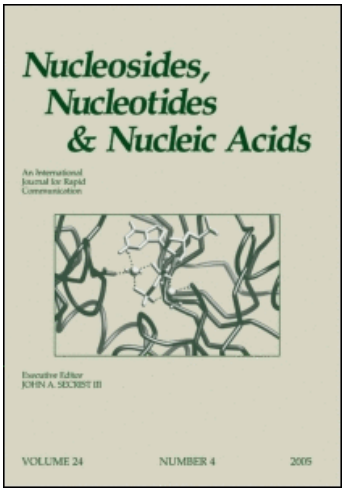

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K. S. Bruzik[ab]; A. Grajkowski[a]; Z. J. Lesnikowski[a]; G. M. Salamonczyk[a]; W. J. Stec[a]
[a] Center of Molecular and Macromolecular Studies, Polish Academy of Sciences, Lodz, Poland [b] Chemistry Department, The Ohio State University, Columbus, OH

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CONFORMATION OF DIASTEREOMERS OF ADENOSINE CYCLIC 3',5'-PHOSPHOROTHIOATE

K. S. Bruzik†,*, A. Grajkowski, Z. J. Lesnikowski, G. M. Salamonczyk and W. J. Stec*

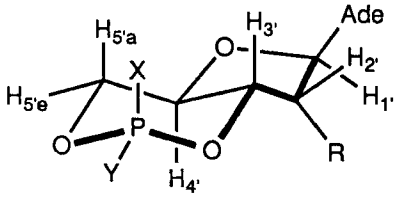
Center of Molecular and Macromolecular Studies, Polish Academy of Sciences, Sienkiewicza 112, 90-363 Lodz, Poland

Abstract: *^{1}H and ^{31}P NMR spectra of cAMP (1) and both diastereomers of cAMPS (2 and 3) were compared with these of structurally related bicyclic phosphate 4 and phosphorothioates 5 and 6. Conformational analysis was also performed by NMR techniques for bicyclic phosphoranilidates 7 and 8 and (Rp)-cdAMP anilidate (9). Chair conformation is predominant for all investigated compounds 1-8, while the phosphoranilidate 9 exists in solution in chair-twist equilibrium. Thus, antagonistic properties of (Rp)-cAMPS with respect to cAMP are inferred by the change in the overall molecular shape caused by the presence of the bulky sulfur atom in the equatorial position of the cAMPS molecule.*

Extensive studies on the chemical synthesis of nucleoside cyclic 3',5'-phosphates and their analogues have been initiated by isolation and purification of adenosine cyclic 3',5'-monophosphate (cAMP, 1) in 1957[1]. In the decades following the discovery of cAMP, over 600 cyclic nucleotide analogues were synthesized[2], and perhaps, the most important goal was to prepare a more potent tissue-specific agonist with a longer biological half-life. The first cAMP antagonist was obtained in 1974 as a mixture of diastereomers of adenosine cyclic 3',5'-phosphorothioate (cAMPS) (2) and (3)[3], but stereospecific synthesis of individual diastereomers and assignment of their absolute configrations was published only in 1979[4,5]. Preliminary studies on stereodependent substrate specificity of cyclic phosphodiesterase towards diastereomers of cAMPS[6,7], demonstrating that (Rp)-cAMPS (2) binds to cAMP-dependent protein kinase

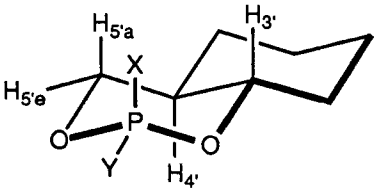
† current address: Chemistry Department, The Ohio State University, Columbus, OH 43210

holoenzyme without causing activation, were published in 1982[8]. The first evidence that (Rp)-cAMPS was a cAMP antagonist and could oppose the action of cAMP was the study done in hepatocytes in 1983[9]. Evidence has been provided that (Rp)-cAMPS is an intracellular inhibitor of cAMP action. Further studies on several cAMP-dependent systems, such as: glucagon - induced lipolysis in adipocytes[10], cAMP-activation of cellular slime mold *Dictyostelium discoideum*[11], cAMP - induced phosphorylation of microtubule associated protein-2 in brain tissue[12], hormone - induced steroidogenesis in cultured granulosa and Leydig cells[13], have shown, that (Rp)-cAMPS, the only known intracellular antagonist of cAMP, provides a means of distinguishing cAMP-dependent from cAMP-independent cellular events[14]. Interestingly, the molecular basis of the stereodifferentiated activity of (Rp)- and (Sp)-diastereomers of cAMPS remains still obscure[15], and the understanding of this phenomenon requires at first the conformational analysis of the individual diastereomers of cAMPS in solution.



1 - 3, 9

1: X=Y=O, R=OH
2: X=O, Y=S, R=OH
3: X=S, Y=O, R=OH
9: X=NHPh, Y=O, R=H



4 - 8

4: X=Y=O
5: X=S, Y=O
6: X=O, Y=S
7: X=S, Y=NHPh
8: X=NHPh, Y=S

A number of studies on the conformation of nucleoside cyclic 3',5'-phosphates and related analogues have been reported[16-21], however none of them dealing with the title compounds. This paper presents the results of comparative nuclear magnetic resonance (NMR) studies on the conformation of cAMP (**1**), (Rp)- and (Sp)-cAMPS (**2** and **3**, respectively). Our results are referenced also to existing literature data on the crystal structure of cAMP[22] and other neutral analogues of cyclic nucleotides[23-25].

RESULTS

In addition to the cAMPS isomers **2** (Rp) and **3** (Sp) and the parent molecule cAMP (**1**), five substituted *trans*-2,4-dioxa-3-thioxo-3-phosphabicyclo[4.4.0]decanes **4**-**8** and *deoxy*-adenosine cyclic 3',5'-phosphoranilidate (**9**) were also investigated as model heterocyclic systems. The bicyclic diesters **4**-**6** were studied as reference compounds owing to pronounced

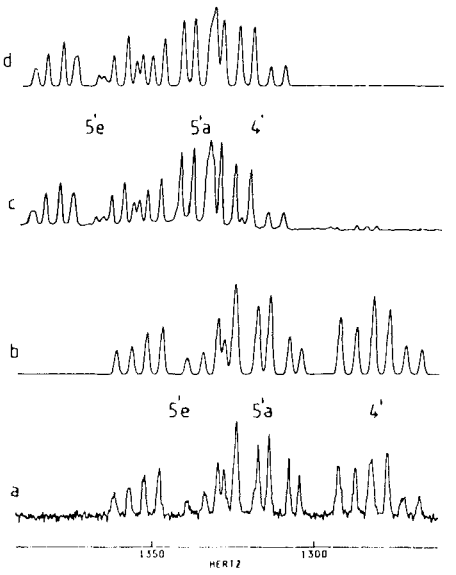


FIGURE 1. 300.13 MHz ^1H NMR spectra of the ribose protons of **2** and **3** in D_2O at 297 K; traces a and c are experimental spectra of **3** and **2**, respectively, and traces b and d are corresponding simulated spectra.

preference of such systems for a double chair conformation[26,27]. The bicyclic anilidates **7** and **8** and the anilidate of *deoxy*-cAMP **9** were used to provide the link to the NMR results obtained previously with neutral cyclic nucleotide derivatives[23,28-33]. Compounds were investigated by ^1H, ^{13}C and ^{31}P NMR in aqueous solution except for **7**, **8** and **9**, which were used as their chloroform solutions.

Spectral data are presented in Tables 1-4. All coupling constants and ^1H NMR shifts were obtained by the iterative simulation of experimental spectra using the PANIC version of the LAOCN3 simulation program[34]. The experimental and simulated spectra of **2** and **3** are presented in Figure 1.

It has been reported, that neutral rigid bicyclic derivatives including those with *trans*-fused 1,3,2-dioxaphosphorinane and tetrahydrofurane rings, can adopt a twisted conformation **11**[23,28,38-40]. This statement holds true also for a number of neutral derivatives of nucleoside 3',5'-cyclic phosphates, such as the cis-N,N-dimethylphosphoramidate of thymidine 3',5'-cyclic phosphate, which were shown to exist in the chair - twist equilibrium[28]. The tendency of the 2-substituted 1,3,2-dioxaphosphorinane ring system to adopt a twist boat conformation is related to

TABLE 1. ^{1}H NMR chemical shifts of 1 - 9 in D_2O solution at 297 K

Compound	1'	2'	3'	4'	5'a	5'e
1	*b*	4.771	*a*	4.398	4.389	4.591
2	6.102	4.725	4.841	4.437	4.489	4.591
3	6.135	4.694	4.907	4.254	4.372	4.449
4	*b*	*b*	4.240	*b*	4.003	4.120
5	*b*	*b*	4.085	*b*	4.004	3.977
6	*b*	*b*	4.085	*b*	3.997	3.939
9	6.314	2.815[c]	5.348	4.113	3.378	4.558

[a] signal overlapped by HDO signal; [b] value not determined; [c] H-2'a proton, H-2'b 2.722 ppm

TABLE 2. ^{1}H-^{1}H and ^{1}H-^{31}P coupling constants for 1 - 9 in D_2O at 297 K

Compound	Coupling constant[c]									
	1'2'	2'3'	3'4'	4'5'e	4'5'a	5'a5'e	1'P	3'P	5'eP	5'aP
1[a]	0.7	5.7	10.1	4.5	11.0	-9.3	0.7	2.2	21.1	1.9
1[b]	0.7	5.7	9.8	4.5	10.7	-9.3		2.2	21.4	1.7
2	1.0	5.1	10.2	4.6	10.0	-9.6	0.7	4.0	20.5	3.4
3	1.0	5.1	9.6	4.8	9.8	-9.2		4.2	23.4	3.3
4				4.6	11.3	-11.3		2.0	22.0	2.1
5[c]			11.0	4.4	11.4	-11.0		3.3[d]	26.0	2.7
6[c]			10.1	4.5	11.5	-10.4		2.0[d]	21.5	2.5
7[c,e,f]		4.3	11.1			-11.8		3.5[d]	26.9	3.1
8[c,e,f]		4.7	10.9			-11.0		1.6[d]	21.0	4.3
9[f]	2.1[g]	7.7[h]	9.1	5.2	10.5	-9.5	0.7	1.3	16.6	5.0

[a] data from ref.16, [b] this work; [c] proton numbering used as in Scheme 1 and 2, in order to maintain analogy with cAMP derivatives, [d] values determined from ^{1}H-^{1}H 2D-J resolved type experiment; [e] values obtained from first order analysis of spectra, [f] in $CDCl_3$ solution, [g] $^3J_{2a'3'}$ coupling, $^3J_{1'2'b}$ 8.6 Hz, [h] $^3J_{1'2a'}$ coupling, $^3J_{2b'3'}$ 10.6 Hz

TABLE 3. ^{13}C NMR chemical shifts and $^3J_{13C-31P}$ couplings for 1 - 3[a]

carbon position	1		2		3[b]	
	δ [ppm]	$J_{13C-31P}$[Hz]	δ [ppm]	$J_{13C-31P}$[Hz]	δ [ppm]	$J_{13C-31P}$[Hz]
1'	94.40	<0.2	94.21		94.37	
2'	75.13	8.0	75.27	7.3	75.06	9.0
3'	74.65	3.7	74.42	5.6	74.56	4.6
4'	80.09	5.0	78.98	6.2	80.00	4.0
5'	70.11	8.0	70.60	9.7	70.34	7.5

[a] digital resolution 0.6 Hz, [b] this work, see also ref. 21

TABLE 4. ^{31}P NMR chemical shifts for compounds **1** - **9**

Compound	Ligand X	Ligand Y	Ligand R	Solvent	δ [ppm]
1[a]	O	O	OH	D_2O	-1.6
2[b]	O	S	OH	H_2O	55.2
3[b]	S	O	OH	H_2O	53.6
4[c]	O	O	-	D_2O	-1.7
5[c]	S	O	-	D_2O	51.6
6[c]	O	S	-	D_2O	55.1
7[c]	S	NHPh	-	$CDCl_3$	64.2
8[c]	NHPh	S	-	$CDCl_3$	60.0
9[d]	NHPh	O	H	C_5H_5N	-3.8

[a] data from ref. 35; [b] data from ref. 36; [c] this work; [d] data from ref. 37

the sustituent's preference to occupy an axial position in the six-membered ring and is most pronounced for *trans*-substituted systems (having a *trans*-singly bonded substituent with respect to H-3') with electronegative substituents. It is a reasonable assumption, that in biological systems, the negative charge of cAMP is at least partially diffused by the protonation of the phosphoryl oxygen or by its complexation with the metal-ion.

In view of this notion, we have measured ^1H and ^{31}P NMR spectra of (Rp)-cAMPS at pH 6.0-1.2 and of (Sp)-cAMPS, in the pH range, 6.4-3.0. Spectra were not measured at a lower pH due to a possible loss of sulfur. It was found, that in the case of both isomers no significant spectral changes occurred upon the decrease in the pH. The most prominent difference in the spectra of **2** was the upfield shift of the H-5'a proton signal (by 0.015 ppm), of H-4' (by 0.02 ppm) and that of ^{31}P NMR signal (by 0.5 ppm). In the case of **3** the corresponding changes were 0.02 ppm, 0.01 ppm and 0.25 ppm. Thus, one can conclude, that in the weakly acidic pH range no meaningful changes in the conformation of cAMPS isomers occur.

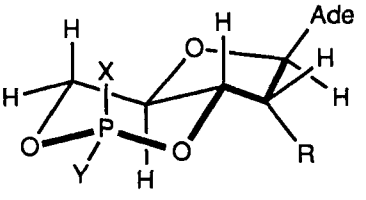
Similarly, we have measured ^1H NMR spectra of **1**, **2** and **3** in the presence of 1 M $MgCl_2$. The differences observed in the case of **2** and **3** were negligible both in the chemical shifts of the H-5'e, H-5'a and H-4' protons and in the magnitude of the coupling constants. Changes observed in the spectrum of **1** resulted merely from a rather insignificant shift of the H-5'e proton position, while the vicinal couplings were retained. It therefore appears, that the conformation of cAMPS is relatively stable under a variety of conditions.

DISCUSSION

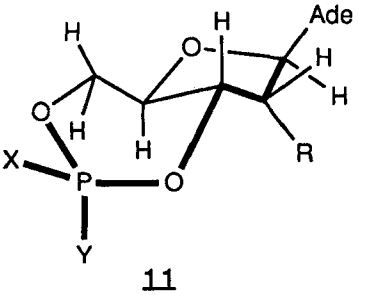
1,3,2-Dioxaphosphorinane ring

The conformation of the 1,3,2-dioxaphosphorinane ring, included in the cAMP molecule, can be judged based on the comparison of the magnitude of the vicinal ^1H-^{31}P and ^{13}C-^{31}P

coupling constants with the values obtained for the reference systems, known to adopt a single conformation. For the assessment of the conformations in cyclic nucleotides it is convenient to use the data reported by Lee and Sarma[16] and by Lapper et al.[21] Since, in general, the value of $^3J_{POCH}$ for phosphorothioates exceeds the analogous values for phosphates[41], we have made use of phosphorothioates **5** and **6** as the reference systems. The highest diagnostic value has the combination of the couplings between phosphorus and protons H-5'a, H-5'e. Large $J_{P5'e}$ (in excess of 20 Hz) and low values of $J_{P-5'a}$, and $J_{P3'}$ (1-3 Hz) are characteristic of the chair conformation. The increase of the $J_{P5'a}$ with the concomitant drop of $J_{P5'e}$ implicates the participation of the twist-conformation. It is common to interpret such changes in terms of the twist - chair equilibrium. The results presented in Table 2 are consistent with the existence of a single chair conformation for compounds **1** - **6**. Interestingly, in all phosphorothioates bearing the sulfur in an axial position the magnitude of $J_{P5'e}$ is ca. 10-30% higher than the analogous value for the second isomer. It is tempting to interpret such differences between the isomers **3**, **5**, **7** (axial sulfur) and **2**, **6**, **8** (equatorial sulfur) as arising from a possible admixture of the twist form in the case of **3**, **6** and **8**. These differences, however, are not matched by the increase of $J_{P5'a}$ in **3**, **5** and **7** and therefore their origin is unclear. A similar behavior is apparent in the closely related bicyclic phosphorothioates[38]. The anilidate **9**, however, displays a lower $J_{P5'e}$ and slightly higher $J_{P5'a}$. It is therefore concluded, that for **9** the chair conformation **10** remains in an equilibrium with the twisted conformation **11**. Assuming the values of vicinal coupling constants for the chair (C) and the twist (T) $J_{P5'e}$ 21.6 Hz (C), $J_{P5'a}$ 0.9 Hz (C), $J_{P5'e}$ 0.9 Hz (T), and $J_{P5'a}$ 21.6 Hz (T)[28] the molar fraction of the twist **11** is estimated as 24 or 20%, depending on which one of the two observed coupling constant with protons H-5'e and H-5'a is used for the calculation.



10



11

X, Y and R as in **1** - **3** and **9**

This conclusion is consistent with the results obtained with the analogous *trans*-dimethyl phosphoramidates[28,38,39]. It remains however in disagreement with the molecular structure of **9** in the crystalline state, in which it exists in the single chair form[25]. It appears, that the solution conformation of the nucleotide analogue differs from its conformation in the single crystal.

The ribose ring and the base

The analysis of the data from Table 2 shows only minor variations in the coupling constants $J_{1'2'}$, $J_{2'3'}$ and $J_{3'4'}$ for protons of the ribose moiety of **1** - **3**. Such differences can not be discussed on the grounds of the conformational change. Hence, we conclude that phosphorothioate analogues do not differ in the conformation of their ribose ring, when compared to the original molecule, and thus assume the C(3')-endo-C(4')-exo conformation.

Rather large differences in the chemical shifts of the 2', 3', 4' and 5' protons of the ribose between **2** and **3** (Table 1) could originate from the diamagnetic shielding of these protons caused by the different time-averaged orientation of the heterocyclic adenine rings in these compounds. Such differences are not observed within the **5**, **6** pair of compounds. Significant differences found among the molecular structures of cyclic 3',5'-nucleotides and of their analogues in the crystalline state further justify such a notion[22-25]. The participation of the twist-form is a less likely cause of these changes, since the recently reported differences in proton chemical shifts in diastereomeric model compounds[38,39] seem to have no correlation with the presence and abundance of this conformer. At present there are, however, no other indications that the sulfur substitution at the phosphorus modifies the angle of rotation of the adenine ring about the C1' - C1 bond in cyclic 3',5'-AMP. The recent theoretical work on the conformation of cAMP and cGMP[43] rules out the existence of the syn-conformer in the former case, however, the longer axial P-S bond in **3** as compared to P-O bond in **1** and **2** could make the water-assisted intramolecular hydrogen bond between sulfur and adenine more feasible.

Thus, despite the grossly different biological properties of the diastereomers of cAMPS, no discernible differences in their conformations could be found. Antagonistic properties of (Rp)-cAMPS with respect to cAMP are most probably inferred by the change in the overall molecular shape caused by the introduction of the bulky sulfur atom into the equatorial position of the six-membered ring[44].

EXPERIMENTAL

All NMR spectra were obtained with a Bruker MSL 300 spectrometer operating at a proton frequency of 300.13 MHz. ^{1}H and ^{13}C spectra were externally referenced to TMS and ^{31}P NMR spectra to external 85% phosphoric acid. ^{15}N NMR spectra are referenced to nitromethane.

(Sp)- and (Rp)-cAMPS were obtained from corresponding anilidates as described recently[45]. cAMP was from Sigma. cdAMP anilidate was obtained according to the previously reported procedure[37]. Cyclic anilidates **7** and **8** were obtained as follows:

2-Hydroxycyclohexyl-1-methanol (2.4 g, 18.5 mmol) was reacted with phosphorus trichloride (1.9 mL, 22 mmol) in methylene chloride at -70° and the reaction mixture was allowed to warm up in the course of 1.5 h. The mixture was concentrated to dryness and a solution of aniline

(4 mL, 42 mmol) in methylene chloride (20 mL) was added. The resulting mixture was maintained at room temperature for 2 h, and was then treated with elemental sulfur (1.2 g). After 12 h at room temperature the mixture was worked-up by filtration of the hydrochloride, washing the solution with HEPES.Na buffer (pH 7.5, 0.1 M) and concentration. The crude mixture was separated by column chromatography on silica gel (gradient of benzene-ether 10:1 / benezene-ether 4:1) giving **7** (15%) and **8** (18%). The absolute stereochemistry of both **7** and **8** was determined according to the empirical rules developed in our laboratory[4,46-48], relating the value of chemical shift in ^{31}P-NMR and absolute value of spin-spin coupling constant between ^{15}N and ^{31}P with the spatial orientation of the anilino group with the respect to the rest of the molecule.

7: ^{31}P NMR (chloroform-d), δ 64.2 ppm; ^{13}C NMR (chloroform-d), δ [ppm, (J_{P-C})] 138.6 (5.6), 129.2, 123.2, 119.5, 119.4, 81.2 (5.1), 71.1 (5.5),41.8 (5.0), 32.6 (9.6) 25.7, 24.6, 24.1 (2.2); ^{15}N NMR (chloroform-d), -296.5 ppm ($^{1}J_{P-N}$ 24.8 Hz)

8: ^{31}P NMR (chloroform-d), δ 60.0 ppm; ^{13}C NMR (chloroform-d), δ [ppm] 140.0, 130.1, 123.1, 119.0, 118.9, 83.7 (8.9), 73.1 (9.0), 41.5 (7.5), 33.2 (8.4), 26.7, 25.1, 24.8; ^{15}N NMR (chloroform-d), δ -300.3 ppm ($^{1}J_{P-N}$ 10.5 Hz)

Phosphorothioates **5** and **6** were obtained in 55 and 60% yield from **7** and **8**, respectively, by the treatment of sodium salts of **7** and **8** with carbon dioxide analogously to the method described previously[49].

5: ^{31}P NMR (D_2O), δ 51.6 ppm; ^{13}C NMR (D_2O), δ [ppm] 84.06 (5.7 Hz), 73.9 (5.8), 44.7 (4.8), 35.2, (9.1), 28.1, 27.2, 26.8

6: ^{31}P NMR (D_2O), δ 55.1 ppm; ^{13}C NMR (D_2O), δ [ppm] 83.0 (7.3), 73.0 (7.9), 44.3 (4.9), 35.4 (8.6), 28.3, 27.1, 26.8

Phosphate **4** (^{31}P NMR (D_2O), -1.67 ppm) was obtained from **5** by its oxidation with butylene oxide as reported from this laboratory[50].

Titration of **1**, **2** and **3** was carried out with trifluoroacetic acid at 20 mM phosphate concentration, in the presence of 20 mM acetate buffer of initial pH 6.0. Measurements of ^{1}H and ^{31}P NMR spectrum was done on the same sample using normal (^{31}P) and decoupling coil (^{1}H).

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