Oligodeoxynucleotides Containing 2′-O-Modified Adenosine: Synthesis and Effects on Stability of DNA:RNA Duplexes

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ABSTRACT: Hybridization thermodynamics were compared for oligonucleotide sequences containing 2′-fluoro dA, 2′-O-methyl A, 2′-O-ethyl A, 2′-O-propyl A, 2′-O-butyl A, 2′-O-pentyl A, 2′-O-nonyl A, 2′-O-allyl A, and 2′-O-benzyl A in place of deoxyadenosine. Although the effect of 2′-modified adenosine on duplex stability is sequence dependent, a clear trend is apparent. For six sequences containing a few 2′-modified adenosines in a background of unmodified deoxynucleotides, the average ΔT_M per substitution ranged from +1.3 °C for 2′-fluoro dA to −2.0 °C for 2′-O-nonyl A. For the 2′-O-alkyl series, the average ΔT_M per substitution correlates well with size of the substituent; the order of stability is 2′-O-methyl A > 2′-O-ethyl A > 2′-O-propyl A > 2′-O-butyl A > 2′-O-pentyl A > 2′-O-nonyl A. This correlation also extends to 2′-fluoro dA, 2′-O-allyl A, and 2′-O-benzyl A if chain length is measured by number of carbon atoms. When examined in the background of 2′-O-methyl ribonucleotides, all 2′-modified adenosines with a substituent no larger than 2′-O-pentyl stabilized the duplex nearly 2 °C per substitution compared to unmodified dA. These thermodynamic results and CD spectra of modified and unmodified hybrids support a model of DNA:RNA hybrids in which the geometry is between that of B-form and A-form.

DNA:RNA hybrids are important in the priming step of DNA replication (Kornberg, 1980), reverse transcription of RNA viruses (Fields & Knippe, 1993), and termination of RNA transcription (Martin & Tinoco, 1980). The structure of these hybrids and forces determining hybrid stability are not well understood (Martin & Tinoco, 1980; Hall & McLaughlin, 1991). Chemical modification of the DNA strand can be used to probe both hybrid structure and the forces affecting hybrid stability. For example, a modification that does not affect duplex stability likely occupies an accessible site in the hybrid and does not interfere with stabilizing interactions. Chemical modifications to DNA have been directed throughout the molecule [reviewed by Uhlmann & Peyman (1990), Cook (1991), Cook (1993), Sanghvi & Cook (1993), and Sanghvi (1993)] including the 2′ position of deoxyribose (Inoue et al., 1985; Inoue et al., 1987; Iribarren et al., 1990; Guinosso et al., 1991; Kawasaki et al., 1993; Sproat & Lamond, 1993; Monia et al., 1993). We chose substitution at the 2′ position for this study because the nature of the 2′ substituent is the primary chemical difference between DNA and RNA and likely plays an important role in relative duplex stability. 2′-O-Alkyl modifications with various chain lengths can be useful for qualitative evaluation of the shape and size of the heteroduplex minor groove.

Effects of 2′ substitution on hybrid stability have been reported previously. Uniform 2′-O-methyl modification (Inoue et al., 1985; Inoue et al., 1987) or uniform 2′-fluoro substitution (Kawasaki et al., 1993) of the DNA strand increases the stability of DNA:RNA hybrids. Uniform 2′-O-allyl and 2′-O-butyl substitution also stabilizes hybrid duplexes, although no direct thermodynamic measurements were described (Iribarren et al., 1990; Sproat & Lamond, 1993). In contrast to the stabilizing effect of these small modifications in the 2′ position, uniform substitution with 2′-O-(3,3-dimethylallyl) ribonucleosides destroyed the ability of an oligonucleotide to bind its complementary sequence (Iribarren et al., 1990).

We set out to extend these studies to include other 2′ adenosine substitutions including 2′-O-$(CH_2)_nCH_3$ (where n = 0, 1, 2, 3, 4, or 8), 2′-O-allyl, 2′-O-benzyl, and 2′-deoxy-2′-fluoro (Scheme I). From one to five modified adenosines containing each of the above substitutions were incorporated into six different oligodeoxynucleotide sequences, and the effect on hybridization to a complementary RNA oligonucleotide was measured. A clear correlation between size of the substituent and its effect on stability of the DNA:RNA hybrid was observed, with small substituents stabilizing and large substituents destabilizing the duplex.

MATERIALS AND METHODS

2′-O-Alkyladenosines. The unprotected requisite 2′-O-alkyladenosine nucleosides were obtained via direct alkylation of unprotected adenosine with alkyl halides in the presence of sodium hydride and dimethylformamide (C. J. Guinosso et al., manuscript in preparation).

2′-O-Alkyl-N6-benzoyladenosine. 2′-O-Alkyladenosines (10 mmol) were silylated with TMSCl[1] in pyridine (Scheme I) and then treated with benzoyl chloride in a manner similar to the transient acylation procedure of Gaffney and Jones (1982). The reaction mixtures were evaporated to dryness *in vacuo*, and the residue was partitioned between water/ethyl acetate. The dried ($MgSO_4$) organic layer was evaporated *in vacuo*, and the resulting residue was chromatographed on

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[1] Abbreviations: A, adenosine; dA, 2′-deoxyadenosine; BzCl, benzoyl chloride; CD, circular dichroism; CEO(Cl)P(diisopropylamine), 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite; DMT, dimethoxytrityl; TEA, triethylamine; HPLC, high-performance liquid chromatography; TMSCl, trimethylsilyl chloride.

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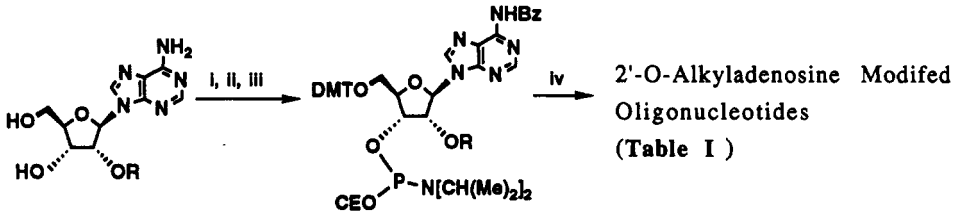
Scheme I: Synthesis of Modified Oligodeoxynucleotides[a]



R = Methyl, Ethyl, Propyl, Butyl, Pentyl, Nonyl, Allyl, Benzyl

[a] (i) TMSCl/Py, BzCl; (ii) DMTCl/Py; (iii) CEO(Cl)P(diisopropylamine)/Py/TEA, (iv) DNA synthesizer.

silica gel (Baker 40 mm, 200 g). The column was eluted with ethyl acetate/methanol (10/1), and the fractions containing pure material were pooled and evaporated *in vacuo* to afford the desired *N6*-benzoyl-protected nucleosides as foams in 50–95% yield. All benzoylated adenosines exhibited satisfactory ^{1}H NMR and elemental analysis data.

2'-O-Alkyl-5'-O-(4',4-dimethoxytrityl)-N6-benzoyladenosine. To a solution of 2'-*O*-alkyl-*N6*-benzoyladenosine (10 mmol) in pyridine (250 mL) was added 4, 4'-dimethoxytrityl chloride (11 mmol) (Scheme I). The reaction mixture was stirred at ambient temperature for 16 h and then added to a mixture of ice/water/ethyl acetate. The organic layer was separated, dried ($MgSO_4$), and concentrated *in vacuo* to a gum. The crude 5'-*O*-(dimethoxytrityl) nucleosides were chromatographed on silica gel (Baker 40 mm, 200 g) and eluted with ethyl acetate/hexane/triethylamine (90/10/1). The pure fractions were pooled and evaporated *in vacuo* to afford colorless foams of the desired materials in 75–85% yield. All DMT-protected adenosines exhibited satisfactory ^{1}H NMR and elemental analysis data.

N6-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-alkyladenosine-3'-O-(N,N-diisopropyl)-β-cyanoethylphosphoramidite. 5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-alkyl-*N6*-benzoyladenosine (3.2 mmol) in anhydrous THF (30 mL) and diisopropylamine (2.5 mL, 14 mmol) was treated with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (2.5 mL, 12 mmol) in a manner similar to the procedure of Seela and Kehne (1987). The reaction was stirred at ambient temperature for 10 h and concentrated to a gum *in vacuo*. The residue was partitioned between methylene chloride and water, separated, dried (Na_2SO_4), and concentrated. Chromatography on silica gel (Baker, 40 mm) with ethyl acetate/hexane/triethylamine (90/10/1) afforded the title compound in 70–80% yield. ^{31}P and ^{1}H NMR and TLC analyses of the resulting foams indicated that the structures were correct and of high purity.

Oligonucleotides. Oligonucleotides containing 2'-*O*-substituted adenosines were synthesized using an Applied Biosystems 380B automated DNA synthesizer and 5'-*O*-(dimethoxytrityl)-3'-*O*-phosphoramidites. For coupling steps involving modified amidites, the standard synthesis cycle was adjusted by increasing the wait step after the pulse delivery of tetrazole to 360 s. Coupling yields for incorporation of 2'-*O*-alkyladenosine were 95% or greater. The oligonucleotides were deprotected at least 16 h at 55 °C in concentrated NH_4OH. Modified oligonucleotides were purified by trityl on reverse-phase HPLC followed by trityl removal and ethanol precipitation. Oligonucleotides were analyzed on a 20% polyacrylamide denaturing gel (Ausubel et al., 1987) and demonstrated to be at least 90% full length.

Synthesis, purification, and characterization of oligoribonucleotides, 2'-*O*-methyl oligoribonucleotides, and oligodeoxyribonucleotides containing 2'-fluoro dA have been described (Chiang et al., 1991; Monia et al., 1992; Kawasaki et al., 1993).

Nucleoside Composition of Oligonucleotides. Nucleoside composition was evaluated by enzymatic degradation and dephosphorylation to the corresponding nucleosides as described previously (Kawasaki et al., 1993), except that digestion reactions were incubated at 37 °C for 24 h. HPLC analysis of the digest was performed as described previously (Kawasaki et al., 1993) for 2'-fluoro-dA nucleosides, except the final gradient from 20 to 60 m was 10% B to 90% B. Relative nucleoside ratios were calculated by converting integrated areas to molar values and comparing all values to cytidine, which was set at its expected value for each oligomer. Extinction coefficients for 2'-*O*-methyl A, 2'-*O*-pentyl A, and 2'-*O*-nonyl A are the same as that reported for dA within experimental error (Dr. P. Davis, personal communication). Therefore, extinction coefficients for all 2'-*O*-modified adenosines and 2'-fluoro dA were assumed to be the same as for adenosine.

Determination of Hybridization Thermodynamics. Absorbance vs temperature curves were obtained as described previously (Monia et al., 1992). A modified oligodeoxynucleotide and its complementary oligoribonucleotide target were combined at 4 μM each strand in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7. Oligonucleotide concentrations were obtained from the absorbance at high temperatures, and extinction coefficients were calculated according to the method of Puglisi and Tinoco (1989). Extinction coefficients for 2'-substituted oligodeoxynucleotides were assumed to be identical to those for unmodified analogs. We felt that this assumption was justified by the observation that the extinction coefficient of dA was unaffected by 2'-*O*-alkyl substitution. T_Ms and free energies of duplex formation were obtained as described previously (Freier et al., 1983; Monia et al., 1992).

Average ΔT_M per substitution for a modification was calculated by summing the ΔT_M values for all oligonucleotides containing that modification and dividing by the total number of substitutions. Averages calculated by this method weigh each oligonucleotide by the number of substitutions it contains.

Circular Dichroism Spectra. CD spectra were recorded in a JASCO J-600 spectropolarimeter. Solutions were identical to those used for the melting curves, except that oligonucleotide concentrations were 3 μM each strand. For each oligonucleotide pair, sample temperature was 40 °C below the T_M reported in Table II. Ellipticities were converted to $\Delta\epsilon$ and are reported per mole residue.

RESULTS

The required 5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-alkyladenosine-3'-*O*-(*N,N*-diisopropyl)-β-cyanoethylphosphoramid-

Table I: Sequences for Evaluation of Hybridization of Oligonucleotides Containing 2′-Modified Adenosine

sequence no.	sequence[a]
1772	aCC GaG GaT CaT GTC GTa CGC
1592	GGa CCG Gaa GGT aCG aG
1732	CGA CTA TGC AAa aaC
1343	CGA CTA TGC AAG TaC
1589	CTC GTA CCa TTC CGG TCC
2256	GaG CTC CCa GGC
3287	G′aG′ C′U′C′ C′C′a G′G′C

[a] a = 2′-substituted A; A, C, T, G = 2′ deoxyribonucleotide; C′, U′, G′ = 2′-*O*-methyl ribonucleotide

Table II: T_Ms (°C) of Oligonucleotides Containing 2′-Substituted Adenosine[a]

	sequence no.						
modification	1772	1592	1732	1343	1589	2256	3287
dA	63.2	57.0	39.0	45.1	63.7	59.9	72.7
2′-*O*-methyl A	63.1	60.8	41.8	45.4	63.7	61.2	77.0
2′-*O*-ethyl A	62.3	59.5	42.1	44.6	63.0	60.8	76.9
2′-*O*-propyl A	61.4	58.2	41.5	44.1	62.6	59.7	76.7
2′-*O*-butyl A	60.4	56.8	40.5	44.1	61.8	58.8	76.3
2′-*O*-pentyl A	58.6	54.1	39.2	43.6	62.0	58.0	76.6
2′-*O*-nonyl A	52.6	43.2	41.5	42.5	60.5	53.8	72.5
2′-*O*-allyl A	61.3	58.6	40.8	44.1	62.2	60.6	76.8
2′-*O*-benzyl A	56.2	50.0	37.7	43.4	60.9	55.8	73.2
2′-fluoro dA	67.7	65.8	43.2	46.4	64.1	64.6	79.0

[a] T_Ms were measured vs RNA complements in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7 at 4 μM strand concentration.

ites were obtained in good yields following standard procedures. Purity and identity of these materials were confirmed by elemental, TLC, and ^1H and ^{31}P NMR analyses.

Every 2′-*O*-alkyladenosine studied was incorporated into each of the seven oligonucleotides listed in Table I. The oligonucleotides range in length from 12 to 21, and the number of substitutions ranges from 1 to 5. For most sequences, the modified A's occur randomly throughout the sequence; 1732, however, contains a "cap" of three consecutive substitutions near the 3′ end. The seventh sequence, 3287, differs from the first six in that the nucleosides other than A are 2′-*O*-methyl-substituted ribonucleosides rather than 2′-deoxyribonucleosides.

Nucleoside composition of 2′-*O*-substituted adenosine containing oligonucleotides was examined in sequence 1732 (Table I). All modified nucleosides were detected in the digestion products, indicating that modified adenosines were successfully incorporated into the oligomers. The relative ratio of nucleosides in each oligomer was in agreement with the expected ratios for all modifications except 2′-*O*-nonyladenosine. 2′-*O*-Nonyladenosine is sparingly soluble in aqueous solutions, and only a fraction of the expected nucleoside was detected. Polyacrylamide gel electrophoresis and anion exchange HPLC of the 2′-*O*-nonyl A-substituted oligonucleotides were consistent with full-length oligomers containing 2′-*O*-nonyladenosine.

Hybridization of each modified oligonucleotide to its RNA complement was evaluated spectroscopically. Typical melting curves are shown in Figure 1, and melting temperatures are listed in Table II. ΔT_M is the increase or decrease in T_M caused by substitution of a modified adenosine for an unmodified dA. Figure 2 plots ΔT_M per substitution for the first six sequences in Table I. These sequences contain a few 2′-modified adenosines in a background of 2′ deoxynucleotides.

Figure 2 shows the thermodynamic effect of each modification depends on sequence. For example, ΔT_M per

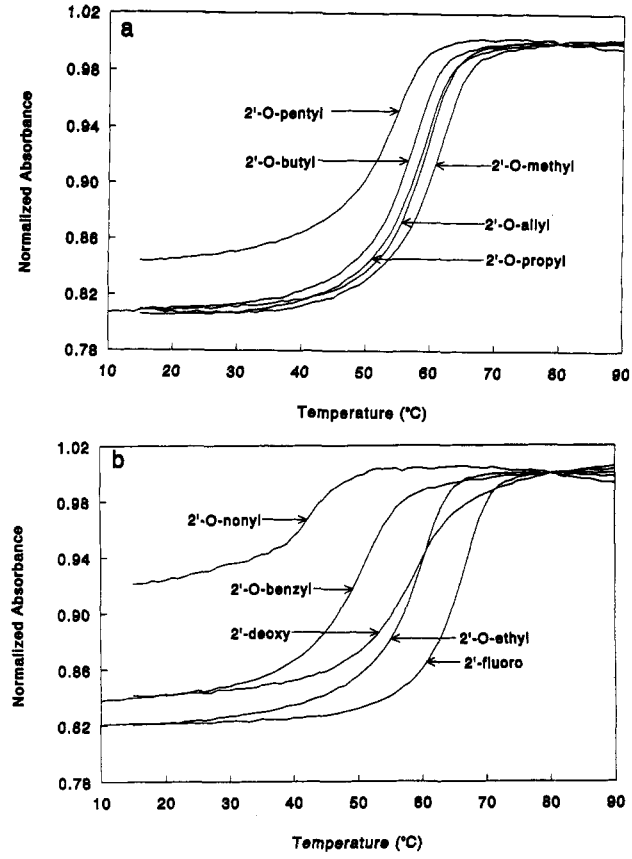


FIGURE 1: Absorbance vs temperature profiles for 1592 (GGaC-CGGaaGGTaCGaG) containing 2′-modified A at the indicated positions hybridizing to complementary RNA. (a) 2′-*O*-Pentyl A, 2′-*O*-butyl A, 2′-*O*-methyl A, 2′-*O*-allyl A, and 2′-*O*-propyl A. (b) 2′-*O*-Nonyl A, 2′-*O*-benzyl A, 2′-deoxy A, 2′-*O*-ethyl A, and 2′-fluoro dA. Absorbance was normalized at 80 °C.

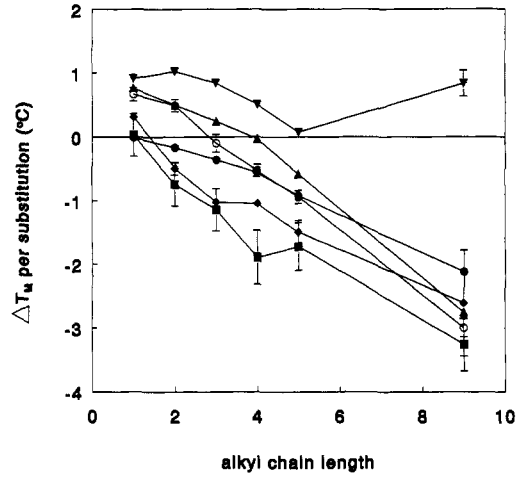


FIGURE 2: (a) ΔT_M per substitution as a function of alkyl chain length for six oligonucleotide sequences containing 2′-*O*-methyl, 2′-*O*-ethyl, 2′-*O*-propyl, 2′-*O*-butyl, 2′-*O*-pentyl, and 2′-*O*-nonyl A substitutions. ●, 1772; ▲, 1592; ▼, 1732, ◆, 1343; ■, 1589; O, 2256. Sequences are listed in Table I. The unmodified control from which ΔT_M is calculated is the fully 2′-deoxy DNA (row 1 of Table II).

substitution for 2′-*O*-ethyl A ranges from –0.7 °C in 1589 to +1.0 °C in 1732. For almost every modification, ΔT_M per substitution is most negative in 1343 and 1589 which contain a single substitution, suggesting that the first 2′ substitution made in an oligonucleotide has a more destabilizing effect than subsequent substitutions. On the other hand, three modifications in a row near the 3′ end of 1732 are the most stabilizing. The increased stabilization may be due to the

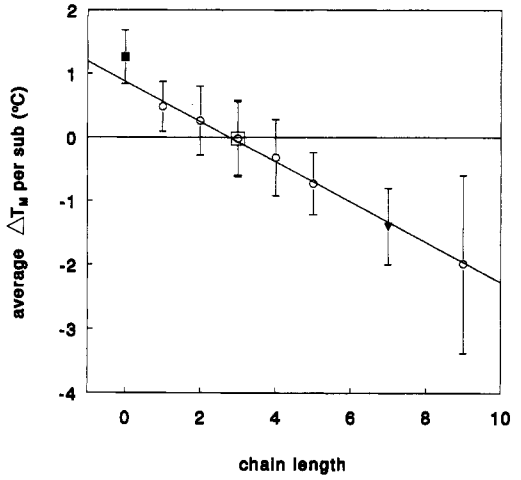


FIGURE 3: ΔT_M per substitution averaged over the six sequences in Figure 2 plotted vs the number of carbon atoms in the modification. O, 2′-*O*-$(CH_2)_nCH_3$ (where n = 0, 1, 2, 3, 4, 8); □, 2′-*O*-allyl; ▼, 2′-*O*-benzyl; ■, 2′-fluoro. The solid line is a best fit for the six substitutions with saturated, linear hydrocarbons.

fact that in this sequence substitutions are consecutive.

In addition to the sequence effects, it is clear that small 2′ substitutions stabilize the duplex and larger 2′ substitutions destabilize the duplex in all sequences except 1732. To better evaluate this effect, ΔT_M per substitution was averaged over the six sequences in Figure 2 and is plotted in Figure 3. The error bars in Figure 3 are not experimental error but are due to the variable sequence effects noted above. In spite of this variability, the average ΔT_M per modification lies on a line with 2′-fluoro and 2′-*O*-methyl modifications stabilizing the duplex and 2′-*O*-benzyl and 2′-*O*-nonyl modifications destabilizing the duplex. The "break-even" point is 2′-*O*-propyl. Averaged over these six sequences, point substitutions of 2′-*O*-propyl A for dA have no effect on duplex stability. Substitutions larger than 2′-*O*-propyl usually destabilize the duplex, and substitutions smaller than 2′-*O*-propyl typically stabilize the duplex.

The sequence dependence of ΔT_M per modification is due to two factors. Firstly, ΔT_M per modification is not a thermodynamic parameter. Even if $\Delta\Delta G°_{37}$ per modification is characteristic only of the modification and is independent of sequence, ΔT_M per modification is expected to depend upon $\Delta H°$ for the particular oligonucleotide studied as well as $\Delta\Delta G°_{37}$ for the modification (Freier et al., 1992). Although ΔT_M is not independent of sequence, it is experimentally more accurate than $\Delta\Delta G°_{37}$ (Freier et al., 1992). Over the range of oligonucleotides studied, ΔT_M is only weakly dependent on oligonucleotide, so we used ΔT_M rather than $\Delta\Delta G°_{37}$ for our structure activity analysis. Plots with $\Delta\Delta G°_{37}$ are similar to Figures 2 and 3, but error bars are larger. The second, and more important, cause of the sequence dependence of ΔT_M per modification is a true sequence dependence of $\Delta\Delta G°_{37}$ per modification. This is likely due to a nearest neighbor effect and is a well-known phenomenon in DNA:DNA and RNA:RNA duplexes (Breslauer et al., 1986; Freier et al., 1986; Kierzek et al., 1986). The effect is sufficiently large that, although the *average* ΔT_M per modification is substantially more positive for 2′-*O*-methyl A than for 2′-*O*-butyl A (+0.5 °C for 2′-*O*-methyl A compared to −0.6 °C for 2′-*O*-butyl A), 2′-*O*-butyl A in 1732 is actually more stabilizing than 2′-*O*-methyl A in 1589. This example merely points out the importance of using the same sequences when comparing thermodynamic effects of various modifications. The averages in Figure 3 were obtained using the same six sequences for each modification and therefore minimize these sequence effects.

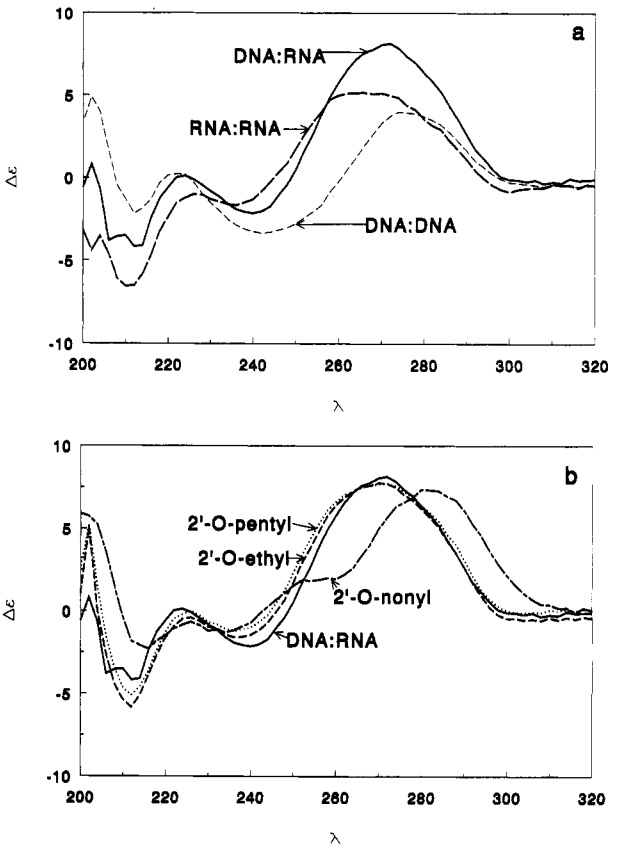


FIGURE 4: Circular dichroism of duplexes of 2′-*O*-modified analogs of 1592 hybridized to RNA. (a) —, DNA·RNA; - - -, DNA·DNA; — — —, RNA·RNA. (b) —, DNA·RNA; — – —, 2′-*O*-nonyl-substituted 1592·RNA; - · -, 2′-*O*-pentyl-substituted 1592·RNA; · · ·, 2′-*O*-ethyl-substituted 1592·RNA.

To determine if thermodynamic effects of these modifications are due to changes in duplex structure, circular dichroism spectra were measured on hybrids of 1592 or its 2′-modified analogs with RNA as well as DNA:DNA, DNA:RNA, and RNA:RNA controls (Figure 4). The DNA:DNA duplex shows a conservative CD spectrum characteristic of B-form helices. The CD of the RNA:RNA duplex is typical of A-form helices, with a positive band near 262 nm and a strong negative band at 210 nm. The maximum of the positive band in the CD spectrum of the DNA:RNA hybrid is at 270 nm, between the maxima of DNA:DNA and RNA:RNA, and, at wavelengths below 250 nm, the ellipticity of the DNA:RNA hybrid is between those of DNA:DNA and RNA:RNA duplexes. These observations suggest that the geometry of the hybrid is intermediate between B-form and A-form.

Figure 4 plots CD spectra of 1592 analogs with five 2′-*O*-ethyl A or five 2′-*O*-pentyl A substitutions. Spectra for 1592 substituted with five substitutions of 2′ fluoro dA, 2′-*O*-methyl A, 2′-*O*-propyl A, 2′-*O*-allyl A, 2′-*O*-butyl A or 2′-*O*-benzyl A were indistinguishable from those reported for 2′-*O*-ethyl A and 2′-*O*-pentyl A (data not shown). Substitution with these 2′ modifications in 1592 does not substantially affect the CD spectrum; position and intensity of the positive band at 268 nm is similar in modified and unmodified heteroduplexes. At wavelengths below 260 nm, spectra of 2′-*O*-modified hybrids fall between those of DNA:RNA and RNA:RNA, suggesting that conformations of 2′-*O*-modified hybrids may be more like A-form than the unmodified hybrid but not as fully A-form as RNA:RNA. The duplex with

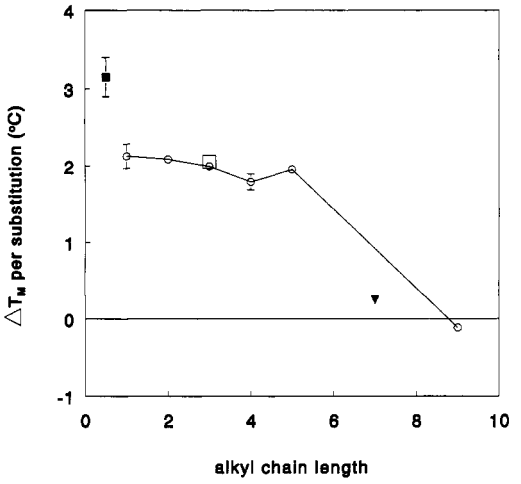


FIGURE 5: ΔT_M per substitution for 3287 analogs plotted vs the number of carbon atoms in the modification. ○, 2′-*O*-$(CH_2)_nCH_3$ where n = 0,1,2,3,4,8; □, 2′-*O*-allyl; ▼, 2′-*O*-benzyl; ■, 2′-fluoro. All of the oligonucleotides contain 2′-*O*-methyl residues in positions 1, 3–8, and 10–11. The unmodified control from which ΔT_M is calculated contains 2′-dA in positions 2 and 9 and 2′-*O*-methyl nucleosides in positions 1, 3–8, and 10–11.

2′-*O*-nonyl-substituted 1592, whose T_M is 14 °C below that of the unmodified duplex, generates a very unique spectrum, suggesting that its conformation is substantially different from that of the other duplexes. Although CD spectra were measured at a temperature well below the T_M of the 2-*O*-nonyl-substituted hybrid, the shape of the spectrum and positions of the bands are suggestive of a melted duplex (Gray et al., 1981).

One objective of this research was to use oligonucleotides containing only a few 2′-*O*-modified A's and unmodified dC, dG, and dT to predict the thermodynamic effect of uniformly 2′-*O*-modified oligonucleotides. This would allow us to evaluate hybridization properties of uniformly modified oligonucleotides without synthesis of modified C, G, and T amidites. An oligonucleotide containing isolated 2′-*O* substitutions separated by 2′ deoxy tracts might not behave like a uniformly 2′-*O*-modified oligonucleotide, however. This concern was supported by the observation that 2′ modifications are less destabilizing in 1732 with three consecutive substitutions than in other sequences with substitutions separated by unmodified residues. To reduce the effect of junctions between 2′-*O*-substituted A residues and 2′-deoxy residues, the 3287 12-mer was synthesized with 2′-*O*-modified A in positions 2 and 9 and 2′-*O*-methyl ribonucleotides in positions 1, 3–8, and 10–11. Figure 5 plots the effect of 2′-*O* A substitutions in the background of a 2′-*O*-methyl oligoribonucleotide on T_M of the hybrid. In the background of a 2′-*O*-methyl oligoribonucleotide, 2′-*O*-ethyl A, 2′-*O*-propyl A, 2′-*O*-allyl A, 2′-*O*-butyl A, and 2′-*O*-pentyl A all stabilize the duplex as much as 2′-*O*-methyl A. 2′-*O*-Benzyl A and 2′-*O*-nonyl A, however, do not stabilize this duplex relative to unmodified dA.

DISCUSSION

A clear structure–activity relationship is evident in Figure 3 in that duplex stability is correlated with substituent size. The effect of 2′-*O*-alkyl-substituted A on DNA:RNA duplex stability is linearly related to length of the alkyl chain; longer substituents are less stabilizing than shorter ones. The line in Figure 3 is a fit of only the six 2′-*O*-alkyl substitutions. Data for 2′-fluoro dA, 2′-*O*-allyl A, and 2′-*O*-benzyl A were added to the plot using number of carbon atoms to calculate "chain length". Results for 2′-*O*-allyl A and 2′-*O*-benzyl A fall on the line for linear hydrocarbon substitutions. 2′-Fluoro dA is even more stabilizing than the line in Figure 3 predicts. It has been suggested (Kawasaki et al., 1993; Olson, 1982) that the stabilization afforded by 2′-fluoro substitutions could be due to the high electronegativity of fluorine, which shifts the sugar equilibrium toward 3′-endo (Uesugi et al., 1979; Guschlbauer & Jankowski, 1980; Ikehara, 1984). Small, electronegative fluorine can act as a hydrogen-bond acceptor (Doddrell et al., 1969; Marler & Hopkins, 1970; Joesten, 1982; Murray-Rust et al., 1983; Karipides & Miller, 1984) and may stabilize interactions of the hybrid with water.

Previous investigators also found that 2′-*O*-methyl and 2′-fluoro substitution caused an increase in T_M of DNA:RNA hybrids (Inoue et al., 1985; Inoue et al., 1987; Kawasaki et al., 1993). Results reported above cannot be compared quantitatively to these earlier reports as different sequences were included in the two studies. In addition, earlier investigations evaluated uniformly modified oligonucleotides, and compounds in this study are modified only at adenosine. Conclusions from the studies, however, are identical. When hybridizing to complementary RNA, the order of stability is 2′-fluoro > 2′-*O*-methyl > 2′-H.

Uniformly substituted 2′-*O*-allyl-, 2′-*O*-(3,3-dimethylallyl)-, and 2′-*O*-butyl-substituted DNAs have been synthesized and evaluated as probes in Northern blots (Iribarren et al., 1990; Sproat & Lamond, 1993). Uniformly modified 2′-*O*-allyl RNA or 2′-*O*-butyl RNA was able to hybridize to RNA complement (Sproat & Lamond, 1993), but uniform substitution with 2′-*O*-(3,3-dimethylallyl) totally disrupted hybrid formation (Iribarren et al., 1990). In the present work, substitution with 2′-*O*-pentyl A did not destroy hybrid formation, suggesting that the branched 2′-*O*-(3,3-dimethylallyl) substitution is more disruptive to helix formation than the linear 5-carbon substitution.

Typically, DNA:RNA hybrids are less stable than RNA:RNA duplexes (Martin & Tinoco, 1980; Hall & McLaughlin, 1991), and for all the sequences in Table I, T_Ms of the DNA:RNA heteroduplexes are less than those of the RNA:RNA duplexes (data not shown). One way to improve hybridization properties of antisense DNA oligonucleotides to RNA targets is to attach electronegative substitutions at the 2′ position, which shifts the sugar conformational equilibrium toward 3′-endo (Uesugi et al., 1979; Guschlbauer & Jankowski, 1980; Ikehara, 1984; Kawasaki et al., 1993). Shifting the conformation of the DNA strand toward 3′-endo will put it in a more RNA-like conformation and may cause the hybridization properties to be more like those of RNA:RNA. Our results confirm that small 2′ substitutions with high electronegativity like 2′-fluoro dA or 2′-*O*-methyl A stabilize the duplex, and this stabilization is correlated with more A-like duplex geometry.

Recent NMR and Raman data (Chou et al., 1989; Katahira et al., 1990) demonstrate that DNA and RNA strands in oligonucleotide heteroduplexes in solution adopt intrinsic B- and A-type conformations, respectively. The sugar conformation of the DNA strand, however, deviates slightly from 2′-endo. CD spectra of the 1592 hybrid and other sequences (data not shown) also imply that the geometry of oligonucleotide DNA:RNA heteroduplexes is between that of B- and A-form, although the degree of conversion to A-form depends on sequence (E. A. Lesnik et al., manuscript in preparation).

Three distinct CD experiments suggest that the intermediate geometry of a DNA:RNA heteroduplex shifts toward A-form as small 2′ substitutions are added. Firstly, CD spectra of

1592 (Figure 4) analogs reveal that substitution with five small 2′-*O*-alkyl substitutions shifts the heteroduplex geometry slightly toward A-type. Secondly, increasing numbers of small 2′ substitutions in a DNA oligonucleotide cause the duplex to become increasingly A-form. Hybrids of a series of 1343 analogs containing 0, 3, 5, 8, 11, or 14 2′-fluoro substitutions exhibit CD spectra that are increasingly like that of the RNA:RNA duplex (E. A. Lesnik & A. M. Kawasaki, unpublished). Thirdly, uniform substitution with 2′-fluoro, 2′-*O*-methyl, 2′-*O*-propyl, or 2′-*O*-pentyl in the 1592 sequence causes complete conversion to A-form; CD spectra of the hybrids are identical to that of RNA:RNA (E. A. Lesnik et al., manuscript in preparation). This shift toward a more A-like structure is consistent with the tendency of nucleosides containing electronegative 2′ substitutions to adopt a 3′-endo conformation (Uesugi et al., 1979; Guschlbauer & Jankowski, 1980; Ikehara, 1984).

Elongation of the alkyl chain on 2′-*O*-substitutions results in reduced duplex stability. Electronegativity of 2′-*O*-alkyl substitutions changes insignificantly with alkyl chain length and, consequently, cannot be the source of decreased stability. Perhaps larger substitutions cause local distortions in the helix, contributing to reduced base stacking energy. The model suggested by CD spectra and thermodynamic data shows that the effect of 2′-*O*-alkyl modifications on heteroduplex stability is the result of two opposing factors: (1) the stabilizing effect of electronegative 2′ substitutions which impart some additional A-like character to the hybrid duplex and (2) the destabilizing effect of local disordered structure at the site of insertion of large alkyl groups in the minor groove of heteroduplexes. CD spectra, however, detect a substantial change in helix geometry of the 1592 heteroduplex only with 2′-*O*-nonyl substitution (Figure 4). We cannot say whether these changes in CD spectra reflect small changes in geometry of the whole duplex or local conformational reorganization at the points of modification. Nevertheless, it is evident that insertion of five very long 2′-*O*-nonyl substitutions in the minor groove of the heteroduplex results in large-scale steric distortion of the structure, which is likely the major cause of the extreme destabilization observed for this modification.

Destabilization by 2′-*O*-alkyl substitutions may also be caused by local restructuring of water around the alkyl group. The effect of alkyl groups on local water structure is responsible for similar behavior of hydrophobic polyelectrolytes (Dubin & Strauss, 1970; Strauss, 1989) and may also play a role in destabilization of hybrid duplexes by large 2′-*O*-alkyl substitutions.

The proposed model explains the different effect of 2′ substitutions incorporated in the same sequence in a background of 2′ deoxynucleotides (2256 analogs) and in a background of 2′-*O*-methyl ribonucleotides (3287 analogs). In the first case, duplex stability depends linearly on substituent size (Figure 3), whereas in a 2′-*O*-methyl oligoribonucleotide background, all 2′-*O*-modifications except for 2′-*O*-benzyl A and 2′-*O*-nonyl A stabilize the duplex to an equal extent (Figure 5). The different effects of 2′-modifications in different backgrounds can be explained by the different geometry of heteroduplexes with 2′-deoxy or 2′-*O*-methyl strands. Arnott et al. (1986) described X-ray data of poly(dA)·poly(rU) and poly(dI)·poly(rC) fibers and reported that some parameters of hybrid helices are characteristic of A- or B-form and others are intermediate between A- and B-helices. In particular, the minor groove of DNA:RNA hybrids is narrower and much deeper than that in A-RNA. Apparently, increasingly large 2′-alkyl substitutions are accommodated in the minor groove of a hybrid duplex with increasing difficulty, thus resulting in decreasing duplex stability. On the other hand, heteroduplexes formed by uniform 2′-*O*-methyl oligoribonucleotides and their RNA complements exhibit CD spectra that coincide with spectra of RNA:RNA duplexes, demonstrating 2′-*O*-methyl:RNA heteroduplexes exist in an A-conformation (Kawasaki et al., 1993). Apparently, the wide, shallow minor groove of the A-form 2′-*O*-methyl:RNA duplex can accommodate 2′-O substitutions as large as pentyl without affecting hybrid stability.

The stabilization by small substitutions such as 2′-fluoro and 2′-*O*-methyl inserted into a 2′-*O*-methyl oligoribonucleotide strand is 1.5–2 times greater than the stabilization caused by the same substitutions in an unmodified DNA strand. This may also be the result of different conformations of modified and unmodified oligonucleotide heteroduplexes. Perhaps isolated 2′-fluoro dA or 2′-*O*-alkyl A residues incorporated in 2′-deoxy tracts adopt an intermediate conformation and only weakly shift the geometry of the heteroduplex to A-form. Such an intermediate conformation was observed for DNA and RNA residues at RNA–DNA and DNA–RNA junctions of a chimeric DNA–RNA–DNA duplex (Chou et al., 1991). On the other hand, 2′-fluoro dA or 2′-*O*-alkyl A inserted in an A-like strand can completely adopt an A-like conformation and thus stabilize the duplex more effectively.

Compared to the effect of 2′-substitutions at adenosine on the stability of DNA:RNA hybrids, the same substitutions are more destabilizing in DNA:DNA duplexes (data not shown). This effect has been reported previously for uniformly substituted 2′-*O*-methyl oligonucleotides (Inoue et al., 1985; Inoue et al., 1987) and is likely due to the tendency of 2′-*O*-substituted nucleosides to adopt a 3′-endo geometry inconsistent with B-form DNA:DNA geometry.

It should be noted the behavior of the 1732 sequence does not fit the model well. All 2′-*O*-alkyl substitutions including 2′-*O*-nonyl stabilize this heteroduplex. The 1732 sequence is unique in that it contains five consecutive dA residues, resulting in a $(dA)_5$·$(rU)_5$ tract at the 3′ end of the heteroduplex (Table I). It is known that oligomeric (dA·rU) regions are particularly unstable due the tendency of dA stretches to maintain the B-conformation (Martin & Tinoco, 1980). This may be correlated with the observation that four consecutive A/T residues facilitate formation of a water spine in the minor groove of B-DNA, stabilizing the duplex (Drew & Dickerson, 1981). It is unknown if the minor groove is hydrated to the same extent in hybrid duplexes. We can only speculate that the stabilizing effect of large 2′-*O*-alkyl substitutions in 1732 is due to shift of the sugar conformation toward 3′-endo and, in addition, is due to displacement of bound water by hydrophobic 2′-*O*-alkyl groups in the minor groove. Perhaps dehydration promotes more complete conversion of the (dA_5)·(rU_5) tract to an A-like conformation and overrides the destabilizing effect of local structure deformation arising at the insertion of large alkyl substitutions.

Finally, the 2′-*O*-sugar position has been proposed to be a potentially useful site for conjugation of pendent groups to nucleic acids (Cook, 1991; Manoharan et al., 1991). Modification at this position places the substituent in the minor groove of a DNA:RNA helix and may allow design of antisense oligonucleotides with pendent groups to initiate cleavage of target RNA (Cook, 1991). The present study suggests that small groups in this position will enhance hybridization to RNA; larger groups may be tolerated less well. These predictions, however, are based on data for only nine modifications, most of which are linear hydrocarbons. Pendent

substituents are likely to be more polar or more polarizable than alkoxy substitutions. They may be charged or may contain moieties (e.g., H-bond donors, intercalators) which can themselves bind to the duplex. All of these features may be more important than substituent size in the effect of 2′-O-pendent modifications on hybridization thermodynamics.

In summary, we have observed a clear correlation between substituent size and duplex stability for the effect of 2′-substituted A on stability of a DNA:RNA duplex. Small substituents stabilize the duplex, whereas large substituents destabilize the duplex. We explain this result as a combination of two effects. Electronegative substitution at the 2′ position stabilizes the duplex by shifting the geometry to a more A-like conformation. Bulk at the 2′ position, however, cannot be accommodated in the minor groove of a hybrid duplex and causes duplex destabilization. We look forward to extending this study to include other types of substituents at the 2′ position as well as uniformly modified oligonucleotides.

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