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Human $P2Y_{14}$ Receptor Agonists: Truncation of the Hexose Moiety of Uridine-5′-Diphosphoglucose and Its Replacement with Alkyl and Aryl Groups

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Uridine-5′-diphosphoglucose (UDPG) activates the $P2Y_{14}$ receptor, a neuroimmune system GPCR. $P2Y_{14}$ receptor tolerates glucose substitution with small alkyl or aryl groups or its truncation to uridine 5′-diphosphate (UDP), a full agonist at the human $P2Y_{14}$ receptor expressed in HEK-293 cells. 2-Thiouracil derivatives displayed selectivity for activation of the human $P2Y_{14}$ vs the $P2Y_6$ receptor, such as 2-thio-UDP **4** (EC_{50} = 1.92 nM at $P2Y_{14}$, 224-fold selectivity vs $P2Y_6$) and its β-propyloxy ester **18**. EC_{50} values of the β-methyl ester of UDP and its 2-thio analogue were 2730 and 56 nM, respectively. β-*tert*-Butyl ester of **4** was 11-fold more potent than UDPG, but β-aryloxy or larger, branched β-alkyl esters, such as cyclohexyl, were less potent. Ribose replacement of UDP with a rigid North or South methanocarba (bicyclo[3.1.0]hexane) group abolished $P2Y_{14}$ receptor agonist activity. α,β-Methylene and difluoromethylene groups were well tolerated at the $P2Y_{14}$ receptor and are expected to provide enhanced stability in biological systems. α,β-Methylene-2-thio-UDP **11** (EC_{50} = 0.92 nM) was 2160-fold selective versus $P2Y_6$. Thus, these nucleotides and their congeners may serve as important pharmacological probes for the detection and characterization of the $P2Y_{14}$ receptor.

Introduction

Purine and pyrimidine mononucleotides and dinucleotides have a role as extracellular signaling molecules, in addition to their diverse intracellular roles.[1] The P2Y family of G-protein-coupled receptors (GPCRs) responds to various extracellular nucleotides to induce intracellular signaling cascades.[2−4] The $P2Y_{14}$ receptor was identified initially as an orphan GPCR activated by uridine 5′-diphosphoglucose (UDPG[a] **1**, Chart 1) and other endogenous UDP sugars. This Gi-coupled receptor is expressed in the brain and in dendritic cells,[5] although no functional role has yet been clearly assigned in these tissues. The $P2Y_{14}$ receptor also is expressed in the placenta, adipose tissue, stomach, intestine, brain, spleen, thymus, lung, and heart.[1] Thus, the $P2Y_{14}$ receptor is of potential therapeutic interest for modulation of the immune system,[7] as well as treatment of pain,[8] diabetes,[9] cystic fibrosis, and other pulmonary diseases.[10,11]

A current goal of our research is to identify and apply potent, selective, and stable $P2Y_{14}$ receptor ligands to define the physiological role(s) of this receptor.[12−14] Although the $P2Y_{14}$ receptor initially was identified as a UDP-sugar-activated GPCR, we recently discovered that UDP is at least as potent as UDP-glucose and other nucleotide sugars for activation of this receptor.[14] Thus, it is perhaps not surprising that our recent detailed SAR (structure−activity relationship) analyses with synthetic analogues of UDPG[12,13] revealed that the glucose moiety is the least restricted region of the structure for substitutions that maintain $P2Y_{14}$ receptor agonist potency. This realization, together with conclusions made from our recent SAR study of UDP analogues at the $P2Y_6$ receptor,[15] suggests that β-phosphate substitution in a new series of UDP analogues might favor activation of the $P2Y_{14}$ receptor. Therefore, with the ultimate goal of identifying highly selective ligands for the $P2Y_{14}$ and $P2Y_6$ receptors, we have synthesized a new series of β-phosphate-substituted analogues of UDP and compared the potencies of these novel molecules as well as a number of previously prepared uracil nucleotide analogues at the human $P2Y_{14}$ and $P2Y_6$ receptors.[15] Although the glucose moiety of UDPG was suggested to interact with multiple H-bonding and/or charged resides within the putative binding site of the $P2Y_{14}$ receptor, its deletion or substitution with smaller phosphoester groups was tolerated at this receptor. Simple alkyl esters at this position and analogues of UDP displayed highly potent agonist activity at the $P2Y_{14}$ receptor. The effects of these modifications to preserve and enhance potency at the $P2Y_{14}$ receptor were additive with the previously identified potency enhancing effect of 2-thio substitution of the uracil moiety, achieving in some cases nanomolar and subnanomolar potency. Importantly, a number of these new UDP analogues exhibit high selectivity for activation of the $P2Y_{14}$ receptor over the $P2Y_6$ receptor.

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[a] Abbreviations: cAMP, 3′,5′-cyclic adenosine monophosphate; CHO Chinese hamster ovary; DCC, dicyclohexylcarbodiimide; DMEM, Dulbecco's modified Eagle medium; DMF, dimethylformamide; HEK, human embryonic kidney; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; PLC, phospholipase C; SAR, structure−activity relationship; TBAP, tributylammonium phosphate; TEAA, triethylammonium acetate; UDP, uridine 5′-diphosphate; UDPG, uridine 5′-diphosphoglucose.

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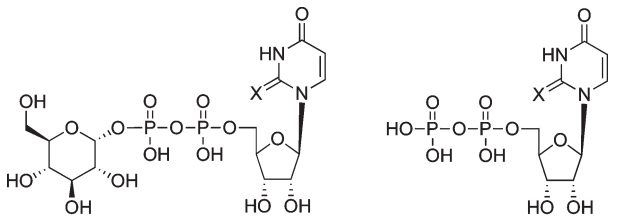
Chart 1. Structures of Two UDP-Sugars (**1**, **2**) That Act as $P2Y_{14}$ Receptor Agonists, UDP **3**, a Naturally Occurring Ligand of the $hP2Y_{14}$ Receptor, and Its Analogue 2-Thio-UDP **4**



1 X = O, UDP-glucose
2 X = S, MRS2690

3 X = O, UDP
4 X = S, 2-thio-UDP

Results

Chemical Synthesis. The principal objective of this study was to structurally simplify the distal end of uracil nucleotide sugars known to activate the $hP2Y_{14}$ receptor and to combine potency-enhancing modifications. By removal of the glucose moiety of UDPG **1**, this agonist is converted into UDP **3**, which we recently discovered is a highly potent agonist of the $hP2Y_{14}$ receptor.[14] The activities of analogues of **1** modified on the glucose moiety have been explored, but intermediate structures and truncated analogues related to UDP have not been systematically examined. We also were searching for leads for UDP analogues that might provide selectivity at the $P2Y_{14}$ receptor over the $P2Y_6$ receptor.

Simple ester and 5′-diphosphate analogues of the $hP2Y_{14}$ receptor agonist UDPG **1** and its 2-thio derivative **2** were synthesized (Table 1). The only modifications of the nucleobase included were thiocarbonyl or methoxyamino substitution of carbonyl groups of the uracil moiety, which were studied previously in $P2Y_{14}$ receptor recognition.[12] Several of the analogues of UDP **3** examined here were reported in studies of SAR at the $P2Y_6$ receptor.[15,17,18] All of the nucleotide analogues were prepared in their ammonium or triethylammonium salt form according to the methods shown in Schemes 1−3 and tested in functional assays of the $hP2Y_{14}$ and $hP2Y_6$ receptors (Table 1).

The uridine-5′-diphospho alkyl or aryl β-ester derivatives were obtained by the following methods. The corresponding alkyl or aryl monophosphate analogues were treated successively with cation-exchange resin and tributylamine. To a solution of the alkyl or aryl monophosphate tributylammonium salt in DMF, commercially available uridine 5′-monophosphate morpholidate 4-morpholine-*N*,*N*-dicyclohexylcarboxamidine salt was added to form uridine-5′-diphospho-β-ester analogues (**6**, **14**, **19**, **20**, **22**−**24**, and **26**−**30**) in a condensation reaction as shown in Scheme 1. 2-Thio UDP **4** and 4-thio UDP **5** were synthesized by using the previously reported procedure.[17] (N)-Methanocarba-UDP **8** and (S)-methanocarba-UDP **9** were synthesized using our previously reported procedures.[15,19,20] α,β-Methylene-UDP **10** and α,β-difluoromethylene-UDP **12** were also prepared using our recently published procedures or modifications thereof. 2-Thio-α,β-methylene-UDP **11** was prepared by the DCC coupling of 2-thiouridine with methylenediphosphonic acid (Scheme 3).[18] Compounds **7** and **13** were synthesized by using a recently developed procedure.[15]

For the preparation of the 2- or 4-thiocarbonyl β-alkyl diphosphate esters (**15**−**18**, **21**, and **25**), we either synthesized the alkyl monophosphate (**42**−**44**)[21] or obtained the commercially available alkyl monophosphates (**41**, **45**). The monophosphates were transformed to the corresponding tributylammonium salts and were then activated with 1,1′-carbonyldiimidazole in DMF for 5 h at room temperature followed by quenching of this reagent with methanol. After removal of the solvent, the residue was dissolved in DMF, and 2-thio or 4-thiouridine 5′-monophosphate tributylammonium salt was added to obtain compounds **15**−**18**, **21**, and **25** as shown in Scheme 2. All the nucleotide analogues were characterized using HPLC, nuclear magnetic resonance (^{1}H NMR, ^{31}P NMR), and high-resolution mass spectrometry.

Quantification of Pharmacological Activity. Inhibition of adenylyl cyclase was quantified in HEK-293 cells stably expressing the $hP2Y_{14}$ receptor. This cell line, as well as a $P2Y_{14}$ receptor-expressing Chinese hamster ovary (CHO) cell line and a $P2Y_{14}$ receptor-expressing C6 rat glioma cell line that we recently developed, provides a physiologically relevant test system, since receptor-promoted responses mediated through a natively expressed heterotrimeric G protein (Gi) and its natively expressed effector protein (adenylyl cyclase) are quantified.[14] We conclude that this system is much preferable to assay methods, employed by us and others, which utilized COS-7 cells transiently overexpressing the $P2Y_{14}$ receptor with an engineered G protein, $G\alpha$-q/i protein ($G\alpha_{qi5}$), that allows coupling of Gi-coupled receptors to activation of phospholipase C (PLC), resulting in inositol lipid hydrolysis.[22−24]

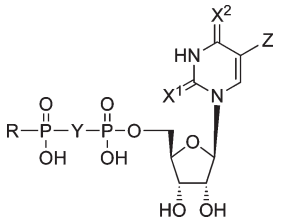
Although UDPG **1** is usually thought of as a specific agonist of the $P2Y_{14}$ receptor, it also was found to activate the $P2Y_2$ receptor ($EC_{50} \approx 10\ \mu M$).[13] We now report EC_{50} values for **1** of 0.40 (Figure 1A) and 16 μM at the $P2Y_{14}$ and $P2Y_6$ receptor, respectively (Table 1). Therefore, although selective for the $P2Y_{14}$ receptor, UDPG also activates other P2Y receptors. Thus, more potent and selective agonists, preferably with a simplified chemical structure, are needed as pharmacological probes of the $P2Y_{14}$ receptor.

UDP **3** and its 4-thio derivative **5** were moderately potent agonists at the $hP2Y_{14}$ receptor. One of the most potent $P2Y_{14}$ receptor agonists in the current set of nucleotides was the 2-thio derivative **4** of UDP, which displayed an EC_{50} value of 1.92 nM. Compound **4** was 83-fold more potent than **3** as a $P2Y_{14}$ receptor agonist and 230-fold selective for the $P2Y_{14}$ receptor in comparison to the $P2Y_6$ receptor. Replacement of a terminal negatively charged oxygen of **3** with an uncharged methyl group in the phosphonate derivative **6** resulted in a molecule with similarly weak potencies at the two receptors. The N^4-methyloxy cytidine derivative **7** was 20-fold less potent than **3** at the $P2Y_{14}$ receptor and consequently displayed selectivity for the $P2Y_6$ receptor, at which its high potency has been described.[15] This finding is consistent with the reported weak $P2Y_{14}$ receptor activity of the corresponding N^4-methyloxy cytidine analogue of UDPG.[13]

Replacement of a ribose moiety of various P2Y receptor agonists with a sterically constrained bicyclic ring has been used to establish the receptor-preferred conformation. Neither of the conformationally constrained methanocarba analogues **8** (North, N) and **9** (South, S) of UDP **3** was active at the $P2Y_{14}$ receptor. Consistent with these findings with analogues of **3**, we have already established that the corresponding (N)- and (S)-methanocarba analogues of UDPG were both inactive at the $P2Y_{14}$ receptor.[13]

The introduction of carbon-bridged substitutions of the phosphate moieties of P2 agonists has led to greater stability

Table 1. In Vitro Pharmacological Data for UDPG **1**, UDP **3**, and Their Analogues (Non-Sugar β-Phosphoesters and Other Derivatives of UDP) in the Inhibition of cAMP Formation at Recombinant hP2Y$_{14}$ Receptors Expressed in HEK-293 Cells Stably Transfected with the hP2Y$_{14}$ and in the Stimulation of PLC at Recombinant hP2Y$_6$ Receptors Stably Expressed in 1321N1 cells[f]



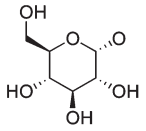
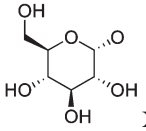
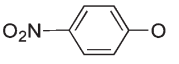
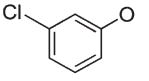
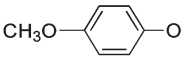
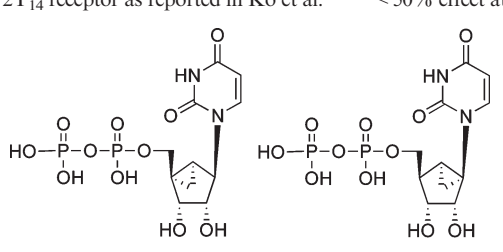
Compound	Modification	Structure R =	EC$_{50}$, nM[a]	
UDP-sugars			**hP2Y$_{14}$**	**hP2Y$_6$**
1	UDP-[1]glucose		400±90	16,000
2[b]	2-thio-UDP-[1]glucose,	 $X^1 = S$	11±5	>10,000[c]
UDP analogues			**hP2Y$_{14}$**	**hP2Y$_6$**
3	UDP	HO	160±40	530±60
4	2-thio-UDP	HO, $X^1 = S$	1.92±0.69	447±100
5	4-thio-UDP	HO, $X^2 = S$	320±130	2360±710
6	Up$_2$-β-Me-phosphonate	R = CH_3	4580±1560	8000±1630
7	N^4-OCH_3-CDP	HO, X^2 = N-OCH_3	3320±1620	70±7[b]
8[d]	(N)-methanocarba UDP (pure enantiomer)	HO	NE	NE
9[d]	(S)-methanocarba UDP (pure enantiomer)	HO	NE	42±8[b]
10	Up-CH_2-p (α,β-methylene UDP)	HO, Y = CH_2	11±6	339±97
11	2-thio-Up-CH_2-p (α,β-methylene-2-thio-UDP)	HO, Y = CH_2, $X^1 = S$	0.92±0.09	1990±370
12[b,e]	Up-CF_2-p (α,β-difluoromethylene UDP)	HO, Y = CF_2	63±9	NE[b]
13	5-I-Up-CF_2-p	HO, Y = CF_2, Z = I	142±74	127±24

Table 1. Continued

Compound	Modification	Structure R =	EC_{50}, nM[a]	
UDP β-esters			**hP2Y$_{14}$**	**hP2Y$_6$**
14	Up_2-OMe	CH_3O	2730±680	>10,000[c]
15	2-thio-Up_2-OMe	CH_3O, X^1 = S	56±19	>10,000[c]
16	4-thio-Up_2-OMe	CH_3O, X^2 = S	NE	10,400±4200
17	2-thio-Up_2-OEt	CH_3CH_2O, X^1 = S	39±20	>10,000[c]
18[e]	2-thio-Up_2-OPr	$CH_3(CH_2)_2O$, X^1 = S	40±6	9100±1430
19	Up_2-$O(CH_2)_2CN$	$O(CH_2)_2CN$	7460±2340	>10,000[c]
20	Up_2-$O(CH_2)_2C{\equiv}CH$	$O(CH_2)_2C{\equiv}CH$	480±161	1520±100
21	2-thio-Up_2-$O(CH_2)_2C{\equiv}CH$	$O(CH_2)_2C{\equiv}CH$, X^1 = S	11.0±1.4	>10,000[c]
22	Up_2-$OCH_2CHOHCH_2OH$	$OCH_2CH(OH)$-CH_2OH	1600±600	>10,000[c]
23	Up_2-$OCH(CH_2OH)_2$	$OCH(CH_2OH)_2$	167±32	>10,000[c]
24	Up_2-$OC(CH_3)_3$	$OC(CH_3)_3$	252±93	10,800±1900
25	2-thio-Up_2-$OC(CH_3)_3$	$OC(CH_3)_3$, X^1 = S	32±1	2040±600
26	Up_2-O-cyclohexyl		5160±1830	>10,000[c]
27	Up_2-OC_6H_5		768±304	7400±860
28	Up_2-OC_6H_4-4-NO_2		1490±490	2760±810
29	Up_2-OC_6H_4-3-Cl		1800±860	7370±530
30	Up_2-OC_6H_4-4-OCH_3		1900±490	>10,000[c]

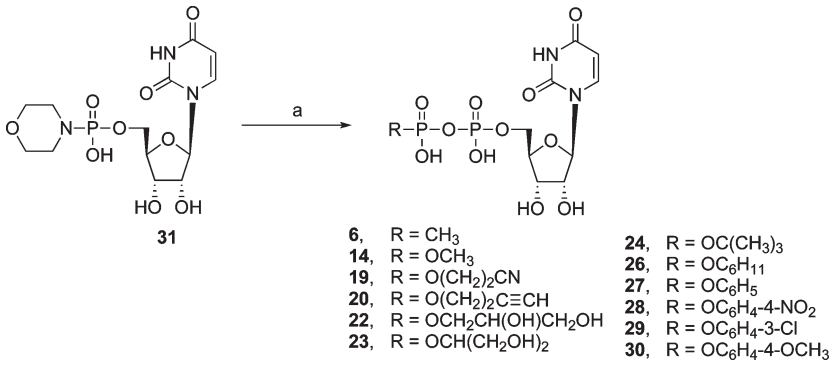
[a] Agonist potencies reflect stimulation of phospholipase C, unless otherwise noted, and were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). EC_{50} values (mean ± standard error) represent the concentration at which 50% of the maximal effect is achieved. Relative efficacies (%) were determined by comparison with the effect produced by a maximal effective concentration of reference agonist (UDP-glucose, **1**) in the same experiment. If no maximal effect is given, then 100% efficacy was achieved. $N = 3$. [b] Potency at $P2Y_6$ receptor as reported by Maruoka et al.,[15] Besada et al.,[17] and Ko et al.[18] Potency at $P2Y_{14}$ receptor as reported in Ko et al.[12,13] [c] < 50% effect at 10 μM. [d] Structure is given below.



8 **9**

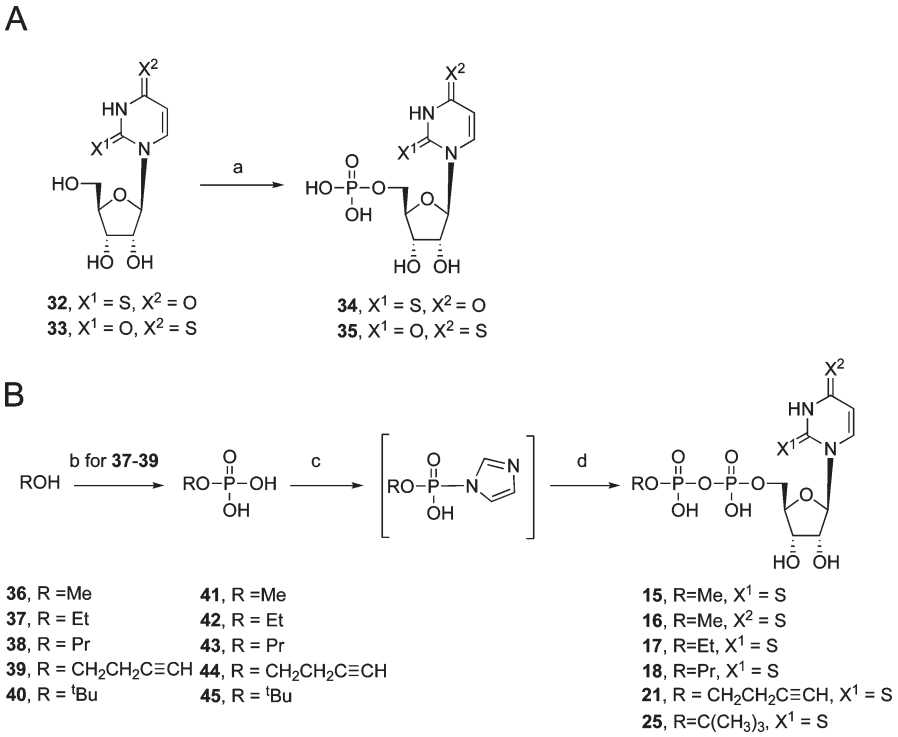
[e] **12**, MRS2802; **18**, MRS2907.[14] [f] Unless otherwise noted: X, Y = O, Z = H. NE: no effect at 10 μM.

Scheme 1. Synthesis of UDPG Analogues Including UDP β-Esters[a]



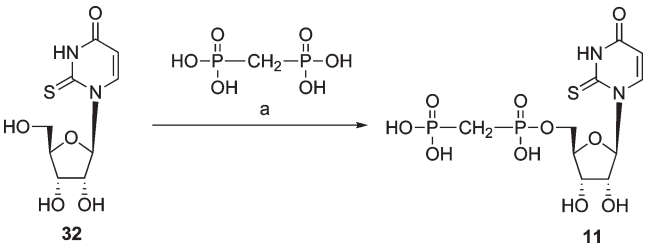
[a] Reagents and conditions: (a) $ROPO_3H_2$, DMF, room temp.

Scheme 2. (A) Synthesis of Thio-UMP Derivatives **34** and **35** and (B) Synthesis of Thio-UDP Derivatives **15**−**18**, **21**, and **25**[a]



[a] Reagents and conditions for part A: (a) (i) $POCl_3$, proton sponge, $PO(OMe)_3$, 0 °C; (ii) 0.2 M triethylammonium bicarbonate, room temp. Reagents and conditions for part B: (b) H_3PO_4, pyridine, Et_3N, Ac_2O; (c) (i) CDI, DMF, room temp; (ii) 5% TEA in 1/1 MeOH/H_2O; (d) 2-thiouridine 5′-monophosphate tributylammonium salt **34** or 4-thiouridine 5′-monophosphate tributylammonium salt **35**, DMF, room temp.

Scheme 3. Synthesis of α,β-Methylene-2-thio-UDP **11**[a]



[a] Reagents and conditions: (a) DCC, DMF, room temp.

in biological systems due to the inability of ectonucleotidases to cleave these groups.[1,27] Replacement of the bridging oxygen of the diphosphate group of UDP with a methylene **10** or difluoromethylene **12** group was well tolerated at the $P2Y_{14}$ receptor, resulting in high potency with EC_{50} values of 11 and 63 nM, respectively (Figure 1B). Indeed, the α,β-difluoromethylene analogue **12** was >2000-fold selective for the $P2Y_{14}$ receptor in comparison to the $P2Y_6$ receptor, at which it was inactive at the concentrations tested. Combining a carbon bridge with a known potency-enhancing uracil modification in 2-thio-α,β-methylene analogue **11** resulted in unprecedented potency at the $hP2Y_{14}$ receptor with an EC_{50} value of 0.92 nM. The selectivity of **11** in comparison to the $hP2Y_6$ receptor was 2160-fold. Combining the α,β-difluoromethylene and 5-iodo modifications in **13** maintained equipotency to UDP **3** at the $P2Y_{14}$ receptor, which stands in contrast with the inactivity of 5-iodo-UDPG and suggests nonidentical modes of uracil binding to the receptor between the two series.[12] However, **13** was not selective for the $P2Y_{14}$ receptor when compared to the $P2Y_6$ receptor.

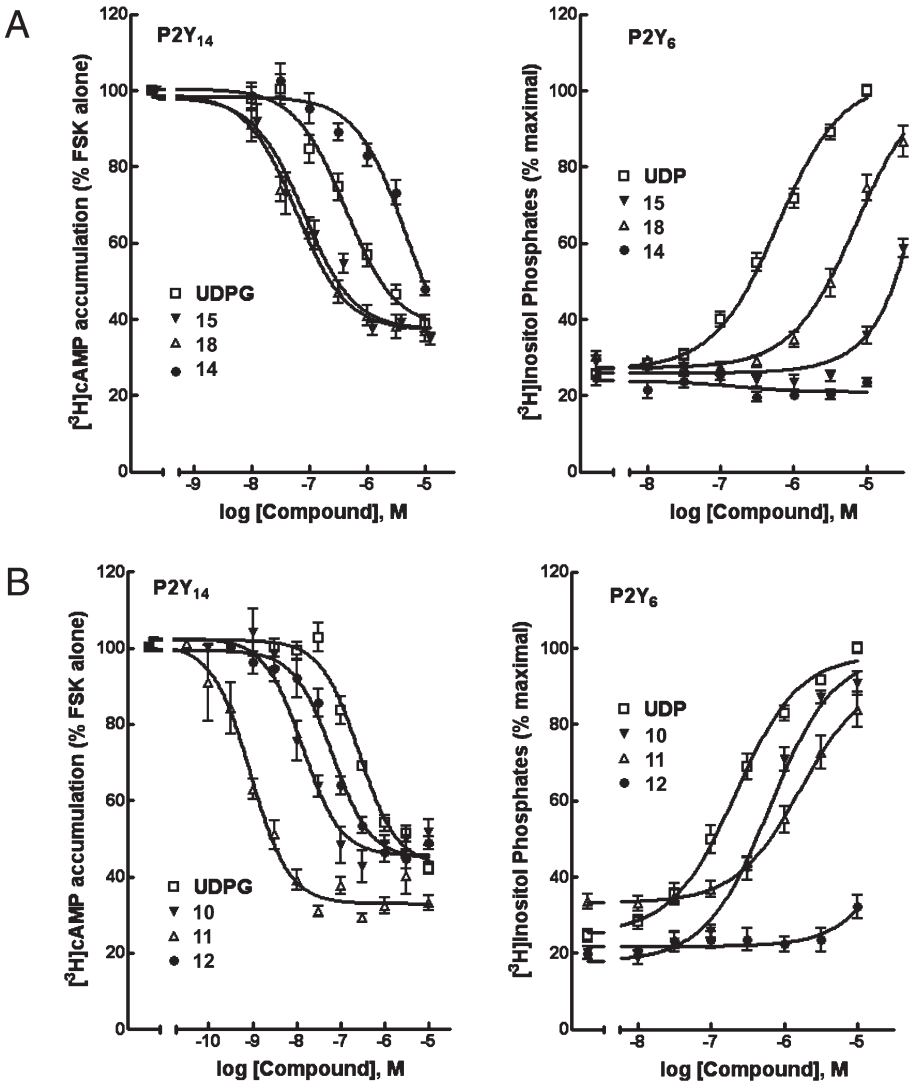


Figure 1. (A) Activation of Gi-coupled $P2Y_{14}$ receptor (left panel) was assessed by quantification of inhibition of forskolin-stimulated [^{3}H]cyclic AMP accumulation in HEK-293 cells stabling expressing the $hP2Y_{14}$ receptor. Activation of the Gq-coupled $P2Y_6$ receptor (right panel) was assessed by quantification of [^{3}H]inositol phosphate accumulation in 1321N1 human astrocytoma cells stably expressing the $hP2Y_6$ receptor by agonists Up_2-OMe **14**, 2-thio-Up_2-OMe **15**, and its *O*-propyl ester **18**. (B) Activation of Gi-coupled $P2Y_{14}$ receptor (left panel) was assessed by quantification of inhibition of forskolin-stimulated [^{3}H]cyclic AMP accumulation in HEK-293 cells stabling expressing the $hP2Y_{14}$ receptor. Activation of the Gq-coupled $P2Y_6$ receptor (right panel) was assessed by quantification of [^{3}H]inositol phosphate accumulation in 1321N1 human astrocytoma cells stably expressing the $hP2Y_6$ receptor by methylene-bridged agonist **10**, its 2-thio analogue **11**, and the difluoromethylene-bridged agonist **12**.

The native agonist **1** is a β-ester of the diphosphate moiety, which prompted us to explore the potency of structurally simpler β-esters at the $P2Y_{14}$ receptor. Since compound **14**, modified with a methyl ester group instead of glucose, weakly activated the $P2Y_{14}$ receptor and was inactive at the $P2Y_6$ receptor, this methyl ester was combined with other modifications to probe the effects on biological activity. The combination with the 2-thio modification in compound **15** increased potency by 42-fold. Thus, compound **15** was >180-fold selective for the $P2Y_{14}$ receptor. The 4-thio modification in methyl ester **16** abolished and greatly reduced potency in comparison to **15** at the $P2Y_{14}$ and $P2Y_6$ receptors, respectively.

In the 2-thio series, homologation of the alkyl ester group of **15** was tolerated at the $P2Y_{14}$ receptor, with the ethyl **17** and propyl **18** derivatives both displaying EC_{50} values of $\sim$40 nM and >200-fold selectivity in comparison to the $P2Y_6$ receptor. A 2-cyanoethyl group in **19** was poorly tolerated at the $P2Y_{14}$ receptor. Substitution of the β-methyl ester with a *tert*-butyl ester group in **25** produced an equipotent agonist, which was 8-fold more potent than the corresponding 2-oxo analogue **24**. The β-*tert*-butyloxy ester of 2-thio-UDP **25** was 11-fold more potent than UDPG. Introduction of a glyceryl moiety esterified through the 2-hydroxyl group in **23** did not diminish the potency of **3**, but an isomeric glyceryl ester **22** was 10-fold less potent than **3**.

Alkynyl ester derivatives **20** (2-oxo) or **21** (2-thio) were either 2-fold less potent or 16-fold more potent, respectively, than **3** at the $P2Y_{14}$ receptor. Compound **21** was >900-fold selective in comparison to the $P2Y_6$ receptor and was designed for coupling to azide-containing molecules by click chemistry.

β-Aryloxy and cycloalkyloxy esters **26**−**30** of UDP were found to only weakly activate the receptor in the order of potency phenyl > 4-nitrophenyl > 3-chloro- and 4-methoxyphenyl > cyclohexyl.

Discussion

In the present study, we have further expanded the range of potent $P2Y_{14}$ receptor ligands through a systematic exploration of the SAR of structurally simple analogues at this receptor, particularly with respect to substitution of the glucose moiety with diverse alkyl moieties. We have found that the introduction of α,β-methylene and difluoromethylene groups is well tolerated at the $P2Y_{14}$ receptor. These carbon-bridged nucleotides, including α,β-methylene-UDP with an EC_{50} value of 11 nM, are expected to display greater stability in biological systems. Methylene-bridged nucleotides tend to have increased stability by impeding breakdown by ecto-nucleotidases present on the cell surface,[27−29] and thus, these carbon-bridged diphosphate analogues have good potential for in vivo applications.

The 2-thio modification, but not the 4-thio modification of the uracil ring, tended to increase potency and selectivity at the $P2Y_{14}$ receptor in comparison to the $P2Y_6$ receptor. The most selective compounds (fold selectivity for $P2Y_{14}$ in comparison to $P2Y_6$) in the present study were UDP analogues **4** (233), **11** (2160), **12** (> 150), **15** (> 180), **17** (> 250), **18** (230), and **21** (> 900). The most potent of these were 2-thio-UDP **4** and α,β-methylene-2-thio-UDP **11**. Compound **4** had a high potency at the $P2Y_{14}$ receptor, with an EC_{50} value of 1.92 nM, and the corresponding α,β-methylene analogue **11** was even twice as potent. Although it was a less potent $P2Y_{14}$ receptor agonist, difluoromethylene-UDP **12** did not activate the $P2Y_6$ receptor at the concentrations of analogue tested. Other compounds that displayed lesser selectivity for the $P2Y_{14}$ receptor were (fold) **25** (64), **23** (59), **24** (43), and **10** (31). Thus, the 2-thio derivatives **4**, **11**, and their congeners may serve as important pharmacological probes for the detection and characterization of the $P2Y_{14}$ receptor.

It is now clear that the glucose moiety of **1** is not required for activation of the $P2Y_{14}$ receptor, although when it is present, there is a SAR pattern related to interaction of specific functionality of this hexose moiety with the receptor.[13,26] We conclude here that UDP and its analogues are potent full agonists of the human $P2Y_{14}$ receptor, consistent with our recent pharmacological studies of this receptor stably expressed in three different cell lines that utilized native Gi proteins for signal transduction.[14] This finding contradicts the original work of Fricks et al. who reported that UDP is a partial agonist/competitive antagonist of the human $P2Y_{14}$ receptor while acting as a full agonist at the rat $P2Y_{14}$ receptor.[16] However, the latter study quantified activity of a recombinant $P2Y_{14}$ receptor transiently coexpressed with an unnatural chimeric G protein, Gαq/i, that couples Gi-activating receptors to activation of PLC-β isozymes. It was suggested that this system favors "a conformation of the $P2Y_{14}$-R..., which results in ligand binding selectivities and agonist activities that are not altogether consistent with activities of the receptor obtained when coupled to its cognate heterotrimeric G protein".[14] Thus, the efficacy of UDP appears to be a function of the G protein signaling system activated by the receptor.

In conclusion, we have identified new analogues of UDP and its simple esters that display enhanced potency and selectivity for the $P2Y_{14}$ receptor and that promise to be useful as pharmacological tools to distinguish the effects of uracil nucleotides acting at $P2Y_{14}$ versus $P2Y_6$ receptors. The nanomolar potency achieved in this series is until now unprecedented for small molecular ligands of the $P2Y_{14}$ receptor. It is now possible to examine these potent agonists on human cells expressing an endogenous $P2Y_{14}$ receptor to aid in delineating a role for this receptor.

Experimental Section

Chemical Synthesis. ^{1}H NMR spectra were obtained with a Varian Gemini 300 spectrometer using D_2O 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 by use of a Varian XL 300 spectrometer (121.42 MHz); orthophosphoric acid (85%) was used as an external standard.

Purity and extent of reaction of nucleotide derivatives was checked 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), linear gradient solvent system of 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)−CH_3CN from 80:20 to 40:60 in 20 min with a flow rate of 1 mL/min (system A), or Zorbax SB-Aq 5 μm analytical column (50 mm × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). Mobile phase for linear gradient solvent system was 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)−CH_3CN from 80:20 to 40:60 in 13 min. The flow rate was 0.5 mL/min (system B). Peaks were detected by UV absorption (254 nm) with a diode array detector.

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. Some of the compounds were additionally purified by HPLC with a Luna 5 μm RP-C18(2) semipreparative column (250 mm × 10.0 mm; Phenomenex, Torrance, CA) and using a linear gradient solvent system of 10 mM TEAA (triethylammonium acetate)−CH_3CN from 100:0 to 95:5 (or up to 99:1 to 90:10) in 30 min with a flow rate of 2 mL/min. The tested nucleotide derivatives were confirmed by HPLC to possess a ≥95% purity. Compounds purified by HPLC were isolated as the triethylammonium salts. Compounds **41** and **45**, other reagents, and solvents were purchased from Sigma-Aldrich (St. Louis, MO).

General Procedure for the Preparation of Uridine 5′-Diphosphoglucose Analogues. The appropriate alkyl or aryl monophosphate was first converted to its tributylammonium salt form by treatment with ion-exchange resin (DOWEX 50WX2-200 (H)), which upon acidification of the supernatant was followed by addition of tributylamine until a basic pH was reached. The aqueous solvent was removed by lyophilization, and the residue was used without further purification. A solution in DMF (1.5 mL) of uridine 5′-monophosphate morpholidate 4-morpholine-*N*,*N*-dicyclohexylcarboxamidine salt (20 mg, 0.029 mmol) and the corresponding monophosphate (0.035 mmol, tributylammonium salt, was stirred at room temperature for 2 days. After that the solvent was removed and the residue was purified by ion-exchange column chromatography using a Sephadex-DEAE A-25 resin (with a linear gradient (0.01−0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase) to provide the corresponding nucleotide as the ammonium salt. Some of the uridine 5′-diphosphoglucose analogues were additionally purified by HPLC as described above.

Diphosphoric Acid 1-β-Methyl (C-P) 2-(Uridine-5′-yl)ester, Triethylammonium Salt (6). Compound **6** (4.0 mg, 23%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.96 (d, J = 8.1 Hz, 1H), 6.00 (d, J = 4.8 Hz, 1H), 5.97 (d, J = 7.8 Hz, 1H), 4.38 (m, 2H), 4.29 (m, 1H), 4.21 (m, 2H), 1.46 (d, J = 16.8 Hz, 3H); ^{31}P NMR (D_2O) δ 17.82 (d, J = 23.9 Hz), −11.06 (d, J = 23.8 Hz); HRMS-EI found 401.0158 $(M - H^+)^-$. $C_{10}H_{15}N_2O_{11}P_2$ requires 401.0151; purity >98% by HPLC (system A, 13.3 min).

Diphosphoric Acid 1-β-Methyl Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (14). Compound **14** (3.9 mg, 30%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.97 (d, J = 8.1 Hz, 1H), 6.01 (dd, J = 3.6, 1.2 Hz,

1H), 5.97 (d, J = 8.1 Hz, 1H), 4.38 (m, 2H), 4.29 (m, 1H), 4.20 (m, 2H), 3.66 (d, J = 11.4 Hz, 3H); ^{31}P NMR (D_2O) δ −9.22 (d, J = 21.4 Hz), −11.03 (d, J = 21.4 Hz); HRMS-EI found 417.0087 $(M - H^+)^-$. $C_{10}H_{15}N_2O_{12}P_2$ requires 417.0100; purity >98% by HPLC (system A, 11.3 min).

Diphosphoric Acid 1-β-(2-Cyanoethyl) Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (19). Compound **19** (3.7 mg, 26%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.99 (d, J = 8.4 Hz, 1H), 5.99 (m, 2H), 4.89 (m, 2H), 4.26 (m, 3H), 4.16 (dd, J = 12.9, 6.0 Hz, 2H), 2.87 (t, J = 6.07 Hz, 2H); ^{31}P NMR (D_2O) δ −11.18 (dd, J = 42.8, 21.4 Hz); HRMS-EI found 456.0212 $(M - H^+)^-$. $C_{12}H_{16}N_3O_{12}P_2$ requires 456.0209; purity >98% by HPLC (system A, 12.8 min).

Diphosphoric Acid 1-β-(3-Butynyl) Ester 2-(Uridine-5′-yl)-ester, Triethylammonium Salt (20). Compound **20** (5.31 mg, 28%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.88 (d, J = 8.1 Hz, 1H), 5.98 (m, 2H), 4.41 (m, 1H), 4.21 (m, 3H), 4.08 (m, 1H), 3.83 (m, 2H), 2.53 (m, 3H); ^{31}P NMR (D_2O) δ −9.61 (d, J = 16.5 Hz), −11.2 (m); HRMS-EI found 455.0257 $(M - H^+)^-$. $C_{13}H_{17}N_2O_{12}P_2$ requires 455.0265; purity >97% by HPLC (system B, 6.89 min).

Diphosphoric Acid 1-α-Glycerol Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (22). Compound **22** (2.2 mg, 15%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.96 (d, J = 8.4 Hz, 1H), 5.60 (m, 2H), 4.40 (m, 2H), 4.26 (m, 3H), 3.99 (m, 3H), 3.68 (m, 2H); ^{31}P NMR (D_2O) δ −10.35 (d, J = 21.4 Hz), −10.97 (d, J = 20.8 Hz); HRMS-EI found 477.0315 $(M - H^+)^-$. $C_{12}H_{19}N_2O_{14}P_2$ requires 477.0312; purity >98% by HPLC (system A, 13.6 min).

Diphosphoric Acid 1-β-Glycerol Ester 2-(Uridine-5′-yl)ester, Triethylammonium Salt (23). Compound **23** (3.1 mg, 16%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.97 (d, J = 7.8 Hz, 1H), 6.01 (m, 1H), 5.98 (d, J = 7.8 Hz, 1H), 4.39 (m, 2H), 4.30 (m, 2H), 4.24 (m, 2H), 3.77 (m, 4H); ^{31}P NMR (D_2O) δ −10.89 (dd, J = 37.9, 21.4 Hz); HRMS-EI found 477.0302 $(M - H^+)^-$. $C_{12}H_{19}N_2O_{14}P_2$ requires 477.0312; purity >98% by HPLC (system A, 11.2 min).

Diphosphoric Acid 1-β-*tert*-Butyl Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (24). Compound **24** (4.4 mg, 31%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.99 (d, J = 7.8 Hz, 1H), 5.99 (m, 2H), 4.39 (m, 2H), 4.29 (m, 1H), 4.24 (m, 2H), 1.44 (d, J = 0.3 Hz, 9H); ^{31}P NMR (D_2O) δ −11.51 (d, J = 21.4 Hz), −14.73 (d, J = 21.4 Hz); HRMS-EI found 459.0565 $(M - H^+)^-$. $C_{13}H_{21}N_2O_{12}P_2$ requires 459.0570; purity >98% by HPLC (system A, 13.6 min).

Diphosphoric Acid 1-β-Cyclohexyl Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (26). Compound **26** (6.2 mg, 41%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 8.00 (d, J = 8.4 Hz, 1H), 6.00 (m, 2H), 4.40 (m, 2H), 4.30 (m, 1H), 4.24 (m, 2H), 4.20 (m, 1H), 1.97 (m, 2H), 1.72 (m, 2H), 1.50−1.10 (m, 6H); ^{31}P NMR (D_2O) δ −11.26; HRMS-EI found 485.0719 $(M - H^+)^-$. $C_{15}H_{23}N_2O_{12}P_2$ requires 485.0726; purity >98% by HPLC (system A, 11.8 min).

Diphosphoric Acid 1-β-Phenyl Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (27). Compound **27** (4.7 mg, 32%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.87 (d, J = 8.4 Hz, 1H), 7.36 (m, 2H), 7.19 (m, 3H), 5.93 (d, J = 5.1 Hz, 1H), 5.81 (d, J = 8.4 Hz, 1H), 4.30 (m, 1H), 4.25 (m, 2H), 4.20 (m, 1H), 4.15 (m, 1H); ^{31}P NMR (D_2O) δ −11.11 (d, J = 21.5 Hz), −15.74 (d, J = 20.8 Hz); HRMS-EI found 479.0247 $(M - H^+)^-$. $C_{15}H_{17}N_2O_{12}P_2$ requires 479.0257; purity >98% by HPLC (system A, 13.1 min).

Diphosphoric Acid 1-β-(4-Nitrophenyl) Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (28). Compound **28** (6.1 mg, 38%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 8.21 (d, J = 9.3 Hz, 2H), 7.76 (d, J = 7.83 Hz, 1H), 7.37 (d, J = 9.0 Hz, 2H), 5.92 (m, 1H), 5.69 (d, J = 7.5 Hz, 1H), 4.25 (m, 4H), 4.14 (m, 1H); ^{31}P NMR (D_2O) δ −11.15 (d, J = 20.8 Hz), −17.12 (d, J = 20.8 Hz); HRMS-EI found 524.0110 $(M - H^+)^-$. $C_{15}H_{16}N_3O_{14}P_2$ requires 524.0108; purity >98% by HPLC (system A, 13.7 min).

Diphosphoric Acid 1-β-(3-Chlorophenyl) Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (29). Compound **29** (3.7 mg, 25%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.85(d, J = 8.1 Hz, 1H), 7.33 (m, 3H), 7.17 (m, 1H), 5.95 (d, J = 4.2 Hz, 1H), 5.76 (d, J = 8.1 Hz, 1H), 4.29 (m, 4H), 4.18 (m, 1H); ^{31}P NMR (D_2O) δ −11.04 (d, J = 21.3 Hz), −16.18 (d, J = 21.3 Hz); HRMS-EI found 512.9867 $(M - H^+)^-$. $C_{15}H_{16}N_2O_{12}ClP_2$ requires 512.9854; purity >98% by HPLC (system B, 8.6 min).

Diphosphoric Acid 1-β-(4-Methoxyphenyl) Ester 2-(Uridine-5′-yl)ester, Ammonium Salt (30). Compound **30** (3.3 mg, 21%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.82 (d, J = 8.1 Hz, 1H), 7.18 (d, J = 8.7 Hz, 2H), 6.93 (d, J = 8.7 Hz, 2H), 5.95 (d, J = 4.8 Hz, 1H), 5.77 (d, J = 8.1 Hz, 1H), 4.29 (m, 4H), 4.16 (m, 1H); ^{31}P NMR (D_2O) δ −11.04 (d, J = 21.3 Hz), −15.37 (d, J = 21.3 Hz); HRMS-EI found 509.0362 $(M - H^+)^-$. $C_{16}H_{19}N_2O_{13}P_2$ requires 509.0353; purity >98% by HPLC (system B, 8.84 min).

General Procedure for the Preparation of Diphosphoric Acid 1-Alkyl Ester 2-(Thiouridine-5′-yl)ester (15−18, 21, and 25). Monophosphates (**42**−**44**) were prepared from the corresponding alcohols (**37**−**39**) using a published procedure,[21] and the spectral data were consistent with the assigned structures.

Each of the prepared monophosphates (**42**−**44**)[21] or commercially available monophosphates (**41**, **45**) was transformed to its tributylammonium salt by treatment with ion-exchange resin followed by the addition of tributylamine. 1,1′-Carbonyldiimidazole (10 mg, 0.06 mmol) was added to the alkyl monophosphate tributylammonium salts (0.017 mmol) (**41**−**45**) in DMF (1 mL). The reaction mixture was stirred at room temperature for 5 h. Methanol (1 mL) was added, and stirring was continued at room temperature for an additional 1 h. After removal of the solvent, the residue was dried under high vacuum and dissolved in DMF (1.5 mL). 2-Thiouridine 5′-monophosphate tributylammonium salt (**34**)[12] (5.7 mg, 0.008 mmol) for **15**, **17**, **18**, **21**, and **25** or 4-thiouridine 5′-monophosphate tributylammonium salt (**35**)[12] (5.7 mg, 0.008 mmol) for **16** was added to the reaction mixture, and it was stirred at room temperature for 2 days. After removal of the solvent, the residue was purified by the same method as the general procedure using Sephadex-DEAE A-25 resin. Compounds **16** and **21** were further purified by HPLC to provide homogeneous products.

Diphosphoric Acid 1-β-Methyl Ester 2-(2-Thiouridine-5′-yl)-ester, Ammonium Salt (15). Compound **15** (0.82 mg, 22%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 8.15 (d, J = 8.1 Hz, 1H), 6.70 (d, J = 3.6 Hz, 1H), 6.24 (d, J = 8.1 Hz, 1H), 4.43 (m, 1H), 3.34 (m, 3H), 4.24 (m, 1H), 3.66 (d, J = 11.7 Hz, 3H); ^{31}P NMR (D_2O) δ −10.68 (dd, J = 21.5, 6.6 Hz), −11.05 (d, J = 21.4 Hz); HRMS-EI found 432.9886 $(M - H^+)^-$. $C_{10}H_{15}N_2O_{11}P_2S$ requires 432.9872; purity >98% by HPLC (system A, 12.9 min).

Diphosphoric Acid 1-β-Methyl Ester 2-(4-Thiouridine-5′-yl)-ester, Triethylammonium Salt (16). Compound **16** (0.91 mg, 18%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 7.9 (d, J = 7.7 Hz, 1H), 6.69 (d, J = 4.3 Hz, 1H), 5.99 (d, J = 7.5 Hz, 1H), 4.40 (m, 3H), 4.30 (m, 2H), 3.69 (m, 3H); ^{31}P NMR (D_2O) δ −10.83 (d, J = 21.3, Hz), −11.12 (d, J = 21.3 Hz); HRMS-EI found 432.9884 $(M - H^+)^-$. $C_{10}H_{15}N_2O_{11}P_2S$ requires 432.9872; purity >98% by HPLC (system B, 7.2 min in negative absorbance).

Diphosphoric Acid 1-β-Ethyl Ester 2-(2-Thiouridine-5′-yl)-ester, Ammonium Salt (17). Compound **17** (7.7 mg, 20%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 8.22 (d, J = 8.1 Hz, 1H), 6.72 (d, J = 3.0 Hz, 1H), 6.32 (d, J = 8.4 Hz, 1H), 4.49 (m, 1H), 3.39 (m, 3H), 4.29 (m, 1H), 4.09 (m, 2H), 1.33 (m, 3H); ^{31}P NMR (D_2O) δ −10.39 (t, J = 21.3, 42.7 Hz), −11.08 (d, J = 21.3 Hz); HRMS-EI found 447.0022 $(M - H^+)^-$. $C_{11}H_{17}N_2O_{11}P_2S$ requires 447.0028; purity >98% by HPLC (system B, 7.09 min).

Diphosphoric Acid 1-β-Propyl Ester 2-(2-Thiouridine-5′-yl)-ester, Ammonium Salt (18). Compound **18** (0.91 mg, 23%) was obtained as a white solid following the general procedure. ^{1}H NMR ^{1}H NMR (D_2O) δ 8.21 (d, J = 8.4 Hz, 1H), 6.69 (d, J = 3.1 Hz, 1H), 6.28 (d, J = 8.4 Hz, 1H), 4.47 (m, 1H), 3.41 (m, 3H), 4.26 (m, 1H), 3.95 (m, 2H), 1.65 (m, 2H); 0.96 (m, 3H); ^{31}P NMR (D_2O) δ −10.68 (d, J = 21.5, 4.), −11.10 (d, J = 21.3 Hz); HRMS-EI found 461.0145 $(M - H^+)^-$. $C_{12}H_{19}N_2O_{11}P_2S$ requires 461.0151; purity >98% by HPLC (system B, 7.4 min).

Diphosphoric Acid 1-β-(3-Butynyl) Ester 2-(2-Thiouridine-5′-yl)ester, Triethylammonium Salt (21). Compound **21**(1.18 mg, 22%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 8.12 (d, J = 8.1 Hz, 1H), 6.79 (d, J = 3.1 Hz, 1H), 6.23 (d, J = 8.1 Hz, 1H), 4.43 (m, 1H), 4.35 (m, 1H), 4.10 (m, 3H), 3.89 (m, 2H), 2.54 (m, 2H), 2.41 (m, 1H); ^{31}P NMR (D_2O) δ −9.63 (d, J = 21.3), −11.12 (d, J = 21.3 Hz); HRMS-EI found 471.0022 $(M - H^+)^-$. $C_{13}H_{17}N_2O_{11}P_2S$ requires 471.0035; purity >97% by HPLC (system B, 6.9 min).

Diphosphoric Acid 1-β-*tert*-Butyl Ester 2-(2-Thiouridine-5′-yl)ester, Ammonium Salt (25). Compound **25** (0.83 mg, 21%) was obtained as a white solid following the general procedure. ^{1}H NMR (D_2O) δ 8.26 (d, J = 8.4 Hz, 1H), 6.68 (d, J = 3.0 Hz, 1H), 6.26 (d, J = 8.1 Hz, 1H), 4.41 (m, 1H), 3.36 (m, 3H), 4.27 (m, 1H), 1.47 (s, 9H); ^{31}P NMR (D_2O) δ −11.00 (d, J = 21.3, Hz), −11.53 (d, J = 21.3 Hz); HRMS-EI found 475.0341 $(M - H^+)^-$. $C_{13}H_{21}N_2O_{11}P_2S$ requires 475.0341; purity >98% by HPLC (system B, 7.16 min).

Preparation of 2-Thiouridine-5′-α,β-methylene Diphosphate, Triethylammonium Salt (11). A solution of 2-thiouridine (13 mg, 0.05 mmol) and DCC (30 mg 0.15 mmol) was stirred in DMF under a nitrogen atmosphere. Methylenediphosphonic acid (13 mg, 0.075 mmol) was added, and the stirring was continued for 48 h. The solvent was removed, and the product was purified by ion exchange column chromatography with a Sephadex-DEAE A-25 resin, followed by semipreparative HPLC as described above. Compound **11** (11.8 mg, 33%) was obtained as a white solid. ^{1}H NMR (D_2O) δ 8.22 (d, J = 7.8 Hz, 1H), 6.65 (d, J = 3.2 Hz, 1H), 6.28 (d, J = 7.8 Hz, 1H), 4.47 (m, 2H), 4.37 (m, 2H), 4.26 (m, 1H), 1.47 (t, J = 19.2 Hz, 2H); ^{31}P NMR (D_2O) δ 16.70, 13.32 (m); HRMS-EI found 416.9935 $(M - H^+)^-$. $C_{10}H_{15}N_2O_{10}P_2S$ requires 416.9923; purity >98% by HPLC (system B, 7.04 min).

Assay of $P2Y_6$ Receptor-Stimulated PLC Activity. Activity at the $hP2Y_6$ receptor was quantified in 1321N1 human astrocytoma cells stably expressing this receptor. Twenty-four hours after plating transfection, the inositol lipid pool of the cells was radiolabeled by incubation in 200 μL of serum-free inositol-free Dulbecco's modified Eagle's medium containing 0.4 μCi of *myo*-[^{3}H]inositol. No changes of medium were made subsequent to the addition of [^{3}H]inositol. Forty-eight hours after transfection, cells were challenged with 50 μL of the 5-fold concentrated solution of receptor agonists in 200 mM HEPES, pH 7.3, containing 50 mM LiCl for 20 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. After 15 min at 4 °C, samples were neutralized with 150 μL of 150 mM NH_4OH. [^{3}H]Inositol phosphates were isolated by ion exchange chromatography on Dowex AG 1-X8 columns as previously described.

Assay of $P2Y_{14}$ Receptor-Inhibited Accumlation of 3′,5′-Cyclic Adenosine Monophosphate (cAMP). Cell Culture. Human embryonic kidney-293 cells stably expressing the $hP2Y_{14}$-R ($P2Y_{14}$-HEK293 cells) were generated as previously described by Fricks et al.[16] $P2Y_{14}$-HEK293 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Genticin (Gibco), and 1% antibiotic−antimocotic (Gibco) at 37 °C in a 5% CO_2 environment.

cAMP Accumulation. $P2Y_{14}$-HEK293 cells were grown in 24-well plates and incubated with 1 μCi [^{3}H]adenine/well in serum-free DMEM for at least 2 h prior to assay. Assays were initiated by addition of HEPES-buffered, serum-free DMEM containing 200 μM 3-isobutyl-1-methylxanthine (IBMX) and 30 μM forskolin, with or without drugs, and incubation continued for 15 min at 37 °C. Incubations were terminated by aspiration of the medium and addition of 450 mL of ice-cold 5% trichloroacetic acid. [^{3}H]cAMP was isolated by sequential Dowex and alumina chromatography and quantified by liquid scintillation counting[14,16] as previously described by Harden et al.[30]

Data Analysis. Agonist potencies (EC_{50} values) were obtained from concentration−response curves by nonlinear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, CA). All experiments were performed in triplicate assays and repeated at least three times. 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.

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Supporting Information Available: NMR spectral data and HPLC traces of selected nucleotide derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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