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Synthesis and potency of novel uracil nucleotides and derivatives as $P2Y_2$ and $P2Y_6$ receptor agonists

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ABSTRACT

The phosphate, uracil, and ribose moieties of uracil nucleotides were varied structurally for evaluation of agonist activity at the human $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. The 2-thio modification, found previously to enhance $P2Y_2$ receptor potency, could be combined with other favorable modifications to produce novel molecules that exhibit high potencies and receptor selectivities. Phosphonomethylene bridges introduced for stability in analogues of UDP, UTP, and uracil dinucleotides markedly reduced potency. Truncation of dinucleotide agonists of the $P2Y_2$ receptor, in the form of Up_4-sugars, indicated that a terminal uracil ring is not essential for moderate potency at this receptor and that specific SAR patterns are observed at this distal end of the molecule. Key compounds reported in this study include **9**, α,β-methylene-UDP, a $P2Y_6$ receptor agonist; **30**, Up_4-phenyl ester and **34**, Up_4-[1]glucose, selective $P2Y_2$ receptor agonists; dihalomethylene phosphonate analogues **16** and **41**, selective $P2Y_2$ receptor agonists; **43**, the 2-thio analogue of INS37217 (P^1-(uridine-5′)-P^4-(2′-deoxycytidine-5′)tetraphosphate), a potent and selective $P2Y_2$ receptor agonist.

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1. Introduction

The P2Y receptor family consists of at least eight human subtypes that are activated by either or both adenine and uracil nucleotides.[1,2] $P2Y_2$ and $P2Y_4$ nucleotide receptors respond to uridine 5′-triphosphate (UTP, **1**) and its analogues, and the $P2Y_6$ receptor responds to uridine 5′-diphosphate (UDP, **2**) and analogues.[3] However, this delineation of agonist selectivities is not absolute. For example, Müller and coworkers and Besada et al. reported that certain 5′-triphosphate derivatives potently activate the $P2Y_6$ receptor.[4,5] The conformational preference of the ribose moiety in binding to uridine nucleotide-activated P2Y receptors has been explored through substitution with the sterically constrained methanocarba (bicyclo[3.1.0]hexane) ring system. $P2Y_2$ and $P2Y_4$ receptors display a North conformational preference, while the $P2Y_6$ receptor prefers the South.[6–8]

Several recent studies have explored structure–activity relationships (SARs) at the $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors.[4–6,9–11] Key pharmacological probes introduced include the nonselective $P2Y_2$ receptor agonists **3** and **4**, which have progressed to clinical studies for dry eye syndrome and pulmonary diseases, the potent and selective $P2Y_2$ receptor agonist **5**,[7] and the selective $P2Y_6$ receptor agonist **6** (Chart 1).[12,13] Modification of the base moiety of UTP to form C-linked nucleotides is possible in $P2Y_2$ receptor agonists and results in enhanced stability.[10] Various dinucleotides tend to activate $P2Y_2$ and $P2Y_4$ receptors (diuridine tetraphosphates) or $P2Y_6$ receptors (diuridine triphosphates) with moderate potency and with greater stability than analogues of UTP and UDP.[9] Also, the pharmacological activity of diadenosine polyphosphates at both P2Y and P2X receptor subtypes has been characterized.[1,2] Diadenosine tetraphosphate is only 3-fold less potent than ATP at the human $P2Y_2$ receptor.

In the present study, we further investigated structure–activity relationships at P2Y receptors through synthesis of molecules with additional substitutions of the uracil, ribose, and phosphate moieties and combinations thereof. These analogues of UDP, UTP, and dinucleotides were assayed for capacity to promote $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor-mediated activation of phospholipase C (PLC).[3] These novel derivatives incorporated groups such as 2-thio, found previously to enhance receptor potency.[4,7] Phosphonomethylene bridges and nonphosphate linkages were introduced to enhance

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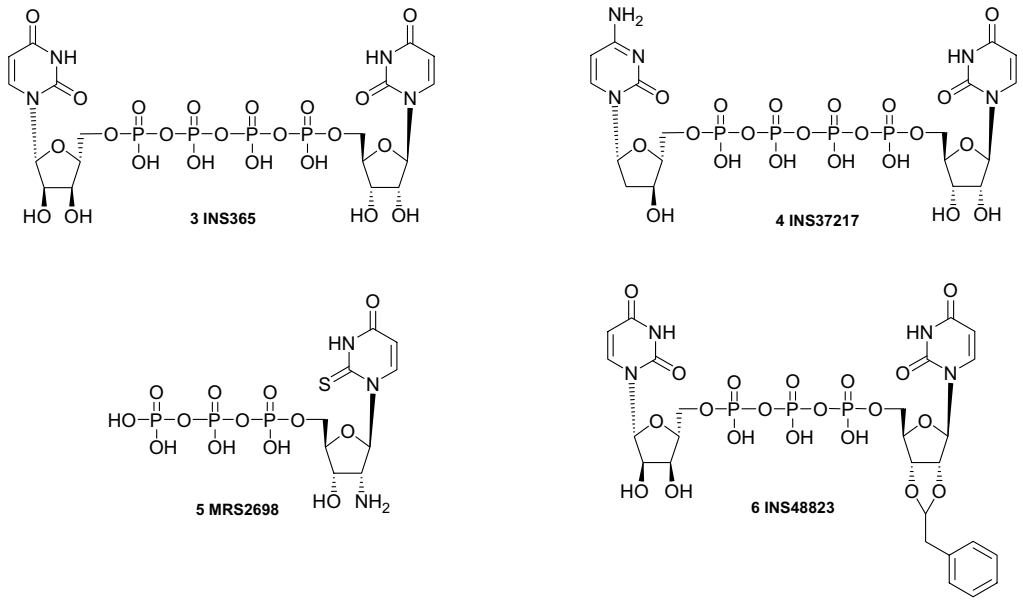


Chart 1. Agonists of the $P2Y_2$ and $P2Y_6$ receptors.

stability of molecules against the action of ectonucleotidases.[14] We also probed the effects of truncation of dinucleotide agonists of the $P2Y_2$ receptor by synthesizing and quantifying the activities of a series of Up_4-sugars.[7]

2. Results and discussion

2.1. Chemical synthesis

The synthetic routes to the novel nucleotide derivatives (Tables 1–3) are shown in Schemes 1–6. The potencies of five known reference compounds are listed in Table 1 ($P2Y_6$ agonist UDP **2**), Table 2 ($P2Y_2$ agonists UTP **1** and MRS2698 **5**), and Table 3 ($P2Y_2$ agonists Up_4U **3** and INS37217 **4**). Types of modifications include UDP analogues containing a methylene-bridged substitute for the diphosphate group (Scheme 1), UTP analogues containing a β,γ-dihalomethylene-bridge in the triphosphate group[4] (Scheme 2), UTP analogues with modified uracil and ribose moieties[7] (Scheme 3), a 5-iodo analogue of INS48823[5,13] (Scheme 4), analogues of uridine 5′-tetraphosphate[7] (Scheme 5) in which the terminal phosphate moiety was condensed with various alcohols, including sugars. Among the derivatives in Scheme 1, compounds **7** and **10** are new compounds, but **8** and **9** were previously reported[15,16] (see Table 1).

The synthesis of UTP analogues **12** and **17**–**25** from the corresponding nucleosides was by standard methods of di-, triphosphate formation.[6] In each case the unprotected nucleoside was first treated with phosphorous oxychloride (Scheme 3). The reaction mixture was either treated immediately with bis(tri-*n*-butylammonium) pyrophosphate (phosphoric acid for **12**) or the isolated 5′-monophosphate was activated with 1,1′-carbonyldiimidazole (CDI) followed by the pyrophosphate salt. An attempt to synthesize 2,4-dithio-UTP led to isolation only of the 4-methylthio analogue **17**. Identification of nucleotide compounds was confirmed by NMR (^1H and ^{31}P) and by high-resolution mass spectrometry (HRMS), and purity was demonstrated with high-performance liquid chromatography (HPLC).

The preferred method of synthesis of 2′-MeUTP (**20**) and 3′-MeUTP (**21**) was through isolation of the monophosphate. 2′-*C*-Methyl-uridine-5′-monophosphate[17] and 3′-*C*-methyl-uridine-5′-monophosphate[18] were obtained as ammonium salts following the Yoshikawa procedure[19] starting from nucleosides 2′-*C*-methyl-uridine (**49**) and 3′-*C*-methyl-uridine (**50**). The nucleotides also were prepared by the one-pot method using a sequential reaction of **49** and **50** with phosphorous oxychloride and pyrophosphoric acid tributylammonium salt, but the yields were lower.

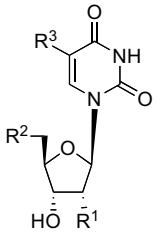
Most of the required nucleoside precursors were readily available, with several exceptions. 2′-Ureido-2′-deoxyuridine **46** was prepared by a one-step method from 2′-amino-2′-deoxyuridine **57** (Scheme 6A).[20] The synthesis of 2,4-dithiouridine **47** is depicted in Scheme 6B. The commercially available β-D-ribofuranose 1,2,3,5-tetraacetate **58** was coupled with silylated 2-thiouracil under $SnCl_4$-catalyzed Vorbrüggen conditions.[21] 4-Thionation of the resulting 2′,3′,5′-tri-*O*-acetyl-2-thiouridine **59** was performed using Lawesson's reagent.[22] Subsequent sugar deprotection of **60** afforded 2,4-dithiouridine **47** in 59% overall yield. The nucleoside 1-(β-D-arabinofuranosyl)-2-thio(1*H*)pyrimidin-4-one **48** was obtained via opening of 2,2′-*O*-anhydrouridine **61**with H_2S and triethylamine in anhydrous DMF (Scheme 6C).[23] The synthetic routes to the nucleosides 2′-*C*-methyl-uridine **49** and 3′-*C*-methyl-uridine **50**, synthesized using the strategy reported by Wolfe & Harry-O'kuru[24] and Mikhailov et al.[25a] with some modifications, are outlined in Scheme 6D.

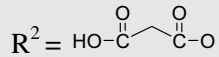
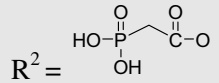
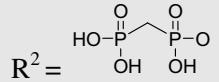
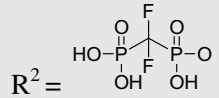
2.2. Pharmacological activity

Activation of PLC by a range of concentrations of each nucleotide derivative (**7**–**44**) was studied in [^{3}H]inositol-labeled 1321N1 human astrocytoma cells stably expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptors (Tables 1–3) by methodology (see Section 3) we have described previously in detail.[1,3,6,26]

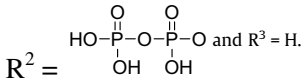
Table 1 illustrates UDP analogues that were designed for possible interaction with the $P2Y_6$ receptor. Compounds **7** and **8** are derivatives substituted with an anionic carboxylic acid or phosphonate acetyl ester moiety with the goal of approximating the charge and electronic characteristics of the diphosphate moiety for interaction with cationic residues in the ligand binding pocket.[8] These molecules were inactive at the $P2Y_6$ receptor. Introduction of an α,β-methylene **9** substitution only slightly reduced potency at the $P2Y_6$ receptor (Fig. 1), while the α,β-difluoromethylene analogue

Table 1
Relative potencies of UDP, **2**, and UDP analogues for activation of the human $P2Y_6$ receptor



Compound	Modification	Structure	EC_{50} (μM) $hP2Y_6$receptor[a]
Nucleosides			
7	Uridine-5′-malonate		NE
8	Uridine-5′-phosphonoacetate		NE
Diphosphates			
2	UDP		0.30 ± 0.06
9[b]	Up-CH_2-p (α,β-Methylene UDP)		0.66 ± 0.11
10	Up-CF_2-p (α,β-difluoromethylene UDP)		NE
11	5-Amino-UDP	$R^3 = NH_2$	0.61 ± 0.17
12	2′-Deoxy-2′-ureido-UDP	$R^1 = NHCONH_2$	4.70 ± 0.44

NE, no effect at 10 μM.

Unless noted: $R^1 = OH$; $R^2 =$  and $R^3 = H$.

[a] Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human $P2Y_6$ receptor. Potencies are presented in the form of EC_{50} values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as means ± standard error and are the average of three to six different experiments with each molecule.

[b] **9**, MRS2782.

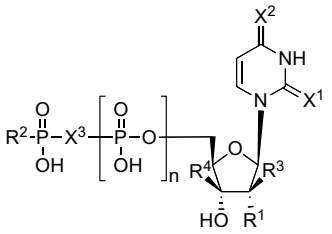
10 was strikingly inactive. Nucleobase substitution was also examined in the UDP series. We previously reported that the higher homologue of **11**, 5-amino-UTP, displays increased potency relative to UTP as a $P2Y_6$ receptor agonist.[7] In contrast, the corresponding diphosphate **11** was ~50-fold less potent than UDP. The introduction of a polar ureido group on the ribose moiety of adenosine derivatives provided tailored analogues for selective recognition by adenosine neoceptors.[27] Therefore, we replaced the 2′-hydroxy group of UDP with a highly H-bonding ureido group. This molecule exhibited a 360-fold reduction of potency at the $P2Y_6$ receptor compared to UDP.

Table 2 illustrates composite data for UTP analogues designed to activate the $P2Y_2$ and $P2Y_4$ receptors. We previously illustrated that substitution of a 2-thio group enhances potency and/or selectivity of UTP analogues (e.g., **5** and **14**) at the $P2Y_2$ receptor.[7] The combination of the 2-thio modification with a β,γ-difluoromethylene **15** or β,γ-dichloromethylene **16** substitution of the triphosphate moiety or 4-methylthio **17** group resulted in analogues that were 50- to 100-fold weaker than **14**. A report of another UTP analogue,[4] 5-bromo-β,γ-dichloromethylene-UTP, that displayed submicromolar $P2Y_2$ receptor potency had suggested the possibility of greater potency in **15** and **16** than we observed experimentally in the current study. Nevertheless, the dichloromethylene group of **16** provides moderate selectivity for the $P2Y_2$ receptor, while the equipotent difluoromethylene derivative **15** was only marginally selective. Replacement of the 2′-hydroxy group of UTP with a ureido group **18** resulted in a 36-fold reduction of potency at the $P2Y_2$ receptor and >100-fold reduction at the $P2Y_4$ receptor.

Methyl groups were placed on the ribose ring of UTP at the 2′ and 3′ positions in **20** and **21**, respectively. Like the methanocarba modification of ribose,[6,28] this approach is a means of conformational control of the ribose ring that has proven effective for achieving selectivity in A_1 adenosine receptor agonists.[29,30] Compound **20** maintains a North conformation of the ribose ring and, as is the case with UTP, activated the $P2Y_2$ and $P2Y_4$ receptors nearly equipotently. However, a 20- to 30-fold decrease in potency relative to UTP was observed. In contrast, **21**, which maintains a South conformation of the ribose ring, was inactive at both $P2Y_2$ and $P2Y_4$ receptors. These results are consistent with previously reported conformational preferences of these receptors deduced from our studies with methanocarba-derivatives of UTP.[6]

No previous studies have reported the potency of nucleoside 5′-tetraphosphates at the $P2Y_2$ receptor, although adenosine 5′-tetraphosphate (Ap_4) was reported to activate a presumed P2X receptor.[31] Therefore, we synthesized and evaluated the activities of 5′-tetraphosphate and pentaphosphate derivatives **22**–**25**. The potency of uridine 5′-tetraphosphate **22** (Up_4) was greatly reduced in

Table 2
Relative potencies of UTP, **1**, and UTP analogues for activation of the human $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors



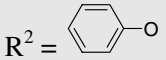
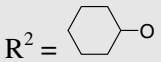
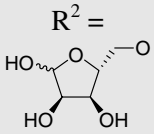
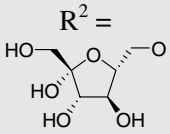
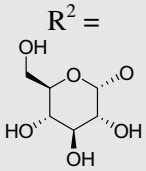
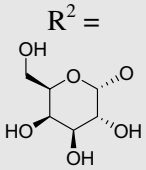
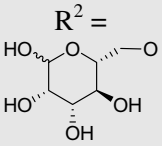
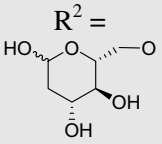
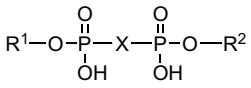
Compound	Modification	Structure	EC_{50} [a] (μM)		
			$hP2Y_2$	$hP2Y_4$	$hP2Y_6$
Triphosphates (n = *2*)					
1[b]	(=UTP)		0.060 ± 0.00	0.090 ± 0.01	>10[d]
13[b]	2′-Deoxy-2′-amino-UTP	$R^1 = NH_2$	0.062 ± 0.008	1.2 ± 0.3	NE
14[b]	2-Thio-UTP	$X^1 = S$	0.035 ± 0.004	0.35 ± 0.10	~1.5[b]
5[b,c]	2-Thio-2′-deoxy-2′-amino-UTP	$X^1 = S$, $R^1 = NH_2$	0.008 ± 0.002	2.4 ± 0.8	NE
15	2-Thio-β,γ-difluoromethylene-UTP	$X^1 = S$, $X^3 = CF_2$	1.63 ± 0.36	8.11 ± 0.69	5.15 ± 0.69
16[c]	2-Thio-β,γ-dichloromethylene-UTP	$X^1 = S$, $X^3 = CCl_2$	2.51 ± 0.65	NE	>10[d]
17	2-Thio-4-methylthio-UTP	$X^1 = S$, $X^2 = S\ CH_3$	0.91 ± 0.06	5.35 ± 1.20	>10[d]
18	2′-Deoxy-2′-ureido-UTP	$R^1 = NHCONH_2$	1.74 ± 0.25	4.64 ± 2.05	>10[d]
19	2-Thio-arabino-UTP	$R^1 = H$, $R^3 = OH$	0.14 ± 0.01	7.93 ± 0.81	NE
20	2′-Methyl-UTP	$R^3 = CH_3$	1.45 ± 0.26	1.26 ± 0.14	NE
21	3′-Methyl-UTP	$R^4 = CH_3$	NE	NE	NE
Tetra- (n = 3) and penta- (n = 4) phosphates					
22	Up_4		2.61 ± 1.39	4.64 ± 2.05	7.56 ± 1.07
23	2-Thio-Up_4	$X^1 = S$	0.60 ± 0.20	5.52 ± 1.75	6.83 ± 1.74
24	4-Thio-Up_4	$X^2 = S$	0.070 ± 0.01	0.28 ± 0.06	6.46 ± 0.41
25	2-Thio-Up_5	$X^1 = S$, $n = 4$	0.57 ± 0.16	5.27 ± 1.24	7.33 ± 0.66
26	Up_4-OMe	$R^2 = OCH_3$	3.95 ± 0.51	2.70 ± 0.43	>10[d]
27	Up_4-δ-Me-phosphonate	$R^2 = CH_3$	4.18 ± 0.43	2.53 ± 0.57	8.16 ± 0.74
28	Up_4-$O(CH_2)_2CN$	$R^2 = O(CH_2)_2CN$	1.70 ± 0.22	1.96 ± 0.53	>10[d]
29	Up_4-$OCH_2CHOHCH_2OH$	$R^2 = OCH_2CHOH\text{-}CH_2OH$	1.87 ± 0.15	1.12 ± 0.04	8.19 ± 0.41
30[c]	Up_4-OC_6H_5	$R^2 =$ 	1.89 ± 0.07	NE	NE
31	Up_4-OC_6H_{11}	$R^2 =$ 	5.86 ± 0.33	>10[d]	>10[d]
Tetraphosphate sugars (n = *3*)					
32	Up_4-[5]ribose	$R^2 =$ 	1.88 ± 0.03	4.78 ± 0.40	>10[d]
33	Up_4-[6]fructose	$R^2 =$ 	3.33 ± 0.42	6.30 ± 0.75	>10[d]
34[c]	Up_4-[1]glucose	$R^2 =$ 	0.30 ± 0.13	2.06 ± 0.18	7.83 ± 0.17
35	Up_4-[1]galactose	$R^2 =$ 	4.85 ± 2.07	1.77 ± 0.41	8.19 ± 0.73

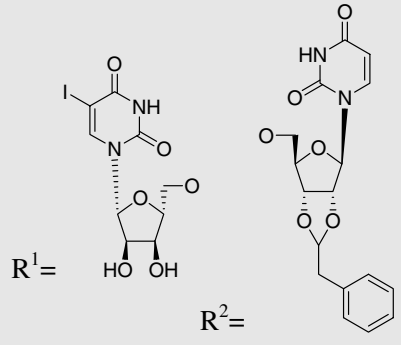
Table 2 (*continued*)

Compound	Modification	Structure	EC_{50} [a] (μM)		
			$hP2Y_2$	$hP2Y_4$	$hP2Y_6$
36	Up_4-[6]mannose		>10[d]	>10[d]	>10[d]
37	Up_4-[6]2′-deoxyglucose		3.54 ± 0.96	4.32 ± 1.50	>10[d]

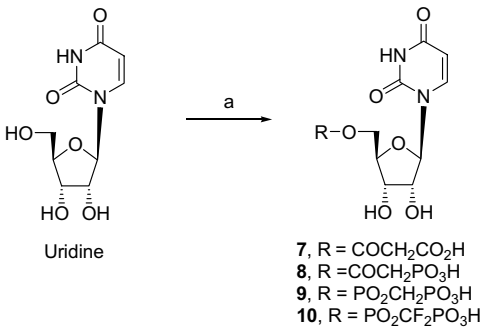
Unless noted: R^1, R^2 = OH; X = O; and R^3, R^4 = H.
NE, no effect at 10 μM.
[a] Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor. Potencies are presented in the form of EC_{50} values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as means ± standard error and are the average of three to six different experiments with each molecule.
[b] Agonist potencies from Refs. 4 and 7.
[c] **5**, MRS2698; **16**, MRS2725; **30**, MRS2768; **34**, MRS2732.
[d] ⩽50% effect at 10 μM.

Table 3
Relative potencies of dinucleotide derivatives for activation of the human $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors

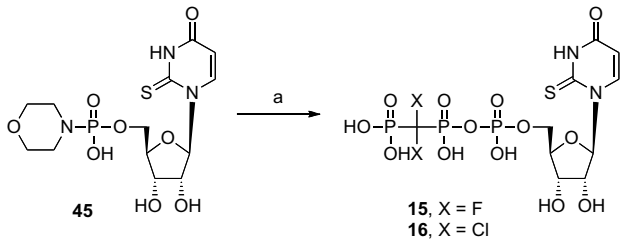


Compound	Modification	Structure	EC_{50}[a] (μM)		
			$hP2Y_2$	$hP2Y_4$	$hP2Y_6$
Dinucleoside triphosphates (n = 1)					
38[b]	Up_3U		1.31 ± 0.21	0.87 ± 0.11	0.27 ± 0.07
39[c]	5-I-Up_3- (2′,3′-phenylethyl acetal)U		9.97 ± 0.95	NE	5.49 ± 0.68
Dinucleoside tetraphosphates (n = 2)					
3	(=Up_4U)		0.21 ± 0.03	0.13 ± 0.01	1.16 ± 0.42
40	4-S-Up_4(4-S-U)	R^1 = R^2 = 4-thio-uridine	0.030 ± 0.010	0.08 ± 0.01	2.03 ± 0.18
41	Up_2-CF_2-p_2-U (β,γ-difluoromethylene Up4U)	X = CF_2	2.27 ± 1.39	>10[d]	>10[d]
42	Up_2-CCl_2-p_2-U (β,γ-dichloromethylene Up4U)	X = CCl_2	7.77 ± 1.39	NE	NE
4	Up_4-2′-dC (INS37217)	R^2 = 2′-deoxy-cytidine	0.14 ± 0.04	0.14 ± 0.04	0.95 ± 0.06
43	2-Thio-Up_4-2′-dC	R^1 = 2-thio uridine R^2 = 2′-deoxy-cytidine	0.08 ± 0.03	0.71 ± 0.15	1.05 ± 0.07
44	Up_4-2′-dG	R^2 = 2′-deoxy-guanosine	0.14 ± 0.04	0.54 ± 0.15	1.03 ± 0.08

Unless noted: R^1, R^2 = Uridine; and X = O.
NE, no effect at 10 μM.
[a] Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor. Potencies are presented in the form of EC_{50} values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as means ± standard error and are the average of three to six different experiments with each molecule.
[b] Reported in Ref. 9.
[c] **39**, MRS2752; **44**, MRS2798; **43**, MRS2657.
[d] ⩽50% effect at 10 μM.



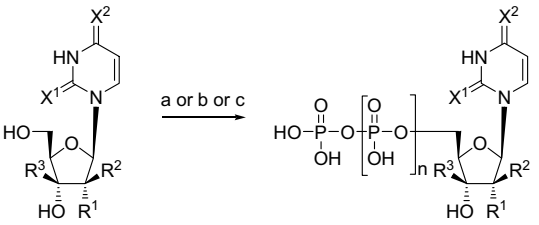
Scheme 1. Synthesis of UDP analogues containing a methylene-bridged substitute for the diphosphate group. Reagent and condition: (a) DCC, ROH, DMF, rt.



Scheme 2. Synthesis of UTP analogues containing a β,γ-dihalomethylene-bridge in the triphosphate group. Reagent and condition: (a) $PO(OH)_2CX_2PO(OH)_2$, DMF, rt.

comparison to UTP **1**. The high $P2Y_2$ receptor potency of 2-thio-UTP **14** was remarkably preserved in the corresponding 4-thio-5′-tetraphosphate analogue **24**; in contrast, the potency of the 2-thio analogue **23** was reduced. Nevertheless, these results illustrate that 2- or 4-thio substitution of Up_4 results in marked increases of potency in tetraphosphate molecules since both **23** and **24** were more potent at the $P2Y_2$ receptor than Up_4 **22**. Homologation of **23** to the pentaphosphate **25** had no effect on potency at the $P2Y_2$ receptor.

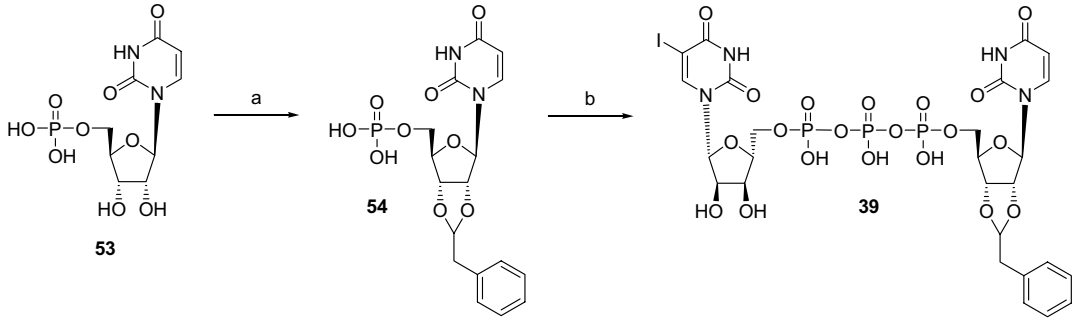
Although dinucleoside tetraphosphates, such as **3** and **4**, are known to activate $P2Y_2$ and $P2Y_4$ receptors with moderate potency,[9] the SAR of the terminal nucleoside moiety mainly has been explored for uridine and other nucleoside units. Therefore, we investigated the effects of substitution of the terminal nucleoside moiety with small organic moieties **26**–**31** or to sugars alone **32**–**37**. Most of these modifications led to $P2Y_2$ receptor potencies in the micromolar range. A terminal methyl phosphoester **26** and the corresponding phosphonate **27** were identical in potency as relatively weak $P2Y_2$ receptor agonists, with EC_{50} values of 4 μM. A terminal cyclohexyl ester **31** was significantly less potent than the corresponding terminal phenyl ester **30**, suggesting that aromatic or hydrophilic groups are more favored than simple hydrophobic groups in this region. Curiously, substitution of an acyclic alkyl phosphate or phosphonate at the terminal position (**26**–**29**) did not favor selective interaction with the $P2Y_2$ versus $P2Y_4$ receptors, while an aryl phosphate ester (**30**) substitution resulted in high selectivity for the $P2Y_2$ receptor (Fig. 2).



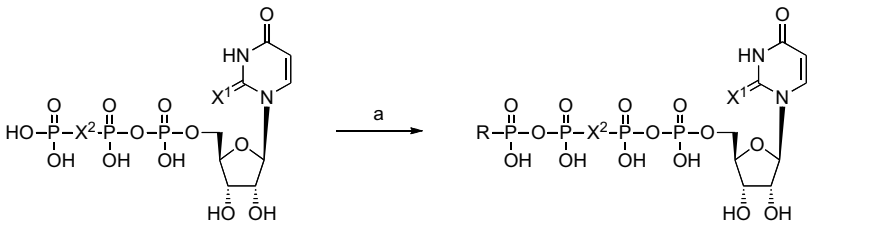
46, X^1 = O, X^2 = O, R^1 = $NHCONH_2$, R^2 = H, R^3 = H
47, X^1 = S, X^2 = S, R^1 = OH, R^2 = H, R^3 = H
48, X^1 = S, X^2 = O, R^1 = H, R^2 = OH, R^3 = H
49, X^1 = O, X^2 = O, R^1 = OH, R^2 = CH_3, R^3 = H
50, X^1 = O, X^2 = O, R^1 = OH, R^2 = H, R^3 = CH_3
51, X^1 = O, X^2 = S, R^1 = OH, R^2 = H, R^3 = H
52, X^1 = S, X^2 = O, R^1 = OH, R^2 = H, R^3 = H

12, X^1 = O, X^2 = O, R^1 = $NHCONH_2$, R^2 = H, R^3 = H, n =1
17, X^1 = S, X^2 = SCH_3, R^1 = OH, R^2 = H, R^3 = H, n = 2
18, X^1 = O, X^2 = O, R^1 = $NHCONH_2$, R^2 = H, R^3 = H, n = 2
19, X^1 = S, X^2 = O, R^1 = H, R^2 = OH, R^3 = H, n = 2
20, X^1 = O, X^2 = O, R^1 = OH, R^2 = CH_3, R^3 = H, n = 2
21, X^1 = O, X^2 = O, R^1 = OH, R^2 = H, R^3 = CH_3, n = 2
23, X^1 = S, X^2 = O, R^1 = OH, R^2 = H, R^3 = H, n = 3
24, X^1 = O, X^2 = S, R^1 = OH, R^2 = H, R^3 = H, n = 3
25, X^1 = S, X^2 = O, R^1 = OH, R^2 = H, R^3 = H, n = 4

Scheme 3. Synthesis of UTP analogues with modified uracil and ribose moieties. Reagents and conditions: (a) i—$POCl_3$, proton sponge, $PO(OMe)_3$, 0 °C; ii—$(Bu_3NH)_2H_2P_2O_7$, Bu_3N, DMF, 0 °C; iii—TEAB 0.2 M rt; (b) i—$POCl_3$, $PO(OMe)_3$, 4 h, 0 °C; ii—concd NH_4OH; iii—Bu_3N, CDI, DMF, **48**; iv—$(Bu_3NH)_2H_2P_2O_7$; TEAB 1 M for **19**; (c) i—$POCl_3$, $PO(OMe)_3$, **49** or **50**, 0 °C; ii—concd NH_4OH; iii—CDI, DMF, rt; iv—$(Bu_3NH)_2H_2P_2O_7$, DMF, rt for **20** or **21**. Note that the UDP analogue **6** (not shown in scheme) was prepared by a similar method to (a), except for use of a phosphoric acid salt in the second step: (ii) $(Bu_3NH)_2H_2PO_4$, Bu_3N, DMF, 0 °C.



Scheme 4. Synthesis of a 5-iodo analogue of INS48823. Reagents and conditions: (a) phenylacetaldehyde dimethylacetal, TFA, rt; (b) 5-iodo-uridine-5′-diphosphoimidazolidate, DMF, rt.



1, X^1 = O, X^2 = O,
14, X^1 = S, X^2 = O,
55, X^1 = O, X^2 = CCl_2
56, X^1 = O, X^2 = CF_2

26, X^1 = O, X^2 = O, R = OCH_3
27, X^1 = O, X^2 = O, R = CH_3
28, X^1 = O, X^2 = O, R = $O(CH_2)_2CN$
29, X^1 = O, X^2 = O, R = $OCH_2CHOHCH_2OH$
30, X^1 = O, X^2 = O, R = OC_6H_5
31, X^1 = O, X^2 = O, R = OC_6H_{11}
32, X^1 = O, X^2 = O, R = [5]ribose
33, X^1 = O, X^2 = O, R = [6]fructose
34, X^1 = O, X^2 = O, R = [1]glucose
35, X^1 = O, X^2 = O, R = [1]galactose
36, X^1 = O, X^2 = O, R = [6]mannose
37, X^1 = O, X^2 = O, R = [6]2'-deoxyglucose
41, X^1 = O, X^2 = CF_2, R = uridine
42, X^1 = O, X^2 = CCl_2, R = uridine
43, X^1 = S, X^2 = O, R = 2'-deoxy-cytidine

Scheme 5. Synthesis of 5′-tetraphosphate analogues, including Up_4-sugars and dinucleotides. The 2′-deoxyguanosine derivative **44** was prepared in the same manner as **43**. Reagents and conditions: (a) i—DCC, DMF, rt; ii—$RPO(OH)_2$, DMF, rt.

Among tetraphosphate sugar derivatives, Up_4-[1] glucose **34** was the most potent with an EC_{50} of 0.3 μM (Fig. 3). The $P2Y_2$ receptor selectivity of **34** in comparison to $P2Y_4$ and $P2Y_6$ receptors was 7- and 26-fold, respectively. Inversion of the chirality of the 4′-hydroxyl group in the sugar, that is, galactose rather than glucose, in **35** reduced potency at the $P2Y_2$ receptor (16-fold), but not at the $P2Y_4$ receptor. The mannose adduct **36** of Up_4[6] was essentially inactive at the $P2Y_2$ receptor. Removal of a single hydroxyl group of **36** to form **37** increased the potency at both $P2Y_2$ and $P2Y_4$ receptors. These results are consistent with our recent $P2Y_2$ receptor modeling study, which proposes specific interactions in the region of the distally-binding uridine moiety of Up_4U.[7]

The SAR of dinucleotides at the $P2Y_2$ and $P2Y_6$ receptors also was further explored (Table 3). An analogue of a known $P2Y_6$ receptor agonist,[9] Up_3U **38**, was designed to combine multiple reported favorable modifications of uracil nucleotides directed toward activation of the $P2Y_6$ receptor based on the agonist INS48823 **6**.[13] The resulting hybrid compound **39**, containing 5-iodo substitution and a 2′,3′-phenylmethylacetyl group on opposite uridine moieties of Up_3U, was a nonselective agonist of the $P2Y_6$ receptor.

Several new dinucleoside tetraphosphate derivatives **41**–**44** were synthesized, and their activities were compared to previously studied compounds **3**, **4**.[9,12] Compound **40** was reported by Shaver et al.[9] The inclusion of a β,γ-difluoromethylene **41** or dichloromethylene **42** bridge in the tetraphosphate moiety resulted in analogues that were at least an order of magnitude weaker at the $P2Y_2$ receptor than Up_4U **3**. The difluoro analogue **41**, which exhibited an EC_{50} of ~2 μM at the $P2Y_2$ receptor, apparently provides a better mimic of the phosphate ester group than does the dichloro substitution in **42**. These results are consistent with electronic effects of the difluoromethylene group noted for other phosphonates.[32] The $P2Y_2$ receptor agonist INS37217 **4** also was prepared for comparison.[12] Substitution of a 2-thio group in **43** resulted in enhanced potency (EC_{50} ~80 nM) and selectivity for the $P2Y_2$ receptor (9-fold in comparison to $P2Y_4$). A 2′-deoxyguanosine analogue **44** also exhibited moderate potency at the $P2Y_2$ receptor, as reported.[12]

Many of the analogues synthesized and studied including Up_4-sugars retained moderate potency at the $P2Y_4$ receptor. However, our work to date has not revealed molecules that exhibit notable selectivity for the $P2Y_4$ receptor subtype over the other uridine nucleotide activated receptors.

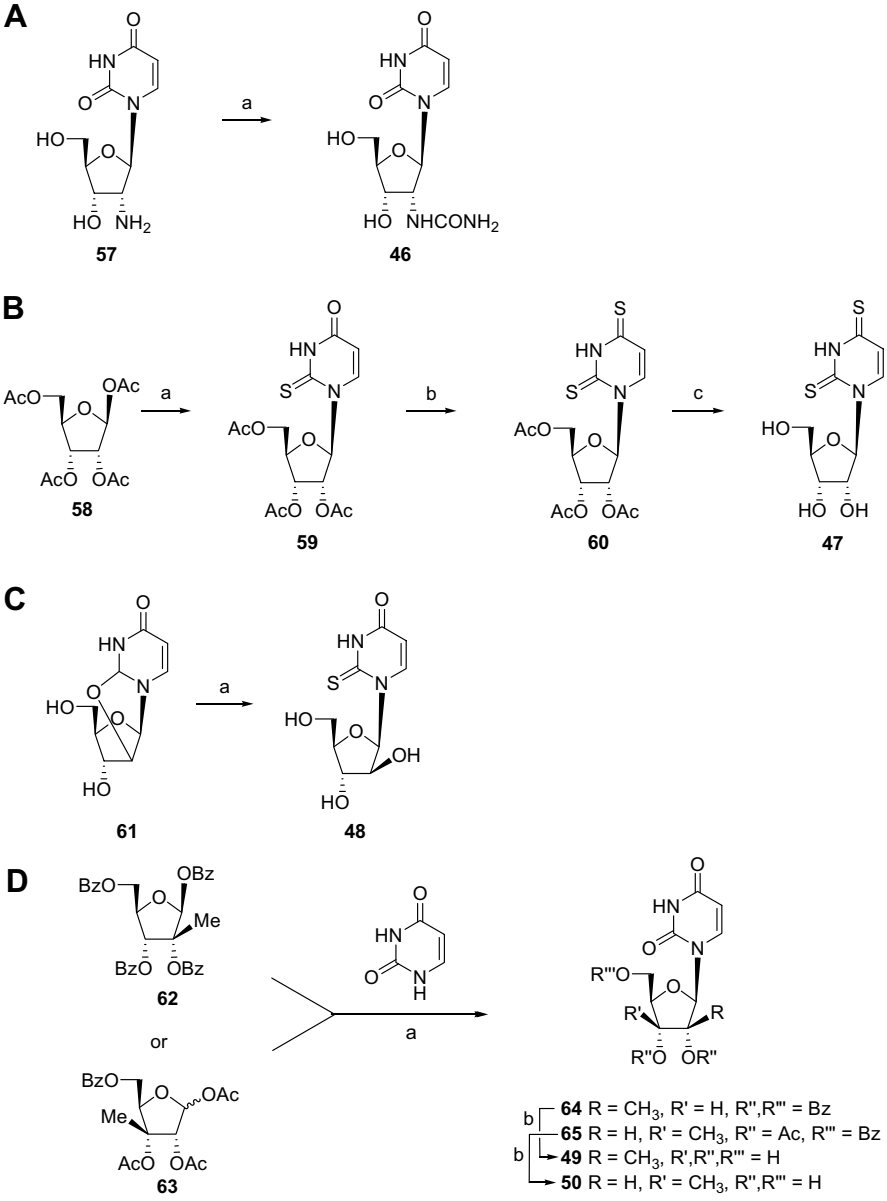
In conclusion, we have synthesized novel uracil nucleotide derivatives that are directed toward activation of the $P2Y_2$ or $P2Y_6$ receptor. Key compounds reported in this study include **34**, Up_4-[1]glucose, which displayed submicromolar potency at the $P2Y_2$ receptor; **16** and **41**, dihalomethylene phosphonate analogues which were selective $P2Y_2$ receptor agonists; and **43**, the 2-thio analogue of INS37217, which was a potent and selective $P2Y_2$ receptor agonist. Thus, the 2-thio modification, found previously to enhance $P2Y_2$ receptor potency, could be combined with other favorable modifications to produce novel molecules that exhibit high potencies and receptor selectivities. Phosphonomethylene bridges introduced for stability in analogues of UDP, UTP, and uracil dinucleotides markedly reduced potency. Truncation of dinucleotide agonists of the $P2Y_2$ receptor, in the form of Up_4-sugars, indicated that a terminal uracil ring is not essential for moderate potency at this receptor and that specific SAR patterns are observed at this distal end of the molecule.

3. Experimental

3.1. Chemical synthesis

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

Purity of compounds was checked using a Hewlett–Packard 1100 HPLC equipped with a Zorbax Eclipse 5 μm XDB-C18 analytical column (250 × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). Mobile phase: linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH_3CN from 80:20 to 40:60 in 20 min; the flow rate was 1 mL/min. Peaks were detected by UV absorption with a diode array detector at 254, 275,



Scheme 6. Synthesis of nucleoside intermediates. Reagents and conditions: **A**, benzotriazole-1-carboxamide, DMF; **B**, (a) silylated 2-thiouracil, $SnCl_4$, $C_2H_4Cl_2$; (b) Lawesson's reagent, toluene, 80 °C, overnight; (c) NaOMe, MeOH, reflux, 4 h; **C**, (a) H_2S, Et_3N, DMF, 200 psi, 2 days; **D**, (a) i—1,2-dichloroethane, hexamethyldisilazane, trimethylsilylchloride, 80 °C, 4 h; ii—$SnCl_4$, rt, 4 h; (b) NH_3/CH_3OH, rt, overnight.

and 280 nm. All derivatives tested for biological activity showed >99% purity by HPLC analysis (detection at 254 nm).

High-resolution mass measurements were performed on Micromass/Waters LCT Premier Electrospray Time of Flight (TOF) mass spectrometer coupled with a Waters HPLC system, unless noted. Purification of the nucleotide analogues for biological testing was carried out on (diethylamino)ethyl (DEAE)-A25 Sephadex columns with a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase. Compounds **16**, **20**, **21**, **39**, and **54** were purified by Sephadex alone (and isolated in the ammonium salt form), and all other compounds were additionally purified by HPLC with a Luna 5 μ RP-C18(2) semipreparative column (250 × 10.0 mm; Phenomenex, Torrance, CA) and using the following conditions: flow rate of 2 mL/min; 10 mM triethylammonium acetate (TEAA)-CH_3CN from 100:0 to 95:5 (or up to 99:1 to 92:8) in 30 min (and isolated in the triethylammonium salt form).

All reagents were of analytical grade. 2′-Amino-2′-deoxyuridine was from Metkinen Chemistry (Kuusisto, Finland). 2,2′-*O*-Anhydrouridine **61** was obtained from Wako Chemicals. 5-Amino-UDP was purchased from ALT, Inc. (Lexington, KY). Other reagents and solvents were purchased from Sigma–Aldrich (St. Louis, MO).

3.1.1. General procedure for the preparation of nucleoside triphosphates (e.g., 14 and 17), tetraphosphates (22–24), pentaphosphate (25). Procedure A

To a solution of nucleoside (0.054 mmol) and Proton Sponge (23 mg, 0.11 mmol) in trimethyl phosphate (1 mL) was added phosphorous oxychloride (0.01 mL, 0.11 mmol) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C and tributylamine (0.03 mL, 0.12 mmol) was added. Tributylammonium pyrophosphate (1.6 moles $C_{12}H_{27}N$ per mole $H_4P_2O_7$, 110 mg, 0.23 mmol) in DMF (0.3 mL) was added at once to the reaction mixture. After 10 min, 0.2 M triethylammonium bicarbonate solution (2 mL) was added, and the clear solution was stirred at rt for 1 h. The latter was lyophilized overnight, and the resulting residue was purified by ion-exchange column chromatography using a Sephadex-

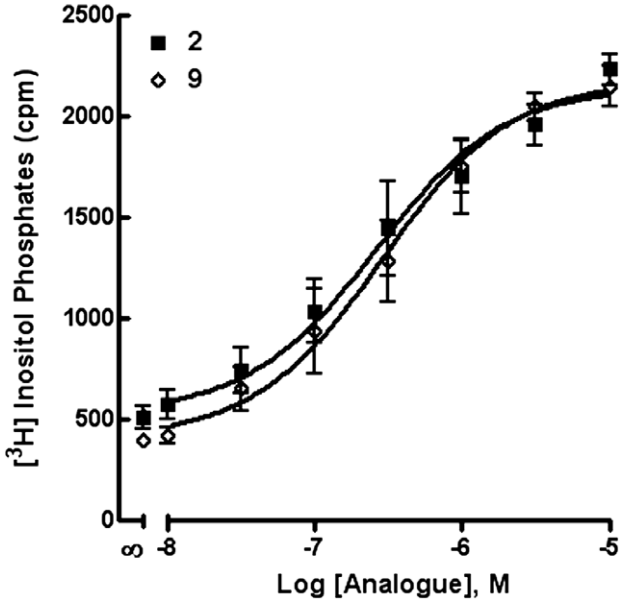


Figure 1. Activity of compounds **2** (the native agonist, UDP) and **9** (α,β-methylene-UDP) at the $P2Y_6$ receptor as indicated by activation of PLC in stably transfected astrocytoma cells.

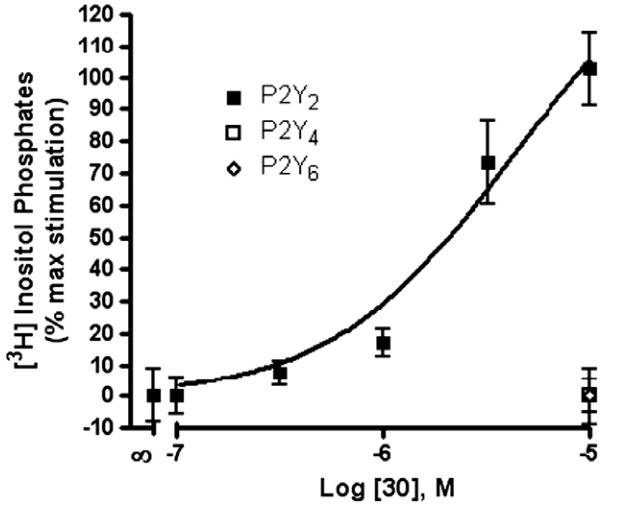


Figure 2. Activity of compound **30** (uridine 5′-tetraphosphate δ-phenyl ester) at $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors as indicated by activation of PLC in stably transfected astrocytoma cells. The effect of UTP corresponds to 100%.

DEAE A-25 resin with a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase to obtain the corresponding nucleotides as the ammonium salts. The collected portions were purified by HPLC again as described above.

3.1.2. General procedure for the preparation of nucleosides tetraphosphate (26–31), nucleoside tetraphosphate sugars (32–37), and dinucleoside tetraphosphates (40–44). Procedure B

Uridine triphosphate trisodium salt (15 mg, 0.027 mmol or UTP analogues for compounds **40**–**44**) and the corresponding monophosphate (0.109 mmol) were converted to the tributylammonium salts by treatment with ion-exchange resin (DOWEX 50WX2-200 (H)) and tributylamine. After removal of the water, the obtained tributylammonium salts were dried under high-vacuum overnight. To a solution of uridine triphosphate tributylammonium salt (0.027 mmol) in DMF (2 mL) was added *N*,*N*′-dicyclohexylcarbodiimide (DCC, 14 mg, 0.07 mmol). After stirring

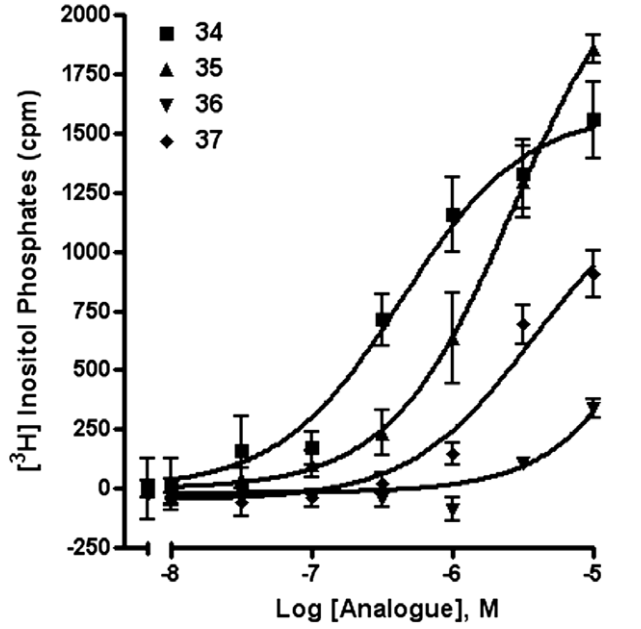


Figure 3. Activity of compounds **34**–**37** (uridine 5′-tetraphosphate δ-sugars) at the $P2Y_2$ receptor as indicated by activation of PLC in stably transfected astrocytoma cells.

the reaction mixture at rt for 1 h, a solution of the corresponding monophosphate tributylammonium salt (0.109 mmol) in DMF (1 mL) was added. The reaction mixture was stirred at rt for 48 h. After removal of the solvent, the residue was purified by ion-exchange column chromatography with a Sephadex-DEAE A-25 resin, followed by a semipreparative HPLC purification as described above. Free nucleoside 5′-tetra- and pentaphosphates were not stable upon prolonged storage at 4 °C, but were stable at −20 °C for at least 3 weeks. Dinucleoside tetraphosphates were stable for several months at −20 °C and later showed signs of gradual decomposition.

3.1.3. General procedure for the preparation of compounds (7–10). Procedure C

To a solution of uridine (25 mg, 0.1 mmol) and DCC (62 mg, 0.3 mmol) in DMF (1.5 mL) was added the appropriate carboxylic acid or phosphonic acid (0.15 mmol): malonic acid for **7**, phosphonoacetic acid for **8**, methylene diphosphonic acid for **9**, difluoromethylene diphosphonic acid for **10**. After stirring the reaction mixture at rt for 24–48 h, the solvent was removed. The residue was purified by ion-exchange column chromatography with a Sephadex-DEAE A-25 resin, followed by a semipreparative HPLC purification as described above.

3.1.4. Uridine-5′-phosphonoacetate triethylammonium salt (8). Procedure C

Compound **8** (13.4 mg, 36%) was obtained as a white solid from uridine (25 mg, 0.1 mmol). ^{1}H NMR (D_2O) δ 7.81 (d, J = 8.1 Hz, 1H), 5.94 (d, J = 8.1 Hz, 1H), 5.91 (d, J = 4.5 Hz, 1H), 4.41 (m, 3H), 4.33 (m, 2H), 2.89 (d, J = 20.4 Hz, 2H); ^{31}P NMR (D_2O) δ 12.23; ^{13}C NMR (D_2O) δ (169.27, 169.19), 164.92, 150.34, 140.53, 101.18, 88.21, 80.14, 72.06, 68.06, 62.87, (36.28, 34.73); HRMS-EI found 367.0658 $(M-H)^-$. $C_{11}H_{16}N_2O_{10}P$ requires 367.0543; purity >99% by HPLC (retention time: 5.9 min).

3.1.5. Uridine-5′-α,β-methylene-diphosphate triethylammonium salt (9). Procedure C

Compound **9** (14 mg, 33%) was obtained as a white solid from uridine (25 mg, 0.1 mmol). ^{1}H NMR (D_2O) δ 8.03 (d, J = 8.1 Hz,

1H), 5.98 (m, 2H), 4.41 (m, 2H), 4.28 (m, 1H), 4.19 (m, 2H), 2.18 (t, J = 19.5 Hz, 2H); ^{31}P NMR (D_2O) δ 19.37 (m), 14.44 (m); HRMS-EI found 403.0245 $(M+H)^-$. $C_{10}H_{17}N_2O_{11}P_2$ requires 403.0308; purity >99% by HPLC (retention time: 12.6 min).

3.1.6. Uridine-5′-α,β-difluoromethylenediphosphate triethylammonium salt (10). Procedure C

Compound **10** (14 mg, 33%) was obtained as a white solid from uridine (25 mg, 0.1 mmol). ^{1}H NMR (D_2O) δ 8.02 (d, J = 8.1 Hz, 1H), 5.98 (m, 2H), 4.40 (m, 2H), 4.26 (m, 1H), 4.19 (m, 2H); ^{31}P NMR (D_2O) δ 4.69 (m), 3.48 (m); HRMS-EI found 436.9977 $(M-H)^-$. $C_{10}H_{13}N_2O_{11}F_2P_2$ requires 436.9963; purity >99% by HPLC (retention time: 14.1 min).

3.1.7. 2′-Deoxy-2′-ureido-uridine-5′-diphosphate triethylammonium salt (12)

A solution of the compound **46** (38 mg, 0.13 mmol) and Proton Sponge (43 mg, 0.20 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then phosphorous oxychloride (25 μL, 0.27 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.25 mL, 1.05 mmol) and a solution 0.35 M of bis(tributylammonium) salt of phosphoric acid in DMF (2.28 mL) was added at once. This salt was prepared by mixing tributylamine (0.4 mL, 1.65 mmol) and phosphoric acid (85 mg, 0.87 mmol) in DMF (2.5 mL). After 10 min, 0.2 M triethylammonium bicarbonate (TEAB) solution (3 mL) was added, and the clear solution was stirred at rt for 30 min. The latter was lyophilized overnight. The residue was purified by Sephadex-DEAE A-25 resin ion-exchange column chromatography, followed by semipreparative HPLC as described above to obtain **12** (8 mg, 8%) as a white solid. ^{1}H NMR (D_2O) δ 7.98 (d, J = 8.1 Hz, 1 H), 6.02 (m, 2H), 4.51 (m, 1H), 4.34 (m, 2H), 4.20 (m, 2H); ^{31}P NMR (D_2O) δ −7.24, −10.14 (d, J = 22.0 Hz); HRMS-EI found 445.0172 $(M-H)^-$. $C_{10}H_{15}N_4O_{12}P_2$ requires 445.0162; purity >99% by HPLC (retention time: 12.7 min).

3.1.8. 2-Thio-uridine-5′-β,γ-difluoromethylene-triphosphate triethylammonium salt (15)

To a solution of 2-thio-uridine 5′-monophosphate morpholidate 4-morpholine-*N,N* dicyclohexylcarboxamidine salt, **45**[33] (10 mg, 0.014 mmol) in DMF (2 mL), difluoromethylene diphosphonate tributylammonium salt (20 mg, 0.021 mmol) was added. After being stirred for 3 days at rt, the reaction mixture was evaporated to remove the solvent and purified by Sephadex-DEAE A-25 resin followed by HPLC purification to give **15**(4 mg, 31%). ^{1}H NMR (D_2O) δ 8.18 (d, J = 8.1 Hz, 1H), 6.69 (d, J = 3.0 Hz, 1H), 6.28 (d, J = 8.1 Hz, 1H), 4.31–4.47 (m, 5H); ^{31}P NMR (D_2O) δ 3.47, −3.80 (m), −10.69 (d, J = 31.2 Hz); HRMS-EI found 532.9375 $(M-H)^-$. $C_{10}H_{14}N_2O_{13}F_2P_3S$ requires 532.9398; purity >99% by HPLC (retention time: 18.1 min).

3.1.9. 2-Thio-uridine-5′-β,γ-dichloromethylene-triphosphate ammonium salt (16)

To a solution of 2-thio-uridine 5′-monophosphate morpholidate 4-morpholine-*N,N* dicyclohexylcarboxamidine salt, **45**[33] (7 mg, 0.01 mmol) in DMF (2 mL), dichloromethylene diphosphonate tributylammonium salt (25 mg, 0.025 mmol) was added. After being stirred for 3 days at rt, the reaction mixture was evaporated to remove the solvent and purified by Sephadex-DEAE A-25 resin to give **16** (5 mg, 80%). ^{1}H NMR (D_2O) δ 8.20 (d, J = 8.1 Hz, 1H), 6.69 (d, J = 2.7 Hz, 1H), 6.29 (d, J = 8.4 Hz, 1H), 4.45 (m, 2H), 4.39 (m, 2H), 4.34 (m, 1H); ^{31}P NMR (D_2O) δ 8.08 (d, J = 17.7 Hz), 1.17 (dd, J = 17.7, 31.2 Hz), −10.60 (d, J = 31.2 Hz); HRMS-EI found 564.8817 $(M-H)^-$. $C_{10}H_{14}N_2O_{13}Cl_2P_3S$ requires 564.8807; purity >99% by HPLC (retention time: 19.5 min).

3.1.10. 2-Thio-4-methylthio-uridine-5′-triphosphate triethylammonium salt (17). Procedure A

Compound **17** (3.2 mg, 6.4%) was obtained as a white solid from 2,4-dithio-uridine, **47** (15 mg, 0.054 mmol). ^{1}H NMR (D_2O) δ 8.51 (d, J = 7.5 Hz, 1H), 7.15 (d, J = 7.2 Hz, 1H), 6.53 (br s, 1H), 4.45 (m, 2H), 4.38 (m, 3H), 2.60 (s, 3H); ^{31}P NMR (D_2O) δ −11.00 (d, J = 19.2 Hz), −21.02 (m); HRMS-EI found 528.9315 $(M-H)^-$. $C_{10}H_{16}N_2O_{13}P_3S_2$ requires 528.9307; purity >99% by HPLC (retention time: 17.1 min).

3.1.11. 2′-Deoxy-2′-ureido uridine-5′-triphosphate triethylammonium salt (18). Procedure A

A solution of the compound **46** (38 mg, 0.13 mmol) and Proton Sponge (43 mg, 0.20 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Phosphorous oxychloride (25 μL, 0.27 mmol) was then added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A solution of tributylammonium pyrophosphate (377 mg, 0.80 mmol) and tributylamine (0.13 mL, 0.53 mmol) in DMF (1 mL) was added and stirring was continued at 0 °C for additional 10 min. Triethylammonium bicarbonate solution (TEAB, 3 mL of 0.2 M) was added, and the reaction mixture was stirred at rt for 30 min. The latter was lyophilized overnight. The residue was purified by Sephadex-DEAE A-25 resin ion-exchange column chromatography, followed by semipreparative HPLC as described above to obtain **18** (9 mg, 7%) as a white solid. ^{1}H NMR (D_2O) δ 7.95 (d, J = 8.4 Hz, 1H), 6.05 (d, J = 7.8 Hz, 1H), 6.01 (d, J = 8.4 Hz, 1H), 4.51 (m, 1H), 4.35 (m, 2H), 4.25 (m, 2H); ^{31}P NMR (D_2O) δ −10.15, −12.15 (d, J = 19.5 Hz), −23.6; HRMS-EI found 524.9812 $(M-H)^-$. $C_{10}H_{16}N_4O_{15}P_3$ requires 524.9825; purity >99% by HPLC (retention time: 16.6 min).

3.1.12. 1-(β-D-Arabinofuranosyl)-2-thio(1H)pyrimidin-4-one 5′-triphosphate triethyl ammonium salt (19)

Solution of **48** (150 mg, 0.58 mmol) in trimethyl phosphate (5.8 mL) was cooled to 0 °C, $POCl_3$ (342 μL, 3.8 mmol) was added dropwise and the mixture was stirred for 4 h at 0 °C and for 30 min at rt. The mixture was poured into ice-water (10 mL), neutralized with concentrated NH_4OH, and evaporated to dryness. The resulting residue was purified by column chromatography (*i*-PrOH:NH_4OH:H_2O 60:30:5). After lyophilization of the collected pure fractions, the 5′-monophosphate of **48** was obtained as a white solid (124 mg, 60%). ^{1}H NMR (D_2O) δ 7.97 (d, J = 8.2 Hz, 1H), 6.78 (d, J = 5.0 Hz, 1H), 6.07 (d, J = 8.2 Hz, 1H), 4.48 (app t, J = 4.8 Hz, 1H), 4.12 (app t, J = 4.8 Hz, 1H), 3.90–4.01 (m, 3H); ^{31}P NMR (D_2O) δ 3.42; HRMS-EI found 363.0271 $[M+Na]^+$. $C_9H_{12}N_2O_8P_1S_1Na$ requires 363.0281. To a solution of 5′-monophosphate of **48** (32 mg, 0.088 mmol) and tributylamine (21 μL, 0.088 mmol) in DMF (3.2 mL) was added CDI (71 mg, 0.44 mmol). After being stirred for 3 h at rt, the reaction mixture was quenched by addition of methanol (14 μL). Bis(tri-*n*-butylammonium)pyrophosphate (228 mg, 0.51 mmol) was added, and the mixture was stirred and subsequently concentrated under reduced pressure. The resulting residue was stirred in 1 M triethylammonium bicarbonate (TEAB) buffer (6 mL, pH 7.4) for 30 min, lyophilized and purified on a preparative HPLC apparatus equipped with a source 15 Q column (100% water → 100% 1 M TEAB/water in 45 min) to yield 10 μmol (11%) of compound **19** after lyophilizing the appropriate fractions. ^{1}H NMR (D_2O) δ 7.90 (d, J = 8.1 Hz, 1H), 6.80 (d, J = 5.2 Hz, 1H), 6.08 (d, J = 8.2 Hz, 1H), 4.48 (app t, J = 5.0 Hz, 1H), 4.16 (m, 3H), 4.03 (m, 1H); ^{31}P NMR (D_2O) δ −9.54 (d, J = 19.6 Hz), −10.35 (d, J = 19.6 Hz), −22.19 (t, J = 19.6 Hz), HRMS-EI found 498.9099 $[M-H]^-$. $C_9H_{14}N_2O_{14}P_3S$ requires 498.9384. Structural assignment was confirmed with COSY. All signals assigned to hydroxyl groups were exchangeable with D_2O. Exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrome-

ter (qToF 2, Micromass, Manchester, UK) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in a *i*-PrOH/water (1:1) mixture at 3 μL/min.

3.1.13. General procedure for the preparation of the nucleoside 5′-triphosphates 2′-MeUTP (20) and 3′-MeUTP (21)

To a solution of 2′-[17] or 3′-*C*-methyl-UMP[18] (0.15 mmol) dissolved in dry DMF (1.5 mL) was added tri-*n*-butylamine (0.15 mmol) and the solution was stirred for 20 min at rt. After evaporation under anhydrous condition, the residue was suspended in 1.4 mL of dry DMF and CDI (122 mg, 0.75 mmol) was added and the mixture was stirred for 6 h at rt. Methanol (49 μl, 1.2 mmol) was added and the mixture was stirred for 30 min. Then 6 mL (3 mmol) of a 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in dry DMF was added. The mixture was stirred for 24 h at rt, and the solvent was removed under high vacuum at rt. The mixture dissolved in water was purified by Sephadex DEAE A-25 resin ion exchange column chromatography with a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate. Compounds **20** and **21** were isolated as ammonium salts (yield 32 and 34%, respectively). Mass spectroscopy was carried out on an HP 1100 series instrument in the negative ion mode using atmospheric pressure electrospray ionization (API-ESI).

3.1.14. 2′-C-Methyl-uridine-5′-triphosphate ammonium salt (20)

^{1}H NMR (D_2O) δ 7.76 (d, *J* = 8.1 Hz, 1H), 5.86 (s, 1H), 5.75 (d, *J* = 8.1 Hz, 1H), 3.85–3.90 (m, 2H), 3.75 (d, *J* = 9.0 Hz, 1H), 3.68 (dd, *J* = 4.3, 13.7 Hz, 1H), 1.05 (s, 3H); ^{31}P NMR (D_2O) δ −4.75 (br s), −19.34 (br s), −20.59 (m); MS *m*/*z* 497.10 $[M-H]^-$.

3.1.15. 3′-C-Methyl-uridine-5′-triphosphate ammonium salt (21)

^{1}H NMR (D_2O) δ 7.78 (d, *J* = 8.1 Hz, 1H), 5.84 (d, *J* = 7.7 Hz, 1H), 5.75 (d, *J* = 8.1 Hz, 1H), 4.03 (d, *J* = 7.7 Hz, 1H), 3.94 (dd, *J* = 3.4, 5.1 Hz, 1H), 3.65 (dd, *J* = 3.4, 12.8 Hz, 1H), 3.58 (dd, *J* = 4.9, 12.6, 1H),1.20 (s, 3H); ^{31}P NMR (D_2O) δ −4.83 (d, *J* = 15.9 Hz), −19.25 (t, *J* = 15.3 Hz), −20.66 (t, *J* = 15.9 Hz); MS *m*/*z* 497.10 $[M-H]^-$.

3.1.16. 2-Thio-uridine-5′-tetraphosphate triethylammonium salt, 2-thio-U_P4 (23). Procedure A

Compound **23** (1.5 mg, 4.5%) was obtained as a white solid from 2-thio-uridine (10 mg, 0.038 mmol). ^{1}H NMR (D_2O) *δ* 8.17 (d, *J* = 8.4 Hz, 1H), 6.73 (d, *J* = 3.0 Hz, 1H), 6.27 (d, *J* = 8.1 Hz, 1H), 4.46 (m, 2H), 4.33 (m, 3H); ^{31}P NMR (D_2O) *δ* −11.18 (d, *J* = 18.9 Hz), −22.55 (m); HRMS-EI found 578.9042 $(M-H)^-$. $C_9H_{15}N_2O_{17}P_4S$ requires 578.9042; purity >99% by HPLC (retention time: 19.8 min).

3.1.17. 4-Thio-uridine-5′-tetraphosphate triethylammonium salt (24). Procedure A

Compound **24** (5.2 mg, 7.6%) was obtained as a white solid from 4-thio-uridine (20 mg, 0.077 mmol). ^{1}H NMR (D_2O) *δ* 7.88 (d, *J* = 7.8 Hz, 1H), 6.66 (d, *J* = 7.8 Hz, 1H), 5.95 (d, *J* = 4.5 Hz, 1H), 4.47 (m, 1H), 4.40 (m, 1H), 4.28 (m, 3H); ^{31}P NMR (D_2O) *δ* −10.91 (d, *J* = 20.2 Hz), −21.97 (m); HRMS-EI found 578.8890 $(M-H)^-$. $C_9H_{15}N_2O_{17}P_4S$ requires 578.9042; purity >99% by HPLC (retention time: 19.2 min).

3.1.18. 2-Thio-uridine-5′-pentaphosphate triethylammonium salt (25). Procedure A

Compound **25** (1.1 mg, 3%) was obtained as a white solid from 2-thio-uridine 52 (10 mg, 0.038 mmol). ^{1}H NMR (D_2O) *δ* 8.15 (d, *J* = 7.8 Hz, 1H), 6.70 (d, *J* = 3.6 Hz, 1H), 6.26 (d, *J* = 8.1 Hz, 1H), 4.43 (m, 2H), 4.32 (m, 3H); ^{31}P NMR (D_2O) *δ* −11.22 (d, *J* = 18.3 Hz), −22.78 (m); HRMS-EI found 658.8782 $(M-H)^-$. $C_9H_{16}N_2O_{20}P_5S$ requires 658.8705; purity >99% by HPLC (retention time: 19.9 min).

3.1.19. Uridine-5′-methyl-tetraphosphate triethylammonium salt (26). Procedure B

Compound **26** (7.8 mg, 33%) was obtained as a white solid using uridine triphosphate (20 mg, 0.036 mmol) and methylphosphate (45 mg, 0.15 mmol). ^{1}H NMR (D_2O) *δ* 8.00 (d, *J* = 8.1 Hz, 1H), 6.03 (d, *J* = 5.7 Hz, 1H), 6.00 (d, *J* = 8.1 Hz, 1H), 4.44 (m, 2H), 4.31 (m, 1H), 4.26 (m, 2H), 3.69 (d,*J* = 11.7 Hz, 3H); ^{31}P NMR (D_2O) *δ* −8.70 (d, *J* = 17.7 Hz), −10.59 (d, *J* = 18.9 Hz), −22.46; HRMS-EI found 576.9375 $(M-H)^-$. $C_{10}H_{17}N_2O_{18}P_4$ requires 576.9427; purity >99% by HPLC (retention time: 18.8 min).

3.1.20. Uridine-5′-methyl$^{(C\text{-}P)}$-tetraphosphate triethylammonium salt (27). Procedure B

Compound **27** (8.6 mg, 34%) was obtained as a white solid using uridine triphosphate (22 mg, 0.04 mmol) and methylphosphoric acid (22 mg, 0.23 mmol). ^{1}H NMR (D_2O) *δ* 7.98 (d, *J* = 8.4 Hz, 1H), 6.02 (d, *J* = 5.4 Hz, 1H), 5.99 (d, *J* = 8.4 Hz, 1H), 4.43 (m, 2H), 4.28 (m, 1H), 4.25 (m, 2H), 1.49 (d, *J* = 17.1 Hz, 3H); ^{31}P NMR (D_2O) *δ* 19.05, −10.57 (d, *J* = 18.3 Hz), −22.34 (d, *J* = 18.9 Hz); HRMS-EI found 560.9476 $(M-H)^-$. $C_{10}H_{17}N_2O_{17}P_4$ requires 560.9478; purity >99% by HPLC (retention time: 19.2 min).

3.1.21. Uridine-5′-(2-cyanoethyl)-tetraphosphate triethylammonium salt (28). Procedure B

Compound **28** (2.5 mg, 13%) was obtained as a white solid using uridine triphosphate (15 mg, 0.027 mmol) and 2-cyanoethyl phosphate (35 mg, 0.11 mmol). ^{1}H NMR (D_2O) *δ* 7.98 (d, *J* = 8.1 Hz, 1H), 5.99 (m, 2H), 4.41 (m, 2H), 4.29 (m, 1H), 4.24 (m, 2H), 4.18 (t, *J* = 6.2 Hz, 2H), 2.88 (t, *J* = 6.2 Hz, 2H); ^{31}P NMR (D_2O) *δ* −10.54 (d, *J* = 17.7 Hz), −10.83 (d, *J* = 18.2 Hz), −22.39; HRMS-EI found 639.9489 $(M+Na-H)^-$. $C_{12}H_{19}N_3O_{18}P_4Na$ requires 639.9512; purity >99% by HPLC (retention time: 19.4 min).

3.1.22. Uridine-5′-α-glycerol-tetraphosphate triethylammonium salt (29). Procedure B

Compound **29** (2.1 mg, 11%) was obtained as a white solid using uridine triphosphate (15 mg, 0.027 mmol) and *D*-*L*-α-glycerol phosphate (35 mg, 0.11 mmol). ^{1}H NMR (D_2O) *δ* 7.99 (d, *J* = 7.8 Hz, 1H), 6.01 (m, 2H), 4.44 (m, 2H), 4.30 (m, 1H), 4.26 (m, 2H), 4.01 (m, 3H), 3.67 (m, 2H); ^{31}P NMR (D_2O) *δ* −9.83 (d, *J* = 17.7 Hz), −10.55 (d, *J* = 18.3 Hz), −22.40; HRMS-EI found 636.9638 $(M-H)^-$. $C_{12}H_{21}N_2O_{20}P_4$ requires 636.9638; purity >99% by HPLC (retention time: 18.9 min).

3.1.23. Uridine-5′-phenyl-tetraphosphate triethylammonium salt (30). Procedure B

Compound **30** (1.1 mg, 6%) was obtained as a white solid using uridine triphosphate (15 mg, 0.027 mmol) and phenyl phosphate (28 mg, 0.11 mmol). ^{1}H NMR (D_2O) *δ* 7.92 (d, *J* = 7.8 Hz, 1H), 7.37 (t, *J* = 7.2 Hz, 2H), 7.25 (d, *J* = 7.8 Hz, 2H), 7.17 (t, *J* = 7.5 Hz, 1H), 5.93 (m, 2H), 4.37 (m, 1H), 4.31 (m, 1H), 4.21 (m, 3H); ^{31}P NMR (D_2O) *δ* −10.51 (d, *J* = 17.7 Hz), −14.80 (d, *J* = 17.1 Hz), −22.43; HRMS-EI found 638.9577 $(M-H)^-$. $C_{15}H_{19}N_2O_{18}P_4$ requires 638.9583; purity >99% by HPLC (retention time: 18.6 min).

3.1.24. Uridine-5′-cyclohexane-tetraphosphate triethylammonium salt (31). Procedure B

Compound **31** (6.7 mg, 20%) was obtained as a white solid using uridine triphosphate (20 mg, 0.036 mmol) and cyclohexene mono-

phosphate tributylammonium salt (20 mg, 0.037 mmol). ^{1}H NMR (D_2O) δ 8.02 (d, J = 8.1 Hz, 1H), 6.03 (m, 2H), 4.42–4.50 (m, 2H), 4.22–4.34 (m, 4H), 2.01 (m, 2H), 1.74 (m, 2H), 1.33 (m, 6H); ^{31}P NMR (D_2O) δ −11.26 (m), −23.10 (m); HRMS-EI found 645.0037 $(M-H)^-$. $C_{15}H_{25}N_2O_{18}P_4$ requires 645.0053; purity >99% by HPLC (retention time: 19.9 min).

3.1.25. Uridine-5′-fructose-6′-tetraphosphate triethylammonium salt (33). Procedure B

Compound **33** (4.2 mg, 19%) was obtained as a white solid using uridine triphosphate (15 mg, 0.027 mmol) and *D*-fructose-6-phosphate (51 mg, 0.12 mmol). ^{1}H NMR (D_2O) δ 7.98 (d, J = 8.3 Hz, 1H), 6.02 (d, J = 5.4 Hz, 1H), 5.99 (d, J = 8.3 Hz, 1H), 4.43 (m, 2H), 4.32–4.06 (m, 7H), 3.95 (m, 1H), 3.63 (m, 1H), 3.56 (m, 1H); ^{31}P NMR (D_2O) δ −10.08 (d, J = 15.3 Hz), −10.54 (d, J = 18.3 Hz), −22.27; HRMS-EI found 724.9796 $(M-H)^-$. $C_{15}H_{25}N_2O_{23}P_4$ requires 724.9799; purity >99% by HPLC (retention time: 19.5 min).

3.1.26. Uridine-5′-glucose-1′-tetraphosphate triethylammonium salt (34). Procedure B

Compound **34** (10 mg, 28%) was obtained as a white solid using uridine triphosphate (25 mg, 0.045 mmol) and *D*-glucose-1-phosphate (68 mg, 0.18 mmol). ^{1}H NMR (D_2O) δ 8.01 (d, J = 8.4 Hz, 1H), 6.02 (m, 2H), 5.66 (m, 1H), 4.45 (m, 2H), 4.32 (m, 1H), 4.28 (m, 2H), 4.01–3.77 (m, 4H), 3.55 (m, 1H), 3.46 (m, 1H); ^{31}P NMR (D_2O) δ −10.53 (d, J = 15.9 Hz), −11.90 (d, J = 16.5 Hz), −22.27; HRMS-EI found 724.9800 $(M-H)^-$. $C_{15}H_{25}N_2O_{23}P_4$ requires 724.9799; purity >99% by HPLC (retention time: 18.9 min).

3.1.27. Uridine-5′-galactose-1′-tetraphosphate triethylammonium salt (35). Procedure B

Compound **35** (4.5 mg, 16%) was obtained as a white solid using uridine triphosphate (20 mg, 0.036 mmol) and *D*-galactose-1-phosphate (51 mg, 0.12 mmol). ^{1}H NMR (D_2O) δ 7.99 (d, J = 8.3 Hz, 1H), 6.03 (d, J = 5.7 Hz, 1H), 6.00 (d, J = 8.3 Hz, 1H), 5.67 (m, 1H), 4.44 (m, 2H), 4.26 (m, 4H), 4.05 (m, 1H), 3.98 (m, 1H), 3.77 (m, 3H); ^{31}P NMR (D_2O) δ −10.54 (d, J = 18.3 Hz), −11.74 (d, J = 18.2 Hz), −22.19; HRMS-EI found 724.9781 $(M-H)^-$. $C_{15}H_{25}N_2O_{23}P_4$ requires 724.9799; purity >99% by HPLC (retention time: 19.0 min).

3.1.28. Uridine-5′-mannose-6′-tetraphosphate triethylammonium salt (36). Procedure B

Compound **36** (2.4 mg, 11%) was obtained as a white solid using uridine triphosphate (15 mg, 0.027 mmol) and *D*-mannose-6-phosphate (23 mg, 0.08 mmol). ^{1}H NMR (D_2O) δ 7.97 (d, J = 8.4 Hz, 1H), 5.99 (m, 2H), 5.18 (m, 3/5H), 4.92 (m, 2/5H), 4.43 (m, 2H), 4.24 (m, 5H), 3.95–3.74 (m, 3H), 3.68 (m, 3/5H), 3.52 (m, 2/5H); ^{31}P NMR (D_2O) δ −9.92 (m), −10.46 (m), −22.20 (m); HRMS-EI found 724.9814 $(M-H)^-$. $C_{15}H_{25}N_2O_{23}P_4$ requires 724.9799; purity >99% by HPLC (retention time: 18.6 min).

3.1.29. Uridine-5′-(2′-deoxy-glucose)-6′-tetraphosphate triethylammonium salt (37). Procedure B

Compound **37** (4.2 mg, 20%) was obtained as a white solid using uridine triphosphate (15 mg, 0.027 mmol) and 2′-deoxy-*D*-glucose-6-phosphate (20 mg, 0.08 mmol). ^{1}H NMR (D_2O) δ 7.99 (d, J = 7.8 Hz, 1H), 6.01 (m, 2H), 5.38 (m, 1/2H), 4.96 (m, 1/2H), 4.44 (m, 2H), 4.33–4.09 (m, 6H), 3.93 (m, 1/2H), 3.73 (m, 1/2H), 3.51 (m, 1H), 2.23 (m, 1/2H), 2.13 (m, 1/2H), 1.74 (m, 1/2H), 1.53 (m, 1/2H); ^{31}P NMR (D_2O) δ −11.60 (d, J = 14.7 Hz), −12.24 (d, J = 18.9 Hz), −23.58, -24.11 (t, J = 12.2 Hz); HRMS-EI found 708.9827 $(M-H)^-$. $C_{15}H_{25}N_2O_{22}P_4$ requires 708.9849; purity >99% by HPLC (retention time: 18.5 min).

3.1.30. (2-Benzyl-1,3-dioxolo-4-yl)uridine 5′-monophosphate ammonium salt (54)

To a solution of uridine 5′-monophosphate (100 mg, 0.27 mmol) in TFA (1 mL) was added phenylacetaldehyde dimethylacetal (0.3 mL, 1.81 mmol). The reaction mixture was stirred at rt for 4 h. After removal of the solvent, the residue was treated with 1 M $NaHCO_3$ (4 mL) and AcOEt (2 mL). Aqueous phase was separated, evaporated, and purified by Sephadex-DEAE A-25 resin as described above to obtain **54** (100 mg, 86%) as a white solid. ^{1}H NMR (D_2O) δ 7.92 (d, J = 8.3 Hz, 1H), 7.36 (m, 5H), 5.90 (d, J = 8.3 Hz, 1H), 5.63 (m, 1H), 5.47 (m, 1H), 4.95 (m, 1H), 4.90 (m, 1H), 4.42 (m, 1H), 3.89 (m, 2H), 3.16 (m, 2H); ^{31}P NMR (D_2O) δ 2.71; HRMS-EI found 425.0748 $(M-H)^-$. $C_{17}H_{18}N_2O_9P$ requires 425.0750; purity >99% by HPLC (retention time: 12.9 min).

3.1.31. P^1-((2-benzyl-1,3-dioxolo-4-yl)uridine 5′) P^3-(5-iodouridine 5′) triphosphate ammonium salt (39)

To a solution of 5-iodouridine 5′-diphosphate[5] (10 mg, 0.017 mmol) in DMF (1 mL) was added CDI (7 mg, 0.04 mmol). The reaction mixture was stirred at rt for 6 h. Methanol (1 mL) was then added, and stirring was continued at rt for an additional 1 h. After removal of the solvent, the residue was dried in high vacuum overnight, was dissolved in DMF (2 mL), and was added compound **54** (11 mg, 0.025 mmol). The reaction mixture was stirred at rt overnight. After removal of the solvent, the residue was purified by Sephadex-DEAE A-25 resin as described above to obtain **39** (2.5 mg, 15%) as a white solid. ^{1}H NMR (D_2O) δ 8.17 (s, 1H), 7.77 (d, J = 8.1 Hz, 1H), 7.35 (m, 5H), 5.88 (m, 2H), 5.53 (m, 1H), 5.44 (m, 1H), 4.92 (m, 2H), 4.45 (m, 1H), 4.31 (m, 2H), 4.19 (m, 5H), 3.13 (m, 2H); ^{31}P NMR (D_2O) δ −10.68 (d, J = 17.7 Hz), −10.97 (d, J = 20.2 Hz), −22.2; HRMS-EI found 936.9611 $(M-H)^-$. $C_{26}H_{29}N_4O_{20}P_3I$ requires 936.9633; purity >99% by HPLC (retention time: 18.5 min).

3.1.32. P^1,P^4-di(uridine 5′-)β,γ-difluoromethylenetetraphosphate triethylammonium salt (41). Procedure B

Compound **41** (1.4 mg, 21%) was obtained as a white solid from uridine-5′-β,γ-difluoromethylenetriphosphate (3.2 mg, 0.0055 mmol) and uridine-5′-monophosphate (3.2 mg, 0.01 mmol). ^{1}H NMR (D_2O) δ 7.97 (br d, J = 6.1 Hz, 2H), 6.01 (m, 4H), 4.41 (m, 4H), 4.28 (m, 6H); ^{31}P NMR (D_2O) δ −6.28 (m), −10.96 (m); HRMS-EI found 822.9910 $(M-H)^-$. $C_{19}H_{25}N_4O_{22}F_2P_4$ requires 822.9879; purity >99% by HPLC (retention time: 19.8 min).

3.1.33. P^1,P^4-di(uridine 5′-)β,γ-dichloromethlyenetetraphosphate triethylammonium salt (42). Procedure B

Compound **42** (2.2 mg, 19%) was obtained as a white solid from uridine-5′-β,γ-dichloromethylenetriphosphate (5 mg, 0.009 mmol) and uridine-5′-monophosphate (6.4 mg, 0.019 mmol). ^{1}H NMR (D_2O) δ 7.99 (d, J = 8.1 Hz, 2H), 5.98 (m, 4H), 4.45 (m, 2H), 4.40 (m, 2H), 4.29 (m, 6H); ^{31}P NMR (D_2O) δ −1.61 (m), −10.94 (m); HRMS-EI found 854.9203 $(M-H)^-$. $C_{19}H_{25}N_4O_{25}Cl_2P_4$ requires 854.9288; purity >99% by HPLC (retention time: 20.0 min).

3.1.34. P^1-(2-thiouridine 5′-)-P^4-(2′-deoxycytidine 5′-)tetraphosphate triethylammonium salt (43). Procedure B

Compound **43** (1.1 mg, 7.1%) was obtained as a white solid from 2-thio-uridine triphosphate tributylammonium salt, (14 mg, 0.013 mmol) and 2′-deoxycytidine mono phosphate tributylammonium salt (27 mg, 0.055 mmol). ^{1}H NMR (D_2O) δ 8.16 (d, J = 8.4 Hz, 1H), 7.97 (d, J = 7.8 Hz, 1H), 6.63 (d, J = 3.0 Hz, 1H), 6.31 (t, J = 6.6 Hz, 1H), 6.26 (d, J = 8.4 Hz, 1H), 6.15 (d, J = 7.2 Hz, 1H), 4.62 (m, 1H), 4.36 (m, 5H), 4.20 (m, 3H), 2.42 (m, 1H), 2.28 (m, 1H); ^{31}P NMR (D_2O) δ −11.10 (m), −22.94 (m); HRMS-EI found 787.9837 $(M-H)^-$. $C_{18}H_{26}N_5O_{20}P_4S$ requires 787.9842; purity >99% by HPLC (retention time: 19.5 min).

3.1.35. 2′-Deoxy-2′-ureidouridine (46)

Benzotriazole-1-carboxamide[20] (20 mg, 0.12 mmol) was added to a solution of 2′-amino-2′-deoxyuridine **57** (20 mg, 0.08 mmol) in DMF (4 mL). The reaction mixture was stirred at rt for 6 h. After removal of the solvent, the residue was purified by preparative thin-layer chromatography (CH_2Cl_2-MeOH, 8:2) to obtain **46** (21 mg, 89%) as a white solid. ^{1}H NMR (CD_3OD) δ 7.99 (d, J = 8.3 Hz, 1H), 5.98 (d, J = 8.1 Hz, 1H), 5.71 (d, J = 8.3 Hz, 1H), 4.41 (m, 1H), 4.20 (m, 1H), 4.03 (m, 1H), 3.75 (m, 2H); HRMS-EI found 285.0859 $(M-H)^+$. $C_{10}H_{13}N_4O_6$ requires 285.0835.

3.1.36. 2′,3′,5′-Tri-*O*-acetyl-2-thiouridine (59)

A suspension of 2-thiouracil (2 g, 15.6 mmol) and trimethylsilyl chloride (1.8 mL) in hexamethyldisilazane (80 mL) was treated with a few crystals of ammonium sulfate and refluxed overnight. The clear greenish solution was evaporated and a solution of β-D-ribofuranose 1,2,3,5-tetraacetate (5.5 g, 17.3 mmol) in 20 mL dry dichloroethane was added. After a few minutes, stannic chloride (2.4 mL, 20.8 mmol) was added and after 1 h, the mixture was poured into a saturated aq $NaHCO_3$ solution under vigorous stirring and then allowed to stand for 1 h. The suspension was filtered over a silica gel pad, which was washed with CH_2Cl_2. The organic layer was separated, dried over $MgSO_4$, and evaporated to dryness. Silica gel chromatography (CH_2Cl_2:MeOH 99:1) yielded 4.75 g (79%) of compound **59**. ^{1}H NMR (DMSO-d_6) δ 10.64 (br s, 1H), 7.49 (d, J = 7.9 Hz, 1H), 6.68 (d, J = 3.8 Hz, 1H), 6.62 (dd, J = 2.3, 7.9 Hz, 1H), 5.45 (dd, J = 3.8, 5.6 Hz, 1H), 5.20 (app t, J = 5.7 Hz, 1H), 4.41–4.47 (m, 2H), 4.31–4.37 (dd, J = 3.2, 13.5 Hz, 1H), 2.09 (s, 6H), 2.06 (s, 3H); HRMS-EI found 387.08604 $(M+H)^+ \cdot C_{15}H_{19}N_2O_8S_1$ requires 387.08620.

3.1.37. 2′,3′,5′-Tri-*O*-acetyl-2,4- dithiouridine (60)

To a solution of **59** (290 mg, 0.75 mmol) in dry toluene (10 mL), Lawesson's reagent (304 mg, 0.75 mmol) was added. After heating the reaction mixture at 80 °C overnight, insoluble materials were filtered off and the filtrate was purified by silica gel chromatography (Hex:EtOAc 65:35), yielding compound **60** as a yellow foam (250 mg, 83%). ^{1}H NMR (DMSO-d_6) δ 9.97, (br s, 1H), 7.65 (d, J = 8.2 Hz, 1H), 6.76 (d, J = 4.1 Hz, 1H), 5.98 (d, J = 8.2 Hz, 1H), 5.37 (dd, J = 4.1, 5.6 Hz, 1H), 5.16 (app t, J = 5.7 Hz, 1H), 4.34–4.40 (m, 2H), 4.25–4.30 (dd, J = 3.5,13.5 Hz, 1H), 2.09 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H); HRMS-EI found 403.06338 $(M+H)^+ \cdot$ $C_{15}H_{19}N_2O_7S_2$. requires 403.06335.

3.1.38. 2,4-Dithiouridine (47)

Compound **60** (240 mg, 0.60 mmol) was dissolved in dry methanol (15 mL) and the warmed solution was treated with 130 μL of a solution of NaOMe (30% w/w) in methanol. The reaction was refluxed for 4 h and then treated with diluted acetic acid to pH 5, evaporated to dryness, and purified on a silica gel column (CH_2Cl_2:MeOH 93:7) to obtain compound **47** as a yellow foam (150 mg, 91%).^{1}H NMR (DMSO-d_6) δ 13.80 (br s, 1H), 8.07 (d, J = 7.7 Hz, 1H), 6.63 (d, J = 7.7 Hz, 1H), 6.40 (d, J = 2.7 Hz, 1H), 5.53 (d, J = 5.3 Hz, 1H), 5.29 (t, J = 5.0 Hz, 1H), 5.11 (d, J = 5.9 Hz, 1H), 4.08 (m, 1H), 3.93 (m, 2H), 3.72–3.78 (m, 1H), 3.58–3.64 (m, 1H); HRMS-EI found 275.01637 $[M-H]^-$. $C_9H_{11}N_2O_4S_2$ requires 275.01602.

3.1.39. 1-β-D-Arabinofuranosyl)-2-thio(1*H*)pyrimidin-4-one (48)

In a parr apparatus 2,2′-*O*-anhydrouridine (**61**, 2 g, 8.8 mmol) was dissolved in dry DMF (40 mL) and triethylamine (6 mL). The solution was saturated with H_2S at −40 °C and allowed to warm to rt resulting in a pressure of 200 psi. After stirring for two days, the remaining H_2S was released and the solvent evaporated to dryness. The brown residue was purified on a silica gel column (CH_2Cl_2/MeOH 96:4), yielding compound **48** (1.6 g, 70%). ^{1}H NMR (DMSO-d_6)δ 12.59 (br s, 1H), 7.72 (d, J = 8.4 Hz, 1H), 6.73 (d, J = 3.9 Hz, 1H), 5.94 (d, J = 8.4 Hz, 1H), 5.59 (d, J = 5.4 Hz, 1H), 5.48 (d, J = 3.9 Hz, 1H), 5.04 (t, J = 5.4 Hz, 1H), 4.18 (m, 1H,), 3.91 (m, 1H), 3.84 (m, 1H), 3.62 (app t, J = 5.1 Hz, 2H); HRMS-EI found 261.0540 $(M+H)^+$. $C_9H_{13}N_2O_5S_1$ requires 261.0545.

3.1.40. General procedure for synthesis of 2′-C-methyl-uridine (49) and 3′-C-methyl-uridine (50)

To dry uracil (0.45 g, 4 mmol) in dry 1,2-dichloroethane (20 mL) were added HMDS (0.68 mL, 0.8 equiv) and trimethylsilyl chloride (TMSCl, 0.3 mL, 0.8 equiv). The reaction mixture was heated at 80 °C for 4 h in the absence of moisture. After cooling to rt, 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-β-D-ribofuranose (**62**)[24] or 1,2,3-tri-*O*-acetyl-5-*O*-benzoyl-3-*C*-methyl-β-D-ribofuranose (**63**)[25b] (1 equiv) in 1,2-dichloroethane (20 mL) was added followed by $SnCl_4$ (0.93 mL, 2 equiv) dropwise. The mixture was stirred at rt for 4 h and quenched by $NaHCO_3$ saturated water solution and extracted with $CHCl_3$ (3× 10 mL). The organic layers were dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give compounds **64** or **65**, which were purified by chromatography on a silica gel column eluting with $CHCl_3$. Compounds **64** and **65** (1 mmol) were treated with methanol (20 mL) saturated with ammonia at 0 °C stirring at rt overnight. Evaporation of the solvent gave the desired compounds **49** and **50**, which were purified by chromatography on a silica gel column.

3.1.41. 1-(2,3,5-Tri-*O*-benzoyl-2-C-methyl-β-D-ribofuranosyl) uracil (64)

The title compound was obtained as a white foam (67% yield). ^{1}H NMR ($CDCl_3$) δ 9.35 (br s, 1H), 8.06 (d, J = 7.7 Hz, 4H), 7.86 (d, J = 7.7 Hz, 2H), 7.4–7.6 (m, 10H), 6.48 (s, 1H), 5.76 (d, J = 5.7 Hz, 1H), 5.66 (d, J = 7.7 Hz, 1H), 4.82 (dd, J = 4.7, 6.2 Hz, 2H), 4.62 (m, 1H), 1.72 (s, 3H).

3.1.42. 1-(2-C-methyl-β-D-ribofuranosyl)uracil (49)

The title compound was obtained as a white foam after chromatography on a silica gel column eluting with $CHCl_3$/MeOH (86:14) (80% yield). ^{1}H NMR (DMSO-d_6) δ 11.35 (br s, 1H), 8.05 (d, J = 8.1 Hz, 1H), 5.78 (s, 1H), 5.58 (d, J = 8.1 Hz, 1H), 5.15 (br s, 2H), 5.10 (s, 1H), 3.40–3.80 (m, 4H), 1.0 (s, 3H).

3.1.43. 1-(2,3-Di-*O*-acetyl-5-*O*-benzoyl-3-C-methyl-β-D-ribofuranosyl)uracil (65)

The title compound was obtained as a white foam (68% yield). ^{1}H NMR ($CDCl_3$): δ 8.70 (br s, 1H), 8.05 (d, J = 7.3 Hz, 2H), 7.62 (t, J = 7.7 Hz, 1H), 7.48 (t, J = 7.9 Hz, 2H), 7.42 (d, J = 8.1 Hz, 1H) 6.22 (d, J = 7.7 Hz, 1H), 5.45 (d, J = 8.1 Hz, 1H), 5.40 (d, J = 7.7 Hz, 1H), 4.90 (t, J = 3.4 Hz, 1H), 4.72 (dd, J = 3.3, 12.6 Hz, 1H), 4.52 (dd, J = 3.8, 12.8, 1H), 2.16 (s, 6H), 1.70 (s, 3H).

3.1.44. 1-(3-C-methyl-β-D-ribofuranosyl)uracil (50)

The title compound was obtained as a white foam after chromatography eluting with $CHCl_3$/MeOH (88:12) (82% yield). ^{1}H NMR (DMSO-d_6) δ 11.3 (br s, 1H), 8.0 (d, J = 8.1 Hz, 1H), 5.85 (d, J = 8.1 Hz, 1H), 5.65 (d, J = 7.7 Hz, 1H), 5.33 (d, J = 6.6 Hz, 1H), 5.10 (t, J = 4.9 Hz, 1H), 4.72 (s, 1H), 3.85 (dd, J = 6.6, 7.7 Hz, 1H), 3.75 (pseudo t, 1H), 3.55 (m, 2H), 1.20 (s, 3H).

3.1.45. Assay of PLC activity stimulated by $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors

Stable cell lines expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor in 1321N1 human astrocytoma cells were generated as described.[3] Agonist-induced [^{3}H]inositol phosphate production was measured in 1321N1 cells plated to 20,000 cells/well on 96-well plates two days prior to assay. Sixteen h before the assay, the inositol

lipid pool of the cells was radiolabeled by incubation in 100 μL of serum-free inositol-free Dulbecco's modified Eagle's medium, containing 1.0 μCi of *myo*-[^{3}H]inositol. No changes of medium were made subsequent to the addition of [^{3}H]inositol. On the day of the assay, cells were challenged with 25 μL of the five-fold concentrated solution of receptor agonists in 200 mM Hepes (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid), pH 7.3 in HBSS, containing 50 mM LiCl for 30 min at 37 °C. Incubations were terminated by aspiration of the drug-containing medium and addition of 30 μL of ice-cold 50 mM formic acid. [^{3}H]Inositol phosphate accumulation was quantified using scintillation proximity assay methodology as previously described in detail.[26]

3.2. Data analysis

Agonist potencies (EC_{50} values) were determined from concentration–response curves by non-linear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, CA). All experiments examining the activity of newly synthesized molecules also included full concentration–effect curves for the cognate agonist of the target receptor: UTP for the $P2Y_2$ receptor, UTP for the $P2Y_4$ receptor, and UDP for the $P2Y_6$ receptor. Each concentration of drug was tested in triplicate assays, and concentration–effect curves for each test drug were repeated in at least three separate experiments with freshly diluted molecule. The results are presented as means ± 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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Supplementary data

NMR spectra and HPLC traces for selected derivatives are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.05.013.

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