Synthesis, Structure, and Reactivity of Adenosine Cyclic 3',5'-Phosphate Benzyl Triesters

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A series of triesters of adenosine cyclic 3',5'-phosphate was synthesized by treatment of the free acid with various diazoalkanes (R = H, CH_3, C_6H_5, o-$NO_2C_6H_4$, p-$NO_2C_6H_4$, p-$CH_3C_6H_4$). The resulting diastereomeric mixtures were separated into their axial and equatorial components. Hydrolysis of the compounds was examined as well as photolysis of the photolabile o-nitrobenzyl ester. All compounds were then tested for their ability to activate the cAMP-dependent protein kinase and for their ability to serve as a substrate for the cAMP phosphodiesterase showing almost no effect on either enzyme. In a biological assay the benzyl triesters were able to penetrate into C 6 rat glioma cells and to induce the typical morphological alteration of the cell shape known for high cellular levels of cAMP. It was concluded that the benzyl triesters of cAMP are useful derivatives which can be efficiently and specifically converted to the parent nucleotide. Benzyl derivatives of biologically active phosphodiesters may provide a useful tool for study in biology and pharmacology.

Adenosine cyclic 3',5'-phosphate (cAMP) plays an important role in regulating a variety of cellular processes in eukaryotic organisms.[1] In order to test cellular response to internal cAMP, one has to overcome the low cell membrane penetrating ability of exogenously added cAMP.[2] This can be accomplished by employing derivatives of cAMP[3] which possess an enhanced lipophilicity and are neutralized in the phosphate moiety in an approach similar to Gohil et al.[4] It was our aim to use such synthesized triesters of cAMP as a transport form to penetrate the cell membrane. Once inside the cell, the derivatives must be easily convertible to the parent cAMP, be it through hydrolysis, photolysis, or enzymatic degradation. Thus we investigated the triesters' reactivity toward hydrolysis and, in the case of the photolabile o-nitrobenzyl ester, toward photolysis. Because two of the most well-known enzymes in cAMP studies are phosphodiesterase,[5] which degrades cAMP, and protein kinase,[6] which is activated by cAMP, we tested our triesters for their ability to interact with these enzymes. Finally, the triesters were examined for their ability to penetrate into intact cells and fulfill the requirements they were constructed for.

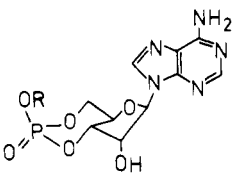
Results and Discussion

Six different triesters of cAMP were prepared according to the simple and straightforward method of Todd et al.[7] through direct treatment of the free acid with diazoalkanes as shown in Table I.

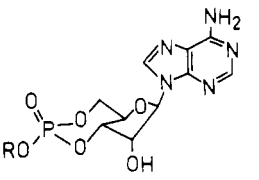
This is a one-step reaction which is easily worked up by chromatography over silica gel to give analytically pure products. The resulting diastereomeric mixtures can be chromatographically separated into the axial (ax) and equatorial (eq) isomers of the underlying dioxaphosphorinan ring. According to an x-ray analysis of the ethyl ester[8] and of the benzyl ester of uridine cyclic 3',5'-monophosphate,[9] the absolute configuration was established. On the basis of the ^{31}P NMR shift differences of all the triesters a structural assignment was possible, since all axial conformers appeared at a higher field relative to 85% H_3PO_4 than the equatorial ones.[10] Although preferential formation of the eq isomer might be expected, the so-called gauche effect[11] leads in this case to predominance of the thermodynamically more stable ax isomer.

The possible differences in reactivity of the two diastereoisomers necessitated their separation before attempting exact kinetic experiments. Once they were separated, the kinetics of their hydrolysis could be investigated. Alkaline hydrolysis of the benzyl ester in 0.1 N NaOH at 37 °C led mainly to cAMP mixed with ring-opened products, while neutral hydrolysis and acidic hydrolysis in 0.1 N HCl gave rise only to cAMP.

Table I. Physical Properties of Adenosine Cyclic 3',5'-Phosphate Triesters



ax



eq

R	λ max, nm[a] (ε × 10^3)	R_f (A)[b]	R_f (B)[b]	^{31}P NMR[c] (δ)
CH_3 [1 (ax + eq)]	258 (11.5)	0.33	0.18	+3.43/ 2.47
C_2H_5 [2 (ax)]	258 (12.1)	0.40	0.30	+6.14
C_2H_5 [2 (eq)]	258 (11.9)	0.35	0.19	+4.43
$CH_2C_6H_5$ [3 (ax)]	258 (12.0)	0.46	0.39	+6.03
$CH_2C_6H_5$ [3 (eq)]	258 (12.7)	0.35	0.25	+4.46
$CH_2C_6H_4$-o-NO_2 [4 (ax)]	258 (16.9)	0.44	0.39	+6.37
$CH_2C_6H_4$-o-NO_2 [4 (eq)]	258 (17.2)	0.39	0.26	+4.87
$CH_2C_6H_4$-p-NO_2 [5 (ax)]	258 (21.2)	0.43	0.34	+6.00
$CH_2C_6H_4$-p-NO_2 [5 (eq)]	258 (20.9)	0.37	0.25	+4.49
$CH_2C_6H_4$-p-CH_3 [6 (ax)]	258 (11.5)	0.45	0.26	+5.61

[a] UV (methanol). [b] A, $CHCl_3$-MeOH (6:1 v/v); B, Cl-CH_2CH_2Cl-EtOH (5:1 v/v). [c] Relative to 85% H_3PO_4 in Me_2SO-d_6.

Table II. Half-Life in Hours of cAMP Triesters at 50 °C in H_2O

Triester	Ax isomer	Eq isomer
$CH_2C_6H_4$-p-CH_3	0.1	a
$CH_2C_6H_5$	2.2	0.5
$CH_2C_6H_4$-o-NO_2	300	83
$CH_2C_6H_4$-p-NO_2	240	73

[a] Too unstable to be measured.

Hydrolysis of all triesters was then carried out in water at 50 °C and observed using liquid chromatography (LC). Kinetic considerations resulted in a pseudo-first-order reaction with a standard deviation of >0.95. The observed half-lives are shown in Table II.

The eq isomer of all triesters was found to react approximately four times faster than the ax isomer. Furthermore, the electron-donating substituent p-CH_3 of the p-methylbenzyl ester increased the reactivity by a factor of 20 over the unsubstituted benzyl ester which was in turn 150 times more reactive than the triesters containing the

Table III. Degradation of cAMP Benzyl Triester; % Composition of Reaction Mixtures after 10 min

Isomer	In presence of	Triester	cAMP	5'-AMP
Ax	H_2O	100 (94)[a]	<1 (6)	– (–)
	Albumin	100 (92)	<1 (8)	– (–)
	PDE	100 (94)	<1 (<1)	<1 (6)
Eq	H_2O	95 (72)	5 (28)	– (–)
	Albumin	94 (70)	6 (30)	– (–)
	PDE	96 (78)	<1 (<1)	4 (22)

[a] Values in parentheses after 60 min.

electron-withdrawing substituents o-NO_2 and p-NO_2. These enormous differences in rate point to hydrolytic attack on the benzylic carbon rather than on phosphorus.[12]

Since the hydrolysis reaction in water becomes more and more acidic through formation of cAMP free acid, it was of interest to measure the hydrolysis in a buffer under conditions which are optimal for enzymes. Treatment of the benzyl ester in Mops buffer, pH 6.5, at 30 °C resulted in a half-life of 9.5 h for the ax isomer and 3.0 h for the eq isomer.

Thereafter, investigation of the triesters' reactivity toward a cAMP specific phosphodiesterase (PDE) from bovine heart was undertaken. When PDE was allowed to react with cAMP in the presence of increasing amounts of triester, no inhibition of the enzyme was observed. It was thought that due to the histidine moieties in PDE the enzyme might possibly catalyze hydrolysis of the triesters before degrading the cAMP thus formed.

PDE was therefore allowed to react with the individual triesters. These proved to be relatively stable even when the enzyme concentration was increased by a factor of 4 over the standard incubation mixture and when the incubation time was prolonged to 60 min. Degradation of the various triesters ranged from 2 to 10% with the exception of the benzyl ester, which showed after 60 min 50% the activity of cAMP toward PDE. These results were measured according to the method of Muneyama et al.[13] In order to overcome the uncertainties involved in this method, the experiment was repeated with the benzyl ester using LC where all products could be unequivocally separated and identified. Bovine serum albumin was chosen as a peptide analogue to PDE. The results are shown in Table III. The analogue peptide has very little catalytic effect on the hydrolysis. The data verify that after simple hydrolysis has taken place PDE will produce an amount of 5'-AMP comparable to the amount of cAMP formed.

The next point to investigate was whether or not cAMP triesters are able to activate a cAMP-dependent protein kinase from bovine brain. The stimulation was calculated from the amount of ^{32}P transferred to histones by this enzyme in the presence of increasing concentrations of the various triesters compared to cAMP. The results are shown in Table IV.

The data indicate that a considerably higher concentration of the triester had to be used to obtain a stimulation of enzyme activity comparable to cAMP. As in the case of PDE this stimulation can probably be accounted for in part by hydrolysis. One experiment was performed on a mixture of the ax and eq isomers of the benzyl ester to see if their reactivities combined other than additively. It was shown that the effect of the two is purely additive, making separation of the two diastereoisomers unnecessary in further experiments.

Finally, photolysis of the photolabile o-nitrobenzyl ester was investigated under irradiation at 366 nm. The half-life, as observed using LC, proved to be 66 min for the ax isomer and 52 min for the eq isomer. The photolysis was then carried out and the stimulation of protein kinase due to the formed cAMP was measured as shown in Table V. After photolysis the stimulation rises as expected from the half-life values to nearly the same level as with cAMP itself. This clearly demonstrates that the by-products formed do not interfere with the enzyme and therefore no trapping reagent is required.

Table IV. Relative Protein Kinase Activity (α) of cAMP Triesters at 10^{-7}–10^{-4} M[a]

R	10^{-7}	10^{-6}	10^{-5}	10^{-4}
CH_3	0	0.05	0.52	0.67
CH_2CH_3 (ax)	0	0	0.06	0.14
CH_2CH_3 (eq)	0	0	0.06	0.58
Bn[b] (ax + eq)	0.07	0.46	0.84	0.66
Bn (ax)	0	0.09	0.59	0.45
Bn (eq)	0.03	0.49	1.00	1.22
o-NO_2Bn (ax)	0	0	0.05	0.34
o-NO_2Bn (eq)	0	0.04	0.59	1.28
p-NO_2Bn (ax)	0	0	0.24	0.54
p-NO_2Bn (eq)	0	0.04	0.49	1.01

[a] α values = (activity of the test compound)/(activity of cAMP). [b] Bn = $C_6H_5CH_2$–.

Table V. Effect of Photolysis on Protein Kinase Activity by o-Nitrobenzyl Ester at 10^{-8}–10^{-4} M (α Value)

R	$h\nu$	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
o-NO_2Bn (ax)	–	0	0	0.05	0.15	0.36
o-NO_2Bn (eq)	–	0	0	0.09	0.64	1.35
o-NO_2Bn (ax)	+	0	0.69	1.31	1.29	0.84
o-NO_2Bn (eq)	+	0	0.61	1.31	1.31	1.23

As a model system to test whether the benzyl esters of cAMP are able to penetrate the cell membrane and to substantiate our idea of using them as a transport form of cAMP, we chose the rat glioma C 6 cell lines for assaying these compounds. Glial cells in culture are able to accumulate large quantities of cAMP in response to catecholamine stimulation.[14,15] This elevation of internal cAMP affects the morphological and biochemical "differentiation" of nerve and glial cells in vitro.[16] The addition of dibutyryl-cAMP alters the morphology of both cell types in primary explants of nerve tissues.[17] Similar observations have also been made with human glial tumor cells.[18] Recently it was shown that the morphological alteration by C 6 rat glioma cells is strictly correlated to the enhanced cellular cAMP concentration induced by noradrenaline.[19] Under normal growth conditions C 6 cells show an irregular flattened shape. After treatment with β-agonistic noradrenaline, the cytoplasma retracts to form a compact cell body with multiple, mainly bipolar processes. This cAMP-induced morphological change occurs within a few minutes after treatment and can be easily studied by microscopic observations. From this internal cAMP response the glial cell system seems to be an accurate method to observe the uptake and intracellular reactivity of cAMP analogues such as has already been shown in the case of dibutyryl-cAMP.[17,19] Before testing all the synthesized triesters of cAMP for their ability to show this morphological effect, we first checked the time dependence of the system with one suitable compound, p-methylbenzyl ester 6 (ax). After 90 min, when the full differentiation had been achieved, the internal cAMP concentration was assayed according to Gilman's procedure;[20] see Table VI. It should be pointed out that the control incubation with corresponding concentration of exogenous cAMP does not produce any alteration of the cells. The effect of all the other triesters on the cell after 90 min was then observed; see Table VII.

Table VI. Morphologically Differentiated Cells (%) at Intervals and Internal cAMP Concentration after 90 min

	M	20 min	50 min	90 min	cAMP, pmol/mg of protein
p-CH_3Bn	10^{-4}	10-15	30-40	80-90	70
[6 (ax)]	10^{-3}	20	60-65	90-95	225
cAMP	10^{-3}	0	0	0	0
Noradrenaline	10^{-4}	50-60	80-90	90-95	250

Table VII. Effect of Various Compounds on Morphological Alteration in C 6 Glioma Cells at 10^{-3} M after 90 min

	Compound					
Isomer	1	2	3	4	5	6
ax	–[a]	–	+[a]	+	+	+
eq	–	–	+	+	+	

[a] +, 90-95%; –, 0% differentiation.

The benzyl esters caused an almost quantitative alteration whereas the methyl and ethyl triester had no effect. After an incubation of 20 h these triesters, nevertheless, also showed some effect. The differences in lipophilicity between the alkyl and the substituted benzyl esters as well as the difference in hydrolysis rates should be weighed when considering the differences in performance. The ethyl triester hydrolyzes with a very long half-life of ~13 days (56 °C) giving a mixture of cAMP and ring-opened alkyl diesters,[4] whereas the benzyl esters hydrolyze exclusively to cAMP. In addition to this chemical evidence that most probably cAMP itself triggers the morphological effect, and the fact that the noradrenaline treatment of the glioma cells leads to a temporary activation of the cytoplasmatic cAMP-dependent protein kinase (Schlaeger, unpublished results), the almost complete inactivity of the triesters toward the enzymes tested makes the conclusion convincing that benzyl triesters are simply a transport form of cAMP. We strongly feel that these results support our hypothesis that only the benzyl esters penetrate into the cell and then measurably and specifically hydrolyze under these condition to cAMP, which produces the morphological differentiation.

Before measuring the internal cAMP concentration according to Gilman's procedure[20] to further verify the cell penetration, the benzyl esters 3 (ax), 3 (eq), and 6 (ax) were tested for their binding ability to protein kinase, giving curves very similar to cAMP itself. Unfortunately the procedure routinely used has too strongly acidic conditions to be sure that no hydrolysis has occurred. A further complication arises from the fact that this glioma system has a remarkably time-dependent excretion of cAMP.[21] Therefore a quantitative treatment by this method will meet with considerable uncertainty.

Conclusion

Triesters of cAMP are currently receiving more and more attention as possible vehicles for transporting cAMP into cell systems. Hydrolytic studies showed that the predominant product of hydrolysis of the benzyl esters is cAMP. This is probably due to attack of water on the benzylic carbon, a conclusion which is also supported by the rate differences of the various benzyl esters. In general these triesters do not react with either enzyme tested. Although this could not be absolutely proven in the protein kinase experiments, it was clearly shown to be the case in the PDE experiments. It is also important to note that the triesters and their hydrolysis products do not inhibit either enzyme and can therefore be metabolized. These esters of cAMP constitute a system with low affinity to protein kinase which can conveniently be transformed to one with high affinity, e.g., through photolysis in the case of the photolabile *o*-nitrobenzyl ester. In the cell assay with glioma C 6 cell lines we could show that the postulated membrane penetration is substantiated. We therefore submit that benzyl triesters are excellent derivatives for studies requiring membrane penetration and specific formation of phosphodiesters in the cell, not only in the case of cAMP but also for biologically active phosphodiesters in general.

Experimental Section

General Methods. Thin-layer chromatography was run on a Schleicher + Schüll silica gel F 1500 LS 254 plate and was developed with either solvent system A, $CHCl_3$–MeOH (6:1 v/v), or B, $ClCH_2CH_2Cl$–EtOH (5:1 v/v). Ultraviolet spectra were determined on a Cary 15 spectrometer. The ^1H nuclear magnetic resonance spectra were recorded on a Jeol JNM MH 100 against Me_4Si and the ^{31}P NMR on a Bruker HX 90 against phosphoric acid (85%). Silica gel for preparative thin-layer chromatography (TLC) was obtained from Merck (60 PF 254). High-performance liquid chromatography (LC) was performed on a Hewlett Packard 1010 A chromatograph with a gradient programmer and a multiple wavelength UV detector (Schöffel). Photolysis was done with a Camag Universal UV lamp (366 nm, 2 × 20 W).

General Procedure. Triesters. Adenosine 3',5'-monophosphate, free acid, is suspended in hexamethylphosphorous triamide (HMPT) with the aid of an ultrasonic bath. To this suspension is added a freshly prepared ethereal solution of the diazo compounds—or, in the case of the substituted phenyldiazomethanes, their crystalline form—and the reaction mixture is stirred until solution is effected. The solution is then concentrated with the aid of an oil pump to a small volume and the product precipitated with ether. After centrifugation and washing with ether the residue is purified first over a short silica gel column (5–7 g), eluted with $CHCl_3$–MeOH (4:1 v/v), and then by preparative TLC with $CHCl_3$–MeOH mixtures. The resulting glass is dissolved in dioxane and freeze-dried to give amorphous powders.

The resulting alkyl triesters of cAMP were separated into their diastereoisomers by preparative TLC on silica gel. The mixture is applied onto 20 × 20 × 0.1 cm or 40 × 20 × 0.2 cm plates and the separation is carried out by developing with either $CHCl_3$–MeOH or $ClCH_2CH_2Cl$–EtOH mixtures. The plates were developed three to four times as indicated below for each compound until a good mechanical separation was possible. The substances were eluted with acetone and freeze-dried from dioxane–water. The purity of the isolated diastereoisomers was checked using LC with either a 50-cm, 10-μm or a 25-cm, 5-μm silica gel column Lichrosorb SI 60 and a $ClCH_2CH_2Cl$–MeOH gradient. With the exception of the methyl ester all the diastereoisomers could be separated with a purity of 98%.

Specific Reaction Conditions. Methyl Ester of 3',5'-cAMP [1 (ax, eq)]. cAMP (164.6 mg, 0.5 mmol) in 10 mL of HMPT and ethereal diazomethane from *N*-methyl-*N*-nitrosourea (5.0 g, 0.48 mol) were stirred for 14 days at 4 °C in the dark: yield 81.0 mg (47%). The product was identical in NMR, IR, and TLC with the one prepared by the method of Gohil et al.[4]

Ethyl Ester of 3',5'-cAMP [2 (ax, eq)]. cAMP (329.2 mg, 1.0 mmol) in 20 mL of HMPT and ethereal diazoethane from *N*-ethyl-*N*-nitrosourea (11.7 g, 0.1 mol) were stirred for 14 days at 4 °C in the dark: yield 151.8 mg (42%). Thin-layer plates were developed four times with $CHCl_3$–MeOH (7:1 v/v) to give 2 (ax) and 2 (eq) in a ratio of 48:52. 2 (ax) was identical in NMR, IR, and TLC with the compound prepared by the method of Gohil et al.[4]

Benzyl Ester of 3',5'-cAMP [3 (ax, eq)]. cAMP (329.2 mg, 1.0 mmol) in 20 mL of HMPT and ethereal phenyldiazomethane from *N*-nitroso-*N'*-nitro-*N*-benzylguanidine[22] (4.5 g, 20 mmol) were stirred for 6 days at room temperature: yield 242.0 mg (58%). The ^1H NMR spectrum showed the benzyl protons as two doublets (J = 5.5 Hz) located at δ 5.26 and 5.20. Thin-layer plates were developed three times with $CHCl_3$–MeOH (9:1 v/v) to give 3 (ax)

and 3 (eq) in a ratio of 65:35. Anal. ($C_{17}H_{18}N_5O_6P \cdot 0.5C_4H_8O_2$) C, H, N, P.

***o*-Nitrobenzyl Ester of 3',5'-cAMP [4 (ax, eq)].** cAMP (329.2 mg, 1.0 mmol) in 20 mL of HMPT and *o*-nitrophenyldiazomethane (1.202 g, 7.4 mmol), freshly prepared by the method of Ried and Ritz,[23] were stirred for 9 days at room temperature in the dark: yield 198.6 mg (43%). The ^1H NMR spectrum showed the benzyl protons as two doublets (J = 5.5 Hz) located at δ 5.62 and 5.63. Thin-layer plates were developed three times with $CHCl_3$–MeOH (9:1 v/v) to give 4 (ax) and 4 (eq) in a ratio of 52:48. Anal. ($C_{17}H_{17}N_6O_8P \cdot H_2O$) C, H, N, P.

***p*-Nitrobenzyl Ester of 3',5'-cAMP [5 (ax, eq)].** cAMP (164.4 mg, 0.5 mmol) in 10 mL of HMPT and *p*-nitrophenyldiazomethane (400 mg, 2.45 mmol) as prepared below were stirred for 17 days at room temperature in the dark: yield 79.0 mg (33%). The ^1H NMR spectrum showed the benzyl protons as two doublets (J = 5.5 Hz) located at δ 5.48 and 5.52. Thin-layer plates were developed four times with $CHCl_3$–MeOH (8:1 v/v) to give 5 (ax) and 5 (eq) in the ratio of 60:40. Anal. ($C_{17}H_{17}N_6O_8P \cdot C_4H_8O_2$) C, N, P; H: calcd, 4.13; found, 4.69.

***p*-Nitrophenyldiazomethane.** *p*-Nitrobenzaltosylhydrazone (3.18 g, 10 mmol) was dissolved in a mixture of 15 mL of 2 N KOH and 200 mL of water and the red-orange solution was warmed in a 50 °C water bath for 3 h. The product, which crystallizes out as orange needles, was filtered, washed with 150 mL of water until neutral, and air-dried: yield 1.42 g (87%); mp 84–85 °C dec (lit.[24] mp 80 °C).

***p*-Methylbenzyl Ester of 3',5'-cAMP [6 (ax, eq)].** cAMP (329.2 mg, 1.0 mmol) in 10.5 mL of HMPT and *p*-tolyldiazomethane,[25] freshly prepared from *p*-methylbenzaltosylhydrazone (5.77 g, 30 mmol), were stirred for 13 days at 4 °C in the dark: yield 109.8 mg (25%). During chromatography on silica gel using $CHCl_3$–MeOH (8:1 v/v) the 6 (eq) isomer decomposed so rapidly that only 6 (ax) could be obtained. Anal. ($C_{18}H_{20}N_5O_6P \cdot 0.5C_4H_8O_2$) C.

Hydrolysis. Hydrolysis was performed on 5 μM triester in Me_2SO–H_2O twice (1:1 v/v) at 50 °C. The rate of production of cAMP was measured by LC. A Partisil 10 SAX column, length 25 cm and diameter 4.6 mm, was used. The compounds were eluted with a flow of 1.6 mL/min 0.03 M KH_2PO_4 + 4% dioxane. A good separation between cAMP and open-chain alkyl esters could be obtained. The UV monitor at 258 nm was coupled to an electronic integrator. The product ratio was either calculated on an internal standard or the complete optical density was registered. The rate of the reaction obeyed a pseudo-first-order kinetic reaction with an average standard deviation of >0.95. On this basis the half-lives were calculated (Table II).

Photolysis. Photolysis[26] was carried out under the following conditions: concentration 4 mM in Me_2SO–phosphate buffer, pH 7.1 (1:10 v/v); volume used 200 μL; irradiation at 366 nm; cut-off for the petri dishes 320 nm; temperature 37 °C; distance from the light source 6 cm. The half-life of the photolysis was estimated by observing the rate of production of cAMP on the LC. The half-life for 4 (ax) was 66 min and for 4 (eq) 52 min.

Biochemical Methods. [^3H]-cAMP was obtained from Amersham-Buchler (Braunschweig), calf thymus histones (type II A) were from the Sigma Chemical Corp. (St. Louis, Mo.), bovine heart cAMP phosphodiesterase and *E. coli* alkaline phosphatase were purchased from Boehringer (Mannheim), and bovine serum albumin (Cohn Frakt. V) was obtained from Serva (Heidelberg).

PDE Assay. The inhibition study of phosphodiesterase was measured with the radioactive method described by Muneyama.[13] The standard incubation mixture contained in 0.25 mL: 40 mM Tris–HCl (pH 7.8), 24 mM $MgCl_2$, and 20 μg of enzyme plus 80 μM [^3H]-cAMP and either 40, 60, or 160 μM triester. The incubation period was 10–60 min at 37 °C.

The enzymatic hydrolysis was performed in the standard incubation mixture plus 80 μM triester and was measured according to Muneyama's colorimetric method.[13] The assay for the experiment followed by LC was performed in 50 μL: 100 mM Tris–HCl (pH 7.6), 40 mM $MgSO_4$, 100 μg of enzyme, and 50 μM triester. The incubation period was 10–60 min at 37 °C.

Protein Kinase Assay. The bovine brain cAMP-dependent protein kinase was purified by DEAE-cellulose chromatography as previously described.[27] Elution was performed with a linear gradient of 5 mM phosphate buffer to 0.5 M NaCl in phosphate buffer. The specific activity of the protein kinase preparation used in the present study was about 1000 units/mg of protein[28] and the stimulation rate as measured in the presence of 5×10^{-6} M cAMP was about 8–10-fold. One unit of enzyme corresponds to the activity which transfers 1 pmol of ^{32}P from [γ-^{32}P]-ATP to acid-precipitable histones in 1 min at 30 °C. The stimulation of the cAMP-dependent protein kinase was assayed as described.[29] The incubation mixture contained in 0.2 mL: 50 mM Mops buffer [3-(*N*-morpholino)propanesulfonic acid, pH 6.5], 5 mM $MgCl_2$, 7 mM mercaptoethanol, 1.5 μM [γ-^{32}P]-ATP, 60 μg of calves thymus histones, 15–25 μg of protein kinase, and various concentrations of cAMP or triester as indicated. The reaction procedure was carried out for 5 min at 30 °C. The specific activity of [γ-^{32}P]-ATP was 1.3 Ci/mmol.[30]

Photolysis was carried out in the above Mops buffer under the irradiation conditions described previously for 1 h.

Cell Assay. The C 6 rat glioma cells, originally isolated by Benda et al.,[31] were obtained from Flow Laboratories. These cells were routinely grown in 60-mm petri dishes with Dulbeccos modified eagle medium (DME) supplemented with 10% newborn calf serum and 50 μg/mL of gentamicin in an atmosphere of 90% air and 10% CO_2 at 37 °C. To study the biological activity of triesters inside the cells, growing C 6 cells were incubated for 90 min with 10^{-3} M of each triester of cAMP in the presence of 10^{-4} M theophylline. All experiments were performed with rarely confluent cultures (about 5–9 $\times 10^5$ cells per plate). During the experiments the dishes were placed on a constantly moving shaking table. The percentage of morphologically altered cells was estimated with an error of 5–10%. The cAMP concentration was assayed using Gilman's method.[20,21] For protein determination[28] bovine serum albumin (Sigma) was used as a standard.

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Antitumor Agents. 25.[1] Synthesis and Antitumor Activity of Uracil and Thymine α-Methylene-γ-lactones and Related Derivatives

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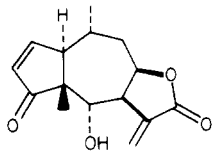
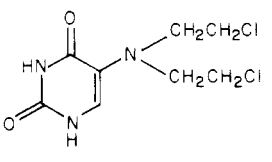
Uracil and thymine α-methylene-γ-lactones and related derivatives have been synthesized as novel potential alkylating antitumor agents. The synthesis of these compounds involved the convenient Reformatsky-type reaction between ethyl α-(bromomethyl)acrylate and the proper pyrimidinyl ketones. Preliminary in vivo tumor assay indicated that these compounds were active against the Walker 256 carcinosarcoma in rats and the P-388 lymphocytic leukemia as well as the B-16 melanotic melanoma in mice at 2.5–25 mg/kg.

Extensive screening of plant extracts has led to the isolation of a large number of sesquiterpene lactones having cytotoxic antitumor activity.[2-5] Our previous investigation of the structure–activity relationships for these compounds has indicated that one of the structural requirements for significant cytotoxic antitumor activity is an O=C—C=CH_2 system as part of an ester as well as a ketone (such as a β-unsubstituted cyclopentenone) or lactone (such as an α-methylene-γ-lactone).[3,6-9] This type of system has been observed, for example, in the cytotoxic antitumor helenalin (1).[6,7] It has been demonstrated that this O=C—C=CH_2 system could act as an alkylating center for the cytotoxic antitumor lactones and ketones, such as elephantopin,[10] vernolepin,[10] helenalin,[11] and tenulin.[11] A Michael-like reaction between biological nucleophiles, such as L-cysteine,[10,11] glutathione[11] or sulfhydryl-containing enzymes (e.g., phosphofructokinase,[12] glycogen synthetase,[13] DNA polymerase[11]), and –C=CH_2 grouping of the γ-lactone or the cyclopentenone moiety of these compounds has been proposed.

Our recent work on the synthesis of compounds derived from the combination of an above active alkylating center and a carrier moiety, such as a steroidal hormone, had led to several novel steroidal α-methylene-γ-lactone alkylating agents which are active against Walker 256 carcinosarcoma in rats.[14] These agents might be tumor specific (e.g., for breast or prostatic cancer) and thus clinically useful. As an extension of this approach we report herein the synthesis of α-methylene-γ-lactone with incorporation of nucleic acid base as a carrier moiety. The introduction of a carrier moiety into an alkylating center has led to compounds, such as uracil mustard (2), of clinical interest as anticancer agents.[15]

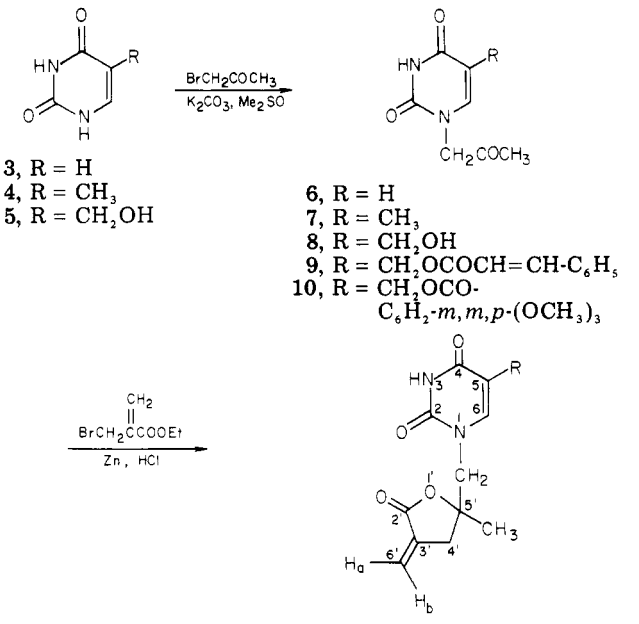


1



2

Scheme I



3, R = H
4, R = CH_3
5, R = CH_2OH

6, R = H
7, R = CH_3
8, R = CH_2OH
9, R = $CH_2OCOCH{=}CH\text{-}C_6H_5$
10, R = $CH_2OCO\text{-}C_6H_2\text{-}m,m,p\text{-}(OCH_3)_3$

11, R = H
12, R = CH_3
13, R = $CH_2OCOCH{=}CH\text{-}C_6H_5$
14, R = $CH_2OCO\text{-}C_6H_2\text{-}m,m,p\text{-}(OCH_3)_3$

Furthermore, a perusal of the literature revealed no record of any investigation on the synthetic α-methylene-γ-lactone bearing nucleic acid bases as alkylating anticancer agents except for the adeninyl- and uracilyl-furanones in which the double bond in the γ-lactone ring is either endocyclic or fully substituted.[16]

Chemistry. The synthesis of these uracil and thymine α-methylene-γ-lactones and their related derivatives (11–14) was carried out using the method similar to our previous work on the preparation of steroidal α-methylene-γ-lactones[14] and is outlined in Scheme I. The preparation of the α-methylene-γ-lactone moiety which