Molecular Recognition of Modified Adenine Nucleotides by the $P2Y_1$-Receptor. 1. A Synthetic, Biochemical, and NMR Approach

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The remarkably high potencies of 2-thioether-adenine nucleotides regarding the activation of the $P2Y_1$-receptor ($P2Y_1$-R) in turkey erythrocyte membranes represent some of the largest substitution-promoted increases in potencies over that of a natural receptor ligand.[9,10] This paper describes the investigation regarding the origin of the high potency of these $P2Y_1$-R ligands over that of ATP. For this study, an integrated approach was employed combining the synthesis of new ATP analogues, their biochemical evaluation, and their SAR analysis involving NMR experiments and theoretical calculations. These experiments and calculations were performed to elucidate the conformation and to evaluate the electronic nature of the investigated $P2Y_1$-R ligands. ATP analogues synthesized included derivatives where C2 or C8 positions were substituted with electron-donating groups such as ethers, thioethers, or amines. The compounds were tested for their potency to induce $P2Y_1$-R-mediated activation of phospholipase C in turkey erythrocytes and Ca^{2+} response in rat astrocytes. 8-Substituted ATP and AMP derivatives had little or no effect on phospholipase C or on calcium levels, whereas the corresponding 2-substituted ATP analogues potently increased the levels of inositol phosphates and $[Ca^{2+}]_i$. AMP analogues were ineffective except for 2-butylthio-AMP which induced a small Ca^{2+} response. $P2Y_1$-R activity of these compounds was demonstrated by testing these ligands also on NG108-15 neuroblastoma × glioma hybrid cells. NMR data together with theoretical calculations imply that steric, rather than electronic, effects play a major role in ligand binding to the $P2Y_1$-R. Hydrophobic interactions and H-bonds of the C2 substituent appear to be important determinants of a $P2Y_1$-R ligand affinity.

Introduction

Extracellular adenine nucleotides exert significant biological actions on various peripheral tissues as well as in the central nervous system.[1] ATP plays a role as a neurotransmitter at neuromuscular junctions and within the central nervous system.[2−4] These effects are mediated via membrane receptors for ATP (P2-R) which comprise two major subtypes termed P2X and P2Y.[5] P2X-Rs are ligand (nucleotide)-gated ion channels, whereas P2Y-Rs are G-protein-coupled receptors which induce the formation of various second messengers. Extracellular ATP can exert morphogenic and mitogenic effects on astrocytes via P2Y-Rs.[6]

Potent, subtype-selective P2-R ligands which are chemically and enzymatically stable are targeted as drugs for treatment of a variety of health disorders involving P2-Rs. Yet, the arsenal of P2-R ligands used by pharmacologists and biochemists is limited.[7] Modifications of the 5′-triphosphate chain of the natural ATP pharmacophore were reported.[8] Recently, we reported the synthesis of potent and subtype-selective P2-R agonists.[9−11] These are ATP analogues modified at either ribose or purine rings[9,10] or at purine and phosphate moieties[11a] or are 'mini-nucleotide' analogues, based on a xanthine alkylphosphate scaffold.[11b] One of these series of analogues represents ATP derivatives bearing a long thioether substitution at the C2 position (2-RS-ATP).[10] Apparently, this substitution renders the molecule stable to enzymatic hydrolysis.[11a,12] Moreover, the 2-thioether moiety increases the potency of the molecules toward P2Y-Rs from 2−5 orders of magnitude compared with ATP as established by measurement of $P2Y_1$-R-promoted phospholipase C activity in turkey erythrocyte membranes and inhibition of c-AMP accumulation by adenylyl cyclase-linked P2Y-Rs in C6 rat glioma cells.[9,10] Furthermore, not only does such substitution render some of the corresponding ATP derivatives the most potent P2Y-R ligands presently known, but it also renders the corresponding AMP derivatives to full agonists, whereas AMP itself is totally inactive in biochemical and physiological assays.[10] These remarkably high potencies are among the largest known substitution-promoted increases in potency compared to the potency of the natural receptor ligand.

The electron-donating character of a thioether moiety, which affects the electronic distribution of the adenine ring system, may be responsible for the higher affinity of these derivatives to $P2Y_1$-R due to favorable interactions. Similar effects have been observed in related ring systems.[13,14] Yet, the electronic nature of the purine ring alone does not necessarily determine high affinity for

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the protein, considering that 2-Cl-ATP is a good $P2Y_1$-R agonist, although poorer than 2-MeS-ATP.[10a] Thus, additional factors, e.g., steric and conformational effects or hydrophobic interactions of the C2 side chain with a hydrophobic pocket in the protein, could play a role.

This paper discusses the origin of the increased potency of 2-thioether-ATP analogues as P2Y-R ligands over the potency of ATP. We attempted to identify the stereoelectronic parameters which determine $P2Y_1$-R recognition of modified-purine ATP analogues. For this purpose, we employed an integrated approach combining the synthesis of a new series of ATP analogues as molecular probes; their biochemical evaluation on $P2Y_1$-R-promoted Ca^{2+} elevation in astrocytes and $P2Y_1$-R activation of phospholipase C in turkey erythrocytes; theoretical calculations evaluating the effect of the substituents on the electronic nature of the purine (see part 2 of this paper); and NMR elucidation of the conformation of these ATP derivatives.

Results

Chemistry. A series of ATP analogues were synthesized, where C2 or C8 positions were substituted with electron-donating groups (EDG) such as ethers, thioethers, or amines (Schemes 1, 2). These molecular probes were selected for the evaluation of resonance and inductive effects of substituents on the purine ring system, by biochemical, spectral, and theoretical means. On the basis of the resonance structures of the abovementioned derivatives, both the C2 and C8 electron-donating substitutions enrich both the pyrimidine and imidazole rings with electrons. A C2-EDG increases electron density on N1, N3, C5, and C8, whereas a C8-EDG increases electron density on C2, C4, C6, and N7. On the other hand, inductive effects at the substitution site could also play a role. The alkyl side chain chosen in all of the substitutions was butyl since previous biochemical results indicated that a relatively long thioether at C2 enhanced P2Y-R activity of the ATP analogue.[9,10] 2-Cl-ATP[10a] and 8-Br-ATP,[9] which we reported earlier, were used as prototypes for comparison with the newly synthesized compounds.

Synthesis of 2-Substituted-ATP Derivatives. Preparation of 2-butylether- and 2-butylamino-ATP derivatives started from 2-Br-adenosine (Scheme 1). The latter was prepared in two steps from 2-amino-6-chloro-9β-(2′,3′,5′-tri-*O*-acetyl)-D-ribofuranosylpurine,[15] by treatment with isoamyl nitrite in bromoform[16] followed by deprotection in ethanolic ammonia for 2 days at room temperature. These conditions provided **1** in a high yield, whereas the corresponding reaction in methanolic ammonia yielded mostly 6-OMe-2-bromo-purine-9-ribosyl.

Heating 2-Br-adenosine in excess butylamine, used as both a reagent and a solvent, produced 2-butylamino-adenosine, **2**, in a quantitative yield. The corresponding 2-butylether derivative could not be obtained by simple displacement of the bromide in **1**, because of side reactions of ribosyl alkoxides, formed under these basic conditions and leading to an insoluble product. Therefore, a prior protection of the ribosyl hydroxyls was required. 2-Br-adenosine was persilylated with *t*-BDMSCl and then treated with an equivalent amount of sodium *n*-butoxide in *n*-BuOH in the presence of Et_3N as a solvent, under reflux conditions. Then, tetrabutylammonium fluoride desilylation and one-pot triphosphorylation[10a,17] of nonprotected nucleosides, **2** and **6**, at the 5′-hydroxyl yielded the desired C2 butylamino- or butylether-substituted ATP derivatives **3a** and **7a**. The corresponding AMP derivatives were isolated as side products, due to incomplete triphosphorylation reaction.

2-Butylthioether-adenosine nucleotides **8a,b** were obtained from 2-SH-adenosine.[18,19] The latter was treated with $NaOH/MeOH/H_2O$ and then the dry thiolate salt was added to butyl bromide in DMF to give **8**, followed by 5′-triphosphorylation.

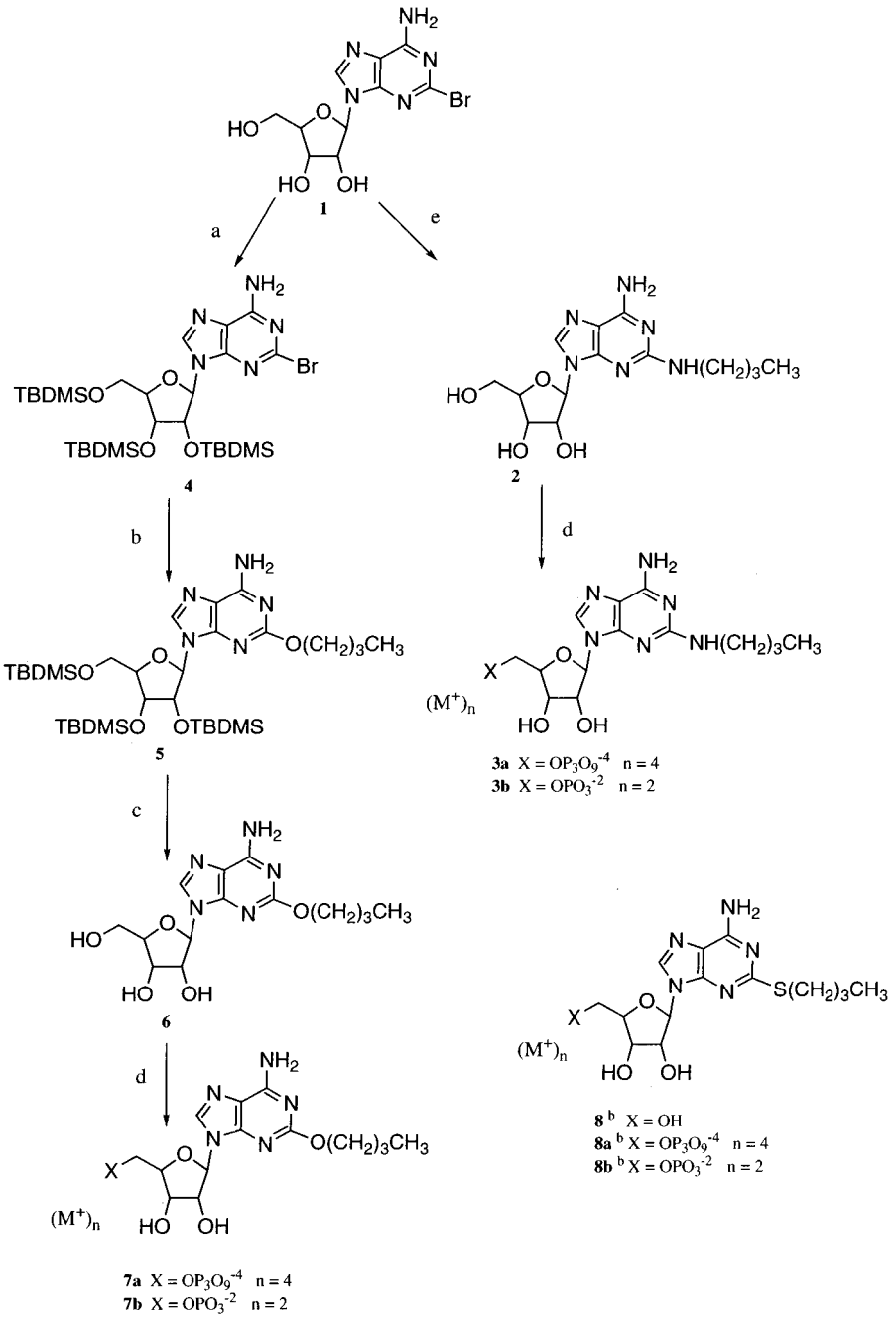
Synthesis of 8-Substituted-ATP Derivatives. The amino and ether derivatives were obtained as described for the corresponding C2 derivatives starting from 8-Br-adenosine (Scheme 2). A prior protection of 2′-, 3′-, and 5′-OH groups was required to avoid formation of the corresponding alkoxides upon reaction of **9** with sodium *n*-butoxide. Protection of **9** with *t*-BDMSCl led, in addition to the trisilylated product **15**, to a tetrasilylated product, **16,** where the N^6 position is protected as well, in a ratio of 4:1, respectively. This phenomenon was not observed in the case of 2-Br-adenosine. This indicates a reduced reactivity of N^6 in the case of 2-Br-adenosine. A difference in the efficiency of the bromide displacement was observed between 2-Br- and 8-Br-adenosine. In the latter case, the reaction was more efficient as reflected for instance by higher yields (86% vs 26%) in the displacement reaction when using sodium *n*-butoxide.

8-Butylthio-adenosine was prepared from 8-SH-adenosine.[14] Isolation of sodium thiolate, **10**, followed by treatment with butyl bromide in DMF afforded 8-butylthio-adenosine in a good yield. Adenosine derivatives **11**, **13,** and **18** were subjected to one-pot phosphorylation conditions to afford both the corresponding 5′-tri- and monophosphate derivatives.

Biochemical Results

Evaluation of the New Compounds as $P2Y_1$-R Ligands in Rat Astrocytes. Brief (30 s) stimulation of astroglia-enriched primary cultures of the brain from newborn rats with 10 μM ATP resulted in a transient rise of the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), as indicated in Figure 1A. To date, five different subtypes of mammalian P2Y-Rs are known.[20] To characterize pharmacologically the subtype(s) expressed in the cell system studied, various purinergic agonists such as ATP, 2-MeS-ATP, UTP, and AMP were used. Results from short-term stimulation (30 s) of the astrocytes with these substances which evoked a transient rise of $[Ca^{2+}]_i$ (Figure 1A−D) were used to derive dose−response curves. The data points were fitted by a three-parameter logistic function yielding EC_{50} values of 6.4×10^{-8}, 7.3×10^{-7}, 4.5×10^{-5}, and 2.1×10^{-5} M for 2-MeS-ATP, ATP, UTP, and AMP, respectively. From the calculated EC_{50} values, we derived an activity sequence: 2-MeS-ATP > ATP > UTP ≫ AMP (Figure 1E). Clearly, the maximum response evoked by 2-MeS-ATP was higher than the maximum response induced by ATP. Application of UTP at a concentration 200 times more than the ATP concentration was required to achieve the maximal Ca^{2+} response (not shown). UTP should activate the $P2Y_2$-R and $P2Y_4$-R but should be ineffective in activating the $P2Y_1$-R. In addition, we calculated the respon-

Scheme 1. 2-Butylamino- and 2-Butylether-ATP Derivatives[a]

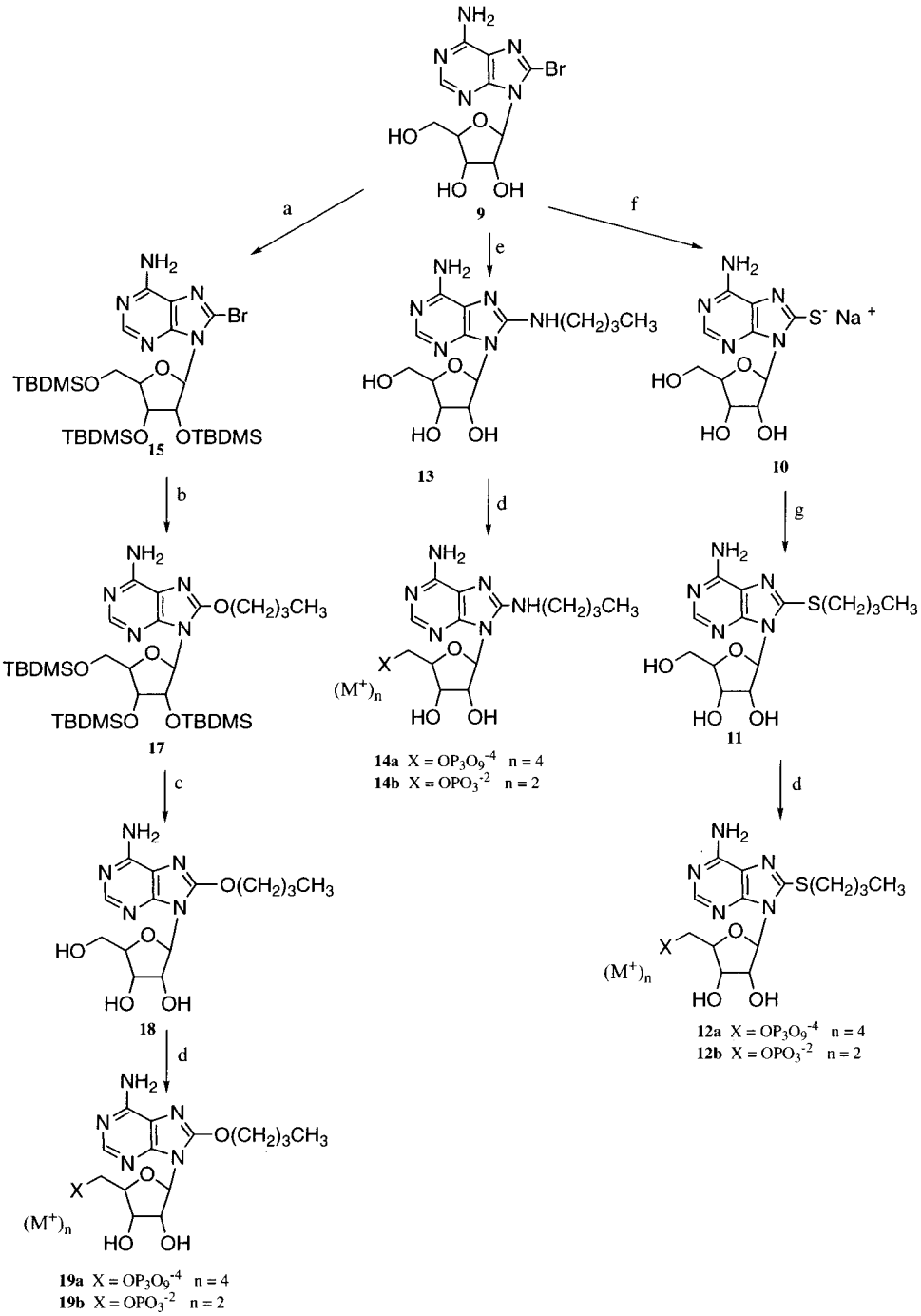


[a] Reagents: (a) tBDMSCl, imidazole; (b) BuONa, BuOH, Et_3N, reflux, 14 days; (c) Bu_4NF, THF, 40 min; (d) 1. $POCl_3$, $PO(OMe)_3$, 2. $(Bu_3NH^+)_2H_2P_2O_7^{2-}$, 3. hydrolysis; (e) $BuNH_2$, reflux. [b] Prepared according to ref 9.

siveness of astrocytes, given as the percentage of cells out of the total number of cells examined, showing an agonist-induced Ca^{2+} response. At submaximal concentrations ATP and 2-MeS-ATP activated 100% of the cells. In contrast with UTP at maximal concentrations, only 90% responsive cells were seen. These results suggest the presence of mainly P2Y$_1$-Rs in astrocytes. In addition, a small contribution of P2Y$_2$-Rs was apparent at high UTP concentrations.[20]

The synthetic ATP and AMP analogues described above were tested for their ability to induce a nucleotide receptor-mediated Ca^{2+} response in primary cultures of rat astrocytes. In a first series of experiments, astrocytes were stimulated for 30 s with the respective ATP and AMP analogues. As summarized in Table 1, ATP and AMP derivatives substituted at C8 with butylamino, -thioether, or -ether proved ineffective in elevating calcium levels. By increasing the concentration of the 8-substituted ATP or AMP derivatives to 50 μM, only 8-BuS-ATP induced a small $[Ca^{2+}]_i$ transient, whereas all the other derivatives caused no change in $[Ca^{2+}]_i$. In contrast, the corresponding 2-substituted ATP analogues evoked a clear transient $[Ca^{2+}]_i$ rise. The dose–response curves and the derived EC_{50} values of 3.4×10^{-7}, 9.0×10^{-7}, and 2.5×10^{-7} M for **3a**, **7a**, and **8a**, respectively, revealed that these analogues possess potencies in the range of 2-MeSATP and ATP. The AMP analogues were ineffective except for 2-butylthio-AMP which induced a small Ca^{2+} response in astrocytes (Table 1).

Scheme 2. 8-Butylamino-, 8-Butylether-, and 8-Butylthioether-ATP Derivatives[a]



[a] Reagents: (a) tBDMSCl, imidazole, DMAP; (b) BuONa, BuOH, Et_3N, reflux, 7 days; (c) Bu_4NF, THF, 40 min; (d) 1. $POCl_3$, $PO(OMe)_3$, 2. $(Bu_3NH^+)_2H_2P_2O_7^{2-}$, 3. hydrolysis; (e) $BuNH_2$, reflux. (f) 1. $(NH_2)_2CS$, DMSO, 2. NaOMe, 3. 0.25 M NaOH, MeOH; (g) $CH_3(CH_2)_3Br$, DMF.

We cannot exclude the activation of a small proportion of $P2Y_2$-Rs in rat astrocytes even at low concentrations of the test compounds. To eliminate such a possible ambiguity in the conclusions about the specificity of the analogues tested, we examined the same substances on neuroblastoma × glioma hybrid cells (clone NG108-15, also designated 108CC15) which are known to express solely the $P2Y_2$-R.[21,22] In the hybrid cells, 100 μM ATP and UTP evoke an identical Ca^{2+} transient (Figure 2). The fact that ATP and UTP but not 2-MeS-ATP (not shown) can induce a $[Ca^{2+}]_i$ response in the neuronal cell line is characteristic for the $P2Y_2$-R subtype. In the concentration range (10−50 μM) tested, C2 substitution with a butyl side chain with the exception of 2-BuS-ATP as well as C8 substitution resulted in ATP analogues with a negligible effect (less than 10% of the UTP response) inducing a Ca^{2+} response in this cell line, (Table 2); thus, the 2-substituted ATP analogues are $P2Y_1$-R- selective ligands being completely ineffective at $P2Y_2$-Rs.

Evaluation of the New compounds as $P2Y_1$-R Ligands in Turkey Erythrocytes. The C2- and C8-substituted analogues of ATP and AMP prepared in this study were also tested for their ability to stimulate phospholipase C activity at the $P2Y_1$-R in turkey erythrocyte membranes. Concentration−effect curves

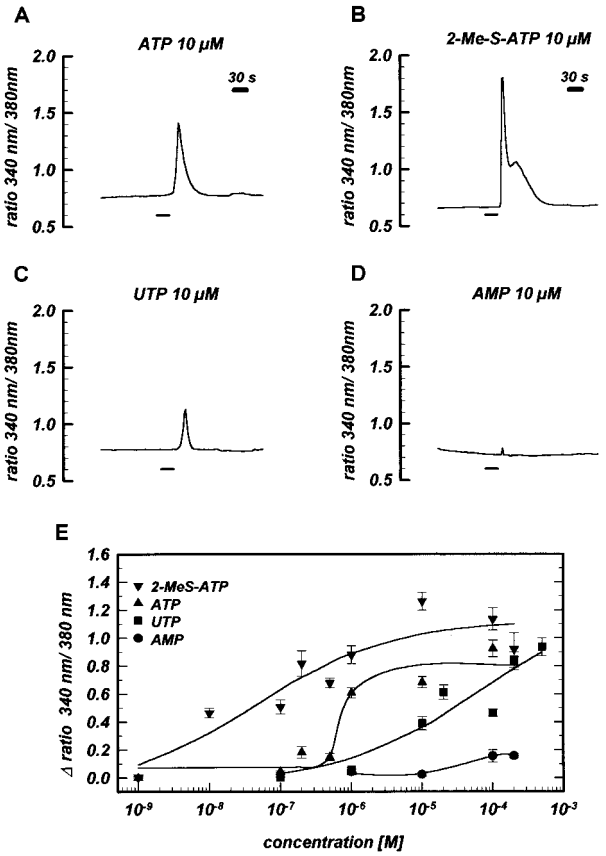


Figure 1. Ca^{2+} response by P2Y-R agonists in rat astrocytes. Fura-2/AM-loaded astrocytes were stimulated for 30 s with different P2-R agonists. The traces shown are representative Ca^{2+} responses obtained for 10 μM ATP (A), 10 μM 2-MeS-ATP (B), 10 μM UTP (C), and 10 μM AMP (D). The changes in $[Ca^{2+}]_i$ are given as changes in the fluorescence ratio F_{340nm}/F_{380nm}. The challenge with the different agonists is indicated by the respective bars. For the concentration−effect curves (E), the maximum change of the ratio (Δ ratio F_{340nm}/F_{380nm}) was plotted against the agonist concentration. The data points represent the mean ± SEM from at least 20 single cells measured, derived from minimally 2 different preparations. The data points were fitted by a three-parameter logistic equation, yielding EC_{50}, maximum response, and the slope of the curve.

Table 1. Amplitude of the $[Ca^{2+}]_i$ Transient Evoked by ATP, AMP, and Synthetic Analogues in Rat Astrocytes[a]

nucleotide analogue C2/C8 substitution	compd no.	Δ ratio, mean ± SEM	*n*	compd no.	Δ ratio, mean ± SEM	*n*
2-BuS	**8a**	0.90 ± 0.06	57	**8b**	0.28 ± 0.02	43
2-BuNH	**3a**	0.89 ± 0.04	40	**3b**	nd	31
2-BuO	**7a**	0.63 ± 0.03	25	**7b**	*	*
8-BuS	**12a**	nd	37	**12b**	nd	24
8-BuNH	**14a**	nd	61	**14b**	nd	58
8-BuO	**19a**	nd	20	**19b**	nd	35

[a] The table summarizes the maximum change of $[Ca^{2+}]_i$ measured, given as Δ ratio (F_{340nm}/F_{380nm}), mean ± SEM, after a brief (30-s) stimulation of rat astrocytes evoked by 10 μM ATP and AMP and the analogues indicated. The number of single cells measured under the respective conditions is given by *n*. The asterisk stands for not tested, and nd means no detectable Ca^{2+} response. For comparison the amplitude obtained with 10 μM ATP was 0.63 ± 0.04 (n = 100) and with 10 μM AMP 0.03 ± 0.01 (n = 20).

were obtained for each compound as indicated in the Methods section. As previously reported, 2-thioether substitution of the ATP molecule, such as 2-butylthio-ATP, resulted in a potent agonist of the P2Y$_1$-R with

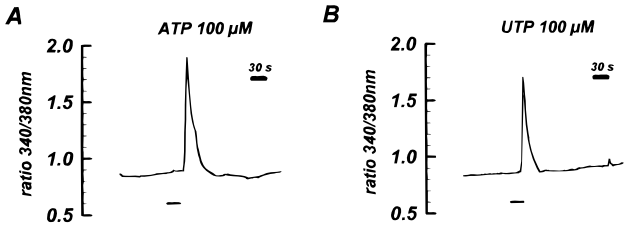


Figure 2. Ca^{2+} response by P2Y-R agonists in NG108-15 neuroblastoma × glioma hybrid cells. For measuring the Ca^{2+} response the cells were loaded with 2 μM fura-2/AM. The plots shown (A, B) are original traces representing the $[Ca^{2+}]_i$ transient, monitored as changes in the fluorescence ratio F_{340nm}/F_{380nm}, induced by a 30-s application of 100 μM ATP and 100 μM UTP, respectively. The presence of the drugs is indicated by the bars.

Table 2. Amplitude of the $[Ca^{2+}]_i$ Transient Evoked by ATP, AMP, and Synthetic Analogues in NG108-15 Neuroblastoma × Glioma Hybrid Cells[a]

nucleotide analogue C2/C8 substitution	compd no.	Δ ratio, mean ± SEM	*n*	compd no.	Δ ratio, mean ± SEM	*n*
2-BuS	**8a**	0.23 ± 0.04	67	**8b**	nd	89
2-BuNH	**3a**	0.05 ± 0.01	79	**3b**	nd	83
2-BuO	**7a**	0.06 ± 0.03	77	**7b**	*	*
8-BuS	**12a**	nd	87	**12b**	nd	84
8-BuNH	**14a**	nd	100	**14b**	nd	85
8-BuO	**19a**	nd	87	**19b**	nd	79

[a] The amplitudes of the Ca^{2+} response induced by 10 μM ATP and AMP analogues are given as mean ± SEM, and *n* represents the number of single cells measured. For comparison the amplitude obtained with 10 μM UTP was 0.66 ± 0.05 (n = 88) and with 10 μM ATP 0.18 ± 0.03 (n = 106). For 2-BuO-AMP, **7b**, no experiments were conducted.

Table 3. Pharmacological Activity of C2 and C8 Substitutions of ATP on the P2Y$_1$-R of Turkey Erythrocytes

analogues	compd no.	EC_{50} ± SEM (μM)
2-BuS-ATP	**8a**	0.018 ± 0.005
2-BuNH-ATP	**3a**	0.039 ± 0.017
2-BuO-ATP	**7a**	0.088 ± 0.027
8-BuS-ATP	**12a**	10[a]
8-BuNH-ATP	**14a**	51[a]
8-BuO-ATP	**19a**	5.71 ± 1.98

[a] n = 2.

potency and efficacy similar to that of the typical P2Y$_1$-R agonist 2-MeS-ADP (Figure 3A and Table 3). The presence of 2-butylamino or 2-butylether substitutions of ATP also resulted in full agonists of slightly lower potency than the 2-butylthio analogue (Table 3). In contrast, the same substitutions at the C8 position on the ATP molecule resulted in compounds with lower potencies for the P2Y$_1$-R than the respective 2-substitutions (Figure 3). The nature of the substitution at the C8 position had little or no effect on the apparent potency of the compound (Figure 3). This finding contrasts with the differential effect on potency of the C2-substituted ATP analogues.

One hundred micromolar concentrations (100 μM) of 2- and 8-substituted AMP produced only between 30% and 60% of the maximal response, and no significant differences in apparent potency were observed between 2- and 8-substitutions, as in the case of the triphosphate analogues (data not shown).

Conformational Studies of the New Compounds by NMR. The possible correlation between the confor-

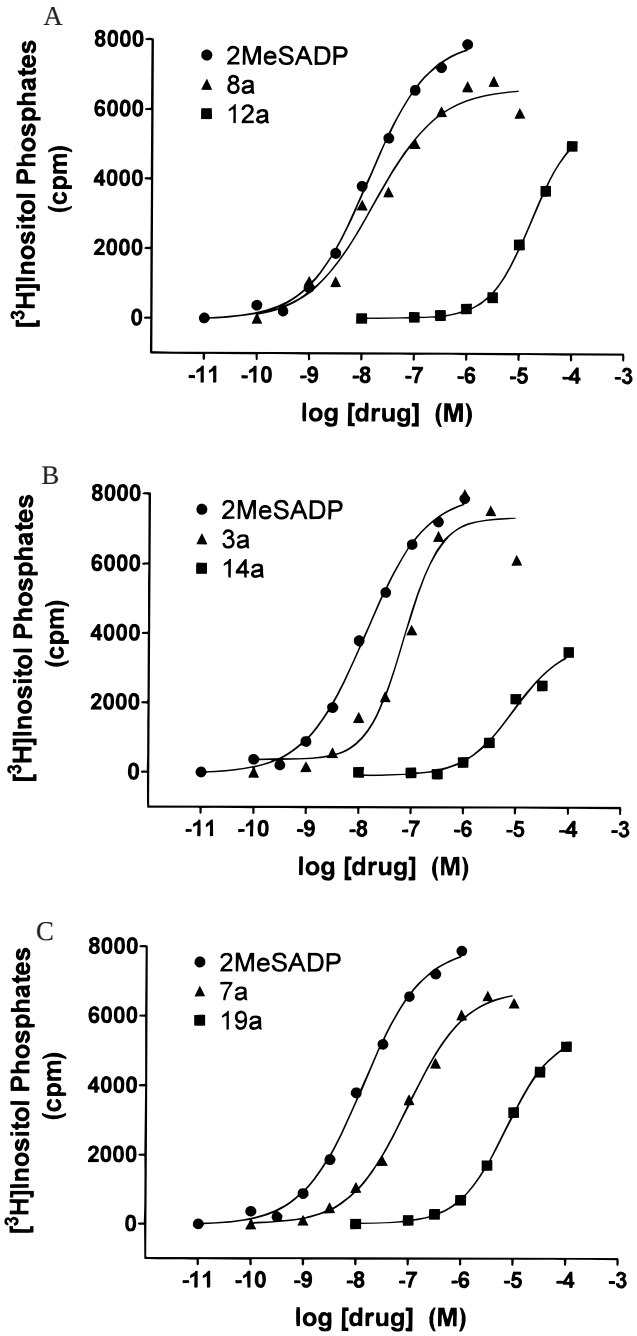


Figure 3. Effects of C2- and C8-substituted ATP analogues on $P2Y_1$-R-mediated inositol lipid hydrolysis in turkey erythrocytes. The capacity of the indicated concentrations of C2- and C8-substituted ATP analogues to stimulate phospholipase C activity was determined as described in the Methods section. Butylthio (A), butylamino (B), and butylether (C) groups were attached at the position C2 (triangles) or position C8 (squares) of ATP. For comparison, the effect of the $P2Y_1$-R agonist 2-MeS-ADP (circles) is also shown. The data presented are from a representative experiment repeated at least three times with different membrane preparations.

mation of the $P2Y_1$-R ligand and its activity was investigated. NOESY NMR experiments have been performed, in D_2O, on the series of 2- and 8-substituted nucleotides to elucidate the conformation around the glycosidic bond.

The NOESY spectrum of 2-SBu-ATP, **8a**, in D_2O indicated clear cross-peaks between H-8 and H-2′ and H-3′, thus proving a predominant *anti* conformation of this nucleotide in aqueous solution. Proton-coupled ^{13}C NMR spectra, measured in D_2O at 600 MHz, provided the values of 4.3 and 2.3 Hz for ^3J(C8,H1′) and ^3J(C4,-H1′), which are consistent with literature values for '*anti*-type' nucleotides.[23a]

Table 4. Chemical Shifts of Purine Carbons in Nucleoside Derivatives in DMSO-d_6 at 75.4 MHz Determined by HMBC/HMQC Experiments

analogue	compd no.	C2	C4	C5	C6	C8
adenosine		152.6	149.2	119.5	156.3	140.2
2-Br-Ad	**1**	144.1	150.1	118.4	156.5	139.8
2-BuS-Ad	**8**	163.7	150.0	116.8	155.4	138.7
2-BuNH-Ad	**2**	158.9	151.5	113.4	155.6	136.6
2-BuO-Ad[a]	**6**	163.3	152.2	116.8	158.2	140.4
8-BuS-Ad	**11**	151.2	150.3	119.5	154.4	148.6
8-BuNH-Ad	**13**	149.3	150.2	117.0	152.4	152.0
8-BuO-Ad	**18**	150.7	148.7	115.0	154.1	154.1

[a] ^{13}C NMR spectrum was measured in MeOH-d_4.

Attempts to elucidate the conformation around the glycosidic bond in 8-SBu-AMP, **12a**, applying a NOESY spectrum in D_2O, failed, due to the absence of significant cross-peaks. However, an indication of a predominant *syn* conformation of this derivative is the downfield shift observed for H-2′ (5.2 ppm) vs H-2′ (4.8 ppm) in 2-SBu-AMP **8a**. A similar effect was observed for 8-OBu-AMP, **19a**. This shift may be due to the anisotropic effect of the purine ring adjacent to the ribose ring. Such downfield shifts for H-2′ were reported for nucleotides in the *syn* conformation.[23b,24a] In addition, a predominant *syn* conformation is indicated in proton-coupled ^{13}C NMR spectrum. The measured values of 5 and 4.5 Hz for ^3J(C8,H1′) and ^3J(C4,H1′) are typical of '*syn*-type' nucleotides.[23a]

Literature reports proposed a preferential *anti* conformation for 8-amino- and 8-alkylamino-adenosine or -AMP analogues based on certain ^1H NMR and crystallographic data.[24] This surprising conclusion was explained by a favorable H-bond between 8-NH and 5′-O, which overcomes the steric hindrance of a large substituent at the C8 position.[24]

Consistent with these reports, we observed that ^3J(C8,H1′) and ^3J(C4,H1′) values measured for 8-NHBu-AMP are 5 and 2.5 Hz, respectively, indicative of '*anti*-type' nucleotides.[24c] Although the NOESY spectrum of this compound in D_2O was inconclusive, the chemical shift of H-2′ is 4.8 ppm, indicative of a preferential *anti* conformation.

Therefore, we conclude that 8-NHBu-AMP in D_2O adopts a preferential *anti* conformation, whereas 8-SBu- and 8-OBu-adenine nucleotides adopt predominantly a *syn* conformation.

Electronic effects of 2- or 8-substitutions on the purine ring system were also evaluated by ^{13}C NMR spectra. Table 4 shows ^{13}C NMR chemical shift values for the purine carbons in 2- or 8-substituted nucleosides in DMSO-d_6. These values, which are at a lower field than those in adenosine, indicate a dominant negative inductive effect, rather than resonance effects, on the substituted carbons (e.g., adenosine C2 and 2-BuS-adenosine C2 appear at 152.6 and 163.7 ppm, respectively; adenosine C8 and 8-BuO-adenosine C8 appear at 140.2 and 154.1 ppm, respectively). The effect on other carbons is negligible.

Discussion

We have previously reported the identification of high-affinity P2Y$_1$-R-selective agonists based on adenine nucleotides modified on the purine ring as chain-extended 2-thioethers (2-RS-ATP).[9,10] The series of 2-RS-ATP analogues stimulated the production of inositol phosphates, promoted by P2Y$_1$-R, with $K_{0.5}$ values of 1.5−770 nM, which are up to 4 orders of magnitude more potent than ATP. In C6 rat glioma cells, some of these analogues inhibited c-AMP accumulation, via adenylyl cyclase-linked P2Y-R, with EC_{50} values of approximately 30 pM. These values represent 100 000-fold increases in potency over ATP.[10b] We also reported P2Y$_1$-R activity of ATP derivatives bearing electron-withdrawing atoms at C2 or C8 positions.[9,10a] Thus, 2-Cl-ATP was approximately 40 times more potent than ATP with $K_{0.5}$ of 72 nM, as established by measurement of P2Y$_1$-R-promoted phospholipase C activity in turkey erythrocyte membranes;[10a] 8-Br-ATP, with $K_{0.5}$ of 47 400 nM, was characterized as a poor P2Y$_1$-R agonist, which is 17 times less potent than ATP.[9]

In the current study, the newly synthesized analogues were evaluated as agonists of the P2Y$_1$-R in the turkey erythrocyte membrane model system; also the physiological Ca^{2+} response of these analogues was tested in fura-2/AM-loaded rat astrocytes and NG108-15 neuroblastoma × glioma hybrid cells. We clearly demonstrated that substitution at the 2-position of the purine ring with SBu, NHBu, or OBu resulted in ATP derivatives capable of inducing activation of phospholipase C and a Ca^{2+} response equipotent to that of 2-MeS-ADP and 2-MeS-ATP, respectively. 2-BuS-ATP and 2-BuNH-ATP are more effective agonists than the corresponding butylether derivative in both phospholipase C activity and Ca^{2+} measurements. However, similar substitutions at the 8-position resulted in compounds with little or no activity at P2Y$_1$-Rs. The pharmacological characterization of the Ca^{2+} responses revealed that astrocytes clearly express the P2Y$_1$-R and to some degree P2Y$_2$-R or P2Y$_4$-R subtypes.[25] However, we cannot completely rule out that the compounds tested also activate, to a small extent, the P2Y$_2$ subtype, as described for rat cortical[26] and striatal astrocytes.[25] To draw conclusions about the specificity of the synthetic compounds, we also tested them on NG108-15 cells. Confirming our previous results[21] we demonstrated that NG108-15 cells express the P2Y$_2$-R. In these cells, neither 8-substituted ATP derivatives nor the 2-substituted derivatives 2-BuOATP and 2-BuNHATP were physiologically active, and 2-BuSATP possesses only a minor Ca^{2+}-inducing efficacy. This demonstrates that **3a**, **7a**, and **8a** are P2Y$_1$-R-selective ligands.

The above-mentioned results of functional assays involving P2Y$_1$-R were used for exploring the origins of the large effect of a 2-thioether substitution on the affinity of an ATP derivative toward P2Y$_1$-R.

The compounds prepared for this study are water soluble, are chemically and enzymatically stable under the assay conditions,[12b] and have no permeability problems regarding their interaction with the membrane receptor. Therefore, a reasonable assumption is that molecular parameters (electronic or steric factors) are those that possibly govern the affinity of the ligand to P2Y$_1$-R.

Both our results and literature reports show that substitution of the C2 position with either electron-donating or -withdrawing groups makes the ATP analogue a good P2Y$_1$-R agonist. However, such substitutions at C8 render the corresponding ATP derivatives poor P2Y$_1$-R agonists or non-agonists. These facts imply that electronic effects do not fully explain the difference in P2Y$_1$-R activity between these two series of purine-modified ATP analogues.

To establish the role of the electronic and steric effects in the recognition of those modified-purine nucleotides by P2Y$_1$-R, these effects were further studied by theoretical calculations (in part 2 of this paper)[27] and NMR experiments.

Ab initio quantum mechanical calculations of properties such as tautomerism, dipole moment, probable protonation sites, and molecular electrostatic potential (MEP) have been performed for adenine models bearing either electron-donating or -withdrawing groups at C2 or C8 positions.[27] These properties help determine potential H-bonding pattern and complementarity of the modified adenine moiety with the protein binding cavity. Apparently, the dominant tautomer was found to be the N^6 amino in all substituted models.[27] This finding implies that the H-bonding pattern of the modified derivatives with the receptor should be similar to that of the parent compound, ATP. Furthermore, we found no correlation between the MEP maps or the size and direction of calculated dipole moments, which are indicative of electron distribution of the title compounds, and the observed trend in the biochemical results. In summary, the large effect of 2-BuS vs 2-H or 8-BuS substitution on their potency regarding P2Y$_1$-R cannot be explained by H-bonding and electronic complementarity of the purine ring and P2Y$_1$-R binding site.

Therefore, steric and conformational differences between 2- and 8-substituted-adenine nucleotides were considered as possible parameters which determine the activity of those compounds.

Free nucleotides in solution, adopt a rather 'standard' geometry: i.e., the base is oriented *anti* with respect to the sugar ($-90° < \chi < -160°$), the sugar pucker is a mixture of either C2′-*endo* or C3′-*endo* conformers, and the γ angle (orientation about C5′−C4′) is ca. 60° (+*sc*).[28] However, large C8 substituents are known to impede the *anti* conformation due to hindrance with the ribose moiety.[28b,23a]

Our NMR structural data support a preferential *anti* conformation for the 2-substituted nucleotide derivatives and *syn* conformation for the 8-SBu and 8-OBu nucleotides in D_2O solution. 8-NHBu-adenine nucleotides, however, adopt a preferential *anti* conformation.

On the basis of the above observations, a possible explanation for the potency of ATP derivatives substituted with an electron-donating or -withdrawing group at C2, as opposed to the corresponding 8-substituted analogues, could be a major contribution of steric effects rather than electronic effects. Modeling studies demonstrate that there is a tight fit between the *anti* conformer of ATP or 2-MeS-ATP and the binding cavity in the cP2Y$_1$-R and hP2Y$_1$-R.[29] The steric incomplementarity of a *syn* adenine nucleotide with the binding site and of a C8-substituted '*anti*-type' analogue might be

the source of inactivity of these compounds, rather than the electronic factor.

However, the nucleotide conformation still cannot explain the large difference in affinity toward the receptor between ATP and the 2-substituted-ATP analogues, where all compounds possess an *anti* conformation. A possible explanation is based on a molecular model of the hP2Y$_1$-R:2-MeS-ATP complex reported recently by Moro et al.[29b] In this model, the MeS group is surrounded by a hydrophobic pocket.[27] Favorable interactions are therefore expected for 2-MeS-ATP and 2-BuS-ATP compared with ATP. Furthermore, the bulky sulfur atom, which is capable of both a tighter fit with the binding site and H-bonding, probably enhances affinity, compared with 2-BuO-ATP and 2-BuNH-ATP.[27] In this context, the difference in enzymatic stability between 2-substituted-ATP analogues and the parent compound is not the reason for the enhanced potency of the former since under the assay conditions (incubation of 30 s at room temperature) only ca. 1% of ATP undergoes degradation.[12]

Why is 2-Cl-ATP still an agonist, although poorer than 2-RS-ATP? A possible answer comes from MEP results showing that a 2-Cl substituent affects the N^6 amine making the hydrogens more positive[27] and therefore more capable of H-bonding relative to the parent compound.

We assume that the same reasoning for the activity of 2-RS-ATP ligands holds for 2-BuNH-ATP and 2-BuO-ATP. The latter is an active P2Y$_1$-R ligand, albeit less potent than 2-BuS-ATP and 2-BuNH-ATP. This difference could be putatively explained on the basis of a tighter fit between the receptor binding cavity and the bulkier side chain in the case of 2-RS-ATP or the presence of an additional H-bond in the case of 2-BuNH-ATP.

Conclusions

The question on the origins of the high affinity of 2-RS-ATP analogues toward P2Y$_1$-R compared with ATP is addressed by a synthetic, biochemical, spectral, and theoretical approach. C2- and C8-substituted-adenine nucleotides were used as molecular probes for the evaluation of stereoelectronic effects on the nucleotide and, consequently, on the affinity toward P2Y$_1$-R. These molecular probes were evaluated biochemically on turkey erythrocyte and rat astrocyte P2Y$_1$-R. 2-BuS-, 2-BuNH-, and 2-BuO-ATP proved to be potent P2Y$_1$-R agonists, whereas the corresponding 8-substituted derivatives were practically inactive. The spectral and theoretical results suggest that a steric factor, rather than an electronic factor, governs molecular recognition of an ATP derivative by P2Y$_1$-R. Thus, the C8-substituted derivatives, e.g., 8-BuS- and 8-BuO-ATP, which adopt a preferential *syn* conformation, rather than the *anti* conformation typical of C2-substituted derivatives, are not recognized by P2Y$_1$-R. Likewise, a C8-substituted '*anti*-type' adenine nucleotide, e.g., 8-NHBu-ATP, is incompatible with the P2Y$_1$-R binding site due to steric hindrance caused by the bulky C8 substituent. 2-BuS-ATP is more active than ATP, although both possess an *anti* conformation, probably because of a tighter fit of the ligand to the binding cavity due to interaction of a C2 substituent with a hydrophobic pocket[27,29] in the receptor binding cavity. Within the series of ATP derivatives substituted at C2 with electron-donating groups, 2-BuS-ATP is the most potent, possibly due to a tighter fit of the bulkier side chain of BuS- and H-bonding of the sulfur atom.[27] The P2Y$_1$-R activity of 2-Cl-ATP can be explained based on MEP results, showing a high H-bonding capability of N^6 hydrogens.

Methods

Chemistry. General. ^{1}H spectra were measured at 200, 300, or 600 MHz and ^{13}C NMR spectra were measured at 75.4 MHz on Bruker AC-200, DPX-300, or DMX-600 spectrometers. The chemical shifts are reported in ppm relative to tetramethylsilane (TMS) as an internal standard. Nucleotides were characterized also by ^{31}P NMR in D_2O using 85% H_3PO_4 as an external reference on a Bruker AC-200 spectrometer. Carbon assignments in nucleosides are based on heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments. COSY (correlated spectroscopy), HMQC, HMBC, and NOESY (nuclear Overhauser and exchange spectroscopy) experiments were recorded on a Bruker DMX-600. Mass spectra were recorded on an AutoSpec-E-FISION VG high-resolution mass spectrometer. Nucleotides were characterized by FAB (fast atom bombardment) and high-resolution FAB using glycerol matrix under FAB negative conditions on an AutoSpec-E-FISION VG high-resolution mass spectrometer. Separation of the newly synthesized nucleotides was achieved on LC (Isco UA-6) using DEAE A-25 Sephadex (HCO_3^- form) anion exchanger as described below. Final purification was done on a HPLC (Merck-Hitachi) system using a semipreparative nucleotide/nucleoside 7U column (1 × 25 cm; Alltech Associates, Inc., Deerfield, IL) and a linear gradient of 0.1 M triethylammonium acetate buffer (TEAA, pH 7.5) and acetonitrile (see below) at 5 mL/min flow rate. For analytical purposes, a LiChroCART LiChrospher 60 RP-select B column (250 mm × 4.6 mm; Merck KGaA) was used applying the same gradient as above at 1 mL/min flow rate. The purity of the nucleotides described below was evaluated on an analytical column in two different solvent systems. One solvent system (I) was 0.1 TEAA/CH_3-CN, 90:10 to 40:60 in 20 min. 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 a gradient of 25% A to 75% A in 20 min.

2-Bromo-adenosine (1). A sealed ampule containing 2-bromo-6-chloro-9β-(2′,3′,5′-tri-*O*-acetyl)-D-ribofuranosylpurine[16] (0.6 g, 1.22 mmol) and NH_3/EtOH 2 M (100 mL) at liquid nitrogen bath was allowed to reach room temperature (RT). After 1 h, a clear yellow solution was formed. Stirring was continued for 2 days at RT; then the solvent was removed under reduced pressure. The residue was purified using silica gel chromatography (elution with $CHCl_3$:MeOH 8:1). The product was obtained as a white solid: 0.348 g (82%); mp 134.5 °C; ^{1}H NMR (CD_3OD, 200 MHz) δ 8.26 (s, 1H, H-8), 5.92 (d, J = 6 Hz, 1H, H-1′), 4.67 (dd, J = 6, 5.5 Hz, 1H, H-2′), 4.32 (dd, J = 5, 3 Hz, 1H, H-3′), 4.15 (q, J = 3 Hz, 1H, H-4′), 3.91 (dd, J = 12.5, 3 Hz, 1H, H-5′), 3.75 (dd, J = 12.5, 3 Hz, 1H, H-5′); ^{13}C NMR (CD_3OD, 300 MHz) δ 157.9 (s, C-6), 151.1 (s, C-2), 145.7 (s, C-4), 142.0 (d, C-8), 120.2 (s, C-5), 90.9 (d, C-1′), 87.8 (d, C-4′), 75.4 (d, C-2′), 72.3 (d, C-3′), 63.3 (t, C-5′); MS (CI/NH_3) m/z 346, 348 MH^+.

When 2 M NH_3/MeOH solution (16 mL) was used for the reaction with 2-bromo-6-chloro-9β-(2′,3′,5′-tri-*O*-acetyl)-D-ribofuranosylpurine (0.1 g, 0.27 mmol), the reaction was completed after 1 day. TLC on a silica gel plate, using $CHCl_3$:MeOH 4:1, indicated the disappearance of the starting material and the formation of two products: 2-bromo-6-methoxy-9β-D-ribofuranosylpurine (R_f = 0.5) and 2-bromo-adenosine as a minor product (R_f = 0.31) in a ratio of 3:1, respectively. The products were purified on a silica gel column ($CHCl_3$:MeOH 10:1).

2-(*n*-Butylamino)adenosine (2). A mixture of 2-bromo-adenosine (330 mg, 0.95 mmol) and *n*-butylamine (10 mL) was

heated under reflux for 2 days. The mixture was concentrated under reduced pressure and the residue was treated with MeOH (5 mL). The suspension was filtered and the filtrate was evaporated to give the product in a quantitative yield as a beige solid: ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.91 (s, 1H, H-8), 6.84 (br s, 2H, NH_2), 6.24 (br s, 1H, N*H*CH$_2$), 5.71 (d, J = 6 Hz, 1H, H-1′), 5.37 (br s, 1H, OH-2′), 5.13 (br d, J = 4 Hz, 2H, OH-3′ & OH-5′), 4.60 (br s, 1H, H-2′), 4.13 (br q, J = 3 Hz, 1H, H-3′), 3.89 (q, J = 3 Hz, 1H, H-4′), 3.63 (dd, J = 12, 4 Hz, 1H, H-5′), 3.52 (dd, J = 12, 4.5 Hz, 1H, H-5′), 3.23 (q, J = 6 Hz, 2H, NHC*H*$_2$), 1.48 (quint, J = 7 Hz, 2H, NHCH$_2$C*H*$_2$), 1.31 (sex, J = 7 Hz, 2H, C*H*$_2$CH$_3$), 0.89 (t, J = 7 Hz, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 158.9 (s, C-2), 155.6 (s, C-6), 151.5 (s, C-4), 136.6 (d, C-8), 113.4 (s, C-5), 87.2 (d, C-1′), 85.3 (d, C-2′), 72.9 (d, C-3′), 70.7 (d, C-4′), 61.8 (t, C-5′), 40.7 (t, NHCH$_2$), 31.4 (d, NHCH$_2$*C*H$_2$), 19.7 (d, *C*H$_2$CH$_3$), 13.9 (q, CH$_3$).

2-Bromo-2′,3′,5′-tri-*O*-(*tert*-butyldimethylsilyl)adenosine (4). 2-Bromo-adenosine (0.4 g, 1.16 mmol), *tert*-butyldimethylsilyl chloride (1.73 g, 10 equiv), and imidazole (1.6 g, 20 equiv) were dissolved in dry THF:DMF solution (5:2 mL). The reaction mixture was stirred under nitrogen at RT overnight and then concentrated under reduced pressure. The residue was dissolved in $CHCl_3$, washed twice with aqueous $NaHCO_3$ and once with water, and then dried over Na_2SO_4. The residue obtained after evaporation was purified on a silica gel column and eluted with $CHCl_3$:MeOH 10:1. The product was obtained as a white solid (0.722 g, 90% yield, mp 148−151 °C): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.50 (s, 1H, H-8), 5.96 (d, J = 4 Hz, 1H, H-1′), 4.60−4.51 (m, 1H, H-2′), 4.27 (t, J = 4.5 Hz, 1H, H-3′), 4.21−4.11 (m, 1H, H-4′), 4.09 (dd, J = 11, 4 Hz, 1H, H-5′), 3.80 (dd, J = 11, 2 Hz, 1H, H-5′), 0.97 (s, 9H, TBDMS), 0.92 (s, 9H, TBDMS), 0.86 (s, 9H, TBDMS), 0.18 (s, 3H, TBDMS), 0.17 (s, 3H, TBDMS), 0.10 (s, 3H, TBDMS), 0.09 (s, 3H, TBDMS), 0.04 (s, 3H, TBDMS), −0.05 (s, 3H, TBDMS); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 154.1 (s, C-6), 148.9 (s, C-2), 146.3 (s, C-4), 137.4 (d, C-8), 114.4 (s, C-5), 89.9 (d, C-1′), 84.9 (d, C-4′), 76.0 (d, C-2′), 70.2 (d, C-3′), 61.5 (t, C-5′), 26.2 (q, 3C, TBDMS), 25.8 (q, 3C, TBDMS), 25.7 (q, 3C, TBDMS), 18.6 (s, TBDMS), 18.0 (s, TBDMS), 17.0 (s, TBDMS), −4.2 (q, TBDMS), −4.8 (q, TBDMS), −4.8 (q, TBDMS), −4.9 (q, TBDMS), −5.1 (q, TBDMS), −5.4 (q, TBDMS).

2-(*n*-Butoxy)adenosine (6). Product **4** (0.79 mmol) was dissolved in Et_3N (4 mL). Sodium butoxide (0.48 mmol Na dissolved in 5 mL *n*-butanol) was added, and the solution was stirred under reflux for 7 days. The mixture was concentrated under reduced pressure. $CHCl_3$ was added to the residue, and the suspension was filtered using a membrane filter. The filtrate was evaporated to dryness and then dissolved in THF (1 mL). Tetrabutylammonium fluoride (1 M in THF, 3 equiv, 2.4 mL) was added, and the solution was stirred for 1.5 h. The residue obtained after evaporation was chromatographed on a silica gel column ($CHCl_3$:MeOH 15:1). Product **6** was obtained in 26% yield: ^{1}H NMR (CD_3OD) δ 8.12 (s, 1H, H-8), 5.91 (d, J = 5.5 Hz, 1H, H-1′), 4.75 (t, J = 5.5 Hz, 1H, H-2′), 4.35 (dd, J = 5.5, 3.5 Hz, 1H, H-3′), 4.29 (t, J = 6.5 Hz, 2H, OCH$_2$), 4.13 (q, J = 3.5 Hz, 1H, H-4′), 3.88 (dd, J = 12.5, 3 Hz, 1H, H-5′), 3.74 (dd, J = 12.5, 3.5 Hz, 1H, H-5′), 1.82−1.30 (m, 4H, OBu), 0.97 (t, J = 7 Hz, 3H, CH$_3$); ^{13}C NMR (CD_3OD, 300 MHz) δ 163.3 (s, C-2), 158.2 (s, C-6), 152.2 (s, C-4), 140.4 (d, C-8), 116.8 (s, C-5), 90.6 (d, C-1′), 87.4 (d, C-4′), 75.2 (d, C-2′), 72.3 (d, C-3′), 68.3 (t, OCH$_2$), 63.3 (t, C-5′), 32.3 (t, OCH$_2$*C*H$_2$), 20.3 (t, *C*H$_2$CH$_3$), 14.2 (q, CH$_3$); MS (CI/NH$_3$) m/z 340 MH$^+$.

2-(*n*-Butylthioether)adenosine (8). A suspension of 2-thioladenosine (0.223 g, 0.747 mmol, in 10 mL MeOH) was dissolved in 0.25 M NaOH (3.35 mL). The clear, yellow solution was stirred at room temperature for 1 h. After freeze-drying, the thiolate sodium salt obtained as a yellowish solid was dissolved in dry DMF (5 mL) and 1-butyl bromide (89 μL, 1.1 equiv) was added. The solution was stirred under nitrogen at RT for 4 h. The solvent was evaporated in vacuo and the yellow residue was coevaporated repeatedly with MeOH, until the residue turned into a yellow solid. The solid was triturated with petroleumether/ether 1:1, then dissolved in MeOH (2 mL), and filtered through a short column of silica (washed with chloroform/methanol 4:1). The pure white solid obtained after evaporation was dried in vacuo for 2 days to give 0.244 g (92%) of **8**: ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.22 (s, 1H, H-8), 7.35 (bs, 2H, NH_2), 5.81(d, J = 6 Hz, 1H, H-1′), 5.45 (d, J = 6 Hz, 1H, OH-2′), 5.20 (d, J = 5 Hz, 1H, OH-3′), 5.07 (t, J = 5.5 Hz, 1H, OH-5′), 4.68−4.56 (m, 1H, H-2′), 4.19−4.09 (m, 1H, H-3′), 3.97−3.87 (m, 1H, H-4′), (H-5′ is hidden by the water peak), 3.15−3.03 (m, 2H, SCH$_2$), 1.73−1.56 (m, 2H, SCH$_2$C*H*$_2$), 1.53−1.32 (m, 2H, C*H*$_2$CH$_3$), 0.92 (t, J = 7 Hz, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 163.7 (s, C-2), 155.4 (s, C-6), 150.0 (s, C-4), 138.7 (d, C-8), 116.8 (s, C-5), 87.3 (d, C-1′), 85.3 (d, C-4′), 73.0 (d, C-2′), 70.4 (d, C-3′), 61.5 (t, C-5′), 31.1 (t, SCH$_2$), 29.7 (t, SCH$_2$*C*H$_2$), 21.3 (t, *C*H$_2$CH$_3$), 13.5 (q, CH$_3$); MS (CI/CH$_4$) m/z 356 MH$^+$.

8-(*n*-Butylthioether)adenosine (11). The compound was prepared as described for 2-thioadenosine and obtained in 90% yield as a white solid: mp 171.5 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.06 (s, 1H, H-2), 7.29 (br s, 2H, NH_2), 5.77 (d, J = 6 Hz, 1H, H-1′), 5.68 (dd, J = 8.5, 3.5 Hz, 1H, OH-5′), 5.42 (d, J = 6.5 Hz, 1H, OH-2′), 5.21 (d, J = 4.5 Hz, 1H, OH-3′), 4.99 (q, J = 6 Hz, 1H, H-2′), 4.19−4.11 (m, 1H, H-3′), 4.00−3.92 (m, 1H, H-4′), 3.74−3.45 (m, 2H, H-5′), (SCH$_2$ is hidden by the water peak), 1.77−1.60 (m, 2H, SCH$_2$C*H*$_2$), 1.52−1.32 (m, 2H, C*H*$_2$CH$_3$), 0.90 (t, J = 7 Hz, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 154.4 (s, C-6), 151.2 (d, C-2), 150.3 (s, C-4), 148.6 (s, C-8), 119.5 (s, C-5), 88.8 (d, C-1′), 86.5 (d, C-4′), 71.2 (d, C-2′), 70.9 (d, C-3′), 62.1 (t, C-5′), 32.0 (t, SCH$_2$*C*H$_2$), 30.8 (t, SCH$_2$), 21.1 (t, *C*H$_2$CH$_3$), 13.3 (q, CH$_3$); MS (CI/CH$_4$) m/z 356 MH$^+$.

8-(*n*-Butylamino)adenosine (13) was prepared as described for **2.** The reaction was completed after 1 day. The product was obtained in a quantitative yield as a beige solid: ^{1}H NMR (DMSO-d_6, 200 MHz) δ 7.88 (s, 1H, H-2), 6.86 (t, J = 5 Hz, 1H, NH), 6.48 (br s, 2H, NH_2), 5.90 (d, J = 7.5 Hz, 1H, H-1′), 5.87 (t, J = 5 Hz, 1H, OH-5′), 5.23 (d, J = 7 Hz, 1H, OH-2′), 5.15 (d, J = 4 Hz, 1H, OH-3′), 4.61 (q, J = 6.5 Hz, 1H, H-2′), 4.13−4.04 (m, 1H, H-3′), 3.97−3.91 (m, 1H, H-4′), 3.64−3.56 (m, 2H, H-5′), (NHCH$_2$ is hidden by the water peak), 2.54−1.46 (m, 2H, NHCH$_2$C*H*$_2$), 1.43−1.23 (m, 2H, C*H*$_2$CH$_3$), 0.90 (t, J = 7 Hz, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 152.4 (s, C-6), 152.0 (d, C-8), 150.2 (s, C-4), 149.3 (d, C-2), 117.0 (s, C-5), 86.8 (d, C-1′), 86.3 (d, C-4′), 71.6 (d, C-2′), 71.2 (d, C-3′), 62.1 (t, C-5′), 42.6 (t, NHCH$_2$), 31.2 (t, NHCH$_2$*C*H$_2$), 20.2 (t, *C*H$_2$CH$_3$), 114.3 (q, CH$_3$); HRMS calcd for $C_{14}H_{23}N_6O_4$ 339.1780, found 339.1800.

8-Bromo-2′,3′,5′-tri-*O*-(*tert*-butyldimethylsilyl)adenosine (15). Product **15** was obtained as described for **4** starting from 8-bromo-adenosine (0.3 g, 0.87 mmol). TLC on a silica gel plate ($CHCl_3$:MeOH 6:1) indicated the formation of **15** (R_f = 0.47) and a byproduct **16** (R_f = 0.85). The products were separated on a silica gel column. Product **15** was obtained in 78% yield (0.47 g): ^{1}H NMR ($CDCl_3$) δ 8.24 (s, 1H, H-2), 6.02 (br s, 2H, NH_2), 5.94 (d, J = 6 Hz, 1H, H-1′), 5.51 (dd, J = 6, 4 Hz, 1H, H-2′), 4.57 (dd, J = 4, 2 Hz, 1H, H-3′), 4.13−4.01 (m, 2H, H-4′ and H-5′), 3.77−3.65 (m, 1H, H-5′), 0.96 (s, 9H, TBDMS), 0.84 (s, 9H, TBDMS), 0.79 (s, 9H, TBDMS), 0.15 (s, 6H, TBDMS), 0.02 (s, 3H, TBDMS), −0.02 (s, 3H, TBDMS), −0.06 (s, 3H, TBDMS), −0.34 (s, 3H, TBDMS); ^{13}C NMR ($CDCl_3$) δ 154.3 (s, C-6), 152.5 (d, C-2), 150.8 (s, C-4), 128.8 (s, C-8), 120.5 (s, C-5), 90.6 (d, C-1′), 85.6 (d, C-4′), 72.2 (d, C-2′), 71.8 (d, C-3′), 62.3 (t, C-5′), 25.9 (q, 3C, TBDMS), 25.8 (q, 3C, TBDMS), 25.7 (q, 3C, TBDMS), 18.3 (s, TBDMS), 18.1 (s, TBDMS), 17.8 (s, TBDMS), −4.5 (q, TBDMS), −4.6 (q, 2C, TBDMS), −5.2 (q, TBDMS), −5.4 (q, TBDMS), −5.6 (q, TBDMS).

Product **16** was obtained in 17% yield (0.117 g): ^{1}H NMR ($CDCl_3$) δ 8.25 (s, 1H, H-2), 5.92 (d, J = 5.5 Hz, 1H, H-1′), 5.52 (dd, J = 5.5, 4.5 Hz, 1H, H-2′), 5.18 (br s, 1H, NHTBDMS), 4.66 (dd, J = 4.5, 3 Hz, 1H, H-3′), 4.15−4.02 (m, 2H, H-4′ & H-5′), 3.76−3.62 (m, 1H, H-5′), 0.96 (s, 9H, TBDMS), 0.86 (s, 9H, TBDMS), 0.82 (s, 9H, TBDMS), 0.81 (s, 9H, TBDMS), 0.35 (s, 6H, TBDMS), 0.16 (s, 6H, TBDMS), 0.00 (s, 3H, TBDMS), −0.04 (s, 6H, TBDMS), −0.31 (s, 3H, TBDMS); ^{13}C NMR

($CDCl_3$) δ 157.0 (s, C-6), 152.2 (d, C-2), 150.4 (s, C-4), 128.3 (s, C-8), 122.4 (s, C-5), 90.7 (d, C-1′), 85.3 (d, C-4′), 72.1 (d, C-2′), 72.0 (d, C-3′), 62.1 (t, C-5′), 26.5 (q, 3C, TBDMS), 25.9 (q, 3C, TBDMS), 25.8 (q, 3C, TBDMS), 25.7 (q, 3C, TBDMS), 18.3 (s, TBDMS), 18.1 (s, TBDMS), 17.8 (s, TBDMS), 17.7 (s, TBDMS), −2.9 (q, TBDMS), −4.4 (q, TBDMS), −4.5 (q, 2C, TBDMS), −4.6 (q, 2C, TBDMS), −5.1 (q, TBDMS), −5.4 (q, TBDMS), −5.6 (q, TBDMS).

8-(*n*-Butoxy)adenosine (18). The reaction was carried out on compound **17** (0.48 mmol) following the same procedure used for **6**. The product was obtained in 86% yield as a white solid: mp 173 °C; ^{1}H NMR (CD_3OD, 200 MHz) δ 8.04 (s, 1H, H-2), 5.91 (d, J = 7 Hz, 1H, H-1′), 4.93 (dd, J = 7, 5 Hz, 1H, H-2′), 4.54 (t, J = 6.5 Hz, 2H, OCH_2), 4.31 (dd, J = 5, 2 Hz, 1H, H-3′), 4.11 (q, J = 2.5 Hz, 1H, H-4′), 3.85 (dd, J = 12.5, 2.5 Hz, 1H, H-5′), 3.69 (dd, J = 12.5, 3 Hz, 1H, H-5′), 1.95−1.78 (m, 2H, OCH_2CH_2), 1.65−1.41 (m, 2H, CH_2CH_3), 1.01 (t, J = 7 Hz, 3H, CH_3); ^{13}C NMR (CD_3OD, 300 MHz) δ 156.1 (s, C-6), 155.3 (s, C-8), 151.4 (d, C-2), 149.6 (s, C-4), 116.7 (s, C-5), 89.0 (d, C-1′), 88.2 (d, C-4′), 73.7 (d, C-2′), 72.7 (d, C-3′), 72.0 (t, OCH_2), 64.0 (t, C-5′), 31.8 (t, OCH_2CH_2), 20.1 (t, CH_2CH_3), 14.0 (q, CH_3); MS (CI/CH_4) m/z 340 MH^+.

General Procedure for Preparation of 2-Substituted- or 8-Substituted-Adenosine Nucleoside 5′-Triphosphate. All phosphorylation reactions were carried out in flame-dried, argon-flushed, two-necked flasks sealed with rubber septa. Nucleosides were dried in vacuo for 2 days. Proton sponge was kept in a desiccator. Phosphorus oxychloride was distilled and kept under N_2. Tri-*n*-butylammonium pyrophosphate solution was prepared as described before.[9]

Typical Procedure. A solution of 2-thiobutyl-adenosine (0.206 g, 0.58 mmol) in dry trimethyl phosphate (1.5 mL) was cooled to 0 °C; then proton sponge (0.195 g, 2 equiv) was added. After 20 min, phosphorus oxychloride (159 μL, 3 equiv) was added dropwise and a purple clear solution was formed. Stirring continued for 2 h at 0 °C. TLC on a silica gel plate (1-propanol/28% NH_4OH/H_2O 11:7:2), indicated the disappearance of starting material and the formation of a polar product (R_f = 0.46). The spot was typically intensely purple under UV light and dark brown in an I_2 chamber. A mixture of Bu_3N (0.57 mL) and 1 M $(Bu_3NH^+)_2P_2O_7H_2$ in DMF (3.7 mL) was added at once. After 1 min 0.2 M TEAB solution (28 mL) was added, and the clear solution was stirred at room temperature for 45 min. The latter was freeze-dried overnight. The semisolid obtained after freeze-drying was chromatographed on an activated Sephadex DEAE-A25 column. The resin was washed with deionized water for 30 min and loaded with the crude reaction residue dissolved in a minimal volume of water. The separation was monitored by UV detection (ISCO, UA-6) at 280 nm. A buffer gradient of 400 mL of water to 400 mL of 0.6 M NH_4HCO_3 was applied. The relevant fractions (of the ATP and AMP derivatives) were pooled and freeze-dried three times to yield a white solid. Final purification was carried out on a semipreparative nucleoside/nucleotide 7U HPLC column. The products, obtained as triethylammonium salts, were generally >92% pure. Finally, the products (dissolved in water) were passed through a Chelex 100 Resin 100−200 mesh column and eluted with deionized water to obtain the corresponding sodium salts after freeze-drying.

2-(*n*-Butylthioether)adenosine 5′-triphosphate (8a) was obtained in 30% yield. Final separation was achieved by applying a linear gradient of TEAA/CH_3CN 10:90 to 50:50 in 20 min (5 mL/min): t_R 9.95 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.38 (s, 1H, H-8), 6.13 (d, J = 5.5 Hz, 1H, H-1′), 4.91−4.80 (m, 1H, H-2′), 4.66−4.55 (m, 1H, H-3′), 4.43−4.32 (m, 1H, H-4′), 4.31−4.14 (m, 2H, H-5′), 3.18 (t, J = 7 Hz, 2H, SCH_2), 1.71 (quint, J = 7 Hz, 2H, SCH_2CH_2), 1.45 (sex, J = 7 Hz, 2H, CH_2CH_3), 0.93 (t, J = 7 Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ −6.86 (d), −10.44 (d), −21.52 (t); UV λ_{max} 278 nm; high-resolution FAB calcd for $C_{14}H_{23}N_5O_{13}P_3S$ 594.0226, found 594.0206; t_R 12.10 min (95% purity) using solvent system I, 14.00 min (99% purity) using solvent system II.

2-(*n*-Butylthioether)adenosine 5′-monophosphate (8b) was obtained in 60% yield. Final separation was achieved by applying a linear gradient of 0.1 M TEAA/CH_3CN 80:20 to 30:70 in 20 min: t_R 5.73 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.44 (s, 1H, H-8), 6.10 (d, J = 5.5 Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.48 (dt, J = 4 Hz, 1H, H-3′), 4.36−4.26 (m, 1H, H-4′), 4.02−3.89 (m, 2H, H-5′), 3.18 (t, J = 7 Hz, 2H, SCH_2), 1.69 (quint, J = 7 Hz, 2H, SCH_2CH_2), 1.43 (sex, J = 7 Hz, 2H, CH_2CH_3), 0.91 (t, J = 7 Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ 4.51 (s); ^{13}C NMR (D_2O, 600 MHz) δ 166.2 (t, J = 5 Hz, C-2), 155.6 (s, C-6), 150.7 (dd, J = 5, 2.3 Hz, C-4), 139.9 (dd, J = 216, 4.3 Hz, C-8), 117.0 (d, J = 11 Hz, C-5), 88.4 (d, J = 160 Hz, C-1′), 84.2 (d, J = 150 Hz, C-4′), 74.7 (d, J = 150 Hz, C-3′), 71.1 (d, J = 150 Hz, C-2′), 65.4 (t, J = 150 Hz, C-5′), 31.7 (t, J = 130 Hz, SCH_2CH_2), 31.5 (t, J = 140 Hz, SCH_2), 22.3 (t, J = 120 Hz, CH_2CH_3), 13.8 (q, J = 120 Hz, CH_3); UV λ_{max} 278 nm; high-resolution FAB calcd for $C_{14}H_{21}N_5O_7PS$ 434.0899, found 434.0850; t_R 11.98 min (98% purity) using solvent system I, 13.81 min (99% purity) using solvent system II.

2-(*n*-Butylamino)adenosine 5′-triphosphate (3a) was obtained in 58% yield. Final separation was achieved by applying a linear gradient of TEAA/CH_3CN 10:90 to 30:70 in 10 min: t_R 7.91 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.13 (s, 1H, H-8), 5.99 (d, J = 6 Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.58 (dd, J = 5, 3.5 Hz, 1H, H-3′), 4.38−4.29 (m, 1H, H-4′), 4.29−4.10 (m, 2H, H-5′), 3.32 (t, J = 7 Hz, 2H, $NHCH_2$), 1.56 (quint, J = 7 Hz, 2H, $NHCH_2CH_2$), 1.36 (sex, J = 7 Hz, 2H, CH_2CH_3), 0.90 (t, J = 7 Hz, 3H, CH_3); ^{31}P NMR (D_2O, pH 9) δ −6.69 (d), −10.47 (d), −21.50 (t); UV λ_{max} 289 nm; high-resolution FAB calcd for $C_{14}H_{24}N_6O_{13}P_3$ 577.0614, found 577.0609; t_R 9.58 min (98% purity) using solvent system I, 10.11 min (99% purity) using solvent system II.

2-(*n*-Butylamino)adenosine 5′-monophosphate (3b) was obtained in 25% yield. Final separation was achieved by applying a linear gradient of 0.1 M TEAA/CH_3CN 89:11 to 70:30 in 10 min: t_R 7.98 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.21 (s, 1H, H-8), 5.99 (d, J = 6 Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.49 (dd, J = 5, 3.5 Hz, 1H, H-3′), 4.30 (bq, J = 3.5 Hz, 1H, H-4′), 3.95 (t, J = 4 Hz, 2H, H-5′), 3.34 (t, J = 7 Hz, 2H, $NHCH_2$), 1.58 (quint, J = 7 Hz, 2H, $NHCH_2CH_2$), 1.37 (sex, J = 7 Hz, 2H, CH_2CH_3), 0.91 (t, J = 7 Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ 4.52 (s); UV λ_{max} 289 nm; high-resolution FAB calcd for $C_{14}H_{22}N_6O_7P$ 417.1288, found 417.1390; t_R 9.90 min (94% purity) using solvent system I, 10.11 min (99% purity) using solvent system II.

2-(*n*-Butoxy)adenosine 5′-triphosphate (7a) was obtained in 53% yield. Final separation was achieved by applying a linear gradient of TEAA/CH_3CN 10:90 to 30:70 in 10 min: t_R 8.47 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.31 (s, 1H, H-8), 6.06 (d, J = 5.5 Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.61 (dd, J = 5, 4 Hz, 1H, H-3′), 4.36 (t, J = 6.5 Hz, 3H, OCH_2 & H-4′ hidden by this peak), 4.30−4.12 (m, 2H, H-5′), 1.74 (quint, J = 7 Hz, 2H, OCH_2CH_2), 1.44 (sex, J = 7 Hz, 2H, CH_2CH_3), 0.93 (t, J = 7 Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ −5.92 (d), −10.43 (d), −21.29 (t); UV λ_{max} 269 nm; high-resolution FAB calcd for $C_{14}H_{23}N_5O_{14}P_3$ 578.0454, found 578.0400; t_R 9.54 min (96% purity) using solvent system I, 11.65 min (99% purity) using solvent system II.

2-(*n*-Butoxy)adenosine 5′-monophosphate (7b) was obtained in 24% yield. Final separation was achieved by applying a linear gradient of TEAA/CH_3CN 10:90 to 40:60 in 10 min: t_R 7.67 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.38 (s, 1H, H-8), 6.06 (d, J = 5.5 Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.51 (bt, J = 4 Hz, 1H, H-3′), 4.44−4.28 (m, 3H, OCH_2 & H-4′), 4.04−3.90 (m, 2H, H-5′), 1.75 (quint, J = 7 Hz, 2H, OCH_2CH_2), 1.46 (sex, J = 7 Hz, 2H, CH_2CH_3), 0.95 (t, J = 7 Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ 4.47 (s); UV λ_{max} 269 nm; high-resolution FAB calcd for $C_{14}H_{21}N_5O_8P$ 418.1128, found 418.1170.

8-(*n*-Butylthioether)adenosine 5′-triphosphate (12a) was obtained in 32% yield. Final separation was achieved by applying a linear gradient of TEAA/CH_3CN 10:90 to 25:75 in 12 min: t_R 9.66 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.16 (s, 1H, H-2), 6.11 (d, J = 6.5 Hz, 1H, H-1′), 5.18 (t, J = 6.5 Hz, 1H, H-2′), 4.64−4.55 (m, 1H, H-3′), 4.39−4.16 (m, 3H, H-4′ & H-5′),

3.41−3.14 (m, 2H, SCH_2), 1.71 (quint, $J = 7$ Hz, 2H, SCH_2CH_2), 1.44 (sex, $J = 7$ Hz, 2H, CH_2CH_3), 0.89 (t, $J = 7$ Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ −5.56 (d), −10.36 (d), −21.29 (t); UV λ_{max} 282 nm; high-resolution FAB calcd for $C_{14}H_{23}N_5O_{13}P_3S$ 594.0226, found 594.0245; t_R 9.55 min (93% purity) using solvent system I, 10.13 min (92% purity) using solvent system II.

8-(*n*-Butylthioether)adenosine 5′-monophosphate (12b) was obtained in 36% yield. Final separation was achieved by applying a linear gradient of 0.1 M TEAA/CH_3CN 10:90 to 40:60 in 10 min: t_R 7.40 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.15 (s, 1H, H-2), 6.07 (d, $J = 6.5$ Hz, 1H, H-1′), 5.19 (t, $J = 6.5$ Hz, 1H, H-2′), 4.51 (dd, $J = 6$, 4.5 Hz, 1H, H-3′), 4.22 (q, $J = 5.5$ Hz, 1H, H-4′), 4.13−3.90(m, 2H, H-5′), 3.40−3.14 (m, 2H, SCH_2), 1.71 (quint, $J = 7$ Hz, 2H, SCH_2CH_2), 1.43 (sex, $J = 7$ Hz, 2H, CH_2CH_3), 0.89 (t, $J = 7$ Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ 4.52 (s); ^{13}C NMR (D_2O, 600 MHz) δ 154.5 (d, $J = 11$ Hz, C-6), 152.7 (d, $J = 204$ Hz, C-2), 152.2 (q, $J = 5$ Hz, C-8), 151.5 (dd, $J = 12$, 4.5 Hz, C-4), 119.86(d, $J = 1.5$ Hz, C-5), 88.7 (d, $J = 160$ Hz, C-1′), 84.2 (d, $J = 150$ Hz, C-4′), 71.2 (d, $J = 150$ Hz, C-3′), 70.4 (d, $J = 150$ Hz, C-2′), 64.6 (t, $J = 150$ Hz, C-5′), 33.2 (t, $J = 140$ Hz, SCH_2), 31.1 (t, $J = 130$ Hz, SCH_2CH_2), 21.7 (t, $J = 120$ Hz, CH_2CH_3), 13.4 (q, $J = 120$ Hz, CH_3); UV λ_{max} 282 nm; high-resolution FAB calcd for $C_{14}H_{21}N_5O_7PS$ 434.0899, found 434.0860; t_R 9.75 min (97% purity) using solvent system I, 10.28 min (98% purity) using solvent system II.

8-(*n*-Butylamino)adenosine 5′-triphosphate (14a) was obtained in 59% yield. Final separation was achieved by applying a linear gradient of TEAA/CH_3CN 95:5 to 80:20 in 12 min: t_R 10.05 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.02 (s, 1H, H-2), 6.07 (d, $J = 8$ Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.64 (dd, $J = 5.5$, 2 Hz, 1H, H-3′), 4.51−4.34 (m, 1H, H-4′), 4.27−4.03 (m, 2H, H-5′), 3.67−3.36 (m, 2H, $NHCH_2$), 1.79−1.56 (m, 2H, $NHCH_2CH_2$), 1.55−1.28 (m, 2H, CH_2CH_3), 0.93 (t, $J = 7.5$ Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ −5.79 (d), −11.28 (d), −21.41(t); UV λ_{max} 282 nm; high-resolution FAB calcd for $C_{14}H_{23}N_6O_{13}P_3$ 576.0536, found 576.0590; t_R 8.47 min (89% purity) using solvent system I, 9.17 min (93% purity) using solvent system II.

8-(*n*-Butylamino)adenosine 5′-monophosphate (14b) was obtained in 23% yield. The product was purified by HPLC applying a linear gradient of TEAA/CH_3CN 90:10 to 30:70 in 20 min: t_R 4.86; ^{1}H NMR (D_2O, 200 MHz) δ 8.03 (s, 1H, H-2), 6.02 (d, $J = 8$ Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.48 (dd, $J = 6$, 1.5 Hz, 1H, H-3′), 4.32 (br s, 1H, H-4′), 4.15−3.88 (m, 2H, H-5′), 3.62−3.37 (m, 2H, $NHCH_2$), 1.78−1.55 (m, 2H, $NHCH_2CH_2$), 1.37 (sex, $J = 7.5$ Hz, 2H, CH_2CH_3), 0.91 (t, $J = 7.5$ Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ 4.26 (s); ^{13}C NMR (D_2O, 600 MHz) δ 152.1 (q, $J = 5$ Hz, C-8), 149.5 (d, $J = 10$ Hz, C-6), 148.9 (dd $J = 11.5$, 2.5 Hz, C-4), 147.1 (d, $J = 205$ Hz, C-2), 115.6 (s, C-5), 86.5 (d, $J = 160$ Hz, C-1′), 84.3 (d, $J = 150$ Hz, C-4′) 70.7 (d, $J = 150$ Hz, C-3′), 69.9 (d, $J = 150$ Hz, C-2′), 64.4 (t, $J = 150$ Hz, C-5′), 42.4 (t, $J = 140$ Hz, $NHCH_2$), 30.5 (t, $J = 130$ Hz, $NHCH_2CH_2$), 19.4 (t, $J = 120$ Hz, CH_2CH_3), 13.2 (q, $J = 120$ Hz, CH_3); UV λ_{max} 282 nm; high-resolution FAB calcd for $C_{14}H_{22}N_6O_7P$ 417.1287, found 417.1270; t_R 7.74 min (98% purity) using solvent system I, 8.79 min (99% purity) using solvent system II.

8-(*n*-Butoxy)adenosine 5′-triphosphate (19a) was obtained in 58% yield. Final separation was achieved by applying a linear gradient of TEAA/CH_3CN 5:95 to 20:80 in 12 min: t_R 10.16 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.12 (s, 1H, H-2), 5.59 (d, $J = 5.5$ Hz, 1H, H-1′), 5.02 (t, $J = 5.5$ Hz, 1H, H-2′), 4.60−4.46 (m, 3H, OCH_2 and H-3′), 4.35−4.03 (m, 3H, H-4′ and H-5′), 1.88 (quint, $J = 7$ Hz, 2H, OCH_2CH_2), 1.48 (sex, $J = 7$ Hz, 2H, CH_2CH_3), 0.96 (t, $J = 7$ Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ −4.99 (d), −10.13 (d), −20.61 (t); UV λ_{max} 260 nm; high-resolution FAB calcd for $C_{14}H_{23}N_5O_{14}P_3$ 578.0454, found 578.0512; t_R 8.34 min (99% purity) using solvent system I, 8.15 min (92% purity) using solvent system II.

8-(*n*-Butoxy)adenosine 5′-monophosphate (19b) was obtained in 19% yield. Final separation was achieved on semipreparative nucleoside/nucleotide column applying a linear gradient of 0.1 M TEAA/CH_3CN 10:90 to 40:60 in 10 min: t_R 5.46 min; ^{1}H NMR (D_2O, 200 MHz) δ 8.12 (s, 1H, H-2), 5.96 (d, $J = 5.5$ Hz, 1H, H-1′), 5.02 (t, $J = 5.5$ Hz, 1H, H-2′), 4.55 (t, $J = 6.5$ Hz, 1H, OCH_2), 4.41 (t, $J = 5.5$ Hz, 1H, H-3′), 4.20 (q, $J = 5.5$ Hz, 1H, H-4′), 4.08−3.80 (m, 2H, H-5′), 1.86 (quint, $J = 7$ Hz, 2H, OCH_2CH_2), 1.47 (sex, $J = 7$ Hz, 2H, CH_2CH_3), 0.95 (t, $J = 7$ Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 9) δ 4.40 (s); UV λ_{max} 260 nm; high-resolution FAB calcd for $C_{14}H_{21}N_5O_8P$ 418.1128, found 418.1150; t_R 8.14 min (97% purity) using solvent system I, 7.90 min (>99% purity) using solvent system II.

Biochemistry. Cell cultures: Primary astrocyte-enriched cell cultures were obtained from 2−3 newborn Wistar rats (Institute breeding stock) using the method of Hamprecht and Löffler.[30] In brief, newborn rats were decapitated; total brains were removed and collected in ice-cold Puck's-D1 solution (137.0 mM NaCl, 5.4 mM KCl, 0.2 mM KH_2PO_4, 0.17 mM Na_2HPO_4, 5.0 mM glucose, 58.4 mM sucrose, pH 7.4). The brains were gently passed through a nylon mesh (250-μm pore width) into 15 mL of ice-cold Puck's-D1 solution. The cell suspension was filtered through nylon mesh (136-μm pore width) and centrifuged at 4 °C for 5 min at 1500 rpm. The cells were resuspended in 10 mL of DMEM supplemented with 10% (v/v) FCS, 20 U/mL penicillin, and 20 μg/mL streptomycin. The cells were plated on round coverslips (22-mm diameter), placed in culture dishes (50-mm diameter) at a density of (3−5) × 10^5 cells/dish, and incubated at 37 °C with 10% CO_2, humidified to saturation. The medium was changed for the first time after 5 days and thereafter every 2−3 days depending on the cell density. For experiments cells were used between days 6 and 11 in culture.

Neuroblastoma × glioma hybrid cells (clone 108CC15, identical with NG 108-15) of passage numbers between 24 and 40 were cultured as described.[31] For the experiments cells were grown on round glass cover slips (22-mm diameter) in Petri dishes for 3−7 days reaching 20−80% confluency, corresponding to 2 × 10^5−10^6 cells/dish.

Cytosolic Ca^{2+} measurements: The free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined using the Ca^{2+}-sensitive fluorescent dye fura-2/AM. For dye loading the cells grown on a coverslip were removed from the culture dish and placed in 1 mL of HEPES-buffered saline (HBS) for 30 min at 37 °C, supplemented with 2 μM fura-2/AM. HBS has the following composition (in mM): NaCl 145, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 25, HEPES 20, pH 7.4, adjusted with tris(hydroxymethyl)aminomethane. Loaded cells were transferred into a perfusion chamber with a bath volume of about 0.2 mL and mounted on an inverted microscope (Zeiss, Axiovert 135, Jena, Germany). During the experiments the cells were continuously superfused with medium heated to 37 °C. The perfusion system was combined with a 6-port valve (Thomachrom, type RH 0112) from Reichelt (Heidelberg, Germany) to allow the switch between solutions containing different agents to be tested. Single-cell fluorescence measurements of $[Ca^{2+}]_i$ were performed using an imaging system from TILL Photonics GmbH (Munich, Germany). Cells were excited alternately at 340 and 380 nm for 20−100 ms at each wavelength with a rate of 0.33 Hz, and the resultant emission was collected above 510 nm. Images were stored on a personal computer, and subsequently the changes in fluorescence ratio F_{340nm}/F_{380nm} were determined from selected regions of interest covering a single cell.

Pharmacological Analyses. P2Y$_1$-R-promoted stimulation of inositol phosphate formation by adenine nucleotide analogues was measured in turkey erythrocyte membranes as previously described.[32,33] The EC_{50} values are the result of three independent experiments carried out for each compound using different membrane preparations. Briefly, 1 mL of washed turkey erythrocytes was incubated with inositol-free DMEM (Gibco, Gaithersburg, MD) with 0.5 mCi of 2-[^{3}H]*myo*-inositol (20 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) for 18−24 h in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. Erythrocyte membranes were prepared by rapid lysis in hypotonic buffer (5 mM sodium phosphate,

pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA) as described. Phospholipase C activity was measured in 25 μL of [^{3}H]inositol-labeled membranes (approximately 175 mg of protein, 200−500 000 cpm/assay) in a medium containing 424 mM $CaCl_2$, 0.91 mM $MgSO_4$, 2 mM EGTA, 115 mM KCl, 5 mM KH_2PO_4, 10 mM Hepes, pH 7.0. Assays (200 μL final volume) contained 1 mM GTPγS and the indicated concentrations of nucleotide analogues. Membranes were incubated at 30 °C for 5 min, and total [^{3}H]inositol phosphates were quantitated by ion-exchange chromatography as previously described.[32,33]

Data Analysis. Agonist potencies were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). EC_{50} values (mean ± standard error) represent the concentration at which 50% of the maximal effect is achieved. All concentration−effect curves were repeated in at least three separate experiments carried out with different membrane preparations using duplicate assays.

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