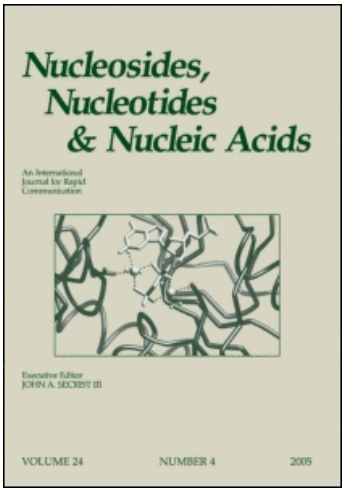

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An Improved Synthesis of 1-(2-Deoxy-β-D-erythro-pentofuranosyl)quinazoline-2,4(3*H*)-dione and Its Incorporation Into G-Rich Triple Helix Forming Oligonucleotides

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AN IMPROVED SYNTHESIS OF 1-(2-DEOXY-β-*D-ERYTHRO*-PENTOFURANOSYL)QUINAZOLINE-2,4(3*H*)-DIONE AND ITS INCORPORATION INTO G-RICH TRIPLE HELIX FORMING OLIGONUCLEOTIDES

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ABSTRACT: A convenient synthesis of 1-(2-deoxy-β-*D-erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (**6**) has been accomplished. The structural conformation of (**6**) was derived by 2D NMR, COSY and NOESY experiments. Nucleoside (**6**) was incorporated into G-rich triplex forming oligonucleotides (TFOs) by solid-support, phosphoramidite method. The triplex forming capabilities of modified TFOs (**S2, S3** and **S4**) has been evaluated in antiparallel motif with a target duplex (duplex-31) 5'd(GTCACTGGCCCTTCCTCCTTCCCGGTCTCAG)3'-5'd(CAGTGACCGGGAAGGAGGAAGGGCCAGAGTC)3' (**D1**) at pH 7.6. The parallel triplex formation of a shorter TFO (**S6**) containing **Q** has also been studied with a target duplex-11 (**D2**) at pH 5.0.

The use of short oligonucleotides for the control of gene expression has become a new type of gene therapy.[1,2] It has been demonstrated that oligonucleotides selectively inhibit gene expression through interference with posttranscriptional events such as splicing and translation.[3-7] It has been shown[8-14] that under suitable conditions, short oligonucleotides will bind in a sequence-specific manner to the major groove of the duplex DNA and form a local three stranded structure or triplex. Triplexes are formed in two different modes, parallel and antiparallel. In parallel binding mode, triple helix formation

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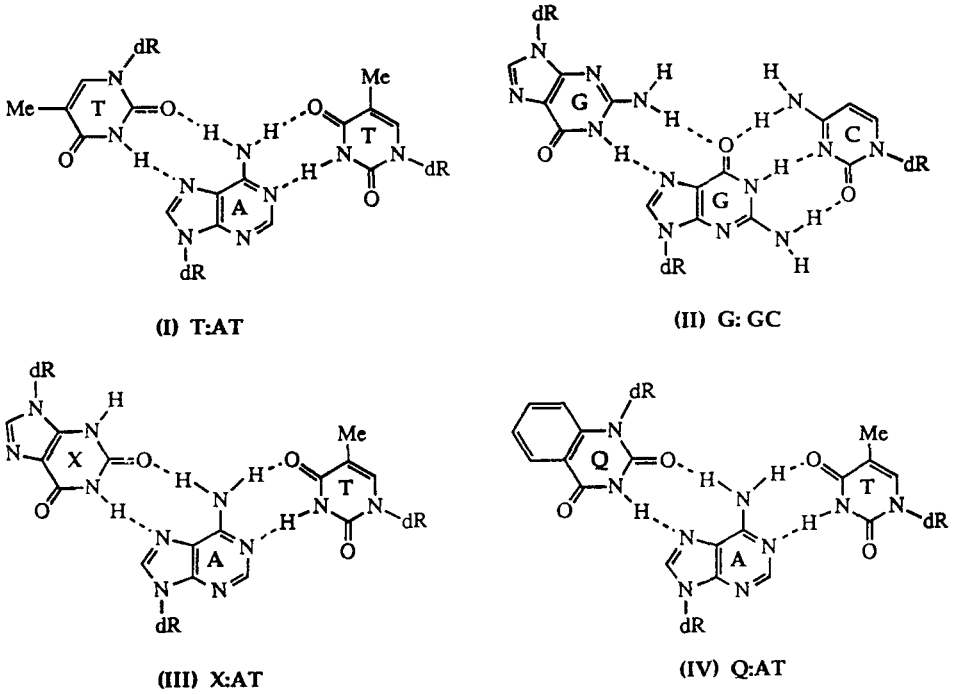


Figure 1. Reverse Hoogsteen triplet interactions for (I) Thymine (T) with AT base pairs; (II) Guanine (G) with GC base pairs; (III) Xanthine (X) with AT base pairs; (IV) Quinazoline-2,4-dione (Q) with AT base pair; dR signifies 2'-deoxyribose.

takes place when T (thymidine) in the third strand binds to AT base pairs (T:AT) and protonated 2'-deoxycytidine (C^+) in the third strand binds to GC base pairs (C^+:GC)[8,15-18] through Hoogsteen hydrogen bonding (*Figure 1*). A major drawback in this mode is that protonation of the third strand C (at N3) is necessary to bind N7 of G (2'-deoxyguanosine) in the duplex, optimally requires a pH of 5-6, which is well below physiological range. In antiparallel motif, T or A (2'-deoxyadenosine) in the third strand binds to AT base pairs and G binds to GC base pairs (G:GC) (*Figure 1*) through reverse Hoogsteen hydrogen bonding. All the bases involved in hydrogen bonding in antiparallel motif are in their normal, uncharged tautomeric forms; thus pH is not a factor in triplex formation.[9,14] Under certain conditions, G and T of third strand bind with high affinity and specificity to the duplex targets with an equilibrium dissociation constant of 10^{-10} M or less.

Despite certain favorable properties of TFOs in antiparallel motif (ap TFOs), there are several limitations that must be overcome. First, triplex

formation ideally requires a homopurine:homopyrimidine target duplex where the ideal duplex site consists entirely of G and A in one strand and paired to its Watson-Crick complementary base in the other. This limits the number of biologically interesting DNA sequences that can be targeted.

A second limitation of apTFOs is that high affinity binding requires G-rich target sequences. It was found empirically that at least 60-65% G residues be present in one strand of the intended target to achieve stable binding.[9] This further limits the ability to target biologically relevant sequences.

Based on the available data on triplex structure, it is possible to make modifications to the third strand that will overcome the above limitations. The requirement for G-rich targets indicates that T:AT triplets are relatively unstable in antiparallel motif. This may be due in part to the known preference of T for forming parallel triplexes.[8,10] This problem may be overcome by replacing T in the third strand with a purine like base analog able to bind to AT base pairs. The 2'-deoxyxanthosine (X) might be an alternative solution but is negatively charged at neutral pH.[19] Analogs of X with relatively stable glycosidic bond and no charge at neutral pH are likely to be good candidates for triplex formation in antiparallel motif. Recently, Milligan et al.[20] have demonstrated that 7-deaza-2'-deoxyxanthosine (c^7X) is a useful substitute for T in apTFOs. It was confirmed[20,21] that replacement of G with c^7G in TFOs substantially destabilizes triplex formation.

Quinazoline-2,4-dione (**Q**) may be looked upon as a benzo-(T) derivative and the replacement of T with **Q** may potentially allow **Q**:AT triplet formation at physiological pH (*Figure 1*). The presence of the benzene ring in the molecule may provide additional opportunities for stacking interactions in the third strand of DNA.

The third strand T residues in both parallel and antiparallel motifs are normally in "anti" glycosidic conformation. Normally, the different conformation model can be reliably described by two-state models "syn" and 'anti"; N/S type sugar puckering.[22] Some of the nucleosides manifest either "syn" or "anti" conformation around the glycosidic bond in the solid state.[23] The nucleosides having the "anti" conformation around the glycosidic bond is the prefered conformation in the third strand of a TFO to form stable triple helices in both parallel and antiparallel motifs. The **Q** nucleoside may reliably have either "syn" or "anti" conformation (*Figure 2*). 1D ^1H Nuclear Overhauser Enhancement Spectroscopy (NOE) technique has been widely used to find qualitative or semi-qualitative information about the preferred conformation[24] of some regular nucleosides and nucleotides in solution. Here we report an improved synthesis

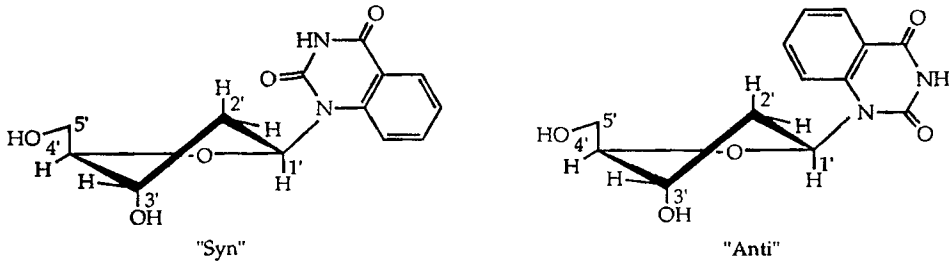


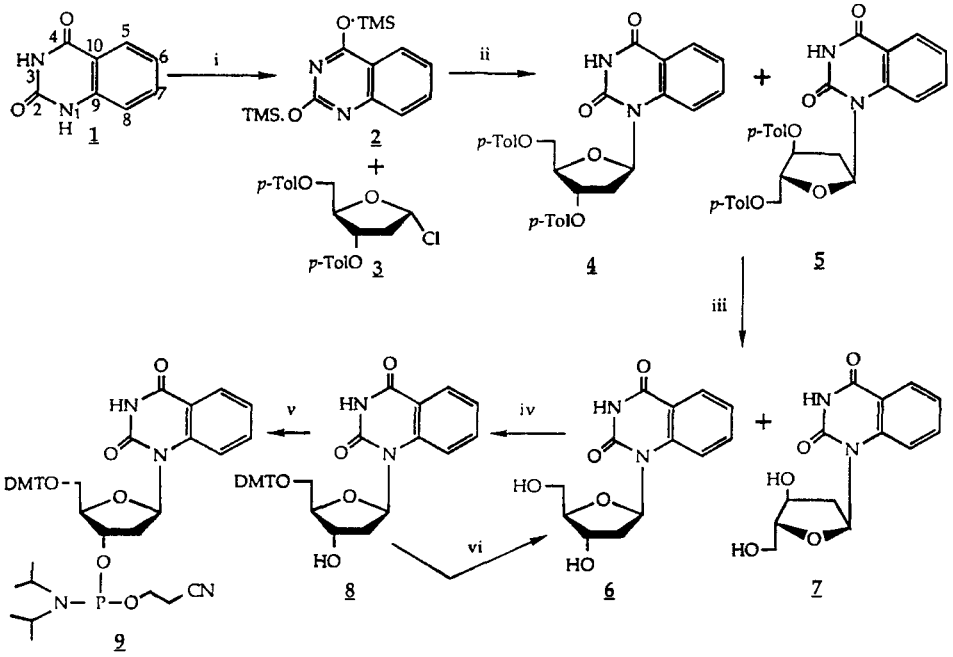
Figure 2. "Syn" and "Anti" conformation of (6)

of 1-(2-deoxy-β-*D-erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (**6**), its conformation in solution by 1D ^{1}H NOE and 2D NOESY experiments and its incorporation into G-rich TFOs employing solid-support, phosphoramidite methodology. The binding abilities of these TFOs have been evaluated with target duplex 31-mer (**D1**) in antiparallel motif at pH 7.6. The capability of triplex formation in parallel motif of a short oligomer containing **Q** has also been evaluated with a short target duplex 11-mer (**D2**) at pH 5.0.

Results and Discussion: The synthesis of 1-(2-deoxy-β-*D-erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (**6**) was first reported by Stout and Robins[25] *via* glycosylation of bis-silyl quinazoline-2,4(1*H*,3*H*)-dione (**2**) with 2-deoxy-3,5-di-*O-p*-toluoyl-α-*D-erythro*-pentofuranosyl chloride (**3**). Dunkel and Pfleiderer[26] later attempted to synthesise (**6**) *via* the glycosylation[25] of (**2**) with (**3**) in the presence of CuI and observed that the glycosylation under these conditions is not stereoselective. It was also observed[26] that the separation of α/β anomeric mixture was rather difficult and not practical for large scale synthesis.

To overcome these difficulties, Dunkel and Pfleiderer[26] synthesised (**6**) *via* a multi-step deoxygenation[27] of quinazoline-2,4-dione ribonucleoside. However, the deoxygenation method is lengthy and suffers from a major drawback in that the phenoxythiocarbonylation of the 2'-hydroxyl group of the 3',5'-tetraisopropyl-protected quinazoline-2,4-dione ribonucleoside led to a mixture of 2'-O-phenoxythiocarbonate derivative along with an anhydronucleoside.[26] In view of these difficulties and some other drawbacks of previous methods[25,26], we used the direct glycosylation procedure as described in *Scheme 1.*

Silylation of quinazoline-2,4(1*H*,3*H*)-dione (**1**)[28] with 1,1,1,3,3,3-hexamethyldisilazane (HMDS) in the presence of $(NH_4)_2SO_4$ and CH_3CONH_2 gave the



(i) HMDS/$(NH_4)_2SO_4$/CH_3CONH_2; (ii) $CHCl_3$; (iii) NH_3/MeOH; (iv) DMT-Cl/Py;

(v) 2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite/DEA/CH_2Cl_2; (vi) 80% AcOH

Scheme 1

bis-trimethylsilyl derivative (2). Treatment of (2) with (3)[29] in anhydrous $CHCl_3$ at ambient temperature and purification of the mixture by column chromatography on silica gel gave a 3:1 β/α mixture of 1-(2-deoxy-3,5-di-*O*-*p*-toluoyl-*D-erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (4) and (5) in 87% yield. The β/α mixture was deblocked with NH_3/MeOH (saturated at 0 °C) at room temperature and the resulting mixture (6 and 7) was tritylated by the treatment with 4,4'-dimethoxytrityl chloride in anhydrous pyridine. The resulting 5'-O-DMT derivatives were separated on a flash silica gel column to obtain fast moving product (R_f = 0.61, CH_2Cl_2:EtOAc; 8:2) 1-{5-O-[(4,4'-dimethoxytrityl)-2-deoxy-β-*D-erythro*-pentofuranosyl]}quinazoline-2,4(3*H*)-dione (8) in a 68% yield. The anomeric proton of (8) resonates at δ 6.89 ppm as a triplet ($J_{1',2'}$ = 8.8 Hz) and overlaps with DMT protons. In case of the α-isomer of (8) (R_f = 0.49; CH_2Cl_2:EtOAc; 8:2) the anomeric proton resonates as a triplet, further down field at δ 6.98 ppm ($J_{1',2'}$ = 7.65 Hz). The β-configuration of (8) was

further proven by detritylation to the free nucleoside (**6**) which on acylation with *p*-toluoyl chloride in pyridine gave 3',5'-di-*O*-*p*-toluoyl drivative (**4**), mp 148 °C, (Lit[26] mp 147-148 °C). The anomeric proton of (**4**) resonates at δ 6.67 ppm (DMSO-d_6) as a triplet, $J_{1',2'}$ = 7.21 Hz and in full agreement with the proven configuration of 1-(3,5-di-*O*-*p*-toluoyl-2-deoxy-β-*D-erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione.[26] Further, β- and α-anomer can be easily distinguished by saturating H1' and measuring the NOE factors at H4' and H3'.[30] It has been established[30] that Occurrence of an NOE at H4' proves β-configuration at anomeric center while α-anomer shows an NOE at H3'. This has been successfully applied to 2'-deoxy-*D*-ribonucleosides and other ribonucleosides.[31] Since the anomeric proton of (**8**) overlaps with proton of DMT, we detritylated (**8**) to get the free nucleoside (**6**). When H1' of (**6**) irradiated, an NOE was observed at H4' (*Figure 3*) and confirms the β-configuration. The occurrence of an NOE proves the the β-configuration of of (**6**) at the anomeric center and this experiment also proves the β-configuration of (**8**). Treatment of **8** with 80% aqueous acetic acid gave a 96% yield of 1-(2-deoxy-β-*D-erythro*-pentofuranosyl)-quinazoline-2,4(3*H*)-dione (**6**) as a crystalline product [mp 184-185 °C; Lit.[26] mp 185-186 °C]. The structure of (**6**) was established unequivocally by 1D ^{1}H NMR, ^{13}C NMR, 2D COSY (homonuclear and heteronuclear) and NOESY studies. The anomeric proton ($C_{1'}H$) of **6** resonates at δ 6.70 ppm as a triplet with $J_{1',2'}$ = 8.0 Hz and $J_{1',2''}$ = 8.02. The detail assignments of the aromatic protons of the benzene ring of (**6**) was not fully characterised previously[26]. Unequivocal assignment of the anomeric proton as well as the benzene protons of (**6**) was confirmed by COSY (homonuclear) and HETCOR spectra.

The assignment of resonances in the ^{1}H and ^{13}C spectra (HETCOR) (*Figure 4*) of (**6**) are self-consistent and find corroboration in 2D correlation data. From the HETCOR spectrum, the peak at δ 84.38 is coupled with one triplet peak at δ 6.70 and assigned C1' and H1' anomeric proton. The peak at δ 116.55 coupled with one doublet peak at 7.89 and assigned as C8 and H8. The peak at 127.45 coupled with one quartet peak at δ 8.04 and assigned as C5 and H5. The initial ambiguity of the assignments[26] of the resonances of H6 and H7 and consequently of C6 and C7 in the ^{13}C NMR spectrum was resolved by the 1D ^{1}H NOE difference study.

An NOE (1D ^{1}H NOE) experiment allowed us to unequivocally assign the H2' (δ 2.31 ppm) and H2" (δ 2.47 ppm) of nucleoside (**6**). The lower field signal at 2.47 ppm (H2") was enhanced by 5.5% on irradiation of anomeric (H1') proton. The higher field signal at 2.31 ppm (H2') was not enhanced. Irradiation of H8 at

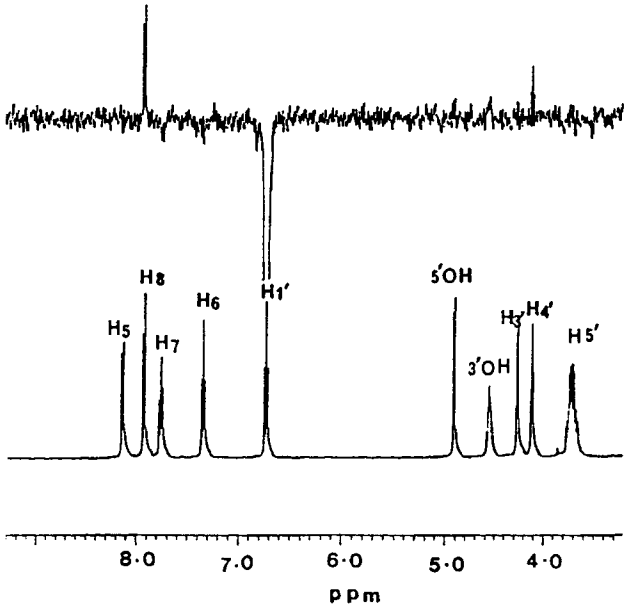


Figure 3. ^{1}H NOE spectrum of (6) in $(CD_3)_2CO$, showing NOE at H4' upon irradiation of H1'.

7.89 ppm led to a 6.9 % enhancement of the resonance at 2.31 ppm (H2'), further confirming that this signal was due to H2'. The NOE difference between H8 and H1' protons (4.5%) was found to be smaller than that of H8 and H2' (*Figure 5*). There was significantly smaller NOE effect between H7 (δ 7.74 ppm) and H2' proton (2.4%). This indicates that H1', H2', H7 and H8 all be in proximity to one another which was also confirmed by 2D NOESY experiments. Distinct correlations for NOE interactions between H2' and H7 as well as H1' and H8 were present (*Figure 6*).

The mutual correlations of 3'-OH proton and 5'-OH proton were very intense and indicated that the C5'O-bond is oriented antiperiplanar to C4'O-bond. The correlations for H8 and H7, H7 and H6, and H6 and H5 are also present confirming signal assignments. Distinct correlations for NOE interactions between H2' and H8, H1' and H8 as well as H2' and H7 clearly indicates the predominance of "anti" conformation of (6) (*Figure 2*) in DMSO-d_6 at room temperature. However, the presence of a small population of "syn" conformer is not ruled out as indicated by some signals in NOESY experiment (*Figure 6*) at room temperature.

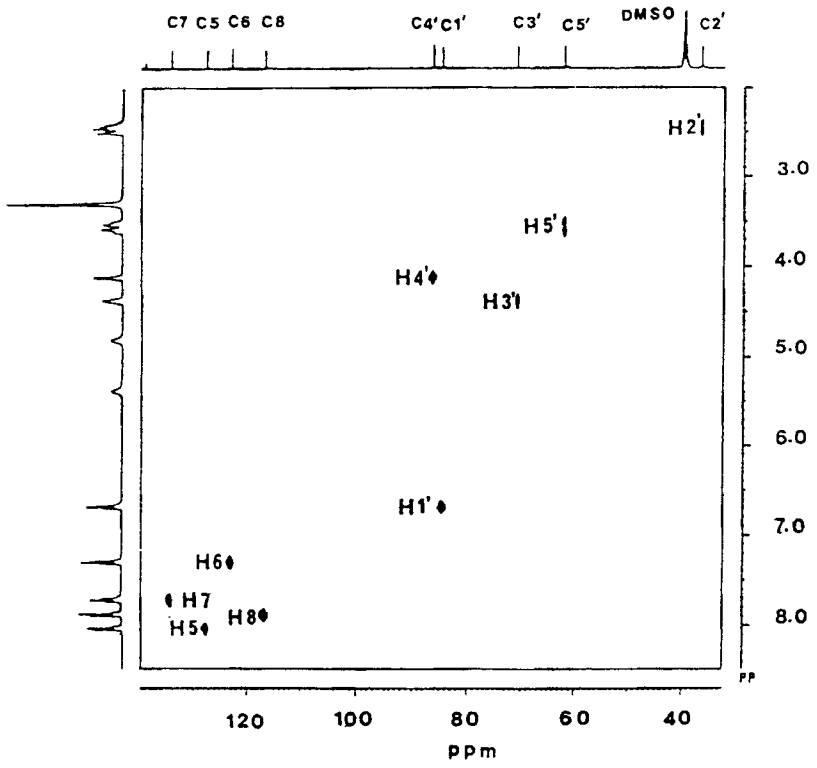


Figure 4. HETCOR spectrum of (6) in DMSO-d_6 at 313 K. For details see experimental section.

Thus, we established that the glycosylation of (2) with (3) in anhydrous $CHCl_3$ is very efficient without the use of CuI. The introduction of the DMT group at 5'-position of an anomeric mixture of nucleosides (6 and 7) has facilitated the separation of the required β-isomer (8) by column chromatography and gave very good overall yield of the free nucleoside (6). Thus, this method is efficient and very practical for the synthesis of (6). The phosphitylation of (8) with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite in dry CH_2Cl_2 in the presence of *N,N*-diisopropylethylamine gave the target 1-{5-O-[4,4'-dimethoxytrityl)-2-deoxy-β-*D-erythro*-pentofuranosyl]}quinazolin-2,4(3*H*)-dione-3'-[2-cyanoethyl bis(1-methylethyl)]-phosphoramidite (9) in a 84% yield.

The phosphoramidite (9) was successfully incorporated into G-rich TFOs by solid-support methodology in an automated DNA-synthesizer. The sequence

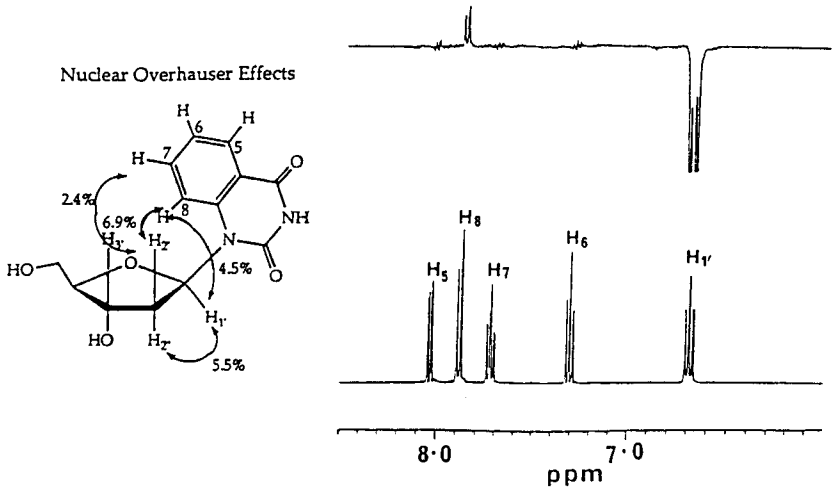


Figure 5. The downfield region of the NOE difference spectrum of (6) in DMSO-d_6 at 313 K..The low power irradiation frequency was cycled every μsec. among the three resonances of H1' triplet centered at 6.70 ppm

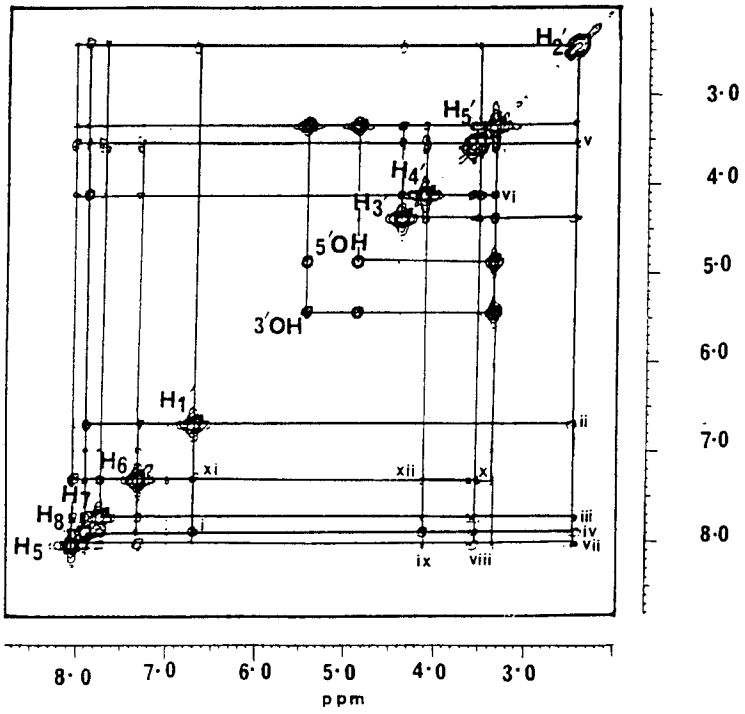


Figure 6. Symmetrized absolute value mode NOESY contour plot of (6) in DMSO-d_6. Labelling peak correlations are: (i) H2'/H7; (ii) H1'/H2'; (iii) H2'/H7;(iv) H2'/H8; (v) H5'/H2'; (vi) H5'/H4'; (vii) H5/H2'; (viii) H5/H5'· (ix) H5/H4'; (x) H6/H5'; (xi) H6/H1'; (xii) H6/H4'. The data was collected in absolute value mode with a D1 = 1 sec; D9 = 500 ms.

of TFOs (**S2, S3, S4** and **S6**) containing quinazoline-2,4-dione (**Q**) has been listed in *Table 1*. These TFOs have been synthesised on 1 μmol scale and were purified by ion-exchange chromatography on a pharmacia column.[32] The purity of the G-rich TFOs was determined by analytical HPLC (Q-sepharose ion-exchange column) and by labeling with ^{32}P-ATP using polynucleotide kinase followed by analysis on a 20% denaturing polyacrylamide gel.[33]

The nucleoside composition analysis of all TFOs (**S2, S3, S4** and **S6**) containing **Q** (**6**) was determined by digestion with P1 nuclease/bacterial alkaline phosphatase. A mixture of standard solutions of the five deoxynucleosides was analysed by a C_{18} reverse-phase HPLC column using 0.05 M KH_2PO_4 buffer and acetonitrile with a flow rate of 2.0 mL/min., which gave the order of elution as **C, G, T, A** and **Q** (*Figure 7A*). The relative amounts of each of the deoxynucleosides in the digestion mixtures were estimated from the peak areas. The HPLC profiles of the digested TFOs have been shown in*Figures 7B-E.* The nucleoside composition analyses are shown to be consistent with the proposed sequences and confirmed that the nucleoside **Q** was stable to the reaction conditions.

The antiparallel triplex formation of the TFOs (**S2, S3** and **S4**) was assayed by the gel mobility shift method.[9,14] The apparent dissociation constant (K_d) for antiparallel triplex formation was estimated as equivalent to the TFO concentration required to bind 50% of the labeled duplex.[9] It was observed that the unmodified oligomer (**S1**) (control) binds to the duplex target (**D1**) at ~3 x 10^{-6}M. In contrast, the TFOs, **S2, S3** and **S4** did not show detectable triplex formation at this micromolar concentration.

In order to evaluate the binding effects of the base **Q** in parallel motif, a TFO (**S6**) containing two **Q**s in place of two Ts of a Oligo (**S5**) (control) was assayed with a short duplex-11 (**D2**) by thermal denaturation method. When the control oligo (**S5**) and the duplex **D2** were mixed in high salt buffer (10μM CH_3COONa, pH 5.0 and 20 μM $MgCl_2$, 1 M NaCl), the formation of a triplex was observed by the presence of two melting transitions. The high temperature transition for the mixture (Tm = 43.5 °C) corresponds to that of the duplex alone (Tm = 43.7 °C). In addition to the duplex transition, the low temperature transition with Tm of 26.4°C corresponds to the melting transition of triplex **S5-D2**. In contrast to the observations with the control oligo (**S5**), no triplex melting transition was detected when the TFO **S6** was mixed with duplex **D2** under identical condition.

Table 1

Oligonucleotides (TFOs)	Sequence
S1	5'-GGGTTGGTGGTTGGG-3'
S2	5'-GGGTTGG**Q**GGTTGGG-3'
S3	5'-GGG**QQ**GGTGG**QQ**GGG-3'
S4	5'-GGG**QQ**GG**Q**GG**QQ**GGG-3'
S5	5'-TTCTTCTT-3'
S6	5'-TTC**QQ**CTT-3'
D1 (Duplex)	5'-GTCACTGGCCCTTCCTCCTTCCCGGTCTCAG-3'
	3'-CAGTGACCGGGAAGGAGGAAGGGCCAGAGTC-5'
D2 (Duplex)	5'-TTCTTCTTCTT-3'
	3'-AAGAAGAAGAA-5'

In summary, we have developed an improved method for the synthesis of 1-(2-deoxy-β-*D-erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (**6**) and successfully incorporaed into G-rich triplex forming oligonucleotides (TFOs) using conventional solid-support methodology. 1D ^{1}H NOE data indicated the predominance of "anti" conformation of (**6**) in DMSO-d_6 solution. However, the presence of a small population of "syn" conformer is not ruled out as indicated by NOESY experiment. The binding results suggests that the replacement of T with **Q** decreased the stability of triple helices in both antiparallel and parallel motifs. A similar observation has been made by Staubli and Dervan[34] in case of non-natural pyrido[2,3-*d*]pyrimidine nucleoside. The decrease in the stability of triple helices might be due to either the bulky benzo group which might prevent **Q** from assuming a conformation compatible with triplex motif or made the "anti" conformation unfavorable.

EXPERIMENTAL

Melting points (uncorrected) were determined in a Thomas-Hoover capillary melting-point apparatus. Elemental analyses were performed by Quantitative Technologies Inc., Whitehouse, NJ. The presence of water as indicated by

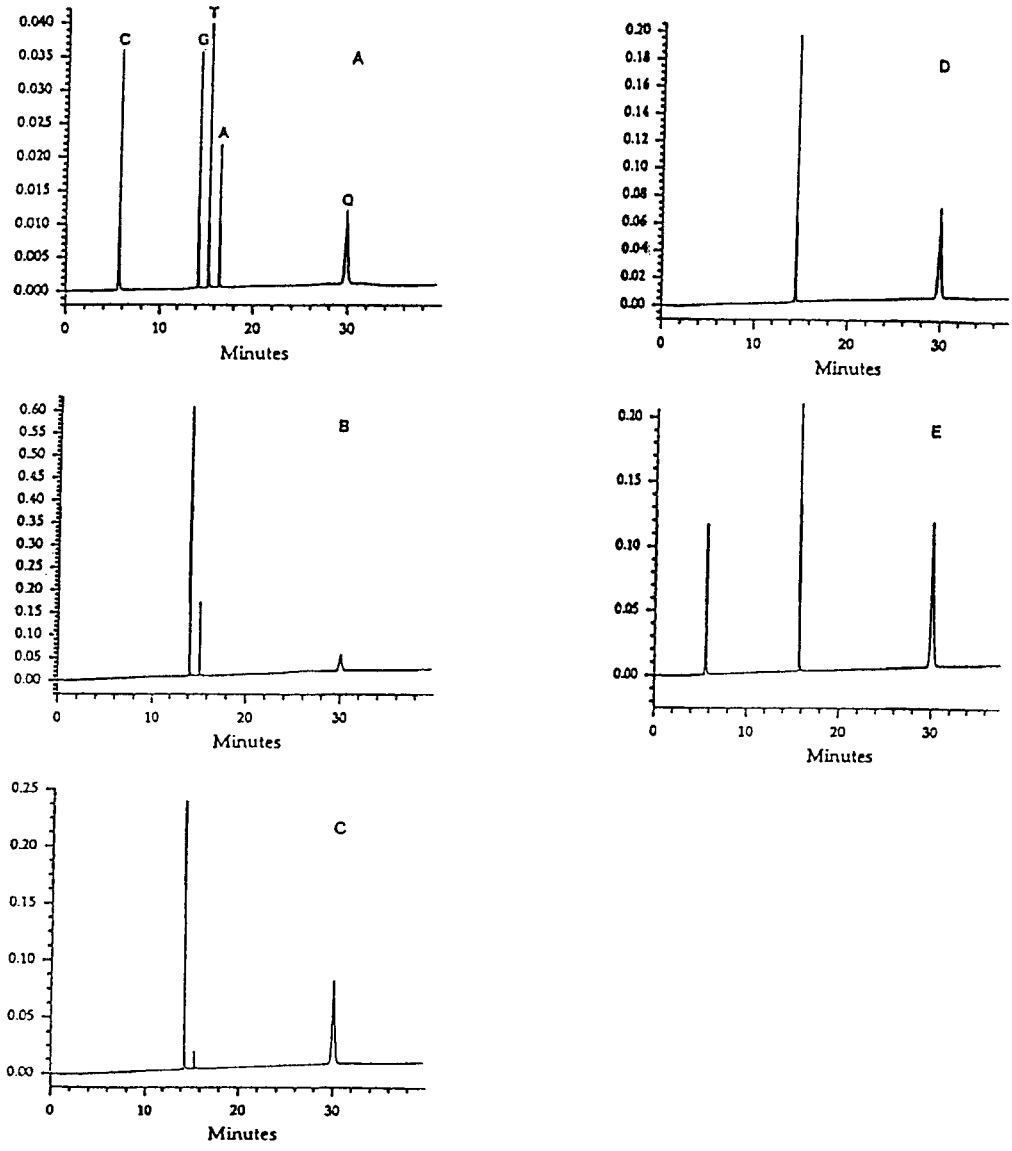


Figure 7. HPLC elution profiles of deoxynucleosides (nucleoside composition analysis) obtained by enzymatic digestion of modified TFOs, A) C, G, T, A, Q; B) (S2); C) (S3); D) (S4) and E) (S6). Separation of deoxynucleosides was performed on a reversed phase SUPELCO, LC-18-DB column using 0.05 M KH_2PO_4 buffer in H_2O/CH_3CN as the eluent, flow rate 2 mL/min.

elemental analysis was verified by ^1H NMR spectroscopy. Thin layer chromatography (TLC) was performed on aluminum plates coated (0.2 mm) with silica gel $60F_{254}$ (EM Science). Silica gel (EM Science, 230-400 mesh) was used for flash column chromatography. All solvents used were reagent grade and the solvent mixtures are in volumes. Detection of nucleoside components on TLC was by uv light, and with 10% H_2SO_4 in MeOH spray followed by heating. Evaporations were conducted under diminished pressure with the bath temperature below 30 °C. Infrared (IR in KBr) spectra were recorded with a Perkin-Elmer 1420 IR spectrophotometer and ultraviolet spectra (UV) were recorded on a Hewlett-Packard 8452 diode array spectrophotometer.

The magnetic resonance spectra were recorded on a Brüker wide bore AM-400 FT-NMR spectrometer operating at a field strength of 9.4 T (400.14 MHz for ^1H, 100.62 MHz for ^{13}C, and 161.98 MHz for ^{31}P). The chemical shift values (δ) are expressed in ppm (parts per million). The ^1H chemical shift is expressed relative to tetramethylsilane (TMS) as the internal standard, ^{31}P shifts are relative to 85% polyphosphoric acid as external standard. The DMSO-d_6 multiplet (39.5 ppm) served as the reference for ^{13}C chemical shifts. The digital resolution employed for ^1H and ^{13}C were 0.66 Hz/pt and 0.65 Hz/pt, respectively, in the 1D spectra (key: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, q = quartet, m = multiplet, br = broad). Data processing was performed with ASPECT 3000 equipped with an array processor. All spectra were run at 313 K. The Homonuclear Double Quantum Filter Correlation Spectroscopy (DQF-COSY) was run in the phase sensitive mode using the standard pulse squence: D1-90°-DO-90°-D3-90°-FID. The data block size was 1024 x 512 with zero-filling in the f1 dimension. The recycle delay D1 was set to 1 sec. and the proton 90 pulse width was 7.4 μsec. The data was processed with a shifted ($\pi/2$) sine bell weighting in both dimensions. The Heteronuclear (^{13}C, ^1H) correlation spectrum (HETCOR) was performed in the absolute value mode using the standard pulse sequence. The 1D ^1H NOE difference spectroscopy was performed using a 20 ppm spectral window with 64 K data points. The 2D Nuclear Overhauser Enhancement Spectroscopy (NOESY) was recorded in the absolute value mode using the standard pulse sequence.

1-(3,5-Di-*O*-*p*-toluoyl-2-deoxy-β-*D*-*erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (4) and 1-(3,5-Di-*O*-*p*-toluoyl-2-deoxy-α-*D*-*erythro*-pentofuranosyl)-quinazoline-2,4(3*H*)-dione (5). Quinazoline-2,4(1*H*,3*H*)-dione (1, 1.0 g, 6.17 mmol) (commercially sold as benzoyleneurea)[28] was added to a mixture of

1,1,1,3,3,3-hexamethyldisilazane (HMDS, 65 mL), $(NH_4)_2SO_4$ (0.10 g) and CH_3CONH_2 (0.15 g). The reaction mixture was heated under reflux with stirring for 12 h with the exclusion of moisture. The mixture was evaporated to dryness and the residue was co-evaporated with anhydrous $CHCl_3$ (2 x 20 mL). The residue was dissolved in dry $CHCl_3$ (75 mL). To the solution was added 2-deoxy-3,5-di-*O*-*p*-toluoyl-α-*D*-*erythro*-pentofuranosyl chloride (**3**)[29] (3.11 g, 6.17 mmol) (26) and the mixture was stirred at ambient temperature for 48 h. The mixture was evaporated to dryness and the residue was dissolved in CH_2Cl_2 (100 mL). The organic solution was washed with a saturated solution of $NaHCO_3$ (250 mL), dried (Na_2SO_4), concentrated and chromatographed on a silica gel column using CH_2Cl_2:EtOAc (96:4) as the eluent to yield 2.75 g (86.7%) of a α/β mixture (**5** + **4**) as a white foam. ^1H NMR (DMSO-d_6): δ 2.51 (m, 1 H, $C_{2'}H$ of **4**), 2.77 (m, 1 H, $C_{2''}H$ of **4**), 2.99 (m, 1 H, $C_{2'}H$ of **5**), 3.01 (m, 1 H, $C_{2''}H$ of **5**), 4.03 (m, 1 H, $C_{3'}H$ of **4**), 4.48-4.68 (m, 5 H, $C_{3'}H$ of **5** , $C_{5'}H_2$ of **4** and **5**), 4.94 (m, 1 H, $C_{4'}H$ of **4**), 5.63 (m, 1 H, $C_{4'}H$ of **5**), 6.77 (t, 1 H, $C_{1'}H$ of **4**), 6.96 (t, 1 H, $C_{1'}H$ of **5**), 7.27-8.08 (m, 12 H, Aromatic) and 11.62 (br s, 2 H, N*H* of **4** and **5**).

1-(2-Deoxy-β-*D*-*erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (6) and 1-(2-deoxy-α-*D*-*erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (7). The above α/β mixture (**4** + **5**, 2.6 g, 5.05 mmol) was treated with NH_3/MeOH (saturated at 0 °C, 80 mL) in a pressure bottle and the mixture was stirred at ambient temperature for 48 h. The reaction mixture was evaporated to dryness and the residue was co-evaporated with MeOH (2 x 25 mL). The residue was triturated with ether (3 x 40 mL). The solid was collected by filtration and crystallized from aqueous EtOH to give 1.30 g (92.5%) of a mixture of **6** and **7**, which was used in the next step without the separation of the anomers.

1-{5-*O*-[(4,4'-Dimethoxytrityl)-2-deoxy-β-*D*-*erythro*-pentofuranosyl]}-quinazoline-2,4(3*H*)-dione (8). The mixture of **6** and **7** (4.50 g, 16.17 mmol) was co-evaporated with dry pyridine (2 x 50 mL) and then dissolved in dry pyridine (250 mL). To this solution 4,4'-dimethoxytrityl chloride (6.02 g, 17.79 mmol) was added and the mixture was stirred at room temperature for 4 h with the exclusion of moisture. The reaction was quenched by the addition of MeOH (4 mL). The mixture was evaporated and the residue was co-evaporated with toluene (3 x 30 mL) to remove the last traces of pyridine. The residue was dissolved in CH_2Cl_2 (125 mL) and the organic solution was washed with a saturated solution of $NaHCO_3$ (250 mL), dried (Na_2SO_4) and evaporated to

dryness. The residue was purified by flash silica gel column chromatography using CH_2Cl_2:EtOAc (8:2) as the eluent to give 6.25 g (66.65%) of **8** as a light yellow solid, mp 130-132 °C; IR ν_{max} 1694 (C=O), 3182-3300 (NH, OH) cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.46 (m, 1 H, $C_{2'}H$), 2.53 (m, 1 H, $C_{2''}H$), 3.11 (q, 1 H, $C_{5'}H$), 3.22 (dd, 1 H, $C_{5''}H$), 3.74 (s, 6 H, 2 OCH_3), 4.34 (m, 2 H, $C_{3'}H$ and $C_{4'}H$), 5.47 (d, J = 4.8 Hz, 1 H, $C_{3'}OH$), 6.89 (t, 1 H, $J_{1',2'}$ = 8.8 Hz, $C_{1'}H$), 6.91-7.44 (m, 14 H, DMT and C_6H), 7.75 (m, 1 H, C_7H), 7.94 (dd, 1 H, C_8H), 8.07 (dd, J = 16 Hz and J = 1.2 Hz, 1 H, C_5H). Anal. Calcd. for $C_{34}H_{32}N_2O_7 \cdot 0.25\ H_2O$: C, 69.79; H, 5.59; N, 4.78. Found: C, 69.47; H, 5.57; N, 4.57.

1-(2-Deoxy-β-*D-erythro*-pentofuranosyl)quinazoline-2,4(3*H*)-dione (6). To a solution of **8** (0.30 g, 0.52 mmol) in MeOH (1.0 mL) wad added 80% AcOH (15 mL) and the mixture was stirred at ambient temperature for 2 h. The mixture was evaporated to dryness and the residue was co-evaporated with MeOH (2 x 10 mL). The residual solid was dissolved in MeOH (2 mL), adsorbed on to silica gel (6 g) and loaded on the top of a pre-packed silica gel column (in CH_2Cl_2). The column was eluted with CH_2Cl_2:MeOH (95:5) and the appropriate homogeneous fractions were pooled. Evaporation of the solvent and crystallization of the residue from water gave 0.15 g (95.5%) of **6** as a crystalline product, mp 184-185 °C [Lit.[27] mp 185-186 °C]; IR ν_{max} 1690 (C=O), 3025-3400 (OH, NH) cm^{-1}; UV λ_{max} nm (ε x 10^{-3}): (pH 1) 224 (76.0), 244 (5.8), 308 (3.0); (pH 7) 226 (16.5), 244 (6.5), 300 (3.3); (pH 11) 224 (15.5), 246 (4.4), 208 (2.4); ^1H NMR (DMSO-d_6): δ 2.47 (m, 2 H, $C_{2'}H$ and $C_{2''}H$), 3.59 (m, 2 H, $C_{5'}H_2$), 4.11 (q, 1 H, $C_{4'}H$), 4.36 (d, 1 H, $C_{3'}H$), 4.86 (s, 1 H, $C_{3'}OH$), 5.43 (s, 1 H, $C_{5'}OH$), 6.70 (t, $J_{1',2'}$ = 8.0 Hz, $J_{1',2''}$ = 8.02 Hz, 1 H, $C_{1'}H$), 7.31 (t, $J_{H6,H5}$ = 4.4 Hz, $J_{H6,H7}$ = 8.0 Hz, 1 H, C_6H), 7.74 (q, 1 H, C_7H), 7.89 (d, $J_{H8,H7}$ = 8.8 Hz, 1 H, C_8H), 8.04 (q, 1 H, C_5H), 11.62 (br s, 1 H, N*H*). ^{13}C NMR (DMSO-d_6): δ 36.38 ($C_{2'}$), 61.72 ($C_{5'}$), 70.52 ($C_{3'}$), 84.38 ($C_{1'}$), 86.03 ($C_{4'}$), 116.45 (C_{10}), 116.55 (C_8), 122.85 (C_6), 127.45 (C_5), 134.23 (C_7), 139.13 (C_9), 149.94 (C_4), 161.30 (C_2). Anal. Calcd. for $C_{13}H_{14}N_2O_5 \cdot 0.5H_2O$: C, 54.34; H, 5.26; N, 9.75. Found: C, 54.45; H, 5.04; N, 9.63.

1-{5-*O*-[(4,4'-Dimethoxytrityl)-2-deoxy-β-*D-erythro*-pentofuranosyl]}-quinazolin-2,4(3*H*)-dione-3'-[2-cyanoethyl bis(1-methylethyl)]phosphoramidite (9). Compound **8** (1.0 g, 1.69 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) and the solution was stirred under argon for 10 min. To this solution, *N,N*-diisopropylethylamine (0.87 g, 6.74 mmol) was added, followed by 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.38 g, 5.83 mmol). The reaction

mixture was stirred at ambient temperature for 45 min. The mixture was diluted with CH_2Cl_2 (50 mL) and partitioned between 15% aqueous solution of $NaHCO_3$ (100 mL). The organic layer was separated, dried (Na_2SO_4) and evaporated to dryness. The residual syrup was dissolved in a mixture of CH_2Cl_2:Et_3N (95:5, 2 mL) and loaded on top of a silica gel column pre-packed in a mixture of CH_2Cl_2:Et_3N (98:2). The column was eluted with CH_2Cl_2:EtOAc:Et_3N (90:8:2), the appropriate homogeneous fractions were pooled and evaporated. The residue was dissolved in CH_2Cl_2 (1 mL) and added with stirring to hexane (100 mL, -40 °C). The cloudy solution was evaporated to dryness to give 1.1 g (83.7%) of **9** as a white foam. ^{31}P NMR (CD_3CN): δ 149.93; ^1H NMR (CD_3CN): δ 1.27{m, 12 H, $N[CH(CH_3)_2]_2$}, 1.97 {m, 2 H, $N[CH(CH_3)_2]_2$}, 2.54 (m, 1 H, $C_2'H$), 2.71 (m, 1 H, $C_{2''}H$), 3.37 (m, 2 H, $C_5'H_2$), 3.58 (m, 2 H, CH_2CN), 3.67 (m, 2 H, OCH_2), 3.77 (s, 6 H, 2 OCH_3), 4.52 (dd, 1 H, $C_4'H$), 4.67 (m, 1 H, $C_3'H$), 6.67 (m, 3 H, $C_1'H$ and DMT), 6.88-7.46 (m, 11 H, DMT), 7.48 (m, 1 H, C_6H), 7.70 (m, 1 H, C_7H), 7.86 (dd, 1 H, C_8H), 8.13 (dd, 1 H, C_5H). Anal. Calcd. for $C_{43}H_{49}N_4O_8P \cdot 0.5\ H_2O$: C, 65.38; H, 6.39; N, 7.09; P, 3.92. Found: C, 65.21, H, 6.76; N, 7.39; P, 3.99.

Synthesis, Purification and Characterization of Oligonucleotides (TFOs): The oligonucleotides (TFOs) were synthesised on Applied Biosystem DNA synthesizer (models 380B or 394) using phosphoramidite method on 1 μmole scale. The phosphoramidite of T (Thymidine), C (2'-deoxycytidine) and G. (2'-deoxyguanosine) were purchased from Chem. Implex International, Inc. The isobutyl-protected G was used for incorporation of G residues into TFOs. The concentration of the phosphoramidites used was 0.1μM and the coupling time was increased to 900 seconds. The coupling efficiency was measured by UV spectrophotometric quantitation of released dimethoxytrityl cation at 498 nm on each synthesis cycle, which indicated that the average coupling efficiency of this synthon (**9**) is more than 98%. After the synthesis, the TFOs were cleaved from the solid-support by treatment with ethanolic ammonia at 56°C for 15-18 h. The purification of the oligonucleotides (TFOs) was carried out by ion-exchange chromatography on a pharmacia column[30] and desalted by passage through a C_{18} sep-pak (Waters) cartridge. The purity of the TFOs was determined by analytical HPLC (Q-sepharose ion-exchange column) using buffer A (0.5 M NaCl in 10 mM NaOH); buffer B (1.5 M NaCl in 10 mM NaOH); flow rate 2.5 mL/min. Initial conditions, A:B = 90:10, 5 min; then A:B = 40:60, 100 min; finally A:B = 0:100, 20 min. The purity of these TFOs was further determined by labeling with

^{32}P-ATP using polynucleotide kinase followed by analysis on a 20% denaturing polyacrylamide gel.[31]

The nucleoside composition of all the modified TFOs containing **Q** was determined by digestion with P1 nuclease/bacterial alkaline phosphatase. A mixture of standard solutions of the five deoxynucleosides was analysed by a C_{18} reverse-phase HPLC column using 0.05 M KH_2PO_4 buffer and CH_3CN with a flow rate of 2.0 mL/min., which gave the order of elution as **C, G, T, A** and **Q**.

Binding Experiments: The antiparallel triplex formation of the TFOs was assayed by the gel mobility shift method.[9,14] The TFOs containing trace concentrations of radiolabeled duplex and increasing concentrations of the TFOs were incubated in 20 μM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10% sucrose, at 37 °C for 18-22 h and were electrophorased in 12% polyacrylamide gels buffered with 89 mM Tris-borate, 10 mM $MgCl_2$. Following electrophoresis, gels were dried and autoradiographed. The apparent dissociation constant (K_d) for antiparallel triplex formation was estimated as equivalent to the TFO concentration required to bind 50% of the labeled duplex.

The parallel triplex formation was assayed by UV-thermal denaturation in a GBC 916 scanning spectrophotometer. Melting was assayed in 10 μM CH_3COONa, pH 5.0, 20 μM $MgCl_2$, 1 M NaCl. Detection was at 260 nm. Each sample was heated from ~5 °C to ~70 °C and back to ~20 °C to ensure that the transitions were reversible. Heating and cooling rates were 0.5 °C/min. Melting temperatures were determined from the maxima of the first derivative of the OD vs. temperature curves.

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