2′-Deoxy Cyclic Adenosine 5′-Diphosphate Ribose Derivatives: Importance of the 2′-Hydroxyl Motif for the Antagonistic Activity of 8-Substituted cADPR Derivatives

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The structural features needed for antagonism at the cyclic ADP-ribose (cADPR) receptor are unclear. Chemoenzymatic syntheses of novel 8-substituted 2′-deoxy-cADPR analogues, including 8-bromo-2′-deoxy-cADPR **7**, 8-amino-2′-deoxy-cADPR **8**, 8-*O*-methyl-2′-deoxy-cADPR **9**, 8-phenyl-2′-deoxy-cADPR **10** and its ribose counterpart 8-phenyl-cADPR **5** are reported, including improved syntheses of established antagonists 8-amino-cADPR **2** and 8-bromo-cADPR **3**. *Aplysia californica* ADP-ribosyl cyclase tolerates even the bulky 8-phenyl-nicotinamide adenine 5′-dinucleotide as a substrate. Structure−activity relationships of 8-substituted cADPR analogues in both Jurkat T-lymphocytes and sea urchin egg homogenate (SUH) were investigated. 2′-OH Deletion decreased antagonistic activity (at least for the 8-amino series), showing it to be an important motif. Some 8-substituted 2′-deoxy analogues showed agonist activity at higher concentrations, among which 8-bromo-2′-deoxy-cADPR **7** was, unexpectedly, a weak but almost full agonist in SUH and was membrane-permeant in whole eggs. Classical antagonists **2** and **3** also showed previously unobserved agonist activity at higher concentrations in both systems. The 2′-OH group, without effect on the Ca^{2+}-mobilizing ability of cADPR itself, is an important motif for the antagonistic activities of 8-substituted cADPR analogues.

Introduction

Cyclic adenosine 5′-diphosphate ribose **1** (cADPR;[a] Figure 1) was first discovered by Lee et al.[1] as a Ca^{2+}-mobilizing metabolite of nicotinamide adenine dinucleotide (NAD^+). cADPR is a potent second messenger,[2] and its Ca^{2+} release is regulated independently of the well-established inositol trisphosphate/Ca^{2+} channel,[1,3] most likely by the ryanodine receptor (RyR).[4] Since its discovery, some useful cADPR derivatives have been synthesized and biologically studied. Among these analogues, 8-substituted derivatives, such as 8-amino-cADPR **2** and 8-bromo-cADPR **3** are antagonists of cADPR/Ca^{2+} signaling, and these two 8-substituted derivatives have been widely used as biological tools in studies of cADPR-mediated Ca^{2+} release.[5] 8-Amino-cADPR **2**, as the most potent competitive antagonist, has been employed in numerous studies in a variety of biological systems, including sea urchin egg fertilization,[6] T cell activation,[7] and smooth muscle[8] and cardiac myocytes.[9] 8-Bromo-cADPR **3**, although not as potent as **2**, has also been a valuable pharmacological tool, particularly because it has proven to be cell-permeant.[10] The related 7-deaza 8-bromo-cADPR has found several biological applications as a more attractive agent, because it is not only membrane-permeant but also more potent in blocking Ca^{2+} release and more resistant to both chemical and enzymatically-induced hydrolysis.[10–13] Although such 8-substituted cADPR analogues have been extensively studied, one central question remains unanswered: which structural features control the agonistic/antagonistic activities?

The early finding that 8-substitution of cADPR converts cADPR from an agonist into an antagonist seemed to indicate that 8-substitution alone could be responsible for this activity. This idea, however, was subsequently found not to be correct. Shuto et al.[14a] reported that some 8-substituted cyclic adenosine diphosphocarbocyclic ribose (cADPcR) analogues are agonists rather than antagonists of Ca^{2+} release in sea urchin egg homogenate, suggesting that the oxygen atom of the "northern" ribose (Figure 1)[b] may also be an important structural feature, contributing to the antagonistic activity of 8-substituted cADPR analogues. Such a substitution in the "southern" ribose also generated an agonist.[14b] A small group of N1-cyclized 8-substituted cyclic inosine 5′-diphosphoribose (cIDPR) analogues were also synthesized in our laboratory.[15,16] Some of the compounds acted as potent agonists in Jurkat T lymphocytes, suggesting that the 6-amino group of the adenine may also play a part in the antagonistic effect of 8-substituted cADPR analogues. These results indicate that the agonistic/antagonistic activity of 8-substituted cADPR derivatives is clearly not only governed by 8-substitution. Most likely, there is a global cooperative conformational effect that governs the whole process, and this can be coordinated by several structural features. We demonstrated that modification of the 3′-hydroxyl of the southern ribose by methylation could also induce antagonism, at least in sea urchin homogenate.[17] A recent conformational study[18] focused on the idea that antagonism by

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[a] Abbreviations: cADPR, cyclic adenosine 5′-diphosphate ribose; cADPcR, cyclic adenosine diphosphocarbocyclic ribose; cIDPR, cyclic inosine 5′-diphosphoribose; NAD^+, nicotinamide adenine dinucleotide; TEAB, triethylammonium bicarbonate; β-NMN^+, β-nicotinamide 5′-mononucleotide; CDI, carbonyldiimidazole; TEP, triethyl phosphate; TMP, trimethylphosphate; ADPRC, ADP-ribosyl cyclase.

[b] The two sugars of cADPR and analogues can be distinguished by a "southern" and "northern" nomenclature and also adopting prime and double-prime notation for the sugar carbons, depending upon their linkage to either N9 or N1 of the adenine, respectively.

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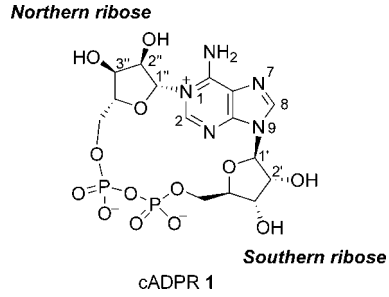
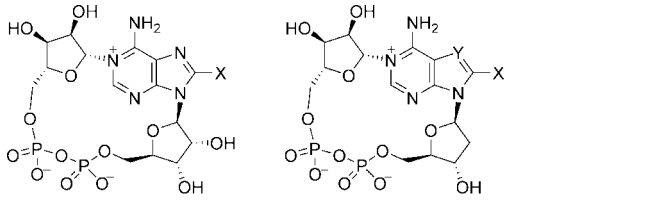


cADPR **1**

Figure 1. cADPR structure and its numbering system.



X = H, cADPR **1**
X = NH_2, 8-NH_2-cADPR **2**
X = Br, 8-Br-cADPR **3**
X = OMe, 8-OMe-cADPR **4**
X = Ph, 8-Ph-cADPR **5**

X = H, Y = N, 2′-deoxy-cADPR **6**
X = Br, Y = N, 8-Br-2′-deoxy-cADPR **7**
X = NH_2, Y = N, 8-NH_2-2′-deoxy-cADPR **8**
X = OMe, Y = N, 8-OMe-2′-deoxy-cADPR **9**
X = Ph, Y = N, 8-Ph-2′-deoxy-cADPR **10**
X = H, Y = C, 7-Deaza-2′-deoxy-cADPR **11**

Figure 2. Structures of the cADPR analogues discussed in the text.

such a 3′-hydroxyl-methylated cADPR analogue might be related to an altered "backbone" conformation, but the evidence was not sufficient to establish this idea. More importantly, it is not known what conformation cADPR analogues may adopt when they actually bind to their receptor. Other work[19,20] explored radical structural modifications of the northern ribose in particular, but only agonistic activities were observed. Modification of the pyrophosphate system led to potent agonist activity for a triphosphate analogue of cADPR,[21] and phosphonate analogues showed decreased agonist activity.[22] The above results illustrate that the global structural features that control the agonistic/antagonistic activity of 8-substituted cADPR derivatives are far from clear and thus encouraged us to pursue further structure-activity relationship (SAR) studies on this class of molecule.

Among southern ribose-modified analogues, 2′-deoxy-cADPR was first synthesized by Ashamu et al.[17] and is almost as potent as cADPR in mediating Ca^{2+} release from cADPR-sensitive stores in sea urchin egg homogenates, indicating that 2′-OH deletion has no or little effect on the agonistic activity of cADPR, at least in the invertebrate assay system. It was not demonstrated, however, whether such a deletion might also potentially affect antagonistic activity. Because deletion of the 2′-OH group also decreases hydrophilicity, this modification might be expected to improve membrane permeability in 8-substituted compounds, which would be highly desirable for pharmacological intervention.

To improve our understanding of SAR in this class of 8-substituted cADPR analogues, we have designed, synthesized, and biologically evaluated a group of as yet unexplored 8-substituted 2′-deoxy-cADPR derivatives (Figure 2). This series of new cADPR analogues includes 8-bromo-2′-deoxy-cADPR **7** and 8-amino-2′-deoxy-cADPR **8**, the 2′-OH deleted version of the well-established antagonists **2** and **3**, and 8-*O*-methyl-2′-deoxy-cADPR **9**, whose parent compound 8-*O*-methyl-cADPR **4** was also exploited previously as an antagonist in T lymphocytes.[12] It was suggested that substitution with a large group at the 8 position might result in a decrease in antagonistic potency.[5] To further explore this idea, we also synthesized 8-phenyl-2′-deoxy-cADPR **10** and its ribose counterpart 8-phenyl-cADPR **5**. Derivatives with a bulky phenyl group at the 8 position were expected to illuminate the relation between agonistic/antagonistic activity, potency, and the size of the substituents in this position. In addition, we investigated the effect of these substitutions upon the cyclization of the corresponding NAD^+ analogues using the ADP-ribosyl cyclase from *Aplysia californica*.

Chemistry

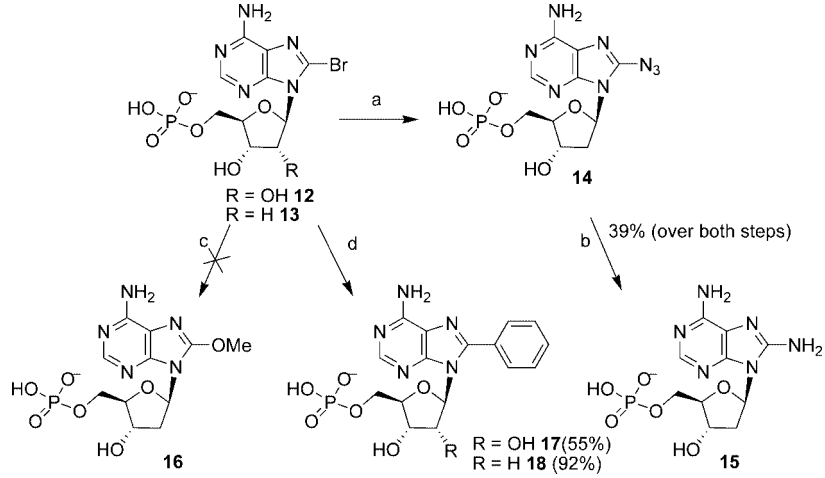
To date, cADPR analogues have been synthesized either by total chemical synthesis[14,19] or a chemoenzymatic method.[15–17] Both of the two approaches have their advantages but also drawbacks. The major drawback of the chemoenzymatic method is the selectivity of the *Aplysia* cyclase, which, although generally promiscuous, cannot always catalyze the transformation of a NAD^+ analogue into the active N1-cyclized product. Hydrolysis or N7 cyclization is the result for some NAD^+ analogues. The total synthetic approach works efficiently for producing carbocyclic cADPcR and other analogues, but just to produce a single analogue, the synthetic route is rather long and difficult.

The earliest reported synthesis of 8-substituted cADPR analogues was through the chemoenzymatic approach, demonstrating the high substrate tolerance of the *Aplysia* cyclase.[23] As we showed earlier that 2′-deoxy-NAD^+ is also recognized as a substrate by this enzyme,[17] we sought to adopt this method in the present study for the preparation of 8-substituted 2′-deoxy-cADPR analogues by incubation of the corresponding NAD^+ derivatives with *Aplysia* ADP ribosyl cyclase. The required NAD^+ analogues can be synthesized by coupling the relevant nucleotides with nicotinamide 5′-mononucleotide (β-NMN^+).

Of the cADPR analogues targeted in this study (Figure 2), the 8-bromo-substituted analogues are the most convenient to synthesize, because bromination of nucleotides has been widely reported in many publications and can be achieved in one step in high yield. On the basis of this consideration, Wagner et al.[16] reported a rapid synthetic route toward a limited number of 8-substituted N1-cIDPR derivatives. This approach was centered on the cyclic inosine 5′-diphosphate ribose (cIDPR) analogue 8-bromo-cIDPR, from which syntheses of other cIDPR analogues were achieved by replacement of bromine with other nucleophiles or by a Suzuki cross-coupling reaction. However, this approach relies heavily on the excellent and unusual stability of N1-cIDPR analogues and is therefore not applicable in our case, because cADPR analogues are known to undergo rapid hydrolysis at the N1−C1″ linkage at raised temperatures. The 8-bromo-NAD^+ analogues are also not ideal for any chemical reactions, because the NAD^+ molecules are prone to lose the nicotinamide moiety even at ambient temperature. 8-Bromonucleotides **12** and **13** are stable under basic conditions and even at elevated temperatures, allowing us to follow the above strategy by employing **12** and **13** as the initial building blocks, from which 8-azido-, 8-amino-, 8-*O*-methyl-, and 8-phenyl-substituted nucleotides were synthesized in one or two steps (Scheme 1).

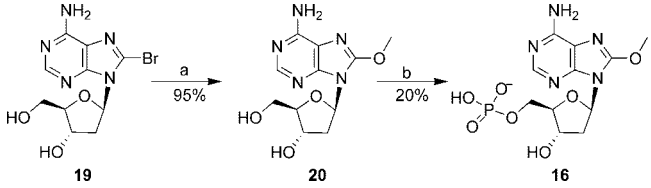
The starting building blocks, 8-bromo-AMP analogues **12** and **13**, were synthesized using the protocol of Ikehara et al.[24,25] Purification using reverse-phase chromatography gave **12** and **13** as their triethylammonium salts in 85 and 55% yield, respectively. Nair et al.[26] reported that treatment of 8-bromo-2′-deoxyadenosine with NaN_3 gave the desired 8-azido-2′-deoxyadenosine in good yield. Application of this reaction to

Scheme 1. Syntheses of 8-Substituted Nucleotides from the Bromo Analogues[a]



[a] Reagents and conditions: (a) NaN_3, H_2O/DMF (20%, v/v), 80 °C, (b) 1,4-dithiol-(D,L)-threitol, 0.05 M TEAB buffer (pH 8), room temperature, (c) NaOMe, MeOH, refluxing, and (d) $Pd(PPh_3)_4$, phenylboronic acid, Na_2CO_3, 2:1 MilliQ/MeCN, 80 °C. Compounds **12**−**18** are in their triethylammonium salt form.

Scheme 2. Synthesis of 8-*O*-Methyl-2′-deoxy-AMP[a]

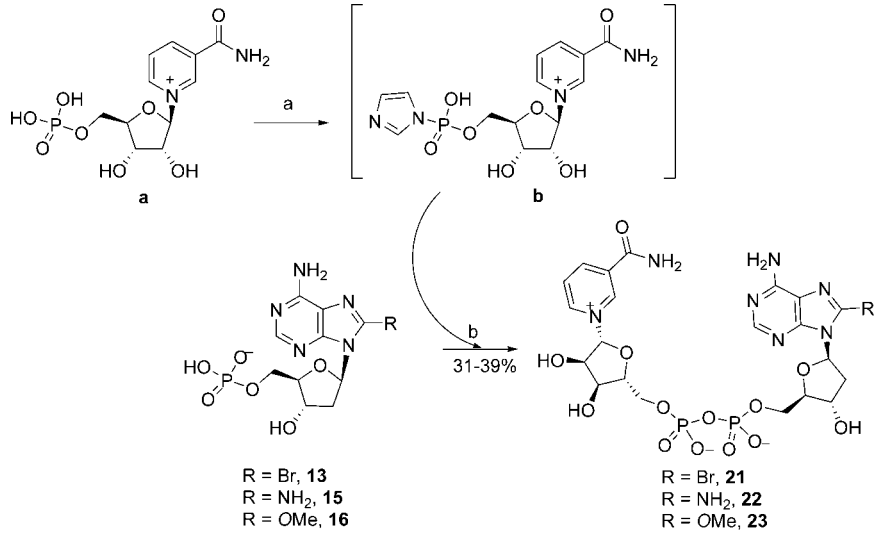


[a] Reagents and conditions: (a) NaOMe, MeOH, refluxing and (b) $POCl_3$, TMP, −20 °C. Compound **16** is in the triethylammonium form.

the 8-bromonucleotide **13** in our case, however, was found not to be successful because the starting material **13** formed a sodium salt upon treatment with NaN_3, which precipitated from the reaction medium (DMF), thus blocking the progress of the reaction. To solve this problem, a H_2O/DMF mixture was used. The sodium salt that formed was successfully solubilized in this polar solvent system, and the reaction was complete after heating overnight. The reaction process could not be monitored by HPLC because **14** has the same retention time as the starting bromo compound **13** in our solvent system. Instead, the reaction was monitored by changes in the maximum UV absorption (UV_{max}). A clear shift from 262 to 282 nm was noticed, indicating the formation of **14**.[27] Reduction of azido **14** into amino **15** was achieved using a dithiothreitol (DTT)-mediated reduction.[27,28] The DTT-mediated reduction is normally performed in aqueous solution at near neutral pH and room temperature. This reaction is thus ideal for reducing polar and unstable molecules, for example, the 8-azido-nucleotides in our case. Treatment of **14** with DTT in TEAB buffer in the dark overnight and purification using reverse-phase chromatography gave **15** in 39% isolated yield based on bromo compound **13**. A shift of UV_{max} from 281 to 274 nm was noticed in this experiment consistent with the observation of Armstrong et al.[27] Attempts to produce 8-methoxy-2′-deoxy-AMP **16** by treatment of **13** with NaOMe failed, because the addition of NaOMe transformed **13** into its sodium salt, which was not soluble in the reaction solvent MeOH. The addition of water to the reaction system was not feasible, because this reaction requires anhydrous conditions. 8-OMe-2′-deoxy-adenosine **20**, however, could be synthesized by treatment of 8-bromo-2′-deoxy-adenosine **19** with NaOMe in over 90% yield[26] (Scheme 2). This encouraged us to perform a phosphorylation reaction on this compound to pursue a synthesis of the desired **16**. It is known that 2′-deoxy-nucleosides are more likely to undergo hydrolysis in acidic conditions than their ribose counterparts.[29] This is probably due to the reason that, under acidic conditions, a 2′-deoxy-nucleoside may form a more stable oxonium intermediate at the ribosyl C-1′ position, thus facilitating a rapid hydrolysis. Because of the lack of acid stability, phosphorylation of **20** using the conventional $POCl_3$/TEP method[30] is problematic, because this method requires an aqueous workup, which will result in an acidic solution. It was indeed found that the 8-substituted 2′-deoxy-AMP that formed in the phosphorylation reaction degraded rapidly during the aqueous workup (data not shown). Different buffer solutions were attempted in the aqueous workup stage, including TEAB buffer and H_2O/pyridine, but all failed. We later found that quenching the phosphorylation reaction by the addition of ice, followed immediately by neutralization with a solution of NaOH could effectively decrease the degradation of the product. Thus, purification using reverse-phase chromatography combined with ion-exchange chromatography gave the desired compound **16** in its triethylammonium form, albeit not in high yield.

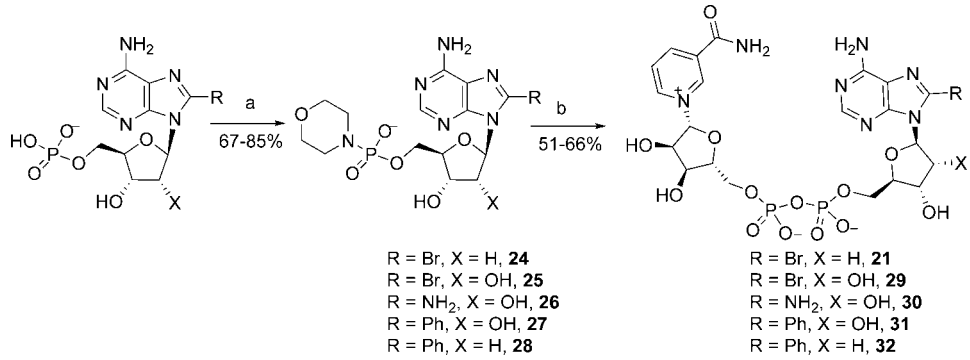
Verlinde et al. demonstrated that 8-phenyl-adenosine could be produced via the Stille-type cross-coupling reaction of a protected 8-bromo-adenosine.[31] A Suzuki cross-coupling reaction was also recently reported,[32,33] facilitating the synthesis of 8-phenyl-nucleosides from the corresponding unprotected 8-bromo-nucleosides. In general, the Suzuki-type coupling reactions are easier to handle than the Stille type because they work under moist or even aqueous solutions and they tolerate the presence of some unprotected functional groups, e.g., the hydroxyl group on the nucleoside ribose. Wagner et al.[16] reported a synthesis of 8-phenyl-cIDPR from 8-bromo-cIDPR by adaptation of the Suzuki cross-coupling reaction, demonstrating the wider application of this reaction to stable nucleotide-type compounds. In the light of this result, it was expected that Suzuki−Miyaura conditions would be compatible with the use of unprotected nucleotides, such as 8-bromo-AMP **12**, and two examples for the Suzuki−Miyaura reaction of unprotected nucleotides have indeed been reported during the course of this study.[34,35] Compound **12** dissolved in a MilliQ water/MeCN mixture was treated with Na_2CO_3, phenylboronic acid, and the palladium catalyst $Pd(PPh_3)_4$ under an argon atmosphere at 80 °C for 3 h (Scheme 1). HPLC analysis indicated that most of the starting material had been consumed and that a new product had formed

Scheme 3. Syntheses of 8-Substituted 2′-Deoxy-NAD$^+$ Analogues Using the CDI Method[a]



[a] Reagents and conditions: (a) CDI, TEA, room temperature, anhydrous DMF and (b) anhydrous DMF, 2 days. Compounds **21**−**23** are in the triethylammonium form.

Scheme 4. Syntheses of NAD$^+$ Analogues Using a Lewis-Acid-Mediated Condensation[a]



[a] Reagents and conditions: (a) PPh_3, dipyridyl disulfide, morpholine, DMSO, room temperature and (b) $MnCl_2$/formamide (0.2 M), $MgSO_4$, β-NMN$^+$, room temperature. Compounds **29**−**32** are in their triethylammonium form.

with a retention time of 12.10 min. Purification using reverse-phase chromatography gave the desired 8-phenyl-AMP **17** in 55% yield as its triethylammonium salt, showing a multiplet (integration 5H) at the aromatic region of its ^{1}H NMR spectrum, representing the phenyl group. It was also noticed that the UV_{max} of the phenyl compound migrated to ca. 276 nm, consistent with the report by Wagner et al.[16] 8-Phenyl-2′-deoxy-AMP **18** was synthesized in the same manner. In this case, an excess of phenylboronic acid (4 equiv) and $Pd(PPh_3)_4$ was added in two stages to the reaction to drive it to completion. RP-HPLC indicated that the starting material had been nearly 100% consumed and that a new compound at 13.7 min was seen as the sole product. Purification using reverse-phase media, however, gave a mixture of the desired compound **18** as its triethylammonium salt (92% yield by ^{1}H NMR) and remaining phenylboronic acid, suggesting that the RP system might not work efficiently to separate these two compounds, which have close polarities. The mixture was not further purified but was directly used in the next step to produce the corresponding morpholidate (as shown in Scheme 4). It was found that the impurity phenylboronic acid was easily removed at the morpholidate step, because it possibly formed a morpholinium salt that dissolved in acetone and was therefore removed in the filtrate together with other reagents, such as triphenylphosphine or dipyridyl disulfide.

The 8-substituted 2′-deoxy-NAD$^+$ analogues were first synthesized by a carbonyldiimidazole (CDI)-mediated condensation between the above 2′-deoxy nucleotides and β-NMN$^+$. An active species **b** was formed in this reaction as shown in Scheme 3.[36,37] In general, β-NMN$^+$ **a** was first treated with triethylamine (TEA) and then with an excess of CDI in anhydrous DMF to provide the activated intermediate **b**, showing a characteristic singlet at ca. −9.0 ppm in the ^{31}P NMR spectrum. After the remaining CDI had been quenched by the addition of methanol, the intermediate was treated with the relevant nucleotide in anhydrous DMF to form the desired NAD$^+$ analogue. 8-Bromo-2′-deoxy-AMP **13** was thus treated with the intermediate **b** in anhydrous DMF for 2 days. Purification of product using ion-exchange chromatography gave the desired 8-bromo-2′-deoxy-NAD$^+$ **21** in 31% yield as its triethylammonium salt, showing a triplet at 6.38 ppm and a doublet at 5.98 ppm in the ^{1}HNMR spectrum, representing the H-1′ and H-1″ anomeric protons, respectively. In the ^{31}P NMR spectrum, two doublets were seen at −11.17 and −10.53 ppm, indicating the formation of the pyrophosphate bond. 8-Amino-2′-deoxy-NAD$^+$ **22** and 8-*O*-methyl-2′-deoxy-NAD$^+$ **23** were synthesized in the same manner. Purification using ion-exchange chromatography gave compounds **22** and **23** both in ca. 40% isolated yield.

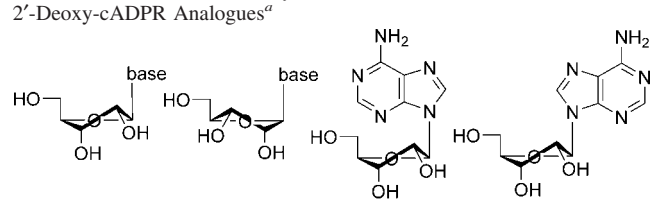
Lee et al.[38] reported that NAD^+ analogues could be synthesized by a Lewis-acid-mediated condensation between activated phosphoromorpholidates and β-NMN^+. This method has been successfully used recently to synthesize 8-substituted NHD^+ analogues in high yield.[15,16] 8-Bromo-2′-deoxy-AMP morpholidate **24** was produced from compound **13** (triethylammonium salt) by treatment with PPh_3, morpholine, and dipyridyl disulfide (Scheme 4).[39] Workup of this reaction gave the desired **24** as a yellow solid in 72% yield, showing a characteristic singlet at 8.20 ppm in the ^{31}P NMR spectrum, representing the formation of morpholidate. The successful synthesis of **24** suggested that the Lewis-acid-mediated condensation could also be used to produce 2′-deoxy-NAD^+ analogues. Thus, morpholidates **25**−**28** (Scheme 4) were synthesized and then condensed with β-NMN^+ in the presence of Lewis acid ($MnCl_2$) in formamide. Purification using reverse-phase chromatography gave the desired dinucleotides, 8-bromo-NAD^+ **29**, 8-amino-NAD^+ **30**, 8-phenyl-NAD^+ **31**, and 8-phenyl-2′-deoxy-NAD^+ **32** as their triethylammonium salts.

8-Substituted NAD^+ analogues **21**–**23** and **29**−**32** were then incubated with *Aplysia* ADP-ribosyl cyclase. Purification of products using ion-exchange chromatography gave the desired 8-substituted cADPR analogues **2**, **3**, **5**, and **7**–**10** (Figure 2) as the triethylammonium salts. The cyclic structures of these cADPR analogues were confirmed by the ^1H NMR spectrum: (1) the aromatic peaks at 8–9 ppm representing nicotinamide had disappeared, indicating the cleavage of the nicotinamide moiety in this reaction; (2) two doublets were observed at ca. 6.0 ppm, representing both the H-1′ and H-1″, demonstrating that these two riboses were connected to the purine base by a glycosyl bond. The enzymatic reaction was monitored by ion-pair HPLC. It was found that the above NAD^+ analogues were turned over by the *Aplysia* cyclase at a roughly similar rate to that of NAD^+ itself. The fact that 8-phenyl-NAD^+ and 8-phenyl-2′-deoxy-NAD^+ were also quickly converted by the *Aplysia* cyclase into the corresponding cyclic compounds further indicates, perhaps somewhat surprisingly, that the enzyme has excellent tolerance toward even very large substituents at the 8 position. Direct chemical modification of an already cyclized precursor was required to achieve a similar end in the cIDPR series,[15] and a similarly bulky 8-*S*-phenyl cADPcR derivative was earlier synthesized by a total synthetic route and, at high concentrations, appeared to act as a competitive antagonist.[14]

Conformational Analysis. To identify the bioactive conformations of the new cADPR analogues, knowledge of their solution structures is required. We have extracted information about the conformation of important partial structures for all 8-substituted cADPR/2′-deoxy-cADPR analogues from their ^1H NMR spectra in D_2O. Of particular interest in the context of this study were potential conformational changes in the southern ribose, resulting from structural modification of positions 2′ and 8. In solution, the furanose in nucleosides and nucleotides generally exists in a conformational equilibrium between the C2′-*endo* (S type) and C3′-*endo* (N type) form (Table 1). In addition, the nucleobase can be oriented toward (*syn*) or away from (*anti*) the ribose ring, depending upon the torsion angle χ about the glycosidic bond. It is apparent that these local conformational changes also affect the global conformation and overall shape of the molecule.

For nucleosides and nucleotides, the magnitude of the H1′−H2′ coupling constant is directly correlated to the ratio of the C2′-*endo*/C3′-*endo* conformers.[40–42] Altona et al. have shown that the percentage of the C2′-*endo* population can be estimated by taking $10J_{1'2'}$.[40] Using Altona's approach, we determined the preferred conformation of the southern ribose for all of the new 8-substituted cADPR/2′-deoxy-cADPR analogues. The results from our analysis are summarized in Table 1. We find that, while the substituent in position 8 has some influence on the conformation of the southern ribose (compare, e.g., 8-amino cADPR **2**, 8-bromo cADPR **3**, and 8-phenyl cADPR **5**), the 2′-deoxy cADPR analogues in this series generally adopt the C2′-*endo* conformation to a higher percentage than their ribose counterparts, irrespective of the nature of the substituent in position 8 (**2** versus **8**, **3** versus **7**, and **5** versus **10**). This slight difference regarding the conformation of the southern ribose in 2′-deoxyribo and ribo nucleotides has also been observed for analogues unsubstituted at position 8, e.g., 2′-deoxy AMP (63–68% C2′-*endo*) and AMP (52% C2′-*endo*).

Table 1. Conformational Analysis of 8-Substituted cADPR/2′-Deoxy-cADPR Analogues[a]



compound	C2′-*endo* (%) ($10J_{1'2'}$)	H-2′ (ppm)	$^{\alpha}\Delta_{H\text{-}2'}$	*syn*/*anti*
2	56	5.36	0.92	*syn*
3	53	5.35	0.91	*syn*
5	60	5.56	1.12	*syn*
7	62–67	3.20 (H-2′a)	0.40	*syn*
8	66–69	3.19 (H-2′a)	0.39	*syn*
9	64–68	3.19 (H-2′a)	0.39	*syn*
10	70	3.46 (H-2′a)	0.66	*syn*

[a] $\Delta_{H\text{-}2'}$ values represent the difference between the chemical shift of H-2′ of the above cyclic compounds and H-2′ of AMP or 2′-deoxy-AMP. H-2′ chemical shifts for AMP and 2′-deoxy-AMP are 4.62 and 2.80 (H-2′a), respectively. Inset shows key descriptors of nucleoside and nucleotide conformation.

In cADPR, the adenine base is oriented in the *syn* conformation both in the crystal structure, with a torsion angle χ of 64 (±1)° about the glycosidic bond,[43] and in solution.[18,42] The chemical shift of the H-2′ signal has been identified as an indicator for the position of the *syn*/*anti* equilibrium in nucleosides and nucleotides, and a significant downfield shift of the H-2′ signal is regarded as indicative of the predominance of the *syn* conformer.[44,45] In cADPR, H-2′ experiences a downfield shift of 0.59 ppm compared to AMP, a nucleotide that exists predominantly in the *anti* form.[44] We observed a similar downfield shift for the H-2′ signal in the ^1H NMR spectra of all of the new 8-substituted cADPR/2′-deoxy-cADPR analogues compared to AMP or 2′-deoxy-AMP (Table 1). These results confirm that all of the new cADPR analogues adopt a global conformation characterized by the *syn* orientation of the nucleobase, similar to that of cADPR itself. This finding is not unexpected. Linear dinucleotides with bulky substituents at position 8 frequently adopt the *syn* conformation, because of the nonbonded repulsion between the substituent in position 8 and the ribose ring,[46,47] and the conformational constraints imposed by the macrocycle in cADPR and similar molecules strongly disfavor the *anti* conformation.

While both 2′-deoxy and ribo analogues in the present series seem to share the same global conformation, we did find, however, that values for $\Delta_{H\text{-}2'}$ were generally lower for the 2′-deoxy analogues than for the ribo derivatives. This suggests that, although the *syn* conformation is preferred in both classes

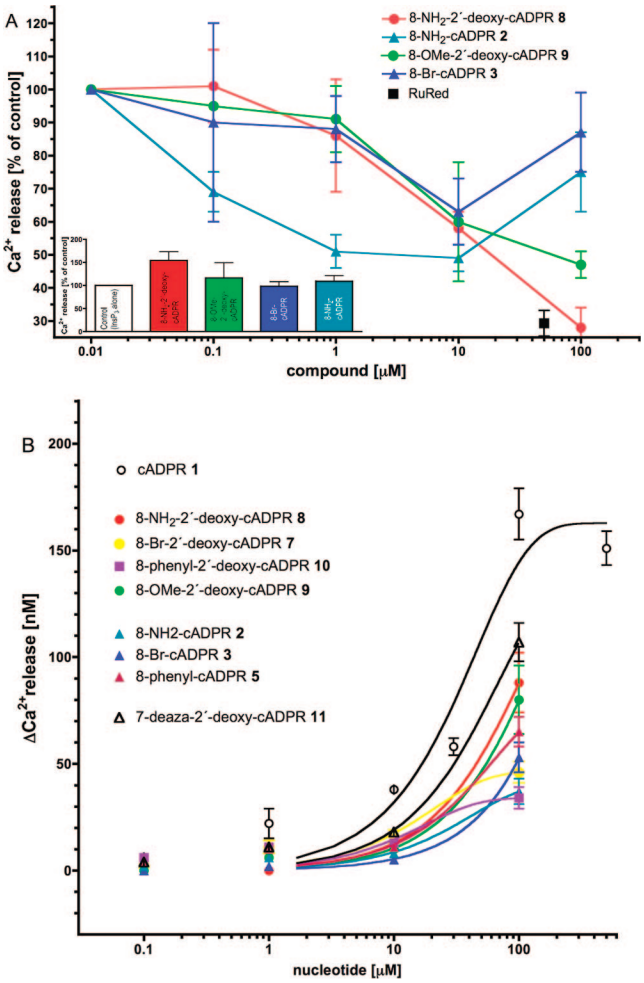


Figure 3. Effects of cADPR analogues on Ca^{2+} release in permeabilized Jurkat T cells. (A) Antagonist activities of 8-amino-cADPR (IC_{50} value of approximately 0.2 μM), 8-amino-2′-deoxy-cADPR (IC_{50} value of approximately 6 μM), 8-*O*-methyl-2′-deoxy-cADPR (IC_{50} value of approximately 8 μM), and 8-bromo-cADPR (IC_{50} value of approximately 10 μM) toward Ca^{2+} release induced by 30 μM cADPR in permeabilized Jurkat T cells. The ryanodine receptor antagonist ruthenium red blocked approximately 70% of Ca^{2+} release evoked by cADPR. The inset shows the effect of 100 μM of the cADPR analogues on Ca^{2+} release induced by $InsP_3$ (4 μM). (B) Ca^{2+} release activity of the 8-substituted cADPR analogues in permeabilized Jurkat T cells. Jurkat T cells were permeabilized, and [Ca^{2+}] was determined using fura2 fluorescence as described in the Experimental Section.

of compounds, the torsion angle χ of the 2′-deoxy analogues may be slightly different from the ribose compounds.

Biological Results

Ca^{2+} Release Antagonism in Jurkat T Cells. First, we investigated the effect of the new cADPR analogues on cADPR-mediated Ca^{2+} release in permeabilized T Jurkat cells. Under these conditions, the known antagonist 8-amino cADPR **2**[23] inhibits Ca^{2+} release with an IC_{50} value of approximately 0.2 μM (Figure 3A). By comparison, all other analogues in this series, including all of the new 2′-deoxy derivatives, displayed decreased potency (e.g., 8-amino-2′-deoxy cADPR **8**, IC_{50} value of approximately 6 μM; 8-methoxy-2′-deoxy cADPR **9**, IC_{50} value of approximately 8 μM; Figure 3A). Interestingly, in the case of the 8-phenyl (**10** versus **5**) and the 8-bromo (**7** versus **3**) derivatives, there is little difference between the 2′-deoxyribo and the ribo congeners with regard to their inhibitory activity. Both 8-phenyl-2′-deoxy cADPR **10** and 8-phenyl cADPR **5** are weak antagonists of Ca^{2+} release, as are 8-bromo-2′-deoxy cADPR **7** and 8-bromo cADPR **3**. On the other hand, in the case of the 8-amino congeners (**8** versus **2**), removal of the 2′-OH group does result in a 30-fold reduction in potency (**8**, IC_{50} value of approximately 6 μM; **2**, IC_{50} value of approximately 0.2 μM; Figure 3A). None of the cADPR analogues antagonized Ca^{2+} release induced by Ins(1,4,5)P_3 (inset in Figure 3A).

These results suggest that there is a role for the 2′-hydroxyl group in antagonizing cADPR-induced calcium release in T cells and that this role seems to be modulated by the nature of the substituent in position 8. Both 8-amino compounds **2** and **8** preferentially adopt a C2′-*endo* conformation at the southern ribose and a *syn* conformation about the N9-glycosyl linkage (see above and Table 1). This probably indicates that the different antagonistic activity of compounds **2** and **8** is not caused by a different global conformation or a different conformation of the southern ribose. There is, however, the possibility that subtle conformational differences between the 8-substituted 2′-deoxy cADPR analogues and their ribose counterparts, e.g., in the torsion angle χ, may explain the differential biological effects seen for compounds **2** and **8**. Alternatively, these differential biological effects may result from differential hydrogen-bonding patterns, and this possibility will be discussed in more detail below.

Ca^{2+} Release Agonism in Jurkat T Cells. At 100 μM concentration, all of the analogues tested in this series (including the well-documented antagonists 8-bromo and 8-amino-cADPR[23,6–10]) stimulate Ca^{2+} release in Jurkat T cells, albeit less strongly than the parent cADPR (Figure 3B). It is noteworthy that this is the first report of the *agonistic* properties of these analogues at higher concentrations. In previous studies, these compounds had been used mostly at concentrations of 10 μM and below, possibly because of their limited availability, which may explain why their agonistic effects had not previously been observed (for discussion *vide infra*).

The most potent agonists in the present study belong to the 2′-deoxyribo series (e.g., 7-deaza-2′-deoxy cADPR **11**, 8-amino-2′-deoxy cADPR **8**, and 8-methoxy-2′-deoxy cADPR **9**), although the 2′-deoxyribo analogues are not generally more potent than the ribo analogues. In those cases that allow a direct comparison between the 2′-deoxyribo and ribo series (8-phenyl, 8-bromo, and 8-amino), little difference in activity was observed for the 8-phenyl (**10** versus **5**) and 8-bromo (**7** versus **3**) pairs, respectively, while the 8-amino pair of compounds once again defies this trend. At a 100 μM concentration, 8-amino-2′-deoxy cADPR **8** stimulates Ca^{2+} release more potently than 8-amino cADPR **2** (Figure 3B). It was noticed that analogues with a large phenyl substitutent at position 8 could also induce Ca^{2+} release, suggesting that the binding site of the receptor is relatively tolerant to the size of a substitutent at this position. This may suggest that cADPR could be attached to a tether at this position while still retaining binding affinity, and such a ligand could be used in, e.g., an affinity chromatography column to detect and purify the cADPR receptor, etc.

When these results are taken together, they may suggest an important role for hydrogen bonding in determining the bioactivity of cADPR and its analogues. Only in those cases where hydrogen bonding is possible between hydrogen donors at position 8 and the cADPR receptor have we found a clear difference in bioactivity for the 2′-deoxyribo and ribo analogues (8-amino-2′-deoxy cADPR **8** versus 8-amino cADPR **2**), at least in T cells. In those cases, where no such hydrogen bonding is possible (8-phenyl and 8-bromo) differences in bioactivity

between the 2′-deoxyribo and ribo analogues are negligible. This trend holds for both agonistic and antagonistic behavior. At 100 μM, 8-amino-2′-deoxy cADPR **8** is a more potent agonist (partial agonist) of cADPR-mediated Ca^{2+} release than 8-amino cADPR **2**. Conversely, at 1 μM concentration, 8-amino cADPR **2** is a much more potent antagonist than 2′-deoxy-8-amino cADPR **8**. Furthermore, the 2′-hydroxy group may be involved in direct interactions with the receptor, e.g., as a hydrogen-bonding acceptor, similar to what has been suggested for the 3′-hydroxy group.[17] In sea urchin homogenates, it was found that 3′-OMe-cADPR blocked the cADPR-induced Ca^{2+} release in a concentration-dependent manner, while 3′-deoxy-cADPR was inactive, suggesting the 3′-OH as an important motif for binding to the receptor.[17] The amino substitutent at position 8 together with the 2′-hydroxyl group may be cooperative in interacting with the receptor by hydrogen bonding, and importantly, both groups are probably required for the outstanding antagonistic activity of **2**. Thus, the deletion of the 2′-OH consequently results in the breakdown of the cooperative hydrogen bonding, and this probably explains the differential bioactivities for analogues **2** and **8**.

Removing the 2′-hydroxyl group and the resulting breakdown of any intermolecular hydrogen bond at this position may further affect Ca^{2+}-release activities. However, in the T-cell system, 7-deaza-2′-deoxy-cADPR **11**[11b] (Figure 3B), similar to its ribose counterpart 7-deaza-cADPR,[48] acted as a partial agonist, consistent with the previous conclusion that, for inducing calcium release, a 2′-hydroxyl group is not crucial. The fact that compound **11** does not exhibit any antagonistic activity further demonstrated that substitution at the 8-position is indeed one of the key requirements for antagonistic activity.

Ca^{2+} Release in Sea Urchin Egg Homogenates (SUH). Many cADPR analogues were investigated in the invertebrate sea urchin egg homogenate (SUH) system, suggesting that the SUH system may be more suitable to test our 8-substituted 2′-deoxy-cADPR analogues. Thus, 8-amino-2′-deoxy-cADPR **8** and the ribose derivative **2** were evaluated in the SUH system (Figure 4). In agreement with observations in T cells, **8**was found to be a weaker antagonist, but it was only 2 times less potent than its ribose counterpart **2**. In comparison to the results in T cells, the effect of 2′-OH deletion in this case was less significant. Nevertheless, this combined with the results in T cells could lead to the conclusion that the 2′-OH group is indeed an important motif for the antagonistic activity, at least for the 8-amino-cADPR derivatives. Removal of the 2′-OH group will result in a decrease of antagonistic activity in both SUH and T-cell systems. Compounds **2** and **8** in SUH were both found to be agonists at high concentrations as observed in T cells (Figure 4), and their potencies for mobilizing Ca^{2+} are shown in Table 2.

As shown in the concentration–response curve (Figure 4), the plateau for **8** could not be defined, suggesting that this compound, unlike the ribose analogue **2**, is possibly a full agonist. Indeed, when 8-bromo-2′-deoxy-cADPR **7** was evaluated for Ca^{2+}-releasing activity in SUH, it was found, surprisingly, that **7** is a weak but almost full agonist (Figure 5A). It was also noticed that **7**, similar to cADPR itself, cross-desensitized the Ca^{2+} release by 500 nM cADPR (Figure 5A). The interesting activity of **7** seems to corroborate our observation that, in T cells, 2′-OH deletion of the 8-substituted cADPR analogues may result in better partial agonists. Because 8-bromo-cADPR **3** is known to be cell-permeant, it is thus expected that compound **7** may also have this property. Incubation of 8-bromo-2′-deoxy-cADPR **7** with intact sea urchin eggs pre-

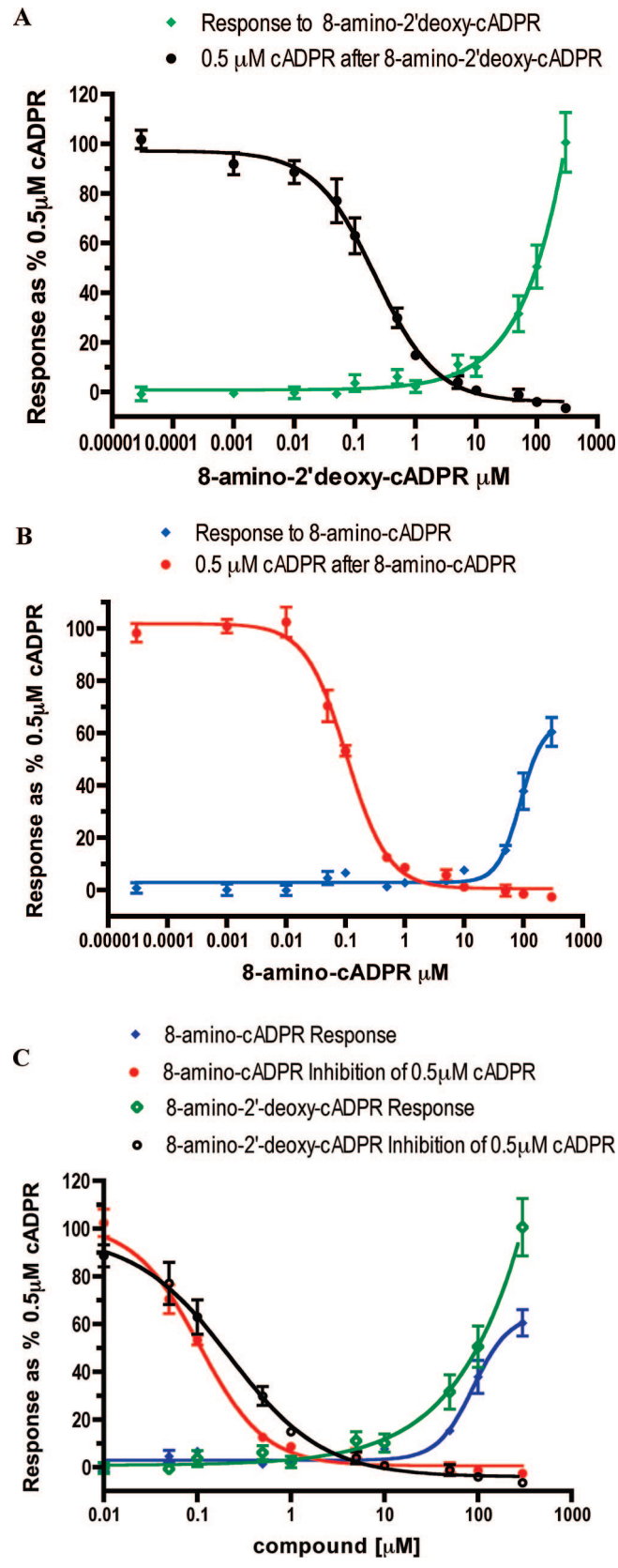


Figure 4. Comparison of the biological activities of 8-amino-cADPR and 8-amino-2′-deoxy-cADPR. (A) 8-NH_2-2′-deoxy cADPR **8**, (B) 8-NH_2-cADPR **2**, and (C) comparison of 8-NH_2-2′-deoxy cADPR **8** and 8-NH_2-cADPR **2**.

loaded with the Ca^{2+} dye rhod dextran revealed that **7** is indeed cell-permeant (Figure 5B); the Ca^{2+} response is relatively small, oscillatory, and cortical compared to that elicited by sperm but

Table 2. Comparison of the Agonistic and Antagonistic Potency of Compound **2** with **8**

compound	EC_{50}	IC_{50}
8-amino-cADPR **2**	89 μM (69–116)	0.105 μM (0.088–0.126)
8-amino-2′-deoxy-cADPR **8**	>89 μM (plateau not defined, constraining to 100% gives 89 μM (73–108)	0.22 μM (0.16–0.3)

significant (interestingly, this is similar to the small oscillatory responses to 5 mM caffeine; data not shown). This result for **7** in intact cells is thus rather promising, and it suggests that further structural modification on this compound could potentially lead to a potent membrane-permeable agonist, which would be a useful tool in the armamentarium to study the role of cADPR in Ca^{2+} release from intracellular stores (*vide infra*). While there have been useful membrane-permeant antagonists available for some time, there is as yet no highly potent membrane-permeant agonist. A few other agents to date have shown promise in this regard, such as 2″,3″-dideoxydidehydro-cADPcR.[42] This compound however only works efficiently in the T lymphocyte but not in the SUH system. Zhang et al.[19,20] reported that replacement of the ribose moiety with an ether bridge could lead to promising membrane-permeant agents, but these compounds are still only weak agonists. 8-bromo-N1-cIDPR was also found to be a cell-permeant agonist in T-lymphocytes, but the agonistic effect was also weak.[49]

Observation of Ca^{2+} release activity for analogues, particularly for the classical antagonists **2** and **3**, at higher concentration in both the Jurkat T cell and SUH systems (Figure 4 and Table 2) is surprising and has not been previously noted. These data may indicate two sites of action of differing affinity, e.g., a lower affinity nucleotide-binding site distinct from the high-affinity cADPR one. There are precedents for low-affinity nucleotide-binding sites, which cause Ca^{2+} release both at RyR,[50] the lower affinity site leading to activation of the channel, and also for the inositol trisphosphate receptor[51] that could potentially be involved here. This site could be nonselective, e.g., an ATP-binding site.[52] While this interpretation is necessarily only speculative at present, our data should stimulate further experiments to explore this effect.

Conclusions

We have synthesized a group of 8-substituted cADPR/2′-deoxy-cADPR analogues using chemoenzymatic methodology. Biological evaluation of these 8-substituted cADPR/2′-deoxy-cADPR analogues revealed that, at least for the 8-amino series, 2′-OH deletion resulted in a decrease of the antagonistic activity in both T cells and in sea urchin egg homogenates. This suggests that the 2′-OH group, although it has no effect on the Ca^{2+}-mobilizing ability of cADPR itself, is an important motif for the antagonistic activities of 8-substituted cADPR analogues. 2′-OH deletion may also result in an increase in the agonistic effects of the 8-substituted cADPR analogues as shown by our studies in both T cells and sea urchin egg systems. A biological study of 8-bromo-2′-deoxy cADPR **7** in intact sea urchin eggs reveals that **7** is not only essentially a full agonist but is also cell-permeant, indicating that **7** might be a potential lead compound toward the design of a potent membrane-permeable cADPR receptor agonist. For the first time, agonist activity at higher concentrations was observed in two diverse systems for the classical cADPR receptor antagonists 8-bromo-cADPR and 8-amino-cADPR, raising the possibility that, in both systems, there could be a lower affinity nucleotide-binding site that could contribute to Ca^{2+} mobilization, possibly distinct from the high-affinity cADPR site.

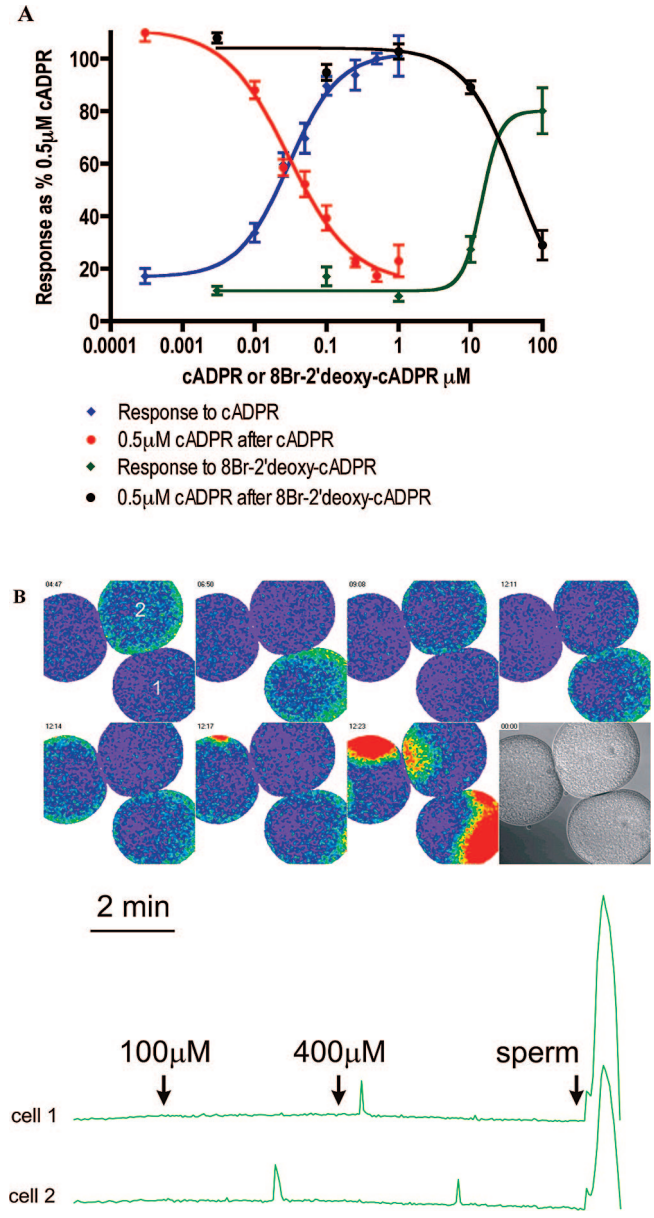


Figure 5. (A) Ca^{2+} mobilization induced by cADPR and 8-bromo-2′-deoxy-cADPR in sea urchin egg homogenate. 8-Bromo-2′-deoxy-cADPR **7** shows weak but almost full agonistic activity (green line) and cross-desensitizes the Ca^{2+} mobilization by cADPR (black line). The agonistic result is compared to cADPR (blue and red lines). (B) Agonistic effect of 8-bromo-2′-deoxy-cADPR in intact sea urchin eggs. The figure shows the Ca^{2+} release in response to the indicating concentrations of the 8-bromo-2′-deoxy-cADPR **7** in sea urchin eggs microinjected with the Ca^{2+} dye rhod dextran (10 μM intracellular concentration). The image is F/F_0, where blue is low Ca^{2+} and red is high Ca^{2+}.

Experimental Section

General Procedure. All reagents and solvents were of commercial quality and were used directly unless otherwise described. *A. californica* ADP-ribosyl cyclase was purchased from Sigma-Aldrich Company Ltd., Gillingham, U.K. Triethylamine was dried over potassium hydroxide, distilled, and kept over potassium hydroxide under argon for use in the CDI-coupling reactions. Morpholine was distilled and stored over potassium hydroxide, and

$MnCl_2$/formamide (0.2 M) was dried over molecular sieves (4 Å) for over 4 days. H_2O was of MilliQ quality. ^1H, ^{13}C, and ^{31}P NMR spectra were collected in DMSO or D_2O, on either a JEOL Delta at 270 MHz (^1H), 68 MHz (^{13}C), or 109 MHz (^{31}P) or a Varian Mercury-vx machine at 400 MHz (^1H) or 100 MHz (^{13}C). Abbreviations for splitting patterns are described below: s (singlet), d (doublet), t (triplet), m (multiplet), etc. UV spectra were collected on either a Perkin-Elmer Lambda EZ 201 or a Lambda 3B spectrophotometer. Low-resolution FAB^- mass spectra were recorded on a Micromass Autospec instrument on samples in a *m*-nitrobenzyl alcohol matrix at the Mass Spectrometry Centre, University of Bath. Accurate mass was recorded either by the Mass Spectrometry Centre at the University of Bath or from the EPSRC National Mass Spectrometry Service Centre at the University of Swansea. HPLC analysis was carried out on a Waters 2695 Alliance module equipped with a photodiode array detector, a Hichrom Guard column, and a Phenomenex Synergi 4 μm MAX-RP 80A column (150 × 4.6 mm). Phosphate buffer [45% (v/v), pH 6.4] in MeOH containing 0.17% (m/v) of cetrimide was used as the solvent system, and samples were eluted at 1 mL/min and monitored at 254 nm. All nucleotides were purified on a Pharmacia Biotech Gradifrac system with a P-1 pump and a UV optical unit (280 nm). The following purification methods were employed: (a) synthetic phosphates were loaded on the Q Sepharose ion-pair column (saturated with MilliQ water) and were eluted with a gradient of 0–50% 1 M TEAB (pH 7.1–7.6) against MilliQ water, and (b) compound was loaded on a LiChroprep RP-18 column which was saturated with 0.05 M TEAB buffer (pH 6.0–6.4) and eluted with a gradient of 0–30% MeCN against 0.05 M TEAB buffer. Cyclic products were quantified using an Ames phosphate assay,[53] from which the extinction coefficients were calculated and used to precisely quantify the concentration of biological solutions.

Determination of Ca^{2+} Release in Permeabilized T Cells. Jurkat T cells were permeabilized by saponin, and experiments were performed as described in an earlier report.[54] Briefly, cells were incubated with saponin in an intracellular buffer (20 mM HEPES, 110 mM KCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, and 10 mM NaCl at pH 7.2) in the absence of extracellular Ca^{2+}. Then, saponin was removed by repeated wash procedures, and the cells were finally resuspended in intracellular buffer. The cells were then kept on ice for 2 h to allow for resealing of intracellular stores. At the start of each individual experiment, the stores of permeabilized cells were reloaded with Ca^{2+} upon the addition of ATP and an ATP-regenerating system consisting of creatine phosphate and creatine kinase.[55] [Ca^{2+}] was determined fluorimetrically by fura2/free acid added to the suspension. Changes in fura2 fluorescence were measured using a Hitachi F-2000 spectrofluorometer (alternating excitation at 340 and 380 nm and emission at 495 nm). When the Ca^{2+} stores were refilled, test compounds were added. Usually, the quality of the permeabilized cell preparation was controlled by its responsiveness to Ins(1,4,5)P_3 and cADPR on each day. Each experiment was calibrated using excess $CaCl_2$ and EGTA/Tris to obtain the maximal and minimal fura2 ratios.

Evaluation of cADPR Analogues in Sea Urchin Egg Homogenates. Sea urchin eggs from *Lytechinus pictus* (Marinus, Long Beach, CA) were obtained by intracoelomic injection of 0.5 M KCl, shed into artificial seawater (435 mM NaCl, 40 mM $MgCl_2$, 15 mM $MgSO_4$, 11 mM $CaCl_2$, 10 mM KCl, 2.5 mM $NaHCO_3$, and 20 mM Tris at pH 8), dejellied by passing through 90 μm nylon mesh, and then washed twice by centrifugation. Homogenates of sea urchin eggs were prepared as described.[1] Briefly, eggs were disrupted in an intracellular-like medium consisting of 250 mM potassium gluconate, 250 mM *N*-methylglucamine, 20 mM HEPES, and 1 mM $MgCl_2$ at pH 7.2, supplemented with 1 mM ATP, 10 units/mL creatine kinase, and 10 mM phosphocreatine and protease inhibitors. Ca^{2+} concentrations were measured with fluo-3 (3 μM) at 17 °C, using 500 μL of continuously stirred homogenate in a fluorimeter (Perkin-Elmer LS-50B) at 506 nm excitation and 526 nm emission.

Evaluation of cADPR Analogues in Intact Sea Urchin Eggs. To intact sea urchin eggs microinjected with Ca^{2+} dye rhod dextran (~10 μM final) was added 100 and 400 μM 2′-deoxy-8-bromo-cADPR. The Ca^{2+} response was monitored at excitation (543 nm) and emission (>560 nm) on a Zeiss LSM510 confocal microscope.

8-Bromo-2′-deoxy-adenosine 5′-Monophosphate (13). Compound **13** was synthesized by adaptation of a literature method.[25] A suspension of 2′-deoxy-AMP disodium salt (100 mg, 0.27 mmol) in NaOAc/AcOH buffer (4 mL, 0.9 M, pH 4.05) was heated strongly with a heat gun for 2 min. The resulting solution was cooled to room temperature, and a solution of bromine in MilliQ water (3.5 mL, 1.3% v/v) was added dropwise over 20 min. The reaction mixture was then stirred at room temperature in the dark for 2 h, after which another portion of a bromine water solution (1 mL) was added. The reaction mixture was stirred for a further hour. The reaction was then quenched by dropwise addition of a saturated solution of $NaHSO_3$ until the color of the solution became just yellowish, and the pH was adjusted to 7 with NaOH (5 M). The crude product was purified by a reverse-phase system and eluted with a linear gradient of 0–30% acetonitrile against 0.05 M TEAB buffer. The appropriate fractions were collected, and the solvent was removed *in vacuo*. Excess TEAB was co-evaporated with MeOH (3×), generating the title compound **13** as a yellow solid in the triethylammonium form (0.15 mmol, 55%). HPLC: 7.87 min at 254 nm. UV (H_2O) λ_{max}: 265.2 nm. ^1H NMR (D_2O, 270 MHz) δ: 8.06 (s, 1H, H-2), 6.43 (t, $J_{1',2'a} = J_{1',2'b} = 7.2$ Hz, 1H, H-1′), 4.73 (m, 1H, H-3′), 4.12 (m, 1H, H-4′), 4.06 (m, 2H, H-5′), 3.26 (m, 1H, H-2′a), and 2.43 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: 1.50 (s). *m/z* (FAB^-): 407.8 [$(M - H)^-$, 95%], 409.8 [$(M - H)^-$, 95%].

8-Bromo-2′-deoxy-adenosine 5′-Monophosphate Morpholidate (24). To a solution of 8-bromo-2′-deoxy-AMP triethylammonium salt (39 mg, 69 μmol) in dry DMSO (0.6 mL) was added in sequence dipyridyl disulfide (180 mg, 0.82 mmol), morpholine (0.1 mL, 1.15 mmol), and triphenylphosphine (214 mg, 0.82 mmol). The resulting yellow solution was stirred at room temperature for 3.5 h and was quenched by dropwise addition of a solution of sodium iodide (0.1 M, 20 mL). The resulting sodium salt precipitate was filtered, washed with acetone, and dried *in vacuo* to give the title compound **26** as a sticky solid (50 μmol, 72%). ^1H NMR (D_2O, 270 MHz) δ: 8.02 (s, 1H, H-2), 6.50 (dd, $J_{1',2'a} = 7.4$ Hz, $J_{1',2'b} = 7.2$ Hz, 1H, H-1′), 4.15 (m, 1H, H-3′), 4.00 (m, 3H, H-4′ and H-5′), 3.38 (m, 5H, 2 × CH_2O and H-2′a), 2.72 (m, 4H, 2 × CH_2N), and 2.48 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: 8.20 (s). HRMS Calcd for $[M - H]^-$ $C_{14}H_{19}{}^{79}BrN_6O_6P^-$ (FAB^-), 477.0287; found, 477.0279. Calcd for $C_{14}H_{19}{}^{81}BrN_6O_6P^-$, 479.0267; found, 479.0290.

8-Bromo-2′-deoxy-nicotinamide Adenine Dinucleotide (21, 8-Br-2′-deoxy-NAD^+). To a suspension of β-NMN^+ (50 mg, 149 μmol) in dry DMF (0.4 mL) was added freshly distilled triethylamine (22 μL) and carbonyldiimidazole (CDI, 100 mg, 0.61 mmol) under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 3 h. A ^{31}P NMR spectrum indicated that all starting β-NMN^+ had been consumed, and a new peak at −9 ppm was formed. The reaction was stopped by addition of dry methanol (few drops), and the solution was stirred at room temperature for a further 10 min. The reaction solution was then concentrated to dryness *in vacuo* and was co-evaporated with dry DMF 3 times to remove any residual methanol. To the resulting yellow residue was added 8-bromo-2′-deoxy-AMP triethylammonium salt (40 mg, 71 μmol, dried over P_2O_5 in *vacuo* for 2 days) and DMF (0.4 mL). The clear yellow solution was stirred at room temperature for 3 days (HPLC analysis indicated that over 80% of the starting material was reacted). DMF was removed in *vacuo*, and the residue was purified by the Q-Sepharose system. The appropriate fractions were pooled and evaporated, and excess TEAB was removed by co-evaporating with MeOH to afford the title compound **21** as a glassy solid in the triethylammonium form (22 μmol, 31%). HPLC: 3.37 min at 254 nm. UV (H_2O) λ_{max}: 265.2 nm. ^1H NMR (D_2O, 400 MHz) δ: 9.29 (s, 1H, H_N-2), 9.09 (d, $J_{6,5} = 6.2$ Hz, 1H, H_N-6), 8.82 (d, $J_{4,5} = 8.2$ Hz, 1H, H_N-4), 8.18 (dd, $J_{5,4} = 8.2$ Hz, $J_{5,6} = 6.2$ Hz, 1H, H_N-5), 8.10 (s, 1H, H-2), 6.38 (dd, $J_{1',2'a} = 7.4$ Hz,

$J_{1',2'b}$ = 7.2 Hz, 1H, H-1′), 5.98 (d, $J_{1'',2''}$ = 4.7 Hz, 1H, H-1″), 4.42–4.15 (m, 9H, H-ribose), 3.33 (m, 1H, H-2′a), and 2.40 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: –11.17 (d, J_{PP} = 18.7 Hz), −10.52 (d, J_{PP} = 18.7 Hz). *m/z* (FAB^-): 725.0, 727.0 [$(M)^-$, 60%]. HRMS Calcd for $[M + H]^+$ $C_{21}H_{27}{}^{79}BrN_7O_{13}P_2{}^+$ (FAB^+), 726.0325; found, 726.0325. Calcd for $[M + H]^+$ $C_{21}H_{26}{}^{81}BrN_7O_{13}P_2{}^+$ (FAB^+), 728.0305; found, 728.0348.

Cyclic 8-Bromo-2′-deoxy-adenosine 5′-Diphosphate Ribose (7, 8-Br-2′-deoxy-cADPR). A solution of 8-bromo-2′-deoxy NAD^+ **21** (19 mg, 22 μmol) in HEPES buffer (25 mM, pH 7.4, 76 mL) was incubated with *Aplysia* cyclase (115 μL) at room temperature for 40 min, after which HPLC analysis of the solution indicated the completion of the reaction (a new peak was produced at ca. 7.78 min). The reaction solution was diluted with MilliQ water (conductivity < 200 μS/cm) and purified by Q-Sepharose ion-exchange chromatography, and the product eluted with a gradient of 0–50% 1 M TEAB buffer against MilliQ water. The fractions containing the title compound were collected and evaporated *in vacuo*. The resulting white residue was co-evaporated with MeOH (3×) to give the title compound **7** in its triethylammonium form (19 μmol, 86%). HPLC: 7.78 min at 254 nm. UV (H_2O, pH 5.2) λ_{max}: 265.2 nm (ϵ/11 300 dm^3 mol^{-1} cm^{-1}). ^{1}H NMR (D_2O, 400 MHz) δ: 8.96 (s, 1H, H-2), 6.55 (dd, $J_{1',2'a}$ = 6.7 Hz and $J_{1',2'b}$ = 6.2 Hz, 1H, H-1′), 6.11 (d, $J_{1'',2''}$ = 4.0 Hz, 1H, H-1″), 4.99 (m, 1H, H-3′), 4.67 (m, 1H, H-2″), 4.61 (m, 1H, H-4″), 4.35 (m, 1H, H-3″), 4.25 (m, 2H, H-5″a and H-5′a), 4.11 (m, 1H, H-4′), 3.88 (m, 1H, H-5″b), 3.79 (m, 1H, H-5′b), 3.20 (m, 1H, H-2′a), and 2.54 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: –10.10 (d, J_{PP} = 14.0 Hz), −10.58 (d, J_{PP} = 14.0 Hz). *m/z* (FAB^+): 604.0 [$(M + H)^+$, 80%], 606.0 [$(M + H)^+$, 80%]. HRMS Calcd for $[M + H]^+$ $C_{15}H_{21}{}^{79}BrN_5O_{12}P_2{}^+$ (FAB^+), 603.9845; found, 603.9838. Calcd for $[M + H]^+$ $C_{15}H_{21}{}^{81}BrN_5O_{12}P_2{}^+$ (FAB^+), 605.9825; found, 605.9840.

8-Amino-2′-deoxy-adenosine 5′-Monophosphate (15, 8-NH_2-2′-deoxy-AMP). To a solution of sodium azide (34 mg, 0.52 mmol) in DMF (5 mL) was added 8-Br-2′-deoxy-AMP (60 mg, 107 μmol) and H_2O (1 mL). After heating at 80 °C in the dark overnight, the resulting yellow solution was evaporated *in vacuo* and purified by reverse-phase column chromatography. The appropriate fractions were collected and concentrated, and the excess TEAB was removed by co-evaporating with MeOH (3×) to give the 8-azido compound **14**. UV (H_2O) λ_{max}: 281.8 nm. ^{1}H NMR (D_2O, 270 MHz) δ: 7.97 (s, 1H, H-2), 6.23 (dd, $J_{1',2'a}$ = 6.8 Hz and $J_{1',2'b}$ = 6.7 Hz, 1H, H-1′), 4.66 (m, 1H, H-3′), 4.06–3.98 (m, 3H, H-4′ and H-5′), 3.00 (m, 1H, H-2′a), and 2.31 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: 1.29 (s). *m/z* (FAB^+): 373.0 [$(M + H)^+$, 65%]. HRMS Calcd. for $C_{10}H_{14}N_8O_6P^+$ $[M + H]^+$, 373.0774; found, 373.0790. To a solution of 8-azido-2′-deoxy-AMP **14** in TEAB buffer (pH 8.0, 0.05 M, 7.5 mL) was added 1, 4-dithiol-(D,L)-threitol (20 mg, 129.9 μmol), and the reaction solution was stirred at room temperature in the dark overnight. HPLC analysis indicated that a new peak was formed at 8.4 min with a $UV_{(max)}$ at 274 nm. The crude product was purified by the reverse-phase system, and excess TEAB was removed by co-evaporating with MeOH. Title compound **15** was obtained as a glassy solid (42 μmol, 39% over two steps). HPLC: 8.41 min at 254 nm. UV (H_2O) λ_{max}: 274.7 nm. ^{1}H NMR (D_2O, 270 MHz) δ: 7.94 (s, 1H, H-2), 6.33 (dd, $J_{1',2'a}$ = 8.1 Hz and $J_{1',2'b}$ = 5.4 Hz, 1H, H-1′), 4.66 (m, 1H, H-3′), 4.19 (m, 1H, H-4′), 4.12 (m, 2H, H-5′), 2.69 (m, 1H, H-2′a), and 2.25 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: 1.55 (s). *m/z* (FAB^+): 346.9 [$(M + H)^+$, 12%]. HRMS Calcd. for $[M + H]^+C_{10}H_{16}N_6O_6P^+$ (FAB^+), 347.0869; found, 347.0881.

8-Amino-2′-deoxy-nicotinamide Adenine Dinucleotide (22, 8-NH_2-2′-deoxy-NAD^+). To a suspension of β-NMN^+ (60 mg, 180 μmol) in DMF (400 μL) was added TEA (30 μL) and CDI (100 mg). This reaction mixture was stirred under argon atmosphere at room temperature for 3 h, after which a ^{31}P NMR spectrum of the reaction mixture indicated that all starting β-NMN^+ was consumed and a new singlet was formed (ca. −9.0 ppm). A few drops of methanol were added, and the reaction mixture was stirred at room temperature for a further 10 min and evaporated *in vacuo*. The resulting yellow residue was co-evaporated with DMF (3 × 2 mL) to remove any remaining methanol. The residue was dissolved in dry DMF (0.4 mL), to which was added 8-amino-2′-deoxy AMP (40 mg, 80 μmol), and the mixture was stirred at room temperature for 2 days. DMF was removed *in vacuo*, and the residue was purified on a Q-Sepharose ion-exchange column, eluted with a gradient of 0–50% 1 M TEAB against MilliQ water. The title compound was produced in its triethylammonium form (31 μmol, 39%). HPLC: 2.95 min at 254 nm. UV (H_2O) λ_{max}: 269.9 nm. ^{1}H NMR (D_2O, 270 MHz) δ: 9.29 (s, 1H, H_N-2), 9.17 (d, $J_{6,5}$ = 5.9 Hz, 1H, H_N-6), 8.77 (d, $J_{4,5}$ = 7.9 Hz, 1H, H_N-4), 8.20 (dd, $J_{5,4}$ = 7.9 Hz and $J_{5,6}$ = 5.9 Hz, 1H, H_N-5), 7.94 (s, 1H, H-2), 6.23 (dd, $J_{1',2'a}$ = 8.1 Hz and $J_{1',2'b}$ = 7.1 Hz, 1H, H-1′), 6.08 (d, $J_{1'',2''}$ = 4.5 Hz, 1H, H-1″), 4.73–4.15 (m, 9H, H-ribose), 2.72 (m, 1H, H-2′a), and 2.23 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: −10.68 (m). *m/z* (FAB^+): 663.1 [$(M + H)^+$, 65%]. HRMS Calcd. for $[M + H]^+$ $C_{21}H_{29}N_8O_{13}P_2{}^+$ (FAB^+), 663.1329; found, 663.1321.

Cyclic 8-Amino-2′-deoxy-adenosine 5′-Diphosphate Ribose (8, 8-NH_2-2′-deoxy-cADPR). A solution of 8-amino-2′-deoxy-NAD^+ (4 mg, 4.8 μmol) and *Aplysia* cyclase (20 μL) in HEPES buffer (25 mM, pH 7.4, 12 mL) was stirred at room temperature for 1 h after which HPLC analysis suggested the formation of a cyclic compound. The reaction mixture was purified as described for **7** to give the title compound **8** as a glassy solid in the triethylammonium form (3.7 μmol, 77%). HPLC: 3.88 min at 254 nm. UV (H_2O, pH 5.0) λ_{max}: 277.0 nm (ϵ/13 400 dm^3 mol^{-1} cm^{-1}). ^{1}H NMR (400 MHz, D_2O) δ: 8.60 (s, 1H, H-2), 6.06 (dd, $J_{1',2'a}$ = 6.9 Hz and $J_{1',2'b}$ = 6.6 Hz, 1H, H-1′), 5.89 (d, $J_{1'',2''}$ = 4.1 Hz, 1H, H-1″), 4.69 (m, 1H, H-3′), 4.55 (m, 2H, H-2″ and H-4″), 4.25 (m, 1H, H-5″a), 4.18 (m, 2H, H-3″ and H-5′a), 4.02 (m, 1H, H-4′), 3.92 (m, 1H, H-5″b), 3.81 (m, 1H, H-5′b), 3.19 (m, 1H, H-2′a), and 2.35 (m, 1H, H-2′b). ^{13}P NMR (D_2O, 109 MHz) δ: −10.09 (m) and −10.56 (m). HRMS Calcd for $[M + H]^+$ $C_{15}H_{23}N_6O_{12}P_2{}^+$, 541.0849 ($ES^+$); found, 541.0826.

8-Methoxy-2′-deoxy-adenosine (20)..[26] To a solution of 8-bromo-2′-deoxy adenosine (267 mg, 0.81 mmol) in dry methanol (11 mL) was added sodium methoxide (114 mg, 2.11 mmol) under an argon atmosphere. The resulting yellow suspension was heated to reflux for 24 h. The reaction solution was evaporated *in vacuo*, and the resulting yellow residue was purified by flash column chromatography, eluted with DCM/methanol (8:1). The title compound was produced as a yellow solid (216 mg, 95%). ^{1}H NMR (DMSO, 270 MHz) δ: 8.00 (s, 1H, H-2), 6.92 (brs, 2H, NH_2), 6.15 (dd, $J_{1',2a'}$ = 6.4 Hz and $J_{1',2b'}$ = 5.6 Hz, 1H, H-1′), 5.24 (m, 2H, 3′-OH and 5′-OH), 4.38 (m, 1H, H-3′), 4.08 (s, 3H, CH_3O), 3.81 (m, 1H, H-4′), 3.50 (m, 2H, H-5′), 2.97 (m, 1H, H-2′a), and 2.08 (m, 1H, H-2′b). *m/z* (FAB^+): 282.1 [$(M + H)^+$, 98%]. HRMS Calcd. for $[M + H]^+$ $C_{11}H_{16}N_5O_4{}^+$ (ES^+), 282.1202; found, 282.1199.

8-Methoxy-2′-deoxy-adenosine 5′-Monophosphate (16). A suspension of 8-methoxy-2′-deoxy-adenosine (165 mg, 0.59 mmol, dried *in vacuo* at 110 °C) in trimethylphosphate (2.0 mL) was heated strongly with a heat gun. The resulting clear solution was cooled to −20 °C, to which was added $POCl_3$ (0.16 mL) under an argon atmosphere. The reaction mixture was stirred for further 2 h, quenched by addition of ice (10 mL), and neutralized immediately with NaOH (5 M). It was extracted with cold ethyl acetate (5 × 20 mL), and the aqueous layers were pooled, dried, and purified on reverse-phase column, eluted with a gradient of 0–30% MeCN against 0.05 M TEAB. The fractions containing the desired compound were pooled, evaporated, and co-evaporated with MeOH to afford the crude 8-methoxy-2′-deoxy-AMP as a glassy solid. It was further purified on Q-Sepharose ion-exchange chromatography giving the pure title compound in its triethylammonium salt (60 mg, 20%). HPLC: 6.21 min. UV (H_2O) λ_{max}: 259.3 nm. ^{1}H NMR (D_2O, 270 MHz) δ: 8.03 (s, 1H, H-2), 6.34 (appt, $J_{1',2'a}$ = $J_{1',2'b}$ = 7.2 Hz, 1H, H-1′), 4.67 (m, 1H, H-3′), 4.17 (s, 3H, OMe), 4.14 (m, 1H, H-4′), 4.02 (m, 2H, H-5′), 3.07 (m, 1H, H-2′a), and 2.38 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: 1.33 (s). *m/z* (FAB^-): 359.1 [$(M - H)^-$, 70%]. HRMS Calcd for $[M - H]^-$ $C_{11}H_{15}N_5O_7P^-$ (FAB^-), 360.0709; found, 360.0652.

8-Methoxy-2′-deoxy-nicotinamide Adenine Dinucleotide (23, 8-OMe-2′-deoxy-NAD$^+$). To a suspension of β-NMN$^+$ (60 mg, 180 μmol) in DMF (400 μL) was added TEA (30 μL) and CDI (100 mg). This reaction mixture was stirred under argon atmosphere at room temperature for 3 h. A few drops of methanol were added, and the reaction mixture was stirred at room temperature for a further 10 min and evaporated under reduced vacuum. The resulting yellow residue was co-evaporated with DMF (3 × 2 mL) to remove any remaining methanol. The residue was dissolved in dry DMF (0.4 mL), to which was added 8-methoxy-2′-deoxy-AMP (40 mg, 77 μmol), and the mixture was stirred at room temperature for 2 days. DMF was removed *in vacuo*, and the residue was purified on Q-Sepharose ion-exchange column. The product eluted with a gradient of 0–50% 1 M TEAB against MilliQ water. The appropriate fractions were pooled, evaporated *in vacuo*, and co-evaporated with MeOH (3×) to generate the desired dinucleotide in its triethylammonium form (31 μmol, 40%). HPLC: 2.92 min. UV (H_2O) λ_{max}: 259.3 nm. ^{1}H NMR (D_2O, 400 MHz) δ: 9.15 (s, 1H, H_N-2), 8.96 (d, $J_{6,5}$ = 6.3 Hz, 1H, H_N-6), 8.62 (d, $J_{4,5}$ = 8.0 Hz, 1H, H_N-4), 8.02 (dd, $J_{5,4}$ = 8.0 Hz and $J_{5,6}$ = 6.3 Hz, 1H, H_N-5), 7.84 (s, 1H, H-2), 6.09 (dd, $J_{1′,2′a}$ = 7.2 Hz and $J_{1′,2′b}$ = 6.9 Hz, 1H, H-1′), 5.88 (d, $J_{1″,2″}$ = 5.0 Hz, 1H, H-1″), 4.50 (m, 1H, H-3′), 4.37 (m, 1H, H-4″), 4.28 (m, 2H, H-2″ and H-3″), 4.18–3.94 (m, 5H, H-ribose), 4.02 (s, 3H, CH_3O), 2.88 (m, 1H, H-2′a), and 2.22 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: −10.53 (d, J_{pp} = 17.1 Hz) and −11.03 (d, J_{pp} = 17.1 Hz). *m/z* (FAB$^-$): 675.9 [(M − H)$^-$, 40%]. HRMS Calcd. for [M + H]$^+$ $C_{22}H_{30}N_7O_{14}P_2^+$ (ES$^+$), 678.1326; found, 678.1321.

Cyclic 8-Methoxy-2′-deoxy-adenosine 5′-Diphosphate Ribose (9, 8-*O*Me-2′-deoxy-cADPR). 8-Methoxy-2′-deoxy-NAD$^+$ (5 mg, 5.9 μmol) and *Aplysia* cyclase (22 μL) were incubated in HEPES buffer (15 mL, pH 7.4, 25 mM) until HPLC analysis indicated that the starting dinucleotide was completely consumed. The reaction mixture was diluted with MilliQ and purified as described for **7** to give the title compound as a glassy solid (5.6 μmol, 95%). HPLC: 3.90 min at 254 nm. UV (H_2O) λ_{max}: 259.3 nm (ϵ/11 500 dm^3 mol^{-1} cm^{-1}). ^{1}H NMR (D_2O, 400 MHz) δ: 8.79 (s, 1H, H-2), 6.28 (dd, $J_{1′,2′b}$ = 6.8 Hz and $J_{1′,2′a}$ = 6.4 Hz, 1H, H-1′), 5.99 (d, $J_{1″,2″}$ = 4.2 Hz, 1H, H-1″), 4.83 (m, 1H, H-3′), 4.69 (m, 1H, H-2″, overlap with HOD signal), 4.62 (m, 1H, H-4″), 4.35 (m, 1H, H-3″), 4.26 (m, 2H, H-5′a and H-5″a), 4.09 (m and s, 4H, H-4′ and OCH_3), 4.01 (m, 1H, H-5″b), 3.86 (m, 1H, H-5′b), 3.19 (m, 1H, H-2′a), and 2.38 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: −10.82 (m), −11.46 (m). *m/z* (FAB$^-$): 553.8 [(M − H)$^-$, 100%]. HRMS Calcd for [M − H]$^-$ $C_{16}H_{22}N_5O_{13}P_2^-$ (FAB$^-$), 554.0689; found, 554.0698.

8-Phenyl-adenosine 5′-Monophosphate (17, 8-Ph-AMP). To a stirred solution of 8-bromo-AMP triethylammonium salt (140 mg, 0.24 mmol), phenylboronic acid (40 mg, 0.33 mmol), and sodium carbonate (76 mg, 0.72 mmol) in 2:1 MilliQ/MeCN (4.1 mL, degassed) was added $Pd(PPh_3)_4$ (40 mg, 35 μmol) under an argon atmosphere. The resulting yellow suspension was vigorously stirred at 80 °C for 3 h. The reaction mixture was filtered, and the filtrate was purified using reverse-phase chromatography. The product eluted with a gradient of 0–30% MeCN against 0.05 M TEAB buffer. The appropriate fractions were collected and evaporated, and the resulting residue was co-evaporated with MeOH (3×) to give the title compound **17** in triethylammonium salt (77 mg, 55%). HPLC: 12.10 min. UV (H_2O) λ_{max}: 275.8 nm. ^{1}H NMR (D_2O, 270 MHz) δ: 8.21 (s, 1H, H-2), 7.66 (m, 2H, ArH), 7.56 (m, 3H, ArH), 5.85 (d, $J_{1′,2′}$ = 6.2 Hz, 1H, H-1′), 5.16 (appt, $J_{2′,1′}$ = $J_{2′,3′}$ = 6.2 Hz, 1H, H-2′), 4.35 (m, 1H, H-3′), and 4.21 (m, 3H, H-4′ and H-5′). ^{31}P NMR (D_2O, 109 MHz) δ: 1.59 (s). HRMS: Calcd for [M + H]$^+$ $C_{16}H_{19}N_5O_7P^+$ (ES$^+$), 424.1022; found, 424.1019.

8-Phenyl-adenosine 5′-Monophosphate Morpholidate (27). To a solution of 8-phenyl-AMP triethylammonium salt (55 mg, 95 μmol) in dry DMSO (0.4 mL) was added triphenylphosphine (113 mg, 431 μmol), morpholine (100 μL, 1.14 mmol), and dipyridyl disulfide (95 mg, 431 μmol). The resulting yellow solution was stirred at room temperature for 2 h, and HPLC analysis indicated that all starting 8-phenyl-AMP was consumed. The reaction was quenched by dropwise addition of a solution of sodium iodide in acetone (0.1 M, 15 mL), and the resulting yellow precipitate was filtered, washed with acetone, and dried *in vacuo*. The title compound **27** was obtained as a yellow solid (70 μmol, 74%). UV (H_2O) λ_{max}: 273.5 nm. ^{1}H NMR (D_2O, 270 MHz) δ: 8.17 (s, 1H, H-2), 7.53 (m, 5H, ArH), 5.79 (d, 1H, $J_{1′,2′}$ = 4.9 Hz, H-1′), 5.21 (m, 1H, H-2′), 4.52 (m, 1H, H-3′), 4.06 (m, 3H, H-4′ and H-5′), 3.42 (m, 4H, 2 × CH_2O), and 2.83 (m, 4H, 2 × CH_2N). ^{31}P NMR (D_2O, 109 MHz) δ: 8.29 (s). HRMS Calcd. for [M + H]$^+$ $C_{20}H_{26}N_6O_6P^+$ (ES$^+$), 493.1601; found, 493.1596.

8-Phenyl-nicotinamide Adenine Dinucleotide (31, 8-Ph-NAD$^+$). A suspension of 8-phenyl-AMP morpholidate (28 mg, 57 μmol), β-NMN$^+$ (23 mg, 68 μmol), and $MgSO_4$ (13 mg, 108 μmol) in a solution of $MnCl_2$ in formamide (0.2 M, 0.42 mL) was stirred at room temperature for 48 h. Precipitation of the product occurred by dropwise addition of MeCN (2 mL); it was filtered and treated with Chelex resin to remove any residual Mn^{2+}. The crude compound was purified on a reverse-phase column, eluted with a 0–30% gradient of MeCN/0.05 M TEAB. The fractions containing the product was pooled and evaporated *in vacuo*, and excess TEAB was co-evaporated with MeOH (5×) to afford the desired dinucleotide as a glassy solid in its triethylammonium form (38 μmol, 66%). HPLC: 4.53 min at 254 nm. UV (H_2O) λ_{max}: 273.5 nm. ^{1}H NMR (D_2O, 400 MHz) δ: 9.21 (s, 1H, H_N-2), 9.05 (d, $J_{6,5}$ = 6.4 Hz, 1H, H_N-6), 8.67 (d, $J_{4,5}$ = 8.1 Hz, 1H, H_N-4), 8.15 (dd, $J_{5,4}$ = 8.1 Hz and $J_{5,6}$ = 6.4 Hz, 1H, H_N-5), 8.13 (s, 1H, H-2), 7.62–7.46 (m, 5H, Ar-H), 5.91 (d, $J_{1″,2″}$ = 5.0 Hz, 1H, H-1″), 5.78 (d, $J_{1′,2′}$ = 5.9 Hz, 1H, H-1′), 5.13 (m, 1H, H-2′), 4.40 (m, 1H, H-3′), 4.34 (m, 1H, H-2″), and 4.38–4.13 (m, 7H, H-ribose). ^{31}P NMR (D_2O, 109 MHz) δ: −11.50 (m). *m/z* (FAB$^-$): 738.1 [(M − H)$^-$, 100%]. HRMS Calcd. for [M − H]$^-$ $C_{27}H_{30}N_7O_{14}P_2^-$ (FAB$^-$), 738.1326; found, 738.1329.

Cyclic 8-Phenyl-adenosine 5′-Diphosphate Ribose (5, 8-Ph-cADPR). To a solution of 8-phenyl-NAD$^+$ (5 mg, 5.5 μmol) in HEPES buffer (25 mM, pH 7.4, 13 mL) was added *Aplysia* cyclase (19.5 μL). The resulting clear solution was stirred at room temperature for 1 h, and the product was purified as described for **7**. The desired compound **5** was obtained as a glassy solid in its triethylammonium form (3.8 μmol, 69%). HPLC: 9.95 min at 254 nm. UV (H_2O) λ_{max}: 275.8 nm (ϵ/20 264 dm^3 mol^{-1} cm^{-1}). ^{1}H NMR (D_2O, 400 MHz) δ: 9.07 (s, 1H, H-2), 7.77–7.62 (m, 5H, Ar-H), 6.20 (d, $J_{1″,2″}$ = 4.2 Hz, 1H, H-1″), 6.07 (d, $J_{1′,2′}$ = 6.0 Hz, 1H, H-1′), 5.56 (dd, $J_{2′,1′}$ = 6.0 Hz and $J_{2′,3′}$ = 5.3 Hz, 1H, H-2′), 4.77 (m, 3H, H-4″, H-2″, and H-3′), 4.48 (m, 3H, H-3″, H-5″a, and H-5′a), 4.29 (m, 1H, H-4′), and 4.13 (m, 2H, H-5″b and H-5′b). ^{31}P NMR (D_2O, 109 MHz) δ: −10.09 (brs) and −10.84 (brs). *m/z* (FAB$^-$): 616.0 [(M − H)$^-$, 100%]. HRMS Calcd for [M − H]$^-$ $C_{21}H_{24}N_5O_{13}P_2^-$ (FAB$^-$), 616.0846; found, 616.0856.

8-Phenyl-2′-deoxy-adenosine 5′-Monophosphate Morpholidate (28). To a stirred solution of 8-Br-2′-deoxy-adenosine 5′-monophosphate (190 mg, 0.34 mmol), phenylboronic acid (80 mg, 0.65 mmol) and Na_2CO_3 (220 mg, 2.07 mmol) in aqueous MeCN (33%, 5 mL, degassed) was added $Pd(PPh_3)_4$ (70 mg, 60 μmol) under an argon atmosphere. The resulting yellow suspension was vigorously stirred at 80 °C for 3 h, after which further portions of phenylboronic acid (80 mg, 0.65 mmol) and $Pd(PPh_3)_4$ (70 mg, 60 μmol) were added. The resulting suspension was stirred at 80 °C for a further 2 h and purified as described for **17**, giving crude 8-phenyl-2′-deoxy AMP **18** as a glassy solid in its triethylammonium form (92% based on ^{1}H NMR). HPLC: 13.71 min at 254 nm. UV (H_2O) λ_{max}: 275.8 nm. ^{1}H NMR (CD_3OD, 270 MHz) δ: 8.22 (s, 1H), 7.77 (m, 2H), 7.53 (m, 3H), 6.25 (appt, $J_{1′,2′a}$ = $J_{1′,2′b}$ = 6.8 Hz, 1H), 4.79 (m, 1H), 4.11 (m, 3H), 3.62 (m, 1H), and 2.20 (m, 1H). *m/z* (ES$^-$): 406.18 [(M − H)$^-$, 100%]. HRMS Calcd for [M + H]$^+$ $C_{16}H_{19}N_5O_6P^+$ (ES$^+$), 408.1073; found, 408.1071. To a solution of **18** in dry DMSO (0.6 mL) was added dipyridyl disulfide (190 mg, 862 μmol), morpholine (0.25 mL, 2.86 mmol), and triphenylphosphine (220 mg, 839 μmol). The resulting green solution was stirred at room temperature for 3 h and quenched with a solution of NaI in acetone (0.1 M, 20 mL). The resulting precipitate was filtered, washed with acetone, and dried *in vacuo*

to afford the desired morpholidate as a yellow solid in 75% yield over both steps. Compound **28** was used in the next step without further purification. ^{1}H NMR (D_2O, 270 MHz) δ: 8.21 (s, 1H, H-2), 7.68 (m, 5H, ArH), 6.26 (appt, $J_{1',2'a} = J_{1',2'b} = 7.4$ Hz, 1H, H-1′), 4.73 (m, 1H, H-3′, overlap with HOD peak), 3.98 (m, 3H, H-4′ and H-5′), 3.35 (m, 5H, 2 × CH_2O, H-2′a), 2.76 (m, 4H, 2 × CH_2N), and 2.27 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: 8.10 (s). *m/z* (ES^-): 475.18 [$(M - H)^-$, 100%]. HRMS Calcd for $[M - H]^- C_{20}H_{24}N_6O_6P^-$ (ES^-), 475.1495; found, 475.1493.

8-Phenyl-2′-deoxy-nicotinamide Adenine Dinucleotide (32, 8-Ph-2′-deoxy-NAD$^+$). To a mixture of 8-Ph-2′-deoxy-AMP morpholidate (30 mg, 63 μmol), β-NMN^+ (25 mg, 75 μmol), and $MgSO_4$ (14 mg, 117 μmol) was added a solution of $MnCl_2$ in formamide (0.2 M, 460 μL). The resulting suspension was stirred at room temperature and monitored by HPLC. After 48 h, the reaction was quenched by the addition of MeCN (2 mL) and the yellow precipitate was filtered, washed with MeCN, and treated with Chelex resin (sodium form) to remove any residual Mn^{2+}. The crude product was further purified on a reverse-phase column, giving the title compound **32** as a glassy solid in its triethylammonium form (38 μmol, 60%). HPLC: 4.62 min. UV (H_2O) λ_{max}: 275.8 nm. ^{1}H NMR (D_2O, 400 MHz) δ: 9.11 (s, 1H, H_N-2), 8.94 (d, $J_{6,5} = 6.1$ Hz, 1H, H_N-6), 8.57 (d, $J_{4,5} = 8.4$ Hz, 1H, H_N-4), 8.05 (dd, 1H, $J_{5,4} = 8.4$ Hz and $J_{5,6} = 6.1$ Hz, H_N-5), 8.03 (s, 1H, H-2), 7.42 (m, 5H, ArH), 6.09 (dd, $J_{1',2'b} = 7.5$ Hz and $J_{1',2'a} = 6.8$ Hz, 1H, H-1′), 5.80 (d, $J_{1'',2''} = 4.7$ Hz, 1H, H-1″), 4.56 (m, 1H, H-3′), 4.24 (m, 1H, H-2″), 4.24–3.96 (m, 7H, H-ribose), 2.89 (m, 1H, H-2′a), and 2.02 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: −11.43 (brs) and −11.76 (brs). *m/z* (ES^-): 599.94 [(M − nicotinamide)$^-$, 100%], 721.94 [$(M - H)^-$, 20%]. HMRS Calcd for $[M - H]^- C_{27}H_{30}N_7O_{13}P_2^-$ (ES^-), 722.1377; found, 722.1382.

Cyclic 8-Phenyl-2′-deoxy-adenosine 5′-Diphosphate Ribose (10, 8-Ph-2′-deoxy-cADPR). To a solution of 8-phenyl-2′-deoxy-NAD^+ (24 mg, 27 μmol) in HEPES buffer (110 mL, pH 7.4, 25 mM) was added *Aplysia* ADP ribosyl cyclase (150 μL). The resulting clear solution was stirred at room temperature for 1 h and was purified on Q-Sepharose ion-exchange column to give the desired compound **10** in its triethylammonium form (16 μmol, 60%). HPLC: 12.72 min at 254 nm. UV (H_2O) λ_{max}: 277 nm (ϵ/21 349 dm^3 mol^{-1} cm^{-1}). ^{1}H NMR (D_2O, 400 MHz) δ: 9.02 (s, 1H, H-2), 7.67 (m, 5H, ArH), 6.41 (appt, $J_{1'2'a} = J_{1'2'b} = 7.0$ Hz, 1H, H-1′), 6.15 (d, $J_{1'',2''} = 3.9$ Hz, 1H, H-1″), 4.94 (m, 1H, H-3′), 4.77 (m, 2H, H-2″ and H-4″), 4.49 (m, 2H, H-3″ and H-5′a), 4.39, (m, 1H, H-5″a), 4.19 (m, 1H, H-4′), 4.14 (m, 1H, H-5″b), 4.06 (m, 1H, H-5′b), 3.46 (m, 1H, H-2′a), and 2.46 (m, 1H, H-2′b). ^{31}P NMR (D_2O, 109 MHz) δ: −9.95 (d, $J_{PP} = 16.4$ Hz) and −10.85 (d, $J_{PP} = 16.4$ Hz). *m/z* (ES^-): 600.06 [$(M - H)^-$, 100%]. HRMS Calcd $[M - H]^- C_{21}H_{24}N_5O_{12}P_2^-$ (ES^-), 600.0897; found, 600.0902.

8-Bromo-adenosine 5′-Monophosphate Morpholidate (25). To a solution of 8-bromo-AMP **12** (126 mg, 220 μmol) in DMSO (0.6 mL) was added triphenylphosphine (200 mg, 763 μmol), morpholine (130 μL, 1.49 mmol), and dipyridyl disulfide (168 mg, 763 μmol). The resulting yellow solution was stirred at room temperature for 3 h and quenched with a solution of sodium iodide in acetone (0.1 M, 20 mL). The resulting precipitate was filtered, washed with acetone, and dried under reduced pressure to afford the desired morpholidate **25** as a yellow solid (187 μmol, 85%). ^{1}H NMR (D_2O, 270 MHz) δ: 8.08 (s, 1H, H-2), 6.00 (d, $J_{1',2'} = 4.7$ Hz, 1H, H-1′), 5.29 (m, 1H, H-2′), 4.70 (m, 1H, H-3′), 4.16 (m, 1H, H-4′), 3.95 (m, 2H, H-5′), 3.39 (m, 4H, 2 × CH_2O), and 2.75 (m, 4H, 2 × CH_2N). ^{31}P NMR (D_2O, 109 MHz) δ: 8.26 (s). *m/z* (ES^-): 493.37 [$(M - H)^-$, 100%], 495.25 [$(M - H)^-$, 100%]. HRMS Calcd for $[M - H]^- C_{14}H_{19}{}^{79}BrN_6O_7P^-$ (ES^-), 493.0236; found, 493.0240.

Cyclic 8-Bromo-adenosine 5′-Diphosphate Ribose (3, 8-Br-cADPR). 8-Bromo-cADPR was synthesized and biologically studied by Lee et al.[23] To a mixture of 8-bromo-AMP morpholidate (73 μmol), β-NMN^+ (31 mg, 92 μmol), and $MgSO_4$ (18 mg, 150 μmol) was added a solution of $MnCl_2$ in formamide (0.2 M, 0.56 mL) under a nitrogen atmosphere. The reaction mixture was then stirred at room temperature for 2 days and quenched by dropwise addition of MeCN (2 mL). The resulting yellow precipitate was filtered, washed with MeCN, and treated with Chelex to remove any residual Mn^{2+}. The crude product was purified on a reverse-phase column, eluted with a gradient of 0–30% MeCN against 0.05 M TEAB buffer. The appropriate fractions were pooled, evaporated *in vacuo*, and co-evaporated with MeOH (3×) to give 8-Br-NAD^+ **29** as a triethylammonium salt. ^{1}H NMR (D_2O, 270 MHz) δ: 9.27 (s, 1H, H_N-2), 9.09 (d, $J_{6,5} = 5.4$ Hz, 1H, H_N-6), 8.79 (d, $J_{4,5} = 8.1$ Hz, 1H, H_N-4), 8.28 (m, 1H, H_N-5), 8.07 (s, 1H, H-2), 5.96 (d, $J_{1',2''} = 3.4$ Hz, 1H, H-1″), 5.93 (d, $J_{1',2'} = 5.5$ Hz, 1H, H-1′), 5.17 (m, 1H, H-2′), and 4.57–4.24 (m, 9H, H-ribose). ^{31}P NMR (D_2O, 109 MHz): −10.46 (brs). *m/z* (ES^-): 739.57 [$(M - H)^-$, 100%, ^{79}Br], 741.45 (^{81}Br). Compound **29** in HEPES buffer (25 mM, pH7.4, 106 mL) was incubated with *Aplysia* ADP ribosyl cyclase (127 μL) at room temperature for 1 h and then purified according to previously described conditions for **7**. The title compound **3** was formed as a triethylammonium salt in 49% yield over two steps. HPLC: 8.39 at 254 min. UV (H_2O) λ_{max}: 265.2 nm. ^{1}H NMR (D_2O, 400 MHz) δ: 8.89 (s, 1H, H-2), 6.02 (d, $J_{1',2'} = 5.3$ Hz, 1H, H-1′), 5.99 (d, $J_{1'',2''} = 4.0$ Hz, 1H, H-1″), 5.35 (dd, $J_{2',1'} = 5.3$ Hz, $J_{2',3'} = 5.0$ Hz, 1H, H-2′), 4.62 (m, 3H, H-2″, H-3′, and H-4″), 4.25 (m, 4H, H-3″, H-5′a, H-5″a, and H-4′), 4.00 (m, 1H, H-5″b), and 3.86 (m, 1H, H-5′b). ^{31}P NMR (D_2O, 109 MHz) δ: −9.81 (brs), −10.75 (brs). HRMS Calcd for $[M - H]^- C_{15}H_{19}{}^{79}BrN_5O_{13}P_2^-$ (ES^-), 617.9638; found, 617.9641.

8-Amino-adenosine 5′-Monophosphate Morpholidate (26). To a solution of 8-amino AMP triethylammonium salt (110 mg, 0.21 mmol) in dry DMSO (0.6 mL) was added a sequence of triphenylphosphine (200 mg, 0.76 mmol), morpholine (0.13 mL, 1.49 mmol), and dipyridyl disulfide (168 mg, 0.76 mmol). The resulting solution was stirred at room temperature for 3.5 h and purified as described for **25**, giving the desired morpholidate **26** as a yellow solid (141 μmol, 67%). HPLC: single peak at 2.70 min at 254 nm. ^{1}H NMR (D_2O, 270 MHz) δ: 7.89 (s, 1H, H-2), 5.92 (d, $J_{1',2'} = 7.2$ Hz, 1H, H-1′), 4.73 (m, 1H, H-2′), 4.42 (m, 1H, H-3′), 4.26 (m, 1H, H-4′), 4.03 (m, 2H, H-5′), 3.58 (m, 4H, 2 × CH_2O), and 2.96 (m, 1H, 2 × CH_2N). ^{31}P NMR (D_2O, 109 MHz) δ: 7.90 (s). *m/z* (ES^-): 430.48 [$(M - H)^-$, 100%]. HRMS Calcd for $[M - H]^- C_{14}H_{21}N_7O_7P^-$ (ES^-), 430.1240; found, 430.1249.

Cyclic 8-Amino-adenosine 5′-Diphosphate Ribose (2, 8-NH_2-cADPR). 8-Amino-cADPR was earlier reported by Lee et al.[23] To a mixture of 8-amino-AMP morpholidate (60 mg, 139 μmol), β-NMN^+ (58 mg, 174 μmol), and $MgSO_4$ (38 mg, 317 μmol) was added a solution of $MnCl_2$ in formamide (0.2 M, 1.25 mL). The resulting suspension was stirred at room temperature for 2 days and purified as described for **29** to form 8-amino-NAD^+ **30** as a triethylammonium salt. ^{1}H NMR (D_2O, 400 MHz) δ: 9.29 (s, 1H, H_N-2), 9.16 (d, $J_{6,5} = 6.3$ Hz, 1H, H_N-6), 8.76 (d, $J_{4,5} = 8.2$ Hz, 1H, H_N-4), 8.16 (dd, $J_{5,4} = 8.2$ Hz and $J_{5,6} = 6.3$ Hz, 1H, H_N-5), 7.96 (s, 1H, H-2), 6.08 (d, $J_{1'',2''} = 5.1$ Hz, 1H, H-1″), 5.84 (d, $J_{1',2'} = 7.1$ Hz, 1H, H-1′), 4.66 (m, 1H, H-2′), 4.43 (m, 1H, H-4″), and 4.42–4.23 (m, 8H, ribose-H). ^{31}P NMR (D_2O, 109 MHz) δ: −10.42 (d, $J_{PP} = 17.1$ Hz) and −11.24 (d, $J_{PP} = 17.1$ Hz). HRMS Calcd for $[M - H]^- C_{21}H_{27}N_8O_{14}P_2^-$ (ES^-), 677.1122; found, 677.1122. Compound **30** and *Aplysia* cyclase (256 μL) in HEPES buffer (25 mM, pH 7.4, 166 mL) was incubated at room temperature for 40 min. The reaction mixture was purified as described for **7** to afford **2** as a glassy solid in the triethylammonium form in 38% yield over two steps. HPLC: 4.39 min at 254 nm. UV (H_2O) λ_{max}: 275.8 nm. ^{1}H NMR (D_2O, 400 MHz) δ: 8.72 (s, 1H, H-2), 6.01 (d, $J_{1'',2''} = 4.0$ Hz, 1H, H-1″), 5.80 (d, $J_{1',2'} = 5.6$ Hz, 1H, H-1′), 5.36 (dd, $J_{2',1'} = 5.6$ Hz and $J_{2',3'} = 4.8$ Hz, 1H, H-2′), 4.65 (m, 3H, H-3′, H-4″, and H-2″), 4.38 (m, 1H, H-3″), 4.29 (m, 2H, H-5′a and H-5″a), 4.22 (m, 1H, H-4′), 4.04 (m, 1H, H-5″b), and 3.94 (m, 1H, H-5′b). ^{31}P NMR (D_2O, 109 MHz) δ: –9.87 (d, $J_{PP} = 11.6$ Hz) and −10.21 (d, $J_{PP} = 11.6$ Hz). HRMS Calcd for $[M - H]^- C_{15}H_{21}N_6O_{13}P_2^-$ (FAB^-), 555.0642; found, 555.0645.

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Supporting Information Available: Table (HPLC %) to show the degree of purity, HPLC traces for all key pyrophosphate compounds, ^{1}H NMR spectra for compounds **2**, **3**, **5**, and **7**−**10**, and ^{13}C data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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