Synthesis and Properties of Adenosine Oligonucleotide Analogues Containing Methylene Groups in Place of Phosphodiester 5′-Oxygens†

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ABSTRACT: The ADP analogue in which the 5′-oxygen has been replaced by a methylene group can be prepared by condensing 5′-deoxy-5′-phosphonomethyladenosine with inorganic phosphate. This analogue readily polymerizes onto the primer A–A in the presence of the enzyme polynucleotide phosphorylase and either Mg^{2+} or Mn^{2+}. The initial products are of the form A–A(–cA)$_n$–cA (where "–" and "–c" stand for the normal phosphodiester linkage and the linkage in which the 5′-oxygen is replaced with the methylene group, respectively). Treatment of these with alkali yields adenosine 2′(3′)-phosphate and the series (A(–cA)$_n$–cA containing only phosphonomethylene linkages. The decamer A(–cA)$_8$–cA interacts with two molecules of U(–U)$_8$–U to form a triple-standard structure that has a stability similar to that exhibited by the analogous complex formed from A(–A)$_8$–A and U(–U)$_8$–U. This property, along with the resistance of these oligomer analogues toward nucleases that cleave phosphodiester linkages between the phosphorus and the 5′-oxygen, should provide a strong rationale for application of phosphonomethylene linkages in schemes for therapeutic drug design that use the antisense strategy.

In the "antisense" strategy of drug development, synthetic oligonucleotide structures are designed to serve as inhibitors of gene expression or other nucleic acid functions by exploiting the capacity of the oligomer's nucleotide sequence to form a stable complex with its Watson–Crick complement within the cell. A large number of oligonucleotide analogue structures have been synthesized and studied for this purpose [reviewed by Uhlmann and Peyman (1990)]. The structural modifications have usually involved changes in the internucleotide linkage which permit the oligomer to conserve its capacity to form base-paired duplexes corresponding to the natural shape while at the same time conferring some resistance to nucleases that would normally destroy the molecule in vivo. The present study [for a preliminary report, see Breaker et al.(1990)] concerns the preparation of adenosine oligomers containing methylene groups in place of their 5′-oxygens, the effects of such substitution on their interaction with uridine oligomers, and their susceptibility to nuclease cleavage.

EXPERIMENTAL PROCEDURES

Materials. Micrococcus luteus polynucleotide phosphorylase, bovine spleen phosphodiesterase, and *Crotalus atrox* snake venom phosphodiesterase were purchased from Pharmacia Biotech Inc. S1 nuclease was obtained from GIBCO BRL. The oligomers A(–A)$_8$–A[1] and U(–U)$_8$–U were constructed on a DNA synthesizer using ribonucleoside 3′-phosphoramidite monomers incorporating the newly developed *o*-nitrobenzyloxymethyl moiety for protection of the 2′-hydroxyl group (Schwartz et al., 1992). The products were deblocked and then purified by anion-exchange HPLC. The appropriate fractions were pooled, concentrated in vacuo, desalted by dialysis, and stored, as described below for the phosphonomethylene oligomers.

Paper Chromatography. Separations were carried out in the descending mode on Whatman 3MM chromatographic paper using the solvent systems: (A) 2-propanol/concentrated NH_4OH/water (7:1:2, v/v); (B) 1-propanol/concentrated NH_4OH/water (55:10:35), v/v); (C) 2-methylpropionic acid/1 M NH_4OH/0.1 M EDTA[1] (100:60:1.6, v/v).

HPLC Analyses and Purification. Products from enzymatic polymerizations and from the chemical or nuclease cleavage of mono- or oligonucleotides were separated by anion-exchange HPLC using a column (1 × 25 cm) of cross-linked poly(ethyleneimine)-silica (Lawson et al., 1983) with a solvent system consisting of 300 mL of 0.05 M KH_2PO_4 containing 30% (v/v) MeOH and a linear gradient of 0–0.9 M $(NH_4)_2SO_4$ at pH 6.0, and with a flow rate of 1 mL/min. Purification of synthetic oligoribonucleotides A(–A)$_8$–A and U(–U)$_8$–U was achieved by using a column (0.4 × 15 cm) of the same material with a solvent system consisting of 100 mL of KH_2PO_4 containing 30% (v/v) MeOH and a linear gradient of 0–0.25 M $(NH_4)_2SO_4$ at pH 6.0, and with a flow rate of 1 mL/min.

5′-Deoxy-5′-Phosphonomethyladenosine. Synthesis of the AMP analogue pcA was carried out following the methods described by Jones and Moffatt (1968, 1973, 1975). 2′,3′-Isopropylidenyladenosine was oxidized to the corresponding 5′-aldehyde, which was then treated with the Wittig reagent diphenyl(triphenylphosphoranylidene)methylphosphonate to yield the unsaturated protected phosphonomethyleneadenosine. For the preparation of the Wittig reagent, chloromethylphosphonic dichloride was converted to diphenyl(chloromethyl)phosphonate by the method of Tsvetkov et al. (1969), and this ester was converted to the desired reagent by the procedure of Jones et al. (1968). The unsaturated protected phosphonomethyleneadenosine was reduced with diimide and the product subjected to alkaline hydrolysis to remove one of

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MES, sodium 2-(*N*-morpholino)ethanesulfonate; pcA, 5′-deoxy-5′-phosphonomethyladenosine; ppcA, the mixed anhydride formed from phosphoric acid and pcA; A–A, two adenosine nucleosides joined by a 3′-5′ phosphodiester linkage; A–cA, the analogue of A–A in which the 5′-oxygen of the second A residue is replaced with a methylene group.

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the phenyl groups, followed by acidic hydrolysis to remove the isopropylidene group. The resulting phenyl ester of 5′-deoxy-5′-phosphonomethyladenosine was purified by preparative paper chromatography using solvent A (R_f = 0.54). The phenyl group was removed by exposure of the ester to the action of snake venom phosphodiesterase, and the product was purified by paper chromatography using solvent B (R_f = 0.40). Finally, the 5′-deoxy-5′-phosphonomethyladenosine was converted to its free acid form by passing it through a column of Dowex ion-exchange resin (50W-X2, H^+).

Phosphonomethylene Analogue of ADP. The ADP analogue (ppcA) was prepared by phosphorylating 5′-deoxy-5′-phosphonomethyladenosine using the procedure described by Bennett and Gilham (1975) for converting 2′,3′-di-*O*-(α-methoxyethyl)nucleoside 5′-phosphates to the corresponding 5′-diphosphates. The product was purified by paper chromatography using solvent C (R_f = 0.66); the chromatogram was dried and washed with ethanol to remove residual 2-methylpropionic acid. The band of product was cut out, eluted with water, and subjected to a second purification on chromatographic paper using solvent B (R_f = 0.41). Conversion to the sodium salt was accomplished by eluting the product band with water and passing it through a column of Dowex ion-exchange resin (50W-X2, Na^+).

Stability of ADP and Its Phosphonomethylene Analogue in the Presence of Mn^{2+}. Solutions of ADP or ppcA (at 2.5 AU_{260}/mL) were prepared in Tris-HCl, pH 9.0, at 25 °C (100 mM), $MnCl_2$ or $MgCl_2$ (5 mM), and incubated at 37 °C. Aliquots of 100 μL were removed for analysis at appropriate times and centrifuged briefly. Any pellet formed was washed twice with water (2 × 100 μL), and the combined supernatants were subjected to analysis by anion-exchange HPLC. Elution volumes determined for pcA, AMP, ppcA, and ADP were 25, 34, 65, and 72 mL, respectively. The extent of hydrolysis was determined by measuring the peak areas obtained from HPLC profiles of the reaction products, and the value for the percent hydrolysis for each time point was corrected for the amount of AMP or pcA present in the sample at the start of the incubation. Plots of the percent hydrolysis versus log time yielded straight lines, and these were used to predict the half-lives.

Polynucleotide Phosphorylase Reactions. All polymerization reactions were conducted in the presence of Tris-HCl, pH 9.0, at 25 °C (100 mM), the primer A–A, ADP or ppcA, and either $MgCl_2$ or $MnCl_2$ as a source of divalent cations. The reactions were incubated at 37 °C and then heated for 5 min at 90 °C to inactivate the enzyme. Any precipitate formed during the polymerization was removed by centrifugation prior to analysis. In the case of the ppcA polymerization, HPLC analysis indicated peaks corresponding to oligomers with chain lengths of up to about 20. The peak elution volumes (mL) are A–A, 24; ppcA, 66; A–A–cA, 41; A–A(–cA)$_n$–cA (where n = 1–9), 51, 63, 76, 90, 104, 117, 131, 144, and 156, respectively. The percent polymerization was derived by measuring HPLC profile peak areas of all species present including unchanged ppcA or ADP (together with their breakdown products pcA or AMP). The number of moles present for each species was calculated from absorptivities at 260 nm derived by assuming a contribution of 15 000 for each adenine residue present in the molecule in the particular HPLC solvent used. Large-scale preparation of phosphonomethylene-linked polymers was carried out in 1 mL of 100 mM Tris-HCl (pH 9.0 at 25 °C) containing 1 mM A–A, 5 mM ppcA, 5 mM $MnCl_2$, and 10 units of polynucleotide phosphorylase. After 8 h at 37 °C, a reaction supplement (0.2 mL of 100 mM Tris-HCl, pH 9.0, at 25 °C) containing 25 mM ppcA, 5 mM $MnCl_2$, and 10 units of the enzyme was added. The polymerization was allowed to proceed for an additional 16 h, and the products were then separated by preparative paper chromatography using solvent B. Bands of A–A and ppcA had R_f values of 0.63 and 0.55, respectively; the material located between the ppcA and the origin was eluted with water. The extract was concentrated to dryness in vacuo, and the residue was dissolved in 0.5 M NaOH (0.4 mL). The solution was kept at 40 °C for 24 h. Separation of the oligomers by chain length was carried out by anion-exchange HPLC. The product peaks were separately desalted by dialysis against water using Spectra/Por CE dialysis tubing (MWCO: 1000) and then concentrated and stored at −20 °C in 0.01 M potassium phosphate, pH 7.0.

Characterization of Oligonucleotides. Some of the purified products from enzymatic polymerizations were subjected to analysis using nuclease digestions. The three enzymes and the concentrations used were (a) snake venom phosphodiesterase (0.4 unit/mL); (b) spleen phosphodiesterase (0.5 unit/mL); and (c) S1 nuclease (1000 units/mL). The corresponding buffers were (a) Tris-HCl (0.1 M, pH 8.7, at 20 °C), $MgCl_2$ (1 mM); (b) 10 mM MES (pH 6.5); and (c) sodium acetate (50 mM, pH 4.6), 0.2 M NaCl, and 1.0 mM $ZnCl_2$. Oligonucleotide concentrations were at 0.5–1.0 AU_{260}/mL, and incubation was set at 37 °C for 30 min to 3 h. Alkaline hydrolyses were performed in 0.5 M NaOH at 40 °C for 24 h; reactions were neutralized with solid CO_2 before analysis. Lead hydrolyses were carried out by heating the sample in solutions containing 40 mg/mL lead acetate at 37 °C for 12 h; Pb^{2+} ions were removed by passing the reaction mixture through a column of Dowex ion-exchange resin (50W-X2, Na^+) before analysis. Products formed in these digestions were identified by their HPLC elution volumes (mL): adenosine, 16; A–cA, 22; A–A, 24; pcA, 25; adenosine-2′(3′) phosphate 33, 34; AMP, 34; A–cA–cA, 40; A(–cA)$_2$–cA, 49; A(–cA)$_3$–cA, 61; ppcA, 65.

Absorbance–Temperature Profiles and Mixing Curves. Measurement of oligomer A_{260} values at various temperatures were conducted in solutions containing 0.01 M potassium phosphate, pH 7.0. Mixing curves were obtained by combining various proportions of U(–U)$_8$–U and A(–A)$_8$–A, or U(–U)$_8$–U and A(–cA)$_8$–cA, such that the total oligomer strand concentration remained constant. The buffer concentration was also kept constant: potassium phosphate, pH 7.0 (0.01 M); NaCl (1.0 M). The mixtures were allowed to stand at 1 °C for 10–15 min before measurement of their A_{260} values. In order to maintain constant strand concentrations in mixing experiments, it was necessary to make estimates of strand absorptivities. The decamer U(–U)$_8$–U displays little hyperchromicity upon heating, and so its ϵ_{260} was taken as 99 000 at 25 °C. For the two decamers A(–A)$_8$–A and A(–cA)$_8$–cA, the ϵ_{260} values at 25 °C were estimated as 131 000 and 135 000, respectively. These absorptivities were calculated by summing the contribution from each A residue (15 000) and reducing the value obtained by the amount of hyperchromicity measured between 25 and 90 °C in each case.

RESULTS

Synthesis and Polymerization of the ADP Analogue. The ADP analogue ppcA was readily prepared by condensing pcA with inorganic phosphate in the presence of 1,1′-carbonyldiimidazole following the procedure used by Bennett and Gilham (1975) to prepare 2′,3′-di-*O*-(α-methoxyethyl)guanosine 5′-diphosphate. Prior to subjecting the analogue to

the action of polynucleotide phosphorylase, it was tested for its chemical stability under the conditions to be used for the enzyme reactions (5 mM Mn^{2+}, pH 9.0, 37 °C). While both ADP and ppcA exhibit slow hydrolysis of their P–O–P bonds under these conditions, there is a considerable difference in the rates. The half-lives of ADP and ppcA are estimated as 5.4 days and 270 days, respectively.

Preliminary experiments with the enzymatic polymerization of ppcA in the presence of the primer A–A showed that Mn^{2+} was more efficient than Mg^{2+} in catalyzing the reaction, especially with respect to the formation of the longer oligonucleotides. In these experiments, comparisons of the polymerization of the phosphonomethylene analogue with that of ADP were also conducted. With ADP, the amount of polymerization reached some 70% after about 4 h, and this level slowly dropped back to 60% over the next 20 h. Under the same conditions, the analogue polymerized at a somewhat slower rate; 24 h were required for the yield to reach 60%. The various products obtained in the latter reaction were well-separated by HPLC, and the assignments of oligomer chain lengths were made on the basis of comparisons with the elution volumes of the series A(–A)$_n$–A (n = 1–10) and confirmed by subjecting a few of the products to analysis. For example, alkaline treatment of the material assigned as the tetramer gave molar equivalents of adenosine 2′(3′)-phosphate and A–cA–cA. The structure of the latter was then confirmed by hydrolysis with snake venom phosphodiesterase to give 1 equiv of adenosine and 2 equiv of pcA. The series A(–cA)$_n$–cA was produced by treating the above polymerization reaction with alkali prior to the HPLC fractionation. Chain lengths were then established by comparing individual products with the material obtained from alkaline treatment of the appropriate member of the A–A(–cA)$_n$–cA series.

The stability of adenosine oligomers containing all phosphonomethylene linkages toward chemical and enzymatic cleavage is as expected. The linkages in these molecules are resistant to cleavage by alkali, by Pb^{2+}, and by enzymes such as ribonuclease T2 and spleen phosphodiesterase that normally cleave the bond between the phosphorus and the 5′-oxygen. On the other hand, the oligomers are readily cleaved by snake venom phosphodiesterase and S1 nuclease, enzymes that cleave the bond between the phosphorus and the 3′-oxygen.

Spectrophotometric Analyses. Heat-induced hyperchromic effects were measured at 260 nm for members of the two series of molecules A(–cA)$_n$–cA and A(–A)$_n$–A. The two octamers A(–cA)$_6$–cA and A(–A)$_6$–A had absorbance ratios (for the two temperatures 90 and 1 °C) of 1.25 and 1.27, respectively. For measurements of A–U complex formation, the two decamers A(–cA)$_8$–cA and A(–A)$_8$–A were chosen. Each of these oligomers was mixed in various proportions with U(–U)$_8$–U for A_{260} measurements at 1 °C. In each of the two mixing experiments, the proportions of the U oligomer and the A oligomer were adjusted such that the total strand concentration was maintained at a constant value (Figure 1). The plots of the various A_{260} values indicate that, as with the normal adenosine decanucleotide, the oligonucleotide containing the phosphonomethylene linkages forms a 1:2 complex with the uridine decanucleotide at 1 °C.

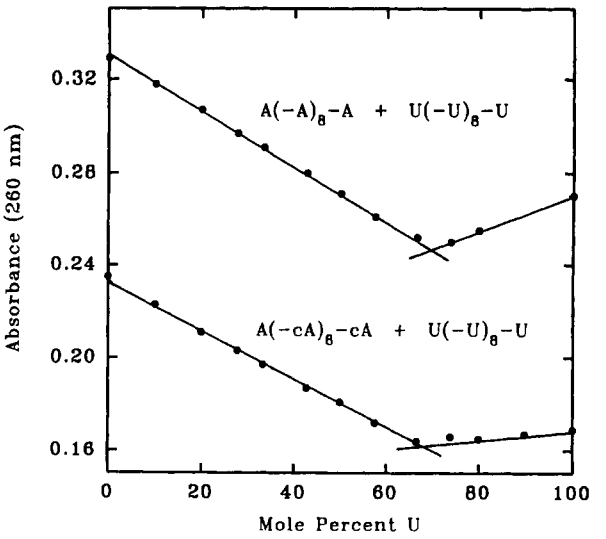

FIGURE 1: Mixing curves. Absorbance values obtained for two sets of mixtures, (a, upper) U(–U)$_8$–U + A(–A)$_9$–A and (b, lower) U(–U)$_8$–U + A(–cA)$_8$–cA, dissolved in 1.0 M NaCl, pH 7.0, at 1 °C. For each set, the total strand concentration in each mixture was kept constant at about 2.6 μM for set a and about 1.8 μM for set b.

DISCUSSION

The choice of Mn^{2+} as the cation for the enzymatic polymerization experiments derives from its previous use in the incorporation of a variety of chemically modified nucleoside 5′-diphosphates into oligomer structures (Mackey & Gilham, 1971; Hawley et al., 1978). However, Mn^{2+} is known to catalyze the hydrolysis of nucleoside diphosphates (Tetas & Lowenstein, 1963), especially at the higher pH values preferred by the enzyme polynucleotide phosphorylase. Since it was expected that the polymerizations would require extended periods of time, it was necessary to study the stability of the monomer substrates under such conditions. The fact that the ADP analogue ppcA displayed considerable stability, with a half-life some 50 times that of ADP itself, is consistent with the hydrolytic mechanism considered by Tetas and Lowenstein for the hydrolysis of ADP to AMP. This mechanism involves an attack of water or hydroxide ion on a complex formed by the divalent cation chelating with the oxygens on the adjacent phosphorus atoms. The attack would be on the β phosphorus with AMP as the leaving group. Assuming that an analogous metal complex can form in the case of ppcA, the corresponding leaving group pcA would be less easily displaced because pcA is likely to be a somewhat weaker acid than AMP. The differences between the pK_a values of the two nucleotides should be similar to those measured for simple phosphonates and phosphates. For example, n-butylphosphonic acid has pK_a values of 2.5 and 8.1 (Freedman & Doak, 1957), while n-butyl phosphate has values of 1.8 and 6.8 (Kumler & Eiler, 1943).

The rates of polymerization of the two substrates ADP and ppcA are quite different (initial rate for ADP is some 7 times that for the analogue, with yields of 70% and 27%, respectively, after 4 h). However, with 24 h of incubation, the amounts of products present in the two reactions are about the same. The explanation for this effect invokes "transnucleotidation", which has been shown to consist of the exchange of terminal nucleotides between the various oligonucleotide products. The mechanism of the exchange is understood to involve the phosphate-catalyzed back-reaction (Sninsky et al., 1974), and in the present case the back-reaction produces ADP which is, as discussed above, subject to Mn^{2+}-catalyzed cleavage to AMP. The net result then is a slow decrease in the amount of the various oligomers present in the reaction mixture. This effect is not seen in the case of the polymerization of the ADP analogue ppcA because it is much more stable to Mn^{2+}-catalyzed hydrolysis.

In the initial experiments on the physical behavior of the oligomers, there does not seem to be any great effect resulting

from the replacement of 5′-oxygens with methylene groups. The observed equivalence in the amounts of heat-induced hyperchromism displayed by corresponding members of the two series A(–cA)$_n$–cA and A(–A)$_n$–A suggests that the two types of oligomer contain about the same amount of base–base stacking. This interpretation does not match the results derived from a study of the optical properties of a number of dinucleoside phosphates (Johnson & Schleich, 1974). In this study, the phosphonomethylene analogue U–cA, in contrast to U–A, was found to display no optical activity attributable to base–base interactions, leading to the conclusion that the absence of detectable stacking demonstrates the importance of backbone stereochemistry to the maintenance of the stacked state. With the results of the present study, however, it is clear that this conclusion is not generally applicable and that it will require measurements on a variety of oligonucleotides with different lengths and sequences before the effects of the phosphonomethylene linkage on stacking can be completely evaluated.

The results of the mixing curves indicate that the oligomer analogue is able to mimic the normal adenosine oligomer in forming triple-stranded structures with 2 equiv of uridine oligomer of the same chain length. The structures formed are probably similar to those first observed by Felsenfeld and Rich (1957) for mixtures of poly(A) and poly(U); the complex formed at high ionic strength comprises one purine strand and two pyrimidine strands. This complex is thought to consist of a Watson–Crick interaction between the poly(A) strand and one of the poly(U) strands, along with an antiparallel Hoogsteen interaction between the poly(A) and the other poly(U) strand (Arnott et al., 1976). If, in the case of the two oligomer triple helices, the structures possess the same level of interaction complexity, the small differences in the phosphonomethylene bond lengths and bond angles compared with the normal phosphodiester linkage must be counterbalanced by other conformational changes in satisfying the structural requirements of the two types of base–base interactions present in the analogue triple helix. It should be possible to define the nature of these changes by carrying out NMR or X-ray crystallographic analyses on appropriate analogue double and triple helices.

Although these studies on oligomers containing the phosphonomethylene linkage are preliminary, it seems likely that molecules of mixed sequences containing part or all of their linkages in the analogue form will be able to mimic the structural interactions of naturally occurring polynucleotides. This property, along with the resistance to many exo- and endonucleases conferred on the oligomer analogues, should make the incorporation of the phosphonomethylene linkage into synthetic molecules an effective strategy in the "antisense" approach to the control of gene expression. Furthermore, the phosphonomethyl group may find use in the study of ribozyme mechanisms, in particular those mechanisms involved in the cleaving of RNA molecules at a bond located between a phosphorus and its associated 5′-oxygen atom. The incorporation of the phosphonomethylene linkage at the point of cleavage could permit the neighboring conformation in the substrate portion to assume the native shape and allow the analysis of the overall structure to be performed without the risk of its self-destruction.

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