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# Chemical Synthesis and Biological Activity of 8-Substituted Adenosine 3′,5′-Cyclic Monophosphate Derivatives*

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ABSTRACT: 8-Bromoadenosine 3′,5′-cyclic monophosphate served as an intermediate for the chemical synthesis of various 8-substituted derivatives of adenosine 3′,5′-cyclic monophosphate (cAMP). Nucleophilic substitution resulted in displacement of bromine at the 8 position of the purine ring without rupture of the 3′,5′-cyclic nucleotide structure. These 8-substituted derivatives of cAMP have been examined as alternate activators of the cAMP-dependent protein kinase isolated and purified from bovine brain. Structure–activity relationships were established with regard to the functional groups present at C-8 and the accompanying biological activity.

All of the analogs studied possessed some activity toward this enzyme, and several were more effective than cAMP itself. The 8-substituted derivatives of cAMP were also examined as alternate substrates for a cAMP phosphodiesterase isolated from rabbit kidney. With a single exception, 8-amino-cAMP, all of the compounds studied were resistant to degradation by this enzyme. In addition, they were found to be inhibitors of the cAMP phosphodiesterase.

Adenosine 3′,5′-cyclic monophosphate (cAMP) has been shown to stimulate the ATP-dependent phosphorylation of a wide variety of proteins; *e.g.*, casein, histone, protamine, phosphorylase *b*-kinase kinase (Walsh *et al.*, 1968), neurotubular protein (Goodman *et al.*, 1970), fat cell lipase (Huttanen *et al.*, 1970), ribosomal protein (Loeb and Blat, 1970), and $\sigma$ factor of bacterial RNA polymerase (Martelo *et al.*, 1970). This enzyme has been isolated from cell-free extracts prepared from nearly every single tissue and organism that has been studied (Miyamoto *et al.*, 1969; Kuo *et al.*, 1970), and has been purified from a wide variety of sources, including bovine brain (Miyamoto *et al.*, 1969). It has been postulated that all of the various biological properties attributed to cAMP may be mediated through the stimulation of these protein kinases (Miyamoto *et al.*, 1969). Furthermore, the regulation of cAMP levels both *in vitro* and *in vivo* is in part accomplished by the activity of a class of cyclic nucleotide phosphodiesterases, isolated from most all tissues, that is capable of hydrolyzing several of the cyclic nucleotides to the 5′-monophosphate (Robison *et al.*, 1968).

Examination of variations in biological activity with analogs of cAMP possessing regularly varied structural modifications has had to await the synthesis of sufficient supplies of the pure cyclic nucleotide derivatives. Some reports have appeared in which cyclic nucleotide derivatives of the naturally occurring nucleosides have been studied as alternate activators of the protein kinase. Kuo *et al.* (1970) have shown that cIMP and cGMP had 32 and 7% the activity of cAMP at concentrations of $5 \times 10^{-7}$ M, using a partially purified preparation of bovine brain protein kinase. The same authors showed that cUMP and cCMP were approximately 7% as active as cAMP, and cdTMP was virtually inactive. Kuo and Greengard (1970a) have also reported on the activity of tubercidin 3′,5′-cyclic monophosphate (cTuMP) which was shown to be about 50% as active as the parent nucleotide, using the same enzyme preparation. Two phosphonate analogs of cAMP (3′-$CH_2$-cAMP and 5′-$CH_2$-homo-cAMP) were also examined and found to be relatively inactive toward the bovine brain enzyme. Drummond and Powell (1970) have similarly reported that these two isomeric cyclic phosphonate compounds and adenosine 3′,5′-phosphorothioate were either extremely weak activators or completely inactive with phosphorylase *b*-kinase kinase.

The stability of several cyclic nucleotides to enzymatic hydrolysis has also been studied. Nair (1966) has shown that cdAMP was hydrolyzed about 30% faster than cAMP, while cIMP and cGMP were cleaved at 60 and 33% the rate of cAMP. The pyrimidine cyclic nucleotides cUMP and cCMP were relatively stable to hydrolysis, using a dog heart phosphodiesterase preparation. Eckstein and Bär (1970) have demonstrated that a thio analog of cAMP (adenosine 3′,5′-phosphorothioate) was resistant to diesterase action.

Studies of the effect of structural analogs and derivatives of cAMP on the above enzyme systems are obviously desirable and should lead to useful information regarding the acti-

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vating effect exerted by cAMP. These derivatives might show sufficient specificity which could lead to the development of useful pharmacological agents that either mimic or antagonize cAMPs activity. The compounds studied in the present investigation were all analogs of adenosine 3′,5′-cyclic monophosphate substituted in the 8 position of the purine nucleus.

Previous methods successful for the introduction of substituents into the 8 position of adenosine (Holmes and Robins, 1964, 1965) suggested that perhaps nucleophilic displacement of the halogen at position 8 might be successful in the case of adenosine 3′,5′-cyclic monophosphate. Success in the synthesis of various 8-substituted 2′-deoxyadenosines (Holmes and Robins, 1964; Long *et al.*, 1967) suggested that selected nucleophiles might react under relatively mild conditions without hydrolysis of the glycosyl bond. The question of the stability of the 3′,5′-cyclic phosphate ring under these conditions remained to be examined. Ikehara and Uesugi (1969) prepared 8-bromoadenosine 3′,5′-cyclic monophosphate (**1**) by direct bromination of cAMP.

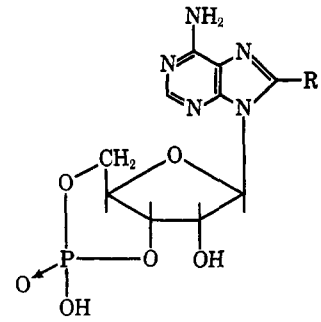
In our laboratory, treatment of **1** with refluxing aqueous methylamine gave 8-methylaminoadenosine 3′,5′-cyclic phosphate (**8**) in 54% yield. The use of dimethylamine in refluxing methanol gave 8-dimethylaminoadenosine 3′,5′-cyclic phosphate after crystallization from water. Similarly benzylamine, ethanolamine, and furfurylamine provided the 8-substituted amino derivatives **5**, **6**, and **9**, respectively. The 8-substituted adenosine 3′,5′-cyclic phosphates were readily purified by crystallization from water, in most instances at pH <3. A comparison of the ultraviolet spectra of these 8-substituted aminocyclic nucleotides with known 8-substituted amino purine-nucleosides and nucleotides confirmed the position of substitution. The nuclear magnetic resonance spectra (in particular $J_{1'-2'} < 1$ Hz) was consistent with the presence of the cyclic phosphate ring (Jardetzsky, 1962).

Treatment of 8-bromoadenosine 3′,5′-cyclic phosphate (**1**) with sodium methoxide in the presence of methanethiol in methanol gave 8-methylthioadenosine 3′,5′-cyclic phosphate (**2**) in good yield. Replacement of methanethiol by ethanethiol, 2-mercaptoethanol, or benzylmercaptan gave **10**, **7**, and **11**, respectively.

Azide displacement of the bromine of 8-bromoadenosine 3′,5′-cyclic phosphate (**1**) gave 8-azidoadenosine 3′,5′-cyclic monophosphate (**14**) which was hydrogenated over palladium on charcoal to yield 8-aminoadenosine 3′,5′-cyclic phosphate (**15**) in good yield.

8-Thioadenosine 3′,5′-cyclic phosphate (**12**) was obtained by treatment of **1** with thiourea in methyl Cellosolve at 90–100°. Treatment of 8-bromoadenosine 3′,5′-cyclic phosphate (**1**) with glacial acetic acid, acetic anhydride and sodium acetate at 100° gave $N^6$,$O^{2'}$-diacetyl-8-oxyadenosine 3′,5′-cyclic phosphate [$\lambda_{max}$ ($H^+$) 287 mμ, $\lambda_{max}$ ($HO^-$) 268 mμ, and $\lambda_{max}$ ($H_2O$) 286 mμ] which was not isolated but was deacetylated with methanolic ammonia to give 8-oxyadenosine 3′,5′-cyclic phosphate (**13**). The elemental analyses, ultraviolet spectra, nuclear magnetic resonance spectra, and thin-layer chromatographic mobilities were consistent with the structure assignments. The properties of these compounds are listed in Table I. The stability of the 3′,5′-cyclic phosphate moiety under the present reaction conditions is indeed noteworthy. The direct utilization of cAMP and other readily available 3′,5′-cyclic nucleotides as intermediates to provide new cyclic nucleotide derivatives has thus been demonstrated. Additional 3′,5′-cyclic nucleotides available in a similar manner will constitute the subject of subsequent communications from these laboratories.

TABLE I: Physical Properties of 8-Substituted Adenosine 3′,5′-Cyclic Phosphates.



| R | $\lambda_{max}$ (mμ) pH 1 | ($\epsilon \times 10^{-3}$) pH 11 | $R_F$ (A) | $R_F$ (B) |
|---|---|---|---|---|
| Br (**1**) | | | 0.47 | 0.47 |
| $SCH_3$ (**2**) | 281 (19.7) | 278 (18.5) | 0.46 | 0.51 |
| $N(CH_3)_2$ (**3**) | 278 (17.8) | 273 (18.4) | 0.57 | 0.48 |
| $OCH_3$ (**4**) | 259 (12.9) | 256 (13.6) | 0.47 | 0.45 |
| $NHCH_2C_6H_5$ (**5**) | 276 (16.9) | 274 (18.6) | 0.64 | 0.61 |
| $NHCH_2CH_2OH$ (**6**) | 275 (15.2) | 274 (17.1) | 0.35 | 0.38 |
| $SCH_2CH_2OH$ (**7**) | 281 (19.9) | 280 (18.0) | 0.42 | 0.44 |
| $NHCH_3$ (**8**) | 273 (14.6) | 274 (16.0) | 0.55 | 0.50 |
| $NHCH_2C_4H_4O$ (**9**) | 275 (16.0) | 273 (17.9) | 0.55 | 0.56 |
| $SCH_2CH_3$ (**10**) | 280 (23.9) | 280 (22.5) | 0.65 | 0.53 |
| $SCH_2C_6H_5$ (**11**) | 283 (19.2) | 283 (16.2) | 0.82 | 0.69 |
| SH (**12**) | 220 (13.4)<br>244 (9.9)<br>308 (25.2) | 224 (17.4)<br>291 (22.4) | 0.38 | 0.13 |
| OH (**13**) | 262 (11.2)<br>284 (10.2) | 278 (15.3) | 0.33 | 0.11 |
| $N_3$ (**14**) | 280 (17.7) | 281 (13.3) | 0.41 | 0.44 |
| $NH_2$ (**15**) | 266 (14.1) | 268 (16.7) | 0.24 | 0.21 |

## Experimental Section

### *Synthetic*

Thin-layer chromatography was run on a Merck cellulose F plate and was developed with either solvent system A (isopropyl alcohol–aqueous $NH_3$–$H_2O$, 7:1:2, v/v) or B (butyl alcohol–acetic acid–$H_2O$, 5:2:3, v/v). Evaporations were accomplished using a Büchler rotating evaporator under reduced pressure with a bath temperature <40°. Ultraviolet spectra were determined on a Cary 15 spectrometer and infrared spectra on a Perkin-Elmer 257 spectrometer. Silica gel for column chromatography was obtained from J. T. Baker Chemical. Charcoal No. 126 was obtained from Barneby Cheney, Los Angeles, Calif. Analytical samples were dried over $CaCl_2$ for 12 hr at 0.01mm (110°).

*8-Bromoadenosine 3′,5′-Cyclic Phosphate* (**1**). To a solution of adenosine 3′,5′-cyclic phosphate (13.2 g, 40 mmoles) in 20 ml of 2 N sodium hydroxide was added 250 ml of 1 M sodium acetate buffer (pH 3.9). A solution of bromine (8.68 g, 2.8 ml) in 300 ml of 1 M sodium acetate buffer (pH 3.9) was added to the nucleotide solution. The solution was stirred overnight and the resulting precipitate was filtered and washed with water to give 11.4 g of chromatographically pure 8-bromoadenosine 3′,5′-cyclic phosphate (**1**). The filtrate was passed through a

charcoal column (140 g) and the column was washed with water. An additional 1.9 g of chromatographically pure **1** was obtained by elution of the column with water–ethyl alcohol–benzene (T. Ueda, 1970, personal communication) (1:1, v/v, saturated with benzene). The total yield of 8-bromoadenosine 3′,5′-cyclic phosphate was 13.1 g (81%).

*8-Methylthioadenosine 3′,5′-Cyclic Phosphate* (**2**). To a solution of methanol (50 ml) containing sodium methoxide (1.4 g, 25 mmoles) and methanethiol (4 ml) was added 2.5 g (6 mmoles) of 8-bromoadenosine 3′,5′-cyclic phosphate (**1**). The reaction mixture was refluxed for 10 hr. The solvent was removed by evaporation and the residue was dissolved in 1 N $NH_4OH$. Adjustment of the pH of the solution to <3 caused crystallization of **2**. The crystals were filtered, washed with water, and dried to give 1.6 g of chromatographically pure **2**.

*Anal.* Calcd for $C_{11}H_{14}N_5O_6PS\cdot 1.5H_2O$: C, 32.83; H, 4.26; N, 17.40; O, 28.82; P, 7.69; S,7.97. Found: C, 32.83; H, 4.38; N, 17.20; O, 29.63; P, 7.82; S, 8.04.

*8-Dimethylaminoadenosine 3′,5′-Cyclic Phosphate* (**3**). A solution of 8-bromoadenosine 3′,5′-cyclic phosphate (**1**) (3 g, 7 mmoles) in methanol (18 ml) and dimethylamine (18 ml) was refluxed for 18 hr. The reaction mixture was evaporated to dryness. To the residue was added silica gel (5 g) and methanol, and the suspension was evaporated to a powder. The powder was added to a silica gel column (30 g, 3 × 25 cm packed in chloroform and washed with chloroform–methanol (3:1, v/v). The nucleotide was eluted with methanol. Evaporation of the appropriate fractions and crystallization of the residue from water gave 2.3 g of **3**.

*Anal.* Calcd for $C_{12}H_{17}N_6O_6P\cdot 2.5H_2O$: C, 34.53; H, 5.31; N, 20.14. Found: C, 34.38; H, 5.24; N, 20.29.

*8-Methoxyadenosine 3′,5′-Cyclic Phosphate* (**4**). A solution of **1** (2.0 g, 5 mmoles) in 30 ml of methanol containing 1.4 g (25 mmoles) of sodium methoxide was refluxed for 10 hr. Evaporation of the solvent gave a residue which was purified as in the procedure for **2**. An additional crystallization as in the procedure for **2** gave 1.3 g of **4**.

*Anal.* Calcd for $C_{11}H_{14}N_5O_7P$: C, 36.77; H, 3.92; N, 19.49. Found: C, 36.96; H, 3.89; N, 19.28.

*8-Benzylaminoadenosine 3′,5′-Cyclic Phosphate* (**5**). To a solution of ethanol (20 ml) and benzylamine (2.1 g, 20 mmoles) was added 2.15 g of **1**. The reaction mixture was heated at 90–100° for 24 hr in a bomb. Evaporation of the solvent gave a residue which was triturated with ether and then dissolved in 1 N $NH_4OH$. Addition of 1 N HCl as in **2** gave 0.75 g of crystalline **5**.

*Anal.* Calcd for $C_{17}H_{19}N_6O_6P\cdot 2H_2O$: C, 43.40; H, 4.92; N, 17.96. Found: C, 43.63; H, 4.70; N, 17.97.

*8-(β-Hydroxylethylamino)adenosine 3′,5′-Cyclic Phosphate* (**6**). A solution of 1.3 g (3 mmoles) of **1** in 2-methoxyethanol (20 ml) and 2-aminoethanol (8 ml) was heated for 12 hr at 100–105° in a bomb. The solution was evaporated and the residue dissolved in water and treated with 10 equiv of Dowex 50 (pyridinium form). The resin was removed by filtration and the filtrate evaporated to dryness. The resulting residue was crystallized as in the procedure for **2** to give 0.6 g of **6**.

*Anal.* Calcd for $C_{12}H_{17}N_6O_7P\cdot 2.5H_2O$: C, 33.25; H, 5.11; N, 19.39. Found C, 33.48; H, 4.84; N, 19.62.

*8-(β-Hydroxyethylthio)adenosine 3′,5′-Cyclic Phosphate* (**7**). A solution of 1.3 g (3 mmoles) of **1** in methanol (30 ml) containing sodium methoxide (0.81g, 15 mmoles) and 2-mercaptoethanol (5 ml) was refluxed for 5 hr. Work-up as in the procedure for **2** gave 1.2 g (90%) of **7**.

*Anal.* Calcd for $C_{12}H_{16}N_5O_7PS\cdot 1.5H_2O$: C, 33.33; H, 4.42; N, 16.19; P, 7.16. Found: C, 33.56; H, 4.19; N, 15.97; P, 7.06.

*8-Methylaminoadenosine 3′,5′-Cyclic Phosphate* (**8**). A solution of 1.3 g (3 mmoles) of **1** in 30% aqueous methylamine (40 ml) was refluxed for 4 hr and worked-up as in the procedure for **2** to give 0.58 g (54%) of **8**.

*Anal.* Calcd for $C_{11}H_{15}N_6O_6P\cdot 2H_2O$: C, 33.50; H, 4.85; N, 21.31. Found: C, 33.75; H, 4.61; N, 21.12.

*8-Furfurylaminoadenosine 3′,'5-Cyclic Phosphate* (**9**). A solution of 1.3 g (3 mmoles) of **1** and furfurylamine (8 ml) in 2-methoxyethanol (50 ml) was heated at 110–115° for 12 hr in a bomb. The reaction mixture was worked up as in the procedure for **2** to give 0.66 g of **9**.

*Anal.* Calcd for $C_{15}H_{17}N_6O_7P\cdot H_2O$: C, 40.72; H, 4.32; N, 19.00. Found: C, 40.88; H, 4.57; N, 18.85.

*8-Ethylthioadenosine 3′,5′-Cyclic Phosphate* (**10**). A solution of 2 g (5 mmoles) of **1** in methanol (50 ml) containing sodium methoxide (1.34 g, 25 mmoles) and ethanethiol (7 ml) was refluxed overnight. Work-up as in the procedure for **2** gave 1.5 g of **10**.

*Anal.* Calcd for $C_{12}H_{16}N_5O_6PS$: C, 37.01; H, 4.14; S, 8.23. Found: C, 36.71; H, 4.30; S, 8.29.

*8-Benzylthioadenosine 3′,5′-Cyclic Phosphate* (**11**). A solution of 2 g (5 mmoles) of **1** in methanol (100 ml) containing sodium methoxide (1.34 g, 25 mmoles) and benzylthiol (7 ml) was refluxed for 5 hr. Work-up as in the procedure for **2** gave 1.1 g (48%) of **11**.

*Anal.* Calcd for $C_{17}H_{18}N_5O_6PS\cdot 0.5H_2O$: C, 44.34; H, 4.15; N, 15.21; S, 6.96. Found: C, 44.11; H, 3.72; N, 14.76; S, 6.95.

*8-Thioadenosine 3′,5′-Cyclic Phosphate* (**12**). A solution of 1 N NaOH (7 ml), thiourea (0.76 g, 10 mmoles) and 2 g of **1** was evaporated to a solid residue. The residue was dissolved in 2-methoxyethanol (100 ml) and was heated at 90–100° for 2 days. The reaction mixture was evaporated to dryness. To the residue was added silica gel (10 g) and water, and the suspension was evaporated to a powder. The powder was added to a silica gel column (60 g, packed with chloroform). The nucleotide was eluted with chloroform–methanol (1:1, v/v). Evaporation of the appropriate fractions and crystallization as in the preparation of **2** gave 0.8 g of **12**.

*Anal.* Calcd for $C_{10}H_{12}N_5O_6PS\cdot H_2O$: C, 31.60; H, 3.72; S, 8.45. Found: C, 32.07; H, 4.04; S, 8.60.

*8-Oxyadenosine 3′,5′-Cyclic Phosphate* (**13**). A solution of **1** (2 g, 5 mmoles), sodium acetate (4.1 g, 50 mmoles), acetic anhydride (10 ml), and glacial acetic acid (100 ml) was heated at 100–120° for 3.5 hr. The reaction mixture was evaporated to dryness. To the residue was added silica gel (20 g) and ethanol, and the suspension was evaporated to a powder. The powder was added to a silica gel column (50 g, packed in chloroform) and the nucleotide was eluted with chloroform–methanol (3:2, v/v). Evaporation of the appropriate fractions gave a residue which was dissolved in methanolic ammonia and heated at 50° for 3 days in a bomb. Evaporation of the solvent and crystallization as in the procedure for **2** gave 0.7 g of **13**; infrared spectrum (KBr) at 1740 $cm^{-1}$ (8-C=O).

*Anal.* Calcd for $C_{10}H_{12}N_5O_7P\cdot H_2O$: C, 33.06; H, 3.88; N, 19.28. Found: C, 33.33; H, 3.62; N, 19.59.

*8-Azidoadenosine 3′,5′-Cyclic Phosphate* (**14**). A dimethylformamide (200 ml) solution of **1** (2 g, 5 mmoles) and sodium azide (0.65 g, 10 mmoles) was heated at 70–80° overnight. Evaporation of reaction mixture gave a crude product which was crystallized as in the procedure for **2**.

*Anal.* Calcd for $C_{10}H_{11}N_8O_6P\cdot H_2O$: C, 30.93; H, 3.37; N, 28.86. Found: C, 30.75; H, 3.66; N, 28.27.

*8-Aminoadenosine 3′,5′-Cyclic Phosphate* (**15**). The crude product from **14** was dissolved in 200 ml of water by the addition of 5 ml of 1 N sodium hydroxide. The solution, in the

presence of 10% palladium on charcoal (1 g), was shaken with $H_2$ at 2–3 atm overnight. The suspension was filtered through diatomaceous earth and evaporated to a small volume. The nucleotide was absorbed on silica gel (15 g) and added to a silica gel column (10 g) as in the preparation of **13**. The nucleotide was eluted with chloroform–methanol (3:2, v/v). Evaporation of the appropriate fractions and crystallization of the residue as in the procedure for **2** gave 0.8 g of **15**.

*Anal.* Calcd for $C_{10}H_{13}N_6O_6P \cdot H_2O$: C, 33.15; H, 4.17; N, 23.20. Found: C, 33.11; H, 4.10; N, 23.41.

*Enzymatic*

The cAMP-dependent protein kinase was purified to the stage of DEAE-cellulose chromatography from bovine brain using the procedure of Miyamoto *et al.* (1969). A preparation having a specific activity of 2500 was obtained. Protein kinase activity was assayed by measuring the incorporation of [$^{32}$P]phosphate into histone from $\gamma$-$^{32}$P-labeled ATP. The incubation mixture contained (final volume of 0.2 ml, amounts in micromoles): sodium glycerol phosphate buffer (pH 6.0, 10) [$\gamma$-$^{32}$P]ATP (about $1 \times 10^6$ cpm, 0.00053), magnesium acetate (9.0), sodium fluoride (2), theophylline (0.1), EDTA (0.06), histone (500 $\mu$g), cAMP or analog as indicated, and purified protein kinase (5–25 $\mu$g). After incubation an aliquot of the reaction mixture (0.1 ml) was transferred to disks of Whatman No. 3MM filter paper, air-dried, and washed with 6% trichloroacetic acid for 15 min at room temperature. After repeated washings with 6% trichloroacetic acid and finally with ether, the air-dried disks were transferred to liquid scintillation vials and radioactivity was determined. One unit of protein kinase activity was defined as that amount of enzyme which will transfer 1 pmole of $^{32}$P from [$\gamma$-$^{32}$P]ATP to histone in 5 min at 30°.

Specific cAMP phosphodiesterase activity was measured using three different methods; however, the basic incubation mixture was the same in all cases. Basic mixture (final volume of 1.0 ml, amounts in micromoles): Tris buffer (pH 7.50, 40), magnesium acetate (25.0), [$^3$H]-, [$^{14}$C]-, or [$^{32}$P]cAMP, unlabeled cAMP, or analog (0.1–1.0 $\mu$mole), enzyme (from 100 to 500 $\mu$g). One unit of activity is that amount of enzyme catalyzing the hydrolysis of 1.0 $\mu$mole in 10 min at 37°. The cAMP phosphodiesterase was a 100,000*g* supernatant preparation isolated from pig brain frontal cortex, or an ammonium sulfate precipitate (30–45%) prepared from rabbit kidney 100,000*g* supernatant.

*Method 1. Radioactive.* The reaction was stopped by the addition of 0.2 ml each of 0.2 M $Ba(OH)_2$ + 0.2 M $H_2SO_4$ + 500 $\mu$g of albumin. The contents were heated to 90° for 2 min and then centrifuged at 1500 rpm for 15 min. The supernatants were withdrawn and 0.1-ml aliquots transferred to scintillation vials containing Bray's counting solution. The amount of 5′-AMP formed was calculated by subtracting the total counts remaining in the supernatant after the reaction, from the total counts present in an incubated control using heat-killed enzyme.

*Method 2. Colorimetric.* After incubation for 5 min at 30° as above, the reaction was heated at 90° for 2 min and then cooled to room temperature. *Escherichia coli* alkaline phosphatase (50 $\mu$g) was added and incubation continued at 37° for 5–10 min. The $P_i$ that was released was measured by the ascorbic acid–molybdate method (Lowry and Lopez, 1946). The results from methods 1 and 2 were in good agreement with each other.

*Method 3. Paper Chromatography.* Incubation of cold cAMP or analog was carried out as above and the reaction was terminated by heating at 90° for 2 min. If necessary, the concentration of cAMP or analog was increased tenfold over that described above. The protein was removed by centrifugation, and an aliquot of the protein-free supernatant was chromatographed on paper using an isopropyl alcohol–$NH_3$–$H_2O$ system (7:1:3, v/v). The ultraviolet-absorbing spots were cut out and eluted with 0.01 N HCl and the absorbance at 260 m$\mu$ was measured. Results from all three methods were comparable, but only the latter two were used in this study to measure degradation of the analogs by phosphodiesterase.

[$^3$H]-, [$^{32}$P]-, or [$^{14}$C]cAMP and [$\gamma$-$^{32}$P]ATP were obtained from International Chemical and Nuclear Corp. *E. coli* alkaline phosphatase was purchased from Nutritional Biochemicals. Fresh bovine brain was obtained from a local slaughterhouse, and pig brain cortices were a generous gift of Dr. K. Swiatek.

TABLE II: Activation of Bovine Brain Protein Kinase by 8-Substituted 3′,5′-cAMP Analogs.

| Concn (M) | pmoles of $^{32}$P Incorpd into Histone in Presence of: cAMP | 8-Thio-cAMP (12) | 8-Oxo-cAMP (13) | 8-$NH_2$-cAMP (15) |
|---|---|---|---|---|
| $10^{-8}$ | 11.2 | 22.4 (2.0)[a] | 11.2 (1.0) | 17.7 (1.70) |
| $10^{-7}$ | 35.0 | 48.2 (1.40) | 42.7 (1.22) | 21.9 (0.62) |
| $10^{-6}$ | 43.7 | 49.7 (1.13) | 45.5 (1.04) | 35.4 (0.81) |
| $10^{-5}$ | 44.0 | 53.4 (1.20) | 50.0 (1.13) | 45.0 (1.0) |
| $10^{-4}$ | 29.2 | 45.2 (1.55) | 45.9 (1.57) | 25.3 (0.85) |

[a] Values in parentheses = $\alpha$, ratio of activity in the presence of analog to activity in the presence of cAMP at the indicated concentration.

## Results

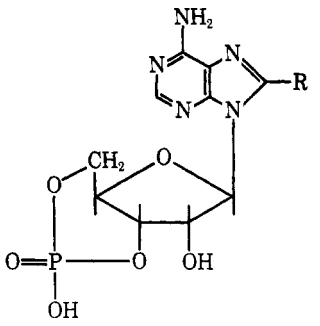
*Protein Kinase Studies.* The results of an initial screening of the compounds used in this study are presented in Tables II–IV. All of the compounds studied were alternate activators of the bovine brain protein kinase, with the exception of 2′,3′-cGMP and adenosyl-5′-methylphosphonate, included as

TABLE III: Activation of Bovine Brain Protein Kinase by 8-Substituted cAMP Analogs.

| Concn (M) | pmoles of $^{32}$P Incorpd into Histone in Presence of: cAMP | 8-$SCH_3$ (2) | 8-$OCH_3$ (4) | 8-$NHCH_3$ (8) |
|---|---|---|---|---|
| $10^{-8}$ | 10.0 | 24.0 (2.4)[a] | 7.0 (0.7) | 8.0 (0.8) |
| $10^{-7}$ | 47.0 | 52.0 (1.10) | 20.0 (0.42) | 30.0 (0.65) |
| $10^{-6}$ | 65.0 | 61.0 (0.94) | 52.0 (0.80) | 48.7 (0.65) |
| $10^{-5}$ | 55.0 | 58.0 (1.15) | 57.0 (1.13) | 57.0 (1.13) |
| $10^{-4}$ | 42.0 | 35.0 (0.88) | 32.0 (0.76) | 49.0 (1.17) |

[a] Values in parentheses = $\alpha$, ratio of activity in the presence of analog to activity in the presence of cAMP at the indicated concentration.

TABLE IV: Relative Protein Kinase Activity ($\alpha$) of 8-Substituted cAMP Analogs at $10^{-8}$–$10^{-4}$ M.



| Compound, R = | $\alpha$ | | | | |
|---|---|---|---|---|---|
| | $10^{-8}$ | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ |
| H | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| $NHCH_2CH_2OH$ (**6**) | 0 | 0 | 0 | 0.18 | 0.16 |
| $SCH_2CH_2OH$ (**7**) | 0 | 0.25 | 0.61 | 0.65 | 0.70 |
| $SCH_2CH_3$ (**10**) | 0.88 | 0.93 | 1.14 | 1.07 | 1.09 |
| $SCH_2C_6H_5$ (**11**) | 0.36 | 1.15 | 0.95 | 0.98 | 0.73 |
| $NHCH_2C_6H_5$ (**5**) | 0 | 0.091 | 0.56 | 0.85 | 0.30 |
| $NHCH_2C_4H_4O$ (**9**) | 0.50 | 0.38 | 0.88 | 0.98 | 1.28 |
| $N(CH_3)_2$ (**3**) | 0.43 | 0.56 | 0.91 | 1.50 | 1.60 |
| $N_3$ (**14**) | 1.57 | 0.63 | 0.80 | 1.0 | 0.86 |
| Br (**1**) | 0.73 | 0.65 | 0.93 | 0.98 | 1.46 |

negative controls. Widely varying degrees of activity were noticed, however, depending on the concentration of the analog being used. If the activities of the analogs were compared at a single concentration, then activity varied with the nature of the grouping at C-8. These compounds were tested over a concentration range of $10^{-4}$–$10^{-8}$ M and their relative effectiveness was compared to that of the parent nucleotide (cAMP). A value for the ratio of activity of the test compound relative to cAMP ($\alpha$) was calculated at each concentration studied.

The data presented in Table II indicate that the presence of either a thio (**12**) or an oxo (**13**) function at C-8 of the purine ring enhances the activity of the compound as compared to cAMP, since both **12** and **13** were more active than the cAMP at each of the concentrations tested. The nitrogen isostere, 8-$NH_2$-cAMP (**15**), on the other hand, was less effective than either the 8-thio (**12**) or the 8-oxo (**13**) derivative. An idea of the quantitative differences between these substances can be obtained if the apparent $K_a$ values (activation constants) for **12**, **13**, **15**, and cAMP are compared (Table V). The numbers

TABLE V: $K_a$ Values for Several Analogs Relative to cAMP.

| R = | $K_a \times 10^8$ | $\frac{K_a \text{ (Test Compound)}}{K_a \text{ (cAMP)}} = K_a'$ |
|---|---|---|
| H | 4.83 (0.999) | |
| SH (**12**) | 1.28 (0.993) | 0.265 |
| OH (**13**) | 1.77 (0.999) | 0.36 |
| $NH_2$ (**15**) | 3.15 (0.999) | 0.65 |

TABLE VI: Inhibition of Pig Brain cAMP Phosphodiesterase by Several 8-Substituted Analogs.

| Compound | $(I_{50})/(S)$ |
|---|---|
| Theophylline | 2.78 |
| 8-Thio-cAMP | 4.55 |
| 8-MeS-cAMP | 0.945 |
| 8-BzlS-cAMP | 0.50 |

in parentheses represent the correlation coefficients obtained using a linear regression analysis of the data derived from Lineweaver–Burk plots of $1/v$ *vs.* $1/(S)$. $K_a$ values for cAMP have ranged from $3.05 \times 10^{-8}$ to $4.83 \times 10^{-8}$ M and for this reason $K_a$ determinations for cAMP are always made at the same time as $K_a$ values for the particular test compound. Accordingly, we have found it convenient to express the $K_a$ values for analogs as a relative $K_a$ value, a ratio of $K_a$ (test compound)/$K_a$(cAMP). In this particular experiment, the $K_a$ value for 8-thio-cAMP was approximately one-fourth that obtained for cAMP ($1.28 \times 10^{-8}$ for **12** *vs.* $4.83 \times 10^{-8}$ for cAMP). The value for 8-oxo-cAMP was intermediate between the two values shown above, and 8-$NH_2$-cAMP had a higher $K_a$ value than the 8-oxo derivative. While the absolute $K_a$ values for a particular compound may vary as much as two-fold from one experiment to another, the relative $K_a$ values ($K_a'$) as well as the relative activity values ($\alpha$) are reproducible.

It next seemed desirable to further substantiate that the presence of a basic nitrogen grouping attached to C-8 of the purine nucleus was less effective than the presence of the more electrophilic sulfur or oxygen atom. As can be seen from Table III, 8-MeS-cAMP (**2**) was more effective than the 8-methoxy analog **4**, and both were slightly more potent than the 8-methylamino derivative **8**.

Several other points deserve comment from the data presented in Table IV. 8-$NHCH_2CH_2OH$ (**6**) was found to be completely inactive at $10^{-6}$ M. At this concentration, the sulfur analog 8-$SCH_2CH_2OH$ (**7**) was 62% as active as cAMP. A similar relationship was noticed when the activity of 8-*S*-Bzl-cAMP (**11**) and 8-*N*-Bzl-cAMP (**5**) were compared at $10^{-7}$ M.

Finally, it should be pointed out that the ethylthio analog of cAMP (**10**) was more active than cAMP itself at $10^{-6}$ M, while the 2-hydroxyethylthio analog (**7**) was only 62% as effective as cAMP. Both 8-azido (**14**) and 8-bromo-cAMP (**1**) possessed activity that was nearly comparable to cAMP over the range of $10^{-7}$–$10^{-4}$ M.

*Phosphodiesterase Studies.* Since we have demonstrated that several of the 8-substituted derivatives were capable of replacing cAMP with at least 100% efficiency in the protein kinase reaction, all of the above compounds were studied for their relative stability to enzymatic hydrolysis by a specific cAMP phosphodiesterase. The methods used (Methods 2 and 3) allowed detection of from 5 to 10% degradation of analog to the corresponding 5′-monophosphate. Only one of the derivatives reported on in this investigation, 8-$NH_2$-cAMP (**15**), was found to be degraded by both the rabbit kidney and pig brain phosphodiesterase, and this was at a rate that was 80% of that observed for cAMP. None of the other derivatives were hydrolyzed by the partially purified rabbit kidney phosphodiesterase.

Finally, several of these analogs were studied for their

inhibitory activity against pig brain cAMP phosphodiesterase. A series of 8-thio-substituted derivatives were chosen for this study. As can be seen from Table VI, 8-thio-cAMP was found to be 60% less effective than theophylline; however, 8-MeS- and 8-BzlS-cAMP were 4.5 and 9.0 times more potent than the unsubstituted 8-thio-cAMP derivative.

## Discussion

It has recently been demonstrated that several of the cyclic 3′,5′-nucleotides of the naturally occurring purine or pyrimidine bases were less effective than cAMP in activating purified protein kinase preparations (Kuo and Greengard, 1970a,-b; Kuo *et al.*, 1970). In fact, when activity of cAMP, cIMP, cGMP, cUMP, cCMP, and cdTMP were compared at $5 \times 10^{-7}$ M using bovine brain protein kinase, none of the compounds mentioned above possessed greater than 32% of the activity of cAMP (Kuo *et al.*, 1970). As can be seen from Tables II to IV, all of the derivatives studied in this report were capable of activating the purified bovine brain enzyme.

Two points become apparent upon examination of Table II. First, several of the derivatives; *i.e.*, 8-thio-cAMP and 8-oxo-cAMP are more effective than cAMP itself, at all concentrations tested, in activating the enzyme. Secondly, it appears that the activity of the compounds decreases as the substituent is changed from thio to oxo to amino at the 8 position. A similar relationship appears to exist even in the case of the alkylated derivatives (Table III), although the differences are not of the same magnitude. A comparison of the relative activities of 8-BzlS- and 8-Bzl$NH_2$-cAMP and of 8-(2-OHEtS)- and 8-(2-OHEt$NH_2$)-cAMP also demonstrates that the presence of an amino nitrogen at C-8 does produce a compound that is less active than cAMP as a protein kinase activator.

The results with 8-MeS-, 8-EtS-, and 8-BzlS-cAMP appear to indicate that there is a fairly good degree of either bulk tolerance or preference for hydrophobic groupings at the 8 position. The apparent low level of activity exhibited by both 8-(2-OHEt$NH_2$)-cAMP and 8-(2-OHEtS)-cAMP as compared to 8-Me$NH_2$-cAMP and 8-EtS-cAMP do indicate that a polar group, such as $CH_2CH_2OH$, is less tolerated than a nonpolar substituent $CH_2CH_3$.

The above findings, as well as the fact that these compounds are extremely resistant to hydrolysis by rabbit kideny cAMP phosphodiesterase presents intriguing possibilities. First, *in vivo*, several of these analogs may be more potent than cAMP (protein kinase activity). Secondly, they would not be susceptible to degradation by the diesterase. Finally, the finding that several of these analogs are capable of inhibiting a cAMP phosphodiesterase to a greater extent than theophylline offers the possibility that as phosphodiesterase inhibitors they may be useful pharmacological agents.

In summary, the present study shows: (1) that several of these nucleotides possess biological activity *in vitro* that is comparable to or surpasses that of the parent nucleotide cAMP; (2) that these compounds (with a single exception) are stable to hydrolysis by a cAMP phosphodiesterase; and (3) that these new compounds possess the ability to inhibit the degradation of cAMP itself by the above enzyme, and in many instances are more potent inhibitors of cAMP phosphodiesterase than theophylline. The possibility exists that these derivatives will possess unique biological activity. Current studies are in progress to examine their properties *in vivo*, in other isolated cell-free systems (phosphodiesterase and protein kinase from other tissues), and in organ and tissue slice preparations.

Since the submission of this work, several 8-substituted derivatives of 3′,5′-cAMP have been studied as substrates for heart phosphodiesterase with results similar to those of the present study (Michal *et al.*, 1970). The chemical syntheses, however, were not described.

## Added in Proof

Since submission of this manuscript, an additional report by Posternak *et al.* (1971) on the synthesis and growth hormone stimulating ability of some C-2- and C-8-substituted cAmp analogs has appeared.

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