Structural Determinants for N1/N7 Cyclization of Nicotinamide Hypoxanthine 5′-Dinucleotide (NHD^+) Derivatives by ADP-Ribosyl Cyclase from *Aplysia californica*: Ca^{2+}-Mobilizing Activity of 8-Substituted Cyclic Inosine 5′-Diphosphoribose Analogues in T-Lymphocytes

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A series of nicotinamide hypoxanthine 5′-dinucleotide (NHD^+) analogues modified at C-8 (**2**−**5**) and 7-deaza-NHD^+ were synthesized, and cyclization in the presence of *Aplysia* ADP-ribosyl cyclase was studied. All 8-substituted NHD^+ analogues were converted into their N1-cyclic forms by the enzyme, while in contrast, 7-deaza-NHD^+ **17** was hydrolyzed into 7-deazainosine 5′-diphosphoribose (7-deaza-IDPR) **25**. Correlations are made showing that the conformation of the NHD^+ substrate is the key to successful cyclization. The pharmacological activities of these novel cIDPR derivatives were evaluated in both permeabilized and intact Jurkat T-lymphocytes. The results show that in permeabilized cells both 8-iodo **1g** and 8-N_3-*N*1-cIDPR **1d** have an activity comparable to that of cADPR, while 8-iodo **1g** and 8-phenyl-*N*1-cIDPR **1c** have a small but significant effect in intact cells and can therefore be regarded as membrane-permeant; thus, cIDPR derivatives are emerging as important novel biological tools to study cADPR-mediated Ca^{2+} release in T-cells.

Introduction

Ca^{2+} is a ubiquitous intracellular messenger that controls a diverse range of cellular functions and plays a pivotal role in processes such as fertilization, gene transcription, muscle contraction, cell proliferation, and secretion of bioactive compounds. These processes occur despite the fact that in other circumstances Ca^{2+} is highly toxic and induces programmed cell death (apoptosis).[1,2] The release of Ca^{2+} from internal stores is mediated through three second messengers: D-*myo*-inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$), nicotinic acid 5′-adenine dinucleotide 2′-phosphate (NAADP), and cyclic adenosine 5′-diphosphate ribose (cADPR[a]). While $Ins(1,4,5)P_3$ acts on the $Ins(1,4,5)P_3$ receptor, pharmacological studies have revealed that cADPR elicits Ca^{2+} release through the ryanodine receptor, though whether this happens by direct receptor binding or via a binding protein remains unclear.[3−6] Over the years a number of cADPR analogues have been synthesized and their biological activities examined in order to characterize the cADPR signaling pathway and identify the binding protein.[7−9]

Hydrolysis of cADPR at the unstable N1 glycosidic bond linkage to give ADP-ribose occurs both under neutral aqueous conditions and, rapidly, in the physiological conditions of the cell.[10,11] This chemical and biological instability has limited studies of the physiological role of cADPR to some extent, and it was thus recognized that cADPR analogues resistant to both chemical and enzymatic hydrolysis were required. The enzyme ADP-ribosyl cyclase (ADPRC) isolated from *Aplysia californica* mainly exerts a cyclase activity (though NAD^+-glycohydrolase has been described too)[12] and has been used extensively to convert NAD^+ and NAD^+ derivatives into cADPR and the corresponding cADPR analogues, respectively.[10,13,14] While NAD^+ and its derivatives are exclusively cyclized at the N1 position by ADPRC, enzymatic cyclization of NHD^+ takes place at N7, leading to biologically inactive *N*7-cIDPR (Figure 1).[15,16]

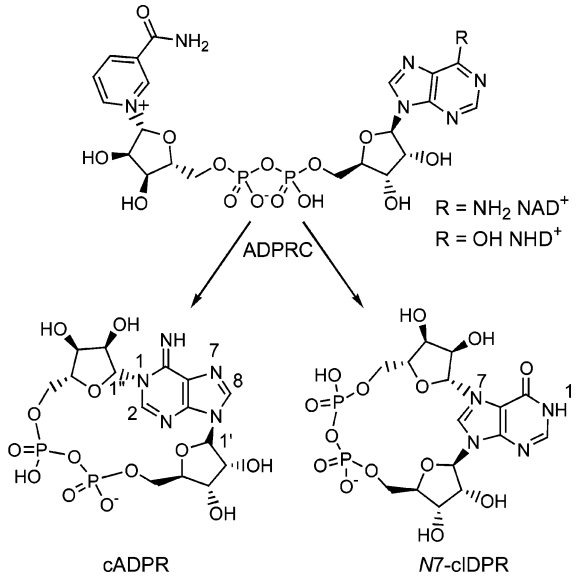

Figure 1. Cyclization behavior of NAD^+ and NHD^+ by ADPRC.

Because of this aberrant cyclization behavior of NHD^+, *N*1-cIDPR analogues have previously been accessible only by total chemical synthesis.[17−20] Recently, we have shown that placement of a bromo substituent at the 8 position of NHD^+ restores

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[a] Abbreviations: cADPR, cyclic adenosine 5′-diphosphoribose; cIDPR, cyclic inosine 5′-diphosphoribose; cIDPRE, N^1-[(2″-*O*-phosphorylethoxy)-methyl]-5′-*O*-phosphorylinosine 2″,5′-cyclicpyrophosphate; NAD^+, nicotinamide adenosine 5′-dinucleotide; NGD^+, nicotinamide guanosine 5′-dinucleotide; NHD^+, nicotinamide hypoxanthine 5′-dinucleotide; ADPRC, ADP-ribosyl cyclase; TEP, triethyl phosphate; β-NMN, β-nicotinamide 5′-mononucleotide; CDI, carbonyldiimidazole; TEAB, triethylammonium bicarbonate; IMP, inosine monophosphate; TDA-1, tris[2-(2-methoxyethoxy)ethyl]amine.

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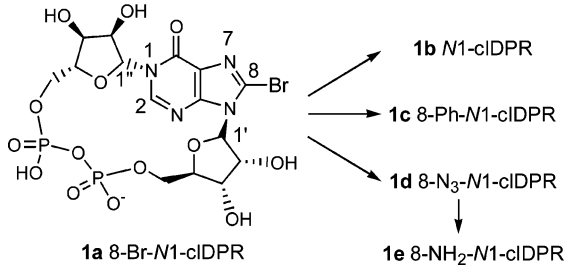


Figure 2. Outline of the synthesis of 8-substituted *N*1-cIDPR analogues directly from 8-bromo-*N*1-cIDPR **1a**.

N1 as the preferred site of cyclization and also confers stability to chemical hydrolysis at neutral pH and elevated temperature.[21] This property was exploited to generate a series of four analogues from the synthetically versatile parent compound (Figure 2).[22] Pharmacological investigation of *N*1-cIDPR **1b** itself has revealed, interestingly, that it is equipotent to cADPR in the release of Ca^{2+} in permeabilized T-cells illustrating the potential of this novel class of analogue.

Current synthetic efforts are directed toward the preparation of further structural analogues of *N*1-cIDPR as novel pharmacological tools for the investigation of cADPR pharmacology. To use the chemoenzymatic strategy on *N*1-cIDPR derivatives to its full potential, it is imperative to understand the structural requirements for N1 cyclization of NHD^+-based ADPRC substrates. Factors influencing the cyclization behavior of structurally different NHD^+ derivatives include the orientation of the hypoxanthine nucleobase in the ADPRC binding site, the respective nucleophilicity of N1/N7, steric bulk at the 8-position, and the conformational equilibrium for rotation about the glycosidic bond. We report here the synthesis of a series of NHD^+ derivatives and describe and analyze their cyclization behavior. We demonstrate that our chemoenzymatic approach provides reliable synthetic access to a structurally diverse family of novel *N*1-cIDPR analogues. We have characterized these analogues pharmacologically and report herein on their Ca^{2+}-mobilizing activity in human T-cells.

Results and Discussion

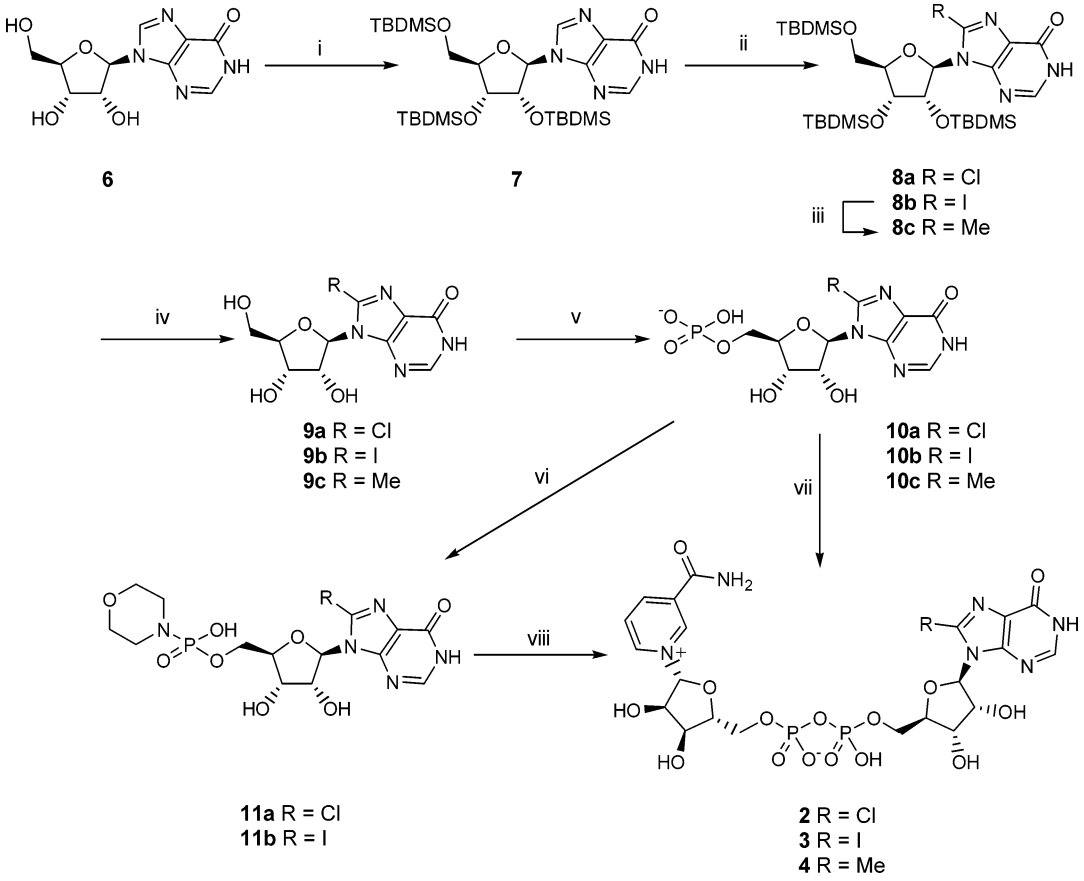
Synthesis of the 8-Chloro-, 8-Iodo-, and 8-Methyl-NHD^+ Analogues. The syntheses of 8-chloro-, 8-iodo-, and 8-methyl-NHD^+ (**2**, **3**, and **4**, respectively) are summarized in Scheme 1. Commercially available inosine **6** was first fully protected with TBDMS groups in 95% yield.[23] A lithio anion at C-8 was then generated with LDA and quenched with toluenesulfonyl chloride[24] or molecular iodine to give the 8-chloro **8a** or 8-iodo **8b** products in 73% and 86% yields, respectively. A Stille coupling of the 8-iodo analogue in the presence of $Pd(PPh_3)_4$ and tetramethyltin afforded the methyl derivative **8c** in 96% yield.[25] Cleavage of the silyl protecting groups with TBAF in THF provided the 8-substituted inosines **9a**−**c**, which were then selectively phosphorylated on the 5′-hydroxyl using $POCl_3$, triethyl phosphate, and water mixture.[26] Interestingly, chromatography of 8-Cl-IMP **10a** on a standard Q Sepharose ion-exchange resin led to cleavage of the glycosidic bond, and under these conditions the free nucleobase 8-Cl hypoxanthine was isolated as the sole product. This destabilizing effect of the electron-withdrawing chloro substituent at position 8 on the glycosidic bond is in marked contrast to the stability previously observed for the 8-bromo congener 8-Br IMP, which could be purified without difficulty on Q Sepharose.[22] Purification of the 8-substituted inosine monophosphates was achieved using reverse-phase Gradifrac chromatography, eluted with a gradient of 0.05M TEAB against MeCN to yield the desired monophosphates **10a**−**c** as their triethylammonium salts. In the following step, to construct the nucleotide, we sought to apply the phosphoramidate approach, which involves the coupling of a monophosphate with a nucleotide phosphoromorpholidate in the presence of a Lewis acid catalyst.[27] This method requires the activation of the appropriate nucleotide monophosphate to its corresponding phosphoromorpholidate *prior* to the coupling reaction. Therefore, both the 8-chloro- and 8-iodo-IMP (**10a** and **10b**) were activated as their respective phosphoromorpholidates by treatment with morpholine, dipyridyl disulfide, and triphenylphosphine.[28,29] The reactions proceeded to completion in about an hour as indicated by ^{31}P NMR spectroscopy (δ_P 6 ppm); the 8-chloro-IMP/8-iodo-IMP morpholidates (**11a** and **11b**) were then precipitated as their sodium salts and were subsequently reacted with β-NMN and $MnCl_2$ in formamide without further purification. The reaction mixtures were stopped after the starting morpholidates had been consumed and then purified by reverse-phase chromatography to yield the desired dinucleotides 8-Cl-NHD^+ **2** and 8-I-NHD^+ **3** as their triethylammonium salts.

The triethylammonium salt of 8-methylinosine 5′-monophosphate **10c**, however, proved to be insoluble in the reaction solvent DMSO, and efficient conversion to the free acid was not practical, with desalination leading to partial degradation of the monophosphate. An alternative method was thus employed for the synthesis of 8-methyl-NHD^+ **4** with a one-pot procedure involving the direct condensation of **10c** and β-NMN in the presence of carbonyldiimidazole.[30,31] Thus, β-NMN was first treated with an excess of carbonyldiimidazole to provide an activated phosphoroimidazolium intermediate, which was confirmed by ^{31}P NMR spectroscopy with the appearance of a new singlet at δ_P −9 ppm. After the remaining CDI was quenched, the phosphoroimidazolium was treated with 8-methyl-IMP **10c** to form 8-methyl-NHD^+ **4** after purification by ion-exchange chromatography on a Q Sepharose column.

Synthesis of 8-Amino NHD^+. We previously reported the synthesis of 8-amino-*N*1-cIDPR directly from the 8-bromo-*N*1-cIDPR **1** via an 8-azido intermediate.[22] To further assess the influence of an electron-donating group in positon 8 on cyclization, as well as for comparison of the product obtained from the cyclization to the 8-amino-*N*1-cIDPR previously synthesized from an alternative route, 8-amino-NHD^+ was conceived as a valuable target in its own right. The synthesis of 8-amino-NHD^+ **5** (Scheme 2) commenced with the fully acetylated inosine **12**, which was brominated in a saturated Br_2/Na_2HPO_4 solution (pH 7)[32] to give the 8-bromoinosine derivative **13** in 84% yield. Removal of the acetate protecting group followed by nucleophilic displacement of the 8-bromo group with sodium azide produced 8-azidoinosine **14b**, which was then selectively phosphorylated at the 5′-hydroxyl position under our standard conditions ($POCl_3$, TEP, and water) to yield 8-N_3-IMP **15a**. The azido compound was then converted into 8-amino-IMP **15b** by treatment with dithiothreitol in 0.05 M TEAB, pH 8.5, overnight. Evidence of the successful reduction of the azido group to the amino group was most clearly demonstrated by the shift in the UV spectrum from λ_{max} = 275.8 nm to λ_{max} = 264.0 nm. Activation of 8-amino-IMP as its phosphoromorpholidate **16**, followed by coupling with β-NMN and $MnCl_2$ in formamide delivered the desired 8-amino NHD^+ **5** in good yield.

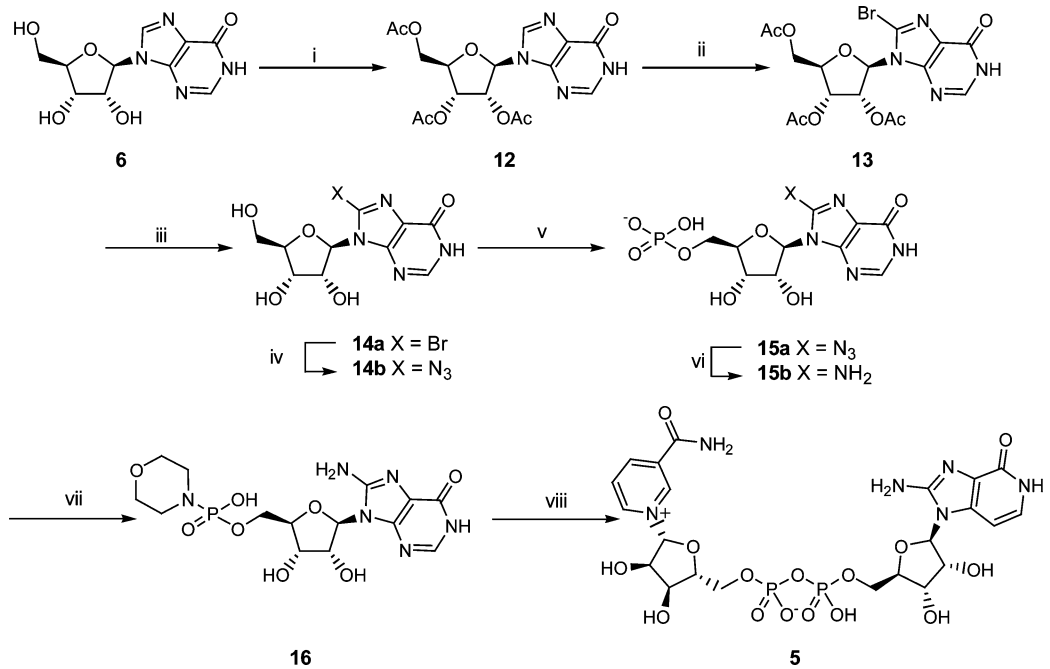
Synthesis of 7-Deaza-NHD^+. A key analogue for our investigations into the cyclase reaction was 7-deaza-NHD^+ **17**. The preferred conformation of **17** was expected to be anti, as in the case of NHD^+, while the formal removal of the ring

Scheme 1. Preparation of 8-X-NHD$^+$ (X = Cl, I, and Me)[a]



[a] Reagents and conditions: (i) TBDMSCl, imidazole, DMF, room temp, o/n; (ii) LDA, THF, −78 °C, 2 h, then I_2 or TsCl, −78 °C, 1 h; (iii) $Pd(PPh_3)_4$, $SnMe_4$, DMF, 100 °C, o/n; (iv) 1 M TBAF in THF, room temp, o/n; (v) $POCl_3$, TEP, 0 °C, 1 h, then H_2O, 0 °C to room temp, 15 min; (vi) morpholine, dipyridyl disulfide, PPh_3, room temp, then 0.1 M NaI in acetone, room temp, 1 h; (vii) carbonyldiimidazole, β-NMN, room temp, 4 days; (viii) β-NMN, $MgSO_4$, 0.2 M $MnCl_2$ in formamide, room temp, o/n.

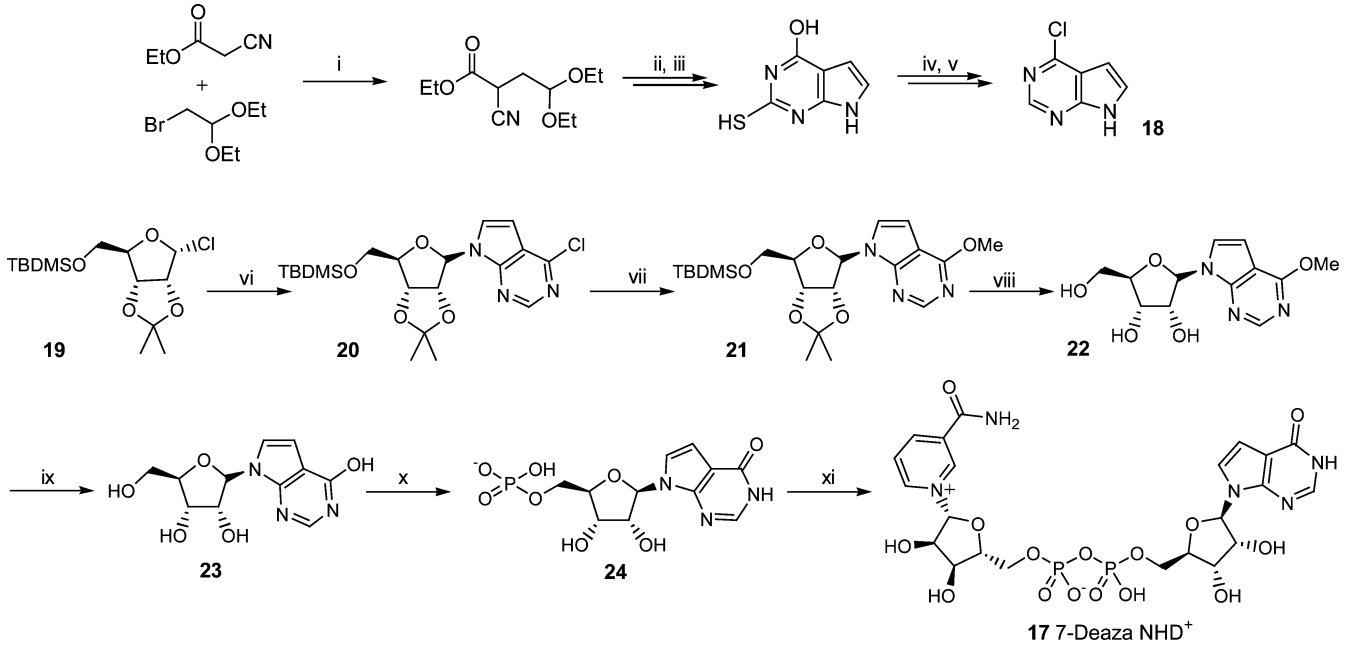
Scheme 2. Preparation of 8-Amino-NHD$^+$ [a]



[a] Reagents and conditions: (i) Ac_2O, pyridine, room temp, 1h; (ii) bromine, 10% aqueous Na_2HPO_4, dioxane, room temp, 5 days; (iii) methanolic ammonia, room temp, o/n; (iv) NaN_3, DMSO, 70 °C, 2 days; (v) $POCl_3$, TEP, H_2O, 0 °C, 1 h; (vi) dithiothreitol, 0.05 M TEAB, room temp, o/n; (vii) morpholine, dipyridyl disulfide, PPh_3, DMSO, room temp, 1 h; (viii) β-NMN, $MgSO_4$, 0.2 M $MnCl_2$ in formamide, room temp, o/n.

nitrogen in position 7 of **17** made the N7 cyclization favored by this conformation impossible. Therefore, **17** represented the perfect tool to investigate whether in those cases where the anti conformation was preferred but N7 cyclization was impossible

Scheme 3. Synthesis of 7-Deaza-NHD⁺ [a]



[a] Reagents and conditions: (i) K_2CO_3, NaI, reflux, 5 h; (ii) thiourea, NaOEt, EtOH, reflux, 6 h; (iii) 0.2 M HCl, room temp, o/n; (iv) Raney nickel, reflux, 6 h; (v) $POCl_3$, reflux, 2 h; (vi) **18**, KOH, TDA-1, HMPT, MeCN, room temp, 4h; (vii) 1 M NaOMe in MeOH, reflux, 6 h; (viii) 75% aqueous TFA, room temp, 30 min; (ix) TMSCl, NaI, MeCN, reflux, 4 h; (x) $POCl_3$, TEP, H_2O, 1 h, 0 °C, then ice/water, 15 min, room temp; (xi) β-NMN, CDI, TEA, DMF, 4 days, room temp.

the enzyme was capable of realigning the substrate in the correct syn conformation for N1 cyclization. If this was indeed the case, we expected 7-deaza-NHD⁺ to show a cyclization behavior different from that of its parent dinucleotide NHD⁺ but potentially similar to that of 7-deaza-NAD⁺ (i.e., to cyclize at N1). To synthesize 7-deaza-NHD⁺, it was first necessary to synthesize the nucleobase **18**. This was achieved in five steps from ethyl cyanoacetate and bromoacetaldehyde diethylacetyl by the method of Davoll and Seela (Scheme 3).[33,34]

The synthesis of the nucleoside then required the regio- and stereospecific glycosylation of the nucleobase with an appropriate α-halogenose to deliver the protected nucleoside intermediate. 1-Chloro-2,3-*O*-isopropylidene-5-*O*-(*tert*-butyl)dimethylsilyl-α-D-ribofuranose **19** has been reported as a reagent for the preparation of anomerically pure ribonucleosides without the problem of neighboring group participation.[35,36] Thus, treatment of 1 equiv of nucleobase **18** with KOH and TDA-1 with 1 equiv of 1-chlororibofuranose **19** gave the desired β-configurated ribonucleoside **20** in 65% yield (Scheme 3). The cryptand TDA-1 was used to increase the nucleophilicity of the nucleobase, thus allowing the use of equimolar quantities of reagents, as reported in the literature.[37] Further conversion of the chloro group into a methoxy group and then deprotection of the TBDMS and isopropylidene groups by treatment with a 75% aqueous TFA solution and subsequent cleavage of the methyl ether afforded the desired 7-deazainosine **23** in a 10% overall yield over 12 steps. Access to the linear dinucleotide 7-deaza-NHD⁺ **17** was then achieved as detailed above with selective phosphorylation at the 5′-hydroxyl followed by a CDI coupling, delivering after purification on Q Sepharose the desired dinucleotide **17** as its triethylammonium salt.

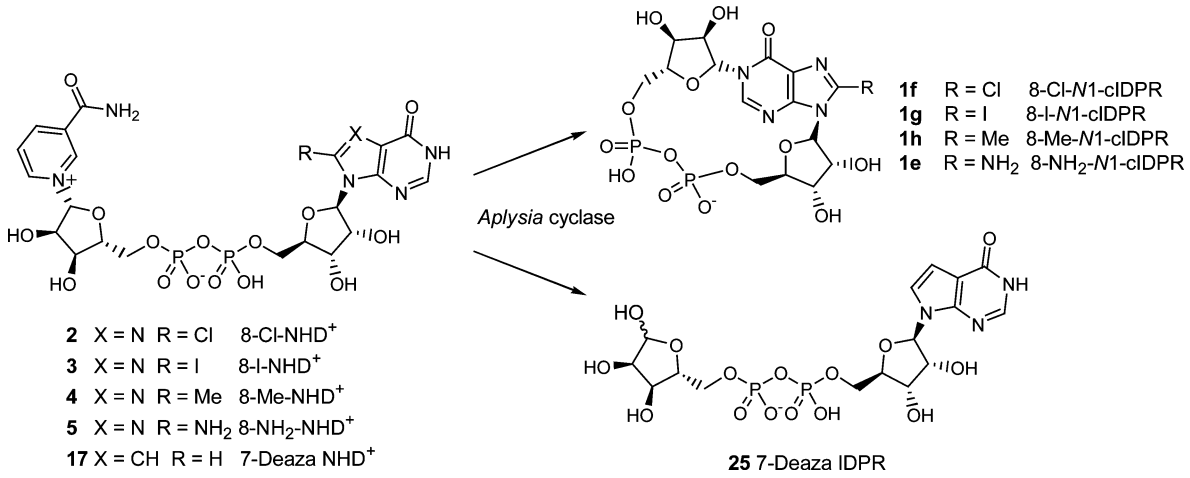
Cyclization Behavior. The four linear NHD⁺ analogues **2**−**5** were incubated with ADP-ribosyl cyclase from *Aplysia californica* to assess the influence of the 8-substituent on the site of cyclization (Scheme 4). We reasoned that an electron-donating substituent at C-8, e.g., CH_3 or NH_2, would enhance nucleophilicity at N7 and might therefore redirect cyclization to this site. In practice, however, all four NHD⁺ derivatives underwent smooth cyclization exclusively at N1 (Scheme 4). This finding suggests that the position of cyclization is independent of the electronic property of the C-8 substituent. It may well be that the steric demands of the C-8 substituents direct cyclization away from the neighboring N7 position.

The structures of the cyclic dinucleotides **1e**−**h** were unambiguously confirmed by mass spectrometry and 2D NMR spectroscopy. The gHMBC experiments revealed crucial cross-peaks for the three-bond coupling of the H-2 proton of the nucleobase with the anomeric carbon of the northern ribose and for the three-bond coupling of the anomeric proton of the northern ribose with C-2 of the nucleobase. Both cross-peaks are indicative of the N1/C-1″ bond characteristic of N1-cyclized dinucleotides. The 8-amino cIDPR **1e** obtained was in every aspect identical with the product previously synthesized by direct transformation of 8-bromo-*N*1-cIDPR **1a**.[22]

In contrast to the 8-substituted NHD⁺ analogues, when 7-deaza-NHD⁺ **17** was incubated with ADPRC, the product obtained from the enzymatic reaction was the linear dinucleotide 7-deaza-IDPR **24** (Scheme 4), as confirmed by the molecular ion of 558 obtained in the electrospray mass spectrum. This is in accord with the mass of a linear dinucleotide rather than the cyclic 7-deaza-cIDPR because this differs by 17 mass units for the additional hydroxyl group. The ^{1}H NMR spectrum also showed the presence of two doublets at 5.28 and 5.16 ppm indicating the β and α configurations of the anomeric protons of the terminal ribose unit. This result was intriguing because it has been reported that the corresponding similarly modified adenine derivative 7-deaza-NAD⁺ does cyclize readily into 7-deaza-cADPR.[13,38,39]

Additional control experiments were carried out to investigate the possibility that 7-deaza-NHD⁺ **17** may not bind to ADPRC at all and may simply be hydrolyzed chemically under the conditions of the cyclization reaction. However, when incubated with HEPES buffer (pH 7.4) alone, dinucleotide **17** was completely stable. This result demonstrates that hydrolysis of

Scheme 4[a]



[a] Reagents and conditions: *Aplysia californica*, 25 mM HEPES, pH 7.4, room temp.

Table 1. ^{1}H NMR Chemical Shifts (δ) of 8-X-IMP Analogues in D_2O

	IMP derivatives[a]						
	10a	**10b**	**10c**	**15b**	8-Br-IMP[b]	**24**	IMP
H-2	8.19	8.11	8.11	7.90	8.13	7.98	8.12
H-1′	6.10 (5.84)	6.02 (5.76)	5.95 (5.78)	5.93 (5.71)	6.08 (5.96)	6.17 (6.04)	6.05 (6.06)
H-2′	5.18 (5.06)	5.32 (5.08)	4.97 (4.90)	4.67 (4.59)	5.24 (5.18)	4.58 (4.53)	4.69 (4.70)
H-3′	4.65	4.63	4.49	4.67	5.24	4.58	4.69
H-4′	4.29(	4.27	4.14	4.25	4.25	4.29	4.34
$\Delta_{1'-2'}$[c]	0.92 (0.78)	0.70 (0.64)	0.98 (0.88)	1.26 (1.12)	0.84 (0.74)	1.59 (1.51)	1.36 (1.36)
conf[d]	syn	syn	syn	anti	syn	anti	anti

[a] The numbers in parentheses represent the resonance values for the corresponding NHD^+ analogues. [b] Data obtained from a previous publication.[22] [c] Difference in chemical shifts between H-1′ and H-2′. [d] Favored glycosidic bond conformation.

7-deaza-NHD^+ **17** requires the presence of ADPRC. Second, when 7-deaza-NHD^+ was co-incubated with ADPRC and a cyclizable substrate, such as NAD^+, cyclization of NAD^+ into cADPR occurred at a much slower rate (data not shown). Indeed, preliminary kinetic data for the enzymatic inhibition of ADPRC by 7-deaza-NHD^+ showed that at 50 μM in HEPES buffer (pH 7.4) and 25 °C the reaction rate is roughly 30 times slower than that of NAD^+ and *Aplysia* cyclase alone under the same conditions. Taken together, these findings support the interpretation that 7-deaza-NHD^+ is indeed recognized by the enzyme but unusually is hydrolyzed rather than cyclized. Therefore, **17** could potentially be used as a template to design inhibitors of ADPRC.

Conformational Analysis. It is well-known that when unsubstituted at C-8, purine nucleosides and nucleotides favor an anti glycosyl bond orientation[40] (e.g., adenosine, inosine, guanosine, 5′-AMP, 5′-IMP, 5′-GMP are all predominantly anti) whereas the syn conformation may be induced by the introduction of bulky substituents at C-8. Early work on conformational analysis developed by both Ikehara[41] and Sundaralingam,[42] using circular dichroism and NMR spectroscopy techniques, has demonstrated that indeed purine nucleosides with bulky substituents at C-8 (such as halogen, SMe, hydroxypropyl, etc.) exist predominantly in the syn conformation in solution. The predominance in the syn conformation is believed to be due to unfavorable steric and electrostatic repulsions between the 8-substituent and the ribose ring. Moreover, Stolarski and Dudycz noted that changes in the base conformation lead to characteristic changes in chemical shifts of the ribose protons, and therefore, the difference in chemical shifts of H-2′ in nucleosides and their corresponding nucleotides can be used as an indicator of the sugar-base orientation.[43,44]

The chemical shift values for the protons common to all IMP analogues prepared during the course of this study, together with reference data for 8-Br-IMP and 5′-IMP, are listed in Table 1, and in agreement with earlier reports, we have found that the NMR resonances of H-2′ can be used very effectively to assign the favored glycosodic bond conformation. The H-2′ chemical shift of δ 4.69 for 5′-IMP is ascribed for nucleotide in the anti conformation, while nucleotides in the syn conformation display H-2′ values further downfield. Thus, the 8-chloro, 8-iodo, 6-methyl, and 8-bromo analogues exhibit a clear preference for the syn conformation as evidenced by their respective H-2′ shifts of >0.3 ppm downfield of the H-2′ value assigned for 5′-IMP. However, the 8-amino IMP is assigned as anti because of its H-2′ resonance value of δ 4.67 being nearly identical to that of 5′-IMP. The anti conformation in this case may well arise from hydrogen bonding between the C-8 NH and the 5′-oxygen and has been extensively investigated.[45,46] The H-2′ NMR resonance of 7-deaza-IMP (δ 4.58) suggests that this nucleotide has significant anti glycosyl bond character, a result that is not surprising in light of its structural resemblance to 5′-IMP. Moreover, $\Delta_{1',2'}$, the difference between H-1′ and H-2′ chemical shifts, appears to be a measure paralleling the trend observed with the H-2′ resonance. Thus, a lower value suggests a syn conformation, whereas a larger one (>1 ppm) suggests anti conformers predominantly.[46]

The glycosidic bond conformation of NHD^+ analogues has not previously been investigated systematically. Our data show a similar trend for the H-2′ resonance values in the NHD^+ series as in the IMP series. Therefore, it seems reasonable to propose that the conformation of the glycosyl bond is unaffected by pyrophosphate bond formation and that, at least in the hypoxanthine series, corresponding mononucleotides and dinucleotides

prefer a similar glycosyl bond orientation (Table 1). Consequently, 8-chloro-, 8-iodo-, and 8-methyl-NHD$^+$ (**2**, **3**, and **4**, respectively) would be predicted to show a clear preference for the syn conformation, whereas both 8-NH_2 and 7-deaza-NHD$^+$ should be predominantly anti.

Mechanistic Study. From the results obtained after incubation with *Aplysia* cyclase we can determine that the cyclase behaves differently in the presence of adenine or hypoxanthine-based substrates. When the nucleobase is adenine, it appears that ADPRC is capable of aligning the substrate in the appropriate conformation for N1 cyclization, regardless of the preferred conformation for the individual linear dinucleotide. NAD$^+$ (predominantly anti configuration) gave cADPR, 8-bromo-NAD$^+$ (predominantly syn conformation) gave 8-bromo-*N*1-cADPR, and 7-deaza-NAD$^+$ (predominantly anti conformation) gave 7-deaza-cADPR. However, when the nucleobase is hypoxanthine, it seems that the enzyme utilizes the substrate in its prearranged conformation and therefore has a low ability to reorient the substrate in its active site in order to deliver the preferred N1 cyclized product. 8-Bromo-NHD$^+$ (predominantly syn conformation) gave 8-bromo-*N*1-cIDPR, NHD$^+$ (predominantly anti conformation) gave *N*7-cIDPR, while 7-deaza-NHD$^+$ (predominantly anti conformation with no nitrogen at N7) gave the hydrolyzed product.

8-NH_2-NHD$^+$ **5** is the one NHD$^+$ analogue that does not follow this trend. **5** shows a preference for the anti conformation and would be expected to cyclize at N7 according to the above interpretation, but cyclization of **5** exclusively gives 8-amino-*N*1-cIDPR **1e**. This result is comprehensible on the basis of the nucleophilicity of N1 and N7 of the 8-amino-NHD$^+$ precursor **5**. It is well known that the N1 in adenine is more nucleophilic than the N1 in hypoxanthine bases, whereas the N7 of hypoxanthine/guanine has greater nucleophilicity than that of adenine bases.[40] It is therefore expected that, as well as the substrate conformation, these differences in nucleobase nucleophilicity could affect the outcome of the cyclization reaction. Indeed, the incubation of both NHD$^+$ and NGD$^+$ with ADPRC generated the N7 cyclic dinucleotide,[16] whereas all the NAD$^+$ substrates cyclized at N1 despite their unfavorable anti conformation (as in 7-deaza-NAD$^+$). In the case of 8-NH_2-NHD$^+$, however, the amino substituent should be significantly electron-donating at the 8-position to make the N1 nucleophilic enough for cyclization at this position and, in addition, most likely the bulkiness of the 8-substituent would also inhibit N7 cyclization. Since space within the N1-cyclized cADPR structure is very crowded, it is very unlikely that an N7-cyclized 8-substituted analogue could be synthesized enzymatically. Even a total synthesis approach is expected to be difficult. Indeed, no such analogue has been synthesized to date by any method. There is most likely insufficient space in the enzyme active site to tolerate a substituent at C-8 in the nucleotide anti conformation, leaving cyclization in the syn conformation as the only possibility. Rearrangement of 8-NH_2-NHD$^+$ **5** from its native anti conformation to the syn conformation required for N1 cyclization would also explain why it takes 24 h for 8-NH_2-NHD$^+$ to cyclize under our conditions, whereas 8-chloro-, 8-iodo-, and 8-methyl-NHD$^+$ (nucleotides that already are in the syn conformation) cyclize in just 5−8 h under comparable conditions.

The hydrolysis of 7-deaza-NHD$^+$ **17** into 7-deaza-IDPR **23** can also be rationalized considering the nucleophilicity of the nitrogen in position 1. According to the ^{1}H NMR data, **17** prefers the anti conformation (condition unfavorable for cyclization) and the N1 would not be nucleophilic enough for cyclization at this position, resulting in the hydrolysis of the substrate.

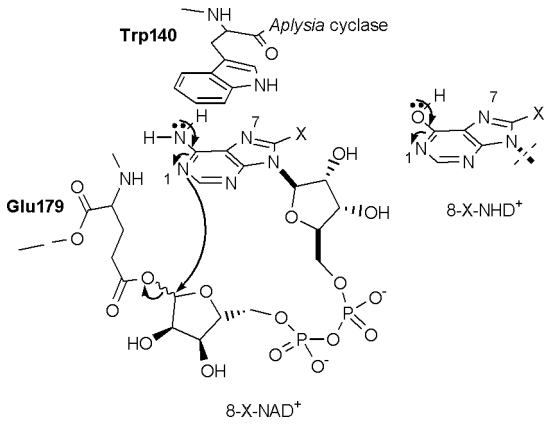


Figure 3. Schematic representation of the mechanism for N1 cyclization.

Crystallographic studies of *Aplysia* cyclase and BST-1, with ADPRC activity, have been obtained and suggest that three residues (Glu 179, Trp 140, and residue 98) play a key role in catalysis. It has been proposed that the mechanism of cyclization (and hydrolysis) proceeds via a covalent intermediate[47] in which the carboxylate group of the residue Glu 179 displaces nicotinamide to form a bond at C-1″. This covalently bound intermediate, directed by a $\pi-\pi$ interaction between Trp 140 and the purine base, then aligns in a hairpin-like conformation to allow a nucleophilic attack on C-1″ by N1 or N7 of the purine to release the cyclic dinucleotide from the enzyme.[48] Residue 98 (glutamate in *Aplysia* cyclase and serine in BST-1) acts as a strong hydrogen bond acceptor to align, through a water molecule, the nucleobase for attack on C-1″ of the ribose ring.[47] ADPRC enzymes catalyze the transformation of numerous substrates to produce a diverse range of products. The cyclization of most adenine-based substrates takes place at N1, while etheno-NAD$^+$, NGD$^+$ (nicotinamide guanosine 5′-dinucleotide), or NHD$^+$ cyclize at N7. The structures of three adenine-based ligands complexed with BST-1 showed that the N1 of ATPγS superimposes with the N7 of both etheno-NAD$^+$ and etheno-NADP$^+$ (ethenonicotinamide adenine 5′-dinucleotide 2′-phosphate), therefore suggesting that residue 98 is crucial for orienting the nucleobase in the correct conformation for cyclization. The direction of cyclization (N1 or N7) may be attributed to the torsion angle of the glycosidic bond, and thus, steric restraints on the alignment of the base in the cleft may account for N1 or N7 selective cyclization or hydrolysis.[48] More recently, the crystal structure of the soluble extracellular domain of the human antigen CD38, which has ectoenzyme ADPRC activity, has demonstrated that this cyclase activity is regulated by Glu 146 (residue conserved in *Aplysia* cyclase but not in BST-1). Model studies have revealed the existence of a hydrogen bond between the N7 atom of the adenine ring and Glu 146, thus showing that Glu 146 helps to fold the purine base for subsequent cyclization after release of nicotinamide.[49]

The parallel reaction courses for the conversion of NAD$^+$ into cADPR and 8-X-NHD$^+$ into 8-X-*N*1-cIDPR implies that ADPRC is presumably capable of stabilizing the 8-X-NHD$^+$ derivatives in their enol form (Figure 3), although hypoxanthine bases predominantly exist in their keto form in aqueous solution.[50] Since 7-deaza-NHD$^+$ and indeed NHD$^+$ itself exist predominantly in the anti conformation and therefore either do not cyclize or cyclize at N7, it would be reasonable to suggest that a substitutent at the 8-position of NHD$^+$ could orient the nucleobase in the appropriate conformation and might also help stabilize the correct tautomer for N1 cyclization.

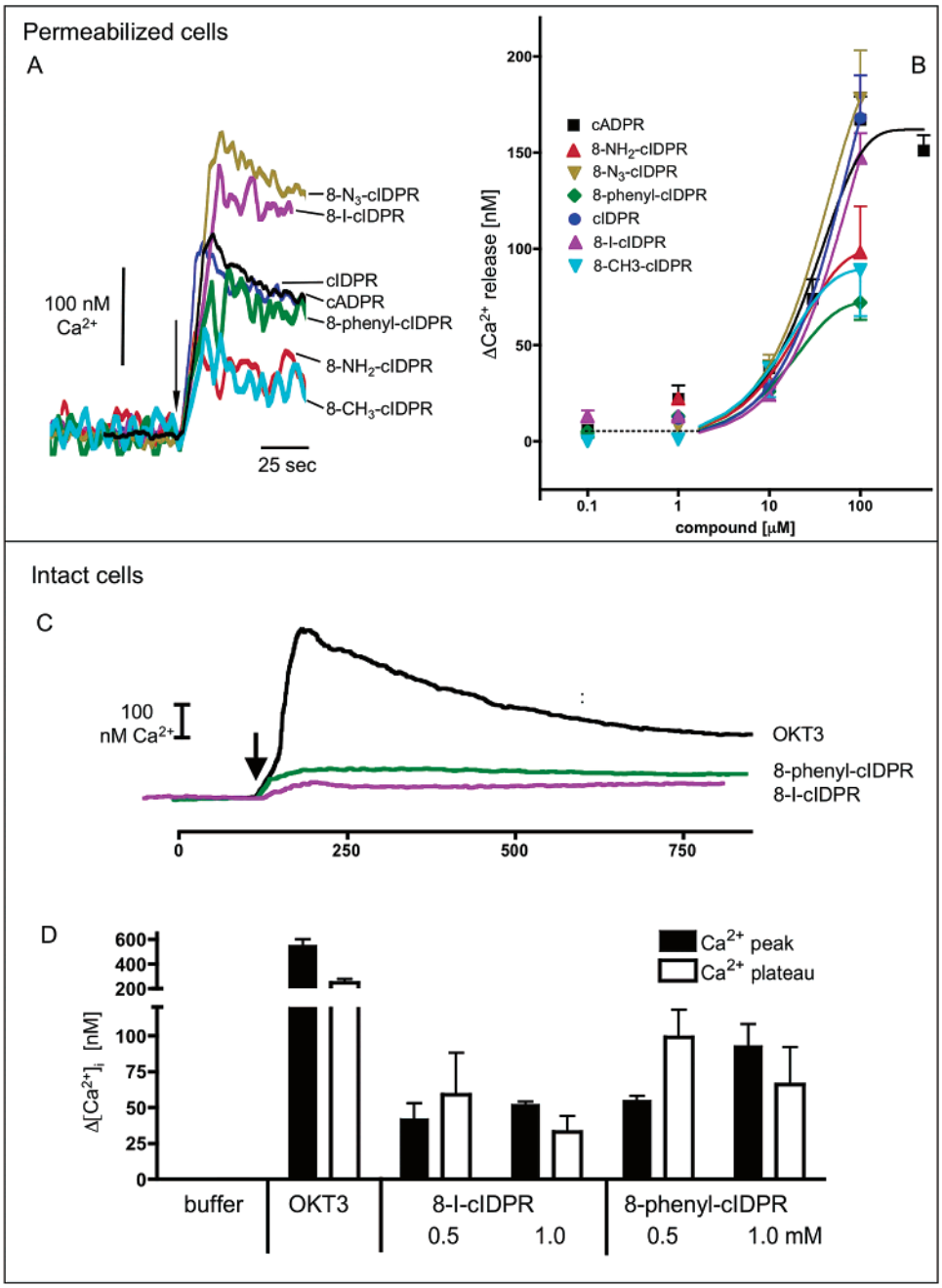


Figure 4. Effect of the 8-substituted *N*1-cIDPR analogues on Ca^{2+} signaling in human T-cells. Jurkat T-cells were permeabilized as described in the Experimental Section. Ca^{2+} stores were loaded using ATP and an ATP-regenerating system. (A) cADPR, *N*1-cIDPR, and its 8-substituted analogues (100 μM) were added as indicated. (B) Combined data are the mean ± SEM ($n \geq 3$; error bars are partially not visible because the values are smaller than the symbol size). (C, D) Intact Jurkat T-cells were loaded with fura2/AM, and $[Ca^{2+}]_i$ was determined fluorimetrically in a cell suspension as described in the Experimental Section. (C) 8-Phenyl-*N*1-cIDPR **1c** or 8-I-*N*1-cIDPR **1g** was added as indicated (final concentration of 1 mM). Representative results from three experiments are displayed. (D) Combined data are given as the mean ± SEM ($n = 3-5$). Addition of buffer (vehicle) did not induced Ca^{2+} signaling in intact cells; thus, no bars are visible.

Since the first step of the cyclization is believed to be the formation of a ribosylated-ADPR intermediate (Figure 3) that subsequently cyclizes, we can assume that the hydrolysis of 7-deaza-NHD^+ implies that limited initial recognition requirements are indeed satisfied, but subsequent syn orientation of the 7-deazahypoxanthine unit is not possible. The higher nucleophilicity of N7 in NHD^+ and NGD^+ has been put forward to explain N7 cyclization for some substrates;[16] however, this is not a factor in **17**. Since 7-deaza-NAD^+ does indeed cyclize into 7-deaza-cADPR, it appears that when there is neither N7 nor 6-NH_2, the enzyme cannot stabilize and orient the purine base for cyclization, and therefore, the substrate gets hydrolyzed. It seems possible that the *N*6-amino group of NAD^+ could be crucial in stabilizing an interaction with the enzyme that orients the adenine in the required conformation for cyclization. This could be via a hydrogen bond donor or acceptor interaction and may only be present in the transition state for this transformation. The hypoxanthine or 7-deazahypoxanthine base has only a potential hydrogen bond acceptor at C-6 in the dominant keto form, indicating that perhaps the deciding interaction for adenine is via hydrogen bond donation from the base. In the case of the 8-halo-NHD^+ analogues, the halo group forces the base into the correct orientation without the stabilizing interaction of the 6-amino group but thus allowing cyclization to occur through

presumably the minor enol tautomer (Figure 3). Alternatively, once the required conformation for cyclization is obtained in 8-bromo-NHD$^+$ by the influence of the 8-bromo group, the enol tautomer at C-6 is stabilized on the enzyme by its mimicry of the N−H motif at adenine N6.

Pharmacology. The pharmacological activity of *N*1-cIDPR **1b** and its 8-substituted analogues was analyzed in permeabilized and intact human Jurkat T-lymphocytes. In permeabilized cells rapid Ca^{2+} release was observed with all analogues reported here. However, while the Ca^{2+} mobilizing activity of *N*1-cIDPR **1b** was almost indistinguishable from that of the endogenous second messenger cADPR,[22] 8-NH_2-*N*1-cIDPR **1e**, 8-CH_3-*N*1-cIDPR **1h**, and 8-phenyl-*N*1-cIDPR **1c** were weaker in their activity (Figure 4A,B). In contrast, 8-I-*N*1-cIDPR **1g** was comparable to cADPR, and 8-N_3-*N*1-cIDPR **1d** showed even a somewhat higher, though statistically not significant, efficiency to release Ca^{2+} from internal stores (Figure 4A,B). These data extend our earlier report showing that *N*1-cIDPR **1b** acts via Ca^{2+} release from internal stores in human Jurkat T-lymphocytes.[22]

Next, the Ca^{2+} mobilizing effect of some selected analogues was analyzed in intact Jurkat T-lymphocytes. Since *N*1-cIDPR **1b**, 8-NH_2-*N*1-cIDPR **1e**, and 8-N_3-*N*1-cIDPR **1d** were considered to be too polar to penetrate the plasma membrane, only the more lipophilic compounds 8-I-*N*1-cIDPR **1g**, 8-phenyl-*N*1-cIDPR **1c**, and 8-CH_3-*N*1-cIDPR **1h** were tested. While 8-CH_3-*N*1-cIDPR **1h** did not much affect $[Ca^{2+}]_i$ (data not shown), there was a small but significant effect of 8-I-*N*1-cIDPR **1g** and 8-phenyl-*N*1-cIDPR **1c** (Figure 4C,D). In contrast to quasi-physiological stimulation by the anti-CD3 mAb OKT3 (antibody to the CD3 antigen of human T-cells), there was no biphasic elevation of $[Ca^{2+}]_i$ by 8-I-*N*1-cIDPR **1g** and 8-phenyl-*N*1-cIDPR **1c**. Instead, both analogues induced a moderate increase of $[Ca^{2+}]_i$, which remained on a more or less constant level for at least 10 min (Figure 4D). This discrepancy in shape and amplitude most likely reflects the complex situation upon quasiphysiological stimulation versus selective activation of a single Ca^{2+} signaling pathway by the cADPR analogues 8-I-*N*1-cIDPR **1g** and 8-phenyl-*N*1-cIDPR **1c.** Upon quasiphysiological stimulation by the anti-CD3 mAb OKT3, a complex pattern of formation and metabolism of at least three Ca^{2+} mobilizing second messengers, NAADP,[51] $InsP_3$,[52] and cADPR,[53] is observed. This complex pattern in conjunction with further Ca^{2+} signaling events, e.g., activation of capacitative Ca^{2+} entry, then results in a complex Ca^{2+} signaling pattern. On the other hand, the second messenger cADPR, mimicked here by 8-I-*N*1-cIDPR **1g** and 8-phenyl-*N*1-cIDPR **1c**, is involved mainly in the *sustained* phase of Ca^{2+} signaling upon T cell receptor/CD3 complex stimulation; thus, the sustained Ca^{2+} signal seen upon stimulation by 8-I-*N*1-cIDPR **1g** and 8-phenyl-*N*1-cIDPR **1c** appears indeed to mimic one component of the physiologically occurring sustained Ca^{2+} signaling phase.

Ca^{2+} mobilizing effects of derivatives of *N*1-cIDPR **1b** in intact cells have been described recently.[19,21,22,54] Interestingly, analogues of *N*1-cIDPR with substitution of either the northern ribose by an ether bridge (cIDPRE, 8-N_3-cIDPRE, 8-NH_2-cIDPRE) or both riboses by two ether bridges (cIDP-DE) induced more pronounced initial Ca^{2+} peaks compared to 8-I-*N*1-cIDPR **1g** and 8-phenyl-*N*1-cIDPR **1c** when added to intact Jurkat T-cells.[19] It is not yet clear why these different types of membrane-permeant cADPR analogues resulted in different initial Ca^{2+} signals; however, this point will be the subject of further investigations. Taken together, in addition to the *N*1-cIDPR analogues with ether bridges, the 8-I-*N*1-cIDPR **1g** and 8-phenyl-*N*1-cIDPR **1c** may be used as novel membrane-permeant cADPR mimics in intact cells. Such tools are very important for cell biology, since in many previous studies cADPR was delivered into cells by either microinjection[55] or infusion via a patch-clamp pipet in the whole-cell configuration.[56] The use of broken-cell preparations such as permeabilized cells[11] or cell homogenates[57] allows evaluation of the Ca^{2+} releasing activity of nonmembrane-permeant compounds; however, only limited conclusions regarding the role of the respective second messenger system in intact cells can be drawn.

Initial experiments indicate that both 8-chloro-*N*1-cIDPR **1f** and 8-bromo-*N*1-cIDPR **1a** behave differently from the other 8-X-substituted cIDPR analogues in permeabilized cells. We have reported 8-bromo-*N*1-cIDPR **1a** to be a membrane-permeant agonist of cADPR-induced Ca^{2+} release in intact cells.[21] A more in-depth investigation of these differences is under way, and the results will be reported in due course.

Regarding the photolabile 8-azido analogue **1d**, it is likely that during the experiment where the cells and the compounds are exposed to strong UV light, the excitation light for the Ca^{2+} dye, covalent bonds between the ligand and its receptor are formed leading to enhanced and prolonged stimulation. A similar effect was observed with 8-N_3-cIDPRE.[19]

Taken altogether, the chemical syntheses of the 8-substituted *N*1-cIDPR analogues reported here result in potentially important and novel tools to study cADPR-mediated Ca^{2+} signaling in permeabilized and intact cells. cIDPR analogues are worthy of further exploration both chemically and biologically. Indeed, we recently reported a novel chemical degradation pathway for 8-bromo- and 8-iodo-cIDPR.[58] The excellent stability of such compounds under biological conditions may, in particular, make them ideal for cocrystallization structure with cADPR binding protein.

Experimental Section

General. All reagents and solvents were of commercial quality and were used without further purification unless described otherwise. Triethylamine and diisopropylethylamine were dried over potassium hydroxide, distilled, and then stored over potassium hydroxide pellets. Morpholine was distilled and dried over KOH, and formamide was distilled and dried over molecular sieves (4 Å). ADP-ribosyl cyclase was purified from the ovotestis of the opithobranch mollusk *Aplysia californica*.[59] H_2O was of MilliQ quality. Unless otherwise stated, all reactions were carried out under an inert atmosphere of nitrogen. All ^1H, ^{13}C, and ^{31}P NMR spectra of final compounds were collected in D_2O, either 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). Prior to the recording of ^{31}P NMR spectra a drop of triethylamine was added to each sample to suppress line broadening and to enhance resolution. Chemical shifts (δ) are reported in parts per million (ppm) relative to HDO as internal standard (δ 4.77 ppm, ^1H) or to $OP(OPh)_3$ (δ −18.0 ppm, ^{31}P) as external standard. 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; etc. UV spectra were collected in aqueous solution on a Perkin-Elmer Lambda EZ 201 or Lambda 3B spectrophotometer. Infrared spectra were recorded from KBr disks on a Perkin-Elmer Spectrum RX1 FT-IR system spectrometer. Peak positions are reported in cm^{-1}, and the following abbreviations are used for peak intensities: w, weak; m, medium; s, strong. FAB mass spectra were recorded on samples in a *m*-nitrobenzyl alcohol matrix with PEG-H/PEG-monomethyl ether as reference substances for accurate mass measurements at the Mass Spectrometry Service Centre, University of Bath, on a Micromass Autospec instrument. EI mass spectra were recorded on a Finnigan MAT 95 XP and Micromass Quattro

II at the EPSRC National Mass Spectrometry Service Centre at the University of Wales Swansea. 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 mm × 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. 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 0.05 M TEAB buffer against MeCN at 5 mL/min; Q Sepharose washed with H_2O, gradient 1 M TEAB buffer (pH 7.1−7.6) against H_2O at 5 mL/min; 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.[60]

Pharmacology. Cell Culture. Jurkat T-lymphocytes (clone JMP) were cultured as described earlier.[61] In brief, cells were cultured in RPMI 1640 supplemented with Glutamax I, 25 mM HEPES, 100 units/mL penicillin, 50 μg/mL streptomycin, and 7.5% (v/v) newborn calf serum.

Loading of Cells with fura2/AM. The cells were loaded with Fura-2/AM as described in an earlier report.[61] Loaded cells were kept in the dark at room temperature until use.

Determination of $[Ca^{2+}]_i$ in Permeabilized Cells. Jurkat T cells were permeabilized by saponin, and experiments were done as described in an earlier report.[11] 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, 10 mM NaCl, 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. Then the cells were 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 addition of ATP and an ATP-regenerating system consisting of creatine phosphate and creatine kinase.[61] $[Ca^{2+}]$ 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, emission 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.

Determination of $[Ca^{2+}]_i$ in Intact Cells. $[Ca^{2+}]_i$ was measured in the presence of 1 mM extracellular Ca^{2+} in Jurkat T-lymphocytes using fura2/AM as described.[61] An F-2000 spectrofluorimeter (Hitachi) was used in the ratio mode as detailed above for permeabilized cells. Intracellular $[Ca^{2+}]$ was calculated after calibration using 0.1% Triton X-100 to obtain the maximal ratio and EGTA/Tris (8mM/60 mM) to obtain the minimal ratio.

Synthesis of 8-Cl-cIDPR (1f). 2′,3′,5′-Tris-*tert*-butyldimethylsilylinosine (7). To a clear solution of *tert*-butyldimethylsilyl chloride (14 g, 93.2 mmol) and imidazole (8.9 g, 130.5 mmol) in dry DMF (30 mL) was added inosine **6** (5 g, 18.6 mmol) in one portion. The reaction mixture was stirred at room temperature overnight, after which TLC analysis ($CHCl_3$/EtOAc, 1:1, $R_f = 0.61$) showed total conversion of starting material in a single product. The white slurry was poured in a bilayer system of water (100 mL) and DCM (100 mL). The organic layer was separated, and the aqueous phase was repeatedly extracted with DCM (3 × 50 mL). The combined organic extracts were dried ($MgSO_4$), filtered, and evaporated under reduced pressure to afford a white solid, which was recrystallized from isopropyl ether/EtOAc (9:1) giving the desired product **7** as a white powder (10.7 g, 95%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.22 (s, 1H, H-2), 8.06 (s, 1H, H-8), 6.0 (d, 1H, $J_{1',2'} = 4.9$ Hz, H-1′), 4.49 (m, 1H, H-2′), 4.29 (m, 1H, H-3′), 4.13−4.10 (m, 1H, H-4′), 3.98 (dd, 1H, $J_{5'a,5'b} = 11.4$ and $J_{5'a,4'} = 3.7$ Hz, H-5′a), 3.78 (dd, 1H, $J_{5'b,5'a} = 11.4$ and $J_{5'b,4'} = 2.5$ Hz, H-5′b), 0.94 (s, 9H, ^{t}Bu), 0.91 (s, 9H, ^{t}Bu), 0.80 (s, 9H, ^{t}Bu), 0.12 (s, 6H, 2 × CH_3), 0.08 (s, 6H, 2 × CH_3), −0.03 (s, 3H, CH_3), and −0.19 (s, 3H, CH_3). MS (FAB^+) *m*/*z* 611.3 [$(M + H)^+$, 40%]. HRMS (FAB^+) calcd for $C_{28}H_{55}N_4O_5P_2Si_3$ 611.3480 $(MH)^+$; found 611.3480. Mp 249−250° (lit.[23] mp 248−249 °C).

8-Chloro-2′,3′,5′-tris-*tert*-butyldimethylsilylinosine (8a). To a solution of diisopropylamine (1.22 mL, 8.71 mmol) in THF (7 mL) at −78 °C was added dropwise ^{n}BuLi (7.7 mL, 8.88 mmol, 1.15 N). A solution of Tris-TBDMS-inosine **7** (1 g, 1.639 mmol) in THF (18 mL) was added dropwise at −78 °C, and the mixture was stirred for further 2 h. Toluenesulfonyl chloride (1.33 g, 6.97 mmol) in THF (5 mL) was added dropwise, and the reaction mixture was stirred until completion (3 h) as shown by TLC ($CHCl_3$/EtOAc, 1:1, $R_f = 0.75$). The mixture was then warmed to room temperature and quenched with 1 N NaOAc/AcOH, pH 4. The aqueous phase was washed with DCM (3 × 50 mL); the combined organic extracts were washed with 0.5 N $NaHCO_3$, dried ($MgSO_4$), filtered, and evaporated under reduced pressure giving a yellow oil that was purified by column chromatography on silica gel (DCM/EtOAc, 9:1) followed by recrystallization with diisopropyl ether to afford the desired product **8a** as a white solid (767 mg, 73%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.20 (s, 1H, H-2), 5.97 (d, 1H, $J_{1',2'} = 6.3$ Hz, H-1′), 5.27 (dd, 1H, $J_{2',1'} = 6.3$ and $J_{2',3'} = 4.5$ Hz, H-2′), 4.45 (dd, 1H, $J_{3',2'} = 4.5$ and $J_{3',4'} = 2.2$ Hz, H-3′), 4.11−4.06 (m, 1H, H-4′), 3.95 (dd, 1H, $J_{5'a,5'b} = 10.9$ and $J_{5'a,4'} = 7.4$ Hz, H-5′a), 3.72 (dd, 1H, $J_{5'b,5'a} = 10.9$ and $J_{5'b,4'} = 4.2$ Hz, H-5′b), 0.94 (s, 9H, ^{t}Bu), 0.85 (s, 9H, ^{t}Bu), 0.79 (s, 9H, ^{t}Bu), 0.13 (s, 6H, 2 × CH_3), 0.03 (s, 3H, CH_3), −0.007 (s, 3H, CH_3), −0.05 (s, 3H, CH_3), and −0.33 (s, 3H, CH_3). MS (FAB^+) *m*/*z* 645.2 [$(M + H)^+$, 80%]. HRMS (FAB^+) calcd for $C_{28}H_{54}N_4O_5Si_3{}^{35}Cl$ 645.3110 $(MH)^+$, found 645.3090; calcd for $C_{28}H_{54}N_4O_5Si_3{}^{37}Cl$ 647.3099, found 647.3061 $(MH)^+$. Mp 96−98 °C (lit.[24] mp 95−97 °C). Anal. ($C_{28}H_{53}ClN_4O_5Si_3$) C, H, N.

8-Chloroinosine (9a). To a solution of 8-chloro-2′,3′,5′-tris-*tert*-butyldimethylsilylinosine **8a** (720 mg, 1.12 mmol) in THF (4 mL) was added 1 M TBAF in THF (1 mL, 4.476 mmol). The mixture was stirred at room temperature until completion of the reaction as shown by TLC (DCM/MeOH, 8:2, $R_f = 0.66$) and was then quenched with MeOH (2 mL). The solvents were removed under reduced pressure, and the crude product was purified by column chromatography on silica gel (DCM/MeOH, 9:1) followed by recrystallization with aqueous MeOH to afford the desired product **9a** as a white solid (260 mg, 77%). ^{1}H NMR (270 MHz, DMSO-d_6) δ 8.16 (s, 1H, H-2), 5.83 (d, 1H, $J_{1',2'} = 6.2$ Hz, H-1′), 5.52 (br s, 1H, OH), 5.27 (br s, 1H, OH), 4.99−4.97 (m, 2H, H-2′ and OH), 4.19−4.17 (m, 1H, H-3′), 3.93−3.91 (m, 1H, H-4′), 3.99−3.93 (m, 1H, H-5′a), and 3.72 (dd, 1H, $J_{5'b,5'a} = 10.9$ and $J_{5'b,4'} = 4.2$ Hz, H-5′b). MS (FAB^+) *m*/*z* 303.0 [$(M + H)^+$, 60%]. HRMS (FAB^+) calcd for $C_{10}H_{12}N_4O_5{}^{35}Cl$ 303.0500 $(MH)^+$, found 303.0496; calcd for $C_{10}H_{12}N_4O_5{}^{37}Cl$ 305.0482, found 305.0466 $(MH)^+$. Anal. ($C_{10}H_{11}ClN_4O_5 \cdot 0.5H_2O$) C, H, N.

8-Chloroinosine 5′-Monophosphate (10a). 8-Chloroinosine **9a** (80 mg, 0.264 mmol) was dissolved in triethyl phosphate (1 mL) by heating with a heat gun. The resulting colorless solution was cooled to 0 °C, water (2 μL) was added followed by $POCl_3$ (0.1 mL, 1.056 mmol), and the mixture was stirred at 0 °C until HPLC analysis showed disappearance of starting material and formation of a single peak ($t_{R(8-Cl-inosine)} = 2.4$ min and $t_{R(8-Cl-IMP)} = 7.4$ min). After 1 h, the reaction mixture was quenched by addition of ice/water (15 mL) and stirred for 15 min at 0 °C, after which it was warmed to room temperature. Triethyl phosphate was extracted with EtOAc (6 × 6 mL), and the aqueous phase was neutralized with 2 N NaOH. It was then applied to a reverse-phase Gradifrac column with elution with a gradient of MeCN in 0.05 M TEAB. The appropriate fractions were collected and lyophilized overnight to afford the desired monophosphate **10a** as its triethylammonium salt. ^{1}H NMR (270 MHz, D_2O) δ 8.17 (s, 1H, H-2), 6.09 (d, $J_{1',2'} = 5.6$ Hz, 1H, H-1′), 5.20 (app t, 1H, $J_{2',1'} = J_{2',3'} = 5.6$ Hz, H-2′), 4.61 (dd, 1H, $J_{3',2'} = 5.6$ and $J_{3',4'} = 4.8$ Hz, H-3′), 4.27 (app q, 1H, $J_{4',3'} = J_{4',5'a} = J_{4',5'b} = 4.8$ Hz, H-4′), and 4.17−4.04 (m, 2H,

H-5′a and H-5′b). ^{31}P (109 MHz, D_2O) δ 1.6 (s). MS (FAB$^-$) *m/z* 381.1 [(M − H)$^-$, 70%]. HRMS (FAB$^-$) calcd for $C_{10}H_{11}N_4O_8P^{35}Cl$ 381.0003 [(M − H)$^-$], found 380.9993; calcd for $C_{10}H_{11}N_6O_8P^{37}Cl$ 382.9973 [(M − H)$^-$], found 382.9966.

Nicotinamide-8-chlorohypoxanthine 5′-Dinucleotide (8-Chloro-NHD$^+$ (2). 8-Cl-IMP **10a** (90 mg, 0.23 mmol) was dissolved in dry DMSO (2 mL) and coevaporated with dry DMF (5 × 2.5 mL). The residue was dissolved in dry DMSO (0.4 mL) to which was added morpholine (104 μL, 1.2 mmol), dipyridyl disulfide (125 mg, 0.55 mmol), and PPh_3 (150 mg, 0.55 mmol), at which point the solution turned bright-yellow. The reaction was completed within 1 h as shown by ^{31}P NMR (δ_P 5.9 ppm). Precipitation occurred by addition of a solution of 0.1 M NaI in acetone. It was then filtered, washed with acetone to remove most of the yellow color, dried under vacuum, and used in the next step without further purification. A small amount was kept for characterization. ^{1}H NMR (270 MHz, D_2O) δ 8.13 (s, 1H, H-2), 6.04 (d, 1H, $J_{1',2'} = 4.5$ Hz, H-1′), 5.24−5.21 (m, 1H, H-2′), 4.69−4.65 (m, 1H, H-3′), 4.20−4.19 (m, 1H, H-4′), 4.03−3.93 (m, 2H, H-5′a and H-5′b), 3.47 (br s, 4H, 2 × CH_2), and 2.82 (m, 4H, 2 × CH_2). ^{31}P (109 MHz, D_2O) δ 8.3. Crude 8-Cl-IMP morpholidate **11a** (35 mg, 0.073 mmol), β-NMN (27 mg, 0.080 mmol), and $MgSO_4$ (17 mg, 0.146 mmol) were dissolved in a 0.2 M solution of $MnCl_2$ in formamide (0.55 mL) and stirred at room temperature overnight, after which HPLC analysis showed completion of the reaction ($t_{R(\beta-NMN)} = 2.1$ min and $t_{R(8-Cl-NHD)} = 4.3$ min). Precipitation of the product occurred by addition of MeCN; it was filtered, dissolved in MilliQ, and applied to a RP-18 Gradifrac column with elution with a gradient of MeCN in 0.05M TEAB. The appropriate fractions were collected and evaporated under reduced pressure to afford the desired product **3** as its triethylammonium salt (23 mg, 46%). ^{1}H NMR (400 MHz, D_2O) δ 9.19 (s, 1H, H_N2), 9.01 (d, 1H, $J_{6,5} = 6.0$ Hz, H_N6), 8.75 (d, 1H, $J_{4,5} = 7.8$ Hz, H_N4), 8.12 (dd, $J_{5,4} = 7.8$ and $J_{5,6} = 6.0$ Hz, 1H, H_N5), 7.95 (s, 1H, H-2), 5.89 (d, 1H, $J_{1',2'} = 4.7$ Hz, H-1″), 5.83 (d, 1H, $J_{1',2'} = 5.4$ Hz, H-1′), 5.07−5.04 (m, 1H, H-2′), 4.50 (app t, 1H, $J_{3',2'} = J_{3',4'} = 5.0$ Hz, H-3′), 4.37−4.25 (m, 5H), and 4.20−4.13 (m, 3H). ^{31}P (109 MHz, D_2O) δ −10.5 (d, $J = 21$ Hz), −11.1 (d, $J = 21$ Hz). UV (H_2O, pH 7.2) λ_{max} 250 nm (ϵ 12 900). MS (FAB$^-$) *m/z* 697.9 [(M − H)$^-$, 50%]. HRMS (FAB$^-$) calcd for $C_{21}H_{24}N_6O_{15}P_2{}^{35}Cl$ 697.0463 [(M − H)$^-$], found 697.0484.

Cyclic 8-chloroinosine 5′-Diphosphate Ribose (8-Chloro-cIDPR, 1f). 8-Chloro-NHD$^+$·2TEA salt **3** (23 mg, 33 μmol) was incubated with *Aplysia* cyclase (0.15 mL) in a 25 mM HEPES buffer (50 mL, pH 4) at room temperature. After 8 h, HPLC analysis showed completion of the reaction ($t_{R(nicotinamide)} = 1.7$ min and $t_{R(8-Cl-cIDPR)} = 11.3$ min). It was then applied to a Q Sepharose ion exchange column with elution with 1 M TEAB buffer. The appropriate fractions were collected and evaporated under vacuum to afford the desired cyclized product as a glaasy solid in its triethylammonium form (13 mg, 68%). ^{1}H NMR (400 MHz, D_2O) δ 8.65 (s, 1H, H-2), 5.93 (s, 1H, H-1″), 5.74 (d, 1H, $J_{1',2'} = 5.8$ Hz, H-1′), 5.28 (app t, 1H, $J_{2',1'} = J_{2',3'} = 5.8$ Hz, H-2′), 4.57−4.56 (m, 1H, H-3′), 4.41−4.38 (m, 1H, H-5′a), 4.34−4.33 (m, 1H, H-5″a), 4.28−4.26 (m, 3H, H-2″, H-3″, and H-4″), 4.20−4.19 (m, 1H, H-4′), 4.07 (d, 1H, $J_{5''a,5''b} = 10.6$ Hz, H-5″a), and 3.97 (d, 1H, $J_{5'b,5'a} = 10.5$ Hz, H-5′b). ^{31}P (109 MHz, D_2O) δ −9.3 (br s) −10.2 (br s). UV (H_2O, pH 5.2) λ_{max} 253 nm (ϵ 10 400). MS (FAB$^-$) *m/z* 574.8 [(M − H)$^-$, 100%], 576.8 [(M − H)$^-$, 37%]. HRMS (FAB$^-$) calcd for $C_{15}H_{18}N_4O_{14}P_2{}^{35}Cl$ 574.9983 [(M − H)$^-$], found 574.9959; calcd for $C_{15}H_{18}N_4O_{14}P_2{}^{37}Cl$ 576.9954 [(M − H)$^-$], found 576.9972.

Synthesis of 8-Iodo-cIDPR (1g). 8-Iodo-2′,3′,5′-tris-*tert*-butyldimethylsilylinosine (8b). To a solution of diisopropylamine (0.57 mL, 4.09 mmol) in THF (4 mL) at −78 °C was added dropwise ^{n}BuLi (3.63 mL, 4.44 mmol, 1.15 N in hexane). The mixture was stirred for 10 min at −78 °C, a solution of Tris-TBDMS-inosine **7** (0.5 g, 0.819 mmol) in THF (9 mL) was added dropwise at −78 °C, and stirring continued for further 2 h. Iodine (0.83 g, 3.276 mmol) in THF (3 mL) was added dropwise until a deep-yellow color settled. It was stirred for 10 min, after which TLC analysis showed completion of the reaction ($CHCl_3$/EtOAc, 1:1, $R_f = 0.63$). The mixture was then warmed to room temperature and quenched with 1 N NaOAc/AcOH, pH 4. The aqueous phase was washed with DCM (3 × 30 mL); the combined organic extracts were washed with 0.5 N $NaHCO_3$, dried ($MgSO_4$), filtered, and evaporated under reduced pressure to give a yellow oil, which was purified by column chromatography on silica gel (DCM/EtOAc, 9:1) to afford the desired product **8b** as a pale-yellow solid (520 mg, 86%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.15 (s,1H, H-2), 5.89 (d, 1H, $J_{1',2'} = 6.5$ Hz, H-1′), 5.35 (dd, 1H, $J_{2',1'} = 6.5$ and $J_{2',3'} = 4.2$ Hz, H-2′), 4.44 (dd, 1H, $J_{3',2'} = 4.2$ and $J_{3',4'} = 2.1$ Hz, H-3′), 4.04 (dd, 1H, $J_{4',3'} = 2.1$ and $J_{4',5'b} = 4.2$ Hz, H-4′), 3.96 (dd, 1H, $J_{5'a,5'b} = 10.6$ and $J_{5'a,4'} = 7.9$ Hz, H-5′a), 3.71 (dd, 1H, $J_{5'b,5'a} = 10.6$ and $J_{5'b,4'} = 4.2$ Hz, H-5′b), 0.95 (s, 9H, ^{t}Bu), 0.85 (s, 9H, ^{t}Bu), 0.78 (s, 9H, ^{t}Bu), 0.13 (s, 6H, 2 × CH_3), 0.029 (s, 6H, 2 × CH_3), −0.07 (s, 3H, CH_3), and −0.35 (s, 3H, CH_3). MS (FAB$^+$) *m/z* 737.3 [(M + H)$^+$, 80%]. HRMS (FAB$^+$) calcd for $C_{28}H_{54}N_4O_5Si_3I$ 737.2467 (MH$^+$), found 737.2446. Mp 105−107 °C. Anal. ($C_{28}H_{53}IN_4O_5Si_3$) C, H, N.

8-Iodoinosine (9b). To a solution of 8-iodo-2′,3′,5′-tris-*tert*-butyldimethylsilylinosine **8b** (1 g, 1.35 mmol) in THF (6 mL) was added 1 M TBAF in THF (1.3 mL, 5.42 mmol). The mixture was stirred at room temperature until completion of the reaction as shown by TLC (DCM/MeOH, 8:2, $R_f = 0.31$) and quenched with MeOH (3 mL). The solvent was removed under reduced pressure, and the product was isolated by column chromatography on silica gel (DCM/MeOH, 9:1) followed by recrystallization with MeOH/H_2O (1:1) to afford the title nucleoside **9b** as a white solid (390 mg, 73%). ^{1}H NMR (270 MHz, DMSO-d_6) δ 7.97 (s, 1H, H-2), 5.73 (d, 1H, $J_{1',2'} = 6.6$ Hz, H-1′), 4.73−4.68 (m, 1H, H-2′), 4.08 (dd, 1H, $J_{3',2'} = 5.5$ and $J_{3',4'} = 3.0$ Hz, H-3′), 3.88 (m, 1H, H-4′), 3.61 (dd, 1H, $J_{5'a,5'b} = 12.0$ and $J_{5'a,4'} = 3.7$ Hz, H-5′a), and 3.51−3.45 (m, 1H, H-5′b). MS (FAB$^+$) *m/z* 395.0 [(M + H)$^+$, 10%]. HRMS (FAB$^+$) calcd for $C_{10}H_{12}N_4O_5I$ 395.9886 (MH$^+$), found 395.9886. IR (KBr disk, cm^{-1}) 3357, 1684, 1105. Mp 210−212 °C. Anal. ($C_{10}H_{11}IN_4O_5$) C, H, N.

8-Iodoinosine 5′-Monophosphate (10b). Compound **10b** was obtained from 8-iodoinosine **9b** following the same procedure as for **10a** (84 mg, 70%). ^{1}H NMR (270 MHz, D_2O) δ 8.11 (s, 1H, H-2), 6.02 (d, 1H, $J_{1',2'} = 5.6$ Hz, H-1′), 5.34−5.30 (m, 1H, H-2′), 4.65−4.61 (m, 1H, H-3′), 4.27 (dd, 1H, $J_{4',3'} = 5.2$ and $J_{4',5'a} = 4.6$ Hz, H-4′), 4.14 (dd, 1H, $J_{5'a,5'b} = 11.4$ and $J_{5'a,4'} = 4.6$ Hz, H-5′a), and 4.09 (dd, 1H, $J_{5'b,5'a} = 11.4$ and $J_{5'b,4'} = 5.4$ Hz, H-5′b). ^{31}P (109 MHz, D_2O) δ 2.23 (s). MS (FAB$^-$) *m/z* 473.0 [(M − H)$^-$, 90%]. HRMS (FAB$^-$) calcd for $C_{10}H_{11}N_4O_8PI$ 472.9354 [(M−H)$^-$], found 472.9349.

Nicotinamide-8-iodohypoxanthine 5′-Dinucleotide (8-Iodo-NHD$^+$, 3). 8-Iodo-IMP·2TEA salt **10b** (70 mg, 0.103 mmol) was dissolved in dry DMSO (2 mL) and coevaporated with dry DMF (5 × 3 mL). The residue was dissolved in DMSO (400 μL) to which was added morpholine (47 μL, 0.536 mmol), dipyridyl disulfide (56 mg, 0.257 mmol), and triphenylphosphine (67 mg, 0.257 mmol), at which point the solution became bright-yellow. It was stirred for 1 h at room temperature, after which ^{31}P NMR showed disappearance of the starting material (δ_P 1.2 ppm) and appearance of a new singlet (δ_P 5.3 ppm) for the morpholidate. Precipitation of the product occurred by dropwise addition of a solution of NaI in acetone (0.1M, 8 mL). The resulting precipitate was filtered and washed with acetone to yield the desired morpholidate **11b** as a pale-yellow solid, which was used in the next step without further purification. ^{1}H NMR (270 MHz, D_2O) δ 7.99 (s, 1H, H-2), 5.88 (d, 1H, $J_{1',2'} = 3.7$ Hz, H-1′), 5.24−5.20 (m, 1H, H-2′), 4.51 (m, 1H, H-3′), 4.12−4.11 (m, 1H, H-4′), 4.0−3.88 (m, 2H, H-5′a and H-5′b), 3.38 (m, 4H, 2 × CH_2) and 2.73 (m, 4H, 2 × CH_2). ^{31}P (109 MHz, D_2O) δ 8.3. MS (FAB$^-$) *m/z* 542.1 [(M − H)$^-$, 100%]. HRMS (FAB$^-$) calcd for $C_{14}H_{18}N_5O_8IP$ 541.9937 [(M − H)$^-$], found 541.9964. Crude 8-iodo-IMP morpholidate **11b** (60 mg, 0.106 mmol), β-NMN (47 mg, 0.139 mmol), and $MgSO_4$ (30 mg, 0.256 mmol) were dissolved in a 0.2 M solution of $MnCl_2$ in formamide (1 mL), and the mixture was stirred at room temperature overnight, after which HPLC analysis showed completion of the reaction ($t_{R(\beta-NMN)} = 2.1$ min and $t_{R(8-I-NHD)} = 4.1$ min). Precipitation of

the product occurred by addition of MeCN; it was filtered, dissolved in MilliQ, and applied to a reverse-phase Gradifrac column with elution with a gradient of 0.05 M TEAB buffer against MeCN. Further treatment with Chelex-100 to remove any paramagnetic particles afforded the desired product **3** as its sodium salt (51 mg, 62% over two steps). ^{1}H NMR (400 MHz, D_2O) δ 9.18 (s, 1H, H_N2), 8.96 (d, 1H, $J_{6,5} = 6.3$ Hz, H_N6), 8.77 (d, 1H, $J_{4,5} = 8.2$ Hz, H_N4), 8.07 (dd, 1H, $J_{5,4} = 8.2$ and $J_{5,6} = 6.3$ Hz, H_N5), 7.98 (s, 1H, H-2), 5.88 (d, 1H, $J_{1'',2''} = 5.8$ Hz, H-1″), 5.76 (d, 1H, $J_{1',2'} = 5.3$ Hz, H-1′), 5.08 (app t, 1H, $J_{2',1'} = J_{2',3'} = 5.3$ Hz, H-2′), 4.48 (dd, 1H, $J_{3',2'} = 5.3$ and $J_{3',4'} = 4.2$ Hz, H-3′), 4.26−4.23 (m, 2H, H-2″ and H-4′), 4.21−4.19 (m, 1H, H-3″), and 4.13−4.05 (m, 5H, H-4″, H5′a, H5′b, H5″a, H5″b). ^{31}P (109 MHz, D_2O) δ −10.5 (d, $J = 19.3$ Hz) −11.0 (d, $J = 19.3$ Hz). MS (FAB^-) *m/z* 789.9 [(M − H)$^-$, 10%]. HRMS (FAB^-) calcd for $C_{21}H_{25}N_6O_{15}P_2I$ 789.9897 [(M − H)$^-$], found 789.9895. UV (H_2O, pH 5.7) λ_{max} 257 nm (ϵ 14 700).

Cyclic 8-iodoinosine 5′-Diphosphate Ribose (8-Iodo-cIDPR, 1g). 8-Iodo-NHD$^+$ **3** (51 mg, 64 μmol) was incubated with *Aplysia* cyclase (160 μL) in a 25 mM HEPES buffer (160 mL, pH 4) at room temperature, and it was monitored by HPLC. It was stopped after 5 h ($t_{R(nicotinamide)} = 1.7$ min and $t_{R(8-I-cIDPR)} = 12.2$ min) and applied to a Q Sepharose column with elution with a linear gradient of 1 M TEAB against MilliQ water. The appropriate fractions were collected, evaporated under vacuum, and coevaporated with MeOH to afford the desired cyclized product **1g** as a glassy solid in the triethylammonium salt form (16 mg, 37%). ^{1}H NMR (400 MHz, D_2O) δ 8.75 (s, 1H, H-2), 5.94 (d, 1H, $J_{1'',2''} = 5.7$ Hz, H-1″), 5.92 (br s, 1H, H-1′), 5.28 (app t, 1H, $J_{2',1'} = J_{2',3'} = 5.7$ Hz, H-2′), 4.59−4.58 (m, 1H, H-3′), 4.45−4.44 (m, 1H, H-5′a), 4.36−4.33 (m, 1H, H-5″a), 4.29−4.26 (m, 4H, H-2″, H-3″, H-4″, and H-4′), 4.07 (d, 1H, $J_{5''a,5''b} = 11.0$ Hz, H-5″a), and 3.97 (d, 1H, $J_{5'b,5'a} = 11.0$ Hz, H-5′b). ^{31}P (109 MHz, D_2O) δ −9.3 (br s) −10.2 (br s). UV (H_2O, pH 5.8) λ_{max} 257 nm (ϵ 10 000). MS (FAB^-) *m/z* 666.8 [(M − H)$^-$, 100%]. HRMS (FAB^-) calcd for $C_{15}H_{18}N_4O_{14}P_2I$ 666.9340 [(M − H)$^-$], found 666.9379.

Synthesis of 8-Methyl-cIDPR (1h). 8-Methyl-2′,3′,5′-tris-*tert*-butyldimethylsilylinosine (8c). In a round-bottomed flask equipped with a condenser was dissolved 8-iodo-2′,3′,5′-tris-*tert*-butyldimethylsilylinosine **8b** (50 mg, 0.068 mmol) in dry DMF. $Pd(PPh_3)_4$ (16 mg, 0.013 mmol) was added followed by tetramethyltin (0.1 mL, 0.684 mmol). The resulting yellow solution was stirred at 100 °C under argon for 16 h, after which TLC analysis showed completion of the reaction ($CHCl_3$/EtOAc, 1:1, $R_f = 0.42$). The mixture was then cooled to room temperature and washed with a saturated aqueous solution of NH_4Cl (20 mL) and extracted with DCM (30 mL). The organic extract was dried (Na_2SO_4), filtered, and evaporated under reduced pressure, giving a brown crude oil which was purified by column chromatography on silica gel (DCM/EtOAc, 9:1) followed by recrystallization with diisopropyl ether to yield the desired protected nucleoside **8c** as a pale-yellow solid (81 mg, 96%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.0 (s, 1H, H-2), 5.76 (d, 1H, $J_{1',2'} = 6.7$ Hz, H-1′), 5.16 (dd, 1H, $J_{2',1'} = 6.7$ and $J_{2',3'} = 4.5$ Hz, H-2′), 4.35 (dd, 1H, $J_{3',2'} = 4.5$ and $J_{3',4'} = 2.0$ Hz, H-3′), 4.02−3.99 (m, 1H, H-4′), 3.93 (dd, 1H, $J_{5'a,5'b} = 10.7$ and $J_{5'a,4'} = 7.2$ Hz, H-5′a), 3.69 (dd, 1H, $J_{5'b,5'a} = 10.7$ and $J_{5'b,4'} = 4.0$ Hz, H-5′b), 2.56 (s, 1H, CH_3), 0.90 (s, 9H, ^{t}Bu), 0.82 (s, 9H, ^{t}Bu), 0.71 (s, 9H, ^{t}Bu), 0.09 (s, 6H, 2 × CH_3), −0.005 (s, 6H, 2 × CH_3), −0.03 (s, 3H, CH_3), and −0.42 (s, 3H, CH_3). MS (FAB^+) *m/z* 625.4 [(M + H)$^+$, 35%]. HRMS (FAB^+) calcd for $C_{29}H_{57}N_4O_5Si_3$ 625.3637 (MH)$^+$, found 625.3586. Mp 245−249 °C. Anal. ($C_{29}H_{56}N_4O_5Si_3$) C, H, N.

8-Methylinosine (9c). To a solution of 8-methyl-2′,3′,5′-tris-*tert*-butyldimethylsilylinosine **8c** (800 mg, 1.28 mmol) in THF (10 mL) was added 1 M TBAF in THF (4 mL, 5.12 mmol). The mixture was stirred at room temperature until completion of the reaction as shown by TLC (DCM/MeOH, 8:2, $R_f = 0.28$) and quenched with MeOH (6 mL). The solvents were removed under reduced pressure, and the crude product was purified by column chromatography on silica gel (DCM/MeOH, 9:1) followed by recrystallization with MeOH/H_2O (1:1) to afford the desired product **9c** as a white solid (350 mg, 97%). ^{1}H NMR (270 MHz, DMSO-d_6) δ 8.01 (s, 1H, H-2), 5.77 (d, 1H, $J_{1',2'} = 6.5$ Hz, H-1′), 5.40 (d, 1H, $J_{OH,2'} = 6.6$ Hz, 2′-OH), 5.24 (d, 1H, $J_{OH-3'} = 4.4$ Hz, 3′-OH), 5.11 (m, 1H, 5′-OH), 4.74 (dd, 1H, $J_{2',1'} = 6.5$ and $J_{2',3'} = 2.9$ Hz, H-2′), 4.12 (m, 1H, H-3′), 3.92 (dd, 1H, $J_{4',3'} = 7.2$ and $J_{4',5'a} = 3.9$ Hz, H-4′), 3.65 (dd, 1H, $J_{5'a,5'b} = 12.0$ and $J_{5'a,4'} = 3.9$ Hz, H-5′a), 3.52 (m, 1H, H-5′b), and 2.50 (s, 3H, CH_3 hidden under DMSO peak). MS (FAB^+) *m/z* 283.0 [(M + H)$^+$, 30%]. HRMS (FAB^+) calcd for $C_{11}H_{15}N_4O_5$ 283.1042 (MH)$^+$, found 283.1053. IR (KBr disk, cm^{-1}) 3495, 1681, 1094. Mp 228−230 °C. Anal. ($C_{11}H_{14}N_4O_5 \cdot 1.5H_2O$) C, H, N.

8-Methylinosine 5′-Monophosphate (10c). Compound **10c** was obtained from 8-methylinosine **9c** following the same procedure as for **10a** (89 mg, 61%). ^{1}H (400 MHz, D_2O) δ 8.11 (s, 1H, H-2), 5.95 (d, 1H, $J_{1',2'} = 5.1$ Hz, H-1′), 4.97 (app t, 1H, $J_{2',1'} = J_{2',3'} = 5.1$ Hz, H-2′), 4.49 (app t, 1H, $J_{3',2'} = J_{3',4'} = 5.1$ Hz, H-3′), 4.14−4.13 (m, 1H, H-4′), 4.03−4.0 (m, 2H, H-5′a and H-5′b), and 2.71 (s, 3H, CH_3). ^{31}P (109 MHz, D_2O) δ 0.6 (s). MS (FAB^-) *m/z* 360.9 [(M − H)$^-$, 85%]. HRMS (FAB^-) calcd for $C_{11}H_{14}N_4O_8P$ 361.0549 [(M − H)$^-$], found 361.0634.

Nicotinamide 8-methylhypoxanthine 5′-Dinucleotide (8-Methyl NHD$^+$, 4). To a solution of β-NMN (81 mg, 0.242 mmol) in dry DMF (600 μL) was added carbonyldiimidazole (149 mg, 0.921 mmol) and triethylamine (37 μL, 0.266 mmol). The mixture was stirred at room temperature for 3 h, after which a small amount of MeOH was added to quench the excess CDI. The solvents were removed under vacuum, and the residue was coevaporated three times with DMF. 8-Me-IMP•1.5 TEA salt **10c** (82 mg, 0.173 mmol) was added along with DMF (600 μL), and the mixture was stirred at room temperature for 5 days. The solvent was removed under reduced pressure, and the residue was applied to Q Sepharose (1 M TEAB) followed by AG MP-1 ion exchange (150 mM TFA) to produce the desired dinucleotide **4** as a free acid (65 mg, 55%). ^{1}H NMR (270 MHz, D_2O) δ 9.20 (s, 1H, H_N2), 9.02 (s, 1H, H_N6), 8.73 (d, 1H, $J_{4,5} = 7.1$ Hz, H_N4), 8.09 (br s, 1H, H_N5), 7.98 (s, 1H, H-2), 5.95 (d, 1H, $J_{1'',2''} = 4.3$ Hz, H-1″), 5.78 (d, 1H, $J_{1',2'} = 5.8$ Hz, H-1′), 4.91−4.88 (m, 1H, H-2′), 4.42−4.09 (m, 9H, H_{sugar}), and 2.43 (s, 3H, CH_3). ^{31}P (109 MHz, D_2O) δ −10.9 (br s). MS (FAB^-) *m/z* 677.1 [(M − H)$^-$, 10%]. HRMS (FAB^-) calcd for $C_{22}H_{27}N_6O_{15}P_2$ 677.1010 [(M − H)$^-$], found 677.1019. UV (H_2O, pH 5.6) λ_{max} 249 nm (ϵ 10 600).

Cyclic 8-Methylinosine 5′-Diphosphate Ribose (8-Methyl cIDPR, 1h). 8-Methyl-NHD$^+$ free acid **4** (90 mg, 96 μmol) was incubated with *Aplysia* cyclase (270 μL) in a 25 mM HEPES buffer (210 mL, pH 4) at room temperature. After 6 h, HPLC analysis showed completion of the reaction ($t_{R(nicotinamide)} = 1.7$ min and $t_{R(8-Me-cIDPR)} = 7.7$ min). It was then applied to a Q Sepharose ion exchange column with elution with 1 M TEAB buffer. The appropriate fractions were collected, evaporated under vacuum, and coevaporated with MeOH to afford the desired cyclized product **1h** as a triethylammonium salt (25 mg, 34%). ^{1}H NMR (400 MHz, D_2O) δ 8.73 (s, 1H, H-2), 5.92 (s, 1H, $J_{1'',2''} = 5.7$ Hz, H-1″), 5.83 (br s, 1H, H-1′), 5.20 (app t, 1H, $J_{2',1'} = J_{2',3'} = 5.2$ Hz, H-2′), 4.52 (dd, 1H, $J_{3',2'} = 5.2$ and $J_{3',4'} = 2.4$ Hz, H-3′), 4.47−4.43 (s, 1H, H-5′a), 4.33−4.30 (m, 1H, H-5″a), 4.26−4.21 (m, 4H, H-2″, H-3″, H-4″, and H-4′), 4.07−4.02 (m, 1H, H-5″b), 3.97−3.93 (m, 1H, H-5′b), and 2.45 (s, 3H, CH_3). ^{31}P (109 MHz, D_2O) δ −10.1 (br s) −10.8 (br s). MS (FAB^-) *m/z* 554.9 [(M − H)$^-$, 60%]. HRMS (ES^+) calcd for $C_{16}H_{23}N_4O_{14}P_2$ 557.0681 (MH)$^+$ found 557.0683. UV (H_2O, pH 5.7) λ_{max} 251 nm (ϵ 15 400).

Synthesis of 8-Amino-cIDPR (1e). 2′,3′,5′-Tri-*O*-acetyl-8-bromoinosine (13). Compound **13** was synthesized according to a known procedure,[32] giving a shiny white solid (84% yield). ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.24 (s, 1H, H-2), 6.21 (dd, 1H, $J_{2',3'} = 5.9$ and $J_{2',1'} = 4.9$ Hz, H-2′), 6.08 (d, 1H, $J_{1',2'} = 4.9$ Hz, H-1′), 5.78−5.74 (m, 1H, H-3′), 4.51−4.46 (m, 1H, H-4′), 4.40−4.27 (m, 2H, H-5′a and H-5′b), 2.14 (s, 3H, OAc), 2.09 (s, 3H, OAc), and 2.05 (s, 3H, OAc).

8-Bromoinosine (14a). 2′,3′,5′-Tri-*O*-acetyl-8-bromoinosine **13** (1.5 g, 3.17 mmol) was dissolved in a methanolic ammonia solution (saturated at 0 °C, 10 mL), and the mixture was stirred at room

temperature for 5 h. The resulting precipitate was filtered and washed with a small portion of methanol, yielding 8-bromoinosine **14a** (1 g, 95%). ^{1}H NMR (270 MHz, DMSO-d_6) δ 8.12 (s, 1H, H-2), 5.81 (d, 1H, $J_{1',2'} = 6.4$ Hz, H-1′), 5.53 (br s, 1H, OH), 5.32 (br s, 1H, OH), 5.02 (m, 1H, H-2′), 4.20−4.17 (m, 1H, H-3′), 3.92 (dd, 1H, $J_{4',3'} = 8.2$ and $J_{4',5'a} = 5.0$ Hz, H-4′), 3.65 (dd, 1H, $J_{5'a,5'b} = 11.8$ and $J_{5'a,4'} = 5.0$ Hz, H-5′a), 3.50 (dd, 1H, $J_{5'b,5'a} = 11.8$ and $J_{5'b,4'} = 4.7$ Hz, H-5′b), and 3.22 (br s, 1H, OH). MS (ES$^-$) *m*/*z* 345 [(M − H)$^-$, 100%]. Mp 196−198 °C (lit.[62] mp 196−198 °C).

8-Azidoinosine (14b). To a solution of 8-bromoinosine **14a** (100 mg, 0.288 mmol) in dry DMSO (1 mL) was added sodium azide (60 mg, 0.922 mmol). The mixture was stirred at 70 °C in the dark for 24 h, after which another portion of sodium azide (60 mg, 0.922 mmol) was added and stirring continued for a further 24 h. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel with elution with DCM/MeOH (8:2) to yield the desired product **14b** as a pale-yellow solid (76 mg, 86%). ^{1}H NMR (270 MHz, DMSO-d_6) δ 8.06 (s, 1H, H-2), 5.64 (d, 1H, $J_{1',2'} = 6.2$ Hz, H-1′), 5.45 (d, 1H, $J_{OH,2'} = 6.2$ Hz, 2′-OH), 5.21 (d, 1H, $J_{OH,3'} = 5.0$ Hz, 3′-OH), 4.90 (t, 1H, $J_{OH,5'} = 5.5$ Hz, 5′-OH), 4.80 (m, 1H, H-2′), 4.13 (dd, 1H, $J_{3',4'} = 8.4$ and $J_{3',2'} = 5.1$ Hz, H-3′), 3.89−3.84 (dd, 1H, $J_{4',3'} = 8.2$ and $J_{4,5'a} = 4.9$ Hz, H-4′), 3.61 (dd, 1H, $J_{5'a,5'b} = 11.9$ and $J_{5'a,4'} = 4.9$ Hz, H-5′a), and 3.48 (dd, 1H, $J_{5'b,5'a} = 11.9$ and $J_{5'b,\ 4'} = 5.2$ Hz, H-5′b). MS (ES$^-$) *m*/*z* 308.3 [(M − H)$^-$, 100%]. Mp 210−212 °C (lit.[62] mp >200 °C).

8-Azidoinosine 5′-Monophosphate (15a). Compound **15a** was obtained from 8-azidoinosine **14b** following the same procedure as for **10a** (67 mg, 84%). ^{1}H NMR (270 MHz, D_2O) δ 8.08 (s, 1H, H-2), 5.88 (d, 1H, $J_{1',2'} = 5.4$ Hz, H-1′), 4.97 (dd, 1H, $J_{2',3'} = 5.7$ and $J_{2',1'} = 5.4$ Hz, H-2′), 4.48 (dd, 1H, $J_{3',2'} = 5.7$ and $J_{3',4'} = 4.9$ Hz, H-3′), 4.15 (app q, 1H, $J_{4',3'} = J_{4',5'} = 4.9$ Hz, H-4′), and 4.04−3.88 (m, 2H, H-5′a and H-5′b). ^{31}P (109 MHz, D_2O), 1.9 (s). MS (ES$^-$) *m*/*z* 388.1 [(M − H)$^-$, 50%]. HRMS (ES$^-$) calcd for $C_{10}H_{11}N_7O_8P$ 388.0412 [(M − H)$^-$], found 388.0411.

8-Aminoinosine 5′-Monophosphate (15b). 8-Azido-IMP **15a** (65 mg, 0.158 mmol) was dissolved in 0.05 M TEAB (100 mL, pH 8.5), and dithiothreitol (243 mg, 1.58 mmol) was added. The reaction mixture was stirred at room temperature in the dark for 18 h, after which HPLC analysis showed completion of the reaction ($t_{R(8-azido-IMP)} = 9.0$ min, $\lambda_{max} = 275.8$ nm and $t_{R(8-amino-IMP)} = 8.4$ min, $\lambda_{max} = 268$ nm). The solvent was removed in vacuo, and the residue was purified on a reverse-phase column with elution with a gradient of MeCN in 0.05 M TEAB to afford the desired product **15b** as its triethylammonium salt (55 mg, 68%). ^{1}H NMR (270 MHz, D_2O) δ 7.90 (s, 1H, H-2), 5.93 (d, 1H, $J_{1',2'} = 7.7$ Hz, H-1′), 4.67 (dd, 1H, $J_{2',1'} = 7.7$ and $J_{2',3'} = 6.0$ Hz, H-2′), 4.48 (dd, 1H, $J_{3',2'} = 6.0$ and $J_{3',4'} = 2.5$ Hz, H-3′), 4.26−4.24 (m, 1H, H-4′), and 4.03−4.06 (m, 2H, H-5′a and H-5′b). ^{31}P (109 MHz, D_2O) δ 1.5 (s). MS (ES$^-$) *m*/*z* 362.0 [(M − H)$^-$, 100%]. HRMS (ES$^-$) calcd for $C_{10}H_{13}N_5O_8P$ 362.0507 [(M − H)$^-$], found 362.0507.

Nicotinamide 8-aminohypoxanthine 5′-Dinucleotide (8-Amino NHD$^+$, 5). 8-Amino-IMP·1.5TEA salt **15b** (42 mg, 0.081 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 was added morpholine (38 μL, 0.440 mmol), dipyridyl disulfide (46 mg, 0.214 mmol), and triphenylphosphine (56 mg, 0.214 mmol), at which point the solution became bright-yellow. The mixture was stirred for 1 h at room temperature, after which HPLC analysis showed completion of the reaction ($t_{R(8-amino-IMP)} = 8.7$ min and $t_{R(8-amino-IMP\ morpholidate)} = 2.8$ min). Precipitation of the product occurred by dropwise addition of a solution of NaI in acetone (0.1 M, 8 mL). The resulting precipitate **16** was filtered, washed with acetone, dried, and then reacted with β-NMN (42 mg, 0.125 mmol) and $MgSO_4$ (27 mg, 0.228 mmol) in a 0.2 M solution of $MnCl_2$ in formamide (0.85 mL) at room temperature overnight, after which HPLC analysis showed completion of the reaction ($t_{R(\beta-NMN)} = 2.1$ min and $t_{R(8-amino-NHD)} = 3.3$ min). Precipitation of the product occurred by addition of MeCN; it was filtered, dissolved in MilliQ water, and applied to a reverse-phase column with elution with a gradient of MeCN in 0.05 M TEAB. Further treatment with Chelex-100 to remove any paramagnetic particles afforded the desired product **5** as a sodium salt (20.2 mg, 37% over two steps). ^{1}H NMR (400 MHz, D_2O) δ 9.19 (s, 1H, H_N2), 9.05 (d, 1H, $J = 6.5$ Hz, H_N6), 8.71 (d, 1H, $J = 7.9$ Hz, H_N4), 8.07 (dd, 1H, $J = 7.9$ and 6.5 Hz, H_N5), 7.81 (s, 1H, H-2), 6.03 (d, 1H, $J_{1'',2''} = 4.5$ Hz, H-1″), 5.71 (d, 1H, $J_{1',2'} = 6.6$ Hz, H-1′), 4.59 (app t, 1H, $J_{2',1'} = J_{2',3'} = 6.6$ Hz, H-2′), 4.47 (br s, 1H, H-4″), 4.39−4.33 (m, 4H, H-2″, H-3′, H-3″, and H-5′a), and 4.18−4.15 (m, 4H, H-4′, H-5′b, H-5″a, and H-5″b). ^{31}P (109 MHz, D_2O) δ −11.6. MS (ES$^-$) *m*/*z* 678.3 [(M − H)$^-$,100%]. HRMS (ES$^-$) calcd for $C_{21}H_{27}N_7O_{15}P_2$ 678.0968 [(M − H)$^-$], found 678.0964. UV (H_2O, pH 6.0) λ_{max} 260 nm (ϵ 12 500).

Cyclic 8-Aminoinosine 5′-Diphosphate Ribose (8-Amino-cIDPR, 1e). 8-Amino-NHD$^+$ **5** (20 mg, 29 μmol) was incubated with *Aplysia* cyclase (60 μL) in a 25 mM HEPES buffer (200 mL, pH 4) at room temperature. After 24 h, HPLC analysis showed completion of the reaction ($t_{R(nicotinamide)} = 1.7$ min and $t_{R(8-amino-cIDPR)} = 8.8$ min). The crude product was then applied to a Q Sepharose ion exchange column with elution with a linear gradient of 1 M TEAB buffer against MilliQ water. The appropriate fractions were collected, evaporated under reduced pressure, coevaporated with MeOH to remove excess triethylammonium salt, and treated with Chelex-100 to afford the desired cyclized product **1e** as a sodium salt (12.5 μmol, 43%). ^{1}H NMR (400 MHz, D_2O) δ 8.73 (s, 1H, H-2), 6.02 (br s, 1H, H-1″), 5.81 (d, $J_{1',2'} = 6.1$ Hz, 1H, H-1′), 5.36−5.33 (m, 1H, H-2′), 4.63 (dd, $J_{3',2'} = 4.8$ and $J_{3',4'} = 2.5$ Hz, 1H, H-3′), 4.52−4.47 (m, 1H, H-5′a), 4.43−4.39 (m, 1H, H-1″a), 4.30 4.26−4.16 (m, 3H, H-2″, H-3″, and H-4″), 4.29−4.27 (m, 1H, H-4′), 4.15 (dd, $J_{5''b,5''a} = 11.7$ and $J_{5''b,4''} = 4.3$ Hz, 1H, H-5″b), and 4.07−4.02 (m, 1H, H-5′b). ^{31}P (109 MHz, D_2O) δ −10.3 (br s) −11.2 (br s). MS (ES$^-$) *m*/*z* 556.1 [(M − H)$^-$, 30%]. HRMS (ES$^-$) calcd for $C_{15}H_{20}N_4O_{14}P_2$ 556.0487 [(M − H)$^-$], found 556.0492. UV (H_2O) λ_{max} 251 nm (ϵ 15 365).

Synthesis of 7-Deaza-NHD$^+$ (17). 4-Chloropyrrolo[2,3-*d*]-pyrimidine (18). Compound **18** was synthesized in five steps by a procedure developed by Davoll,[33] giving a gray solid (28% yield over five steps). ^{1}H (270 MHz, DMSO-d_6) δ 8.58 (s, 1H, H-2), 7.69 (s, 1H, H-7) and 6.60 (d, 1H, $J = 3.8$ Hz, H-8). MS (EI) *m*/*z* 153.0 [M$^+$, 15%]. HRMS (ES$^+$) calcd for $C_6H_5N_3{}^{35}Cl$ 154.0167 (MH)$^+$, found 154.0166. Mp 184−186 °C (lit.[33] mp 189−190 °C).

5-*O*-*tert*-Butyldimethylsilyl-2,3-*O*-isopropylidene-β-D-ribofuranose (19). Compound **19** was synthesized in two steps by known procedures,[63,64] giving a white crystalline solid (71% yield over two steps). ^{1}H (270 MHz, $CDCl_3$) δ 5.36 (d, 1H, $J_{1,2} = 11.9$ Hz, H-1), 4.77 (d, 1H, $J_{2,3} = 5.9$ Hz, H-2), 4.57 (d, 1H, $J_{3,2} = 5.9$ Hz, H-3), 4.44 (br s, 1H, H-4), 3.83 (m, 2H, H-5a and H-5b), 1.56 (s, 3H, CH_3), 1.40 (s, 3H, CH_3), 1.0 (s, 9H, ^{t}Bu), and 0.27 (s, 6H, 2 × CH_3). MS (FAB$^+$) *m*/*z* 287 (M$^+$ + H − OH, 100%). Mp 47−49 °C (lit.[63] mp 47 °C).

4-Chloro-7-(5′-*O*-*tert*-butyldimethylsilyl-2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)pyrrolo[2,3-*d*] pyrimidine (20). A solution of 5-*O*-*tert*-butyldimethylsilyl-2,3-*O*-isopropylidene-D-ribofuranose **19** (500 mg, 1.644 mmol) and CCl_4 (240 μL, 2.545 mmol) in THF (6 mL) was cooled to −78 °C and treated dropwise with hexamethylphosphorus triamide (380 μL, 2.134 mmol) over 15 min. After being stirred at −78 °C for 2 h, the mixture was slowly warmed to room temperarure, evaporated to half its volume, and used as such in the next step (hexane/EtOAc, 8:2, $R_f = 0.8$). A suspension of finely powdered KOH (169 mg, 3.01 mmol), TDA-1 (8 μL, 0.027 mmol), and 4-chloropyrrolo[2,3-*d*]pyrimidine **18** (209 mg, 1.369 mg) in dry MeCN (8 mL) was stirred for 15 min, after which the freshly prepared solution of the chloro sugar **19** (530 mg, 1.643 mmol, assuming 100% yield) was added and the mixture was stirred for 6 h at room temperature under nitrogen. The solvents were removed under reduced pressure, and the crude mixture was purified on silica gel with elution with hexane/EtOAc (9:1) to produce the desired protected nucleoside **20** as a pale-yellow oil (391 mg, 65%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.63 (s, 1H, H-2), 7.54 (d, 1H, $J_{7,8} = 3.8$ Hz, H-7), 6.59 (d, 1H, $J_{8,7} = 3.8$ Hz, H-8), 6.38 (d, 1H, $J_{1',2'} = 3.0$ Hz, H-1′), 5.04 (dd, 1H, $J_{2',3'} = 7.2$ and $J_{2',1'} = 3.0$ Hz, H-2′), 4.93 (dd, 1H, $J_{3',2'} = 7.2$ and $J_{3',4'} = 3.0$ Hz,

H-3′), 4.35−4.31 (m, 1H, H-4′), 3.87 (dd, 1H, $J_{5'a,5'b}$ = 11.4 and $J_{5'a',4'}$ = 3.5 Hz, H-5′a), 3.77 (dd, $J_{5'b,5'a}$ = 11.4 and $J_{5'b,4'}$ = 3.8 Hz, 1H, H-5′b), 1.62 (s, 3H, CH_3), 1.36 (s, 3H, CH_3), 0.85 (s, 9H, ^{t}Bu), and 0.03 (s, 6H, 2 × CH_3). R_f = 0.66 (hexane/EtOAc, 8:2). MS (FAB$^+$) *m*/*z* 440.1 [(M + H)$^+$, 65%]. HRMS (FAB$^+$) calcd for $C_{20}H_{31}N_3O_4Si^{35}Cl$ 440.1772 (MH)$^+$, found 440.1747; calcd for $C_{20}H_{31}N_3O_4Si^{37}Cl$ 442.1742 [(M + H)$^+$], found 442.1732.

4-Methoxy-7-(5′-*O*-*tert*-butyldimethylsilyl-2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (21). 4-Chloro-7-(5′-*O*-*tert*-butyldimethylsilyl-2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine **20** (560 mg, 1.27 mmol) was added to a freshly prepared solution of 1 N NaOMe (58 mg, 2.55 mmol). The resulting solution was then refluxed for 30 min, after which TLC analysis showed completion of the reaction (hexane/EtOAc, 8:2, R_f = 0.56). The mixture was then neutralized with Dowex 50WX4-50 ion-exchange and filtered. The filtrate was concentrated under reduced pressure to leave an oil, which was purified by flash chromatography with elution with hexane/EtOAc (9:1) to produce the title compound **21** as a colorless oil (368 mg, 67%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.43 (s, 1H, H-2), 7.23 (d, 1H, $J_{8,7}$ = 3.6 Hz, H-8), 6.49 (d, 1H, $J_{7,8}$ = 3.6 Hz, H-7), 6.33 (d, 1H, $J_{1',2'}$ = 2.9 Hz, H-1′), 5.08 (dd, 1H, $J_{2',3'}$ = 6.4 and $J_{2',1'}$ = 2.9 Hz, H-2′), 4.92 (dd, 1H, $J_{3',2'}$ = 6.4 and $J_{3',4'}$ = 3.2 Hz, H-3′), 4.26 (dd, 1H, $J_{4',5'a}$ = 4.1 and $J_{4',3'}$ = 3.2 Hz, H-4′), 4.07 (s, 3H, OMe), 3.82 (dd, 1H, $J_{5'a,5'b}$ = 11.2 and $J_{5'a,4'}$ = 4.1 Hz, H-5′a), 3.73 (dd, 1H, $J_{5'b,5'a}$ = 11.2 and $J_{5'b,4'}$ = 4.1 Hz, H-5′b), 1.59 (3H, s, CH_3), 1.34 (3H, s, CH_3), 0.85 (9H, s, ^{t}Bu), and 0.008 (6H, s, 2 × CH_3). MS (FAB$^+$) *m*/*z* 436.5 [(M + H)$^+$, 10%]. HRMS (ES$^+$) calcd for $C_{21}H_{34}N_3O_5Si$ 436.2262 (MH)$^+$, found 436.2263.

4-Methoxy-7-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (22). 4-Methoxy-7-(5′-*O*-*tert*-butyldimethylsilyl-2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine **21** (300 mg, 0.686 mmol) was dissolved in a 75% aqueous TFA solution (10 mL) and stirred at 0 °C for 30 min, after which TLC analysis showed completion of the reaction (DCM/MeOH, 9:1, R_f = 0.48). It was then evaporated to dryness, and the residue was coevaporated three times with MeOH to remove traces of TFA and purified by column chromatography with elution with DCM/MeOH (95:5) to give the deprotected nucleoside **22** as a white solid (190 mg, 98%). ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.43 (s, 1H, H-2), 7.67 (d, 1H, $J_{8,7}$ = 3.6 Hz, H-8), 6.59 (d, 1H, $J_{7,8}$ = 3.6 Hz, H-7), 6.15 (d, 1H, $J_{1',2'}$ = 6.2 Hz, H-1′), 4.41 (app t, 1H, $J_{2',3'}$ = $J_{2',1'}$ = 6.2 Hz, H-2′), 4.11 (m, 1H, H-3′), 4.04 (s, 3H, OMe), 3.90 (m, 1H, H-4′), 3.62 (dd, 1H, $J_{5'a,5'b}$ = 11.9 and $J_{5'a,4'}$ = 3.7 Hz, H-5′a), 3.53 (dd, 1H, $J_{5'b,5'a}$ = 11.9 and $J_{5'b,4'}$ = 3.9 Hz, H-5′b). MS (FAB$^+$) *m*/*z* 282.3 [(M + H)$^+$, 80%]. HRMS (ES$^+$) calcd for $C_{12}H_{16}N_3O_5$ 282.1084 (MH)$^+$, found 282.1084.

4-Hydroxy-7β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (7-Deazainosine, 23). A solution of 4-methoxy-7β-D-ribofuranosyl)-pyrrolo[2,3-*d*] pyrimidine **22** (195 mg, 0.448 mmol) in MeCN (15 mL) was treated with sodium iodide (69 mg, 0.461 mmol) and trimethylsilyl chloride (59 μL, 0.461 mmol). The reaction mixture was heated to reflux for 4 h, after which TLC analysis showed the reaction was completed (DCM/MeOH, 9:1, R_f = 0.12). The mixture was then cooled to 0 °C, and the precipitate was filtered and recrystallized from aqueous MeOH to yield the title compound **23** as a white solid (85 mg, 71%). ^{1}H NMR (270 MHz, DMSO-d_6) δ 11.95 (s, 1H, NH), 7.90 (d, 1H, $J_{2,NH}$ = 3.2 Hz, H-2), 7.36 (d, 1H, $J_{8,7}$ = 3.6 Hz, H-8), 6.51 (d, 1H, $J_{7,8}$ = 3.6 Hz, H-7), 5.99 (d, 1H, $J_{1',2'}$ = 6.1 Hz, H-1′), 5.29 (d, 1H, $J_{OH,2'}$ = 6.1 Hz, 2′-OH), 5.10 (d, 1H, $J_{OH,3'}$ = 4.6 Hz, 3′-OH), 4.99 (t, 1H, $J_{OH,5'}$ = 4.8 Hz, 5′-OH), 4.30 (app q, 1H, $J_{2',1'}$ = $J_{2',3'}$ = $J_{2',OH}$ = 6.1 Hz, H-2′), 4.05 (m, 1H, H-3′), 3.87 (dd, 1H, $J_{4',5'a}$ = 7.4 and $J_{4',3'}$ = 3.9 Hz, H-4′), and 3.63−3.46 (2H, m, H-5a′ and H-5′b). Mp 240−243 °C. MS (FAB$^+$) *m*/*z* 268.0 [(M + H)$^+$, 10%]. HRMS (FAB$^+$) calcd for $C_{11}H_{14}N_3O_5$ 268.0933 (MH)$^+$, found 268.0955. Anal. ($C_{11}H_{13}N_3O_5$·0.5NaI) C, H, N.

7-Deazainosine 5′-Monophosphate (24). Compound **24** was obtained from 7-deazainosine **23** following the same procedure as for **10a** (82 mg, 85%). ^{1}H (270 MHz, D_2O) δ 7.98 (s, 1H, H-2), 7.40 (d, 1H, $J_{8,7}$ = 3.7 Hz, H-8), 6.68 (d, 1H, $J_{7,8}$ = 3.7 Hz, H-7), 6.17 (d, 1H, $J_{1',2'}$ = 6.5 Hz, H-1′), 4.60−4.56 (m, 1H, H-2′), 4.40 (dd, 1H, $J_{3',2'}$ = 5.2 and $J_{3',4'}$ = 3.0 Hz, H-3′), 4.29−4.28 (m, 1H, H-4′), and 4.09−4.07 (m, 2H, H-5′a and H-5′b). ^{31}P (109 MHz, D_2O) δ 0.73 (s). MS (FAB$^-$) *m*/*z* 346.3 [(M − H)$^-$, 10%]. HRMS (FAB$^-$) calcd for $C_{11}H_{13}N_3O_8P$ 346.0440 [(M − H)$^-$], found 346.0438.

Nicotinamide 7-deazahypoxanthine 5′-Dinucletide (7-Deaza-NHD$^+$, 17). To a solution of β-NMN (60 mg, 0.179 mmol) in dry DMF (500 μL) was added carbonyldiimidazole (110 mg, 0.680 mmol) and triethylamine (27 μL, 0.197 mmol). The mixture was stirred at room temperature for 3 h, after which a small amount of MeOH was added to quench the excess CDI. The solvents were removed under vacuum, and the residue was coevaporated three times with DMF. 7-Deaza-IMP (50 mg, 0.143 mmol) was added along with DMF (400 μL), and the mixture was stirred at room temperature for 4 days. The solvent was removed under reduced pressure, and the residue was applied to a Q Sepharose (1 M TEAB) ion-exchange column with elution with a linear gradient of 1 M TEAB against MilliQ water. The appropriate fractions were combined and evaporated under vacuum. The residue was coevaporated with MeOH to remove excess salt and lyophilized overnight to afford the desired dinucleotide **17** as a triethylammonium salt (35 mg, 0.055 mmol, 39%). ^{1}H NMR (400 MHz, D_2O) δ 9.24 (s, 1H, H_N2), 9.09 (d, 1H, $J_{6,5}$ = 6.2 Hz, H_N6), 8.72 (dt, 1H, $J_{4,5}$ = 8.2 and $J_{4,6}$ = 1.4 Hz, H_N4), 8.10 (dd, 1H, $J_{5,4}$ = 8.2 and $J_{5,6}$ = 6.2 Hz, H_N5), 7.93 (s, 1H, H-2), 7.32 (d, 1H, $J_{8,7}$ = 3.8 Hz, H-8), 6.55 (d, 1H, $J_{7,8}$ = 3.8 Hz, H-7), 6.06 (d, 1H, $J_{1',2'}$ = 6.5 Hz, H-1″), 6.04 (d, 1H, $J_{1'',2''}$ = 5.2 Hz, H-1′), 4.53 (dd, 1H, $J_{2',1'}$ = 6.5 and $J_{2',3'}$ = 5.4 Hz, H-2′), 4.48−4.46 (m, 1H, H-4′), 4.40 (app t, $J_{2'',1''}$ = $J_{2'',3''}$ = 5.2 Hz, 1H, H-2″), 4.37−4.35 (m, 1H, H-3′), 4.33 (dd, 1H, $J_{3'',2''}$ = 5.2 and $J_{3'',4''}$ = 3.8 Hz, H-3″), 4.27−4.25 (m, 1H, H-5′a), 4.23 (app q, 1H, J = 2.7 Hz, H-4″), 4.14−4.08 (m, 3H, H-5″a, H-5′b and H-5″b). ^{31}P (109 MHz, D_2O) δ −10.5 (d, J = 18.8 Hz), and −10.9 (d, J = 18.8 Hz). MS (ES$^-$) *m*/*z* 662.3 [(M − H)$^-$, 100%]. HRMS (ES$^-$) calcd for $C_{22}H_{26}N_5O_{15}P_2$ 662.0895 [(M − H)$^-$], found 662.0897. UV (H_2O, pH 5.8) λ_{max} 259 nm (ε 12 200).

Enzymatic Hydrolysis of 17. 7-Deaza-NHD$^+$ sodium salt **17** (35 mg, 0.055 mmol) was incubated with *Aplysia* cyclase (150 μL) in a 25 mM HEPES buffer (125 mL, pH 4) at room temperature. After 4 days at room temperature, HPLC analysis showed completion of the reaction ($t_{R(product)}$ = 11.1 min). The mixture was then applied to a Q Sepharose ion-exchange column with elution with a lineat gradient of 1 M TEAB buffer against MilliQ water. The appropriate fractions were collected, evaporated under vacuum, and coevaporated with MeOH to afford the hydrolyzed product 7-deaza-IDPR **25** as a glassy solid in the triethylammonium form. ^{1}H NMR (400 MHz, D_2O) δ 8.05 (s, 1H, H-2), 7.51 (d, 1H, $J_{8,7}$ = 3.8 Hz, H-8), 6.76 (d, 1H, $J_{7,8}$ = 3.8 Hz, H-7), 6.22 (d, 1H, $J_{1',2'}$ = 6.7 Hz, H-1′), 5.28 (d, 0.5H, $J_{1'',2''}$ = 4.0 Hz, H_β-1″), 5.16 (d, 0.5H, $J_{1'',2''}$ = 2.2 Hz, H_α-1″), 4.64 (dd, 1H, $J_{2',1'}$ = 6.7 and $J_{2',3'}$ = 5.4 Hz, H-2′), 4.47−4.45 (m, 1H, H-4′), 4.29−4.21 (m, 2H), 4.12−4.09 (m, 2H), 4.05−3.94 (m, 3H), and 3.86−3.82 (m, 1H). ^{31}P (109 MHz, D_2O) δ −10.5 (br s) and −10.7 (br s). MS (ES$^-$) *m*/*z* 558.2 [(M − H)$^-$, 100%]. HRMS (ES$^-$) calcd for $C_{16}H_{22}N_3O_{15}P_2$ 558.0532 [(M − H)$^-$], found 558.0538. UV (H_2O, pH 6.1) λ_{max} 260 nm (ε 14 900).

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Supporting Information Available: Elemental analysis data for compounds **8**, **9**, and **23**; ^{1}H NMR and HPLC data for targets compounds **1e**−**h** and **25**; 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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