Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 15, pp. 1701-1706, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00288-X

ENZYMATIC CYCLIZATION OF 1,N^6-ETHENO-NICOTINAMIDE ADENINE DINUCLEOTIDE

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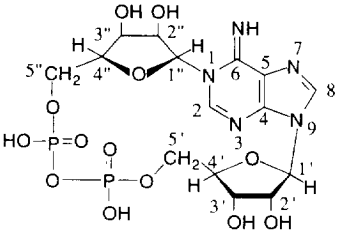
ABSTRACT: The ADP-ribosyl cyclase from *Aplysia californica* catalyzed the transformation of 1,N^6-etheno-nicotinamide-adenine dinucleotide (**2**) into a novel cyclic nucleotide, **3**. The newly formed glycosyl linkage is attached onto the N-1 position of the etheno-adenine ring corresponding to the N-7 position of the adenine nucleus. This alternative mode of cyclization gives more versatility in the biosynthesis of novel analogs of cADPR (**1**).

Cyclic ADP-ribose (cADPR), **1**, is a naturally occurring metabolite of NAD and a potent Ca^{2+}-mobilizing agent.[1] It has been implicated as a novel second messenger in the regulation of calcium homeostasis.[2]



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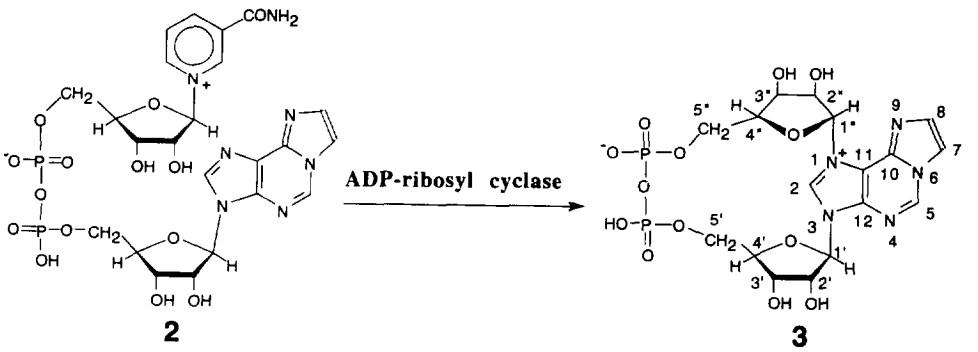
ADP-ribosyl cyclase (cyclase), the enzyme that catalyzes the biosynthesis of cADPR from NAD, is widely distributed in mammalian and invertebrate tissues.[3] The ovotestis of *Aplysia californica* is uniquely rich in cyclase activity. This cyclase has been purified, sequenced[4] and appears to have broad substrate specificity. It is now commercially available from Sigma.

Etheno(ϵ)-bridged nucleotides have served as valuable probes for the structural diagnosis of tRNA and DNA and carcinogenesis.[5] Moreover, many enzyme-coenzyme interactions have been examined by virtue of the fluorescent properties of etheno-substituted nucleotides, which have provided useful

information regarding binding sites, distances between cofactor or substrate and selected amino acids, induced conformational change, differentiation between regulatory and catalytic sites, and location of proximate amino acids by additional affinity labeling. Hence, a cyclic etheno analog of cADPR could serve as an useful probe for our investigation of cADPR binding proteins. However, it is uncertain whether ADP-ribosyl cyclases would accept $1,N^6$-etheno-nicotinamide adenine dinucleotide (ϵNAD), **2**, as a substrate since its N-6 position, corresponding to the N-1 position of the adenine ring, is substituted. In this paper, we report the isolation and identification of a metabolite after exposure of **2** to ADP-ribosyl cyclases. It is a novel cyclic nucleotide whose N-glycosyl bond is attached onto the N-1 position of the $1,N^6$-etheno-adenine nucleus or the N-7 position of the adenine ring as shown in **3**.



2 **3**

In contrast to other ADP-ribosyl cyclases, the *Aplysia* cyclase is virtually devoid of NADase and cADPR hydrolase activities. This is reflected in the HPLC profile of an incubation mixture of ϵNAD (**2**) with the *Aplysia* cyclase (Fig. 1). cϵADPR, **3**, was obtained in 91% yield accompanied by nicotinamide and a small quantity of residual **2** but no significant quantity of ϵADP-ribose (ϵADPR, **4**) was detected.

In a typical experiment, 6.5 mg (9.5 μmol) of **2** was incubated with 100 μg of the *Aplysia* cyclase (specific activity 50 units/mg) in 6.5 mL of 0.1 M potassium phosphate buffer, pH 5.3. The reaction mixture was incubated for 8 hrs at 24 °C and the enzyme protein was removed by ultrafiltration. The filtrate was concentrated under reduced pressure and then subjected directly to reverse phase HPLC separation since no **4** was detectable in the mixture (Fig. 1). The reverse phase chromatography was performed on a Hitachi L-6200A instrument equipped with a L-3000 diode array spectrometric detector set at 260 and 290 nm. The column was a Waters Nova-Pak C18 (100 x 8 mm i.d., 4 μm) from Millipore. Solvent A was 3 mM trifluoroacetic acid (TFA) and solvent B was 5% acetonitrile in 3 mM TFA. The column was first eluted with solvent A at a flow rate of 0.9 mL/min for 5 min, followed by solvent B at a flow rate of 3 mL/min. The retention times (r.t.) for nicotinamide, **2** and **3** were 6.4, 7.6, and 4.3 min

respectively. Fractions containing **3** (r.t. 4.3 min) were combined and evaporated to dryness under reduced pressure to yield 4.9 mg of 3 (91%) as a white solid. ^{1}H NMR (300 MHz, D_2O, pH 3.5) δ3.97-4.17 (2H, m), 4.24-4.37 (4H, m), 4.43-4.57 (4H, m), 6.36 (1H, d, J=4.0 Hz, H-1′ or H-1″), 6.53 (1H, d, J=3.5 Hz, H-1″ or H-1′), 7.65 (1H, d, J=1.8 Hz, H-8), 8.07 (1H, d, J=1.8 Hz, H-7), 9.30 (1H, s, H-5), 9.34 (1H, s, H-2) ppm. UV λ_{max} (pH 1.0 and 7.0) 277 nm (ϵ 8,000). FAB MS m/e 566 (M^+); 606 $(M+K+H)^+$.

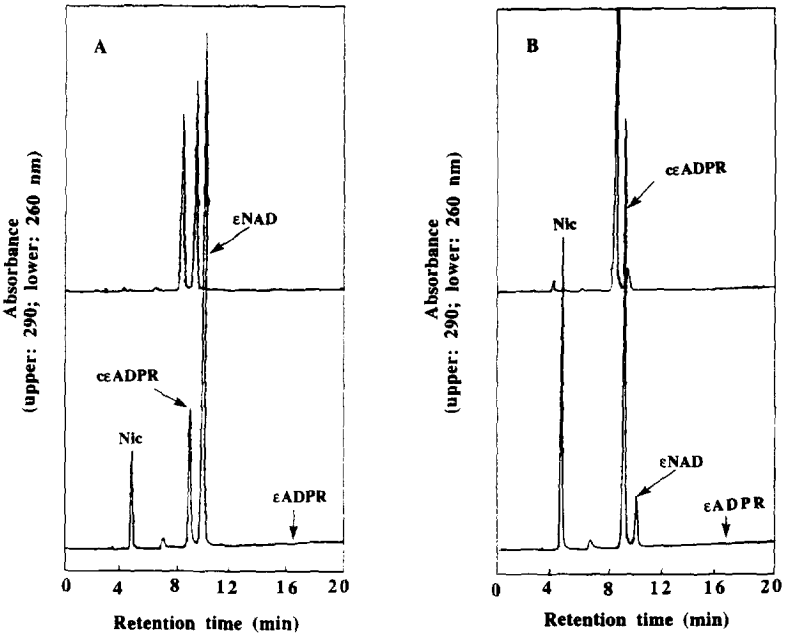


FIGURE 1. Synthesis of cεADPR (**3**) from **2** using *Aplysia* cyclase. **2** (6.5 mg, 9.5 μmol) was incubated with 5 units of cyclase in 6.5 mL of 0.1 M phosphate buffer, pH 5.3. Samples were analyzed by anion-exchange HPLC on a Synchropak AX-100 column (250 x 7.8 mm i.d., 5 μm) immediately after 1 hr (A) and 8 hr (B) of incubation. Nic is nicotinamide. The column was eluted using a linear gradient of 0.1 M and 1.0 M ammonium formate (solvent A and B) to 30% for 12 min at a flow rate of 2 mL/min, followed by elution using solvent B at a flow rate of 3 mL/min until 20 min. The retention time for Nic, **3**, **2**, and **4** were 4.6, 9.2, 10.0, and 17.0 min respectively.

Since a stoichiometric quantity of nicotinamide is released, by analogy to the transformation of NAD to **1**, we surmised that a cyclic nucleotide is similarly generated via the formation of a new glycosyl linkage. This supposition is supported by its ^{1}H NMR spectrum showing the presence of the singlets at δ9.30 and 9.34, corresponding to the H-5 and H-2 protons of the 1,N^6-etheno-adenine (ϵ-adenine) nucleus, and the FAB mass spectrum exhibited a molecular ion, m/e at 566. There are four sites that the anomeric carbon of the ribose could conceivably attach onto the ϵ-adenine nucleus: N-1, N-4, N-6, and N-9. It is well documented that alkylation of 1,N^6-ethenoadenosine derivatives with alkylating agents

always yield N-9-alkylated products.[6] Thus, it is reasonable to make the supposition that the enzymatic cyclization of **2** is likely to proceed via the linkage of the C-1″ position of the ribose to the N-9-nitrogen of the 1,N^6-etheno-adenine ring. However, a comparison of the ^1H and ^{13}C NMR spectra of **3** to the compound 9-methyl-β-D-ribofuranosylimidazo[2,1-i]purinium chloride[6a] showed considerable differences. Consequently, a series of NMR experiments was conducted to deduce its chemical identity.

In the 2D-NOESY spectrum, it was observed that one aromatic proton at δ9.34 correlated with the protons on both of the ribosyl units. This can only occur if the newly formed glycosyl bond is attached to the N-1 position. Additional features of the spectrum are also consistent with this assignment. For example, the H-7 and H-8 correlated with each other and H-7 also correlated with H-5. The structure of **3** was further confirmed by the 2D HMBC spectrum[7] which showed that the two anomeric protons (H-1′ and H-1″) exhibited intense cross-peaks due to their respective three-bond couplings with C-2 at δ134.8. The main ^1H-^{13}C connectivities for **3** are summarized in Table 1, from which the skeletal structure of **3** was unambiguously assigned.

TABLE 1. ^1H-^{13}C connectivities for **3** established by HMBC spectrum

	^{13}C (δ)	H-2	H-5	H-7	H-8	H-1′ or H-1″	H-1″ or H-1′
C-2	134.8	HC				HCNC	HCNC
C-5	142.6		HC	HCNC			
C-7	116.2			HC	HCC		
C-8	135.4			HCC	HC		
C-10	135.6		HCNC	HCNC	HCNC		
C-11	115.0	HCNC					
C-12	137.4	HCNC	HCNC				
C-1′ or C-1″	92.0	HCNC				HC	
C-1″ or C-1′	94.6	HCNC					HC

Recently, Lee and coworkers[8] reported the conversion of nicotinamide guanine dinucleotide (NGD) into cyclic GDP-ribose (cGDPR) with a structure similar to cADPR except guanine replacing adenine. In contrast, the newly formed glycosyl bond in **3** is attached to a position corresponding to the N-7 position of the adenine ring. This latter mode of cyclization is similar to the biomimetic cyclization recently developed in our laboratory.[9] Both NGD^+ and nicotinamide hypoxanthine dinucleotide (NHD) were cyclized nonenzymically and the glycosyl bonds of the products are likewise attached onto the N-7 positions of the purine rings. It is likely that the mechanism of both type of cyclizations proceeds through

a common oxocarbenium intermediate.

The metabolic enzymes of cADPR are rather heterogeneous. One extreme of this class is the *Aplysia* cyclase, catalyzing mainly the formation of cADPR from NAD whereas the NAD glycohydrolases (NADases) and the CD38[10]-like enzymes produce mainly ADPR and only small quantities of cADPR. For example, the ratio of NADase:cyclase:hydrolase activities for the CD38 from human erythrocyte membranes was found to be 100:1:10,[11] and the enzyme from canine spleen microsomes was 100:2:30.[12] Thus, the low levels of cyclase activities among these enzymes have made their detection difficult and the failure of previous studies in detecting cADPR biosynthesis is probably the result of the less sensitive assays used.

We have found pig brain NADase and human erythrocyte CD38 are capable of catalyzing the transformation of **2** into **3** in yields approximately twice higher than the corresponding conversions of NAD into cADPR. Moreover, **3** was very stable to the action of cADPR hydrolase from pig brain extract at 37 °C. Thus, when **2** is used as a substrate instead of NAD, the cyclic product **3**, less susceptible to hydrolysis, is released. The excitation and emission spectra of **3** are shown in Fig. 2A and B respectively. The fluorescence intensity of **3** at 410 nm (excitation at 300 nm) was about ten times higher than **2**.

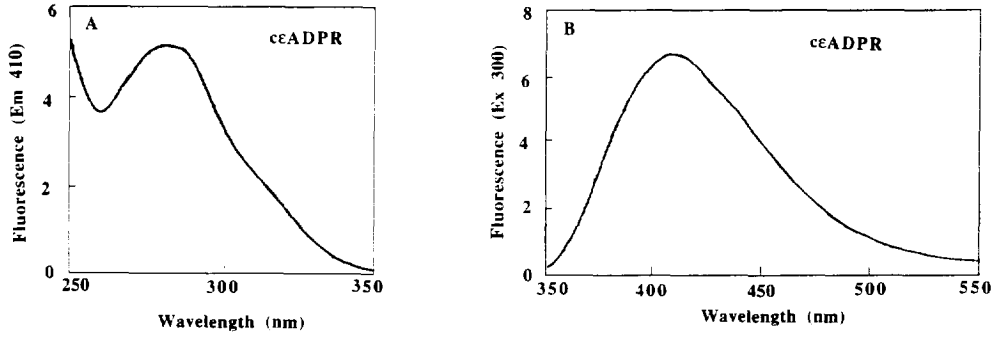


FIGURE 2. Fluorescence properties of **3**. A, the excitation spectrum of 25 μM of **3** was measured in 10 mM phosphate buffer, pH 7.0. The emission wavelength (Em) was set at 410 nm. B, emission spectrum of 25 μM of **3** with the excitation wavelength (Ex) set at 300 nm.

In conclusion, we have shown for the first time that the *Aplysia* cyclase catalyzed the transformation of **2** into a novel cyclic nucleotide, **3**, wherein the newly formed glycosyl bond is attached onto the N-1 position of the etheno-adenine ring, corresponding to the N-7 position of the adenine nucleus. This alternative mode of cyclization provides one with more versatility for the biosynthesis of novel analogs of cADPR. Also, the unique fluorescent properties of **3** and its less susceptibility to hydrolysis by cADPR hydrolases provide the investigator with a useful tool to investigating the cyclase activities of metabolic enzymes of cADPR in crude homogenates.

Acknowledgements: This work was supported by National Institutes of Health grant GM 331449. 2D NMR studies were carried out at the National Magnetic Resonance Facility at Madison (operation subsidized by the NIH Biomedical Research Technology Program under grant RR 02301).

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(*Received in USA* 12 *May* 1995; *accepted* 27 *June* 1995)