# Synthesis and Properties of the Dinucleoside Monophosphates Containing Adenine *S*-Cyclonucleosides and Adenosine. Factors Determining the Stability and Handedness of the Stacking Conformation in a Dinucleoside Monophosphate[1]

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**Abstract:** Ten dinucleoside monophosphates containing 8,2′-anhydro-8-mercapto-9-β-D-arabinofuranosyladenine (A$^s$, $\chi$ = 122°), 8,3′-anhydro-8-mercapto-9-β-D-xylofuranosyladenine (As, $\chi$ = 75°), 8,5′-anhydro-8-mercapto-9-β-D-ribofuranosyladenine ($_s$A, $\chi$ = 42°), and adenosine (A, $\chi$ = 0°) residues were synthesized. They are A$^s$pAs, AspA$^s$, AspAs, (2′–5′)- and (3′–5′)-sApAs, A$^s$pA, AspA, ApAs, and sApA. Analyses of these molecules by UV, CD, hybridization with poly(U), and molecular model buildings suggest that (1) AspAs, the homodimer of As, can take a left-handed stacked conformation at low temperature; (2) A$^s$pAs and AspA$^s$, the heterodimers of A$^s$ and As, may take mainly a left-handed stacked conformation, the stability of which is in-between those of A$^s$pA$^s$ and AspAs; (3) A$^s$pA, AspA, and sApA, the dimers containing a 5′-linked adenosine and a 3′-linked cycloadenosine residue, take a right-handed stacked conformation; (4) ApA$^s$ and ApAs, the dimers containing a 5′-linked cycloadenosine and a 3′-linked adenosine residue, take a left-handed stacked conformation; (5) sApAs's may take a conformation other than ordinary stacking. Explanations for these results by considering the least strained arrangement of two nucleoside residues for base stacking in a dimer are described. Factors determining the stability and handedness of a dinucleoside monophosphate are also discussed.

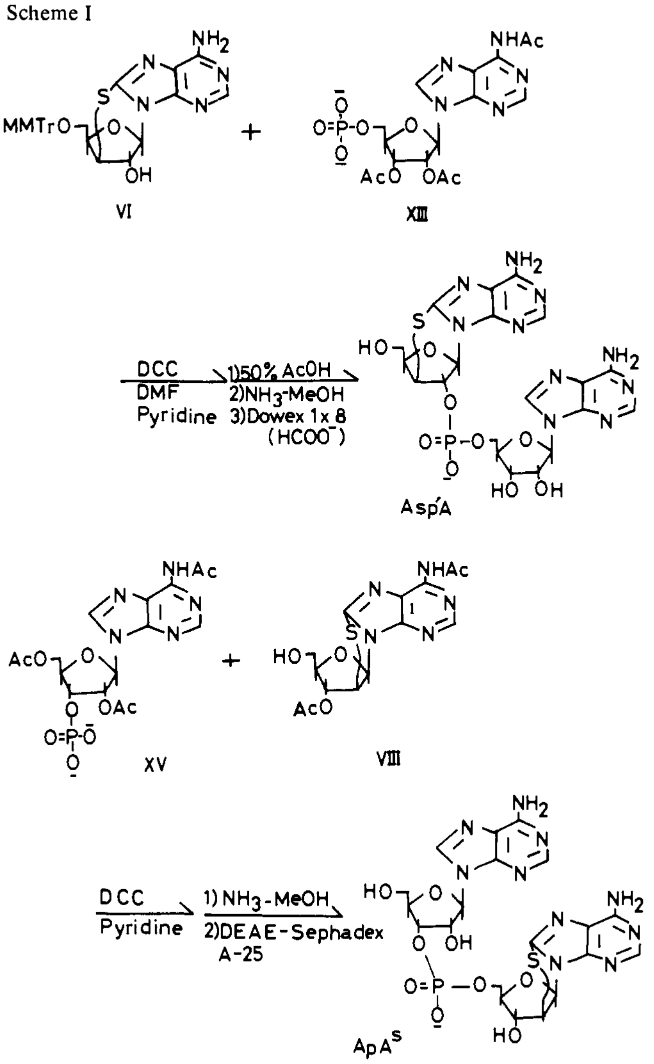
We have been studying oligomers containing cyclonucleosides with fixed torsion angles about their glycosidic bonds to elucidate the effect of the glycosidic torsion angle on the conformational properties of oligo- and polynucleotides.[2-7] The dinucleoside monophosphate of 8,2′-anhydro-8-mercapto-9-β-D-arabinofuranosyladenine (8,2′-*S*-cycloadenosine, A$^s$),[8] A$^s$pA$^s$ (Ia), was shown to have a stable, stacked conformation with a left-handed screw axis by UV, CD, and $^1$H NMR studies.[2] The fixed torsion angle ($\chi$)[9] about the glycosidic bond in a 8,2′-*S*-cycloadenosine (II) residue is 122°,[10] while the adenosine residue in ApA, which is assumed to have a right-handed stack,[11-13] is thought to take an $\chi$ value near 0°.[14] Homooligonucleotides of A$^s$ [3] and the dinucleoside monophosphate of 8,2′-*O*-cycloadenosine, A$^o$pA$^o$ [4] (Ib), also gave CD spectra of similar patterns suggesting left-handed stacking. These oligomers with a left-handed stack did not form a complex with the right-handed helix of poly(U). But the octamer of pA$^s$ did form complexes with the octamer of 6,2′-anhydro-6-oxy-1-β-D-arabinofuranosyluracil 5′-phosphate (6,2′-*O*-cyclouridine 5′-phosphate, pU$^o$) to give either a double or a triple helix of left-handedness.[6] 6,2′-*O*-Cyclouridine[15] is the complementary, pyrimidine counterpart of 8,2′-*S*-cycloadenosine. The (pA$^s$)$_8$ was also shown to form a complex with poly(laurusin phosphate).[7] It is thought that the glycosidic torsion angle of laurusin, which is a deaminated derivative of the antibiotic *C*-nucleoside, formycin, can be changed easily.[16] Poly(laurusin phosphate) has also been shown to form complexes with polymers containing natural nucleotides.[17] From these results, it is concluded that oligo- and polynucleotides containing nucleoside residues with $\chi$ values in the anti-syn boundary region (100–120°) have a tendency to form a left-handed helix. As a next step, we were interested in the heterodimers which contain two nucleoside residues with different $\chi$ values. In this paper, we wish to report on the synthesis and properties of ten dinucleoside monophosphates which contain 8,2′-*S*-cycloadenosine (A$^s$, $\chi$ = 122°), 8,3′-anhydro-8-mercapto-9-β-D-xylofuranosyladenine (8,3′-*S*-cycloadenosine, As, $\chi$ = 75°), 8,5′-anhydro-8-mercapto-9-β-D-ribofuranosyladenine (8,5′-*S*-cycloadenosine, sA, IV, $\chi$ = 42°),[19] and adenosine (A, $\chi$ = 0°). The dimers are A$^s$pAs (XVI), Asp′A$^s$ [20] (XVIII), Asp′As (XIX), (2′–5′)- and (3′–5′)-sApAs (XXIa,b), A$^s$pA (XVII), ApA$^s$ (XXIII), Asp′A (XX), ApAs (XXIV), and sApA (XXII). This paper contains the synthetic methods, identification and characterization, susceptibility to enzymic digestion and hydrolytic conditions, ultraviolet absorption, circular dichroic properties, and hydridization experiments with poly(U). From these results probable stacking conformations are proposed for the dimers. The plausibility of the proposed conformations was tested by molecular model building. Finally, general conclusions on factors governing the stability and handedness of stacking in a dinucleoside monophosphate are discussed.

**Synthesis of Monomer Units.** The syntheses of dinucleoside monophosphates were performed by two routes: (1) condensation of 5′-protected nucleosides and 5′-mononucleotides and (2) condensation of 3′-mononucleotides and 2′- and (or) 3′-protected nucleosides. For this purpose a variety of monomer components having suitable protecting groups were synthesized. 5′-*O*-Monomethoxytrityl-A$^s$ (MMTrA$^s$, V) and -As (MMTrAs, VI) were synthesized by the reaction of A$^s$ [21] (II) and As[21] (III) with monomethoxytrityl chloride in DMF in yields of 37 and 48%, respectively. The compound V was alternatively synthesized by the monomethoxytritylation of 8-Br-2′-TPS-adenosine,[21] followed by cyclization using NaSH. As a protected A$^s$, the 5′-*O*-trityl-$N^6$-dimethylaminomethylene derivative[2] VII was also used. These 5′-protected cyclonucleosides and unprotected sA[22] (IV) were used as the 5′-end nucleoside components. Compounds V and VI were acetylated with acetic anhydride in pyridine (or in pyridine–DMF) and treated with 80% acetic acid to give either $N^6$,3′-*O*-diacetyl-A$^s$ (VIII) or $N^6$,2′-*O*-diacetyl-As(IX), respectively. These 5′-free nucleosides as well as 2′,3′-*O*-ethoxymethylidene-A[23] (X) were used as the 3′-end nucleoside components.

As 3′-end component mononucleotide units, *N*,3′-*O*-diacetyl-A$^s$ 5′-phosphate (XI), *N*,2′-*O*-diacetyl-As 5′-phosphate (XII), and *N*,2′,3′-*O*-triacetyl-A 5′-phosphate (XIIIa) were used. Compound XII was synthesized by acetylation of As 5′-phosphate which was obtained starting from 5′-*O*-monomethoxytrityl-As (VI) by benzoylation, detritylation, and phosphorylation with cyanoethyl phosphate.[24] As the 3′-phosphate, $N^6$,2′-*O*-diacetyl-sA 3′-phosphate (XIV) was synthesized from sAp[25] by acetylation using acetic anhydride and tetraethylammonium acetate in a yield of 57%. $N^6$,5′2′-*O*-Triacetyl-Ap (XV) was synthesized according to the method of Khorana et al.[26]

**Synthesis of Dinucleoside Monophosphates.** Dinucleoside monophosphates were synthesized according to reactions shown in Scheme I. 5′-Protected nucleosides V, VI, VII, and

Scheme I




III were condensed with 5′-phosphates having suitable protecting groups, XI, XII, or XIIa,b, using dicyclohexylcarbodiimide (DCC) as the condensing reagent. By these reactions 8,2′-*S*-cycloadenylyl-(3′-5′)-8,3′-*S*-cycloadenosine ($A^s$pAs, XVI), 8,2′-*S*-cycloadenylyl-(3′-5′)-adenosine ($A^s$pA, XVII), 8,3′-*S*-cycloadenylyl-(2′-5′)-8,2′-*S*-cycloadenosine (Asp′$A^s$ [20] XVIII), 8,3′-*S*-cycloadenylyl-(2′-5′)-8,3′-*S*-cycloadenosine (Asp′As, XIX), 8,3′-*S*-cycloadenylyl-(2′-5′)-adenosine (Asp′A, XX), and 8,5′-*S*-cycloadenylyl-(2′-5′)- and -(3′-5′)-8,3′-*S*-cycloadenosine (sApAs and sAp′As, XXIa and XXIb) were obtained in yields of 9–59% (see Chart I). Characterization of these compounds was performed by paper chromatography (PPC), paper electrophoresis (PEP) (Table I), and enzymatic digestion as described below. In the condensation reaction to yield XXIa,b, an unusually low yield due to decomposition of the product was found. As the by-product, 8,5′-*S*-cycloadenosine 2′,3′-cyclic phosphate and $N^6$,2′-*O*-diacetyl-As (VIII) were found in 30% yield. The decomposition was also observed on alkaline treatment of the compound XXI with concentrated ammonia at room temperature with $\tau_{1/2}$ equal to 4 h. There was no difference in the rate of decomposition between 2′-5′ and 3′-5′ isomers. This type of easy decomposition of a trinucleotide having 8,5′-*S*-cycloadenosine at the 5′ end, sApUpG, has been noted as previously reported.[25] Although the reason for this lability is not clearly understood as yet, an unusual conformation of compound XXI[27] may be the cause of this phenomenon. For this reason deprotection of the compound XXI should be conducted by using methanolic ammonia at 0 °C for 6 h.[28] From the 3′-phosphate, XIV and XV, and the 5′-OH free nucleoside, VIII-X, using DCC as the condensing reagent, we obtained 8,5′-*S*-cycloadenylyl-(3′-5′)-adenosine (sApA, XXII), adenylyl-(3′-5′)-8,2′-*S*-cycloadenosine (Ap$A^s$, XXIII), and adenylyl-(3′-5′)-8,3′-*S*-cycloadenosine (ApAs, XXIV) in yields of 36–63%. Characterization was also performed by PPC, PEP (see Table I), and enzymatic digestion.

**Table I.** Chromatographic Properties of Dinucleoside Monophosphates

| | Linkage | PPC ($R_f$) B[a] | PPC ($R_f$) C[a] | PPC ($R_f$) D[a] | PEP, $R_m$ (pA-A)[b] |
|---|---|---|---|---|---|
| $A^s$p$A^s$ | 3′-5′ | 0.24 | 0.23 | 0.26 | 0.30 |
| Asp′As | 2′-5′ | 0.14 | 0.28 | 0.17 | 0.32 |
| $A^s$pAs | 3′-5′ | 0.18 | 0.28 | 0.20 | 0.34 |
| Asp′$A^s$ | 2′-5′ | 0.18 | 0.30 | 0.20 | 0.25 |
| Ap$A^s$ | 3′-5′ | 0.12 | 0.27 | 0.19 | 0.14 |
| ApAs | 3′-5′ | 0.10 | 0.22 | 0.16 | 0.27 |
| $A^s$pA | 3′-5′ | 0.17 | 0.29 | 0.16 | 0.35 |
| Asp′A | 2′-5′ | 0.12 | 0.36 | 0.17 | 0.38 |
| sApAs | 3′-5′ | 0.10 | 0.13 | 0.21 | 0.15 |
| sAp′As | 2′-5′ | 0.16 | 0.19 | 0.21 | 0.30 |
| sApA | 3′-5′ | 0.12 | 0.26 | 0.14 | 0.21 |
| A | | 0.52 | 0.48 | 0.44 | 0.0 |
| pA | | 0.07 | 0.10 | 0.14 | 1.0 |

[a] B, EtOH-1 M $NH_4OAc$ (7:3); C, *i*-PrOH-$NH_4OH$-$H_2O$ (7:1:2); D, *n*-BuOH-AcOH-$H_2O$ (5:2:3). [b] 0.05 M triethylammonium bicarbonate buffer pH 7.5, 900 V/25 cm. $R_m$ (pA-A) stands for migration ration to pA (1.0) and adenosine (0.0).

Chart I

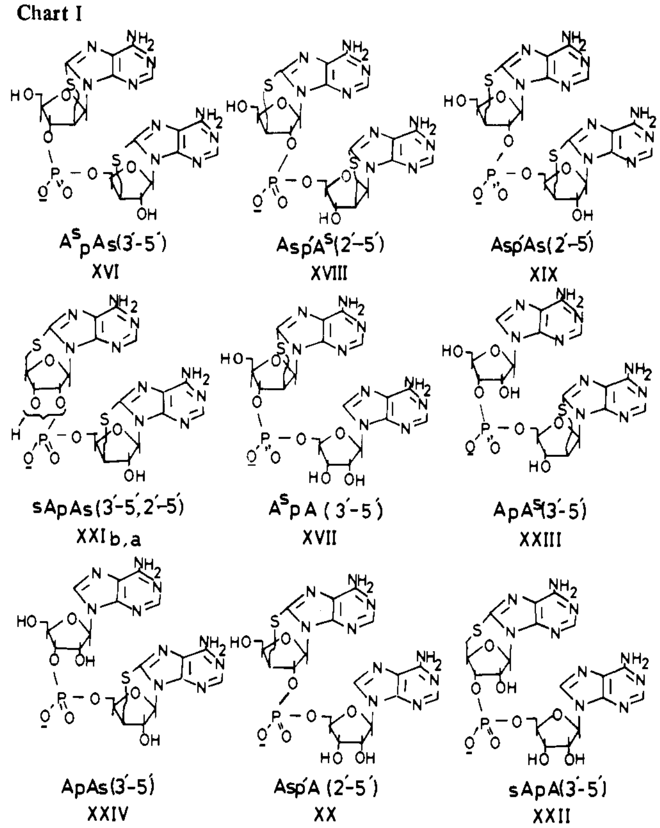


**Table II.** $\epsilon$(p) Values and Results of Hydrolysis of Dinucleoside Monophosphates[a]

| | Linkage | $\epsilon(p) \times 10^{-3}$ at $\lambda_{max}$ (nm) | Enzymatic hydrolysis: Venom phosphodiesterase[b] | Spleen phosphodiesterase[c] | RNase M[d] | Alkaline hydrolysis: 28% $NH_4OH$[e] | 0.3 N KOH[f] |
|---|---|---|---|---|---|---|---|
| $A^{s}pA^{s}$ | 3′-5′ | 32.6 (271.0) | − | − | − | − | − |
| Asp′As | 2′-5′ | 38.0 (281.0) | − | − | − | − | − |
| $A^{s}pAs$ | 3′-5′ | 38.6 (275.5) | − | − | − | − | − |
| $Asp'A^{s}$ | 2′-5′ | 33.8 (279.0) | − | − | − | − | − |
| $ApA^{s}$ | 3′-5′ | 29.8 (266.5) | − | − | + | − | + |
| ApAs | 3′-5′ | 26.2 (272.0) | − | − | + | − | + |
| $A^{s}pA$ | 3′-5′ | 21.2 (262.0) | + | − | − | − | − |
| Asp′A | 2′-5′ | 21.0 (266.5) | + | − | − | − | − |
| sApAs | 3′-5′ | 33.5 (281.5) | − | − | + | + | + |
| sApAs | 2′-5′ | 33.4 (282.0) | − | − | − | + | + |
| sApA | 3′-5′ | 21.2 (266.0) | + | − | + | + | + |
| $pA^{s}$ | | 20.3 (275.0) | | | | | |
| pAs | | 22.0 (282.5) | | | | | |
| sAp(3′) | | 17.0 (285.5) | | | | | |
| pA | | 15.4 (258.0) | | | | | |

[a] A plus in this table means that the dinucleoside monophosphate was hydrolyzed completely to its component monomers under the condition indicated. [b] Sample 5 OD enzyme, 20 $\mu$g; 1 M $(NH_4)_2CO_3$, 20 $\mu$L; $H_2O$, 80 $\mu$L. [c] Sample 5 OD enzyme, 0.4 unit; 1 M $NH_4OAc$, 20 $\mu$L; $H_2O$, 80 $\mu$L. [d] Sample 3 OD enzyme, 20 $\mu$g; 0.05 M $NH_4OAc$ (pH 5.0), 400 $\mu$L. For footnotes *a–d*, incubation at 37 °C, 3 h. [e] 37 °C, 24 h. [f] 37 °C, 18 h.

**Alkali and Enzymatic Hydrolysis of Dinucleoside Monophosphates.** Results of hydrolysis and $\epsilon$(p)[29] values are summarized in Table II. Each dimer has an $\epsilon$(p) value nearly twice as large as that of the component monomers, suggesting that these were in fact dimers with only two nucleoside units. As to the enzymatic digestion of dimers, the following points may be emphasized.

(1) Dimers having adenylic acid at the 3′ end, XVII, XX, and XXII, were completely hydrolyzed by snake venom phosphodiesterase to give a cyclonucleoside and 5′-AMP in each case.

(2) Dimers which contain 5′-end nucleotides having 2′-OH's, XXIb, XXII, XXIII, and XXIV, were completely hydrolyzed by RNase M to give a nucleoside 3′-phosphate and a nucleoside in each case. They were also hydrolyzed completely with 0.3 N KOH.

(3) Spleen phosphodiesterase did not catalyze the hydrolysis of any dimers.

(4) Dimers having a cyclonucleoside as the 3′-end unit could hardly be hydrolyzed by the snake venon phosphodiesterase. These features may be reasonably explained by difficulties in recognizing cyclonucleosides as suitable substrate for these enzymes except for RNase M.[30]

**Properties of Dinucleoside Monophosphates Containing Only Cyclonucleoside Residues.** Although large and clearly split CD bands were observed in $A^{s}pA^{s}$,[2] another dinucleoside monophosphate containing only cyclonucleoside residues showed CD spectra of small difference from the corresponding average of the component monomer spectra. In the case of $A^{s}pAs$ (XVI), in which 8,3′-*S*-cycloadenosine is the 5′-linked nucleoside, the CD spectrum at 60 °C is very similar to the average of the monomer spectra in shape but shows a small decrease of magnitude in both bands, a positive one at long wavelength and a negative one at short wavelength (Figure 1). At 0 °C, there is a further decrease of magnitude in both bands. In the case $Asp'A^{s}$ (XVIII), the sequence isomer of $A^{s}pAs$ with a 2′–5′ phosphodiester linkage, the CD spectrum is rather different from that of the average of the monomer spectra both in shape and magnitude (Figure 2). As in the case of $A^{s}pAs$, the intensity of the CD band changes in the negative direction around 280 nm and in the positive direction around 220 nm, in going from the average of the monomer spectra to the CD spectrum at 0 °C. From CD data, it seems that these two sequence isomers have some kind of base–base interactions and that Asp′As has stronger interactions. The UV absorption data support this conclusion. $Asp'A^{s}$ shows $\lambda_{max}$ at 279 nm ($\epsilon$ 16.9 $\times 10^3$) and calculated hypochromicity of 15% (Table III). $A^{s}pAs$ has $\lambda_{max}$ at 275.5 nm ($\epsilon$ 19.3 $\times 10^3$) and its hypochromicity is only 3.5%. In the case of Asp′As (XIX), a homodimer of 8,3′-*S*-cycloadenosine with a 2′-5′ phosphodiester linkage, at 25 °C it gives a CD spectrum very similar to that of the monomer (Figure 3). However at 0 °C it gives a clearly different CD spectrum possessing a negative band around 300 nm and a larger positive band around 270 nm. The difference spectrum between spectra at 0 and 25 °C shows a negative band around 300 nm and a positive band around 270 nm with a crossing point at 286 nm ($\lambda_{max}$) of the dimer (Figure 4). This type of conservative spectrum, having a pair of bands with opposite signs and equal magnitude, is assumed to arise from an exciton coupling of two stacked chromophores.[31,32] From the arrangement pattern of signs of the pair, a negative band in the long-wavelength region and a positive one in the short-wavelength region, it is suggested that a greater fraction of Asp′As molecules may take a left-handed stacked conformation at lower temperature. The UV absorption spectrum of

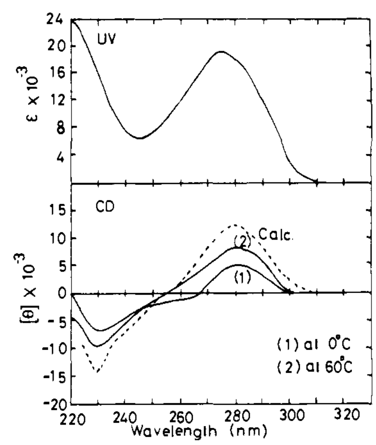



Figure 1. UV absorption and CD spectra of $A^{s}pAs$ (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and the average of the monomer spectra (- - -).

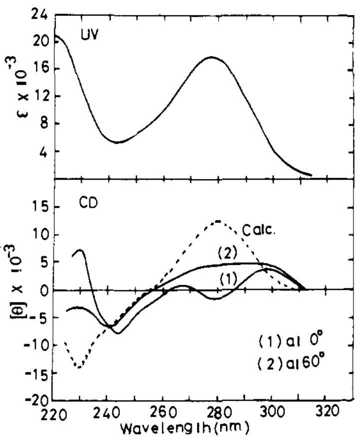



Figure 2. UV absorption and CD spectra of Asp′A$^s$ (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and the average of the monomer CD spectra (- - -).

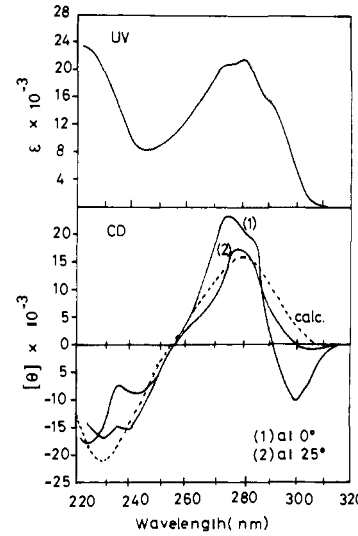



Figure 3. UV absorption and CD spectra of Asp′As (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and the monomer CD spectrum (- - -).

Table III. Hypochromicity[a] of the Dinucleoside Monophosphates

| Compound | Phosphodiester linkage | % hypochromicity |
|---|---|---|
| A$^s$pA$^s$ (Ia) | 3′-5′ | 15[b] |
| A$^s$pAs (XVI) | 3′-5′ | 3.5[b] |
| AspA$^s$ (XVIII) | 2′-5′ | 13[b] |
| AspAs (XIX) | 2′-5′ | 3[b] |
| sApAs (XXIa) | 2′-5′ | 12[c] |
| sApAs (XXIb) | 3′-5′ | 8.5[c] |
| A$^s$pA (XVII) | 3′-5′ | 8[d] |
| ApA$^s$ (XXIII) | 3′-5′ | 7[d] |
| AspA (XX) | 2′-5′ | 14[d] |
| ApAs (XXIV) | 3′-5′ | 6.5[d] |
| sApA (XXII) | 3′-5′ | 3[d] |

[a] Comparison of ultraviolet absorption was made at the $\lambda_{max}$ of the dimer and the mixture of its components. [b] Calculated from $\epsilon$ values obtained by phosphorus analysis. [c] Obtained from the results of alkaline hydrolysis. [d] Obtained from the results of enzymic hydrolysis.

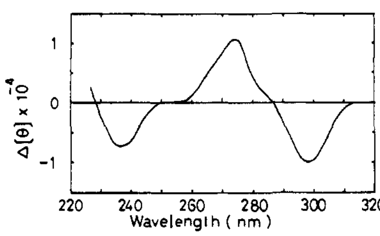



Figure 4. CD difference spectrum for Asp′As. $\Delta[\theta]$ represents $[\theta]$ at 0 °C − $[\theta]$ at 25 °C.

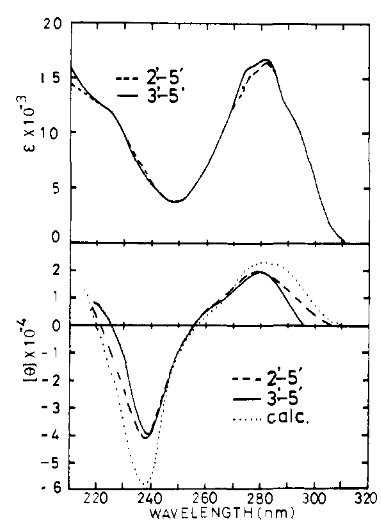



Figure 5. UV absorption and CD spectra of (2′-5′)-sAp′As (- - -) and (3′-5′)-sApAs (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) at 25 °C and the average of the monomer CD spectra (· · · ·).

Asp′As at room temperature is quite similar to that of the monomer with only 1.5 nm of hypsochromic shift of $\lambda_{max}$ and 3% of hypochromicity. These values are small as compared with those (5 nm and 15%) of A$^s$pA$^s$, the homodimer of 8,2′-*S*-cycloadenosine. So it may be concluded that Asp′As does not have a stacking conformation at room temperature but at low temperature it has a tendency to take a left-handed, stacked conformation. In the case of sApAs (XXIa,b), CD spectra of both isomers of different phosphodiester linkages are quite similar to the average of the monomer spectra and have little dependence on temperature (Figure 5). Upon alkaline hydrolysis, they give relatively large hyperchromism. Hypochromicity was 12% for sAp′As (2′-5′) and 8.5% for sApAs (3′-5′). So some kind of base-base interaction is expected for both isomers at room temperature and they are not sensitive to temperature. Of five dinucleoside monophosphates studied, only the homodimer, Asp′As, shows clearly a pair of CD bands, which are assumed to arise from exciton coupling, at low temperature. Other heterodimers fail to give clearly split bands but have some kind of base-base interaction. sApAs could be in a stacked conformation with parallel arrangement of transition moments of two bases resulting in the same CD spectrum as that of the average of the monomer spectra but with considerable hypochromicity.

**Properties of Dinucleoside Monophosphates Containing a Cyclonucleoside and an Adenosine Residue.** As described above, the homodimers A$^s$pA$^s$ and Asp′As seem to take a left-handed stacked conformation under certain conditions. As a next step, we were interested in hybrid dimers which contain both cyclonucleoside and adenosine residues. A$^s$pA (VXII) exhibits a CD spectrum entirely different from that of A$^s$pA$^s$ but quite similar to that of ApA (Figure 6). It is a conservative spectrum containing a positive band in the long-wavelength region and a negative one in the short-wavelength region with a crossing point at around $\lambda_{max}$ of the absorption band. This CD pattern is characteristic of right-handed stacking of chromophores. By raising the temperature, the magnitude of both bands de-

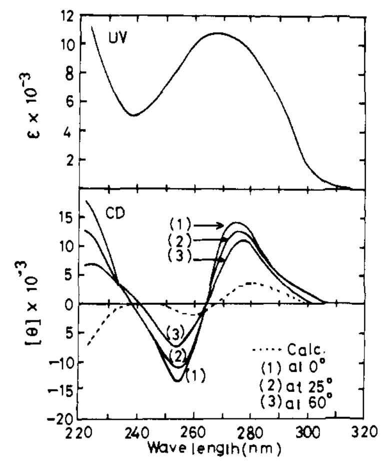



Figure 6. UV absorption and CD spectra of A$^{s}$pA (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and the average of the monomer CD spectra (- - -).

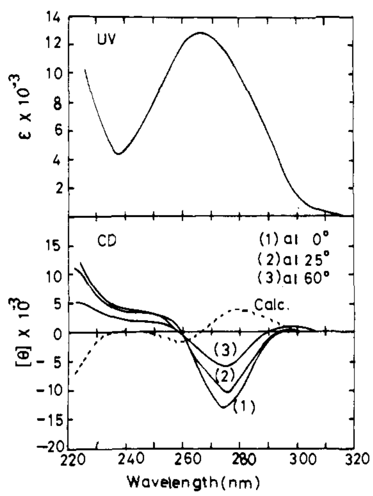



Figure 7. UV absorption and CD spectra of ApA$^{s}$ (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and the average of the monomer CD spectra (- - -).

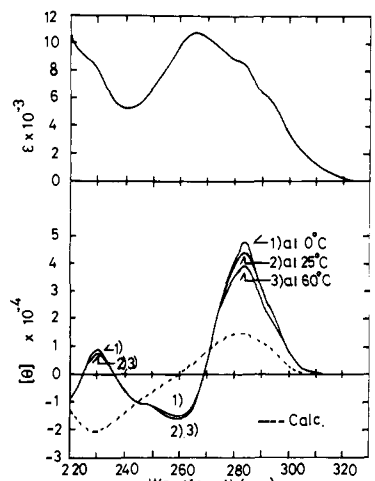



Figure 8. UV absorption and CD spectra of Asp′A (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and the average of the monomer CD spectra (- - -).

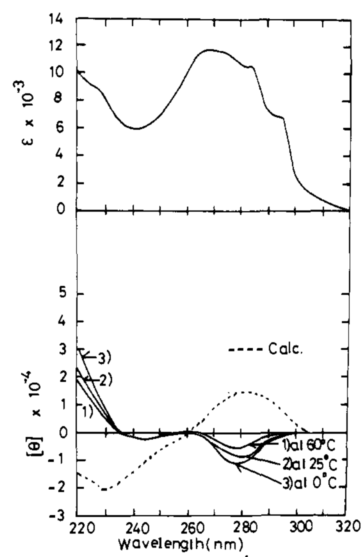



Figure 9. UV absorption and CD spectra of ApAs (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and the average of the monomer CD spectra (- - -).

creases simultaneously, suggesting that this pair of bands could arise from exciton coupling. In sharp contrast to A$^{s}$pA, the sequence isomer, ApA$^{s}$ (XXIII), exhibits a CD spectrum quite similar to that of A$^{s}$pA$^{s}$ (Figure 7). The CD spectrum at 25 °C shows a large negative band at around 275 nm and a small positive one at around 250 nm with a crossing point near $\lambda_{max}$ of the absorption band. The magnitudes of both bands decrease or increase simultaneously on changing the temperature. These results suggest again that this pair of adjacent bands could arise from exciton coupling though the splitting pattern of the bands is opposite to that of A$^{s}$pA. This reversal of splitting pattern could mean a direct reversal of handedness of the stacking conformation, if the difference in directions of transition moments between cycloadenosine and adenosine is small. This point will be discussed later. Hypochromicity obtained from enzymic hydrolysis experiments is 8% for A$^{s}$pA and 7% for ApA$^{s}$, suggesting considerable stacking interactions in the dimers.

Two sequence isomers of dimer which contain an 8,3′-*S*-cycloadenosine and an adenosine residue revealed very similar properties to those of the dimers containing an 8,2′-*S*-cycloadenosine and an adenosine residue. Asp′A (XX) exhibits the same pattern of CD spectrum as that of A$^{s}$pA (Figure 8) though it is less conservative. The difference spectrum against the average of the monomer spectra contains a positive band at around 285 nm and a negative band at around 260 nm with an intersection point near the wavelength of maximum absorption. The magnitude of both bands decreases simultaneously on increasing the temperature. The counterpart ApAs (XXIV) gives a CD spectrum quite similar to that of ApA$^{s}$, though the positive band at around 250 nm in the difference spectrum against the average monomer spectra is smaller than that of ApA$^{s}$ (Figure 9). The CD bands of ApAs show a dependency on temperature in the same way as ApA$^{s}$. Hypochromicity obtained from enzymic hydrolysis experiments is 14% for AspA and 6.5% for ApAs, suggesting relatively strong base-base interactions. A considerable difference is noted in the UV absorption spectra of the two sequence isomers. AspA has $\lambda_{max}$ at shorter wavelength, presumably showing that it has some hypsochromicity as well as hypochromicity. The same phenomena were also observed in the case of A$^{s}$pA and ApA$^{s}$. At this stage, we could conclude that the relative position of cycloadenosine in the heterodimer with adenosine plays an important role in determining the mode of base stacking and most probably the handedness. sApA (XXII) which contains 8,5′-*S*-cycloadenosine as the 3′-linked nucleoside residue gives a CD spectrum very similar to the average of the monomer spectra (Figure 10). The difference spectrum against the average of monomer spectra shows a pair of adjacent bands, a

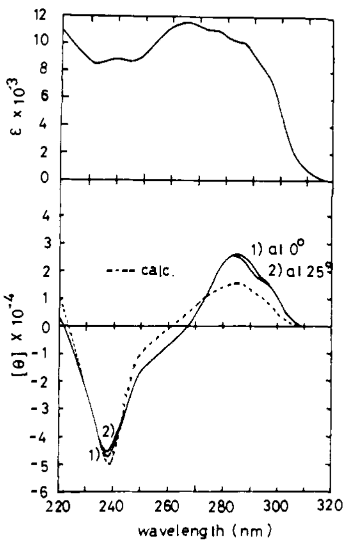



Figure 10. UV absorption and CD spectra of sApA (—) in 0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and the average of the monomer CD spectra (- - -)

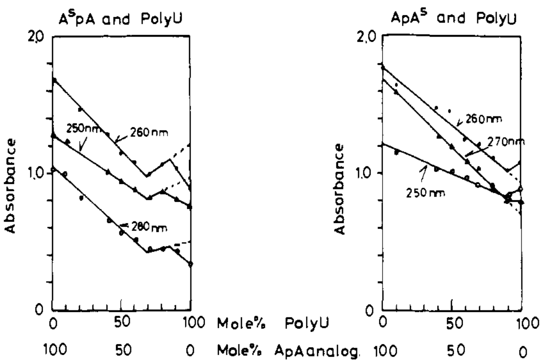



Figure 11. Mixing curves for A$^s$pA–poly(U) (on the left) and ApA$^s$–poly(U) (on the right) measured by UV absorption in 0.01 M $MgCl_2$, 0.01 M Tris-HCl buffer (pH 7.5) at 0 °C. The total nucleotide concentration was maintained at 0.15 mM.

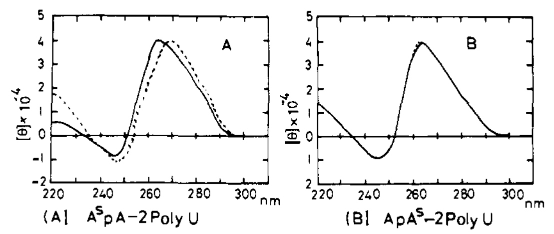



Figure 12. CD spectra of A$^s$pA–poly(U) (1:2) mixture (A) and ApA$^s$–poly(U) (1:2) mixture (B) in 0.01 M $MgCl_2$, 0.01 M Tris-HCl buffer (pH 7.5) at −5 °C: before mixing (- - -); after mixing (—).

positive band at long wavelength and a negative one at short wavelength with a crossing point near the $\lambda_{max}$ of the absorption band. Thus the circular dichroic properties of sApA are similar to those of Asp′A or A$^s$pA, in which cycloadenosine is the 3′-linked nucleoside component. Upon lowering the temperature, the magnitude of the positive band increases slightly. Only a small hypochromicity (3%) is observed for sApA, whereas A$^s$pA and Asp′A give significantly greater hypochromicities.

**Hybridization Experiments with Poly(U).** As discussed above, a right-handed stack was suggested for the heterodimers containing 3′-linked cycloadenosine residues and a left-handed stack was postulated for the heterodimers containing 5′-linked cycloadenosine residues from CD data. But the CD spectral pattern may not be correlated directly to handedness of stack in heterodimers because some difference in the direction of transition moments is expected between adenosine and cycloadenosine. To obtain additional support for our assignment, A$^s$pA and ApA$^s$ were tested for their ability to form a complex with poly(U). It is known that ApA[33] (2′-5′ and 3′-5′) and d-ApA[34] having a right-handed stack form a 1A–2U complex with poly(U) having also right-handed helical structure in 0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.5) at low temperature. Although L-ApA having a left-handed stack is reported to hybridize with poly(U), an inversion of handedness or stack must take place in that case.[33] A$^s$pA$^s$ [2] and A$^o$pA$^o$,[4] which were thought to have relatively rigid left-handed stacks, failed to hybridize with poly(U) under similar conditions. Mixing experiments of A$^s$pA and ApA$^s$ with poly(U) were carried out in 0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.5) at 0 °C. The results are shown in Figure 11. The mixing curves at three different wavelengths for A$^s$pA, which is assumed to have a right-handed stack, exhibit the existence of 1A–2U association [another discontinuity observed around 80–90 mol % poly(U) may be due to a self-complex of poly(U) as mentioned by Tazawa et al.[33]]. On the other hand, the mixing curves for ApA$^s$ at three different wavelengths show neither 1A–1U nor 1A–2U complex formation. The CD spectrum of the 1:2 mixture of A$^s$pA and poly(U) shows a considerable difference from that of the addition spectrum at −5 °C, confirming formation of a complex (Figure 12). Upon complex formation $[\theta]$ in the long-wavelength region (above 267 nm) changes to the negative direction, $[\theta]$ in the medium-wavelength region (240–267 nm) changes to the positive direction, and in the short-wavelength region $[\theta]$ changes again to the positive direction. This tendency of change is the same as in the case of the ApA–2-poly(U) complex.[33] On the other hand, the ApA$^s$–2-poly(U) mixture gives no difference in CD spectrum with respect to the addition spectrum, suggesting again no interaction under the same conditions. The A$^s$pA–2-poly(U) complex seems less stable than the ApA–2-poly(U) complex, judging from hypochromicity and differences in CD spectra. The results described above are consistent with assigned handedness of stack, right-handed stack for A$^s$pA, and left-handed stack for ApA$^s$, though we cannot exclude the possibility for conversion of handedness upon complex formation.

**Relation between CD Spectrum and Handedness of Stack.** We would like to discuss our explanation of the CD data. The magnitude and sign of a CD band of a dinucleoside monophosphate are dependent on the angle between the transition moments of two bases. In the very simplified model of a homodimer they are shown to be dependent on $\sin\gamma\cos\gamma$ where $\gamma$ stands for the angle between the two bases.[35] In this model the magnitude of a CD band becomes zero at 0 or ±90° and the sign of the CD band changes between these angles. A detailed calculation on the dependence of optical activity of dinucleoside monophosphates on $\gamma$ has been reported by Bush and Tinoco.[36] They discuss the relationship between optical activity and $\gamma$ not only in homodimers, such as ApA, but also in heterodimers, such as ApU and UpA. In the latter case, the angle between the two bases ($\gamma$) is different from the angle between the two transition moments ($\beta$) which actually determine the sign of a CD band. When we have a dinucleoside monophosphate, $B_1pB_2$, and the directions of the transition moments relative to a certain base coordinate system are $\beta_1$ and $\beta_2$ for each base, $\beta = \gamma + \beta_1 - \beta_2$.[36] If we have $ApB_2$ and choose $\beta_1$ for A as zero, $\beta = \gamma - \beta_2$. Similarly, for $B_1pA$ we can write $\beta = \gamma + \beta_1$. According to the discussion by Bush et al., in the case of ApU and UpA where $\beta_1 = \beta_2 = \beta_u$, the CD band in the longer wavelength region should be positive when $\gamma > \beta_u$ for ApU and when $\gamma > -\beta_u$ for UpA. The same argument could be applied to the case of ApA$^s$ and A$^s$pA because both A and A$^s$ have a relatively isolated absorption band around

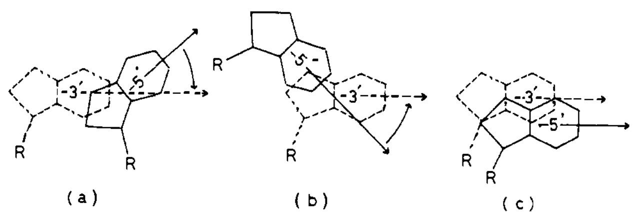



Figure 13. Schematic presentation of three types of base stacking: (a) a right-handed stack; (b) a left-handed stack; (c) nonhelical stack. The 3′-linked residue below the plane of the paper is illustrated in broken lines. The direction of base rotation which is directly related to the handedness of stack is illustrated using the geometrical principal axes of the bases.

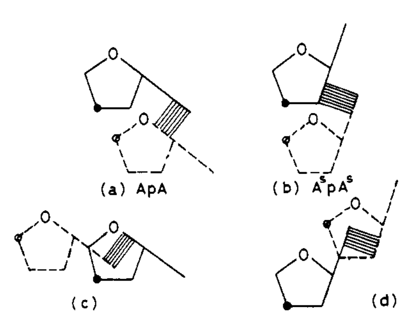



Figure 14. Schematic illustration of stacking conformations of ApA and $A^spA^s$ for examination of base–sugar and sugar–sugar interactions: (a) a right-handed stack for ApA; (b) a left-handed stack for $A^spA^s$; (c) a left-handed stack for ApA; (d) a right-handed stack for $A^spA^s$. The sugar residues is represented by a pentagon and the base residue is represented by a line through C-1′. The 5′-linked nucleoside residue is illustrated in broken lines. The 3′ (or 2′) position of the 3′- (or 2′-) linked residue is marked by a closed circle and the 4′ (and approximately 5′) position of the 5′-linked residue is marked by a open circle. The shaded area between the bases shows the site of base overlapping.

260–280 nm and may have a similar set of transitions around the same region as evidenced by their magnetic CD spectrum.[37] In that case, where $\beta_l = \beta_2 = \beta_s$, if $\beta_s > 0$, $ApA^s$ may give a negative band at longer wavelength when $\gamma < \beta_s$ and $A^spA$ may give a positive band in the same region when $\gamma > -\beta^s$. That means when the absolute value of $\gamma$ is smaller than $\beta_s$ the sign of the longer wavelength CD band does not reflect the handedness of stack, which is determined by the sign of $\gamma$. But in the most probable case, namely, when $\beta_s$ (difference in direction of transition moment relative to adenosine) is smaller than the absolute value of $\gamma$ which is thought to be around 30–40° for usual oligonucleotides and polynucleotides, the splitting pattern of CD bands could be correlated directly to the handedness of stack. When $\beta_s$ is negative, a negative longer wavelength band may be observed for $ApA^s$ where $\gamma < \beta_s < 0$ and a positive one for $A^spA$ where $0 < -\beta_s < \gamma$. So in this case the observed pattern of CD bands is directly related to the handedness of stack. The direction of the $\pi$–$\pi^*$ transition at around 275 nm of 9-methyladenine has been determined to be almost parallel to the short axis ($C_4$–$C_5$).[38] A negative $\beta_s$ means that the direction of the transition moment in $A^s$ is rotated counterclockwise from this direction. In conclusion, from CD data and hybridization experiments we might say that dimers containing 5′-linked adenosine and 3′-linked cycloadenosine have a tendency to take a right-handed stacked conformation and dimers containing 5′-linked cycloadenosine and 3′-linked adenosine tend to take a left-handed stacked conformation.

**Examination of Stacking Conformation with a Molecular Model.** From the results described above, the following conclusions may be made: (1) Asp′As, the homodimer of As ($\chi = 75°$), can take a left-handed stacked conformation at low temperature, which is much less stable than that of $A^spA^s$, the homodimer of $A^s$ ($\chi = 122°$); (2) Asp′$A^s$ and $A^s$pAs, the heterodimers containing $A^s$ and As, have a stacked conformation, probably of left-handedness, the stability of which is in-between that of $A^spA^s$ and Asp′As; (3) $A^spA$ and Asp′A, the dimers containing a 5′-linked adenosine residue ($\chi = 0°$), take a right-handed stacked conformation; (4) $ApA^s$ and ApAs, the dimers containing a 5′-linked cyclonucleoside residue, take a left-handed stacked conformation; (5) sApAs's and sApA, the dimers containing sA ($\chi = 42°$), have a stacked conformation of no helical turn or of little stability. Close examination of these molecules with CPK models gave support for these conclusions.

Before beginning a detailed analysis, some schematic visualization of concepts involved may be helpful for the discussion. In Figure 13, three types of base stacking in purine nucleoside dimers are illustrated. They are viewed from a direction perpendicular to the parallel base planes. Type (a) is a right-handed stack which is common for natural nucleic acids and ordinary oligo- and polynucleotides. Type (b) is a left-handed stack such as was assigned to $A^spA^s$. In the present discussion, the lower base is of the 3′-linked nucleoside and the upper base is of the 5′-linked nucleoside. For stabilization in water, the hydrophobic base chromophores tend to stack on each other. The extent of base overlapping is restricted by repulsions between base and sugar, between sugar and sugar, and sugar–phosphate backbone (length and torsion angles involved) if the torsion angles about glycosidic bonds are given. To avoid steric distortion, bases may be partially stacked, mainly with an overlap between the pyrimidine ring of one nucleoside and imidazole ring of the other nucleoside.[39] A right-handed stack of ApA and a left-handed stack of $A^spA^s$ are illustrated schematically from a different viewpoint, in Figure 14 (a,b) where a nucleoside residue is represented by a combination of a sugar plane and a base plane and base stacking is viewed from the direction parallel to the base planes and approximately perpendicular to the sugar planes. In this manner, the steric relation between base–base and base–sugar and the feasibility of phosphodiester bond formation between two sugar residues can be examined. For comparison, a left-handed stack of ApA with the same torsion angle ($\chi$) and a right-handed stack of $A^spA^s$ are also shown in Figure 14 (c,d). It is immediately obvious that a right-handed stack is the sterically preferable conformation for ApA and a left-handed stack is favorable for $A^spA^s$ with respect to their counterparts. Careful examination of these molecules with a CPK model also leads to this conclusion. In model building, reasonable ranges of torsion angles involved in the sugar–phosphate backbone ($\varphi'$, $\omega'$, $\omega$, $\varphi$, and $\psi$)[9] were always used. From x-ray analysis of oligo- and polynucleotides, prefered ranges of torsion angles are known.[9]

Now we would like to consider the conformations of the dinucleoside monophosphates containing cyclonucleosides one by one. For AspAs, a left-handed stack seems to be preferable to a right-handed stack as in the case of $A^spA^s$ [Figure 15 (a)]. But in this case, the two bases must stack over some part of the sugar residue of the 2′-linked nucleoside and the 2′-H becomes an obstruction for parallel stacking. Moreover, the 2′–5′ phosphodiester linkage in AspAs is unfavorable for a left-handed stack when compared with the 3′–5′ phosphodiester linkage in $A^spA^s$. In other words, an ideal, left-handed stack in Asp′As requires a greater distance between $O_{2'(3')}$ of the 2′- (or 3′-) linked nucleoside and the $O_{5'}$ (in gg conformation) of 5′-linked nucleoside than that in $A^spA^s$. These considerations can explain the observed results: the very unstable nature of the left-handed stack in Asp′As. For $A^s$pAs, a left-handed stack seems to be more favorable than the alternatives [Figure 15 (b)].

In the case of dinucleoside monophosphates containing an adenosine residue in addition to a cyclonucleoside, it may be reasonable to assume that the adenosine residue changes its torsion angle about the glycosidic bond to adopt a stable,

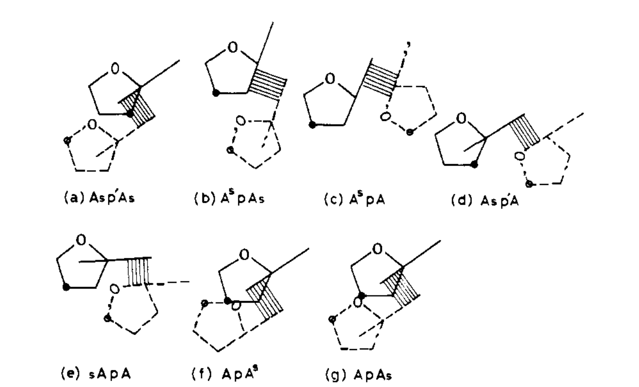



**Figure 15.** Schematic illustration of the preferable stacking conformations for various dinucleoside monophosphates. See the legend for Figure 14.

stacked conformation with the cyclonucleoside residue. Sundaralingam et al. have proposed the concept of a "rigid" 5′-nucleotide, suggesting that 5′-nucleotides are conformationally far more rigid than either nucleosides or 3′-nucleotides and that they occur exclusively in the gg conformation about the $C_{4'}$–$C_{5'}$ bond and in the anti glycosidic conformation.[40,41] This constraint in the conformation of 5′-nucleotides is assumed to be due predominantly to the coulombic interactions between the 5′-phosphate group with the base and ribose residues. So in the dinucleoside monophosphates containing one adenosine residue, changing the torsion angle of the 5′-linked adenosine may be more difficult than that of the 3′-linked adenosine. For A$^{s}$pA, a right-handed stack is preferable to a left-handed one when the $\chi$ value is maintained close to 0° [Figure 15 (c)]. But an ideal stacking requires a longer phosphodiester linkage and this implies less stable stacking of A$^{s}$pA with respect to that of ApA. For Asp′A, a right-handed stack seems to be difinitely preferable [Figure 15 (d)]. The (2′–5′)-phosphodiester linkage favors the right-handed stack and there is no need to change the glycosidic torsion angle of the adenosine residue. From CD data, the stacking conformation of Asp′A has been shown to be quite stable and model building gives support to this observation. For sApA, a right-handed stack also seems to be preferable to the alternative [Figure 15 (e)]. In the case of dinucleoside monophosphates containing 3′-linked adenosine, stacked conformations of either handedness seem to be unfavorable when the glycosidic torsion angle remains near 0°. If the adenine base of 3′-linked adenosine rotates to take a higher $\chi$ value, however, a left-handed stack becomes preferable [Figure 15 (f,g)]. In the schematic illustrations, $\chi$ of 3′-linked adenosine is assumed to be near 70°. X-Ray crystallographic results on phenylalanine tRNA show that glycosidic torsion angles of some nucleoside residues in a polynucleotide chain can be changed to very high values (up to 120°) to form the most stable conformation of the whole molecule.[42] Even a syn conformation ($\chi = -163°$) is assumed for an adenosine residue which is involved in a tertiary hydrogen bonding interaction.[42] These conformations of ApA$^{s}$ and ApAs are similar to those of Asp′A$^{s}$ and Asp′As in some respects. But the former contains the (3′–5′)-phosphodiester linkage instead of the (2′–5′) linkage of the latter and the (3′–5′) linkage is more favorable for a left-handed stack. This may explain the higher stability of stacking in ApA$^{s}$ and ApAs than that in Asp′A$^{s}$ and Asp′As. For sApAs's, no ordinary stacked conformation seems to be favorable.

**Factors Determining Handedness and Stability of Stacked Conformation in Dinucleoside Monophosphates.** The results and discussion described above taken together with previous knowledge allow us to make the following general conclusions. The handedness and stability of a stacking conformation in a dinucleoside monophosphate depend mainly on (1) the glycosidic angles ($\chi$) of the two nucleoside residue, (2) the positions of the two nucleoside residues [3′(2′)-linked or 5′-linked] with respect to the phosphate-linkage, and (3) the type of phosphodiester linkage (3′–5′ or 2′–5′). In a series of homodimers, ApA, Asp′As, and A$^{s}$pA$^{s}$, and in a series of dimers with 3′–5′ phosphodiester linkage, ApA, sApAs, A$^{s}$pAs, and A$^{s}$pA$^{s}$ or ApA, sApA, and A$^{s}$pA, the glycosidic torsion angles ($\chi$) of the two nucleoside components or one nucleoside component increase from ca. 0 to 120° and the stacked conformations of the dimers at both ends of the series (the first one and the last one) are much more stable than those of the middle ones. In other words, the existence of nucleoside(s) with medium $\chi$ value(s) (40–80°) in a dinucleoside monophosphate produces some difficulty in stacking of the bases. In stacked oligo- and polynucleotides with ribo sugar and natural bases, the $\chi$ value of purine nucleoside residue is always very small (2–22°) with a positive sign.[43-47] For double helical RNA's, similar values of $\chi$ (9–14°) are reported.[48] On the other hand, the adenosine residue in unstacked UpA has a high value (37 or 44°) of $\chi$.[49,50] Further evidence can be collected from recent results from yeast phenylalanine tRNA.[42] Most of the stacked nucleoside residues in this molecule possess $\chi$ values in a narrow range (−10 to 30°) near 0° and most of nucleoside residues which do not stack with the nearest neighbor residues have higher $\chi$ values (30–120°). As seen from the discussion described above, small $\chi$ values, where the base plane is over one side ($O_{1'}$–$C_{1'}$) of the ribose ring, are required for a stable right-handed stacking. Another stable stacking with opposite handedness can occur at $\chi$ values near 120°, where the base plane is over the other side ($C_{2'}$–$C_{1'}$) of the sugar ring.

A group of dimers, sApA, Asp′A, and A$^{s}$pA, which have 5′-linked adenosine seem to take a right-handed stack, and ApAs and ApA$^{s}$ which have 3′-linked adenosine seem to take a left-handed stack. This phenomena can be explained partly by the concept of rigidity in 5′-nucleotides.[40,41] What kind of differences are there between 5′-linked nucleoside and 3′-linked nucleoside in addition to this? In ordinary stacking conformations of dinucleoside monophosphates, the bases and sugars are aligned pointing at approximately the same direction and a face of one base overlaps with a different face of the other base. When we take a 8,5′-*S*-cycloadenosine molecule, for instance, we can call the face in the $O_{1'}$ side of sugar A face and the face in the $C_{2'}$ side of the sugar B face. Thus, in a dinucleoside monophosphate, the B face of the 3′-linked nucleoside contacts with the A face of the 5′-linked nucleoside base. More precisely speaking, base–base overlap is mainly between the pyrimidine (Py) ring of one base and the imidazole (Im) ring of the other base. This is actually the case in purine–purine stacking in double helical RNA's.[45-48] A typical right-handed stack, as assumed in ApA,[39] contains B(Py)–A(Im) contact and a typical left-handed stacking, as assumed in A$^{s}$pA$^{s}$, contains a B(Im)–A(Py) contact. So it may be considered that the pyrimidine ring, B(Py), of a 3′-linked nucleoside is involved in overlapping for a right-handed stack and the imidazole ring, B(Im), of same one is involved for a left-handed stack. Similarly, the imidazole ring, A(Im), of a 5′-linked nucleoside is involved for a right-handed stack and the pyrimidine ring, A(Py), of the same one is involved for a left-handed stack. The glycosidic torsion angle of each nucleoside residue affects the feasibility of each part's being involved in stacking. Figure 16 is included to explain this argument. In 3′-linked adenosine, the B(Py) part is far more accessible for stacking than the B(Im) part, because the B(Py) part has a space in the B side whereas the B(Im) part is deep in the back of the sugar residue with respect to approach of the base of the 5′-linked nucleoside. In 5′-linked adenosine, both A(Py) and A(Im) parts have free space on the A side of the plane. A combination of 3′-linked adenosine and 5′-linked adenosine, therefore, should result in B(Py)–A(Im) contact to give a right-handed stack when the phosphodiester backbone can allow it. On the other hand, in the case of 8,2′-*S*-cycloadenosine ($A^s$), B(Py) and B(Im) parts in the 3′-linked residue are accessible for stacking and only the A(Py) part may be accessible in the 5′-linked nucleoside. Under these restrictions, B(Im)–A(Py) contact to form a left-handed stack may be much more preferable than the alternative. In the case of 8,3′-*S*-cycloadenosine (As), the B(Py) part is more accessible than the B(Im) part in the 3′-linked nucleoside and the A(Py) part is much more accessible than A(Im) in the 5′-linked nucleoside. In As′pAs B(Im)–A(Py) contact seems to have a small advantage over B(Py)–A(Im) contact so that a weak, left-handed stack can be predicted. In the case of 8,5′-*S*-cycloadenosine (sA), which can be only the 3′(2′)-linked nucleoside, the B(Py) part is much more accessible than the B(Im) part. Similar consideration of the dimers containing cycloadenosine and adenosine residues and on the remaining dimers, $A^s$pAs and Asp′$A^s$, predict that the assumed stacking conformation for each dimer is the preferable one. As to the third factor, the type of phosphodiester linkage (3′–5′ or 2′–5′), changing the linkage from (3′–5′) to (2′–5′) may have two steric effects. One is displacement of the 5′-linked nucleoside reside to the $C_{1'}$–$C_{2'}$ side of the 3′-linked sugar relative to the $C_{4'}$–$C_{3'}$ side to favor stacking interaction between the pyrimidine ring of the 3′-linked nucleoside and the imidazole ring of the 5′-linked nucleoside. Another is release of repulsion between sugar groups by increasing the distance between them and placing in a staggered position. More stable, right-handed stacking of Asp′A than that of $A^s$pA may be explained by these effects. The disadvantage of a (2′–5′) linkage for left-handed stacking in Asp′As and Asp′$A^s$ can be also explained.

The left-handed stacking conformation postulated by us does not require an unusual backbone conformation by any means. Examination with a CPK model shows that $g^-$–t or $g^-$–$g^-$ (combination of torsion angles about P–O bonds, $\omega'$ and $\omega$) conformation can afford a left-handed stack. According to the results of calculations by Sasisekharan on possible combinations of torsion angles (sugar puckering, $\psi$, $\omega'$, and $\omega$) for double helical polynucleotides, a combination of $C_{3'}$-endo, gg, and $g^-$–$g^-$ and a combination of $C_{3'}$-endo, gg, and $g^-$–t can make up both right-handed and left-handed helices.[51] Similar results have been reported by Yathindra et al.[52] Quite recently, it was confirmed by energy calculations that a left-handed stack with $g^-$–$g^-$ or $g^-$–t conformations of the phosphodiester backbone is the most favorable one for $A^s$p$A^s$.[53]

## Experimental Section

**General Procedures.** UV absorption spectra were recorded on a Hitachi EPS-3T recording spectrophotometer. In mixing experiments, measurements were made by a Hitachi 124 spectrophotometer equipped with a Komatsu Solidate SPD-H-124 thermostated cell. CD spectra were recorded on a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment and a thermojacketed cell. The instrument was calibrated with an aqueous solution of *d*-10-camphorsulfonic acid. The molar ellipticity, [$\theta$], and molar extinction coefficient, $\epsilon$, are presented in terms of per residue value if not cited. Paper chromatography was performed on Toyo filter paper No. 51A using the following solvent systems: solvent A, saturated ammonium sulfate–water–2-propanol (79:12:2); solvent B, ethanol–1 M ammonium acetate (7:3); solvent C, 2-propanol–concentrated ammonium hydroxide–water (7:1:2); solvent D, 1-butanol–acetic acid–water (5:2:3). Paper electrophoresis was performed for 1 h with a voltage gradient of 35 V/cm on Toyo filter paper No. 51A using 0.05 M triethylammonium bicarbonate buffer (pH 7.5). Thin-layer chromatography was performed on silica gel plates with Merck Kieselgel $HF_{254}$ using mixed solvent systems of chloroform and ethanol.

**5′-*O*-Monomethoxytrityl-8,2′-*S*-cycloadenosine (V).** Well-dried 8,2′-*S*-cycloadenosine (434.5 mg, 1.54 mmol) was dissolved in DMF (3.5 mL) and monomethoxytrityl chloride (370 mg, 1.2 equiv) was added. The mixture was stirred for 2 days at room temperature in a dark place. The reaction mixture was added dropwise to saturated aqueous $NaHCO_3$ (150 mL) and precipitates were collected by filtration. After washing with *n*-hexane, the precipitates were recrystallized from $H_2O$–MeOH: mp 141 °C; yield 361 mg (0.65 mmol, 42%); UV $\lambda_{max}^{EtOH}$ 277 nm; UV $\lambda_{max}^{H^+}$ 279 nm; UV $\lambda_{max}^{OH^-}$ 277.5 nm. This compound gave a single spot on TLC [$CHCl_3$–EtOH (10:1), $R_f$ 0.41], which developed a yellow color on spraying with 30% sulfuric acid and heating. Anal. Calcd for $C_{30}H_{27}O_4N_5S$: C, 65.08; H, 4.92; N, 12.65; S, 5.79. Found: C, 65.15; H, 5.01; N, 12.63; S, 5.72.

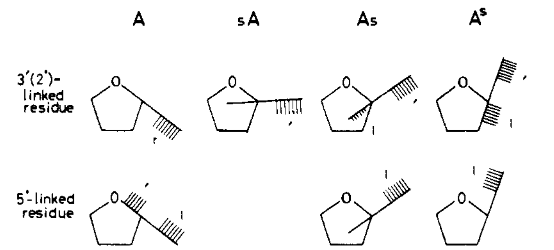



Figure 16. Schematic illustration of the accessible areas for stacking interactions in adenosine and cycloadenosines. The shading represents the accessible areas of base and the letters, r and l, show the corresponding handedness of stack.

**5′-*O*-Monomethoxytrityl-8,3′-*S*-cycloadenosine (VI).** (i) Well-dried 8,3′-*S*-cycloadenosine (501 mg, 1.8 mmol) was dissolved in DMF (6.5 mL) and monomethoxytrityl chloride (680 mg, 2.66 mmol) was added. The mixture was kept at room temperature for 3 days in a dark place. The reaction mixture was added dropwise to saturated aqueous $NaHCO_3$ (200 mL) and precipitates were collected by filtration. After thorough washing with $H_2O$, the precipitates were dried and dissolved in pyridine (5 mL). Addition of this solution to ether (200 mL) gave precipitates, which were recrystallized from EtOH: yield 490.8 mg (0.87 mmol, 48.2%); UV $\lambda_{max}^{EtOH}$ 278, 295 nm (sh); TLC ($CHCl_3$–EtOH, 20:1) $R_f$ 0.24 (8,3′-*S*-cyclonucleoside, $R_f$ 0.04). Anal. Calcd for $C_{30}H_{27}O_4N_5S$: C, 65.05; H, 4.91; N, 12.65; S, 5.80. Found: C, 64.46; H, 4.72; N, 12.39; S, 5.94.

(ii) 8-Bromo-3′-triisopropylbenzenesulfonyladenosine (3.1 g, ~5 mmol) was dissolved in DMF (50 mL) and monomethoxytrityl chloride (2.5 g, 1.2 equiv) was added. The mixture was kept in a dark place for 2 days. The reaction mixture was added dropwise to $H_2O$ (500 mL) containing 2% concentrated $NH_4OH$. Precipitates were collected by filtration: UV $\lambda_{max}^{50\% EtOH}$ 231, 265 nm; UV $\lambda_{max}^{H^+}$ 230, 263 nm; UV $\lambda_{max}^{OH^-}$ 230, 265 nm; TLC ($CHCl_3$–EtOH, 10:1) $R_f$ 0.39 (the starting material, $R_f$ 0.26), which was revealed by yellow color by straying with 30% $H_2SO_4$ and heating. The precipitates were dissolved in DMF (150 mL), bubbled through with $N_2$ gas, and freshly prepared 50% aqueous NaSH (18 mmol) was added. The flask was tightly stoppered and heated at 70 °C for 24 h. The reaction mixture was neutralized with 1 N HCl added dropwise to $H_2O$ (1.5 L), and precipitates were collected by filtration. Recrystallization of this material gave crystals in a yield of 1.16 g (2.1 mmol, 42.0%). This specimen was identical with that obtained in (i).

**$N^6$,3′-*O*-Diacetyl-8,2′-*S*-cycloadenosine (VIII).** 5′-*O*-Monomethoxytrityl-8,2′-*S*-cycloadenosine (170.5 mg, 0.31 mmol) was dissolved in pyridine–$Ac_2O$ (2:1 v/v, 5 mL) and kept at 35 °C for 12 h. The solvent was evaporated in vacuo and the residue was treated with 80% AcOH (3 mL) at room temperature for 3 h. Acetic acid was evaporated; the residue was dissolved in pyridine (2 mL) and poured into ether (100 mL). Precipitates were collected by filtration, washed with $Et_2O$, and dried over $P_2O_5$ in vacuo: yield 25.3 mg (~22%); UV $\lambda_{max}^{50\% EtOH}$ 231.5, 294 nm; UV $\lambda_{max}^{H^+}$ 303, 315 nm (sh); UV $\lambda_{max}^{OH^-}$ 295 nm. Anal. Calcd for $C_{14}H_{15}O_5N_5S$: C, 46.02; H, 4.14; n, 19.17; S, 8.78. Found: C, 45.88; H, 4.56; N, 19.03; S, 8.54. This compound gave a single spot on TLC [$CHCl_3$–EtOH (10:1), $R_f$ 0.28] which gave a negative result in the color test for the trityl group. It was used for the condensation reaction without further purification.

**$N^6$,2′-*O*-Diacetyl-8,3′-*S*-cycloadenosine (IX).** 5′-*O*-Monomethoxytrityl-8,3′-*S*-cycloadenosine (270.8 mg, 0.49 mmol) was dissolved in a mixture of DMF–pyridine–$Ac_2O$ (1:2.5:2 v/v, 5.5 mL) and stirred for 12 h at room temperature. The solvent was evaporated and the residue was treated with 80% AcOH (4 mL) at room temperature for 3 h. The work-up as in the case of VIII gave 114.8 mg (~67%) of diacetyl-8,3′-*S*-cycloadenosine: UV $\lambda_{max}^{H^+}$ 304 nm; UV $\lambda_{max}^{H_2O}$ 294, 301 nm; UV $\lambda_{max}^{OH^-}$ 298 nm. Anal. Calcd for $C_{14}H_{15}O_5N_5S \cdot H_2O$: C, 45.05; H, 4.43; N, 18.26; S, 8.35. Found: C, 45.32; H, 4.31; N, 17.87; S, 8.16. This compound gave a single spot on TLC [$CHCl_3$–EtOH (10:1), $R_f$ 0.29] which gave a negative result

**Table IV.** Synthesis of Dinucleoside Monophosphates

| Product | Starting material (mg, mmol) | Solvent (mL) | Reagent (mg, mmol) | Addition (mg) | Time, h | Column chromatography (elution buffer) | Yield, % | Remarks |
|---|---|---|---|---|---|---|---|---|
| $A^spA^s$ (XVI) | MMTr-$A^s$ (65, 0.12) $N$,2′-$AC_2$-$pA_s$ (60.8, 0.1) | DMF (1), Py[b] (0.5) | DCC (126, 0.6) | Dowex 50-X8 (500) | 48 | Dowex 1-X8 (0–0.15 M HCOOH) | 960 $A_{275.5}$ (50) | |
| $A^spA$ (XVII) | Tr-$A^s$(DMM)[a] (29, 0.05) $N$,2′,3′-$Bz_3$-pA (37 + 38) | Py (2) | DCC (100 + 200) | Dowex 50W-X8 (50) | 120 + 72 | Dowex 1-X8 (0.03 N HCOOH), DEAE-cellulose (0.05 M TEAB)[c] | 323 $A_{260}$ (30) | |
| Asp′$A^s$ (XVIII) | $A_S$ (75.5, 0.136) $N$,2′-$Ac_2$-$pA^s$ (75, 0.136) | DMF (0.5), Py (0.2) | DCC (168.8, 0.78) | | 48 | Dowex 1-X2 (0–0.15 M HCOOH) | 2600 $A_{280}$ (57) | |
| Asp′As (XIX) | MMTr-As (65, 0.12) $N$,2′-$Ac_2$-pAs (60.8, 0.1) | DMF (1), Py (0.5) | DCC (126, 0.6) | Dowex 50W-X8 (500) | 48 | Dowex 1-X8 (0–0.15 M HCOOH) | 610 $A_{280}$ (32) | |
| Asp′A (XX) | MMTr-As (88.9, 0.16), $N$,2′,3′-$Ac_3$-pA (55, 0.1) | DMF (0.2), Py (0.6) | DCC (126, (126, 0.6) | Dowex 50W-X8 (500) | 22.5 | Dowex 1-X8 (0–0.15 M HCOOH) | 621 $A_{264}$ (59) | |
| sApAs (XXIa,b) | $^s$A (37.5, 0.12) $N$,2′-$Ac_2$-pAs (58, 0.11) | DMF (0.5), Py (0.5) | DCC (155, 9.75) 0.75) | Dowex 50W-X8 (500) | 27 | DEAE-Sephadex A-25 (0.002–0.15 M TEAB) | XXIa 136 $A_{280}$ (6.5), XXIb 45 $A_{280}$ (2.1) | e |
| sApA (XXII) | $N$,2′-$Ac_2$-sAp (58.2, 0.11) A (EM)[d] (117.3, 0.36) | Py (0.9) | DCC (125, 0.6) | | 20 | DEAE-Sephadex A-25 (0.002–0.12 M TEAB) | 408 $A_{268}$ (35.5) | f |
| $ApA^s$ (XXIII) | $N$,3′-$Ac_2A^s$ (25.3, 0.07) $N$,2′,5′-$Ac_3$-Ap (55, 0.1) | Py (0.8) | DCC (79.8, 0.38) | | 48 | DEAE-Sephadex A-25 (0.02–0.1 M TEAB) | 559.5 $A_{268}$ (62.8) | |
| ApAs (XXIV) | $N$,2′-$Ac_2$As (74.4, 0.2) $N$,2′,5′-$Ac_3$-Ap (55, 0.1) | Py (0.9) | DCC (148, 0.71) | | 72 | DEAE-Sephadex A-25 (0.02–0.15 M TEAB) | 613.7 $A_{272}$ (55.0) | g |

[a] DMM stands for dimethylaminomethylene. [b] Py stands for pyridine. [c] TEAB stands for triethylammonium bicarbonate buffer (pH 7.5). [d] EM stands for ethoxymethylidene. [e] sA cyclic 2′,3′-phosphates (600 $A_{280}$) were obtained. [f] sA cyclic 2′,3′-phosphates (60%) were obtained. [g] The final product was purified by PPC.

in the color test for the trityl group. It was used for the condensation reaction without further purification.

**8,3′-*S*-Cycloadenosine 5′-Phosphate.** 5′-*O*-Monomethoxytrityl-8,3′-*S*-cycloadenosine (145 mg, 0.24 mmol) was dissolved in pyridine (3 mL) and benzoyl chloride (0.53 mL) was added. The mixture was kept at room temperature for 2 h and added dropwise to a saturated aqueous $NaHCO_3$ (50 mL) solution. The products were extracted with $CHCl_3$ and washed with water, and $CHCl_3$ was evaporated. To the residue AcOH (15 mL) and $H_2O$ (1.7 mL) were added and the solution was kept at room temperature for 12 h. The solvent was evaporated and traces of AcOH were azeotropically removed. The residue was recrystallized from EtOH. $N$,2′-*O*-Dibenzoyl-8,3′-*S*-cycloadenosine was obtained in a yield of 89 mg (75%); UV $\lambda_{max}^{pH2}$ 237, 302 nm; UV $\lambda_{max}^{pH7}$ 236.5, 301.5 nm; UV $\lambda_{max}^{pH10}$ 314 nm; $A_{236.5}^{pH7}/A_{301.5}^{pH7} = 1.75$; TLC ($CHCl_3$-EtOH, 19:1) $R_f$ 0.41 (As, 0.01). This compound gave a negative result in the color test for the trityl group.

This material (213 mg, 0.44 mmol) and cyanoethyl phosphate pyridinium salt (2 mmol) were rendered anhydrous by evaporation several times with pyridine. The residue was dissolved in pyridine (5 mL) and DCC (618 mg, 2.5 mmol) was added. The mixture was kept at room temperature for 2 days; 50% pyridine (5 mL) was added and the solution was kept at room temperature for 3 h. Dicyclohexylurea was removed by filtration and unreacted DCC was extracted with $n$-pentane. The aqueous solution was evaporated and the residue was made anhydrous by evaporation with pyridine. The residue was dissolved in methanolic ammonia (saturated at 0 °C, 20 mL) and heated at 40 °C for 3 days. The solvent was evaporated and the residue was dissolved in pyridine and precipitated in ether. Powdery precipitates were dissolved in $H_2O$, adjusted to pH 3, and applied to a column (2 × 4 cm) of charcoal. After thorough washing with $H_2O$, the product was eluted with 50% EtOH containing 2% concentrated $NH_4OH$. The eluents (450 mL) were evaporated in vacuo to give 8,3′-*S*-cycloadenosine 5′-phosphate[54] in a yield of 6450 $OD_{280}$ (0.3 mmol, 68%). The $\epsilon$(p) value is presented in Table II.

**$N^6$,2′-*O*-Diacetyl-8,3′-*S*-cycloadenosine 5′-Phosphate (XII).** 8,3′-*S*-Cycloadenosine 5′-phosphate pyridinium salt (137 mg, 0.3 mmol) was rendered anhydrous by evaporation with added pyridine and the residue was dissolved in pyridine (10 mL) and $Ac_2O$ (5 mL). The mixture was heated at 45 °C for 24 h. The solvent was evaporated and the residue was treated with 50% pyridine (5 mL) at room temperature for 6 h. The solvent was removed by evaporation and the residue was made anhydrous by evaporation several times with added pyridine. The residue was dissolved in pyridine (10 mL) and added dropwise to ether–pentane (3:2 v/v, 200 mL): yield 142 mg (~90%); UV $\lambda_{max}^{H^+}$ 303 nm; UV $\lambda_{max}^{H_2O}$ 231.5, 293.5, 300.5 nm; UV $\lambda_{max}^{OH^-}$ 298 nm; PPC $R_f$ (B) 0.45 (pAs 0.17). Anal. Calcd. for $C_{24}H_{19}O_5N_5S$: C, 58.88; H, 3.91; N, 14.31; S, 6.55. Found: C, 59.03; H, 3.91; N, 14.31; S, 6.62.

**8,5′-*S*-Cycloadenosine 3′-Phosphate.** 8,5′-*S*-Cycloadenosine (567.3 mg, 2 mmol) and cyanoethyl phosphate pyridinium salt (4 mmol) were dissolved in anhydrous DMF (20 mL) and pyridine (10 mL). DCC (2.476 g, 12 mmol) was added to the solution and it was kept at room temperature for 3 days. $H_2O$ (30 mL) was added and after 1 h at room temperature dicyclohexylurea was filtered off. The filtrate was extracted with $n$-hexane and the water layer was evaporated. The residue was dissolved in 0.5 M LiOH and heated at 100 °C for 15 min. Precipitates were filtered off and the filtrate was neutralized with Dowex 50 ($H^+$ form). The solution was passed through a column (1 × 30 cm) of Dowex 50 ($H^+$ form) and eluents were evaporated to a volume of

45 mL. Neutralization with $Ba(OH)_2$ to pH 7.5 gave a powder of the 3′-phosphate Ba salt (940.2 mg). The powder was dissolved in $H_2O$ (10 mL) by shaking with Dowex 50 ($H^+$ form) resin and applied to a column of Dowex 50 ($H^+$ form). Elution with $H_2O$ gave eluents, which were made weakly alkaline with concentrated $NH_4OH$. After concentration to ~20 mL, the solution was applied to a column (1.4 × 21 cm) of Dowex 1-X2 (formate form). Elution was carried out using a linear gradient of formic acid (0.05–0.2 M, total 2 L). Fractions of 14 mL each were collected at 28-min intervals. Fractions containing pure 3′-phosphate (fraction no. 190–230), which appeared after a peak of the 2′-phosphate (fraction no. 140–175), were collected and desalted through a charcoal column. After washing with $H_2O$, the column was eluted with 50% EtOH containing 2% concentrated $NH_4OH$: yield 5360 $OD_{282}$ (0.3 mmol, 15%); UV: $\lambda_{max}{}^{H^+}$ 237 (sh), 275 (sh), 283, 294 nm (sh); UV $\lambda_{max}{}^{H_2O,OH^-}$ 236.5 (sh), 276 (sh), 285, 295 nm (sh); PPC $R_f$ (A) 0.16, $R_f$ (B) 0.12, $R_f$ (D) 0.18; PEP $R_{pA-A}$ 0.98. This compound was identical with that prepared previously by a different route. The $\epsilon$(p) value is presented in Table II.

**$N^6$,2′-*O*-Diacetyl-8,5′-*S*-cycloadenosine 3′-Phosphate** (XIV). 8,5′-*S*-Cycloadenosine 3′-phosphate (0.3 mmol) and tetraethylammonium acetate buffer (1.2 mmol) were rendered anhydrous by evaporation several times with added pyridine. After evaporation with added toluene, the residue was dissolved in $Ac_2O$ (0.3 mL) and kept at room temperature in a dark place for 4 days. Pyridine–MeOH (4:1 v/v, 5 mL) was added to the solution and it was kept at room temperature for 12 h. The solution was passed through a column (1 × 25 cm) of Dowex 50W-X8 (pyridinium form) and eluents were evaporated in vacuo. The residue was dissolved in pyridine and added dropwise to $Et_2O$ (20 vol). Precipitates were collected by centrifugation: yield 92.3 mg (~57%); UV $\lambda_{max}{}^{H^+}$ 303.5 nm; UV $\lambda_{max}{}^{H_2O}$ 244 (sh), 284, 303 nm (sh); PEP $R_{pA-A}$ 0.93; PPC $R_f$ (B) 0.43 (sAp 0.16, sA 0.39).

**General Procedure for the Synthesis of Dinucleoside Monophosphates.** Starting materials (amount as listed in Table IV) were dissolved in pyridine (1–2 mL) (if the material was insoluble in pyridine, DMF was added) and evaporated in vacuo. This process was repeated at least three times. The residue was dissolved in anhydrous pyridine and DMF (amount as in Table IV) and DCC was added. The additions were done in a drybox. The tightly stoppered flask containing the reaction mixture was kept at room temperature in a dark place. After times as listed in Table IV, the reaction was stopped by addition of 50% pyridine (~5–10 mL) and the solution was kept at room temperature for 12 h. Dicyclohexylurea was removed by filtration and the filtrate was washed with *n*-pentane to remove unreacted DCC. The $H_2O$ solution was evaporated in vacuo. Removal of protecting groups was performed with 80% acetic acid (for trityl and monomethoxytrityl group) and methanolic ammonia (for acetyl group). For the removal of the dimethylaminomethylene, methanolic ammonia for 2 days at room temperature was used. For the removal of the ethoxymethylidene group, 50% AcOH for 24 h at room temperature was used. The solution was evaporated and the residue was rendered anhydrous by evaporation with added pyridine. The residue was dissolved in $H_2O$ or 0.02 M TEAB buffer (pH 7.5) and applied to the column of appropriate ion exchanger as summarized in Table IV. Eluting buffers and yields of dinucleoside monophosphates are listed in Table IV.

**Enzymatic Digestion of Dinucleoside Monophosphates.** Conditions and results were as listed in Table II.

**Supplementary Material Available**: detailed chromatographic conditions and chromatograms for separation of the dinucleoside monophosphates (8 pages). Ordering information is given on any current masthead page.

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