lution of N,N'-diaminopiperazine dihydrate (3.80 g, 0.028 mol) in water (50 ml). The addition was made over the period of 1 hr. The reaction was stirred for an additional 1 hr after the addition. Half of the reaction volume was evaporated under vacuum. The remaining portion was extracted once with chloroform (100 ml). The chloroform solution was evaporated under vacuum affording the product as an orange powder: yield 0.98 g (83%); tlc R_f 0.45 (ethanol); uv (in ethanol) 237 nm (ϵ 25,500), 340 (21,200), 479 (11,500).

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Synthesis and Biological Activity of Certain Carbamoyl and Alkoxycarbonyl Derivatives of Adenosine 3′,5′-Cyclic Phosphate

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Received April 5, 1973

N-Methyl, -*n*-propyl, and -phenyl N^6,2′-*O*-bis(carbamoyl)adenosine 3′,5′-cyclic phosphates were prepared from adenosine 3′,5′-cyclic phosphate (cAMP) and the corresponding isocyanates. Selective basic hydrolysis of the N^6,2′-*O*-bis(carbamoyl)-cAMP derivatives gave N^6-(*N*-methylcarbamoyl)-cAMP, N^6-(*N*-*n*-propylcarbamoyl)-cAMP, and N^6-(*N*-phenylcarbamoyl)-cAMP, respectively. N^6,2′-*O*-Bis(*N*-methylcarbamoyl)-8-bromo-cAMP and 8-benzylthio-N^6-(*N*-phenylcarbamoyl)-cAMP were prepared similarly from the respective isocyanate and the 8-substituted cAMP derivative. Treatment of N^6,2′-*O*-bis(*N*-methylcarbamoyl)-8-bromo-cAMP with thiourea gave N^6,2′-*O*-bis(*N*-methylcarbamoyl)-8-thio-cAMP. cAMP and ethyl chloroformate gave N^6-ethoxycarbonyl-cAMP. The protein kinase and phosphodiesterase structure–activity relationships of these derivatives are discussed. The K_a for activation of protein kinase for N^6-(*N*-phenylcarbamoyl)-cAMP was one-fifth the K_a for cAMP. Other derivatives had K_a values either similar to the K_a of cAMP or considerably higher where a 2′-O substituent was present. The N^6-ethoxycarbonyl and carbamoyl derivatives were poor substrates (<10%) for rabbit kidney high K_m phosphodiesterase. Varying degrees of inhibition of the beef heart and rabbit lung low K_m phosphodiesterases by the derivatives were observed. No unusual cytotoxicity of the derivatives to KB cells was observed, with the possible exception of N^6,2′-*O*-bis(*N*-phenylcarbamoyl)-cAMP.

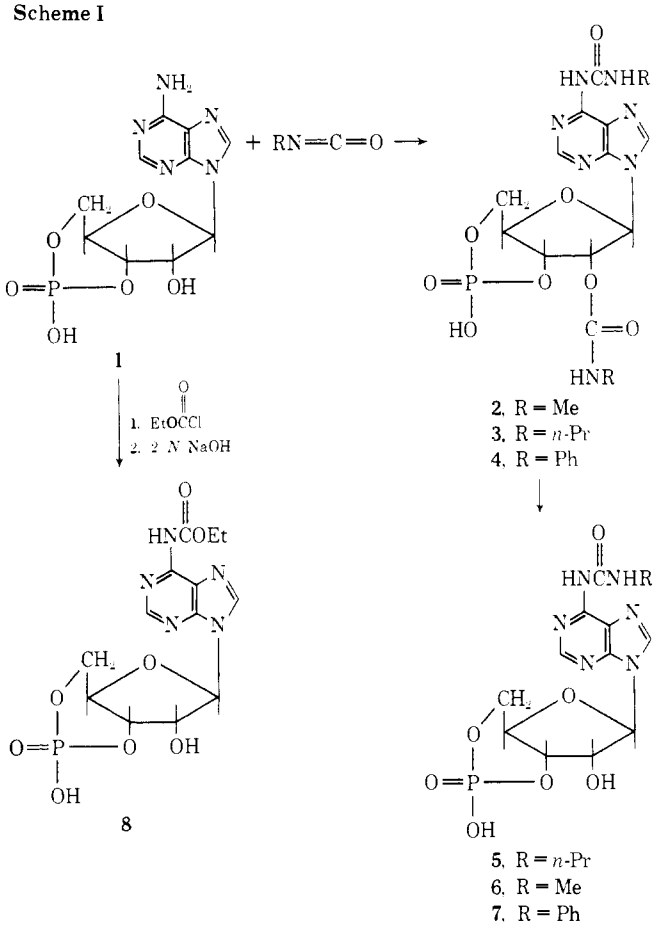
The ureidopurines, *N*-(purin-6-ylcarbamoyl)threonine, *N*-(purin-6-ylmethylcarbamoyl)threonine,[1] and *N*-(purin-6-ylcarbamoyl)glycine, have been found to occur naturally as the β-D-ribofuranosyl derivative in tRNA.[2-5] *N*-(Purin-6-ylcarbamoyl)threonine has also been identified in human urine.[6] Several ureidopurine derivatives related to *N*-(purin-6-ylcarbamoyl)threonine have been synthesized and found to have cytokinin-like growth-promoting properties.[7,8] Other ureido compounds, such as *N*-phenyl-*N*′-allylurea and phenylurea analogs, induce cell division.[9] Adenosine 3′,5′-cyclic phosphate (cAMP, 1) has been well established as a mediator of many hormonal effects. cAMP and some 8-substituted cAMP derivatives have also been found to control cell growth in tumor cell lines, as well as to effect differentiation of tumor cell lines.[10] It was of interest, therefore, to incorporate the ureido moiety into cAMP analogs and examine their biological activity.

As a measure of biological activity, the carbamoyl and ethoxycarbonyl cAMP derivatives were examined for their ability to activate a purified cAMP-dependent protein kinase isolated from bovine brain. Kuo and Greengard[11] have postulated the activation of this protein kinase may indeed explain the mechanism of action of cAMP in most biological systems. It has been shown that several 6- and 8-substituted derivatives of cAMP inhibit cAMP phosphodiesterase while exhibiting resistance to hydrolysis by the enzyme.[12,13] Examination of the present ureido derivatives has been made for their stability toward enzymatic hydrolysis and for inhibition of two different cAMP phosphodiesterases. All of these compounds were also examined for their toxicity to tumor cells (KB cells) in tissue culture.

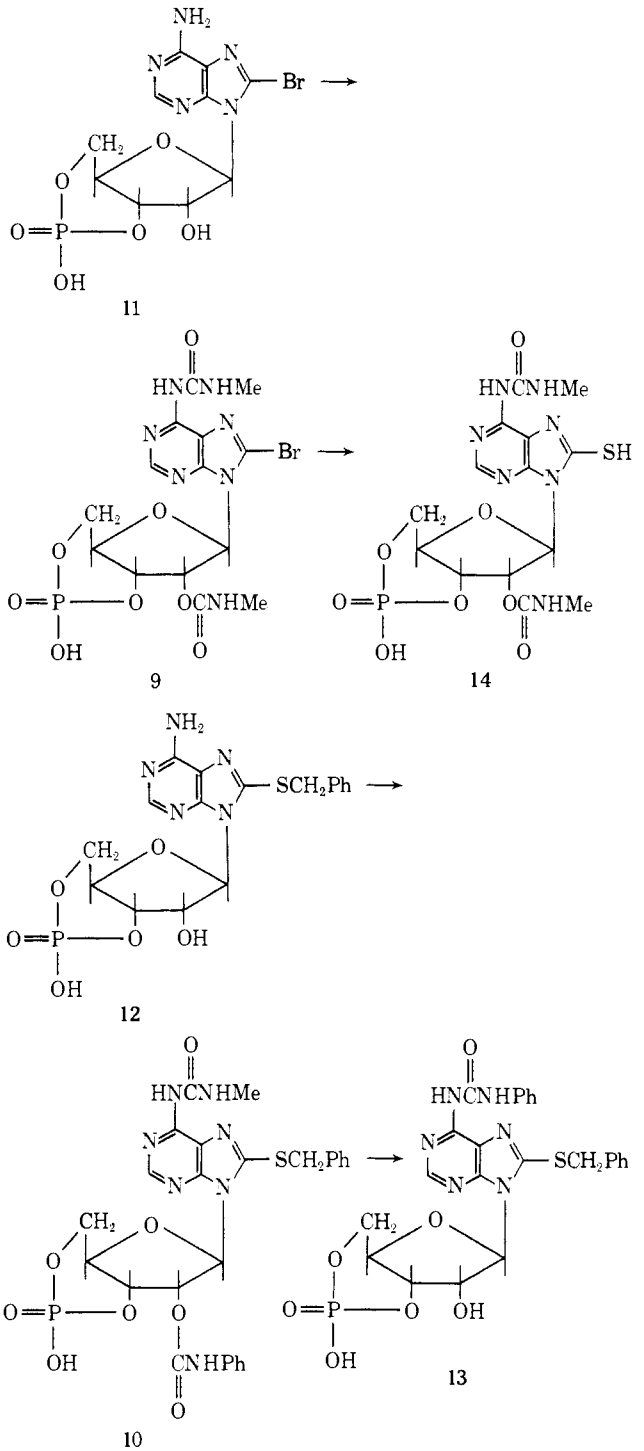
Chemistry. The *N*-methyl, -*n*-propyl, and -phenyl N^6,2′-*O*-bis(carbamoyl)adenosine 3′,5′-cyclic phosphates **2**, **3**, and **4** were prepared by treatment of adenosine 3′,5′-cyclic phosphate (cAMP, 1) with the respective isocyanates at 60 to 80°. These N^6,2′-*O*-bis(carbamoyl)adenosine 3′,5′cyclic phosphates **2**, **3**, and **4** were then selectively deblocked with either refluxing $NaOCH_3$ or aqueous NaOH at room temperature to give the respective N^6-carbamoyladenosine 3′,5′-cyclic phosphates **6**, **5**, and **7**. Similarly, treatment of cAMP (1) with ethyl chloroformate and then 2 *N* NaOH gave N^6-ethoxycarbonyl-cAMP (**8**) (see Scheme I).

Muneyama, *et al.*,[12] and Bauer, *et al.*,[14] have shown that 8 substituents, *e.g.*, bromo and benzylthio, of cAMP increase the relative potency of cAMP to activate protein kinases and to stimulate glycogenolysis. Attempts to

Scheme I



Scheme II



brominate N^6-(*N*-methylcarbamoyl)-cAMP (6) with bromine,[15] *N*-bromosuccinimide,[16] or *N*-bromoacetamide[17] were unsuccessful, presumably due to the electron-withdrawing effect of the N^6-methylcarbamoyl moiety. The N^6,2′-*O*-bis(*N*-methylcarbamoyl)-8-bromo-cAMP (9) and 8-benzylthio-N^6,2′-*O*-bis(*N*-phenylcarbamoyl)-cAMP (10) were obtained in good yield by direct treatment of 8-bromo-cAMP (11) and 8-benzylthio-cAMP (12)[12] with the respective isocyanate. Basic hydrolysis of 10 gave 8-benzylthio-N^6-(*N*-phenylcarbamoyl)-cAMP (13). The synthesis of N^6,2′-*O*-bis(N-substituted carbamoyl)-8-bromo-cAMP derivatives provides a useful route to other 8-substituted N^6-mono- and N^6,2′-*O*-bis(N-substituted carbamoyl)-cAMP derivatives by nucleophilic substitution of the 8-bromo group. For example, treatment of N^6,2′-*O*-bis(*N*-methylcarbamoyl)-8-bromo-cAMP (9) with thiourea gave N^6,2′-*O*-bis(*N*-methylcarbamoyl)-8-thio-cAMP (14) (see Scheme II). The structures of the ethoxycarbonyl and carbamoyl derivatives of cAMP were verified by pmr and uv spectra,[7] elemental analyses, and tlc.

Experimental Section

Syntheses. Evaporations were performed *in vacuo* at <40°. Uv spectra were determined on a Cary 15 spectrometer. Silica gel for column chromatography was E. Merck reagent silica gel 60 (particle size 0.063–0.200 nm). The eluates from column chromatography were monitored at 254 nm to detect the presence of uv-absorbing compounds. All samples were dried over $CaSO_4$ at 100° under high vacuum. Tlc of the NH_4 salt of the compounds was run on E. Merck silica gel F-254 plate and developed with CH_3CN–0.1 *M* NH_4Cl (5:1), $R = R_f$ (compound)/R_f **1**.

N^6,2′-*O*-**Bis(*N*-methylcarbamoyl)adenosine 3′,5′-Cyclic Phosphate Sodium Salt (2).** A solution of 1.0 g (3.2 mmol) of cAMP (1), 1 ml of Et_3N, and 3 ml of methyl isocyanate in 50 ml of DMF was heated at 80° overnight in a bomb. The solution was poured into 200 ml of Et_2O and the Et_2O decanted from the oil. The oil was again titurated with Et_2O and decanted. The residue was dissolved in $CHCl_3$ and placed onto a 30-g column (2.5-cm diameter) of silica gel (packed in $CHCl_3$). The column was washed with $CHCl_3$ and the product eluted with MeOH–$CHCl_3$ (15:85). An aqueous solution of the product was passed through a Dowex 50 (Na^+) column and the aqueous eluate evaporated to dryness. The residue was dissolved in MeOH, 2 vol of EtOH were added at boiling, and the volume was reduced until solid separated. The suspension was cooled and the solid filtered to yield 412 mg (28%) of **2**: λ_{max} (pH 1) 276 nm, 285 sh (ϵ 23,100, 17,600); λ_{max} 267 nm, 275 sh (ϵ 22,500, 21,500); λ_{max} (pH 11) 267 nm, 275 sh (ϵ 21,900, 18,600); R = 1.8. *Anal.* ($C_{14}H_{17}N_7O_8NaP$) C, H, N, Na.

N^6,2′-*O*-**Bis(*N*-*n*-propylcarbamoyl)adenosine 3′,5′-Cyclic Phosphate Sodium Salt (3).** 3 was prepared from 7 g (21.3 mmol) of cAMP, 5 ml of Et_3N, and 14 ml of *N*-propyl isocyanate in 80 ml of DMF and purified on an 80-g silica gel column as in the procedure of **2**. Precipitation of 3 from MeOH with EtOAc

gave 5.8 g (52%) of **3**: λ_{max} (pH 1) 276 nm, 285 sh (ϵ 24,900, 19,400); λ_{max} (pH 7) 267 nm, 275 sh (ϵ 23,500, 20,100); λ_{max} (ph 11) 267 nm, 275 sh (ϵ 23,500, 20,100); R = 3.5. *Anal.* ($C_{18}H_{25}N_7O_8$-NaP·H_2O) C, H, N, Na.

N^6,2′-*O*-Bis(*N*-phenylcarbamoyl)adenosine 3′,5′-Cyclic Phosphate Sodium Salt (4). A solution of 12.0 g (36.5 mmol) of cAMP (**1**), 10 ml of Et_3N, and 25 ml of phenyl isocyanate in 400 ml of DMF was stirred overnight in a 70° oil bath. The resulting solution was concentrated to 0.5 vol and diluted with 200 ml of H_2O and 200 ml of EtOAc. The aqueous phase was heated on a steam bath and the hot solution extracted four times with EtOAc. The aqueous phase was warmed on a steam bath, 50 ml of a saturated NaCl solution was added, and the solution was cooled. The resulting crystals were filtered, washed with a small amount of cold H_2O, and recrystallized from H_2O–EtOH (1:4) to yield 10 g (47%) of **4**: λ_{max} (pH 1) 284 nm (ϵ 20,300); λ_{max} (pH 7) 231 nm, 276 (ϵ 18,200, 22,100); λ_{max} (pH 11) 233 nm, 276 (ϵ 17,200, 21,500); λ_{max} (pH 13) 307 nm (ϵ 29,100); R = 5.4. *Anal.* ($C_{24}H_{21}N_7O_8NaP$) C, H, N, Na.

N^6-(*N*-*n*-Propylcarbamoyl)adenosine 3′,5′-Cyclic Phosphate (5). **3** (2.6 g, 5.0 mmol) and 3 g of NaOMe in 200 ml of absolute MeOH was refluxed for 9 hr. The solution was evaporated and the residue dissolved in H_2O and placed onto a 25-ml column (2-cm diameter) of Dowex 1-X2 (formate, 100–200 mesh). The column was washed with H_2O and then eluted with a gradient of 650 ml of 0–6 *N* formic acid. Elution was continued with 300 ml of 6 *N* formic acid. Appropriate fractions were coevaporated with EtOH. The residue was slurried in EtOH and filtered to yield 1.06 g (51%) of **5**: λ_{max} (pH 1) 277 nm, 285 sh (ϵ 23,800, 18,600); λ_{max} (pH 7) 268 nm, 276 sh (ϵ 22,700, 19,200); λ_{max} (pH 11) 268 nm, 276 sh (ϵ 22,200, 18,900); λ_{max} (pH 12.65) 277 nm, 296, 269 sh (ϵ 14,900, 16,600 13,800); R = 2.3. *Anal.* ($C_{14}H_{19}N_6O_7P$) C, H, N.

N^6-(*N*-Methylcarbamoyl)adenosine 3′,5′-Cyclic Phosphate (6). cAMP (5.0 g, 15 mmol) and 5 ml of Et_3N and 10 ml of methyl isocyanate were treated as for compound **2** above. The solution was evaporated and the resulting residue was treated with methanolic NaOMe (5 g) and charged onto a column (4-cm diameter) of 54 ml of Dowex 1-X2 (formate, 100–200 mesh) as for compound **5** above. The column was eluted with 1.6 l. of 0–6 *N* formic acid and the appropriate fractions were coevaporated with EtOH. The resulting solid was crystallized from EtOH to give 2.66 g (50%) of **6**: λ_{max} (pH 1) 276 nm, 285 sh (ϵ 22,900, 17,200); λ_{max} (pH 7) 267 nm, 275 sh (ϵ 22,300, 19,000); λ_{max} (pH 11) 267 nm, 275 sh (ϵ 21,800, 18,500); R = 1.7. *Anal.* ($C_{12}H_{15}N_6O_7P$·0.75H_2O) C, H, N.

N^6-(*N*-Phenylcarbamoyl)adenosine 3′,5′-Cyclic Phosphate (7). **4** (9.0 g, 15 mmol) in 75 ml of DMF and 45 ml of 2 *N* NaOH was stirred at room temperature for 2.5 hr. The solvent was evaporated and the residue was partitioned between 100 ml of H_2O and 100 ml of Et_2O. The aqueous phase was extracted three times with Et_2O and then was acidified to pH 1 with 1 *N* HCl. The resulting solid was filtered, washed with H_2O, and dried to yield 6.2 g (85%) of **7**: λ_{max} (pH 1) 285 nm (ϵ 28,300); λ_{max} (pH 7) 277 nm (ϵ 29,000); λ_{max} (pH 11) 277 nm (ϵ 28,600); R = 3.2. *Anal.* ($C_{17}H_{17}N_6O_7P$·1.5H_2O) C, H, N.

N^6-Ethoxycarbonyladenosine 3′,5′-Cyclic Phosphate Sodium Salt (8). A hot solution of 6.0 g (18 mmol) of cAMP (**1**) and 5.2 g of 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine in aqueous pyridine was coevaporated with pyridine to dryness. The residue was dissolved in 200 ml of pyridine and cooled to 0° and 15 ml of ethyl chloroformate was added dropwise. The reaction mixture was allowed to warm to room temperature overnight and then 50 g of ice was added. After 4 hr the solution was evaporated and the residue coevaporated with EtOH–toluene to remove traces of pyridine. The residue was taken up in H_2O and the pH adjusted to 11 with 2 *N* NaOH. After 0.5 hr the solution was neutralized with AcOH and placed onto a 500-ml column (4-cm diameter) of Dowex 50 X8 (H^+, 100–200 mesh). The column was eluted with H_2O. The appropriate fractions containing the product were pooled, reduced in volume *in vacuo*, and passed through a 100-ml column of Dowex 50 (Na^+). The aqueous eluate was evaporated and the residue was dissolved in boiling MeOH. Two volumes of EtOH were added slowly at boiling. The volume was reduced at boiling until solid appeared. After cooling, the solid was filtered to yield 2.15 g (28%) of **8**: λ_{max} (pH 1) 275 nm (ϵ 20,500); λ_{max} (pH 7) 267 nm (ϵ 19,000); λ_{max} (pH 11) 268 nm, 289, 275 sh (ϵ 14,500, 9500, 13,900); λ_{max} (pH 12) 289 nm (ϵ 24,100); R = 1.9. *Anal.* ($C_{13}H_{15}N_5O_8NaP$) C, H, N, Na.

N^6,2′-*O*-Bis(*N*-methylcarbamoyl)-8-bromoadenosine 3′,5′-Cyclic Phosphate (9). A solution of 1.0 g (2.4 mmol) of 8-bromoadenosine 3′,5′-cyclic phosphate (**11**),[12] 1 ml of Et_3N, and 2 ml methyl isocyanate in 40 ml of DMF was heated at 70° overnight. The solution was diluted with 150 ml of H_2O and extracted three times with 150 ml of EtOAc. The aqueous phase was placed onto a 150-ml column (2-cm diameter) of Dowex 1-X2 (formate, 100–200 mesh). The column was eluted with a gradient of 1.2 l. of 0–6 *N* formic acid to yield, after appropriate evaporations, 550 mg (43%) of the pure **9**. The Na salt of **9** (*via* Dowex 50, Na^+) was crystallized from EtOH for analysis: λ_{max} (pH 1) 281 nm, 291 sh (ϵ 21,800, 15,500); λ_{max} (pH 7) 277 nm, 280 (ϵ 22,400, 19,200); λ_{max} (pH 11) 277 nm, 280 (ϵ 22,400, 19,200); λ_{max} (pH 13) 299 nm (ϵ 21,600); R = 2.5. *Anal.* ($C_{14}H_{16}N_7O_8BrNaP$·H_2O) C, H, N, Na.

8-Benzylthio-N^6,2′-*O*-bis(*N*-phenylcarbamoyl)adenosine 3′,5′-Cyclic Phosphate Sodium Salt (10). 8-Benzylthioadenosine 3′,5′-cyclic phosphate sodium salt (2 g, 4.5 mmol) (**12**)[12] and 3 ml of phenyl isocyanate in 50 ml of DMF were allowed to react and were extracted (150 ml of H_2O) as for compound **4** above. The aqueous phase was cooled and the solid was filtered, washed with cold H_2O, and recrystallized from EtOH to yield 1.4 g (43%) of **10**: λ_{max} (pH 1) 309 nm (ϵ 17,500); λ_{max} (pH 7) 295 nm (ϵ 20,000); λ_{max} (pH 11) 298 nm (ϵ 24,800); λ_{max} (pH 12.8) 321 nm (ϵ 31,000). Solutions of **10** were opaque and ϵ values are therefore minimum values; R = 5.7. *Anal.* ($C_{31}H_{27}N_7O_8NaPS$·0.5H_2O) C, H, N, Na, S.

8-Benzylthio-N^6-(*N*-phenylcarbamoyl)adenosine 3′,5′-Cyclic Phosphate (13). A solution of 0.6 g (0.83 mmol) of **10** in 6 ml of DMF and 4 ml of 2 *N* NaOH was stirred at room temperature for 2.5 hr. The solvent was removed *in vacuo* and the residue was partitioned between 50 ml of hot H_2O and 50 ml of EtOAc. The hot aqueous phase was extracted twice with 50 ml of EtOAc. EtOH (50 ml) was added to the aqueous solution and the pH was adjusted to 2 with 1 *N* HCl. The gelatinous solid which formed was filtered, washed with 10 ml of H_2O, and dissolved in 20 ml of EtOH. The solution volume was reduced to 10 ml and cooled and the solid filtered to yield 220 mg (45%) of **13**: λ_{max} (pH 1) 308 nm (ϵ 27,900); λ_{max} (pH 7) 296 nm (ϵ 31,200); λ_{max} (pH 11) 296 nm (ϵ 30,300); λ_{max} (pH 12.75) 320 nm (ϵ 32,800); R = 5.0. *Anal.* ($C_{24}H_{23}N_6O_7PS$·H_2O) C, H, N, S.

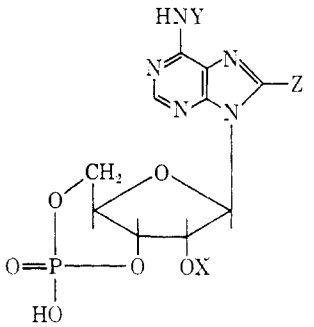
N^6,2′-*O*-Bis(*N*-methylcarbamoyl)-8-thioadenosine 3′,5′-Cyclic Phosphate Sodium Salt (14). A solution of 700 mg of **9** and 700 mg of thiourea in 50 ml of H_2O containing 5 drops of formic acid was refluxed for 1 hr. After cooling, the pH was adjusted to 8.5 with 1 *N* NaOH. The solution was evaporated and 3 g of silica gel added to the residue. The mixture was suspended in MeOH and the MeOH evaporated. The final residue was added to a 15-g column (2.5-cm diameter) of silica gel (packed in $CHCl_3$). The column was washed with $CHCl_3$ and then MeOH–$CHCl_3$ (1:3) to remove product. The appropriate fractions were evaporated and the resulting residue was suspended in EtOH and filtered to yield 300 mg (45%) of **14**: λ_{max} (pH 1) 244 nm, 313 (ϵ 19,800, 33,800); λ_{max} (pH 7) 233 nm, 312 (ϵ 17,800, 30,800); λ_{max} (pH 11) 233 nm, 311 (ϵ 17,500, 29,600); R = 2.5. *Anal.* ($C_{14}H_{17}N_7O_8NaPS$·1.75H_2O) C, H, N, S.

Biochemical. The cAMP phosphodiesterases were purified from a 30,000g supernatant of beef heart, rabbit lung, or rabbit kidney. The procedure, which was used for all three tissues, involved ammonium sulfate fractionation (the 0–50% of saturation fraction was used) and DEAE-cellulose chromatography (the protein eluting from the column between 0.08 and 0.4 *M* KCl was used). The details of the purifications have been reported previously.[18] Bovine brain cAMP-dependent protein kinase was purified through the DEAE cellulose step as previously described.[11]

The assay for inhibition of cAMP phosphodiesterase contained in 1.0 ml: 50 μmol of Tris HCl, pH 7.5; 10 μmol of $MgCl_2$; 10–200 μg of phosphodiesterase protein; 100 nmol of 8-[^{3}H]-cAMP (350,000 cpm); and varying concentrations of the nucleoside cyclic phosphate 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′-phosphate product to a nucleoside. The untreated nucleoside cyclic phosphate was absorbed onto Dowex 1 and the radioactivity of the nucleoside fraction determined.

When testing cAMP derivatives as substrates for cAMP phosphodiesterase, the standard reaction mixture contained in 0.60 ml: 3.0 μmol of cAMP or cAMP derivative; 30 μmol of Tris HCl, pH 7.5; 6 μmol of $MgCl_2$; and 0.1–0.3 mg of phosphodiesterase protein. After an appropriate incubation period, the reaction was terminated by heating and treated with bacterial alkaline phos-

Table I. Effect of Carbamoyl and Alkoxycarbonyl Derivatives of cAMP on Bovine Brain cAMP-Dependent Protein Kinase and Beef Heart and Rabbit Lung cAMP Phosphodiesterases[a]



Compd	Substitution X	Substitution Y	Substitution Z	Protein kinase activation,[b] K_a'	Phosphodiesterase inhibition,[c] I_{50}, μM: Beef heart	Phosphodiesterase inhibition,[c] I_{50}, μM: Rabbit lung
1	H	H	H	1.0		
6	H	C(=O)NHCH$_3$	H	0.72	140	260
2	C(=O)NHCH$_3$	C(=O)NHCH$_3$	H	0.002	650	290
9	C(=O)NHCH$_3$	C(=O)NHCH$_3$	Br	0.001	260	130
14	C(=O)NHCH$_3$	C(=O)NHCH$_3$	SH	0.001	1300	1000
5	H	C(=O)NHC$_3$H$_7$	H	1.1	2300	330
3	C(=O)NHC$_3$H$_7$	C(=O)NHC$_3$H$_7$	H	0.001	2300	290
7	H	C(=O)NHC$_6$H$_5$	H	4.9	22	160
4	C(=O)NHC$_6$H$_5$	C(=O)NHC$_6$H$_5$	H	0.015	93	190
10	C(=O)NHC$_6$H$_5$	C(=O)NHC$_6$H$_5$	SCH$_2$C$_6$H$_5$	0.010	22	10
13	H	C(=O)NHC$_6$H$_5$	SCH$_2$C$_6$H$_5$	1.6	14	21
8	H	C(=O)OC$_2$H$_5$	H	0.29	130	210
12	H	H	SCH$_2$C$_6$H$_5$[d]	1.9	22	60
	Theophylline				130	230

[a] The technical assistance of Mieka B. Scholten is gratefully acknowledged. [b] Values reported are K_a' values, where K_a' = (K_a for cAMP)/(K_a for test compound). K_a is the activation constant determined from a Lineweaver–Burk plot. K_a for cAMP was 4×10^{-8} M. [c] I_{50} is the concentration of compound causing 50% inhibition of the phosphodiesterase. [d] Reference 12.

phatase, and the phosphate released assayed colorometrically. The details of the phosphodiesterase substrate and inhibition assayed have been previously described.[18] The assay for stimulation of protein kinase by cAMP analogs and the method for the determination of K_a values were performed as previously described.[12]

Cytotoxicity was determined by microscopic examination of the drug-exposed cell layers 3 days after incubation at 37° in the presence of varying concentrations of each drug. Numbers of floating cells, cell granulation, and alteration of cell shape were used as indications of toxicity.

Results and Discussion

Effect of cAMP-Dependent Protein Kinase. In order to determine the effect the addition of an N^6-carbamoyl or an N^6-alkoxycarbonyl moiety to the cAMP molecule has on the biological activity of the derivative, the resulting compounds were tested for their ability to stimulate a cAMP-dependent protein kinase from bovine brain.[12] The results (Table I) show that substitution on cAMP of an N^6-carbamoyl group was preferred over an N^6-ethoxycarbonyl group for activation of protein kinase. Furthermore, there appears to be a strong preference by the active site on protein kinase for a phenyl group compared to an alkyl (methyl or propyl) group as a substituent on the ureido nitrogen. This is shown by the ability of N^6-(N-phenylcarbamoyl)-cAMP (7, K_a' = 4.9) to activate the protein kinase five times better than cAMP, whereas N^6-(N-methylcarbamoyl)-cAMP (6) and N^6-(N-propylcarbamoyl)-cAMP (5) had K_a' values not appreciably different from cAMP. That the mere presence of a phenyl group is not the only determinant for the high K_a' value of 7 is suggested by the lower K_a' values of N^6-benzyl-cAMP (K_a' = 0.82),† N^6-benzyloxy-cAMP (K_a' = 1.0),† and 6-benzylthio-9-β-D-ribofuranosylpurine 3′,5′-cyclic phosphate (K_a' = 1.3).[13]

†R. B. Meyer, J. P. Miller, D. A. Shuman, and R. K. Robins, unpublished results.

In addition, other N^6-substituted derivatives of cAMP containing small nonaromatic N^6 substituents were less active than cAMP at stimulating bovine protein kinase: N^6-ethyl-cAMP (K_a' = 0.50),[13] N^6-methoxy-cAMP (K_a' = 0.65),† and N^6-ethoxy-cAMP (K_a' = 0.44).†

Our previous studies[12] have demonstrated that the substitution of a benzylthio group at C-8 enhanced the ability of the derivative to activate protein kinase [K_a' of 8-benzylthio-cAMP (12) = 1.9]. Although N^6-(N-phenylcarbamoyl)-cAMP (7) had a K_a' of 4.9, an additive effect was not seen when a benzylthio moiety was placed in the 8 position. Indeed, the resulting compound, 8-benzylthio-N^6-(N-phenylcarbamoyl)-cAMP (13) was one-third as active (K_a' = 1.6) as 7. Additionally, the significantly decreased ability to activate protein kinase by the 2′-O-carbamoyl derivatives 2–4, 9, 10, and 14 was not enhanced by substitution at the 8 position by benzylthio, Br, or SH (compounds 9, 10, and 14).

These studies demonstrate again the necessity of a free 2′-OH (β-D-ribo) group for activation of a cAMP-dependent protein kinase[18] and further report on the synthesis of the most potent activator of protein kinase to date, namely, N^6-(N-phenylcarbamoyl)-cAMP (7).

cAMP Phosphodiesterase Studies. All the derivatives listed in Table I were tested for hydrolysis by rabbit kidney high K_m cAMP phosphodiesterase at a substrate concentration of 5×10^{-3} M. When incubated under the same conditions as those for cAMP, hydrolysis could be detected only with N^6-ethoxycarbonyl-cAMP (8). It was hydrolyzed at a rate that was only 8% that of cAMP.

These same compounds were also tested as inhibitors of beef heart and rabbit lung low K_m cAMP phosphodiesterase. The results are summarized in Table I. Theophylline, which was included for comparison, demonstrated approximately the same I_{50} against both enzymes. N^6-(N-Methylcarbamoyl)-cAMP (6), N^6,2′-O-bis(N-methyl-

carbamoyl)-cAMP (2), N^6,2′-O-bis(N-methylcarbamoyl)-8-bromo-cAMP (9), N^6,2′-O-bis(N-phenylcarbamoyl)-cAMP (4), and N^6-ethoxycarbonyl-cAMP (8) had I_{50} values within the same range as those of theophylline and showed little difference in inhibitory activity between the lung and heart enzymes. N^6,2′-O-Bis(N-methylcarbamoyl)-8-thio-cAMP (14), N^6-(N-n-propylcarbamoyl)-cAMP (5), and N^6,2′-O-bis(N-n-propylcarbamoyl)-cAMP sodium salt (3) were significantly more active against the lung enzyme, while N^6-(N-phenylcarbamoyl)-cAMP (7) was appreciably more active against the heart enzyme. 8-Benzylthio-N^6,2′-O-bis(N-phenylcarbamoyl)-cAMP (10) and 8-benzylthio-N^6-(N-phenylcarbamoyl)-cAMP (13) were the most active of all the compounds tested in this group, being 5 to 20 times more potent than theophylline. The low K_m cAMP phosphodiesterase was most strongly inhibited by those cAMP derivatives which contained a phenyl substitution. This structural specificity was similar to that observed for activation of the protein kinase.

The effects of the present series of compounds on tumor cell growth in tissue culture have been examined. No unusual cytotoxicity was seen, with the single possible exception of N^6,2′-O-bis(N-phenylcarbamoyl)-cAMP (4), which was cytotoxic to KB cells between 1 and 3.2 μg/ml. All of the other derivatives were nontoxic to the cells at doses nearly ten times greater (10–32 μg/ml).

Future studies are aimed at determining the pharmacological properties and biological effects in whole cell systems of these interesting derivatives.

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Hydrolysis of Hydroxybenzotrifluorides and Fluorinated Uracil Derivatives. A General Mechanism for Carbon-Fluorine Bond Labilization

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Received March 16, 1973

The kinetics of hydrolysis of o- and p-hydroxybenzotrifluoride (1 and 2) and 1-trifluoromethyl-2-(4-hydroxyphenyl)-ethylene (3) and the uracil derivatives 5-difluoromethyluracil (5) and 1-methyl-5-difluoromethyluracil (6) are described. Compounds 1 and 2 hydrolyze to the corresponding hydroxybenzoic acids and 3 gives p-coumaric acid upon hydrolysis. Reduction of the ethylenic double bond of 3 gives the hydrolytically stable 1-trifluoromethyl-2-(4-hydroxyphenyl)ethane (4). Compounds 5 and 6 hydrolyze to the corresponding 5-formyluracil derivatives. The data obtained from these reactions implicate the anchimeric assistance of the phenolate or uracil anion in the hydrolysis mechanisms. A general mechanism for carbon-fluorine bond labilization is presented which is predictively useful for the study of enzyme mechanisms and perhaps in the design of effective chemotherapeutic drugs.

Our previous studies on the mechanisms of hydrolysis of various 5-trifluoromethyluracil derivatives[1] led us to begin studies on 5-difluoromethyluracils to determine if there was any relation between the chemistry of these two groups of compounds. The data from these studies suggested we consider the generality and utility of such reactions; that is, are such carbon-fluorine bond labilization reactions general enough that they may be utilized in the design of potential medicinal agents and/or can they be used in the determination of enzyme mechanisms?

Organic fluorine compounds have been utilized to a great extent in the study of biological systems. The replacement of hydrogen by fluorine in biologically active materials confers upon these molecules properties which may be used to gain insight into the means by which normal substrates react. Frequently, such altered compounds are enzyme inhibitors and use has been made of their pharmacological activities. The more notable of these are 5-fluorouracil and 5-trifluoromethyluracil and their derivatives,[1,2] which have been used in cancer chemotherapy, and fluorinated analogs of compounds involved in the tricarboxylic acid cycle[3] used to study that system.

In most cases studied, the carbon-fluorine bond appears unreactive to displacement or elimination reactions; however, there are systems in which it is substantially more labile than would be expected from the nature of "normal" carbon-fluorine bonds. The general properties of organofluorine compounds and carbon-fluorine bonds are well documented[4] and will be described only briefly. The

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