Structure−Activity Relationships of Uridine 5′-Diphosphate Analogues at the Human $P2Y_6$ Receptor

Pedro Besada,[†] Dae Hong Shin,[†] Stefano Costanzi,[‡] Hyojin Ko,[†] Christophe Mathé,[§] Julien Gagneron,[§] Gilles Gosselin,[§] Savitri Maddileti,[#] T. Kendall Harden,[#] and Kenneth A. Jacobson*[,†]

Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, Computational Chemistry Core Laboratory, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, Laboratoire de Chimie Organique Biomoléculaire de Synthese, UMR 5625 CNRS-UM II, Université Montpellier II, Place E. Bataillon, 34095 Montpellier, Cedex 5, France, and Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Received April 25, 2006

The structure−activity relationships and molecular modeling of the uracil nucleotide activated $P2Y_6$ receptor have been studied. Uridine 5′-diphosphate (UDP) analogues bearing substitutions of the ribose moiety, the uracil ring, and the diphosphate group were synthesized and assayed for activity at the human $P2Y_6$ receptor. The uracil ring was modified at the 4 position, with the synthesis of 4-substituted-thiouridine 5′-diphosphate analogues, as well as at positions 2, 3, and 5. The effect of modifications at the level of the phosphate chain was studied by preparing a cyclic 3′,5′-diphosphate analogue, a 3′-diphosphate analogue, and several dinucleotide diphosphates. 5-Iodo-UDP **32** ($EC_{50} = 0.15\ \mu M$) was equipotent to UDP, while substitutions of the 2′-hydroxyl (amino, azido) greatly reduce potency. The 2- and 4-thio analogues, **20** and **21**, respectively, were also relatively potent in comparison to UDP. However, most other modifications greatly reduced potency. Molecular modeling indicates that the β-phosphate of 5′-UDP and analogues is essential for the establishment of electrostatic interactions with two of the three conserved cationic residues of the receptor. Among 4-thioether derivatives, a 4-ethylthio analogue **23** displayed an EC_{50} of 0.28 μM, indicative of favorable interactions predicted for a small 4-alkylthio moiety with the aromatic ring of Y33 in TM1. The activity of analogue **19** in which the ribose was substituted with a 2-oxabicyclohexane ring in a rigid (S)-conformation ($P = 126°$, 1′-exo) was consistent with molecular modeling. These results provide a better understanding of molecular recognition at the $P2Y_6$ receptor and will be helpful in designing selective and potent $P2Y_6$ receptor ligands.

Introduction

P2 nucleotide receptors consist of two families: G-protein-coupled receptors (GPCRs) designated P2Y, which include eight subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$−$P2Y_{14}$), and ligand-gated cation channels designated P2X, which include seven subtypes ($P2X_1$−$P2X_7$). All of these subtypes have been cloned and functionally characterized.[1−10] The family of P2Y receptors can be further divided in two different subgroups based on overall sequence similarity, coupling to specific G proteins, and second messenger responses. The $P2Y_1$-like subgroup ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) is G_q-coupled and stimulates phospholipase C (PLC), and the $P2Y_{12}$-like subgroup ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) is G_i-coupled and inhibits adenylyl cyclase (AC).[11] The distribution of P2Y receptors is broad, and certain of these are of therapeutic interest including antithrombotic therapy, modulation of the immune system and cardiovascular system, inflammation, pain, diabetes, and treatment of cystic fibrosis and other pulmonary diseases.[12−15] Whereas P2X receptors tend to be activated principally by adenine nucleotides, P2Y receptors are activated by adenine and/or uracil nucleotides. The $P2Y_2$ receptor is activated by both uridine 5′-triphosphate (UTP) and adenosine 5′-triphosphate (ATP), the $P2Y_4$ receptor by UTP, the $P2Y_{14}$ by uridine 5′-diphosphate glucose, and the $P2Y_6$ receptor by uridine 5′-diphosphate (UDP, **1**, Chart 1).[16]

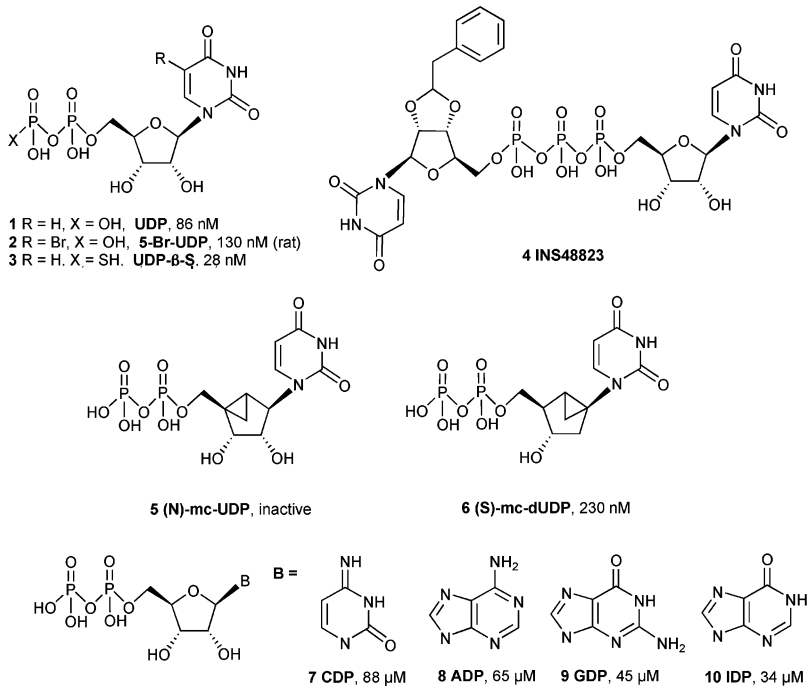
The $P2Y_6$ receptor is distributed in various tissues including lung, heart, thymus, aorta, bone, spleen, digestive tract, placenta, and brain. This receptor has been implicated in protection against apoptosis induced by TNFα,[17] enhancement of osteoclast survival through NF-κB activation,[18] regulation of electrolyte transport in the airways,[19] production of proinflammatory cytokines and chemokines,[20,21] and growth and contraction of vascular muscles.[22]

The SAR (structure−activity relationship) of nucleotides in activating the human $P2Y_6$ receptor has been probed (Chart 1). 5-Br-UDP **2**, UDP-β-S **3**, and the dinucleotide triphosphate INS48823 (P1-((2-benzyl-1,3-dioxolo-4-yl)uridine 5′) P3-(uridine 5′) triphosphate) **4** are potent and/or stable agonists of the $P2Y_6$ receptor.[5,18,19,22] We have reported that the South (S) conformation of the ribose moiety is preferred for ligand recognition by the $P2Y_6$ receptor.[23,35] The conformationally constrained (N)-methanocarba (mc) derivative **5** was inactive, and the (S)-methanocarba derivative **6** was moderately potent in activating the $P2Y_6$ receptor. Replacement of the uracil moiety with other nucleobases **7**−**10** greatly reduced the potency at the human $P2Y_6$ receptor.[24] To investigate more extensively the SAR at the $P2Y_6$ receptor, we synthesized a series of UDP analogues, where modifications at the level of the uracil moiety, ribose ring, and/or phosphate chain have been introduced. Molecular modeling was carried out to predict sites of interaction of the ligands with the $P2Y_6$ receptor and to provide hypotheses for the design of additional analogues.

* To whom correspondence should be addressed. Phone: 301-496-9024. Fax: 301-480-8422. E-mail: kajacobs@helix.nih.gov.
[†] Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases.
[‡] Computational Chemistry Core Laboratory, National Institute of Diabetes and Digestive and Kidney Diseases.
[§] Université Montpellier II.
[#] University of North Carolina School of Medicine.

10.1021/jm060485n CCC: $33.50
Published on Web 08/04/2006

Chart 1. Structures of UDP and Various Analogues and the Reported EC_{50} Values in Stimulation of PLC through the Recombinant $P2Y_6$ Receptor (Human, Unless Noted)[16,22−24,35,a]



[a] The EC_{50} for INS48823 is similar to that for UDP.[18]

To facilitate the comparison among receptors, throughout this paper we use the GPCR residue indexing system, as explained in detail elsewhere.[11]

Results

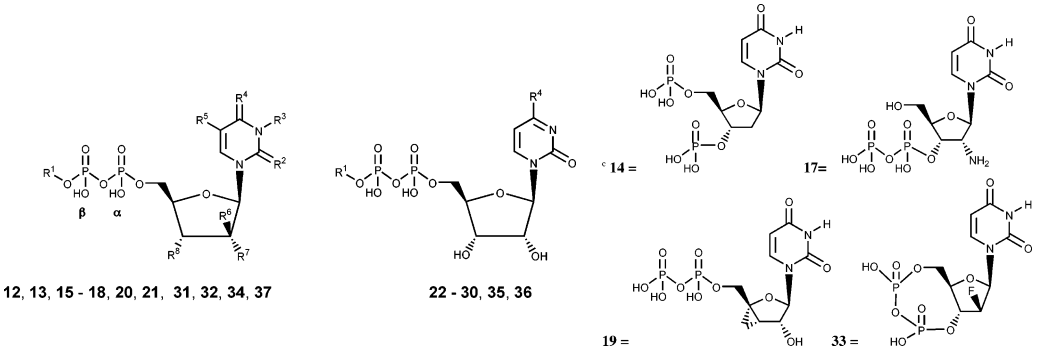
Chemical Synthesis. Analogues of UDP **1** with modifications in the ribose ring, uracil moiety, and phosphate chain, as well as dinucleotides (Table 1), were synthesized. The ribose ring was modified by removal of the hydroxyl group at the 3′ position, **13**, replacement of the hydroxyl at the 2′ position with various functional groups, **15**−**18**, and with the substitution of a 2-oxabicyclohexane ring (2-OBH) in place of the ribose ring,[25] which fixed the sugar moiety in a rigid (S)-conformation, **19**. The uracil ring was modified at the 2 and 4 positions, with the synthesis of 2-thiouridine diphosphate **20** and 4-substituted-thiouridine diphosphate analogues **22**−**30**, and at the 3 and 5 positions with **31** and **32**, respectively. Finally, the synthesis of 2′-deoxy-2′-aminouridine 3′-diphosphate **17**, the cyclic 3′,5′-diphosphate **33**, and the dinucleotides **34**−**37** as byproducts has allowed us to study modifications at the level of the phosphate chain. All the nucleotide analogues were prepared in their ammonium or triethylammonium salt forms according to the methods shown in Schemes 1−5 and tested in functional assays of the $P2Y_6$ receptor (Table 1). The nucleotide analogues were characterized using nuclear magnetic resonance (^1H, ^{13}C, and ^{31}P NMR and COSY) and high-resolution mass spectrometry.

The nucleotide diphosphates were obtained following two different methods of phosphorylation. The one-pot method using a sequential reaction with phosphorus oxychloride and phosphoric acid tributylammonium salt[26] provided the 5′-diphosphate analogues **13**, **15**, **16**, **18**−**20** in moderate yields (Schemes 1 and 2). The phosphorylation of the 2′-amino-2′-deoxyuridine **40** using the one-pot method provided the 3′-diphosphate derivative **17**. The ^1H NMR of compound **17** showed peaks at 5.01 ppm (H-3′) and 3.89 ppm (H-5′), whereas the chemical shifts of 5′-diphosphate uridine analogues are usually at 4.65−4.35 ppm (H-3′) and 4.40−4.15 ppm (H-5′). These results combined with ^{31}P NMR and HRMS indicated that for **17** the diphosphate group is in the 3′ position. The synthesis of 2′-deoxy-2′-aminouridine 5′-diphosphate **16** was accomplished after the acylation of the amino group in **40** with a trifluoroacetyl group to obtain **41**. Phosphorylation of the 2′-trifluoroacetyl-amino derivative **41** followed by an in situ deprotection of the 2′-amine provided the 5′-diphosphate analogue **16**. Compound **16** was also obtained by reduction of the 2′-deoxy-2′-azidouridine 5′-diphosphate **15** with H_2 and Pd/C. The NMR spectrum for **16** shows peaks at 4.61 ppm (H-3′) and 4.24, 4.16 ppm (H-5′) in accordance with other 5′-diphosphates derivatives. The preparation of compounds **28**, **30**, and **32** was accomplished by another method, i.e., activation of the 5′-monophosphate by reaction with 1,1′-carbonyldiimidazole followed by the addition of phosphoric acid tributylammonium salt[23] to provide the corresponding diphosphate (Schemes 4 and 5).

The synthesis of various 4-substituted-thiouridine nucleosides and nucleotides from uridine was described previously,[27−29] and various methods of *S*-alkylation were applied to a purine nucleoside, 2-thioadenosine.[26,30] By adapting these alkylation procedures, we have developed a more direct synthesis of 4-substituted-thiouridine nucleotides starting with the commercially available 4-thiouridine 5′-diphosphate (4-thio-UDP) **21** that allowed us to obtain the final compounds in only two steps (Scheme 3).

The treatment of 4-thio-UDP with 0.25 M NaOH in methanol for 2 h at room temperature gave the corresponding sodium thiolate salt, which appeared as a yellow solid after the solvent was lyophilized. The nucleotide diphosphate sodium salt was selectively *S*-alkylated by reacting with an excess of the corresponding alkyl halide at room temperature and using dimethylformamide as solvent. Compounds **22**−**27** and **29** were obtained following this procedure. Because of stability problems, the ester derivatives **45** and **46** were directly subjected to hydrolysis without purification by treatment with 0.25 M NaOH at room temperature to afford the corresponding acid derivatives **26** and **29**, respectively.

Table 1. In Vitro Pharmacological Data for UDP, **1**, and Its Analogues in the Stimulation of PLC at Recombinant Human $P2Y_6$ Receptors Expressed in 1321N1 Astrocytoma Cells[a]



compd	modification	structure	EC_{50} at $hP2Y_6$ receptor,[b] μM
1	(=UDP)		0.013 ± 0.004
11	(=UMP)		NE[d]
ribose-modified			
12	2′-deoxy	$R^7 = H$	1.72 ± 0.76
13	3′-deoxy	$R^8 = H$	2.5 ± 0.8
14	(=2′-deoxyuridine bisphosphate)	see structure above	NE[d]
15	2′-deoxy-2′-azido	$R^7 = N_3$	1.5 ± 0.4
16	2′-deoxy-2′-amino	$R^7 = NH_2$	3.9 ± 0.9
17	2′-deoxy-2′-amino 3′-diphosphate	see structure above	NE[d]
18	2′-fluoro-2′-deoxyara	$R^7 = H$, $R^6 = F$	5.5 ± 0.5
5	(N)-methanocarba	*e*	NE[d]
6	(S)-methanocarba-2′-deoxy	*e*	0.23 ± 0.05
19	3′,4′-cyclopropyl[c]	see structure above	3.5 ± 0.8
uracil-modified			
20	2-thio	$R^2 = S$	0.06 ± 0.01
21	4-thio	$R^4 = S$	0.08 ± 0.04
22	4-methylthio	$R^4 = SCH_3$	2.3 ± 0.6
23	4-ethylthio	$R^4 = SCH_2CH_3$	0.28 ± 0.02
24	4-allylthio	$R^4 = SCH_2{-}CH{=}CH_2$	0.56 ± 0.09
25	4-benzylthio	$R^4 = SCH_2C_6H_5$	0.80 ± 0.05
26	4-carboxymethylthio	$R^4 = SCH_2CO_2H$	1.7 ± 0.3
27	4-carboxamidomethylthio	$R^4 = SCH_2CONH_2$	18 ± 9
28	4-carboxyethylthio	$R^4 = S(CH_2)_2COOH$	1.1 ± 0.2
29	4-carboxypropylthio	$R^4 = S(CH_2)_3COOH$	8.8 ± 2.0
30	4-hexylthio	$R^4 = S(CH_2)_5CH_3$	4.9 ± 0.2
31	3-methyl	$R^3 = CH_3$	3.3 ± 1.0
32[f]	5-iodo	$R^5 = I$	0.015 ± 0.002
phosphate-and-ribose-modified			
33	cyclic 3′,5′-diphosphate and 2′-ara-F	see structure above	NE[d]
dinucleotides			
34	Up_2U	R^1 = uridine-5′-	2.3 ± 0.9
35	U(4-ethylthio)p_2U(4-ethylthio)	R^1 = 4- SCH_2CH_3-uridine-5′-, $R^4 = SCH_2CH_3$	<50% at 10 μM
36	U(4-benzylthio)p_2U(4-benzylthio)	R^1 = 4- $SCH_2C_6H_5$-uridine-5′-, $R^4 = SCH_2C_6H_5$	<50% at 10 μM
37	Cp_2C	R^1 = cytidine-5′-, R^4 = NH	NE[d]

[a] Unless otherwise noted, the groups are as follows: R^1, R^3, R^5, R^6 = H; R^2, R^4 = O; R^7, R^8 = OH. [b] Agonist potencies were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). EC_{50} values (mean ± standard error) represent the concentration at which 50% of the maximal effect is achieved. Relative efficacies (%) were determined by comparison with the effect produced by a maximal effective concentration of reference agonist (UDP) in the same experiment. If no maximal effect is given, then 100% efficacy was achieved. [c] Oxabicyclohexane ring system (2-OBH).[25] [d] NE: no effect at 10 μM. [e] See Chart 1. [f] **32**, MRS2693.

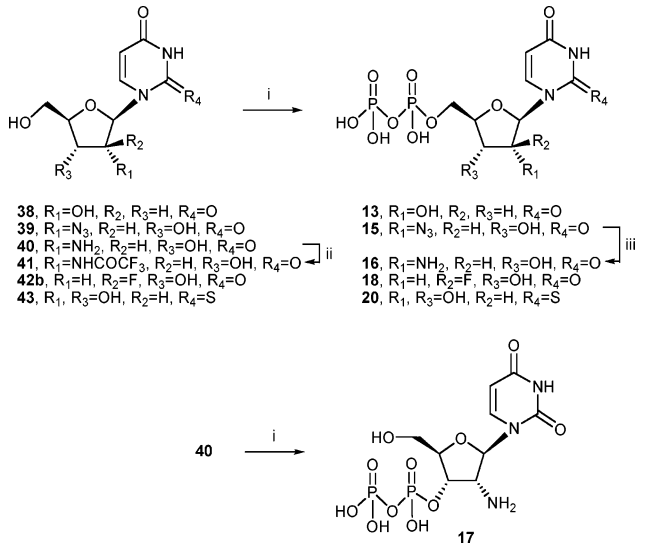
Nucleoside 5′-diphosphates are generally less stable than the corresponding monophosphates. For this reason, for alkyl halides in which heating is necessary for the alkylation reaction, the starting material was the commercially available 4-thiouridine 5′-monophosphate (4-thio-UMP) **47** (Scheme 4). After the formation of the sodium thiolate salt of the 4-thio-UMP, the thio group of the uracil moiety was alkylated following the same procedure as in Scheme 3. The alkylthio monophosphates obtained were converted to the corresponding diphosphates through a phosphorylimidazolide intermediate, which reacted with the phosphoric acid tributylammonium salt.[23] Compounds **28** and **30** were obtained following this procedure. The ester derivatives **48** and **49**, because of stability problems, were directly subjected to the next reaction without purification.

The nucleotides were selectively *S*-alkylated using the procedures described above. The ^{13}C NMR spectrum of the amide derivative **27** shows the peak corresponding to the methylene of the acetamide at 33.0 ppm, typical of a carbon−sulfur bond.

Pharmacological Activity. Activation of PLC by the nucleotide derivatives was studied in 1321N1 astrocytoma cells stably expressing the human $P2Y_6$ receptor.[16,31] Removal of the 2′- or 3′-hydroxyl group of UDP led to a >100-fold decrease in potency in **12** and **13**, respectively. Substitution of the 2′-hydroxyl group of UDP with an azido group and amino group in **15** and **16**, respectively, greatly reduced potency. The $P2Y_6$ receptor is highly selective for 5′-diphosphate derivatives. Thus, uridine 5′-monophosphate **11**, 2′-deoxyuridine 3′,5′-bisphosphate **14**,[32] and a uridine 3′-diphosphate derivative **17** were inactive as either agonists or antagonists.

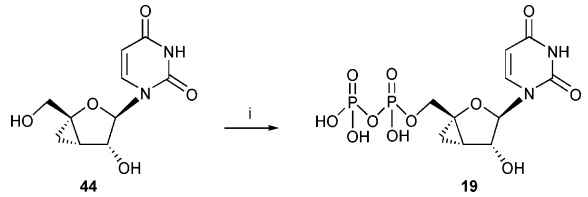
Rigid ring systems also were used to determine the preferred ribose conformation at the $P2Y_6$ binding site. As previously

Scheme 1. Preparation of Ribose- and Uracil-Modified UDP Analogues[a]



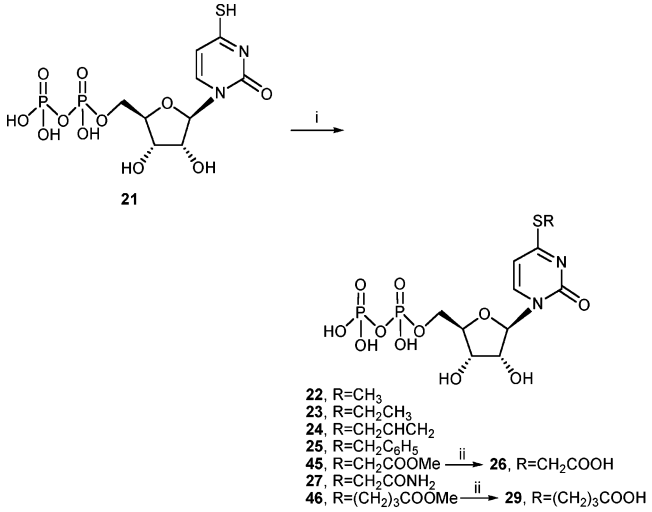
[a] Reagents and conditions: (i) (1) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C; (2) $(Bu_3NH^+)_2PO_4H^{2-}$, Bu_3N, DMF, 0 °C; (ii) CF_3COOEt, DIEA, DMF, room temp; (iii) H_2, Pd/C, MeOH, room temp.

Scheme 2. Preparation of 2-OBH UDP Analogue[a]



[a] Reagents and conditions: (i) (1) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C; (2) $(Bu_3NH^+)_2PO_4H^{2-}$, Bu_3N, DMF, 0 °C.

Scheme 3. Preparation of 4-Substituted-thio-UDP Analogues: Procedure B[a]



[a] Reagents and conditions: (i) (1) 0.25 M NaOH, MeOH, room temp; (2) RX, DMF, room temp; (ii) 0.25 M NaOH, H_2O, room temp.

reported, a strong preference for the (S)-conformation was indicated (compare **6** with **5**). Another rigid ring system (2-OBH) in **19** was tolerated at the $P2Y_6$ binding site.

Among uracil-modified compounds, 2-thio-UDP **20** and 4-thio-UDP **21** were tested in order to probe the effect of the electronegativity as well as the size of the exocyclic atoms at positions 2 and 4. Both derivatives were 5-fold less potent than the parent compound UDP. Among uncharged alkylated derivatives of **21**, the highest potency was observed with the *S*-ethyl analogue **23**, which was 20-fold less potent than UDP. In the series of carboxyalkylthio ethers, the highest potency was observed with the *S*-carboxyethylthio analogue **28**. Substitution at the 3 position in **31** markedly decreased potency, while halogenation of the 5 position in the iodo analogue **32** resulted in a molecule that was equal in potency to UDP. By analogy, a 5-bromo analogue was previously reported to be roughly equipotent to UDP.[16]

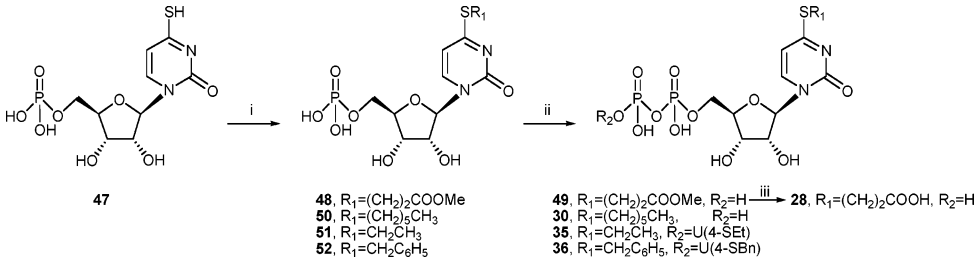
The cyclic 3′,5′-diphosphate **33**, which can be considered a closely related cyclized analogue of bisphosphate **14**, was inactive at the $P2Y_6$ receptor. The dinucleotide **34** was moderately potent at the $P2Y_6$ receptor. 4-Thioether modification at both nucleobases in **35** and **36** reduced potency. A 4-O or 4-S was required for $P2Y_6$ receptor activation, since the corresponding NH analogue **37** was inactive.

Within a series of uracil dinucleotide 5′,5′-polyphosphates, the triphosphates were the most potent and selective at the human $P2Y_6$ receptor. However, dinucleotide 5′,5′-diphosphates also showed selectivity for the $P2Y_6$ receptor.[33,34] Therefore, several dinucleotide diphosphates **34**−**37**, including 4-alkylthio analogues, were compared in this study. Only the simple Up_2U **34** displayed substantial micromolar potency at the $P2Y_6$ receptor.

Molecular Modeling. Docking studies of selected compounds were conducted at the model of the membrane-embedded human $P2Y_6$ receptor.[35] The binding mode of each studied compound was obtained by subjecting the complex to two distinct conformational searches, performed sequentially. These searches were intended to exhaustively explore the possible docking poses of the ligand by means of rotations and translations of the small molecule within the binding pocket. Simultaneously, the conformation of the residues lining the pocket was rearranged to minimize the energy of interaction with the ligand.

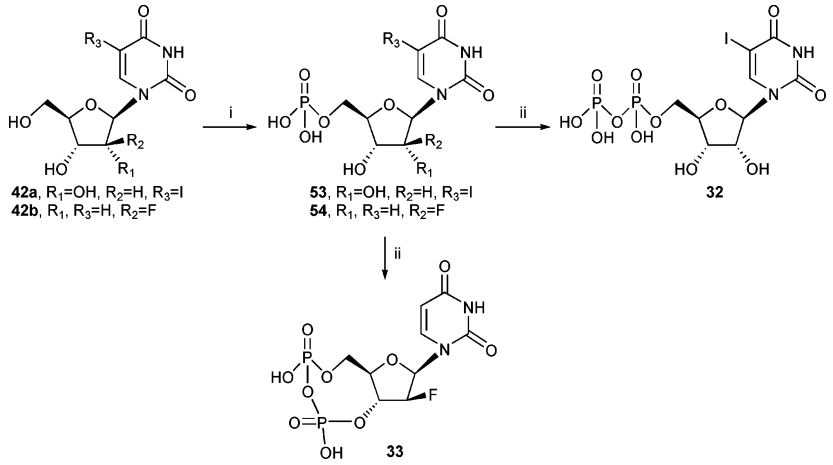
A schematic representation of the entire structure of the $P2Y_6$ receptor complexed with **32** is given in Figure 1a, while Figure 1b shows the details of the receptor−ligand interactions. In analogy with our previous $P2Y_6$−UDP complex,[35] the diphosphate moiety of **32** is coordinated by three cationic residues from transmembrane domain 3 (TM3), TM6, and TM7, namely, 3.29, 6.55, and 7.39. The O at position 2 and the NH at position 3 of the uracil ring are H-bonded with S291(7.43) and Y33(1.33), respectively. Consistent with the equipotency of **32** and UDP, the 5-iodo group does not show any sort of steric or electronic incompatibility with the features of the $P2Y_6$ binding pocket. Figure 1c shows compound **6** docked into the putative binding pocket of the $P2Y_6$ receptor model. The locked puckering of the pseudoribose ensures that the phosphate and the nucleobase moiety of **6** maintain the optimal geometry for the interaction with the $P2Y_6$ binding pocket. The donation of an H-bond from the NH at position 3 of the uracil ring to Y33(1.39), even though not essential for the recognition of the nucleotides, apparently contributes to enhanced potency at the $P2Y_6$ receptor. Compound **31**, which is incapable of donating an H-bond to Y33(1.39) because of the methyl substituent at the N3 position (Figure 1d), exhibited a 250-fold lower potency than UDP. A decreased partial negative atomic charge on the N3 might contribute to the decreased potency of 4-thio-UDP (**21**) as a consequence of a weaker H-bond donation to Y33(1.39). Moreover, all the 4-thio substituted compounds, which are incapable of donating an H-bond to Y33(1.39), exhibited lower potency than the parent compound 4-thio-UDP (**21**). Among these molecules, 4-ethylthio-UDP (**23**), 4-allylthio-UDP

Scheme 4. Preparation of 4-Substituted-thio-UDP Analogues: Procedure C[a]



[a] Reagents and conditions: (i) (1) 0.25 M NaOH, MeOH, room temp; (2) R_1X, DMF, 90 °C; (ii) (1) 1,1′-carbonyldiimidazole, DMF, room temp; (2) Et_3N 5% in 1/1 H_2O/MeOH, room temp; (3) $(Bu_3NH^+)_2PO_4H^{2-}$, Bu_3N, DMF, room temp; (iii) 0.25 M NaOH, H_2O, room temp.

Scheme 5. Preparation of 5-Halo and Phosphate- and Ribose-Modified UDP Analogues[a]



[a] Reagents and conditions: (i) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C; (ii) (1) 1,1′-carbonyldiimidazole, DMF, room temp; (2) Et_3N 5% in MeOH, room temp; (3) $(Bu_3NH^+)_2PO_4H^{2-}$, Bu_3N, DMF, room temp.

(**24**), and 4-benzylthio-UDP (**25**) showed the highest potency. Molecular modeling results suggest that the hydrophobic substituents at position 4 of the uracil ring establish favorable interactions with the aromatic ring of Y33(1.39) and with the hydrophobic pocket located between TM1 and TM2 (Figure 1e). The substitution at the 4 position with carboxyalkylthio ethers (**26**, **28**, **29**) is also tolerated by the receptor. These target structures were designed on the basis of molecular modeling results to engage an electrostatic interaction with K204(7.36) (Figure 1f).

Discussion

As a result of mutagenesis and molecular modeling studies, we proposed that nucleotides bind to the P2Y receptor with the sugar moiety accommodated between TM3 and TM7, with the base pointing toward TM1 and TM2 and with the polyphosphate moiety pointing in the direction of TM6 (Figure 1a).[11,35,36] According to this model, the electrostatic interaction of the phosphate moiety with three cationic residues from TM3, TM6, and TM7 (3.29, 6.55, and 7.39) is fundamental for the recognition of nucleotides. Our current modeling data (Figure 1) suggest that the β-phosphate of UDP and related analogues is fundamental for the establishment of electrostatic interactions with two of the three cationic residues, namely, K259(6.55) and R287(7.39). Not surprisingly, compounds **11**, **14**, **17**, and **33**, which lack a β-phosphate linked through the 5′ position of ribose, were inactive at the $P2Y_6$ receptor.

The puckering of the ribose ring, described by the phase angle of pseudorotation (*P*, Figure 2), is of fundamental importance for the biological activity of nucleosides and nucleotides. We recently discovered that the (S)-conformation of the ribose moiety is preferred for ligand recognition by the $P2Y_6$ receptor.[35] Our molecular modeling studies suggested that a pure (S)-conformation ($P = 180°$, 2′-endo and 3′-exo) would confer to the phosphate and nucleobase moieties of UDP the most favorable orientation for establishment of interactions with the residues from TM1, TM3, TM6, and TM7. Compound **6**, which is locked in the (S)-conformation ($P = -162°$, 3′-exo) by a (S)-methanocarba (mc) ring, proved to be about 10 times more potent than **12**. Conversely, **5**, locked in the (N)-conformation by a (N)-methanocarba ring, was found to be completely inactive. Compound **19**, which is locked in a (S)-conformation ($P = 126°$, 1′-exo) by a 2-OBH ring system but is further away from pure (S)-conformation than **6**, maintained agonist activity at the $P2Y_6$ receptor but was less potent than the corresponding flexible analogues. The proposed conformation of **19** was calculated using quenched molecular dynamics and energy minimization. The cyclopropane ring, fused between C3′ and C4′, forces the C1′ into the exo conformation in order for the six-membered ring to adopt a pseudoboat conformation.

Table 2 shows the relative EC_{50} values with respect to the native ligands at three P2Y receptors for the same changes in the base or nucleotide, applied to the 5′-diphosphate derivative ($P2Y_6$ receptor) and to the 5′-triphosphate derivative ($P2Y_2$ and $P2Y_4$ receptors). In most cases, there is a parallel reduction of potency at $P2Y_2$ and $P2Y_4$ receptors. However, there tended to be a greater loss of potency at the $P2Y_6$ receptor, for example, for 2′-ribose modifications. The removal of the 2′- or 3′-hydroxyl group was more poorly tolerated at the $P2Y_6$ receptor than at the two other subtypes. The reduction of potency of the 2′-deoxyarabino-2′-fluoro analogue **18** was particularly pronounced at the $P2Y_6$ receptor. The low potency of the 2′-amino analogue **16** is in sharp contrast with the high potency of the corresponding triphosphate at the $P2Y_2$ receptor.[37] The 2- and 4-thio

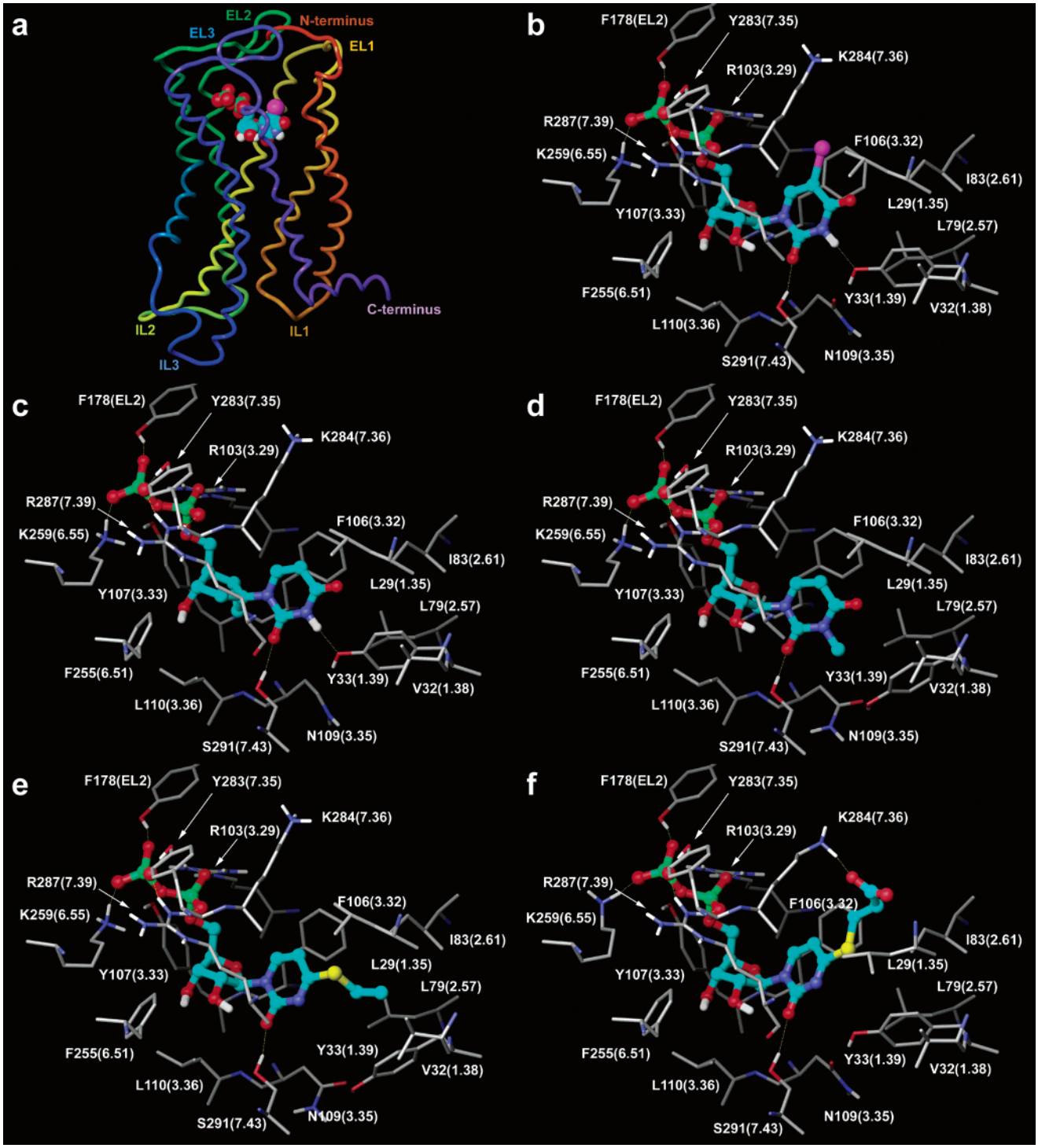


Figure 1. Molecular models of the complexes of the human $P2Y_6$ receptor with UDP analogues **32** (a and b, 5-iodo), **6** (c, (S)-methanocarba), **31** (d, 3-methyl), **23** (e, 4-ethylthio), and **28** (f, 4-carboxyethylthio). In all cases, the diphosphate moiety of the nucleotide is coordinated by three cationic residues from TM3 (3.29), TM6 (6.55), and TM7 (7.39). The donation of an H-bond from the NH at position 3 of the uracil ring to Y33(1.39) seems to contribute to higher potency in the activation of the $P2Y_6$ receptor. To facilitate the comparison, in the six panels of this figure the receptor maintains the same orientation relative to the plane of the membrane. In the schematic representation of the $P2Y_6$ receptor complexed with **32** (a), the tube represents the backbone of the receptor and is colored according to residue positions, with a spectrum of colors that ranges from red (N-terminus) to purple (C-terminus): TM1 is in orange, TM2 in ochre, TM3 in yellow, TM4 in green, TM5 in cyan, TM6 in blue, TM7 in purple.

modifications tended to preserve potency at all subtypes but most effectively at the $P2Y_2$ and $P2Y_4$ receptors. Strikingly, the 5-iodo modification preserves potency most effectively at the $P2Y_6$ receptor, and 5-halo substitution of the uracil ring may therefore be a basis for achieving selectivity at this subtype.

Conclusions

The P2Y receptor family is unusual in comparison to other pharmacologically defined clusters of GPCR sequences because they respond to varied and diverse nucleotide ligands. Within the same phylogenetic cluster are receptors for other anionic

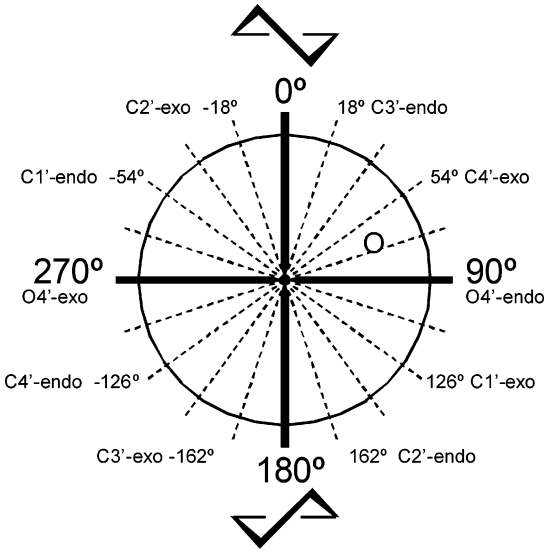


Figure 2. Graphical representation of the pseudorotational cycle,[48] which defines all of the possible ribose ring conformations. The phase angle of pseudorotation (*P*) describes the geometry of the ribose puckering. The (N)-methanocarba ring system adopts a C2′-exo conformation, and the (S)-methanocarba ring system adopts a C3′-exo conformation. The 2-OBH ring system maintains a C1′-exo conformation, in the (S)-region.

ligands: lysophosphatidylserine (GPR34), succinic acid (GPR91), and α-keto-glutarate (GPR80).[1,11] The challenge for the medicinal chemist is to understand the structural basis for the P2Y ligand selectivities and to use that information to design much needed pharmacological probes to act as selective agonists and antagonists. We have recently focused on the influence of the ribose conformation on subtype selectivity. In this study of SAR at the human $P2Y_6$ receptor following diverse structural changes of UDP, we have identified various modifications that deselect for this receptor subtype in relation to other uracil nucleotide-responsive receptors and several modifications (i.e., 5-iodo and the (S)-mc ring) that provide $P2Y_6$ selectivity. Combinations of these various modifications should achieve higher potency and selectivity and in conjunction with molecular modeling and receptor docking studies should lead to high-affinity agonists that are highly selective for the $P2Y_6$ receptor.

Experimental Section

Chemical Synthesis. 2′-Deoxyuridine-5′-diphosphate (**12**) was purchased from Jena Bioscience USA (San Diego, CA). 3′-Deoxyuridine-5′-triphosphate was purchased from TriLink BioTechnologies (San Diego, CA). 3′-Deoxyuridine (**38**) was purchased from T.R.C., Inc. (North York, Ontario, Canada). 2′-Azido-2′-deoxyuridine (**39**) and 2′-amino-2′-deoxyuridine (**40**) were purchased from CMS Chemicals Ltd. (Oxfordshire, U.K.). 2′-Arafluoro-2′-deoxyuridine (**42b**) was purchased from R.I. Chemical, Inc. (Orange, CA). 2-Thiouridine (**43**) was purchased from Berry & Associates, Inc. (Dexter, MI). Compounds **21**, **47**, and **53**, reagents, and solvents were purchased from Sigma-Aldrich (St. Louis, MO). Compounds **5**, **6**, **14**, and **31** were synthesized in our laboratory as described.[23,32,35,37] Compounds **34**, **37**, and **44** were synthesized as reported.[25,38,39]

^{1}H NMR spectra were obtained with a Varian Gemini 300 spectrometer using D_2O as a solvent. The chemical shifts are expressed as relative ppm from HOD (4.78 ppm). ^{13}C NMR spectra were recorded at room temperature by use of the Varian XL 300 spectrometer (75 MHz); methanol-d_4 was used as an external standard. ^{31}P NMR spectra were recorded at room temperature by use of the Varian XL 300 spectrometer (121.42 MHz); orthophosphoric acid (85%) was used as an external standard. The complete assignment of the signals was performed by COSY experiments obtained with the Varian XL 300 spectrometer.

The purity of compounds was checked using a Hewlett-Packard 1100 HPLC equipped with a Luna 5 μm RP-C18(2) analytical column (250 mm × 4.6 mm; Phenomenex, Torrance, CA). System A parameters were as follows: linear gradient solvent system of 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)−CH_3CN from 80:20 to 40:60 in 20 min, then isocratic for 2 min; the flow rate was 1 mL/min. System B parameters were as follows: linear gradient solvent system of 10 mM TEAA (triethylammonium acetate)−CH_3CN from 100:0 to 85:15 in 20 min, then isocratic for 2 min; the flow rate was 1 mL/min. System C parameters were as follows: linear gradient solvent system of 10 mM TEAA−CH_3-CN from 90:10 to 60:40 in 20 min, then isocratic for 2 min; the flow rate was 1 mL/min. The purity of compounds **13**, **15**−**17**, **20**, **25**, **29**, **32**, **50**, and **51** in system B or C was checked using a Zorbax Eclipse 5 μm XDB-C18 analytical column (250 mm × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). Peaks were detected by UV absorption with a diode array detector. All derivatives tested for biological activity showed >98% purity in the HPLC systems.

High-resolution mass measurements were performed on Micromass/Waters LCT Premier electrospray time-of-flight (TOF) mass spectometer coupled with a Waters HPLC system. Purification of the nucleotide analogues for biological testing was carried out on (diethylamino)ethyl (DEAE) A-25 Sephadex columns as described below. Compounds **13** and **15**−**17** were additionally purified by HPLC using system D (10 mM TEAA−CH_3CN from 100:0 to 90:10 in 30 min, then isocratic for 2 min; the flow rate was 2 mL/min) with a Luna 5 μm RP-C18(2) semipreparative column (250 mm × 10.0 mm; Phenomenex, Torrance, CA).

General Procedure for the Preparation of Nucleoside 5′-Diphosphates. Procedure A. A solution of the corresponding nucleoside (0.04−0.19 mmol) and Proton Sponge (1.5 equiv) in trimethyl phosphate (2 mL) was stirred for 10 min at 0 °C. Then phosphorus oxychloride (2 equiv) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (9 equiv) and a solution of 0.35 M bis(tributylammonium) salt of phosphoric acid in DMF (6 equiv) was added at once. This salt was prepared by mixing tributylamine (0.16 mL, 0.66 mmol) and phosphoric acid (34 mg, 0.35 mmol) in DMF (1 mL). After 6 min, 0.2 M triethylammonium bicarbonate solution (3 mL) was added, and the clear solution was stirred at room temperature for 45 min. The latter was lyophilized overnight. The residue was purified by ion-exchange column chromatography using a Sephadex-DEAE A-25 resin with a linear gradient (0.01−0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase. The corresponding nucleoside diphosphates were collected, frozen, and lyophilized as the ammonium salts.

(2*R*,3*R*,5*S*)-1-(5-(Diphosphoryloxymethyl)-3-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione, Triethylammonium Salt (13). Procedure A. Compound **13** (2 mg, 7%) was obtained as a white solid from **38** (10 mg, 0.04 mmol). ^{1}H NMR (D_2O) δ 8.04 (d, J = 8.1 Hz, 1H), 5.94 (d, J = 8.1 Hz, 1H), 5.85 (d, J = 1.5 Hz, 1H), 4.68 (m, 1H), 4.55 (m, 1H), 4.35 (m, 1H), 4.14 (m, 1H), 2.25 (m, 1H), 2.10 (m, 1H); ^{31}P NMR (D_2O) δ −10.33 (d, J = 20.8 Hz), −10.79 (d, J = 19.6 Hz); HRMS *m*/*z* found 386.9995 $(M - H^+)^-$. $C_9H_{13}N_2O_{11}P_2$ requires 386.9995; HPLC (system A) 13.7 min (98%), (system B) 6.7 min (98%).

(2*R*,3*R*,4*S*,5*R*)-1-(3-Azido-5-(diphosphoryloxymethyl)-4-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione, Triethylammonium Salt (15). Procedure A. Compound **15** (12 mg, 35%) was obtained as a white solid from **39** (32 mg, 0.12 mmol). ^{1}H NMR (D_2O) δ 7.99 (d, J = 8.1 Hz, 1H), 6.04 (d, J = 5.1 Hz, 1H), 5.99 (d, J = 8.1 Hz, 1H), 4.63 (m, 1H), 4.40 (m, 1H), 4.29 (m, 1H), 4.25 (m, 2H); ^{31}P NMR (D_2O) δ −9.63, −10.81; HRMS *m*/*z* found 427.9993 $(M - H^+)^-$. $C_9H_{12}N_5O_{11}P_2$ requires 428.0009; HPLC (system A) 13.9 min (99%), (system B) 9.8 min (99%).

(2*R*,3*R*,4*S*,5*R*)-*N*-(2-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-3-yl)-2,2,2-trifluoroacetamide (41). To a solution of **40** (20 mg, 0.08 mmol) in DMF (1 mL) was added DIEA (0.04 mL, 0.25 mmol) and ethyl

Table 2. Relative Effects of Parallel Structural Modifications of Uracil Nucleotide Analogues on Their Potency in the Stimulation of PLC at Recombinant Human $P2Y_2$, $P2Y_4$, and $P2Y_6$ Receptors[a]

compd no. of 5′-diphosphate	modification (as di- or triphosphate)	structure[e]	EC_{50} ratio of 5′-diphosphate/UDP, $hP2Y_6$ receptor	EC_{50} ratio of 5′-triphosphate/UTP, $hP2Y_2$ receptor	EC_{50} ratio of 5′-triphosphate/UTP, $hP2Y_4$ receptor
substitution of base moiety					
1	UDP, UTP	*e*	1	1	1
7	CDP, CTP	*e*	1000[b]	110	NA[g]
8	ADP, ATP	*e*	>7000	1.7	NA[g]
9	GDP, GTP	*e*	500[b]	54	90
10	IDP, ITP	*e*	400[b]	13[c]	9.1[c]
ribose modifications					
5	(N)-methanocarba	*e*	>7000	2.0[d]	1.7[d]
12	2′-deoxy	$R^7 = H$	130	22	26
13	3′-deoxy	$R^8 = H$	190	17[f]	42[f]
15	2′-azido-2′-deoxy	$R^7 = N_3$	120	100	15
16	2′-amino-2′-deoxy	$R^7 = NH_2$	300	1.3	16
18	2′-deoxyarabino-2′-fluoro-	$R^7 = H$, $R^6 = F$	420	11	7.1
uracil modifications					
20	2-thio	$R^2 = S$	4	0.71	4.8
21	4-thio	$R^4 = S$	6	0.53	0.32
31	3-methyl	$R^3 = CH_3$	250	24	47
32[h]	5-iodo	$R^5 = I$	1.1	17	55

[a] The EC_{50} ratios at the $P2Y_6$ receptors are derived from the data in Table 1 for derivatives of the native agonist, i.e., 5′-diphosphates. The EC_{50} ratios of the corresponding 5′-triphosphates at human $P2Y_2$ and $P2Y_4$ receptors expressed in astrocytoma cells are derived from published data,[23,37] unless otherwise noted. In each case, the endogenous agonist (UDP at $P2Y_6$ receptors, UTP at $P2Y_2$ and $P2Y_4$ receptors) is assigned the value of 1. [b] Robaye et al., 1997.[24] [c] Kennedy et al., 2000.[47] [d] Kim et al., 2002.[23] [e] See Table 1 and Chart 1. [f] The EC_{50} values of 3′-deoxy-UTP were determined to be 0.81 ± 0.09 μM at $P2Y_2$ receptors and 3.1 ± 1.1 μM at $P2Y_4$ receptors, using reported methods.[37] [g] NA: no activation at 10 μM. [h] **32**, MRS2693.

trifluoroacetate (0.03 mL, 0.25 mmol). The reaction mixture was stirred at room temperature for 16 h. The solvent was removed in vacuo, and the residue was purified by preparative thin-layer chromatography (CH_2Cl_2−MeOH, 85:15) to afford **41** (24 mg, 86%) as a white solid. ^{1}H NMR (CD_3OD) δ 8.01 (d, J = 8.1 Hz, 1H), 6.13 (d, J = 7.8 Hz, 1H), 5.73 (d, J = 8.1 Hz, 1H), 4.60 (m, 1H), 4.31 (m, 1H), 4.01 (m, 1H), 3.77 (m, 2H); HRMS *m*/*z* found 338.0587 $(M - H^+)^-$. $C_{11}H_{11}N_3O_6F_3$ requires 338.0600.

(2*R*,3*R*,4*S*,5*R*)-1-(3-Amino-5-(diphosphoryloxymethyl)-4-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione (16). Procedure A. Compound **16** in the triethylammonium salt (7 mg, 17%) was obtained as a white solid from **41** (20 mg, 0.06 mmol). Alternatively, compound **16** (0.6 mg, 75%) was obtained by treatment of a solution of the triethylammonium salt of compound **15** (1.5 mg, 0.002 mmol) in MeOH (1 mL) with 10% Pd/C (0.5 mg) and H_2 at atmospheric pressure for 1 h at room temperature. ^{1}H NMR (D_2O) δ 7.99 (d, J = 7.5 Hz, 1H, H-6 pyrimidine), 6.12 (d, J = 7.2 Hz, 1H, H-2), 6.00 (d, J = 7.5 Hz, 1H, H-5 pyrimidine), 4.61 (m, 1H, H-4), 4.36 (m, 1H, H-5), 4.24 (m, 1H, *H*CHO), 4.16 (m, 1H, HC*H*O), 3.88 (m, 1H, H-3); ^{31}P NMR (D_2O) δ −6.77, −10.57; HRMS *m*/*z* found 402.0126 $(M - H^+)^-$. $C_9H_{14}N_3O_{11}P_2$ requires 402.0104; HPLC (system A) 6.0 min (99%), (system B) 7.1 min (99%).

(2*R*,3*R*,4*S*,5*R*)-1-(3-Amino-4-(diphosphoryloxy)-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione, Triethylammonium Salt (17). Procedure A. Compound **17** (6 mg, 8%) was obtained as a white solid from **40** (25 mg, 0.10 mmol). ^{1}H NMR (D_2O) δ 7.88 (d, J = 8.1 Hz, 1H, H-6 pyrimidine), 6.29 (d, J = 6.9 Hz, 1H, H-2), 5.91 (d, J = 8.1 Hz, 1H, H-5 pyrimidine), 5.01 (m, 1H, H-4), 4.46 (m, 1H, H-5), 4.14 (m, 1H, H-3), 3.89 (m, 2H, CH_2O); ^{13}C NMR (D_2O) δ 167.3, 152.9, 142.4, 103.9, 87.9, 87.5, 74.7, 62.0, 56.3; ^{31}P NMR (D_2O) δ −7.73 (d, J = 22.0 Hz), −11.16 (d, J = 22.0 Hz); HRMS *m*/*z* found 402.0094 $(M - H^+)^-$. $C_9H_{14}N_3O_{11}P_2$ requires 402.0104; HPLC (system A) 10.6 min (98%), (system B) 6.7 min (98%).

(2*R*,3*S*,4*R*,5*R*)-1-(5-(Diphosphoryloxymethyl)-3-fluoro-4-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione, Ammonium Salt (18). Procedure A. Compound **18** (2.1 mg, 32%) was obtained as a white solid from **42b** (9 mg, 0.04 mmol). ^{1}H NMR (D_2O) δ 7.79 (d, J = 7.9 Hz, 1H), 6.36 (m, 1H), 5.93 (d, J = 7.9 Hz, 1H), 5.53 (apparent dt, J_{H-F} = 69.0 Hz, 1H), 4.42 (m, 1H), 4.19 (m, 3H); ^{31}P NMR (D_2O) δ −8.89, −10.81; HRMS *m*/*z* found 404.9892 $(M - H^+)^-$. $C_9H_{12}N_2O_{11}P_2F$ requires 404.9900; HPLC (system A) 15.1 min (99%), (system B) 10.9 min (99%).

(1*S*,3*R*,4*R*,5*S*)-1-(4-Hydroxy-1-(diphosphoryloxymethyl)-2-oxabicyclo[3.1.0]hexan-3-yl)pyrimidine-2,4(1*H*,3*H*)-dione, Ammonium Salt (19). Procedure A. Compound **19** (4 mg, 31%) was obtained as a white solid from **44** (10 mg, 0.04 mmol). ^{1}H NMR (D_2O) δ 7.80 (d, J = 8.1 Hz, 1H), 5.79 (d, J = 8.1 Hz, 1H), 5.60 (d, J = 8.1 Hz, 1H), 4.65 (m, 1H), 4.07(m, 1H), 3.67 (m, 1H), 1.81 (m, 1H), 1.37 (m, 1H), 0.84 (m, 1H); ^{31}P NMR (D_2O) δ −7.08, −10.55; HRMS *m*/*z* found 398.9985 $(M - H^+)^-$. $C_{10}H_{13}N_2O_{11}P_2$ requires 398.9995; HPLC (system A) 14.5 min (99%), (system B) 10.2 min (99%).

(2*R*,3*R*,4*S*,5*R*)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)-tetrahydrofuran-2-yl)-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one, Ammonium Salt (20). Procedure A. Compound **20** (21 mg, 23%) was obtained as a white solid from **38** (50 mg, 0.19 mmol). ^{1}H NMR (D_2O) δ 8.19 (d, J = 8.1 Hz, 1H), 6.65 (d, J = 2.7 Hz, 1H), 6.27 (d, J = 8.1 Hz, 1H), 4.45 (m, 1H), 4.32 (m, 4H); ^{31}P NMR (D_2O) δ −9.35 (d, J = 20.8 Hz), −10.84 (d, J = 20.8 Hz); HRMS *m*/*z* found 418.9712 $(M - H^+)^-$. $C_9H_{13}N_2O_{11}P_2S$ requires 418.9715; HPLC (system A) 15.0 min (98%), (system B) 8.4 min (98%).

General Procedure for the Preparation of 4-Substituted-thio-UDP Analogues. Procedure B. To a suspension of 4-thiouridine 5′-diphosphate (5 mg, 0.01 mmol) in MeOH (1 mL) was added 0.25 M NaOH (0.14 mL). The reaction mixture was stirred at room temperature for 2 h, and then the solvent was eliminated under high vacuum. The 4-thio-UDP sodium salt obtained was suspended in dry DMF (1.5 mL), and an excess of the corresponding alkyl halide (50 equiv) was added. The reaction mixture was stirred at room temperature for 24 h, and the progress of the reaction was monitored by analytical HPLC (system A). After removal of the solvent, the residue was purified using the same method as in procedure A, and the corresponding nucleotide diphosphates were obtained as the ammonium salts.

Procedure C. To a solution of 4-thiouridine 5′-monophosphate (10 mg, 0.03 mmol) in MeOH (1 mL) was added 0.25 M NaOH (0.13 mL). The reaction mixture was stirred at room temperature for 2 h, and then the solvent was eliminated under high vacuum. The 4-thio-UMP sodium salt obtained was suspended in dry DMF (2 mL), and an excess of the corresponding alkyl halide (50 equiv) was added. The reaction mixture was stirred at 90 °C for 4−8 h,

and the progress of the reaction was monitored by analytical HPLC (system A). The solvent was removed in vacuo to obtain the corresponding 4-substituted-thio-UMP as a crude solid, which then was used directly in the next step without further purification. To a solution of the crude solid containing 4-substituted-thio-UMP in DMF (1.5 mL) was added 1,1′-carbonyldiimidazole (26 mg, 0.16 mmol). The reaction mixture was stirred at room temperature for 6 h. Then 5% triethylamine solution in 1/1 water/methanol (3 mL) was added and stirring was continued at room temperature for an additional 2 h. After removal of the solvent, the residue was dried in high vacuum and dissolved in DMF (1 mL). To this solution was successively added tributylamine (0.11 mL, 0.46 mmol) and a solution of 0.35 M bis(tributylammonium) salt of phosphoric acid in DMF (0.4 mL). The reaction mixture was stirred at room temperature for 2 days, and then 0.2 M triethylammonium bicarbonate was added. After removal of the solvent, the residue was purified using the same method as in procedure A, and the corresponding nucleotide diphosphates were obtained as the ammonium salts.

(2*R*,3*R*,4*S*,5*R*)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)-tetrahydrofuran-2-yl)-4-(methylthio)pyrimidin-2(1*H*)-one, Ammonium Salt (22). Procedure B. Compound **22** (7 mg, 80%) was obtained as a white solid from **21** (10 mg, 0.02 mmol) and using iodomethane as alkylating agent. ^{1}H NMR (D_2O) δ 8.25 (d, J = 7.1 Hz, 1H), 6.76 (d, J = 7.1 Hz, 1H), 5.94 (d, J = 2.4 Hz, 1H), 4.41 (m, 1H), 4.32 (m, 4H), 2.54 (s, 3H); ^{13}C NMR (D_2O) δ 182.4, 156.8, 142.0, 106.4, 91.9, 83.8, 76.0, 69.6, 65.0, 14.0; ^{31}P NMR (D_2O) δ −7.15 (d, J = 23.1 Hz), −10.67 (d, J = 23.1 Hz); HRMS *m*/*z* found 432.9888 $(M - H^+)^-$. $C_{10}H_{15}N_2O_{11}P_2S$ requires 432.9872; HPLC (system A) 14.9 min (99%), (system B) 11.1 min (99%).

(2*R*,3*R*,4*S*,5*R*)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)-tetrahydrofuran-2-yl)-4-(ethylthio)pyrimidin-2(1*H*)-one, Ammonium Salt (23). Procedure B. Compound **23** (2 mg, 44%) was obtained as a white solid using iodoethane as alkylating agent and stirring the reaction mixture at 70 °C for 4 h. ^{1}H NMR (D_2O) δ 8.24 (d, J = 7.4 Hz, 1H), 6.75 (d, J = 7.4 Hz, 1H), 5.94 (d, J = 2.4 Hz, 1H), 4.41 (m, 1H), 4.32 (m, 4H), 3.15 (q, J = 7.5 Hz, 2H), 1.36 (t, J = 7.5 Hz, 3H); ^{31}P NMR (D_2O) δ −7.49, −10.73; HRMS *m*/*z* found 447.0032 $(M - H^+)^-$. $C_{11}H_{17}N_2O_{11}P_2S$ requires 447.0028; HPLC (system A) 14.7 min (99%), (system B) 14.5 min (99%).

(2*R*,3*R*,4*S*,5*R*)-4-(Allylthio)-1-(3,4-dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1*H*)-one, Ammonium Salt (24). Procedure B. Compound **24** (3.4 mg, 73%) was obtained as a white solid using allyl bromide as alkylating agent. ^{1}H NMR (D_2O) δ 8.26 (d, J = 7.5 Hz, 1H), 6.76 (d, J = 7.5 Hz, 1H), 5.99 (m, 1H), 5.93 (m, 1H), 5.40 (d, J = 17.1 Hz, 2H), 5.23 (d, J = 10.2 Hz, 2H), 4.43 (m, 1H), 4.32 (m, 4H), 3.84 (d, J = 6.6 Hz, 2H); ^{31}P NMR (D_2O) δ −6.08 (d, J = 21.9 Hz), −10.55 (d, J = 21.9 Hz); HRMS *m*/*z* found 459.0039 $(M - H^+)^-$. $C_{12}H_{17}N_2O_{11}P_2S$ requires 459.0028; HPLC (system A) 16.0 min (99%), (system B) 16.2 min (99%).

(2*R*,3*R*,4*S*,5*R*)-4-(Benzylthio)-1-(3,4-dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1*H*)-one, Ammonium Salt (25). Procedure B. Compound **25** (3.3 mg, 65%) was obtained as a white solid using benzyl bromide as alkylating agent. ^{1}H NMR (D_2O) δ 8.21 (d, J = 7.2 Hz, 1H), 7.52 (m, 2H), 7.39 (m, 3H), 6.73 (d, J = 7.2 Hz, 1H), 5.95 (d, J = 2.7 Hz, 1H), 4.46 (s, 2H), 4.34 (m, 4H), 4.23 (m, 1H); ^{31}P NMR (D_2O) δ −9.41, −10.93; HRMS *m*/*z* found 509.0171 $(M - H^+)^-$. $C_{16}H_{19}N_2O_{11}P_2S$ requires 509.0185; HPLC (system A) 16.8 min (98%), (system C) 7.9 min (98%).

(2*R*,3*R*,4*S*,5*R*)-2-(1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-ylthio)-acetic Acid, Ammonium Salt (26). Procedure B. Compound **45** (HPLC (system A) 15.6 min) was obtained as a crude solid using methyl bromoacetate as alkylating agent. The residue that contained compound **45** was dissolved in H_2O (1 mL), and 0.25 M NaOH (0.5 mL) was added. The reaction mixture was stirred at room temperature for 2 h. After removal of the solvent, the residue was purified following the general procedure to give compound **26** (2.4 mg, 49% from **21**) as a white solid. ^{1}H NMR (D_2O) δ 8.21 (d, J = 7.1 Hz, 1H), 6.75 (d, J = 7.1 Hz, 1H), 5.96 (d, J = 2.4 Hz, 1H), 4.35 (m, 4H), 4.25 (m, 1H), 3.88 (s, 2H); ^{31}P NMR (D_2O) δ −10.37 (d, J = 20.6 Hz), −10.92 (d, J = 20.6 Hz); HRMS *m*/*z* found 476.9773 $(M - H^+)^-$. $C_{11}H_{15}N_2O_{13}P_2S$ requires 476.9770; HPLC (system A) 18.3 min (99%), (system B) 9.0 min (99%).

(2*R*,3*R*,4*S*,5*R*)-2-(1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-ylthio)-acetamide, Ammonium Salt (27). Procedure B. Compound **27** (3.8 mg, 79%) was obtained as a white solid using iodoacetamide as alkylating agent. ^{1}H NMR (D_2O) δ 8.31 (d, J = 7.4 Hz, 1H), 6.79 (d, J = 7.4 Hz, 1H), 5.92 (d, J = 1.5 Hz, 1H), 4.42 (m, 1H), 4.32 (m, 4H), 3.96 (s, 2H); ^{13}C NMR (D_2O) δ 177.9, 173.9, 156.0, 141.9, 105.8, 91.2, 83.0, 75.1, 68.3, 63.8, 33.0; ^{31}P NMR (D_2O) δ −7.63, −10.66 (d, J = 21.9 Hz); HRMS *m*/*z* found 475.9935 $(M - H^+)^-$. $C_{11}H_{16}N_3O_{12}P_2S$ requires 475.9930; HPLC (system A) 14.6 min (99%), (system B) 8.6 min (99%).

(2*R*,3*R*,4*S*,5*R*)-3-(1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-ylthio)-propanoic Acid, Ammonium Salt (28). Procedure C. Compound **49** (HPLC (system A) 16.0 min) was obtained as a crude solid using methyl 3-bromopropionate and stirring at 90 °C for 8 h in the alkylation reaction. The residue that contained compound **49** was dissolved in H_2O (1 mL), and 0.25 M NaOH (2 mL) was added. The reaction mixture was stirred at room temperature for 6 h. After removal of the solvent, the residue was purified following the general procedure to give compound **28** (2.8 mg, 20% from **47**) as a white solid. ^{1}H NMR (D_2O) δ 8.21 (d, J = 7.2 Hz, 1H), 6.76 (d, J = 7.2 Hz, 1H), 5.99 (d, J = 2.7 Hz, 1H), 4.38 (m, 4H), 4.26 (m, 1H), 3.39 (t, J = 6.9 Hz, 2H), 2.73 (t, J = 6.9 Hz, 2H); ^{31}P NMR (D_2O) δ −10.39, −10.84; HRMS *m*/*z* found 490.9951 $(M - H^+)^-$. $C_{12}H_{17}N_2O_{13}P_2S$ requires 490.9927; HPLC (system A) 18.5 min (99%), (system B) 9.6 min (99%).

(2*R*,3*R*,4*S*,5*R*)-4-(1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-ylthio)-butanoic Acid, Ammonium Salt (29). Procedure B. Compound **46** (HPLC (system A) 16.4 min) was obtained as a crude solid using methyl 4-iodobutyrate as alkylating agent and stirring the reaction mixture at 50 °C for 20 h. The residue that contained compound **46** was dissolved in H_2O (2 mL), and 0.25 M NaOH (2 mL) was added. The reaction mixture was stirred at room temperature for 2 h. After removal of the solvent, the residue was purified following the general procedure to give compound **29** (2.0 mg, 16% from **21**) as a white solid. ^{1}H NMR (D_2O) δ 8.19 (d, J = 6.8 Hz, 1H), 6.75 (d, J = 6.8 Hz, 1H), 5.96 (m, 1H), 4.35 (m, 4H), 4.24 (m, 1H), 3.19 (d, J = 7.1 Hz, 1H), 2.40 (d, J = 7.5 Hz, 1H), 1.99 (m, 2H); ^{31}P NMR (D_2O) δ −10.22, −10.98; HRMS *m*/*z* found 505.0096 $(M - H^+)^-$. $C_{13}H_{19}N_2O_{13}P_2S$ requires 505.0083; HPLC (system A) 18.2 min (99%), (system B) 9.2 min (99%).

(2*R*,3*R*,4*S*,5*R*)-1-(3,4-Dihydroxy-5-(monophosphoryloxymethyl)tetrahydrofuran-2-yl)-4-(hexylthio)pyrimidin-2(1*H*)-one, Ammonium Salt (50). To a solution of 4-thiouridine 5′-monophosphate (10 mg, 0.03 mmol) in MeOH (1 mL) was added 0.25 M NaOH (0.13 mL). The reaction mixture was stirred at room temperature for 2 h, and then the solvent was eliminated under high vacuum. The 4-thio-UMP sodium salt obtained was suspended in dry DMF (2 mL), and 1-iodohexane (0.19 mL, 1.28 mmol) was added. The reaction mixture was stirred at 90 °C for 4 h, and the progress of the reaction was monitored by analytical HPLC (system A). The solvent was removed in vacuo to obtain compound **50** as a crude solid, which then was used directly in the next step without further purification. For characterization purposes a small portion of compound **50** was purified by ion-exchange column chromatography as described in procedure A. ^{1}H NMR (D_2O) δ 8.21 (d, J = 7.5 Hz, 1H), 6.73 (d, J = 7.5 Hz, 1H), 5.97 (m, 1H), 4.34 (m, 3H), 4.29 (m, 1H), 4.14 (m, 1H), 3.18 (t, J = 7.1 Hz, 2H), 1.74 (m, 2H), 1.46 (m, 2H), 1.33 (m, 4H), 0.89 (t, J = 6.9 Hz, 3H); ^{31}P NMR (D_2O) δ 0.60; HRMS *m*/*z* found 423.0995 $(M - H^+)^-$. $C_{15}H_{24}N_2O_8PS$ requires 423.0991; HPLC (system A) 15.1 min (99%), (system C) 13.7 min (99%).

(2*R*,3*R*,4*S*,5*R*)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)-tetrahydrofuran-2-yl)-4-(hexylthio)pyrimidin-2(1*H*)-one, Am-

monium Salt (30). To a solution of the crude solid containing compound **50** in DMF (1.5 mL) was added 1,1′-carbonyldiimidazole (26 mg, 0.16 mmol). The reaction mixture was stirred at room temperature for 6 h. Then 5% triethylamine solution in 1/1 water/methanol (3 mL) was added and stirring was continued at room temperature for an additional 2 h. After removal of the solvent, the residue was dried in high vacuum and dissolved in DMF (1 mL). To this solution was successively added tributylamine (0.11 mL, 0.46 mmol) and a solution of 0.35 M bis(tributylammonium) salt of phosphoric acid in DMF (0.4 mL). The reaction mixture was stirred at room temperature for 2 days, and then 0.2 M triethylammonium bicarbonate was added. After removal of the solvent, the residue was purified using the same method as in procedure A to afford **30** (3.4 mg, 21% from **47**) as a white solid. ^{1}H NMR (D_2O) δ 8.21 (d, J = 7.4 Hz, 1H), 6.75 (d, J = 7.4 Hz, 1H), 5.96 (d, J = 2.7 Hz, 1H), 4.36 (m, 4H), 4.25 (m, 1H), 3.17 (t, J = 7.2 Hz, 2H), 1.73 (m, 2H), 1.45 (m, 2H), 1.32 (m, 4H), 0.88 (t, J = 7.1 Hz, 3H); ^{31}P NMR (D_2O) δ −9.67, −10.83; HRMS *m*/*z* found 503.0606 $(M - H^+)^-$. $C_{15}H_{25}N_2O_{11}P_2S$ requires 503.0654; HPLC (system A) 18.9 min (99%), (system C) 13.8 min (99%).

(2*R*,3*R*,4*S*,5*R*)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)-tetrahydrofuran-2-yl)-5-iodopyrimidine-2,4(1*H*,3*H*)-dione, Ammonium Salt (32). To a solution of **53** (8 mg, 0.02 mmol) in DMF (2 mL) was added 1,1′-carbonyldiimidazole (16 mg, 0.10 mmol). The reaction mixture was stirred at room temperature for 6 h. Then 5% triethylamine solution in 1/1 water/methanol (2 mL) was added and stirring was continued at room temperature for an additional 2 h. After removal of the solvent, the residue was dried in high vacuum and dissolved in DMF (2 mL). To this solution was successively added tributylamine (0.07 mL, 0.29 mmol) and a solution of 0.35 M bis(tributylammonium) salt of phosphoric acid in DMF (0.3 mL). The reaction mixture was stirred at room temperature for 3 days, and then 0.2 M triethylammonium bicarbonate (2 mL) was added. The mixture was stirred for 30 min. After removal of the solvent, the residue was purified using the same method as in procedure A to afford **32** (1.8 mg, 19%) as a white solid. ^{1}H NMR (D_2O) δ 8.28 (s, 1H), 5.93 (d, J = 4.2 Hz, 1H), 4.42 (m, 2H), 4.25 (m, 3H); ^{31}P NMR (D_2O) δ −6.89, −10.60; HRMS *m*/*z* found 528.8901 $(M - H^+)^-$. $C_9H_{12}N_2O_{12}P_2I$ requires 528.8910; HPLC (system A) 14.7 min (99%), (system B) 8.8 min (99%).

(2*R*,3*S*,4*R*,5*R*)-1-(3-Fluoro-4-hydroxy-5-(monophosphoryloxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione, Ammonium Salt (54). A solution of 2′-fluoro-2′-deoxy-β-D-arabinofuranosyluridine (15 mg, 0.06 mmol) and Proton Sponge (26 mg, 0.12 mmol) in trimethyl phosphate (2 mL) was stirred for 10 min at 0 °C. Then phosphorus oxychloride was added dropwise (0.01 mL, 0.12 mmol), and the reaction mixture was stirred for 2 h at 0 °C. Then 0.2 M triethylammonium bicarbonate solution (7 mL) was added, and the clear solution was stirred at room temperature for 45 min. The latter was lyophilized overnight. The residue was purified using the same method as in procedure A to afford **54** (7 mg, 60%) as a white solid. ^{1}H NMR (D_2O) δ 7.90 (d, J = 8.1 Hz, 1H), 6.31 (dd, J = 15.9, 4.2 Hz, 1H), 5.89 (d, J = 8.1 Hz, 1H), 5.21 (apparent dt, J_{H-F} = 51.6 Hz, 1H), 4.52 (apparent dt, J_{H-F} = 19.2 Hz, 1H), 4.13 (m, 3H); ^{31}P NMR (D_2O) δ 4.01; HRMS *m*/*z* found 325.0240 $(M - H^+)^-$. $C_9H_{11}N_2O_8FP$ requires 325.0237; HPLC (system A) 10.1 min (99%), (system B) 6.2 min (99%).

(2*R*,3*S*,4*R*,5*R*)-1-(3-Fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione Cyclic-4,5-diphosphate, Ammonium Salt (33). To a solution of **54** (5 mg, 0.02 mmol) in DMF (2 mL) was added 1,1′-carbonyldiimidazole (12 mg, 0.08 mmol). The reaction mixture was stirred at room temperature for 8 h. Then 5% triethylamine in methanol was added and stirring was continued at room temperature for an additional 2 h. After removal of the solvent, the residue was dried in high vacuum and dissolved in DMF (1 mL). To this solution was successively added tributylamine (0.06 mL, 0.25 mmol) and a solution of 0.35 M bis(tributylammonium) salt of phosphoric acid in DMF (0.23 mL). The reaction mixture was stirred at room temperature for 3 d, and then 0.2 M triethylammonium bicarbonate (2 mL) was added. The mixture was stirred for 30 min. After removal of the solvent, under reduced pressure at 50 °C, the residue was purified using the same method as in procedure A to afford **33** (1.6 mg, 29%) as a white solid. ^{1}H NMR (D_2O) δ 7.78 (d, J = 8.1 Hz, 1H), 6.35 (dd, J = 13.1, 5.3 Hz, 1H), 5.92 (d, J = 8.1 Hz, 1H), 5.50 (apparent dt, J_{H-F} = 52.2 Hz, 1H), 4.47 (m, 1H), 4.25 (m, 3H); ^{31}P NMR (D_2O) δ −7.15, −10.37; HRMS *m*/*z* found 386.9783 $(M - H^+)^-$. $C_9H_{10}N_2O_{10}P_2F$ requires 386.9795; HPLC (system A) 13.2 min (99%), (system B) 8.6 min (99%).

(2*R*,3*R*,4*S*,5*R*)-1-(3,4-Dihydroxy-5-(monophosphoryloxymethyl)tetrahydrofuran-2-yl)-4-(ethylthio)pyrimidin-2(1*H*)-one, Ammonium Salt (51). To a solution of 4-thiouridine 5′-monophosphate (10 mg, 0.03 mmol) in MeOH (1 mL) was added 0.25 M NaOH (0.13 mL). The reaction mixture was stirred at room temperature for 2 h, and then the solvent was eliminated under high vacuum. The 4-thio-UMP sodium salt obtained was suspended in dry DMF (2 mL), and iodoethane (0.1 mL, 1.28 mmol) was added. The reaction mixture was stirred at 70 °C for 2 h, and the progress of the reaction was monitored by analytical HPLC (system A). The solvent was removed in vacuo to obtain compound **51** as a crude solid, which then was used directly in the next step without further purification. For characterization purposes a small portion of compound **51** was purified by ion-exchange column chromatography as described in procedure A. ^{1}H NMR (D_2O) δ 8.26 (d, J = 7.4 Hz, 1H), 6.73 (d, J = 7.4 Hz, 1H), 5.96 (m, 1H), 4.33 (m, 3H), 4.24 (m, 1H), 4.09 (m, 1H), 3.15 (q, J = 7.4 Hz, 2H), 1.36 (t, J = 7.4 Hz, 3H); ^{31}P NMR (D_2O) δ 1.48; HRMS *m*/*z* found 367.0377 $(M - H^+)^-$. $C_{11}H_{16}N_2O_8PS$ requires 367.0365; HPLC (system A) 8.5 min (98%), (system B) 6.7 min (98%).

***P*^{1},*P*^{2}-Bis-5-[(2*R*,3*R*,4*S*,5*R*)-1-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-4-(ethylthio)pyrimidin-2(1*H*)-one]pyrophosphate, Ammonium Salt, (4-SEt)Up2(4-SEt)U (35).** To a solution of the crude solid containing compound **51** in DMF (1.5 mL) was added 1,1′-carbonyldiimidazole (26 mg, 0.16 mmol). The reaction mixture was stirred at room temperature for 6 h. Then 5% triethylamine solution in 1/1 water/methanol (3 mL) was added and stirring was continued at room temperature for an additional 2 h. After removal of the solvent, the residue was dried in high vacuum and dissolved in DMF (1 mL). To this solution was successively added tributylamine (0.11 mL, 0.46 mmol) and a solution of 0.35 M bis(tributylammonium) salt of phosphoric acid in DMF (0.4 mL). The reaction mixture was stirred at room temperature for 2 days, and then 0.2 M triethylammonium bicarbonate was added. After removal of the solvent, the residue was purified using the same method as in procedure A to afford **35** (5 mg, 52% from **47**) as a white solid. ^{1}H NMR (D_2O) δ 8.10 (d, J = 7.4 Hz, 2H), 6.57 (d, J = 7.4 Hz, 2H), 5.95 (d, J = 2.7 Hz, 2H), 4.45 (m, 2H), 4.28 (m, 8H), 3.10 (q, J = 7.5 Hz, 4H), 1.35 (t, J = 7.5 Hz, 6H); ^{31}P NMR (D_2O) δ −10.23; HRMS *m*/*z* found 717.0711 $(M - H^+)^-$. $C_{22}H_{31}N_4O_{15}P_2S_2$ requires 717.0703; HPLC (system A) 14.9 min (99%), (system C) 6.8 min (99%).

***P*^{1},*P*^{2}-Bis-5-[(2*R*,3*R*,4*S*,5*R*)-4-(benzylthio)-1-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1*H*)-one]pyrophosphate, Ammonium Salt, (4-SBn)Up2(4-SBn)U (36). Procedure C.** Compound **36** (5 mg, 45% from **47**) was obtained as a white solid using benzyl bromide and stirring overnight at room temperature in the alkylation reaction. ^{1}H NMR (D_2O) δ 8.11 (d, J = 7.2 Hz, 2H), 7.47 (m, 4H), 7.37 (m, 6H), 6.55 (d, J = 7.2 Hz, 2H), 5.92 (d, J = 2.4 Hz, 2H), 4.31 (m, 14H); ^{31}P NMR (D_2O) δ −10.81; HRMS *m*/*z* found 841.0991 $(M - H^+)^-$. $C_{32}H_{35}N_4O_{15}P_2S_2$ requires 841.1016; HPLC (system A) 19.2 min (99%), (system C) 16.8 min (99%).

Assay of $P2Y_6$ Receptor-Stimulated PLC Activity. A stable cell line expressing the human $P2Y_6$ receptor in 1321N1 human astrocytoma cells was generated as previously described in detail.[16] Agonist-induced [^{3}H]inositol phosphate production was measured in 1321N1 cells grown to confluence on 48-well plates. Twelve hours before the assay, the inositol lipid pool of the cells was radiolabeled by incubation in 200 μL of serum-free inositol-free Dulbecco's modified Eagle's medium, containing 0.4 μCi of *myo*-

[^{3}H]inositol. No changes of medium were made after the addition of [^{3}H]inositol. On the day of the assay, cells were challenged with 50 μL of the 5-fold concentrated solution of receptor agonists in 200 mM Hepes (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid), pH 7.3, containing 50 mM LiCl for 20 min at 37 °C. Incubations were terminated by aspiration of the drug-containing medium and addition of 450 μL of ice-cold 50 mM formic acid. After 15 min at 4 °C, samples were neutralized with 150 μL of 150 mM NH_4OH. [^{3}H]Inositol phosphates were isolated by ion exchange chromatography on Dowex AG 1-X8 columns as previously described.[31]

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

Molecular Modeling. Molecular mechanics calculations have been carried out by means of the Discover3 module of InsightII,[40] using the CFF91 force field.[41] The receptor model used for the docking experiments was the $P2Y_6$−UDP complex that we previously built[11] and optimized by means of a molecular dynamics (MD) simulation in an explicit fully hydrated lipid bilayer.[35]

Before the docking experiments were performed, the ligands were first fully optimized by means of quenched molecular dynamics followed by energy minimization. An NVT (constant-volume/constant-temperature) molecular dynamics simulation was carried out at a constant temperature of 300 K for 10 ps, using a time step of 1 fs. During the simulation, snapshots of the system were taken at regular intervals of 1 ps. The structures in each snapshot were energy-minimized (BFGS Newton method) until a root-mean-squared deviation (rmsd) of 0.000 01 kcal mol^{-1} Å^{-1} on the gradient was reached. For each ligand, the conformation showing the lowest energy was promoted to the next phase of the study.

Subsequently, the ligands were flexibly superimposed to the bound conformation of **1**, as derived from our MD simulation.[35] The flexible superimpositions have been carried out by means of the FieldFit program of the Search Compare module of InsightII,[40] giving equal weight to the steric and the electrostatic factors. The bound conformation of **1** was kept rigid during the calculation, while the structures to be superimposed were fully flexible. An alignment of the molecules based on their dipole and quadruple moments was used as the starting position.

The docking experiments were carried out by means of the Monte Carlo minimization approach implemented in the Affinity module of InsightII.[40] The binding site was defined as all the residues within a distance of 6 Å from the ligand. Full flexibility was granted to the ligands and to the residues of the binding site. The scaling factor for the van der Waals term was set at 0.1, while the Coulombic term was set at 1. The maximum movement in each random translation and rotation of the ligand were set to 0.1 Å and 1°, respectively.

After the docking procedure, the receptor−ligand complexes were optimized by means of a Monte Carlo multiple minimum (MCMM)[42] conformational search as implemented in MacroModel.[43,44] The search was performed on the ligand and the residues located within a distance of 6 Å from the ligand, while the remaining residues were conformationally frozen. The following parameters were employed for the conformational search: number of steps = 100; number of structures to save for each search = 100; energy window for saving structures = 1000.0 kJ/mol. The calculations were conducted with the MMFF force field,[45] using water as implicit solvent (GB/SA model as implemented in MacroModel)[46] and a molecular dielectric constant of 1. For the energy minimizations the Polak−Ribier conjugate gradient was used with a convergence threshold on the gradient of 0.05 kJ Å^{-1} mol^{-1}.

Acknowledgment. Dr. Pedro Besada and Dr. Dae Hong Shin thank the Cystic Fibrosis Foundation, Bethesda, MD, for financial support. Mass spectral measurements were carried out by Dr. John Lloyd and NMR by Wesley White (NIDDK). We thank Kanna Palaniappan for proofreading this manuscript. This research was supported in part by the Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases. This work was supported by National Institutes of Health Grants GM38213 and HL34322 to T. K. Harden.

Supporting Information Available: Coordinates of the complex of the human $P2Y_6$ receptor with compound **32** (5-iodoUDP) in pdb format. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Fredholm, B. B.; Abbracchio, M. P.; Burnstock, G.; Dubyak, G. R.; Harden, T. K.; Jacobson, K. A.; Schwabe, U.; Williams, M. Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol. Sci*. **1997**, *18*, 79−82.

(2) North, R. A.; Barnard, E. A. Nucleotide receptors. *Curr. Opin. Neurobiol.* **1997**, *7*, 346−357.

(3) von Kügelgen, I.; Wetter, A. Molecular pharmacology of P2Y-receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2000**, *362*, 310−323.

(4) Jacobson, K. A.; Costanzi, S.; Ohno, M.; Joshi, B. V.; Besada, P.; Xu, B.; Tchilibon, S. Molecular recognition at purine and pyrimidine nucleotide (P2) receptors. *Curr. Top. Med. Chem*. **2004**, *4*, 805−819.

(5) von Kügelgen I. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol. Ther.* **2006**, *110*, 415−432.

(6) Lustig, K. D.; Shiau, A. K.; Brake, A. J.; Julius, D. Expression cloning of an ATP receptor from mouse neuroblasoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5113−5117.

(7) Communi, D.; Pirotton, S.; Parmentier, M.; Boeynaems, J. M. Cloning and functional expression of a human uridine nucleotide receptor. *J. Biol. Chem.* **1995**, *270*, 30849−30852.

(8) Nguyen, T.; Erb, L.; Weisman, G. A.; Marchese, A.; Heng, H. H. Q. Cloning, expression, and chromosomal localization of the human uridine nucleotide receptor gene. *J. Biol. Chem.* **1995**, *270*, 30845−30848.

(9) Chang, K.; Hanaoka, K.; Kumada, M.; Takuwa, Y. Molecular cloning and functional analysis of a novel P2 nucleotide receptor. *J. Biol. Chem.* **1995**, *270*, 26152−26158.

(10) Communi, D.; Suarez Gonzalez, N.; Detheux, M.; Brezillon, S.; Lannoy, V.; Parmentier, M.; Boeynaems, J. M. Identification of a novel human ADP receptor coupled to Gi. *J. Biol. Chem.* **2001**, *276*, 41479−41485.

(11) Costanzi, S.; Mamedova, L.; Gao, Z.-G.; Jacobson, K. A. Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis, and homology modeling. *J. Med. Chem.* **2004**, *47*, 5393−5404.

(12) Shaver, S. R. P2Y receptors: biological advances and therapeutic opportunities. *Curr. Opin. Drug Discovery Dev.* **2001**, *4*, 665−670.

(13) Yerxa, B. R.; Sabater, J. R.; Davis, C. W.; Stutts, M. J.; Lang-Furr, M.; Picher, M.; Jones, A. C.; Cowlen, M.; Dougherty, R.; Boyer, J.; Abraham, W. M.; Boucher, R. C. Pharmacology of INS37217 [P(1)-(uridine 5′)-P(4)-(2′-deoxycytidine 5′)tetraphosphate, tetrasodium salt], a next-generation $P2Y_2$ receptor agonist for the treatment of cystic fibrosis. *J. Pharmacol. Exp. Ther.* **2002**, *302*, 871−880.

(14) Burnstock, G. Purinergic P2 receptors as targets for novel analgesics. *Pharmacol. Ther.* **2006**, *110*, 433−454.

(15) Jacobson, K. A.; Jarvis, M. F.; Williams, M. Purine and pyrimidine (P2) receptors as drug targets. *J. Med. Chem.* **2002**, *45*, 4057−4093.

(16) Nicholas, R. A.; Lazarowski, E. R.; Watt, W. C.; Li, Q.; Harden, T. K. Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol. Pharmacol.* **1996**, *50*, 224−229.

(17) Kim, S. G.; Soltysiak, K. A.; Gao, Z. G.; Chang, T. S.; Chung, E.; Jacobson, K. A. Tumor necrosis factor α-induced apoptosis in astrocytes is prevented by the activation of $P2Y_6$, but not $P2Y_4$ nucleotide receptors. *Biochem. Pharmacol.* **2003**, *65*, 923−931.

(18) Korcok, J.; Raimundo, L. N.; Du, X.; Sims, S. M.; Dixon, S. J. $P2Y_6$ nucleotide receptors activate NF-κB and increase survival of osteoclasts. *J. Biol. Chem.* **2005**, *280*, 16909−16915.

(19) Schreiber, R.; Kunzelmann, K. Purinergic $P2Y_6$ receptors induce Ca^{2+} and CFTR dependent Cl^- secretion in mouse trachea. *Cell. Physiol. Biochem.* **2005**, *16*, 99−108.

(20) Cox, M. A.; Gomes, B.; Palmer, K.; Du, K.; Wiekowski, M.; Wilburn, B.; Petro, M.; Chou, C.-C.; Desquitado, C.; Schwarz, M.; Lunn, C.; Lundell, D.; Narula, S. K.; Zavodny, P. J.; Jenh, C.-H. The pyrimidinergic $P2Y_6$ receptor mediates a novel release of proinflammatory cytokines and chemokines in monocytic cells stimulated with UDP. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 467−473.

(21) Khine, A. A.; Del Sorbo, L.; Vaschetto, R.; Voglis, S.; Tullis, E.; Slutsky, A. S.; Downey, G. P.; Zhang, H. Human neutrophil peptides induce interleukin-8 production via $P2Y_6$ signaling pathway. *Blood* **2006**, *107*, 2936−2942.

(22) Hou, M.; Harden, T. K.; Kuhn, C. M.; Baldetorp, B.; Lazarowski, E.; Pendergast, W.; Möller, S.; Edvinsson, L.; Erlinge, D. UDP acts as a growth factor for vascular smooth muscle cells by activation of $P2Y_6$ receptors. *Am. J. Physiol.: Heart Circ. Physiol.* **2002**, *282*, H784−H792.

(23) Kim, H. S.; Ravi, R. G.; Marquez, V. E.; Maddileti, S.; Wihlborg, A. K.; Erlinge, D.; Malmsjö, M.; Boyer, J. L.; Harden, T. K.; Jacobson, K. A. Methanocarba modification of uracil and adenine nucleotides: High potency of Northern ring conformation at $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$, but not $P2Y_6$ receptors. *J. Med. Chem.* **2002**, *45*, 208−218.

(24) Robaye, B.; Boeynaems, J. M.; Communi, D. Slow desensitization of the human $P2Y_6$ receptor. *Eur. J. Pharmacol*. **1997**, *329*, 231−236.

(25) Gagneron, J.; Gosselin, G.; Mathe, C. Synthesis of nucleoside analogues bearing the five naturally occurring nucleic acid bases built on a 2-oxabicylo[3.1.0]hexane scaffold. *J. Org. Chem*. **2005**, *70*, 6891−6897.

(26) Fischer, B.; Boyer, J. L.; Hoyle, C. H. V.; Ziganshin, A. U.; Brizzolara, A. L.; Knight, G. E.; Zimmet, J.; Burnstock, G.; Harden, T. K.; Jacobson, K. A. Identification of potent, selective P_{2Y}-purinoceptor agonists: Structure activity relationships for 2-thioether derivatives of adenosine-5′-triphosphate. *J. Med. Chem.* **1993**, *36*, 3937−3946.

(27) Shaver, S. R.; Pendergast, W.; Siddiqi, S. M.; Yerxa, B. R.; Croom, D. K.; Dougherty, R. W.; James, M. K.; Jones, A. C.; Rideout, J. L. 4-Substituted uridine 5′-triphosphates as agonists of the $P2Y_2$ purinergic receptor. *Nucleosides Nucleotides* **1997**, *16*, 1099−1102.

(28) Aviño, A.; Guimil Garcia, R.; Eritja, R. Synthesis of oligoribonucleotides containing 4-thiouridine using the convertible nucleoside approach and the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl group. *Nucleosides, Nucleotides Nucleic Acids* **2004**, *23*, 1767−1777.

(29) Xu, Y.-Z.; Zheng, Q.; Swann, P. F. Synthesis of DNA containing modified bases by post-synthetic substitution. Synthesis of oligomers containing 4-substituted thymine: O^4-alkylthymine, 5-methylcytosine, N^4-dimethylamino-5-methylcytosine, and 4-thiothymine. *J. Org. Chem.* **1992**, *57*, 3839−3845.

(30) Fischer, B.; Chulkin, A.; Boyer, J. L.; Harden, K. T.; Gendron, F. P.; Beaudoin, A. R.; Chapal, J.; Hillaire-Buys, D.; Petit, P. 2-Thioether 5′-*O*-(1-thiotriphosphate)adenosine derivatives as new insulin secretagogues acting through P2Y-receptors. *J. Med. Chem.* **1999**, *42*, 3636−3646.

(31) Brown, H. A.; Lazarowski, E. R.; Boucher, R. C.; Harden, T. K. Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5′-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.* **1991**, *40*, 648−655.

(32) Nandanan, E.; Camaioni, E.; Jang, S.-Y.; Kim, Y. C.; Cristalli, G.; Herdewijn, P.; Secrist, J. A., III; Tiwari, K. N.; Mohanram, A.; Harden, T. K.; Boyer, J. L.; Jacobson, K. A. Structure activity relationships of bisphosphate nucleotide derivatives as $P2Y_1$ receptor antagonists and partial agonists. *J. Med. Chem.* **1999**, *42*, 1625−1638.

(33) Pendergast, W.; Yerxa, B. R.; Douglass, J. G., 3rd; Shaver, S. R.; Dougherty, R. W.; Redick, C. C.; Sims, I. F.; Rideout, R. L. Synthesis and P2Y receptor activity of a series of uridine dinucleoside 5′-polyphosphates. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 157−160.

(34) Shaver, S. R.; Rideout, J. L.; Pendergast, W.; Douglass, J. G.; Brown, E. G.; Boyer, J. L.; Patel, R. I.; Redick, C. C.; Jones, A. C.; Picher, M.; Yerxa, B. R. Structure−activity relationships of dinucleotides: Potent and selective agonists of P2Y receptors. *Purinergic Signalling* **2005**, *1*, 183−191.

(35) Costanzi, S.; Joshi, B. V.; Maddileti, S.; Mamedova, L.; Gonzalez-Moa, M. J.; Marquez, V. E.; Harden, T. K.; Jacobson, K. A. Human $P2Y_6$ receptor: Molecular modeling leads to the rational design of a novel agonist based on a unique conformational preference. *J. Med. Chem*. **2005**, *48*, 8108−8111.

(36) Ohno, M.; Costanzi, S.; Kim, H. S.; Kempeneers, V.; Vastmans, K.; Herdewijn, P.; Maddileti, S.; Gao, Z.-G.; Harden, T. K.; Jacobson, K. A. Nucleotide analogues containing 2-oxa-bicyclo[2.2.1]heptane and L-α-threofuranosyl ring systems: interactions with P2Y receptors. *Bioorg. Med. Chem.* **2004**, *12*, 5619−5630.

(37) Jacobson, K. A.; Costanzi, S.; Ivanov, A. A.; Tchilibon, S.; Besada, P.; Gao, Z.-G.; Maddileti, S.; Harden, T. K. Structure activity and molecular modeling analyses of ribose- and base-modified uridine 5′-triphosphate analogues at the human $P2Y_2$ and $P2Y_4$ receptors. *Biochem. Pharmacol*. **2006**, *71*, 540−549.

(38) Christie, S. M. H.; Elmore, D. T.; Kenner, G. W., Todd, A. R.; Weymouth, F. J. Syntheses of P^1P^2-diadenosine-5′ and P^1P^2-diuridine-5′ pyrophosphates. *J. Chem. Soc*. **1953**, 2947−2953.

(39) Baddiley, J.; Buchanan, J. G.; Fawcett, C. P. Syntheses of cytidine diphosphate ribitol. *J. Chem. Soc.* **1959**, 2192−2196.

(40) *InsightII*, version 2000.1; Accelrys (formerly MSI): San Diego, CA.

(41) Maple, J. R.; Hwang, M. J.; Stockfisch, T. P.; Dinur, U.; Waldman, M.; Ewig, C. S.; Hagler, A. T. Derivation of class II force fields. 1. Methodology and quantum force field for the alkyl functional group and alkane molecules. *J. Comput. Chem*. **1994**, *15*, 162.

(42) Chang, G.; Guida, W.; Still, W. C. An internal coordinate Monte Carlo method for searching conformational space. *J. Am. Chem. Soc.* **1989**, *111*, 4379−4386.

(43) *MacroModel*, version 9.1; Schrödinger L.L.C.: New York.

(44) Mohamadi, F. N.; Richards, G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. MacroModel, an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. *J. Comput. Chem.* **1990**, *11*, 440−467.

(45) Halgren, T. A. MMFF VII. Characterization of mmff94, mmff94s and other widely available force fields for conformational energies and for intermolecular interaction energies and geometries. *J. Comput. Chem*. **1999**, *20*, 730−748.

(46) Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. A general treatment of solvation for molecular mechanics. *J. Am. Chem. Soc.* **1990**, *112*, 6127.

(47) Kennedy, C.; Qi, A. D.; Herold, C. L.; Harden, T. K.; Nicholas, R. A. ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. *Mol Pharmacol*. **2000**, *57*, 926−931.

(48) Marquez, V. E.; Siddiqui, M. A.; Ezzitouni, A.; Russ, P.; Wang, J.; Wagner, R. W.; Matteucci, M. D. Nucleosides with a twist. Can fixed forms of sugar ring pucker influence biological activity in nucleosides and oligonucleotides? *J. Med. Chem.* **1996**, *39*, 3739−3747.

JM060485N