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Pyrimidine Ribonucleotides with Enhanced Selectivity as $P2Y_6$ Receptor Agonists: Novel 4-Alkyloxyimino, (S)-Methanocarba, and 5′-Triphosphate γ-Ester Modifications†

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The $P2Y_6$ receptor is a cytoprotective G-protein-coupled receptor (GPCR) activated by UDP (EC_{50} = 0.30 μM). We compared and combined modifications to enhance $P2Y_6$ receptor agonist selectivity, including ribose ring constraint, 5-iodo and 4-alkyloxyimino modifications, and phosphate modifications such as α,β-methylene and extension of the terminal phosphate group into γ-esters of UTP analogues. The conformationally constrained (S)-methanocarba-UDP is a full agonist (EC_{50} = 0.042 μM). 4-Methoxyimino modification of pyrimidine enhanced $P2Y_6$, preserved $P2Y_2$ and $P2Y_4$, and abolished $P2Y_{14}$ receptor potency, in the appropriate nucleotide. N^4-Benzyloxy-CDP (**15**, MRS2964) and N^4-methoxy-Cp_3U (**23**, MRS2957) were potent, selective $P2Y_6$ receptor agonists (EC_{50} of 0.026 and 0.012 μM, respectively). A hydrophobic binding region near the nucleobase was explored with receptor modeling and docking. UTP-γ-aryl and cycloalkyl phosphoesters displayed only intermediate $P2Y_6$ receptor potency but had enhanced stability in acid and cell membranes. UTP-glucose was inactive, but its (S)-methanocarba analogue and N^4-methoxycytidine 5′-triphospho-γ-[1]glucose were active (EC_{50} of 2.47 and 0.18 μM, respectively). Thus, the potency, selectivity, and stability of pyrimidine nucleotides as $P2Y_6$ receptor agonists may be enhanced by modest structural changes.

Introduction

The P2Y receptors are G-protein-coupled receptors (GPCRs) that respond to naturally occurring extracellular nucleotides including ATP, ADP, UTP,[a] UDP, and UDP-glucose.[1] Five of the eight subtypes, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors, are preferentially coupled through Gq to activate phospholipase C-β (PLC-β) and represent a distinct structural subfamily.[2] The remaining $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors are coupled to inhibition of adenylate cyclase. Activation of the $P2Y_6$ receptor by its native ligand UDP **1** (Chart 1) is associated with vasoconstriction and cytoprotection.[3−6] We reported the effects of $P2Y_6$ receptor agonists to counteract apoptosis induced by tumor necrosis factor α in astrocytoma cells stably expressing the human $P2Y_6$ receptor and in mouse C2C12 skeletal muscle cells and MIN6 pancreatic cells that express an endogenous $P2Y_6$ receptor.[4−6] Furthermore, in vivo administration of 5-iodo-UDP (MRS2693) **3**, which is a selective $P2Y_6$ receptor agonist, induced protection in a model of ischemia in hindleg skeletal muscle.[6] The dinucleoside triphosphate INS48823 **4** activates the $P2Y_6$ receptor in bone and increased survival of osteoclasts.[7] A role for the $P2Y_6$ receptor in inducing phagocytosis in microglial cells was recently discovered, suggesting the possible application of $P2Y_6$ receptor ligands for the treatment of neurodegenerative conditions.[8] The $P2Y_6$ receptor is up-regulated when neurons are damaged and in intestinal inflammation.[8,9] UDP applied topically to the cornea reduced intraocular pressure, suggesting the use of $P2Y_6$ receptor agonists for the treatment of ocular hypertension and glaucoma.[10] Studies of mice lacking the $P2Y_6$ receptor recently demonstrated a defective response to UDP in macrophages, endothelial cells, and vascular smooth muscle cells.[11] Thus, pharmacological modulation of the $P2Y_6$ receptor is of increasing interest for future therapeutics.[12]

Potent $P2Y_6$ receptor agonists, such as 3-phenacyl-UDP **5** with an EC_{50} value of 70 nM, have been reported.[13−16] Recently, we reported that UDP also activates the $P2Y_{14}$ receptor, and therefore, agonists that delineate between these two subtypes are needed.[17,18] We have adopted a structure-based approach to developing $P2Y_6$ receptor agonist ligands with improved potency, selectivity, and stability. In the absence of an X-ray structure, rhodopsin-based homology modeling has predicted a putative binding site for nucleotides in the human $P2Y_6$ receptor.[13] The receptor recognition of the

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[a] Abbreviations: CDI, *N*,*N*-carbonyldiimidazole; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIC, *N*,*N*′-diisopropylcarbodiimide; DMF, dimethylformamide; HEK, human embryonic kidney; HBSS, Hank's balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; PLC, phospholipase C; SAR, structure−activity relationship; TBAP, tetrabutylammonium dihydrogen phosphate; TEAA, triethylammonium acetate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate.

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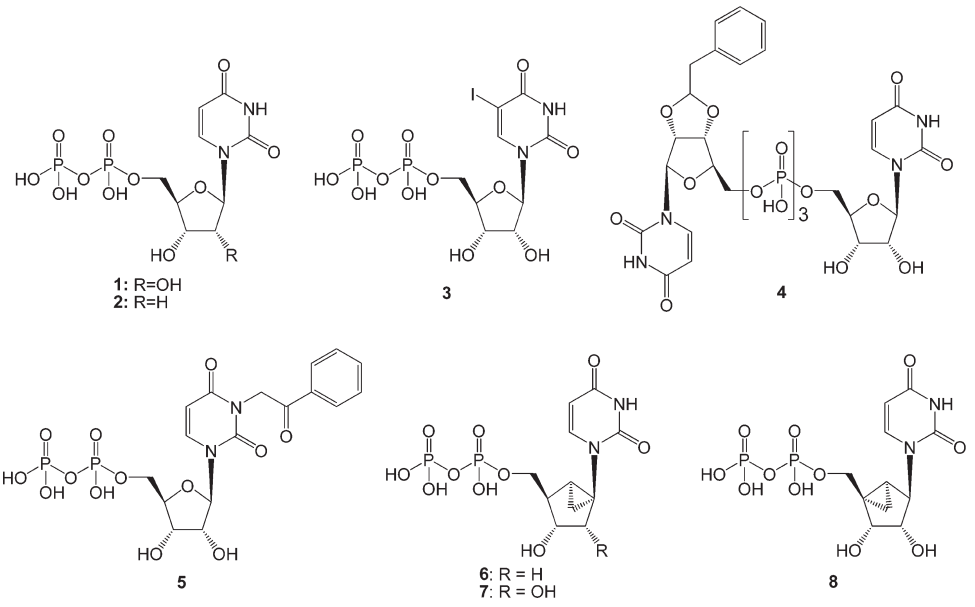
Chart 1. Structures of Prototypical Agonist Ligands for Studying $P2Y_6$ Receptors



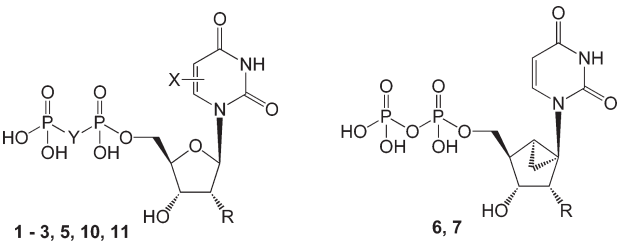
5′-diphosphate group is associated with three cationic residues in the transmembrane region, which serve as counterions and which are conserved in charge across the five Gq-coupled P2Y receptors.[2] A Monte Carlo analysis of conformations of receptor-docked UDP molecules, followed by molecular dynamics of the receptor−ligand complex in a lipid bilayer model, has predicted that the South (S) conformation of the ribose ring is preferred at the $P2Y_6$ receptor.[13] This is the only Gq-coupled P2Y subtype at which the (S)-conformation of the ribose or a ribose-like moiety of nucleotide ligands appears to be favored, and our comparison of the weak agonist 2′-deoxy-UDP **2** and the corresponding 2′-deoxy (S)-methanocarba analogue **6**[13,14] supported this idea.

We recently reported the first synthetic route to the enantiomerically pure (S)-methanocarba-uridine,[19] and in this study we examined the hypothesis that the (S)-conformation is preferred at the $P2Y_6$ receptor in the more potent 2′-hydroxynucleotide series. Modifications of the pyrimidine ring of UDP, including 5-iodo and 4-alkyloxyimino, and of the phosphate moiety, including α,β-methylene, and extension of the terminal phosphate group were compared and combined in an effort to further enhance selectivity for the $P2Y_6$ receptor.[14,16] Chemical and metabolic instability of mononucleotides is well-documented.[20] Mononucleotides are degraded enzymatically by ectonucleotidases into the lower nucleotides and eventually into the nucleoside. Chemical degradation of nucleotides is also evident at pH ranges less than 4 or greater than 10. The degradation products of nucleotides may interact with multiple P2Y receptors or with adenosine receptors and lead to side effects. Since various dinucleotides, such as diuridine triphosphates, show greater stability than analogues of mononucleotides,[21,22] we hypothesized that blockade of the terminal phosphate with an alkyl or aryl group may also increase its stability.

Results

Chemical Synthesis. Known (**1**−**6**, **8**, **10**, and **11**) and newly synthesized derivatives of UDP (**7**, **9**) and CDP (**12**−**16**) were assembled and compared (Tables 1 and 2). Novel analogues of UTP and Up_3U (**18**, **20**, and **22**−**33**) also were included in this structure−activity relationship (SAR) analysis (Table 3). The chemical synthesis of ribonucleoside 5′-diphosphate and triphosphate derivatives (Scheme 1) was performed by

Table 1. Potencies of (S)-Methanocarba Analogues **6**, **7**, and **9** in Relation to Known Uridine 5′-Diphosphate Derivatives for Activation of the Human $P2Y_6$ Receptor[a]

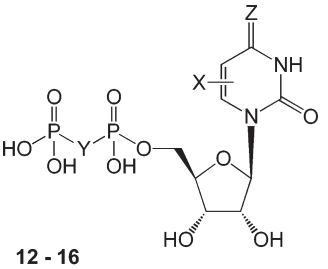


X = H; Y = O, R = OH, unless noted.

compd	modification	structure	EC_{50}, μM[b] hP2Y$_6$
1[f]	UDP		0.30 ± 0.06
2	dUDP	R = H	1.72 ± 0.76
3[c]	5-I-UDP	X = 5-I	0.015 ± 0.002
5[c]	phenacyl-UDP	X = 3-Ph-$COCH_2$	0.070 ± 0.006
6[c,d]	(S)-methanocarba-dUDP	R = H	0.23 ± 0.05
7[d]	(S)-methanocarba-UDP		0.042 ± 0.008
8[c,e]	(N)-methanocarba-UDP		NE
9	(S)-methanocarba U-cyclic-3′,5′-diphosphate		NE
10[c,f]	α,β-CH_2-UDP	Y = CH_2	0.70 ± 0.11
11[f]	α,β-CH_2-5-I-UDP	X = 5-I, Y = CH_2	0.13 ± 0.02

[a] Unless otherwise noted, R = uridine and X = O. NE: no effect at 10 μM. [b] Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human $P2Y_6$ receptor. Potencies are presented in the form of EC_{50} values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as the mean ± standard error and are the average of three to six different experiments with each molecule. [c] Values reported in literature: **3**, Besada et al.;[14] **5**, El-Tayeb et al.;[8] **6** and **8**, Costanzi et al.;[13] **10**, Ko et al.[16] [d] **6**, MRS2633; **7**, MRS2795. [e] Structure of **8** given in Chart 1. [f] EC_{50} values[33] (μM) reported in activation of the $hP2Y_{14}$ receptor expressed in HEK-293 cells: **1**, 0.16 ± 0.04; **10**, 0.011 ± 0.005; **11**, 0.14 ± 0.07.

Table 2. Potencies of N^4-Alkyloxycytidine 5′-Diphosphate Derivatives for Activation of the Human $P2Y_2$, $P2Y_4$, and $P2Y_6$ Receptors[a]



12 - 16

X = H; Y = O, unless noted.

			EC_{50}, μM[b]		
compd	modification	structure	$hP2Y_2$	$hP2Y_4$	$hP2Y_6$
12[c]	N^4-OMe-CDP	Z = *N*-OCH_3	3.60 ± 1.08	6.45 ± 1.55	0.070 ± 0.007
13	N^4-OEt-CDP	Z = *N*-OCH_2CH_3	0.28 ± 0.015	0.18 ± 0.02	0.104 ± 0.023
14	N^4-O′Bu-CDP	Z = *N*-$OC(CH_3)_3$	3.51 ± 1.52	6.23 ± 1.88	2.55 ± 0.70
15	N^4-OBn-CDP	Z = *N*-OCH_2Ph	2.13 ± 0.64	1.15 ± 0.15	0.026 ± 0.002
16	α,β-CH_2-N^4-OMe-CDP	Y = CH_2, Z = *N*-OCH_3	> 10[d]	NE	0.678 ± 0.018

[a] ND: not determined. NE: no effect at 10 μM. [b] Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor. Potencies are presented in the form of EC_{50} values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as the mean ± standard error and are the average of three to six different experiments with each molecule. [c] EC_{50} value[33] reported in activation of the $hP2Y_{14}$ receptor expressed in HEK-293 cells: **12**, 3.32 ± 1.62 μM. [d] ≤50% effect at 10 μM.

standard phosphorylation methods using the unprotected nucleoside precursors.[23,24] Various 5′-diphosphate derivatives were then extended to form dinucleoside triphosphates or other 5′-triphosphate γ-esters (Scheme 2). (S)-Methanocarba nucleotides were synthesized as shown in Scheme 3. The synthesis of several of the pyrimidine nucleotides was reported in previous studies of the P2Y receptor (**3**, **5**, **6**, **8**, **10**, **19**, **21**, and **26**).[14−16]

5-Iodouridine **34** was available from commercial sources, while the 4-methoxyimino derivative **36** and (S)-methanocarbauridine **41** were prepared as described,[19,25] and compounds **37**−**39** were prepared by the same procedure as **36**. Compounds **34** and **36** were converted to the α,β-methylene diphosphonate analogues **11** and **16**, respectively, by a carbodiimide condensation. N^4-Alkoxycytidines **36**−**39** were converted to the corresponding 5′-diphosphates **12**−**15** and N^4-methoxycytidine **36** to the 5′-triphosphate **20** using phosphorus oxychloride followed by either phosphate or pyrophosphate.[24] Various 5′-diphosphates (**1**, **3**, **5**, **10**, and **12**) were converted to the corresponding 5′-triphosphate γ-esters by condensation with the appropriate monophosphate derivative in DMF.

Nucleotides **18** and **28** derived from (S)-methanocarbauridine **40** were prepared by similar phosphorylation of the unprotected nucleoside (Scheme 3).[24] The 3′,5′-cyclic-diphosphate **9** was isolated as a side product in the synthesis of **28**. A similar cyclic diphosphate was isolated following phosphorylation of other uridine analogues.[14]

Pharmacological Evaluation. The nucleotide analogues (Tables 1−3) were assayed for capacity to promote activation of phospholipase C (PLC) in 1321N1 human astrocytoma cells stably expressing the human $P2Y_6$ receptor.[26] Receptor selectivity of the novel analogues of CDP and UTP in Tables 2 and 3 was assessed by quantification of PLC activation in 1321N1 cells stably expressing either the $P2Y_2$ receptor or the $P2Y_4$ receptor. Previously reported data for several analogues (**2**−**6**, **8**, and **10**) were included for comparison.

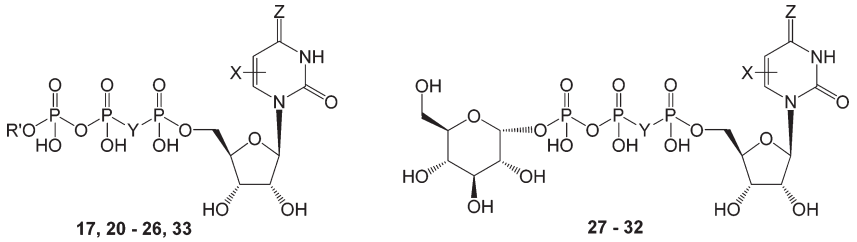
The enantiomerically pure (S)-methanocarba-UDP **7** was more potent at the $P2Y_6$ receptor than either UDP **1** (7-fold) or the previously reported (S)-methanocarba-2′-deoxy-UDP **6** (5-fold). In contrast, the North (N)-methanocarba conformationally locked UDP analogue **8** was previously found to be inactive at the $P2Y_6$ receptor, confirming a strong conformational preferrence in $P2Y_6$ receptor recognition.[13,14] The (S)-methanocarbauridine 3′,5′-cyclic-diphosphate **9** was also inactive at the $P2Y_6$ receptor.

The carbon-bridged analogue α,β-methylene-UDP **10** was recently reported to be nearly as potent as UDP at the $P2Y_6$ receptor.[16] Compound **11** contained the 5′-α,β-methylene-diphosphonate modification, which would increase the stability of a nucleotide in biological systems, and the 5-iodo modification (as in **3**), both of which were previously noted to preserve potency at the $P2Y_6$ receptor.[14] This combination of modifications resulted in a molecule **11** that potently activated the $P2Y_6$ receptor with an EC_{50} value of 127 nM.

Introduction of the 4-methoxyimino modification of UDP, i.e., N^4-methoxy-CDP **12**, resulted in high potency at the $P2Y_6$ receptor (EC_{50} = 70 nM), which represents a 4-fold gain of potency over UDP (Table 2, Figure 1A). Compound **12** was 47-fold selective for activation of PLC via the $P2Y_6$ receptor in comparison to inhibition of cyclic AMP accumulation in HEK-293 cells expressing the Gi-coupled human $P2Y_{14}$ receptor.[18] The native agonist for $P2Y_{14}$, UDP-glucose, did not tolerate the 4-methoxyimino substitution.[27] However, the 4-methoxyimino modification of the pyrimidine ring in the 5′-triphosphate derivative **20** produced a gain of potency (EC_{50} = 80 nM) of >100-fold over UTP **17** at the $P2Y_6$ receptor (Table 3). The 4-methoxyimino modification of **20** was also well-tolerated at the $P2Y_2$ and $P2Y_4$ receptors, with EC_{50} values of 50 nM. Thus, compound **20** was a nonselective agonist at these three P2Y receptor subtypes.

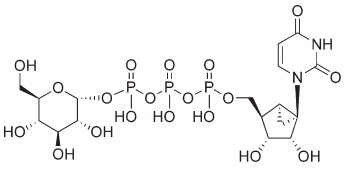
In the series of 4-alkyloxyimino 5′-diphosphate analogues, the progression from MeO to larger *O*-alkyl substituents, **12**−**15**, first decreased then increased the $P2Y_6$ receptor potency. The sterically bulky *O*-*tert*-butyl group of **14** was not well tolerated. However, the *O*-benzyl analogue **15** was

Table 3. Potencies of Nucleotide (5′-Triphosphate) Derivatives for Activation of the Human $P2Y_2$, $P2Y_4$, and $P2Y_6$ Receptors[a]



compd	modification	structure	EC_{50}, μM[b]		
			$hP2Y_2$	$hP2Y_4$	$hP2Y_6$
17	UTP		0.060 ± 0.00	0.090 ± 0.01	> 10[e]
18	(S)-methanocarba-UTP		0.08 ± 0.02	0.30 ± 0.10	1.37 ± 0.05
19[c]	(N)-methanocarba-UTP		0.016 ± 0.01	0.085 ± 0.005	NE
20	N^4-OMe-CTP	Z = *N*-OCH_3	0.05 ± 0.00	0.05 ± 0.02	0.08 ± 0.02
21[c]	Up_3U	R′ = [5′]uridine	1.31 ± 0.21	0.87 ± 0.11	0.27 ± 0.07
22	α,β-CH_2-Up_3U	R′ = [5′]uridine, Y = CH_2	> 10[e]	NE	1.30 ± 0.18
23	N^4-OMe-Cp_3U	Z = *N*-OCH_3 R′ = [5′]uridine	0.17 ± 0.04	0.79 ± 0.12	0.012 ± 0.0024
24	N^4-OMe-Cp_3(N^4-OMe)C	Z = *N*-OCH_3 R′ = [5′](N^4-methoxy)cytidine	2.12 ± 0.66	0.56 ± 0.04	0.063 ± 0.002
25	Up_3-*O*-cyclohexyl	R′ = cyclohexyl	2.28 ± 0.1	NE	6.99 ± 0.18
26	Up_3-*O*-phenyl	R′ = phenyl	> 10[e]	NE	2.13 ± 0.18
27	Up_3-gluc		NE	NE	> 10[e]
28[d]	(S)-methanocarba-Up_3-gluc		NE	NE	2.47 ± 0.39
29	5-I-Up_3-gluc	X = 5-I	NE	NE	4.69 ± 1.68
30	3-phenacyl-Up_3-gluc	X = 3-Ph-$COCH_2$	NE	NE	> 10[e]
31	α,β-CH_2-Up_3-gluc	Y = CH_2	NE	NE	> 10[e]
32	N^4-OMe-Cp_3-gluc	Z = *N*-OCH_3	> 10[e]	NE	0.18 ± 0.04
33	N^4-OMe-Cp_3-*O*-Ph	R′ = phenyl, Z = *N*-OCH_3	> 10[e]	NE	3.49 ± 1.63

[a] NE: no effect at 10 μM. ND: not determined. [b] Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor. Potencies are presented in the form of EC_{50} values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as the mean ± standard error and are the average of three to six different experiments with each molecule. [c] Values reported in literature: **19**, Kim et al.;[26] **21**, Ko et al.[16] [d] Structure of **28**:



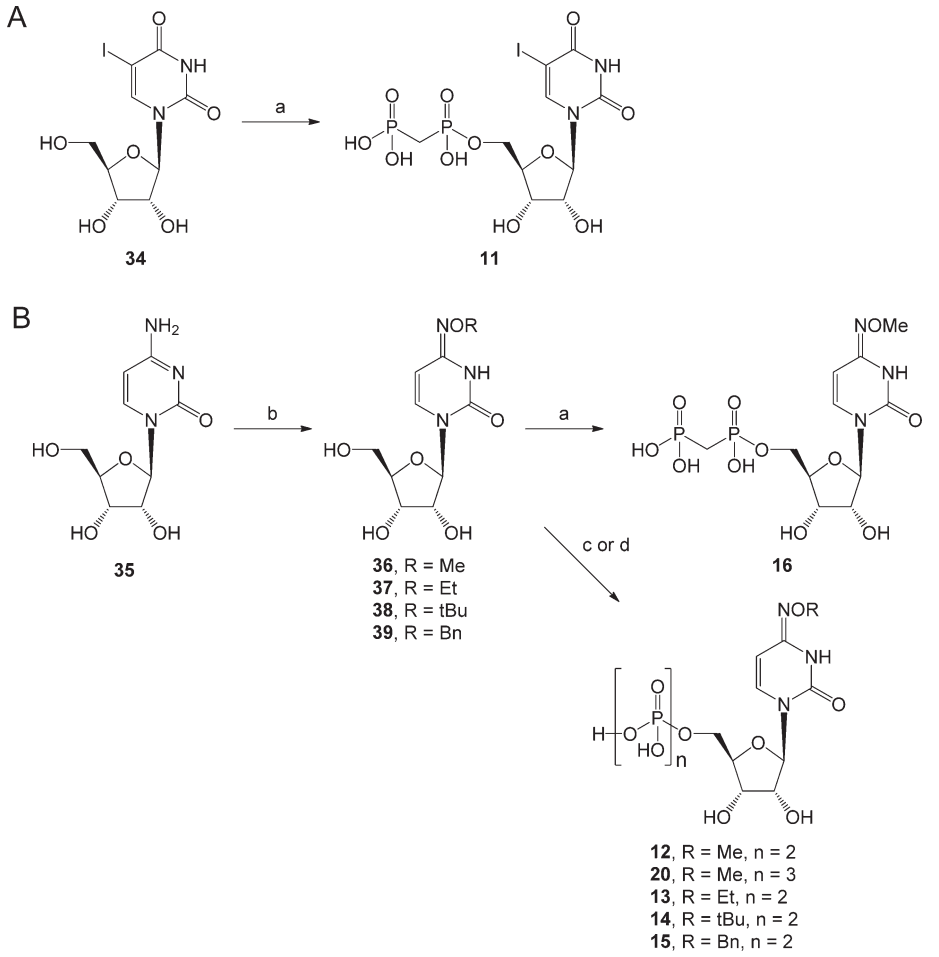
[e] ≤50% effect at 10 μM.

the most potent $P2Y_6$ receptor agonist in the series of 4-imino 5′-diphosphate analogues with 82- and 44-fold selectivity vs $P2Y_2$ and $P2Y_4$ receptors, respectively. Curiously, the *O*-ethyl analogue **13** was potent at $P2Y_2$ and $P2Y_4$ receptors and was thus a nonselective $hP2Y_{2/4/6}$ agonist. The potency-enhancing 4-methoxyimino modification was combined with α,β-methylene substitutions in compound **16**. However, there was no enhancement by the 4-methoxyimino modification, as **10** and **16** were equipotent at the $P2Y_6$ receptor.

Up_3U **21** slightly favored activation of the $P2Y_6$ receptor, in comparison to the $P2Y_2$ and $P2Y_4$ receptors, with an EC_{50} value of 270 nM.[16] Introduction of a carbon bridge in the corresponding α,β-methylene analogue **22** reduced $P2Y_6$ receptor potency 5-fold but with increased selectivity. Given the $P2Y_6$ receptor potency of extended phosphate derivatives, such as dinucleoside triphosphates related to Up_3U **21** (including **4**), we tested other γ-phosphoesters of UTP for $P2Y_6$ receptor activity, including alkyl and aryl esters and Up_3-sugar derivatives. The analogue of Up_3U containing a single 4-methoxyimino modification **23** displayed 22-fold enhanced potency at the $P2Y_6$ receptor with an EC_{50} value of 12 nM and selectivity versus the $P2Y_2$ and $P2Y_4$ receptors of 14- and 66-fold, respectively. The γ-cyclohexyl **25** and γ-phenyl **26** esters of UTP displayed moderate potency at the $P2Y_6$ receptor, with EC_{50} values of 6.99 and 2.13 μM, respectively. However, Up_3-[1]glucose **27** only weakly activated the $P2Y_6$ receptor, and an EC_{50} value was not determined. The introduction of the potency enhancing (S)-methanocarba modification in the corresponding ring-constrained analogue **28** increased potency, as predicted. Nevertheless, only a moderate potency of 2.47 μM was attained.

Combinations of Up_3-glucose with other known modifications that are tolerated or favored at the $P2Y_6$ receptor were evaluated. Introduction of the 5-iodo modification in **29** only slightly enhanced potency in comparison to **27**. The corresponding 3-phenacyl **30** and α,β-methylene **31** analogues were only weakly active at the $P2Y_6$ receptor. However, the $P2Y_6$ receptor potency of the corresponding N^4-methoxycytidine

Scheme 1. Synthesis of Various Pyrimidine Ribonucleoside 5′-Diphosphate and Triphosphate Derivatives[a]



[a] Reagents and conditions: (a) DCC, methylene bisphosphonate, rt, overnight, 11% for **11**, 48% for **16**; (b) $RONH_2$, pyridine, 90 °C, overnight, 55−100%; (c) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C, 2 h, followed by tributylammonium phosphate, rt, 0.5 h (for **12**−**15**), 14−47%; (d) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C, 2 h, followed by tributylammonium pyrophosphate, rt, 0.5 h, 31% (for **20**).

analogue **32** was greatly increased with an EC_{50} value of 180 nM, which also displayed > 100-fold selectivity versus $P2Y_2$ and $P2Y_4$ receptors. This finding suggested another analogue, N^4-methoxycytidine 5′-triphosphate γ-phenyl ester **33**, but an additive effect on potency was absent, as it weakly activated the $P2Y_6$ receptor (EC_{50} = 3.49 nM).

Selected nucleotide analogues were also examined for the ability to raise levels of intracellular calcium ions in 1321N1 human astrocytoma cells expressing the human $P2Y_6$ receptor (Figure 1B). The rank order of potency was **23**, **3** > **21**, **24**, which was similar to the order of potency in PLC activation.

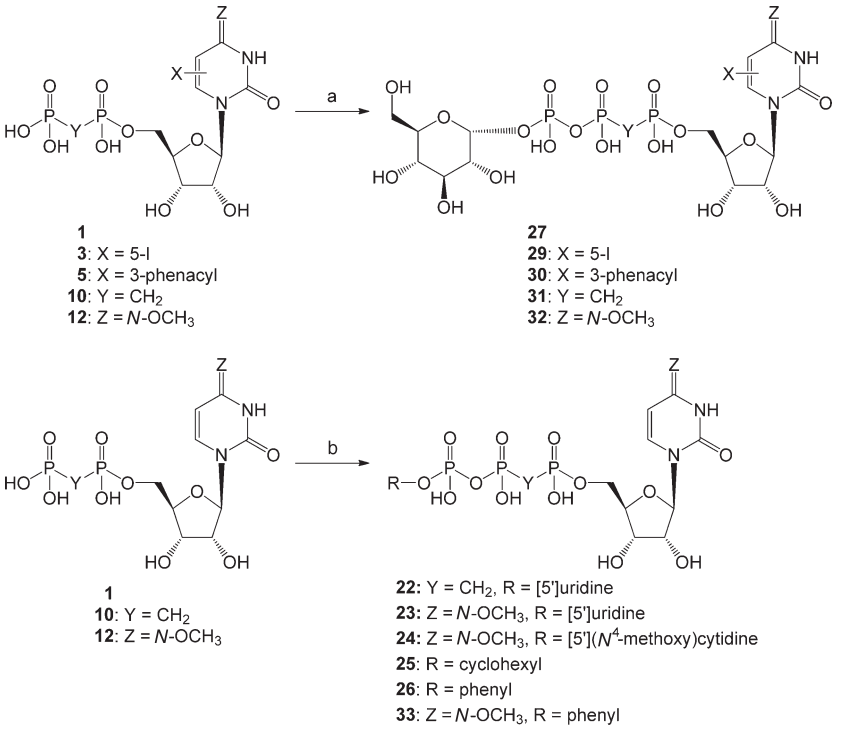
Stability of Nucleotide Derivatives. The chemical and enzymatic instability of nucleotide analogues in biological systems is a major limitation in their use as pharmacological probes. Therefore, to assess their potential for broad use, selected nucleotide derivatives were evaluated for stability during prolonged exposure to two different conditions. The derivatives were incubated at 37 °C in aqueous medium at low pH or in the presence of 1321N1 astrocytoma membranes, which are known to contain ectonucleotidases, as a representative mammalian cell of the central nervous system. At regular intervals aliquots were taken for HPLC analysis (Table 4 and Supporting Information). The injection sample was prediluted with 5 mM tetrabutylammonium dihydrogen phosphate (TBAP) to allow complete equilibration with the mobile phase in order to avoid the early HPLC elution at around 1 min.

The stability in the presence of cell membranes was **25**, **22** > **12** > **21**, **26** > **17**, **27** > **15**, **23** > **31**. The potent $P2Y_6$ receptor agonist **12** was more stable than the related N^4-benzyloxycytidine 5′-diphosphate **15**. The γ-cyclohexyl ester **25** was more stable than either Up_3U **21**, which has a uridyl group at this terminal position, or the γ-phenyl ester **26**. The α,β-methylene analogue of Up_3U **22** was more stable than the parent Up_3U **21** in the presence of cell membranes, which may indicate that a α,β-methylene bridge blocks enzymatic breakdown, as has been observed for other P2 receptor agonists containing phosphonates.[16,28,29] However, the introduction of a methylene bridge into Up_3U unfortunately also reduced potency at the $P2Y_6$ receptor 5-fold.

In acidic solution, the CDP derivatives **12** and **15** and the dinucleoside triphosphates were of enhanced chemical stability. Also, the γ-phenyl phosphoester **26** was relatively stable. However, the Up_3U analogue **22** containing a α,β-methylene bridge was significantly less stable than the parent Up_3U **21**. Likewise, the Up_3-[1]glucose analogue **31** containing a α,β-methylene bridge was less stable than the parent Up_3-[1]glucose **27**, which itself was very labile. Since both of the glucose derivatives **27** and **31** were rapidly degraded, the introduction of glucose was not a favored modification.

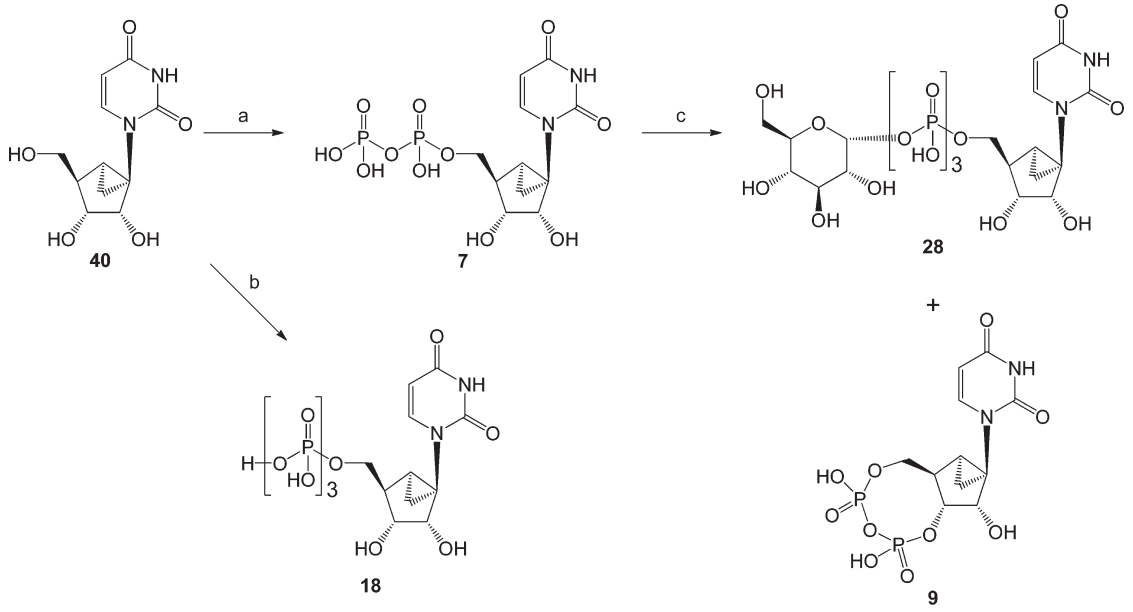
Molecular Modeling. A docking of the semirigid (S)-methanocarba analogue **7** in complex with our rhodopsin-based $P2Y_6$ receptor model[13] (Figure 2A) suggested that **7**

Scheme 2. Synthesis of Various Pyrimidine Ribonucleoside 5′-Triphosphates and Dinucleoside Triphosphates[a]



[a] Reagents and conditions: (a) DIC, $MgCl_2$, glucose-1-monophosphate tributylammonium salt, DMF, rt, overnight, 9−80%; (b) DIC, $MgCl_2$, respective monophosphate tributylammonium salt, e.g., uridine 5′-monophosphate, **35**, cyclohexylmonophosphate[16] or phenylmonophosphate, in DMF, rt, overnight, 9−80%. Unless otherwise noted, X = H and Y, Z = O.

Scheme 3. Synthesis of Uracil Ribonucleoside Phosphate Derivatives Constrained with a (S)-Methanocarba Ring[a]



[a] Reagents and conditions: (a) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C, 2 h; then tributylammonium phosphate, rt, 0.5 h, 45%; (b) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C, 2 h; then tributylammonium pyrophosphate, rt, 0.5 h, 56%; (c) CDI, DMF, rt, 1 h, then glucose-1-monophosphate tributylammonium salt, overnight, 23% for **28**, 51% for **9**.

establishes with the receptor the same putative interactions characteristic of UDP. In particular, three cationic residues located in transmembrane domain 3 (TM3), TM6, and TM7 coordinate the diphosphate of **7**, namely, R103(3.29), K259(6.55), and R287(7.39), while the base is coordinated by Y33(1.39) and S291(7.43), located in TM1 and TM7, respectively (see Experimental Section for residue indexing). The higher potency of **7** compared to UDP is induced by the rigid methanocarba scaffold, which constrains the ribose in the bioactive conformation, likely with a consequent gain in the entropic component of the binding affinity. A hydrogen bond is possibly formed between the ribose 2′-hydroxy group and the uracil 2-oxo group of **7** (Figure 2A). Furthermore, as shown in Figure 2A, the molecular dynamics simulation

predicted the access of several water molecules to the receptor binding pocket from the extracellular milieu. These molecules surround the portion of the ligand exposed to the extracellular opening of the binding site, including the 5 position of the uracil ring. For entropic reasons, substituents capable of displacing these water molecules from the binding cavity to the bulk of the solvent are expected to enhance the affinity of the compounds. The increase in affinity noted upon the introduction of the iodo-substituent at the 5-position of the uracil ring (see compounds **1** and **3**) is consistent with this reasoning and supports the orientation of this moiety of the ligand toward the extracellular opening of the helical bundle of the receptor as suggested by our models.

A molecular model of N^4-methoxy-CDP **12** docked in the $P2Y_6$ receptor showed similar coordination to that of UDP; i.e., S291(7.43) coordinates the base, and four cationic residues coordinate the diphosphate moiety (Figure 2B). The methyl group at the 4 position seems to fit well in a sterically restricted hydrophobic pocket, which consisted of V32(1.38), Y33(1.39), I83(2.61), and P288(7.40). These hydrophobic interactions may be the reason for the increased activity of **12** (Table 2).

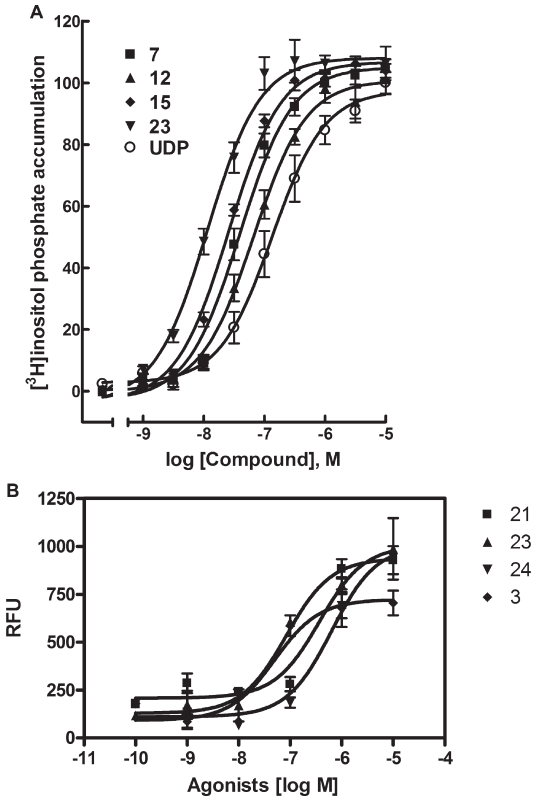


Figure 1. (A) Activity of agonists **7**, **12**, **15**, and **23** at the human $P2Y_6$ receptor as indicated by activation of PLC in stably transfected 1321N1 human astrocytoma cells. The effect of UDP corresponds to 100%. (B) Mobilization of intracellular calcium in astrocytoma cells expressing the human $P2Y_6$ receptor (EC_{50} values in nM) by nucleotide agonists **3** (50), **21** (370), **23** (83), and **24** (630). The EC_{50} value of **1** was 200 nM.

Discussion

The present set of analogues provides new insights into the structural requirements for activation of the $P2Y_6$ receptor at each of the three regions modified: ribose, nucleobase, and terminal phosphate. In addition to studying the SAR of pyrimidine nucleotides in receptor activation, we have also confirmed that blockade of the terminal phosphate with an alkyl or aryl group or other groups may increase its stability.

Using sterically constrained carbocyclics, we have extended to the ribo (2′-hydroxy) series the prediction that the (S)-conformation of a ribose-like moiety is preferred for recognition at the human $P2Y_6$ receptor, consistent with previous activity of the 2′-deoxy analogues. The first synthesis of enantiomerically pure (S)-methanocarbauridine enabled the preparation of nucleotide derivatives in the 2′-hydroxy series (**7**, **18**, and **28**). These (S)-methanocarba nucleotides exhibited enhanced potency over the corresponding natural ribose analogue in each case.

In homology-based modeling of the $P2Y_6$ receptor and ligand docking, an intramolecular hydrogen bond was detected between the 2′-hydroxy group and the uracil 2-oxo group of the conformationally constrained, potent analogue of UDP **7**. We suggest that this interaction might further restrain **7** in its bioactive conformation and thus contribute to the greater potency of **7** over the corresponding 2′-deoxy analogue **6**.

One of the striking findings of this study was that the 4-methoxyimino and 4-benzyloxyimino modifications of the pyrimidine ring in the appropriate nucleotide derivative served to enhance potency at the $P2Y_6$ receptor. This is one of the few modifications of the uracil ring that is tolerated for activation of the $P2Y_6$ receptor. CDP itself is nearly inactive at the human $P2Y_6$ receptor, and its EC_{50} at the $P2Y_{14}$ receptor is $> 10\ \mu M$.[17,30] The 4-methoxyimino 5′-diphosphate analogue **12** displayed a selectivity of 51- and 47-fold versus the $P2Y_2$ and $P2Y_{14}$ receptors, respectively, and a substituent as large as *O*-benzyl **15** was tolerated. In the 5′-triphosphate analogue **20**, the 4-methoxyimino modification also preserved potency at $P2Y_2$ and $P2Y_4$ receptors and abolished potency at the $P2Y_{14}$ receptor.[27] In Schemes 1 and 2, the imino group of N^4-methoxycytidine is depicted as the iminooxo tautomer, based on an early study of the poly-C derivative after treatment with

Table 4. Half-Lives of Nucleotide (5′-Triphosphate) Derivatives When Incubated at 37°C in Acidic Aqueous Solution or in the Presence of Mammalian (1321N1 Human Astrocytoma) Cell Membranes, Determined by HPLC

compd	$t_{1/2}$, h (pH 1.2)	$t_{1/2}$, h (membranes)	pharmacological properties at $hP2Y_6$ receptor
12, N^4-MeO-CDP	116	55	selective, potent agonist
15, N^4-BnO-CDP	126	26	selective, potent agonist
17, UTP	23	31	~inactive
21, Up_3U	218	42	moderately potent agonist, only 4- to 5-fold selective
22, α,β-me-Up_3U	34	62	weak agonist, selective
23, N^4-MeO-Cp_3U	120	23	selective, potent agonist
25, Up_3-*O*-cyclohexyl	193	62	weak agonist, nonselective vs $P2Y_2$ receptor
26, Up_3-*O*-Ph	164	43	weak agonist, selective
27, Up_3-gluc	5.1	29	~inactive
31, α,β-Me-Up_3-gluc	2.8	19	~inactive

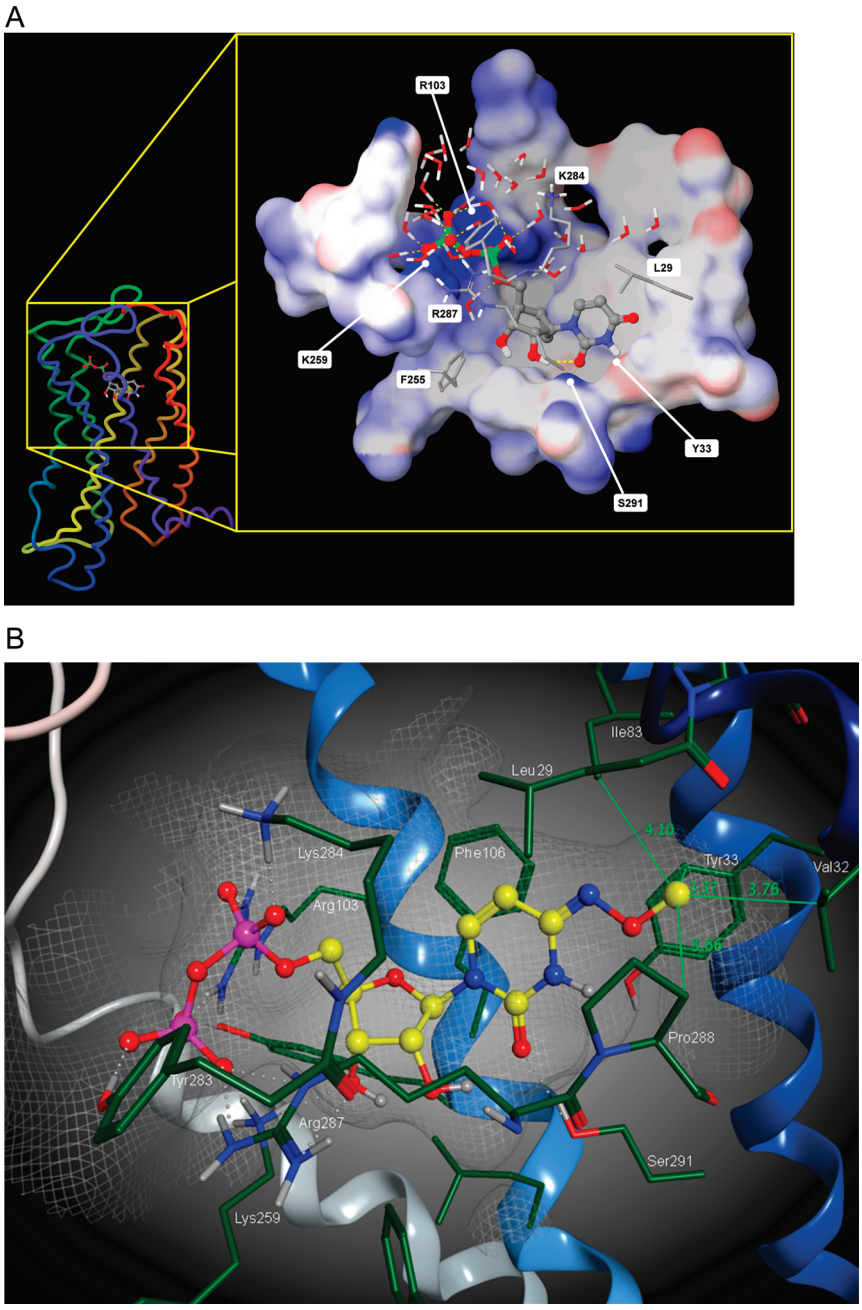


Figure 2. Molecular modeling of the human $P2Y_6$ receptor. (A) Details of the nucleotide binding site of the receptor complexed with the agonist **7** as obtained after a fully flexible Monte Carlo conformational search. The ligand is represented as balls and sticks, colored by element. The binding pocket is represented as a van der Waals surface colored according to the electrostatic potential, with positive charges in blue and negative charges in red. For clarity, the residues located in front of the ligand are represented as sticks, colored by element. Labels indicate all of the residues represented as sticks (adjacent), and the key residues are represented as van der Waals surfaces (trough arrows). A schematic representation of the receptor−ligand complex is given in the lower left inset. In the tube representations the receptor is colored according to residue positions, with colors that range from red (N-terminus) to purple (C-terminus): TM1 is in orange, TM2 in ochre, TM3 in yellow, TM4 in green, TM5 in cyan, TM6 in blue, TM7 in purple. (B) Docking of the potent agonist N^4-methoxycytidine 5′-diphosphate **12**. A hydrophobic binding pocket for the N^4-alkyloxy substituent is defined in this docking model. Distances in Å from the methoxy carbon atom to neighboring amino acid residues are shown.

methoxyamine.[31] This predominant form, which resembles the major lactam (keto) tautomer of UDP, was used in the molecular modeling. However, the amino tautomeric form, which is found in native cytosine in DNA, might also be present. The 4-methoxyimino group in a γ-[1]glucose triphosphate ester **32** also enhanced potency. However, the γ-[1]glucose triphosphate in another analogue **27** suffered from low stability, and therefore, **32** is not a likely candidate for generation of a potent and stable agonist.

The introduction of a phenyl or cyclohexyl ring at the terminal phosphate of UTP in compounds **25** and **26**, respectively, enhanced stability in both acidic solution and in the presence of membranes to at least the same extent as Up_3U. Both **25** and **26** containing terminal hydrophobic groups showed higher potency than UTP **17** at the $P2Y_6$ receptor, and the more hydrophilic Up_3-[1]glucose **27** was nearly inactive. Thus, the nature of the terminal group can greatly affect agonist activity. Since Up_4-[1]glucose is a potent agonist of the

$P2Y_2$ receptor and since Up_2-[1]glucose is a native agonist of the $P2Y_{14}$ receptor, we speculated that the intermediate triphosphate homologue **27** might be an effective agonist of the $P2Y_6$ receptor. However, this receptor is not as tolerant of a terminal glucose moiety as are the $P2Y_2$ and $P2Y_{14}$ receptors. Nevertheless, the introduction of a rigid (S)-methanocarba ring system in **18** and **28** resulted in agonists that exceeded the $P2Y_6$ potency of both UTP and Up_3-[1]glucose.

The combination of two modifications in the present set of compounds either produced an additive effect on $P2Y_6$ receptor potency, as in γ-[1]glucose triphosphate ester **32**, or failed to increase potency, as in the γ-phenyl ester **33** and the α,β-methylene analogue **16**. The inconsistent effects of combination of various structural modifications on receptor recognition suggested multiple modes of binding of these nucleotide series.

The homology modeling and docking, interpreted in light of the biological activity, indicate conformational cross-talk between the nucleobase and the terminal phosphate substituent. Although the molecular model of N^4-methoxy-CDP **12** docked in the $P2Y_6$ receptor showed coordination similar to that of UDP, Up_3-*O*-phenyl **26** docked a different mode (not shown). The base of **26** did not coordinate with Y33(1.39) and S291(7.43) because the position of the base had shifted within the binding site. Also, the ribose ring showed a planar rather than a (S)-conformation. The terminal phenyl group of **26** seemed to fit in a hydrophobic region in the model, which might be the reason for the dislocation of the nucleobase, i.e., by withdrawal of the entire molecule. On the other hand, the docked model of N^4-methoxy-Cp_3-*O*-phenyl **33** in the receptor showed a position and coordination similar to N^4-methoxy-CDP **12**, but the phenyl ring did not interact with the distal hydrophobic region occupied by **26**. The strong interaction of the methyl group of **33** in a hydrophobic pocket near the nucleobase may cause the phenyl group to exit its large hydrophobic binding region. This interpretation is consistent with the lack of enhanced activity of compound **33** in comparison to Up_3-*O*-phenyl **26**, despite the presence of methoxyimino moiety at the 4 position.

The modeling and receptor docking also predicted that various water molecules are located in proximity of the 5 position of the ligand. The enhanced affinity of the 5-iodo-substituted analogues could be a result of the fact that the halogen displaced some of the structured water molecules present in the binding site, with a consequent entropic gain.

Conclusions

We have identified novel nucleotides, such as N^4-alkoxycytidine derivatives, that are more potent, selective, and potentially more stable in vivo than both the native agonist and previously reported synthetic agonists of the $P2Y_6$ receptor. Thus, N^4-methoxycytidine nucleotides **12**, **15**, and **23** were relatively potent and selective agonists, and moreover, compound **23** displayed favorable stability. Thus, in future SAR studies (S)-methanocarba-(α,β-CH_2)-N^4-OMe-CDP could be a suitable combination to maximize the potency while retaining selectivity and stability.

The $P2Y_6$ receptor is implicated in neurodegeneration, osteoporosis, ischemic effects in skeletal muscle, and possibly diabetes.[4,32,33] Therefore, new potent and selective ligands are of interest in the search for new treatments for these conditions. Agonists of the $P2Y_6$ receptor are predicted to produce beneficial responses in, for example, muscle wasting and possibly neurodegeneration, and several compounds such as **23** from the present SAR analysis could provide useful pharmacological tools.

Experimental Section

Synthesis. ^{1}H NMR spectra were obtained with a Varian Gemini 300 or Varian Mercury 400 spectrometer using D_2O, $CDCl_3$, or DMSO-d_6 as a solvent. The chemical shifts are expressed as relative ppm from HOD (4.80 ppm). ^{31}P NMR spectra were recorded at room temperature (rt) by use of Varian XL 300 (121.42 MHz) or Varian Mercury 400 (162.10 MHz) spectrometers; orthophosphoric acid (85%) was used as an external standard. In several cases the signal of the terminal phosphate moiety was not visible because of high dilution.

Purity of compounds was checked using a Hewlett-Packard 1100 HPLC instrument equipped with a Zorbax Eclipse 5 μm XDB-C18 analytical column (250 mm × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). For system A, the mobile phase was a linear gradient solvent system of 5 mM TBAP−CH_3CN from 80:20 to 40:60 in 20 min; the flow rate was 1 mL/min or Zorbax SB-Aq 5 μm analytical column (50 mm × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). For system B, the mobile phase was a linear gradient solvent system of 5 mM TBAP−CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min. Peaks were detected by UV absorption with a diode array detector at 254, 275, and 280 nm. All derivatives tested for biological activity showed >99% purity by HPLC analysis (detection at 254 nm).

High-resolution mass measurements were performed on a Micromass/Waters LCT Premier electrospray time of flight (TOF) mass spectrometer coupled with a Waters HPLC system unless otherwise noted. Purification of the nucleotide analogues for biological testing was carried out on (diethylamino)ethyl (DEAE)-A25 Sephadex columns with a linear gradient (0.01−0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase. Compounds **7** were purified by Sephadex alone (and isolated in the ammonium salt form), and all other compounds were purified by HPLC with a Luna 5 μm RP-C18(2) semipreparative column (250 mm × 10.0 mm; Phenomenex, Torrance, CA) and using the following conditions: flow rate of 2 mL/min; 10 mM triethylammonium acetate (TEAA)−CH_3CN from 100:0 to 95:5 (or up to 100:0 to 75:25) in 20−40 min (and isolated in the triethylammonium salt form).

All reagents were of analytical grade. 3-Phenacyl-UDP **5** was purchased from Tocris. 5-Iodouridine **34** was purchased from Berry Associates (Ann Arbor, MI). Compound **36** was synthesized as reported.[19,25] Other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO).

(S)-Methanocarbauridine 5′-Diphosphate Triethylammonium Salt (7). A solution of the (S)-methanocarbauridine **40** (1-[(1*S*,2*S*,5*S*,3*R*,4*R*)-2,3-dihydroxy-4-(hydroxymethyl)bicyclo-[3.1.0]hexyl]-1,3-dihydropyrimidine-2,4-dione, 10 mg, 0.04 mmol, ref 13) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then phosphorus oxychloride (0.008 mL, 0.08 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.8 mL, 0.35 mmol) and a solution 0.35 M of the bis-(tributylammonium) salt of phosphoric acid in DMF (0.7 mL) was added at once. After 10 min, 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **7** (1.3 mg, 45%) as a white solid. ^{1}H NMR (D_2O) δ 7.79 (d, J = 7.8 Hz, 1H), 5.86 (d, J = 8.1 Hz, 1H), 4.69 (m, 1H), 4.15 (m, 3H), 2.39 (t, J = 6.3 Hz, 1H), 1.87 (m, 1H), 1.71 (t, J = 6.0 Hz, 1H), 1.30 (ddd, J = 2.1, 6.3, 8.4 Hz, 1H); ^{31}P NMR (D_2O) δ −5.87 (d, J = 22.0 Hz), −10.10 (d, J = 23.2 Hz); HRMS-EI found 413.0170 $(M - H^+)^-$. $C_{11}H_{15}N_2O_{11}P_2$ requires 413.01511; purity >99% by HPLC (system A, 11.1 min).

5-Iodouridine 5′-α,β-Methylenediphosphate Triethylammonium Salt (11). Methylene diphosphonic acid (14 mg, 0.08 mmol) and DCC (25 mg, 0.121 mmol) were added to a solution of 5-iodouridine **34** (15 mg, 0.040 mmol) in DMF (1.0 mL). The mixture was stirred for 2 days at rt under a nitrogen atmosphere. Solvent was evaporated and the residue was purified on Sephadex-DEAE A-25 resin and HPLC using the same method as the general procedure, to give the compound **11** (2 mg, 11%) as a white powder. ^{1}H NMR (D_2O) δ 8.26 (s, 1H), 5.98 (d, J = 6.1 Hz, 1H), 4.39−4.46 (m, 2H), 4.29−4.34 (m, 1H), 4.19−4.24 (m, 2H), 2.23 (t, J = 19.1 Hz, 2H); ^{31}P NMR (D_2O) δ 19.02 (d, J = 8.34 Hz), 15.04 (d, J = 8.34 Hz); HRMS-EI found 526.9108 (M − H). $C_{10}H_{15}IN_2O_{11}P_2$ requires 527.9109.

***N*^{4}-Methoxycytidine 5′-Diphosphate Ammonium Salt (12).** A solution of the N^4-methoxycytidine **36** (10 mg, 0.037 mmol) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then phosphorus oxychloride (0.008 mL, 0.08 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.8 mL, 0.35 mmol) and a solution 0.35 M of the bis(tributylammonium) salt of phosphoric acid in DMF (0.7 mL) was added at once. After 10 min 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **12** (4.4 mg, 25%) as a white solid. ^{1}H NMR (D_2O) δ 7.23 (d, J = 8.1 Hz, 1H), 5.92 (d, J = 5.7 Hz, 1H), 5.79 (d, J = 7.8 Hz, 1H), 4.35 (m, 2H), 4.21 (m, 1H), 4.15 (m, 2H), 3.78 (s, 3H); ^{31}P NMR (D_2O) δ −7.74 (d, J = 21.4 Hz), −10.6 (d, J = 21.4 Hz); HRMS-EI found 432.0207 $(M - H^+)^-$. $C_{10}H_{16}N_3O_{12}P_2$ requires 432.0209; purity >98% by HPLC (system A, 13.6 min).

***N*^{4}-Ethoxycytidine 5′-Diphosphate Ammonium Salt (13).** A solution of the cytidine **35** (200 mg, 0.82 mmol) and *O*-ethylhydroxylamine hydrochloride (160 mg, 1.64 mmol) in pyridine (2 mL) was stirred overnight at 100 °C. The reaction mixture was concentrated, and the residue was purified on a silica gel column ($CHCl_3$/MeOH = 10/1) to obtain compound **37** (230 mg, 97%) as a yellow amorphous solid. ^{1}H NMR (acetone-d_6) δ 7.32 (d, J = 8.2 Hz, 1H), 5.86 (d, J = 5.1 Hz, 1H), 5.62 (d, J = 8.2 Hz, 1H), 4.27−4.21 (m, 2H), 4.07−3.95 (m, 3H), 3.79 (dd, J = 12.0, 2.8 Hz, 1H), 3.73 (dd, J = 12.0, 2.8 Hz, 1H), 1.22 (t, J = 7.0 Hz, 3H); HRMS-EI found 288.1196 $(M + H^+)^-$. $C_{11}H_{18}N_3O_6$ requires 288.1185

A solution of the N^4-ethoxycytidine **37** (85 mg, 0.30 mmol) and Proton Sponge (95 mg, 0.44 mmol) in trimethyl phosphate (2.5 mL) was stirred for 10 min at 0 °C. Then phosphorus oxychloride (0.054 mL, 0.59 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.56 mL, 2.4 mmol) and a 0.35 M solution of the bis(tributylammonium) salt of phosphoric acid in DMF (6.6 mL) was added at once. After 10 min 0.2 M triethylammonium bicarbonate solution (6.9 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to obtain compound **13** (21.3 mg, 14%) as a white amorphous solid. ^{1}H NMR (D_2O) δ 7.24 (d, J = 8.2 Hz, 1H), 5.96 (d, J = 5.6 Hz, 1H), 5.82 (d, J = 8.2 Hz, 1H), 4.42−4.33 (m, 2H), 4.26−4.14 (m, 3H), 4.05 (dd, J = 14.1, 7.0 Hz, 2H); HRMS-EI found 446.0366 $(M - H^+)^-$. $C_{10}H_{16}N_3O_{12}P_2$ requires 446.0373; purity >98% by HPLC (system B, 7.54 min).

***N*^{4}-*tert*-Butyloxycytidine 5′-Diphosphate Ammonium Salt (14).** A solution of the cytidine **35** (200 mg, 0.82 mmol) and *O*-*tert*-butylhydroxylamine hydrochloride (207 mg, 1.64 mmol) in pyridine (2 mL) was stirred overnight at 100 °C. The reaction mixture was concentrated, and the residue was purified on a silica gel column ($CHCl_3$/MeOH = 10/1) to obtain compound **38** (143 mg, 55%) as a white solid. ^{1}H NMR (acetone-d_6) δ 7.18 (d, J = 8.2 Hz, 1H), 5.85 (d, J = 5.7 Hz, 1H), 5.56 (d, J = 8.2 Hz, 1H), 4.27−4.17 (m, 2H), 3.95 (dd, 1H, J = 6.1, 3.0 Hz), 3.80−3.68 (m, 2H), 1.26 (s, 9H); HRMS-EI found 316.1509 $(M + H^+)^-$. $C_{13}H_{22}N_3O_6$ requires 316.1516.

A solution of the N^4-*tert*-butyloxycytidine **38** (39 mg, 0.12 mmol) and Proton Sponge (40 mg, 0.19 mmol) in trimethyl phosphate (1.2 mL) was stirred for 10 min at 0 °C. Then phosphorus oxychloride (0.023 mL, 0.25 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.24 mL, 1.0 mmol) and a 0.35 M solution of the bis(tributylammonium) salt of phosphoric acid in DMF (2.8 mL) was added at once. After 10 min 0.2 M triethylammonium bicarbonate solution (3 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to obtain compound **14** (12.5 mg, 21%) as a white amorphous solid. ^{1}H NMR (D_2O) δ 7.12 (d, J = 8.3 Hz, 1H), 5.84 (d, J = 6.0 Hz, 1H), 5.74 (d, J = 8.3 Hz, 1H), 4.32−4.23 (m, 2H), 4.13−4.03 (m, 2H), 3.08 (dd, J = 14.7, 7.4 Hz, 12H), 1.18 (s, 9H), 1.16 (t, J = 7.3 Hz, 18H); HRMS-EI found 474.0679 $(M - H^+)^-$. $C_{13}H_{22}N_3O_{12}P_2$ requires 474.0669; purity >98% by HPLC (system B, 8.06 min).

***N*^{4}-Benzyloxycytidine 5′-Diphosphate Ammonium Salt (15).** A solution of the cytidine **35** (200 mg, 0.82 mmol) and *O*-benzylhydroxylamine hydrochloride (263 mg, 1.64 mmol) in pyridine (2 mL) was stirred overnight at 100 °C. The reaction mixture was concentrated, and the residue was purified on a silica gel column ($CHCl_3$/MeOH = 10/1) to obtain compound **39** (288 mg, 100%) as a pale-yellow amorphous product. ^{1}H NMR (acetone-d_6) δ 7.40−7.26 (m, 5H), 7.23 (d, J = 8.2 Hz, 1H), 5.85 (d, J = 5.6 Hz, 1H), 5.52 (d, J = 8.2 Hz, 1H), 5.00 (s, 2H), 4.27−4.18 (m, 2H), 3.96 (dd, J = 6.1, 3.0 Hz, 1H), 3.82−3.69 (m, 2H); HRMS-EI found 350.1352 $(M + H^+)^-$. $C_{16}H_{19}N_3O_6$ requires 350.1356.

A solution of the N^4-benzyloxycytidine **39** (80 mg, 0.23 mmol) and Proton Sponge (74 mg, 0.34 mmol) in trimethyl phosphate (2.4 mL) was stirred for 10 min at 0 °C. Then phosphorus oxychloride (0.042 mL, 0.46 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.43 mL, 1.83 mmol) and a 0.35 M solution of the bis(tributylammonium) salt of phosphoric acid in DMF (5.1 mL) was added at once. After 10 min 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to obtain compound **15** (54 mg, 47%) as a white amorphous solid. ^{1}H NMR (D_2O) δ 7.50−7.39 (m, 5H), 7.26 (d, J = 8.4 Hz, 1H), 5.95 (d, J = 5.9 Hz, 1H), 5.77 (d, J = 7.8 Hz, 1H), 5.07 (s, 2H), 4.46−4.43 (m, 1H), 4.37 (t, J = 5.7 Hz, 1H), 4.27−4.11 (m, 3H); ^{31}P NMR (D_2O) δ −7.33 (s), −11.04 (s), −11.18(s); HRMS-EI found 508.0522 $(M - H^+)^-$. $C_{16}H_{20}N_3O_{12}P_2$ requires 508.0507; purity >98% by HPLC (system B, 9.45 min).

***N*^{4}-Methoxycytidine 5′-α,β-Methylenediphosphate Triethylammonium Salt (16).** Methylene diphosphonic acid (13 mg, 0.07 mmol) and DCC (23 mg, 0.11 mmol) were added to a solution of N^4-methoxycytidine **36** (10 mg, 0.037 mmol) in DMF (1.0 mL). The mixture was stirred for 2 days at rt under a nitrogen atmosphere. Solvent was evaporated and the residue was purified on Sephadex-DEAE A-25 resin and HPLC using the same method as the general procedure, to give the compound **16** (7.5 mg, 48%) as a white powder. ^{1}H NMR (D_2O) δ 7.30 (d, J = 8.1 Hz, 1H), 5.97 (br, 1H), 5.84 (d, J = 8.1 Hz, 1H), 4.42−4.39 (m, 2H), 4.28−4.23 (m, 1H), 4.17−4.12 (m, 2H), 3.83 (s, 3H), 2.20 (t, J = 19.5 Hz, 2H); ^{31}P NMR (D_2O) δ 18.88 (br), 14.75 (br); HRMS-EI found 430.0425 $(M - H^+)^-$. $C_{11}H_{18}N_3O_{11}P_2$ requires 430.0417; purity >98% by HPLC (system B, 6.93 min).

(S)-Methanocarbauridine 5′-Triphosphate Triethylammonium Salt (18). A solution of (S)-methanocarbauridine **41** (10 mg, 0.04 mmol) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then phosphorus

oxychloride (0.008 mL, 0.08 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. Tributylammonium pyrophosphate (1.6 mol of $C_{12}H_{27}N$ per mol of $H_4P_2O_7$, 110 mg, 0.23 mmol) in DMF (0.3 mL) was added at once to the reaction mixture. After 10 min, 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **18** (2.0 mg, 56%) as a white solid. ^{1}H NMR (D_2O) δ 7.78 (d, J = 7.8 Hz, 1H), 5.85 (d, J = 8.1 Hz, 1H), 4.67 (m, 1H), 4.16 (m, 3H), 2.38 (t, J = 6.3 Hz, 1H), 1.86 (m, 1H), 1.71 (t, J = 6.0 Hz, 1H), 1.30 (ddd, J = 2.1, 6.3, 8.4 Hz, 1H); ^{31}P NMR (D_2O) δ −6.76 (m), −10.7 (d, J = 20.2 Hz), −22.63 (app t); HRMS-EI found 492.9847 $(M - H^+)^-$. $C_{11}H_{16}N_2O_{14}P_3$ requires 492.9814; purity >98% by HPLC (system A, 17.5 min).

N^4-Methoxycytidine 5′-Triphosphate Ammonium Salt (20). A solution of N^4-methoxycytidine **36** (10 mg, 0.037 mmol) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then phosphorus oxychloride (0.008 mL, 0.08 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. Tributylammonium pyrophosphate (1.6 mol of $C_{12}H_{27}N$ per mol of $H_4P_2O_7$, 110 mg, 0.23 mmol) in DMF (0.3 mL) was added at once to the reaction mixture. After 10 min, 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **20** (3.9 mg, 31%) as a white solid. ^{1}H NMR (D_2O) δ 7.25 (d, J = 8.1 Hz, 1H), 5.97 (d, J = 6.3 Hz, 1H), 5.83 (d, J = 8.4 Hz, 1H), 4.40 (m, 2H), 4.22 (m, 3H), 3.81 (s, 3H); ^{31}P NMR (D_2O) δ −8.82 (d, J = 20.2 Hz), −11.0 (d, J = 20.2 Hz), −22.40 (t, J = 19.5 Hz); HRMS-EI found 511.9873 $(M - H^+)^-$. $C_{10}H_{17}N_3O_{15}P_3$ requires 511.9857; purity >98% by HPLC (system A, 17.2 min).

P^1,P^3-Di(uridine 5′-)α,β-Methylenetriphosphate Triethylammonium Salt (22). Compound **10** (3 mg, 0.006 mmol) and UMP (1.6 mg, 0.006 mmol) were converted to the corresponding tributylammonium salts by ion exchange. A solution of compound **10**, tributylammonium salt, and *N,N′*-diisopropylcarbodiimide (DIC, 2.8 μL, 0.018 mmol) in DMF (0.06 mL) was stirred for 3 h at rt. Then a solution of the UMP tributylammonium salt in DMF (0.04 mL) and a solution of $MgCl_2$ (1.7 mg, 0.018 mmol) in DMF (0.02 μL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (0.6 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **22** (1.2 mg, 27%) as a white solid. ^{1}H NMR (D_2O) δ 8.03 (d, J = 8.1 Hz, 1H), 7.98 (d, J = 8.1 Hz, 1H), 6.02−5.94 (m, 4H), 4.43−4.35 (m, 2H), 4.32−4.17 (m, 3H), 2.37 (t, J = 20.1 Hz, 1H); ^{31}P NMR (D_2O) δ 17.35 (s), 7.42 (d, J = 27.6 Hz), −10.93 (d, J = 27.6 Hz; HRMS-EI found 751.0009 $(M + 2Na^+ - 3H^+)^-$. $C_{19}H_{24}N_4Na_2O_{19}P_3$ requires 751.0043; purity >99% by HPLC (system B, 9.3 min).

P^1-Uridine 5′- P^3-Cyclohexyltriphosphate Triethylammonium Salt (25). Compound **1** (4 mg, 0.010 mmol) was exchanged to the tributylammonium salt form. A solution of compound **1**, tributylammonium salt, and DIC (4.6 μL, 0.030 mmol) in DMF (0.08 mL) was stirred for 3 h at rt. Then a solution of cyclohexylphosphate tributylammonium salt in DMF (0.08 mL) and a solution of $MgCl_2$ (2.8 mg, 0.030 mmol) in DMF (0.04 mL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (0.4 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **26** (1.35 mg, 18%) as a white solid. ^{1}H NMR (D_2O) δ 7.92 (d, J = 8.1 Hz, 1H), 7.39 (dd, J = 8.2, 8.7 Hz, 2H), 7.26 (d, J = 8.7 Hz, 2H), 7.19 (t, J = 8.2 Hz, 1H), 5.94 (d, J = 4.5 Hz, 1H), 5.92 (d, J = 8.7 Hz, 1H), 4.38−4.32 (m, 1H), 4.30−4.18 (m, 4H); ^{31}P NMR (D_2O) δ −11.22 (d, J = 19.1 Hz), −15.71 (d, J=19.9 Hz), −23.07 (t, J = 19.1 Hz); HRMS-EI found 558.9928 $(M - H^+)^-$. $C_{15}H_{18}N_2O_{15}P_3$ requires 558.9920; purity >99% by HPLC (system B, 8.9 min).

P^1-Uridine 5′-P^3-Phenyltriphosphate Triethylammonium Salt (26). Compound **1** (4 mg, 0.010 mmol) and sodium phenylphosphate dibasic dihydrate (5.0 mg, 0.020 mmol) were cation-exchanged to the tributylammonium salts. A solution of compound **1**, tributylammonium salt, and DIC (4.6 μL, 0.030 mmol) in DMF (0.08 mL) was stirred for 3 h at rt. Then a solution of phenylphosphate tributylammonium salt in DMF (0.08 mL) and a solution of $MgCl_2$ (2.8 mg, 0.030 mmol) in DMF (0.04 mL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (0.4 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **25** (1.24 mg, 16%) as a white solid. ^{1}H NMR (D_2O) δ 8.02 (d, J = 8.7 Hz, 1H), 6.07−6.00 (m, 2H), 4.49−4.40 (m, 2H), 4.35−4.15 (m, 4H), 4.01−3.77 (m, 4H), 2.06−1.96 (m, 2H), 1.79−1.69 (m, 2H), 1.59−1.14 (m, 6H); ^{31}P NMR (D_2O) δ −11.5 (br, 2H), −23.1 (br, 1H); HRMS-EI found 565.0405 $(M - H^+)^-$. $C_{15}H_{24}N_2O_{15}P_3$ requires 565.0390; purity >99% by HPLC (system B, 9.8 min).

P^1-Uridine 5′-P^3-[1]Glucose-1′-Triphosphate Triethylammonium Salt (27). Compound **1** (10 mg, 0.025 mmol) and α-D-glucose-1-phosphate disodium salt hydrate (10 mg, 0.038 mmol) were exchanged to the tributylammonium salts. A solution of compound **1**, tributylammonium salt, and DIC (12 μL, 0.075 mmol) in DMF (0.10 mL) was stirred for 3 h at rt. Then a solution of the α-D-glucose-1-phosphate tributylammonium salt in DMF (0.10 mL) and a solution of $MgCl_2$ (7 mg, 0.075 mmol) in DMF (0.05 μL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (0.4 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **27** (1.8 mg, 9%) as a white solid. ^{1}H NMR (D_2O) δ 7.97 (d, J = 7.8 Hz, 1H), 6.01 (d, J = 4.5 Hz, 1H), 5.99 (d, J = 7.8 Hz, 1H), 5.63 (dd, J = 7.2, 3.6 Hz, 1H), 4.45−4.37 (m, 2H), 4.32−4.22 (m, 3H), 3.97−3.73 (m, 4H), 3.52 (dt, J = 9.9, 3.3 Hz, 1H), 3.45 (t, J = 9.6 Hz); ^{31}P NMR (D_2O) δ −11.27 (d, J = 19.8 Hz), −12.65 (d, J = 19.8 Hz), −22.70 (d, J = 19.8 Hz); HRMS-EI found 645.0100 $(M - H^+)^-$. $C_{15}H_{24}N_2O_{20}P_3$ requires 645.0135; purity >99% by HPLC (system B, 8.8 min).

(S)-Methanocarbauridine 3′,5′-Cyclic-diphosphate Triethylammonium Salt (9) and (S)-Methanocarbauridine 5′-Glucose-1′-triphosphate Triethylammonium Salt (28). To a solution of compound **7** (2.6 mg, 0.0036 mmol) in DMF (2 mL), 1,1′-carbonyldiimidazole (3.0 mg, 0.018 mmol) was added. The reaction mixture was stirred at rt for 5 h. Then 5% triethylamine solution in 1/1 water/methanol (1 mL) was added and stirring was continued at rt for an additional 2 h. After removal of the solvent, the residue was dried under high vacuum and dissolved in DMF (2 mL). Glucose-1-monophosphate tributylammonium salt (4 mg, 0.006 mmol) in DMF (0.2 mL) was added to this mixture. The reaction mixture was stirred at rt for 2 days. After removal of the solvent, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **28** (0.8 mg, 23%) and **9** (1.1 mg, 51%) additionally. Compound **28**: ^{1}H NMR (D_2O) δ 7.79 (d, J = 8.1 Hz, 1H), 5.86 (d, J = 8.1 Hz, 1H), 5.64 (dd, J = 3.3, 7.2 Hz, 1H), 4.62 (m, 1H), 4.15 (t, J = 6.3 Hz, 2H), 3.86 (m, 4H), 3.50 (m, 3H), 2.39 (t, J = 6.3 Hz, 1H), 1.88 (dd, J = 4.8, 9.9 Hz, 1H), 1.72 (t, J = 5.7 Hz, 1H), 1.39 (m, 1H); ^{31}P NMR (D_2O) δ −10.87 (d, J = 21.4 Hz), −12.58 (d, J = 20.76 Hz); HRMS-EI found 655.0326 $(M - H^+)^-$. $C_{17}H_{26}N_2O_{19}P_3$ requires 655.0343; purity >98% by HPLC (system A, 16.9 min). Compound **9**: ^{1}H NMR (D_2O) δ 7.81 (d, J = 8.1 Hz, 1H), 5.84 (d, J = 8.1 Hz, 1H), 5.30 (m, 1H), 4.08 (m, 3H), 2.63 (t, J = 6.3 Hz, 1H), 2.03 (m, 1H), 1.71 (t, J = 6.0 Hz, 1H), 1.50 (ddd, J = 2.1, 6.3, 8.4 Hz, 1H); ^{31}P NMR (D_2O) δ −9.55 (app d), −22.66 (app t); HRMS-EI found 395.0044 $(M - H^+)^-$. $C_{11}H_{13}N_2O_{10}P_2$ requires 395.0045; purity >98% by HPLC (system A, 12.0 min).

5-Iodouridine 5′-Glucose-1′-triphosphate Triethylammonium Salt (29). Compound **3** (2 mg, 0.003 mmol) and α-D-glucose-1-phosphate disodium salt hydrate (2 mg, 0.005 mmol) were exchanged to the tributylammonium salts. A solution of compound **3**, tributylammonium salt, and DIC (1.3 μL, 0.008 mmol) in DMF (0.04 mL) was stirred for 3 h at rt. Then a solution of the α-D-glucose-1-phosphate tributylammonium salt in DMF (0.04 mL) and a solution of $MgCl_2$ (0.8 mg, 0.008 mmol) in DMF (0.02 μL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (0.2 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **29** (0.35 mg, 13%) as a white solid. ^{1}H NMR (D_2O) δ 8.26 (s, 1H), 6.01 (d, J = 4.8 Hz, 1H), 5.68 (dd, J = 3.0, 6.9 Hz, 1H), 4.49−4.40 (m, 2H), 4.36−4.25 (m, 3H), 4.03−3.80 (m, 4H), 3.62−3.47 (m, 2H); ^{31}P NMR (D_2O) δ −11.46 (d, J = 19.1 Hz), −12.60 (d, J = 19.1 Hz), −22.60 (t, J = 19.1 Hz); HRMS-EI found 770.9131 $(M - H^+)^-$. $C_{15}H_{23}IN_2O_{20}P_3$ requires 770.9102; purity >99% by HPLC (system B, 9.7 min).

3-Phenacyluridine 5′-Glucose-1′-triphosphate Triethylammonium Salt (30). Compound **5** (2 mg, 0.005 mmol) and α-D-glucose-1-phosphate disodium salt hydrate (2 mg, 0.010 mmol) were exchanged to the tributylammonium salts. A solution of compound **5**, tributylammonium salt, and DIC (2 μL, 0.015 mmol) in DMF (0.04 mL) was stirred for 3 h at rt. Then a solution of the α-D-glucose-1-phosphate tributylammonium salt in DMF (0.04 mL) and a solution of $MgCl_2$ (1 mg, 0.015 mmol) in DMF (0.02 μL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (0.2 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **30** (1.0 mg, 29%) as a white solid. ^{1}H NMR (D_2O) δ 8.08−8.04 (m, 3H), 7.80 (t, J = 7.2 Hz, 1H), 7.65 (t, J = 7.2 Hz, 2H), 6.17 (d, J = 7.8 Hz, 1H), 6.04 (d, J = 4.5 Hz, 1H), 5.66 (dd, J = 3.6, 6.9 Hz), 5.55 (s, 2H), 4.49−4.43 (br, 2H), 4.37−4.29 (br, 3H), 4.01−3.77 (m, 4H), 3.60−3.50 (br, 1H), 3.48 (t, J = 9.1 Hz, 1H); ^{31}P NMR (D_2O) δ −11.24 (d, J = 25.4 Hz), −12.63 (d, J = 25.4 Hz), −22.65 (d, J = 25.8 Hz); HRMS-EI found 763.0554 $(M - H^+)^-$. $C_{23}H_{30}N_2O_{21}P_3$ requires 763.0589; purity >99% by HPLC (system B, 9.9 min).

Uridine 5′-Glucose-1′-α,β-methylenetriphosphate Triethylammonium Salt (31). Compound **10** (2.4 mg, 0.004 mmol) and α-D-glucose-1-phosphate disodium salt hydrate (3.6 mg, 0.012 mmol) were exchanged to the tributylammonium salts. A solution of compound **10**, tributylammonium salt, and DIC (1.8 μL, 0.012 mmol) in DMF (0.04 mL) was stirred for 3 h at rt. Then a solution of the α-D-glucose-1-phosphate tributylammonium salt in DMF (0.04 mL) and a solution of $MgCl_2$ (2.6 mg, 0.028 mmol) in DMF (0.02 μL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (0.4 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **31** (2.7 mg, 80%) as a white solid. ^{1}H NMR (D_2O) δ 8.07 (d, J = 7.8 Hz, 1H), 6.06−5.99 (m, 2H), 5.70−5.63 (m, 1H), 4.49−4.43 (m, 2H), 4.35−4.27 (m, 1H), 4.27−4.21 (m, 2H), 4.00−3.77 (m, 4H), 3.60−3.43 (m, 2H), 2.43 (t, J = 19.8 Hz, 2H); ^{31}P NMR (D_2O) δ 17.32 (d, J = 9.1 Hz), 8.27 (dd, J = 9.1, 26.7 Hz), −12.23 (d, J = 26.7 Hz); HRMS-EI found 643.0348 $(M - H^+)^-$. $C_{16}H_{26}N_2O_{19}P_3$ requires 643.0343; purity >99% by HPLC (system B, 9.2 min).

N^4-Methoxycytidine 5′-Glucose-1′-triphosphate Triethylammonium Salt (32). Compound **12** (4.9 mg, 0.009 mmol) and α-D-glucose-1-phosphate disodium salt hydrate (8.4 mg, 0.028 mmol) were exchanged to the tributylammonium salts. A solution of compound **12**, tributylammonium salt, and DIC (4.3 μL, 0.028 mmol) in DMF (0.1 mL) was stirred for 3 h at rt. Then a solution of the α-D-glucose-1-phosphate tributylammonium salt in DMF (0.1 mL) and a solution of $MgCl_2$ (2.6 mg, 0.028 mmol) in DMF (50 μL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (1 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **32** (0.62 mg, 10%) as a white solid. ^{1}H NMR (D_2O) δ 7.28 (d, J = 8.4 Hz, 1H), 5.99 (d, J = 5.4 Hz, 1H), 5.86 (d, J = 8.4 Hz, 1H), 5.65 (dd, J = 3.6, 7.2 Hz, 1H), 4.45−4.36 (m, 2H), 4.30−4.15 (m, 3H), 3.97−3.77 (m, 4H), 3.82 (s, 3H), 3.57−3.50 (m, 1H), 3.47 (t, J = 9.3 Hz, 1H); ^{31}P NMR (D_2O) δ −11.27 (d, J = 19.1 Hz), −12.65 (d, J = 19.1 Hz), −22.71 (t, J = 19.1 Hz); HRMS-EI found 674.0387 $(M - H^+)^-$. $C_{16}H_{27}N_3O_{20}P_3$ requires 674.0401; purity >99% by HPLC (system B, 9.3 min).

N^4-Methoxycytidine 5′-Phenyltriphosphate Triethylammonium Salt (33). Compound **12** (6.9 mg, 0.011 mmol) and sodium phenylphosphate dibasic dihydrate (8.3 mg, 0.033 mmol) were exchanged to the tributylammonium salts. A solution of compound **12** tributylammonium salt and DIC (5.0 μL, 0.033 mmol) in DMF (0.07 mL) was stirred for 3 h at rt. Then a solution of the phenylphosphate tributylammonium salt in DMF (0.07 mL) and a solution of $MgCl_2$ (3.1 mg, 0.033 mmol) in DMF (0.07 mL) were added. After the reaction mixture was stirred at rt for 16 h, the solvent was removed and water (1 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **33** (0.9 mg, 14%) as a white amorphous solid. ^{1}H NMR (D_2O) δ 7.44−7.36 (m, 2H), 7.31−7.17 (m, 4H), 5.92 (d, J = 6.0 Hz, 1H), 5.77 (d, J = 8.4 Hz, 1H), 4.38−4.28 (m, 2H), 4.24−4.15 (m, 3H); HRMS-EI found 588.0186 $(M - H^+)^-$. $C_{16}H_{22}N_3O_{15}P_3$ requires 588.0199; purity >99% by HPLC (system B, 10.3 min).

Pharmacological Analyses. UDP was purchased from Sigma (St. Louis, MO). *myo*-[^{3}H]Inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Measurement of intracellular calcium ion concentration in response to nucleotides was performed as described.[4]

Assay of PLC Activity Stimulated by $P2Y_2$, $P2Y_4$, and $P2Y_6$ Receptors. Stable cell lines expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor in 1321N1 human astrocytoma cells were generated as described.[26] Agonist-induced [^{3}H]inositol phosphate production was measured in 1321N1 cells plated to 20 000 cells/well on 96-well plates 2 days prior to assay. Sixteen hours before the assay, the inositol lipid pool of the cells was radiolabeled by incubation in 100 μL of serum-free inositol-free Dulbecco's modified Eagle's medium, containing 1.0 μCi of *myo*-[^{3}H]inositol. No changes of medium were made subsequent to the addition of [^{3}H]inositol. On the day of the assay, the cells were challenged with 25 μL of a 5-fold concentrated solution of receptor agonists in 200 mM HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid), pH 7.3 in HBSS, containing 50 mM LiCl for 30 min at 37 °C. Incubations were terminated by aspiration of the drug-containing medium and addition of 450 μL of ice-cold 50 mM formic acid. [^{3}H]Inositol phosphate accumulation was quantified using scintillation proximity assay methodology as previously described in detail.[26]

Data Analysis. Agonist potencies (EC_{50} values) were determined from concentration−response curves by nonlinear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, CA). All experiments examining the activity of newly synthesized molecules also included full concentration effect curves for the cognate agonist of the target receptor: UTP for the $P2Y_2$ receptor, UTP for the $P2Y_4$ receptor, and UDP for the $P2Y_6$ receptor. Each concentration of drug was tested in triplicate assays, and concentration effect curves for each test drug were repeated in at least three separate experiments with freshly diluted molecule. The results are presented as the mean ± SEM from multiple experiments or in the case of concentration effect curves from a single experiment carried out with triplicate assays that were representative of results from multiple experiments.

Stability of Nucleotide Derivatives. UTP was purchased from Sigma (St. Louis, MO). Up_3U **19** was synthesized by the procedure previously reported.[21] HCl buffer (pH 1.2) solution was prepared as follows. Concentrated HCl (7 mL, 12 N) was

added to 2.0 g of sodium chloride and diluted with water to a volume of 1000 mL. Membranes were prepared as follows. $P2Y_1$ receptor-expressing astrocytoma cells were grown to 80% confluence and then harvested. The cells were homogenized, and the nuclear fraction was removed by centrifuging at 100*g* for 5 min. The pellet was resuspended in 50 mM tris(hydroxymethyl)-aminomethane (Tris) hydrochloride buffer (pH 7.4). The suspension was homogenized with a Polytron homogenizer (Brinkmann) for 10 s and was ultracentrifuged at 20000*g* for 20 min at 4 °C. The resultant pellets were resuspended in Tris buffer (pH 7.4), and the membrane was stored at −80 °C until the experiments. The protein concentration of the membrane preparation was measured by Bradford assay.[34] The membrane preparation was diluted with 4 mL of Dulbecco's phosphate buffered saline (pH 7.4) and homogenized before use for the stability check. The final protein concentration was 14.9 μg/mL.

An amount of 10 μL of a 2 mg/mL aqueous solution of each nucleotide derivative was mixed with 90 μL of HCl buffer (pH 1.2) or the membrane suspension and incubated at 37 °C. At regular intervals, a 6 μL aliquot of the above mixture was mixed with 54 μL of water or 5 mM TBAP. Then 50 μL of the mixure was injected into the HPLC.

AUC of compounds were measured using a Hewlett-Packard 1100 HPLC equipped with a Zorbax Eclipse 5 μm XDB-C18 analytical column (250 mm × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). The mobile phase was a linear gradient solvent system of 5 mM TBAP−CH_3CN from 80:20 to 40:60 in 6.5 min, and the flow rate was 1 mL/min (system C) or system B. Peaks were detected by UV absorption with a diode array detector at 254, 275, and 280 nm, and AUC values were calculated based on the peak at 254 nm.

Molecular Modeling. $P2Y_6$−Compound 7 Complex. Molecular modeling was based on our previously published model of the complex between UDP and the rhodopsin-based $P2Y_6$ receptor, obtained through Monte Carlo conformational searches and molecular dynamics in a hydrated lipid bilayer.[13]

Here, compound **7** was sketched from the docked UDP and subsequently subjected to a series of energy minimizations allowing flexibility to increasingly larger portions of the model, followed by a conformational search. In particular, the minimizations were performed on the cyclopropane moiety of the ligand, the ribose moiety of the ligand, and the entire ligand. The final minimization and the conformational search were performed on the entire ligand plus all the $P2Y_6$ residues and the water molecules located within a radius of 5 Å. All of the atoms within an additional shell of a radius of 3 Å were also taken into account in the calculations as a frozen environment.

All the calculations were conducted using MacroModel, version 9.6, as implemented in the Schrödinger package, with the OPLS_2005 force field and water as implicit solvent (GB/SA model).[35] The minimizations were based on the Polak−Ribier conjugate gradient algorithm and were performed with a threshold on the gradient of 0.05 kJ/mol. The conformational search was based on 1000 steps of extended Monte Carlo multiple minimum (MCMM) torsional sampling. The sampling regarded the torsional angles of residues and ligand, as well as the rotation and translation of the ligand.

$P2Y_6$−Compound 12, 26, or 33 Complex. Molecular modeling was based on the same structure described in the above section. Compounds **12**, **26**, and **33** were sketched from the docked UDP and subsequently subjected to a series of energy minimizations allowing flexibility to increasingly larger portions of the model, followed by a conformational search. All the conformational searches were performed on each entire ligand plus all the $P2Y_6$ residues located within a radius of 6 Å from the initial ligand, UDP. All of the atoms within an additional shell of a radius of 4 Å were also taken into account in the calculations as a frozen environment.

All the calculations were conducted using MacroModel, version 9.7.1, as implemented in the Schrödinger package, with the OPLS_2001 force field.[36] The minimizations were based on the Polak−Ribier conjugate gradient algorithm and were performed with a threshold on the gradient of 0.05 kJ/mol. The conformational search was based on 100 steps of extended Monte Carlo multiple minimum (MCMM) torsional sampling. The sampling regarded the torsional angles of residues and ligand, as well as the rotation and translation of the ligand.

Residue Indexing. To facilitate the comparison among receptors, residues are identified through the GPCR residue indexing system. In each TM, the index *X*.50, where *X* is the TM number, is assigned to the position hosting the most conserved residue among class A GPCRs. All other positions in the TM are numbered relative to the *X*.50 position.[37]

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Supporting Information Available: Time course of decomposition/hydrolysis of selected pyrimidine nucleotide derivatives in acidic solution and in the presence of cell membranes; stick representation of Figure 2A. This material is available free of charge via the Internet at http://pubs.acs.org.

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