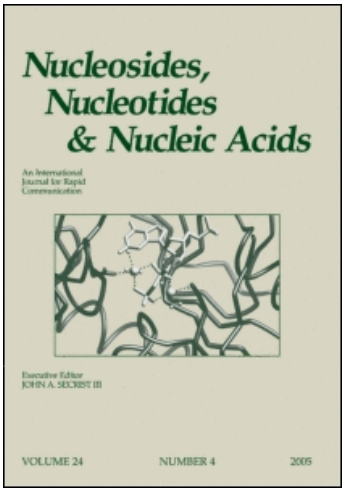

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NMR STUDIES AND SEMI-EMPIRICAL ENERGY CALCULATIONS FOR CYCLIC ADP-RIBOSE

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ABSTRACT

A possible pH-dependent conformational switch was investigated for cyclic ADP-ribose. NMR signals for the exchangeable protons were observed in H_2O at low temperature, but there was no direct evidence for the protonation of N-3 at neutral pH that has previously been postulated. MNDO calculations indicated that pH dependent ^{31}P chemical shift changes are attributable to protonation of the phosphate adjacent to the N-1 of adenine, and not due to trans-annular hydrogen bonding with a protonated N-3.

INTRODUCTION

Cyclic ADP-ribose (cADPR) was initially discovered as a potent mediator of calcium release from sea urchin egg microsomes[1]. In sea urchins this nucleotide activates membrane calcium channels distinct from those activated by inositol trisphosphate[2], and a physiological role in the fertilization of sea urchin eggs has been demonstrated[3,4]. In mammals, cADPR has been shown to induce calcium release from membrane preparations of various rat

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tissues including pituitary gland[5], heart, and brain[6]. The nucleotide appears to activate calcium channels associated with microsomal[6], mitochondrial[7], and plasma membrane fractions[8].

cADPR is synthesized from NAD^+ by cleaving the nicotinamide-ribose bond and cyclysing the ribose moiety to the N-1 position of the adenine ring[9–11]. The enzyme NAD(P)ase from the marine mollusk *Aplysia californica* that catalyzes the synthesis of cADPR has been purified and characterized[12–14]. The *A. californica* enzyme is inefficient in catalyzing the hydrolysis of cADP-ribose to ADP-ribose, in contrast to the mammalian enzyme, and is therefore referred to as ADP-ribose cyclase rather than an NAD(P)ase.

While cADPR is a potent activator of membrane calcium channels, free ADP-ribose is totally inactive[2]. Therefore, cADPR must possess unique structural features that promote channel activation.

Spectroscopic studies of cADPR using ^1H[15,16] and ^{31}P NMR[16,17] have been reported previously. It was recently noted that the resonance frequency of one of the two ^{31}P resonances is pH dependent (pK_a 8.3)[17]. The pH profile was also recorded by circular dichroism (CD) studies, and were interpreted in terms of a pH-dependent conformational switch[18]. One hypothesis is that the key pK_a for the conformational switch is protonation/deprotonation of N-3[17]. Trans-annular hydrogen bond formation involving the N-3 proton may then result in ^{31}P chemical shift perturbation. The objective of the study was to confirm the assignment of the pH dependent and pH independent ^{31}P resonances, ascertain whether N-3 is protonated at neutral pH, and investigate the conformational preferences at high and neutral pH. The protonation state of N-3 should be evident from the multiplicity of H-2, which occurs as a singlet in D_2O but would be split into a doublet by *J*-coupling with the N-3 proton in H_2O. In addition, a conformational search was performed to obtain more insight into the pH dependence of the ^{31}P NMR spectra.

RESULTS AND DISCUSSION

The synthesis of cADPR from NAD involves cleavage of the linkage between nicotinamide and ribose and cyclization to N-1 of the adenine ring[9–11]. It has been suggested that the structure has a formal charge on the adenine ring at neutral pH which is lost upon dissociation of a proton with a pK_a of about 8.3[9,17]. Figure 1 shows the deprotonated form of cADPR and the atom numbering designation.

^1H-^1H correlation spectra (COSY-45 and TOCSY) were acquired to verify the ^1H resonance assignments previously reported for cADPR. The ^1H assignments at pH 7.0 (Table 1) were almost identical to those of Wada *et al.*[16] (pH not given). Measured ^1H spin-coupling constants were also consistent with the previously reported values[16]. $^3J_{H4''\text{-}H5''}$ values indicated

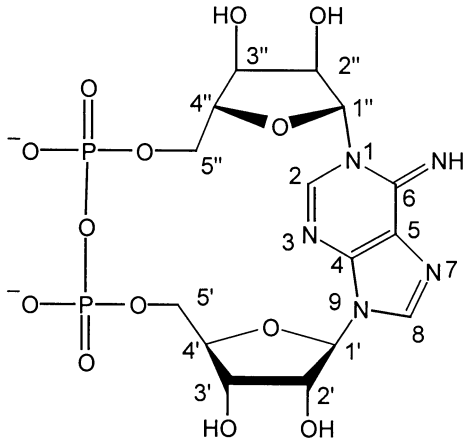

Figure 1. Structure and atom numbering for cyclic ADP-ribose.

Table 1. ^{1}H NMR Spectral Assignments for Cyclic ADP-Ribose in D_2O

	Proton Chemical Shift (ppm from DSS)				Spin-Coupling Constant (Hz)		
	a	b	c		a	b	c
Ribose Unit							
H-1″	6.14	6.17	6.06	$J_{H1''\text{-}H2''}$	3.7	3.9	2.7
H-2″	4.74	4.76(59)	4.54	$J_{H2''\text{-}H3''}$	5.1	5.3	4.7
H-3″	4.49	4.49	4.46	$J_{H3''\text{-}H4''}$	2.7	2.4	4.3
H-4″	4.73	4.75	4.56	$J_{H4''\text{-}H5a''}$	2.2	2.4	2.0
H-5a″	4.41	4.40	4.40	$J_{H4''\text{-}H5b''}$	2.2	2.4	2.0
H-5b″	4.15	4.14	4.15	$J_{H5a''\text{-}H5b''}$	−11.9	−11.9	−11.9
				$J_{H5a''\text{-}P}$	2.2	3.2	4.3
				$J_{H5b''\text{-}P}$	3.7	4.0	4.6
Adenosine Unit							
H-1′	6.08	6.09	5.99	$J_{H1'\text{-}H2'}$	5.6	5.9	6.3
H-2′	5.36	5.37	5.29	$J_{H2'\text{-}H3'}$	5.1	5.2	4.9
H-3′	4.76	4.76(57)	4.68	$J_{H3'\text{-}H4'}$	3.2	2.5	2.9
H-4′	4.38	4.37	4.36	$J_{H4'\text{-}H5a'}$	6.8	6.7	7.4
H-5a′	4.53	4.52	4.56	$J_{H4'\text{-}H5b'}$	2.6	2.5	2.7
H-5b′	4.07	4.06	4.08	$J_{H5a'\text{-}H5b'}$	−11.0	−11.4	−10.9
H-2	8.97	9.00	8.75	$J_{H5a'\text{-}P}$	3.9	4.2	4.3
H-8	8.38	8.39	8.17	$J_{H5b'\text{-}P}$	3.4	3.7	4.8

a. results from reference 16, unspecified pD, 13°C.
b. results from current study, pD 6.9, 20°C (0.4 Hz per point digital resolution).
c. results from current study, pD 9, 20°C (0.4 Hz per point digital resolution).

that the C-4″-C-5″ rotamer (for the ribose unit adjacent to N-1) was in the *gauche-gauche* orientation (H-4″ *syn*-clinal to both H-$5''_{proR}$ and H-$5''_{proS}$). In contrast for the C-4′-C-5′ rotamer, the estimated dihedral angles are $\sim 35°$ for H-4′-H-5a′ and $\sim 100°$ for H-4′-H-5b′, although the two C-5′ protons could not be assigned stereospecifically. The rotamer distribution indicated by the experimental data is not consistent with the conformational model proposed by Wada *et al*[16].

^{1}H-^{1}H NOESY experiments showed no trans-annular NOE, but there were correlations between H-2 and H-1″, and H-8 and H-1′ (Table 2). Comparison of NOESY spectra at pH 7 and at pH 9 showed an identical pattern (and relative intensity) of NOEs, indicating that there is no substantial pH-dependent conformational switch affecting the relative orientation of the adenine and ribose units.

At pH 9 there were some small but significant changes in proton chemical shifts and *J*-coupling constants (Table 1). The largest changes of 0.2 ppm were noted for δ H-2″ and δ H-4″, and of 1−2 Hz for $^3J_{H1''\text{-}H2''}$ and $^3J_{H3''-H4''}$, consistent with a minor change in the ring pucker of the ribose attached to N-1. A maximum change of approximately 15° in the dihedral angles was inferred from the changes in *J*-coupling constants, using Haasnoot's form of the Karplus approximation, which includes a compensation for the effects of substituent electronegativities[19]. There was no significant change in C-4-C-5 bond rotamers for either ribose unit, and the changes in $^3J_{P\text{-}H}$ values for all of the C-5 protons was insignificant. Hence it is difficult to discern any gross conformational changes from the NMR spectra. Proton chemical shifts in the adenine moiety showed upfield shifts of 0.2 ppm for both H-2 and H-8 on changing to higher pH.

Assignment of ^{13}C resonances was verified from a ^{1}H-^{13}C correlation spectrum (HSQC), and was consistent with the previous assignment[16], with the exception of the reversed assignment of C-2″ and C-3′. ^{13}C chemical shifts in the ribose attached to N-9 were virtually invariant to pH (Table 3), whereas those in both the adenine and the N-1 ribose showed changes between 0.3 and 3.0 ppm.

Table 2. NOE Observed for Cyclic ADP-Ribose at Neutral pH, 20°C, 85% H_2O, 15% Acetone-d_6 (from NOESY with 500 ms mixing time)

From	To			
H-2	H-1″ (weak)	H-3″ (weak)	H-5a″ (medium)	H-2″ or H-4″ (weak)
H-8	H-1′ (strong)	H-2′ (weak)		
H-1′	H-2′ (strong)	H-3′ (weak)	H-4′ (weak)	
H-2′	H-1′ (strong)	H-3′ (strong)	H-5a′ (weak)	H-8 (weak)
H-1″	H-2″ (strong)	H-3″ (weak)		

Table 3. Differences in ^{13}C Chemical Shift (ppm) for Cyclic ADP-Ribose, $\delta_{pH\,9}$-$\delta_{pH\,7}$, at 20°C in D_2O

C-1″	C-2″	C-3″	C-4″	C-5″	C-1′	C-2′	C-3′	C-4′	C-5′	C-2	C-4	C-5	C-6	C-8
−1.8	−0.7	−1.8	−1.5	−0.9	−0.2	−0.1	−0.2	−0.1	+0.1	+0.3	−2.8	+1.6	+3.0	−2.1

At pH 7, two strongly-coupled ^{31}P doublets ($^2J_{P-P} = 14.5 \pm 0.6$ Hz) were observed with apparent chemical shifts of −10.16 and −10.86 ppm, corresponding to the pH independent and pH dependent resonances, respectively, as previously reported[17]. At pH 9, the value of $^2J_{P\text{-}P}$ was slightly lower (13.3 ± 0.6 Hz). A ^1H-^{31}P COSY experiment correlated the downfield ^{31}P resonance (−10.16 ppm) with H-5′ resonances at 4.52 and 4.06 ppm. The higher field ^{31}P resonance showed correlations to H-5″ resonances at 4.40 and 4.14 ppm, and an additional correlation to H-4″ at 4.75 ppm. These results indicate that the pH dependent ^{31}P signal arises from P1, the phosphate nearest the ribose attached to N-1 of the adenine ring as shown by Wada *et al.*[16]

In order to observe the exchangeable protons (Fig. 2), a separate solution of cADPR was prepared in 85% H_2O, 15% $(CD_3)_2CO$ adjusted to

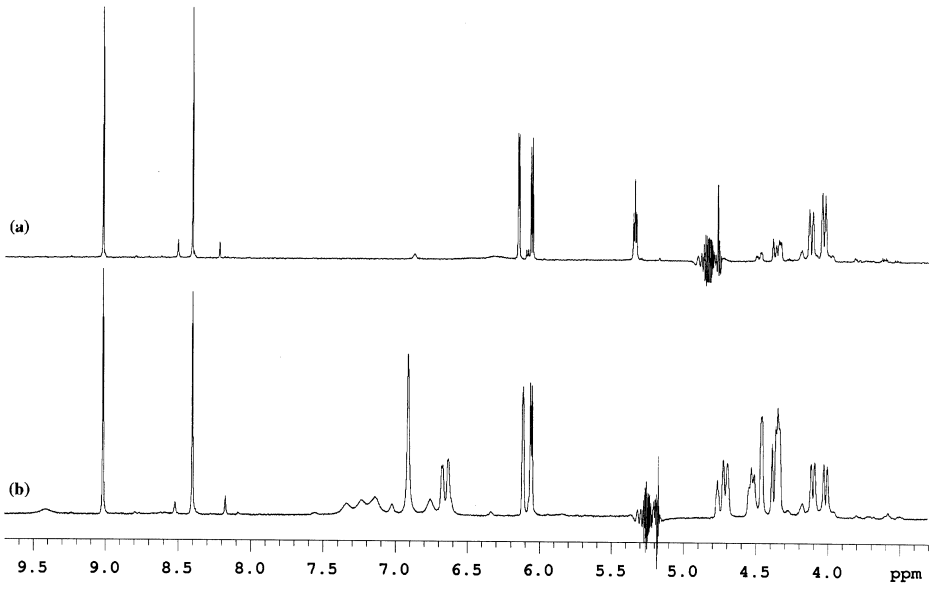

Figure 2. Partial ^1H NMR spectrum of cyclic-ADP ribose in 85% H_2O 15% acetone-d_6 at pH 6−6.5; (a) at 20°C, (b) at −15°C, revealing additional resonances from several exchangeable protons. Both spectra were recorded with water suppression, using the technique of double pulsed field gradient spin echo.

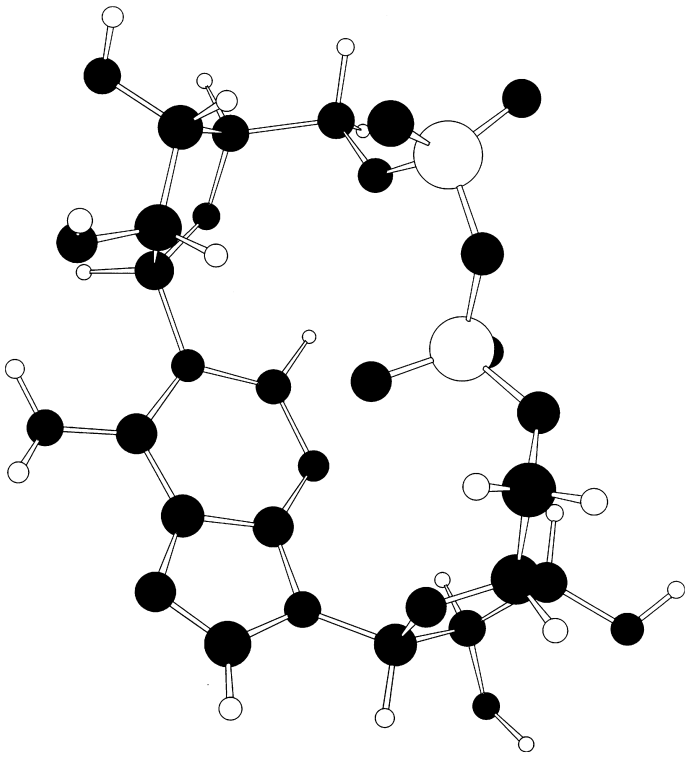

Figure 3. Minimum energy conformation for cyclic-ADP ribose, derived from a full conformational search using MNDO semi-empirical energy calculations.

approximately pH 6–6.5. ^{1}H NMR spectra were acquired with excitation sculpting[20] for water suppression. The sample temperature was reduced to −15°C in order to slow the rate of chemical exchange. The total integral of the exchangeable protons corresponds to seven proton equivalents (relative to the integral for the peak of the non-exchangeable H-2 proton), and there are at least seven distinct sites (Fig. 2), one of which integrates to two protons. All of the resonances in the region between 6.5 and 7.3 ppm produced exchange cross-peaks with the water signal in a NOESY experiment with 300 ms mixing time. From a COSY experiment the relatively sharp signals at 6.55 and 6.51 ppm were assigned to HO-2′ and HO-3′, respectively, and the broader signal at 6.64 ppm was assigned to HO-3″. A sharp signal at 6.79 ppm, integrating to two proton equivalents, was assigned to the N-6 amino-protons, consistent with observations of a similar signal for this amine group in analogous systems such as cyclic-AMP.

There were a further four partially resolved unassigned signals between 6.8 and 7.3 ppm, and a broader signal at 9.3 ppm, none of which showed any

correlations in the two-dimensional spectra (other than for exchange with water). The peaks at 9.3 and 6.9 ppm showed significantly lower intensity, and may originate from the decomposition product. The HO-3″ peak integral indicated 0.5 proton equivalents, and it is tentatively suggested that the three unassigned peaks may correspond to the remaining signals from hydroxyl protons of the N-1 ribose. Both of these protons occupy at least two distinct sites.

In the absence of any supporting evidence from the 2D NMR spectra it could not be excluded that one or more of these signals arises from a protonated nitrogen. There was no splitting of the C-2 proton signal by *J*-coupling with an N-3 proton, and hence no direct evidence to indicate protonation of N-3. Clearly, the identity of the protonation sites was equivocal, and semi-empirical energy calculations were used to assess the feasibility of the tentative model.

Heats of formation of several tautomers of cADPR were calculated using the MNDO parameter set. Global energy minima for each of the tautomers is given in Table 4. There is a clear preference ($\sim 70\,\text{kcal mol}^{-1}$) for protonation at one or other of the phosphate groups over either of the two adenine nitrogen atoms (N-3 or N-6). However, since the calculated energy difference for protonation of the two phosphate groups is less than $2\,\text{kcal mol}^{-1}$, it is not possible to differentiate which should have the lower pK_a on the basis of the energy calculations alone. That the lowest energy tautomer was in agreement with the ^{31}P NMR observations (in which P1 is protonated preferentially over P2) is fortuitous, given the uncertainty associated with such small energy differences in MNDO calculations. There need only be a minor preference for protonation of P1 for the first protonation to be selective; protonation of both of the phosphate groups is considerably disfavoured. It is interesting to note that the N-3 tautomer proposed in previous work is the least stable of the tautomers. Seven conformers of the N-3 tautomer show hydrogen bonding from H-N-3 to the phosphate groups, either to P1 ($-466.24\,\text{kcal mol}^{-1}$) or P2 ($-473.43\,\text{kcal mol}^{-1}$) terminal oxygen atoms, but also to the P1-ribosyl linking oxygen atom ($-476.10\,\text{kcal mol}^{-1}$).

Table 4. MNDO Heats of Formation Calculated for Singly Negatively Charged Tautomers of Cyclic ADP-Ribose

Tautomer	Energy (kcal mol^{-1})
N-6	− 483.38
N-3	− 476.10
P-1	− 544.64
P-2	− 542.75

MNDO is known to reproduce poorly hydrogen-bond geometries and energies but it unlikely that this could account for the 70 kcal mol^{-1} energy difference.

CONCLUSION

The previous hypothesis of intramolecular hydrogen bonding between a protonated N-3 and phosphate in cADPR was not supported by NMR data or semi-empirical energy calculations. The charge state of the diphosphate group does not exert conformational control that can be detected in the NMR spectra for cADPR. Since there is no pH dependence of the ^{31}P chemical shifts in acyclic ADP-ribose in the range pH 6.4–9.4[17] we suggest that conformational restriction in the cyclic form of ADP-ribose modulates the charge state of the phosphate, which appears to be critical to calcium channel activation by cADPR, in contrast to the acyclic compound.

Wong *et al*. recently reported that 3-deaza-cADPR releases calcium through the same mechanism as cADPR[21], but the nitrogen to carbon substitution at the 3-position of the purine increased the calcium releasing activity. The absence of a trans-annular hydrogen bonding capability to the adenine 3-position evidently did not result in an inactive conformation of the ligand.

EXPERIMENTAL

cADPR was prepared as described by Hellmich and Strumwasser[12]. The purity of the final preparation was initially greater than 98% as judged by HPLC[9] with the remaining material being free ADP-ribose. Upon storage at neutral pH at $< -20°C$, there was a slow conversion of cADPR to free ADP-ribose. The identity of the cADPR was also confirmed by FAB-mass spectrometry. The m/z values for the positive and negative ions were 540 and 542, respectively.

A D_2O (100 atom %) buffer solution, was prepared containing 50 mM tris-d_{11} (perdeuterated tris(hydroxymethyl)methylamine), 20 mM EDTA and DSS (sodium salt of dimethyl silapentane sulfonate). The buffer was adjusted to neutral pH using deuterium chloride. cADPR (2.46 mg, 9.1 mM) was dissolved in 500 µl buffer, and a glass pH probe inserted into the NMR tube indicated pD 6.9 (corrected for D_2O isotope effect by subtracting 0.4 from the meter reading).

All NMR experiments were performed at a nominal probe temperature of 20.0°C, except where stated otherwise. Spectra were recorded using a Varian Unity-*plus* spectrometer operating at 500.3 MHz ^1H, 202.5 MHz ^{31}P,

and 125.8 MHz ^{13}C. ^{1}H and ^{31}P chemical shifts were measured from one-dimensional spectra (digital resolution 0.4 Hz/pt for ^{1}H and 0.6 Hz/pt for ^{31}P). Chemical shifts were referenced to internal DSS at 0.00 ppm for ^{1}H, ^{13}C and external orthophosphoric acid (85% v/v in H_2O) in a coaxial glass capillary at 0.00 ppm for ^{31}P. For the ^{1}H acquisition a repetition time of 11.4 s ensured complete relaxation between successive transients. The ^{31}P spectrum was recorded with 1.3 s repetition time, and with ^{1}H-decoupling (WALTZ, 3.2 kHz field).

In all two-dimensional NMR experiments the ^{1}H spectral width was 5912 Hz (2.9 Hz/pt digital resolution in the processed data), the recycle time between successive transients (relaxation delay + acquisition time) was 1.8 s, and the data were zero-filled in the f_1 dimension. The COSY-45 was processed with unshifted sine-bell weighting in both dimensions, with 5.8 Hz/pt digital resolution in f_1. For all other spectra, cosine-bell weighting was applied to both dimensions prior to transformation. The ^{1}H-^{1}H total correlation spectroscopy (TOCSY) experiment was recorded with 100 ms MLEV-17 spin-lock in a 10.0 kHz B_1 field. The digital resolution was 5.8 Hz/pt in f_1. The ^{1}H-^{31}P COSY experiment[22] was acquired with digital resolution was 3.0 Hz/pt in f_1. The heteronuclear single quantum coherence[23] (HSQC) ^{1}H-^{13}C correlation experiment was tuned for optimum sensitivity at $J_{CH} = 150$ Hz ($\Delta = 3.3$ ms), and included GARP decoupling of ^{13}C during t_2. The f_1 digital resolution was 45.4 Hz/pt.

All modelling calculations were performed with the MNDO parameter set[24,25] and hamiltonian using MOPAC. It was found that more recent NDDO type methods such as AM1 and PM3 gave incorrect geometries for the diphosphate. Optimisation of simple diphosphates with AM1 and PM3 gave a P-O-P bond angle of 180° whereas MNDO optimisation results in a P-O-P bond angle of around 140°, comparable with X-ray data. As optimisers will only find the closest minimum to any given starting point it is necessary to consider a good many different starting points in order to be sure of finding the global minimum energy conformation. This can be problematic with comparatively small rings of limited flexibility.

The heterocycle was broken between the two phosphate groups to generate a linear analogue, X. Consideration of the available minima for all the rotatable bonds generated 2916 separate conformations. Only those conformations (111 in total) with both phosphate groups on the same face of the adenine and reasonably close to each other (P-O distance ≤ 3.8 Å). These conformations were cyclised via the shortest P-O contact and subjected to molecular mechanics optimisation (AMBER force field, no charges) to quickly correct bond lengths and angles. 7 duplicates and 20 close similarities were eliminated at this stage, leaving 84 conformations for semi-empirical MO optimisation.

MOPAC optimisation allowed the elimination of a further 39 conformations (7 gave incorrectly bonded structures, 23 gave duplicate con-

formations and 9 gave conformations with close similarities to others) resulting in 45 starting points for each of the tautomers.

This range of starting points allows us to identify global energy minima with confidence and also indicates the degree of conformational variation between the tautomers.

We have only calculated energies and conformations for the singly charged tautomers and not the doubly charged species as the latter could not reliably reach self-consistency in the MO calculations. As a result we cannot identify computationally any conformational changes occurring as a result of the deprotonation at pH 8.3, though we can identify which proton is lost. A full conformational analysis was carried out for tautomers with the N-6 proton transferred to N-3 and each of the phosphate oxygen atoms.

ABBREVIATIONS

NMR, nuclear magnetic resonance; D_2O, deuterium oxide, COSY, correlated spectroscopy; ADP-ribose, adenosine diphosphoribose; NOE, nuclear Overhauser effect, HSQC, heteronuclear single quantum coherence; TOCSY, total correlation spectroscopy; DSS, dimethyl silapentane sulfonate; tris-d_{11} (perdeuterated tris(hydroxymethyl) methylamine; cADPR, cyclic adenosine diphosphoribose.

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