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Physicochemical properties of phosphorothioate oligodeoxynucleotides

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ABSTRACT

We have recently shown that phosphorothioate (PS) oligodeoxynucleotide (ODN) analogs, unlike their normal congeners, exhibit significant anti-HIV activity (Matsukura *et al.*, (1987) *Proc.Natl.Acad.Sci.USA* **84**, 7706-7710). We now report the syntheses, melting temperatures (Tm), and nuclease susceptibilities of a series of phosphorothioate ODN analogs. These include all-PS duplexes, duplexes with one normal chain and the other chain either all-PS, or end-capped with several PS groups at both 3' and 5' ends. The DNase susceptibilities of the S-ODNs are much less than the normal phosphodiesters, but by contrast duplexes of poly-rA with S-dT_{40} are much more susceptible to RNase H digestion. The Tm's for AT base pairs of S-ODNs are significantly depressed relative to normals, while GC base pairs show much less Tm depression. The Tm's of S-dT oligomers with poly-rA are reduced relative to the duplexes with normal dA oligomers. These results have significance for the biological properties of these analogs as anti-message inhibitors of gene expression, and provide a rational basis for the S-dC/G sequences as potential effective anti-AIDS agents.

INTRODUCTION

Oligodeoxynucleotides and their analogs are potential anti-sense inhibitors of selected gene expression (1). We have recently shown that phosphorothioate oligodeoxynucleotide analogs have anti-HIV activity (2). We have also investigated the activity of specific sequences of these analogs against selected gene expression in other retro-viruses, particularly lentiviruses (Dahlberg, J., Stein, C., and Cohen, J.S., unpublished results), as well as against oncogenes in HL60 cells (3).

One reason postulated for the effectiveness of these analogs is the fact that the phosphorothioate diester bond is reported to be more stable to nucleases than the normal phosphodiester bond in oligomers (4). However, very little is known about the actual stability and hybridization of these analogs. The synthesis and some properties of the two stereo-isomers (*Rp* and *Sp*) of an oligomer with a single phosphorothioate substitution have been reported (5). But, the stereospecific

synthesis is not convenient, and is not adapted to the use of the automated synthesizer to produce large quantities of these analogs for biological testing.

In this paper we report the routine synthesis of these analogs, as well as their physico-chemical properties, including melting temperatures and nuclease susceptibilities to a number of endo- and exo-nucleases. In each case we have compared the phosphorothioate analog with the normal oligomer, either as single strand (nuclease susceptibility) or duplex (melting). In view of the fact that these compounds may function by interaction with m-RNA, we have also compared the melting temperatures of the homopolymers of S-dC_n, which was found to have activity against HIV with n>14 (2), with poly-rI, and of S-dT_n with poly-rA, as well as with oligo-dA.

The results of these studies are shown to have relevance to the biological function of these analogs. Notably, the results indicate that GC-rich base sequences of moderate length (20-30 bases) can be expected to provide sufficiently high Tm's and sufficiently low rates of nuclease digestion for phosphorothioate oligomers to be potential inhibitors of gene expression.

MATERIALS AND METHODS

Synthesis of Phosphorothioate Oligomers.

All phosphorothioates were synthesized on the Applied Biosystems 380B DNA Synthesizer using an adaptation of the method of Stec *et al.* (6). The standard oxidation cycle using iodine was substituted by a sulfurization step: A 5% solution was prepared by dissolving 7.5 g elemental sulfur (Aldrich) in 71.5 ml carbon disulfide. To this was added 71.5 ml of colorless pyridine that had been dried over molecular sieve for at least 1 h, and 7.5 ml triethylamine. The deep yellow solution turns red with time and/or on exposure to moisture, and cannot be used in the sulfurization step without unacceptable product loss. The sulfur solution must be remade for each synthesis. Before and after the oxidation step, the column is washed repeatedly with a 1:1 solution of carbon disulfide and pyridine to remove any residual sulfur which may preciptate in the line. The sulfur oxidation is not as rapid as the iodine oxidation and a wait step of 450 seconds, as compared to 30 seconds, is required. Oligodeoxynucleotides with blocks of phosphorothioates at the 3' or 5' ends were synthesized by automatically changing the oxidation cycle at the required point. After cleavage from the column and deblocking in aqueous ammonia (60°, 10h), phosphorothioate oligomers and block co-polymers were purified via reverse phase HPLC (PRP-1 column, 0.1M triethylammonium acetate buffer (pH 7) - acetonitrile (20%, increase to 40% at 20 min). Detritylation was accomplished in 3% acetic acid (10 min), and the solution was extracted with two

equal volumes of ethyl acetate, frozen in dry ice, and lyophilized. The solids were dissolved in 0.3 ml of 1M NaCl, and the product precipitated by addition of 3.5 volumes of absolute ethanol. The triethylammonium salts of some phosphorothioate oligomers, particularly the homopolymer dC_{28}, are extremely insoluble in 1M NaCl. Introduction of a small amount of ammonia vapor (not aqueous ammonia) by a Pasteur pipette solubilized all the solids. The yield determined from absorbance at λmax was ca. 50% for 14-mers and ca 30% for 28-mers.

Melt Temperatures.

Poly-rA was obtained from Pharmacia. All optical measurements were made on a Shimadzu UV-160 recording spectrophotometer coupled to a CPS Controller thermostat. Values of absorbance were recorded at 260 nm in either 10 mM sodium cacodylate/140 mM NaCl buffer (pH 7.0) or in 10 mM HEPES/140 mM NaCl (physiologic saline, pH 7.5). All duplexes were formed in 1:1 mixtures of a strand with its complement. All samples were pre-melted at 75-80° to destroy scondary structure, and then allowed to thermally equilibrate. Each melt curve is composed of a minimum of 20 individual temperature points.

NMR measurements:

NMR spectra were recorded on a Varian XL-400 spectrometer at 400 MHz for ^{1}H and 162 MHz for ^{31}P at 22°C. Recycle times were 2-3 sec and the number of scans was 64-200 for ^{1}H and up to 3000 for ^{31}P. Integration was performed using the Varian program.

Enzyme Kinetics.

RNase H, S1 nuclease and P1 nuclease were obtained from BRL. Snake venom phosphodiesterase was obtained from Pharmacia. All reactions were run in a total volume of one ml at 37°. The value of absorbance was measured at λmax. The RNAse H reaction buffer consisted of 20mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1mM dithiothreitol and 5% (w/v) sucrose. The final concentration of enzyme was 7 units/ml. S1 nuclease (1000 u/ml) was diluted 1:10 or 1:100 in reaction buffer, which consisted of 30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM zinc acetate and 5% (v/v) glycerol. The final concentration of enzyme was 100 u/ml (for the homopolymer-dC) or 10 u/ml (for all other sequences). P1 nuclease (40u/ml) was diluted 1:10 in reaction buffer, which consisted of 50 mM sodium acetate (pH 5.3). The final enzyme concentration was 4 u/ml. Snake venom phosphodiesterase (46 u/mg solid) was dissolved in 500 mL water. One lambda of this solution was added to the reaction mixture, which contained 100 mM Tris-HCl (pH 8.9), 100 mM NaCl and 14 mM $MgCl_2$.

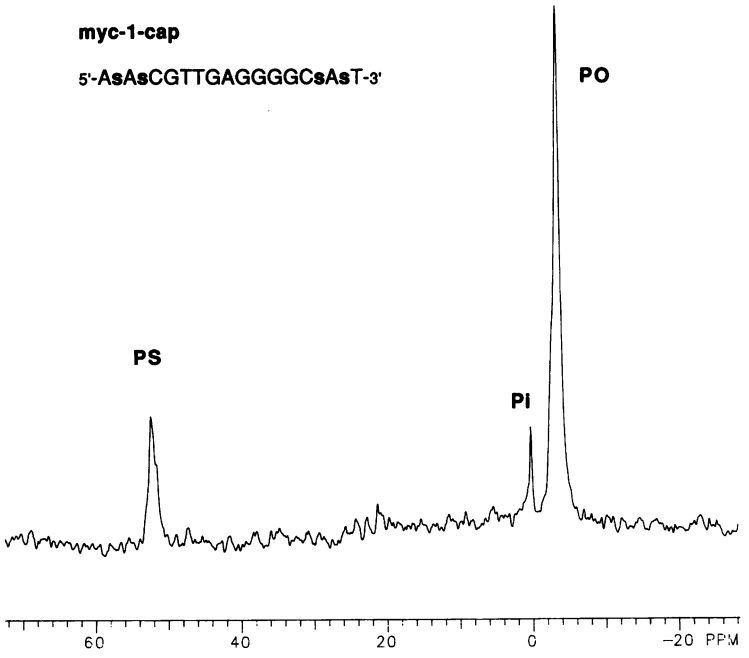

Figure 1. ^{31}P NMR spectrum at 162 MHz of the myc-1 oligomer sequence indicated, containing two PS bonds at each end of the 15-mer.

Data Analysis.

All data was analyzed using the MLAB program on the DEC PDP10 computer of the NIH Computer Center. A simple exponential was fitted to the nuclease digestion data (absorption vs. time), and a sigmoidal curve of the form,

$$OD(T) = \varepsilon_A/(1+k) + \varepsilon_B/(1-k) \qquad (1)$$

where, $k = \exp[\Delta H(T-Tm)/(RTm^2)]$, and ΔH is the van't Hoff enthalpy, was fitted to the melting curves (normalized absorption vs. temperature; see ref. 7 for details).

RESULTS

Characterization of Phosphorothioates.

The ^{31}P NMR spectrum of a phosphorothioate oligodeoxynucleotide 15-mer (myc-1) consists of a single downfield shifted peak (6). The absence of any significant phosphate peak indicated insignificant (<5%) oxidation. The ^{31}P spectrum of the 3',5'-phosphorothioate end-capped myc-1 with two PS groups at both ends shown as an example gave the correct integrated PO/PS peak ratio of

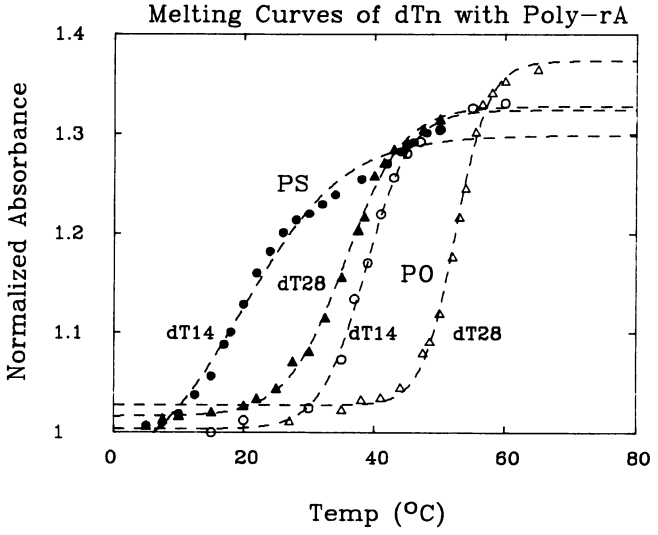

Figure 2. Melting curves of dTn with Poly-rA. Absorbance at 260 nm was normalized and fitted using the MLAB program to a sigmoidal curve shown in equation *1*. Measurements were performed in either 10 mM Na cacodylate/140 mM NaCl (pH 7.0) or in 10 mM HEPES/140 mM NaCl (pH 7.5). Closed symbols are phosphorothioates, open symbols normal DNA.

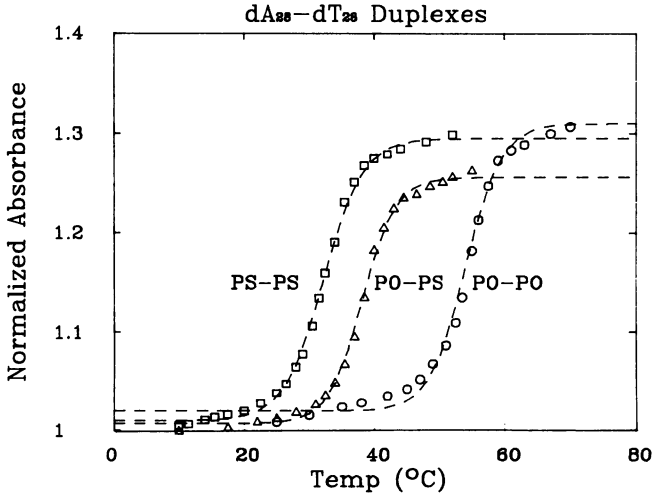

Figure 3. Melting curves of deoxy-homo A-T 28-mer duplexes. Circles represent the all-oxygen normal duplex. Triangles represent the mixed phosphorothioate - oxygen duplex. Also shown is a duplex in which both strands are all-phosphorothioate (squares).

Table 1. Melting Temperatures with Polyribonucleotides[1]

Poly-rX	PO-Oligo	Tm	ΔH	PS-Oligo	Tm	ΔH	ΔTm
Poly-rA	O-dT14	39	57	S-dT14	20	22	19
	O-dT15	39	55	S-dT15	23	31	16
	O-dT21	48	75				
	O-dT28	52	84	S-dT28	36	43	16
	O-dT40	58.5	101	S-dT40	43.5	67	15
				2S-cap-dT15	36	43	3
				4S-cap-dT21	43	56	5
				5S-cap-dT23	44		
Poly-rI	O-dC15	29	83	S-dC15	20	85	9
	O-dC28	41	107	S-dC28	31	92	10

[1] Tm is in °C, and ΔH in kcal/mol

2.5:1 (Fig. 1). With longer mixed PO/PS sequences (4-PS capped dT_{21} and 5-PS capped dT_{23}) the integrated areas of the NMR peaks indicated reduced sulfurization by about one sulfur per molecule. This probably results from the repetitive oxidation cycles that the 3' end of the oligomer undergoes in these mixed PS/PO oligos resulting in substitution of PS by PO in the presence of iodine.

Table 2. Melting Temperatures of Oligodeoxynucleotides[1]

Duplex	Tm	ΔH	Duplex	Tm	ΔH	ΔTm
O-dT14/O-dA14	36	50	S-dT14/O-dA14	20	53	17
O-dT28/O-dA28	54	77	S-dT28/O-dA28	38	74	15
			S-dT28/S-dA28	32	61	22
O/O-33%GC15	50	39	S/O-33%GC15	41	42	9
O/O-53%GC15	62	37	S/O-53%GC15	55	35	7
O/O-67%GC15	65	43	S/O-67%GC15	54	49	11
O/O-70%GC20	>70		S/O-70%GC20	67	36	
			S/S-70%GC20	58	32	

[1] Tm is in °C, and ΔH in kcal/mol; sequences are:
33%GC15 = d-TAGCTTATGTAATGC
53%GC15 = d-AACGTTGAGGGGCAT (myc-1)
67%GC15 = d-CCGGGTATCCGATGC
70%GC20 = d-CAGCGTGCGCCATCCTTCCC

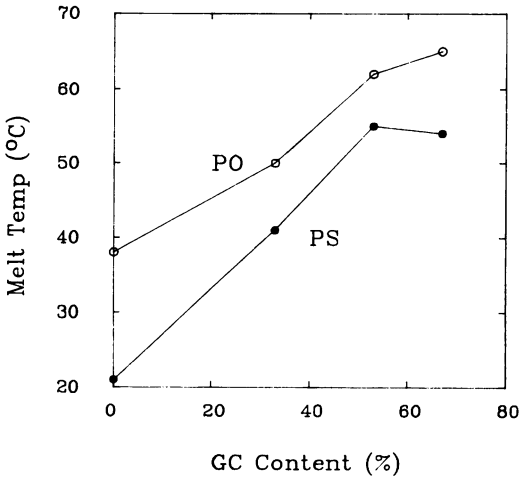

Figure 4. Melt temperature vs. G-C content (%) of oligodeoxynucleotide duplexes. Note the increasing value of Tm with increasing G-C content until it levels off at GC = 50%. Closed symbols represent phosphorothioate complexes: open symbols are normal DNA.

Melt Temperatures.

The fact that equation 1, that is based on a two-state transition model, fits the data as well for the mixtures of all-PS oligos with normal complement as for the all-normal duplexes, indicates that these oligos are also forming stable duplexes at low temperature (Figs. 2, 3). The change in absorbance (ε_A - ε_B) and the slopes (ΔH) on melting are also quite comparable to the normal duplexes, only the Tm's are significantly reduced. Had the slopes been significantly broadened this would have indicated formation of many hybrids of relatively unstable duplexes due to the stereoisomerism. However, the melting data indicate a well-behaved two-state transition.

Melt temperatures of duplexes of oligo-dT of various lengths both PS and PO were determined with poly-rA (Table 1 and Fig. 2). Melt temperatures of oligo-dG with poly-rC and oligo-dC with poly-rG could not be determined due to the insolubility of the complexes. In place of these oligo-dC with poly-rI was used (Table 1). In general the value of Tm was reduced by ca. 15-20°C for the PS relative to the normal PO oligomer of equal length, with the difference diminishing slightly at longer oligomer length. In addition, the value of ΔH is about 30-40 kcal/mole less for the phosphorothioate oligomers, indicating diminished binding cooperativity and increased local melting with polyribonucleotides at temperatures

Table 3. Nuclease Susceptibilities of Oligomers, $t_{1/2}$ (sec)[1]

Sequence	S1	Ratio	P1	Ratio	SV	Ratio
O-dC15	822		1810		35	
S-dC15	11000	134	27700	15.3	133000	3800
O-dC28	3910		3160		70	
S-dC28	7990	2	48600	15	>100000	>1400
O-ODN-4	203		72		20	
S-ODN-4	8370	41	1370	19	92500	4625
O-myc-1	36		69		28	
S-myc-1	330	9	249	4	12400	443
myc-1-cap	1530	43	807	12	4230	151

[1]Ratio = $t_{1/2}$PS-oligo/$t_{1/2}$PO-oligo

ODN-4 = d-TCGTCGCTGTCTCCGCTTCTTCCTGCCA

myc-1 = d-AACGTTGAGGGGCAT

below the Tm. The S-capped molecules studied here had intermediate values of Tm and ΔH.

When dT-dA duplexes were examined a similar decrease in Tm was noted for S-dT, but there was little change in ΔH (Table 2 and Fig. 3). Because of the relatively low Tm's of oligomers containing only AT base pairs, a series of oligomers were synthesized containing varying proportions of GC base pairs (Table 2 and Fig. 4). It is apparent that the difference in Tm between the PS and PO oligomers decreases with increasing GC content up to 50%, when it levels off.

Nuclease Susceptibilities.

Several oligodeoxynucleotides were studied with regard to DNase sensitivity (Table 3 and Fig. 5). These include cytidine homopolymers, ODN-4 (an anti-message 28-mer complementary to the 3' region of the *art/trs* region of HIV BH10 clone)(2), and myc-1 (a 15-mer complementary to the initiation codon region of the C-*myc* oncogene)(4). The DNases employed were the predominantly endonuclease S1, the exo- and endonuclease P1, and snake venom (SV) phosphodiesterase. The concentration of S1 nuclease was ten-fold higher (100 μM/ml) for reactions of oligo-dC, since both the normal and PS analog were degraded extremely slowly by this enzyme. S1 and P1 nuclease digestion proceeded at 2-45 times slower for the S-ODNs than for the normal oligomers, with the 15-mer being somewhat more readily digested than the 28-mer. The 2S-capped myc-1 species behaved similarly to the all-PS compounds.

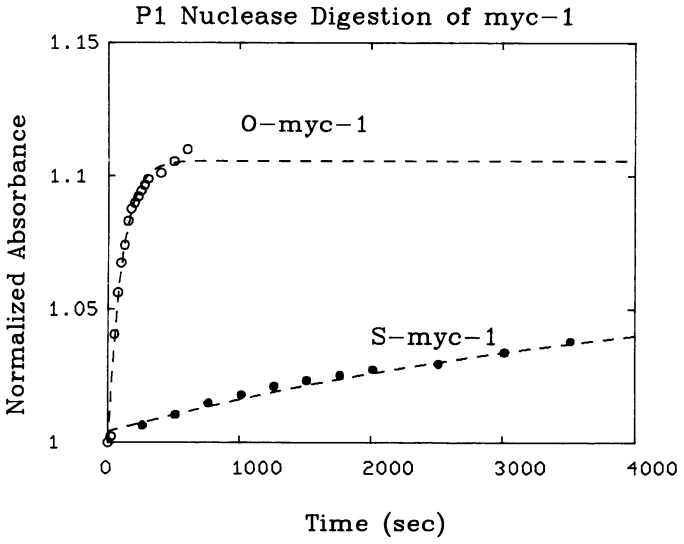

Figure 5. P1 nuclease digestion of O-myc-1 (open symbols) and S-myc-1 (closed symbols)(see legend to Table 3 for sequence). Normalized absorbance is plotted vs. time. Reactions were carried out in 50 mM Na acetate (pH 5.3). Data was fitted to a simple exponential function using the MLAB program.

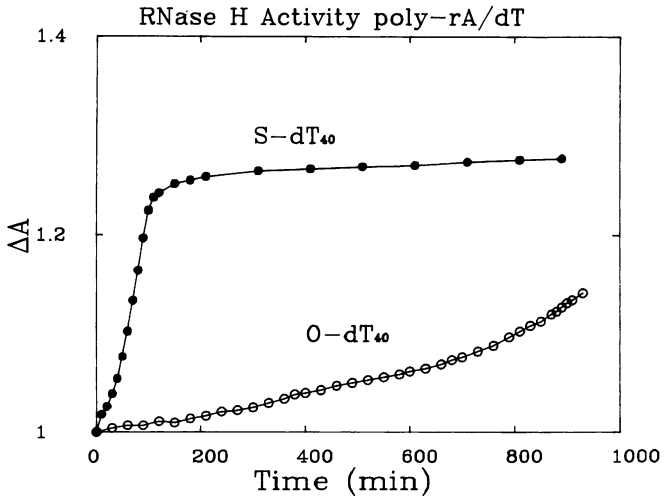

Figure 6. RNase H digestion of poly-rA/oligodeoxy-dT duplexes. Plotted are time vs. normalized absorbance at 260 nm. The relatively faster rate of digestion of the phosphorothioated (closed circles) vs. the normal species (open circles) is clearly shown.

The S-ODNs are all but impervious to the effects of SV phosphodiesterase, and in this case the differences from normal oligos are quite dramatic. For the homopolymers, a half-life of $>10^5$ sec was determined, which represents a three-log decrease of the rate versus the normal oligomer. Similar results were found with myc-1 and ODN-4. Digestion of 2S-cap-myc-1 by SV phosphodiesterase was also slowed (Table 3), but not as markedly as some of the other species. However, the half-life of 3',5'-2S-cap-myc-1 in 50% human serum (as measured by ^{31}P NMR) is >1 month vs 2-3 days for normal myc-1.

In Fig. 6 is shown the RNase H digestion of duplexes of dT_{40} with poly-rA. For the phosphorothioate species the reaction is over in about 100 min, whereas digestion of the normal duplex clearly proceeds far more slowly.

DISCUSSION

Modification of the phosphodiester backbone in order to produce a nuclease-resistant oligodeoxynucleotide has resulted in the preparation of methylphosphonate (8-10) and phosphorothioate (6,11) analogs. These compounds are stereoisomeric at phosphorus, but the syntheses are usually not stereospecific. Nevertheless, LaPlanche et al. (5) have reported elegant work in which they described the stereospecific synthesis of a single modified residue in an oligomer. They isolated the *R*p-*R*p and *S*p-*S*p diastereoisomers of the duplex $d(GGsAATTCC)_2$ and showed that the Tm of the *R*p-*R*p duplex was ca. 2.4° lower than the parent compound. However, the automated synthesis of all-phosphorothioate DNA is not stereospecific, and cannot be rendered so. Indeed, for the homopolymer S-dC_{28}, there are theoretically 2^{27} different stereoisomers. Thus, it is not possible to extrapolate the results of LaPlanche et al (5) to the systems we have studied. Parenthetically, it should be noted that the negative impact of stereoisomerism on hybridization would be expected to depend on the steric interference of the groups substituted at phosphorus.

We have shown that phosphorothioate oligodeoxynucleotides (S-ODNs) are effective anti-HIV agents (2). Several mechanisms have been hypothesized for that activity, including inhibition of reverse-transcriptase (Majumdar, C., Stein, C., Wilson, S., and Cohen, J.S., unpublished results), as well as anti-sense inhibition in which specific sequence phosphorothioate oligomers have been found to selectively inhibit HIV gene expression (Matsukura, M., Stein, C., Broder, S. and Cohen, J.S., unpublished results). In order to understand the length and sequence dependence (or lack thereof) of these S-ODNs on HIV expression, we wished to determine the actual Tm's of all-phosphorothioate analogs, prepared under the same conditions as

used for the biological experiments. The results presented here allow us to make some relevant conclusions.

The Tm of AT base pairs with phosphorothioate substitution show a greater reduction than exhibited by GC base pairs. Consequently, a high GC content is preferable if hybridization is operative for the biological function of such an oligomer. Furthermore, at least where AT base pairs are concerned, the values of ΔH are all significantly lower than for the normal analogs. This implies (equation *1*) that at any given temperature below the Tm there is more local melting for the PS-AT base pairs than for the PO-AT sequences. Whether this trend carries over to GC base pairs is uncertain, however. Values of ΔH for PS-CI base pairs are not appreciably lower than the PO-CI sequences, at least in the limited number of cases studied here, although values of Tm still remained depressed by about 10^{o}.

The DNase sensitivity of S-ODNs is far less than for their normal congeners. This parallels the results of Eckstein *et al.* (4), who noted that S1 hydrolysis of a phosphorothioate (dTpsdA) was 14-times slower than for the corresponding oxy compound. In this case, only the *Sp* diastereomer was cleaved by the enzyme. P1 nuclease was also shown (12) to accept only this stereoisomer. On the other hand, SV phosphodiesterase, which hydrolyzes normal DNA 100-fold faster than phosphorothioates, preferentially cleaves the *Rp* diastereomer (13). Burgers *et al.* (14,15) have noted that the relative rate of *Rp*/*Sp* hydrolysis is at least $9x10^3$. Furthermore, Stec et al. (6) have reported that in oligodeoxynucleotides having two adjacent phosphorothioates, SV phosphodiesterase did not hydrolyze *Rp*-*Sp* and *Sp*-*Sp* combinations. It is thus not surprising that the half-time for digestion of all-phosphorothioate DNA is exceedingly long; perhaps what is more surprising is that it can be digested at all. The slower rates seen with S1 and P1 nucleases are also understandable in terms of their diastereomer selectivity, but it is unclear why the myc-1-cap compound should be digested slower than the the all-phosphorothioate oligomer. Also of interest is that the dC homopolymers are much less sensitive to the effects of several nucleases than are the AT-containing oligos, with the PS being more slowly digested than the PO in each case. This is reminiscent of the inhibitory effect of dC_n on reverse transcriptase, with PS>>PO (Majumdar, C., Stein, C., Wilson, S., and Cohen, J.S., unpublished results).

The 3',5'-capped PS compounds seem to retain the nuclease resistance properties of the all-PS species, while having values of Tm and ΔH close to those of the all-oxygenated species. In addition they are extremely stable in 50% human serum, and on the basis of HPLC elution characteristics are somewhat more polar than the all PS-ODNs. These properties make the end-capped S-ODNs appear to be

ideal candidates as reagents for gene-specific inhibition, and studies are currently underway to evaluate them.

RNase H activity of duplexes of S-dT$_{40}$ with poly-rA is much greater than for the normal O-dT40. This observation may be explained by appreciation of the physico-chemical properties of the S-ODNs described above. S-ODNs with AT base pairs have Tm and ΔH depressed relative to normal ODNs. It can be shown from equation *1* that at 37^{o} the S-dT$_{40}$·poly-rA duplex (Tm=43.5^{o}) is about 10% melted. RNase H scission of the poly-rA strand would create duplex fragments that would melt at the assay temperature (37^{o}). Cleavage of poly-rA would have to occur at multiple sites for the O-dT$_{40}$·poly-rA duplex to become as unstable. This probably explains the apparent paradox of increased RNase H activity for S-dT$_{40}$.

While the mechanism of inhibition of gene expression by phosphorothioate oligomers in retroviral or oncogene systems is unknown, the factors of length and GC content are important ones when hybridization or an "anti-message" mechanism are operative. In fact, the results observed for HIV, that S-dC$_n$>>S-dT$_n$>S-dA$_n$ for base composition effectiveness, and n=>14 for length dependence of inhibition (2), can be understood simply in terms of the hybridization results presented here. (Note that it was not possible to test S-dG$_n$ because of the insolubility of these oligomers.)

Thus, although various complex biological mechanisms of action of these compounds may be invoked, we favor at this point a fairly simple explanation, namely that at 37^{o}C, the extent of duplex formation for S-dA/T with any HIV RNA (either viral or m-RNA) is inadequate, and this is presumably also true for S-dC$_{14}$. However, with S-dC$_{28}$, by comparison with the results for the S-dT oligomers, one can calculate (assuming the same ΔH) that there would be 90% duplex formation at 37^{o}. It has also been found that specific sequence oligomers antisense to regions of the HIV genome and with high GC content and length of 28 bases are effective inhibitors of HIV function (Matsukura, M., Stein, C., Broder, S. and Cohen, J.S., unpublished results).

The novel finding presented here that the RNase H activity of duplexes of poly-rA with S-dT$_{40}$ is much greater than that of the normal O-dT$_{40}$ (Fig. 6) also helps to rationalize the genetic inhibition results (2). Thus, while the PS compounds are expected to remain stable to DNases *in vivo* because of their general superior resistance to endo- and exo-phosphodiesterases (Table 3), the increased susceptibility of RNAs to which they hybridise to RNase H cleavage increases the biological potency of the S-ODNs as "anti-message" inhibitors of gene expression. In this case, we presume that either the intrinsic RNase H activity of the S-ODN-RNA duplexes is greater because of differences in degree of hybridization

(flexibility or local melting), or the breakup of the duplexes occurs more readily once an RNA break occurs opposite an S-ODN, or both.

Although many other issues, such as rate of membrane transport, pharmacokinetics and toxicology*in vivo*, and cost of production in large quantities, are still matters to be resolved, the results presented here go some way in helping to shed light on the possible mode of action of the phosphorothioate oligomers as potential agents for selective gene inhibition.

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