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2′-O-Appended Polyamines that Increase Triple-Helix-Forming Oligonucleotide Affinity are Selected by Dynamic Combinatorial Chemistry

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The sequence-specific recognition of double-stranded DNA by triplex-forming oligonucleotides (TFOs) has potential application as therapeutics in the antigene strategy as well as tools in molecular biology.[1] TFOs bind to the major groove of DNA either in a parallel or antiparallel orientation according to their base composition. TFOs composed of pyrimidine bases bind parallel to the purine strand of oligopurine–oligopyrimidine duplexes through T-AT and C^+-GC triplet formation. However, the use of TFOs under physiological pH and ionic strength is hampered by their weak binding to DNA duplexes mainly due to the requirement for cytosine protonation and charge repulsion between the three negatively charged strands. Spermine and related polyamines are largely protonated at physiological pH and are known to promote triplex stabilization both upon external addition[2] as well as upon conjugation at the 5′-terminus,[3] C5 of dU,[4] and C4 of 5-methyl-dC.[5] In contrast, attachment of spermine on a 2′-position in a TFO was found to have a deleterious effect on triplex stability.[6] In this case, replacement of one internal dT unit by an *ara* U 2′-phosphorylpropylspermine within an oligo dT was found to abolish its capacity to form a triplex with an oligo-dA·oligo-dT duplex; this suggests that tethering position, *ara* C2′, or/and the linker phosphorylpropyl was inappropriate. However, Cuenoud et al. showed later that TFOs containing 2′-*O*-aminoethyl ribonucleotides (2′-AE-TFOs) formed stabilized triplexes owing to dual recognition of DNA targets by base–base contacts and concomitant salt-bridge formation between positively charged ammonium groups on the TFO and DNA phosphates.[7a,b] This dual recognition approach would be further improved if amino groups present on 2′ positions of a 2′-AE-TFO were substituted by polyamines, thus generating 2′-polycationic chains that are able to strongly interact with several DNA phosphate groups and bring additional stabilization to the triplex.

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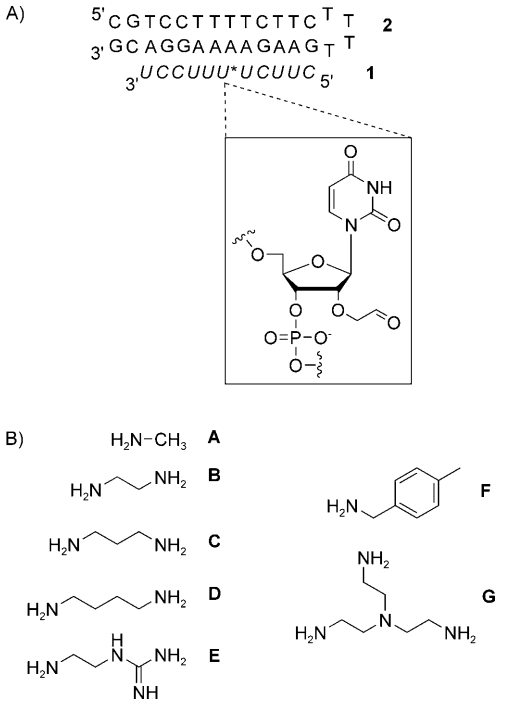
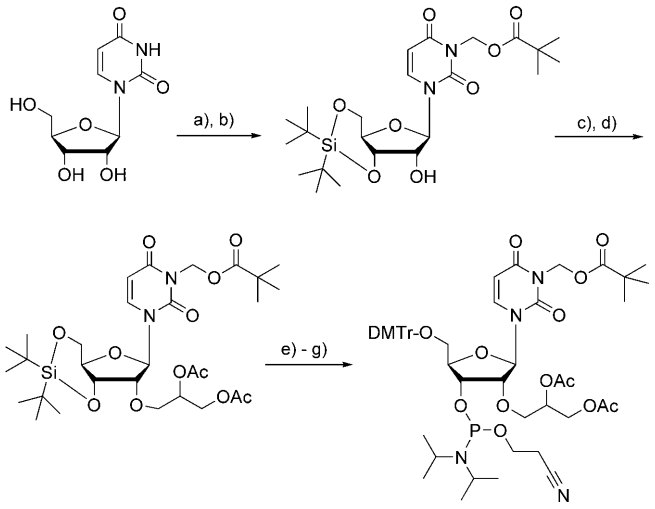
In this context, dynamic combinatorial chemistry (DCC) appears to be a method of choice for the identification of such 2′-O-appended polyamines.[8] DCC has attracted increasing interest over recent years as an alternative approach to traditional combinatorial chemistry that combines in a single step the library build-up and screening processes. DCC involves the use of reversible reactions between different building blocks to generate an equilibrating mixture of compounds that is able to respond through noncovalent interactions to the addition of a target molecule. The preferential binding of one member of the mixture to the target induces a shift in the equilibrium towards that particular compound. Thus DCC offers in situ screening of the combinatorial library simply by comparing its composition in the absence and presence of the target. DCC experiments have been performed by using various biological targets including nucleic acids.[9] In previous studies, we have established that DCC can be used to identify covalently appended small molecules that stabilize oligonucleotide complexes.[9e,f] For that purpose, equilibrating imines formed from 2′-aminonucleotide incorporated into an oligonucleotide ligand and a small set of aldehydes were submitted to the template effect of a nucleic acid target. These studies were carried out in the context of DNA and RNA duplexes as well as an RNA–RNA kissing complex. In each case, after reduction of the imines, a chemically stable conjugated ligand with an increased affinity for its target was identified that corresponded to the most amplified compound. Here, by using an "inverted" imine reaction, we report an application of DCC for the screening of various amines and polyamines for their ability upon reaction with a 2′-linked aldehyde group present in an internal ribonucleoside unit of a TFO to stabilize the triplex formed with a DNA target.

Eleven-base-long 2′-*O*-methyl TFO **1** bearing a central 2′-*O*-(2-oxoethyl)uridine and able to form a parallel triplex with the stem of DNA hairpin **2** was synthesized (Scheme 1 A). TFO **1** was obtained by periodate-mediated oxidation of precursor 2′-*O*-Me oligonucleotide containing a 2′-*O*-(2,3-dihydroxypropyl)-uridine in position 6 and synthesized by the phosphoramidite method.[10] The fully protected phosphoramidite of this latter nucleoside was synthesized (Scheme 2) by using a slightly modified procedure as described by Zatsepin et al.[11] Starting from uridine, the less-expensive 3′,5′-di-*tert*-butyl disiloxane protecting group was preferred to Markewicz's reagent; protection of the 3-N position by pivaloyloxymethyl (Pom) group was necessary for further selective 2′-O allylation. Palladium-assisted allylation proceeded in good yield, and the *vic*-diol was subsequently produced through oxidation by osmium tetroxide/4-methylmorpholine *N*-oxide (NMO). The diol was protected through acetylation, and the resulting uridine derivative was 3′-O phosphitylated.



Scheme 1. Structures of A) DNA hairpin target **2** and TFO **1** (italicized letters indicate 2′-*O*-methyl-ribonucleotides); B) reacting amines (**A**–**G**) that were used for generating the dynamic library.



Scheme 2. Synthesis of fully-protected 2′-*O*-(2,3-dihydroxypropyl)uridine phosphoramidite. a) tBu$_2$SiCl$_2$, AgNO$_3$, DMF; b) Pom-Cl, Bu$_4$NHSO$_4$, CH$_2$Cl$_2$/aq Na$_2$CO$_3$; c) allylmethyl carbonate, tris(dibenzylidene acetone)Pd$_2$, PPh$_3$, THF; d) NMO, OsO$_4$, THF then Ac$_2$O, *N*,*N*-dimethyl-4-aminopyridine, pyridine; e) 3(HF)Et$_3$N, THF; f) 4,4′-dimethoxytritylchloride (DMTr-Cl), pyridine; g) (iPr)$_2$NEt, chloro(2-cyanoethoxy)(diisopropylamino)phosphine, iPr$_2$EtN, CH$_2$Cl$_2$.

For the build up of the dynamic combinatorial library, we selected seven amines **A**–**G** (Scheme 1 B) differing in chain length and number of positive charges present at physiological pH and exhibiting various potential for hydrogen bonding and electrostatic interactions. Previously reported DCC experiments involving imine libraries were based on the assumption that formation of interconverting imines and their interaction with template (the target) are rapid processes compared to their reduction into stable secondary amines.[9e,12] Because formation is a considerably slower process for triplex than for duplex[13] and to avoid a possible kinetic bias, a triplex between TFO **1** and target **2** was allowed to form before amines **A**–**G** and sodium cyanoborohydride were added.[14] Under these conditions, interconvertion of imines is likely to occur on extensively target-bound TFO in a similar way to that operating in the tethering fragment-based drug-discovery method,[15] thereby amplifying the template effect of DNA target **2**. Attempts to analyze the resulting mixture of conjugated TFOs **1A**–**G** by RP- or ion-paired RP-HPLC failed due to considerable peak overlap. Therefore, we turned our attention to the use of MALDI-Tof mass spectrometry under conditions similar to those reported by Sarracino et al. for the quantitative detection of oligonucleotides.[16] Before analysis, samples were extensively desalted and no internal standard was added because the relative quantification of conjugates **1A**–**G** was sufficient for our purpose. Typical mass spectra obtained from conjugate mixture generated in the presence or absence of target **2** are presented in Figure 1 A. Proportions of **1A**–**G** were estimated from the corresponding peak heights and their change induced by the presence of DNA target **2** are reported in Figure 1 B. There was a clear amplification of conjugates **1E** (9%) and **1G** (29%) derived from (2-aminoethyl)guanidine and tris(2-aminoethyl)-amine, respectively, which occurred at the expense of other conjugates. Conjugates **1B–D** were unchanged or moderately repressed (1 to −8%) whereas conjugates **1A** and **1F** were more strongly repressed (−33 and −29%, respectively).

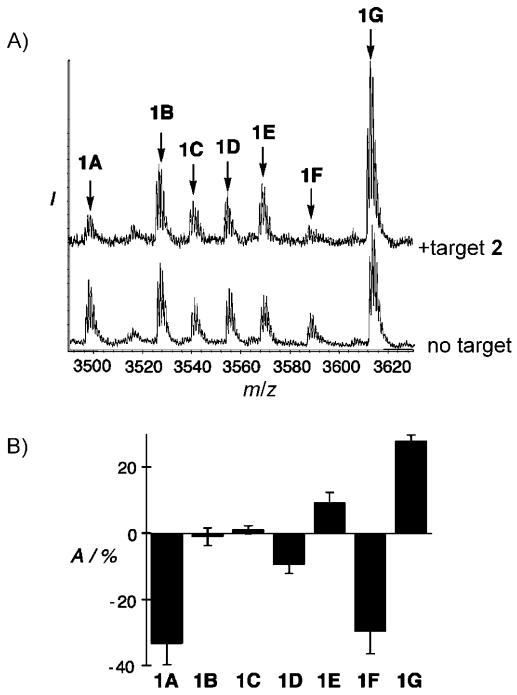


Figure 1. Expensions of the MALDI-Tof mass spectrum of A) untemplated and templated dynamic combinatorial library after reduction by NaBH$_3$CN and B) relative amplifications (as estimated from relative MS peak heights) of 2′-O-conjugated TFOs. Data shown represent the mean ± s.d. of three DCC experiments

This discrimination suggests that electrostatic interactions between positively charged 2′-O-appending chains at pH 6 and the polyanionic DNA target have mainly driven the selection. Moreover a terminal guanidinium group (in **1E**) contributes more to this process than a terminal ammonium group (in **1B**–**D**). In addition, when comparing data corresponding to conjugates **1B**–**D**, which are derived from diamines, a chain-length effect on selection is apparent that suggests a better fitting of conjugates derived from 1,2-diaminoethane and 1,3-diaminopropane over the one derived from putrescine. Next, to ascertain that the selection occurring during the DCC process was related to the thermodynamic stabilities of the triplexes formed with DNA target **2**, conjugates **1A**–**G** were separately re-synthesized and UV-monitored melting experiments were carried out.

Of the two transitions observed during the melting of each triplex, the one with the higher T_m value (around 72 °C), which was the same for all complexes, corresponded to the melting of the hairpin DNA target **2**. The one with lower T_m value corresponded to the dissociation of the third strand (Figure 2). In all cases, the presence of a 2′-*O* cationic chain in the third strand of the triple helices had a stabilizing effect compared to the starting triplex **1**+**2** which exhibited a T_m value (36.4 °C) similar to that of the triplex obtained with the all-2′-*O*-methyl version of **1**, **1Me** (U* = 2′-*O*-methyl-U (Scheme 1) and **2** (T_m = 36.3 °C). The strongest effects were observed with TFOs **1E** (ΔT_m = 10.6 °C) and **1G** (ΔT_m = 10.9 °C) in accordance with their amplifications. The 2′-*O* chain present in TFO **1G** resulted from conjugation with tris(2-aminoethyl)amine (pK_a values 8.56, 9.59, 10.29) and was the highest positively charged (+3 at pH ≤ 7) group submitted to the selection. The high ΔT_m that was observed reflected its capacity to interact with negatively charged target phosphates. Although, only two positive charges are expected on the 2′-*O* chain present in TFO **1E**, the presence of a planar and highly basic guanidinium group (pK_a = 12.5) capable of inducing directionality in H-bonding interactions might account for efficient stabilization of triplexes.[17] When considering the stabilizing effect of 2′-*O* linked diamines in **1B–D**, 1,3-diaminopropane was slightly more efficient than putrescine as reflected by a higher amplification of **1C** during the selection. In contrast, the lower stabilizing effect observed upon conjugation with 1,2-diaminoethane was at variance with the amplification ranking of **1B** and might be tentatively explained by the fact that although the selection operated on unstable imines, binding properties were measured with the corresponding reduced compounds **1A–G**. This could also account for the discrepancy observed between a sharp difference in amplification values for **1E** and **1G** (9 and 29%, respectively) and their close T_m values.[8c] Finally, conjugation with methylamine afforded the lowest stabilizing effect with a ΔT_m value consistent with previously reported data.[7a] In this latter case, replacing the methyl group by a 4-methylbenzyl group as in **1F** increased the stabilizing effect, suggesting that contributions to the stability of triplex other that electrostatic interactions and H-bonding might occur with 2′-O-appending groups.

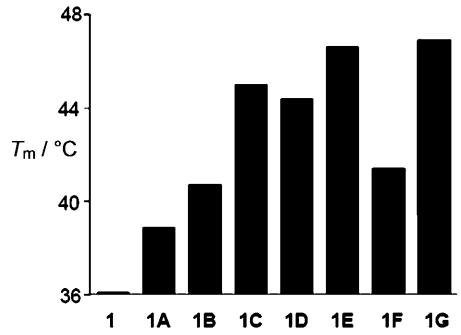


Figure 2. Triple-helix stabilizing effects obtained upon conjugation of TFO **1** with amines **A**–**G**. The T_m values (±0.5 °C) are averages of the values determined from three separate melting experiments by using a temperature increase of 0.4 °C.min^{-1}. Each strand concentration was 1 μM in 10 mM cacodylate buffer (pH 5.5) with 100 mM NaCl and 0.1 mM EDTA).

Binding properties of selected TFO **1G** were further studied. The stability at pH 5.5 of triplex **1G**+**2** containing a single tetramine chain at micromolar concentration was higher than that of triplex **1Me**+**2** in the presence of millimolar concentration of externally added tetramine **G** (T_m 44.7 °C). In contrast, the attachment of tetramine in **1G** had no effect on the stability of a duplex formed with complementary oligoribonucleotide (data not shown); this illustrates the target specificity of the selection that occurred during the DCC experiment. We also examined TFO **1G** binding with a mismatched triplet by inverting the AT base pair in the DNA target opposite to the tetramine-linked U. This produced an unstable U-TA in place of U-AT. A decrease in triplex T_m of 30.1 °C was observed that was higher than that obtained with **1Me** (24.9 °C; Table 1).

This is a clear demonstration that the increased thermal stability provided upon 2′-*O* conjugation of a TFO with tetramine **G** does not compromise its selectivity. The binding of TFO **1G** was tested at pH 7. Upon these conditions, deprotonation of cytosines is known to destabilize C-GC triplets. At neutral pH, triplex **1G**+**2** was still observed with a T_m value of 20.7 °C, whereas triplex **1**+**2** was no longer detected. The extra binding energy that resulted from interactions with a single 2′-O-appended tetramine was sufficient to allow the formation of the triple helix.

In conclusion, we have described the successful use of DCC for the rapid identification of cationic 2′-O-appended groups that increase TFO affinity. Remarkably, a good correlation of the most amplified conjugates with the best binders was found in spite of the fact that all conjugates subjected to se-

Table 1. Effects of pH and mismatch on triplex stability (T_m).[a]

5′ C G T C C T T X T C T T C T T
3′ G C A G G A A Y A G A A G T T

2 (XY = TA)
2inv (XY = AT)

TFO	pH 5.5	pH 7.0[b]
1Me[c]	36.3 (11.4)[d]	< 5
1G	47.2 (17.1)[d]	20.7

[a] The experiments were performed as reported in Figure 2. [b] Buffer pH 7.0: 10 mM cacodylate buffer (pH 7.0), 100 mM NaCl, 0.1 mM EDTA. [c] **1Me** (U* = 2′-*O*-methyl-U, Scheme 1) corresponds to the full 2′-*O*-methyl version of **1**. [d] Values in brackets correspond to T_m of triplexes formed with hairpin DNA target **2inv**.

lection were found to be better binders than unconjugated TFO. The selected tetramine linked to 2′-O position of a uridine within the TFO binds to AT base pair with high affinity and specificity, permitting stable triplex formation at pH 7. Increasing the number of 2′-*O*-(2-oxoethyl)nucleotides in the TFO and broadening the library of appended molecules with intercalating agents would allow identification of multi-modified TFOs that are able to form highly stable triplexes.

Experimental Section

DCC experiment: A mixture of amines **A** (250 mM), **B** (2 mM), **C** (15 mM), **D** (52,5 mM), **E** (8 mM), **F** (70 mM), and **G** (4 mM) was obtained from neutralized (pH 5–6, with diluted HCl) stock solutions in H_2O.[14] TFO **1** (0.23 mM) was incubated (1 min at 90 °C then 12 h at 4 °C) in the absence or in presence of DNA target **2** (0.23 mM) in cacodylate buffer (23 mM, pH 6.0, 85 μL), NaCl (23 mM), KCl (165 mM), and EDTA (0.12 mM). A mixture of amines (10 μL) was added and after 30 min at 25 °C a freshly prepared solution of $NaBH_3CN$ (100 mM, 5 μL) in H_2O was added. The resulting mixture was stirred for 24 h at 25 °C. Aliquots (50 μL) from reaction mixtures were drop-dialyzed (twice) over V series membranes (Millipore) against 0.1 M aqueous ammonium citrate and then analyzed on a MALDI-Tof mass spectrometer (Reflex III, Brucker) operated in the reflectron mode with a 20 kV acceleration voltage and a 23 kV reflector voltage. A mixture of oligonucleotides d(T12)-d(T18) (Sigma) was used for external calibration. The matrix used was a 4:1 mixture of 2,4,6-trihydroxyacetophenone (10 mg mL^{-1}) in EtOH and 100 mM aq ammonium citrate. Samples (0.8 μL, aq. oligonucleotides (~50 μM)/matrix 1:1) were spotted on a stainless steel target and air-dried before analysis. For each sample, a MALDI mass spectrum was acquired by accumulating the ion signals from 100 UV laser shots with a constant laser power.

Experimental details for synthesis of fully-protected 2′-*O*-(2,3-dihydroxypropyl)uridine 3′-*O* phosphoramidite, its incorporation into oligonucleotides, synthesis and characterization of conjugated TFOs, and UV-melting experiments are described in the Supporting Information.

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Keywords: amination · combinatorial chemistry · DNA recognition · oligonucleotides · self-assembly

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