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Azole substituted oligonucleotides promote antiparallel triplex formation at non-homopurine duplex targets

Ross H. Durland+, T. Sudhakar Rao, Veeraiah Bodepudi, Dale M. Seth, Krishna Jayaraman and Ganapathi R. Revankar*

Triplex Pharmaceutical Corporation, 9391 Grogans Mill Road, The Woodlands, TX 77380, USA

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ABSTRACT

The ability of certain azole substituted oligodeoxyribonucleotides to promote antiparallel triple helix formation with duplex targets having CG or TA interruptions in the otherwise homopurine sequence was examined. 2′-Deoxyribonucleosides of the azoles, which include pyrazole, imidazole, 1,2,4-triazole and 1,2,3,4-tetrazole were synthesized using the stereospecific sodium salt glycosylation procedure. These nucleosides were successfully incorporated using solid-support, phosphoramidite chemistry, into oligonucleotides designed to interact with the non-homopurine duplex targets. The interaction of these modified oligonucleotides with all four possible base pairs was evaluated and compared to similar data for a series of natural oligonucleotides. The oligonucleotides containing simple azoles enhanced the triplex forming ability considerably at non-homopurine targets. Binding of these modified oligonucleotides to duplex targets containing TA inversion sites was particularly noteworthy, and compare favorably to unmodified oligonucleotides for binding to duplex targets containing CG as well as TA base pairs. The selectivity exhibited by certain azoles is suggestive of base pair specific interactions. Thus, the azoles evaluated during this study show considerable promise for efforts to develop generalized triplex formation at non-homopurine duplex sequences.

INTRODUCTION

Binding of oligonucleotides to appropriate duplex DNA targets can result in the formation of a local triple helix structure, or triplex (1–4). Ideally, the duplex portion of the triplex consists of a homopurine strand and a homopyrimidine strand. The third strand binds in the major groove of the duplex by hydrogen bonding to the purine bases in the homopurine strand. Several different types of triplexes have been described (1). However, the triplex stability is often severely reduced by the presence of one or more pyrimidines in the purine-rich strand of the duplex (5–12). This prevents triplex formation at mixed sequence duplex targets.

Numerous attempts have been made to extend triplex formation to include non-homopurine duplexes. One approach has been to test natural nucleosides to identify those that exhibit significant ability to bind to sites where a pyrimidine interrupts an otherwise homopurine region of a duplex (henceforth termed an inversion site). Examples of this include the parallel G•TA triplet (5,11,13–15) and the antiparallel T•CG triplet (9,12). However, these triplets are significantly less stable than the canonical ones. Steric constraints imposed by CG or TA base pairs force natural nucleosides in the third strand to be displaced from the ideal position. Although favorable interactions may sometimes be accommodated, the disruption imposed by the steric problem is likely to limit the utility of natural nucleosides.

A second approach has been the *de novo* design of novel nucleoside analogs intended to bind to CG or TA base pairs (16–18). Although this approach has had some success, the observed binding was not based on the formation of hydrogen bonded triplets, as originally proposed (19). This is likely due to the difficulty of designing a complex nucleoside capable of binding to CG or TA base pairs with appropriate affinity and specificity.

An alternative approach has been to use non-nucleosidic linkers to connect separate oligonucleotides such that they can bind to nearby homopurine targets separated by a non-homopurine intervening sequence (20–23). A drawback of this approach is that there is no interaction between the linked oligonucleotides and the intervening sequence. In the case of a single pyrimidine embedded within a homopurine target, use of an oligonucleotide containing a linker will disrupt potential stacking interactions across the inversion site. Thus, none of the current strategies for binding to non-homopurine targets is ideal.

We describe in this paper a unique approach to triplex formation at non-homopurine targets, utilizing the modified oligonucleotides containing simple azole 2′-deoxyribonucleo-

* To whom correspondence should be addressed

+Present address: Gene Medicine, Inc., 8080 N. Stadium Drive, Suite 2100, Houston, TX 77054-1823, USA

Table 1. Physical properties of azole-2′-deoxyribonucleosides

compd	mp, °C	yield, %	^{1}H NMR, δ (ppm)	formula	anal
3a	110	67.6	2.37, 2.40 (2s, 2CH_3), 2.67 (m, 2′*H*), 3.11 (m, 2″*H*), 4.38 (m, 4′*H*), 4.50 (m, 5′H_2), 5.76 (m, 3′*H*), 6.31 (t, C_4H), 6.36 (t, J = 6.2 Hz, 1′*H*), 7.29-7.36 (m, *Tol*), 7.59 (s, C_3H), 7.86-7.95 (m, *Tol*, C_5H).	$C_{21}H_{24}N_2O_3$	C, H, N
4a	gum	80.0	2.20 (m, 2′*H*), 2.57 (m, 2″*H*), 3.42 (m, 5′*H*), 3.52 (m, 5″*H*), 3.80 (m, 4′*H*), 4.35 (m, 3′*H*), 4.84 (t, 5′O*H*), 5.20 (d, 3′O*H*), 6.10 (t, J = 6.3 Hz, 1′*H*), 6.27 (t, C_4H), 7.51 (s, C_2H), 7.91 (d, C_5H).	$C_8H_{12}N_2O_3$	C, H, N
5a	54-56	64.8	2.22 (m, 2′*H*), 2.48 (m, 2″*H*), 3.08 (m, 5′H_2), 3.73 (s, 2OCH_3), 3.91 (m, 4′*H*), 4.37 (m, 3′*H*), 5.10 (s, 3′O*H*), 6.10 (t, J = 5.6 Hz, 1′*H*), 6.24 (t, C_4H), 6.81-6.84 (m, Ar*H*), 7.18-7.36 (m, Ar*H*), 7.44 (d, C_3H), 7.79 (d, C_5H).	$C_{28}H_{29}N_2O_5$	C, H, N
6a	foam	86.5	1.04-1.22 (m, CH_3 of isopropyls), 2.49-2.70 (m, 2′*H*, OCH_2CH_2CN), 2.85 (m, 2″*H*), 3.10 (m, *CH* of isopropyl), 3.22 (m, *CH* of isopropyl), 3.50-3.80 (m, 5′H_2, OCH_2CH_2CN), 3.75 (s, 2OCH_3), 4.10 (m, 4′*H*), 4.75 (m, 3′*H*), 6.11 (t, 1′*H*), 6.25 (t, C_4H), 6.81-6.83 (m, Ar*H*), 7.18-7.46 (m, Ar*H*, C_3H), 7.68 (d, C_5H). ^{31}P NMR (CD_3CN): δ 149.36	$C_{38}H_{47}N_4O_6P\cdot 0.5H_2O$	C, H, N, P
3b	112-114	64.6	2.38, 2.40 (2s, 2CH_3), 2.70-2.79 (m, 2′*H*, 2″*H*), 4.48-4.57 (m, 4′*H*, 5′H_2), 5.64 (m, 3′*H*), 6.28 (t, 1′*H*), 6.94 (s, C_4H), 7.32-7.37 (m, *Tol*, C_5H), 7.86-7.95 (m, *Tol*, C_2H).	$C_{24}H_{24}N_2O_5$	C, H, N
4b	140-142	81.2	2.20 (m, 2′*H*), 2.33 (m, 2″*H*), 3.48 (m, 5′H_2), 3.78 (m, 4′*H*), 4.28 (m, 3′*H*), 4.83 (t, 5′O*H*), 5.21 (d, 3′O*H*), 6.02 (t, J = 6.9 Hz, 1′*H*), 6.90 (s, C_4H), 7.31 (s, C_5H).	$C_8H_{12}N_2O_3$	C, H, N
5b	84-86	54.2	2.28 (m, 2′*H*), 2.37 (m, 2″*H*), 3.11 (d, 5′H_2), 3.78 (s, 2OCH_3), 3.91 (m, 4′*H*), 4.27 (m, 3′*H*), 5.27 (d, 3′O*H*), 6.07 (t, J = 6.4 Hz, 1′*H*), 6.84-6.90 (m, Ar*H* , C_4H), 7.19-7.37 (m, Ar*H*, C_5H), 7.79 (s, C_2H).	$C_{29}H_{30}N_2O_5\cdot 0.1H_2O$	C, H, N
6b	foam	80.0	1.04-1.24 (m, CH_3 of isopropyls), 2.46-2.64 (m, 2′*H*, 2″*H*, OCH_2CH_2CN), 3.20 (m, *CH* of isopropyl), 3.56-3.75 (m, 5′H_2, OCH_2CH_2CN), 3.75 (s, 2OCH_3), 4.10 (m, 4′*H*), 4.60 (m, 3′*H*), 6.04 (t, J = 6.6 Hz, 1′*H*), 6.82-6.85 (m, Ar*H*), 6.92 (s, C_5H), 7.10 (s, C_4H), 7.25-7.43 (m, Ar*H*), 7.63 (s, C_2H). ^{31}P NMR (CD_3CN): δ 149.39, 149.64.	$C_{38}H_{47}N_4O_6P$	C, H, N, P
3c	84-86	71.1	2.36, 2.39 (2s, 2CH_3), 2.75 (m, 2′*H*), 3.05 (m, 2″*H*), 4.30-4.57 (m, 4′*H*, 5′H_2), 5.77 (m, 3′*H*), 6.48 (t, J = 6.1 Hz, 1′*H*), 7.27-7.34 (m, *Tol*), 7.70-7.91 (m, *Tol*), 8.03 (s, C_3H), 8.69 (s, C_5H).	$C_{23}H_{23}N_3O_5$	C, H, N
4c	gum	86.0	2.35 (m, 2′*H*), 2.60 (m, 2″*H*), 3.60 (m, 5′H_2), 3.90 (m, 4′*H*), 4.40 (m, 3′*H*), 4.65 (s, 5′O*H*), 5.15 (s, 3′O*H*), 6.22 (t, J = 6.2 Hz, 1′*H*), 7.98 (s, C_3H), 8.66 (s, C_5H).	$C_7H_{11}N_3O_3\cdot 0.2H_2O$	C, H, N
5c	68-70	59.0	2.30 (m, 2′*H*), 2.60 (m, 2″*H*), 3.10 (m, 5′H_2), 3.73 (s, 2OCH_3), 3.96 (m, 4′*H*), 4.40 (m, 3′*H*), 5.25 (d, 3′O*H*), 6.27 (t, 1′*H*), 6.80 (m, Ar*H*), 7.10-7.40 (m, Ar*H*), 7.96 (s, C_3H), 8.66 (s, C_5H).	$C_{28}H_{28}N_3O_5$	C, H, N
6c	foam	88.0	1.10 (m, CH_3 of isopropyls), 2.40-2.85 (m, 2′*H*, 2″*H*, OCH_2CH_2CN), 3.05-3.30 (m, *CH* of isopropyl), 3.50-3.75 (m, 5′H_2, OCH_2), 3.75 (s, 2OCH_3), 4.15 (m, 4′*H*), 4.75 (m, 3′*H*), 6.25 (dd, 1′*H*), 6.80 (m, Ar*H*), 7.10-7.45 (m, Ar*H*), 7.83 (s, C_3H), 8.34 (s, C_5H). ^{31}P NMR (CD_3CN): δ 149.50, 149.65.	$C_{37}H_{44}N_5O_6P$	C, H, N, P
8	144-146	45.0	2.38, 2.40 (2s, 2CH_3), 2.96 (m, 2′*H*), 3.26 (m, 2″*H*), 4.40 (m, 5′*H*), 4.52 (m, 5′*H*), 4.70 (m, 4′*H*), 5.87 (m, 3′*H*), 6.96 (t, J = 5.9 Hz, 1′*H*), 7.33 (m, *Tol*), 7.87 (m, *Tol*), 9.10 (s, C_4H).	$C_{22}H_{22}N_4O_5$	C, H, N
9	168-170	38.0	2.37, 2.40 (2s, 2CH_3), 2.90 (m, 2′*H*), 3.15 (m, 2″*H*), 4.45 (m, 5′H_2), 4.67 (m, 4′*H*), 5.80 (m, 3′*H*), 6.73 (t, J = 5.9 Hz, 1′*H*), 7.33 (m, *Tol*), 7.85 (m, *Tol*), 9.61 (s, C_5H).	$C_{22}H_{22}N_4O_5$	C, H, N
10	80-82	70.0	2.45 (m, 2′*H*), 2.75 (m, 2″*H*), 3.50 (m, 5′H_2), 3.90 (m, 4′*H*), 4.40 (d, 3′*H*), 4.85 (br s, 5′O*H*), 5.35 (br s, 3′O*H*), 6.50 (t, J = 5.3 Hz, 1′*H*), 9.55 (s, C_5H).	$C_6H_{10}N_4O_3$	C, H, N
11	70-72	71.0	2.45 (m, 2′*H*), 2.75 (m, 2″*H*), 3.05 (m, 5′H_2), 3.73 (s, 2OCH_3), 4.02 (m, 4′*H*), 4.45 (m, 3′*H*), 5.39 (d, 3′O*H*), 6.52 (dd, 1′*H*), 6.82 (m, Ar*H*), 7.15-7.29 (m, Ar*H*), 9.52 (s, C_5H).	$C_{27}H_{28}N_4O_5$	C, H, N
12	foam	81.6	1.10 (m, CH_3 of isopropyls), 2.45-2.70 (m, 2′*H*, OCH_2CH_2CN), 2.29 (m, 2″*H*), 3.15 (m, *CH* of isopropyl), 3.30 (m, *CH* of isopropyl), 3.55 (m, 5′H_2, OCH_2CH_2CN), 3.79, 3.80 (2s, 2OCH_3), 4.22 (m, 4′*H*), 4.75 (m, 3′*H*), 6.45 (m, 1′*H*), 6.85 (m, Ar*H*), 7.15-7.39 (m, Ar*H*), 8.97 (s, C_5H). ^{31}P NMR (CD_3CN): δ 149.69, 149.80.	$C_{36}H_{45}N_6O_6P$	C, H, N, P

sides. The azoles tested include pyrazole, imidazole, 1,2,4-triazole and 1,2,3,4-tetrazole.

MATERIALS AND METHODS

Nucleoside phosphoramidite synthesis

General procedure for glycosylation of azoles with the α-halogenose 2. To a solution of the azole (**1a–c**, **7**, 20 mmol) in dry CH_3CN (200 ml) was added NaH (20 mmol, 80% dispersion in oil). After stirring the mixture at room temperature for 30 min, 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (**2**, 20 mmol) was added in four portions at 20 min intervals. After a total of 2 h stirring, the solvent was evaporated and the residue was diluted with CH_2Cl_2 (150 ml). The organic solution was washed with H_2O (50 ml), and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was purified by chromatography on a silica gel column (2.5 × 25 cm) using a gradient of increasing concentration of EtOAc in CH_2Cl_2 as the eluent to yield the corresponding blocked nucleosides (**3a–c**, **8**, **9**) (Table 1).

General procedure for deblocking 3a–c and 9. The blocked nucleoside (7.12 mmol) was dissolved in MeOH/NH_3 (200 ml, MeOH saturated with NH_3 at 0°C) and the mixture was stirred at room temperature for 18 h. The solvent was evaporated and the residue was purified by chromatography on a silica gel column (2.5 × 15 cm) using a gradient of increasing concentration of MeOH in CH_2Cl_2 to give the corresponding free nucleosides (**4a–c**, **10**) (Table 1).

*General procedure for 5′-O-tritylation of **4a–c** and **10**.* The free nucleoside (4.09 mmol) was dried by co-evaporation with dry pyridine (2 × 15 ml) and dissolved in dry pyridine (10 ml). 4,4′-Dimethoxytrityl chloride (DMT-Cl, 1.8 g, 5.3 mmol) was added and the mixture was stirred at room temperature for 3 h under an argon atmosphere. The reaction mixture was diluted with CH_2Cl_2 (150 ml) and the organic solution was washed with water (25 ml). The organic layer was dried (Na_2SO_4) and evaporated. The residue was co-evaporated with toluene (2 × 10 ml) to remove traces of pyridine. The product was purified by chromatography on a silica gel column (2.5 × 25 cm) using a gradient of increasing concentration of MeOH in CH_2Cl_2 as the eluent to yield the corresponding 5′-*O*-(4,4′-dimethoxytrityl) derivatives (**5a–c**, **11**) (Table 1).

*General procedure for phosphitylation of **5a–c** and **11**.* To a solution of the 5′-*O*-trityl nucleoside (1.52 mmol) in a mixture of anhydrous CH_2Cl_2 (15 ml) and *N,N*-diisopropylethylamine (1.07 ml, 6.1 mmol) was added 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.43 ml, 1.98 mmol) under an argon atmosphere. After stirring the reaction mixture at ambient temperature for 30 min, it was diluted with EtOAc (50 ml) and the organic solution was washed with aqueous saturated $NaHCO_3$ solution (30 ml). The organic layer was dried (Na_2SO_4) and evaporated to dryness. The residue was purified by chromatography on a silica gel column (2.5 × 10 cm) packed in a mixture of CH_2Cl_2:EtOAc:NEt_3 (75:23:2, v/v). The product was eluted using the same solvent mixture and the eluate containing the desired product was collected and evaporated to dryness. The residue was dissolved in CH_2Cl_2 (5 ml) and the solution was added slowly to rapidly stirred cold pentane (100 ml). The supernatant was decanted from the precipitated solid. The solid was dried *in vacuo* until free of residual solvents to yield the corresponding 3′-phosphoramidites (**6a–c**, **12**) (Table 1).

Oligonucleotide synthesis

Phosphoramidites of deoxyguanosine and thymidine were purchased from Applied Biosystems or Glen Research. Pyrazole, imidazole, triazole and tetrazole 2′-deoxyribonucleosides were tested for their stability under DNA synthesis conditions before incorporating them into oligonucleotides. With the exception of pyrazole-2′-deoxyribonucleoside, all nucleosides were stable to synthesis and deblocking conditions (30% NH_4OH, 56°C, 16 h). By TLC analysis, it appeared that 5–10% of the pyrazole-2′-deoxyribonucleoside was degraded under these conditions. In contrast, 16 h incubation in concentrated NH_4OH at room temperature did not result in any detectable degradation of pyrazole-2′-deoxyribonucleoside.

Oligonucleotides were synthesized on Applied Biosystems DNA synthesizers (models 380B or 394) employing the phosphoramidite methodology, on 0.2 or 1 mmol scale. Standard coupling times and phosphoramidite concentrations were used. Oligonucleotide **17**, containing pyrazole-2′-deoxyribonucleoside, was prepared using the 2-*N*-dimethylformamidine-5′-*O*-DMT-3′-*O*-phosphoramidite of 2′-deoxyguanosine (Applied Biosystems). Stepwise coupling efficiency for non-natural nucleosides ranged from 93 to 99%. After cleavage from the support with concentrated NH_4OH at room temperature for 2 h, the oligomer was deblocked at room temperature overnight with concentrated NH_4OH. All other oligonucleotides were prepared using dG phosphoramidites containing the standard 2-*N*-isobutyryl blocking group, and were deblocked with concentrated NH_4OH at 56°C for 16 h. Oligonucleotides were purified by denaturing 20% polyacrylamide, 7 M urea, gel electrophoresis and desalted using C18 Sep-Pack (Waters) column. Aliquots of purified oligonucleotides were analyzed by gel electrophoresis to confirm the expected length and purity.

Oligonucleotides containing azole nucleosides were analyzed by electrospray mass spectrometry. Observed molecular weights were within 0.013% of the calculated values. We further analyzed a pentamer containing imidazole using fast atom bombardment mass spectrometry. The molecular ion had the expected molecular weight, and analysis of the fragmentation pattern demonstrated the presence and position of a nucleoside monophosphate with a molecular weight identical to that expected for imidazole-2′-deoxyribonucleoside monophosphate. These data were taken as evidence that the nucleosides in this study could be routinely incorporated into oligonucleotides without substantial degradation or modification.

Binding assays

Duplex DNA targets were prepared by annealing complementary single stranded oligonucleotides and purifying by nondenaturing polyacrylamide gel electrophoresis. Duplexes were labeled using polynucleotide kinase and [γ-^{32}P]ATP (Amersham). Triplex binding assays (2,4) were performed by mixing radiolabeled duplex (0.01–0.1 nM) with varying concentrations of oligonucleotide in 20 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 10%

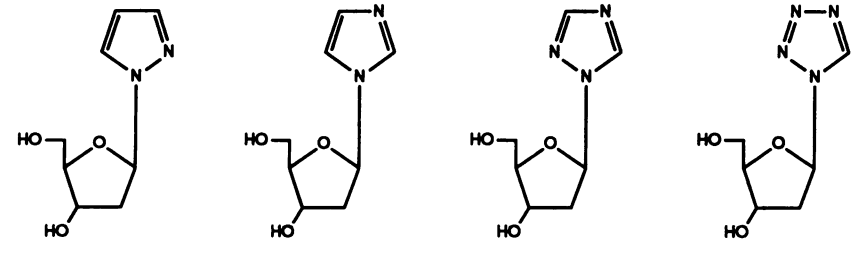

Figure 1. Structures of non-natural nucleosides used in this study.

sucrose, and incubating for 24–48 h at 37°C. Samples were separated on polyacrylamide gels buffered with TBM (4). The radioactivity in the duplex and triplex bands was quantified using a Beta-Scope, corrected for background, and plotted as:

$$F_{triplex} = \frac{c.p.m._{triplex}}{c.p.m._{duplex} + c.p.m._{triplex}} \quad (1)$$

Data points were fit to the equation

$$F_{triplex} = F_{sat}\frac{K[O_{tot}]}{1 + K[O_{tot}]} \quad (2)$$

where F_{sat} is the apparent fraction triplex at saturating amounts of third strand, K is the apparent macromolecular association constant for triplex formation, and O_{tot} is the total concentration of added third strand oligonucleotide. F_{sat} ranged from 0.75 to 1.0, with most values in the 0.85–0.9 range. This equation assumes that the total added third strand oligonucleotide is always in substantial excess over labelled duplex. The curve fitting function of SigmaPlot was used to determine best fit values for F_{sat} and K. Values reported in Table 2 and depicted in Figure 5 are the K values determined by this procedure.

RESULTS AND DISCUSSION

Synthesis of non-natural nucleosides

Figure 1 shows the non-natural nucleosides evaluated in this study. These include 2′-deoxyribofuranosyl derivatives of pyrazole (**4a**), imidazole (**4b**), 1,2,4-triazole (**4c**) and 1,2,3,4-tetrazole (**10**). Several criteria were considered in selecting these analogues. Their small size and lack of exocyclic functional groups should minimize potential steric clash with bases of the duplex at sites of CG or TA inversion. The number and position of ring nitrogens was varied to empirically determine the optimal arrangement for potential stacking, electrostatic and hydrogen bonding interactions.

Total synthesis of the azole nucleosides was achieved by utilizing the stereospecific sodium salt glycosylation procedure (24). Thus, reaction of the sodium salt of pyrazole (**1a**) or imidazole (**1b**) (generated *in situ* by the treatment with NaH) with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (**2**) (25) in dry acetonitrile (CH_3CN) afforded the corresponding protected nucleosides (**3a** and **3b**) (Fig. 2). Removal of the protecting toluoyl groups of **3a** and **3b** was accomplished by the treatment with methanolic ammonia at ambient temperature and the corresponding free nucleosides **4a** and **4b** were isolated in good yields. The free nucleosides were converted to the corresponding 5′-*O*-(4,4′-dimethoxytrityl) derivatives by treatment with 4,4′-dimethoxytrityl chloride (DMT-Cl) in anhydrous pyridine. Purification of the reaction product by silica gel column chromatography provided pure **5a** and **5b**. Conventional phosphitylation of **5a** and **5b** with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in CH_2Cl_2 in the presence of *N*,*N*-diisopropylethylamine gave the corresponding phosphoramidites **6a** and **6b**.

In the case of pyrazole and imidazole, glycosylation at either ring nitrogen results in identical compounds. However, in the case of triazole (**1c**) or tetrazole (**7**), glycosylation could lead to the formation of two positional isomers. Reaction of the sodium salt of **1c** with **2** in dry CH_3CN, work-up and purification of the reaction mixture by silica gel column chromatography gave mainly the positional isomer **3c**. The structure of **3c** was readily apparent since two singlets (each for C_3H and C_5H) were observed in the ^1H NMR spectrum at δ 8.03 and 8.69, establishing N^1 as the site of glycosylation (26). The ^1H NMR spectrum of the isomeric 4-(2-deoxy-β-D-*erythro*-pentofuranosyl)-1,2,4-triazole is expected to exhibit a singlet for the C_3 and C_5 protons due to symmetry (27). Toluoyl protecting groups of **3c** were removed to give the free nucleoside (**4c**), which was converted to the target phosphoramidite (**6c**) *via* the intermediate **5c** by the conventional procedure.

Treatment of the sodium salt of tetrazole (**7**) with **2** in dry CH_3CN gave a reaction mixture comprising two positional isomers (**8** and **9**; Fig. 3). These were separated by silica gel column chromatography. NOE enhancement studies (28) showed that irradiation of $C_1'H$ of **9** increased the signal intensity of C_5H

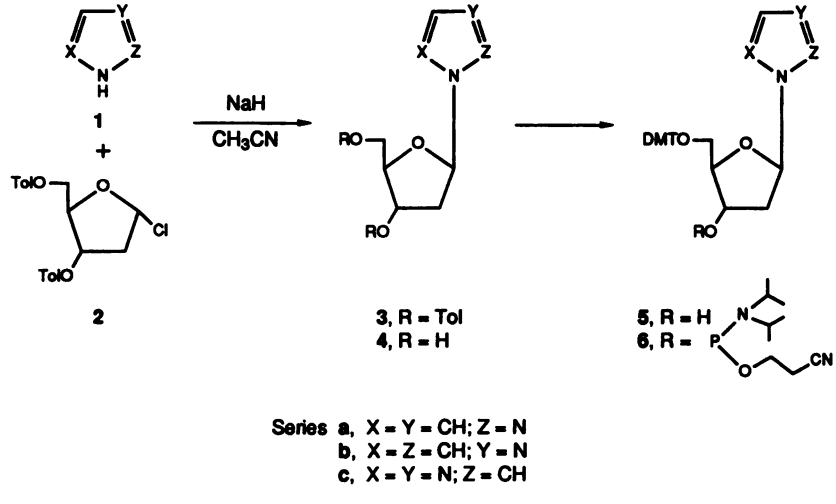

Figure 2. Synthesis of pyrazole, imidazole and 1,2,4-triazole 2′-deoxyribonucleoside phosphoramidites.

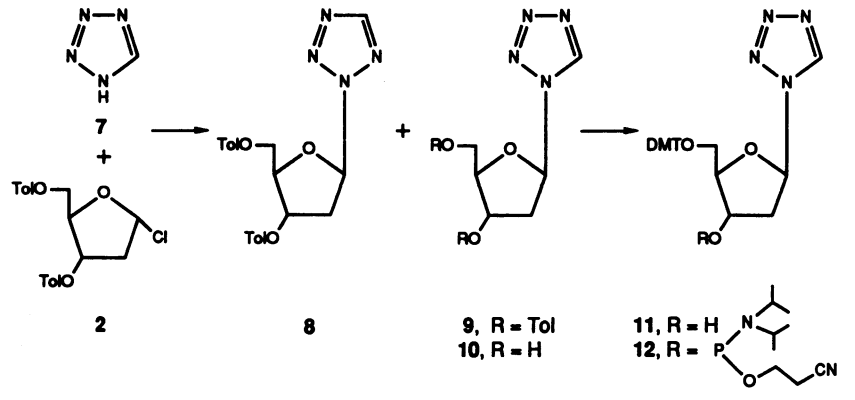

Figure 3. Synthesis of 1,2,3,4-tetrazole 2′-deoxyribonucleoside phosphoramidite.

by 3.5%, establishing the indicated structure of **9**. No such NOE enhancement of C_5H was observed when $C_1'H$ of **8** was irradiated. Removal of the protecting groups of **9** by treatment with methanolic ammonia provided **10**, which was converted to the DMT-phosphoramidite **12** via **11** as described above.

Binding properties of azole-2′-deoxyribonucleosides

In order to evaluate binding of azole 2′-deoxyribonucleosides in antiparallel triplexes, we tested the ability of the modified oligonucleotides **17–20** to bind to a series of duplex targets, **21–24** (Fig. 4). The sequence of these targets varies at the positions complementary to the sites of incorporation of the azoles in oligonucleotides **17–20**. Binding was tested by the electrophoretic band shift assay (2,4). As a positive control for triplex formation, we first tested the binding of triplex forming oligonucleotide (TFO) **13** to **21** and TFO **15** to **22**. In each case, the target is a perfect homopurine-homopyrimidine sequence, and the oligonucleotides are designed to bind to their respective targets in the antiparallel orientation (relative to the purine strand of the duplex), forming a series of G·GC and T·AT triplets. Triplexes **13•21** and **15•22** formed with high affinity (Fig. 5A). Estimated association constants for the duplex to triplex transition were in the range of 2–5 × 10^9 M^{-1}. In contrast, combinations of **13** + **24**, or **15** + **23**, did not lead to significant triplex formation (Fig. 5B). It is apparent that the presence of three CG inversions in **24** prevents binding of **13**. Similarly, the three TA inversions in **23** prevents binding of **15**. These comparisons illustrate the difficulties of forming triplexes at non-homopurine duplexes.

Binding of oligonucleotides **13–20** to duplex **23** is shown in Figure 6. No detectable triplex formation was observed for any of the natural nucleosides (oligonucleotides **13–16**). However, all of the oligonucleotides containing azoles (oligonucleotides **17–20**) showed considerable binding to duplex **23**. Oligonucleotides containing azole nucleosides (**17–20**), bind at least 100–1000-fold more tightly to duplex **23** (with association constants 8E7–5E8) than oligonucleotides containing only natural nucleosides (**13–16**). However, the association constants of **17–20** to **23** are 10–30 times less than those of the 'natural' triplexes **13•21** and **15•22**. These results indicate that the use of azole nucleosides can significantly expand the range of duplex targets amenable to antiparallel triplex formation.

Table 2 summarizes apparent association constants determined from these experiments. A number of interesting features deserve

```
13      5'-ggggttgggggggttgggggttgggggg-3'

14      5'-ggggttgggaggttggaggttgaggg-3'

15      5'-ggggttgggtggttggtggttgtggg-3'

16      5'-ggggttgggcggttggcggttgcggg-3'

17      5'-ggggttgggyggttggyggttgyggg-3'

18      5'-ggggttgggmggttggmggttgmggg-3'

19      5'-ggggttgggrggttggrggttgrggg-3'

20      5'-ggggttgggeggttggeggttgeggg-3'

21      3'-ggggaaggggggaaggggaaggggg-5'
        5'-ccccttccccccttccccttccccc-3'

22      3'-ggggaagggaggaaggaggaagaggg-5'
        5'-ccccttcccctccttcctccttctccc-3'

23      3'-ggggaagggtggaaggtggaagtggg-5'
        5'-ccccttcccaccttccaccttcaccc-3'

24      3'-ggggaagggcggaaggcggaagcggg-5'
        5'-ccccttcccgccttccgccttcgccc-3'
```

Figure 4. Oligonucleotides and duplexes used in this study. Abbreviations are: g, 2′-deoxyguanosine; a, 2′-deoxyadenosine; t, thymidine; c, 2′-deoxycytidine; y, pyrazole 2′-deoxyribonucleoside (**4a**); m, imidazole 2′-deoxyribonucleoside (**4b**); r, 1,2,4-triazole 2′-deoxyribonucleoside (**4c**); e, 1,2,3,4-tetrazole 2′-deoxyribonucleoside (**10**); Duplexes **21–24** are composed of annealed, complementary oligonucleotides as shown. Note that oligonucleotides are shown in the standard 5′ to 3′ direction, while the top strand of each duplex is shown in the opposite 3′ to 5′ direction. These are the relative orientations expected in an antiparallel triplex.

comment. First a comparison of binding affinities for oligonucleotides **13–16**, containing all natural nucleosides, shows that G has the highest affinity for GC base pairs, and A and T have the highest affinities for AT base pairs. This is in general accord with previous data (9,12,29). All of the TFOs containing simple azole-2′-deoxyribonucleosides demonstrate some propensity to bind to duplex targets containing GC base pairs, although the affinities are substantially lower than that of G. Surprisingly, none of the TFOs containing azoles exhibit any binding to duplex targets containing AT base pairs.

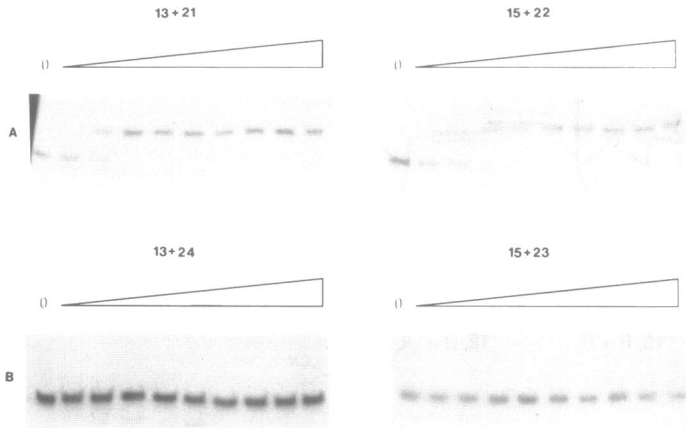

Figure 5. (**A**) Electrophoretic band shift analysis of triplex formation between **13** and **21** (left), and between **15** and **22** (right). (**B**) Similar analysis of interactions between **13** and **24** (left) and **15** and **23** (right). In all cases, the duplex DNA migrates as the faster moving band. Binding of the third strand oligonucleotide results in formation of a triplex, which migrates more slowly (upper bands). Duplex concentrations were ~3 × 10^{-11} M. Oligonucleotide concentrations in each gel are, from left to right, 0, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 nM.

As described above, none of the TFOs containing natural nucleosides bind with appreciable affinity to TA base pairs, whereas all of the modified TFOs bind strongly. T binds to CG base pairs to form T•CG triplets (**15•24**) as previously described (9,12). TFOs containing imidazole and tetrazole also show substantial binding to duplexes containing CG base pairs, while triazole substitution exhibits weaker binding, and the pyrazole substituted TFO is apparently unable to bind.

Inspection of the binding affinities for oligonucleotides containing the simple azole nucleosides reveals that there are two classes. Oligonucleotide **17**, containing pyrazole-2′-deoxyribonucleoside, binds only to duplexes **21** and **23**, containing GC and TA base pairs, respectively, and the binding is ~4-fold stronger to the duplex containing TA base pairs. In contrast, TFOs containing imidazole, triazole and tetrazole exhibit binding to duplexes containing GC, CG and TA base pairs, but are apparently unable to bind to duplexes containing AT base pairs. This degree of specificity was not anticipated, as these azoles were not specifically designed to recognize particular base pairs in the duplex. Further study is warranted to determine the molecular basis for these observations.

Table 2. Apparent association constants for oligonucleotides **13–20** binding to duplexes **21–24**[a]

	Third strand[b]							
Duplex[c]	**13**(g)	**14**(a)	**15**(t)	**16**(c)	**17**(y)	**18**(m)	**19**(r)	**20**(e)
21(gc)	4E9	9E8	2E8	3E8	1E8	2E8	1E8	3E7
22(at)	4E8	5E9	2E9	4E8	–	–	–	–
23(ta)	–	–	–	–	5E8	3E8	3E8	8E7
24(cg)	–	–	1E9	–	–	2E8	2E7	1E8

[a]Listed values are apparent association constants for triplexes formed by the indicated combination of oligonucleotide and duplex. Values are averages of at least two determinations, with duplicate values agreeing within a factor of two or less. Where values are not given (–), little or no triplex was detected, and the apparent association constant is estimated to be $< 10^6$.

[b]The letter in parentheses represents the nucleoside of interest (bold letters in Fig. 4) found in the indicated third strand oligonucleotide. Nucleoside abbreviations are described in the legend to Figure 4.

[c]The letters in parentheses indicate the base pair of interest (bold letters in Fig. 4) for the indicated duplex.

CONCLUSIONS

In conclusion, we have demonstrated that TFOs containing simple azole 2′-deoxy-ribonucleosides are capable of associating with duplexes containing CG and TA base pairs in antiparallel triplexes with relatively high affinity. In general, these nucleosides are superior to natural nucleosides in this regard. Although the current data do not address the mechanism of association, it is likely that multiple factors contribute. The potential for these azoles to maintain some degree of stacking interaction with neighboring base triplets seems likely to contribute to the observed association properties. In addition, the small size of azoles relative to natural bases may reduce or eliminate unfavorable steric hindrance when the modified TFO was bound to the duplex having CG or TA base pairs.

Azole 2′-deoxyribonucleosides seem to be promising compounds for incorporation into TFOs for generalizing antiparallel

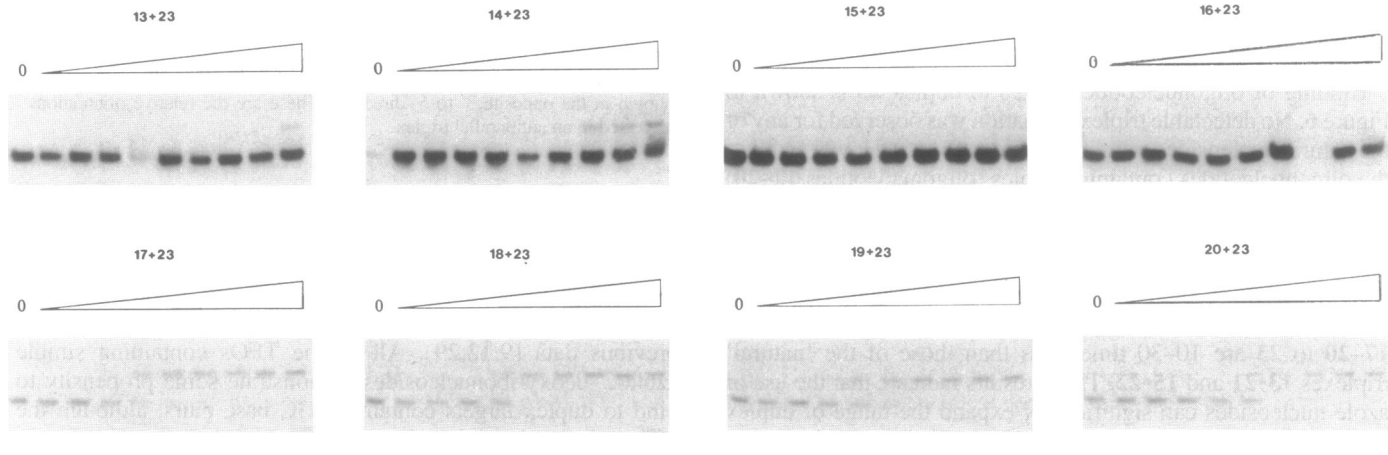

Figure 6. Band shift analysis of binding of oligonucletides **13–20** to duplex **23**. DNA and oligonucleotide concentrations were as described in Figure 5.

triple helix formation at non-homopurine targets. It is abundantly clear that the azole substitution permit binding of the modified TFO to the duplex sequence containing three TA base pairs. Pyrazole-2′-deoxyribonucleoside is of particular interest due to its strong discrimination against AT and CG base pairs, and weaker discrimination against GC base pairs. This is probably the first example of a nucleoside that is relatively specific for duplexes containing TA base pairs to form antiparallel triplexes. Although it is likely that sequence context effects will be observed, we expect that these compounds will also enhance antiparallel triplex formation with other targets containing TA (and CG) base pairs.

Based on the current data, monocyclic azoles could be viewed as a starting point in the design of more refined nucleosides. Addition of suitable exocyclic groups, perhaps designed to provide hydrogen bonding to the duplex bases, might enhance both affinity and specificity for a given base pair. Future design of such compounds would benefit greatly from an understanding of the molecular structure of an azole bound in a triplex. In that regard, we are presently exploring the use of ^{1}H NMR to determine the mechanism of binding for one or more of these analogues.

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