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Polynucleotides. LII.[1] Synthesis and properties of poly(2'-deoxy-2'-fluoroadenylic acid)

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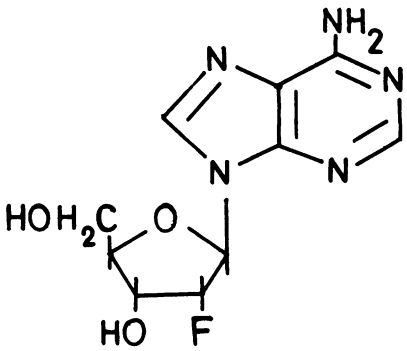
ABSTRACT

2'-Deoxy-2'-fluoroadenosine was chemically transformed to its 5'-diphosphate and polymerized with polynucleotide phosphorylase to give poly(2'-deoxy-2'-fluoroadenylic acid) [poly(Af)]. Polymerization proceeded smoothly as in the case of poly(A) and the yield of the polymerization was 55%. The UV absorption spectra of poly(Af) closely resembled those of poly(A) and the hypochromicity was 32% at pH 7.0. The CD profile at 25° and neutrality showed similar pattern to that of other poly(2'-deoxy-2'-halogenoadenylic acids) with somewhat larger [θ] values both in the positive and negative maxima. Acid titration of poly(Af) showed a transition point at pH 5.2 and the Tm of the acid form was 37° which was significantly lower than that of poly(A), but similar to that of poly(2'-azido-2'-deoxyadenylic aicd). Poly(Af) formed 1:1 and 1:2 complexes with poly-(U) having Tm of 49° and 62° at 0.04M and 0.15M Na^+ concentration, respectively. Poly(Af) also formed a 1:2 complex with poly(I) and its Tm was 36° at 0.05M Na^+ concentration. These data showed that poly(Af) has rather similar properties to those of poly(A),but not to poly(dA).

INTRODUCTION

Recently we have reported the synthesis and properties of poly(2'-deoxy-2'-azido-[2], chloro-[3] and bromoadenylic acid).[3] The general feature on introducing aprotic and polarizable group such as azido, chloro or bromo at the 2'-position instead of the OH of poly(A) is that the physical properties of these plynucleotides are rather similar in spite of their lacking proton donors, which are thought to stabilize ribopolynucleotide conformations.[4,5] Moreover, it was found that poly(2'-azido-2'-deoxyinosinic acid)[6] was active as an interferon inducer when complexed with poly(C).[7]

In this paper we report a method for synthesis of poly(Af) from 2'-deoxy-2'-fluoroadenosine[8] and data on its physical pro-



2'-Deoxy-2'-fluoro-adenosine

perties such as UV and CD spectra, Tm, acid titration of single stranded form and formation of complexes with poly(U) and poly-(I).

MATERIALS AND METHODS

2'-Deoxy-2'-fluoroadenosine 5'-monophosphate

2'-Deoxy-2'-fluoroadenosine[8] (40.3 mg, 0.15 mmole) was stirred with $POCl_3$ (0.1 ml, 1.1 mmole) and $(EtO)_3PO$ (1 ml) at 0° for 3 hrs. The mixture was poured in ice-water (ca. 200 ml) and the solution was applied to a column of charcoal (ca. 2 ml). After the water-wash the nucleotide was eluted with a mixture of EtOH-H_2O-c.NH_4OH (50:50:1, vol/vol, 50 ml) and eluents were evaporated in vacuo. The residue was dissolved in H_2O and applied to a column of Dowex 1x2 (formate form, 2 ml). After the water-wash, elution with 0.1N formic acid gave a peak of Af 5'-MP. The yield was 1607 A_{260} (0.11 mmole, 76%). Paper chromatography : Rf(A) 0.23, Rf(C) 0.13. Paper electrophoresis (at pH 7.5): $R_{A\text{-}pA}$ 1.04. When Af5'-MP (3 A_{260} units) was incubated with 0.1M $MgCl_2$ 5 µl, 1M Tris.HCl (pH 8.5) 4 µl, crude snake venom (10 mg/ml) 30 µl and H_2O 10 µl at 37° for 4 hrs, it was completely dephosphorylated. Thus the position of phosphorylation was confirmed as 5'.

2'-Deoxy-2'-fluoroadenosine 5'-diphosphate

Af 5'-MP (1600 A_{260}, 0.11 mmole) and morpholine (0.1 ml, 1.1 mmole) were dissolved in t-BuOH (1.5 ml) and H_2O (1.5 ml). While this solution was refluxed, DCC (235 mg) dissolved in t-BuOH (2 ml) was added dropwise in 40 min. After 1.5 hrs refluxing, morpholine (0.1 ml, 1.1 mmol) and DCC (235 mg) were

added and the refluxing was continued for a further 2 hrs. H_2O (ca. 20 ml) was added, dicyclohexylurea was removed by filtration, and the solution was extracted with ether (10 ml x 3). The aqueous solution was evaporated in vacuo and evaporated three times with added pyridine. Inorganic phosphoric acid (80%, 40 µl, 0.58 mmole) and $(n\text{-But})_3N$ (0.14 ml, 0.58 mmole) were rendered anhydrous by evaporation three times with pyridine. Both residues were dissolved in DMF (1 ml) and kept at 31° for 3 days. H_2O (ca. 60 ml) was added and the acidic solution was applied to a column of charcoal (20 ml). After a water-wash, the nucleotide was eluted with methanolic ammonia (50 ml) as was described before. Eluents were evaporated in vacuo and the residue applied to a column (1.5 x 38 cm) of DEAE-Sephadex A-25 (bicarbonate form). Elution with triethylammonium bicarbonate buffer of 0M to 0.3M in a linear gradient gave three peaks. The last peak which eluted at 0.15M buffer concentration was pooled and evaporated in vacuo. The yield was 705 A_{260} (0.05 mmole, 46%). Paper chromatography : Rf(A) 0.13, Rf(B) 0.44. Paper electrophoresis (at pH 7.5) : $R_{A\text{-}pA}$ 1.35.

Poly(2'-deoxy-2'-fluoroadenylic acid) [poly(Af)]

A solution (5 ml) containing 2'-deoxy-2'-fluoroadenosine 5'-DP 4 mM, $MgCl_2$ 2 mM, Tris.HCl (pH 8.5) 100 mM and *E. coli* polynucleotide phosphorylase 4.5 units/ml was incubated at 37° for 24 hrs. Inorganic phosphate (0.68 µmol/0.25 ml of the incubation mixture) was liberated. The mixture was extracted with i-AmOH-$CHCl_3$ (1:3, vol/vol) mixture and the water-layer was lyophilized. The powder thus obtained was filtered through a column (2.7 x 95 cm) of Sephadex G-50 (540 ml). The material wich was excluded in the void volume was collected. The yield was 108 A_{260} (0.011 mmole, 55% regardless of hypochromicity). Digestion of this polynucleotide with snake venom phosphodiesterase showed only Af 5'-P and 2'-deoxy-2'-fluoroadenosine was not detected on a paper chromatogram of the digest. This means that the chain length of the polynucleotide is greater than 100 nucleotide units.

Physical measurements

U.V. absorption spectra were taken with a Hitachi 124

spectrophotometer equipped with a thermostated cell. CD spectra were measured with a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment. Tm was measured with a Hitachi 124 spectrophotometer equipped with a themostated cell. The temperature inside the cell was measured with a thermocouple.

Paper chromatography and electrophoresis

Paper chromatography was performed in solvent systems: A, n-BuOH-AcOH-H_2O (5:2:3) and B, i-PrOH-conc.NH_4OH-H_2O (7:1:2)by the descending technique. Paper electrophoresis was performed in 0.05M triethylammonium bicarbonate buffer (pH 8.5) at 900 V/20 cm for 1 hr.

Enzymes

Polynucleotide phosphorylase was prepared by the method described by Williams and Grunberg-Manago.[9] Crude snake venom was a gift from Kagoshima Prefecture Hygienic Institute to which our thanks are due. Purified snake venom phosphodiesterase was purchased from Worthington Biochem. Co.

RESULTS AND DISCUSSION

Polymerization of AfDP catalyzed by polynucleotide phosphorylase

The polymerization of AfDP using E. coli polynucleotide phosphorylase proceeded smoothly to the extent of 65% in 24 hrs. This rate is comparable to that of ADP plymerization, while AzDP[2], AclDP[3] and AbrDP[3] polymerized in the same conditions to extents of only 13-25%. This may mean that the conformation of AfDP in the incubation mixture is very similar to that of ADP. It is wothwhile mentioning that in spite of the small size of the 2'-fluoro atom, AfDP completely mimics ADP rather than dADP, which is known to be an inhibitor of the polymerization.[10]

U.V. absorption properties of poly(Af)

The U.V. absorption spectrum of poly(Af) at pH 7.0 and 25° is shown in Fig. 1 together with that of Af 5'-MP. The spectrum of poly(Af) has λ max at 255 nm and the ε_{max} was 9,700. The hypsochromic shift (4 nm)is significantly larger than that of poly(A) and ε value is smaller than that of poly(A) measured in the same conditions (Table I).

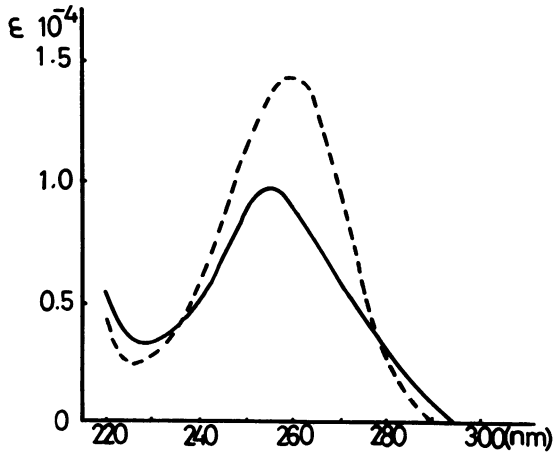


Fig. 1 U. V. absorption spectra of poly(Af) and Af 5'-MP. Poly(Af) ———, Af-5'-MP ----.

These facts may indicate that in poly(Af) the adenine residues stacked more strongly than in poly(A), presumably because of the large polarity and small size of fluorine atom at the 2'-position. The hypochromicity calculated from the ε value of the monomer is 32%, which is also somewhat smaller than that of poly(A). This is in accordance with our previous findings[2,3] that the size and polarity of 2'-substituents influence the UV spectral properties of polynucleotides.

CD spectrum of poly(Af)

The CD spectrum of poly(Af) is shown in Fig. 2 together with that of poly(Acl) and poly(Abr). The shape of the spectra closely resmble each other and that of poly(A), suggesting that these 2'-hogenated polynucleotides are in very similar conformations in solution at pH 7.0, which is analogous to that of poly(A). The $[\theta]_{peak}$ of poly(Af) at 264 nm is 6,3000 and $[\theta]_{trough}$ at 248 nm is -49,000 (see Table II).

Comparing these values with those of poly(Acl) and poly-(Abr), it was shown that the total [θ] values are in the order

Table I

Polynuclotide	ε	(λmax)	εMonomer	(λmax)	Hypochromicity (%)
Poly(A)	10,000	(257 nm)	15,400	(259 nm)	35
Poly(Af)	9,700	(255)	14,300	(259.5)	32
Poly(Acl)	10,500	(257)	15,500	(259)	32
Poly(Abr)	10,700	(257)	15,000	(259)	29

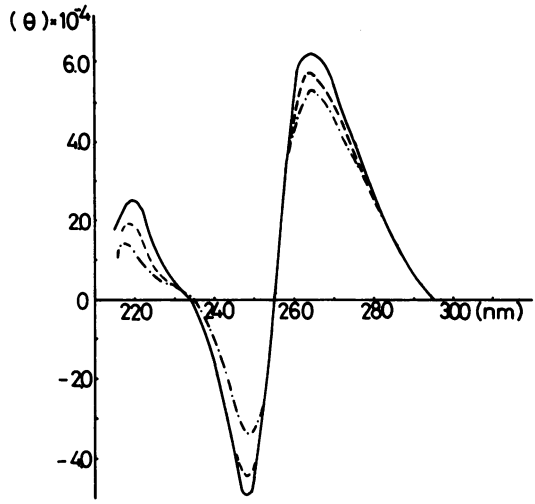


Fig. 2 CD spectra of poly(Af), poly(Acl) and poly(Abr). Poly(Af) ——, poly(Acl) ----, poly(Abr)—.—.—.

Af > Acl > Abr, which is inversely parallel to the size of the 2'-substituents. This fact may indicate that, if the nature of the substituents is similar, the size of the 2'-atom is an important factor for determining [θ] values in CD spectra, which are caused by coupling of transition moments[11] of adenine bases stacked one on another in the polynucleotide array.

Acid titration of poly(Af)

When poly(Af) was titrated with 0.1N HCl at 25° in the presence of 0.15M Na^+, a titration curve having a transition point at pH 5.2 was obtained. Thia fact indicated that poly-(Af) changed from its neutral form to an acid form as has been found in the case of poly(A).[12] As shown in Fig. 3, the CD curve of poly(Af) acid form showed a curve of similar shape, but different [θ] values to that of poly(A) acid form.

This may indicate that the introduction of the strongly electron-withdrawing fluoro atom at the 2'-position instead of OH, changed the stacking conformation of the acid form significantly. This may be seen in Table III by comparing the Tm

Table II

Polynucleotide	$[\theta]_{peakI}$ (nm)	$[\theta]_{trough}$ (nm)	$[\theta]_{peakII}$ (nm)
Poly(Af)	63,000 (264)	-49,000 (248)	25,000 (220)
Poly(Acl)	57,000 (264)	-44,000 (248)	19,000 (218)
Poly(Abr)	53,000 (264)	-34,000 (249)	14,000 (217)

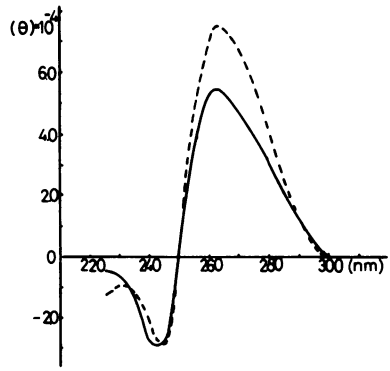


Fig. 3 CD spectra of poly(Af) and poly(A) acid form. Poly(Af) ———, poly(A) ----.

values of the acid form of poly(Af) with that of other polynucleotides. Introduction of aprotic substituents such as halogen or azido groups markedly decreases the Tm of the acid forms by as much as 20-40°. This may suggest that the proton at 2'-OH plays an important role in stabilizing the conformation of the poly(A) acid form.

Complex formation of poly(Af) with poly(U)

As all 2'-substituted polynucleotides so far examined formed two- and three-stranded complexes[2,3] with poly(U) as in the case of poly(A),[13] the complex formation of poly(Af) was studied.

In conditions of 0.15M Na^+ concentration, pH 7.0 and 25° poly(Af) formed a three-stranded complex with poly(U) as indicated by the continuous variation curves shown in Fig. 4. Measurement of U.V. spectra at percentages from 0-30% poly(Af) showed isosbestic points at 232, 283 and 300 nm, while at percentages between 40-100% isosbestic points were observed at 222, 281 and 300 nm. This suggests that only one three-stranded complex is present in this solution.

Table III

Polynucleotide	Tm at pH 4.5 in the presence of 0.10M NaCl(at 24-26°)
Poly(A)	over 80°
Poly(Af)	37°
Poly(Acl)	63°
Poly(Abr)	56°
Poly(Az)	38°

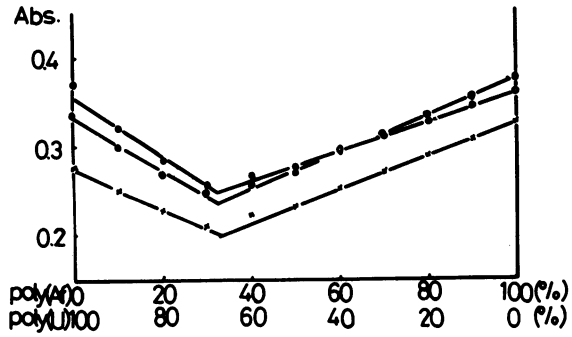


Fig. 4 Mixing curves of poly(Af) with poly(U) measured at 25° in the presence of 0.10M NaCl and 0.05M Na Cacodylate (pH 7.0).
At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

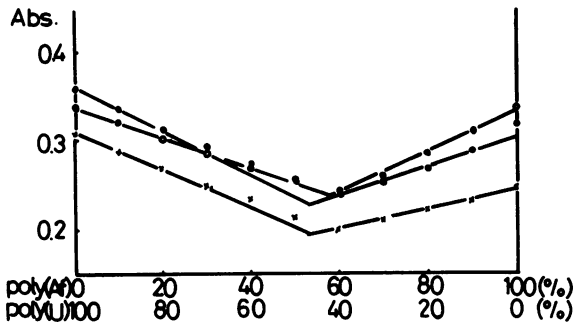


Fig. 5 Mixing curves of poly(Af) with poly(U) in the presence of 0.04M Na Cacodylate (pH 7.0) at 25°.
At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

At Na^+ concentration of 0.05M mixing curves (Fig. 5) showed 1:1 complexing of poly(Af) and poly(U) only after 30 min annealing. These curves gradually changed to those of a 1:2 complex after 7 days at 25°. Therefore, the 1:1 complex is relatively unstable in this condition and gradually rearranges to the 1:2 complex even in these low salt conditions.

CD curves before and after the mixing of poly(Af) and poly(U) showed significant changes (data not shown) and the complex formation has been substantially confirmed.

The Tm's of A-U complexes of various 2'-halogeno polynucleotides are summarized in Table IV. It is shown that the sizes of Tm values are inversely parallel to those of the halogen atoms in the order of F > Cl > Br for both 1:1 and 1:2

Table IV

Polynucleotide	Tm at 0.04M Na^+	Tm at 0.15M Na^+
Poly(Af)+poly(U)	49°	64°
Poly(Acl)+poly(U)	46°	56°
Poly(Abr)+poly(U)	45°	53°

complexes. Again the size of 2'-substituents semms to influence the Tm of the hetero complexes. Electronegativity of the halogen atoms might also be a consideration of the stacking conformations.

Complex formation of poly(Af) with poly(I)

Poly(A) was reported to form a triple stranded complex with poly(I).[14] When poly(Af)-poly(I) complexing was tested by the continuous variation method, curves as shown in Fig. 6 were obtained. These curves clearly showed inflection points at poly(Af):poly(I) ratios equal to 33:67 indicating that a 1:2 complex was formed. U. V. absorption curves measured in poly(Af) concentration ranges 0-30% showed isosbestic points at 227, 260 and 300 nm and those between 40-100% showed isosbestic points at 246, 283 and 300 nm. These facts indicated that only a 2:1 complex was present in the mixture.

Fig. 7 shows the melting curves of various complexes of poly(A) analogs with poly(I). All these complexes had sharp transition points and hypochromicities before and after the melting are of the same order. As summarized in Table V, the

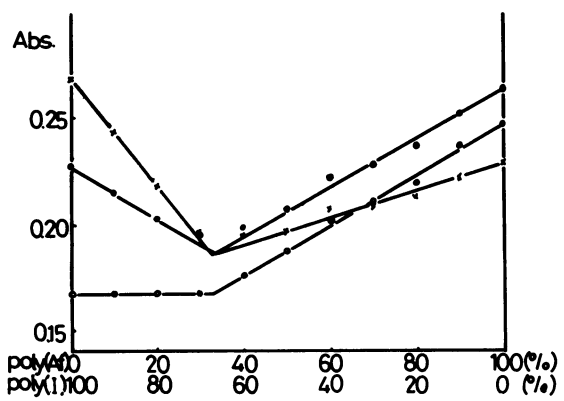


Fig. 6 Mixing curves of poly(Af) with poly(I) in the presence of 0.10M NaCl and 0.05M Na Cacodylate (pH 7.0) at 25°. At 250 nm x-x-x, 255 nm ●-●-●, 260 nm o-o-o.

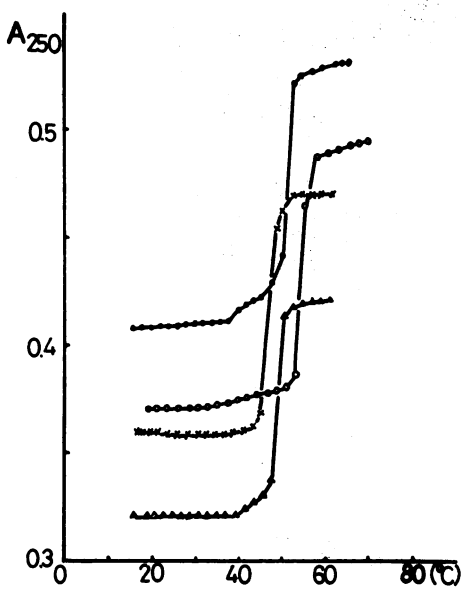


Fig. 7 Melting profiles of poly(A)-poly(I) complexes measured in the presence of 0.10M NaCl, 0.05M Na Cacodylate (pH 7.0) and 10mM $MgCl_2$.
Poly(Af)·2poly(I) ●-●-●, poly(A)·2poly(I) o-o-o, poly-(Abr)·2poly(I) x-x-x, poly(Abr)·2poly(I) △-△-△.

Table V

Complex	Tm at 0.15M Na^+	+10mM Mg^{2+}
Poly(A)·2poly(I)	42°	54°
Poly(Af)·2poly(I)	36°	51°
Poly(Acl)·2poly(I)	36°	49°
Poly(Abr)·2poly(I)	35°	47°

Tm differences among the 2'-halogeno polynucleotide complexes are rather small and somewhat lower than that of poly(A)·2poly-(I). However, in the presence of 10mM Mg^{2+} in addition to 0.15M Na^+, the differences increased and the order of Tms was Af $\rangle$ Acl $\rangle$ Abr. The tendency for a decrease in thermal stability with size of the substituents was again observed in these three-stranded, all purine polynucleotide complexes.

CONCLUDING REMARKS

We have presented evidence that poly(A) analogs having 2'-halogen atoms instead of OH show quite similar physical properties to those of poly(A). Even when the size of the

halogen is very small (as in the case of F), the polynucleotide poly(Af) showed similar properties to poly(rA) rather than poly(dA). These facts clearly demonstrate that 2'-substituents of nucleosides in polynucleotides must be involved as governing factor(s) of polynucleotide conformation and the size as well as polarity of these substituents have influence on their stability. If the nature of the 2'-substituents is similar, for instance halogen atoms, the conformational stability decreases as the size of the substituent increases.

Although the true mechanism by which 2'-substituents exert their stabilization effects must await further investigations, some involvement of solvent molecules cannot be excluded.

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