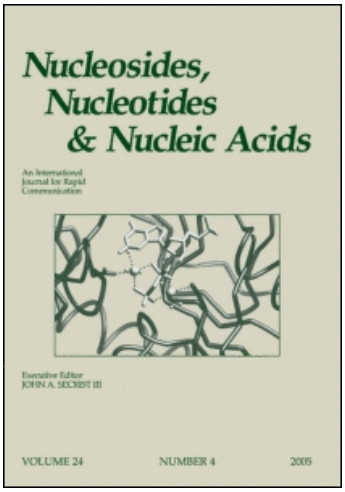

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Synthesis and Hybridization Studies of Oligonucleotide Sequences with Modified Fluorescent Nucleoside Analogs

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SYNTHESIS AND HYBRIDIZATION STUDIES OF OLIGONUCLEOTIDE SEQUENCES WITH MODIFIED FLUORESCENT NUCLEOSIDE ANALOGS

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ABSTRACT

Two complementary oligodeoxynucleotide hexamers CATGAA and TTCATG and a pentamer with a fluorescent nucleoside analog viz. 9-N-(2'-deoxy-ß-D-ribofuranosyl) carbazole (C*) incorporated into it, TTC*ATG were synthesized and characterised by spectroscopic and chromatographic studies. The comparative fluorescent studies of the two nucleoside analogs viz. 9-N-(2'-deoxy-ß-D-ribofuranosyl) acridone and its carbazole analog (C*) have been carried out under different experimental conditions. The effect on fluorescence by incorporation of (C*) into the sequence and its subsequent hybridization with the complementary sequence have been studied.

INTRODUCTION

Nonradioactive labelled synthetic oligonucleotides have recently attracted worldwide attention as nucleic acids probes for study of fluorescent in situ hybridization (FISH)[1], detection and isolation of specific genes in DNA sequencing[2-3], medical diagnostics[4] or clinical genetics[5-6] and DNA profiling,[6] as affinity binding molecules, in electron microscopy and as photoactivatable cross linking agents for modulation of gene expression[7]. Some fluorescent nucleosides and their analogs have also been incorporated into oligonucleotides[8-11]. They are used as probes for detection of nucleic acids hybridization[12], DNA sequencing[13], interaction of nucleic acids with proteins and other ligands. The derivatization of nucleosides and their substitution with other appropriate moieties leading to their use for diagnostic purpose is a field of recent interest. In this paper we report the comparative fluorescence studies on two deoxyribosides of carbazole and acridone, incorporation of the former into a pentamer as nucleoside analog and subsequent effect of hybridization on relative fluorescence.

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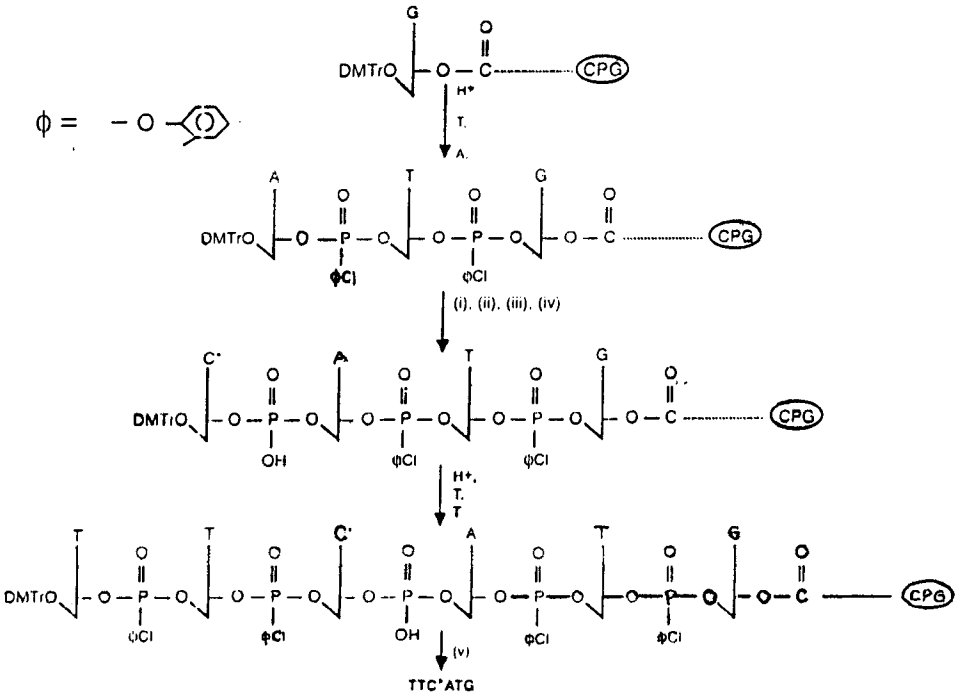
RESULTS AND DISCUSSION

The present study is an effort towards development of self diagnostic antisense oligonucleotides, which by virtue of their hybridization with any target DNA or RNA sequence in specific viral life cycle can inhibit the translation process i.e. formation of virions. Past study has shown that for this purpose oligos of at least 15-20 base length can be effective. However, we have selected only hexamer for the preliminary study. The main aim was to introduce a fluorescent moiety in the form of a modified nucleoside base into the sequence, so that it can act as self diagnostic unit, thus avoiding the covalent attachment of separate reporter group through linkers. An assessment of fluorescence intensity at oligo level can indicate the number of such units required to be introduced for a specific
length of oligonucleotide for necessary required detection. However, such incorporation of modified units has to be carried out in a manner so that it does not effect hybridisation significantly.

We have selected hexamer TTCATG since it is most oft repeat sequence in the sensitive tat initiator region of HIV-1 genome. Its complementary sequence CATGAA was synthesised in order to study the hybridization of the two by recording melting curves. CATGAA, TTCATG and TTC*ATG **(SCHEME 1)** were synthesized on solid phase via phosphotriester approach[14] while the incorporation of 9-N-(2'-deoxy-3'-H-phosphonyl-5'-O-DMT-ß-D-ribofuranosyl) carbazole (C*) in between the sequence of oligonucleotide, TTC*ATG was carried out by H-phosphonate approach. Long chain alkylamino CPG with a loading of 34.5 µM/ g was used as the polymer support. Two synthesis were carried out simultaneously on dual column DNA bench synthesiser with alternating wash and coupling cycles. After each coupling step the DMTr group was removed by 3% TCA in DCM which imparts orange colour. The percentage coupling yield at each step was estimated spectrophotometrically which was approximately 95-96 % .

The 9-N-(2'-deoxy-ß-D-ribofuranosyl) carbazole **(1c)** was prepared by glcosylation of carbazole. The 5'-tritylated moiety **(1e)** was phosphonylated at 3'-OH resulting in formation of 9-N-(2'-deoxy-3'-H-phosphonyl-5'-O-DMTr-ß-D-ribofuranosyl) carbazole **(1f) (FIG 1)**. The corresponding glycoside of acridone i.e. 9-N-(2'-deoxy-ß-D-ribofuranosyl) acridone **(1d)** was also prepared and its fluorescence studied but only the former was incorporated into oligo sequence, because of solubility problem.



SCHEME 1 : Synthesis of modified hexamer (TTC* ATG)
(i) TCA (ii) carbazole - H - phosphonate (iii) CH_3CN / pivaloyl chloride
(iv) I_2 in Pyr/NMI / H_2O / THF (v) Conc NH_3 (15 M)

The deprotection of base exocyclic amino protecting groups (benzoyl, isobutyryl) and phosphate protecting group (2-chlorophenyl) as well as cleavage from the resin was achieved by treatment with oximate followed with concentrated ammonia solution.

The relative fluorescence of (**1c**) and (**1d**) in water, aq. sodium carbonate, dioxane and methanol was recorded at initial concentration of 10 µmol/L and subsequent dilution to 7.5 µmol/L, 5.0 µmol/L and 2.5 µmol/L solutions. The excitation wavelengths were worked out as 292 nm for (**1d**) and 337 nm for (**1c**). A gradual decrease in fluorescence was observed with dilution in all these solvents. When concentration was kept constant, the fluorescence intensity appeared to depend on the nature of the solvents and it decreased with the change of the solvent from water to methanol, dioxane and aq. sodium carbonate in compound (**1d**) and from methanol to dioxane, water and aq. sodium carbonate respectively for compound (**1c**). These results showed that water is the best solvent for compound (**1d**) and methanol

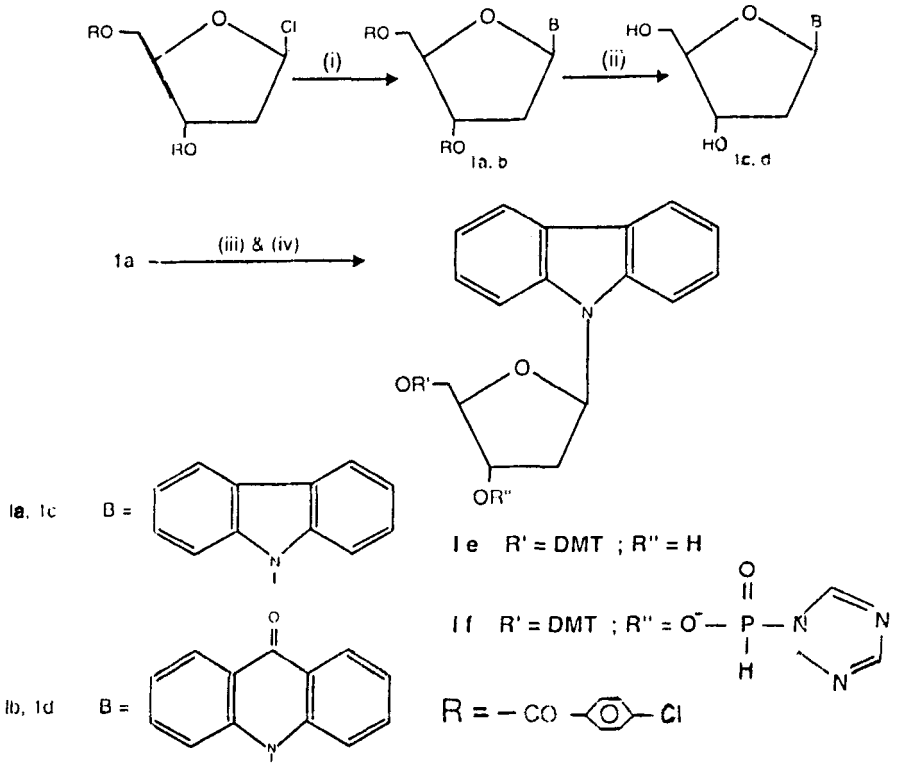


FIG. 1 : (i) carbazole / acridone in benzene / heating for 2 hr. (ii) Dowex H^+ / CH_3ONa (iii) DMTrCl / pyr. (iv) PCl_3, triazole / TEAB buffer.

is the best solvent for compound **(1c)**, while aq. sodium carbonate is least useful for both. It seems that fluorescence quenching is higher in the presence of Na^+ ions.

The compound **(1d)** and **(1c)** possess fluorescence detectable at the concentration of 10^{-4} mol/lit i.e. within significant limits of detection. The fluorescence intensity of the monomer i.e. carbazole derivative **(1c)** and the modified oligo i.e. (TTC*ATG) at similar concentration i.e. at 0.05 OD was found to be 99% and 68.3% respectively thus showing that after incorporation of C* into the ODN's the fluorescence was quenched conclusively **(FIG. 2)**. The introduction of one modified unit per five normal monomer units in the oligomer of any length can suffice for detection based fluorescence.

The purity of all sequences CATGAA, TTCATG and TTC*ATG in a gradient of 0-50% CH_3CN in 0.15M aq. triethyl amm. acetate buffer shows Retention time 9.58, 9.83 and 11.23 min. respectively **(FIG. 3)**.

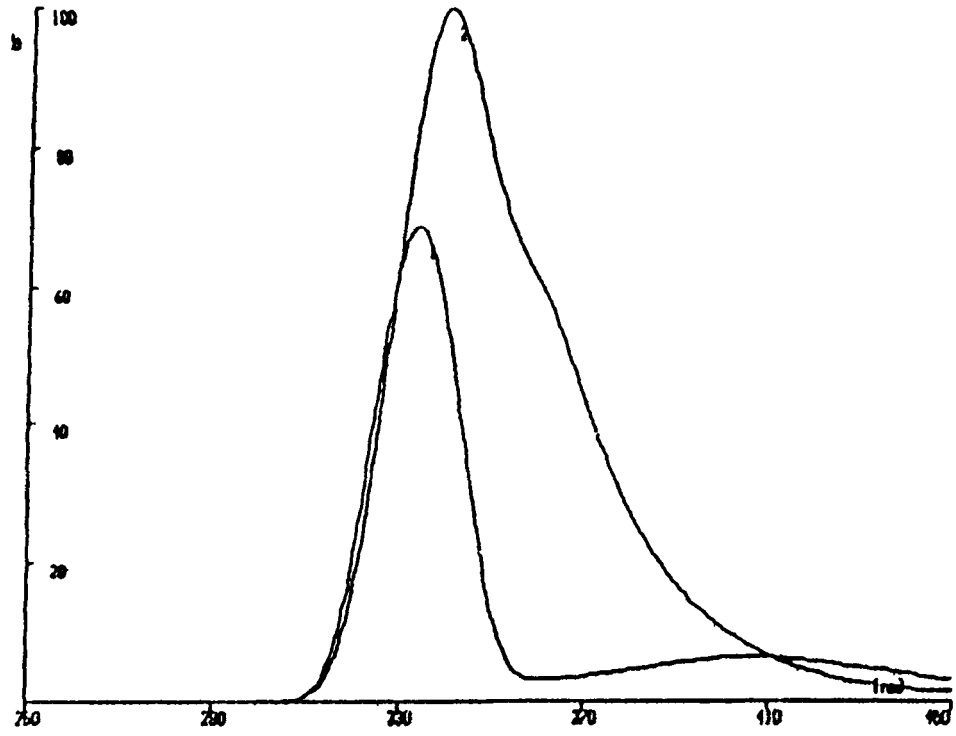


FIG.2 : Comparative fluorescence emission spectra of modified oligomer (TTC*ATG) (1) and 9-N-(2'- deoxy - β - D - ribofuranosyl) carbazole (2) at 0.05 OD concentration in 0.15 M ammonium acetate buffer.

The hybridization of two normal complementary sequences viz. d(CATGAA) and d(TTCATG) and that of the modified sequence (TTC*ATG) with the former was studied by mixing 0.8351 OD in 6X SSC buffer (100 mM, Na^+ pH 7.0, citrate) solution of these at 260 nm and heating at 60°C and then cooling to 0°C.

However, no definite Tm's could be obtained, probably due to short length of the sequences, inadequate concentration of salt or insufficient binding due to nature of terminal bases. This study reveals that incorporation of the fluorescent monomer at regular spacing in any oligo so that hybridization is not significantly affected can result in the development of a suitable self diagnostic sequence.

MATERIAL AND METHODS

All the four deoxynucleotides, DMTrCl, MSNT, tetramethyl guanidine and 4-nitro benzaldoxime were purchased from Sigma chemical Co. 2-Deoxy-D-ribose,

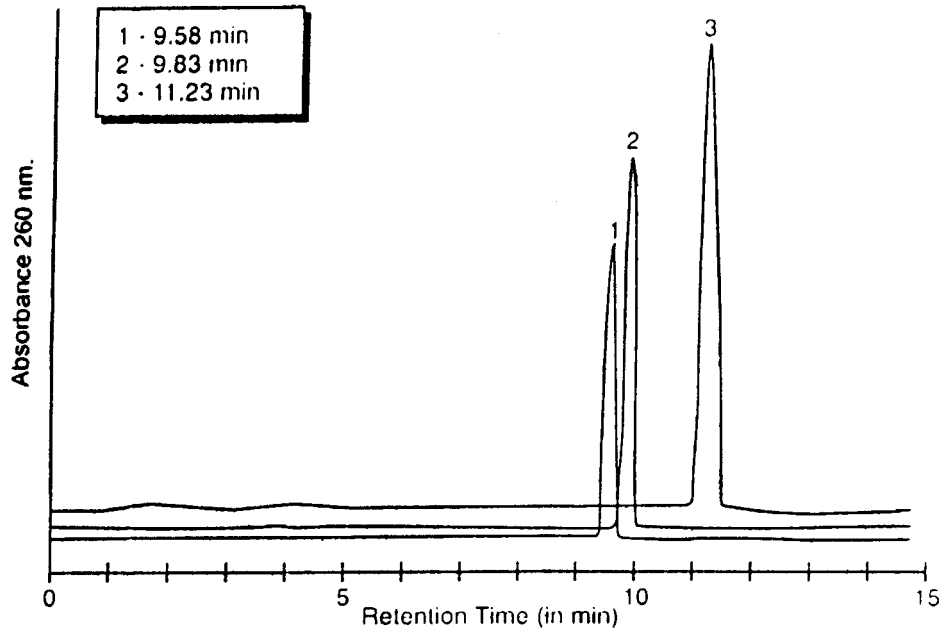


FIG.3 : HPLC Profile diagram of (1) hexamer CATGAA, (2) hexamer TTCATG & (3) modified hexamer (TTC*ATG), Gradient 0 - 50 % CH_3CN in 0.15 M aq. triethyl ammonium acetate buffer. Temp. 25°C, column (RPC C_{18})

carbazole, trichloroacetic acid, pyridine, water, dioxane and methanol (HPLC grade) were purchased from E. Merck India Ltd.

All melting points reported are uncorrected. UV absorption and emission spectra were recorded on Hitachi 220 S spectrophotometer and Kontron SFM 25 spectrofluorometer respectively. ^1H NMR spectra were recorded on Perkin Elmer E-32. Purity of compounds was tested by TLC (silica gel G) and HPLC (Pharmacia model LKB, DBF) using RPC C18 column. Oligonucleotides were synthesized on Omnifit Dual column DNA bench synthesizer using LCAA CPG as solid support.

Triethyl ammonium bicarbonate (TEAB) buffer was prepared in laboratory by passing CO_2 in cold aq. TEA solution. 2-Deoxy-3,5-di-O-p-chlorobenzoyl-α-D-ribofuranosyl chloride was synthesized by condensing 2-deoxy-D-ribose (containing 0.27 N HCl-MeOH) in p-chlorobenzoyl chloride.

9-N-(2'-deoxy-3',5'-di-O-p-chlorobenzoyl-ß-D-ribofuranosyl) carbazole (1a): 2-Deoxy-3,5-di-O-p-chlorobenzoyl-α-D-ribofuranosyl chloride (prepared according

to published procedure[15]) (0.43 g, 1 mmol) and carbazole (0.167 g, 1 mmol) were taken in benzene (5 ml) and refluxed for 2 h. After the reaction was complete, the reaction mixture was taken up in benzene (15 ml) and water (5 ml) was added to it. The heterogeneous mixture was evaporated to dryness and taken up in toluene (6 ml). The mixture was heated to boiling and filtered. Fractional crystallization gave the β-isomer as the major product (95%). Repeated crystallization was required to get the pure product. Homogeneity of the product was confirmed on RPC C_{18} column (single peak with Retention Time 4.98 min). Yield 55% , R_f 0.15 (Hex:Bz:: 8:2 v/v), UV (λ in nm) 337, 323, 292 and 235 nm. ^1H NMR 7.5 (d, 2H, J= 5.8 & 2.2), 7.0 (m, 2H), 6.89 (m, 2H) 7.1 (dd, 2H J= 5.8 & 2.0).

9-N-(2'-deoxy-3',5'-di-O-p-chlorobenzoyl-ß-D-ribofuranosyl)acridone (1b): 2-Deoxy-3, 5- di-O-p-chlorobenzoyl-α-D-ribofuranosyl chloride (0.43 g, 1 mmol) and acridone (0.196 g,1 mmol) were refluxed in benzene (5 ml) for 2 h. The resulting mixture was worked up as given above. R_f 0.27 (Hex:Bz:: 8:2 v/v), UV (λ in nm) 395, 378, 292 and 255. HPLC, Retention Time 3.43 min. ^1H NMR 7.6 (d, 2H, J=6 cps & 2.1 cps), 7.2 (m, 2H), 7.35 (m, 2H) and 7.5 (d, 2H, J=6.1 & 2 cps).

9-N-(2'-deoxy-ß-D-ribofuranosyl)carbazole (1c): To a suspension of **(1a)** (0.05 mmol) in methanol (1.25 ml) under nitrogen atmosphere was added a solution of sodium (3 g atoms) in methanol (0.25 ml) and the resulting solution was stirred at r. t. under nitrogen for 2 h. Dowex 50 W resin (H^+ form, 0.025 mg) previously washed with MeOH was added and after an additional 5 min of stirring the solution was filtered from the resin and glacial AcOH (0.025 ml) was added to the filtrate. The solution was evaporated in vacuo bath (39°C) to 2 ml volume, and the product was pptd. with the addition of ether (2.5 ml). After standing for several hours at r.t. the product was collected and washed with ether to give the compound. Yield 55%, R_f 0.12 (Hex:Bz:: 8:2 v/v), UV (λ max in MeOH) 337, 323, 292 and 235 nm. HPLC Retention Time 4.63 min. (eluent MeOH). Anal. Calcd. for $C_{17}H_{17}NO_3$: C, 71.6; H, 6.02; N, 5.0 found C, 70.8; H, 5.08; N, 4.8.

9-N-(2'-deoxy-ß-D-ribofuranosyl)acridone (1d): To a suspension of **(1a)** (0.05 mmol) in methanol (1.25 ml) under nitrogen atmosphere was added to a solution of sodium (3 g atoms) in methanol (0.25 ml) and the resulting solution was stirred at r.t. under nitrogen for 2 h. The cleavage of p-chlorobenzoyl group was worked up as described above. Yield 56%, R_f 0.24 (Hex:Bz:: 7.9:2.1 v/v), UV (λ max in MeOH)

395, 378, 292 and 255 nm. HPLC Retention Time 3.23 min (eluent MeOH). Anal. Calcd. for $C_{18}H_{17}NO_4$: C, 70.5; H, 4.5; N. 5.5 found C, 69.9; H, 4.4; N, 5.4.

Fluorescence Studies of (1c) and (1d) using different solvent systems: 9-N-(2'-deoxy-ß-D-ribofuranosyl) acridone **(1d)** (0.031 g, 0.01 mmol) and 9-N-(2'-deoxy-ß-D-ribofuranosyl) carbazole**(1c)** (0.028g, 0.01 mmol) were dissolved in 100 ml each of water, aq. sodium carbonate (10 mM), dioxane and methanol (10 µmol/l). These were then diluted to make the concentration 7.5 µmol/l, 5.0 µmol/l and 2.5 µmol/l. Excitation wavelength for these molecules were fixed i.e. 292 nm for compound **(1d)** and 337 nm for compound **(1c)**. Emission spectra were recorded between the wavelengths 600 and 200 nm in both the cases.

Relative Fluorescence in different solvents was as follows,

	Water	Sod.Carbonate	Dioxane	Methanol
(1c)	23.2	11.2	62.1	99.8
(1d)	99.6	14.7	29.9	64.8

9-N-(2'-deoxy-5'-O-DMTr-ß-D-ribofuranosyl)carbazole (1e): The 9-N-(2'-deoxy-ß-D-ribofuranosyl) carbazole **(1c)** (28.1 mg, 0.1 mM) was dissolved in pyridine (4.0 ml) and to this was added DMAP (0.06 mg, 0.05 eq.), triethylamine (1.4 eq. 0.02 ml) and DMTrCl (1.2 eq. 4.1 mg) and stirred the solution for 3 h at r.t. Reaction mixture was evaporated to dryness. The residue was dissolved in $CHCl_3$ (5ml) and then extracted with 5% aq. $NaHCO_3$ followed by NaCl. Organic phase was separated and combined phases were dried on anhyd. Sod. sulphate and evaporated to a solid residue. Yield 90%, R_f 0.14 (benzene).

9-N-(2'-deoxy-3'-H-phosphonyl-5'-O-DMTr-ß-D-ribofuranosyl)carbazole (1f): To a stirred solution of 1,2,4-triazole (12.0 mg) and triethylamine (0.07 ml) in anhy. DCM (0.5 ml) was added PCl_3 (45 µL) at r.t. under nitrogen. After 30 min the reaction mixture was cooled to 0°C and **(1e)** (5.8 mg) dried by evaporation from pyridine in DCM (0.15 ml) was added dropwise over 10-15 minute, the mixture stirred for 15 min at 0°C allowed to warm at ambient temperature and was poured into 1M TEAB (0.75 ml, pH 8.5) with stirring. The mixture was transferred to a seperatory funnel, the phases were separated and aq. phase was extracted with DCM (1.5 ml). The combined organic phase was washed with 1M TEAB (1.75 ml), dried over sodium sulphate and evaporated in to a foam. Yield 6.12 mg (90 %), HPLC Retention Time 6.43 min (eluent MeOH).

Synthesis of Hexanucleotides d(CATGAA) and d(TTCATG) : The synthesis was performed on 1 μmol scale by using phosphotriester approach[14]. The coupling yield of the intermediate steps were estimated by spectroscopic analysis of the dimethoxy trityl liberated from support. The average coupling yield was approx. 95-96%. The sequence was treated with a solution of 4-nitro benzaldoxime (70 mg) in 1 ml of dioxane:water (1:1 v/v) to which 1,1,3,3 tetramethyl guanidine (50 μL, 0.4 mM) was added and then concentrated ammonia (15 M) (5.0 ml) at 60°C for 16h. The purification of oligos with 5'-DMT intact was done by dissolving 5-10 μg of each one in water and loading it on RPC C_{18} column. The elution was done by using buffer A(0.15 M triethyl amm. acetate, pH 7.5) and buffer B(acetonitrile). HPLC, Retention Time of the oligomers (CATGAA) and (TTCATG) was 9.58 and 9.83 min. respectively **(FIG.3)**.

Synthesis of modified oligomer (TTC*ATG): 3'-Guanosine attached CPG support (25mg) loading 34.5 μmol/g was taken in the column. The cycle of the solvents was followed in the sequence

	Solvents	Function	Time (in min)
1.	Pyridine	wash	2.0
2.	DCM	wash	1.5
3.	3% TCA in DCM	Detritylation	0.5
4.	DCM	wash	1.5
5.	Pyridine	wash	2.5
6.	Coupling	addition of nucleotides	15.0

A solution of triethyl ammonium salt of 5'-O-DMT-dT-3'-O-chlorophenyl phosphate (20 μM) in anhyd. pyridine (0.2 ml) freshly activated with MSNT (40 μM) and 1-methyl imidazole (0.01 ml) was injected to the column.

After the coupling of nucleotide A the flow rate of solvent was adjusted to about to 1ml/min, washed with CH_3CN and treated with 3% TCA in DCM in order to deprotect the DMTr group from 5'-position of nucleotide bound to support. After pyridine/CH_3CN wash was added the activated monomer solution of **(1f)** (10.5 mg, 13 μM) containing trimethyl acetyl chloride (pivaloyl chloride; 7.8 mg, 65 μM) in pyridine/CH_3CN (1:1, 100 μL). The coupling took place in two min and it was followed by oxidation with I_2/water. The coupling yield was approximately 90% . The reaction cycle is as follows

	Solvents	Function	Time (in min)
1.	Anhy. CH_3CN	wash	2.0
2.	3% TCA In DCM	Detritylation	0.5
3.	Anhy.Pyr/CH_3CN	wash	1.5
4.	carbazole-H-phosphonate/pivaloyl chloride/Pyr./CH_3CN	coupling	2.0
5.	0.1 M I_2in Pyr/NMI/water/THF (5:1:4:90)	Oxidation of H-phosphonate	2.5

This was followed by two more couplings with triethyl ammonium salt of 5'-O-DMTr-dT-3'-o-chlorophenyl phosphate in sequence. The oligomer was treated with 4-nitro benzaldoxime (70 mg) in 1 ml of dioxane:water (1:1 v/v) to which 1,1,3,3 tetramethyl guanidine (50 ul, 0.4 μM) was added for 16-20 h at 37°C and then concentrated ammonia (5.0 ml) at 60°C for 16 h.

The purity was checked by HPLC, the elution was done by using gradient (0-50%) of buffer A (0.15 M triethylammonium acetate, pH 7.5) and acetonitrile. The modified oligomer has Retention Time 11.23 min **(FIG.3)**.

Fluorescence Study of Modified Oligomer (TTC*ATG): The modified oligomer (TTC*ATG) was dissolved in 0.15 M ammonium acetate buffer and emission spectra was recorded by fixing its excitation wavelengths at 337 nm at 0.05 OD concentration.

In another set of experiment comparative fluorescence of **(1a)** and modified oligomer was recorded in 0.15 M ammonium acetate buffer keeping the same concentration of both i.e. on 0.05 OD scale. The excitation wavelengths was fixed at 337 nm in both cases and emission spectra was recorded in between 600 to 200 nm. **(FIG.2)**. The relative fluorescence for **(1a)** was 99% and that for the hexamer was 68.3%.

Hybridization Study of Oligomers: The oligomers d(CATGAA), d(TTCATG) and d(TTC*ATG) were separately dissolved in 6X SSC buffer (100 mM, Na^+, pH 7.0, citrate). The mixtures of d(CATGAA) and d(TTCATG) and d(CATGAA) and d(TTC*ATG) were heated at 60°C and allowed to cool to 0°C. After 5 min at that temp., the samples were again heated with a rise of 1°C /min. The melting temperature

(Tm) value were obtained as the maxima of first derivative of absorbance/temp function.

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