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Aberrant Cyclization Affords a C-6 Modified Cyclic Adenosine 5′-Diphosphoribose Analogue with Biological Activity in Jurkat T Cells

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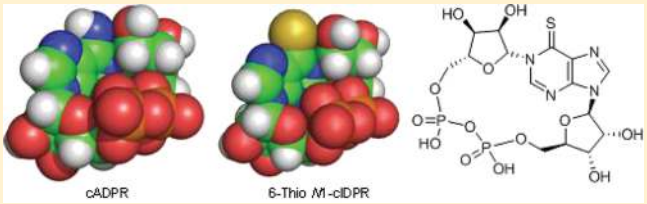
Supporting Information

ABSTRACT: Two nicotinamide adenine dinucleotide (NAD^+) analogues modified at the 6 position of the purine ring were synthesized, and their substrate properties toward *Aplysia californica* ADP-ribosyl cyclase were investigated. 6-*N*-Methyl NAD^+ (6-*N*-methyl nicotinamide adenosine 5′-dinucleotide **10**) hydrolyzes to give the linear 6-*N*-methyl ADPR (adenosine 5′-diphosphoribose, **11**), whereas 6-thio NHD^+ (nicotinamide 6-mercaptopurine 5′-dinucleotide, **17**) generates a cyclic dinucleotide. Surprisingly, NMR correlation spectra confirm this compound to be the *N*1 cyclic product 6-thio *N*1-cIDPR (6-thio cyclic inosine 5′-diphosphoribose, **3**), although the corresponding 6-oxo analogue is well-known to cyclize at *N*7. In Jurkat T cells, unlike the parent cyclic inosine 5′-diphosphoribose *N*1-cIDPR **2**, 6-thio *N*1-cIDPR antagonizes both cADPR- and *N*1-cIDPR-induced Ca^{2+} release but possesses weak agonist activity at higher concentration. **3** is thus identified as the first C-6 modified cADPR (cyclic adenosine 5′-diphosphoribose) analogue antagonist; it represents the first example of a fluorescent *N*1-cyclized cADPR analogue and is a new pharmacological tool for intervention in the cADPR pathway of cellular signaling.



INTRODUCTION

Cyclic adenosine 5′-diphosphoribose (cADPR, **1**, Figure 1), discovered by Lee et al. in 1987,[1] is one of the principal second messenger molecules that mobilize intracellular Ca^{2+} in a different way to the well-established D-*myo*-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$),[2] by gating the ryanodine receptor.[3,4] The cADPR/Ca^{2+} signaling system is active in diverse mammalian cellular systems such as cardiac muscle, acinar cells, and plant cells.[5] cADPR is a metabolite of nicotinamide adenine dinucleotide (NAD^+) and is produced enzymatically by ADP-ribosyl cyclases (ADPRC). Its structure was fully characterized by Lee et al.[6] as a cyclic 18-membered dinucleotide featuring two glycosidic bonds and a pyrophosphate linkage. Several excellent reviews dealing with the chemistry of cADPR and the cADPR/Ca^{2+} signaling system have appeared in recent years.[3,5,7−11]

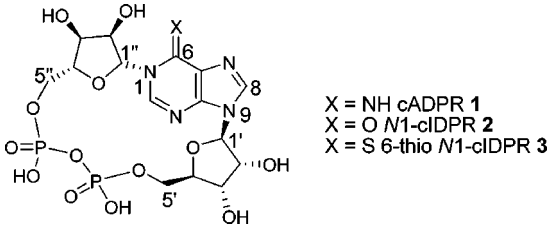


Figure 1. C-6 modified cADPR analogues and numbering system.

cADPR is readily hydrolyzable at the *N*1 glycosidic bond linkage to give ADPR in both neutral aqueous solution and under physiological conditions,[12,13] thus rendering chemical synthesis of analogues challenging. The main choice has been between total chemical synthesis[14] and a chemoenzymatic approach[15,16] developed earlier and modeled on the biosynthesis of cADPR from NAD^+. By combination of both approaches, a large number of cADPR analogues have been synthesized over the years and their Ca^{2+} release activities examined in several systems, such as sea urchin egg homogenate (SUH) and T cells inter alia. Modification at the 8-position with 8-amino and 8-bromo groups in particular has been shown to convert cADPR from an agonist to an antagonist,[16−19] although at high concentrations this does not apparently hold.[20] However, 8-substituted cyclic adenosine 5′-diphosphocarbocyclic ribose (8-X cADPcR) analogues developed by Shuto[21] were reported to be agonists in sea urchin egg homogenates, indicating that 8-substitution alone may not be responsible for antagonist activity. 3′-*O*-Methyl cADPR was also found to have antagonist properties, at least in SUH.[13] The hydrolysis-resistant 7-deaza 8-bromo cADPR has found several biological applications[22,23] as a cell permeant competitive antagonist.[17,24,25] Analogues with structural modifications of the "northern" ribose[12] showed agonist activities in T cells, including those with more radical changes.[26,27]

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Recently, we have also pioneered a synthesis of novel cADPR derivatives that are highly stable both chemically and enzymatically. The 8-bromo *N*1-cyclic inosine 5′-diphosphoribose (8-Br-*N*1-cIDPR) thus obtained proved to be a novel agonist of Ca^{2+} release in intact cells.[28,29] The opposed bioactivities of the classical antagonist 8-Br-cADPR and 8-Br-cIDPR thus show that the smallest of structural changes (i.e., $NH_2 \rightarrow C{=}O$ at C-6, as with changes at C-8 and C-3′) can transform a cADPR antagonist into an agonist. Furthermore, the enhanced stability of 8-Br-cIDPR allows direct chemical modification at the 8-position, including to the parent *N*1-cIDPR, (**2**, Figure 1), which is roughly equipotent to cADPR in permeabilized T cells.[30] This stable analogue was recently cocrystallized with the wild-type ectoenzyme CD38, a multifunctional enzyme responsible for the formation and metabolism of cADPR, providing insight into substrate binding and the catalytic process.[31]

The chemoenzymatic approach relies on the selectivity of *Aplysia* cyclase that, while promiscuous, may not always recognize the required NAD^+ analogue as substrate. Moreover, while NAD^+ analogues generally cyclize at *N*1, some are hydrolyzed to the corresponding linear ADPR analogue. Another exception, ethenonicotinamide adenine 5′-dinucleotide, in which the *N*1 position is substituted, cyclizes through the *N*7 nitrogen by ADPRC.[32] NHD^+, a close analogue of NAD^+ but bearing a 6-keto group on the purine ring rather than an amino group, cyclizes at *N*7, giving the fluorescent, biologically inactive *N*7-cIDPR, as indeed does the

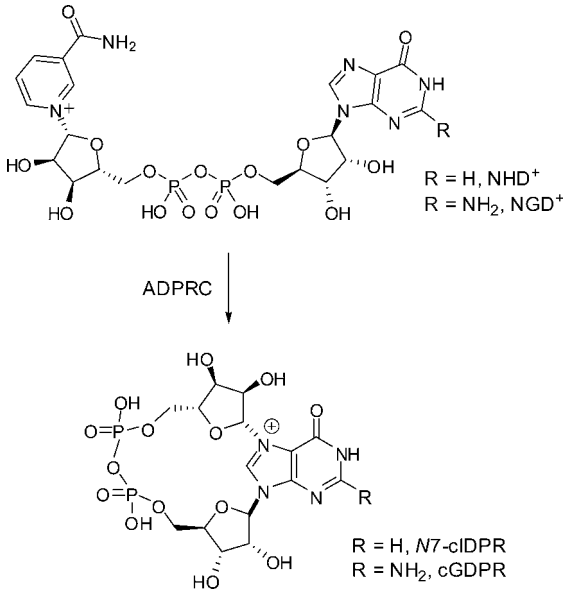

Figure 2. *N*7 cyclization of NHD^+ by *Aplysia* cyclase.

guanosine congener NGD^+ (Figure 2).[33] The reasons for this are not fully understood. 6-Thio NHD^+ (where a 6-mercaptopurine replaces the hypoxanthine ring of NHD^+) has yet to be explored as a potential substrate for ADP-ribosyl cyclase. Since the only modifications at position 6 in the cyclic nucleotide explored to date (NH_2 and C=O) result in major changes in biological activity, we anticipated that the 6-thio (C=S) derivative in particular would be of significant interest.

The synthesis of C-6 modified cADPR analogues has never been straightforward. NAD^+ analogues with substituents at C-6 such as SMe, $NH(CH_2)_6NH_2$ or H all fail to cyclize when incubated with ADPRC.[7] The corresponding hydrolyzed product is formed instead. The simple *N*-methylated analogue, 6-*N*-methyl nicotinamide adenosine 5′-dinucleotide (6-*N*Me-NAD^+) has, however, not yet been investigated. *N*1-cIDPR was prepared via both 8-Br-NHD^+ and 8-Br-*N*1-cIDPR to bypass the problem encountered with *N*7 cyclization.[30] To the best of our knowledge, even a total synthesis was never attempted for such a class of compound, presumably because of the difficulty of incorporating an intact "northern" ribose into the molecule. In order to further investigate the requirements for *N*1 versus *N*7 cyclization inter alia, we report here syntheses of both 6-NMe-NAD^+ and 6-thio NHD^+ and an investigation of their cyclization behavior.

RESULTS AND DISCUSSION

Synthesis of 6-*N*-Methyl NAD^+. 6-*N*Me adenosine **7** was earlier synthesized from 1-*N*-methyladenosine via a Dimroth rearrangement that involves an opening and recyclization of the adenine ring.[34] We synthesized **7** by a different synthetic route that could provide wider flexibility for other modifications longer term. 6-Chloropurine **4** was first activated with TMS-triflate, then condensed with tetraacetyl ribose **5** to produce the 6-chloro protected nucleoside **6** in its β configuration (Scheme 1). Treatment with methylamine hydrochloride followed by acetate removal with methanolic ammonia produced 6-*N*Me adenosine **7** in good yield. Phosphorylation was achieved by using the established $POCl_3$/TEP method but, in contrast to other nucleosides, both the 5′-monophosphate **8a** and 3′,5′-bisphosphate **8b** were formed as a mixture. Subsequent treatment with triphenylphosphine, morpholine, and dipyridyl disulfide produced two morpholidates. The desired monomorpholidate **9a** was then easily isolated by ion-exchange chromatography and condensed with β-NMN^+ to form the target 6-*N*Me-NAD^+ **10**.

Incubation of 6-*N*Me-NAD^+ **10** with *Aplysia* cyclase generated only the hydrolyzed product 6-*N*Me-ADPR **11**, confirmed by ^1H NMR spectroscopy and a molecular ion of 572 in the electrospray mass spectrum. The latter is in accord with the structure of a linear nucleotide rather than the cyclic 6-*N*Me-cADPR, as it differs by 17 mass units for the additional hydroxyl group. Moreover, the presence of two NMR doublets at 5.24 and 5.14 ppm represents a typical anomeric proton pattern for the terminal ribose hydroxyl in the α and β configurations. This result is perhaps not too surprising based upon another report of the hydrolysis of C-6 substituted NAD^+ analogues, but these had more radical changes.[7]

It was initially assumed that cyclization of 6-*N*Me-NAD^+ is most likely blocked by the steric hindrance brought about by the free rotation of the methyl group. However, an early review[7] reported that the compound with an H at C-6 rather than an amino group is apparently also unable to cyclize, indicating that the cyclization catalyzed by *Aplysia* cyclase might (not surprisingly) involve the participation of the C-6 amino group. It may be possible that the steric hindrance of the methyl group disturbs the electrophilic attack at the *N*1 position and therefore blocks the cyclization. It seems likely that the steric bulk of the NMe might interfere with the approach to the known covalent E179 enzyme−ribose intermediate for cyclization.[35] For hydrolysis to occur requires the formation of the 6-*N*Me-NAD^+−enzyme covalent complex, which allows water to attack the ribosyl C-1″ rather than *N*1. Another explanation could be interference with the actual binding of 6-*N*Me-NAD^+ to *Aplysia* cyclase by reducing the H-bonding sites in the active site.

Synthesis of 6-Thio NHD^+. 6-Thio NHD^+ was first reported by Atkinson et al.[36] and synthesized by coupling 6-thio IMP (**14**) with nicotinamide mononucleotide with dicyclohexylcarbodiimide, a method developed by Todd et al.[37] The low

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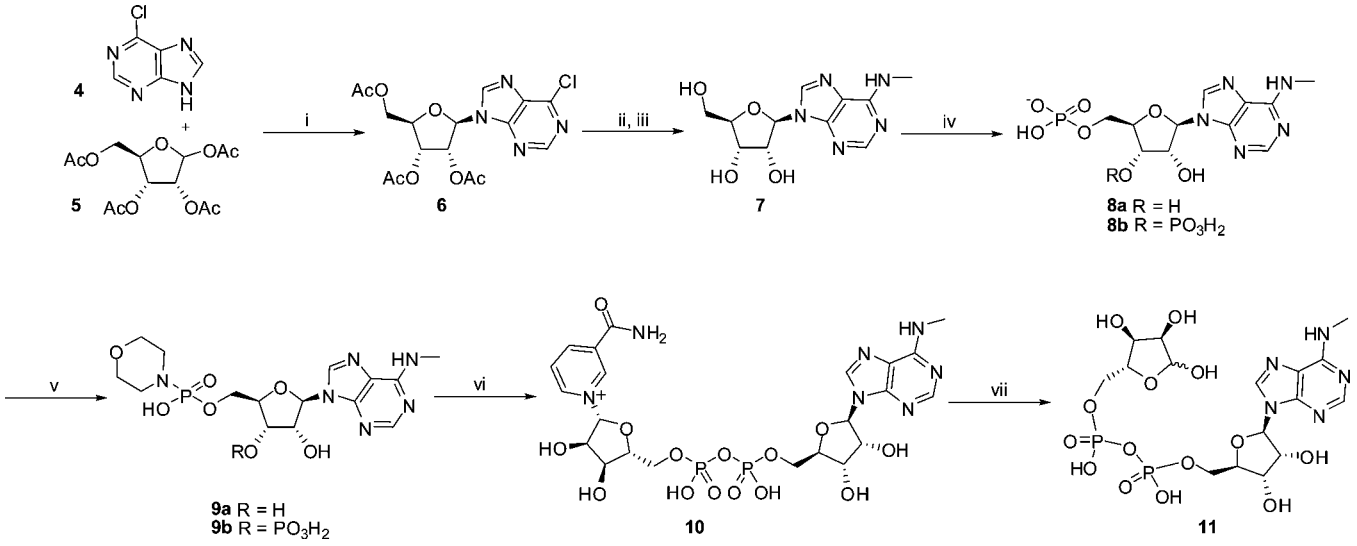
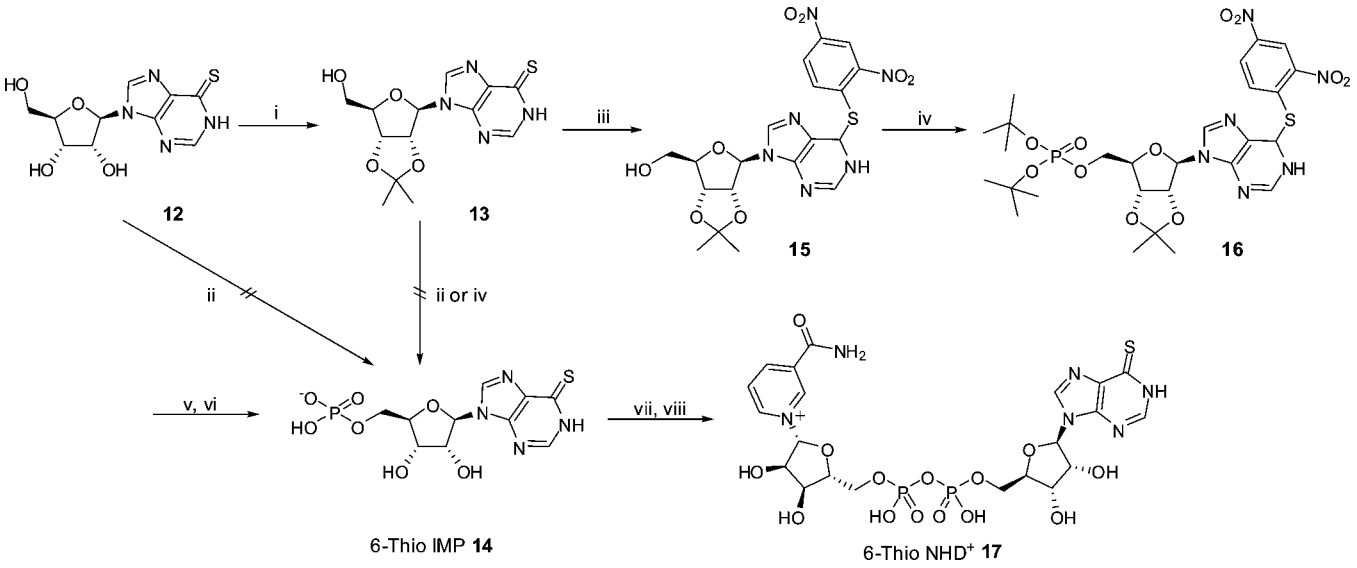
Scheme 1. Synthesis of 6-*N*Me NAD$^+$[a]



[a]Reagents and conditions: (i) TMSOTf, DBU, MeCN, 60 °C, 1 h; (ii) methylamine hydrochloride, DCM/EtOH/Et_3N, 60 °C, overnight; (iii) NH_3/MeOH, 0 °C, 3 h; (iv) $POCl_3$, TEP, H_2O, 0 °C, 3 h; (v) PPh_3, dipyridyl disulfide, morpholine, room temp, 2 h; (vi) β-NMN$^+$, $MnCl_2$ in formamide, room temp, 48 h; (vii) *Aplysia californica*, 25 mM HEPES (pH 7.4), room temp.

Scheme 2. Synthesis of 6-Thio NHD$^+$ [a]



[a]Reagents and conditions: (i) dimethoxypropane, acetone, $HClO_4$, 20 min, room temp; (ii) $POCl_3$, TEP, H_2O, 0 °C, 1 h; (iii) dinitrofluorobenzene, Et_3N, MeCN, room temp, 1 h; (iv) diisopropyl-di-*tert*-butylphosphoramidite, tetrazole, DCM, room temp, 1 h, then mCPBA, −78 °C, 10 min; (v) 10% mercaptoethanol in MeCN + 1% DIPEA, room temp, 1 h; (vi) 50% aq TFA, room temp, overnight; (vii) morpholine, PPh_3, dipyridyl disulfide, DMSO, room temp, 1 h; (viii) β-NMN$^+$, 0.2 M $MnCl_2$ in formamide, room temp, overnight.

yield and lack of structural data in the initial report prompted us to investigate a new, reliable and more modern, route to 6-thio NHD$^+$. The key compound is **14**, which was previously synthesized mostly in the 1960s more or less successfully.[38,39] We initially reasoned that the synthesis of **14** should be easily accomplished by selective phosphorylation of 6-thioinosine **12** by adaptation of a published method[40] that we have used very successfully on a wide range of nucleosides. However, treatment of 6-thioinosine with $POCl_3$ in TEP was unsuccessful because of the poor solubility of the starting nucleoside in TEP (Scheme 2). An isopropylidene protecting group at the 2′,3′-hydroxyls was then considered to have the double advantage of solving the solubility issue and being removable during the phosphorylation procedure, which is carried out under acidic conditions. Phosphorylation at the 5′-position proved to be very difficult. Indeed, HPLC analysis of the quenched reaction showed a complex mixture of products that could not be isolated by reverse-phase chromatography. We reasoned that there was a mixture of protected and deprotected material as well as products phosphorylated at the 5′-OH, the sulfur atom, or both, since the sulfur is more nucleophilic than the oxygen.

Phosphoramidite chemistry has been reported on 2′,3′-*O*-isopropylidene-6-thioinosine **13**[41] using *N*,*N*-diisopropyl-di-*tert*-butylphosphoramidite as a phosphitylating reagent.[42] Both

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tert-butyl and isopropylidene protecting groups could then be cleaved under acidic conditions. However, in our hands the phosphorylation step could not be repeated, as the starting material is insoluble in most organic solvents except DMF and DMSO, and therefore only the starting material was recovered after 24 h with no sign of product.

Thio-substituted nucleotides are particularly useful in molecular biology, as they have been incorporated into oligonucleotides by chemical methods and used for postsynthetic modification.[43−46] During the former process, the sulfur is always protected to avoid unnecessary side reactions during phosphitylation and oxidation of the phosphite.[47] Since the synthesis of 6-thio IMP is hampered by the lack of solubility of 6-thioinosine, protecting the sulfur should further solve the solubility issue encountered. Various protecting groups have been used such as cyanoethyl, which can be removed by treatment with DBU. Although the cyanoethyl group has been used extensively, we decided to utilize the 2,4-dinitrophenyl (DNP) group, as previous studies have shown that it can easily be removed with mercaptoethanol under very mild alkaline conditions.[48]

The synthesis of 6-thio NHD$^+$ **10** is outlined in Scheme 2. 2′,3′-*O*-Isopropylidene-6-thioinosine **13** was prepared in a very high yield (86% over five steps) using published methods starting from inosine **12**.[41,49] Protection of the sulfur was achieved by treatment of 2′,3′-*O*-isopropylidene-6-thioinosine with triethylamine and 2,4-dinitrofluorobenzene. The yellow product **15** obtained after flash chromatography could then be selectively phosphitylated at the 5′-hydroxyl using the *tert*-butyl protected phosphitylating reagent in high yield to give **16**. Removal of the DNP protecting group was accomplished by treatment with mercaptoethanol under mild alkaline conditions. Careful purification by flash chromatography led to a very clean product in 75% yield. Next, both the isopropylidene and *tert*-butyl protecting groups were removed very cleanly by treatment with 50% aqueous TFA to generate the desired 6-thio IMP **14** in 86% yield, without further purification. This clearly is the advantage of the DNP group, as an ion-exchange purification step is avoided during which, from our experience, some product often gets unavoidably lost. **14** was thus obtained in a satisfying 48% yield after a four-step procedure from 2′,3′-*O*-isopropylidene-6-thioinosine.

With 6-thio IMP in hand, we proceeded to synthesize the key intermediate 6-thio NHD$^+$ **17**. The synthesis of the pyrophosphate was achieved using a procedure initially reported by Moffatt[50] and later improved by Lee et al.[51] that relies on the coupling of a sugar phosphate with a nucleotide phosphoromorpholidate in the presence of a Lewis acid. We have previously used this method to successfully generate various NAD$^+$ and NHD$^+$ analogues in relatively high yield.[30,52] 6-Thio IMP was first activated using a combination of morpholine/dipyridyl disulfide and triphenylphosphine to yield the morpholidate that was then condensed with β-NMN$^+$ with $MnCl_2$ as Lewis acid. A ^{31}P NMR shift of around −10 ppm clearly indicated the formation of a pyrophosphate linkage. Purification on reverse-phase chromatography afforded the desired 6-thio NHD$^+$ **17** as the sole product.

6-Thio NHD$^+$ was then incubated with *Aplysia californica* cyclase. There are several possible outcomes for this reaction (Figure 3), i.e., formation of 6-thio *N*1-cIDPR, 6-thio *N*7-cIDPR as for the oxo congener, 6-thio IDPR (6-thioinosine 5′-diphosphate ribose), and it is possible to envisage cyclization on to the sulfur to give the 6-thio-S-cIDPR. The new product displayed a molecular ion of 557 in the ES$^-$ mass spectrum, which ruled out the formation of both the hydrolyzed product 6-thio IDPR and surprisingly also the *N*7 cyclized product 6-thio *N*7-cIDPR, for which a mass of 575 and 558, respectively, would be expected. ^{1}H NMR spectroscopy showed a chemical shift of 5.2 ppm for H-2′, a typical value for a nucleotide in the *syn* conformation, which also ruled out the formation of 6-thio *N*7-cIDPR. Additionally, a C-6 ^{13}C chemical shift of 175 ppm is typical of a thione form (thiol, ∼155 ppm), therefore ruling out the putative sulfur-cyclized product 6-thio *S*-cIDPR as a possibility. Crucially, a gHMBC spectrum showed cross-peaks between the H-2 proton of the purine ring (δ 9.32 ppm) and the anomeric carbon C-1″ of the "northern" ribose (δ 94.9 ppm) and between the anomeric

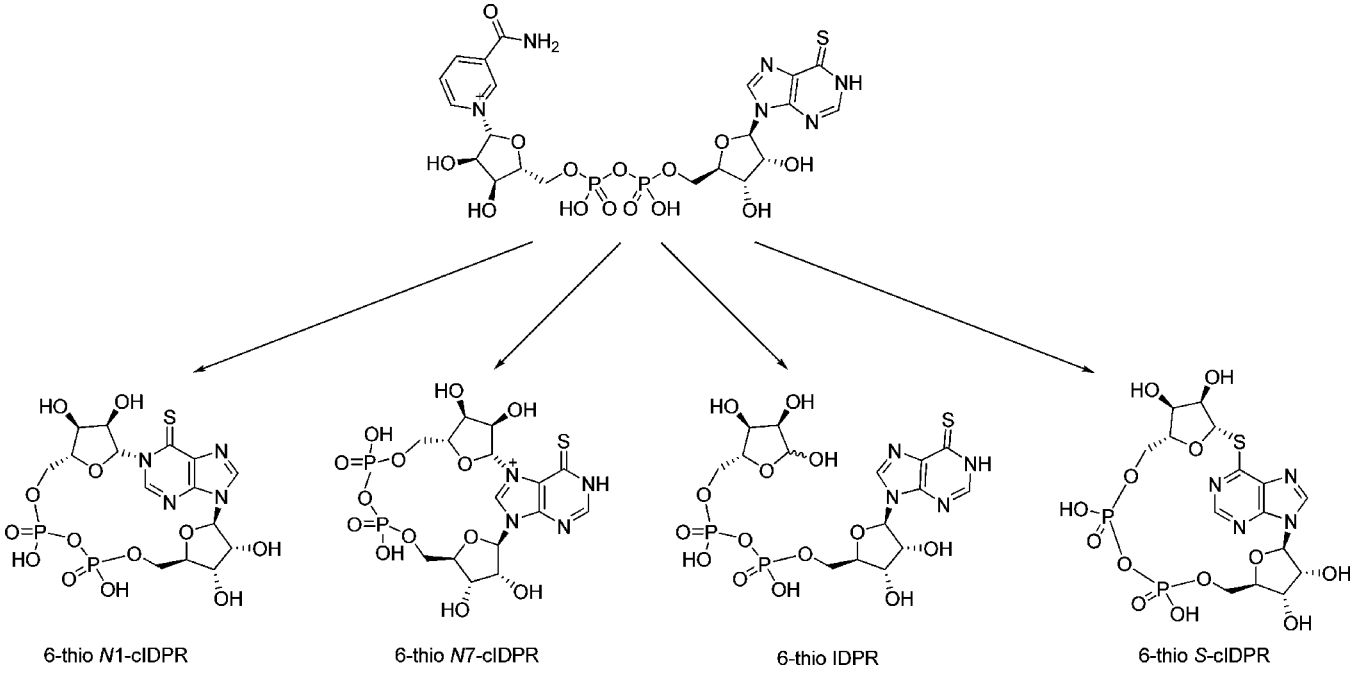

Figure 3. Four possible outcomes from the incubation of 6-thio NHD$^+$ with ADPRC.

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proton H-1″ (δ 6.65 ppm) with the carbon C-2 (δ 144.9 ppm) and C-6 (δ 175.5 ppm) of the nucleobase (Figure 3). Finally, the $^{31}P-^{31}P$ coupling constant for the pyrophosphate linkage in **3** showed J = 11.8 Hz, similar to that of cADPR and cIDPR (13.5 [53] and 12.5 [30] Hz, respectively), whereas the $^{31}P-^{31}P$ coupling of the linear IDPR generally has a much higher frequency (~20 Hz). These analytical data thus provide evidence of a successful and surprising cyclization of 6-thio NHD$^+$ into the corresponding 6-thio *N*1 cIDPR **3** (Figure 4).

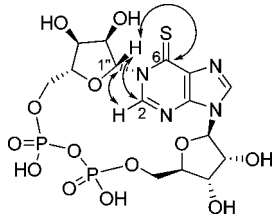

Figure 4. $^1H/^{13}C$ correlations based on gHMBC spectrum supporting the formation of the N1−C1″ bond.

The photochemical properties of 6-thio *N*1-cIDPR **3** were also examined. While adenine and guanosine based nucleotides have an absorbance maximum of around 260 nm, replacement of the oxygen by a sulfur atom shifts the UV absorbance spectrum to 320−340 nm. 6-Thio cIDPR seems to be no exception. The UV spectrum in water exhibited intense absorption at 320 nm (λ_{max}) with an extinction coefficient (ε) of 18 600 mol^{-1} dm^3 cm^{-1} (data not shown). A smaller peak at 267 nm was also observed. More interestingly, 6-thio cIDPR displays fluorescent properties in water at room temperature. When it is excited at 335 nm (just above the maximum UV absorption), an emission spectrum with a single peak at 415 nm was observed (see Supporting Information). In contrast, no fluorescence was observed by the related *N*1-cIDPR (data not shown). Thus far, only *N*7-cyclized dinucleotides such as *N*7-cIDPR, *N*7-cGDPR, and etheno cyclic ADP diphosphoribose have been demonstrated to be fluorescent, and this property was used to rule out the formation of *N*7-cyclic product when no fluorescence was observed.[33] To the best of our knowledge, 6-thio *N*1-cIDPR is therefore the first fluorescent *N*1-cyclized cADPR analogue and this property may find applications in cADPR binding protein biochemistry.

Conformational Analysis. There have been reports that the Ca^{2+}-release activity and antagonism[54,55] of cADPR analogues may be linked to their conformation in solution. The major puckering mode of cADPR is C2′ endo in the *N*9 ribose moiety with a *syn* conformation about the *N*9 glycosidic bond.[6] The furanose ring is in equilibrium between C2′ endo/C3′ endo forms, and the ratio can be calculated from ^1H NMR data following the equation $[C2'\ endo] = [J_{1',2'}/(J_{1',2'} + J_{3',4'})] \times 100$.[56] Also, the H-2′ chemical shift can be used as an indicator of glycosidic bond conformation.[57,58] Therefore, from our NMR data, 6-thio cIDPR exhibits a 74% C2′ endo puckering with a *syn* conformation about the glycosidic bond (Table 1), which is consistent with that observed by both cADPR and *N*1-cIDPR.

Mechanistic Study. ADP-ribosyl cyclase can use different substrates to produce structurally distinct products. The cyclization of NAD$^+$ analogues usually takes place at the *N*1 position of the adenine ring. However, NAD$^+$ analogues, which have their *N*1 position blocked (e.g., etheno NAD$^+$), cyclize at *N*7.[32] If *N*1 is free but is electronically deactivated (e.g., in 2-fluoro NAD$^+$[62]) and the purine is still adenine, then this seems

Table 1. Conformational Analysis of NAD$^+$ Analogues and Their Respective Cyclic Dinucleotides

	H-1′[a]	H-2′[a]	$\Delta_{1'-2'}$[b]	conf[c]	$J_{1',2'}$[a]	$J_{3',4'}$[a]	C2′ endo (%)
cADPR	5.80	5.20	0.6	*syn*	5.6	3.2	64
*N*7-cIDPR	6.19	3.90	2.29	*anti*	3.0	nd	30
*N*1-cIDPR	5.89	5.18	0.71	*syn*	6.1	nd	61
*N*7-cGDPR	6.07	4.63	1.44	*anti*	2.8	nd	30
6-thio *N*1-cIDPR	6.0	5.2	0.8	*syn*	6.3	2.2	74

[a]Data obtained from various sources: cADPR;[59] *N*7-cIDPR and *N*7-cGDPR;[59−61] *N*1-cIDPR.[30] [b]Difference in chemical shifts between H-1′ and H-2′. [c]Preferred glycosidic bond conformation.

to be correctly aligned in the active site but the substrate is hydrolyzed rather than cyclized at *N*7. The enzyme can also cyclize NHD$^+$, NGD$^+$, and NXD$^+$ to their respective *N*7 cyclized dinucleotide *N*7-cIDPR, *N*7-cGDPR, and *N*7-cXDPR.[33] In those cases, the most nucleophilic *N*7 nitrogen in guanine/hypoxanthine is often invoked to rationalize cyclization at this position.[33]

However, nucleophilicity is not the only factor to consider. We previously made correlations showing that the conformation about the glycosidic bond of NHD$^+$ analogues appears to play a role in their cyclization.[52] We argued that a *syn* conformation about the glycosidic bond is key for a successful cyclization at *N*1, i.e., that the enzyme seems to utilize the linear precursor in its prearranged conformation (at least in the case of the hypoxanthine series and presumably the guanosine series as well). Indeed, NHD$^+$ (*anti*) cyclizes at *N*7 (product also in *anti* conformation), 8-X-NHD$^+$ (*syn*) cyclizes at *N*1 (products in *syn* conformation), and 7-deaza NHD$^+$ (*anti* and no possibility to cyclize at *N*7) hydrolyzes to the linear 7-deaza IDPR.[52] However, the NAD$^+$ analogues cyclize at *N*1 regardless of their conformation; NAD$^+$ (*anti*), 8-X NAD$^+$ (*syn*), and 7-deaza NAD$^+$ (*anti*) cyclize at *N*1, all forming products in their *syn* conformation. When 6-thio NHD$^+$ is used as a substrate, the enzyme converts it into 6-thio *N*1-cIDPR, therefore suggesting that the enzyme utilizes this substrate in the same way as NAD$^+$ but clearly differently from NHD$^+$, although NHD$^+$ and 6-thio NHD$^+$ are structurally very similar.

Lee et al. have intensively investigated the structures of both *Aplysia* ADP-ribosyl cyclase and human CD38 to unravel the catalytic mechanism of the NAD$^+$ cyclization and cADPR hydrolysis reactions. Recently, they suggested that the cyclization reaction of NAD$^+$ analogues occurs through a four-step sequence where both residues Tyr-81 and Phe-174 play an instrumental role by stabilizing the nucleobase through π-stacking interactions in a folded conformation so that cyclization can occur.[63,64] Mutagenesis confirmed that Phe-174 was likely to be responsible for folding the linear substrate in the correct conformation. However, this is only true for adenine-based substrates; mutagenesis did not affect the cyclization of NGD$^+$ to *N*7-cGDPR. Therefore, it is likely that two residues are responsible for the different base cyclization sites (*N*1 versus *N*7) and that residue Phe-174 may also be involved in the cyclization of 6-thio NHD$^+$ to 6-thio *N*1-cIDPR.

With the help of molecular modeling, we investigated further how NAD$^+$ cyclization to cADPR may occur. The ligand in the ribo-2′-F-NAD$^+$·ADPRC structure (PDB code 3I9O)[63] was manually manipulated by rotating individual bonds to approximate the position the adenine would have to be in to attack C-1″ and form the *N*1-cyclized product (Figure 5). In order for NAD$^+$ to cyclize at *N*1, the adenine must rotate around the

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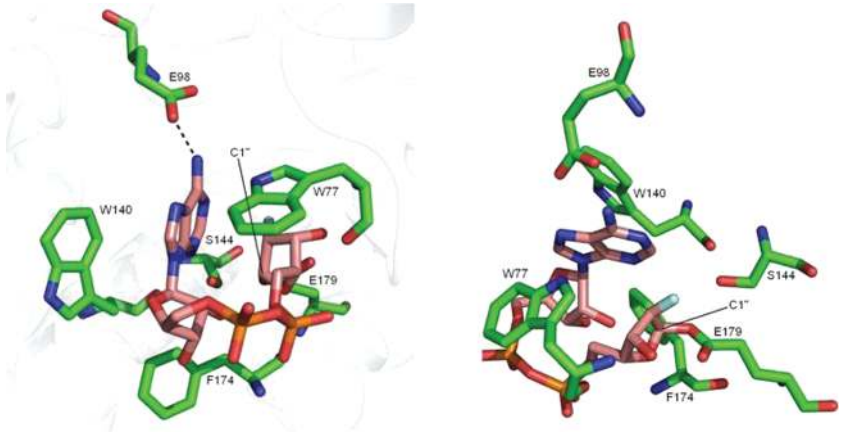

Figure 5. Two views of a model of the position of the adenine of the intermediate from ribo-2′-F-NAD$^+$·ADPRC complex immediately prior ring closure. The hydrogen bond from the base to Glu-98 is shown. The model was built by rotating individual bonds in the PDB 3I9O structure (ribo-2′-F-NAD$^+$·ADPRC) using the Schrödinger software running under Maestro, version 9.0.111. The color scheme for the ligand is as follows: pink, carbon; blue, nitrogen; red, oxygen; white, hydrogen; light blue, fluorine; orange, phosphorus. The color scheme for the residues is the same as for the ligand except the carbons are green.

*N*9/C-1′ bond to adopt the *syn* configuration. It can then approach the C-1″ by stacking with Trp-140 and having the 6-amino group forming at least one hydrogen bond to Glu-98 to further orient and hold the adenine in position. This proposed mechanism has some support from the kinetic data for Glu-98 mutants, as indeed mutation of this residue was shown to reduce cyclase activity.[65] NHD$^+$ (or NGD$^+$) would be unable to form the hydrogen bonds to Glu-98, and the lack of stabilization may partly explain the lack of cyclization at *N*-1.

Exactly why 6-thio NHD$^+$ cyclizes at *N*1 is unknown at present. It is intuitively clear that in order for 6-thio NHD$^+$ to cyclize, the enzyme should be capable of stabilizing it in its thiol form, although the mercaptopurine base normally exists predominantly in its thione form in solution.[66] In the case where the protonation state plays a role in the reaction mechanism, the pK_a values of *N*1, *N*7, and X1 (NH$_2$, OH, SH) and X2 (=O, =NH, =S) were calculated computationally (Figure 6; see

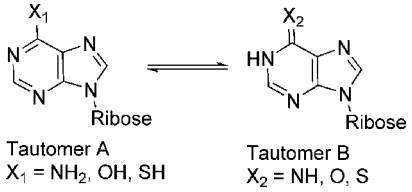

Figure 6. Tautomeric form of purines.

also Supporting Information). The calculations were done on the nucleoside (adenosine, inosine, and 6-thioinosine) and each respective tautomer; we presume that it is reasonable to propose that these values would follow the same trend for the corresponding NAD/NHD analogue.

For three of the possible ionization states, the program was unable to calculate a pK_a, presumably because they are too extreme. At physiological pH, most of these ionizable groups are going to be either fully protonated or deprotonated, the exception being for the oxygen-containing compound (see Supporting Information). In the A tautomer the X$_1$ ionization (OH → O$^-$) has a pK_a of 6.56, and in the B tautomer the N1 ionization (NH → N$^-$) has a pK_a of 8.67. These two values are within the range of physiological pH, which means that at this pH there will be a mixture of four tautomeric forms for the oxygen-containing compound (Figure 7) while there will

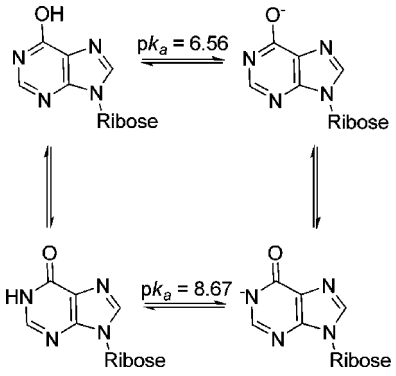

Figure 7. Presumed different forms of the oxygen containing compound that may be present at physiological pH.

be only two forms of the nitrogen- and sulfur-containing compound.

Previously, we argued that the deciding interaction through the adenine ring was via hydrogen bond donation from the base, since the hypoxanthine base has only hydrogen acceptor character in its keto form. However, the 6-thiopurine base has virtually no hydrogen bond acceptor or donor character, which may imply that hydrogen bonding is unimportant at C-6 and that cyclization is influenced by other factors such as the size of the substituent (the sulfur atom is larger than the nitrogen and oxygen atoms; see Supporting Information), the conformation about the glycosidic bond (for the oxygen containing nucleotides), electronic properties, or a combination of these. It may be that an H-bonding interaction with the enzyme locks the purine of NHD$^+$ in the *anti* form, leading to *N*7 cyclization. However, this interaction could be very weak or nonexistent with the 6-thiopurine, allowing it to rotate to facilitate *N*1 cyclization. In the case of 8-Br NHD$^+$ the strong orienting effect of the bromo group may be enough to overcome such H-bonding. Protein crystallography with such a ligand may reveal those interactions as important for catalysis to occur.

Pharmacology. The Ca^{2+} release activity of the newly synthesized 6-thio cIDPR **3** was evaluated fluorimetrically in

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permeabilized Jurkat T cells, and the results are shown in Figure 8. Because of the intrinsic fluorescence of 6-thio cIDPR,

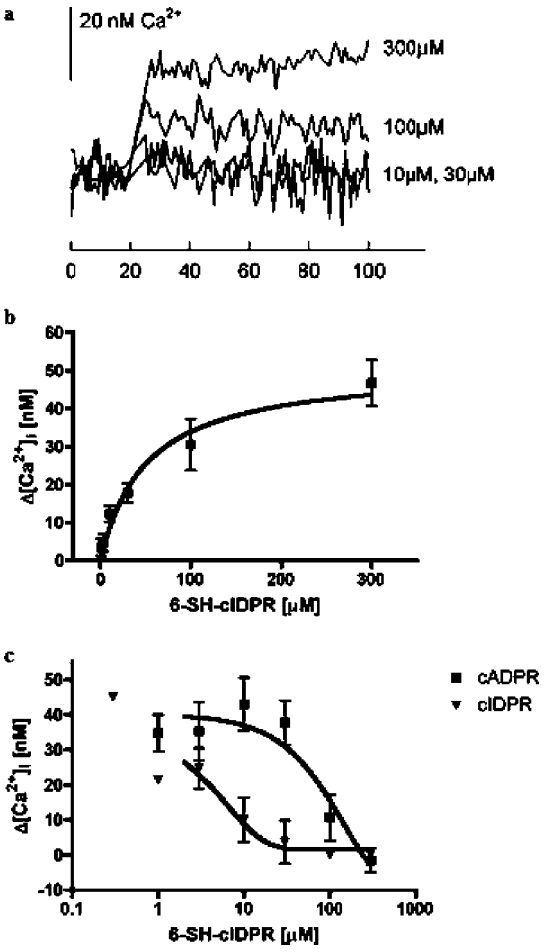

Figure 8. 6-Thio-cIDPR-induced Ca^{2+} release and effect of 6-thio-cIDPR on cADPR and cIDPR-induced Ca^{2+} release in permeabilized Jurkat T cells. Jurkat T cells were permeabilized, and $[Ca^{2+}]$ was measured in the presence of Fluo-3, ATP, and an ATP regenerating system as detailed in the Experimental Section. (a) Ca^{2+} release induced by addition of 6-thio-cIDPR. Characteristic tracings from representative experiments are shown. (b) Concentration−response curve of 6-thio-cIDPR induced Ca^{2+} release. Results represent Ca^{2+} increase over baseline (Δ values) expressed as mean ± SD (n = 4−7) of single tracings. (c) The inhibitory effect of 6-thio-cIDPR was estimated by previous addition of 6-SH-cIDPR and subsequent addition of either cIDPR or cADPR. Since 6-thio-cIDPR also elicited a weak agonist effect on its own (a, b), calculation of its antagonist effect on cADPR (30 μM) or cIDPR (30 μM) was carried out by subtracting the corresponding agonist data (data in part b). Concentration−response curves represent the mean ± SD (n = 3) of single tracings. A one-phase exponential decay was used to fit curves; r^2 was 0.6486 for cADPR and 0.7107 for cIDPR.

Fluo3 was used as a Ca^{2+} indicator to avoid any possible interference. At low concentration, 6-thio cIDPR **3** did not stimulate Ca^{2+} release in T cells. Ca^{2+} release was, however, observed at significantly higher concentration (100 μM and above, Figure 8a and Figure 8b). In comparison with *N*1-cIDPR **2**, weaker agonist activity was observed for 6-thio cIDPR **3**; however, in the current series of experiments the permeabilized cell preparations responded also somewhat more weakly to the naturally occurring cADPR **1**. Regarding antagonism by 6-thio cIDPR **3** a concentration-dependent effect on cADPR and *N*1-cIDPR **2** induced Ca^{2+} release was observed (Figure 8c). While the IC_{50} for cADPR induced Ca^{2+} release was between 30 and 100 μM, a somewhat better inhibition of Ca^{2+} release induced by *N*1-cIDPR **2** was obtained ($IC_{50} \approx 3$ μM). We thus demonstrate here that a simple modification from a C═O to C═S bond at C-6 weakens agonist activity but enhances antagonist activity in Jurkat T cells. Antagonist activity was specifically strong when *N*1-cIDPR **2** was used to trigger Ca^{2+} release, indicating that the oxygen group in cIDPR can be replaced at lower 6-thio cIDPR **3** concentrations compared to the situation where Ca^{2+} release was induced by cADPR (Figure 8c). The most likely explanation for this differential effect of 6-thio cIDPR **3** is differences in protonation pattern and hydrogen bonding interactions. The amino group in cADPR can be a hydrogen bond donor or acceptor, whereas the oxygen group in cIDPR is a good hydrogen bond acceptor. Obviously, 6-thio cIDPR **3** competes more effectively with cIDPR for binding to the cADPR receptor.

Taken together, these results show that replacement of the keto moiety by a more hydrophobic thione does interfere with the functional consequences of binding of the ligand to its receptor. There is an obvious role in the C-6 group in antagonizing Ca^{2+} release, if not directly then through subtle conformational effects or other. It may well be that a change from a hypoxanthine to a mercaptopurine results in a different protonation pattern and hydrogen bonding interactions. The amino group in cADPR can be a hydrogen bond donor or acceptor, whereas the oxygen group in cIDPR is a good hydrogen bond acceptor, as opposed to the sulfur atom. A C2′-endo/*syn* conformation is obviously not the sole factor that governs agonist/antagonist activity, since all three nucleotides cADPR, cIDPR, and 6-thio cIDPR have the same global conformation, although one can suggest that a C3′-endo/*anti* conformation (as in *N*7-cIDPR and *N*7-cGDPR, Table 1) would most likely lead to inactive compounds. It is also well-known that the hydrophobicity of sulfur leads to differences in the associated water shell. Thus, it may be that differential arrangements of additional water molecules in the receptor binding site can influence the receptor machinery.

Although surprising, this is not the first time that Ca^{2+} release has been observed at higher concentrations. Indeed, we previously reported that the classical and widely used antagonists such as 8-bromo cADPR and 8-amino cADPR also show such unexpected agonist activity at high concentration.[20] One of the reasons invoked to explain this earlier unobserved effect is that there may be two previously unrecognized different sites of action, such as an additional lower affinity nucleotide binding site, which causes Ca^{2+} mobilization distinct from the high affinity cADPR site. This would not have been noticed with the parent cIDPR, since it only acts as a potent agonist, but is again revealed with the structurally related 6-thio cIDPR.

The C-6 position in cADPR may therefore be revealed as an attractive new site to imbue antagonistic properties in a cADPR analogue. Its proximity to the crucial N1−C1″ linkage makes it likely that structural modifications could lead to modulation of activity of cADPR binding proteins. As such, it will be of interest to develop new ways to approach the synthetic problem of C-6 modification. Although the present result has been achieved somewhat unexpectedly via a chemoenzymatic route, it seems most likely that greater flexibility in this regard will be offered by total synthetic approaches in the future.

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CONCLUSION

In summary, we present a synthetic focus upon enzymatic C-6 structural modifications with a view to their enzymatic incorporation into cADPR. The substituted *N*6-NAD^+ analogue 6-*N*Me-NAD^+, upon incubation with *Aplysia* cyclase, hydrolyzes to the linear product 6-*N*Me-ADPR, presumably because of steric hindrance and/or reduction of hydrogen bonding interaction with the enzyme. A high yielding route also generated another C-6 modified analogue, 6-thio NHD^+. Surprisingly, this analogue cyclizes at *N*1 in a similar manner to NAD^+, to give the novel fluorescent cADPR analogue 6-thio *N*1-cIDPR, although the parent oxo-congener NHD^+ cyclizes at *N*7. A mechanistic study implies a more complex explanation than simply a hydrogen bonding interaction, as the enzyme recognizes this thio derivative as a normal substrate, although it is structurally closer to NHD^+ than it is to NAD^+. Biological evaluation in permeabilized Jurkat T cells reveals that this compound is decreased in its agonist activity relative to cIDPR but possesses new antagonist activity against both cADPR- and cIDPR-induced Ca^{2+} release, showing that substitution of the oxygen of the hypoxanthine ring for sulfur interferes with the functional consequences of ligand binding to its receptor. Like two known classical antagonists, 6-thio cIDPR shows agonist activity at high concentrations. Antagonist activity in cADPR analogues has thus now been demonstrated in compounds substituted at the 8-position of the purine, the 3′-hydroxyl group of the "southern" ribose, and now for the first time in a compound with a C-6 structural modification. Both the fundamental causes of antagonism and the switch to agonism at higher concentration in some cases thus seem highly complex and seem to defy a simple explanation. The present study demonstrates, however, that future synthetic efforts to facilitate further C-6-structural modification should be a fruitful enterprise in elucidating cADPR SAR and defining useful new tools for dissection of its signaling pathway.

EXPERIMENTAL SECTION

General. All reagents and solvents were of commercial quality and were used without further purification unless described otherwise. Triethylamine and morpholine were dried over potassium hydroxide, distilled, and then stored over potassium hydroxide pellets. ADP-ribosyl cyclase was purified from the ovotestis of *Aplysia californica*.[67] H_2O was of Milli-Q quality. All ^1H, ^{13}C, and ^{31}P NMR spectra of final compounds were collected in D_2O on a JEOL Delta machine at 270 MHz (^1H) or 109 MHz (^{31}P) or on a Varian Mercury-vx system at 400 MHz (^1H) or 100 MHz (^{13}C). All ^1H and ^{13}C NMR assignments are based on gCOSY, gHMBC, gHMQC, and DEPT experiments. Abbreviations for splitting patterns are as follows: br, broad; s, singlet; d, doublet; t, triplet; m, multiplet. UV spectra were collected in aqueous solution on a Perkin-Elmer Lambda EZ 201 or Lambda 3B spectrophotometer. High resolution time-of-flight mass spectra were obtained on a Bruker Daltonics micrOTOF mass spectrometer using electrospray ionization (ESI). HPLC analyses were carried out on a Waters 2695 Alliance module equipped with a Waters 2996 photodiode array detector (210−350 nm). The chromatographic system consisted of a Hichrom guard column for HPLC and a Phenomenex Synergi 4 μm MAX-RP 80A column (150 × 4.60 mm), with elution at 1 mL/min with the following ion-pair buffer: 0.17% (m/v) cetrimide and 45% (v/v) phosphate buffer (pH 6.4) in MeOH. The purities of all phosphate containing compounds were determined by HPLC, and they were in all cases over 95%. All other compounds were analyzed using a Phenomenex Gemini column 5 μm C18 (150 mm × 4.6 mm), with elution at 1 mL/min with a MeCN/H_2O gradient (5−65% over 20 min). Preparative chromatography was performed on a Pharmacia Biotech Gradifrac system equipped with a peristaltic P-1 pump and a fixed wavelength UV-1 optical unit (280 nm). The following purification methods were employed: LiChroprep RP-18 equilibrated with 0.05 M TEAB buffer (pH 6.0−6.4), gradient of 0.05 M TEAB buffer against MeCN at 5 mL/min, Q-Sepharose washed with H_2O, gradient of 1 M TEAB buffer (pH 7.1−7.6) against H_2O at 5 mL/min, and AG MP-1 washed with H_2O, gradient 150 mM TFA against H_2O at 3 mL/min. Synthetic phosphates were assayed by an adaptation of the Briggs phosphate test.[68]

Computational Details. cADPR, *N*1-cIDPR, and 6-thio *N*1-cIDPR were built using Sybyl-X 1.1.1, and the volumes were calculated. The pK_a values were calculated using the Sparc online calculator http://sparc.chem.uga.edu/sparc/.

Pharmacology. Materials. Fluo-3 was purchased from Molecular Probes. Saponin and KH_2PO_4 were obtained from Fluka. ATP, creatine phosphate, EGTA, Tris, and NaCl were provided from Sigma Aldrich. $MgCI_2$, $CaCl_2$, and KCl were procured from Merck Chemicals. HEPES was purchased from Biomol. Creatine kinase was obtained from Roche. Culture medium reagents were supplied by Invitrogen or Biochrom.

Cell Culture. Jurkat T-lymphocytes (subclone JMP) were cultured as described previously[69] at 37 °C in the presence of 5% CO_2 in RPMI 1640 medium containing Glutamax I and HEPES (25 mM) and supplemented with 7.5% (v/v) NCS (newborn calf serum), 100 units/mL penicillin, and 100 μg/mL streptomycin.

Ca^{2+} Release Experiments in Permeabilized Cells. Permeabilized cells were prepared as described,[70] and the Ca^{2+} concentration was measured by the use of Fluo-3. In brief, cells were permeabilized in the presence of saponin (55 μg/mL) for 20 min in an intracellular buffer (20 mM HEPES, 110 mM KCI, 2 mM $MgCI_2$, 5 mM KH_2PO_4, 10 mM NaCI, pH 7.2) at 37 °C. An aliquot containing 1×10^8 cells was transferred to a cuvette, and fluorescence was measured in a Hitachi F-2000 spectrofluorometer (excitation 504 nm, emission 524 nm) at 37 °C in the presence of Fluo-3 (1 μM) with continuous stirring. Reuptake of Ca^{2+} into stores was achieved by addition of ATP (1 mM), creatine phosphate (20 mM), and creatine kinase (20 U/mL). At the end of each experiment, the free Ca^{2+} concentration was calibrated by addition of $CaCl_2$ and subsequently by addition of EGTA/Tris and calculated by using the following equation: $[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$ where F_{min} is the fluorescence intensity in the absence of Ca^{2+}, F_{max} is the fluorescence intensity of the Ca^{2+}-saturated indicator, F is the fluorescence during the measurement, and K_d is the dissociation constant of Fluo-3. K_d of Fluo-3 (503 nM) was determined on the basis of the calcium calibration buffer kit (Molecular Probes) for intracellular buffer containing 1 mM ATP, 20 mM creatine phosphate, and 20 U/mL creatine kinase at 37 °C, pH 7.06.

6-Chloroadenosine 2′,3′,5′-Triacetate (6). Compound **6** was synthesized according to a modified Vorbrüggen condensation. To a vigorously stirred solution of 6-chloropurine (500 mg, 3.23 mmol), β-D-ribofuranose 1,2,3,5-tetraacetate (926 mg, 2.91 mmol), and DBU (1.3 mL, 8.70 mmol) in dry MeCN (22 mL) was added TMSOTf (2.12 mL) at 0 °C under an argon atmosphere. The reaction mixture was heated at 60 °C for 1 h and carefully quenched by addition of a saturated solution of $NaHCO_3$ (100 mL). The crude compound was extracted with DCM (3 × 100 mL), and the organic layers were combined, dried over $MgSO_4$, and evaporated under reduced pressure. The yellow residue obtained was further purified by flash column chromatography, eluting with DCM−MeOH, 20:1, to give the desired compound as a yellow oil (960 mg, 77%). ^1H ($CDCl_3$, 270 MHz) δ 8.76 (s, 1H, H-8), 8.28 (s, 1H, H-2), 6.21 (d, 1H, $J_{1',2'}$ = 5.2 Hz, H-1′), 5.92 (dd, 1H, $J_{2',3'}$ = 5.4 Hz and $J_{2',1'}$ = 5.2 Hz, H-2′), 5.62 (dd, 1H, $J_{3',2'}$ = 5.4 Hz and $J_{3',4'}$ = 4.9 Hz, H-3′), 4.37 (m, 3H, H-4′ and H-5′), 2.14 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), and 2.07 (s, 3H, CH_3).

6-*N*-Methyladenosine 5′-Acetate. The title compound was synthesized by adaptation of a literature protocol.[71] To a suspension of 6-chloroadenosine triacetate **6** (800 mg, 1.94 mmol) and methylamine hydrochloride (573 mg, 8.42 mmol) in a mixture of DCM (20 mL) and ethanol (4.2 mL) was added triethylamine (3.8 mL). The resulting mixture was stirred at 60 °C overnight. TLC analysis indicated that the starting material was completely consumed and three more polar spots were given. The reaction mixture was transferred into a pressure tube

with triethylamine (4 mL), methylamine hydrochloride (500 mg, 7.35 mmol), and ethanol (4 mL). The mixture was heated at 60 °C for 6 h and left at room temperature for 2 days. TLC of the mixture indicated only one major spot at R_f = 0.25 (DCM−MeOH, 10:1). The solvent was removed and the crude compound was purified by flash column chromatography, eluting with DCM−MeOH, 30:1, to give a mixture of the desired compound and the methylamine hydrochloride. Pure title compound was obtained as a white solid by washing the resulting mixture with Milli-Q water (420 mg, 67%). ^{1}H (DMSO-d_6, 270 MHz) δ 8.35 (s, 1H, H-8), 8.24 (brs, 1H, H-2), 7.81 (brs, 1H, NH), 5.91 (d, 1H, $J_{1',2'}$ = 5.0 Hz, H-1′), 5.61 (m, 1H, 2′-OH), 5.42 (m, 1H, 3′-OH), 4.67 (m, 1H, H-2′), 4.31−4.18 (m, 4H, H-3′, H-4′, H-5′), 2.95 (brs, 3H, CH_3N), 1.95 (s, 3H, CH_3); HRMS (ES$^+$) calcd for $C_{13}H_{18}N_5O_5$ 324.1308 (MH)$^+$, found 324.1302.

6-*N*-Methyladenosine (7). A suspension of 6-*N*-methyladenosine 5′-acetate (400 mg, 1.23 mmol) in a saturated methanolic ammonia (50 mL) was stirred at room temperature for 3 h, after which the solvent was removed in vacuo. The residue obtained was purified by flash column chromatography, eluting with DCM−methanol, 10:1, to produce the title compound as a white solid (300 mg, 86%); mp 178−180 °C; ^{1}H (DMSO-d_6, 270 MHz, D_2O shake) δ 8.31 (s, 1H, H-8), 8.20 (brs, 1H, H-2), 5.85 (d, 1H, $J_{1',2'}$ = 6.4 Hz, H-1′), 4.57 (dd, 1H, $J_{2',1'}$ = 6.4 Hz and $J_{2',3}$ = 5.2 Hz, H-2′), 4.12 (dd, 1H, $J_{3',2'}$ = 5.2 Hz and $J_{3',4'}$ = 3.0 Hz, H-3′), 3.97 (app.q, 1H, $J_{4',5'a}$ = $J_{4',5'b}$ = $J_{4',3'}$ = 3.0 Hz, H-4′), 3.64 (dd, $J_{5'a,5'b}$ = 12.9 Hz and $J_{5'a,4'}$ = 3.0 Hz, 1H, H-5′a), 3.55 (dd, 1H, $J_{5'b,5'a}$ = 12.9 Hz and $J_{5'b,4'}$ = 3.0 Hz, H-5′b), and 2.94 (brs, 3H, CH_3N); HRMS (ES$^+$) calcd for [M + H]$^+$ $C_{11}H_{16}N_5O_4$ 282.1202 (MH$^+$), found 282.1201.

6-*N*-Methyladenosine 5′-Monophosphate (8a, 6-*N*-Methyl AMP). A suspension of 7 (110 mg, 0.39 mmol, dried in vacuo at 100 °C for 2 h) in triethyl phosphate (1.4 mL), was heated strongly with a heat gun for 5 min. To the resulting clear solution were added $POCl_3$ (0.3 mL, 3.20 mmol) and H_2O (1 μL) at 0 °C, and the mixture was stirred for 1 h. The reaction mixture was quenched by addition of ice (15 mL). The resulting solution was extracted with cold ethyl acetate (5 × 20 mL). The aqueous layer was neutralized with NaOH (5 M) and loaded onto a reverse phase column, eluting with a gradient of 0−30% MeCN against 0.05 M TEAB. Fractions containing the desired compound were pooled, evaporated and excess TEAB was coevaporated with MeOH (3×) to give a mixture of title compound **8a** (150 mg, 0.20 mmol, 51%) and the bis-phosphate **8b**. ^{1}H (D_2O, 270 MHz) δ 8.17 (s, 1H, H-8), 7.71 (s, 1H, H-2), 5.83 (d, 1H, $J_{1',2'}$ = 5.7 Hz, H-1′), 4.57 (m, 1H, H-2′), 4.37 (m, 1H, H-3′), 4.25 (m, 1H, H-4′), 4.03 (m, 2H, H-5′) and 2.78 (m, 3H, CH_3N); ^{31}P (D_2O, 109 MHz) δ 1.12 (s). Bisphosphate **8b**: ^{1}H (D_2O, 270 MHz) δ 8.13 (s, 1H, H-8), 7.81 (s, 1H, H-2), 6.06 (d, 1H, $J_{1',2'}$ = 4.5 Hz, H-1′), 5.25 (m, 1H, H-2′), 5.05 (m, 1H, H-3′), 4.75 (m, 1H, H-4′ half overlap with HOD signal), 4.55 (m, 2H, H-5′), and 2.80 (m, 3H, CH_3).

6-*N*-Methyladenosine 5′-Monophosphate Morpholidate (9). A suspension of 6-*N*-methyl-AMP and the bisphosphate as above **8a**/**8b** (0.20 mmol, calculated by ^{1}H NMR integration) in dry DMSO (0.9 mL) was evaporated with dry DMF (3 × 2 mL). To the residue were added in sequence triphenylphosphine (280 mg, 1.07 mmol), morpholine (0.15 mL, 1.72 mmol), and dipyridyl disulfide (235 mg, 1.07 mmol). The resulting yellow solution was stirred at room temperature for 4 h, after which a solution of sodium iodide in acetone (0.2 M, 15 mL) was added and the resulting precipitate was filtered and washed with acetone. The crude product was further purified by ion-exchange chromatography, eluting with a gradient of 0−50% 1 M TEAB against Milli-Q water. The solvent was evaporated in vacuo and excess TEAB was coevaporated with MeOH (3 times) to give the title compound as its triethylammonium salt (93 mg, 78%). HPLC, t_R = 2.8 min at 254 nm; UV (H_2O) λ_{max} 266.4 nm; ^{1}H (D_2O, 270 MHz) δ 8.23 (s, 1H, H-8), 7.98 (s, 1H, H-2), 5.93 (d, 1H, $J_{1',2'}$ = 4.9 Hz, H-1′), 4.68 (m, 1H, H-2′), 4.42 (m, 1H, H-3′), 4.26 (m, 1H, H-4′), 3.94 (m, 2H, H-5′), 3.44 (m, 4H, 2 × CH_2O), 2.91 (m, 3H, CH_3N), and 2.80 (m, 4H, 2 × CH_2N); ^{31}P (D_2O, 109 MHz) δ 8.10 (s); HRMS (ES$^+$) $C_{15}H_{24}N_6O_7P$ 431.1444 calcd for MH$^+$, found 431.1441.

6-*N*-Methyl nicotinamide Adenine Dinucleotide (6-*N*-Methyl NAD$^+$, 10). To a mixture of **9** (56 mg, 95 μmol), β-NMN$^+$ (55 mg, 165 μmol), and $MgSO_4$ (38 mg, 317 μmol) was added a solution of $MnCl_2$ in formamide (0.2 M, 1.18 mL). The resulting suspension was stirred at room temperature for 48 h under a nitrogen atmosphere, and the reaction mixture was quenched by dropwise addition of MeCN (2 mL). The yellow precipitate was filtered, washed with acetone, and dissolved in small amount of Milli-Q water. The aqueous solution was treated with Chelex 100 (sodium form) to remove any residual manganese and purified by reverse-phase chromatography, eluting with a gradient of 0.05 M TEAB against MeCN. The title compound was isolated as a glassy solid in the triethylammonium form (51 mg, 64%). HPLC, t_R = 3.4 min at 254 nm; UV (H_2O) λ_{max} 262.5 nm (ε/dm^3 mol^{-1} cm^{-1} 14 560); ^{1}H (D_2O, 400 MHz) δ 9.25 (s, 1H, H_N-2), 9.07 (d, 1H, $J_{6,5}$ = 5.9 Hz, H_N-6), 8.71 (d, 1H, $J_{4,5}$ = 8.2 Hz, H_N-4), 8.26 (s, 1H, H-8), 8.09 (dd, 1H, $J_{5,4}$ = 8.2 Hz and $J_{5,6}$ = 5.9 Hz, H_N-5), 7.97 (s, 1H, H-2), 5.99 (d, 1H, $J_{1'',2''}$ = 5.5 Hz, H-1″), 5.91 (d, 1H, $J_{1',2'}$ = 5.9 Hz, H-1′), 4.70 (dd, 1H, $J_{2',1'}$ = 5.9 Hz and $J_{2',3'}$ = 5.5 Hz, H-2′), 4.48 (m, 1H, H-4″), 4.44 (m, 2H, H-3″ and H-3′), 4.37 (m, 1H, H-2″), 4.32−4.16 (m, 5H, H-4′, H-5′, and H-5″), and 2.93 (CH_3N); ^{31}P (D_2O, 109 MHz) δ −10.73 (brs); HRMS (ES$^-$) calcd for $C_{22}H_{28}N_7O_{14}P_2$ 676.1169 [M − H]$^-$, found 676.1158.

6-*N*-Methyladenosine 5′-Diphosphate Ribose (6-*N*-Methyl ADPR, 11). To a solution of **10** (20 mg, 24 μmol) in HEPES buffer (25 mM, pH 7.4, 60 mL) was added *Aplysia* ADP-ribosyl cyclase (80 μL). The resulting solution was stirred at room temperature until RP-HPLC indicated that all the starting material had reacted. The solution was diluted, and then product was purified by ion-exchange chromatography, eluting with a gradient of 0−50% 1 M TEAB buffer against Milli-Q water. The appropriate fractions were collected, evaporated and excess TEAB was removed by coevaporating with MeOH (3 times) to give the compound as a glassy solid in its triethylammonium form (10 mg, 59%). HPLC, t_R = 11.8 min at 254 nm; UV (H_2O) λ_{max} 265.1 nm (ε/dm^3 mol^{-1} cm^{-1} 13 780); ^{1}H (D_2O, 270 MHz) δ 8.37 (s, 1H, H-8), 8.10 (s, 1H, H-2), 6.02 (d, 1H, $J_{1',2'}$ = 5.9 Hz, H-1′), 5.24 (d, 0.3H, $J_{1'',2''}$ = 4.0 Hz, H-1″$_\beta$), 5.14 (m, 0.7H, H-1″$_\alpha$), 4.70−3.94 (m, 10H, H-ribose), and 2.87 (m, 3H, CH_3N); ^{31}P (D_2O, 109 MHz) δ −10.6 (brs); HRMS (ES$^-$) calcd for $C_{16}H_{24}N_5O_{14}P_2$ 572.0795 [M − H]$^-$, found 572.0792.

2′,3′-*O*-Isopropylidene-6-(2,4-dinitrophenyl)thioinosine, 15. To a suspension of **13** (80 mg, 0.236 mmol) in dry MeCN (5 mL) were added triethylamine (197 μL, 1.416 mmol) and dinitrofluorobenzene (36 μL, 0.283 mmol). The resulting solution was stirred at room temperature for 1 h after the solvent was removed under reduced pressure and the residue obtained was purified by flash chromatography on silica gel (EtOAc/hexane, 1:1) to yield the desired product as a yellow foam (96 mg, 83%). HPLC, t_R = 14.6 min at 260 nm; ^{1}H (270 MHz, $CDCl_3$) δ 8.98 (d, 1H, J = 2.5 Hz, Ar-H), 8.65 (s, 1H, H-2), 8.38 (dd, 1H, J = 8.8 and 2.5 Hz, Ar-H), 8.16 (s, 1H, H-8), 7.97 (d, 1H, J = 8.8 Hz, Ar-H), 5.94 (d, 1H, $J_{1',2'}$ = 4.7 Hz, H-1′), 5.21−5.17 (m, 1H, H-2′), 5.12−5.08 (m, 1H, H-3′), 4.55−4.54 (m, 1H, H-4′), 3.98−3.76 (m, 2H, H-5′), 1.64 (s, 3H, CH_3), and 1.37 (s, 3H, CH_3); HRMS (ES$^+$) calcd for $C_{19}H_{19}N_6O_8S$ 4910.0980 (MH$^+$), found 491.0957; R_f = 0.3 (EtOAc/hexane, 6:4).

2′,3′-*O*-Isopropylidene-5′-*O*-di-*tert*-butylphosphoramidite-6-(2,4-dinitrophenyl)thioinosine, 16. To a solution of **15** (70 mg, 0.138 mmol) in dry DCM (3 mL) were added tetrazole (20 mg, 0.276 mmol) and *N*,*N*-diisopropyl-di-*tert*-butylphosphoramidite (66 μL, 0.208 mmol). The reaction mixture was stirred at room temperature for 1 h, after which time TLC analysis (hexane/EtOAc, 6:4) indicated conversion of starting material to a single phosphite. The mixture was cooled to −78 °C, and mCPBA (47 mg, 0.276 mmol) was added. After 20 min, 10% aqueous Na_2SO_3 (10 mL) was added and the mixture was warmed to room temperature. The organic layer was separated and washed with a saturated solution of $NaHCO_3$ (15 mL) and brine (15 mL), dried (Na_2SO_4), filtered, and evaporated under reduced pressure to leave an oil which was purified by column chromatography on silica gel (hexane/EtOAc, 1:1) to give the title compound **16** as a yellow oil (85 mg, 90%). HPLC, t_R = 12.1 min at 260 nm; ^{1}H (270 MHz, $CDCl_3$) δ 8.98 (d, 1H, J = 2.5 Hz, Ar-H), 8.74 (s, 1H, H-2), 8.35 (d, 1H, J = 2.5 Hz, Ar-H), 8.33 (s, 1H, H-8), 7.92 (d, 1H, J = 8.8 Hz, Ar-H), 6.23 (d, 1H, $J_{1',2'}$ = 2.7 Hz, H-1′), 5.33 (dd, 1H,

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$J_{2',3'}$ = 6.0 Hz and $J_{2',1'}$ = 2.7 Hz, H-2′), 5.04 (dd, 1H, $J_{3',2'}$ = 6.0 Hz and $J_{3',4'}$ = 2.4 Hz, H-3′), 4.55−4.54 (m, 1H, H-4′), 4.16−4.12 (m, 2H, H-5′), 2.03 (s, 3H, CH_3), 1.63 (s, 3H, CH_3), 1.44 (s, 9H, ^{t}Bu), and 1.40 (s, 9H, ^{t}Bu); ^{31}P (decoupled, 109 MHz, $CDCl_3$) δ −9.3 (s); HRMS (ES^+) calcd for $C_{27}H_{36}N_6O_{11}PS$ 683.1895 (MH^+), found 683.1871; R_f = 0.14 (hexane/EtOAc, 6:4).

2′,3′-*O*-Isopropylidene-5′-*O*-(di-*tert*-butylphosphoramidite)-6-thioinosine. 16 (80 mg, 0.116 mmol) was stirred in a solution of MeCN (10 mL) containing 10% (v/v) 2-mercaptoethanol (1 mL) and 1% DIPEA (0.1 mL) for 1 h. Water (15 mL) and DCM (30 mL) were then added. The organic layer was washed several times with water to remove the excess 2-mercaptoethanol. The organic phase was dried, filtered, and evaporated in vacuo to leave an oil which was purified by column chromatography on silica gel (DCM/acetone, 7:3) to give the title compound as a white waxy solid (45 mg, 75%). ^{1}H (270 MHz, $CDCl_3$) δ 8.38 (s, 1H, H-2), 8.36 (s, 1H, H-8), 6.14 (d, 1H, $J_{1',2'}$ = 2.2 Hz, H-1′), 5.52 (dd, 1H, $J_{2',3'}$ = 6.1 Hz and $J_{2',1'}$ = 2.1 Hz, H-2′), 5.04 (dd, 1H, $J_{3',2'}$ = 6.1 Hz and $J_{3',4'}$ = 2.5 Hz, H-3′), 4.56−4.54 (m, 1H, H-4′), 4.23−4.17 (m, 2H, H-5′), 1.63 (s, 3H, CH_3), 1.44 (s, 18H, 2 × ^{t}Bu), and 1.42 (s, 3H, CH_3); ^{31}P (decoupled, 109 MHz, $CDCl_3$) δ −9.3 (s); HRMS (ES^+) calcd for $C_{21}H_{34}N_4O_7PS$ 517.1880 (MH^+), found 517.1863.

6-Thioinosine 5′-Monophosphate (6-SH IMP, 14). 2′,3′-*O*-Isopropylidene-5′-*O*-(di-*tert*-butylphosphoramidite)-6-thioinosine (35 mg, 0.067 mmol) was stirred in a 50% aqueous TFA solution (2 mL) at room temperature for 24 h. The solvent was removed under reduced pressure and coevaporated several times with MeOH to remove any residual TFA. The residue was dissolved in water and worked up with EtOAc (2 × 10 mL). The aqueous layer was evaporated to dryness to produce the desired monophosphate **14** as a glassy solid (21 mg, 86%). HPLC, t_R = 2.9 min at 320 nm; ^{1}H (270 MHz, D_2O) δ 8.94 (s, 1H, H-2), 8.23 (s, 1H, H-8), 6.05 (d, 1H, $J_{1',2'}$ = 3.9 Hz, H-1′), 4.60 (dd, 1H, $J_{2',3'}$ = 4.7 Hz and $J_{2',1'}$ = 3.9 Hz, H-2′), 4.41 (app t, 1H, J = 5.0 Hz, H-3′), 4.27 (dd, 1H, $J_{4',3'}$ = 5.0 Hz and $J_{4',5'a}$ = 2.5 Hz, H-4′), 4.17 (ddd, 1H, $J_{5'a,5'b}$ = 11.8 Hz, $J_{5'a,P}$ = 4.4 Hz, and $J_{5''a,4}$ = 2.5 Hz, H-5′a), and 4.05 (ddd, 1H, $J_{5'a,5'b}$ = 11.8 Hz, $J_{5'a,P}$ = 5.5 Hz, and $J_{5''a,4}$ = 2.8 Hz, H-5′b); ^{31}P (decoupled, 109 MHz, D_2O) δ 0.51 (s); HRMS (ES^+) calcd for $C_{10}H_{14}N_4O_7PS$ 365.0315 (MH^+), found 365.0310.

Nicotinamide 6-Mercaptopurine 5′-Dinucleotide (6-SH NHD$^+$, 17). 14 (13 mg, 0.036 mmol) was dissolved in dry DMSO (1 mL) and coevaporated with dry DMF (5 × 3 mL). The residue was dissolved in DMSO (400 μL), to which were added morpholine (16 μL, 0.187 mmol), dipyridyl disulfide (19 mg, 0.089 mmol), and triphenylphosphine (24 mg, 0.089 mmol), at which point the solution became bright yellow. It was stirred for 1 h at room temperature, after which HPLC analysis showed completion of the reaction. Precipitation of the product occurred by dropwise addition of a solution of NaI in acetone (0.1M, 8 mL). The resulting precipitate was filtered, washed with acetone, and dried (δ_P 6.7 ppm and HPLC, t_R = 5.9 min at 320 nm). It was then reacted with β-NMN$^+$ (17 mg, 0.050 mmol) and $MgSO_4$ (11 mg, 0.092 mmol) in a 0.2 M solution of $MnCl_2$ in formamide (0.35 mL) at room temperature overnight, after which HPLC analysis showed completion of the reaction ($t_{R(\beta\text{-NMN})}$ = 2.1 min and $t_{R(6\text{-SH-NHD})}$ = 7 min). Precipitation occurred by dropwise addition of MeCN. The precipitate was filtered, dissolved in Milli-Q, and applied to a reverse-phase column, eluting with a gradient of MeCN in 0.05 M TEAB. Further treatment with Chelex 100 to remove any paramagnetic particles afforded the desired dinucleotide **17** as a glassy solid (10 mg, 15.2 μmol, 31% from 6-thio IMP). HPLC, t_R = 6.9 min at 320 nm; ^{1}H (270 MHz, D_2O) δ 9.17 (s, 1H, H_N2), 8.96 (d, 1H, $J_{6,5}$ = 6.1 Hz, H_N6), 8.65 (d, 1H, $J_{4,5}$ = 7.7 Hz, H_N4), 8.30 (s, 1H, H-2), 8.08 (s, 1H, H-8), 8.02−7.96 (m, 1H, H_N5), 6.03 (d, 1H, $J_{1'',2''}$ = 6.5 Hz, H-1″), 5.86 (d, 1H, $J_{1',2'}$ = 5.8 Hz, H-1′), 4.65 (app t, 1H, $J_{2',1'}$ = $J_{2',3'}$ = 5.5 Hz, H-2′), and 4.41−4.07 (m, 9H, H_{sugar}); ^{31}P (decoupled, 109 MHz, D_2O) δ −10.5 (d, J = 19.7) and −10.8 (d, J = 19.7); HRMS (ES^+) calcd for $C_{21}H_{27}N_6O_{14}P_2S$ 681.0776 (MH^+), found 681.0752; UV (H_2O, pH 7.3) λ_{max} 322 nm (ε/dm^3 mol^{-1} cm^{-1} 20 320).

Cyclic 6-Thioinosine 5′-Diphosphate Ribose (6-Thio cIDPR, 3). **17** (13.5 μmol) was incubated with *Aplysia* cyclase (150 μL) in a 0.1 M $NaHCO_3$ buffer (30 mL, pH 7.6) at room temperature. After 30 h, HPLC analysis showed total consumption of starting material and formation of a new large peak at 11 min. The water was evaporated to a minimum, and the residue was applied on a RP-18 column, eluting with a gradient of MeCN in 0.05 M TEAB (0−65% over 300 mL). The product came through with 18% MeCN. The appropriate fractions were collected and evaporated under reduced pressure. The residue was passed through a small Chelex column previously washed with Milli-Q water to bring the pH down to 8. The product was washed off with Milli-Q water. It was then lyophilized to afford the desired cyclic dinucleotide as its sodium salt (7 μmol, 52%). HPLC, t_R = 10.7 min at 320 nm; ^{1}H (270 MHz, D_2O) δ 9.32 (s, 1H, H-2), 8.29 (s, 1H, H-8), 6.65 (br s, 1H, H-1″), 5.99 (d, 1H, $J_{1',2'}$ = 6.3 Hz, H-1′), 5.20 (dd, 1H, $J_{2',1'}$ = 6.3 Hz and $J_{2',3'}$ = 5.0 Hz, H-2′), 4.64 (dd, 1H, $J_{3',2'}$ = 5.0 Hz and $J_{3',4'}$ = 2.2 Hz, H-3′), and 4.59−4.07 (m, 8H, H-4′, H-5′, H-2″, H-3″, H-4″, and H-5″); ^{31}P (decoupled, 109 MHz, D_2O) δ −9.2 (d, AB system, J = 11.8 Hz), −10.5 (d, AB system, J = 11.8 Hz); HRMS (ES^-) calcd for $C_{15}H_{19}N_4O_{13}P_2S$ 557.0150 (MH^-), found 557.0161; UV (H_2O, pH 7.2). λ_{max} 322 nm (ε/dm^3 mol^{-1} cm^{-1} 18 600).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full NMR characterization spectra, fluorescence spectrum, HPLC traces, computational data for target compound **3**, and ^{13}C NMR data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

cADPR, cyclic adenosine 5′-diphosphoribose; cIDPR, cyclic inosine 5′-diphosphoribose; cADPcR, cyclic adenosine 5′-diphosphocarbocyclic ribose; cIDPRE, N^{1-}[(2″-*O*-phosphoryl-ethoxy)methyl]-5′-*O*-phosphorylinosine 2″,5′-cyclic pyrophosphate; NAD^+, nicotinamide adenosine 5′-dinucleotide; 6-NMe NAD^+, 6-*N*-Methyl nicotinamide adenosine 5′-dinucleotide; NGD^+, nicotinamide guanosine 5′-dinucleotide; NHD^+, nicotinamide hypoxanthine 5′-dinucleotide; 6-thio NHD^+, nicotinamide 6-mercaptopurine 5′-dinucleotide; ADPRC, ADP-ribosyl cyclase; TEP, triethylphosphate; β-NMN^+, β-nicotinamide 5′-mononucleotide; CDI, carbonyldiimidazole; TEAB, triethylammonium bicarbonate; IMP, inosine 5′-monophosphate; ribo-2′-F-NAD^+, ribosyl-2′-fluoro-2′-deoxy-nicotinamide adenine dinucleotide

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