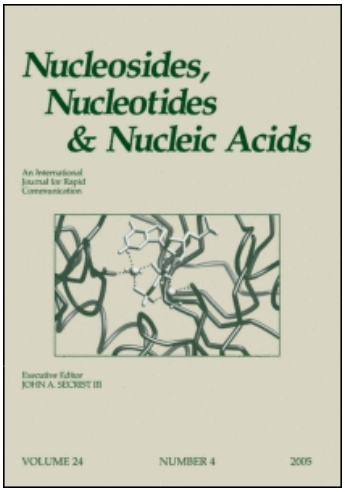

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Synthesis and Properties of 2'4'- and 3'-5'-Linked Ribodinucleoside Monophosphates Containing 2-Aminoadenosine and Uridine

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SYNTHESIS AND PROPERTIES OF 2'-5'- AND 3'-5'-LINKED RIBODINUCLEOSIDE MONOPHOSPHATES CONTAINING 2-AMINOADENOSINE AND URIDINE

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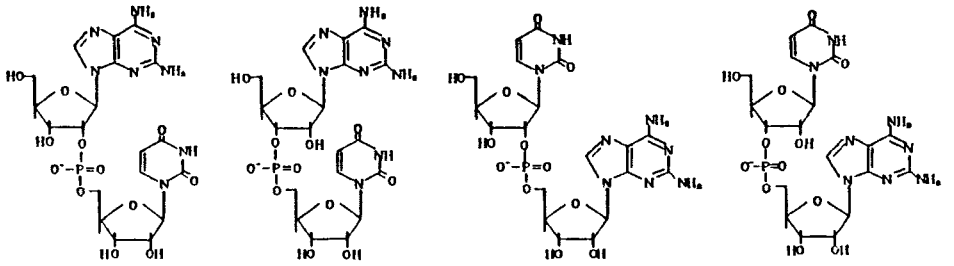
ABSTRACT: Self complementary diribonucleoside monophosphates containing 2-aminoadenosine (n^2A) and uridine (U) residues, (2'-5') n^2ApU (**1**), (3'-5') n^2ApU (**2**), (2'-5') Upn^2A (**3**) and (3'-5') Upn^2A (**4**), were synthesized by condensation of suitably protected nucleoside and nucleotide units using dicyclohexylcarbodiimide (DCC). The dimers, (**3**) and (**4**), were also obtained from uridine 2',3'-cyclic phosphate and unprotected 2-aminoadenosine using 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) as the condensing agent. The conformational properties of these dimers were examined by UV, CD and NMR spectroscopy. The results reveal that the 2'-5' isomers take a stacked conformation, which contains a larger base-base overlap and is more stable against thermal perturbation with respect to the 3'-5' isomers. The n^2ApU isomers have more stacked structure than the Upn^2A isomers.

It was reported that 2-aminoadenosine nucleotides possess interesting biological as well as physicochemical properties. A 2-aminoadenine residue can form a base pair containig three hydrogen bonds of the Watson-Crick type with a uracil residue. In the ribopolynucleotide, poly(2-aminoadenylic acid) forms double and triple helices with poly(uridylic acid)[1-3]. Kirnos et al. [4, 5] have found that the DNA of cyanophage S-2L has all its adenine residues replaced by 2-aminoadenine and formed a complementary base pair with thymine.



1 2 3 4

(2'-5')n^2ApU (3'-5')n^2ApU (2'-5')Upn^{2}A (3'-5')Upn^{2}A

FIG. 1.

It is worthwhile to investigate the physicochemical properties of dinucleoside monophosphates, because they are the simplest models of polynucleotide. Recently we reported[6] synthesis and properties of self complementary dinucleoside monophosphates containing 8,2'-anhydro-9-β-D-arabinofuranosyl-8-mercapto-2-aminoadenine (n^2A^S) and 6,2'-anhydro-1-β-D-arabinofuranosyl-6-hydroxyuracil (U^O), $n^2A^SpU^\circ$ and $U^\circ pn^2A^S$. We found that $U^\circ pn^2A^S$ takes a stable stacked comformation but $n^2A^SpU^\circ$ does not like A^SpU°[7]. The present paper describes synthesis and properties of 2'-5' and 3'-5' linked four ribonucleoside monophosphates which consist of 2-aminoadenosine (n^2A) and uridine (U), 2-aminoadenylyl(2'-5')uridine ((2'-5')n^2ApU)(**1**), 2-aminoadenylyl(3'-5')uridine ((3'-5')n^2ApU)(**2**), uridylyl(2'-5')-2-aminoadenosine ((2'-5')Upn^{2}A)(**3**) and uridylyl(3'-5')-2-aminoadenosine ((3'-5') Upn^{2}A)(**4**) (FIG. 1) ***.

Synthesis of the Dimers

The synthesis of 2'-5' and 3'-5' linked ribodinucleoside monophosphates, **1**, **2**, **3** and **4**, were carried out by condensation of a nucleoside 5'-phosphate and a nucleoside having free 2' and 3' hydroxyl groups with dicyclohexylcarbodiimide (DCC) as the condensing agent. $\underline{N}^2$,$\underline{N}^6$-diacetyl-2-aminoadenosine[8] was protected

***Most of the abbreviations and symbols follow the recommendation by IUPAC-IUB Commission on Biochemical Nomenclature: Biochemistry **9**, 4022 - 4027 (1970).

with monomethoxytrityl chloride in pyridine to give 5'-O-monomethoxytrityl-N^2,N^6-diacetyl-2-aminoadenosine and condensed with 2',3'-O-diacetyl uridine 5'-phosphate using DCC. The reaction mixture was treated successively with methanolic ammonia, 40 % methylamine and 80 % acetic acid to remove the protecting groups. The mixture was subjected to anion-exchange chromatography on a column of Dowex 1X2 (formate form) to separate the 2'-5' and 3'-5' linked isomers. These isomers were contaminated with small amounts of 2-acetylaminoadenylyl(2'-5')uridine and 2-acetylaminoadenylyl(3'-5')uridine, respectively. The pure isomers can be purified by rechromatography on a column of DE-23 cellulose (bicarbonate form). The desired isomers **1** and **2** were obtained in yields of 26 % and 10 %, respectively. Similarly, 5'-O-monomethoxytrityluridine[9] was allowed to react with N^2,N^6,2',3'-O-tetraacetyl-2-aminoadenosine 5'-phosphate synthesized from 2-aminoadenosine 5'-phosphate[3] in the presence of DCC as the condensing agent. The reaction mixture was treated in the same manner as described above. The isomeric mixture of **3** and **4** were separated by Dowex 1X2 (formate form) and then DE-23 cellulose (bicarbonate form) column chromatography. The 2'-5' and 3'-5' linked isomers, **3** and **4**, were obtained in yields of 23 % and 9 %, respectively.

Compounds **3** and **4** were also synthesized by an alternative method. Acetylated uridine 2',3'-cyclic phosphate[10] and unprotected 2-aminoadenosine were condensed with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl). After deacetylation with methanolic ammonia, 2'-5' and 3'-5' linked isomers **3** and **4** were isolated by chromatography on a column of Dowex 1X2 (formate form) in yields of 30 % and 16 %, respectively. Some degradation products, n^2A and U, of the intermediate were detected as the by-products.

The identification and characterization of the ribodinucleoside monophosphate isomers were performed by enzymic hydrolysis, paper electrophoresis (PEP), HPLC, UV, CD and ^{1}H-NMR spectra. Rf values in paper chromatography (PC) and mobilities in PEP are shown in TABLE 1 and 2.

TABLE 1. Chromatographic Properties for Related Compounds

Compound	PC Rf			PEP
	A*	B**	C***	$Rm._pU=1.0$
n^2A	0.46	0.44	0.15	0.14
U	0.33	0.68	0.63	0.39
pn^2A	0.00	0.06		0.79
UMP	0.06	0.14	0.45	1.00
(2'-5')n^2ApU	0.15	0.16	0.22	0.57
(3'-5')n^2ApU	0.10	0.15	0.10	0.49
(2'-5')Upn^2A	0.11	0.16	0.12	0.53
(3'-5')Upn^2A	0.09	0.16	0.48	0.54

*A; 2-propanol-conc.ammonia-water (7:1:2 v/v/v)
**B; ethanol-1M ammonium acetate, pH7.5 (7:3 v/v)
***C; ammonium salfate-water-2-propanol (66:100:2 w/v/v)

TABLE 2. UV Absorption Data for Related Compounds

Compound	pH 7		pH 2		pH14	
	λ_{max} (nm)	(εP)	λ_{max} (nm)	(εP)	λ_{max} (nm)	(εP)
(2'-5')n^2ApU	258	(16000)	256	(18100)	258	(14700)
	275	sh(14100)	300	sh(7500)	275	sh(12400)
(3'-5')n^2ApU	258	(17000)	257	(19300)	258	(15900)
	275	sh(14600)	290	sh(9500)	275	sh(13400)
(2'-5')Upn^2A	258	(13800)	256	(15200)	258	(12400)
	275	sh(11000)	290	sh(7400)	279	sh(9500)
(3'-5')Upn^2A	258	(15200)	257	(16900)	259	(14300)
	275	sh(12100)	292	sh(7700)	275	sh(11700)

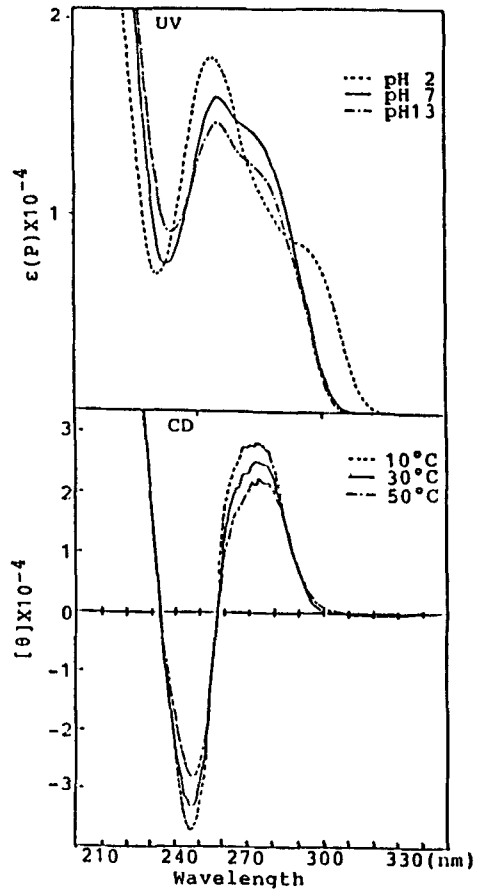


FIG. 2. UV and CD Spectra of $(2'\text{-}5')n^2ApU(1)$

UV and CD Spectral Properties of the Dimers

UV and CD spectra of the dimers are shown in FIG. 2 - 5. The UV spectra at pH 7 show a peak at around 260 nm and a shoulder at around 270-280 nm. It appears that the shoulder is more characteristic for the 2'-5' isomers than for the 3'-5' isomers.

The spectra at pH 2 and pH 12 display characteristic changes suggesting the presence of both uracil and 2-aminoadenine residues.

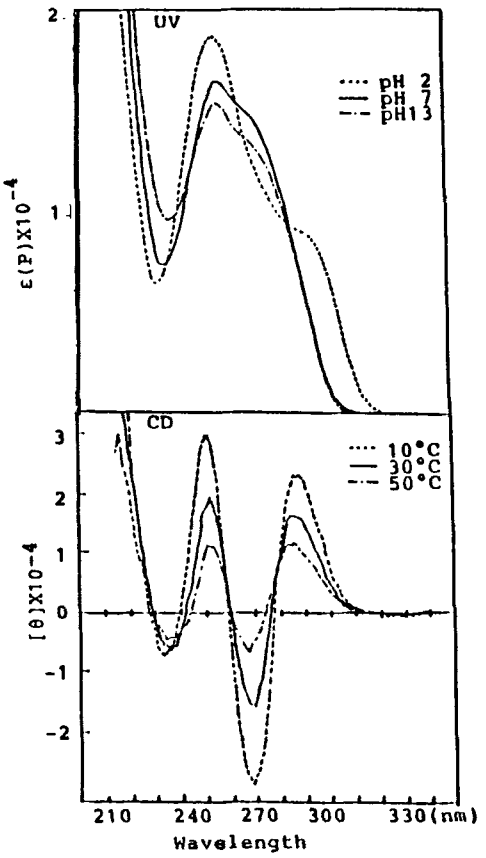


FIG. 3. UV and CD Spectra of (3'-5')n^{2}ApU(2)

The CD spectra of (2'-5')n^{2}ApU show very large difference from those of the 3'-5' isomer (FIG. 2 and 3). The latter exhibits two positive bands and a negative band at around 285 nm, 250 nm and 270 nm, respectively whereas the former exhibits a positive band and a negative band at around 270 nm and 245 nm, respectively, in the 230-310 nm region. It is also noted that the spectra of (3'-5')n^{2}ApU are much more sensitive to thermal perturbation than those of the 2'-5' isomer. The same phenomenon is observed for linkage isomers of ApA and ApC[11]. These results

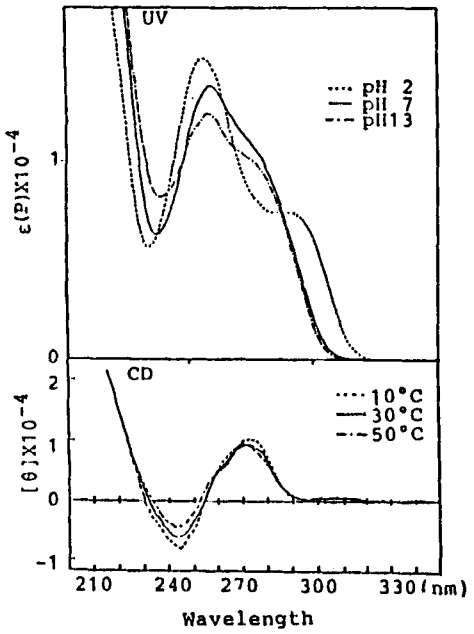


FIG. 4. UV and CD Spectra of (2'-5')Upn^{2}A(3)

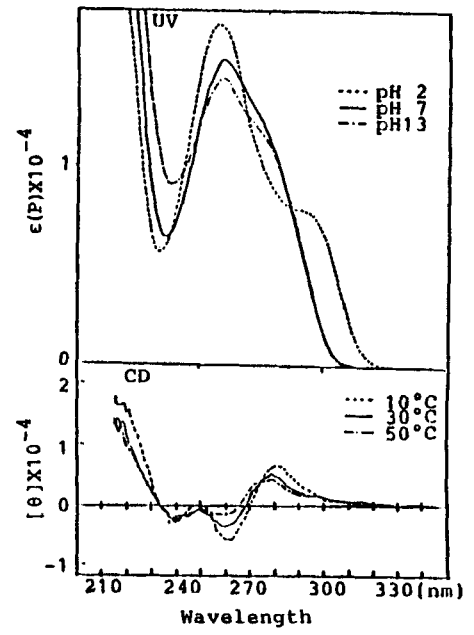


FIG. 5. UV and CD Spectra of (3'-5')Upn^{2}A(4)

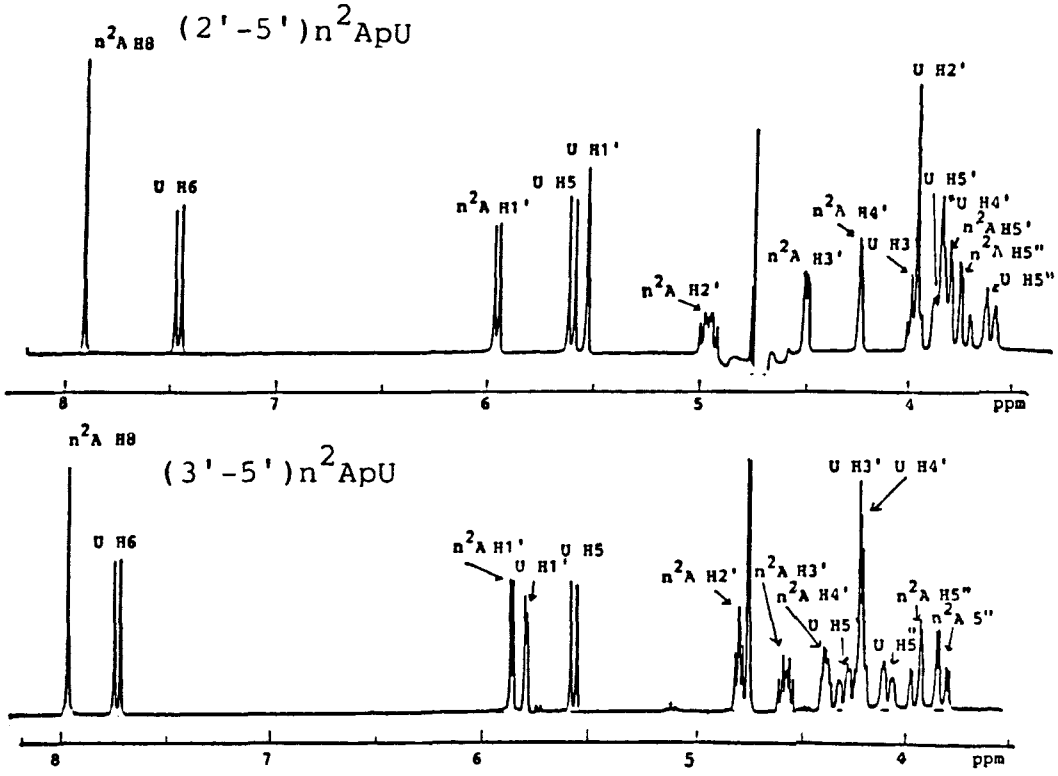


FIG. 6. ^{1}H-NMR Spectra of 1 (2'-5')n^{2}ApU and (3'-5')n^{2}ApU

may be explained by the difference in geometry of base stacking between the 2'-5' and 3'-5' isomers. The 2'-5' phosphodiester bond allows the two bases to stack with a larger twist angle and also with a larger overlap with respect to the 3'-5' counterpart[11].

The UpA analogues show considerably smaller CD bands than the ApU analogues (FIG. 4 and 5). The same phenomenon is observed for the 3'-5' isomers of ApU and UpA[12, 13]. It can be explained by the weaker stacking in UpA that is expected from the mode of base-base interactions in a right-handed stack[3]. The spectral patterns for each set of the sequence isomers with the same phosphodiester linkage are rather similar. However, the positive band in the 240 nm region of the spectra of (3'-5')n²ApU

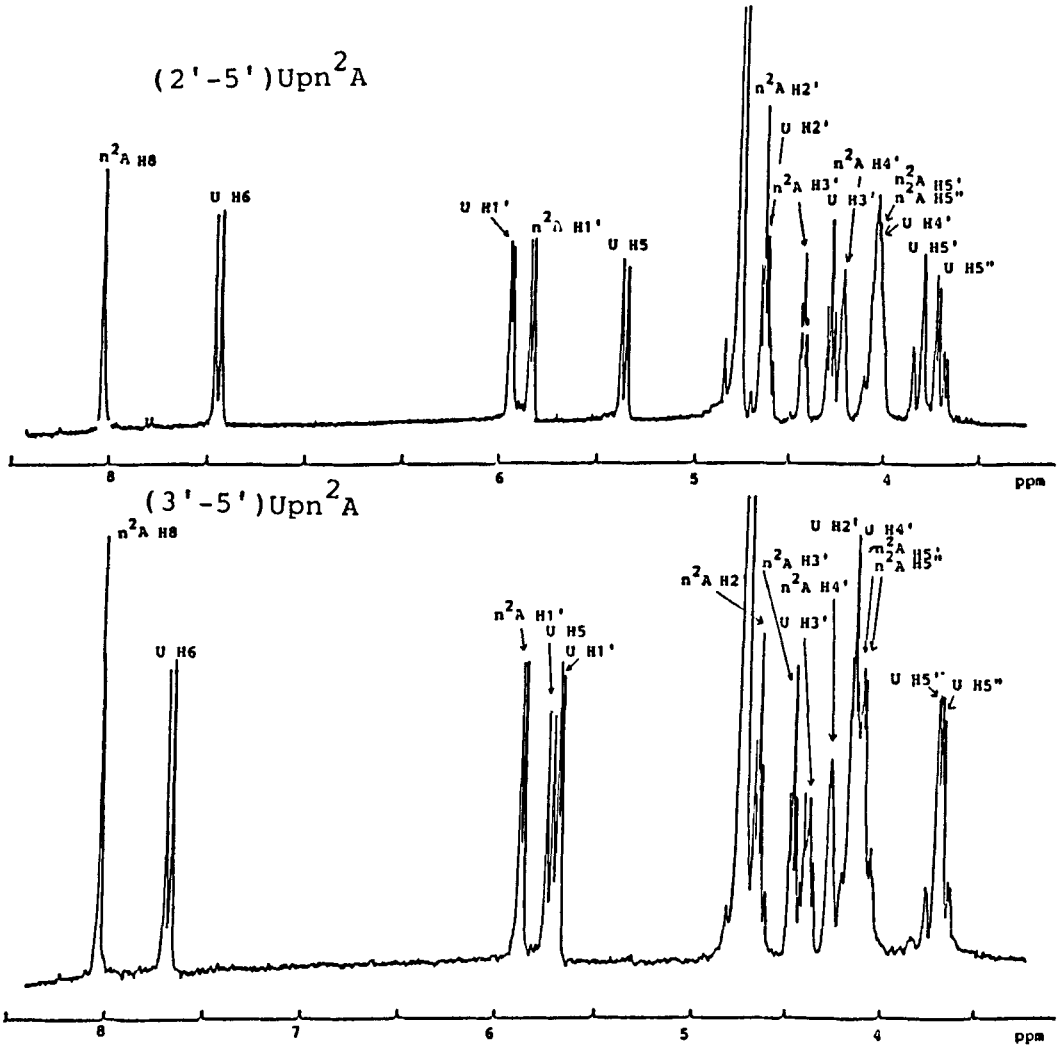


FIG.7. ^{1}H-NMR Spectra of (2'-5')Upn^{2}A and (3'-5')Upn^{2}A

is missing in the spectra of (3'-5')Upn²A. Similar difference is observed between the spectra of ApU and UpA in the region around 220 nm[12]. It should also be noted that the spectra of (3'-5')Upn²A are more sensitive to temperature than those of the 2'-5' isomer as observed for the isomers of n²ApU.

TABLE 3. ^{1}H-NMR Data for The Dimers

Compound		Chemical Shift (ppm) H8	H6	H5	H1'	H2'	H3'	H4'	H5'	H5"
(2'-5')n^{2}ApU	n^{2}Ap-	7.90			5.95	4.96	4.50	4.25	3.83	3.73
	-pU		7.41	5.59	5.52	3.93	3.97	3.82	3.87	3.57
(3'-5')n^{2}ApU	n^{2}Ap-	7.99			5.88	4.83	4.60	4.41	3.94	3.84
	-pU		7.76	5.59	5.81	4.23	*	*	4.33	4.10
(2'-5')Upn^{2}A	Up-		7.43	5.37	5.95	4.64	4.29	4.05	3.79	3.70
	-pn^{2}A	8.04			5.84	4.66	4.43	4.22	4.08	4.02
(3'-5')Upn^{2}A	Up-		7.70	5.77	5.73	4.19	4.43	4.14	3.77	3.69
	-pn^{2}A	8.07			5.91	4.70	4.50	4.36	4.21	4.11

* We were not able to assign H3' and H4' resonances of (3'-5') n^{2}ApU because of serious overlapping of the signals.

TABLE 4. Coupling Constants for the Dimers

Compound		Coupling Constant (Hz) 56	1'2'	2'3'	3'4'	4'5'	4'5"	5'5"	2'P	3'P	5'P	5"P
(2'-5') n^{2}ApU	n^{2}Ap-		6.9	5.1	1.8	2.2	2.6	-12.9				
	-pU	7.9	2.9	5.1	6.9	2.5	3.7	-12.0				
(3'-5') n^{2}ApU	n^{2}Ap-		3.7	4.5	4.8	2.4	3.4	-12.9		8.3		
	-pU	8.3	3.2								3.9	3.4
(2'-5') Upn^{2}A	Up-	7.8	4.4	5.9	5.6	2.9	4.6	-12.7	2.7			
	-pn^{2}A		5.4	5.4	5.4							
(3'-5') Upn^{2}A	Up-	8.1	4.4	5.1	5.9	2.4	3.9	-13.1		8.3		
	-pn^{2}A		4.9	5.1	5.4						3.9	3.9

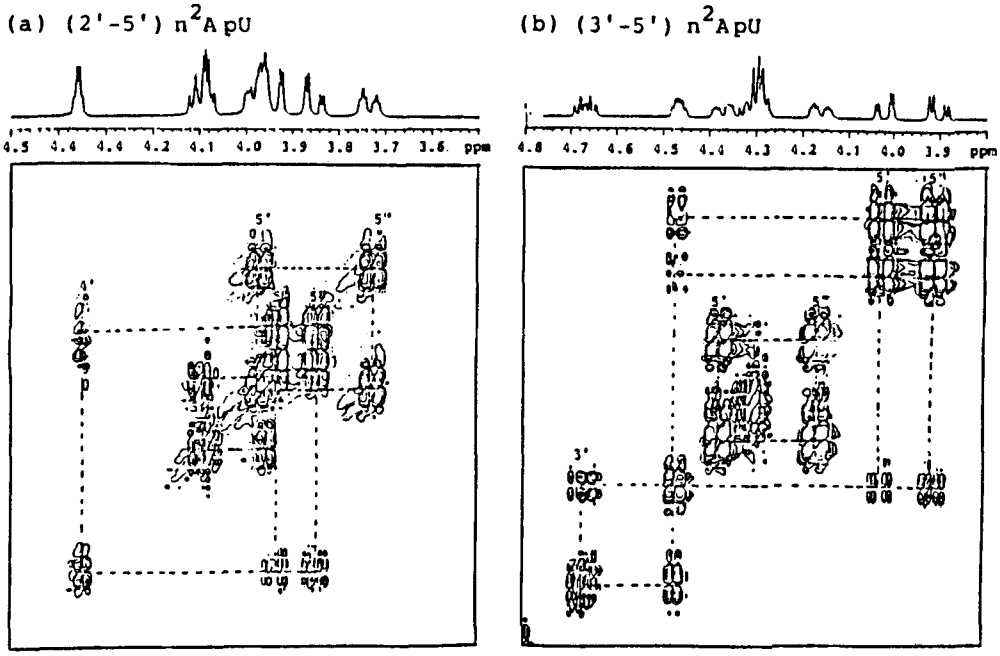


FIG. 8. The DQF-COSY spectra of n^2ApU taken by JEOL GSX-400 (400 MHz for ^{1}H) at 30° which are recorded on a phase sensitive mode. (a) and (b) are the spectra of 2'-5' and 3'-5' dinucleoside monophosphates, respectively. The figure shows only the crowded regions of ribose moieties. The severe overlapping signals with strong couplings prohibit the first order spectral analyses. An alternative approach to the spectral simulation was also unsuccessful. Thus, the unequivolcal assignment of these compounds awaits for further studies.

^{1}H-NMR Spectra of the Dimers

The proton NMR spectral data of the dimers are presented in FIG. 6, 7, TABLE 3 and 4. Spectral assignments were mainly made by the decoupling method with some help of nuclear Overhauser effect (NOE) and two-dimensional COSY experiments (FIG. 8). When chemical shift data (TABLE 3) for (2'-5')n²ApU and (3'-5')n²ApU are compared, H6 and H1' resonances of the -pU residue in the 2'-5' isomer show considerable upfield shifts with respect to those of the 3'-5' isomer (also see FIG. 6). This result suggests that (2'-5')n²ApU has more stacked structure than (3'-5')n²ApU. Similarly, H5 and H6 resonances of the (2'-5')Upn²A show an

upfield shift with respect to those of (3'-5')Upn²A (FIG. 7). It should be noted that H5'' resonance of the -pU residue in (2'-5')n²ApU appears in the highest field of the spectrum whereas the presence of the phosphate group on C5' causes a marked downfield shift of H5' and H5'' resonances (FIG. 6). This result indicates that the H5'' is close to the 2-aminoadenine residue and receives its shielding effect. A similar upfield shift of H5'' resonance of the 5'-linked residue (-pU or -pC residue) is also observed for (2'-5')GpU and (2'-5')ApC [14]. These results suggest that the 2'-5' isomers have a larger base-base overlap than the 3'-5' isomers as expected also from the CD data.

It is known that the population of furanose puckering conformation, which is in an equilibrium between 2'-endo and 3'-endo forms, can be calculated from the data of $J_{1'2'}$ and $J_{3'4'}$[15]. The ratio of 2'-endo puckering is proportional to $J_{1'2'}$ and that of 3'-endo is proportional to $J_{3'4'}$. The coupling constant data (TABLE 4) show that n²Ap- residue in (2'-5')n²ApU particularly prefer 2'-endo puckering. A similar marked preference for 2'-endo conformation is also observed for Gp- and Ap- residues in 2'-5' isomers of GpU and ApC [14]. The quasiequatorial orientation of the C2'-O2' bond in 2'-endo conformation may accommodate a larger overlap between the pyrimidine part of the purine base and the pyrimidine base which produces strong stacking interactions. Such preference is not observed either for (2'-5')Upn²A or for UpG and CpA[14].

EXPERIMENTAL

General Methods: UV absorption spectra were measured with a Hitachi 340 spectrometer. NMR spectra were taken, otherwise mentioned, with a Bruker WH-270 at ambient probe temperature (23° C). Samples were used as the Na salt in D_2O. NOE difference spectra were taken directly subtracting the FIDs for each scan. DQF-COSY spectra were measured using a JEOL GSX-400 at 30°C. CD spectra were measured with a JASCO J-500A spectropolarimeter. Analytical HPLC was performed on a Hitachi's HPLC system apparatus on a Senshu ODS-2251-N chromatographic column. The ε(P)

value was determined by phosphorus analysis. Paper chromatography (PC) was performed on Toyo filter paper No. 51A. Thin-layer chromatography (TLC) was performed on plates of silica gel (Merck 60 F_{254}). For column chromatography, silica gel (Merck 60H) was used. Paper electrophoresis (PEP) was performed using 0.05 M triethylammonium bicarbonate (TEAB) buffer (pH 7.5) at 900 V/40 cm for 1 hr on Toyo filter paper No. 51A.

5'-O-Monomethoxytrityl-N^2,N^6-diacetyl-2-aminoadenosine

A mixture of N^2, N^6-diacetyl-2-aminoadenosine[8] (1.1 g, 3 mmole) and monomethoxytrityl chloride (1.21 g, 3.6 mmole) in pyridine (3 ml) was stirred at 25°C for 16 hr. The mixture was concentrated in vacuo. The residue was dissolved in chloroform and purified by silica gel column chromatography using chloroform -methanol (20:1) elution system to give a foamy material (1.50 g, 90.5 %) mp. 176-178°C. TLC $CHCl_3$:EtOH (20:1) Rf 0.48, UV: $\lambda_{max}^{50\%EtOH}$ 288, 292(sh) and 303 nm, $\lambda_{max}^{H^+}$ 265 and 273 nm at pH 2, $\lambda_{max}^{OH^-}$ 236 and 285 nm at pH 12. Anal. Calc. for $C_{34}H_{34}O_7N_6 \cdot 1/2H_2O$: C, 63.05; H, 5.44; N, 12.98. Found: C, 63.00; H, 5.44; N, 12.85.

Pyridinium N^2,N^6,2',3'-O-tetraacetyl-2-aminoadenosine 5'-phosphate

2-Aminoadenosine 5'-phosphate[3] (79 mg, 0.23 mmole) was dissolved in 10 % aqueous pyridine (10 ml) and passed through a column (1.1 X 7 cm) of Dowex 50X2 (100-200 mesh, pyridinium form). The column was washed with 10 % pyridine (100 ml). The eluent and washing were combined and evaporated in vacuo to dryness at 30°C and the residue was coevaporated with pyridine three times. It was treated with acetic anhydride (1.2 ml) in pyridine (3.5 ml) for 18 hr at 25°C with stirring and volatile materials were removed by evaporation. The residue was dissolved in 50 % aqueous pyridine under cooling in an ice bath. After 2 hr at room temperature, the solution was concentrated. The residue was rendered anhydrous by coevaporation with pyridine and the anhydrous pyridine solution was added to ether with vigorous stirring. The precipitate was centrifuged and washed with ether three times. The yield was nearly quantitative. UV: λ_{max}^{H2O} 235, 262 and 287 nm. λ_{max}^{H+} 232, 270 and 302 nm at pH 2, $\lambda_{max}^{OH^-}$ 244 and 288 nm at pH 12.

(2'-5')n²ApU (1) and (3'-5')n²ApU (2)

A mixture of 5'-O-monomethoxytrityl-N^2,N^6-diacetyl-2-aminoadenosine (191.6 mg, 0.3 mmole) and pyridinium 2',3'-O-diacetyluridine 5'-phosphate[10] (145.9 mg, 0.3 mmole) was coevaporated with pyridine three times and the residue was dissolved in pyridine (2 ml). It was treated with DCC (309.5 mg, 1.5 mmole) for 60 hr at 30°C. Water (2 ml) was added and the solution was kept for 16 hr at 30°C. Dicyclohexylurea was removed by filtration and the filtrate was washed with n-pentane three times and concentrated. The residue was treated with 15 N methanolic ammonia (15 ml) for 16 hr at 30°C. The volatile materials were removed by evaporation and the residue was treated with 40 % methylamine (4 ml) for 2 hr at 25°C. After evaporation, the residue was treated with 80 % acetic acid (15 ml) for 2 hr at 25°C and concentrated. The residue was dissolved in water and extracted with ether. The aqueous solution (70 ml) was adjusted with ammonia to pH 8 and applied to a column (1.5 X 45 cm) of Dowex 1 X 2 (formate form, 200-400 mesh). The column was washed with water and eluted with a linear gradient of formic acid (0 - 0.5 M, total 4 l). Compound **1** was eluted at 0.13 M formic acid containing a small amount of N^2-acetoamidoadenylyl-(2'-5')-uridine. The 3'-5'linked isomer **2** was eluted at the 0.16 M formic acid also contained a small amount of N^2-acetamidoadenylyl-(3',5')-uridine. Each fraction was further applied to a column (1.5 X 45 cm) of DE-23 cellulose (bicarbonate form) respectively. After washing with water, the each column was eluted with a linear gradient of TEAB buffer (0 - 0.1 M, total 2 l). Chromatographycally pure 2'-5' linked **1** (1095, A_{258} at 0.032 M TEAB buffer) and 3'-5' linked **2** (475, A_{258} at 0.074 M TEAB buffer) were obtained as the triethylammonium salts .

(2'-5')Upn²A (3) and (3'-5')Upn²A (4)

Method(A). From nucleoside 5'-phosphate: A mixture of pyridinium N^2,N^6,2',3'-O-tetraacetyl-2-aminoadenosine 5'-phosphate (92.4 mg, 0.15 mmole) and 5'-O-monomethoxytrityluridine[11] (75.1 mg, 0.15 mmole) was coevaporated with pyridine three times and the residure was dissolved in pyridine (1 ml). It was treated with DCC (155 mg, 0.75 mmole) for 100 hr at 30°C. The mixture was

worked up in the manner described for **1** and **2**. The residue was dissolved in water (100 ml). The solution was adjusted with 1 M ammonia to pH 8 and applied to a column (1.8 X 35 cm) of Dowex 1X2 (formate form). The column was washed with water and eluted with a linear gradient of formic acid (0 - 1.0 M, total 4 l). The 2'-5' linked **3** (854 A_{260} units) was eluted at 0.23 N formic acid contained uridylyl-(2'-5')-$\underline{N}^2$-acetamidoadenosine. The 3'-5' linked **4** (510 A_{260} units) was eluted at around 0.27 M formic acid. This peak also contained uridylyl-(3'-5')-$\underline{N}^2$-acetamidoadenosine. Each fraction was further applied to a column (1.5 X 31.5 cm) of DE-23 cellulose (bicarbonate form). After washing with water, the column was eluted with a linear gradient of TEAB buffer (0 - 0.2 M, total 4 l). The pure 2'-5'linked **3** (551 A_{260} units) was eluted at 0.04 M TEAB buffer. The 3'-5' linked **4** (246 A_{260} units) was eluted at 0.04 M TEAB buffer.

Method (B). From nucleoside 2',3'-cyclic phosphate: To the solution of tri-$\underline{n}$-butylammonium 5'-O-acetyluridine 2',3'-cyclic phosphate[7] (0.3 mmole) in DMF (1.5 ml) was added TPS-Cl (0.9 mmole) and stirred for 10 min, then 2-aminoadenosine (0.17 g, 0.6 mmole) was added. The mixture was stirred until the solution became clear in about 20 min and allowed to stand at 25°C for 2 hr. To the reaction mixture, water (5 ml) and tri-$\underline{n}$-butylamine (2 ml) were added and kept for 30 min at 25°C to hydrolyze TPS-Cl and the nucleotide triester. Water (30 ml) was further added and the mixture was extracted with ether three times and the aqueous layer was concentrated to dryness. The residue was treated with 15 N methanolic ammonia (30 ml) for 16 hr at 25°C and concentrated. The residue was dissolved in water and it was adjusted with 1 M ammonia to pH 8.0 and applied to a column (1.8 X 26 cm) of Dowex 1X2 (formate form, 200 - 400 mesh). After washing with water (2 l), elution was carried out with a linear gradient of formic acid (0 - 0.05 M, total 4 l). Fractions of 7.8 ml were collected at 6 min intervals. The 2'-5' linked **3** (1196 A_{260} units, yield 30%) was eluted at 0.025 M formic acid and 3'-5' linked **4** (720 A_{260} units, yield 16%) was eluted at 0.03 M formic acid.

Hydrolysis of the Dimers with Snake Venom Phosphodiesterase

Each dimer samples (5 A_{max} units) was incubated with snake venom phosphodiesterase (1 mg/ml 5 µl), in 0.05 M TEAB buffer (pH 7.5 200 µl) at 37°C for 5 hr. After quenching the reaction by heating at 100°C for 5 min, the digest was subjected to PEP at pH 7.5 and determined by UV absorption measurement. Each dimer (**1, 2, 3 and 4**) was hydrolyzed to give approximately equal amounts of the nucleoside and nucleotide.

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