Adenine Nucleotide Analogues Locked in a Northern Methanocarba Conformation: Enhanced Stability and Potency as $P2Y_1$ Receptor Agonists

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Preference for the Northern (N) ring conformation of the ribose moiety of nucleotide 5′-triphosphate agonists at $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{11}$ receptors, but not $P2Y_6$ receptors, was established using a ring-constrained methanocarba (a 3.1.0-bicyclohexane) ring as a ribose substitute (Kim et al. *J. Med. Chem.* **2002**, *45*, 208−218.). We have now combined the ring-constrained (N)-methanocarba modification of adenine nucleotides with other functionalities known to enhance potency at P2 receptors. The potency of the newly synthesized analogues was determined in the stimulation of phospholipase C through activation of turkey erythrocyte $P2Y_1$ or human $P2Y_1$ and $P2Y_2$ receptors stably expressed in astrocytoma cells. An (N)-methanocarba-2-methylthio-ADP analogue displayed an EC_{50} at the $hP2Y_1$ receptor of 0.40 nM and was 55-fold more potent than the corresponding triphosphate and 16-fold more potent than the riboside 5′-diphosphate. 2-Cl−(N)-methanocarba-ATP and its N^6-Me analogue were also highly selective, full agonists at $P2Y_1$ receptors. The (N)-methanocarba-2-methylthio and 2-chloromonophosphate analogues were full agonists exhibiting micromolar potency at $P2Y_1$ receptors, while the corresponding ribosides were inactive. Although β,γ-methylene-ATP was inactive at P2Y receptors, β,γ-methylene-(N)-methanocarba-ATP was a potent $hP2Y_1$ receptor agonist with an EC_{50} of 160 nM and was selective versus $hP2Y_2$ and $hP2Y_4$ receptors. The rates of hydrolysis of Northern (N) and Southern (S) methanocarba analogues of AMP by rat 5′-ectonucleotidase were negligible. The rates of hydrolysis of the corresponding triphosphates by recombinant rat NTPDase1 and 2 were studied. Both isomers were hydrolyzed by NTPDase 1 at about half the rate of ATP hydrolysis. The (N) isomer was hardly hydrolyzed by NTPDase 2, while the (S) isomer was hydrolyzed at one-third of the rate of ATP hydrolysis. This suggests that new, more stable and selective nucleotide agonists may be designed on the basis of the (N)-conformation, which greatly enhanced potency at $P2Y_1$ receptors.

Introduction

The P2 nucleotide receptors consist of two families: G-protein-coupled receptors (GPCR) termed P2Y, of which seven mammalian subtypes ($P2Y_{1,2,4,6,11,12,13}$) have been cloned, and ligand-gated cation channels termed P2X, of which seven mammalian subtypes ($P2X_{1-7}$) have been cloned.[1−3] Adenine nucleotides are required for activation of $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ subtypes, while uracil nucleotides activate $P2Y_2$, $P2Y_4$, and $P2Y_6$ subtypes. P2Y receptors may couple to multiple second messengers, but all except the recently cloned[3−5] $P2Y_{12}$ and $P2Y_{13}$ receptors lead to activation of phospholipase C (PLC) via G_q, resulting in a rise in intracellular calcium. The G_i-coupled $P2Y_{12}$ and $P2Y_{13}$ receptors occur on a more distant branch of the sequence dendrogram than the other members of the P2Y family[6] and are closer in sequence to a newly characterized GPCR that is selective for sugar nucleotides (e.g. UDP-glucose).[7]

The medicinal chemistry of P2 receptors is underdeveloped compared to many other GPCRs.[8−10] Reasons for the lack of progress in this field include nonselectivity, chemical instability, heterogeneity, and nonbioavailability of known ligands. Other difficulties in characterizing the receptors include the enzymatic instability of the phosphate groups of nucleotides to form biologically active metabolites. Indeed, most of the known P2 antagonists also inhibit ecto-nucleotidase activity, thus augmenting the effects of the endogenous agonist(s). Receptor heterogeneity,[11] oligomerization,[12] and desensitization[13] are further complications.

Despite the above-mentioned difficulties, the SAR of ATP analogues (e.g., **1**−**5**; Chart 1) at P2 receptors has been probed.[8−10] Selective agonists ($P2Y_1$, $P2Y_6$)[14,15] and antagonists ($P2Y_1$, $P2Y_{12}$, $P2X_1$, $P2X_7$)[14,16−19] have been reported for only a few of the subtypes. 2-Methylthio-ATP (**1**, 2-MeSATP) is one of the most potent agonists at a number of P2Y and P2X receptors.[10] It was formerly regarded as selective for P2Y receptors; however, by adjustment for its lability in classical smooth muscle assays in which P2X receptors have been assayed, it is a highly potent agonist within the P2X superfamily.[20] At the $P2Y_1$ receptor, which is present in the heart,

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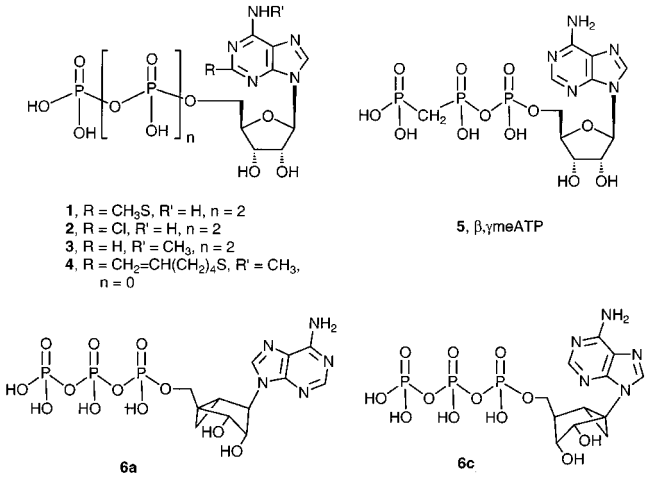
Chart 1



skeletal and various smooth muscles, prostate, ovary, and brain,[21] the potency order for activation is 2-methylthioadenosine 5′-diphosphate (2-MeSADP) > 2-MeSATP > ADP > ATP, and AMP and UTP are inactive. 2-Chloro-ATP and N^6-methyl-ATP (**2** and **3**, respectively) are moderately potent agonists at $P2Y_1$ receptors.[22] MRS 2055 **4** (N^6-methyl-2-(5-hexenyl)thioadenosine 5′-triphosphate) is an agonist with micromolar potency at a glial adenylate cyclase coupled P2Y receptor (now identified as identical to $P2Y_{12}$)[23,24] but is inactive at $P2Y_1$ receptors. β,γ-Methylene ATP **5** activates $P2X_1$, but not $P2Y_1$, receptors.[21,22]

P2Y receptor ligands are being explored for therapeutic applications in the cardiovascular, endocrine, and other systems. A selective $P2Y_1$ receptor agonist may have potential as an antihypertensive or antidiabetic agent.[25−27] A selective antagonist of the $P2Y_1$ receptor may be potentially useful in antithrombotic therapy,[28,29] since activation of this subtype is associated with the initial shape change during platelet aggregation.[29] ATP analogues have been shown to be effective insulin secretagogues.[30] A $P2Y_1$ receptor in osteoclasts has been linked to bone disintegration.[31] First cloned from chick brain,[32] the distribution of the $P2Y_1$ receptor in the human brain has been described[33] and abnormalities in $P2Y_1$ receptor levels have been detected in the Alzheimer's brain.[34] A linkage between $P2Y_1$ receptors and the MAP kinase/apoptosis pathway has been proposed.[35] Selective agonists and antagonists at the $P2Y_1$ receptor have been reported.[10,14]

3′,5′-Bisphosphate nucleotides have been explored as selective antagonists of the $P2Y_1$ receptor, and nearly nanomolar affinities have been achieved.[14] The ribose moiety of $P2Y_1$ receptor antagonists may be replaced with carbocylics, smaller and larger rings, acyclics, and conformationally constrained rings,[14] resulting in retention of affinity for the receptor. The ribose rings of nucleosides and nucleotides can adopt a range of conformations, although their target receptors likely prefer specific conformations. For $P2Y_1$ antagonists we have replaced the ribose moiety with a carbocyclic ring locked in a preferred conformation, a fusion of cyclopropane and cyclopentane rings known as the methanocarba modification.[36−38] Two structural variations, depending on the position of the cyclopropane ring, restrict the ring pucker, i.e., hold the ribose-like ring (pseudosugar) in either a Northern (N, 2′-exo) **6a** or Southern (S, 2′-endo) **6c** envelope conformation (Chart 1), as defined in the pseudorotational cycle. Preference for the Northern (N) ring conformation of the ribose moiety of nucleoside 5′-triphosphate agonists at $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{11}$ receptors, but not $P2Y_6$ receptors, was established using a constrained methanocarba (bicyclic) ring.[39]

In the present study we combined the ring-constrained (N)-methanocarba modification of adenine nucleotides with other functionalities known to enhance potency at P2 receptors. In several cases, the constrained ring conferred dramatic increases in agonist potencies at $P2Y_1$ receptors, and generally the modification preserved the potencies at $P2Y_2$ receptors.

Results

Chemical Synthesis. We prepared methanocarbocyclic analogues of various synthetic adenine nucleotides (Table 1) in which fused cyclopropane and cyclopentane rings fixed the pseudoribose moiety in a rigid (N)-envelope conformation. The unsubstituted ATP **6a** and AMP **7a** analogues were reported in a previous study.[39] The new nucleotide analogues **8a**−**15a** and **17a** were prepared in the ammonium salt form according to the methods shown in Schemes 1 and 2 and were tested biologically as agonists at various P2Y receptors (Table 1). Identity was confirmed using NMR (^{1}H and ^{31}P) and high-resolution mass spectrometry, and purity was demonstrated using high-pressure liquid chromatography (HPLC) in two different solvent systems (Table 2).

As in the previous study,[39] since the phosphorus oxychloride method[40,41] was unsatisfactory for phosphorylation of (N)-methanocarba nucleosides at the 5′-hydroxyl group, an alternative multistep process was used. This consisted of the formation of a monophosphate using the phosphoramidite method followed by condensation with additional phosphate or pyrophosphate groups using carbonyldiimidazole. By this method, the monophosphate analogues were cleanly converted to either di- or triphosphate via a phosphorimidazolidate intermediate.[41] During the sequence, it was necessary to hydrolyze the cyclic carbonates[42] **38**−**41**, which were formed at the 2′,3′-dihydroxy groups, using a 5% triethylamine solution.

The (N)-methanocarba analogues of various ATP derivatives substituted at the 2- and/or 6-positions of the purine base moiety or at the β,γ position of the triphosphate moiety were prepared by this approach. To construct the pseudoribose ring leading to the (N)-methanocarbaadenosine precursors, a 3.1.0-bicyclohexane structure was prepared by the general approach of Lee et al.,[43] which utilized ring closure metathesis. An adenine precursor **18** or **19** was condensed with the protected bicycloheptane derivative **20**[43] using Mitsunobu conditions to give **21**[39] or **22** (Scheme 1). To introduce an amino or methylamino group at the 6-position, it was necessary to substitute 6-Cl with the corresponding amine following the Mitsunobu reaction to yield **23**−**26**. Deprotection of the 5′-benzyl group of **23**−**26** was carried out smoothly using Pd black/formic acid, even in the presence of 2-chloro substitution, to

Table 1. Biological Potencies of Methanocarba Derivatives of Adenine Nucleotides and Their Corresponding Riboside Analogues (Stimulation of Phospholipase C through Activation of P2Y Receptors)

compd	analogue	subtype	**a**, methanacarba EC_{50} (nM) of (N)-methanocarba analogue[a]	**b**, riboside EC_{50} (nM) of corresponding ribose analogue[a]	ref
6	ATP	$tP2Y_1$	14 ± 3	2800 ± 700	
		$hP2Y_1$	52 ± 22	1500 ± 200	
		$hP2Y_2$	91 ± 5	85 ± 12	
		$hP2Y_4$	32% at 10 μM	*c*	
		$hP2Y_{11}$	35 ± 5% at 10 μM	17300 ± 2800	
7	AMP	$tP2Y_1$	*d*	*d*	
8	2-MeSATP	$tP2Y_1$	3.7 ± 1.7	8 ± 2	40
		$hP2Y_1$	23 ± 11	34 ± 11	
9	2-MeSADP	$tP2Y_1$	0.88 ± 0.37	6 ± 3	40
		$hP2Y_1$	0.40 ± 0.23	6.4 ± 3.5	
		$hP2Y_2$	54 ± 8% at 10 μM	*d*	
		$hP2Y_4$	*d*	*d*	
		$hP2Y_{11}$	*d*	*d*	
10	2-MeSAMP	$tP2Y_1$	1190 ± 500	15% at 100 μM	
		$hP2Y_1$	590 ± 20	*d*	
		$hP2Y_{11}$	*d*	*d*	
11	2-MeS(O)AMP	$tP2Y_1$	36.6 ± 12.5	*d*	
		$hP2Y_1$		*d*	
12	2-C1-ATP	$tP2Y_1$	3 ± 1	720 ± 20	22
		$hP2Y_1$	7 ± 1	770 ± 120	46
		$hP2Y_2$	1900[b]	2300 ± 300	
13	2-C1-AMP	$tP2Y_1$	3900 ± 2900	*d*	
		$hP2Y_1$	1890 ± 770	*d*	
14	N^6-Me-ATP	$tP2Y_1$	92 ± 32	19000 ± 6000	22
		$hP2Y_1$	33[b]		
		$hP2Y_2$	197 ± 2		
15	2-C1-N^6-MeATP	$tP2Y_1$	6 ± 3		
		$hP2Y_1$	9 ± 1		
		$hP2Y_2$	8400 ± 3600		
16	2-C1-N^6-MeAdo-5′- CONH-Me	$tP2Y_1$	*d*		
17	β,γ-Me-ATP	$tP2Y_1$	11,300 ± 1700	*d*	22
		$hP2Y_1$	158[b]	*d*	
		$hP2Y_2$	*d*	*d*	
		$hP2Y_4$	*d*		

[a] Average ± SEM data for **6a** and **7a** reported in ref 39. A percentage refers to percent of maximal effect. [b] Average of two determinations. [c] ATP is an antagonist at $hP2Y_4$ receptors.[50] [d] No effect as either agonist or antagonist at 10 μM.

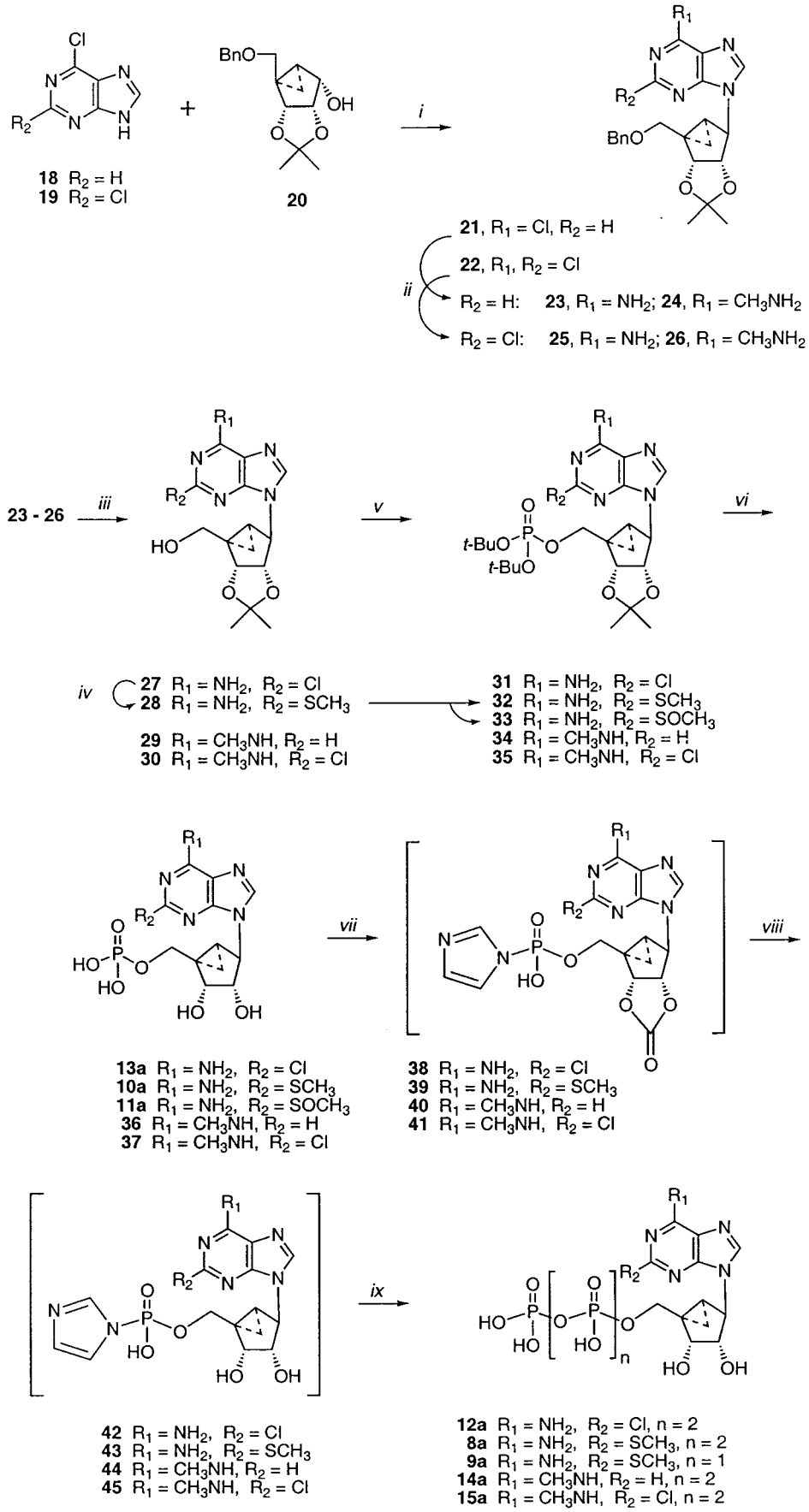
give **27**, **29**, and **30**. This was followed by 5′-phosphorylation using a phosphoramidite method[44] to give the di-*tert*-butyl protected monophosphates **31**, **34**, and **35**. Alternatively, the 2-chloro group of **27** was replaced with methylthio to give the nucleoside **28**, leading to the protected monophosphate **32**. In the process of synthesis of the 2-methylthio-AMP derivative **32**, the corresponding sulfoxide **33** was isolated as a byproduct. The monophosphates **31**−**35** were deprotected and either tested biologically or carried further to the di- and triphosphates by the method shown in Scheme 1. The (N)-methanocarba β,γ-methylene triphosphate analogue **17a** was prepared from the unsubstituted AMP equivalent **7a**[39] using methylene diphosphonate instead of pyrophosphate (Scheme 2).

Three riboside 5′-monophosphates **10b**, **11b**, and **13b** were prepared using methods shown in Scheme 3 for comparison to the corresponding (N)-methanocarba derivatives. The isopropylidene group was used as protection for the 2′- and 3′-hydroxyl groups, and phosphorylation was carried out using the phosphoramidite method, as above.

Biological Activity. (N)-Methanocarba analogues (denoted "**a**", Table 1) were tested for agonist activity by measuring $P2Y_1$-receptor-promoted phospholipase C activity in turkey erythrocyte membranes and by measuring inositol phosphate accumulation in 1321N1 human astrocytoma cells stably expressing the human $P2Y_1$, $P2Y_2$, $P2Y_4$, or $P2Y_{11}$ receptors. In each case, full concentration effect curves were generated and EC_{50} values from at least three separate experiments were determined. The agonist potencies of the corresponding ribose-containing nucleotides (denoted "**b**") were simultaneously determined for comparison purposes.

Given the high potencies observed with (N)-methanocarba analogues of ATP and UTP and the much greater potency of the (N)-methanocarba as opposed to (S)-methanocarba conformation, we extended our studies to (N)-methanocarba derivatives synthesized containing various functionalities that we and others have shown to produce potent or receptor-selective adenine nucleotide analogues.[8−10] As has been observed for adenine nucleotides, substitution at the 2-position, such as 2-methylthio, in the (N)-methanocarba-nucleotide series enhanced $P2Y_1$ receptor potency. Thus, the high $P2Y_1$ potency of 2-MeSATP **8b** (Figure 1) was preserved in the corresponding (N)-methanocarba analogue **8a**. The most potent $P2Y_1$ agonist in this series was the 2-methylthio diphosphate analogue **9a** (Figure 2), which displayed an EC_{50} of 0.88 nM at the turkey $P2Y_1$ receptor and was 7-fold more potent than the corresponding ribose analogue **9b** and 26-fold more potent than **8a**. At other receptors **9a** was inactive ($P2Y_4$, $P2Y_{11}$) or weakly active ($P2Y_2$). The (N)-methanocarba-2-methylthio monophosphate analogue **10a** and the (N)-methanocarba-2-chloro monophosphate analogue **13a** were full agonists exhibiting micromolar potency at the turkey and human $P2Y_1$ receptors. Oxidation of the

Scheme 1. Synthesis of (N)-Methanocarbaadenosine 5′-Phosphate Derivatives[a]



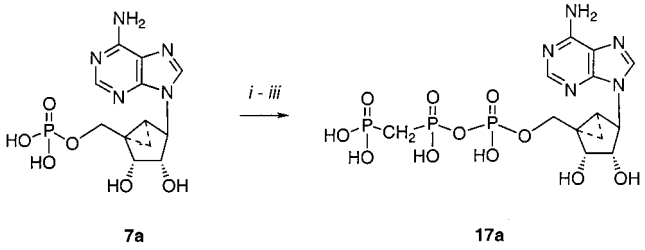
[a] Reagents: (i) DEAD, Ph_3P, THF; (ii) NH_3 or CH_3NH_2, THF; (iii) palladium black, HCO_2H, methanol; (iv) $NaSCH_3$, DMF, 90−100 °C; (v) di-*tert*-butyl *N*,*N*-diethylphosphoramidite, tetrazole, THF, room temp, 20 min and then *m*-CPBA, −78 °C to room temp; (vi) DOWEX 50x8-200, methanol, 60−70 °C, ~80%; (vii) carbonyldiimidazole, DMF, room temp, 6 h; (viii) 5% triethylamine/H_2O/MeOH, 2 h; (ix) tributylammonium pyrophosphate or tributylammonium phosphate, DMF, 3 days.

Table 2. Synthetic Data for Nucleotide Derivatives, Including Structural Verification Using High-Resolution Mass Spectrometry and Purity Verification Using HPLC

		FAB (M − H$^+$) *m/z*		HPLC retention time in min (purity in %[a])		
compd	formula	calcd	found	system A	system B unless otherwise noted	yield[b] (%)
8a	$C_{13}H_{20}N_5O_{12}P_3S$	561.9964	561.9966	11.0 (99)	19.7 (99)	35
9a	$C_{13}H_{19}N_5O_9P_2S$	482.0301	482.0291	10.5 (98)	19.4 (98)	40
10a	$C_{13}H_{18}N_5O_6PS$	402.0637	402.0650	9.0 (98)	15.6 (99)	60
10b	$C_{11}H_{16}N_5O_7PS$	394.0586	394.0591	14.7 (98)	16.2 (99)	16
11a	$C_{13}H_{18}N_5O_7PS$	418.0586	418.0607	6.3 (97)	12.7 (97)	65
11b	$C_{11}H_{16}N_5O_8PS$	410.0535	410.0545	11.2 (97)	17.1 (97)	16
12a	$C_{12}H_{17}N_5O_{12}P_3Cl$	551.9667	551.9673	7.4 (97)	19.1 (98)	30
13a	$C_{12}H_{15}N_5O_6PCl$	390.0370	390.0384	7.7 (99)	11.1(99)	66
13b	$C_{10}H_{13}N_5O_7PS$	382.0319	382.0322	9.2 (99)	17.5 (99)	42
14a	$C_{13}H_{20}N_5O_{12}P_3$	530.0243	530.0247	6.0 (98)	8.8 (98)	25
15a	$C_{13}H_{19}N_5O_{12}P_3Cl$	563.9853	563.9866	11.2 (98)	6.6 (98)	32
16a	$C_{14}H_{17}N_6O_3Cl$	353.1129	353.1139	10.4 (98)	10.3[c] (99)	*d*
17a	$C_{13}H_{20}N_5O_{11}P_3$	514.0294	514.0306	3.2 (98)	15.6 (97)	32

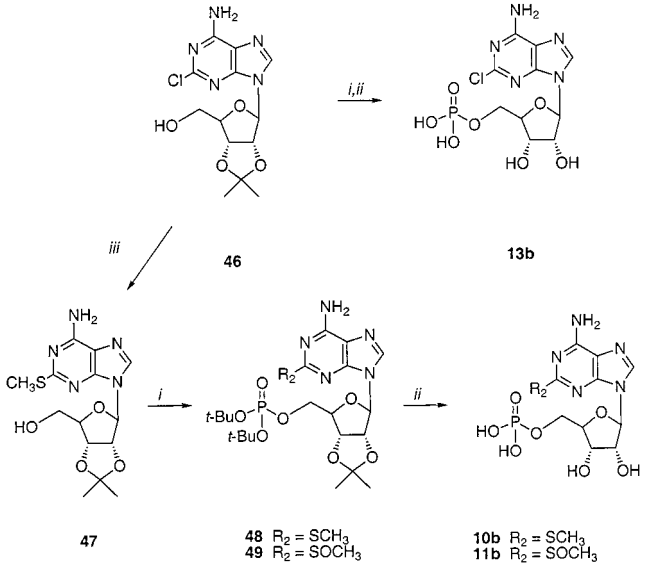
[a] Purity of each derivative was ≥97%, as determined using HPLC with two different mobile phases. System A: gradient of 0.1 M TEAA/CH_3CN from 95/5 to 40/60. System B: gradient of 5 mM TBAP/CH_3CN from 80/20 to 40/60. System C: gradient of 0.1 M TEAB/CH_3CN from 100/0 to 90/10 in 20 min. [b] The percent yields refer to the overall yield for each phosphorylation sequence. [c] System C. [d] Synthesis described in ref 45.

Scheme 2. Synthesis of the (N)-Methanocarba Analogue of β,γ-Me-ATP[a]



[a] Reagents: (i) carbonyldiimidazole, DMF, room temp, 6 h; (ii) triethylamine/H_2O/MeOH (1:10:10), 2 h; (iii) methylenediphosphonic acid, triethylamine, DMF, 3 days.

Scheme 3. Synthesis of Adenosine 5′-Monophosphate Analogues[a]



[a] Reagents: (i) tetrazole, di-*tert*-butyl *N,N*-diethylphosphoramidite, THF, then *m*-CPBA, CH_2Cl_2; (ii) DOWEX 50Wx8-200, H_2O/MeOH; (iii) $NaSCH_3$, DMF, 90 °C.

thioether to a sulfoxide, in **11a**, reduced tP2Y$_1$ receptor potency by 30-fold. The corresponding 5′-monophosphate ribosides **10b**, **11b**, and **13b** were inactive at P2Y$_1$ receptors. The (N)-methanocarba-2-chloro 5′-triphosphate analogue **12a** was 240-fold more potent at the turkey P2Y$_1$ receptor than the corresponding riboside **12b**.

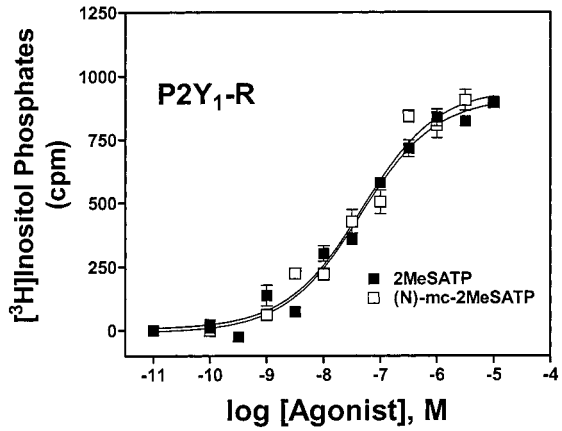


Figure 1. Effects of the (N)-methanocarba modification of 2-MeS-ATP on phospholipase C activity in 1321N1 astrocytoma cells expressing the hP2Y$_1$ receptor. Concentration-dependent stimulation of inositol phosphate formation by compounds **8a** (□) and **8b** (■) is shown. The data shown are typical curves for at least three experiments.

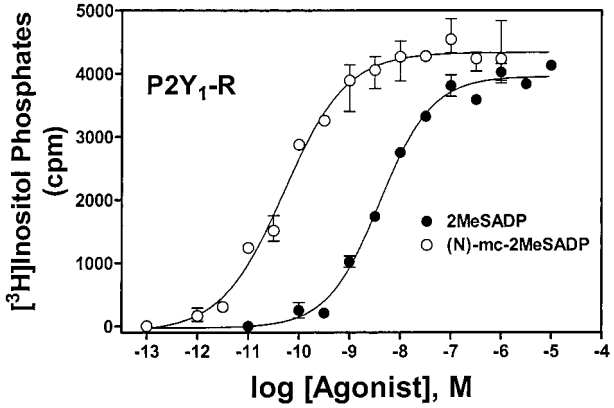


Figure 2. Effects of the (N)-methanocarba modification of 2-MeS-ADP on phospholipase C activity in 1321N1 astrocytoma cells expressing the hP2Y$_1$ receptor. Concentration-dependent stimulation of inositol phosphate formation by compounds **9a** (○) and **9b** (●) is shown. The data shown are typical curves for at least three experiments.

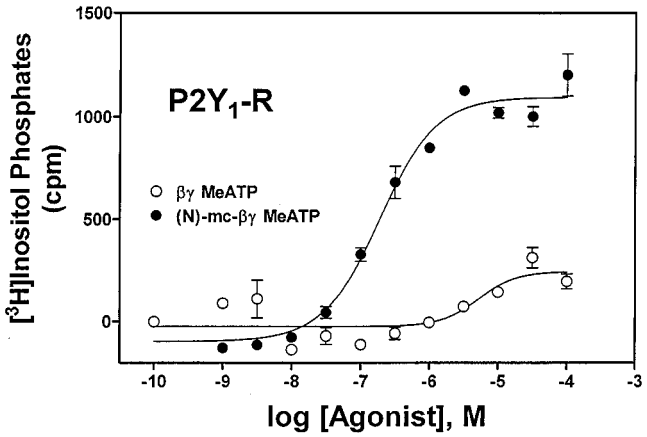
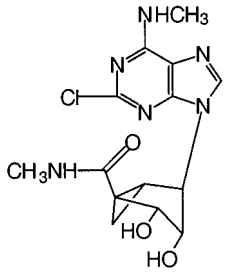


Figure 3. Effects of the (N)-methanocarba modification in triphosphate-modified adenine nucleotides on activation of phospholipase C in 1321N1 astrocytoma cells expressing $hP2Y_1$ receptors, showing concentration-dependent stimulation of inositol phosphate formation by compounds **14a** (●) and **14b** (○). The data shown are typical curves for at least three experiments.

As for the riboside series, substitution at the exocyclic amine of adenine was tolerated for (N)-methanocarba analogues acting as P2Y receptor agonists. The N^6-methyl 5′-triphosphate analogue **14a** was a potent agonist at the turkey and human $P2Y_1$ receptors and was 5-fold more potent as a full agonist at the $hP2Y_1$ than at the $hP2Y_2$ receptor. Combination of N^6-methyl and 2-chloro modifications in **15a** resulted in a very potent agonist for the turkey and human $P2Y_1$ receptors and selectivity for the $hP2Y_1$ receptor versus $hP2Y_2$ receptor of 930-fold. None of the (N)-methanocarba analogues of adenine nucleotide derivatives studied here were agonists at the human $P2Y_4$ receptor (data not shown).

Since the 2-chloro-N^6-methyl 5′-triphosphate analogue **15a** was a potent $P2Y_1$ receptor agonist, compound **16a**[45] was designed to evaluate the feasibility of replac-



16a

ing the triphosphate moiety with an uncharged moiety. However, it was inactive either as an agonist or as an antagonist at the turkey $P2Y_1$ receptor. β,γ-Me-ATP **17b** was inactive as an agonist at the $P2Y_1$ receptor.[46,47] In contrast, and consistent with the potency enhancement observed for other nucleotide analogues, the (N)-methanocarba-β,γ-methylene analogue **17a** was a relatively potent (EC_{50} = 11 μM) full agonist at the turkey $P2Y_1$ receptor and a very potent (EC_{50} = 160 nM) agonist at the $hP2Y_1$ receptor (Figure 3). In contrast, this analogue was inactive at the $P2Y_2$ and $P2Y_4$ receptors.

Because of the well-established lability of nucleotide analogues in pharmacological studies, the enzymatic stability of the simple (N)-methanocarba and (S)-methanocarba analogues of AMP and ATP was investigated (Tables 3 and 4). Neither the (N)-methanocarba nor (S)-methanocarba 5′-monophosphate derivatives **7a** and **7c**, respectively, were substrates for recombinant rat ecto-5′-nucleotidase (Table 3).[48] Hydrolysis of the (N)-methanocarba and (S)-methanocarba derivatives of ATP **6a** and **6c**, respectively, by recombinant rat ecto-apyrase (NTPDase1) and ecto-ATPase (NTPDase2)[49] stably expressed in CHO cells was also examined (Table 4). The (N)-methanocarba-ATP analogue was resistant to hydrolysis by NTPDase2 (4% as effective as ATP as a substrate), whereas the corresponding (S)-methanocarba-ATP was hydrolyzed by NTPDase2 at one-third (34%) the rate of ATP hydrolysis. In contrast, both molecules were good substrates for NTPDase1, at about half the rate of ATP hydrolysis (52% and 57%, respectively).

Table 3. Hydrolysis Rates of Monophosphate Derivatives as Substrates of Purified Recombinant Rat Ecto-5′-nucleotidase

substrate	activity[a] (%)
AMP	100
7a, (N)-methanocarba-AMP[39]	0.14
7c, (S)-(±)-methanocarba-AMP[39]	0.0

[a] Values are means of two experiments with duplicate determinations in each. The catalytic activity in the presence of AMP (100%) corresponded to 5.6 nmol/(min μg) protein.

Table 4. Hydrolysis of the Triphosphate Derivatives as Substrates of Rat NTPDase1 (Ecto-apyrase) and NTPDase2 (Ecto-ATPase) Stably Expressed in CHO Cells

	activity[a] (%)	
substrate	NTPDase1	NTPDase2
ATP	100	100
6a, (N)-methanocarba-ATP[39]	52	4
6c, (S)-(±)-methanocarba-ATP[39]	57	34

[a] The hydrolysis rate with the two derivatives as substrate is compared to that of ATP (100%) for each enzyme. Values are means of two experiments with duplicate determinations in each. Nontransfected CHO cells were analyzed as control. They contained no significant activity for the extracellular hydrolysis of ATP. 100% values correspond to the following: NTPDase1, 42.1 nmol/(min 10^6 cells); NTPDase2, 13.5 nmol/(min 10^6 cells).

Discussion

In the previous study,[39] we concluded that substitution of the ribose moiety with fused cyclopentane and cyclopropane rings in a pseudorotational (N)-conformation produced agonists at certain adenine and uracil nucleotide activated P2Y receptors. At $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{11}$ receptors, but not $P2Y_6$ receptors, an (N)-methanocarba analogue was much more potent than the corresponding molecule constrained in the Southern conformation. Here, we have extended the synthesis to multiple substituted analogues of ring-constrained (N)-methanocarba-ATP, ADP, and AMP and illustrated that these derivatives exhibit as high or even higher potency for activation than the corresponding riboside, depending on the P2Y subtype involved. In some cases, alreadypotent ATP analogues were increased in potency upon (N)-methanocarba modification. In other cases, an inactive compound became biologically active upon such substitution.

A key finding in this study is that remarkably potent agonists, especially at the $P2Y_1$ receptor, can be pro-

duced by introducing the (N)-methanocarba modification into molecules having other substitutions known to increase P2Y receptor potency. This was very notable in the (N)-methanocarba analogues of 2-Cl-ATP **13a** and N^6-Me-ATP **14a**, which exhibited increases in potency in the (N)-methanocarba form versus ribosides of >200-fold. However, perhaps the most remarkable enhancement of activity occurred with β,γ-Me-ATP, although a full agonist at P2X receptors is not an agonist (or antagonist) at the $P2Y_1$ and other P2Y receptors. However, the (N)-methanocarba analogue of β,γ-Me-ATP was a full agonist at the turkey and human $P2Y_1$ receptors, exhibiting an EC_{50} for the human receptor of 160 nM. We have not yet extended this analysis to a direct comparison of relative activities of (N)- versus (S)-conformers to molecules that also possess other functional groups, e.g., 2-MeS or N^6-CH_3 substitution, known to increase binding affinity and selectivity for P2Y receptor agonists.

As we have observed with many different molecules that were synthesized and tested previously, results in human and turkey $P2Y_1$ receptors were similar. The potencies determined in assays of phospholipase C in turkey erythrocyte membranes corresponded closely to the EC_{50} values determined for the same molecules at the recombinant human $P2Y_1$ receptor stably expressed in 1321N1 human astrocytoma cells. This correlation was closest with a series of analogues containing the (N)-methanocarba substitution with various functional groups, e.g., 2-Cl, 2-MeS, N^6-CH_3, that we and others had previously demonstrated to increase P2Y receptor agonist potency in the riboside series. An explanation for the relatively large difference in apparent potency of the (N)-methanocarba analogue of β,γ-Me-ATP at the turkey versus human $P2Y_1$ receptor is not readily apparent.

Although not studied in detail here, the (N)-methanocarba analogues exhibit resistance to hydrolysis relative to naturally occurring nucleotides. This observation may be particularly significant for 5′-monophosphate derivatives, since we have shown that they potently and selectively activate $P2Y_1$ receptors but may be somewhat stable to dephosphorylation by 5′-nucleotidase. However, it should be pointed out that this resistance to hydrolysis would at most only make a minor contribution to the very large difference in potencies seen between certain (N)-methanocarba compounds and their natural ribose counterpart. That is, the K_m/V_{max} values of ecto-nucleotidases on 1321N1 cells do not result in sufficient nucleotide hydrolysis to result in large shifts in the activation curves of, for example, ATP or UTP. More work will be required to obtain a clearer view on the capacity of the (N)-methanocarba molecules to serve as substrates for nucleotide-metabolizing ectoenzymes.

Thus, we established that constraining the ribose-like ring as methanocarba analogues was an important principle for producing P2Y receptor-subtype selectivity, similar to our previous demonstration of this phenomenon for $P2Y_1$ receptor antagonists. The (N)-methanocarba analogues generally displayed increased potency over the corresponding nucleotide agonists at the $P2Y_1$ and $P2Y_2$ receptors. The high potency of several of the derivatives (e.g., the selective $P2Y_1$ receptor agonist **9a**) suggested the possibility of preparation of high-affinity radioligands in the (N)-methanocarba series. The potent and selective $P2Y_1$ agonists (EC_{50} in nM at the $tP2Y_1$ receptor unless otherwise noted) found in the present study were the following: **9a** (0.88), **12a** (3), **15a** (9), **8a** (23), **17a** (160 at human), and **10a** (590). Compound **14a** was roughly equipotent at $P2Y_1$ and $P2Y_2$ receptors.

In conclusion, remarkable $P2Y_1$ potency was generated in derivatives that combined the (N)-methanocarba modification with other functional groups known to enhance potency and/or selectivity in P2 receptor agonists. These molecules provide a new armamentarium for the study of P2Y receptors. Moreover, they provide an attractive new chemical backbone on which to build highly potent and selective P2Y receptor agonists and antagonists that, in lacking a ribose moiety, are not nucleotides, and therefore, they are less likely to bind to the myriad of nucleotide binding proteins that are not P2Y receptors. Furthermore, enhanced stability to some nucleotidase enzymes is indicated in the methanocarba series. These findings promise to be useful for defining the microscopic determinants of the binding sites on these receptors and for designing novel pharmacological probes and/or therapeutic agents.

Experimental Section

1. Chemical Synthesis. Nucleosides and synthetic reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich (Milwaukee, WI). 2,6-Dichloropurine was obtained from Sigma. The protected intermediate **21** was synthesized in our laboratory as described.[43,45] (N)-Methanocarba analogues of ATP **6a** and AMP **7a** and the corresponding (±)-(S)-methanocarba analogues **6c** and **7c**, respectively, were prepared as described.[39] Compound **16a** was prepared as described.[45]

^{1}H NMR spectra were obtained with a Varian Gemini-300 spectrometer (300 MHz) using D_2O/$CDCl_3$ and CD_3OD 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 mm × 4.6 mm; Separation Methods Technologies, Inc., Newark, DE) in two solvent systems.

System A was a linear gradient solvent system consisting of 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 was a linear gradient solvent system consisting of 5 mM TBAP/CH_3CN from 80/20 to 40/60 in 20 min, and the flow rate was 1 mL/min. System C was a linear gradient solvent system consisting of 0.1 M TEAB/CH_3CN from 100/0 to 90/10 in 20 min, and the flow rate was 1 mL/min.

Peaks were detected by UV absorption using a diode array detector. All derivatives tested for biological activity showed ≥97% 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 was carried out with a VG7070F mass spectrometer 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 the nucleotide analogues for biological testing was carried out on DEAE-A25 Sephadex columns as described above.

General Phosphorylation Procedure: Synthesis of (N)-Methanocarbaadenosine Derivatives (23−26). (1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-9*H*-purin-9-yl)-1-[(phenylmethoxy)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (23). To a solution of **21** (0.2 g, 0.47 mmol) in *i*-PrOH

(3 mL) was added NH_3 (2 M solution in *i*-PrOH, 5 mL, 10 mmol), and the reaction mixture was heated at 90 °C in a closed tube for 15 h for complete reaction. The resulting mixture was concentrated under reduced pressure, and the residue obtained was purified by flash chromatography using 9/1 $CHCl_3$/MeOH to furnish 0.182 g of **23** (95%). ^{1}H NMR ($CDCl_3$) δ 8.35 (s, 1H), 8.30 (s, 1H), 7.37 (s, 5H), 5.87 (bs, 2H), 5.31 (d, 1H, J = 6.8 Hz), 5.13 (s, 1H), 4.64 (qAB, 2H, J = 11.7, 20.5 Hz), 4.51 (d, 1H, J = 6.8 Hz), 3.97 (d, 1H, J = 10.7 Hz), 3.35 (d, 1H, J = 10.7 Hz), 1.72−1.62 (m, 1H), 1.55 (s, 3H), 1.32−1.26 (m, 1H), 1.23 (s, 3H), 1.00−0.93 (m, 1H).

Compounds **24** and **25** were synthesized from **21,** and **26** was synthesized from **22**. All were produced in 90−95% yields.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-9*H*-purin-9-yl)-1-[(phenylmethoxy)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (24): ^{1}H NMR ($CDCl_3$) δ 8.41 (s, 1H), 8.22 (s, 1H), 7.40−7.30 (m, 5H), 6.01 (bs, 1H), 5.30 (d, 1H, J = 7.2 Hz), 5.12 (s, 1H), 4.62 (qAB 2H, J = 12.1, 20.9 Hz), 4.51 (d, 1H, J = 7.2 Hz), 3.94 (d, 1H, J = 9.9 Hz), 3.35 (d, 1H, J = 9.9 Hz), 3.21 (s, 3H), 1.70−1.62 (m, 1H), 1.55 (s, 3H), 1.29−1.25 (m, 1H), 1.22 (s, 3H), 0.98−0.92 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-chloro-9*H*-purin-9-yl)-1-[(phenylmethoxy)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (25): ^{1}H NMR ($CDCl_3$) δ 8.22 (s, 1H), 7.40−7.33 (m, 5H), 6.48 (bs, 2H), 5.32 (d, 1H, J = 7.15 Hz), 5.07 (s, 1H), 4.61 (qAB, 2H, J = 12.1, 19.2 Hz), 4.51 (d, 1H, J = 7.2 Hz), 3.94 (d, 1H, J = 9.9 Hz), 3.42 (d, 1H, J = 9.9 Hz), 1.64−1.56 (m, 1H), 1.55 (s, 3H), 1.30−1.26 (m, 1H), 1.24 (s, 3H), 0.98−0.933 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-2-chloro-9*H*-purin-9-yl)-1-[(phenylmethoxy)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (26): ^{1}H NMR ($CDCl_3$) δ 8.15 (s, 1H), 7.40−7.28 (m, 5H), 6.29 (bs, 1H), 5.32 (d, 1H, J = 7.1 Hz), 5.05 (s, 1H), 4.61 (qAB, 2H, J = 12.4, 17.6 Hz), 3.90 (d, 1H, J = 10.2 Hz), 3.42 (d, 1H, J = 10.2 Hz), 3.17 (bs, 3H), 1.60−1.56 (m, 1H), 1.54 (s, 3H), 1.30−1.26 (m, 1H), 1.24 (s, 3H), 0.96−0.92 (m, 1H).

Procedure for Debenzylation of 24−26. See ref 39.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-chloro-9*H*-purin-9-yl)-1-[hydroxymethyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (27): ^{1}H NMR ($CDCl_3$) δ 7.90 (s, 1H), 5.56 (d, 1H, J = 7.4 Hz), 4.79 (s, 1H), 4.66 (d, 1H, J = 7.4 Hz), 4.26 (d, 1H, J = 11.5 Hz), 3.37 (d, 1H, J = 11.5 Hz), 1.78−1.70 (m, 1H), 1.55 (s, 3H), 1.26 (s, 3H), 1.20−1.14 (m, 1H), 1.20−0.96 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-9*H*-purin-9-yl)-1-[hydroxymethyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (29): ^{1}H NMR ($CDCl_3$) δ 8.36 (s, 1H), 7.77 (s, 1H), 5.61 (d, 1H, J = 7.4 Hz), 4.78 (s, 1H), 4.65 (d, 1H, J = 7.4 Hz), 4.33 (d, 1H, J = 11.5 Hz), 3.25 (d, 1H, J = 11.5 Hz), 3.20 (bs, 3H), 1.79−1.74 (m, 1H), 1.55 (s, 3H), 1.25 (s, 3H), 1.17−1.13 (m, 1H), 1.01−0.96 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-2-chloro-9*H*-purin-9-yl)-1-[hydroxymethyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (30): ^{1}H NMR (CD_3OD) δ 8.14 (s, 1H), 5.30 (d, 1H, J = 7.1 Hz), 4.87 (s, 1H), 4.61 (d, 1H, J = 7.1 Hz), 3.91 (d, 1H, J = 11.5 Hz), 3.52 (d, 1H, J = 11.5 Hz), 3.00 (bs, 3H), 1.64−1.60 (m, 1H), 1.44 (s, 3H), 1.18 (s, 3H), 1.08−1.04 (m, 1H), 0.92−0.87 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′S)-4-(6-Amino-2-methylthio-9*H*-purin-9-yl)-1-[hydroxymethyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (28). Compound **27** (30 mg, 0.086 mmol) was dissolved in DMF (1.5 mL) and treated with sodium thiomethoxide (90 mg, 1.3 mmol). The reaction mixture was heated in a sealed tube at 90 °C for 1.5 h. The solvent was removed under vacuum, and the product was purified by preparative thin-layer chromatography (9/1 $CHCl_3$/MeOH) to furnish 25 mg of **28** (80%). ^{1}H NMR ($CDCl_3$) δ 7.76 (s, 1H), 5.82 (bs, 2H), 5.56 (d, 1H, J = 7.1 Hz), 4.78 (s, 1H), 4.71 (d, 1H, J = 7.1 Hz), 4.25 (d, 1H, J = 11.5 Hz), 3.35 (d, 1H, J = 11.5 Hz), 2.59 (s, 3H), 1.71−1.66 (m, 1H), 1.55 (s, 3H), 1.26 (s, 3H), 1.18−1.14 (m, 1H), 0.99−0.93 (m, 1H).

Protected (*N*)-Methanocarbaadenosine 5′-Monophosphate Derivatives (31−35). These monophosphate derivatives were synthesized by procedures similar to those used to prepare the unsubstituted adenine analogue.[39]

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-chloro-9*H*-purin-9-yl)-1-[(di-*tert*-butyl phosphate)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (31): ^{1}H NMR ($CDCl_3$) δ 8.23 (s, 1H), 6.91 (bs, 2H), 5.41 (d, 1H, J = 7.1 Hz), 5.16 (s, 1H), 4.64 (d, 1H, J = 7.1 Hz), 4.59 (dd, 1H, J = 5.5, 11.0 Hz), 3.98 (dd, 1H, J = 6.3, 11.0 Hz), 1.84−1.76 (m, 1H), 1.63 (s, 3H), 1.61 (s, 9H), 1.59 (s, 9H), 1.42−1.37 (m, 1H), 1.33 (s, 3H), 1.18−1.13 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-methylthio-9*H*-purin-9-yl)-1-[(di-*tert*-butyl phosphate)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (32) and (1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-methylsulfoxy-9*H*-purin-9-yl)-1-[(di-*tert*-butyl phosphate)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (33). To a mixture of **28** (20 mg, 0.055 mmol) and tetrazole (0.012 g, 0.165 mmol) in anhydrous THF (2 mL) was added di-*tert*-butyl-*N*,*N*-diethylphosphoramidite (0.023 mL, 0.083 mmol), and the mixture was stirred at room temperature overnight. The reaction mixture was cooled to −78 °C, and a solution of 70% mCPBA (0.012 g, 0.066 mmol) in 1 mL of CH_2Cl_2 was warmed to room temperature. A total of 1 mL MeOH was added, and the mixture was concentrated and purified by preparative TLC using 10% MeOH in $CHCl_3$ to furnish **32** (12 mg) and **33** (12 mg).

32: ^{1}H NMR ($CDCl_3$) δ 7.98 (s, 1H), 5.87 (bs, 2H), 5.31 (d, 1H, J = 7.2 Hz), 5.07 (s, 1H), 4.59 (d, 1H, J = 7.2 Hz), 4.49 (dd, 1H, J = 5.8, 11.0 Hz), 3.83 (dd, 1H, J = 6.3, 11.0 Hz), 2.59 (s, 3H), 1.75−1.70 (m, 1H), 1.55 (s, 3H), 1.49 (s, 18H), 1.30−1.27 (m, 1H), 1.23 (s, 3H), 1.05−1.00 (m, 1H).

33: ^{1}H NMR ($CDCl_3$) δ 8.26 (s, 1H), 6.66 (bs, 2H), 5.33 (d, J = 7.14 Hz, 1H), 5.22 (s, 1H), 4.60−4.50 (m, 2H), 3.81−3.71 (m, 2H), 2.93 (s, 3H), 1.76−1.70 (m, 1H), 1.54 (s, 3H), 1.50 (s, 9H), 1.34−1.30 (m, 1H), 1.25 (s, 3H), 1.23 (s, 9H), 1.07−1.02 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-9*H*-purin-9-yl)-1-[(di-*tert*-butyl phosphate)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (34): ^{1}H NMR ($CDCl_3$) δ 8.45 (s, 1H), 8.05 (s, 1H), 6.04 (bs, 1H), 5.30 (d, 1H, J = 7.2 Hz), 5.08 (s, 1H), 4.57 (d, 1H, J = 7.2 Hz), 4.47 (dd, 1H, J = 5.8, 11.0 Hz), 3.90 (dd, 1H, J = 6.3, 11.0 Hz), 3.20 (bs, 3H), 1.79−1.71 (m, 1H), 1.54 (s, 3H), 1.51 (s, 9H), 1.50 (s, 9H), 1.29−1.26 (m, 1H), 1.22 (s, 3H), 1.08−1.03 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-2-chloro-9*H*-purin-9-yl)-1-[(di-*tert*-butyl phosphate)methyl]bicyclo[3.1.0]hexane-2,3-(*O*-isopropylidene) (35): ^{1}H NMR ($CDCl_3$) δ 8.04 (s, 1H), 5.38 (d, 1H, J = 7.1 Hz, 1H), 5.04 (s, 1H), 4.58 (d, 1H, J = 7.1 Hz), 4.42−4.52 (m, 1H), 3.90−4.08 (m, 1H), 3.20 (bs 3H), 1.79−1.71 (m, 1H), 1.54 (s, 3H), 1.51 (s, 9H), 1.50 (s, 9H), 1.29−1.26 (m, 1H), 1.22 (s, 3H), 1.08−1.03 (m, 1H).

General Procedure for Deprotection of (N)-Methanocarbaadenosine 5′-Monophosphate Derivatives (Synthesis of 13a, 10a, 11a, 36, and 37). Deprotection was carried out by procedures similar to those used to prepare the unsubstituted adenine analogue.[39]

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-methylthio-9*H*-purin-9-yl)-1-[phosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (10a): ^{1}H NMR (D_2O) δ 8.53 (s, 1H), 4.91 (s, 1H), 4.48 (dd, 1H, J = 4.7, 11.0 Hz), 4.05 (d, 1H, J = 6.6 Hz), 3.69 (dd, 1H, J = 4.7, 11.0 Hz), 2.63 (s, 3H), 1.92−1.84 (m, 1H), 1.58−1.40 (m, 1H), 1.18−0.98 (m, 1H). ^{31}P NMR (D_2O) δ 0.613.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-methylthiooxy-9*H*-purin-9-yl)-1-[phosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (11a): ^{1}H NMR (D_2O) δ 8.83 (s, 1H), 5.46 (d, J = 6.04 Hz, 1H), 4.52 (dd, J = 4.94, 10.71 Hz, 1H), 4.06 (d, J = 6.04 Hz, 1H), 3.72 (dd, J = 4.94, 10.71 Hz, 1H), 2.99 (s, 3H), 2.04−1.92 (m, 1H), 1.26−1.22 (m, 1H), 1.04−0.98 (m, 1H). ^{31}P NMR (D_2O) δ 0.623.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-chloro-9*H*-purin-9-yl)-1-[phosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (13a): ^{1}H NMR (D_2O) δ 8.60 (s, 1H), 4.72 (s, 1H), 4.48−4.40 (m, 1H), 3.99 (d, 1H, J = 6.6 Hz), 3.62−3.76 (m, 1H), 1.94−1.82 (m, 1H), 1.60−1.52 (m, 1H), 1.08−0.95 (m, 1H).

The imidazolate **42**, derived from **13a**, displayed a single ^{31}P NMR resonance in D_2O at δ −11.824.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-9*H*-purin-9-yl)-1-[phosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (36): ^{1}H NMR (D_2O) δ 8.47 (s, 1H), 8.26 (s, 1H), 4.87 (s, 1H), 4.76 (d, 1H, J = 6.6 Hz), 4.46 (dd, 1H, J = 5.0, 11.0 Hz), 3.99 (d, 1H, J = 6.6 Hz), 3.66 (dd, 1H, J = 5.0, 11.0 Hz), 3.12 (bs, 3H), 1.88−1.82 (m, 1H), 1.54−1.48 (m, 1H), 1.00−0.92 (m, 1H).

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-2-chloro-9*H*-purin-9-yl)-1-[phosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (37): ^{1}H NMR (D_2O) δ 8.42 (s, 1H), 4.71 (s, 1H), 4.52 (m, 1H), 3.97 (d, 1H, J = 6.3 Hz), 3.72−3.62 (m, 1H), 3.01 (bs, 3H), 1.72−1.80 (m, 1H), 1.62−1.48 (m, 1H), 1.04−0.94 (m, 1H).

General Method of Synthesis of (*N*)-Methanocarba-ATP Analogues (8a, 12a, 14a, and 15a). These triphosphate derivatives were synthesized by procedures similar to the procedure used to prepare the unsubstituted adenine analogue[39] and the procedure for 5′-diphosphate analogue **9a**.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-methylthio-9*H*-purin-9-yl)-1-[diphosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (9a). A mixture of **10a** (14 mg, 0.035 mmol) and 1,1′-carbonyldiimidazole (28 mg, 0.175 mmol) in 1 mL of anhydrous DMF was stirred at room temperature for 6 h. Et_3N/H_2O/MeOH (1:10:10 by volume, 2 mL) was added, and the mixture was stirred at room temperature for an additional 2 h. The mixture was concentrated to dryness under vacuum. To the residue, phosphoric acid (20 mg, 0.21 mmol) was added and dried under high vacuum. This was suspended in anhydrous DMF (2 mL), and Et_3N (0.1 mL) was added. The mixture was stirred at room temperature for 3 days. Triethylammonium bicarbonate (2 mL) was added, all of the solvent was removed under vacuum, and the residue was purified using Sephadex column chromatography, eluting with 150 mL of water and 150 mL of ammonium bicarbonate to furnish **9a** (5.0 mg). ^{1}H NMR (D_2O) δ 8.34 (s, 1H), 4.87 (s, 1H), 4.71 (d, J = 6.2 Hz, 1H), 4.54−4.48 (m, 1H), 4.02 (d, J = 6.2 Hz, 1H), 3.85−3.78 (m, 1H), 2.58 (s, 3H), 1.91−1.86 (m, 1H), 1.54−1.46 (m, 1H), 1.02−0.96 (m, 1H). ^{31}P NMR (D_2O) δ −9.63, −10.70.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-methylthio-9*H*-purin-9-yl)-1-[triphosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (8a): ^{1}H NMR (D_2O) δ 8.15 (s, 1H), 4.10−3.98 (m, 1H), 3.90−3.74 (m, 1H), 3.67−3.61 (m, 1H), 3.57−3.52 (m, 1H), 2.59 (s, 3H), 2.02−1.84 (m, 1H), 1.62−1.46 (m, 1H), 1.06−0.94 (m, 1H). ^{31}P NMR (D_2O) δ 0.02, −9.6, −10.8.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-2-chloro-9*H*-purin-9-yl)-1-[triphosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (12a): ^{1}H NMR (D_2O) δ 8.22 (s, 1H), 4.86 (s, 1H), 4.72 (d, 1H, J = 5.2 Hz), 3.84−3.74 (m, 2H), 2.14−2.02 (m, 1H), 1.62−1.54 (m, 1H), 1.06−0.96 (m, 1H). ^{31}P NMR (D_2O) δ −10.55, −10.93, −22.79.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-9*H*-purin-9-yl)-1-[triphosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (14a): ^{1}H NMR (D_2O) δ 8.44 (s, 1H), 8.24 (s, 1H), 4.85 (d, 1H, J = 5.2 Hz), 4.75 (s, 1H), 4.57 (dd, 1H, J = 5.2, 11.3 Hz), 4.01 (d, 1H, J = 5.2 Hz), 3.83 (dd, 1H, J = 4.7, 11.3 Hz), 3.09 (bs, 3H), 1.96−1.86 (m, 1H), 1.56−1.48 (m, 1H), 1.04−0.96 (m, 1H). ^{31}P NMR (D_2O) δ −10.03, −10.67, −22.54.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Methylamino-2-chloro-9*H*-purin-9-yl)-1-[triphosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (15a): ^{1}H NMR (D_2O) δ 8.42 (s, 1H), 4.86 (s, 1H), 4.74 (s, 1H), 4.64−4.55 (m, 1H), 4.00 (s, 1H), 3.95−3.72 (m, 1H), 3.08 (bs, 3H), 1.96−1.82 (m, 1H), 1.58−1.44 (m, 1H), 1.08−0.96 (m, 1H). ^{31}P NMR (D_2O) δ −8.05, −10.73, −22.19.

(1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(6-Amino-9*H*-purin-9-yl)-1-[*b*,*g*-methylenetriphosphoryloxymethyl]bicyclo[3.1.0]hexane-2,3-diol (17a). A mixture of compound **7a** (11 mg, 0.03 mmol)[39] and 1,1′-carbonyldiimidazole (24 mg, 0.15 mmol) in DMF (2 mL) was stirred at room temperature for 6 h. Et_3N/H_2O/MeOH (1:10:10 by volume, 1 mL) was added, and the mixture was stirred for 2 h. All of the solvent was removed under vacuum to dryness. The residue and methylene diphosphonic acid (0.32 g, 0.18 mmol) were dried under high vacuum for 1 h and suspended in anhydrous DMF (2 mL), 0.1 mL Et_3N was added, and the mixture was stirred at room temperature for 3 days. Triethylammonium bicarbonate (2 mL) was added, and all of the solvent was removed under vacuum and purified using Sephadex column chromatography, eluting with 100 mL of water and 100 mL of ammonium bicarbonate to furnish **17a** (4.0 mg). ^{1}H NMR (D_2O) δ 8.70 (s, 1H), 8.41 (s, 1H), 4.98 (s, 1H), 4.59 (dd, J = 4.95, 10.72 Hz, 1H), 4.00 (d, J = 6.04 Hz, 1H), 3.82 (dd, J = 4.95, 10.72 Hz, 1H), 2.36 (bs, 2H), 1.98−1.86 (m, 1H), 1.58−1.52 (m, 1H), 1.06−0.98 (m, 1H). ^{31}P NMR (D_2O) δ 15.454, 9.024, −10.341.

Phosphoric Acid Mono[(2*R*,3*R*,4*S*,5*R*)-5-(6-amino-2-chloro-9*H*-purin-9-yl]-3,4-dihydroxytetrahydrofuran-2-ylmethyl]ester (13b). This monophosphate was synthesized by procedures similar to those used to prepare the unsubstituted adenine analogue.[39] ^{1}H NMR (D_2O) δ 8.23 (s, 1H), 5.93 (d, J = 6.04 Hz, 1H), 4.39 (s, 1H), 4.27 (s, 1H), 3.92−3.82 (m, 2H).

(2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-methylsulfanyl-9*H*-purin-9-yl]tetrahydrofuran-3,4-(*O*-isopropylidene) (47). Compound **46** (30 mg, 0.088 mmol) was dissolved in DMF (1.5 mL) and treated with sodium thiomethoxide (30 mg, 0.44 mmol). The reaction mixture was heated in a sealed tube at 90 °C for 1.5 h. The solvent was removed under vacuum, and the product was purified using preparative thin-layer chromatography (9/1 $CHCl_3$/MeOH) to furnish 20 mg of **47** (65%). ^{1}H NMR ($CDCl_3$) δ 7.7 (s, 1H), 5.81 (d, J = 4.39 Hz, 1H), 5.62 (s, 2H), 5.34−5.28 (m, 1H), 5.12 (d, J = 4.39 Hz, 1H), 4.47 (s, 1H), 3.98−3.76 (m, 2H), 2.56 (s, 3H), 1.63 (s, 3H), 1.38 (s, 3H).

Phosphoric Acid Mono[(2*R*,3*R*,4*S*,5*R*)-5-(6-amino-2-methylsulfanyl-9*H*-purin-9-yl]-3,4-dihydroxytetrahydrofuran-2-ylmethyl]ester (10b) and Phosphoric Acid Mono-[(2*R*,3*R*,4*S*,5*R*)-5-(6-amino-2-methylsulfinyl-9*H*-purin-9-yl]-3,4-dihydroxytetrahydrofuran-2-ylmethyl]ester (11b). Di-*tert*-butyl-*N*,*N*-diethylphosphoramidite (0.02 mL, 0.075 mmol) was added to a mixture of **47** (18 mg, 0.05 mmol) and tetrazole (17 mg, 0.05 mmol) in anhydrous THF (2 mL), and the mixture was stirred at room temperature overnight. The reaction mixture was cooled to −78 °C, treated with a solution of 70% mCPBA (15 mg, 0.06 mmol) in 1 mL of CH_2Cl_2, and warmed to room temperature. A total of 1 mL of MeOH was added, and the mixture was concentrated and purified by preparative TLC using 10% MeOH in $CHCl_3$ to furnish **48** (10 mg) and **49** (10 mg). These were treated with 30 mg of Dowex 50x8-200 in 1/1 H_2O/MeOH at 80 °C for 1 h. The Dowex resin was filtered, and the filtrate was concentrated to furnish **10b** (3 mg, 15%) and **11b** (3 mg, 15%).

10b: ^{1}H NMR (D_2O) δ 8.39 (s, 1H), 6.11 (d, J = 5.5 Hz, 1H), 4.82−4.95 (m, 1H), 4.45 (s, 1H), 4.26 (s, 1H), 3.92−3.82 (m, 2H), 2.59 (s, 3H).

11b: ^{1}H NMR (D_2O) δ 8.49 (s, 1H), 6.11 (d, J = 5.5 Hz, 1H), 4.81−4.94 (m, 1H), 4.45 (s, 1H), 4.26 (s, 1H), 3.92−3.82 (m, 2H), 2.82 (s, 3H).

2. Enzymatic Analyses. 2.1. Hydrolysis of 5′-Triphosphate Derivatives by Recombinant Rat Ecto-5′-nucleotidase. Catalytically active recombinant ecto-5′-nucleotidase was expressed in insect cells using the baculovirus system and purified as described in ref 48. Ecto-5′-nucleotidase (0.5 μg) was incubated in Tris-buffered saline (TPS: 10 mM Tris, 150 mM NaCl, 0.4 mM $MgCl_2$, pH 7.4) with the corresponding substrate (AMP, **7a**, or **7c**) at a final concentration of 100 μM. After 10 min of incubation at 37 °C, the reaction was terminated by addition of 5% TCA. The sample was centrifuged for 10 min at 12000*g* (4 °C). Inorganic phosphate was determined in the supernatant fraction according to the method of Lanzetta et al.[51]

2.2. Hydrolysis of 5′-Triphosphate Derivatives by Rat NTPDase1 (Ectoapyrase) and NTPDase2 (Ecto-ATPase) Stably Expressed in CHO Cells. Cell Transfection. CHO cells stably transfected with a plasmid DNA containing NTPDase1 (ecto-apyrase) or NTPDase2 (ecto-ATPase) have previously been described.[49] They were cultured in in HAM's FC-12 medium containing 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Eggenstein, Germany) as described in ref 52.

2.3. Measurement of Ecto-nucleotidase Activity. Transfected CHO cells were seeded in multiwell plates (20 000 cells per well, 1.88 cm^2). Surface-located enzyme activity of intact cells was determined 24 h later at 37 °C. Cells were washed twice with physiological saline solution (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM Hepes, pH 7.4) and subsequently incubated in 500 μL of the identical saline solution containing 50 mM of nucleotide substrate (ATP, **6a**, or **6c**). Aliquots of the culture supernatant were collected at varying time points and subjected to high-performance liquid chromatography (HPLC) analysis as previously described.[49] The absorbance at 260 nm was continuously monitored, and the nucleotide and nucleoside concentrations were determined from the area under each absorbance peak.

4. Pharmacological Analyses. P2Y-receptor-promoted stimulation of inositol phosphate formation was measured at human $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{11}$ receptors stably expressed in 1321N1 human astrocytoma cells as previously described.[46,50] The $K_{0.5}$ values were averaged from 3 to 8 independently determined concentration−effect curves for each compound. Turkey erythracytes were incubated in inositol-free medium (DMEM; Gibco, Gaithersburg, MD) with 0.5 mCi of 2-[^{3}H]*myo*-inositol (20 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) for 18−24 h in a humidified atmosphere of 95%/5% air/CO_2 at 37 °C. t$P2Y_1$ receptor-promoted 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. Membranes were incubated at 30 °C for 5 min, and total [^{3}H]inositol phosphates were quantified by anion exchange chromatography as previously described.[47,53]

5. 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 UTP or UDP in the same experiment.

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

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