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REVIEW ARTICLE

Bioorganic Chemistry of Cyclic ADP-ribose (cADPR)†

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Abstract—The objective of this brief review is to present an overview of the bioorganic chemistry of cyclic-ADP-ribose (cADPR) with special emphasis on the methodology used for the synthesis of analogues of cADPR. New structural analogues of cADPR can be prepared using either the biomimetic method or ADP-ribosyl cyclase from *Aplysia californica*. 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 analogues of NAD^+ into their corresponding cyclic nucleotides. Several of these novel analogues are more potent than cADPR in inducing calcium release and are also more stable towards degradative enzymes. They could serve as valuable affinity probes for the isolation of cADPR-binding proteins. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

The importance of NAD^+ and $NADP^+$ as coenzymes for cellular oxidation–reduction reactions is well known. In addition, NAD^+ plays non-oxidation–reduction reaction roles by serving as an ADP-ribose donor in the posttranslational modification of proteins.[1] The ADP-ribosylation of proteins has been implicated in the regulation of cell recovery from DNA damage,[2] protein trafficking and secretion,[3] and membrane signal transduction.[4] In addition, NAD^+ has been shown to be the precursor to cyclic ADP-ribose (cADPR), a nucleotide with potent Ca^{2+}-release activity. Much evidence has accumulated suggesting that cADPR regulates the Ca^{2+}-induced Ca^{2+}-release by ryanodine-type Ca^{2+} release channels.[5,6] However, the mechanism by which the endogenous levels of cADPR are regulated is still not well understood. Since several excellent review articles[5,7–11] dealing with the physiological functions of cADPR have appeared recently, we shall limit our discussions to the bioorganic chemistry of cADPR with emphasis to results originated in our laboratory.

Discovery of cADPR

Cyclic ADP-ribose (cADPR) was discovered in 1987 when Lee and co-workers[12] examined various metabolites for their abilities to induce calcium release in sea urchin egg homogenates. Because pyridine nucleotide levels fluctuated markedly after fertilization, they tested NAD^+ for its ability to induce Ca^{2+} release. Unexpectedly, NAD^+ released almost as much calcium as IP_3, but there was a time lag of 1–4 min. Since NAD^+ itself does not induce calcium release, the delayed response was later shown to reflect the time for its enzymatic conversion to a novel metabolite, which was isolated by HPLC in quantities sufficient for structural determination. Using NMR and mass spectroscopy, Lee and co-workers suggested that this metabolite is a cyclic compound with the adenine ring of the NAD^+ molecule forming a N-glycosidic linkage between the N^6-amino group of the adenine with the anomeric carbon of the terminal ribosyl unit by displacing the nicotinamide group.[13] Since the metabolite is a cyclic compound and can be hydrolyzed to ADP-ribose (ADPR), the name cyclic-ADP-ribose was assigned to this compound with the proposed gross structure as shown in Figure 1. The absolute stereochemistry of the linkage between the adenine and the terminal ribose (α or β-linkage) was left unassigned.

This structural assignment was based on the observation that, under their unspecified conditions, cADPR had a UV spectrum similar to that of NAD^+ and ADPR. They reasoned that linkage to any of the ring nitrogens such as the N-1 position would introduce a positive charge on the adenine nucleus and that the UV spectra of such compounds would differ from those of NAD^+ and ADPR. However, it was found that the UV spectrum of cADPR, notably the ratio of absorbancies at 260/290 nm was clearly different from that of

Key words: Cyclic-ADP-ribose; ADP-ribosyl cyclase; chemoenzymatic synthesis; biomimetic synthesis; affinity probes.

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† In memory of Sir Derek H. R. Barton, 1918–1998.

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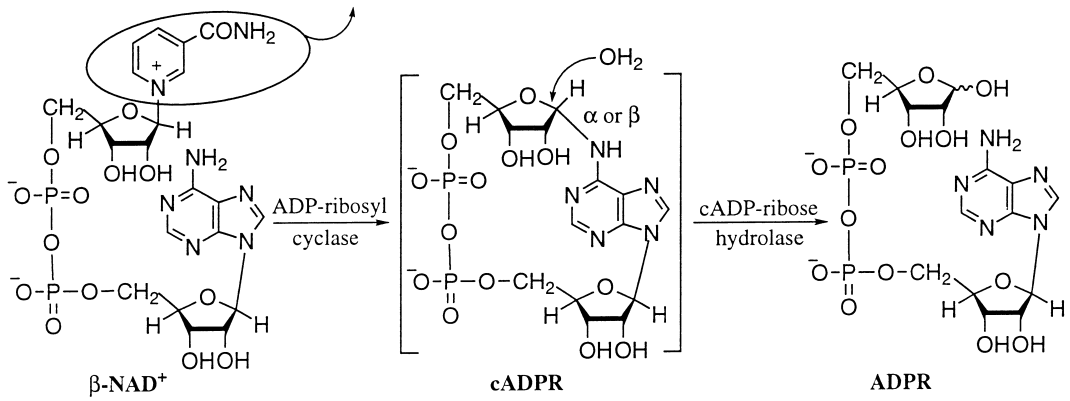


Figure 1. Lee's first proposed structure of cADPR.

ADPR.[14] Using N^1-(5′-phosphoribosyl)ATP (N^1-P-rib-ATP) and N^6-(5′-phosphoribosyl)ATP (N^6-P-rib-ATP) as model compounds, it was found that the spectroscopic properties of N^1-P-rib-ATP coincided very closely to that of cADPR whereas N^6-P-rib-ATP did not. A similar conclusion was reached by Jacobson and his co-workers[15] using N^1 and N^6-substituted adenosine derivatives. This spectral behavior strongly suggested that the newly formed N–glycosyl bond is attached onto the N^1-nitrogen of the adenine ring rather than to the N^6-nitrogen as proposed. Confirmation of the structure and stereochemistry of cADPR were achieved by its synthesis[14] from N^1-P-rib-ATP using NAD^+ pyrophosphorylase (Fig. 2), an enzyme that normally catalyzes the formation of NAD^+ from nicotinamide mononucleotide (NMN) and ATP (Fig. 3). The product obtained was found to be identical to a sample of cADPR prepared by the exposure of NAD^+ to pig brain acetone powder. The structural assignment has now been further confirmed by NMR[16] and X-ray crystallography.[17]

Stability of cADPR

The cADPR molecule is quite stable but it is slowly hydrolyzed to ADPR at room temperature under slightly acidic conditions with a half-life of about 10 days. At 37 °C, the half-life for spontaneous hydrolysis is about 24 h. The pH titration curve of cADPR revealed that it has a pK_a of 8.2 and $\varepsilon = 3200$ at 290 nm. The absence of a pK_a at pH 4.0, corresponding to the pK_a of the amino group of ADPR or AMP, indicates that cADPR, like N^1-P-rib-ATP forms an imino base rather than an amino base[14] (Fig. 4).

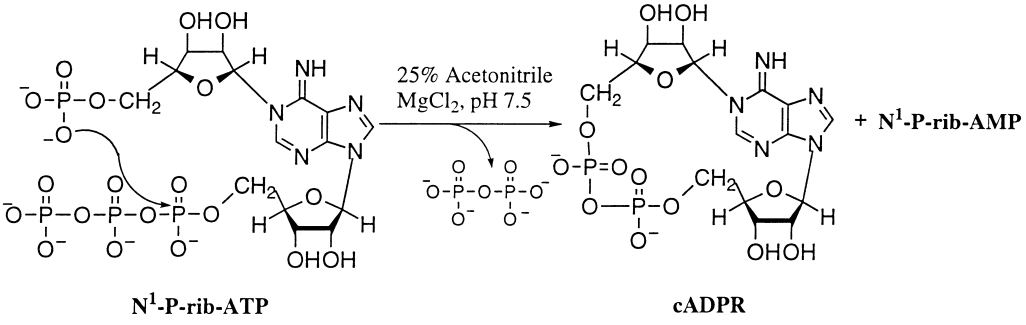


Figure 2. cADPR (**1**) via NAD^+ pyrophosphorylase-catalyzed intramolecular cyclization of N^1-phosphoribosyl ATP.

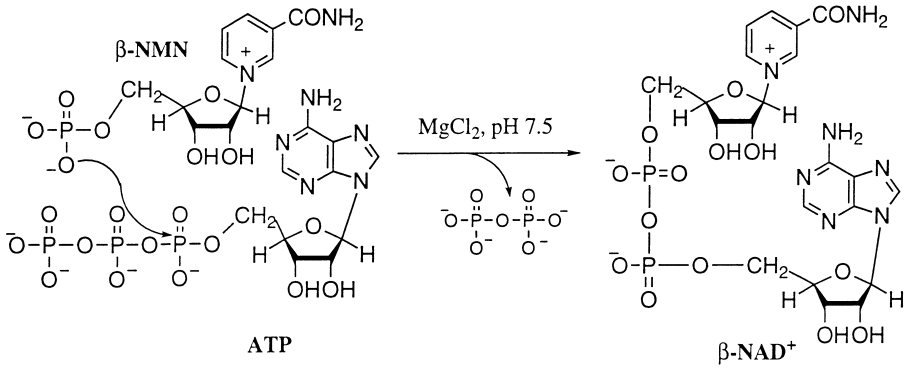


Figure 3. NAD^+ pyrophophorylase catalyzed synthesis of β-NAD^+.

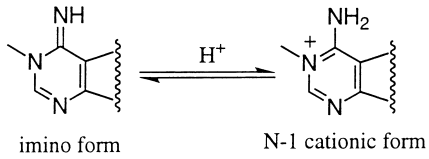


Figure 4. Isomerization of cADPR and N^1-P-rib-ATP under acidic conditions.

It is well documented that N^1-alkylated adenosine derivatives undergo the Dimroth rearrangement in base to give the N^6-derivatives.[18] Numerous mechanistic studies, including ^{15}N-isotope labeling, have shown that this rearrangement proceeds through adenine ring opening without cleavage of the N^1–alkyl bond. Indeed, Ames[19] has observed that N^1-P-rib-ATP, an open ring analogue of cADPR, underwent such a rearrangement at pH 10 (Fig. 5). On the other hand, it is interesting to note that cADPR is very stable in base and does not undergo the Dimroth rearrangement at pH 10. The pyrophosphate bond in cADPR is also moderately resistant to alkaline hydrolysis, but can be cleaved to N^1-P-rib-AMP with potassium *t*-butoxide in dimethyl sulfoxide (Fig. 6).[14] Although cADPR was formed when N^1-P-rib-AMP was treated with 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC) in 1.5 M Hepes buffer (pH 6.8), the yield was only of the order of 1%. cADPR is readily hydrolyzed to ADPR by NAD^+ glycohydrolases, enzymes that are widely distributed among mammalian tissues. In the presence of a suitable nucleophile, these enzymes can catalyze the synthesis of NAD^+ derivatives from cADPR.[20] The pyrophosphate linkage in cADPR is resistant to the action of pyrophosphatases in contrast to ADPR, which is readily attacked by these enzymes.[14]

Enzymatic Synthesis of cADPR Derivatives

There are three types of enzyme systems known for the conversion of NAD^+ into cADPR (cyclase) or ADPR (NADase) and the hydrolysis of cADPR to ADPR (hydrolase): (1) A membrane-bound NAD^+ glycohydrolase (NADase) was purified to homogeneity from canine spleen microsomes, which contained three enzyme activities. The ratio of NADase:cyclase:hydrolase activities for this enzyme was found to be 100:2:30.[21] The NAD^+ glycohydrolase purified from bovine spleen showed similar activities and was extensively studied.[22] It catalyzed the formation of cADPR (in low yield) from NAD^+ and cyclic GDP-ribose from NGD^+. (2) The human and rodent CD38 antigen also possess cyclase and hydrolase activities. An ectoenzyme was purified to homogeneity from solubilized human erythrocyte membranes and exhibited a similar ratio of enzyme activities (100:1:10).[23] (3) In contrast, the enzyme derived from the invertebrate, *Aplysia californica* is very rich in cyclase activity and has little NADase and hydrolase activities[24] (Fig. 7).

This enzyme was named as ADP-ribosyl cyclase, and is now commercially available from Sigma in a highly purified state, and has been widely used for the laboratory preparation of cADPR and its analogues. The crystal structure of the cyclase was reported in 1996.[25] Very recently, this enzyme has been cloned in the methylotrophic yeast, *Pichia pastoris* that expressed high

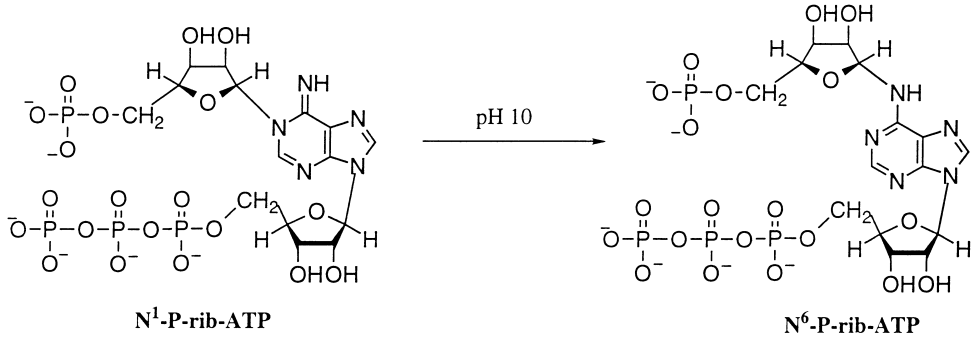


Figure 5. Base-catalyzed transformation of N^1-P-rib-ATP to N^6-P-rib-ATP.

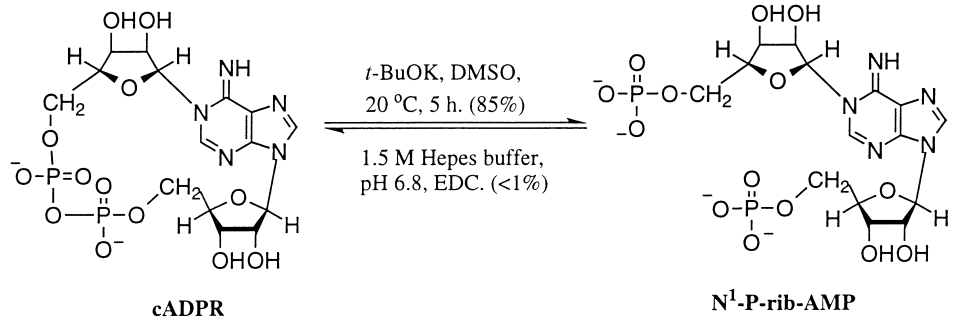


Figure 6. Base-catalyzed cleavage of cADPR.

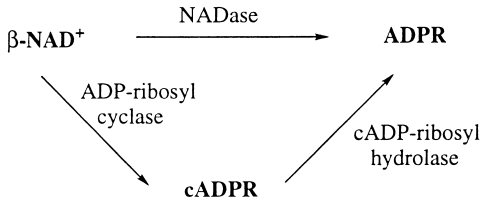


Figure 7. Activities of cADPR-forming enzymes.

levels of this enzyme.[26] Using high biomass fermentations up to 300 milligram per liter of cyclase was achieved. SDS-PAGE analysis revealed that the heterologous protein comprised 90–95% of the total protein secreted extracellularly. The properties of the recombinant enzyme compared favorably to those of the native enzyme. The yeast expression system can produce gram quantities of this cyclase. Hence the supply of the enzyme for synthetic applications is no longer a problem. The ADP-ribosyl cyclase of *Aplysia californica* converts β-NAD^+ analogues into three different types of products depending on the structural features of substrates. In all cases, α-NAD^+ derivatives are not cyclized or hydrolyzed by this enzyme (Fig. 8).

Cyclization at N-1

The ADP-ribosyl cyclase efficiently converts β-NAD^+ into cADPR in over 90% yield because this enzyme has little NADase and hydrolase activities. In a similar manner, it transforms $NADP^+$ into the corresponding cADPRP (**2**), 3′-$NADP^+$ into 3′-cADPRP (**3**) and 2′,3′-cyclic $NADP^+$ into 2′,3′-cADPRP (**4**).[27,28] cADPRP stimulates calcium release two times more effectively than cADPR, but by a mechanism distinct from both cADPR and IP_3. The other two analogues are inactive in inducing calcium release. cADPRP can also be generated by incubation of $NADP^+$ with pig brain NADase. The occurrence of cADPRP in mammalian tissues was also demonstrated.[29] Due to the wide distribution of $NADP^+$ in mammalian systems and its unique calcium releasing property, several papers on the role of cADPRP in the regulation of Ca^{2+} homeostasis and its characterization have been reported recently.[20,30–33]

The 2′-deoxy, 3′-deoxy and 3′-O-methyl derivatives of NAD^+ were converted into their respective cADPR derivatives.[34] 2′-Deoxy-cADPR (**5**) is similar to cADPR in inducing Ca^{2+} release, whereas 3′-deoxy-cADPR (**6**) is 100-fold less potent. However, 3′-O-methyl-cADPR (**7**) inhibited the cADPR-induced Ca^{2+} release in a dose-dependent manner. This suggested that the 3′-hydroxy, but not the 2′-hydroxy group was essential for the calcium releasing activity. Cyclic aristeromycin diphosphate ribose (cArisDPR, **8**), the first carbocyclic analogue of cADPR, was prepared via the cyclization of $NArisD^+$.[35] Interestingly, while retaining a similar calcium release profile to that of cADPR, cArisDPR showed higher stability towards enzymatic hydrolysis. All the above results indicated that the cyclase can

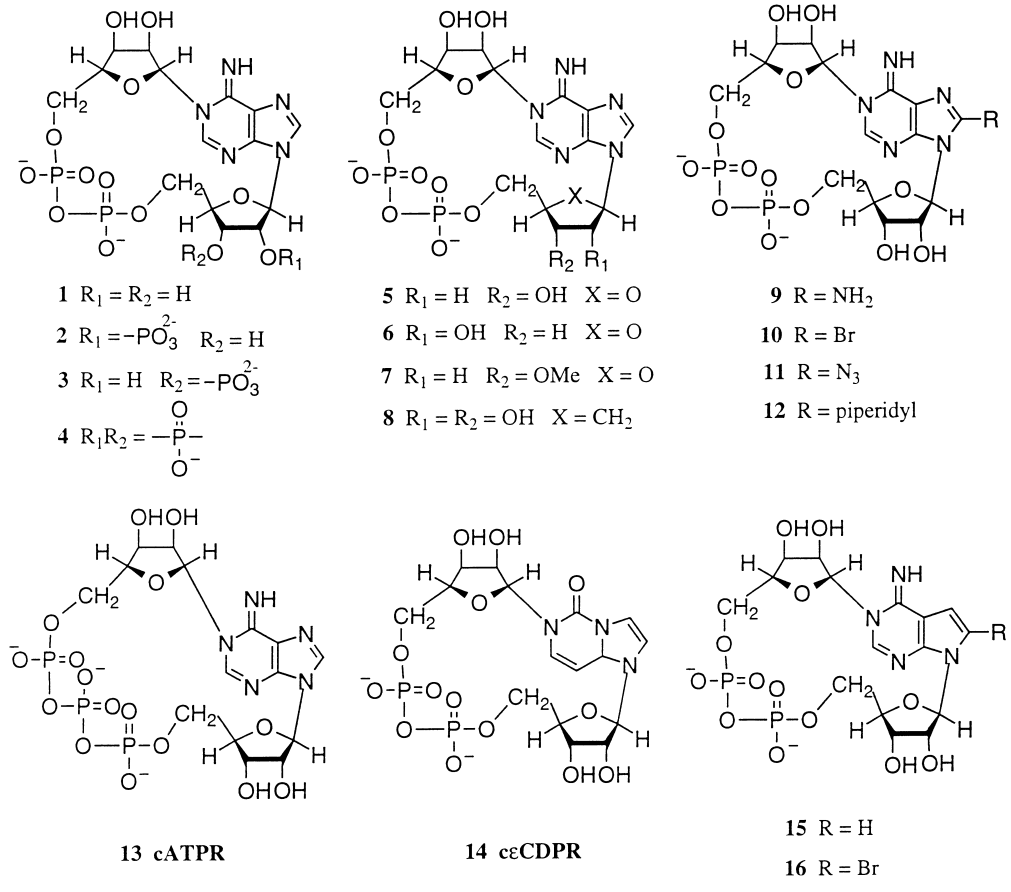


Figure 8. cADPR analogues cyclized at the N-1 position.

tolerate substantial changes around the ribosyl moiety of the adenosine portion of the NAD^+ derivatives.

As the cyclase enzyme can accommodate large substituents at the 8-position of the adenine ring of NAD^+ analogues, a series of 8-substituted cADPR analogues were prepared. Among these, 8-amino-cADPR (**9**) was the strongest antagonist of cADPR, whereas 8-Br- and 8-azido-cADPR (**10**, **11**) were less potent as antagonists,[36] and 8-piperidyl-cADPR (**12**) was inactive.[37]

The triphosphate analogue of cADPR (cATPR, **13**) was formed via the cyclization of 5′-triphosphopyridine nucleotide (5′-TPN).[38] cATPR was about 20 times more potent in inducing Ca^{2+} release and was more resistant than cADPR to hydrolysis by NAD-glycohydrolase. More importantly, cATPR and cADPR modulate the Ca^{2+} release via the same mechanism. These properties enabled cATPR to be used as a suitable molecular probe for the study of cADPR binding proteins.

Since no cyclic analogue containing a pyrimidine nucleus has been prepared, we attempted the cyclization of nicotinamide cytosine dinucleotide using the *Aplysia* cyclase. Somewhat to our surprise, no cyclized product was detected, which suggested that a bicyclic ring system may be required for cyclization. This assumption led to the deployment of 3,N^4-ethenocytosine ring system as a mimic of the adenine ring of NAD^+. Indeed, this nicotinamide-3,N^4-ethenocytosine diphosphate analogue was converted into a new cyclic nucleotide, cyclic etheno-CDP-ribose (cε-CDPR, **14**), in 92% yield.[39] This result poignantly reflected the importance of substrate structure in dictating product formation. Although cε-CDPR was found to be inactive in inducing Ca^{2+} release, it is the first cADPR analogue with a modified purine ring. Comparing the structural features of the 3,N^4-ethenocytosine ring versus that of the adenine ring, it is worth noting that the cyclization site of cε-CDPR (N-1) corresponded to the N-9 position of adenine ring. This means the direction of cyclization of NAD^+ analogues could occur not only from N-9 to N-1 but also possibly from N-1 to N-9 for the generation of unique cADPR analogues. cADPR analogues modified at N-7, such as 7-deaza-cADPR (**15**)[40] and 7-deaza-8-bromo-cADPR (**16**)[41,42] were also prepared via cyclization of the corresponding NAD^+ analogues. The former (**15**) was a partial agonist whereas **16** was an antagonist of cADPR. However, both were resistant to chemical and enzymatic hydrolyses and possessed the important feature of membrane permeability.

Before concluding the discussion of the N-1 cyclized cADPR analogues, we would like to highlight the caged cADPR derivative. When cADPR was treated with 1-(2-nitrophenyl)diazoethane at pH 2.3, a monocaged cADPR (**17**) was produced, in which the 2-(nitrophenyl)ethyl group was attached to one of the two phosphates (Fig. 9).[43] Compared to cADPR, **17** has several advantages in studying the mechanism of calcium mobilization induced by cADPR. The caged cADPR is not active until the caging group is cleaved by UV light photolysis. It is more permeable to the cells due to the hydrophobic nature of the 2-(nitrophenyl)ethyl group. The unique features of **17** enable it to be a valuable tool for investigating calcium release induced by cADPR in vitro as well as in vivo.

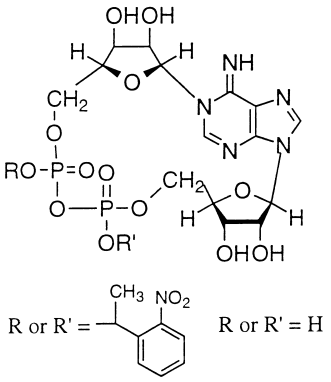


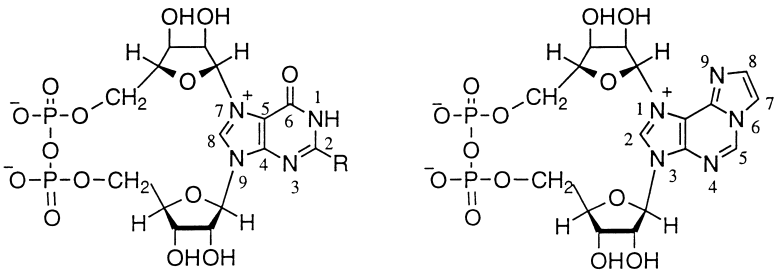
Figure 9. Caged cADPR (**17**).

Cyclization at N-7

The cyclase of *Aplysia californica* catalyzed an alternative mode of cyclization of nicotinamide guanine dinucleotide (NGD^+) and nicotinamide hypoxanthine dinucleotide (NHD^+) to form cyclic GDP-ribose (cGDPR, **18**) and cyclic HDP-ribose (cHDPR, **19**), respectively (Fig. 10).[44] In these cyclic nucleotides, the newly formed glycosyl bonds were shown to be attached onto the N-7 nitrogen of the purine rings and not to the N-1 position of the guanine ring as was previously proposed.[45] The cyclase also catalyzed the transformation of nicotinamide-1,N^6-etheno-adenine dinucleotide into a novel cyclic nucleotide (cεADPR, **20**) whose N-glycosyl bond was attached onto the N-1 position of the 1,N^6-etheno-adenine nucleus corresponding to the N-7 position of the adenine ring.[46] While all of the three N-7 cyclized cADPR analogues were much less active than cADPR in inducing calcium release in the rat microsomal system, their novel ring structures were more stable than cADPR towards heat and NADase hydrolysis. Furthermore, their unique fluorescent properties provide investigators with a useful tool for monitoring the cyclase reaction especially in crude homogenates.[45] The cyclization of NGD^+ to cGDPR may be used as a continuous spectrofluorimetric assay of cyclase activity. This assay method has several advantages. NGD^+ is cyclized to cGDPR in yield higher than that of NAD^+ to cADPR, the fluorescent chromophore is formed only upon cyclization offering low background and high sensitivity, the assay is continuous and is adaptable to spectrophotometric or fluorimetric detection.

Hydrolysis to ADPR analogues

The above results showed that the cyclase has a relaxed substrate specificity, which has provided investigators with options to prepare various N-1 and N-7 cyclized cADPR analogues in good to high yields by the chemoenzymatic method. However, the enzymatic method still has inherent limitations because of its intrinsic substrate specificity. In our experiments, the NAD^+ analogues



18 R = NH_2
19 R = H

20

Figure 10. cADPR analogues cyclized at N-7.

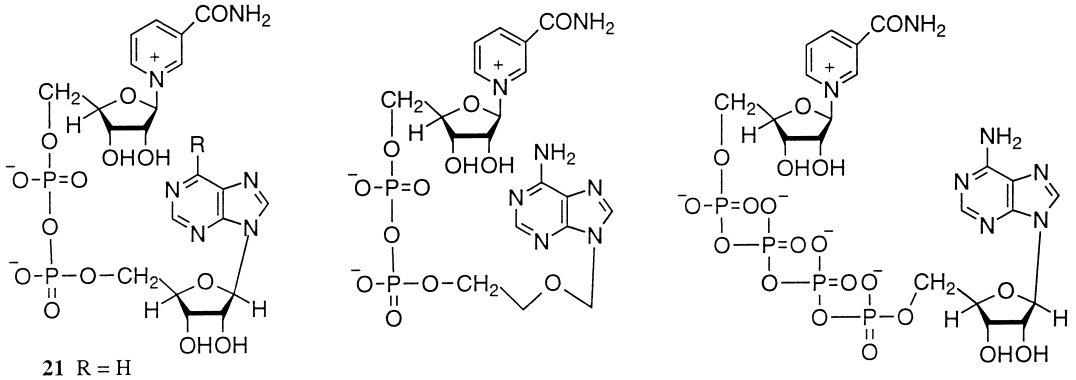
(**21**–**26**) shown in Figure 11 could not be cyclized by the cyclase to the corresponding cADPR analogues. In the presence of an excess of the cyclase, these NAD^+ analogues were hydrolyzed to their respective ADPR analogues. The hydrolysis may be due to the inherent NADase activity possessed by the cyclase.

Non-Enzymatic Syntheses of cADPR Analogues

Since the discovery of cADPR, much attention has been paid to its synthesis by non-enzymatic methods. The goal of the non-enzymatic methods was not only to verify the structure of cADPR, but also to prepare stable cADPR analogues or analogues that were not possible to prepare by the enzymatic method. Until now, two strategies have been developed for the synthesis of cADPR and its analogues:

Total synthesis of cADPR analogues via intramolecular condensation reaction

This method was first used in the authors' laboratory to prepare cADPR in order to confirm its structure.[14] As shown in Figure 6, the key intermediate N^1-P-rib-AMP was prepared by a chemo-enzymatic method. Its cyclization to cADPR in the presence EDC was attempted but was not successful due to the low cyclization yield (< 1%). Later, several hydrolysis-resistant carbocyclic analogues of N^1-P-rib-AMP (Fig. 12) were prepared by two different laboratories.[47,48] The ring closure of **27** was attempted but failed.[47] Successful cyclization of analogues of N^1-P-rib-AMP was achieved only recently using a modified method of Shuto et al.[49] It was surmised that the intramolecular cyclization would depend on the conformations of the nucleotides, for the nucleotides could exist as the *anti* and *syn* conformers (Fig. 13).[50] In the *syn* conformer, the two phosphate groups are close to each other, which should facilitate the intramolecular condensation reaction. Unfortunately, it is well known that the *anti* conformation predominates in most natural nucleotides, which may be the reason for the unsuccessful cyclization of N^1-P-rib-AMP. By introducing a bulky group at the 8-position of purine nucleosides, the conformation of the nucleotide may be restricted to the *syn* form due to the steric interaction between the bulky group and the ribose moiety. Based on this assumption, Shuto et al.[49] synthesized compound **30**, an 8-bromo substituted analogue of N^1-P-rib-IMP, which was successfully cyclized to give **31** in 23% yield. After removal of the 8-bromo substituent and the protecting groups, the cADPR analogue (**32**) was obtained. This constituted the first cADPR analogue synthesized via a total synthesis approach, and provided a general method for the preparation of stable cADPR analogues (Fig. 14).

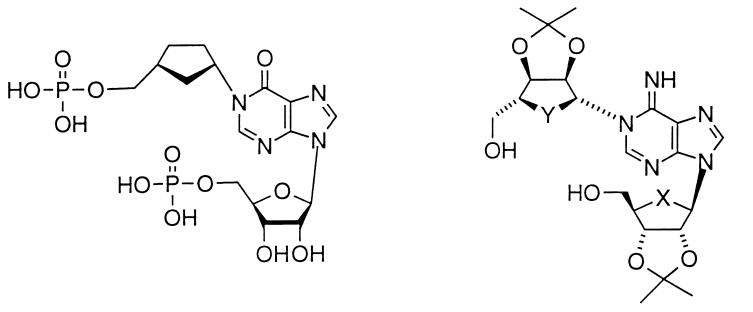


21 R = H
22 R = SMe
23 R = $NHCH_2CO_2H$
24 R = $NH(CH_2)_6NH_2$

25

26

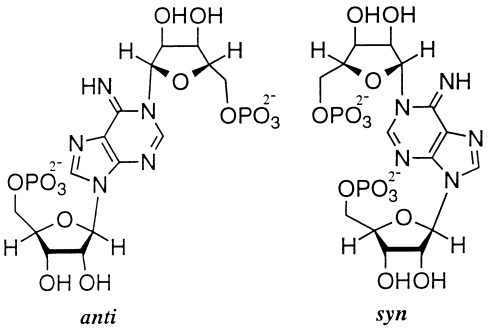
Figure 11. NAD^+ analogues not cyclized by ADP-ribosyl cyclase.



27

28 X = CH_2, Y = CH_2
29 X = O, Y = CH_2

Figure 12. Carbocyclic analogues of N^1-P-rib-AMP.

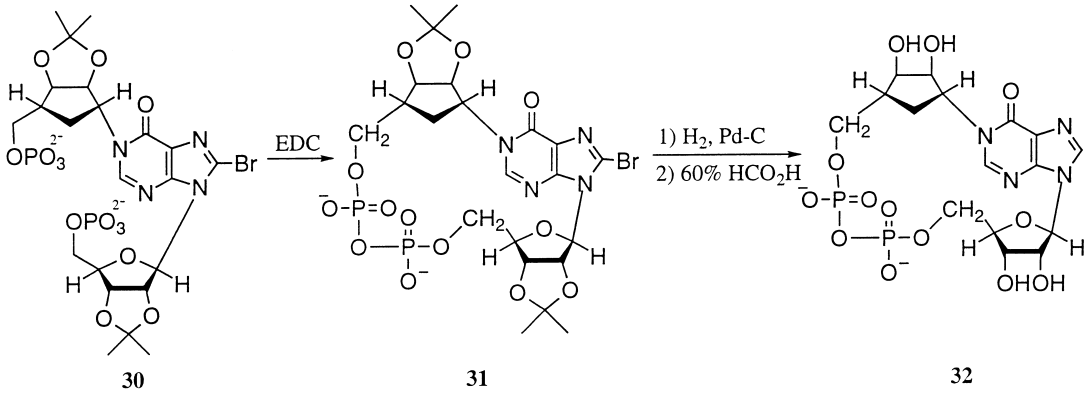


anti *syn*

Figure 13. Possible conformations of N^1-P-rib-AMP.

Biomimetic synthesis of cADPR

Since de novo synthesis of cADPR would require extensive chemoselective protection and deprotection of functional groups, Yamada et al.[51] developed a biomimetic cyclization of α and β-NAD^+. It was discovered that by reacting β-NAD^+ with NaBr and triethylamine in dry DMSO at 70 °C, cADPR was obtained in 28% yield (Fig. 15). Lower yields were obtained using other halide salts. Nicotinamide, residual β-NAD^+ and the hydrolysis product, ADPR were readily separated from cADPR by HPLC. It is noteworthy that this intramolecular cyclization was completely stereoselective as cADPR was obtained as the sole isomer. As α-NAD^+ and β-NAD^+ were both transformed into cADPR as the only isomer, it is likely that these cyclizations proceeded via a common oxo-carbenium ion intermediate (Fig. 16). To determine the versatility of this cyclization reaction, a series of NAD^+ analogues were treated under the same reaction conditions. In most cases, the biomimetic route gives similar product profiles to that of enzymatic method, but higher yields are generally obtained with the latter method. 2′-Deoxy-cADPR, cεCDPR and cATPR were obtained via N-1 cyclization, whereas cGDPR and cHDPR were obtained via N-7 cyclization (Fig. 17). However, one exception was when nicotinamide-1,N^6-etheno-adenine dinucleotide (εNAD^+) was subjected to the condition of biomimetic synthesis, no formation of cεADPR (**20**) was detected. Instead, a new cyclic nucleotide (9-cεADPR, **33**) was obtained.[39] This cyclic nucleotide, **33**, differed from cεADPR with respect to retention time on HPLC, UV, and NMR spectra. Because of MS and 2-D NMR spectra, its structure was assigned as a cyclic product in which the newly formed glycosyl bond was attached to N-9 instead of the N-1 position as shown in Figure 18. Compound **33** was about two times more potent than cADPR in inducing Ca^{2+} release. Along with its unique fluorescence behavior, 9-cεADPR provides investigators with a useful probe for the study of cADPR-binding proteins. This is the first time that we have observed that the enzymatic and biomimetic methods proceeded via different reaction pathways to give different cyclization products, thereby, demonstrating the usefulness of the biomimetic method.



30 31 32

Figure 14. The first total synthesis of a cADPR analogue (**32**).

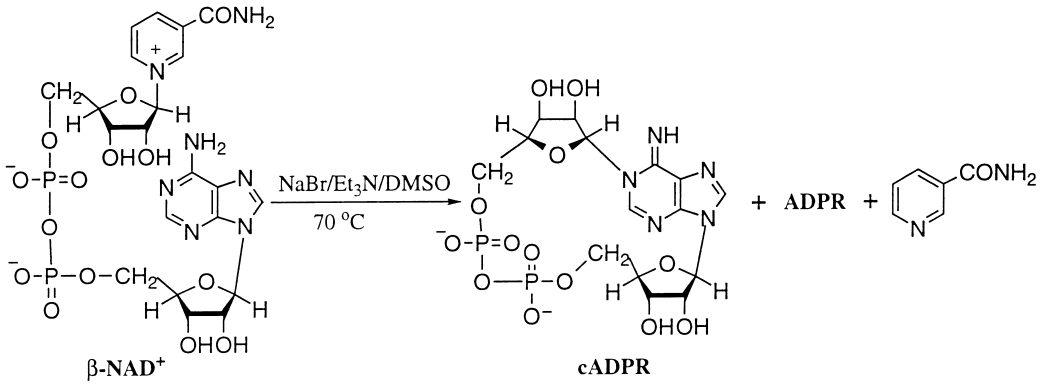


Figure 15. Biomimetic cyclization of β-NAD$^+$.

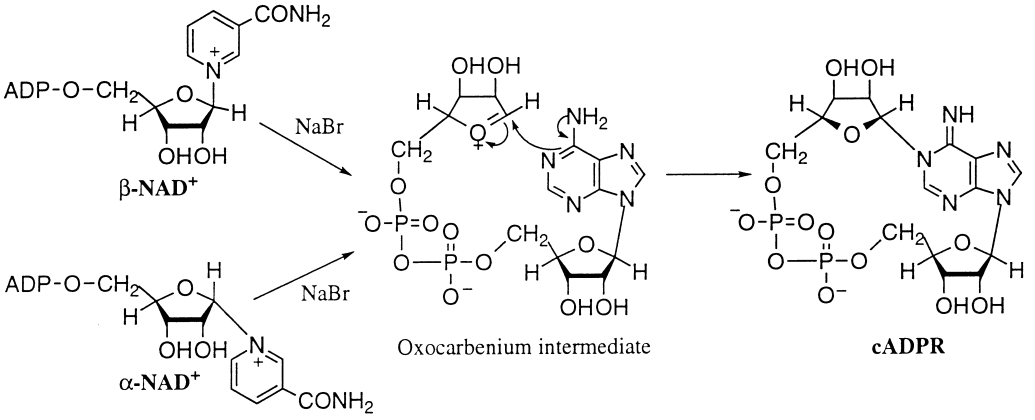


Figure 16. Mechanism of biomimetic cyclization of α and β-NAD$^+$.

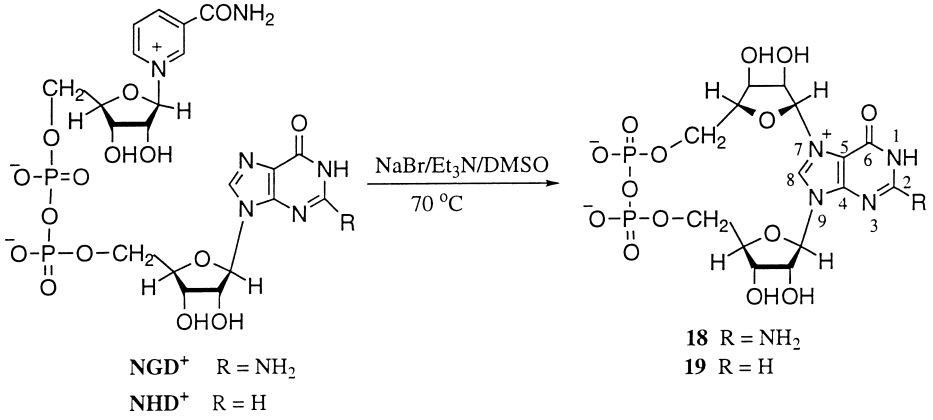


Figure 17. Biomimetic cyclization of NGD$^+$ and NHD$^+$.

Preparation of Affinity Probes

To gain insights into the physiological functions of cADPR, it is necessary to isolate and characterize the cADPR-binding proteins, which in turn may shed light on the putative role of cADPR as a second messenger. Affinity column chromatography is a useful method for the isolation of ligand binding proteins. We have designed and prepared three 8-(6-aminohexyl) amino substituted cADPR analogues as potential affinity probes (Fig. 19) based on the following considerations. First, many 8-substituted cADPR analogues[36] have been used as antagonists of cADPR and one of them, photolabeled 8-azido-cADPR, was used for the characterization of cADPR-binding proteins in sea urchin egg homogenates.[52] Further, although a cADPR affinity probe should be of the first choice to purify cADPR-binding proteins, our previous studies[35] showed that cADPR was easily hydrolyzed by NAD-glycohydrolases (NADases), which could complicate the purification of the binding proteins. On the other hand, cyclic ATPR, an analogue of cADPR was found to be more potent than cADPR in inducing Ca^{2+} release and was remarkably more stable to the hydrolytic action of NADases.

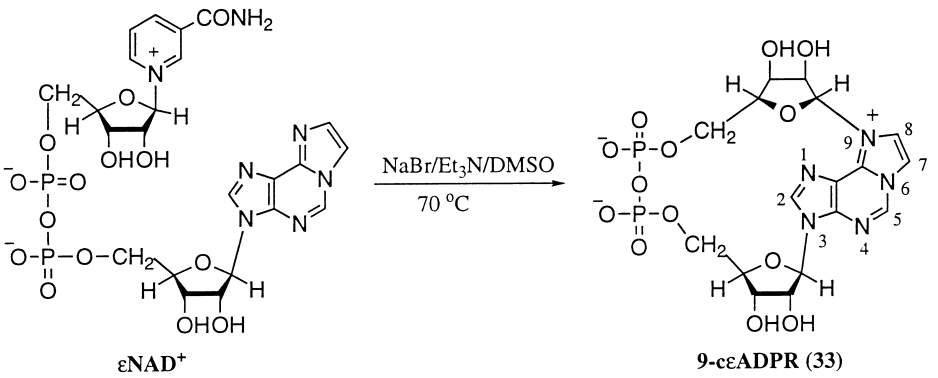


Figure 18. Biomimetic cyclization of εNAD$^+$.

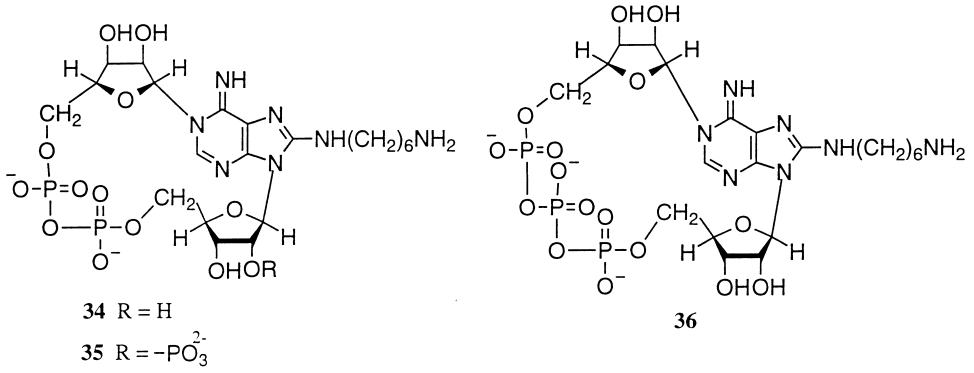


Figure 19. Affinity probes of cADPR, cADPRP and cATPR.

More importantly both cADPR and cATPR induced Ca^{2+} release in the rat brain microsomal system via the same mechanism,[38] which suggested that it should be possible to isolate and characterize cADPR-binding proteins using the more stable and active analogue, cATPR, as the affinity probe. Finally, although cADPRP is structurally similar to cADPR, it induced calcium release by a different mechanism than that of cADPR.[24] Thus, a comparison of the protein-binding profiles of the three aforementioned affinity probes may provide more reliable and valuable information.

8-(6-Aminohexyl) amino-cADPR (**34**)[53] was easily obtained by treating 8-(6-aminohexyl) amino-NAD$^+$ (from Sigma) with the cyclase under the usual reaction conditions. A new and efficient approach to the synthesis of 8-(6-aminohexyl) amino-cATPR was developed recently.[54] The key intermediate, 8-(6-aminohexyl)-amino-ADP (**37**) was prepared in a two-step procedure from ADP in 75% yield. The coupling of **37** to β-nicotinamide mononucleotide gave 8-(6-aminohexyl)amino-5′-triphosphopyridine nucleotide (**38**) (35%), which was then cyclized by exposure to the cyclase to give **36** in 81% yield (Fig. 20).

The synthesis of 8-(6-aminohexyl) amino-cADPRP has also been achieved in our laboratories very recently (unpublished results). It was synthesized in a six-step procedure using NADP$^+$ as starting material. Briefly, NADP$^+$ in water was treated with excessive bromine in the dark for 3 h and the reaction was shown to be complete by HPLC.[55] The unreacted bromine was removed by repeated extraction with CCl_4 and the aqueous layer was lyophilized to dryness. The lyophilized powder

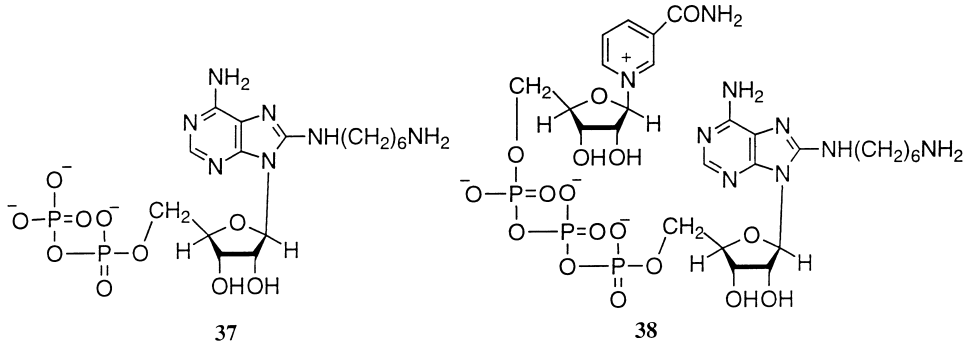


Figure 20. Intermediates for the synthesis of affinity probes of cATPR.

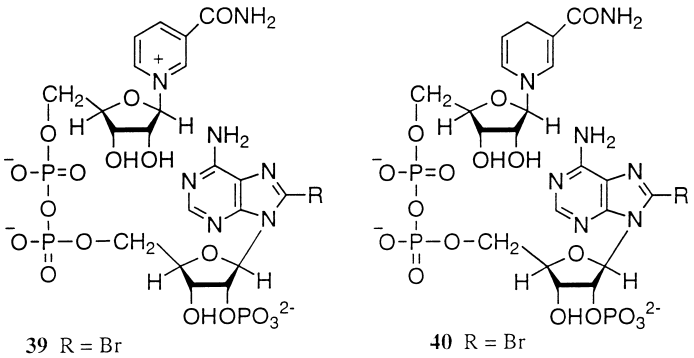


Figure 21. Intermediates for the synthesis of 8-(6-aminohexyl)-cADPRP.

(8-bromo-NADP$^+$, **39**)[56] was dissolved in 20 mM Tris buffer (pH 9.0) and then reduced to **40** in the presence of NADP$^+$-dependent alcohol dehydrogenase from *T. brokii* (from Sigma) and isopropanol at 43 °C.[57] After **39** was completely converted to **40**, to the reaction mixture was added 1,6-diaminohexane (50 equivalent) and the reaction mixture was stirred at 70 °C for 3 h. Then the solvent was evaporated under reduced pressure. The residue was washed with ethanol and centrifuged. The solid (**41**) was collected and dissolved in 0.2 M phosphate buffer (pH 7.6). This solution was incubated with glutathione reductase (from Sigma) and glutathione (oxidized form) at 23 °C for 1 h.[58] The reaction mixture was lyophilized and the residue was purified on HPLC55 to afford **42**[59] in 42% yield for the above 5 steps. Compound **42** was converted to **35**[60] with the cyclase in 80% yield (Fig. 21).

With these three affinity probes in hand, the study of the binding proteins can now be conducted. The free amino groups in these compounds will provide researchers with many options for conjugation to fluorescent and radiolabelled probes as well as to agarose resins.

Summary

Considerable progress has been made in the synthesis of cADPR analogues using enzymatic and non-enzymatic methods. The enzyme from *Aplysia californica* is commercially available, easily attached onto a solid support, and may be reused for many cycles.[20] The enzyme accepts various analogues of NAD$^+$ as substrates but the products of the reaction depend on the site of substituents on the adenosine portion of the NAD$^+$ molecule. Three types of reaction products may be formed: for cyclization to occur at the N-1 position, it is necessary to have an adenine nucleus. When adenine is replaced by hypoxanthine or guanine, cyclization occurred at the N-7 position instead. Substitution at the 6-position of the adenine ring or making the NAD$^+$ molecule more flexible by removing the 2- and 3-carbons of the ribose ring led to the formation of only hydrolysis products. For the synthesis of affinity probes, the most suitable position on the NAD$^+$ molecule for attachment is the 8-position because the cyclase can accommodate very large substituents at this site. Hence, it is not surprising that so many 8-substituted analogues have been made. As a rule, the product profiles of the biomimetic synthetic method were the same as the enzymatic method but in lower yields (Fig. 22).

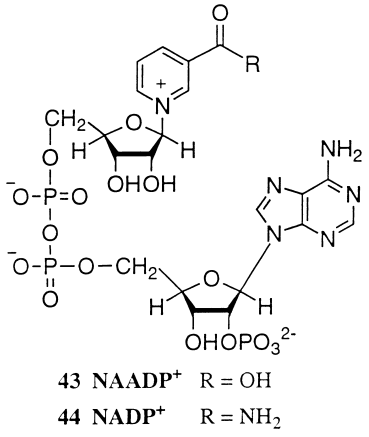


Figure 22. Structures of NAADP$^+$ and NADP$^+$.

More recently, nicotinic acid adenine dinucleotide phosphate (NAADP$^+$) (**43**) was found to be a new putative second messenger of intramolecular calcium release.[8,9] NAADP$^+$ could be produced by alkaline treatment of NADP$^+$ (**44**),[61,62] or via base-exchange reaction of **44** with nicotinic acid catalyzed by a number of enzymes,[62–64] or by reaction of cADPRP (**2**) with nicotinic acid in the presence of the ADP-ribosyl cyclase.[20] The NAADP$^+$-dependent calcium release mechanism was found to be independent of the cADPR- and IP_3-pathways.

The discovery and identification of cADPR and NAADP$^+$ as calcium releasing agents are important developments in the current investigations of cellular signaling mechanisms. These intracellular messengers provide chemists with an opportunity for developing more efficient methodologies for the synthesis of more active and stable analogues of cADPR and NAADP$^+$.

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55. All the reactions are monitored by HPLC. For the reduced compounds (**40** and **41**) there is a strong absorbency at 340 nm. All samples were analyzed or purified by an anion-exchange HPLC on a Synchropak AX-100 column (25×7.8 mm i.d., 5 μm). The column was eluted using a linear gradient of 0.1 M KH_2PO_4 and 1.5 M NaCl in 0.1 M KH_2PO_4 (solvent A and B, pH 4.0) to 80% for 16 min at a flow rate of 2 mL/min, followed by elution using solvent B at a flow rate of 3 mL/min until 26 min. The retention time for $NADP^+$, **39**, **40**, **41**, **42**, and **35** were 15.8, 16.6, 25.2, 19.0, 9.6, and 10.0 min, respectively. Compound **42** and **35** were further purified on a Waters Nova-Pak C18 column (100×8 mm i.d., 4 μm). The column was first eluted with 3 mM trifluoroacetic acid at a flow rate of 0.8 mL/min for 6 min, followed by 5% acetonitrile in 3 mM TFA at a flow rate of 3 mL/min. The retention time for **42** and **35** were 5.0 and 8.6 min, respectively.
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60. **35**: ^{1}H NMR (D_2O, 300 MHz, pH 3.0) δ 1.45 (m, 4H), 1.71 (m, 4H), 3.00 (m, 2H), 3.48 (m, 2H), 4.05–4.90 (m, 9H), 5.49 (m, 1H), 6.10 (d, J = 4.5 Hz, 1H), 6.14 (d, J = 4.0 Hz, 1H), 8.87 (s, 1H); UV λ_{max} (pH 6.0) 280 (ε 15,300) nm; FAB MS *m*/*e* 733 ($[M + 2H]^+$), 770 ($[M + K]^+$).
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