2-Thioether 5′-*O*-(1-Thiotriphosphate)adenosine Derivatives as New Insulin Secretagogues Acting through P2Y-Receptors

Bilha Fischer,*,† Ana Chulkin,† Jose L. Boyer,‡ Kendall T. Harden,‡ Fernand-Pierre Gendron,§ Adrien R. Beaudoin,§ Jeannie Chapal,⊥ Dominique Hillaire-Buys,⊥ and Pierre Petit⊥

Department of Chemistry, Gonda-Goldschmied Medical Research Center, Bar-Ilan University, Ramat-Gan 52900, Israel, Department of Pharmacology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27599, Biology Sciences, University of Sherbrooke, J1K 2R1 Canada, and Laboratory of Pharmacology, Research Unit UPRES EA 1677, School of Medicine, University of Montpellier I, 34060 Montpellier Cedex 1, France

Received April 1, 1999

P2-Receptors (P2-Rs) represent significant targets for novel drug development. P2-Rs were identified also on pancreatic B cells and are involved in insulin secretion. Therefore, novel P2Y-R ligands, 2-thioether 5′-*O*-phosphorothioate adenosine derivatives (2-RS-ATP-α-S), were synthesized as potential insulin secretagogues. An efficient synthesis of these nucleotides and a facile method for separation of the chiral products are described. The enzymatic stability of the compounds toward pig pancreas type I ATPDase was evaluated. The rate of hydrolysis of 2-hexylthio-5′-*O*-(1-thiotriphosphate)adenosine (2-hexylthio-ATP-α-S) isomers by ATPDase was 28% of that of ATP. Some 2-thioether 5′-(monophosphorothioate)adenosine derivatives (2-RS-AMP-S) exerted an inhibitory effect on ATPDase. The apparent affinity of the compounds to $P2Y_1$-R was determined by measurement of P2Y-R-promoted phospholipase C activity in turkey erythrocyte membranes. 2-RS-ATP-α-S derivatives were agonists, stimulating the production of inositol phosphates with $K_{0.5}$ values in the nanomolar range. 2-RS-AMP-S derivatives were full agonists, although 2 orders of magnitude less potent. All the compounds were more potent than ATP. The effect on insulin secretion and pancreatic flow rate was evaluated on isolated and perfused rat pancreas. A high increase, up to 500%, in glucose-induced insulin secretion was due to addition of 2-hexylthio-ATP-α-S in the nanomolar concentration range, which represents 100-fold enhancement of activity relative to ATP. 2-Hexylthio-AMP-S was 2.5 orders of magnitude less effective.

Introduction

P2-Receptors (P2-Rs) are membrane proteins that lead to inhibitory or excitatory effects upon binding ADP, ATP, or, in some subtypes, UTP.[1] A distinction was made between G-protein-coupled receptors and ligand-gated ion-channel receptors as the basis of the separation of P2-R into two broad classes: P2Y and P2X, respectively.[2] P2-Rs represent significant targets for novel drug development for a variety of pathophysiological conditions.[3] Moreover, the large heterogeneity of P2-R subtypes in different tissues opens the possibility of developing selective organ- or tissue-specific P2-R-targeted drugs.

P2-Rs have been characterized on pancreatic B cells[4] including P2Y-R subtypes.[5] Their activation by extracellular ATP results in stimulation of insulin secretion. Various selective ligands have been shown to increase insulin secretion and decrease glycemia in vivo.[6] Therefore, P2Y-R agonists may be considered as novel insulin-releasing compounds, with potential for the treatment of type 2 diabetes (non-insulin-dependent diabetes, NIDDM).[7]

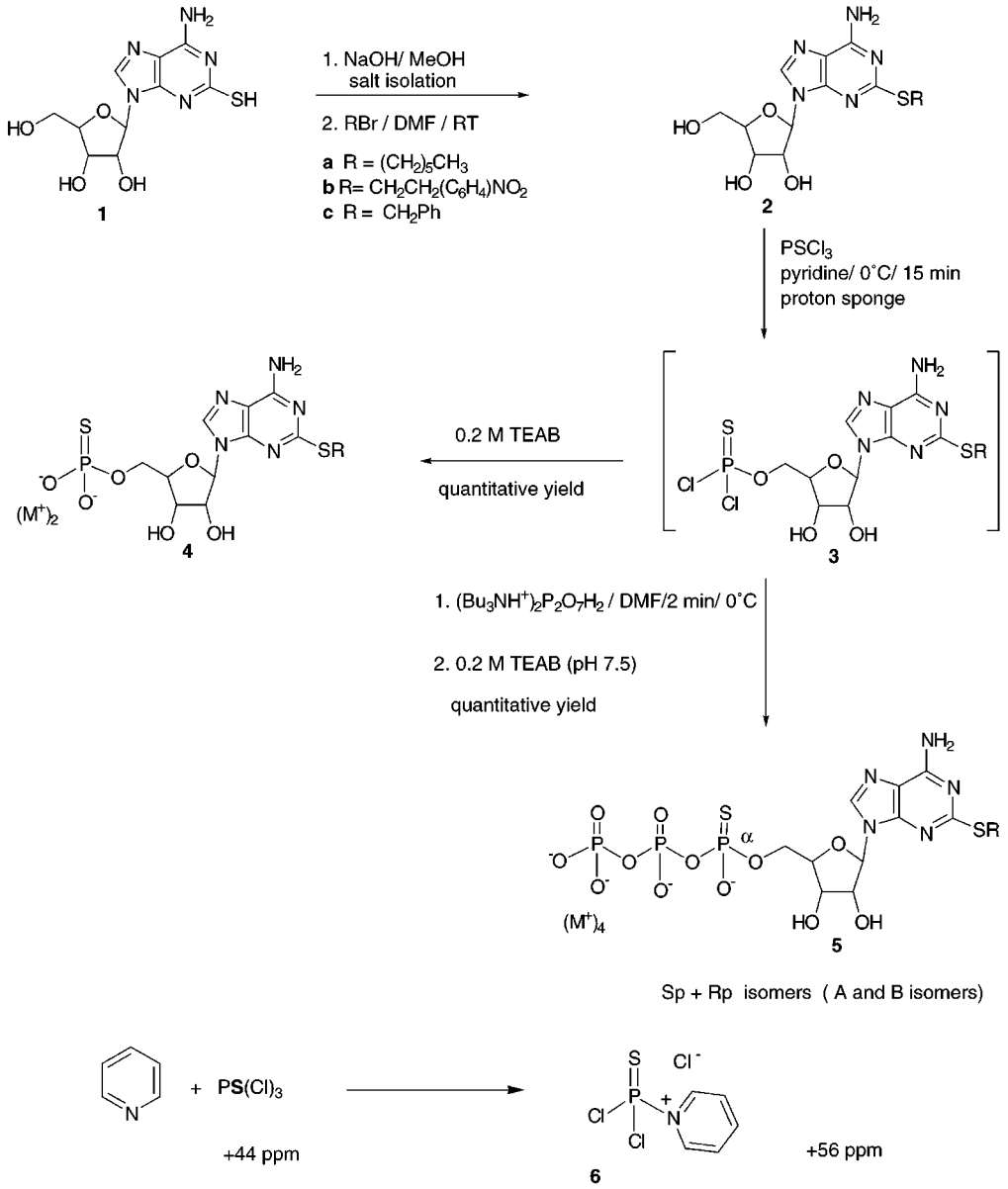
In previous studies, we showed that long thioether chain substitutions at the C2 position of ATP resulted in a large increase in potency (2−5 orders of magnitude) toward $P2Y_1$-R compared with ATP.[8−10] For instance, some 2-thioether ATP derivatives stimulated the production of inositol phosphates in turkey erythrocyte membranes, through the activation of $P2Y_1$-R, with $K_{0.5}$ values in the nanomolar range.[8a] Other 2-thioether ATP derivatives inhibited c-AMP accumulation in C6 rat glioma cells with EC_{50} values in the picomolar range.[10] Some 2-thioether AMP derivatives proved to be full agonists, whereas AMP was practically inactive.[8] Moreover, a long C2 thioether substitution on ATP rendered the molecule relatively stable to enzymatic hydrolysis[11] and also affected the receptor subtype selectivity, making these derivatives active mainly at the P2Y-R subtypes.[8a,9]

The relative metabolic stability of nucleoside-5′-phosphorothioates is well-documented. For instance, AMP-S is relatively resistant to enzymatic transformations by adenylate deaminase, adenylate kinase, and 5′-nucleotidase,[12,13] and ATP-α-S diastereoisomers exhibit selective metabolic stability.[14] In addition to their relative metabolic stability, phosphorothioates are also characterized by their higher acidity relative to phosphates.[12,15−17] Since electrostatic interactions of the ATP phosphate chain with positively charged amino acid residues at the P2Y-R binding site are likely a major determinant for binding,[18] it is expected that the higher acidity of nucleoside-phosphorothioates will enhance binding affinity to P2Y-R. Indeed, ATP-α-S, Rp

* Address correspondence to Bilha Fischer at Bar-Ilan University. Fax: 972-3-5351250. Tel: 972-3-5318303. E-mail: bfischer@mail.biu.ac.il.
† Bar-Ilan University.
‡ University of North Carolina.
§ University of Sherbrooke.
⊥ University of Montpellier I.

10.1021/jm990158y CCC: $18.00
Published on Web 08/17/1999

Scheme 1. Synthesis of 2-Thioether 5′-*O*-(Phosphorothioate)adenosine Derivatives



isomer, a P2Y-R selective ligand, was 50 times more potent than ATP at inducing relaxation of guinea pig taenia coli.[19]

This paper describes the synthesis and functional investigation of novel insulin secretagogues which amplify glucose-stimulated insulin secretion from pancreatic B cells by binding to P2Y-Rs in the membrane of these cells. We report here a highly efficient, rapid synthesis and diastereomeric separation of 2-thioether 5′-*O*-(1-thiotriphosphate)adenosine-nucleotide (2-RS-ATP-α-S) derivatives and their corresponding monophosphorothioate (2-RS-AMP-S) analogues. The high potency of these compounds as $P2Y_1$-R ligands in turkey erythrocytes and as insulin secretagogues in rat pancreas is described, as well as their relative enzymatic stability.

Results

Ligand Synthesis. Scheme 1 outlines the synthesis of 2-RS-ATP-α-S nucleotides and their lower homologues, starting at 2-SH-adenosine, **1**.[20] The latter was treated with NaOH/MeOH, and the isolated sodium thiolate salt was selectively S-alkylated with an alkyl bromide in DMF. The selection of the side chains at C2 is based on our previous results.[8−10] For the preparation of the corresponding 5′-phosphorothioate-nucleosides, the resulting 2-thioether adenosine derivatives **2** were used with no further 2′,3′-OH protection.

The reported preparations of 5′-monothiophosphate-nucleosides involved treatment of a suitably protected nucleoside with various phosphorothioate reagents,[21a,c,22] phosphitylation and subsequent addition of sulfur,[21b] or, alternatively, a direct thiophosphorylation of unprotected nucleosides using thiophosphoryl chloride in trimethyl phosphate.[12,15] Conventional phosphorylation methods of nonprotected nucleosides require the use of trimethyl phosphate (TMP) as a solvent,[23,24] since it dissolves both polar nucleosides and nonpolar phosphorylating reagents. In our hands, however, applying these conditions[12] to thiophosphorylated 2-RS-adenosine derivatives **2** did not lead to the expected 5′-phosphorothioate nucleoside in good yield but rather to a mixture

of 2-RS-AMP and 2-RS-AMP-S in a ratio of 3:2. Moreover, when these conditions were applied to the synthesis of 2-RS-ATP-α-S derivatives, inseparable mixtures of phosphorylated and thiophosphorylated nucleosides (e.g., 2-RS-AMP, **4**, 2-RS-ATP, and 2-RS-ATP-α-S(Rp+Sp), **5**) formed. The phosphate side products might stem from a reaction of thiophosphoryl chloride with TMP. Monitoring the reaction by FAB spectroscopy revealed species where the sulfur atom was exchanged with an oxygen. Thus, when $P(S)Cl_3$ and $PO(OMe)_3$, in a 1:48 ratio, as in the reaction mixture, were added to the nucleoside, only a ′regular′ phosphate product (2-RS-AMP), was observed after 5 min at 0 °C. The phosphorothioate product **4** was identified as a minor product (phosphate:phosphorothioate ratio 5:1) only after 20 min.

Alternative solvents such as acetonitrile,[25] *m*-cresol, nitrobenzene,[26] or trimethyl thiophosphate[27] were considered. However, no product was obtained in suspensions with these solvents. Apparently, the dissolution of the nucleoside is essential for the occurrence of the reaction.

Dry pyridine was found to be a superior alternative to TMP. Not only does it dissolve both the thiophosphorylation reagent and the polar nucleoside **2** and lead to the product in a quantitative yield, but it also speeds up the reaction, which is completed within 15 min, instead of 12 h, when using TMP. This effect is attributed to formation of a reactive species: (S)P-(pyridinium)$Cl_2^+Cl^-$, **6**. This species was observed, as the only new signal, in the ^{31}P NMR spectrum taken 10 min after the addition of excess dry pyridine to $P(S)Cl_3$ at 20 °C. The signal for the reagent appeared at 44 ppm and shifted to 56 ppm upon pyridine addition. After 10 min at 20 °C, the ratio of (S)P(pyridinium)$Cl_2^+Cl^-$:$P(S)Cl_3$ was 1:3. Moreover, the basic environment, produced by pyridine and the addition of 1,8-bis-(dimethylamino)naphthalene (proton sponge), prevents the desulfurization process typical of phosphorothioate diesters at very low or high pH.[28a,29]

Although a nonprotected nucleoside is used, phosphorylation takes place selectively at the 5′-position as evidenced in the ^1H NMR spectrum. This selectivity is achieved when the reaction is carried out at 0 °C. At higher temperature, e.g., at 6 °C, the 2′- and 3′-hydroxyls also undergo thiophosphorylation, in addition to the 5′-OH.

2-Thioether 5′-*O*-(1-thiotriphosphate)adenosine-nucleotides were prepared in a one-pot synthesis and were obtained as the only product in high yield (Scheme 1). These conditions were superior to reported syntheses of ATP-α-S.[30,31] A short reaction time with pyrophosphate and neutral pH at the hydrolysis step are imperative for obtaining the product in quantitative yield. This procedure prevents the formation of side products such as nucleoside-monophosphorothioate, ′regular′ triphosphate, tetraphosphate, pentaphosphate, ADP-α-S, and cyclic phosphorothioates. DMF plays an essential role in the second synthetic step. When the pyrophosphate salt was dissolved in pyridine instead of DMF, product **5** was not obtained. Apparently, DMF forms a Vilsmeier type intermediate, effective in the condensation reaction of pyrophosphate with **3**.[32]

2-RS-ATP-α-S derivatives **5** are obtained as a mixture of two diastereoisomers due to chirality of the α-phosphorothioate. These diastereoisomers are almost indistinguishable by ^1H NMR, except for the H-8 signal which appears at 8.46 and 8.53 ppm for the two diastereoisomers. The latter difference is due to the proximity of the phosphorothioate sulfur atom to H-8 in one of the isomers (in the anti conformation, which is expected for 2-RS-ATP derivatives). A larger difference between the two diastereomers is observed in the ^{31}P NMR spectrum, where the Pα appears as two doublets at 44.56 and 44.21 ppm, $J = 13.5$ Hz.

Many enzymes show a strong preference for one of the diastereomers of ATP-α-S.[16] A similar preference was observed for GTP-α-S in activating various G-proteins.[33b,c] It was expected, therefore, that such stereoselectivity would prevail with the P2Y-Rs as well. Therefore, diastereoisomers of **5** were separated on a reverse-phase HPLC column applying a linear gradient of triethylammonium acetate (TEAA):CH_3CN. Separation was improved upon addition of 0.01% (w/w) $MgCl_2$. This solvent system is a better alternative to the reported phosphate or Tris-HCl buffers[34] which cannot be removed by freeze-drying. The separated diastereoisomers are designated **A** and **B**, e.g., **5aA** and **5aB**, and their absolute configuration has not been yet established. **A** isomer is the one with a shorter retention time. Any **A** isomer is of the same chirality in all analogues, based on correlation of retention time with ^1H NMR data for H-8.

Some of the newly synthesized derivatives were evaluated biochemically as described below. Only **4a**, **5aA**, and **5aB** which were the most promising $P2Y_1$-R agonists were evaluated in all the biochemical assays.

Enzymatic Stability toward Type I ATPDase. Analysis of the potency of the new compounds as P2Y-R ligands may be complicated by the presence of ecto-nucleotidases in the preparation. The relative potency of some P2-R ligands is related to their resistance to hydrolysis.[35] Therefore, an evaluation of the enzymatic stability of the new compounds is an essential step in the process of the development of P2-R ligands as potential leads.

Up until recently it was believed that ATP was converted to adenosine by ecto-ATPase, ecto-ADPase, and 5′-nucleotidase.[36] However, in many organs and tissues including endothelial, smooth muscle, and pancreatic cells,[37] ATP-diphosphorohydrolase, ATPDase (EC 3.6.1.5), is probably responsible for most of the hydrolysis of extracellular triphospho- and diphospho-nucleosides.[35] ATPDase hydrolyzes pyrophosphate bonds of nucleoside di- and triphosphates in the presence of Ca^{2+} or Mg^{2+}.[38]

In the present study, we evaluated the resistance of 2-thioether adenosine-5′-phosphorothioate derivatives toward hydrolysis by semipurified preparation of pig pancreas type I ATPDase.[38a] The enzymatic stability of the novel phosphorothioate analogues, bearing modifications on both the purine and the phosphate chain, was also compared with analogues bearing only one modification on either the purine or the phosphate chain.

All phosphorothioate analogues tested interacted with the pig pancreas type I ATPDase. Thus, the hydrolysis rate of 2-hexylthio-ATP-α-S **5aA** and **5aB** isomers by the pancreas type I ATPDase was 28% of that of ATP and about 50% and 35% of that of ATP-α-S and

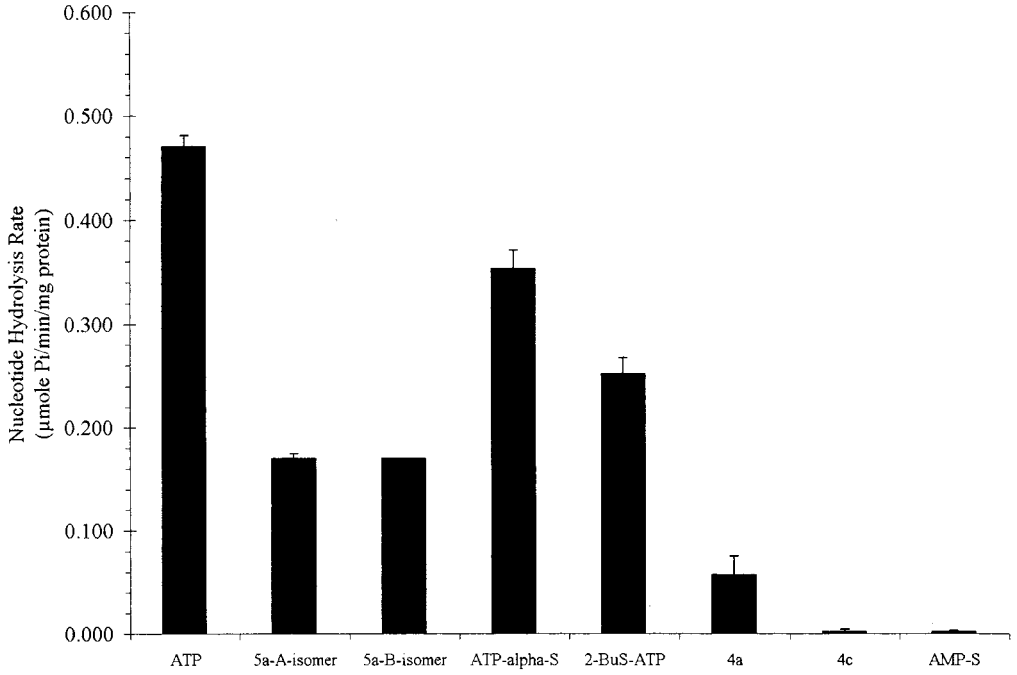


Figure 1. Hydrolysis rate of ATP and its analogues by type I ATPDase. Assays were carried out for 10 min at 37 °C in the presence of 1.9 μg of proteins. ATP and analogues were used at 100 μM concentration: 2-hexylthio-AMP-S (**4a**); 2-benzylthio-AMP-S (**4c**); 2-hexylthio-ATP-α-S-A isomer (**5aA**); 2-hexylthio-ATP-α-S-B isomer (**5aB**); ATP-α-S Sp isomer (ATP-S); and 2-butylthio-ATP (2-BuS-ATP).[39] **5aA** and **5aB** isomers were hydrolyzed at 28% the rate of ATP, whereas the monophosphorothioate derivatives were not hydrolyzed by ATPDase. All the experiments were carried out in triplicate, and results are expressed as the mean ± SEM.

2-butylthio-ATP (2-BuS-ATP),[39] respectively (Figure 1). This result suggests that both the C2 hexylthio substituent and the phosphorothioate moiety play an important role in protecting ATP analogues against ATPDase hydrolysis. Estimated apparent K_m and V_{max} for 2-hexylthio-ATP-α-S (**5aA**) were 41 μM and 0.23 μmol Pi/min/mg protein, respectively (Figure 2). 2-Hexylthio-ATP-α-S, **5aB**, exhibited low affinity for ATPDase as shown by a slow rate of hydrolysis. At concentrations as high as 100 μM, no saturation of the enzyme was observed (Figure 2A). The apparent K_m and V_{max} for ATP-α-S, in which the C2 hexylthio substituent is missing, were 18 μM and 0.45 μmol Pi/min/mg protein, respectively (Figure 3A). 2-Butylthio-ATP (Figure 3B), in which there is a phosphate instead of phosphorothioate chain, exhibited apparent K_m and V_{max} of 18 μM and 0.27 μmol Pi/min/mg protein, respectively. For comparison purposes, the estimated apparent K_m and V_{max} for ATP (data not shown) were 9 μM and 0.5 μmol Pi/min/mg of protein, respectively.

As expected, the monophosphate derivatives 2-hexylthio-AMP-S, **4a**, and 2-benzylthio-AMP-S, **4c**, were not hydrolyzed by type I ATPDase (Figure 1). In the case of **4a**, there was a substantial inorganic phosphorus background (Figure 1). The latter was a weak competitive inhibitor of ATPDase, with an estimated K_i of 75 μM (Figure 4). **4c** and AMP-S, which were stable in the assay medium (Figure 1), exerted an effect on ATPDase that could be interpreted as a mixed type of inhibition (Figures 5 and 6).

Evaluation of the New Compounds as $P2Y_1$-R Ligands. Previous work has demonstrated that turkey erythrocytes are a valuable model system for the study and development of agonists and antagonists of $P2Y_1$-Rs.[40] The 5′-phosphorothioate analogues of adenine nucleotides prepared in this study were tested for their ability to stimulate phospholipase C activity through activation of the $P2Y_1$-R in turkey erythrocyte membranes. The substitution of a nonbridging oxygen on the α-phosphate of ATP analogues by sulfur results in chirality, and both diastereoisomers of 2-hexylthio-ATP-α-S, **5aA** and **5aB**, produced a marked concentration-dependent activation of phospholipase C activity with EC_{50} values of 17 ± 3 and 21 ± 7 nM for **5aA** and **5aB**, respectively (Figure 7A and Table 1). These values are almost identical to that of the corresponding triphosphate analogue 2-hexylthio-ATP (10 ± 4 nM).[8a] As illustrated in Figure 7A,B, the potency for activation of $P2Y_1$-Rs by the two diastereoisomers, either **5aA** and **5aB** or **5bA** and **5bB**, was identical, suggesting that the absolute configuration of Pα is not important in conferring high-affinity binding of the ATP molecule with the receptor. In contrast to 2-hexylthio-ATP-α-S derivatives, **5a**, which are equipotent with 2-thioether ATP analogues, 2-hexylthio-AMP-S, **4a**, was 46-fold less potent than 2-hexylthio-AMP, EC_{50} 2790 ± 980 and 60 ± 9 nM, respectively[8b] (Figure 7A). However, 2-RS-AMP-S derivatives are still $P2Y_1$-R agonists, whereas the parent compounds AMP and AMP-S were ineffective in stimulating this receptor (not shown). These data confirm our earlier observations that 2-thioether substitution of the adenine base results in compounds of high potency for $P2Y_1$-Rs.[8] In contrast to 2-hexylthio-ATP-α-S, **5a**, and 2-hexylthio-AMP-S, **4a**, the 2-*p*-nitrophenethylthio substitution in **5bA** and **5bB** resulted in 42−74-fold decreases in potency for activation of $P2Y_1$-Rs (Figure 7B). The ineffectiveness of 2-*p*-nitrophenethylthio substitution in producing potent $P2Y_1$-R ligands is also reflected in the low potency of 2-*p*-nitrophenethylthio-AMP. This compound has an EC_{50} value of 3000 nM compared with

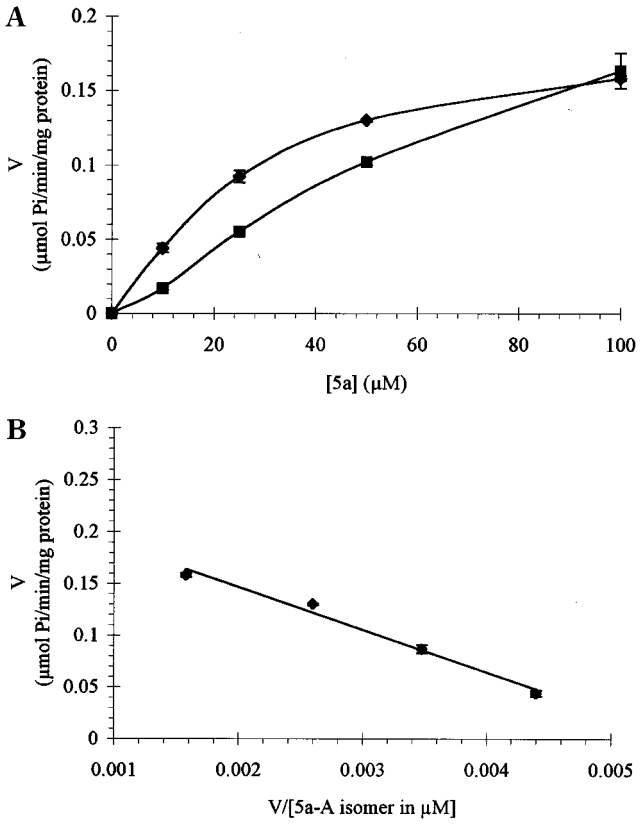


Figure 2. **5aA** and **5aB** isomer hydrolysis by pig pancreas type I ATPDase. **5aA** isomer (◆) and **5aB** isomer (■) were used at 100 μM concentration. (A) Hydrolysis rate of **5aA** and **5aB** isomers. The enzyme was not saturated, even at 100 μM **B** isomer. (B) Woolf−Augustinson−Hofstee representation of **5aA** isomer hydrolysis by ATPDase. The apparent K_m and V_{max} were 41 μM and 0.23 μmol Pi/min/mg protein, respectively. All the experiments were carried out in triplicate.

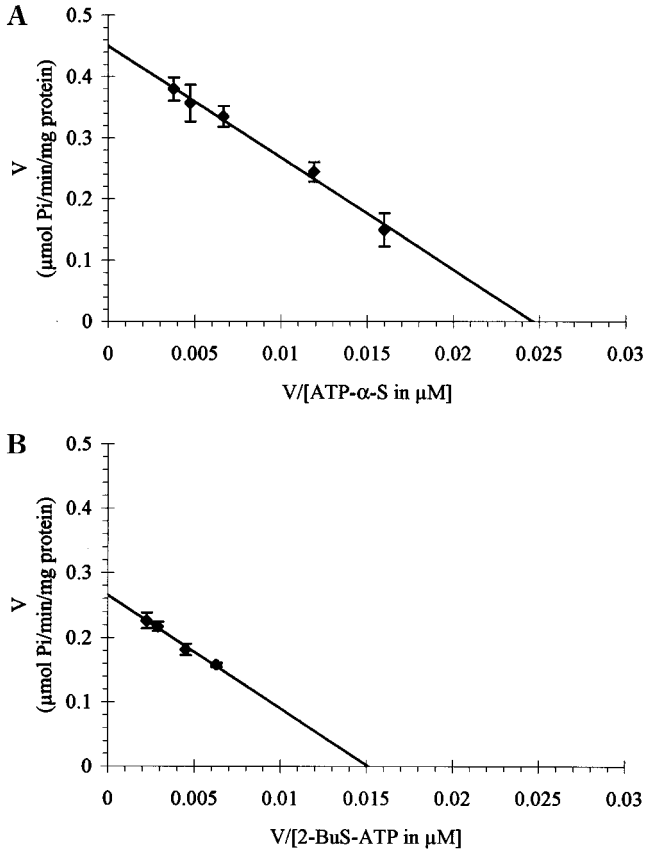


Figure 3. Woolf−Augustinson−Hofstee representation of the hydrolysis of ATP-α-S (panel A) and 2-BuS-ATP[39] (panel B) by type I ATPDase. (A) The apparent K_m and V_{max} for ATP-α-S were 18 μM and 0.45 μmol Pi/min/mg protein, respectively. (B) The apparent K_m and V_{max} for 2-BuS-ATP were 18 μM and 0.27 μmol Pi/min/mg protein, respectively. All the experiments were carried out in triplicate.

60 nM of the corresponding 2-hexylthio-AMP[8] (Table 1). Likewise, a 5′-phosphorothioate moiety in 2-*p*-nitrophenethylthio-AMP-S, **4b**, does not compensate for the loss of activity due to this C2 substitution (Figure 7B and Table 1).

Functional Investigations in Isolated Rat Pancreas. The newly synthesized analogues were evaluated as insulin secretagogues on isolated and perfused rat pancreas. Insulin in the pancreatic effluent was measured by radioimmunoassay. Variations in flow rate, which reflect the changes in vascular resistance, were registered throughout the experiment.

When 2-hexylthio-AMP-S was administered, a moderate increase in glucose-induced insulin secretion was observed only with a concentration of 15 μM, while 1.5 μM concentration was ineffective (Figure 8A). This response is similar to that previously obtained with AMP.[4a] In addition, this compound induced a concentration-dependent decrease in pancreatic flow rate (Figure 8B). However, 2-hexylthio-ATP-α-S (**A** isomer) induced a large biphasic and concentration-dependent increase in glucose-induced insulin secretion, in a much lower range of concentrations: 0.05−1.5 μM (Figure 9A). At all concentrations tested a transient vasoconstriction is consistently observed (Figure 9B). At the concentrations of 0.5 and 1.5 μM, the insulin-secreting response is not significantly different, suggesting that these values approach the maximal effective concentrations. In addition, the **B** isomer of 2-hexylthio-ATP-α-S is equipotent to the **A** isomer (Figure 10). These compounds are about 100 times more potent than ATP and approximately as potent as ADP-β-S.[5d]

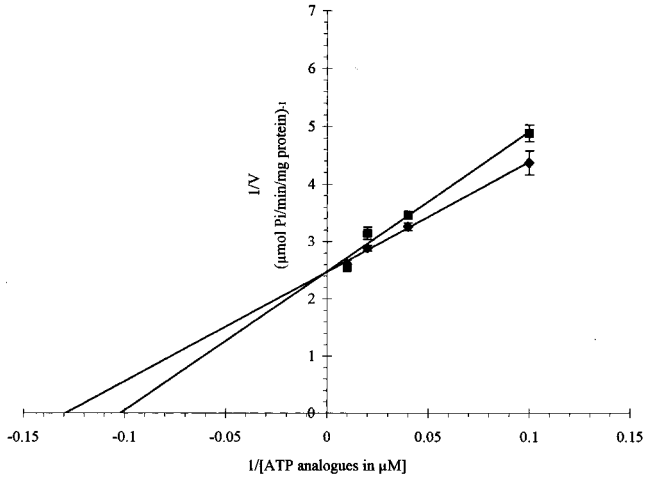


Figure 4. Lineweaver−Burk plot representation of the inhibitory effect of 2-hexylthio-AMP-S, **4a**, on ATP hydrolysis by type I ATPDase. ATP concentration ranged from 10 to 100 μM. Assays were carried out in the presence of 20 μM **4a** (■) or in the absence of ATP analogue (◆). **4a** exerted an effect on type I ATPDase that could be interpreted as a competitive type of inhibition, with an estimated K_i of 75 μM. All the experiments were carried out in triplicate.

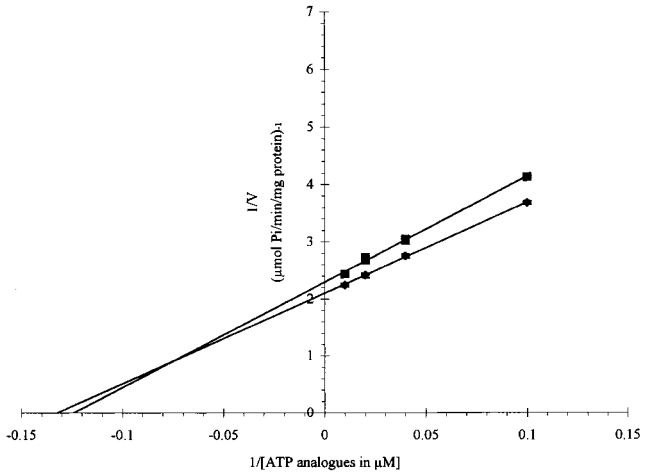


Figure 5. Lineweaver−Burk plot representation of the inhibitory effect of 2-benzylthio-AMP-S, **4c**, on ATP hydrolysis. ATP concentration ranged from 10 to 100 μM. Assays were carried out in the presence of 30 μM **4c** (■) or in the absence of the inhibitor (◆). **4c** exerted an effect on type I ATPDase that could be interpreted as a mixed type of inhibition. All the experiments were carried out in triplicate.

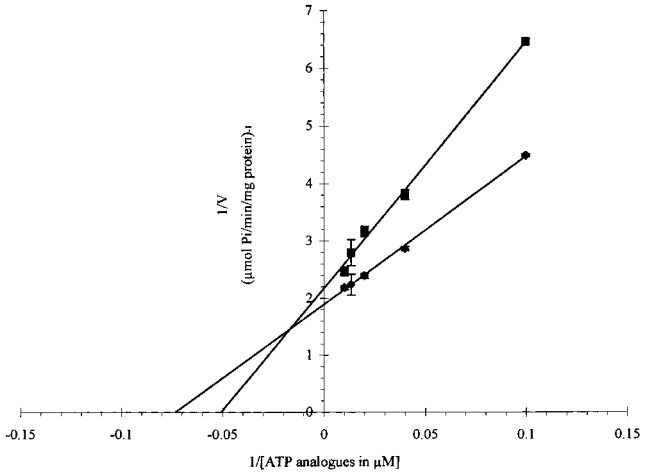


Figure 6. Lineweaver−Burk plot representation of the inhibitory effect of AMP-S on ATP hydrolysis by type I ATPDase. ATP concentration ranged from 10 to 100 μM. Assays were carried out in the presence of 100 μM AMP-S (■) or in the absence of inhibitor (◆). AMP-S exerted an effect on type I ATPDase that could be interpreted as a mixed type of inhibition. All the experiments were carried out in triplicate.

Discussion

The main determinants that control the level of extracellular nucleotides in vivo are their release process from cells and the presence of ectonucleotidases. The ectoenzymes involved in the conversion of ATP to adenosine have been reviewed by Zimmermann.[41] Ecto-ATPase, ecto-ATPDase, and the 5′-nucleotidase play a key role in the extracellular metabolism of ATP and other nucleotides. The relative importance of ecto-ATPase and ATPDase may vary from one system to the other. Picher et al. have shown that some ATP analogues are hydrolyzed whereas others act as inhibitors of the enzyme.[35] From our previous experiments on a limited number of analogues, it appears that analogues modified on a phosphate chain, mainly on the β- or γ-phosphate, were not hydrolyzed whereas those modified on the other parts of the molecule were hydrolyzed at different rates.[35]

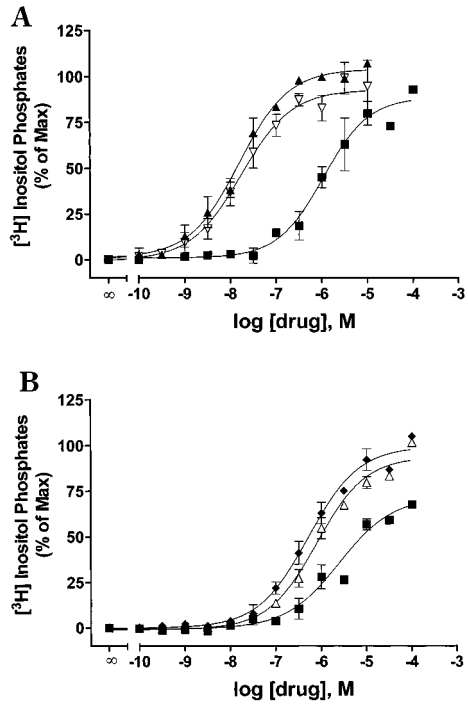


Figure 7. Effects of 2-RS-5′-(phosphorothioate)adenosine-nucleotides on $P2Y_1$-R-mediated inositol lipid hydrolysis in turkey erythrocytes. The capacity of the indicated concentrations of these nucleotides to stimulate phospholipase C activity was determined as described in Methods: (A) 2-hexylthio-ATP-α-S **A** isomer (▲), 2-hexylthio-ATP-α-S **B** isomer (Δ), and 2-hexylthio-AMP-S (■); (B) 2-(*p*-NO_2-phenethyl)thio-ATP-α-S **A** isomer (▲), 2-(*p*-NO_2-phenethyl)thio-ATP-α-S **B** isomer (Δ), and 2-(*p*-NO_2-phenethyl)thio-AMP-S (■). The data presented are from a representative experiment repeated at least three times with different membrane preparations.

Table 1. 2-Thioether 5′-*O*-(Thiophosphate)adenosine Derivatives Are Potent $P2Y_1$-R Agonists

compound	EC_{50} (nM)
2-hexylthio-ATP[8a]	10 ± 4
2-hexylthio-ATP-α-S-A, **5aA**	17 ± 3
2-hexylthio-ATP-α-S-B, **5aB**	21 ± 7
2-hexylthio-AMP[8a]	60 ± 9
2-hexylthio-AMP-S, **4a**	2790 ± 980
2-*p*-nitrophenethylthio-ATP[8a]	12 ± 4
2-*p*-nitrophenethylthio-ATP-α-S-A, **5bA**	510 ± 120
2-*p*-nitrophenethylthio-ATP-α-S-B, **5bB**	890 ± 340
2-*p*-nitrophenethylthio-AMP[8a]	3000 ± 1200
2-*p*-nitrophenethylthio-AMP-S, **4b**	2360 ± 980
ATP	6353 ± 2464
ATP-α-S (Sp isomer)[a]	6275 ± 2900
AMP-S[a]	no effect

[a] A commercial compound.

In this study, using a semipurified preparation of zymogen granule of pig pancreas (type I ATPDase), we have shown that the 5′-*O*-phosphorothioate moiety of ATP-α-S did not suppress the catalytic activity of the enzyme. This substitution increased the apparent K_m, whereas V_{max} was not significantly modified. Interestingly, substitution at C2 of ATP-α-S by a thioether (butylthio or hexylthio) caused a significant reduction of catalytic activity expressed by an increase in K_m and a decrease in V_{max}. Comparison of the results with those obtained with 2-substituted ATP derivatives suggests a possible steric hindrance at the level of the catalytic site. Indeed, the K_m of 2-hexylthio-ATP-α-S **A** isomer,

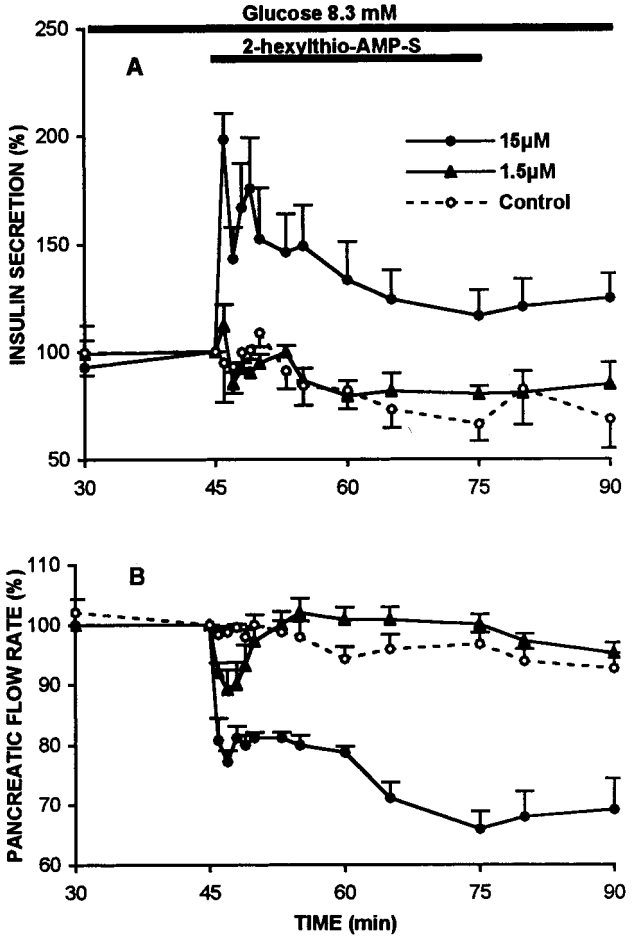


Figure 8. Effects of 2-hexylthio-AMP-S, **4a**, at (●) 15 μM and (▲) 1.5 μM on (A) insulin secretion and (B) pancreatic flow rate, from the isolated perfused pancreas of rat. Each point represents the mean of four experiments, with SEM shown by vertical lines.

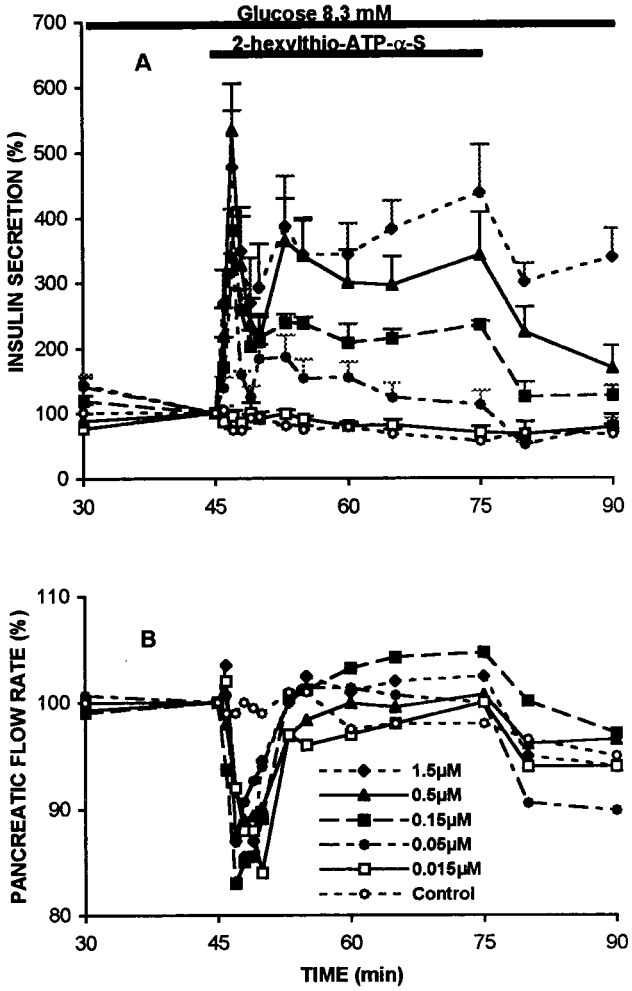


Figure 9. Effects of 2-hexylthio-ATP-α-S, **5aA**, on (A) insulin secretion and (B) pancreatic flow rate, from the isolated perfused pancreas of rat. Each point represents the mean of four experiments, with SEM shown by vertical lines.

5aA, is increased by a factor of 2 with no significant modification of V_{max}, compared to 2-butylthio-ATP.

The 2-RS-AMP-S derivatives, which are not hydrolyzed by this enzyme, are potential ATPDase inhibitors. Indeed, among the various analogues tested, 2-hexylthio-AMP-S, **4a**, is a weak competitive inhibitor, with a K_i in the order of 75 μM, whereas 2-benzylthio-AMP-S, **4c**, and AMP-S show a mixed type of inhibition.

These results indicate that substitution at the C2 position of the purine ring strongly affects hydrolysis by ATPDase, thus turning these new analogues into relatively stable P2-R ligands.

The apparent affinity of the compounds to $P2Y_1$-R was determined by measurement of P2Y-R-promoted phospholipase C activity in turkey erythrocyte membranes. 2-Hexylthio-ATP-α-S isomers, **5aA** and **5aB**, were found to be good agonists, stimulating the production of inositol phosphates with $K_{0.5}$ values in the nanomolar range. 2-RS-AMP-S derivatives were also full agonists, although 2 orders of magnitude less potent. All of the compounds were more potent than ATP. A difference of about 0.5 order of magnitude was observed for **5aA** and **5aB** diastereoisomers.

A high increase, up to 500%, in glucose-induced insulin secretion was due to addition of 2-hexylthio-ATP-α-S **A** isomer, **5aA**, in the nanomolar concentration range, to an isolated and perfused rat pancreas. **5aA**

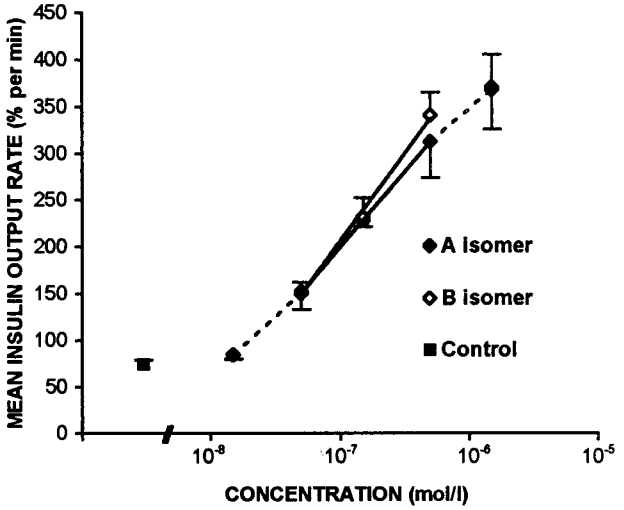


Figure 10. Concentration−response curves for 2-hexylthio-ATP-α-S, **5a**: (◆) **A** isomer, (◇) **B** isomer.

and **5aB** isomers are about 100 times more potent than ATP[4a] and approximately as potent as ADP-β-S.[5b] In the same preparation, 2-methylthio-ATP has been shown to be only 45 times more potent than ATP.[5a] Consequently, these new P2Y-R ligands may be of potential interest for the development of antidiabetic drugs that

promote insulin release. 2-Hexylthio-AMP-S was less potent than ATP, showing similar activity as AMP.[4a] These results on insulin secretion from rat pancreas show some discrepancies with those observed on inositol lipid hydrolysis in turkey erythrocyte membranes. They suggest that the P2Y receptor subtypes implicated in these two preparations may be different. On the other hand, concerning the vascular effects of these new derivatives of AMP and ATP, it may be hypothesized that they result from activation of a P2X-R or P2Y-R subtype sensitive to UTP, as has been previously observed in the perfused pancreas.[42]

In summation, 2-thioether 5′-*O*-(1-thiotriphosphate)-adenine-nucleotides are highly potent $P2Y_1$-R ligands in turkey erythrocytes, and they are highly effective insulin secretagogues active at the nanomolar concentration range. Moreover, these compounds exhibit relative enzymatic stability regarding type I ATPDase. However, their poor selectivity for the insulin-secreting cell, illustrated by their ability to induce vascular effects, prevents their further use as potential antidiabetic agents. Therefore, the development of improved generation, tissue-specific agents is currently being investigated by us.

Methods

Chemistry. General. All air- and moisture-sensitive reactions were carried out in flame-dried, argon-flushed, two-necked flasks sealed with rubber septa, and the reagents were introduced with a syringe. TLC analysis was performed on precoated Merck silica gel plates (60F-254). New compounds were characterized (and resonances assigned) by proton nuclear magnetic resonance using Bruker DPX-300, DMX-600, or AC-200 spectrometers. ^{1}H NMR spectra was recorded in D_2O, and the chemical shifts are reported in ppm relative to HDO (4.78 ppm) as an internal standard. Phosphorylated products were isolated as either Na^+, H^+, or Et_3NH^+ salts. Chemical shifts of Et_3NH^+ counterion are 3.18 ppm (q, J = 7.2 Hz, CH_2) and 1.26 ppm (t, J = 7.2 Hz, CH_3). The number of equivalents of triethylammonium counterions was defined by integration of their peaks, in the ^{1}H NMR spectrum, relative to those of the nucleotide. Nucleotides were characterized also by ^{31}P NMR in D_2O, using 85% H_3PO_4 as an external reference, on an AC-200 spectrometer at pH 8. All final products were characterized on an AutoSpec-E FISION VG high-resolution mass spectrometer by chemical ionization and high-resolution mass spectrometry. Nucleotides were desorbed from a glycerol matrix under FAB (fast atom bombardment) conditions in low and high resolution. Primary purification of the nucleotides was achieved on an LC (Isco UA-6) system using a Sephadex DEAE-A 25 column, which was swelled in 1 M $NaHCO_3$ in the cold for 1 day. The resin was washed with deionized water before use. Separation was monitored by UV, detection at 280 nm. A buffer gradient of 0−0.8 or 0−0.9 M NH_4HCO_3 (350 mL of H_2O:350 mL of buffer) was applied. Final purification and separation of diastereoisomers, in the cases of chiral products, were done on a HPLC (Merck−Hitachi) system using a semipreparative reverse-phase Nucleotide/Nucleoside 7U column (1 × 25 cm; Alltech Associates, Deerfield, IL) in different solvent systems for each product. The purity of all newly synthezised nucleotides was evaluated on an analytical column in different solvent systems. Peaks were detected by UV absorption using a diode array detector. 2RS-AMP-S and 2RS-ATP-α-S derivatives were generally >95% pure by HPLC. Minute contaminants are not nucleotides, as shown by their UV spectrum.

2-Thioether Adenosine Derivatives. General Non-aqueous Alkylation Procedure. A suspension of 2-SH-adenosine[20] (0.1 g, 0.335 mmol, in 10 mL of MeOH) was dissolved in 0.25 M NaOH (1.25 mL) and stirred at room temperature for 1 h. The solvent was evaporated under high vacuum. 2-Thioadenosine sodium salt, obtained as a yellow solid after freeze-drying, was dissolved in dry DMF (10 mL), and 1 equiv of alkyl bromide was added. The clear, yellow reaction mixture was stirred for 2 h at room temperature. TLC ($CHCl_3$:MeOH, 4:5) indicated that all starting material reacted and a major product was formed. The solvent was evaporated under high vacuum, and the residue was washed with MeOH (10 mL) several times and evaporated, until the residue turned to a yellow solid. The solid was triturated three times with a solution of ether:hexane (1:1), and TLC indicated that the product was pure. The product was dried under high vacuum.

2-(6-Amino-2-hexylsulfanyl-7*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (2a). 2a was obtained in 95% yield: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.23 (s, 1H, H-8), 7.34 (brs, 2H, NH_2), 5.82 (d, J = 6 Hz, 1H, H-1′), 4.62 (“t”, J = 5.5 Hz, 1H, H-2′), 4.14 (dd, J = 5, 3.5 Hz, 1H, H-3′), 3.91 (q, J = 4 Hz, 1H, H-4′), 3.64 (dd, J = 12, 4 Hz, 1H, H-5′), 3.52 (dd, J = 12,4 Hz, 1H, H-5′), 3.07 (ABq, J = 6.5, 13 Hz, 2H, CH_2−S), 1.65 (quintet, J = 7 Hz, 2H, $CH_2\beta$-S), 1.42 (quintet, J = 7 Hz, 2H, $CH_2\gamma$-S), 1.28 (m, 4H, 2 × (CH_2)), 0.87 (t, J = 7 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 163.78 (s, C-2), 155.48 (s, C-6), 150.10 (s, C-4), 138.81 (d, C-8), 116.91 (s, C-5), 87.35 (d, C-1′), 85.40 (d, C-4′), 73.15 (d, C-2′), 70.47 (d, C-3′), 61.60 (t, C-5′), 30.86 (t, CH_2−S), 30.10 (t, CH_2), 29.08 (t, CH_2), 27.98 (t, CH_2), 22.03 (t, CH_2), 13.90 (q, CH_3); MS (CI, ammonia) 384 (MH^+).

2-(6-Amino-2-(*p*-nitrophenethyl)sulfanyl-7*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (2b). 2b was obtained in 96% yield: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.31 (s, 1H, H-8), 8.20 (AA′XX′, J = 8 Hz, 2H, Ar), 7.65 (AA′XX′, J = 8 Hz, 2H, Ar), 7.42 (brs, 2H, NH_2), 5.91 (d, J = 6 Hz, 1H, H-1′), 5.53 (d, J = 6 Hz, 1H, OH(H-2′)), 5.26 (d, J = 5 Hz, 1H, OH (H-3′)), 4.60 (dd, J = 6, 5.5 Hz, 1H, H-2′), 4.14 (dd, J = 5, 3.5 Hz, 1H, H-3′), 3.95 (q, J = 4.5 Hz, 1H, H-4′), 3.56 (dd, J = 12, 5 Hz, 1H, H-5′), 3.56 (dd, J = 12, 5 Hz, 1H, H-5′), 3.50 (CH_2−S under the water signal), 3.13 (t, J = 7 Hz, 2H, CH_2−Ar); ^{13}C NMR (DMSO-d_6, 200 MHz) δ 163.12 (s, C-2), 155. 52 (s, C-6), 150.20 (s, C-4), 148.75 (s, C−NO_2), 146.05 (s, C_{arom}-CH_2), 138.44 (d, C-8), 130.03 (d, CH_{ortho} to NO_2), 123.37 (d, CH_{meta} to NO_2), 116.82 (s, C-5), 87.04 (d, C-1′), 85.83 (d, C-4′), 73.49 (d, C-3′), 70.42 (d, C-2′), 61.43 (t, C-5′), 35.31 (t, CH_2−C_{arom}), 31.06 (t, CH_2−S); FAB (negative) 449 (MH^+).

2-(6-Amino-2-benzylsulfanyl-7*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (2c). 2c was obtained in 97% yield: ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.25 (s, 1H, H-8), 7.42 (m, 4H, CH_{ortho}+NH_2), 7.29(m, 3H, $2H_{meta}$+H_{para}), 5.89 (d, J = 6 Hz, 1H, H-1′), 4.59 (dd, J = 6, 5 Hz, 1H, H-2′), 4.48 (s,1H, CH_2), 4.14 (dd, J = 5, 3.5 Hz, 1H, H-3′), 3.93 (q, J = 4 Hz, 1H, H-4′), 3.51(AB dq J = 12, 4 Hz, 2H, H-5′); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 163.18 (s, C-2), 155.49 (s, C-6), 150.10 (s, C-4), 138.64 (d, C-8 + s, C_{arom}-CH_2), 129.65 (d, C_{meta}), 128.30 (d, C_{ortho}), 126.83 (d, C_{para}), 116.91 (s, C-5), 87.24 (s, C-1′), 85.43 (C-4′), 73.50 (s, C-2′), 70.39 (s, C-3′), 61.44 (t, C-5′), 34.35 (t, CH_2−S); HRMS (DCI, CH_4) calcd for $C_{17}H_{19}N_5O_4S$ (MH^+) 390.1235, found 390.1230.

Thiophosphorylation Reaction. General Procedure. Nucleosides were dried for 2 days in a vacuum oven (40−50 °C). Anhydrous solvents (pyridine, DMF) were used. Thiophosphoryl chloride was distilled and kept under argon.

2-(6-Amino-2-hexylsulfanyl-7*H*-purin-9-yl)-5-(monothiophosphate-methyl)tetrahydrofuran-3,4-diol (4a). A suspension of 2-(hexylthio)adenosine, **2a** (0.1 g, 0.2 mmol), in dry pyridine was heated for several minutes until a clear solution was attained and then cooled to room temperature. Proton sponge was added (0.12 g, 2 equiv), and the solution was stirred for 10 min at 0 °C. Thiophosphoryl chloride was added dropwise (40 μL, 2 equiv, 0.4 mmol). TLC taken after 15 min (2-propanol:H_2O:NH_4OH, 16.5:5:1) indicated the complete disappearance of starting material and the formation of a single polar product (R_f = 0.48). After 15 min at 0 °C, a solution of 0.2 M TEAB (25 mL) was added; the clear solution was stirred at room temperature for 45 min and then freeze-dried.

The semisolid residue, dissolved in a minimal volume of water/pyridine, was chromatographed at room temperature on an activated Sephadex DEAE-A25 column. A buffer gradient of 0−0.8 M NH_4HCO_3 was applied, and about 100 10-mL fractions were collected, then the column was washed with 2 M NH_4HCO_3. The relevant fractions were pooled and freeze-dried three times to yield the product as a white solid (87% yield). Final separation was achieved on a semipreparative HPLC column applying a linear gradient of 0.1 M TEAA (pH 7.5)/CH_3CN, 80:20 to 50:50, in 20 min (flow rate 5 mL/min). Retention time: 12.63 min. The product was 98.6% pure: ^{1}H NMR (D_2O, 200 MHz) δ 8.45 (s, 1H, H-8), 6.13 (d, J = 6 Hz, 1H, H-1′), (H-2′ is hidden by the water signal), 4.63 ("t", J = 4 Hz, 1H, H-3′), 4.39 (br. s, 1H, H-4′), 4.06 (m, 2H, H-5′), 3.25 (m, 2H, CH_2−S), 1.72 (quintet, J = 7 Hz, 2H, $CH_2\beta$-S), 1.44 (quintet, J = 7 Hz, 2H, $CH_2\gamma$-S), (2 × (CH_2) is hidden by the counterion signal), 0.84 (t, J = 7 Hz, 3H, CH_3); ^{31}P NMR (D_2O, 200 MHz, pH 8) δ 45.70 (s); FAB (negative) 478 (MH^+); UV λ_{max} 278 nm.

2-(6-Amino-2-(*p*-nitrophenethyl)sulfanyl-7*H*-purin-9-yl)-5-(monothiophosphate-methyl)tetrahydrofuran-3,4-diol (4b). 4b was obtained in 80% yield. Final purification was achieved on a semipreparative column applying a linear gradient of 0.1 M TEAA (pH 10)/CH_3CN, 80:20 to 85:25, in 20 min. Retention time: 11.79 min. Product **4b** was 98.8% pure: ^{1}H NMR (D_2O, 200 MHz) δ 8.48 (s, 1H, H-8), 7.98 (AA′XX′, J = 8 Hz, 2H, Ar), 7.44 (AA′XX′, J = 8 Hz, 2H, Ar), 6.03 (d, J = 6 Hz, 1H, H-1′), (H-2′ is hidden by the water signal), 4.53 (t, J = 4 Hz, 1H, H-3′), 4.39 (m, 1H, H-4′), 4.05 (m, 2H, H-5′), 3.55 (m, 4H, CH_2−S, CH_2−Ar); ^{31}P NMR (D_2O, 200 MHz, pH 8) δ 43.89 (s); FAB (negative) 543 (MH^+), 565 (MW + Na^+); HRFAB calcd for $C_{18}H_{20}N_6O_8PS_2$ 543.0521, found 543.0570; UV λ_{max} 278 nm.

2-(6-Amino-2-benzylsulfanyl-7*H*-purin-9-yl)-5-(monothiophosphate-methyl)tetrahydrofuran-3,4-diol (4c). 4c was obtained in 84% yield. Final purification was achieved on a semipreparative column applying a linear gradient of 0.1 M TEAA (pH 7.5)/CH_3CN, 80:20 to 50:50, in 20 min. Retention time: 9.85 min. Product **4c** was >98% pure: ^{1}H NMR (D_2O, 200 MHz) δ 8.89 (s, 1H, H-8), 7.85 (m, 2H, CH_{ortho}), 7.30 (m, 3H, $2H_{meta}$+H_{para}), 6.10 (d, J = 6 Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.49 (t, J = 4 Hz, 1H, H-3′), 4.40 (s, 3H, CH_2+ H-4′), 4.03 (m, 2H, H-5′); ^{31}P NMR (D_2O, 200 MHz, pH 8) δ 44.19 (s); FAB (negative) 484 (MH^+); HRFAB calcd for $C_{17}H_{19}N_5O_6PS_2$ 483.0436, found 483.0509. UV λ_{max} 278 nm.

General Procedure for the Preparation of 5′-*O*-(1-Thiotriphosphate)nucleosides. 2-(6-Amino-2-hexylsulfanyl-7*H*-purin-9-yl)-5-*O*-(1-thiotriphosphate-methyl)tetrahydrofuran-3,4-diol (5a). A suspension of 2-(hexylthio) adenosine, **2a** (0.1 g, 0.2 mmol), in dry pyridine was heated for several minutes until a clear solution was attained and then cooled to room temperature. Proton sponge was added (0.12 g, 2 equiv), and the solution was stirred for 10 min at 0 °C. Thiophosphoryl chloride was added dropwise (40 μL, 2 equiv, 0.4 mmol). After 15 min, a mixture of Bu_3N (0.25 mL) and 1 M $(Bu_3NH^+)_2P_2O_7H_2$ in DMF[8a] (2 mL) was added. After 2 min, a solution of 0.2 M TEAB (25 mL) and proton sponge (2 equiv) was added, and the clear solution was stirred at room temperature for 45 min in pH 8 and freeze-dried. The semisolid obtained, dissolved in a minimal volume of water/pyridine, was chromatographed at room temperature on an activated Sephadex DEAE-A25 column. A buffer gradient of 0−0.9 M NH_4HCO_3 was applied, and 130 10-mL fractions were collected; then the column was washed with 2 M NH_4HCO_3. The relevant fractions were pooled and freeze-dried three times to yield a white solid (87% yield). Final purification was achieved on a semipreparative HPLC column applying a linear gradient of 0.1 M TEAA + 0.01% (w/w) $MgCl_2$ (pH 7.5)/CH_3CN, 80:20 to 60:40, in 20 min (flow rate 5 mL/min). Retention time: 19.71 min for isomer **A** and 23.15 min for isomer **B**. The purity of each diastereoisomer was >95%: ^{1}H NMR (for **A** & **B** isomers) (D_2O, 300 MHz) δ 8.53 (s, 1H, H-8 for diastereomer *A*), 8.46 (s, 1H, H-8 for diastereomer *B*), 6.13 (d, J = 6 Hz, 1H, H-1′), 4.87 (m, 1H, H-2′), 4.64 (t, J = 4.5 Hz, 1H, H-3′), 4.40 (m, 1H, H-4′), 4.30 (m, 2H, H-5′), 3.21 (m, 2H, CH_2−S), 1.75 (quintet, J = 7 Hz, 2H, $CH_2\beta$-S), 1.43 (quintet, J = 7 Hz, 2H, $CH_2\gamma$-S), (2 × (CH_2) is hidden by the counterion peak), 0.87 (t, J = 7 Hz, 3H, CH_3); ^{31}P NMR (**A** isomer) (D_2O, 200 MHz, pH 8) δ 45.90 (d, J = 32 Hz), −5.16 (d, J = 23 Hz), −20.08 (dd, J = 32, 23 Hz); ^{31}P NMR (**B** isomer) (D_2O, 200 MHz, pH 8) δ 45.70 (d, J = 32 Hz), −4.91 (d, J = 23 Hz), −19.87 (dd, J = 32, 23 Hz); FAB (negative) 660 (MW + $2H^+$ + Na^+), 682 (MW + H^+ + $2Na^+$), 704 (MW + $3Na^+$); HRFAB calcd for $C_{16}H_{24}N_5O_{12}P_3S_2$ 660.0130, found 660.0230; UV λ_{max} 278 nm.

2-(6-Amino-2-(*p*-nitrophenethyl)sulfanyl-7*H*-purin-9-yl)-5-(triphospho-α-thioate-methyl)tetrahydrofuran-3,4-diol (5b). 5b was obtained in 78% yield. Purification of the product on a semipreparative HPLC column applying a linear gradient of 0.1 M TEAA (pH 7.5)/CH_3CN, 80:20 to 65:35, in 20 min (flow rate 5 mL/min). Final separation of the two diastereomers was achieved on a semipreparative column applying a linear gradient of 0.1 M TEAA (pH 7.5)/CH_3CN, 80:20 to 75:25, in 20 min. Retention time: 16.09 min for **A** isomer, 17.99 min for **B** isomer. **A** isomer was 85% pure; **B** isomer was >95% pure: ^{1}H NMR (for **A** & **B** isomers) (D_2O, 600 MHz) δ 8.49 (s, 1H, H-8 for diastereomer *A*), 8.40 (s, 1H, H-8 for diastereomer *B*), 7.97 (AA′XX′, J = 8 Hz, 2H, Ar), 7.45 (AA′XX′, J = 8 Hz, 2H, Ar), 6.02 (d, J = 6 Hz, 1H, H-1′), (H-2′ is hidden by the water signal), 4.58 (m, 1H, H-3′), 4.40 (m,1H, H-4′), 4.28 (m, 2H, H-5′), 3.52 (m, 4H, CH_2−S, CH_2−Ar); ^{31}P NMR (for **A** & **B** isomers) (D_2O, 200 MHz, pH 8) δ 44.35 (d, J = 32 Hz), −5.1 (d, J = 24 Hz), −20.55 (dd, J = 32, 24 Hz); FAB (negative) 660 (MW + $2H^+$ + Na^+), 682 (MW + H^+ + $2Na^+$), 704 (MW + $3Na^+$); UV λ_{max} 278 nm.

2-(6-Amino-2-benzylsulfanyl-7*H*-purin-9-yl)-5-(triphospho-α-thioate-methyl)tetrahydrofuran-3,4-diol (5c). 5c was obtained in 83% yield. Purification of the product on a semipreparative column applying a linear gradient of 0.1 M TEAA (pH 7.5) + 0.01% $MgCl_2$/CH_3CN, 80:20 to 60:40, in 20 min (flow rate 5 mL/min). Final separation of the two diastereomers was achieved on a semipreparative column applying a linear gradient of 0.1 M TEAA (pH 7.5)/CH_3CN, 80:20 to 55:35, in 20 min. Retention time: 10.08 min for **A** isomer, 10.59 min for **B** isomer. The purity of each diastereoisomer was >96%: ^{1}H NMR (for **A** & **B** isomers) (D_2O, 300 MHz) δ 8.50 (s, 1H, H-8 for diastereomer *A*), 8.45 (s, 1H, H-8 for diastereomer *B*), 7.56 (m, 2H, CH_{ortho}), 7.35 (m, 3H, $2H_{meta}$+H_{para}), 6.14 (d, J = 6 Hz, 1H, H-1′), (H-2′ is hidden by the water signal), 4.55 (t, J = 4 Hz, 1H, H-3′), 4.43 (s, 3H, CH_2+ H-4′), 4.27 (m, 2H, H-5′); ^{31}P NMR (D_2O, 200 MHz, pH 8) δ 44.10 (d, J = 32 Hz), −5.52 (d, J = 22 Hz), −21.99 (t, J = 27 Hz); FAB (negative) 643 (MH^+); HRFAB calcd $C_{17}H_{19}N_5O_{12}P_3S_2$ for 643.9841, found 643.9830; UV λ_{max} 278 nm.

Evaluation of Enzymatic Stability. ATPDase assays were carried out with a purified zymogen granule membrane preparation from pig pancreas.[38a] This preparation was devoid of any other nucleotidase activities.[43] The enzyme preparation had a specific activity of about 0.5 μmol Pi/min/mg protein. Enzyme activity was measured at 37 °C in 1 mL of the following incubation medium: 8 mM $CaCl_2$, 5 mM tetramisole, 50 mM Tris, and 50 mM imidazole, buffered at pH 8.0. The reaction was started by the addition of 100 μM nucleotide substrate or as otherwise indicated. Inorganic phosphorus release was evaluated by the malachite green method as described by Baykov et al.[44] Protein concentration was determined by the Bradford microplate assay[45] using bovine albumin as a standard reference. All the experiments were carried out in triplicate. The deviation of the triplicate means was within 5%.

Evaluation of the New Compounds as P2Y-R Ligands. $P2Y_1$-R-promoted stimulation of inositol phosphate formation by adenine nucleotide analogues was measured in turkey erythrocyte membranes as previously described.[46,47] 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]myoinositol (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, and 10 mM Hepes, pH 7.0. Assays (200 mL 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.[46, 47]

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.

Insulin Release from Isolated Rat Pancreas. The pancreas was isolated from anesthetized male Wistar rats (320−350 g) and perfused with a Krebs-bicarbonate buffer (containing 8.3 mM glucose) at a constant pressure (40−50 cm water). In all the experiments, a 30-min adaptation period was allowed before taking the first two samples for insulin assay and flow rate measurement. The new synthetic derivatives, dissolved in a physiological medium containing 0.01 mM dithiotreitol (DTT), were perfused for 30 min. Samples were taken every minute during 5 min and then at 8, 10, 15, 20, and 30 min; each sample was collected during 1 min. Insulin in the pancreatic effluent was measured by radioimmunoassay. The variations in the flow rate, which reflect the changes in vascular resistance, were registered throughout the experiment.

Acknowledgment. The Israeli group thanks the Israeli Ministry of Health for financial support. J.C., D.H.B., and P.P. also wish to thank S. Dietz, N. Linck, and V. Montesinos for technical assistance. J.L.B. and K.T.H. are indebted to Arvind Mohanram for excellent technical assistance. This work was supported by USPHS Grants GM38213 and HL54889.

References

(1) (a) Bhagwat, S. S.; Williams, M. P2 purine and pyrimidine receptors: emerging superfamilies of G-protein and ligand-gated ion channels receptors. *Eur. J. Med. Chem.* **1997**, *32*, 183−193. (b) King, B. F.; Townsend-Nicholson, A.; Burnstock, G. Metabotropic receptors for ATP and UTP: exploring the correspondence between native and recombinant nucleotide receptors. *Trends Pharmacol. Sci.* **1998**, *19*, 506−514.

(2) Abbracchio, M. P.; Burnstock, G. Purinoceptors: are there families of P2X and P2Y Purinoceptors? *Pharmacol. Ther.* **1994**, *64*, 445−475.

(3) (a) Chan, C. M.; Unwin, R. J.; Burnstock, G. Potential functional roles of extracellular ATP in kidney and urinary tract. *Exp. Nephrol.* **1998**, *6*, 200−207. (b) Boarder, M. R.; Hourani, S. M. O. The regulation of vascular function by P2 receptors: multiple site and multiple receptors. *Trends Pharmacol. Sci.* **1998**, *19*, 99−107. (c) Barnard, E. A.; Simon, J.; Webb, T. E. Nucleotide receptors in the nervous system- An abundant component using diverse transduction mechanisms. *Mol. Neurobiol.* **1997**, *15*, 103−129. (d) Inoue, K. The function of ATP receptors in the hippocampus. *Pharmacol. Res.* **1998**, *38*, 323−331. (e) Abbrachio, M. P. P1 and P2 receptors in cell growth and differentiation. *Drug Dev. Res.* **1996**, *39*, 393−406. (f) Fischer, B. Therapeutic applications of ATP−(P2)-receptors agonists and antagonists. *Exp. Opin. Ther. Patents* **1999**, *9*, 385−399.

(4) (a) Loubatières-Mariani, M. M.; Chapal, J.; Lignon, F.; Valette, G. Structural specificity of nucleotides for insulin secretory action from the isolated perfused rat pancreas. *Eur. J. Pharmacol.* **1979**, *59*, 277−286. (b) Chapal, J.; Loubatières-Mariani, M. M. Effects of phosphate-modified adenine nucleotide analogues on insulin secretion from perfused rat pancreas. *Br. J. Pharmacol.* **1981**, *73*, 105−110.

(5) (a) Bertrand, G.; Chapal, J.; Loubatières-Mariani, M. M.; Roye, M. Evidence for two different P_2-purinoceptors on β cell and pancreatic vascular bed. *Br. J. Pharmacol.* **1987**, *102*, 783−787. (b) Bertrand, G.; Chapal, J.; Puech, R.; Loubatières-Mariani, M. M. Adenosine-5′-O-(2-thiodiphosphate) is a potent agonist at P_2 purinoceptors mediating insulin secretion from perfused rat pancreas. *Br. J. Pharmacol.* **1991**, *102*, 627−630. (c) Petit, P.; Hillaire-Buys, D.; Manteghetti, M.; Debrus, S.; Chapal, J.; Loubatières-Mariani, M. M. Evidence for two different types of P2 receptors stimulating insulin secretion from pancreatic B cell. *Br. J. Pharmacol.* **1998**, *125*, 1368−1374.

(6) (a) Ribes, G.; Bertrand, G.; Petit, P.; Loubatières-Mariani, M. M. Effects of 2-methylthio ATP on insulin secretion in the dog in vivo. *Eur. J. Pharmacol.* **1988**, *155*, 171−174. (b) Hillaire-Buys, D.; Bertrand, G.; Chapal, J.; Puech, R.; Ribes, G.; Loubatières-Mariani, M. M. Stimulation of insulin secretion and improvment of glucose tolerance in rat and dog by the P_{2y} purinoceptor agonist, adenosine-5′-O-(2-thiodiphosphate). *Br. J. Pharmacol.* **1993**, *109*, 183−187.

(7) (a) Petit, P.; Loubatières-Mariani, M. M.; Keppens, S.; Sheehan, M. J. Purinergic receptors and metabolic function. *Drug Dev. Res.* **1996**, *39*, 413−425. (b) Loubatières-Mariani, M. M.; Hillaire-Buys, D.; Chapal, J.; Bertrand, G.; Petit, P. P_2 purinoceptor agonists: new insulin secretagogues potentially useful in the treatment of noninsulin-dependent diabetes mellitus. *Purinergic Approaches Exp. Ther.* **1997**, *13*, 253−260.

(8) (a) Fischer, B.; Boyer, J. L.; Hoyle, C. H. V.; Ziganshin, A. V.; Brizzolara, A. L.; Knight, G. E.; Zimmet, J.; Burnstock, G.; Harden, T. K.; Jacobson, K. A. Identification of potent, selective P2Y-purinoceptor agonists: structure−activity relationships for 2-thioether derivatives of adenosine 5′-triphosphate. *J. Med. Chem.* **1993**, *36*, 3937−3946. (b) Boyer, J. L.; Siddiqi, S.; Fischer, B.; Romero-Avila, T.; Jacobson, K. A.; Harden, T. K. Identification of potent P2Y-purinoceptor agonists that are derivatives of adenosine 5′-monophosphate. *Br. J. Pharmacol.* **1996**, *118*, 1959−1964.

(9) Burnstock, G.; Fischer, B.; Hoyle, C. H. V.; Maillard, M.; Ziganshin, A. V.; Brizzolara, A. L.; von Isakovics, A.; Boyer, J. L.; Harden, T. K.; Jacobson, K. A. Structure activity relationship for derivatives of adenosine 5′-triphosphate as agonists at P2-purinoceptors: heterogeneity within P2X and P2Y subtypes. *Drug Dev. Res.* **1994**, *31*, 206−219.

(10) Boyer, J. L.; O'Tuel, J. W.; Fischer, B.; Jacobson, K. A.; Harden, T. K. 2-Thioether derivatives of adenine nucleotides are exceptionally potent agonists at adenylyl cyclase-linked P2Y-purinoceptors. *Br. J. Pharmacol.* **1995**, *116*, 2611−2616.

(11) Zimmet, J.; Järlebark, L.; van Galen, P. J. M.; Jacobson, K. A.; Heilbronn, E. Synthesis and biological activity of novel 2-thio derivatives of ATP. *Nucleosides Nucleotides* **1993**, *12*, 1−20.

(12) Murray, A. W.; Atkinson, M. R. Adenosine 5′-phsphorothioate. A nucleotide analogue that is a substrate, competitive inhibitor, or regulator of some enzymes that interact with adenosine 5′-phosphate. *Biochemistry* **1968**, *11*, 4023−4029.

(13) Eckstein, F.; Sternbach, H. Nucleoside 5′-O-phosphorothioates as inhibitors for phosphatases. *Biochim. Biophys. Acta* **1967**, *146*, 618−619.

(14) Eckstein, F.; Goody, R. S. Synthesis and properties of diastereomers of adenosine 5′-(O-1-thiotriphosphate) and adenosine 5′-(O-2-thiotriphosphate). *Biochemistry* **1976**, *15*, 1685−1691.

(15) Hampton, A.; Brox, L. W.; Bayer, M. Analogues of inosine 5′-phosphate with phosphorus−nitrogen and phosphorus−sulfur bonds. Binding and kinetic studies with inosine 5′-phosphate dehydrogenase. *Biochemistry* **1969**, *8*, 2303−2311.

(16) Eckstein, F. Nucleoside phosphorothioates. *Annu. Rev. Biochem.* **1985**, *54*, 367−402 and references therein.

(17) Jaffe, E. K.; Cohn, M. ^{31}P nuclear magnetic resonance spectra of the thiophosphate analogues of adenine nucleotides; effects of pH and Mg^{2+} binding. *Biochemistry* **1978**, *17*, 652−657.

(18) (a) Van Rhee, M. A.; Fischer, B.; van Galen, P. G. M.; Jacobson, K. A. Modelling the P2Y purinoceptor using rhodopsin as template. *Drug Des. Discovery* **1995**, *13*, 133−154. (b) Jiang, Q.; Guo, D.; Lee, B. X.; van Rhee, A. M.; Kim, Y.-C.; Nicholas, R. A.; Schachter, J. B.; Harden, T. K.; Jacobson, K. A. A mutational analysis of residues essential for ligand recognition at the human $P2Y_1$-receptor. *Mol. Pharmacol.* **1997**, *59*, 499−507. (c) Moro, S.; Guo, D.; Camaioni, E.; Boyer, J. L.; Harden, T. K.; Jacobson, K. A. Human $P2Y_1$-receptor: molecular modeling and site-directed mutagenesis as tools to identify agonist and antagonist recognition sites. *J. Med. Chem.* **1998**, *41*, 1456−1466.

(19) Burnstock, G.; Cusack, N. J.; Meldrum, L. A. Effects of phosphorothioate analogues of ATP, ADP and AMP on guinea-pig taenia coli and urinary bladder. *Br. J. Pharmacol.* **1984**, *82*, 369−374.

(20) Kikugawa, K.; Suehiro, H.; Yanase, R.; Aoki, A. Platelet aggregation inhibitors. IX. Chemical transformation of adenosine into 2-thio adenosine derivatives. *Chem. Pharm. Bull.* **1977**, *25*, 1959−69 and references therein.

(21) (a) Eckstein, F. Nucleoside phosphorothioates. *J. Am. Chem. Soc.* **1966**, *88*, 4292−4294. (b) Chen, J.-T.; Benkovic, S. J. Synthesis and separation of diastereomers of deoxynucleoside 5′-O-(1-thio)-triphosphates. *Nucleic Acids Res.* **1983**, *11*, 3737−3751. (c) Reese, C. B.; Shek, L. H. K.; Zhao, Z. Aminolysis and alkaline hydrolysis of protected 1-hydroxybenzo-triazol-1-yl esters of adenosine 5′-phosphorothioate and -phosphorodithioate. *J. Chem. Soc., Perkin Trans. 1* **1995**, 3077−3084.

(22) Cook, A. F. Nucleosides S-alkyl phosphorothioates. IV. Synthesis of nucleoside phosphorothioates monoesters. *J. Am. Chem. Soc.* **1970**, *92*, 190−195.

(23) Yoshikawa, M.; Kato, T.; Takenishi, T. Studies of phosphorylation. III. Selective phosphorylation of unprotected nucleosides. *Bull. Chem. Soc. Jpn.* **1969**, *42*, 3505−3508.

(24) Ikemoto, T.; Haze, A.; Hatano, H.; Kitamoto, Y.; Ishida, M.; Nara, K. Phosphorylation of nucleosides with phosphorous oxychloride in trialkyl phosphate. *Chem. Pharm. Bull.* **1995**, *43*, 210−215.

(25) Sowa, T.; Ouchi, S. The facile synthesis of 5′-nucleotides by the selective phosphorylation of a primary hydroxyl group of nucleosides with phosphoryl chloride. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 2084−2090.

(26) Imai, K.-I.; Fujii, S.; Takanohashi, K.; Furukawa, Y.; Masuda, T.; Honjo, M. Studies on phosphorylation. IV. Selective phosphorylation of the primary hydroxyl group in nucleosides. *J. Org. Chem.* **1969**, *34*, 1547−1550.

(27) Efimov, A. E.; Kalinkina, A. L.; Chakhmakhcheva, O. G.; Schmaltz Hill, T.; Jayaraman, K. New efficient sulfurizing reagents for the preparation of oligo-deoxynucleotide phosphorothioate analogues. *Nucleic Acids Res.* **1995**, *23*, 4029−4033.

(28) (a) Ora, M.; Oivanen, M.; Almer, H.; Stromberg, R.; Lönnberg, H. Hydrolytic reactions of the diastereomeric phosphoromonothioate analogues of uridylyl (3′,5′)uridine: kinetics and mechanisms for desulfurization, phosphoester hydrolysis, and transestrification to the 2′,5′-isomers. *J. Org. Chem.* **1995**, *60*, 5620−5627. (b) Ora, M.; Oivanen, M.; Lönnberg, H. Hydrolysis and desulfurization of the diastereomeric phosphoromonothioate analogues of uridine 2′,3′-cyclic monophosphate. *J. Org. Chem.* **1996**, *61*, 3951−3955. (c) Ora, M.; Oivanen, M.; Lönnberg, H. Phosphoester hydrolysis and intramolecular transesterification of ribonucleoside 2′- and 3′-phosphoromonothioate triesters: kinetics and mechanisms for the reactions of 5′-O-methyluridine 2′- and 3′-dimethylphosphoromonothioates. *J. Org. Chem.* **1997**, *62*, 3246−3253.

(29) Kodra, J. T.; Kehler, J.; Dahl, O. Stability of oligodeoxynucleoside phosphorodithioates and phosphorothioates in aqueous ammonia. *Nucleic Acids Res.* **1995**, *23*, 3349−3350.

(30) (a) Yee, D.; Armstrong, V. W.; Eckstein, F. Mechanistic studies on deoxyribonucleic acid dependent ribonucleic acid. Polymerase from *Escherichia coli* using phosphorothioate analogues. 1. Initiation and pyrophosphate exchange reactions. *Biochemistry* **1979**, *18*, 4116−4120. (b) Ludwig, J.; Eckstein, G. Rapid and efficient synthesis of nucleoside 5′-O-(1-thiotriphosphate), 5′-triphosphates and 2′,3′-cyclophosphorothioates using 2-chloro-4H-1,3,2-benzodioxa-phosphorin-4-one. *J. Org. Chem.* **1989**, *54*, 631−635.

(31) Eckstein, F.; Goody, R. S. Synthesis and properties of diastereoisomers of adenosine 5′-(O-1-thiotriphosphate) and adenosine 5′-(O-2-thiotriphosphate). *Biochemistry* **1976**, *15*, 1685−1691.

(32) Mishra, N. C.; Broom, A. D. A novel synthesis of nucleoside 5′-triphosphates. *J. Chem. Soc., Chem. Commun.* **1991**, 1276−1277.

(33) (a) Jones, D. T.; Masters, S. B.; Bourne, H. R.; Reed, R. R. Biochemical characterization of three stimulatory GTP-binding proteins. *J. Biol. Chem.* **1990**, *265*, 2671−2676. (b) Yamanaka, G.; Eckstein, F.; Stryer, L. Stereochemistry of the guanyl nucleotide binding site of transducin probed by phosphorothioate analogues of GTP and GDP. *Biochemistry* **1985**, *24*, 8094−8101. (c) Paris, S.; Eckstein, F. Activation of G proteins by (Rp) and (Sp) diastereomers of guanosine 5′-[β-thio]triphosphate in hamster fibroblasts. *Biochem. J.* **1992**, *284*, 327−332.

(34) Marquetant, R.; Goody, R. S. An improved separation of diastereomers of nucleoside phosphorothioates using reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **1983**, *280*, 386−389.

(35) Picher, M.; Sevigny, J.; D′Orleans-Juste, P.; Beaudoin, A. R. Hydrolysis of P2-purinoceptor agonists by a purified ectonucleotidase from the bovine aorta, the ATP-diphosphohydrolase. *Biochem. Pharmacol.* **1996**, *51*, 1453−1460.

(36) Pearson, J.; Carleton, J. S.; Gordon, J. L. Metabolism of adenine nucleotides by ectoenzymes of vasculare endothelial and smooth-muscle cells in culture. *Biochem. J.* **1980**, *190*, 421−429.

(37) LeBel, D.; Poirier, G. G.; Phaneuf, S.; St.-Jean, P.; Laliberte, J. F.; Beaudoin, A. R. Characterization and purification of a calcium-sensitive ATP diphosphohydrolase from pig pancreas. *J. Biol. Chem.* **1980**, *255*, 1227−1233.

(38) (a) Sevigny, J.; Cote, Y. P.; Beaudoin, A. R. Purification of pancreas Type I ATP diphosphohydrolase and identification by affinity labeling with 5′-p-fluorosulfonyl benzoyl adenosine ATP analogue. *Biochem. J.* **1995**, *312*, 351−356. (b) Christofordis, S.; Papamarcaki, T.; Galaris, D.; Kellner, R.; Tsolas, O. Purification and properties of human placental ATP diphosphohydrolase. *Eur. J. Biochem.* **1995**, *234*, 66−74.

(39) Fischer, B.; Halbfinger, E.; Major, D. T.; Ritzmann, M.; Ubl, J.; Reiser, G.; Boyer, J. L.; Harden, K. T. Molecular recognition of modified adenine nucleotides by the $P2Y_1$-receptor. Part I. A synthetic, biochemical, and NMR approach. Submitted for publication.

(40) (a) Boyer, J. L.; Schachter, J. B.; Sromek, S. M.; Palmer, R. K.; Jacobson, K. A.; Nicholas, R. A.; Harden, T. K. Avian and human homologues of the $P2Y_1$ receptor: pharmacological, signaling, and molecular properties. *Drug Dev. Res.* **1996**, *39*, 253−261. (b) Boyer, J. L.; Romero, T.; Schachter, J. B.; Harden, T. K. Identification of competitive antagonists of the $P2Y_1$ receptor. *Mol. Pharmacol.* **1996**, *50*, 1323−1329.

(41) Zimmermann, H. Extracellular purines metabolism. *Drug Dev. Res.* **1996**, *39*, 337−352.

(42) (a) Hillaire-Buys, D.; Chapal, J.; Petit, P.; Loubatières-Mariani, M. M. Dual regulation of pancreatic vascular tone by P2X and P2Y purinoceptor subtypes. *Eur. J. Pharmacol.* **1991**, *199*, 309−314. (b) Hillaire-Buys, D.; Chapal, J.; Petit, P.; Loubatières-Mariani, M. M. Vasoconstrictor effect of uridine triphosphate on pancreatic vascular bed. *C. R. Soc. Biol.* **1995**, *189*, 333−338.

(43) Laliberte, J.-F.; Beaudoin, A. R. Sequential hydrolysis of the gamma and beta-phosphate groups of ATP by the ATP diphosphohydrolase from pig pancreas. *Biochim. Biophys. Acta* **1983**, *742*, 9−15.

(44) Baykov, A. A.; Evtushenko, O. A.; Avaeva, S. M. A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-base enzyme immunoassay. *Anal. Biochem.* **1988**, *171*, 266−270.

(45) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(46) Boyer, J. L.; Downes, C. P.; Harden, T. K. Kinetics of activation of phospholipase C by P2Y purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.* **1989**, *264*, 884−890.

(47) Harden, T. K.; Hawkins, P. T.; Stephens, L.; Boyer, J. L.; Downes, C. P. Phosphoinositide hydrolysis by guanosine 5′-[gamma-thio]triphosphate-activated phospholipase C of turkey erythrocyte membranes. *Biochem. J.* **1988**, *252*, 583−593.

JM990158Y