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Synthesis of uracil nucleotide analogs with a modified, acyclic ribose moiety as $P2Y_2$ receptor antagonists

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ABSTRACT

A series of new uracil nucleotide analogs (monophosphates, triphosphates, and phosphonates) was synthesized, in which the ribose moiety was replaced by acyclic chains, including branched or linear alkyl or dialkylether linkers. 1-ω-Bromoalkyluracil derivatives (**2**) were converted to the corresponding alcohols by treatment with sodium hydroxide and subsequently phosphorylated using phosphorus oxychloride followed by hydrolysis to yield the monophosphates, or by coupling with diphosphate to form the triphosphates. Reaction of **2** with triethyl phosphite followed by deprotection with trimethylsilyl bromide led to the ω-phosphonylalkyluracil derivatives. These products could be further phosphorylated by converting them into their imidazolides and subsequent treatment with diphosphate yielding the corresponding UTP analogs. Nucleoside analogs with an oxygen atom in the 2′-position, which are more similar to the natural ribosides, were synthesized from silylated uracil and trimethylsilyl iodide-treated 1,3-dioxolane, or 1,3-dioxane, respectively, and subsequently phosphorylated by standard procedures. The nucleotide analogs were investigated in a functional assay at NG108-15 cells, a neuroblastoma × glioma hybrid cell line which expresses the UTP- and ATP-activated nucleotide receptor subtype $P2Y_2$. The acyclic nucleotide analogs were generally weaker ligands than UTP, and—in contrast to UTP—they were antagonistic. The most potent compound was diphosphoric 5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)pentylphosphonic anhydride (**5c**) with an IC_{50} value of 92 μM showing that the replacement of the α-phosphate by phosphonate, which leads to enhanced stability, was well tolerated.

1. Introduction

The pyrimidine nucleotides UTP and UDP have been recognized as important signalling molecules activating G protein-coupled membrane receptors (GPCRs) of the P2 (nucleotide) receptor family.[1–3] P2Y receptors are currently subdivided into eight members, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$.[3] While the human $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ receptors are activated exclusively by adenine nucleotides, the human P2Y subtypes $P2Y_{4,6,14}$ are only activated by uracil nucleotides. The human $P2Y_2$ receptor responds to ATP and UTP.[2,3] Species differences exist, for example, in contrast to the human $P2Y_4$ receptor (a UTP receptor), the rat orthologue is potently activated by UTP and ATP.[2–4] P2Y receptors have been found to be implicated in a variety of physiological functions and pathophysiological conditions. Therefore P2Y receptors are of interest as drug targets. Irreversible $P2Y_{12}$ antagonists are already in use as antithrombotic drugs, for example, clopidogrel (**I**).[5] The ATP analogue AR-C69931 MX (cangrelor, **II**) was studied in clinical trials as a competitive $P2Y_{12}$ antagonist for antithrombotic therapy;[6] further $P2Y_{12}$ antagonists are currently evaluated.[7] Another P2Y receptor subtype which is of considerable interest as a new drug target, is the $P2Y_2$ receptor subtype.[3,8–11] $P2Y_2$ agonists are being developed for the treatment of cystic fibrosis (INS37217, denufosol, **III**) and dry eye syndrome (INS 365, diquafosol, **IV**), see Figure 1.[12–14] However, **III** and **IV** are moderately potent and weakly selective $P2Y_2$ agonists. $P2Y_2$ receptor antagonists possess therapeutic potential for the treatment of inflammatory conditions, pain, coronary vasospastic disorders, and neurodegeneration.[2,3] However, potent and selective $P2Y_2$ antagonists are currently not available.[3]

In previous studies, we modified the uracil base and the phosphate residue of UDP and UTP.[15,16] The resulting compounds were agonists at $P2Y_2$ receptors. In the present study we modified the ribose part of UTP with the goal to develop novel $P2Y_2$ receptor ligands. We selected UTP over ATP, as a lead structure due to the expected higher selectivity of pyrimidine nucleotides versus the other P2 receptor subtypes that are activated by the adenine nucleotides ATP or ADP. Furthermore, the degradation products of ATP analogs (the nucleoside adenosine and the nucleobase adenine) may interact with adenosine (P1) or adenine (P0) receptors and thus lead to unwanted side-effects.[3] Our goal was to investigate the consequences of replacing the ribose moiety in uracil nucleotides by various acyclic ribose-mimetic structures.

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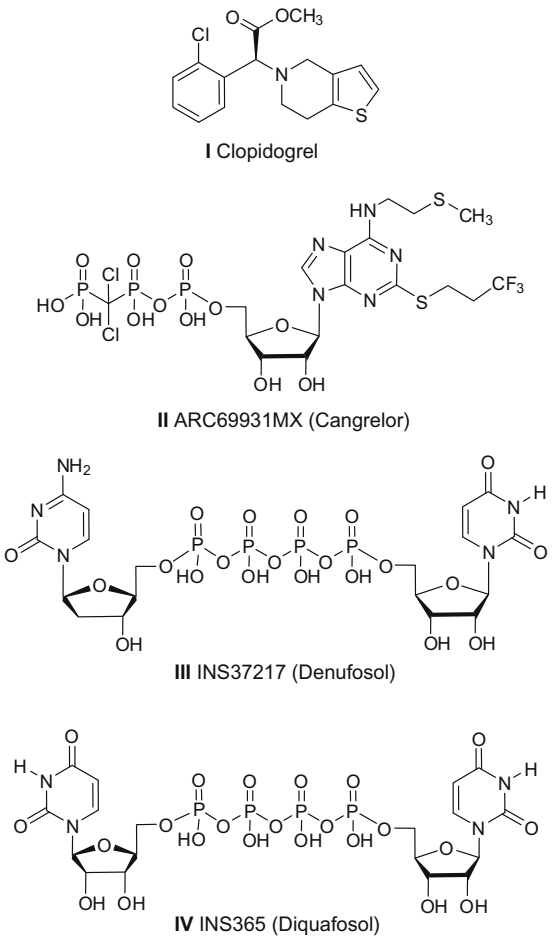


Figure 1. Selection of biologically active P2Y receptor ligands.

Several acyclic analogs of adenine nucleotides have been described and evaluated;[17,18] some have been reported to be moderately potent $P2Y_1$ receptor antagonists,[19,20] while a series of acyclic adenine nucleotide analogs was found to inhibit platelet aggregation by blocking $P2Y_{12}$ receptors.[21] Furthermore, acyclic nucleotide analogs have been investigated as antiviral, cytostatic, antiparasitic and immunomodulatory agents.[22,23] Three compounds bearing a phosphonate residue, namely cidofovir, adefovir, and tenofovir, are therapeutically used as antiviral drugs for the treatment of hepatitis B, AIDS, and various diseases caused by DNA viruses.[23] A large number of acyclic nucleoside analogs has been developed, especially adenosine and guanosine derivatives, such as the antiviral drug acyclovir (9-(2-hydroxyethoxymethyl)guanine).[24] Because of their antiviral effects the interest in such compounds has been high.[25,26] Due to the recent discovery of the important roles of small interfering ribonucleic acids (siRNAs), a novel area of drug development focuses on modified small RNAs.[27] In this respect, the newly synthesized acyclic nucleotide analogs may be useful for the preparation of artificial, stabilized siRNAs and RNA chain terminators.

2. Results and discussion

2.1. Chemistry

A series of uridine analogs in which the ribose moiety was replaced by a 'spacer' was synthesized and converted to analogs of uracil nucleotides. Our goal was to investigate the binding of such acyclic nucleotides to $P2Y_2$ receptors and investigate the role of the sugar moiety on the interaction of UTP with $P2Y_2$ receptors. As a replacement of the ribose moiety, short aliphatic chains with a terminal hydroxyl group or acyclic ribose fragments were considered. To stabilize the compounds, the α-phosphate was replaced by phosphonate in some derivatives.

1-Phosphonylalkyluracil derivatives, **4b–d**, were prepared as metabolically stable nucleotide analogs according to the procedure described in Scheme 1. The synthesis of 1-bromoalkyluracil and 1-bromoalkylthymine derivatives had previously been described.[28–31] Modification of the reported methods led to compounds **2a–d** via silylation of uracil **1** by excess hexamethyldisilazane under reflux condition. Due to steric effects the N1-position of the uracil ring is more reactive than the N3-position. As shown in Scheme 1, the N1-substituted 1-bromoalkyluracil derivatives **2a–d** were obtained after reaction times of a few days in acceptable yields. As described in the literature,[32] by-products, such as dimers, can be formed during the reaction. Therefore chromatographic purification was required to obtain the pure products. The 1-bromoalkyluracil derivatives **2b–d** were refluxed with triethyl phosphite yielding products **3b–d** by Arbuzov reaction. In order to hydrolyze the ethyl ester functions, compounds **3b–d** were dissolved in acetonitrile and reacted with trimethylsilyl bromide under mild conditions to afford **4b–d** in good yields. Compounds **4b–d** could be converted to their sodium salts via ion exchange resin Dowex WX8 (Na^+ form).

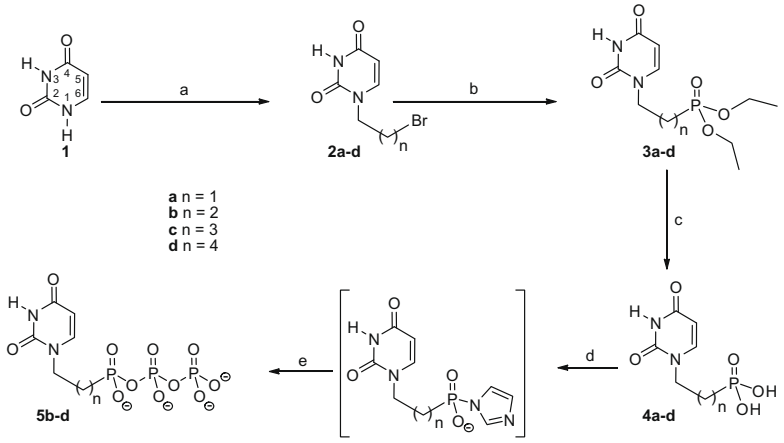
The phosphorylation of **4b–d** to obtain UTP analogs **5b–d** was achieved according to the method described by Moffat[33] for the preparation of nucleoside triphosphates. The phosphonic acids **4b–d** were activated with 1,1′-carbonyldiimidazole under argon in the presence of tributylamine as a base to yield phosphonic acid imidazolides which were not isolated but immediately reacted with 5 equiv of tributylammonium diphosphate in DMF to give the corresponding diphosphoryl phosphonates **5b–d** (Scheme 1).

A number of by-products were formed during these reaction steps. In Scheme 2 the main side products observed during the formation of **5d** are shown as an example. When the reaction time was extended to more than 30 min, dimerization occurred and the corresponding tetraphosphate derivative was formed. This dimerization product was analyzed by ^{31}P NMR spectroscopy and identified by comparison with literature data for similar compounds.[34] The same dimerization reaction was also observed during the synthesis of **5b** and **5c**. After anion exchange chromatography of the reaction mixture, the desired triphosphate analog **5d** (see Scheme 2) was isolated as the main product. In addition, the diphosphate analog **6d** was formed after hydrolysis of the dimeric tetraphosphate during the purification process. Both products, **5d** and **6d** were obtained at a ratio of 4:1 (Scheme 2).

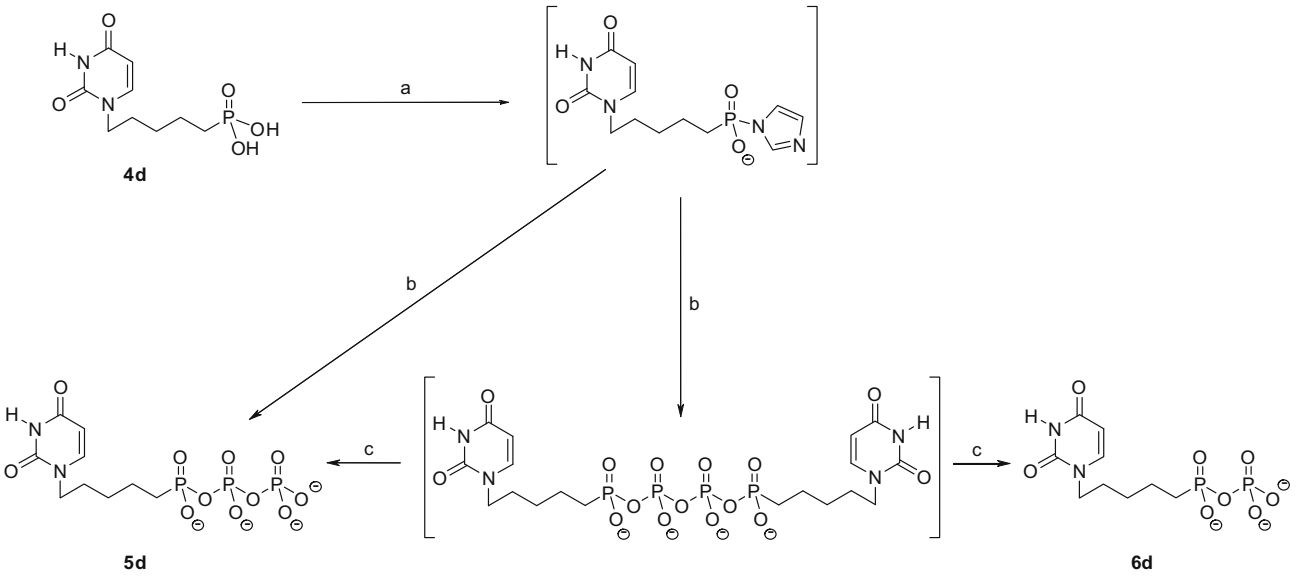
For the synthesis of uracil-1-alkyl mono- and triphosphates 1-(ω-bromoalkyl)uracils **2a**,**c–d** were reacted with 1 N aqueous sodium hydroxide solution to obtain the corresponding 1-(ω-hydroxyalkyl)uracils **7a**,**c–d** as previously described (see Scheme 3).[35] If a higher concentration of sodium hydroxide or higher temperatures than 50 °C were applied, the alkyl residues were split off and the formation of uracil was observed as a side reaction.

Furthermore, we introduced oxygen-containing dialkyl ether linkers as more ribose-like replacements of the sugar moiety. Silylation of uracil was performed by treatment with bis(trimethylsilyl)acetamide in acetonitrile followed by reaction with the alkylating reagent formed in situ by treatment of 1,3-dioxolane, or 1,3-dioxane, respectively, with trimethylsilyl iodide. 1-(2-Hydroxyethoxymethyl)uracil (**8a)** and 1-(3-hydroxypropoxymethyl)uracil (**8b)** were obtained in 25% and 35% yields, respectively (Scheme 3).

The synthesized nucleoside analogs **7a**,**c–d** and **8a**,**b** were susceptible to phosphorylation yielding the corresponding



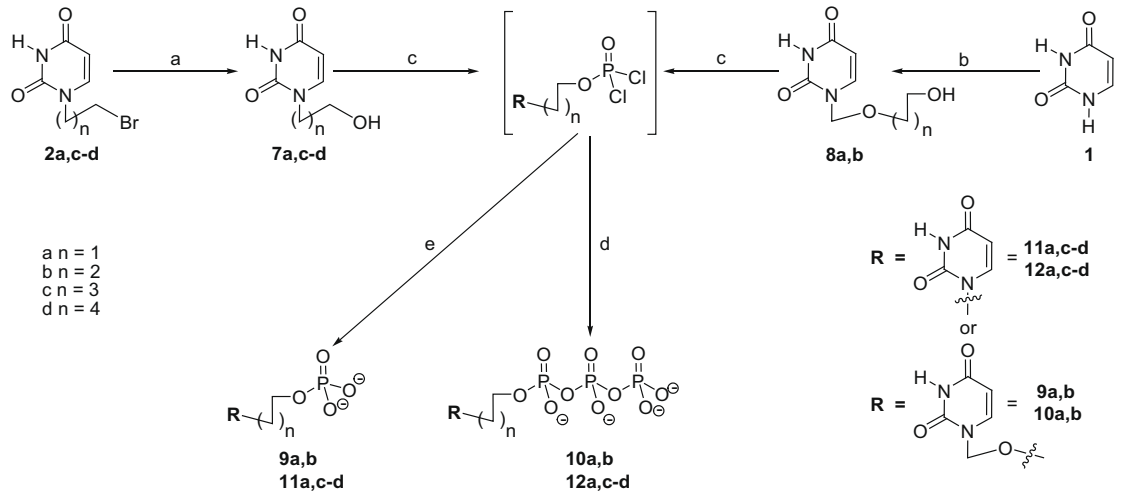
Scheme 1. Synthesis of 1-phosphonylalkyluracil and 1-(ω-phosphonylalkyl)uracil diphosphate derivatives **5b–d**. Reagents and conditions: (a) (1) HMDS, Me_3SiCl, 12 h, 110 °C; (2) $Br(CH_2)nBr$, I_2, 4–7 d, 60 °C, 21–46%; (b) $P(OEt)_3$, 3 h, 150 °C; (c) Me_3SiBr, acetonitrile, 12 h, rt, 82–85%; (d) Bu_3N, 1,1′-carbonyldiimidazole, 12 h, rt; (e) 5 equiv $(Bu_3NH^+)_4P_2O_7$, Bu_3N, DMF, 30 min, rt.



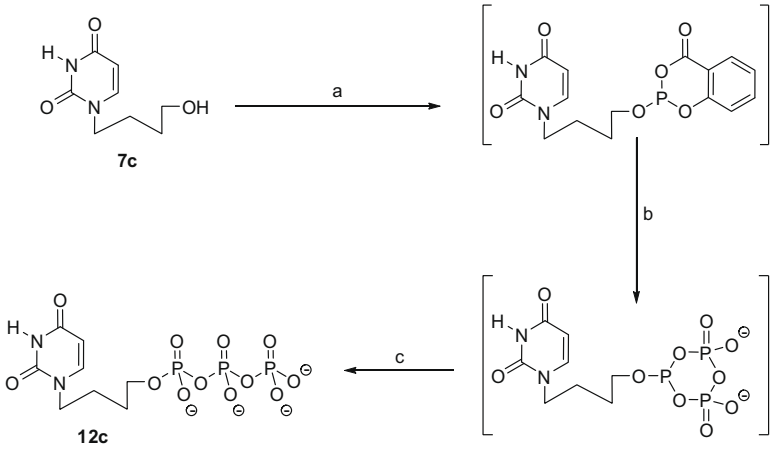
Scheme 2. Observed side reactions during the synthesis of 1-(ω-phosphonylalkyl)uracil diphosphates (example **6d**). Reagents and conditions: (a) Bu_3N, DMF, 1,1′-carbonyldiimidazole, 12 h, rt; (b) 1.5 equiv $(Bu_3NH^+)_4P_2O_7$, Bu_3N, DMF, 2 h, rt; (c) hydrolysis, DEAE-A25 Sephadex chromatography.

triphosphates. The acyclic nucleoside analogs **8a**,**b** were dissolved in dry trimethylphosphate under an argon atmosphere, and 'proton sponge' (1,8-bis(dimethylamino)naphthaline) was added in order to prevent the lowering of the pH value of the reaction mixture as described by Ludwig.[36] After stirring the solution at 0–4 °C for some minutes, phosphorus oxychloride was added. After several hours of stirring the 'activated' monophosphate was formed, which was either hydrolyzed with triethylammonium hydrogencarbonate (pH 7.5) to yield the monophosphates **9a**,**b**, or further reacted with the tributylammonium salt of diphosphate (dissolved in DMF) to afford, after hydrolysis, the triphosphates **10a**,**b** (Scheme 3). The same synthetic procedure was also used for the phosphorylation of 1-(ω-hydroxyalkyl)uracil derivatives **7a**,**c–d** (Scheme 3). The monophosphates **11a**,**c–d** were obtained due to the hydrolysis of the phosphorodichloridate intermediate during the reaction and isolated as side products during the synthesis of the triphosphates **12a**,**c–d**. The yield ratio between the triphosphates **12a**,**c–d** and the corresponding monophosphates **11a**,**c–d** was about 4:1. After chromatographic purification, the total yield of all described triphosphates synthesized by the Ludwig method ranged between 30% and 40%.

As an alternative to the phosphorylation using $POCl_3$, the Ludwig–Eckstein phosphorylation method[37] was applied to phosphorylate compound **7c**. This was done in order to investigate whether higher yields could be achieved with that procedure, which was, however, not the case. As shown in Scheme 4, the first reaction step was the formation of phosphate using 2-chloro-4*H*-1,3,2-benzodioxaphosphorine-4-one as a phosphitylating agent. The resulting nucleoside phosphite was immediately coupled with tributylammonium diphosphate in DMF in the presence of tributylamine leading to the displacement of the salicylate resulting in the formation of a cyclic intermediate (Scheme 4). This reaction was monitored by ^{31}P NMR spectroscopy. The signal for the trivalent α-phosphorus atom in the cyclic intermediate showed a typical shift of +104 ppm and appeared as a triplet with a coupling constant of 43 Hz as a result of the coupling with the two phosphate groups in the cyclic intermediate (Scheme 4). As it is an achiral molecule, the two cyclic phosphate groups showed a single signal and appeared as a doublet (J = 43 Hz) with a characteristic shift of −20 ppm. The addition of iodine in aqueous solution led to oxidation of the α-phosphorus atom and ring opening to the linear triphosphate **12c**. As in the other phosphorylation methods,



Scheme 3. Synthesis of acyclic nucleoside analogs **7a**,**c**–**d** and **8a**,**b** and acyclic nucleotide analogs **9a**,**b**, **11a**,**c**–**d** (monophosphates) and **10a**,**b**, **12a**,**c**–**d** (triphosphates). Reagents and conditions: (a) 1 N NaOH, 48 h, 50 °C, 45–70%; (b) (1) bis(trimethylsilyl)acetamide, acetonitrile, 3 h, rt; (2) KI, Me_3SiI, 1,3-dioxolane or 1,3-dioxane, 24 h, rt; **8a** (25%), **8b** (35%); (c) $POCl_3$, $OP(OMe)_3$, 1 h, 0–4 °C; (d) (1) $(Bu_3NH^+)_4P_2O_7$, Bu_3N, DMF, 1 h, 0–4 °C; (2) $(Et_3NH)HCO_3$, 25–30%; (e) $(Et_3NH)HCO_3$; **9a**,**b** (50–60%), **11a**,**c**–**d** (10–15%).



Scheme 4. Ludwig–Eckstein phosphorylation procedure for the synthesis of nucleoside triphosphate **12c**. Reagents and conditions: (a) 2-chloro-4*H*-1,3,2-benzodioxaphosphorine-4-one, dioxane/DMF, 0–4 °C; (b) $(Bu_3NH^+)_4P_2O_7$, Bu_3N, DMF; (c) I_2, H_2O, 25%.

this reaction is characterized by formation of numerous, mostly unidentified by-products, so that the total yield after chromatographic purification was even lower in comparison with the Ludwig method.

Since the phosphorylation reactions are usually complicated by the formation of side products, chromatographic purification of all synthesized mono- and triphosphates (and phosphonates) was performed in two steps: anion exchange chromatography was applied in order to separate the formed mono- from the triphosphates; in a second step, preparative HPLC was applied, in order to remove inorganic impurities. The purity of the final products was controlled by ^{31}P NMR spectroscopy, capillary electrophoresis, and HPLC–ESI-MS analysis (see Supplementary data) and found to be >95% in all cases.

3. Biological investigations

3.1. $P2Y_2$-antagonistic effect

The potency of the test compounds to inhibit UTP-induced intracellular calcium release in NG108-15 cells (mouse neuroblastoma × rat glioma hybrid cell line) was determined by a fluorescence method using the calcium-chelating fluorophor Oregon Green® as previously described.[8–10] NG108-15 cells express the mouse $P2Y_2$ receptor, which is coupled to phospholipase C activation and subsequent intracellular calcium release.[38] They also express UDP-activated $P2Y_6$ receptors, but not UTP-sensitive $P2Y_4$ receptors as shown by reverse transcriptase polymerase chain reaction.[39] Therefore the addition of UTP will only activate the $P2Y_2$ receptor subtype in NG108-15 cells.

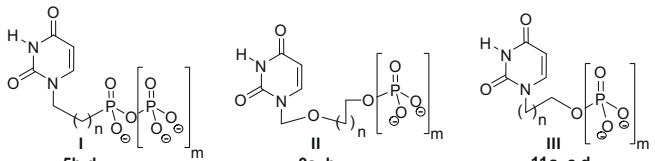
$P2Y_2$ agonists as well as antagonists have been shown to inhibit a subsequent receptor stimulation, agonists by inducing receptor desensitization, and antagonists by a blockade of the receptors.[40] The mouse $P2Y_2$ receptor is very similar to the human subtype due to high sequence homology (89% identity in amino acid sequence).[41] Therefore data from mouse $P2Y_2$ receptors are highly predictive of activity at human $P2Y_2$ receptors.[9] As an agonist, UTP induced a concentration-dependent increase in $[Ca^{2+}]_i$ with an EC_{50} value of 1.26 ± 0.45 μM (n = 10). This relatively high EC_{50} value is probably due to the moderate receptor expression level in the NG108-15 cells. In contrast, UTP usually shows a much lower EC_{50} value in artificial cell lines with high receptor expression

levels.[8,16,18] In antagonist assays, a UTP concentration of 1 μM corresponding to the EC_{50} value was used for stimulation. Initial screening of the nucleotide analogs for inhibition of UTP- (1 μM) induced calcium mobilization was carried out at concentrations of 10, 100, and 500 μM. For selected compounds full concentration–response curves were determined and IC_{50} values were calculated. The results are depicted in Table 1. Typical concentration–inhibition curves are shown in Figure 2.

UTP preincubation led to a dose-dependent inhibition of UTP-stimulated intracellular calcium release due to receptor desensitization. UTP showed an IC_{50} value of 1.51 ± 0.60 μM (n = 4) as an inhibitor of UTP- (1 μM) induced calcium mobilization (Table 1), a value that was very close to the EC_{50} value determined for its $P2Y_2$-agonistic effect (1.26 μM).

All acyclic nucleotide analogs were less potent than UTP. Structure II (Table 1) is most similar to the natural nucleotides. In this set of compounds a chain length of n = 2 (chain of 6 atoms to P_α) was superior to n = 1 (5 atom chain), and monophosphates were similarly potent as triphosphates (compare **9b**/**10b**). Activity of nucleoside monophosphates had previously also been found for 2-substituted adenosine 5′-monophosphate derivatives at the $P2Y_1$ and the $P2Y_{12}$ receptor subtypes.[11,42] The best compound in this series was **10b**, which was 175-fold less potent than UTP (IC_{50} = 264 μM) in inhibiting or preventing $P2Y_2$ stimulation by UTP. In series III, where the oxygen atom corresponding to position 2′ of ribose was lacking, a similar trend could be observed. The optimal chain length of six carbon atoms (n = 4) was best tolerated by the receptor in combination with a monophosphate substitution (**11d**). This compound had a similar potency as the corresponding oxygen-containing analog **9b**. The best compounds were found among the phosphonates (structure I). In this series a shorter chain length was favorable (4 carbon atoms, n = 3, **5c**), while 5 atoms (**5d**) were also well tolerated. Three phosph(on)ate groups (UTP analogs) were superior to only two (UDP analog; compare **5d**/**6d**). The most potent compound of the present study was **5c**, diphosphoric 5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)pentylphosphonic anhydride, with an IC_{50} value of 92 μM. The compound was 61-fold less potent than UTP (preincubation) in preventing UTP-induced $P2Y_2$ activation.

Table 1
Inhibitory potency of nucleotide analogs at $P2Y_2$ receptors expressed in NG108-15 cells (UTP = 1 μM)



5b-d
6d

9a, b
10a, b

11a, c-d
12a, c-d

Compd	Structure	n	m	IC_{50}[a] (μM) (% inhibition ± SEM)
UTP	—	—	—	1.51 ± 0.60
5b	I	2	2	≈500 (52 ± 1%)
5c	I	3	2	92 ± 1
5d	I	4	2	166 ± 6
6d	I	4	1	≈500 (55 ± 1%)
9a	II	1	1	>500 (16 ± 1%)
9b	II	2	1	338 ± 2
10a	II	1	3	>500 (16 ± 3%)
10b	II	2	3	264 ± 4
11a	III	1	1	>500 (22 ± 7%)
11c	III	3	1	⩽500 (60 ± 3%)
11d	III	4	1	267[b]
12a	III	1	3	⩽500 (60 ± 7%)
12c	III	3	3	596 ± 0[c]
12d	III	4	3	≈500 (50 ± 1%)

[a] n = 2–5, each in quadruplicate.
[b] n = 1.
[c] UTP = 3 μM.

3.2. $P2Y_2$-agonistic effect

The next step was to investigate whether the new compounds were agonists or antagonists at $P2Y_2$ receptors. All compounds were investigated in concentrations of up to 100 μM. Cells were treated with UTP (agonist) or test compound and changes in intracellular calcium concentrations were recorded. While UTP showed a concentration-dependent increase in $[Ca^{2+}]_i$ with an EC_{50} value of 1.26 ± 0.45 μM, the acyclic nucleotide analogs—with the exception of **9b**—did not show any effect indicating that they were antagonists rather than agonists at $P2Y_2$ receptors (data not shown). Compound **9b** showed an increase in fluorescence at 100 μM (ca. 40% of the maximal effect observed with UTP, data not shown), however the increase in fluorescence was untypically slow and may have been caused by a $P2Y_2$ receptor-independent effect.

4. Conclusions

In conclusion, we have synthesized a series of uracil nucleotide analogs, in which the ribose moiety was replaced by acyclic structures. In some of the compounds, the α-phosphate was replaced by a phosphonate to enhance chemical and metabolic stability. Most of the compounds were new derivatives not previously described in the literature. Evaluation at $P2Y_2$ receptors showed that—in contrast to the parent UTP—the acyclic nucleotide analogs were antagonists. The α-phosphonate modification was well tolerated. Despite their moderate potency they might serve as a starting point for further optimization. Furthermore, some of the new compounds may be of interest as building blocks for the preparation of modified RNAs.

5. Experimental

5.1. General information

The ^1H and ^{13}C NMR spectra were measured on a Bruker AC-200, or an AMX-300 spectrometer. Coupled ^1H–^{31}P NMR spectra were measured on a Bruker Avance 500 spectrometer. Tetramethylsilane was used as an internal standard for the spectra measured in $CDCl_3$. ^{31}P NMR spectra were determined on a Bruker AMX300 or an AMX-400 spectrometer. Phosphoric acid (85%) was used as an external standard. The chemical shifts are given in ppm relative to the external standard (^{31}P NMR) or relative to the remaining protons of the deuterated solvents used as internal standard in case of ^1H and ^{13}C NMR spectra (for spectra in DMSO-d_6: δ ^1H: 2.51, δ ^{13}C: 39.7, in D_2O: ^1H 4.65). Melting points were measured on a Büchi 510 melting point apparatus and are uncorrected. CHN elemental analyses were determined at the Institute of Inorganic Chemistry, University of Würzburg, Germany, using a Carlo Erba Elemental Analyzer. The reactions were monitored and the purity of the compounds was checked by thin layer chromatography (TLC) using aluminum sheets with Silica Gel 60 F_{254} (Merck). As solvent systems dichloromethane/methanol (9:1), or for all phosphates and phosphonates, isopropanol/NH_3/H_2O (6:3:1) were used.

A GradiFrac system (Pharmacia-Biotech) with a high-load pump P50 and Sephadex DEAE A25 (Pharmacia) was used for anion exchange chromatography. Preparative HPLC was performed using a Knauer instrument (HPLC pump 64) on a Eurospher column (100, C18: 7 μm, 20 mm) applying the software package Eurochrom 2000. The purity control of the synthesized nucleotides

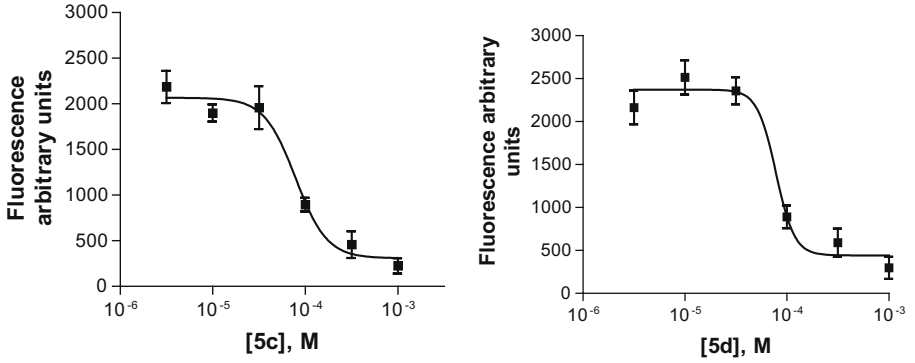


Figure 2. Typical concentration–inhibition curves for compounds **5c** and **5d**. Data points represent mean ± SEM ($n = 4$).

was performed by capillary electrophoresis using a Beckmann P/ACE system with a 5500 P/ACE diode array detector. The purity of the nucleotides was examined in a second chromatography system by dissolving 1 mg/ml of compound in H_2O/MeOH = 1:1, containing 2 mM ammonium acetate. A sample of 10 µl was injected into an HPLC instrument (Agilent 1100) using a Phenomenex Luna 3µ C18 column. Elution was performed with a gradient of water: methanol (containing 2 mM ammonium acetate) from 90:10 to 0:100 for 30 min at a flow rate of 250 µl/min, starting the gradient after 10 min. UV absorption was detected from 190 to 400 nm using a diode array detector. The demineralized water used for chromatography was obtained from a Purelab-plus instrument (<0.055 µS/cm).

The synthesis of compounds **2a–d**,[28–30] **7a**,**c–d**[35] and **8a**[43] has previously been described. Compounds **9a**,[44] **10a**,[45,46] **11a**,**d**,[47] **11c**[48] and **12c**[47] have also been reported in the literature but without physicochemical or spectral data.

5.2. General procedure for the preparation of phosphonic acid diethyl ester derivatives 3b–d

Compound **2b**, **c**, or **d** (200 mg) was dissolved in 10 ml triethyl phosphite and the reaction mixture was refluxed for 3 h. After the reaction was completed, the solvent was distilled off and the residue was dried under vacuum. Coevaporation of the sticky residue with absolute ethanol for several times afforded compounds **3b–d** as waxy substances.

5.3. General procedure for the preparation of phosphonic acid derivatives 4b–d

Compound **3b**, **c**, or **d** (150 mg) was dissolved in 10 ml of absolute acetonitrile at rt, a 5–6-fold excess of trimethylsilyl bromide was added and the reaction mixture was stirred overnight. To the resulting solution 10 ml of methanol/water (5:1) was added dropwise followed by stirring for a few minutes. Then the solvent was removed under reduced pressure, and the resulting yellow oily product was dissolved in 5 ml of water and subsequently lyophilized. The product was recrystallized from isopropanol to yield pure compounds as solid colorless materials.

5.4. General procedure for preparation of the diphosphoryl phosphonate derivatives 5b–d

Compound **4b**, **c**, or **d** (0.4 mmol) was dissolved in 15 ml DMF followed by the addition of 0.15 ml tributylamine. Carbonyldiimidazole (300 mg, 2 mmol) was added and the reaction mixture was stirred at rt for 12 h while protecting it from light. Tributylammonium diphosphate solution in DMF (2 ml of 0.5 M, 3 equiv) and 0.5 ml tributylamine were added to the reaction mixture. After 30 min of stirring under ice-cooling 15 ml of 1 M triethylammonium hydrogen carbonate (TEAB, pH 7.5) was added to the reaction mixture. After 1 h the solvent was removed under high vacuum at 40 °C and the residue was further purified by ion exchange chromatography and subsequently by preparative reversed phase HPLC.

5.5. General procedure for the preparation of 1-(2-hydroxyethoxymethyl) uracil (8a) and 1-(3-hydroxypropoxymethyl)uracil (8b)

Uracil (1.12 g, 10 mmol) was suspended in 15 ml of absolute acetonitrile, bis(trimethylsilyl)acetamide (5.4 ml, 22 mmol) was added, and the reaction mixture was stirred at rt for 3 h while protecting it from light until a clear solution was obtained. Then KI (1.7 g, 10 mmol) and trimethylsilyl chloride (1.38 ml, 10 mmol) were added, and after 15 min of stirring, 10 mmol of 1,3-dioxolane, or 1,3-dioxane, respectively, were added. The reaction mixture was stirred for 24 h at rt and then quenched by the addition of 20 ml of methanol and subsequent neutralization with 4 g of sodium bicarbonate. After filtration of the solid residue, the filtrate was evaporated under reduced pressure and the obtained residue was dissolved in 3 ml of methanol, applied to a silica gel-column and eluted with chloroform/methanol (95:5). The products were then recrystallized from isopropanol to yield pure products.

5.6. General procedure for the preparation of 1-(2-hydroxyethoxymethyl)uracil monophosphate (9a) and 1-(3-hydroxypropoxymethyl)uracil monophosphate (9b)

Compound **8a** or **b** (0.5 mmol) was dissolved in 2.5 ml of trimethyl phosphate (dried over molecular sieve). The reaction was cooled to 0–4 °C and to it 0.15 g (0.75 mmol) of 1,8-bis(dimethylamino)naphthaline were added while keeping the reaction mixture under argon. After 15 min of stirring, 0.075 ml (0.8 mmol) $POCl_3$ was added and stirring was continued for 1 h at 0–4 °C. Hydrolysis was achieved by addition of 15 ml of 1 M TEAB (pH 7.5) to the reaction mixture. Trimethyl phosphate was removed by extraction with *t*-butylmethyl ether (three times) and the aqueous phases were combined and dried under high vacuum at 40 °C until dryness. The residue was dissolved in 2 ml of 1 M TEAB and applied to a DEAE-A25 Sephadex column for purification.

5.7. General procedure for the preparation of 1-(2-hydroxyethoxymethyl)uracil triphosphate (10a) and 1-(3-hydroxypropoxymethyl)uracil triphosphate (10b)

Acyclic nucleoside analog **8a** or **8b** (0.5 mmol) was dissolved in 2.5 ml trimethyl phosphate (dried over molecular sieve) under argon. The reaction mixture was cooled down to 0–4 °C and 0.15 g

(0.75 mmol) 1,8-bis(dimethylamino)naphthaline was added upon stirring. After 15 min, $POCl_3$ (0.075 ml, 0.8 mmol) was added to the reaction mixture. After stirring for 1 h, a mixture of 0.3 ml (2 mmol) tributylamine and 2.5 ml (1.25 mmol) 0.5 M tributylammonium diphosphate in DMF was added and the stirring was continued for another 30 min at 0–4 °C. Hydrolysis was achieved by addition of 15 ml of 0.5 M TEAB (pH 7.5). Trimethyl phosphate and proton sponge were removed from the reaction mixture by extraction with *t*-butylmethyl ether. The aqueous phase was dried under high vacuum at 40 °C and the crude nucleotides were purified using ion exchange chromatography followed by preparative HPLC.

5.8. Ludwig method[36] for the preparation of 1-(ω-hydroxyalkyl)uracil monophosphates (11a,c–d) and 1-(-hydroxyalkyl)uracil triphosphates (12a,c–d)

1-(ω-Hydroxyalkyl)uracil derivative **7a**, **c**, or **d** (0.5 mmol) was dissolved, at 0–4 °C, in 2.5 ml trimethylphosphate (dried over molecular sieve) under argon and 0.15 g (0.75 mmol) of 1,8-bis(dimethylamino)naphthaline was added. After 15 min, 0.075 ml (0.8 mmol) $POCl_3$ was added. After 1 h of stirring, a mixture of 0.3 ml (2 mmol) tributylamine and 2.5 ml (1.25 mmol) of a 0.5 M solution of tributylammonium diphosphate in DMF was added and the stirring was continued for another 30 min at 0–4 °C. Hydrolysis was achieved by addition of 15 ml of 0.5 M TEAB solution (pH 7.5). Trimethyl phosphate and proton sponge were removed from the reaction mixture by extraction with *t*-butylmethyl ether. The aqueous phase was dried under high vacuum at 40 °C and the crude nucleotides were subsequently purified by ion exchange chromatography to separate mono- and triphosphates followed by further purification with preparative HPLC.

5.9. Alternative procedure for the synthesis of 1-(4-hydroxybutyl)uracil triphosphate (12c) according to the Ludwig–Eckstein method[37]

1-Hydroxybutyluracil **7c** (30 mg, 0.16 mmol) was dissolved in 3 ml of dry DMF at rt under argon. Then, 0.175 ml (0.17 mmol) of a freshly prepared 1 M solution of 2-chloro-4*H*-1,2,3-dioxaphosphorine-4-one in dry dioxane was added. After 15 min of stirring, 0.5 ml of a 0.5 M tri-*n*-butylammonium diphosphate solution in DMF was added and stirring was continued for an additional 30 min followed by the addition of an aqueous solution of I_2. After 1 h, the reaction was hydrolyzed by addition of 15 ml of 0.5 M TEAB (pH 7.5) and the product was isolated as described above.

5.10. Purification of nucleotides

5.10.1. Ion exchange chromatography

The crude nucleotides were purified by ion exchange chromatography on a GradiFrac system (Pharmacia-Biotech) with the high-load pump P50 with an XK 26 mm/20 cm length column (Pharmacia) using Sephadex DEAE A-25 gel HCO_3^--form swelled in a 1 M solution of TEAB at 4 °C. After equilibration of the column with deionized water, the crude product was dissolved in 2 ml of aqueous triethylammonium hydrogencarbonate solution. The column was washed with deionized water, followed by a solvent gradient of 0–500 mM TEAB using approximately 1000 ml of solvent to elute the triphosphates at a flow rate of 1 ml/min. Fractions were collected and appropriate fractions pooled, diluted in water, and lyophilized.

5.10.2. Preparative HPLC

Lyophilized nucleotides obtained after ion exchange chromatography purification were dissolved in 5 ml of deionized water and injected into an RP-HPLC column (Knauer 20 mm ID, Eurospher-100, C18, 7μ, 25 cm). The column was eluted with a solvent gradient of 0–5% of acetonitrile in 50 mM aqueous ammonium formate solution for 20–30 min at a flow rate of 15 ml/min (10 MPa). The UV absorption was detected at 254 nm. Fractions were collected and appropriate fractions pooled, diluted with water and lyophilized several times to remove the remaining buffer yielding the final pure nucleotides as white powders.

5.10.2.1. [3-(2,4-Dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)propyl]-phosphonic acid diethyl ester (3b). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.23 (t, 3H, J = 7.0 Hz), 1.66–1.81 (m, 4H), 3.71 (t, 2H, J = 6.21 Hz), 3.91–4.03 (m, 2H), 5.56 (dd, 1H, $J_{5,6}$ = 7. 9 Hz, $J_{5,3}$ = 2.2 Hz), 7.64 (d, 1H, J = 7.9 Hz), 11.23 (s, 1H). ^{31}P NMR (120 MHz, DMSO-d_6) δ 30.97 (s, P_α). ^{13}C NMR (125 MHz, DMSO-d_6) δ 16.44 (d, J = 6.1 Hz), 21.87 (d, J = 140.4 Hz), 22.01 (d, J = 4.57 Hz), 48.05 (d, J = 19.9 Hz), 61.23 (d, J = 6.1 Hz), 101.19, 145.85, 151.16, 163.96.

5.10.2.2. [4-(2,4-Dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)butyl]-phosphonic acid diethyl ester (3c). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.22 (t, 3H, J = 7.0 Hz), 1.41–1.84 (m, 6H), 3.66 (t, 2H, J = 6.58 Hz), 3.89–4.01 (m, 2H), 5.54 (dd, 1H, $J_{5,6}$ = 7. 8 Hz, $J_{5,3}$ = 2.3 Hz), 7.65 (d, 1H, J = 7.9 Hz), 11.23 (s, 1H). ^{31}P NMR (120 MHz, DMSO-d_6) δ 31.67 (s, P_α). ^{13}C NMR (125 MHz, DMSO-d_6) δ 16.44 (d, J = 6.1 Hz), 19.20 (d, J = 6.1 Hz), 24.19 (d, J = 138.9 Hz), 29.25 (d, J = 16.8 Hz), 46.98, 61.02 (d, J = 6.1 Hz), 101.01, 145.79, 151.13, 163.87.

5.10.2.3. [5-(2,4-Dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)pentyl]-phosphonic acid diethyl ester (3d). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.22 (t, 3H, J = 7.0 Hz), 1.29–1.79 (m, 8H), 3.64 (t, 2H, J = 7.1 Hz), 3.89–4.04 (m, 2H), 5.54 (dd, 1H, $J_{5,6}$ = 7. 9 Hz, $J_{5,3}$ = 2.2 Hz), 7.65 (d, 1H, J = 7.9 Hz), 11.21 (s, 1H). ^{31}P NMR (120 MHz, DMSO-d_6) δ 31.89 (s, P_α). ^{13}C NMR (125 MHz, DMSO-d_6) δ 16.47 (d, J = 6.1 Hz), 21.95 (d, J = 4.6 Hz), 24.49 (d, J = 138.9 Hz), 26.68 (d, J = 16.8 Hz), 28.12, 60.49 (d, J = 6.1 Hz), 100.95, 145.82, 151.10, 163.90.

5.10.2.4. [3-(2,4-Dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)propyl]-phosphonic acid (4b). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.41–1.58 (m, 2H), 1.65–1.87 (m, 2H), 3.70 (t, 2H, J = 6.67 Hz), 5.56 (d, 1H, J = 7. 7 Hz), 6.20 (s, 2H), 7.66 (d, 1H, J = 7.9 Hz), 11.24 (s, 1H). ^{31}P NMR (120 MHz, DMSO-d_6) δ 25.66 (s, P_α). ^{13}C NMR (125 MHz, DMSO-d_6) δ 22.70 (d, J = 4.6 Hz), 24.65 (d, J = 138.9 Hz), 48.22 (d, J = 18.3 Hz), 101.01, 145.92, 151.10, 163.93. Calcd for $C_7H_{11}N_2O_5P$ (234.159 g/mol): C, 35.91; H, 4.74; N 11.96. Found: C, 35.52; H, 4.83; N 11.74.

5.10.2.5. [4-(2,4-Dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)butyl]-phosphonic acid (4c). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.41–1.68 (m, 6H), 3.66 (t, 2H, J = 6.9 Hz), 5.55 (d, 1H, J = 7. 7 Hz), 7.66 (s, 2H), 7.65 (d, 1H, J = 7.7 Hz), 11.23 (s, 1H). ^{31}P NMR (120 MHz, DMSO-d_6) δ 26.51 (s, P_α). ^{13}C NMR (125 MHz, DMSO-d_6) δ 19.88 (d, J = 4.6 Hz), 27.27 (d, J = 137.3 Hz), 29.56 (d, J = 16.8 Hz), 47.19, 100.98, 145.79, 151.10, 163.89. Calcd for $C_8H_{13}N_2O_5P$ (248.18 g/mol): C, 38.68; H, 5.24; N, 11.28. Found: C, 38.51; H, 5.35; N, 10.91.

5.10.2.6. [5-(2,4-Dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)pentyl]-phosphonic acid (4d). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.24–1.64 (m, 8H), 3.63 (t, 2H, J = 7.1 Hz), 5.504 (d, 1H, J = 7. 1 Hz), 7.05 (s, 2H), 7.64 (d, 1H, J = 7.9 Hz), 11.21 (s, 1H). ^{31}P NMR (120 MHz, DMSO-d_6) δ 27.08 (s, P_α). ^{13}C NMR (125 MHz, DMSO-d_6) δ 22.58 (d, J = 4.6 Hz), 26.70 (d, J = 15.3 Hz), 27.51 (d, J = 137.3 Hz), 28.30, 47.46, 101.80, 145.82, 151.10, 163.93. Calcd for $C_9H_{15}N_2O_5P$

(262.20 g/mol): C, 41.23; H, 5.77; N, 10.68. Found: C, 40.87; H, 5.74; N 10.34.

5.10.2.7. Diphosphoric 3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)propylphosphonic anhydride (5b). ^{1}H NMR (300 MHz, D_2O) δ 1.72–1.91 (m, 4H), 3.79 (t, 2H, J = 7.0 Hz), 5.78 (d, 1H, J = 7. 7 Hz), 7.66 (d, 1H, J = 7.7 Hz). ^{31}P NMR (120 MHz, D_2O) δ −22.8 (t, P_β, J = 21.6 Hz), −7.7 (d, P_γ, J = 20.4 Hz), 17.8 (d, P_α, J = 24.1 Hz).

5.10.2.8. Diphosphoric 4-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)butylphosphonic anhydride (5c). ^{1}H NMR (300 MHz, D_2O) δ 1.52–1.80 (m, 6H), 3.73 (t, 2H, J = 7.3 Hz), 5.74 (d, 1H, J = 7.7 Hz), 7.60 (d, 1H, J = 7.7 Hz). ^{31}P NMR (120 MHz, D_2O) δ −23.5 (t, P_β, J = 24.2 Hz), −11.2 (d, P_γ, J = 17.8 Hz), 19.7 (d, P_α, J = 25.4 Hz).

5.10.2.9. Diphosphoric 5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)pentylphosphonic anhydride (5d). ^{1}H NMR (300 MHz, D_2O) δ 1.32–1.76 (m, 8H), 3.71 (t, 2H, J = 7.3 Hz), 5.73 (d, 1H, J = 7. 7 Hz), 7.58 (d, 1H, J = 7.7 Hz). ^{31}P NMR (120 MHz, D_2O) δ −23.5 (t, P_β, J = 23.5 Hz), −10.9 (d, P_γ, J = 20.4 Hz), 20.6 (d, P_α, J = 26.7 Hz).

5.10.2.10. 1-((3-Hydroxypropoxy)methyl)pyrimidine-2,4(1*H*,3*H*)-dione (8b). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.63 (q, 2H, J = 6.4 Hz), 3.41 (t, 2H, J = 6.5 Hz), 3.51 (t, 2H, J = 6.5 Hz), 4.44 (s, 1H), 5.06 (s, 2H), 5.61 (d, 1H, J = 7.8 Hz), 7.69 (d, 1H, J = 7.8 Hz), 11.31 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 32.63, 57.65, 65.89, 76.57, 101.79, 145.18, 151.28, 163.86. Elemental Anal. Calcd for $C_8H_{12}N_2O_4$ (200.20): C, 48.00; H, 6.04; N, 13.99. Found: C, 47.81; H, 5.99; N, 13.99.

5.10.2.11. 2-((2,4-Dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)methoxy)ethyl monophosphate (9a)[44]. ^{1}H NMR (300 MHz, D_2O) δ 3.48 (m, 4H), 5.09 (s, 2H), 5.61 (d, 1H, J = 7. 8 Hz), 7.71 (d, 1H, J = 7.8 Hz). ^{31}P NMR (120 MHz, D_2O) δ 0.40 (s, P_α).

5.10.2.12. 3-((2,4-Dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)methoxy)-propyl monophosphate (9b). ^{1}H NMR (300 MHz, D_2O) δ 1.66 (q, 2H, J = 6.3 Hz), 3.47 (t, 2H, J = 6.3 Hz), 3.66 (m, 2H), 4.96 (s, 2H), 5.60 (d, 1H, J = 7. 9 Hz), 7.49 (d, 1H, J = 7.9 Hz). ^{31}P NMR (120 MHz, D_2O) δ 0.25 (s, P_α).

5.10.2.13. 2-((2,4-Dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)methoxy)-ethyl triphosphate (10a)[45,46]. ^{1}H NMR (300 MHz, D_2O) δ 3.48 (m, 4H), 5.09 (s, 2H), 5.61 (d, 1H, J = 7. 8 Hz), 7.71 (d, 1H, J = 7.8 Hz). ^{31}P NMR (120 MHz, D_2O) δ −23.6 (t, P_β, J = 15.9 Hz), −11.5 (d, P_γ, J = 20.3 Hz), −11.1 (d, P_α, J = 20.4 Hz).

5.10.2.14. 3-((2,4-Dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)methoxy)-propyl triphosphate (10b). ^{1}H NMR (300 MHz, D_2O) δ 1.37 (m, 2H), 3.15 (t, 2H, J = 6.3 Hz), 3.46 (m, 2H), 4.65 (s, 2H), 5.30 (d, 1H, J = 7. 9 Hz), 7.17 (d, 1H, J = 7.9 Hz). ^{31}P NMR (120 MHz, D_2O) δ −22.4 (t, P_β, J = 15.9 Hz), −11.4 (d, P_γ, J = 19.1 Hz), −8.1 (d, P_α, J = 14.4 Hz).

5.10.2.15. 2-(2,4-Dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)ethyl monophosphate (11a).[47] ^{1}H NMR (300 MHz, D_2O) δ 3.89 (t, 2H, J = 4.9 Hz), 4.1 (m, 2H), 5.70 (d, 1H, J = 7. 9 Hz), 7.60 (d, 1H, J = 7.9 Hz). ^{31}P NMR (120 MHz, D_2O) δ 1.9 (s, P_α).

5.10.2.16. 4-(2,4-Dioxo-3,4-dihydropyrimidin-1(2H)-yl)butyl monophosphate (11c).[48] ^{1}H NMR (300 MHz, D_2O) δ 1.60–1.83 (m, 4H), 3.82 (t, 2H, J = 7.2 Hz), 3.89 (m, 2H), 5.82 (d, 1H, J = 7. 9 Hz), 7.66 (d, 1H, J = 7.9 Hz). ^{31}P NMR (120 MHz, D_2O) δ 0.7 (s, P_α).

5.10.2.17. 5-(2,4-Dioxo-3,4-dihydropyrimidin-1(2H)-yl)pentyl monophosphate (11d).[47] ^{1}H NMR (300 MHz, D_2O) δ 1.15–1.53 (m, 6H), 3.54–3.65 (m, 4H), 5.60 (d, 1H, J = 7. 9 Hz), 7.43 (d, 1H, J = 7.9 Hz). ^{31}P NMR (120 MHz, D_2O) δ 0.1 (s, P_α).

5.10.2.18. 2-(2,4-Dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)ethyl triphosphate (12a). ^{1}H NMR (300 MHz, D_2O) δ 3.95 (t, 2H, J = 5.0 Hz), 4.08 (m, 2H), 5.71 (d, 1H, J = 7. 9 Hz), 7.61 (d, 1H, J = 7.9 Hz). ^{31}P NMR (120 MHz, D_2O) δ −20.7 (t, P_β, J = 18.5 Hz), −10.1 (d, P_γ, J = 19.7 Hz), −6.5 (d, P_α, J = 17.2 Hz).

5.10.2.19. 4-(2,4-Dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)butyl triphosphate (12c)[47]. ^{1}H NMR (300 MHz, D_2O) δ 1.24–1.82 (m, 4H), 3.81 (t, 2H, J = 7.1 Hz), 4.01 (m, 2H), 5.81 (d, 1H, J = 7.7 Hz), 7.69 (d, 1H, J = 7.7 Hz). ^{31}P NMR (120 MHz, D_2O) δ −20.4 (t, P_β, J = 19.1 Hz), −11.1 (d, P_γ, J = 19.1 Hz), −8.4 (d, P_α, J = 21.6 Hz).

5.10.2.20. 5-(2,4-Dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)pentyl triphosphate (12d). ^{1}H NMR (300 MHz, D_2O) δ 0.98–1.38 (m, 6H), 3.43 (t, 2H, J = 7.2 Hz), 3.62 (m, 2H), 5.45 (d, 1H, J = 7. 9 Hz), 7.31 (d, 1H, J = 7.9 Hz). ^{31}P NMR (120 MHz, D_2O) δ −23.5 (t, P_β, J = 19.1 Hz), −11.1 (d, P_γ, J = 17.8 Hz), −10.9 (d, P_α, J = 20.4 Hz).

5.11. Chemicals for biological experiments

Uridine-5′-triphosphate (UTP), dimethyl sulfoxide (DMSO), constituents of Krebs–Hepes buffer (sodium chloride, potassium chloride, potassium dihydrogenphosphate, sodium hydrogencarbonate, anhydrous glucose, sodium hydroxide (for pH adjustment of Krebs–Hepes buffer to pH 7.4), Hepes (free acid), calcium chloride and magnesium sulfate), Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin and fetal bovine serum for cell culture were obtained from Sigma (Taufkirchen, Germany). HAT (hypoxanthine, aminopterin, thymidine) supplement was from Gibco Life Technologies (Karlsruhe, Germany).

5.12. Cell culture

NG108-15 (mouse neuroblastoma × rat glioma) hybrid cells were cultured as previously described[8,10,49] in Dulbecco's modified Eagle's medium (containing 100 μg/ml streptomycin, 100 U/ml penicillin G, 10% fetal bovine serum (Sigma–Aldrich) and HAT (1.0×10^{-5} M hypoxanthine, 4.0×10^{-8} M aminopterin, 1.6×10^{-6} M thymidine) until cells were 80% confluent. Cells were incubated at 37 °C with 5% CO_2 and 95% relative humidity.

5.13. Calcium measurements

Ca^{2+} fluorescence measurements were performed using a FLUOstar® plate reader, equipped with one injector (BMG LabTechnologies, Offenburg, Germany) as previously described.[8–10,40] Cells were loaded for 1 h at 25 °C (continuous shaking at 600 rpm, exclusion of light) with 3 μM of Oregon Green 488 BAPTA-1/AM (Molecular Probes), which had been mixed with a 20% solution of Pluronic F-127 (Molecular Probes) in DMSO at a ratio of 1:1 immediately before use. After incubation, cells were rinsed twice with Krebs–HEPES buffer, diluted and evenly plated into black 96-well plates (Packard Optiplate™ HTRF-96; Packard BioScience) at a density of 17,000 cells/well. To determine agonistic activity, test compounds dissolved in buffer were injected sequentially into separate wells and fluorescence intensity was measured at 520 nm (bandwidth 25 nm) for 60 intervals of 0.4 s each. Excitation wavelength was at 485 nm (bandwidth 20 nm). Antagonistic potency of test compounds was initially determined by preincubation of the cells with two concentrations of test compound (100 and 500 μM) at 37 °C for at least 30 min prior to injection of the agonist UTP (1 μM). The final volume was 200 μl. Data points within one assay were determined in quadruplicate. For compounds that appeared to be

potent in the initial screening tests (repeated at least once), dose–response curves were recorded and IC_{50} values were calculated by nonlinear regression with a sigmoidal dose–response equation (variable Hill slope) using the computer program Prism®3.0 (Graph Pad Software, Inc., San Diego, CA, USA).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.05.062.

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