# Diadenosine and Diuridine Poly(borano)phosphate Analogues: Synthesis, Chemical and Enzymatic Stability, and Activity at $P2Y_1$ and $P2Y_2$ Receptors†

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Dinucleoside polyphosphates, $Np_nN'$, exert their physiological effects via P2 receptors. They are attractive drug targets as they offer better stability and specificity compared to nucleotides, the most common P2-receptor ligands. To further improve the properties of $Np_nN'$, which are still pharmacologically unsatisfactory, we developed novel boranophosphate isosteres of dinucleoside polyphosphates, denoted as $Np_n(B)N$. These analogues were obtained in a facile and efficient synthesis as the exclusive products in a concerted reaction of two nucleoside phosphorimidazolides and inorganic boranophosphate. This unusual reaction is due to the preorganization of three reactant molecules by the $Mg^{2+}$ ion. We found that $Ap_{3/5}(\beta/\gamma\text{-B})A$ analogues were potent $P2Y_1$-R agonists. $Ap_5(\gamma\text{-B})A$ was equipotent to 2-MeS-ADP ($EC_{50}$ $6.3 \times 10^{-8}$ M), thus making it one of the most potent $P2Y_1$-R agonists currently known. Moreover, $Ap_5(\gamma\text{-B})A$ did not activate $P2Y_2$-R. In contrast, $Up_{3/5}(\beta/\gamma\text{-B})U$ analogues were extremely poor agonists of both $P2Y_1$-R and $P2Y_2$-R. $Np_n(B)N$ analogues exhibited remarkable chemical stability under physiological conditions. Under conditions mimicking gastric juice, $Np_3(\beta\text{-B})N$ analogues exhibited a half-life ($t_{1/2}$) of 1.3 h, whereas $Np_5(\gamma\text{-B})N$ degraded at a much faster rate ($t_{1/2}$ 18 min). The hydrolysis of $Ap_3(\beta\text{-B})A$ by human nucleotide pyrophosphatase phosphodiesterases (NPP1 and NPP3) was slowed by 40% and 59%, respectively, as compared to $Ap_3A$. However, this effect of the boranophosphate was position-dependent, as $Np_5(\gamma\text{-B})N$ was degraded at a rate comparable to that of $Np_5N$. In summary, $Ap_5(\gamma\text{-B})A$ appears to be a highly potent and selective $P2Y_1$-R agonist, as compared to the parent compound. This promising scaffold will be applied in the design of future metabolically stable analogues.

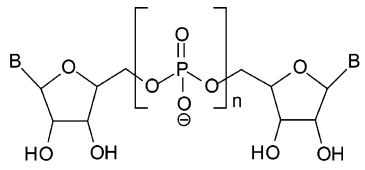
## Introduction

Naturally occurring dinucleoside polyphosphates, $Np_nN'$ (**1**)[1−3] represent a diverse group of extracellular and intracellular mediators controlling various physiological functions.[4] Diadenosine polyphosphates, $Ap_nA$, the most studied $Np_nN'$ analogues, inhibit key enzymes such as adenosine kinase and adenylate kinase,[5] stimulate NO release from endothelial cells,[6] inhibit platelet aggregation,[7] and act as neurotransmitters.

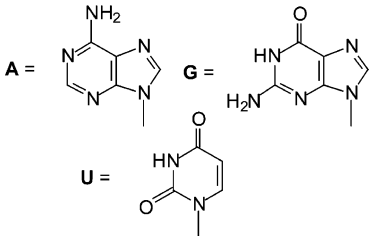
Extracellular effects of $Np_nN'$ analogues are mediated by the adenosine and uridine nucleotide receptors, P2-Rs, and possibly by dinucleotide receptors.[8] The existence of dinucleoside receptors has been suggested, but no receptor has been cloned and clearly characterized.[9]

The members of the P2-receptor superfamily, consisting of ligand-gated ion channels (P2X-Rs) and G-protein coupled receptors (P2Y-Rs), are activated generally by ATP. Some P2-Rs are also activated by ADP, UTP, or UDP.[10] In addition, various P2X- and P2Y-Rs respond potently to several dinucleoside polyphosphates.[8] For instance, $Up_4U$, a naturally occurring analogue, is an effective agonist of $P2Y_2$-R that may also activate other P2-Rs.[11]

P2Y-Rs are attractive pharmaceutical targets due to their involvement in the modulation of various functions in many tissues and organs under both normal and pathophysiological conditions.[12−14] Currently, P2Y-R agonists proposed as drugs consist of a nucleotide scaffold, mostly ATP.[12,14] Yet, this scaffold suffers from inherent limitations, as ATP is recognized by numerous proteins and cannot confer any selectivity. Furthermore, the nucleotide scaffold is enzymatically and chemically unstable.




**1** n = 2-8 ; B = A, G, U




Since $Np_nN'$ analogues are more protein-selective and metabolically stable,[15] as compared to naturally occurring nucleotides, these analogues represent alternative therapeutic agents targeting P2Y-Rs.

To date, several $Np_nN'$ analogues have been administered in human clinical trials.[16] These analogues include $Ap_4A$ injected intravenously (iv) for lowering blood pressure during anesthesia, $Up_4U$ applied as a treatment for dry eye disease, and $Up_4dC$

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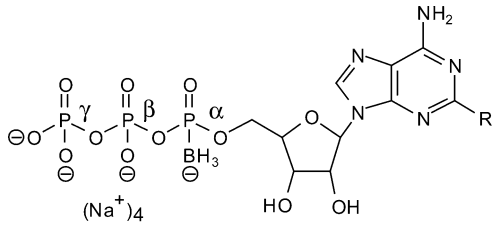
(uridine-tetraphosphate-2′-deoxycytidine) used for the treatment of cystic fibrosis and retinal detachment.

Although several endogenous dinucleoside polyphosphates have a good pharmacological activity, their in vivo half-life is relatively short.[9] For instance, the half-life of $Up_4U$ in the mucosal surface of human nasal epithelial cells in culture is 50 min.[15] Although this endogenous dinucleotide has a significantly longer life than UTP (half-life of ca. 3 min), prolongation of in vivo half-life of pharmacologically active $Np_nN'$ analogues would be highly desirable. In addition, the enhancement of the affinity and selectivity of endogenous $Np_nN'$ analogues to the target P2 receptors are other important challenges.

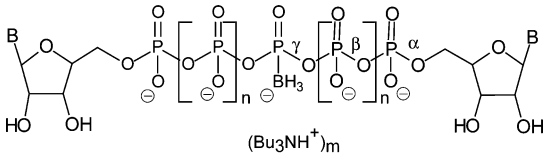
Recently, we demonstrated the beneficial effect of the boranophosphate bioisostere in ATP-α-B analogues (**2**) in improving both receptor-subtype selectivity and chemical and metabolic stability of the P2Y-R endogenous ligand, ATP.[17,18]



**2** R = H, Cl, SMe

Therefore, here, we targeted dinucleoside poly(borano)-phosphate bioisosteres, $Np_n(B)N$, in an attempt to improve the pharmacological properties of dinucleoside polyphosphate P2Y-R ligands.

We report, here, on the synthesis of several diadenosine and diuridine tri- and penta(borano)phosphate analogues (**3**−**6**), their activity at P2Y-Rs, their chemical stability, and their enzymatic hydrolysis by human nucleotide pyrophosphatase phosphodiesterases NPP 1 and 3.

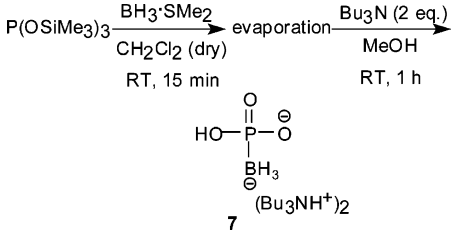


**3** n = 0 ; B = A ; m = 3
**4** n = 0 ; B = U ; m = 3
**5** n = 1 ; B = A ; m = 5
**6** n = 1 ; B = U ; m = 5

## Results

**Synthesis of Dinucleoside Poly(borano)phosphate Analogues 3−6.** Several chemical methods have been developed to form the pyrophosphate bond in dinucleoside polyphosphates. Dinucleoside polyphosphates are conventionally prepared via the activation of 5′-terminal phosphate of nucleotide, thus forming a phosphoryl donor (P-donor) followed by a reaction with nonactivated nucleotides (phosphoryl acceptor, P-acceptor). Nucleotides activated with 5′-phosphoroimidazolide,[19] 5′-phosphomorpholidate,[19] or phosphoropiperididate,[20] are used as phosphoryl donors for the synthesis. Phosphoroimidazolide was found to be the most reactive donor in the presence of divalent metal ions, such as $Mg^{2+}$, $Zn^{2+}$, and $Mn^{2+}$. The best catalyst for pyrophosphate bond formation between GDP and methyl phosphorimidazolide in anhydrous DMF was $Zn^{2+}$.[20] A pyrophosphate bond was formed also in aqueous solution starting from adenosine 5′-phosphorimidazolide and ADP by use of $Mn^{2+}$ and $Cd^{2+}$ metal ion catalysts.[21]

**Scheme 1**



Phosphoroimidazolides may be generated in situ or isolated by use of carbonyl diimidazole (CDI) prior to the reaction with the corresponding nucleotides.[22] Additional nucleotide coupling procedures include the use of dicyclohexylcarbodiimide (DCC)[23] or *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC).[24] By implementation of these procedures, dinucleoside polyphosphates with a phosphate linker ranging from 2 to 6 phosphates have been synthesized.[21,16]

The synthesis of the target $Np_3(B)N$ and $Np_5(B)N$ analogues (**3**−**6**) required a novel building block, inorganic boranophosphate, BPi (**7**), as a phosphoryl acceptor. We recently reported on the preparation of boranophosphate salts (**7**) and the exploration of their properties.[25,26] BPi salts were easily prepared in excellent yield in a one-pot two-step reaction from tris-(trimethylsilyl)phosphite (Scheme 1) and were characterized by X-ray crystal structure, IR, and $^1H$ and $^{31}P$ NMR.

Substitution of the phosphate, $P_i$, oxygen by the $BH_3$ group results in a significant change of the chemical nature of $BP_i$ as compared to $P_i$. Specifically, the reducing nature of the $BH_3$ moiety is drastically decreased in $BP_i$. Likewise, the nucleophilicity of $BP_i$'s oxygen diminishes as compared to $P_i$, possibly due to the extensive H-bonded clustering in organic solvents.[26] On the basis of its water solubility, acid−base character, H-bonding properties, and high stability, $BP_i$ appears as a perfect mimic of $P_i$ and is an attractive alternative to the known phosphate isosteres.[26]

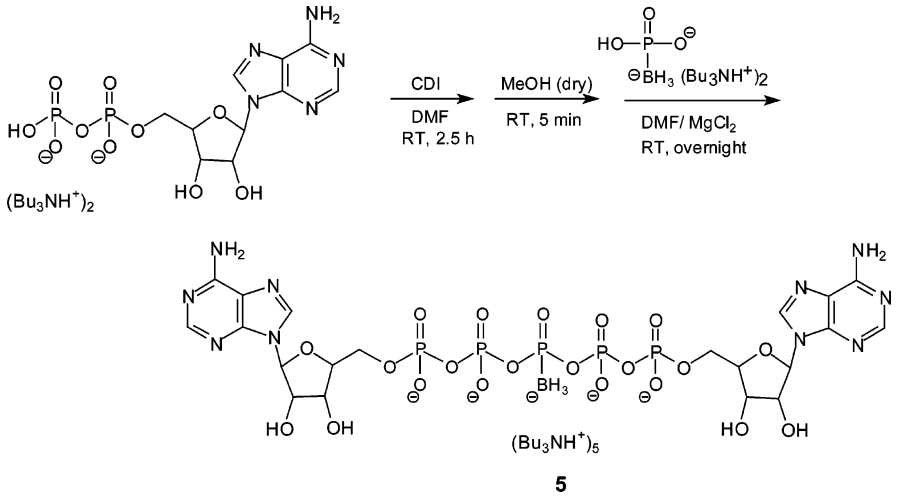
Therefore, for the synthesis of analogues **3**−**6** $BP_i$ was employed as P-acceptor and nucleoside phosphoroimidazolides as P-donors. Yet, due to the low nucleophilicity of $BP_i$, its application as a P-acceptor (a nucleophile) requires preactivation of $BP_i$. For this purpose, we have added $Mg^{2+}$ ions to the reaction mixture. These ions coordinate with $BP_i$ and break the extensive H-bonded clustering of $BP_i$ in organic solvents, which reduces $BP_i$'s nucleophilicity.

Briefly, the synthesis of analogues (**3**−**6**) involved the activation of a nucleotide (e.g., AMP) tributylammonium salt with CDI in dry DMF at room temperature for 2.5 h, followed by the addition of $BP_i$ and $MgCl_2$, and stirring at room temperature for ca. 20 h (a typical reaction is depicted in Scheme 2). Analogues (**3**−**6**) were formed as the exclusive product in good to high yields. The reaction proceeded equally well with adenosine and uridine nucleotides. Yet, formation of $Np_5(B)N$ products (**5** and **6**) was less efficient than that of $Np_3(B)N$ analogues (**3** and **4**).

No product was formed when $BP_i$ was preactivated by CDI. Thus, $BP_i$ cannot be applied as a P-donor in the preparation of analogues **3**−**6**. In this reaction, three reactant molecules (two P-donors and one P-acceptor) react concertedly. Yet, even at a 4-fold reaction dilution or with the addition of a higher number of $BP_i$ equivalents (4 equiv), $Np_n(B)N$ analogues were still the sole products formed. No $Np_n(B)$ products resulting from the reaction of one P-donor and one P-acceptor were formed under these conditions.

Various divalent metal ions, such as $Zn^{2+}$, $Cd^{2+}$, $Mn^{2+}$, and $Mg^{2+}$, were reported to facilitate nucleotide and dinucleoside

**Scheme 2**




**5**

**Table 1.** Activity of Diadenosine and Diuridine Poly(borano)phosphate Analogues at $P2Y_1$ and $P2Y_2$ Receptors

| | $EC_{50}$ (M) | |
|---|---|---|
| agonist | $P2Y_1$ | $P2Y_2$ |
| 2-MeS-ADP | $1.0 \times 10^{-7}$ (R), $1.1 \times 10^{-8}$ (Hb) | ND |
| $Ap_3A$ | $6.0 \times 10^{-8}$ (R) | weak response (Ha) |
| $Ap_3(\beta$-B)A, **3** | $9.0 \times 10^{-7}$ (R) | weak response (Ha) |
| $Ap_5A$ | $\geq 5 \times 10^{-6}$ (R) | weak response (Ha) |
| $Ap_5(\gamma$-B)A, **5** | $6.3 \times 10^{-8}$ (R) | weak response (Ha) |
| UTP | ND | $1.3 \times 10^{-7}$ (R), $4.5 \times 10^{-7}$ (Ha) |
| $Up_3(\beta$-B)U, **4** | $8 \times 10^{-7}$ (Hb)[b] | no response (R), no response (Ha) |
| $Up_5(\gamma$-B)U, **6** | $8 \times 10^{-7}$ (Hb)[b] | $>7 \times 10^{-6}$ (R), $3 \times 10^{-5}$ (Ha)[c] |

[a] (R) Rat $P2Y_1$-GFP transfected in HEK 293 cells; (Ha): A549 cells, a human cell line expressing endogenously $P2Y_2$ but not $P2Y_1$ receptor; (Hb) human $P2Y_1$-GFP transfected in 1321N1 cells; ND, not determined. [b] Less than 20% of the maximal response obtained with 2-MeS-ADP. [c] Less than 10% of the maximal response obtained with UTP.

polyphosphate synthesis from the corresponding P-acceptor and P-donor.[27] However, here, the reaction to produce $Np_n$(B)N is metal-ion-specific. Only $Mg^{2+}$ ions have been shown to be able to act as metal-ion activator. Specifically, the synthesis of $Np_n$(B)N analogues was attempted with various other metal ions. Replacing $Mg^{2+}$ by $Ca^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ produced no $Np_n$(B)N product.

**Activity of Analogues 3−6 at $P2Y_1$ and $P2Y_2$ Receptors.** To test the novel analogues as P2Y receptor agonists, we used HEK 293 cells transfected with rat P2Y (rP2Y)-GFP constructs. The functional expression of $rP2Y_1$-GFP receptor in HEK 293 cells[28] was confirmed with the agonist 2-MeS-ADP. These cells were then stimulated with different concentrations of analogues **3** and **5**. The $Ca^{2+}$ response of $Ap_{3/5}(\beta/\gamma$-B)A derivatives was compared with that of the commercially available diadenosine polyphosphates, $Ap_3A$ and $Ap_5A$, respectively. As indicated in Figure 1A, $Ap_3(\beta$-B)A (**3**) is less potent than either $Ap_3A$ or 2-MeS-ADP. Analogue **3** is an agonist about 10 times less potent at the $rP2Y_1$-GFP receptor than $Ap_3A$, which is reflected in the $EC_{50}$ values of $9.0 \times 10^{-7}$ and $6.0 \times 10^{-8}$ M, respectively (Table 1). The most evident change in receptor affinity was observed for the $\gamma$-borano modification of $Ap_5A$ (**5**), which greatly increased the potency of the agonist at the $rP2Y_1$-GFP receptor by about 2 orders of magnitude (Figure 1B). $Ap_5(\gamma$-B)A (**5**) is slightly more potent at the $rP2Y_1$-GFP receptor than the most potent $P2Y_1$-R agonist currently known, 2-MeS-ADP, as reflected by the respective $EC_{50}$ values of $6.3 \times 10^{-8}$ and $1.0 \times 10^{-7}$ M (Table 1).

The diuridine derivatives **4** and **6** were tested on HEK 293 cells stably expressing functional $rP2Y_2$-GFP,[18] as shown in Figure 2A. In addition, these analogues were tested on A549 cells, a human cell line endogenously expressing $P2Y_2$ but not $P2Y_1$-R,[29] as a control for effectiveness on the $P2Y_2$-R. The results obtained with A549 cells are displayed in Figure 2B. Analogue **4** is completely ineffective at both the rat and human $P2Y_2$ receptors (Figure 2). From our results obtained with transfected HEK 293 cells, it appears that analogue **6** might be a low-potency agonist at the rat $P2Y_2$-GFP receptor (Figure 2A). However, from the data in Figure 2B we conclude that **6** certainly is not effective at the human $P2Y_2$ receptor. Analogue **6** induces at most 10% of the effect of UTP in A549 cells, even at high concentrations (Figure 2B).

The very low potency of analogue **6** in inducing a $Ca^{2+}$ response in HEK 293 cells transfected with $rP2Y_2$-GFP receptor ($EC_{50}$ value $>7 \times 10^{-6}$ M) indicates that **6** could activate some endogenous receptors in HEK 293 cells, probably $hP2Y_1$ receptors. Our analysis of P2Y receptor expression in the HEK 293 cells used in the present study[29] had shown that in these cells the $P2Y_6$ receptor is absent and the $P2Y_4$ receptor is weakly expressed. On the $P2Y_4$ receptor, $Up_3U$ and $Up_5U$ were virtually inactive.[11] Therefore, we investigated the remaining possibility, whether $hP2Y_1$ receptors might be activated by **6,** using 1321N1 cells stably expressing the $hP2Y_1$-GFP receptor. The 1321N1 cells do not endogenously express any P2Y receptors. The $P2Y_1$ receptor agonist 2-MeS-ADP caused a substantial $Ca^{2+}$ response with an $EC_{50}$ value of $1 \times 10^{-8}$ M in the transfected 1321N1 astrocytoma cells (Figure 3). In these $hP2Y_1$-GFP receptor-transfected 1321N1 cells, **4** and **6** induced a weak $Ca^{2+}$ response (Figure 3). Only for the highest concentrations of **4** and **6** did we obtain about 20% of the 2-MeS-ADP-induced response amplitude with $EC_{50}$ values at about $1 \times 10^{-6}$ M (Figure 3). From these results we can infer that **6** might be a very weak agonist at the $P2Y_1$ receptor and some other receptors that are endogenously present in HEK 293 cells.

Furthermore, the possible effect of the $Ap_n$(B)A analogues **3** and **5** on the $P2Y_2$ receptor was tested in A549 cells, as these cells do not endogenously express the $P2Y_1$ receptor. We observed that both these substances are very weak agonists at the $P2Y_2$ receptor. This was clear from the $Ca^{2+}$ response that we obtained on stimulation of the A549 cells with 100 $\mu$M **3** and **5**, which was $0.26 \pm 0.05$ and $0.30 \pm 0.03$, respectively

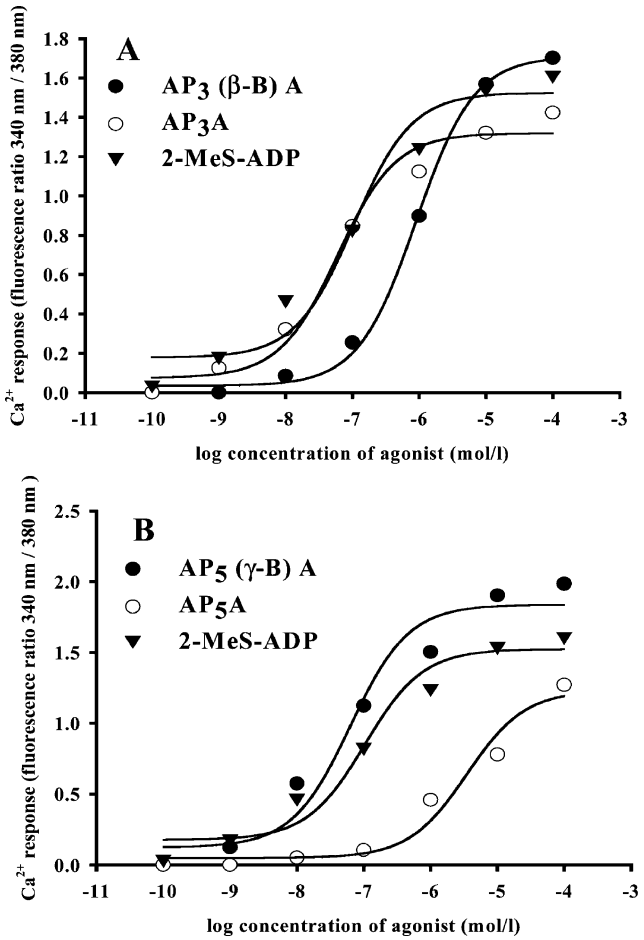



**Figure 1.** Concentration−response curves for $Ca^{2+}$ rise induced by diadenosine polyphosphate analogues in HEK 293 cells stably expressing the $rP2Y_1$-GFP receptor. HEK 293 cells stably expressing $rP2Y_1$-GFP receptor were grown in serum-containing medium. For measurements, the cells were preloaded with Fura-2/AM and then superfused with NaHBS containing $Ca^{2+}$ and stimulated for 1 min with different concentrations of agonist. The concentration-dependent rise in $Ca^{2+}$ is measured as the increase in the ratio between the fluorescence obtained at 340 and 380 nm upon stimulation with different concentrations of (A) 2-MeS-ADP (▼), $Ap_3A$ (○), and **3** (●) or (B) 2-MeS-ADP (▼), $Ap_5A$ (○), and **5** (●). The data are from $Ca^{2+}$ recordings in single cells and represent the mean ± SD from at least 50 cells for each value.

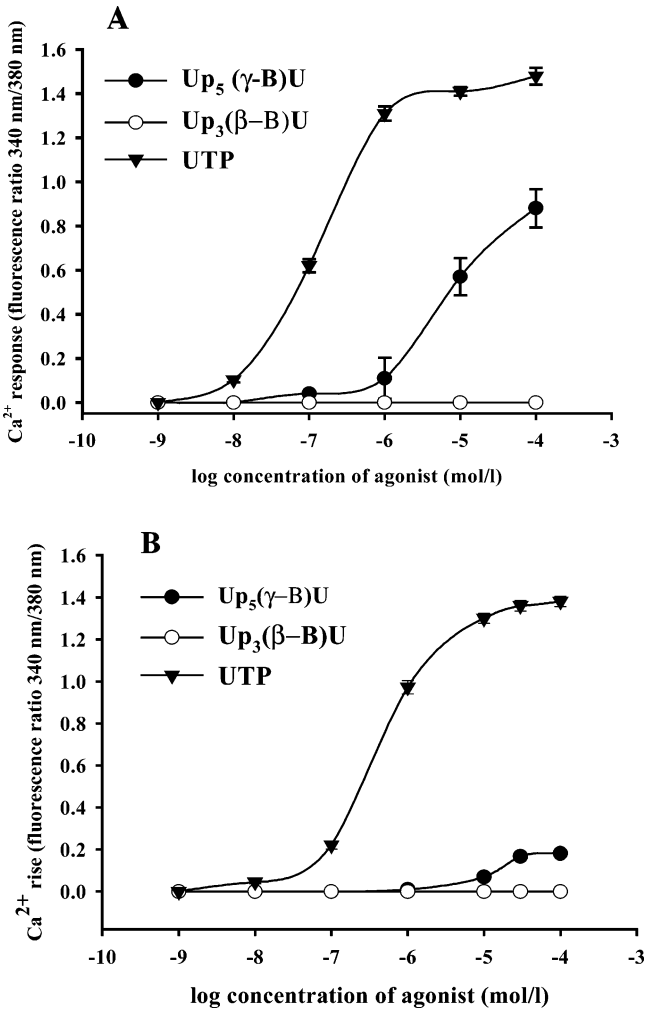



**Figure 2.** Concentration−response curves for $Ca^{2+}$ rise induced by diuridine polyphosphate analogues in HEK 293 cells expressing the $P2Y_2$-GFP receptor (A) and in A549 cells (B). HEK 293 cells stably expressing $rP2Y_2$-GFP were grown in serum-containing medium. For measurements the cells were preloaded with Fura-2/AM and then superfused with NaHBS containing $Ca^{2+}$ and stimulated for 1 min with different concentrations of agonist. The concentration-dependent rise in $Ca^{2+}$ is measured as the increase in the ratio between the fluorescence obtained at 340 and 380 nm upon stimulation with different concentrations of UTP (▼), **6** (○), and **4** (●). (B) $Ca^{2+}$ rise induced by UTP (▼), **6** (○), and **4** (●) in A549 cells. Cells were loaded with Fura-2 and then stimulated as $rP2Y_2$-GFP cells, with different concentrations of agonists, and the rise in $[Ca^{2+}]_i$ was measured. The data are from single-cell measurements and represent the mean ± SD from at least 50 cells for each value.

(Figure 4). These responses to **3** and **5** are almost negligible, compared to the $Ca^{2+}$ response of 1.5 that is obtained with stimulation of the cells with 100 $\mu$M UTP or ATP, which are the natural agonists at the $P2Y_2$ receptor.

**Hydrolytic Stability of Analogues 3−6.** To explore the suitability of the new P2Y-R agonists (**3**−**6**) as drug candidates, we evaluated their hydrolytic stability. The effect of the borane group on the hydrolytic stability of $Np_n(B)N$ analogues was monitored by $^{31}P$ NMR spectroscopy under physiological conditions, pH 7.4/37 °C, and conditions simulating gastric juice, pH 1.4/37 °C.

Thus, $^{31}P$ NMR spectra of **4** and **5** tri- and pentasodium salts, respectively, in Tris/HCl buffer (in $H_2O$), pH 7.4, were recorded during 4 weeks at 1−3-day intervals at 37 °C. Under these conditions, compounds **4** and **5** exhibited unusual stability. The decomposition of compound **5** was less than 15% as judged by the signal for $P_\alpha$ of ADP product at 2.5 ppm, and no decomposition at all was observed for compound **4**. In addition, no deboranation products could be detected.

Chemical stability under conditions simulating gastric juice (pH 1.4/37 °C) was evaluated with **3**, **4**, and **5** sodium salts in KCl/HCl buffer. $^{31}P$ NMR spectra were recorded during 3 h at 10 min intervals (e.g., Figure 5). For compounds **3** and **4**, increasing amounts of AMP/UMP were observed in addition to starting material **3** or **4**. However, $^{31}P$ NMR spectrum did not show a quartet at ca. 95 ppm typical of protonated inorganic $BP_i$, in addition to the AMP/UMP phosphate singlet.[26] Mass spectrometric analysis (FAB negative) of the hydrolytic mixture of **3** after neutralization showed, in addition to a peak at 778.6 *m*/*z* ($M + Na^+ + 2H^+$), a peak at 423.5 *m*/*z* corresponding to ADP-$\beta$-B. Namely, hydrolysis does not occur symmetrically to produce two AMP/UMP molecules and one $BP_i$. Instead, hydrolysis occurs at the $P_\beta$ position to provide one AMP/UMP molecule and one ADP-$\beta$-B/UDP-$\beta$-B molecule.

The intensity changes of the nucleoside monophosphate (NMP) $^{31}P$ signal ($\delta = 1$ ppm) with time were fit to a pseudo-first-order exponential decay rate equation with respect to **3**/**4**

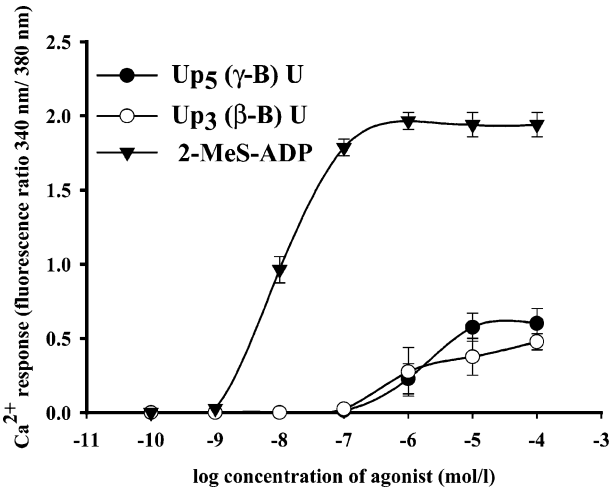



**Figure 3.** Concentration−response curves for $Ca^{2+}$ rise induced by diuridine polyphosphate analogues in 1321N1 cells stably expressing $hP2Y_1$-GFP receptor. 1321N1 cells stably expressing hP2Y1-GFP receptor were grown in serum-containing medium. For measurements, the cells were preloaded with Fura-2/AM and then superfused with NaHBS containing $Ca^{2+}$ and stimulated for 1 min with different concentrations of agonist. The concentration-dependent rise in $Ca^{2+}$ is measured as the increase in the ratio between the fluorescence obtained at 340 and 380 nm upon stimulation with different concentrations of 2-MeS-ADP (▼), **6** (○), and **4** (●).

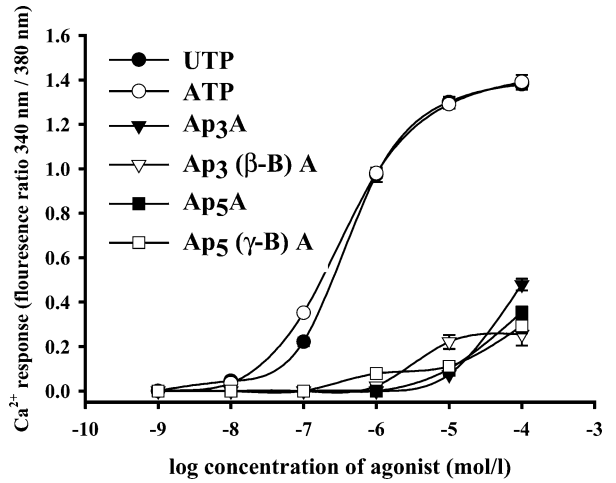



**Figure 4.** Concentration−response curves for $Ca^{2+}$ rise induced by diadenosine polyphosphate analogues in A549 cells. A549 cells were grown in serum-containing medium. For measurements, the cells were preloaded with Fura-2/AM and then superfused with NaHBS containing $Ca^{2+}$ and stimulated for 1 min with different concentrations of agonist. The concentration-dependent rise in $Ca^{2+}$ is measured as the increase in the ratio between the fluorescence obtained at 340 and 380 nm upon stimulation with different concentrations of UTP (●), ATP (○), $Ap_3A$ (▼), **3** (▽), $Ap_5A$ (■), and **5** (□). The data are from $Ca^{2+}$ recordings in single cells and represent the mean ± SD from at least 50 cells for each value.

concentration. The calculated hydrolysis rate constants determined at pH 1.4/37 °C for **3** and **4** were $1.52 \times 10^{-4}$ $s^{-1}$ and $1.65 \times 10^{-4}$ $s^{-1}$, respectively, corresponding to $t_{1/2}$ of 1.3 and 1.2 h.

Compound **5** was also subjected to hydrolysis at pH 1.4/37 °C, and $^{31}P$ NMR spectra were recorded during 1 h at 10 min intervals (Figure 5). However, unlike compounds **3** and **4**, compound **5** proved to be less stable. $^{31}P$ NMR spectra indicated the gradual increase of an inorganic protonated $BP_i$ signal at ca. 95 ppm and the concomitant decrease of $P_\beta$ signal of **5**. The hydrolysis rate constant for **5**, determined at pH 1.4/37 °C, was

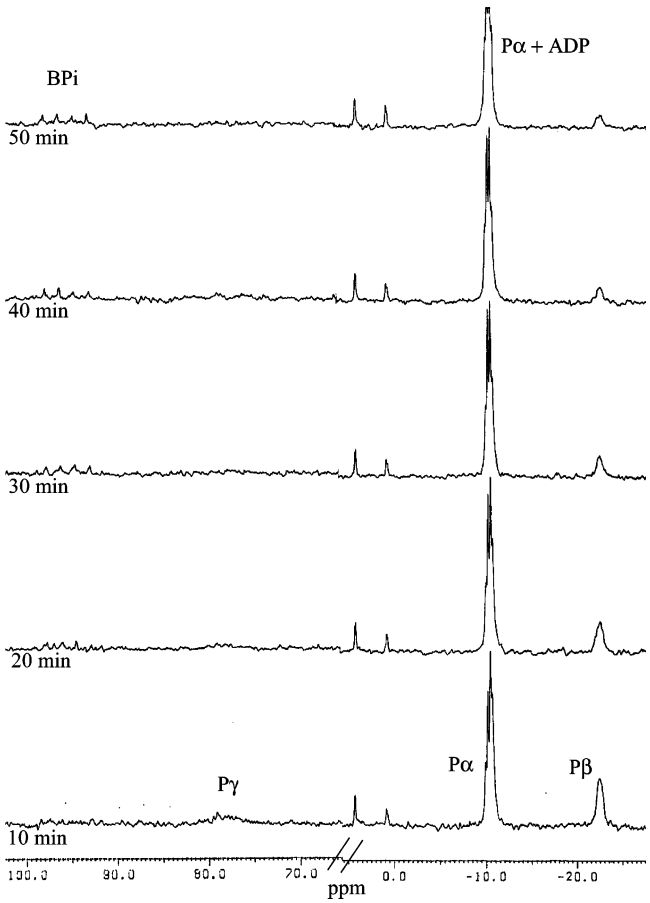



**Figure 5.** Hydrolysis of $Ap_5(\gamma$-B)A, **5**, under gastric juice-simulating conditions monitored by $^{31}P$ NMR at 81 MHz. $^{31}P$ NMR spectra of hydrolysis of 0.016 M **5** pentasodium salt, in KCl/HCl buffer at pH 1.4 and 37 °C, were recorded for 3 h at 10 min intervals.

based on the absolute intensity changes of the $P_\beta$ signal of **5** with time fit to a pseudo-first-order exponential decay rate equation. The calculated hydrolysis rate constant for **5** was $6.48 \times 10^{-4}$ $s^{-1}$, corresponding to a $t_{1/2}$ of 17.8 min.

**Hydrolysis of $Np_n$(B)N Analogues by Human NPP1 and NPP3.** Dinucleoside polyphosphates are substrates of certain members of the ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) family.[30] We, therefore, investigated if derivatives (**3**−**6**) could be hydrolyzed by human NPP1 or NPP3. First, COS-7 cells were transfected with plasmids encoding each enzyme, and crude cell lysates were prepared and tested for NPP activity. While control cells did not metabolize $Ap_3A$ or $Ap_5A$ (data not shown), NPP-transfected cell lysates hydrolyzed all dinucleotide polyphosphates tested [$Ap_3A$, $Ap_5A$, and $Np_n$-(B)N derivatives (**3**−**6**); Figure 6]. Human NPP1 and NPP3 hydrolyzed $Np_5(\gamma$-B)N to NMP + $Np_4(\gamma$-B). The latter degradation product appeared as two symmetrical peaks in the reverse-phase HPLC chromatogram, corresponding to the two diastereoisomers of chiral $Np_4(\gamma$-B).

For $Np_3(\beta$-B)N, the NMP increase corresponded to double the amount of substrate decrease as measured by HPLC analysis. This unexpected high amount of NMP could be due to the hydrolytic instability of $Np_2(\beta$-B) in the acidic medium added to stop the enzymatic reaction. Indeed, acidic hydrolysis of $Np_2$-($\beta$-B) results in the formation of NMP and inorganic boranophosphate.

Compound **3** was hydrolyzed by NPP1 and NPP3 at a velocity of 106 ± 7 and 65 ± 17 nmol of $Np_nN$ $min^{-1}$ $mg^{-1}$, respectively, corresponding to decreases of about 40% ± 2%

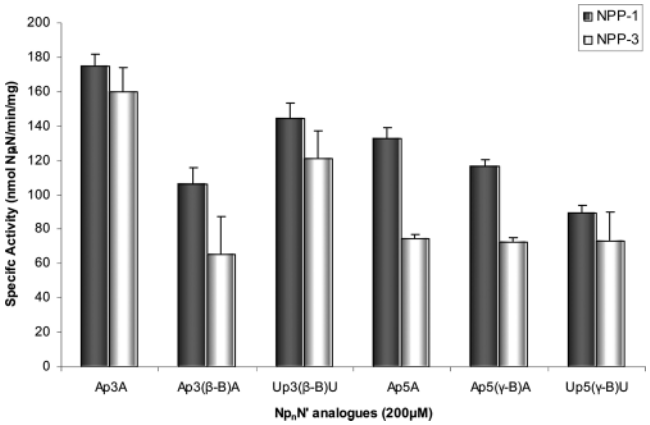



**Figure 6.** Hydrolysis of dinucleotide poly(borano)phosphate analogues by human NPP1 and NPP3. Enzymatic assays were carried out with protein extracts from transiently transfected COS-7 cells in the presence of 0.2 mM diadenosine polyphosphates ($Ap_3A$, $Ap_5A$, and analogues **3** and **5**) and diuridine polyphosphates (analogues **4** and **6**), as described under Materials and Methods. Human NPP1 (dark bars) and human NPP3 (light bars) hydrolyzed all dinucleoside poly(borano)phosphates tested. Specific activity (nanomoles of $Np_nN$ per minute per milligram) corresponds to substrate decrease and is expressed as the average ± SD of two separate experiments performed in triplicate.

and 59% ± 8% of the hydrolysis rate of $Ap_3A$ (175 ± 7 and 159 ± 18 for NPP1 and NPP3 respectively). When the borano modification was placed further from the cleavage site, the activity was only slightly reduced. Compound **5** was hydrolyzed at 88% ± 6% and 9% ± 3% the rate of $Ap_5A$, for NPP1 and NPP3, respectively. The modified diuridine polyphosphates (compounds **4** and **6**) were hydrolyzed by human NPP1 and NPP3, like diadenosine polyphosphates, with comparable rates of hydrolysis (Figure 6).

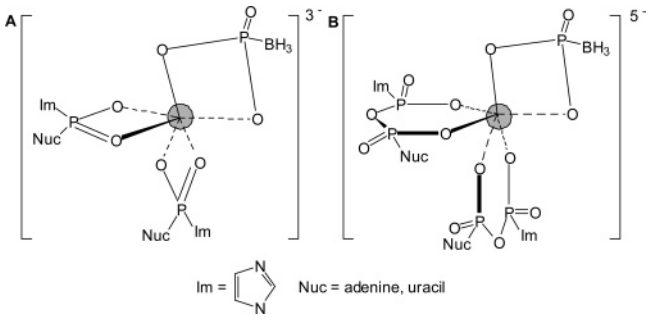
## Discussion

Diadenosine polyphosphates represent an important class of biomolecules that regulate a large number of biological functions ranging from control of vascular tone[31] and neurotransmission[32] to apoptosis and cell cycle.[33] Diadenosine and diuridine polyphosphates are assumed to act via the P2X- and the P2Y-Rs or via other specific receptors.[34,35] In an attempt to identify potent and stable dinucleoside polyphosphate $P2Y_1$/$P2Y_2$-R ligands, we developed a novel series of boranophosphate bioisosteres of $Np_nN$ analogues.

**Synthesis of $Np_n$(B)N Analogues.** Boranophosphate bioisosteres of diadenosine and diuridine polyphosphate (**3**−**6**) were synthesized from the corresponding nucleoside phosphoroimidazolides (NMP-Im/NDP-Im) and an inorganic building block, $BP_i$, in the presence of $Mg^{2+}$ ions.

Dinucleoside poly(borano)phosphate products **3**−**6** result probably due to a *preorganization* of the P-acceptor ($BP_i$) and two P-donors (nucleoside phosphoroimidazolides) coordinated with one $Mg^{2+}$ ion. The $Mg^{2+}$ ion probably stabilizes a folded structure (Scheme 3) involving two NMP-Im/NDP-Im and one $BP_i$ ion. This structure provides the correct orientation and proximity for nucleophilic attack of $BP_i$ on both nucleoside phosphoroimidazolide molecules. In addition, the $Mg^{2+}$ ion partially masks the phosphate negative charges, thus exposing the phosphate electrophilic center. The three reactant molecules occupy all six octahedral positions around the $Mg^{2+}$ ion (Scheme 3). Coordination of the hard $Mg^{2+}$ ion occurs exclusively at the oxygen atoms with no coordination with N7 of the adenine ring.[36] Clearly, this $Mg^{2+}$ ion provides only a partial neutralization of the phosphoryl donor and acceptors. Full neutralization is provided by the tributylammonium counterions.

**Scheme 3.** Proposed Structures for $Mg^{2+}$ Complexes Producing Products **3** and **4** (A) and Products **5** and **6** (B)




In this way, the template effect of the $Mg^{2+}$ ion dictates the exclusive formation of $Np_n$(B)N products and not nucleoside boranophosphate, $Np_n$(B), products. This template effect is observed for $Mg^{2+}$ ions but not for $Zn^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ ions. $Mg^{2+}$ ions coordinate only with the phosphate residue, whereas softer metal ions such as $Zn^{2+}$/$Ni^{2+}$/$Cu^{2+}$ coordinate with the adenine N7 in addition to the phosphate residues. Therefore, no preorganization of the reactants with medium-soft metal ions is possible.

$Ca^{2+}$ ion also does not catalyze the formation of $Np_n$(B)N, although it does not coordinate with the adenine N7. The difference between the roles of $Ca^{2+}$ and $Mg^{2+}$ ions in the reaction may be due to their different coordination numbers, 7 and 6, respectively. The relatively larger $Ca^{2+}$ ion has a higher coordination number (usually 7)[37] and probably cannot induce the desired orientation of P-donor and acceptors required for the formation of $Np_n$(B)N.

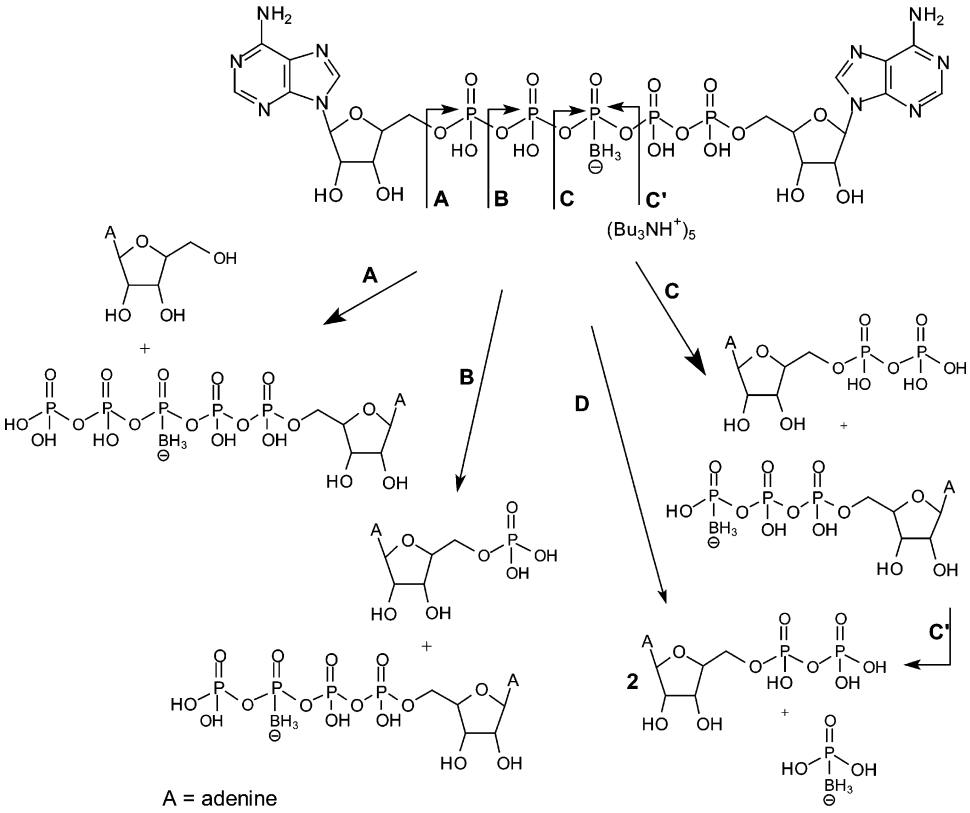
In contrast to our results, $Mg^{2+}$ was reported to induce ADP/ATP but not $Ap_3A$/$Ap_5A$ formation from adenosine 5′-phosphorimidazolide and inorganic phosphates in aqueous solutions.[21] Furthermore, when we repeated the reaction in buffer solution (pH 7), neither $Np_n$(B)N nor $Np_n$(B) products were formed. The reason for that may be $Mg^{2+}$ outer-sphere complexes that may be formed in water. Thus, in various complexes, for example, in tRNA, coordination of $Mg^{2+}$ occurs via bridging water molecules (outer-sphere coordination).[36] In such a complex, the reactants (P-donors and -acceptor) are too distant from each other, and no $Np_n$(B)N can be formed. However, in DMF, in which no outer-sphere complexes are formed, the P-donors and -acceptor are closer to each other and a nucleophilic attack of $BP_i$ on the phosphoroimidazolide is feasible, resulting in the $Np_n$(B)N product.

On the basis of the above template hypothesis, the coordination of $Mg^{2+}$ to both the phosphoroimidazolide oxygen and phosphoroimidazolide nitrogen is unlikely. Therefore, it seems that $Mg^{2+}$ does not play a direct role in the phosphorimidazole P−N bond cleavage.

The $Mg^{2+}$ ion template effect was observed only with $BP_i$ and not with $P_i$. As of yet, the origin of this difference is not clear. However, this effect may be correlated with the unique clustering tendency of $BP_i$ as opposed to that of $P_i$.[26]

**Activity of $Np_n$(B)N Analogues at P2Y-Rs.** We first measured the effect of $Ap_3$($\beta$-B)A and $Ap_5$($\gamma$-B)A to elicit a $Ca^{2+}$ release in HEK 293 cells stably expressing the $rP2Y_1$-GFP receptor, as $Ap_3A$ and $Ap_5A$ are more potent in elevating intracellular $Ca^{2+}$ via the $P2Y_1$ receptor than via the $P2Y_2$ receptor[38] (unpublished results). In the case of **3**, the borano modification reduces the potency of the substance at the $P2Y_1$ receptor in terms of $Ca^{2+}$ response, when compared with the

**Scheme 4.** Possible Hydrolytic Pathways of Dinucleoside Poly(borano)phosphate Analogues **3**−**6**




commercially available 2-MeS-ADP and $Ap_3A$. In the case of $Ap_5A$, the inclusion of the borano modification dramatically improves the potency of the compound. This is reflected in the $EC_{50}$ value obtained for **5** ($6.3 \times 10^{-8}$ M), which is slightly lower than that of 2-MeS-ADP ($1.0 \times 10^{-7}$ M). We tested the above substances also for their efficacy to stimulate $P2Y_2$ receptors in A549 cells. All adenine dinucleotides with or without the borano modification were virtually inactive at $P2Y_2$ receptors.

The modified diuridine polyphosphates were tested on HEK 293 cells stably expressing $rP2Y_2$-GFP receptor or on A549 cells. Diuridine polyphosphates have been shown before to be active on the $hP2Y_2$ receptor with the following order of potency: $Up_4U > Up_5U > Up_7U > Up_6U \gg Up_3U$. $Up_2U$ was inactive at the $P2Y_2$ receptor, and all the aforementioned $Up_nUs$ were inactive at the $hP2Y_1$ receptor.[11]

We observed that the borano-modified $Up_5U$ elicits a very weak $Ca^{2+}$ response in the A549 cells, which primarily express the $P2Y_2$ receptor and are $P2Y_1$ receptor-negative.[29] Analogue **6** causes a low-affinity response in the HEK 293 cells stably expressing the $P2Y_2$-GFP receptor, which seems to be due to some endogenous receptor that is different from the $P2Y_2$ receptor. Thus, the borano modification of $Up_5U$ completely diminished its ability to stimulate the $P2Y_2$ receptor. Since the amplitude of the $Ca^{2+}$ response for 100 $\mu$M **6** in HEK 293 cells stably expressing $P2Y_2$-GFP receptor was higher than that in A549 cells, this indicates that **6** does stimulate other endogenous P2Y receptors. In the case of HEK 293 cells, there is a high level of endogenous $P2Y_1$ receptor expression.[29] Therefore, we tested the effect of **6** on 1321N1 cells stably expressing $hP2Y_1$-GFP receptor. This analogue proved a weak agonist at the $hP2Y_1$ receptor, achieving only 20% of the response induced by 2-MeS-ADP.

Analogue **4** was not active at the $P2Y_2$ receptor in A549 cells or in HEK 293 cells. This indicates that the borano modification completely abolishes the potency of $Up_3U$ on the $P2Y_2$ receptor. On stimulation of the 1321N1 cells stably expressing the $hP2Y_1$-GFP with the borano-modified $Up_3U$, a weak $Ca^{2+}$ response compared to 2-MeS-ADP was observed. This indicates that $Up_3$-($\beta$-B)U functions as a weak agonist at the $P2Y_1$ receptor compared to $Up_3U$, which is totally inactive at this receptor in terms of $Ca^{2+}$ signaling.[11]

**Hydrolytic Stability of $Np_n$(B)N Analogues.** $Np_n$(B)N analogues may undergo several hydrolytic pathways (Scheme 4): (A) hydrolytic cleavage of the phosphodiester bond at $P_\alpha$, to produce the free nucleoside (N) and $Np_n(\beta/\gamma\text{-B})$; (B) hydrolytic cleavage of the phosphodiester bond at $P_\beta$, to release $Np_{n-1}(\beta/\gamma\text{-B})$ and NMP; (C) hydrolytic cleavage of the phosphodiester bond at the central boranophosphate moiety, $P_\beta/P_\gamma$, to release $Np_{2/3}(\beta/\gamma\text{-B})$ and $Np_{1/2}$; and (D) hydrolytic cleavage of the phosphodiester bond at the central boranophosphate moiety, $P_\beta/P_\gamma$, to release $BP_i$ and two molecules of $Np_{1/2}$.

Hydrolysis of the N-glycosidic bond in analogues **3**−**6** under gastric juice conditions was not considered here. This is because in a previous study we found that no degradation products could be observed for adenosine and 2-benzylthioadenosine under these conditions, even after 14 days.[39] Likewise, deboranation of the $Np_n$(B)N analogue to the corresponding $Np_n$N analogue was not considered here, since we showed previously that the deboranation reaction at pH 1.4 proceeds relatively slowly (at room temperature, pH 2, $k = 7 \times 10^{-7}$ s$^{-1}$, $t_{1/2} = 275$ h)[17] as compared to the hydrolysis of the phosphodiester bond.

Although the signals of ADP-$\beta$-B/UDP-$\beta$-B hydrolytic products were hidden by the signals of $P_\beta$ and $P_\alpha$ of compounds **3**/**4**, mass spectrometric analysis revealed that the hydrolytic mechanism of analogues **3** and **4** is asymmetric, corresponding to path C in Scheme 4. Hydrolysis of compound **5**, however, did not follow path C. The hydrolytic reaction of **5** results in the formation of two molecules of ADP and one $BP_i$ molecule. These products may result from either paths C and C′ or path

D in Scheme 4. A stepwise hydrolysis of **5** may occur by asymmetric hydrolytic cleavage to form $Ap_3$($\gamma$-B) and ADP, followed by hydrolysis of the former to ADP and $BP_i$. Alternatively, a concerted hydrolysis may occur (path D), releasing two ADP molecules and $BP_i$. On the NMR time scale it is impossible to differentiate between these two mechanisms. ATP-$\gamma$-B was not detected, although it might have been formed but underwent rapid hydrolysis to ADP and $BP_i$. The disappearance of $P_\beta$ and the formation of inorganic boranophosphate occur at the same rate in the NMR time scale.

Apparently, nucleophilic attack of a water molecule on compound **5** occurs preferentially on $P_\gamma$. This phosphorus atom is probably more electrophilic than $P_\alpha$ and $P_\beta$, although $P_\alpha$ and $P_\beta$ are neutral at pH 1.4. The reason is probably the P−$BH_3$ coordinate bond that reduces electron density on P as compared to phosphate.

A preferential nucleophilic attack on the boranophosphate moiety at pH 1.4 was observed also for compounds **3** and **4**. Previously, we observed similar hydrolytic behavior of a related scaffold, adenosine α-borano-triphosphate (ATP-α-B) (**2**). The hydrolytic site at this triphosphate chain was also the boranophosphate moiety. Hydrolysis of ATP-α-B analogues yielded $BP_i$, $PP_i$, and nucleoside.[17]

The base, either adenine or uracil, does not affect the hydrolytic stability of analogues **3**−**6**. Yet the length of the polyphosphate chain in these analogues makes a difference. Under gastric juice conditions, **5** is ca. 4-fold less hydrolytically stable as compared to its lower homologue, **3**. Analogue **3** in turn is slightly (2-fold) less stable than ATP-α-B analogues **2**, as reported before.[17]

However, under physiological conditions, **4** is by far more stable than ATP-α-B analogues ($t_{1/2}$ of the latter is ca. 1400 h), showing no decomposition even after 1 month. This extreme hydrolytic stability of **4** may be due to the lack of a terminal phosphate.

The higher stability of analogues **3**−**6** at pH 7.4 versus 1.4 is due to the predominant polyanion form that retards the nucleophilic attack of a water molecule.

**Enzymatic Stability of $Np_n$(B)N Analogues.** Dinucleoside polyphosphates have considerably longer half-lives in vivo than nucleotides (NTP, NDP, and NMP),[4] which are rapidly metabolized by members of various enzyme families: nucleoside triphosphate diphosphohydrolases (NTPDases), ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs), calcium-activated nucleotidase (CAN), and alkaline phosphatases.[4,40−43] Naturally occurring dinucleoside polyphosphates are substrates of only a few NPPs.[30] The dinucleoside poly(borano)phosphate derivatives (**3**−**6**) were hydrolyzed by both human NPP1 and NPP3. Substitution of the oxygen atom on the central phosphate of the dinucleoside polyphosphates by a $BH_3^-$ group did not interfere with the hydrolysis of **5**, while it slowed the hydrolysis of **3** by about 50%, as compared to the parent compound. This suggests that the presence of $BH_3^-$ near the cleavage site (NPPs cut after a NMP moiety)[41] confers some stability to the dinucleoside polyphosphates regarding enzymatic hydrolysis by NPP1 and NPP3. Compounds **4** and **6** could not be compared with $Up_3U$ and $Up_5U$, as they are not available commercially.

Analogues **3**−**6** can be converted by NPPs to NMP and NDP-$\beta$-B, for analogues **3** and **4**, or to NMP plus $Np_4$($\gamma$-B) for analogues **5** and **6**. Further hydrolysis of NMP by alkaline phosphatase or ecto-5′-nucleotidase to nucleoside, like adenosine, could activate P1 receptors (A1, $A_{2A}$, $A_{2B}$, or A3). Moreover, the degradation products NDP-$\beta$-B and $Np_4$($\gamma$-B) could possibly activate some P2-Rs. Such potential effects should be considered prior to the use of these molecules for in vivo studies.

## Conclusion

The beneficial effect of the boranophosphate bioisostere has been demonstrated here in achieving one of the most potent $P2Y_1$-R agonists currently known, **5**. Furthermore, this analogue is practically inactive at $P2Y_2$-R. The effect of the boranophosphate bioisostere on improving metabolic stability (resistance to NPP), as compared to the parent compound, was observed for **3**. Apparently, the effect of the borano modification is pronounced only in the vicinity of the cleavage site ($P_\alpha$). We will apply the promising scaffold of $Ap_5$($\gamma$-B)A in the design of future metabolically stable analogues.

## Experimental Details

**General.** All air- and moisture-sensitive reactions were performed in flame-dried, nitrogen-flushed, two-neck flasks sealed with rubber septa, and the reagents were introduced with a syringe. Progress of reactions was monitored by thin-layer chromatography (TLC) on precoated Merck silica gel plates (60F-254). Compounds were characterized by nuclear magnetic resonance on an AC-200 spectrometer. $^1$H NMR spectra were measured in $D_2O$, and the chemical shifts are reported in parts per million (ppm) relative to HOD (4.78 ppm) as an internal standard. Dinucleotides were characterized also by $^{31}$P NMR in $D_2O$, with 85% $H_3PO_4$ as an external reference. Dinucleotides were desorbed from a glycerol matrix under FAB (fast atom bombardment) conditions at low and high resolution. Primary purification of the dinucleotides was achieved on a liquid chromatography (LC) (Isco UA-6) system with a Sephadex DEAE-A25 column, which was swelled in 1 M $NaHCO_3$ in the cold for 1 day. Final purification of the nucleotides was achieved on a high-performance liquid chromatography (HPLC) (Merck-Hitachi) system with a semipreparative reverse-phase (LiChrospher 60, RP-select-B) column. Conditions for LC and HPLC separation are described below. For analytical purposes, a LiChroCART LiChrospher 60 RP-select B column (250 mm × 4 mm) was used with a flow rate of 1 mL/min. The purity of the dinucleotides was evaluated on an analytical column in two different solvent systems. Solvent system I was (A) $CH_3OH$ and (B) 0.1 M TEAA, with isocratic elution. Solvent system II was (A) 5 mM tetrabutylammonium phosphate (TBAP) in methanol and (B) 60 mM ammonium phosphate and 5 mM TBAP in 90% water/10% methanol, with isocratic elution. Apparent pH values were measured on a Hanna Instruments pH-meter (HI 8521) equipped with an Orion microcombination pH electrode (9802). AMP·$H_2O$ (free acid) was purchased from Sigma−Aldrich Co. ADP (free acid) was purchased from ICN Biomedicals, Inc. UMP (free acid) and UDP ($Na^+$ salt) were purchased from MP Biochemicals, Inc.

**Typical Procedure for the Preparation of Boranated Dinucleoside Polyphosphate Derivatives (3−6).** ADP($Bu_3NH^+$)$_2$, AMP($Bu_3NH^+$)$_2$, and UMP($Bu_3NH^+$)$_2$ were prepared from the corresponding free acids and $Bu_3N$ (2 equiv) in EtOH. UDP($Bu_3NH^+$)$_2$ was prepared from UDP disodium salt. The latter salt was passed through a column of activated Dowex 50WX-8 200 mesh, $H^+$ form. The column eluate was collected in an ice-cooled flask containing tributylamine (2 equiv) and EtOH. The resulting solution was freeze-dried to yield UDP($Bu_3NH^+$)$_2$ as a viscous oil.

Bis(tributylammonium) AMP/UMP/ADP/UDP salt (0.23 mmol) was dissolved in dry DMF (3 mL), and CDI (186.5 mg, 1.15 mmol) was added. The resulting solution was stirred at room temperature for 2.5 h. Dry MeOH (74.6 $\mu$L, 1.15 mmol) was added. After 5 min, $BP_i$($Bu_3NH^+$)$_2$ (**7**) (214 mg, 0.46 mmol) in dry DMF (3 mL), and $MgCl_2$ (8 equiv) were added. The resulting solution was stirred at room temperature overnight. The semisolid obtained after evaporation of the solvent was chromatographed at room temperature on a Sephadex DEAE-A25 column, which was swelled in 1 M $NaHCO_3$. The separation was monitored by UV detection (Isco, UA-5) at 280 nm. A buffer gradient of water (800 mL) to 0.7 or

0.5 M $NH_4HCO_3$ (800 mL) was applied. The relevant fractions were pooled and freeze-dried to yield a white solid. Final purification was achieved on a semipreparative HPLC column.

**Reverse-Phase HPLC Purification.** The dinucleotide purification was achieved with a semipreparative reverse-phase Lichro-CART 250-10 column and isocratic elution [MeOH/100 mM triethylammonium acetate (TEAA), pH 7] with a flow rate of 6 mL/min. Fractions containing the same compound (similar retention time) were freeze-dried. The excess buffer was removed by repeated freeze-drying cycles, with the solid residue dissolved each time in deionized water. The triethylammonium counterions were exchanged for $Na^+$ by passing the pure dinucleoside poly(borano)-phosphate analogue through a Sephadex-CM C-25 ($Na^+$ form) column.

**Ap$_5$($\gamma$-B)A (5)** was obtained from ADP($Bu_3NH^+$)$_2$ (182 mg, 0.228 mmol) in 38% (43 mg) yield. The product was purified by HPLC isocratic elution with methanol/TEAA (11:89). Retention time was 9.25 min. $^1$H NMR ($D_2O$, 200 MHz): $\delta$ 8.45 (s, H-8, 1H), 8.13 (s, H-2, 1H), 6.00 (d, H-1′, 1H), H-2′ is hidden by the water signal, 4.60 (m, H-3′, 1H), 4.38 (m, H-4′, 1H), 4.30 (m, H-5′, 2H), 0−1.30 (m, $BH_3$, 3H) ppm. $^{31}$P NMR ($D_2O$, 81 MHz, pH 7): $\delta$ 77.0 (m, P$\gamma$, 1P), −10.5 (d, P$\alpha$, 2P), −22.0 (t, P$\beta$, 2P) ppm. HR FAB (negative) calcd for $C_{20}H_{31}{}^{11}BN_{10}O_{21}P_5$ 913.045, found 913.056 ($M^-$). Retention time 8.71 min (95% purity) with solvent system I (A:B 15:85); retention time 7.85 min (95% purity) with solvent system II (A:B 20:80).

**Ap$_3$($\beta$-B)A (3)** was obtained from AMP($Bu_3NH^+$)$_2$ (180 mg, 0.25 mmol) in 88% (90 mg) yield. The final purification was achieved by HPLC isocratic elution with methanol/TEAA (13:87). Retention time was 9.46 min. $^1$H NMR ($D_2O$, 200 MHz): $\delta$ 8.30 (s, H-8, 1H), 8.07 (s, H-2, 1H), 5.98 (d, H-1′, 1H), 4.60 (m, H-2′, 1H), 4.48 (m, H-3′, 1H), 4.31 (m, H-4′, 1H), 4.28 (m, H-5′, 2H), −0.9−1.2 (m, $BH_3$, 3H) ppm. $^{31}$P NMR ($D_2O$, 81 MHz, pH 7): $\delta$ 78.5 (m, P$\beta$, 1P), −10.8 (d, P$\alpha$, 2P) ppm. HR FAB (negative) calcd for $C_{20}H_{29}{}^{11}BN_{10}O_{15}P_3$ 753.112, found 753.123 ($M^-$). Retention time 6.65 min (99% purity) with solvent system I (A:B 17:83); retention time 5.88 min (97% purity) with solvent system II (A:B 23:77).

**Up$_5$($\gamma$-B)U (6)** was obtained from UDP ($Bu_3NH^+$)$_2$ (170.5 mg, 0.22 mmol) in 43% (46 mg) yield. The final purification was achieved by HPLC isocratic elution with methanol/TEAA (4:96). Retention time was 17 min. $^1$H NMR ($D_2O$, 200 MHz): $\delta$ 7.95 (d, $J$ = 8.2 Hz, H-5, 1H), 5.99 (d, $J$ = 5.1 Hz, H-1′, 1H), 5.97 (d, $J$ = 8.2 Hz, H-6, 1H), H-2′ and H-3′ are hidden by the water signal, 4.41 (m, H-4′, 1H), 4.25 (m, H-5′, 2H), −0.90−1.20 (m, $BH_3$, 3H) ppm. $^{31}$P NMR ($D_2O$, 81 MHz, pH 7): $\delta$ 77.0 (m, P$\gamma$, 1P), −10.7 (d, P$\alpha$, 2P), −22.6 (dd, P$\beta$, 2P) ppm. HR FAB (negative) calcd for $C_{18}H_{28}{}^{11}BN_4O_{25}Na_2P_3$ 910.965, found 910.966 ($M - 2H + 2Na)^-$. Retention time 6.70 min (98% purity) with solvent system I (A:B 6:94); retention time 6.17 min (99% purity) with solvent system II (A:B 12:88).

**Up$_3$($\beta$-B)U (4)** was obtained from UMP ($Bu_3NH^+$)$_2$ (177.2 mg, 0.255 mmol) in 78% (75 mg) yield. The final purification was achieved by HPLC isocratic elution with methanol/TEAA (6:94). Retention time was 6.33 min. $^1$H NMR ($D_2O$, 200 MHz): $\delta$ 8.00 (d, $J$ = 8.2 Hz, H-5, 1H), 5.99 (d, $J$ = 5.1 Hz, H-1′, 1H), 5.97 (d, $J$ = 8.2 Hz, H-6, 1H), H-2′ and H-3′ are hidden by the water signal, 4.38 (m, H-4′, 1H), 4.25 (m, H-5′, 2H), −0.90−1.20 (m, $BH_3$, 3H) ppm. $^{31}$P NMR ($D_2O$, 81 MHz, pH 7): $\delta$ 75.0 (m, P$\beta$, 1P), −11.0 (d, P$\alpha$, 2P) ppm. HR FAB (negative) calcd for $C_{18}H_{27}{}^{11}BN_4O_{19}P_3$ 707.057, found 707.062 ($M^-$). Retention time 6.86 min (98% purity) with solvent system I (A:B 5:95); retention time 8.34 min (95% purity) with solvent system II (A:B 10:90).

**Evaluation of Chemical Stability of Dinucleoside Poly-(borano)phosphates 3−6.** The stability of derivatives **3**−**6** in the appropriate buffer solutions (pH 7.4 or 1.4) was evaluated at 37 °C. $^{31}$P NMR was used for monitoring phosphate-chain degradation products. Dinucleoside polyboranophosphates tri- or pentasodium salts were dissolved in Tris/HCl buffer (pH 7.4, 0.45 mL), and $D_2O$ (0.6 mL). The pH was then adjusted to 7.4 with HCl and measured with a pH-meter. Final analogue concentration was ca. 0.02 M. $^{31}$P NMR spectra were recorded during 4 weeks at 3−7 day intervals at 37 °C. Percentage of phosphoester hydrolysis of Ap$_3$($\beta$-B)A/Up$_3$($\beta$-B)U is based on absolute integrations of monophosphate signal (2.5 ppm). Percentage of phosphodiester hydrolysis of dinucleoside pentaphosphate is based on absolute integrations of $P_\alpha$ signal of ADP or UDP products.

To evaluate chemical stability at pH 1.4, sodium salts of dinucleoside polyboranophosphate analogues were dissolved in KCl/HCl buffer (pH 1.4, 0.44 mL) and $D_2O$ (0.06 mL). The final pH was then adjusted with HCl and measured with a pH-meter (final concentration 0.016 M). $^{31}$P NMR spectra of Ap$_3$($\beta$-B)A and Up$_3$-($\beta$-B)U were recorded during 3 h at 10 min time intervals at 37 °C. Number of scans in all experiments was 1200; number of experiments was 17. The hydrolysis rate was determined by measurements of absolute integration of $P_\alpha$ (2.5 ppm) signal as a function of time and was fitted to a pseudo-first-order reaction model.

$^{31}$P NMR spectra of Ap$_5$($\gamma$-B)A were recorded during 1 h at 10 min time intervals at 37 °C. Number of scans in all experiments was 1200; number of experiments was 6. The hydrolysis rate was determined by measurement of the absolute integration of $P_\beta$ signal as a function of time and was fitted to a pseudo-first-order reaction model.

**Biochemical Assays: (A) Materials.** Geneticine (G418 sulfate) was purchased from Calbiochem; poly-L-lysine (PLL), UTP, 2-MeS-ADP (Sigma, Deisenhofen); Ham's-F12, Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (10 000/10 000 units/mL), trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05%/0.02%), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium-methyl sulfate (DOTAP), FuGENE 6 (Roche, Germany); FCS (fetal calf serum) (Seromed, Biochrom, Berlin); cell culture dishes (Nunc, Wiesbaden); coverslips (22 mm) (OmniLab); Fura 2-AM (Biomol, Hamburg/Molecular Probes). Plasmids encoding NPP1 and NPP3 were obtained from Dr. Goding and Dr. Sano, respectively.[44,45]

**(B) Cell Culture and Transfection.** The rP2Y$_2$ receptor cDNA[46] was subcloned into pEGFPN2 (Clontech), with the GFP tag on the C-terminus. HEK 293 cells were used for transfection, as these cells endogenously express this receptor subtype. Cell culture and transfection of HEK 293 was carried out as described.[28] The HEK 293 cells were transfected with 5 $\mu$g/mL DNA in serum-free medium with DOTAP for 8 h. After 8 h the medium was replaced with growth medium, and positive cells were selected with 1 mg/mL G418. Transfected cells were grown in medium consisting of DMEM/Ham's-F12 (1:1), supplemented with 10% FCS, 100 IU/mL penicillin, and 100 IU/mL streptomycin in a 5% $CO_2$/95% air humidified atmosphere at 37 °C. The cells were plated at a density of $5 \times 10^5$ to $1 \times 10^6$ cells/dish (Ø = 50 mm) containing poly-L-lysine (PLL) (0.01%) precoated coverslips (Ø =22 mm). HEK 293 cells expressing P2Y$_1$-GFP[28] were handled in the same way. A549 cells were grown in DMEM medium supplemented with 10% FCS, 100 IU/mL penicillin, and 100 IU/mL streptomycin in a 5% $CO_2$/95% air humidified atmosphere at 37 °C.

1321N1 cells were grown in DMDEM medium supplemented with 5% FCS, 100 IU/mL penicillin, and 100 IU/mL streptomycin in 10% $CO_2$/90% air humidified atmosphere at 37 °C. The cDNA for hP2Y1 receptor was generated from HEK 293 cells and was subcloned into pEGFPN1 (Clontech) with the GFP on the C-terminus. The cells were transfected with 1 $\mu$g/mL DNA in serum-free medium by use of FuGENE-6 for 8 h. After 8 h the medium was replaced with growth medium and positive cells were selected with 1 mg/mL G418.

NPPs were produced by transiently transfecting COS-7 cells in 10 cm plates by use of Lipofectamine (Invitrogen), as previously described.[47] Briefly, 70−90% confluent cells were incubated for 5 h at 37 °C in DMEM in the absence of fetal bovine serum (FBS) with 6 $\mu$g of plasmid DNA and 24 $\mu$L of Lipofectamine reagent. Then, an equal volume of DMEM containing 20% FBS was added, and 40−44 h later cells were collected for analysis.

**(C) $[Ca^{2+}]_i$ Measurements.** The cells were plated on PLL-coated plates and single cell measurement was done after 3 days, when the cells were 40−60% confluent. The changes in free intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) were measured, as described before,[48]

after preincubation of the cells with 2 $\mu$M Fura-2AM for 30 min in NaHBS [HEPES-buffered saline solution: 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, and 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)/Tris pH 7.4]. Then the cells were stimulated under continuous superfusion of prewarmed NaHBS at 37 °C with different concentrations of UTP or the different agonist at 37 °C. Fluorescence intensity was recorded alternately at 340 and 380 nm excitation and 520 nm emission. Changes were monitored in single cells bathed in a perfusion chamber, which was placed on the microscope stage of a fluorescence imaging system from TILL Photonics with a 40× oil immersion objective and a flow rate of 1 mL/min.[18]

**(D) Preparation of Membrane Fractions.** NPP1- and NPP3-transfected cells were washed three times with Tris−saline buffer at 4 °C, harvested by scraping in 95 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 45 mM Tris, pH 7.5, and washed twice by centrifugation at 300*g* for 5 min at 4 °C. Cells were resuspended in the harvesting buffer containing 10 $\mu$g/mL aprotinin and sonicated. Nuclear and cellular debris were discarded after another centrifugation as described above. Glycerol was added to the resulting supernatant at a final concentration of 7.5%. Samples were kept at −80 °C until used. Protein concentration was estimated by the Bradford microplate assay, with bovine serum albumin as the standard reference.[49]

**(E) Measurement of Enzymatic Hydrolysis of Dinucleoside Poly(borano)phosphate Analogues.** The hydrolysis of $Ap_nA$ and compounds **3**−**6** was measured in membrane preparations from NPP1- and NPP3-transfected cells. Enzyme activity was measured at 37 °C in 0.2 mL of the following incubation medium: 1 mM $CaCl_2$, 200 mM NaCl, 10 mM KCl, and 100 mM Tris, pH 8.5, as described by Vollmayer et al.[30] Enzyme preparation was added to the incubation mixture and preincubated for 3 min at 37 °C. Reaction was started with 0.2 mM substrate ($Ap_3A$, $Ap_5A$ or analogues **3**−**6**), stopped after 20 min by transferring an aliquot of 100 $\mu$L from the reaction mixture to 125 $\mu$L of ice-cold 1 M perchloric acid. The samples were centrifuged for 5 min at 10000*g*. Supernatants were neutralized with 1 M KOH (4 °C) and centrifuged for 5 min at 10000*g*. An aliquot of 20 $\mu$L was separated by reverse-phase HPLC to evaluate the nucleotide content of each reaction sample. Substrate decrease was measured for the dinucleotide analogues **3** and **4**. For compounds **5** and **6,** the detection of NMP increase was evaluated. In the latter case, better precision was obtained for NMP determination as the peaks of compounds **5** and **6** appeared at longer retention times. For all substrates used in this study, the final specific activities are reported as substrate decrease and are expressed as micromoles of $Np_nN$ per minute per milligram and correspond to the average ± SD of two separate experiments performed in triplicate.

**(F) Separation and Quantification of Nucleotides by HPLC.** Diadenosine polyphosphates ($Ap_3A$, $Ap_5A$, and analogues **3** and **5**) were eluted from a 15 cm × 4.6 mm, 3 $\mu$m Supelcosil LC-18-T column (Supelco) with a mobile phase composed of 25 mM TBA, 5 mM EDTA, and 100 mM $KH_2PO_4/K_2HPO_4$ plus the indicated concentration of MeOH, pH 7.0, with a step gradient of methanol at a flow rate of 1 mL/min. The step gradient was carried out for 25 min with the mobile phase with 2% MeOH, then 10% MeOH for 15 min, and finally equilibrated with 2% MeOH for 15 min before the next run. Diuridine polyphosphates (analogues **4** and **6**) were resolved with a Supelcosil LC-18-T column (25 cm × 4.6 mm, 5 $\mu$m, Supelco) under isocratic conditions and eluted with 16.7 mM TBA, 3.3 mM EDTA, and 66.7 mM $KH_2PO_4/K_2HPO_4$ at pH 7.0 in 5% (v/v) methanol.

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