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UV SPECTROSCOPY STUDY ON COMPLEXES OF PHOSPHONATE ApA ANALOGS WITH POLY(U): PROMISING STEP IN PREDICTION OF OLIGONUCLEOTIDE ANALOG PROPERTIES?

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Abstract: The isopolar nonisosteric phosphonate analogs of ApA differing in the position of extra methylene group introduced into the sugar-phosphate backbone, featuring both possible 2',5'- and 3',5'- pairs as well as their conformationally restricted congeners, were investigated for their ability to form complexes with polyU. The results may lead to the specification of candidates for synthesis of novel oligonucleotides.

The general concept of the use of oligonucleotides as a tool for specific regulation of gene expression has brought about a chance for new, highly specific chemotherapeutics[1,2,3]. An effective *in vivo* use of oligonucleotides requires a sufficient affinity to the target and ability to penetrate across the cell wall, as well as the stability towards nucleases. Because natural oligonucleotides do not secure the latter requirement, various analogs with modifications of the internucleotide linkage have been prepared[4,5]. Besides continuing studies on oligodeoxynucleotides with modified 3'-5' internucleotide linkage, an increasing attention has been turned towards their 2'-5' congeners both in 3'-deoxyribo and ribo series because of their higher binding selectivity to RNA than to ssDNA[6].

Earlier studies on interaction of the 2'-5' and 3'-5' adenine diribonucleoside monophosphates and their enantiomers[7], the 2,6-diaminopurine derivatives[8], and the neutral phosphotriesters derived from the d(ApA)[9] (including the d(ApA) methylphosphonate[10]) with polyU revealed the ability of these shortest oligonucleotides to form complexes of a 1:2 stoichiometry with polyU. These important findings which point to triple stranded, nucleic acid-like structures were obtained from CD data and UV spectroscopic measurements of T_m characteristics.

The present work was undertaken as a part of thorough structural studies[11,12,13,14,15] on the phosphonate mimic of the phosphodiester bond in oligonucleotides. Our idea was to examine the role of several specific modifications of the internucleotide linkage in influencing the stability of duplex or triplex formation, first at the level of modified diribonucleoside monophosphates[16,17,18,19] which are more readily accessible than longer modified oligoribonucleotides. In this respect, the diribonucleoside monophosphate analogs represent the shortest oligonucleotides featuring the fundamental attributes, i.e. base-stacking and base-pairing, of the longer oligonucleotides that would come into consideration and possess the same modifications.

Thus, we have chosen here the unique set of phosphonate ApA analogs[17] **3-9 (Fig. 1)** prepared earlier in our

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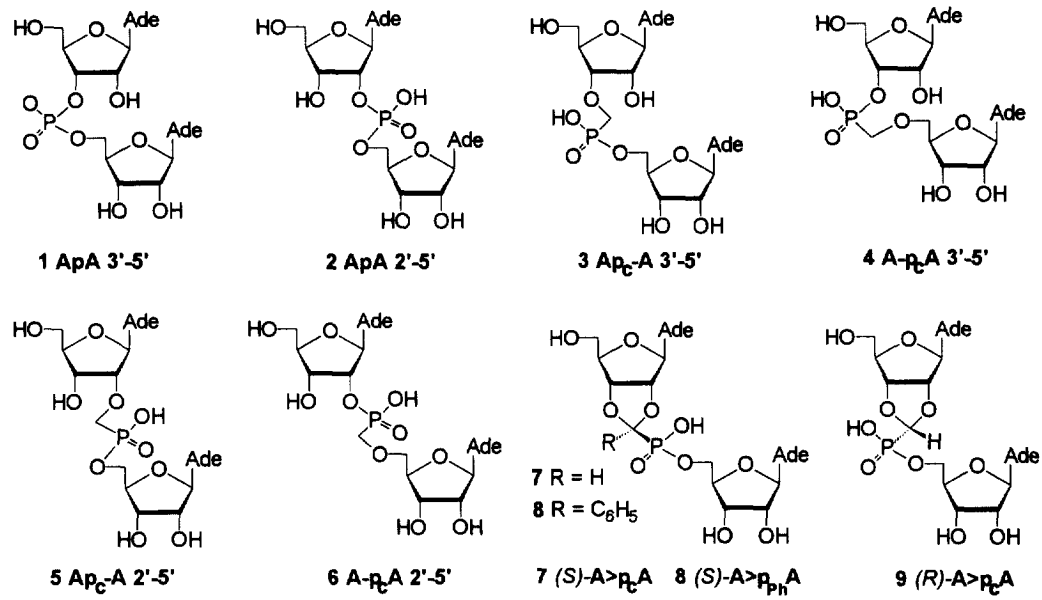
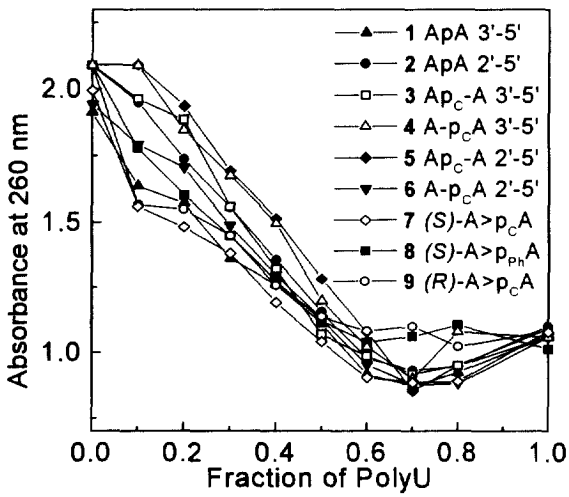


Figure 1. Structures of phosphonate analogs of ApA

laboratory containing both the 3'-5' pair **3**,**4** and the 2'-5' pair **5**,**6** as well as the conformationally restricted congeners[18] **7-9** of compounds **3** and **5** to investigate their ability to form complexes with polyU and compare them with the natural 3'-5' ApA **1** and the 2'-5' ApA isomer **2** with the aim to make it possible to specify suitable candidates for oligoribonucleotide construction. In this respect, undoubtedly, the question of conformational adaptability of the isopolar but nonisosteric, phosphonate-based internucleotide linkage was the one to be answered.

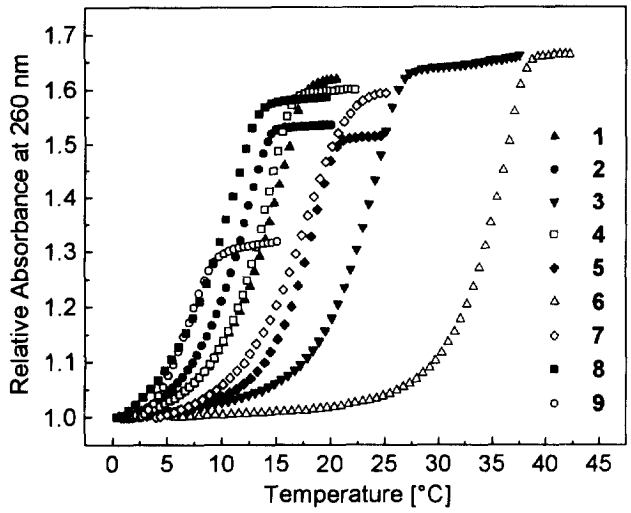
Figure 2. Mixing Curves of ApA analog with polyU



In order to determine the stoichiometry of the interaction of polyU and ApA analog, the hypochromicity of their various mixtures was measured at 260 nm and at 3 °C in 10mM Tris-HCl buffer pH 7.6 with 10 mM $MgCl_2$. The formation of complexes was followed by continuous variation method[7]. Mixing curves of all complexes were expressed as the plot of

absorbance *versus* molar fraction of polyU in the mixture of polyU and ApA analog at total concentration of $1.5x10^{-4}$ M (calculated for nucleobases). All mixing curves of both natural ApA compounds and their phosphonate analogs showed, under experimental conditions, flat minimum with the midpoint at 0.7 fraction of polyU (**Fig.2**). There is, therefore, an evidence for a 1:2 stoichiometry with polyU, the same as described for the natural 3'-5' and 2'-5' ApA[8,20]. Thermal stability of the formed triple-stranded complexes at fraction of polyU 0.7 was examined by measuring the T_m characteristics, i.e., absorbance *versus* temperature plots[21] at 260 nm in the temperature range of 0-40 °C (slope, 0.5 °C/min) (**Fig.3**). The maxima values obtained from the differentiated melting curves (data not shown) were taken as T_m values (**Table 1**). The hyperchromic effect values of almost all complexes (except the complex of compound **9**) were found to be in the narrow range of relative absorbance. All complexes showed sharp transition profiles with T_m values within a relatively wide range of 7-37 °C , which indicates a high sensitivity of the complex formation towards geometry of the internucleotide linkage. The T_m values for most complexes were found to be higher than for both natural ApA dimers **1** and **2** (T_m 15.7 °C and 12.6 °C, resp.)[8,19]. Distinctly out of this range, however, lie the T_m values for two ApA analogs containing methylene group located closer to the 5'-*end* of the modified sugar-phosphate backbone, namely, the 3'-5' A-p_cA **4** (T_m 36.7 °C) and 2'-5' A-p_cA **6** (T_m 24.5 °C). Surprisingly, their isomers **3** and **5** (with methylene group located closer to the 3'-*end* of the sugar-phosphate backbone) exhibit significantly lower T_m values (14.8 and 19.1 °C, resp.) but still comparable with (or higher than) those of the natural ApA dimers. The difference in T_m is much higher between 3'-5' isomers **3** and **4** than between 2'-5' isomers **5** and **6**.

Figure 3 T_m Characteristics of Complexes of ApA analogs with polyU



As for the remaining, conformationally restricted dimers **7**, **8** and **9**, the lowest T_m value was found for the (*R*)-A>p_cA **9** (7.2 °C), indicating that spatial arrangement of this structure and/or its added rigidity probably strongly restrict formation of the complex. Its epimer (*S*)-A>p_cA **7**, showed a T_m value (17.9 °C) higher to those of unmodified dimers **1** and **2**. Finally, a significant 6.2 °C drop in T_m value in comparison with dimer **7** was found for its congener, the (*S*)-A>p_{Ph}A dimer **8** (T_m 11.7 °C) containing a bulky phenyl group oriented in an opposite direction than phosphonate moiety.

Table 1. T_m Values and Thermodynamic Data for the 1:2 Complexes of ApA analog with polyU

ApA analog	1 ApA 3'-5'	2 ApA 2'-5'	3 Ap_c-A 3'-5'	4 A-p_cA 3'-5'	5 Ap_c-A 2'-5'	6 A-p_cA 2'-5'	7 (*S*) A>p_cA	8(*S*) A>p_{Ph}A	9 (*R*) A>p_cA
T_m [1]	15.7	12.6	14.8	36.7	19.1	24.5	17.9	11.7	7.2
ΔH_{VH} [2]	-330±27	-336±13	-416±26	-544±30	-569±40	-275±23	-118±4	-386±49	-167±21
ΔS_{Tm} [3]	-114±9	-118±5	-144±9	-176±10	-195±14	-92±8	-41±1	-135±17	-60±8

[1] in °C; [2] van't Hoff transition enthalpy in kcal.mol^{-1}; [3] entropy at T_m in 10^2.kcal.mol^{-1}.K^{-1}; ± standard deviation

There is obviously one fundamental question: *Why do these ApA analogs exhibit comparable or higher thermal stability of their complexes with polyU than their natural counterparts despite the fact that the modified internucleotide linkage is one CH_2 group longer than the natural one?* Although the lengthening of the linkage by introduction of methylene group brings one more degree of freedom to the whole molecule, the sugar puckering for both sugar parts of ApA analogs **3-6** was earlier found to be similar to natural ApA[11]. In other words, the conformational flexibility of the O-P-CH_2-O internucleotide linkage can favor the population of thermodynamically stable conformers exhibiting the "better" and/or more "tightly" stacked structure of adenines in modified ApA which could be more advantageous for hydrogen bond base-pairing with polyU.This hypothesis need not be at variance with findings[22] indicating that increased flexibility in oligonucleotide analog containing 3',4'-*seco*-thymidine unit resulted in decreased affinity for natural complementary strand. In our case, the modification concerns "only" the internucleotide linkage and it is not as deep as the change caused by the opening of sugar residue which increases tremendously the degree of freedom of the 3',4'-*seco*-thymidine nucleotide unit.

The compounds **7** and **9** can be considered as two possible conformers of dimer **3** or **5** and, therefore, one of them could be entropically favored with respect to the complexation properties with polyU because of the conformationally restricted sugar-phosphonate backbone[23]. It is noticeable that the complex of dimer **7** with polyU exhibits the T_m value just lying between those for compounds **3** and **5**.

The complex stability can be also influenced by the distribution of negative electric charge along the backbone of "pseudo-RNA" composed of modified ApA units as well as by the repulsive forces between both chains. Therefore, the presence of monovalent or divalent cations, especially magnesium ions to neutralize a net negative charge of the RNA helix, was strictly required. And indeed, no sharp transition profiles (sigmoid curves) were obtained in the absence of magnesium ions (data not shown). The arrangement of magnesium ion orbitals (tetragonal bipyramide) allows to coordinate up to six ligands, e.g. six molecules of water or the hydroxyls and nitrogen- or phosphorus-containing group(s) of ApA analog. These known facts could contribute to the explanation of enhanced thermal stability of studied complexes. Thus, e.g. the simultaneous coordination of 2'-hydroxyl group, 5'-ether-type of oxygen atom, and phosphorus hydroxyl group *via* magnesium ion could be capable of enhancing the stability of "correct" conformation of ApA analog.

We also attempted to analyse the shapes of equilibrium melting curves in order to calculate the values of the

van't Hoff transition enthalpy ΔH_{VH} for each complex of ApA analog. Marky and Breslauer[24] suggested to extract the thermodynamic data from the shape of the differenciated equilibrium transition curves of oligomeric or polymeric nucleic acids for any molecularity of the reaction. The ΔH_{VH} (summarized in **Table 1**) was obtained as an average value from two ΔH_{VH} calculated from two general forms of van't Hoff equation[25]. On the assumption that (i) the ΔH_{VH} is temperature independent in the range of 0 to 40°C and (ii) ΔG at T_m is zero ($\Delta G = -RT_m \ln K_{Tm} = 0$) because the dissociation constant K_{Tm} at equilibrium (at T_m) should equal one (the rate of association and dissociation of the complex is equal), we can use the expression $\Delta G = \Delta H_{VH} - T_m \Delta S_{Tm} = 0$ for calculation of entropy, ΔS_{Tm} at T_m (see **Table 1**). Although the adopted simplification has obviously its limitation, these values could serve for evaluation of the degree of order or disorder of the equilibrium complex, and for comparison of the various complexes. Thus, the complexes of dimers **7** and **9** but also "unrestricted" dimer **6** seem to exhibit more ordered structures at equilibrium than remaining compounds **1-5** including restricted dimer **8**; however, it does not necessarily mean the higher stability of the complex.

With the presented experimental results obtained with this limited but unique set of compounds, it seems that we get answer to the question of principal importance for any future work with this kind of nonisosteric phosphonate oligonucleotides: indeed, this internucleotide bond which features remarkable enzymatic stability but is one CH_2 group longer than natural one is evidently conformationally flexible enough to enable hybridization with natural polynucleotide strand, and even marked stabilization of the structures, as in the case of **4**, **5** and **6**, could occur. We are aware of the fact that, in certain attributes, longer oligomers may differ from related dimeric units in their complexation properties, as described for 20-mers of oligoA methylphosphonates where only duplex but no triplex was found[26] but it was the case of *non-charged* oligomers. Thus, without going into further speculations on the nature of the complexes, the observed direct relation of the T_m values to the introduced structural changes suggests potential usefulness of presented approach for making predictions on hybridization properties of longer chains and on their modulations. Obviously, to verify this idea in practice means the necessity to synthesize a set of oligonucleotides containing both modified and natural internucleotide linkages, e.g. in an alternating mode. Such work as well as detailed NMR and CD spectroscopy study of diverse types of ApA analogs is now underway.

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