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Phosphorothioate-modified oligodeoxyribonucleotides. III. NMR and UV spectroscopic studies of the R_p-R_p, S_p-S_p, and R_p-S_p duplexes, [d($GG_sAATTCC$)]$_2$, derived from diastereomeric *O*-ethyl phosphorothioates

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ABSTRACT

2D-NOE and ^{1}H NMR chemical shift data obtained for the title oligonucleotides were compared with similar data previously reported [Broido et al. (1985) Eur. J. Biochem. 150, 117-128] for the unmodified "parent" structure, [d(GGAATTCC)]$_2$. The spectroscopically detectable structural perturbations caused by replacement of phosphate oxygen with sulfur were mostly localized within the G_sA moiety, and were greater for the R_p configuration wherein sulfur is oriented into the major groove of the B-helix. UV-derived T_m measurements gave the following order of stability for the duplexes in 0.4 M NaCl: unmodified (33.9±0.1 °C) ~ S_p-S_p (34.1 °C) > R_p-R_p (31.7 °C). The title compounds were prepared by a new and convenient synthetic route which utilized HPLC to separate the diastereomeric O-ethyl phosphorothioate precursors, (R_p)- and (S_p)-d[GG(S,Et)AATTCC], for subsequent de-ethylation by ammonia in water.

INTRODUCTION

Phosphorothioate analogues of nucleotides and nucleic acid polymers have proven to be very useful in studies that deal with a wide variety of subjects, such as the conformational properties of DNA,[1-4] stereochemistry and mechanisms of enzyme reactions,[5-9] recognition of DNA by proteins,[10] molecular biology,[11] and antiviral agents.[12] With regard to internucleotide linkages in DNA, substitution of one of the diastereotopic oxygens with sulfur leads to a pair of phosphorothioate-containing diastereomers whose absolute configuration at phosphorus is designated as either R_p or S_p. These diastereomers necessarily have different structural and electronic features, especially at the site of modification; however, the magnitude of these differences are unknown, and their physico-chemical effects have been studied in only a few cases.[1-4,7,8] Analyses of experimental evidence regarding bond order and charge delocalization in thiophosphate anions have led to the conclusion[13] that the negative charge is localized mainly on sulfur, which can be represented by the partial valence-bond structure $O{=}P{-}S^-$. X-Ray

crystallographic data for a phosphorothioate-modified oligonucleotide has been very recently obtained (F. Eckstein, private communication) and will provide the first quantitative information regarding molecular structure in the solid state. Nevertheless, inspection of molecular models clearly indicates that the P-S^- bond-axis for the R_p configuration is oriented into the major groove,[3,4] whereas in the S_p configuration this bond-axis points away from helix with sulfur thus being positioned on the face of the sugar-phosphate back-bone.[4] The conformational effects of these alternative orientations can be studied in solution by NMR and UV methods that have been applied to numerous synthetic oligonucleotides and base modified versions thereof;[14] far fewer reports of this sort have dealt with backbone modifications involving sulfur[1-4] or alkyl groups.[15,16]

We are unaware of any conformational investigations of a mono-phosphorothioate analogue of DNA. This together with the following additional factors led us to select the title duplexes as prototypal compounds for examining the orientational effects of sulfur substitution. EcoRI endonuclease-catalyzed cleavage of the parent duplex, $[d(GGAATTCC)]_2$, and base-modified variants have been investigated in considerable detail.[17,18] Moreover, the action of this enzyme is known to be remarkably sensitive to both the location and stereochemistry of sulfur within phosphorothioate analogues of this octanucleotide.[6d,7-9,18] A complete proton-assignment and detailed conformational analysis of the parent duplex using the two-dimensional nuclear Overhauser effect (2D-NOE) technique and a complete relaxation matrix approach[19] have been reported by Broido et al.,[20] and the results were recently compared to an ethyl phosphorotriester modified analogue, $\{d[GGAA(Et)TTCC]\}_2$, as a way of identifying substituent-induced physico-chemical differences.[16,21] Finally, it was of interest that conformational studies[1-4] of poly-phosphorothioate analogues of DNA have indicated marked nearest-neighbor effects; however, the cases analyzed to date have dealt mostly with enzymatically derived R_p thioates, and to our knowledge none of the examples have included a sequence wherein the the modified linkage is flanked by two purine residues, such as that in the title compounds.

For all of the aforementioned reasons, and as part of our ongoing studies of thioate[6d,8,22] and triester[16,21] analogues of DNA, we describe here a new and relatively efficient synthetic approach to the individual diastereomers of $d(GG_sAATTCC)$, which were studied previously by Connolly et al.[7] and Stec et al.[8] The present data reveals greater conformational adjustments and destabilization caused by the inward-oriented R_p vs. outward-oriented S_p

P-S^- moiety in the B-helix of these diastereomeric analogues of DNA.

EXPERIMENTAL

General procedures[23,24] for automated (Applied Biosystems Models 380A and 380B) solid-phase synthesis using commercially available (Applied Biosystems) long-chain alkylamine controlled pore glass support, O-methyl, O-β-cyanoethyl, and home-made[18,21] O-ethyl phosphoramidite reagents, with inclusion of a sulfurization step,[8] have been described in the cited references. Analysis and isolation of oligonucleotides by HPLC using previously reported methods[25,26] employed the following columns, eluents, and gradients: column 1, μBondapak C_{18} (Waters, 7.8 mm x 30 cm); column 2, Zorbax ODS (DuPont, 21.1 mm x 25 cm); column 3, PRP-1 (Hamilton, 7 mm x 30 cm); eluent A, acetonitrile; eluent B, 0.1 M triethylammonium acetate, pH 7.0; gradient I, 20-30% A *vs.* B at 1%/min for 10 min followed by isocratic elution, 4 mL/min; gradient II, 5-25% A *vs.* B at 1%/min for 20 min, 4 mL/min; gradient III, 10-20% A *vs.* B at 0.5%/min for 20 min, 13.5 mL/min; gradient IV, 10-13% A *vs.* B at 0.075%/min for 40 min, 3 mL/min.

Preparation of the Diastereomeric Mixture (R_p,S_p)-d($GG_sAATTCC$). A 10-μmol scale synthesis was carried out using a 3-min coupling time for O-methyl phosphoramidite reagents, a 1-h sulfurization step at *ca.* 50-55 °C, and backbone demethylation with PhSH-Et_3N. HPLC analysis (column 1, gradient I) of the crude 5'-dimethoxytrityl (DMT) derivative led to elution of an apparent single peak at 13.2 min, which was then preparatively collected upon injection of the entire sample (column 2, gradient I, 13.5 mL/min, product elution time 12-14 min). The DMT-bearing material was lyophilized, detritylated, and then analyzed by HPLC (column 1, gradient II), which showed the presence of a 17:83 area-ratio of peaks identified as d(GGAATTCC)[20] (12.2 min) and d($GG_sAATTCC$)[8] (12.8 min) by comparison (elution time) with authentic samples. The latter material was preparatively collected (2 injections, column 2, gradient III, product elution time 9-10 min), lyophilized, precipitated twice from 2 mL of 30:70 v/v 0.2 M NaCl-EtOH, and then eluted with water from a column of Chelex-100 (4-mL bed, Na^+-form) to afford 322 OD_{260}-units (38% isolated yield based on starting nucleoside) of a *ca.* 1:1 mixture of (R_p,S_p)-d($GG_sAATTCC$), which was identified by ^{31}P NMR analysis.[7,8]

Preparation of Diastereomerically Pure (R_p)- and (S_p)-d($GG_sAATTCC$). Three 1-μmol scale syntheses were carried out in parallel using a 20-sec coupling time for both the O-β-cyanoethyl- (0.1 M) and 5'-DMT-N^2-isobutyryl-2'-deoxyguanosine 3'-O-ethyl-N,N-diisopropyl-phosphoramidite (0.2 M) reagents,

and a 1-h sulfurization step at 55-60 °C.[21] Treatment with PhSH-Et_3N was excluded prior to cleavage from the support with conc. NH_4OH (1 h, 25 °C) and backbone/base-deprotection with conc. NH_4OH (48 h, 25 °C). Each of the crude 5'-hydroxyl O-ethyl phosphorothioate products was separately purified by HPLC (4 injections, column 3, gradient IV) to give the fast-eluted (24 min) and slow-eluted (26 min) diastereomers of the desired precursor, d[GG(S,Et)AATTCC], which were pooled, lyophilized, and then desalted (Sephadex G-25M PD-10 column) to afford a total of ca. 30 OD_{260}-units of each material (12% isolated yield based on starting nucleoside). These fast- and slow-eluted O-ethyl phosphorothioates (ca. 25 OD_{260}-units) were separately heated at 70 °C in conc. NH_4OH (1 mL) for 22.5 h, and the lyophilized products were identified as (R_p)- and (S_p)-d($GG_sAATTCC$), respectively, by previously reported enzymatic methods[8,22] and by ^{31}P NMR analysis.[7,8]

NMR Measurements. One-dimensional ^1H (400 MHz) and ^{31}P (161 MHz) NMR spectral data were obtained with a JEOL GX-400 NMR spectrometer. The 2D-NOE data were obtained with a Nicolet NM500 spectrometer equipped with a Nicolet 1280 computer. Pure-absorption mode spectra were obtained as described previously for the parent octamer.[20] The sample for 2D-NOE data collection (5.0 mM single-strand concentration) contained NaCl (0.180 M), sodium phosphate (0.100 M), and EDTA (0.2 mM); pH* ≅ 7. ^1H and ^{31}P chemical shifts were referenced to internal residual HDO = 4.84 ppm, and external aqueous trimethylphosphate = 0.0 ppm, respectively.

Tm Measurements. Oligonucleotide UV spectral analysis and melting experiments were performed with a Cary 219 spectrophotometer interfaced to an Apple IIe microcomputer as described previously.[16] A total of 10 absorbance readings were collected and averaged for each point on the absorbance vs. temperature (Tm) curve. Tm measurements were initiated at ≤ 0 °C and the temperature ramp was 0.5 °C/min. Samples (1.5×10^{-4} M oligomer) contained PIPES buffer (10 mM), EDTA (1 mM) and NaCl (PIPES 00 = no added NaCl; PIPES 10 = 0.1 M NaCl; PIPES 20 = 0.2 M NaCl; PIPES 40 = 0.4 M NaCl; PIPES 100 = 1 M NaCl); pH = 7.0.

RESULTS AND DISCUSSION

Synthesis. Unlike the DNA polymerase-mediated synthesis of phosphorothioate analogues which produces only R_p internucleotide linkages,[1,2,5] chemical routes to these polymers can afford both configurations at phosphorus. Among the various synthetic methods[4,7,8,12,27,28] that have been reported to date, block-coupling[4,7] of

pre-separated diastereomerically pure dinucleoside phosphorothioate derivatives offers the advantage of directly providing chirally pure oligomer products. On the other hand, sulfurization[7,8,12,28] of epimeric phosphite triester intermediates can be fully automated.[8] The successful use of HPLC to separate the diastereomers which are produced by the latter method has been found[8,26] to be dependent upon the number of such isomers, chain length, location of the phosphorothioate linkage, and the presence or absence of a 5'-DMT or 5'-phosphate group, as well as the chromatographic conditions. Thus, Connolly *et al.*[7] were unable to resolve (R_p)- and (S_p)-d($GG_sAATTCC$) while other investigators found[26] that partial separation could be obtained at least with analytical-scale injections. Improved separation and the capability for preparative-scale chromatography were obtained with either the corresponding 5'-DMT[8] or 5'-phosphate[7] derivatives. The results of more recent studies[18,21] of ethyl phosphotriester analogues of DNA have indicated that even better separations are possible with 5'-hydroxyl *O*-ethyl phosphorothioates, relative to the 5'-DMT and 5'-phosphate derivates. Moreover, the ethyl group can be stereospecifically removed by ammoniolysis with complete retention of configuration to give a chirally pure phosphorothioate linkage.[16,21]

The 2D-NOE experiments reported here were carried out with the mixture of R_p-R_p, R_p-S_p, and S_p-S_p diastereomers of the duplex, $[d(GG_sAATTCC)]_2$, which was obtained by HPLC without fractionation of the previously reported[8] 5'-DMT derivatives. To prepare the diastereomerically pure samples of this octamer, a combination of *O*-β-cyanoethyl and *O*-ethyl[18,21] phosphoramidite reagents were used to construct the support-bound fully protected sulfur-containing oligomer.

Base- and backbone-deprotection were carried out with conc. NH_4OH under mild conditions (25 °C, 48 h) so as to preserve the R_p and S_p *O*-ethyl phosphorothioate linkages in the desired precursor, d[GG(S,Et)AATTCC]. The extent of loss of these diastereomers by competing de-ethylation was relatively small (*ca.* 5-10%). The *O*-ethyl phosphorothioate diastereomers were readily separated by reversed-phase HPLC, and they were individually reacted with conc. NH_4OH under conditions (70 °C, 22.5 h) which corresponded to *ca.* 5-times the estimated[21] half-life for de-ethylation. Thus, ammoniolysis of the fast- and slow-eluted *O*-ethyl phosphorothioate diastereomers quantitatively afforded (R_p)- and (S_p)-d($GG_sAATTCC$), respectively, which were configurationally assigned based on the results of separate treatments with

snake venom phosphodiesterase and nuclease P1 ($\underline{R}_p$ and $\underline{S}_p$ selectivity, respectively[7,8,21]).

Despite the prolonged exposure to hydroxide ion during the aforementioned ammoniolytic de-ethylation reaction, there was no detectable (<5%) loss of sulfur, as judged by ^{31}P NMR signal integrations. Other advantages of this synthetic route to internucleotide phosphorothioate linkages are that the de-ethylation reaction with ammonia is very simple, essentially quantitative, and does not require subsequent work-up other than the removal of volatile materials *in vacuo*, which are significant improvements over the previously reported[18] use of $PhSH-Et_3N$.

NMR Findings. ^{31}P chemical shifts for the phosphodiester and phosphorothioate linkages in the stereochemically pure $\underline{R}_p$-$\underline{R}_p$ and $\underline{S}_p$-$\underline{S}_p$ duplexes were in agreement with the values previously reported. The downfield shift for the $\underline{R}_p$ signal relative to the $\underline{S}_p$ signal followed the established trend.[7,8] The ^{31}P NMR spectrum of the duplexes derived from ($\underline{R}_p$,$\underline{S}_p$)-d(GG_s-AATTCC) showed two rather than three phosphorothioate signals, consistent with the relatively large distance between these linkages in the three possible duplexes, $\underline{R}_p$-$\underline{R}_p$, $\underline{S}_p$-$\underline{S}_p$, and $\underline{R}_p$,$\underline{S}_p$ (see below). As observed previously,[7] some of the ^{31}P signals in the ca. 4.0-4.5 ppm region exhibited slightly different shifts for the $\underline{R}_p$-$\underline{R}_p$ and $\underline{S}_p$-$\underline{S}_p$ duplexes, suggesting that the modifications have at least some effect on the conformation of the neighboring nucleotides.

Localized structural perturbations in modified oligonucleotides (*e.g.*, ethyl phosphotriesters[16] have been monitored directly by comparison of ^1H chemical shifts to those of the unmodified, parent oligonucleotide. Since many of the *ca.* 100 ^1H-resonance signals for each of the diastereomeric octamers reported here were unresolved in the 1D spectrum, 2D spectroscopy was employed. The 2D-NOE data were obtained for a *ca.* 50:50 mixture of the $\underline{R}_p$ and $\underline{S}_p$ oligomers, with the expectation of obtaining some resolved signals for the $\underline{R}_p$-$\underline{R}_p$, $\underline{R}_p$-$\underline{S}_p$ (= $\underline{S}_p$-$\underline{R}_p$) and $\underline{S}_p$-$\underline{S}_p$ duplexes. Interestingly, except for two protons (G2-H_8 and A3-H_3'), only one set of ^1H NMR signals was observed. This somewhat unexpected finding suggested that the structural and electronic effects of the stereochemical orientation of $O=P-S^-$ moiety were highly localized in the oligonucleotide duplexes, *i.e.*, the presence of either a $\underline{R}_p$ or $\underline{S}_p$ phosphorothioate linkage at the G2-P-A3 phosphate position did not detectably influence the ^1H chemical shifts of the adjacent, H-bonded T6 or C7 base residues.

8 7 6 5 4 3 2 1
3' C C T T A A_S G G 5'
5' G G_S A A T T C C 3'
1 2 3 4 5 6 7 8

The ^{1}H NMR chemical shifts were assigned using conventional procedures[20b,29] and the values are summarized in Table I, together with those for the parent duplex. To facilitate comparison of these values, the chemical shift differences for signals of the thioate-modified and parent duplexes is shown graphically in Fig. 1. As found previously,[16] signals for the terminal residues (G1 and C8) display some variations in shift, possibly due to residual triethylammonium acetate (from HPLC) in the samples which can catalyze NH-N proton exchange and may affect the nature of the Watson-Crick hydrogen bonding.[30]

All of the remaining ^{1}H chemical shifts were very similar to those measured for the parent octamer (Fig. 1), except for the nucleotide residues adjacent to the site of substitution. One set of resonance signals was

Table I. ^{1}H NMR chemical shift assignments for [d($GG_sAAATTCC$)]$_2$ and [d(GGAATTCC)]$_2$ [a]

Residue[b]		H_8	H_6	H_5	CH_3	H_1'	H_2'	H_2''	H_3'	H_4'	H_5'	H_5''
G1	R,S	7.79				5.67	2.43	2.70	4.84	4.24	3.71	3.62
	P	7.80				5.62	2.47	2.68	4.80	4.19	3.66	3.66
G2	R,S	7.80				5.23	2.62	2.80	5.10	4.32	4.25	4.05
	P	7.80				5.42	2.65	2.74	5.00	4.33	4.13	4.05
A3	R	8.37				6.10	(2.77	3.00)	5.11	4.51	4.31	4.26
	S	8.24				6.14	(2.80	3.02)	5.15	4.54	4.32	4.32
	P	8.16				6.04	2.74	2.96	5.09	4.48	(4.22	4.16)
A4	R,S	8.18				6.20	(2.62	2.96)	5.07	4.52	4.30	4.30
	P	8.16				6.20	2.61	2.96	5.03	4.50	4.33	4.28
T5	R,S		7.14		1.29	5.94	2.01	2.58	4.83	4.38	4.23	4.15
	P		7.20		1.29	5.93	2.02	2.58	4.80[c]	4.37	4.22	4.14
T6	R,S		7.38		1.55	6.14	2.14	2.57	4.92	4.23	4.22	4.12
	P		7.40		1.58	6.12	2.19	2.58	4.91	4.22	4.22	4.13
C7	R,S		7.52	5.68		5.97	2.12	2.42	4.86	4.10	4.23	4.17
	P		7.54	5.68		6.02	2.16	2.47	2.84	4.10[c]	(4.22	4.14)
C8	R,S		7.42	5.32		6.18	2.27	2.31	4.57	4.23	4.06	3.99
	P		7.50	5.51		6.18	2.28	2.28	4.56	4.19	4.05	4.01

[a] Data for the parent oligonucleotide taken from ref. 20 except as noted; T=20 °C; referenced to internal residual HDO (4.84 ppm). [b] The letters R, S, and P refer to the R_p- , S_p- and parent sequences, respectively. [c] It is highly likely that the chemical shift assignments reported by Broido, et al., for T5-H_3' (4.48 ppm) and C7-H_4' (4.37 ppm) are incorrect. One of the present authors (M.F.S.) has analyzed a total of 8 derivatives of the parent oligonucleotide, where the modification sites are well separated from the above protons, and in each case the chemical shifts are in the range of 4.80 ± 0.05 ppm for T5-H_3' and 4.10 ± .06 ppm for C7-H_4'. Examination of the limited spectra published by Broido, et al., supports the new values given in Table I.

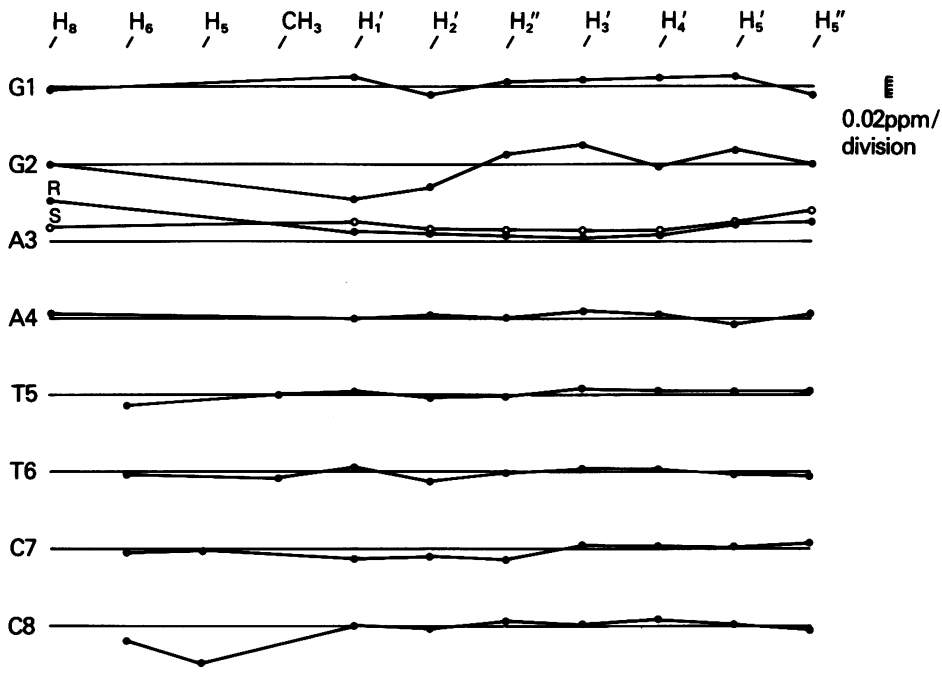

Figure 1. Diagram showing the chemical shift differences for protons of the $\underline{R}_p$- and $\underline{S}_p$- isomers of $[d(GG_8AAATTCC)]_2$ relative to the parent oligonucleotide, $[d(GGAATTCC)]_2$. The letters R and S refer to signals for the $\underline{R}_p$- and $\underline{S}_p$ isomers, respectively (see text for numbering).

observed for the G2 base moiety whereas two distinct sets of signals were observed for the A3 residue. That the chemical shifts for G2-H_3' and A3-H_5', H_5'' were significantly different from those of the parent duplex was reasonable since these protons are proximate to the phosphothioate moiety. Although the G2-H_8 signal was unperturbed, the sugar protons of the G2 residue were shifted significantly relative to the parent octamer.

The ribose proton chemical shifts for the A3 residue were relatively unaffected by the phosphorothioate group (except for H_5', H_5'' as discussed above). However, the A3-H_8 signal of the $\underline{R}_p$ isomer was shifted significantly to lower field relative to the parent octamer, whereas the shift for the $\underline{S}_p$ isomer was only slightly affected. This finding was significant, as it was consistent with the absolute stereochemistry of the phosphorothioate linkages: for a B-type geometry, the sulfur of the $\underline{R}_p$ isomer is directed inward toward the major groove of the duplex and is close to A3-H_8, whereas the sulfur in the $\underline{S}_p$ duplex is directed outward toward the solvent.

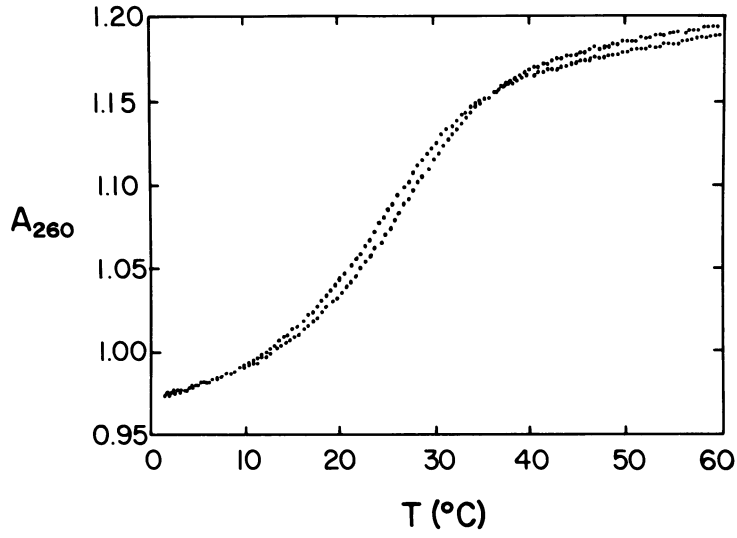

Figure 2. Plots of absorbance at 260 nm as a function of temperature for the $\underline{R}_p$-$\underline{R}_p$ (lower Tm) and $\underline{S}_p$-$\underline{S}_p$ (higher Tm) duplexes. The oligomer concentration was 1.5 x 10(-4)M and the buffer was PIPES 10.

Melting Behavior. UV melting studies revealed a small but significant difference in the relative stabilities of the stereochemically pure $\underline{R}_p$-$\underline{R}_p$ and $\underline{S}_p$-$\underline{S}_p$ duplexes under standard conditions (1.5 x 10^{-4} M oligomer, pH 7.0). Representative melting curves for these duplexes in PIPES 10 buffer are shown in Fig. 2. Broad melting curves were observed since the helix to single-strand transitions have relatively low cooperativity in short oligomers. The curves are monophasic and thus indicate that no significant amount of hairpin[14e] or other conformation were formed under these conditions as the temperature was increased. Each curve was asymmetric on the low temperature side which suggested that terminal base pair unstacking occurs before duplex dissociation. In the PIPES 40 buffer the $\underline{S}_p$-$\underline{S}_p$ duplex Tm (34.1 °C) was virtually identical to that of the parent duplex (33.9±1 °C), while the Tm for the $\underline{R}_p$-$\underline{R}_p$ duplex (31.7 °C) was ca. 2.4 °C lower than that of the parent duplex. Although Tm values have been reported previously for these[7] and other[3,4] phosphorothioate-modified oligonucleotides, differences in concentration, temperature, buffers, and salts employed preclude meaningful comparisons.

Similar melting curves were obtained for the oligomers over NaCl concentrations from 0.05 to 1.0 M. Monophasic melting transitions were obtained at all salt concentrations. The Tm values are plotted as a function

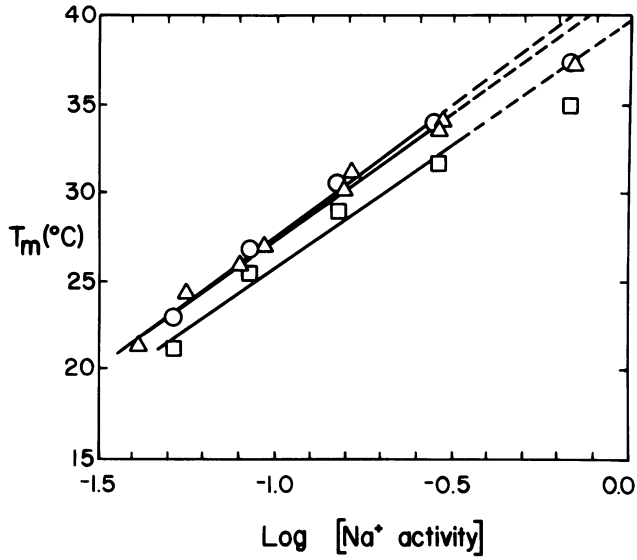

Figure 3. Oligomer Tm values as a function of log (Na^+ activity) for the parent (Δ), $\underline{R_p}$-$\underline{R_p}$ (□), and $\underline{S_p}$-$\underline{S_p}$ (○) duplexes. The points for the parent and $\underline{S_p}$-$\underline{S_p}$ oligomers overlap extensively with the line for the thioate derivative having a slightly greater slope. The Tm values for the 1M NaCl experiments fall significantly below the least-squares line for the other salt concentrations and this point was not included in calculating the best-fit lines in the plot. The lines in the high salt region of the figure are, therefore, indicated with a dashed line.

of ionic strength in Fig. 3. As previously demonstrated for ethyl phosphotriester modified oligonucleotides,[16] the Tm values for 1 M NaCl are less than expected and the results obtained at that ionic strength were not used in calculating the slope of the Tm vs. log (Na^+ activity) data. From Fig. 3 it can be seen that all of the oligonucleotides gave virtually the same slope (14.6), which was as expected since these oligomers all possess the same total charge. This is in contrast to the ethyl phosphotriester modified oligonucleotides[16] where the reduced charge gave a reduced slope.

CONCLUSIONS

An important feature of Fig. 3 was that the difference in Tm for the $\underline{R_p}$-$\underline{R_p}$ duplex relative to the parent and $\underline{S_p}$-$\underline{S_p}$ duplexes was maintained at all salt concentrations. This strongly suggested that the ca. 2.4 °C destabilization of the $\underline{R_p}$-$\underline{R_p}$ duplex was not simply the result of an electrostatic effect. In fact, it was most interesting that the orientation of the sulfur in the $\underline{R_p}$-$\underline{R_p}$ duplex (pointing into the major groove) lead to

both destabilization and to significant ^1H NMR chemical shift deviations (vide supra) for protons near the modification site. Thus, the downfield shift of A3-H_8 suggested that A3-H_8 and the inward oriented sulfur are close in space, perhaps close enough that the destabilization may be the result of a steric effect. In light of recent reports which conclude that, in phosphorothioate-modified DNA, the negative charge resides almost exclusively at sulfur and not oxygen, and given that the effective radius of S^- should be greater than that of a neutral oxygen, the above conclusion seems reasonable, albeit simplistic. By application of the presently reported synthetic methods, it is possible to isolate multi-milligram quantities of stereochemically pure R_p and S_p phosphorothioate-modified oligonucleotides. With such samples, we hope to obtain more quantitative information on the nature of the structural perturbations caused by phosphorothioate modifications using the quantitative 2D-NOE distance determination approach. The results of these future studies may provide insights regarding the intriguing phenomenon of "remote control of diastereoselectivity"[6d] of nucleases towards phorphorothioate analogues of oligonucleotides.

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