Adenosine 5′-*O*-(1-Boranotriphosphate) Derivatives as Novel $P2Y_1$ Receptor Agonists

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P2-receptors (P2-Rs) represent important targets for novel drug development. Most ATP analogues proposed as potential drug candidates have shortcomings such as limited receptor-selectivity and limited stability that justify the search for new P2-R agonists. Therefore, a novel series of nucleotides based on the adenosine 5′-*O*-(1-boranotriphosphate) (ATP-α-B) scaffold was developed and tested as $P2Y_1$-R agonists. An efficient four-step one-pot synthesis of several ATP-α-B analogues from the corresponding nucleosides was developed, as well as a facile method for the separation of the diastereoisomers (A and B isomers) of the chiral products. The potency of the new analogues as $P2Y_1$-R agonists was evaluated by the agonist-induced Ca^{2+} release of HEK 293 cells stably transfected with rat-brain $P2Y_1$-R. ATP-α-B A isomer was equipotent with ATP ($EC_{50} = 2 \times 10^{-7}$ M). However, 2-MeS− and 2-Cl− substitutions on ATP-α-B (A isomer) increased the potency of the agonist up to 100-fold, with EC_{50} values of 4.5×10^{-9} and 3.6×10^{-9} M, compared to that of the ATP-α-B (A isomer). Diastereoisomers A of all ATP-α-B analogues were more potent in inducing Ca^{2+} release than the corresponding B counterparts, with a 20-fold difference for 2-MeS-ATP-α-B analogues. The chemical stability of the new $P2Y_1$-R agonists was evaluated by ^{31}P NMR under physiological and gastric-juice pH values at 37 °C, with rates of hydrolysis of 2-MeS-ATP-α-B of 1.38×10^{-7} s^{-1} ($t_{1/2}$ of 1395 h) and 3.24×10^{-5} s^{-1} ($t_{1/2} = 5.9$ h), respectively. The enzymatic stability of the new analogues toward spleen NTPDase was evaluated. Most of the new analogues were poor substrates for the NTPDase, with ATP-α-B (A isomer) hydrolysis being 5% of the hydrolysis rate of ATP. Diastereoisomers A and B exhibited different stability, with A isomers being significantly more stable, up to 9-fold. Furthermore, A isomers that are potent $P2Y_1$-R agonists barely interact with NTPDase, thus exhibiting protein selectivity. Therefore, on the basis of our findings, the new, highly water-soluble, $P2Y_1$-R agonists may be considered as potentially promising drug candidates.

Introduction

ATP serves as an important extracellular signaling molecule, transmitting its signal to the cell through the ubiquitous family of ATP receptors (P2-Rs): P2Y-Rs, which are G-protein-coupled receptors; P2X-Rs, which are ion channels.[1] ATP exerts significant biological actions on various tissues and organs.[2] Therefore, P2-Rs represent important targets for novel drug development regarding different pathophysiological conditions.[3]

The development of P2-Rs agonists as potential drugs involves the identification of potent and receptor-selective agonists, usually nucleotide derivatives, that are chemically and metabolically stable. Several ATP analogues have been proposed as potential drug candidates.[3,4] Yet, most of these analogues have shortcomings such as limited receptor selectivity and limited stability.[3b] Thus, the search for new, potent, and stable P2-R agonists continues.

In previous reports, we and others established molecular recognition determinants of agonists in the $P2Y_1$-R.[5,6] We demonstrated that neither tautomerism nor electronic distribution changes of the adenine ring, due to substitution of the ring, play a major role in determining binding specificity of adenine nucleotides to the receptor. We concluded that steric, rather than electronic, effects play a major role in ligand binding to the $P2Y_1$-R. Furthermore, hydrophobic interactions and H-bonding of a C2 substituent appear to be major determinants of $P2Y_1$-R ligand affinity, enhancing significantly the potency of C2-substituted ATP analogues relative to ATP.[5,7]

In this study, we aimed at developing novel, potent, chemically, and enzymatically stable $P2Y_1$-R agonists as potential drugs. C2-substitution of the ATP scaffold, which has been proven to be essential for enhancement of potency and stability of the agonist, was retained in the new series of analogues. In addition, we aimed at improving binding interactions of $P2Y_1$-R with the 5′-triphosphate moiety of ATP by altering the properties of the triphosphate chain.

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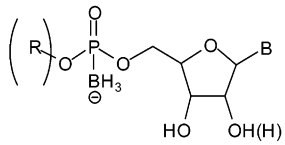
P-boronated nucleotides, **1**, were proposed in the pioneering work of Ramsay-Shaw et al. as important tools for biochemists.[8] Substitution of BH_3 for nonbridging O in nucleotides introduces a center of chirality into the nucleotide. Therefore, nonterminal P-boronated nucleotides exist as a pair of diastereoisomers that can exhibit quite different behavior toward enzymes.[9]



1 B = A, T, G, C
R = diphosphate, nucleoside

The borane moiety is isoelectronic and isosteric with oxygen. Yet, boranophosphate analogues have a different charge distribution and polarity than the corresponding natural nucleotides.[10] Furthermore, this moiety is not expected to form classical H bonds or to coordinate with metals.[11] Although nucleoside boranophosphate analogues are highly soluble in water, the borane group brings about a significant change in the nucleotide lipophilicity.[10]

In addition, the boranophosphate moiety is expected to improve metabolic and chemical stability of the nucleotide derivative. Although borane derivatives are not natural constituents of the organism, they are not toxic.[10] This fact, in addition to their unique properties, turns them into potential therapeutic agents.

Therefore, we planned a new series of P2Y$_1$-R agonists bearing two modifications at two locations on the ATP scaffold: a modification at the C2 position of the adenine ring and a second modification of the 5′-triphosphate chain, replacing it by a boranophosphate group (**8a**−**c**, Scheme 1).

Here, we report on the efficient synthesis and diasteroisomers separation of a new series of adenosine 5′-*O*-(1-boranotriphosphate) (ATP-α-B) analogues, **8a**−**c**, and the evaluation of the new derivatives as P2Y$_1$-R agonists. In addition, we report on the evaluation of the chemical and enzymatic stability of those ATP-α-B analogues.

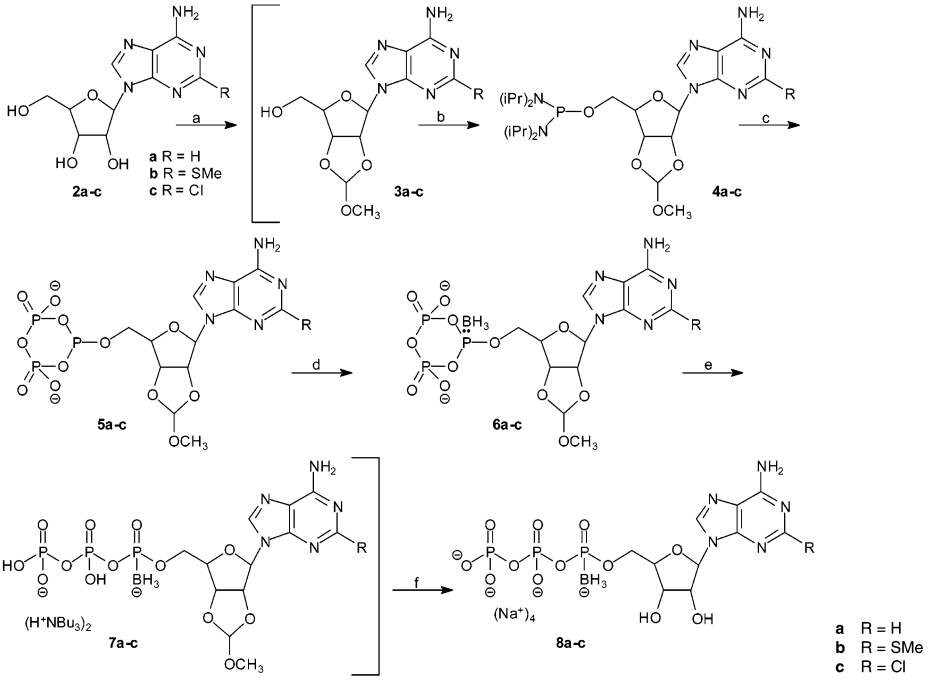
Results

Synthesis. Although the synthesis of ATP-α-BH_3 (ATP-α-B) has been reported by Ramsay-Shaw et al.,[12] we aimed at developing an improved and shorter one-pot synthesis of several ATP-α-B analogues. Our one-pot method is outlined in Scheme 1. The nucleoside starting materials were selected on the basis of our previous studies.[5,7] Since 2-MeS-ATP and 2-Cl-ATP are among the most potent P2Y-Rs agonists, we chose 2-MeS-adenosine[13] **2b**, 2-Cl-adenosine,[14] and **2c**, as the starting materials for the preparation of 2-MeS-ATP-α-B, **8b**, and 2-Cl-ATP-α-B, **8c**, respectively. ATP-α-B was prepared from adenosine for the evaluation of the effect of the borane group on biochemical activity relative to ATP. In addition, the effect of 2-MeS- and 2-Cl-ATP-α-B substitutions on biochemical activity was compared to that of ATP-α-B.

The use of phosphitylation and boronation reagents in the synthetic method (Scheme 1) requires the use of protected nucleosides. Yet, one would like to minimize as much as possible the number of synthetic steps leading to the protected starting material.

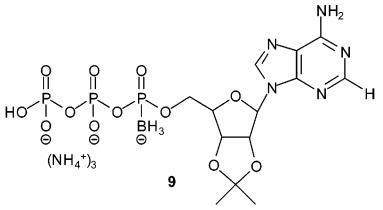
To minimize the number of synthetic steps, we focused on protective groups that are selective to 2′,3′-hydroxyls, thus avoiding protection and deprotection of 5′-OH. In addition, the protective groups should be removed under mild conditions so that the formed boranophosphate moiety or the *N*-glycosidic bond is not

Scheme 1[a]



[a] (a) $HC(OMe)_3$, *p*-TsOH, DMF, room temp, 24 h; (b) $(^iPr)_2NEt$, $[(^iPr)_2N]PCl$, $CHCl_3$, 0 °C, 2 h; (c) $H_2P_2O_7^{2-}$ $(H^+NBu_3)_2$ (1 M in DMF), room temp, 4 h; (d) BH_3·SMe_2 (2 M in THF), room temp, 15 min; (e) H_2O, room temp, 45 min; (f) pH 2.3, room temp, 3 h, followed by pH 9, room temp, 40 min.

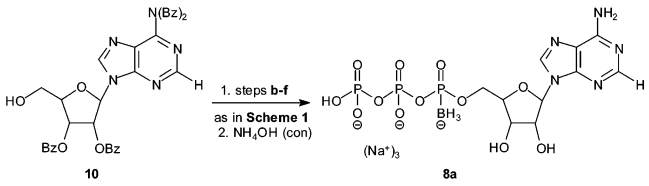
harmed. Thus, for instance, the reaction to form acetonide-protected ATP-α-B, **9**, occurred with relatively high overall yield (Scheme 1, steps b−e, 53% yield).



However, deprotection of **9** under various conditions resulted in degradation of the boranophosphate moiety.

Methoxymethylidene proved to be a highly useful protective group for adenosine 2′,3′-hydroxyls. This protective group remained stable throughout the entire synthesis and was efficiently and relatively rapidly removed at the last step. Removal of a methoxymethylidene group involved a hydrolysis step at pH 2.3 and another at pH 9,[15] at which products **8a**−**c** were stable. Furthermore, both of these steps were relatively rapid, lasting less than 4 h compared with 24−48 h required for the removal of the alternative acetate protecting groups. However, for the preparation of ATP-α-B, **8a**, the protection of 2′,3′-OH in adenosine with benzoyl groups, **10**, resulted in a higher overall yield of **8a** (20%) than the yield obtained with a methoxymethylidene-protected adenosine, **3a** (4%) (Scheme 2).

Scheme 2



To avoid additional protection of the adenine N^6 position, we selected relatively mild reagents and conditions for the phosphoboronation reaction as described below.

Formation of the adenosine 5′-(1-boranotriphosphate), **8**, from **3** involved the use of a phosphytilation reagent (step b). Salicyl phosphochloridite[16] and 2-cyanoethyl diisopropylchlorophosphoramidite[17] have been proposed before for the preparation of nucleoside 5′-boranophosphate in 25−31% yield. To avoid protection of the N^6 position and to retain a reasonably reactive phosphorus reagent, we investigated several tervalent phosphorus reagents such as diethylphosphoramidous dichloride and bis(diisopropylamino)chlorophosphine. Our reagent of choice was the latter, leading to optimal yields. Methoxymethylidene-protected nucleoside **3** was treated with bis(diisopropylamino)chlorophosphine in dry chloroform to form a phosphitylated product **4**. This product was not isolated, and to this reaction mixture bis(tributylammonium) pyrophosphate salt was added at room temperature (1.5 equiv), yielding after 4 h intermediate **5**, which underwent a subsequent boranation step to yield **6**.

From borane complexes reacting as Lewis acids, we selected borane dimethyl sulfide as a boronation reagent that was effective under mild conditions (15 min, room temperature). This mild reagent is applicable to variously substituted adenosine analogues. The tervalent phosphorus intermediate **5**, reacting as a Lewis base, coordinated with the electron-deficient borane to form **6**.

In the same pot, intermediate **6** was hydrolyzed at room temperature to form a protected ATP-α-B analogue **7**. Finally, after purification of **7** on an anion exchanger, the methoxymethylidene protective group was removed as described above to yield product **8**.

The order of the addition of reagents is important. Thus, with a reverse order of addition, namely, first borane dimethyl sulfide and then pyrophosphate salt, the yield of product **7** was very low.

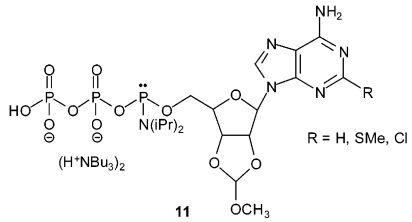
The advantages of this synthesis of P-boronated nucleosides are the following: (a) four synthetic steps (b−e) are performed in one-pot without a need to separate intermediate products; (b) good overall yields of the five synthetic steps (b−f) leading to products **8a**−**c** (31−43%); (c) mild conditions applicable to a relatively wide range of nucleoside analogues; (d) the reaction is relatively short and steps b−f are completed within about 7 h; (e) there is no need to protect the N^6 position; (f) there is no need to protect and then deprotect 5′-OH for the preparation of the starting nucleoside.

The identity and purity of the products were established by ^{1}H and ^{31}P NMR, high-resolution FAB MS, and HPLC in two solvent systems. ^{31}P NMR spectra of products **8a**−**c** showed a typical P_α signal as a multiplet at about 84 ppm. ^{1}H NMR spectra of **8a**−**c** showed borane hydrogen atoms as a very broad signal at about 0.4 ppm.

In both ^{1}H and ^{31}P NMR spectra, there was a slight difference between the chemical shifts for the two diastereoisomers of **8a**−**c**. For instance, for **8b** diastereisomers, two sets of signals were observed for H8, at 8.46 and 8.42 ppm, and for P_α, at 82.7 and 83.9 ppm.

For a more distinct differentiation between the diastereoisomers in ^{1}H and ^{31}P NMR spectra, we added metal ions that upon coordination with the phosphate groups are likely to cause a shift of the phosphate chemical shifts (and hence of H8) and to increase $\Delta\delta$ between P_α or H8 signals of the diastereoisomer pair. When a mixture of ATP-α-B isomers in aqueous solution was titrated with $MgCl_2$, up to 1 mM concentration, no change was observed in ^{1}H and ^{31}P NMR spectra. However, when 2-Cl-ATP-α-B, **8c**, was titrated with the shift reagent $Eu(NO_3)_3$, the signals broadened considerably, up to coalescence after the addition of 0.14 equiv of Eu salt. Thus, this shift reagent provided no efficient means of discriminating between the diastereoisomers. Furthermore, the Eu(III) salt promoted hydrolysis of the phosphate chain.[18]

Elucidation of the Structure of Intermediate 5. Addition of a pyrophosphate salt to intermediate **4** is expected to bring about displacement of either two or one diisopropylamino groups to form either intermediate **5** or **11** containing a cyclic or linear phosphate moiety, respectively. To elucidate the structure of the intermediate, we monitored this reaction step with ^{31}P NMR. Intermediate **4b** was taken from the reaction mixture (6.5 mM) into an NMR tube, and bis(tributylammonium) pyrophosphate salt in DMF (1.5 equiv) was added. ^{31}P NMR spectra were recorded at 15 min intervals for 1.5 h at room temperature. The phosphite signal of inter-



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mediate **4** at 124 ppm decreased gradually, and new signals, a triplet at 104 ppm and a doublet at −21 ppm, emerged, indicating the formation of a cyclic intermediate **5**. The latter intermediate was formed because of the displacement of both amine groups in **4** by pyrophosphate.

Separation of Diastereoisomers of ATP-α-B Analogues. ATP-α-B analogues **8a**−**c** contain a chiral center at P_α. The diastereoisomer pairs of **8a**−**c** have been efficiently separated on reverse-phase HPLC applying 100 mM triethylammonium acetate/MeOH isocratic elution, having about 2 min difference in their retention times. The first eluting isomer was designated the A isomer, and the other was designated the B isomer.

To assign absolute configuration of **8a**−**c** diastereoisomers, we attempted the crystallization of purified A or B isomer of the tetrasodium salt **8b**. Yet, all attempted crystallization conditions failed to provide crystals. Likewise, attempts to assign the absolute configuration around the P_α of A and B isomers by nuclear Overhauser enhancement (NOE) and nuclear Overhauser effect spectrometry (NOESY) experiments also failed. No cross-peaks between H8 and the borane hydrogen atoms could be observed,[9,19] possibly because of the large flexibility of compounds **8**. Furthermore, even expected cross-peaks between H8 and H5′ or H3′ [5a] were not observed, possibly because of a competing relaxation mechanism involving the boron nucleus.

Lipophilicity. Although nucleoside boranophosphate analogues are highly soluble in water, the borane group brings about a significant change in the lipophilicity of the nucleotide.[10] To evaluate the effect of the borane group on the lipophilicity of ATP-α-B analogues **8a**−**c** relative to ATP, we attempted to determine the octanol/water partition coefficient (P_{OW}) of those compounds. The concentrations of the nucleotides in both phases were analyzed by UV spectroscopy and HPLC. Analogue **8b**, like ATP, was detected only in the aqueous phase. Namely, borane substitution does not change the overall lipophilicity of analogues **8** compared to ATP.

Evaluation of ATP-α-B Analogues as P2Y$_1$-R Agonists. To examine the efficacy of analogues **8a**−**c** in a functional assay, we tested them by the agonist-induced Ca^{2+} release in HEK 293 cells stably transfected with rat brain P2Y$_1$ receptor[20] (Figure 1). Those P2Y$_1$-R transfected cells were shown to be suitable for pharmacological characterization.[21] Concentration−Ca^{2+} response data obtained from average values from 60 to 100 single cells were analyzed to derive EC_{50} values (half-maximal response). EC_{50} indicates the respective affinity of the test substance used for P2Y$_1$-R. The standard reference compound for receptor activation was ATP.[21]

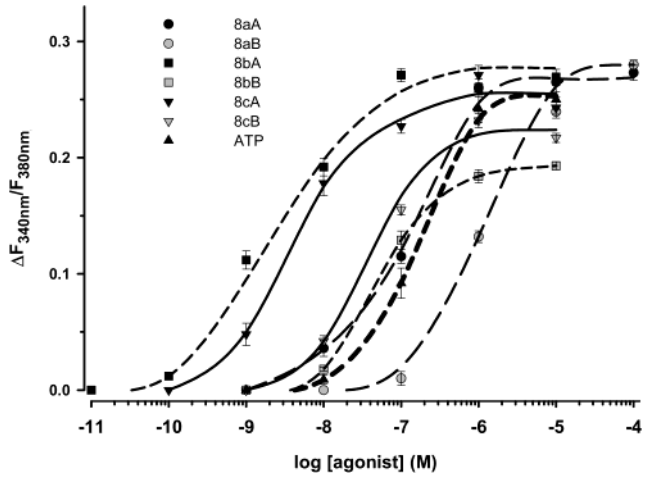


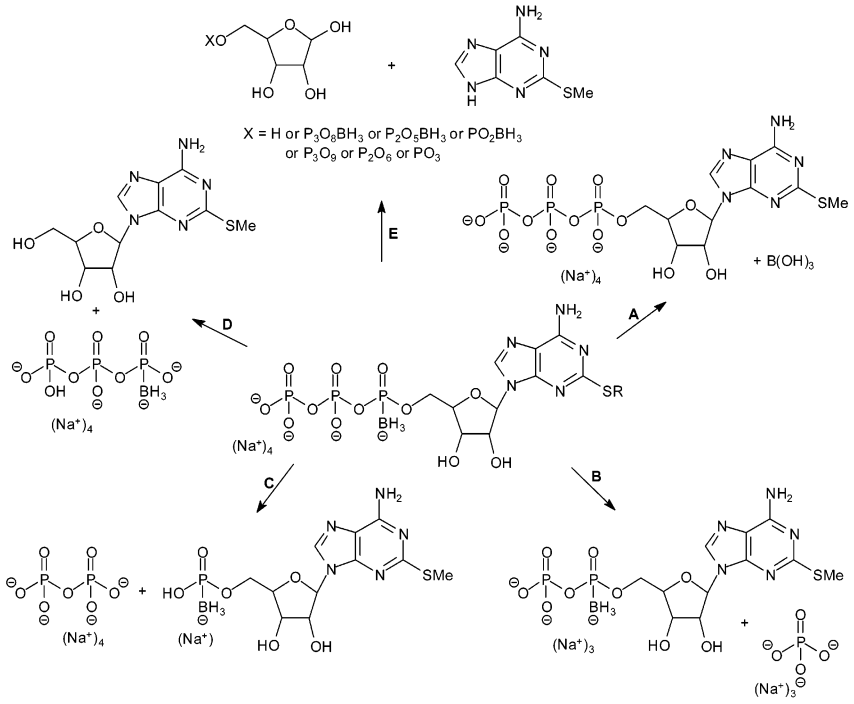
Figure 1. Concentration−response curves for ATP-α-B analogues **8a**−**c** by measuring Ca^{2+} release in HEK293 cells expressing the rP2Y$_1$-receptor. The potency of the indicated concentrations of **8aA** (black circle), **8aB** (gray circle), **8bA** (black square), **8bB** (gray square), **8cA** (black triangle down), **8cA** (gray triangle down) to raise the $\Delta F_{340}/F_{380}$ ratio was determined as described in Experimental Section. Triangles up represent the concenration−response curve for ATP. Data show the mean values and standard error (SE) from 60 to 100 single cells and are representative for results obtained in at least three separate experiments. Standard errors are omitted when they are smaller in the size than the symbols used.

The ATP-α-B A isomer, with an EC_{50} of 2×10^{-7} M, was found to be equipotent with ATP. However, substitution at C2 of ATP-α-B by an electron-donating group increased the potency of the agonist in inducing Ca^{2+} release. The agonist potency of the analogue **8b** A isomer was increased 100-fold compared with the ATP-α-B A isomer. In the case of an electron-withdrawing group at C2, analogue **8c**, the change in electron distribution of the adenine ring in 2-Cl-ATP-α-B did not affect significantly the ligand potency, as noted by comparison of its EC_{50} value with that of 2-MeS-ATP-α-B ($EC_{50} = 4.5 \times 10^{-9}$ vs 3.6×10^{-9} M). This result agrees with data regarding the potency of variously C2-substituted ATP analogues.[22]

Moreover, we found that the A diastereoisomers of all ATP-α-B analogues are more potent in inducing Ca^{2+} release than the corresponding B diastereoisomers. Thus, the ATP-α-B A isomer ($EC_{50} = 2 \times 10^{-7}$ M) and 2-Cl-ATP-α-B A isomer ($EC_{50} = 4.5 \times 10^{-9}$ M) have approximately a 10-fold increased potency in comparison to the corresponding B isomers ($EC_{50} = 2 \times 10^{-6}$ and 3.6×10^{-8} M, respectively). The 2-MeS-ATP-α-B A isomer ($EC_{50} = 2.6 \times 10^{-9}$ M) is even 20-fold more potent than the corresponding B isomer ($EC_{50} = 5.3 \times 10^{-8}$ M). Apparently, the A configuration allows a tighter receptor fit.

Chemical Stability. To explore the suitability of the new P2Y$_1$-R agonists **8a**−**c** as drug candidates for oral administration, we evaluated their chemical stability. An ATP-α-B analogue might undergo several hydrolytic pathways (Scheme 3): (A) deboranation of the ATP-α-B analogue to the corresponding ATP analogue; (B) hydrolysis of the terminal phosphate ester bond of ATP-α-B to produce ADP-α-B; (C) hydrolysis of the phosphate ester bond of ATP-α-B to release a pyrophosphate moiety and AMP-α-B; (D) hydrolysis of the entire boranophosphate chain of ATP-α-B to form the free

Scheme 3



nucleoside; (E) deglycosylation reaction to yield an adenine derivative and a boranophosphorylated ribose (or ribose and phosphate).

The major difference between the ^{31}P NMR chemical shifts of boranophosphates and phosphates, about 85 ppm, enables easy monitoring of the above-mentioned degrading reactions by ^{31}P NMR spectrometry.

The effect of the borane group on the hydrolytic stability of ATP-α-B derivatives was monitored under physiological conditions (pH 7.4 and 37 °C) and under conditions simulating gastric juice (pH 1.4 and 37 °C). Thus, ^{31}P NMR spectra of a diastereoisomeric mixture of 2-MeS-ATP-α-B tetrasodium salt in Tris-HCl buffer (in H_2O), pH 7.4, were recorded for 3 weeks at 1- to 3-day intervals at 37 °C. Under these conditions, path B (Scheme 3) was the only hydrolytic pathway observed. A signal for inorganic phosphate emerged at 1.7 ppm, while the signal for Pγ (−7.5 ppm) decreased, indicating that the terminal phosphate was lost under these conditions. The intensity changes of the ^{31}P peaks with time were fit to a pseudo-first-order exponential decay rate equation with respect to 2-MeS-ATP-α-B concentration. The calculated hydrolysis rate constant determined at pH 7.4 and 37 °C was 1.38×10^{-7} s^{-1}, $t_{1/2}$ = 1395 h. No deboranation products could be detected. Previously we made a similar observation for hydrolysis of an ATP-α-S analogue under the same conditions. A comparable rate was also obtained for hydrolysis of 2-benzylthioether-ATP-α-S ($(7.29 \pm 0.15) \times 10^{-7}$ s^{-1}).[4b]

Chemical stability under conditions simulating gastric juice (pH 1.4 and 37 °C) was evaluated with diastereoisomeric mixtures of 2-MeS-ATP-α-B and 2-Cl-ATP-α-B tetrasodium salts, **8b**,**c**, in KCl/HCl buffer. ^{31}P NMR spectra were recorded for 17 h at 0.5 h intervals (Figure 2). The signals for P$_\alpha$ (87 ppm) and P$_\beta$ (−23 ppm) gradually decreased with a concomitant increase of a new well-resolved boranophosphate signal at 96 ppm and a singlet at −10 ppm (overlapping with the P$_\gamma$ signal). The ^{1}H NMR spectrum after 17 h showed a sharp dABq at 3.70 ppm for H-5′ with no $^3J_{PH}$; namely, the hydrolytic product of **8b**,**c** was the corresponding nucleoside. These changes in the ^{31}P and ^{1}H NMR spectra imply that at pH 1.4 the hydrolytic pathway is different than that at neutral pH. At pH 1.4, hydrolysis involves the concomitant release of a pyrophosphate moiety (singlet at −10 ppm), inorganic boranophosphate (96 ppm), and 2-MeS-adenosine or 2-Cl-adenosine. The rate of consumption of C2-substituted ATP-α-B analogue was calculated on the basis of the intensity changes of the ^{31}P peaks of starting material with time that were fit to a pseudo-first-order reaction model. Rate constants of 3.24×10^{-5} s^{-1} ($t_{1/2}$ = 5.9 h), and 7.2×10^{-5} sc^{-1} ($t_{1/2}$ = 2.7 h) were established for **8b** and **8c**, respectively.

The disappearance of P$_\alpha$ and P$_\beta$ and the formation of inorganic boranophosphate and pyrophosphate occur at the same rate on the NMR time scale. This can be explained by three possible mechanistic pathways that cannot be differentiated by ^{31}P NMR (Scheme 4). Pathway A involves an intramolecular nucleophilic attack of the terminal phosphate on P$_\alpha$ to form a cyclic trimetaphosphate intermediate **12** and nucleoside **2**. Intermediate **12** may undergo a fast hydrolytic decomposition to pyrophosphate and inorganic boranophosphate. Pathway B involves a slow attack of a water molecule primarily at C5′ [23] to release **2** and inorganic boranotriphosphate, which is rapidly hydrolyzed to pyrophosphate and inorganic boranophosphate. Alternatively, via pathway C, a water molecule may attack at P$_\alpha$, releasing pyrophosphate and the AMP-α-B analogue. This nucleotide then may undergo fast hydrolysis to **2** and inorganic boranophosphate.

A tandem, much slower, hydrolytic pathway was also observed. New singlets appeared at 2, 5, and 8 ppm, indicating the formation of H-phosphonate and inorganic phosphate species. Under these highly acidic

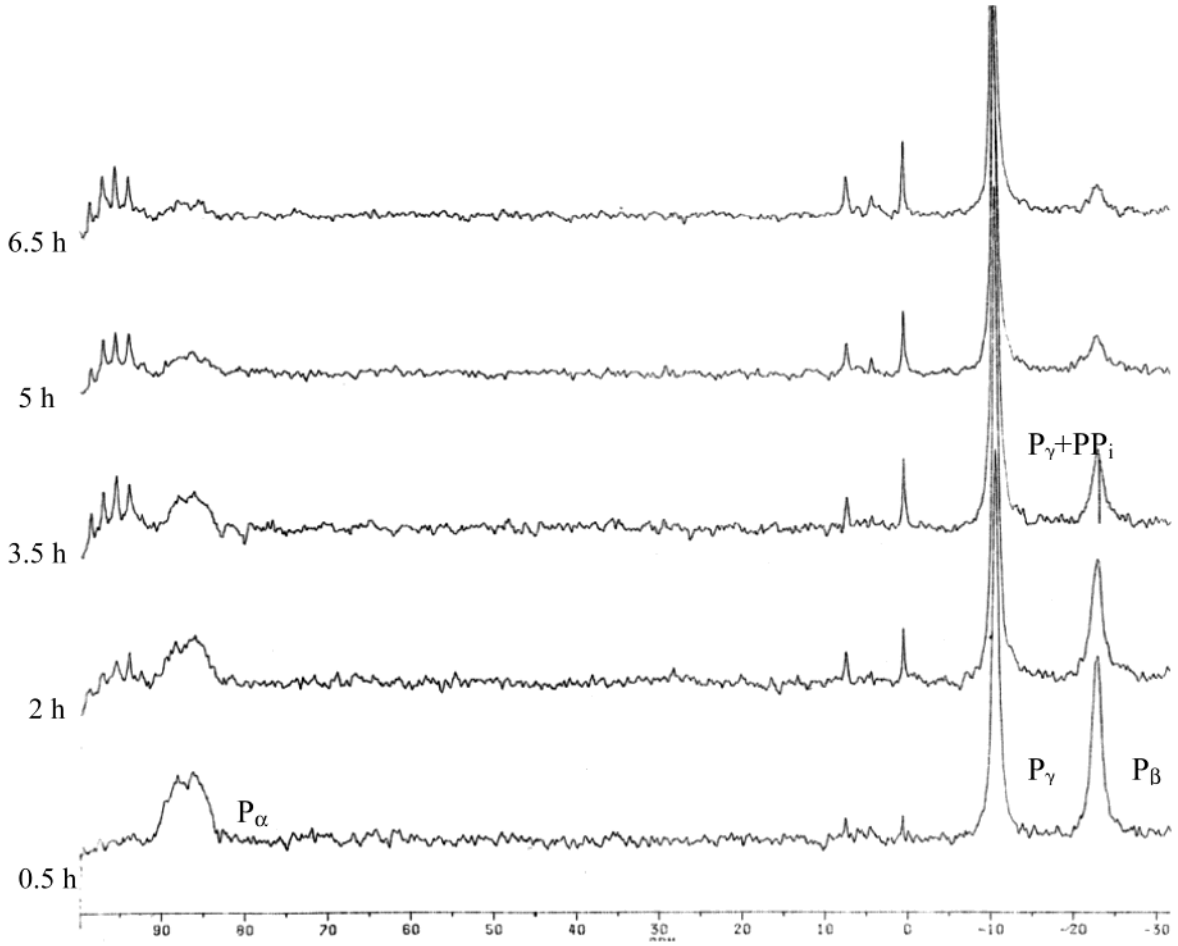
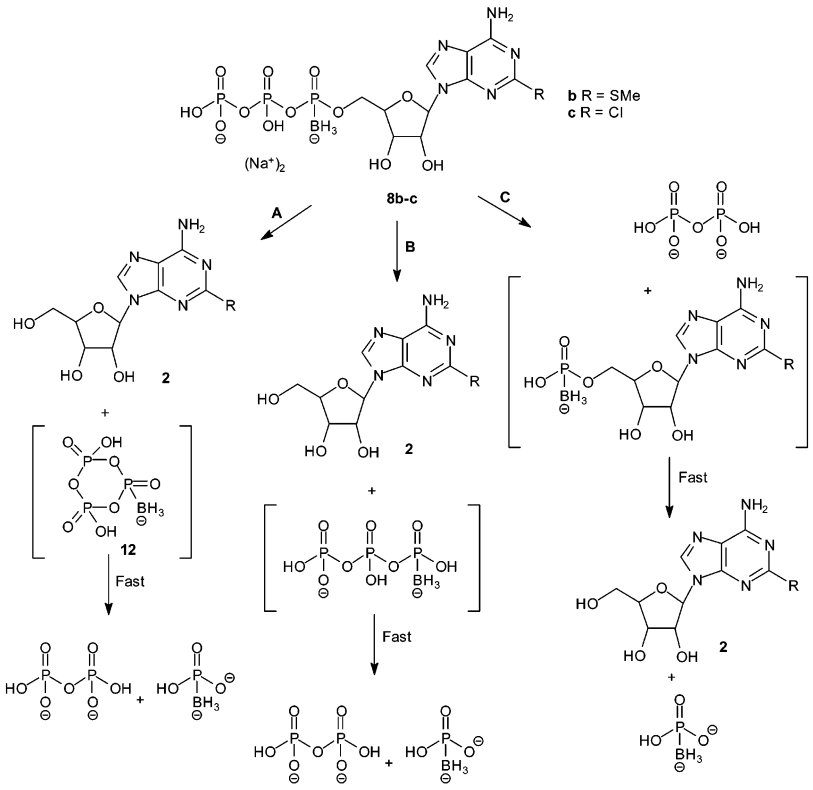


Figure 2. Rate of hydrolysis of **8c** at gastric-juice stimulating conditions monitored by ^{31}P NMR at 81 MHz. ^{31}P NMR spectra of hydrolysis of 0.03 M solution of 2-Cl-ATP-α-B tetrasodium salt in KCl/HCl buffer at pH 1.4 and 37 °C were recorded for 17 h at 0.5 h intervals.

Scheme 4



conditions, additional hydrolysis of inorganic boranophosphate to the corresponding H-phosphonate and boric acid occurred to a minor extent.[24] In addition, the pyrophosphate hydrolytic product was further hydrolyzed to inorganic phosphate.

Hydrolysis of the *N*-glycosidic bond in C2-substituted ATP-α-B (path E, Scheme 3) 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-benzylthioether adenosine under these conditions, even after 14 days.[4b]

The higher stability of 2-MeS-ATP-α-B at pH 7.4 vs 1.4 is due to the predominant existing tetraanion form that retards the nucleophilic attack of water molecule.

Evaluation of Enzymatic Stability Regarding NTPDase. Extracellular concentration of nucleotides and nucleotide analogues, which act as P2-R ligands, is determined by their resistance to hydrolysis by exo- and ectoenzymes.[25] For instance, addition of an exogenous ATP diphosphohydrolase reverses platelet aggregation induced by ADP.[26] Therefore, the evaluation of the enzymatic stability of new compounds is an essential step in the development of P2-R ligands as potential drug candidates. Furthermore, it is necessary to determine if these ligands can inhibit ectonucleotidase-catalyzed hydrolysis of nucleoside tri- and diphosphate to nucleoside monophosphate. Such an inhibition would presumably result in an accumulation of these nucleotides in the extracellular space. Hence, this would potentially lead to P2-R desensitization and reduction of the cellular response.

Until 1980, it was widely believed that mammalian cells could sequentially dephosphorylate ATP to ADP, to AMP, and to adenosine, each step being catalyzed by a discrete enzyme.[25,27] It is now known that the major player in the conversion of extracellular 5′-triphosphate and diphosphate nucleosides to monophosphate nucleosides is an ectonucleotidase named NTPDase1 (EC 3.6.1.5).[28,29] It is also known that the hydrolysis of the γ- and the β-phosphate diester bonds of nucleoside 5′-di- and triphposphates by this enzyme is strongly stimulated by Ca^{2+} or Mg^{2+}.[29]

In the present study, all ATP-α-B analogues tested interacted with the bovine spleen NTPDase1. This was tested according to the following two different approaches: (1) resistance to hydrolysis by NTPDase and (2) interference with ATP hydrolysis by this enzyme.

We first evaluated the resistance of ATP-α-B derivatives toward hydrolysis by the semipurified preparation of bovine spleen NTPDase1. Enzymatic stability of the novel boranophosphate analogues, bearing modifications at both purine and phosphate chain, **8a**−**c**, was also compared to the corresponding ATP analogues, bearing modifications only on the purine ring (Table 1). As shown in Figure 3, all ATP-α-B analogues were hydrolyzed at a rate inferior to that of ATP. The A isomers of analogues **8a**−**c** were poor substrates for the spleen NTPDase1. The rate of hydrolysis of the **8a-A** isomer was 5% of the ATP rate, whereas **8b-A** and **8c-A** were hydrolyzed at 14% and 23% of the rate of ATP's hydrolysis, respectively. In contrast, the B isomers of **8a**−**c** were significantly hydrolyzed (52%, 57%, and 53%, respectively). From these results, the introduction of the boranophosphate moiety apparently plays an important role in protecting ATP analogues against NTPDase hydrolysis, especially for the A isomers. Because of their low hydrolysis, the kinetic parameters for these isomers could not be calculated.

Table 1. Kinetic Parameters of Bovine Spleen NTPDase1 Hydrolysis of ATP Analogues **13a**−**c** and ATP-α-B Analogues **8a**−**c** B Isomers[a]

analogue	compd	V_{max}, μmol min^{-1} mg^{-1} protein	$K_{m_{app}}$, μM
ATP	**13a**	2.4 ± 0.1	15.9 ± 0.2
2-MeS-ATP	**13b**	1.9 ± 0.3	62.0 ± 12.0
2-Cl-ATP	**13c**	1.9 ± 0.1	12.9 ± 0.4
ATP-α-B	**8a-B**	0.78 ± 0.09	13.8 ± 3.0
2-MeS-ATP-α-B	**8b-B**	0.89 ± 0.03	17.4 ± 0.9
2-Cl-ATP-α-B	**8c-B**	0.88 ± 0.04	7.2 ± 1.7

[a] Kinetic parameters for **8a**−**c** A isomers are absent because their hydrolysis rates are insignificant (see Figure 3). Experiments were performed in triplicate, and the results were obtained by the best fit obtained with GraFit 4 software (Erithacus, U.K.). Apparent K_m and V_{max} were estimated from the Woolf−Augustinson−Hofstee representation (Figure 4) using a concentration ranging from 10 to 100 μM. Values are the means ± SEM of two experiments, each run in triplicate.

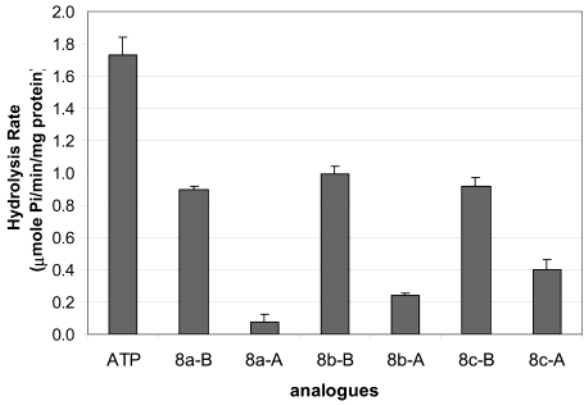
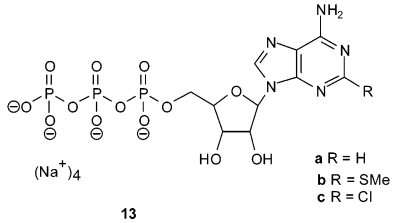


Figure 3. Hydrolysis rate of ATP-α-B analogues **8a**−**c** by bovine spleen NTPDase1. Assays were performed for 7 min at 37 °C in the presence of 1.0 μg of protein. ATP and analogues were used at 100 μM concentration: ATP-α-B (**8a-A** and **8a-B**); 2-methylthio-ATP-α-B (**8b-A** and **8b-B**); 2-chloro-ATP-α-B (**8c-A** and **8c-B**). The A isomers of analogues **8a**−**c** were poor NTPDase1 substrates, being hydrolyzed at 5%, 14%, and 23% the rate of ATP. In contrast, the B isomers **8a**−**c** were better substrates, being hydrolyzed at 52%, 57%, and 53% the rate of ATP. Experiments were performed in triplicate. Deviations between replicates were generally within 10% from their mean.



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A more detailed analysis was conducted, and kinetic parameters K_m and V_{max} were determined for the B isomers of **8a**−**c** according to the Woolf−Augustinson−Hofstee plots (Figure 4, Table 1). The apparent K_m of these analogues is very close to that of ATP, whereas the apparent V_{max} is quite different. $K_{m_{app}}$ values of ATP and the ATP analogues bearing one modification on the purine ring, **13b**−**c** (Table 1), were not significantly

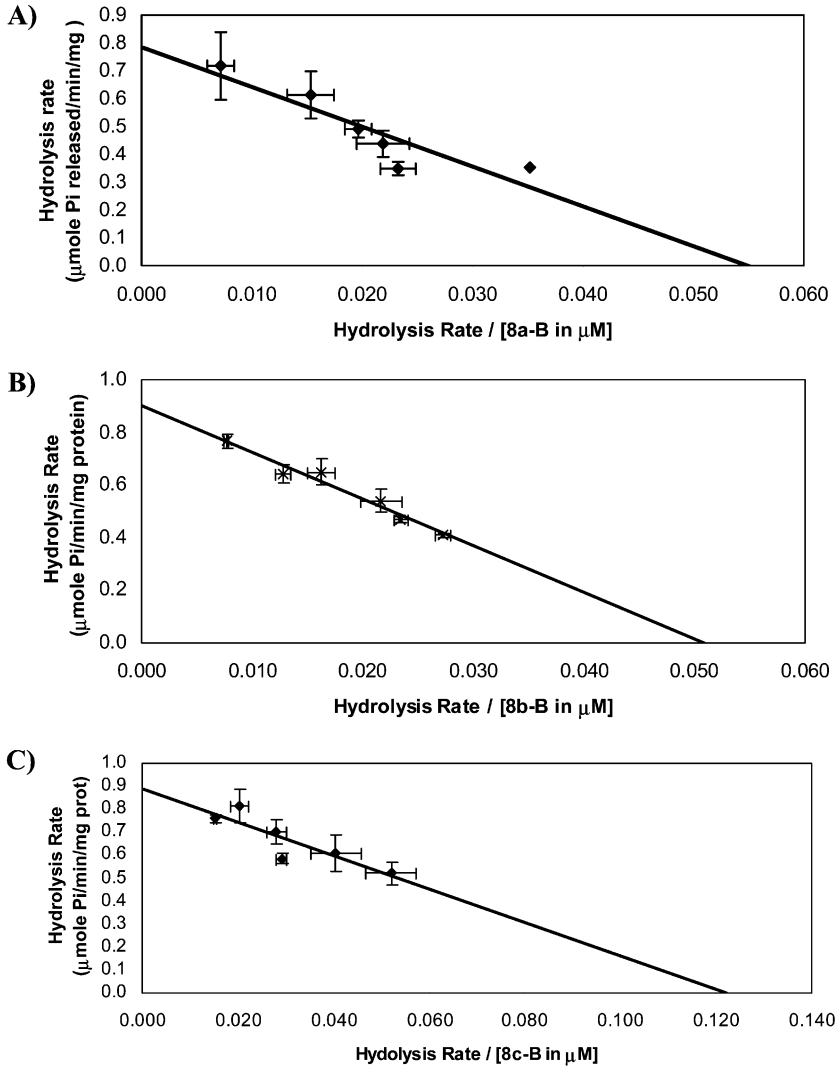

Figure 4. Woolf−Augustinson−Hofstee representation of the hydrolysis of **8a-B** (A), **8b-B** (B), and **8c-B** (C) by bovine spleen NTPDase1. The apparent K_m and V_{max} were 14 μM and 0.79 μmol of Pi min^{-1} mg^{-1} protein for **8a-B** (A), 18 μM and 0.91 μmol Pi min^{-1} mg^{-1} protein for **8b-B** (B), and 7 μM and 0.88 μmol Pi min^{-1} mg^{-1} protein for **8c-B** (C), respectively. Experiments were performed in triplicate, and the maximum deviation was within 10% of the mean.

different from those of ATP analogues bearing modifications on the phosphate chain and/or the purine ring and **8a**−**c**, with the exception of 2-MeS-ATP, **13b**. Here, the $K_{m_{app}}$ is higher than for **8b-B** (Table 1). We conclude that the borano modification on the phosphate chain did not affect NTPDase1 affinity for these analogues.

In a second series of experiments, the influence of ATP-α-B derivatives **8a**−**c** B isomers on ATP hydrolysis by NTPDase1 was studied (Figure 5). The catalytic activity of spleen NTPDase1 was significantly reduced by the poorly hydrolyzed A isomers. Indeed, Figure 5 shows that the combination of ATP-α-B analogues with ATP results in one of two types of behavior: either inhibition in the case of A isomers **8a**−**c** and **8b-B** or stimulation of Pi release for **8a-B** and **8c-B**. One has to consider the contribution of the analogue as a substrate for the enzyme, which is the case for the B isomers. Nevertheless, the stimulation occurred when the analogues were introduced at a concentration of 10 μM (36% and 30%, respectively). At 10 μM, there is no significant contribution to Pi release from the hydrolyses of these analogues (data not shown).

Discussion

In this study, we designed and evaluated a new series of adenosine 5′-(1-boranotriphosphate) analogues, **8a**−**c**, as P2Y$_1$-R agonists. For this purpose, we developed an efficient and short synthesis of **8a**−**c** from the corresponding methoxymethylidene-protected adenosine analogues **3a**−**c**. The preparation of **8a**−**c** was achieved via a four-step one-pot synthesis. The selection of mild reaction conditions and methoxymethylidene protecting group provided an efficient and general method involving a minimal number of protection and deprotection steps.

In this nonstereospecific reaction, P_α Rp and Sp isomers of ATP-α-B analogues **8a**−**c** were obtained in a 1:1 ratio. These diastereoisomers exhibited a different affinity to the HPLC column, eluting with a 2 min difference. Yet, their spectral characteristics were very similar except for more significant changes for H8 and P_α in ^{1}H and ^{31}P NMR spectra (H8, at 8.46 and 8.42 ppm; P_α, at 82.7 and 83.9 ppm).

We attempted to assign the absolute configuration around P_α in **8a**−**c** by crystallography or NMR experiments. However, the enormous solubility of these com-

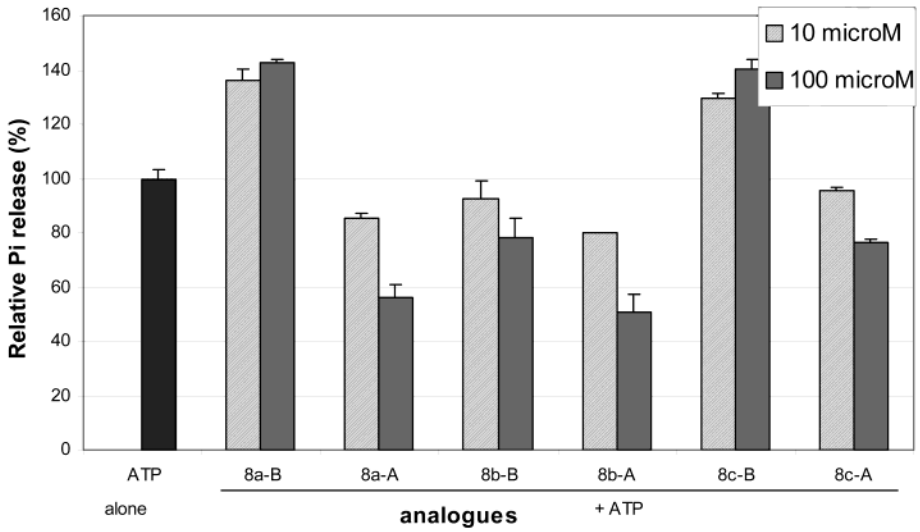

Figure 5. Influence of ATP-α-B analogues **8a**−**c** on the release of inorganic phosphate by NTPDase1. Assays were performed for 7 min at 37 °C in the presence of 1.0 μg of protein, and analogues were added at concentrations of either 10 or 100 μM in the incubation medium. Reactions were started by adding the substrate. All the analogues exerted an influence on ATP hydrolysis by NTPDase1. A significant increase in Pi release is observed with **8a-B** (43%) and **8c-B** (40%), whereas a significant decrease is observed with **8a-A** (44%) and **8b-A** (49%). There is no major change with **8b-B** and **8c-A** derivatives (almost 20% decrease). Results are the mean of triplicate samples. The maximum deviation from the mean was within 5%.

pounds in water complicated their crystallization. Likewise, their high structural flexibility in solution, and possibly a competing relaxation mechanism by the boron nucleus, prevented absolute configuration assignment by NOE and NOESY experiments. Currently, we are still unable to provide absolute configuration data for **8a**−**c**. Therefore, we denoted the first HPLC eluting isomer as A and the other one as B.

Although the borane group was reported to bring about a significant change in the lipophilicity of the nucleoside boranophosphate,[10] we found, on the basis of the determination of P_{OW} of compounds **8**, that the borane substitution did not change the overall lipophilicity of ATP-α-B analogues compared to ATP.

Analogues **8a**−**c** were evaluated in a functional assay as $P2Y_1$-R agonists. We determined the nucleotide-promoted Ca^{2+} response of HEK293 cells stably transfected at high density with $P2Y_1$-R. All analogues activated the $P2Y_1$-R with EC_{50} values in the nanomolar range for the 2-substituted analogues **8b**,**c**. Comparison of EC_{50} values of ATP and the ATP-α-B A isomer, which are about 2×10^{-7} M, revealed that the borane group has no significant effect on affinity to the $P2Y_1$-R. Likewise, this phenomenon was also observed for the 2-MeS-ATP-α-B A isomer, which is equipotent with 2-MeS-ATP (2.6×10^{-9} vs 1×10^{-9} M).[21]

Yet, the most significant influence, up to 100-fold, on the affinity of the ligand toward the $P2Y_1$-R stems from the chemical modification at C2 in **8b**,**c**, either from electron-donating or -withdrawing groups. In both cases the affinity was highly increased.

The borane substitution had no significant effect on the affinity of the ligand toward $P2Y_1$-R compared with the corresponding ATP analogues. Nevertheless, introduction of a chiral center at P_α, due to borane substitution, caused a significant differentiation of the diastereoisomers' affinity toward the receptor of up to a 20-fold difference.

The therapeutic potential of these new potent $P2Y_1$-R agonists is related to their chemical stability and their resistance to enzymatic hydrolysis. Indeed, analogue **8b**, subjected to physiological or gastric-juice pH at 37 °C, exhibited high stability, with half-lives of 1395 and 5.9 h, respectively. Furthermore, isomer **8a-A** was highly stable in terms of NTPDase hydrolysis, being hydrolyzed at a rate that is 5% of that of ATP.

8a−**c** A isomers were more potent $P2Y_1$-R agonists than the corresponding B isomers, up to a 20-fold difference. Furthermore, this stereoselectivity was observed also for NTPDase with the A isomers being more stable than their B counterparts (Figure 3). Therefore, the binding domain of $P2Y_1$-R, which interacts preferentially with A isomers, apparently has a stereoselectivity that is opposite to the stereoselectivity of the catalytic site in NTPDase (interacting preferentially with B isomers). In other words, $P2Y_1$-R potent A isomer agonists barely interact with NTPDase, thus exhibiting protein selectivity. This stereoselectivity observed for ATP-α-B analogues **8a**−**c**, in both $P2Y_1$-R and NTPDase, was not observed for the corresponding phosphorothioates analogues (ATP-α-S derivatives) that also possess a chiral center at P_α.[4] The stereochemical requirements of $P2Y_1$-R for molecular recognition of ATP-α-B and ATP-α-S analogues are currently under investigation and will be published in due course.

These ATP-α-B analogues that on one hand stimulate $P2Y_1$-R at the nanomolar range and on the other hand do not inhibit NTPDase at a concentration below 10 μM would allow induction of a specific physiological effect without disturbing other less sensitive receptors. A good theoretical example could be platelet aggregation and control of vascular tone involving several purinergic receptors. The addition of the ATP-α-B analogue in the nanomolar range could, on one hand, induce the release of NO via $P2Y_1$-R on endothelial cells,[29] whereas on the other hand, the same concentration would not exert any inhibitory effect on the NTPDase. This enzyme hydrolyzes ADP to AMP, thereby preventing or reversing

normal platelet aggregation[26,30] induced by the platelet receptor P2Y$_{12}$. The P2Y$_{12}$ receptor responds to ADP in the micromolar range.[31] Namely, using these ATP-α-B analogues, one can selectively activate a specific P2Y-R subtype.

An interesting and significant aspect of the biochemical activity of those ATP-α-B analogues is NTPDase1 stimulation by B isomers **8a-B** and **8c-B**. Indeed, an unexpected rise in Pi release was observed for **8a-B** and **8c-B** even when these analogues were introduced at only 10 μM. To the best of our knowledge, no other ATP analogues have been found to increase NTPDase activity. It would be premature at this stage to suggest an explanation such as the presence of an allosteric site that would respond to these analogues. However, experiments from previous studies have indicated that some ATP analogues exhibit a mixed-type of competitive and uncompetitive inhibition.[32] This would argue again in favor of another binding site at minimal concentration, exerting allosteric effects.

In summary, potent and stable P2Y$_1$-R agonists were developed on the basis of the adenosine 5′-*O*-(1-boranotriphosphate) scaffold. Some of these compounds are highly potent with EC$_{50}$ values in the nanomolar range. Furthermore, they are highly chemically stable under physiological conditions with a half-life of 1395 h. Even under gastric-juice simulating conditions, the half-life of 2-MeS-ATP-α-B was 5.9 h. In addition, these compounds proved to be stable in terms of hydrolysis by spleen NTPDase, with the ATP-α-B A isomer hydrolysis being 5% compared to ATP hydrolysis. In addition, A isomers exhibited protein selectivity for the nucleotide binding site in the P2Y$_1$-R as opposed to the NTPDase hydrolyzing enzyme. Therefore, the new, highly water-soluble P2Y$_1$-R agonists may be considered as potentially attractive drug candidates.

Absolute configuration assignment and therapeutic applications of these analogues will also be published in due course.

Experimental Section

General. All air- and moisture-sensitive reactions were carried out in flame-dried, nitrogen-flushed, two-neck flasks sealed with rubber septa, and the reagents were introduced with a syringe. The progress of the reactions was monitored by TLC on precoated Merck silica gel plates (60F-254). Column chromatography was performed with Merck silica gel 60 (230−400 mesh). Compounds were characterized by nuclear magnetic resonance using Brucker DPX-300, DMX-600, and AC-200 spectrometers. ^{1}H NMR spectra were measured in D_2O, and the chemical shifts are reported in ppm relative to HOD (4.78 ppm) as an internal standard. Nucleotides were also characterized by ^{31}P NMR in D_2O, using 85% H_3PO_4 as an external reference. All final products were characterized on an AutoSpec-E FISION VG high-resolution mass spectrometer by chemical ionization. Nucleotides were desorbed from a glycerol matrix under FAB (fast atom bombardment) conditions in both low and high resolution. Primary purification of the nucleotides was achieved on an LC (Isco UA-6) system using a Sephadex DEAE-A25 column, which was swelled in 1 M $NaHCO_3$ in the cold for 1 day. Final purification of the nucleotides and separation of the diastereoisomer pair were achieved on an HPLC (Merck-Hitachi) system using a semi-preparative reverse-phase (LiChrospher 60, RP-select-B) column. Conditions for LC and HPLC separation are described below. The purity of the nucleotides described below was evaluated on an analytical column in two different solvent systems. A LiChroCART LiChrospher 60 RP-select B column (250 mm × 4 mm) was used with a flow rate of 1 mL/min. One solvent system (I) was 0.1 M TEAA/CH_3OH, 84:16, with isocratic elution. The second 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, applying an isocratic elution of A/B, 16:84. 2-Methylthioadenosine was synthesized from 2-SH-adenosine as described earlier.[13] 2-SH-adenosine was obtained from adenosine in three steps according to literature procedure.[33] 2-Chloroadenosine was prepared in four steps from guanosine through 2,6-Cl-9β-(2′,3′,5′-tri-*O*-acetyl)-D-ribofuranosylpurine[14] by treatment of the latter with NH_3 in EtOH in a sealed ampule at 100 °C for 24 h.

Typical Procedure for the Preparation of 2′,3′-*O*-Methoxymethylidene Adenosine Derivatives (3a−c). *p*-TsOH (2 mmol, 2 equiv) was added to a dry adenosine derivative (1 mmol, 1 equiv) (**2a−c**) in a two-neck flask under N_2, followed by the addition of dry DMF (4 mL). Then, trimethyl orthoformate (50 equiv) was added and the resulting solution was stirred at room temperature for 1 day. The mixture was cooled to 0 °C, and Dowex MWA-1 (weakly basic anion exchanger, 6 equiv) was added. Stirring continued at room temperature for an additional 3 h. The Dowex resin was filtered out in vacuo; the filtrate was concentrated under reduced pressure and coevaporated several times with MeOH to remove residual DMF. The residue was dissolved in $CHCl_3$ and extracted with saturated $NaHCO_3$. The organic phase was dried with Na_2SO_4 and evaporated to give pure protected adenosine derivative **3**.

2′,3′-*O*-Methoxymethylidene Adenosine 3a. 3a was obtained from adenosine and trimethyl orthoformate in 80% yield. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.42, 8.38 (2s, H-8, 1H), 8.21, 8.20 (2s, H-2, 1H), 7.42 (br s, NH_2, 2H), 6.30, 6.20 (2d, J = 3 Hz and J = 2.8 Hz, H-1′, 1H), 6.22, 6.11 (2s, C*H*−OCH_3, 1H), 5.53, 5.47 (2dd, J = 2.8, 6 Hz and J = 3, 7 Hz, H-2′, 1H), 5.26, 5.20 (2t, J = 5.5 Hz, OH-5′, 1H), 5.11, 5.03 (2dd, J = 2.8, 6 Hz and J = 3, 7 Hz, H-3′, 1H), 4.31, 4.24 (2dt, J = 3, 5 Hz, H-4′, 1H), 3.51−3.68 (m, H-5′, 2H), 3.40 (s, O−CH_3, 3H) ppm. ^{13}C NMR (DMSO-d_6, 300 MHz): δ 156.16 (C-6), 152.73 (CH-2), 148.88, 148.84 (C-4), 139.79, 139.69 (CH-8), 119.04, 119.00 (C-5), 118.44, 116.93 (*C*H−OMe), 89.40, 88.76 (CH-1′), 86.93, 85.90 (CH-2′), 83.47, 82.45 (CH-3′), 81.08, 80.72 (CH-4′), 61.58, 61.32 (CH_2-5′), 51.87, 50.40 (OCH_3) ppm. MS CI/NH_3 *m*/*z*: 310 (MH^+).

2-Methylthio-(2′,3′-*O*-methoxymethylidene)adenosine, 3b. 3b was obtained from 2-methylthioadenosine and trimethyl orthoformate in 75% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.96, 7.93 (2s, H-8, 1H), 6.17, 5.91 (2d, J = 3.6 Hz, J = 3.9 Hz, H-1′, 1H), 6.05, 5.97 (2s, C*H*−OMe, 1H), 5.47, 5.39 (2dd, J = 3.9, 6 Hz, H-2′ and J = 3.6, 7 Hz, H-2′, 1H), 5.18−5.22 (m, H-3′, 1H), 4.55, 4.48 (2 "q", J = 2.5 Hz, J = 1.8 Hz, H-4′, 1H), 3.18−4.03 (m, H-5′, 2H), 3.46, 3.35 (2s, OCH_3, 3H), 2.57, 2.56 (2s, S−CH_3, 3H) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz): δ 165.86 (C-2), 154.31, 154.24 (C-6), 149.52 (C-4), 139.35 (C-8), 119.48, 117.74 (*C*H−OMe), 117.41, 117.33 (C-5), 92.33, 92.03 (CH-1′), 87.41, 86.10 (CH-2′), 83.87, 82.72 (CH-3′), 80.97, 80.73 (CH-4′), 62.79, 62.72 (CH_2-5′), 52.95, 51.72 (O−CH_3), 14.47, 14.41 (S−CH_3) ppm. FAB (positive mode) *m*/*z*: 356.035 (MH^+). HR FAB (positive mode) *m*/*z*: calcd for $C_{13}H_{17}N_5O_5S$ (MH^+) 356.1028, found 356.1038.

2-Chloro-(2′,3′-*O*-methoxymethylidene)adenosine, 3c. 3c was obtained from 2-chloroadenosine and trimethyl orthoformate in 81% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.70 (H-8, 1H), 6.69 (s, NH_2, 2H), 6.16, 5.85 (2d, J = 3.6 Hz, J = 3.9 Hz, H-1′, 1H), 6.03, 5.95 (2s, C*H*−OMe, 1H), 5.35−5.17 (2m, H-2′ and H-3′, 2H), 4.53, 4.49 (2 "br s", H-4′, 1H), 4.02−3.8 (m, H-5′, 2H), 3.47, 3.32 (2s, OCH_3, 3H) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz): δ 163.54 (C-2), 156.45 (C-6), 154.15 (C-4), 130.86 (C-8), 119.54, 117.67 (*C*H−OMe), 117.85 (C-5), 92.71, 92.44 (CH-1′), 87.55, 85.94 (CH-2′), 83.98, 82.68 (CH-3′), 80.95, 80.73 (CH-4′), 62.97, 62.88 (CH_2-5′), 53.04, 51.72 (O−CH_3) ppm. MS CI/NH_3 *m*/*z*: 344 (MH^+). HRMS *m*/*z*: calcd for $C_{12}H_{14}ClN_5O_5$ 343.0683, found 343.0671.

General Procedure for the Preparation of Derivatives of Adenosine 5′-*O*-(1-Boranotriphosphate), 8a−c. Protected nucleoside **3** (0.5 mmol) was dissolved in dry $CHCl_3$ (7 mL) in a flame-dried two-neck flask under N_2. $(^iPr)_2NEt$ (0.11 mL, 1.3 equiv) was added at room temperature, and the solution was stirred for 30 min. The mixture was cooled to 0 °C, and to this mixture, $[(^iPr)_2N]_2PCl$ (148 mg, 1.1 equiv) dissolved in $CHCl_3$ (2 mL) was slowly added with a syringe to give intermediate **4**. The resulting solution of derivative **4** was stirred at 0 °C for 2 h followed by the addition of a 1 M solution of $H_2P_2O_7^{2-}$ $(^+HNBu_3)_2$ in DMF (0.75 mL, 1.5 equiv) to produce intermediate **5**. This solution was kept at room temperature for an additional 4 h and then cooled to 0 °C. A 2 M solution of BH_3-SMe_2 complex in THF (2.52 mL, 10 equiv) was added. After the mixture was stirred at room temperature for 15 min, deionized water (8 mL) was added, and the resulting mixture was stirred for 1 h and then freeze-dried. Compound **6,** obtained as a semisolid, was dissolved in water and extracted with $CHCl_3$. The aqueous phase was freeze-dried, and the resulting residue was applied on an activated Sephadex DEAE-A25 column (0−0.7 M NH_4HCO_3, total volume greater than 2000 mL). The relevant fractions were collected and freeze-dried, and excess NH_4HCO_3 was removed by repeated freeze-drying with deionized water to yield compound **7** as the trisammonium salt. The methoxymethylidene protecting group was removed by acidic hydrolysis (10% HCl solution was added until pH 2.3 was obtained). After 3 h at room temperature, the pH was rapidly raised to 9 by the addition of NH_4OH solution (pH 11), and the solution was kept at room temperature for 40 min. The desired adenosine 5′-*O*-(1-boranotriphosphate) derivative **8** was obtained after freeze-drying the solution. Final purification and separation of diastereoisomers of **8** was achieved on a semipreparative HPLC column. The triethylammonium counterions were exchanged for Na^+ by passing the pure diastereoisomer through a Sephadex-CM C-25 column.

ATP-α-B, 8a. 8a was obtained according to the above procedure starting from tetrabenzoyladenosine **9**. The yield was 19%.

2-SMe-ATP-α-B, 8b. 8b was obtained in 38% yield according to the above procedure starting from 2-thiomethyl-(2′,3′-*O*-methoxymethylidene)adenosine.

2-Cl-ATP-α-B, 8c. 8c was obtained in 43% yield according to the above procedure starting from 2-chloro-(2′,3′-*O*-methoxymethylidene)adenosine.

Reverse-Phase HPLC Separation of Diastereoisomers of Adenosine 5′-*O*-(1-Boranotriphosphate) Derivatives 8a−c. The separation of diastereoisomers was accomplished using a semipreparative reverse-phase Lichro CART 250−10 column and isocratic elution [100 mM triethylammonium acetate (TEAA), pH 7 (A)/MeOH (B), 84:16] with a flow rate of 6 mL/min. Fractions containing the same isomer (similar retention time) were freeze-dried. The excess buffer was removed by repeated freeze-drying cycles, dissolving each time the solid residue with deionized water. The isomer with the shorter retention time is designated the A isomer, and the other is designated the B isomer.

ATP-α-B, 8a, A Isomer. Retention time: 10.4 min. ^1H NMR (D_2O, 200 MHz, pH 6.5): δ 8.62 (s, H-8, 1H), 8.25 (s, H-2, 1H), 6.16 (d, $J = 7$ Hz, H-1′, 1H), 4.79 (m, H-2′, 1H), 4.65 (m, H-3′, 1H), 4.42 (m, H-4′, 1H), 4.25 (m, H-5′, 2H), 0.36 (m, BH_3, 3H) ppm. ^{31}P NMR (D_2O, 200 MHz, pH 6.5): δ 83.88 (m, $P_\alpha-BH_3$), −9.42 (d, P_γ), −22.23 (t, P_β) ppm. UV: $\lambda_{max} = 260.5$ nm. FAB (negative mode) *m*/*z*: 526.162 ($M^{4-} + 2H^+ + Na^+$). Retention time: 10.65 min (97% purity) using solvent system I. Retention time:14.92 min (92% purity) using solvent system II.

ATP-α-B, 8a, B Isomer. Retention time: 12.4 min. ^1H NMR (D_2O, 200 MHz, pH 6.5): δ 8.58 (s, H-8, 1H), 8.25 (s, H-2, 1H), 6.15 (d, $J = 7$ Hz, H-1′, 1H), 4.77 (m, H-2′, 1H), 4.56 (m, H-3′, 1H), 4.41 (m, H-4′, 1H), 4.23 (m, H-5′, 2H), 0.36 (m, BH_3, 3H) ppm. ^{31}P NMR (D_2O, 200 MHz): δ 84.5 (m, $P_\alpha-BH_3$), −9.34 (d, P_γ), −22.2 (t, P_β) ppm. UV: $\lambda_{max} = 260.5$ nm. FAB (negative mode) *m*/*z*: 504.094. Retention time: 15.05 min (95% purity) using solvent system I. Retention time:15.99 min (97% purity) using solvent system II.

2-SMe-ATP-α-B, 8b, A Isomer. Retention time: 13.4 min. ^1H NMR (D_2O, 300 MHz, Na^+ form, pH 7.5): δ 8.46 (s, H-8, 1H), 6.14 (d, $J = 5.3$ Hz, H-1′, 1H), 4.69 (dd, $J = 3.8$, 4.9 Hz, H-3′, 1H), 4.38 (m, H-4′, 1H), 4.35, 4.14 (αm, H-5′, 2H), 2.59 (s, C*H*$_3$−S, 3H), 0.47 (m, BH_3, 3H) ppm. ^{31}P NMR (D_2O, 200 MHz): δ 82.7 (m, $P_\alpha-BH_3$), −6.5 (d, P_γ), −21.5 (t, P_β) ppm. UV: $\lambda_{max} = 275.7$ nm. FAB (negative mode) *m*/*z*: 550.172. Retention time: 8.85 min (98% purity) using solvent system I. Retention time: 15.31 min (92% purity) using solvent system II.

2-SMe-ATP-α-B, 8b, B Isomer. Retention time: 15.6 min. ^1H NMR (D_2O, 300 MHz, Na^+ form, pH 7.5): δ 8.42 (s, H-8, 1H), 6.13 (d, $J = 5.6$ Hz, 1H), 4.86 (dd, $J = 5$, 5.6 Hz, H-2′, 1H), 4.61 (dd, $J = 3.6$, 5 Hz, H-3′, 1H), 4.39 (q, $J = 3.6$, 6 Hz, H-4′, 1H), 4.29 (ddd, $J = 2.9$, 7.4, 11.8 Hz, H-5′, 1H), 4.19 (ddd, $J = 2.9$, 5.5, 11.8 Hz, H-5′, 1H), 2.59 (s, C*H*$_3$−S, 3H), 0.46 (m, BH_3, 3H) ppm. ^{31}P NMR (D_2O, 200 MHz) δ 83.9 (m, $P_\alpha-BH_3$), −6.8 (d, P_γ), −21.6 (t, P_β). UV: $\lambda_{max} = 275.7$ nm. FAB (negative mode) *m*/*z*: 550.202. Retention time: 8.52 min (99.5% purity) using solvent system I. Retention time: 15.04 min (91% purity) using solvent system II.

2-Cl-ATP-α-B, 8c, A Isomer. Retention time: 10.2 min. ^1H NMR (D_2O, 300 MHz, Na^+ form, pH 7.5): δ 8.59 (s, H-8, 1H), 6.07 (d, $J = 5$ Hz, H-1′, 1H), 4.69 (dd, $J = 3.6$, 4.5 Hz, H-3′, 1H), 4.41 (m, H-4′, 1H), 4.17, 4.37 (m, H-5′, 2H), 0.5 (m, BH_3, 3H) ppm. ^{31}P NMR (D_2O, 200 MHz) δ 82.9 (m, $P_\alpha-BH_3$), −6.01 (d, P_γ), −21.4 (t, P_β) ppm. UV: $\lambda_{max} = 264.5$ nm. FAB (negative mode) *m*/*z*: 559.023 ($M^{4-} + H^+ + Na^+$). Retention time: 5.86 min (99.5% purity) using solvent system I. Retention time: 13.79 min (94% purity) using solvent system II.

2-Cl-ATP-α-B, 8c, B Isomer. Retention time: 12.6 min. ^1H NMR (D_2O, 300 MHz, Na^+ form, pH 7.5): δ 8.54 (s, H-8, 1H), 6.04 (d, $J = 5.6$ Hz, H-1′, 1H), 4.57 (dd, $J = 3.5$, 4.7 Hz, H-3′, 1H), 4.40 (m, H-4′, 1H), 4.30 (ddd, $J = 2.6$, 7.5, 11.5 Hz, H-5′, 1H), 4.18 (ddd, $J = 2.9$, 5, 11.5 Hz, H-5′, 1H), 0.45 (m, BH_3, 3H) ppm. ^{31}P NMR (D_2O, 200 MHz): δ 84.0 (m, $P_\alpha-BH_3$), −6.4 (d, P_γ), −21.0 (t, P_β) ppm. UV: $\lambda_{max} = 264.5$ nm. FAB (negative mode) *m*/*z*: 559.765 ($M^{4-} + H^+ + Na^+$). Retention time: 5.61 min (95% purity) using solvent system I. Retention time: 14.69 min (92% purity) using solvent system II.

Evaluation of Chemical Stability of ATP-α-B Derivatives. The stability of ATP-α-B derivatives in the appropriate buffer solutions (pH 7.4 or 1.4) was evaluated by ^{31}P NMR at 37 °C for monitoring possible dephosphorylation products. NMR spectra were recorded on a Bruker AC-200 spectrometer with a ^{31}P NMR probe (isotope frequency of 81 MHz) using 85% H_3PO_4 as an external reference.

2-SMe-ATP-α-B tetrasodium salt was dissolved in 0.6 mL of Tris-HCl buffer (pH 7.4), and 0.1 mL of D_2O was added. Spectra were recorded for 3 weeks at 1−3 days intervals at 37 °C. The percentage of phosphate ester hydrolysis is based on integrations of the P_γ signal (−7.5 ppm) and the inorganic phosphate signal (1.7 ppm). The hydrolysis rate was determined by measuring changes in the integration of the respective NMR signals with time.

To evaluate chemical stability at pH 1.4, 2-Cl-ATP-α-B tetrasodium salt was dissolved in 0.54 mL of KCl/HCl buffer (pH 1.4) and D_2O (0.06 mL) was added. The final pH was adjusted to pH 1.4. pH measurements were performed with an Orion microcombination pH electrode and a Hanna Instruments pH meter. Spectra were recorded for 17 h at 0.5 h time intervals at 37 °C. The number of scans in all experiments was 1800, and the number of experiments was 34. The hydrolysis rate was determined by measuring the changes in the absolute intensity of the P_β signal as a function of time and was fit to a pseudo-first-order reaction model.

Evaluation of P_{OW} of ATP-α-B Derivatives. Tris-HCl buffer (pH 7.4) and analytic grade *n*-octanol were stirred together at room temperature for 24 h for presaturation of the solvents. ATP tetrasodium salt and 2-SMe-ATP-α-B, **8b**, tetrasodium salt were each vigorously stirred for 30 min in the presaturated octanol buffer two-phase solution. ATP or

2-SMe-ATP-α-B in buffer solution (6 mL) was shaken with water-saturated octanol (6 mL). After the phase separation, the concentration of ATP and **8b** in the aqueous phase was measured by UV spectroscopy (for ATP, $\lambda_{max} = 260$ nm, $\epsilon = 14393$; for 2-SMe-ATP-α-B, $\lambda_{max} = 276$ nm, $\epsilon = 12637$).

The P_{OW} measurements were also performed using analytical reverse-phase HPLC with isocratic elution of TEAA/MeOH (84:16) for ATP and TEAA/MeOH (80:20) for 2-SMe-ATP-α-B. ATP or 2-SMe-ATP-α-B buffer solution (120 μL) was shaken with octanol (120 μL), and an aliquot of each phase (20 μL) was injected into the HPLC instrument. The concentration of each phase was determined on the basis of the peak area of both the sample and the standard solutions.

NTPDase Assays. Reagents. ATP, tetramisole, imidazole, calcium chloride, malachite green, bovine serum albumin (BSA), and Tris-base were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Cl-ATP and 2-MeS-ATP were purchased from RBI Inc. (Natick, MA). Bradford reagent was obtained from Bio-Rad Laboratories (Mississauga, Ontario, Canada).

NTPDase Assays. Enzyme activity was measured by the release of inorganic phosphate with the malachite green colorimetric assay.[34] Relative activity and resistance to hydrolysis were determined at 37 °C in 1 mL of the following incubation medium: 8 mM $CaCl_2$, 5 mM tetramisole, 50 mM imidazole, and 50 mM Tris-base buffered at pH 7.5. The enzyme preparation (1.1 μg), consisting of a bovine spleen particulate fraction isolated as previously described,[35] was added to the incubation medium and preincubated for 3 min. The reaction was started by adding the substrate, either 100 μM of ATP or its boron analogues, and stopped 7 min later with 250 μL of the malachite green reagent. Controls were run in parallel by adding the enzyme after the malachite green reagent. Enzyme hydrolysis was expressed as units, i.e., micromoles of P_i released per minute per milligram of protein (μmol min^{-1} mg^{-1}).[29] Protein concentration was estimated by the Bradford microplate assay, using bovine serum albumin as a reference standard.[36] All experiments were performed in triplicate.

$[Ca^{2+}]_i$ Measurements. For Ca^{2+} measurements, cells grown on coverslips were loaded for 30 min with 2 μM Fura 2-AM in HEPES buffered saline (HBS) containing 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, and 20 mM HEPES, pH 7.4. Cells were assayed under continuous superfusion of 35 °C prewarmed HBS (1 mL/min) in the presence of varying concentrations of different nucleotides, as indicated.

The relative enzymatic stability of the analogues tested, in addition to the fast superfusion system used to apply the different ATP analogues, precludes any appreciable enzymatic conversion. Thus, problems related to responses that may result from degraded or released nucleotides are circumvented. Constant superfusion of the prewarmed buffer excluded unspecific Ca^{2+} responses caused by mechanical stress, temperature variation, or different components of the buffer.

Fluorescence changes of single cells were detected with an imaging system (TILL Photonics GmbH) attached to a Zeiss axioscope, using alternative excitation at 340/380 nm and emission at 500 nm. The fluorescence emission ratio was converted to intracellular calcium concentration $[Ca^{2+}]_i$ using the equation of Grynkiewicz et al.[37] Concentration−response data were analyzed with the Excel program applying $\Delta F_{340nm}/F_{380nm}$ before and after the addition of the agonist. Curve fitting was performed by adjusting a four parameters smoothed sigmoidal curve, using the SigmaPlot program. EC_{50} values represent the agonist concentration at which 50% of the maximal effect is achieved.

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