SAR Analysis of Adenosine Diphosphate (Hydroxymethyl)pyrrolidinediol Inhibition of Poly(ADP-ribose) Glycohydrolase

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Received December 3, 2002

Polyadenosine diphosphoribose glycohydrolase (PARG) catalyzes the intracellular hydrolysis of adenosine diphosphoribose polymers. Because structure−activity data are lacking for PARG, the specific inhibitor adenosine diphosphate (hydroxymethyl)pyrrolidinediol (ADP-HPD) was utilized to determine the effects of structure on inhibitor potency using PARG isolated from bovine thymus (bPARG) and recombinant bovine PARG catalytic fragment (rPARG-CF). Both enzymes were strongly inhibited by submicromolar levels of ADP-HPD, but ADP and the phosphorylated pyrrolidine displayed no activity. Utilizing ADP-HPD analogues containing 2-, N^6, or 8-adenosyl substituents or guanine instead of adenine, the importance of adenine ring recognition as well as a correlation between loss of PARG inhibition and the length and bulkiness of 8-adenosyl substituents was shown. Utilization of ADP-HPD analogues lacking one or both pyrrolidine *cis*-hydroxyls demonstrated their importance for inhibitor binding. Last, the similarity between naturally occurring bPARG and heterologously expressed rPARG-CF was demonstrated. Therefore, readily available rPARG-CF is suitable for use in future studies to determine the structural aspects of PARG.

Introduction

The metabolism of adenosine diphosphoribose (ADP-ribose) polymers is of importance to the maintenance of genomic integrity of the multicellular organism and in regulating the response to DNA damage. Polymers are synthesized by the poly(ADP-ribose) polymerase (PARP) family of enzymes, which utilize nicotinamide adenine dinucleotide (NAD^+) as substrate to covalently modify acceptor proteins with ADP-ribose polymers.[1−3] The typical characteristics of these polymers, which can attain lengths of 200 ADP-ribose residues and contain several branches,[1] include a high negative charge and the ability to mimic DNA for noncovalent interactions.[4] PARP-1, the original and best studied PARP, is a DNA damage sensing protein specifically activated by DNA strand breaks.[5] ADP-ribose polymer metabolism following PARP-1 activation facilitates the recovery of dividing cells from DNA damage.[6] Accordingly, inhibition of the metabolism of ADP-ribose polymers was recognized as a strategy for treatment of malignancy.[6] However, two significant discoveries led to the expansion of this therapeutic potential. First, PARP-1 inhibition was shown to prevent massive cell necrosis following exposure of cells to oxidative damage.[7] Second, the discovery of a diverse family of PARP isozymes suggests novel functions for ADP-ribose polymer metabolism.[2] Targeting these cycles offers the additional therapeutic potential to treat telomere-associated immortality,[8] cardiac or cerebral ischemia,[9] diabetes,[10] and Parkinson's disease.[11]

Poly(ADP-ribose) glycohydrolase (PARG) catalyzes the hydrolysis of the O-linked α(1″→2′) or α(1‴→2″) ribosyl−ribose linkages of ADP polymers to produce free ADP-ribose.[3,12] The efficient action of PARG results in the rapid turnover of ADP-ribose polymers,[13−15] thereby reversing ADP ribosylation.

Bovine PARG (bPARG) has been shown to originate from a single copy gene,[16] and to date, no other PARG homologues have been identified within the mammalian genome. Therefore, preliminary evidence suggests that PARG is required to complete the ADP-ribose polymer cycles initiated by the entire PARP family of enzymes. While therapies targeting the metabolism of ADP-ribose polymers have primarily focused on PARP-1 inhibition,[17,18] PARG is also predicted to be a therapeutic target due to its essential function, its aforementioned uniqueness, its presence at low levels, and the unique structure of its substrate within the cell.

Our laboratory recently deduced the cDNA sequence encoding full-length bPARG, which allowed the elucidation of the domain organization of bPARG and enabled the bacterial expression and production of the recombinant PARG catalytic fragment (rPARG-CF).[16] In addition, prior studies identified adenosine diphosphate (hydroxymethyl)pyrrolidine diol (**1**, ADP-HPD), a nitrogen in the ring analogue of ADP-ribose, as a potent and specific inhibitor of PARG with a partial noncompetitive mechanism of inhibition.[19] Thus, the ability to specifically and effectively inhibit PARG has allowed ADP-HPD to be utilized as a versatile tool to further study

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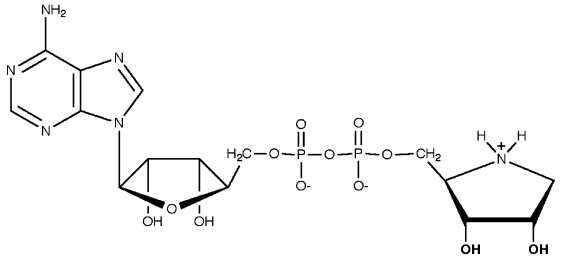
10.1021/jm020541u CCC: $25.00
Published on Web 08/29/2003

1, ADP-HPD



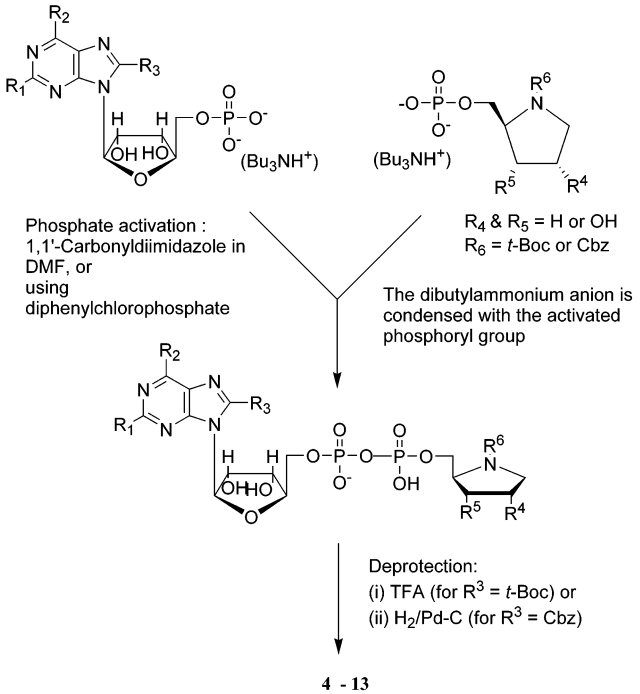
this enzyme. Because structure−activity relationships (SARs) of PARG are poorly understood, we investigated the SAR of naturally occurring bPARG and rPARG-CF obtained from heterologous bacterial expression of the cDNA encoding the bPARG catalytic fragment using ADP-HPD as the lead inhibitor. This paper presents the results of a detailed investigation into the molecular interactions necessary for optimal binding to the PARG active site. We first examined the ability of the major partial structures of **1** to function as PARG inhibitors by determining the potency of ADP and pyrrolidines **2** and **3** as PARG inhibitors. Next, the effect of deoxygenation of the pyrrolidine ring on inhibitor potency is determined using deoxy compounds **4** and **5**. Last, the effect of modification of the purine ring, particularly with respect to 8-substitution, is evaluated using compounds **6**−**13**. Our results offer insight into the interaction between ADP-HPD and its binding site. Furthermore, we show that the active sites of both enzymes are similar as demonstrated by SAR analysis. Therefore, the readily available rPARG-CF may be used in future studies designed to determine the structural aspects of PARG.

Results

Chemistry. The syntheses of ADP-HPD (**1**), pyrrolidine (**2**), and pyrrolidine phosphate (**3**) were performed as described in our previous papers,[20,21] with some modification as noted in the Experimental Section. The synthesis of the novel ADP-HPD analogues **4**−**13** followed the strategy depicted in Scheme 1. 5′-Mononucleotides obtained commercially or through synthesis are converted to their *n*-butylammonium salts, and the phosphate is activated. Activation was accomplished by treating the phosphate anion with carbonyldiimidazole, converting it to the phosphoimidazolide,[22] or by treating it with diphenylchlorophosphate, converting it to the mixed anhydride.[23] The phosphate-activated 5′-mononucleotide was condensed with the butylammonium salt of a pyrrolidine phosphate in which the nitrogen was protected with either a *t*-Boc or a Cbz group. The initial coupling products in which the pyrrolidine nitrogen is protected were purified using anion exchange chromatography and completely characterized. Removal of protection using either acid treatment or hydrogenolysis resulted in the production of analogues **4**−**13**.

Synthesis of the monodeoxygenated ADP-HPD derivative **4** required the production of a new 4-monohydroxylated pyrrolidine, **17** (Scheme 2). This substance was readily available from commercial 4-hydroxy-L-proline using the sequence of reactions depicted in Scheme 2. In this scheme, the proline is first completely protected as **14**, and the methyl ester **14** is converted

Scheme 1. Synthesis of ADP-HPD Analogues



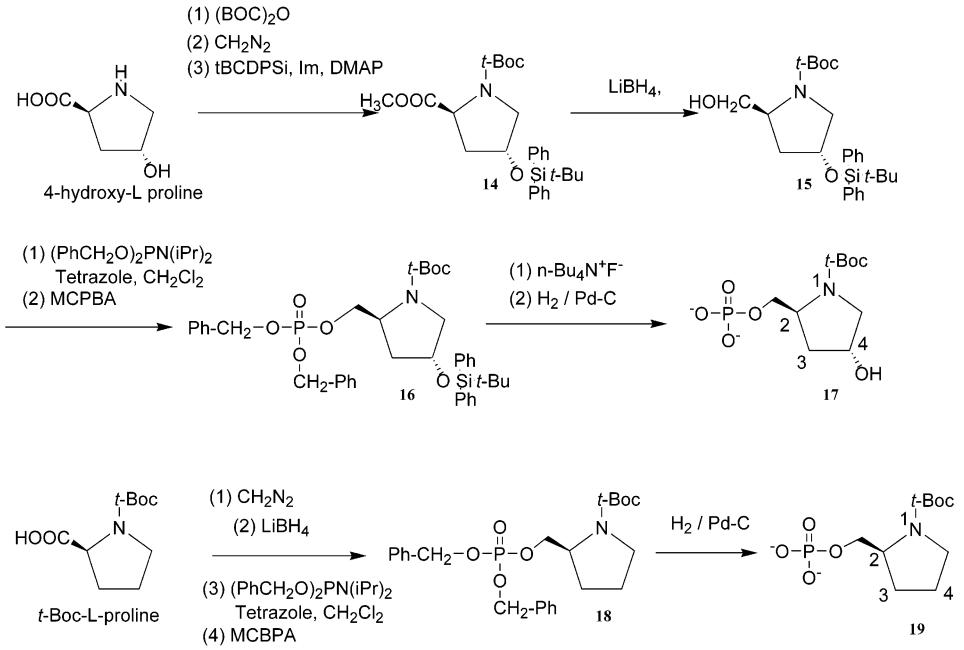
to the alcohol **15** by borohydride reduction. The phosphate is introduced by reaction of the protected alcohol with *N*,*N*-diisopropyldibenzylphosphoramidite, followed by oxidation producing phosphoester **16** in protected form. The dibenzyl-protected phosphate ester **16** was purified easily and in large quantity using flash chromatography on silica gel. It was quantitatively deprotected yielding **17** and converted to its tributylammonium salt for coupling with nucleotides. The dideoxypyrrolidine derivative **19** was similarly available from *t*-Boc-L-proline (Scheme 2) through the intermediacy of the dibenzyl phosphate **18**.

Compounds **8**−**12** required substituted AMP derivatives that are not commercially available. The derivative required for the production of both **8** and **9** was synthesized from commercial 8-(6-aminohexyl)amino-AMP by protection of the amine as a trifluoroacetyl derivative. The synthesis of the 5′-nucleotides required for synthesis of **10**−**12** began with the commercially available nucleosides, 8-bromoadenosine and 6-chloropurine ribofuranoside (Scheme 3). The 6- or 8-substituent was introduced by nucleophilic displacement of the halogen, and the phosphate was introduced by selective phosphorylation of the 5′-hydroxyl using $POCl_3$ in trimethyl phosphate.[24] The final products **10**−**12** were obtained by coupling of the phosphate ester to the protected pyrrolidine phosphate and subsequent deprotection as stated above and previously described in Scheme 1.[23,25]

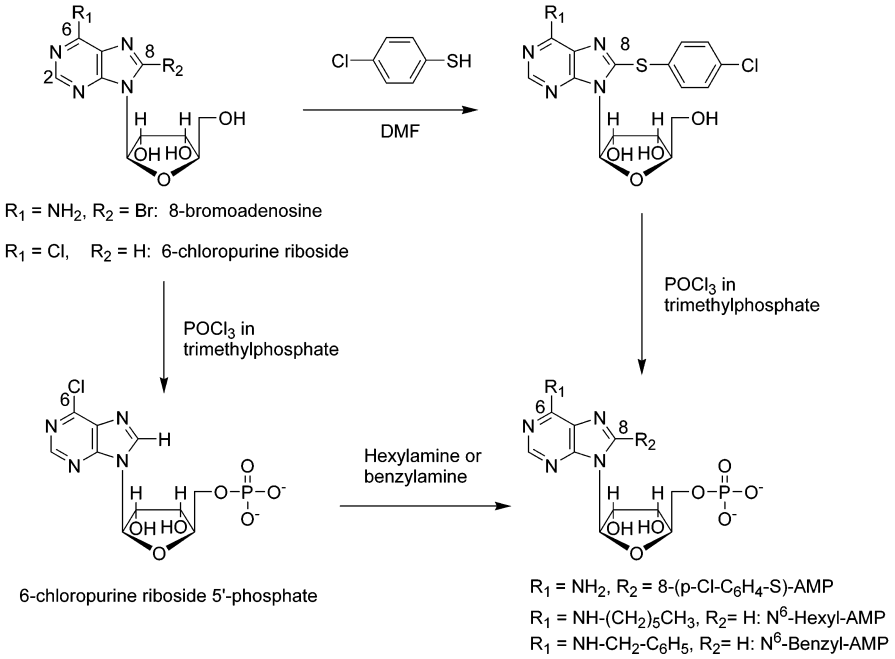
SAR Studies. The rPARG-CF and bPARG utilized were similar in size and antigenicity to polyclonal anti-bPARG antibodies,[26] and each was catalytically active (see below). Because of these similarities, each was used to characterize the pattern of PARG catalytic activity inhibition in order to interpret the molecular requirements needed to produce optimal interaction between substrate and enzyme by SAR analysis.

Elucidation of PARG Active Site Molecular Interactions with Substrate. Several PARG inhibi-

Scheme 2. Synthesis of Modified Pyrrolidines



Scheme 3. Synthesis of Modified Adenosines



tors have been reported,[27,28] but the most specific and potent inhibitor reported to date is ADP-HPD (**1**). ADP-HPD was a potent inhibitor of bPARG with an approximate IC_{50} of 0.33 μM (Figure 2A, open squares), which is in agreement with the previously reported value.[20] Several compounds were then assayed to provide initial insight into the structural requirements needed for optimal interaction of ADP-HPD with its PARG binding site. The first series of compounds utilized were the constitutive portions of ADP-HPD. The inability of ADP to inhibit bPARG enzymatic activity, even up to a concentration of 1 mM (Figure 2A, closed squares), indicated the need for the pyrrolidine region of ADP-HPD in order to optimally bind with PARG. Likewise, the inability of HPD **2** (Figure 2A, open circles) or P-HPD **3** (Figure 2A, closed circles), compounds that each constitute the remaining portion of ADP-HPD, to inhibit bPARG activity demonstrated the requirement for the ADP portion as well. Thus, only the sum of these parts displayed inhibition of bPARG activity. The same potencies for these compounds were observed when they were tested as inhibitors of rPARG-CF (Figure 2B), where an $IC_{50} = 1.4$ μM was measured for the inhibition of rPARG-CF by **1**. Because of the inability of the constitutive portions of ADP-HPD to independently inhibit the activity in either enzyme, it was concluded that affinity for the PARG active site requires major binding interactions with both the ADP and the HPD regions of the ADP-HPD molecule.

Significance of the Pyrrolidine *cis*-Hydroxyls in the Interaction of Substrate with the bPARG and rPARG-CF Active Sites. The PARG active site was

Table 1. Activities of Test Compounds as Inhibitors of bPARG and rPARG-CF

	bPARG		rPARG-CF	
compd	logIC_{50} ± error (μM)	IC_{50} (μM)	logIC_{50} ± error (μM)	IC_{50} (μM)
1, ADP-HPD	−0.48 ± 0.03	0.33	+0.148 ± 0.07	1.4
4, ADP-HPM	+0.48 ± 0.05	3.07	+0.623 ± 0.08	4.2
5, ADP-HP	+1.28 ± 0.13	19.2	+1.80 ± 0.18	63.
6, 8-N_3-ADP-HPD	−0.41 ± 0.02	0.39	−0.035 ± 0.02	0.44
7, 2-N_3-ADP-HPD	+2.45 ± 0.04	290	+2.17 ± 0.07	148
13, GDP-HPD	>3	>1000	+2.99 ± 0.06	970

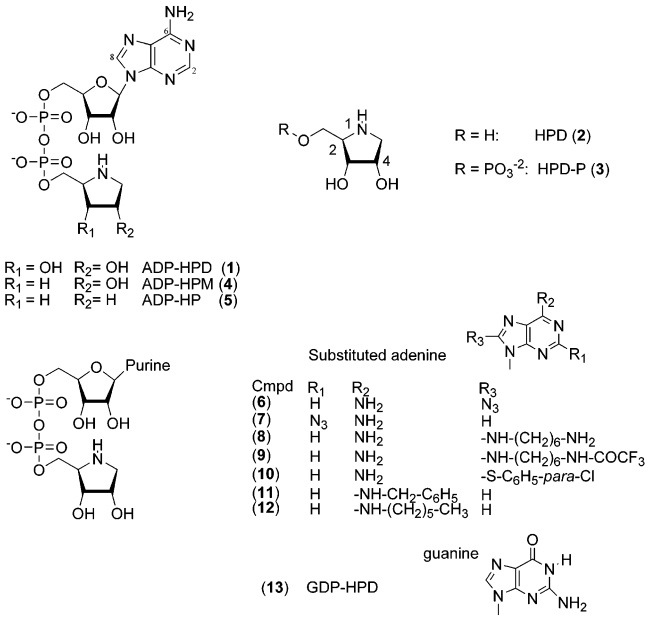


Figure 1. Structures of ADP-HPD and related compounds tested as PARG inhibitors.

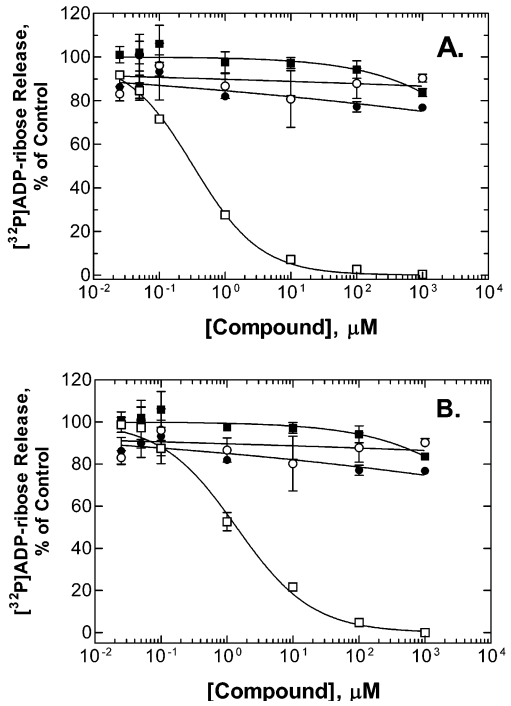


Figure 2. Elucidation of bPARG and rPARG-CF active site molecular interactions. SAR analyses of bPARG (A) and rPARG-CF (B) were performed by adding varying amounts of the inhibitor to the enzyme assay conducted as described in the Experimental Section. Inhibitor concentrations of 0.025, 0.05, 0.1, 1, 10, 100, and 1000 μM were used. The compounds tested as inhibitors were ADP-HPD (open squares), ADP (closed squares), HPD (open circles), and P−HPD (closed circles).

investigated further by utilizing compounds designed to determine the importance of the pyrrolidine *cis*-hydroxyls of ADP-HPD. ADP-HPM (**4**) (Table 1), identical to ADP-HPD but devoid of the pyrrolidine 3-hydroxyl group, was a less potent inhibitor of bPARG catalytic activity as ADP-HPD, since the IC_{50} of this compound was approximately 3 vs 0.3 μM for ADP-HPD (**1**) (Table 1). A more profound effect on inhibition of bPARG activity was seen by removing both pyrrolidine hydroxyls of ADP-HPD to produce **5**, since the IC_{50} value observed for this compound was 19 μM, 6-fold higher than **4** and 60-fold higher than **1**. A similar pattern of inhibition was seen with rPARG-CF, where the IC_{50} value of **4** was 4 μM while the same value for **5** was 63 μM, 15-fold higher. Because the results demonstrated significant differences in inhibition by comparing the effect of **1** vs **4** and **5** on the enzymatic activity of bPARG and rPARG-CF, it was concluded that the proximal *cis*-hydroxyls of ADP-HPD are key structural requirements for interactions with the PARG active site.

Significance of the Adenine Ring in the Interaction of Substrate with the bPARG and rPARG-CF Active Sites. To investigate the contribution that the adenine ring of ADP-HPD makes to inhibitor binding, the next group of ADP-HPD-related compounds contained variations in the purine ring. ADP-HPD (**1**) and 8-N_3-ADP-HPD (**6**) were approximately equipotent as inhibitors of bPARG (Table 1). Interestingly, 8-N_3-ADP-HPD (**6**) displayed even greater potency for inhibition of rPARG-CF activity than did ADP-HPD. Both of these observations suggested that limited modification of the 8-adenosyl position would not decrease the inhibitory potency of ADP-HPD. The IC_{50} measured for 2-N_3-ADP-HPD (**7**) was approximately 300 μM (Table 1) or 100-fold higher than the IC_{50} value for ADP-HPD. Similar results were seen using rPARG-CF, where an IC_{50} value of 150 μM was determined for 2-N_3-ADP-HPD (**7**). Clearly, this substitution at the 2-position had a profound negative effect on inhibition of bPARG and rPARG-CF activity. The effect of replacing adenine with guanine in ADP-HPD to produce GDP-HPD (**13**) resulted in the least potent inhibitor in this group of compounds. No significant inhibition of bPARG or rPARG-CF catalytic activity was observed using GDP-HPD concentrations up to 100 μM, while the highest concentration utilized (1 mM) inhibited bPARG activity by 10% and rPARG-CF activity by 50% (data not shown). However, the inability of GDP-HPD to inhibit catalytic activity greater than 50% at high concentrations suggested that this compound did not efficiently bind the bPARG or rPARG-CF active site with high affinity. In summary, the results demonstrated the requirement for an adenine moiety to facilitate optimal

Table 2. Activities of Purine-Substituted Compounds on rPARG-CF

	rPARG-CF	
compd	logIC_{50} ± error (μM)	IC_{50} (μM)
1, ADP-HPD[a]	−0.00 ± 0.08	1.
8	+0.97 ± 0.13	9.5
9	+2.75 ± 0.07	570
10	+2.07 ± 0.25	120
11	>3	>1000
12	+2.71 ± 0.27	510

[a] The IC_{50} for ADP-HPD was remeasured with the determination of the IC_{50} values for **8**−**12**.

interaction with the PARG active site, while the substituents in the 8-adenosyl position did not adversely affect this interaction.

Effect of 8-Substituents on the Inhibition of rPARG-CF Catalytic Activity by ADP-HPD. The next set of compounds was designed to determine the tolerance of PARG to modifications at the 8-position of the adenine ring. Three additional 8-substituted ADP-HPD analogues, **8**−**10**, were assayed for their ability to inhibit rPARG-CF enzymatic activity, since bPARG and rPARG-CF were demonstrated to be similar in all previous SAR analyses. All were less potent inhibitors of rPARG-CF activity than was either ADP-HPD (**1**) or 8-N_3-ADP-HPD (**6**), but the most profound reduction was observed with **9** and **10** (Table 2). The IC_{50} value of ADP-HPD (**1**) was 1 μM, that for **8** was 10 μM, the IC_{50} value for **10** approached 120 μM, and that for **9** was almost 600-fold higher at 570 μM. In summary, although 8-N_3-ADP-HPD (**6**) did not significantly affect the inhibition of rPARG-CF activity by parent compound, **8**−**10** decreased the inhibition to varying degrees.

Effect of N^6-Substituents on the Inhibition of rPARG-CF Catalytic Activity by ADP-HPD. The last set of compounds was designed to investigate yet another site on the adenine ring of ADP-HPD to determine their effects on inhibition of rPARG-CF. Two lipophilic substituents were coupled to the N^6-position of adenine to produce **11** and **12**. Both significantly reduced the inhibition of rPARG-CF activity by ADP-HPD since a dose of 100 μM, the highest dose utilized for each compound, only produced 33−34% inhibition of enzymatic activity (Table 2). The results provide additional evidence of the requirement for optimal interaction of adenine with the PARG active site, with substituents at the N^6-position significantly affecting this interaction.

Discussion

To date, there have been no detailed papers on structure−activity characterizations of PARG. In this paper, SAR studies utilized ADP-HPD (**1**) as the lead inhibitor, not only because of its high potency but also because of its demonstrated high specificity.[20] The results of the SAR analysis provided significant insight into the substrate structural requirements for optimal affinity for the PARG active site. Both the ADP and the HPD portions were required for high affinity binding to the bPARG active site since ADP, HPD (**2**), or P-HPD (**3**) alone did not significantly inhibit bPARG activity. More specifically, the *cis*-diols of the pyrolidine moiety were shown to be important for high potency binding, since the magnitude of bPARG inhibition by ADP-HPD and analogues resulted in the following succession: **1** > **4** ≫ **5**. In the ADP portion of ADP-HPD, the bPARG active site was shown to selectively recognize the adenine moiety of ADP-HPD since GDP-HPD (**13**) did not inhibit bPARG catalytic activity until the highest concentration of compound was utilized. If the mechanism of PARG hydrolysis of ADP-ribose polymers is similar to that of other glycosyl hydrolases or even a reverse of the PARP-1 catalytic mechanism, then the possibility exists that the 3‴- and 4‴-ribosyl hydroxyls are critical sites of hydrogen bond acceptors/donors within the active site in order to properly hold and orient the substrate for catalysis.[29,30] In addition, the demonstrated importance of the adenine ring may suggest its involvement in initial substrate recognition by PARG, with either a hydrophobic or a hydrogen-bonding interaction with an active site residue(s). A direct interaction between the adenine ring of ADP-HPD and the Tyr[796] of PARG is supported by the results of our recent photoaffinity labeling study.[31] However, further high resolution studies are needed to substantiate these assertions.

Interesting effects were observed when substitutions were introduced into the adenine ring. Both **11** and **12** were shown to greatly affect the inhibition of PARG catalytic activity by ADP-HPD. However, differing effects were seen when 2- and 8-adenosyl substituents were utilized. The difference of bPARG inhibition observed between 2-N_3-ADP-HPD (**7**) and 8-N_3-ADP-HPD (**6**) possibly reflects a conformational requirement in order to efficiently bind the active site, since purine nucleotides containing substituents in the 8-position favor the syn configuration[32] while those in the 2-position favor the anti.[33] However, lack of potent inhibition exhibited by 2-N_3-ADP-HPD may also reflect a decrease in active site access due to the steric effects elicited after cyclization of the azido group with the adenosyl-N^3 nitrogen at physiologic pH to produce a tetrazolo isomer.[33] Nevertheless, it was concluded that substituents in the N^6- and 2-adenosyl positions of ADP-HPD greatly decrease access to the PARG active site, probably by obstructing adenine ring binding.

A further investigation of substituents at the 8-adenosyl position of ADP-HPD (**1**) provided useful information. Loss of inhibitor potency for both rPARG-CF and bPARG is associated with the introduction of large substituents at the 8-adenosyl as in compounds **8**−**10**. Because **8** contains a functionalized spacer arm 8-atoms in length vs 3-atoms in **6**, loss of potency seems to be associated with introduction of large substituents. Because a greater loss of potency was seen with **10**, it seems that large, bulky substituents in the 8-adenosyl position produce an even larger decrease in inhibition potency. However, because the greatest effect on rPARG-CF inhibition was demonstrated by **9**, it was concluded that the combination of a long and bulky substituent at the 8-adenosyl position of ADP-HPD would most significantly decrease the inhibition of PARG catalytic activity by parent compound. Compound **10** was synthesized for the explicit purpose of utilization as a cell-permeable inhibitor of PARG as previously reported for other nucleotides with this modification.[34−36] However, whenever a spacious aliphatic group was directly at-

tached to the adenine ring (**10**) or linked via an 8-atom spacer arm (**9**), profound reductions were seen in the inhibition of rPARG-CF activity as compared to the parent compound ADP-HPD. Although the exact mechanism of this effect on ADP-HPD inhibition of rPARG-CF was not known, it is possible that the steric hindrance created by these large aliphatic groups obstructed binding of the compound to the active site, thereby interfering with its ability to inhibit PARG activity. On the other hand, the ability of 8-N_3-ADP-HPD (**6**) to inhibit PARG activity comparable to ADP-HPD suggested that the azido group did not create any hindrance for the compound to optimally bind the active site. Therefore, it was concluded that limited modifications of the 8-adenosyl position of ADP-HPD should not extensively affect the high affinity binding of inhibitor to the PARG active site. Although the IC_{50} of **8** is lower than that of the parent compound **1**, it is still sufficiently potent at 10 μM to enable **8** to be successfully used as a ligand for developing biospecific absorbents for PARG.

Structural studies of PARG are lacking due to the limited availability of the enzyme isolated from tissue. Furthermore, the rPARG-CF obtained from heterologous bacterial expression in other published papers has been utilized only qualitatively in order to verify that the protein translated from the cloned PARG cDNA from various sources exhibited PARG catalytic activity.[16,37] Because it is now clear that PARG isolated from tissue is derived from proteolysis of a 111 kDa precursor, rPARG-CF may further differ both structurally and in its enzymatic activity from the enzyme isolated from natural sources. The present study not only provides a detailed analysis of substrate structural requirements needed in order to properly bind PARG, but it also demonstrated the similarity between the conventionally purified and recombinant enzyme. Because similar patterns of inhibition, or lack thereof, utilizing ADP-HPD and related compounds were observed for bPARG and rPARG-CF (Figure 1, Table 1), it was concluded that the inhibitor binding sites were similar as demonstrated by SAR analysis. Close similarity between bPARG and rPARG-CF is further supported by similarity of k_{cat} and K_m values for the proteins. For bPARG, $k_{cat} = 31$ μmol/min/mg and $K_m = 0.38$ μM (in ADP-ribose residues), while for rPARG-CF $k_{cat} = 10$ μM/min/mg and $K_m = 0.52$ μM. rPARG-CF is readily available, so it will be utilized in future PARG structural studies, such as the mapping of its active site, the identification of essential catalytic residues within its active site, and the determination of the three-dimensional structure of its active site by X-ray crystallographic analysis.

Biological studies regarding PARG are lacking due in part to the absence of cell permeable specific PARG inhibitors. ADP-HPD is highly polar and therefore is not expected to readily traverse the plasma membrane. Therefore, its use in determining the in vivo effects of intracellular PARG inhibition is not warranted. The information presented in this study, which suggests that an appropriate hydrophobic 8-substituent might be tolerated, should prove useful when designing potential cell permeable PARG inhibitors using ADP-HPD, the most specific inhibitor of PARG known, as a template.

In summary, we present the first insight into the molecular interactions of the PARG active site with substrate using SAR analysis. Furthermore, we demonstrate the similarity of naturally isolated and recombinant enzymes. The information presented here should facilitate both structural and biological studies of PARG in the future in order to elucidate the structural biology of PARG, establish its molecular mechanism of catalyzing ADP-ribose polymer hydrolysis, facilitate the rational structure-based inhibitor design for this potentially novel therapeutic target, and determine the exact biological role of PARG within the cell.

Experimental Section

Materials. Recombinant PARP-1 was prepared as previously described,[38] as was rPARG-CF[16] and bovine thymus PARG,[39] except that steps nos. 6 (DNA-agarose) and 7 (Heparin-Sepharose) were omitted and a Red-Sepharose step was added.[40] [α-^{32}P]NAD^+ at high specific activity was purchased from ICN. ADP was purchased from Sigma. ADP-HPD (**1**), 2-N_3-ADP-HPD (**7**), and 8-N_3-ADP-HPD (**6**) were synthesized as previously described.[20,25] Compounds **8** and **9** were produced starting from 8-(6-aminohexyl)amino-AMP (Sigma) by converting it to 8-(6-trifluoroacetylaminohexyl)amino-AMP using *S*-ethyltrifluorothioacetate treatment and coupling the resulting AMP derivative to *t*-Boc-protected pyrrolidine (**3**). A detailed synthetic procedure will be published separately.

General Methods. ^{1}H NMR spectra were determined at 300 mHz at ambient probe temperature, at a concentration of ca. 10 mg/mL. ^{13}C NMR spectra were determined at 75 mHz. ^{31}P NMR spectra were acquired on a Varian Gemini-200 spectrometer at 80.95 mHz using 5 mm tubes. Chemical shifts are referenced to an external standard of 85% aqueous H_3PO_4. Mass spectral analysis (electron impact, fast atom bombardment (FAB), or MALDI) was performed at the University of Kentucky Mass Spectrometry Facility, Lexington, KY.

High-performance liquid chromatography (HPLC) was performed using a high-pressure gradient system and a detector operating at 260 nm. Reversed-phase separations were performed using a 3.9 mm × 300 mm reversed-phase column (C18 Bondapak,15−20 μm, 125 Å; Waters, Milford, MA) using an ion-pairing technique at a constant flow rate of 1.5 mL/min. The solvents were (A) 20 mM $NaH_2PO_4 \cdot H_2O$ + 2 mM $Bu_4NH_2PO_4$, pH 6, and (B) 50% (v/v) solvent A and acetonitrile. A mobile phase of 80% A and 20% B was changed linearly to 50% A over 5 min and thereafter maintained isocratically for 25 min. Under these conditions, AMP eluted at 2.7 min and GMP eluted at 3.8 min.

(2*R*,3*R*,4*S*)-1-(Benzyloxycarbonyl)-2-(hydroxymethyl)-pyrrolidine-3,4-diol-3,4-O-isopropylidine Acetal. The precursor for **2** and **3** was synthesized as described by Goli et al.[21]

(2*R*,3*R*,4*S*)-2-Hydroxymethylpyrrolidine-3,4-diol hydrochloride (HPD, 2). 1-Benzyloxycarbonyl-2-(hydroxymethyl)pyrrolidine-3,4-diol 3,4-O-isopropylidene acetal (250 mg, 0.81 mmol) was dissolved in 4 mL of water at 35 °C, 1 mL of trifluoroacetic acid (TFA) was added, and the reaction was stirred at 35 °C for 3 h. Thin-layer chromatography (TLC) (silica gel, ethyl acetate/methanol 99:1) verified the complete conversion of the starting material to a lower R_f product. The solvent was evaporated in vacuo and chased by 5 mL of toluene. The product was purified by flash column chromatography (silica gel 35−70 μm, 100 g, ethyl acetate/methanol 98:2, 225 mL followed by ethyl acetate/methanol 95:5, 200 mL) to obtain a yellow-colored oil. The benzyloxycarbonyl group was removed by dissolving the oil in 15 mL of methanol, adding 50 mg of 5% palladium on carbon, and hydrogenating at 30 psi for 4 h. TLC (silica gel, ethyl acetate/methanol 95:5) verified complete consumption of the starting material. The catalyst was removed by filtration over Celite filter aid. Evaporation of solvent in vacuo yielded a black residue. The product was stirred in 2 mL of 1 M HCl for 30 min and lyophilized to obtain a gray-colored, flaky solid (80 mg, 58%). TLC (silica gel, ethyl acetate/methanol 95:5): $R_f = 0.07$. ^{1}H

NMR (D_2O, referenced to HDO at δ 4.67 ppm): δ 3.20−3.25 (dd, 1 H, CH_2N), 3.34−3.38 (dd, 1 H, CH_2N), 3.34−3.35 (m, 1 H, CHN), 3.65−3.71 (dd, 1 H, CH_2OH), 3.80−3.86 (dd, 1 H, CH_2OH), 4.04−4.08 (m, 1 H, CHOH), 4.22−4.26 (m, 1 H, CHOH). ^{13}C NMR (D_2O, referenced to CD_3OD as external reference δ 49.0 ppm): δ 50.56 (CH_2N), 58.94 (CH_2OH), 62.74 (CHN), 70.37 (CHOH), 72.12 (CHOH). FAB mass spectrum (positive ion): calcd for (M+) ($C_5H_{12}O_3N$), *m*/*z* 134; found, *m*/*z* 134.

(2*R*,3*R*,4*S*)-1-(Benzyloxycarbonyl)-2-[(phosphooxy)-methyl]pyrrolidine-3,4-diol (Cbz-3). Freshly distilled $POCl_3$ (0.71 mL, 7.6 mmol) was cooled in an ice bath, and water (68 μL, 3.8 mmol) followed by pyridine (0.6 mL, 7.6 mmol) was added with stirring. The reaction mixture solidified but liquified upon the addition of acetonitrile (0.85 mL). The starting alcohol, (2*S*,3*R*,4*S*)-1-(benzyloxycarbonyl)-2-(hydroxymethyl)pyrrolidine-3,4-diol-3,4-O-isopropylidine acetal (0.59 g, 1.9 mmol), was added as a solution in 0.85 mL of acetonitrile. The mixture was stirred at 4 °C for 2 h, at which time several small pieces of ice were added to destroy the excess $POCl_3$. Four milliliters of 1 M HCl was added, and the reaction was stirred at ambient temperature for 45 min to remove the acetonide protecting group. The product was desalted by chromatography on a column of Amberchrome CG 71ms resin (1.5 cm × 45 cm), a reversed-phase type adsorbent (Toso Haas Inc.). The chromatography was developed with water. A large peak of pyridine preceded two smaller peaks. The test for inorganic phosphate (Ames, 1966) showed that most of the inorganic phosphate had coeluted with pyridine. The latter two UV absorbing peaks were pooled, and the pH was adjusted to 7.5.

The monophosphate was purified by anion exchange chromatography on a 1.5 cm × 43 cm column of DE-52 cellulose (Whatman), developed by the application of a linear gradient formed between 400 mL of 0.01 M NH_4HCO_3 (pH 7.5) and 400 mL of 0.2 M NH_4HCO_3 (pH 7.5). Fractions (7.5 mL) were collected, and absorbance at 254 nm was measured. A single major peak eluting approximately midway through the gradient was pooled and lyophilized. After several lyophilizations, the monophosphate was obtained as a white amorphous solid (0.51 g, 77% yield). The structure was confirmed by comparison to authentic material[20] using TLC, HPLC, and NMR.

(2*R*,3*R*,4*S*)-2-[(Phosphooxy)methyl]pyrrolidine-3,4-diol (3). Cbz-**3** (0.27 g, 0.77 mmol) was dissolved in 10 mL of water and 30 mL of methanol and subjected to catalytic hydrogenation (50 psi, 5% Pd on carbon, 340 mg) for 4 h. TLC verified that the starting material had been completely consumed at this time. The catalyst was removed by filtering through a Celite filter aid. The solvent was evaporated under reduced pressure to obtain an oil in quantitative yield that was dissolved in water and lyophilized. TLC (silica gel, 2-propanol/concd NH_4OH/water, 6:3:1): R_f = 0.13. ^1H NMR (D_2O): δ 3.33−3.38 (d, 1H, CH_2N), 3.45−3.50 (dd, 1H, CH_2N), 3.68−3.73 (m, 1H, CHN), 3.97−4.06 (m, 1H, CH_2OP), 4.19−4.27 (m, 1H, CH_2OP), 4.37−4.41 (m, 2H, 2CHOH). ^{13}C NMR (D_2O): δ 52.20 (C-5), 62.95 and 63.01 (C-6), 64.02 and 64.08 (C-2), 72.59 and 73.86 (C-3 and C-4). FAB mass spectrum (positive ion): calcd for $(M + H)^+$ ($C_5H_{13}O_6NP$), *m*/*z* 214; found, *m*/*z* 214.

Synthesis of Adenosine Diphosphate (Hydroxymethyl)pyrrolidine Monoalcohol (ADP-HPM, 4). *N*-*tert*-Butyloxycarbonyl-*trans*-4-hydroxy-L-proline. A three-necked round-bottom flask (500 mL), equipped with a magnetic stirring bar, a dropping funnel, and a reflux condenser, was charged with a solution of NaOH (3.4 g, 84 mmol) in water (85 mL). Stirring was then initiated, and *trans*-4-hydroxy-L-proline (10 g, 76.2 mmol) was added at ambient temperature and diluted with *tert*-butyl alcohol (50 mL). A solution of di-*tert*-butyl dicarbonate (16.6 g, 76.2 mmol) was added to the stirred solution of the amino acid (pH 12−12.5), and the mixture was allowed to stir at room temperature overnight. The pH of the solution at this point was 8.5. The reaction mixture was then extracted with pentane (2 × 50 mL), and the combined organic phase was back-extracted with saturated aqueous $NaHCO_3$ solution (3 × 20 mL) and mixed with the aqueous layer. The combined aqueous layer was acidified to approximately pH 1.5 with 10% H_2SO_4 solution while maintaining the temperature to 0−5 °C (ice bath) and extracted with ether (4 × 50 mL). The combined ether extracts were washed with water (2 × 30 mL) and brine (1 × 50 mL) and dried (Na_2SO_4). Evaporation of the solvent gave a sticky, colorless oil (11 g, 62%). ^1H NMR ($CDCl_3$): δ 1.45 (d, J = 24.8 Hz, 9H), 2.2 (m, 1H), 2.4 (m, 1H), 3.52 (d, J = 3.2 Hz, 1H), 3.58 (m, 1H), 4.41 (m, 1H), 4.47 (br, 2H). ^{13}CNMR ($CDCl_3$): δ 28.27, 38.35, 52.17, 54.68, 69.46, 80.21, 152.84, 172.19.

***N*-*tert*-Butyloxycarbonyl-*trans*-4-hydroxy-L-proline Methyl Ester.** To a solution of *N*-*tert*-butyloxycarbonyl-*trans*-4-hydroxy-L-proline (11.00 g, 47.6 mmol) in ether containing 5% CH_3OH (200 mL) was added a solution of diazomethane (CH_2N_2, prepared from 21.5 g Diazald according to the manufacturer's directions) slowly with swirling, until a yellow color persisted. The yellow color disappeared with a few drops of glacial acetic acid (1 mL), and the ethereal solution was extracted with saturated $NaHCO_3$ solution (2 × 50 mL). It was washed with water (1 × 100 mL) and saturated brine and dried (Na_2SO_4). Evaporation of the solvent gave a colorless oil (11.00 g, 95%); R_f = 0.07 (silica gel, 7:3 hexane:ethyl acetate); R_f = 0.28 (silica gel, 1:1 hexane:ethyl acetate). ^1H NMR ($CDCl_3$): δ 1.42 (d, J = 19.6 Hz, 9H), 1.76 (s, br, 1H), 2.06 (m, 1H), 2.28 (m, 1H), 3.50 (dd, J = 42 Hz, 11.6 Hz, 1H), 3.62 (dd, J = 11.6 Hz, 3.6 Hz, 1H), 3.72 (d, J = 4.4 Hz, 3H), 4.4 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 28.24, (38.48, 39.14), (52.02, 52.21), 54.71, (57.45, 57.86), (69.54, 70.26), 80.33, 153.92, 173.59. MS (MALDI): *m*/*e* 268 (MNa^+). Anal. ($C_{11}H_{19}NO_5$) C, H, N.

(2*S*,4*R*)-*N*-*tert*-Butyloxycarbonyl-4-[(*tert*-butyldiphenylsilyl)oxy]pyrrolidine-2-carboxylic Acid, Methyl Ester (14). A solution of *N*-*tert*-butyloxycarbonyl-*trans*-4-hydroxy-L-proline methyl ester (3.50 g, 14.28 mmol) in anhydrous dimethyl formamide (DMF; 10 mL), which had been flushed with N_2, was treated with *tert*-butylchlorodiphenylsilane (5.88 g, 21.4 mmol) with stirring followed by imidazole (2.43 g, 35.7 mmol), and the mixture was allowed to stir at room temperature overnight under N_2. TLC indicated the presence of both of the starting materials; hence, DMAP (12 mg) was added, and the mixture was stirred for another 4 h. A complete disappearance of the silane was observed by TLC (although some alcohol was still present); hence, the reaction was stopped by partitioning between $KHSO_4$ (5%, 100 mL) and $CHCl_3$ (100 mL). The layers were separated, the aqueous layer was extracted with $CHCl_3$ (2 × 100 mL), and the combined organic phase was washed with water (100 mL) and saturated brine (100 mL) and dried (Na_2SO_4). The solvent was then evaporated to give the crude silyl ether, which upon flash column chromatography on silica gel using hexane:ethyl acetate (90:10) as the elutant gave 3.07 g of the pure product (45%) as a sticky oil; R_f = 0.52 (silica gel, 7:3 hexane:ethyl acetate); R_f = 0.78 (silica gel, 1:1 hexane:ethyl acetate). ^1H NMR ($CDCl_3$): δ 1.05 (s, 9H), 1.44 (d, J = 10.4 Hz, 9H), 1.85 (m, 1H), 2.2 (m, 1H), 3.45 (m, 2H), 3.68 (d, J = 9.6 Hz, 3H), 4.42 (m, 2H), 7.40 (m, 6H), 7.63 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 26.67, 28.10, (38.10, 39.52), (51.43, 51.90), (54.52, 55.00), (57.62, 58.10), (70.48, 71.19), 79.28, 127.62, 130.00, (133.33, 133.81), 135.71, (152.86, 153.81), 172.62. MS (MALDI): *m*/*e* 506 (MNa^+). Anal. ($C_{27}H_{37}NO_5$ Si) C, H, N.

(2*S*,4*R*)-*N*-*tert*-Butyloxycarbonyl-4-[(*tert*-butyldiphenylsilyl)oxy]-2-hydroxymethyl Pyrrolidine (15). To a solution of *N*-*tert*-butyloxycarbonyl-4-[(*tert*-butyl diphenyl silyl)-oxy]pyrrolidine-2-carboxylic acid (methyl ester, 3.00 g, 6.21 mmol) in anhydrous THF (30 mL), $LiBH_4$ (0.27 g, 12.4 mmol) was added with stirring under N_2. The mixture was allowed to stir at room temperature for 2 h, at which point a complete disappearance of the starting material was verified by TLC (hexane:ethyl acetate 80:20). The excess $LiBH_4$ was then inactivated with glacial acetic acid, and the mixture was partitioned between $NaHCO_3$ (5%, 100 mL) and $CHCl_3$ (100 mL). The layers were separated, the aqueous layer was extracted with $CHCl_3$ (2 × 100 mL), and the combined organic phase was washed with water (100 mL) and saturated brine

(100 mL) and dried (Na_2SO_4). Stripping of the solvent gave pure product as a colorless oil (2.86 g, 100%); $R_f = 0.22$ (silica gel, 8:2 hexanes:ethyl acetate); $R_f = 0.57$ (silica gel, 1:1 hexanes:ethyl acetate). ^{1}H NMR ($CDCl_3$): δ 1.05 (s, 9H), 1.45 (s, 9H), 2.02 (m, 1H), 3.15 (dd, $J = 12$ Hz, 1.7 Hz, 1H), 3.50 (dd, $J = 12$ Hz, 1.7 Hz, 2H), 3.71 (m, 2H), 4.29 (s, br, 2H), 4.95 (d, $J = 9$ Hz, 1H), 7.42 (m, 6H), 7.65 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 19.06, 26.79, 28.41, 37.68, 55.83, 59.19, 67.34, 70.84, 80.41, 127.75, 129.84, 133.60, 135.59, 135.64, 157.51. MS (MALDI): *m/e* 478 (MNa^+). Anal. ($C_{26}H_{37}NO_4$ Si) C, H, N.

(2*S*,4*R*)-*N*-*tert*-Butyloxycarbonyl-4-[(*tert*-butyldiphenylsilyl)oxy]-2-(dibenzylphosphoxymethyl) Pyrrolidine (16). 1-H-Tetrazole (0.65 g, 9.32 mmol) was added to a solution of *N*-*tert*-butyloxycarbonyl-4-[(*tert*-butyl diphenyl silyl)oxy]-2-hydroxymethyl pyrrolidine (2.82 g, 6.21 mmol) in CH_2Cl_2 (80 mL), and the suspension was stirred under N_2 for 5 min. *N*,*N*-Diisopropyldibenzylphosphoramidite (3.22 g, 9.32 mmol) was added, and the reaction mixture was stirred at room temperature for 3 h under N_2. The reaction mixture was cooled to −40 °C (in dry ice−acetonitrile bath), and *m*-chloroperoxybenzoic acid (mCPBA) (2.14 g, 12.4 mmol) in CH_2Cl_2 (20 mL) was added. The bath was replaced by an ice bath, and the mixture was stirred at 0 °C for 1 h, diluted with CH_2Cl_2 (100 mL), washed with $NaSO_3$ (10%, 2 × 50 mL), $NaHCO_3$ (saturated, 2 × 50 mL), water (100 mL), and saturated brine (100 mL), and dried ($MgSO_4$). The solvent was evaporated to give crude product, which on flash column chromatography on silica gel using hexane:ethyl acetate (70:30) as the elutant, gave 4.4 g of pure product (100%) as a colorless oil; $R_f = 0.22$ (silica gel, 7:3 hexane:ethyl acetate); $R_f = 0.49$ (silica gel, 1:1 hexane:ethyl acetate). ^{1}HNMR ($CDCl_3$): δ 1.05 (s, 9H), 1.45 (s, 9H), 1.80 (m, 1H), 2.03 (m, 2H), 3.09 (m, 1H), 3.50 (m, 1H), 3.95 (m, 1H), 4.30 (m, 1H), 4.93 (m, 4H), 5.00 (m, 1H), 7.29 (m, 16H), 7.62 (d, $J = 6$ Hz, 4H). ^{13}CNMR ($CDCl_3$): δ 19.05, 26.79, 28.40, (36.80, 37.74), (54.85, 55.42), (55.54, 55.65), (67.72, 68.60), 69.22, (70.67, 71.33), (79.57, 79.91), 127.61, 127.73, 127.92, 128.53, 129.81, 133.74, 135.43, 135.57, 135.63, 155.20. Anal. ($C_{40}H_{50}NO_7PSi$) C, H, N.

(2*S*,4*R*)-*N*-*tert*-Butyloxycarbonyl-4-hydroxy-2-(dibenzylphosphoxymethyl) Pyrrolidine (17). *N*-*tert*-Butyloxycarbonyl-4-[(*tert*-butyl diphenyl silyl)oxy]-2-(dibenzylphosphoxymethyl) pyrrolidine (4.2 g, 5.87 mmol) was dissolved in THF (40 mL) in a 500 mL round-bottomed flask, and a 1 M solution of tetra-*n*-butylammonium fluoride in THF (23.5 mL, 23.5 mmol) was added with stirring. The mixture was allowed to stir for 2 h at room temperature, and the solvent was evaporated in vacuo to give crude product appearing as a colorless oil. Flash column chromatography on silica gel (using hexane:ethyl acetate 40:60 as the elutant) gave a colorless, clear oil (2.60 g, 92.8%); $R_f = 0.24$ (silica gel, 2:8 hexanes:ethyl acetate); $R_f = 0.35$ (silica gel, ethyl acetate). ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H), 2.0 (m, 2H), 2.48 (br, 1H, D_2O exchangeable), 3.40 (m, 2H), 4.18 (m, 4H), 5.03 (d, $J = 9.9$ Hz, 4H), 7.35 (s, 10H). ^{13}CNMR ($CDCl_3$): δ 28.36, (36.54, 37.12), 54.94, (55.32, 55.44), (67.67, 68.05), 69.30, 80.10, 127.91, 127.95, 128.56, 135.70, 154.55. ^{31}P NMR ($CDCl_3$, external reference, H_3PO_4): δ −0.10, −0.32. FAB mass spectrum (positive-ion): calcd *m/z* 477; found, *m/z* 478 (MH^+). Anal. ($C_{24}H_{32}NO_7P$) C, H, N.

Adenosine Diphosphate *N*-(*tert*-Butyloxycarbonyl)-hydroxymethyl Pyrrolidine Mono-ol (*t*-Boc-4). Adenosine-5′-monophosphate (AMP) (70 mg, 0.2 mmol) was suspended in methanol (2 mL), and tri-*n*-butylamine (48 μL, 0.2 mmol) was added. The mixture was heated gently in a water bath to obtain a clear solution (5 min). Methanol was removed in vacuo, and DMF (2 mL) was added. The clear solution obtained was evaporated in vacuo at 35 °C to yield a white solid. This was suspended in another 2 mL portion of DMF, and the solvent was evaporated. The resulting white solid was suspended in DMF (2 mL), and 1,1′-carbonyl diimidazole (160 mg, 1 mmol) in DMF was added with stirring. The mixture was allowed to stir at room temperature overnight. The mixture was then treated with methanol (66 μL, 1.6 mmol) to quench the reaction and stirred at room temperature for 30 min.

N-*tert*-Butyloxycarbonyl-4-hydroxy-2-(dibenzylphosphoxymethyl) pyrrolidine (300 mg, 0.62 mmol) was dissolved in methanol (10 mL) with stirring, and 5% Pd−C (40 mg) was added. The reaction flask was purged with H_2 and allowed to stir overnight at room temperature under H_2. It was filtered through Celite filter aid, and the solvent was evaporated to give a white solid. It was suspended in methanol (2 mL), and tributylamine (116.6 mg, 150 μL, 0.62 mmol) was added. The mixture was stirred for 5 min, and the methanol was evaporated in vacuo and chased once with DMF (2 mL). This solid was suspended in DMF and added to the AMP−imidazolide solution obtained as above. The mixture was allowed to stir for 5 days at room temperature until the completion of the reaction was verified by TLC.

The reaction was quenched by adding methanol (25 mL), and the solvents were evaporated in vacuo. The mixture was diluted with water (100 mL), the pH was adjusted to 7.5 with 1 M NH_4OH, and the sample was applied to a benzyl DEAE cellulose column (40 mL; 1.2 cm × 35 cm) previously equilibrated with 10 mM NH_4HCO_3. The column was developed with a linear gradient formed between 400 mL of 10 mM NH_4HCO_3 and 400 mL of 400 mM NH_4HCO_3, pH 7.5. Fractions (10 mL) were collected, and the product was eluted in fractions 61−79. This was found to be a mixture of three components as detected by analytical reversed-phase HPLC (C18 Bondapak).

The combined fractions were evaporated in vacuo at 35 °C, and the white solid obtained was dissolved in a minimum volume of water and acidified to pH 1.7 with dilute HCl. The mixture was applied to a reversed-phase column (Amberchrome CG-71 md, 1.2 cm × 47 cm) and eluted with deionized water. Fractions containing the product were pooled and analyzed by reversed-phase HPLC, which showed the presence of a small amount of AMP. Hence, the solution was concentrated and reapplied to the Amberchrome column. The fractions containing the pure product were pooled, adjusted to pH 7.2 with 1 M NH_4OH, and lyophilized to give the pure product (50 mg, 40%). HPLC (reversed-phase C18 Bondapak, ion pair), retention time = 12.5 min, retention time of AMP = 2.7 min; $R_f = 0.52$ (silica gel, 6:3:1 2-propanol:concentrated ammonia:water). ^{1}H NMR (D_2O): δ 1.25 (s, 9H, *tert*-butyl), 1.86 (m, 1H, pyrrolidine H-3′), 2.00 (m, 1H, pyrrolidine H-3′), 3.21 (m, 2H, pyrrolidine H-1′), 3.83 (m, 3H, pyrrolidine H-4′ and H-5′), 4.05 (s, 2H, adenosine H-5′), 4.22 (s, 2H, adenosine H-4′ and pyrrolidine H-2′), 4.35 (m, 1H, adenosine H-3′), 4.61 (m, 1H, adenosine H-2′), 5.97 (d, $J = 5.6$ Hz, 1H, adenosine H-1′), 8.10 (s, 1H, adenine H-2), 8.37 (s, 1H, adenine H-8). ^{13}CNMR (D_2O): δ 28.60 (*tert*-butyl), 37.11 (pyrrolidine C-3′), 55.02 (pyrrolidine C-1′), 56.60 (pyrrolidine C-4′), 66.05 (pyrrolidine (C-5′), 67.42 (adenosine C-5′), 69.84 (pyrrolidine C-2′), 71.30 (adenosine C-3′), 75.27 (adenosine C-2′), 82.57 (*tert*-butyl 4° carbon), 84.97 (adenosine C-4′), 88.07 (adenosine C-1′), 119.57 (adenine C-5), 140.38 (adenine C-8), 148.68 (adenine C-4), 155.54 (adenine C-2), 157.30 (adenine C-6). ^{31}P NMR (D_2O, external reference, H_3PO_4): δ −10.40 (multiplet). FAB mass spectrum (positive-ion): calcd *m/z* 626; found, *m/z* 627 (MH^+).

Adenosine Diphosphate (Hydroxymethyl)pyrrolidine Monoalcohol (ADP-HPM, 4). *t*-Boc-ADP-HPM (40 mg, 0.064 mmol) was mixed with TFA (5 mL) and stirred at room temperature for 30 min. TFA was then evaporated in vacuo, the residue was dissolved in water (100 mL), and the pH was adjusted to basic (9.7) with 1 M NH_4OH. The sample was then applied as a dilute solution to an 83 mL column of benzyl DEAE cellulose (1.5 cm × 47 cm), previously equilibrated with 10 mM NH_4HCO_3 and eluted with a linear gradient formed between 10 mM NH_4HCO_3 and 300 mM NH_4HCO_3 (400 mL each) at a flow rate of 1 mL/min. Fractions of 10−11 mL each were collected. The chromatography produced two well-separated peaks. Fractions 52−66 were pooled together and lyophilized twice to give pure, salt free product (30 mg, 90%). HPLC (reversed phase C18 Bondapak, ion pair), retention time = 5.5 min, retention time of AMP = 2.7 min. ^{1}H NMR (D_2O): δ 1.91 (m, 2H, pyrrolidine H-3′), 3.14 (d, $J = 12.51$ Hz, 1H, pyrrolidine H-1′), 3.25 (dd, $J = 12.5$ Hz, 3.32 Hz, 1H, pyrrolidine H-1′), 3.88 (m, 1H, pyrrolidine H-4′), 4.07 (m, 4H,

pyrrolidine H-5′ and adenosine H-5′), 4.23 (s, 1H, adenosine H-4′), 4.35 (t, J = 3.75 Hz, 1H, adenosine H-3′), 4.47 (s, 1H, pyrrolidine H-2′), 4.57 (t, J = 5.2 Hz, 1H, adenosine H-2′), 5.92 (d, J = 5.4 Hz, 1H, adenosine H-1′), 7.97 (s, 1H, adenine H-2), 8.28 (s, 1H, adenine H-8). ^{13}C NMR (D_2O) δ 35.34 (pyrrolidine C-3′), 53.67 (pyrrolidine C-1′), 59.19 (pyrrolidine C-4′), 65.13 (pyrrolidine C-5′), 66.28 (adenosine C-5′), 70.71 (pyrrolidine C-2′), 71.24 (adenosine C-3′), 75.23 (adenosine C-2′), 84.65 (adenosine C-4′), 88.02 (adenosine C-1′), 114.84 (adenine C-5), 140.32 (adenine C-8), 150.38 (adenine C-4), 153.41 (adenine C-2), 156.21 (adenine C-6). ^{31}P NMR (D_2O, external reference, H_3PO_4): δ −10.38 (multiplet). FAB mass spectrum (positive-ion): calcd m/z 526; found, m/z 527 (MH^+). Anal. ($C_{15}H_{24}N_6O_{11}P_2 \cdot 1/4NH_4^+ \cdot 1/2H_2O$) C, H, N.

Synthesis of Adenosine Diphosphate (Hydroxymethyl)pyrrolidine (ADP-HP, 5). *N*-(*tert*-Butyloxycarbonyl)-L-proline, Methyl Ester. *N*-(*tert*-Butyloxycarbonyl)-L-proline (2.60 g, 12.00 mmol) was dissolved in a mixture of ether (50 mL) and methanol (5 mL) in a wide-mouthed conical flask, and a solution of diazomethane (16 mmol) in ether (made from Diazald, according to the manufacturers instructions) was added with swirling. The solvents were then evaporated to give a colorless oil (2.78 g, 100%). An analytically pure sample was obtained by bulb-to-bulb distillation; R_f = 0.35 (silica gel, 70:30 hexane:ethyl acetate); R_f = 0.58 (silica gel, 50:50 hexane:ethyl acetate). ^{1}H NMR ($CDCl_3$): δ 1.44 (d, J = 15 Hz, 9H), 1.93 (m, 3H), 2.21 (m, 1H), 3.48 (m, 2H), 3.74 (s, 3H), 4.28 (ddd, J = 30 Hz, 8.2 Hz, 4.1 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ (23.35, 24.01), 28.08, (29.59, 30.54), (45.98, 46.22), (51.54, 51.66), (58.41, 58.76), 79.39, (153.424, 154.06), (173.11, 173.37). MS: m/e 229 (M^+). Anal. ($C_{11}H_{19}NO_4$) C, H, N.

(2*S*)-*N*-(*tert*-Butyloxycarbonyl)-2-hydroxymethylpyrrolidine. *N*-(*tert*-Butyloxycarbonyl)-L-proline (methyl ester, 2.76 g, 12 mmol) was dissolved in dry THF (20 mL), and the solution was flushed with N_2. Lithium borohydride (0.53 g, 24 mmol) was then added under a blanket of N_2, and the mixture was stirred under N_2 for 4 h at room temperature. TLC indicated that the reaction was complete, so excess $LiBH_4$ was destroyed with glacial acetic acid (5 mL) and the solvents were evaporated in vacuo. The residue was partitioned between $NaHCO_3$ (5%, 100 mL) and $CHCl_3$ (100 mL), the aqueous layer was extracted with $CHCl_3$ (2 × 100 mL), and the combined organic layers were backwashed with water (100 mL), saturated brine (100 mL), and dried (Na_2SO_4). Evaporation of the solvent gave a colorless oil (2.03 g, 84%); R_f = 0.22 (silica gel, 70:30 hexane:ethyl acetate). ^{1}H NMR ($CDCl_3$): δ 1.48 (s, 9H), 1.81 (m, 3H), 2.02 (m, 1H), 3.30 (m, 1H), 3.45 (m, 1H), 3.59 (m, 2H), 3.95 (m, 1H), 4.76 (s, br, 1H). ^{13}C NMR ($CDCl_3$): δ 23.90, 28.34, 28.54, 47.40, 60.01, 67.33, 80.03, 156.95. MS: m/e 201 (M^+). Anal. ($C_{10}H_{19}NO_3$) C, H, N.

(2*S*)-*N*-(*tert*-Butyloxycarbonyl)-2-(dibenzylphosphoxymethyl)pyrrolidine (18). To a solution of *N*-(*tert*-butyloxycarbonyl)-2-(hydroxymethyl)pyrrolidine (0.500 g, 2.5 mmol) in CH_2Cl_2 (50 mL), 1-H-tetrazole (0.525 g, 7.5 mmol) was added and the suspension was stirred for 5 min. *N*,*N*-Diisopropyldibenzylphosphoramidite (1.3 g, 3.75 mmol) was then added under N_2, and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was cooled to −40 °C in dry ice−acetonitrile bath, mCPBA (0.85 g, 5 mmol, 57−86% grade) in CH_2Cl_2 (20 mL) was added all at once, the cooling bath was removed, and the mixture was allowed to stir at 0 °C for 1 h. The reaction mixture was diluted with CH_2Cl_2 (100 mL), washed with Na_2SO_3 solution (10%, 2 × 50 mL), saturated $NaHCO_3$ solution (2 × 50 mL), water (100 mL), and saturated brine (100 mL), and dried ($MgSO_4$). Evaporation of the solvent gave the crude product as a colorless oil (1.5 g), which was purified by flash column chromatography on silica gel using hexanes:ethyl acetate (70:30) as the elutant to yield a product appearing as a colorless oil (0.63 g, 55%); R_f = 0.35 (silica gel, 50:50 hexane:ethyl acetate). ^{1}H NMR ($CDCl_3$): δ 1.45 (s, 9H), 1.77 (m, 2H), 1.89 (m, 2H), 3.31 (m, 2H), 3.96 (m, 3H), 5.04 (d, J = 8.1 Hz, 4H), 7.34 (s, 10H). ^{13}C NMR ($CDCl_3$): δ (22.60, 23.72), 27.44, 28.60, (46.42, 47.16), 56.50, 67.67, 69.77, (79.53, 80.00), 127.90, 128.60, 135.80, 154.20. ^{31}P NMR ($CDCl_3$, external reference, H_3PO_4): δ −0.30 (d, J = 15 Hz). MS: m/e 461 (M^+). Anal: ($C_{24}H_{32}NO_6$ P) C, H, N.

(2*S*)-*N*-(*tert*-Butyloxycarbonyl)-2-(phosphoxymethyl) Pyrrolidine (19). To a solution of *N*-(*tert*-butyloxycarbonyl)-2-(dibenzylphosphoxymethyl) pyrrolidine (0.63 g, 1.36 mmol) in methanol (25 mL), 5% Pd−C catalyst (60 mg) was added with stirring, and the flask was purged with H_2. The mixture was then allowed to stir under H_2 for 16 h at which point the TLC indicated a complete absence of starting material. The mixture was filtered through a Celite pad and washed with methanol, and the combined filtrate and washings were evaporated in vacuo to yield a colorless oil (0.63 g, 100%). ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 9H), 1.72 (m, 4H), 3.07 (m, 2H), 3.71 (m, 3H).

Adenosine Diphosphate *N*-(*tert*-Butyloxycarbonyl)-2-(hydroxymethyl)pyrrolidine (*t*-Boc-ADP-HP, *t*-Boc-5). Adenosine-5′-monophosphate (AMP) (70 mg, 0.2 mmol) was suspended in methanol (2 mL), and tri-*n*-butylamine (48 μL, 0.2 mmol) was added. The mixture was heated gently in a water bath to yield a clear solution (5 min). Methanol was removed in vacuo, and DMF (2 mL) was added. The clear solution obtained was evaporated in vacuo at 35 °C to yield a white solid. This was then suspended in another 2 mL portion of DMF, and the solvent was evaporated. The resulting white solid was suspended in DMF (2 mL), and 1,1′-carbonyl diimidazole (81 mg, 0.500 mmol) in DMF was added with stirring. The mixture was allowed to stir at room temperature overnight. The mixture was then treated with methanol (33 μL, 0.8 mmol) to destroy excess carbonyl diimidazole and stirred at room temperature for 30 min.

(2S)-*N*-(*tert*-Butyloxycarbonyl)-2-(phosphoxymethyl)pyrrolidine (monotributylamine salt, 232.5 mg, 0.5 mmol) in DMF (5 mL) was added to the reaction mixture, and the mixture was stirred at room temperature for 24 h. Methanol (5 mL) was added, and the solvents were evaporated in vacuo until dryness.

The product was purified by anion exchange chromatography. The residue was dissolved in water (100 mL), and the pH was adjusted to 7.5 with NH_4OH solution and applied to a 50 mL column of benzyl DEAE cellulose (1.2 cm × 45 cm) previously equilibrated with 10 mM NH_4HCO_3, pH 7.5. The column was then developed with a linear gradient formed between 400 mL of 10 mM NH_4HCO_3 and 400 mL of 400 mM NH_4HCO_3 buffered at pH 7.5. Fractions (10 mL) were collected, and the product eluted into fractions 70−90. Pooled fractions were evaporated in vacuo at 35 °C and lyophilized to give the dinucleotide, with very little contamination of AMP. This was then repurified by preparative anion exchange HPLC to yield pure dinucleotide (92 mg, 74.8%); R_f = 0.56 (silica gel, 6:3:1 2-propanol:NH_4OH:H_2O); R_f = 0.59 (silica gel, 7:1:2 2-propanol:triethylamine:H_2O); HPLC (reversed phase C18 Bondapak, ion pair), retention time = 12.5 min, retention time of AMP = 2.7 min. ^{1}H NMR (D_2O): δ 1.10 (s, 9H, *tert*-butyl), 1.59 (m, 4H, pyrrolidine H-2′ and H-3′), 2.97 (m, 2H, pyrrolidine H-1′), 3.58 (m, 3H, pyrrolidine H-4′ and H-5′), 3.95 (s, 2H, adenosine H-5′), 4.12 (s, 1H, adenosine H-4′), 4.26 (m, 1H, adenosine H-3′), 4.53 (dd, J = 11 Hz, 5.6 Hz, 1H, adenosine H-2′), 5.86 (d, J = 5.7 Hz, 1H, adenosine H-1′), 7.97 (s, 1H, adenine H-2), 8.24 (s, 1H, adenine H-8). ^{13}C NMR ($CDCl_3$): δ 24.58, 26.56, 28.69, 30.56, 46.63, 60.66, 65.42, 66.17, 71.23, 75.60, 82.29, 85.05, 85.16, 88.95, 119.54, 149.35, 150.95, 157.37. ^{31}P NMR (D_2O, external reference, H_3PO_4): δ −10.50 (multiplet). MS (MALDI): m/e 609 ($M - H)^-$.

Adenosine Diphosphate (Hydroxymethyl)pyrrolidine (ADP-HP, 5). Adenosine diphosphate *N*-(*tert*-butyloxycarbonyl)-2-(hydroxymethyl) pyrrolidine (*t*-boc-ADP-HP) (80 mg, 0.13 mmol) was mixed with TFA (5 mL) and stirred at room tempearature for 30 min. TFA was evaporated in vacuo, and the mixture was dissolved in water (20 mL) and purified by preparative anion exchange HPLC (Waters, Accell Plus, quaternary methylamine [QMA] anion exchange media, 500 Å, 1.2 cm × 10 cm, volume = 10 mL; elutant buffers, 10 mM NH_4HCO_3 and 400 mM NH_4HCO_3). The fractions containing the product were pooled and lyophilized to give pure ADP-HP

(60 mg, 90%); $R_f = 0.30$ (silica gel, 6:3:1 2-propanol:NH_4OH:H_2O); $R_f = 0.21$ (silica gel, 7:1:2 2-propanol:triethylamine:H_2O). ^{1}H NMR (D_2O): δ 1.61 (m, 1H, pyrrolidine H-2′), 1.88 (m, 3H, pyrrolidine H-2′ and H-3′), 3.15 (t, $J = 7$ Hz, 2H, pyrrolidine H-1′), 3.74 (m, 1H, pyrrolidine H-4′), 3.86(m, 1H, pyrrolidine H-5′), 4.07 (m, 3H, pyrrolidine H-5′ and adenosine H-5′), 4.24 (s, 1H, adenosine H-4′), 4.37 (m, 1H, adenosine H-3′), 4.59 (dd, $J = 11$ Hz, 5.6 Hz, 1H, adenosine H-2′), 5.97 (d, $J = 5.8$ Hz, 1H, adenosine H-1′), 8.07 (s, 1H, adenine C-2), 8.32 (s, 1H, adenine H-8). ^{13}CNMR (D_2O): δ 24.28 (pyrrolidine C-2′), 27.05 (pyrrolidine C-3′), 46.76 (pyrrolidine C-1′), 60.62 (pyrrolidine C-4′), 65.95 (pyrrolidine C-5′), 67.10 (adenosine C-5′), 71.38 (adenosine C-3′), 75.00 (adenosine C-2′), 84.76 (adenosine C-4′), 87.81 (adenosine C-1′), 119.80 (adenine C-5), 140.02 (adenine C-8), 149.82 (adenine C-4), 155.46 (adenine C-2), 159.13 (adenine C-6). ^{31}P NMR (D_2O, external reference, H_3PO_4): δ −10.5 (multiplet). MS (FAB mass spectrum, positive ion): calcd m/z 510; found, m/z 511 (MH^+).

8-Chlorophenylthioadenosine 5′-Diphosphate (Hydroxymethyl)pyrrolidinediol (10). 8-Bromoadenosine (2 g, 5.8 mmol), *p*-chlorobenzenethiol (2 g, 14 mmol), and $LiOCH_3$ (1.3 g) were reacted in 40 mL of anhydrous DMF at 60 °C for 4 h. The solvent was evaporated in vacuo, and the residue was purified by chromatography on silica gel developed in methylenechloride/methanol (10:1) to obtain 8-CPT-adenosine. 8-CPT-AMP was prepared by the method of Yoshikawa et al.[24] 8-CPT-adenosine (2 g) was dissolved in 20 mL of trimethyl phosphate followed by the addition of 25 mmol of $POCl_3$. The mixture was left at 0 °C overnight. Purification was obtained on a DEAE−cellulose column using a linear gradient of 0−0.25 M NH_4HCO_3. (2*R*,3*R*,4*S*)-1-(*tert*-Butyloxycarbonyl)-2-[(phosphooxy)methyl]pyrrolidine-3,4-diol was prepared according to Ramsinghani et al.[25] 8-CPT-AMP was coupled to (2*R*,3*R*,4*S*)-1-(*tert*-butyloxycarbonyl)-2-[(phosphooxy)methyl]pyrrolidine-3,4-diol by a method based on the procedure of Michelson[23] and purified by DEAE−cellulose anion exchange chromatography. The *tert*-butyloxycarbonyl group was removed by reacting in 10% TFA in methylenechloride at room temperature for 30 min to obtain the desired product. TLC (silica gel, 2-propanol/concd NH_4OH/water 6:3:1) $R_f = 0.57$. ^{1}H NMR (D_2O, referenced to HDO at δ 4.67): δ 3.20−3.3.25 (d, 1H, CH_2N), 3.37−3.47 (d, 1H, CH_2N), 3.65 (broad s, 1H, CHN), 4.08−4.28 (m, 7H), 4.48−4.53 (t, 1H,CHOH), 5.14−5.22 (t, 1H, CHOH), 6.10−6.22 (d, 1H, anomeric H), 7.16−7.43 (d, 2H, PhH), 7.45−7.61 (d, 2H, PhH), 8.19 (s, 1H, adenosyl H).

***N*^{6}-Benzyladenosine 5′-Diphosphate (Hydroxymethyl)-pyrrolidinediol (11).** *N*^{6}-Benzyladenosine was phosphorylated to obtain *N*^{6}-benzyl-AMP as described above. *N*^{6}-Benzyl-AMP was coupled to Cbz-**3** as described above. The benzyloxycarbonyl group was removed by catalytic hydrogenation to obtain the desired product. TLC (silica gel, 2-propanol/concentrated NH_4OH/water 6:3:1) $R_f = 0.57$. ^{1}H NMR (D_2O, 1% w/w DSS): δ 8.39 (s,1H), δ 8.16 (s,1H), δ 7.31−7.23 (m,5H), δ 6.04 (d,1H), δ 4.26−4.12 (m, 4H), δ 3.68−3.42 (m,4H), δ 3.38−3.22 (m, 4H).

***N*^{6}-Hexyladenosine 5′-Diphosphate (Hydroxymethyl)-pyrrolidinediol (12).** 6-Chloropurine riboside was phosphorylated to obtain 6-chloropurine riboside 5′-monophosphate as described above. *N*^{6}-Hexyl-AMP was prepared by nucleophilic aromatic substitution with hexylamine at 60 °C.[41] *N*^{6}-Hexyl-AMP was coupled to Cbz-**3** as described above. The benzyloxycarbonyl group was removed by catalytic hydrogenation to obtain the desired product. TLC (silica gel, 2-propanol/concd NH_4OH/water 6:3:1) $R_f = 0.59$. ^{1}H NMR (D_2O, 1% w/w DSS): δ 8.32 (s,1H), δ 8.11(s,1H), δ 6.02(d,1H), δ 4.58−4.65 (m,1H), δ 4.52−3.90 (m, 3H), δ 3.28−3.25 (m, 4H), δ 2.91−2.75 (m, 4H), δ 1.68−1.62 (m, 4H), δ 1.17−1.12 (m, 4H),δ 0.73−0.52 (m, 3H).

Guanosine 5′-Diphosphate (Hydroxymethyl)pyrrolidinediol (GDP-HPD, 13). Guanosine-5′-monophosphate (GMP, 72.64 mg, 0.2 mmol) was suspended in methanol (2 mL), and tri-*n*-butylamine (37 mg, 48 μL, 0.2 mmol) was added. The mixture was heated gently in a water bath to yield a clear solution (5 min). Methanol was removed in vacuo, and DMF (2 mL) was added. The clear solution obtained was evaporated in vacuo at 35 °C to give a white solid. This was then suspended in another 2 mL portion of DMF, and the solvent was evaporated. The resulting white solid was suspended in DMF (2 mL), and 1,1′-carbonyldiimidazole (160 mg, 1 mmol) in DMF was added with stirring. The mixture was allowed to stir at room temperature overnight. The mixture was treated with methanol (66 μL, 1.6 mmol) to destroy excess carbonyldiimidazole and stirred at room temperature for 30 min.

To this mixture, a solution of Cbz-HPD phosphate (NH_4^+ salt) in DMF (2 mL) was added, and the mixture was allowed to stir at room temperature for 7 days, at which time TLC indicated the completion of the reaction. Methanol (2 mL) was then added, and the solvents were evaporated in vacuo at 35 °C, and the residue was dissolved in water and acidified with dilute HCl. This mixture was then loaded onto a reversed-phase column (Amberchrome CG-71md, 1.5 cm × 100 cm, 175 mL) with deionized water elution. Fractions (10−11 mL) were collected, and fractions 18−40 were pooled, which appeared as three peaks on analytical reversed-phase HPLC (Bondapak C18) analysis. The combined fractions were made basic (pH 9.0) with 1 M NH_4OH and purified by anion exchange chromatography (benzyl-DEAE cellulose, 1.5 cm × 47 cm, volume = 83 mL) and developed using with a linear gradient formed between 10 mM NH_4HCO_3 (400 mL) and 300 mM NH_4HCO_3 (400 mL). The chromatography produced well-separated peaks, from which the fractions containing the dinucleotide product were pooled and lyophilized to give a chromatographically pure product.

The product was dissolved in water (20 mL), catalyst 5% Pd−C was added (10 mg), and the mixture was allowed to stir at room temperature under H_2 overnight. The mixture was then filtered through a Celite pad, the solvent was evaporated, and the residue was dissolved in water (100 mL) with the pH adjusted to 7.5 and purified by anion exchange chromatography using benzyl-DEAE cellulose (1.5 cm × 47 cm, 83 mL). The column was developed with a linear gradient of 10 mM NH_4HCO_3 and 250 mM NH_4HCO_3 (400 mL each). Lyophilization of the fractions containing the product yielded the pure dinucleotide (70 mg, 62%). HPLC (reversed-phase C18 Bondapak, ion pair), retention time = 2.4 min, retention time of GMP = 3.8 min. ^{1}H NMR (D_2O): δ 3.18 (d, $J = 11.5$ Hz, 1H, pyrrolidine H-1′), 3.32 (dd, $J = 12$ Hz, 2 Hz, 1H, pyrrolidine H-1′), 3.57 (m, 1H, pyrrolidine H-4′), 4.06 (m, 3H), 4.20 (m, 4H), 4.36 (t, $J = 4$ Hz, 1H, guanosine H-3′), 4.60 (m, 1H, guanosine H-2′), 5.78 (d, $J = 4.8$ Hz, 1H, guanosine H-1′), 7.95 (s, 1H, guanine H-8).

SAR Studies. PARG inhibition assays were performed in at least triplicate as described previously,[42] using 0.25 ng of bPARG or 5 ng of rPARG-CF with selected inhibitors included in reaction mixes (except for control) in the presence of 10 μM [α-^{32}P]ADP-ribose polymers in a total volume of 30 μL. The catalytic activity of PARG was measured in the presence of varying concentrations of inhibitors. Data were fit to the equation describing a sigmoidal dose response relation between % activity (100 to 0%) and log[inhibitor] using GraphPad prism version 3.02 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com. The procedure resulted in calculated values for logIC_{50} and for the standard error in logIC_{50}, which are reported.

Acknowledgment. Supported by research grants from the Ohio Division of the American Cancer Society and de Arce Memorial Endowment Fund to J.T.S. and the National Institutes of Health Grant CA 43894 to M.K.J.

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JM020541U