Effects of pendant groups at phosphorus on binding properties of d-ApA analogues

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ABSTRACT

The interaction of several synthetic analogues of d-ApA with Poly U and Poly dT was examined to explore the effects of substituents at phosphorus on binding properties of oligonucleotides. These analogues contained a bulky, lipophilic group (2,2,2-trichloroethoxy or 2,2,2-trichloro-1,1-dimethylethoxy) a small, uncharged hydrogen-bonding group (amido), or a cationic phosphoramidate (2-aminoethylamido, protonated in neutral aqueous media) in place of the anionic oxygen of the internucleotide phosphate. As determined by "melting curves" each formed a complex with Poly U more stable than the Poly U·d-ApA complex. Binding to Poly dT was comparable or in some cases stronger. Checks on composition (mixing curves) revealed the expected stoichiometry of 1dA:2U (or 2dT). Stereochemistry at phosphorus influenced stability of the complexes, but the effect was not a major one. These results suggest that oligonucleotides containing large, lipophilic groups, as well as small non-ionic groups (e.g., the methyl phosphonates) or polar groups, could be useful as probes in hybridization experiments.

INTRODUCTION

Defined oligonucleotides possessing pendant groups linked covalently to specified phosphorus atoms in the backbone chain have potential as tools in molecular biology. The pendant groups could serve as fluorescent markers, as signals to stop enzymatic reactions at selected sequences, as lipophilic centers to enhance interaction with membranes, as factors that stabilize hybridization, and as sites for triggering cross-linking reactions. Pioneering studies with small pendant groups (methyl and ethyl esters and methyl phosphonates) were made by Miller and Ts'o and co-workers,¹⁻³ who made the significant observation that such non-ionic oligomers would hybridize to complementary oligonucleotides and inhibit certain cellular processes. Other backbone modifications in polynucleosides include anionic thiophosphate⁴ and phosphoramidate⁵ internucleotide links.

This paper introduces several studies directed toward development of synthetic procedures for oligonucleotides with a variety of modified backbones and elucidation of the properties of these substances. To guide

research on the more complex materials we first explored some deoxyadenylyl-deoxyadenosine derivatives with modifications at phosphorus. As shown for nucleotide esters and phosphonates,^{1,2} complexes derived from small adenylate oligomers and polyuridylic acid (Poly U) serve as useful models for interactions in polynucleotide analogues.

We report here on complexes of compounds I-V with Poly U and polythymidylic acid (Poly dT). Compounds I, II and V are prototypes of polynucleotides with bulky, lipophilic groups at phosphorus. Compound III was selected to explore the effect of a cationic substituent at phosphorus on binding properties. Compound IV tests the effect of a small substituent (NH₂) which is uncharged yet forms hydrogen bonds in aqueous media.

COMPOUND	DESIGNATION	X	n
I	d-A(TCE)A	-OCH ₂ CCl ₃	1
II	d-A(TCDM)A	-OC(CH ₃) ₂ CCl ₃	1
III	d-A(NHC ₂ H ₄ NH ₂)A	-NHCH ₂ CH ₂ NH ₂	1
IV	d-A(NH ₂)A	-NH ₂	1
V	d-A(TCE)A(TCE)A	-OCH ₂ CCl ₃	2

MATERIALS AND METHODS

General Methods. Analytical thin layer chromatography (TLC) was carried out on Eastman 6060 or EM Merck silica plates or reverse phase plates (Whatman MKC18F). Electrophoreses were run on Analtech Avicel 250 μ plates using a Savant flat plate 1000 volt instrument; migration relative to dPT is reported as the R_m value. To facilitate solubility of oligonucleotide derivatives protected with trityl groups, a buffer consisting of 1:1 dioxane:0.2 M aq. NaH₂PO₄ (pH 6.8) was used to wet the Avicel plate, which was connected to the solvent troughs with paper wicks moistened with 0.1 M NaH₂PO₄ buffer. Nucleosidic material was visualized under a UV lamp. Dimethoxytrityl ethers gave an orange color when exposed to HCl vapor or sprayed with 10% aq. HClO₄.

Ultraviolet spectral data were obtained with a Perkin-Elmer Coleman 270 spectrophotometer equipped with a digital temperature controller. ³¹P NMR spectra were recorded at 36.20 MHz with a JEOL-90 Q spectrometer with triphenyl phosphate as an internal standard (chemical shifts are reported as ppm relative to triphenyl phosphate). The solvent was *t*-butyl alcohol/water (2:1, v/v) unless otherwise noted.

Pyridine was distilled from *p*-toluenesulfonyl chloride, redistilled from

CaH₂ and stored over Linde 4-A Molecular Sieves under nitrogen. Tetrahydrofuran (THF) was distilled from LiAlH₄ and stored similarly. Ethylenediamine was distilled from CaH₂ and stored in sealed vials at 0°C or in the frozen state. Homopolyribonucleotides were purchased from Miles Laboratories. The extinction coefficients used in preparing solutions were: ϵ_{265} 9430 for Poly U, ϵ_{265} 8520 for poly dT, ϵ_{258} 9180 for poly A, and ϵ_{258} 12,700 for d-ApA. Determination of extinction coefficients for di- and trinucleotide derivatives followed previously described methods.¹

2,2,2-Trichloroethyl Ester of Deoxyadenylyl-(3'-5')-deoxyadenosine (d-A(TCE)A). 2,2,2-Trichloroethyl phosphorodichloridite (0.14 mL, 1 mmol) was added dropwise with stirring to 1,2,4-triazole (0.34 g, 5 mmol) in THF-pyridine (2:1, v/v, 3 mL) at -78°C. After 10 min. of stirring the following reagents were added in succession (time of reaction and temperature indicated in the last parenthesis): dimethoxytrityldeoxyadenosine (d-DMTA) (0.50 g, 0.9 mmol) in THF-pyridine (2:1 v/v, 1.5 mL) (30 min. at -78°C); a slurry of deoxyadenosine (0.73 g, 2.7 mmol) in THF-pyridine (2:1 v/v, 3 mL) (20 min. at 0°C); and iodine (0.25 g, 1 mmol) in THF-H₂O (2:1 v/v, 3 mL) (20 min. at 0°C). Excess I₂ was reduced with Na₂S₂O₅, and the products were partitioned between CH₂Cl₂ and H₂O. Concentration of the organic layer and chromatography of the residue (2000 μ Analtech silica plate, 15% CH₃OH in CH₂Cl₂) afforded the epimers of d-DMTA(TCE)A as overlapping bands, which were eluted together: 0.39 g (44%); UV (95% C₂H₅OH) $\lambda_{\max}$ 238 nm (ϵ 21,670) and 259 nm (ϵ 23,250); $\lambda_{\min}$ 225.5 nm (ϵ 17,480) and 244.5 nm (ϵ 20,050). Anal. (after precipitation from CH₂Cl₂ into pentane). Calcd. for C₄₃H₄₄N₁₀O₁₀PCl₃: C, 51.74; H, 4.44; N, 14.03. Found: C, 51.61; H, 4.46; N, 14.30. For isolation of the epimers at phosphorus the d-DMTA(TCE)A sample was rechromatographed on 250 μ Merck silica plates by developing with 15% CH₃OH in CH₂Cl₂ until a good separation of bands was achieved. Individual bands were eluted with 25% C₂H₅OH in CH₂Cl₂ and the products were lyophilized from *t*-butyl alcohol. d-DMTA(TCE)A from the fast running band (designated Hrf isomer) showed R_f 0.27 (15% CH₃OH in CH₂Cl₂) and a peak at 13.5 ppm in the ³¹P NMR spectrum; that in the slower running band (designated Lrf isomer) showed R_f 0.20 (15% CH₃OH in CH₂Cl₂) and a peak at 13.9 ppm in the ³¹P NMR spectrum. The DMT group was removed from each isomer by treating with 80% aq. acetic acid (2 h, room temperature). Concentration, extraction with ether to remove dimethoxytritanol, and lyophilization from *t*-butyl alcohol afforded the separate isomers of d-A(TCE)A as fluffy white solids: Hrf sample, $\lambda_{\max}$ 258.5 nm (ϵ 26,200), $\lambda_{\min}$ 226.5 nm (ϵ 6,420); Lrf

sample, $\lambda_{\max}$ 258 (ϵ 26,800); $\lambda_{\min}$ 226.5 (ϵ 6,100).

For further characterization a sample of d-DMTA(TCE)A (72 mg), prepared similarly, was allowed to stand with 80% aq. acetic acid (2 mL) until fully detritylated (TLC test); then the solution was concentrated and chromatographed on a silica plate (developed first with 20% and then 33% CH₃OH in CHCl₃) to give 45 mg (89%) of the mixed isomers of d-A(TCE)A; R_f 0.16 (silica, 20% CH₃OH in CHCl₃). Anal. Calcd. for C₂₂H₂₆Cl₃N₁₀O₈P•H₂O: C, 37.01; H, 3.96; N, 19.62. Found: C, 37.33; H, 3.92; N, 19.74. A portion of this compound was deprotected by treatment with zinc in acetylacetone-pyridine,⁶ followed by precipitation of ZnS by H₂S. On hydrolysis with snake venom phosphodiesterase⁷ this d-ApA was completely converted to dpA and dA (1.06:1.00), confirming the 3'-5' linkage in the d-ApA sample and therefore in the parent phosphotriester.

This synthesis employing d-DMTA (unprotected NH₂) and deoxyadenosine (unprotected NH₂ and 3'OH) directly in the phosphitilation reactions depends on the high selectivity of the triazolyphosphine reagents at -78°C. Attack on the adenine NH₂ is very slow under these conditions, and reaction at the 5'OH is much faster than reaction at the 3'OH of the nucleoside. Excess deoxyadenosine was used in the second condensation to ensure a favorable ratio of 5' to 3'OH groups throughout the course of the reaction. The low reactivity of the adenine NH₂ groups at -78°C has been noted previously for reactions of trichloroethyl phosphorodichloridite.⁸

Bis-2,2,2-Trichloroethyl Ester of Deoxyadenylyl-(3'-5')-deoxyadenylyl-(3-5')-deoxyadenosine [d-A(TCE)A(TCE)A]. d-DMTA(TCE)A(TCE)A was prepared from d-DMTA(TCE)A (0.2 mmol), Cl₂POCH₂CCl₃ (0.4 mmol), triazole (2 mmol) and dA (0.6 mmol) by the same procedure used to make d-A(TCE)A. The crude mixture was separated by preparative chromatography (2000 μ Analtech plates; 30% CH₃OH in CH₂Cl₂). Elution of the main band (R_f 0.3) and precipitation of the product from hexane gave the desired triester as a mixture of stereoisomers (115 mg, 40%). On rechromatography on silica (250 μ Merck plate; 25% CH₃OH in CH₂Cl₂) the products resolved into three bands. Repeated development gave a good separation. Recovery by elution yielded the individual products, designated HRf (R_f in 25% CH₃OH in CH₂Cl₂ 0.39), MRf (R_f 0.34) and LRF (R_f 0.25) fractions. Each was then subjected to 80% aq. acetic acid (12 h, room temperature) for detritylation. The products were isolated by chromatography (250 μ Analtech plates) followed by elution with 95% CH₂H₅OH. UV spectral data are shown in Table I. Values for the ³¹P chemical shifts are: HRf sample, 14.1 and 13.7 ppm; MRf sample, 14.1, 13.9, and 13.7 ppm;

and Lrf sample, 14.0 and 13.6 ppm. Four isomers of d-A(TCE)A(TCE)A, arising from chirality at the two phosphorus atoms, are possible. Presumably two of these did not separate or else one of the possible isomers did not form. For further characterization, one of the fractions (Lrf) was converted to d-ApApA by reductive cleavage with zinc⁶ and the trinucleoside diphosphate was hydrolyzed with snake venom phosphodiesterase. Two bands were found on chromatography on an Avicel 250 μ plate with $\underline{1}$ -C₃H₅OH-NH₄OH-H₂O (7:1:2), corresponding to dA (Rf 0.62) and dpA (Rf 0.10) in the ratio of 1.0:1.6.

Aminoethylphosphoramidate of Deoxyadenylyl-(3'-5')-deoxyadenosine, d-A(NHC₂H₄NH₂)A. d-DMTA(TCE)A (0.105 g, 0.1 mmol of mixed epimers) in ethylene diamine (5 mL) was incubated at 40°C for 7 h, then diluted with t-butyl alcohol and lyophilized to remove the solvent. The resulting solid was chromatographed on preparative silica plates with repeated development with 5% NH₄OH in CH₃OH until a good separation of bands was achieved. The three main bands were eluted separately with 5% NH₄OH in CH₃OH. Each solution was evaporated to dryness and the residue was taken up in CH₂Cl₂-CH₃OH and centrifuged to remove silica. The solvent was evaporated, and the product was detritylated (80% aq. acetic acid at room temperature, 1 h).⁹ Concentration, washing with ether, and lyophilization of an aqueous solution yielded the products as white solids. The product from the fastest running band (Rf 0.74 in 5% NH₄OH in CH₃OH on silica; Rf 0.05 in water on DE81-DEAE cellulose) was dApA, 8.5 mg (15%), as shown by electrophoresis on Avicel (R_m +0.46), by ³¹P NMR (16.7 ppm relative to triphenyl phosphate), and by a negative ninhydrin test. The other two materials were the aminoethylphosphoramidate epimers. Both gave a positive ninhydrin test characteristic of a primary aliphatic amine, and in a neutral buffer both migrated as singly positively charged fragments (R_m -0.6 relative to dpT). The Hrf isomer (faster chromatographically) amounted to 16 mg (25%); $\lambda_{\max}$ 258 nm (ϵ 12,700), $\lambda_{\min}$ 225.5 nm (ϵ 3,050). The slower running isomer (Lrf) weighed 31 mg (49%); $\lambda_{\max}$ 258.5 nm (ϵ 12,900), $\lambda_{\min}$ 226.5 nm (ϵ 3,400). The proton NMR spectra, taken in d₆-DMSO at 270 MHz on a JEOL-270 spectrometer, were consistent with the assigned structures.

To gain information on the course of the substitution reaction, samples (7 mg) of the separated Hrf and Lrf d-DMTA(TCE)A fractions were converted to the corresponding aminoethylamidates by the procedure previously described. The products were identified by TLC and the ³¹P NMR spectra. The Hrf ester afforded a single amidate, Hrf d-DMTA(NHC₂H₄NH₂)A (Rf on silica gel in 5% NH₄OH in CH₃OH 0.58; ³¹P 27.6 ppm) along with d-DMTApA (Rf 0.84); ³¹P

Figure 1. Dissociation of complex formed from Poly U and Hrf d-A(TCE)A (open data points) or Lrf d-A(TCE)A (closed data points): standard conditions with no added salt (circles), with 10 mM MgCl_2 (squares), with 1 mM ethylenediamine (triangles).

16.7 ppm). The molar ratio of amidate to diester determined by the relative heights of the ^{31}P signals was 5.4:1. Similarly, Lrf d-DMTA(TCE)A yielded a single amidate. Lrf d-DMTA($\text{NHC}_2\text{H}_4\text{NH}_2$)A (Rf on silica gel in 10% NH_4OH in CH_3OH 0.46; ^{31}P 28.3 ppm) along with d-DMTApA. The amidate:diester ratio in this case was 6.2:1. The ^{31}P resonance at about 28 ppm from triphenyl phosphate is characteristic for diester phosphoramidates and provides good evidence for the presence of that functional group in these molecules.

Since ethylenediamine was found to enhance binding of d-A(TCE)A to Poly U (Figure 1), care was taken to insure that samples of d-A($\text{NHC}_2\text{H}_4\text{NH}_2$)A were not contaminated by this amine. Both ethylenediamine and d-DMTA($\text{NHC}_2\text{H}_4\text{NH}_2$)A give a positive test with ninhydrin. Using this test, no evidence for ethylenediamine in the aminoethylamidate samples could be found on thin layer chromatography or analysis on a Durrum 500 Aminoacid Analyzer. The sensitivity of the latter is sufficient that one can place an upper limit of 5 mole percent for ethylenediamine in the samples of d-A($\text{NHC}_2\text{H}_4\text{NH}_2$)A or 5×10^{-5} M for the concentration of any contaminating ethylenediamine in the binding experiments. At this concentration ethylenediamine would have no detectable effect on the melting curves.

Figure 2. Dissociation of complex formed from Poly U and HRF d-A(TCDM)A (open data points) or the mixed isomers of d-A(NH₂)A (closed data points): standard conditions, with no added salt (circles), with 10 mM MgCl₂ (squares).

The 2,2,2-Trichloro-1,1-dimethylethyl Ester of Deoxyadenylyl-(3'-5')-deoxyadenosine [d-A(TCDM)A]¹⁰ and the phosphoramidate d-A(NH₂)A were prepared and provided by Dr. Toshiki Tanaka. For d-A(NH₂)A only one resonance was observed in the ³¹P NMR spectrum (29.8 ppm relative to triphenyl phosphate) and no separation of stereoisomers could be achieved by chromatography. It is possible that only one of the two epimers was present; however, in light of the method of preparation (reaction of ammonia on the *o*-chlorophenyl ester of deoxyadenyl-(3'-5')-deoxyadenosine in the presence of CsF) it is more likely that both isomers had formed but had very similar properties.

Sensitivity to Nucleases. The new nucleotide derivatives were incubated individually with snake venom phosphodiesterase and spleen phosphodiesterase for 16 h at 37°C in standard buffers.⁷ Under these conditions d-ApA was completely hydrolyzed by each enzyme, but none of the triester derivatives was attacked. d-A(NH₂)A was hydrolyzed to the extent of 14% by snake venom phosphodiesterase, as indicated by chromatographic separation of the products, and to the extent of 8% by spleen phosphodiesterase. d-A(NHC₂H₄NH₂)A was not degraded by either enzyme under these conditions.

Figure 3. Dissociation of complex formed from Poly U and HRF d-A(NHC₂H₄NH₂)A under standard conditions with no added salt (circles), with 10 mM MgCl₂ (squares), with 1 mM ethylenediamine (triangles).

RESULTS

Dissociation of the complexes of the deoxyadenosine derivatives with Poly U and Poly dT was followed spectrophotometrically. The concentrations correspond to 3.3×10^{-5} dA units and 6.7×10^{-5} U (or dT) units. The pH was 6.8 (10 mM Tris-HCl buffer) and in designated cases ethylenediamine (1 mM) or MgCl₂ (10 mM) was present. Data are presented graphically (Figures 1-3) and in terms of T_m values (temperature at change in slope of the absorbance versus temperature curves) (Table I).

Results on the binding of d-A(TCE)A to Poly U at three different sets of salt conditions are given in Figure 1. Little interaction was observed in the "low salt" media (10 mM Tris-HCl buffer), but relatively strong binding (T_m 14-16°C) occurred in the "high salt" solutions (10 mM MgCl₂ and 10 mM Tris-HCl). Protonated ethylenediamine has been reported to serve as an effective cation in stabilizing duplex polynucleotide strands.¹¹ As shown in Figure 1, protonated ethylenediamine, even at a concentration of 1 mM, also stabilizes the complex formed between d-A(TCE)A and Poly U. The two isomers of d-A(TCE)A differing in chirality of phosphorus behaved similarly in these systems, although small differences in stability of the complexes were found.

Table I. UV Spectral and Binding Data

Compound	$10^3 \epsilon_{\max}$ per A	% hypochrom- icity in oligomers	T _m of Poly U Oligomer Complex °C		T _m of Poly dT Oligomer Complex °C	
			low salt ^b	high salt ^c	low salt ^b	high salt ^c
(d-ApA ^d)	(12.7)	(17)	(<0)	(7)		(9)
d-A(TCE)A						
HRf	13.1	7	<0	14		28
LRF	13.4	5	<0	16		25
d-A(TCDM)A						
HRf	10.7	24	7.3	15		
LRF	11.2	21	7.6	16		
d-A(NH ₂)A	12.1	15	11	19		22
d-A(NHC ₂ H ₄ NH ₂)A						
HRf	12.7	10	10	26	25	26
LRF	12.9	9	8	21	27	28
d-A(TCE)A(TCE)A						
HRf	11.7	17	17	30	36	44
MRF	11.4	13	13	29		
LRF	11.3	11	11	35		

(a) $\lambda_{\max}$ in 258-258.5 nm range in all cases. (b) 10 mM Tris-HCl. (c) 10 mM Tris-HCl, 10 mM MgCl₂. (d) Data for d-ApA from reference 2.

Melting curves for complexes of Poly U with d-A(TCDM)A and d-A(NH₂)A are presented in Figure 2, and similar data for a complex of d-A(NHC₂H₄NH₂)A are given in Figure 3. As with d-A(TCE)A, epimers at phosphorus gave similar patterns. The graphs have been simplified, therefore, by including data for just one of the isomers in each case. T_m values derived from these curves and data from related experiments are summarized in Table I. In each case the complex formed by the nucleotide analogue was more stable than that formed by d-ApA.

A few experiments were carried out with Poly dT in place of Poly U. The data in Table I show that this change had little effect on T_m values for complexes of d-ApA, d-A(NH₂)A, and d-A(NHC₂H₄NH₂)A in 10 mM MgCl₂ solutions. On the other hand significant enhancement (Δ T_m 8-15°C) was found for complexes of Poly dT with the trichloroethyl and trichlorodimethylethyl phospho-

Figure 4. Mixing curve for d-A(TCE)A and Poly dT at 0°C in 10 mM MgCl₂ and 10 mM Tris-HCl. The total nucleotide concentration was 0.08 mM.

triesters in 10 mM MgCl₂ and with d-A(NHC₂H₄NH₂)A in the low salt medium.

In all previous cases in which a stable complex was observed between a polynucleotide and d-ApA or a non-ionic derivative, the adeny:pyrimidyl ratio was found to be 1:2.^{1,2} As a check on the stoichiometry in the present examples the composition of several of the more stable complexes was examined by the continuous variation method of Job. Plots relating absorbance at 260 nm to mole percent of U in mixtures at a given total nucleotide concentration were obtained for the three fractions of d-A(TCE)A(TCE)A and the HRF fraction of d-A(NHC₂H₄NH₂)A under both low salt and high salt conditions. Similar data were collected for the complex of HRF d-A(TCE)A and Poly dT (high salt conditions). In each case a break was found at about 67% U or dT, showing the expected adeny:pyrimidyl ratio of 1:2. Data for d-A(TCE)A and Poly dT are shown in Figure 4.

DISCUSSION

The phosphotriesters d-A(TCE)A, d-A(TCE)A(TCE)A, and d-A(TCDM)A were prepared by a phosphite-triester route utilizing d-DMTA, the appropriate alkyl phosphoro-bis-triazolide, and excess unprotected deoxyadenosine. Epimers at phosphorus were distinguished by the ^{31}P chemical shifts and were separated chromatographically. For preparation of the aminoethylamide, d-A(NHC₂H₄NH₂)A, we employed the reaction of ethylenediamine with d-A(TCE)A. This displacement reaction, developed for synthesis of analogous thymidine derivatives,¹² proceeds satisfactorily although not quantitatively; about 15% of the d-DMTA(TCE)A is converted to d-ApA. The reaction is stereospecific in that one isomer of d-A(TCE)A cleanly yields one isomer of the amide. The absolute stereochemistry at phosphorus for these isomers has not yet been established; however, since other direct nucleophilic substitutions of phosphoryl compounds proceed with inversion in configuration, inversion is probably the course here. In that case, HRf d-A(TCE)A corresponds in configuration to LRf d-A(NHC₂H₄NH₂)A.

Hypochromicity values for these deoxyadenosine dinucleotide analogues range from 5-7% for the isomers of d-A(TCE)A to 21-24% for the isomers of d-A(TCDM)A (Table I). Kondo *et al.*¹³ have correlated hypochromicity in related dimers with overlap of the purine rings. On this basis the extent of base stacking in d-A(TCE)A is low compared to that in d-ApA whereas base stacking in d-A(TCDM)A is unusually high. Insufficient information is available to permit a meaningful interpretation at this time; however, it is reasonable that the bulky methyl groups buttressed by three chlorine atoms in the trichlorodimethylethyl group could have a significant effect on the conformation assumed by the adenine fragments.

The principal result of the binding studies is that all the deoxyadenosine derivatives bind to the complementary polynucleotides, and some of the complexes are surprisingly stable. At the high extreme are the adducts of Poly dT with HRf d-A(TCE)A and d-A(TCE)A(TCE)A in 10 mM MgCl₂, for which the T_m values are 28 and 44°C, respectively. For comparison, T_m reported for the complex of Poly dT with d-A(CH₃)A (the methyl phosphonate) under similar conditions is 19°C.² Data for interaction of Poly U with d-A(TCDM)A and d-A(NH₂)A in low ionic strength solutions are equally striking (T_m 7.3-7.6° and 11°C, respectively). Association of d-ApA derivatives with Poly U has not previously been observed under these conditions; for binding of d-ApA and the corresponding methyl or ethyl ester or methyl phosphonate,^{1,2} as also for d-A(TCE)A, high ionic strength is necessary to enable the two

strands of Poly U to come together in the triplex.

Several conclusions can be drawn from these data. (1) A bulky group at phosphorus does not destabilize the complexes formed between these small oligo-dA derivatives and Poly U or Poly dT. Indeed, the bulky trichloro-dimethylethyl group actually serves as a stabilizing factor in this system. The supposition that increasing the size of the side chain in triester dimer derivatives will destabilize complexes formed with homopolymers² is not borne out in the present study. (2) Increasing the triester length from two to three nucleotide units enhances binding to Poly U. Thus T_m for the complexes formed from Poly U and the d-A(TCE)A(TCE)A fractions ranged from 29°C to 35°C compared to 14° to 16°C for the isomers of d-A(TCE)A. Although one cannot safely extrapolate from the trimer to long chains built exclusively with bulky triester links, for which in any case solubility in water becomes limiting, the results with this model system suggest that incorporation of several bulky, lipophilic ester fragments at phosphorus in the backbone chain of an oligonucleotide may not seriously interfere with binding to complementary oligomers. (3) A phosphoramidate internucleotide link ($-\text{OP}(\text{O})(\text{NH}_2)\text{O}^-$) stabilizes binding of a deoxyadenosine dimer derivative to Poly U, as demonstrated by the relatively high T_m for the complex of d-A(NH₂)A. The effect is most noticeable in low ionic strength solution (T_m 11°C compared to well below 0°C for the corresponding complex of d-ApA). This feature and the favorable solubility characteristics of the phosphoramidate may be attributed at least in part to the fact that the $-\text{NH}_2$ group is small, is uncharged in aqueous solution, and forms hydrogen bonds readily. (4) The aminoethylphosphoramidate (in d-A(NHC₂H₄NH₂)A) also facilitates formation of complexes in this system. Since in neutral aqueous media the basic aminoethyl group is protonated, electrostatic attraction for the anionic Poly U strands is no doubt a stabilizing factor. It may be noted, however, that the non-ionic $-\text{NH}_2$ group and the charged $-\text{NHC}_2\text{H}_4\text{NH}_3^+$ have roughly comparable effects on stability of the complexes. An interesting feature of the data for the aminoethyl derivative is the relatively small hyperchromicity observed on dissociation of the complex in the presence of magnesium ion--about 39% compared to 67% in presence of the ethylenediamine cation or in buffer in absence of magnesium ion (see Figure 3). These results suggest a change in conformation of the complex in the presence of magnesium ion.

These model studies provide synthetic leads for incorporating pendant groups at phosphorus in oligonucleotides. The binding studies suggest that

such oligomers, even those containing quite large lipophilic side chains, will hybridize to complementary polynucleotide sequences. In subsequent papers we shall report extension of this chemistry to duplex structures containing mixed base sequences.

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