R_f 0.37 ($CHCl_3$/MeOH, 7 : 3), MALDI-TOF (2.5-DHBA): $[M + H]^+$ calcd. 619.4, found 618.6, $[M + Na]^+$ calcd. 642.4, found 643.3, $[M + K]^+$ calcd. 658.4, found 659.3. ^{1}H NMR (δ, ppm): 11.38 (1 H, s, H3), 7.11 (1 H, t, J 5.8 Hz, NHOCO), 3.28–3.34 (12 H, m, $-NHCH_2-CH_2-NH-$), 2.85 (6 H, s, NCH_3), 4.57 (1 H, br, SO_2NH), 8.01 (1 H, t, naphthoyl), 8.52–7.87 (3 H, m, naphthoyl), 6.44–6.50 (2 H, m, H1', naphthoyl), 7.59 (1 H, d, *J* 7.9 Hz, H6), 5.16 (1 H, d, *J* 8.1 Hz, H5), 5.68 (1 H, d, H1'), 5.49 (1 H, m, H2'), 4.87 (1 H, m, H3'), 3.91 (1 H, m, H4'), 5.57 (1 H, m, 3'-OH), 5.21 (1 H, m, 5'-OH), 3.60 (2 H, m, H5').

3'-*O*-(*N*,*N*-Diisopropylamino-2-cyanoethoxyphosphinyl)-5'-*O*-(4,4'-dimethoxytrityl)uridine-2'-yl carbamates (7). Uridin-2'-yl carbamate was first treated with 4,4'-dimethoxytrityl chloride (DMTr-Cl) in presence of dimethylaminopyrimidine (DMAP) and TEA to get 5'-*O*-(4,4'-dimethoxytrityl) uridin-2'-yl carbamate (**6**). To the well-stirred solution of (**6**) (1.3 mM, 1.2 g) in anhydrous dichloromethane (12 ml), 2-cyanoethyl-*N*,*N*,*N*',*N*''-tetraisopropylphosphoramidite (Bis-reagent) (3.5 mM, 1.12 ml) was added at ambient temperature. Pyridinium trifluoroacetate (Py · TFA) (3.5 mM, 0.82 g) was added to the reaction mixture and stirred for 3 h. After complete consumption of starting material (monitored on TLC), methanol (3 ml) was added, and the resulting solution was washed with 10% $NaHCO_3$ (3 × 15 ml). Organic layer was dried over sodium sulfate and directly applied to a short silica gel column. Compound was eluted with dichloromethane/ethyl acetate/Et_3N (4.5 : 4.5 : 1, v/v/v). The desired fractions were pooled and concentrated under reduced pressure to afford title compound as amorphous yellow powder. Yield 1.24 g (85%). R_f 0.63 (CH_2Cl_2/EtOAc/Et_3A: 4.5 : 4.5 : 1, v/v/v). λ_{max} 256, 335 nm. ^{1}H NMR (δ, ppm): 11.42 (1 H, s, NH), 8.39 (1 H, d, *J* 9.1 Hz, naphthoyl), 8.22–8.27 (2 H, m, naphthoyl + H6), 7.59–7.96 (2 H, d, *J* 9 Hz, naphthoyl), 7.28–7.37 (7 H, m, naphthoyl, C5, Ar), 7.08–7.78 (8 H, d, *J* 8.5 Hz, DMTr), 7.53 (1 H, t, *J* 5.9 Hz, NHCOO), 6.65 (1 H, d, *J* 9 Hz, naphthoyl), 6.39–6.43 (1 H, m, H1'), 5.03 (1 H, m, H2'), 4.57 (1 H, br, SO_2NH), 4.14–4.17 (2 H, m, H3', H4'), 4.01–4.03 (2 H, m, H5'), 3.91 (2 H, t, P–O–CH_2), 3.87 (6 H, s, $-OCH_3$), 3.21–3.32 (4 H, m, $NHCH_2$), 3.14 (2 H, m, NCH-), 2.93 (6 H, s, $-NCH_3$), 2.62 (2 H, t, CH_2CN), 1.92–1.71 (8 H, m, $NHCH_2(CH_2)_4CH_2NH$), 1.22 (12 H, d, $-NCH(CH_3)_2$). MALDI-TOF (2.5-DHAP):

$[M + H]^+$ calcd. 1124.3, found 1124.1, $[M + Na]^+$ calcd. 1147.3, found 1148.5, $[M + K]^+$ calcd. 1163.3, found 1164.1. ^{31}P NMR (CD_3CN) 150.3 ppm, 149.9 ppm (diastereoisomers).

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