8-Substituted Derivatives of Adenosine 3′,5′-Cyclic Phosphate Require an Unsubstituted 2′-Hydroxyl Group in the Ribo Configuration for Biological Activity†

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ABSTRACT: Derivatives of adenosine 3′,5′-cyclic phosphate (cAMP) with modifications in both the 2′ and the 8 positions were synthesized and their enzymic activities as activators of cAMP-dependent protein kinase and as substrates for and inhibitors of cAMP phosphodiesterases were determined. Three types of derivatives were investigated: 8-substituted derivatives of $O^{2'}$-Bt-cAMP, 8-substituted derivatives of 9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate (ara-cAMP), and 8-substituted derivatives of 8,2′-anhydro-9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate (8,2′-anhydro-cAMP). The 8-substituted $O^{2'}$-Bt-cAMP derivatives were synthesized by acylation of the preformed 8-substituted cAMP (8-HS-cAMP, 8-MeS-cAMP, and 8-$PhCH_2S$-cAMP). 8-Br-$O^{2'}$-tosyl-cAMP was used as an intermediate for the preparation of 8,2′-anhydro-cAMP derivatives (8-HO-, 8-SH-, 8-H_2N-, and 8-H_3 CHN derivatives of 8,2′-anhydro-cAMP). 8-Substituted ara-cAMP derivatives were obtained by ring opening of 8-HO-8,2′-anhydro-cAMP with H^+/H_2O, NH_3/MeOH, or MeONa/MeOH (to yield the 8-HO-, 8-H_2N-, and 8-MeO-ara-cAMP derivatives). All of these *doubly* modified derivatives of cAMP were less than one-hundredth as active as cAMP at activating protein kinase and did not serve as substrates for the phosphodiesterase. These data show that the general inactivity of 2′ derivatives of cAMP with the kinase was not overcome by addition of an 8-substituent, even though many 8-substituted derivatives of cAMP activate the kinase more efficiently than does cAMP itself. In addition they show that while 2′-modifications were tolerated by the phosphodiesterase, addition of an 8-substituent countermanded the allowable 2′-modification. The 8-substituted derivatives of $O^{2'}$-Bt-cAMP were found in general to be slightly better inhibitors of phosphodiesterase than the parent compounds containing no $O^{2'}$-Bt substitution. As a group, the 8-substituted ara-cAMP derivatives were poorer inhibitors of phosphodiesterase than 8-substituted cAMP derivatives while the 8,2′-anhydro-cAMP derivatives were much poorer inhibitors than the 8-substituted ara-cAMP derivatives.

We have been interested in the effects of modification of the purine, carbohydrate, and phosphate moieties of 3′,5′-cyclic nucleotides on their interaction with various enzymes of cyclic nucleotide metabolism (for review, see Simon et al., 1973; Meyer and Miller, 1974). We have previously shown that many 8-substituted analogs of cAMP[1] activated bovine brain cAMP-dependent protein kinase more efficiently than did cAMP (Muneyama et al., 1971; Miller et al., 1973a). Any 2′ substitution of cAMP was not tolerated by this enzyme and a free 2′-hydroxyl group in the ribo configuration was essential for its activation (Miller et al., 1973b). In contrast, 8-substituted derivatives of cAMP were very poor substrates for rabbit kidney phosphodiesterase (Muneyama et al., 1971; Miller et al., 1973a), whereas the 2′ derivatives were hydrolyzed at substantial rates by this enzyme (Miller et al., 1973b).

We have now begun to investigate the effects of *multiple* substitutions of cAMP on its biological activity. Some of these studies on purine 5′-thio-5′-deoxynucleoside 3′,5′-cyclic phosphorothioates have suggested that the interaction of cAMP with the above two enzymes involved binding of both the 6-amino group and the 5′ oxygen in a synergistic manner to the enzymes (Shuman et al., 1973). We here report the synthesis of cAMP analogs with modifications in *both* the $O^{2'}$ and 8 positions. Three different types of derivatives were investigated: (1) 8-substituted 8,2′-anhydro-cAMP derivatives, (2) 8-substituted ara-cAMP derivatives, and (3) 8-substituted $O^{2'}$-Bt-cAMP derivatives. The in vitro enzymic properties of these derivatives were investigated by studying their ability to serve as substrates for and inhibitors of cyclic nucleotide phosphodiesterases and to activate a cAMP-dependent protein kinase.

Experimental Section

Synthetic. 8-Substituted cAMP analogs were synthesized as previously described (Muneyama et al., 1971). 8-Br-$O^{2'}$-tosyl-cAMP (**2**) (Khwaja et al., 1972) was used as a versatile intermediate for the preparation of 8,2′-anhydro-cAMP derivatives (**11–13**) by reaction with an appropriate nucleophile: NaSH, NH_3, or CH_3NH_2 (Scheme I). 8-Substituted ara-cAMP derivatives (**15, 16,** and **18**) were obtained by ring opening 8,2′-anhydro-9-β-D-arabinofuranosyl-8-hydroxyadenine 3′,5′-cyclic phosphate (**10,** 8-HO-8,2′-anhydro-cAMP) (Khwaja et al., 1972; Mian et al., 1974) with methanolic ammonia, dilute acid, or methanolic sodium methylate (Scheme II). 9-β-D-Arabinofuranosyladenine 3′,5′-cyclic phosphate (**14,** ara-cAMP) and 8-thio-9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate (**17,** 8-HS-ara-cAMP) were synthesized as previously described (Khwaja et al., 1972; Mian et al., 1974). The ^1H nuclear magnetic resonance (^1H NMR) spectra of these arabinose cyclic phosphates exhibit characteristic doublets (δ 6–7) for

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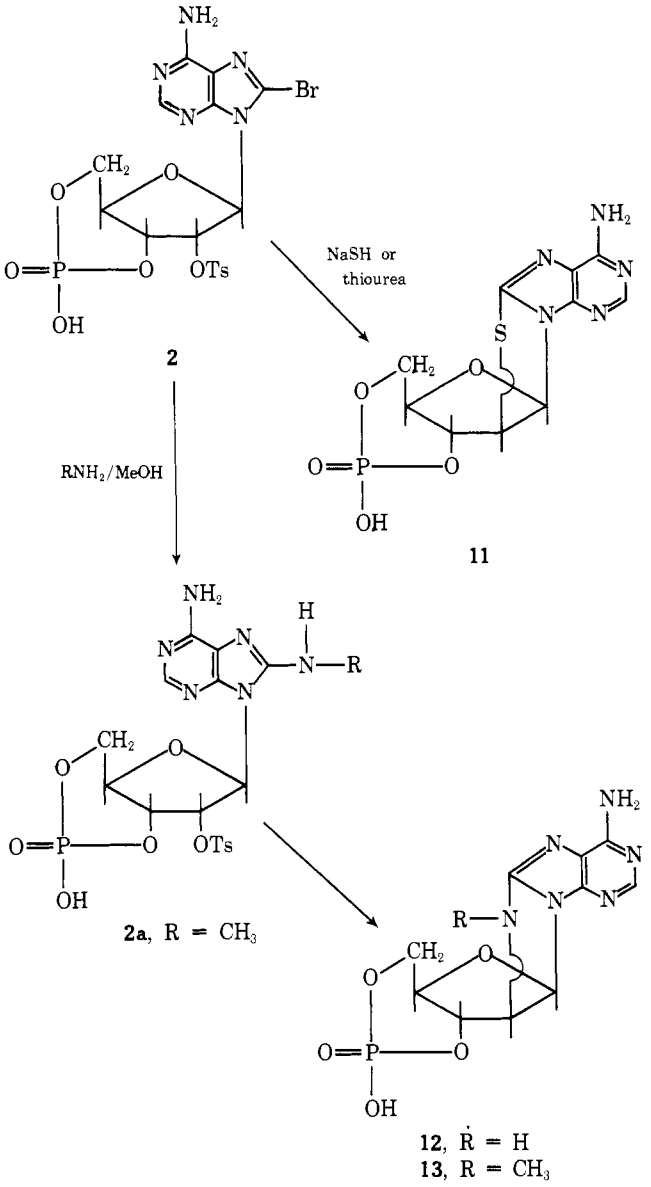
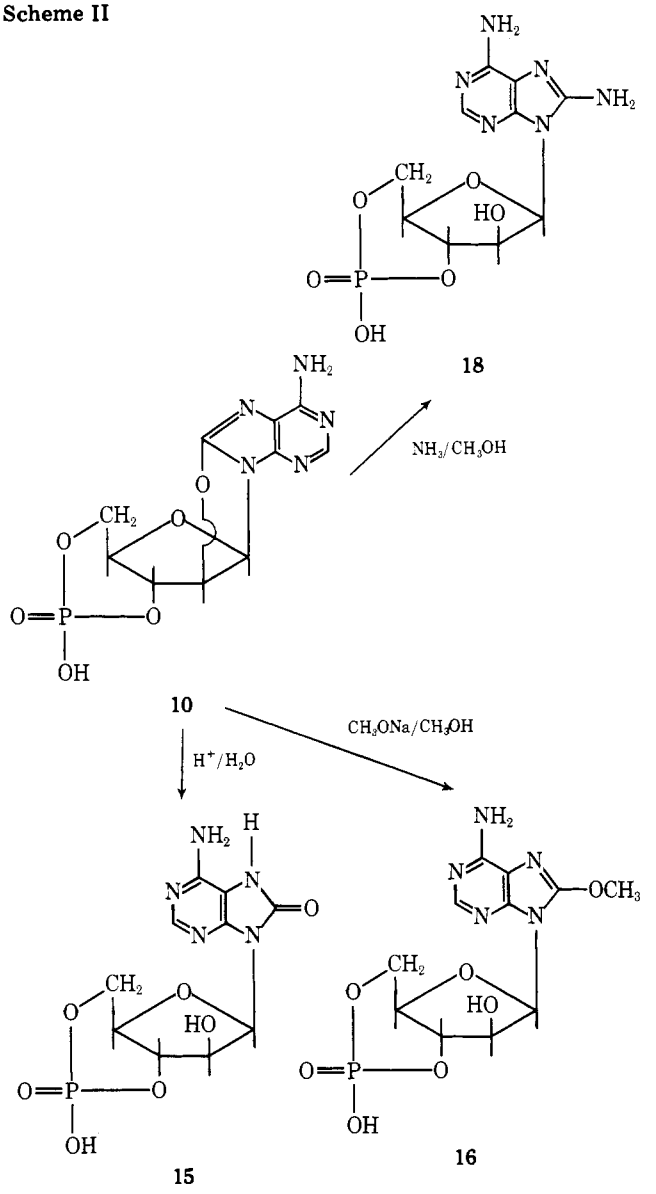
[1] Abbreviations used are: cAMP, adenosine 3′,5′-cyclic phosphate; ara-cAMP, 9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate; 8,2′-anhydro-cAMP, 8,2′-anhydro-9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate.

Scheme I



Scheme II



their anomeric protons (Khwaja et al., 1972) with $J_{1',2'}$ coupling in the range of 6–7 Hz. 8-Substituted $O^{2'}$-Bt-cAMP derivatives (**20–22**) were acylated in pyridine using butyric anhydride (Posternak et al., 1962). These compounds were characterized by usual uv, ^{1}H NMR, and analytical techniques. $O^{2'}$-Bt-cAMP was purchased from Sigma.

8,2′-Anhydro-9-β-D-arabinofuranosyl-8-mercaptoadenine 3′,5′-Cyclic Phosphate (**11**, *8-HS-8,2′-anhydro-cAMP*). Method 1. Compound **2** (5.86 g, 10 mmol) was suspended in dimethylformamide (60 ml), 1 *N* NaSH (30 ml) was added, and the stirred solution was left at room temperature for 24 hr. Then the solution was diluted with EtOH (50 ml) and carefully evaporated under reduced pressure. The residual gum was absorbed on a silicic acid (Mallinckrodt 100 mesh) column (6 × 38 cm). The column was eluted with $CHCl_3$ (1200 ml) followed by a mixture of $CHCl_3$/MeOH (1:1, v/v). Several uv absorbing peaks appeared; the first major peak consisted of *p*-toluenesulfonic acid. Product **11** appeared as the second major peak and the corresponding fractions were collected and evaporated under reduced pressure to obtain a gum. The gum was dissolved in a minimum volume of Me_2SO, and filtered, and a large excess of $CHCl_3$ was added to precipitate **11** as a pure colorless powder, yield 3.3 g, 91%. The product moved as a homogeneous component in several thin-layer chromatography (TLC) systems. ^{1}H NMR showed the anomeric proton as a doublet ($J_{1',2'}$ = 6.2 Hz) centered at δ 6.54 (determined in Me_2SO) and the $H_{2'}$ as a triplet located at δ 4.98. Ultraviolet absorption: λ_{max}(pH 1) 276 nm (ϵ 20,900); λ_{max}(pH 11) 275 nm (ϵ 21,200). Anal. Calcd for $C_{10}H_{10}N_5O_5PS \cdot 1.12H_2O$: C, 33.04; H, 3.33; N, 19.26. Found: C, 32.91; H, 3.30; N, 19.16.

Method 2. Compound **2** (8.71 g, 15.5 mmol, ammonium salt) was suspended in 1-butanol (275 ml) and refluxed (bath temperature 130°) for 30 min until most of the starting material dissolved. Thiourea (1.725 g) was added and the stirred solution refluxed for 3.5 hr. The reaction mixture was cooled and left at 0° overnight. The resulting precipitate was filtered, washed with 1-butanol, and dried under vacuum at 80° to obtain **11** as a chromatographically pure material. The product was dissolved in aqueous NH_4OH and crystallized with addition of acetone, yield 3.9 g, 70%. The spectral properties of the product were identical with those of **11** obtained by Method 1.

8,2′-Anhydro-9-β-D-arabinofuranosyl-8-aminoadenine

3',5'-Cyclic Phosphate (**12,** *8-H_2N-8,2'-anhydro-cAMP*). Compound **2** (5.84 g, 10 mmol) was suspended in methanolic ammonia and heated (bath temperature 80°) in a stainless steel bomb for 24 hr. The resulting solution was evaporated to dryness and the residue was dissolved in 50 ml of MeOH/$CHCl_3$ (1:1, v/v) and the solution was absorbed onto a silicic acid (Mallinckrodt, 100 mesh) column (5.4 × 22.0 cm). The column was eluted with a gradient of MeOH in $CHCl_3$ (0–20%, 2500 ml), followed by 35% MeOH in $CHCl_3$ (3000 ml), and finally 50% MeOH in $CHCl_3$. Several ultraviolet absorbing peaks appeared. The product (**12**) was eluted with 50% MeOH in $CHCl_3$ as the last peak. The appropriate fractions were collected and evaporated. The residue was dissolved in MeOH, acidified with 2 *N* HCl, and precipitated with EtOH–ether as a chromatographically homogeneous white powder, yield 150 mg. The product showed no ir absorption band at 1185 cm^{-1} indicating the absence of the sulfonyl moiety in the $O^{2'}$ position.

Paper electrophoretic mobility (pH 7) showed the presence of only one negative charge on the phosphate (molybdate spray positive) group indicating the retention of the cyclic phosphate moiety. The ^1H NMR spectrum showed the anomeric proton as a doublet located at 6.42 ($J_{1',2'}$ = 6.5 Hz) confirming the arabinose configuration. Ultraviolet absorption: λ_{max}(pH 1) 272 nm (ε 15,800); λ_{max}(pH 11) 274 nm (ε 14,500). Anal. Calcd for $C_{10}H_{13}N_6O_5P$: C, 38.82; H, 3.82; N, 24.70. Found: C, 38.50; H, 4.01; N, 24.55.

8,2'-Anhydro-9-β-D-arabinofuranosyl-8-methylaminoadenine 3',5'-Cyclic Phosphate (**13,** *8-MeHN-8,2'-anhydro-cAMP*). Compound **2** (0.586 g, 1 mmol) was suspended in CH_3OH (20 ml) containing 3 ml of methylamine. The solution was heated under reflux for 2 hr. The cooled reaction was evaporated to dryness under reduced pressure and the residual gum coevaporated with EtOH. The final residue was dissolved in EtOH (8 ml) and the solution acidified with 2 *N* HCl. The precipitated material was left in a freezer for 4 hr, filtered, and washed with EtOH to obtain 0.54 g of 8-MeHN-$O^{2'}$-tosyl-cAMP (**2a**) as a white powder. This material was chromatographically homogeneous. The ir spectrum showed the presence of a sulfonyl band at 1185 cm^{-1}. The ^1H NMR spectrum showed the presence of the $-NCH_3$ protons at δ 2.94 as a three-proton singlet. Ultraviolet absorption: λ_{max}(pH 1) 276 nm; λ_{max}(pH 11) 274 nm.

The 8-MeHN-$O^{2'}$-tosyl-cAMP (0.27 g, 0.5 mmol) was dissolved in dry dimethylformamide (15 ml) and heated (bath temperature 125°) with sodium acetate (0.20 g, 2.5 mmol) for 24 hr. The resulting solution was cooled and evaporated to dryness under reduced pressure. The residue was dissolved in water (2 ml) and absorbed on a Dowex 50 H^+ (100–200 mesh) column (1.5 cm × 18 cm). The column was eluted with water to remove all ultraviolet absorbing impurities. Subsequent elution with 9 *M* formic acid gave **13** as a broad peak; the appropriate fractions were collected and evaporated carefully under reduced pressure to obtain a gum. The gum was precipitated from aqueous EtOH with Et_2O to provide 20 mg of **13** as a dry white powder. The product was homogeneous in several TLC systems and its ir spectrum showed no sulfonyl band at 1185 cm^{-1}. The ^1H NMR spectrum showed the presence of $-NCH_3$ protons as a three-proton singlet at δ 2.96. The anomeric proton was a doublet ($J_{1',2'}$ = 6.3 Hz) indicating an arabinose configuration. Ultraviolet absorption: λ_{max}(pH 1) 278 nm (ε 13,800); λ_{max}(pH 11) 277 nm (ε 14,400).

8-Hydroxy-9-β-D-arabinofuranosyladenine 3',5'-Cyclic Phosphate (**15,** *8-HO-ara-cAMP*). Compound **15** was obtained as a by-product during the reductive cleavage of **10** with liquid H_2S (Khwaja et al., 1972). Compound **10** (1.31 g, 4 mmol) was suspended in dimethylformamide (3 ml) and pyridine (40 mg). To this liquid H_2S (40 ml) was added and the mixture heated in a stainless steel bomb (bath temperature 100°) for 18 hr. The reaction solution was cooled and carefully evaporated under reduced pressure. The residue was dissolved in MeOH and absorbed onto a silicic acid column. The column was eluted with $CHCl_3$ (950 ml) and then with a mixture of MeOH/$CHCl_3$ (1:1, v/v). The first major peak consisted of 9-β-D-arabinofuranosyl-8-mercaptoadenine 3',5'-cyclic phosphate (**17**) (Khwaja et al., 1972). The fractions corresponding to the second peak were collected and evaporated under reduced pressure. The white residue was dissolved in MeOH and acidified with 2 *N* HCl to obtain **15** as a white powder (192 mg). The ^1H NMR spectrum (determined in NaOD) showed the anomeric proton as a doublet ($J_{1',2'}$ = 6.5 Hz) located at δ 6.34. Ultraviolet absorption: λ_{max}(pH 1) 263 nm (ε 10,020) and 286 nm (ε 9800); λ_{max}(pH 11) 278 nm (ε 14,100). Anal. Calcd for $C_{10}H_{12}N_5O_7P \cdot H_2O$: C, 33.07; H, 3.88; N, 19.28. Found: C, 33.34; H, 3.55; N, 19.60.

8-Methoxy-9-β-D-arabinofuranosyladenine 3',5'-Cyclic Phosphate (**16,** *8-MeO-ara-cAMP*). Compound **10** (1.0 g) was dissolved in MeOH (100 ml) containing 1 g of sodium methoxide and the solution heated under reflux for 48 hr. The reaction contents were evaporated, the residue was dissolved in H_2O (20 ml), and the solution was acidified with 2 *N* HCl. The precipitated **16** was filtered and washed with H_2O (5 ml). ^1H NMR showed the presence of a three-proton singlet (δ 4.2) indicating the presence of the methoxy group. The anomeric proton was a doublet ($J_{1',2'}$ = 6.9 Hz) located at δ 6.32. Ultraviolet absorption: λ_{max}(pH 1) 260 nm (ε 11,200); λ_{max}(pH 11) 260 nm (ε 11,800). Anal. Calcd for $C_{10}H_{11}N_6O_5P \cdot H_2O$: C, 34.88; H, 3.78; N, 24.41. Found: C, 34.71; H, 3.90; N, 24.32.

8-Amino-9-β-D-arabinofuranosyladenine 3',5'-Cyclic Phosphate (**18,** *8-H_2N-ara-cAMP*). Compound **10** (0.98 g, 3 mmol) was dissolved in saturated methanolic ammonia in a bomb and the bomb was maintained at 120° for 48 hr. At the end of this period the reaction solution was evaporated to dryness and the residue was dissolved in MeOH and absorbed onto a silicic acid (Mallinckrodt, 100 mesh) column (4 × 28 cm). The column was washed with $CHCl_3$ (500 ml) and subsequently eluted with a mixture of $CHCl_3$ and MeOH (1:1, v/v). The first peak consisted of unreacted starting material which was followed by a major peak of the product **18.** The corresponding fractions were pooled and evaporated to dryness. The residue was dissolved in MeOH (50 ml) and acidified with 2 *N* HCl to crystallize the product (**18**) as the pure free acid (0.72 g, 66%). The ^1H NMR spectrum showed the anomeric proton as a doublet ($J_{1',2'}$ = 6.9 Hz) located at δ 6.26. Ultraviolet absorption: λ_{max}(pH 1) 271 nm (ε 13,450); λ_{max}(pH 11) 270 nm (ε 16,800). Anal. Calcd for $C_{10}H_{16}N_7O_6P \cdot H_2O$: C, 33.15; H, 4.17; N, 23.20. Found: C, 33.30; H, 3.99; N, 23.47.

2'-O-Butyryl-8-mercaptoadenosine 3',5'-Cyclic Phosphate[2] (**20,** *8-HS-$O^{2'}$-Bt-cAMP*). To a solution of 8-HS-cAMP (**5**) (Muneyama et al., 1971) (3.0 g, 8.2 mmol) in 10 ml of H_2O was added 20 ml of pyridine followed by the slow addition of 200 ml of a 1:1 mixture of butyric anhydride–pyridine. The resulting mixture was allowed to slowly

[2] After the completion of this work, Giao et al. (1974) reported the synthesis of 8HS-$O^{2'}$ Bt-cAMP by essentially the same method.

cool to room temperature overnight. Unreacted starting material was filtered off and set aside. The solution was diluted with 100 g of ice and then stirred for 2 hr. The filtrate was evaporated and the resulting residue was codistilled with H_2O until a solid formed. The solid was recrystallized from H_2O to yield 350 mg of the product (**20**). The unreacted starting material, from the filtration, was recycled to yield an additional 791 mg of the product; total yield 1.14 g (32%). Ultraviolet absorption: λ_{max}(pH 1) 308 nm (ϵ 26,200); λ_{max}(pH 11) 295 nm (ϵ 22,950). Anal. Calcd for $C_{14}H_{18}N_5O_7PS$: C, 38.95; H, 4.20; N, 16.23; S, 7.43. Found: C, 38.82; H, 4.14; N, 16.01; S, 7.28.

2′-O-Butyryl-8-methylthioadenosine 3′,5′-Cyclic Phosphate (**21**, *8-MeS-$O^{2'}$-Bt-cAMP*). 8-MeS-cAMP·$2H_2O$ (**6**) (Muneyama et al., 1971) (4.0 g, 10.4 mmol) was dissolved in 100 ml of boiling pyridine and then evaporated to dryness. The residue was redissolved in pyridine and evaporated three more times. The final residue was dissolved in a solution of pyridine (50 ml) and butyric anhydride (40 ml). The solution was stirred overnight at room temperature and then diluted with 100 g of ice and then stirred for 2 hr. The solvent was evaporated and the residue triturated with 300 ml of boiling H_2O. The resulting solid which formed was collected by filtration and suspended in 500 ml of boiling H_2O. After the solution was cooled, the solid was filtered off and dried to give 3.4 g (73%) of the product (**21**). Ultraviolet absorption: λ_{max}(pH 1) 280 nm (ϵ 22,600); λ_{max}(pH 11) 279 nm (ϵ 20,900). Anal. Calcd for $C_{15}H_2N_5O_7PS$: C, 40.45; H, 4.52; N, 15.72. Found: C, 40.33; H, 4.65; N, 15.59.

2′-O-Butyryl-8-benzylthioadenosine 3′,5′-Cyclic Phosphate. (**22**, *8-$PhCH_2S$-$O^{2'}$-Bt-cAMP*). A solution of 8-$PhCH_2S$-cAMP (**7**) (Muneyama et al., 1971) (3.0 g, 6.2 mmol) and 4-morpholine-*N,N′*-dicyclohexylcarboxamidine (1.8 g, 6.2 mmol) in 150 ml of dry pyridine and 75 ml of butyric anhydride was stirred overnight at room temperature. The solution was diluted with 100 g of ice and after stirring an additional 2 hr the solution was evaporated. The residue was triturated with 100 ml of H_2O and then dissolved in 800 ml of boiling H_2O. The solid, which formed on cooling, was collected by filtration and recrystallized from H_2O to give 1.57 g (46%) of the desired product (**22**). Ultraviolet absorption: λ_{max}(pH 1) 285 nm (ϵ 17,700); λ_{max}(pH 11) 286 nm (ϵ 14,900). Anal. Calcd for $C_{21}H_{24}N_5O_7PS\cdot 1.5H_2O$: C, 45.98; H, 4.96, N, 12.76; S, 5.84. Found: C, 45.98; H, 4.77; N, 12.88; S, 5.76.

Biochemical Methods

The rabbit lung, beef heart, and rabbit kidney phosphodiesterases were prepared as previously described (Miller et al., 1973b). The assay for the inhibition of the rabbit lung and beef 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 the corresponding nucleoside. The untreated 3′,5′-cyclic nucleotide was absorbed onto Dowex-1 and the radioactivity of the nucleoside fraction determined. When testing cAMP derivatives as substrates for rabbit kidney phosphodiesterase, the standard reaction mixture contained in 0.60 ml: 3.0 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 previously described (Miller et al., 1973b).

The bovine brain cAMP-dependent protein kinase was purified through the DEAE-cellulose step as described by Kuo and Greengard (1969). The assay for the stimulation of the cAMP-dependent protein kinase (Muneyama et al., 1971) 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, 0.05 ml of each reaction mixture was absorbed onto Whatman 3MM paper discs, and the discs were dried. The discs were washed sequentially in 10% Cl_3CCO_2H, 5% Cl_3CCO_2H (three times), EtOH, and Et_2O. After thorough drying, the radioactivity in each disc was determined in a liquid scintillation spectrometer.

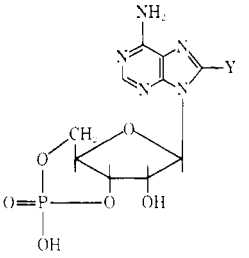
Results

Protein Kinase Activation. All of the 8,2′-disubstituted derivatives were analyzed for their ability to activate bovine brain cAMP dependent protein kinase and were compared with analogous 8-substituted derivatives of cAMP containing no 2′ modifications (**1** and **3–9**). The results in Table I show that regardless of whether the 2′ modification was an arabino configuration of the sugar (**14–18**), an $O^{2'}$-Bt substitution (**19–22**), or an 8,2′-anhydro configuration (**10–13**), the compounds were very poor activators of the protein kinase.

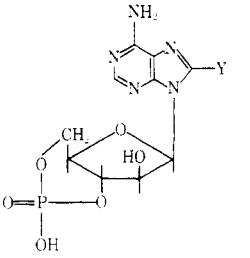
cAMP Phosphodiesterase Studies. All of the derivatives were tested for their ability to be hydrolyzed by rabbit kidney cAMP phosphodiesterase as described in Biochemical Methods. None of the 8,2′-disubstituted cAMP derivatives were hydrolyzed by the enzyme. Even increasing the amount of the enzyme (tenfold) or the time of incubation (fivefold) did not result in any detectable hydrolysis products. Of a series of 8-substituted cAMP derivatives, only 8-H_2N-cAMP (**8**) was found to be hydrolyzed by this phosphodiesterase (Muneyama et al., 1971). By comparison, 8-H_2N-ara-cAMP (**18**) and 8-H_2N-8,2′-anhydro-cAMP (**12**) did not serve as substrates for the enzyme. As previously reported (Miller et al., 1973b), ara-cAMP (**14**) and $O^{2'}$-Bt-cAMP (**19**) were hydrolyzed at 17 and 37% the rate of cAMP (**1**), respectively, by the rabbit kidney enzyme.

The ability of the 8,2′-disubstituted analogs of cAMP to inhibit the hydrolysis of cAMP by the cAMP phosphodiesterases from beef heart and rabbit lung was also studied and the results are summarized in Table II. The ability of 8-substituted cAMP derivatives containing the same 8 substituents were also tested as inhibitors. The results show that, in general, the 8-substituted $O^{2'}$-Bt-cAMP derivatives (**20–22**) were in the same order of magnitude of potency as the 8-substituted cAMP analogs containing no $O^{2'}$ substitution (**5–7**). The 8-substituted ara-cAMP derivatives (**14–18**) were somewhat less potent as inhibitors of cAMP hydrolysis than the latter two classes of compounds, and the 8-substituted 8,2′-anhydro-cAMP derivatives were very poor inhibitors.

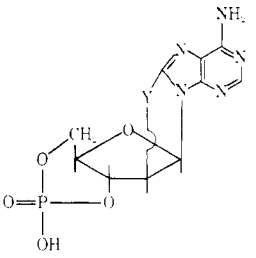
Table I: Effect of 8-Substituted Derivatives of cAMP with Various Chemical Modifications in the 2′ Position on cAMP-Dependent Protein Kinase.[a]



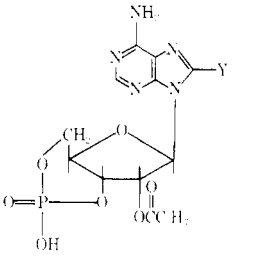
8-Substituted cAMP



8-Substituted Ara-cAMP



8-Substituted 8,2′-Anhydro-cAMP



8-Substituted 2′-*O*-Butyryl-cAMP

Y Substituent[b]	Compd No.	K_a'	Compd No.	K_a'	Compd No.	K_a'	Compd No.	K_a'
–H	1	1.0	14	0.001			19	
–OH (–O–)	3	2.8	15	0.004	10	0.001		
$-OCH_3$	4	0.29	16	0.003				
–SH (–S–)	5	3.8	17	0.004	11	0.002	20	0.004
$-SCH_3$	6	2.4					21	0.002
$-SCH_2C_6H_5$	7	2.1					22	0.001
$-NH_2$ (–N(H)–)	8	1.5	18	0.002	12	0.001		
$-NHCH_3$ (–N(CH_3)–)	9	0.38			13	0.0007		

[a] The assay for protein kinase activation was performed as described in Biochemical Methods. The values reported are K_a' values, where K_a' is K_a for cAMP (2.0×10^{-7} *M*)/K_a for the test compounds. K_a is the activation constant determined from a Lineweaver–Burk plot. [b] The form of the substituent for the 8,2′-anhydro derivatives is given in parentheses.

All of the 8-substituted cAMP derivatives, with the exception of 8-HO-cAMP (**3**), demonstrated essentially the same I_{50} values. 8-HO-cAMP had, on the average, I_{50} values about an order of magnitude higher than the other 8-substituted cAMP derivatives. A similar comparison of the 8-substituted ara-cAMP derivatives shows that both 8-HO-ara-cAMP (**15**) and 8-MeO-ara-cAMP (**16**) were significantly poorer inhibitors of cAMP hydrolysis than were ara-cAMP (**14**), 8-HS-ara-cAMP (**17**), and 8-H_2N-ara-cAMP (**18**). In addition, while each of the 8-substituted ara-cAMP derivatives inhibited the phosphodiesterases from lung and heart with about equal potency, ara-cAMP (**14**) was tenfold better as an inhibitor of the heart enzyme than of the lung enzyme. 8-HO-8,2′-anhydro-cAMP (**10**) was the only one of the 8-substituted 8,2′-anhydro-cAMP derivatives which was a significant inhibitor of cAMP hydrolysis. In fact, 8-HO-8,2′-anhydro-cAMP (**10**) was a better inhibitor than 8-HO-ara-cAMP (**15**) and was about equal in inhibitory potency to 8-HO-cAMP (**3**). All of the 8-substituted $O^{2'}$-Bt-cAMP derivatives (**20–22**) were less potent than $O^{2'}$-Bt-cAMP (**19**) as phosphodiesterase inhibitors.

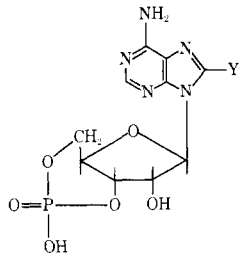
Discussion

The effect of modifications of the purine (Muneyama et al., 1971, 1974; Meyer et al., 1972; Boswell et al., 1973; Miller et al., 1973a,c), carbohydrate (Miller et al., 1973b), and cyclic phosphate (Meyer et al., 1973; Shuman et al., 1973; Eckstein et al., 1974; Jones et al., 1970; Jastorff and Bär, 1973) moieties of cAMP on the interaction of the resulting compounds with the enzymes of cAMP metabolism suggests that all these portions of the cAMP molecule are interacting in some manner with these enzymes. Disubstituted derivatives of cAMP present the possibility of studying how the different portions of the cAMP molecule interact together with the binding site on an enzyme which utilizes cAMP.

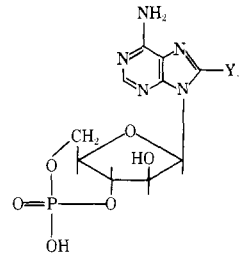
Previous results have indicated that modification of both the 6 and 5′ positions of cAMP was not tolerated, while either modification by itself does not cause a radical change in the ability of the resulting compounds to be utilized by cAMP-dependent protein kinase or cAMP phosphodiesterase (Shuman et al., 1973). We have also shown that many N^6-substituted cAMP analogs are efficient activators of protein kinase (Meyer et al., 1972; Miller et al., 1973b; Boswell et al., 1973). Studies on several N^6,$O^{2'}$-bis(*N*-alkylcarbamoyl)adenosine 3′,5′-cyclic phosphate derivatives have shown that the N^6,$O^{2'}$-disubstituted compounds did not activate protein kinase, while several of the corresponding N^6-monosubstituted derivatives demonstrated K_a' values greater than unity (Boswell et al., 1973). N^6,$O^{2'}$-Dibutyryladenosine 3′,5′-cyclic phosphate does not activate protein kinase, but N^6-butyryladenosine 3′,5′-cyclic phosphate exhibits a K_a' of 0.84 (Miller et al., 1973b). In these instances, the $O^{2'}$ substitution abrogated any enhancive effect of the N^6 substitution on kinase activation. The data on the activation of protein kinase by the 8,2′-disubstituted derivatives of cAMP show that the general inactivity of 2′ derivatives of cAMP (Miller et al., 1973b) with the kinase was not overcome by addition of an 8 substituent, even though many 8-substituted derivatives of cAMP activate the kinase more efficiently than does cAMP itself.

With respect to their interaction with the phosphodiesterase, the 2′ derivatives of cAMP are substrates for the enzyme (Miller et al., 1973b), and the 8-substituted analogs generally are not (Muneyama et al., 1971, 1974; Miller et al., 1973a). The 8,2′ doubly modified derivatives reported here are resistant to enzymic hydrolysis. In this case, the substitution of the 8 position countermanded the tolerated 2′ modification. The studies on the inhibition of phosphodiesterase produced divergent results which depended on the type of 2′ modification. Changing the ribo configuration to arabino (compounds **14–18**) decreased the ability of the resulting compounds to inhibit the enzymes, while a $O^{2'}$ sub-

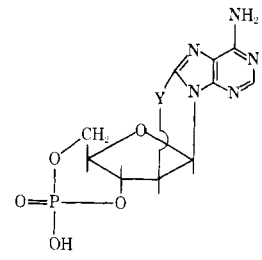
Table II: Effect of 8-Substituted Derivatives of cAMP with Various Chemical Modifications in the 2' Position on cAMP Hydrolysis.[a]



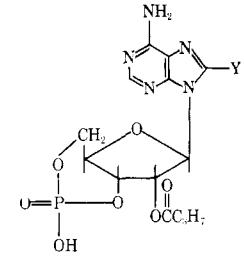
8-Substituted cAMP



8-Substituted Ara-cAMP



8-Substituted 8,2'-Anhydro-cAMP



8-Substituted 2'-*O*-Butyryl-cAMP

Y Substituent[b]	Compd No.	I_{50} (μM) Rabbit Lung	I_{50} (μM) Beef Heart	Compd No.	I_{50} (μM) Rabbit Lung	I_{50} (μM) Beef Heart	Compd No.	I_{50} (μM) Rabbit Lung	I_{50} (μM) Beef Heart	Compd No.	I_{50} (μM) Rabbit Lung	I_{50} (μM) Beef Heart
–H	1			14	230	23				19	5.6	5.6
–OH (–O–)	3	230	630	15	2700	1600	10	670	230			
$-OCH_3$	4	60	50	16	2600	1800						
–SH (–S)	5	21	51	17	140	300	11	>100,000	>100,000	20	130	130
$-SCH_3$	6	70	26							21	32	70
$-SCH_2C_6H_5$	7	52	18							22	12	13
$-NH_2$ (–N(H)–)	8	80	36	18	340	300	12	>100,000	>100,000			
$-NHCH_3$ (–N(CH_3)–)	9	77	36				13	>100,000	15,000			

[a] The assay for the inhibition of cAMP hydrolysis was performed as described in Biochemical Methods. Results are reported as I_{50} values, where I_{50} is the concentration of compound that produces 50% of the uninhibited rate. [b] The form of the substituent for the 8,2'-anhydro derivatives is given in parentheses.

stitution (compounds **20–22**) generally did not affect the inhibitory activity of the resulting compounds. The 8-substituted 8,2'-anhydro-cAMP derivatives, with the exception of the 8-HO-8,2'-anhydro-cAMP (**10**), were such poor inhibitors that in most cases the I_{50} value could not be determined.

Both of the cyclic nucleotide preparations were examined for their molecular heterogeneity and substrate specificity. When subjected to gel filtration according to Thompson and Appleman (1971), both the rabbit lung and the beef heart preparations contained primarily what these authors refer to as "Fraction II" type activity. The preparations used here demonstrated the same K_m values for cAMP as did the "Fraction II" obtained from subjecting these preparations to gel filtration. The K_m for cAMP with the rabbit lung phosphodiesterase was 0.32 μM and with the beef heart phosphodiesterase was 0.085 μM. I_{50} values were determined using the "Fraction II" activity from each preparation for compounds **3, 5, 15, 17, 10, 11,** and **20,** and were found not to be significantly different from those reported in Table II using the preparations described in Biochemical Methods. The detailed properties of these phosphodiesterase preparations will be the subject of a subsequent report.

X-Ray data (Travale and Sobell, 1970) indicate that crystals of 8-substituted purine nucleoside exist as syn conformers. Circular dichroism studies (Ikehara et al., 1972) suggest that 8-substituted purine nucleosides retain this syn conformation in aqueous solution. X-Ray analysis of crystals of cAMP (**1**) revealed two molecules per unit cell, one in anti and the other in the syn conformation (Watenpaugh et al., 1968). ^1H NMR studies (Schweizer and Robins, 1973) indicate that substitution of cAMP (**1**) or ara-cAMP (**14**) in the 8 position with a bulky group forces the resulting derivatives to exist mainly in the syn form while the parent compounds show a preference for the anti form. In addition, a comparison of the vicinal $^{13}C_8$–$^1H_{1'}$ coupling constants for cAMP, 8-Br-cAMP, and 8-HO-cAMP (Dea et al., 1973) can be interpreted to suggest that a solution of cAMP contains some anti conformers. ^1H NMR experiments utilizing the nuclear Overhauser effect were also consistent with this conclusion (Barry et al., 1974). This possibility of cAMP existing in two conformations in aqueous solution raises the questions, whether cAMP will exhibit its biological activity in only the syn or only the anti configuration, and whether an 8-substituted derivative of cAMP when fixed in an anti configuration will also show cAMP-like activity.[3] 8-Substituted 8,2'-anhydro-cAMP derivatives (**10–13**) which are fixed in an anti-like conformation did not activate bovine brain cAMP dependent protein kinase. However, since these 8-substituted 8,2'-anhydro-cAMP analogs (**10–13**) lack a 2'-hydroxyl group in the ribose configuration, which has been shown to be required for protein kinase activation (Miller et al., 1973b) by cAMP and its analogs, the question of the activity of cAMP in the anti conformation in such a system remains open.

The 8-substituted 8,2'-anhydro cAMP derivatives probably exist with the C-8 positioned over the C-2' and remain fixed in that position. The other classes of 8-substituted cAMP derivatives, while being syn-like in conformation, still are significantly less hindered in rotation about the glycosidic bond than the covalently fixed 8-substituted 8,2'-anhydro-cAMP derivatives. The torsional angle of the glycosidic bond of cAMP while it is bound to phosphodiesterase may well be such that the C-8 is not positioned over the C-2'. The data, in fact, suggest this. Hampton et al. (1972,

[3] Two reports on the conformation of cAMP in solution using lanthanide ions as ^1H NMR probes (Barry et al., 1974; Lavallee and Zeltmann, 1974) suggest that cAMP exists in the syn conformer at pH 5.3–5.5 in the presence of lanthanide ions. Without knowing the effects of lanthanide ions on the conformation, we prefer to consider the data from the nonperturbed systems (Schweizer and Robins, 1973; Dea et al., 1973).

1973) have shown that 8,5′-cycloadenosine 5′-phosphate is a good substrate for many 5′-AMP utilizing enzymes while 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine 5′-phosphate and 8,3′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine 5′-phosphate were essentially inactive with the same enzymes. The compound 8,5′-cyclo-cAMP would indeed provide additional insight into the conformeric form of enzyme-bound cAMP.

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