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Synthesis and Enzymatic Activity of Adenosine 3′,5′-Cyclic Phosphate Analogs[1]

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2-Mono- and 2,6-di-substituted cAMP (cAMP: adenosine 3′,5′-cyclic phosphate) were derived from the corresponding adenosine analogs *via* the 5′-phosphates. 2-Substituted cAMP and aristeromycin 3′,6′-cyclic phosphate were led to the 8-bromo derivatives. 8-(3,5-Dimethylpyrazol-1-yl)-cAMP was derived from 8-hydrazinoadenosine. 8-(2-Hydroxypropyl-2)-cAMP was prepared by a γ-ray irradiation of cAMP in isopropanol. N^6-Butyl-2-phenyl-cAMP was derived from 2-phenylinosine. 2- or 8-Substituted cAMP was led to its dibutyryl derivative. The activities of these new analogs were assessed with cAMP-dependent protein kinases (PK) and cAMP phosphodiesterases (PDE). 8-Bromo-2-chloro-, 2-chloro-, 2-bromo-, 2-phenylthio-, 8-carbamoyl- and 8-carboxy-cAMP were better activators of PK than cAMP, while 2-substituted analogs were significant substrates and inhibitors of PDE. 2-Phenyl-, benzyl-, phenoxy-, chloro- and bromo-cAMP or aristeromycin 3′,6′-cyclic phosphate had an inhibitory effect on the binding of cAMP to PK equal to or more than cAMP.

Keywards——cyclic AMP; phosphodiesterase; protein kinase; structure–activity relationship; aristeromycin; enzyme activation; substrate; enzyme inhibitor; *syn-anti* conformation; cAMP analogs

The prominent regulatory role of adenosine 3′,5′-cyclic phosphate (cAMP) as a second messenger in animal tissues has been well established. Several research groups have modified the cAMP molecule in different positions of the purine base, the ribose and the phosphate moiety with the expectation of creating more specific and higher biological activity and cell penetration than cAMP itself.[3] This paper deals with the synthesis of 2-substituted analogs[4] and other novel cAMP analogs possessing 8-mono-, N^6,2-di-, 2,8-di- and 8,2′-di-, and N^6, 8,2′-tri- and 2,8,2′-tri-substituents, as well as their activities to cAMP-dependent protein kinases and cAMP phosphodiesterases.

Synthesis

2-Mono- and N^6, 2-di-substituted adenosine were selectively phosphorylated at the 5′-position by treating the corresponding nucleosides with pyrophosphoryl chloride in aceto-

1) A part of this paper was presented at the 3rd Symposium on Nucleic Acid Chemistry, Sapporo, September 1975.

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3) P. Greengard and G.A. Robison (ed.), "Advances in Cyclic Nucleotide Research," Vol. 3, Raven Press, New York, N.Y., 1973, p. 247.

4) When we began our work, 2-amino-cAMP was the only known 2-substituted analog. After we had finished our work and applied for a Japanese patent [Japanese Patent Publication (unexamined) 1974—11896 (filing data; May 15, 1972)], Jastorff and Freist[5] published the synthesis and biological activity of 2-halogeno-cAMP, and Robins, *et al.*[6] reported some 2-substituted analogs, which were prepared by a route different from that of ours.

5) *a*) B. Jastorff and W. Freist, *Angew. Chem.*, **84**, 711 (1972); *b*) *Idem*, *Bioorg. Chem.*, **3**, 103 (1973).

6) *a*) R.B. Meyer, Jr., D.A. Shuman, and R.K. Robins, *J. Am. Chem. Soc.*, **96**, 4962 (1974); *b*) R.B. Meyer, Jr., H. Uno, R.K. Robins, L.N. Simon, and J.P. Miller, *Biochem.*, **14**, 3315 (1975).

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nitrile[7] or with phosphoryl oxychloride in triethyl phosphate.[8] The resulting 5′-nucleotides were cyclized with dicyclohexylcarbodiimide (DCC) in boiling pyridine[9] to the 3′,5′-cyclic phosphates (Chart 1). 2-Substituted cAMP and aristeromycin 3′,6′-cyclic phosphate were treated with bromine in a buffer solution[10] to give the corresponding 8-bromo derivatives (Chart 2). 8-Hydrazinoadenosine was allowed to react with acetylacetone to afford 8-(3,5-dimethylpyrazol-1-yl)adenosine, which was led to the 3′,5′-cyclic phosphate (Chart 3). cAMP was irradiated in isopropanol with ^{60}Co γ-rays[11] to give 8-(2-hydroxypropyl-2)-cAMP (Chart 4). 2-Phenylinosine was converted to N^6-butyl-2-phenyladenosine *via* 6-chloro-2-phenylnebularine, and the nucleoside was led to the 3′,5′-cyclic phosphate (Chart 5). 2- And 8-substituted cAMPs were acylated with butyric anhydride in pyridine[12] to give the corresponding N^6, 2′-O-dibutyryl derivatives, while 8-bromo-2-chloro-cAMP afforded the 2′-O-monobutyryl derivative under the same conditions. Introduction of two halogen atoms to the purine ring affected the electron density[13] of the 6-amino group, thus bringing about resistance against

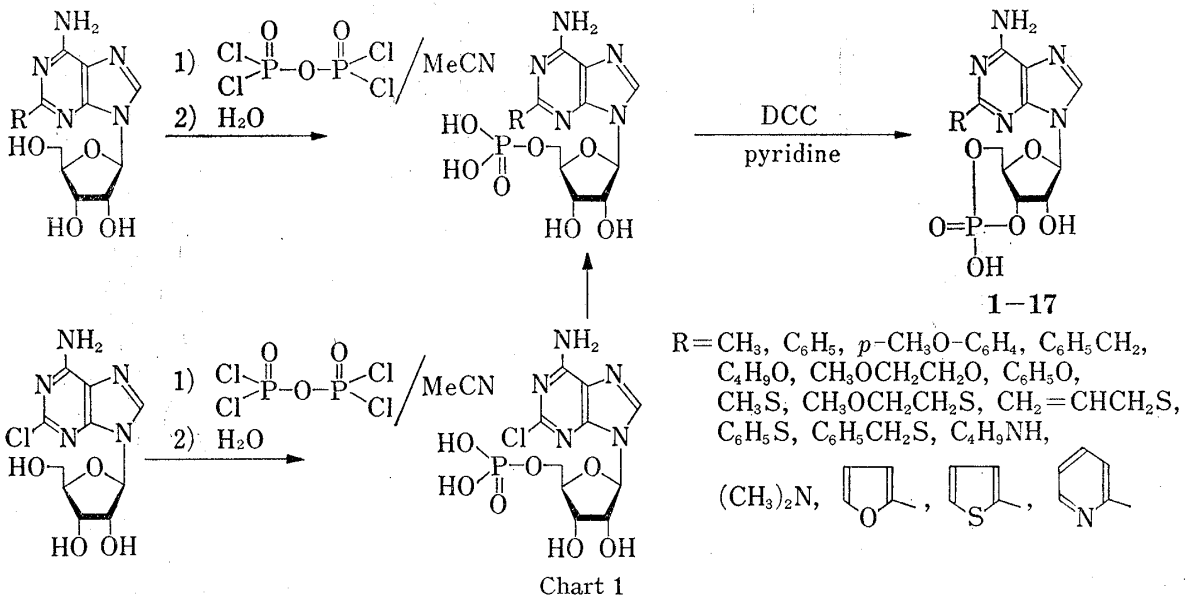

Chart 1

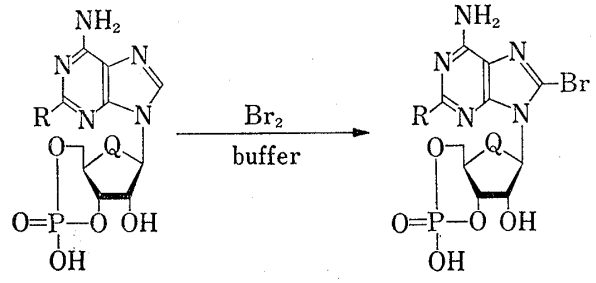

Chart 2

7) K. Imai, S. Fujii, K. Takanohashi, Y. Furukawa, T. Masuda, and M. Honjo, *J. Org. Chem.*, 34, 1547 (1969).
8) M. Yoshikawa, T. Kato, and T. Takenishi, *Tetrahedron Lett.*, **1967**, 5065.
9) R. Lohrmann and H.G. Khorana, *J. Am. Chem. Soc.*, **88**, 829 (1966).
10) M. Ikehara and S. Uesugi, *Chem. Pharm. Bull.* (Tokyo), **17**, 348 (1969).
11) *a*) H. Steinmaus, I. Rosenthal, and D. Elad, *J. Am. Chem. Soc.*, **91**, 4291 (1969); *b*) D. Lenow and D. Elad, *J. Org. Chem.*, **39**, 1470 (1974); *c*) M. Ikehara, S. Uesugi, and K. Yoshida, *Biochem.*, **11**, 830 (1972).
12) Th. Posternak, E.W. Sutherland, and W.F. Henion, *Biochim. Biophys. Acta*, **65**, 558 (1962).
13) The effect was also observed by the down-field shift of the N^6-amino group in the nuclear magnetic resonance (NMR) spectrum of 8-bromo-2-chloro-adenosine. NMR (d_6-DMSO) δ: 8.38 (1H, s, NH_2 of 8-Br-2-Cl-adenosine) [7.50 (1H, s, NH_2 of adenosine)].

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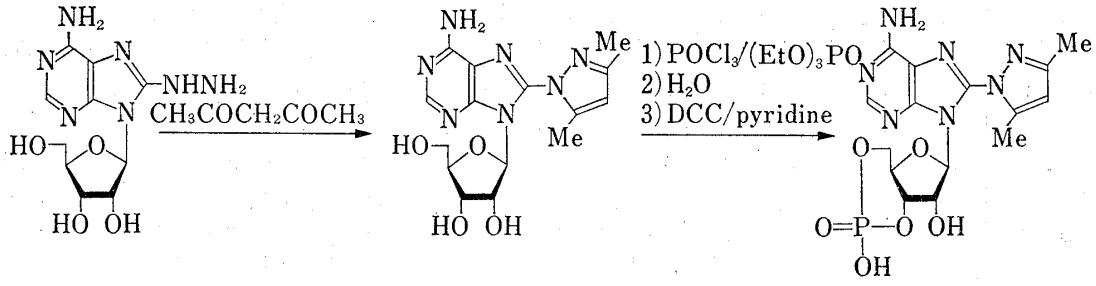

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Chart 3

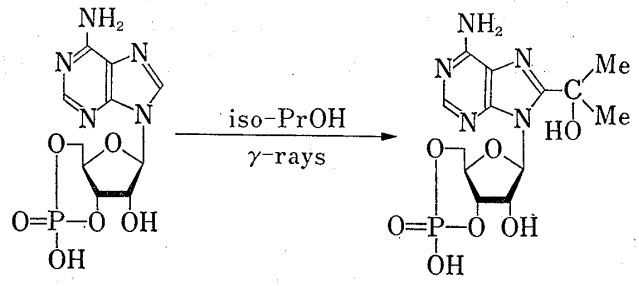

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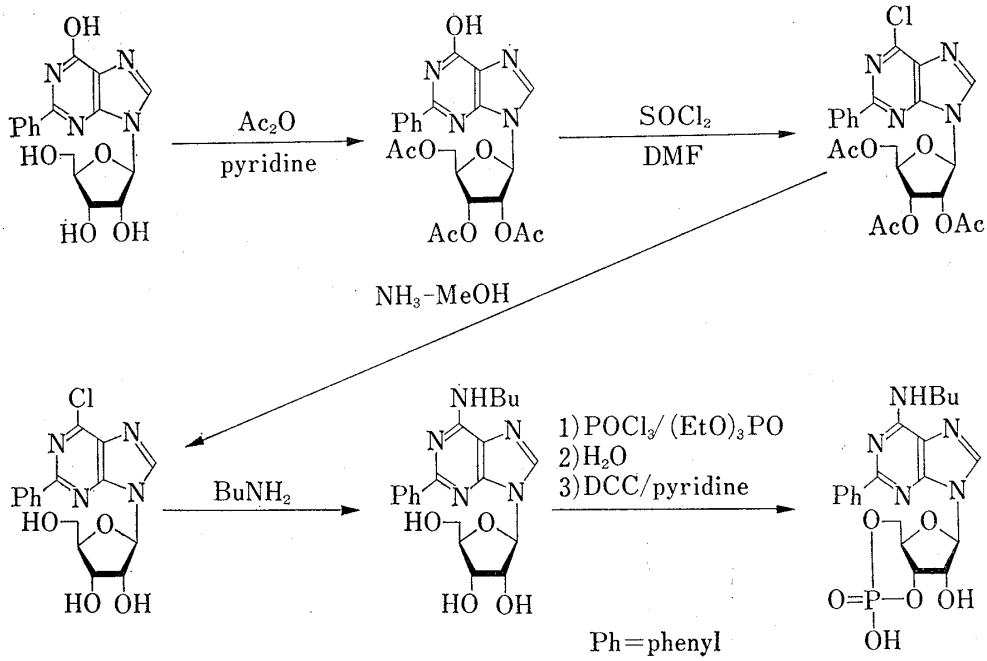
Chart 4



Ph=phenyl

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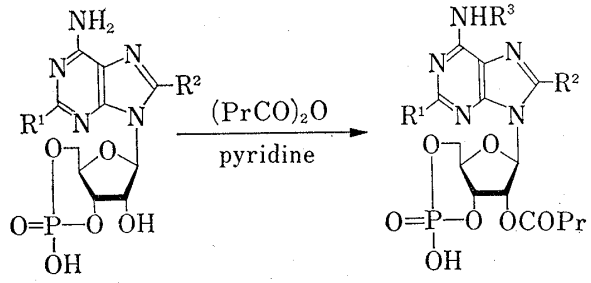
Chart 5



23 : $R^1=C_6H_5$, $R^2=H$, $R^3=PrCO$
24 : $R^1=p$-$MeOC_6H_4$, $R^2=H$, $R^3=PrCO$
25 : $R^1=H$, $R^2=CONH_2$, $R^3=PrCO$
26 : $R^1=Cl$, $R^2=Br$, $R^3=H$

Chart 6

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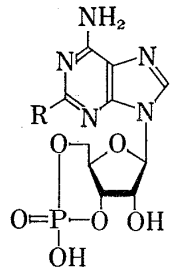
TABLE I. Physical Properties and Elemental Analyses of cAMP Analog



Compd.	R	mp (°C)	Synthetic route	$[\alpha]_D^{25}$ in 1N NaOH (Concentration, %)	UV λ_{max} nm (pH)	Formula	Analysis (%) Calcd. (Found) C	H	N	P	S
1	CH_3		1	−41.2° (0.8)	261 (1); 265 (13)	$C_{11}H_{13}N_5O_6P \cdot NH_4 \cdot EtOH \cdot H_2O$	36.80 (36.62	5.94 5.10	19.80 19.69	7.30 7.59)	
2	C_6H_5	240	1	−2.6° (1.0)	270, 295 (1); 239, 268 (13)	$C_{16}H_{16}N_5O_6P \cdot H_2O$	45.39 (45.36	4.28 4.00	16.54 16.47	7.33 7.72)	
3	p-CH_3OC_6H	229	1	+3.3° (1.0)	267 (7)	$C_{17}H_{18}N_5O_7P \cdot \frac{1}{2}H_2O$	45.95 (46.12	4.30 4.76	15.76 15.37	6.98 6.23)	
4	$C_6H_5CH_2$		1		265 (MeOH)	$C_{17}H_{14}N_4O_6P \cdot NH_4 \cdot 1\frac{1}{2}H_2O$	44.06 (43.66	5.22 4.55	18.14 16.82	6.68 6.68)	
5	n-C_4H_9O		1	−37.4° (1.0)	248, 276 (2); 253[a], 268 (13)	$C_{14}H_{19}N_5O_7P \cdot NH_4 \cdot \frac{1}{2}(CH_3)_2CO \cdot H_2O$	39.99 (39.81	6.06 5.45	18.05 18.03	6.66 7.36)	
6	$CH_3OCH_2CH_2O$		1	−33.4° (1.0)	248, 273 (2); 266 (13)	$C_{13}H_{17}N_5O_8P \cdot NH_4 \cdot (CH_3)_2CO$	38.24 (38.51	5.27 5.07	19.11 19.53	7.05 7.48)	
7	C_6H_5O		1		266 (MeOH)	$C_{16}H_{15}N_5O_7P \cdot NH_4 \cdot 2\frac{1}{2}H_2O$	39.75 (39.28	5.00 4.55	17.39 17.97	6.54 7.06)	
8	CH_3S		1		271 (2); 235, 276 (12)	$C_{11}H_{13}N_5O_6PS \cdot Et_3NH$	42.84 (42.35	6.13 6.09	17.64 17.35	6.51 6.53)	
9	$CH_3OCH_2CH_2S$–		1	−13.5° (1.0)	225[a], 265, 285[a] (2); 235, 275 (13)	$C_{13}H_{17}N_5O_7PS \cdot NH_4 \cdot \frac{1}{3}(CH_3)_2CO$	36.89 (36.55	5.08 5.13	18.44 18.37	6.81 6.59)	
10	CH_2=$CHCH_2S$		1	−16.3° (1.0)	225[a], 271, 290[a] (2); 235, 276 (H_2O)	$C_{13}H_{15}N_5O_6PS \cdot NH_4 \cdot \frac{1}{2}(CH_3)_2CO$	38.96 (39.19	4.95 4.90	18.80 18.44	6.94 6.96)	
11	C_6H_5S		1	−9.0° (1.0)	235, 280 (2); 285 (13)	$C_{16}H_{15}N_5O_6PS \cdot NH_4 \cdot H_2O$	40.67 (41.20	4.48 4.41	17.78 17.57	6.56 6.96)	

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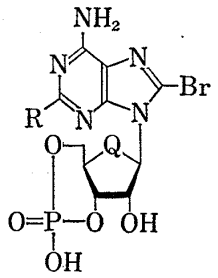
Compd.	R	mp (°C)	Synthetic route	$[\alpha]_D^{25}$ in 1N NaOH (Concentration, %)	UV λ_{max} nm (pH)	Formula	Analysis (%) Calcd. (Found) C	H	N	P	S
12	$C_6H_5CH_2S$	220	1	+14.3° (1.0)	235[a], 273 (2); 236, 276 (13)	$C_{17}H_{18}N_5O_6PS$	45.23 (45.16	4.02 4.47	15.52 14.79	6.87 5.94	7.10 7.28)
13	*n*-C_4H_9NH		1	−4.4° (1.0)	265 (2); 264 (13)	$C_{14}H_{21}N_6O_6P \cdot 2H_2O$	38.52 (38.54	5.77 5.61	19.26 19.84	7.11 7.28)	
14	$(CH_3)_2N$–	254—265	1		210, 260, 306 (2); 224, 262.5, 294.5 (13)	$C_{12}H_{17}N_6O_6P \cdot H_2O$	36.93 (36.98	4.91 4.83	21.53 21.46	7.94 7.68)	
15			1	+4.6° (1.0)	285, 320 (2): 253[a], 259, 287[a] (13)	$C_{14}H_{14}N_5O_7P \cdot H_2O$	40.58 (40.99	3.89 3.92	16.90 16.98	7.48 7.84)	
16			1		213, 274, 324 (2); 253, 308 (13)	$C_{14}H_{14}N_5O_6PS \cdot Et_3NH \cdot H_2O$	45.27 (45.66	5.88 5.71	15.84 15.83	5.88 5.89)	
17			1		233, 263, 329 (1); 232, 263, 289 (13)	$C_{15}H_{15}O_6N_6P \cdot 1.2(CH_3)_2CO \cdot H_2O$	45.22 (46.06	4.94 5.03	17.01 17.04	6.27 6.44)	



Compd.	R	Q	mp (°C)	Synthetic route	UV λ_{max} nm (pH)	Formula	Analysis (%) Calcd. (Found) C	H	N	P	Br
18	C_6H_5	O		2	275, 305[a] (1); 245, 280—290 (12)	$C_{16}H_{14}BrN_5O_6P \cdot Et_3NH$				5.29 (5.27	13.65 13.71)
19	H	CH_2	240	2	265 (2); 267 (12)	$C_{11}H_{15}BrN_5O_5P \cdot NH_4 \cdot 3H_2O$	27.68 (27.40	4.64 4.33	17.61 18.01	6.50 6.27)	

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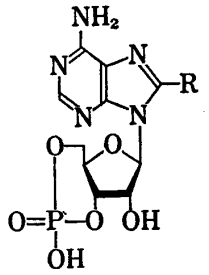
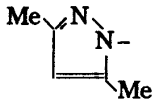
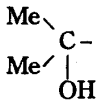
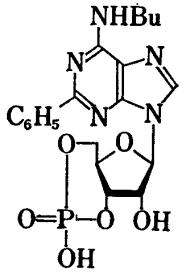


Compd.	R	Synthetic route	UV λ_{max} nm (pH)	Formula	Analysis (%) Calcd. (Found) C	H	N	P
20		3	270 (1, 7)	$C_{15}H_{17}N_7O_6P \cdot NH_4 \cdot 3\frac{1}{2}H_2O$	35.85 (35.88	5.62 5.37	22.30 22.71	6.17 5.84)
21		4	260 (1, 12)	$C_{13}H_{18}N_5O_7P \cdot H_2O$	38.51 (38.59	4.97 5.23	17.27 17.03)	



Compd.	Synthetic route	UV λ_{max} nm (Solvent)	Formula	Analysis (%) Calcd. (Found) C	H	N	P
22	5	249.5, 270[a], 283[a], 290, 301[a] (MeOH)	$C_{20}H_{23}N_5O_6P \cdot NH_4 \cdot H_2O$	48.38 (48.56	5.48 5.74	16.93 16.63	6.24 6.18)

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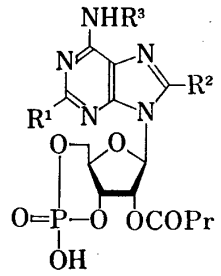

Compd.	R^1	R^2	R^3	Synthetic route	UV λ_{max} nm (Solvent)	Formula	Analysis (%) Calcd. (Found) C	H	N	P
23	C_6H_5	H	PrCO	6	247, 287 (MeOH)	$C_{24}H_{27}N_5O_8P \cdot Et_3NH \cdot H_2O$	54.20 (54.55	6.82 6.61	12.64 12.63	4.67 4.83)
24	*p*-MeO·C_6H_4	H	PrCO	6	263, 280[a], 298, 315[a] (MeOH)	$C_{25}H_{29}N_5O_9P \cdot Na \cdot H_2O$	48.78 (48.87	5.08 5.20	11.38 10.75	5.04 4.76)
25	H	H_2NCO	PrCO	6						
26	Cl	Br	H	6	266 (MeOH)	$C_{14}H_{15}BrClN_5O_7P \cdot NH_4$	31.74 (32.59	3.62 4.27	15.87 16.02	5.85 5.94)

a) Shoulder.

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the acylation at this position (Chart 6). Physical properties of the analogs are listed in Table I. The following 2-substituted cAMP: methyl-, phenyl-, (thienyl-2)-, methylthio-, allylthio-, benzylthio- and dimethylamino-cAMP were synthesized from the corresponding nucleosides by the Chart 1, but there has already been reported another method[6] for the synthesis of these analogs. The standard cAMP analogs unless otherwise noted, were prepared by the method, which were described in the literatures cited in the foot-notes of Tables.

Results and Discussion

Activation of Rat Brain, Adrenal or Liver cAMP-Dependent Protein Kinase

The results are summarized in Table II. The 2-substituted analogs [phenylthio-[14] (**11**), chloro- and bromo-cAMP], 8-substituted analogs [carbamoyl- and carboxy-cAMP, and bromo-

Table II. Relative Activity of cAMP Analogs of Rat Protein Kinases

Compd.	Relative activity[a]		
	Brain	adrenal	Liver
cAMP	1	1	1
1	7		4
2	8	7	
3	9	9	
4	6		3
5	6	7	
7	1.5		1.8
8	8		3
9	11	10	
10	7	6	
11	5		0.4
12	5	2	
13	10	11	
14	21		5
15	19		15
16	14		4
18	6		3
19	0.8		0.5
20	150		106
21	78		126
22	1.6		1.3
23	200		>500
8-Br-cAMP[b]	0.3	0.6	0.3
8-SH-cAMP[b]	0.6	0.2	0.4
8-$CONH_2$-cAMP[c]	0.8		1.5
8-CO_2H-cAMP[c]	2.3		0.9
2-Br-cAMP[d]	3		0.8
2-Cl-cAMP[d]	3		0.2
8-Br-2-Cl-cAMP[d]	0.2		0.1
Aristeromycin 3′,6′-cyclic phosphate[d]	5		4
cUMP[e]		137	
Pyrimidopyrimidinium Compound[f]			1.0

a) K_a for the analog/K_a for cAMP. K_a: Molar concentration of the analog causing 50% activation of cAMP dependent protein kinase. K_a for cAMP: rat brain (8×10^{-8} M), rat adrenal (6.7×10^{-8} M), rat liver (3.5×10^{-8} M).
b) K. Muneyama, *et al.*, *Biochem* **10**, 2390 (1971).
c) T. Naka and M. Honjo, *Chem. Pharm. Bull.* (Tokyo), **24**, 2052 (1976).
d) R. Marumoto, *et al.*, *Chem. Pharm. Bull.* (Tokyo), **23**, 2295 (1975).
e) Purchased from Boehringer Mannheim GmbH (Germany).
f) Y. Furukawa, *et al*, *Chem. Pharm. Bull.* (Tokyo), **22**, 2552 (1974). Compd. XIII.

14) This sample was prepared from the corresponding 2-substituted adenosine[15] by the route 1.
15) R. Marumoto, Y. Yoshioka, O. Miyashita, S. Shima, K. Imai, K. Kawazoe, and M. Honjo, *Chem. Pharm. Bull.* (Tokyo), 23, 759 (1975).

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aristeromycin 3′,6′-cyclic phosphate (**19**)] and 2,8-di-substituted analog (8-bromo-2-chloro-cAMP) were more effective than cAMP, and particularly 8-bromo-2-chloro-cAMP was the most active. Introduction of the bromine atom to the 8-position of the 2-substituted (*e.g.* phenyl- or chloro-) cAMP or aristeromycin 3′,6′-cyclic phosphate resulted in an increase of the activity [8-bromo-2-phenyl- (**18**), 8-bromo-2-chloro-cAMP and **19**]. This fact suggests that the oxygen in the ribofuranose ring would have little effect on the activity, and the assumption[16] that the *syn*-conformation forced by the bulky 8-substituent might be required for the activation of the protein kinase. 2-Alkylamino- [*e.g.* butylamino- (**13**) or dimethylamino- (**14**)] cAMP, 2-alkoxy- [*e.g.* butoxy- (**5**)] cAMP and 2-higher alkylthio- [*e.g.* allylthio- (**10**) or methoxyethylthio- (**9**)] cAMP were less effective than cAMP. The result would be explained by a predominant contribution of the *anti*-conformation owing to the steric hindrance between the bulky long chain of the 2-substituent and the plane of the ribose moiety.

Inhibition of Binding of cAMP to Bovine Muscle Protein Kinase

Our attention was next focused on the inhibitory effect of the analogs on the binding of cAMP to the protein kinase, as a measure of their relative affinity for the enzyme as compared to cAMP. The results are presented in Table III, which proved that the inhibition of the analogs on the cAMP binding to protein, *i.e.* the affinity of the analogs for the protein, correlated with the activation of the protein kinase except for 2-phenyl- (**2**) and 2-benzyl- (**4**) cAMP. The two analogs had a potent inhibitory activity on the cAMP binding but a relatively weak activity on the activation of the protein kinase. Compounds, **2**, **4**, **7**, 2-chloro-, 2-bromo-cAMP and aristeromycin 3′,6′-cyclic phosphate had an inhibitory effect equal to or more than cAMP. Butyrylation of cAMP and its analogs led to a decrease in the potency

Table III. Inhibition of cAMP Analogs on [^{3}H] cAMP[a] Binding to Bovine Muscle Protein Kinase

Compd.	Relative inhibitory activity[b]	Compd.	Relative inhibitory activity[b]
cAMP	1	**19**	55
N^6-Butyryl-cAMP[c]	3.3	**20**	1875
Dibutyryl-cAMP[c]	53	**21**	269
1	11	**22**	6
2	0.8	**24**	>1000
3	17	**25**	>5000
4	1.1	**26**	239
5	3	8-Br-cAMP	3
7	1	8-SH-cAMP	2
8	12	Dibutyryl-8-SH-cAMP[d]	586
9	7	8-$CONH_2$-cAMP	6
10	6	2-Br-cAMP	1.2
11	1.8	2-Cl-cAMP	1.1
12	6	8-Br-2-Cl-cAMP	4
13	1.7	Aristeromycin 3′,6′-cyclic phosphate	0.7
14	280	cCMP[c]	1773
15	23	cUMP	279
16	24		

a) 20 μM.
b) I_{50} for the analog/I_{50} for cAMP, I_{50}: molar concentration of the analog causing 50% inhibition of cAMP binding to bovine muscle protein kinase.
c) Purchased from Boehringer Mannheim GmbH (Germany).
d) Berg. Patent 802151.

16) W.B. Anderson, R.L. Perlman, and I. Pastan, *J. Biol. Chem.*, 247, 2721 (1972).

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to inhibit the cAMP binding [cAMP, 2-*p*-methoxypheny- (3), 8-mercapto- and carbamoyl-cAMP *vs.* the corresponding N^6, 2′-O-dibutyryl derivatives].

Hydrolysis by Rat and Bovine Heart cAMP Phosphodiesterases

The results are shown in Table IV. Introduction of the substituents at the 2-, 8- or 2,8-positions brought about the resistance against hydrolysis by the phosphodiesterase. The rate of hydrolysis was in the following order: cAMP 2-substituted cAMP, 2,8-disubstituted cAMP, 8-substituted cAMP. This fact supports the assumption[17] that the *syn*-conformer such as 8-substituted cAMP[18] would be resistant against the hydrolysis by phosphodiesterase and the *anti*-conformer such as cAMP[19] would be required for the enzyme, and also suggests that 2-substituted cAMPs would share the anti-conformation to a lesser extent than cAMP.

Table IV. Hydrolysis of cAMP Analoge[a] by Cardiac cAMP Phosphodiesterase (PDE)

Compd.	Rat PDE[b]	Bovine PDE[c]
cAMP	1	1
N^6-Butyryl-cAMP	0.75	
Dibutyryl-cAMP	0.45	0.19
2	0.36	0.33
3	0.36	
4	0.86	0.82
5	0.53	
7	0.95	0.83
8	0.79	0.78
9	0.93	0.76
10	0.68	0.84
11	0.89	0.93
12	0.69	0.60
13	0.49	
14	0.57	0.37
16	0.49	0.75
19	0.07	
20	0.22	
21	0.00	
22	0.27	
23	0.25	
24	0.00	
25	0.08	
26	0.06	
8-Br-cAMP	0.14	0.22
8-SH-cAMP	0.21	
Dibutyryl-8-SH-cAMP	0.16	
8-$CONH_2$-cAMP	0.00	
2-Br-cAMP	0.68	
2-Cl-cAMP	0.72	
8-Br-2-Cl-cAMP	0.28	0.26
Pyrimidopyrimidinium compound[d]	0.85	
Aristeromycin 3′,6′-cyclic phosphate	0.15	

a) Rate of hydrolysis of the analog/rate of hydrolysis of cAMP.
b) See the experimental section.
c) Purchased from Boehringer Mannheim GmbH (Germany).
d) See Table II.

17) G. Michal, K. Mühlegger, M. Nelböck, C. Thiessen, and G. Weimann, *Pharmacol. Res. Commun.*, **6**, 243 (1974).
18) 8-Substituted cAMP, cIMP and cGMP are resistant against the phosphodiesterase.[17]
19) P. Greengard and G.A. Robison (ed.), "Advances in Cyclic Nucleotide Research," Vol. 3, Raven Press, New York, N.Y., 1973, p. 240.

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Butyrylation of the analogs led to an increase of resistance against the hydrolysis by the enzyme.

Inhibition of cAMP Hydrolysis by Rat Heart or Brain cAMP Phosphodiesterase

The results are shown in Table V. The 2-substituted analogs [*p*-methoxyphenylmethylthio- (**8**), chloro- or bromo-cAMP] had a great inhibitory effect on cAMP hydrolysis, while the 8-substituted analogs (mercapto-, bromo- or carbamoyl-cAMP) had no practical effect. The effect of the 2-substituted analogs could be explained by the competitive inhibition, because they are potent substrates to the enzyme. Butyrylation of the analogs led to a decrease of the inhibitory effect on cAMP hydrolysis.

Table V. Inhibitory Activity of cAMP Analog on Hydrolysis by Rat cAMP Phosphodiesterase[a]

Compd.	I_{50} (μM)[b]	
	Heart	Brain
N^6-Butyryl-cAMP	>1000	>1000
Dibutyryl-cAMP	>1000	>1000
1	13	44
2	150	56
3	6	24
4	37	64
7	44	54
8	3	36
9	9	44
10	9	33
11	20	44
12	13	34
14	800	>1000
15	120	22
16	430	34
19	190	>1000
20	>1000	>1000
21	>1000	>1000
22	500	>1000
23	63	50
24	30	55
25	55	190
26	24	223
8-Br-cAMP	150	>1000
8-SH-cAMP	116	>1000
Dibutyryl-8-SH-cAMP	>1000	>1000
8-$CONH_2$-cAMP	>1000	>1000
2-Br-cAMP	5	28
2-Cl-cAMP	4	30
8-Br-2-Cl-cAMP	23	74
Pyrimidopyrimidinium compound[c]	>1000	>1000
cGMP[d]	>1000	136

a) The low K_m values for cAMP on rat heart and brain cAMP PDE were 0.6 and 1.3 μM, respectively.
b) Molar concentration of the analog causing 50% inhibition of the rate of cAMP hydrolysis (cAMP concentration was 1 μM).
c) See Table II.
d) Purchased from Boehringer Mannheim GmbH (Germany).

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Experimental

Synthesis[20]——2-Benzyl-cAMP (**4**): To a suspension of 2-benzyladenosine[15] (3.57 g, 10 mmol) in CH_3CN (100 ml) was added pyrophosphoryl chloride (4 ml) at 0—5°.[7] The mixture was stirred at these temperatures for 3 hr and poured into ice-water (500 ml). After standing overnight, the solution was adjusted to pH 5 with Et_3N and applied to a column of activated charcoal (30 g). The column was washed with H_2O and eluted with a mixture of BuOH–Et_3N–conc. NH_4OH–EtOH–H_2O (5: 2: 2: 45: 46 v/v). The eluate was evaporated to dryness *in vacuo*, and a portion of the residue was examined by PE (borate), which afforded a major UV absorbing spot at $M_{5'\text{-AMP}}$[21] 0.9.

The crude 2-benzyladenosine 5′-phosphate-Et_3N salt (yield, 95%) was dissolved in pyridine (300 ml), and the solution was added dropwise over 2 hr to a boiling solution of DCC (8.24 g, 40 mmol) in pyridine (300 ml). The mixture was refluxed for 3 hr, and then evaporated to dryness *in vacuo*. The residue was dissolved in water (500 ml) and an insoluble material was filtered off. To the filtrate were added water (500 ml) and MeOH (1 l) and the solution was passed through a column of DEAE-cellulose (HCO_3^-, 800 ml). The column was eluted with 5% MeOH (7 l) and then with 0.05 M NH_4HCO_3 in 50% MeOH (3 l). The desired fractions were collected and evaporated to dryness *in vacuo*. The residue was treated with EtOH to afford crystals (NH_4 salt), of which UV spectrum was superimposable upon that of 2-benzyladenosine.

2-(*p*-Methoxyphenyl)-cAMP (**3**), 2-(2-Furyl)-cAMP (**15**), 2-Butoxy-cAMP (**5**), 2-Methoxyethoxy-cAMP (**6**), 2-Phenoxy-cAMP (**7**), 2-(Methoxyethylthio)-cAMP (**9**) and 2-Phenylthio-cAMP (**11**): These 3′,5′-cyclic phosphates were prepared from the corresponding adenosine analogs[15] in a manner similar to that described in the preceding section.

2-Butylamino-cAMP (**13**): 2-Chloroadenosine[15] (0.3 g, 1 mmol) was treated with pyrophosphoryl chloride (0.4 ml) in CH_3CN (10 ml) to afford 2-chloroadenosine 5′-phosphate·NH_4 salt. To this salt were added water (2 ml) and $BuNH_2$ (4 ml). The mixture was refluxed for 10 hr and evaporated to dryness. The residue was dissolved in water (100 ml) and chromatographed on a column of DEAE cellulose (HCO_3^-, 60 ml) to obtain 2-butylaminoadenosine 5′-phosphate (NH_4 salt, 250 mg). The nucleotide was cyclized with DCC in pyridine to the desired 3′,5′-cyclic phosphate (free, 150 mg).

8-(3,5-Dimethylpyrazol-1-yl)-cAMP (**20**): To a suspension of 8-bromoadenosine (0.6 g, 1.7 mmol) in methyl cellosolve (2 ml) was added $NH_2NH_2 \cdot H_2O$ (2.5 ml, 50 mmol). The mixture was stirred at room temperature for 2.5 hr to give a homogeneous solution. The solution was evaporated to dryness *in vacuo*, the residue was dissolved in methyl cellosolve (4 ml), and the solution was evaporated again to dryness. This procedure was repeated 3 times to remove $NH_2NH_2 \cdot H_2O$. 8-Hydrazinoadenosine thus obtained was dissolved in methyl cellosolve (4 ml) and after the addition of acetylacetone (0.5 ml) the solution was kept at room temperature for 1 hr. Evaporation of the solution to dryness afforded a syrup, which was chromatographed on a column of silica gel (25 g) with $CHCl_3$–MeOH (10: 1 v/v) as eluent to afford a pale yellow syrup of 8-(3,5-dimethylpyrazol-1-yl)-adenosine (650 mg). UV λ_{max}^{pH1} nm: 270; λ_{max}^{pH7} nm: 270.

This nucleoside (3.5 g) was dissolved in $(EtO)_3PO$ (100 ml) and to the ice-cooled solution was added dropwise $POCl_3$ (3 ml) and the solution was stirred at 0—3° for 3 hr.[8] The reaction mixture was poured into ice-water (1.5 l), the solution was adjusted to pH 7 with $NaHCO_3$, and passed through a column of activated charcoal (30 g). The column was eluted with a mixture of BuOH–conc. NH_4OH–H_2O–EtOH (1: 1: 9: 9 v/v, 4 l). The eluate was evaporated to dryness *in vacuo* to afford a brown syrup, which was washed with ether to remove $(EtO)_3PO$. The 5′-phosphate was dissolved in Et_3N (4 ml) and the solution was evaporated to dryness, and a trace of water was removed by an azeotropic distillation with pyridine (150 ml). The resulting Et_3N salt was cyclized with DCC in pyridine to 3′,5′-cyclic phosphate which was chromatographed on a column of DEAE cellulose (HCO_3^-, 400 ml) to obtain a brown oil. The oil was dissolved in EtOH (30 ml) and to the solution was added a small amount of acetone to separate out a precipitate. A solution of the precipitate in a small amount of EtOH was filtered to remove an insoluble material, and the filtrate was evaporated to dryness. The residue was dissolved in hot BuOH and to the solution was added acetone to afford a yellow powder (NH_4 salt, 420 mg).

8-(2-Hydroxypropyl-2)-cAMP (**21**): A solution of cAMP-Et_3N salt (1.2 g) in a mixture of iso-PrOH (100 ml) and acetone (100 ml) was irradiated under a nitrogen atmosphere for 48 hr with a total dose of 2.4×10^{21} ev/ml/hr (^{60}Co γ-rays, dose rate, 5×10^{19} ev/ml/hr).[11] The reaction mixture was evaporated to dryness *in vacuo* to afford a yellow syrup. A solution of the syrup in H_2O was adjusted to pH 3 with dil. HCl and

20) Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. NMR spectra were recorded on a Hitachi Model R-24 spectrometer using tetramethylsilane as an internal standard. Chemical shifts were expressed in δ values. Ultraviolet (UV) spectra were measured on a Hitachi Model EPU-2A. Paper electrophoresis (PE) was run on Whatman No. 1 paper at a constant voltage of 22 v/cm in 0.05 M borate buffer (pH 9.2). Kieselgel 60 (0.063—0.200 mm, E. Merck) was used for a silica gel column chromatography. All preparations of cAMP analogs were proved to be pure by showing a single UV absorbing spot on paper electrograms.

21) Mobility ratio to 5′-AMP.

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passed through a column of activated charcoal (12 g). The column was washed with H_2O and then MeOH, and eluted with 5% Et_3N in MeOH. The eluate was evaporated to dryness to afford a powder (500 mg). A solution of the powder in H_2O was passed through a column of DEAE cellulose (HCO_3^-, 150 ml), which was washed with H_2O and eluted with 0.05 M NH_4HCO_3 to give the desired product (250 mg). UV $\lambda_{max}^{pH\,1}$ nm: 260; $\lambda_{max}^{pH\,1}$ nm: 231; $\lambda_{max}^{pH\,13}$ nm: 260; $\lambda_{max}^{pH\,13}$ nm: 230. NMR (d_6-DMSO): 1.65 (6H, s, $-C(HO)(Me)(Me)$), 6.85 (1H, s, H_1'), 8.25 (1H, s, 2-H).

N^6-Butyl-2-phenyl-cAMP (**22**): A mixture of 2-phenylinosine[22] (25 g), pyridine (150 ml) and Ac_2O (100 ml) was allowed to react at room temperature for 2 hr. The reaction mixture was evaporated to dryness, and the residue was dissolved in $CHCl_3$ (250 ml). The solution was washed with H_2O (300 ml × 2) and evaporated to dryness to give a syrup (35 g) of 2-phenyl-2′,3′,5′-tri-O-acetylinosine. This showed a single UV absorbing spot at *Rf* 0.2 (TLC, E. Merck: DC-Alufolien Kieselgel 60 F 254, layer thickness 0.2 mm, $CHCl_3$–MeOH 19: 1 v/v).

The tri-acetate described above (23 g) was dissolved in $CHCl_3$ (100 ml) and after the addition of DMF (7.5 ml) and $SOCl_2$ (22.5 ml) the solution was refluxed for 2 hr. The reaction mixture was evaporated to dryness *in vacuo*, and the residue was dissolved in $CHCl_3$ (250 ml). To the solution was added ice-water (250 ml) and the stirred mixture was adjusted to pH 7 by adding 5% aq. $NaHCO_3$.

The $CHCl_3$ layer was dried over Na_2SO_4 and evaporated to dryness. A solution of the residue in 20% NH_3–MeOH (500 ml) was kept in a refrigerator for 20 hr, and concentrated to give colorless needles of 6-chloro-2-phenylnebularine [mp 189—195° (dec.), 17 g]. The nucleoside (5 g) was dissolved in a mixture of methyl cellosolve (100 ml) and *n*-$BuNH_2$ (3 ml). The solution was heated at 120° for 3 hr, and evaporated to dryness *in vacuo*. The residue was recrystallized from EtOH to give crystalls of 6-butylamino-2-phenylnebularine (mp 156—160°, 5 g). To an ice-cooled solution of the nucleoside (1 g) in $(EtO)_3PO$ (20 ml) was added $POCl_3$ (2 ml). The mixture was kept at 0—3° for 5 hr and poured into ice-water. On adjustment to pH 5 with 1 N NaOH, a precipitate of free N^6-butyl-2-phenyladenosine 5′-phosphate separated out. The precipitate was collected by filtration, washed with water and dried. The nucleotide suspended in MeOH was dissolved immediately by the addition of Et_3N. The solution was evaporated to dryness *in vacuo*. A solution of the residue in pyridine (100 ml) was added dropwise over 1 hr to a boiling solution of DCC (2.5 g) in pyridine (200 ml). The mixture was refluxed for 2 hr and evaporated to dryness. A suspension of the residue in H_2O was stirred for 20 hr and extracted with ether to remove unreacted DCC. The aqueous layer (500 ml) was passed through a column of DEAE cellulose (HCO_3^-, 300 ml) and the fractions eluted with 0.2 M NH_4HCO_3 (3 l) was evaporated to dryness. The residue was triturated with ether to give a pale yellow powder (160 mg) of the desired 3′,5′-cyclic phosphate.

8-Bromo-2-phenyladenosine 3′,5′-Cyclic Phosphate (**18**): To a stirred and ice-cooled solution of 2-phenyl-cAMP (**2**)[14] (180 mg) in 1 M acetate buffer (pH 4.4, 10 ml) was dropwise added Br_2 (100 mg). The mixture was stirred for 30 min and excess Br_2 was decomposed by addition of 2% $NaHSO_3$. The solution was poured into water (100 ml) and desalted with a column of activated charcoal (2 g). The eluate (100 ml) was evaporated to dryness *in vacuo*. A solution of the residue in water (50 ml) was applied to a column of DEAE cellulose (HCO_3^-, 50 ml). The fractions (1.5 l) eluted with 0.1 M NH_4HCO_3 were collected and evaporated to dryness. To the residue were added Et_3N (1 ml) and water (2 ml), and the mixture was evaporated to dryness. After this procedure was repeated twice, the residue was dissolved in a small amount of MeOH. Acetone was added to the solution to deposit a precipitate (90 mg).

8-Bromoaristeromycin 3′,6′-Cyclic Phosphate (**19**): To a solution of aristeromycin 3′,6′-cyclic phosphate[23] (200 mg) in 1 M acetate buffer (pH 4, 20 ml) was added saturated Br_2–water (10 ml). The mixture was stirred for 2 hr and aq. Na_2SO_3 solution was added to decompose excess Br_2. The solution was desalted with a column of activated charcoal (4 g). The desalted solution was applied to a column of DEAE cellulose (HCO_3^-, 200 ml). The fractions (500 ml) eluted with 0.05 M NH_4HCO_3 were evaporated to dryness. To the residue was added 50% MeOH and the mixture was evaporated to dryness. This procedure was repeated twice and to the residue was added EtOH to afford colorless needles (84 mg).

N^6,2′-O-Dibutyryl-2-phenyl-cAMP (**23**): A solution of 2-phenyl-cAMP (**2**) (free, 0.5 g) in a mixture of MeOH (5 ml) and Et_3N (2 ml) was evaporated to dryness. The residue (Et_3N salt) was dried over P_2O_5 at room temperature and dissolved in pyridine (30 ml). To the solution was added butyric anhydride (10 ml).[12] The mixture was stirred for 4 days, until a fluorescent spot on the paper chromatogram (BuOH–AcOH–H_2O, 4: 1: 1 v/v) had disappeared. The solution was evaporated to dryness. The residue was dissolved in a small amount of acetone and to the solution was added ether to deposit a syrup. This procedure was repeated twice and the syrup thus obtained was dried over P_2O_5 at 60° to give a light brown foam (400 mg). UV λ_{max}^{MeOH} nm (ε): 247 (28400), 287 (18100); λ_{max}^{MeOH} nm: 227, 262.

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N^6,2′-O-Dibutyryl-2-(*p*-methoxyphenyl)-cAMP (**24**): To a solution of 2-(*p*-methoxyphenyl)-cAMP (3)[14] (3 g) in pyridine (200 ml) was added butyric anhydride (60 ml). The mixture was kept at 45° for 2 days, and evaporated to dryness. The residue was dissolved in acetone (2 ml), and to the solution was added ether (100 ml) to deposit a solid, which was filtered and dried. The powder thus obtained was dissolved in 50% EtOH (50 ml), and the solution was applied to a column of (IR)-120 (Na^+, 50 ml), and the column was washed with 50% EtOH (20 ml). The passing solution and washings were combined. After evaporation of the solution to dryness, the residue was dissolved again in acetone (2 ml) and to the solution was added ether (100 ml) to deposit a light brown powder (3 g).

N^6,2′-O-Dibutyryl-8-carbamoyl-cAMP (**25**): To a stirred suspension of 8-carbamoyl-cAMP[24]·Et_3N salt (350 mg) in pyridine (35 ml) was added dropwise butyric anhydride (15 ml) and the mixture was allowed to stand for 5 days. The brown solution was evaporated to dryness *in vacuo* to give a syrup, which yielded a brown powder by trituration with ether. A solution of the powder (500 mg) in 50% EtOH (10 ml) was passed through a column of IR-120 (Na^+, 5 ml). The passing solution and washings were combined and concentrated to deposit a light brown powder (450 mg).

Paper chromatography of the sample gave a single UV absorbing spot of *Rf* 0.8 (8-carbamoyl-cAMP *Rf* 0.3, 0.5 M $AcONH_4$–EtOH 2: 5 v/v).

2′-O-Butyryl-8-bromo-2-thloro-cAMP (**26**): A mixture of 8-bromo-2-chloro-cAMP[23] (5 g), pyridine (250 ml) and butyric anhydride (100 ml) was stirred at room temperature for 2 days, and evaporated to dryness *in vacuo*. The residue was dissolved in 50% MeOH and the solution was applied to a column of IR-120 (NH_4, 200 ml). The passing solution was evaporated to dryness *in vacuo* and to the residue was added ether to deposit a solid, which was filtered and washed thoroughly with acetone to give a pale brown powder (3.5 g).

Biochemical Methods——Activation of cAMP-Dependent Protein Kinase (PK): The $100000\times g$ supernatant fluid of rat brain, adrenal or liver homogenate was used as cAMP dependent PK preparation. Activation of PK by cAMP analogs was determined according to the method of Yamamura, *et al.*[25] using ATP-^{32}P and histone as substrate. The K_a value was expressed as the concentration required to activate the PK activity by 50%.

Inhibition of cAMP-^{3}H Binding to cAMP Dependent PK: PK was purified from the bovine skeletal muscle according to the method of Gilman.[26] cAMP binding activity of the PK preparation purified by DEAE-cellulose column chromatography was 90 pmol per mg of protein. For an evaluation of inhibitory effect of compounds on cAMP-^{3}H binding to PK, the following reaction mixture was used: the reaction medium of 200 μl total volume consisted of 2 pmol of cAMP-^{3}H (about 5×10^4 dpm), 2 μg of PK, 20 μmol of acetate buffer (pH 4.0) and 2—2000 μmol of compounds. The mixture was stood at 4° for 40 min and after addition of 10 mM potassium phosphate buffer (pH 6.0, 3 ml) filtered through Millipore (HAWP, pore size 0.45 mμ). The radioactivity of cAMP-^{3}H complex retained on the filter was counted.

Hydrolysis of Compounds by cAMP Phosphodiesterase (PDE): Heart of male Sprague–Dawley rat was homogenized with 9 times volume of 0.32 M sucrose (w/v) containing 10 mM Tris-Cl buffer (pH 7.5), and the homogenate was centrifuged at $1000\times g$ for 5 min. The supernatant fluid was dialyzed until inorganic phosphate became undetectable, and the solution was used as PDE preparation in this experiment. Reaction mixture of 500 μl total volume contained 1 mM test compound, 0.04 M Tris–Cl buffer (pH 7.5), 2.5 mM $MgSO_4$ and 1 mg protein of the enzyme, and it was incubated at 30° for 20 min. The reaction was stopped by heating at 100° for 2.5 min, and then the mixture was incubated at 30° with 0.25 U of 5′-nucleotidase (*Crotalus adamanteus* venom, Sigma grade II) for further 10 min. The reaction was stopped by addition of 55% trichloroacetic acid (50 μl), and inorganic phosphate released from the test compound was determined according to the method of Fiske and Subbarow.[27]

Inhibition of PDE Activity: PDE activity was assayed according to the method of Brooker, *et al.*[28] The $100000\times g$ supernatant fluid of brain or heart of male Sprague–Dawley rats was used as PDE preparation in this assay system. The reaction mixture of 500 μl total volume contained 2×10^5 dpm of cAMP-^{3}H (specific activity, 12 Ci/mmol), 1 μM cAMP, 5 mM $MgCl_2$, 0.04 M Tris-Cl buffer (pH 8.0), 3.75 mM β-mercaptoethanol, PDE and 1—1000 μM test compound.

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