

Fluorescent N²,N³-ε-Adenine Nucleoside and Nucleotide Probes: Synthesis, Spectroscopic Properties, and Biochemical Evaluation**

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Abstract

N¹,N⁶-ethenoadenine, ε-A, nucleos(t)ides have been previously applied as fluorescent probes in numerous biochemical systems. However, these ε-A analogues lack the H-bonding capability of adenine. To improve the fluorescence characteristics while preserving the H-bonding pattern required for molecular recognition, we designed a novel probe: N²,N³-etheno-adenosine, (N²,N³-ε-A). Here, we describe four novel syntheses of the target ε-nucleoside and related analogues. These methods are short, facile, and provide the product regioselectively. In addition, we report the absorption and emission spectra of N²,N³-ε-A and the dependence of the spectral features on the pH and polarity of the medium. Specifically, maximum emission of N²,N³-ε-A in water is observed at 420 nm (φ=0.03, excitation at 290 nm). The biochemical relevance of the new probe was evaluated with respect to the P2Y₁ receptor and NTPDases 1 and 2. N²,N³-ε-ATP was found to be almost equipotent with ATP at the P2Y₁ receptor and was hydrolyzed by NTPDases 1 and 2 at about 80% of the rate of ATP. Furthermore, protein binding does not seem to shift the fluorescence of N²,N³-ε-ATP. Based on the fluorescence and full recognition by ATP-binding proteins, we propose N²,N³-ε-ATP and related nucleos(t)ides as unique probes for the investigation of adenine nucleos(t)ide-binding proteins as well as for other biochemical applications.

Keywords

fluorescent probes; NTPDase; nucleosides; nucleotides; P2Y₁ receptor

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Introduction

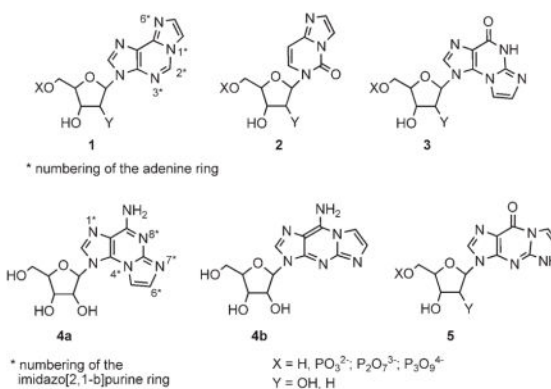
Adenine has poor fluorescence properties.^[1] However, bridging the adenine N1,N⁶-positions with an etheno moiety, **1**, improves the fluorescence of the parent adenine system (e.g., for **1**: $\lambda_{\text{max}}=415$ nm; $\phi=0.56$).^[2, 3] Over the past three decades, N1,N⁶-etheno-adenosine, **1** (ϵ -A), N3,N⁴-etheno-cytidine, **2** (ϵ -C), N²,N3-etheno-guanosine, **3** (ϵ -G), and the corresponding nucleotide analogues have been extensively studied as fluorescent nucleos(t)ide probes. All these probes bear an etheno bridge that causes minimal perturbation to the natural system.^[4, 5]

N1,N⁶-Etheno-adenine nucleotides are commonly applied as fluorescent probes for various biochemical studies, such as the structure and function of nucleic acids,^[2, 4, 6–8] protein visualization,^[7] enzymatic studies,^[2, 6, 7, 9–11] investigation of nucleotide-binding sites,^[2, 12–14] conformational analysis of nucleotides,^[10] and pharmacology of nucleosides/nucleotides.^[5, 15]

Although useful probes, N1,N⁶- ϵ -A nucleos(t)ides cannot be applied to biochemical systems requiring H-bonding-based molecular recognition. Specifically, the engagement of the N1 and N⁶ nitrogens of ϵ -A in an imidazole ring results in the loss of the adenine natural H-bonding capability. Consequently, the molecular recognition of these probes by target proteins or nucleic acids is reduced or lost.

So far, no fluorescent analogue of adenosine or the corresponding nucleotides with free N1,N⁶-positions has been investigated.^[16] To address this challenge, we decided to extend the natural adenine chromophore at the C2,N3-positions to form analogue **4a**. Specifically, we considered substitution of an amino group at the adenine C2-position, as substituents that donate electrons to the π system enhance the absorption of light and increase fluorescence. A good example is 2-aminopurine, which is a fluorescent purine analogue with a quantum yield of 0.6 in water and an emission maximum at 370 nm.^[17] Next, we planned the incorporation of the adenine C2-amino group into an imidazole ring fused to the adenine ring, to form 9-amino-3- β -D-ribofuranosylimidazo[2,1-b]purine (**4a**), denoted here N²,N3-etheno-adenosine or N²,N3- ϵ -A.

Here, we describe four novel syntheses of the target ϵ -nucleoside, **4a** and related analogues. These methods are short (3- to 4-step syntheses), facile, and provide the product regioselectively in a reasonable yield. We also report on the spectral properties of the novel analogue, including absorption and emission spectra, and the dependence of the spectral features on the pH and polarity of the medium. Finally, we report on the biochemical relevance of the new fluorescent nucleotide, N²,N3- ϵ -ATP, and related analogues with respect to several ATP-binding proteins: the P2Y₁ receptor and NTPDases 1 and 2.



Results and Discussion

Synthesis

The extension of natural nucleobases to form fluorophores **1–3** is achieved by treating the nucleobases with α -haloaldehydes at pH 4.5.^[3, 18] For instance, the fluorescent angular isomer, N²,N³-e-G (**3**; emission maximum in water at 410 nm)^[19] is obtained from *O*⁶-benzyl-guanosine and bromoacetaldehyde at pH 4.5.^[20] However, at an elevated pH, a nonfluorescent^[19, 21] linear isomer, N¹,N²-e-G(**5**) is obtained from guanosine.^[21, 22]

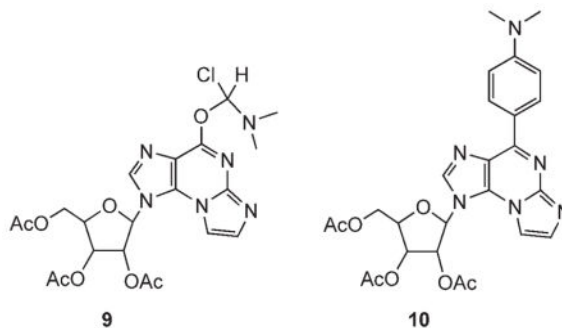
Since we targeted the novel angular heterocyclic system N²,N³-e-A (**4a**) rather than the related angular N¹,N⁶-e-A (**1**) or linear N¹,N²-e-A (**4b**) isomers, we had to direct the regiochemistry of the ring closure to N²,N³- rather than to the N¹,N⁶- or N¹,N²-positions. For this purpose, we selected 6-Cl-, 6-NHAr-, or 6-SMe-purine riboside analogues as starting materials that would direct the reaction with bromoacetaldehyde, at pH 4.5, to the purine N²,N³-positions. With these starting materials, we developed several efficient syntheses of the target compound **4a** and its analogues.

Thus, in the first synthesis, 6-chloro-2-aminopurine riboside **6** was treated with freshly prepared bromoacetaldehyde at pH 4.5 and 38°C for 30 h (Scheme 1), and N²,N³-e-guanosine (**3**) was obtained in 86% yield. Initially, N²,N³-e-6-chloropurine riboside was formed and then hydrolyzed to the corresponding guanosine derivative **3**. Apparently, formation of the imidazole ring is faster than the hydrolysis of the chloro substituent, as guanosine is not reactive at all with α -haloaldehyde at pH 4.5.^[19]

The replacement of the C6 carbonyl oxygen in **3** by NH₂ was achieved in three steps: 1) The ribose hydroxyls were peracetylated to provide product **7**; 2) This product was then chlorinated with phosphoryl chloride in the presence of *N,N*-dimethylaniline to provide **8**; 3) Finally, **8** was treated with ethanolic NH₃ at 100 °C to provide product **4a**.

The moderate yield of the chlorination step prompted us to explore more drastic conditions for this conversion.^[23] Thus, **7**, in neat SOCl₂ solution and one drop of DMF, was heated at 100°C for 3 h. Product **9** was obtained almost quantitatively, resulting from the reaction of **7** with a Vilsmeier reagent formed in situ from thionyl chloride and DMF.^[24] Likewise, treatment of **7** in neat phosphoryl chloride solution in the presence of *N,N*-dimethylaniline

and tetraethylammonium chloride at 120°C for 3 h resulted in the quantitative formation of product **10**, due to the attack of *N,N*-dimethylaniline on the initially formed chloro product **8**.



To avoid the loss of the chloro substituent at C6, due to hydrolysis (conversion of **6** to **3**), we attempted the N²,N³-ring closure reaction under anhydrous conditions. Instead of using the aqueous bromoacetaldehyde solution, we attempted in situ generation of the aldehyde under anhydrous conditions in dry acetonitrile from bromoacetaldehyde diethyl acetal, BF₃/etherate, and NaI under reflux.^[25] Peracetylated 2-amino-6-chloropurine riboside **12** was then added to the solution at 38 °C; this gave rise to a complex mixture of products.

Therefore, instead of working under anhydrous conditions, we repeated the reaction of **6** with bromoacetaldehyde in CHCl₃/buffer (pH 4.5) solution (Scheme 2). To increase the solubility of **6** in chloroform, we used 2',3',5'-triacetylated-2-amino-6-chloropurine riboside **12**, obtained by chlorination of peracetylated guanosine **11** or acetylation of **6**. Thus, **12** in CHCl₃ was treated with bromoacetaldehyde and tetrabutylammonium bromide buffer (pH 4.5) at 38 °C for 48 h. The desired product, **8**, was obtained in a 38% yield as the major product. In addition, etheno-guanosine analogue **7** was obtained in an 8% yield. This minor product was converted back to **8** in neat POCl₃ in the presence of *N,N*-dimethylaniline at 120 °C for 20 min. Heating product **8** in ethanolic ammonia replaced C6-chloro by amine, and removed the acetate protective groups to furnish **4a** in 43% yield.

To improve the overall yield of **4a**, we attempted alternative pathways. The first approach involved the early substitution of the C6-Cl by an aryl amine to yield **13**, followed by treatment with bromoacetaldehyde to form etheno analogue **14**, and finally, attempted removal of the N⁶-amine protecting group to produce **4a** (Scheme 3).

We selected 2,4-(dimethoxy)benzyl as an amine protective group due to the relatively mild conditions needed for its removal compared to those for the *N*-benzyl group. Our preliminary attempts to remove a benzyl protective group from N⁶-exocyclic amine in **14a** included hydrogenation at ~5 atm over Pd/C with or without HOAc,^[26, 27] and oxidation of the benzyl carbon by RuO₂/NaIO₄^[28] or NH₄S₂O₈.^[29] However, these attempts were unsuccessful as **14a** remained unchanged under the hydrogenation conditions, and oxidation with persulfate caused complete cleavage of the etheno bridge. Alternatively, **12** was treated with 2,4-(dimethoxy)benzylamine in EtOH to provide **13b** in 91% yield. Product **13b** was in turn treated with bromoacetaldehyde, as described above, to provide, regiospecifically, the

angular etheno product **14b** in a 73% yield. However, the attempted removal of the aryl protective group of **14b** by oxidation with 2,3-dichloro-5,6-(dicyano)-benzoquinone (DDQ)^[30] in CHCl₃/H₂O caused complete cleavage of both the glycosidic bond and the etheno bridge in **14b**, whereas the 2,4-(dimethoxy)benzylamino group remained intact.

Nonetheless, nucleotide analogues of **14a**, **b**, and **c** (the last was prepared from **12** and dibenzylamine) were synthesized (Scheme 6, below) to evaluate their molecular recognition by our target proteins.

The best synthetic approach, for both directing the ring cyclization to N²,N³-positions and facilitating the eventual substitution of C6 by an amine, involved purine riboside as the starting material (compound **16**; Scheme 4). Compound **16** was prepared by treating **6** with NaSMe in DMSO.^[31] Alternatively, **16** was obtained in a 84% yield by methylation of thioguanosine **15**.^[32] Bromoacetaldehyde solution was added to **16** in EtOH buffer (pH 4.5) at 60 °C for 8 h to provide the etheno product **17a** in a 64% yield and absolute regioselectivity. Finally, product **17a** was treated with ethanolic ammonia at 100°C for 14 h to provide product **4a** in a 68% yield.

Regiochemistry of the ring-cyclization reaction

To assign the regiochemistry of the ring-closure product as either **4a** or **4b**, we recorded the ¹H,¹H NOESY spectra of intermediate ϵ -products **7**, **14a** (Figure 1), and **17a**. A clear cross peak between the etheno H5 and the ribose H1' proved the angular geometry of these products. A cross peak between H2 and H2' indicated the *anti* geometry of the products.

Mechanism and regiochemistry control of the cyclization reaction

In all the synthetic routes we attempted, we observed regioselective N²,N³-ring cyclization of the 2-aminopurine riboside analogue with bromoacetaldehyde. To elucidate the origin of this regioselectivity, we repeated the cyclization reaction (Scheme 4) at an elevated pH (pH 6.4) and compared our observations with related cyclization reactions (Table 1).

In our ring-closure reactions of various purine riboside derivatives with bromoacetaldehyde at pH 4.5 (Schemes 1–4), we observed that the nature of the C6 substituent, either Cl, NHCH₂Ar, or SMe (as in **12**, **13**, and **16**, respectively), plays no role in directing the ring closure. All these reactions occurred with complete regioselectivity, forming only the N²,N³- ϵ -purine riboside analogue.

Previous reports indicated that treatment of guanosine with chloroacetaldehyde at pH 4.5 resulted in no cyclization product.^[19] However, at elevated pH (pH 6.5), the linear N¹,N²- ϵ -G product **5** was formed in a low yield after several days (Table 1). To control the regiochemistry of this reaction, Leonard et al. selected *O*⁶-benzylguanine to direct the reaction with chloroacetaldehyde to guanosine N² and N³ positions by sterically hindering N¹.^[19]

In a related report, Singer et al. treated 5'-phosphate-*O*⁶-benzyl-2'-deoxyguanosine with chloroacetaldehyde at pH~8. 2'-Deoxy-N²,N³- ϵ -G, **3**, was obtained in only 18% yield,

accompanied by several by-products.^[33] In another reaction, the linear nucleotide, 2'-deoxy-N1,N²- ϵ -G, **5**, was isolated in 55% yield at basic pH.^[22]

Based on our observations, and those of others, we suggest that the regioselectivity of the reaction is controlled by pH, rather than by steric or electronic characteristics of the purine reactant. Specifically, at pH 4.5, the N1-position of analogues **13** and **16**, which is the most basic position in this ring system,^[34] is protonated and is not available for nucleophilic attack, thus making the N3 nitrogen the favored nucleophilic site. For the chloro analogue **12**, probably no N1 protonation occurs at pH 4.5 due to the most significant electron-withdrawing effect of the adjacent chloro group.^[35] However, the chloro substituent also reduces the nucleophilicity of N1; that is, the N3 nitrogen is the preferred nucleophilic site also in analogue **12**.

Indeed, when we repeated the reaction of **16** and bromoacetaldehyde at pH 6.4, the major product was N1,N²- ϵ -6-SMe-purine riboside, **17b** (47% yield), accompanied by some **17a** (15% yield; Scheme 4). At an elevated pH, N1 in **16** is not protonated. Furthermore, electron donations from the two electron-rich substituents *ortho* to N1 result in higher electron density at N1 than at N3. Therefore, at pH 6.4 the major product is the N1,N²- ϵ -analogue.

The mechanism of ring cyclization to form etheno products **8**, **14**, and **17** involves, first, the displacement of the bromoacetaldehyde bromide by the pyrimidine N3 nitrogen, rather than the exocyclic nitrogen (Scheme 5). This step in the mechanism is supported by literature data on the reaction of 2-aminopyridines with α -halo ketones.^[36] The cyclic nitrogen is the favored nucleophilic site in 2-aminopyridine, since the positive charge that develops in the transition state is stabilized by a resonance interaction with the exocyclic amine, as shown by Hand.^[36] The displacement of bromide by the pyrimidine N1 nitrogen (at pH 4.5) is impossible, as mentioned above.

In the second step, the N²-exocyclic amine in **12**, **13**, and **16** reacts with the aldehyde moiety;^[37] followed by elimination of a water molecule.

Spectral properties of N²,N3- ϵ -A

The absorption spectrum of compound **4a** at pH 7.0 showed two bands at 234 and 274 nm. The absorption of **4a** at pH 12.3 was almost identical to that at neutral pH (Table 2). However, at pH 1.6, there was a blue shift, due to protonation of **4a**.

Upon excitation of **4a** in water at 290 nm, an emission maximum was observed at 420 nm (quantum yield is 0.03). This value represents a red-shift compared to that of N²,N3- ϵ -G (emission maximum 410 nm, excitation at 280 nm).^[19] At basic pH, the emission spectrum of **4a** was identical to that at pH 7; however, at acidic pH, a blue-shift and reduced quantum yield were observed (Table 2).

In organic solvents, the emission spectrum of **4a** is linearly dependent on the polarity of the medium (Table 3). Thus, increasing the solvent polarity from dioxane ($\epsilon_d=2.2$) to DMSO ($\epsilon_d=47.6$) resulted in increases of both λ_{max} (from 434 to 458 nm) and ϕ (from 0.042 to 0.085). This polarity dependence of **4a** is very different from that of the related N²,N3- ϵ -G.

The wavelength of the emission maximum of the latter does not change significantly with decreasing solvent polarity. On the other hand, the quantum yields of N²,N³-ε-Gincrease substantially with decreasing solvent polarity.^[19]

Spectral properties of N², N³-ε-6-methylthio-purine riboside, **17a**

Among the etheno intermediates **8**, **14a**, and **17a**, the last analogue exhibited promising spectral properties. The absorption spectrum of compound **17a** in water showed four bands at 227, 247, 307, and 345 nm (Table 4). The absorption of **17a** at pH 12.3 was similar to that at neutral pH; however, at pH 1.6, there was a blue shift of the absorption spectrum due to protonation of **17a**.

Upon excitation of **17a** in water at 330 nm, maximum emission was observed at 494 and 518 nm. At both acidic and basic pH (1.6 and 12.3), reduced quantum yield was observed (Table 4).

Biochemical evaluation of the novel probes

The biochemical relevance of the new fluorescent analogues was evaluated with respect to three different ATP-binding proteins. Nucleotide probes were evaluated either as ligands of the P₂Y₁ receptor or substrates/inhibitors of NTPDase1 and NTPDase2. For this purpose, nucleotide analogues of **18a–f** and **19a–f** were prepared (Scheme 6).

Evaluation of the probes as P₂Y₁-R agonists

The use of fluorescent ATP analogues in biochemical research has brought new insights into enzymatic mechanisms and protein–nucleotide interactions.^[38–40] So far, only limited information is available about the interaction of such compounds with nucleotide receptors (P₂ receptors). Therefore, several of the novel fluorescent ATP analogues were evaluated as potential P₂Y₁ receptor agonists.

Analogues **18a**, **d**, and **f** were tested in a functional assay to induce an intracellular Ca²⁺ release in HEK293 cells stably transfected with rat brain P₂Y₁ receptor (P₂Y₁-R; Figure 2).^[41] These P₂Y₁-R-transfected cells were shown to be suitable for pharmacological characterization.^[42] Analogue **18a**, with an EC₅₀ of 3.90±0.31×10⁻⁷ M, was found to be almost equipotent with ATP, which has an EC₅₀ value of 1.30±0.04×10⁻⁷ M. However, a benzyl substitution at N⁶ of the fluorescent ATP compounds, as in analogue **18d**, reduced the ligand's potency; this resulted in a clear drop by about two orders of magnitude in the EC₅₀ value, which can only be estimated to be >1.00×10⁻⁵ M as concentrations higher than 10 μM were not investigated. A dimethoxybenzyl substituent at N⁶, as in analogue **18f**, resulted in a similar reduction of the potency of the ligand. For **18f**, the EC₅₀ value was also estimated to be >1.00×10⁻⁵ M.

Analysis of the Ca²⁺ responses in the presence of the selective P₂Y₁ receptor antagonist MRS2179 upon stimulation with **18a** (1 μM) clearly showed interaction of the novel compound with the P₂Y₁ receptor. At a concentration of 100 μM (*n*=45), and even at 10 μM (*n*=33), MRS2179 completely abolished the response to **18a**.

Whereas the well-established fluorescent probe N1,N⁶-etheno-ATP (e-ATP) needs further modifications to gain an affinity for the binding site of P2Y₁-R,^[43] analogue **18a** was found to be almost equipotent with ATP. Namely, the introduction of the 2,3-aminoetheno group to create a fluorophore has no negative effect on the potency of ATP at the P2Y₁ receptor. The potency of analogues **18d** and **18f** was weak; this emphasized the receptor's requirement for H-bonding interactions with N⁶. In addition, the substituents at N⁶ might hinder the binding to P2Y₁-R. The decreased potency due to benzyl substitution in **18d** and **f** is consistent with a previous report for N⁶-alkyl or -chloro substituted adenine nucleotide at P2Y₁-R.^[44]

Next, we examined whether the specific binding of **18a** to P2Y₁-R has any influence on the fluorescence properties of the substrate. Firstly, we measured the fluorescence of **18a** at a concentration (10 μM) that yielded a maximal response for agonist-induced Ca²⁺ release (see Figure 2). Then, we repeated the measurements in the presence of 1321N1 cells. These cells were found to be P2Y-R negative.^[45,46] This should exclude any possible influence of binding of the substance to proteins of the cell surface other than P2Y-R.

Finally, we measured the fluorescence in the presence of HEK293 cells expressing the P2Y₁-GFP receptor. A maximal response is obtained when all receptors are occupied by the ligand. Therefore, for measuring the effect of **18a** binding to the P2Y₁ receptor on its fluorescence spectrum, we used a concentration of the agonist that elicited a maximal response in the pharmacological assay. We can assume that, under these conditions, a significant amount of the ligand is bound to the receptor and that we would be able to detect any perturbation of its spectrum if binding to the receptor affected it. Importantly, the spectrum of **18a** when bound to P2Y₁-R was not significantly different from the one measured without cells or when incubated with 1321N1 cells. Nucleotide **18a** displayed an almost superimposable spectrum in all cases with λ_{max}=430 nm (data not shown). Therefore, we assume that the specific binding of the compound to the receptor has a minor effect on its fluorescence properties.

Evaluation of the probes as substrates/inhibitors of NTPDases 1 and 2

We first evaluated analogues **18a–f** as potential substrates of human NTPDases 1 and 2. These two enzymes were selected due to their different substrate preferences. NTPDase1 hydrolyzes di- and triphosphonucleosides equally well to generate the corresponding monophosphonucleoside, while NTPDase2 prefers triphosphonucleosides.^[47] All the analogues tested were hydrolyzed by NTPDases 1 and 2. Analogues **18a** and **18c** were hydrolyzed at about 80% of the rate of the parent compound, ATP or GTP, respectively (Figure 3). Apparently, N²,N³-etheno modification of the purine ring did not significantly affect the recognition of **18a** or **c** by NTPDases 1 and 2. However, NTPDase1 was more affected by C6 substitutions (**18b**, **18d**, and **18f**) on the nucleotides, as these analogues were hydrolyzed less efficiently by NTPDase1 than by NTPDase2. Only **18e**, bearing dibenzyl substituents at N⁶ affected both enzymes equally, with hydrolysis of 27±2% and 22±1% for NTPDases 1 and 2, respectively (Figure 3). Taken together, these results suggest that the N²,N³-etheno modification of the adenine ring to generate fluorescent probes did not alter the interaction with NTPDases significantly.

Secondly, we also tested whether **18e** and monophosphonucleoside analogues **19a–f** could block NTPDase1 activity. Comparison of ATP hydrolysis with or without **18e** showed that this nucleotide blocked ATPase activity of human NTPDase1 by $69\pm 6\%$ ($p < 0.05$; Figure 4). The real inhibition might be underestimated, as **18e** can also be hydrolyzed by NTPDase1 at 27% of the rate of ATP (Figure 3A) and as the malachite green assay does not discriminate phosphate origin. Analogue **19e** also blocked human NTPDase1 activity by a significant $46\pm 5\%$ ($p < 0.05$). However, none of the other monophosphonucleosides tested (AMP, GMP, and **19a–f**) affected ATP hydrolysis by NTPDase1.

Next, we measured the fluorescence spectra of **18a**, **18d**, **18f**, and their monophosphate homologues, **19a**, **19d**, and **19f**, in the presence or absence of human NTPDase2. We chose NTPDase2 over NTPDase1 for these experiments as the former was not affected by the etheno modification on the adenine ring (Figure 3) and, more importantly, because NTPDase2 dephosphorylates triphosphonucleoside to the diphosphonucleoside derivative with little formation of monophosphate nucleoside.^[47] This reaction should therefore be easier to interpret when compared to the more complex NTPDase1 reaction with triphosphonucleosides. For the three analogues tested (**18a**, **18d**, and **18f**), we observed that the fluorescence intensity (Figure 5A) and emission spectra (Figure 5B) remained constant over the 20 min tested while they were hydrolyzed by human NTPDase2, as measured by the malachite green method (data not shown). Effectively, even with 54% hydrolysis, the fluorescence intensity of **18a** did not change significantly (data not shown). This observation excludes a lower fluorescent intensity of formed product or a rapid intensity decay of the probe. Moreover, maximum emission for the free nucleotide does not shift upon the addition of the enzyme (Figure 5B); this implies that the catalytic site does not provide an acidic environment (cf. Table 2). These observations indicate that the fluorescence of the modified nucleotides does not appear to change in the presence of NTPDase2, at least not in a significant manner.

Conclusion

We have developed three short, facile, and regiospecific syntheses of N^2,N^3 - ϵ -A, **4a**, starting from 2-amino-6-chloropurine riboside (**6**) or 2-amino-6-thiomethylpurine riboside (**16**). Our synthetic procedures also proved efficient for the known N^2,N^3 - ϵ -G (**3**). In earlier syntheses, N^2,N^3 - ϵ -G was achieved in a 25–48% yield,^[23,48] whereas in our procedure (Scheme 1), this compound was obtained in an 86% yield. The best synthetic pathway for the preparation of **4a** was a three-step synthesis starting from thioguanosine **15** (Scheme 4).

We have demonstrated the superior fluorescent properties of N^2,N^3 - ϵ -A analogues compared to those of adenosine. Analogue **4a** has $\lambda_{\max}=420$ nm in water ($\phi=0.03$) and has similar fluorescence properties to those of N^2,N^3 - ϵ -G (**3**) and N^1,N^6 - ϵ -A (**1**).^[3] However, unlike the latter, **4a** exhibits significant fluorescence even at acidic pH. Analogue, N^2,N^3 - ϵ -6-methylthiopurine riboside (**17a**) exhibits remarkable fluorescence ($\lambda_{\max}=518$ nm in water, $\phi=0.13$).

N^2,N^3 - ϵ -ATP (**18a**) proved to be nearly equipotent with ATP at P2Y₁-R and NTPDases 1 and 2. Particularly, the extension at the ATP's C2 and N3 positions is tolerated by both types

of protein. Furthermore, **18a** apparently preserves the H-bonding pattern of ATP, as the major interactions of these proteins with the adenine moiety are at the N1 and N⁶ positions. The importance of H-bonding interactions of the N⁶ amine with the protein binding pocket is demonstrated by the significant reduction of the affinities of **18d** and **18f** to P2Y₁-R and of **18e** to NTPDase2. In addition, analogues bound to NTPDase2 and the P2Y₁ receptor appeared to be fluorescent.

Based on the unique fluorescent and H-bonding properties, we propose the novel adenosine and ATP analogues **4a** and **18a**, respectively, as useful probes for studies of adenosine- and ATP-binding proteins. Furthermore, the novel analogues may be applied to the detection and exploration of nucleic acids. These applications will be reported in due course.

Experimental Section

General

New compounds were characterized and resonances were assigned by proton and carbon NMR spectroscopy on Bruker AC-200/DPX-300 and DMX-600 spectrometers. The HOD signal (4.78 ppm) was used as a reference for samples in D₂O. Nucleoside derivatives were characterized by ESI/MS on a Q-TOF micro mass spectrometer (Micromass-Waters, Corp., UK). High-resolution mass spectra were obtained on an ApexIII Bruker mass spectrometer (ESI/MS).

Bromoacetaldehyde, used for the synthesis of the etheno derivatives, was prepared as follows: a mixture of bromoacetaldehyde diethyl acetal (5 mL), HCl (1 M, 15 mL), and ethanol (5 mL) was stirred for 3 days at 37°C. No phase separation occurred when the mixture was brought to room temperature. The final solution contained approximately 1.3 mmol bromoacetaldehyde in 1 mL. For the preparation of the etheno products, we used a NH₄HCO₃/HCl buffer (pH 4.5). This buffer proved superior to the commonly used NaOAc/HOAc, as the former can be removed by freeze-drying and enables simple and easy purification of the etheno product. Chlorination and amination reactions were performed in flame-dried flasks under Ar. Reagents were predried in a vacuum oven overnight. POCl₃, acetonitrile and aromatic amines were freshly distilled. DMSO was dried over activated 4 Å molecular sieves. Compounds **17a** and **4a** were separated on an HPFC automated flash purification system (SP1, Biotage Inc.).

N²,N³-Ethenoguanosine **3**

2-(4-(O-benzyl)-1H-imidazo[2,1-e]purine-1-yl)-tetrahydro-5-(hydroxymethyl) furan-3,4-diol: A solution of bromoacetaldehyde (1.3 M, 0.9 mL, 6 equiv) in NH₄HCO₃/HCl buffer (6 mL, pH 4.5) was added to a solution of 6-chloro-2-aminopurine riboside (**6**; 50 mg, 0.16 mmol) in EtOH (3 mL). The reaction mixture was stirred at 38°C for 30 h, then evaporated in vacuo to about half its original volume, and EtOH was removed. A few drops of aqueous sodium hydrogen carbonate (1 M) were added until pH 8 was attained. The mixture was diluted with water (100 mL) and freeze-dried. The product was purified on a silica-gel column and eluted with MeOH/CHCl₃ (1:1). Product **3** was obtained as a white solid in 86% yield (44 mg). ¹H NMR (600 MHz, D₂O): δ=8.03 (s, 1 H), 7.40 (d,

$J=2.8$ Hz, 1H), 7.21 (d, $J=2.8$ Hz, 1H), 5.89 (d, $J=3.3$ Hz, 1H), 4.75 (dd, $J=3.3$, 5.0 Hz, 1 H), 4.42 (t, $J=5.0$, 6.3 Hz, 1H), 4.23 (m, $J=3.0$, 3.6, 6.3 Hz, 1 H), 3.9 (dd, $J=3.0$, 11.8 Hz, 1 H), 3.83 ppm (dd, $J=3.6$, 11.8 Hz, 1H); ^{13}C NMR (600 MHz, D_2O): $\delta=153.1$, 150.0, 145.5, 139.7, 115.8, 107.5, 117.6, 88.7, 85.9, 73.9, 71.2, 62.1 ppm; HRMS calcd for $\text{C}_{12}\text{H}_{13}\text{N}_5\text{O}_5$: 307.1150, found 307.09166.

2',3',5'-Tri-O-acetylated-N²,N³-ethenoguanosine 7

2-(4-(oxo)-1H-imidazo[2,1-e]purine-1-yl)-tetrahydro-2,3,5-tri-O-acetyl-furan: A solution of **3** (200 mg, 0.58 mmol) in pyridine (0.8 mL) and acetic anhydride (124 μL , 8 equiv) was stirred at RT for 48 h, then the pyridine and acetic acid were evaporated in vacuo. The residue was washed with EtOH (4 \times), and the resulting solution was evaporated and purified on a silica-gel column by elution with MeOH/ CHCl_3 (1:1). Product **7** was obtained as yellow oil in 63% yield (178 mg). ^1H NMR (600 MHz, CHCl_3): $\delta=7.90$ (s, 1H; H-8), 7.67 (d, $J=2.4$ Hz, 1H; etheno), 7.26 (d, $J=2.4$ Hz, 1H; etheno), 6.03 (d, $J=4.8$ Hz, 1H; H-1'), 6.10 (dd, $J=4.8$, 5.4 Hz, 1H; H-2'), 5.80 (dd, $J=4.5$, 5.4 Hz, 1H; H-3'), 4.42 (dd, $J=3.0$, 11.4 Hz, 1H; H-4'), 4.39 (dd, $J=5.4$, 11.4 Hz, 1H; H-5'), 4.32 ppm (m, $J=3.0$, 4.5, 5.4 Hz, 1H; H-5'); ^{13}C NMR (600 MHz, CHCl_3): $\delta=170.9$ (CH_3), 169.8 (CH_3), 169.5 (CH_3), 151.6 (C-6), 146.0 (C-4), 145.9 (C-2), 137.6 (C-8), 114.9 (C-5), 107.6 (etheno), 115.9 (etheno), 87.1 (C-1'), 79.8 (C-4'), 72.3 (C-2'), 70.6 (C-3'), 63.0 (C-5'), 20.7 (CO), 20.5 (CO), 20.4 ppm (CO); MS: m/z : 434 [$M+\text{H}$] $^+$.

N²,N³-Etheno-6-chloro-2',3',5'-tri-O-acetyluracil riboside 8

2-(4-(chloro)-1H-imidazo[2,1-e]purine-1-yl)-tetrahydro-2,3,5-tri-O-acetyl-furan: Phosphoryl chloride (448 μL , 6 equiv) and *N,N*-dimethylaniline (101 μL , 1 equiv) were added to a solution of predried **7** (347 mg, 0.81 mmol) in dry acetonitrile (3 mL). The reaction flask was then placed in an oil bath preheated to 70 $^\circ\text{C}$, and the solution was stirred and heated for 1 h. Volatile materials were then evaporated immediately in vacuo. The residue was dissolved in CHCl_3 (5 mL), and the solution was stirred vigorously with crushed ice for 15 min. The layers were separated, and the aqueous phase was extracted with CHCl_3 (3 \times 5 mL). The combined organic phase was kept cold by the addition of crushed ice and washed with cold water (6 \times 5 mL), 5% NaHCO_3 (5 mL) and water (2 \times 5 mL) to pH 7. The organic phase was dried over MgSO_4 and filtered, and the solvent was evaporated in vacuo. Product **8** was obtained as a yellow oil in 45% yield (154 mg). ^1H NMR (300 MHz, CDCl_3): $\delta=7.97$ (d, $J=1.5$ Hz, 1H; etheno), 7.26 (d, $J=1.5$ Hz, 1H; etheno), 5.98 (d, $J=2.7$ Hz, 1H; H-1'), 5.91 (dd, $J=2.7$, 5.4 Hz, 1H; H-2'), 4.42 (dd, $J=5.4$, 3.6 Hz, 1H; H-3'), 4.23 (ddd, $J=2.4$, 3.0, 3.6 Hz, 1H; H-4'), 3.9 (dd, $J=2.4$, 12.6 Hz, 1H; H-5'), 3.61 (dd, $J=3.0$, 12.6 Hz, 1H; H-5'), 2.10–2.12 ppm (s, 9H; CH_3CO); ^{13}C NMR (600 MHz, CDCl_3): $\delta=170.3$ (C-6), 169.5 (CO), 169.4 (CO), 169.2 (CO), 150.0 (C-4), 145.5 (C-2), 139.7 (C-8), 115.8 (C-5), 107.5 (etheno), 117.6 (etheno), 88.7 (C-1'), 85.9 (C-4'), 73.9 (C-2'), 71.2 (C-3'), 62.1 ppm (C-5'); HRMS calcd for $\text{C}_{18}\text{H}_{17}\text{N}_5\text{O}_7\text{Cl}$: 450.1450, found 450.08110.

Attempts to chlorinate compound 7 under drastic conditions

A) SOCl_2 (1 mL) was added to a solution containing predried **7** (70 mg, 0.16 mmol) and DMF (1 drop). The reaction flask was placed in an oil bath preheated to 100 $^\circ\text{C}$ for 3 h.

Volatile materials were evaporated in vacuo. The residue was dissolved in CH₂Cl₂, and the solution was stirred vigorously with crushed ice. The layers were separated, and the organic phase was washed with 5% NaHCO₃ (5 mL), dried over MgSO₄, and filtered. The solvent was evaporated in vacuo. Product **9** was obtained in a quantitative yield. MS: *m/z*: 522 [*M*+H]⁺.

B) *N,N*-dimethylaniline (21 μL, 1 equiv) and phosphoryl chloride (91 μL, 6 equiv) were added to predried **7** (70 mg, 0.16 mmol) and predried tetraethylammonium chloride (53 mg, 2 equiv) at RT. The reaction flask was placed in an oil bath preheated to 120 °C, and the solution was heated under reflux with stirring for 3 h. Volatile materials were evaporated in vacuo. The residue was dissolved in CHCl₃ (5 mL), and the solution was stirred vigorously with crushed ice for 15 min. The layers were separated, and the aqueous phase was extracted with CHCl₃ (3×5 mL). The combined organic phase was kept cold by the addition of crushed ice. The organic phase was washed with cold water (6×5 mL), 5% NaHCO₃ (5 mL), and water (2×5 mL) to pH 7. The organic phase was dried over MgSO₄ and filtered, and the solvent was evaporated in vacuo. Product **10** was obtained in a quantitative yield. MS: *m/z*: 537 [*M*+H]⁺.

N²,N³-Etheno-6-chloro-2',3',5'-tri-O-acetyluracil riboside **8 by two-phase synthesis**

A solution of bromoacetaldehyde in HCl(aq) and EtOH (1.3 M, 3.2 mL, 6 equiv) and tetrabutylammonium bromide (2 equiv) were added to 2',3',5'-triacetylated-2-amino-6-chloropurine riboside (**12**; 300 mg, 0.7 mmol) dissolved in CHCl₃ (10 mL) and EtOH (15 mL). The pH of the mixture was adjusted to 4.5 throughout the entire reaction time by the dropwise addition of NH₄HCO₃ (1 M) and 10% HCl. The reaction mixture was stirred at 38 °C for 48 h. At the end of the reaction, a few drops of saturated aqueous NaHCO₃ were added until pH 8 was attained. Water (100 mL) was added to the reaction mixture, which was then freeze-dried and purified on a silica-gel column (elution with CHCl₃). Product **8** was obtained in 38% yield (12 mg) as yellow oil. In addition, by-product **7** was isolated in 8% yield (2.4 mg) as a white solid.

2',3',5'-Tri-O-acetylated-6-chloro-2-aminopurine riboside **12**

5-((2-amino-6-chloro-9H-purine-9-yl)-tetrahydro-2,3,5-tri-O-acetyl)furan: A) A solution of **6** (300 mg, 1 mmol) in pyridine (2 mL) and acetic anhydride (94 μL, 4 equiv) was stirred at RT for 48 h, then the pyridine and acetic acid were evaporated in vacuo. The residue was washed with EtOH (4×) and purified on a silica-gel column (elution with diethyl ether/EtOAc, 2.5:1). Product **12** was obtained as a white solid in 68% yield (287 mg). ¹H NMR (200 MHz, CDCl₃): δ=8.27 (s, 1H; H-8), 5.97 (d, *J*=5.5 Hz, 1H; H-1'), 4.4 (dd, *J*=5.2, 5.5 Hz, 1H; H-2'), 4.20 (dd, *J*=3.3, 5.2 Hz, 1H; H-3'), 5.40 (ddd, *J*= 3.0, 3.3, 5.6 Hz, 1H; H-4'), 4.35 (dd, *J*=3.0, 12.6 Hz, 1H; H-5'), 4.07 (dd, *J*=5.6, 12.6 Hz, 1H; H-5'), 2.10–2.12 ppm (s, 9H; CH₃CO); MS: *m/z*: 428 [*M*+H]⁺.

B) Alternatively, product **12** was obtained by chlorination of per-acetylated guanosine **11**. *N,N*-dimethylaniline (1.55 mL, 1 equiv) and phosphoryl chloride (6.83 mL, 6 equiv) were added to predried peracetylated guanosine (5 g, 0.012 mmol) in dry acetonitrile (25 mL). The flask was then placed in an oil bath preheated to 70 °C, and the solution was heated with

stirring for 1 h. Volatile materials were evaporated immediately in vacuo. The residue was dissolved in CHCl_3 (50 mL), and the solution was stirred vigorously with crushed ice for 15 min. The layers were separated, and the aqueous phase was extracted with CHCl_3 (5 \times). The combined organic phase was kept cold by the addition of crushed ice. It was then washed with cold water, 5% NaHCO_3 , and water to pH 7. The organic phase was dried over MgSO_4 and filtered, and the solvent was evaporated in vacuo. The residue was purified on a silica-gel column (elution with EtOAc /diethyl ether, 7:3). Product **12** was obtained as a white solid in 49% yield (2.6 g).

6-Methylthio-2-aminopurine riboside 16

5-(2-amino-6-methylthio-9H-purine-9-yl)tetrahydro-2-(hydroxymethyl)-furan-3,4-diol:

A) Sodium thiomethoxide (138 mg, 1.97 mmol, 3 equiv) was added to a solution of **6** (200 mg, 0.66 mmol) in dry DMSO (6 mL). The solution was stirred at 60 °C for 40 h. A new spot on TLC plates (CHCl_3 /MeOH 8:2) at $R_f=0.53$ indicated the formation of product **16**. The solvent was evaporated in vacuo, and the crude residue was purified on a silica-gel column (elution with CHCl_3 /MeOH 95:5). Product **16** was obtained in 26% yield (53.6 mg) as a light yellow solid. ^1H NMR (200 MHz, CDCl_3): $\delta=8.19$ (s, 1H; H-8), 5.87 (d, $J=6.3$ Hz, 1H; H-1'), 4.69 (t, $J=6.3, 11.4$ Hz, 1H; H-2'), 4.30 (m, 1H; H-3'), 4.12 (m, 1H; H-4'), 3.82 (dd, $J=2.1, 12.3$ Hz, 1H; H-5'), 3.78 (dd, $J=2.4, 12.3$ Hz, 1H; H-5'), 2.61 ppm (s, 3H; CH_3); MS: m/z . HRMS calcd for $\text{C}_{11}\text{H}_{16}\text{N}_5\text{O}_4\text{S}$: 314.3444, found 314.0918.

B) 6-Thioguanosine (300 mg, 1 mmol) was treated with 0.4 M sodium hydroxide and methyl iodide, as described before for 6-thioinosine.^[23] A new spot on TLC plates (CHCl_3 /MeOH 8:2) at $R_f=0.53$ indicated the quantitative formation of product **16**. The solution was freeze-dried, and the crude residue was purified on a silica-gel column (elution with CHCl_3 /MeOH 93:7). Product **16** was obtained in 84% yield (264.2 mg) as a white solid.

N²,N³-etheno-6-thiomethylpurine riboside 17a

Tetrahydro-2-(hydroxymethyl)-5-(4-methylthio-1H-imidazo[2,1-e]purin-1-yl)furan-3,4-diol:

Bromoacetaldehyde (1.3 M, 1.47 mL, 1.92 mmol, 6 equiv) was added to NH_4HCO_3 /HCl buffer (pH 4.5), and this solution was added to **16** (0.1 g, 0.32 mmol) in EtOH (1.75 mL). The resulting solution was stirred at 60 °C for 8 h. TLC (CHCl_3 /MeOH 8:2) showed a new spot at $R_f=0.46$. A few drops of aqueous NaHCO_3 (1 M) were added until pH 8 was attained. The mixture was then diluted with water (100 mL) and freeze-dried. Finally, the residue was purified on a silica-gel column and eluted with CHCl_3 /MeOH 75:25. Product **17a** was obtained in 64% yield (69 mg) as a light yellow solid. ^1H NMR (300 MHz, CD_3OD): $\delta=8.62$ (s, 1H; H-8), 7.82 (ABq, 1H; etheno), 7.71 (ABq, 1H; etheno), 6.01 (d, $J=6.3$ Hz, 1H; H-1'), 4.79 (dd, $J=5.4, 6.3$ Hz, 1H; H-2'), 4.41 (dd, $J=4.8, 5.4$ Hz, H-3', 1 H), 4.16 (dd, $J=2.4, 4.8$ Hz, 1H; H-4'), 3.89 (dd, $J=2.4, 12.3$ Hz, 1H; H-5'), 3.85 (dd, $J=4.8, 12.3$ Hz, 1H; H-5'), 3.23 ppm (s, 3H; CH_3); MS: m/z . HRMS calcd for $\text{C}_{13}\text{H}_{16}\text{N}_5\text{O}_4\text{S}$: 338.3664, found 338.0917.

N¹,N²-etheno-6-thiomethylpurine riboside 17b

Tetrahydro-2-(hydroxymethyl)-5-(9-methylthio-3H-imidazo[1,2-f]furan-3-ol): Product **17b** was obtained by following the same conditions as for product **17a**, starting from **16**

(0.56 mmol). The pH of the reaction was kept at 6.4 throughout the entire reaction time. TLC (CHCl₃/MeOH 8:2) showed a new spot at $R_f=0.37$ (**17b**). The reaction residue was purified on a silica-gel column by elution with CHCl₃/MeOH (92:8). Product **17b** was obtained in 47% yield (83.7 mg) as a light yellow solid, in addition some **17a** (15% yield, 24 mg) was formed. ¹H NMR (300 MHz, CD₃OD): $\delta=8.49$ (s, 1H; H-8), 8.17 (d, $J=1.5$ Hz, 1H; etheno), 7.83 (d, $J=1.5$ Hz, 1H; etheno), 6.33 (d, $J=5.1$ Hz, 1H; H-1'), 4.67 (t, $J=4.8, 5.1$ Hz, 1H; H-2'), 4.43 (t, $J=4.5, 4.8$ Hz, 1H; H-3'), 4.28 (m, $J=3.0, 3.6, 4.5$ Hz, 1H; H-4'), 3.86 (dd, $J=3.0, 12.3$ Hz, 1H; H-5'), 3.82 (dd, $J=3.6, 12.3$ Hz, 1H; H-5'), 3.35 ppm (s, 3H; CH₃); MS: m/z : 338 [$M+H$]⁺.

N²,N³-Ethenoadenosine (**4a**)

Tetrahydro-2-(hydroxymethyl)-5-(4-amino-1H-imidazo[2,1-e]purin-1-yl)furan-3,4-diol:

A) Product **8** (25 mg, 0.055 mmol) was dissolved in a solution of NH₃ (2 M) in EtOH (10 mL) in a thick, sealed ampoule. The solution was stirred at 100°C for 24 h, and then at room temperature overnight. A new spot on TLC plates (MeOH) at $R_f=0.63$ indicated the formation of product **4a**. The solvent was evaporated to dryness in vacuo, and the residue was separated on a silica-gel column (CHCl₃/MeOH 1:1). Product **4a** was obtained in 43% yield (7.3 mg) as a light yellow solid. ¹H NMR (300 MHz, CD₃OD): $\delta=8.37$ (s, 1H; H-8), 7.78 (d, $J=1.8$ Hz, 1H; etheno), 7.26 (d, $J=1.8$ Hz, 1H; etheno), 6.25 (d, $J=4.8$ Hz, 1H; H-1'), 4.63 (dd, $J=4.8, 5.1$ Hz, 1H; H-2'), 4.38 (dd, $J=4.5, 5.1$ Hz, 1H; H-3'), 4.25 (ddd, $J=3.0, 3.3, 4.5$ Hz, 1H; H-4'), 3.85 (dd, $J=3.0, 12.3$ Hz, 1H; H-5'), 3.82 ppm (dd, $J=3.3, 12.3$ Hz, 1H; H-5'); ¹³C NMR (300 MHz, CD₃OD): $\delta=191.7$ (C-6), 130.6 (C-4), 136.9 (C-2), 137.9 (C-8), 118.2 (C-5), 125.6 (etheno), 109.8 (etheno), 87.9 (C-1'), 49.56 (C-4'), 76.7 (C-2'), 71.7 (C-3'), 62.1 ppm (C-5'); MS: m/z : HRMS calcd for C₁₂H₁₅N₆O₄: 307.2885, found 307.1149.

B) Alternatively, **17a** (63 mg, 0.186 mmol) was dissolved in a solution of NH₃ (2 M) in EtOH (15 mL) in a thick, sealed ampoule. The solution was stirred at 100°C for 14 h. A new spot on TLC plates (CHCl₃/MeOH 8:2) at $R_f=0.07$ indicated the formation of product **4a**. The solvent was evaporated to dryness in vacuo, and the crude residue was purified on a silica-gel column (elution with CHCl₃/MeOH 6:4). Product **4a** was obtained in 68% yield (38.5 mg) as a light yellow solid.

General procedure for the treatment of 2-amino-6-chloropurine riboside **12** with aromatic amines for the preparation of **13a–d**

Aromatic amine analogue (6 equiv) was added to a solution of the 2-amino-6-chloropurine riboside **12** (0.33 mmol) in EtOH (10 mL). The reaction mixture was heated at 100°C for 18 h. Once TLC (CHCl₃/MeOH 9:1) indicated that the reaction was completed, the mixture was concentrated under reduced pressure. The crude products were purified by flash column chromatography on silica gel with 90% CHCl₃/MeOH. Yields: **13a**: 92% (112.9 mg), **13b**: 91% (167.8 mg), **13c**: 91% (130.0 mg), and **13d**: 90% (137.5 mg).

2-Amino-N⁶-benzylpurine riboside **13a**

5-(2-amino-6-(N-benzyl)-9H-purine-9-yl)tetrahydro-2-(hydroxymethyl)-furan-3,4-

diol: ¹H NMR (600 MHz, CDCl₃): $\delta=7.39$ (s, 1H; H-8), 7.29 (m, 5H; benzyl), 5.66 (d, $J=9$

Hz, 1H; H-1'), 5.09 (s, 2H; CH₂), 4.86 (t, *J*=9, 18 Hz, 1H; H-2'), 4.30 (m, 1H; H-3'), 3.79 (m, 1H; H-4'), 3.64–3.57 ppm (m, 2H; H-5'); MS: *m/z*: 372 [*M*+H]⁺; HRMS calcd for C₁₇H₂₁N₆O₄: 373.3866, found 373.1624.

2',3',5'-Tri-O-acetylated-2-amino-N⁶-(2,3-dimethoxybenzyl)purine riboside 13b

5-(2-amino)-6-(2,3-dimethoxybenzyl)-9H-purin-9-yl)tetrahydro-2,3,5-(tri-O-acetyl)furan: ¹H NMR (200 MHz, CD₃OD): δ=7.86 (s, 1H; H-8), 7.57 (m, 2H; benzyl), 7.30 (s, 1H; benzyl), 6.41 (d, *J*=5.0 Hz, 1H; H-1'), 5.98 (t, *J*=5.0, 5.6 Hz, 1H; H-2'), 5.84 (t, *J*=5.1, 5.6 Hz, 1H; H-3'), 5.27 (s, 2H; CH₂), 4.36 (m, 2H; H-4', H-5'), 3.86 (s, 3H; OCH₃), 3.77 (s, 3H; OCH₃), 2.07–2.11 ppm (s, 9H, CH₃); MS: *m/z*: HRMS calcd for C₂₅H₃₁N₆O₉: 559.5529, found 559.2147.

2-Amino-N⁶-(2,3-dimethoxybenzyl)purine riboside 13c

5-(2-amino-6-(2,3-dimethoxybenzyl)-9H-purine-9-yl)tetrahydro-2-(hydroxymethyl)furan-3,4-diol: ¹H NMR (300 MHz, CDCl₃): δ=7.68 (s, 1H; H-8), 7.25 (m, 3H; benzyl), 6.05 (d, *J*=6.6 Hz, 1H; H-1'), 5.88 (s, 2H; CH₂), 5.36 (m, 1H; H-2'), 5.19 (m, 1H; H-3'), 4.72 (m, 1H; H-4'), 4.51 (m, 2H; H-5'), 4.18 (s, 3H; CH₃), 4.15 ppm (s, 3H; CH₃); MS: *m/z*: 433 [*M*+H]⁺; HRMS calcd for C₁₉H₂₄N₆O₆: 432.4307, found 432.1757.

2-Amino-N⁶,N⁶-dibenzylpurine riboside 13d

5-(2-amino-6-(N-dibenzyl)-9H-purine-9-yl)tetrahydro-2-(hydroxymethyl)furan-3,4-diol: ¹H NMR (600 MHz, CDCl₃): δ=7.83 (s, 1H; H-8), 7.39 (m, 5H; benzyl), 5.68 (d, *J*=7.5 Hz, 1H; H-1'), 4.93 (s, 2H; CH₂), 4.38 (s, 2H; CH₂), 5.18 (m, 1H; H-2'), 4.33 (m, 1H; H-3'), 3.95 (m, 1H; H-4'), 3.87 ppm (m, 2H; H-5'); MS: *m/z*: 463 [*M*+H]⁺; HRMS calcd for C₂₄H₂₆N₆O₄: 462.4012, found 462.2016.

Reaction of 6-aromatic amine-2-aminopurine riboside derivatives 13a–d with bromoacetaldehyde to produce N²,N³-etheno products 14a–d

Bromoacetaldehyde (1.3 M, 6 equiv) was added to a solution of 6-aromatic amine-2-aminopurine riboside (0.30 mmol) in a solution of H₂O/NH₄HCO₃/HCl (6 mL, pH 4.5) and EtOH (3 mL). The reaction mixture was stirred at 38°C for 20 h. TLC showed the disappearance of starting material, and the presence of a new spot of the product at *R*_F=0.63, 0.35, 0.36, or 0.46, respectively. The mixture was evaporated in vacuo to about half its original volume to remove EtOH. A few drops of saturated aqueous NaHCO₃ were added until pH 8 was attained. Then the mixture was diluted by adding water (100 mL) and freeze-dried overnight. Yields: **14a**: 87% (103.6 mg), **14b**: 73% (68.0 mg), **14c**: 61% (89.1 mg), and **14d**: 73% (99.8 mg).

2',3',5'-Tri-O-acetylated-N⁶-(2,3-dimethoxybenzyl)-N²,N³-ethenopurine riboside 14b

2-(4-(2,3-dimethoxybenzyl)-1H-imidazo[2,1-e]purin-1-yl)tetrahydro-2,3,5-(tri-O-acetate)furan: ¹H NMR (300 MHz, CD₃OD): δ=8.436 (s, 1H; H-8), 8.02 (d, *J*=3.9 Hz, 1H; etheno), 7.73 (d, *J*=3.9 Hz, 1H; etheno), 6.95 (m, 5H; benzyl), 6.14 (d, *J*=5.4 Hz, 1H; H-1'), 5.67 (2H; CH₂), 4.96 (s, 3H; CH₃), 4.80 (s, 3H; CH₃), 4.71 (t, *J*=4.8, 5.4 Hz, 1H; H-2'), 4.43

(t, $J=4.8, 5.1$ Hz, 1H; H-3'), 4.17 (m, $J=3.0, 3.3, 5.1$ Hz, 1H; H-4'), 3.88 ppm (dd, $J=3.0, 3.3, 15.3$ Hz, 2H; H-5'); ^{13}C NMR (300 MHz, CD_3OD): $\delta=154.2$ (C-6), 131.9 (C-4), 142.6 (C-2), 125.3 (C-8), 121.5 (C-5), 113.8 (etheno), 108.2 (etheno), 89.8 (C-1'), 62.8 (C-4'), 87.2 (C-2'), 75.7 (C-3'), 72.0 (C-5'), 56.3 (s, CH_3), 49.6 (s, CH_3) 44.7 (benzyl) 49.8–48.1 (m, benzyl), 129.2–128.6 ppm (benzyl); MS: m/z : HRMS calcd for $\text{C}_{27}\text{H}_{31}\text{N}_6\text{O}_9$: 583.5749, found 583.2147.

N^6 -benzyl- N^2 , N^3 -ethenopurine riboside 14a

2-(4-benzyl-1H-imidazo[2,1-e]purin-1-yl)tetrahydro-5-(hydroxymethyl)-furan-3,4-diol: ^1H NMR (600 MHz, CD_3OD): $\delta=8.49$ (s, 1H; H-8), 8.19 (ABq, 1H; etheno), 7.75 (ABq, 1H; etheno), 6.08 (d, $J=2.4$ Hz, 1H; H-1'), 5.52 (dd, $J=7.5, 9$ Hz, 2H; CH_2), 4.70 (t, $J=2.4, 5.4$ Hz, 1H; H-2'), 4.38 (t, $J=5.4, 5.7$ Hz, 1H; H-3'), 4.15 (m, 1H; H-4'), 3.84 (dd, $J=1.5, 6$ Hz, 1H; H-5'), 3.76 ppm (dd, $J=1.5, 6.3$ Hz, 1H; H-5'); ^{13}C NMR (600 MHz, CD_3OD): $\delta=154.61$ (C-6), 138.71 (C-4), 145.63 (C-2), 142.75 (C-8), 113.78 (C-5), 108.93 (etheno), 121.34 (etheno), 89.68 (C-1'), 44.30 (C-4'), 75.81 (C-2'), 71.85 (C-3'), 62.73 (C-5'), 48.49 (CH_2), 128.32–130.09 ppm (benzyl); MS: m/z : 397 [$M+H$] $^+$; HRMS calcd for $\text{C}_{19}\text{H}_{21}\text{N}_6\text{O}_4$: 397.2492, found 397.1642.

N^6 -dibenzyl- N^2 , N^3 -ethenopurine riboside 14d

2-(4-dibenzyl-1H-imidazo[2,1-e]purin-1-yl)tetrahydro-5-(hydroxymethyl)furan-3,4-diol: ^1H NMR (600 MHz, CD_3OD): $\delta=8.46$ (s, 1H; H-8), 8.09 (ABq, 1H; etheno), 7.63 (ABq, 1H; etheno), 6.27 (d, $J=2.4$ Hz, 1H; H-1'), 4.73 (dd, $J=2.4, 5.1$ Hz, 1H; H-2'), 4.38 (dd, $J=2.4, 4.5$ Hz, 1H; H-3'), 4.27 (dd, $J=4.5, 6.0$ Hz, 1H; H-4'), 3.81 (dd, $J=1.5, 6.3$ Hz, 1H; H-5'), 3.75 ppm (dd, $J=1.5, 6.0$ Hz, 1H; H-5'); ^{13}C NMR (600 MHz, CD_3OD): $\delta=154.31$ (C-6), 137.42 (C-4), 144.82 (C-2), 136.71 (C-8), 119.51 (C-5), 119.32 (etheno), 111.12 (etheno), 90.88 (C-1'), 79.43 (C-4'), 76.38 (C-2'), 71.69 (C-3'), 61.97 (C-5'), 49.85–48.15 (2 CH_2), 128.63–129.20 ppm (benzyl); MS: m/z : 487 [$M+H$] $^+$; HRMS calcd for $\text{C}_{26}\text{H}_{27}\text{N}_6\text{O}_4$: 487.53065, found 487.2094.

N^6 -(2,3-dimethoxybenzyl)- N^2 , N^3 -ethenopurine riboside 14c

2-(4-(2,3-dimethoxybenzyl)-1H-imidazo[2,1-e]purin-1-yl)tetrahydro-5-(hydroxymethyl)furan-3,4-diol: ^1H NMR (300 MHz, CD_3OD): $\delta=8.436$ (s, 1H; H-8), 8.02 (ABq, 1H; etheno), 7.73 (ABq, 1H; etheno), 6.95 (m, 5H; benzyl), 6.14 (d, $J=5.5$ Hz, 1H; H-1'), 5.67 (s, 2H; CH_2), 4.96 (s, 3H; CH_3), 4.80 (s, 3H; CH_3), 4.71 (dd, $J=4.5, 5.5$ Hz, 1H; H-2'), 4.43 (dd, $J=4.5, 5.8$ Hz, 1H; H-3'), 4.17 (m, 1H; H-4'), 3.88 ppm (m, 2H; H-5'); ^{13}C NMR (300 MHz, CD_3OD): $\delta=154.18$ (C-6), 131.99 (C-4), 142.60 (C-2), 125.31 (C-8), 121.46 (C-5), 113.77 (etheno), 108.18 (etheno), 89.76 (C-1'), 62.82 (C-4'), 87.22 (C-2'), 75.75 (C-3'), 72.03 (C-5'), 56.30 (s, CH_3), 49.56 (s, CH_3) 44.71 (benzyl) 49.85–48.15 (m, benzyl), 128.63–129.20 ppm (benzyl); MS: m/z : 456 [$M+H$] $^+$; HRMS calcd for $\text{C}_{21}\text{H}_{24}\text{N}_6\text{O}_6$: 456.4521, found 456.1757.

General procedure for the preparation of N^2 , N^3 -ethenonucleotides 18a–f

All phosphorylation reactions were carried out in flame-dried, argon-flushed, two-necked flasks sealed with rubber septa. Nucleosides were dried in vacuo for 2 days. The proton

sponge was kept in a desiccator. Phosphorus oxychloride was distilled and kept under N₂. Tri-*n*-butylammonium pyrophosphate solution was prepared as described before.^[49]

A solution of N²,N³-ethenoguanosine (**3**; 0.1 g, 0.32 mmol) in dry trimethyl phosphate (1.0 mL) was cooled to 0°C, then proton sponge (0.140 g, 2 equiv) was added. After 20 min, phosphorus oxychloride (91 mL, 3 equiv) was added dropwise. Stirring was continued for 2 h at 0°C. TLC on a silica gel plate (propan-1-ol 28%/NH₄OH/H₂O 11:7:2) indicated the disappearance of starting material and the formation of a polar product (*R*_F=0.35). A mixture of Bu₃N (0.31 mL) and (Bu₃NH⁺)₂P₂O₇H₂ (1 M) in DMF (2.0 mL) was added at once. After 1 min, a solution of triethylammonium bicarbonate (TEAB) in water (0.2 M 15.45 mL) was added, and the clear solution was stirred at RT for 45 min. The solution was then freeze-dried overnight. The semisolid obtained after freeze-drying was chromatographed on an activated Sephadex DEAE-A25 column. The resin was washed with deionized water for 30 min and loaded with the crude reaction residue dissolved in a minimal volume of water. The separation was monitored by UV detection (ISCO, UA-6) at 280 nm. A buffer gradient of H₂O (500 mL) to NH₄HCO₃ (0.5 M, 500 mL) was applied. The relevant fractions of the ATP and AMP derivatives were pooled and freeze-dried three times to yield a white solid. The final purification of the products was achieved on an HPLC system (Merck–Hitachi) by using a semipreparative reversed-phase LichroCART lichrospher 60, RP-select B column (Merck, Darmstadt, Germany) and a linear gradient of acetonitrile and 0.1 M triethylammonium acetate buffer (pH 7; solvent system I: linear gradient of acetonitrile/0.1 M TEAA buffer 15:85 to 45:55 over 25 min) with a flow rate of 6 mL min⁻¹. These reaction conditions provided the nucleoside triphosphate products in yields ranging from 31 to 63%. The corresponding nucleoside monophosphate analogues (**19a–f**) were obtained as by-products in all reactions. Triethylammonium salts of mono- and trinucleotides, obtained after HPLC separation, were passed through Sephadex-CM C-25 and eluted with deionized water to obtain the corresponding sodium salts after freeze drying.

N²,N³-ethenoadenosine-5'-triphosphate (**18 a**)

2-(4-amino-1H-imidazo[2,1-e]purin-1-yl)-3,4-dihydroxytetrahydrofuran- 5-triphosphate: Yield: 49% (24 mg); ¹H NMR (200 MHz, D₂O, pH 6): δ=8.51 (s, 1H; H-8), 7.78 (ABq, 2H; etheno), 7.65 (ABq, 2H; Ar), 6.12 (d, *J*=7.5 Hz, 1H; H-1'), H-2', H-3', and H-4' are hidden by the water peak, 4.42 ppm (m, 2H; H-5'); ³¹P NMR (200 MHz, D₂O, pH 6): δ=-9.91 (d), -10.92 (d), -22.58 ppm (t); *t*_R=3.41 min, solvent system I, 2:98–65:35, purity >95%, *t*_R=2.99 min, solvent system II: 5 mM tetrabutylammonium dihydrogenphosphate (TBAP) in MeOH/60 mM ammonium dihydrogenphosphate +5 mM TBAP in H₂O/MeOH (90:10) 25:75 to 75:25 over 20 min, purity >95%; FAB (negative mode): *m/z*: 545 [*M*⁴⁻+3H]⁺; HRFAB calcd for C₁₂H₁₆N₆O₁₃P₃: 544.9988, found 544.9993.

6-SMe-N²,N³-ethenopurine riboside-5'-triphosphate **18b**

2-(4-thiomethyl-1H-imidazo[2,1-e]purin-1-yl)-3,4-dihydroxytetrahydrofuran- 5-triphosphate: Yield: 58% (62 mg); ¹H NMR (200 MHz, D₂O, pH 6): δ=8.90 (s, 1H; H-8), 8.01 (ABq, 2H; etheno), 7.91 (ABq, 2H; Ar), 6.18 (d, *J*=2.1 Hz, 1H; H-1'), H-2' and H-3' are hidden by the water peak, 4.48 (m, 1H; H-4'), 4.33 (m, 2H; H-5'), 3.12 ppm (s, 3H;

SMe); ^{31}P NMR (200 MHz, D_2O , pH 6): δ = -9.85 (d), -10.85 (d), -22.32 ppm (t), t_{R} = 9.47 min, solvent system I, 2:98–65:35, purity >94%, t_{R} = 2.72 min, solvent system II, purity >95%. FAB (negative mode): m/z : 577 [$M^A - 3\text{H}$] $^+$.

N^2, N^3 -ethenoguanosine-5'-triphosphate (18 c)

2-(4-oxo-1H-imidazo[2,1-e]purin-1-yl)-3,4-dihydroxytetrahydrofuran-5-

triphosphate: Yield: 31% (19 mg); ^1H NMR (200 MHz, D_2O , pH 6): δ = 8.34 (s, 1H; H-8), 8.89 (ABq, 2H; etheno), 7.42 (ABq, 2H; Ar), 6.12 (d, J = 9.0 Hz, 1H; H-1'), 5.01 (dd, J = 7.2, 9.0 Hz, 1H; H-2'), H-3' and H-4' are hidden by the water peak, 4.43 ppm (m, 2H, H-5'); ^{31}P NMR (200 MHz, D_2O , pH 6): δ = -5.43 (d), -10.39 (d), -21.15 ppm (t); t_{R} = 3.71 min, solvent system I: 2:98–65:35, purity >95%, t_{R} = 2.24 min, solvent system II, purity >97%; FAB (negative mode): m/z : 546 [$M^A - \text{H}$] $^+$; HRFAB calcd for $\text{C}_{12}\text{H}_{15}\text{N}_5\text{O}_{14}\text{P}_3$: 545.9828, found 545.9857.

N^6 -Benzyl- N^2, N^3 -ethenoadenosine-5'-triphosphate (18 d)

2-(4-benzyl-1H-imidazo[2,1-e]purin-1-yl)-3,4-dihydroxytetrahydrofuran-5-

triphosphate: Yield: 63% (28 mg); ^1H NMR (200 MHz, D_2O , pH 6): δ = 8.18 (s, 1H; H-8), 7.65 (ABq, 2H; Ar), 7.32 (ABq, 2H; Ar), 6.12 (d, J = 8.1 Hz, 1H; H-1'), 4.72 (m, 1H; H-2'), 4.60 (m, 1H; H-3'), 4.43 (m, 1H; H-4'), 4.21 ppm (m, 2H; H-5'); ^{31}P NMR (200 MHz, D_2O , pH 6): δ = -6.23 (d), -10.44 (d), -21.24 ppm (t); t_{R} = 4.11 min, solvent system I, 15:85–45:55, purity >98%, t_{R} = 6.56 min, solvent system II, purity >95%; FAB (negative mode): m/z : 546 [$M^A - 3\text{H}$] $^+$; HRFAB calcd for $\text{C}_{19}\text{H}_{22}\text{N}_6\text{O}_{13}\text{P}_3$: 635.33172, found 635.0463.

N^6, N^6 -Dibenzyl- N^2, N^3 -ethenoadenosine-5'-triphosphate (18 e)

2-(4-dibenzyl-1H-imidazo[2,1-e]purin-1-yl)-3,4-dihydroxytetrahydrofuran-5-

triphosphate: Yield: 59% (33 mg); ^1H NMR (200 MHz, D_2O , pH 6): δ = 8.42 (s, 1H; H-8), 8.00 (ABq, 2H; Ar), 7.78 (ABq, 2H; Ar), 7.35 (m, 10H; benzyl), 6.30 (d, J = 8.3 Hz, 1H; H-1'), 5.27 (s, 4H; benzyl), H-2' and H-3' are hidden by the water peak, 4.50 (m, 1H; H-4'), 4.25 ppm (m, 2H; H-5'); ^{31}P NMR (200 MHz, D_2O , pH 6): δ = -5.37 (d), -10.67 (d), -21.27 ppm (t); t_{R} = 9.79 min, solvent system I, purity >99 %, 25:75–40:60, t_{R} = 13.44 min, solvent system II, 40:60–90:10, purity >95 %; FAB (negative mode): m/z : 725 [$M^A + 3\text{H}$] $^+$; HRFAB calcd for $\text{C}_{26}\text{H}_{28}\text{N}_6\text{O}_{13}\text{P}_3$: 725.3391, found 725.0932.

N^6 -2,3-Dimethoxybenzyl- N^2, N^3 -ethenoadenosine-5'-triphosphate (18 f)

2-(4-(2,3-dimethoxybenzyl)-1H-imidazo[2,1-e]purin-1-yl)-3,4-

dihydroxytetrahydrofuran-5-triphosphate: Yield: 58% (42 mg); ^1H NMR (200 MHz, D_2O , pH 6): δ = 8.30 (s, 1H; H-8), 7.71 (ABq, 2H; Ar), 7.57 (ABq, 2H; Ar), 6.87 (m, 3H; benzyl), 6.00 (d, J = 8.1 Hz, 1H; H-1'), 5.24 (s, 2H; benzyl), H-2', H-3', and H-4' are hidden by the water peak, 4.26 (m, 2H; H-5'), 3.78 (s, 3H; OCH_3), 3.72 ppm (s, 3H; OCH_3); ^{31}P NMR (200 MHz, D_2O , pH 6) δ = -6.59 (d), -10.40 (d), -21.28 ppm (t); t_{R} = 6.13 min, solvent system I, purity >99 %, 15:85–45:55, t_{R} = 11.87 min, solvent system II, purity >95 %; FAB (negative mode): m/z : 695 [$M^A + 3\text{H}$] $^+$; HRFAB calcd for $\text{C}_{21}\text{H}_{26}\text{N}_6\text{O}_{15}\text{P}_3$: 695.0668 found, 695.06713.

Absorption spectra

Absorption spectra of compounds **4a** and **17a** were determined in dilute HCl or NaOH over the pH range 1.6–12.3. The concentration of samples of **4a** and **17a** in aqueous solutions and in organic solvents was of the order of 4.85×10^{-5} M.

Emission spectra

The measurement conditions of compounds **4a** and **17a** included 740 V sensitivity and a 4- or 2-nm slit for the emission spectra in aqueous or organic medium, respectively. Emission spectra were corrected by the subtraction of the medium's emission spectrum. Spectra were determined in dilute HCl or NaOH over the pH range 1.6–12.3. The concentration of the aqueous or organic samples was of the order of 4.85×10^{-5} M. All organic solvents were freshly distilled prior to emission measurements. Measurements of the emission of the nucleoside analogues were conducted with subtraction of the solvent emission. The quantum yield of each compound was calculated from the observed absorbance at 290 nm and the area of the fluorescence emission band. Quinine sulfate was used as reference compound, and a quantum yield value of 0.55 was assumed.^[50]

Fluorescence studies of the **18a**–NTPDase2 complex

Emission spectra of **18a**, **18d**, **18 f**, and their monophosphate homologues (**19a**, **19d**, **19 f**) were measured in the presence of human NTPDase2 by using an Aminco–Bowman series 2 Luminescence Spectrometer. Measurements were done at 37°C at various excitation wavelengths (**18a**: 290 nm, **18d**: 315 nm, **18 f**: 334 nm) and 740–780 V sensitivity. The concentration of the samples was 2×10^{-4} M in NTPDase incubation mixture (5 mM CaCl₂ and 80 mM Tris/HCl, pH 7.4). Measurements of the emission spectra (**18a**: 425 nm, **18d**: 445 nm, **18 f**: 442 nm) were performed in fluorescent nucleosides (1.5 mL, 200 μM) without enzyme. A sample of NTPDase2-transfected COS-7 cells lysate (3.6 μg) was added, and then emission spectra were taken at 5 min intervals. Samples of the reaction mixture (200 μL) were taken before each emission measurement and added to malachite green reagent (50 μL) to estimate Pi released (see NTPDases activity assay).

Fluorescence studies of **P2Y₁**-R-bound **18a**

Fluorescence spectra were taken of **18a** in the presence of P2Y-receptor-expressing and P2Y-receptor-negative cells. Samples were excited at 330 nm, and the fluorescence intensity was detected from 340 to 520 nm by using a Perkin–Elmer LS50B fluorimeter. **18a** (10 μM) was first measured in physiological buffer (pH 7.4), then in the presence of HEK293 cells expressing the P2Y₁-GFP receptor. Cells were suspended in the buffer (NaHBS), which was used for the physiological experiments (100000 cells per mL). Finally, the fluorescence spectrum of **18a** was measured in the presence of 1321N1 cells (100000 cells per mL), which are known to lack endogenous P2Y receptor expression. Buffer and cells showed no fluorescence in the range determined (data not shown).

[Ca²⁺]_i measurements

The HEK293 cells expressing the rat P2Y₁ receptor at high density^[41] were plated on PLL-coated plates, and single cell measurements were made after 3 days, when the cells were 40–

60% confluent. The changes in free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were measured, as described before,^[51, 52] after preincubation of the cells with fura-2AM (2 μM) and with varying concentrations of MRS2179 (for the test of the P2Y_1 -receptor specificity of the fluorescent analogues) for 30 min at 37 °C in NaHBS (HEPES buffered saline solution: 145 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 25mM glucose, and 20 mM HEPES/Tris pH 7.4). Then the cells were stimulated under continuous superfusion of prewarmed NaHBS at 37°C with different concentrations of the different agonists and MRS2179 in 1 min agonist pulses at 37°C. Fluorescence intensity was recorded alternately at 340 and 380 nm excitation and 520 nm emission. Changes were monitored in single cells bathed in a perfusion chamber with a volume of 500 μL which was placed on the microscope stage of a fluorescence imaging system from TILL Photonics with a 40X/NA 1.30 oil immersion objective and a flow rate of 1 mL min^{-1} .^[53]

NTPDases activity assay

Protein extracts were obtained from COS-7 cells transiently transfected with human NTPDase 1 or 2, as previously described.^[47] Contaminating activity of intracellular ATPases or other ectonucleotidases was minimal; untransfected cells had no detectable ADPase activity and low intrinsic ATPase activity (less than 5% compared to transfected COS-7 cells lysate). The enzymatic assays were performed for 20 min at 37 °C in CaCl_2 (5 mM) and Tris/HCl (80 mM) incubation mixture (200 μL , pH 7.4) with the protein extracts in the presence of ATP, GTP, or N^2, N^3 - ϵ -adenosine triphosphates (100 μM) or an equal concentration of ATP and N^2, N^3 - ϵ -adenosine monophosphates (100 μM each) as indicated. For hydrolysis assay, reaction was started with ATP, GTP, or N^2, N^3 - ϵ -adenosine triphosphates (10 μL) and stopped with malachite green reagent (50 μL). For inhibition assays, enzyme extracts were preincubated for 3 min with monophosphate (**19a–f**) or **18e**, and reaction was started with ATP (100 μM) and stopped with malachite green reagent. Inorganic phosphate (P_i) liberated during the enzymatic reaction was quantified according to Baykov et al.^[54] Enzymatic activity was expressed as micromoles of P_i released per min per mg of protein. Protein concentration was estimated by Bradford microplate assays with bovine serum albumin as a standard of reference.^[55]

Statistical analysis

All measurements were made in three independent experiments, each performed in triplicate. The mean and the standard error of the mean (SEM) were calculated from the mean specific activity of enzyme in presence of nucleotides. Data were compared for difference by Student's t-test. All analyses were performed according to the Microsoft Excel software. *p* values of less than 0.01 were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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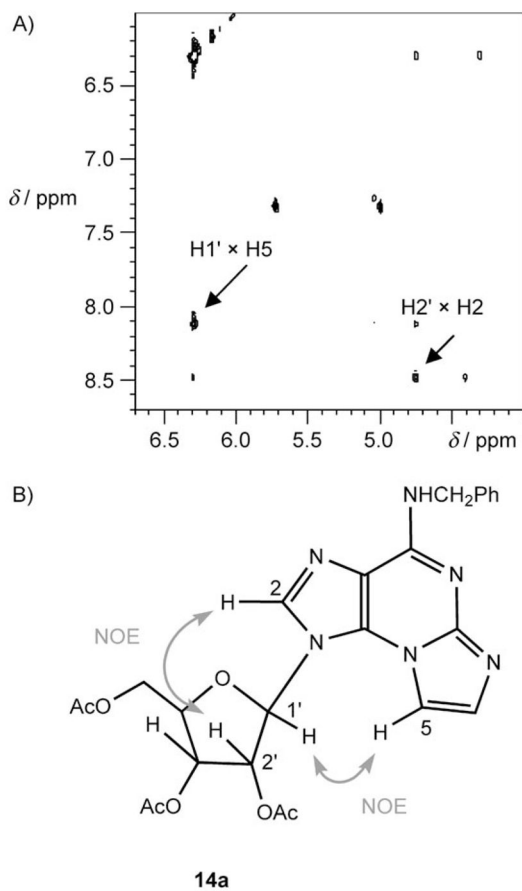


Figure 1. Assignment of the regiochemistry of an N²,N³-e-A product. A) ¹H,¹H-NOESY spectrum of product **14a**; B) NOE interactions in product **14a**.

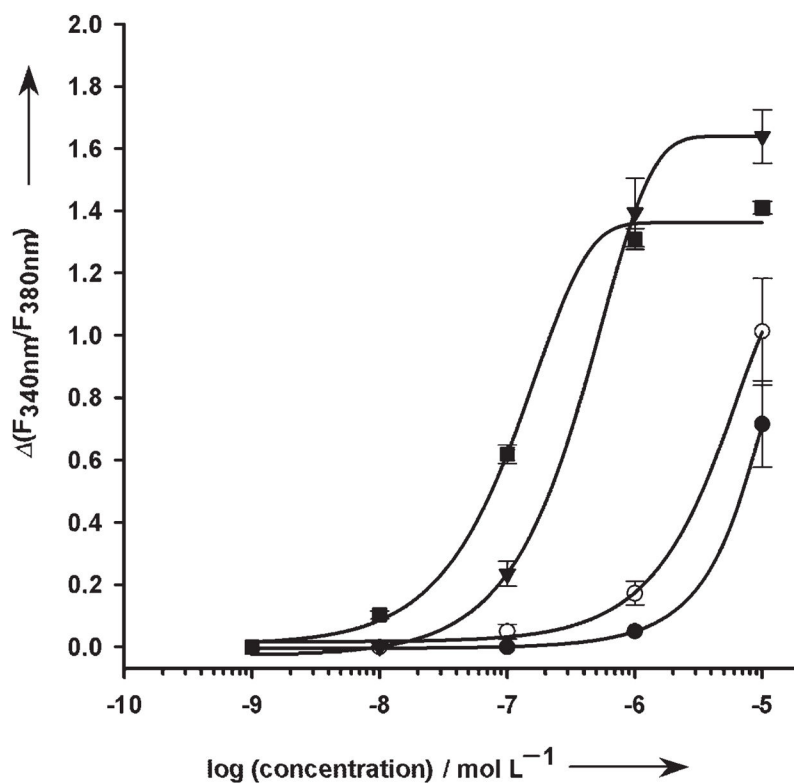


Figure 2. Concentration-response curves for fluorescent ATP analogues. Intracellular $[Ca^{2+}]$ release in HEK293 cells stably expressing the rP2Y₁ receptor was induced by the analogues. The potencies found for **18d** (○), **18f** (●), and **18a** (▼) are compared to the potency of ATP (■) at this receptor. Measurements of the intracellular $[Ca^{2+}]$ release were made as described in the Experimental section. Data represent the mean values and standard error of the mean obtained from 60–100 cells, which were measured in at least 3 independent experiments.

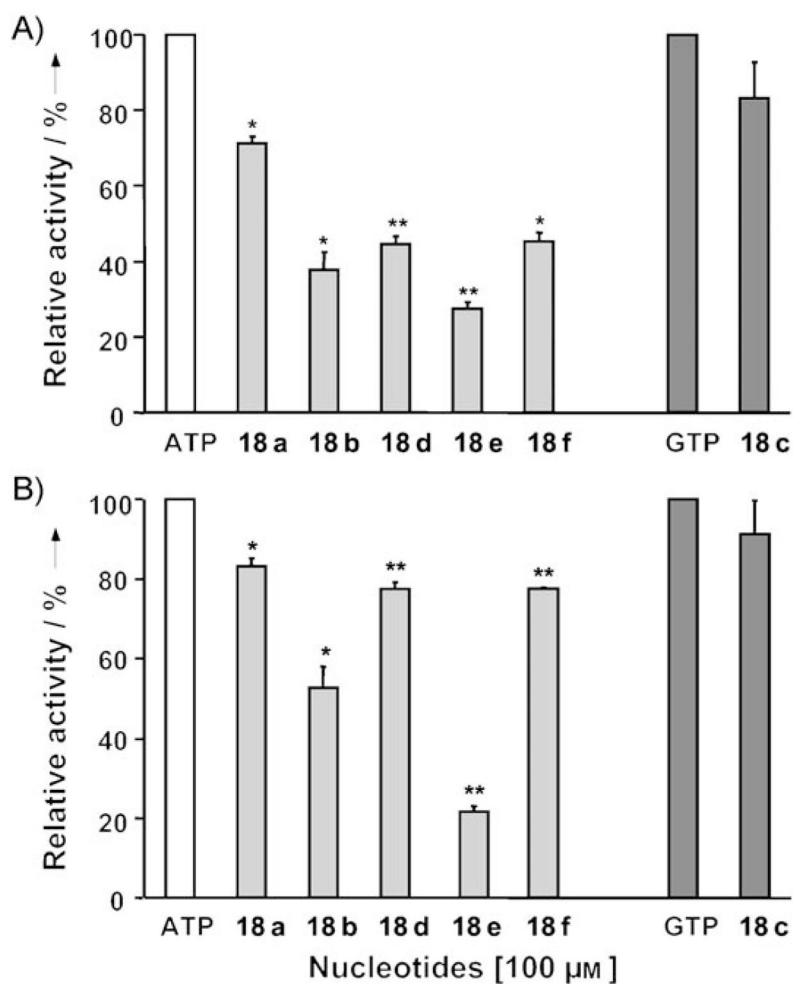


Figure 3. Hydrolysis of analogues **18a–f** by human A) NTPDase1 and B) NTPDase2. Analogues **18a–f** were incubated for 20 min at 37°C in the presence of lysates of COS-7 cells transfected with NTPDase1 or 2. Bars represent the mean±SEM of three independent experiments performed in triplicate, and significant differences compared to ATP (white bar), adenine nucleotide derivatives (light gray bars) or guanine nucleotide derivatives (dark gray bars) are indicated: * $p < 0.01$ and ** $p < 0.001$; 100% of ATP hydrolysis= $0.44 \pm 0.07 \mu\text{mol Pi min}^{-1} \text{mg}^{-1}$ for NTPDase1 and $0.7 \pm 0.2 \mu\text{mol Pi min}^{-1} \text{mg}^{-1}$ for NTPDase2 protein extracts; 100% of GTP hydrolysis= $0.5 \pm 0.1 \mu\text{mol Pi min}^{-1} \text{mg}^{-1}$ for NTPDase1 and $0.23 \pm 0.06 \mu\text{mol Pi min}^{-1} \text{mg}^{-1}$ for NTPDase2.

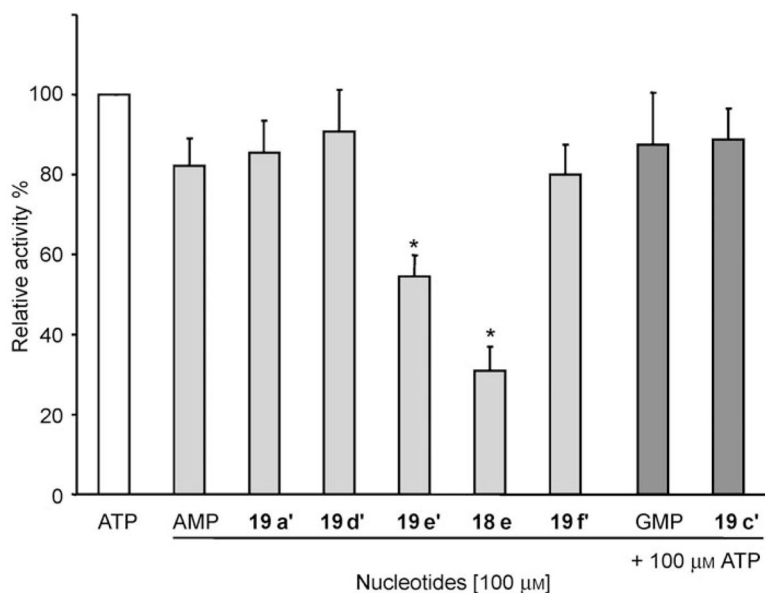


Figure 4. Hydrolysis of ATP by human NTPDase1 in the presence of N^2,N^3 -e-purine-5'-monophosphates (**19a**, **19c-f**)^[*] or triphosphate (**18e**). Each analogue was incubated for 20 min at 37°C in the presence of the substrate ATP and lysates of COS-7 cells transfected with NTPDase1. Hydrolysis of ATP alone ($0.56 \pm 0.06 \mu\text{mol Pi min}^{-1} \text{mg}^{-1}$ of NTPDase1-transfected protein extracts) was set as 100%. Bars represent the mean \pm SEM of three independent experiments each performed in triplicate. Only **18e** and **19e** significantly inhibited NTPDase1 (* $p < 0.01$).

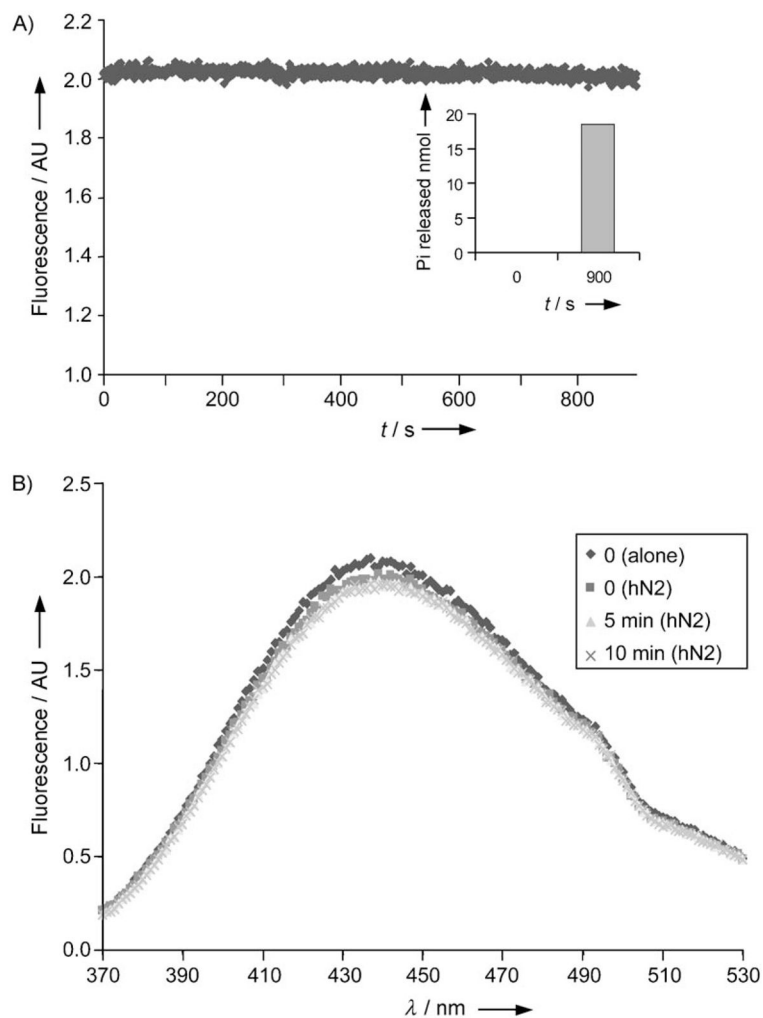
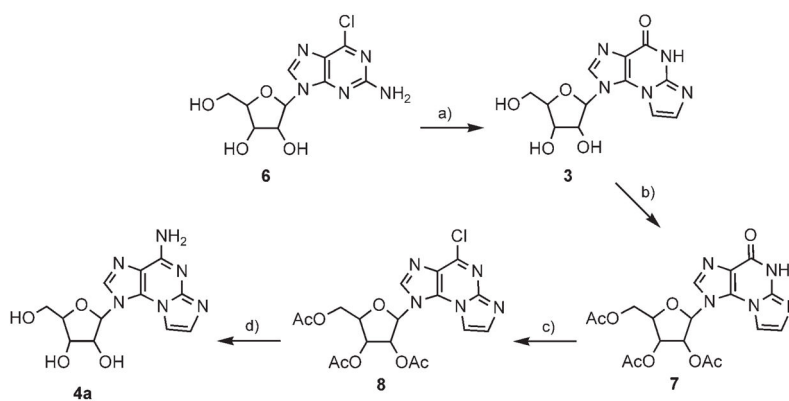
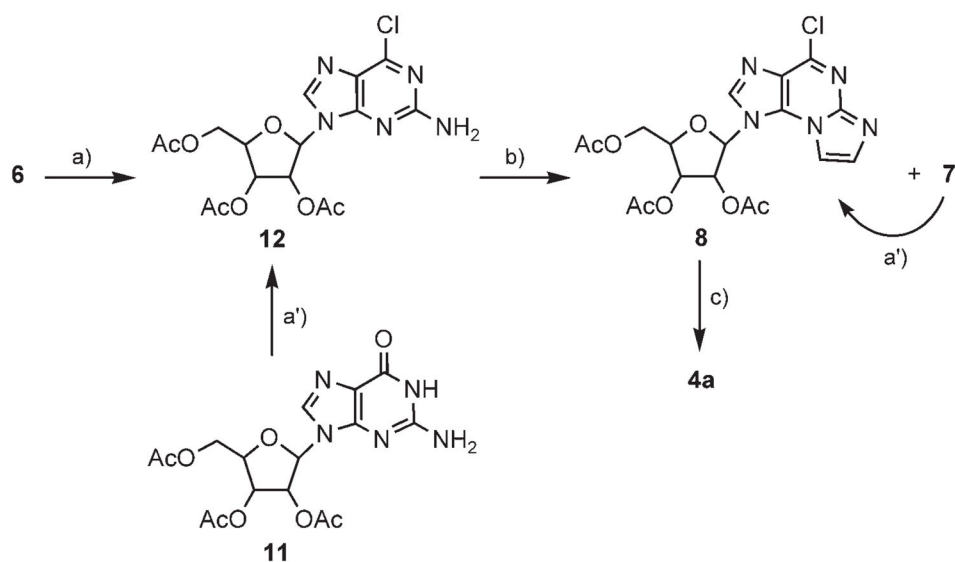


Figure 5.

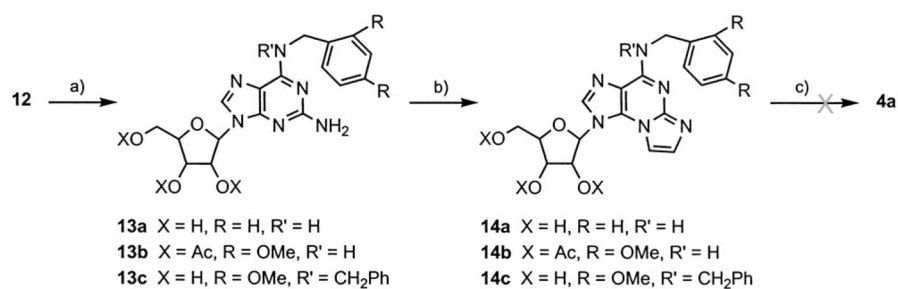
Fluorescence properties of **18d** in the presence of NTPDase2. A) Fluorescence was measured for 15 min during the hydrolysis of **18d** by human NTPDase2. Inset: Pi released reveals that about 10% of **18d** was hydrolyzed during the course of this reaction. B) Emission spectra of **18d**, excitation at 315 nm before the addition of human NTPDase2 (◆) and in the presence of NTPDase2 after 0, 5, and 10 min hydrolysis. A full spectrum was measured in 160 s. The spectra were almost identical. For both assays, protein extracts of COS-7 cells transiently transfected with human NTPDase2 (3.1 μ g) were added to 200 μ M **18d** in assay buffer (80 mM Tris-base, 5 mM CaCl_2 , pH 7.4).

**Scheme 1.**

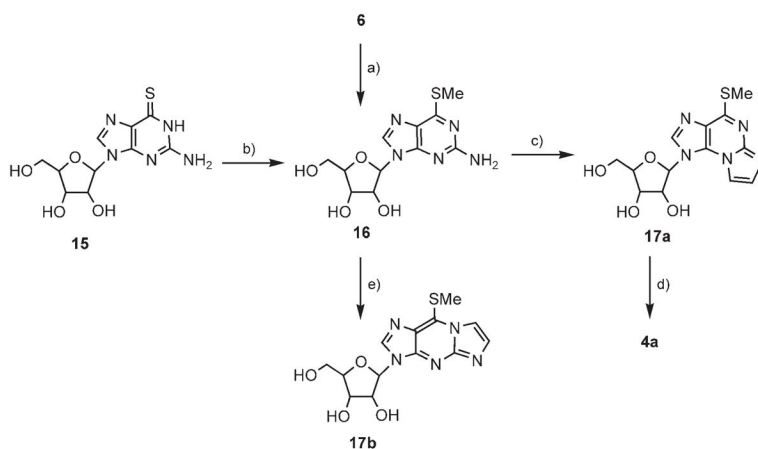
Synthesis of $N^2,N^3\text{-}\epsilon\text{-A}$ from 2-amino-6-chloro-purine riboside. Reagents and conditions: a) BrCH_2CHO , EtOH/buffer (pH 4.5), 38°C , 30 h (86%); b) $\text{Ac}_2\text{O/Py}$, RT, 48 h (63%); c) POCl_3 , N,N -dimethylaniline, CH_3CN , 70°C , 1 h (45%); d) 2 M NH_3/EtOH , 100°C , 24 h (43 %).

**Scheme 2.**

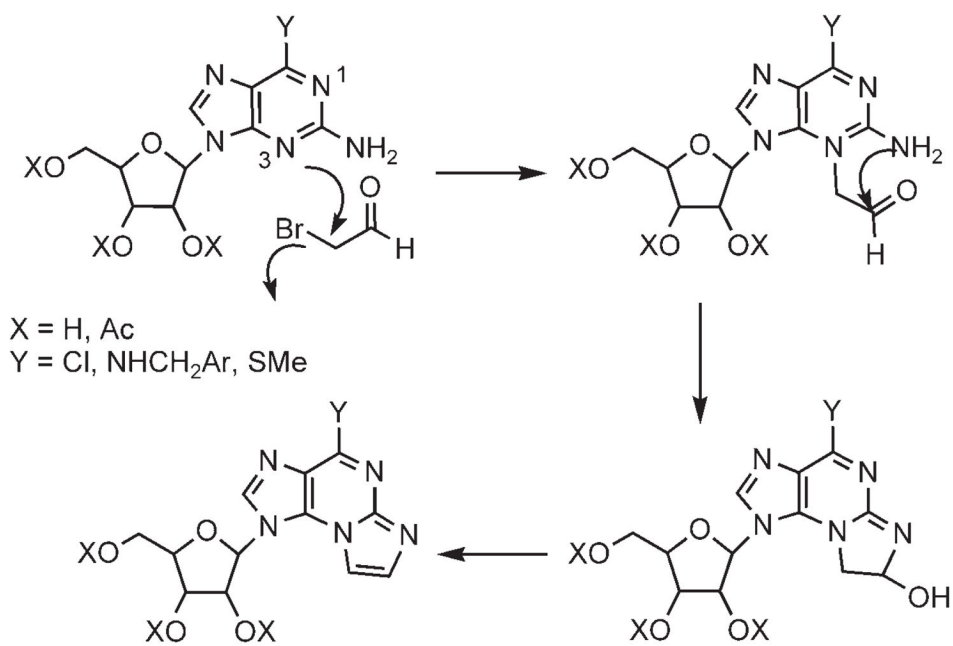
Two-phase synthesis of N²,N³-e-A from 2-amino-6-chloropurine riboside. Reagents and conditions: a) Ac₂O/Py, RT, 48 h (68 %); a') POCl₃, *N,N*-dimethylaniline, CH₃CN, 70°C, 1 h (49 %); b) BrCH₂CHO, CHCl₃/EtOH/buffer (pH 4.5), 38°C, 48 h (**8**: 38%, **7**: 8%); c) 2 M NH₃/EtOH, 100 °C, 24 h (43 %).

**Scheme 3.**

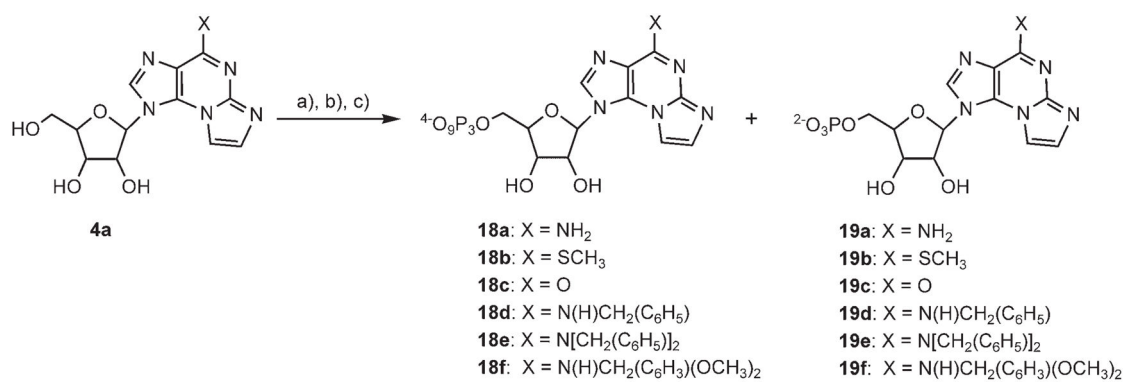
Attempted synthesis of **4a** from 2-amino-6-arylaminopurine riboside. Reagents and conditions: a) 2,4-di(*O*-methyl)benzylamine, EtOH, 60°C, 36 h (91%); b) BrCH₂CHO, CHCl₃, buffer (pH 4.5), 38 °C, 30 h (73 %); c) DDQ, CHCl₃, H₂O, RT, 12 h.

**Scheme 4.**

Synthesis of **4a** from 2-amino-6-(thiomethyl)purine riboside. Reagents and conditions: a) NaSMe, DMSO, 60°C, 40 h (26%); b) NaOH(aq), MeI (84%); c) BrCH₂CHO, EtOH/buffer (pH 4.5), 60°C, 8 h (64%); d) 2 M NH₃/EtOH, 100°C, 14 h (68%); e) BrCH₂CHO, EtOH/buffer (pH 6.4), 38°C, 30 h (47 %).



Scheme 5.
Mechanism of regiospecific formation of **4a**.

**Scheme 6.**

Preparation of nucleotides **18a–f**. Reagents and conditions: a) POCl₃, PO(OMe)₃, proton sponge; b) (Bu₃NH⁺)₂H₂P₂O₇²⁻; c) 0.2 M TEAB.

Table 1

N1,N² versus N²,N3- ϵ -cyclization of 2-amino purine riboside analogues.

Purine riboside C6-substituent	Product formed at	
	pH 4.5	pH 6.4
O	no reaction ^[a]	N1,N ² - ϵ -guanosine (5 ; 8%) ^[a]
OR	N ² ,N3- ϵ -purine riboside ^[a]	N ² ,N3- ϵ -purine riboside (18%+by-products) ^[b] N1,N ² - ϵ -purine riboside (55%) ^[c]
SR	N ² ,N3- ϵ -purine riboside	N1,N ² - ϵ -purine riboside (17b ; 47%) and N ² ,N3- ϵ -purine riboside (17a ; 15%)
NHCH ₂ Ar	N ² ,N3- ϵ -purine riboside	–
Cl	N ² ,N3- ϵ -purine riboside	–

^[a] ref. [19].

^[b] ref. [33].

^[c] ref. [22].

Table 2

Absorption and emission data of **4a** [4.85×10^{-5} M] at several pH values.

pH	Absorption		Emission	
	λ_{\max}	$\epsilon \times 10^3$	λ_{\max}	ϕ
1.6	224	6.17	396	0.021
	268	14.22		
7.0	234	12.58	420	0.032
	274	3.31		
12.3	234	13.89	420	0.031
	276	3.69		

Table 3

Emission data of **4a** at 4.85×10^{-5} M in various organic solvents.

Solvent	ϵ_d	ϕ	λ_{\max}
DMSO	47.6	0.085	458
DMF	37.6	0.082	448
dioxane	2.2	0.042	434

Table 4

Absorption and emission data of **17a** [4.85×10^{-5} M] at several pH values.

pH	Absorption		Emission	
	λ_{\max}	$\epsilon \times 10^3$	λ_{\max}	ϕ
1.6	227	16.52	492	0.013
	243	13.57	516	0.022
	301	7.39		
	331	7.13		
7.0	231	16.09	494	0.046
	247	13.25	518	0.107
	307	7.07		
	345	2.90		
12.3	233	19.29	494	0.021
	307	5.95	520	0.063