Synthesis and Structure−Activity Relationships of Uracil Nucleotide Derivatives and Analogues as Agonists at Human P2Y$_2$, P2Y$_4$, and P2Y$_6$ Receptors

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A series of UTP, UDP, and UMP derivatives and analogues were synthesized and evaluated at the human pyrimidinergic P2Y receptor subtypes P2Y$_2$, P2Y$_4$, and P2Y$_6$ stably expressed in 1321N1 astrocytoma cells. Substituents at N3 of UTP were poorly tolerated by P2Y$_2$ and P2Y$_4$ receptors. In contrast, a large phenacyl substituent at N3 of UDP was well tolerated by the P2Y$_6$ receptor, yielding a potent and selective P2Y$_6$ receptor agonist (3-phenacyl-UDP, EC$_{50}$ = 70 nM, >500-fold selective). The most potent and selective P2Y$_2$ receptor agonist of the present series was 2-thio-UTP (EC$_{50}$ = 50 nM, ≥30-fold selective vs P2Y$_4$ and P2Y$_6$). All modifications at the uracil base of UTP led to a decrease in potency at the P2Y$_4$ receptor. A β,γ-dichloromethylene modification in the triphosphate chain of 5-bromo-UTP was tolerated by all three receptor subtypes, thus opening up a new strategy to obtain ectonucleotide diphosphohydrolase- and phosphatase-resistant P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptor agonists.

Introduction

The pyrimidine nucleotides UTP and UDP have been recognized as important signalling molecules activating G-protein-coupled membrane receptors (GPCRs) of the P2 (nucleotide) receptor family.[1,2] P2 receptors are subdivided into a subfamily of ligand-gated ion channels (P2X receptors) activated by ATP[3] and a family of GPCRs that consists of at least eight members, P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{11}$, P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$.[4] While the human P2Y$_1$, P2Y$_{11}$, P2Y$_{12}$, and P2Y$_{13}$ receptors are solely activated by adenine nucleotides (P2Y$_{1,12,13}$ by ADP, P2Y$_{11}$ by ATP), the other P2Y subtypes may additionally (P2Y$_2$) or are exclusively activated by uracil nucleotides. The P2Y$_2$ and P2Y$_4$ receptors respond to UTP (**1**), whereas the P2Y$_6$ receptor is activated by UDP (**2**) and the P2Y$_{14}$ receptor by the nucleotide sugar UDPglucose.[2,4] On the basis of structural similarities, two distinct clusters of P2Y receptor subtypes can be identified: P2Y$_{12,13,14}$ receptors that are coupled to inhibition of adenylate cyclase, and P2Y$_{1,2,4,6,11}$ receptors that are coupled to activation of phospholipase C leading to the formation of inositol trisphosphate (IP$_3$) and subsequent mobilization of intracellular calcium.[5]

P2Y receptors have been found to be implicated in a variety of pathophysiological states such as lung diseases, cancer progression, as well as vascular, inflammatory, and immune diseases.[4,9,10] P2Y$_2$ receptor agonists are currently undergoing clinical evaluation for the symptomatic treatment of cystic fibrosis (denufosol, **5**)[11] and dry eye syndrome (diquafosol, **4**).[12] However, both compounds are only moderately selective (Figure 1). The lack of specific and metabolically stable ligands (agonists and antagonists) for each of the P2Y receptor subtypes has hampered a full evaluation of the potential of the P2Y receptors as drug targets. In the present study we focused our attention on the UTP- and UDP-activated P2Y receptor subtypes P2Y$_2$, P2Y$_4$, and P2Y$_6$. Only limited information on the structure−activity relationships for agonists at these uracil nucleotide receptor subtypes has been available.[4,13] The physiological agonist UTP (**1**) is one of the most potent P2Y$_2$ agonists, but the nucleotide is also very active at P2Y$_4$ receptors (see Figure 1). Dinucleotides such as Up$_4$U (diquafosol, **4**) and denufosol (**5**) are somewhat weaker and also exhibit only low selectivity for P2Y$_2$ versus P2Y$_4$ but show increased metabolic stability. Up$_4$U has been postulated to be a physiological agonist at those receptors acting as a novel endothelium-derived vasoconstrictive factor.[14] Other UTP derivatives that are relatively potent P2Y$_2$ receptor agonists include the sulfur-containing nucleotides UTPγS and 4-thio-UTP and the ribose-modified UTP analogue 2′-deoxy-2′-amino-UTP, all of which are only moderately selective or nonselective versus P2Y$_4$ receptors.[4,15] Little is known about the structure−activity relationships of UTP derivatives and analogues at P2Y$_4$ receptors, while only few potent agonists for the UDP-sensitive P2Y$_6$ receptor subtype have been described, including UDPβS and 2′-deoxy-(*S*)-methanocarba-UDP (**3**).[4,6]

In the present study, we (re)synthesized a series of base-(pyrimidine-) modified UTP, UDP, and UMP derivatives as potential ligands for P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptors. We selected UTP rather than ATP as a lead compound in the case of the UTP- and ATP-activated P2Y$_2$ receptor because of the expected higher selectivity of pyrimidine nucleotides versus the other P2 receptors that are activated by the purine nucleotide ATP or ADP. In addition, the degradation products of ATP and its analogues (the nucleoside adenosine and analogues) may interact with adenosine (P1) receptors and thus lead to unwanted side effects.[16] The present study led, among others, to the discovery of a new potent and selective P2Y$_6$ receptor agonist, 3-phenacyl-UDP (**10c**). Furthermore, we found that a β,γ-dichloromethylene modification of the triphosphate chain in 5-bromo-UTP, resulting in enhanced metabolic stability, is well tolerated by P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptors.

Synthesis

Base-modified uridine derivatives and analogues were synthesized according to published procedures with minor modifications (for details, see Supporting Information). The following base-modified uridine derivatives (N1-nucleosides) were ob-

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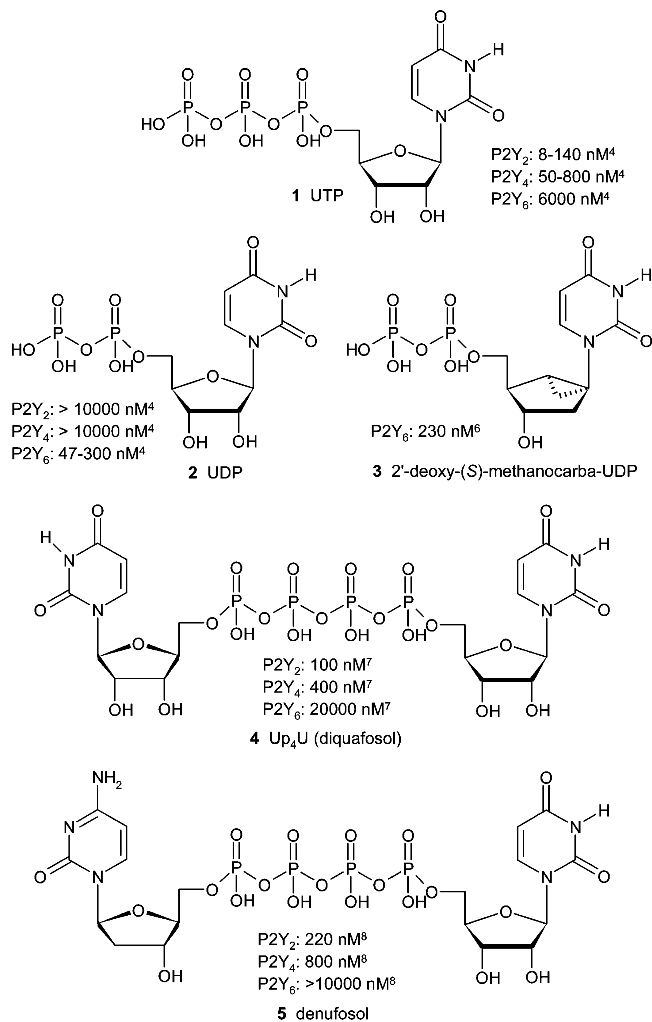


Figure 1. Structures of physiological and synthetic agonists at $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. Data are from functional assays (intracellular calcium mobilization or inositol trisphosphate accumulation) at human receptor subtypes.

tained: 2-thiouridine (2-thio-1-β-D-ribofuranosyl(3*H*)pyrimidine-2,4-dione, **6a**),[17] *N*3-methyluridine (3-methyl-1-β-D-ribofuranosylpyrimidine-2,4-dione, **6b**),[18] *N*3-phenacyluridine (3-phenacyl-1-β-D-ribofuranosylpyrimidine-2,4-dione, **6c**),[19] and 6-hydroxyuridine (1-β-D-ribofuranosyl-(3*H*)-pyrimidine-2,4,6-trione, barbituric acid riboside, **6e**).[20] In addition, two 6-substituted *N*3-ribosides were synthesized: the 6-methyluridine isomer 6-methyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione **(6f)**[21] and the 6-propyluridine isomer 6-propyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione (**6g**).[22]

The synthesized nucleosides and the commercially available 5-bromouridine (nucleosides **6a**−**g)** were subjected to phosphorylation according to the Ludwig procedure[23] with minor modifications (Scheme 1). The dry nucleosides (**6a**−**g**), which had been subjected to lyophilization, were dissolved in trimethylphosphate and reacted with phosphorus oxychloride in the presence of 1,8-bis(dimethylamino)naphthaline ("Proton Sponge") to yield the reactive 5′-dichlorophosphate intermediates **7a**−**g**. Those were immediately reacted with a mixture of 1 equiv of tri-*n*-butylamine and a 6-fold excess of bis(tri-*n*-butylammonium) pyrophosphate in DMF to afford the cyclotrimetaphosphate intermediates **8a**−**g**. Hydrolysis of **8a**−**g** with triethylammonium hydrogen bicarbonate buffer yielded the desired nucleoside triphosphates **9a**−**g**.

The phosphorylation process yielded a number of side products, including the monophosphates **11a**−**g** and, in some cases, the corresponding diphosphates (less than 10% with respect to the triphosphates). The formation of diphosphates may be explained either by hydrolysis of the pyrophosphate buffer, which can then react with the nucleoside 5′-dichlorophosphates (**7**), or by reaction of phosphorus oxychloride with **7** to afford the diphosphate derivatives, while the monophosphates may be formed by hydrolysis of the dichlorophosphates **7**. Further isolated and identified side products included compounds **12** and **13** (Figure 2).

The structures of the isolated compounds **12** and **13** were elucidated by mass spectrometry and NMR spectroscopy. Compound **12** showed four peaks in the ^{31}P NMR spectrum, three of which correspond to the 5′-triphosphate group (−11.69 (d, 1P, $J = 19.68$ Hz, P_α), −23.40 (t, 1P, $J = 20.91$ Hz, P_β), −10.73 (d, 1P, $J = 19.68$ Hz, P_γ), determined in methanol-d_4), and the fourth peak at 19.02 ppm integrating as one phosphorus atom representing the 2′,3′-cyclic phosphate (d, 1P, $J_{P,H2',3'} = 14.76$ Hz), in agreement with published data for related compounds.[24,25]

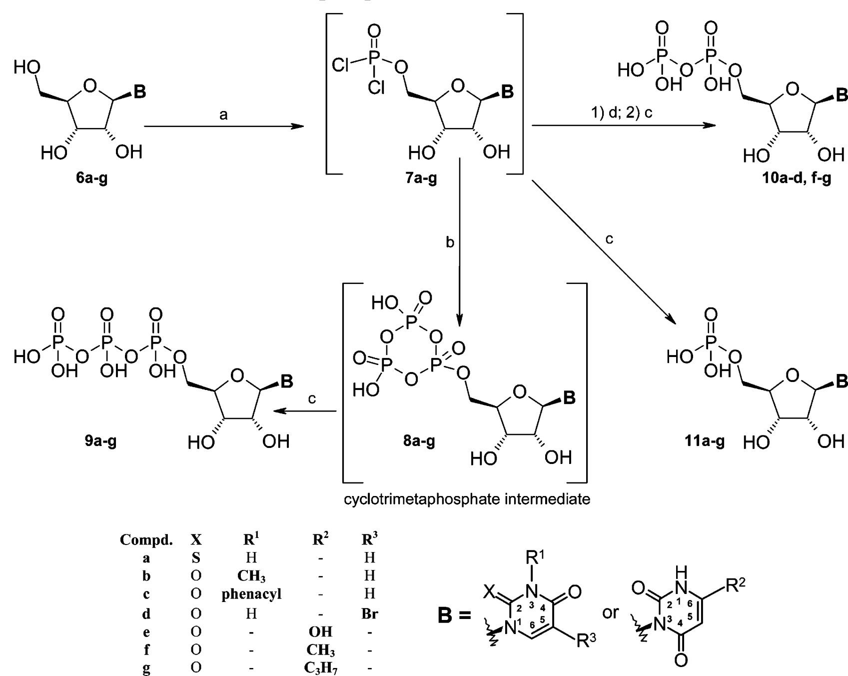
Compound **13** also showed four peaks in the ^{31}P NMR spectrum, three of them corresponding to the 5′-triphosphate group (−11.60 (d, 1P, $J = 19.68$ Hz, P_α), −22.50 (t, 1P, $J = 18.45$ Hz, P_β), −9.67 (d, 1P, $J = 17.22$ Hz, P_γ), determined in methanol-d_4), and the fourth peak at 18.43 ppm integrating as one phosphorus atom representing the 2′-phosphate (t, 1P, $J_{P,H} = 8.61$ Hz).[26] The ^{13}C NMR spectrum of **13** showed a doublet for C′1 at 93.73 ppm ($J_{C1',P2'} = 4.48$ Hz) and a downfield shift of the C1′ of 2.53 ppm in comparison with the signal for the 2′-unsubstituted derivative **9c**, indicating the phosphorylation of the 2′- rather than the 3′-hydroxyl group.[27] The ^1H NMR spectrum of **13** was also consistent with the proposed structure (see Experimental Section).[28]

In a previous study, in which 5-substituted UTP derivatives were synthesized, we had observed that phosphorylation of the 2′- and 3′-hydroxy groups as a side reaction was associated with bulky substitution of the pyrimidine base.[29] The phosphorylation of compounds such as 5-butyluridine had yielded a particularly high percentage of side products, including 5-butyl-UTP 2′-phosphate, 3′-phosphate, and 2′,3′-cyclophosphate. Electronic effects as well as steric interaction of the bulky butyl residue with the 5′-hydroxyl function in polar aprotic solvents might be responsible for the increased rate of phosphorylation of the secondary hydroxyl groups at C2′and C3′.[29] In the present study we observed the formation of the 2′,3′-cyclic phosphate in addition to the 5′-triphosphorylation of 6-propyl-3-β-D-ribofuranosyluracil (product **12**). In the case of *N*3-phenacyluridine we obtained the 2′-phosphorylated *N*3-phenacyl-UTP (**13**) as a side product. Enhanced formation of such side products was associated with bulky substituents in the 6 or N3 positions of the pyrimidine moiety.

Nucleoside diphosphates (**10a**−**d**,**f**,**g**, Scheme 1) were synthesized analogously by reaction of the nucleoside 5′-dichlorophosphates **7a**−**g** with tri-*n*-butylammonium phosphate buffer in dimethylformamide. Curiously, in some cases small amounts of triphosphates were produced during the synthesis of the diphosphates. Similar side reactions have previously been observed.[30] Nucleoside monophosphates (**11a**−**g**, Scheme 1) were obtained by hydrolyzing the nucleoside 5′-dichlorophosphates **7a**−**g** by treatment with triethylammonium hydrogen bicarbonate buffer.

A triphosphate-analogous structure containing a $P_\beta P\gamma$-dichloromethylene bridge was successfully introduced into 5-bromouridine (**6e**), yielding the nucleotide analogue **15**, by using the same procedure of phosphorylation as for triphosphates[23] with

Scheme 1. Synthesis of Nucleoside Mono-, Di-, and Triphosphates[a]



Compd.	X	R¹	R²	R³
a	S	H	-	H
b	O	CH_3	-	H
c	O	phenacyl	-	H
d	O	H	-	Br
e	O	-	OH	-
f	O	-	CH_3	-
g	O	-	C_3H_7	-

[a] Reagents and conditions: (a) $(OCH_3)_3PO$, $POCl_3$, 1,8-bis(dimethylamino)naphthalene (Proton Sponge), 5 h, 0 °C ;(b) bis(tri-*n*-butylammonium) pyrophosphate in DMF, 5 min, 0 °C; (c) triethylammonium hydrogen bicarbonate buffer, pH 7.4−7.6, room temp, 1 h; (d) tri-*n*-butylammonium phosphate in DMF, 5 min, 0 °C.

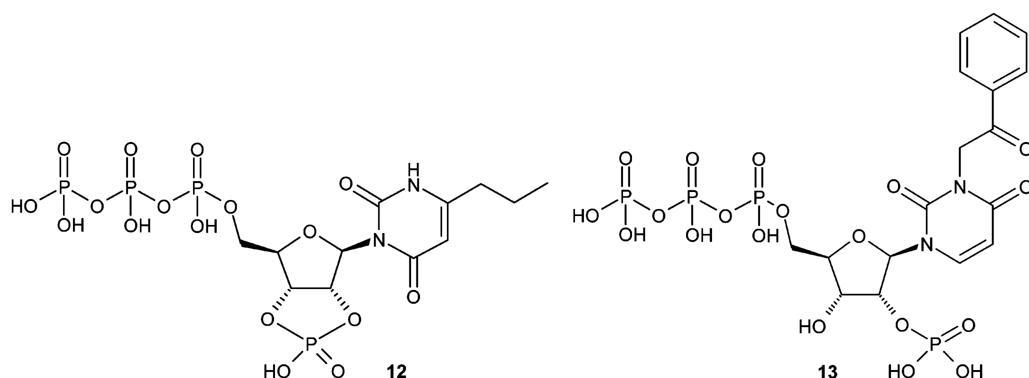


Figure 2. Structures of nucleotides **12** and **13** obtained as side products during the synthesis of nucleoside triphosphates **9g** and **9c.**

some modifications as shown in Scheme 2. Instead of bis(tri-*n*-butylammonium) pyrophosphate used for the synthesis of triphosphates, bis(tri-*n*-butylammonium)dichloromethylene diphosphonate in DMF was reacted with 5-bromouridine 5′-dichlorophosphate intermediate **7e** yielding **15**.

The performed syntheses of nucleotides generally yielded mixtures of products and side products. Therefore, purification represented the most important step in obtaining the target nucleotides and nucleotide analogues as pure products. In the first step the synthesized nucleotides were purified by anion exchange chromatography on Sephadex diethylaminoethyl (DEAE) A-25 gel using a fast protein liquid chromatography (FPLC) apparatus. The separation took place at 4 °C. The negatively charged nucleotides interact with the positively charged gel and are eluted from the gel by applying a linear gradient (0−100%, pure water → 0.5 M aqueous triethylammonium hydrogen carbonate buffer) according to the number of charges in the following sequence: monophosphates, diphosphates, triphosphates, and finally oligo- and polyphosphates.

In addition to various nucleotides, the products also contained large amounts of inorganic salts, such as inorganic phosphates, e.g., phosphate (PO_4^{3-}), pyrophosphate ($P_2O_7^{4-}$), linear triphosphate ($P_3O_{10}^{5-}$), and cyclotrimetaphosphate ($P_3O_9^{3-}$), as well as buffer components. Therefore, the products were further purified by high-performance liquid chromatography (HPLC) on reversed-phase C18 material. The stable cyclotrimetaphosphate ($P_3O_9^{3-}$) impurity had previously been reported to be difficult to remove by standard chromatographic methods because of its coelution with UTP derivatives,[29] but in the present study we did not detect it by ^{31}P NMR as an impurity in the final products.

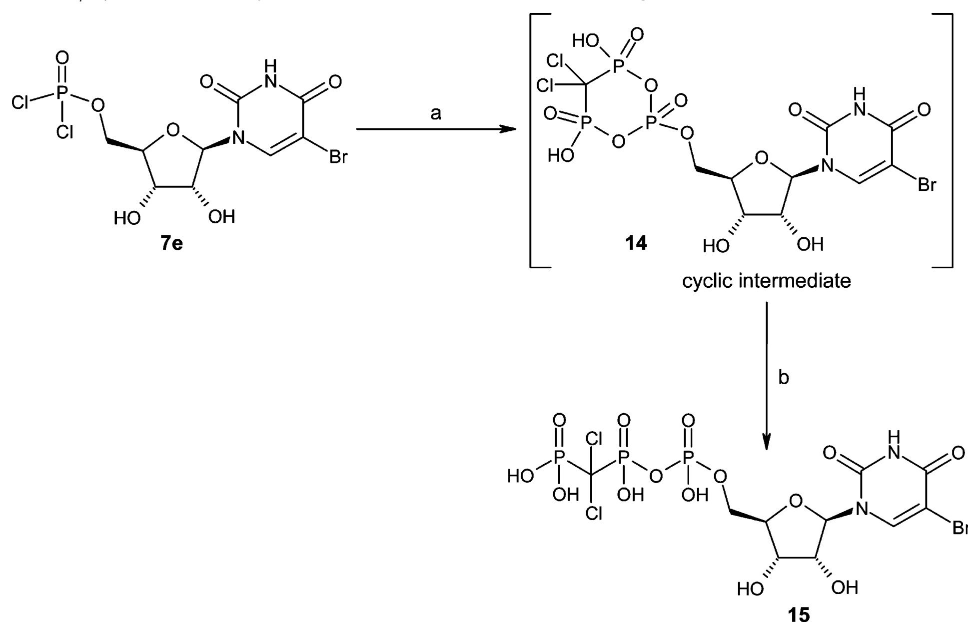
The structures of the synthesized nucleotides, some of which have previously been described (see Experimental Section), and the nucleotide analogue **15** were confirmed by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, in addition to HPLC analysis coupled to electrospray ionization mass spectrometry (LC/ESI-MS) performed in both positive and negative modes.

The purity of the products was generally >95%, as confirmed by analytical HPLC analyses in two different HPLC separation systems (for details, see Supporting Information).

Biological Activity

The synthesized nucleotides and nucleotide analogues were investigated for their potency to activate human P2Y$_2$, P2Y$_4$,

Scheme 2. Synthesis of $P_\beta P_\gamma$-Dichloromethylene-Substituted 5-Bromo-UTP Analogue **15**[a]



[a] Reagents and conditions: (a) bis(tri-*n*-butylammonium)dichloromethylene diphosphonate in DMF, 5 min, 0 °C; (b) triethylammonium hydrogen bicarbonate buffer, pH 7.4−7.6, room temp, 1 h.

and $P2Y_6$ receptors. Stimulation of the phospholipase C coupled receptors leads to an increase in the intracellular inositol phosphate (IP) concentration. Accumulation of [^{3}H]inositol phosphates induced by the test compounds was measured in 1321N1 human brain astrocytoma cells stably expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor using a scintillation proximity assay (SPA).[31] Since some of the nucleotides were tested as their triethylammonium salts, we also investigated the effects of triethylammonium phosphate for comparison; the compound was found to be inactive at all three receptor subtypes at concentrations up to 100 μM. The nucleotides were initially tested at three different concentrations: 10 nM, 1 μM, and 100 μM. For potent compounds full concentration−response curves were determined and EC_{50} values were calculated. The physiological agonists (UTP for $P2Y_2$ and $P2Y_4$, UDP for $P2Y_6$) were tested under the same conditions for comparison. Potential antagonistic effects (inhibition of agonist-induced IP accumulation) of selected nucleotides were also investigated using the same assays.

Structure−Activity Relationships

The results of the screening of the compounds as agonists at the human UTP receptors $P2Y_2$ and $P2Y_4$ and the human UDP receptor $P2Y_6$ in comparison with the native agonists (set at 100% maximal stimulation) are shown in Figure 3. The sequence of the compounds in the figures as well as in Table 1 is as follows: nucleoside triphosphates (**9a−g**, **12**, and **13**), nucleoside diphosphates (**10a−d**,**f**,**g**), followed by nucleoside monophosphates (**11a−g**) and finally the stable nucleoside triphosphate analogue 5-bromo-β,γ-dichloromethylene-UTP (**15**). The screening results already showed that the latter compound (**15**) was quite potent at all receptor subtypes (Figure 3). Nucleoside monophosphates exhibited only very low potency with one exception: **11d**, 5-bromo-UMP, was a relatively potent agonist at $P2Y_6$ receptors (43% stimulation at 1 μM, 124% stimulation at 100 μM; estimated EC_{50} value of ~2 μM). **11d** was also the most potent nucleoside monophosphate of the present series at $P2Y_2$ receptors (70% stimulation at 100 μM, estimated EC_{50} value ca. 40 μM), but it was much weaker at $P2Y_2$ than at $P2Y_6$ receptors (~20-fold). The compound was inactive at $P2Y_4$ receptors in concentrations up to 100 μM. As expected, nucleoside triphosphates were generally more potent at the UTP receptor subtypes $P2Y_2$ and $P2Y_4$, while the UDP receptor $P2Y_6$ preferred nucleoside diphosphates. Figure 3 also shows that several of the investigated compounds were highly active at $P2Y_2$ and $P2Y_6$ receptors, while the $P2Y_4$ receptor hardly tolerated any base-modified UTP or UDP derivatives, and none of the compounds reached the potency of the physiological agonist UTP at this receptor subtype.

Selected compounds that had shown good efficacy in the screening assays at 1 μM were further evaluated. Concentration-response curves were obtained as shown in Figures 4. All nucleotides and nucleotide analogues investigated at $P2Y_2$, $P2Y_4$, and/or $P2Y_6$ receptors were shown to be full agonists at the respective receptors in comparison with the physiological agonists (Figures 4). Differences in their maximal effects were not statistically significant. Data presented in Figure 3 appeared to indicate that some compounds might be more efficacious than the physiological agonists UTP and UDP; however, this was not confirmed by dose-response curves and averaged data from three independent experiments. Figure 4 shows that compounds for the $P2Y_2$ and the $P2Y_6$ receptors were identified (parts A and C of Figure 4) exhibiting similarly high potency as the physiological agonists, while in case of the $P2Y_4$ receptor subtype UTP was by far the most potent agonist and all modifications led to an at least 20-fold reduction in potency. EC_{50} values calculated from the concentration-response curves are collected in Table 1. For the less potent compounds, percent inhibition at high concentrations is given. For a few of the compounds that were active at higher micromolar concentrations, EC_{50} values were roughly estimated based on three data points spanning 5 orders of magnitude of concentrations (Table 1).

In contrast to K_i values obtained from radioligand binding studies, EC_{50} values measured in functional assays strongly depend on the receptor density.[36] Therefore, literature EC_{50} values for the same compound may show large differences; e.g., for UTP, EC_{50} values ranging from 8 to 140 nM have been

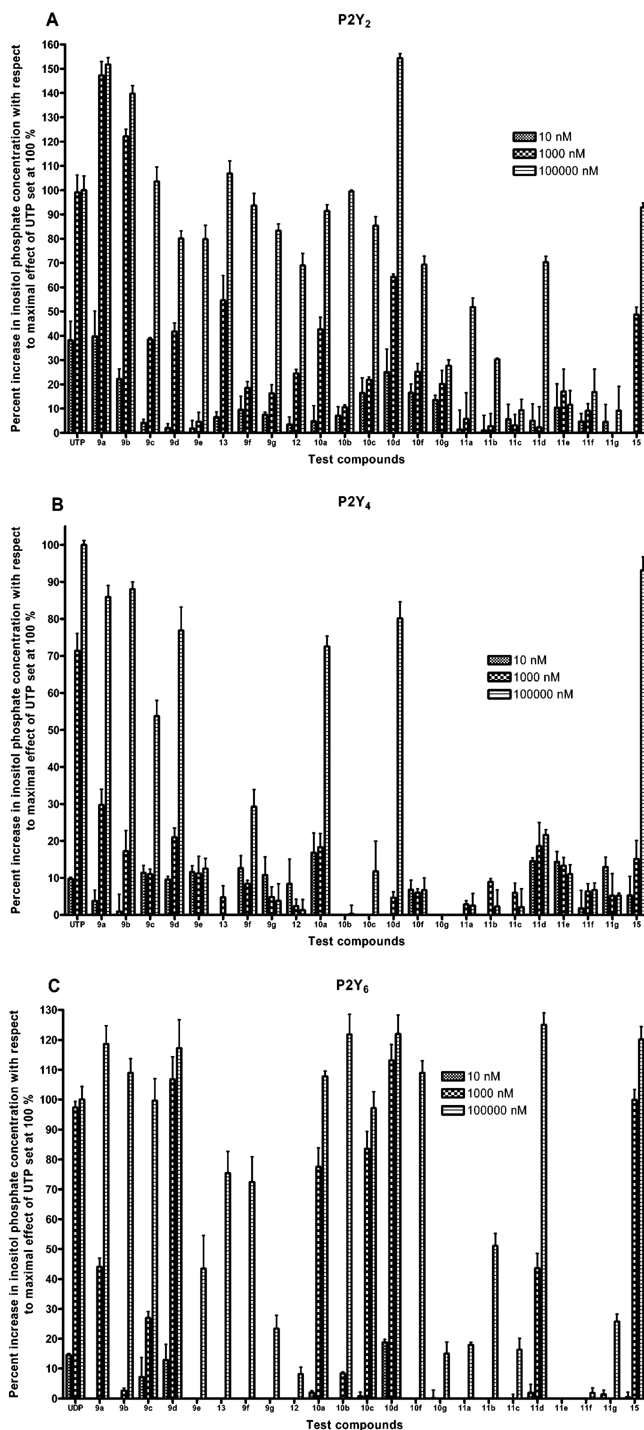


Figure 3. Results of initial screening of test compounds at human $P2Y_2$ (A), $P2Y_4$ (B), and $P2Y_6$ (C) receptors at three different concentrations, 10 nM, 1 μM, and 100 μM. Increase in inositol phosphates was measured by scintillation proximity assay in recombinant 1321N1 astrocytoma cells transfected with the appropriate receptor subtype. The maximal effect of the physiological nucleotide (UTP for $P2Y_2$ and $P2Y_4$, UDP for $P2Y_6$) was set at 100%, and the effects of the test compounds relative to that effect are shown. Data shown are from single experiments out of three independent experiments performed in triplicate.

reported at the human $P2Y_2$ receptor (Table 1).[36] Unfortunately, radioligands for $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors have not been developed yet. In order to be able to compare our data for the new compounds, we tested standard agonists under identical conditions. We kept the incubation time short (10 min) to prevent enzymatic degradation of the nucleotides. In general, uracil nucleotides are degraded much more slowly than adenine nucleotides,[37] but it cannot be excluded that a small percentage of some of the nucleotides are enzymatically hydrolyzed under the assay conditions.

Three of the compounds prepared in this study (**9a**, **9b**, **9d**) have also been investigated by another group, and data at human $P2Y_2$ and $P2Y_4$ receptors were recently published.[15] Those data are in good agreement with the data that we obtained in our assays for the compounds that we had independently synthesized ourselves (see Table 1; published data[15] are given for comparison). We now provide additional data for those compounds at $P2Y_6$ receptors. Structure−activity relationships are summarized in Figure 5.

UTP Derivatives and Analogues

The parent compound UTP is a potent $P2Y_2$ ($EC_{50} = 43$ nM) and $P2Y_4$ agonist ($EC_{50} = 87$ nM) and highly selective versus $P2Y_6$ receptors (Table 1). Exchange of the oxygen atom at the 2-position of the uracil nucleus by a sulfur atom (in **9a**) is well tolerated by the $P2Y_2$ receptor, but less well by the $P2Y_4$ receptor (20-fold reduction). As a triphosphate, compound **9a** is only a weak agonist at $P2Y_6$ receptors (estimated EC_{50} value of ∼1500 nM); however, it appears to be somewhat more potent than UTP, showing that a 2-thio substitution is favorable for $P2Y_6$ receptor activity of uracil nucleotides, a finding that is confirmed in the diphosphate series (compound **10a**, see below). Thus, the $P2Y_2$ as well as the $P2Y_6$ receptor, in contrast to the $P2Y_4$ receptor, may not depend on a strong hydrogen-accepting group in that position. With the next two compounds we investigated whether the hydrogen atom at N3 is required for hydrogen bonding or if it can be replaced. A small methyl group (**9b**) or a large phenacyl residue (**9c**) was introduced in order to probe the bulk tolerance of the receptors in that region. At $P2Y_2$ and $P2Y_4$ receptors both modifications resulted in a decrease in potency (compared with UTP), which was more dramatic at $P2Y_4$ than at $P2Y_2$ receptors. The smaller methyl group was better tolerated at both receptor subtypes (13-fold reduction of EC_{50} value at $P2Y_2$, 34-fold reduction at $P2Y_4$) than the large phenacyl substituent (47-fold at $P2Y_2$, >1000-fold at $P2Y_4$). Interestingly, a different trend was observed for the $P2Y_6$ receptor: N3-methylation of UTP did not result in a further reduction in potency (compare UTP (**1**) and **9b**), and the larger N3-phenacyl-substituted derivative **9c** appeared to be even somewhat more potent. A similar observation, namely, that the $P2Y_6$ receptor tolerates large N3 substituents, could be made in the series of UDP derivatives, which are generally much more potent at $P2Y_6$ receptors than UTP derivatives (compound **10c**; see below). In the next step we investigated the effect of a bromine atom at the uracil C5-position. The voluminous atom in that position reduced potency at $P2Y_2$ receptors by about 8-fold and at $P2Y_4$ receptors by about 40-fold. This result confirmed our previous study in which we had shown that large alkyl substitutents at the 5-position of UTP were also not tolerated by $P2Y_2$ receptors, and the potency decreased with an increase in bulk.[29] In contrast, the potency of UTP at $P2Y_6$ receptors is largely increased by C5 bromination, and **9d** (although being a UTP rather than a UDP derivative) is a very potent $P2Y_6$ agonist ($EC_{50} = 291$ nM). It is equally potent at $P2Y_2$ and $P2Y_6$ receptors and about 10-fold less potent at the $P2Y_4$ receptor subtype. Compound **9e** can be envisaged as 6-hydroxy-substituted UTP, in which the uracil moiety of UTP has been replaced by barbituric acid. However, barbituric acid derivatives prefer the trioxo tautomeric form in neutral solution. Barbituric acid nucleotide **9e** was inactive or only weakly active

Table 1. Potency of Uracil Nucleotides and Analogues as Agonists at Human $P2Y_2$, $P2Y_4$, and $P2Y_6$ Receptors

N1-nucleotides **N3-nucleotides** **15**

compd	X	R^3	R^5	R^6	EC_{50} (SEM [nM] (% effect with respect to the maximal effect of the physiological agonist (set at 100%) at concentration indicated), IP accumulation human recombinant P2Y receptors expressed in 1321N1 astrocytoma cells ($n \geq 3$)		
					$P2Y_2$	$P2Y_4$	$P2Y_6$
					Nucleoside Triphosphates ($m = 2$) N1-Nucleotides		
1 (UTP)	O	H	H	H	43 ± 4 (8−140)[4]	87 ± 5 (50−800)[4]	nd (6000,[32] >10000)[7]
9a	S	H	H	H	50 ± 4 (35)[15]	1770 ± 140 (350)[15]	>1000 (∼1500)[a] (44% at 1 μM, 118% at 100 μM)
9b	O	methyl	H	H	564 ± 26 (1200)[15]	2920 ± 160 (3400)[15]	>1000 (∼8000)[a] (2% at 1 μM, 109% at 100 μM)
9c	O	phenacyl	H	H	>1000 (∼2000)[a] (38% at 1 μM, 103% at 100 μM)	>1000 (∼ 90000)[a] (10% at 1 μM, 54% at 100 μM)	>1000 (∼3000)[a] (27% at 1 μM, 100% at 100 μM)
9d	O	H	Br	H	347 ± 27(750)[15]	3460 ± 200(2100)[15]	291 ± 19 (800)[32]
9e[b,d]	O	H	H	OH	>1000 (∼25000)[a] (6% at 1 μM, 80% at 100 μM)	>100000 (11% at 1 μM, 12% at 100 μM)	>100000 (0% at 1 μM, 43% at 100 μM)
					2′-Phosphate		
13	O	phenacyl	H	H	≤1000 (∼900)[a] (54% at 1 μM, 107% at 100 μM)	>100000 (0% at 100 μM)	>1000 (∼35000)[a] (0% at 1 μM, 75% at 100 μM)
					N3-Nucleotides		
9f[b,d]				methyl	>1000 (∼35000)[a] (18% at 1 μM, 93% at 100 μM)	>100000 (8% at 1 μM, 29% at 100 μM)	>1000 (∼40000)[a] (0% at 1 μM, 72% at 100 μM)
9g[b,d]				propyl	>1000 (∼40000)[a] (16% at 1 μM, 83% at 100 μM)	>100000 (4% at 100 μM)	>100000 (0% at 1 μM, 23% at 100 μM)
					2′,3′-Cyclophosphate		
12				propyl	>1000 (∼50000)[a] (24% at 1 μM, 69% at 100 μM)	>100000 (1% at 100 μM)	>100000 (8% at 100 μM)
					Nucleoside Diphosphates ($m = 1$) N1-Nucleotides		
2 (UDP)	O	H	H	H	nd (16500)[33]	nd (14000)[34]	42 ± 5 (47−300)[4]
10a	S	H	H	H	>1000 (∼50000)[a] (42% at 1 μM, 91% at 100 μM)	>1000 (∼40000)[a] (18% at 1 μM, 73% at 100 μM)	239 ± 20
10b	O	methyl	H	H	>1000 (∼7000)[a] (10% at 1 μM, 99% at 100 μM)	>100000 (0% at 100 μM)	>1000 (∼8000)[a] (8% at 1 μM, 121% at 100 μM)
10c	O	phenacyl	H	H	>1000 (∼40000)[a] (22% at 1 μM, 85% at 100 μM)	>100000 (11% at 100 μM)	70 ± 6
10d	O	H	Br	H	3670 ± 120	7200 ± 210	151 ± 15 (130 nM)[c,35]
					N3-Nucleotides		
10f				methyl	>1000 (∼50000)[a] (25% at 1 μM, 69% at 100 μM)	>100000 (7% at 100 μM)	>1000 (∼10000)[a] (0% at 1 μM, 108% at 100 μM)
10g[d]				propyl	>100000 (28% at 100 μM)	>100000 (0% at 100 μM)	>100000 (23% at 100 μM)
					Nucleoside Monophosphates ($m = 0$) N1-Nucleotides		
11a	S	H	H	H	≥100000 (6% at 1 μM, 51% at 100 μM)	>100000 (2% at 100 μM)	>100000 (18% at 100 μM)
11b	O	methyl	H	H	>100000 (3% at 1 μM, 30% at 100 μM)	>100000 (3% at 100 μM)	≥ 100000 (0% at 1 μM, 51% at 100 μM)
11c[b,d]	O	phenacyl	H	H	>100000 (9% at 100 μM)	>100000 (2% at 100 μM)	>100000 (16% at 100 μM)
11d	O	H	Br	H	>1000 (∼40000)[a] (2% at 1 μM, 70% at 100 μM)	>100000 (21% at 100 μM)	>1000 (∼2000)[a] (43% at 1 μM, 124% at 100 μM)
11e	O	H	H	OH	>100000 (11% at 100 μM)	>100000 (11% at 100 μM)	>100000 (0% at 100 μM)
					N3-Nucleotides		
11f				methyl	>100000 (17% at 100 μM)	>100000 (6% at 100 μM)	>100000 (2% at 100 μM)
11g				propyl	>100000 (9% at 100 μM)	>100000 (5% at 100 μM)	>100000 (26% at 100 μM)
					Nucleoside Triphosphate Analogue (β,γ-Dichloromethylene-5-bromo-UTP Analogue)		
15					354 ± 27	3990 ± 120	120 ± 13

[a] Roughly estimated EC_{50} value (from three test concentrations spanning 5 orders of magnitude) [b] Compound (100 μM) did not antagonize UTP (10 μM) induced accumulation of IP at $P2Y_2$ and $P2Y_4$ receptors or UDP (1 μM) induced IP accumulation at $P2Y_6$ receptors. [c] Data from rat $P2Y_6$ receptor. [d] Compound (100 μM) did not antagonize UDP (1 μM) induced IP accumulation at $P2Y_6$ receptors.

at all three receptor subtypes; the modification was best tolerated by the $P2Y_2$ receptor ($EC_{50} \approx 25\,000$ nM).

A side product obtained during the phosphorylation of *N*3-phenacyluridine that could be isolated and purified was the 2′-phosphate of **9c**, compound **13**. The 2′-phosphorylation of **9c** was tolerated by the $P2Y_2$ receptor, but potency at the $P2Y_4$ and the $P2Y_6$ was reduced (compare **13** and **9c**).

Three *N*3-riboside derivatives were investigated, in which the ribose moiety is attached to the N3- rather than the N1-position. This modification was combined with alkyl (methyl or propyl substitution) at C6. Those compounds are lacking the oxo group (C4═O in N1 nucleosides), which is replaced by an alkyl group, and they feature an oxo group in the position that corresponds to C6 in N1-nucleosides instead. All of the investigated N3-

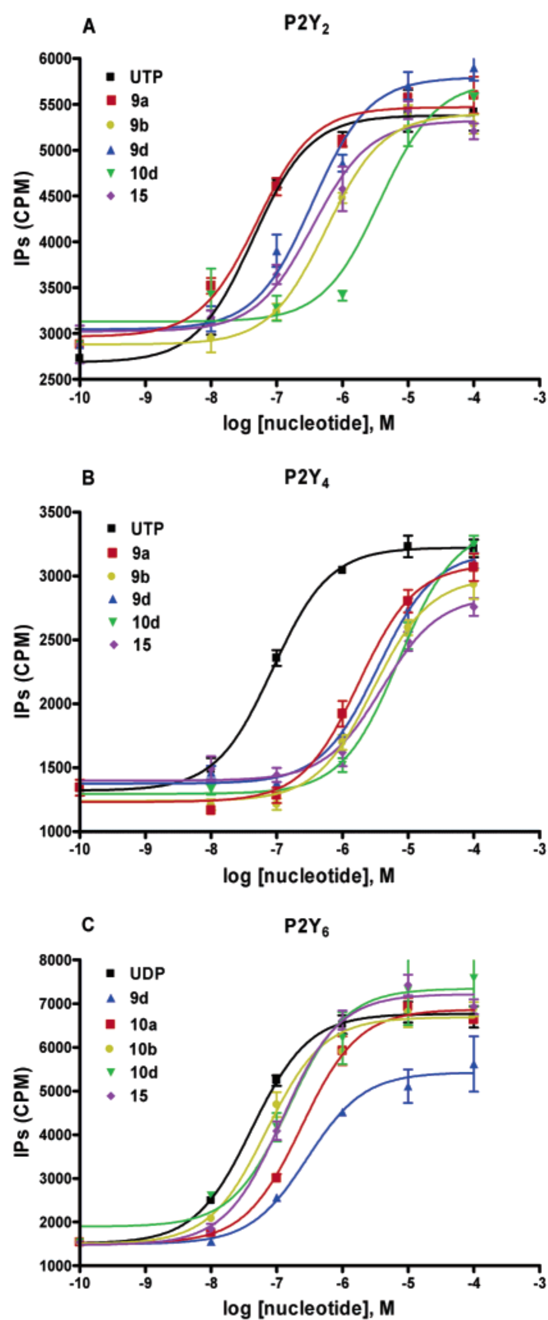


Figure 4. Concentration-response curves of pyrimidine nucleotides as agonists at pyrimidinergic P2Y receptor subtypes. Dose-dependent stimulation of inositol phosphate accumulation was determined in 1321N1 human astrocytoma cells stably transfected with (A) the human $P2Y_2$ receptor, (B) the human $P2Y_4$ receptor, and (C) the human $P2Y_6$ receptor.

nucleoside triphosphates (**9f**, **9g**, **12**), as well as the corresponding diphosphates (**10f**, **10g**) and monophosphates (**11f**, **11g**), showed low potency or were inactive. The $P2Y_2$ and the $P2Y_6$ receptor tolerated these base modifications better than the $P2Y_4$ receptor. While methyl and propyl substitutions resulted in similar low potency at $P2Y_2$ receptors ($EC_{50} \approx 35$ μM for **9f**; $EC_{50} \approx 40$ μM for **9g**), the $P2Y_6$ receptor only tolerated the methyl derivative, in the triphosphate (**9f**, $EC_{50} \approx 40$ μM) as well as in the diphosphate series (**10f**, $EC_{50} \approx 10$ μM). The 2′,3′-cyclophosphate (**12**) of the propyl-substituted N3-nucleoside **9g** was obtained as a byproduct and could be isolated, purified, and tested at the receptors. It was slightly less potent at $P2Y_2$ receptors than the parent compound **9g** ($EC_{50} \approx 50$ μM), indicating that the $P2Y_2$ receptor may tolerate such a modification.

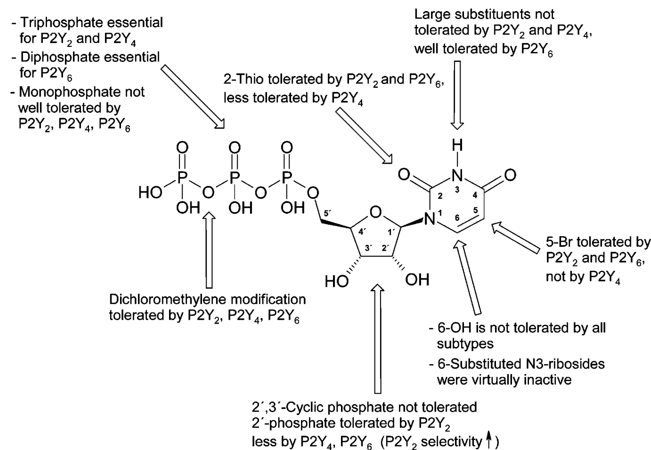


Figure 5. Structure–activity relationships of uracil nucleotides as agonists at $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors.

UDP Derivatives and Analogues

Uridine diphosphate (UDP, **2**) is a very potent $P2Y_6$ receptor agonist ($EC_{50} = 42$ nM) and highly selective versus the UTP receptors $P2Y_2$ and $P2Y_4$ (>300-fold). All investigated diphosphates exhibited low potency at the UTP receptors with EC_{50} values greater than 1000 nM. The best tolerated modifications of UDP at $P2Y_2$ receptors were bromination in the 5-position (**10d**, $EC_{50} = 3670$ nM) and N3-methylation (**10b**, $EC_{50} \approx 7000$ nM). However, the corresponding UTP derivatives were at least 10-fold more potent. 5-Bromo-UDP (**10d**) was also the most potent UDP derivative at $P2Y_4$ receptors ($EC_{50} \approx 7200$ nM), and it was only 2.5-fold less potent than 5-Br-UTP. Thus, 5-bromination is less unfavorable in UDP derivatives than in UTP derivatives for the UTP-activated receptor subtypes $P2Y_2$ and $P2Y_4$, and it may even attenuate the negative effects of the lack of a third phosphate group perhaps by inducing a slightly different binding mode of the nucleotides in the receptor binding pockets.

Replacement of the 2-oxo by a 2-thio group yielding 2-thio-UDP (**10a**) was tolerated by the $P2Y_6$ receptor, resulting in a 6-fold decrease in potency (**10a**, $EC_{50} = 239$ nM). Since 2-thio-UDP was only very weakly active at $P2Y_2$ and $P2Y_4$ receptors, it is a potent and highly selective (>150-fold) $P2Y_6$ receptor agonist. N3-Alkylation of UDP was investigated: 3-methylation (**10b**) was unfavorable ($EC_{50} \approx 8000$ nM), while the larger phenacyl residue at N3 was very well tolerated exhibiting a similar potency as UDP at $P2Y_6$ receptors ($EC_{50} = 70$ nM). 3-Phenacyl-UDP (**10c**) was the most potent and most selective $P2Y_6$ receptor agonist of the present series. The large phenacyl substituent appeared to be much less well tolerated by $P2Y_2$ and especially by $P2Y_4$ receptors and thus enhanced $P2Y_6$ selectivity. Introduction of a 5-bromo substituent (compound **10d**) was tolerated by the $P2Y_6$ receptor; the resulting 5-Br-UDP (**10d**) was only 3.6-fold less potent ($EC_{50} = 151$ nM) than UDP. 5-Bromo substitution also increased potency of UDP at $P2Y_2$ and $P2Y_4$ receptors, and therefore, selectivity was reduced (24-fold selectivity for $P2Y_6$ vs $P2Y_2$, 48-fold vs $P2Y_4$) in comparison with UDP. The N3-nucleotides **10f** and **10g**, which were methyl- or propyl-substituted in the 6-position, were weak agonists at $P2Y_6$ receptors. The smaller methyl group (**10f**) was better tolerated (**10g**, $EC_{50} \approx 10$ μM) than the larger propyl residue ($EC_{50} > 100$ μM).

UMP Derivatives and Analogues

Uridine monophoshate (UMP) has been reported to be inactive at $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors.[13,36] Likewise, AMP has also been shown to be inactive at ATP- and ADP-activated P2 receptor subtypes.[13,36] However, by modification of the adenine base, it had been possible to obtain AMP derivatives, e.g., 2-hexylthio-AMP and related compounds, that were highly potent agonists at P2Y receptors expressed in a C6 rat glioma cell line.[38] Cristalli and colleagues reported on 2-hexynyl-AMP and 2-phenylethynyl-AMP as $P2Y_1$ receptor antagonists.[39] In the present study most of the UMP derivatives were found to be inactive at concentrations up to 100 μM. None of the UMP derivatives showed any activity at $P2Y_4$ receptors. The only compound that exhibited appreciable activity at the $P2Y_2$ receptor with an EC_{50} value below 100 μM was 5-bromo-UMP (**11d**, $EC_{50} \approx 40$ μM). The same compound was also the best UMP derivative at $P2Y_6$ receptors with an EC_{50} value of about 2 μM. It was as $P2Y_6$-selective as the more potent 5-bromo-UDP (**10d**), 20-fold versus $P2Y_2$ and >50-fold versus $P2Y_4$.

β,*γ*-Dichloromethylene-Bridged UTP Analogue

Nucleotides are hydrolytically stable molecules in the pH range of usually about 4−11,[40] but they can be rapidly degraded by enzymatic hydrolysis. A major family of UTP- and UDP-degrading enzymes are the ectonucleotidases (ectonucleotide diphosphohydrolases, E-NTPDases). They attack the terminal phosphate group and can thus convert UTP to UDP, or UDP to UMP, or UTP via UDP to UMP,[41] resulting in very short half-lives for UTP and UDP in vivo. Fischer and colleagues recently proposed borano analogues of dinucleoside (diuridine and diadenosine) polyphosphates as P2Y agonists with enhanced metabolic stability.[42] However, the synthesized analogues of diuridine triphosphate and diuridine pentaphosphate, $Up_3(\beta\text{-B})U$ and $Up_5(\gamma B)U$, had shown only very low activity at $P2Y_2$ receptors and no test results have been reported for $P2Y_4$ and $P2Y_6$ receptors. For application in biological systems, especially for in vivo studies, stable receptor agonists are preferred. Therefore, we investigated whether the UTP and UDP receptors ($P2Y_2$, $P2Y_4$, $P2Y_6$) would tolerate UTP analogues in which the oxygen bridge between the β- and the γ-phosphate group was replaced by a dichloro-substituted methylene bridge. Such compounds would not be susceptible to hydrolysis by NTPDases or phosphatases.[43] Other replacements had previously been investigated, including an unsubstituted β,γ-methylene bridge, a β,γ-difluoromethylene bridge, and a β,γ-imido bridge, yielding the corresponding hydrolysis-resistant UTP analogues.[44] Those compounds had been investigated as agonists at human $P2Y_2$ receptors and were shown to exhibit only low potency (β,γ-methylene-UTP $EC_{50} = 73.3$ μM, β,γ-difluoromethylen-UTP $EC_{50} = 8.92$ μM, and β,γ-imido-UTP $EC_{50} = 1.45$ μM).[44] In the present study we introduced the dichloromethylene group as a bioisosteric replacement of the oxygen bridge because of similar electronic properties, which are important for the pK_a value and thus the protonation state of the γ-phosphate/phoshonate. The same modification had been successfully used in a series of ATP analogues that had been developed as potent antagonists at the platelet $P2Y_{12}$ receptor.[43,45] 5-Bromo-UTP (**9d**) was selected for investigating the consequences of such a phosphate chain modification on the activity at $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors because **9d** was relatively potent at all three receptor subtypes. The resulting 5-bromo-β,γ-dichloromethylene-UTP analogue was found to be a full agonist at all three receptor subtypes. In comparison with the parent compound 5-bromo-UTP (**9d**), analogue **15** exhibited nearly identical potency at the $P2Y_2$ (**9d** $EC_{50} = 347$ nM; **15** $EC_{50} = 354$ nM) and the $P2Y_4$ receptor (**9d** $EC_{50} = 3460$ nM; **15** $EC_{50} = 3990$ nM). At the $P2Y_6$ receptor, the enzymatically stable analogue **15** was even more potent (2.4-fold) than the parent 5-bromo-UTP (**9d**), exhibiting an EC_{50} value of 120 nM (versus 291 nM for **9d**). The new UTP analogue **15** is somewhat selective for the $P2Y_6$ receptor (33-fold vs $P2Y_4$, 3-fold vs $P2Y_2$). Thus, the β,γ-dichloromethylene modification appears to be a possibility for obtaining potent and metabolically stable $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor agonists. This should contribute to the future design of not only potent and metabolically more stable compounds but also subtype-selective $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor agonists by combining the β,γ-dichloromethylene modification with appropriate substituents on the ribose moiety and/or the uracil ring.

Antagonistic Activity

A selection of compounds that had shown no or only weakly agonistic activity at UTP and UDP receptors were additionally investigated for antagonistic activity, i.e., their potency to inhibit agonist-induced activation of inositol phosphate accumulation at a high concentration of 100 μM. The following compounds were investigated at all three receptor subtypes: the triphosphates **9e**, **9f**, **9g**, and the monophosphate **11c**. In addition, diphosphate **10g** was investigated at $P2Y_6$ receptors only. None of the compounds showed an antagonistic effect at any of the investigated receptors.

Conclusions

In conclusion, we have synthesized a series of UTP, UDP, and UMP derivatives and analogues modified in the uracil part of the molecules. Furthermore, a 5-bromo-UTP analogue containing a modified triphosphate chain conferring enhanced metabolic stability was obtained. Structure−activity relationships as agonists at the pyrimidinergic $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor subtypes were explored. All three receptor subtypes tolerated a β,γ-dichlormethylene modification of the triphosphate chain very well. 6-Alkyl-substituted N3-nucleotides were virtually inactive at all receptor subtypes. 3-Phenacyl-UDP (**10c**) was found to be a potent and highly selective $P2Y_6$ receptor agonist. It is interesting to note that all modifications performed at the uracil moiety and the triphosphate chain led to full agonists (or inactive compounds), but none of the selected compounds tested showed antagonistic activity at $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptors.

Experimental Section

All commercially available reagents were obtained from various producers (Acros, Aldrich, Fluka, Merck, and Sigma) and used without further purification. Solvents were used without additional purification or drying, unless otherwise noted. The reactions were monitored by thin layer chromatography (TLC) using aluminum sheets with silica gel 60 F_{254} (Merck). Column chromatography was carried out with 0.060−0.200 mm silica gel, pore diameter of ~6 nm. Mass spectra were recorded on an API 2000 (Applied Biosystems, Darmstadt, Germany) mass spectrometer (turbo ion spray ion source) coupled with a Waters HPLC system (Agilent 1100) using a Phenomenex Luna 3 μm C18 column. ^1H, ^{31}P, and ^{13}C NMR spectra were performed on a Bruker Avance 500 MHz spectrometer. $CDCl_3$, DMSO-d_6, MeOD-d_4, or D_2O were used as solvents as indicated below. ^{31}P NMR spectra were recorded at room temperature; orthophosphoric acid (85%) was used as an external standard. Shifts are given in ppm relative to the external standard (^{31}P NMR) or relative to the remaining protons of the deuterated solvents used as internal standard (^1H, ^{13}C). Elemental microanalyses were performed on a VarioEL apparatus at the Pharmaceutical Institute, Bonn-Endenich, University of Bonn.

Melting points were determined on a Büchi 530 melting point apparatus and are uncorrected. Purity of the prepared nucleosides was checked by TLC on silica gel 60 F_{254} (Merck) aluminum plates, using dichloromethane/methanol (9:1) or dichloromethane/methanol (3:1) as the mobile phase. Purity of the prepared nucleotides was confirmed by HPLC on an RP-HPLC column (Knauer 20 mm i.d., Eurospher-100 C18). The column was eluted with a solvent gradient of 0−10% of acetonitrile in 50 mM aqueous NH_4HCO_3 buffer for 25 min at a flow rate of 5 mL/min. UV absorption was detected at 254 nm. The purity of the nucleotide samples was checked in a second chromatography system by dissolving 1 mg/mL in 1:1 H_2O/MeOH, containing 2 mM ammonium acetate. A sample of 10 μL was injected into an HPLC instrument (Agilent 1100) using a Phenomenex Luna 3 μm C18 column. Elution was performed with a gradient of water/methanol (containing 2 mM ammonium acetate) from 90:10 to 0:100 for 30 min at a flow rate of 250 μL /min, starting the gradient after 10 min. UV absorption was detected from 190 to 400 nm using a diode array detector.

General Procedure of Phosphorylation. Preparation of Bis(tri-*n*-butylammonium) Pyrophosphate. Sodium pyrophosphate decahydrate 6.69 g (15 mmol) was dissolved in 150 mL of deionized water. Excess of Dowex ion-exchange resin 50 mm × 8 mm, 20−50 mesh, proton form, prewashed several times with water, was added to the solution of sodium pyrophosphate, and the mixture was gently stirred for 60 min. A mixture of 60 mL of ethanol and 7.14 mL of tributylamine in a flask was placed in an ice−water bath, and the pyrophosphate solution was filtered directly into the flask. The resin was repeatedly washed with water until the filtrate was no longer acidic. The solvent was then evaporated under reduced pressure at 40 °C to give a thick, nearly colorless syrup. This residue was treated twice with 100 mL of C_2H_5OH and then evaporated. The residue was taken up in 40 mL of anhydrous DMF and evaporated again. This residue was dissolved in 30 mL of anhydrous DMF, yielding 30 mL of a 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in DMF. The solution was stored, sealed, and cooled at 4 °C until use.[29]

Preparation of Tri-*n*-butylammonium Phosphate. The same procedure was used as in case of the preparation of bis(tri-*n*-butylammonium) pyrophosphate using sodium monophosphate instead of sodium pyrophosphate.

Preparation of Triethylammonium Hydrogen Carbonate Buffer (TEAB). A 1 M solution of TEAB was prepared by bubbling CO_2 through a 1 M triethylamine solution in water at 0−4 °C for several hours (pH of approximately 7.4−7.6).[29]

General Procedure for the Synthesis of Nucleotides. Lyophilized nucleoside (1 mmol) was dissolved in 5 mL of trimethyl phosphate (dried over 10 Å molecular sieves). The mixture was stirred at room temperature under argon and then cooled to 4 °C. Dry 1,8-bis(dimethylamino)naphthalene (Proton Sponge, 0.32 g, 1.5 mmol) was added, followed by 0.20 g (1.3 mmol) of $POCl_3$ 5 min later. After several hours of stirring at 0−4 °C, tri-*n*-butylamine (0.1 mL, 0.72 mmol) was added to the solution followed by 10 mL (5 mmol) of 0.5 M bis(tri-*n*-butylammonium) pyrophosphate (or phosphate) solution in DMF. After 2−5 min the mixture was poured into a cold 0.5 M aqueous TEAB solution (30 mL, pH 7.5) and stirred at 0−4 °C for several minutes. The solution was allowed to reach room temperature upon stirring and then left standing for 1 h. Trimethylphosphate was extracted with *tert*-butyl methyl ether, and the aqueous solution was evaporated and lyophilized to yield glassy colorless oils. The reactions were controlled by TLC using a freshly prepared solvent system (2-propanol/NH_4OH/water = 6 : 3 : 1). TLC plates were dried before UV absorption was detected, and the plates were subsequently sprayed with a phosphate reagent.[29]

Purification of Nucleotides. Ion Exchange Chromatography. The crude nucleoside 5′-triphosphates were purified by ion exchange chromatography on an FPLC instrument (ÄKTA FPLC, from Amersham Biosciences) with an XK 26 mm × 20 cm length column (Pharmacia) using Sephadex DEAE A-25 gel, HCO_3^- form, swelled in a 1 M solution of TEAB at 4 °C. After equilibration of the column with deionized water, the crude product was dissolved in 2 mL of aqueous triethylammonium hydrogen carbonate buffer. The column was washed with deionized water, followed by a solvent gradient of 0−800 mM TEAB buffer using approximately 3000 mL of solvent to elute the triphosphates. Fractions were collected and appropriate fractions pooled, diluted in water, and lyophilized.

Preparative HPLC. Lyophilized nucleoside 5′-triphosphates obtained from FPLC purification were dissolved in 5 mL of deionized water and injected into an RP-HPLC column (Knauer 20 mm i.d., Eurospher-100 C18). The column was eluted with a solvent gradient of 0−10% of acetonitrile in 50 mM aqueous NH_4HCO_3 buffer for 25 min at a flow rate of 5 mL/min. The UV absorption was detected at 254 nm. Fractions were collected and appropriate fractions pooled, diluted with water, and lyophilized several times to remove the NH_4HCO_3 buffer, yielding the nucleotides as white powders.

Preparation of the Sodium Salts. Nucleotide tributylammonium salts (50 mmol) were dissolved in absolute methanol (dried over Mg) and evaporated. This procedure was repeated twice. The solid was then dissolved in 5 mL of absolute methanol with stirring. A 1.5-fold excess of a 1 M sodium iodide solution in acetone was added dropwise. The solution was then diluted with absolute acetone. The formed precipitate was filtered off and washed several times with absolute acetone. The white solid was dried under high vacuum. Most nucleotides, however, were stored and tested as tributylammonium salts because of enhanced stability in comparison with the sodium salts. The tributylammonium cation was shown to have no effect on the test system.

2-Thio-1-*β*-D-ribofuranosyl(3*H*)pyrimidine-2,4-dione 5′-Triphosphate (9a).[46] ^{1}H NMR (500 MHz, MeOD) δ 4.26 (m, 2H), 4.28 (m, 1H), 4.34 (m, 1H), 4.41 (m, 1H), 6.25 (d, 1H, J = 8.19 Hz), 6.68 (d, 1H, J = 2.83 Hz), 8.23 (d, 1H, J = 8.51 Hz). ^{31}P NMR (202 MHz, MeOD) δ −23.74 (t, J = 17.22 Hz), −12.31 (d, J = 19.68 Hz), −10.83 (d, J = 17.22 Hz). ^{13}C NMR (125 MHz, MeOD) δ 65.41, 69.99, 76.27, 84.55, 94.47, 107.91, 143.42, 163.54, 177.69. LC/ESI-MS: negative mode 499 ([M − H]$^-$), positive mode 501 ([M + H]$^+$).

3-Methyl-1-*β*-D-ribofuranosylpyrimidine-2,4-dione 5′-Triphosphate (9b).[15] ^{1}H NMR (500 MHz, MeOD) δ 3.33 (s, 3H), 4.20 (m, 2H), 4.31 (m, 2H), 4.42 (t, 1H, J = 4.72 Hz), 5.98 (d, 1H, J = 7.88 Hz), 6.03 (d, 1H, J = 4.72 Hz), 8.07 (d, 1H, J = 7.88 Hz). ^{31}P NMR (202 MHz, MeOD) δ −23.31 (t, J = 19.68 Hz), −10.18 (d, J = 19.68 Hz), −6.04 (d, J = 19.68 Hz). ^{13}C NMR (125 MHz, MeOD) δ 28.46, 66.39, 71.31, 75.90, 85.16, 90.91, 102.81, 141.10, 153.37, 165.87. LC/ESI-MS: negative mode 497 ([M − H]$^-$), positive mode 499 ([M + H]$^+$).

3-Phenacyl-1-*β*-D-ribofuranosylpyrimidine-2,4-dione 5′-Triphosphate (9c). ^{1}H NMR (500 MHz, MeOD) δ 4.18 (m, 2H), 4.28−4.37 (m, 2H), 4.47 (t, 1H, J = 4.88 Hz), 5.44 (d, 2H, J = 1.57 Hz), 6.01 (m, 2H), 7.57 (m, 5H), 8.21 (d, 1H, J = 8.19 Hz). ^{31}P NMR (202 MHz, MeOD) δ −21.09 (t, J = 14.76 Hz), −10.55 (d, J = 14.76 Hz), −5.93 (d, J = 12.30 Hz). ^{13}C NMR (125 MHz, MeOD) δ 47.47, 66.33, 71.31, 76.14, 85.29, 91.20, 102.61, 129.16−136.59, 141.73, 152.96, 164.98, 194.13. LC/ESI-MS: negative mode 601 ([M − H]$^-$), positive mode 623 ([(M − H + Na) - H]$^-$).

5-Bromo-1-*β*-D-ribofuranosyl(3*H*)pyrimidine-2,4-dione 5′-Triphosphate (9d).[47] ^{1}H NMR (500 MHz, D_2O) δ 4.26 (m, 2H), 4.30 (m, 1H), 4.40 (t, 1H, J = 5.04 Hz), 4.43 (t, 1H, J = 4.72 Hz), 5.95 (d, 1H, J = 5.04 Hz), 8.25 (s, 1H). ^{31}P NMR (202 MHz, D_2O) δ −20.94 (t, J = 15.99 Hz), −10.67 (d, J = 17.22 Hz), −6.12 (d, J = 17.22 Hz). ^{13}C NMR (125 MHz, D_2O) δ 67.69, 72.19, 76.63, 86.19−86.26, 91.65, 99.92, 143.74, 154.36, 165.18. LC/ESI-MS: negative mode 561 ([M − H]$^-$), 583 ([(M − H + Na) - H]$^-$), positive mode 463 ([M + H]$^+$).

1-*β*-D-Ribofuranosyl(3*H*)pyrimidine-2,4,6-trione 5′-Triphosphate (9e). ^{1}H NMR (500 MHz, D_2O) δ 4.04 (m, 4H), 4.25 (m, 1H), 4.46 (t, 1H, J = 6.77 Hz), 4.73 (m, 1H), 6.19 (d, 1H, J = 3.15 Hz). ^{31}P NMR (202 MHz, D_2O) δ −22.51 (t, J = 19.68 Hz), −10.72 (d, J = 19.68 Hz), −9.29 (d, J = 19.68 Hz). ^{13}C NMR (125 MHz, D_2O) δ 47.35, 66.34, 70.04, 72.32, 81.74, 88.04, 153.68,

167.46. LC/ESI-MS: negative mode 499 ([M − H]$^-$), positive mode 501 ([M + H]$^+$).

6-Methyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione 5′-Triphosphate (9f). ^{1}H NMR (500 MHz, MeOD) δ 2.14 (d, 3H), 4.04 (m, 2H), 4.30 (m, 1H), 4.52 (t, 1H, *J* = 6.77 Hz), 4.65 (m, 1H), 5.50 (s, 1H), 6.28 (d, 1H, *J* = 3.15 Hz). ^{31}P NMR (202 MHz, MeOD) δ −23.79 (t, *J* = 20.91 Hz), −11.57 (d, *J* = 19.68 Hz), −10.89 (d, *J* = 19.68 Hz). ^{13}C NMR (125 MHz, MeOD) δ 18.70, 67.52, 71.84, 73.60, 84.33, 89.80, 100.21, 152.80, 154.57, 165.76. LC/ESI-MS: negative mode 497 ([M − H]$^-$), positive mode 499 ([M + H]$^+$).

6-Propyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione 5′-Triphosphate (9g). ^{1}H NMR (500 MHz, D_2O) δ 0.92 (t, 3H, *J* = 6.62 Hz), 1.60 (m, 2H), 2.39 (t, 2H, *J* = 7.56 Hz), 4.10 (m, 2H), 4.26 (q, 1H, *J* = 6.41 Hz), 4.51 (t, 1H, *J* = 6.62 Hz), 4.74 (m, 1H), 5.67 (s, 1H), 6.24 (d, 1H, *J* = 2.20 Hz). ^{31}P NMR (202 MHz, D_2O) δ −22.05 (t, *J* = 19.68 Hz), −10.64 (d, *J* = 19.68 Hz), −7.13 (d, *J* = 19.68 Hz). ^{13}C NMR (125 MHz, D_2O) δ 15.33, 22.99, 36.64, 68.22, 72.05, 74.31, 84.49, 90.51, 101.50, 155.00, 160.91, 168.33. LC/ESI-MS: negative mode 525 ([M − H]$^-$), positive mode 527 ([M + H]$^+$).

2-Thio-1-β-D-ribofuranosyl(3*H*)pyrimidine-2,4-dione 5′-Diphosphate (10a).[48] ^{1}H NMR (500 MHz, MeOD) δ 4.26 (m, 2H), 4.29 (m, 1H), 4.34 (m, 1H), 4.41 (m, 1H), 6.25 (d, 1H, *J* = 8.19 Hz), 6.64 (d, 1H, *J* = 3.46 Hz), 8.25 (d, 1H, *J* = 7.88 Hz). ^{31}P NMR (202 MHz, MeOD) δ −11.51 (d, *J* = 14.76 Hz), −9.55 (d, *J* = 14.76 Hz). ^{13}C NMR (125 MHz, MeOD) δ 64.91, 69.68, 76.20, 84.36, 94.56, 107.88, 143.43, 163.72, 177.45. LC/ESI-MS: negative mode 419 ([M − H]$^-$), positive mode 421 ([M + H]$^+$).

3-Methyl-1-β-D-ribofuranosylpyrimidine-2,4-dione 5′-Diphosphate (10b).[49] ^{1}H NMR (500 MHz, MeOD) δ 3.33 (s, 3H), 4.18 (m, 2H), 4.27 (q, 1H, *J* = 4.25 Hz), 4.32 (m, 1H), 4.44 (t, 1H, *J* = 5.35 Hz), 5.97 (m, 2H), 8.11 (d, 1H, *J* = 8.19 Hz). ^{31}P NMR (202 MHz, MeOD) δ −11.26 (d, *J* = 14.76 Hz), −7.17 (d, *J* = 14.76 Hz). ^{13}C NMR (125 MHz, MeOD) δ 28.41, 65.49, 70.65, 76.19, 84.89, 91.52, 102.57, 141.16, 153.23, 165.91. LC/ESI-MS: negative mode, 417 ([M − H]$^-$), positive mode 419 ([M + H]$^+$).

3-Phenacyl-1-β-D-ribofuranosylpyrimidine-2,4-dione 5′-Diphosphate (10c). ^{1}H NMR (500 MHz, D_2O + NaOD) δ 3.42 (m, 2H), 3.77 (m, 2H), 3.90 (m, 1H), 5.28 (m, 2H), 5.32 (m, 2H), 7.27−7.76 (m, 5H), 8.11 (m, 1H). ^{31}P NMR (202 MHz, D_2O + NaOD) δ −14.72 (d, *J* = 19.68 Hz), −10.21 (d, *J* = 19.68 Hz). ^{13}C NMR (125 MHz, D_2O) δ 50.36, 66.53, 72.01, 75.80, 85.47, 91.83, 104.09, 131.27−137.91, 142.92, 154.18, 166.84, 187.73. LC/ESI-MS: negative mode 521 ([M − H]$^-$), positive mode 523 ([M + H]$^+$).

5-Bromo-1-β-D-ribofuranosyl(3*H*)pyrimidine-2,4-dione 5′-Diphosphate (10d).[50,51] ^{1}H NMR (500 MHz, D_2O) δ 4.21 (m, 2H), 4.25 (m, 1H), 4.37 (m, 2H), 5.92 (d, 1H, *J* = 4.72 Hz), 8.24 (s, 1H). ^{31}P NMR (202 MHz, D_2O) δ −10.94 (d, *J* = 22.14 Hz), −7.90 (d, *J* = 17.22 Hz). ^{13}C NMR (125 MHz, D_2O) δ 67.21, 72.01, 76.72, 86.12, 91.88, 99.84, 143.76, 154.16, 165.05. LC/ESI-MS: negative mode 481 and 483 ([M − H]$^-$), positive mode 483 and 485 ([M + H]$^+$).

6-Methyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione 5′-Diphosphate (10f). ^{1}H NMR (500 MHz, D_2O) δ 2.15 (s, 3H), 3.97 (m, 2H), 4.08 (m, 1H), 4.50 (t, 1H, *J* = 6.46 Hz), 4.66 (m, 1H), 5.64 (s, 1H), 6.22 (d, 1H, *J* = 2.83 Hz). ^{31}P NMR (202 MHz, D_2O) δ −10.50 (d, *J* = 17.22 Hz), −8.42 (d, *J* = 19.68 Hz). ^{13}C NMR (125 MHz, D_2O) δ 20.71, 67.96, 72.15, 74.33, 84.47, 90.52, 102.08, 157.39, 160.76, 168.15. LC/ESI-MS: negative mode 417 ([M − H]$^-$), positive mode 419 ([M + H]$^+$).

6-Propyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione 5′-Diphosphate (10g). ^{1}H NMR (500 MHz, MeOD) δ 1.03 (t, 3H, *J* = 7.25 Hz), 1.65 (m, 2H), 2.38 (t, 2H, *J* = 7.56 Hz), 4.0 (m, 2H), 4.24 (m, 1H), 4.58 (t, 1H, *J* = 6.77 Hz), 4.66 (m, 1H), 5.67 (s, 1H), 6.29 (d, 1H, *J* = 2.83 Hz). ^{31}P NMR (202 MHz, MeOD) δ −11.20 (d, *J* = 17.22 Hz), −10.02 (d, *J* = 19.68 Hz). ^{13}C NMR (125 MHz, MeOD) δ 15.29, 22.97, 36.58, 68.12, 72.27, 74.32, 84.51, 90.53, 101.38, 155.00, 160.76, 168.33. LC/ESI-MS: negative mode 445 ([M − H]$^-$), positive mode 447 ([M + H]$^+$).

2-Thio-1-β-D-ribofuranosyl(3*H*)pyrimidine-2,4-dione 5′-Monophosphate (11a).[46,48,52] ^{1}H NMR (500 MHz, MeOD) δ 4.02 (m, 2H), 4.26 (m, 1H), 4.34 (m, 1H), 4.41 (m, 1H), 6.26 (d, 1H, *J* = 7.88 Hz), 6.71 (d, 1H, *J* = 3.46 Hz), 8.36 (d, 1H, *J* = 8.19 Hz). ^{31}P NMR (202 MHz, MeOD) δ 1.62 (s). ^{13}C NMR (125 MHz, MeOD) δ 63.52, 70.24, 76.29, 85.10, 94.49, 107.96, 143.73, 163.84, 178.02. LC/ESI-MS: negative mode 339 ([M − H]$^-$), positive mode 341 ([M + H]$^+$).

3-Methyl-1-β-D-ribofuranosyl-pyrimidine-2,4-dione 5′-Monophosphate (11b).[53] ^{1}H NMR (500 MHz, MeOD) δ 3.33 (s, 3H), 4.01 (m, 2H), 4.20 (q, 1H, *J* = 1.78 Hz), 4.36 (m, 2H), 6.03 (m, 2H), 8.19 (d, 1H, *J* = 8.19 Hz). ^{31}P NMR (202 MHz, MeOD) δ 1.03 (s). ^{13}C NMR (125 MHz, MeOD) δ 28.69, 65.41, 71.51, 75.69, 90.77, 98.75, 102.95, 138.96, 141.40, 149.80. LC/ESI-MS: negative mode 337 ([M − H]$^-$), positive mode 339 ([M + H]$^+$).

3-Phenacyl-1-β-D-ribofuranosylpyrimidine-2,4-dione 5′-Monophosphate (11c). ^{1}H NMR (500 MHz, D_2O) δ 4.10 (m, 2H), 4.29 (m, 1H), 4.33 (t, 1H, *J* = 4.86 Hz), 4.37 (t, 1H, *J* = 4.86 Hz), 5.51 (s, 2H), 5.98 (d, 1H, *J* = 4.47 Hz), 6.07 (d, 1H, *J* = 8.15 Hz), 7.60−8.09 (m, 6H). ^{31}P NMR (202 MHz, D_2O) δ 0.60 (s). ^{13}C NMR (125 MHz, D_2O) δ 50.65, 67.53, 72.08, 76.83, 86.08, 92.31, 104.67, 131.16−137.80, 143.22, 154.65, 165.18, 188.66. LC/ESI-MS: negative mode 441 ([M − H]$^-$), positive mode 443 ([M + H]$^+$).

5-Bromo-1-β-D-ribofuranosyl(3*H*)pyrimidine-2,4-dione 5′-Monophosphate (11d).[54] ^{1}H NMR (500 MHz, D_2O) δ 4.07 (m, 2H), 4.28 (m, 1H), 4.32 (t, 1H, *J* = 4.73 Hz), 4.36 (t, 1H, *J* = 5.13 Hz), 5.95 (d, 1H, *J* = 4.99 Hz), 8.26 (s, 1H). ^{31}P NMR (202 MHz, D_2O) δ 1.09 (s). ^{13}C NMR (125 MHz, D_2O) δ 66.83, 72.57, 76.79, 86.46, 91.71, 99.89, 143.78, 154.09, 164.88. LC/ESI-MS: negative mode 401 ([M − H]$^-$), 423 ([(M − H + Na) - H]$^-$), positive mode 403 ([M + H]$^+$).

1-β-D-Ribofuranosyl(3*H*)pyrimidine-2,4,6-trione 5′-Monophosphate (11e).[55] ^{1}H NMR (500 MHz, D_2O) δ 3.86 (m, 2H), 3.98 (m, 3H), 4.39 (m, 2H), 6.159 (d, 1H, *J* = 3.15 Hz). ^{31}P NMR (202 MHz, D_2O) δ 1.56 (s). ^{13}C NMR (125 MHz, D_2O) δ 47.35, 66.34, 69.56, 72.08, 81.32, 87.98, 153.68, 167.46. LC/ESI-MS: negative mode 339 ([M − H]$^-$), positive mode 441 ([M + H]$^+$).

6-Methyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione 5′-Monophosphate (11f).[56] ^{1}H NMR (500 MHz, MeOD) δ 2.15 (s, 3H), 3.98 (m, 2H), 4.14 (m, 1H), 4.40 (t, 1H, *J* = 6.46 Hz), 4.65 (m, 1H), 5.51 (s, 1H), 6.27 (d, 1H, *J* = 3.15 Hz). ^{31}P NMR (202 MHz, MeOD) δ −1.70 (s). ^{13}C NMR (125 MHz, MeOD) δ 18.70, 67.52, 72.32, 73.44, 84.33, 89.80, 100.21, 152.80, 154.57, 165.76. LC/ESI-MS: negative mode: 337 ([M − H]$^-$), positive mode: 339 ([M + H]$^+$).

6-Propyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione 5′-Monophosphate (11g). ^{1}H NMR (500 MHz, MeOD) δ 1.01 (t, 3H, *J* = 7.40 Hz), 1.64 (m, 2H), 2.36 (t, 2H, *J* = 7.72 Hz), 3.98 (m, 2H), 4.14 (m, 1H), 4.43 (t, 1H, *J* = 6.46 Hz), 4.67 (m, 1H), 5.51 (s, 1H), 6.28 (d, 1H, *J* = 3.15 Hz). ^{31}P NMR (202 MHz, MeOD) δ 0.47 (s). ^{13}C NMR (125 MHz, MeOD) δ 13.98, 22.16, 35.47, 67.19, 72.38, 73.43, 84.44, 89.75, 99.52, 152.97, 158.09, 165.95. LC/ESI-MS: negative mode 365 ([M − H]$^-$), positive mode 367 ([M + H]$^+$).

6-Propyl-3-β-D-ribofuranosyl(1*H*)pyrimidine-2,4-dione 2′,3′-(Cyclic phosphate) 5′-Triphosphate (12). ^{1}H NMR (500 MHz, MeOD) δ 1.02 (t, 3H, *J* = 7.40 Hz), 1.65 (m, 2H), 2.38 (t, 2H, *J* = 7.72 Hz), 4.17 (m, 2H), 4.41 (q, 1H, *J* = 5.88 Hz), 5.06 (m, 1H), 5.23 (m, 1H), 5.53 (s, 1H), 6.54 (s, 1H). ^{31}P NMR (202 MHz, MeOD) δ −23.40 (t, *J* = 20.91 Hz), −11.69 (d, *J* = 19.68 Hz), −10.73 (d, *J* = 19.68 Hz), 19.02 (d, *J* = 14.76 Hz). ^{13}C NMR (125 MHz, MeOD) δ 14.04, 22.13, 35.59, 67.60, 80.46, 82.60, 86.65, 88.79, 99.45, 152.81, 158.42, 163.39. LC/ESI-MS: negative mode 587 ([M − H]$^-$), positive mode 589 ([M + H]$^+$).

3-Phenacyl-1-β-D-ribofuranosylpyrimidine-2,4-dione 2′-Monophosphate 5′-Triphosphate (13). ^{1}H NMR (500 MHz, MeOD) δ 4.32 (m, 2H), 4.49 (m, 1H), 5.04 (m, 2H), 5.44 (d, 2H, *J* = 3.15 Hz), 5.99 (d, 1H, *J* = 8.19 Hz), 6.19 (d, 1H, *J* = 3.46 Hz), 7.57−8.11 (m, 6H). ^{31}P NMR (202 MHz, MeOD) δ −22.50 (t, *J* = 18.45 Hz), −11.60 (d, *J* = 19.68 Hz), −9.67 (d, *J* = 17.22 Hz), 18.43 (t,

J = 8.61 Hz). ^{13}C NMR (125 MHz, MeOD) δ 47.76, 66.72, 78.95, 82.60, 85.91−85.99, 93.73, 102.94, 129.46−136.58, 142.49, 152.45, 164.82, 194.06. LC/ESI-MS: negative mode 681 ([M − H]$^-$), positive mode 683 ([M + H]$^+$).

5-Bromouridine-5′-uridylic Acid (1,1-Dichloro-1-phosphonomethyl-1-phosphonyl)anhydride (15). ^{1}H NMR (500 MHz, D_2O) δ 4.22 (m, 3H), 4.38 (m, 2H), 5.95 (d, 1H, J = 5.04 Hz), 8.24 (s, 1H). ^{31}P NMR (202 MHz, D_2O) δ −10.92 (d, J = 31.99 Hz), 1.33 (dd, J^1 = 22.14 Hz, J^2 = 49.22 Hz), 7.52 (dd, J^1 = 27.07 Hz, J^2 = 59.06 Hz). ^{13}C NMR (125 MHz, D_2O) δ 67.91, 72.40, 76.69, 80.42, 86.40, 91.53, 99.94, 143.74, 154.11, 164.88. LC/ESI-MS: negative mode 627, 629, 631 ([M − H]$^-$), positive mode 646, 648, 649 ([M + H + NH_4^+]$^+$).

Functional Assays at $P2Y_2$, $P2Y_4$, and $P2Y_6$ Receptors: Determination of Inositol Phosphate Accumulation by SPA Beads. Assays were performed using 1321N1 astrocytoma cells recombinantly expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor subtype (Department of Pharmacology, University of North Carolina). Cells were seeded at a density of 30 000 cells in 100 μL medium/well in 96-well plates 3 days before assays. Cells were incubated with [^{3}H]inositol medium, (1 μCi/40 μL)/well, overnight. The test compounds were added in 10 μL of a 5-fold concentrated solution in 250 mM HEPES/50 mM LiCl buffer, pH 7.25. Following a 10 min incubation at 37 °C, the medium was aspirated and the assay was terminated by adding 30 μL of ice-cold formic acid (50 mM) to the cells. The cells were lysed for at least 30 min. Scintillation proximity assay (SPA) beads (from Amersham) suspended in water were put into a 96-well TopCount plate (70 μL resin, 0.2 mg/well) and kept at 4 °C. All 30 μL of cell lysates was transferred from the culture plates to the TopCount plates, and the TopCount plates were shaken for at least 1 h at 4 °C. Then the TopCount plate was allowed to settle down for at least 1 h at room temperature and subsequently counted. Data were analyzed with PRISM 4.0 (GraphPad Software Inc., San Diego, CA). Three separate experiments were performed, and each data point was determined in triplicate.

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Supporting Information Available: Synthetic procedures, ^{1}H and ^{13}C NMR spectral data, and elemental analysis results for nucleosides **6a−g** and HPLC purity data for the synthesized nucleotides in two different HPLC chromatography systems. This material is available free of charge via the Internet at http://pubs.acs.org.

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