Synthesis, Biological Activity, and Molecular Modeling of Ribose-Modified Deoxyadenosine Bisphosphate Analogues as P2Y$_1$ Receptor Ligands

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The structure−activity relationships of adenosine-3′,5′-bisphosphates as P2Y$_1$ receptor antagonists have been explored, revealing the potency-enhancing effects of the N^6-methyl group and the ability to substitute the ribose moiety (Nandanan et al. *J. Med. Chem*. **1999**, *42*, 1625−1638). We have introduced constrained carbocyclic rings (to explore the role of sugar puckering), non-glycosyl bonds to the adenine moiety, and a phosphate group shift. The biological activity of each analogue at P2Y$_1$ receptors was characterized by measuring its capacity to stimulate phospholipase C in turkey erythrocyte membranes (agonist effect) and to inhibit its stimulation elicited by 30 nM 2-methylthioadenosine-5′-diphosphate (antagonist effect). Addition of the N^6-methyl group in several cases converted pure agonists to antagonists. A carbocyclic N^6-methyl-2′-deoxyadenosine bisphosphate analogue was a pure P2Y$_1$ receptor antagonist and equipotent to the ribose analogue (MRS 2179). In the series of ring-constrained methanocarba derivatives where a fused cyclopropane moiety constrained the pseudosugar ring of the nucleoside to either a Northern (N) or Southern (S) conformation, as defined in the pseudorotational cycle, the 6-NH_2 (N)-analogue was a pure agonist of EC_{50} 155 nM and 86-fold more potent than the corresponding (S)-isomer. The 2-chloro-N^6-methyl-(N)-methanocarba analogue was an antagonist of IC_{50} 51.6 nM. Thus, the ribose ring (N)-conformation appeared to be favored in recognition at P2Y$_1$ receptors. A cyclobutyl analogue was an antagonist with IC_{50} of 805 nM, while morpholine ring-containing analogues were nearly inactive. Anhydrohexitol ring-modified bisphosphate derivatives displayed micromolar potency as agonists (6-NH_2) or antagonists (N^6-methyl). A molecular model of the energy-minimized structures of the potent antagonists suggested that the two phosphate groups may occupy common regions. The (N)- and (S)-methanocarba agonist analogues were docked into the putative binding site of the previously reported P2Y$_1$ receptor model.

Introduction

P2 receptors, which are activated by purine and/or pyrimidine nucleotides, consist of two families: G protein-coupled receptors termed P2Y, of which five mammalian subtypes have been cloned, and ligand-gated cation channels termed P2X, of which seven mammalian subtypes have been cloned.[1,2] The P2Y$_1$ receptor, which is present in the heart, skeletal and various smooth muscles, prostate, ovary, and brain,[3] was the first P2 subtype to be cloned.[4] The nomenclature of P2 receptors and their various ligand specificities have been reviewed.[5−7]

Nucleotide agonists binding at P2Y$_1$ receptors induce activation of phospholipase C (PLC), which generates inositol phosphates and diacylglycerol from phosphatidylinositol (4,5)bisphosphate,[8] leading to a rise in intracellular calcium. A P2Y$_1$ receptor in platelets is involved in ADP-promoted aggregation.[9−11] Thus, a selective P2Y$_1$ receptor antagonist may have potential as an antithrombotic agent, while a selective P2Y$_1$ receptor agonist may have potential as an antihypertensive or antidiabetic agent.[12,13]

Recently, progress in the synthesis of selective P2 receptor antagonists has occurred.[11,14−21] Adenosine-3′,5′- and -2′,5′-bisphosphates were recently shown to be selective antagonists or partial agonists at P2Y$_1$ receptors.[19,20] Other classes of P2 antagonists include pyridoxal phosphate derivatives,[16,21] isoquinolines,[15] large aromatic sulfonates related to the trypanocidal drug suramin[22] and various dyestuffs,[4] and 2′,3′-nitrophenyl nucleotide derivatives.[14] SAR (structure−activity relationship) studies of analogues of adenosine bis-

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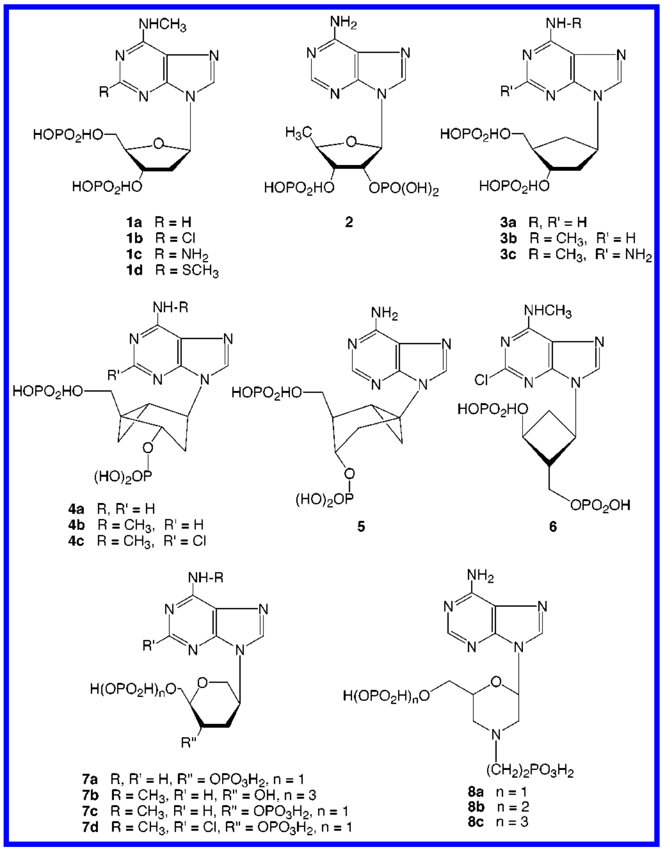
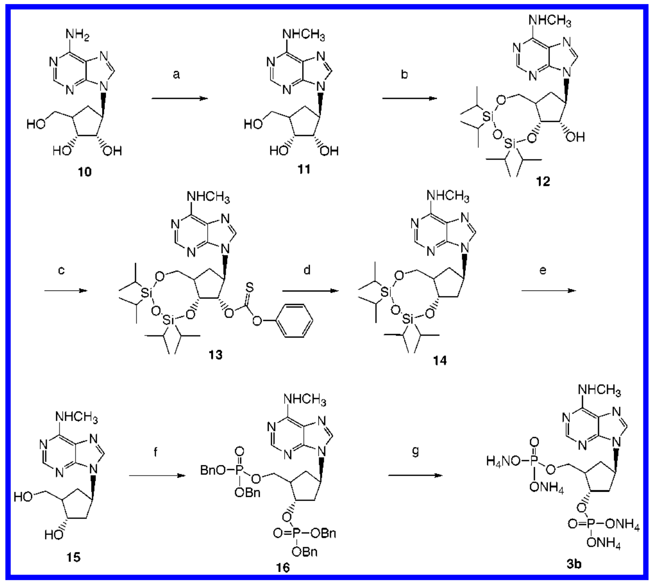


Figure 1. Structures of ribose-modified nucleotide analogues synthesized as ligands for the P2Y$_1$ receptor. The corresponding ammonium salts were synthesized and tested for biological activity. Rigid compounds **4** and **5** are in the $_2$E (N) and $_3$E (S) conformations, respectively, as defined by the pseudorotational cycle.[29] The anti-conformation (not shown) of **4** is favored.

phosphates[17,18] have resulted in N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (**1a**, MRS 2179; Figure 1), a competitive antagonist at human and turkey P2Y$_1$ receptors, with a K_B value of approximately 100 nM.[18] The presence of an N^6-methyl group and the absence of a 2′-hydroxyl group both enhanced affinity and decreased agonist efficacy, thus resulting in a pure antagonist at both turkey and human P2Y$_1$ receptors. The corresponding 2-Cl analogue (**1b**)[19] was slightly more potent than **1a** as an antagonist at turkey P2Y$_1$ receptors, with an IC$_{50}$ value of 0.22 μM in blocking the effects of 10 nM 2-methylthioadenosine-5′-diphosphate (2-MeSADP). Compound **1a** was inactive at P2Y$_2$, P2Y$_4$, and P2Y$_6$ subtypes, at the adenylyl cyclase-linked P2Y receptor in C6 glioma cells (J. Boyer, personal communication),[23] at a novel avian P2Y receptor that inhibits adenylyl cyclase,[24] and at the canine P2Y$_{11}$ receptor (B. Torres, A. Zambon, and P. Insel, unpublished observations). However, the selectivity of certain nucleotide antagonists for the P2Y$_1$ receptor is not absolute, since **1a** also displayed considerable antagonist affinity at P2X$_1$ receptors (IC$_{50}$ 1.15 μM) and at P2X$_3$ receptors (IC$_{50}$ 12.9 μM), but not at P2X$_2$ and P2X$_4$ receptors.[25]

To move away from the nucleotide structure of **1a** and thereby increase biological stability and selectivity for the receptors in the present study, further structural modifications of the ribose moiety have been carried out. In a previous study[19] it was found that carbocyclic[26] and

Scheme 1. Synthesis of N^6-Methyl-2′-deoxyaristeromcyin Derivatives[a]



[a] Reagents: (a) (1) MeI, DMF, (2) MH$_4$OH, 90 °C; (b) DCTIDS/imidazole, DMF; (c) PhOCSCl/DMAP, CH$_3$CN; (d) *n*-Bu$_3$SnH/AIBN, toluene; (e) *n*-Bu$_4$NF, THF; (f) LDA/TBPP; (g) (1) H$_2$/Pd−C, (2) NH$_4$HCO$_3$.

anhydrohexitol[27] modifications of the ribose ring (Figure 1) are tolerated at P2Y$_1$ receptors. We have further explored the SAR of these two series and introduced other major modifications of the ribose moiety. These modifications include fixing the ring pucker conformation in the carbocyclic series using a bridging cyclopropane ring,[28,29] ring enlargement with introduction of a nitrogen atom,[30] and ring contraction.[31]

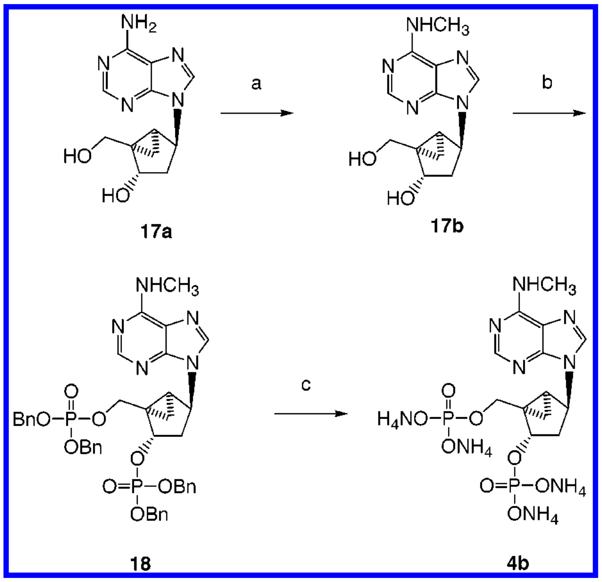
Results

Chemical Synthesis. We have explored structural modifications (Figure 1) of N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (**1a**) in order to increase P2Y$_1$ receptor affinity and selectivity and to probe the relationship between ribose structure and agonist efficacy. 2-Position adenine modifications (e.g., chloro, **1b**; amino, **1c**; and methylthio, **1d**) known to be favorable for antagonist potency[19] have been included for comparison. Synthetic routes are shown in Schemes 1−7, and characterization and yields of the nucleotide derivatives prepared are summarized in Table 1.

An isomer of **1a**, in which a phosphate group is shifted from the 5′- to the 2′-position, **2**, was synthesized by phosphorylation of 5′-deoxyadenosine by the tetrabenzyl pyrophosphate method,[32,33] followed by hydrogenation to remove the benzyl groups. We have also prepared 2′-deoxyadenosine bisphosphate analogues containing four- and five-membered carbocyclic ring (**3**−**6**) and six-membered ring (**7** and **8**) modifications of the ribose moiety.

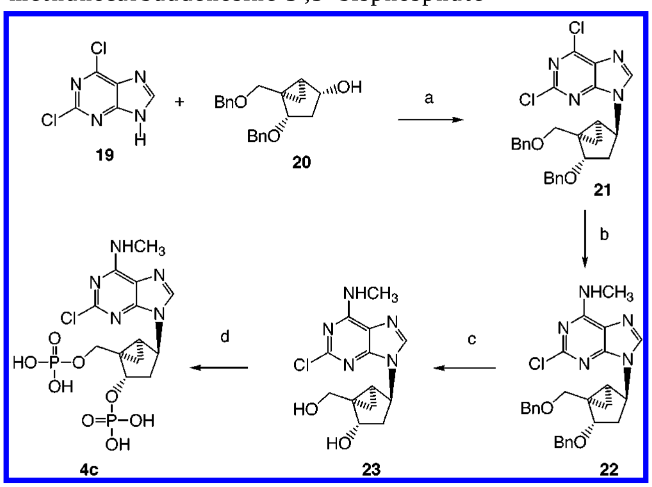
Some of the nucleosides utilized for phosphorylation were obtained commercially, such as the 5′-deoxy analogue leading to compound **2**. The carbocyclic 2′-deoxyadenosine bisphosphate **3a** was reported in the previous study. The N^6-methyl group in **3b** and **3c** was introduced in the carbocyclic series by the Dimroth rearrangement (Scheme 1), starting with aristeromycin, **10**. The 2′-hydroxyl group was removed selectively using

Scheme 2. Synthesis of N^6-Methyl-(N)-methanocarbaadenosine-3′,5′-bisphosphate[a]



[a] Reagents: (a) (1) MeI, DMF, (2) NH_4OH, 90 °C; (b) LDA/TBPP/THF; (c) (1) H_2/Pd−C, (2) NH_4HCO_3.

Scheme 3. Synthesis of 2-Chloro-N^6-methyl-(N)-methanocarbaadenosine-3′,5′-bisphosphate[a]

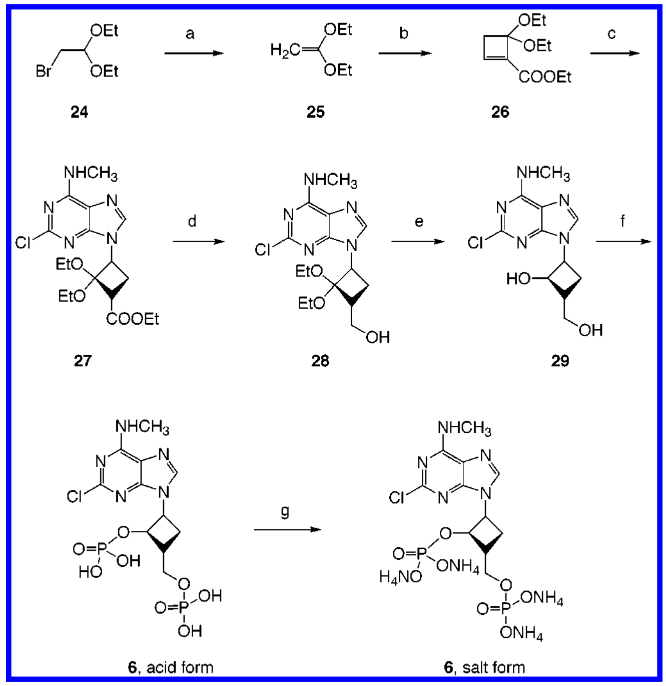


[a] Reagents: (a) DEAD, Ph_3P, THF; (b) $MeNH_2$; (c) BCl_3; (d) (1) LDA/TBPP, (2) BCl_3.

tributyltin hydride and 2,2′-azobis[isobutyronitrile] (AIBN) on the 3′,5′-protected nucleoside **13**. Compound **13** was deprotected and phosphorylated using tetrabenzyl pyrophosphate, leading to **3a**.

The methanocarbocyclic 2′-deoxyadenosine analogues in which the fused cyclopropane ring fixes the conformation of the carbocyclic nucleoside into a rigid Northern (N) or Southern (S) envelope conformation, as defined in the pseudorotational cycle, were synthesized as precursors of nucleotides **4** and **5** by the general approach of Marquez and co-workers.[28,29,34,35] Again, the N^6-methyl group was introduced by the Dimroth rearrangement (Scheme 2). 2-Position adenine modifications were further introduced in the (N)-conformation series as shown in Scheme 3. The 9-cyclobutyladenine analogue **29** (Scheme 4) was synthesized by an adaptation of the method of Schneller et al.[31] and phosphorylated by the phosphorus oxychloride method,[19] leading to compound **6**.

Scheme 4. Synthesis of a 9-Cyclobutyladenine Bisphosphate Derivative[a]



[a] Reagents: (a) KO*t*-BU, heat; (b) HCC−COOEt, CH_2Cl_2, 50 °C; (c) DBU, DMF, rt; (d) $LiAlH_4$, THF, 0 °C; (e) acetone, HCl, rt, $NaBH_4$, MeOH, 0 °C; (f) $POCl_3$, Proton Sponge, $(CH_3O)_3PO$, 0 °C; (g) NH_4HCO_3.

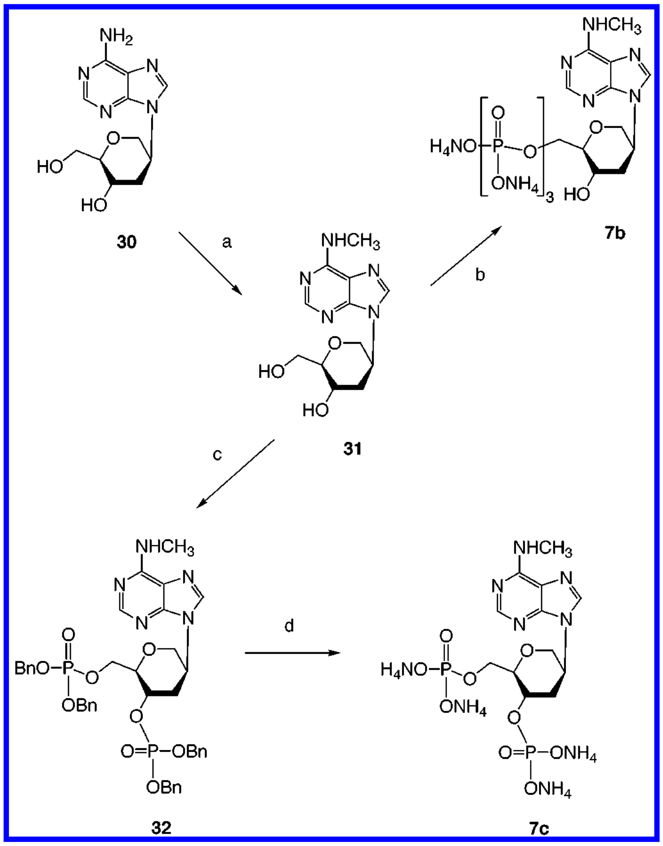
A deoxyanhydrohexitol adenine nucleoside was prepared by the method of Verheggen et al.[27] as the precursor of the unsubstituted bisphosphate **7a**, reported previously.[19] The corresponding N^6-methyl analogue **7c** was synthesized as shown in Scheme 5, leading also to compound **7b**, a triphosphate derivative. A 2-chloro analogue, **7d** was also prepared. Displacement by 2-chloro-N^6-methyladenine, **9b**, of a tosyl derivative, **9a**, of the protected sugar provided the nucleoside starting material for the bisphosphate derivative **7d** (Scheme 6).

Morpholine rings were prepared by periodate oxidative opening of the ribose ring of adenosine-5′-monophosphate,[30] followed by reductive amination with sodium cyanoborohydride (Scheme 7). A phosphonate group could be introduced directly in the reductive amination step using aminoethylphosphonic acid.

Biological Activity. Adenine nucleotides markedly stimulate inositol lipid hydrolysis by phospholipase C in turkey erythrocyte membranes,[36] through activation of a $P2Y_1$ receptor.[37] The agonist used in screening these analogues, 2-MeSADP, has a higher potency than the corresponding triphosphate[53] for stimulation of inositol phosphate accumulation in membranes isolated from [^{3}H]inositol-labeled turkey erythrocytes.

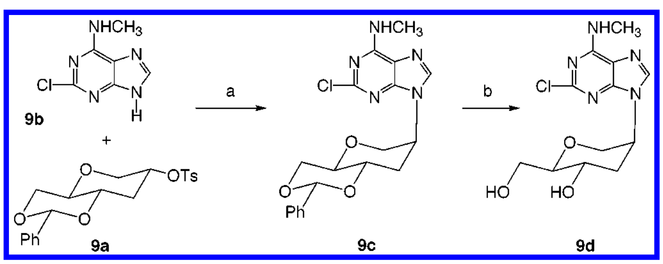
As in our previous studies,[17−19] the deoxyadenosine bisphosphate nucleotide analogues prepared in the present study were tested separately for agonist and antagonist activity in the PLC assay at the $P2Y_1$ receptor in turkey erythrocyte membranes, and the results are reported in Table 2. Concentration−response curves were obtained for each compound alone and in combination with 30 nM 2-MeSADP. Concentration−response curves for representative compounds are shown in Figure 2.

Scheme 5. Synthesis of an 9-Anhydrohexitol Adenine Bisphosphate Derivative[a]



[a] Reagents: (a) (1) MeI, DMF, (2) NH_4OH, 90 °C; (b) $POCl_3$, $(MeO)_3PO$, Proton Sponge; (c) LDA/TBPP, THF; (d) (1) H_2/Pd−C, (2) NH_4HCO_3.

Scheme 6. Synthesis of an 9-Anhydrohexitol 2-Chloro-N^6-methyladenine Bisphosphate Nucleoside Intermediate[a]

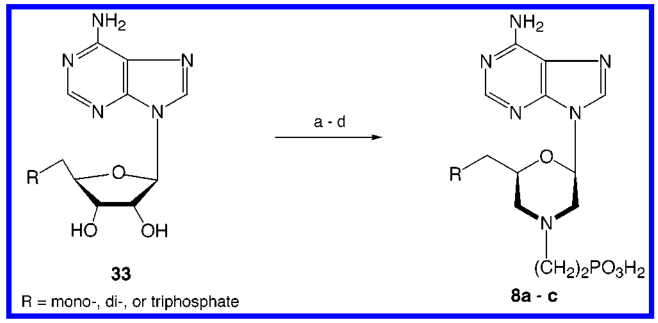


[a] Reagents: (a) LiH, DMF; (b) 80% acetic acid, 60 °C.

For comparison purposes the previously reported N^6-methyl analogues **1a**−**d** are shown in Table 2. All are antagonists, with the potency of 2-substituted analogues decreasing in the order Cl > SCH_3 > NH_2. The analogue in which formalistically a phosphate group of the previous bisphosphates has been shifted from the 5′- to the 2′-position, **2**, was a very weak antagonist at $P2Y_1$ receptors.

Analogues lacking a glycosyl bond, **3**, **4**, **6**, **7**, and **9**, demonstrated that numerous non-ribose structures are recognized at the $P2Y_1$ receptor binding site. Addition of the N^6-methyl group in several cases converted pure agonists to pure antagonists (cf. **4a** and **4b**; **7a** and **7c**). Among simple carbocyclic adenosine analogues **3a**−**c**, the presence of the N^6-methyl group in several cases converted a partial agonist, e.g., **3a**, to a pure antagonist, **3b**, and at the same time increased potency.

Scheme 7. Synthesis of Phosphonate Derivatives of Adeninylmorpholine[a]



[a] Reagents: (a) $NaIO_4$, H_2O; (b) $NH_2(CH_2)_2PO_3H_2$; (c) $NABH_3CN$; (d) NH_4HCO_3.

Table 1. Synthetic Data for Nucleotide Derivatives, Including Structural Verification Using HRMS and Purity Verification Using HPLC

		FAB (M − H+)		HPLC (rt; min)[a]		method,
no.	formula	calcd	found	sys A	sys B	yield (%)[b]
2	$C_{10}H_{15}O_9N_5P_2$	410.0267	410.0269	3.53	10.72	B, 21.7
3b	$C_{12}H_{19}O_8N_5P_2$	422.0631	422.0664	3.41	8.21	B, 8.0
4a	$C_{12}H_{17}O_8N_5P_2$	420.0474	420.0482	3.92	7.30	A, 5.5
4b	$C_{13}H_{19}O_8N_5P_2$	434.0631	434.0622	5.91	7.83	B, 8.3
4c	$C_{13}H_{18}O_8N_5P_2Cl$	468.0241	468.0239	8.05	8.54	B, 2.3
5	$C_{12}H_{17}O_8N_5P_2$	420.0474	420.0481	4.02	6.84	A, 7.5
6	$C_{11}H_{16}O_8N_5P_2Cl$	442.0084	442.0070	6.67	6.82	A, 24.3
7b	$C_{12}H_{20}O_{12}N_5P_3$	518.0237	518.0243	4.98	12.74	A, 1.8
7c	$C_{12}H_{19}O_9N_5P_2$	438.0580	438.0580	4.63	9.36	B, 50.1
7d	$C_{12}H_{18}O_9N_5P_2Cl$	472.0201	472.0190	5.67	9.97	B, 31.3
8a	$C_{12}H_{20}O_8N_6P_2$	437.0740	437.0721	2.37	8.78	8.0
8b	$C_{12}H_{21}O_{11}N_6P_3$	517.0403	517.0404	2.42	9.23	7.2
8c	$C_{12}H_{22}O_{14}N_6P_4$	597.0066	597.0053	2.96	10.02	4.0

[a] Purity of each derivative was ≥95%, as determined using HPLC with two different mobile phases: system A, gradient of 0.1 M TEAA/CH_3CN from 95/5 to 40/60, and system B, gradient of 5 mM TBAP/CH_3CN from 80/20 to 40/60. [b] Phosphorylation methods: method A refers to use of phosphorus oxychloride, and method B refers to use of tetrabenzyl pyrophosphate/lithium diisopropylamide followed by hydrogenation. The percent yields refer to overall yield for each phosphorylation sequence. For the method of synthesis of **8** refer to Experimental Section.

Compound **3b** was equipotent to the ribose analogue **1a**. Curiously, the concurrent presence of a 2-amino group, in **3c**, entirely canceled the effect of the N^6-methyl group, resulting in a mixed agonist/antagonist.

Marquez and co-workers[28,29] have introduced the concept of ring-constrained carbocyclic nucleoside analogues, based on cyclopentane rings constrained in the (N)- and (S)-conformations by fusion with a cyclopropane (methanocarba) ring. In the series of ring-constrained (N)-methanocarba derivatives, the 6-NH_2 analogue **4a** was a pure agonist of EC_{50} 155 nM and 86-fold more potent than the corresponding (S)-isomer **5**, also an agonist. Thus, the ribose ring (N)-conformation appeared to be favored in recognition at $P2Y_1$ receptors. The N^6-methyl- and 2-chloro-N^6-methyl-(N)-methanocarba analogues **4b** and **4c** were antagonists having IC_{50} values of 276 and 53 nM, respectively. A 2-chloro-N^6-methyl-9-cyclobutyl analogue **6** was a pure antagonist of IC_{50} 805 nM.

Six-membered ring anhydrohexitol bisphosphate analogues displayed micromolar potency at $P2Y_1$ receptors, as either agonists (**7a**, 6-NH_2) or antagonists (**7c**, N^6-methyl). The triphosphate analogue **7b** was principally an antagonist of moderate potency, with an IC_{50} of 2.37 μM. The 2-chloro-N^6-methyl analogue **7d** was a more

Table 2. In Vitro Pharmacological Data for Stimulation of PLC at Turkey Erythrocyte $P2Y_1$ Receptors (agonist effect) and Inhibition of PLC Stimulation Elicited by 30 nM 2-MeSADP (antagonist effect), for at Least Three Separate Determinations

no.	agonist effect, % of maximal increase[a]	EC_{50}, μM[a]	antagonist effect, % of maximal inhibition[b]	IC_{50}, μM[b] (*n*)
1a[c,e]	NE		99 ± 1	0.331 ± 0.059 (5)
1b[c,e]	NE		95 ± 1	0.206 ± 0.053
1c[e]	4	*d*	96 ± 2	1.85 ± 0.74
1d[e]	6 ± 2	*d*	94 ± 2	0.362 ± 0.119
2	NE		47 ± 2[f]	small decrease (4)
3a[e]	27 ± 11	7.21 ± 4.40	73 ± 11	2.53 ± 0.57
3b	NE		100	0.148 ± 0.069 (5)
3c[e]	26 ± 3	6.51 ± 2.75	74 ± 3	5.42 ± 2.13
4a	92 ± 5	0.155 ± 0.021	NE	
4b	NE		100	0.157 ± 0.060
4c	NE		100	0.0516 ± 0.0008
5	50 ± 4	18.9 ± 5.7	42 ± 10%[f]	~40
6	NE		100	0.805 ± 0.349 (5)
7a[e]	100	2.99 ± 0.35	NE	
7b	18 ± 5	9.35 ± 3.43	82 ± 5	2.37 ± 0.54
7c	NE		100	1.64 ± 0.43
7d	NE		100	0.566 ± 0.224
8a	NE	37 ± 6[f]	37 ± 6[f]	small decrease
8b	11 ± 1	*d*	NE	
8c	NE		41 ± 12%[f]	small decrease

[a] Agonist potencies were calculated using a four-parameter logistic equation and the GraphPad softaware package (GraphPad, San Diego, CA). EC_{50} values (mean ± standard error) represent the concentration at which 50% of the maximal effect is achieved. Relative efficacies (%) were determined by comparison with the effect produced by a maximal effective concentration of 2-MeSADP in the same experiment. [b] Antagonist IC_{50} values (mean ± standard error) represent the concentration needed to inhibit by 50% the effect elicited by 30 nM 2-MeSADP. The percent of maximal inhibition is equal to 100 minus the residual fraction of stimulation at the highest antagonist concentration. [c] **1a**, MRS 2179; **1b**, MRS 2216; **3b**, MRS 2267; **4a**, MRS 2268; **4c**, MRS 2279; **5**, MRS 2266; **6**, MRS 2264; **7a**, MRS 2255; **7c**, MRS 2269; **7d**, MRS 2283. [d] EC_{50} was not calculated for increases of ≤15% at 100 μM. [e] Values from refs 17, 19. NE, no effect at 100 μM.

potent antagonist activity, with an IC_{50} of 0.57 μM. Another set of six-membered rings, morpholino (mono-, di-, or triphosphate) analogues **8a**−**c** containing an aminophosphonic acid, was nearly inactive at $P2Y_1$ receptors.

Molecular Modeling. To better understand the role of the sugar puckering on the human $P2Y_1$ agonist and antagonist activities, we carried out a molecular modeling study of this new generation of ribose-modified ligands. Such modifications include cyclopentyl rings constrained in the (N)- and (S)-conformations with cyclopropyl (methanocarba) groups, six-membered rings (morpholino and anhydrohexitol analogues), and cyclobutyl nucleotides. We have recently developed a model of the human $P2Y_1$ receptor, using rhodopsin as a template, by adapting a facile method to simulate the reorganization of the native receptor structure induced by the ligand coordination (*cross-docking* procedure).[38] Details of the model building are given in the Experimental Section. We have also reported the hypothetical molecular basis for recognition by human $P2Y_1$ receptors of the natural ligand ATP and the new potent, competitive antagonist 2′-deoxy-N^6-methyladenosine-3′,5′-bisphosphate (**1a**). Both ATP and **1a** are present in the hypothetical binding site with a (N)-sugar ring conformation. In the present work, the sterically constrained (N)- and (S)-methanocarba agonist analogues **4a** and **5**, respectively, were docked into the putative binding site of our previously reported $P2Y_1$ receptor model. According to their structural similarity, the *cross-docking* procedure demonstrated that the receptor architecture found for binding of ATP and **1a** was energetically appropriate also for the binding of both **4a** and **5**. However, the (N)-methanocarba/$P2Y_1$ complex appeared more stable than the (S)-methanocarba/$P2Y_1$ complex by approximately 20 kcal/mol (does not include entropic and solvation effects). Figure 3A represents the lowest energy docked complex of (N)-methanocarba agonist in the proposed ligand binding cavity. In this model, the side chain of Gln307(TM7) is within hydrogen-bonding distance of the N^6 atom at 1.8 Å and the side chain of Ser314(TM7) is positioned at 2.0 Å from the N1 atom and at 3.4 Å from the N^6 of the purine ring. As already reported, another three amino acids are important for the coordination of the phosphate groups in this antagonist: Arg128(TM3), Lys280(TM6), and Arg310(TM7). As shown in Figure 3A Lys280 may interact directly with both 3′- and 5′-phosphates (1.7 Å, O3′; and 1.7 Å, O5′), whereas Arg128(TM3) and Arg310(TM7) are within ionic coupling range to both the O2 and O3 atoms of the 5′-phosphate. As shown in Figure 3B, a poor superimposition (rms = 1.447) between the (N)- and (S)-methanocarba agonist analogues has been found inside the receptor binding domain. In particular, the adenine moiety and 5′-phosphate of the (S)-methanocarba derivative are shifted out of position with respect to those presented by the (N)-methanocarba isomer, decreasing the stability of the (S)-methanocarba/$P2Y_1$ complex. This fact might be correlated with the difference of their biological activity (see Table 3).

Using the information that a common binding site could be hypothesized among these deoxyadenosine bisphosphate analogues, a superimposition analysis of the energy minimization of the more potent antagonists has been performed. In this analysis we have used **1a** as a reference compound, and we have defined three

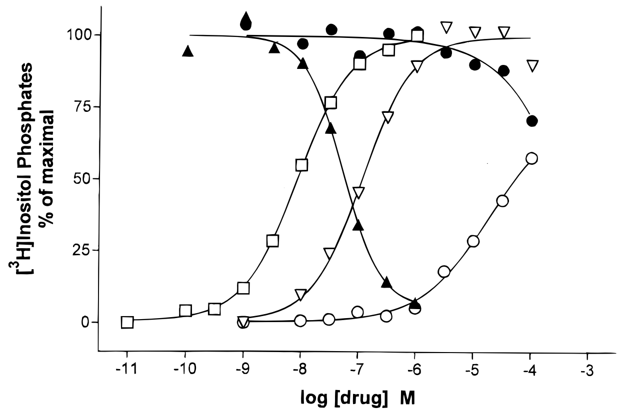


Figure 2. Effects of deoxyadenosine bisphosphate derivatives on $P2Y_1$ receptor-activated phospholipase C activity in turkey erythrocyte membranes: concentration-dependent stimulation of inositol phosphate formation by 2-MeSADP (□), compound **4a** (▽), and compound **5** (○) and its inhibition in the presence of 30 nM 2-MeSADP by compound **4c** (▲) and compound **5** (●). Membranes from [^{3}H]inositol-labeled erythrocytes were incubated for 5 min at 30 °C in the presence of the indicated concentrations of 2-MeSADP or of test compound, either alone or in combination with 30 nM 2-MeSADP. The data shown are typical curves for at least three experiments carried out in duplicate using different membrane preparations.

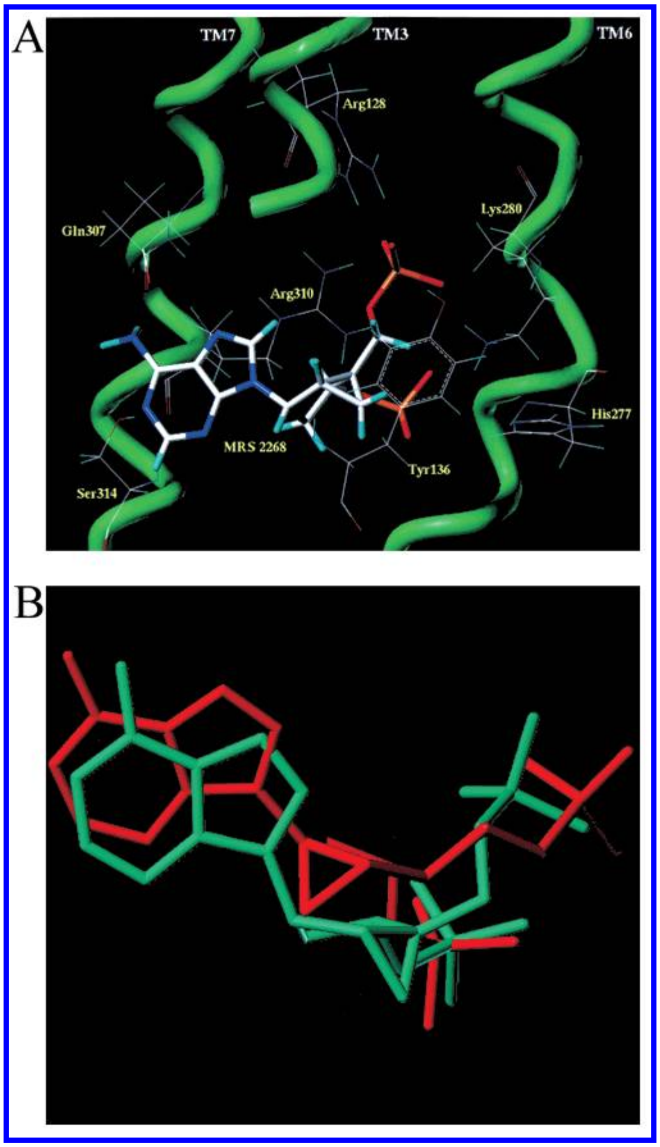


Figure 3. (A) Side view of the **4a**/P2Y$_1$ receptor complex model. The side chains of the important residues in proximity to the docked **4a** molecule are highlighted and labeled. Residues in proximity (≤5 Å) to the docked **4a** molecule: Arg128(TM3), Tyr136(TM3), His277(TM6), Lys280(TM6), Gln307(TM7), Arg310(TM7), and Ser314(TM7). (B) Alignments generated by "Fit Atoms" analysis between **4a** (green) and **5** (red).

Table 3. Superimposition rms Values of Bisphosphate Agonist and Antagonist Derivatives (agonist reference structure: **4a**; antagonist reference structure: **1a**)

no.	rms, Å	EC$_{50}$, μM[a]	IC$_{50}$, μM[b]
		Agonists	
4a		0.152	
5	1.447	13.3	
		Antagonists	
1a			0.331
4b	0.359		0.157
6	0.777		0.805
7c	0.799		1.64
c	0.829		1.60

[a] See footnote *a* of Table 2. [b] See footnote *b* of Table 2. [c] 2-[2-(6-Methylaminopurin-9-yl)ethyl]propane-1,3-bisoxy(diammonium phosphate).[41]

matching pairs of atoms, corresponding to the N^6 atom of the purine ring and the P atoms of both 3′- and 5′-

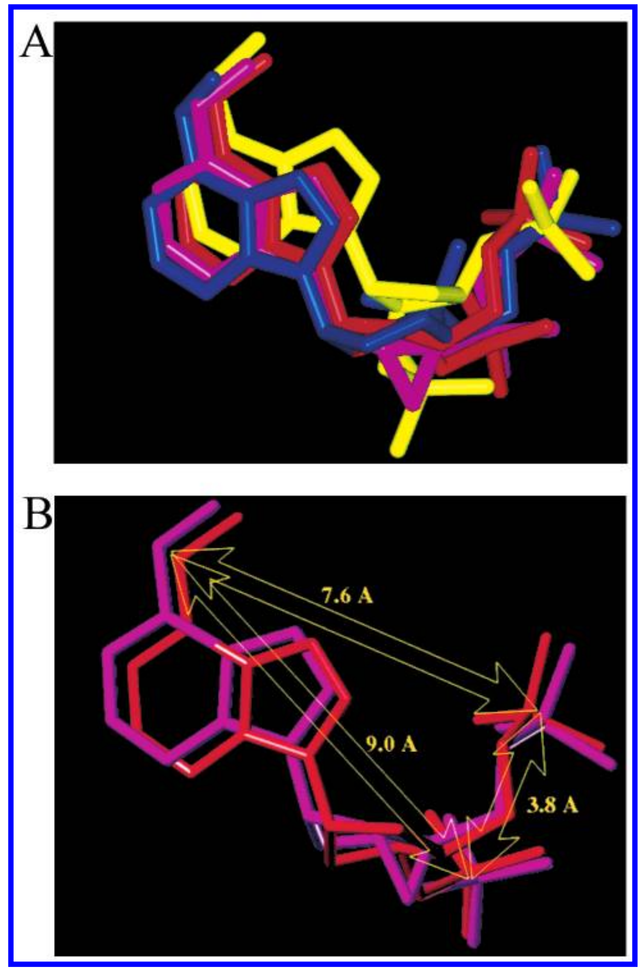


Figure 4. (A) Superposition of docked P2Y$_1$ antagonist structures, all of which contain an N^6-Me group. The transmembrane helical bundle is not highlighted, but it conserves the same arrangement shown in Figure 3A: red = **1a;** magenta = **4b**; yellow = **6**; blue = **7c**. (B) Alignment of **4b** (magenta) generated by "Fit Atoms" analysis using **1a** (red) as reference structure.

phosphate groups, to carry out the superimposition analysis. As reported in Table 3, acceptable rms values have been obtained for all the antagonists compared with the **1a** structure. As shown in Figure 4A, this superimposition study suggested that the two phosphate groups may occupy common receptor regions, and a general pharmacophore model for bisphosphate antagonists binding to the human P2Y$_1$ receptor can be extrapolated (see Figure 4B). The model defines approximate distances between phosphate groups (3.8 Å) and between 5′- and 3′-phosphates and the exocyclic amine (7.6 and 9.0 Å, respectively).

Discussion

In conclusion, the present study has identified new pharmacological probes of P2Y$_1$ receptors, including full agonists, partial agonists, and antagonists. Such probes may be useful, for example, in characterizing antithrombotic effects in platelets, in which at least three P2 receptors coexist.[9−11] The selectivity of the present compounds for P2Y$_1$ receptors is being explored. While compound **1a** was moderately potent at P2X$_1$ receptors, antagonists **1b**, **3b**, and **7c** did not block P2X$_1$ or P2X$_3$ receptors at 10 μM.[25]

The SAR of **1a** indicates that the ribose ring oxygen may be readily substituted with carbon, as in **3**. Furthermore, analogues of constrained conformation, e.g., the (*N*)-methanocarba analogues **4**, displayed enhanced receptor affinity. Additional 2-chloro and N^6-methyl substitution is favorable for affinity at $P2Y_1$ receptors, and nearly pure antagonism is maintained provided that the N^6-methyl group is present. Thus, in this respect, the carbocyclic analogues behaved similar to the ribose series of $P2Y_1$ receptor antagonists,[19] in which 2-substitution tended to increase the potency. Similarly, in the agonist series of 5′-AMP and 5′-ATP derivatives,[39,45] 2-thioether substitution increased potency at $P2Y_1$ receptors. The 2-amino analogue **3c** was a mixed agonist/antagonist.

Among anhydrohexitol adenine bisphosphate derivatives, **7a** was a pure agonist of potency similar to ATP, while the N^6-methyl analogues **7c** and **7d** were pure antagonists. The triphosphate **7b** displayed both agonist and antagonist properties. Thus, the biological potency and efficacy of this series of bisphosphates appeared to be highly dependent on subtle conformational factors, which would influence the orientation of the phosphate groups within the receptor binding site.

The sugar moiety of nucleosides and nucleotides in solution is known to exist in a rapid, dynamic equilibrium between extreme (N)-(2′-*exo*/3′-*endo*) and (S)-(2′-*endo*/3′-*exo*) conformations[29,40] as defined in the pseudorotational cycle. While the energy gap between (N)- and (S)-conformations is in the neighborhood of 4 kcal/mol, such a disparity can explain the difference between micromolar and nanomolar binding affinities. Using a molecular modeling approach, we have analyzed the sugar conformational requirements for a new class of bisphosphate ligands binding to the human $P2Y_1$ receptor. As experimentally shown, the ribose ring (N)-conformation appeared to be favored in recognition at the human $P2Y_1$ receptor (see Table 3). We have found new support to our recently presented hypothesis in which three important recognition regions are present in the bisphosphate molecular structures: the N1 atom of the purine ring and the P atoms of both 3′- and 5′-phosphate groups. The (N)-conformation seems to be essential to maximize the electrostatic interactions between the negatively charged phosphates and the positively charged amino acids present in the receptor binding cleft, as well as Arg128(TM3), Lys280(TM6), and Arg310(TM7), as shown in Figure 3A.

Our hypothesis is that the conformationally rigid pseudosugar of **4** serves as a scaffold to position the phosphate groups in the correct orientation relative to the adenine ring for optimal interaction. Other differences between **4** and **5** that may in principle contribute to the (N)-methanocarba effect on receptor affinity include the barrier to pseudoglycosyl bond rotation ((N)-conformation favors an anti-conformation at the glycosidic bond[54]) and the steric and hydrophobic properties of the cyclopropane methylene group. In this regard, it is to be noted that the methylene carbon does not protrude outside the region of the five-membered ring into the binding regions of the base or hydroxyl groups due to the constraints of the pseudoboat shape of the bicyclo[3.1.0]hexane (Figure 1).

Interestingly, we have already reported that these electrostatic contacts are also crucial for the recognition of bisphosphate antagonists. Using superimposition analysis, a general pharmacophore model for the bisphosphate antagonists binding to the $P2Y_1$ receptor has been proposed (see Figure 4B). According to the pharmacophore map, recognition of all bisphosphate antagonists at a common region inside the receptor binding site and, consequently, a common electrostatic potential profile is possible. As well as for the agonists, the (N)-conformation seems to be essential to maximize the electrostatic interactions between the negatively charged phosphates and the positively charged amino acids present in the receptor binding cleft. As we predicted using the previously reported $P2Y_1$ receptor model,[38] the sugar moiety does not seem to be crucial for the ligand recognition process. This is indicated by the affinity of carbocyclic antagonists **4b** and **6** (Figure 4A) and acyclic nucleotides which are $P2Y_1$ antagonists, such as 2-[2-(6-methylaminopurin-9-yl)ethyl]propane-1,3-bisoxy(diammonium phosphate).[41] In our pharmacophore model, the sugar moiety plays the role of appropriate spacer between the N^6 position of the purine system and the two phosphate groups (3′ and 5′, respectively). Consequently, the sugar ring can be replaced without drastically losing biological activities.

As described above, the simple addition of the N^6-methyl group in several cases converted pure agonists to antagonists. From a pharmacological point of view, this is really a unique situation. Generally, how agonist binding transforms a resting GPCR into its active form and the microscopic basis of binding site blockade by an antagonist are still unclear. Also in this specific case, there are not enough data to appropriately answer the question. We know that Gln307(TM7) and Ser314(TM7) are positioned, in our model, in the vicinity of the N^6-amine of the adenine moiety. We speculate that these two amino acids may be involved in the recognition of the nucleotide base in the agonist structure. The important role of HN^6 of the adenine moiety, putatively through Gln307 and Ser314, as a hydrogen-bond acceptor has been demonstrated using a doubly alkylated N^6-derivative of ATP, for which no agonist activity was observed.[17,42] Moreover, a markedly reduced response of 2-MeSADP was observed for the Q307A and S314A mutant receptors compared with the wild-type receptor.[38] Rotations and translations of the TM domains are crucial factors in the ligand recognition and activation process in different GPCRs, as recently described from Moro et al.[38] We hypothesize that the N^6-amine of the adenine moiety could simultaneously make a double hydrogen-bonding interaction with both Gln307(TM7) and Ser314(TM7), and this double hydrogen-bonding interaction could be the trigger of the activation process. With the addition of the N^6-methyl group it is not possible to have a double hydrogen-bonding interaction, and consequently, the activation pathway is blocked. However, for all the N^6-methyl antagonists the possibility to participate in at least one of the two possible hydrogen bonds appears to be very important for the increase in affinity at the $P2Y_1$ receptor.

Experimental Section

Chemical Synthesis. Nucleosides and synthetic reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and

Aldrich (St. Louis, MO). 6-Chloro-2′-deoxypurine riboside was obtained from Sigma. Compound **1a** was synthesized in our laboratory as described.[17] Several 2′-deoxy nucleosides, including an anhydrohexitol adenine nucleoside[25] and 2′-deoxyaristeromycin,[19] were synthesized as reported.

^{1}H NMR spectra were obtained with a Varian Gemini-300 spectrometer using D_2O as a solvent. ^{31}P NMR spectra were recorded at room temperature by use of a Varian XL-300 spectrometer (121.42 MHz); orthophosphoric acid (85%) was used as an external standard.

Purity of compounds was checked using a Hewlett-Packard 1090 HPLC apparatus equipped with an SMT OD-5-60 RP-C18 analytical column (250 × 4.6 mm; Separation Methods Technologies, Inc., Newark, DE) in two solvent systems. System A: linear gradient solvent system: 0.1 M TEAA/CH_3CN from 95/5 to 40/60 in 20 min and the flow rate was 1 mL/min. System B: linear gradient solvent system: 5 mM TBAP/CH_3CN from 80/20 to 40/60 in 20 min and the flow rate was 1 mL/min. Peaks were detected by UV absorption using a diode array detector. All derivatives showed more than 95% purity in the HPLC systems.

Low-resolution CI-NH_3 (chemical ionization) mass spectra were carried out with Finnigan 4600 mass spectrometer and high-resolution EI (electron impact) mass spectrometry with a VG7070F mass spectrometry at 6 kV. High-resolution FAB (fast atom bombardment) mass spectrometry was performed with a JEOL SX102 spectrometer using 6-kV Xe atoms following desorption from a glycerol matrix.

Purification of most of the nucleotide analogues for biological testing was carried out on DEAE-A25 Sephadex columns as described above. However, compounds **7b** and **8a**−**c** required HPLC purification (system A, semipreparative C18 column) of the reaction mixtures.

General Procedure of Phosphorylation. Method A: The nucleoside (0.1 mmol) and Proton Sponge (107 mg, 0.5 mmol) were dried for several hours in high vacuum at room temperature and then suspended in 2 mL of trimethyl phosphate. Phosphorus oxychloride (Aldrich; 37 μL, 0.4 mmol) was added, and the mixture was stirred for 1 h at 0 °C. The reaction was monitored by analytical HPLC (eluting with a gradient consisting of buffer:CH_3CN in the ratio 95:5 to 40:60, in which the buffer was 0.1 M triethylammonium acetate (TEAA); elution time was 20 min; flow rate was 1 mL/min; column was SMT OD-5-60 RP-C18; detector was by UV in the E_{max} range of 260−300 nm). The reaction was quenched upon addition of 2 mL of triethylammonium bicarbonate buffer and 3 mL of water. The mixture was subsequently frozen and lyophilized. Purification was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, a linear gradient (0.01 to 0.5 M) of 0.5 M ammonium bicarbonate was applied as the mobile phase, and UV and HPLC were used to monitor the elution. All nucleotide bisphosphates were collected, frozen and lyophilized as the ammonium salts. All synthesized compounds gave correct molecular mass (high-resolution FAB) and showed more than 95% purity (HPLC, retention times are reported in Table 1).

Method B: Nucleoside (0.1 mmol) dried for several hours in high vacuum at room temperature was dissolved in 2 mL of dry THF. Lithium diisopropylamide solution (Aldrich; 2.0 M in THF, 0.4 mmol) was added slowly at −78 °C. After 15 min, tetrabenzyl pyrophosphate (Aldrich; 0.4 mmol) was added and the mixture was stirred for 30−60 min at −78 °C. The reaction mixture was warmed to 0 °C to room temperature and stirred for an additional period ranging from 2 to 24 h. Chromatographic purification (pTLC, $CHCl_3$:CH_3OH, 10:1) gave the tetrabenzyl phosphorylated compound. This compound (20 mg) was dissolved in a mixture of methanol (2 mL) and water (1 mL) and hydrogenated over a 10% Pd-on-C catalyst (10 mg) at room temperature for 62 h. The catalyst was removed by filtration and the methanol was evaporated. The residue was treated with ammonium bicarbonate solution and subsequently frozen and lyophilized. Purification, if necessary, was by the same procedure as in method A.

5′-Deoxyadenosine-2′,3′-bis(diammonium phosphate) (2). 24.3 mg (0.0967 mmol) of 5′-deoxyadenosine reacted with tetrabenzyl pyrophosphate following the general procedure B to give 26.9 mg (0.0348 mmol, 36.0% yield) of the desired compound, 5′-deoxyadenosine-2′,3′-bis(dibenzyl phosphate): ^{1}H NMR (CD_3OD) δ 1.35 (3H, d, J = 6.8 Hz, 4′-CH_3), 4.22 (1H, m, H-4′), 4.98 (1H, m, H-2′), 5.10 (2H, d. J = 9.8 Hz, CH_2), 5.75 (1H, m, H-3′), 6.10 (1H, d, J = 4.9 Hz, H-1′), 7.24 (5H, m, C_6H_5), 8.12 (1H, bs, H-2, H-8); ^{31}P NMR (CD_3OD) δ −1.15 (bs).

For final deprotection of the phosphate groups, 20.0 mg (0.0259 mmol) of the tetrabenzyl intermediate was converted to the corresponding phosphoric acid analogue by catalytic hydrogenation as described in the general procedure B to give 7.5 mg (0.0156 mmol, 60.4% yield) of the desired compound: ^{1}H NMR (D_2O) δ 1.41 (3H, d, J = 6.8 Hz, 4′-CH_3), 4.44 (1H, m, H-4′), 4.62 (1H, m, H-2′), 5.17 (1H, m, H-3′), 6.20 (1H, d, J = 4.9 Hz, H-1′), 8.25 (1H, s, H-2), 8.42 (1H, s, H-8); ^{31}P NMR (D_2O) δ 2.78, 3.06 *(2s, 2′-P, 3′-P).*

Carbocyclic *N*⁶-Methyl-2′-deoxyadenosine-3′,5′-bis(diammonium phosphate) (3b) [*N*⁶-Methyl-2′-deoxyaristeromycin-3′,5′-bis(diammonium phosphate)]. 17.9 mg (0.0228 mmol) of compound **16** was converted to the corresponding phosphoric acid analogue using hydrogenation following the general procedure B. Purification was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, linear gradient (0.01 to 0.5 M) of 0.5 M ammonium bicarbonate was applied as the eluent to give 3.0 mg (0.0061 mmol, 26.8% yield) of the desired compound:^{1}H NMR (D_2O) δ 1.85−2.61 (1H, m, H-4′, 2H, m, CH_2-2′, 2H, m, CH_2-4′), 3.07 (3H, s, N^6-CH_3), 3.94 (2H, m, CH_2-5′), 4.67 (1H, m, H-3′), 5.06−5.11 (1H, m, H-1′), 8.22 (1H, s, H-2), 8.27 (1H, s, H-8); ^{31}P NMR (D_2O) δ 1.20, 2.34 *(2s, 3′-P, 5′-P).*

(*N*)-Methanocarba-2′-deoxyadenosine-3′,5′-bis(diammonium phosphate) (4a) [(*1R,2S,4S,5S*)-1-[(Phosphato)methyl]-4-(6-aminopurin-9-yl)bicyclo[3.1.0]hexane-2-phosphate Tetraammonium Salt]. Starting from 16 mg (0.06 mmol) of (N)-methanocarba-2′-deoxyadenosine and following the general phosphorylation procedure A we obtained 1.8 mg (0.0037 mmol, 5.5% yield) of the desired compound: ^{1}H NMR (D_2O) δ 0.90 (1H, m, CH_2-6′), 1.10 (1H, m, CH_2-6′), 1.82 (1H, m, CH-5′), 1.91 (1H, m, CH_2-3′) 2.23 (1H, m, CH_2-3′), 3.49 (1H, d, J = 11.7 Hz, CH_2O), 4.16 (1H, d, J = 11.7 Hz, CH_2O), 4.90−4.97 (1H, m, CH-4′), 5.12 (1H, d, J = 6.9 Hz, CH-2′), 8.39 (1H, s, H-2), 8.54 (1H, s, H-8); ^{31}P NMR (D_2O) δ 0.43 (s, 5′-P), −0.19 (s, 3′-P).

(N)-Methanocarba-*N*⁶-methyl-2′-deoxyadenosine-3′,5′-bis(diammonium phosphate) (4b) [(*1R,2S,4S,5S*)-1-[(Phosphato)methyl]-4-(6-methylaminopurin-9-yl)bicyclo[3.1.0]-hexane-2-phosphate Tetraammonium Salt]. Compound **18** (13.5 mg, 0.0170 mmol) was converted to the corresponding phosphoric acid analogue using hydrogenation following the general procedure B. Purification was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, eluting with a linear gradient of ammonium bicarbonate (0.01 M to 0.5 M) to give 3.0 mg (0.0060 mmol, 35.3% yield) of the desired compound: ^{1}H NMR (D_2O) δ 0.93−0.98 (1H, m, CH_2-6′), 1.17 (1H, m, CH_2-6′), 1.86−1.88 (1H, m, CH-5′), 1.94−1.98 (1H, m, CH_2-3′), 2.23−2.31 (1H, m, CH_2-3′), 3.09 (3H, bs, N^6-CH_3), 3.61−3.64 (1H, m, CH_2O), 4.51−4.55 (1H, m, CH_2O), 5.01−5.03 (1H, m, CH-4′), 5.19−5.21 (1H, m, CH-2′), 8.22 (1H, s, H-2), 8.51 (1H, s, H-8); ^{31}P NMR (D_2O) δ 1.26, 1.92 (2s, 3′-P, 5′-P).

(N)-Methanocarba-*N*⁶-methyl-2-chloro-2′-deoxyadenosine-3′,5′-bis(diammonium phosphate) (4c) [(*1R,2S,4S,5S*)-1-[(Phosphato)methyl]-4-(2-chloro-6-aminopurin-9-yl)bicyclo[3.1.0]hexane-2-phosphate Tetraammonium Salt]. The nucleoside, compound **23**, reacted with tetrabenzyl pyrophosphate, as in method B, followed by an alternative deprotection procedure. Starting from 10 mg (0.0323 mmol) of (N)-methanocarba-*N*⁶-methyl-2-chloro-2′-deoxyadenosine and following the general phosphorylation procedure (method B) we obtained 9.5 mg (0.0114 mmol, 35% yield) of the desired compound, (N)-methanocarba-*N*⁶-methyl-2-chloro-2′-deoxyadenosine-3′,5′-bis(dibenzyl phosphate): ^{1}H NMR ($CDCl_3$) δ

0.75–0.81 (1H, m, CH_2-6′), 1.03–1.08 (1H, m, CH_2-6′), 1.49–1.51 (1H, m, CH-5′), 1.84–1.94 (1H, m, CH_2-3′), 1.99–2.10 (1H, m, CH_2-3′), 3.12 (3H, bs, N^6-CH_3), 4.11–4.20 (1H, m, CH_2O), 4.50–4.55 (1H, m, CH_2O), 4.90–4.98 (8H, m, $-OCH_2$), 4.99–5.01 (1H, m, CH-4′), 5.23–5.30 (1H, m, CH-2′), 5.90 (1H, bs, NH), 7.20–7.29 (20H, m, C_6H_5), 7.82 (1H, s, H-8); ^{31}P NMR (D_2O) δ −0.58 (s, 5′-P); −1.06 (s, 3′-P); MS (CI-NH_3) (M + 1) 830; HRMS (FAB−) (M + Cs) calcd 962.1252, found 962.1252.

The tetrabenzyl-protected intermediate (9.5 mg, 0.0114 mmol) was added to dry CH_2Cl_2 (1.0 mL) and cooled to −78 °C under argon. The mixture was treated with 100 μL of boron trichloride solution (1 M in CH_2Cl_2) and 100 μL of anisole.[43] The reaction mixture was stirred for 24 h at 0 °C, allowed to warm to room temperature, and quenched with 1 M triethylammonium bicarbonate solution (Sigma). The CH_2Cl_2 was removed in vacuo, and the aqueous residue was lyophilized. Purification was performed on an ion-exchange column packed with Sephadex-DEAE A-25 resin, eluting with a linear gradient of 0.01 to 0.5 M ammonium bicarbonate to give 0.4 mg (0.0007 mmol, 7% yield) of the desired compound, **4c**: ^{1}H NMR (D_2O) δ 0.91–0.96 (1H, m, CH_2-6′), 1.12–1.16 (1H, m, CH_2-6′), 1.80–1.84 (1H, m, CH-5′), 1.85–1.98 (1H, m, CH_2-3′), 2.20–2.50 (1H, m, CH_2-3′), 3.08 (3H, bs, N^6-CH_3), 3.57–3.60 (1H, m, CH_2OH), 4.52–4.67 (1H, m, CH_2OH), 4.94–4.96 (1H, m, CH-4′), 5.18–5.21 (1H, m, CH-2′), 8.52 (1H, s, H-8); ^{31}P NMR (D_2O) δ 1.82, 2.52 (2s, 3′-P, 5′P).

(*S*)-Methanocarba-2′-deoxyadenosine-3′,5′-bis(diammonium phosphate) (5) [(*1S,3S,4R,5S*)-4-[(Phosphato)-methyl]-1-(6-aminopurin-9-yl)bicyclo[3.1.0]hexane-3-phosphate Tetraammonium Salt]. Starting from 16 mg (0.06 mmol) of (*S*)-methanocarba-2′-deoxyadenosine and following the general phosphorylation procedure A, we obtained 2.1 mg (0.0043 mmol, 7.5% yield) of the desired compound **5**: ^{1}H NMR (D_2O) δ 1.36 (1H, m, CH_2-6′), 1.53 (1H, t, J = 4.8 Hz, CH_2-6′), 2.05 (1H, m, CH_2-5′), 2.30 (1H, m, CH-4′), 2.46 (2H, m, CH_2-2′), 3.97 (2H, m, CH_2OH), 4.45 (1H, d, J = 6.6 Hz, CH-3′), 8.16 (1H, s, H-2), 8.30 (1H, s, H-8); ^{31}P NMR (D_2O) δ 0.85 (bs, 5′-P), 0.31 (bs, 3′-P).

2′-(2-Chloro-6-methylaminopurin-9-yl)cyclobutane-1′,5′-bis(diammonium phosphate) (6). Starting from 15 mg (0.052 mmol) of 2′-(2-chloro-6-methylaminopurin-9-yl)cyclobutane, **29**, and following the general phosphorylation procedure A, we obtained 5.6 mg (0.0126 mmol, 24.3% yield) of the desired compound: ^{1}H NMR (D_2O) δ 2.55 (1H, m, H-2′), 3.07 (3H, s, $NHCH_3$), 3.33 (2H, m, CH_2-4′), 4.10 (2H, t, J = 5.1 Hz, CH_2-5′), 4.48 (2H, m, H-1′ and H-3′), 8.29 (1H, s, H-8); ^{31}P NMR (D_2O) δ 0.99 (s, 4′-P), −0.53 (d, J = 2.4 Hz, 2′-P).

***N*⁶-Methyl-1,5-anhydro-2-(adenin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol-6-triphosphate Tetraammonium Salt (7b).** Starting from 26.6 mg (0.0952 mmol) of **31**, and following the general phosphorylation procedure A. Purification was performed by semipreparative HPLC using system A, and we obtained 1.0 mg (0.0017 mmol, 1.8% yield) of the desired compound: ^{1}H NMR (D_2O) δ 2.09 (1H, m, H-3′), 2.51 (1H, m, H-3′), 3.09 (3H, s, N^6-CH_3), 3.63 (1H, m, H-5′), 3.88 (1H, m, H-4′), 4.10–4.30 (1H, m, H-1′), 4.27 (2H, m, H-6′), 8.25 (1H, s, H-2), 8.43 (1H, s, H-8); ^{31}P NMR (D_2O) δ −7.96 (m), −10.39 (m), −22.22 (m).

***N*⁶-Methyl-1,5-anhydro-2-(adenin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol-4,6-bis(diammonium phosphate) (7c).** Compound **32** (25.0 mg, 0.0312 mmol) was converted to the corresponding phosphoric acid analogue using hydrogenation following the general procedure B to give 14.2 mg (0.0280 mmol, 89.7% yield) of the desired compound: ^{1}H NMR (D_2O) δ 2.12–2.22 (1H, m, H-3′), 2.64–2.68 (1H, m, H-3′), 3.04 (3H, s, N^6-CH_3), 3.66–3.75 (1H, m, H-5′), 3.88–3.96 (1H, m, H-4′), 4.08–4.09 (2H, m, CH_2-6′), 3.96–4.30 (2H, m, H-1′), 4.86 (1H, bs, H-2′), 8.17 (1H, s, H-2), 8.39 (1H, s, H-8); ^{31}P NMR (D_2O) δ 3.97, 2.55 (*2s, 3′-P, 5′-P).*

1,5-Anhydro-2-(2-chloro-*N*⁶-methyladenin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol-4,6-bis(diammonium phosphate) (7d). The 2-chloro-N^6-methylanhydrohexitol nucleoside, **9d**, reacted with tetrabenzyl pyrophosphate, as in method B, followed by an alternative deprotection procedure. Starting from 15.0 mg (0.048 mmol) of **9d** and following the general phosphorylation procedure of method B, we obtained 30.0 mg (0.036 mmol, 75.0% yield) of the desired compound, 1,5-anhydro-2-(2-chloro-N^6-methyladenin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol-4,6-(dibenzyl phosphate): ^{1}H NMR ($CDCl_3$) δ 1.99–2.09 (1H, m, H-3′), 2.60–2.64 (1H, m, H-3′), 3.20 (3H, s, N^6-CH_3), 3.55–3.60 (1H, m, H-5′), 3.83 (1H, dd, J = 2.0,12.7, H-1′), 4.07–4.13 (1H, m, H-4′), 4.18 (1H, d, J = 12.7, H-1′), 4.32–4.38 (2H, m, CH_2-6′), 4.98 (8H, 4d, J = 7.9 Hz, O−CH_2), 6.11 (1H, bs, H-2′), 7.03 (1H, bs, NH), 7.30 (20H, m, C_6H_5), 8.08 (1H, s, H-8); ^{31}P NMR (CD_3Cl) −0.76, −1.77 (2s, 4′-P, 6′-P).

30.0 mg (0.036 mmol) of the tetrabenzyl-protected intermediate added to dry CH_2Cl_2 (1.0 mL) was cooled to −78 °C under argon and treated with 400 μL of boron trichloride solution (1 M in CH_2Cl_2) and 400 μL of anisole. The reaction mixture was stirred for 12 h at 0 °C to room temperature and added with triethylammonium bicarbonate (1.0 mL) in ice bath. After the removal of CH_2Cl_2 under nitrogen stream, the reaction mixture was lyophilized. Purification was performed on Sephadex ion-exchange column chromatography described in general procedure A to afford 8.0 mg (0.015 mmol, 41.7% yield) of the desired compound, 1,5-anhydro-2-(2-chloro-N^6-methyladenin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol-4,6-bis(diammonium phosphate): ^{1}H NMR (D_2O) δ 2.13–2.23 (1H, m, H-3′), 2.65–2.69 (1H, m, H-3′), 3.06 (3H, s, N^6-CH_3), 3.68–3.72 (1H, m, H-5′), 3.98–4.06 (1H, m, H-1′) 4.09–4.11 (2H, m, CH_2-6′), 4.17–4.20 (1H, m, H-4′), 4.30 (1H, d, J = 12.7, H-1′), 4.82 (1H, m, H-2′), 8.40 (1H, s, H-8); ^{31}P NMR (D_2O) δ 3.61, 2.07 (*2s, 4′-P, 6′-P);* HRMS (FAB−) calcd 472.0201, found 472.0190; HPLC 5.67 min (sys A).

Phosphoric Acid Mono[(*2S*)-6-(6-aminopurin-9-yl)-4-(2′-phosphonoethyl)morpholin-2-ylmethyl] Ester (8a). Adenosine 5′-monophosphate sodium salt (240.0 mg, 0.481 mmol), sodium periodate (102.8 mg, 0.481 mmol) and 2-aminoethylphosphonic acid (72.2 mg, 0.577 mmol) were dissolved in 2.0 mL of water. The reaction mixture was stirred at room temperature for 1.5 h. Sodium cyanoborohydride (71.1 mg, 0.962 mmol) was added, and the reaction mixture was stirred for an additional 30 min and passed through Amberlite CG50 (H^+ form) with an elution of water. The acidic fractions were collected, neutralized with triethylamine, purified by Sephadex ion-exchange column chromatography and semipreparative HPLC described in general procedure A to afford 19.5 mg (0.038 mmol, 8.0% yield) of the desired compound: ^{1}H NMR (D_2O) δ 2.03–2.11 (2H, m, CH_2), 3.16 (1H, t, J = 11.7 Hz, 5-CH_2), 3.82 (1H, d, J = 11.7 Hz, 5-CH_2), 3.39–3.43 (2H, m, CH_2), 3.62–3.70 (2H, m, 3-CH_2), 4.08–4.10 (2H, m, 2-CH_2), 4.42–4.45 (1H, m, H-2), 6.18–6.22 (1H, d, J = 10.7 Hz, H-6), 8.25 (1H, s, H-2), 8.33 (1H, s, H-8); ^{31}P NMR (D_2O) δ 19.93, 0.84 (*2s, 2-P, 4-P).*

Phosphoric Acid Di[(2*S*)-6-(6-aminopurin-9-yl)-4-(2′-phosphonoethyl)morpholin-2-ylmethyl] Ester (8b). Adenosine 5′-diphosphate monopotassium salt (140.2 mg, 0.280 mmol), sodium periodate (59.8 mg, 0.280 mmol) and 2-aminoethylphosphonic acid (42.0 mg, 0.336 mmol) were dissolved in 2.0 mL of water. The reaction mixture was stirred at room temperature for 1.5h. Sodium cyanoborohydride (41.3 mg, 0.559 mmol) was added, and the reaction mixture was stirred for an additional 30 min and passed through Amberlite CG50 (H^+ form) with an elution of water. The acidic fractions were collected, neutralized with triethylamine, purified by Sephadex ion-exchange column chromatography and semipreparative HPLC described in general procedure A to afford 12.2 mg (0.0202 mmol, 7.2% yield) of the desired compound:^{1}H NMR (D_2O) δ 1.94–2.06 (2H, m, CH_2), 2.90 (1H, t, J = 11.7 Hz, 5-CH_2), 3.59–3.62 (1H, d, J = 11.7 Hz, 5-CH_2), 3.17–3.25 (2H, m, CH_2), 3.35–3.52 (2H, m, 3-CH_2), 4.12–4.18 (2H, m, 2-CH_2), 4.41–4.45 (1H, m, H-2), 6.13 (1H, dd, J = 10.7, 2.9 Hz, H-6), 8.19 (1H, s, H-2), 8.26 (1H, s, H-8); ^{31}P NMR (D_2O) δ 19.54 (t, J = 7.3 Hz, 4-P), −10.05,−10.90 (2d, J = 20.6 Hz, 2-POP).

Phosphoric Acid Tri[(2*S*)-6-(6-aminopurin-9-yl)-4-(2′-phosphonoethyl)morpholin-2-ylmethyl] Ester (8c). Adenosine 5′-triphosphate disodium salt (100.0 mg, 0.181 mmol), sodium periodate (38.8 mg, 0.181 mmol) and 2-aminoethylphosphonic acid (27.2 mg, 0.218 mmol) were dissolved in 2.0 mL of water. The reaction mixture was stirred at room temperature for 1.5 h. Sodium cyanoborohydride (26.8 mg, 0.363 mmol) was added, and the reaction mixture was stirred for an additional 30 min and passed through Amberlite CG50 (H^+ form) with an elution of water. The acidic fractions were collected, neutralized with triethylamine, purified by Sephadex ion-exchange column chromatography and semipreparative HPLC described in general procedure A to afford 5.0 mg (0.0071 mmol, 4.0% yield) of the desired compound: ^{1}H NMR (D_2O) δ 1.82−1.93 (2H, m, CH_2), 2.53 (1H, t, J = 10.8, 5-CH_2) 3.36−3.40 (1H, d, J = 10.8 Hz, 5-CH_2), 2.90−3.24 (4H, m, CH_2, 3-CH_2), 4.11−44.13 (2H, m, 2-CH_2), 4.27−4.31 (1H, m, H-2), 6.01 (1H, d, J = 8.8 Hz, H-6), 8.25 (1H, s, H-2), 8.37 (1H, s, H-8); ^{31}P NMR (D_2O) δ 21.28 (pt, 4-P), −8.68 (d, J = 18.9 Hz), −10.83 (d, J = 19.6 Hz), −22.34 (t, J = 20.2 Hz).

1,5-Anhydro-2-(2-chloro-6-methylaminopurin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol (9d). A mixture of 2-chloro-6-methylaminopurine (**9b**; 37 mg, 0.2 mmol), lithium hydride (1.6 mg, 0.2 mmol), and 1,5-anhydro-4,6-*O*-benzylidene-3-deoxy-2-*O*-(*p*-tolylsulfonyl)-D-*ribo*-hexitol (**9a**; 78 mg, 0.2 mmol) in DMF (4 mL) was stirred at 80 °C for 48 h. A second amount of 1,5-anhydro-4,6-*O*-benzylidene-3-deoxy-2-*O*-(*p*-tolylsulfonyl)-D-*ribo*-hexitol (78 mg, 0.2 mmol) was added and the mixture was further heated for 48 h at 80 °C. After addition of water (0.5 mL), the reaction mixture was evaporated, diluted with CH_2Cl_2 (20 mL), filtered and washed with water (20 mL). The organic layer was dried and purified by column chromatography on silica (CH_2Cl_2:CH_3OH, 97.5:2.5). The resulting compound (70 mg, 87%) was directly deprotected by treatment with 80% of acetic acid at 60 °C for 6 h. Evaporation, followed by preparation thin-layer chromatography (CH_2Cl_2−CH_3OH 90:10) yielded 20 mg (65%) of the title compound: MS (LSIMS) *m*/*z*: 314 (M + H^+); ^{13}C NMR (DMSO-d_6) δ 27.2 (CH_3), 35.9 (C3′), 50.4 (C2′), 60,5 (C6′), 60.7 (C4′), 67.9 (C1′), 83.0 (C5′), 117.9 (C5), 140.0 (C8), 149.4 (C4), 153.2 (C2), 155.6 (C6); ^{1}H NMR (DMSO-d_6) δ 1.89 (ddd, J = 4.4, 11.4 and 13.4 Hz, H-3′a), 2.26 (br d, J = 13.2 Hz, H-3′e), 2.93 (d, J = 4.2 Hz, CH_3), 3.20 (ddd, J = 2.2, 4.4 and 9.0 Hz, H-5′), 3.52 (tt, J = 5.1 and 10.2 Hz, H-4′), 3.60 (dt, J = 5.8 and 11.7 Hz, H-6′a), 3.69 (ddd, J = 2.0, 5.1 and 11.7 Hz, H-6′e), 3.85 (dd, J = 2.7 and 12.7 Hz, H-1′a), 4.17 (d pst, J = 2.3 and 12.7 Hz, H-1′e), 4.62 (dd, J = 6.4 and 5.6 Hz, 6′-OH), 4.73 (br s, H-2′), 4.89 (d, J = 5.6 Hz, 4′-OH), 8.19 (br, NH), 8.28 (s, H-8). Anal. ($C_{12}H_{16}N_5O_3Cl$) Calcd for (MW 313.74) C, 45.94; H, 5.14; N, 22.32. Found: C, 45.71; H, 5.03; N, 22.17.

Carbocyclic *N*⁶-Methyladenosine (*N*⁶-Methylaristeromycin) (11). The Dimroth rearrangement (Scheme 1) of carbocyclic adenosine (**10**; 450 mg, 1.81 mmol) gave compound **11** as a light yellowish solid (400 mg, 1.52 mmol, 84.0%). Specifically carbocyclic adenosine was heated at 40 °C with methyl iodide (672 μL, 10.8 mmol) in dry DMF (4.0 mL) for 48 h. The solvent was evaporated under reduced pressure, and the residue was heated at 90 °C with ammonium hydroxide (4.0 mL) for 4 h. The water was evaporated, and the residue was purified by pTLC using MeOH:$CHCl_3$ (1:9): ^{1}H NMR (CD_3OD) δ 1.87−2.50 (3H, m, H-4′, 4′-CH_2), 3.10 (3H, bs, N^6-CH_3), (1H, m, H-4′), 3.30 (1H, d, J = 2.0 Hz, CH_2-5′), 3.69 (2H, d, J = 4.9 Hz, CH_2-5′), 4.05 (1H, m, H-2′), 4.48−4.52 (1H, m, H-1′), 8.15 (1H, s, H-2), 8.22 (1H, s, H-8); MS (CI-NH_3) 280 (M^+ + 1).

Carbocyclic *N*⁶-Methyl-3′,5′-*O*-(tetraisopropyldisiloxa-1,3-diyl)adenosine (12). A solution of carbocyclic N^6-methyladenosine (**11**; 350 mg, 1.25 mmol) in dry DMF (2.0 mL) was treated with imidazole (340 mg, 4.99 mmol) followed by 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (481 μL, 1.50 mmol). The reaction mixture was stirred at 24 °C under nitrogen for 24 h. The solvent was evaporated under reduced pressure, and the residue was purified by pTLC using MeOH:$CHCl_3$ (1:10) to afford a yellowish liquid (409 mg, 0.783 mmol, 62.5%): ^{1}H NMR ($CDCl_3$) δ 0.76−1.06 (28H, m, $CH(CH_3)_2$), 1.96−2.19 (2H, m, 4′-CH_2), 2.22 (1H, m, H-4′), 3.07 (3H, bs, N^6-CH_3), 3.75 (1H, dd, J = 11.7, 4.9 Hz, CH_2-5′), 3.94 (1H, dd, J = 11.7, 2.9 Hz, CH_2-5′), 4.29−4.32 (1H, m, H-2′), 4.53−4.59 (1H, m, H-3′), 4.60−4.65 (1H, m, H-1′), 6.84 (1H, d, J = 4.9 Hz, 2′-OH), 7.73 (1H, s, H-2), 8.20 (1H, s, H-8); MS (CI-NH_3) 522 (M^+ + 1); HRMS (FAB−) calcd 521.2853, found 521.2850.

Carbocyclic N^6-Methyl-2′-*O*-[phenoxy(thiocarbonyl)]-3′,5′-*O*-(tetraisopropyldisiloxa-1,3-diyl)adenosine (13). A suspension of compound 12 (400 mg, 0.766 mmol) and 4-(dimethylamino)-pyridine (280 mg, 2.29 mmol) in dry acetonitrile (2.0 mL) was treated with phenyl chlorothionoformate (127 μL, 0.918 mmol). The suspended solid slowly went into solution when stirred at 24 °C under nitrogen. After 12 h, the solvent was evaporated under reduced pressure, and the residue was purified by pTLC using MeOH:$CHCl_3$ (1:15) to afford a yellowish liquid (450 mg, 0.684 mmol, 89.3%): ^{1}H NMR ($CDCl_3$) δ 0.85−1.09 (28H, m, $CH(CH_3)_2$), 2.15−2.22 (3H, m, H-4′, 4′-CH_2), 3.13 (3H, bs, N^6-CH_3), 3.90 (2H, dd, J = 61.1, 11.7 Hz, CH_2-5′), 4.80−4.85 (1H, m, H-3′), 5.04−5.09 (1H, m, H-1′), 5.86 (1H, bs, NH), 5.93−5.95 (1H, m, H-2′), 7.01−7.35 (5H, m, C_6H_5), 7.66 (1H, s, H-2), 8.24 (1H, s, H-8); MS (CI-NH_3) 658 (M^+ + 1); HRMS (FAB−) calcd 657.2836, found 657.2812.

Carbocyclic *N*⁶-Methyl-2′-deoxy-3′,5′-*O*-(tetraisopropyldisiloxa-1,3-diyl)adenosine (14). Compound **13** (450 mg, 0.684 mmol) was dissolved in dry toluene (0.2 mL). After degassing with oxygen-free argon for 20 min, tributyltin hydride (368 μL, 1.37 mmol) and azobis[isobutyronitrile] (40.1 mg, 0.244 mmol) were added. The reaction mixture under argon was heated at reflux for 3 h. The solvent was evaporated under reduced pressure, and the residue was purified by pTLC using MeOH:$CHCl_3$ (1:10) to afford a yellowish liquid (284 mg, 0.562 mmol, 82.1%):^{1}H NMR ($CDCl_3$) δ 0.90−1.05 (28H, m, $CH(CH_3)_2$), 0.78−1.28 (2H, m, H-2′), 1.88−2.04 (2H, m, 4′-CH_2), 2.25−2.33 (1H, m, H-4′), 3.12 (3H, bs, N^6-CH_3), 3.70 (1H, dd, J = 11.7, 4.9 Hz, CH_2-5′), 3.97 (1H, dd, J = 11.7, 2.9 Hz, CH_2-5′), 4.56−4.64 (1H, m, H-3′), 4.95−5.01 (1H, m, H-1′), 7.71 (1H, s, H-2), 8.31 (1H, s, H-8); MS (CI-NH_3) 506 (M^+ + 1); HRMS (FAB−) calcd 505.2904, found 505.2880.

Carbocyclic *N*⁶-Methyl-2′-deoxyadenosine (15). A solution of compound **14** (280 mg, 0.554 mmol) in dry tetrahydrofuran (2.5 mL) was treated with tetrabutylammonium fluoride (210 mg, 0.664 mmol). The reaction mixture was stirred at room temperature for 30 min. The solvent was evaporated under reduced pressure, and the residue was purified by pTLC using MeOH:$CHCl_3$ (1:10) to afford a yellowish liquid (90.2 mg, 0.343 mmol, 61.9%): ^{1}H NMR (CD_3OD) δ 1.89−1.96 (1H, m, 2′-CH_2), 2.20−2.27 (2H, m, 4′-CH_2), 2.39−2.46 (1H, m, 2′-CH_2), 2.52−2.56 (1H, m, H-4′), 3.12 (3H, bs, N^6-CH_3), 3.35−3.78 (2H, m, CH_2-5′), 3.78−4.33 (1H, m, H-3′), 5.10−5.20 (1H, m, H-1′), 8.17 (1H, s, H-2), 8.25 (1H, s, H-8); MS (EI) 263 (M^+); HRMS (FAB−) calcd 263.1382, found 263.1375.

Carbocyclic *N*⁶-Methyl-2′-deoxyadenosine-3′,5′-bis(dibenzyl phosphate) (16). Carbocyclic N^6-methyl-2′-deoxyadenosine (**15**; 20.0 mg, 0.0759 mmol) was phosphorylated following the general procedure B to give 17.9 mg (0.0228 mmol, 30.0% yield) of the desired compound **16**: ^{1}H NMR (CD_3OD) δ 1.71−2.38 (2H, m, 2′-CH_2), 3.03 (3H, s, N^6-CH_3), 3.69−3.73 (1H, m, H-4′), 4.00−4.04 (2H, m, 5′-CH_2), 4.83−4.86 (1H, m, H-3′), 4.96 (2H, d, J = 8.8 Hz, CH_2), 7.24 (5H, m, C_6H_5), 7.91 (1H, s, H-2), 7.98 (1H, s, H-8), 8.12 (1H, m, H-1′); ^{31}P NMR (CD_3OD) 0.36, −0.60 (2s, 3′-P, 5′-P); HRMS (FAB−) calcd 783.2586, found 783.2568.

(1*R*,2*S*,4*S*,5*S*)-1-[(Hydroxy)methyl]-2-hydroxy-4-(6-methylaminopurin-9-yl)bicyclo[3.1.0]hexane (17b). The Dimroth rearrangement (Scheme 2) was carried out on (N)-methanocarba-2′-deoxyadenosine. Specifically, the (N)-methanocarba-2′-deoxyadenosine (**17a**; 50.0 mg, 0.191 mmol) was heated at 40 °C with methyl iodide (71.5 μL, 1.15 mmol) in dry DMF (2.0 mL) for 48 h. The solvent was evaporated under reduced pressure, and the residue was heated at 90 °C with ammonium hydroxide (4.0 mL) for 4 h. The water was evaporated, and the residue was purified by pTLC using MeOH:$CHCl_3$ (1:9) to afford compound **17b** as a colorless solid (40 mg, 0.15 mmol, 76%): ^{1}H NMR (CD_3OD) δ 0.77−0.81 (1H, m, CH_2-6′), 1.03−1.07 (1H, m, CH_2-6′), 1.68−1.72 (1H, m, CH-5′), 1.79−1.89 (1H, m, CH_2-3′), 2.00−2.07 (1H, m, CH_2-3′), 3.12

(3H, bs, N^6-CH_3), 3.33 (1H, d, J = 11.7 Hz, CH_2OH), 4.29 (1H, d, J = 11.7 Hz, CH_2OH), 4.89−4.92 (1H, m, CH-4′), 5.02 (1H, d, J = 6.9 Hz, CH-2′), 8.24 (1H, s, H-2), 8.49 (1H, s, H-8); MS (CI-NH_3) 276 (M + 1); HRMS (FAB−) calcd 275.1382, found 275.1389.

(*N*)-Methanocarba-*N*⁶-methyl-2′-deoxyadenosine-3′,5′-bis(dibenzyl phosphate) (18) [(*1R,2S,4S,5S*)-1-[(Dibenzylphosphato)methyl]-4-(6-methylaminopurin-9-yl)-bicyclo[3.1.0]hexane-2-(dibenzyl phosphate)]. Starting from 20.0 mg (0.0726 mmol) of (N)-methanocarba-N^6-methyl-2′-deoxyadenosine, **17b,** and following the general phosphorylation procedure (method B) we obtained 13.5 mg (0.0170 mmol, 23.4% yield) of the desired protected intermediate **18** (Scheme 2): ^{1}H NMR ($CDCl_3$) δ 0.73−0.78 (1H, m, CH_2-6′), 0.94−0.98 (1H, m, CH_2-6′), 1.53−1.54 (1H, m, CH-5′), 1.81−1.91 (1H, m, CH_2-3′), 2.05−2.13 (1H, m, CH_2-3′), 3.15 (3H, bs, N^6-CH_3), 3.70−3.83 (1H, m, CH_2OP), 4.49−4.55 (1H, m, CH_2OP), 4.89−5.00 (8H, m, $-OCH_2$), 5.02−5.06 (1H, m, CH-4′), 5.27−5.32 (1H, m, CH-2′), 5.86 (1H, bs, NH), 7.21−7.23 (20H, m, C_6H_5), 7.86 (1H, s, H-2), 8.31 (1H, s, H-8); ^{31}P NMR (D_2O) δ −0.56, −1.05 (2s, 3′-P, 5′-P); HRMS (FAB−) (M + Cs) calcd 928.1641; found 928.1700.

(*1R,2S,4S,5S*)-1-[(Benzyloxy)methyl]-2-benzyloxy-4-(2,6-dichloropurin-9-yl)bicyclo[3.1.0]hexane (21). To an ice-cold solution of triphenylphosphine (278 mg, 1.06 mmol) in dry THF (2 mL) was added diethyl azadicarboxylate (170 μL, 1.06 mmol) dropwise under a nitrogen atmosphere, and the mixture was stirred for 20 min until the solution turned red orange (Scheme 3). This mixture was added dropwise to a cold stirred mixture of the starting alcohol (135 mg, 0.417 mmol) and 2,6-dichloropurine (157 mg, 0.883 mmol) under a nitrogen atmosphere. The reaction mixture was stirred in an ice bath for 30 min and then allowed to warm to room temperature, and stirring continued for 12 h. Solvent was removed by nitrogen purge, and the residue was purified by pTLC using EtOAc:petroleum ether (1:1) to afford a thick liquid (132 mg, 0.263 mmol, 64%):^{1}H NMR (CD_3OD) δ 0.85 (m, 1H), 1.13 (m, 1H), 1.59 (m, 1H), 1.68 (m, 1H), 2.06 (m, 1H), 3.17 (d, J = 10.8 Hz, 1H), 4.11−4.57 (m, 5H), 5.20 (d, J = 6.9 Hz, 1H), 6.6 (bs, 1H), 7.23−7.37 (m, 10H), 8.98 (s, 1H); MS (EI) 494 (M^+).

(1*R*,2*S*,4*S*,5*S*)-1-[(Benzyloxy)methyl]-2-benzyloxy-4-(2-chloro-6-methylaminopurin-9-yl)bicyclo[3.1.0]hexane (22). Compound **21** (100 mg, 0.202 mmol) was dissolved in methylamine in methanol (30% solution, 3 mL) and was stirred at room temperature for 12 h under a nitrogen atmosphere. The solvent was evaporated, and the crude product was purified by pTLC using EtOAc:petroleum ether (6:4) to afford **22** as a light yellow solid (86 mg, 0.176 mmol, 88%): ^{1}H NMR (CD_3OD) δ 0.70 (m, 1H), 1.06 (m, 1H), 1.50 (m, 1H), 1.76 (m, 1H), 1.96 (m, 1H), 3.01 (s, 3H), 3.08 (m, 2H), 4.03 (m, 4H), 4.45 (bs, 1H), 5.02 (bs, 1H), 8.38 (s, 1H); MS (CI) 490 (M + 1).

(1*R*,2*S*,4*S*,5*S*)-1-[(Hydroxy)methyl]-2-hydroxy-4-(2-chloro-6-methylaminopurin-9-yl)bicyclo[3.1.0]hexane (23). Compound **22** (40 mg, 0.0816 mmol) was dissolved in dry CH_2Cl_2 (1.0 mL), and hydrogenated using BCl_3 (1 M in CH_2Cl_2, 175 μL) for 50 min at −78 °C under argon. The solvent was evaporated, and the crude product was purified by pTLC using $CHCl_3$:MeOH (10:1) to afford **23** as a light yellow solid (10.0 mg, 0.0323 mmol, 39.6%): ^{1}H NMR (CD_3OD) δ 0.77−0.81 (1H, m, CH_2-6′), 1.02−1.05 (1H, m, CH_2-6′), 1.65−1.68 (1H, m, CH-5′), 1.78−1.91 (1H, m, CH_2-3′), 1.99−2.07 (1H, m, CH_2-3′), 3.08 (3H, bs, N^6-CH_3), 3.37 (1H, d, J = 11.7 Hz, CH_2OH), 4.27 (1H, d, J = 11.7 Hz, CH_2OH), 4.89−4.91 (1H, m, CH-4′), 4.97 (1H, d, J = 6.8 Hz, CH-2′), 8.46 (1H, s, H-8); MS (CI-NH_3) 310 (M + 1); HRMS (FAB−) calcd 309.0992, found 309.0991.

Ketene Diethylacetal (25). Ketene diethylacetal (Scheme 4) was prepared from bromoacetaldehyde diethylacetal **(24)** (Aldrich Chemical Co.) and potassium *tert*-butoxide (Aldrich Chemical Co.) by direct mixing and distillation at a 110 °C bath temperature. Specifically, in a 100 mL two-neck round-bottomed flask equipped with a Vigreux column distillation apparatus were added bromoacetaldehyde diethyl acetal (19.71 g, 0.1 mol) and potassium *tert*-butoxide (11.2 g, 0.1 mol) under a nitrogen atmosphere at room temperature. *tert*-Butyl alcohol was removed from reaction mixture by distillation in vacuo at 65−70 °C (bath temperature 110 °C). The bath temperature was raised to 120−140 °C, and product was distilled out from the reaction mixture at 85−95 °C in vacuo. Exposure to moisture in the air was avoided, since the product was highly reactive and polymerized to a white solid: ^{1}H NMR ($CDCl_3$) δ 1.18−1.35 (m, 6 H, 2 x CH_3), 3.09 (s, 2 H, CH_2=), 3.83 (q, 4 H, 2 x CH_2).

3,3-Diethoxy-2-(ethoxycarbonyl)cyclobutene (26). A mixture of ketene diethyl acetal (3.28 g, 28.2 mmol) and ethyl propiolate (3 mL, 30.1 mmol) in dry dichloromethane (25 mL) was heated at 50 °C under a nitrogen atmosphere for 28 h. After the reaction mixture was concentrated to dryness, the residue was purified by quick short distillation to give compound **25** as a colorless liquid at 51 °C/0.09 Torr (lit.[44] bp 40 °C/0.003 Torr): ^{1}H NMR ($CDCl_3$) δ 1.22 (t, J = 7.1 Hz, 3 H, OCH_2CH_3), 1.29 (t, J = 7.1 Hz, 3 H, OCH_2CH_3), 2.67 (d, J = 1.3 Hz, 2 H, $-CH_2$-), 3.67−3.78 (m, 4 H, 2 x OCH_2CH_3), 4.22 (q, J = 7.1 Hz, 2 H, $COOCH_2CH_3$), 7.15 (t, J = 1.3 Hz, CH=); MS (CI) *m/e* 215 (MH^+).

(±)-2-Chloro-9-[(1α,2*β*)-3,3-diethoxy-2-(ethoxycarbonyl)cyclobutyl]-*N*⁶-methyladenine (27). 2-Chloro-N^6-methyladenine was prepared as follows: A solution of 2,6-dichloropurine (1.38 g, 7.32 mmol) in 40% methylamine in water (12 mL) was heated at 100 °C for 1 h in a sealed bottle. Solid precipitate was filtered, washed with cold water, and dried to give the title compound (1.25 g, 93.5%): ^{1}H NMR (DMSO-d_6) δ 2.91 (br s, 3 H, CH_3), 8.09 (s, 1 H, H-8), 7.90 (br s, 1 H, N*H*), 13.07 (br s, 1 H, N*H*).

To a mixture of 3,3-diethoxy-2-(ethoxycarbonyl)cyclobutene (**26**; 1.07 g, 5.0 mmol) and 2-chloro-N^6-methyladenine (0.658 g, 3.58 mmol) in anhydrous DMF (21 mL) was added DBU (0.536 mL, 3.58 mmol) at 0 °C under a nitrogen atmosphere. After the reaction mixture was stirred at room temperature for 18 h, DMF was evaporated by rotary evaporation. The residue was dissolved in chloroform (30 mL) and washed with saturated $NaHCO_3$, brine, dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by silica gel column chromatography (hexanes:EtOAc, 5:1→3:1→1:1→1:3) to give compound **27** (1.1 g, 77%) as a white solid: R_f = 0.24 (hexanes:EtOAc, 1:1), 0.27 ($CHCl_3$:MeOH, 20:1); ^{1}H NMR ($CDCl_3$) δ 1.19 (t, J = 6.8 Hz, 3 H, CH_3), 1.26 (t, J = 6.8 Hz, 3 H, CH_3), 1.28 (t, J = 6.8 Hz, 3 H, CH_3), 2.79 (dd, J = 12.4, 8.8 Hz, 1 H, $-CH_{2a}$-), 2.96 (dd, J = 12.4, 8.8 Hz, 1 H, $-CH_{2b}$-), 3.18 (br s, 3 H, $NHCH_3$), 3.50 (m, 2 H), 3.67 (m, 1H), 3.82 (m, 1 H), 3.98 (d, J = 7.8 Hz, 1 H), 4.20 (m, 2 H), 5.15 (dt, J = 8.8 Hz, 1 H), 6.00 (br s, 1 H, N*H*), 7.81 (s, 1 H, H-8); low-resolution MS (CI) *m/e* 398 (MH^+).

(±)-2-Chloro-9-[(1α,2*β*)-3,3-diethoxy-2-(hydroxymethyl)cyclobutyl]-*N*⁶-methyladenine (28). To a solution of compound **27** (1.036 g, 2.6 mmol) in anhydrous THF (15 mL) was added 1.0 M $LiAlH_4$ in THF (4.03 mL, 4.03 mmol) at 0 °C under a nitrogen atmosphere. The reaction mixture was stirred at 0 °C for 1 h and 20 min and quenched by the addition of H_2O (0.2 mL), 5 M NaOH (0.2 mL), and H_2O (0.5 mL). After the mixture was stirred vigorously for 20 min, a solid was removed by filtration, and the filtrate was concentrated to dryness. The white solid was dissolved in H_2O (0.5 mL) and chloroform (30 mL), and the aqueous layer was extracted with chloroform (30 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by silica gel column chromatography ($CHCl_3$:MeOH, 20:1) to give compound **28** (833 mg, 90%) as a white solid: R_f = 0.21 ($CHCl_3$:MeOH, 20:1); ^{1}H NMR (DMSO-d_6) δ 1.15 (t, J = 6.0 Hz, 3 H), 1.17 (t, J = 6.0 Hz, 3 H), 2.49 (m, 1 H), 2.81 (dd, J = 11.7, 8.8 Hz, 1 H), 2.91 (d, J = 2.9 Hz, 3 H, $NHCH_3$), 3.10 (m, 1 H), 3.33−3.61 (m, 5 H), 3.72 (m, 1 H), 4.34 (dt, J = 8.8 Hz, 1 H), 8.21 (br d, J = 2.9 Hz, 1 H), 8.29 (s, 1 H, H-8); ^{1}H NMR ($CDCl_3$) δ 1.22 (t, J = 7.1 Hz, 3 H, CH_3), 1.24 (t, J = 7.1 Hz, 3 H, CH_3), 2.57 (ddd, J = 12.7, 7.8, 1.5 Hz, 1 H), 3.01 (m, 2 H), 3.2 (br s, 3 H, $NHCH_3$), 3.44−3.61 (m, 4 H), 3.74 (br s, 1 H), 3.91 (m, 1 H), 4.00 (m, 1H), 4.67 (dt, 8.5, 7.8 Hz, 1 H), 6.03 (br s, 1 H, NH), 7.84 (s, 1 H, H-8); low-resolution MS (CI) *m/e* 356 (MH^+).

(±)-2-Chloro-9-[(1α,2β,3α)-3-hydroxy-2-(hydroxymethyl)cyclobutyl]-N^6-methyladenine (29). To a solution of compound **28** (833 mg, 2.34 mmol) in acetone (117 mL) was added 1 N HCl (22 mL) slowly. The reaction mixture was stirred at room temperature for 2 days. After acetone was removed by rotary evaporation, the residue was treated with 5 N NaOH to neutral. It was extracted with EtOAc (3 × 30 mL), and the combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness to give the crude ketone as a white solid: $R_f = 0.09$ ($CHCl_3$:MeOH, 20:1); ^{1}H NMR (DMSO-d_6) δ 2.5 (m, 1 H), 2.92 (s, 3 H, NHCH_3), 3.46 (ddd, $J = 17.8$, 8.8, 2.2 Hz, 1 H), 3.61−3.79 (m, 2 H), 4.16 (m, 1 H), 5.05 (t, $J = 4.4$ Hz, exchangeable with D_2O, 1 H, OH), 5.20 (dt, $J = 8.6$, 6.8 Hz, 1 H), 8.30 (br s, 1 H, NH), 8.41 (s, 1 H, H-8); low-resolution MS (CI) *m/e* 282 (MH^+).

The above crude product was dissolved in anhydrous MeOH (45 mL) and $NaBH_4$ (177 mg, 4.68 mmol) was added in three portions at 0 °C. The reaction mixture was stirred at that temperature for 1 h before the reaction was quenched by the addition of acetone (2 mL). After the mixture was stirred for another 20 min, it was concentrated to dryness. The residue was purified by silica gel column chromatography ($CHCl_3$:MeOH, 10:1) to give compound **29** (350 mg, 52.7%) as a white solid: ^{1}H NMR (DMSO-d_6) δ 2.20 (m, 1 H), 2.72 (dt, $J = 10.8$, 7.3 Hz, 1 H), 2.82 (m, 1 H), 2.90 (s, 3 H, NHCH_3), 3.55 (m, 2 H), 3.82 (m, 1 H), 4.26 (dt, $J = 8.1$, 8.8 Hz, 1 H), 4.69 (t, $J = 4.0$ Hz, exchangeable with D_2O, 1 H, OH), 5.29 (d, $J = 6.4$ Hz, exchangeable with D_2O, 1 H, OH), 8.18 (br s, exchangeable with D_2O, 1 H, NH), 8.27 (s, 1 H, H-8); low-resolution MS (CI) *m/e* 284 (MH^+).

N^6-Methyl-1,5-anhydro-2-(adenin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol (31). The Dimroth rearrangement (Scheme 5) of 1,5-anhydro-2-(adenin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol (40.0 mg, 0.151 mmol) gave compound **31** as a colorless solid (30.0 mg, 0.107 mmol, 71.2%). Specifically, 1,5-anhydro-2-(adenin-9-yl)-2,3-dideoxy-D-*arabino*-hexitol the was heated at 40 °C with methyl iodide (56.3 μL, 0.905 mmol) in dry DMF (2.0 mL) for 48 h. The solvent was evaporated under reduced pressure, and the residue was heated at 90 °C with ammonium hydroxide (4.0 mL) for 4 h. The water was evaporated, and the residue was purified by pTLC using MeOH:$CHCl_3$ (1:9): ^{1}H NMR (CD_3OD): δ 1.96−2.07 (1H, m, H-3′), 2.39−2.49 (1H, m, H-3′), 3.14 (3H, bs, N^6-CH_3), 3.36 (1H, m, H-5′), 3.58−3.67 (1H, m, H-4′), 3.78 (1H, dd, $J = 11.7$, 4.9 Hz, H-6′), 3.90 (1H, dd, $J = 11.7$, 2.0 Hz, H-6′), 4.04 (1H, d, $J = 13.4$ Hz, H-1′), 4.37 (1H, d, $J = 13.4$ Hz, H-1′), 4.79 (1H, m, H-2′), 8.29 (1H, s, 2-H), 8.46 (1H, s, 8-H); MS (CI-NH_3) 280 ($M^+ + 1$); HRMS (FAB−) calcd 279.1331, found 279.1319.

1,5-Anhydro-2-(adenin-9-yl)-N^6-methyl-2,3-dideoxy-D-*arabino*-hexitol-4,6-bis(dibenzyl phosphate) (32). Starting from 23.0 mg (0.0726 mmol) of 1,5-anhydro-2-(adenin-9-yl)-N^6-methyl-2,3-dideoxy-D-*arabino*-hexitol and following the general phosphorylation procedure (method B) we obtained 32.4 mg (0.0405 mmol, 55.8% yield) of the desired compound: ^{1}H NMR (CD Cl_3) δ 2.04−2.17 (1H, m, H-3′), 2.70−2.75 (1H, m, H-3′), 3.23 (3H, s, N^6-CH_3), 3.58−3.63 (1H, m, H-5′), 3.87 (1H, dd, $J = 12.7$, 2.9 Hz, H-1′), 4.25 (1H, d, $J = 12.7$ Hz, H-1′), 4.08−4.16 (1H, m, H-4′), 4.32−4.38 (2H, m, CH_2-6′), 4.72 (8H, 4d, $J = 7.9$ Hz, CH_2), 5.95 (1H, bs, H-2′), 7.00 (1H, bs, NH), 7.30 (20H, m, C_6H_5), 8.10 (1H, s, H-2), 8.38 (1H, s, H-8); ^{31}P NMR (CD_3Cl) δ −0.76, −1.78 (2s, 3′-P, 5′-P); MS (SIMS) 800 ($M^+ + 1$); HRMS (FAB−) (M + Cs) calcd 932.1590, found 932.1608.

Pharmacological Analyses. $P2Y_1$ receptor-promoted stimulation of inositol phosphate formation by adenine nucleotide analogues was measured in turkey erythrocyte membranes as previously described.[8,36] The $K_{0.5}$ values were averaged from 3−8 independently determined concentration−effect curves for each compound. Briefly, 1 mL of washed turkey erythrocytes was incubated in inositol-free medium (DMEM; Gibco, Gaithersburg, MD) with 0.5 mCi of 2-[^{3}H]*myo*-inositol (20 Ci/mmol; American Radiolabelled Chemicals, Inc., St. Louis, MO) for 18−24 h in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Erythrocyte ghosts were prepared by rapid lysis in hypotonic buffer (5 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA) as described.[36] Phospholipase C activity was measured in 25 μL of [^{3}H]inositol-labeled ghosts (approximately 175 μg of protein, 200−500000 cpm/assay) in a medium containing 424 μM $CaCl_2$, 0.91 mM $MgSO_4$, 2 mM EGTA, 115 mM KCl, 5 mM KH_2PO_4, and 10 mM Hepes, pH 7.0. Assays (200 μL final volume) contained 1 μM GTPγS and the indicated concentrations of nucleotide analogues. Ghosts were incubated at 30 °C for 5 min, and total [^{3}H]inositol phosphates were quantified by anion-exchange chromatography as previously described.[8,36]

Data Analysis. Agonist potencies were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). EC_{50} values (mean ± standard error) represent the concentration at which 50% of the maximal effect is achieved. Relative efficacies (%) were determined by comparison with the effect produced by a maximal effective concentration of 2-MeSADP in the same experiment.

Antagonist IC_{50} values (mean ± standard error) represent the concentration needed to inhibit by 50% the effect elicited by 30 nM 2-MeSADP. The percent of maximal inhibition is equal to 100 minus the residual fraction of stimulation at the highest antagonist concentration.

All concentration−effect curves were repeated in at least three separate experiments carried out with different membrane preparations using duplicate or triplicate assays.

Molecular Modeling. All calculations were performed on a Silicon Graphics Indigo 2 R8000 workstation. All the ligand structures were constructed using the "Sketch Molecule" of SYBYL 6.5.[46] Semiempirical molecular orbital calculations were done using the AM1 Hamiltonian[47] as implemented in MOPAC 6.0[48] (keywords: PREC, GNORM = 0.1, EF, MMOK if necessary).

Relative to the published X-ray structures of the parent methanocarba nucleosides,[35,49] bisphosphate analogues **4a** and **5** retained their characteristic (N) ($P = 342$) and (S) ($P = 198$) orientations, respectively, in the pseudorotational cycle.[54] However, the maximum degree of puckering, ν_{max}, which defines the extent that the ring deviates from planarity showed that the bisphosphate analogues were significantly more planar ($\nu_{max} = 10$) than the parent compounds ($\nu_{max} = 28$).[49] Whether this flattening of the ring is a direct consequence of the attraction between the two phosphate groups and/or is a consequence of the semiempirical methodology used, perhaps in combination with the lack of aqueous environment in the calculation, will be the subject of a separate investigation.

Superimposition of these geometry-optimized ligand structures was carried out using the "Fit Atoms" method implemented in SYBYL. The quality of the fit is represented by the rms value computed for the matched atoms.

The three-dimensional human $P2Y_1$ receptor model was built and optimized using SYBYL 6.5 and Macromodel 6.0,[50] respectively, based on the approach described by Moro et al.[38] Briefly, the seven-transmembrane helical domains were identified with the aid of Kyte−Doolittle hydrophobicity[51] and E_{mini}[51] surface probability parameters. The helices were built and energy-minimized for each transmembrane sequence. The minimized helices were then grouped together to form a helical bundle matching the overall characteristics of the electron density map of rhodopsin. The helical bundle was energy-minimized using the AMBER[52] all-atom force field, until the rms value of the conjugate gradient (CG) was <0.1 kcal/mol/Å. A fixed dielectric constant = 4.0 was used throughout these calculations.

The structures of **4a** and **5** were rigidly docked into the helical bundle using graphical manipulation with continuous energy monitoring (Dock module of SYBYL). Both local energy-minimized receptor−ligand complexes were subjected to an additional CG minimization run of 300 steps. Partial atomic charges for the ligands were taken from the MOPAC output files. We have recently introduced the *cross-docking* procedure to obtain energetically refined structures of GPCR/ligand complexes.[38] We applied this technique to predict the structure of both **4a**/P2Y_1 and **5**/P2Y_1 receptor complexes. Cross-docking allows possible ligand-induced rearrangements of the 7TM bundle to be explored by sampling 7TM conformations in the presence of the docked ligands. Small translations and rotations were applied to each helix relative to its original position until a new lower energy geometry was obtained. These manual adjustments were followed by 25 ps of molecular dynamics (MD module of Macromodel) performed at a constant temperature of 300 K using a time step of 0.001 ps and a dielectric constant = 4.0. This procedure was followed by another sequence of CG energy minimization to a gradient threshold of <0.1 kcal/mol/Å. Energy minimization of the complexes was performed using the AMBER all-atom force field in MacroModel.

The interaction energy values were calculated as follows: $E(\text{complex}) = E(\text{complex}) - (E(\text{L}) + E(\text{receptor}))$. These energies are not rigorous thermodynamic quantities, but can only be used to compare the relative stabilities of the complexes. Consequently, these interaction energy values cannot be used to calculate binding affinities since changes in entropy and solvation taken into account.

Abbreviations: AIBN, 2,2′-azobis[isobutyronitrile]; ATP, adenosine-5′-triphosphate; CG, conjugate gradient; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCTIDS, 1,3-dichlorotetraisopropyl-1,1,3,3-disiloxane; DEAD, diethyl azadicarboxylate; DEAE, diethylaminoethyl; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide, DMSO, dimethyl sulfoxide; FAB, fast atom bombardment (mass spectroscopy); HPLC, high-pressure liquid chromatography; MS, mass spectroscopy; HRMS, high-resolution mass spectroscopy; LDA, lithium diisopropylamide; 2-MeSADP, 2-methylthioadenosine-5′-diphosphate; TBAP, tetrabutylammonium phosphate; TBPP, tetrabenzyl pyrophosphate; TEAA, triethylammonium acetate; THF, tetrahydrofuran; pTLC, preparative thin-layer chromatography.

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