Selectivity and affinity of DNA triplex forming oligonucleotides containing the nucleoside analogues 2′-*O*-methyl-5-(3-amino-1-propynyl)uridine and 2′-*O*-methyl-5-propynyluridine†

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Triplex forming oligonucleotides (TFOs) containing the nucleoside analogues 2′-*O*-methyl-5-propynyluridine (**1**) and 2′-*O*-methyl-5-(3-amino-1-propynyl)uridine (**2**) were synthesized. The affinity and selectivity of triplex formation by these TFOs were studied by gel shift analysis, T_m value measurement, and association rate assays. The results show that the introduction of **1** and **2** into TFOs can improve the stability of the triplexes under physiological conditions. Optimized distribution of **1** or **2** in the TFOs combined with a cluster of contiguous nucleosides with 2′-aminoethoxy sugars resulted in formation of triplexes with further enhanced stability and improved selectivity.

Introduction

A DNA triple helix can form when a third strand binds in the major groove of an intact polypurine:polypyrimidine duplex.[1,2] The structure is stabilized by Hoogsteen hydrogen bonds between the third strand bases and the duplex purines. These interactions are sequence specific and a triplex binding code has been elaborated.[3] The third strand may consist of purines or pyrimidines, depending on the target sequence. The absence of a requirement for a protein or enzyme cofactor, coupled with the sequence stringency, support the long standing view that triple helix forming oligonucleotides (TFOs) have the potential to become effective gene targeting reagents in living cells.[4,5]

There are, however, a number of impediments to the activity of TFOs against polypurine:polypyrimidine targets *in vivo*. These include the instability of triplexes with pyrimidine third strands at physiological pH due to the requirement for cytosine protonation, the repulsion between third strand and duplex phosphates, and the conformational restrictions on both third strand and duplex (reviewed in refs. 4, 6). Although these are significant challenges, base and sugar analogues have been described that provide promising solutions to these problems.

RNA analogue sugar substitutions have been of interest ever since the demonstration that RNA third strands formed more stable triplexes than their deoxy counterparts.[7] This observation prompted analysis of third strands with 2′-*O*-methyl sugars, which have been shown to impose relatively little distortion on the underlying duplex.[8] TFOs with other analogues, such as locked or bridged (LNA/BNA) ribose, make very stable triplexes.[9,10] The 2′-aminoethoxy (2′-AE) modification, which imparts a positive charge to an RNA analogue sugar, has been shown to improve both the kinetics of formation and the stability of pyrimidine motif triplexes. Additionally, we have shown that appropriate use of this modification supports gene targeting activity by TFOs in living cells.[11]

Base analogues in TFOs also improve triplex stability. For example, 5-methylcytosine has been used for many years as a replacement for cytosine in TFOs, with a partial amelioration of the pH limitation on triplexes with pyrimidine third strands.[12] Other cytosine replacements have been discussed more recently.[13,14] The 3-propynyl derivative of uridine, as a replacement for thymidine enhances the stability of pyrimidine motif triplexes.[15] This work has been substantially extended by the synthesis and characterization of the 5-(3-amino-1-propynyl)uridine.[16] This analogue adds a favorable electrostatic interaction *via* the positive charge of the amino group to the stacking interactions of the propynyl group. The importance of positively charged residues in TFOs, either sugar or base, on triplex stabilization has been noted previously.[16–22]

Typically, studies on the contribution of a base analogue to the activity of a TFO vary the extent and distribution of the particular modification in the context of a DNA third strand, that is, with deoxy sugars. In this format the influence of the individual modification can be assessed against the background of a relatively weak baseline. Experiments with TFOs carrying combinations of base and sugar analogues are less common. However it is likely that biologically active TFOs will require multiple modifications, as some of our previous work suggests.[23] In these circumstances the contribution of an individual analogue, previously characterized in a deoxyoligonucleotide, may not be predictable. Here we have addressed this issue and describe the influence on TFO activity of combinations of propynyl or aminopropynyl uridine, with 2′-*O*-methyl and/or 2′-*O*-aminoethyl ribose sugars.

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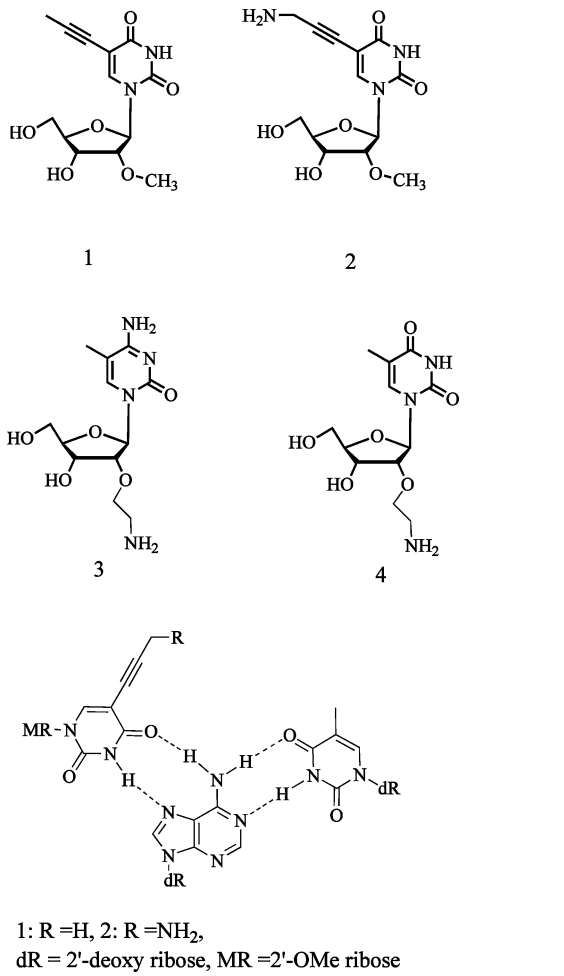
† Electronic supplementary information (ESI) available: Table S1 Maldi-TOF data of oligos and Fig. S1 The profile of non-denaturing gel electrophoresis of triplexes. See DOI: 10.1039/b810709b

Results

The nucleoside analogues 2′-*O*-methyl-5-propynyluridine (**1**), 2′-*O*-methyl-5-(3-amino-1-propynyl)uridine (**2**) and the corresponding phosphoramidites were synthesized according to published procedures.[24,25] The structure of the **1** or **2**·A:T triplet is shown in Scheme 1. The triplex target sequence is 19 mer duplex (**5**:**6**) from Intron 4, adjacent to Exon 5, of the *Hprt* gene in the Chinese Hamster genome, and consists of an uninterrupted 17 nt polypurine: polypyrimidine element.[26] The TFOs examined in this report contained 2′-*O*-Me ribose and the additional modifications, **1**, **2**, and 2′-AE (**3**, **4**) as shown in Table 1.



Scheme 1 Chemical structures of **1**, **2**, **3**, **4** and the triplet base pair of **1** or **2**·AT.

Non-denaturing gel analysis

The ability of the TFOs to form triplexes was examined by neutral gel electrophoresis. In this assay the duplex purine strand **6** was labeled by ^{32}P, and then the labeled duplex target was incubated with the TFOs. The shift of the labeled strand towards slower mobility demonstrates the extent of triplex formation. After incubation of each of the TFOs in this study the duplex was completely converted to the triplex (supplementary materials†). Thus all the modified TFOs in our work could form stable triplexes at pH 7.0. We then measured the K_d value of TFO-**7** and TFO-**9**

Table 1 The upper part is the duplex target sequence in the Chinese hamster *Hprt* gene. The target lies in Intron 4 adjacent to Exon 5. Also shown is the modification pattern of the TFOs, in which T is 2′-*O*-Me-5-methyl dU and C is 2′-*O*-Me-5-methyl dC

Duplexes	
5′-TTTCTCTTTTTTCTTCT AC-3′	**5**
3′-AAAGAGAAAAAAGAAGA TG-5′	**6**
TFOs	
3′-TTTCTCTTTTTTCTTCT-5′	**7**
3′-TTTCTC**1**TTTT**1**C**1**TC**1**–5′	**8**
3′-TTTCTCT**1**T**1**T**1**C**1**TCT-5′	**9**
3′-TTTCTCT**1111**TCTTCT-5′	**10**
3′-TTTCTC**2**TTTT**2**C**2**TC**2**–5′	**11**
3′-TTTCTCT**2**T**2**T**2**C**2**TCT-5′	**12**
3′-TTTCTCT**2222**TCTTCT-5′	**13**
3′-T**4434**CTTTTTTCTTCT-5′	**14**
3′-T**4434**CT**2**T**2**T**2**C**2**TCT-5′	**15**
3′-T**4434**C**22**TTTTC**22**CT-5′	**16**
3′-T**4434**C**2**T**2**TTTCT**2**C**2**–5′	**17**

by gel shift. The gel profile of the TFO concentration dependent forming of triplex **12**·**5**:**6** is shown in Fig. 1 (top). The relative intensity of the duplex and the triplex bands was quantified by Phosphorimager analysis and the corresponding data were converted to binding isotherm curves (Fig. 1b). The **2** modified TFO-**12** shows higher binding affinity with lower K_d (321 nM) value than that of TFO-**7** (427 nM). The improvement in K_d value of the **2** modified TFO is not as great as observed previously with TFOs with 2′-aminoethoxy-5-(3-aminoprop-1-ynyl) uridine.[21,27] This is because the reference TFO-**7** contained 2′-OMe sugars, a modification that confers relatively high affinity at pH 7.0.

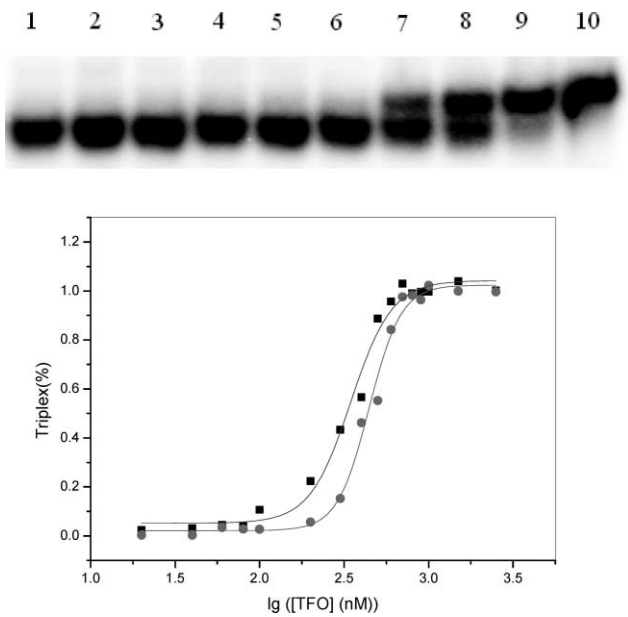


Fig. 1 (Top) Typical non-denaturing gel profile of the TFO concentration dependent forming of triplex **12**·**5**:**6**. Concentration of duplex target **5**:**6** was fixed at 5 nM. Lane 1–10, TFO-**12** concentration (nM): 0, 20, 40, 60, 80, 100, 200, 400, 600, 800. (Bottom) Binding isotherms of TFOs **7** (circles) and **12** (squares) in Hepes buffer (20mM, pH 7.2) with 10 mM $MgCl_2$.

Thermal stability measurements

We investigated the thermostability of the **1** or **2** modified triplexes at pH 7.0. As discussed before,[23] the stability of the triplex formed

Table 2 T_m Values and associate rates of triplexes (TFOs **7–13**)

TFOs	T_m/°C	k_{on}/l s^{-1} mol^{-1}
3′-TTTCTCTTTTTTCTTCT-5′ **7**	54	2000
3′-TTTCTC**1**TTTT**1**C**1**TC**1**-5′ **8**	59	2400
3′-TTTCTCT**1**T**1**T**1**C**1**TCT-5′ **9**	62	3400
3′-TTTCTCT**1111**TCTTCT-5′ **10**	63	3500
3′-TTTCTC**2**TTTT**2**C**2**TC2-5′ **11**	59	2800
3′-TTTCTCT**2**T**2**T**2**C**2**TCT-5′ **12**	65	3600
3′-TTTCTCT**2222**TCTTCT-5′ **13**	65	3600

T_m Buffer: 100 mM NaCl. 2.0 mM $MgCl_2$ and 10 mM Na-cacodylate, pH 7.0. Kinetic buffer: 150 mM KCl. 1.0 mM $MgCl_2$ and 10 mM Na-cacodylate, pH 7.2.

by the 2′-*O*-Me TFO (**7**) was 54 °C, which is higher than the duplex target alone. Therefore, only one transition in the melting curve was detected during the denaturation process, and that transition reflected the T_m value of the triplex. TFOs with 2′-*O*-Me ribose at all positions and with four substitutions of **1** or **2** formed triplexes with T_m values greater than formed by **7** (Table 2). The stabilizing effect of **1** and **2** was similar when they were distributed throughout the TFO. Thus the T_m values of **8**·**5**:**6** and **11**·**5**:**6** were both 59 °C. When **1** or **2** were introduced in the middle of the TFOs, either separated by one base or adjacent to each other, the triplexes modified by **2** were slightly more stable than the triplexes containing **1**. For example the T_m value of **12**·**5**:**6** was 65 °C, 2 °C higher than that of **9**·**5**:**6**. Consequently the positively charged analogue made only a modest contribution to triplex stability. Furthermore, clustering, or close alternation yielded greater stability than a more dispersed distribution. One possible reason is in the triplexes **8**·**5**:**6** and **11**·**5**:**6** one of the modified nucleosides was introduced at the end of the TFOs. Generally base analogues show weaker stabilizing effect when located at the end of the sequence than in the middle of the strand. Another explanation is the arrangement of modified residues is important for maximum stability, as observed previously.[13] In the optimal configuration, the increment of additional stability provided by **1** was 2.25 °C per residue, while that from **2** was 2.75 °C per residue.

Association rate measurements

The relative association rates for the TFOs were measured. As shown in Table 2, the k_{on} of the 2′-*O*-Me TFO (**7**) was 2000 l s^{-1} mol^{-1}. Additionally modified TFOs with **1** or **2** showed enhanced k_{on} values. With the modification at the 5 prime end, in both cases the k_{on} increased to 2500 l s^{-1} mol^{-1}. The TFOs with a middle cluster of **1** or **2** showed the highest k_{on}, 3500–3600 l s^{-1} mol^{-1}. As with thermal stability, the positive charge added only a modest increment to the k_{on} value.

Discrimination of duplex targets with sequence interruptions by TFOs with 2′-OMe and 2 modifications

In the light of the increased stability of the triplexes formed by TFOs containing **2** and 2′-*O*-Me ribose, it was important to examine the influence of the combined modifications on the specificity of triplex formation. Consequently we measured the thermal stability of triplexes formed by TFO-**12** with four duplexes containing a different base pair at one position. The variable base pair was located at the site of one of the **2** substitutions. For comparison we determined the stability of triplexes formed by the variable duplex targets and TFO-**7**. The typical T_m values for **12**·**5**:**6** with **2** placed at different X:Y base pairs are shown in Table 3. The T_m value for the triplex with the **2**·A:T pair was 65 °C. As expected, the triplexes formed with duplexes containing the other base pairs showed declines in thermal stability. The greatest discrimination was with the triplex containing the **2**·T:A pairing (49 °C, ΔT_m between **2**·A:T and **2**·T:A is 16 °C), while that with the **2**·G:C combination was relatively more stable with a T_m value of 57 °C. The **2**·C:G pairing was of intermediate stability (53 °C). It was noteworthy that the least stable triplex formed by the third strand with 2′-OMeT was against the duplex target with the C:G interruption (36 °C), in contrast to the result with TFO-**12**. Apparently the presence of **2** changed the affinity relationships and stabilized the interaction with the C:G interruption of the polypurine:polypyrimidine target.

Table 3 T_m values of triplexes with mismatch base pairs (TFO **7** and TFO **12**)

	N			
	OMe T (TFO **7**)		**2** (TFO **12**)	
	T_m/°C	ΔT_m/°C	T_m/°C	ΔT_m/°C
N·AT	54	N/A	65	N/A
N·TA	41	13	49	16
N·GC	47	7	57	8
N·CG	36	18	53	12

Duplex target 3′-AAAGAGAAAAAXGAAGATC, 5′-TTTCTCTTTT-TYCTTCTAG. X, Y = each base A, G, T, C in turn. Δ T_m (°C): the T_m value difference between the N·AT and mismatch base pairs.

TFOs with 2′AE, 2′-OMe, and 2

The preceding results indicated that the introduction of **1** or **2** into TFOs increased triplex stability and enhanced association rates relative to TFO **7**. In our earlier work we found that a patch of 3–4 contiguous 2′-AE residues made an important contribution towards the bioactivity of TFOs containing 2′-*O*-Me ribose sugars.[23,28] Accordingly we synthesized TFO-**15** with 2′-*O*-Me ribose, four contiguous 2′-AE residues at the 3′ end, and the alternating distribution pattern of **2** found in TFO-**12**. The T_m value of the resultant triplex was 72 °C, an improvement over the 63 °C obtained with the **14**·**5**:**6** triplex. This result was consistent with expectations, and indicated that the increase in stability due to the added **2** was 2.25 °C per residue. However, when we determined the k_{on} value for TFO-**15** we found a pronounced decline relative to the TFO-**14** control. This was unexpected since both the 2′-AE cluster,[28] and the alternating pattern of **2** (Table 2) enhance association rate relative to TFOs containing only the 2′-*O*-Me modification. Given the importance of the association rate as a correlate of TFO bioactivity, we examined the properties of two additional TFOs containing 2′-AE and 2′-*O*-Me ribose. In these the four residues of **2** were well separated such that there were two groups of two, either immediately adjacent or alternating (TFO-**16**, -**17**). One of these, TFO-**16** (12000 l s^{-1} mol^{-1}) had a k_{on} slightly greater than the reference TFO-**14** (1000 l s^{-1} mol^{-1}), while the T_m value of **16**·**5**:**6** (67 °C) was greater than that of the **14**·**5**:**6** triplex (Table 4).

Table 4 T_m values and association rates of triplexes (TFO **14–17**)

TFOs	T_m/°C	k_{on}/l s^{-1} mol^{-1}
3′-T**4434**CTTTTTTCTTCT-5′ **14**	63	11000
3′-T**4434**CT**2**T**2**T**2**C**2**TCT-5′ **15**	72	5500
3′-T**4434**C**22**TTTTC**22**CT-5′ **16**	67	12000
3′-T**4434**C**2**T**2**TTTCT**2**C**2**–5′ **17**	66	8500

T_m buffer: 100 mM NaCl. 2.0 mM $MgCl_2$ and 10 mM Na-cacodylate, pH 7.0. Kinetic buffer: 150 mM KCl. 1.0 mM $MgCl_2$ and 10 mM Na-cacodylate, pH 7.2.

Discrimination of duplex targets with sequence interruptions by TFOs containing 2′-AE, 2′-*O*-Me, and 2 modifications

The preceding results indicated that TFO-**16** formed triplexes with a slightly improved k_{on} and increased thermal stability relative to the reference TFO-**14**. We then asked if this TFO, with three modifications would display the same selectivity seen with TFO-**12**. This analysis was performed with two different duplex targets. In one the X:Y position, as before, was at the position corresponding to a residue of **2** in the TFO. In the other we used a duplex with the X:Y pair placed between two residues of **2** in TFO-**16**. This experiment was performed to ask if the improved stability afforded by **2** would influence selectivity when sequence interruptions in the duplex were located adjacent to the canonical triplet formed by **2**. We found that the differential between the triplex with the **2**·A:T triplet and the most stable alternate, the **2**·G:C triplet, actually increased from 6 °C to 10 °C as compared to the T_m values obtained with the reference TFO-**14** (Table 5). Apparently the introduction of substitutions of **2** had the effect of increasing the overall stability of the triplex, but also of improving the relative selectivity. The same trend was observed with the triplexes in which **2**·A:T triplets flanked T·X:Y triplets. The differentials were enhanced relative to the triplexes formed by TFO-**14**. Consequently, substitution by **2** in a TFO also containing 2′-*O*-Me and 2′-AE, whether opposite or adjacent to "interrupting" duplex pairs, increased stability without loss, even slight improvement, in target sequence selectivity.

Table 5 T_m values of triplexes with mismatch base pairs (TFO **14** and TFO **16**)

	N					
	OMe T (TFO **14**)		**2** (TFO **16**)		OMe T (TFO **16**)	
	T_m/°C	ΔT_m/°C	T_m/°C	ΔT_m/°C	T_m/°C	ΔT_m/°C
N·AT	63	N/A	67	N/A	67	N/A
N·TA	50	13	49	18	48	19
N·GC	57	6	57	10	57	10
N·CG	53	10	52	15	50	17

Duplex target for the mismatch of OMe T in TFOs **14** and **16**: 3′-**AAAGAGAAAAAXGAAGATC**, 5′-**TTTCTCTTTTTYCTTCTAG**. Duplex target for the mismatch of **2** in TFOs **16**: 3′-**AAAGAGAXAAAAGAAGATC**, 5′-**TTTCTCTYTTTTCTTCTAG**. X, Y = each base A, G, T, C in turn. Δ T_m (°C): the T_m value difference between the N·AT and mismatch base pairs.

Discussion

A central goal of triple helix research is the identification of base and sugar modifications that overcome the challenges to triplex stability under physiologically relevant conditions. There is now a wealth of base and sugar analogues that improve TFO affinity, and in some cases, triplex formation kinetics. Most of these studies describe the consequences of a particular analogue in the context of deoxyoligonucleotides. However, there is ample evidence that RNA analogue sugar modifications can greatly improve TFO performance.[8–10,18,29] Among the base analogues that show promise are propynyl and aminopropynyl uridine, both of which have been shown to enhance triplex stability in deoxyoligonucleotide TFOs.[16,30] In this study we have examined the influence of these analogues on TFOs additionally containing 2′-*O*-Me, and 2′-AE substitutions.

We found that the addition of **1** or **2** improved the stability of triplexes formed by TFOs also containing uniform 2′-*O*-Me ribose substitution. In previous work with deoxyoligonucleotide TFOs it was found that propynyl deoxyuridine residues increased T_m values by about 2 °C per residue,[15] while the increase for deoxyaminopropynyluridine was about 3 °C per residue.[17] In the work presented here we found that, despite the gain in thermal stability derived from the 2′-*O*-Me modification, further improvement from the addition of **1** or **2** was possible, and this was comparable to earlier findings. There was only a modest difference in increment between the two modifications, suggesting that the major contribution comes from the propynyl moiety. This has been ascribed, by the Feigon group, to an increase in stacking interactions between the propynyl group and the aromatic ring of the 5′ adjacent base in the TFO. In addition, these workers have pointed to the stabilizing influence of the localized hydrophobic environment resulting from the propynyl substitution.[30]

A second observation of note was that the increased stability did not come at the expense of relative selectivity. The T_m value differentials between the triplexes formed by TFOs **7** or **12** on the "perfect" match duplex target, and the "mismatched" target supporting the most stable triplex (the G:C target) were quite similar (Table 3). Furthermore, an even greater differential between the perfect match triplex and the most stable interrupted triplex (again the G:C replacement) was observed with the TFOs containing the cluster of 2′-AE residues (Table 3). This was independent of whether **2** was placed at, or adjacent to, the "mismatch" base pair (Table 3). Thus, although there was an elevation of the T_m value when **2** was incorporated into TFOs with 2′-*O*-Me, or 2′-AE and 2′-*O*-Me, relative selectivity was maintained. This is in agreement with studies of TFOs using 2′-aminoethoxy-5-(3-aminoprop-1-ynyl)uridine (BAU) for recognizing AT base pairs in duplex DNA, in which BAU showed high selectivity and conferred enhanced stability on BAU·A:T triplets relative to T·A:T.[21,27] It will be important to determine if this selectivity is maintained in assays of TFO bioactivity in living cells.

The measurement of the association rates revealed an interesting paradox. When residues of **2** were clustered or alternated in the TFOs with only 2′-*O*-Me ribose there was an increase in association rate. This was consistent with our earlier study in which clustered 2′-AE, residues were found to increase k_{on}.[23] However, when alternating residues of **2** were placed on TFO-**15**, also containing clustered 2′-AE substitutions, the association

rate declined, despite an increase in the thermal stability of the triplex. Disruption of the cluster of **2**, at the expense of the T_m value, was required to slightly improve the association rate relative to that of TFO-**14**. There may be multiple reasons for this. In light of the relatively simple target sequence, two closely spaced groups of positive charges may increase the frequency of incorrectly positioned nucleation events. This would have the effect of slowing the rate of formation of correct nucleation events, and thus the overall rate of triplex formation. It is also possible that the conformational and structural adjustments imposed by the propynyl side chain on the duplex, and resultant triplex,[30] contribute to the slowed association. However, if this were true TFO-**9** would have been expected to show a slower k_{on} than the reference TFO-**7**, rather than the increased rate that was observed (Table 2). It is of interest that in other work TFOs with the nucleoside analogue, 2′-*O*-(2-aminoethyl)-5-(3-amino-1-propynyl)uridine did not show an enhanced association rate, although analogues with either modification alone would have increased the rate.[22]

The conclusion that we draw from these studies is that multiple base and sugar modifications clearly improve TFO performance. However, it is also apparent that we have much to learn regarding the optimal organization of these modifications. At this time we are in an empirical phase. Eventually principles with predictive value should emerge.

Experimental

Materials

Phosphoramidites and CPG Supports. N^4-Formamido-5′*O*-(4,4′-dimethoxytrityl)-5-methylcytidine-2′-*O*-methyl-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite, 5′-*O*-(4,4′dimethoxytrityl)-5-methyluridine 2′-*O*-methyl-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite and 5′-*O*-(4,4′-dimethoxytrityl)-5methyluridine-2′-*O*-methyl-3′-*O*-succinamido-N^6-hexanamidoN^3-propyl-CPG (controlled pore glass) support were purchased from Chemgenes, Ashland, MA. Synthesis of N^4-(*N*-methylpyrrolidinoamidino)-5′-*O*-(4,4′-dimethoxytrityl)-5-methylcytidine 2′-*O*-(2-aminoethyl)-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite and 5′-*O*-(4,4′-dimethoxytrityl)-5-methyluridine, 2′-*O*-(2-aminoethyl)-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite was reported previously.[18,29] The compounds **1**, **2** and the corresponding building block were synthesized according to published work.[24,25]

Synthesis, deprotection and purification of TFOs. The oligonucleotides were synthesized on a 1 μmol scale on CPG supports (1000 Å) using an Expedite 8909 synthesizer as previously described.[11] The phosphoramidites were dissolved at a concentration of 0.1 M. The coupling times for the phosphoramidite monomers of protected 2′-*O*-methylthymidine, 2′-*O*-methyl-5-methylcytidine, **1** and **2** were 360 s. The coupling times for the phosphoramidite monomers of protected 2′-aminoethylthymidine and 2′-aminoethyl-5-methylcytidine were 900 s. The TFOs were deprotected with equal ratios (1 : 1) of 0.5 mL of 28% aqueous NH_3 and 0.5 mL of 40% aqueous methylamine (Aldrich Chemicals) solution for 90 min at room temperature, immediately evaporated to dryness, and resuspended in HPLC grade H_2O. Purification of TFOs was carried out by an ion-exchange HPLC using a Dionex DNAPac PA-100 column (column sizes are 4.0 mm × 250 mm for analysis and 9.0 mm × 250 mm for purification, respectively) on a Shimadzu HPLC system (LC-10ADvp) with a dual wavelength detector (SPD-10AVvp) and an auto injector (SIL10ADvp). HPLC conditions were as follows: linear gradient, % buffer B 0–50% for 45 min, ~100% for 60 min, 1.5 mL min^{-1}; buffer A consisted of 100 mM Tris (pH 7.8) containing 10% CH_3CN; buffer B consisted of 1.0 M NaCl containing 100 mM Tris (pH 7.8) and 10% CH_3CN; UV monitor, 254 and 315 nm (λ_{max} for psoralen). The oligonucleotides were collected, lyophilized, and desalted using a Sep-Pak Plus C18 cartridge (Waters Corp.). The masses of the chemically modified oligonucleotides were determined by the positive ion mode using MALDI-TOF (matrix-assisted laser desorption ionization time of flight) mass spectroscopy on a Voyager Applied Biosystem Instrument. The matrix used for preparing the MALDI-TOF samples is a mixture of 3-hydroxypicolinic acid (50 mg mL^{-1} in 50% CH_3CN) and ammonium citrate (50 mg mL^{-1} in HPLC grade water).

Thermal denaturation

The thermal melting experiments were carried out using TFOs with a 19-mer *HPRT* hamster duplex target (5′-GTAGAAGAAAAAAGAGAAA and 3′-CATCTTCTTTTTTC-TCTTT) which had a T_m value of 53.5 °C. A 1.0 μM stock solution of target duplex was prepared in a buffer containing 100 mM NaCl, 2 mM $MgCl_2$, and 10 mM sodium cacodylate at pH 7.0. The solution was heated to 80 °C for 15 min, allowed to reach room temperature over 4 h, and then stored in the refrigerator at 4 °C. An aliquot of concentrated TFO solution was added to 1.0 mL of the stock duplex to make the final concentration of the TFO is 2.0 μM, and the mixture was incubated at room temperature overnight. Thermal melting determinations were carried out using a Cary 3E UV-Vis spectrophotometer fitted with a thermostat sample holder and a temperature controller. The triplex solution was heated from 25 to 85 °C at a rate of 0.4 °C min^{-1}, and the absorbance at 260 nm was recorded as a function of the temperature. The raw data were processed using Origin 7.0 software to determine the first derivative curve, from which the T_m value was obtained. All analyses were performed at least twice with an error of no more than 0.5 °C.

Association rate analysis

The TFO + duplex to triplex transition is accompanied by a decrease in UV absorbance. To measure the association rate kinetics of TFOs, we used a 19-mer linear duplex target (5′-GTAGAAGAAAAAAGAGAAA and 3′-CATCTTCTTT-TTTCTCTTT). A stock duplex solution (2.0 μM for each strand) was prepared in a kinetic buffer containing 150 mM KCl, 1 mM $MgCl_2$, and 10 mM sodium cacodylate at pH 7.2. 0.5 ml TFO solution (2.0 μM) was added with vigorous mixing to 0.5 ml of the duplex stock solution. The mixing process was no longer than 10 s. The experiments were carried out on a Cary dual-beam spectrophotometer fitted with a Peltier temperature controller at 25 °C. The UV decay curves were fitted to second-order kinetics using software supplied with the instrument, where

association rate constants were obtained. The rate constants reported here were taken from an average of three individual experiments.

Polyacrylamide gel electrophoresis

Purine strand (5′-GTAGAAGAAAAAAGAGAAA) (0.5 μM) was ^{32}P-end labeled using [γ-^{32}P] ATP (NEN) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and annealed with pyrimidine strand (3′-CATCTTCTTTTTTCTCTTT) (5.0 μM). Then the ^{32}P-end labeled duplex was diluted 20-fold with 2 × triplex buffer (40 mM Tris, 20 mM $MgCl_2$, pH 7.0) to make a stock duplex solution. Ten microliters of stock duplex oligos were incubated with 10 μL of each TFO (4.0 μM stock in water) at room temperature overnight. The final concentrations of both duplex strands were negligible relative to the third strand concentration. For native gel electrophoresis, the dye (0.1% xylene cyanol, 0.1% bromophenol blue, 5.0 μL) was added to each of the triplex mixtures, and the mixtures were immediately loaded onto a 12% polyacrylamide gel in 1× TAE buffer and run for 4 h at 150 V. Gels were visualized using a Fuji Phosphorimager and quantified using ImageQuant software.

K_d Determination

5 nM of labeled duplex was incubated with various concentrations of the TFOs in buffer consisting of 20 mM Hepes, pH 7.2, and mM $MgCl_2$ as indicated, at room temperature for 24 h. Samples were then loaded on a 15% acrylamide gel in Hepes buffer, pH 7.2, containing 10 mM $MgCl_2$ and electrophoresed for 16 h. The relative intensity of the duplex and triplex bands was determined by Phosphorimager analysis. The K_d values were determined by Hill′s equation [$y = ax^b/(c^b + x^b)$], where y = percent triplex formation, x = TFO concentration, a = maximum percent value of triplex formation, b = Hill's coefficient, c = approximate value of K_d. The assumptions on the basis of which the Hill's equation was used are (1) TFOs do not interact with themselves and (2) the concentration of the duplex target is too small to influence the equilibrium of triplex formation. The data were processed with origin 8.0 software.

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