Cyclic ADP-Ribose via Stereoselective Cyclization of β-NAD[†]

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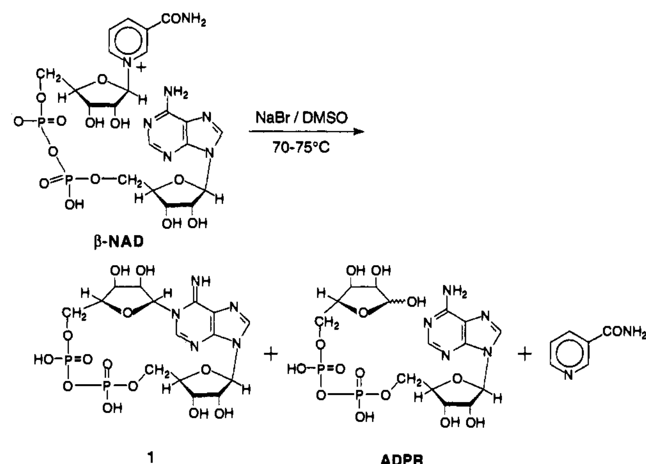
Cyclic ADP-ribose (cADPR) is a naturally occurring cyclic nucleotide and a potent mediator of calcium mobilization in many mammalian tissues.[1] Recent studies suggest that cADPR may play an important role as a second messenger, analogous to inositol triphosphate (IP_3)[2] in Ca^{2+} signaling, but targeting a different intracellular Ca^{2+} channel, the ryanodine receptor.[3]

cADPR is a metabolite of β-NAD^+, and its synthesizing enzyme, β-NAD^+ cyclase, has been shown to be widely distributed among mammalian tissues.[4] In 1989, Lee and coworkers[5] proposed that the metabolite is a cyclized ADP-ribose having an N-glycosyl bond between the anomeric carbon of the terminal ribose unit and the N^6-amino group of the adenine moiety. However, the spectral data were not in accord with the



proposed structure.[6,7] Recently we established that cADPR has the anomeric carbon of the ribose attached onto the N^1-nitrogen of the adenine nucleus via a β-N-glycosyl linkage as shown in **1**.[7] This structural assignment was made by correlating cADPR to N^1-(5′-phosphoribosyl)AMP, a known intermediate of histidine biosynthesis.[8] This transformation was achieved by cleaving the pyrophosphate bond under conditions not perturbing the integrity of the C–N-glycosyl linkage. In addition, cADPR was successfully synthesized by the cyclization of N^1-(5′-phosphoribosyl)ATP,[8] catalyzed by NAD^+ pyrophosphorylase in an organic solvent–aqueous medium.

Although cADPR may be prepared from β-NAD^+ by the use of ADP-ribosyl cyclase from *Aplysia californica*, which has been purified[9,11] and cloned,[10] this enzyme has not been generally accessible.[11] Alternatively, a small quantity of cADPR may be synthesized using β-NAD^+ glycohydrolases,[7,12] enzymes that catalyze the synthesis of cADPR from β-NAD^+ and the hydrolysis of cADPR to ADP-ribose. Herein, we report the development of the first nonenzymatic stereoselective synthesis of cADPR from β-NAD^+. This new cyclization procedure provides another alternative for the synthesis of cADPR as well as analogs and affinity ligands for receptor protein(s) that are not accessible by enzymatic means.

Table 1. Effect of Metal Halides on the Cyclization of β-NAD^+ [a]

reagent	β-NAD^+ (mM)	time (h)	cADPR[b] (%)
LiBr	1.5	2	2.1
LiI	1.5	2	0.5
NaBr	1.5	2	27.8
NaBr	7.5	2	14.8
NaBr	15	1	11.5
NaI	1.5	2	5.6
KI	1.5	2	4.2

[a] The reaction mixture contained metal halide, 747 mM; β-NAD^+; triethylamine, 1 μL/mg β-NAD^+; 5 mL of DMSO. After the solution was stirred at 70 °C for 1–2 h, the DMSO was evaporated using a stream of nitrogen. The residue was dissolved in 100 μL of water and analyzed by HPLC on a SynChropak AX-100 column as described in the text. [b] The yield of cADPR was calculated from HPLC peak area using its molar extinction coefficient at 260 nm, pH 3.0, of 13 700. ^{1}H NMR (D_2O): δ 4.05–4.60 (9H, m), 5.39 (1H, t, J = 5.62 Hz, H_A2'), 6.11 (1H, d, J = 5.62 Hz, H_A1'), 6.20 (1H, d, J = 7 Hz, H1′), 8.44 (1H, s, H_A2), 9.05 (1H, s, H_A8).

Since any de novo synthesis of cADPR would require extensive chemoselective protection and deprotection of functional groups, we elected to focus our attention on the biomimetic cyclization of β-NAD^+ because the conciseness of this approach outweighs any potential disadvantages. As the quaternary pyridinium group in β-NAD^+ is a good leaving group, we first attempted the cyclization of β-NAD^+ in different anhydrous solvents in the presence of nonnucleophilic bases. Because no trace of cADPR was detected in any of these trials, we used metal halides to exchange the quaternary pyridinium ion for a different leaving group. While there are no reports of nucleophilic substitutions of quaternary pyridinium groups by halides, it was our contention that the formation of a glycosyl halide may be possible using strong nucleophiles such as LiI. We found that by reacting β-NAD^+ with LiI and triethylamine in DMSO at 70 °C, a small quantity of cADPR was formed. This observation encouraged us to examine other metal halides, and the best yield of cADPR was obtained with NaBr (28%), as shown in Table 1. Nicotinamide, remaining β-NAD^+, and the side product, ADP-ribose (ADPR), were readily separated from cADPR by HPLC chromatography. More importantly, it was enlightening to see that the cyclization was stereoselective, as cADPR was obtained as the sole isomer. As with most intramolecular cyclization reactions, the yield of cADPR decreased as the concentration of β-NAD^+ increased.

A representative procedure for the preparation of cADPR is as follows: β-NAD^+ (50 mg, 0.075 mmol) 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 14 h). To this solution was added triethylamine (25 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

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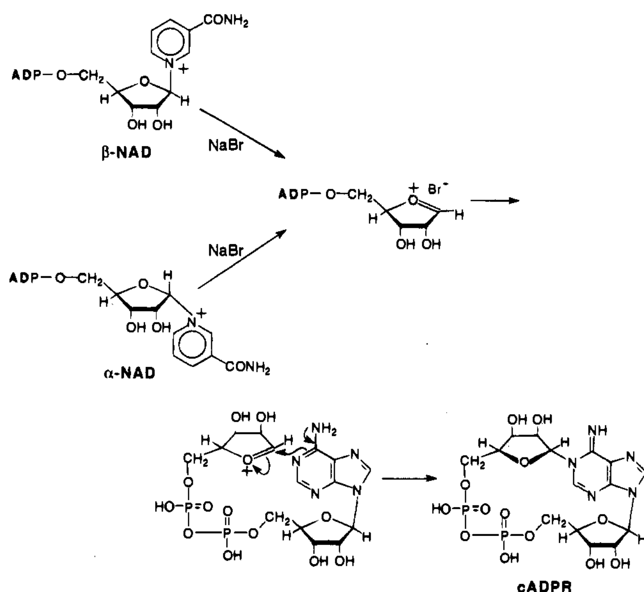
Table 2. Cyclization of NAD Analogs[a]

substrate (concn, mM)	NaBr (mM)	Et_3N (μL)	DMSO (mL)	time (h)	cyclized product (%)
β-NAD (14.9)	747	50	5	2	cADPR (27.8)
α-NAD (14.9)	747	50	5	4	cADPR (16.8)
NHD (14.9)	742	20	2	2	cHDPR (5.6)
NGD (14.9)	742	20	2	2	cGDPR (11.5)
5′-TPN (11.5)	636	50	3	2	cATPR (10.0)

[a] All reactions were conducted at 70 °C. cADPR, cHDPR, and cATPR were isolated on a Synchropak AX-100 column (retention times were 13.7, 15.0, and 24.5−26 min, respectively) and were further purified on a C_{18} column as described in the text. cGDPR was isolated directly on a Waters Nova-pak C_{18} column, which was eluted isocratically with 2 mM TFA at a flow rate of 1 mL/min. The retention time of cGDPR was 4.5 min.

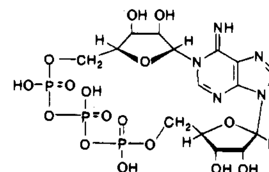
Scheme 1. Proposed Mechanism of the Formation of cADP-Ribose



mL). After drying, the residue was dissolved in 1 mL of H_2O, and the product was purified on a SynChropak AX-100 column (250 × 7.8 mm i.d., 5 μm) using a linear gradient of 0−1.05 M NaCl in 0.1 M KH_2PO_4 in 14 min, followed by an isocratic elution using 1.05 M NaCl in 0.1 M KH_2PO_4 until 20 min at a flow rate of 1 mL/min.[7] Fractions containing cADPR (retention time, 13.7 min) were combined and further purified on a Waters Nova-Pak C_{18} column (100 × 8 mm i.d., 4 μm). The column was eluted isocratically with 2 mM TFA at a flow rate of 1 mL/min. Evaporation of the solvent afforded 4.7 mg (11.5%) of cADPR, whose ^{1}H NMR and mass spectra were found to be identical to those of the cADPR, prepared by enzymatic methods.

To gain an insight into the mechanism of this intramolecular cyclization reaction, we subjected α-NAD$^+$ to the same reaction conditions. In this case, the reaction rate was found to be considerably slower than that of β-NAD$^+$ as manifested by the quantity of recovered α-NAD$^+$, but again, cADPR was obtained as the only isomer (Table 2). These observations suggested that both cyclization reactions proceeded via a common oxo-carbenium intermediate (Scheme 1), similar to the proposed carbocation catalytic mechanism of phosphoribosyl transferases.[13] However, at this stage we are uncertain as to whether a glycosyl halide is formed in the reaction.

To determine the versatility of this cyclization reaction, we first prepared 5′-triphosphopyridine nucleotide (5′-TPN) in 70% yield by coupling β-nicotinamide mononucleotide (β-NMN) to ADP using 3-ethyl-1-(3-(dimethylamino)propyl)carbodiimide (EDC).[14] When 5′-TPN was subjected to the same cyclization conditions, cyclic ATP-ribose (cATPR) (**2**) was isolated in about 10% yield. Its ^{1}H NMR (D_2O) spectrum exhibited peaks at δ



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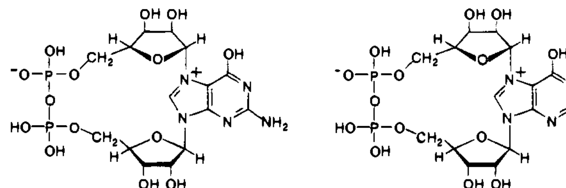

3 **4**

4.2−4.7 (9H, m), 5.36 (1H, t, J = 5.0 Hz, H_A2'), 6.13 (1H, d, J = 5.33 Hz, H_A1'), 6.23 (1H, d, J = 4.3 Hz, H1′), 8.47 (1H, s, H_A2), and 9.11 (1H, s, H_A8), and its UV (A_{260}/A_{290}) ratio was similar to that cADPR at various pH values.[7] These data are consistent for the expected cyclic structure having the glycosyl bond attached onto the N^1-nitrogen of the adenine ring. This analog will be useful for examining the influence of ring size on Ca^{2+} mobilization. Also, we have successfully cyclized the NAD analogs nicotinamide guanine dinucleotide (NGD) and nicotinamide hypoxanthine dinucleotide (NHD) using the same reaction conditions. However, their ^{1}H NMR and UV spectral data[15] suggested that cyclization may have occurred with the N^7-nitrogen of the purine ring to furnish cGDPR (**3**) and cHDPR (**4**) in 11.5 and 5.6% yields, respectively.

In summary, we have developed for the first time a stereoselective synthesis of cADPR from β-NAD$^+$. The substrate specificity of this nonenzymatic intramolecular cyclization is being compared to that of the *Aplysia* ADP-ribosyl cyclase.[9,11] We are continuing to examine the factors that affect this biomimetic cyclization reaction and its applicability to the synthesis of novel analogs with interesting biological properties as well as affinity ligands for the cADPR receptor protein(s).

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(15) The ^{1}H NMR (D_2O) of cHDPR: δ 4.1−4.9 (10H, m), 6.36 (1H, d, J = 4.1 Hz, H_H1'), 6.71 (1H, d, J = 4.8 Hz, H1′), 8.43 (1H, s, H_H2), 9.25 (1H, s, H_H8). The ^{1}H NMR (D_2O) of cGDPR: δ 4.1−4.8 (10H, m), 6.16 (1H, d, J = 4.1 Hz, H_G1'), 6.60 (1H, d, J = 4.9 Hz, H1′), 8.92 (1H, s, H_G8). The C-8 protons of the purines appear at lower fields (δ 8.92−9.25) in the cyclized structure due to deshielding as compared to those in the open structure (δ 8.4−8.62). The UV (H_2O) λ_{max} of cHDPR was at 259 nm, as compared to those of 1-methylinosine (λ_{max} 251)[16] and 7-methylinosine (λ_{max} 261).[17] For cGDPR, the UV (H_2O) λ_{max} was at 278 nm, as compared to those of 1-methyldeoxyguanosine (λ_{max} 257),[18] 7-methyldeoxyguanosine (λ_{max} 280),[18] and 7-methylguanosine (λ_{max} 275).[17]

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