Phosphoramidate analogues of DNA: synthesis and thermal stability of heteroduplexes

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ABSTRACT:

The deoxyoligonucleotide 5' AATCGGGCATGGATT (15-mer) was synthesized containing 12 phosphoramidate linkages derived from 2 primary and 2 secondary amines. The oligonucleotides were purified by reverse phase HPLC and characterized by PAGE. The thermal stability of the duplexes derived from these compounds, when hybridized to the complementary diester linked oligomer, were determined and compared to the diester and methanephosphonate oligomer. The results indicated that all analogue oligomers form less stable duplexes than the diester oligomer. A large difference was observed between primary and secondary amine derived phosphoramidates.

INTRODUCTION:

Deoxyoligonucleotides (DNA) containing internucleotide phosphate analogues have shown anti-viral activity *in vitro*. Methanephosphonate linked deoxyoligonucleotides have been shown to inhibit viral replication in mammalian cells by the specific inhibition of mRNA translation¹. Phosphorothioates have recently shown anti-HIV activity by competitive inhibition of reverse transcriptase². Phosphate diester linked deoxyoligonucleotides have also shown anti-viral activity when an intercalator function such as acridine is attached to the oligomer³. A recent report has shown that gene expression is affected by anti-sense oligonucleotides *in vitro* and a comparison of alkyl phosphotriester, methylphosphonate and phosphorothioate analogues was made⁴. The phosphoramidate linkage, which can serve as an attachment to DNA for reporter groups, intercalating agents and cross-linking agents, is an attractive possibility for non-ionic analogues of DNA. The phosphoramidate linkage has been shown to be resistant to nucleases such as snake venom phosphodiesterase and spleen phosphodiesterase^{5,6}. Stability to nucleases is a desirable property especially when *in vivo* applications are considered⁷. It has been shown that internucleotide phosphate analogues of polythymidine are readily prepared by the H-phosphonate method of DNA synthesis⁶.

Described herein is the synthesis and characterization of oligonucleotides containing internucleotide phosphoramidate linkages. A 15-mer of defined sequence (Figure 1a) was prepared containing 12 phosphoramidate linkages derived from two primary (**1b**, **1c**) and two secondary amines (**1d**, **1e**). The stability of the duplex derived from each of these analogues, when hybridized to the diester linked complementary oligomer (**2**), was assessed by thermal denaturation. The

phosphoramidate analogues were compared to the diester linked (**1a**) and methanephosphonate linked (**1f**) deoxyoligonucleotide.

MATERIALS AND METHODS:

Synthesis of Deoxyoligonucleotides:

Polymer bound polynucleoside H-phosphonates were prepared as previously described on control pore glass using the DBU salt of the protected nucleoside H-phosphonate ^{8,9} (Figure 1b). The diester linkages were generated by aq. I₂ oxidation and the amidate linkages by amine/CCl₄ oxidation. The preparation of **1c** will be described as a representative example. After 12 couplings the polynucleoside H-phosphonate was oxidized with a solution of 2-methoxyethylamine in Pyr/CCl₄ (1/5/5) (20 min) followed by two more couplings and oxidation with aq. I₂ (0.1M in N-methyl morpholine/water /THF, 5/5/90) to generate a 15-mer containing 12 phosphoramidate linkages and two diester linkages. The oligomer was removed from the solid support and deprotected with conc. NH₄OH (45°C/18 hr), evaporated, dissolved in DMF/H₂O (1/1) and purified by reverse phase HPLC (C₈) using an acetonitrile (CH₃CN) gradient in 25 mM aqueous TEAP. The product fraction (~5 mL) was diluted with 5 mL of 1.0 M NaCl and loaded onto a SPE C₈ cartridge (500 mg, J. T. Baker), washed with 30 mL H₂O, 15 mL 2% CH₃CN then eluted with 2 mL of 30% aq. CH₃CN, 2 mL of 60% aq. CH₃CN and evaporated. Approximately 1 µg of purified product was 5'-end labeled with T4 polynucleotide kinase and γ-³²P ATP for further characterization. Polyacrylamide gel electrophoresis (10% PAGE, 7 M urea) of the samples is shown in Figure 3. The T₁₄ and T₁₅ standards, containing one, two and three diester linkages, were prepared and purified separately as described above.

Hydrolysis of the Phosphoramidate Linkage to a Phosphate Diester Linkage:

The 5'-end labeled sample (~0.1 µg) was incubated with 0.5 mL of a solution containing 10% isoamyl nitrite in water/acetic acid/ethanol (1/4/5) at 45°C for 5 hr, diluted with 1 mL of water and extracted with diethyl ether (2 x 1 mL), ethyl acetate (2 x 1 mL) and again with ether (2 x 1 mL). After evaporation the residue was dissolved in formamide/water (1/1) and analyzed by PAGE (20% / 7 M urea).

Hypochromicity Measurements:

Hypochromicity of the heteroduplexes was measured at 260 nm with a KON TRON Uvikon 810 spectrophotometer in a 1 cm masked cuvette. The samples were degassed with He, heated to 80°C for 20 min, allowed to cool to room temperature and maintained at 10°C overnight. The absorbance of the samples was monitored from 10°C to 80°C, increasing the temperature at a rate of 0.2°C/min. The T_m of each oligomer was determined in high and low salt [150 mM NaCl/10 mM Na₂HPO₄ (pH = 7.2) and 15 mM NaCl/1 mM Na₂HPO₄ (pH = 7.2)] at a concentration of approximately 4 µM. The data from separate runs (3-5) was normalized to % denaturation [$\% \text{ denaturation} = \frac{A_0 - A_i}{A_f - A_i} \times 100\%$ (A₀ = observed Abs.; A_i = initial Abs. @ 10°C; A_f = final Abs. @ 80°C)] and combined to obtain a melting curve. A linear least square analysis of this data (with RS/E) gave a slope of

RESULTS and DISCUSSION:

FIGURE 2: HPLC chromatogram of crude phosphoramidate linked oligomer **1c**, Partisil Cg (10 μ), 2.5 mL/min, 0.5 aufs @ 254 nm Solvent A = 5% CH₃CN/25 mM TEAP (pH=6.6) Solvent B = 75% CH₃CN/25 mM TEAP (pH=8.0)

introduction¹⁰ and has lead to extensive assessment of their biological properties. Phosphoramidate analogues have only recently been investigated⁵. In this case, only dimers and trimers were investigated because of synthetic limitations.

We have prepared phosphoramidate linked deoxyoligonucleotides in high yield via H-phosphonate intermediates. The synthetic scheme is shown in Figure 1b with the H-phosphonate linked oligomer synthesized in standard fashion⁸. This chain is then oxidized in the presence of a primary or secondary amine yielding a phosphoramidate linkage at all sites. For this study, two diester linkages were included at the 5'-end of compound **1** to allow for electrophoresis and phosphorylation with T₄ kinase. The phosphoramidate linkage derived from primary and secondary amines is stable to the conditions used to remove the protecting groups on the exocyclic amino group of the heterocyclic bases (conc. NH₄OH/45°C/18 hr).

Phosphoramidate linked oligonucleotides are readily purified by reverse phase HPLC. The chromatogram of **1c**, shown in Figure 2, is a representative example of the product released from the silica support by ammonia. The major peak was collected and isolated by desalting on a C₈ cartridge followed by evaporation. Compounds **1b-e** were purified in this manner. The purified oligomers were 5'-end labeled with T₄ polynucleotide kinase and γ -³²P ATP and characterized by gel electrophoresis. Examination of Figure 3 shows that the substituent amine has a slight effect on electrophoretic mobility. Included in Figure 3 are 5'-end labeled T₁₅ and T₁₄ phosphoramidate standards derived from methoxyethylamine that contain one, two and three diester linkages at their

FIGURE 3: Audioradiogram of 10% PAGE / 7 M urea derived from 5'-end labeling of purified oligomers. Lane 1: T_{14} standards; Lane 2: T_{15} standards; Lane 3: N-methylphosphoramidate (**1b**); Lane 4: N-(2-methoxyethyl)phosphoramidate (**1c**); Lane 5: phosphormorpholidate (**1d**); Lane 6: N,N-dimethylphosphoramidate (**1e**); Lane 7: methanephosphonate (**1f**).

5'-ends. These standards show that electrophoretic mobility is dependent on the number of diesters and that under these conditions oligomers of the same size can be resolved on the basis of the number of phosphoramidate linkages they contain. PAGE therefore allows the assessment of homogeneity with respect to the number of amidate linkages, but the length of the fragment cannot be absolutely assessed.

In order to confirm the length of the fragment it is necessary to hydrolyze the phosphoramidate linkage to a phosphate diester linkage and compare to the known standard by PAGE. Selective hydrolysis of the phosphoramidate linkage to the phosphate diester linkage was previously carried out ⁶ with 85% formic acid at 95°C. These conditions would lead to hydrolysis of the glycosidic bond in purine containing deoxyoligonucleotides, therefore isoamyl nitrite was used for the selective hydrolysis of the phosphoramidate ^{11,12}. The isoamyl nitrite treated oligomers can be compared to the parent diester by PAGE. Compounds **1b-e** can be selectively hydrolyzed to a compound comigrating with the parent diester **1a** (data not shown).

The thermal stability of the oligomers (**1a-e**) duplexed with **2** was assessed by hypochromicity at 260 nm from 10°C to 80°C at a concentration of approximately 4 μ M. Figure 4 shows the curves of each, obtained in 150 mM NaCl. Table I shows the T_m values and the slope of transition for

FIGURE 4: T_m curves in 150 mM NaCl; plotted as a function of temperature in degrees C (abscissa) vs. percent denaturation (ordinate).

Table I
 T_m values and slope of transition (high and low salt)

Oligomer	150 mM NaCl		15 mM NaCl	
	T_m	Slope	T_m	Slope
1a	60.5	0.61	46.1	0.74
1b	45.0	0.60	43.0	0.60
1c	46.3	0.54	45.5	0.57
1d	36.7	0.58	32.4	0.54
1e	35.7	0.46	32.5	0.54
1f	52.5	0.51	51.1	0.62

both the 150 mM NaCl and 15 mM NaCl experiments. The analogues derived from secondary amines give very unstable duplexes with T_m 's significantly below those derived from primary amines. The methanephosphonate sequence had a T_m about 6-7° higher than the sequence derived from the primary amines. The T_m of the diester control was about 8° higher than the methanephosphonate. All samples showed similar hypochromicity (~20%) except for those derived from secondary amines (~18%).

The neutral analogues all have T_m 's which show a small salt dependence relative to the diester. This small dependence is probably because of the two diester linkages (12 analogue linkages) in the analogue oligomers. The reason for the lack of salt dependence of T_m in neutral analogues is well known¹³. There is no electrostatic repulsion between the two strands of the duplex that is masked by higher salt concentrations.

The oligomers presented above contain 12 chiral phosphate linkages leading to 4096 (2¹²) possible diastereomeric oligomers. If the chirality at phosphorus is important for duplex stability a broad melting curve would be expected. The lower T_m of oligomers containing methanephosphonate linkages has been attributed to the mixture of diastereomers¹³. Examination of Figure 4 demonstrates that, with the exception of the secondary amines, the curves are sigmoidal, monophasic and fairly sharp. These results indicate that the mixture of diastereomers is probably not the main reason for a lower T_m of the analogue oligomers derived from primary phosphoramidates and methanephosphonates. The reason for the consistently lower T_m of the neutral analogues relative to diesters is somewhat puzzling. It has been suggested that the neutral analogues significantly disturb the spine of hydration around the phosphates thereby destabilizing the duplex (Miller, P.S., personal communication). This idea is supported by X-ray crystal structure studies on DNA oligomer duplexes which show highly ordered water molecules around the phosphates^{14,15}.

The phosphoramidate analogues derived from secondary amines give lower T_m 's, much broader melting curves and lower hypochromicity as compared to those derived from primary amines. The lower hypochromicity suggests that the secondary phosphoramidates are not completely hybridized at the lowest temperature studied. The reason for the duplex instability is likely due to the increased steric hindrance and restriction of phosphoramidate bond rotation.

Previous T_m data on phosphoramidate/diester duplexes demonstrated enhanced stability relative to diester/diester duplexes⁵. These studies were performed with short sequences (dimers and trimers) of deoxyadenosine. We observe less stability in the case of the analogue duplexes. This difference could be attributed to either the length or sequence of the duplexes. It is likely that analogue duplexes of enhanced stability are the exception rather than the rule. We also observe a lower T_m for the methanephosphonate oligomer/diester oligomer duplex relative to the diester/diester control. Previous studies of the stability of methanephosphonate/diester duplexes have shown enhanced T_m 's in the case of homopolymers^{16,17,18}. The stability of mixed sequences has been discussed qualitatively but no quantitative data has been presented¹⁹.

We have shown the facile synthesis of phosphoramidate analogues of DNA and demonstrated the formation of stable duplexes derived from primary amines. Secondary amine derived phosphoramidates form duplexes that are significantly less stable than those derived from primary amines. At present, the duplex stabilities observed are below that of a diester-diester interaction. The synthetic ease in which modifications can be made to an amidate side chain allows for investigations aimed at increasing the stability of the analogue duplex and introducing biologically active ligands into synthetic DNA.

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Abbreviations: DMT, 4,4'-dimethoxytrityl; DCA, dichloroacetic acid; Pyr, pyridine; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TEAP, triethylammonium phosphate; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene.

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