action at other loci. In vivo, where pharmacology is superimposed upon intrinsic activity, IIb is substantially more effective than III and does not have the objectionable tissue-staining properties of I.

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Articles

Synthesis and Enzymatic and Inotropic Activity of Some New 8-Substituted and 6,8-Disubstituted Derivatives of Adenosine Cyclic 3′,5′-Monophosphate

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The synthesis of certain new 8-(arythio)- and 8-(alkylthio)-cAMP derivatives and N^6-alkyl- and N^6-dialkyl-8-(arylthio) and -8-(alkylthio) derivatives of cAMP is reported. On the basis of activation of protein kinase, several N^6-alkyl-8-(benzylthio)-cAMP derivatives were selected for evaluation as inotropic agents using cat papillary muscle in vitro. Activity in these studies resulted in the selection of several analogues for in vivo studies in the anesthetized dogs. The best inotropic agent selected on the basis of in vivo studies was N^6-butyl-8-(benzylthio)-cAMP (**26**), which exhibited an increase in blood-flow rate of 85% with no increase in heart rate. A large-scale synthesis of **26** from cAMP is reported via N^1-alkylation, followed by a Dimroth rearrangement, reduction, bromination, and nucleophilic displacement via benzyl mercaptan. The N^6-alkyl-8-substituted-cAMP derivatives represent a new class of potent inotropic agents. The direct mechanism of action of **26** suggests the possible utility of this cyclic nucleotide to treat clinical myocardial infarction by rapid intravenous infusion.

The concept of designing nucleoside cyclic 3′,5′-monophosphates as potential medicinal agents was outlined in our first paper[1] in 1971. Progress of our studies in this area has recently been reviewed.[2,3] Amer and McKinney[4,5] have pointed out in some detail the potential of cyclic nucleotides as mediators of drug action in a number of areas of medicinal research. The possible sites in the system of cyclic nucleotide biochemistry which may serve as targets for drug design have recently been reviewed.[2,3,6] Our own approach to this field has been one of designing cyclic nucleotides which would either mimic or antagonize the action of cAMP or cGMP and which would be resistant to enzymatic degradation by phosphodiesterase[2,3] (PDE).

Ischemic heart disease is the most common serious health problem of our contemporary society.[7] In the United States alone, more than 675 000 patients die each year from ischemic heart disease and its complications. Approximately 1 300 000 patients each year have a myocardial infarction, and countless more suffer from congestive heart failure secondary to ischemic myocardial damage.[7] Thus, ischemic heart disease and its complications are by far the most common cause of death in the developed world.[7] The rapid decline in heart muscle contractility induced by ischemia is one of the major problems of clinical cardiovascular pharmacology. In 1962, Sutherland and co-workers[8] first suggested that cAMP was involved in the inotropic function of the heart when they showed that the in vivo potency of several catecholamines could be correlated with their ability to stimulate adenyl cyclase in membrane preparations of dog heart. Robison

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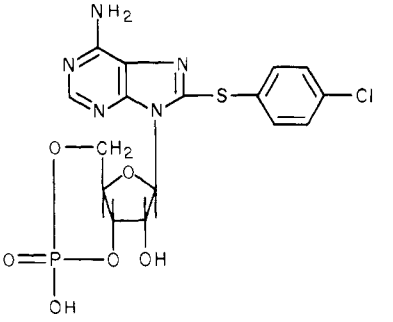
et al.[9] in 1965 reported that epinephrine injected into perfused rat hearts caused a rapid rise in cAMP, which was followed by an increased contractile force. Additional studies in other animal systems[10a] provide substantial evidence that cAMP mediates the positive inotropic effect of catecholamines. One of the difficulties in proving that cAMP is involved in regulating cardiac contractility is the failure to produce positive effects when the cyclic nucleotide is applied directly, since heart cells are highly impermeable to cAMP itself.[10a] Dibutyryl-cAMP, however, which is considered to cross cellular membranes more readily, has shown a positive inotropic effect in perfused hearts of guinea pigs, rats, and rabbits.[10b] These studies were later extended to the canine heart, with dibutyryl-cAMP showing similar results.[10c] Several recent reviews have appeared dealing with the role of cAMP and its regulatory function in contractility[10a,d,e] which has now become rather firmly established.

In considering the possibility of synthesizing a derivative or analogue of cAMP which would be superior to cAMP itself as a desirable inotropic agent, several problems were considered. First, the cAMP derivative should be rather lipid soluble so that it would transport satisfactorily into the cardiac cell. Secondly, it should be resistant to degradation by the cellular phosphodiesterases which rapidly convert cAMP to 5'-AMP. Thus, one would hope for a more lasting inotropic effect. Thirdly, the design of such a derivative would hopefully be quite specific in activating the cAMP-dependent protein kinase of the cells in the heart muscle without simultaneously causing a number of undesirable physiological responses in other organs. Studies from our laboratory[11] have shown that N^6-ethyl- and N^6,N^6-diethyl-cAMP were essentially as effective as cAMP in activating bovine brain protein kinase and were surprisingly resistant to cAMP phosphodiesterase isolated from rabbit kidney.[11] In an effort to prepare cAMP derivatives with superior potency and longer duration of action, we decided to prepare a series of N^6-alkyl-8-(alkylthio)-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphates,[12,13] since earlier studies from our laboratory[1] indicated that 8-substituted cAMP analogues are also resistant to hydrolysis by cAMP phosphodiesterase. The present work is a study of the enzymatic and inotropic activity of these and related 6,8-disubstituted purine nucleoside cyclic 3',5'-monophosphates. An 8-substituted cAMP derivative, 8-chloro-cAMP,[14] has recently been shown to mimic the adrenal steroidogenic action of ACTH in vivo.[15] 8-Chloro-cAMP elicited the secretion of corticosterone to normal plasma levels in both betamethasone treated and hypophysectomized rats.[15]

The synthesis of additional 8-(alkylthio)- and 8-(arylthio)-cAMP derivatives described in the present work was prompted by the fact that 8-[(*p*-chlorophenyl)thio]-cAMP[16] (I) is 18 times more potent than cAMP[16] as an activator of bovine brain cAMP-dependent protein kinase in vitro. 8-[(*p*-Chlorophenyl)thio]-cAMP (I) is 50 times more potent than dibutyryl-cAMP as an inducer of tyrosine aminotransferase in rat liver in vivo.[17] I inhibits the



I

spontaneous firing of 92% of rat cerebellar Purkinje cells.[18] Hall, Barnes, and Dousa[19] have recently reported that I mimics the effect of vasopressin in mammalian kidney tissue and is 100 times more potent in this system than cAMP. More recently, Stadel and Goodman[20] have shown that I mimics ADH (antidiuretic hormone) in the toad urinary bladder. The hydroosmotic response of I is equal in magnitude to oxytocin.

Synthesis of 8-(Arylthio)- or 8-(Alkylthio)-cAMP Derivatives. The 8-aryl or 8-(alkylthio) derivatives of cAMP were prepared by one of two methods. Method A consisted of the treatment of 8-bromo-cAMP with the requisite arylthiol or alkylthiol as described in our previous publications.[1,16] Method B consisted of the treatment of 8-thio-cAMP[1] with an appropriate alkyl or aryl halide to give the corresponding 8-alkyl- or 8-(arylthio)-cAMP derivative, as listed in Table I.

Synthesis of 6,8-Disubstituted Derivatives of cAMP. A general route to the synthesis of N^6-substituted 8-(alkylthio)- or 8-(arylthio)-cAMP derivatives was outlined in an earlier publication from this laboratory.[13] Certain new compounds of this general structure are listed in Table II and their syntheses described under Experimental Section.

8-(Benzylthio)-6-(methylamino)-9-(β-D-ribofuranosyl)-purine cyclic 3',5'-phosphate (**20**) was prepared by alkylation of 8-(benzylthio)-cAMP (**16**) with methyl iodide to give the intermediate N^1-methyl-8-(benzylthio)-cAMP (**16**), which was converted to **20** via the Dimroth rearrangement in base. By a similar procedure, 6-(benzylamino)-8-(benzylthio)-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate (**35**) was also prepared from **16** and benzyl bromide.

2'-*O*-Acetyl-8-(benzylthio)-6-chloro-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate[13] and piperidine at room temperature gave 6-(benzylthio)-6-piperidino-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate (**43**) in 59% yield. This procedure was also utilized to prepare **22**, **31**, **42**, and **26**.

When 8-(benzylthio)-6-(*n*-butylamino)-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate (**26**) was required

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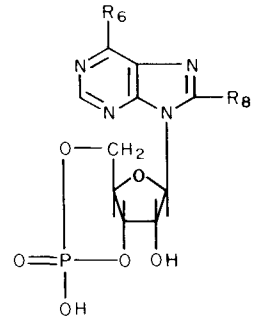
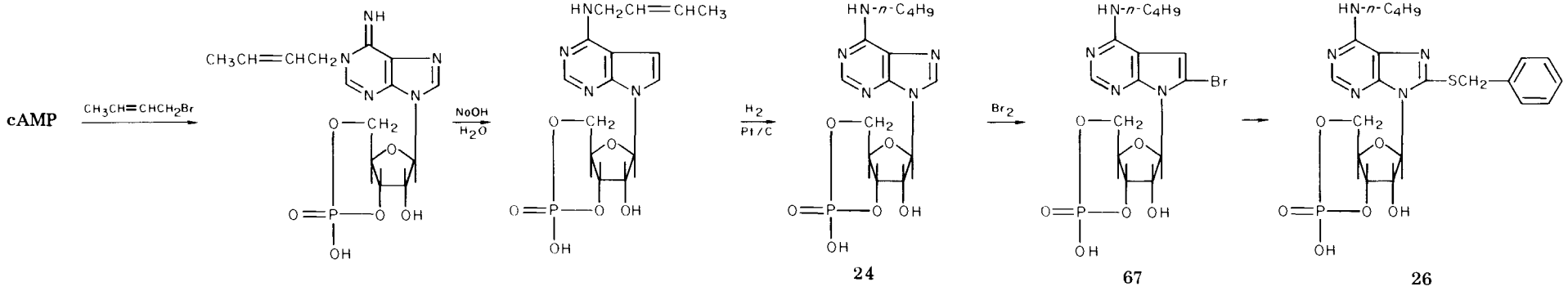
Table I. Synthesis and Physical Properties of Certain 8-(Alkylthio)- and 8-(Arylthio)-cAMP Derivatives



no.	R	mol formula for which anal. agreed	meth of synth[a]	reaction solvent	base	reaction time, h	reaction temp, °C	recryst solvent	UV absorption: λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 1	UV absorption: λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 11	^{1}H NMR H_1' proton singlet (ppm), δ
46	$-CH_2COONa^+$	$C_{12}H_{13}N_5NaO_8PS \cdot 3H_2O$	B	H_2O	Na_2CO_3	1	0	H_2O/MeOH	278 (19.1)	279 (18.1)	5.28
49	$-(CH_2)_2CH_3$	$C_{13}H_{18}N_5O_6PS \cdot H_2O$	A	MeOH	$NaOCH_3$	16	4		283 (20.0)	280 (18.8)	5.90
50	$-CH(CH_3)_2$	$C_{13}H_{18}N_5O_6PS$	A	MeOH	$NaOCH_3$	16	4		282 (16.6)	281 (16.2)	5.94
51	$-CH_2CH{=}CH_2$	$C_{13}H_{16}N_5O_6PS$	B	MeOH	NaOAc	16	25	0.1 N HCl	282 (18.5)	280 (16.5)	5.89
52	$-(CH_2)_3CH_3$	$C_{14}H_{20}N_5O_6PS$	A	MeOH	$NaOCH_3$	16	4	H_2O	282 (20.1)	280 (18.6)	5.88
53	$-(CH_2)_4CH_3$	$C_{15}H_{22}N_5O_6PS \cdot 1.5H_2O$	A	MeOH	$NaOCH_3$	16	4		282 (20.6)	280 (19.0)	5.89
54	-cyclohexyl	$C_{16}H_{22}N_5O_6PS \cdot H_2O$	A	EtOH	NaOAc	16	4		283 (19.0)	281 (17.3)	5.81
55	$-CH_2$-(pyrid-3-yl)·HCl	$C_{16}H_{17}N_6O_6PS \cdot HCl \cdot 1.5H_2O$	B	EtOH	NaOAc	120	25	H_2O/EtOH	276 (17.4)	283 (14.1)	5.74
56	$-CH_2$Ph-o-CH_3	$C_{18}H_{20}N_5O_6PS$	B	MeOH	NaOAc	16	25	H_2O/EtOH	283 (17.4)	283 (15.0)	5.87
57	$-CH_2$Ph-*p*-CH_3	$C_{18}H_{20}N_5O_6PS$	B	MeOH	NaOAc	16	25	H_2O/EtOH	283 (17.6)	283 (15.3)	5.84
58	$-CH_2$Ph-*p*-OCH_3	$C_{18}H_{20}N_5O_7PS$	A	MeOH	$NaOCH_3$	2	4	H_2O/EtOH	283 (17.8)	283 (15.6)	5.81
59	$-CH_2$Ph-*m*-NO_2	$C_{17}H_{17}N_6O_8PS$	B	MeOH	NaOAc	16	25	H_2O/EtOH	278 (21.3)	279 (18.4)	5.86
60	$-CH_2$Ph-*p*-NO_2	$C_{17}H_{17}N_6O_8PS$	B	MeOH	NaOAc	4	4	H_2O/EtOH	272 (24.7)	276 (23.6)	5.81
61	$-CH_2$Ph-*p*-Cl	$C_{17}H_{17}ClN_5O_6PS$	B	MeOH	NaOAc	16	25	H_2O/EtOH	283 (18.1)	282 (15.6)	5.85
62	$-CH_2$Ph-*p*-F	$C_{17}H_{17}FN_5O_6PS$	B	MeOH	NaOAc	16	25	H_2O/EtOH	282 (17.5)	283 (15.1)	5.83
63	-Ph	$C_{16}H_{16}N_5O_6PS \cdot H_2O$	A	EtOH	NaOAc	16	4	methoxyethanol	282 (17.5)	282 (15.6)	6.05
64	$-PhOCH_3$	$C_{17}H_{18}N_5O_6PS \cdot 1.5H_2O$	A	EtOH	NaOAc	16	ref	methoxyethanol	281 (17.0)	281 (15.5)	6.12
65	-Ph-*p*-OCH_3	$C_{17}H_{18}N_5O_7PS \cdot 2H_2O$	A	EtOH	NaOAc	16	4		281 (18.2)	282 (17.0)	6.05
66	-Ph-*p*-Br	$C_{16}H_{15}BrN_5O_6PS \cdot H_2O$	A	EtOH	NaOAc	16	4		280 (17.7)	283 (16.3)	6.14

[a] See Experimental Section for details. [b] All compounds analyzed correctly for C, H, N within 0.3% for the empirical formulas indicated. Compounds 55-60 were analyzed for phosphorous and checked within 0.25%.

Scheme I



in large quantities (100 g) for pharmacological studies, the direct alkylation of 8-(benzylthio)-cAMP (**16**) with *n*-butyl bromide was investigated. This route failed, however, since after 2 days the alkylation at N^1 was less than 50% complete. Efforts to raise the reaction temperature to ensure completion of the reaction resulted in a number of undesired byproducts, presumably due to alkylation at sites other than N^1. Attempts to employ *n*-butyl iodide also resulted in very low yields of the desired rearranged product **26**. In view of these difficulties, another route was devised from cAMP.

The readily available crotyl bromide was employed to alkylate cAMP at N^1 in the presence of DMF and DBU (Scheme I). This product could be rearranged without isolation to give N^6-crotyl-cAMP. Hydrogenation with a platinum on carbon catalyst in the presence of hydrogen gave a good yield of N^6-*n*-butyl-cAMP (**24**). Bromination of **24** in a buffered media gave N^6-*n*-butyl-8-bromo-cAMP (**67**). Treatment of **67** with benzyl mercaptan at pH 12 gave the desired product N^6-*n*-butyl-8-(benzylthio)-cAMP (**26**). This route proved very successful and adaptable to large-scale synthesis, since the steps of rearrangement, reduction, bromination, and nucleophilic displacement with benzyl mercaptan could be accomplished without isolation of the product, **26**, until the final step. Over 100 g of **26** was successfully prepared by this procedure.

When 2′-*O*-acetyl-6-chloro-8-[(*p*-chlorophenyl)thio]-9-(β-D-ribofuranosyl)purine cyclic 3′,5′-phosphate[13] was treated with aniline in water and ethanol, 2′-*O*-acetyl-6-anilino-8-[(*p*-chlorophenyl)thio]-9-(β-D-ribofuranosyl)-purine cyclic 3′,5′-phosphate was obtained. Deacetylation was accomplished with 2 N NH_4OH and methanol to give the desired product (**36**). When 2′-*O*-acetyl-6-chloro-8-[(*p*-chlorophenyl)thio]-9-(β-D-ribofuranosyl)purine cyclic 3′,5′-phosphate was treated with piperidine in water, deacetylation and nucleophilic displacement occurred simultaneously to yield 8-[(*p*-chlorophenyl)thio]-6-piperidino-9-(β-D-ribofuranosyl)purine cyclic 3′,5′-phosphate (**44**).

The N^6-substituted 8-[(*p*-chlorophenyl)thio]-cAMP derivatives **27** and **28** were similarly prepared by nucleophilic displacement of the 6-chloro group with the requisite amine.

Experimental Section

Synthesis of 8-Alkyl- and 8-(Arylthio)-cAMP Derivatives in Table I. The synthesis of the 8-alkyl- and 8-(arylthio)-cAMP derivatives was accomplished in a manner similar to that previously reported.[1,16] The syntheses consisted of two general procedures, methods A and B. In method A, 8-bromo-cAMP[1] was treated with an appropriate alkyl- or arylthiol. In method B, 8-thio-cAMP[1] was treated with an appropriate alkyl or aryl halide. Both methods follow the general procedure outlined.

General Procedure. Equal portions by weight of 8-bromo- or 8-mercaptoadenosine cyclic 3′,5′-phosphate and the indicated base were stirred in approximately 40 mL of solvent/g of nucleotide. Alkyl or aryl mercaptan, or alkyl or aryl halide, in an amount equal to three-fourths by weight of nucleotide was added and the resulting mixture stirred for the specified time at the indicated temperature. In instances wherein the product was insoluble in the solvent, the crude product was collected by filtration and washed with solvent and then ether to remove excess mercaptan or halide. In instances wherein the product is soluble in the solvent, the solvent was removed by evaporation under reduced pressure and the residue washed with ether to remove excess mercaptan or halide. In either case, the resulting crude residue was dissolved in water and acidified with dilute HCl to pH 1. The resulting solid product was collected by filtration, washed with a small portion of water, and further purified by recrystallization. Recrystallization from aqueous alcohol was from approximately a 1:1 mixture.

8-(Benzylthio)-6-(methylamino)-9-(β-D-ribofuranosyl)-purine Cyclic 3′,5′-Phosphate (20). Method A. A solution of 8-(benzylthio)adenosine cyclic 3′,5′-phosphate sodium salt[1] (1.32 g, 2.7 mmol), 1,5-diazabicyclo[5.4.0]undec-5-ene (0.67 mL, 4.05 mmol), and methyl iodide (0.5 mL) in 4 mL of Me_2SO was stirred at room temperature overnight. The reaction was added to 150 mL of H_2O containing $NaHCO_3$ (1.3 g) and Na_2CO_3 (1.0 g) and heated on a steam bath for 3 h. After adjusting the pH to 1.5 with concentrated HCl, the reaction was placed under an aspirator vacuum for 1 h. The pH was readjusted to 8.5 with 2 N NaOH, and the solution was chromatographed on Dowex 1 × 2 (100–200 mesh, formate column 30 mL, 3 × 4.5 cm) and gradient eluted with 500 mL of water–500 mL of 2 N formic acid. The major band from the column was suspended in MeOH and filtered to yield 375 mg (28%) of **20**. An analytical sample was obtained by dissolving a sample in a large volume of MeOH–H_2O, filtering, and evaporating to dryness. The residue was suspended in H_2O, filtered, and dried: UV λ_{max}, pH 1, 287 nm (ε 20 200), pH 11, 291 (17 600). Anal. Calcd for $C_{18}H_{20}N_5O_6PS \cdot 1.5H_2O$: C, 43.90; H, 4.70; N, 14.22. Found: C, 43.72; H, 4.36; N, 14.36.

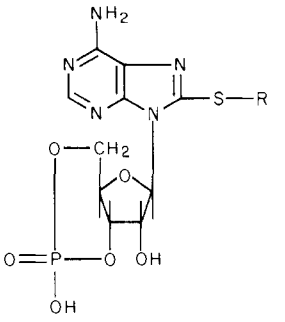
8-(Benzylthio)-6-(ethylamino)-9-(β-D-ribofuranosyl)purine Cyclic 3′,5′-Phosphate (22). A solution of 2′-*O*-acetyl-8-(benzylthio)-6-chloro-9-(β-D-ribofuranosyl)purine cyclic 3′,5′-phosphate sodium salt[13] (5 g, 8.8 mmol) in 60 mL of 35% aqueous ethylamine was stirred for 2 days at room temperature. The solvent was evaporated, and the residue was dissolved in H_2O and acidified to pH 2 with 1 N HCl. The liquid was decanted from the gum which formed. After trituration with hot water, the gum solidified. The solid was filtered, washed with water and boiling EtOH, and dried to yield 2.4 g (56%) of **22**. A sample was recrystallized from EtOH–H_2O (1:1) for analysis: UV λ_{max}, pH 1, 286 nm (ε 20 650), pH 7, 291 (17 000). Anal. Calcd for $C_{19}H_{22}N_5O_6PS \cdot 0.75H_2O$: C, 46.29; H, 4.76; N, 14.20; S, 6.50. Found: C, 46.23; H, 4.73; N, 14.08; S, 6.63.

8-(Benzylthio)-6-(*n*-propylamino)-9-(β-D-ribofuranosyl)-purine Cyclic 3′,5′-Phosphate (23). 2′-*O*-Acetyl-8-(benzylthio)-6-chloro-9-(β-D-ribofuranosyl)purine cyclic 3′,5′-phosphate sodium salt,[13] 300 mg, was stirred overnight in 5 mL of *n*-propylamine and 20 mL of H_2O. The resulting solution was evaporated, and the residue was triturated two times with Et_2O, dissolved in H_2O, and acidified to pH 1 with 2 N HCl. The resulting solid was filtered, washed with H_2O, EtOH, and Et_2O, and dried to give 205 mg of **23**: UV λ_{max}, pH 1, 287 nm (ε 19 000), pH 7, 292 (16 000). Anal. Calcd for $C_{20}H_{24}N_5O_6PS$: C, 48.68; H, 4.90; N, 14.19. Found: C, 48.62; H, 5.08; N, 13.98.

6-(Benzylamino)-8-(benzylthio)-9-(β-D-ribofuranosyl)-purine Cyclic 3′,5′-Phosphate (35). Aliquots of α-bromotoluene (0.2 mL) were added to a solution of 8-(benzylthio)adenosine cyclic 3′,5′-phosphate sodium salt[1] (442 mg, 1 mmol) and 1,5-diazabicyclo[5.4.0]undec-5-ene (0.2 mL, 1.2 mmol) in 1 mL of Me_2SO at times 0 and 1 h. After the mixture was stirred an additional 18 h, 50 mL of H_2O and 1.0 g of Na_2CO_3 were added and the resulting solution was heated on a steam bath for 2 h. After the mixture cooled, the pH was adjusted to 1.5 with HCl. The precipitate was filtered and reprecipitated from dilute alkali with concentrated HCl to yield 326 mg (66%) of **35**; the product could be recrystallized from methanol: UV λ_{max}, pH 1, 289 nm (ε 20 600), pH 11, 290 (18 300). Anal. Calcd for $C_{24}H_{24}N_5O_6PS$: C, 53.23; H, 4.46; N, 12.93. Found: C, 53.14; H, 4.46; N, 13.05.

8-(Benzylthio)-6-piperidino-9-(β-D-ribofuranosyl)purine Cyclic 3′,5′-Phosphate (43). A solution of 2′-*O*-acetyl-8-(benzylthio)-6-chloro-9-(β-D-ribofuranosyl)purine cyclic 3′,5′-phosphate sodium salt[13] (1.3 g) in 20 mL of water and 10 mL of piperidine was stirred overnight at room temperature. The solvent was evaporated and the residue triturated twice with ether. The final residue was dissolved in water and placed onto a Dowex 50 (H^+, 100–200 mesh) 2.5 × 10 cm column which had been prewashed with methanol–water (1:1). The column was washed with water followed by 100 mL of methanol–water (1:4), 100 mL of methanol–water (1:1), and finally methanol–water (3:1) until all the product was eluted. The fractions containing the product were collected, combined, and evaporated to dryness. The residue was dissolved in boiling ethanol, 5 volumes of water was added, and the solution was concentrated to one-fifth the volume. Upon cooling, 700 mg (59%) of white crystals of **43** deposited from the aqueous solution. Anal. Calcd for $C_{22}H_{26}N_5O_6PS$: C, 50.86; H,

Table II. Enzymatic Properties of 6,8-Disubstituted cAMP Analogues



no.	substituents R_6	substituents R_8	protein kinase activation: K_a'[a]	hydrolysis phosphodiesterase: α[a]	inhibn of phosphodiesterase: I_{50}, μM[a] rabbit lung	inhibn of phosphodiesterase: I_{50}, μM[a] bovine heart	ref
1	-Cl	-H	1.4	1.2	20	77	11
2	-Cl	-OH	1.0	<0.05	>400	330	13
3	-Cl	-NH_2	1.7	0.74	200	170	13
4	-Cl	-NEt_2	0.12	0.10	80	200	13
5	-SH	-H	0.20	1.1	53	29	11
6	-SH	-NH_2	0.30	0.30	83	50	13
7	-SMe	-H	0.90	0.46	80	83	11
8	-SMe	-SMe	1.7	<0.05	50	67	13
9	-SCH_2Ph	-H	1.9	0.54	25	53	11
10	-SCH_2Ph	-SCH_2Ph	0.04	<0.05	4.6	8.3	13
11	-NH_2	-H	1.0	1.0			
12	-NH_2	-Cl	3.1	<0.05	59	22	14
13	-NH_2	-Br	2.9	<0.05	40	7.7	1, 16
14	-NH_2	-OH	2.8	<0.05	150	500	1, 16
15	-NH_2	-SMe	2.4	<0.05	69	24	1, 16
16	-NH_2	-SCH_2Ph	2.1	<0.05	53	17	1, 16
17	-NH_2	-SPh-*p*-Cl	18.0	<0.05	200	22	16
18	-NH_2	-NH_2	1.5	0.80	83	33	1, 16
19	-NHMe	-H	1.3	0.22	50	33	11
20	-NHMe	-SCH_2Ph	1.8	<0.05	120	170	*b*
21	-NHEt	-H	0.80	0.20	400	130	11
22	-NHEt	-SCH_2Ph	1.9	<0.05	2.5	8.3	*b*
23	-NH-*n*-Pr	-SCH_2Ph	2.2	<0.05	3.6	12	*b*
24	-NH-*n*-Bu	-H	1.8	0.21	33	67	*b*
25	-NH-*n*-Bu	-SCH_3	3.6	0.26	53	80	*b*
26	-NH-*n*-Bu	-SCH_2Ph	1.8	<0.05	20	50	*b*
27	-NH-*n*-Bu	-SCH-*p*-Cl	7.8	<0.05	40	75	*b*
28	-NH-*i*-Bu	-SPh-*p*-Cl	3.5	<0.05	1.2	3.1	*b*
29	-NH-*n*-pent	-SCH_2Ph	5.0	<0.05	16	10	*b*
30	-NH-*i*-pent	-SCH_2Ph	3.3	<0.05	2.0	3.7	*b*
31	-NH-*n*-C_6H_{13}	-SCH_2Ph	2.5	<0.05	30	56	*b*
32	-$NHCH_2Ph$	-H	0.82	0.30	130	37	2
33	-$NHCH_2Ph$	-Br	1.7	<0.05	61	67	13
34	-$NHCH_2Ph$	-SMe	1.7	0.19	17	32	13
35	-$NHCH_2Ph$	-SCH_2Ph	1.4	<0.05	29	56	*b*
36	-NHPh	-SPh-*p*-Cl	4.3	<0.05	36	29	*b*
37	-NEt_2	-H	1.4	0.09	67	33	11
38	-NEt_2	-Cl	4.5	0.10	18	9.1	13
39	-NEt_2	-SCH_2Ph	1.1	<0.05	3.6	5.6	13
40	-NEt_2	-SPh-*p*-Cl	4.0	<0.05	3.5	5.6	13
41	-NEt_2	-NEt_2	0.06	<0.05	13	21	13
42	-c-NC_4H_8	-SCH_2Ph	16	<0.05	5.0	3.6	*b*
43	-c-NC_5H_{10}	-SCH_2Ph	7.0	<0.05	4.8	7.7	*b*
44	-c-NC_5H_{10}	-SPh-*p*-Cl	5.4	<0.05	10	5.6	*b*
45	-NH_2	-SH	3.8	<0.05	20	48	13
46	-NH_2	-$SCH_2COO^-Na^+$	0.004	<0.05	400	290	*b*
47	-NH_2	-SCH_2CH_3	2.0	<0.05	33	77	13
48	-NH_2	-SCH_2CH_2OH	0.54	<0.05	20	33	13
49	-NH_2	-$S(CH_2)_2CH_3$	0.80	<0.05	37	310	*b*
50	-NH_2	-$SCH(CH_3)_2$	1.3	<0.05	56	200	*b*
51	-NH_2	-$SCH_2CH{=}CH_2$	1.0	<0.05	29	33	*b*
52	-NH_2	-$S(CH_2)_3CH_3$	3.4	<0.05	25	160	*b*
53	-NH_2	-$S(CH_2)_4CH_3$	0.82	<0.05	15	290	*b*
54	-NH_2	-S-c-C_6H_{11}	3.5	<0.05	13	33	*b*
55	-NH_2	-SCH_2-(pyrid-3-yl)	0.26	<0.05	100	250	*b*
56	-NH_2	-SCH_2Ph-o-CH_3	3.2	<0.05	33	77	*b*
57	-NH_2	-SCH_2Ph-*p*-CH_3	2.4	<0.05	13	13	*b*
58	-NH_2	-SCH_2Ph-*p*-OCH_3	1.4	<0.05	30	33	*b*

Table II (*Continued*)

no.	substituents R_6	substituents R_8	protein kinase activation: K_a'[a]	hydrolysis phosphodiesterase: α[a]	inhibn of phosphodiesterase: I_{50}, μM[a] rabbit lung	inhibn of phosphodiesterase: I_{50}, μM[a] bovine heart	ref
59	$-NH_2$	$-SCH_2Ph$-*m*-NO_2	0.71	<0.05	25	11	*b*
60	$-NH_2$	$-SCH_2Ph$-*p*-NO_2	0.67	<0.05	27	14	*b*
61	$-NH_2$	$-SCH_2Ph$-*p*-Cl	1.2	<0.05	11	11	*b*
62	$-NH_2$	$-SCH_2Ph$-*p*-F	1.4	<0.05	38	19	*b*
63	$-NH_2$	-SPh	3.0	<0.05	57	33	*b*
64	$-NH_2$	-SPh-*o*-CH_3	0.62	<0.05	29	18	*b*
65	$-NH_2$	-SPh-*p*-OCH_3	0.82	<0.05	48	27	*b*
66	$-NH_2$	-SPh-*p*-Br	1.6	<0.05	240	60	*b*
67	-NH-*n*-C_4H_9	-Br	3.8	<0.05			*b*
68	$-N(CH_3)_2$	$-SCH_2Ph$	2.5	<0.05			*b*
69	$-N(CH_3)$-*n*-C_4H_9	$-SCH_2Ph$	3.7	<0.05			*b*
70	$-N(n\text{-}C_3H_7)_2$	$-SCH_2Ph$	15.0	<0.05			*b*

[a] The protein kinase activation assay and the phosphodiesterase inhibition assay were performed as described. K_a' is the ratio K_a for cAMP/K_a for the analogue, where the K_a values are determined from a Lineweaver-Burk plot. The K_a for cAMP was 0.2 μM. The value of α is the ratio of the rate of hydrolysis of the analogue to the rate of hydrolysis of cAMP at a substrate concentration of 4 mM. The I_{50} is the concentration of the analogue that resulted in a 50% inhibition of the rate of cAMP hydrolysis at a substrate concentration of 0.16 μM. [b] Described in the present work.

5.04; N, 13.48; S, 6.17. Found: C, 51.01; H, 5.11; N, 13.52; S, 6.05.

8-(Benzylthio)-6-pyrrolidino-9-(β-D-ribofuranosyl)purine Cyclic 3',5'-Phosphate (42). A solution of 2'-*O*-acetyl-8-(benzylthio)-6-chloro-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate[13] (2 g) in 20 mL of H_2O containing 5 mL of pyrrolidine was treated as for the synthesis of compound **43**. Water was added to the residue from the column, and the resulting crystals were filtered and dried to give 947 mg (51%) of **42**. Anal. Calcd for $C_{21}H_{24}N_5O_6PS\cdot1.5H_2O$: C, 47.36; H, 5.11; N, 13.15. Found: C, 47.05; H, 5.26; N, 12.94.

8-(Benzylthio)-6-(*n*-hexylamino)-9-(β-D-ribofuranosyl)-purine Cyclic 3',5'-Phosphate (31). Five grams of 2'-*O*-acetyl-8-(benzylthio)-6-chloro-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate sodium salt[13] was stirred in 15 mL of *n*-hexylamine and 100 mL of H_2O overnight. The solvent was evaporated and the residue triturated three times with Et_2O. The remaining gum was dissolved in H_2O/methylcellosolve and acidified to pH 1 with 2 N HCl to yield 1.61 g of **31** in three crops. A small sample was recrystallized from EtOH/H_2O for analysis: UV λ_{max}, pH 1, 288 nm (ε 18 200), pH 11, 292 (15 300). Anal. Calcd for $C_{23}H_{30}N_5O_6PS\cdot0.25H_2O$: C, 50.32; H, 5.79; N, 13.19. Found: C, 50.29; H, 5.99; N, 12.80.

6-Anilino-8-[(*p*-chlorophenyl)thio]-9-(β-D-ribofuranosyl)purine Cyclic 3',5'-Phosphate (36). Ethanol was added dropwise to a mixture of 2'-*O*-acetyl-6-chloro-8-[(*p*-chlorophenyl)thio]-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate sodium salt[13] in 20 mL of H_2O and 3 mL of aniline until a clear solution was obtained. After the mixture was stirred overnight, the solid which had separated from the solution was filtered and washed with EtOH. A second crop of solid was filtered and washed. The combined crops were dissolved in hot EtOH/H_2O with the addition of a small amount of 1 N NH_4OH. The solution was acidified to pH 1 with 2 N HCl. The resulting solid was filtered, washed, and dried to yield 406 mg of 2'-*O*-acetyl-6-anilino-8-[(*p*-chlorophenyl)thio]-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate. This 2'-*O*-acetyl intermediate, 150 mg, was dissolved in 25 mL of 2 N NH_4OH by the addition of MeOH. After the mixture was stirred for 2 h, the solvent was evaporated, and the residue was dissolved in H_2O and put onto a Dowex 50 (H^+, 2 × 5 cm) column. The column was washed with H_2O and the product was eluted with 1:1 MeOH/H_2O. The column eluate was evaporated to yield 90 mg of **36**. Anal. Calcd for $C_{22}H_{19}N_5O_6ClPS\cdot1.5H_2O$: C, 45.95; H, 3.85; N, 12.18. Found: C, 45.88; H, 3.70; N, 11.96.

8-[(*p*-Chlorophenyl)thio]-6-piperidino-9-(β-D-ribofuranosyl)purine Cyclic 3',5'-Phosphate (44). A solution of 2'-*O*-acetyl-6-chloro-8-[(*p*-chlorophenyl)thio]-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate sodium salt[13] (1.0 g, 1.8 mmol) in 20 mL of H_2O containing 3 mL of piperidine was stirred overnight at room temperature. The solvent was evaporated and the residue was triturated twice with ether. The final residue was dissolved in H_2O and the pH adjusted to pH 1 with 1 N HCl. The liquid was decanted from the gum which formed. The gum was triturated with H_2O and then dissolved in hot H_2O. Upon cooling the solution, a semisolid separated out of solution, and upon standing overnight it solidified. The solid was filtered and dried to yield 670 mg (70%) of **44**: UV λ_{max}, pH 1, 294 nm (ε 18 500), pH 11, 305 (16 000). Anal. Calcd for $C_{21}H_{23}N_5O_6ClPS$: C, 46.71; H, 4.29; N, 12.97. Found: C, 46.55; H, 4.28; N, 12.80. 6-(*n*-Butylamino)-8-[(*p*-chlorophenyl)thio]-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate (**27**) and 8-[(*p*-chlorophenyl)thio]-6-(isobutylamino)-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate (**28**) were prepared, isolated, and purified in a similar fashion.

8-(Benzylthio)-6-(*n*-butylamino)-9-(β-D-ribofuranosyl)-purine Cyclic 3',5'-Phosphate (26). Method A. A solution of 2'-*O*-acetyl-8-(benzylthio)-6-chloro-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate sodium salt[13] (3.5 g, 5.25 mmol) in 50 mL of H_2O and 10 mL of *n*-butylamine was stirred overnight at room temperature. The solvent was evaporated and the residue triturated twice with ether, dissolved in H_2O, and acidified to pH 1 with 1 N HCl. The crude solid was filtered and dissolved in EtOH. Five volumes of ether was added to the EtOH and the precipitated solid collected: recrystallization from EtOH–H_2O (1:1); yield 1.68 g (61%) of **26**; UV λ_{max}, pH 1, 288 nm (ε 22 300), pH 11, 291 (18 100). Anal. Calcd for $C_{21}H_{26}N_5OPS\cdot H_2O$: C, 47.99; H, 5.32; N, 13.32; S, 6.10. Found: C, 47.77; H, 5.48; N, 13.24; S, 6.30.

Method B. In a 2-L, three-neck flask equipped with a thermometer, drying tube, and reflux condensor, a suspension of adenosine cyclic 3',5'-phosphate (230 g, 0.70 mol) and 1,5-diazabicyclo[5.4.0]undec-5-ene (115 g, 0.75 mol) in 600 mL of DMF was stirred at 60 °C (internal temperature) until a solution was obtained. 2,6-Lutidine (37.4 g, 240 mL) was added dropwise, followed by the slow addition of crotyl bromide (135 g, 1 mol). The reaction mixture was stirred for an additional 5 h at 60 °C. The product which had slowly crystallized from solution was filtered, washed with a small volume of DMF and MeOH, and dried to yield 146 g of N^1-crotyl-cAMP.

N^1-Crotyl-cAMP (140 g, 0.365 mol) was dissolved in 800 mL of 1 N NaOH and heated at 75 °C for 2 h. The reaction mixture was cooled and 24 mL of glacial acetic acid added. After the addition of 4.0 g of 5% Pt on carbon, the reaction mixture was shaken under H_2 (40 psi for 5 h) until 87 lb of H_2 was taken up (91 lb theoretical). The catalyst was filtered and washed with 200 mL of hot H_2O. To the combined filtrate plus wash (1 L) was added Br_2 (61 g, 0.38 mol) in two portions at 30-min intervals. Forty grams of NaOAc was added and the solution was stirred at room temperature. After 4 h, an additional 10 mL of Br_2 was added, and stirring was continued for 3 days.

The solution was purged with N_2 for 2 h followed by the addition of $NaBH_4$ until the solution gave a negative KI–starch test. The pH was adjusted to pH 12 with 30% NaOH, and 40 mL of

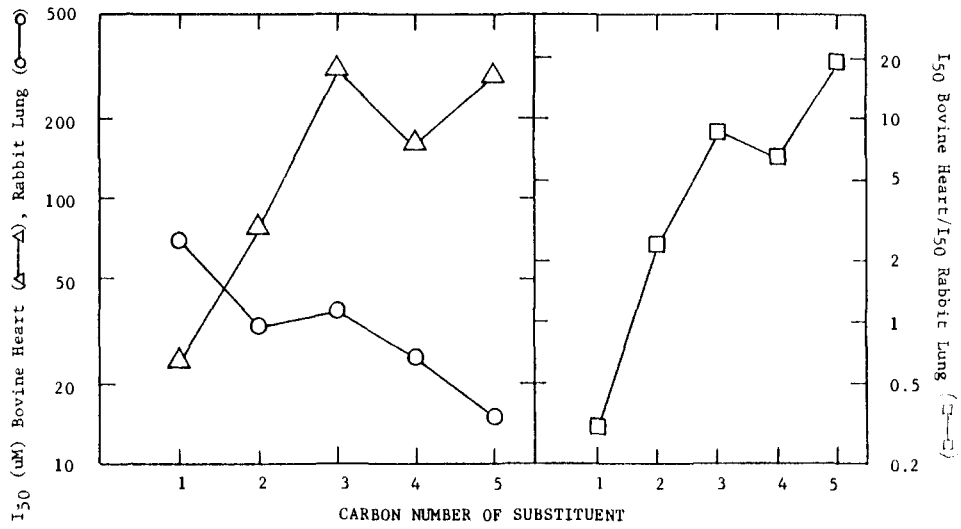


Figure 1.

benzyl mercaptan was added. The resulting solution was heated at 70 °C for 1 h and then acidified to pH 1.8 with concentrated HCl. The acidic solution was stirred and allowed to slowly cool. The crude product was filtered and washed with H_2O and then EtOH. The crude material was dissolved in $CHCl_3$ by the addition of triethylamine. The $CHCl_3$ solution was added to a column of 200 g of dry silica gel. The column was washed with $CHCl_3$ and the product eluted with 4% EtOH in $CHCl_3$. The appropriate column fractions were pooled and evaporated. The triethylammonium salt was dissolved in 100 mL of warm 2 N NH_4OH, 500 mL of MeOH was added, and the solution was acidified to pH 2 with concentrated HCl. After the solution was cooled, the resulting solid was filtered, washed with H_2O and EtOH, and dried, yielding 101 g of **26** which was identical with that prepared by method A.

8-Bromo-6-(*n*-butylamino)-9-(β-D-ribofuranosyl)purine Cyclic 3′,5′-Phosphate (67). N^1-Crotyl-cAMP (14 g) was treated as described above, up to and including purging with N_2. The resulting solution was percolated through a Dowex 50 (H^+, 5 × 20 cm) column. The column was eluted with H_2O. The appropriate fractions containing the product were pooled and evaporated to yield 5.1 g of **67**: UV λ_{max}, pH 1, 293 nm (ε 20 300), pH 11, 296 (16 700). Anal. Calcd for $C_{14}H_{19}BrN_5O_6P$: C, 36.22; H, 4.12; N, 15.08. Found: C, 36.20; H, 4.12; N, 15.05.

Compounds **29**, **30**, **68**, **69**, and **70** were each prepared from 2′-*O*-acetyl-8-(benzylthio)-6-chloro-9-(β-D-ribofuranosyl)purine cyclic 3′,5′-phosphate[13] (sodium salt) and the requisite amine in a manner similar to that described for 8-(benzylthio)-6-(*n*-butylamino)-9-(β-D-ribofuranosyl)purine cyclic 3′,5′-phosphate (**26**), method A.

Enzyme Studies. 8-Monosubstituted cAMP Derivatives. Enzymatic Activity. Activation of Bovine Brain Type II cAMP-Dependent Protein Kinase by 8-Monosubstituted cAMP Analogues. Each of the 8-(alkylthio)- and 8-(arylthio)-cAMP analogues was examined for its ability to activate a partially purified type II cAMP-dependent protein kinase from bovine brain. The relative potency of each analogue as an activator of this kinase is expressed as a K_a' value, which is the ratio of the apparent K_a value for the analogue to the apparent K_a value for cAMP, where the K_a value for cAMP is 78 nM. The K_a' values for the analogues are listed in Table II. For comparison, the K_a' values for the previously reported 8-(alkylthio)- and 8-(arylthio)-cAMP analogues, that are structurally similar to the new analogues reported here, are also included in Table II.

The only analogue exhibiting a K_a considerably less than cAMP was 8-($^+Na^-OOCCH_2S$)-cAMP (**46**; $K_a' = 0.004$), indicating that the cAMP binding site on protein kinase does not tolerate a charged group in the 8 position. The only analogue found to be significantly more potent than cAMP was 8-(*p*-ClPhS)-cAMP (**17**; $K_a' = 18$). In contrast, the closely related 8-(*p*-BrPhS)-cAMP (**66**; $K_a' = 1.6$) was not significantly different than cAMP. The reason for this considerable difference in activity between **17** and **66** is presently unknown.

The remaining analogues **47–66** (Table II) were all approximately equal in potency (K_a' values from 0.54 to 3.5). This observation allows the following conclusions to be drawn. The cAMP binding site on protein kinase that is adjacent to the 8 position of cAMP can apparently tolerate considerable bulk, since 8-(H_3CS)-cAMP (**15**, $K_a' = 2.4$) and 8-(*p*-$H_3COPhCH_2S$)-cAMP (**58**; $K_a' = 1.4$) are essentially equal in activity. There does not appear to be a significant interaction between the phenyl group in these analogues and protein kinase, since the K_a' values of the analogues are not significantly changed by either (1) saturating the phenyl ring [compare 8-(cyclohexylthio)-cAMP (**54**; $K_a' = 3.5$) with 8-(PhS)-cAMP (**63**, $K_a' = 3.0$)], (2) changing the juxtaposition of the phenyl ring of the substituent relative to the purine ring of cAMP [compare 8-(PhS)-cAMP (**63**; $K_a' = 3.0$) with 8-($PhCH_2S$)-cAMP (**16**, $K_a' = 2.1$)], or (3) substitution of the phenyl ring with groups expected to affect its electronic nature [for example, compare 8-(*p*-$H_3COPhCH_2S$)-cAMP (**58**; $K_a' = 1,4$), 8-(*p*-O_2NPhCH_2S)-cAMP (**60**; $K_a' = 0.67$), and 8-(*p*-$ClPhCH_2S$)-cAMP (**61**; $K_a' = 1.2$)]. The above results, taken together, suggest that perhaps even larger 8 substitutents may be tolerated by the protein kinase.

Resistance of 8-Substituted cAMP Analogues to Hydrolysis by Rabbit Kidney cAMP Phosphodiesterase. Each of the newly prepared 8-(alkylthio)- and 8-(arylthio)-cAMP analogues (Table II) was examined for susceptibility to be hydrolyzed to the corresponding 5′-phosphate by a partially purified cAMP phosphodiesterase from rabbit kidney. Under the standard conditions for this assay (described under Experimental Section), the rate of hydrolysis of cAMP was 1.5 μmol of 5′-AMP formed 10 min^{-1} (mg of protein)$^{-1}$ at 30 °C. Under the standard conditions of the assay, none of the 8-alkyl- and 8-(arylthio)-cAMP analogues, **47–66**, were hydrolyzed to a measurable extent. None of the following changes in the conditions of the assay resulted in the detection of hydrolysis products with any of these analogues: (1) increasing the concentration of the cAMP analogue by 2-, 4-, 10-, 20-, or 50-fold (where solubility allowed); (2) increasing the concentration of enzyme 2-, 5-, 10-, or 25-fold; (3) increasing the incubation time from 10–60 min to 2, 8, or 24 h; or (4) increasing the incubation temperature from 30 to 37 °C. A few of the new analogues (**49**, **53**, **54**, **57**, **60**, **63**, and **66**) were also examined for their ability to be hydrolyzed by partially purified cAMP phosphodiesterases from bovine heart and rat liver; none of these analogues exhibited any measurable hydrolysis with these enzymes.

Inhibition of cAMP Phosphodiesterases by 8-Substituted cAMP Analogues. Each of the 8-(alkylthio)- and 8-(arylthio)-cAMP analogues was examined for its ability to inhibit partially purified low K_m cAMP phosphodiesterase preparations from bovine heart and rabbit lung at a substrate (cAMP) concentration of 0.14 μM. The most potent inhibitors were capable of producing 50% inhibition of these phosphodiesterases at concentrations approximately 100 times greater than that of the cAMP substrate. The best inhibitors were those analogues

containing a phenylthio or a benzylthio moiety. The poorest inhibitor was 8-($^+Na^-OOCCH_2S$)-cAMP (**46**), indicating that the phosphodiesterases, like the protein kinase, do not tolerate a charged group in the 8 position.

The nature of the substituent in position 8 affected the ratio of the I_{50} values for inhibition of the lung phosphodiesterase to I_{50} values for inhibition of the heart phosphodiesterase. The analogues lacking a phenyl ring in the 8 position were, in general, more potent inhibitors of the lung enzyme than of the heart enzyme, while the analogues containing a phenyl ring in the 8 position were generally more potent inhibitors of the heart enzyme than of the lung enzyme. In addition, among the straight-chain alkylthio analogues (**15**, **47**, **49**, **52**, and **53**), it was found that with increasing chain length the potency of the analogues as inhibitors of the lung enzyme increased while at the same time the potency of the analogues as inhibitors of the heart enzyme decreased. This observation is demonstrated graphically in Figure 1.

Enzyme Studies of the 6,8-Disubstituted cAMP Analogues. Activation of Bovine Brain Type II cAMP-Dependent Protein Kinase by 6,8-Disubstituted cAMP Analogues. Each of the 6,8-disubstituted cAMP analogues was examined for its ability to activate a partially purified type II cAMP-dependent protein kinase from bovine brain. The relative potency of each analogue as an activator of this kinase is expressed as K_a' value, as described above. The K_a' values for the analogues are listed in Table II. Also included in Table II are the K_a' values for certain related similar 6- and 8-substituted cAMP analogues for comparison. An assignment of the various 6,8-disubstituted analogues can be made into one of four groups. These groups are defined by the relative effect of the double substitution on the K_a' values of the corresponding 6- or 8-substituted analogues.

The first group consists of 6,8-disubstituted cAMP analogues with K_a' values that are approximately the same as those for cAMP and for both the corresponding 6- and 8-substituted cAMP analogues. For example, a comparison of the K_a' values for the monosubstituted analogues—6-Cl-c-NMP (**1**; $K_a' = 1.4$) (c-NMP is an abbreviation for nebularine cyclic 3′,5′-phosphate), 8-HO-cAMP (**14**; $K_a' = 2.8$), and 8-H_2N-cAMP (**18**; $K_a' = 1.5$)—with those for the corresponding disubstituted analogues—6-Cl-8-HO-cNMP (**2**; $K_a' = 1.0$) and 6-Cl-8-H_2N-cNMP (**3**; $K_a' = 1.7$)—shows that the protein kinase can simultaneously tolerate replacement of the 6-amino group by a chlorine and substitution of the 8 position by an oxo or an amino function. Similarly, the K_a' values for 6,8-$(MeS)_2$-cNMP (**8**, $K_a' = 1.7$), 6-(MeS)-cNMP (**7**; $K_a' = 0.90$), and 8-(MeS)-cAMP (**15**; $K_a' = 2.4$) are all similar. In a similar comparison, the following N^6-alkyl- and N^6,N^6-dialkyl-8-(alkylthio)-cAMP analogues—N^6-Me-8-($PhCH_2S$)-cAMP (**20**; $K_a' = 1.8$), N^6-Et-8-($PhCH_2S$)-cAMP (**22**; $K_a = 1.9$), N^6-*n*-Bu-8-(MeS)-cAMP (**25**; $K_a' = 3.6$), N^6-*n*-Bu-8-($PhCH_2S$)-cAMP (**26**; $K_a' = 1.8$), N^6-(PhH_2C)-8-(MeS)-cAMP (**34**; $K_a' = 1.7$), N^6-(PhH_2C)-8-($PhCH_2$)-cAMP (**35**; $K_a' = 1.4$), and 6-(Et_2N)-8-($PhCH_2S$)-cNMP (**39**; $K_a' = 1.1$)—exhibited K_a' values that were approximately equal to those for the corresponding N^6-alkyl- and N^6,N^6-dialkyl-cAMP analogues and 8-(alkylthio)-cAMP analogues—N^6-Me-cAMP (**19**; $K_a' = 1.3$), N^6-Et-cAMP (**21**; $K_a' = 0.80$), N^6-*n*-Bu-cAMP (**24**; $K_a' = 1.8$), N^6-(PhH_2C)-cAMP (**32**, $K_a' = 0.82$), 6-(Et_2N)-cNMP (**37**; $K_a' = 1.4.4$), 8-(MeS)-cAMP (**15**; $K_a' = 2.4$), and 8-($PhCH_2S$)-cAMP (**16**; $k_a' = 2.1$). A similar comparison could not be made for the other N^6-alkyl- and N^6,N^6-dialkyl-8-(alkylthio)-cAMP analogues (**23**, **29–31**, **42**, and **43**) because the corresponding 6- and 8-substituted cAMP analogues have not been tested by us as kinase activators. In addition, the N^6-alkyl- and N^6,N^6-dialkyl-8-halo-cAMP analogues—N^6-(PhH_2C)-8-Br-cAMP (**33**; $K_a' = 1.7$) and 6-(Et_2N)-8-Cl-cNMP (**38**; $K_a' = 4.5$)—were found to possess K_a' values in the same range as those for the corresponding monosubstituted analogues—N^6-(PhH_2C)-cAMP (**32**; $K_a' = 0.82$), 6-Et_2-cNMP (**37**; $K_a' = 1.4$), 8-Br-cAMP (**13**; $K_a' = 2.9$), and 8-Cl-cAMP (**12**; $K_a' = 3.1$).

The second group consists of 6,8-disubstituted cAMP analogues with K_a' values approximately the same as those for the corresponding 8-monosubstituted cAMP analogues, where the latter analogues have K_a' values significantly greater than both cAMP and the corresponding 6-monosubstituted analogues. The analogues in this group, in general, are approximately an order of magnitude more potent than cAMP as protein kinase activators. The compounds in this group are N^6-alkyl- and N^6,N^6-dialkyl-8-(arylthio)-cAMP analogues—N^6-*n*-Bu-8-(*p*-ClPhS)-cAMP (**27**; $K_a' = 7.8$) and 6-(Et_2N)-8-(*p*-ClPhS)-cNMP (**40**; $K_a' = 4.0$)—and their K_a' values are approximately equal to the corresponding 8-(arylthio)-cAMP analogue—8-(*p*-ClPhS)-cAMP (**17**; $K_a' = 18$)—and are significantly greater than the corresponding N^6-alkyl- and N^6,N^6-dialkyl-cAMP analogues—N^6-(PhH_2C)-cAMP (**32**; $K_a' = 0.82$) and 6-(Et_2N)-cNMP (**37**; $K_a' = 1.4$). A similar comparison could not be made for the other N^6-alkyl- and N^6,N^6-dialkyl-8-(arylthio)-cAMP analogues (**28**, **36**, and **44**) because the corresponding 6- and 8-substituted cAMP analogues are not available.

The third group consists of 6,8-disubstituted cAMP analogues with K_a' values that are significantly less than that for cAMP and where one of the two corresponding 6- or 8-substituted cAMP analogues has a K_a' value that is similar to that for the 6,8-disubstituted cAMP analogue, while the other of the two corresponding 6- or 8-substituted cAMP analogues is approximately equal in potency to cAMP. For example, a comparison of the K_a' value for 6-HS-8-H_2N-cNMP (**6**; $K_a' = 0.30$) with the K_a' values for the corresponding monosubstituted analogues—6-HS-cNMP (**5**; $K_a' = 0.20$) and 8-H_2N-cAMP (**18**; $K_a' = 1.5$)—indicates that the detrimental effect of the 6-thio substituent on the K_a' value is also observed in the presence of an 8-amino function. In a similar comparison, 6-Cl-8-(Et_2N)-cNMP (**4**; $K_a' = 0.12$) was approximately one order of magnitude less active than cAMP, while 6-Cl-cNMP (**1**; $K_a' = 1.4$) has a K_a' approximately equal to that for cAMP. Even though the corresponding 8-(Et_2N)-cAMP has not been synthesized for comparison, the related 8-(Me_2N)-cAMP has a K_a' of 0.59 (ref 16), suggesting that the lower potency of the 6-Cl-8-(Et_2N)-cAMP (**4**) as a protein kinase activator compared to the other 6-Cl-8-substituted-cNMP analogues (**2** and **3**) may be at least partially a result of the 8-(NEt_2) substituent, although the possiblity of a detrimental effect on the kinase activation by a combination of the 6-Cl and the 8-(NEt_2) functions cannot be ruled out.

The fourth group consists of 6,8-disubstituted cAMP analogues that are significantly less active than cAMP and where both of the corresponding 6- and 8-substituted cAMP analogues are approximately equal in potency to cAMP. For example, the disubstituted 6,8-$(PhCH_2S)_2$-cNMP (**10**; $K_a' = 0.04$) is one to two orders of magnitude less active than either 6-($PhCH_2S$)-cNMP (**9**, $K_a' = 1.9$) or 8-($PhCH_2S$)-cAMP (**16**; $K_a' = 2.1$). Similarly, 6,8-$(Et_2N)_2$-cNMP (**41**; $K_a' = 0.06$) is more than an order of magnitude less active than 6-(Et_2N)-cNMP (**37**; $K_a' = 1.4$). While 8-(Et_2N)-cAMP has not been synthesized, the similar 8-(Me_2N)-cAMP has a K_a' of 0.59 (ref 16), which is 10 times more active than 6,8-$(Et_2N)_2$-cNMP (**41**). It is interesting to note the difference in activity between 6,8-$(PhCH_2S)_2$-cNMP (**10**; $K_a' = 0.04$) and N^6-(PhH_2C)-8-($PhCH_2S$)-cAMP (**35**; $K_a' = 1.4$), where the only difference between these two analogues is the replacement of the 6-NH moiety by a 6-S moiety. The reason for this difference is unknown.

Hydrolysis of 6,8-Disubstituted cAMP Analogues by Rabbit Kidney cAMP Phosphodiesterase. Each of the new 6,8-disubstituted cAMP analogues was examined for its ability to be hydrolyzed to its corresponding 5′-phosphate by a partially purified cAMP phosphodiesterase from rabbit kidney. Most of the 6,8-disubstituted analogues were resistant to enzymatic hydrolysis; those analogues that did undergo hydrolysis at a measurable rate are listed in Table II, along with their relative rates of hydrolysis, α, which is the ratio of the rate at which the analogue was hydrolzyed to the rate at which cAMP was hydrolyzed. The rate of hydrolysis of cAMP was 1.5 μmol of 5′-AMP formed 10 min^{-1} (mg of protein)$^{-1}$ at 30 °C. In addition, the values of α are listed for the corresponding 6- and 8-substituted cAMP analogues.

A comparison of the α values for the 6,8-disubstituted analogues with the α values for the corresponding 6- and 8-substituted cAMP analogues resulted in the assignment of the various disubstituted analogues to one of three general groups. These groups are defined by the relative effect of the double substitution of the α values of the corresponding monosubstituted analogues.

The first group consists of 6,8-disubstituted cAMP analogues that are hydrolyzed at significant rates and that exhibit α values that are approximately the same as those for both the corresponding 6- and 8-substituted cAMP analogues. The analogues with these properties are 6-Cl-8-H_2N-cNMP (**3**; $\alpha = 0.74$) and

6-HS-8-H_2N-cNMP (**6**; α = 0.30); the corresponding monosubstituted analogues— 6-Cl-cNMP (1; α = 1.2), 6-HS-cNMP (5; α = 1.1), and 8-H_2N-cAMP (**18**, α = 0.80)—are all hydrolyzed at rates comparable to that for cAMP. These results show that the rabbit kidney cAMP phosphodiesterase can tolerate the 6-chloro or 6-thio and 8-amino substituents simultaneously.

The second group consists of 6,8-disubstituted cAMP analogues that are hydrolyzed at approximately the same rate as the corresponding 6-substituted cAMP analogues. For example, while 8-(MeS)-cAMP (**15**) is apparently resistant to hydrolysis, the N^6-alkyl-8-(MeS)-cAMP analogues—N^6-*n*-Bu-8-(MeS)-cAMP (**25**, α = 0.26) and N^6-(PhH_2C)-8-(MeS)-cAMP (**34**; α = 0.19)—are hydrolyzed at rates similar to their corresponding N^6-alkyl-cAMP analogues—N^6-*n*-Bu-cAMP (**24**; α = 0.21) and N^6-(PhH_2C)-cAMP (**32**, α = 0.30). Interestingly, this relationship did not hold true for 6,8-$(MeS)_2$-cNMP (8; α < 0.05); even though 6-(MeS)-cNMP (7, α = 0.46) is hydrolyzed at a significant rate, the 6,8-disubstituted analogue is apparently resistant to hydrolysis. A last example of this second group is 6-(Et_2N)-8-Cl-cNMP (**38**; α = 0.10), which is hydrolyzed at the same rate as 6-(Et_2N)-cNMP (**37**; α = 0.09) and where the corresponding 8-substituted analogue—8-Cl-cAMP (**12**)—is apparently resistant to hydrolysis. These data indicate that, while the presence of an 8-(SMe) or 8-Cl substitution eliminates the ability of cAMP to be enzymatically hydrolyzed, the presence of these 8 substituents have no effect on the ability of N^6-alkyl- and N^6,N^6-dialkyl-cAMP analogues to be hydrolyzed by phosphodiesterase.

The third group is made up of 6,8-disubstituted cAMP analogues which, like the corresponding 8-substituted cAMP analogues, are apparently resistant to hydrolysis, although the corresponding 6-substituted cAMP analogues are hydrolyzed at significant rates. Compounds in this group include the 6,8-bis(alkylthio)purine-cNMP analogues (**8** and **10**) and the N^6-alkyl- and N^6,N^6-dialkyl-cAMP analogues containing the following 8 substituents: Br (**33**), SCH_2Ph (**20**, **22**, **26**, **35**, and **39**), or SPh-*p*-Cl (**27** and **40**). All of the other N^6-alkyl-8-($PhCH_2S$)- or N^6-alkyl-8-(*p*-ClPhS)-cAMP analogues (**23**, **28**–**31**, **36**, and **42**–**44**) are also apparently resistant to hydrolysis, but the relative rates of hydrolysis of the corresponding N^6-alkyl-cAMP derivatives are unknown. A last example of this group is 6-Cl-8-HO-cNMP (**2**); in this case, the 8-oxo substitution quite dramatically eliminates the enzymatic hydrolysis of the corresponding 6-Cl-cNMP (1; α = 1.4) by PDE.

Inhibition of cAMP Phosphodiesterases by 6,8-Disubstituted cAMP Analogues. Each of the 6,8-disubstituted cAMP analogues was examined for its ability to inhibit partially purified low K_m cAMP phosphodiesterase preparations from bovine heart and rabbit lung at a substrate (cAMP) concentration of 0.14 μM. The most potent inhibitors were capable of producing 50% inhibition of these phosphodiesterases at concentrations 10 to 50 times greater than that of the cAMP substrate. With both of the phosphodiesterase preparations, the N^6-alkyl-8-(alkylthio)- and N^6-alkyl-8-(arylthio)-cAMP analogues were, in general, equal or better inhibitors than the corresponding 6- or 8-substituted cAMP analogues. Two examples—N^6-Me-8-($PhCH_2S$)-cAMP (**20**) and N^6-(PhH_2C)-8-($PhCH_2S$)-cAMP (**35**)—appear to be exceptions to this generalization. In addition, the data show that, while the 6- or 8-substituted cAMP analogues were, in general, better inhibitors of the heart phosphodiesterase than of the lung phosphodiesterase, the 6,8-disubstituted cAMP analogues were, in general, better inhibitors of the lung phosphodiesterase than of the heart phosphodiesterase. This observation suggests that the larger 6,8-disubstituents of the cAMP analogues may be better able to interact with the cAMP binding site on the lung phosphodiesterase than the corresponding site on the heart phosphodiesterase.

Enzyme Assays. The bovine brain cAMP-dependent protein kinase was purified through the DEAE-cellulose step;[22] it eluted from the DEAE-cellulose at 0.15–0.20 M NaCl and had the properties of a type II cAMP-dependent protein kinase.[23,24,26] Protein kinase assays were performed using the paper-disk method previously described.[16,25]

The assay for the kinase contained in 0.1 mL: 5 μmol of NaOAc, pH 6.0; 1 μmol of $MgCl_2$; 15 μg of histone (Worthington HLY); 0.5 nmol of [γ-^{32}P]ATP (150000 cpm); 3.7 pmol of protein kinase; and various concentrations of the cAMP analogue being tested as an activator (10^{-9}–10^{-3} M). The concentration of the holoenzyme was based on the cAMP binding capacity of each enzyme preparation.[11,26,27] The catalytic activity of the kinase was measured in the presence of a number of concentrations (at least seven) of the cAMP analogue being tested as a stimulator, varied over a least a 100-fold concentration range. The amount of product formed was determined at a number (at least three) of time points (3–12 min) to assure that the data were kinetically valid. The K_a for each analogue was determined from the x intercept (calculated from linear-regression analysis) of a line described by a double-reciprocal plot of the above data (pmoles of phosphate transfered to histone)$^{-1}$ vs. (concentration of cyclic nucleotide analogue)$^{-1}$ (ref 14 and 16). The K_a is a measure of the apparent affinity of the cAMP analogue for the cAMP binding site on the regulatory subunit of protein kinase. Three separate K_a determinations were made for each analogue with each protein kinase. K_a values were considered adequately precise when the results of the three experiments yielded values within 10% of each other. The K_a' value (defined as the ratio of the apparent K_a for cAMP to the apparent K_a for the analogue) was calculated for each analogue using the K_a values for cAMP of 78 nM.

The "high K_m" cAMP phosphodiesterase from rabbit kidney was purified as previously described.[16,25] The "low K_m" cAMP phosphodiesterase from bovine heart and rabbit lung was prepared by gel filtration using the method of Thompson and Appleman[28] and corresponded to the "fraction II" enzymes described by these authors. The reaction mixture for the determination of the rate of hydrolysis of the analogues with the "high K_m" phosphodiesterase contained in 0.60 mL: 2.4 μmol of cyclic nucleotide; 30 μmol of Tris-HCl, pH 7.5; 6 μmol of $MgCl_2$; and phosphodiesterase protein (0.10–0.50 mg). After an appropriate incubation time (10–60 min, previously determined from pilot assays to give kinetically valid results), the reaction was terminated by heating and treated with bacterial alkaline phosphatase, and the phosphate released was assayed colorimetrically as previously described.[16,25] The cyclic nucleotide derivatives had no effect on the alkaline phosphatase step or on the phosphate assay. The α value (defined as the ratio of the rate at which cAMP is hydrolyzed to the rate at which the analogue is hydrolyzed) was calculated for each analogue using the rate of cAMP hydrolysis of 1.5 μmol of 5′-AMP formed 10 min^{-1} (mg of protein)$^{-1}$.

The reaction mixture for the determination of the I_{50} values of the analogues as inhibitors of the "low K_m" phosphodiesterases contained in 0.5 mL: 25 μmol of Tris-HCl, pH 7.5; 5 μmol of $MgCl_2$; 1 μmol of 2-mercaptoethanol; 20–200 μg of phosphodiesterase protein; 80 pmol of [8-^{3}H]cAMP (460000 cpm); and varying concentrations (1–1000 μM) of the cyclic nucleotide analogue being tested as an inhibitor. After an appropriate incubation time (4–20 min, previously determined from pilot assays to give kinetically valid results), the mixture was heat inactivated to terminate the reaction and treated with 5′-nucleotidase and Dowex-1, and the radioactivity in the nucleoside fraction was determined as previously described.[16,25] The cyclic nucleotide analogues had no effect on the nucleotidase step or the Dowex absorption step. At least six different cyclic nucleotide analogue

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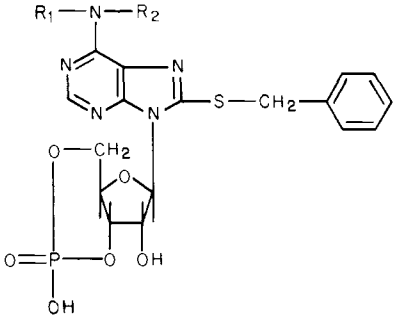
Table III. Inotropic Effect of Intravenous Administration of Certain N^6-Substituted 8-(Benzylthio) 3',5'-Cyclic Nucleotides (3.1 mg/kg) to Methoxyflurane-Anesthetized Dogs



no.	R_1	R_2	no. of animals	dP/dt max, max % change	aortic BP (sys/dias) mmHg change[a]	heart rate, beats/min change[a]
26	-H	-*n*-C_4H_9	4	+84	+2/-5	0
23	-H	-*n*-C_3H_7	1	+127	+6/-19	+24
39	-C_2H_5	-C_2H_5	1	+45	-4/-9	+5
22	-H	-C_2H_5	1	+80	-6/-22	+10
68	-CH_3	-CH_3	1	+61	-8/-25	+15
20	-H	-CH_3	1	+71	0/-6	0

[a] Values obtained at the time of maximal percent change in dP/dt max.

concentrations (in triplicate), varied over at least a 50-fold concentration range, were used for each I_{50} determination.

The I_{50} values were determined graphically by plotting the percent inhibition vs. the log of the inhibitor (cAMP analogue) concentration and reading the 50% inhibitory concentration from the graph. The I_{50} value of theophylline was determined in each experiment as an internal control; I_{50} values for theophylline were consistently 180–200 and 95–110 μM for the rabbit lung and bovine heart, respectively.

Pharmalogical Studies. Inotropic Studies with Cat Papillary Muscle. Method. Cats were anesthetized with ether, their chests were opened, and their hearts were removed and placed into Krebs–Henseleit solution C as described by Thorp and Cobbin.[29] The papillary muscles from the right ventricle were mounted in a tissue holder and placed in a 100-mL bath with Kreb–Henseleit solution at 37 °C. The papillary muscles were stimulated electrically with square wave shocks of 6-ms duration, 10 V and 1/s frequency. Contractile force was recorded through Statham UC3 universal transducing cells on a Hewlett-Packard Type 7700 recording system. Under these conditions, the contractile force of the the papillary muscles in solution C declines rapidly and reaches 25 to 30% of original force within 1.5 to 2 h. At that time, the test drug was introduced into the bath and the contractile force was recorded.

8-(Benzylthio)-6-(*n*-butylamino)-9-(β-D-ribofuranosyl)purine cyclic 3',5'-phosphate (**26**) in vitro showed a significant (23%) increase in cardiotonic activity at 2.5 μg/mL and higher concentrations on isolated cat heart papillary muscle. Its duration exceeded 30 min. Its effect was not blocked by sotalol, a β-adrenergic blocking agent, at 20 μg/mL and was not abolished by pretreatment of cats with reserpine at 0.5 mg/kg ip 18 h prior to the experiment.

Inotropic Studies in Dogs. Methods. Adult mongrel dogs of either sex were anesthetized with sodium methohexital and maintained in a steady state of anesthesia with methoxyflurane. These animals were permitted to breathe spontaneously. Body temperature was maintained at 37 ± 1 °C. Aortic and left ventricular pressures were measured with catheter-tip transducers. In these studies, the heart was electrically paced with a bipolar electrode catheter placed in the right atrium. Lead II ECG, aortic blood pressure, and left ventricular pressure were monitored on an oscilloscope, recorded on a magnetic tape recorder, and subsequently analyzed by an electronic computer. Measurement of dP/dt max of the left ventricular pressure was used as the index of change in myocardial contractility. Spontaneous heart rate was obtained by turning off the electrical pacer periodically. Drug administration was via the femoral vein.

Inspection of Table III shows that a number of N^6-alkyl-8-(benzylthio) derivatives of cAMP exhibit significant inotropic activity in the anesthetized dog. The single N^6,N^6-dialkyl-substituted analogue (**39**) was less active than any of the N^6-alkyl-substituted analogues tested. Although the N^6-*n*-propyl analogue **23** exhibited a great dP/dt maximum, it also increased heart rate by 24%. Since N^6-*n*-butyl-8-(benzylthio)-cAMP (**26**) showed no increase in heart rate when studied in four dogs and yet showed an increase in blood flow rate of 84%, this derivative was selected for detailed study in dogs. A more detailed report of the study of **26** has appeared.[21] N^6-*n*-Butyl-8-(benzylthio)-cAMP (**26**) showed an increase in mesenteric, renal, and carotid arterial flow in dogs at doses as low as 1.0 mg/kg iv. The most interesting aspect of these studies is that the positive inotropic effect observed in dogs was maintained even in the presence of β-receptor blockade.[21] It has been concluded by Imai and co-workers[10c] that dibutyryl-cAMP passes the surface membrane and exerts its inotropic effect after conversion to cAMP within the cell. Thus, like cAMP we postulate that **26** acts within the cardiac cell, unlike the β-receptor agonists such as glucagon which act at the cell-surface membrane to stimulate adenylate cyclase, thus resulting in conversion of ATP to cAMP within the cell.[10d] Thus, it is quite likely that **26** stimulates cardiac protein kinase directly within the cell, resulting in the physiological response of contraction of the cardiac muscle. The selection of the N^6-(alkylamino)-8-(benzylthio)-cAMP derivatives for evaluation in animals was based on a good value for activation of protein kinase ($K_a' = 1.8$; Table II) and a preliminary in vitro screening of a large number of derivatives of cAMP for those compounds which showed specificity of action and were not as broad in their action as cAMP itself. It should be noted that compound **26** like glucagon causes hyperglycemia and release of insulin. No significant changes in plasma sodium or glycerol were seen. Plasma glucose levels peaked in dogs after 20 min and gradually decreased toward control values in 60 min. This is unlike sympathomimetic agents which inhibit insulin release. If compound **26** releases insulin in human subjects as it does in dogs, it would indeed be beneficial to the patient. It should be noted that dibutyryl-cAMP also causes release of insulin from normal subjects.[30] The pharmacological profile of **26** is quite similar to glucagon; however, since glucagon produces its inotropic effect by acting on a membrane receptor site which stimulates adenyl cyclase and generates cAMP and since glucagon loses its inotropic effects in chronic congestive heart failure, there may be many advantages to the use of N^6-*n*-butyl-8-(benzylthio)-cAMP (**26**) which has a more direct effort on the cardiovascular system. For example, it may prove that **26** would be more effective in treating hemorrhagic shock than glucagon, since **26** acts directly on myocardial protein kinase, and a change in membrane responsiveness should not lessen its action. This new type of inotropic agent would beem to have a number of potential advantages over currently available drugs. These data strongly suggest clinical evaluation of **26** as an attractive candidate for the management of acute clinical myocardial failure.

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