Synthesis and Biological Activity of Several 6-Substituted 9-β-D-Ribofuranosylpurine 3′,5′-Cyclic Phosphates†

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ABSTRACT: We report on a new synthesis of several novel 6-substituted derivatives of 9-β-D-ribofuranosylpurine 3′,5′-cyclic phosphate. These analogs were shown to be more readily cleaved by rabbit kidney and pig brain phosphodiesterase than the corresponding 8-substituted compounds (*viz.*, 8-SH, SCH_3, SC_2H_5, $SCH_2C_6H_5$) previously reported upon. In addition, several of the 6-alkylthio derivatives were up to two times more active than cAMP as alternate activators of the purified cAMP-dependent bovine brain protein kinase. These same derivatives were less active than guanosine 3′,5′-cyclic phosphate in stimulating a cGMP-dependent protein kinase partially purified from lobster muscle. Finally, one of these derivatives, 9-β-D-ribofuranosylpurine-6-thione 3′,5′-cyclic phosphate was nearly 50% less active than cAMP *vs.* bovine brain kinase (cAMP dependent), but was 70% more effective than cGMP *vs.* the lobster kinase (cGMP dependent).

The central role of adenosine 3′,5′-cyclic phosphate (cAMP) as a second messenger in many diverse biological systems is well established. That cAMP exerts its regulatory function *via* the stimulation of an ATP-dependent protein kinase is a key postulation in the explanation of the role of cAMP. The synthesis of cyclic nucleotide derivatives which vary in structure from cAMP by substitution in the base or sugar portion of the molecule raises the possibility of creating either alternate activators (agonists) or inhibitors (antagonists) of cAMP's activity. In the first two papers of this series (Muneyama *et al.*, 1971; Bauer *et al.*, 1971), we reported on the synthesis and biological activity of several 8-substituted cAMP derivatives. These compounds were shown to be alternate activators of a protein kinase isolated and purified from bovine brain. In addition, we found that with a single exception (8-amino-cAMP), all of the analogs appeared to be relatively stable to enzymatic hydrolysis by cAMP phosphodiesterases isolated from several different tissues.

In 1969, Posternak *et al.* reported on the synthesis of several 6-substituted derivatives of cAMP. These compounds were reported to act as agonists of cAMP and were able to stimulate the release of growth hormone and prolactin from slices of the anterior pituitary gland (Cehovic *et al.*, 1970). Du Plooy *et al.* (1971) have studied several 6-substituted derivatives of 9-β-D-ribofuranosylpurine 3′,5′-monophosphate as activators of glycogen phosphorylase in rat liver homogenates. These derivatives were also found (Michal *et al.*, 1970) to be more stable to enzymatic hydrolysis by heart phosphodiesterase or brain phosphodiesterase relative to cAMP.

In this paper we wish to report on a new method of synthesis of 6-substituted derivatives of cAMP and describe their ability to activate a purified cAMP-dependent protein kinase prepared from bovine brain and a purified cGMP-dependent protein kinase prepared from lobster muscle. We have found that several of these derivatives substituted at position 6 are capable of inhibiting the binding of cAMP to the bovine brain protein kinase. Data will be presented to show that structural specificity of the functional group present at the 6 position of the purine nucleus is not the same for the cAMP-dependent and cGMP-dependent protein kinases.

Experimental Section

Synthetic. Thin-layer chromatography was run on Merck cellulose F plates developed with either solvent system A (isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2, v/v)) or solvent system B (1-butanol–acetic acid–water (5:2:3, v/v)). Evaporations were performed under diminished pressure at less than 40°. Ultraviolet spectra were determined on a Cary 15 spectrometer. Silica gel for column chromatography was J. T. Baker Chemical Co., cat. no. 3405. All samples were dried over P_2O_5 at 76° using high vacuum.

Inosine 3′,5′-Cyclic Phosphate Hydrate (II). To an ice-cooled suspension of 20.0 g (0.061 mole) of cAMP (I) in 150 ml of water and 30 ml of HOAc was added 25 g (0.372 mole) of $NaNO_2$. The flask was loosely covered and stirred 4 hr in the ice bath. The bath was removed and the solution was stirred overnight at room temperature, then allowed to stand open for 24 hr. The solution was evaporated *in vacuo*, co-evaporated with 10 ml of HOAc, taken up in a minimum of water, and applied to a 5 × 44 cm column containing 700 ml of Dowex 50-X8 (H^+, 100–200 mesh). The column was eluted with water, and the fractions containing uv-absorbing material (1000 ml) were evaporated *in vacuo*. The residue was stirred with EtOH for several hours and filtered, giving 15.4 g (73%) after drying at high vacuum over P_2O_5. *Anal.* Calcd for $C_{10}H_{11}N_4O_7P \cdot H_2O$: C, 34.49; H, 3.76; N, 16.09. Found: C, 34.55; H, 3.68; N, 16.13.

6-N,N-Diethylamino-9-β-D-ribofuranosylpurine 3′,5′-Cyclic Phosphate (VI). To a stirred solution of 0.65 g (6.4 mmoles) of triethylamine, 15 ml of acetic anhydride, and 15 ml of pyridine was added 2.0 g (5.75 mmoles) of cIMP (II). After stirring overnight, 10 ml of water was added dropwise with ice-cooling and the solution was stirred an additional hour. The solution was evaporated *in vacuo*, and twice a 50-ml portion of toluene–ethanol (5:1, v/v) was added and evaporated. The thoroughly dried residue was refluxed 3 min with 20 ml of phosphoryl chloride and 2 ml of *N,N*-diethylaniline. After the volatiles were evaporated *in vacuo*, the residue was dissolved in 20 ml of dioxane and poured into 150 g

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of ice and 20 g of sodium bicarbonate and stirred 2 hr. It was occasionally necessary to add additional sodium bicarbonate to maintain the pH at about 7.

To this mixture was added 10 ml of *N,N*-diethylamine, and the mixture stirred at 70° for 2 hr. After extraction of the cooled mixture with three times 100-ml portions of ether, the pH of the resultant aqueous solution was adjusted to 2 with concentrated hydrochloric acid and applied to a column containing 100 ml of activated charcoal. The charcoal was washed with water until the eluate was chloride and phosphate free, and the product was eluted with ethanol–water–triethylamine (50:50:4, v/v). This solution was evaporated and the residue was dissolved in a small amount of chloroform and applied to a column containing 40 g of silica gel packed with chloroform. After washing with 500 ml of chloroform and 500 ml of chloroform–methanol (9:1, v/v), the product was eluted with chloroform–methanol (4:1, v/v). After the evaporation of the solvent, the residue was taken up in 20 ml of ethanol and brought to pH 2 with concentrated hydrolchloric acid. The product crystallized, was collected on a filter, and dried at 76° under high vacuum; yield, 0.37 g (17%). *Anal.* Calcd for $C_{14}H_{20}N_5O_6P$: C, 43.64; H, 5.23; N, 18.17. Found: C, 43.35; H, 4.96; N, 17.98.

6-Ethylamino-9-β-D-ribofuranosylpurine 3′,5′-Cyclic Phosphate (V). Cyclic IMP (II) (4.0 g, 11.5 mmoles) was acetylated and treated with phosphoryl chloride and diethylaniline as described for VI. After evaporation of the phosphoryl chloride, the residue was dissolved in 50 ml of dioxane and added to 200 ml of 0.5 M disodium hydrogen phosphate. The mixture was stirred 2 hr, the pH was adjusted to 8 with 4 N sodium hydroxide, and the mixture was extracted with three times 100-ml portions of chloroform. After adjusting the pH to 2, the mixture was desalted on a column of 200 ml of activated charcoal. Elution was accomplished with ethanol–water–triethylamine (50:50:4, v/v), and the residue after evaporation was stirred overnight with 30 ml of monoethylamine and 100 ml of ethanol. This was evaporated and the residue was chromatographed on 100 g of silica gel in the manner described for VI. The solvent was evaporated from the appropriate fractions and the residue was dissolved in 10 ml of water. After addition of 75 ml of ethanol, concentrated hydrochloric acid was added to bring the pH to 2 and the mixture was chilled. The product was collected on a filter; yield, 0.76 g (17.5%). *Anal.* Calcd for $C_{12}H_{16}N_5O_6P \cdot H_2O$: C, 38.41; H, 4.83; N, 18.69. Found: C, 38.48; H, 4.89; N, 18.69.

9-β-D-Ribofuranosylpurine-6-thione 3′,5′-Cyclic Phosphate (VII). Cyclic IMP (II) (5.0 g, 14.5 mmoles) was acetylated, then treated with phosphoryl chloride, and desalted as described for the preparation of V. The residual triethylammonium salt of IV was stirred overnight with 2.0 g of sodium hydrosulfide (Technical grade) and 100 ml of ethanol. After acidification with acetic acid, the solution was evaporated, taken up in a small amount of water, and passed through 300 ml of Dowex 50 (triethylammonium form). The residue, after evaporation, was chromatographed on silica gel as in the preparation of VI. The organic solvent was removed from the appropriate fractions, and the residue was taken up in water, passed through a column of 50 ml of Dowex 50 (H^+), evaporated, and triturated with ethanol to give 0.61 g (11%). *Anal.* Calcd for $C_{10}H_{11}N_4O_6PS \cdot H_2O$: C, 32.97; H, 3.60; N, 15.40. Found: C, 33.02; H, 3.46; N, 15.40.

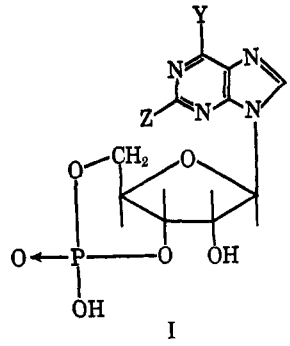
6-Methoxy-9-β-D-ribofuranosylpurine 3′,5′-Cyclic Phosphate (VIII). Cyclic IMP (II) (7.9 g, 22.8 mmoles) was acetylated as in the procedure for VI. The residue was refluxed 15 min with 100 ml of phosphoryl chloride. After removal of the volatiles, 200 ml of 0.5 M disodium hydrogen phosphate was added. After stirring 30 min, the pH was adjusted to 7 with 4 N sodium hydroxide, stirring was continued 1 hr more, and the pH was adjusted to 2. The product was absorbed on 200 ml of activated charcoal and washed until salt free, then eluted with ethanol–water–concentrated ammonium hydroxide (50:50:2, v/v). After evaporation, the residue was taken up in water and passed through a column of 50 ml of Dowex 50 (Na^+). The water was evaporated and the residue dried over P_2O_5 in high vacuum. To this was added 100 ml of methanol and 2.0 g of sodium methoxide, and after 1-hr stirring the solution was neutralized with acetic acid and evaporated. Cations were removed on Dowex 50 (H^+), and the product was chromatographed on a 5 × 40 cm column of Brinkman Avicel microcrystalline cellulose, packed in 1-butanol–acetic acid–water (5:2:3, v/v), and eluted with the same solvent. Evaporation of the solvent gave the pure nucleotide; yield, 0.25 g (3%). *Anal.* Calcd for $C_{11}H_{13}N_4O_7P \cdot H_2O$: C, 36.47; H, 4.17; N, 15.47. Found: C, 36.80; H, 4.16; N, 15.51.

6-Methylthio-9-β-D-ribofuranosylpurine 3′,5′-Cyclic Phosphate (IX). A solution of 9.1 g (25 mmoles) of 9-β-D-ribofuranosylpurine-6-thione 3′,5′-cyclic phosphate hydrate (VII) and 6.2 g (50 mmoles) of sodium carbonate hydrate in 25 ml water was diluted with 100 ml of methanol and 15 ml of methyl iodide. The mixture was stirred 1 hr and evaporated to a small volume, then applied to a column containing 800 ml of Dowex 50-X8 (H^+, 100–200 mesh). The column was eluted with water and 23-ml fractions were collected. Impurities were eluted first, and fractions containing pure product were evaporated *in vacuo* to dryness. The residue was taken up in methanol. Addition of ether gave a white solid which was collected on a filter and dried; yield, 8.08 g (85%). A sample was recrystallized from ethanol–water for analysis. *Anal.* Calcd for $C_{11}H_{13}N_4O_6PS \cdot 1.25H_2O$: C, 34.51; H, 4.08; N, 14.64. Found: C, 34.40; H, 3.66; N, 14.53.

6-Ethylthio-9-β-D-ribofuranosylpurine 3′,5′-Cyclic Phosphate (X). A solution of 6.0 g (16.5 mmoles) of 9-β-D-ribofuranosylpurine-6-thione 3′,5′-cyclic phosphate hydrate (VII) and 3.7 g (33 mmoles) of sodium carbonate hydrate in 25 ml of water was diluted with 100 ml of methanol and 10 ml of ethyl iodide. After 10-min stirring, the pH of the mixture was adjusted to 1 with Dowex 50-X8 (H^+), then back to 7 with NaOH. The resin was filtered and the filtrate was applied to a 2.5 × 20 cm column of Dowex 1-X2 (formate form, 100–200 mesh). After washing well with water, the product was eluted with 2 N ammonium formate and 23-ml fractions were collected. The fractions containing product were passed through a column of 1000 ml of Dowex 50-X8 (H^+, 100–200 mesh). Solvent was removed *in vacuo*, the residue was taken up in MeOH, and the product was precipitated by addition of ether. The product was collected on a filter and dried; yield, 3.1 g (50%). *Anal.* Calcd for $C_{12}H_{15}N_4O_6PS$: C, 38.50; H, 4.04; N, 14.97. Found: C, 38.22; H, 4.46; N, 14.60.

6-Benzylthio-9-β-D-ribofuranosylpurine 3′,5′-Cyclic Phosphate (XI). A solution of 4.82 g (13.2 mmoles) of 9-β-D-ribofuranosylpurine-6-thione 3′,5′-cyclic phosphate hydrate (VII) and 3.28 g (26.4 mmoles) of sodium carbonate hydrate in 20 ml of water was diluted with 80 ml of methanol and 1.9 ml (16 mmoles) of benzyl bromide. The mixture was stirred 1 hr, then 3 ml of formic acid was added. The methanol was removed *in vacuo*, 150 ml of water was added, and the solution was extracted with 100 ml of ether. The aqueous phase was applied to the top of a column containing 1000 ml of Dowex 50-X8 (H^+, 100–200 mesh). The column was eluted

TABLE I: Properties of the 6-Substituted 9-β-D-Ribofuranosylpurine 3′,5′-Cyclic Monophosphates.



I

No.	Y (Z = H)	λ_{max} (mμ) (ε × 10^{-3}) pH 1	pH 11	R_{cAMP}[a] A	B
II	OH	247.5 (11.8)	252 (12.8)	0.69	0.55
IV	Cl	265		1.79	1.55
V	NHC_2H_5	262 (18.7)	266 (17.5)	1.77	1.45
VI	$N(C_2H_5)_2$	268 (17.7)	277 (17.2)	1.95	1.52
VII	SH	321 (23.7)	310 (22.2)	0.37	0.75
VIII	OCH_3	248 (11.3)	248 (10.8)	1.85	2.0
IX	SCH_3	292 (16.4)	288 (18.0)	1.72	2.25
X	SC_2H_5	293 (16.3)	290 (18.4)	1.96	2.75
XI	$SCH_2C_6H_5$	292 (17.0)	291 (18.3)	2.27	3.30

[a] R_{cAMP} = mobility relative to that of cAMP in solvent systems A and B.

with 1000 ml of water, and 23-ml fractions were collected. The product began to appear after 600 ml of eluate. Elution of the column with 1000 ml of 50% aqueous ethanol removed the rest of the product. The fractions containing product were pooled and evaporated *in vacuo*. Addition of hot ethyl acetate induced crystallization of the residue, which was then filtered and dried; yield, 4.52 g (75%). *Anal.* Calcd for $C_{17}H_{17}N_4O_6PS \cdot H_2O$: C, 44.93; H, 4.22; N, 12.33. Found: C, 44.88; H, 4.16; N, 11.97.

Biochemical

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). The cGMP-dependent enzyme isolated from lobster muscle was purified to the stage of DEAE-cellulose chromatography using previously published procedures (Kuo and Greengard, 1970). The cAMP phosphodiesterases used in this study were ammonium sulfate precipitates of 100,000g supernatants prepared from tissue homogenates of rabbit kidney or pig brain frontal cortex. Protein kinase activity was assayed by measuring the incorporation of [^{32}P]phosphate into histone from γ-^{32}P-labeled ATP. The incubation mixture contained (amounts in micromoles): sodium glycerol phosphate buffer (pH 6.5), 10; [γ-^{32}P]ATP, ~2 × 10^6 cpm, 0.005; magnesium acetate, 3; sodium fluoride, 2; EDTA, 0.06; histone, 500 μg; cAMP, cGMP, or analog as indicated; purified protein kinase, 5–10 μg in a final volume of 0.2 ml. One unit of protein kinase activity was defined as that amount of enzyme which will transfer 1 pmole of ^{32}P from [γ-^{32}P]ATP to histone in 5 min at 30°. K_a' is defined as the relative activation constant and is the ratio of K_a of cAMP (or cGMP)/K_a analog.

The ability of the analogs to serve as substrates for the cAMP phosphodiesterase was measured using our previously described method (Muneyama *et al.*, 1971). Inorganic phosphate, released from the 5′-monophosphate formed upon treatment of the analog with phosphodiesterase, was determined colorimetrically. The inorganic phosphate release was effected with snake venom 5′-nucleotidase of *E. coli* alkaline phosphatase. The basic mixture contained the following (amounts in micromoles): Tris buffer (pH 7.5), 40; magnesium acetate, 25; cAMP or analog, 0.1; enzyme, 100–500 μg in a final volume of 1.0 ml. One unit of activity was defined as that amount of enzyme catalyzing the hydrolysis of 1.0 μmole in 10 min at 37°.

Inhibition of binding of cAMP to bovine brain binding protein (protein kinase) was carried out at 0°. The assay procedures described by Gilman (1970) were adopted with little alteration. The binding reaction was carried out in a total volume of 0.2 ml in 0.05 M sodium acetate (pH 4). Adenosine 3′,5′-cyclic [8-^{3}H]phosphate was added to each assay at a concentration of 8 × 10^{-8} M and a specific activity of approximately 2000 cpm/pmole. The concentration of various analogs varied from 6 × 10^{-8} to 6 × 10^{-6} M. Protein (46 μg) was added to initiate the reaction. All assays were performed in duplicate.

The tubes were incubated at 0° in an ice bath for 1 hr at which time they were filtered through Millipore (HAWP D25 00) membrane filters which had been presoaked at least 1 hr in 0.02 M potassium phosphate buffer (pH 6). The vacuum pressure used in the filtration was maintained at approximately 30–40 cm of water. Each filter was washed three times with 2.5 ml of 0.02 M potassium phosphate buffer (pH 6). The filters were placed in scintillation vials and 3 ml of Bray's dioxane-based scintillation cocktail was added. The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. A basic reaction mixture (minus enzyme) was included in each experiment as a filtration blank.

Results

As can be seen from Tables I and II, all of the 6-substituted compounds studied were capable of activating the cAMP-dependent protein kinase isolated from bovine brain. A survey of the degree of activation relative to cAMP revealed that only the 6-alkylthio analogs appeared to be better activators than cAMP of the bovine brain enzyme. The data presented in Table III further substantiate this fact and showed that the relative K_a values for each of these substances was less than 1.0, with the exception of the 6-alkylthio derivatives IX, X, and XI, which had relative K_a (K_a') values of >1.0 (K_a' = K_a(cGMP)/K_a(analog)).

Our attention was next focused on the effect of the analogs on the binding of cAMP to the binding protein isolated from bovine brain, in the same manner as described for the cAMP-dependent protein kinase, as another measure of their relative affinity for the enzyme as compared to cAMP. Table IV indicates that all of these compounds with the exception of VII were relatively weak with regard to their ability to bind to the kinase.

Since compounds possessing an oxo function in the 6 position may be considered as analogs of cGMP in which the 2-amino function is missing (2-desamino-cGMP), all of the compounds were tested for their ability to activate the cGMP-dependent protein kinase purified from lobster muscle. All of

TABLE II: Relative Ability of Some 6-Substituted Purine Ribonucleoside 3′,5′-Cyclic Phosphates to Activate a Purified Bovine Brain Protein Kinase.[a]

Compound	Y (Z = H)	α[b] 10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
I	NH_2 (cAMP)	1.0 (26.1)[c]	1.0 (112)	1.0 (169)	1.0 (175)	1.0 (115)
V	NHC_2H_5	0.66	0.95	1.05	0.92	2.2
VI	$N(C_2H_5)_2$	0.98	1.0	1.08	1.3	2.4
VII	SH	0.48	0.52	0.09	0.97	1.48
VIII	OCH_3	0.68	0.77	0.99	1.07	1.63
II	OH	0.35	0.31	0.75	1.06	1.60
IX	SCH_3	1.2	1.0	1.1	1.45	2.0

[a] See Table I for structure. [b] α = Ratio of pmoles of incorporated by test compound/pmoles of incorporated by cAMP. [c] pmoles of ^{32}P incorporated into histone.

these derivatives were capable of activating this kinase over a wide range of concentrations. Determination of the relative K_a values allowed for a direct comparison of the affinity of this cGMP-dependent protein kinase for cGMP and the analogs (Table V). Cyclic IMP was approximately four times less effective than cGMP (K_a' = 0.26) in activating the cGMP-dependent protein kinase from lobster muscle, while VII had a K_a' value of 1.85. 6-Methoxy-9-β-D-ribofuranosylpurine 3′,5′-cyclic phosphate (VIII) was less effective than cGMP itself (K_a' = 0.41). The 6-alkylthio derivatives IX, X, and XI were all less effective than cGMP as activators of the lobster muscle enzyme as judged from their higher K_a (lower K_a') values.

Most all of the 8-substituted derivatives of cAMP studied previously (Muneyama *et al.*, 1971) were relatively stable to hydrolysis by the cAMP phosphodiesterase from pig brain frontal cortex and rabbit kidney. The results presented in Table VI show that while cIMP was cleaved at a rate that was 46% that of cAMP, the 6-thio analog of cIMP, 9-β-D-ribofuranosylpurine-6-thione 3′,5′-cyclic phosphate was cleaved at a slightly greater rate than cAMP, and twice as fast as cIMP. Substitution of an alkyl radical in the SH, OH, or NH_2 moiety at C_6 to form the SR, OR, or HNR derivatives led to compounds which were more resistant to cleavage than the unsubstituted compounds (Table VI).

TABLE III: K_a' (Relative Affinity Constants) Values for 6-Substituted Purine 3′,5′-Cyclic Nucleotides against cAMP-Dependent Bovine Brain Protein Kinase.

Compound	Y (Z = H)	K_a'[a]
V	NHC_2H_5	0.50
VII	SH	0.56
II	OH	0.59
VI	$N(C_2H_5)_2$	0.66
VIII	OCH_3	0.66
XI	$SCH_2C_6H_5$	1.25
IX	SCH_3	1.45
X	SC_2H_5	2.0

[a] K_a' = K_a for cAMP/K_a for analog.

TABLE IV: Inhibition of [^{3}H]cAMP Binding to Bovine Brain Binding Protein by 6-Substituted Purine 3′,5′-Cyclic Nucleotides.

Compound	Y (Z = H)	I_{50}[a]	I'_{50}[b]
I	NH_2	5×10^{-8}	1.0
VII	SH	5×10^{-9}	10.0
II	OH	2.7×10^{-8}	1.85
VIII	OCH_3	8×10^{-8}	0.63
VI	$N(C_2H_5)_2$	9×10^{-8}	0.55
V	HNC_2H_5	1.5×10^{-7}	0.33
IX	SCH_3	8×10^{-8}	0.63

[a] I_{50} = molar concentration of substance causing 50% inhibition of cAMP binding to bovine brain binding protein. [b] I'_{50} = I_{50} cAMP/I_{50} analog.

TABLE V: Activation of cGMP-Dependent Protein Kinase from Lobster Muscle.

Compound	Y[c]	Z[c]	K_a (M × 10^8)	K_a'[a]	r[b]
cGMP	OH	NH_2	3.16	1.0	0.99
I	NH_2	H	43.5	0.07	0.99
II	OH	H	12.10	0.26	0.94
VII	SH	H	1.70	1.85	0.99
VIII	OCH_3	H	7.69	0.41	0.99
V	NHC_2H_5	H	2.21	1.41	0.95
VI	$N(C_2H_5)_2$	H	14.30	0.22	0.98
IX	SCH_3	H	7.5	0.42	0.99
XI	$SCH_2C_6H_5$	H	11.7	0.27	0.94
X	SC_2H_5	H	7.95	0.40	0.96

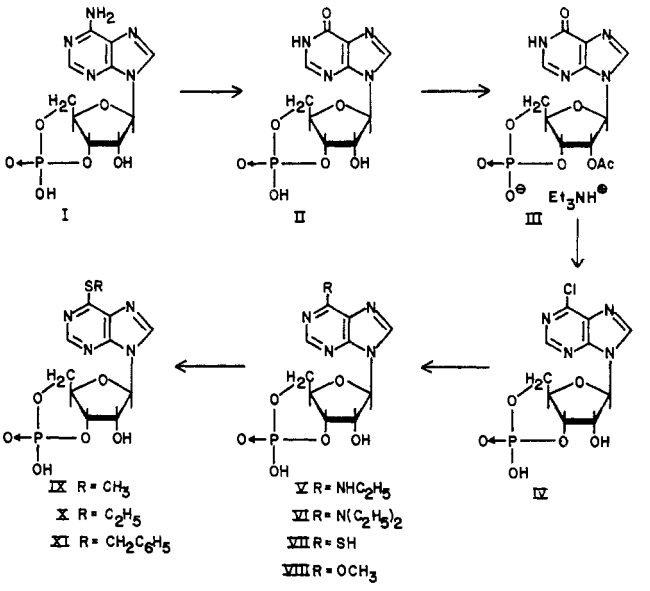
[a] K_a' = K_a for cGMP/K_a for analog. [b] r = correlation coefficient for linear regression analysis of Lineweaver–Burk double-reciprocal plots. [c] See Table I for structure.

TABLE VI: Effect of cAMP Phosphodiesterases on Some 6-Substituted Purine Ribonucleoside 3',5-'Monophosphates.

Compound	Y (Z = H)	Pig Brain Frontal Cortex α^a	Rabbit Kidney α
I	NH_2	1.0	1.0
II	OH	0.46	
VII	SH	1.04	1.06
VIII	OCH_3	0.57	0.53
V	NHC_2H_5	0.32	0.20
VI	$N(C_2H_5)_2$	0.10	0.09
IX	SCH_3	0.52	0.41
X	SC_2H_5		0.57
XI	$SCH_2C_6H_5$		0.54

[a] α = rates of hydrolysis of test compounds/rates of hydrolysis of cAMP.

SCHEME I



Discussion

Previous methods of synthesis of 9-β-D-ribofuranosylpurine 3',5'-cyclic phosphates with substituents on the aglycon have involved cyclization of a suitably substituted 9-β-D-ribofuranosylpurine 5'-phosphate (Thomas and Montgomery, 1968; Posternak *et al.*, 1969; Hanze, 1968). The cyclization was mediated by *N,N'*-dicyclohexylcarbodiimide in refluxing pyridine (Smith *et al.*, 1961; Drummond *et al.*, 1964), or accomplished by treatment of the nucleoside 5'-*p*-nitrophenyl phosphate with potassium *tert*-butoxide in dimethyl sulfoxide (Borden and Smith, 1966). In the present work, a facile method of synthesis of 6-substituted 9-β-D-ribofuranosylpurine 3',5'-cyclic phosphates from a precursor with a preformed cyclic phosphate moiety was sought. Methods for the chlorination of blocked derivatives of inosine or guanosine involve treatment with refluxing phosphoryl chloride and *N,N*-diethylaniline (Gerster *et al.*, 1963; Honjo *et al.*, 1968), or thionyl chloride and dimethylformamide in refluxing chloroform (Zemlicka and Sorm, 1965; Ikehara and Uno, 1965). These procedures, and in particular the successful synthesis in this manner of 6-chloro-9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-purine 3',5'-cyclic phosphate (Honjo *et al.*, 1968), suggested a similar route *via* 6-chloro-9-β-D-ribofuranosylpurine 3',5'-cyclic phosphate to various 6-substituted purine cyclic nucleotides.

Deamination of cAMP (I) with sodium nitraite in aqueous acetic acid gave inosine 3',5'-cyclic phosphate (cIMP, II) (Lipkin *et al.*, 1959) in good yield (Scheme I). Acetylation of cIMP in the presence of acetic anhydride, pyridine, and triethylamine gave 2'-*O*-acetyl-cIMP (III) as the triethylammonium salt. Compound III was then treated with refluxing phosphoryl chloride in the presence of *N,N*-diethylaniline and, after removal of the excess phosphoryl chloride *in vacuo*, the residue was hydrolyzed by an aqueous buffer. Adsorption of the product on a charcoal column to remove inorganic salts gave 6-chloro-9-β-D-ribofuranosylpurine 3',5'-cyclic phosphate (IV). The reaction of III with phosphoryl chloride occurred without added *N,N*-diethylaniline, but was more efficient in its presence.

Treatment of IV with mono- or diethylamine at room temperature yielded, respectively, the desired 6-(mono- or diethylamino)-9-β-D-ribofuranosylpurine 3',5'-cyclic phosphates (V and VI). The products were readily purified by column chromatography on silica gel, followed by crystallization from aqueous ethanol at pH 2. Treatment of IV with sodium hydrosulfide in ethanol gave 9-β-D-ribofuranosylpurine-6-thione 3',5'-cyclic phosphate (VII), previously synthesized by an alternate route (Thomas and Montgomery, 1968). Treatment of an anhydrous solution of IV with methanolic sodium methoxide at room temperature gave 6-methoxy-9-β-D-ribofuranosylpurine 3',5'-cyclic phosphate (VIII), which was purified by column chromatography on cellulose. The preparation of several 6-alkylthio derivatives of VII was achieved by the alkylation of VII with methyl iodide, ethyl iodide, or benzyl bromide to produce the 6-methylthio- (IX), 6-ethylthio- (X), and 6-benzylthio- (XI) 9-β-D-ribofuranosylpurine 3',5'-cyclic phosphates, respectively.

The physical properties of the nucleotides are shown in Table I. All compounds were verified to be diesters of phosphoric acid by electrophoresis at pH 7.2, in which case they had mobilities similar to cAMP. Additionally, the proton magnetic resonance (pmr) spectrum of each compound showed the anomeric hydrogen to be an apparent singlet, indicating the $J_{1'-2'} < 1$ Hz, as has been observed in the pmr spectra of cAMP (Jardetzky, 1962). The key intermediate, IV, may be used in the preparation of a wide variety of 6-substituted purine cyclic ribonucleotides, and such studies are currently in progress in this laboratory.

Previous reports have discussed the ability of various 6-substituted derivatives of purine nucleoside 3',5'-cyclic monophosphates to stimulate activation of glycogen phosphorylase in liver homogenates and to elicit the release of growth hormone from pituitary slices. In this report we present information relating 6 substitution to the stimulation of two purified protein kinases.

In our previous paper we demonstrated that several substitutions at the 8 position of the purine nucleus led to compounds which possessed significantly greater ability to activate a cAMP-dependent protein kinase. Thus 8-bromo-cAMP was more effective than cAMP itself (Muneyama *et al.*, 1971). In some recent experiments we have found that the K_a' for 8-bromo-cGMP is 1.5 for cGMP-dependent protein kinase isolated from lobster muscle.

In examining the relative activity of several naturally occurring 2- and 6-substituted purine 9-β-D-ribofuranosyl 3′,5′-cyclic phosphates with the cAMP and cGMP-dependent protein kinase, it became obvious that substitution of an oxo function at C_6 (cIMP) lowered the ability of the compound to bind to the cAMP-dependent kinase (II was four times less effective than cAMP). When the same compound (II, cIMP) was compared to cAMP with regard to its ability to bind to the cGMP-dependent kinase, it as found to be some 10–20 times more effective than cAMP although it was still less potent than cGMP. Several of the 6-alkylthio derivatives (VII, IX, X, and XI) were examined using the cGMP-dependent kinase and compared to their effect on the cAMP-dependent kinase. The results with these analogs dramatically demonstrate that the cGMP- and cAMP-dependent kinases have vastly different specificity with regard to structural alterations at position 6 of the purine nucleus. In fact, a derivative of cGMP lacking the 2-amino group (VII, 9-β-D-ribofuranosylpurine-6-thione 3′,5′-cyclic phosphate) is more effective than cGMP itself in the cGMP-dependent system, while being less active than cAMP *vs.* the brain kinase. It should be pointed out that 6-methylthio-9-β-D-ribofuranosylpurine 3′,5′-cyclic phosphate (IX) behaves more nearly like a cAMP analog than a cGMP analog since it is more active than cAMP using the cAMP-dependent enzyme, and less active than cGMP using the lobster kinase. The ability of 6-thiopurine derivatives to act more like guanosine analogs and 6-methylthiopurine derivatives to be treated as adenosine analogs has been reported previously with regard to the mechanism of action of 6-mercaptopurine and 6-methylthiopurine as antitumor agents (Ho, 1971; Montgomery, 1970).

We have verified the recent results of Michal *et al.* (1970) who found that 6-alkylamino derivatives of cAMP were relatively resistant to phosphodiesterase as compared to cAMP. In addition, we have extended these findings and have shown that several 6-alkylthiopurine ribonucleoside 3′,5′-monophosphate derivatives are more susceptible to hydrolysis by rabbit kidney phosphodiesterase than their nitrogen isosteres. On the other hand, while cIMP is cleaved less readily than cAMP, the sulfur analog of cIMP (VII) is hydrolyzed at a rate that is over two times greater than cIMP and some 6% greater than cAMP itself. These studies were all performed with the high K_m cyclic nucleotide phosphodiesterase isolated from rabbit kidney or pig brain frontal cortex, which we have found to have a greater specificity for cGMP ($K_m = 4.0 \times 10^{-5}$ M) than cAMP ($K_m = 1.0 \times 10^{-4}$ M). It is interesting to point out that the same structural feature (presence of a 6-thione grouping, VII) which elicits high cGMP-like activity with a cGMP-dependent protein kinase also leads to a compound which is more nearly handled like cGMP by the cGMP-specific phosphodiesterase from rabbit kidney.

In summary, it thus appears that the presence of a bulky methylthio, ethylthio, or benzylthio moiety as in IX, X, and XI actually increases the activity of the compound for a cAMP-dependent protein kinase as compared to the parent nucleotide (cAMP). The presence of the same substituents, methylthio, ethylthio, and benzylthio causes a *decrease* in activity for the cGMP-dependent kinase from lobster muscle. While the relatively high susceptibility of some of these analogs (VII, IX, X and XI) to cleavage by a cyclic nucleotide phosphodiesterase as compared to the 8-substituted compounds (Muneyama *et al.*, 1971) is undesirable from the standpoint of agonist-like activity of a cAMP analog, we have recently shown that extracts of KB cells (a cell line grown from a human tumor isolated from the nasopharynx) were capable of cleaving the 6-thione derivative (VII) to 6-mercaptopurine 5′-ribonucleotide. LePage (LePage and Hersh, 1972) has recently demonstrated that the 3′,5′-cyclic phosphate derivatives of 9-β-D-arabinofuranosyladenine, 6-mercaptopurine ribonucleoside, and 6-methylthiopurine ribonucleoside were capable of inhibiting the growth of ascites cells *in vivo* when tumor cells which were resistant to 9-β-D-arabinofuranosyladenine, 6-mercaptopurine, and 6-methylthiopurine were utilized. The implications of these findings for effective antitumor therapy are currently being investigated.

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