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New Highly Active Antiplatelet Agents with Dual Specificity for Platelet P2Y₁ and P2Y₁₂ Adenosine Diphosphate Receptors

Ivan B. Yanachkov^{†,*}, Hung Chang^{&,#}, Milka I. Yanachkova[†], Edward J. Dix[†], Michelle A. Berny-Lang[†], Thomas Gremmel[‡], Alan D. Michelson^{‡,&}, George E. Wright[†], and Andrew L. Frelinger III^{‡,&}

[†]GLSynthesis, Inc. Worcester, MA

[‡]Center for Platelet Research Studies, Division of Hematology/Oncology, Boston Children's Hospital, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA

[&]Center for Platelet Function Studies, Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA

[#]Hematology Division, Chang Gung Memorial Hospital, Chang Gung University, Taipei, Taiwan

Abstract

Currently approved platelet adenosine diphosphate (ADP) receptor antagonists target only the platelet P2Y₁₂ receptor. Moreover, especially in patients with acute coronary syndromes, there is a strong need for rapidly acting and reversible antiplatelet agents in order to minimize the risk of thrombotic events and bleeding complications. In this study, a series of new P¹,P⁴-di(adenosine-5') tetraphosphate (Ap₄A) derivatives with modifications in the base and in the tetraphosphate chain were synthesized and evaluated with respect to their effects on platelet aggregation and function of the platelet P2Y₁, P2Y₁₂, and P2X₁ receptors. The resulting structure-activity relationships were used to design Ap₄A analogs which inhibit human platelet aggregation by simultaneously antagonizing both P2Y₁ and P2Y₁₂ platelet receptors. Unlike Ap₄A, the analogs do not activate platelet P2X₁ receptors. Furthermore, the new compounds exhibit fast

CORRESPONDING AUTHOR: Ivan B. Yanachkov, GLSynthesis, Inc., One Innovation Drive, Worcester, MA 01605, phone: (+1) 508 754 6700 x117, Fax: (+1) 508 754 7075, ivan.yanachkov@glsynthesis.com.

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Supporting Information

Additional chemical procedures and NMR spectra.

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onset and offset of action and are significantly more stable than Ap₄A to degradation in plasma, thus presenting a new promising class of antiplatelet agents.

Graphical Abstract

Keywords

Antiplatelet therapy; Antithrombotics; Adenosine diphosphate; Cardiovascular disease; Acute coronary syndromes; P2Y₁; P2Y₁₂; P2X₁; dinucleoside polyphosphates; bis-nucleoside polyphosphates; inhibitors; platelets

1. INTRODUCTION

Platelets play critical roles in hemostasis and its pathophysiology [1]. Undesired platelet activation can be a result of many common pathologies or interventions e.g. atherosclerotic plaque rupture, surgeries, percutaneous interventions, or major traumas, and may lead to excessive platelet aggregation and generation of occlusive thrombi. The ischemic events that follow, such as acute myocardial infarction and stroke, are leading causes of death and incapacitation in the developed world and are major contributors to health care costs. It is also increasingly recognized that platelets are an essential and integral part of the immune system [2–4], and abnormal platelet activation can be a contributing factor to vascular inflammation and associated vascular injury and atherosclerosis [5]. As a result, therapeutics that control platelet reactivity have achieved significant use in clinical practice, and the development of new drugs of the class has been a major focus of the research community and the pharmaceutical industry [6,7].

ADP plays a major role in the process of platelet activation [8]. It is released by activated platelets [9], and is an agonist at two platelet purinergic G-protein coupled transmembrane receptors – the G_q coupled P2Y₁ and the G_i coupled P2Y₁₂. A third platelet P2 receptor, P2X₁, is an ATP-activated ion channel. P2Y₁ activation initiates ADP-induced platelet aggregation, and is responsible for platelet shape change [10]. However, without P2Y₁₂ activation, the result is a small and reversible platelet aggregation. P2Y₁₂ activation results in amplification and stabilization of the aggregation response. There is a complex interplay between P2Y₁ and P2Y₁₂ receptors [11], and co-activation of both is necessary for full platelet aggregation [12]. The role of P2X₁ may be associated with platelet shape change in response to ATP, and may contribute to the activation of platelets by low collagen

concentrations and high shear stress, thus playing a role in localized thrombus formation in small arteries [13].

The P2Y₁₂ receptor is the most important platelet drug target. It is irreversibly inhibited by the major class of antiplatelet agents – the thienopyridines [14,15]. It is also the target of the newer reversible antiplatelet drugs ticagrelor [16], cangrelor [17], elinogrel [18], and other drug candidates [19] in various stages of development. P2Y₁-selective antagonists have been identified [20,21], but the lack of clinical candidates contrasts with the essential role of this receptor in platelet aggregation [22]. By using selective inhibitors, Nylander et al. [23] found that simultaneous targeting of P2Y₁ and P2Y₁₂ is highly synergistic. We [24,25] have reported that Ap₄A and its phosphonate analogs inhibit both human platelet P2Y₁ and P2Y₁₂ receptors and that the IC₅₀s for inhibition of ADP-induced human platelet aggregation were lower than the IC₅₀s for each of the receptors.

Both ADP and ATP scaffolds have been heavily modified in search of new P2 receptor agonists and antagonists (for reviews, see [22,26–29]), and the efforts resulted in the discovery of the highly potent P2Y₁₂ antagonists cangrelor [17] and ticagrelor [16], and highly potent P2Y₁ agonists and antagonists [30,31].

P¹,P⁴-Di(adenosine-5') tetraphosphate (Ap₄A) is the most important member of the group of dinucleoside polyphosphates. It is found in a variety of cells, is secreted extracellularly, and is involved in the regulation of variety of intra- and extracellular physiological functions [32]. In platelets Ap₄A is stored in dense granules and is therefore released along with ADP and ATP upon platelet activation [33]. It is well known that Ap₄A inhibits ADP-induced platelet activation [34], and modifications of Ap₄A's tetraphosphate chain have been shown to improve on this effect and to increase the biological stability [35–37]. We recently reported that Ap₄A and its tetraphosphate chain modified (P¹- and/or P⁴-thio, and P²,P³-chloromethylene) analogs inhibit platelet aggregation by targeting both P2Y₁ and P2Y₁₂ receptors [24,25].

While the modifications of the tetraphosphate chain of the Ap₄A scaffold have been extensively explored [24, 25, 35–37], and some modifications of the ribose moiety, namely, the 2',3'-*O*-benzylidene derivative have been made [38], no exploration of the base modifications has been attempted. This is due, in part, to the lack of a chemical method allowing rapid synthesis in good yield of new Ap₄A analogs. We have recently reported synthesis and properties of new reagents, diimidazolyl derivatives of diphosphoric and (methylene)bisphosphonic acids, which allow rapid synthesis of dinucleoside tetraphosphates in high yields [39]. We explored this method to prepare a number of new Ap₄A analogs with modifications in the adenosine base and the tetraphosphate moiety, and now report on their synthesis, their properties as platelet aggregation inhibitors and their activities toward platelet purinergic (P2) receptors. This SAR effort resulted in the discovery of highly potent platelet aggregation inhibitors which selectively target both platelet ADP receptors – P2Y₁ and P2Y₁₂, and have potential as novel antiplatelet drugs distinct from current antiplatelet agents, including the ATP analog cangrelor, which target only P2Y₁₂.

2. RESULTS AND DISCUSSION

2.1. Selection of Modifications

Introduction of certain substituents at position 2 of the adenine greatly enhances the agonist potency of ADP, and the antagonist potency of ATP toward platelet ADP receptors [19]. For instance, the EC₅₀ of ADP and 2-MeSADP toward P2Y₁ in a functional assay are 8000 nM and 6 nM, respectively, and toward P2Y₁₂ are 69 nM and 0.3 nM, respectively. In development of ATP type inhibitors of P2Y₁₂, Ingall et al. [40] synthesized a homologous series of 2-substituted ATP analogs. According to the authors, any substitution at this position increases receptor affinity, but the effect is most pronounced when the substituent is a non-polar group attached through a sulfur atom. For instance, going from adenosine 5'-(P²,P³-dichloromethylenetriphosphate) to its 2-ethylthio analog decreased the IC₅₀ 1000 fold. Replacement of EtS group with *n*-PrS increased the inhibitory potency another 100 fold. Further increase of the chain length or introduction of additional substitution did not provide additional advantage [40].

In development of P2Y₁ antagonists, the group of Jacobson prepared a series of C-2, and N⁶ substituted carbocyclic adenosine-3',5'-diphosphate analogs (the so called "methanocarba" analogs) [41]. The effect of C-2 substitution at the adenine base on the antagonist potency was in the order I>Br>Me>Cl>H>F. In contrast to P2Y₁₂ (see above), increasing the size of a C-2 alkyl group or attaching it through a sulfur atom decreased the potency.

Substitutions at the N⁶ amino group of the adenine moiety also proved to be beneficial in development of P2Y₁ and P2Y₁₂ agonist or antagonists. In Ingall's SAR study [40] mono-alkylation of N⁶ improved the antagonist potency of the ATP analogs toward P2Y₁₂, although the effect was less pronounced than that of the substitution at C-2 position. The optimal length of the alkyl chain was 3–4 carbon atoms, and dialkylation or acylation of N⁶ significantly reduced the activity. The effect of the N⁶ alkylation proved to be additive with the effect of the C-2 substitution, and led to the discovery of the ATP based antiplatelet drug candidate cangrelor. In contrast to P2Y₁₂, the P2Y₁ receptor is less tolerant to bulky substituents at N⁶, and the optimal modification appears to be mono-methylation [42]. Both P2Y₁ and P2Y₁₂ poorly tolerate substitutions at C-8 of adenine [43].

Modifications in the polyphosphate groups of ADP and ATP have also been extensively explored. Replacement of a phosphate oxygen by sulfur is well tolerated by both P2Y₁ and P2Y₁₂, and leads in many cases to more potent analogs [26]. Actually, P²-thioADP is a better agonist of P2Y₁ than ADP [30], and almost as effective as ADP toward P2Y₁₂ receptor [26]. An additional benefit of this modification is the improved stability of the thiophosphates toward enzymatic degradation. Replacement of the oxygen atom between P² and P³ by halomethylene groups improves the inhibitory potency of the ATP scaffold in respect to P2Y₁₂, as well as the degradation stability [40]. All these observations are summarized in Fig. 1.

Assuming a related mode of binding of Ap₄A and ADP/ATP to P2Y₁₂/P2Y₁ receptors we chose to investigate the effects of substitutions at C2 and N⁶ of the adenine base, with or without modifications in the tetraphosphate chain of the Ap₄A scaffold, on platelet

aggregation and on platelet P1Y₁, P2Y₁₂, and P2X₁ receptors. To this end the new Ap₄A analogs **2-16** shown in Fig. 2 have been synthesized and studied. The known, base unsubstituted compound **1** has been prepared and studied for reference purposes, too [24].

2.2 Chemistry

The C-2 and N⁶ substituted adenosine derivatives were prepared as follows: 2-alkylthioadenosines, precursors for compounds **2-9** and **16**, were prepared by S-alkylation of 2-thioadenosine [44]. N⁶-Methyladenosine, precursor of compound **13**, was prepared from commercially available 6-chloropurine riboside and methylamine. N⁶-Propyladenosine, the precursor of **14**, was prepared from inosine by adaptation of the general method of Wan et al.[45]. 2-Methylthio-N⁶-methyladenosine, the precursor of compound **15**, was synthesized in three steps from 2-methylthioadenosine by adaptation of a method described by Ingall et al.[40].

The precursor nucleosides for compounds **10** and **12**, 2-chloro-, and 2-iodoadenosine, are commercially available. 2-Bromoadenosine [43], needed for compound **11**, was prepared in 3 steps from guanosine through 2',3',5'-tri-*O*-acetylguanosine, 2-amino-6-chloro-9-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)purine [46], and 2-bromo-6-chloro-9-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)purine [47], as described.

The nucleosides were directly phosphorylated to 5'-monophosphates with phosphoryl chloride in trimethylphosphate [48], or to 5'-thiomonophosphates with thiophosphoryl chloride in pyridine [49]. Coupling of the mono-, or thiomonophosphates with the diimidazolides of diphosphoric or (monochloromethylene)bisphosphonic acids in the presence of zinc chloride (Scheme 1) afforded the corresponding dinucleoside tetraphosphates [39].

The attempt to prepare compound **16** by the diimidazolidine method was not successful. The diimidazolidine of (dichloromethylene)bisphosphonic acid was prepared and characterized successfully, yet it failed to react with 2-methylthioadenosine 5'-thiomonophosphate (MeSAMP(S)) under all conditions, and catalytic systems we tried. This is probably due to the unexpectedly high steric hindrance by the dichloromethylene group on the phosphorous atoms. Compound **16** was prepared, albeit in low yield, by zinc chloride catalyzed coupling of the cyclic trimetaphosphate **17** with MeSAMP(S) (Scheme 2) [50].

2.3. Platelet aggregation inhibition

All new Ap₄A analogs (compounds **2-16**) inhibited ADP-stimulated human platelet shape change (by optical aggregometry, data not shown) and both primary and secondary platelet aggregation (Fig. 3). We studied the concentration effect of this inhibition using the 96-well microplate method [51]. This is a simple, rapid, low volume method to simultaneously measure platelet aggregation of multiple samples, thereby avoiding the problem of platelet aging and inter-patient variability. Typical antagonist concentration – optical transmission curves for different concentrations of compound **5** are presented in Fig. 3.

To compare the ability of different Ap₄A derivatives to rapidly and competitively inhibit human platelet activation by ADP, in one set of experiments platelets were exposed to a

mixture of ADP (3 μM) and different concentrations of each Ap_4A derivative (i.e. platelets were not pre-incubated with the drugs). In other set of experiments, platelets were exposed to the Ap_4A derivatives for 15 min to achieve steady-state inhibition, and then challenged with 5 or 20 μM ADP. The corresponding IC_{50} s for each set of experiments are summarized in Table 1. Typical antagonist concentration – aggregation inhibition curves are presented in Fig. 4.

Introduction of small thioalkyl substituents at position 2 of the base significantly increased platelet aggregation inhibition activity, and the effect gradually diminished with the increase of the length of the alkyl substituent, the optimal size being between methyl and ethyl. A halogen substituent at this position also increased the inhibitory activity in the order $\text{I} > \text{Br} > \text{Cl}$. Contrarily, N^6 -methyl and N^6 -propyl substituents significantly reduced the inhibitory activity. Similarly to our previous finding for base unsubstituted Ap_4A analogs [24], introduction of P^1, P^4 -dithio, and P^2, P^3 -monochloromethylene substitutions on the polyphosphate chain of the base substituted (2-MeS) analog (compounds **3**, **4**, and **5**) improved the inhibitory activity, albeit the effect of both substituents was not clearly additive. In contrast to the monochloromethylene group, the P^2, P^3 -dichloromethylene group decreased the antagonist activity (2-MeS base substitution, compound **16** vs. compounds **3** and **5**). These observations clearly differ from the effect of analogous modifications in the ATP scaffold on platelet aggregation inhibition, where bulky alkyl substituents at C2 and N^6 of the adenine base and the P^2, P^3 -dichloromethylene group were well tolerated and resulted in significant increase of activity, indicating a distinct mode of interaction of Ap_4A analogs with their respective platelet targets.

2.4. Effect on platelet purinergic receptors

2.4.1. Effect on P2Y_1 —The effect of the new Ap_4A analogs on human platelet P2Y_1 receptors was determined by measuring the P2Y_1 -mediated cytosolic Ca^{2+} increase from intraplatelet stores after stimulation with ADP (3 μM) as previously described [24]. All of the compounds reduced the effect of ADP on the cytosolic Ca^{2+} , i.e. inhibited P2Y_1 . None of the compounds caused increase of the cytosolic Ca^{2+} in absence of ADP, i.e. none showed agonist effect toward P2Y_1 . Typical concentration/inhibition effect curves are presented in Fig. 5A, and the corresponding IC_{50} s are listed in Table 2. The 2-methylthio base substitution increased the antagonist properties of Ap_4A , from 32.5 μM (IC_{50} of Ap_4A [24]) to 4.9 μM (IC_{50} of the di-2-MeS analog of Ap_4A , compound **2**, Table 2). The same effect of the 2-MeS group was observed for the analogs with modifications in the polyphosphate chain. For instance, the P^1, P^4 -dithio- P^2, P^3 -monochloromethylene derivative (**1**, base unsubstituted) had IC_{50} of 10.2 μM [24] and compound **5** (2-MeS base substitution) had IC_{50} of 2.15 μM . Further increase of the size of the thioalkyl group appeared to reduce the inhibitory potency, yet the trend was not clearly defined, and the di-*n*-pentylthio derivative (**9**) had IC_{50} of $4.21 \pm 1.98 \mu\text{M}$. The N^6 -methyl substitution (**13**) also resulted in an increase in the P2Y_1 inhibitory properties, with IC_{50} of 1.97 μM , but this effect decreased with the increase of the alkyl chain size to *n*-propyl (**14**, 8.8 μM). The positive effect of 2-MeS and N^6 -Me groups on P2Y_1 inhibition was additive (compound **15**, IC_{50} of 0.36 μM). The effect of the polyphosphate chain substitutions follows previously observed effects on the base-unsubstituted Ap_4A analogs [24]: the P^1, P^4 -dithio substitution significantly

increased P2Y₁ inhibition, and the P²,P³-monochloromethylene substitution decreased it (compound **2** vs. compounds **3**, and **4**). Interestingly, the P²,P³-dichloromethylene group appeared to be better tolerated than the monochloromethylene (**5** vs. **16**).

2.4.2. Effect on P2Y₁₂—The effect of Ap₄A analogs on human platelet P2Y₁₂ receptors was determined by measuring P2Y₁₂-mediated decrease in intraplatelet phosphorylated vasodilator stimulated phosphoprotein (VASP) [52]. VASP phosphorylation was measured by flow cytometry using a kit (BioCytex, Marseilles, France) after P2Y₁₂ activation with 3 μM ADP. None of the test compounds caused decrease of VASP phosphorylation in absence of ADP, i.e. none showed agonist effect toward P2Y₁₂, and all of them reduced the effect of ADP on VASP phosphorylation, i.e. showed antagonist effects. Typical concentration-inhibition curves are presented in Fig. 5B, and the corresponding IC₅₀s are listed in Table 2. Methylthio substitution at C2 of the base had a significant effect on inhibitory activity: while Ap₄A is only a partial antagonist of P2Y₁₂ with a IC₅₀ above 250 μM [25], compound **2** (2-MeS Ap₄A analog) was a full antagonist with IC₅₀ of 1.4 μM. In contrast to the ATP scaffold, increase of the alkyl chain above ethyl did not result in increase of the inhibitory potency. Also, in contrast with the ATP scaffold, the N⁶-alkyl substituents were poorly tolerated, and both N⁶-methyl and N⁶-propyl substitutions (compounds **13** and **14**) significantly reduced the inhibitory activity. Interestingly, introduction of a 2-MeS group together with N⁶-Me group almost completely abolished the negative effect of the latter, and resulted in relatively potent P2Y₁₂ inhibitor (compound **15**). Introduction of a monochloromethylene group between P² and P³ had a significantly increased inhibitory activity (**4** vs **2** and **5** vs. **3**, Table 2). The dichloromethylene group between P² and P³ was less effective than the monochloromethylene group (compound **16** vs. compound **5**).

2.4.3. Effect on P2X₁—Ap₄A is an effective P2X₁ receptor agonist [25]. Since P2X₁ plays a role in the process of platelet activation [53] and in vasoconstriction [54], such an agonist effect would be undesirable for potential antithrombotic agents. Therefore, we tested the effect of the new base substituted Ap₄A analogs on the activation of platelet P2X₁ receptors. Because P2X₁ is an ion channel whose activation causes an influx of extracellular Ca²⁺, this was done by flow cytometric measurement of the cytosolic Ca²⁺ increase after P2X₁ stimulation. At the same time control experiments with a selective P2Y₁ inhibitor were done to control for possible P2Y₁ mediated Ca²⁺ increase. The effects of the test compounds were compared with that of 20 μM of a standard stable P2X₁ agonist, P²,P³-methyleneATP, and are presented as percent of that of P²,P³-methyleneATP in Table 2. Methylthio substitution at C2 decreased the agonist properties (198% for 1 μM of Ap₄A [24] vs. 48% for compound **2**). P¹,P²-Dithio, and especially P²,P³-monochloromethylene substitutions reduced the Ap₄A scaffold agonist properties toward P2X₁, and compound **5** was practically devoid of agonist activity. This activity re-appeared, however, with further increase of the alkyl chain length. The 2-halo, and N⁶-alkyl substitutions effectively reduced the P2X₁ agonist activity. Interestingly, the doubly base substituted 2-MeS and N⁶-Me analog (compound **15**) was an effective P2X₁ agonist.

Examining the data in Tables 1 and 2 shows that human platelet aggregation is inhibited with IC₅₀s lower than the IC₅₀s for inhibition of both P2Y₁ and P2Y₁₂. A synergistic effect

from the simultaneous inhibition of both ADP receptors was reported [23] after studying the combined use of selective P2Y₁ and P2Y₁₂ receptors antagonists on platelet aggregation, and can be explained by the complex interplay between the two receptors and the signaling pathways they trigger [11,56]. We still cannot, however, completely exclude the contribution of additional, yet unknown, mechanism(s) of ADP induced platelet aggregation inhibition by the test compounds.

2.5. Inhibitor pre-incubation effect and reversibility of platelet aggregation inhibition

Comparing the IC₅₀ data from Table 1 for ADP induced human platelet aggregation inhibition in which the inhibitor is pre-mixed with the activator (3 μM ADP) with that in which the inhibitor is added to the platelets 15 min before their activation (5 μM ADP) reveals that in many cases, but not all, the IC₅₀s in the second case are lower, even though the platelets are activated at higher ADP concentration, indicating that pre-incubation of the platelets with the inhibitor contributes, in some cases, to its activity. To shed light on this phenomenon, in a separate experiments we examined by optical aggregometry the inhibition of ADP induced human platelet aggregation by three of the compounds (**5**, **6**, and **11**) at 20 μM ADP concentration. In Fig. 6 are presented the corresponding concentration-inhibition curves and IC₅₀s for an experiment without pre-incubation (panel A) and with 15 min pre-incubation of the platelets with the inhibitor (panel B). While the pre-incubation of the platelets with compounds **5** and **6** (2-MeS, and 2-EtS substitutions, resp.) resulted in an almost 50-fold decrease of their IC₅₀s (from 2.56 μM to 63.6 nM for **5**, and from 3.32 μM to 54.3 nM for **6**), it had little effect with compound **11** (2-Br substitution, 6.22 μM to 4.52 μM).

In a separate experiment we tested the effect of the varied pre-incubation times (0, 5 min, 15 min) on the aggregation inhibition of compound **5** (at 25 and 100 nM concentration), and found that full pre-incubation effect develops within 15 min of pre-incubation (Data not shown).

In order to confirm that this effect was not due to irreversible receptor inhibition, we tested the reversibility of platelet aggregation inhibition after their exposure to compound **5**. Expression of the activation markers GPIIb-IIIa and P-selectin on the surface of human platelets was used to determine the reversibility of their activation by 20 μM ADP. Compound **5** inhibited the expression of both markers with remarkable potency (IC₅₀s of 1.1 nM for GPIIb-IIIa, and 0.6 nM for P-selectin, Fig. 7A), especially considering the high activator (ADP) concentration used in this experiment. Figure 7B shows that, after pre-incubation with **5** and dilution (100x) with platelet poor plasma, the platelets recovered ca. 80–90% of their ability to express the activation markers after stimulation indicating that the inhibitory effect of **5**, and presumably the rest of the series, is reversible.

2.6. Platelet aggregation inhibition by individual stereoisomers of compound **5**

Thio substitution at P¹ and P⁴ renders the corresponding phosphorous atoms chiral. Therefore, compounds **3** and **16** exist as 3 stereoisomers with absolute configurations at P¹ and P⁴ phosphorous atoms R_PR_P, S_PS_P, and R_PS_P = S_PR_P (the subscript “P” indicates that this absolute configuration applies to an asymmetric phosphorous and not to a carbon atom).

Those three stereoisomers are diastereomeric to each other because of the chiral character of the ribose moiety. The stereochemical character of the P²,P³-monochloromethylene group in compounds **1** and **5-15** depends on the absolute configuration at P¹ and P⁴. When P¹ and P⁴ have the same configuration (R_pR_p, or S_pS_p) the carbon atom of the chloromethylene group has two identical substituents, and is not asymmetric. However, when those two atoms are in different absolute configurations (R_pS_p = S_pR_p,) the carbon atom of the monochloromethylene group becomes pseudo-asymmetric and can exist in two absolute configurations designated with r and s (the prefix “pseudo” indicates that this carbon contains two substituents that differ only in their stereo-configuration, and lower case r and s, instead of upper case R and S are used for the same reason) [60]. Therefore compounds **1** and **5-15** can have 4 different stereoisomers with configurations R_pR_p, S_pS_p, R_prS_p = S_prR_p, and R_psS_p = S_psR_p. In the past we isolated the four diastereomers of base unsubstituted compound **1** and found a statistically significant difference in their inhibition of ADP induced human platelet aggregation [61]. Using the same technique (preparative reverse phase HPLC) we separated the four diastereomers of compound **5** (Fig. 8) and studied their individual inhibition of ADP induced human platelet aggregation by optical aggregometry. The concentration dependence of the aggregation inhibition for each diastereomer is plotted in Fig. 9, and the corresponding IC₅₀s are listed in Table 3. There was no statistical significant difference in the IC₅₀ values of the individual diastereomers and the diastereomeric mixture (compound **5**). One possible explanation of this difference between compounds **1** and **5** is an increased contribution of the substituted base to the inhibitor–receptor interaction, with corresponding reduction of the relative contribution of the polyphosphate chain.

2.7. Stability and metabolism in plasma

Unmodified dinucleoside polyphosphates are quickly hydrolyzed by plasma ecto-nucleotide pyrophosphatase/phosphodiesterase activity (NPP1, PC-1; NPP2, autotaxin; NPP3, Gp130) [62]. For instance, Ap₄A is degraded in human plasma and whole blood with half-lives of 2.0 and 4.4 min, respectively [63]. The hydrolysis is non-symmetric between P¹ and P² (or P³ and P⁴), generating one molecule of mono- and one molecule of triphosphate [63,64]. Modification of P¹ and P⁴ phosphate groups to thiophosphates significantly increases the plasma stability of dinucleoside tetraphosphates [35]. For instance, in rat plasma, Ap₄A had a half-life of 2.99 min, whereas its P¹,P⁴-dithio analog had a half-life of 154 min [65]. While the P²,P³-chloromethylene substitution did not have a significant impact on plasma stability (the half-life of P²,P³-chloromethylene Ap₄A analog was 6.29 min) [65], it significantly improved the chemical stability. The combination of P¹,P⁴-dithio, and P²,P³-chloromethylene modifications on Ap₄A resulted in derivatives that had remarkable enzymatic and chemical stability in plasma [35].

By using very long incubation times we were able to observe enzymatic degradation of compound **5** in rat and human plasmas (the absence of chemical degradation was confirmed by control incubations with heat denaturated plasma). A representative chromatogram of HPLC analysis after incubation in human plasma for 77 h at 37 °C is shown in Fig. 10. The four diastereomers of **5** (D1–D4) are indicated on the chromatogram. Also indicated in the chromatogram are the two detected metabolites (2-methylthioadenosine 5'-

thiomonophosphate, MeSAMP(S) and 2-methylthioadenosine, MeSAdo), which were identified by analysis of synthetic reference compounds, and confirmed by LCMS analysis of representative incubation mixtures. The internally normalized peak areas of the four diastereomers of **5** (by the order of their elution, D1, D2, D3, and D4) and the two detected metabolites (MeSAMP(S) was not detected in human plasma) are plotted in Fig. 11 panel A (rat plasma) and panel B (human plasma).

The kinetics of degradation of **5** in both plasmas did not follow 1st order, most likely due to loss of plasma activity during the long incubation times. Indeed, in a control experiment the rate of degradation of **5** in rat plasma which was aged for 24 h at 37 °C was the same as in fresh plasma after 24 h incubation with the test compound (data not shown). The first three time points (0, 1 and 4 hours) were used to estimate the initial first order elimination constants and the associated half-lives (by non-linear regression using GraphPad Prism) and the results are presented in Table 4.

Compound **5** was degraded much faster in rat than in human plasma. Also the stability of the individual diastereomers was markedly different – D1 being most and D4 least stable. D2 and D3 had close and intermediate stability. This is in agreement with our previous studies [61] of the plasma stability of the individual diastereomers of the base-unsubstituted compound **1**, and confirms our previous conclusion that phosphorothioates in the S_p absolute configuration resist hydrolysis by plasma pyrophosphatase/phospho-diesterases. The final metabolite in both rat and human plasma was 2-methylthioadenosine. Also, the intermediate 2-methylthioadenosine 5-monothiophosphate was observed, albeit at lower levels (it was below the limit of quantification in human plasma). The intermediate triphosphate analog was not detected.

3. CONCLUSION

In conclusion, we have prepared for the first time a series of base-substituted Ap₄A analogs (with and without polyphosphate chain substitution) and studied their platelet related properties. We used this information to establish structure-activity relations for platelet aggregation and purinoreceptor inhibition by this class of compounds. Some of the compounds showed very potent (nanomolar level) inhibition of ADP induced human platelet aggregation (Table 1) and simultaneous inhibition of both P2Y₁ and P2Y₁₂ receptors (Table 2). Compound **5** reversibly inhibited ADP-stimulated expression of platelet activation markers GPIIb-IIIa and P-selectin with remarkably low IC₅₀ values of 1.1 and 0.6 nM, respectively. Also this compound did not have an effect on human platelet P2X₁ receptors (Table 2) and on human P2Y₂, P2Y₄, and P2Y₆ receptors (data not shown). Simultaneous targeting of platelet P2Y₁ and P2Y₁₂ receptors might have a synergistic effect, and may provide an antithrombotic drug with superior efficacy and safety profiles. The only rapidly reversible, injectable antiplatelet drug currently available for clinical use in patients who are in need of immediate antiplatelet therapy and may develop bleeding complication, or be in need of urgent surgical intervention is the ATP derivative cangrelor. Taking into account the rapid elimination from circulation of the nucleoside polyphosphates, the present class of compounds may serve as a basis for the development of antiplatelet agents with rapid onset and offset of the pharmacological effect, and with a unique mechanism of action to fill an

important unmet clinical need. Compound **5** is currently undergoing preclinical testing for this purpose.

4. EXPERIMENTAL SECTION

4.1. Chemistry

All solvents and reagents used were obtained commercially and used as such unless noted otherwise. ^1H and ^{31}P NMR spectra were recorded in CDCl_3 , $\text{DMSO}-d_6$, or D_2O solutions at 298 °K using a Bruker Avance 300 instrument. Chemical shifts are reported as parts per million (ppm) relative to tetramethylsilane (TMS) for ^1H and phosphoric acid for ^{31}P NMR. All ^{31}P spectra were proton decoupled unless otherwise noted. Spin multiplicities are given as s (singlet), br s (broad singlet), ms (multiple singlets), d (doublet), t (triplet), q (quartet), or m (multiplet). All chemical reactions and the purity of the intermediates were analyzed using TLC (silica gel 60 F₂₅₄ plates, UV or I₂ visualization) and LCMS (ThermoFinnigan ICQ Advantage with Surveyor LC system) with a XBridge C18 3.5 μm 2 $\times$ 50 mm column and a gradient from 0.1 to 100% acetonitrile in 0.1% formic acid (unless otherwise indicated in procedure) at a flow rate of 0.2 ml/min. Detection was at 210–420 nm for UV and electrospray ionization in the positive and the negative ionization modes (ESI+, ESI–) for MS. Preparative silica gel chromatography was performed using an ISCO Flash chromatography system and RediSep flash cartridges (particle size: 35–70 μm , Teledyne ISCO) or PuriFlash cartridges (particle size: 50 μm , spherical, Interchim). Preparative reverse phase HPLC was carried out using Waters XBridge C18 5 μm OBD 19 $\times$ 250 mm column on a Varian ProStar instrument. All nucleotides were purified by preparative ion-exchange HPLC using a Varian Load&Lock 50 $\times$ 300 mm column packed with TSKgel SuperQ-5PW (20 μm , Tosoh). The elution was carried out with a gradient from 20 mM to 500 mM (monophosphates), or from 20 mM to 2 M (tetraphosphates) triethylammonium bicarbonate (TEAB, pH 8) containing 10% acetonitrile (MeCN) for 180 min, at a flow rate of 35 ml/min. TEAB was prepared by saturation of water/triethylamine mixture with carbon dioxide. All final compounds (**1**–**16**) were determined to be greater than 95% pure via analysis by ion-exchange (IE) HPLC on a DNAPac PA200 4 $\times$ 250 mm column (Dionex) eluted with a gradient from 10% MeCN in water to 1 M ammonium bicarbonate and 2% MeCN in water for 15 min at a flow rate of 1 ml/min and UV detection at 260 nm (273 nm for the 2-alkylthio analogs), and by reverse phase (RP) HPLC on a XBridge C18 3.5 μm 2 $\times$ 50 mm column, eluted with a gradient of MeCN in 20 mM TEAB at a flow rate of 0.2 ml/min, and UV detection (190–420 nm). The exact gradient parameters are indicated in each procedure.

Compounds **3** and **16** exist as mixtures of 3 diastereomers, and compounds **5**–**15** as mixtures of 4 diastereoisomers. Where those diastereomers are separated the individual retention times are given. 2-Thioadenosine [66], 2-bromoadenosine [43], (monochloromethylene)bisphosphonic acid [67], the P¹,P²-diimidazolidine of (monochloromethylene)bisphosphonic acid [39], and 2-methylthioadenosine 5'-monophosphate [68] were prepared as previously described (See Supporting Information for details). The procedures for synthesis of all other nucleoside, and nucleoside monophosphate analogs, and their characterization information are presented in the Supporting Information.

4.1.1. General procedure for synthesis of modified P¹,P⁴-di-(5'-adenosine) tetraphosphates, sodium salts (compounds 2-15)—

Bis-triethylammonium salt of modified adenosine 5'-monophosphate or 5'-monothiophosphate (0.30 mmol) was dissolved in anhydrous DMF (5 ml), and the solvent was evaporated under vacuum (0.2 mm Hg) at 35 °C to produce a foam. The residue was dissolved in anhydrous DMF (5 ml) under Ar. Disodium di-(1-imidazolyl)pyrophosphate or disodium di-(1-imidazolyl)chloromethylene-*bis*-phosphonate [39] (0.1 mmol) was added to this solution, followed by anhydrous zinc chloride (408.9 mg, 3.0 mmol). The mixture was stirred protected from moisture for 30 min, and then added to a stirred mixture of Chelex® 100 resin in the sodium form (10 ml; Sigma-Aldrich) and 0.1 M triethylammonium bicarbonate buffer (20 ml, pH 8). After stirring for 15 min the mixture was filtered, and the resin was washed twice with water (10 ml each). The combined filtrate and washings were loaded on a preparative ion-exchange HPLC column (5 × 30 cm, Load&Lock®, Varian) packed with SuperQ-5PW resin (Tosoh Inc.) in the triethylammonium form, which was pre-equilibrated with 2 column volumes of 0.02 M TEAB buffer, pH 8, containing 10% (v/v) MeCN. A linear gradient of TEAB containing 10% (v/v) MeCN, from 0.02 to 2 M for 180 min, was passed through the column at a rate of 35 ml/min. The fractions containing the product were combined and evaporated under vacuum. The residue was re-evaporated three times from methanol (50 ml each), dissolved in methanol (0.5 ml), and mixed with a 2.0 M solution of sodium perchlorate in acetone (5 ml). The mixture was diluted with acetone (15 ml) and stirred for 2 h. The colorless solid was collected by centrifugation, and washed by suspending in acetone (20 ml), centrifugation, and decanting. This acetone washing was repeated two more times, and the colorless solid was dried first under a stream of nitrogen, and then for 6 h under high vacuum.

4.1.2. P¹,P⁴-di-(2-Methylthio-5'-adenosine) tetraphosphate, sodium salt (2)—

Prepared by the above procedure from 2-methylthioadenosine 5'-monophosphate bis-triethylammonium salt (179 mg, 0.30 mmol) and disodium di-(1-imidazolyl)pyrophosphate (32.2 mg, 0.1 mmol). Yield: 68 mg, 67%; ¹H NMR (300 MHz, D₂O) δ: 8.15 (s, 2H, H-8), 5.94 (d, J = 5.8 Hz, 2H, H-1'), 4.72 (m, partially overlaps with solvent peak, H-2'), 4.49 (dd, J₁ = 3.7 Hz, J₂ = 5.0 Hz, 2H), 4.29 (m, 2H, H-3'), 4.20 (m, 4H, H-5',5''), 2.46 (s, 6H, SCH₃); ³¹P NMR (121 MHz, D₂O) δ: -11.2 (m, P¹+P⁴), -22.6 (m, P²+P³); MS (ESI-), observed, m/z: 927.2 (100.0%), 928.1 (26.3%), 929.1 (15.9%), 930.1 (3.9%), 931.1 (0.9%); calculated for [M-H]⁻, C₂₂H₃₁N₁₀O₁₉P₄S₂⁻, m/z: 927.0 (100.0%), 928.0 (30.2%), 929.0 (17.4%), 930.0 (4.2%), 931.0 (1.2%); purity 97.8%, RP HPLC RT: 9.30 min (gradient from 0 to 30% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 8.8 min.

4.1.3. P¹,P⁴-di-(2-methylthio-5'-adenosine) P¹,P⁴-dithiotetraphosphate, sodium salt (3)—Prepared by the above procedure from 2-methylthioadenosine 5'-monothiophosphate bis-triethylammonium salt (183 mg, 0.30 mmol) and disodium di-(1-imidazolyl)pyrophosphate (32.2 mg, 0.1 mmol). This compound was isolated as a mixture of 3 diastereomers in ratio 1:2:1 due to the different stereo configurations at P¹ and P⁴. Yield: 66 mg, 63%; ¹H NMR (300 MHz, D₂O): δ 8.24 (s, 0.5H, diast. 1 H-8), 8.19 (s, 0.5H, diast. 2 H-8), 8.18 (s, 1H, diast. 3 H-8), 5.90 (d, 2H, J = 5.8 Hz, H-1'), 4.77 – 4.64 (m, 2H, H-2'),

4.52 – 4.44 (m, 2H, H-3'), 4.30 – 4.24 (m, 2H, H-4'), 4.24 – 4.15 (m, 4H, H-5'), 2.445 (s, 3H, diast. 3 SCH₃), 2.435 (s, 1.5H, diast. 1 or 2 SCH₃), 2.427 (s, 1.5H, diast. 2 or 1 SCH₃); ³¹P NMR (121 MHz, D₂O), ppm: 43.53 – 42.94 (m, P¹+P⁴), –23.93 – –24.39 (m, P²+P³); MS (ESI[–]), observed, m/z: 958.9 (100.0), 959.9 (27.3), 960.9 (23.9), 961.9 (6.6), 962.9 (2.2); calculated for [M–H][–], C₂₂H₃₁N₁₀O₁₇P₄S₄[–], m/z: 959.0 (100.0%), 960.0 (31.7%), 961.0 (26.4%), 962.0 (7.2%), 963.0 (3.0%); purity 97.1%, RT RP HPLC: 9.75, 9.95, 10.41 min (gradient from 0 to 30% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 9.1 min.

4.1.4. P¹,P⁴-di-(2-methylthio-5'-adenosine) P²,P³-

chloromethylenetetraphosphate, sodium salt (4)—Prepared by the above procedure from 2-methylthioadenosine 5'-monophosphate bis-triethylammonium salt (179 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 83 mg, 79%; ¹H NMR (300 MHz, D₂O): δ 8.14, 8.12 (2H, s, H-8), 5.95 (2H, d, *J* = 5.5 Hz, H-1'), 4.73 – 4.67 (2H, m, H-2'), 4.51 – 4.47 (2H, m, H-3'), 4.28 – 4.23 (2H, m, H-4'), 4.18 (1H, t, ²*J*_{P–H} = 15.0 Hz, CHCl), 4.21 – 4.09 (4H, m, H-5'), 2.449, 2.445 (6H, s, SCH₃). ³¹P NMR (121 MHz, D₂O), ppm: 2.30 – 1.72 (m, P²+P³), –10.55 – –11.05 (m, P¹+P⁴); MS (ESI[–]), observed, m/z: 959.2 (100.0%), 960.2 (33.1%), 961.1 (50.5%), 962.1 (13.5%), 963.1 (6.6%), 964.1 (1.4%); calculated for [M–H][–], C₂₃H₃₂ClN₁₀O₁₈P₄S₂[–], m/z: 959.0 (100.0%), 960.0 (31.2%), 961.0 (49.4%), 962.0 (14.3%), 963.0 (6.8%), 964.0 (1.6%); purity 97.4%, RT RP HPLC: 9.47 min (gradient from 0 to 30% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 11.85, 12.12, 12.38 min.

4.1.5. P¹,P⁴-di-(2-methylthio-5'-adenosine) P¹,P⁴-dithio-P²,P³-

chloromethylenetetraphosphate, sodium salt (5)—Prepared by the above procedure from 2-methylthioadenosine 5'-monothiophosphate bis-triethylammonium salt (183 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 88 mg, 81%; ¹H NMR (300 MHz, D₂O) δ 8.35 – 8.11 (ms, 2H, H-8), 6.09 – 5.94 (m, 2H, H-1'), 4.90 – 4.35 (1H, multiple t, CHCl), 4.82 – 4.70 (m, 2H, H-2'), 4.60 – 4.50 (m, 2H, H-3'), 4.37 – 4.13 (m, 6H, H-4'+H'5'), 2.53 – 2.47 (ms, 6H, SCH₃). ³¹P NMR (121 MHz, D₂O), ppm: 43.15 – 42.02 (m, P¹+P⁴), 2.00 – 1.22 (m, P²+P³); MS (ESI[–]), observed, m/z: 990.9 (100.0%), 991.9 (29.5%), 992.9 (56.7%), 993.9 (16.6%), 994.8, (10.0%); calculated for [M–H][–], C₂₃H₃₂ClN₁₀O₁₆P₄S₄, m/z: 990.95 (100.0%), 991.96 (32.8%), 992.95 (57.5%), 993.95 (17.1%), 994.94 (11.5%); purity 99.2%, RT RP HPLC: 3.55, 4.07, 4.88, 5.58 min (gradient from 0 to 12% MeCN in 20 mM triethylammonium acetate for 12 min); IE HPLC: 8.9, 9.1 min.

4.1.6. P¹,P⁴-di-(2-ethylthio-5'-adenosine) P¹,P⁴-dithio-P²,P³-

chloromethylenetetraphosphate, sodium salt (6)—Prepared by the above procedure from 2-ethylthioadenosine 5'-monothiophosphate bis-triethylammonium salt (188 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 60 mg, 54%; ¹H NMR (300 MHz, D₂O) δ 8.15 – 8.02 (multiple s, 2H, H-8), 5.88 – 5.83 (m, 2H, H-1'), 4.78 – 4.35 (1H, multiple t, CHCl), 4.67 – 4.55 (m, 2H, H-2'), 4.42 – 4.35 (m, 2H, H-3'), 4.22 – 4.13 (m, 2H, H-4'), 4.13 – 3.98 (m, 4H, H-5'), 3.00 – 2.86 (m, 4H, SCH₂), 1.21 – 1.12 (mt, 6H, CH₃); ³¹P NMR (121 MHz, D₂O), ppm: 44.32 – 42.78

(m, P¹+P⁴), 2.79 – 2.12 (m, P²+P³); MS (ESI[−]), observed, m/z: 1019.1 (100%), 1020.1 (38.0%), 1021.0 (53.8%), 1022.0 (19.5%), 1022.9 (11.8%), 1023.9 (3.1%); calculated for [M−H][−], C₂₅H₃₆ClN₁₀O₁₆P₄S₄[−], m/z: 1018.98 (100.0%), 1019.99 (33.0%), 1020.98 (59.3%), 1021.98 (19.1%), 1022.98 (12.0%), 1023.98 (2.3%); purity 99.2%, RT RP HPLC: 11.05, 11.98 min (gradient from 0 to 30% MeCN in 20 mM triethylammonium acetate for 15 min); IE HPLC: 8.4, 8.7 min.

4.1.7. P¹,P⁴-di-(2-propylthio-5'-adenosine) P¹,P⁴-dithio-P²,P³-

chloromethylenetetraphosphate, sodium salt (7)—Prepared by the above procedure from 2-propylthioadenosine 5'-monothiophosphate bis-triethylammonium salt (192 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 80 mg, 70%; ¹H NMR (300 MHz, D₂O) δ 8.29 – 7.95 (ms, 2H, H-8), 5.89 – 5.82 (m, 2H, H-1'), 4.81 – 4.31 (1H, multiple t, CH-Cl), 4.67 – 4.55 (m, 2H, H-2'), 4.43 – 4.33 (m, 2H, H-3'), 4.24 – 4.16 (m, 2H, H-4'), 4.16 – 4.00 (m, 4H, H-5'), 2.98 – 2.81 (m, 4H, SCH₂), 1.63 – 1.42 (m, 4H, SCH₂CH₂), 0.87 – 0.78 (multiple t, 6H, CH₃); ³¹P NMR (121 MHz, D₂O), ppm: 43.8 – 42.5 (m, P¹+P⁴), 3.1 – 1.8 (m, P²+P³); MS (ESI[−]), observed, m/z: 1047.0 (100%), 1048.0 (36.7%), 1048.9 (55.3%), 1049.9, (18.4%), 1050.9, (10.8%), 1051.9 (4.6%); calculated for [M−H][−], C₂₇H₄₀ClN₁₀O₁₆P₄S₄[−], m/z: 1047.0 (100.0%), 1048.0 (37.2%), 1049.0 (60.0%), 1050.0 (20.5%), 1051.0 (12.4%), 1052.0 (3.6%); purity, 98.6%, RT RP HPLC: 9.99, 10:25 min (gradient from 0 to 50% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 8.4, 8.6 min.

4.1.8. P¹,P⁴-di-(2-(3,3,3-trifluoropropylthio)-5'-adenosine) P¹,P⁴-dithio-P²,P³-

chloromethylenetetraphosphate, sodium salt (8)—Prepared by the above procedure from 2-(3,3,3-trifluoropropylthio)adenosine 5'-monothiophosphate bis-triethylammonium salt (208 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloromethylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 76 mg, 61%; ¹H NMR (300 MHz, D₂O) δ 8.28 – 8.10 (ms, 2H, H-8), 5.98 – 5.91 (m, 2H, H-1'), 4.95 – 4.40 (1H, multiple t, CHCl), 4.67 – 4.60 (m, 2H, H-2'), 4.53 – 4.43 (m, 2H, H-3'), 4.33 – 4.25 (m, 2H, H-4'), 4.25 – 4.11 (m, 4H, H-5'), 3.24 – 3.12 (m, 4H, CF₃CH₂), 2.70 – 2.50 (m, 4H, SCH₂); ³¹P NMR (121 MHz, D₂O), ppm: 44.2 – 42.6 (m, P¹+P⁴), 3.0 – 2.1 (m, P²+P³); MS (ESI[−]), observed, m/z: 1155.1 (100%), 1156.1 (35.2%), 1157.0 (54.9%), 1158.0 (18.4%), 1159.0 (10.2%), 1159.9 (3.2%); calculated for [M−H][−], C₂₇H₃₄ClF₆N₁₀O₁₆P₄S₄[−], m/z: 1155.0 (100.0%), 1156.0 (37.1%), 1157.0 (60.0%), 1158.0 (20.4%), 1159.0 (11.1%), 1160.0 (3.3%); purity, 96.1%, RT RP HPLC: 11.59, 11.87 min (gradient from 0 to 50% MeCN in 20 mM triethylammonium acetate for 15 min); IE HPLC: 8.2, 8.5 min.

4.1.9. P¹,P⁴-di-(2-pentylthio-5'-adenosine) P¹,P⁴-dithio-P²,P³-

chloromethylenetetraphosphate, sodium salt (9)—Prepared by the above procedure from 2-pentylthioadenosine 5'-monothiophosphate bis-triethylammonium salt (200 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 75 mg, 64%; ¹H NMR (300 MHz, D₂O) δ 8.26 – 8.12 (multiple s, 2H, H-8), 5.98 – 5.93 (m, 2H, H-1'), 4.89 – 4.39 (1H, multiple t, CH-Cl), 4.73 – 4.64 (m, 2H, H-2'), 4.52 – 4.45 (m, 2H, H-3'), 4.32 – 4.23 (m, 2H, H-4'), 4.23 – 4.11 (m, 4H, H-5'), 3.03 – 2.92 (m, 4H, SCH₂), 1.63 – 1.50 (m, 4H, SCH₂CH₂), 1.34 – 1.12 (m, 8H, CH₂CH₂CH₃), 0.82 –

0.72 (m, 6H, CH₃); ³¹P NMR (121 MHz, D₂O), ppm: 44.05 – 42.83 (m, P¹+P⁴), 2.96 – 2.13 (m, P²+P³); MS (ESI[−]), observed, m/z: 1103.3 (100%), 1104.3 (43.1%), 1105.1 (61.8%), 1106.1 (21.8%), 1107.0 (11.0%), 1108.0 (3.2%); calculated for [M–H][−], C₃₁H₄₈ClN₁₀O₁₆P₄S₄[−], m/z: 1103.1 (100.0%), 1104.1 (41.6%), 1105.1 (61.8%), 1106.1 (23.1%), 1107.1 (13.3%), 1108.1 (4.1%); purity, 95.2%, RT RP HPLC: 12.08, 12.50, 12.67 min (gradient from 0 to 50% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 8.4, 8.6 min.

4.1.10. P¹,P⁴-di-(2-chloro-5'-adenosine) P¹,P⁴-dithio-P²,P³-

chloromethylenetetra-phosphate, sodium salt (10)—Prepared by the above procedure from 2-chloroadenosine 5'-monothiophosphate bis-triethylammonium salt (180 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 71.5 mg, 69%; ¹H NMR (300 MHz, D₂O) δ 8.39 – 8.26 (multiple s, 2H, H-8), 5.91 – 5.86 (m, 2H, H-1'), 4.90 – 4.39 (1H, multiple t, CH-Cl), 4.70 – 4.61 (m, 2H, H-2'), 4.53 – 4.46 (m, 2H, H-3'), 4.36 – 4.27 (m, 2H, H-4'), 4.27 – 4.11 (m, 4H, H-5'); ³¹P NMR (121 MHz, D₂O), ppm: 43.75 – 42.42 (m, P¹+P⁴), 2.63 – 1.95 (m, P²+P³); MS (ESI[−]), observed, m/z: 967.0 (92.2%), 968.0 (27.0%), 969.0 (100.0%), 970.0 (28.9%), 971.0 (42.0%), 972.0 (10.3%), 973.0 (8.6%), 973.9 (1.8%); calculated for [M–H][−], C₂₁H₂₆Cl₃N₁₀O₁₆P₄S₂[−], m/z: 966.9 (89.1%), 967.9 (25.8%), 968.9 (100.0%), 969.9 (28.1%), 970.9 (42.3%), 971.9 (11.4%), 972.9 (8.3%), 973.9 (2.1%); purity, 99.3%, RT RP HPLC: 7.60, 7.88, 8.12, 8.33 min (gradient from 0 to 30% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 6.7, 6.9 min.

4.1.11. P¹,P⁴-di-(2-bromo-5'-adenosine) P¹,P⁴-dithio-P²,P³-

chloromethylenetetra-phosphate, sodium salt (11)—Prepared by the above procedure from 2-bromoadenosine 5'-monothiophosphate bis-triethylammonium salt (193 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 76.7 mg, 66%; ¹H NMR (300 MHz, D₂O) δ 8.48 – 8.33 (multiple s, 2H, H-8), 6.02 – 5.96 (m, 2H, H-1'), 5.01 – 4.50 (1H, multiple t, CH-Cl), 4.78 – 4.72 (m, 2H, H-2'), 4.63 – 4.57 (m, 2H, H-3'), 4.46 – 4.37 (m, 2H, H-4'), 4.37 – 4.22 (m, 4H, H-5'); ³¹P NMR (121 MHz, D₂O), ppm: 44.05 – 42.58 (m, P¹+P⁴), 2.92 – 2.02 (m, P²+P³); MS (ESI[−]), observed, m/z: 1055.1 (42.0%), 1056.2 (10.0%), 1057.0 (100.0%), 1058.0 (29.6%), 1058.9 (77.3%), 1059.9 (20.6%), 1060.8 (24.4%), 1061.8 (6.8%), 1062.8 (2.0%); calculated for [M–H][−], C₂₁H₂₆Br₂ClN₁₀O₁₆P₄S₂[−], m/z: 1054.8 (41.2%), 1055.8 (11.9%), 1056.8 (100.0%), 1057.8 (28.6%), 1058.8 (80.3%), 1059.8 (22.4%), 1060.8 (24.1%), 1061.8 (6.3%), 1062.8 (2.8%); purity, 97.8%, RT RP HPLC: 7.05, 7.29 min (gradient from 0 to 50% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 6.8, 7.1 min.

4.1.12. P¹,P⁴-di-(2-iodo-5'-adenosine) P¹,P⁴-dithio-P²,P³-chloromethylenetetra-phosphate, sodium salt (12)

—Prepared by the above procedure from 2-iodoadenosine 5'-monothiophosphate bis-triethylammonium salt (208 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 90.6 mg, 73%; ¹H NMR (300 MHz, D₂O) δ 8.38 – 8.16 (multiple s, 2H, H-8), 5.89 – 5.84 (m, 2H, H-1'), 4.93 – 4.39 (1H, multiple t, CH-Cl), 4.64 – 4.57 (m, 2H, H-2'), 4.52 – 4.45 (m, 2H,

H-3'), 4.34 – 4.27 (m, 2H, H-4'), 4.27 – 4.14 (m, 4H, H-5'); ^{31}P NMR (121 MHz, D_2O), ppm: 43.95 – 42.45 (m, P^1+P^4), 3.13 – 2.02 (m, P^2+P^3); MS (ESI^-), observed, m/z : 1151.0 (100.0%), 1152.0 (29.7%), 1152.9 (46.2%), 1153.9 (13.7%), 1154.9 (5.8%), 1155.9 (1.3, %); calculated for $[\text{M}-\text{H}]^-$, $\text{C}_{21}\text{H}_{26}\text{ClI}_2\text{N}_{10}\text{O}_{16}\text{P}_4\text{S}_2^-$, m/z : 1150.8 (100.0%), 1151.8 (28.9%), 1152.8 (48.3%), 1153.8 (13.1%), 1154.8 (6.3%), 1155.8 (1.4%); purity, 96.4%, RT RP HPLC: 9.32, 9.75, 9.95, 10.12 min (gradient from 0 to 30% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 6.8, 7.1 min.

4.1.13. P^1, P^4 -di-(N^6 -methyl-5'-adenosine) P^1, P^4 -dithio- P^2, P^3 -

chloromethylenetetraphosphate, sodium salt (13)—Prepared by the above procedure from N^6 -methyladenosine 5'-monothiophosphate bis-triethylammonium salt (174 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 67.1 mg, 66%; ^1H NMR (300 MHz, D_2O) δ 8.38 – 8.16 (2H, ms, H-8), 7.98 – 7.92 (ms, 2H, H-2), 5.98 – 5.91 (2H, m, H-1'), 5.03 – 4.42 (1H, mt, $\text{CH}-\text{Cl}$), 4.77 – 4.68 (2H, m, H-2'), 4.55 – 4.47 (2H, m, H-3'), 4.37 – 4.29 (2H, m, H-4'), 4.29 – 4.08 (4H, m, H-5'), 2.90 – 2.82 (6H, ms, NCH_3); ^{31}P NMR (121 MHz, D_2O), ppm: 43.97 – 42.43 (m, P^1+P^4), 3.03 – 1.92 (m, P^2+P^3); MS (ESI^-), observed, m/z : 927.1 (100.0%), 928.1 (31.9%), 929.1 (47.0%), 930.1 (19.3%), 931.0 (8.1%); calculated for $[\text{M}-\text{H}]^-$, $\text{C}_{23}\text{H}_{32}\text{ClN}_{10}\text{O}_{16}\text{P}_4\text{S}_2^-$, m/z : 927.0 (100.0%), 929.0 (49.0%), 928.0 (31.1%), 930.0 (14.2%), 931.0 (6.6%); purity, 99.2%, RT RP HPLC: 11.13, 11.63, 11.84, 12.28 min (gradient from 0 to 15% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 6.6, 6.8 min.

4.1.14. P^1, P^4 -di-(N^6 -propyl-5'-adenosine) P^1, P^4 -dithio- P^2, P^3 -

chloromethylenetetraphosphate, sodium salt (14)—Prepared by the above procedure from N^6 -propyladenosine 5'-monothiophosphate bis-triethylammonium salt (182 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloro-methylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 74.0 mg, 69%; ^1H NMR (300 MHz, D_2O) δ 8.38 – 8.19 (2H, multiple s, H-8), 7.95 – 7.90 (multiple s, 2H, H-2), 5.98 – 5.91 (2H, m, H-1'), 4.98 – 4.42 (1H, multiple t, $\text{CH}-\text{Cl}$), 4.77 – 4.64 (2H, m, H-2'), 4.55 – 4.47 (2H, m, H-3'), 4.36 – 4.27 (2H, m, H-4'), 4.27 – 4.09 (4H, m, H-5'), 3.30 – 3.11 (4H, multiple b s, NCH_2), 1.61 – 1.46 (m, 4H, NCH_2CH_2), 0.92 – 0.83 (m, 6H, CH_3); ^{31}P NMR (121 MHz, D_2O), ppm: 44.02 – 42.41 (m, P^1+P^4), 2.97 – 2.03 (m, P^2+P^3); MS (ESI^-), observed, m/z : 983.1 (100.0%), 984.1 (33.6%), 985.1 (48.3%), 986.1 (15.0%), 987.0 (6.9%), 988.1 (1.5%); calculated for $[\text{M}-\text{H}]^-$, $\text{C}_{27}\text{H}_{40}\text{ClN}_{10}\text{O}_{16}\text{P}_4\text{S}_2^-$, m/z : 983.1 (100.0%), 984.1 (35.6%), 985.1 (50.4%), 986.1 (16.4%), 987.1 (7.3%), 988.1 (1.9%); purity, 98.8%, RT RP HPLC: 10.95 min (gradient from 0 to 30% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 6.7, 6.9 min.

4.1.15. P^1, P^4 -di-(2-methylthio- N^6 -methyl-5'-adenosine) P^1, P^4 -dithio- P^2, P^3 -

chloromethylenetetraphosphate, sodium salt (15)—Prepared by the above procedure from 2-methylthio- N^6 -methyladenosine 5'-monothiophosphate bis-triethylammonium salt (188 mg, 0.30 mmol) and disodium di-(1-imidazolyl)chloromethylene-bis-phosphonate (35.5 mg, 0.1 mmol). Yield: 79.9 mg, 72%; ^1H NMR (300 MHz, D_2O) δ 8.10 – 7.90 (2H, ms, H-8), 5.90 – 5.83 (2H, md, H-1'),

5.03 – 4.35 (1H, mt, CH-Cl), 4.75 – 4.63 (2H, m, H-2'), 4.52 – 4.43 (2H, m, H-3'), 4.29 – 4.21 (2H, m, H-4'), 4.21 – 4.02 (4H, m, H-5'), 2.88 – 2.71 (6H, ms, SCH₃), 2.40 – 2.25 (6H, ms, NCH₃); ³¹P NMR (121 MHz, D₂O), ppm: 41.96 – 40.32 (m, P¹+P⁴), 0.69 – -0.16 (m, P²+P³); MS (ESI⁻), observed, m/z: 1019.0 (100.0%), 1020.0 (36.6%), 1020.9 (53.8%), 1021.9 (18.0%), 1022.9 (11.3%); calculated for [M-H]⁻, C₂₅H₃₆ClN₁₀O₁₆P₄S₄⁻: 1019.0 (100.0%), 1020.0 (35.0%), 1021.0 (59.3%), 1022.0 (19.2%), 1023.0 (12.0%); purity, 95.8%, RT RP HPLC: 8.89, 9.19, 9.56 min (gradient from 0 to 50% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 8.3, 8.6 min.

4.1.16. P¹,P⁴-di-(2-methylthio-5'-adenosine) P¹,P⁴-dithio-P²,P³-

dichloromethylenetetraphosphate, sodium salt (16)—2-Methylthioadenosine (1.88 g, 6 mmol) was dissolved in dry DMF (24 ml) under Ar. *p*-Toluenesulfonic acid (2.30 g) was added on stirring, followed by trimethyl orthoformate (31.3 g, 32.3 ml, 300 mmol). The resulting solution was kept at rt under Ar for 18 h. The *p*-toluenesulfonic acid was removed by addition of Dowex MWA-1 anion exchange resin in the OH⁻ form (10.3 g). The resin was filtered and washed twice with DMF (10 ml each). The combined filtrate and washings were evaporated under vacuum, and the residue was dried under high vacuum at rt for 6 h to give 2-methylthio-2',3'-(methoxymethylene)adenosine as a colorless foam. Yield: 1.90 g, 89% as two diastereomers; ¹H NMR (300 MHz, DMSO-d₆) δ 8.13, (0.5H, s, diast. 1 H-8), 8.09 (0.5H, s, diast. 2 H-8), 7.28 (2H, bs, NH₂), 6.10 (0.5H, d, *J* = 2.9 Hz, diast. 1 H-1'), 6.06 (0.5H, s, diast. 2 CHOMe), 6.01 (0.5H, d, *J* = 2.5 Hz, diast. 2 H-1'), 5.96 (0.5H, s, diast. 1 CHOMe), 5.41 (0.5H, dd, *J* = 2.5, 6.3 Hz, diast. 2 2'), 5.37 (0.5H, dd, *J* = 2.9, 7.1 Hz, diast. 1 2'), 5.00 – 4.94 (1.5H, m, diast. 2 H-3' + OH), 4.87 (0.5H, dd, diast. 1 H-3'), 4.15 (0.5H, m, diast. 2 H-4'), 4.05 (0.5H, m, diast. 1 H-4'), 3.41 (2H, m, H-5'), 3.25 (1.5H, s, diast. 1 OCH₃), 3.13 (1.5H, s, diast. 2 OCH₃), 2.36 (1.5H, s, diast. 2 SCH₃), 2.35 (1.5H, s, diast. 1 SCH₃); MS (ESI⁻): 354.3 [M-H]⁻; purity, 95.3%, RT RP HPLC: 20.53 min (gradient from 0 to 50% MeCN in 20 mM aqueous triethylammonium acetate for 15 min).

2-Methylthio-2',3'-(methoxymethylene)adenosine (178 mg, 0.5 mmol) was evaporated twice from dry DMF (5 ml each). The flask was equipped with a stir bar, sealed and flushed with Ar. Dry THF (5 ml) was added via a syringe followed by diisopropylethylamine (155 mg, 209 μl, 1.2 mmol). The contents were stirred until solids dissolved and cooled to -10 °C. 2-Chloro-1,3,2-benzodioxaphosphorin-4-one (salicyl chlorophosphite, 122 mg, 0.6 mmol) was dissolved in dry THF (3 ml) under Ar, and the solution was added by a syringe dropwise and with stirring to the cooled nucleoside solution at -10 – 0 °C. The cooling bath was removed and the reaction mixture was left for 10 min at rt.

(Dichloromethylene)bisphosphonic acid (147 mg, 0.6 mmol) was dissolved in methanol (4 ml). Tributylamine (334 mg, 428 μl, 1.8 mmol) was added and the mixture was evaporated under vacuum. The residue was rendered anhydrous by repeated evaporation from dry DMF (3×10 ml) under vacuum. The residue was dissolved under Ar in 3 ml anhydrous DMF and was added by a syringe dropwise with stirring to the cooled (-10 °C) reaction mixture at -10 – 0 °C. The cooling bath was removed and the reaction mixture was left for 30 min at rt under Ar. A suspension of sulfur (fine powder, 32 mg, 1 mmol) in 2 ml dry DMF was added by a syringe. After another 30 min stirring at rt under Ar a solution of 2-methylthioadenosine 5'-monothiophosphate bis-triethylammonium salt (459 mg, 0.75 mmol,

rendered anhydrous by repeated evaporation from dry DMF under vacuum) and tributylamine (370 mg, 475 μ l, 2 mmol) in 4 ml dry DMF were added by a syringe with stirring, followed by a solution of anhydrous zinc chloride (340 mg, 2.5 mmol) in 3 ml dry DMF. The reaction mixture was concentrated under vacuum at rt to half of its volume and stirred under Ar overnight. TEAB (0.1 M, 50 ml) and Chelex[®] ion exchange resin in the sodium form (20 ml) were added, and the mixture was stirred for 2 h and then filtered. The filtrate was extracted with ether containing 1% triethylamine (100 ml) and then evaporated under vacuum to dryness. The residue was treated with 20 ml methanol and filtered. (The filtrate contained mostly unreacted monothiophosphate.) The solid was dissolved in 8 ml water and subjected to preparative ion-exchange chromatography on TSKgel SuperQ-5PW as described in the general section, and the fractions were analyzed by LCMS. During the reaction partial loss of the 2',3'-methoxymethylene protecting group was observed (most likely caused by the ZnCl₂ catalyst). The major products were the corresponding protected and unprotected triphosphates. The peaks containing the products (P¹-(2-methylthio-2',3'-(methoxymethylene)-5'-adenosine)-P⁴-(2-methylthio-5'-adenosine)-P¹,P⁴-dithio-P²,P³-dichloromethylenetetraphosphate and the deprotected **16**) eluted at 143 and 152 min, respectively, were combined, evaporated under vacuum, and stripped from the residual buffer by repeated evaporation from methanol. The residual 2',3'-methoxymethylidene protection was removed by treatment with 10% hydrochloric acid (1 ml) for 3 h. The acid was neutralized with triethylamine to pH 9, and the solution was loaded on a XBridge Prep C18 5 μ m 20 $\times$ 250 mm column and eluted with a gradient from 50 mM TEAB to 50 mM TEAB in 50% aqueous MeCN for 30 min at 15 ml/min and 273 nm UV detection. The fractions containing the product were evaporated under vacuum, and repeatedly evaporated from methanol (3 $\times$ 30 ml). The product was converted to the sodium form by dissolving in water (1 ml) and passing through a column of Dowex 50WX2 in the sodium form (30 $\times$ 10 mm), elution with water (5 ml), concentration under vacuum to 1 ml and lyophilization. Yield: 33.5 mg, 6%; ¹H NMR (300 MHz, D₂O) δ 8.23, 8.20, 8.17 (2H, ms, H-8), 5.99 – 5.94 (2H, md, H-1'), 4.79 – 4.71 (2H, m, H-2'), 4.55 – 4.46 (2H, m, H-3'), 4.34 – 4.27 (2H, m, H-4'), 4.27 – 4.11 (4H, m, H-5'), 2.48, 2.47 (6H, two s, SCH₃); ³¹P NMR (121 MHz, D₂O), ppm: 43.79 – 42.89 (m, P¹+P⁴), 1.53 – -1.26 (m, P²+P³); MS (ESI⁻), observed, m/z: 1025.1 (100%), 1026.1 (31.7%), 1027.0 (91.7%), 1028.0 (28.5%), 1028.9 (31.4%), 1030.0 (8.6%), 1030.9 (3.8%), 1032.0 (0.9%); calculated for [M-H]⁻, C₂₃H₃₁Cl₂N₁₀O₁₆P₄S₄: 1024.9 (100.0%), 1026.9 (90.5%), 1025.9 (32.7%), 1028.9 (30.3%), 1027.9 (28.3%), 1029.9 (8.8%), 1030.9 (4.9%), 1031.9 (1.3%); purity, 96.5%, RT RP HPLC: 8.53 min (gradient from 0 to 50% MeCN in 20 mM aqueous triethylammonium acetate for 15 min); IE HPLC: 9.3 min.

4.1.17. Separation of the diastereomers of 5—Analytically the diastereomers of **5** were separated by reverse phase HPLC on a XBridge RP C18 column, 3.5 μ m, 4.6 $\times$ 150 mm (Waters) at 30 °C with isocratic elution with 15% methanol in 20 mM potassium phosphate buffer pH 8.5 containing 10 mM EDTA, at a flow rate of 1 ml/min and UV detection at 273 nm (Fig. 8). Preparative separations (12.5 mg per injection) were done on a 20 $\times$ 250 mm XBridge C18 column 5 μ m column at rt, eluted isocratically for 60 min with the same mobile phase, and then a linear gradient to 30% methanol for 60 min, at a flow rate of 10 ml/min. The fractions, after concentration to 1/5 of their original volume under vacuum, were

desalted by loading on the same column equilibrated with 0.2 M TEAB buffer, pH 8, and then eluted with a gradient from the equilibration buffer to 50% methanol in the equilibration buffer at 15 ml/min for 30 min. After desalting, the fractions were evaporated on a rotary evaporator, and the residual TEAB was removed by repeated evaporation from methanol. Finally the diastereomers were converted to the sodium salts by passing through a column of Dowex W50X2 (20 × 5 mm) in the sodium form, elution with two column volumes of water, and lyophilization. The purity of each diastereomer was above 95% as judged by analytical RP HPLC.

Diastereomer D1: Yield: 1.7 mg, 15%; ¹H NMR (300 MHz, D₂O) δ 8.29 (1H, s, H-8), 8.18 (1H, s, H-8), 6.00 (1H, d, *J* = 5.1 Hz, H-1'), 5.99 (1H, d, *J* = 6.1 Hz, H-1'), 4.80 – 4.71 (2H, m, H-2'), 4.67 (1H, t, *J* = 17.6 Hz, CH-Cl), 4.56 – 4.51 (2H, m, H-3'), 4.33 – 4.25 (2H, m, H-4'), 4.25 – 4.08 (4H, m, H-5'), 2.495, (3H, s, SCH₃), 2.477 (3H, s, SCH₃); ³¹P NMR (121 MHz, D₂O), ppm: 43.84 – 42.23 (m, P¹+P⁴), 2.29 – 1.52 (m, P²+P³); RT RP HPLC: 6.76 min; IE HPLC: 8.9 min.

Diastereomer D2: Yield: 3.3 mg, 29%; ¹H NMR (300 MHz, D₂O) δ 8.26 (1H, s, H-8), 8.15 (1H, s, H-8), 5.96 (1H, d, *J* = 5.7 Hz, H-1'), 4.76 – 4.70 (2H, m, H-2'), 4.54 – 4.47 (2H, m, H-3'), 4.45 (1H, t, *J* = 17.4 Hz, CH-Cl), 4.32 – 4.27 (2H, m, H-4'), 4.27 – 4.12 (4H, m, H-5'), 2.475, (3H, s, SCH₃), 2.461 (3H, s, SCH₃); ³¹P NMR (121 MHz, D₂O), ppm: 43.58 – 42.97 (m, P¹+P⁴), 2.31 – 1.69 (m, P²+P³); RT RP HPLC: 7.52 min; IE HPLC: 9.1 min.

Diastereomer D3: Yield: 3.8 mg, 34%; ¹H NMR (300 MHz, D₂O) δ 8.56 (1H, s, H-8), 8.11 (1H, s, H-8), 5.99 (1H, d, *J* = 5.1 Hz, H-1'), 5.98 (1H, d, *J* = 6.1 Hz, H-1'), 4.82 (1H, t, *J* = 17.6 Hz, CH-Cl), 4.80 – 4.72 (2H, m, H-2'), 4.55 – 4.50 (2H, m, H-3'), 4.34 – 4.23 (2H, m, H-4'), 4.23 – 4.09 (4H, m, H-5'), 2.485, (3H, s, SCH₃), 2.473 (3H, s, SCH₃); ³¹P NMR (121 MHz, D₂O), ppm: 43.14 – 42.46 (m, P¹+P⁴), 2.53 – 1.70 (m, P²+P³); RT RP HPLC: 10.62 min; IE HPLC: 8.9 min.

Diastereomer D4: Yield: 2.4 mg, 21%; ¹H NMR (300 MHz, D₂O) δ 8.20 (1H, s, H-8), 8.17 (1H, s, H-8), 5.97 (1H, d, *J* = 5.8 Hz, H-1'), 4.77 – 4.71 (2H, m, H-2'), 4.52 (1H, t, *J* = 17.5 Hz, CH-Cl), 4.55 – 4.49 (2H, m, H-3'), 4.32 – 4.26 (2H, m, H-4'), 4.26 – 4.10 (4H, m, H-5'), 2.479, (3H, s, SCH₃), 2.473 (3H, s, SCH₃); ³¹P NMR (121 MHz, D₂O), ppm: 43.51 – 42.62 (m, P¹+P⁴), 2.32 – 1.71 (m, P²+P³); RT RP HPLC: 12.41 min; IE HPLC: 9.1 min.

4.2. Stability and metabolism in rat and human plasma

Commercial frozen, pooled, heparin-anticoagulated rat or human plasma (Bioreclamation, Westbury, NY) was thawed upon arrival, aliquoted (3 ml) in sterile polypropylene vials, re-frozen in dry ice, and stored at –45 °C. At the time of testing the plasma aliquots were thawed and incubated at 37 °C for 5 min. Compound **5** (15 µl of 10 mM solution in physiological saline, 50 µM final concentration) was added, the sample was mixed briefly, and incubated at 37 °C. Aliquots (250 µl) were taken at the specified times, mixed with 250 µl MeCN, vortexed briefly, and then centrifuged at 15000 xG for 15 min. The supernatant was filtered through centrifugal ultrafiltration device (Nanosep®, Pall, 10K membrane, 15 min at 14000 xG). The filtrates (250 µl) were evaporated on a Speed-Vac (4 h, 3 mm Hg, 35

°C), reconstituted in 50 µL of 50 mM potassium phosphate 10 mM EDTA buffer pH 8.5, centrifuged (5 min, 15000 xG) and the supernatants were transferred into micro-insert equipped HPLC vials for analysis. Blank rat or human plasma (3 ml) was incubated for 5 min or for 24 h at 37 °C, spiked with water (15 µl), and then processed as above to prepare blank samples. The processed samples were analyzed by HPLC on a XBridge Shield C18, 3.5 µm, 150×4.6 mm column (Waters) with 5 µl injection and isocratic elution with 20% methanol in 50 mM potassium phosphate buffer pH 8.4 containing 10 mM EDTA at a flow rate of 1 ml/min and UV detection at 273 nm. The quantification was done by internal normalization of the peak areas of the four diastereomers and the metabolites. The rate constants for degradation in plasma and the associated half-lives were estimated by a non-linear fitting of a first order elimination model to the internally normalized chromatographic peak areas of the first three time points.

4.3. Platelets and platelet receptors studies

4.3.1. General—MRS2179, probenecid, adenosine 5'-(P²,P³-methylene)triphosphate (β, γ-CH₂-ATP), EGTA and apyrase (grade VII) were from Sigma-Aldrich, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem, FLUO-4 was from Invitrogen, ADP was from Bio/Data, and CD41-phycoerythrin-Cy5 was from Beckman Coulter.

4.3.2. Ethics Statement—The studies involving human volunteers were approved by the University of Massachusetts Medical School and Boston Children's Hospital Institutional Review Boards (IRB). Written IRB-approved informed consent was obtained prior to blood collection.

4.3.3. Blood collection and sample preparation—Human blood samples were taken from healthy volunteer donors free from aspirin or other non-steroidal anti-inflammatory drugs for more than 7 days. Blood was drawn from antecubital veins into tubes containing 3.2% sodium citrate. For platelet aggregation assays the blood was centrifuged at 110 × g for 12 minutes, and platelet rich plasma (PRP) was immediately removed. Centrifugation 1 at 1650 × g for 10 minutes was to obtain platelet-poor plasma (PPP).

4.3.4. ADP-induced platelet aggregation—The 96-well microplate method for the detection of ADP-induced platelet aggregation and the concentration dependence of its inhibition by the tested compounds was used as previously described [24,25], thereby avoiding the problem of platelet aging [51]. In brief, PRP at 37° C was added to a pre-warmed 96-well microplate containing ADP (3 µM final concentration) and test compounds (various concentrations) or vehicle (10 mM Hepes, 0.15 M NaCl, pH 7.4). Light transmission at 580 nm was recorded immediately and at 11 second intervals for 6 min at 37° C with intermittent programmed shaking of the plate in a Molecular Devices microplate reader. Within each experiment all samples were run in duplicate and each experiment was repeated 3 – 5 times with PRP from different donors.

4.3.5. P2Y₁₂-mediated vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay—VASP phosphorylation was measured by flow cytometry using

a BioCytex kit, essentially according to the manufacturer's recommendations, except that a small volume of the test compound solution or vehicle (HEPES-saline) was added to each assay tube as previously described [24,25]. Analysis was performed in a FACSCalibur (Becton Dickinson) flow cytometer.

4.3.6. P2Y₁-mediated cytosolic Ca²⁺ increase assay—ADP-dependent, P2Y₁-mediated increase in platelet cytosolic Ca²⁺ was measured by detecting changes in FLUO-4 fluorescence as previously described [24,25]. In brief, citrated whole blood was added to a loading solution consisting of 5 μM FLUO-4, CD41-PE-Cy5 and 1 mM probenecid, and the mixture was incubated for 30 minutes at room temperature. Samples were diluted 36-fold in 10 mM HEPES, 0.15 M NaCl, pH 7.4 and analyzed in a FACSCalibur flow cytometer. After obtaining a 30 second baseline recording, the acquisition was paused, and 60 μL of ADP (3 μM final concentration) and test compound solutions at various concentrations or ADP plus vehicle (HEPES-saline) were quickly added, the sample mixed, and the acquisition resumed (total pause time less than 10 seconds). FLUO-4 fluorescence before and after addition of ADP (3 μM final concentration) and test compound solutions was monitored. The mean FLUO-4 fluorescence of the baseline 30-second interval and of 10-second post-stimulant intervals was calculated. The cytosolic Ca²⁺ increase was calculated as the ratio of the maximal post-stimulant FLUO-4 fluorescence to the baseline FLUO-4 fluorescence. The percent inhibition of ADP-induced Ca²⁺ increase due to the addition of the test compounds was calculated relative to 3 μM ADP plus vehicle (HEPES-saline).

4.3.7. P2X1-mediated entry of extracellular Ca²⁺—Measurement of P2X1-mediated entry of extracellular Ca²⁺ based on changes in FLUO-4 fluorescence was performed as previously described [25]. The non-hydrolyzable ATP analog β,γ-CH₂-ATP (20 μM) was used as a positive control. To confirm that any increases in intracellular Ca²⁺ observed were unrelated to P2Y₁ activation, experiments were repeated with 100 μM MRS2179, a selective P2Y₁ inhibitor, in the ambient buffer. The ability of high concentrations of the test compounds to antagonize P2X1 activation by 20 μM β,γ-CH₂-ATP was also tested.

4.3.8. Statistical analysis—The results were analyzed using GraphPAD Prism software, version 4.00 for Windows (GraphPad Software, San Diego, CA). All data are expressed as mean followed by 95% confidence interval (95% CI). Student's t-test was used to determine statistical significance when two groups of data were compared. One-way ANOVA and Bonferroni's multiple comparison tests were used when three or more groups of data were compared.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

Ap₄A	P ¹ ,P ⁴ -di(adenosine-5') tetraphosphate
bs	broad singlet
dd	doublet of doublets
MeCN	acetonitrile
2-MeSADP	2-methylthioadenosine 5'-diphosphate
MeSAMP	2-methylthioadenosine 5'-monophosphate
MeSAMP(S)	2-methylthioadenosine 5'-monothiophosphate
PRI	Platelet Reactivity Index
PRP	Platelet-rich plasma
PPP	platelet-poor plasma
VASP	Vasodilator-stimulated phosphoprotein

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HIGHLIGHTS

- Effect of Ap₄A modification on human platelet aggregation and P2Y₁ and P2Y₁₂ receptor inhibition.
- SAR within Ap₄A scaffold for platelet aggregation and P2Y₁/P2Y₁₂ inhibition was established.
- Some Ap₄A derivatives potently and reversibly inhibit ADP induced human platelet aggregation.
- Some Ap₄A derivatives synergistically inhibit both platelet P2Y₁ and P2Y₁₂ receptors.

Figure 1.
Summary of the SAR in modification of the adenine nucleotide scaffold.

No.	R ₁	R ₂	X	Y	No.	R ₁	R ₂	X	Y
1	H	H	S	CHCl	9	<i>n</i> -C ₅ H ₁₁ S	H	S	CHCl
2	MeS	H	O	O	10	Cl	H	S	CHCl
3	MeS	H	S	O	11	Br	H	S	CHCl
4	MeS	H	O	CHCl	12	I	H	S	CHCl
5	MeS	H	S	CHCl	13	H	Me	S	CHCl
6	EtS	H	S	CHCl	14	H	Pr	S	CHCl
7	PrS	H	S	CHCl	15	MeS	Me	S	CHCl
8	CF ₃ (CH ₂) ₂ S	H	S	CHCl	16	MeS	H	S	CCl ₂

Figure 2.
Structures of Ap₄A analogs.

Figure 3.

Concentration-dependent inhibition of human platelet aggregation by **5**. Platelet-rich plasma (PRP) was incubated with compound **5** for 15 min at 37°C. Aggregation was initiated by addition of 5 μ M ADP and 10 seconds shaking. Absorbance at 580 nm was read at 27 second intervals with 10 seconds shaking between reads. Hundred percent aggregation was established using platelet-poor plasma and 0% aggregation was established using PRP.

Figure 4. Inhibition of platelet aggregation by selected Ap₄A derivatives. Platelet aggregation with human platelet rich plasma (PRP) was stimulated with 3 μ M ADP in the presence or absence of test compounds (no pre-incubation of PRP with inhibitors). Changes in optical density were monitored on a microplate reader.

Figure 5.

Inhibition of platelet P2Y₁ and P2Y₁₂ receptors by selected Ap₄A derivatives. Panel **A**, P2Y₁-mediated platelet calcium flux. Citrate anticoagulated human whole blood was loaded with the calcium indicator FLUO-4. Changes in cytoplasmic calcium induced by the addition of 3 μ M ADP were measured by flow cytometry. Panel **B**, P2Y₁₂-mediated changes in VASP phosphorylation. Sodium citrate-anticoagulated human blood was treated with PGE₁ in the absence or presence of 3 μ M ADP. The resulting levels of VASP phosphorylation, and the effect of the test compounds on them were measured flow cytometrically and used to calculate the Platelet Reactivity Index (PRI).

Figure 6. Concentration-effect curves for ADP (20 μ M) induced human platelet aggregation inhibition by compounds 5, 6, and 11 without (panel A) and with (panel B) pre-incubation (15 min) with the inhibitor.

Figure 7.

Inhibition of ADP-stimulated platelet surface activated GPIIb-IIIa and P-selectin expression and reversal after removal of drug. Panel A: Compound **5** concentration-dependent inhibition of 20 μ M ADP-stimulated platelet surface activated GPIIb-IIIa (circles) and P-selectin (triangles) mean fluorescence intensity (MFI). Panel B: ADP-stimulated platelet surface activated GPIIb-IIIa (gray columns) and P-selectin (black columns) mean fluorescence intensity after 30 min incubation with 10 nM of compound **5**, followed by 100-fold dilution in plasma with, or without 10 nM compound **5**. Results are the means $\pm$ SEM of 3 experiments.

Figure 8.
Separation of the four diastereomers of compound **5** by reverse phase chromatography. See Experimental Section for details

Figure 9. Platelet aggregation inhibition by the individual diastereomers of compound **5** (D1-D4) and the diastereomeric mixture.

Figure 10.

Representative chromatogram of the incubation of **5** in human plasma, 77 h at 37 °C.

MeSAdo, 2-methylthioadenosine; MeSAMPs, 2-methylthioadenosine 5-monothiophosphate.

Figure 11.

Effect of the incubation of compound **5** with rat (panel A) and human (panel B) plasma on the relative concentration of the four diastereomers of compound **5**, and its metabolites (MeSAdo, 2-methylthioadenosine, and MeSAMPs, 2-methylthioadenosine 5-monothiophosphate).

Scheme 1.
Synthesis of dinucleoside tetraphosphates.

Scheme 2.
Synthesis of compound **16**.

Table 1

ADP induced human platelet aggregation inhibition of new Ap₄A derivatives.

No.	Modifications ^a				IC ₅₀ , nM (95% Confidence Interval) ^b		
	R ₁	R ₂	X	Y	no pre-incubation 3 μM ADP	pre-incubation with test compound 5 μM ADP	20 μM ADP
1 ^c	H	H	S	CHCl	437 (273 – 699)	757 (519 – 1100)	7680 (4680 – 12600)
2	MeS	H	O	O	71.3 (49.1 – 104)	1020 (640 – 1620)	3950 (2030 – 7660)
3	MeS	H	S	O	61.8 (31.0 – 123)	70.3 (57.4 – 86.0)	731 (506 – 1050)
4	MeS	H	O	CHCl	33.1 (20.8 – 52.8)	8.89 (7.32 – 10.8)	32.6 (23.1 – 45.9)
5	MeS	H	S	CHCl	36.1 (27.5 – 47.3)	11.6 (9.46 – 14.3)	27.6 (20.9 – 36.3)
6	EtS	H	S	CHCl	46.1 (26.7 – 79.6)	8.48 (6.85 – 10.5)	13.4 (12.3 – 14.6)
7	PrS	H	S	CHCl	113 (76.0 – 167)	23.4 (19.0 – 28.7)	44.6 (34.3 – 58.0)
8	CF ₃ (CH ₂) ₂ S	H	S	CHCl	144 (95.9 – 216)	15.0 (12.4 – 18.1)	26.6 (21.2 – 33.3)
9	<i>n</i> -C ₅ H ₁₁ S	H	S	CHCl	1090 (925 – 1290)	90.7 (73.0 – 113)	172 (155 – 190)
10	Cl	H	S	CHCl	140 (83.4 – 234)	369 (286 – 476)	2690 (2030 – 3560)
11	Br	H	S	CHCl	87.8 (69.2 – 111)	173 (148 – 201)	936 (792 – 1110)
12	I	H	S	CHCl	49.9 (41.6 – 59.8)	48.5 (37.7 – 62.3)	416 (346 – 499)
13	H	Me	S	CHCl	5870 (4110 – 8380)	10700 (8070 – 14100)	37200 (28500 – 48600)
14	H	Pr	S	CHCl	3930 (2570 – 6010)	12000 (7910 – 18100)	22400 (–)
15	MeS	Me	S	CHCl	616 (524 – 724)	757 (519 – 1100)	7680 (4680 – 12600)
16	MeS	H	S	CCl ₂	295 (223 – 389)	1020 (640 – 1620)	3950 (2030 – 7660)

^aRefer to Fig. 2 for structures.

^bThree to five donors per compound. Two experiments with each donor.

^cThis known compound [24,35] was tested for control and comparison purposes.

Table 2

Platelets P2Y₁, and P2Y₁₂ receptors inhibition, and P2X₁ receptor activation by new Ap₄A derivatives.

No.	Modifications ^a				Effect		
	R ₁	R ₂	X	Y	P2Y ₁ inhibition	P2Y ₁₂ inhibition	P2X ₁ activation ^c
					IC ₅₀ ±SEM ^b , μM	IC ₅₀ ±SEM, μM	%±SEM
2	MeS	H	O	O	4.9 ± 1.3	1.4 ± 0.7	48 ± 12
3	MeS	H	S	O	0.16 ± 0.07	2.4 ± 1.5	3.0 ± 0.9
4	MeS	H	O	CHCl	29 ± 5.6	0.31 ± 0.13	ND ^d
5	MeS	H	S	CHCl	2.15 ± 0.67	0.22 ± 0.04	0.24 ± 0.64
6	EtS	H	S	CHCl	9.6 ± 6.3	0.14 ± 0.02	23.8 ± 4.9
7	PrS	H	S	CHCl	6.30 ± 0.74	1.02 ± 0.058	31(N=1)
8	CF ₃ (CH ₂) ₂ S	H	S	CHCl	5.20 ± 2.29	0.21 ± 0.03	4.7 ± 2.4
9	<i>n</i> -C ₅ H ₁₁ S	H	S	CHCl	4.21 ± 1.98	3.1 ± 0.43	4.7 ± 1.0
10	Cl	H	S	CHCl	7.37 ± 1.84	9.3 ± 3.0	0.07 ± 1.0
11	Br	H	S	CHCl	2.24 ± 0.98	0.34 ± 0.05	1.8 ± 0.9
12	I	H	S	CHCl	2.65 ± 0.46	1.35 ± 0.47	0.69 ± 0.89
13	H	Me	S	CHCl	1.97 ± 0.42	>200	0.31 ± 1.31
14	H	Pr	S	CHCl	8.8 (N=1)	156 (N=1)	-0.01 ± 0.91
15	MeS	Me	S	CHCl	0.36 (N=1)	0.73 (N=1)	92 (N=1)
16	MeS	H	S	CCl ₂	1.02 ± 0.41	1.12 ± 0.20	3.3 ± 1.3
MRS2179 ^e					2.25 ± 0.40	Not inhibitor	ND
Cangrelol ^f					Not inhibitor	0.0183 ± 0.0063	ND

^a Refer to Fig. 2 for structures.

^b N=3

^c Intracellular Ca²⁺ increase by 1 μM of test compound due to extracellular Ca²⁺ uptake measured by whole blood flow cytometry, relative to the effect of 20 μM β,γ-CH₂-ATP (considered as 100%).

^d Not done.

^e Control P2Y₁ selective inhibitor

Control P2Y₁₂ selective inhibitor

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Table 3

ADP induced human platelet aggregation inhibition by the diastereomeric mixture of compound **5**, and its individual diastereomers (D1-D4).

Compound	IC ₅₀ , μ M (95% Conf. Int.)
Diast. mixture	0.079 (0.040 – 0.154)
Diastereomer 1	0.076 (0.034 – 0.171)
Diastereomer 2	0.083 (0.042 – 0.165)
Diastereomer 3	0.064 (0.028 – 0.144)
Diastereomer 4	0.110 (0.034 – 0.361)

Table 4

First order elimination rate constants and half-lives of compound **5** and its 4 diastereomers in rat and human plasmas.

	5	D1	D2	D3	D4
Rat					
k, h ⁻¹	0.147	0.00690	0.137	0.128	0.508
t _{1/2} , h	4.73	100.5	5.06	5.40	1.37
Human					
k, h ⁻¹	0.0221	0.00976	0.0171	0.0217	0.0424
t _{1/2} , h	31.3	71.0	40.6	31.9	16.4