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Selective recognition of CG interruption by 2′,4′-BNA having 1-isoquinolone as a nucleobase in a pyrimidine motif triplex formation☆

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Abstract—To develop a novel nucleoside analogue for the effective recognition of CG interruption in a homopurine–homopyrimidine tract of double-stranded DNA (dsDNA), we succeeded in the synthesis of a triplex-forming oligonucleotide (TFO) containing a novel 2′,4′-BNA (Q^B) bearing 1-isoquinolone as a nucleobase, and the triplex-forming ability and sequence-selectivity of the TFO (TFO-Q^B) were examined. On melting temperature (T_m) measurements, it was found that the TFO-Q^B formed a stable triplex DNA in a highly sequence-selective manner under near physiological conditions.

1. Introduction

Certain homopyrimidine or homopurine oligonucleotides [triplex-forming oligonucleotides (TFOs)] are well-known to bind with double-stranded DNA (dsDNA) to form a triplex DNA in a sequence-specific manner, and they have attracted widespread attention, due to their potentiality as a practical tool to control a specific gene-expression in vitro and in vivo.[1–5] In a pyrimidine motif triplex formation, the sequence-specificity is derived from a Hoogsteen-type interaction of T and C^+ with AT and GC base pairs, respectively (Fig. 1(a)). On the other hand, in a purine motif triplex DNA, the formation of the reverse Hoogsteen-type base triads, A·AT, T·AT and G·GC, also results in the sequence-specific interaction (Fig. 1(b)). Thus, the TFOs in both motifs bind to only the homopurine–homopyrimidine tract of dsDNA, and pyrimidine–purine interruption in a homopurine–homopyrimidine region leads to a drastic decrease in the stability of a triplex. In addition, a triplex is well known to be much more unstable than a duplex. Therefore, many attempts to develop nucleoside analogues to effectively recognize pyrimidine–purine interruption and to stabilize a triplex have been made to date;[6–14] however, most of the approaches have not reached the practical level.

For several years, we have been developing novel nucleic acid analogues, bridged nucleic acids (BNAs).[15] One of these analogues, 2′-*O*,4′-*C*-methyleneribonucleic acid (2′,4′-BNA) has a fixed N-type sugar conformation (Fig. 2) and its oligonucleotides were found to exhibit extraordinarily high binding affinity for not only single-stranded RNA or DNA but also homopurine–homopyrimidine dsDNA.[16–22] Moreover, we demonstrated a 2′,4′-BNA derivative bearing a 2-pyridone nucleobase (P^B), nicely recognized CG interruption in a homopurine–homopyrimidine tract.[23,24] In this triad, the carbonyl oxygen in a 2-pyridone nucleobase (P) would play an important role in the recognition of CG interruption through hydrogen bonding with the 4-amino hydrogen in C (Fig. 3(a)).

However, P^B was found to also interact with an AT base pair though the binding affinity was weak compared with that for a CG base pair (Fig. 3(a)). For discrimination between a CG and AT base pair, we focused on a bulky 5-methyl group of T. The 5-methyl group is located in the major groove of the DNA duplex; therefore, an appropriate substitution of P^B nucleobase would decrease the stability for the AT base pair by a steric hindrance of the methyl group. After due consideration, we selected 1-isoquinolone (Q)[25] as a nucleobase to recognize the CG base pair in triplex formation (Fig. 3(b)). We expected that the steric repulsion between a 4-hydrogen of Q and the 5-methyl group of T would prevent Q·AT triad formation, while a 2-carbonyl oxygen of Q would make a hydrogen bond with a 4-amino hydrogen of C to form a stable Q·CG triad. In this paper, the synthesis of the TFO containing a novel 2′,4′-BNA analogue bearing 1-isoquinolone (Q^B) and the selective recognition of CG interruption by Q^B are described.

☆ Y. H. and S. O. have contributed equally to this work.

Keywords: nucleic acid analogues; molecular recognition; triplex; antigene.

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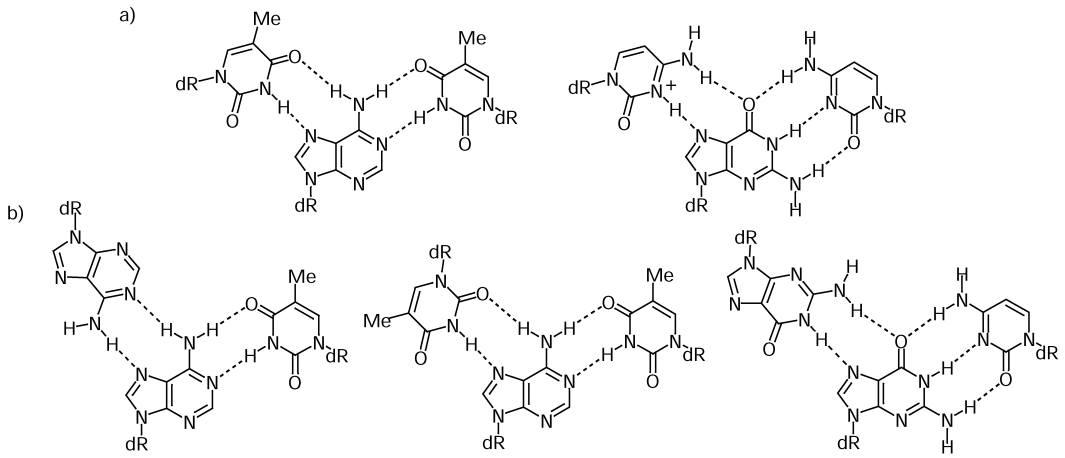


Figure 1. Hydrogen bonding styles of base triads. (a) T·AT and C^{+}·GC triads in a pyrimidine motif triplex; (b) A·AT, T·AT and G·GC triads in a purine motif triplex.

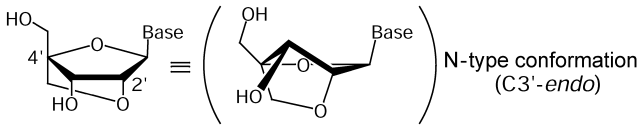


Figure 2. The chemical structure of 2′,4′-BNA.

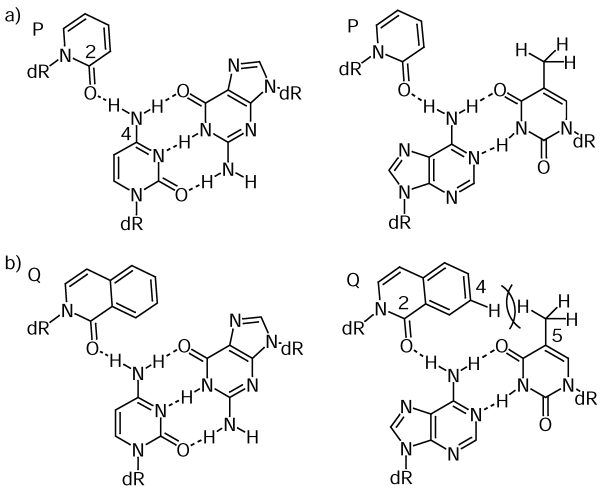
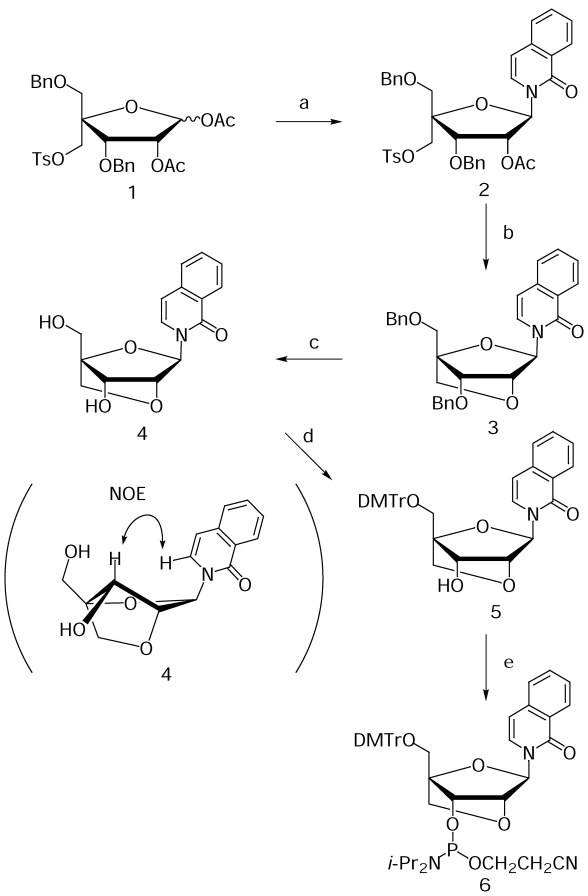


Figure 3. The proposed hydrogen bonding styles of base triads. (a) P·CG and P·AT triads; (b) Q·CG and Q·AT triads.

2. Results and discussion

2.1. Synthesis of a TFO containing Q^{B}

As shown in Scheme 1, the starting material **1**,[26] which was prepared from D-glucose, was treated with 1-isoquinolone, *N*,*O*-bis(trimethylsilyl)acetamide (BSA) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) to give the β-isomer **2** in 83% yield according to Vorbrüggen's procedure.[27] The bicyclic nucleoside analogue **3** was obtained in 95% yield by removal of the 2′-*O*-acetyl group and the following ring-closure of **2**. Hydrogenolysis of **3** produced the desired compound **4** in 95% yield. From ^{1}H NMR measurements, all hydrogens at the 1′-, 2′- and 3′-positions in **4** were observed to be singlet signals, indicating that the sugar puckering in **4** was fixed in an N-type conformation.[28] Furthermore, the orientation of 1-isoquinolone as the nucleobase moiety was determined to be *anti*-orientation from the observation of NOE between a hydrogen at the 3′-position in the sugar moiety and a hydrogen at the 3-position in 1-isoquinolone. The result showed that the carbonyl group in 1-isoquinolone was placed in a suitable direction for the formation of a Hoogsteen-type hydrogen bond with a CG base pair.



Scheme 1. *Reagents and conditions*: (a) 1-isoquinolone, *N*,*O*-bis(trimethylsilyl)acetamide, TMSOTf, 1,2-dichloroethane, reflux, 83%; (b) K_2CO_3, MeOH, room temperature, 95%; (c) 20% $Pd(OH)_2$–C, cyclohexene, EtOH, reflux, 95%; (d) DMTrCl, pyridine, room temperature, quant.; (e) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, MeCN–THF, room temperature, 97%.

TFOs
TFO-Q^B : 5'-TTTTTCTQ^BTCTCTCT-3'
TFO-P^B : 5'-TTTTTCTP^BTCTCTCT-3'
TFO-H^B : 5'-TTTTTCTH^BTCTCTCT-3'
TFO-T : 5'-TTTTTCTTTCTCTCT-3'
(C = 2'-deoxy-5-methylcytidine)

dsDNA targets
Duplex-XY (XY = CG, GC, TA and AT):
5'-GCTAAAAAGAXAGAGAGATCG-3'
3'-CGATTTTTCTYTCTCTCTAGC-5'

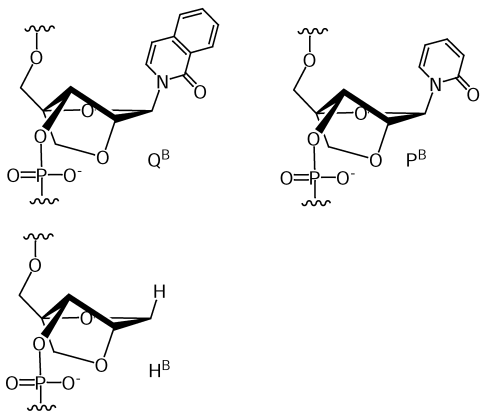


Figure 4. The sequence of TFOs and targeted DNA duplexes.

Protection of a primary alcohol of **4** with DMTrCl in pyridine afforded **5** quantitatively. The phosphoramidite **6** was obtained in 97% yield by phosphitylation of **5**, then the phosphoramidite **6** was successfully introduced into an oligonucleotide (TFO-Q^B) on an automated DNA synthesizer using standard phosphoramidite chemistry (Fig. 4). The purity of the TFO-Q^B was verified using reversed-phase HPLC, and the composition was determined by MALDI-TOF-Mass.

2.2. Triplex-forming ability of TFO containing Q^B

Triplex-forming ability of TFO-Q^B was evaluated by an analysis of the melting temperature (T_m) as shown in Table 1 and Figure 5. All T_m measurements were carried out under 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl, 10 mM $MgCl_2$. Since thymine is well known to form the most stable traid with the CG base pair in all four natural nulceobases,[29,30] the T_m value of the synthesized oligonucleotide TFO-Q^B was compared with that of the natural oligonucleotide TFO-T. Comparing the T_m values of the triplexes TFO-Q^B/Duplex-XY with those of the triplexes TFO-P^B/Duplex-XY and TFO-H^B[31]/Duplex-XY, the strict sequence-selectivity of Q^B was also

Table 1. T_m values (°C) of DNA triplexes

	Duplex-CG	Duplex-TA	Duplex-GC	Duplex-AT
TFO-T	25	17	20	44
TFO-Q^B	29(+5)	16(−4)	20(±0)	15(−1)
TFO-P^{Ba}	33(+9)	14(−6)	19(−1)	23 (+7)
TFO-H^{Ba}	24	20	20	16

Conditions: 7 mM sodium phosphate buffer (pH 7.0), 140 mM KCl, 10 mM $MgCl_2$, the concentration of a triplex=1.5 μM. The changes in T_m values between a TFO and TFO-H^B were shown in the parentheses.
[a] Our previous result.[23]

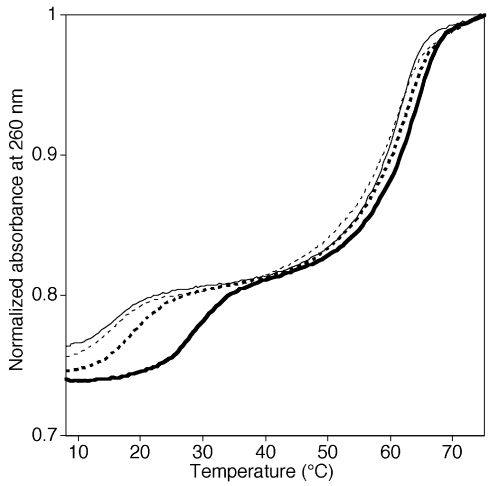


Figure 5. The dissociation curves of the triplexes TFO-Q^B/Duplex-XY. XY=CG (a solid line), GC (a dash line), TA (a thin dash line) and AT (a thin solid line).

clarified. As we have previously reported,[23] the nucleoside analogue P^B effectively recognized CG interruption in the homopurine–homopyrimidine region; however, the difference in T_m values between the triplexes TFO-P^B/Duplex-AT and TFO-H^B/Duplex-AT was relatively large (+7°C). It seems that P^B has a tendency to interact not only with a CG base pair but also with an AT base pair, though the gross T_m value of TFO-P^B/Duplex-AT was not so large. On the other hand, almost no difference was observed in the T_m values between the triplexes TFO-Q^B/Duplex-AT and TFO-H^B/Duplex-AT. Moreover, thermal stability of the triplexes TFO-Q^B/Duplex-GC and TFO-Q^B/Duplex-TA was the same as or less than that of the triplexes TFO-H^B/Duplex-GC and TFO-H^B/Duplex-TA, respectively. These results clearly demonstrate that the 1-isoquinolone moiety of Q^B made stable interaction with a CG base pair, but not with AT, TA and GC base pairs. Compared with the triplex TFO-P^B/Duplex-CG, slight decrease in T_m value was observed for the triplex TFO-Q^B/Duplex-CG probably due to steric hindrance of 1-isoqinolone. However, the hindered 1-isoqinolone of Q^B efficiently discriminated the CG base pair from the other three base pairs (Fig. 3(b)).

3. Conclusion

In this report, we have accomplished the synthesis of 2′,4′-BNA (Q^B) bearing 1-isoquinolone as a nucleobase. Under near physiological conditions, the Q^B incorporated into a TFO efficiently recognized CG interruption rather than the three others in the homopurine–homopyrimidine tract of dsDNA. We believe Q^B to be a promising candidate to overcome one of the crucial inherent problems in a TFO, namely, limitation of the sequence of targeted dsDNA.

4. Experimental

4.1. General

All melting points were measured on a Yanagimoto micro melting points apparatus and are uncorrected. Optical

rotations were recorded on a JASCO DIP-370 instrument. IR spectra were recorded on a JASCO FT/IR-200 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL EX-270 (^{1}H, 270 MHz; ^{13}C, 67.8 MHz) and ^{31}P NMR spectrum was recorded on a Varian VXR-200 (^{31}P, 81.0 MHz). Mass spectra of nucleoside analogues were recorded on a JEOL JMS-D300 or JMS-600 mass spectrometer. For flash column, Fuji Silysia BW-300 (200–400 mesh) was used. MALDI-TOF-Mass spectra were recorded on a Perceptive Inc. Voeyger®-DE.

4.1.1. 2-[2-*O*-Acetyl-3,5-di-*O*-benzyl-4-(*p*-toluenesulfonyloxymethyl)-β-D-ribofuranosyl]-1-isoquinolone (2). Under a nitrogen atmosphere 1-isoquinolone (308 mg, 2.12 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (0.61 ml, 2.48 mmol) were added to a solution of compound **1**[26] (1.06 g, 1.77 mmol) in anhydrous 1,2-dichloroethane (20 ml) at room temperature and the mixture was refluxed for 1 h. TMSOTf (0.19 ml, 1.06 mmol) was added to the reaction mixture at room temperature and the mixture was refluxed for 2 h. The reaction was quenched by addition of a saturated aqueous solution of $NaHCO_3$. The mixture was extracted with AcOEt. The organic phase was washed with water and brine and dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [CH_2Cl_2/AcOEt (50:1, v/v)] to give compound **2** (1.00 g, 83%) as a colorless oil. $[\alpha]_D^{27}$=+26.3 (*c* 0.58, $CHCl_3$). IR ν_{max} (KBr): 1747, 1661, 1364, 1230, 1181, 1101 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 2.02 (3H, s), 2.40 (3H, s), 3.59, 3.81 (2H, AB, *J*=10 Hz), 4.21, 4.27 (2H, AB, *J*=11 Hz), 4.37–4.56 (5H, m), 5.43 (1H, dd, *J*=5, 5 Hz), 6.20–6.23 (2H, m), 7.20–7.48 (12H, m), 7.62 (1H, dd, *J*=8, 8 Hz), 7.75 (2H, d, *J*=8 Hz), 8.34 (1H, d, *J*=8 Hz). ^{13}C NMR ($CDCl_3$) δ: 20.8, 21.7, 69.3, 70.3, 73.7, 74.4, 75.1, 77.4, 85.2, 87.9, 106.2, 125.7, 125.8, 126.8, 127.0, 127.7, 127.8, 127.8, 127.9, 128.0, 128.3, 128.4, 129.7, 132.3, 132.5, 136.7, 137.1, 137.2, 144.9, 161.6, 169.5. Mass (EI): *m*/*z* 683 (M^+, 3.5), 91 (100). Anal. calcd for $C_{38}H_{37}NO_9S$: C, 66.75; H, 5.45; N, 2.05; S, 4.69. Found: C, 66.46; H, 5.47; N, 1.93; S, 4.62.

4.1.2. 2-(3,5-Di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)-1-isoquinolone (3). To a solution of compound **2** (1.00 g, 1.46 mmol) in MeOH (15 ml) was added K_2CO_3 (600 mg, 4.34 mmol) at room temperature and the mixture was stirred for 9 h. The solvent was concentrated under reduced pressure. After addition of water, the residue was extracted with AcOEt. The organic phase was washed with water and brine and dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [*n*-hexane/AcOEt (4:1, v/v)] to give compound **3** (562 mg, 95%) as a white powder. Mp 118–119°C. $[\alpha]_D^{27}$=+198.5 (*c* 0.79, $CHCl_3$). IR ν_{max} (KBr): 1654, 1059 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 3.80, 3.90 (2H, AB, *J*=11 Hz), 3.94, 4.08 (2H, AB, *J*=8 Hz), 4.07 (1H, s), 4.43, 4.58 (2H, AB, *J*=12 Hz), 4.62–4.73 (3H, m), 6.04 (1H, s), 6.44 (1H, d, *J*=8 Hz), 7.22 (4H, m), 7.37 (4H, m), 7.49–7.53 (3H, m), 7.62–7.69 (3H, m), 8.40 (1H, d, *J*=8 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 64.9, 72.1, 72.3, 73.7, 76.2, 76.7, 87.1, 88.0, 105.9, 125.6, 125.7, 125.8, 126.7, 127.4, 127.5, 127.7, 127.8, 128.2, 128.4, 132.4, 136.7, 137.0, 137.6, 161.4. Mass (EI): *m*/*z* 469 (M^+, 16.1), 91 (100). Anal. calcd for $C_{29}H_{27}NO_5$: C, 74.18; H, 5.80; N, 2.98. Found: C, 74.15; H, 5.91; N, 2.96.

4.1.3. 2-(2-*O*,4-*C*-Methylene-β-D-ribofuranosyl)-1-isoquinolone (4). A solution of compound **3** (533 mg, 1.14 mmol), 20% $Pd(OH)_2$–C (250 mg) and cyclohexene (5.80 ml, 57.3 mmol) in EtOH (20 ml) was refluxed for 1 h. The mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [$CHCl_3$/MeOH (23:1, v/v)] to give compound **4** (37 mg, 95%) as a white powder. Mp 91–94°C. $[\alpha]_D^{22}$=+139.3 (*c* 0.84, CH_3OH). IR ν_{max} (KBr): 3388, 1650, 1270, 1060 cm^{-1}. ^{1}H NMR (CD_3OD) δ: 3.85, 4.01 (2H, AB, *J*=8 Hz), 3.97 (2H, s), 4.14 (1H, s), 4.36 (1H, s), 5.92 (1H, s), 6.75 (1H, d, *J*=8 Hz), 7.63–7.75 (2H, m), 7.79 (1H, d, *J*=8 Hz), 8.29 (1H, d, *J*=8 Hz). ^{13}C NMR (CD_3OD) δ: 57.9, 70.4, 72.6, 80.9, 89.0, 90.2, 107.5, 126.3, 126.7, 127.3, 127.8, 128.0, 133.9, 138.4, 163.1. Mass (EI): *m*/*z* 289 (M^+, 32.3), 145 (100). Anal. calcd for $C_{15}H_{15}NO_5 \cdot H_2O$: C, 58.63; H, 5.58; N, 4.56. Found: C, 58.90; H, 5.43; N, 4.56.

4.1.4. 2-[5-*O*-(4,4′-Dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]-1-isoquinolone (5). Under a nitrogen atmosphere DMTrCl (152 mg, 0.45 mmol) was added to a solution of compound **4** (100 mg, 0.35 mmol) in anhydrous pyridine (2 ml) at room temperature and the mixture was stirred at room temperature for 3 h. The reaction was quenched by addition of a saturated aqueous solution of $NaHCO_3$. The mixture was extracted with AcOEt. The organic phase was washed with water and brine and dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [*n*-hexane/AcOEt (4:3, v/v)] to give compound **5** (205 mg, 100%) as a white powder. Mp 105–109°C. $[\alpha]_D^{22}$=+57.9 (*c* 1.34, $CHCl_3$). IR ν_{max} (KBr): 3373, 3007, 2952, 1650, 1593, 1508, 1458, 1251, 1178, 1056 cm^{-1}. ^{1}H NMR (CD_3COCD_3) δ: 3.55, 3.61 (2H, AB, *J*=11 Hz), 3.81 (6H, s), 3.84, 3.96 (2H, AB, *J*=8 Hz), 4.40 (1H, s), 4.49 (1H, d, *J*=4 Hz), 4.77 (1H, d, *J*=4 Hz), 5.93 (1H, s), 6.61 (1H, d, *J*=8 Hz), 6.93 (4H, d, *J*=9 Hz), 7.27–7.71 (12H, m), 7.95 (1H, d, *J*=8 Hz), 8.31 (1H, d, *J*=8 Hz). ^{13}C NMR (CD_3COCD_3) δ: 55.5, 59.6, 70.6, 72.4, 80.2, 87.1, 88.4, 88.6, 105.8, 113.8, 126.6, 126.9, 127.1, 127.3, 127.5, 127.7, 128.5, 128.8, 130.8, 130.8, 133.1, 136.3, 136.5, 137.8, 145.8, 159.5, 161.5. Mass (FAB): *m*/*z* 614 (MNa^+). Mass (FAB): *m*/*z* 598 (MLi^+). Anal. calcd for $C_{36}H_{33}NO_7 \cdot 1/2H_2O$: C, 71.99; H, 5.71; N, 2.33. Found: C, 71.73; H, 5.91; N, 2.38.

4.1.5. 2-[3-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5-*O*-(4,4′-dimethoxytrityl)-2-*O*-4-*C*-methylene-β-D-ribofuranosyl]-1-isoquinolone (6). Under a nitrogen atmosphere 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (43 μl, 0.14 mmol) was added to a solution of compound **5** (40 mg, 68 μmol), diisopropylammonium tetrazolide (14 mg, 82 μmol) in anhydrous MeCN–THF (3:1, 1.2 ml) at room temperature and the mixture was stirred at room temperature for 5 h. The solvent was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography [*n*-hexane/AcOEt/Et_3N (75:25:1, v/v/v)] to give compound **6** (52 mg, 97%) as a white powder. Mp 76–79°C. ^{31}P NMR ($CDCl_3$) δ: 148.6, 149.5.

4.1.6. Synthesis of TFO-Q^B. The modified oligonucleotide

TFO-Q^B was synthesized on a 0.2 μmol scale on Pharmacia Gene Assembler® Plus according to the standard phosphoramidite protocol. The solid supported oligonucleotide, which was protected by 5′-terminal DMTr group, was treated with concentrated ammonium hydroxide at 60°C for 18 h, and the solvents were concentrated. After simple purification through NENSORB™ PREP, the oligonucleotide was purified by reversed-phase HPLC (ChemcoPak® CHEMCOSORB 300-5C18, 4.6 mm×250 mm) with a 11% MeCN in 0.1 M triethylammonium acetate buffer (pH 7.0). MALDI-TOF-Mass data for TFO-Q^B $[M-H]^-$: found 4542.59, calcd 4543.11.

4.2. T_m measurements

UV melting experiments were carried out on a Beckmann DV-650 spectrophotometer equipped with T_m analysis accessary. The profiles were recorded in 7 mM sodium phosphate buffer (pH 7.0), 140 mM KCl and 10 mM $MgCl_2$ at a scan rate of 0.5°C/min at 260 nm. The final concentration of each oligonucleotide used was 1.5 μM. A T_m value was designated the maximum of the first derivative calculated from the UV melting profile.

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