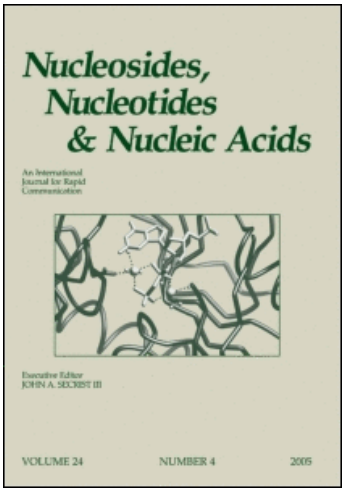

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Structural Characterization of Cyclic ADP-Ribose by NMR Spectroscopy

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STRUCTURAL CHARACTERIZATION OF CYCLIC ADP-RIBOSE BY NMR SPECTROSCOPY

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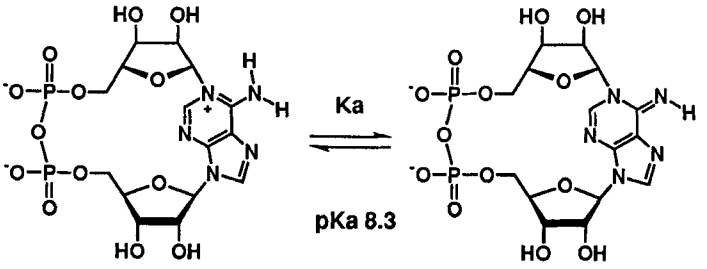
ABSTRACT: Structure of cyclic adenosine diphosphoribose (cADPR) was reinvestigated by using ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. The ^{1}H-^{1}H coupling constants and NOE data suggested that the adenosine and ribose moieties have a predominant C2'-endo conformation and an unusual flat conformation, respectively.

INTRODUCTION

Cyclic adenosine diphosphoribose (cyclic ADP-ribose, cADPR) discovered in 1990 by Lee *et al.*[1] has proved to serve as a new type of potent calcium-releasing second messenger.[2-4] On the basis of spectroscopic studies using ^{1}H NMR and FAB mass, the structure of cADPR was first described as a compound having an *N*-glycosyl linkage between the anomeric carbon of the ribose unit and the 6-*N*-amino group of the adenosine unit with the α-configuration.[5] A recent structural study of cADPR using UV spectroscopy reported by Jacobson *et al.* revealed that the anomeric carbon of the ribose unit is bound to the 1-*N* position of the adenine ring (Scheme 1).[6]

On the other hand, we have already reported a preliminary result of the structural determination of cADPR in aqueous solution by NMR spectroscopy suggesting that cADPR has a 1-*N*-glycosyl linkage with the β-configuration.[7] Quite recently, Lee *et al.* have reported that the crystal structure of free acid of cADPR by X-ray crystallography which is consistent with our results.[8] However, only limited information about the torsion angles of this molecule was given in their preliminary paper. The X-ray analysis of cADPR stimulated us to describe in more detail the structural analysis of cADPR in aqueous solution by using NMR spectroscopy.



Scheme 1

RESULTS AND DISCUSSION

Assignment of the ^{1}H NMR spectra and conformational analysis

From chemical and biological points of view, structural analysis of cADPR in aqueous solution is of great importance. However, complete assignment of the ^{1}H NMR signals along with the coupling constants of cADPR has not been reported. Lee *et al.* has originally assigned all ^{1}H resonance signals of the free acid of cADPR on the basis of the analysis of the ^{1}H-^{1}H COSY spectrum in D_2O at room temperature.[5] However, determination of several resonance signals was equivocal since they were hidden under the large HOD signal. Therefore, we measured the ^{1}H NMR spectra of cADPR in such a manner that the HOD signal was shifted upfield or downfield by changing temperatures from 273K to 313K. As the result of these experiments, several resonance signals (H_A8, H_A2, H_A4', H_R4', H_A5' and H_R5') were found to be incorrectly assigned (H_A and H_R refer to protons of the adenosine and the ribose units, respectively).

The ^{1}H NMR spectrum of the free acid of cADPR measured at 313K revealed that three protons overlapped with the HOD signal at room temperature. Unfortunately, it was found that the free acid of cADPR gradually decomposed in D_2O even at room temperature. Therefore, to avoid an additional complication derived from the decomposition, the sodium salt of cADPR was prepared for all experiments. Interestingly, there was no significant difference between the ^{1}H NMR spectra of the free acid and the sodium salt of cADPR .

In the 500 MHz ^{1}H NMR spectrum of cADPR (sodium salt) in D_2O at 286K, two resonance signals were observed at 8.38 and 8.97 ppm in the aromatic proton region (FIG. 1A). The resonance at 8.38 ppm was assigned to the H_A8 proton by phase-sensitive 2D ROESY experiments (FIG. 3A), since a strong NOE was observed with the H_A1' proton. This

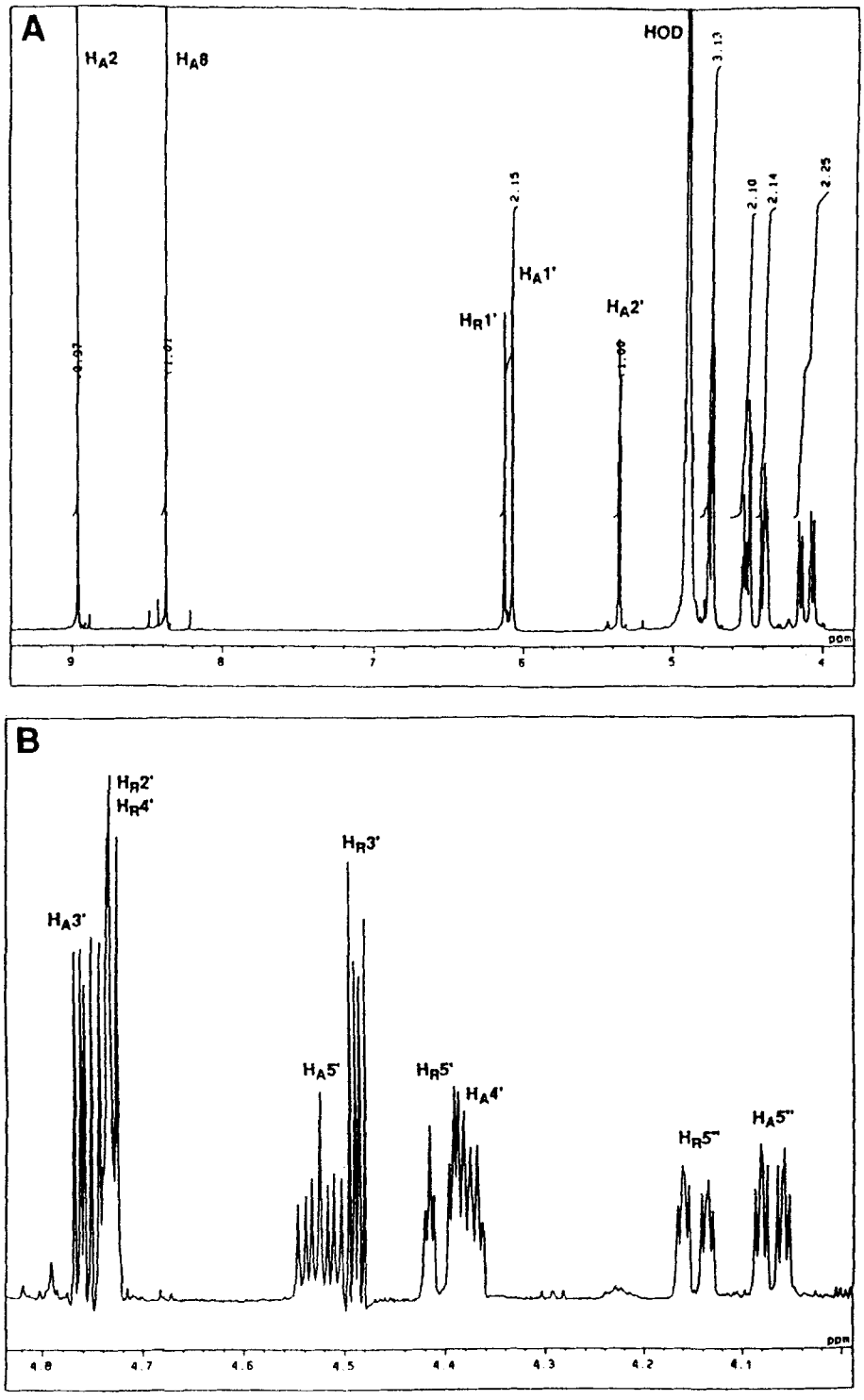


FIG. 1. (A) 500 MHz ^1H NMR spectrum of cADPR in D_2O at 286K. (B) Expanded highfield region of the spectrum (A).

strong NOE suggested the glycosyl torsion angle was fixed at a *syn* conformation in the adenosine unit. In the crystal structure of free acid of cADPR, the glycosyl torsion angle χ(O1'-C1'-N9-C4) was found to be 64(±1)° with the *syn* conformation[8] which is consistent with the NOE data.

Interestingly, compared with the H8 protons of usual adenosine derivatives, the H_A8 proton of cADPR was observed in upper field than the H_A2 signal. The large downfield shift of the H_A2 proton ($\Delta\delta$ = +0.85 ppm relative to that of 5'-AMP)[9] might be a characteristic feature of 1-*N* substituted adenine derivatives.[10]

Another characteristic feature of the ^1H NMR spectrum of cADPR is a large downfield shift of the H_A2' proton ($\Delta\delta$ = +0.59 ppm relative to that of 5'-AMP)[9]. This fact may be attributed to the anisotropic effect of the lone pair electrons on the 3-*N* atom in the adenine ring which has a fixed *syn* conformation.[11] The complete assignment of the other resonance signals was made on the basis of the analysis of the DQF-COSY spectrum as shown in FIG. 2. The chemical shifts and coupling constants of cADPR are summarized in TABLE 1 along with 5'-AMP as a reference compound.[9]

According to the analysis of the coupling constants between the sugar protons as shown in TABLE 1, populations of the conformers having different sugar puckerings could be estimated. The populations of the C2'-endo and C3'-endo conformers in the adenosine unit were calculated to be 64% and 36%, respectively, by the use of the values of $J_{1',2'}$ and $J_{3',4'}$ of the adenosine unit.[12] The pseudorotational analysis using the $J_{4',5'}$ and $J_{4',5''}$ values showed a preference for a *t* conformer about the torsion angle γ(O5'-C5'-C4'-C3'). The populations of the g^+, g^- and *t* conformers were estimated to be 39%, 1% and 60%, respectively.[13] In the phase-sensitive 2D ROESY experiments, the ROEs between the H_A2' and both the H_A5' and H_A5'' protons were observed (FIG. 3B). This result strongly suggested the predominance of a C2'-endo sugar puckering and a *t* conformation around the C4'-C5' bond in the adenosine unit. The ROEs observed in the 2D ROESY experiments are summarized in TABLE 2. On the other hand, pseudorotational analysis about the torsion angle β(P-O5'-C5'-C4') by using the values of $J_{4',5'}$ and $J_{4',5''}$ indicated that the *t* conformer predominantly exists (89%) over the other two conformers.[14]

In the case of the ribose unit, the populations of the C2'-endo and C3'-endo conformers were calculated to be 59% and 41%, respectively, by the use of the values of $J_{1',2'}$ and $J_{3',4'}$.[12] It is well known that the value of $J_{1',2'} + J_{3',4'}$ is nearly 10 Hz in general.[15] However, the sugar protons of the ribose unit showed an extremely unusual value of $J_{1',2'} + J_{3',4'}$ (6.4 Hz). This result might be explained in terms of the presence of furanose ring conformers diverse from the usual C2'-endo and C3'-endo conformers. Generally, there are linear correlations between the $J_{1',2'}$ and $J_{3',4'}$ values of nucleoside derivatives.[10] It should be noted that the $J_{1',2'}$ and $J_{3',4'}$ values of cADPR show a

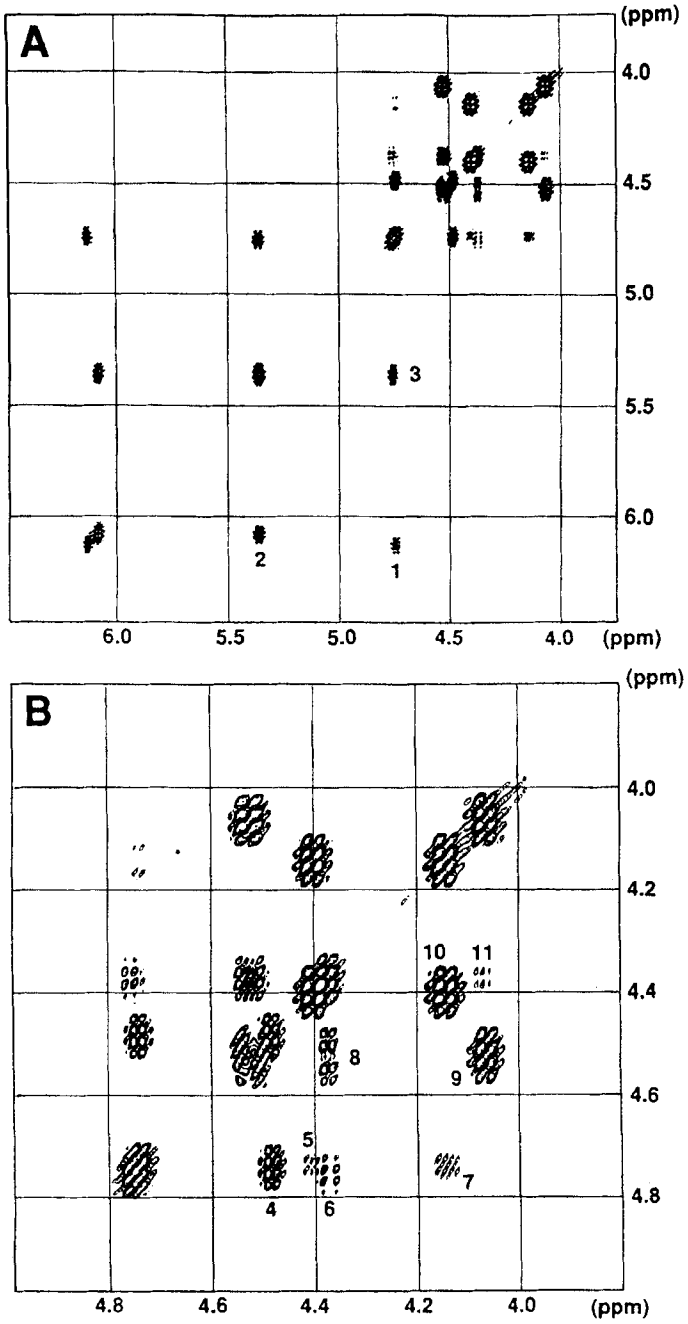


FIG. 2. (A) DQF-COSY spectrum of cADPR in D_2O at 286K. (B) Expanded highfield region of the spectrum (A). Cross peak assignments: (1) H_R1'-H_R2', (2) H_A1'-H_A2', (3) H_A2'-H_A3', (4) H_R2'-H_R3', H_R4'-H_R3', (5) H_R4'-H_R5', (6) H_A3'-H_A4', (7) H_R4'-H_R5'', (8) H_A5'-H_A4', (9) H_A5'-H_A5'', (10) H_R5'-H_R5'', (11) H_A4'-H_A5''.

TABLE 1. ^1H chemical shifts and coupling constants of cADPR in D_2O at 286K.

	adenosine unit	ribose unit	5'-AMP[b]
^1H chemical shifts (ppm)[a]			
2	8.97	—	8.12
8	8.38	—	8.55
1'	6.08	6.14	6.04
2'	5.36	4.74	4.77
3'	4.76	4.49	4.46
4'	4.38	4.73	4.35
5'	4.53	4.41	4.01
5"	4.07	4.15	4.01
^1H-^1H and ^1H-^{31}P coupling constants (Hz)			
1', 2'	5.6	3.7	5.9
2', 3'	5.1	5.1	5.0
3', 4'	3.2	2.7	3.6
4', 5'	6.8	2.2	3.2
4', 5"	2.6	2.2	3.2
5', 5"	-11.0	-11.9	—
5', P	3.9	2.2	4.3
5", P	3.4	3.7	4.3

[a] DSS was used as an external standard .
[b] See reference 9.

tendency similar to those of 2',3'-*O*-isopropylidene nucleosides.[11,16] It is well known that 2',3'-cyclic nucleosides such as 2',3'-*O*-isopropylidene and 2',3'-cyclic phosphate derivatives have flat conformations in the furanose rings.[17-22] In the case of cADPR, such a flat conformer or an equilibrium mixture of unusual furanose ring conformers may be present because of steric strain of the intramolecular diphosphate bridge. In fact, in the crystal structure of cADPR, endocyclic sugar torsion angle ν_1(C4'-O1'-C1'-C3') is 6° and furanose ring has an unusual flat conformation.[8] If the ribose unit has a flat furanose conformation, the distances between the H_A2 and both the H_R2' and H_R3' protons should be short enough to observe ROEs. The phase-sensitive 2D ROESY

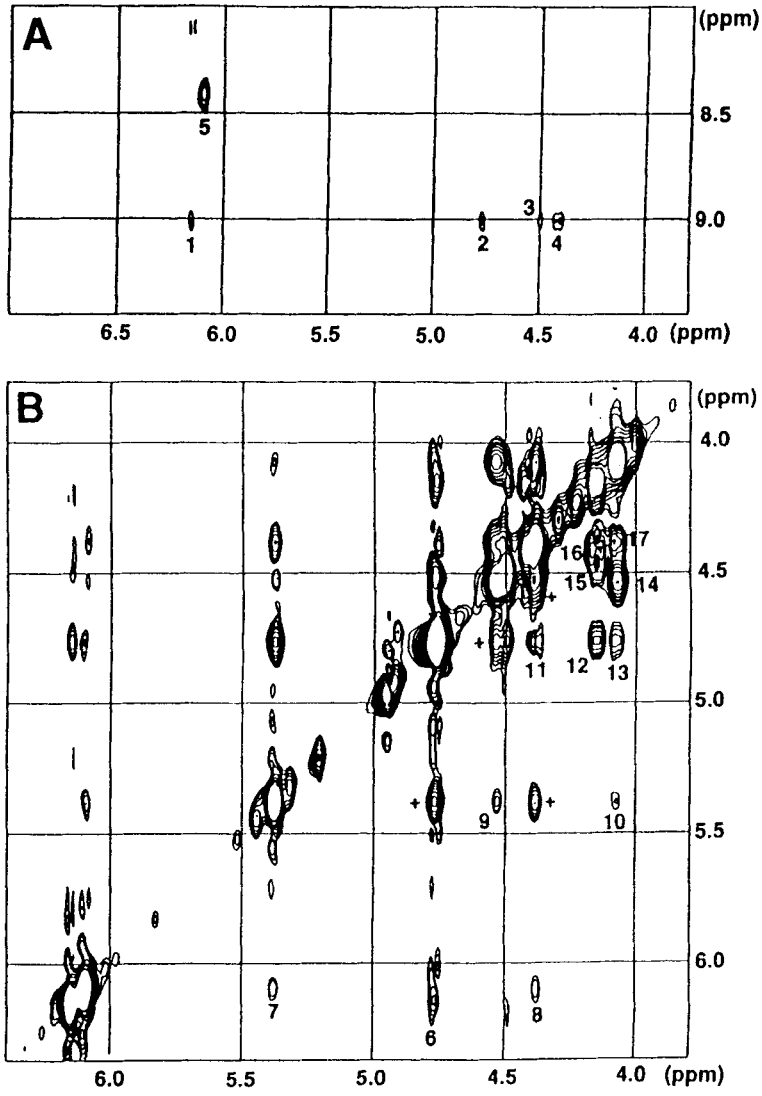


FIG. 3. Phase-sensitive 2D ROESY spectra of cADPR in D_2O. The spectra were obtained at 283K with a mixing time of 250 ms. (A) Spectrum of aromatic proton region (F_1) and sugar proton region (F_2). (B) Spectrum of sugar proton region. Cross peak assignments: (1) H_A2-H_R1', (2) H_A2-H_R2', (3) H_A2-H_R3', (4) H_A2-H_R5', (5) H_A8-H_A1', (6) H_R1'-H_R2'/H_R1'-H_R4', (7) H_A1'-H_A2', (8) H_A1'-H_A4', (9) H_A2'-H_A5', (10) H_A2'-H_A5'', (11) H_R4'-H_R5' and H_A3'-H_A4', (12) H_R4'-H_R5'', (13) H_A3'-H_A5'', (14) H_A5'-H_A5'', (15) H_R3'-H_R5'', (16) H_R5'-H_R5'', (17) H_A4'-H_A5''. Cross peaks labeled + refer to HOHAHA transition peaks.

TABLE 2[a]. Relative intensity of the ROE cross peaks[b] obtained by the 2D ROESY experiment.

$_A$\$^R$	2	8	1'	2'	3'	4'	5'	5"
2			vw	vw	w	-	s	-
8			-	-	-	-	-	-
1'	-	s		w	-	w[d]	-	-
2'	-	-	m		c	c	e	e
3'	-	-	-	c		c	c	s
4'	-	-	w	-	m		s	vs
5'	-	-	-	m	c	c		vs
5"	-	-	-	w	s	vs	vs	

[a] Above and below diagonal of the table show the observed ROEs of the protons in the ribose and adenosine units, respectively.

[b] Intensity of the ROEs was estimated on the basis of the volumes of the cross peaks. Abbreviations: vs: very strong; s: strong; m: medium, w: weak; vw: very weak.

[c] ROE cross peaks might overlap with HOHAHA cross peaks.

[d] Because the signals of the H_R2' and H_R4' protons could not be separated, these cross peaks could be assigned to the H_R2' and/or H_R4' protons.

[e] ROE cross peaks might overlap with other strong cross peaks.

spectrum (FIG. 3A) of cADPR indicated that the ROEs between H_A2 and both the H_R2' and H_R3' protons were clearly observed. This result strongly indicates that existence of the flat conformation is highly plausible. If the ribose unit has a C2'-endo conformation, the ROE should be observed between the H_A2 and H_R2' protons but the distance between the H_A2 and the H_R3' protons is too long to observe ROE. In the case of the ribose unit having a C3'-endo conformation, the distance between the H_A2 and H_R3' protons is short enough to observe the ROE but the distance between the H_A2 and the H_R2' protons is too long to observe the ROE. However, if C2'-endo and C3'-endo conformers exist in rapid equilibrium, the ROEs between the H_A2 and both the H_R2' and H_R3' protons may be also observed. Therefore, this possibility can not be ruled out.

In the ribose unit, the pseudorotational analysis about the torsion angle γ(O5'-C5'-C4'-C3') using the values of $J_{4',5'}$ and $J_{4',5''}$ indicated a predominance of the g^+ conformer (92%).[13] Since the distance between the H_R4' and H_R5' protons is almost equal to that of the H_R4' and H_R5'' protons in the g^+ conformer, strong ROEs with similar intensities should be observed between pairs of protons. However, the intensity of the ROE cross peak observed between the H_R4' and H_R5'' protons was stronger than that of the H_R4' and

H_R5' protons (FIG. 3B and TABLE 2). In the case of the *t* conformer around the C4'-C5' bond in the ribose unit, the distance of H_R4'-H_R5'' is shorter than that of H_R4'-H_R5'. This fact suggests that the *t* conformation around the C4'-C5' bond in the ribose unit is more suitable for the ROE data. This result is consistent with that of the X-ray analysis of cADPR reported by Lee *et al.*[8] On the other hand, pseudorotational analysis about the torsion angle β(P-O5'-C5'-C4') in the ribose unit by using the values of $J_{4',5'}$ and $J_{4',5''}$ indicated that the *t* conformer predominantly exists in 96%.[14] This result is consistent with the X-ray data.[8] However, if the sugar puckering is flat, the distribution of g^+, g^- and *t* conformers about the torsion angle β and γ can not be exactly estimated by the well known equation reported by Altona.[13]

Consequently, a probable structure of cADPR which is consistent with the NMR data is presented in FIG. 4.

^{13}C and ^{31}P NMR spectra

^{13}C and ^{31}P NMR spectroscopic studies of cADPR have not been reported. FIG. 5 shows the ^{13}C NMR spectrum of cADPR in D_2O at 277K. All the ^{13}C resonance signals of cADPR in D_2O at 277K could be assigned by using the HSQC experiments (See experimental section). The chemical shifts and coupling constants are summarized in TABLE 3, where those of 5'-AMP are listed as reference data.[24,25]

One of the characteristic features of the ^{13}C spectrum of cADPR is the significant large downfield shift of the anomeric carbon C_R1' compared with those of normal nucleotide derivatives ($\Delta\delta$ = +11.1 ppm relative to that of 5'-AMP). Jacobson reported that the pKa value of cADPR is 8.3.[6] This fact indicates that cADPR exists as the 6-*N* protonated form in neutral or acidic solution (Scheme 1). The electron-withdrawing effect of the 6-*N*-protonated adenine ring bearing a positive charge on the 1-*N* atom may cause a large deshielding effect on the anomeric carbon. The anomeric carbon C_A1' also showed the downfield shift ($\Delta\delta$ = +6.3 ppm) which may be resulted from the same reason. The other sugar carbons of the adenosine unit also showed the downfield shifts ($\Delta\delta$ = +3.3 to +4.9 ppm). Interestingly, the C_R4' carbon showed a relatively large downfield shift ($\Delta\delta$ = +6.5 ppm). Other characteristic features of the ^{13}C NMR spectrum of cADPR are the large upfield shift of the C_A2 carbon ($\Delta\delta$ = -7.3 ppm) and the large downfield shifts of the C_A8 and C_A5 carbons ($\Delta\delta$ = +8.0 and +5.0 ppm, respectively) relative to 5'-AMP. The phenomena may reflect the substituent effect of the adenine ring at the 1-*N* position. In contrast, the ^{13}C chemical shifts of 6-*N*-substituted adenine derivatives such as 6-*N*-methyladenosine did not change essentially compared with those of adenosine.[25]

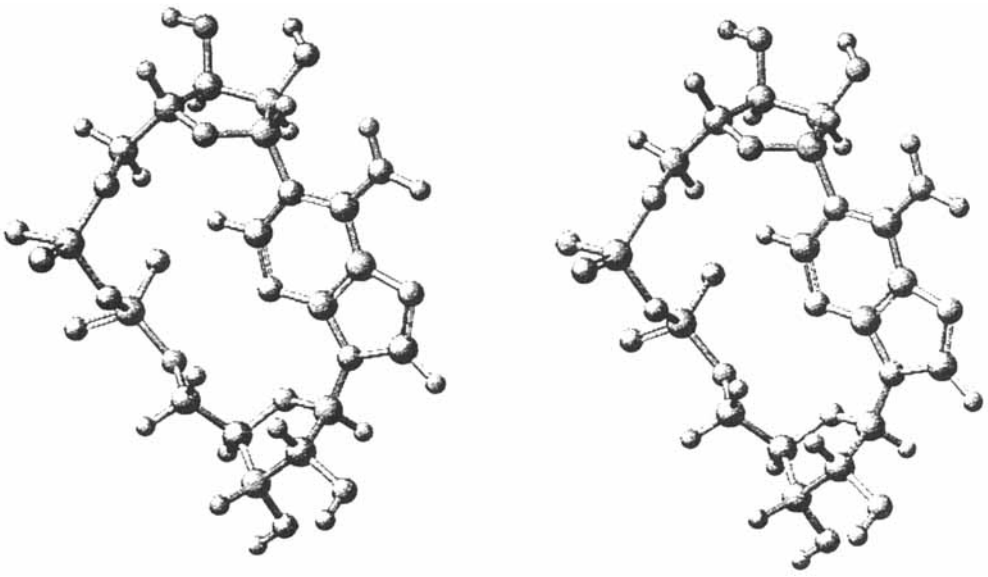


FIG 4. A probable structure of cADPR in neutral aqueous solution.

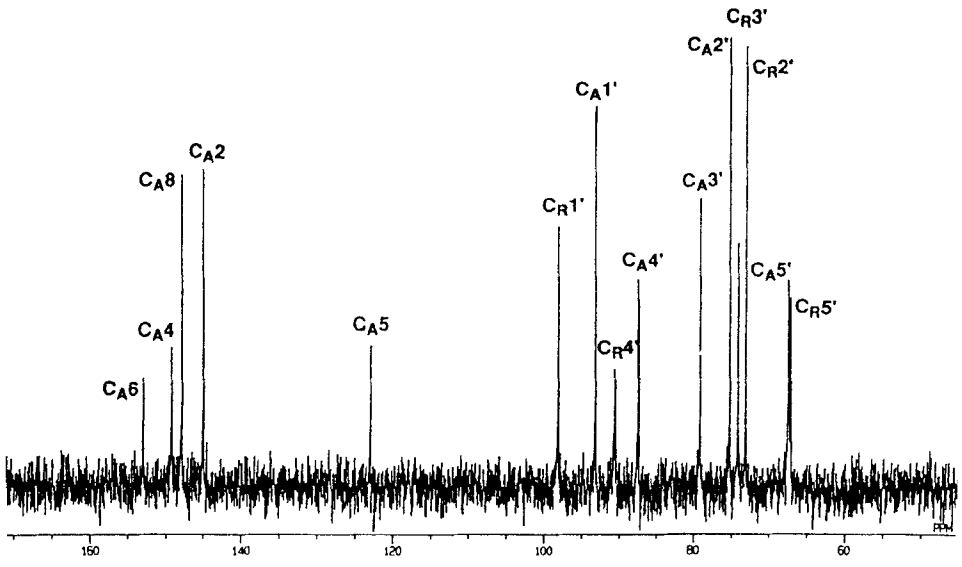


FIG 5. 67.8 MHz ^{13}C NMR spectrum of cADPR in D_2O at 227K.

TABLE 3. ^{13}C and ^{31}P NMR data of cADPR in D_2O.

	adenosine unit	ribose unit	5'-AMP[b]
^{13}C chemical shifts (ppm)[a]			
2	145.01	—	152.4
4	149.29	—	148.4
5	123.00	—	118.0
6	153.04	—	155.0
8	147.94	—	139.9
1'	93.26	98.11	87.0
2'	75.38	73.21	70.5
3'	79.28	74.25	74.5
4'	87.54	90.65	84.2
5'	67.55	67.26	63.5
^{13}C-^{31}P coupling constants (Hz)			
4', P	9.7	4.9	4.9[c]
5', P	8.5	d	8.5[c]
^{31}P chemical shifts (ppm)[e]			
	-9.92	-10.67	—
^{31}P-^{31}P coupling constants (Hz)			
	14.5	14.5	—

[a] DSS was used as an external standard at 227K.
[b] See reference 24.
[c] See reference 25.
[d] The coupling constant could not be determined.
[e] H_3PO_4 was used as an external standard at 286K.

The ^{31}P NMR spectrum of cADPR showed two resonance signals at -9.92 ppm and -10.67 ppm which are typical for pyrophosphate derivatives. The assignment of the chemical shifts was carried out by the use of ^{1}H-^{31}P COSY experiments (See experimental section). These results are summarized in TABLE 3.

In conclusion, analysis of the coupling constants of the sugar protons and the NOE data obtained by the 2D ROESY experiments suggested that sugar puckering of the adenosine unit prefers a C2'-endo conformation. In the ribose unit, the NOE data and the conformational analysis indicated that the furanose ring has an unusual flat conformation which is similar to those of 2',3'-*O*-isopropylidene nucleosides. It is also possible to propose the existence of a rapid equilibrium between the C2'-endo and C3'-endo conformers in the ribose unit. On the basis of the NOE data, it was concluded that cADPR in aqueous solution has a *t* conformation around both the C_A4'-C_A5' and the C_R4'-C_R5' bonds. Detailed study of constrained molecular dynamics of this molecule will be reported elsewhere. These results described here would be useful for designing cADPR analogs as potential antagonists. We are now studying the chemical synthesis of modified cADPR derivatives as well as cADPR. These results will be shortly reported.

EXPERIMENTAL

Materials

ADP-ribosyl cyclase was purified from ovotestes of *Aplysia kurodai*, a common species around the Japanese coast, by the reported method.[26] Cyclic ADP-ribose was prepared from β-NAD treated with the purified ADP-ribosyl cyclase. The crude product was purified by HPLC using a AG MP-1 resin (Bio Rad) and a Whatman Partisil 5 SAX column (0.46 x 10 cm), sequentially, to give the free acid of cADPR.[5] The free acid of cADPR (23 mg) was passed through a column of Dowex 50W x 8 (Na^+ form, 1.0 x 20 cm) and the eluate was lyophilized from water to afford the sodium salt of cADPR as a white powder. The material prepared showed a single peak in HPLC and was further characterized by ^{1}H NMR[5] and Ca^{2+}-release assay with microsomal fractions of sea urchin eggs.[2] The material manifested the potential activity of releasing Ca^{2+} ion from intracellular calcium store existing in sea urchin eggs *in vitro*.

NMR experiments

Free acid or sodium salt of cADPR (20 mg) was repeatedly lyophilized from D_2O and dissolved in 500 μl of D_2O (99.95%, Merck). One dimensional ^{1}H NMR spectra including difference NOE and homo-spin decoupled spectra were obtained at 270.0 MHz on a JEOL-EX-270 spectrometer or at 500.0 MHz on a JEOL-α-500 spectrometer with sodium 4,4-dimethyl-4-silapentane sulfonate (DSS) as an external standard in D_2O.

Resolution enhancement of spectra was carried out using a shifted sine-bell function. A two dimensional double quantum filtered COSY (DQF-COSY) spectrum was obtained on a JEOL-α-500 spectrometer using a phase-sensitive mode with the spectral width of 5998.80 Hz. The spectrum was acquired with 512 and 2048 data points in t_1 and t_2 domains, respectively. The data matrix was zero-filled to 2K x 2K and processed with a shifted sine-square function. ^{13}C NMR spectra were obtained at 67.8 MHz on a JEOL-EX-270 spectrometer with DSS as an external standard in D_2O. ^{31}P NMR spectra were obtained at 109.25 MHz on a JEOL-EX-270 spectrometer with 85% H_3PO_4 as an external standard in D_2O. A two dimensional heteronuclear single quantum correlation (HSQC) spectrum was obtained on a JEOL-α-500 spectrometer with the spectral width of 33898.31 and 5998.80 Hz for the ^{13}C and ^{1}H dimensions, respectively. The spectrum was acquired with 512 and 2048 data points in t_1 and t_2 domains, respectively. The data matrix was zero-filled to 1K x 2K and processed with a shifted-sine square function. A two dimensional ^{1}H-^{31}P COSY spectrum was obtained on a JEOL-EX-270 spectrometer with the spectral width of 5736.0 and 4000.0 Hz for the ^{1}H and ^{31}P dimensions, respectively. The spectrum was acquired with 256 and 2048 data points in t_1 and t_2 domains, respectively. The data matrix was zero-filled to 1K x 2K and processed with a shifted sine-bell function. A two dimensional rotating frame overhauser effect spectroscopy (ROESY) experiment was carried out on a JEOL-α-500 spectrometer using a phase-sensitive mode with the spectral width of 5998.80 Hz. The spectrum was acquired with 512 and 2048 data points in t_1 and t_2 domains, respectively. The data matrix was zero-filled to 2K x 2K and processed with a shifted sine-square function.

ACKNOWLEDGMENTS

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