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Polynucleotides. LXI.[1] Synthesis and Properties of Dinucleoside Monophosphates Containing 8,2′-S-Cycloadenosine and 8,2′-S-Cycloinosine Residues. Sequence Dependency of the Stability of the Stacking Conformation

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Three dinucleoside monophosphates containing 8,2′-anhydro-8-thio-9-β-D-arabinofuranosyladenine (A^s) and its hypoxanthine derivative (I^s), A^spI^s, I^spA^s and I^spI^s, were synthesized. Examination of their properties by ultraviolet absorption, circular dichroism and ^1H nuclear magnetic resonance measurements and comparison with the properties of A^spA^s, which has been shown to take a left-handed stacked conformation, showed that all these dimers take a left-handed stacked conformation, and the order of extent of stacking is $A^spA^s \approx I^spA^s > A^spI^s \approx I^spI^s$. This sequence dependency of stability of stacking can be explained in terms of the mode of base-base overlap in a left-handed stack. A similar explanation may be applicable to the corresponding sequence dependency of natural dimers with a right-handed stack.

Keywords——cycloadenosine; cycloinosine; dinucleoside monophosphate; glycosidic torsion angle; conformation; stacking; NMR; CD; UV

Introduction

We have been studying oligonucleotides containing cyclonucleoside residues, in which the glycosidic torsion angle (χ)[3] is fixed, in order to elucidate the effects of the glycosidic conformation on oligonucleotide structure.[4–8] These studies revealed that oligonucleotides containing cyclonucleosides with χ in the high *anti* region ($\chi \approx 120$) have a tendency to take a left-handed stacked conformation in contrast to the right-handed stacking of natural nucleic acids.[9] Thus, the dinucleoside monophosphate (**14**, A^spA^s) of 8,2′-anhydro-8-thio-9-β-D-arabionofuranosyladenine (**1**, A^s) takes a stable, left-handed stacked conformation.[4a,b] The oligomers of pA^s take a left-handed helical structure[4c] and form left-handed multi-stranded helices with the oligonucleotide of 6,2′-anhydro-6-oxy-9-β-D-arabinofuranosyluracil (U^o), which also has a high *anti* glycosidic torsion angle.[5] Recently, we synthesized self-complementary dinucleoside monophosphates, A^spU^o and U^opA^s, and found that U^opA^s takes a stable stacked conformation but A^spU^o does not.[8] This phenomenon can be explained in terms of their tendency to take a left-handed stack and the mode of overlap between the bases in the stack. To investigate further the effects of base sequence and the effects of the nature of the base on oligonucleotide conformation, dinucleoside monophosphates containing 8,2′-

1) Part LX of this series: S. Uesugi, Y. Takatsuka, M. Ikehara, D.M. Cheng, L.S. Kan, and P.O.P. Ts' o, *Biochemistry*, submitted.
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3) M. Sundaralingam, *Biopolymers*, **7**, 821 (1969).
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7) M.M. Dhingra, R.H. Sarma, S. Uesugi, and M. Ikehara, *J. Am. Chem. Soc.*, **100**, 4669 (1978).
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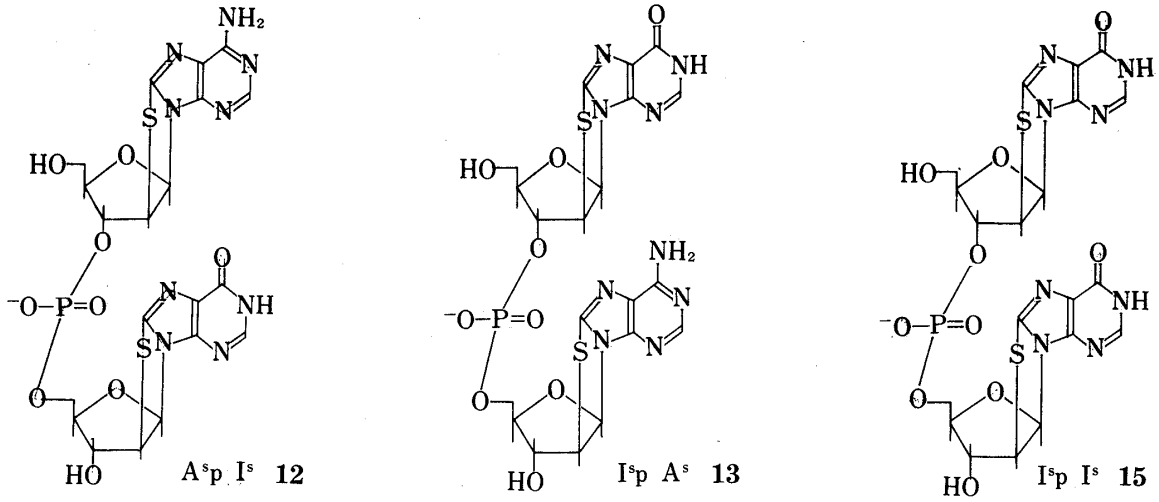

Chart 1

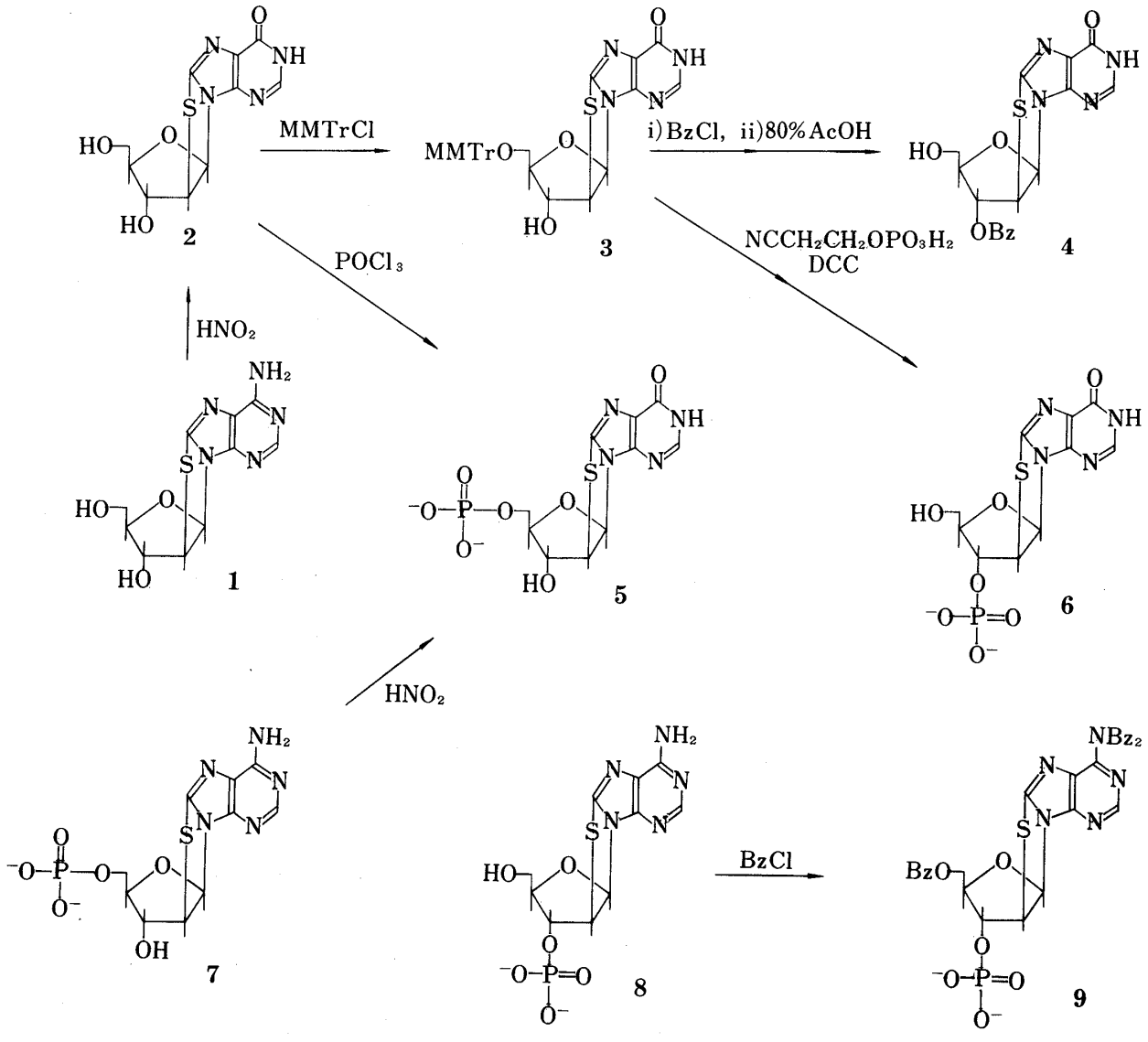

Chart 2

anhydro-8-thio-9-β-D-arabinofuranosylhypoxanthine (**2**, I^s) residues, A^spI^s, I^spA^s and I^spI^s (Chart 1), were synthesized and their properties were examined by ultraviolet (UV), circular dichroism (CD), and ^1H nuclear magnetic resonance (^1H NMR) spectroscopy. It is known that poly(inosinic acid) forms double and triple complexes with poly(adenylic acid).[10,11] A^spI^s and I^spA^s are also self-complementary dimers. Quite recently, a left-handed double helical structure was found in oligo- and polynucleotide duplexes containing a pyrimidine-purine alternating sequence.[12]

Synthesis of the Monomer Derivatives

The synthetic scheme for the monomer derivatives is shown in Chart 2. It was expected that the nucleotide derivatives of cycloinosine, which have no protecting group on the base moiety, might have relatively low solubilities in organic solvents such as pyridine or dimethylformamide (DMF). Therefore, we chose cycloinosine derivatives as nucleoside components and cycloadenosine derivatives as nucleotide components for condensation to form dimers.

I^s (**2**)[13] was obtained from A^s (**1**)[14] by deamination with barium nitrite-2N acetic acid. 5′-*O*-MMTr-I^s (**3**) was synthesized by treatment of I^s with monomethoxytrityl chloride (MMTr-Cl) in pyridine in 60% yield. Benzoylation of **3** with benzoyl chloride and subsequent detritylation with 80% acetic acid gave 3′-*O*-benzoyl-I^s (**4**) in 86% yield. The structures of these compounds were confirmed by UV spectral and chromatographic measurements and elemental analysis. *N*,*N*,5′-*O*-tribenzoyl-A^s (**9**) was synthesized by benzoylation of A^s-3′-phosphate (**8**)[15] with benzoyl chloride in pyridine in 67% yield. Its identity was confirmed by UV spectral and chromatographic measurements.

As reference compounds for ^1H NMR studies, 3′- and 5′-phosphates (**6** and **5**) of I^s were synthesized. The 3′-phosphate (**6**) was obtained by phosphorylation of **3** with β-cyanoethyl-phosphate[16] and dicyclohexylcarbodiimide (DCC) and subsequent deprotection in 68% yield. The 5′-phosphate (**5**) was synthesized by two routes. I^s was phosphorylated specifically with phosphoryl chloride in triethyl phosphate[17] and **3** was isolated by DEAE-cellulose column

Table I. Chromatographic Properties of the Dimers and Related Compounds

Compound	PPC *Rf*				PEP R_m (pA–A)
	A	B	C	D	
A	0.53	0.60	0.49	0.64	0.0
A^s	0.53	0.54	0.48	0.61	−0.05
I^s	0.39	0.59	0.37	0.57	0.42
pA	0.08	0.19	0.13	0.35	1.00
pA^s	0.07	0.15	0.16	0.31	0.96
pI^s	0.05	0.18	0.19	0.33	1.15
A^sp	0.08	0.16	0.16	0.36	1.02
I^sp	0.05	0.16	0.16	0.29	1.19
A^spA^s	0.17	0.19	0.17	0.38	0.32
I^spA^s	0.08	0.16	0.12	0.31	0.62
A^spI^s	0.11	0.19	0.12	0.35	0.62
I^spI^s	0.06	0.19	0.11	0.35	0.81

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11) S. Arnott and P.J. Bond, *Science*, **181**, 68 (1973).
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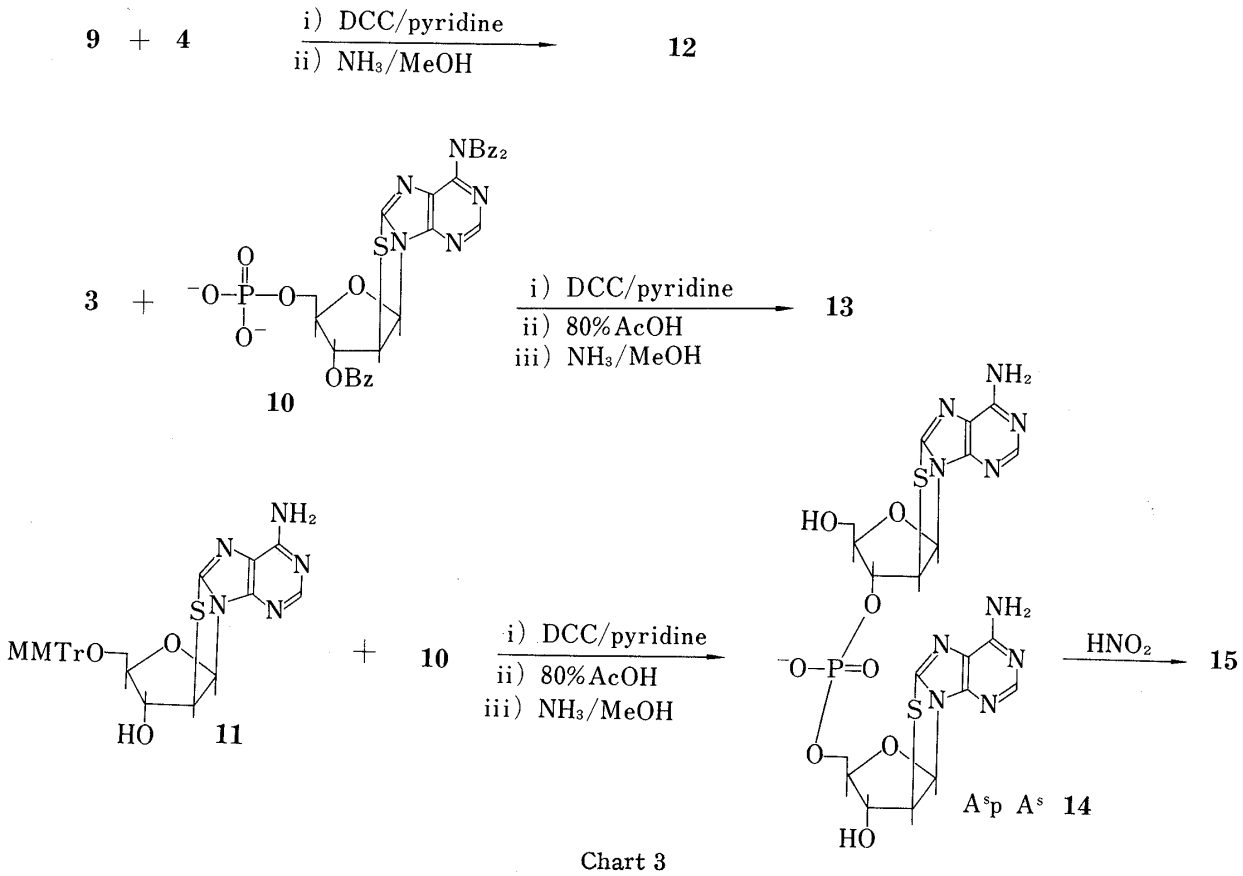

Chart 3

chromatography in 66% yield. On the other hand, the 5′-phosphate of A^s (pA^s, **7**)[18] was deaminated with barium nitrite in 2N acetic acid and the product was isolated by Dowex 1 resin column chromatography in 85% yield. These compounds were identified by UV spectral and chromatographic measurements (Table I), phosphorus analysis and ^1H NMR (Table III).

Synthesis of the Dimers

The synthetic scheme for the dimers is shown in Chart 3. A^spI^s (**12**) was synthesized by condensation of **9** and **4** with DCC (6 eq.) in anhydrous pyridine at room temperature for 7 days. After debenzoylation with methanolic ammonia, A^spI^s was isolated by chromatography on a DEAE-cellulose column in a yield of 70% (Fig. 1). I^spA^s (**13**) was synthesized by condensation of **3** and *N*,*N*,3′-*O*-benzoyl-pA^s (**10**)[8] with DCC (5 eq.) in anhydrous pyridine at 32° for 4 days. After deprotection with 80% acetic acid and methanolic ammonia, I^spA^s was isolated by chromatography on a DEAE-cellulose column under the conditions used for A^spI^s in a yield of 94% (Fig. 1). It is noteworthy that I^spA^s is eluted slightly earlier than A^spI^s. A similar phenomenon has been noted in the case of U^opA^s and A^spU^o, the former being eluted earlier, and it is assumed to result from a more folded conformation of U^opA^s than of A^spU^o.[8] I^spI^s (**15**) was synthesized by deamination of A^spA^s (**14**), which was prepared by a route different from the original one.[4a,b] A^spA^s was synthesized by condensation of 5′-*O*-MMTr-A^s[6] (**11**, 1.4 eq.) and **10** with DCC in anhydrous pyridine at 31° for 3 days. After deprotection, chromatography on a DEAE-cellulose column provided A^spA^s in a yield of 58% (Fig. 1). A^spA^s was treated with sodium nitrite in 8N acetic acid at room temperature for 20 hr. The solution was desalted with a charcoal column, then I^spI^s was isolated by column chromatography on DEAE-cellulose under the same conditions as above in a yield of 87% (Fig. 1). The

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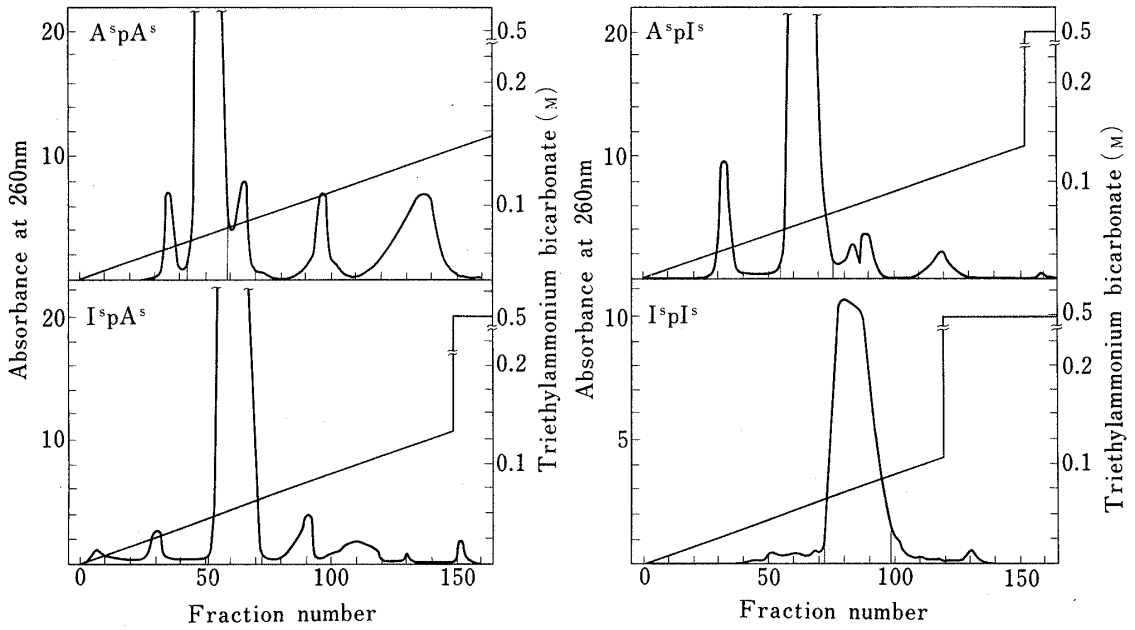

Fig. 1. Elution Profiles of the Condensation Products in DEAE-cellulose Column Chromatography

A column (1.8×37 cm or 1.8×38 cm) of DE-23 (bicarbonate form) was used. Elution was carried out with a linear gradient of triethylammonium bicarbonate buffer (0—0.2 M, total 4 l). Fractions of 18 ml were collected at 15 min intervals.

structures of these dimers were confirmed by their UV, CD and ^{1}H NMR spectra, chromatographic properties (Fig. 1 and Table I), and the results of phosphorus analysis, enzymic hydrolysis and chemical conversion to deoxy-dimers.

Enzymic Hydrolysis and Desulfurization of the Dimers

Hydrolysis of the four dinculeoside monophosphates with snake venom phosphodiesterase was attempted, and the results are shown in Table II. All dimers containing cyclonucleoside residues were rather resistant to the enzyme, and the extent of hydrolysis was 20—50% after 24 hr at 37°, whereas ApA was almost completely hydrolyzed in 4 hr. The products were identified as the corresponding nucleoside and nucleoside 5′-phosphate in a 1:1 ratio. It seems that the dimers containing an A^s residue are resistant to hydrolysis with the phsphodiesterase.

A^{s}pI^s and I^{s}pA^s were desulfurized with Raney Ni to dApdI and dIpdA, respectively, and these products were hydrolyzed with snake venom phosphodiesterase to give the corresponding deoxynucleoside and deoxynucleoside 5′-phosphate in a 1:1 ratio.

UV Absorption and CD Spectra

The UV and CD spectra of A^{s}pI^s, I^{s}pA^s and I^{s}pI^s are shown in Fig. 2—4. The CD spectra of ApI and IpA[24a] are included in Figs. 2 and 3 for comparison. The UV spectra of A^{s}pI^s and I^{s}pA^s at pH 2 are different from those at pH 7, whereas the spectra of I^{s}pI^s at the two pH's are identical. This result indicates that the heterodimers contain an adenine base which

Table II. Hydrolysis (%) of the Dimers with Snake Venom Phosphodiesterase[a]

Incubation time (hr)	ApA	A^{s}pA^s	A^{s}pI^s	I^{s}pA^s	I^{s}pI^s
5	94	9	9	15	24
24	100	23	22	26	47

a) Incubation conditions are given in the experimental section.

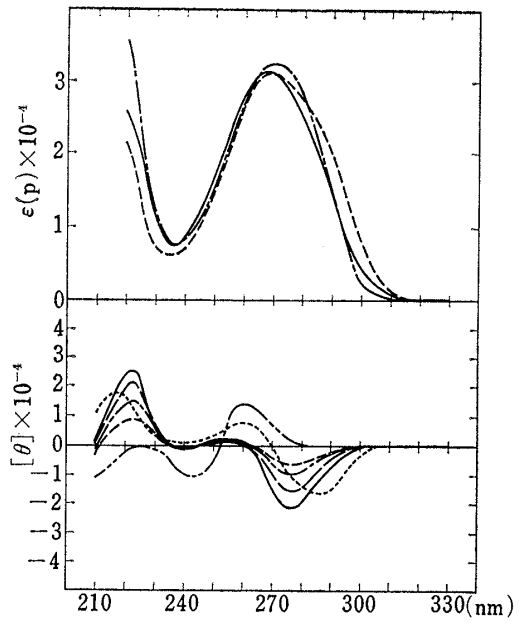

Fig. 2. UV Absorption and CD Spectra of A^spI^s

UV: ------, pH 2; ——, pH 7; – – –, pH 12. CD: ——, 3°; – – –, 20°; – – –, 40°; ------, 60° in 0.1 M NaCl, 0.01 M sodium cacodylate buffer (pH 7.0); ·····, 20° in 0.1 M NaCl, 0.01 N HCl; – · – · –, ApI, 21° in 0.1 M NaCl (pH 7.3) (taken from ref. 24a).

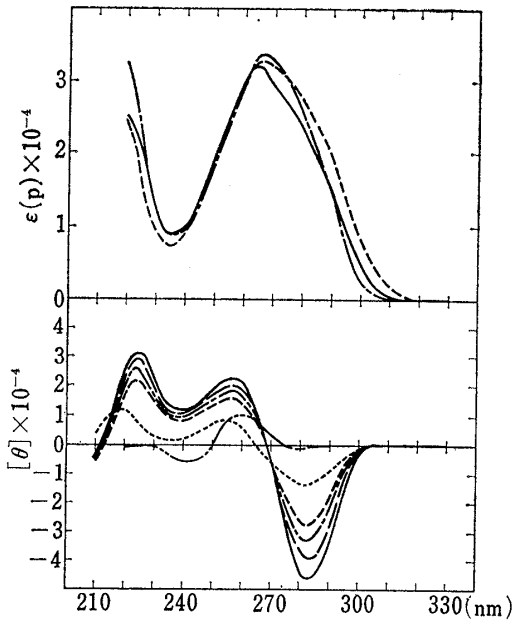

Fig. 3. UV Absorption and CD Spectra of I^spA^s

UV: ------, pH 2; ——, pH 7; – – –, pH 12. CD: ——, 3°; – – –, 20°; – – –, 40°; ------, 60° in 0.1 M NaCl, 0.01 M sodium cacodylate buffer (pH 7.0); ·····, 20° in 0.1 M NaCl, 0.01 N HCl; – · – · –, IpA, 25° in 0.1 M NaCl (pH 7.3) (taken from ref. 24a).

is protonated at pH 2. Close examination of the spectra at pH 7 of A^spI^s and I^spA^s reveals that the spectral patterns around 275 nm are different and that I^spA^s has λ_{max} at shorter wavelength (264.5 nm) than that (266.5 nm) for A^spI^s. In other words, I^spA^s shows a hypsochromic shift of λ_{max} and a larger hypochromicity in the region of 265—285 nm. I^spI^s also shows a hypsochromic shift (1.5 nm) of λ_{max} though it is much smaller than that (5 nm) of A^spA^s.[4a,b] Hypochromicity calculated from the ε's at λ_{max}'s of the monomer (averaged value for pI^s and I^sp) and the dimer is 3%, while the corresponding value for A^spA^s is 15%.[4b] These results suggest much weaker stacking in I^spI^s than in A^spA^s.

All three dimers exhibit qualitatively similar CD spectral patterns at pH 7, which are also similar to that of A^spA^s, *i.e.*, a negative band in the long-wavelength region and two adjacent positive bands in the middle- and short-wavelength regions, but entirely different from those of ApI and IpA (Fig. 2—4). However, the magnitude and position of each band are quite different amond the four dimers. Thus, $[\theta]_{min}$ values of the negative band around 280 nm at 20° are: -3.7×10^4 (290 nm) for A^spA^s; -3.9×10^4 (283 nm) for I^spA^s; -1.5×10^4

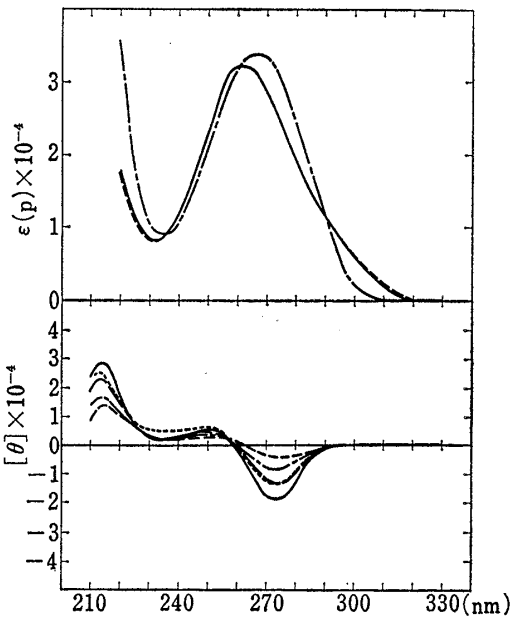

Fig. 4. UV Absorption and CD Spectra of I^spI^s

UV: ------, pH 2; ——, pH 7; – – –, pH 12. CD: ——, 3°; – – –, 20°; – – –, 40°; ------, 60° in 0.1 M NaCl, 0.01 M sodium cacodylate buffer (pH 7.0); ·····, 20° in 0.1 M NaCl, 0.01 N HCl.

(277 nm) for A^spI^s; -1.4×10^4 (273 nm) for I^spI^s. These data show that I^spA^s has a more stacked conformation than A^spI^s, and that I^spI^s has a less stacked conformation than A^spA^s. The temperature dependencies of the CD spectra at pH 7 are also shown in Fig. 2—4. When the temperature is raised within the range of 0—60°, the magnitudes of all the bands decrease linearly. This result indicates that the observed CD bands are indeed associated with stacking conformation of the dimers. The decreases in the absolute value of $[\theta]_{min}$ around 280 nm are 38% for A^spA^s,[4b] 43% for I^spA^s, 69% for A^spI^s and 74% for I^spI^s over the range of 0° to 60°. Therefore, the stacked conformations of A^spA^s and I^spA^s are much more stable to thermal perturbation than those of A^spI^s and I^spI^s. The CD spectra of these dimers at pH 2 and at 20° are also included in Fig. 2—4. In the case of I^spI^s, no change was seen in the UV spectrum and the CD spectrum showed essentially no change upon acidification. As already mentioned, these results are consistent with the fact that I^spI^s has no protonation site on the base residues. On the other hand, both A^spI^s and I^spA^s, which have a protonation site on the adenine residue, both exhibited definite changes in the absorption spectra. In the CD spectra, I^spA^s shows CD bands with markedly decreased magnitudes while A^spI^s shows CD bands at altered positions but of similar magnitudes upon acidification. These results suggest that the base stacking in I^s pA^s is profoundly disrupted whereas that of I^spA^s is almost unaffected upon protonation of the adenine residue. It is known that protonation on both base residues causes profound unstacking in a dinucleoside monophosphate[4c,19,20] and protonation on one of the base residues can sometimes still afford a relatively stable stacked conformation.[20,21]

Table III. ^{1}H NMR Data for the Dimers and Related Compounds[a]

Compound		Chemical hifts[b] and $\Delta\delta$[c] (ppm)		$J_{1'2'}$ (Hz)
		H-2	H-1′	
pA^s		8.46	7.01	6.9
A^sp		8.34	6.92	6.6
pI^s		8.53	7.12	7.0
I^sp		8.53	7.11	7.1
A^spI^s	A^sp-	8.31(0.03)	6.68(0.24)	7.0
	-pI^s	8.27(0.26)	6.96(0.16)	6.9
I^spA^s	I^sp-	8.49(0.04)	6.48(0.63)	6.9
	-pA^s	8.16(0.30)	6.85(0.16)	6.9
I^spI^s	I^sp-	8.53(0.00)	6.81(0.30)	6.8
	-pI^s	8.39(0.14)	7.03(0.09)	6.8

a) 0.2 M solution (sodium salt) in D_2O except for I^spI^s (0.1 M). Measured at pD 7.5 (dimer) and pD 5.5 (monomer).
b) Chemical shifts are given relative to external TMS.
c) shown in parentheses: $\Delta\delta=\delta$(monomer)$-\delta$(dimer).

^{1}H NMR Spectra of the Dimers

The ^{1}H NMR data are presented in Table III. The spectra of the monomers were measured at pD 5.5 to minimize the secondary phosphate dissociation. H-2 and H-1′ of the monophosphates of I^s and A^s exhibit similar chemical shifts and can be easily distiguished from the other protons. The assignment of the two H-1' signals of each dimer was carried out by an approach based on the specific broadening effect of Mn^{2+} upon binding.[19,22] Upon

19) M.M. Warshaw and I. Tinoco, Jr., *J. Mol. Biol.*, **13**, 54 (1965).
20) *a*) M.D. Topal and M.M. Warshow, *Biopolymers*, **15**, 1755 (1976); *b*) N. Ogasawara, and Y. Inoue, *J. Am. Chem. Soc.*, **98**, 7048 (1976).
21) N.P. Johnson and T. Schleich, *Biochemistry*, **13**, 981 (1974).
22) *a*) S.I. Chan and J.H. Nelson, *J. Am. Chem. Soc.*, **91**, 168 (1968); *b*) K.N. Fang, N.S. Kondo, P.S. Miller, and P.O.P. Ts'o, *ibid.*, **93**, 6647 (1971).

addition of Mn^{2+} ($2—4\times10^{-4}$ M), the H-1' signal at higher field was specifically broadened for all three dimers containing I^s residues. This result suggests that the H-1' with the smaller chemical shift is the H-1' of the 3'-linked nucleoside residue which is closer to the Mn^{2+} bound to the internucleotidic phosphate group than the 5'-linked residue. Thus the H-1' of the 3-linked residue is more shielded than that of the 5'-linked residue. This situation is consistent with a dimer in a left-handed stack, where the H-1' of the 3'-linked residue is more shielded by the ring-current of the base of the 5'-liked residue (see Fig. 5). The left-handed stack is also supported by CD results. The two H-2 signals of each dimer were assigned on the basis of this mode of stacking (illustrated in Fig. 5). Thus the H-2 of the 5'-linked residue is more shielded than the other. The dimerization shifts, $\Delta\delta=\delta$(monomer)$-\delta$(dimer), according to the above assignments are also presented in Table III. These $\Delta\delta$'s cannot be compared directly among the dimers since the ring-current effects of the adenine and hypoxanthine rings are different and the adenine ring has more shielding power.[23] However, the following comparison may be reasonable, because shieldings by the same base are considered. The $\Delta\delta$ (0.63 ppm) of H-1' of the I^sp- residue in I^spA^s is comparable to that (0.59 ppm)[4b] of the A^sp-residue in A^spA^s. The $\Delta\delta$(0.24 ppm) of H-1' of the A^sp- residue in A^spI^s is comparable to that (0.30 ppm) of the I^sp- residue in I^spI^s. The $\Delta\delta$ (0.30 ppm) of H-2 of the -pA^s residue in I^spA^s is significantly larger than that (0.14 ppm) of the -pI^s residue in I^spI^s. The $\Delta\delta$ (0.37 ppm)[4b] of H-2 of the -pA^s residue is larger than that (0.26 ppm) of the -pI^s residue in A^spI^s. These results indicate that stacking of A^spA^s and I^spA^s is more extensive than that of A^spI^s and I^spI^s.

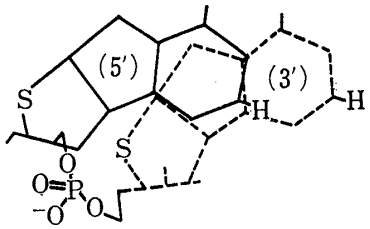

Fig. 5. Schematic Illustration of Left-Handed Stacking of a Dimer Containing Cyclonucleoside Residues

The 5'-linked nucleoside residue (solid line) is above the 3'-linked nucleoside residue (broken line).

Conclusions

All the UV absorption, CD and ^1H NMR studies suggest that the order of the extent of base stacking is $A^spA^s\approx I^spA^s>A^spI^s\approx I^spI^s$. The stacking order of $A^spA^s>I^spI^s$ is simply explained by the stronger stacking power of the adenine base relative to the hypoxanthine base. IpI and even poly(I) have much weaker stacked conformations than ApA.[24] The order of $I^spA^s>A^spI^s$ can be explained by the mode of overlapping of bases in a left-handed stack, as illustrated in Fig. 5. In the case of I^spA^s, base-base overlap is mainly between the imidazole part of I^s and the pyrimidine part of A^s. On the other hand, the imidazole part of A^s and the pyrimidine part of I^s mainly overlap in A^spI^s. The difference between adenine and hypoxanthine lies in the pyrimidine part of the purine ring. Therefore, a stacking involving the pyrimidine part of A^s may be more stable, and I^s pA^s is expected to have more stable stacking than A^spI^s. A similar explanation has been applied to the case of A^spU^o and U^opA^s, where only U^opA^s has a stacked conformation under similar conditions. Such an explanation may also be valid for the sequence isomers of dimer containing natural nucleoside residues which show a sequence preference for stacking. In this case, a reversed sequence dependency will be observed because they take a right-handed stack. In fact, it is known that purine-pyrimidine dimers generally stack better than the corresponding pyrimidine-purine isomers,

23) *a*) C. Giessner-Prettre, B. Pullman, P. Borer, L.S. Kan, and P.O.P. Ts'o, *Biopolymers*, **15**, 2277 (1976); *b*) C. Giessner-Prettre and B. Pullman, *Biochem. Biophys. Res. Commun.*, **70**, 578 (1976); *c*) *Idem*, *J. Theor. Biol.*, **27**, 87 (1970).

24) *a*) C. Formoso and I. Tinoco, Jr., *Biopolymers*, **10**, 531 (1971); *b*) S. Tazawa, I. Tazawa, J.L. Alderfer, and P.O.P. Ts'o, *Biochemistry*, **11**, 3544 (1972).

as judged by CD[25] and ^{1}H NMR[26] studies. In the case of ApI and IpA, ApI exhibits significantly larger CD bands[24] (see Figs 2 and 3) and, therefore, the same principle may be applied.

Different stabilities of the base stacking in A^spI^s and I^spA^s against perturbation by protonation of the adenine residue may also be explained as follows. The stacking of I^spA^s, which contains mainly pyrimidine(-pA^s)-imidazole(I^sp-) overlap, must be more vulnerable to perturbation by protonation than that of A^spI^s, which contains mainly pyrimidine(-I^sp)-imidazole-(A^sp-) overlap, because the protonation occurs at N(1) of the adenine base. A similar phenomenon has been observed with natural ApU and UpA, where unstacking at acidic pH occurs in ApU but not UpA.[21] This trend, which is opposite to the case of cyclonucleotide dimers, can be explained by the mode of base overlap in a right-handed stack.

Experimental

General Procedures——UV absorption spectra were recorded on a Hitachi 124 spectrophotometer. CD spectra were recorded on a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment. The molar ellipticity, $[\theta]$, and molar extinction coefficient, ε, are presented as the per residue values. Paper chromatography (PPC) was performed by the descending technique on Whatman No. 1 paper with the following solvent systems: solvent A, 2-propanol–conc. ammonia–water (7:1:2 v/v); solvent B, ethanol-1 M ammonium acetate (pH 7.5) (7:3 v/v); solvent C, 1-butanol–acetic acid–water (5:2:3 v/v); solvent D, 1-propanol–conc. ammonia–water (55:10:35 v/v). Paper electrophoresis (PEP) was performed for 1 hr at a voltage gradient of 35 V/cm on Toyo filter paper No. 51A with 0.05 M triethylammonium bicarbonate buffer (pH 7.5). Thin-layer chromatography (TLC) was performed on a silica gel (Merck HF_{254}) plate with $CHCl_3$–95% EtOH mixture as a solvent. Snake venom phosphodiesterase was purchased from Worthington Biochemical Co. Charcoal for chromatography was supplied by Wako Junyaku Ltd.

^{1}H NMR Measurement——An aqueous solution of each compound (Na salt, 0.08 mmol) was passed through a column of Chelex 100 resin. The lyophilized compound was dissolved in D_2O and pD was adjusted with NaOD or DCl to 7.5 (for the dimers) or 5.5 (for the monomers). After lyophilization from D_2O (three times), the compound was finally dissolved in D_2O (99.8% ^{2}H, 0.4 ml). ^{1}H NMR spectra were recorded with a Hitachi R-22 spectrometer (90 MHz, ambient probe temperature, 34°). Chemical shifts were measured fom an external tetramethylsilane (TMS) capillary.

5′-*O*-Monomethoxytrityl-8,2′-S-cycloinosine (3)——Monomethoxytrityl chloride (296 mg, 1.2 eq) was added to a stirred suspension of I^s[13] (2, 226 mg, 0.8 mmol) in pyridine (5 ml) at 0°. The mixture was kept at room temperature with stirring. After 24 hr, further monomethoxytrityl chloride (250 mg, 1 eq.) was added. After 2 days, the reaction mixture was poured into dilute aqueous ammonia in an ice-bath. The mixture was extracted with $CHCl_3$ (30 ml × 3) and the organic layer was dried over $MgSO_4$. After removal of the solvent by evaporation the residue was rendered anhydrous by repeated evaporation with added puridine (twice). The product was precipitated with ether–hexane (1:1 v/v) from its solution in pyridine (3 ml). The precpitate was collected by centrifugation and washed with ether, then a solution of the precipitate in $CHCl_3$ was applied to a column (2.8 × 8 cm) of silica gel G (25 g). Elution was performed with $CHCl_3$–EtOH (20:1). **3** was obtained as a powder by precipitation with ether–hexane (4:1) from its solution in pyridine. The yield was 267 mg (0.48 mmol, 60%), mp 185—187°. *Anal.* Calcd for $C_{30}H_{26}N_4O_5S$: C, 64.96; H, 4.73; N, 10.10; S, 5.78. Found: C, 64.19; H, 4.59; N, 9.92; S, 5.64. UV λ_{max} nm (ε): 264 (15400), 232 (16300). TLC: *Rf* ($CHCl_3$–EtOH, 10:1) 0.14. PPC: *Rf*(B) 0.91 (I^s, 0.66).

3′-*O*-Benzoyl-8,2′-S-cycloinosine (4)——A solution of **3** (555 mg, 1 mmol) in pyridine (8 ml) was cooled in an ice-bath and benzoyl chloride (0.35 ml, 3.6 eq.) was added. The resulting solution was maintained at room temperature for 18.5 hr then poured into an aqueous $NaHCO_3$ solution with stirring. The mixture was extracted with $CHCl_3$ (60 ml). The organic layer was washed with aqueous $NaHCO_3$ (30 ml × 3) and water (30 ml × 2), then evaporated to dryness *in vacuo*. After removing traces of pyridine by coevaporation with toluene, the residue was treated with 80% AcOH (40 ml) at room temperature for 2.5 hr. During this period, precipitation of the detritylated compound was observed. The solvent was evaporated off *in vacuo* and residual AcOH was removed by coevaporation with water (twice) to give **4** as a powder, 333 mg (0.86 mmol, 86%), mp 250° (dec.). *Anal.* Calcd for $C_{17}H_{14}N_4O_5S$: C, 52.84; H, 3.66; N, 14.50; S, 8.30. Found: C, 52.83; H, 3.70; N, 13.84; S, 7.84. UV λ_{max} 260.5, 233 nm. PPC: *Rf*(B) 0.78 (I^s, 0.66).

8,2′-S-Cycloinosine 5′-Phosphate (5)——a) From 8,2′-S-Cycloinosine: Compound **2** (287 mg, 1 mmol) was dissolved in triethyl phosphate (6 ml) with heating and the solution was cooled to 0°. A mixture of triethyl phosphate (1 ml) and $POCl_3$ (0.4 ml) was added. The resulting solution was kept at 0° for 4.5 hr and

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26) F.S. Ezra, C.H. Lee, N.S. Kondo, S.S. Danyluk, and R.H. Sarma, *Biochemistry*, **16**, 1977 (1977).

added dropwise to a stirred ice-water (150 ml) mixture. The solution was diluted to 700 ml with water and applied to a column (1.5×11 cm) of charcoal for chromatography. After being thoroughly washed with water, the column was eluted with conc. ammonia–95% EtOH–water (5: 50: 45 v/v). Volatile materials were evaporated off, then the residue was dissolved in water and applied to a column (1.8×30.5 cm) of DE-23 (bicarbonate form). After being washed with water, the column was eluted with a linear gradient of triethylammonium bicarbonate (TEAB) buffer (pH 7.5) (0—0.2 M, total 4 l). **5** was eluted in a major peak at around 0.08 M TEAB concentration. The yield was 10800 A_{265} units (66%).

b) From 8,2′-S-Cycloadenosine 5′-Phosphate: 4 N AcOH (5 ml) and $Ba(NO_2)_2 \cdot H_2O$ (350 mg, 3.1 eq.) in water (2.5 ml) were added to a solution of the sodium salt of 8,2′-S-cycloadenosine 5′-phosphate[18] (**9**, 0.45 mmol) in water (5 ml) with stirring. The mixture was stirred at room temperature for 15 hr, diluted to 150 ml with water and applied to a column (1.8×45 cm) of charcoal for chromatography. After being thoroughly washed with water, the column was eluted with conc. ammonia–95% EtOH–water (5: 50: 45). Volatile materials were evaporated off, and the residue was dissolved in water and applied to a column (1.3×15 cm) of Dowex 1×2 (formate form). The column was washed with water and eluted stepweise with aqueous formic acid. **5** was eluted with 0.4 N formic acid. The yield was 6250 A_{265} units (0.38 mmol, 85%). Removal of the solvent by evaporation, coevaporation with water *in vacuo* and lyophilization gave the free acid as a crystalline powder. UV λ_{max}^{pH2} nm (ε): 263.5 (16300). λ_{max}^{pH7}: 264 (16300). λ_{max}^{pH12}: 265.5 (16700). Chromatographic properties are shown in Table I. ^{1}H NMR data are presented in Table III.

8,2′-S-Cycloinosine 3′-Phosphate (6)——The barium salt of β-cyanoethyl phosphate[16] (750 mg, 2.6 mmol) was converted to the pyridinium salt by passage through a column of Dowex 50 (pyridinium form). A mixture of the pyridinium salt and **3** (420 mg, 0.76 mmol) was rendered anhydrous by evaporation with added pyridine (3 times) and then dissolved in anhydrous pyridine (5 ml). Dicyclohexylcarbodiimide (DCC) (434 mg, 5 eq.) was added and the mixture was kept at 31° for 3 days. 50% aqueous pyridine was added and the solution was left to stand at room temperature overnight. Dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness *in vacuo* and the residual pyridine was removed by coevaporation with toluene. The residue was treated with 80% AcOH (45 ml) at 60° for 1 hr. The solvent was evaporated off, and a solution of the residue in 30% aqueous pyridine (40 ml) was washed with ether then evaporated to dryness. The residue was treated with conc. ammonia (40 ml) at 65° for 2 hr. Ammonia was largely removed by bubbling N_2 gas through the solution, and the solvent was removed by evaporation. Water (200 ml) was added to the residue and insoluble materials were removed by filtration. After pH adjustment to 4, the filtrate was applied to a column of charcoal for chromatography (20 ml). The column was washed thoroughly with water, and eluted with conc. ammonia–95% EtOH–water (5: 50: 45). Volatile materials were evaporated off, and a solution of the residue in water (100 ml) was applied to a column (1.3×28 cm) of DE-23 (bicarbonate form). The column was washed with water and eluted with a linear gradient of TEAB buffer (0—0.15 M, total 4 l). **6** was eluted in a major peak at around 0.04 M TEAB concentration. The yield was 8760 A_{265} units (0.52 mmol, 68%). UV λ_{max}^{pH2} nm (ε): 263.5 (16900). λ_{max}^{pH7}: 264 (17000). λ_{max}^{pH12}: 266 (17300). The chromatographic properties are shown in Table I. ^{1}H NMR data are presented in Table III.

***N*,*N*,5′-*O*-Tribenzoyl-8,2′-S-cycloadenosine 3′-Phosphate (9)**——The pyridinium salt of 8,2′-S-cycloadenosine 3′-phosphate[15] (**8**, 1.35 mmol) was rendered anhydrous by evaporation with added pyridine (3 times) and finally suspended in anhydrous pyridine (15 ml). Benzoyl chloride (3.2 ml, 27.7 mmol) was added. The mixture was stirred at room temperature for 1.5 hr and added dropwise to a saturated $NaHCO_3$ solution (50 ml) cooled in an ice-bath, with stirring. The product was extracted with $CHCl_3$ (30 ml×3). The organic layer was washed with saturated $NaHCO_3$ solution (50 ml) and water (30 ml×2) and evaporated to dryness. The residue was rendered anhydrous by evaporation with added pyridine (twice) and treated with pyridine–acetic anhydride (12 ml—12 ml) at room temperature overnight. MeOH was added to the residue cooled in an ice-bath. The solution was allowed to stand for a while at room temperature, and the solvent was evaporated off. After being dried by evaporation with added pyridine (twice), **9** was precipitated with ether–hexane (100 ml—20 ml) from its solution in pyridine (2 ml). Yield was 754 mg (0.90 mmol, 67%). UV $\lambda_{max}^{H^+}$ nm: 291.5, 223.5. $\lambda_{max}^{H_2O}$: 291.5, 223.5. $\lambda_{max}^{OH^-}$: 291, 223. PPC: *Rf*(B) 0.65 (A, 0.65, pA, 0.22); *Rf*(C) 0.81 (A, 0.52, pA, 0.12). This compound was fluorescent under a UV lamp.

8,2′-S-Cycloadenylyl-(3′—5′)-8,2′-S-cycloinosine (A^spI^s, 12)——A mixture of **9** (335 mg, 0.4 mmol) and **4** (163 mg, 0.42 mmol) was rendered anhydrous by evaporation with added pyridine (4 times) and dissolved in anhydrous pyridine (7 ml). DCC (501 mg, 6 eq.) was added. The mixture was stirred at room temperature for 7 days and 50% aqueous pyridine (30 ml) was added. The solution was allowed to stand at room temperature overnight, dicyclohexylurea was removed by filtration and the solvent was evaporated off. The residue was treated with methanolic ammonia (30 ml) at 32° overnight. Ammonia was largely removed by bubbling N_2 gas through the solution and the solvent was removed in *vacuo*. The residue was dissolved in 50% aqueous pyridine (50 ml) and washed with ether. The aqueous layer was evaporated to dryness. Water was added to the residue and insoluble materials were filtered off. The filtrate was diluted to 1.5 l with water and applied to a column (1.8×37 cm) of DE-23 (bicarbonate form). The column was washed with water, and elution was carried out with a linear gradient of TEAB buffer (0—0.2 M, total 4 l). Fractions of 18 ml were collected at 15 min intervals. **12** was eluted in a major

peak (fraction No. 56—76) around 0.06 M TEAB concentration (Fig. 1). The buffer was removed by repeated coevaporation with water and 95% EtOH and finally by lyophilization. The yield was 8660 $A_{266.5}$ units (0.28 mmol, 70%). UV λ_{max}^{pH2} nm (ε): 268 (15400). λ_{max}^{pH7}: 266.5 (15600). λ_{max}^{pH12}: 269 (16500). Chromatographic properties are shown in Table I. UV absorption and CD spectra are shown in Fig. 2. ^{1}H NMR data are presented in Table III.

8,2′-S-Cycloinosinyl-(3′—5′)-8,2′-S-cycloadenosine (I^{s}pA^s, 13)——A mixture of **3** (235 mg, 0.42 mmol) and pyridinium *N*,*N*,3′-*O*-tribenzoyl-8,2′-S-cycloadenosine 5′-phosphate[8] (**10**) (334 mg, 0.40 mmol) was rendered anhydrous by evaporation with added pyridine (3 times) and dissolved in anhydrous pyridine (5 ml). DCC (402 mg, 5 eq.) was added and the resulting solution was stirred for a while. Dicyclohexylurea began to precipitate. The mixture was kept at 32° for 4 days, then 50% aqueous pyridine was added. The mixture was kept at 32° overnight and evaporated to dryness after removal of the solid materials by filtration. The residue was treated with 80% AcOH (50 ml) at room temperature for 3 hr. The solvent was evaporated off, and the residual AcOH was removed by coevaporation with water and 1-butanol–water (1: 1). The residue was treated with methanolic ammonia (40 ml) at 32° overnight. Ammonia was largely removed by bubbling N_2 gas through the solution, then volatile materials were evaporated off with an aspirator. 50% aqueous pyridine (50 ml) was added and insoluble materials were removed by filtration. The filtrate was washed with ether (35 ml) and evaporated to dryness. The residue was dissolved in water (1 l) and subjected to column chromatography under the conditions described for A^{s}pI^s. **13** was eluted in a major peak (fraction No. 53—73) at around 0.057 M TEAB concentration (Fig. 1). The yield was 12100 $A_{264.5}$ units (0.38 mmol, 94%). UV λ_{max}^{pH2} nm (ε): 267 (16300). λ_{max}^{pH7}: 264.5 (16100), 275 (shoulder). λ_{max}^{pH12}: 266.5 (16800). Chromatographic properties are shown in Table I. UV absorption and CD spectra are shown in Fig. 3. ^{1}H NMR data are presented in Table III.

8,2′-S-Cycloadenylyl-(3′—5′)-8,2′-S-cycloadenosine (A^{s}pA^s, 14)——A mixture of 5′-*O*-monomethoxytrityl 8,2′-S-cycloadenosine (**11**)[6] (310 mg, 0.56 mmol) and the pyridinium salt of **10**[8] (334 mg, 0.4 mmol) was rendered anhydrous by evaporation with added pyridine (3 times), then dissolved in anhydrous pyridine (5 ml). DCC (403 mg, 5 eq.) was added. The resulting solution was kept at 31° for 3 days. The mixture was worked up in the manner described for I^{s}pA^s. Detritylation was carried out with 80% AcOH at 60° for 2 hr. After work-up, the residue was dissolved in water (500 ml) and subjected to column chromatography under the conditions described for A^{s}pI^s. **14** was eluted in a major peak (fraction No. 45—60) at around 0.048 M TEAB concentration (Fig. 1). The yield was 8810 A_{271} units (0.23 mmol, 58%). This compound was identical with an authentic sample[4a,b] as judged from UV absorption CO and ^{1}H NMR spectra and chromatographic properties (Table I).

8,2′-S-Cycloinosinyl-(3′—5′)-8,2′-S-cycloinosine (I^{s}pI^s, 15)——$NaNO_2$ (1038 mg, 100 eq) and 16 N AcOH (6.3 ml) were added successively to a solution of **14** (triethylammonium salt, 5100 A_{271} units, 0.134 mmol) in water (6.3 ml). The solution was kept at room temperature for 20 hr then evaporated to dryness *in vacuo*. The residue was dissolved in water (200 ml) and applied to a column of charcoal for chromatography (3 ml). The column was washed thoroughly with water, and elution was performed with conc. ammonia–water–EtOH (5: 45: 50). Volatile materials were evaporated off, then the residue was dissolved in water (100 ml) and subjected to column chromatography under the conditions described for A^{s}pI^s. **15** was eluted in a major peak (fraction No. 74—100) around 0.078 M TEAB concentration. The yield was 3770 A_{263} units (0.117 mmol, 87%). UV λ_{max}^{pH2} nm (ε): 262 (16100). λ_{max}^{Hp17}: 262.5 (16200). λ_{max}^{pH12}: 265 (17000). Chromatographic properties are shown in Table I. UV absorption and CD spectra are shown in Fig. 4. ^{1}H NMR data are presented in Table III.

Hydrolysis of the Dimers with Snake Venom Phosphodiesterase——In the case of dimers containing cyclonucleoside residues, the incubation mixture (200 μl) contained a dimer (10A_{max} units), snake venom phosphodiesterase (0.2 mg/ml) and TEAB buffer (0.04 M). In the case of ApA, half the amount of the enzyme was used. Incubation was performed at 37°. After 5 hr and 24 hr, 100 μl of the reaction mixture was taken out and heated at 100° for 2 min. The products were separated by paper electrophoresis at pH 7.5 and determined by UV absorption measurement after extraction with water. The molar ratios of nucleoside to 5′-nucleotide were close to 1. The extents of hydrolysis are shown in Table II.

Desulfurization of A^{s}pI^s and I^{s}pA^s——A solution of A^{s}pI^s or I^{s}pA^s (2—4 mg) in water (2—3 ml) was refluxed after the addition of a small amount of Raney Ni for 2 hr. The product was isolated by paper chromatography in solvent A or D and purified further by paper electrophoresis at pH 7.5. From A^{s}pI^s, dApdI was obtained. UV $\lambda_{max}^{H_2O}$: 253 nm. PEP: R_m(pA–A) 0.50. From I^{s}pA^s, dIpdA was obtained. UV $\lambda_{max}^{H_2O}$: 252 nm. PEP: R_m(pA–A) 0.49. Hydrolysis of these dimers with snake venom phosphodiesterase gave the corresponding nucleoside and 5′-nucleotide in a 1: 1 ratio as examined by paper electrophoresis.