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NOVEL ANALOGS OF CYCLIC-ADP-RIBOSE: 9-CYCLIC ETHENO-ADP-RIBOSE AND CYCLIC ETHENO-CDP-RIBOSE

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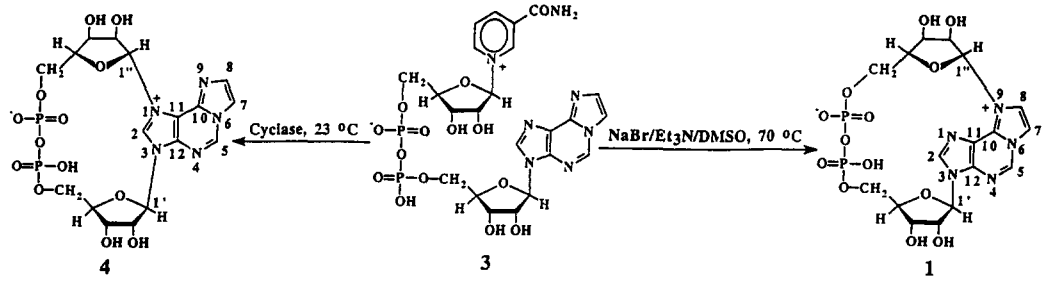
Abstract: Two novel analogs of cyclic ADP-ribose (cADPR), 9-cyclic etheno-ADP-ribose (**1**) and cyclic etheno-CDP-ribose (**2**) were synthesized. We have shown for the first time that enzymatic and biomimetic methods may proceed by different reaction pathways. 1,N^6-Etheno-nicotinamide-adenine dinucleotide (**3**) was converted into **1** using the biomimetic procedure, whereas ADP-ribosyl cyclase transformed **3** into **4**. The unique fluorescence property and the strong Ca^{2+} mobilizing activity of **1** provide investigators with a useful probe for the study of cADPR-binding proteins.

Cyclic ADP-ribose (cADPR) is a novel metabolite of NAD and its synthesizing enzyme, ADP-ribosyl cyclase, has been shown to be widely distributed among mammalian and invertebrate tissues.[1] Currently, there is considerable interest in defining the biological roles of cADPR as a second messenger candidate in Ca^{2+} signaling[2] and insulin release.[3] The structure and absolute stereochemistry of cADPR have now been firmly established by X-ray crystallography[4] and by synthesis.[5]

New structural analogs of cADPR can now be prepared using either the biomimetic route[5b] or the ADP-ribosyl cyclase from *Aplysia californica*.[6] For the most part, both procedures give similar product profiles, but higher yields are generally obtained with the enzymatic method. These synthetic methodologies have allowed the transformation of a variety of structurally modified analogs of NAD into their corresponding cyclic nucleotides with the newly formed glycosyl bonds attached to the N-1 nitrogen of the purine rings as in cADPR.[7] However, when the adenine ring in NAD was replaced by guanine or hypoxanthine, an alternative mode of cyclization was observed wherein the N-7 nitrogen of the purine rings were used to form the glycosyl bonds.[8]

Our continuing interest in the synthesis of more active and stable analogs of cADPR as fluorescent affinity probes for investigations of cADPR-binding proteins led us to the preparation of two novel analogs. Herein, we describe the experimental details used in the synthesis and characterization of 9-cyclic etheno-ADP-ribose, **1** and cyclic etheno-CDP-ribose, **2**.

We have previously reported that ADP-ribosyl cyclase catalyzed the conversion of 1,N^6-etheno-Nicotinamide-adenine dinucleotide (**3**) into the cyclic nucleotide (**4**) whose N-glycosyl bond is attached onto the N-1 position of the 1,N^6-etheno-adenine nucleus corresponding to the N-7 position of the adenine ring as shown.[9] However, when **3** was subjected to the conditions of biomimetic synthesis,[5b] we were unable to detect the formation of **4**. Instead, a novel fluorescent cyclic nucleotide was isolated in 6% yield.



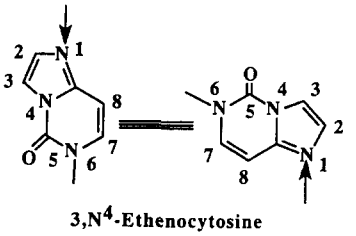
In a representative experiment, εNAD (**3**) (25 mg, 36 μmol) and NaBr (388 mg, 3.77 mmol), dried over P_2O_5 in a vacuum desiccator, were dissolved in 5 mL of freshly distilled DMSO (dried by refluxing over CaH_2 for 20 h). To this solution was added triethylamine (12.5 μL), and the mixture was stirred at 70 °C for 1 h under an atmosphere of argon. After the solution was cooled, the products were precipitated by the addition of cold ethanol (25 mL). The solid was collected by centrifugation and was purified on a Synchropak AX300 column (250 × 10 mm i.d., 5 μm) using a linear gradient of 0.1-0.31 M ammonium formate in 12 min at a flow rate of 2 mL/min, followed by an isocratic elution using 1 M ammonium formate until 20 min at a flow rate of 3 mL/min. Fractions containing 9-cεADPR (**1**) (retention time 8.2 min) were combined and further purified on a Waters Nova-Pak C18 column (100 × 8 mm i.d., 4 μm). The column was eluted isocratically with 1 mM TFA at a flow rate of 0.8 mL/min and **1** (retention time 5.8 min) was obtained in 6% yield. ^{1}H NMR (300 MHZ, D_2O, pH 3.0): δ 3.85 (1H, d, J = 7.5 Hz), 4.0-4.20 (3H, m), 4.34 (1H, brs, H4″), 4.39 (1H, brs, H4′), 4.49 (1H, t, J = 4.5 Hz, H3″), 4.57 (1H, t, J = 4.5 Hz, H3′), 5.12 (1H, d, J = 4.0 Hz, H2′), 5.30 (1H, t, J = 4.0Hz, H2″), 6.25 (1H, d, J = 2.0 Hz, H1″), 6.29 (1H, s, H1′), 8.03 (1H, d, J = 2.0 Hz, H8), 8.25 (1H, d, J = 2.0Hz, H7), 8.69 (1H, s, H2), 9.32 (1H, s, H5). ^{13}C NMR (300 MHZ, D_2O, pH 3.0) δ 145.3 (C12), 143.8 (C2), 138.8 (C5), 137.8 (C10), 124.1 (C8), 120.5 (C11), 115.4 (C7), 93.4 (C1″), 91.7 (C1′), 85.2 (C4″), 84.8 (C4′), 75.5 (C2′), 74.3 (C4″), 70.8 (C3′), 70.1 (C3″), 64.5 (C5′ and C5″). UV λ_{max} (pH 7.0) 278 (ε 9,000) nm. FAB MS *m/e* 564 ($[M-H]^+$).

It is well documented that alkylation of 1,N^6-ethenoadenosine derivatives with various alkylating agents gave N-9 alkylated products. Since the UV, ^{1}H and ^{13}C NMR spectra of **1** were very similar to those of N-9 methylated ethenoadenosine derivatives,[10,11] it is reasonable to assume that the gross structure of **1** is a cyclized product in which the newly formed glycosyl bond is attached to the N-9 instead of the N-1 position. This proposition was confirmed by the following NMR data: the 1D proton spectrum of **1** showed two singlets at δ 9.32 and 8.69 corresponding to H5 and H2, two doublets at δ 8.25 and 8.03 (H7 and H8), two signals at δ 6.29 and 6.25 for H1′ and H1″, and signals for the other ribosyl protons in the region of δ 3.85-5.30. The distinctions between H5 and H2 and between H8 and H7 were made by the 2D NOESY spectrum which showed the correlations between H7 and H5, H8 and H1″ and H2 and H1′. These data are consistent with the view that

Table 1. ^{1}H-^{13}C connectivities for 9-cεADPR established by HMBC spectrum

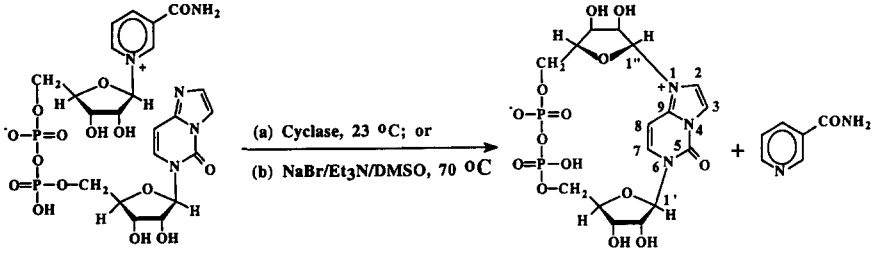
	^{13}C (δ)	H2	H5	H7	H8	H1′	H1″
C2	143.8	HC				HCNC	
C5	138.8		HC	HCNC			
C7	115.4			HC	HCC		
C8	124.1			HCC	HC		HCNC
C10	137.8		HCNC	HCNC	HCNC		HCNC
C11	120.5	HCNC					
C12	145.3	HCNC	HCNC				
C1′	91.7	HCNC				HC	
C1″	93.4						HC

cyclization of **3** proceeded via the union of the C1″ position of the ribose to the N-9 of the 1, N^6-ethenoadenine ring. The assignments of the ribosyl protons were made from the H-H COSY spectrum. For example, H1′ correlated with H2′ (δ 5.12), H2′ correlated to H3′ (δ 4.57), and H3′ to H4′ (δ 4.39), etc. However, the correlation between H5′ and H4′, as well as H5″ and H4″, was not detectable. With all the protons assigned, the corresponding carbons were deduced from the HSQC (Heteronuclear single-bond correlation) spectrum and the assignments are listed in the text. The structural assignment of **1** was further substantiated by the three-bond couplings shown in its HMBC (heteronuclear multibond correlation) spectrum (Table 1).



3,N^4-Ethenocytosine

Since no cADPR analog containing a pyrimidine nucleus has been prepared, we attempted the cyclization of nicotinamide cytosine dinucleotide using the biomimetic approach, as well as the Aplysia cyclase. To our surprise, in both cases, no cyclized product was detected, which suggested that a bicyclic ring system may be required for cyclization. This led us to the consideration of the deployment of 3,N^4-ethenocytosine nucleus as a mimic of the adenine ring.



5 2

Indeed, when **5**[12] was exposed to the Aplysia cyclase,[6] a new metabolite (**2**) was generated in 92% yield, accompanied by a stoichiometric quantity of nicotinamide. Upon treatment of **5** using the biomimetic method as described for εNAD (**2**) was also obtained, but in a lower yield (8%). It was purified as follows: the reaction mixture was first chromatographed on a Synchropak AX300 column (250 × 10 mm i.d., 5 μm) using a linear gradient of 0.1-0.37 M ammonium formate in 12 min at a flow rate of 2 mL/min, followed by isocratic elution using 1 M ammonium formate until 20 min at a flow rate of 3 mL/min. Fractions containing cεCDPR (retention time 8.4 min) were then combined and further purified on a Waters Nova-Pak C18 column (100 × 8 mm i.d., 4 μm). The column was eluted isocratically with 1 mM TFA at a flow rate of 0.9 mL/min and cεCDPR (retention time, 4.0 min) was obtained as a white solid. ^{1}H NMR (D_2O, 300 MHZ, pH 3.0) δ 4.15-4.80 (10 H, m), 5.99 (1 H, d, J = 6.0 Hz, H1″), 6.07 (1H, s, H1′), 7.76 (1H, d, J = 8.2 Hz, H8), 7.93 (1H, d, J = 2.4 Hz, H2), 8.09 (1H, d, J = 2.4 Hz, H3), 8.79 (1H, d, J = 8.2 Hz, H7). ^{13}C NMR (D_2O, 300 MHZ, pH 3.0) δ 145.0 (C5), 144.4 (C9), 138.8 (C7), 124.7 (C2), 114.8 (C3), 94.9 (C1″), 94.5 (C8), 93.2 (C1′), 86.5, 84.3, 75.4, 75.2, 70.4, 68.6, 65.7, 63.7. UV λ_{max} (pH 1.0 or 7.0) 294 (ε12,000) nm. ESI MS *m/e* 540 ([M-H]$^-$).

Table 2. ^{1}H-^{13}C connectivities for cεCDPR established by HMBC spectrum

	^{13}C (δ)	H2	H3	H7	H8	H1′	H1″
C2	124.7	HC	HCC				HCNC
C3	114.8	HCC	HC				
C5	145.0			HCNC			
C7	138.8			HC	HCC	HCNC	
C8	94.5			HCC	HC		
C9	144.4	HCNC	HCNC	HCCC	HCC		HCNC
C1′	93.2			HCNC		HC	
C1″	94.9						HC

Table 3. ^1H-^1H connectivities for cεCDPR established by NOESY spectrum

	^1H (δ)	H2	H3	H7	H8	H1′	H1″
H2	7.93		+				+
H3	8.09	+					
H7	8.79				+	+	
H8	7.76			+			+
H1′	6.07			+			
H1″	5.99	+			+		

The mass spectrum of **2** showed a molecular ion at 540 ([M-H]$^-$) and its UV, ^1H and ^{13}C NMR spectra were very similar to 1-alkylated 3,N^4-ethenocytidinium chloride,[11,13] suggesting that this compound could be a new cyclic nucleotide with the newly formed glycosyl bond attached to the N-1 position of the ethenocytosine ring. This structural assignment was further verified by a series 2D NMR experiments (COSY, NOESY and HMBC). As shown in Table 2, three-bond couplings were observed between H1″ and C2 and C9; the NOESY spectrum showed the correlations of H1″ with H2 and H8 (Table 3).

Both **1** and **2** exhibited unique fluorescence properties. The emission maximum of **1** at pH 7.0 was at 358 nm upon excitation at 290 nm and the intensity was about two times higher than that of εNAD. When excited at 290 nm, **2** showed an emission maximum at 356 nm with an intensity about half of that of εNAD. Under the same conditions, the fluorescence of εNCD was negligible. No significant changes in the fluorescence properties of **1** and **2** were observed when the pH value of the solution was lowered from 7.0 to 1.0.

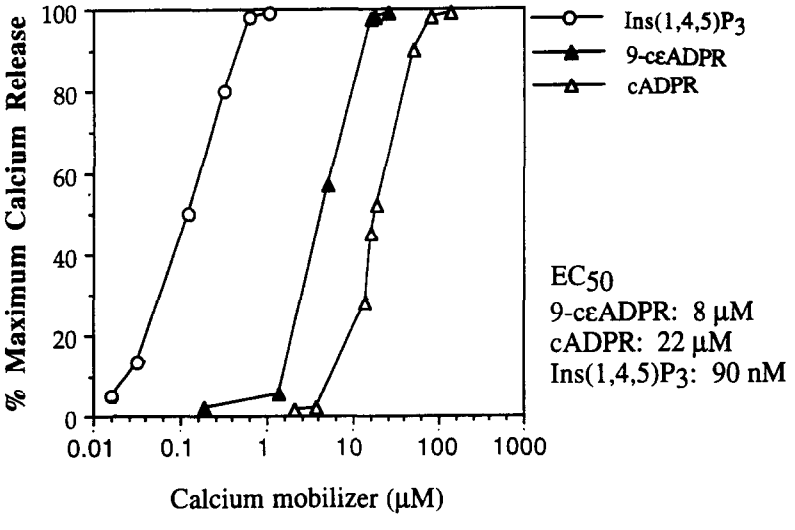


Figure 1. Dose response curve of Ca^{2+} release from rat brain microsomes

Ca^{2+} release in the rat brain microsomal system was measured as described by White et al.[14] As shown in Figure 1, the half-maximal effective concentration (EC_{50}) of 9-cεADPR (**1**) was 8 μM, which is about two times more potent than cADPR. When the microsomes were first desensitized by cADPR, further addition of 9-cεADPR did not induce Ca^{2+} release, but these desensitized microsomes still responded to IP_3. This result indicated that **1** and cADPR modulated Ca^{2+} release through the same mechanism, but different from that of IP_3. No Ca^{2+} release activity was observed with cεCDPR (**2**).

In conclusion, we have successfully synthesized two new analogs of cADPR, **1** and **2**. For the first time, we have observed that enzymatic and biomimetic methods can proceed by different reaction pathways to give different cyclization products. We surmise that in the enzymatic cyclization of **3**, product formation was determined by the conformation of the enzyme-substrate complex whereas in the biomimetic route, product formation depended on the electrophilic properties of the nitrogens of the heterocyclic ring. The importance of substrate structure in dictating product formation was further demonstrated by the ability of the cyclase to transform **5** into **2**. The unique fluorescence behavior and the strong Ca^{2+} mobilizing activity of **1** provide investigators with a useful probe for the study of cADPR-binding proteins.

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