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4′-Thioadenosine 3′,5′-Cyclic Phosphate and Derivatives. Chemical Synthesis and Hydrolysis by Phosphodiesterase†

A. K. M. Anisuzzaman, William C. Lake, and Roy L. Whistler*

ABSTRACT: 9-(2,3-*O*-Isopropylidene-4-thio-β-D-ribofuranosyl)-adenine is phosphorylated with cyanoethyl phosphate and the isopropylidene group removed to yield 9-(4-thio-β-D-ribofuranosyl)adenine 5′-phosphate (**1**), which is cyclized to the desired 4′-thioadenosine 3′,5′-cyclic phosphate (**2**). Reaction of **2** with butyric anhydride gives the N^6,2′-*O*-dibutyryl derivative. 6-Chloro-9-(2,3,5-tri-*O*-acetyl-4-thio-β-D-ribofuranosyl)-purine is allowed to react with *n*-butylamine to produce N^6-(*n*-butyl)-9-(4-thio-β-D-ribofuranosyl)adenine (**4**). The 2′,3′-*O*-isopropylidene derivatives of **4** are phosphorylated and hydrolyzed to the N^6-(*n*-butyl) derivative, which is cyclized to give N^6-(*n*-butyl)-s$^{4'}$cAMP. s$^{4'}$cAMP and its derivatives are examined as alternate substrates for cAMP phosphodiesterase from bovine heart muscle. s$^{4'}$cAMP is hydrolyzed at 95% of the rate observed for cAMP with K_m = 56.8 μM and V_m = 2.08 μmol of P_i per mg of protein per minute. Thus, substitution of sulfur as the heteroatom in the D-ribose ring does not affect the binding or hydrolysis of cAMP by phosphodiesterase. The N^6-(*n*-butyl) and N^6,2′-*O*-dibutyryl derivatives are hydrolyzed at a rate of 12 and 0%, respectively, relative to cAMP. All three of the s$^{4'}$cAMP phosphate analogs are effective inhibitors of the action of phosphodiesterase on cAMP.

The plethora of interest in adenosine 3′,5′-cyclic phosphate has induced us to prepare and examine the analog with sulfur replacing the oxygen atom in the D-ribofuranosyl ring. In addition, we have prepared certain derivatives of this sulfur analog which are analogous to other derivatives showing biochemically interesting properties. We have previously shown that sugar analogs (Shankland *et al.*, 1968; Hoffman and Whistler, 1968), nucleoside analogs (Bobek *et al.*, 1970), and certain nucleotide analogs (Hoffman and Whistler, 1970) having sulfur replacing the ring oxygen atom of the sugar have especially interesting and intriguing biological properties among which is low toxicity (Hoffman and Whistler, 1968). These analogs are proving useful tools in elucidating the biochemistry of the naturally occurring compounds (Hellman *et al.*, 1973; Whistler and Lake, 1972; Barnett *et al.*, 1970).

In the present work 9-(2,3-*O*-isopropylidene-4-thio-β-D-ribofuranosyl)adenine (Hoffman and Whistler, 1970) is phosphorylated and the isopropylidene blocking group removed to give 9-(4-thio-β-D-ribofuranosyl)adenine 5′-phosphate (**1**) in 46% yield. Cyclization of **1** to 9-(4-thio-β-D-ribofuranosyl)-adenine 3′,5′-cyclic phosphate (**2**) (s$^{4'}$cAMP) in 63% yield is accomplished by modification of the method of Smith and coworkers (1961). Reaction of s$^{4'}$cAMP with butyric anhydride produces N^6,2′-*O*-dibutyryl-9-(4-thio-β-D-ribofuranosyl)adenine 3′,5′-cyclic phosphate in 50% yield.

6-Chloro-9-(2,3,5-tri-*O*-acetyl-4-thio-β-D-ribofuranosyl)-purine (Bobek *et al.*, 1970) reacts with *n*-butylamine to give N^6-(*n*-butyl)-9-(4-thio-β-D-ribofuranosyl)adenine (**4**) in 63% yield. Compound **4** exhibits a negative Cotton effect consistent with the β-D configuration (Emerson *et al.*, 1966; Nishimura *et al.*, 1968). The 2′,3′-hydroxyl groups of **4** are protected by isopropylidene blocking to give N^6-(*n*-butyl)-9-(2,3-*O*-isopropylidene-4-thio-β-D-ribofuranosyl)adenine (**5**) in 79%

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TABLE I: Physical Properties of 4′-Thio-Cyclic Nucleotides and Nucleosides.

Compound	λ_{max} (nm)	E_{max}	R_F C	D	E
4′-Thioadenosine	259	14,310	0.62	0.85	0.67
s$^{4'}$AMP	259	14,200	0.10	0.06	0.46
s$^{4'}$cAMP	260	14,170	0.47	0.30	0.44
Dibutyryl-s$^{4'}$cAMP	268	17,200	0.81	0.83	0.79
N^6-(*n*-Butyl)-4′-thioadenosine	268	27,900	0.76	0.82	0.89
N^6-(*n*-Butyl)-2′,3′-*O*-isopropylidene-4′-thioadenosine	267	17,050	0.87	0.91	0.96
N^6-(*n*-Butyl)-s$^{4'}$AMP	268	17,300	0.68	0.37	0.53
N^6-(*n*-Butyl)-s$^{4'}$cAMP	268	23,600	0.89	0.75	0.81

yield. **5** is then phosphorylated and hydrolyzed to N^6-(*n*-butyl)-9-(4-thio-β-D-ribofuranosyl)adenine 5′-phosphate (**6**) which is obtained in 41% yield. N^6-(*n*-Butyl-9-(4-thio-β-D-ribofuranosyl)adenine 3′,5′-cyclic phosphate (**7**) is produced in 70% yield by cyclization of **6**.

Experimental Section

Synthetic

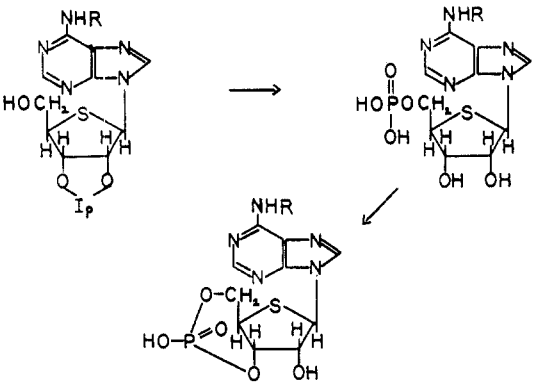
Thin-layer chromatography was performed on silica gel G (Merck Chemical Co.) coated glass plates irrigated with (A) chloroform–acetone (9:2) or (B) chloroform–methanol (6:1). Descending paper chromatography was performed using Whatman No. 1 paper, but for preparative paper chromatography Whatman No. 3 paper was used. Irrigants for paper chromatography were: (C) isopropyl alcohol–concentrated ammonia–water (7:1:2); (D) isopropyl alcohol–0.5 M ammonium acetate (5:2); (E) ethyl alcohol–0.5 M ammonium acetate (5:2).

Solvent ratios are based on volumes and nucleosides and nucleotides were detected by irradiating with ultraviolet light. Values for R_F and λ_{max} are given in Table I.

Optical rotatory dispersions were made with a Bendix Model 460-C spectropolarimeter. The measurements were taken in 1-cm cylindrical cells from Optical Cell Co., Brentwood, Md. The ammonium hydroxide solutions had optical densities between 1 and 2.

9-(4-Thio-β-D-ribofuranosyl)adenine 5′-Phosphate (**1**). A mixture of 470 mg of 9-(2,3-*O*-isopropylidene-4-thio-β-D-ribofuranosyl)adenine (Hoffman and Whistler, 1970), 272 mg of cyanoethyl phosphate (Tener, 1961), and 2.5 g of *N,N′*-dicyclohexylcarbodiimide was stirred at 25° for 4 days. Water (10 ml) was added and the mixture was stirred for 1 hr and evaporated to dryness. The residue was heated with 40 ml of 30% aqueous ammonium hydroxide for 30 min at 60° and concentrated. The residue was heated with 40 ml of 20% aqueous acetic acid for 90 min at 100° and concentrated. The reaction mixture was purified by preparative paper chromatography using irrigant E. s$^{4'}$cAMP was converted to the free acid by passing through a column of Dowex-50 (H^+) resin (see Scheme I); yield, 211 mg. *Anal.* Calcd for $C_{10}H_{14}N_5O_6PS \cdot H_2O$: C, 32.88; H, 4.41; P, 8.48. Found: C, 32.67; H, 4.41; P, 8.55.

SCHEME I



R=H ; $-CH_2CH_2CH_2CH_3$

9-(4-Thio-β-D-ribofuranosyl)adenine 3′,5′-Cyclic Phosphate (**2**). A mixture of 60 mg of 4′-thioadenosine 5′-phosphate, 80 mg of 4-morpholine-*N,N′*-dicyclohexylcarboxamidine (Moffatt and Khorana, 1961), 30 ml of pyridine, and 5 ml of water was concentrated. The residue was dried by co-evaporation two times with 20-ml portions of dry pyridine. It was then dissolved in 250 ml of dry pyridine and added dropwise to a stirred solution of 1.3 g of dicyclohexylcarbodiimide in 125 ml of pyridine at 100°. The mixture was kept at 100° for 6 hr. Pyridine was removed and the dicyclohexylcarbodiimide was extracted from the residue with ether. Ether-insoluble material was suspended in water and the mixture was filtered. The filtrate was evaporated to a syrup which, after purification by preparative paper chromatography in irrigant C, gave the ammonium salt of **2**, which was crystallized from water–ethanol (4:1, v/v) at pH 2.0. The yield was 45 mg (63%) but was only 25% when the cyclization reaction was performed at 80°. *Anal.* Calcd for $C_{10}H_{12}N_5O_5PSNH_3 \cdot 0.5H_2O$: C, 32.35; H, 4.35; N, 22.63; P, 8.34. Found: C, 32.37; H, 4.78; N, 22.10; P, 8.65.

N^6,2′-O-Dibutyryl-9-(4-thio-β-D-ribofuranosyl)adenine 3′,5′-Cyclic Phosphate (**3**). A 40-mg portion of the ammonium salt **2** was converted to the dibutyryl derivative (Falbriard *et al.*, 1967). N^6,2′-*O*-Dibutyryl-s$^{4'}$cAMP was purified by preparative paper chromatography using irrigant D. Segments of the paper containing the dibutyryl derivative were eluted with water and the eluent was adjusted to pH 2 by the addition of Dowex 50 (H^+) resin. Resin was removed by filtration and the filtrate was evaporated to give the crystalline dibutyryl derivative **3** in 50% yield. *Anal.* Calcd for $C_{18}H_{24}N_5O_7PS \cdot H_2O$: P, 6.15. Found: P, 5.85.

N^6-(n-Butyl)-9-(4-thio-β-D-ribofuranosyl)adenine (**4**). To 2 g of 6-chloro-9-(2,3,5-tri-*O*-acetyl-4-thio-β-D-ribofuranosyl)purine (Bobek *et al.*, 1970) was added 100 ml of methanol containing 8.0 g of *n*-butylamine. The reaction mixture was stirred at 50° for 30 min and then at 25° for 18 hr. Progress of the reaction was monitored by tlc with irrigant B. Crystals which separated were removed by filtration and recrystallized from 90% methanol to give the nucleoside **4**; yield, 1.0 g; mp 194°; $[\alpha]^{25}D$ −85.3° (*c* 0.4, Me_2SO). The optical rotatory dispersion (ORD) spectrum of **4** showed a negative Cotton effect: $[\phi]_{292}$ −6780°, $[\phi]_{281}$ 0°, $[\phi]_{270}$ +6026° (*c* 3.6 × 10^{-3}, MeOH). *Anal.* Calcd for $C_{14}H_{21}N_5O_3S \cdot 1.5H_2O$: C, 45.95; H, 6.56; N, 19.10. Found: C, 45.95; H, 6.51; N, 18.87.

N^6-(n-Butyl)-9-(2,3-O-isopropylidene-4-thio-β-D-ribofuranosyl)adenine (**5**). To 500 mg of **4** in 50 ml of dry acetone was

added 0.6 g of *p*-toluenesulfonic acid. After stirring for 7 hr, the reaction mixture was neutralized with solid sodium bicarbonate, filtered, and evaporated. The residue was extracted with chloroform and the extract washed with water, dried (Na_2SO_4), and evaporated to a syrup, which was purified by column chromatography on silica gel using chloroform–acetone (20:1, v/v) as the eluent. The isopropylidene derivative was obtained as a syrup which was homogeneous on tlc (irrigant A); yield, 440 mg. *Anal.* Calcd for $C_{17}H_{25}N_5O_3S$: C, 53.80; H, 6.64. Found: C, 53.37; H, 6.11.

N^6-(*n*-*Butyl*)-9-(*4-thio-β-*D-*ribofuranosyl*)*adenine 5′-Phosphate* (**6**). A mixture of 400 mg of the nucleoside **5** and 226 mg of cyanoethyl phosphate in 15 ml of pyridine and 2 g of dicyclohexylcarbodiimide was stirred at 25° for 4 days. The reaction mixture was then treated as in the preparation of 4′-thioadenosine 5′-phosphate (**1**) to give 182 mg of the free acid of **6**. *Anal.* Calcd for $C_{14}H_{22}N_5O_6SP$: C, 40.11; H, 5.29. Found: C, 40.09; H, 5.52.

N^6-(*n*-*Butyl*)-9-(*4-thio-β-*D-*ribofuranosyl*)*adenine 3′,5′-Cyclic Phosphate* (**7**). The pyridinium salt of **6** (200 mg) and 105 mg of 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine were dissolved in 10 ml of pyridine containing 1 ml of water and the solution was concentrated to dryness. After removal of water by co-evaporation with pyridine, the residue was dissolved in 250 ml of pyridine and added dropwise to a solution of 2 g of dicyclohexylcarbodiimide in 150 ml of pyridine at 100°. The reaction mixture was heated at 100° for 6 hr, and then treated as in the preparation of s$^{4'}$cAMP **2** to give the cyclic nucleotide **7** which was purified by preparative paper chromatography using irrigant C. Segments of the paper containing the cyclic nucleotide **7** were eluted with water to give the ammonium salt of **7**; yield, 101 mg. *Anal.* Calcd for $C_{14}H_{20}N_5O_5SP \cdot NH_3$: P, 7.42. Found: P, 7.01.

Optical Rotatory Dispersion. Comparison of the optical rotatory dispersion of cAMP and s$^{4'}$cAMP gave the following values: cAMP, $[\phi]_{295}$ −1500°, $[\phi]_{275}$ −2800° trough, $[\phi]_{265}$ −1700°, $[\phi]_{260}$ 0°, $[\phi]_{255}$ +1800°; s$^{4'}$cAMP, $[\phi]_{295}$ −1300°, $[\phi]_{279}$ −3600° trough, $[\phi]_{265}$ −400°, $[\phi]_{263}$ 0°; $[\phi]_{260}$ +1000°.

Enzymatic

Adenosine 3′,5′-cyclic phosphoric acid, lyophilized *Crotalus atrox* snake venom, and 3′,5′-cyclic nucleotide phosphodiesterase from beef heart were purchased from Sigma Chemical Co. [8-^{3}H]Adenosine 3′,5′-cyclic phosphate (21.8 Ci/mmol) was obtained from International Chemical and Nuclear Corporation. All other reagents were commercial preparations of A.R. grade.

Two assay systems were used in the measurement of cyclic nucleotide phosphodiesterase activity. The first utilizes the release of inorganic phosphate as a measure of phosphodiesterase activity and was first described by Butcher and Sutherland (1962). Assay was conducted in a volume of 0.9 ml containing 40 μmol of Tris-Cl (pH 7.5), 1 μmol of $MgCl_2$, 1 μmol of 3′,5′-cyclic nucleotide, and the phosphodiesterase preparation. After temperature equilibration at 30° for 3 min, the reaction was initiated by the addition of enzyme. Release of phosphate was linear over the total incubation time of 30 min. After 20 min, 0.1 mg of snake venom in 0.1 ml of 10 mM Tris-Cl (pH 7.5) was added and incubation continued for the remaining 10 min. At the conclusion of the incubation period, 0.1 ml of cold 55% trichloroacetic acid was added to stop the reaction. After centrifugation, a 0.5-ml portion of the supernatant solution was analyzed for inorganic phosphate by the method of Fiske and SubbaRow (1925) modified as described by Butcher and Sutherland (1962). Control values were obtained by replacing the cyclic nucleotide phosphodiesterase solution with water or with a previously boiled enzyme preparation. No differences were noted between the two controls. For kinetic studies various concentrations of the nucleotide were incubated with phosphodiesterase for 20 min, and the reaction stopped by heating in a boiling water bath for 3 min. This was followed by a separate incubation with 0.1 mg of snake venom for 10 min at 30°. Reaction with snake venom was terminated by the addition of 0.1 ml of cold 55% trichloroacetic acid.

A second assay procedure was used to determine the effect of thio cyclic nucleotide analogs on the hydrolysis of cAMP by phosphodiesterase. Assay was in a volume of 0.9 ml containing 40 μmol of Tris-Cl (pH 7.5), 1 μmol of $MgCl_2$, 1 μmol of ^{3}H-labeled cAMP, 0.5 μmol or 1 μmol of the thio analog to be tested, and the enzyme preparation. Reaction was initiated by the addition of enzyme after temperature equilibration at 30° for 3 min. Following a 20-min incubation, 0.1 ml of cold 55% trichloroacetic acid was added to terminate the reaction. Carrier AMP and cAMP were added to the reaction mixture and a 0.1-ml sample of the solution was put on Whatman No. 3 preparative paper for descending chromatography in irrigant C. AMP spots were located by ultraviolet illumination, removed, and counted in a Beckman CPM-100 liquid scintillation counter.

Scintillation fluid contained 0.5% 2,5-diphenyloxazole and 10% naphthalene in dioxane. Control values were obtained by replacing the enzyme with water or pretreatment of the reaction medium with 0.1 ml of cold 55% trichloroacetic acid. No differences were noted between the two controls.

Results

s$^{4'}$cAMP Nucleotides as Substrates for Phosphodiesterase. Cyclic nucleotide phosphodiesterase hydrolyzed the s$^{4'}$cAMP nucleotide monophosphate analogs with the following rates relative to cAMP (100): s$^{4'}$cAMP, 95; *N*^{6}-(*n*-butyl)-s$^{4'}$cAMP, 12; *N*^{6},2′-*O*-dibutyryl-s$^{4'}$cAMP, 0. All of the 4′-thio-5′-monophosphate analogs were hydrolyzed by snake venom.

The affinity of phosphodiesterase toward s$^{4'}$cAMP is shown in Figure 1. As determined graphically by the method of Lineweaver and Burk (1934), the value for K_m was 56.8 μM and that for V_m was 2.08 μmol of P_i released per milligram of protein per minute at pH 7.5 with Tris-Cl buffer.

Effect of s$^{4'}$cAMP Nucleotides on the Hydrolysis of cAMP. To determine the inhibitory activity of the s$^{4'}$cAMP analogs, their effect upon the rate of hydrolysis of [8-^{3}H]adenosine 3′,5′-cyclic phosphate was investigated. In the presence of 1.0 mM s$^{4'}$cAMP, the rate of hydrolysis of cAMP by phosphodiesterase was 56% inhibited. Addition of 1.0 mM *N*^{6},2′-*O*-dibutyryl-4′-s$^{4'}$cAMP or 1.0 mM *N*^{6}-(*n*-butyl)-s$^{4'}$cAMP inhibited the rate of hydrolysis by 53 and 30%, respectively. In the presence of 0.5 mM dibutyryl- or *N*^{6}-(*n*-butyl)-s$^{4'}$cAMP, inhibition was 27 and 10%, respectively.

Discussion

Clarification of the structural requirements for the action of cyclic nucleotide phosphodiesterase on cAMP has been assisted by the preparation of a number of derivatives and analogs of the nucleotide. The majority of these compounds, however, differ in the base moiety. Only two cAMP analogs with modification of the sugar moiety have been prepared. With the synthesis of s$^{4'}$cAMP further investigations on the role of the sugar moiety are possible.

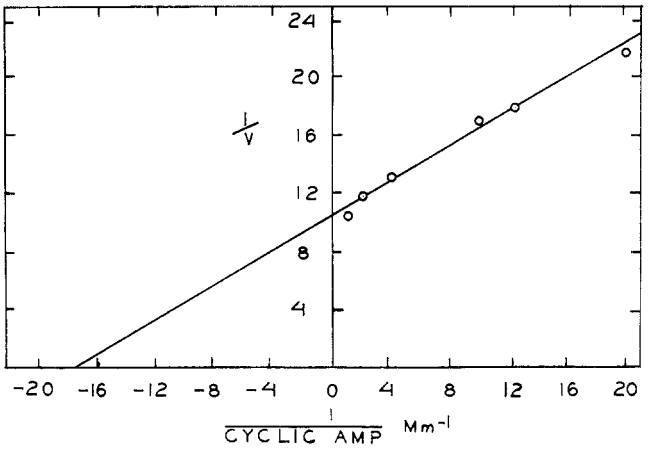


FIGURE 1: Lineweaver–Burk plot of s^4'cAMP hydrolysis by phosphodiesterase. The K_m value is 56.8 μM and the V_{max} is 2.08 μmol of P_i per mg of protein per min.

Several investigators (Nair, 1966; Michal *et al.*, 1970; Song and Cheung, 1971) have reported that cyclic nucleotide phosphodiesterase shows marked preference for purine cyclic nucleotide analogs over pyrimidine cyclic nucleotide analogs. None of these analogs, however, is hydrolyzed at a rate corresponding to the rate of hydrolysis of cAMP. In the case of cGMP, the only cyclic nucleotide found to occur naturally besides cAMP, a separate phosphodiesterase specific for cGMP has been reported (Thompson and Appleman, 1971). Muneyama *et al.* (1971) and Michal *et al.* (1970) have prepared a large number of cAMP derivatives substituted in position 8 of the purine base. All of these derivatives possess very low activities or are inactive as substrates for phosphodiesterase. Some of the derivatives, however, are more effective as activators of bovine brain protein kinase than cAMP (Muneyama *et al.*, 1971). Derivatives of cAMP substituted in position 6 of the purine base also show very low substrate activities (Michal *et al.*, 1970).

Adenosine 3′,5′-cyclic phosphorothioate and two isomeric cyclic phosphonate derivatives were not effective substrates for phosphodiesterase (Drummond and Powell, 1970). Only tubericidin 3′,5′-cyclic phosphate, among the base-modified analogs, has proved to be a more effective substrate than cAMP (Drummond and Powell, 1970).

Two analogs with a modified sugar moiety have previously been tested as substrates for phosphodiesterase. Deoxyadenosine 3′,5′-cyclic phosphate was hydrolyzed at a rate of 130% relative to cAMP (Nair, 1966) while the rate of hydrolysis of D-xylofuranosyladenine 3′,5′-cyclic phosphate was 4% relative to cAMP (Drummond and Powell, 1970). Both of these analogs were ineffective as activators of phosphorylase *b* kinase (Drummond and Powell, 1970).

In the present work, s^4'cAMP is hydrolyzed by phosphodiesterase at 95% of the rate of cAMP. Acylation of s^4'cAMP to give N^6,2′-*O*-dibutyryl-s^4'cAMP makes the nucleotide completely resistant to hydrolysis. Similar inactivity has been reported for the dibutyryl derivative of cAMP. We know of no report concerning N^6-(*n*-butyl)-cAMP as a substrate for phosphodiesterase, but the corresponding N^6-(*n*-butyl)-s^4'cAMP shows low substrate activity with a hydrolysis rate relative to cAMP of 12%.

The high rate of hydrolysis of s^4'cAMP and its Michaelis constant of 56.8 μM, compared to a value of 60 μM for cAMP, indicates that s^4'cAMP is a close analog of cAMP. It appears also that the heteroatom of the D-ribose ring is not an important effector of binding or hydrolysis of the cyclic nucleotide by phosphodiesterase.

Investigations of 4′-thio-D-xylose (Rao *et al.*, 1963) and 5-thio-D-glucose (Suzuki and Whistler, 1972) have shown that substitution of sulfur in the sugar ring has essentially no effect on the conformation of these sugars. This is interesting since replacement of the ring oxygen atom by the much larger but less negative sulfur atom could be expected to affect the conformation. The analog of reduced nicotinamide adenine dinucleotide (NADH) containing a 4′-thioadenosine moiety showed a greater degree of interaction between the bases of the analog than observed in NADH (Hoffman and Whistler, 1970). This was attributed to a restricted rotation of adenine about the C–N glycosyl bond caused by electronic repulsion between the nonbonding electrons of the N^3 and the sulfur ring. Optical rotatory dispersion data indicate also that the adenine rings of 4′-thioadenosine and 4′-thioadenosine 5′-phosphate have a more restricted movement than in adenosine or adenosine 5′-phosphate (Hoffman and Whistler, 1970). Thus, although the substitution of sulfur in the sugar ring does not appear to affect the conformation of the sugar, incorporation of the thio sugar into a nucleoside may affect the conformation of the nucleoside. Comparison of ORD spectra of cAMP and s^4'cAMP indicates that the adenine ring of the thio cyclic nucleotide is also more restricted in its rotation than in cAMP. The restriction in the freedom of rotation is, however, not as great as that observed in 4′-thioadenosine and s^4'cAMP. This is undoubtedly due to the additional rigidity conferred upon the molecule by the presence of the six-membered phosphate ring. This small restriction in the rotation of the adenine ring of s^4'cAMP apparently has no effect upon the hydrolysis of the analog by phosphodiesterase.

The substitution of sulfur in the sugar ring of several nucleosides (Bobek *et al.*, 1970) and in D-glucose (Hoffman and Whistler, 1968; Hellman *et al.*, 1973; Whistler and Lake, 1972) has been shown to confer upon the analogs' unusual biological properties, different from those of the naturally occurring compounds.

All three of the s^4'cAMP analogs are effective inhibitors of the action of phosphodiesterase on cAMP and may, thus, prove to be useful antagonists against the cellular degradation of cAMP by phosphodiesterase. This inhibition may be useful in prolonging the action of cAMP in biological systems. In addition, the close similarity between s^4'cAMP and cAMP may allow the sulfur analog to mimic or substitute for the naturally occurring nucleotide in biological processes.

Investigations are currently under way to further define the biochemical properties of these analogs.

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Kinetics of Phenylalanine Hydroxylase with Analogs of Tetrahydrobiopterin†

J. E. Ayling,* G. R. Boehm, S. C. Textor, and R. A. Pirson

ABSTRACT: Phenylalanine hydroxylase catalyzes the conversion of phenylalanine to tyrosine using molecular oxygen and a tetrareduced pteridine, tetrahydrobiopterin. In order to clarify the mode of interaction of tetrahydrobiopterin in this hydroxylation, the effects of ten differently substituted tetrahydropteridines on the action of partially purified rat liver phenylalanine hydroxylase were determined. Activity was assayed (1) by a specific spectrophotometric assay in which the phenylalanine-dependent oxidation of the pteridine cofactor is monitored at 330 nm (Ayling, J. E., Pirson, R., Pirson, W., and Boehm, G. (1973), *Anal. Biochem. 51*, 80), and (2) from the amount of tyrosine formed in the presence of dithiothreitol as a cofactor regenerating system. No activity or inhibition was found with any nonreduced pteridines, or with 2,4-diketo-6,7-dimethyltetrahydropteridine (H_4Pt), 2-amino-4-keto-6-carboxyl-H_4Pt, or tetrahydroaminopterin. Four of the tetrareduced pteridines were inhibitory. In order of increasing inhibitory potency these were 4-amino-, 4-keto-, 4-keto-6,7-dimethyl-, and 2-amino-4-keto-6,7-diphenyl-H_4Pt. The inhibition with respect to cofactor was mixed for the first two and competitive for the last two inhibitors. All showed mixed inhibition with respect to phenylalanine, except 4-keto-6,7-dimethyl-H_4Pt, which was noncompetitive. Tetrahydropteridines active as cofactors were 2-amino-4-keto-, 2-amino-4-keto-6,7-dimethyl-, 2,4-diamino-6,7-dimethyl-H_4Pt, and tetrahydrobiopterin, in order of increasing apparent maximum velocity. Except for 2-amino-4-keto-H_4Pt, each reacted stoichiometrically with phenylalanine to form tyrosine. With 2-amino-4-keto-H_4Pt only about half the predicted amount of tyrosine was formed. These results indicate that a 2-amino group, or possibly a similar electron donor, is essential for cofactor activity. At the 4 position a keto or an amino group produces an equally effective cofactor. The affinity of tetrahydropteridines for phenylalanine hydroxylase is affected by the substituent at the 6 position. With a methyl or a hydrogen at this position the affinity is less than that of tetrahydrobiopterin, which has a dihydroxypropyl, and with a carboxyl or *p*-aminobenzoylglutamate (as in aminopterin) there is no binding at all. In addition, a substituent at the 6 position appears to be necessary for tetrahydropteridines to bind in a specific manner at the active site, since in the absence of a 6 substituent inhibition is mixed, rather than competitive, with inhibitory compounds, and for pteridines with cofactor activity the normal stoichiometry is not observed. The inhibition observed with 2-amino-4-keto-6,7-diphenyl-H_4Pt suggests that the position of phenylalanine in the complex is adjacent to the tetrahydrobiopterin 7 position.

Phenylalanine hydroxylase, in catalyzing the hydroxylation of phenylalanine to tyrosine, utilizes molecular oxygen and a reducing cofactor, tetrahydrobiopterin. The immediate pteridine product of the reaction is unstable, and, in the absence of NADH and dihydropteridine reductase, or a nonenzymatic regenerating system, stabilizes to 7,8-dihydrobiopterin. The autoxidation of tetrahydrobiopterin to 7,8-dihydrobiopterin

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