BIOORGANIC CHEMISTRY 8, 9–16 (1979)

Synthesis and Reactivity of Guanosine 3′,5′-Phosphoric Acid Alkyl Esters[1]

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Received March 27, 1978

Guanosine 3′,5′-phosphoric acid alkyl esters were synthesized by reaction of the free acid of guanosine 3′,5′-phosphate (cGMP) with the appropriate diazoalkane. The resulting pair of diastereoisomers was separated chromatographically, and the conformations were assigned on the basis of ^{31}P-nmr. Hydrolysis of the compounds in water was measured, and their half-lives were determined. The ability of the compounds to act as substrates for phosphodiesterase from beef heart was tested, and their inhibition of this enzyme was measured.

Guanosine 3′,5′-phosphate (*2*) (cGMP) is argued to be similar to adenosine 3′,5′-phosphate (cAMP) in that it seemingly regulates various cellular functions. Increasing evidence indicates a partially antagonistic behavior for these two effectors (*3*). Esterification of 3′,5′-cyclophosphates renders them membrane-transportable, and use of the benzyl group allows a subsequent selective hydrolytic cleavage. It could be shown in the case of adenosine 3′,5′-phosphoric acid benzyl ester that external application to glial cells produces the morphological alteration of cell shape known to be specific for internal cAMP (*4*). Furthermore, a positive inotropic effect in heart muscle analogous to that obtained through noradrenaline stimulation is also observable (*5*). Supported by these results, we were interested in applying this concept to benzyl esters of cGMP to help clarify the question of its role in biological regulation.

RESULTS AND DISCUSSION

The guanosine 3′,5′-phosphoric acid esters were prepared by reaction of the appropriate diazoalkane with the free acid of cGMP in hexamethylphosphoric triamide (HMPT). Since the starting material **1** is relatively insoluble, the reaction is carried out in suspension, and the mixture is stirred until solution is effected. The purity of the solvent is decisive.

The alkylation of guanine (*6*) occurs as a side reaction, decreasing in extent, however, in the series diazomethane, phenyldiazomethane, substituted phenyldiazomethane; so that only the methylated compounds **7–9** were investigated.

They were shown chromatographically, electrophoretically, and in ^{31}P-nmr to be triesters. Assignment of the position of the methyl group on guanine was accomplished with the help of uv and nmr spectroscopy. Table 1 shows the data for **7** and **8** and an

[1] Paper VII in a series on Cyclic Phosphates.

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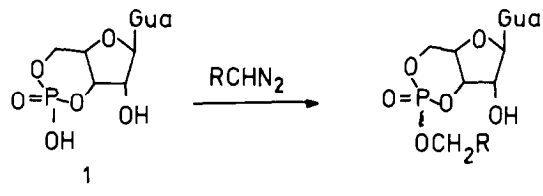
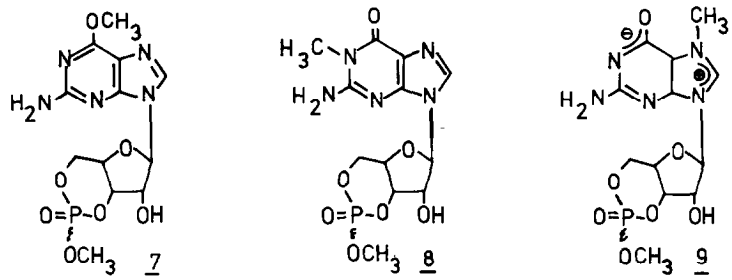


1

	2	3	4	5	6
R	H	C_6H_5	$C_6H_4CH_3$-(4)	C_6H_4Cl-(4)	$C_6H_4NO_2$-(2)



7 8 9

authentic sample of *N*-7-methylguanosine (*7*), along with literature data (*6*) for *N*-1- and *O*-6-ethylguanosine. On the basis of the characteristic uv spectrum, **7** can clearly be identified as *O*-6-methylguanosine. Since the spectra of *N*-1-ethyl- and *N*-7-methylguanosine are not very different, it is difficult to determine whether **8** is the *N*-1 or the *N*-7 derivative. Still, **8** lacks the long-wave absorption in water which is present for *N*-7-methylguanosine. The position of its methyl signal in ^{1}H-nmr is also different than that of *N*-7-methylguanosine. Finally, its chromatographic mobility and lack of fluorescence at 366 nm point to the presence of a non-zwitterionic species. It was therefore concluded that **8** is methylated in the *N*-1 position.

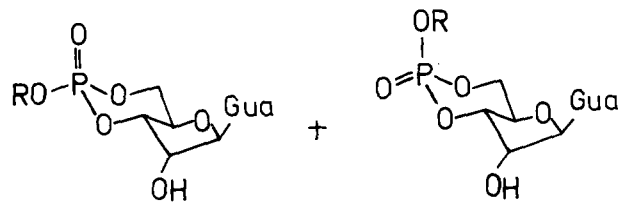
TABLE 1

UV AND NMR SPECTRA OF ALKYLATED cGMP

	pH 1		H_2O		pH 13		
	max (nm)	min (nm)	max (nm)	min (nm)	max (nm)	min (nm)	^{1}H-nmr CH_3(δ, ppm)
1-Ethylguanosine	261		257		258		
	[272][a]	235	[270]	237	[270]	239	
O-6-Ethylguanosine	244	233	248	229	247	233	
	286	260	277	261	278	261	
7-Methylguanosine	257		257				
	[278]	228	280	243			4.01
7	245	234	246	230	245	235	
	287	260	279	261	276	261	3.95
8	260		257		258		
	[280]	234	[272]	235	[268]	236	3.30

[a] [ ] = shoulder.

Due to an asymmetric center on phosphorus, all synthesized triesters occur as diastereomeric mixtures, small quantities of which can be chromatographically separated. Assignment of the equatorial *e* and axial *a* conformation was based on ^{31}P-nmr and ir data in analogy to the *e* and *a* isomers of uridine 3′,5′-phosphoric acid esters (*8*, *9*).



e a

The ^{31}P-nmr signal at higher field corresponds to the axial isomer, that at lower field to the equatorial; this phenomenon probably arises from the different charge distributions of the axial and equatorial phosphoryl oxygen (*10*). Whether the absolute configuration in this case applies will have to be determined by an X-ray analysis.

The ^{31}P-nmr chemical shift differences of the *e* and *a* isomers in DMSO amount to 1–1.5 ppm. It is interesting to note the solvent dependence in this connection. In dioxane, a solvent with a low dielectric constant, the shift amounts to 3.2 ppm instead of 1.1 in DMSO for **3**. The special position of dioxane was also apparent in two other respects. First, the addition of small amounts of dioxane to the solvent increased the chromatographic separation of the isomers, which leads to the conclusion that the two are differently solvated. Second, the presence of dioxane in our samples made it very difficult to obtain uniform elementary analyses. Because of solubility problems, the samples were freeze-dried from dioxane/water; but even after extensive drying over P_4O_{10} at 35–40°C and 0.01 Torr, the nmr spectrum still showed the presence of a dioxane peak at δ 3.5 ppm. A differential thermal analysis with simultaneous mass spectroscopic investigation indicated the strong affinity between dioxane and these substances. Compound **5** lost dioxane in a temperature range from 60 to 180°C before decomposing exothermally. Unfortunately, we were not able to determine accurately from the weight loss the amount of bound dioxane, since this method requires large amounts of the substance. The hydrolysis of the benzyl esters **3–6** was examined, the most interesting result being their clean autohydrolysis (*11*) to the parent cyclic phosphate. A possible explanation was found in the investigation of the hydrolysis reaction mechanism of adenosine 3′,5′-phosphoric acid benzyl esters with $H_2{}^{18}O$ (*12*). It has been shown that under neutral and moderate acidic conditions water attacks the benzyl carbon, not phosphorus. The compound **4** treated with $H_2{}^{18}O$ in the same way as described for cAMP benzyl ester (*12*) did fully incorporate the label in the 4-methylbenzyl alcohol. Based on the significant rate differences for compounds **2** and **4** in water and following the arguments listed in (*12*), we favour an S_N1 mechanism for the neutral and acidic hydrolysis of phosphoric acid benzyl triesters. The half-lives of the pseudo-first-order hydrolysis of **2–5** in water at 50°C are shown in Table 2. Here again a drastic difference between an electron-donating **2** and an electron-attracting **4** sub-

TABLE 2

HALF-LIVES OF 2–5 AT 50°C IN H_2O

$t_{1/2}$ (min)		2	3	4	5
	a	~6000	235	10	192
	e	~4200	105	4	93

stituted compound by a factor of 600 could be observed. Therefore the different hydrolytic labilities in conjunction with different substituents on the benzyl ring should result in an array of cGMP precursors.

An enzyme essential to control of the cGMP level in cells is phosphodiesterase (PDE), which degrades cyclic phosphates to 5′-phosphates. The triesters **2–5** were tested for their ability to serve as substrates for raw extracts of this enzyme from beef heart. As was also true of cAMP esters, the results of this test were negative.

In the case of PDE from rat liver, it could be shown that at least three different types of PDE are present (*13*): an enzyme with high activity toward cGMP; one with high activity toward cAMP, which is inhibited by cGMP; and one with negative cooperative activity toward cAMP. This is probably also true of PDE extracts from other organs (*14*). Therefore, in testing the inhibition of PDE by our triesters, we provided first cGMP as a substrate, and then cAMP. The triesters (mixtures of both *e* and *a* isomers) and the substrate were added in equal molar amounts. The results are shown in Table 3. Since the inhibition is approximately equal in the presence of both substrates, it is not possible to make any statement about the relative effect on each sort of PDE. An investigation of the purified PDE specific for cGMP would be of interest here.

No attempt was made to test the effect of the triesters on protein kinase because, similar to that of esters of cAMP, hydrolysis occurs fast enough to provide the cyclic phosphate necessary for stimulation of the enzyme.

Using cAMP esters as a transport form of cAMP, it is possible to produce a positive inotropic effect in guinea pig papillary muscle, corresponding to that caused by norepinephrine (*5*). In the same way it was hoped that esters of cGMP could be used as a transport form of cGMP. It has been suggested that increases in cGMP levels are responsible for the negative inotropic effects of acetylcholine in the heart. Therefore we tested the *p*-methylbenzyl ester of cGMP **4** on the papillary muscle of the guinea pig.

TABLE 3

PERCENTAGE OF PDE INHIBITION[a]

	PDE (heart) cGMP	PDE (heart) cAMP
2*a*,*e*	25	17
3*a*,*e*	58	58
4*a*,*e*	23	65
5*a*,*e*	41	42

[a] Substrate and triester added in equal molar amounts, 1/1 (millimoles).

Unfortunately the ester did not show any significant effect at all, and thus the correlation between cGMP levels and negative inotropic effect has to be questioned, as has been done elsewhere (*15*).

EXPERIMENTAL

The uv spectra were recorded with a Cary recording spectrometer, Model 1115/15 and 118 from Applied Physics Corp. Thin-layer chromatography was run on Schleicher and Schüll silica gel F1500 LS 254 plates. Silica gel for preparative thin-layer chromatography was obtained from Merck (60 PF 254). High performance liquid chromatography (hplc) was performed on a Hewlett-Packard 1010 A chromatograph equipped with a variable wavelength uv detector (Schöffel). The 25-cm steel columns, 4 mm internal diameter, were packed with Nucleosil 10 SB from Macherey–Nagel and with Partisil SAX from Reeve & Angel. The ^{31}P-nmr spectra were recorded on a Bruker HX 90 spectrometer using FT and broad-band decoupling without susceptibility correction. The ^{1}H-nmr spectra were recorded on a Jeol JNM-MH-100 spectrometer. Differential thermal analysis was carried out on the instrument STA Model 429 from Netzsch (Selb) coupled with a quadrupole mass spectrometer QMG 511 from Balzers (Liechtenstein).

GENERAL PROCEDURE—TRIESTERS

Guanosine 3′,5′-phosphate **1**. Guanosine 3′,5′-phosphate **1** (*16*) dried at 40°C and 0.01 Torr was suspended in freshly distilled hexamethylphosphoric triamide (HMPT) with the aid of an ultrasonic bath. Solutions of freshly prepared diazoalkanes in ether or pentane, or in the case of diazomethane, the gas itself, were added to the suspension, and the volatile solvent was evaporated. The reaction was then stirred in the dark until solution was effected. In several instances the treatment with fresh diazo compound had to be repeated. When reaction was complete, the HMPT was evaporated in vacuum to 1–2 ml, and the product was precipitated with ether and pentane. After centrifugation the product was dissolved in chloroform/methanol, 1/1, and applied to a short silica gel column (5–7 g) which was first eluted with chloroform to remove side products of the diazo compound and then eluted with chloroform/methanol, 4/1, to obtain the raw product. The crude material was then dissolved in a little DMF and applied to 40 × 20 × 0.1-cm silica gel plates which were first developed with ether and then with the mixture stated below. The product zone was eluted with chloroform/methanol and the product was freeze-dried from dioxane/water.

To separate the resulting mixtures of diastereoisomers, 10 mg dissolved in DMF were applied to a 20 × 20 × 0.1-cm plate and developed with ether and then repeatedly with benzene/isopropanol, 2/1, until a reasonable separation was achieved.

Guanosine 3′,5′-phosphoric acid methyl ester **2**. A total of 181.7 mg (0.5 mmol) of **1** in 20 ml of HMPT cooled in ice was treated with gaseous diazomethane prepared from 3.25 g (15 mmol) of *p*-tolylsulfonylnitrosamide according to de Boer and Backer (*17*).

Due precautions should be taken to prevent explosion of gaseous diazomethane. The reaction was stirred for 4 days in the dark at 4°C. The raw product was applied to two silica gel plates and developed with chloroform/methanol, 4/1. The four zones were eluted with chloroform/methanol, 3/1. The main product was located in the two lower zones, the two diastereoisomers **2a** and **2e** (65.9 mg, 30%).

Anal. Calcd for $C_{11}H_{14}N_5O_7P \cdot C_4H_8O_2$ (447.3): C, 40.28; H, 4.95; N, 15.65; P, 6.94. Found: C, 40.37; H, 5.12; N, 15.79; P, 6.67.

O-6-Methylguanosine 3',5'-phosphoric acid methyl ester **7**. The highest zone in the previously described chromatogram (30.7 mg, 13%) was again applied to a 20 × 20 × 0.1-cm silica gel plate and developed twice with chloroform/methanol, 6/1. By freeze-drying from dioxane/water, 5.1 mg colorless powder were obtained. With the help of uv, nmr, and ir, structure **7** could be assigned.

Anal. Calcd for $C_{12}H_{16}N_5O_7P \cdot C_4H_8O_2$ (461.3): P, 6.73. Found: P, 6.49.

N-1-Methylguanosine 3',5'-phosphoric acid methyl ester **8**. The second highest zone in the previously described chromatogram was again applied to a 20 × 20 × 0.1-cm silica gel plate and developed with chloroform/methanol, 6/1. Freeze-drying from dioxane/water yielded 9.4 mg (4%) of colorless powder, to which with the help of uv, nmr, and ir structure **8** was assigned.

Guanosine 3',5'-phosphoric acid benzyl ester **3**. In this procedure, 363.2 mg (1.0 mmol) of **1** in 20 ml of HMPT and phenyldiazomethane from 3.35 g (15 mmol) of *N*-nitroso-*N'*-nitro-*N*-benzylguanidine (*18*) were stirred at room temperature in the dark for 10 days. The raw product was applied to two 40 × 20 × 0.1-cm silica gel plates and developed with chloroform/methanol, 6/1. The product was eluted with chloroform/methanol, 3/1, and freeze-dried from dioxane/water to yield 127.1 mg (28%) of colorless powder.

Anal. Calcd for $C_{17}H_{18}N_5O_7P \cdot H_2O$ (453.3): C, 45.04; H, 4.44; N, 15.45; P, 6.83. Found: C, 44.67; H, 4.20; N, 15.67; P, 6.55.

Guanosine 3',5'-phosphoric acid 4-methylbenzyl ester **4**. A mixture of 160.0 mg (0.44 mmol) of **1** in 10 ml of HMPT and *p*-tolyldiazomethane from 7.2 g (59 mmol) of *p*-tolylaldehyde, hydrazine, and mercury-II oxide (*19*) was stirred at room temperature in the dark for 11 days. The raw product was applied to two 20 × 20 × 0.1-cm silica gel plates and developed with chloroform/methanol, 4/1. The product was eluted with chloroform/methanol, 4/1, and freeze-dried from dioxane/water to yield 112.5 mg (57%) of colorless powder.

Anal. Calcd for $C_{18}H_{20}N_5O_7P \cdot 1/2C_4H_8O_2 \cdot 2H_2O$ (529.3): C, 45.38; H, 5.33; N, 13.23; P, 5.85. Found: C, 45.14; H, 5.75; N, 12.98; P, 6.31.

Guanosine 3',5'-phosphoric acid 4-chlorobenzyl ester **5**. A mixture of 181.6 mg (0.5 mmol) of **1** in 20 ml of HMPT and 4-chlorophenyldiazomethane from 8.5 g (60 mmol) of 4-chlorobenzaldehyde, hydrazine, and mercury-II oxide (*18*) was stirred at room temperature in the dark for 7 days. The raw product was applied to two 40 × 20 × 0.1-cm silica gel plates and developed with chloroform/methanol, 5/1. The product was eluted with chloroform/methanol, 3/1, and freeze-dried from dioxane/water to yield 61 mg (21%) of colorless powder.

Anal. Calcd. for $C_{17}H_{17}Cl\ N_5O_7P \cdot H_2O \cdot 1/2\ C_4H_8O_2$ (549.9): C, 40.77; H, 4.58; N, 12.73; P, 6.64; Found: C, 41.22; H, 5.01; N, 13.12; P, 5.32.

Guanosine 3',5'-phosphoric acid-2-nitrobenzyl ester **6** is described in Ref. (*1*). The physical properties of guanosine 3',5'-phosphoric acid esters are summarized in Table 4.

TABLE 4

PHYSICAL PROPERTIES OF GUANOSINE 3',5'-PHOSPHORIC ACID ESTERS

Substance	uv spectra (MeOH) (nm)		tlc on SiO_2 (R_f values)[a]		^{31}P-nmr[b]	ir (KBr) P=O (cm^{-1})	^{1}H-nmr [D_6]DMSO/TMS (ppm) CH_2Ph(d)[c]
	λ_{max}	lg ε	$CHCl_3$/MeOH (4/1)	Benzene/isopropanol (2/1)			
2	256	4.39	(*a*) 0.37	0.07	4.93	1295	3.80[d]
			(*e*) 0.51	0.18	4.18	1255	3.74
3	256	4.16	(*a*) 0.74	0.34	5.76	1275	5.16
			(*e*) 0.78	0.43	4.62	1245	5.14
4	256	4.15	(*a*) (*e*) 0.78	0.40	5.39	1280	5.12
				0.46	4.44	1255	5.06
5	256	4.20	(*a*) 0.75	0.37	5.93	1295	5.19
			(*e*) 0.78	0.45	4.70		5.14
6[e]	256	4.26	(*a*) 0.73	0.30	5.91	1290	5.54
			(*e*) 0.77		4.67	1252	5.52
7	246[f]	4.23	(*a*) (*e*) 1.0	0.48	4.43		3.80[d]
	279	4.14		0.69	3.51		3.74
8	257[f]	4.32	0.64	0.13	3.57		3.73[d]

[a] Plates from Schleicher & Schüll, F 1500 LS 254.

[b] Positive sign relative to 85% H_3PO_4 in [D_6]DMSO.

[c] The CH_2 group appears as a doublet due to coupling with ^{31}P.

[d] $POCH_3$ instead of CH_2Ph.

[e] For synthesis see Ref. (*1*).

[f] In water.

HYDROLYSIS

About 1 μmol of each diastereoisomer was dissolved in 50 μl of dimethyl sulfoxide (DMSO), 100 μl water were added, and the probe was incubated at 50°C. The rate of production of cGMP was measured by hplc using cytidine or uridine 5'-phosphate as internal standard. The rate of reaction obeyed pseudo-first-order kinetics, giving a least squares line with standard deviation 0.98. The hplc conditions were isocratic, using either 0.025 *M* KH_2PO_4 and 4% dioxane for Partisil or 0.05 *M* KH_2PO_4 and 2% dioxane for Nucleosil with a flow of 1.4 ml/min. The uv detector measured at 258 nm, and integration of the peaks was performed electronically by a Minigrator from Perkin–Elmer.

For the $H_2{}^{18}O$ experiment 0.5 μmol of **4** were suspended in 20 μl of $H_2{}^{18}O$ (95% ^{18}O, Amersham Buchler). After incubation at 37°C for 1 hr the 4-methylbenzylalcohol (M^+ = 122 *m/e*) and the ^{18}O 4-methylbenzylalcohol (M^+ = 124 *m/e*) were identified in the mass spectrometer (CH 7, Varian MAT at 70 eV). The relative intensities of $(124)^+ : (122)^+$ were 86 : 14 ±4%.

ENZYME STUDY

The PDE from beef heart (Boehringer) was employed without further purification. For the substrate test, the following amounts were used: 70 nmol of triester, 0.1 mg of PDE, 4 μmol of $MgSO_4$, 1.5 μmol of Tris–buffer, pH 7.3, and 4% DMSO in a total volume of 40 μl. For the inhibition study the following amounts were used: 10 μl of PDE solution (10 mg/ml), 10 μl of 0.4 *M* $MgCl_2$, 10 μl of 15 m*M* cGMP or cAMP in Tris–buffer, 30 μl of 5 m*M* triester in DMSO/Tris, 1/8, and 40 μl of 0.1 *N* Tris–buffer, pH 7.3. The probes were incubated at 37°C for 5 min, and the amount of cGMP and 5′-GMP was measured using hplc. The inhibition due to DMSO was also established and subtracted from the values found above.

ACKNOWLEDGMENTS

Many thanks are due to Rolf Reidys, who in the course of his engineer thesis laid the groundwork for this paper; to Jeanne Hoftiezer for her technical assistance; as well as to the Deutsche Forschungsgemeinschaft for financial support.

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