Synthesis and properties of some cyclic AMP alkyl phosphotriesters

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ABSTRACT

Cyclic AMP was converted to its phosphotriesters according to the classical approach of phosphate activation with a sulfonyl chloride, followed by esterification with an alcohol. The methyl, ethyl, propyl, butyl and cetyl triesters were prepared, and some of their physical-chemical properties determined. Alkaline hydrolysis of these alkyl phosphotriesters resulted predominantly in ring opening. On the other hand, nucleophilic attack by thiourea led to the formation of cAMP as the main product. The conclusion can be drawn from these results that cAMP phosphotriesters could serve as suitable storage forms of cAMP, and cyclic triesters may be the best vehicle of transporting nucleotides through biological membranes.

INTRODUCTION

The central role of cAMP in the regulation of several metabolic processes has made this compound the target of drug design. The first chemical work on cAMP was directed towards producing metabolically stable precursors, the best of which is dibu-cAMP.¹⁺ Later a large number of analogs were synthesized,² representing modifications of the carbohydrate, the base and phosphate moieties, in the hope that these compounds could mimic cAMP action. Indeed, the *in vitro* performance of some analogs, particularly, the 8-substituted ones, compares favorably with cAMP. One drawback remains, however, with both precursors and analogs: their uptake into the cells is inefficient owing to the negative charge. Because of the rapid hydrolysis by diesterases, large concentrations of cAMP derivatives have to be employed in many studies, thus complicating the evaluation of results.

It seems logical that neutralization of the phosphate moiety would produce a more stable derivative with a better chance to penetrate cellular membranes. Efforts along these lines have been scarce, or remained regret-

+ Abbreviations: dibu-cAMP, 2',N⁶-dibutyryl-adenosine-3':5'-phosphate; Me-cAMP, Et-cAMP, Pr-cAMP, Bu-cAMP are the corresponding P-O-alkyl triesters of cAMP; Me-diac-cAMP, methyl ester of 2',N⁶-diacetyl cAMP.

tably unpublished, although announcements on the synthesis of methyl and ethyl phosphotriesters were made.^{1,3} Neutral phosphoramidates of cAMP proved to be too stable to chemical hydrolysis at physiological pH.⁴ In contrast cyclic phosphotriesters are known to undergo general-base catalyzed hydrolysis, but the dominant reaction is ring opening.⁵

The first description of a nucleoside 3':5'-cyclic phosphotriester dealt with the chemically simpler and biologically inactive 2'-deoxythymidine derivatives.⁶ We have recently reported on the synthesis and some properties of Me-diac-cAMP and Et-diac-cAMP.⁷ The present paper is concerned with the synthesis and chemical behavior of methyl, ethyl, propyl, butyl, and cetyl triesters of unprotected cAMP.

R: a, METHYL; b, ETHYL; c, PROPYL;
d, BUTYL; e, CETYL

RESULTS

We found that cAMP could be converted to its phosphotriesters according to the classical approach of phosphate activation in anhydrous pyridine solution, followed by esterification with a suitable alcohol.^{8,9} Several sulfonyl chlorides were explored as activating agents. The use of the more reactive methane- and ethanesulfonyl chloride had the principal advantage of easy product purification. They reacted too fast with methanol and ethanol, and also with the 2'-OH group, but the higher alcohols gave better yields. With the use of p-toluenesulfonyl chloride and, especially, triisopropylbenzenesulfonyl chloride in the cold, the reactive intermediate (I) could be generated without much side reaction prior to the addition of the alcohol. The yields were better, above 50%, but the separation of the sulfonates and sulfonyl esters required repeated chromatography on silica gel and Sephadex columns. Under no conditions could the triesterification

of cAMP be carried out to completion even with renewed addition of activating agent and excess alcohol. In addition to unreacted cAMP, one observed the formation of a more polar compound, apparently zwitter-ionic, which has not yet been characterized. This compound probably derives directly from the mixed anhydride I because the triesters II are relatively stable under these conditions. Some cAMP persists because the sulfur is quite susceptible to nucleophilic attack, and sulfonyl esters could be isolated in the first fraction of silica gel column chromatography.

Of the five triesters (IIa-IIe) prepared, only the ethyl triester has been crystallized so far. This compound also gave the best elementary analysis while the others contained a few percent inorganic impurities from the silica gel and some solvent. Further proof of structure rests on the following facts.

1. All compounds behaved neutral on electrophoresis at pH 4.5 and 7.5 and were not bound to ion-exchange resins.
2. The NMR spectra reveal the presence of the base protons, the very characteristic 1' proton¹⁰ and the respective alkyl protons. Spin-spin coupling of triesterified phosphorus¹¹ to the α protons is clearly apparent in the methyl ester.
3. Hydrolysis of triesters gave diesters and AMP.

The outstanding physical-chemical property of triesterified cAMP's IIa - IIe is their good solubility in organic solvents such as alcohol and acetone. While the methyl and ethyl compounds exhibited good solubility in water (> 50 mg/ml), the butyl ester was almost completely insoluble. The cetyl ester formed a turbid, soap-like colloid aggregate in water which was quantitatively retained on millipore filter. Et-cAMP was also shown to be capable of micellar associations; in the presence of 0.05 M Na-laurate its elution volume from a Sephadex G-25 column was near $2 \times V_0$ while in distilled water V_e was three times higher. Some binding also takes place with the weakly surface active triisopropylbenzene sulfonate ion in course of purification.

The chemical reactivity of cAMP phosphotriesters is determined by the presence of a highly electrophilic phosphorus and two electrophilic carbon atoms. The stereochemistry of hydrolysis is of particular interest both from a pure chemical point of view and for the possible biological usefulness of these cAMP derivatives. The questions arise whether hydrolysis takes place under P-O or C-O cleavage, and which one of the possible linkages participates in the hydrolysis. In course of our preliminary investi-

gation on the alkaline hydrolysis of Me-diac-cAMP we observed the formation of one diester fraction which was completely cleaved by beef heart phosphodiesterase.⁷ The conclusion that only exocyclic cleavage took place was now found to be in error. By an improved TLC separation it was possible to isolate methyl AMP as the major product which, however slowly, was also hydrolysed by the enzyme. Synthetic methyl 2'(3')-AMP proved to be quite labile to alkali giving rise to 2'(3')-AMP following the intermediary formation of 2':3'-cAMP which also complicates the evaluation. We determined the relative amounts of cyclic and alkyl AMP's following a short exposure of triesters to 0.3 N KOH at 20°, i.e., under circumstances when negligible amounts of AMP were formed. Even in this case, the cAMP fraction consisted chiefly of 2':3'-cAMP, and the 3':5'-cAMP content had to be determined by the protein kinase assay.¹² These experiments are summarized in Table I. It can be seen that the times necessary for 50% hydrolysis were less than 10 min. The percentage of exocyclic hydrolysis (cAMP/total diester) is very low, 3 to 5%.

Table I. Hydrolysis of cAMP phosphotriesters

Products ^a	Percentages			
	Me-cAMP	Et-cAMP	Pr-cAMP	Bu-cAMP
triester	4	25	28	
alkyl AMP's	82	62	58	
cAMP's	14	13	14	
% exo ^a		3.0	4.5	4.6
% exo ^b		44	26	

^a Alkaline hydrolysis of cAMP alkyl phosphotriesters. One μ mole of triester was treated with 0.2 ml of 0.3 N KOH at 20° for 10 min. and the products analyzed as described in Methods. ^b Treatment with thiourea.

From the biological point of view, the specific base catalysis of hydrolysis is less important than a general nucleophilic catalysis. For this reason we measured the action of thiourea on the ethyl and propyl esters at pH 7.0; methyl and butyl esters were also used in a few qualitative experiments. The disappearance of triester in this case probably was due to C-O cleavage, i.e., S-alkylation reactions, which could involve both the exocyclic and the 5' carbons. In the case of IIa and IIc two diesters were observed; one of them migrated like cAMP. On the other hand, the treatment of the ethyl triester yielded only one diester which on TLC be-

haved identically to cAMP. To eliminate the uncertainty inherent in chromatographic identifications, we have also identified cAMP by the more characteristic protein kinase assay.¹² According to this method, all triester hydrolysates contain cAMP in much higher quantities (25 to 50%) than in the KOH hydrolysis.

In light of these facile S-alkylation reactions, one expected that intermolecular and/or intramolecular N-alkylations would also take place. We have previously shown that 2':3'-cyclic triesters undergo intramolecular rearrangement accompanied by ring opening.¹³ It is surprising that Et-cAMP was found stable in dimethylformamide at 70° for 3 hr. The stereo model of cAMP as revealed by X-ray crystallography¹⁴ suggests that the 5' carbon in its trans-gauche conformation may not be available for intramolecular N-alkylation.

DISCUSSION

Several cyclic triesters of phosphoric acid have been prepared and studied by Westheimer and associates¹⁵ in course of their classical work on phosphate ester hydrolysis. The general conclusion that cyclic phosphotriesters are more labile than acyclic ones allows for a wide range of difference between the various ring structures with regard to general-base-catalyzed hydrolysis. In light of the fact that nucleoside 2':3'-cyclic phosphate triesters¹⁶ are too labile to be isolated, and the enthalpy of hydrolysis of 3':5'-cAMP was greater than that of 2':3'-cAMP¹⁷, the relative stability of 3':5'-cAMP triesters was somewhat unexpected. In absence of acidic, general-base and micellar catalysis, these triesters are stable in dilute solution at room temperature for days. Although detailed kinetic studies have yet to be done, the alkaline hydrolysis of IIa, IIb and IIc seems comparable in a semi-quantitative sense to the hydrolysis of methyl ethylene cyclic phosphate.⁵ In both instances, the relief of ring strain through ring opening is the dominant reaction, but some exocyclic hydrolysis is also taking place. We have not tried to assess the proportions of 3' and 5' alkyl AMP's but only a very small fraction of the diester was stable to alkali. cAMP itself yielded predominantly 3'-AMP.¹⁸

Experiments with thiourea were conducted as a model system which could approximate the nucleophilic catalysis by sulfhydryl groups in biological media. It is an interesting finding that in IIa and IIb the methyl and ethyl groups, are primarily removed, while the sterically more hindered IIc and IId also undergo ring opening. These experiments and results are of utmost importance for our chief concern of drug development aiming at a

suitable storage form of cAMP. They also provide the basis for transporting other biologically potent nucleotides into the mammalian cells via the triesters as a vehicle.⁹

At present one obstacle stands in the way of exact kinetic experiments. Since the synthesis is not expected to be stereospecific, one deals with an undetermined mixture of two diastereomers, endo and exo, which differ only in configuration at the phosphorus. The ratio of two isomers may well depend on the size of alkyl groups, and, therefore, no accurate comparison can be drawn. The two diastereomers may have different reactivity both on carbons and phosphorus. Et-cAMP in crystals exists in the endo form as it was determined by X-ray crystallography (F.A. Cotton, E.E. Hazen, Jr., A.G. Stanislawski and P.W. Tucker, unpublished).

In spite of some uncertainties of rate determination, these experiments illustrate the great reactivity of this novel ring system under relatively mild conditions. One can predict that similar reactions i.e., cAMP formation and S-alkylation may also take place in vivo. The biological activities of some of these triesters⁷ which exceed those of exogenously applied cAMP are indicative that such reactions are indeed taking place. The capacity of cAMP phosphotriesters to serve as a storage form of cAMP, coupled with increased membrane penetration and lipid solubility, opens up the best prospect for a rational chemotherapy of proliferative skin diseases and some tumors.¹⁹ The effects of these triesters as enzyme inhibitors and their action on some solid tumors will be published shortly.

EXPERIMENTAL

General methods

All synthetic operations were carried out under rigorously anhydrous conditions. cAMP was dried by six evaporations from anhydrous pyridine, all evaporations were done in vacuo at temperatures below 25°. Elementary analyses were conducted by M-H-W Laboratories.

Analytical scale separations by thin layer chromatography (TLC) were performed on Eastman Chromagram sheets with fluorescent indicator in the solvents 'A': toluene-ethanol (85:15), and 'B': 1-butanol-0.5 M ammonium acetate - 95% ethanol (72:16:12). For preparative separations, the silica gel (Davison Chem. Co., 60-200 mesh) was deactivated by washing with dil. $\text{Et}_3\text{NH}\cdot\text{HCO}_3$ and acetone.

NMR spectrum of Me-cAMP was recorded by a Varian HA-100 spectrometer at ambient temperature in D_2O -acetone- d_6 1:1 with TMS as external standard. The spectra of the other triesters were obtained by a Varian T-60 instrument in D_2O -acetone- d_6 1:9. UV spectra were taken by Gilford or Cary 15 spectro-

photometers.

For enzymatic degradation, cyclic nucleotide phosphodiesterase from beef heart (Sigma), 0.05 units, was added to 0.5 μ mole of nucleotide in 0.2 ml of 0.04 M Tris·HCl-0.02 M MgSO_4 , pH 7.5, and the solution incubated at 37° for 16 hr. The result was evaluated by TLC in solvent 'B'.

Cyclic AMP was determined using the binding assay of Gilman¹² in a volume of 200 μ l using beef heart protein kinase purified by the method of Kuo and Greengard.²⁰ The magnesium-containing buffer of Erlichman et al.²¹ was used.

Alkaline hydrolysis of triesters.

Approximately 1 μ mole (15 O.D.²⁶⁰ units) quantities of triesters were dissolved in 0.2 ml 0.3 N KOH at 20°. They were neutralized after 10 min. with Dowex 50 resin (pyridinium form) and applied to TLC. The chromatograms were developed in solvent B using appropriate standards. The following R_f values were measured: cAMP 0.35, Me-AMP 0.40, Et-AMP 0.42, Pr-AMP 0.45, Bu-AMP 0.50. The bands were eluted with 50% ethanol, and the amounts of UV absorbing materials were determined. The quantitation of cAMP was difficult because of its small percentage and poor separation relative to alkyl AMP, and duplicates varied by 20%. Because of the uncertainty of the characterization by UV, the total diester fraction was also subjected to the biochemical binding assay (values listed in Table I). A similar treatment of 2'(3') propyl AMP resulted in the formation of some 2':3'-AMP.

Reaction of thiourea with triesters.

Solutions containing 15 O.D.²⁶⁰ units of the triester (IIa, IIb, and IIc) and 10 mg thiourea in 0.2 ml 50% ethanol-0.05 M Tris·HCl, pH 7.0 were incubated at 40° for 16 hr. The products were separated by TLC in solvent B, eluted with water and subjected to Gilman's binding assay for cAMP. About 20 to 30% of the triesters reacted with thiourea, while in the absence of thiourea only IIa hydrolysed to a small extent. In case of IIa, two bands were found similar to cAMP, but only the major band responded positive in the binding assay, although both compounds were degraded by phosphodiesterase. In the case of IIb, only one diester was observed on TLC and it gave positive binding test. In contrast, IIc produced somewhat less cAMP; the other product remained unidentified. In parallel experiments, the diester products were eluted from 0.5 x 4 cm DEAE cellulose column with 0.05 M triethylammonium bicarbonate and subjected to the cAMP binding assay.

Materials

cAMP¹⁸ and diac-cAMP¹ were prepared by the established procedures, and the Bu₃N⁺H salts were obtained via ion exchange through Dowex 50 resin. 2'(3')- Alkyl AMP's were synthesized according to Michelson.²²

Triesterification, general procedure

cAMP, Bu₃N⁺H salt, 1.00 g (28,000 O.D.₂₆₀ units, 1.92 mmoles) was rendered anhydrous by six consecutive evaporations in vacuo of its solution in dry pyridine (20 ml each); finally it was dissolved in a mixture of 5 ml pyridine and 0.9 ml tri-n-butylamine. To this solution 0.730 g (3.84 mmoles) of p-toluenesulfonyl chloride in 2 ml pyridine was added at 0°, followed after a period of 2 min. by the addition of 8 mmoles of the anhydrous alcohol. The reaction mixture was allowed to stand at 5° for 30 min., whereafter its volume was reduced in vacuo by one third for removal of the alcohol. The reaction cycle was repeated once more using 1.09 g (5.76 mmole) of p-toluenesulfonyl chloride in 5 ml pyridine, 1.2 ml tri-n-butylamine, and 12 mmoles of alcohol with the temperature rising from 0° to 20° during 30 min. The progress of the reaction was determined by thin layer chromatography, and a third activation cycle was employed if the triester to cAMP ratio was smaller than 2. Otherwise, the reaction was terminated by pouring a great excess of alcohol into the flask. The solution was evaporated to a gum which was extracted twice with toluene - 1% Et₃N (20 ml total). The combined extracts, containing mostly sulfonyl ester with traces of triester, were passed into a silica gel column (50 x 2 cm). The remaining gum was mixed with 10 g silica gel with the help of toluene-ethanol 1:1, the mixture was dried and loaded onto the same column. The column was washed first with 100 ml of toluene, which removed 90% of sulfonate esters, followed by elution with 200 ml toluene-20% ethanol and 800 ml of toluene-30% ethanol. The appearance of the triester was observed on thin layer chromatography developed in the same solvent. The triesters were eluted with the toluene-ethanol mixtures at V_e = 200 to 700 ml, but they were generally contaminated with sulfonate ions. Therefore, this fraction was evaporated and the material was passed through an Amberlite CG-400 column (45x2 cm, C1 form) using 50% pyridine-H₂O as eluant. Desalting could be best achieved by passing the concentrated solution through a Sephadex G-25 column (2 x 100 cm) in distilled water. Owing to the poor solubility of the triesters, their elution volume is significantly higher (V_e > 500 ml) than those of the contaminants. The methyl and ethyl esters could be eluted with water, but the propyl and butyl esters required a

change to 20% alcohol following the passage of 400 ml water. After freeze-drying, white amorphous powder was obtained (yield 15 to 50%) which was once more freeze-dried from dioxane and dried over P_2O_5 for analysis. Triesterifications were also carried out with methanesulfonyl chloride and triisopropylbenzenesulfonyl chloride as the activating agents.

Me-diac-cAMP

The above process was employed with methanesulfonyl chloride as the activating agent. Isolated yield 15%; white powder which could not be crystallized. $\lambda_{\max}(H_2O)$ 273 nm. NMR spectrum: 8.60 (s, 1, H_8), 8.43 (s, 1, H_2), 6.23 (s, 1, H-1'), 4.01 (d, 3, OCH_3 , $J_{H-P} = 17$ cps), 2.21 ppm (s, 3, CH_3-CO-N). The *O*-acetyl group was partially lost during purification. Anal. for $C_{15}H_{18}N_5O_8P \cdot 3 H_2O$ (480.51) calcd C 37.50, H 4.83, N 14.58, P 6.45% found C 37.77, H 4.42, N 15.48, P 6.34.

Me-cAMP

Best yield (33%) was obtained with triisopropylbenzenesulfonyl chloride. Amorphous powder, labile at room temperature, stable in frozen solution. NMR spectrum: 8.58 (s, 1, H_8), 8.51 (s, 1, H_2), 6.51 (d, 1, $J = 0.5$ cps, H-1'), 4.37 (d, 3, $J = 11$ cps, OCH_3). Anal. for $C_{11}H_{14}N_5O_6P \cdot H_2O + SiO_2 + 1/2 C_4H_8O_2$ (dioxane), MW = 465.39, calcd C 33.55, H 4.33, N 15.05, P 6.65; found C 34.02, H 4.17, N 14.75, P 6.58%. Thin layer chromatography $R_f(A)$ 0.15, $R_f(B)$ 0.63.

Et-cAMP

This compound was prepared exactly as described in the general procedure and it could be crystallized by slow evaporation of water at $+5^\circ$. Best yield 45%. $\lambda_{\max}(H_2O)$ 259 nm, $\epsilon(P)$ 15,200. Anal. for $C_{12}H_{16}N_5O_6P \cdot H_2O$ (357.27), calcd C 38.41, H 4.83, N 18.66, P 8.25; found C 37.95, H 4.39, N 17.94, P 8.13. Thin layer chromatography: $R_f(A)$ 0.17, $R_f(B)$ 0.70.

Pr-cAMP was obtained by the general procedure as an amorphous powder (22% yield). NMR spectrum exhibits the signals characteristic of cAMP and the *O*-propyl group. Anal. for $C_{13}H_{18}N_5O_6P + H_2O + 1/2 SiO_2$ (419.35), calcd C 37.23, H 4.81, N 16.70, P 7.38; found C 37.05, H 4.59, N 14.77, P 6.65. Thin layer chromatography: $R_f(A)$ 0.20.

Bu-cAMP

Methanesulfonyl chloride was found preferable as activating agent over triisopropylbenzenesulfonyl chloride for reasons of purification when following the general procedure. Initial yield of 65% dropped to 20% after repeated chromatography on silica gel which was necessary to separate

some 2'-O-mesyl derivative. NMR spectrum contains the signals of cAMP and various multiplets of the n-butyl group. Anal. for $C_{14}H_{20}N_5O_6P + 2H_2O$ (385.32), calcd C 39.91, H 5.74, N 16.62, P 7.35; found C 40.29, H 5.34, N 14.73, P 6.97; $\epsilon(P)$ 14,300. Thin layer chromatography: $R_f(A)$ 0.22.

Cetyl-cAMP was prepared once in small yield (5%). It was purified on Sephadex LH-20 column (3 x 76 cm) using 2-propanol as eluant (V_e 900 ml). The gum-like material was homogenous on thin layer chromatography: $R_f(A)$ 0.12. It formed a turbid soapy solution in water from which optically pure water was obtain by filtration through millipore.

Cyanoethyl-cAMP could not be prepared without the presence at least 10% cAMP.

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