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INHIBITORY EFFECT OF 8-SUBSTITUTED ADENOSINE DERIVATIVES ON Ca^{++} AND MODULATOR PROTEIN-DEPENDENT PHOSPHODIESTERASE ACTIVITY

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Summary: 8-Substituted adenosine and cyclic AMP derivatives exhibited some negative Cotton effects in circular Dichroism at B_{2u} band in pH 7.5 solution, suggesting that these derivatives take *syn* conformation. The adenosine derivatives, as well as cyclic AMP derivatives, competitively inhibited the cyclic AMP hydrolyzing activity in Ca^{++} and modulator protein-dependent phosphodiesterase preparation from hog brain cortex. The inhibitory potential of an adenosine derivative was lower than that of the cyclic AMP derivative having the same substituent by the lack of the phosphate moiety for which affinity was 0.5 kcal / mol. These results may suggest that the cyclic AMP hydrolyzing site on the enzyme requires the *syn* conformation of purine riboside.

We have reported the inhibitory effect of 8-substituted cyclic AMP derivatives on the phosphodiesterase activity in the partially purified preparation from hog brain cortex (1). Almost all of the derivatives were found to be competitive inhibitor of the enzyme; 8-hexylthio-cyclic AMP was the strongest inhibitor. A cyclic AMP derivatives having a bulky group at the 8 position is considered to take *syn* conformation, while cyclic AMP takes both

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Abbreviations used are; 8,2'-O-anhydro-c-AMP, 8,2'-anhydro-9- -D-arabinofuranosyl-8-hydroxyadenine 3',5'-monophosphate; 8,2'-CH_3N-anhydro-c-AMP, 8,2'-anhydro-9- -D-arabino-furanosyl-8-methylaminoadenine 3',5'-monophosphate; 8,2'-O-anhydro-As, 8,2'-anhydro-9- -D-arabinofuranosyl-8-hydroxyadenine.

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syn and *anti* conformations in aqueous solution (2,3,4,5). These facts may suggest that the cyclic AMP hydrolyzing site of the phosphodiesterase requires the *syn* conformation of cyclic AMP derivatives as inhibitor.

To investigate the above concept, we have tested the effect of 8-substituted adenosine derivatives on the cyclic AMP hydrolyzing activity of the Ca^{++} and modulator protein-dependent phosphodiesterase prepared from hog brain cortex, and have examined the circular dichroism of the adenosine derivatives.

MATERIAL and METHODS

Cyclic AMP and 8-substituted cyclic AMP derivatives were prepared according to the method described previously (1). This method was applied to the synthesis of 8-substituted adenosine deivatives. 8-Bromoadenosine (2.8 g; 8 mmol) was refluxed in 70 ml of ethanol containing 3.4 ml of n-butanethiol (32 mmol) and sodium methoxide (24 mmol) for 1 h, and then the reaction mixture was dried and dissolved in the minimum volume of water. The solution was adjusted to pH 8.5 and stirred at cold room overnight. The obtained crystallite was further recrystallized in tha same manner until indicating one spot in paper chromatography and electrophoresis. 2.32 g of 8-butylthioadenosine was obtained in a 81 % yield: λ_{max} (pH 2.0) 285 nm (ϵ 19700), λ_{max} (pH 13) 283 nm; R_f on paper chromatography (n-butanol,acetate,water=4:1:5) 0.60; melting point, 182 °C; Mass spectrum, m/e 355 for 8-butylthioadenosine, 223 for 8-butylthioadenine, 167 for 8-hydrothioadenine. The other derivatives were synthesized in the same way. 8-Pentylthioadenosine; λ_{max} (pH2.0) 285 nm (ϵ 21000), λ_{max} (pH13), 283 nm; R_f, 0.80; melting point,183 °C. 8-Hexylthioadenosine; λ_{max} (pH2.0) 285 nm (ϵ 20200), λ_{max} (pH13) 283 nm; R_f, 0.87; melting point, 182 °C. 8-Heptylthioadenosine; λ_{max} (pH2.0) 285 nm (ϵ 20500), λ_{max} (pH13) 283 nm; R_f, 0.88; melting point, 185 °C.
8,2'-Anhydro-9-β-D-arabinofuranosyl-8-hydroxyadenine 3',5'-monophosphate(8,2'-O-anhydro-c-AMP), and 8,2'-anhydro-9-β-D-arabinofuranosyl-8-methylaminoadenine 3',5'-monophosphate (8,2'-CH_3N-anhydro-c-AMP) were synthesized by Khwaja's method (10).
8,2'-Anhydro-9-β-D-arabinofuranosyl-8-hydroxyadenine(8,2'-O-anhydro-As) was synthesized by Ikehara's method (6) with a minor modification.

Circular dichroism determination was performed at appropriate concentrations of derivatives (0.08 mM to 0.1 mM) at 24 °C in a 10 mm pathlength cell using a JASCO Model J-20 Spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo). The solution was 50 mM Tris-Cl (pH 7.5).

Modulator protein-deficient phosphodiesterase was prepared by Teo and Wang's method (7) with a minor modification; the procedure included fractionation of 105000 x g supernatant with 55 % $(NH_4)_2SO_4$, and repeated runs of DEAE column chromatography with linear gradient elution from 0.08 M to 0.4 M NaCl (in the presence of 0.2 mM glycoletherdiamine-teraacetic acid (EGTA)).

Modulator protein, prepared by Yazawa's method (8), showed a single band upon polyacrylamide-gel electerophoresis.

Phosphodiesterase activity was measured by Weiss' method (9). The reaction was initiated by adding the enzyme preparation to the mixture containing 80 mM Tris-Cl (pH 7.5), 2.0 mM $MgCl_2$, 0.4 mM $CaCl_2$, 2.0 mM dithiothreitol (DTT), 20 μg of bovine serum albumin (BSA), 0.45 μg of modulator protein, various concentrations of cyclic AMP, and various concentrations of inhibitor. After 15 min incubation at 30 °C, the reaction was stopped by boiling for 2 min. 5'-AMP produced was then converted to ATP by the second stage incubation (2 hs at 37 °C): the reaction was carried out by adding 0.5 ml of mixture containing 50 mM Tris-Cl (pH 7.5), 1.0 mM $MgCl_2$, 0.5 mM EGTA, 5.0 mM DTT, 0.2 mM phosphoenolpyruvate, 10 nM ATP, 25 μg of BSA, 5.0 μg of pyruvate kinase, and 2.5 μg of myokinase. ATP thus produced was determined in luciferin-luciferase system using ATP photometer Model 2000 (SAI Co., USA).

Firefly extract was purchased from Sigma (No,FLE-50). Pyruvate kinase, myokinase and phosphoenolpyruvate were purchased from Boehringer Mannheim-Yamanouchi (Tokyo, Japan).

RESULT and DISCUSSION

Fig. 1 shows the circular dichroism (CD) spectra of cyclic AMP, adenosine, and their derivatives in pH 7.5 solution. Cyclic AMP or its derivatives show a negative Cotton effect at B_{2u} band (258 nm or 278 nm). The CD profiles of these compounds are similar to one another. However, the magnitude of the negative Cotton effect of derivatives is about one and half times larger than that of cyclic AMP (Table I). Similar results were obtained with adenosine and its derivatives. The magnitude of the effect of adenosine derivatives is about four times larger than that of adenosine. On the other hand, the magnitude of the effect of adenosine derivatives is larger than that of cyclic AMP derivatives, and that of adenosine itself is lower than that of any nucleosides and nucleotides used in this experiment.

It is well established that the 8-substituted derivatives of purine nucleoside showing large negative Cotton effect at B_{2u} band take *syn* conformation in aqueous solution, because of steric interference between the 8-substituent and the furanosyl moiety (2,3,9). While, cyclic AMP or adenosine shows a small negative

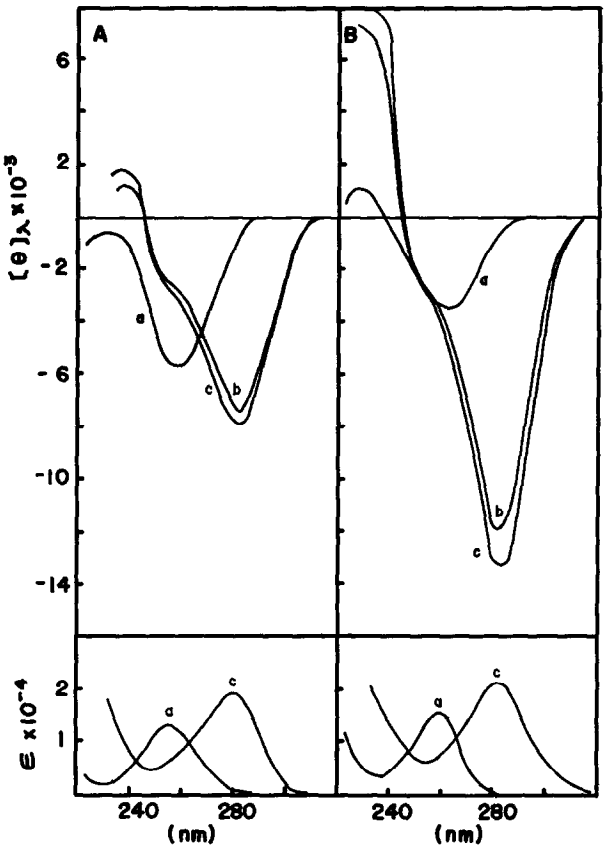

Fig. 1. CD profiles of cyclic AMP, adenosine, and their derivatives in 50 mM Tris-Cl (pH 7.5).
(A): (a)- cyclic AMP; (b)- 8-butylthio-cyclic AMP; (c)- 8-heptylthio-cyclic AMP.
(B): (a)- adenosine; (b)- 8-butylthioadenosine; (c)- 8-heptylthioadenosine.

Cotton effect in aqueous solution, suggesting that these take both *syn* and *anti* comformations. Taking only either *syn* or *anti* conformation, the compound should exhibit a large negative or positive Cotton effect at B_{2u} band (10,11,12).

Ikehara and his colleagues have reported the relationship between the magnitude of Cotton effect and the torsion angle of purine riboside, using anti-form adenosine derivatives (13). Ikehara's result may suggest that the difference between the mag-

Table I Circular Dichroism Properties of Cyclic AMP, Adenosine, and Their Derivatives

Substituent	UV Peak (B_{2U}) at pH 7.5	Sign	Amplitude $[\theta]_{\lambda} \times 10^{-3}$
H- (c-AMP)	258 nm	(−)	5.66
$CH_3(CH_2)_3S$-	283	(−)	8.29
$CH_3(CH_2)_4S$-	283	(−)	7.98
$CH_3(CH_2)_5S$-	283	(−)	6.77
$CH_3(CH_2)_6S$-	283	(−)	8.06
H- (adenosine)	262 nm	(−)	3.36
$CH_3(CH_2)_3S$-	282	(−)	11.2
$CH_3(CH_2)_4S$-	282	(−)	14.0
$CH_3(CH_2)_5S$-	282	(−)	13.1
$CH_3(CH_2)_6S$-	282	(−)	13.3

nitude of the cyclic AMP derivative and adenosine derivative results from the difference in the torsion angle.

Effects of 8-substituted cyclic AMP or adenosine derivatives on the cyclic AMP hydrolyzing activity of the Ca^{++} and modulator protein-dependent phosphodiesterase from hog brain cortex were examined. Fig. 2 shows that 8-heptylthio-cyclic AMP competitively inhibits the phosphodiesterase activity. Other cyclic AMP derivatives were also found to inhibit the activity competitively. Table II exhibits that the inhibitory potential of derivatives increases according to the elongation of substituent, and that 8-heptylthio-cyclic AMP has the highest potential, as observed in previous report using the modulator protein-deficient phosphodiesterase (1).

Similar result was obtained with 8-substituted adenosine derivatives (Fig. 2 and Table II). The inhibitory potential of an adenosine derivative was found to be lower than that of the cyclic AMP derivative having the same substituent. This difference in

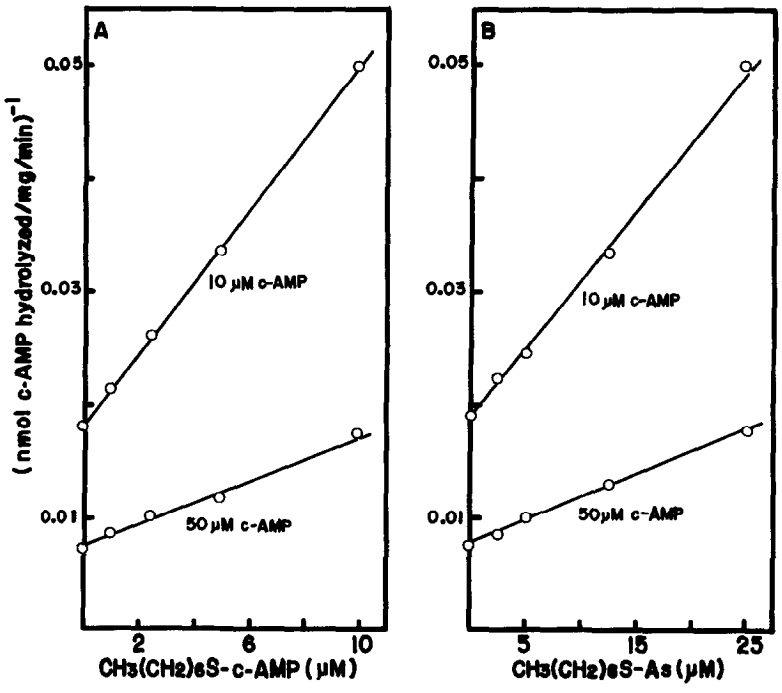

Fig. 2. Inhibitory effect of cyclic AMP or adenosine derivative on the cyclic AMP hydrolizing activity of $Ca^{++}$and modulator protein-dependent phosphodiesterase. The procedures are described in the text. The substrate concentrations were 10 μM and 50 μM. c-AMP; adenosine 3',5'-monophosphate(cyclic AMP).

Table II Apparent Ki Value and Standard Affinity of Derivatives for Phosphodiesterase

Substituent	Cyclic AMP Derivative		Adenosine Derivative		Difference[1] (kcal/mol)
	Apparent Ki (μM)	Standard Affinity (kcal/mol)	Apparent Ki (μM)	Standard Affinity (kcal/mol)	
H-	(Km; 20)	6.49	3000	3.48	
$CH_3(CH_2)_3S$-	25.9	6.33	69.3	5.74	0.59
$CH_3(CH_2)_4S$-	13.4	6.72	34.5	6.16	0.56
$CH_3(CH_2)_5S$-	7.4	7.08	16.3	6.61	0.47
$CH_3(CH_2)_6S$-	4.1	7.43	9.9	6.91	0.52

[1] This value was calculated as the difference between the standard affinity of cyclic AMP derivative and adenosine derivative having the same substituent. The mean value of Difference is 0.54±0.045(kcal/mol).

the inhibitory potential should be based on the affinity for the phosphate moiety of cyclic AMP derivative, because these cyclic

AMP and adenosine derivatives were similar in inhibitory effect and CD pattern. Table II shows the standard affinities of derivatives, which are calculated from the apparent Ki value of derivatives described previously (1), and the difference between the standard affinity of cyclic AMP derivative and adenosine derivative having the same substituent. This difference is almost constant (0.54 ± 0.045 kcal / mol). This may interpret the above concept.

On the other hand, anti-form derivatives, 8,2'-O-anhydro-c-AMP, 8,2'-CH_3N-anhydro-c-AMP, and 8,2'-O-anhydro-As, were confirmed to be no inhibitor (11). Adenosine was also confirmed to be a poor inhibitor and to have more than 3000 μM of apparent Ki (15).

These facts may suggest that 8-substituted derivatives taking *syn* conformation share the cyclic AMP hydrolyzing site on the phosphodiesterase, and also may suggest that the cyclic AMP hydrolyzing site requires the *syn* conformation of purine nucleoside.

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