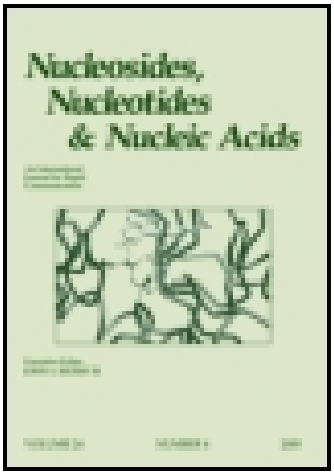

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Synthesis, Mechanism and Fluorescence Properties of 8-(Aryl)-3-β-D-Ribofuranosylimidazo[2,1-i]purine 5′-Phosphate Derivatives

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(5&6), 1033–1054 (2000)

SYNTHESIS, MECHANISM AND FLUORESCENCE PROPERTIES OF 8-(ARYL)-3-β-D-RIBOFURANOSYLIMIDAZO[2,1-i]PURINE 5'-PHOSPHATE DERIVATIVES

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ABSTRACT: The synthesis of new fluorescent nucleotides is described. This synthesis comprises two parallel reactions, the Kornblum oxidation and imidazole formation, which lead to 8-(aryl)-3-β-D-ribofuranosylimidazo[2,1-*i*]purine 5'-phosphates **2** from AMP or ATP. A detailed mechanism is proposed based on monitoring the reaction by ^{1}H- and ^{13}C-NMR spectroscopy, MS, FAB, HPLC, and pH meter. The spectral and fluorescent properties of the new derivatives at various pH values are described. Excitation and emission maxima for **3** were observed at 290 and 420 nm, respectively, in both basic and neutral media. In acidic media, the emission maximum shifted to 410 nm, however, the fluorescence intensity increased 1.5-fold. ATP analogues **2b** and **3b** exhibited relative stability regarding hydrolysis by type II ATPDase. Compound **3b** is relatively chemically stable at pH 10.4 and 7.4.

N1,N^6-Etheno adenine nucleotides (ε-adenine nucleotides) are commonly applied as fluorescent probes for various biochemical studies, such as: structure and function of nucleic acids [1-3], protein visualization [1b], enzymatic studies [1,2,4-6], investigation of nucleotide binding-site [2,7], conformational analysis of nucleotides [6], and pharmacology of nucleosides/nucleotides [8a,b,3b]. The synthesis of ε-adenine nucleotides [5,8-12] and their fluorescent properties [3b,10,13] are well- documented.

Fluorescence spectra of N1,N^6-etheno adenine nucleotides exhibit excitation and emission maxima at 275 and 410 nm, respectively, at pH 7 [10]. Fluorescent adenine nucleotide derivatives emitting at higher wavelengths are desirable for various biochemical applications. Such compounds allow excitation at a wavelength range which

is outside the range of absorption of proteins and nucleic acids. Moreover, a longer excitation wavelength allows excitation and emission measurements using plain glass instead of quartz equipment.

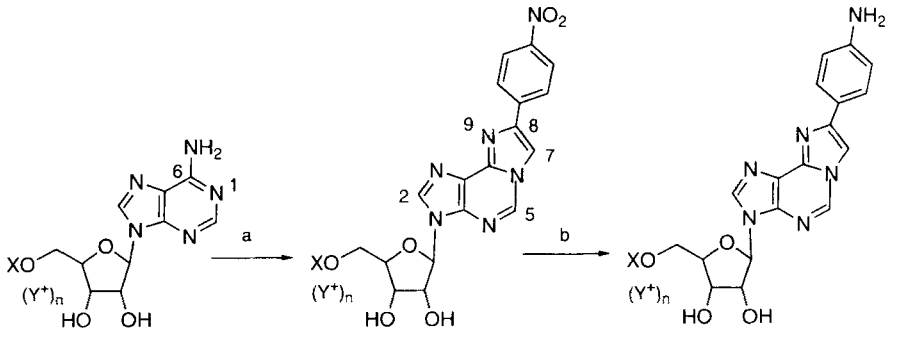
Extended conjugation of heterocycles, e.g., at the 2- and 5-positions of furan or oxazole rings, is known to give a bathochromic shift in UV spectra due to mesomeric effects [14]. Therefore, an extension of the conjugation of the $N1,N^6$-etheno-bridge by an auxochrome, e.g., an aryl moiety, is desirable.

This paper describes the extension of the fluorophore of $N1,N^6$-etheno adenine nucleotides for producing molecular probes **2** and **3** with unique characteristics, starting from AMP and ATP. The synthesis of compounds **2** exploits the Kornblum oxidation [15] reaction for the extension of the adenine chromophore. A detailed mechanism is suggested based on monitoring the reaction by spectral means, HPLC, and pH meter. The spectral and fluorescent properties, and the chemical and enzymatic stability of the new derivatives were evaluated. Based on their unique properties, compounds **3** are proposed, among other numerous biochemical applications, as indicators of acidity in a protein nucleotide binding-site.

RESULTS

Synthesis of 8-(aryl)-3-β-D-ribofuranosylimidazo[2,1-*i*]purine 5'-phosphates. The synthesis of ε-nucleotides, from chloroacetaldehyde and the corresponding nucleotide, is generally performed in aqueous media [5,8-12]. However, the extension of $N1,N^6$-etheno adenine nucleotide chromophore with conjugated double bonds or aromatic rings, requires the reaction of the nucleotide with non-polar reagents, e.g., an aromatic-α-haloketone (Scheme 1). Thus, a solubility problem arises as the nucleotide reactants are water soluble. Furthermore, extension of the adenine chromophore by fusion of an imidazole, requires an acidic aqueous solution [8-12], or acidic solutions of $EtOH/H_2O$ [5] or DMF/H_2O [11], at elevated temperatures for several days. These conditions cause partial hydrolysis of the nucleotide phosphate chain, and isolation of the product in low yields.

We propose here a new synthesis, for solving the above-mentioned problems in the preparation of aryl-substituted $N1,N^6$-etheno adenine nucleotides **2**. The Kornblum oxidation reaction [15,16] was chosen to promote the imidazole formation in the product



1a X= PO_3^{2-} Y = H^+ n = 2 **2** Y = Na^+ n = 2, 3, 4 **3**
1b X = $P_3O_9^{4-}$ Y = Bu_3NH^+ n = 4
1c X = $P_2O_6^{3-}$ Y = Bu_3NH^+ n = 3

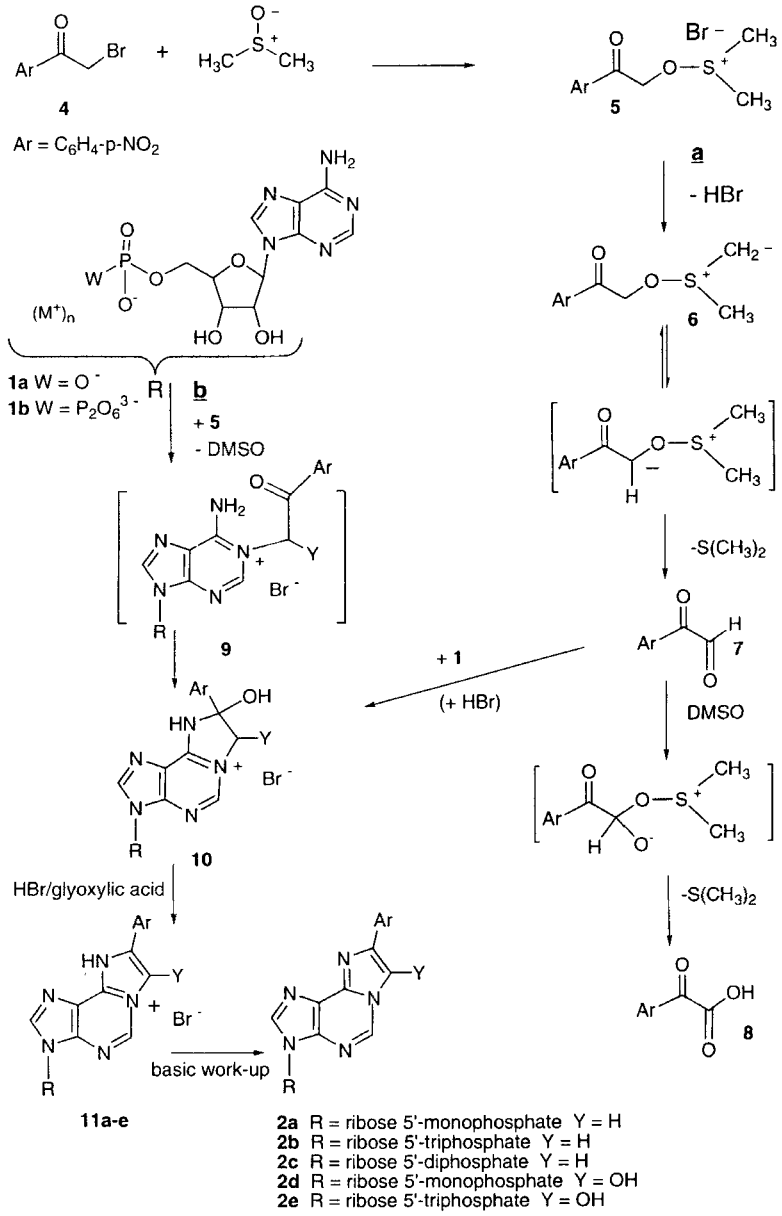
a. p-NO_2-C_6H_4-(CO)CH_2Br, DBU, DMSO b. H_2/PtO_2

SCHEME 1. Synthesis of fluorescent aryl-e-adenosine nucleotides

2. The Kornblum reaction, known to oxidize α-bromoketones to the corresponding glyoxals by DMSO, assists the synthesis of **2** since it provides a reactive reagent, **5**, the acidic medium required for imidazole formation and the conditions needed for solubilizing both the polar and non-polar reactants, **4** and **1** (Scheme 2).

In a typical procedure, AMP free acid was dissolved in DMSO together with an 10 eq. of 2-Br-(*p*-nitro)-acetophenone, and pH 4.5 was maintained by a dropwise addition of DBU. After 12 h at room temperature almost all starting nucleotide was consumed, and product **2a** was isolated in 62% yield under mildly basic conditions, as the only regioisomer [3b,12]. Due to an incomplete reaction, intermediate **10** was also isolated, in its neutral form as a by-product.

As AMP and ATP sodium salts are insoluble in DMSO, AMP free acid and tetrakis-tetrabutylammonium ATP, which are DMSO-soluble, were used as reactants. The reaction is pH-dependent. An optimal pH value of 4.5-5.0 [12] is required for this reaction, which is maintained by the gradual addition of a non-nucleophilic base, e.g., DBU. The yield is optimal at a temperature range of 20-30 °C. Higher temperatures accelerate the reaction of the α-bromo-ketone with DMSO which result in very low pH values (Fig. 1). The reaction time (12h) is shorter than that reported in related reactions [e.g., 4,10].



SCHEME 2. Mechanism of nucleotides **2** formation assisted by the Kornblum-oxidation

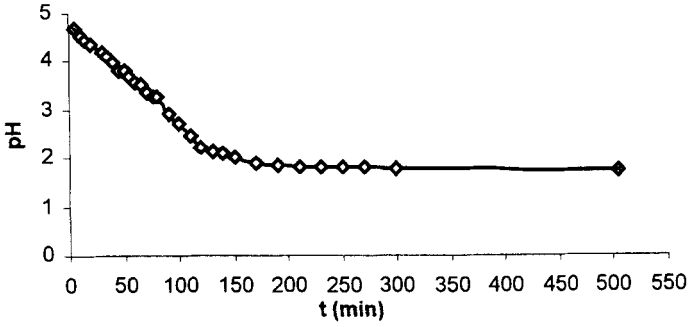


FIG. 1: Rapid acidification of the reaction mixture is observed upon mixing 2-Br-(*p*-nitro)-acetophenone in DMSO at room temperature.

^{1}H-NMR spectrum of product **2a** in D_2O (pH 8.2) showed three sharp singlets (8.64, 7.82, 7.32 ppm) which belong to the new heterocyclic system, that were assigned as H-2, H-5 and H-7 respectively, based on NOESY spectrum. Upon lowering the pH of the NMR sample solution to 4.5, line-broadening occurred. This occurrence indicates an equilibrium between the protonated and non-protonated species **11** and **2**, and is consistent with the pKa of 3.8 for 5'-ε-AMP [1].

For improving both the fluorescence properties (i.e., quantum yield, longer emission maximum wavelength) and the affinity of derivatives **2** to nucleotide-binding proteins, the corresponding *p*-amino-phenyl-etheno-adenine nucleotides, **3**, were prepared by catalytic hydrogenation of **2** over PtO_2.

Reaction Mechanism. A detailed mechanism is proposed based on monitoring the reaction by ^{1}H-, and ^{13}C-NMR spectroscopy, and by mass and FAB spectroscopies, HPLC, and pH meter. Observations, made during the reaction course, support an 'intertwining mechanism', resulting in product **11/2** (Scheme 2).

Acidic pH (ca. 4.5) is obtained instantaneously upon mixing 2-Br-(*p*-nitro)-acetophenone in DMSO. Within 150 min at room temperature the mixture turns strongly acidic (pH 1.8), and then the pH value remains almost constant (Fig. 1). The reaction between 2-Br-(*p*-nitro)-acetophenone and DMSO was also monitored by ^{1}H-NMR in DMSO-d_6 at 22 °C during 120 min. After 30 min, a signal emerged at 9.5 ppm, indicating the formation of an aldehyde. After 90 min, an additional signal at 13 ppm, indicative of a

carboxylic acid, was observed. Likewise, when this reaction was monitored by mass spectroscopy, signals indicating the formation of both *p*-nitro-phenylglyoxal **7** and *p*-nitro-phenylglyoxylic acid **8**, were noticed. After 6 h at room temperature, the ratio of arylglyoxylic acid: arylglyoxal was 3:2, and after 52 h the ratio changed to 3:1, correspondingly. An instantaneous formation of the adduct, 2-(dimethylsulfonium)-(*p*-nitro)-acetophenone bromide salt, **5**, (M^+-Br^-, 242), was noticed by fast-atom-bombardment (FAB) spectroscopy when DMSO and 2-Br-(*p*-nitro)-acetophenone were mixed at room temperature. The relatively short reaction time reflects the higher reactivity of dimethylsulfonium intermediate **5** vs. α-bromo-ketone **4**.

The observation of rapid acidification of the reaction mixture and formation of various intermediates, detected by pH meter, ^{1}H-NMR, MS and FAB spectroscopy, support the mechanism, described in Scheme 2. In this reaction, DMSO is not only a solvent capable of dissolving both the non-polar α-bromo-ketone and the polar nucleotide, but also a reagent, reacting with excess α-bromo-ketone, **4**, to form a reactive intermediate, 2-(dimethylsulfonium)-(*p*-nitro)-acetophenone bromide salt, **5**. This intermediate is consumed by either reaction a or b. In reaction a, **5** instantaneously loses a proton to give ylid **6** [16] and HBr. Intermediate **6**, leads to the corresponding aryl-glyoxal, 7, upon dimethylsulfide elimination, as clearly observed in ^{1}H-NMR spectra (9.5 ppm). Further oxidation to the aryl-glyoxylic acid occurs upon reaction of 7 with a second equivalent of DMSO. This explains the rapid acidification of the reaction mixture which is enhanced throughout the reaction course. The reaction mixture routinely reaches pH 1.6 after 8 h at room temperature.

Reaction b, intertwines with reaction a at several junctions. The adenine nucleotide N1 acts as a nucleophile [1,17] reacting with the activated alkylating agent **5**. The N1-alkylated product, **9**, was not isolated. Apparently, this product undergoes a rapid ring closure to form **10**, isolated in 22% yield, as indicated by UV and FAB spectrum. Product **11**, is obtained upon acid (HBr and arylglyoxylic acid) catalyzed dehydration.

The possible involvement of arylglyoxal in this reaction was also explored, since aqueous glyoxals are known to form labile adducts with guanosine [18] at pH 4-7. Arylglyoxal is an electrophilic reagent, that can compete with intermediate **5** upon reacting with N1 of the nucleotide. Therefore, phenylglyoxal was added under the reaction conditions (DMSO, pH 4.5, room temperature) to AMP free acid and the reaction was monitored by ^{1}H NMR and HPLC. An HPLC chromatogram of the reaction mixture

after 29 h at room temperature, indicated the appearance of a tiny new peak, the UV spectrum of which (253, 290 nm) differs from a fused imidazopurine system. The reaction was investigated also by FAB spectroscopy. After 18 h at room temperature, most of AMP remained unchanged. Minute amounts of AMP:glyoxal adduct, **10d** (MW 482), and of its dehydration product, **2d** (MW 463) were identified. ^{1}H NMR spectrum indicated rapid disappearance of the aldehyde signal at 9.5 ppm. These data indicate that phenyl glyoxal reacts preferentially with DMSO rather than with AMP, i.e., follows reaction a and not b.

UV Spectroscopy. UV spectrum of **2a** showed four maxima (238, 271, 304, 352 nm) at pH 7.0 (Table 1, Fig. 2A). In a basic medium (pH 12.3), only minute changes were observed in spectrum maxima and ε. However, in an acidic medium (pH 1.6), in which the nucleotide is still stable for several hours [see also 1a], a blue shift of 31 nm is observed. A more pronounced blue shift of 41 nm was observed in the UV spectrum of **3a** at pH 1.6 (Table 1, Fig. 2B). This shift indicates an equilibrium between protonated and non-protonated species. The extension of the adenine chromophore in product **3a**, is indicated by the shift in UV spectrum, from absorption at 260 nm for the starting adenine nucleotide, **1**, or at 275 nm for N1, N^6-etheno-adenine [6], to 331 nm for product **3a** at neutral pH (Table 1).

Fluorescence Studies. Excitation and emission spectra, as well as quantum yields were measured for **2a** and **3a** (the triphosphate analogues **2b**, **3b** have similar fluorescent properties) at various pH values. The excitation spectrum for **3a** at pH 1.6 (Fig. 3) was identical with its UV spectrum. Excitation of **2a** and **3a** at 290-300 nm resulted in maximum fluorescence at all pH values, whereas at 350 nm no fluorescence was observed. Emission spectra for compounds **2a, 3a** were pH dependent (Fig. 4A-B). Emission maxima for compound **3a** were observed at 420 nm in basic and neutral media (Fig. 4B), and were detected at a low concentration of 2.5×10^{-7} M. In acidic medium, emission maximum shifted to 410 nm, however, fluorescence intensity increased 1.5-fold (Fig. 4B). The increase in fluorescence is reflected in the higher quantum yield, 42% at acidic pH, compared to 16% at basic and neutral pH. This increase indicates that both neutral and protonated species are the origin of fluorescence. This observation is exceptional since the fluorescence of **2a**, under acidic conditions almost disappeared (Fig.

TABLE 1. UV Spectra of **2a** (1.86×10^{-5} M) and **3a** (1.97×10^{-5} M) at various pH values:

3a	**3a**	**2a**	**2a**	Cmp. No.
$\varepsilon \times 10^{-3}$	λmax (nm)	$\varepsilon \times 10^{-3}$	λmax (nm)	pH
2.87	290	3.10	321	**1.6**
5.43	249	3.5	285	
		3.60	258	
		5.32	232	
1.62	331	2.35	352	**7.0**
6.31	273	3.15	304	
		3.38	271	
		6.45	238	
2.03	333	2.42	348	**12.3**
7.70	274	3.16	305	
		3.24	272	
		6.61	235	

4C), relative to its fluorescence in neutral and basic pH (Fig. 4A). Furthermore, ε-adenosine · HCl or ε-ATP [19] have practically no fluorescence at pH 1.6 [1]. Unlike emission spectra for **3a**, no shift in the position of the band due to pH changes is observed for **2a**, suggesting that only one structure, the neutral form, is responsible for the fluoresence [1]. The quantum yields for compound **2a**, relative to quinine sulfate [20], are 6.5% at basic and neutral pH and 0.35% at acidic pH.

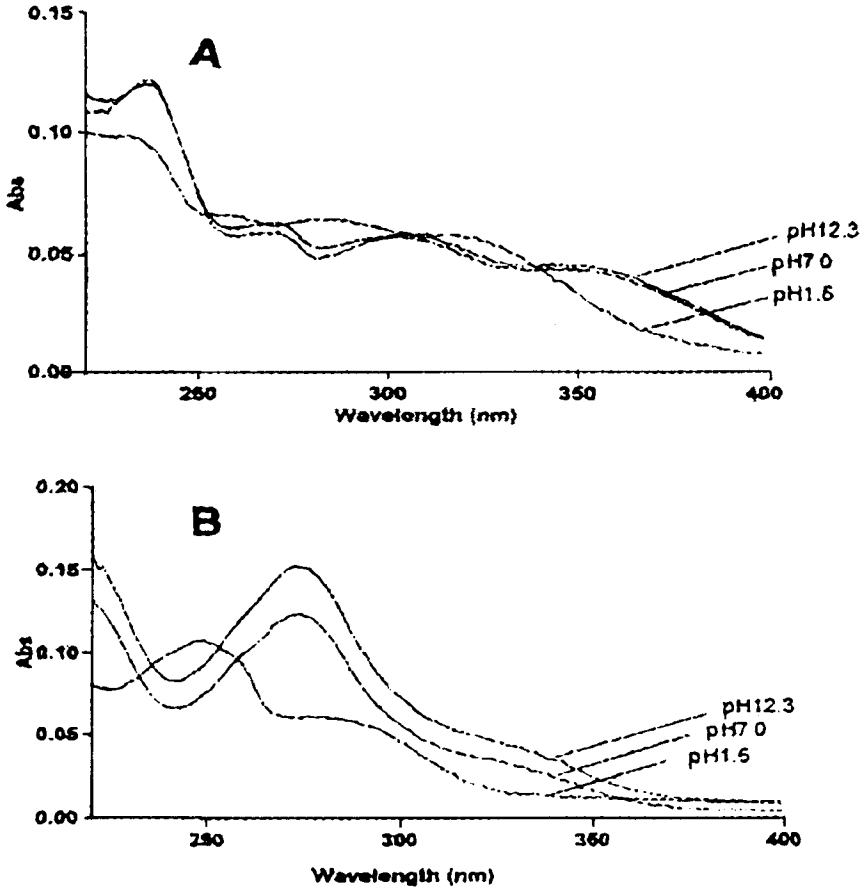


FIG. 2: pH-Dependence of UV spectra of **2a** (panel A) and **3a** (panel B) at 18.6 μM, in buffered solutions, at room temperature.

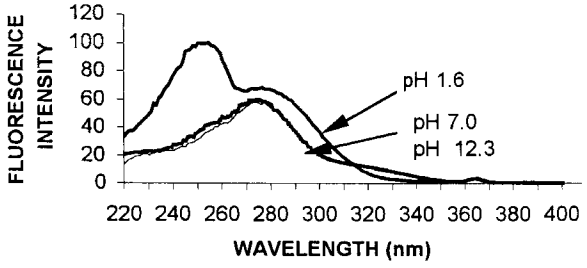


FIG. 3: Corrected fluorescence excitation spectra of **3a** at 1.97 μM as a function of pH.

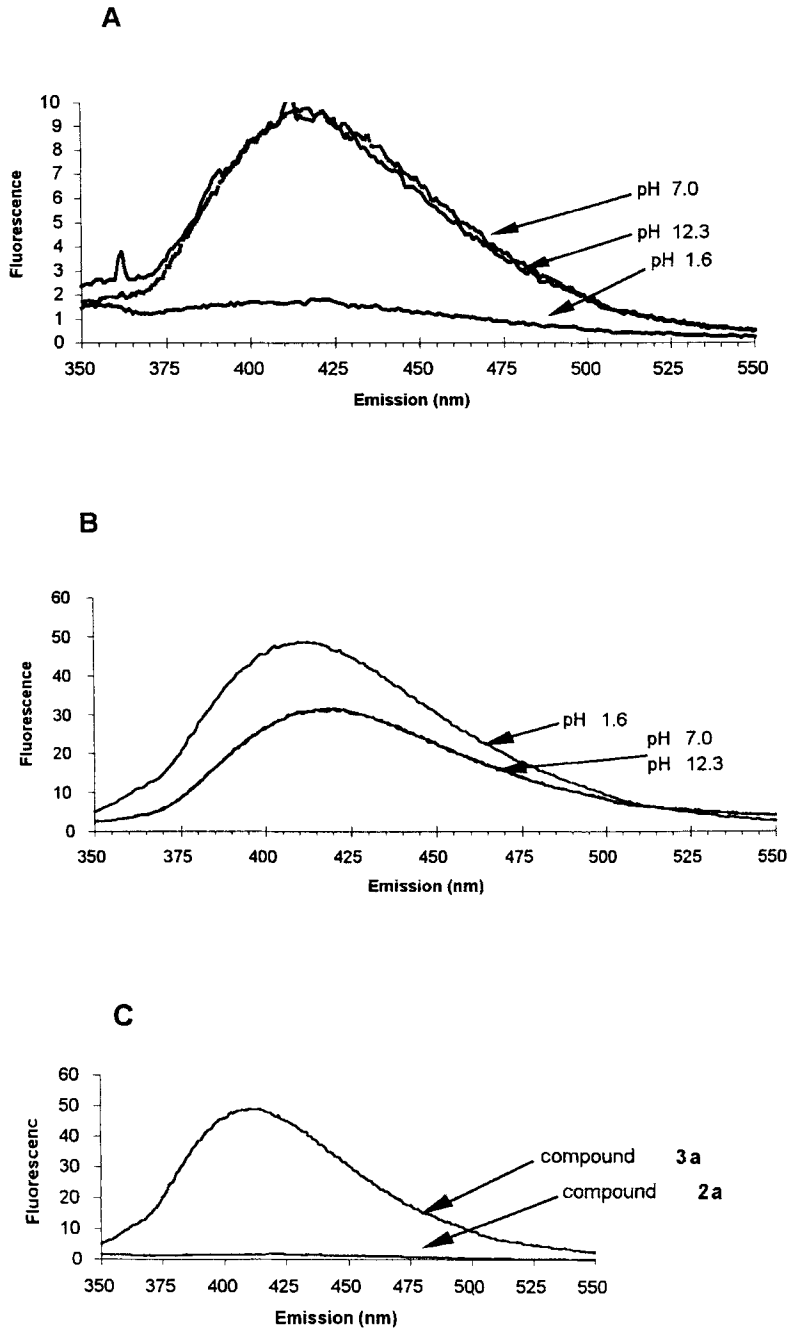


FIG. 4: Corrected fluorescence emission spectra of **2a** (panel A) and **3a** (panel B) at 1.97 μM as a function of pH, excitation at 290 nm. Panel A: emission maximum at 420 nm is observed at both basic and neutral buffered solutions. In acidic buffer (pH 1.6) fluorescence is diminished. Panel B: emission maximum at 420 nm is observed at both basic and neutral buffered solutions. In acidic buffer (pH 1.6) fluorescence is enhanced 1.5 fold, and emission maximum is shifted to 410 nm. Panel C: comparison of fluorescence emission spectra of **2a** and **3a** at pH 1.6.

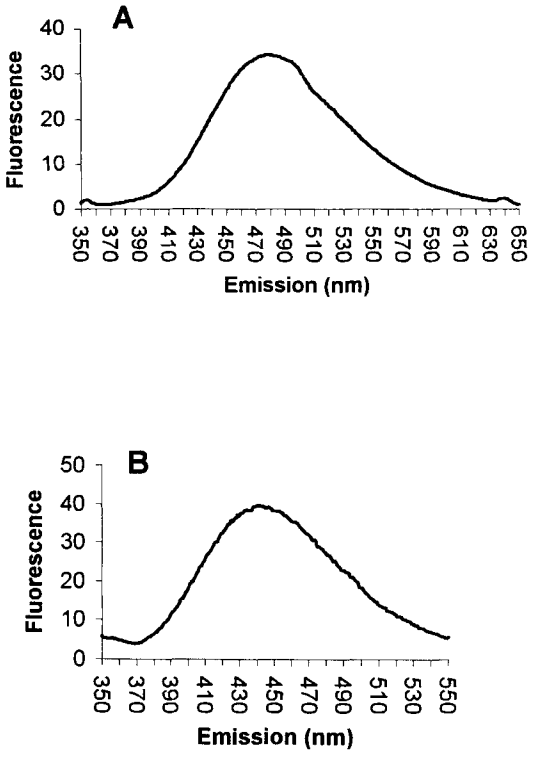


FIG. 5: Corrected fluorescence emission spectra of **3a** at 1.97 μM in DMSO (panel A) and dioxane (panel B). Panel A: emission maximum at 480 nm, excitation at 320 nm. Panel B: emission maximum at 440 nm, excitation at 310 nm.

Literature reports suggest that the primary protonation site of aqueous ε-AMP is N9 [3c]. Leonard et al. concluded from the pH dependent emission spectra of ε-adenosine that the neutral form is responsible for the fluorescence, while the 9-protonated form has vanishing fluorescence efficiency [19]. However, the pronounced increase in fluorescence of compound **3**, is due to its protonated form. The main contribution to fluorescence probably stems from protonation of the anilino moiety.

Emission maxima of **3a** in dioxane and DMSO were shifted, due to solvent effects, to 440 and 480 nm, with quantum yields of 76% and 64%, respectively (Fig. 5).

Chemical stability. The relative stability of the heterocyclic system in **2b** was monitored by ^{1}H-NMR in D_2O, at pH 10.4 at 22 °C for 24 h. Virtually no change of the

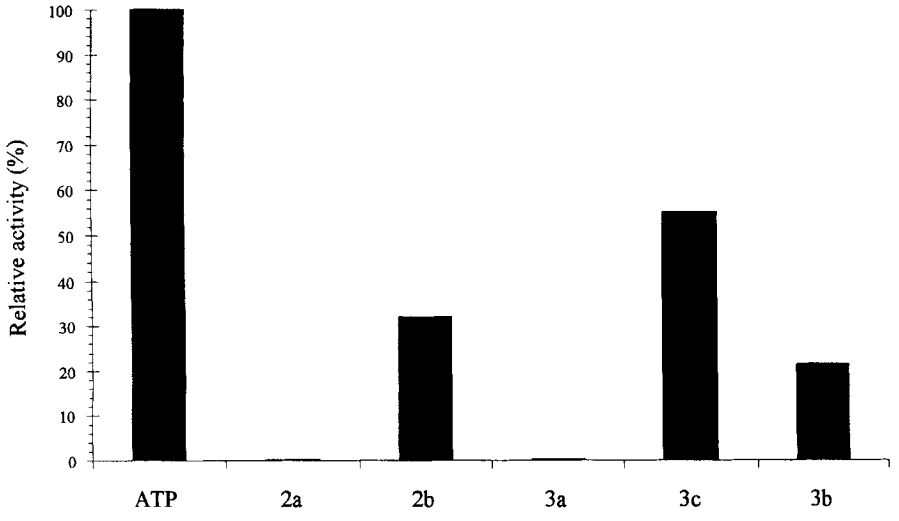


FIG. 6: Hydrolysis rate of ATP and its analogues by type II ATPDase. ATP and analogues were used at a concentration of 100 μM. Experiments were performed in triplicate.

chemical shifts of imidazopurine protons was observed after 24 h at pH 10.4. The slight change in the ^{1}H-NMR spectrum is due to formation of the corresponding diphosphate derivative because of basic hydrolysis of the triphosphate moiety, as observed by ^{31}P NMR. Under physiological conditions, pH 7.4 at 37 °C, **2b** and **3b** are stable for at least six days.

Enzymatic stability. The enzymatic stability of the new compounds was investigated for estimating their suitability for various *in-vivo* studies.

Until recently it was believed that ATP was converted to adenosine by ecto-ATPase, ecto-ADPase and 5'-nucleotidase [21]. However, in many organs and tissues [22] such as endothelial, smooth muscle and pancreatic cells, ATP diphosphohydrolase [ATPDase; EC 3.6.1.5] and ecto-ATPase are probably responsible for most of the hydrolysis of extracellular triphospho- and diphosphonucleosides [23]. ATPDase hydrolyzes pyrophosphate bonds of nucleoside di- and triphosphates in the presence of Ca^{2+} and Mg^{2+} [22,24]. In this work, the resistance of fluorescent derivatives **2a,b**, and **3a-c** towards hydrolysis by type II ATPDase was therefore evaluated [24a].

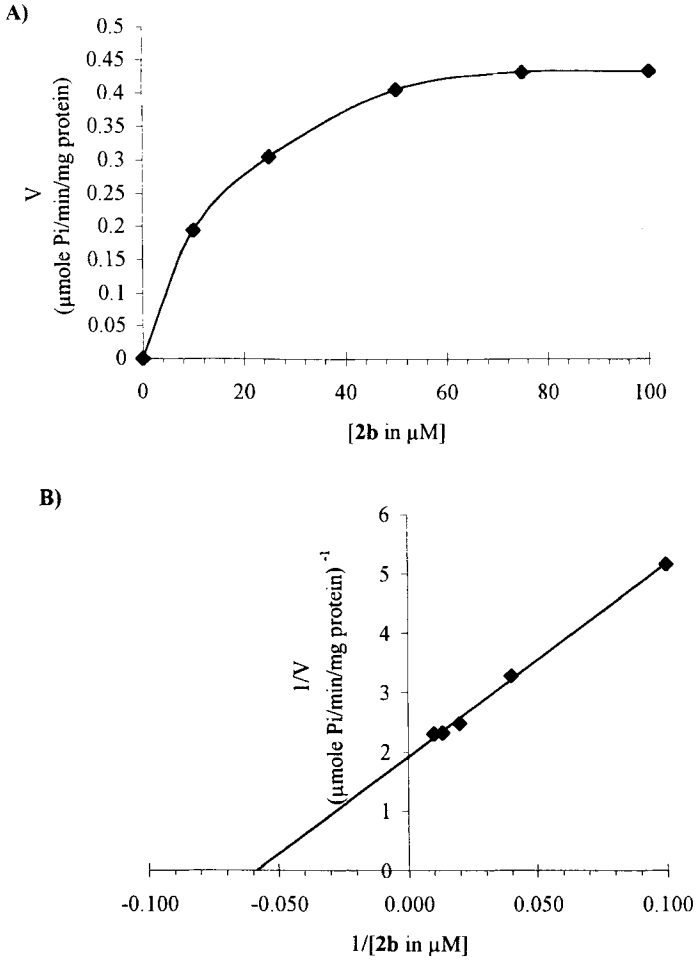


FIG. 7: Hydrolysis of **2b** by bovine spleen type II ATPDase. Panel A: reaction rate of **2b** as a function of substrate concentration. Panel B: Lineweaver-Burk plot of **2b** hydrolysis by ATPDase. Apparent Km and Vmax were 17 µM and 0.52 µmole Pi/min/mg protein, respectively. Experiments were perfomed in triplicate.

The enzymatic stability of the novel analogues towards type II ATPDase, was compared with ATP at a substrate concentration of 100 µM. The rate of hydrolysis of analogues **2b**, **3b**, and **3c** were 32%, 22% and 56% of the comparable ATP rate (Fig. 6). A more detailed analysis of the kinetic parameters for **2b** shows a Km, app. of 17 µM and Vmax of 0.52 µmoles Pi/min/mg protein (Fig. 7) as compared to the values obtained with ATP: Km, app. of 15 µM and Vmax of 1.65 µmole Pi/min/mg protein. In contrast,

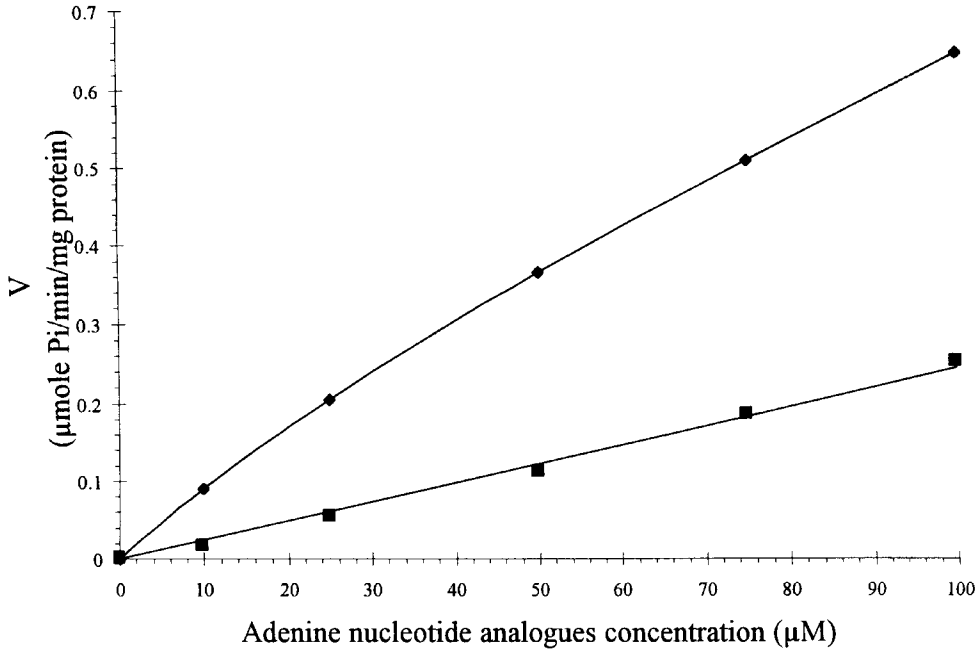


FIG. 8: Reaction rate of **3c** (♦) and **3b** (■) as a function of substrate concentration. Experiments were performed in triplicate.

with the **3c** and **3b** analogues, no substrate saturation was obtained up to 100 µM (Fig. 8).

As expected, the monophosphate derivatives, **2a** and **3a**, were not hydrolysed. However, kinetic analysis shows that **2a** is a weak competitive inhibitor with an estimated Ki of 220 µM (Fig. 9A). This finding is confirmed by the parallel curves of the Cornish-Bowden representation (Fig. 9B) [25]. Analogue **3a** did not inhibit the enzyme.

CONCLUSION:

A new synthesis, based on assistance by the Kornblum oxidation, was used for the preparation of fluorescent nucleotides **2**.

The extension of the adenine chromophore affects UV absorption and fluorescence emission. Thus, product **3a** absorbs at 331 nm, whereas the starting adenine nucleotide absorbs at 260 nm. UV absorption spectra **2a** and **3a** are pH dependent. Extension of the

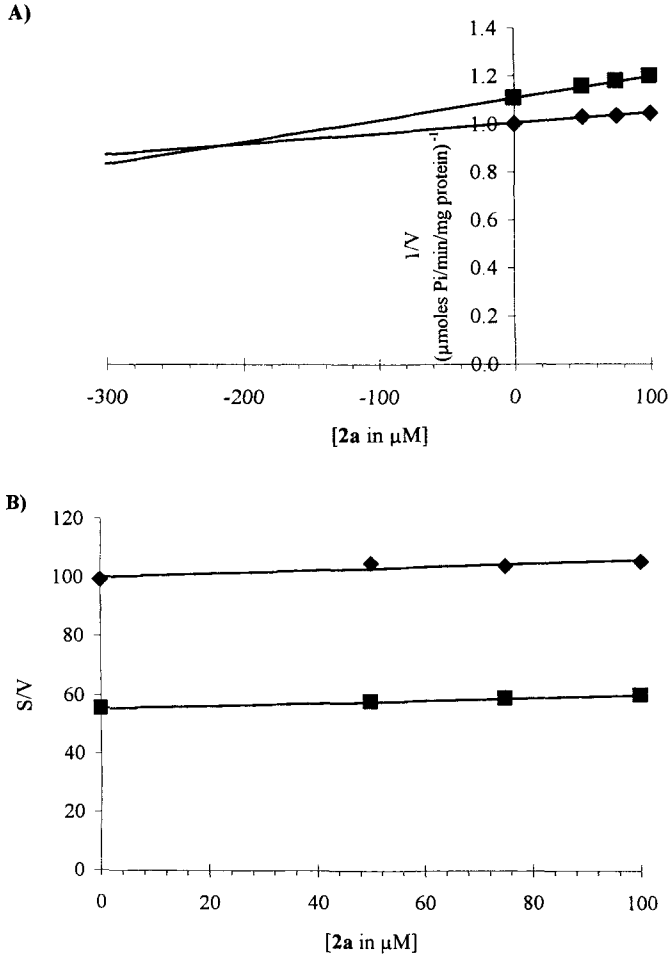


FIG. 9: Panel A: Dixon plot of the inhibitory effect of **2a** on ATP hydrolysis by type II ATPDase. Assays were carried out in the presence of 50 μM ATP (■) or 100 μM of ATP (♦). Panel B: Cornish-Bowden plot showing competitive inhibition of **2a**. Experiments were performed in triplicate.

fluorophore shifts excitation and emission wavelengths to higher values relative to N1,N^6-etheno adenine nucleotides [10]. Products **2** and **3** are excited at 290-300 nm and emit at 420 nm at pH 7.0, and can be detected as low as 10^{-7} M concentrations. Emission spectra for compounds **2a, 3a** were pH dependent. In acidic medium, the emission maximum for **3a** shifted to 410 nm, however, fluorescence intensity increased 1.5-fold, and the quantum yield increased to 42%. This increase might indicate that both neutral

and protonated species are the origin of fluorescence. This observation is exceptional since the fluorescence of **2a** and ε-ATP [1], almost disappeared under these conditions. Unlike emission spectra for **3a**, no shift in the position of the band due to pH changes is observed for **2a**, suggesting that only one structure, the neutral form, is responsible for the fluoresence. At acidic pH the quantum yield of **3a** is 42%, representing an enhancement of fluorescence of 120 fold compared with **2a** and ε-ATP under these conditions [19]. The electronic transitions responsible for the increase of fluorescence of **3** in acidic pH, compared to the vanishing fluorescence of ε-adenine nucleotides under the same conditions, are currently investigated by us by computational means.

Compounds **2** and **3** proved to be relatively chemically stable at pH 7.4 and 10.4. Their relative stability towards hydrolysis by ATPDase, might be attributed to the bulky aryl-etheno moiety, which turns these derivatives into poor substrates, or might indicate that a free N1, N^6 region is required for H-bonding. These compounds may still be applicable to protein studies where a free N1, N^6 region is not essential for binding [4].

To the best of our knowledge, compounds **3** are the first examples of N1,N^6-etheno-adenine nucleotides which highly fluoresce at acidic pH. Previous examples include only etheno-cytidine derivatives, the fluorescence of which is low [14]. Therefore, due to the large fluorescence enhancement of compounds **3** in acidic pH, these compounds are proposed as indicators of acidity in a protein nucleotide binding-site [7c]. This application is currently under investigation.

In conclusion, the novel nucleotides **2** and **3** are easily prepared and are relatively chemically stable. These compounds are also relatively stable towards enzymatic hydrolysis by ATPDase. In addition, their fluorescent properties: excitation wavelengths outside the range of proteins and nucleic acids, emission maxima at relatively long wavelengths enabling the use of glass equipment, and reasonable to good quantum yields, justify their use as fluorescent molecular probes for various biochemical applications.

EXPERIMENTAL:

General. New compounds were characterized and resonances assigned by proton and carbon nuclear magnetic resonance using Bruker AC-200, DPX-300 or DMX-600 NMR spectrometers. HOD signal was used as a reference, at 4.78 ppm, for samples in D_2O.

Nucleotides were characterized also by ^{31}P NMR in D_2O using 85% H_3PO_4 as an external reference. Samples were treated with CHELEX-100 (BioRad, Richmond, CA) prior to spectral measurement. Mass spectra were recorded on AutoSpec-E-Fision VG high resolution Mass Spectrometer. Nucleotide derivatives were desorbed from a glycerol matrix under fast atom bombardment (FAB), and high resolution FAB, negative conditions using 6 kV Xe atoms. ATP disodium salt, which is insoluble in DMSO, was converted to the corresponding ATP-tetrakis(tetrabutylammonium) salt by eluting ATP disodium salt on tetrabutylammonium ion- exchanger. The latter was prepared by elution of tetrabutylammonium bromide (12 equivalents) through CHELEX-100. The ion-exchange column was then washed with 10 volumes of distilled water. Purification of nucleotides was achieved on an Isco UA-6 LC system using DEAE A-25 Sephadex (HCO_3^- form) columns and a linear gradient of 0-0.5 M NH_4HCO_3. Signals were detected by UV absorption at 280 nm using a UV detector. The final purification of the products was done on a Merck-Hitachi HPLC system using an analytical LichroCART lichropher 60 RP-select B column 250x4 mm (Merck, Darmstadt, Germany) and a linear gradient of acetonitrile (A): 0.1 M TEAA buffer (pH 7) (B) (A:B) 25:75-45:55 in 25 min (solvent system I) with a flow rate of 1 mL/min. The purity of the nucleotides was evaluated on an analytical column in two different solvent systems. Solvent system (I), described above, and solvent system II, consisting of 5 mM tetrabutylammonium dihydrogenphosphate (TBAP) in MeOH (A) and 60 mM ammonium dihydrogenphosphate and 5 mM TBAP in 90% H_2O/10% MeOH (B). A concentration gradient from 75% B to 25% B in 20 min was applied. Diode array detector was used and peaks absorbing at 351 nm (compound **2**) or at 331 nm (compound **3**) were collected. In order to improve the water solubility of products **2** and **3** they were converted to their corresponding di- or tetra-sodium salts after HPLC separation.

8-(p-Nitro-phenyl)-3-β-D-ribofuranosylimidazo[2,1-*i*]purine-5'-mono-phosphate (2a): Adenosine 5'-phosphoric acid (0.05 g, 0.136 mmol) was added to a solution of 2-bromo-4'-nitro-acetophenone (0.33 g, 1.36 mmol) in DMSO (10 mL) at pH 4.5. DBU (0.09 g, 0.62 mmol) was added dropwise in order to maintain a pH value of 4.5 during the course of the reaction. The reaction mixture was stirred at RT for 12 h. TLC (iso-propanol : H_2O : NH_4OH, 65 : 30 : 5) indicated that the reaction was completed. A

yellow spot at Rf 0.75, is typical of product **2a**. Acetone (20 mL) was added to the reaction mixture followed by addition of water (10 mL), a clear solution was obtained and pH was adjusted to 6.5 by addition of DBU. The solution was extracted with ether (5x4 mL) to remove non-polar compounds. The aqueous solution was filtered and separated on an LC system using Sephadex DEAE-25 column. A linear gradient 0-0.5 NH_4HCO_3 (pH 7.0), total 1 L, was applied. The relevant fractions were freeze dried four times. Final separation was achieved on HPLC. Product **2a**, 62% yield, appeared at retention time: 4.68 min, purity >95% in solvent system I, in the solvent system II retention time was: 3.36 min, purity >95%. ^{1}H NMR: (300 MHz, D_2O, pH 5.5) δ: 8.74 (s, 1H, H-5), 8.48 (s, 1H, H-2), 7.94 (s, 1H, H-7), 7.66 (ABq, 2H, Ar), 7.44 (ABq, 2H, Ar), 6.00 (d, 1H, H-1'), 4.66 (t, 1H, H-2'), 4.52 (t, 1H, H-3'), 4.37 (q, 1H, H-4'), 4.16 (m, 2H, H-5') ppm. ^{31}P-NMR (200 MHz, D_2O, pH 5.5): 1.7 ppm. FAB (Neg.): 491 (MH^+). HRFAB: m/z calcd for $C_{18}H_{15}N_6O_9P$ (MH^+) 491.0716, found 491.074.

8-(p-Nitro-phenyl)-8-hydroxy-3-β-D-ribofuranosylimidazolino[2,1-*i*]purine-5'-mono-phosphate (10a): was obtained as a by product due to incomplete reaction in 22% yield (15 mg). UV (λ max): 242, 264 nm. FAB (Neg.): m/z calcd for $C_{18}H_{18}N_6O_{10}P$ (MH^+) 509, found 509.

8-(p-Amino-phenyl)-3-β-D-ribofuranosylimidazo[2,1-*i*]purine-5'-mono-phosphate (3a): compound **2a** (10 mg, 0.02 mmol) dissolved in 5 mL H_2O was hydrogenated (at 61 psi) overnight at room temperature over PtO_2 catalyst. TLC (iso-propanol : H_2O : NH_4OH, 65 : 30 : 5) indicated that the reaction was completed, showing a typical spot at Rf 0.57. After removal of the catalyst by centrifugation, the product was purified by HPLC. Product **3a**, obtained in a quantitative yield (9.0 mg), appeared at retention time: 2.89 min in solvent system I, purity >94%, and at retention time: 6.00 min in the solvent system II, purity >95%. ^{1}H NMR (300 MHz, D_2O, pH 6) δ: 9.01 (s, 1H, H-5), 8.71 (s, 1H, H-2), 8.16 (s, 1H, H-7), 7.7 (ABq, 2H, Ar), 6.88 (ABq, 2H, Ar), 6.22 (d, 1H, H-1'), 4.65 (t, 1H, H-2'), 4.55 (t, 1H, H-3'), 4.32 (q, 1H, H-4'), 4.14 (m, 2H, H-5') ppm. ^{31}P-NMR (200 MHz, D_2O, pH 6): 3.7 ppm. FAB (Neg.): 461 (MH^+). HRFAB: m/z calcd for $C_{18}H_{17}N_6O_7P$ (MH^+) 461.0974, found 461.1072.

8-(p-Nitro-phenyl)-3-β-D-ribofuranosylimidazo[2,1-*i*]purine-5'-tri-phosphate (2b): This compound was prepared as compound **2a**, starting with 400 mg (0.27 mmol) ATP tetrakis-(tetrabutylammonium) salt. Product **2b** was obtained in 37% yield (72 mg). retention time: 3.53 min in solvent system I, purity >95%, and at retention time: 2.21 min in the solvent system II, purity >95%. ^{1}H NMR (300 MHz, D_2O, pH 7.5) δ: 9.00 (s, 1H, H-5), 8.60 (s, 1H, H-2), 8.25 (s, 1H, H-7), 8.05 (ABq, 2H, Ar), 7.85 (ABq, 2H, Ar), 6.15 (d, 1H, H-1'), 4.82 (t, 1H, H-2'), 4.72 (t, 1H, H-3'), 4.62 (q, 1H, H-4'), 4.53 (m, 2H, H-5') ppm. ^{31}P-NMR: (200 MHz, D_2O, pH 7.5) δ: -7 (d), -10 (d), -21 (t) ppm. FAB (Neg.): 652 ($M+4H^+$). HRFAB: m/z calcd for $C_{18}H_{19}N_6O_{15}P_3$ ($M+4H^+$) 652.0121, found 652.0210.

8-(p-Nitro-phenyl)-3-β-D-ribofuranosylimidazo[2,1-*i*]purine-5'-diphosphate (2c): was obtained as a by-product, due to hydrolysis of the phosphate chain, in 12% yield (21 mg). ^{1}H NMR (300 MHz, D_2O, pH 7.00) δ: 9.00 (s, 1H, H-5), 8.55 (s, 1H, H-2), 8.25 (s, 1H, H-7), 8.05 (ABq, 2H, Ar), 7.85 (ABq, 2H, Ar), 6.15 (d, 1H, H-1'), 4.85 (t, 1H, H-2'), 4.60 (t, 1H, H-3'), 4.40 (q, 1H, H-4'), 4.25 (m, 2H, H-5') ppm. ^{31}P-NMR: (200 MHz, D_2O, pH 7.00) δ: -7.80 (d), -10.00 (d) ppm.

8-(p-Amino-phenyl)-3-β-D-ribofuranosylimidazo[2,1-*i*]purine-5'-tri-phosphate (3b): This compound was prepared as compound **3a** starting with **2b** (20 mg, 0.027 mmol). The product was separated on HPLC. Product **3b** (98%, 18.77 mg) appeared at retention time: 7.75 min in solvent system I, purity >96%, and at retention time: 6.25 min in the solvent system II, purity >96%. ^{1}H NMR (300 MHz, D_2O, pH 7.00) δ: 9.07 (s, 1H, H-5), 8.64 (s, 1H, H-2), 8.13 (s, 1H, H-7), 7.73 (ABq, 2H, Ar), 6.68 (ABq, 2H, Ar), 6.25 (d, 1H, H-1'), 4.85 (t, 1H, H-2'), 4.73 (t, 1H, H-3'), 4.55 (q, 1H, H-4'), 4.30 (m, 2H, H-5') ppm. ^{31}P-NMR: (200 MHz, D_2O, pH 7.00) δ: -6.00 (d), -10.47 (d), -20.81 (t) ppm. FAB (Neg.): m/z calcd for $C_{18}H_{21}N_6O_{13}P_3$ ($M+4H^+$) 622, found 622. Product **3c** was obtained from **2c** in the same way.

Spectroscopic Measurements:

UV: The new compounds were characterized by UV using a Varian Cary-1E UV-Visible spectrometer. Absorption spectra were determined in buffers at several pH values. Three equal aliquots were withdrawn and placed in volumetric flasks. These

were diluted to give three solutions of known, equal volume: one solution in 0.025 M phosphate (KH_2PO_4) buffer (pH 7.0), one in 0.05 M HCl (pH 1.6), and in 0.05 M NaOH (pH 12.3). In all aliquots the concentration of the tested compound was of the order of (1-2) x 10^{-5} M. All spectra were determined against the appropriate blank using a matched set of sample and reference cells.

Excitation and Emission Spectra: of the new compounds were measured using a Aminco-Bowman series 2 Luminescence Spectrometer (slit: 4 nm, sensitivity: 770 V). Molecular fluorescence emission and fluorescence excitation corrected spectra were determined in buffers at several pH values: one solution in 0.025 M phosphate (KH_2PO_4) buffer (pH 7.0), one in 0.05 M HCl (pH 1.6), and in 0.05 M NaOH (pH 12.3). In all aliquots the concentration of the tested compound was of the order of (1-2) x 10^{-5} M. 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 a reference compound assuming a quantum yield value of 0.55 [20].

Enzymatic studies:

ATPDase assays were carried out with a semi-purified membrane preparation from bovine spleen [26]. This preparation was devoid of any other nucleotidase activities [27]. Enzyme activity was measured at 37 °C in 1 mL of the following incubation medium: 8 mM $CaCl_2$, 5 mM tetramisole, 50 mM Tris and 50 mM imidazole, buffered at pH 7.6. Reaction was started by the addition of the nucleotide substrate (100 μM) or as otherwise indicated. Inorganic phosphorus release was evaluated by the malachite green method as described by Baykov et al [28]. Protein concentration was determined with the Bradford [29] microplate assay, using bovine serum albumin as a standard of reference.

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REFERENCES:

1. a. Secrist III, J. A.; Barrio, J. R.; Leonard, N. J.; Weber, G. *Biochemistry,* **1972**, *11*, 3499-3506. b. Nakayama, H.; Yamaga, T. *Biophys. Chem.* **1998**, *75*, 1-6.

2. Thomas, R. W.; Leonard, N. J. *Heterocycles,* **1976**, *5*, 839-882.

3. a. Lee, C. H.; Wetmur, J. G. *Biochem. Biophys. Res. Commun.* **1973**, *50*, 879-885. b. Kusmierek, J. T.; Singer B. *Biochemistry,* **1982**, *21*, 5717-5722. c. Leonard, N. J. *CRC Crit. Rev. Biochem.* **1984**, 15, 125.

4. Secrist III, J. A.; Barrio, J. R.; Leonard, N. J. *Science,* **1972**, *175*, 646-647.

5. Meyer Jr., R. B.; Shuman, D. A.; Robins, R. K.; Miller, J. P.; Simon, L. N. *J. Med. Chem.* **1973**, *16*, 1319-1323.

6. a. Tolman, G. L.; Barrio, J. R.; Leonard, N. J. *Biochemistry,* **1974**, *13*, 4869-4878. b. Gualix, J.; Abal, M.; Pintor, J.; MirasPortugal, M. T. *FEBS Lett.* **1996**, *391*, 195-198.

7. a. Harvey, S. C.; Cheung, H. C. *Biochem. Biophys. Res. Commun.* **1976**, *73*, 865-868. b. Worthington, R. A.; Hansen, M. A.; Bennett, M. R.; Barden, J. A.; Balcar, V. J. *Biochem. Biophys. Res. Commun.* **1998**, *249*, 166-171. c. Caiolfa, V. R.; Gill, D.; Parola, A. H. *Biophys. Chem.* **1998**, *70*, 45-56.

8. a. Schram, K. H.; Townsend, L. B. *Tetrahedron Lett.* **1974**, 1345-1348. b. Leonard, N. J. *Chemtracts-Biochem.-Biochem. Mol. Biol.* **1992**, *3*, 273-297.

9. Kochetkov, N. K.; Shibaev, V. N.; Kost A. A.; Zelinsky, N. D. *Tetrahedron Lett.* **1971**, 1993-1996.

10. Barrio, J. R.; Secrist III, J. A.; Leonard, N. J. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 597-604.

11. Jones, J. H.; Murthy, D.V. K.; Tegg, D.; Golling, R.; Moffatt, J.G. *Biochem. Biophys. Res. Commun.* **1973**, *53*, 1338-1343.

12. Biernat, J.; Ciesiolka, J.; Gornicki, P.; Adamiak, R. W.; Krzyzosiak, W. J.; Wiewiorowski, M. *Nucleic Acids Res.*, **1978**, *5*, 789-804.

13. Secrist III, J. A.; Barrio, J. R.; Leonard, N. J.; Villar-Palasi, C.; Gilman, A. G. *Science,* **1972**, *177*, 279-280.

14. Barrio, J. R.; Sattsangi, P. D.; Gruber, B. A.; Dammann, L. G.; Leonard, N. J. *J. Am. Chem. Soc.* **1976**, *98*, 7408-7414.

15. a. Kornblum, N.; Powers, J. W.; Anderson, G. J.; Jones, W. J.; Larson, H. O.; Levand, O.; Weaver, W. M. *J. Am. Chem. Soc.* **1957**, *79*, 6562; b. Kornblum, N.; Jones, W. J.; Anderson, G. J. *J. Am. Chem. Soc.* **1959**, *81*, 4113-4114.

16. Torssell, K. *Acta Chem. Scand.* **1967**, *21*, 1-14.

17. Srivastava, P. C.; Robins, R. K.; Meyer, R. B. *Chemistry of nucleosides and nucleotides* Ed. Townsend, L. B. Plenum Press, NY, 1988, pp 203-208.

18. a. Shapiro, R.; Hachmann, J. *Biochemistry,* **1966**, *5*, 2799-2807. b. Shapiro, R.; Sodum, R. S.; Everett, D. W.; Kundu, S. K. *IARC. Sci. Publ.* **1986**, *70*, 165-73. c. Seidel, W.; Pischetsrieder, M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2017-2022. d. Broude, N. E., Budowsky, E. I. *Biochim. Biophys. Acta.* **1971**, *254*, 380-8. e. Yuki, H.; Sempuku, C.; Park, M.; Takiura, K. *Anal. Biochem.* **1972**, *46*, 123-128.

19. Spencer, R. D.; Weber, G.; Tolman, G. L.; Barrio, J. R.; Leonard, N. J. *Eur. J. Biochem.* **1974**, *45*, 425-429.

20. Udenfriend, S. *Fluorescence assay in biology and medicine*; Academic Press, New York, 1969, pp. 1-41.

21. Pearson, J.; Carleton, J. S.; Gordon, J. L. *Biochem. J.* **1980**, *190*, 421-429.

22. LeBel, D.; Poirier, G. G.; Phaneuf, S.; St.-Jean, P.; Laliberte, J. F.; Beaudoin, A. R. *J. Biol. Chem.* **1980**, *255*, 1227-1233.

23. Picher, M.; Sevigny, J.; D'Orleans-Juste, P.; Beaudoin, A. R. *Biochem. Pharmacol.* **1996**, *51*,1453-1460.

24. a. Sevigny, J.; Cote, Y. P.; Beaudoin, A. R. *Biochem. J.* **1995**, *312*, 351-356. b. Christoforidis, S.; Papamarcaki, T.; Galaris, D.; Kellner, R.; Tsolas O. *Eur. J. Biochem.* **1995**, *234*, 66-74.

25. Dixon, M.; Webb, E. C. *Enzymes* 3th edition; Academic Press: New-York, NY, 1979.

26. Sevigny, J.; Levesque, F.P.; Grondin, G.; Beaudoin, A.R. *Biochim. Biophys. Acta* **1997**, *1334*, 73-88.

27. Laliberte, J. F.; Beaudoin, A.R. *Biochim. Biophys. Acta* **1983**, *742*, 9-15.

28. Baykov, A. A.; Evtushenko, O. A.; Avaeva, S. M. *Anal. Biochem.* **1988**, *171*, 266-270.

29. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.

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