Duplex stabilities of phosphorothioate, methylphosphonate, and RNA analogs of two DNA 14-mers

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ABSTRACT

The duplex stabilities of various phosphorothioate, methylphosphonate, RNA and 2'-OCH_3 RNA analogs of two self-complementary DNA 14-mers are compared. Phosphorothioate and/or methylphosphonate analogs of the two sequences d(TAATTAATTAATTA) [D1] and d(TAGCTAATTAGCTA) [D2] differ in the number, position, or chirality (at the 5' terminal linkage) of the modified phosphates. Phosphorothioate derivatives of D1 are found to be less destabilized when the linkage modified is between adenines rather than between thymines. Surprisingly, no base sequence effect on duplex stabilization is observed for any methylphosphonate derivatives of D1 or D2. Highly modified phosphorothioates or methylphosphonates are less stable than their partially modified counterparts which are less stable than the unmodified parent compounds. The 'normal' (2'-OH) RNA analog of duplex D1 is slightly destabilized, whereas the 2'-OCH_3 RNA derivative is significantly stabilized relative to the unmodified DNA. For the D1 sequence, at approximately physiological salt concentration, the order of duplex stability is 2'-OCH_3 RNA > unmodified DNA > 'normal' RNA > methylphosphonate DNA > phosphorothioate DNA. D2 and the various D2 methylphosphonate analogs investigated all formed hairpin conformations at low salt concentrations.

INTRODUCTION

Chemically modified oligonucleotides have attracted considerable recent attention for a variety of reasons,[1-3] including their potential use as chemotherapeutic agents. Among the many types of oligonucleotide analogs,[2,4] methylphosphonates[5-9] and phosphorothioates[10-16] have been widely investigated and compared.[17-23] The relatively large amount of attention given to these type analogs in particular, derives from their enhanced nuclease resistance,[1,14,24,25] ability to permeate cell membranes,[1,3,14,26] accessibility by means of simple automated synthesis[27-29] with commercially available monomers, and purification by preparative HPLC.[30,31] In addition, studies[17-22] involving both methylphosphonates and phosphorothioates suggest that their chemical properties may be manipulated to achieve desired biological and physical results. Specifically, oligomer length[17,19,22,32,33] and base sequence[1,33-37] as well as the number[18,20,21,38], position[36,38-41] and chirality[36,41-46] of modified linkages can affect the activity and stability of complexes formed with these compounds. Studies with modified oligonucleotides have already provided useful and unique information on nucleic acid stabilities, and interactions with ions and proteins.[39,43]

With the nonself-complementary duplex $dA_{19}\cdot dT_{19}$, we have found significant sequence effects in stereorandom methylphosphonate[39] and phosphorothioate (Kibler-Herzog, Zon, Wilson, manuscript in preparation) substitutions. Derivatives with the dA chain modified, for example, are considerably more stable than those with the dT chain modified. Cosstick and Eckstein,[36] in their investigation of $d(GC)_4$ and $d(CG)_4$, and Suggs and Taylor,[53] with poly (dA-dT)·poly (dA-dT), have also observed sequence dependent effects with modified nucleic acids, but no systematic analysis of these effects has been reported. To further elucidate the magnitude and nature of methylphosphonate and phosphorothioate substituent effects on nucleic acid stability, with a view toward defining more reliable physicochemical models, we have utilized two sequences [d(TAATTAATTAATTA) and d(TAGCTAATTAGCTA)] which are self-complementary and allow systematic placement of substituents between a wide range of nearest-neighbor base pairs that are repetitive but effectively noninteractive, due to the distance of separation (Scheme 1). The substituent effects are therefore amplified yet factorable. In addition, more limited studies provide information on how the extent and stereochemistry of substitution affect duplex stability.

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Scheme I. Sites of methylphosphonate (★), phosphorothioate (S) and 2'-O-methyl (O*) substitution in d(TAATTAATTAATTA), d(TAGCTAATTAGCTA) and r(UAAUUAAUUAAUUA).

Oligomer: 5'd(	T	A	A	T	T	A	A	T	T	A	A	T	T	A)3'
D1	--	--	--	--	--	--	--	--	--	--	--	--	--	--
D1-A*A	--	★	--	--	--	★	--	--	--	★	--	--	--	--
D1-A*T	--	--	★	--	--	--	★	--	--	--	★	--	--	--
D1-T*T	--	--	--	★	--	--	--	★	--	--	--	★	--	--
D1-T*A(Fa)[a]	★	--	--	--	★	--	--	--	★	--	--	--	--	--
D1-T*A(Sl)[a]	★	--	--	--	★	--	--	- -	★	--	--	--	--	--
D1-12*(5')	★	★	★	★	★	★	★	★	★	★	★	★	--	--
D1-ASA	--	S	--	--	--	S	--	-	--	S	--	--	--	--
D1-TST	--	--	--	S	--	--	--	S	--	--	--	S	--	--
D1-12S	S	S	S	S	S	S	S	S	S	S	S	S	--	--

Oligomer: 5'd(	T	A	G	C	T	A	A	T	T	A	G	C	T	A)3'
D2	--	--	--	--	--	--	--	--	--	--	--	--	--	--
D2-A*G(A)	--	★	--	--	--	★	--	--	--	★	--	--	--	--
D2-G(A)*C(T)	--	--	★	--	--	--	★	--	--	--	★	--	--	--
D2-C(T)*T	--	--	--	★	--	--	--	★	--	--	--	★	--	--
D2-T*A(Fa)[a]	★	--	--	--	★	--	--	--	★	--	--	--	--	--
D2-T*A(Sl)[a]	★	--	--	--	★	--	--	--	★	--	--	--	--	--
D2-12*(5')	★	★	★	★	★	★	★	★	★	★	★	★	★	--
D2-12*(3')	--	★	★	★	★	★	★	★	★	★	★	★	★	★

Oligomer: 5'r(	U	A	A	U	U	A	A	U	U	A	A	U	U	A)3'
R1	--	--	--	--	--	--	--	--	--	--	--	--	--	--
R1-2'O*	O*	O*	O*	O*	O*	O*	O*	O*	O*	O*	O*	O*	O*	O*

[a]D1-T*A(Fa) and D1-T*A(Sl) are assumed (based on earlier work[30]) to be diastereomers with only the Rp or Sp configuration at the 5' terminal methylphosphonate. These HPLC separated samples are designated (Fa) for "fast" or (Sl) for "slow". The same applies for D2-T*A(Fa) and D2-T*A(Sl). In neither case was the configurational assignment attempted. Previous studies[41] have shown that HPLC "fast" and "slow" products of this type can correspond to the 'R' and 'S' diastereomers, respectively.

RNA oligomers substituted with 2'-OCH_3 groups provide another possible agent for chemotherapeutic exploration.[47–49] These derivatives can be prepared in a straightforward manner by machine synthesis, and they exhibit significant nuclease resistance.[50] Phosphorothioate analogs of 2'-OCH_3 RNA oligomers have exhibited[51] significant inhibitory activity against the human immunodeficiency virus (HIV-1) in tissue culture.[10,52] Preliminary studies[47] of duplexes containing 2'-OCH_3 strands suggested that these duplexes might not show the sequence dependent Tm decreases observed with methylphosphonate and phosphorothioate derivatives. However, the stability of such duplexes and their biological properties have not been well studied. The 2'-OCH_3 subclass deserves more detailed investigation. For this reason, we have included the 'normal' (2'-OH) RNA and 2'-OCH_3 RNA analogs of D1 in our investigation of the effects of modifications on nucleic acid duplex stabilities.

Oligomer modifications and description

The unmodified self-complementary DNA 'parent' compounds d[TAATTAATTAATTA] and d[TAGCTAATTAGCTA] are referred to as D1 and D2, respectively. The two RNA analogs investigated are referred to as R1 for the RNA counterpart of D1 and R1-2'O* for the RNA analog in which the 2'-OH is replaced by -OCH_3. Methylphosphonate and phosphorothioate analogs contain various normal phosphodiester linkages replaced stereorandomly by 5'-O-P(O)CH_3-O-3' or 5'-O-P(O)S-O-3', respectively. The location and extent of substitution for each analog is shown in Scheme I. As with our previous study of $dA_{19}\cdot dT_{19}$, our initial focus was placed on evaluation of nearest neighbor basepair effects for stereorandom mixtures of these diastereomeric oligonucleotide analogs. Such mixtures having R and S configurations at phosphorus are provided by currently available methods of automated synthesis. Only when the methylphosphonate was incorporated at the end of these sequences were diastereomers able to be separated by HPLC.

EXPERIMENTAL

Synthesis of D1, D2, and their methylphosphonate analogs

An Applied Biosystems Model 380B automated DNA synthesizer was employed using the manufacturer's columns (1-μmol scale), monomer reagents (*O*-β-cyanoethyl phophoramidites and methylphosphonamidites), cycles, and recommended protocols for preparing oligonucleotides with phosphodiester and methylphosphonate linkages.[54] In each case, following anhydrous cleavage and deprotection with ethylenediamine-ethanol[55] at 55°C for 55 min,[54] the 5'-dimethoxytrityl (5'-DMT) derivative was isolated by reversed-phase HPLC[30,54] using a C_8 column (4.6×150 mm) that was eluted for 30 min with an increasing linear-gradient (1%/min) of acetonitrile vs. 0.1 M triethylammonium acetate, beginning at 20% acetonitrile; flow-rate = 1 mL/min. Retention times (min) for each of the collected HPLC fractions were as follows: D1, 17.3; D1-A*A, 17.9; D1-A*T, 17.3; D1-T*T, 16.9; D1-T*A(Fa), 'fast', 18.2; D1-T*A(Sl), 'slow', 20.2; D1-12*(5'), multiple peaks, 22–24; D2, 16.0; D2-A*G(A), 17.0; D2-G(A)*C(T), multiple peaks, 15–16; D2-C(T)*T, multiple peaks, 16–17; D2-T*A(Fa), 'fast', 17.2; D2-T*A(Sl), 'slow', 20.0; D2-12*(5'), multiple peaks, 21–24; D2-12*(3'), multiple peaks, 20–23. Following detritylation with acetic acid,[54] the resultant 5'-hydroxyl products were subjected to gel filtration,[54] except for D1-12*(5'), which was isolated in final form using a C_{18} cartridge[55] The yield ranged from *ca.* 20–40 OD_{260}-units. In view of the established nature of these syntheses[41] and purification[30,54] methods, the compounds were used without further purification or analysis, except for representatives D1-A*A and D1-T*T, which were analyzed as follows: Analysis of D1-A*A by capillary electrophoresis (Applied Biosystems Model 270A) using a Microgel-100™ gel-filled column led to detection (260 nm) of a single peak, which had an elution time (11.3 min) comparable to a *ca.* 22-mer reference oligonucleotide with phosphodiester linkages throughout. Interestingly, the same analytical method applied to D1-T*T led to resolution of 4 equal-intensity peaks, Which had elution times (11.7–12.1 min) comparable to 24- through 27-mer reference oligonucleotides with phosphodiester linkages throughout.

Synthesis of phosphorothioate analogs of D1

Compounds D1-ASA and D1-TST. The aforementioned sythesizer and columns were employed using the manufacturer's recommended cycle for coupling *O*-β-cyanoethyl phosphoramidites. In the appropriate cycle, just before oxidation with iodine/2,6-lutidine/water, the synthesis was interrupted, and the column was removed for manual sulfurization (30 min) with a solution prepared from elemental sulfur (0.25 g) dissolved in a mixture of CS_2 (2.4 mL), pyridine (2.4 mL), and Et_3N (0.2 mL).[56] The used sulfurization solution was discarded and the column was then thoroughly washed, sequentially, with CS_2-pyridine (1:1 v/v) and acetonitrile, before continuation of the automated synthesis. Compound D1-ASA was isolated by reversed-phase HPLC[30] collection of its 5'-DMT derivative and then conventional detritylation. Analysis of the resultant D1-ASA by reversed-phase HPLC[30] gave rise to a single peak. Similar processing afforded D1-TST that was further purified at the 5'-hydroxyl stage in order to remove a minor, faster-eluted, shoulder-peak.

Compound D1-12S. The aforementioned synthesizer was employed using the manuractuer-supplied hydrogen-phosphonate

monomers, ancillary reagents/solvents, and cycle.[57] The 1-μmol scale column that was used had been previously incorporated into a single cycle of conventional chemistry with *O*-β-cyanoethyl phosphoramidite monomer to incorporate what would become the single phophodiester linkage at the 3′ end of the target compound. Manual sulfurization[57,58] and then OPC™ purification[58] were carried out according to the referenced procedures.

Synthesis of the ribo analog of D1

The aforementioned synthesizer was employed using commercially available monomers (Peninsula Laboratories) in conjunction with standard operating procedures.[59] Products were purified by polyacrylamide gel electrophoresis and then recovered by conventional methods.[60]

Synthesis of the 2′-OCH_3 ribo analog of D1

The aforementioned synthesizer was employed with manufacturer-supplied reagents, solvents, and cycle for conventional 2′-deoxy *O*-β-cyanoethyl phosphoramidites, except for substitution of the corresponding 2′-OCH_3 ribo amidites and 1-μmol column, which were kindly supplied by Ajinomoto Co., Inc. Cleavage, deprotection, and reversed-phase HPLC purification were carried out in the conventional manner.[60]

Oligomer solutions and buffer

Stock solutions were prepared by dissolving lyophilized samples of oligomers in small amounts of deionized water and were frozen when not in use. Concentrations of oligomer stock solutions were determined at 25°C by using the following extinction coefficients (per single strand of oligomer): 1) ϵ(D1) = 1.50×10^5 L/mol-cm.; 2) ϵ(D2) = 1.42×10^5 L/mol-cm.; 3) ϵ(R1)= 1.60×10^5 L/mol-cm, calculated by the nearest-neighbor method.[61] The same extinction coefficients were used to calculate concentrations of stock solutions of modified D1, D2, and the R1-2′O* oligonucleotides, respectively. Oligomers were prepared for UV spectroscopy experiments by addition of a concentrated aqueous stock solution to 1.000 mL of PIPES buffer (0.01 M [piperazine-N, N′-bis {2-ethane sulfonic acid}], 0.001 M disodium EDTA adjusted to pH 7.0 with NaOH and filtered through a 0.45 micron teflon filter prior to use). The desired ionic strength was obtained for individual samples by additions of either a 4 M solution or solid NaCl.

UV spectral and Tm measurements

Oligomer UV spectral analyses and melting temperature experiments were performed as previously described.[39] Unless otherwise noted, the reported Tm for each transition is the temperature corresponding to $\alpha = 0.5$, where a is the fraction of single strands. A plot of α versus temperature and a Tm can be obtained by directly fitting experimental absorbance versus temperature curves to the following equations with a non-linear least squares computer program:[62,63]

$$A = C_T\alpha\epsilon_{ss}l + C_T(1-\alpha)\epsilon_{ds}l$$
$$\epsilon_{ss} = m_{ss}T + b_{ss}$$
$$\epsilon_{ds} = m_{ds}T + b_{ds}$$
$$K = \exp(-\Delta H°/R + \Delta S°/RT)$$

where A = absorbance; C_T = total oligomer strand concentration in moles per liter; ϵ_{ss}, m_{ss} and b_{ss} = the oligomer extinction coefficient, upper baseline slope and y intercept for the single-stranded (ss) form, respectively; ϵ_{ds}, m_{ds} and b_{ds} = the oligomer extinction coefficient, lower baseline slope and y intercept for the double-stranded (ds) form, respectively; l = the sample cell pathlength; T = temperature on the kelvin scale; and K = the equilibrium constant, $\Delta H°$ = the enthalpy, and $\Delta S°$ = the entropy for duplex dissociation. This curve fitting method for Tm evaluation was chosen because it gives accurate values for Tm even with relatively broad curves. The ΔH or ΔS obtained are apparent values since each methylphosphonate or phosphorothioate oligomer actually exists as 2^n different isomers (where n is the number of chiral linkages per oligomer).

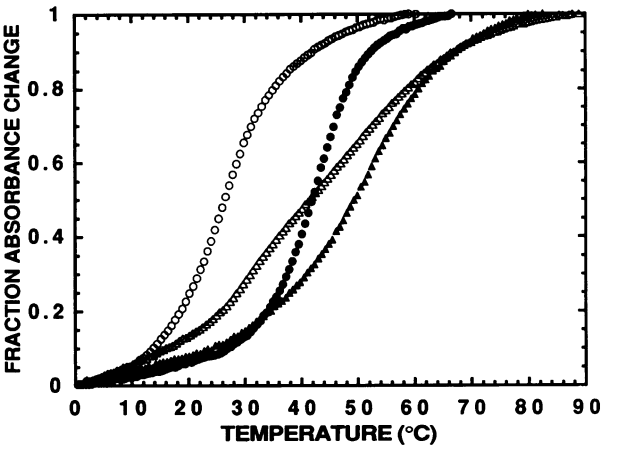

Figure 1. Melting curves for 5×10^{-6} M D1 in PIPES buffer at 0.07 M NaCl (○) and 0.8 M NaCl (●); and for 5×10^{-6} M D2 in PIPES buffer at 0.07 M NaCl (△) and 0.8 M NaCl (▲).

Cooperative melting curves were obtained for all oligomers, and plots of α versus temperature could be calculated for almost all of the oligomers investigated. For some oligomers, α versus temperature plots could not be obtained due to 1) the occurrence of more than a single transition or 2) the lack of sufficient high or low temperature baselines. In the latter case, a 'Tm' value is estimated as the temperature where the derivative of absorbance with respect to temperature is maximum. 'Tm' values obtained from this method of dAbs/dT were consistently slightly higher (around 2°C) than those found from a plots, as expected[64]. It should be noted that since the discrepancies in the methods are systematic, slopes of Tm versus log (sodium ion activity) plots were approximately identical regardless of which method was employed.

Since α versus temperature plots cannot be obtained for all melting profiles, for the purpose of comparative visualization, absorbances were converted to fraction absorbance change (defined as $A_T-A_{LT}/A_{HT}-A_{LT}$ where A_T = the absorbance at some temperature T, A_{LT} = the absorbance for the associated complex at the low temperature extreme investigated, and A_{HT} = the absorbance for the denatured complex in the form of single strands at the high temperature extreme investigated) and plotted versus T. Hyperchromicities were calculated for oligomer denaturations by first obtaining linear sloping baselines in both the high and low temperature regions of each melting curve. When taken at the Tm, the absorbance difference between these baselines divided by the upper absorbance value is defined as the hyperchromicity. Transition breadth is defined as the change in temperature from $\alpha = 0.2$ to $\alpha = 0.8$. Log (sodium ion activity) is calculated from log $\{(\gamma_{Na}{}^+)\times([\gamma Na^+])\}$, where $\gamma_{Na}{}^+$ is the activity coefficient for Na^+ [65].

Table I. Tm values (°C) at various salt concentrations, plus average melting transition breadths (Transition B) and hyperchromicities for D1, D2 and some derivatives; slopes of linear fits from Tm versus log (sodium ion activity) plots shown in Figures 2-A, 2-B and 2-C.

	Sample Identification[a]							
[Na+]/Log a_{Na+}	D1	D1-A*A	D1-A*T	D1-T*T	[Na+]/Log a_{Na+}	D1-T*A(Sl)	D1-T*A(Fa)	D1-12*(5')[b]
0.07/-1.25	24.3	22.5	22.8	23.2	0.07/-1.25	24.2	23.4	20.6
0.12/-1.05	29.0	25.2	26.0	26.0	0.12/-1.05	26.7	25.8	21.0
0.21/-0.82	33.8	28.5	29.0	29.3	0.21/-0.82	30.6	29.4	20.7
0.40/-0.55	38.6	31.2	31.1	31.5	0.41/-0.55	33.6	32.5	20.8
0.60/-0.39	40.9	32.7	32.4	32.4	0.61/-0.39	34.7	33.5	20.7
0.80/-0.27	42.0	34.1	33.6	33.4	0.81/-0.27	35.9	34.4	21.1
Transition B (°C):	12	12	15	17	Transition B (°C):	11	11	----
Slope (°C):	19.2	11.9	10.9	10.7	Slope (°C):	12.0	12.3	0
Hyperchromicity:	0.17	0.20	0.19	0.19	Hyperchromicity:	0.21	0.20	----

	Sample Identification[a]							
[Na+]/Log a_{Na+}	D2	D2-A*G(A)	D2-G(A)*C(T)	D2-C(T)*T	D2-T*A(Sl)	D2-T*A(Fa)	D2-12*(5')[b]	D2-12*(3')[b]
0.82/-0.26	50.7	42.2	42.6	41.4	43.1	42.5	17.5	16.3
Hyperchromicity:	0.13	0.15	0.15	0.14	0.15	0.15	----	----

	Sample Identification[a]					Sample Identification[c]		
[Na+]/Log a_{Na+}	D1-ASA	D1-TST	D1-12S[b]	R1-2'O*[a]	[Na+]/Log a_{Na+}	D1	R1	R1-2'O*
0.07/-1.25	----	15.4	----	----	0.07/-1.25	20.7	18.4	33.8
0.12/-1.04	25.1	19.0	----	40.5	0.12/-1.04	24.9	22.6	37.8
0.17/-0.89	29.1	22.9	9.7	43.6	0.22/-0.89	29.8	27.5	42.9
0.37/-0.58	34.6	28.9	15.6	49.4	0.42/-0.58	34.4	32.1	47.7
0.57/-0.40	37.2	30.7	17.5	52.2	0.62/-0.40	36.2	34.9	50.5
0.82/-0.26	37.9	32.0	18.3	54.3	0.82/-0.26	38.0	----	52.1
1.02/-0.17	38.3	32.9	19.0	55.2	1.02/-0.17	38.3	36.7	54.2
Transition B (°C):	9	10	----	12	Transition B (°C):	12	13	12
Slope (°C):	19.1	19.0	17.2	18.6	Slope (°C):	18.1	18.8	19.2
Hyperchromicity:	0.18	0.19	----	0.25	Hyperchromicity:	0.22	0.15	0.33

[a,c] Sample concentrations averaged around: [a]5 x 10^{-6} M and [c]7 x 10^{-7} M per single strand of oligomer.
[b] "Tm" values for these transitions were determined from the maximum in the derivative of a plot of absorbance vs temperature.

RESULTS

Melting behavior of unmodified DNA oligomers

Representative melting curves for duplexes formed from the unsubstituted self-complementary oligomers d(TAATTAATT-AATTA) [D1] and d(TAGCTAATTAGCTA) [D2] are shown in Figure 1 at 0.07 M and 0.8 M NaCl. D1 melting curves are cooperative and appear monophasic at all salt concentrations. At salt concentrations of around 0.2 M and higher, melting curves for D2 duplexes are similar in shape, but broader, than those for D1 duplexes, and only the single transition for the duplex to denatured single-strand equilibrium can be seen. At salt concentrations below 0.2 M, D2 has a fairly sharp low temperature transition and a very broad transition at higher temperatures. This behavior is characteristic of a duplex to hairpin transition at low temperatures and a hairpin to denatured strand transition at higher temperatures.[66,67] Similar behavior has been observed with other self-complementary oligomer sequences where GC base pairs flank central AT sequences.[68] Absorbance versus temperature plots were identical when samples of D1, D2 or any derivatives investigated were heated, slowly cooled, and then reheated under the same experimental conditions.

Tm values for duplex to denatured-strand transitions of D1 at various salt concentrations are listed in Table I and are plotted in Figure 2 as a function of log (sodium ion activity). Tm values increase with increasing sodium ion activity and the slope of the linear fit shown in Figure 2 for D1 is listed in Table I. The average hyperchromicity, as well as the average transition breadth from melting profiles of this complex at all salt concentrations investigated are also given in Table I.

The Tm for the unmodified D2 duplex melting transition at a salt concentration of 0.8 M NaCl is listed in Table I. This oligomer has two transitions at low salt concentrations with significant transition overlap at intermediate salt concentrations, and duplex to denatured single-strand Tm values cannot be obtained under these conditions. As expected, the value obtained for the duplex melting transition of D2 at high salt concentration is greater than that obtained for the transition of D1 (containing only A and T).

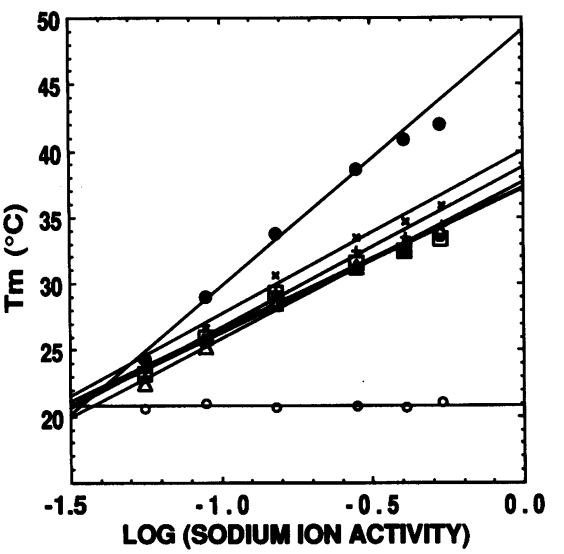

Figure 2. The effect of sodium ion activity on Tm for 5×10^{-6} M D1 (●), D1-A*A (△), D1-A*T (■), D1-T*T (□), D1-T*A(Sl) (×), D1-T*A(Fa) (+) and D1-12*(5') (○). Linear fits include data from log activities of sodium ion less than −0.3. Slopes of linear fits are given in Table I. Symbols used in Figure 2 correspond to those in Figure 3.

Melting behavior of methylphosphonate DNA oligomers

Melting profiles for D1 methylphosphonate duplexes at 0.2 M NaCl are shown in Figure 3. At all salt concentrations, curves of similar or slightly broader shape were obtained when three (of thirteen total) normal phosphodiester linkages were replaced by methylphosphonates in D1. Substitution of twelve linkages

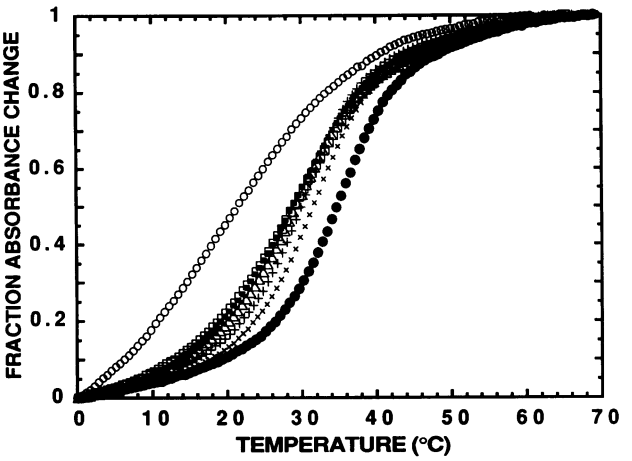

Figure 3. Melting curves in PIPES buffer at 0.2 M NaCl for 5×10^{-6} M D1 (●), D1-A*A (△), D1-A*T (■), D1-T*T (□), D1-T*A(Sl) (×), D1-T*A(Fa) (+) and D1-12*(5′) (○).

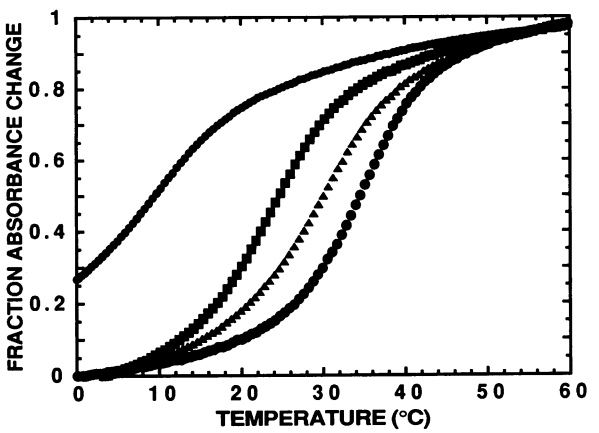

Figure 4. Melting curves in PIPES buffer at 0.21 M NaCl for 5×10^{-6} M D1 (●), and at 0.17 M NaCl for 5×10^{-6} M D1-ASA (▲), D1-TST (■) and D1-12S (●).

with methylphosphonates [i.e., D1-12*(5′)] resulted in a much broader melting curve. As observed with the unsubstituted 'parent' oligomer, methylphosphonate derivatives of D2 exhibit biphasic behavior characteristic of hairpin formation at salt concentrations below approximately 0.2 M, but above 0.2 M only a single transition was observed. Except for the highly substituted methylphosphonate analog, Tm values for methylphosphonate derivatives of D1 increase with increasing sodium ion activity (Table 1 and Figure 2). The Tm of D1-12*(5′) is approximately 21°C regardless of salt concentration. Slopes of linear fits from Figure 2, average hyperchromicities and transition breadths are given in Table I along with Tm values and hyperchromicities for D2 derivatives at 0.8 M NaCl.

Tm values for duplex melting transitions of all methylphosphonate derivatives investigated are lower than those for transitions of the respective unmodified parent compound and depend on the extent of substitution. For each type of oligomer (D1 or D2) at any given salt concentration, all derivatives in which three of the thirteen normal phosphodiester linkages were

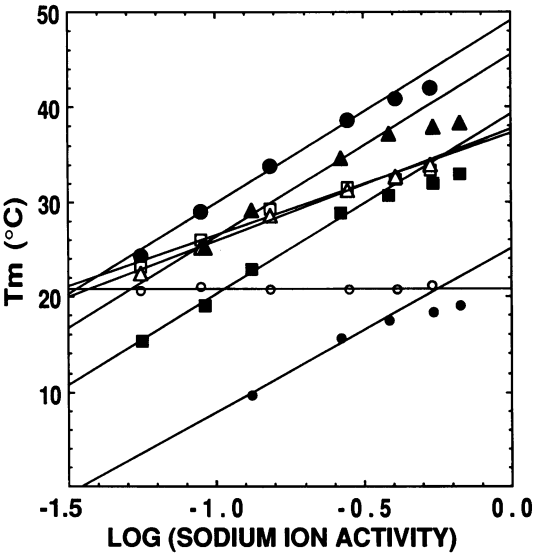

Figure 5. The effect of sodium ion activity on Tm for 5×10^{-6} M D1 (●), D1-ASA (▲), D1-TST (■), D1-12S (●), D1-A*A (△), D1-T*T (□) and D1-12*(5′) (○). Linear fits include data from log activities of sodium ion less than −0.3. Slopes of linear fits are given in Table I. Symbols used in Figure 5 correspond to those in Figures 3 and 4.

replaced with methylphosphonates formed duplexes of approximately equal stability, regardless of the location or 5′ terminal chirality of the methylphosphonate (see Table I and Figure 3). Similarly, modifications at twelve of thirteen linkages in two samples of D2 yielded duplexes of approximately equal stability.

Melting behavior of phosphorothioate DNA oligomers

Melting profiles are shown in Figure 4 for D1 and D1 phosphorothioate derivatives at 0.21 M and 0.17 M NaCl, respectively. Transition curves for both D1 phosphorothioates with three modified linkages have approximately the same shape. Substitution of twelve normal linkages with phosphorothioates [i.e., D1-12S] resulted in a melting curve that was even broader than that of the analogous highly substituted methylphosphonate [i.e., D1-12*(5′)]. Tm values of duplex to denatured strand transitions are listed in Table I along with hyperchromicities and transition breadths. Plots of Tm versus log (sodium ion activity) are shown in Figure 5. Tm values increase with increasing sodium ion activity. Slopes of linear fits from Figure 5 plots (see Table 1) are essentially identical for D1 and all phosphorothioate derivatives investigated, as expected from their charge equivalencies. Plots for analogous methylphosphonate oligomers are included in Figure 5 for direct comparison.

Replacement of normal phosphodiester linkages in D1 with phosphorothioates resulted in melting transitions with lowered Tm values relative to those of unmodified D1 at all salt concentrations considered (Table I). The amount of destabilization caused by this type modification depends on both the sequence and extent of substitution. At a given salt concentration, Tm values for complexes modified at three of the thirteen positions are lower when the phosphorothioate is between two adenines [D1-ASA] than when it is between two thymines [D1-TST]. Complexes with modifications at twelve positions have lower Tm values than their partially modified counterparts at all salt concentrations.

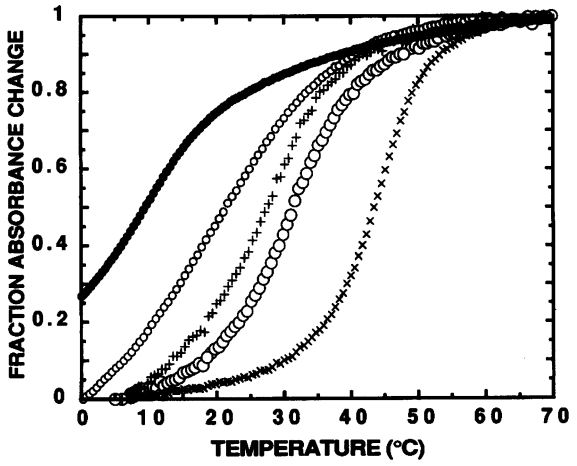

Figure 6. Melting curves in PIPES buffer at 0.2 M NaCl for 7×10^{-7} M D1 (○), R1 (+) and R1−2′O* (×); and for 5×10^{-6} M duplex D1-12S (●) and D1-12*(5′) (○).

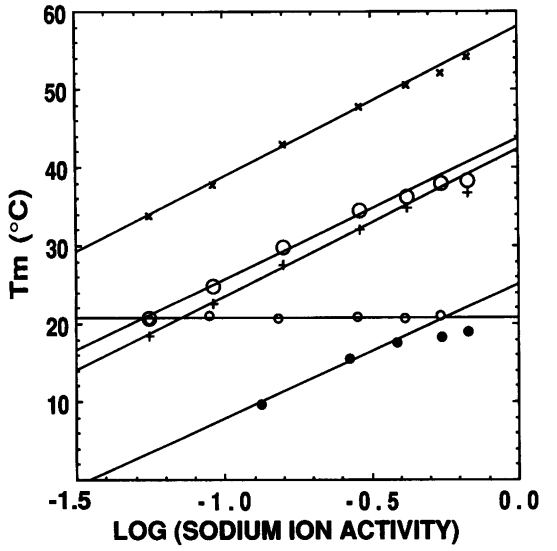

Figure 7. The effect of sodium ion activity on Tm for 7×10^{-7} M D1 (○), R1 (+) and R1−2′O* (×); and for 5×10^{-6} M D1-12S (●) and D1-12*(5′) (○). Linear fits include data from log activities of sodium ion less than −0.3. Slopes of linear fits are given in Table I. Symbols used in Figure 7 correspond to those in Figure 6.

Melting behavior of RNA oligomers

For comparative purposes, Figure 6 shows example melting curves of D1 and R1, as well as the similarly substituted 2′-OCH_3 RNA [R1-2′O*], and the DNA methylphosphonate [D1-12*(5′)] and phosphorothioate [D1-12S] compounds at approximately 0.2 M NaCl. Melting curves obtained for both RNA analogs were similar in shape to those obtained for the unmodified DNA oligomer at all salt concentrations considered. Tm values obtained for the RNA oligomers increase with increasing sodium ion activity (Table 1). Plots of Tm versus log (sodium ion activity) for both RNA oligomers are compared in Figure 7 to plots for the unmodified DNA and the highly substituted methylphosphonate and phosphorothioate oligomers. Tm values for R1 transitions are slightly lower and for R1-2′O* transitions are higher than those for the unmodified DNA oligomer at all salt concentrations considered. Slopes (in Figure 7 and Table 1) for the RNA oligomers are similar to those for the unmodified and phosphorothioate derivatives of DNA. Melting curves for low concentrations of D1 and R1-2′O* are similar in shape to those for higher concentrations of the same samples.

DISCUSSION

Effects of methylphosphonate, phosphorothioate, 2′-OH and 2′-OCH_3 substitution on duplex stability

Both RNA analogs, as well as the partially and highly substituted methylphosphonate and phosphorothioate strands of D1, form duplexes which have cooperative melting transitions, and at 0.07 M NaCl and above, all such duplexes, except the 2′-OCH_3 analog, are destabilized relative to the unmodified DNA oligomer. Our results indicate that, in some respects, the destabilization from phosphorothioate substitution is sequence dependent in a manner similar to that observed with duplexes of $dA_{19}\cdot dT_{19}$ containing methylphosphonates.[39] With $dA_{19}\cdot dT_{19}$ derivatives in 1.0 M NaCl, we observed a Tm decrease of approximately 20°C (about 1°C per methylphosphonate group) when only the dT_{19} strand was substituted with methylphosphonates, a slight increase in Tm (approximately 1°C per duplex) when only dA_{19} was substituted, and a decrease of approximately 22°C per duplex when both chains were fully substituted with methylphosphonates. Although, quantitatively the amount of decrease or increase in Tm relative to that of the unsubstituted oligomer was dependent upon salt concentration (i.e., the stabilizing effect was larger at lower salt concentrations), the relative sequence effects were consistent from 0.07 M to 0.8 M NaCl. In a similar manner, phosphorothioate substitution in D1 between thymines is destabilizing relative to substitution between adenines. In contrast to our previous results with methylphosphonates, however, phosphorothioate substitution between adenines did not stabilize the duplex. The Tm of duplex D1-ASA with three phosphorothioate groups per strand is decreased relative to D1 by approximately 4°C (about 1°C per phosphorothioate group in the duplex) at all salt concentrations. For duplex D1-TST, this relative decrease is approximately 10°C (about 2°C per phosphorothioate group). These results agree with those of previous studies involving phosphorothioate derivatives of poly (dA-dT)·poly (dA-dT)[53] and poly (dA)·poly (dT)[69] in the presence of Na^+, where a phosphorothioate diester 5′ to a purine caused less destabilization (a 1°C or 6°C decrease in Tm, respectively) than when it was 5′ to a pyrimidine (a 12°C or 17°C decrease in Tm, respectively).

Surprisingly, unlike our results with $dA_{19}\cdot dT_{19}$ duplexes, varying the location of methylphosphonate linkages in D1 or D2, has no significant sequence dependent effects on duplex destabilization. At 0.8 M NaCl, all duplexes with three methylphosphonate linkages per strand have approximately an 8°C decrease in Tm relative to the 'parent' compounds D1 and D2, (about 1°C per methylphosphonate group in the duplex). In comparison, substitution of three phosphodiester linkages with methylphosphonates in two DNA 14-mers containing A, T, G and C resulted in duplexes which were only slightly more destabilized (by 11 or 13°C per duplex) relative to their parent compounds when the methylphosphonate was 3′ to a T or G than

when it was 3′ to an A or C (9 or 11°C per duplex)[70]. However, few conclusions about the sequence dependence of methylphosphonates can be drawn from these results because bases on the 3′ side of methylphosphonate groups varied from position to position within each oligomer.

From the limited results with substitutions of methylphosphonate groups in different sequences, no clear pattern has emerged that defines the sequence dependent effects of these subtituents on duplex stability, and no complete understanding of the molecular basis of these substituent effects can be obtained until enough modified sequences have been evaluated to define the substituent-sequence pattern. It is clear, however, that in the sequences we have evaluated up to this time, substitution of methylphosphonate for normal phosphate groups causes a decrease in duplex stability, and the degree of destabilization is dependent upon the extent of substitution.

The two RNA counterparts of D1, R1 and R1-2′O*, have significantly different duplex stabilities at all salt concentrations. Complete substitution of U for T bases plus -OH for -H at the 2′ position [R1] destabilizes the duplex by approximately 2°C relative to D1. However, substituting U for T plus replacing the 2′-H with 2′-OCH_3 [R1-2′O*], increases duplex stability by approximately 14°C relative to D1 (or by approximately 16°C relative to R1). Since the methyl group in thymine is known[71] to stablize the helix, but the extent of this stabilization in different sequences has not been quantified, we cannot determine the effect of the 2′-OH group alone from these results. On the other hand, the 2′-OCH_3 analog is more stable than D1 (around 1°C per 2′-OCH_3) in spite of the T to U base changes. Previous studies[47] involving nonanucleotides also showed that changes from U to T and from 2′-H to 2′-OCH_3 increased duplex thermal stability relative to the DNA 'parent' compound by around 1°C per 2′-OCH_3 group. These studies, however, found that the 'normal' (2′-OH) RNA compound was similar in stability to the 2′-OCH_3 RNA derivative (more stable than DNA). From all of these results it is clear that the 2′-OCH_3 is stablizing, but effects of the 2′-OH are less clear and may be quite sequence dependent. We conclude that in our sequence, containing only A and T or U bases, a 2′-OCH_3 residue does make a significant contribution to duplex stability, but further studies are needed to investigate the effects of base sequence on the stabilities of other modified RNA duplexes.

For drug design considerations, we note that with three (of thirteen total) linkages modified between two adenines, the D1-ASA phosphorothioate duplex is more stable at low salt concentrations and less stable at high salt concentrations than the corresponding D1 methylphosphonate duplex [i.e., D1-A*A] (see Figure 5). When modified between two thymines, the partially modified phosphorothioate [i.e., D1-TST] is always less stable than the corresponding methylphosphonate duplex [i.e., D1-T*T], although their relative stabilities are dependent upon salt concentration. This latter relationship also applies for the highly substituted D1-12S and D1-12*(5′). In all instances, highly modified methylphosphonate or phosphorothioate duplexes are less stable than those partially modified which are less stable than the unmodified parent oligomers. Notice, that at approximately physiological salt concentration (0.2 M NaCl), D1 duplexes highly modified with methylphosphonates are more stable than those highly modified with phosphorothioates, but are less stable than either the unmodified DNA or 'normal' RNA configurations; the R1-2′O* duplex is significantly more stable than any of them (Figure 7).

Effects of salt concentration on the stability of modified and unmodified DNA and RNA duplexes

An average slope of Tm versus log (sodium ion activity) of 18.7°C for the unmodified D1 duplex is in good agreement with other results for AT rich duplexes[39,50,71,72,73]. By using counterion condensation theory as previously described[39], with an enthalpy of melting of 1.04×10^5 kcal/mole duplex[74], we calculated a value of 4.2 Å for b of the D1 single strand (b is the average spacing between phosphates based on modeling the oligonucleotide as a linear array of negative charges). This value is intermediate between our previously determined values of 3.5 Å and 5.6 Å for b of single-stranded dA_{19} and dT_{19}, respectively[39], and seems reasonable since D1 is 50% dA and 50% dT.

In contrast to our previous studies with dA_{19} and dT_{19} methylphosphonates[39], all D1 duplexes containing an equivalent number (i.e. three per strand) of methylphosphonate linkages have approximately equal slopes of Tm versus log (sodium ion activity) plots, regardless of location of the methylphosphonate. As expected, these slopes are decreased relative to that for unmodified D1. Essentially the same value was calculated for the phosphate spacing (b = 4.3 Å) in the single strands of all the partially substituted oligomers, and this was the same value as that calculated for the unmodified D1 oligomer. Differences in slopes for $dA_{19}\cdot dT_{19}$ methylphosphonate duplexes have been explained by differences in base stacking in the charged single strands. The partially neutralized D1 methylphosphonate single strands appear to have no significant differences in base stacking when methylphosphonates are substituted at various positions in the sequence. The slope of Tm versus log (sodium ion activity) for D1-12*(5′) is 0, as expected, since the low charge density of the duplex prevents ion condensation.

Slopes of Tm versus log (sodium ion activity) plots for fully charged modified D1 duplexes [D1 oligomers containing phosphorothioates, R1 and R1-2′O*] are essentially identical to the slope for unmodified D1. Regardless of the position or extent of substitution, a value of 4.4 Å was calculated for the phosphate spacing of single strands containing phosphorothioate linkages. This is essentially the same value as that calculated for the unmodified and methylphosphonate modified D1 single strands (4.2 Å and 4.3 Å, respectively). However, using 8.9×10^4 kcal/mole duplex for ΔH[75] and a value of 1.36 Å for b of the RNA duplexes[76], the phosphate spacing in the single strands of both RNA oligomers is calculated to be approximately 3.6 Å. The difference in calculated values of b for single-stranded D1 and R1 compounds agrees with general views of greater flexibility of DNA relative to RNA and is consistent with other studies which demonstrate how statistical chain dimensions of single strands are affected by differences in puckering of the ribose ring due to the 2′ substituent.[64]

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