Polynucleotides. XXIV¹. Synthesis and properties of a dinucleoside monophosphate derived from uridine 6,2'-cyclonucleoside²

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ABSTRACT

A dinucleoside monophosphate, 6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyl-uracil-phosphoryl-(3'-5')-6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyluracil (I) was synthesized by the condensation reaction using DCC from 5'-monomethoxy-trityl derivative(VII) and 3'-acetyl-5'-phosphate(X) of the monomer units. Yield was ca.70%. UV of compound I showed no hypochromicity and its CD spectrum showed only a trough at 252 nm. No temperature dependency of ϵ and $[\theta]$ values was observed. These results suggested an unstacked random conformation for I.

Complex formation of I with cycloadenosine oligomer could not be detected under the experimental condition.

Previously we have shown³⁻⁵ that oligonucleotides of 8,2'-cycloadenosine had unique features of stacking to form a left-handed helix and of extremely high melting temperature. Because of these properties, 8,2'-cycloadenosine oligomers could not form double helical complexes with oligomers of usual bases such as poly(U), despite the fact that the left-handed L-ApA could form a complex with poly(U)⁶.

In order to test a hypothesis that oligonucleotides having the same (or nearly same) torsion angle ϕ_{CN} ⁷ form helical complexes, we attempted to synthesize a dinucleoside monophosphate, 6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyl-uracil-phosphoryl-(3'-5')-6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyluracil ($U^O p U^O$) (I). Since the torsion angle of U^O must be around -120° *, this dimer should be expected to form the complex with oligomers of adenine 8,2'-cyclo-nucleosides, which have the torsion angle equal to -122° ⁸.

Synthesis of $U^O p U^O$ (I) was conducted as in Chart I. Starting from uridine (II), arabinofuranosyluracil(IV) was synthesized by a method of Hampton and Nichol⁹. Compound IV was brominated at position 5 by the method reported for bromination of uridine¹⁰ to give compound V, which was cyclized to 6,2'-O-cyclonucleoside(VI) as reported by Otter et al¹¹.

* 5-Iodo derivative of U^O has $\phi_{CN} = -104^\circ$, private communication from Dr. M. Nishikawa.

Chart I.

Compound VI was tritylated in pyridine to give 5'-O-trityl derivative (VII) in a yield of 71%. Phosphorylation of VII using cyanoethyl phosphate and DCC¹² and subsequent detritylation with 80% acetic acid gave 6,2'-cyclo-uridine 3'-phosphate(VIII).

When compound VI was phosphorylated with phosphoryl chloride in trimethyl phosphate¹³, a phosphorylated product(IX) was obtained in a yield of 70% after purification on a Dowex (formate) column. Comparison of IX with the 3'-phosphate obtained as above showed complete difference of behaviors in paper chromatography and in enzymatic hydrolysis. Compound IX was thus confirmed to be 6,2'-O-cyclo-uridine 5'-phosphate. Compound IX was protected on 3'-OH by an acetyl group to give compound X. The condensation reaction of compound X and VII was performed in an absolutely anhydrous condition using DCC as the condensating agent at 27° for 60 hr.. Appropriate work-up and column chromatography on DEAE-cellulose (carbonate form), which was eluted with a linear gradient of 0.1 M triethylammonium bicarbonate buffer, gave $U^O p U^O$ (I) in a yield of 70%. This sample was digested partly (17% of TpT) by snake venom phosphodiesterase and the digested products were U^O and $p U^O$ in a ratio of 1:1. Together with other physicochemical properties described below, the structure of I was confirmed to be 6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyluracil-phosphoryl-(3'-5')-6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyluracil.

Properties of $p U^O$ and $U^O p U^O$ toward enzymatic hydrolysis

$p U^O$ was completely hydrolyzed to U^O and Pi with E. coli alkaline phosphatase, but resisted to the hydrolysis with crude snake venom 5'-nucleotidase or potato 3'-nucleotidase. This properties may be because of an unusual cyclo-nucleoside conformation leading to difficulties in substrate-enzyme binding as previously found in cycloadenosine phosphate³.

$U^O p U^O$ could be digested with snake venom phosphodiesterase to an extent of 17% in the condition of 100% hydrolysis of TpT by 4 hr incubation. The incubation for 24 hr gave 37% hydrolysis of $U^O p U^O$. Analysis of the product by paper electrophoresis and paper chromatography showed only $p U^O$ and U^O in addition to unreacted $U^O p U^O$. The ratio of $p U^O$ and U^O was nearly equal to 1:1. Therefore, the hydrolyzed material at least was shown to be $U^O p U^O$ having the 3'-5' phosphodiester linkage. Spleen phosphodiesterase could not hydrolyze $U^O p U^O$ even after 25 hrs' incubation. This fact suggests again an unusual conformation of $U^O p U^O$, which is unable to bind to the enzyme active site(s). Purine cyclooligomers also have not been hydrolyzed with these enzymes³.

UV and CD spectra of pU^O and U^OpU^O

As shown in Fig.1, pU^O and U^OpU^O have almost the same ϵ of 1.6×10^4 at λ_{max} 's 254 nm showing no hypochromicity. This fact indicates that in the molecule of U^OpU^O , the monomer unit U^O exists in an unstacked form.

In the CD spectra (Fig.2), both nucleotides also have nearly the same $[\theta] = -38000 - -40000$ and their troughs appear only at 251 nm. This negative value is as predicted by Ulbricht et al.¹⁴ for 6,2'-cyclouridine. However, in the CD spectrum of U^OpU^O splitting¹⁵ of B-band into a couple of + and - bands was not observed. When temperature dependency of UV and CD was measured as to U^OpU^O (Fig.3), there observed no change between 0° and 80° for ϵ and 0°-60° for $[\theta]$ values.

These facts suggested that there is almost no interaction of bases in the molecule of U^OpU^O and the compound itself exists in a random coil form in this condition (in presence of 0.1 M KF and pH 7.3). For a dimer UpU having usual torsion angle, some interaction of bases was observed^{16,17} and $[\theta]$ value decreased 30% between 0° and 50°. Therefore, it may be deduced that in U^OpU^O an unusual conformation of the monomer units due to bases fixed at $\phi_{CN} \approx -120^\circ$, caused unfavorable effect towards the stacking. This point was also supported by investigation of a CPK molecular model, in which overlapping of the U^O bases was extremely difficult because of the fixation of the bases at certain angle by means of the anhydro linkages.

This fact is rather unexpected, because in the case of purine cyclo-nucleoside oligomers extremely thermostable conformations have been found^{3,4}. In the purine cyclooligomers, however, fixation of the bases at -120° may favor the stacking of bases, because point of the fixation exists far from the pyrimidine portion of the purine ring, which is important for the stacking. For the pyrimidine cyclooligomer, bases are directly fixed by the anhydro linkage and the situation of two bases is significantly different from that of the purine oligomers.

Complex formation of U^OpU^O with oligo(A^S) or poly(A)

Possibility of the complex formation of U^OpU^O either with oligo(A^S) having $\phi_{CN} \approx -120^\circ$ or with poly(A) having $\phi_{CN} \approx -30^\circ - -40^\circ$ were examined by the method of mixing of various molar ratios of two complementary oligomers.

By the criteria of UV absorption, neither oligo(A^S) nor poly(A) would show the tendency to form complexes with U^OpU^O . From our previous experiments with poly(U) and oligo(A^S)⁴, it may be predicted that U^OpU^O and poly(A) may not form the complex, because ϕ_{CN} is largely different and poly(A) exists in

a right-handed stacked conformation¹⁸. In the case of octamer of A^S, however, there is no unfavorable factor for the complex formation, because both oligomers have almost the same $\phi_{CN} \approx -120^\circ$. Moreover, a left-handed helical conformation for A^S oligomer and a random coil conformation for U^OpU^O were assigned. In the case of L-ApA⁶ also a very flexible conformation of the backbone would be favorable for complexing with poly(U). A preliminary experiments² between a A^S octamer and a U^O octamer showed formation of a stable complex, which melted at below 15°. Therefore, it would be deduced that the desired complex between U^OpU^O and oligo(A^S) has a very low T_m presumably below 5° and the indication of the complex formation has not been observed by the present experiments.

Synthesis of the U^O oligomer and its properties will be published shortly.

EXPERIMENTS

UV spectra were taken with a Hitachi EPS-3T or 124 spectrophotometer, CD spectra with a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment. T_m and mixing curves were measured with Komatsu SPD-H-124 equipped in Hitachi 124 spectrophotometer or with a low temperature apparatus of JASCO for CD instrument. Temperature inside of the cell was measured with Cu-constantan thermocouple at 10 mm light path. $[\theta]$ and ϵ were represented as per residue value. Phosphate analysis was performed by Allen's method. NMR spectra were taken with Hitachi R20 (90 MHz) or R22 (60 MHz) spectrometer in d₆-DMSO using tetramethylsilane as external standard. Paper electrophoresis (PEP) and paper chromatography (PPC) were performed on Toyo filter paper No. 51A. PEP was done in 0.05 M triethylammonium bicarbonate buffer (pH 7.5) or 0.05 M borate buffer (pH 6.0) at 35 V/cm for 30 min to 1 hr. Solvent of PPC was as follows: A, ethanol-1 M ammonium acetate (7:3); B, n-butanol-acetic acid-water (5:2:3); C, isopropanol-conc.NH₄OH-water (7:1:2); D, n-propanol-conc.NH₄OH-water (55:10:35); E, saturated ammonium sulfate-water-isopropanol (74:19:2). All PPC were done by the descending technique.

1-β-D-Arabinofuranosyluracil (IV)—— 2,2'-Anhydro-1-β-D-arabinofuranosyluracil⁹ (III) (3.48 g, 15.4 mmoles) was dissolved in 0.1 N NaOH (10 ml) and kept at room temperature for 1 hr. After completion of the hydrolysis was confirmed by TLC, Dowex 50 (H⁺ form, 60 ml) was added. The resin was removed by filtration and filtrate and washings (H₂O, 100 ml x 2) were evaporated to dryness in vacuo. Residue was recrystallized from methanol. IV m.p. 225.5°-

Fig. 1

Fig. 2

Fig. 1 UV spectra of pU^O and $U^O pU^O$, taken at room temperature with 0.1 M KF and 0.01 M phosphate buffer, pH 7.3. --- pU^O , — $U^O pU^O$

Fig. 2 CD spectra of pU^O and $U^O pU^O$, taken at room temperature with 0.1 M KF and 0.01 M phosphate buffer, pH 7.3. --- pU^O , — $U^O pU^O$

Fig. 3 Temperature dependency of UV absorption and circular dichroism of $U^O pU^O$. ●—● CD, ○—○ UV absorption

227° was obtained in a yield of 3.47 g (92%). UV, $\lambda_{\max}^{\text{H}^+}$ 263.5 nm, $\lambda_{\max}^{\text{OH}^-}$ 265 nm. NMR, $J_{\text{H}_1, -\text{H}_2} = 4.2$ Hz (uridine, $J_{\text{H}_1, -\text{H}_2} = 4.6$ Hz). PEP (borate pH 6.0) $R_{\text{uridine}} 0.7$. PPC, Rf(A) 0.69, Rf(B) 0.54, Rf(E) 0.59.

1- β -D-Arabinofuranosyl-5-bromouracil(V)----- Compound IV (3.42 g, 14 mmoles) was dissolved in ethanol (50 ml) and Br_2 (0.85 ml, 15 mmoles) was added dropwise under gentle stirring. The solution was refluxed for 2 hr. Br_2 (0.1 ml) was added again dropwise and the refluxing was further maintained for 45 min. The mixture was concentrated in vacuo and crystalline material which appeared during overnight storing was filtered. Crystals were recrystallized from 95% ethanol to give 3.7 g of V (yield 82%), m.p. 237°-237.5°. UV, $\lambda_{\max}^{\text{H}^+}$ 281 nm, $\lambda_{\max}^{\text{H}_2\text{O}}$ 281.5 nm, $\lambda_{\max}^{\text{OH}^-}$ 279 nm. NMR, $\text{H}_6 = 8.06$ ppm. PPC, Rf(A) 0.78, Rf(B) 0.67. Beilstein test: (+).

6,2'-Anhydro-6-oxy-1- β -D-arabinofuranosyluracil(VI)----- Compound V (1.38 g, 4.25 mmoles) was dissolved in methanol (200 ml), containing Na (490 mg). The solution was refluxed for 25 hr under exclusion of moisture and neutralized with Dowex 50 (H^+ form, 40 ml). The resin was filtered and filtrate and washings were evaporated. Residue was crystallized from ethanol to give VI (520 mg, 51%), m.p. 254°-256°. UV, $\lambda_{\max}^{\text{H}^+}$ 253 nm, $\lambda_{\max}^{\text{H}_2\text{O}}$ 253 nm, $\lambda_{\max}^{\text{OH}^-}$ 255 nm. PPC, Rf(A) 0.69, Rf(B) 0.54, Rf(C) 0.53, Rf(E) 0.63.

5'-O-Monomethoxytrityl-6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyluracil(VII)----- Compound VI (480 mg, 2 mmoles) and monomethoxytrityl chloride (740 mg, 2.4 mmoles) were dissolved in anhydrous pyridine (10 ml) and kept at room temperature for 43 hr under stirring. The reaction mixture was poured into dilute ammonia in ice-water (75 ml) and the nucleoside was extracted with chloroform (40 ml x 4). Chloroform layer was dried over MgSO_4 and evaporated in vacuo. Residue was dissolved in a small amount of pyridine and added dropwise to a mixture of ether-n-pentane (1:1). Solvent was removed by centrifugation and pellet was triturated with ether-pentane (2:1) mixture. Powdered material was washed with ether to give 662 mg of crude VII. Ether-pentane layer was evaporated and the residue was crystallized from benzene (253 mg). Recrystallization of both crops from benzene gave 724 mg of VII (71%). M.p. 154°-155°, anal. calcd. for $\text{C}_{29}\text{H}_{26}\text{O}_7\text{N}_2 \cdot 1/2 \text{ C}_6\text{H}_6$, C, 69.43; H, 5.28; N, 5.06. Found: C, 69.60; H, 5.14; N, 5.00. UV, $\lambda_{\max}^{\text{H}^+}$ 235.5 nm ($\epsilon 1.96 \times 10^4$), 250 nm (sh) ($\epsilon 1.56 \times 10^4$), 280 nm (sh), $\lambda_{\max}^{95\% \text{ EtOH}}$ 235.5 nm ($\epsilon 2.11 \times 10^4$), 250 nm (sh) ($\epsilon 1.61 \times 10^4$), 280 nm (sh), $\lambda_{\max}^{\text{OH}^-}$ 237.5 nm ($\epsilon 1.95 \times 10^4$), 252 nm (sh) ($\epsilon 1.33 \times 10^4$), 280 nm (sh). TLC (Kieselgel, CHCl_3 -EtOH 1:2) showed one spot.

6,2'-Anhydro-6-oxy-1-β-D-arabinofuranosyluracil 3'-phosphate(VIII)-----

Compound VII (25 mg, 0.05 mmole) and cyanoethylphosphate (pyridinium salt, 0.125 mmole) were dissolved in anhydrous pyridine (1 ml) and evaporated in vacuo. This procedure was repeated 4 times. To the residue were added pyridine (0.5 ml) and DCC (100 mg, 0.5 mmole), and the mixture was kept at room temperature for 4 days. Water (0.1 ml) was added and the solution was kept at room temperature for 4 hr. After evaporation, 80% AcOH (1 ml) was added and the solution was kept for 4 hr at room temperature. AcOH was removed by evaporation and the residue was coevaporated twice with water and finally with pyridine. Residue was dissolved in methanol previously saturated with ammonia (2 ml) at 0° and kept for 3 days at room temperature.

Solvent was evaporated and the residue was purified by PEP. Material revealed at R_{U-PU} 1.16 was extracted. UV, $\lambda_{max}^{H^+}$ 253.5 nm, $\lambda_{max}^{H_2O}$ 253 nm, $\lambda_{max}^{OH^-}$ 255 nm. PEP, R_{U-PU} 1.16. PPC, Rf(B) 0.11, Rf(C) 0.06, Rf(E) 0.74.

6,2'-Anhydro-6-oxy-1-β-D-arabinofuranosyluracil 5'-phosphate(IX)----- Compound

VI (363 mg, 1.5 mmole) was dissolved in trimethylphosphate (3 ml) with slight heating. Into the solution was added POCl₃ (0.55 ml) dissolved in trimethylphosphate (0.8 ml). The reaction mixture was cooled at 0° and kept for 6 hr under stirring. The mixture was added dropwise in ether-pentane and the precipitates were collected by centrifugation. To the precipitates was added water under cooling with ice, and the solution was neutralized with 1 N NaOH. Nucleotidyl material was applied to a column (1.5 x 34 cm) of Dowex 1X2 (formate) and eluted stepwise with 0.04 N formic acid + 0.004 M ammonium formate (0.9 l.), 0.08 N formic acid + 0.008 M ammonium formate (1.3 l.) and 0.12 N formic acid + 0.012 M ammonium formate (1 l.) at a rate of one fraction 15 ml/ 15 min. Compound IX was eluted in the last peak (Fig. 4), which was collected and applied to a charcoal column. Elution with 50% ethanol containing 2% conc. NH₄OH and evaporation of the eluents gave IX (17000 OD₂₅₂, ca. 70%). UV, $\lambda_{max}^{0.001 M HCl}$ 253 nm (ϵ 1.58 x 10⁴), $\lambda_{max}^{pH 7.0}$ 252.5 nm (ϵ 1.64 x 10⁴), $\lambda_{max}^{0.001 N NaOH}$ 254.5 nm (ϵ 1.18 x 10⁴). PPC, Rf(B) 0.21, Rf(C) 0.10, Rf(E) 0.73. PEP, R_{U-PU} 1.01.

3'-O-Acetyl-6,2'-anhydro-6-oxy-1-β-D-arabinofuranosyluracil 5'-phosphate(X)---

Compound IX (1600 OD₂₅₂, 0.1 mmole) was passed through a column of Dowex 50 (pyridinium form) to convert it to the pyridinium salt. Effluents were evaporated and dried by coevaporation 3 times with added pyridine. Residue was dissolved in 0.5 ml of pyridine, acetic anhydride (0.3 ml) was added, and kept for 9 hr in a dark place. Solvent was evaporated and the residue was dis-

Fig. 4 Column chromatography of 6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyl-uracil 5'-phosphate. Three small peaks were unidentified.

solved in 50% pyridine (1 ml) under cooling. After it was kept at room temperature overnight, solvent was evaporated. Residue was dried by evaporation several times with added pyridine, dissolved in a small amount of pyridine, and dropped into ether (30 ml). Powdered material was collected by centrifugation and washed with ether. PEP, R_{pU} 0.93. PPC, $R_f(A)$ 0.27. This material contained 7% of 5',5'-pyrophosphate of pU^O -(oAc).

6,2'-Anhydro-6-oxy-1- β -D-arabinofuranosyluracil-phosphoryl-(3'-5')-6,2'-anhydro-6-oxy-1- β -D-arabinofuranosyluracil(I)----- Compound VII (55 mg, 0.1 mmole) and X (1544 OD₂₅₂, 0.09 mmole) were dissolved in pyridine and evaporated several times with added pyridine. Residue was dissolved in pyridine (1 ml) and DOC (103 mg, 0.5 mmole) was added. The reaction mixture was kept in a thermostated box at 27° for 60 hr. Into the solution was added 50% aqueous pyridine, dicyclohexylurea was filtered off, and the filtrate was washed twice with 15 ml of pentane. Water layer was kept overnight and evaporated in vacuo. After coevaporation with toluene, 80% acetic acid (20 ml) was added and the solution was kept at room temperature for 4 hr. The solvent was evaporated, residue was dissolved in water (30 ml), and extracted with ether (10 ml x 3). Water layer was evaporated and methanolic ammonia (20 ml) was added. After kept it overnight methanol was evaporated and the residue was dissolved in water (100 ml) containing a small amount of pyridine. The solution was applied to a column (2 x 35 cm) of DEAE-cellulose (bicarbonate form). After washing with water (1 l.), the column was eluted with water (1.5 l.) and 0.1 M triethylammonium bicarbonate buffer (1.5 l.) by a linear gradient technique. One fraction (15 ml) was collected in 15 min. Appropriate fractions were collected, evaporated in vacuo, and coevaporated with methanol.

UV, $\lambda_{\max}^{0.001 \text{ N HCl}}$ 253 nm (ϵ 3.52×10^4), $\lambda_{\max}^{\text{pH } 7.3}$ 252 nm (ϵ 3.2×10^4),
 $\lambda_{\max}^{0.001 \text{ N NaOH}}$ 255 nm (ϵ 2.5×10^4). PEP, $R_{\text{U-pU}}$ 0.56. PPC, Rf(B) 0.19,
 Rf(C) 0.12, Rf(D) 0.43, Rf(E) 0.34.

Enzymatic hydrolysis of pU^{O} and $\text{U}^{\text{O}}\text{pU}^{\text{O}}$ ———

E. coli alkaline phosphatase ——— Incubation mixture (120 μl) containing pU^{O} 5 OD, 1 M NH_4HCO_3 10 μl , enzyme (0.4 mg/ml) 10 μl was incubated at 37° for 4 hr. The digest was applied to a paper electrophoresis. Only pU^{O} and Pi were detected.

Crude snake venom (5'-nucleotidase) ——— Incubation mixture (100 μl) containing pU^{O} or pU 5 OD, 0.1 M MgCl_2 10 μl , 1 M Tris-HCl (pH 8.7) 10 μl , enzyme (20 mg/ml) 4 μl was incubated at 37° for 4 hr. pU was completely hydrolyzed to give uridine. pU^{O} was not hydrolyzed even after 24 hr incubation.

Potato 3'-nucleotidase ——— Incubation mixture (260 μl) containing pU^{O} or Up 5 OD, 0.25 M Tris-HCl (pH 8.1) 100 μl , enzyme (10 mg/ml) 100 μl was incubated at 37° for 27 hr. Up was hydrolyzed completely. pU^{O} was not hydrolyzed at all.

Snake venom phosphodiesterase ——— Incubation mixture (200 μl) containing $\text{U}^{\text{O}}\text{pU}^{\text{O}}$ or TpT 10 OD, 1 M $(\text{NH}_4)_2\text{CO}_3$ 50 μl , enzyme (1 mg/ml) 40 μl was incubated at 37° for 4 hr. TpT was completely hydrolyzed in this condition. $\text{U}^{\text{O}}\text{pU}^{\text{O}}$ was hydrolyzed for an extent of 17%. After 24 hr $\text{U}^{\text{O}}\text{pU}^{\text{O}}$ was hydrolyzed to 37%.

Incubation mixture was applied to PEP and monophosphate band was applied to PPC(E). Only pU^{O} was detected. The ratio of U^{O} : pU^{O} was 1:1.

Spleen phosphodiesterase ——— Incubation mixture (100 μl) containing $\text{U}^{\text{O}}\text{pU}^{\text{O}}$ or TpT 5 OD, 1 M NH_4OAc (pH 6.0) 20 μl , enzyme (20 units/ml) 20 μl was incubated at 37° for 5 hr. TpT was hydrolyzed completely. $\text{U}^{\text{O}}\text{pU}^{\text{O}}$ was not hydrolyzed even after 25 hr incubation.

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