[Chem. Pharm. Bull. 20(11)2394—2401(1972)]

UDC 547.963.32.057 : 547.857.7.04

Polynucleotides. XII.[1] Synthesis and Properties of Dinucleoside Monophosphates containing 8-Bromoadenosine

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(Received April 24, 1972)

Attempt to obtain 8-bromoadenylyl-(3′-5′)-8-bromoadenosine (BrApBrA) (III) by the condensation of N^6,$O^{2'}$,$O^{3'}$-tribenzoyl-8-bromoadenosine 3′-phosphate (V) and 2′,3′-O-ethoxymethylidene-8-bromoadenosine (VI) using dicyclohexylcarbodiimide (DCC) gave 8-oxyadenylyl-(3′-5′)-8-bromoadenosine (HOApBrA) (VII) after removal of protecting groups. Structure of VII was confirmed by ultraviolet (UV) and circular dichroism (CD) spectra as well as enzymatic hydrolysis.

Condensation of N^6,O^5-diacetyl-8-bromoadenosine 2′(3′)-phosphate (IVa) and compound VI using diphenylphosphorochloridate gave 2′-5′ (X) and 3′-5′ (III) isomers of 8-bromoadenylyl-8-bromoadenosine, which were separated and purified by column chromatography, paper electrophoresis and paper chromatography. Structure of compounds X and III was elucidated by optical properties and enzymatic digestions. Hypochromicities of X and III were 14% and 10%, respectively. From the CD spectra of both compounds, a *syn-syn*, left-handed structure of these dinucleoside monophosphates was predicted.

We have reported on syntheses and properties of 8-substituted adenosine,[3,4] its 5′-phosphates,[5–7] 5′,5′-pyrophosphates[8] and polynucleotides.[9] As to 8-bromoadenosine, the base moiety was elucidated as in *syn* conformation either in the crystal form[10] or in the solution.[7] Since adenylyl-(3′-5′)-adenosine (ApA) (I) was almost established as in *anti-anti*, right-handed stacked form,[11] linking of two *syn* type nucleosides, 8-bromoadenosine (BrA), by a 3′-5′ phosphodiester bond may be interesting to obtain informations about relationship of the torsion angle[12] and the mode of stacking. In this connection we have already reported on a dinucleoside monophosphate from two 8,2′-S-cycloadenosines[13] (A^spA^s) (II), which had the torsion angle about −108°, and it was found that this compound had stacking of left-handed screw axis.

The synthesis of 8-bromoadenylyl-(3′-5′)-8-bromoadenosine (BrApBrA)[14] (III) was attempted first by the method using benzoyl as protecting groups. 8-Bromoadenosine 3′-phosphate[6] (IVa) was obtained from 2′(3′)-adenylic acid by bromination in sodium acetate buffer at pH 4.0 followed by separation of 3′-phosphate with Dowex-I column chromatography. Compound IV was treated with benzoic anhydride as described by Khorana, *et al.*[15] to give

1) Part XI: M. Ikehara and M. Hattori, *Biochim. Biophys. Acta*, **269**, 27 (1972).
2) Location: *6-1-1, Toyonaka, Osaka.*
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4) M. Ikehara and S. Yamada, *Chem. Pharm. Bull.* (Tokyo), **19**, 104 (1971).
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6) M. Ikehara and S. Uesugi, *Chem. Pharm. Bull.* (Tokyo), **17**, 348 (1969).
7) M. Ikehara, S. Uesugi, and K. Yoshida, *Biochemistry*, **11**, 830 (1972).
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9) M. Ikehara, I. Tazawa, and T. Fukui, *Biochemistry*, **8**, 736 (1969).
10) S.S. Travale and H.M. Sobell, *J. Mol. Biol.*, **48**, 109 (1970).
11) N.S. Kondo, H.M. Holmes, L.M. Stempel, and P.O.P. Ts'o, *Biochemistry*, **9**, 3479 (1970).
12) J. Donohue and N.K. Trueblood, *J. Mol. Biol.*, **2**, 363 (1960).
13) M. Ikehara, S. Uesugi, and M. Yasumoyo, *J. Am. Chem. Soc.*, **92**, 4335 (1970).
14) Abbreviations: BrA stands for 8-bromoadenosine and HOA for 8-hydroxyadenosine.
15) Y. Lapidot and H.G. Khorana, *J. Am. Chem. Soc.*, **95**, 3857 (1963).

$N^6,O^{2'},O^{3'}$-tribenzoyl-8-bromoadenosine 3′-phosphate (V). Condensation of compound V with 2′,3′-O-ethoxymethylidene-8-bromoadenosine (VI) using dicyclohexylcarbodiimide (DCC) as condensing agent was performed at room temperature for 20 days. Separation of products by DEAE-cellulose column chromatography gave a dinucleoside phosphate (VII), which had ultraviolet (UV) absorption λ_{max}'s 265 nm at pH 7 and 268 nm at pH 12, as a major product. The bathochromic shift of alkaline maximum to 270 nm suggested the presence of an alkali dissociable residue in the compound VII, which could not be expected from BrApBrA. Coumpound VII was then hydrolyzed with RNase M,[16] which will give component nucleoside and nucleoside 3′-phosphate. Application of the incubation mixture to paper chromatography and paper electrophoresis showed two spots corresponding to 8-bromoadenosine (VIII) and 8-oxyadenosine 3′-phosphate (IXa) in a ratio of nearly 1:1. Digestion with spleen phosphodiesterase[17] gave the same results. Hydrolysis using venom phosphodiesterase[18] gave a nucleotide (IXb), which migrated more slowly than 8-bromoabenosine 3′-phosphate (BrAp) in electrophoresis and had UV absorption properties same as those of 8-oxyadenosine 5′-monophosphate.[19] Since nucleotide IXb was hydrolyzed by ribonuckase RNase M to give 8-oxyadenosine 3′-monophosphate IXa, this was assumed to be 8-oxyadenosine 2′,3′-cyclic phosphate. This type of cyclic esterification by spleen phsophodiesterase was reported previously by Heppel and Whitfield.[20] From these evidences the original dinucleoside monophosphate (VII) was confirmed to be 8-oxyadenylyl-(3′-5′)-8-bromoadenosine. Introduction of benzoyl group to 8-bromoadenine 6-NH_2 group may be responsible for labilization of 8-bromo atom towards acidic treatment in the deprotection procedures as shown previously by Holmes and Robins.[21]

Hyperchromicity in the enzymic digestion was calculated to be 11% and suggested a stacked conformation to 8-oxyadenylyl-(3′-5′)-8-bromoadenosine (HOApBrA) (VII). This was also supported by circular dichroism (CD) spectrum of compound VII. As shown in Fig.1, a negative Cotton band at 280 nm and a positive one at 260 nm constituted a pair of bands, which presumably arose from a coupling[22] of transition moments in B region of both nucleosides.

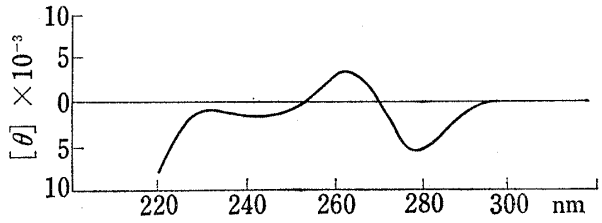


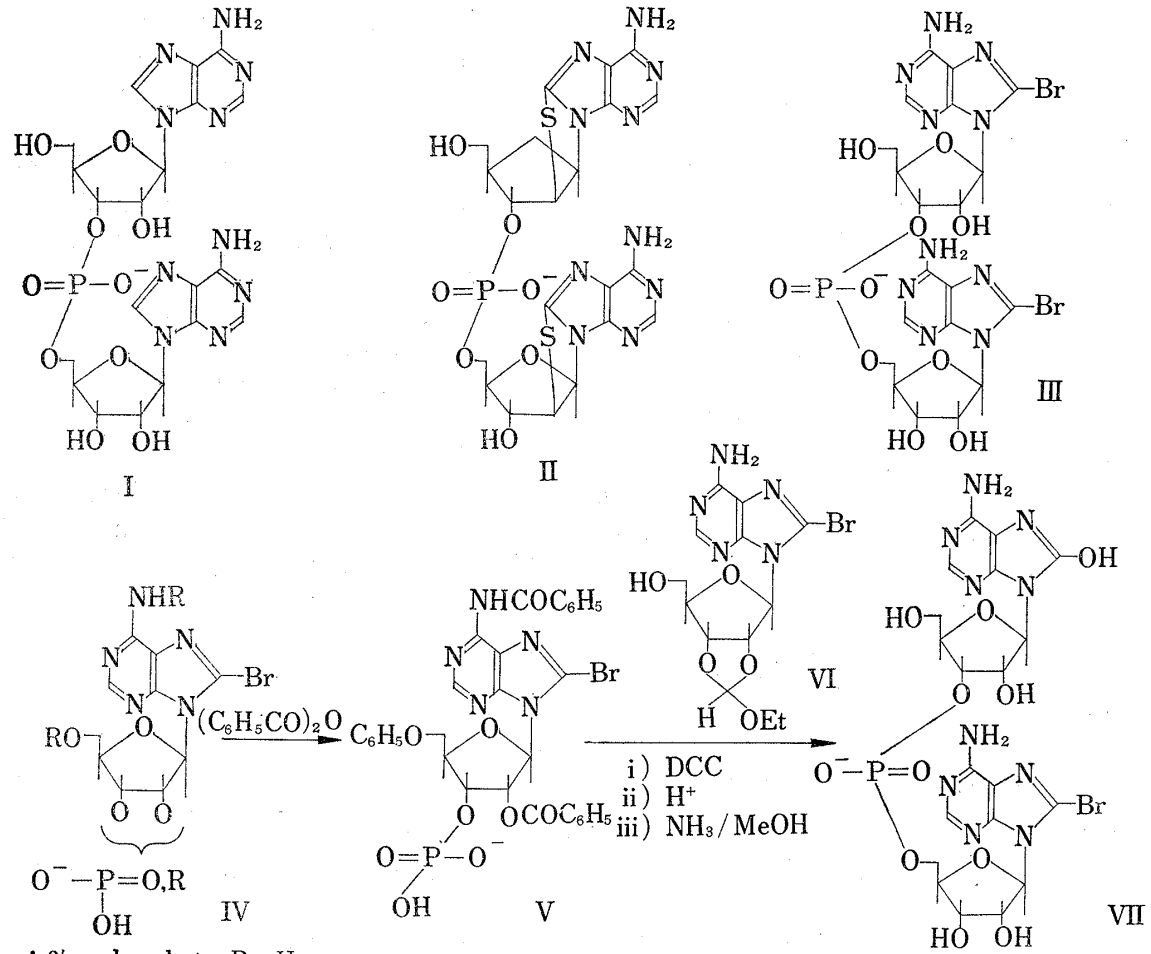
Fig. 1. Circular Dichroism Spectrum of HOApBrA

taken in 0.05M phosphate buffer (pH 7.0)

As we failed to obtain desired BrApBrA (III) by the above method, we attempted to synthesize it by way of anhydride exchange method of Michelson.[23] Starting from 8-bromoadenosine 2′,3′-cyclic phosphate (IVb), we obtained $N^6,O^{5'}$-diacetyl 2′,3′-cyclic phosphate (IVc) by treatment with acetic anhydride in the presence of tri-*n*-butylamine, followed by treatment with ethyl chloroformate, and IVc was condensed with 2′,3′-ethoxymethylidene-8-bromoadenosine (VI) using diphenyl phosphorochloridate. Subsequent column chromatography on Dowex 1×2 resin gave peaks thought to be corresponding to BrApBrA (III) and its 2′,5′-isomer (X) in a ratio of 320:330 OD units (Fig. 2). Nucleotides obtained from these peaks were further purified by paper electrophoresis. Both dinucleoside monophos-

16) M. Irie, *J. Biochem.*, **62**, 509 (1967).
17) R.W.E. Razzel and H.G. Khorana, *J. Biol. Chem.*, **236**, 1144 (1961).
18) E.J. Williams, S.-C. Sung, and M. Laskowski, *Sr.*, *J. Biol. Chem.*, **236**, 1130 (1961).
19) M. Ikehara and K. Murao, *Chem. Pharm. Bull.* (Tokyo), **16**, 1330 (1968).
20) L.A. Heppel and P.R. Whitfield, *Biochem. J.*, **60**, 1 (1955).
21) R.E. Holmes and R.K. Robins, *J. Am. Chem. Soc.*, **87**, 1772 (1965).
22) I. Tinoco, Jr., *J. Am. Chem. Soc.*, **86**, 297 (1964).
23) A.M. Michelson, *J. Chem. Soc.*, **1959**, 3655.

phates had UV absorption maxima around 264—244.5 nm and R_{pA-A}[24] in electrophoresis 0.34—0.35. These properties are consistent with the structure of BrApBrA. However, in the hydrolytic experiments using RNase M and spleen phosphodiesterase the sample corre-



a : 3′—phosphate, R=H
b : R=H

Chart 1

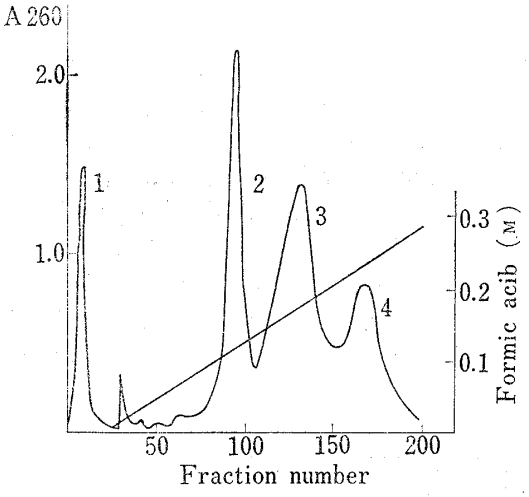


Fig. 2. Column Chromatography of 2′-5′ and 3′-5′ BrApBrA on Dowex 1 Column

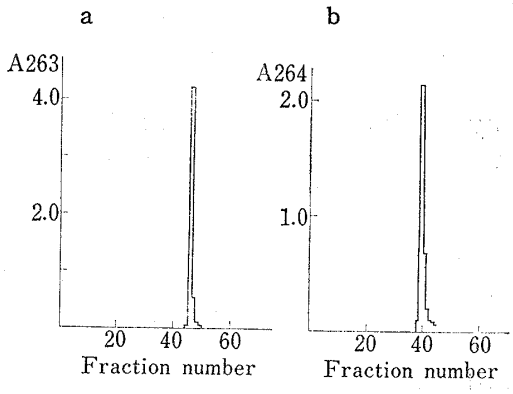


Fig. 3a. Purification of BrApBrA on Biogel P-2 Column

Fig. 3b. Purification of BrApBrA on Sephadex G-25 Column

24) Explanation of this abbreviation is in the experimental.

sponding to BrApBrA (III) was cleaved only to the extent of 70%. Since HOApBrA (VII) was completely digested by these enzymes, this result may be ascribed to a contaminant. Accordingly, the BrApBrA fraction was chromatographed further on Biogel P-2 and Sephadex G-25 columns. As shown in Fig. 3a and 3b, there was obtained BrApBrA in clearly resolved single peaks. These fractions were combined and purified by charcoal column to remove slightly contaminating inorganic salts.

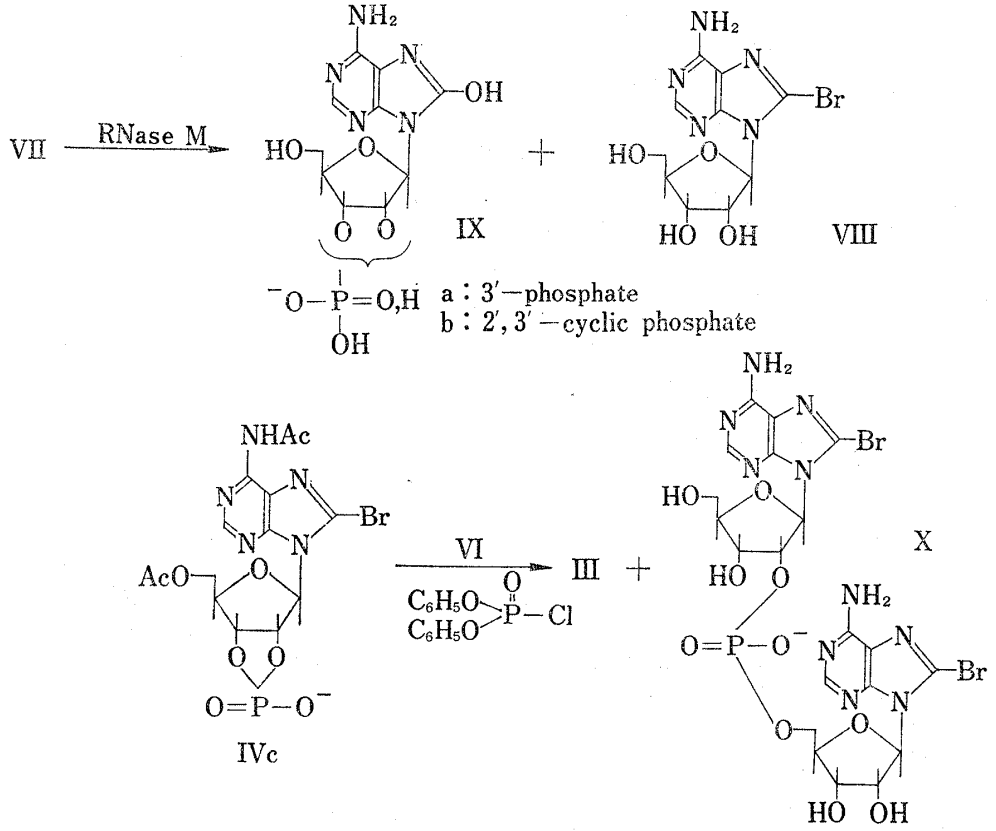
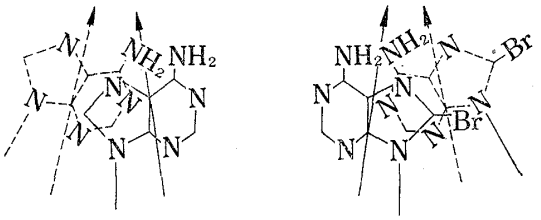


Chart 2

Pure samples of BrApBrA (III), thus obtained, and BrA-2′-p-5′-BrA (X) were analyzed then by hydrolytic enzymes. By snake venom phosphodiesterase both compounds were hydrolyzed completely to bromoadenosine (VIII) and its 5′-phosphate. In the hydrolysis using spleen phosphodiesterase 2′-5′ isomer (X) showed complete resistance even in the presence of a large excess enzymes. In contrast to this, BrApBrA (III) was hydrolyzed completely by RNase M to bromoadenosine and its 3′-phosphate in 1:1 ratio. In the case of using spleen phosphodiesterase extent of the hydrolysis was 87% as compared with the complete digestion of ApA in this condition. Hypochromicity calculated from these enzymatic digestion was 14% and 10% for X and III, respectively. A larger hypochromicity of 2′-5′ than that of 3′-5′ isomer was analogous in the case of ApA and its 2′-5′-isomer.[11]

Together with the enzymatic hydrolyses of HOApBrA, these experiments showed that even when the base moieties were in *syn* conformation, if the substrate had 3′-5′ phosphodiester bond, these enzymes catalyzed the hydrolysis as in natural substrates. CD spectra of BrApBrA (III) and its 2′-5′-isomer (X) were recorded in Fig. 4a and b. While the spectrum of compound III had two troughs at 280 and 255 nm and a peak at 220 nm, which arose from the coupling of transition moments of the monomer, that of X had a trough at 275 nm and two peaks at 255 and 220 nm. $[\theta]$ value of compound X was much larger than that of III. Although spectra taken at 2° and 25° did not show a large difference in B-region, curves around 220 nm were fairy different in shape. From these evidences and hypochromicity data

we could predict a stacked conformation of bases in these dinucleoside monophosphates and the degree of stacking might be larger in 2′-5′ (X) than in 3′-5′ isomer (III). Furthermore, if we assume the *syn* conformation in both nucleosides, as in the case of P^1,P^2-di-5′-bromoadenosine pyrophosphate,[8] this stacking would be along left-handed screw axis in contrary to the case of ApA. As shown in Fig. 5, in the case of ApA (*anti*-right-handed)[11] major transition moments of two adenines coupled each other as indicated by arrows and gave positive-negative Cotton bands from long wave length in CD. In contrast, BrApBrA (III) showed negative–positive Cotton bands and therefore, according to Tinoco's theory,[25] arrows, whose directions were predicted as in the Fig. 5,[8] should be coupled in an opposite way to those of



ApA (*anti* -*anti*) BrApBrA (*syn*-*syn*)

Fig. 5. Schematic Representation of Mode of Stacking of ApA and BrApBrA

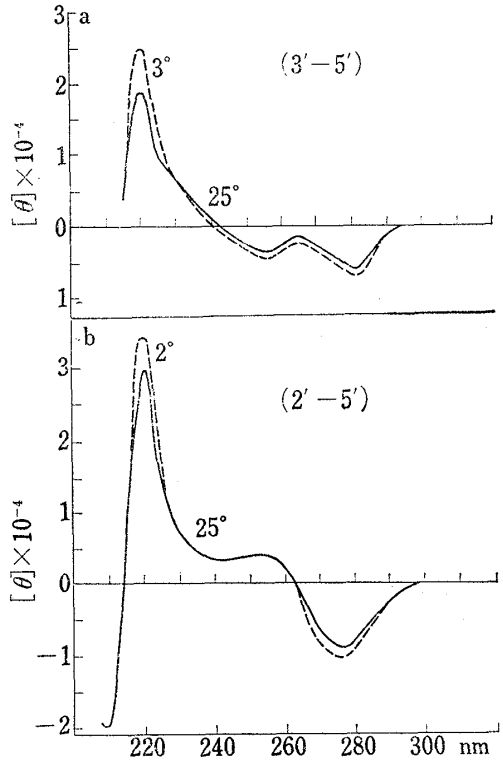


Fig. 4. Circular Dichroism Spectra of BrApBrA

taken in 0.05M phosphate buffer (pH 7.0)

ApA. From the examination of CPK molecular model,[26] this type of coupling could be allowed only in left-handed stacking with two *syn*-type nucleosides. Therefore, BrApBrA (III) and its 2′-5′-isomer (X) are the first couple of dinucleoside monophosphates, in which two bases are maintained in the *syn* form and stacked along the left-handed helical axis. These compounds may be suitable for the elucidation of the conformation of nucleic acids, especially in the case of *syn-anti* ambiguities.

Experimental[27]

Paper Chromatography——All paper chromatographies were performed by descending technique on Toyo filter paper No. 51A. Solvent used were: A, EtOH–M NH_4OAc (7: 3); B, iso-PrOH–conc. NH_3–H_2O (7:1: 2); C, *n*-BuOH–AcOH–H_2O (5: 2: 3); D, sat. $(NH_4)_2SO_4$–H_2O–iso-PrOH (79:19: 2), and represented as R_{pA} or $R_{pA-A}\cdot R_{pA}$ stands for migration ratio relative to pA. R_{pA-A} stands for migration ratio assuming pA=1.0 and adenosine=0.0.

N^6,$O^{2'}$,$O^{3'}$-Tribenzoyl-8-bromoadenosine 3′-Phosphate——8-Bromoadenosine 3′-phosphate[6] (pyridinium salt, 0.35 mmole) was dissolved in water (5 ml) containing tetraethylammonium benzoate (3.5 mmoles). The solution was evaporated four times with added pyridine and further four times with added toluene. Into the residue was added benzoic anhydride (1.58 g, 7 mmoles) and the mixture was heated at 40° for 60 min and at 28° for 6 days. After the reaction was stopped with 50% aqueous pyridine (10 ml), the solution was extracted with *n*-pentane and chloroform. Chloroform layer was evaporated, made anhydrous by

25) C.A. Bush and I. Tinoco, Jr., *J. Mol. Biol.*, 23, 601 (1967).
26) W.L. Koltun, *Bioplymers*, 3, 665 (1965).
27) UV spectra were taken with a Hitachi EPS-3T recording spectrophotometer, CD spectra were taken with a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment in 10 mm path-length at OD max/ml 1—2. Calibration was made by d-10-camphorsulfonic acid.

evaporation with added pyridine, and finally taken up in a small amount of pyridine. Dropwise addition of the solution into *n*-pentane–ether (2: 3) mixture gave white precipitates, which were collected by centrifugation. The precipitate was dried by evaporation with added pyridine, dissolved in pyridine (1 ml), and acetic anhydride (0.5 ml) was added. After it was kept at room temperature for 10 hr, 50% pyridine was added to stop the reaction. The solution was kept at room temperature for 2 hr and applied to a column of Dowex 50 (pyridinium form). Elution with 25% aqueous pyridine gave tribenzoyl-8-bromoadenosine 3′-phosphate fraction, which was evaporated, dried by evaporation with added pyridine, and finally precipitated in *n*-pentane–ether (2:3) mixture. UV: $\lambda_{max}^{H^+}$ 234, 288 nm; $\lambda_{max}^{H_2O}$ 234, 288 nm; $\lambda_{max}^{OH^-}$ 316 nm. PPC: *Rf* (A) 0.63 (Ap, 0.22). PEP: R_{Ap-A} (A) 0.87. This sample gave only 8-bromoadenosine 3′-phosphate by treatment with ammonia-methanol.

8-Hydroxyadenylyl-(3′-5′)-8-bromoadenosine——N^6,$O^{2'}$,$O^{5'}$-Tribenzoyl-8-bromoadenosine (0.35 mmole) and 2′,3′-O-ethoxymethylidne-8-bromoadenosine (134 mg, 0.30 mmole) were dissolved in anhydrous pyridine and evaporated three times. Finally the residue was dissolved in pyridine (3 ml). Into this solution was added Dowex 50 (pyridinium form) resin (110 mg) and DCC (434 mg, 2.1 mmoles). After evaporation of pyridine to its half volume, the mixture was stoppered and kept at 25—28° for 20 days. 50% pyridine was added to the reaction mixture, extracted with *n*-pentane, and water layer was evaporated. After traces of pyridine were removed by evaporation with added water, the residue was dissolved in 80% acetic acid (5 ml) and kept at room temperature for 49 hr. Acetic acid was removed by distillation and evaporated repeatedly with added ethanol. Residue, thus obtained, was dissolved in ammonia–methanol (saturated at 0°) and kept at room temperature for 1 day. After addition of a few drops of triethylamine, the solvent was evaporated. Residue was dissolved in water and applied to a column (1.5 × 25 cm) of DEAE-cellulose (bicarbonate form). The column was eluted with 0—0.1M triethylammonium bicarbonate (1.5 liter—1.5 liter) by a linear gradient technique. Fractions were collected in 10 ml each (see Fig. 6). Peak (Fr. No. 163—250) was evaporated and purified by paper chromatography in solvent A. Band at *Rf* 0.31 was cut and extracted with water. This ma terial was further purified by paper electrophoresis in solvent A. Behaviors in PPC and PEP were summarized in Table I. UV: $\lambda_{max}^{pH\,2}$ 263 nm ($\varepsilon^{28)}$ 28400); $\lambda_{max}^{pH\,7}$ 265 nm (26400); $\lambda_{max}^{pH\,12}$ 268 nm (26400); $\lambda_{min}^{pH\,2}$ 234.5 nm (8100); $\lambda_{min}^{pH\,7}$ 234.5 nm (9300); $\lambda_{min}^{pH\,12}$ 238 nm (8200). CD taken in 0.1M phosphate buffer (pH 7) was shown in Fig.

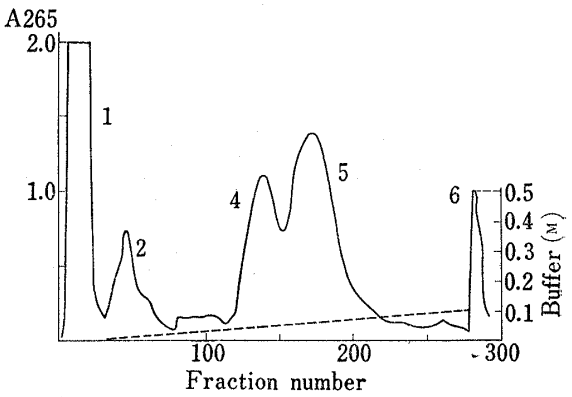


Fig. 6. DEAE-Cellulose Chromatography of HOApBrA

Enzymatic Hydrolysis of HOApBrA——i) Venom Phosphodiesterase: Incubation mixture (100 μl) contained 1M ammonium bicarbonate 20 μl, enzyme (1 mg/ml) 20 μl and nucleotide 3 OD_{max} units. Incubation was carried out at 37° for 4 hr. Examination of the reaction mixture by paper electrophoresis (solvent A) showed complete splitting to 8-oxyadenosine ($R_ABr_{p-A}Br$ 0.38, $\lambda_{max}^{H_2O}$ 271 nm, OD $_{max}^{H_2O}$ 0.22) and 8-bromoadenosine 5′-phosphate ($R_ABr_{p-A}Br$ 1.00, $\lambda_{max}^{H_2O}$ 264 nm, OD $_{max}^{H_2O}$ 0.32, A^{OH}: pA^{Br}=1:1.09).

ii) Spleen Phosphodiesterase: Incubation mixture (100 μl) contained 1M ammonium acetate (pH 5.7) 20 μl, enzyme (20 units/ml) 20 μl and nucleotide 3 OD_{max} units. Incubation was carried out at 37° for 4 hr. Examination of the reaction mixture by paper electrophoresis showed complete digestion to 8-bromoadenosine ($R_{Ap}Br_{-A}Br$ 0.02, $\lambda_{max}^{H_2O}$ 264.5 nm, $OD_{max}^{H_2O}$ 0.45), 8-oxyadenosine 2′,3′-cyclic phosphate ($R_{Ap}Br_{-A}Br$ 0.78, $\lambda_{max}^{H_2O}$ 271 nm, OD $_{max}^{H_2O}$ 0.14) and 8-oxyadenosine 3′-phosphate ($R_{Ap}Br_{-A}Br$ 1.22, $\lambda_{max}^{H_2O}$ 271.5 nm, OD $_{max}^{H_2O}$ 0.23). The cyclic phosphate converted to 3′-phosphate by the treatment with RNase M. Ap^{OH}: A^{Br}=1.0: 0.91.

iii) RNase M: Incubation mixture (100 μl) contained 1M ammonium acetate (pH 6.3) 10 μl, enzyme (1 mg/ml) 20 μl and nucleotide 3 OD_{max} units. Incubation was carried out at 37° for 4 hr. Examination by paper electrophoresis showed a complete digestion to 8-oxyadenosine 3′-phosphate ($R_{Ap}Br_{-A}Br$ 0.02, $\lambda_{max}^{H_2O}$ 264.5, $OD_{max}^{H_2O}$ 0.625) and 8-bromoadenosine ($R_{Ap}Br_{-A}Br$ 1.18, $\lambda_{max}^{H^+}$ 265 nm, $\lambda_{max}^{H_2O}$ 271.5 nm, $\lambda_{max}^{OH^-}$ 282 nm, $OD_{max}^{H_2O}$ 0.477). Ap^{OH}:A^{Br}=0.99:1.0. PPC (A) showed two spots having *Rf* 0.14 (A^{Br}) and *Rf* 0.69 (Ap^{OH}).

Hyperchromicity of A^{OH} pA^{Br}——Substrate (1.5 OD_{max}) was digested with venom phosphodiesterase at 37° for 4 hr. The mixture was diluted with 0.05M phosphate buffer (pH 7.0) and UV spectra were taken. Comparison with an untreated mixture gave hyperchromicity of 11% and hypochromicity of 10%.

8-Bromoadenosine 2′(3′)-phosphate——Adenosine 2′(3′)-phosphoric acid (1.460 g, 4 mmoles as monohydrate) was dissolved in 1N KOH (8 ml) and 0.5M acetate buffer (pH 4, 80 ml). Into this solution was added saturated bromine-water (80 ml) in several portions. After confirmation of complete disappearing

28) ε was presented as per residue value.

of the starting material by PPC, N_2 was bubbled through the mixture for 2 hr. Color of the solution was discharged by the addition of $NaHSO_3$ (do not add too much) and $Ba(OH)_2$, $8H_2O$ (1.38 g, 4.4 mmoles) was added. Precipitates were filtered off and filtrate was evaporated to dryness *in vacuo*. Water was added to the residue and evaporated again. Finally the residue was dissolved in a small amount of water and ethanol was added to cause precipitation. Precipitates were collected by centrifugation, washed well with aqueous ethanol, and dried over P_2O_5 in a desiccator *in vacuo*. Yield was 1.82 g (2.23 mmoles, 56%).

O^5,N^6-Diacetyl-8-bromoadenosine 2′,3′-Cyclic Phosphate——Barium salt obtained as above was suspended in water and shaked with added Dowex 50 (pyridinium) resin. The solution was passed through column of the same resin. Eluents were evaporated *in vacuo* and evaporated again with tri-*n*-butylamine and pyridine-water. Tributylammonium salt (0.09 mmole), thus obtained, was dissolved in water (0.3 ml) and tri-*n*-butylammine (0.18 ml) and ethyl chloroformate (20 μl) were added. After vigorous shaking the mixture for 20 min, the solvent was evaporated several times with added ethanol to obtain anhydrous 8-bromoadenosine 2′,3′-cyclic phosphate. Paper electrophoresis (buffer A): R_{pA-A} 0.56. The 2′,3′-cyclic phosphate was dissolved in DMF (0.2 ml) and dioxane (2 ml). Into the solution was added tri-*n*-butylamine (0.14 ml) and acetic anhydride (50 μl). The reaction was performed at 40° for 2 days. Solvent was evaporated, the residue was washed twice with petroleum ether and with ether. The residue was dissolved in dioxane and lyophilized. Examination by PPC in solvent A showed two spots having *Rf* 0.87 and 0.69 (adenosine 0.58, 5′-AMP 0.17). The compound of *Rf* 0.87 showed UV $\lambda_{max}^{H_2O}$ 277 nm and $\lambda_{min}^{H_2O}$ 240 nm, which is consistent with N^6,$O^{5'}$-diacetylstructure. The compound of *Rf* 0.69 had $\lambda_{max}^{H_2O}$ 266 nm, $\lambda_{min}^{H_2O}$ 236 nm and suggested to have $O^{5'}$-monoacetyl structure. The ratio of these compounds were estimated roughly 1: 3.

8-Bromoadenylyl-(2′-5′)-8-bromoadenosine and 8-Bromoadenylyl-(3′-5′)-8-bromoadenosine——Acetylated 8-bromoadenosine 2′,3′-cyclic phosphate, obtained above, and 2′,3′-ethoxymethylidene-8-bromoadenosine (60 mg) were dissolved in dioxane and lyophilized. The residue was dissolved in dioxane (0.5 ml) and tri-*n*-butylamine (0.18 ml), diphenylphosphorochloridate (70 μl) were added. After shaking the flask well, it was kept at room temperature for 24 hr. Solvent was removed by evaporation *in vacuo* and washed twice with petroleum-ether. The residue was dissolved in pyridine-water and extracted with petroleum-ether and twice with ether. Water layer was adjusted to pH 10 with conc. NH_3, stoppered and kept at 37° for 24 hr. Solvent was evaporated to its 1/3 volume and evaporated again with addition of few drops of tri-*n*-butylamine. After further evaporation with added 50% ethanol twice, the residue was kept at room temperature overnight. Solvent was evaporated twice by vacuum pump with addition of water. Residue, thus obtained, was dissolved in water (50 ml), made alkaline with 1 drop of conc. NH_3, and applied to a column (0.9×10 cm) of Dowex 1×2 (formate from, 100—200 mesh). After washing with water (300 ml), the column was eluted with 0—0.3N formic acid (1 liter—1 liter) by linear gradient technique. Fraction was collected in 10 ml each and flow rate was 40 ml/hr. Result was shown in Fig. 2. Peak 3 (fraction No. 85—102) and peak 3 (fraction No. 109—143) were collected separately, neutralized with 1N NaOH, and applied to a column (0.7×3.5 cm) of active charcoal. Washing with water (200 ml) and elution with 50% ethanol containing 2% conc. NH_3, followed by concentration *in vacuo* and lyophilization gave pure 2′-5′ isomer from the peak 2 and 3′-5′ isomer contaminated with 30% 2′-5′ isomer from the peak 3. 2′-5′ isomer was further purified by paper electrophoresis (R_{pA-A} 0.35) in buffer A. Properties were summarized below with 3′-5′ isomer.

Further Purification of Bromoadenylyl-(3′-5′)-bromoadenosine——Lyophilized powder of 3′-5′ isomer (*ca.* 30 OD_{263} units) were applied to paper electrophoresis in buffer A and band at R_{pA-A} 0.34 was cut and eluted with water. This was applied to a paper chromatography in solvent D for 1.5 day. Band at R_{BrAp}=0.15 was cut and eluted with water (total OD_{263}=15.8). The eluate was concentrated to *ca.* 3 ml and applied to a column (1.7×80 cm, bed volume 161 ml) of Biogel P-2. Elution was carried out with water made slightly alkaline with NH_3. Fraction was collected in 3 g each and flow rate was 3.5 g/10 min. Fraction No. 47 and 48 were pooled and evaporated, during which crystalline material precipitated. The precipitate was dissolved in water and applied to a column (1.1×56 cm, bed volume 50 ml) of Sephadex G-25 (200—400 mesh). Elution was performed with slightly alkaline water. Fractions were collected each 1 g in a flow rate of 1 g/5 min. Fractions 40 and 41 were evaporated and applied to paper electrophoresis. Yield was 8 OD_{264} units.

The rest of the lyophilized powder of 3′-5′ isomer (80 OD_{263} units) was applied to preparative paper electrophoresis and band at R_{pA-A} 0.34 was cut and eluted with water. The dimer portion, thus obtained, was applied to preparative paper chromatography (solvetnt D) and band appeared at R_{ApBr} 0.14 was eluted with water 3 times (40+20+20 ml). Nucleotide (OD_{263}44) was concentrated to *ca.* 50 ml and applied to a column (0.7×5 cm) of active charcoal (Wako, for chromatography). The column was washed with water (100 ml) and 50% ethanol (20 ml). Elution with 50% ethanol containing 0.2% conc. NH_3 (60 ml) gave $A^{Br}pA^{Br}$ in a yield of OD_{263}41. This material was further purified with paper electrophoresis in buffer A.

Properties of Bromoadenylyl-(2′-5′)-and-(3′-5′)-bromoadenosine——i) 2′-5′ Isomer: UV: λ_{max}^{pH2} 262.5 nm (ε 15800); λ_{max}^{pH7} 264.5 nm (13100); λ_{min}^{pH2} 232 nm (3800); λ_{min}^{pH7} 235 nm (3900). ε (p) calcd. was 2.62×10^4. PPC and PEP were summarized in Table I. Hyperchromicity obtained by venom phosphodiesterase digestion was 17%. Hyperchromicity was 14%. CD was recorded in Fig. 4b. Properties in paper chromato-

Table I. Properties of Different Compounds in Paper Chromatography and Paper Electrophoresis

Compound	Paper elec tro-phoresis[a]	Paper chromatography[b]			
	A	A	B	C	D
8-Bromoadenosine	0.00	0.67	0.60	0.72	0.08
8-Bromoadenosine 2′-monophosphate	1.00				0.18
8-Bromoadenosine 3′-monophosphate	1.00	0.20	0.22	0.47	0.11
8-Bromoadenyl-(2′-5′)-8-bromoadenosine	0.42	0.35	0.36	0.45	0.05
8-Bromoadenyl-(3′-5′)-8-bromoadenosine	0.35	0.36	0.38	0.51	0.02
8-Oxyadenosine	0.37	0.64	0.36	0.61	0.26
8-Oxyadenosine 5′-monophosphate	1.15	0.15	0.05	0.29	0.47
8-Oxyadenylyl-(3′-5′)-8-bromoadenosine	0.53	0.31	0.18	0.39	0.05

a) Conditions see text, R_{BrAp} values were presented.
b) Solvents see text, *Rf* values were presented.

graphy were shown in Table I.

ii) 3′-5′ Isomer: UV: $\lambda_{max}^{pH\,2}$ 262.5 nm (16200); $\lambda_{max}^{pH\,2}$ 263.5 nm (14500); $\lambda_{min}^{pH\,2}$ 232 nm (3700); $\lambda_{min}^{pH\,2}$ 232.5 nm (4000). ε (p) calcd. was 2.90×10^4. Hyperchromicity was 11% and hypochromicity was 10%. CD was recorded in Fig. 4a. Properties in paper chromatography were shown in Table I.

Enzymatic Digestion of Bromoadenylyl-(2′-5′)- and -(3′-5′)-bromoadenosine——i) Venom Phosphodiesterase: Incubation condition was same as in the case of HOA_pBrA. Both isomers showed complete diges tion to give bromoadenosine (R_{ApBr} 0.05, A_{266} 0.45) and bromoadenosine 5′p (R_{BrAp} 1.11, A_{264} 0.46). The ratio of pBrA: BrA=1.00: 0.97.

ii) Spleen Phosphodiesterase: Incubation mixture (100 μl) contained 1M NH_4OAc (pH 5.7) 20 μl, enzyme (20 units/ml) 20 μl and nucleotide 3 OD_{264} units. Incubation was carried out at 37° for 4 hr. Incubation mixture was applied to PEP in buffer A. 2′-5′ isomer was almost completely resistant to the hydrolysis. 3′-5′ isomer was hydrolyzed to an extent of 87% to give BrAp (R_{ApBr_ABr} 0.97, λ_{max} 267.5 nm, A_{max} 0.07), BrAp (R_{ApBr_ABr} 0.59, λ_{max} 266 nm, A_{max} 0.29) and BrA (R_{ApBr_ABr} 0.02, λ_{max} 264.5 nm, OD_{max} 0.34). BrAp was completely digested with RNase M at 37° for 4 hr to produce BrAp. The ratio BrAp+BrAp>:BrA=1.0:1.1.

iii) RNase M: Incubation mixture (100 μl) contained 1 M NH_4OAc (pH 6.3) 10 μl, enzyme (1 mg/ml) and nucleotide 3 OD_{max} units. Incubation was carried out at 37° for 4 hr. 2′-5′ isomer was resistant to hydrolysis. 3′-5′ isomer was completely hydrolyzed to give BrAp (R_{ArBr_ABr} 0.94, λ_{max} 266 nm, A_{max} 0.40) and BrA (R_{ApBr_ABr} 0.14, λ_{max} 263.5 nm, A_{max} 0.412). Ratio of BrA:BrAp=1.0:1.0.