2-Substituted Derivatives of Adenosine and Inosine Cyclic 3′,5′-Phosphates. Synthesis, Enzymic Activity, and Analysis of the Structural Requirements of the Binding Locale of the 2-Substituent on Bovine Brain Protein Kinase†

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ABSTRACT: A number of 2-substituted cyclic nucleotide derivatives were synthesized and investigated as activators of cAMP-dependent protein kinase and as substrates for and inhibitors of cAMP phosphodiesterase. Ring closure of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide cyclic 3′,5′-phosphate (**1**) with various aldehydes according to a new procedure (Meyer, R. B., Jr., Shuman, D. A., and Robins, R. K. (1974), *J. Am. Chem. Soc. 96,* 4962) gave new derivatives of adenosine cyclic 3′,5′-phosphate with the following 2-substituents: *n*-propyl, *n*-hexyl, *n*-octyl, *n*-decyl, styryl, *o*-methoxyphenyl, and 2-thienyl. Alkylation of 2-mercaptoadenosine cyclic 3′,5′-phosphate (**20,** Meyer et al., 1974) gave new cAMP derivatives with the following 2-substituent: ethylthio, *n*-propylthio, isopropylthio, allylthio, *n*-decylthio, and benzylthio. Deamination of 2-methyl-, 2-*n*-butyl-, and 2-ethylthioadenosine cyclic 3′,5′-phosphate with sodium nitrite in aqueous HCl gave the corresponding 2-substituted inosine cyclic 3′,5′-phosphate. Using multiple regression analysis, a striking relationship was found between the relative potency of the compounds as activators of bovine brain cAMP-dependent protein kinase and parameters describing the hydrophobic, steric, and electronic character of the substituents on these compounds. All compounds were substrates for a cyclic nucleotide phosphodiesterase preparation from rabbit kidney. Additionally, the compounds were, as a group, good inhibitors of the hydrolysis of cAMP by phosphodiesterase preparations from rabbit lung, beef heart, and dog heart.

The preparation and testing of modified analogs of adenosine cyclic 3′,5′-phosphate (cAMP) have been undertaken in several laboratories, including our own, with the expectation of providing new therapeutic and biochemical tools for use in correcting physiological defects and probing biochemical mechanisms related to cAMP (for a review, see Simon et al., 1973). This promise received early fulfillment in the utility of N^6,2′-*O*-dibutyryladenosine cyclic 3′,5′-phosphate (Robison et al., 1971).

A great many new cyclic nucleotides have been reported in the past 6 years, some of which are markedly more potent than cAMP in their ability to stimulate cAMP-dependent protein kinase preparations and are resistant to cleavage by phosphodiesterase (Simon et al., 1973; Meyer and Miller, 1974). It has also been shown that this ability of various cyclic nucleotide analogs to stimulate protein kinase and to be resistant to hydrolysis by phosphodiesterase was directly related to the ability of the analogs to elicit a cAMP-related effect, the induction of hepatic tyrosine aminotransferase, in whole animals (Miller et al., 1975).

For these reasons, we have been conducting a systematic investigation into the effect of substituents in various positions of the molecule on the biochemical activity of the analogs of cAMP. We have extensively covered the effects of single substituents (see Simon et al. (1973) and Meyer and Miller (1974) for comprehensive reviews) at virtually all accessible positions of the cAMP molecule except the 2 position of the purine ring. This report deals with the results of substitution at that position on biochemical activity and the results of a multiple-parameter structure–activity analysis which revealed a great deal about the effects of the structure of the 2-substituent upon the activation of the protein kinase.

Chemistry

The ready availability of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamidine cyclic 3′,5′-phosphate (**1**) (Meyer et al., 1973) in four steps from cAMP has made possible the direct synthesis of a variety of 2-substituted cAMP derivatives. Some of these, in addition to a novel closure of the purine ring, have been reported earlier (Meyer et al., 1974a). All of the additional 2-aryl- and 2-alkyl-cAMP derivatives in this report were prepared by this method, which involves condensation of the appropriate aldehyde with **1** to yield the 2-substituted 2,3-dihydroadenosine cyclic 3′,5′-phosphate (**2**). This intermediate was oxidized in situ by either chloranil or refluxing the solution in the presence of Pd/C to give the fully aromatized adenine derivative. The new cyclic nucleotides prepared in this fashion (see Scheme I) were 2-propyl- (**5**), 2-hexyl- (**8**), 2-octyl- (**9**), 2-decyl- (**10**), 2-styryl- (**11**), 2-(*o*-methoxy)phenyl- (**14**), and 2-(2-thienyl)adenosine cyclic 3′,5′-phosphate (**16**). The physical constants of all new compounds are given in Table I.

Reduction of 2-styryl-cAMP over Pt/C catalyst gave 2-(phenethyl)adenosine cyclic 3′,5′-phosphate (**12**).

Treatment of 2-mercaptoadenosine cyclic 3′,5′-phosphate (**20**) (Meyer et al., 1974a) with various alkyl halides gave the respective 2-(alkylthio)adenosine cyclic 3′,5′-phosphates

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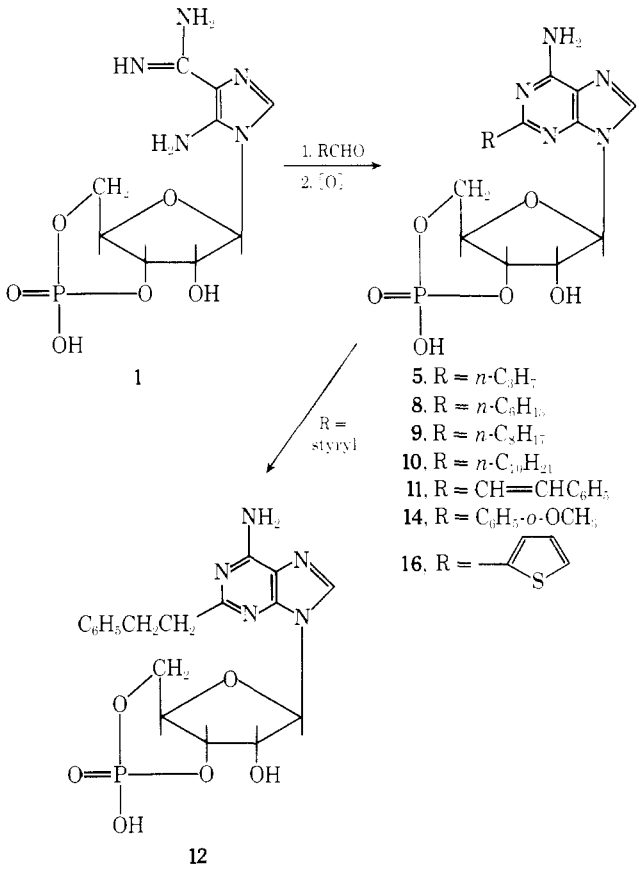
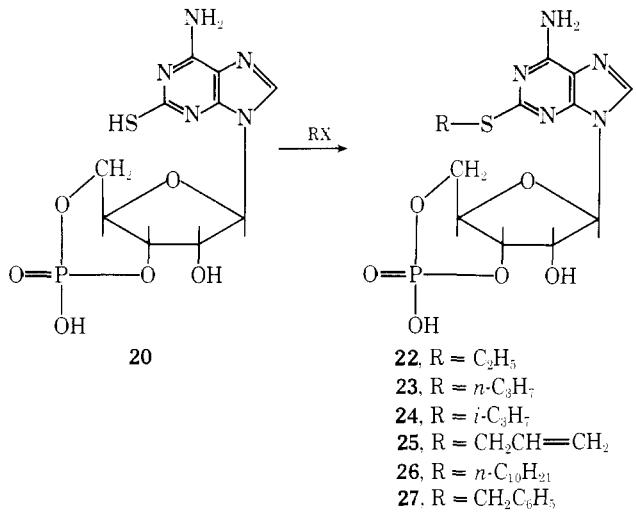
Scheme I



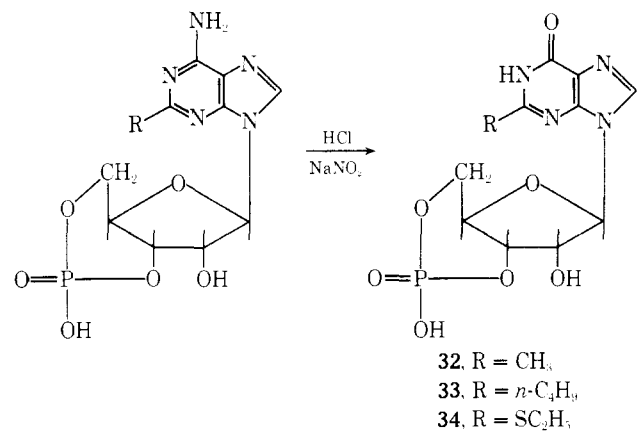
Scheme II



(**21–27**) (Scheme II). 2-(Methylthio)adenosine cyclic 3′,5′-phosphate has been previously reported (Meyer et al., 1974a; Jastorff and Freist, 1974). The derivatives prepared in this fashion were 2-(ethylthio)- (**22**), 2-(propylthio)- (**23**), 2-(isopropylthio)- (**24**), 2-(allylthio)- (**25**), 2-(decylthio)- (**26**), and 2-(benzylthio)adenosine cyclic 3′,5′-phosphate (**27**).

The 2-substituted derivatives of inosine cyclic 3′,5′-phosphate (cIMP) were prepared from the corresponding cAMP derivative by deamination with $NaNO_2$ in cold dilute HCl (Scheme III). 2-Methyl- (**32**), 2-butyl- (**33**), and 2-(ethylthio)inosine cyclic 3′,5′-phosphate (**34**) were prepared in this manner. The synthesis of the remaining cyclic nucleotides in Table II has been previously described (Meyer et al., 1974a, 1975).

Scheme III



Experimental Section

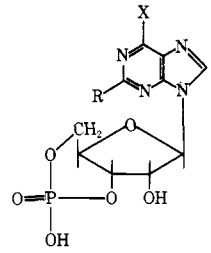
Ultraviolet (uv) spectra were recorded on a Cary 15 and are reported in Table I. The ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Hitachi-Perkin-Elmer R-20A and were in each case consistent with the assigned structure. In particular, the anomeric proton of each cyclic nucleotide was an apparent singlet ($J_{1'-2'} < 1$ Hz), which is indicative of the presence of the cyclic 3′,5′-phosphate (Jardetzky, 1962). Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., or by Het-Chem-Co, Harrisonville, Mo. Thin-layer chromatograms were developed in either solvent system A (MeCN–0.1 *N* NH_4Cl, 3:1) on ICN-Woelm silica gel F254 plates or B (*i*-PrOH–concentrated NH_4OH–H_2O, 7:1:2) on E. M. Laboratories Cellulose F plates. The R_f values are recorded in Table I. Compounds were dried under high vacuum at 60°.

2-Propyladenosine Cyclic 3′,5′-Phosphate (**5**). A mixture of 1.0 g (3.1 mmol) of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamidine cyclic 3′,5′-phosphate (**1**, Meyer et al., 1973), 1.5 ml of 2 *N* NaOH, 5 ml of H_2O, and 15 ml of dimethylformamide was stirred until solution was complete. Butyraldehyde (2 ml) was added and the solution was stirred 3 hr; then 1.0 g of freshly recrystallized chloranil in 15 ml of dimethylformamide was added. After 1 hr of stirring, the mixture was evaporated to dryness and partitioned between 50 ml of 0.2 *N* NH_4OH and 50 ml of EtOAc, and the aqueous layer was extracted with 50 ml of EtOAc. The aqueous phase was diluted with 50 ml of MeOH and passed through a column containing 150 ml of Dowex 50-X2 (H^+, 100–200 mesh), packed in and eluted with 50% aqueous MeOH. Product-containing fractions were evaporated to dryness giving 0.69 g (59%) of product. Anal. Calcd for $C_{13}H_{18}N_5O_6P \cdot H_2O$: C, 40.10; H, 5.18; N, 17.99. Found: C, 40.25; H, 5.07; N, 17.97.

2-(2-Thienyl)adenosine cyclic 3′,5′-phosphate (**15**) was prepared from **1** (1 g, 3.1 mmol) and thiophene-2-carboxaldehyde (1 ml) as in the preparation of **5**, except that the product was directly crystallized from the aqueous phase by acidification to pH 2 with HCl after EtOAc extraction; yield 0.44 g (33%). Anal. Calcd for $C_{14}H_{14}N_5O_6PS \cdot 1.5H_2O$: C, 38.35; H, 3.91; N, 15.98. Found: C, 38.09; H, 4.06; N, 16.09.

2-(2-Methoxyphenyl)adenosine cyclic 3′,5′-phosphate (**13**) was prepared from **1** (1.0 g, 3.1 mmol) and *o*-anisaldehyde (1 g) as in the preparation of **15**; yield, 0.83 g

Table I: Physical Constants of the New 2-Substituted Adenosine and Inosine Cyclic 3′,5′-Phosphates.[a,b]



No.	R	X	λ_{max} (nm) ($\epsilon \times 10^{-3}$) pH 1	pH 7	pH 11	R_f[c] A	B
5	n-C_3H_7	NH_2	256 (14.1)	261 (14.6)	261 (14.6)	0.36	0.73
16	C_4H_3S	NH_2	272 (13.0)	252 (16.6)	252 (17.3)	0.69	0.51
			323 (16.1)	307 (15.3)	307 (15.8)		
14	C_6H_4-o-OCH_3	NH_2	272 (14.6)	244 (13.4)	244 (13.4)	0.45	0.68
			308 (12.6)	264 (15.8)	264 (15.8)		
8	n-C_6H_{13}	NH_2	256 (14.2)	261 (14.9)	261 (14.7)	0.63	0.89
9	n-C_8H_{17}	NH_2	256 (13.1)	261 (13.6)	261 (13.9)	0.69	0.93
10	n-$C_{10}H_{21}$	NH_2	255 (12.4)	260 (12.7)	260 (13.3)	0.75	0.94
11	$CH{=}CHC_6H_5$	NH_2	274 (12.8)	265 (17.2)	265 (17.2)	0.65	0.80
			333 (17.1)	316 (16.1)	316 (16.1)		
12	$CH_2CH_2C_6H_5$	NH_2	257 (13.7)	261 (13.9)	261 (13.6)	0.53	0.79
22	SC_2H_5	NH_2	269 (16.7)	234 (22.0)	235 (18.3)	0.60	0.65
				273 (14.6)	273 (14.9)		
23	SC_3H_7-n	NH_2	270 (16.4)	235 (21.9)	236 (21.3)	0.68	0.78
				274 (14.7)	274 (14.6)		
24	SC_3H_7-i	NH_2	270 (15.9)	235 (21.2)	236 (20.6)	0.69	0.80
				275 (14.3)	275 (14.3)		
25	$SCH_2CH{=}CH_2$	NH_2	269 (15.8)	233 (22.2)	233 (21.6)	0.69	0.74
				273 (14.7)	273 (15.0)		
26	$SC_{10}H_{21}$	NH_2	270 (13.8)	240 (18.2)	241 (18.3)	0.87	0.95
				274 (12.3)	275 (12.8)		
27	$SCH_2C_6H_5$	NH_2	269 (16.6)	234 (25.7)	235 (25.4)	0.80	0.83
				273 (15.4)	273 (15.7)		
32	CH_3	OH	249 (12.7)	248 (13.0)	253 (13.0)	0.27	0.35
33	n-C_4H_9	OH	250 (13.2)	248 (13.7)	252 (13.7)	0.48	0.65
34	SC_2H_5	OH	264 (14.0)	262 (14.0)	230 (16.8)	0.36	0.43
					269 (14.2)		

[a] Physical data for the remaining compounds are in Meyer et al. (1974a) and Meyer et al. (1975). [b] The technical assistance of Ms. Milda Strikaitis is acknowledged. [c] See Experimental Section.

(59%). Anal. Calcd for $C_{17}H_{18}N_5O_7P{\cdot}H_2O$: C, 45.04; H, 4.45; N, 15.45. Found: C, 45.30; H, 4.47; N, 15.61.

2-Hexyladenosine Cyclic 3′,5′-Phosphate (**8**). A mixture of 3.19 g (10 mmol) of **1**, 5 ml of 2 *N* NaOH, 20 ml of MeOH, 0.25 g of 10% Pd/C, and 1.6 ml (1.37 g, 12 mmol) of heptaldehyde was refluxed 1 hr and then filtered and evaporated to dryness. The residue was taken up in 100 ml of H_2O and heated to 80°, and 5 ml of 2 *N* HCl was added. The crystalline product was filtered off after cooling. Recrystallization was achieved by dissolution in 100 ml of H_2O containing 5 ml of 2 *N* NH_4OH and treating the boiling solution with 5 ml of 2 *N* HCl. Cooling gave 2.35 g (52%) of product. Anal. Calcd for $C_{16}H_{24}N_5O_6P{\cdot}2H_2O$: C, 42.76; H, 6.28; N, 15.58. Found: C, 42.62; H, 6.26; N, 15.59.

2-Octyladenosine cyclic 3′,5′-phosphate (**9**) was prepared from **1** (4.8 g, 15 mmol) and nonaldehyde (3.45 ml, 20 mmol) as in the preparation of **8**, except that recrystallization was accomplished by acidification of a hot solution of the product in EtOH–MeOH (1:1) containing 7.5 ml of 2 *N* NH_4OH; yield, 3.83 g (58%). Anal. Calcd for $C_{18}H_{28}N_5O_6P$: C, 48.98; H, 6.39; N, 15.87. Found: C, 48.80; H, 6.61; N, 15.66.

2-Decyladenosine Cyclic 3′,5′-Phosphate (**10**). A mixture of 0.80 g (2.5 mmol) of **1**, 1.4 ml of 2 *N* NaOH, 20 ml of MeOH, 1 ml of undecaldehyde, and 100 mg of 10% Pd/C was refluxed for 6 hr and filtered. The solution was heated to boiling and 1.5 ml of 2 *N* HCl was added. Upon standing, 0.60 g (68%) of product crystallized. Anal. Calcd for $C_{20}H_{32}N_5O_6P{\cdot}3.5H_2O$: C, 45.11; H, 7.38; N, 13.15. Found: C, 44.71; H, 7.13; N, 13.69.

2-Styryladenosine cyclic 3′,5′-phosphate (**11**) was prepared from **1** (9.6 g, 30 mmol) and cinnamaldehyde (4.5 g, 34 mmol) as in the preparation of **8**; yield, 2.25 g (16%). Anal. Calcd for $C_{18}H_{18}N_5O_6P{\cdot}2H_2O$: C, 46.26; H, 4.75; N, 14.98. Found: C, 46.21; H, 4.72; N, 15.19.

2-(Phenethyl)adenosine Cyclic 3′,5′-Phosphate (**12**). A mixture of 0.90 g (1.93 mmol) of **11**, 3 ml of 2 *N* NaOH, 200 ml of H_2O, and 200 mg of 5% Pt/C was shaken at 40–50° under 2–3 atm of H_2 pressure for 24 hr. The filtered solution was evaporated to dryness, and the residue was taken up in 20 ml of hot H_2O. After adjustment of the pH to 2, 0.57 g (71%) of product crystallized. Anal. Calcd for $C_{18}H_{20}N_5O_6P$: C, 49.89; H, 4.65; N, 16.16. Found: C, 49.42; H, 4.93; N, 15.79.

2-(Ethylthio)adenosine 3′,5′-Cyclic Phosphate (**22**). 2-Mercaptoadenosine cyclic 3′,5′-phosphate (**20**, Meyer et al., 1974a) (0.50 g, 1.3 mmol as the monohydrate) was dissolved in 1.4 ml of 2 *N* NaOH and 10 ml of H_2O. Ethyl bromide (0.2 ml, dissolved in 10 ml of MeOH) was added

to the solution gradually and the mixture was stirred overnight at room temperature. The solvent was evaporated and the residue was taken up in 20 ml of H_2O. The solution was passed onto a column of Dowex 1-X2 (formate, 3 × 9 cm). The column was washed with H_2O (200 ml) and then the nucleotide was eluted with an 800-ml linear gradient from 0 to 3 *N* HCOOH in 50% ethanol. Evaporation of fractions containing product gave 0.25 g (47%) of **22.** Anal. Calcd for $C_{12}H_{16}N_5O_6PS \cdot H_2O$: C, 35.38; H, 4.45; N, 17.19. Found: C, 35.14; H, 4.56; N, 16.97.

The following compounds were prepared in the same manner from **20** and the specified organic halide (yield).

2-(Propylthio)adenosine cyclic 3′,5′-phosphate (**23**); *n*-C_3H_7Br (38%). Anal. Calcd for $C_{13}H_{18}N_5O_6PS \cdot H_2O$: C, 37.05; H, 4.78; N, 16.62. Found: C, 37.08; H, 4.72; N, 16.46.

2-(Isopropylthio)adenosine cyclic 3′,5′-phosphate (**24**); *i*-C_3H_7I (9%). Anal. Calcd for $C_{13}H_{18}N_5O_6PS \cdot H_2O$: C, 37.05; H, 4.78; N, 16.62. Found: C, 37.09; H, 4.73; N, 16.43.

2-(Allylthio)adenosine cyclic 3′,5′-phosphate (**25**); $CH_2{=}CHCH_2Br$ (26%). Anal. Calcd for $C_{13}H_{16}N_5O_6PS \cdot H_2O \cdot 0.5C_2H_5OH$: C, 38.00; H, 4.78; N, 15.83. Found: C, 38.38; H, 4.48; N, 16.16.

2-(Decylthio)adenosine cyclic 3′,5′-phosphate (**26**); *n*-$C_{10}H_{21}Br$ (11%). Anal. Calcd for $C_{20}H_{32}N_5O_6PS \cdot H_2O$: C, 46.23; H, 6.60; N, 13.48. Found: C, 46.46; H, 6.64; N, 13.27.

2-(Benzylthio)adenosine cyclic 3′,5′-phosphate (**27**); $C_6H_5CH_2Br$ (28%). Anal. Calcd for $C_{17}H_{18}N_5O_6PS \cdot H_2O$: C, 43.49; H, 4.29; N, 14.92. Found: C, 43.18; H, 4.09; N, 14.96.

2-Methylinosine Cyclic 3′,5′-Phosphate (**32**). A solution of 0.50 g (7.2 mmol) of $NaNO_2$ in 10 ml of H_2O was added dropwise with stirring to a solution of 0.50 g (1.4 mmol as the hydrate) of 2-methyladenosine cyclic 3′,5′-phosphate (**2,** Meyer et al., 1974a) in 20 ml of 2 *N* HCl which had been pre-cooled to and was maintained at 0–5° in an ice bath. After 5 hr of cooling and stirring, an additional 0.20 g (2.9 mmol) of $NaNO_2$ in 5 ml of H_2O was added. After standing 20 hr at ambient temperature, the solution was evaporated, and the residue was co-evaporated with 20 ml of H_2O. The residue was dissolved in 20 ml of H_2O and passed through a 1.5 × 9 cm column of Dowex 50-X8 (H^+, 100–200 mesh) and the product was eluted with H_2O. The appropriate fractions were evaporated and triturated with acetone; yield 0.32 g (60%). Anal. Calcd for $C_{11}H_{13}N_4O_7P \cdot H_2O$: C, 36.47; H, 4.17; N, 15.46. Found: C, 36.31; H, 4.09; N, 15.33.

The following compounds were prepared in the same manner.

2-Butylinosine cyclic 3′,5′-phosphate (**33**) from 2-butyladenosine cyclic 3′,5′-phosphate (**6**) (Meyer et al., 1974a) in 50% yield. Anal. Calcd for $C_{14}H_{19}N_4O_7P \cdot H_2O$: C, 41.58; H, 5.23; N, 13.85. Found: C, 41.83; H, 5.09; N, 14.01.

2-(Ethylthio)inosine cyclic 3′,5′-phosphate (**34**) from **22** in 18% yield. Anal. Calcd for $C_{12}H_{15}N_4O_7PS \cdot 1.5H_2O$: C, 34.53; H, 4.34; N, 13.42. Found: C, 34.33; H, 4.51; N, 13.32.

Biochemical Methods. Enzyme Preparations. The cAMP phosphodiesterases were purified from a 30,000*g* supernatant of beef heart, dog heart, rabbit lung, or rabbit kidney. The procedure, which was used for all three tissues, involved $(NH_4)_2SO_4$ fractionation (the 0–50% of saturation fraction was used) and DEAE-cellulose chromatography (the protein eluting from the column between 0.08 and 0.4 *M* KCl was used). The details of the purifications have been reported previously (Miller et al., 1973b). Bovine brain cAMP-dependent protein kinase was purified through the DEAE-cellulose step as previously described (Kuo and Greengard, 1970).

Enzyme Assays. The assay for inhibition of cAMP hydrolysis by the rabbit lung, beef heart, and dog heart enzymes contained in 0.5 ml: 25 μmol of Tris-HCl (pH 7.5), 5 μmol of $MgCl_2$, 20–200 μg of phosphodiesterase protein, 80 nmol of 8-[^{3}H]cAMP (350,000 cpm), and varying concentrations of the 3′,5′-cyclic nucleotide being tested as an inhibitor. The incubation times were determined from pilot assays to give kinetically valid data. The mixture was heat inactivated to terminate the reaction, and treated with 5′-nucleotidase (crude *Crotalus atrox* venom) to convert the 5′-nucleotide to a nucleoside. The unreacted 3′,5′-cyclic nucleotide was absorbed onto Dowex 1-X2 and the radioactivity of the nucleoside fraction determined (Thompson and Appleman, 1971a).

When testing cAMP derivatives as substrates for rabbit kidney phosphodiesterase, the standard reaction mixture contained in 0.60 ml: 3.0 μmol of cyclic nucleotide, 30 μmol of Tris-HCl (pH 7.5), 6 μmol of $MgCl_2$, and 0.1–0.3 mg of phosphodiesterase protein. After an appropriate incubation period (usually 10–60 min), the reaction was terminated by heating and treated with bacterial alkaline phosphatase, and the phosphate released was assayed colorimetrically. The details of the phosphodiesterase substrate and inhibition assays have been published (Miller et al., 1973b). The data for the inhibition of the phosphodiesterases are presented as I_{50} values, where the I_{50} is the concentration of inhibitor which produces a 50% inhibition of the rate of cAMP hydrolysis. The data for the hydrolysis of the cyclic nucleotide analogs by phosphodiesterase are presented relative to cAMP as an α value, where α = rate of hydrolysis of the analog/rate of hydrolysis of cAMP.

The assay for the stimulation of the cAMP-dependent protein kinase contained in 0.1 ml: 5 μmol of NaOAc (pH 6.0), 1 μmol of $MgCl_2$, 20 μg of histone (Worthington HLY), 0.5 nmol of [γ-^{32}P]ATP, protein kinase enzyme (20–200 μg), and various concentrations of the 3′,5′-cyclic nucleotide being tested as an activator (10^{-9}–10^{-3} *M*). After a suitable incubation time to give kinetically valid data (4–20 min), 0.05 ml of each reaction mixture was assayed for the amount of ^{32}P covalently bound to histone by the filter paper disk method, the details of which have been previously described (Miller et al., 1973a).

The cyclic nucleotide being tested as an activator was assayed at no fewer than seven different concentrations (in duplicate) and the apparent K_a was determined from the intercept [= (−apparent K_a)$^{-1}$] on the abscissa of the straight line generated by plotting (picomoles of ^{32}P transferred)$^{-1}$ vs. [cyclic nucleotide]$^{-1}$. The intercept on the abscissa was determined by regression analysis of the data and only those apparent K_a values which resulted from lines with a correlation coefficient (r) ≥ 0.980 were judged acceptable.

Results and Discussion

The activation of protein kinase by cAMP has been established to proceed via binding of cAMP to the regulatory subunit of the inactive holoenzyme to yield an active catalytic subunit (Langan, 1973). Under conditions where the substrates (ATP and histone) are saturating and the sub-

strates by themselves do not significantly modify the equilibrium of the binding of cAMP to the holoenzyme, the rate of histone phosphorylation should be proportional to the concentration of the active catalytic subunit. Therefore, activation of the protein kinase by the analogs will be discussed in terms of the factors influencing binding to the holoenzyme.

The activation of bovine brain cAMP-dependent protein kinase by the 2-substituted cAMP derivatives, as seen in Table II, showed a very definite relationship of structure of the side chain to biological activity when analyzed using the multiparameter regression analysis approach of Hansch (1971). The factors that affected side-chain binding were clearly segregated when the data were fit to a multiple regression analysis equation which expressed log (K_a') as a function of the sum of the contributions of the hydrophobic, steric, and electronic parameters of the substituent in the 2 position. K_a' is the ratio of the apparent K_a for cAMP to the apparent K_a for the analog. These apparent K_a values are determined graphically as described in the section on Biochemical Methods, and under the conditions of the protein kinase assay should be proportional to K_d, the dissociation constant for the equilibrium between the inactive holoenzyme, cyclic nucleotide, and active catalytic subunit. In addition, K_a' is directly proportional to the inverse of the molar activation constant of the analogs. The parameters used were π, the Hansch hydrophobic constant from the benzene system (Fujita et al., 1964), the Taft steric parameter E_s (Taft, 1956) or estimates thereof (Kutter and Hansch, 1969), and the inductive ($\mathfrak{F}$) and resonance ($\mathfrak{R}$) constants derived by Swain and Lupton (1968) as tabulated by Hansch et al. (1973). Only those compounds for which these constants were available or readily estimable were used in the regression analysis with two exceptions. 2-Decyl-cAMP (**10**) and 2-decylthio-cAMP (**26**) gave calculated K_a' values much below the expected level, as discussed below.

Multiple regression analysis of the data in Table III yielded eq 1:

$$\log (K_a') = -0.72(\pm 0.21) + 0.39(\pm 0.11)\pi + 0.63(\pm 0.13)E_s + 0.65(\pm 0.60)\mathfrak{F} + 2.58(\pm 1.06)\mathfrak{R} \quad (1)$$

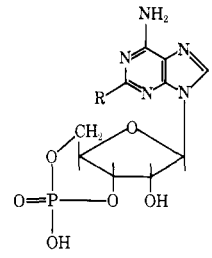
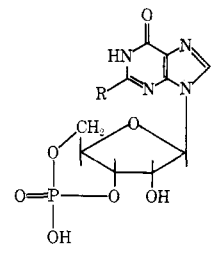
$$n = 14,\ r = 0.967,\ s = 0.149$$

where n is the number of data points, r is the coefficient of multiple correlation, and s is the standard deviation. The numbers in parentheses are the 95% confidence intervals. The values for K_a' calculated for each compound using eq 1 and expressed as the antilogs are tabulated in Table III.

Equation 1 gave an excellent picture of the binding of the side chain in the 2 position of cAMP to the bovine brain protein kinase. There appeared to be a hydrophobic slot next to the binding locale of the 2 position which could accommodate a straight hydrophobic chain. The steric requirements of this site were very strict in the area occupied by the first two atoms of the chain; the initial loss in binding from introduction of 2-Me or 2-Et on cAMP was gradually overcome as additional hydrophobic binding was observable with longer chain substituents. Additionally, chain branching [2-phenyl (**23**), 2-isobutyl (**7**), and 2-isopropylthio (**24**)] in this region led to significant decrease in activity.

This "linear hydrophobic slot" was vividly portrayed by the correlation of only the π and E_s values of cAMP and its straight-chain-substituted analogs (**2, 4–6, 8, 9**), for which electronic effects are virtually the same, to log (K_a') as

Table II: Interaction of 2-Substituted Adenosine and Inosine Cyclic 3′,5′-Phosphate Derivatives with Some Enzymes of cAMP Metabolism.[a]

No.	R	Protein Kinase K_a'[b]	Phosphodiesterase: Substrate, α[c]	Phosphodiesterase: Inhibitor I_{50} (μM)[d]: Rabbit Lung	Beef Heart	Dog Heart
						
2	Me	0.16	0.83	1.6	1.1	5.0
3	F_3C	0.43	0.43	3.2		1.6
4	Et	0.25	0.40	1.7	71	
5	*n*-Pr	0.24	0.89	2.0	50	4.0
6	*n*-Bu	0.46	0.64	3.0	10	2.0
7	*i*-Bu	0.05	0.86	8.4	10	
8	*n*-Hexyl	0.91	0.76	6.9		2.0
9	*n*-Octyl	1.6	0.41	2.0		2.4
10	*n*-Decyl	0.28	0.28			
11	PhCH=CH	1.7	0.67	2.8		0.42
12	$PhCH_2CH_2$	0.55				
13	Ph	0.024	0.32	4.2	15	0.7
14	*o*-CH_3OPh	0.070	0.76	8.0		1.8
15	*o*-ClPh	0.090	0.71	3.5	4.6	
16	2-Thienyl	0.23	0.30	2.4	40	2.0
17	2-Furyl	0.14	0.53	10.		
18	2-Pyridyl	0.009	0.76	15.	110	0.90
19	Ferrocenyl	0.050	0.51	2.0		6.5
20	HS[e]	0.001	0.15	8.4	3.4	7.2
21	MeS	0.20	0.55	2.4	4.5	
22	EtS	0.18	1.0	1.0	4.6	2.2
23	*n*-PrS	0.30	0.74	0.70	0.55	
24	*i*-PrS	0.13	0.62	2.2	4.8	
25	CH_2=$CHCH_2S$	0.20	0.40	1.2	1.2	
26	*n*-Decyl·S	0.16	0.40	0.86	1.3	0.65
27	$PhCH_2S$	0.52	0.63	0.90	0.65	1.1
28	H_2N[e]	0.12	0.93			
29	Me_2N[e]	0.070	0.24	6.0	65	2.0
30	HO[e]	0.056	0.66	74		
31	Cl[e]	0.39	0.85	0.66	0.50	4.0
						
cIMP	H	0.59	0.46	100	3.9	
32	Me	0.006	0.21	36	0.85	2.4
33	*n*-Bu	0.020	0.32	11	5.5	
34	EtS	0.040	0.46	10	1.3	

[a] The technical assistance of Ms. Mieka Scholten is acknowledged. [b] K_a' = (apparent K_a of cAMP/apparent K_a of the analog), where the apparent K_a is determined as described in the section on Biochemical Methods. [c] α = (rate of hydrolysis of analog/rate of hydrolysis of cAMP), determined with the rabbit kidney phosphodiesterase as described in the section on Biochemical Methods. [d] Inhibitor concentration giving 50% inhibition of the rate of hydrolysis of cAMP (concentration = 1.7×10^{-7} M), determined as described in the section on Biochemical Methods. [e] Protein kinase K_a' and phosphodiesterase α previously reported (Meyer et al., 1975).

Table III: Structural Parameters[a] and Calculated K_a' Values of the 2-Substituted cAMP Derivatives.

No.	2-Substituent on cAMP	π	E_s	$\mathcal{F}$	$\mathcal{R}$	K_a' Obsd[b]	K_a' Calcd[c]
cAMP	H	0	1.24	0	0	1	1.15
2	Me	0.5	0	−0.04	−0.13	0.16	0.13
4	Et	1.0	−0.07	−0.05	−0.10	0.25	0.21
5	*n*-Pr	1.5	−0.36	−0.06	−0.08	0.24	0.24
6	*n*-Bu	2.0	−0.39	−0.06	−0.11	0.46	0.30
8	*n*-Hexyl	3.0[d]	−0.4[d]	−0.06[d]	−0.11[d]	0.91	0.73
9	*n*-Octyl	4.0[d]	−0.4[d]	−0.06[d]	−0.11[d]	1.6	1.80
13	Ph	2.13	−2.58	0.08	−0.08	0.024	0.021
7	*i*-Bu	1.8[e]	−0.93[h]	−0.06[d]	−0.11[d]	0.05	0.11
21	MeS	0.61	0.17[f]	0.20	−0.18	0.20	0.19
22	EtS	1.07	−0.12[g]	0.23	−0.18	0.18	0.20
23	*n*-PrS	1.57	−0.15[g]	0.23[d]	−0.18[d]	0.30	0.30
30	Cl	0.71	0.27	0.41	−0.15	0.39	0.40
3	CF_3	0.88	−1.16	0.38	0.19	0.43	0.42

[a] All parameters taken from Hansch et al. (1973), unless otherwise indicated. The meaning of the parameters is defined in the text. [b] K_a' defined in footnote *b*, Table II. [c] Expressed as the antilog of the log (K_a') values calcd from eq 1. [d] Estimated. [e] Taken from Hansch (1972). [f] Estimated from a sulfur radius of 1.80 Å (Bondi, 1964) and eq 1 in Kutter and Hansch (1969). [g] Estimated by extrapolation of the E_s for MeS using the decrease in E_s for the series Et–Pr–Bu (Taft, 1956). [h] Taken from Taft (1956).

shown in eq 2. The data are from Table III.

$$\log (K_a') = -0.90(\pm 0.17) + 0.36(\pm 0.092)\pi + 0.71(\pm 0.22)E_s \quad (2)$$

$$n = 7, r = 0.984, s = 0.082$$

Past the first two atoms in the side chain, the presence of chain branching appeared to have little effect on binding of the analogs to the kinase. This fact made the Taft E_s values particularly useful in reflecting the effects of steric hindrance from these positions, since these constants are derived from rate changes in hydrolysis of aliphatic esters, and the greatest steric effect on these rates is seen with substituents on the carbons α and β to the ester function.

The validity of the correlation was tested by estimating the parameters of two of the compounds, **12** and **27**, which were not in the calculations, and calculating their K_a'. For compound **12**, 2-phenethyl-cAMP, $\pi = 2.66$ (Hansch et al., 1973), $E_s = -0.38$ (Taft, 1956), $\mathcal{F} = -0.06$, and $\mathcal{R} = -0.08$, which gives a calculated K_a' of 0.68, in good agreement with the observed value of 0.55. Estimating the parameters for 2-benzylthio-cAMP (**27**) to be $\pi = 2.3$, $E_s = -0.15$, $\mathcal{F} = 0.23$, and $\mathcal{R} = -0.18$, a calculated K_a' of 0.56 was obtained, again in good agreement with the experimental value of 0.52.

It was fairly clear, however, from initial inspection of the data that steric interference was present in the first two atoms of the side chain and that hydrophobic binding was observable in longer chain substituents. What was not obvious from simple inspection of the data was the contribution of the electronic character of the 2-substituent. Clearly an electron-withdrawing ($\mathcal{F}^+$) substituent at C-2 on the purine ring gave increased binding. The apparently large contribution of the resonance ($\mathcal{R}$) term could be misleading; only one $\mathcal{R}^+$compound (2-F_3C-cAMP) was included in the fit. The data seemed to show, however, that the enzyme required a 2-substituent which stabilized proton donation rather than protonation.

Other substituted cAMP derivatives were not included in the fit because their substituent constants were unknown or difficult to accurately estimate. The 2-HS- and 2-HO-cAMP's probably exist in the keto tautomer and were not included for that reason. As mentioned above, the 2-decyl- and 2-(decylthio)-cAMP's were not included because their K_a' values were anomalously low, probably due to coiling of the side chain, to aggregation in aqueous solution, or to an abrupt end to the "hydrophobic slot". The extinction coefficients in Table I, showing hypochromicities of 13% for 2-decyl-cAMP vs. 2-Pr-cAMP and 14% for 2-decylthio-cAMP vs. 2-*n*-PrS-cAMP, suggested that the long-chain compounds might be aggregated in aqueous solution.

The factors derived from this analysis gave a clear picture of the structural requirements of a 2-substituent on cAMP for the activation of the bovine brain protein kinase by the 2-substituted derivatives. This information should definitely be of use in the future design of cAMP analogs.

As can be seen in Table II, the 2-substituted derivatives of cIMP (**31–33**) were considerably less active as protein kinase activators than the corresponding derivatives of cAMP. Addition of the methyl, butyl, or ethylthio substituent to cIMP caused a greater loss in activation than did the addition of the same substituent to the cAMP molecule, indicating that possibly the hypoxanthine moiety has a slightly different binding mechanism than the adenine moiety.

Hydrolysis by Phosphodiesterase. All of the 2-substituted derivatives of cAMP were substrates for a crude cyclic nucleotide phosphodiesterase preparation isolated from rabbit kidney (Miller et al., 1973b). The 2-substituted cIMP analogs were also good substrates for this phosphodiesterase preparation. We (Muneyama et al., 1971, 1974; Miller et al., 1973a,b; Boswell et al., 1973; Meyer et al., 1972, 1973) and others (Michal et al., 1970; DuPlooy et al., 1971) have previously observed that an 8- or 6-substituent on the purine ring totally eliminates or substantially reduces the ability of the corresponding cyclic nucleotide to serve as a substrate for phosphodiesterase. Apparently the introduction of a 2-substituent onto the cAMP or cIMP molecule has little or no such effect.

Inhibition of Phosphodiesterase. Some of the 2-substituted cAMP analogs described here proved to be significantly better phosphodiesterase inhibitors than any cyclic nucleotides described to date. The inhibition of cAMP hydrolysis by three different phosphodiesterase preparations was compared using beef heart, dog heart, and rabbit lung

phosphodiesterase. 2-Cl-cAMP (**30**) was the best inhibitor of two of these preparations, having an I_{50} of 0.66 μM against the rabbit lung preparation and 0.5 μM against the beef heart preparation when assayed at a cAMP concentration of 1.6×10^{-7} M. With these two enzyme preparations, the order of inhibition effectiveness among the analogs was fairly similar, with the straight-chain alkyl and alkylthio compounds having the lower I_{50} values and the bulkier substituents imparting diminished activity. Interestingly, this trend was reversed for the dog heart preparation. The aromatic substituents imparted the best inhibition ability upon the cAMP molecule, while 2-Cl-cAMP was a tenfold worse inhibitor than the best of the inhibitors (2-styryl-cAMP, **11**) of this preparation.

Conclusive structure–activity relationships were impossible to draw in these cases of phosphodiesterase inhibition because the preparations probably contained more than one enzyme (Thompson and Appleman, 1971b; Monn and Christiansen, 1971; Hrapchak and Rasmussen, 1972). We have performed some preliminary kinetic experiments, however, and shown that 2-Cl- (**30**), 2-Me- (**2**), 2-MeS- (**21**), and 2-HO-cAMP (**29**) exhibited competitive inhibition of the hydrolysis of cAMP with the beef heart and rabbit lung preparations as judged by a double reciprocal plot of $1/V$ vs. 1/[cAMP] at several inhibitor concentrations (Miller et al., 1974; Meyer et al., 1974b). The inhibition kinetics of these potent phosphodiesterase inhibitors on a purified enzyme from dog heart will be the subject of a forthcoming report.

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