Intramolecular Stacking Association of Three Dinucleoside Monophosphates Containing Naturally-occurring 1-Methyladenosine Residue(s): m¹ApA, Apm¹A, and m¹Apm¹A[1)]

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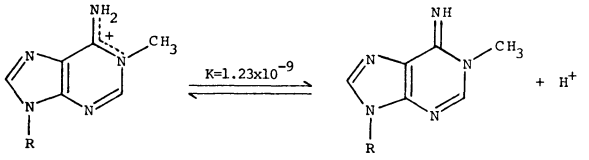
Three modified dinucleoside monophosphates containing naturally-occurring 1-methyladenosine residue, m¹ApA, Apm¹A, and m¹Apm¹A, were prepared. Intramolecular stacking association properties of these dimers were studied quantitatively using two independent methods. Thermal denaturation and acid-base titration experiments reached to the same conclusion that both $m^1\overset{+}{A}pA$ and $Apm^1\overset{+}{A}$ were less stacked than ApA. Acid-base titration experiment also showed that the three modified dimers, in their neutral forms, were stacked to almost the same extent as ApA. These results indicate the N-1 methylation of an adenine base does not enhance stacking interaction of dimers, in sharp contrast to the N^6 methylation of the same base. UV hypochromicity and CD measurements were also made for the three dimers, and the results were found to be consistent with those of the stacking quotient determination.

As a part of studies on the intramolecular stacking interaction of modified base-containing dinucleoside monophosphates,[2-7)] dimers containing 1-methyladenosine (m¹A) were studied in order to see the effect of a methyl substitution at a ring nitrogen atom of an adenine base on stacking association. In view of a wide occurrence of m¹A residue in eukaryotic tRNA molecules,[8)] it is of interest to study the effect of this residue on the stacking properties and compare it with that of other methylated base residues.

It is well established that a variety of methylated dinucleoside monophosphates exhibit stronger stacking tendency as compared to the unmethylated corresponding parent dimers. For example results from our laboratory and those from others have revealed that N^6 methylation of an adenine base enhances stacking interaction in dimers, as did so for m^6Apm^6A,[6)] $m_2^6Apm_2^6A$,[7,9)] m^6ApU,[9,10)] and m_2^6ApU.[9,10)] Little is known, however, about the effect of a m¹A residue on stacking of dimers. Danyluk *et al.* first reported that $m^1\overset{+}{A}pU$ and $Upm^1\overset{+}{A}$ were less stacked than unmethylated ApU and UpA, respectively, as studied by ^1H NMR spectroscopy.[11)] Their results are noteworthy in view of the general stacking-enhancing properties of a methyl substituent and the fact that the $m^7\overset{+}{G}$ residue is known to exert such an effect as observed in our previous work for $m^7\overset{+}{G}pU$[3)] and $m^7\overset{+}{G}pG$.[4)]

We have undertaken a detailed study on the effect of m¹A residue on the stacking properties of dimers using three dinucleoside monophosphates, m¹ApA, Apm¹A, and m¹Apm¹A. Using two independent methods, thermal denaturation and acid-base titration experiments, the stacking quotients were estimated for these three dimers and compared with those for the parent molecule, ApA. Because m¹A exists in the protonated form (Fig. 1) in neutral aqueous solutions (basic pK of m¹A is 8.76, see Table 2), the stacking properties of the monoprotonated dimers were studied together with those of the neutral forms. Optical properties (UV and CD spectra) of the three dimers were also studied, and results were compared with those of the stacking quotients determined.



($m^1\overset{+}{A}$) (m^1A)

R = Ribosyl residue

Fig. 1. Acid-base equilibrium in 1-methyladenosine,

Experimental

Materials. *m^1Apm^1A, m^1ApA, and Apm^1A*: To ApAp (3′) (9 μmol), dissolved in 5 ml of 0.1 M NaOAc buffer (pH 5.0, 1 M = 1 mol dm^{-3}) was added 100 μl of dimethyl sulfate and the solution was kept under stirring for 3 h at room temperature. During the reaction the solution was maintained at pH 3—5 by gradual addition of 1 M NaOH solution. Figure 2 shows chromatographic separation of the products with a Dowex 1X2 anion exchanger (1.3 cm × 50 cm) which afforded three modified dinucleotides and unreacted ApAp(3′). m^1Apm^1Ap(3′), m^1ApAp(3′), and Apm^1Ap(3′)

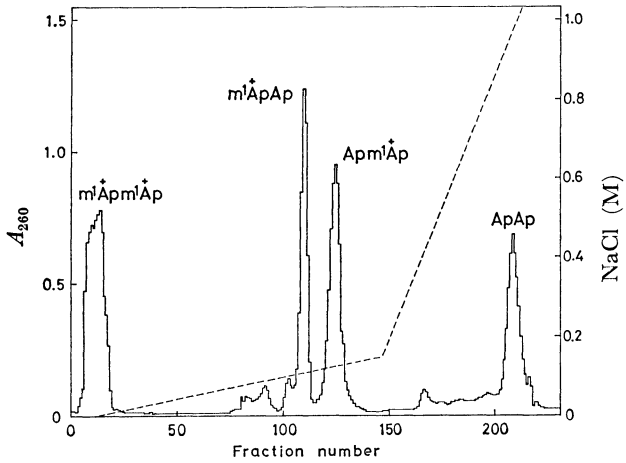


Fig. 2. Chromatogram of a partially methylated ApAp (3′) on a column of Dowex 1X2 (1.3 cm × 50 cm) with linear gradients of NaCl in 5 mM NaOAc (pH 5,0) as indicated. Fraction volume was 10 ml.

thus obtained were treated with *E. coli* alkaline phosphatase (Boehringer, Mannheim) at 39 °C and pH 7 for 4 h to give m¹Apm¹A, m¹ApA, and Apm¹A, respectively. The dimers were susceptible to degradation by ribonuclease T_2 and gave a mixture of the corresponding nucleoside 3′-phosphate and nucleoside. Desalting of the dimer preparations was effected by gel filtration using Sephadex G-10.

m^1A, $m^1Ap\text{-}OCH_3$, and $CH_3O\text{-}pm^1A$: A, Ap-OCH_3,[12] and CH_3O-pA[13] were treated with dimethyl sulfate in a manner similar to that described for ApAp(3′).

Methods. Thermal Denaturation of $m^1\overset{+}{A}pA$ and $Apm^1\overset{+}{A}$: In the present work, methods and calculations followed closely those described previously.[5,14] 3,3-Dimethylglutarate buffer (10 mM; pH 5.5) was used. The dimer concentrations were about 5×10^{-5} mol/l. For each dimer solution, 6 repeated denaturation cycles were performed in the temperature range 0—70 °C, and changes of the difference in absorbance (dimer *vs.* constituent monomers) at 262 nm with temperature were recorded using a recording millivolt meter (Watanabe Sokki MC642).

p*K Determinations of Nucleosides, Nucleoside Methyl Phosphates, and Dinucleoside Monophosphates*: All ionization constants were determined spectrophotometrically at 25 °C and ionic strength 0.1, by the method described previously.[15]

Optical Measurements: UV spectra were recorded with a Hitachi 124 recording spectrophotometer. CD spectra were taken with a JASCO J40 spectropolarimeter with a 1-cm quartz cell at 25 °C and ionic strength 0.1 (We thank Dr. T. Oshima, Mitsubishi-Kasei Institute of Life Sciences, for making the apparatus available to us). Spectra of the neutral dimers were not measured directly because of the lability of m¹A residues in high pH region ($t_{1/2}$ for Dimroth rearrangement of m¹A is about 5 h at pH 11 and 25°). Instead, spectra at several different pH values (as indicated in caption for Fig. 3) were measured and employed to compute the spectra of the neutral species with the knowledge of pK values of the dimers (Table 2).

Results and Discussion

Thermal Denaturation Experiments to Obtain Intramolecular Stacking Equilibrium Quotient, s_1, of $m^1\overset{+}{A}pA$ and $Apm^1\overset{+}{A}$. The thermal denaturation experiments were performed at pH 5.5 to obtain the stacking quotients for the monoprotonated form in which the dimers existed under the physiological conditions (see Table 2). Both dimers showed hyperchromism at 262 nm on heating their solutions. The thermal denaturation profiles were analyzed based on the two-state equilibrium model shown below for a specific case of $m^1\overset{+}{A}pA$.

$$(m^1\overset{+}{A}pA)_{unstacked} \overset{s_1}{\rightleftharpoons} (m^1\overset{+}{A}pA)_{stacked}$$

Here the equilibrium quotient, s_1, for the reversible association of an unstacked conformer, $(m^1\overset{+}{A}pA)_{unstacked}$, to give the stacked conformer, $(m^1\overset{+}{A}pA)_{stacked}$, is defined as

$$s_1 = \frac{[(m^1\overset{+}{A}pA)_{stacked}]}{[(m^1\overset{+}{A}pA)_{unstacked}]}$$

The stacking quotient, s_1, at a given temperature and thermodynamic functions, $\Delta H°$ and $\Delta S°$, were then calculated according to Eq. 1 with the assumption that the parameters, ε_u, ε_s, $\Delta H°$, and $\Delta S°$ are all temperature-independent.

$$\ln s_{1i} = \ln\frac{\varepsilon_u-\varepsilon_i}{\varepsilon_i-\varepsilon_s} = -\frac{\Delta H°}{RT_i}+\frac{\Delta S°}{R} \quad (1)$$

where ε_i, ε_s, and ε_u are the observed molar absorption coefficients at the ith of n temperature readings, and those for the extreme stacked and unstacked conformers, respectively. In this case, the parameters ε_u, ε_s, $\Delta H°$, and $\Delta S°$ were found by an iterative nonlinear least-squares approximation on a HITAC M-200H computer at the Computer Center of the University of Tokyo. The formalism and further details are available in Ref. 18

Table 1. Thermodynamic parameters and intramolecular stacking equilibrium quotients (s_1 or s_0)[a] of $m^1\overset{+}{A}pA$, $Apm^1\overset{+}{A}$, and ApA[b] (25°, I=0.1)

Dimer	$\Delta H°$ (σ)[c] / kJ mol^{-1}	$\Delta S°$ (σ)[c] / J mol^{-1} deg^{-1}	s_1 or s_0(σ)[c]
$m^1\overset{+}{A}pA$	−18.5(0.5)	−64.9(1.4)	0.70(0.02)
$Apm^1\overset{+}{A}$	−23.0(0.5)	−81.0(1.6)	0.62(0.02)
ApA	−21.1(0.3)	−70.1(0.9)	1.10(0.01)

a) s_1 for $m^1\overset{+}{A}pA$ and $Apm^1\overset{+}{A}$ (at pH 5.5). s_0 for ApA (at pH 7.0). s_1 and s_0 standing for the stacking quotients for monoprotonated and neutral speceis, respectively. b) Taken from our previous paper.[7] c) The standard deviations (σ) were computed from the root mean square (rms) values between experimental and calculated melting profiles as described in a previous paper.[18]

Table 1 lists the values of $\Delta H°$, $\Delta S°$, and stacking equilibrium quotient thus determined at 25 °C for $m^1\overset{+}{A}pA$ and $Apm^1\overset{+}{A}$, together with those for ApA (at pH 7.0)[7] for comparison. From s values in Table 1 it is concluded that $m^1\overset{+}{A}pA$ and $Apm^1\overset{+}{A}$ are stacked to a similar extent, and considerably less stacked than the parent molecule, ApA. The same stacking-diminishing effect by $m^1\overset{+}{A}$ has been observed by Danyluk *et al.*[13] in $m^1\overset{+}{A}pU$ and $Upm^1\overset{+}{A}$. The present conclusion is rather surprising because the methyl group, when introduced to a nitrogen atom, has almost invariably been found stacking-enhancing in dimers.[3,4,6,7,9,10] To substantiate the above conclusion the acid-base titration experiments were performed.

Acid-base Titration Experiments to Obtain Stacking Equilibrium Quotients, s_1 and s_0 of m^1ApA, Apm^1A, and m^1Apm^1A. We have developed a procedure that can give an estimate of stacking equilibrium quotients of homodimers for both neutral and monoprotonated (or monoionized) species from their ionization constants and those of constituent monomers.[5,6,15,16] We now extend the method to heterodimers (XpY) in which X and Y are both acids or bases. For a diacidic base one can write the following schemes.

$$(\overset{+}{X}pY\overset{+}{)}_{u+s} \overset{K_2}{\rightleftharpoons} (XpY)^+_{u+s} \overset{K_1}{\rightleftharpoons} (XpY)_{u+s}$$

$$\begin{array}{ccccc} (\overset{+}{\mathrm{X}}\mathrm{pY})_s & \overset{K_{s2}}{\rightleftharpoons} & (\mathrm{XpY})_s^+ & \overset{K_{s1}}{\rightleftharpoons} & (\mathrm{XpY})_s \\ \downarrow\uparrow s_2 & & \downarrow\uparrow s_1 & & \downarrow\uparrow s_0 \\ (\overset{+}{\mathrm{X}}\mathrm{pY})_u & \overset{K_{u2}}{\rightleftharpoons} & (\mathrm{XpY})_u^+ & \overset{K_{u1}}{\rightleftharpoons} & (\mathrm{XpY})_u \end{array}$$

where subscripts, u and s, denote unstacked and stacked conformations, respectively, and u+s denotes composite of unstacked and stacked conformations. From these schemes the following equations can be derived which relate ionization constants of a dimer to its stacking equilibrium quotients at various stages of protonation.

$$\mathrm{p}K_2 = \mathrm{p}K_{u2} - \log(1+s_1)/(1+s_2) \quad (2)$$

$$\mathrm{p}K_1 = \mathrm{p}K_{u1} - \log(1+s_0)/(1+s_1) \quad (3)$$

Since s_2 is considered to be very small because of the electrostatic repulsion between two positively charged bases,[15,17,18] Eq. 2 can be simplified to

$$\mathrm{p}K_2 = \mathrm{p}K_{u2} - \log(1+s_1) \quad (4)$$

K_{u1} and K_{u2} are the intrinsic ionization constants for the unstacked residues which must be estimated in some way to solve Eqs. 3 and 4 for s_1 and s_0. For homodimers (X=Y), K_{u1} and K_{u2} have been approximated in the following way using ionization constants of the constituent monomers.[15]

$$K_{u1} = \frac{1}{2}K_0, \quad K_{u2} = 2K_0 \quad (5)$$

$$\mathrm{p}K_0 = \frac{1}{4}[\mathrm{p}K_{3'\text{-nucleotide}} + \mathrm{p}K_{5'\text{-nucleotide}} + 2\mathrm{p}K_{\text{nucleoside}}] \quad (6)$$

For heterodimers in which X and Y have similar basicity, estimation of the intrinsic K's is a complicated matter. However, if they differ markedly in their basicity (say, $|\mathrm{p}K_X - \mathrm{p}K_Y| > 4$), the intrinsic K's can be approximated in the same way as those of homodimers. That is, if $\mathrm{p}K_X > \mathrm{p}K_Y$, it follows that $[(\overset{+}{\mathrm{X}}\mathrm{pY})_u] \gg [(\mathrm{X}\overset{+}{\mathrm{p}}\mathrm{Y})_u]$. Consequently, K_{u1} and K_{u2} are regarded as the intrinsic K's of X and Y residues, respectively, which can be approximated by K_0 in Eq. 6 for X and Y, respectively.

In the present study, because of the large difference in basicity of m^{1}A and A residues in the dimers, values of s_1 and s_0 were obtained by acid-base titration of the dimers and the corresponding constituent monomers. The $\mathrm{p}K_1$ and $\mathrm{p}K_2$ values of m^{1}ApA, Apm^{1}A, and m^{1}Apm^{1}A determined by spectrophotometric titration are given in Table 2 together with the basic pK values of the constituent monomers [The latter values were used to estimate $\mathrm{p}K_{u1}$ and $\mathrm{p}K_{u2}$ values in Table 2]. For comparison, relevant values for ApA[7] are also included in the table.

Values of s_1 and s_0 for three dimers were calculated from the pK values in Table 2 using Eqs. 3 and 4, and listed in Table 3 together with those for ApA. From the numerical data in Table 3 we can draw the following conclusion:

(1) s_1 values of m$^1\overset{+}{\mathrm{A}}$pA (0.87) and Apm$^1\overset{+}{\mathrm{A}}$ (1.11) are similar to each other and they are smaller than s_0 of ApA (1.51). This result substantiates a conclusion reached from the thermal denaturation experiments, and shows that N-1 methylation of an adenine residue decreases the extent of intramolecular stacking interaction.

(2) s_1 values of m$^1\overset{+}{\mathrm{A}}$pA (0.87) and Apm$^1\overset{+}{\mathrm{A}}$ (1.11) are not significantly different from that of (ApA)$^{\pm}$ (1.14). This suggests that the presence of the methyl group itself does not influence the stacking interaction in these monoprotonated dimers.

(3) All four dimers have similar s_0 values. This suggests that in neutral form introduction of a methyl group neither enhances nor diminishes the extent of the stacking interaction of ApA.

Optical Studies of m^{1}ApA, Apm^{1}A, and m^{1}Apm^{1}A. UV absorption and hypochromicity data of the four dimers are shown in Table 4. In agreement with the conclusion from the stacking quotient measurements hypochromicity data indicate the stacking in m$^1\overset{+}{\mathrm{A}}$pA and Apm$^1\overset{+}{\mathrm{A}}$ being a little bit weaker than in ApA. The value for m$^1\overset{+}{\mathrm{A}}pm^1\overset{+}{\mathrm{A}}$ was found to be negligibly small, and this is also in line with the previous assumption of $s_2=0$.

The CD spectra of m$^1\overset{+}{\mathrm{A}}pA, Apm^1\overset{+}{\mathrm{A}}$, and their un-

TABLE 2. IONIZATION CONSTANTS (AS pK) OF m^{1}ApA, Apm^{1}A, m^{1}Apm^{1}A, ApA, AND THE CONSTITUENT NUCLEOSIDES, NUCLEOSIDE MONOPHOSPHATES, AND NUCLEOSIDE METHYL PHOSPHATES (25°, I=0.1), AND CALCULATED INTRINSIC pK's[a,b]

Dimer	pK_1	pK_2	pK_{u1}	pK_{u2}
m^{1}ApA	8.66 (0.02)	3.41 (0.03)	8.80 (0.01)	3.68 (0.01)
Apm^{1}A	8.73 (0.01)	3.36 (0.02)	8.80 (0.01)	3.68 (0.01)
m^{1}Apm^{1}A	8.91 (0.03)	8.29 (0.04)	9.10 (0.01)	8.50 (0.01)
ApA[c]	3.91 (0.03)	3.05 (0.07)	3.98 (0.01)	3.38 (0.01)

Monomer	pK	Monomer	pK
m^{1}A[d]	8.76 (0.01)	A[c]	3.61 (0.02)
m^{1}Ap-OCH$_3$[d]	8.79 (0.02)	Ap[c]	3.66 (0.01)
CH$_3$O-pm^{1}A[d]	8.90 (0.01)	pA[c]	3.85 (0.02)

a) Standard deviations of experimental values are shown in parentheses. b) For homodimers $\mathrm{p}K_{u1}$ and $\mathrm{p}K_{u2}$ were calculated by Eqs. 5 and 6. For heterodimers Eq. 6 was used, K_0 representing either K_{u1} or K_{u2}. c) Revised values from those given in our previous paper.[19] d) Methyl esters of 3'- and 5'-nucleotides were used to avoid an influence of an additional negative charge on the phosphate group in pH region near the pK values.

TABLE 3. ESTIMATED STACKING EQUILIBRIUM QUOTIENTS FROM THE DATA BASED ON TITRATION STUDIES (25°, I=0.1)[a]

Dimer	s_1	s_0
m^{1}ApA	0.87±0.15	1.62±0.23
Apm^{1}A	1.11±0.17	1.48±0.21
m^{1}Apm^{1}A	0.63±0.13	1.56±0.28
ApA[b]	1.14±0.35	1.51±0.43

a) The probable errors given for s_1 and s_0 values are based on errors in $\mathrm{p}K_{u1}$, $\mathrm{p}K_{u2}$, $\mathrm{p}K_1$, and $\mathrm{p}K_2$. b) Revised values from those given in our previous paper.[19]

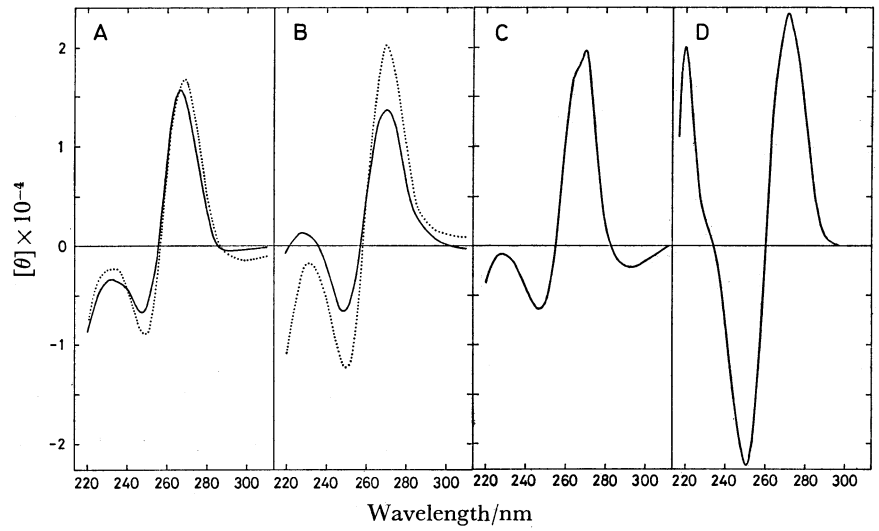


Fig. 3. Difference CD spectra of m¹ApA, Apm¹A, m¹Apm¹A, and ApA (25 °C, I=0.1). Spectra of the constituent monomers have been subtracted from those of dimers. Ellipticity is expressed per mole of monomer residues.
(A) ——, $m^1\overset{+}{A}pA$ at pH 6.0; ----, m¹ApA calculated from spectra at pH 6.0 and 8.9; (B) ——, $Apm^1\overset{+}{A}$ at pH 6.0; ----, Apm¹A calculated from spectra at pH 6.0 and 9.1; (C) m¹Apm¹A calculated from spectra at pH 5.0, 8.1, 9.2, and 10.1; (D) ApA at pH 7.0.

Table 4. UV spectral and hypochromicity data of dimers (25°, I=0.1)

Dimer	pH	λ_{max} / nm	H[a] / %
$m^1\overset{+}{A}pA$	5.5	258	10
$Apm^1\overset{+}{A}$	5.5	258	10
$m^1\overset{+}{A}pm^1\overset{+}{A}$	5.5	258	1
ApA	7.0	258	12

a) Hypochromicity is measured at the λ_{max} of monomers.

protonated species at 25° are shown in Figs. 3A and 3B together with that of ApA for comparison (Fig. 3D). Cation formation of m¹ApA produces only minor changes in the CD spectrum (Fig. 3A) while deprotonation of $Apm^1\overset{+}{A}$ considerably enhances the intensity of the CD spectrum (Fig. 3B). Another feature of interest in the CD spectra shown in Fig. 3 is that the CD spectrum of $m^1\overset{+}{A}pA$ is almost quantitatively similar to that of $Apm^1\overset{+}{A}$ and the intensities of the CD spectra for this pair of sequence isomers are considerably smaller than that for ApA. These observations may be taken as *prima facie* evidence for weaker base-base stacking interactions in $m^1\overset{+}{A}pA$ and $Apm^1\overset{+}{A}$ than ApA. Thus the correlation with the stacking quotient data listed in Tables 1 and 3 is striking. It should be noted on comparing the vastly different CD spectra[4] of a pair of sequence isomers $m^7\overset{+}{G}pG$ and $Gpm^7\overset{+}{G}$ that m¹A-containing dimers differ considerably from other methylated dimers in the molecular conformations.

Concerning the stacking association of m¹Apm¹A, titrations gave an s_0 value of 1.56 at 25 °C. Although acid-base titrations are seen to overestimate the stacking quotient s_0,[5,14] the value for m¹Apm¹A is by no means greater than that for the parent unmethylated ApA. Supporting evidence for this comes from the comparison of the CD spectrum extracted for m¹Apm¹A (Fig. 3C) and that of ApA (Fig. 3D). The presently observed effect for m¹A is thus in contrast to the general pattern of the stacking-enhancing effect of methyl substitution as previously observed in methyl-substituted dinucleoside monophosphates.[3,4,6,7,9,10]

In consequence, m¹A-containing dinucleoside monophosphates stack intramolecularly less extensively than the corresponding parent unmethylated dimers, and differ from other methyl-substituted dimers in the stacking equilibrium ratios. Such differences can be accounted empirically for by the fact that m¹A possesses an amidine structure with a quasi-ortho-quinonoid form unfavoring the stacking-enhancing effect of the methyl group. Incidentally, the CD spectrum of m¹Apm¹A is quite similar in shape to that of IpI[19,20] which has the quasi-ortho-quinonoid amide structure.

Finally, it should be noted that the methylation of N^6 position of an adenine base increased stacking interaction in dimers.[6,7,9,10] Considering the wide existence of these methylated adenine residues in nucleic acid molecules (m¹A in tRNA;[8] m^6A in tRNA[8] and mRNA;[21] $m_2{}^6$A in rRNA[22]), it is of interest to note that a methyl group can exert various effects on the stacking of dimers depending on the site of its introduction. Thus, the present study has shown that methylation of a nucleic acid base is one of effective means for the fine controll of a nucleic acid conformation.

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1) Abbreviations:—A dimer means a dinucleoside monophosphate. XpY represents a dimer having a 3′-5′ phosphodiester bond, X-3′-p-5′-Y. "p" to the left of a nucleoside symbol indicates a 5′-phosphate, and to the right indicates a 3′-phosphate. CH_3O- and -OCH_3 attached to "p" mean the phosphate is esterified by a methyl group. "+" on a nucleoside symbol indicates the nucleoside has a positive charge. Nucleoside symbols used for modified bases are the following: m^1A, 1-methyladenosine; m^6A, N^6-methyladenosine; m_2^6A, N^6,N^6-dimethyladenosine; m^7G, 7-methylguanosine; I, inosine.

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