Structure activity relationship of uridine 5′-diphosphate analogues at the human P2Y6 receptor

Pedro Besada[a], **Dae Hong Shin**[a], **Stefano Costanzi**[b], **Hyojin Ko**[a], **Christophe Mathé**[c], **Julien Gagneron**[c], **Gilles Gosselin**[c], **Savitri Maddileti**[d], **T. Kendall Harden**[d], and **Kenneth A. Jacobson**[a],*

[a]Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

[b]Computational Chemistry Core Laboratory, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

[c]Laboratoire de Chimie Organique Biomoléculaire de Synthese, UMR 5625 CNRS - UM II, Université Montpellier II, Place E. Bataillon, 34095 Montpellier, cedex 5 France

[d]Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA

Abstract

The structure activity relationships and molecular modeling of the uracil nucleotide-activated P2Y6 receptor have been studied. A series of UDP analogues bearing substitutions of the ribose moiety, the uracil ring, and the diphosphate group was synthesized and assayed for activity at the human P2Y6 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 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** (EC50 0.15 µM) was equipotent to UDP, while substitutions of the 2′-hydroxyl (amino, azido) greatly reduce potency. 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 analogs 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 EC50 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 P2Y6 receptor and will be helpful in designing selective and potent P2Y6 receptor ligands

*Corresponding author: Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA. Tel. 301-496-9024; FAX 301-480-8422; kajacobs@helix.nih.gov.

Supporting Information Available: Coordinates of the complex of the human P2Y6 with compound **32** (5-iodoUDP) are included. This material is available free of charge via the Internet at http://pubs.acs.org.

Keywords

G protein-coupled receptor; nucleotides; thionucleotides; phospholipase C; pyrimidines; homology modeling

Introduction

P2 nucleotide receptors consist of two families: G protein-coupled receptors designated P2Y, which include 8 subtypes (P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{11}$-P2Y$_{14}$), and ligand-gated cation channels designated P2X, which include 7 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 UDP-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] and production of pro-inflammatory 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] In order to investigate more extensively the SAR at the P2Y$_6$ receptor, we synthesized a series of UDP analogues, where modifications at the level of 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 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 diphosphates 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 triethyl ammonium salt form 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 (NMR, ^{1}H, ^{13}C, ^{31}P 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 phosphorous oxychloride and phosphoric acid tributylammonium salt[26] provided the 5′-diphosphate analogues **13, 15, 16, 18-20** in moderate yields (Schemes 1, 2). The phosphorylation of the 2′-amino-2′-deoxyuridine **40** using the one-pot method provided the 3′-diphosphate derivative **17**. The ^{1}H NMR of compound **17** shows peaks at 5.01 ppm (H-3′) and 3.89 ppm (H-5′), when 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 indicate that for **17** the diphosphate group is in 3′ position. The synthesis of 2′-deoxy-2′-aminouridine-5′-diphosphate **16** was accomplished after the acetylation of the amino group in **40** with a trifluoroacetyl group to obtain **41**. Phosphorylation of the 2′-trifluoroacetylamino 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 others 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, 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.

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 phosphoryl imidazolide intermediate, which reacted with 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 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 or amino groups 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 reported, a strong preference for the (S) conformation was indicated (cf. **6** vs. **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 prepared and 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 five-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 μmolar 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 (TM) 3, 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. Consistently 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) due to 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 (**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 an hydrophobic pocket located between TM1 and TM2 (Figure 1e). The substitution at the 4-position with carboxyalkylthioethers (**26**, **28**, **29**) is also well 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, the base pointing toward TM1 and TM2, and 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 analogs 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, 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 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) 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 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 in that they respond to varied and diverse nucleotide ligands. Within the same phylogenetic cluster are receptors for other anionic 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, UK). 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, 53** and reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). Compounds **5, 6, 14, 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 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 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 a Varian XL 300 spectrometer.

Purity of compounds was checked using a Hewlett–Packard 1100 HPLC equipped with a Luna 5 μ RP-C18(2) analytical column (250 X 4.6 mm; Phenomenex, Torrance, CA). System A: linear gradient solvent system: 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: linear gradient solvent system: 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: linear gradient solvent system: 10 mM TEAA-CH_3CN from 90:10 to 60:40 in 20 min, then isocratic for 2 min; the flow rate was 1 mL/min. 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 X 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)-A25 Sephadex columns as described below. Compounds **13, 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 μ RP-C18(2) semipreparative column (250 X 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 phosphorous oxychloride (2 equiv) was added dropwise, and the reaction mixture was stirred for 2 hours at 0 °C. A mixture of tributylamine (9 equiv) and a solution 0.35 M of 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.

(2R,3R,5S)-1-(5-(Diphosphoryloxymethyl)-3-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-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%).

(2R,3R,4S,5R)-1-(3-Azido-5-(diphosphoryloxymethyl)-4-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-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%).

(2R,3R,4S,5R)-N-(2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-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 ethyltrifluoroacetate (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.

(2R,3R,4S,5R)-1-(3-Amino-5-(diphosphoryloxymethyl)-4-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-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%).

(2R,3R,4S,5R)-1-(3-Amino-4-(diphosphoryloxy)-5-(hydroxymethyl)-tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-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$_2$O) δ -7.73 (d, J=22.0 Hz), -11.16 (d, J=22.0 Hz); HRMS m/z found 402.0094 (M – H$^+$)$^-$. C$_9$H$_{14}$N$_3$O$_{11}$P$_2$ requires 402.0104; HPLC (System A) 10.6 min (98%), (System B) 6.7 min (98%).

(2R,3S,4R,5R)-1-(5-(Diphosphoryloxymethyl)-3-fluoro-4-hydroxytetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-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$_2$O) 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$_2$O) δ -8.89, -10.81; HRMS m/z found 404.9892 (M – H$^+$)$^-$. C$_9$H$_{12}$N$_2$O$_{11}$P$_2$F requires 404.9900; HPLC (System A) 15.1 min (99%), (System B) 10.9 min (99%).

(1S,3R,4R,5S)-1-(4-Hydroxy-1-(diphosphoryloxymethyl)-2-oxabicyclo[3.1.0]hexan-3-yl)pyrimidine-2,4(1H,3H)-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$_2$O) 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$_2$O) δ -7.08, -10.55; HRMS m/z found 398.9985 (M – H$^+$)$^-$. C$_{10}$H$_{13}$N$_2$O$_{11}$P$_2$ requires 398.9995; HPLC (System A) 14.5 min (99%), (System B) 10.2 min (99%).

(2R,3R,4S,5R)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-2-thioxo-2,3-dihydropyrimidin-4(1H)-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$_2$O) 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$_2$O) δ -9.35 (d, J=20.8 Hz), -10.84 (d, J=20.8 Hz); HRMS m/z found 418.9712 (M – H$^+$)$^-$. C$_9$H$_{13}$N$_2$O$_{11}$P$_2$S 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, 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 than in procedure A, and the corresponding nucleotide diphosphates were obtained as the ammonium salts.

Procedure C. To a solution of 4-thiouridine 5′-monophsate (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, 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 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 0.35 M of 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 than in procedure A, and the corresponding nucleotide diphosphates were obtained as the ammonium salts.

(2R,3R,4S,5R)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-4-(methylthio)pyrimidin-2(1H)-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$_2$O) 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$_2$O) 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$_2$O) 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$_2$O$_{11}$P$_2$S requires 432.9872; HPLC (System A) 14.9 min (99%), (System B) 11.1 min (99%).

(2R,3R,4S,5R)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-4-(ethylthio)pyrimidin-2(1H)-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$_2$O) 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$_2$O) δ -7.49, -10.73; HRMS m/z found 447.0032 (M − H$^+$)$^-$. C$_{11}$H$_{17}$N$_2$O$_{11}$P2S requires 447.0028; HPLC (System A) 14.7 min (99%), (System B) 14.5 min (99%).

(2R,3R,4S,5R)-4-(Allylthio)-1-(3,4-dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1H)-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$_2$O) 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$_2$O) 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$_2$O$_{11}$P$_2$S requires 459.0028; HPLC (System A) 16.0 min (99%), (System B) 16.2 min (99%).

(2R,3R,4S,5R)-4-(Benzylthio)-1-(3,4-dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1H)-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$_2$O) 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$_2$O) δ -9.41, -10.93; HRMS m/z found 509.0171 (M − H$^+$)$^-$. C$_{16}$H$_{19}$N$_2$O$_{11}$P$_2$S requires 509.0185; HPLC (System A) 16.8 min (98%), (System C) 7.9 min (98%).

(2R,3R,4S,5R)-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$_2$O (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%)

(2R,3R,4S,5R)-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%).

(2R,3R,4S,5R)-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 8h 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%).

(2R,3R,4S,5R)-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^{+})[-]. $C_{13}H_{19}N_2O_{13}P2S$ requires 505.0083; HPLC (System A) 18.2 min (99%), (System B) 9.2 min (99%).

(2R,3R,4S,5R)-1-(3,4-Dihydroxy-5-(monophosphoryloxymethyl)tetrahydrofuran-2-yl)-4-(hexylthio)pyrimidin-2(1H)-one, Ammonium salt (50)

To a solution of 4-thiouridine 5′-monosphate (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, 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$_2$O) 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$_2$O) δ 0.60; HRMS *m/z* found 423.0995 (M – H$^+$)$^-$. C$_{15}$H$_{24}$N$_2$O$_8$PS requires 423.0991; HPLC (System A) 15.1 min (99%), (System C) 13.7 min (99%).

(2R,3R,4S,5R)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-4-(hexylthio)pyrimidin-2(1H)-one, Ammonium 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 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 0.35 M of 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 than in procedure A to afford **30** (3.4 mg, 21% from **47**) as a white solid. ^{1}H NMR (D$_2$O) 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$_2$O) δ -9.67, -10.83; HRMS *m/z* found 503.0606 (M – H$^+$)$^-$. C$_{15}$H$_{25}$N$_2$O$_{11}$P$_2$S requires 503.0654; HPLC (System A) 18.9 min (99%), (System C) 13.8 min (99%).

(2R,3R,4S,5R)-1-(3,4-Dihydroxy-5-(diphosphoryloxymethyl)tetrahydrofuran-2-yl)-5-iodopyrimidine-2,4(1H,3H)-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 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 0.35 M of 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, and stirred for 30 min. After removal of the solvent, the residue was purified using the same method than in procedure A to afford **32** (1.8 mg, 19%) as a white solid. ^{1}H NMR (D$_2$O) 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$_2$O) δ -6.89, -10.60; HRMS *m/z* found 528.8901 (M – H$^+$)$^-$. C$_9$H$_{12}$N$_2$O$_{12}$P$_2$I requires 528.8910; HPLC (System A) 14.7 min (99%), (System B) 8.8 min (99%).

(2R,3S,4R,5R)-1-(3-Fluoro-4-hydroxy-5-(monophosphoryloxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-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 phosphorous oxychloride was added dropwise (0.01mL, 0.12 mmol), and the reaction mixture was stirred for 2 hours 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 than in procedure A to afford **54** (7 mg, 60%) as a white solid. ^{1}H NMR (D$_2$O) 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$_2$O) δ 4.01; HRMS m/z found 325.0240 (M – H$^+$)$^-$. C$_9$H$_{11}$N$_2$O$_8$FP requires 325.0237; HPLC (System A) 10.1 min (99%), (System B) 6.2 min (99%).

(2R,3S,4R,5R)-1-(3-Fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-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 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 0.35 M of bis(tributylammonium) salt of phosphoric acid in DMF (0.23 mL). The reaction mixture was stirred at room temperature for 3 days and then 0.2 M triethylammonium bicarbonate (2 mL) was added, and stirred for 30 min. After removal of the solvent, under reduced pressure at 50 °C, the residue was purified using the same method than in procedure A to afford **33** (1.6 mg, 29%) as a white solid. ^{1}H NMR (D$_2$O) 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$_2$O) δ -7.15, -10.37; HRMS m/z found 386.9783 (M – H$^+$)$^-$. C$_9$H$_{10}$N$_2$O$_{10}$P$_2$F requires 386.9795; HPLC (System A) 13.2 min (99%), (System B) 8.6 min (99%).

(2R,3R,4S,5R)-1-(3,4-Dihydroxy-5-(monophosphoryloxymethyl)tetrahydrofuran-2-yl)-4-(ethylthio)pyrimidin-2(1H)-one, Ammonium salt (51)

To a solution of 4-thiouridine 5′-monosphate (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, 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$_2$O) 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$_2$O) δ 1.48; HRMS m/z found 367.0377 (M – H$^+$)$^-$. C$_{11}$H$_{16}$N$_2$O$_8$PS requires 367.0365; HPLC (System A) 8.5 min (98%), (System B) 6.7 min (98%).

P^1,P^2-Bis-5-[(2R,3R,4S,5R)-1-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-4-(ethylthio)pyrimidin-2(1H)-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 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 0.35 M of 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 than in procedure A to afford **35** (5 mg, 52% from **47**) as a white solid. ^{1}H NMR (D$_2$O) 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$_2$O) δ -10.23; HRMS

m/z found 717.0711 (M − H$^+$)$^-$. C$_{22}$H$_{31}$N$_4$O$_{15}$P$_2$S$_2$ requires 717.0703; HPLC (System A) 14.9 min (99%), (System C) 6.8 min (99%).

P^1,P^2-Bis-5-[(2R,3R,4S,5R)-4-(benzylthio)-1-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1H)-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$_2$O) 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$_2$O) δ -10.81; HRMS *m/z* found 841.0991 (M − H$^+$)$^-$. C$_{32}$H$_{35}$N$_4$O$_{15}$P$_2$S$_2$ 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 h 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 subsequent to the addition of [^{3}H]inositol. On the day of the assay, cells were challenged with 50 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, 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$_4$OH. [^{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 non-linear 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 mean ± SEM from multiple experiments or in the case of concentration effect curves from a single experiment carried out with triplicate assays that were representative of results from multiple experiments.

Molecular Modeling

Molecular mechanics calculations have been carried out by means of the Discover3 module of InsightII,[40] using the CFF91 forcefield.[41] The receptor model used for the docking experiments was the P2Y$_6$/UDP complex which we previously built[11] and optimized by means of a molecular dynamics (MD) simulation in an explicit fully hydrated lipid bilayer.[35]

Before performing the docking experiments, the ligands were first fully optimized by means of quenched molecular dynamics followed by energy minimization. An NVT (constant-volume/constant-temperature) molecular dynamic simulation was carried out at 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 an RMSD (root mean squared) of 0.00001 kcal/mol/Å 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 at 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 MMFFs 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/mol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson KA, Schwabe U, Williams M. Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol Sci. 1997; 18:79–82. [PubMed: 9133776]

2. North RA, Barnard EA. Nucleotide receptors. Curr Opin Neurobiol. 1997; 7:346–357. [PubMed: 9232809]

3. von Kügelgen I, Wetter A. Molecular pharmacology of P2Y-receptors. Naunyn Schmiedeberg's Arch Pharmacol. 2000; 362:310–323. [PubMed: 11111826]

4. Jacobson KA, Costanzi S, Ohno M, Joshi BV, Besada P, Xu B, Tchilibon S. Molecular recognition at purine and pyrimidine nucleotide (P2) receptors. Curr Top Med Chem. 2004; 4:805–819. [PubMed: 15078212]

5. von Kügelgen I. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. Pharmacology Therapeutics. 2006; 110:415–432. [PubMed: 16257449]

6. Lustig KD, Shiau AK, Brake AJ, Julius D. Expression cloning of an ATP receptor from mouse neurablasoma cells. Proc Natl Acad Sci USA. 1993; 90:5113–5117. [PubMed: 7685114]

7. Communi D, Pirotton S, Parmentier M, Boeynaems JM. Cloning and functional expression of a human uridine nucleotide receptor. J Biol Chem. 1995; 270:30849–30852. [PubMed: 8537336]

8. Nguyen T, Erb L, Weisman GA, Marchese A, Heng HHQ. Cloning, expression, and chromosomal localization of the human uridine nucleotide receptor gene. J Biol Chem. 1995; 270:30845–30848. [PubMed: 8537335]

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. [PubMed: 7592819]

10. Communi D, Suarez Gonzalez N, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM. Identification of a novel human ADP receptor coupled to Gi. J Biol Chem. 2001; 276:41479–41485. [PubMed: 11546776]

11. Costanzi S, Mamedova L, Gao Z-G, Jacobson KA. Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis, and homology modeling. J Med Chem. 2004; 47:5393–5404. [PubMed: 15481977]

12. Shaver SR. P2Y receptors: biological advances and therapeutic opportunities. Curr Opin Drug Disc. 2001; 4:665–670.

13. Yerxa BR, Sabater JR, Davis CW, Stutts MJ, Lang-Furr M, Picher M, Jones AC, Cowlen M, Dougherty R, Boyer J, Abraham WM, Boucher RC. 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. [PubMed: 12183642]

14. Burnstock G. Purinergic P2 receptors as targets for novel analgesics. Pharmacology Therapeutics, Therapeutics. 2006; 110:433–454.

15. Jacobson KA, Jarvis MF, Williams M. Perspective: Purine and pyrimidine (P2) receptors as drug targets. J Med Chem. 2002; 45:4057–4093. [PubMed: 12213051]

16. Nicholas RA, Lazarowski ER, Watt WC, Li Q, Harden TK. 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. [PubMed: 8700127]

17. Kim SG, Soltysiak KA, Gao ZG, Chang TS, Chung E, Jacobson KA. 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. [PubMed: 12623123]

18. Korcok J, Raimundo LN, Du X, Sims SM, Dixon SJ. $P2Y_6$ nucleotide receptors activate NF-κB and increase survival of osteoclasts. J Biol Chem. 2005; 280:16909–16915. [PubMed: 15722352]

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. [PubMed: 16121038]

20. Cox MA, Gomes B, Palmer K, Du K, Wiekowski M, Wilburn B, Petro M, Chou C-C, Desquitado C, Schwarz M, Lunn C, Lundell D, Narula SK, Zavodny PJ, 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. [PubMed: 15796906]

21. Khine AA, Del Sorbo L, Vaschetto R, Voglis S, Tullis E, Slutsky AS, Downey GP, Zhang H. Human neutrophil peptides induce interleukin-8 production via $P2Y_6$ signaling pathway. Blood. 2006; 107:2936–2942. [PubMed: 16322472]

22. Hou M, Harden TK, Kuhn CM, 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. [PubMed: 11788430]

23. Kim HS, Ravi RG, Marquez VE, Maddileti S, Wihlborg AK, Erlinge D, Malmsjö M, Boyer JL, Harden TK, Jacobson KA. 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. [PubMed: 11754592]

24. Robaye B, Boeynaems JM, Communi D. Slow desensitization of the human $P2Y_6$ receptor. Eur J Pharmacol. 1997; 329:231–236. [PubMed: 9226417]

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. [PubMed: 16095310]

26. Fischer B, Boyer JL, Hoyle CHV, Ziganshin AU, Brizzolara AL, Knight GE, Zimmet J, Burnstock G, Harden TK, Jacobson KA. 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. [PubMed: 8254622]

27. Shaver SR, Pendergast W, Siddiqi SM, Yerxa BR, Croom DK, Dougherty RW, James MK, Jones AC, Rideout JL. 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 PF. 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 JL, Harden KT, Gendron FP, Beaudoin AR, 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. [PubMed: 10479295]

31. Brown HA, Lazarowski ER, Boucher RC, Harden TK. 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. [PubMed: 1944236]

32. Nandanan E, Camaioni E, Jang S-Y, Kim YC, Cristalli G, Herdewijn P, Secrist JA III, Tiwari KN, Mohanram A, Harden TK, Boyer JL, Jacobson KA. Structure activity relationships of bisphosphate nucleotide derivatives as P2Y$_1$ receptor antagonists and partial agonists. J Med Chem. 1999; 42:1625–1638. [PubMed: 10229631]

33. Pendergast W, Yerxa BR, Douglass JG 3rd, Shaver SR, Dougherty RW, Redick CC, Sims IF, Rideout RL. Synthesis and P2Y receptor activity of a series of uridine dinucleoside 5'-polyphosphates. Bioorg Med Chem Lett. 2001; 11:157–60. [PubMed: 11206448]

34. Shaver SR, Rideout JL, Pendergast W, Douglass JG, Brown EG, Boyer JL, Patel RI, Redick CC, Jones AC, Picher M, Yerxa BR. Structure–activity relationships of dinucleotides: Potent and selective agonists of P2Y receptors. Purinergic Signalling. 2005; 1:183–191. [PubMed: 18404503]

35. Costanzi S, Joshi BV, Maddileti S, Mamedova L, Gonzalez-Moa MJ, Marquez VE, Harden TK, Jacobson KA. 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. [PubMed: 16366591]

36. Ohno M, Costanzi S, Kim HS, Kempeneers V, Vastmans K, Herdewijn P, Maddileti S, Gao Z-G, Harden TK, Jacobson KA. 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. [PubMed: 15465340]

37. Jacobson KA, Costanzi S, Ivanov AA, Tchilibon S, Besada P, Gao Z-G, Maddileti S, Harden TK. 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. [PubMed: 16359641]

38. Christie SMH, Elmore DT, Kenner GW, Todd AR, Weymouth FJ. Syntheses of P^{1}P^2-diadenosine-5' and P^{1}P^2-diuridine-5' pyrophosphates. J Chem Soc. 1953:2947–2953.

39. Baddiley J, Buchanan JG, Fawcett CP. Syntheses of cytidine diphosphate ribitol. J Chem Soc. 1959:2192–2196.

40. InsightII, version 2000.1, Accelrys (former MSI). San Diego, CA:

41. Maple JR, Hwang MJ, Stockfisch TP, Dinur U, Waldman M, Ewig CS, Hagler AT. 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 WC. 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;

44. Mohamadi FN, Richards GJ, Guida WC, Liskamp R, Lipton M, Caufield C, Chang G, Hendrickson T, Still WC. MacroModel - an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. J Comput Chem. 1990; 11:440–467.

45. Halgren TA. 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 WC, Tempczyk A, Hawley RC, Hendrickson T. A general treatment of solvation for molecular mechanics. J Am Chem Soc. 1990; 112:6127.

47. Kennedy C, Qi AD, Herold CL, Harden TK, Nicholas RA. ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. Mol Pharmacol. 2000; 57:926–931. [PubMed: 10779375]

48. Marquez VE, Siddiqui MA, Ezzitouni A, Russ P, Wang J, Wagner RW, Matteucci MD. 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. [PubMed: 8809162]
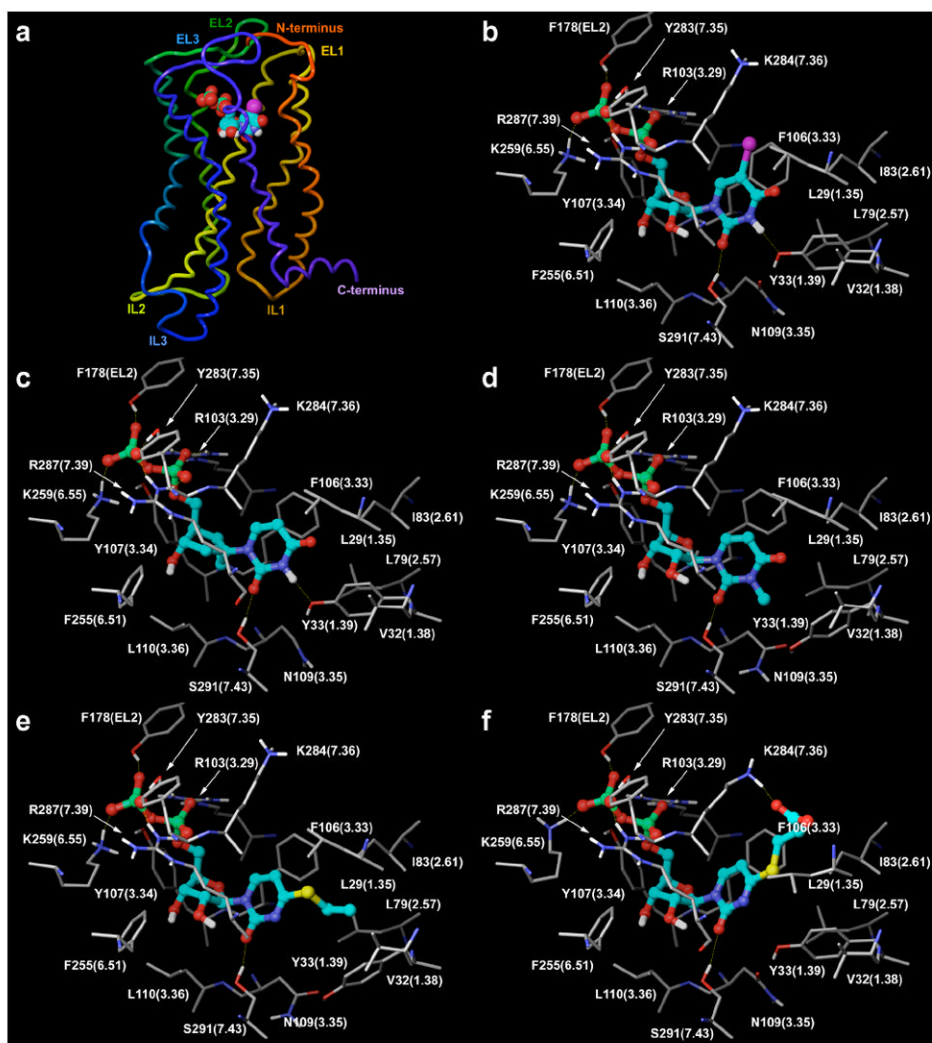
Figure 1.
Molecular models of the complexes of the human P2Y$_6$ receptor with UDP analogues 32, (a and b, 5-iodo), **6** (c, (S)-methanocarba), **31** (d, 3-methyl), **23** (e, 4-ethylthio), and **28** (f, 4-carboxyethylthio). In all cases, the diphosphate moiety of the nucleotide is coordinated by 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 Cpd. **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 (Cterminus): TM1 is in orange, TM2 in ochre, TM3 in yellow, TM4 in green, TM5 in cyan, TM6 in blue, TM7 in purple.
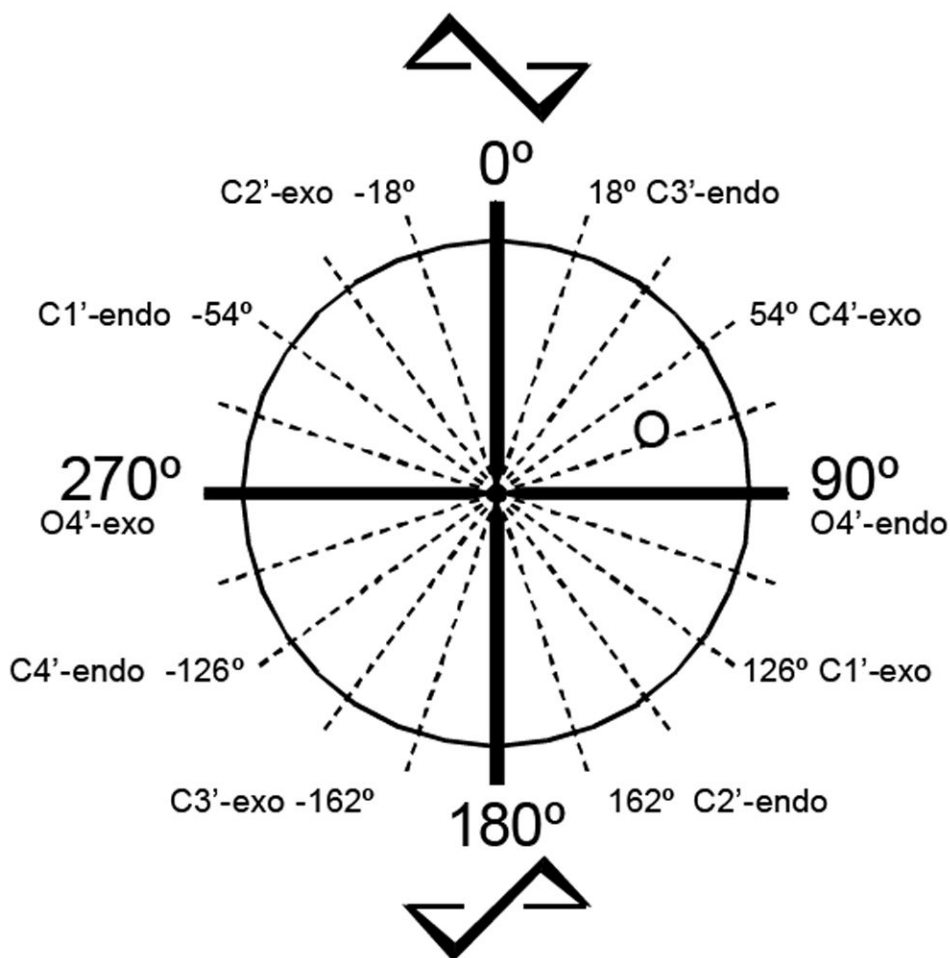
Figure 2.
Graphical representation of the pseudorotational cycle,[48] which defines all of the possible ribose ring conformations. The phase angle of pseudorotation (P) describes the geometry of the ribose puckering. The (N)-methanocarba ring system adopts a C2′-exo conformation and the (S)-methanocarba ring system a C3′-exo conformation. The 2-OBH ring system maintains a C1′-exo conformation, in the (S) region.
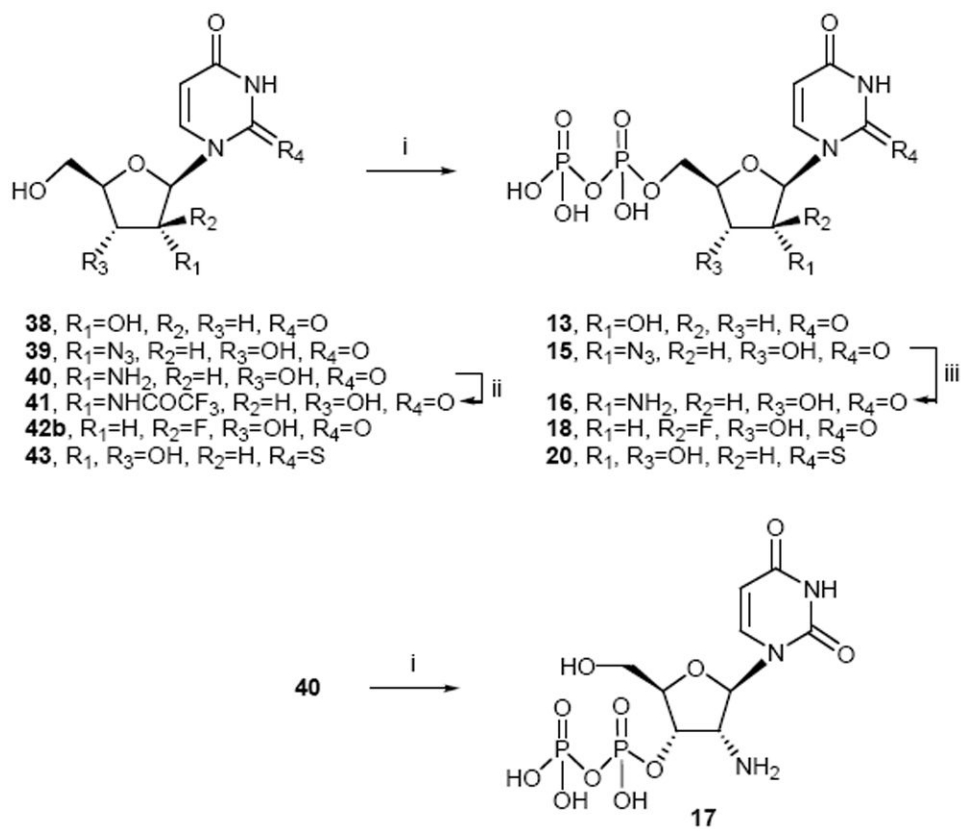
Scheme 1.
Preparation of ribose- and uracil-modified UDP analogues. *Reagents and conditions*: (i) (1) POCl$_3$, proton sponge, PO(OMe)$_3$, 0 °C; (2) (Bu$_3$NH$^+$)$_2$PO$_4$H, Bu$_3$N, DMF, 0 °C; (ii) CF$_3$COOEt, DIEA, DMF, rt. (iii) H$_2$, Pd/C, MeOH, rt.
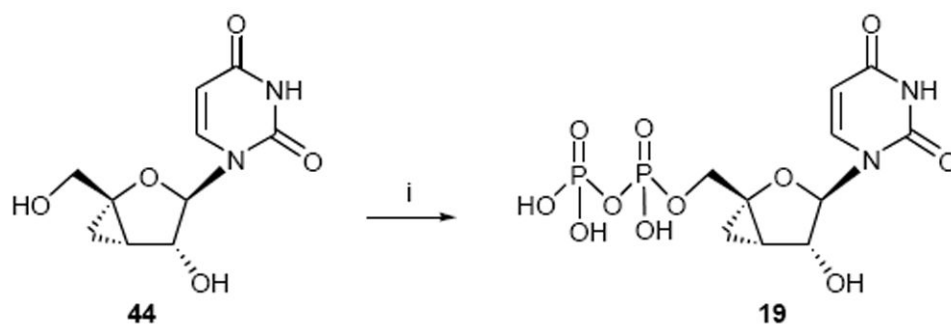
Scheme 2.
Preparation of 2-OBH UDP analogue. *Reagents and conditions*: (i) (1) POCl$_3$, proton sponge, PO(OMe)$_3$, 0 °C; (2) (Bu$_3$NH$^+$)$_2$PO$_4$H, Bu$_3$N, DMF, 0 °C.
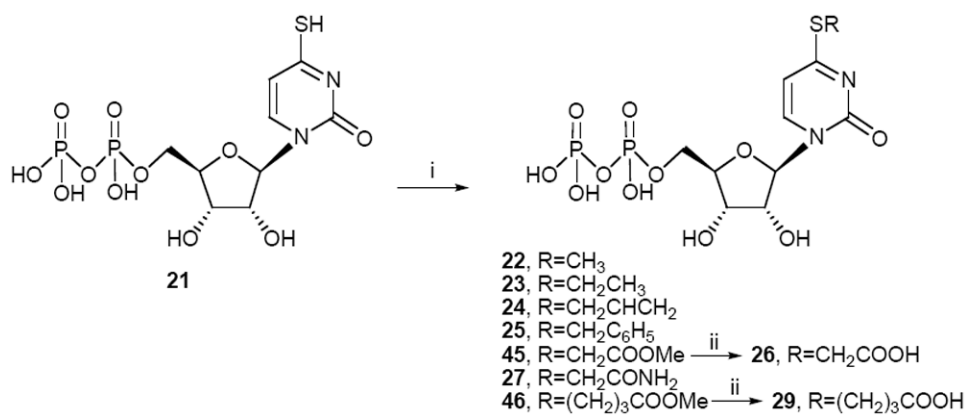
Scheme 3.
Preparation of 4-substituted-thio-UDP analogues. Procedure B. *Reagents and conditions*: (i)
(1) 0.25 M NaOH, MeOH, rt; (2) RX, DMF, rt; (ii) 0.25 M NaOH, H_2O, rt.
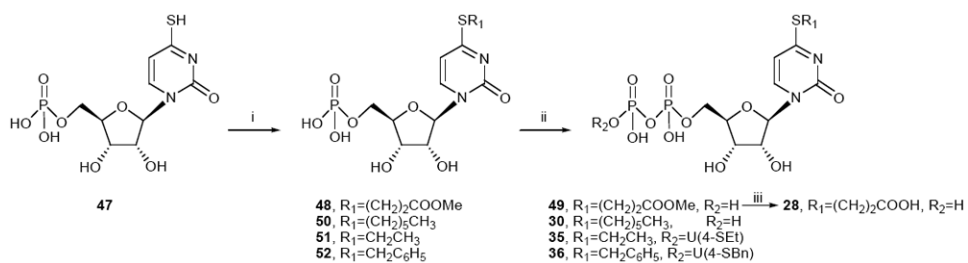
Scheme 4.

Preparation of 4-substituted-thio-UDP analogues. Procedure C. *Reagents and conditions*: (i) (1) 0.25 M NaOH, MeOH, rt; (2) R_1X, DMF, 90 °C; (ii) (1) 1,1'-carbonyldiimidazole, DMF, rt; (2) Et_3N 5% in H_2O/MeOH 1/1, rt; (3) $(Bu_3NH^+)_2PO_4H$, Bu_3N, DMF, rt; (iii) 0.25 M NaOH, H_2O, rt.
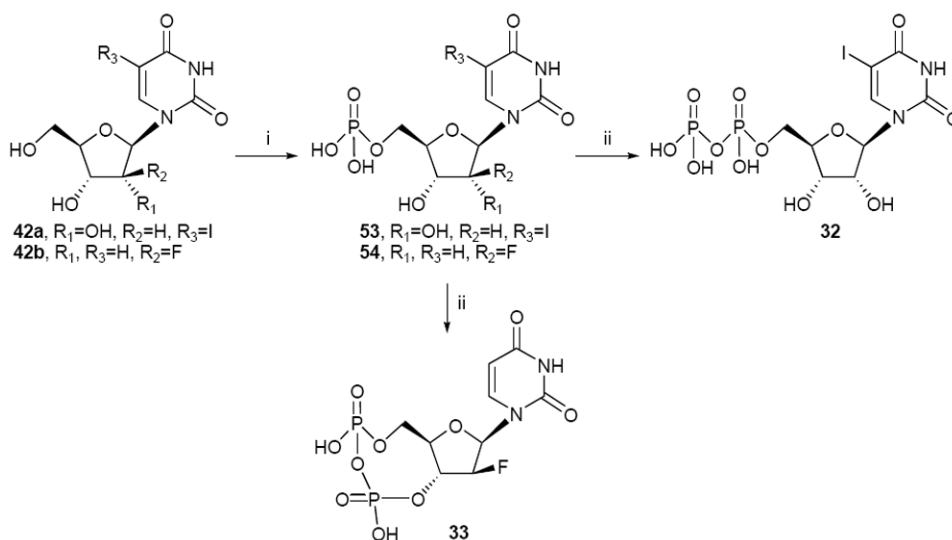
Scheme 5.

Preparation of 5-halo and phosphate- and ribose-modified UDP analogues. *Reagents and conditions*: (i) POCl$_3$, proton sponge, PO(OMe)$_3$, 0 °C; (ii) (1) 1,1'- carbonyldiimidazole, DMF, rt; (2) Et$_3$N 5% in MeOH, rt; (3) (Bu$_3$NH$^+$)$_2$PO$_4$H^{2-}, Bu$_3$N, DMF, rt.

1 R = H, X = OH, **UDP**, 86 nM
2 R = Br, X = OH, **5-Br-UDP**, 130 nM (rat)
3 R = H, X = SH, **UDP-β-S**, 28 nM

4 INS48823

5 (N)-mc-UDP, inactive

6 (S)-mc-dUDP, 230 nM

B =

7 CDP, 88 µM   **8 ADP**, 65 µM   **9 GDP**, 45 µM   **10 IDP**, 34 µM
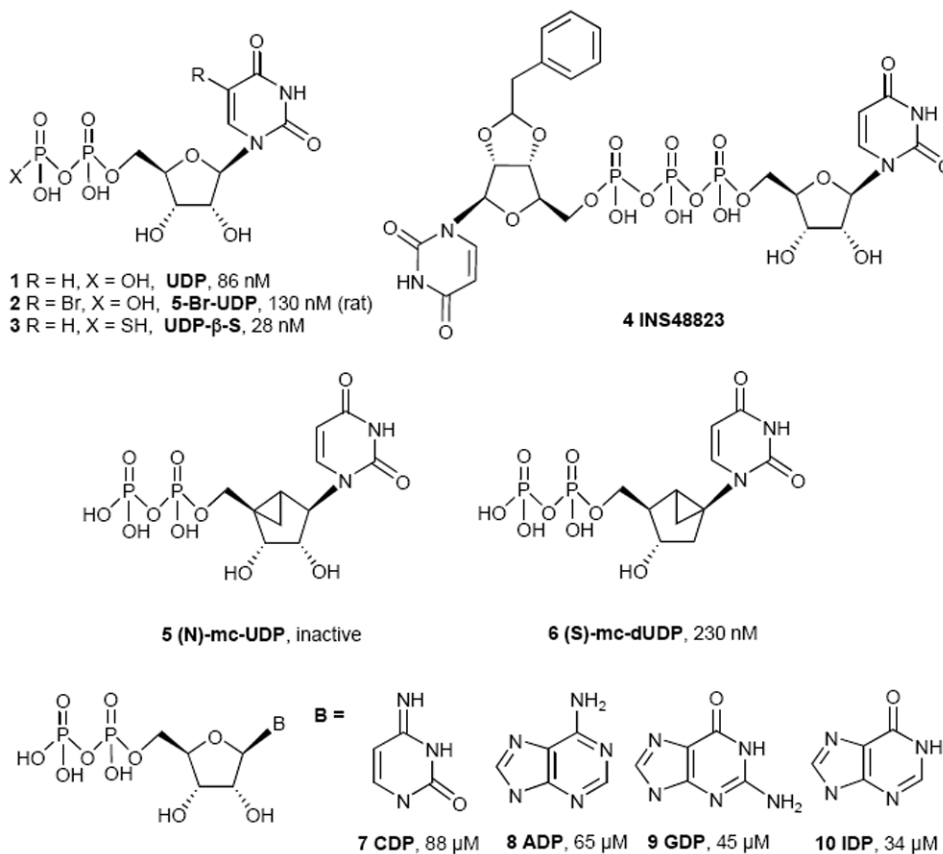
Chart 1.
Structures of UDP and various analogues and the reported EC_{50} values in stimulation of PLC through the recombinant $P2Y_6$ receptor (human, unless noted).[16,22,23,24,35] The EC_{50} for INS48823 is similar to UDP.[18]

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. Unless noted: R^1, R^3, R^5, R^6 = H; R^2, R^4 = O; and R^7, R^8 = OH.

12, 13, 15 - 18, 20, 21, 31, 32, 34, 37   22 - 30, 35, 36
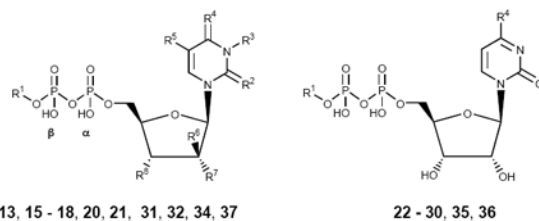
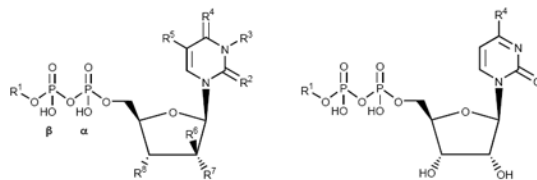| Compound	Modification	Structure	EC$_{50}$ at hP2Y$_6$ receptor, μM^a
1	(= UDP)		0.013 ± 0.004
11	(= UMP)		NE
Ribose-modified			
12	2′-deoxy	R^7 = H	1.72 ± 0.76
13	3′-deoxy	R^8 = H	2.5 ± 0.8
14	(= 2′-deoxyuridine-bisphosphate)	c	NE
15	2′-deoxy-2′-azido	R^7 = N$_3$	1.5 ± 0.4
16	2′-deoxy-2′-amino	R^7 = NH$_2$	3.9 ± 0.9
17	2′-deoxy-2′-amino 3′-diphosphate	c	NE
18	2′-fluoro-2′-deoxyara	R^7 = H, R^6 = F	5.5 ± 0.5
5	(N)-methanocarba	d	NE
6	(S)-methanocarba-2′-deoxy	d	0.23 ± 0.05
19	3′,4′-cyclopropyl^b	c	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$_2$CH$_3$	0.28 ± 0.02
24	4-allylthio	R^4 = SCH$_2$-CH=CH$_2$	0.56 ± 0.09
25	4-benzylthio	R^4 = SCH$_2$C$_6$H$_5$	0.80 ± 0.05
26	4-carboxymethylthio	R^4 = SCH$_2$CO$_2$H	1.7 ± 0.3
27	4-carboxamido-methylthio	R^4 = SCH$_2$CONH$_2$	18 ± 9
28	4-carboxyethylthio	R^4 = S(CH$_2$)$_2$COOH	1.1 ± 0.2
29	4-carboxypropylthio	R^4 = S(CH$_2$)$_3$COOH	8.8 ± 2.0
30	4-hexylthio	R^4 = S(CH$_2$)$_5$CH$_3$	4.9 ± 0.2
31	3-methyl	R^3 = CH$_3$	3.3 ± 1.0
32	5-iodo	R^5 = I	0.015 ± 0.002
Phosphate-and ribose modified			

12, 13, 15 - 18, 20, 21, 31, 32, 34, 37   22 - 30, 35, 36

Compound	Modification	Structure	EC$_{50}$ at hP2Y$_6$ receptor, μM[a]
33	Cyclic 3′,5′-diphosphate and 2′-ara-F	c	NE
Dinucleotides			
34	Up$_2$U	R^1 = uridine-5′-	2.3 ± 0.9
35	U(4-ethylthio) p$_2$U(4-ethylthio)	R^1 = 4-SCH$_2$CH$_3$-uridine-5′-, R^4 = SCH$_2$CH$_3$	<50% at 10 μM
36	U(4-benzylthio) p$_2$U(4-benzylthio)	R^1 = 4-SCH$_2$C$_6$H$_5$-uridine-5′-, R^4 = SCH$_2$C$_6$H$_5$	<50% at 10 μM
37	Cp$_2$C	R^1 = cytidine-5′-, R^4 = NH	NE

[a]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.

[b]oxabicyclohexane ring system (2-OBH)[25].

NE - no effect at 10 μM

[c]

[d]see Chart 1.

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. 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 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.

Compound Number of 5´-Diphosphate	Modification (as di- or triphosphate)	Structure[d]	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	d	1	1	1
7	CDP, CTP	d	1000[a]	110	NA
8	ADP, ATP	d	>7000	1.7	NA
9	GDP	d	500[a]	54	90
10	IDP	d	400[a]	13[b]	9.1[b]
Ribose modifications					
5	(N)-d methanocarba	d	>7000	2.0[c]	1.7[c]
12	2´-deoxy	R^7 = H	130	22	26
13	3´-deoxy	R^8 = H	190	17[e]	42[e]
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´-deoxy-arabino-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	5-iodo	R^5 = I	1.1	17	55

[a]Robaye et al, 1997.[24]

[b]Kennedy et al., 2000.[47]

[c]Kim et al., 2002.[23]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

d
see Table 1 and Chart 1.

*e*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]

NA - no activation at 10 μM.