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Chemoenzymatic Synthesis of Cytokinins from Nucleosides: Ribose as a Blocking Group

Vladimir E. Oslovsky†, Pavel N. Solyev†, Konstantin M. Polyakov, Cyril S. Alexeev and Sergey N. Mikhailov*

Nucleoside phosphorylases are involved in the salvage pathways of nucleoside biosynthesis and catalyze the reversible reaction of the nucleobase with α-D-ribose-1-phosphate to yield the nucleoside and inorganic phosphate. The equilibrium of these reactions is shifted towards nucleosides, especially in the case of purines. Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) is widely used in labs and industry for the synthesis of nucleosides of practical importance. Bacterial PNPs have relatively broad substrate specificity utilizing a wide range of purines with different substituents to form corresponding nucleosides. To shift the reaction in the opposite direction we have used arsenolysis instead of phosphorolysis. This reaction is irreversible due to the hydrolysis of the resulting α-D-ribose-1-arsenate. As a result, heterocyclic bases are formed in quantitative yields and can be easily isolated. We have developed a novel method for preparation of cytokinins based on the enzymatic cleavage of the N-glycosidic bond of N^6-substituted adenosines in the presence of PNP and Na_2HAsO_4. According to the HPLC analysis the conversion proceeds in quantitative yields. In the proposed strategy the ribose residue acts as a protective group. No contamination of the final products with AsO_4^{3-} has been detected via HPLC-HRMS; simple analytical arsenate detection via ESI-MS has been proposed.

Introduction

Purine is the most widely distributed heterocycle in nature – the core component of purine alkaloids, cytokinins, purine nucleoside antibiotics that exhibit profound and diverse biological activities [1]. Being one of the most promising heterocyclic scaffolds, purine often serves as a source for drug design and synthesis of biologically active compounds [2-3].

Cytokinins (CKs) is an important group of phytohormones that play a crucial role in numerous biochemical processes in plants: stimulation of cell division, increase of cell volume, shoot differentiation, activation of metabolite attraction, influence on the biosynthesis of pigments, etc. Naturally occurring CKs are adenine derivatives that carry either an isoprene-derived side chain or an aromatic side chain at the N^6-terminus (Figure 1) [4]. The first CK synthesis was accomplished more than 60 years ago and since that time efficient chemical procedures of CK preparation have been developed [5, 6]. One of the most reliable method is the reaction of 6-chloropurine with benzylamine in n-butanol in the presence of triethylamine at elevated temperatures [6-7].

Our aim is to develop ribose as a new blocking group which can be cleaved enzymatically under very mild conditions in quantitative (or near to quantitative) yields.

Literature analysis reveals that the ribose residue can be removed by acid or enzymatic hydrolysis. Acid hydrolysis of cytokinin nucleosides with aromatic residues leads to CKs in high yields. At the same time, the isoprenyl side chain is rather labile under these conditions [8]. The enzymatic hydrolysis of the N-glycosidic bond can be a method of choice for the preparation of such CK derivatives starting from N^6-substituted adenosines. There are some benefits of using nucleosides as starting compounds for the preparation of CKs and their derivatives. In the proposed strategy, the ribose residue acts as a protective group at the N^9 position of purine, increases
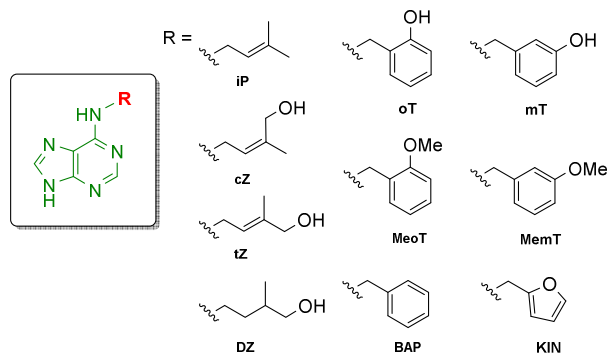
Figure 1. Structure of natural cytokinins (iP – isoprenyladenine; cZ – *cis*-zeatin; tZ – *trans*-zeatin; DZ – dihydrozeatin; mT – *meta*-topolin; oT – *ortho*-topolin; MeoT – *O*-methyl-*ortho*-topolin; MemT – *O*-methyl-*meta*-topolin; BAP – N^6-benzylaminopurine; KIN – kinetin).

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov St., 119991 Moscow, Russian Federation.
Correspondence: smikh@eimb.ru; Tel.: +7-499-135-9733
†   The authors contributed equally.

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the regioselectivity of the electrophilic reactions and facilitates nucleophilic substitutions compared to the unprotected purine derivatives. Isolation and purification of the protected nucleoside derivatives by adsorption chromatography on silica gel is less laborious than that of the corresponding bases. In addition, the cost of natural nucleosides is comparable or in some cases even lower than the cost of the corresponding heterocyclic bases.

Recently, we have developed a useful and versatile approach for the preparation of N^6-modified adenosine derivatives by the regioselective alkylation of N^6-acetyl-2',3',5'-tri-O-acetyladenosine with alcohols under the Mitsunobu reaction conditions or by the base-promoted reaction with alkyl halides (Scheme 1) [9-12]. The main advantage of N^6-acetyl-2',3',5'-tri-O-acetyladenosine as a substrate is the possibility to use both alkyl halides and alcohols for the N^6-modification, that is especially important in the case when an amine is not stable or is hardly available. Using this method, several hundred N^6-substituted adenosines have been synthesized in our laboratory. The described approach compliments the traditional one for the preparation of N^6-alkylated or N^6-arylated adenosines starting from commercially available 6-chloropurine riboside with alkyl- or arylamines [7] (Scheme 1).
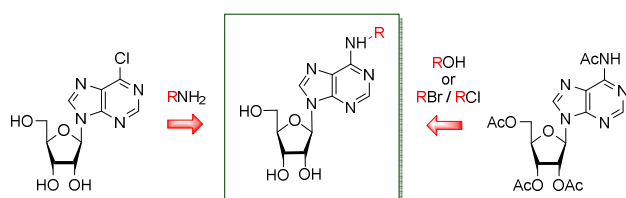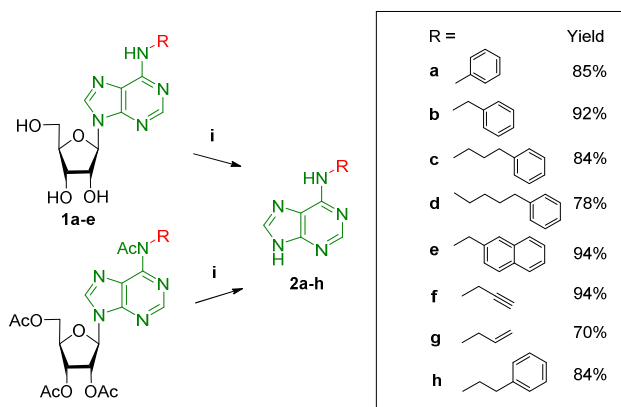

Scheme 1. Methods of choice for the preparation of N^6-substituted adenosine derivatives

Results and discussion

Acid hydrolysis

First, we have examined the acid hydrolysis of a number of N^6-substituted adenines (**2a**-**h**) in aqueous 0.5M HCl at 100°C. According to TLC, most of the reactions were complete in 3 h,

they were cooled and neutralized to pH 7 with 25% aqueous ammonia (Scheme 2). It should be noted that the hydrolysis of N^6-phenyladenosine (**1a**) proceeds within 20 min. However, this method is suitable only for the preparation of aromatic and non-labile saturated CK derivatives [8] and cannot be used in case of unstable 6-substituents. The acid hydrolysis of the fully acetylated N^6-substituted adenosines (**1f**-**h**) proceeds with the formation of N^6-substituted adenines (**2f**-**h**) during 8 h. The prolongation of the reaction time is needed for the additional cleavage of the acetyl group at the N^6-position. Similarly, as in case of the unprotected adenosines, the final products are precipitated from the reaction mixture after neutralization in high yields (70-94%).

Catalyst characteristics

Nucleoside phosphorylases are important biocatalysts for the reversible phosphorolysis of nucleosides to the corresponding nucleobases with the formation of α-D-ribose-1-phosphate (Scheme 3).
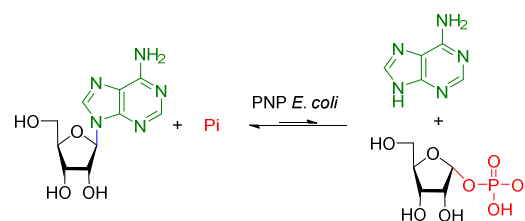
Scheme 3. Reversible phosphorolysis of adenosine. Pi – inorganic phosphate.

The equilibrium of these reactions is shifted towards the nucleosides, especially in the case of purine derivatives [13]. The active site of PNP from *Bacillus cereus* complex with adenosine and sulphate is shown on Figure 2. The active center of the enzyme is formed by two adjacent subunits and the purine base is bound to the hydrophobic pocket. Examination of the crystallographic structure of the complex with nucleosides reveals that there is a large space around the 2- and 6-positions of purine that can accommodate rather bulky substituents (Figure 2) [14].
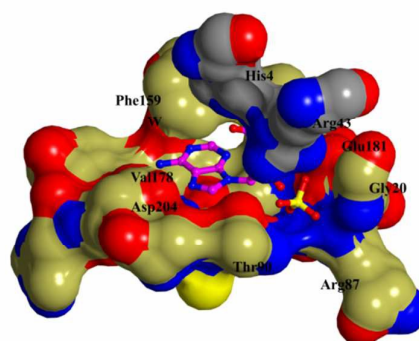

Scheme 2. Synthesis of N^6-substituted adenines (**2a-h**) by acid hydrolysis of unprotected (**1a-e**) and acetylated (**1f-h**) N^6-substituted adenosines. *Reagents and conditions:* i – 0.5 M HCl, 100°C, 20 min – 3 h in the case of the unprotected adenosines, 8 h in the case of the peracetylated adenosines

Figure 2. The active site structure of PNP from Bacillus cereus complex with adenosine (magenta color) and sulfate (yellow color) (3UAW - PDB ID) [14]. The protein is shown as solvent access surface coloured by atom type (the carbon atoms for His4 and Arg43 from the second subunit of the dimer are coloured by grey)

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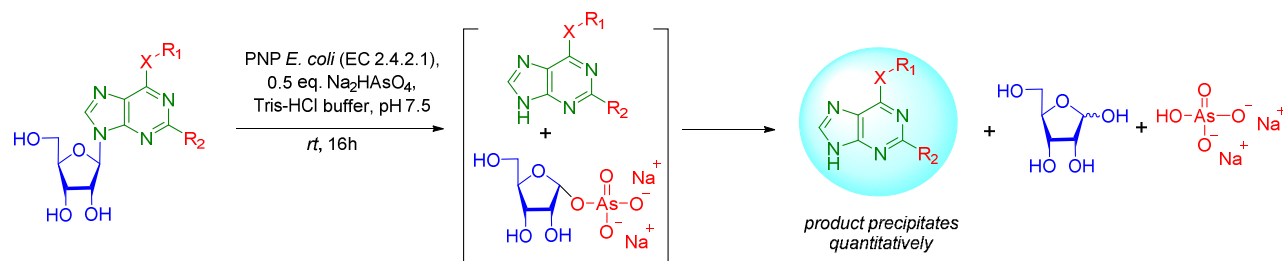
Scheme 4. Irreversible enzymatic arsenolysis of purine nucleosides; X = NH, O; R_2 = NH$_2$, H; R_1 = aromatic or unsaturated aliphatic substituents

Several complexes of PNP with nucleosides were solved with a resolution of 1.9-2.5Å. As a more accurate model for the interaction of an enzyme with a nucleoside, a PNP structure from *Bacillus cereus* was selected because this structure is determined with a resolution at 1.2Å and exhibits significant similarities with the PNP from *E. Coli* in overall structure as well as in active site composition and substrate-binding geometry [14].

Enzymatic hydrolysis

PNPs are widely used for the preparation of differently substituted purine nucleosides. Purines with bulky groups in the 2- and 6-position may be converted by PNP to the corresponding nucleosides. Bacterial PNPs have broader specificity than mammalian ones and may utilize a wide range of purines including adenine, guanine, hypoxanthine and some

other heterocycles including imidazole, triazole, benzimidazole, allopurinol and some others [16, 17]. The unprecedentedly broad substrate specificity of PNP was recently extended to the pyrimidine nucleosides [17, 18]. Broad specificity and bulk tolerance of PNP makes it a valuable tool for the preparation of cytokinin nucleosides.

Earlier we have used PNP for the preparation of CKs starting from the corresponding nucleosides [9]. This reaction is reversible and the equilibrium is shifted towards the formation of nucleosides (Scheme 3). To ensure the completeness of phosphorolysis, alkaline phosphatase is added to the reaction mixture to hydrolyze α-D-ribose-1-phosphate [9]. Using this procedure CKs were prepared in moderate yields (50-68%).

Here, we present another approach based on enzymatic arsenolysis of purine nucleosides with PNP. This reaction is irreversible due to hydrolysis of the resulting α-D-ribose-1-arsenate (Scheme 4) [18].

We have shown that the PNP-catalyzed arsenolysis reactions of the N^6-substituted purine nucleoside derivatives in the presence of Na$_2$HAsO$_4$×7H$_2$O in subequimolar quantities proceed with the formation of the corresponding purine derivatives with quantitative yields according to the HPLC analysis (Figure 3).

Reactions were conducted in 50 mM Tris-HCl buffer solution, pH 7.5 at 50°C for 16 h. The increased temperature is necessary due to the poor solubility of the starting nucleoside. The enzyme, nevertheless, remains stable and soluble under these conditions [19]. After cooling to the ambient temperature the reaction products crystallize directly from the reaction mixture with high yields (Scheme 5).

The advantage of the proposed method in comparison with the acid hydrolysis is the possibility of obtaining purines containing substituents that are unstable under acid hydrolysis, such as isoprenoid or other labile unsaturated substituents [8]. Moreover, the proposed method is useful for the preparation of labile O^6-substituted hypoxanthine and guanine derivatives, which can also be applied as synthetic cytokinin analogues. Thus, we have developed mild and efficient enzymatic method for the cleavage of the D-ribofuranosyl group which might be of general value for the conversion of nucleosides into corresponding heterocycles.

The advantage of the enzymatic arsenolysis as compared to the enzymatic phosphorolysis is the possibility of carrying out
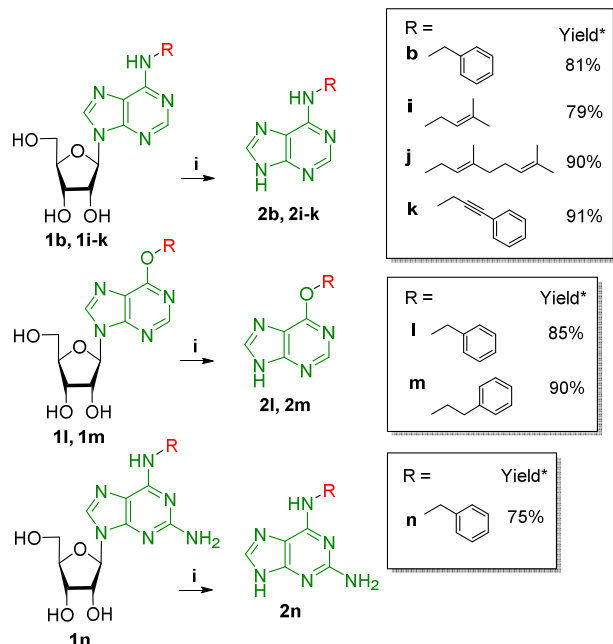
Scheme 5. Synthesis of N^6-substituted adenines (2b, 2i-k, 2n) and O^6-substituted hypoxanthines (2l,m) by enzymatic arsenolysis. Reagents and conditions: i – Na$_2$HAsO$_4$×7H$_2$O (0.5 eq.), PNP, Tris-HCl buffer, pH 7.5, 50°C, 16 h. *The reaction proceeds quantitatively according to HPLC; yields are given for compounds after isolation and purification.

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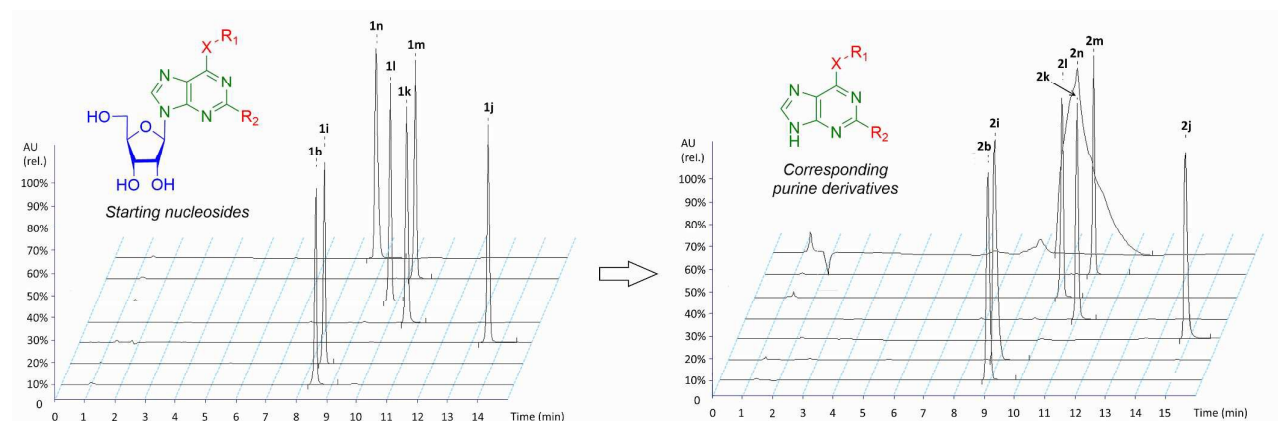
Figure 3. HPLC analysis of the enzymatic arsenolysis of the purine nucleosides **1b, 1i-m**. The reaction was triggered by the addition of 2.8 μl of 1 mg/ml solution of PNP *E. coli* (0.1 activity units) to a nucleoside (0.25-0.29 μmol) in the presence of $Na_2HAsO_4 \times 7H_2O$ (0.5 eq. to nucleoside) in Tris-HCl buffer, pH 7.5 at 50°C (HPLC parameters are described in the Experimental section)

reactions in a short period of time (16 h) using only one enzyme PNP and a small amount of arsenate, while the phosphorolysis reaction requires an excess of phosphate and an additional use of alkaline phosphatase [9].

Products precipitated during the reaction were conveniently separated by the filtration and washed with water several times. To be sure in the absence of toxic sodium arsenate in the products we decided to quantify the possible contamination of the CK samples by HPLC-HRMS.

Determination of the product purity via HPLC-HRMS.

All the products obtained by the enzymatic arsenolysis (Scheme 5) were characterized for purity and homogeneity. The absence of arsenate was additionally confirmed, since inorganic arsenic compounds are the most toxic forms of this element and can be readily absorbed and methylated to methanearsonate or cacodylate *in vivo* [20].

Currently, a combination of several detectors and methods is considered as the most powerful strategy for blind detection of As species [21], and ICP-MS is 3 orders of magnitude more sensitive than ESI-MS. However, the latter type of spectrometer is more common in organic chemistry laboratories. In our case, the nature of the As-containing ion was known (as arsenate) and there was no need for additional tandem MS/MS experiments for precursor ions ([AsO_3]$^+$ and [AsO_2]$^+$). Instead, we conducted further optimization of parameters in the HPLC-MS technique of single ionization for fast and effective detection.

The addition of quaternary ammonium salts is a known technique in MALDI mass-spectrometry for proteins and phosphate group ionization. These dopants create noncovalent complexes with anionic groups of the molecular ion and help to reduce alkali metal "messy" ions. Usually ammonium acetate or formate are added to the buffer solution, and its pH is generally affected; as a result, a suitable acid additive is needed to reach the pH range; changes in retention times should be considered [22].

We proposed a convenient procedure for the preparation of samples with anionic groups where ionization is very poor: a dopant of triethylamine to the sample vial (instead of the ion-pairing additive to the buffer). In our case triethylamine additive to a sample dramatically enhanced arsenate and phosphate ionization by several orders.

The optimization of the MS method has been studied in previous works [23]. We observed the same trends in the negative ion mode: with higher capillary voltage the intensity of the arsenate molecular ion was greater.

We empirically optimized some other parameters for our equipment. There was no marked dependence of RF amplitude of the ion funnels on the low *m/z* cutoff, so they were set at 400 Vpp. Ion transmission in the collision cell was most effective at 750 Vpp (lower RF voltages produced less intensity gain, and >1000 Vpp notably reduced the arsenate molecular ion peak) with collision cell energy of 45 eV. The pre-pulse storage of 10 μsec ditched most of the unnecessary ions of higher masses and gained more 100-150 Da peaks. Good implementation was achieved using quadrupole ion energy of 10 eV, no additional MRM or isCID experiments were carried out.

The sensitivity of the method was evaluated by establishing a limit of detection (LOD – the lowest concentration of an analyte that could be detected with a signal-to-noise ratio of at least 3) and a limit of quantification (LOQ – the same as LOD but with a signal-to-noise ratio of 10) for arsenate ions. The linear dynamic range at five positive control concentrations (containing 1.0×10^{-5} M, 1.0×10^{-6} M, 1.0×10^{-7} M, 1.0×10^{-8} M and 1.0×10^{-9} M solutions of Na_2HAsO_4) and three repeated experiments for each allowed us to assess LOD (AsO_4^{3-}) as 12 ± 2 nM concentration, approximately equivalent to 15 ng/ml, and LOQ (AsO_4^{3-}) as 50 ng/ml. The samples were treated and analyzed immediately in 1 h after their dilution.

As a result of the HPLC-HRMS analysis, none of the tested samples contained AsO_4^{3-} in it in the quantities exceeding LOQ.

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Conclusions

We have shown that nucleosides may be used as the starting compounds for the synthesis of CKs and their derivatives. In the suggested strategy, the ribose residue acts as a protective group. We have compared chemical and enzymatic cleavage of the *N*-glycosidic bond in nucleosides.

The chemical hydrolysis results in CKs in high yields. At the same time it has the evident limit in the case of preparation of labile CK derivatives. This can be solved by the enzymatic hydrolysis which proceeds quantitatively under mild conditions and may be applied to a wide range of nucleosides. The presence of subequimolar quantities of arsenate in the reaction mixture makes the enzymatic hydrolysis irreversible. The final products are wholly separated from arsenate and other co-reactants – the purity of products has been confirmed by HPLC-HRMS. Using the arsenolysis reaction the broad specificity of PNP makes it a valuable tool not only for preparation of modified nucleosides but also for heterocyclic bases with bulky aromatic or isoprenoid substituents.

Experimental

General

The solvents and materials were reagent grade and were used without additional purification. TLC was performed on Alugram SIL G/UV254 (Macherey-Nagel) with UV visualization. Melting points were determined with Electrothermal Melting Point Apparatus IA6301 and are uncorrected.

^{1}H (400 MHz) and ^{13}C (with complete proton decoupling, 100.6 MHz) NMR spectra were recorded on a Bruker AMX 400 NMR instrument at 300K. Chemical shifts in ppm were measured relative to the residual solvent signals as internal standards (CDCl$_3$, ^{1}H: 7.26 ppm, ^{13}C: 77.1 ppm; DMSO-d_6, ^{1}H: 2.50 ppm, ^{13}C: 39.5 ppm), spin-spin coupling constants (*J*) are given in Hz. Double resonance technique was applied for assigning the resonances.

High resolution mass spectra (HRMS) were registered on a Bruker Daltonics micrOTOF-Q II instrument using electrospray ionization (ESI). The measurements were done in a negative ion mode. Interface capillary voltage: 4700 V; mass range from m/z 50 to 3000; external calibration (Electrospray Calibrant Solution, Fluka); nebulizer pressure: 0.4 Bar; flow rate: 3 μL/min; dry gas: nitrogen (4L/min); interface temperature: 200°C. Samples were injected into the mass spectrometer chamber from the Agilent 1260 HPLC system equipped with Agilent Poroshell 120 EC-C18 column (3.0 × 50 mm; 2,7 μm) and an identically packed security guard, using an autosampler. The samples were injected from the 50% acetonitrile (LC-MS grade) in water (MilliQ ultrapure water, Merck Millipore KGaA, Germany) solution in the concentration of 0.1 mg/ml (350 μl) with 50 μl dopant solution of 5% triethylamine. The autosampler syringe was washed before and after injection two times each with 1% formic acid in acetonitrile (wash 1) followed by 20% methanol in water (wash 2). The column temperature was 30°C and 15 μl of the sample solution was injected. The column was eluted in a gradient of

concentrations of A (acetonitrile) in B (water) with the flow rate of 400 μl/min in the following gradient parameters: 0-15% A for 6.0 min, 15%-85% A for 1.5 min, 85%-0% A for 0.1 min, 0% A for 2.4 min. Retention times were as follows: arsenate (analytical detection) – 0.7 min, **2a** – 4.0 min; **2b** – 3.9 min; **2c** – 4.6 min; **2d** – 4.9 min; **2e** – 4.7 min; **2f** – 3.1 min; **2g** – 3.1 min; **2h** – 4.2 min; **2i** – 4.0 min; **2j** – 5.5 min; **2k** – 4.5 min; **2l** – 4.2 min; **2m** – 4.4 min; **2n** – 4.0 min.

HPLC analysis of the test reaction was performed on a Dr. Maisch Reprosil-Pur C$_{18}$-AQ column (4×150mm, 5μm, 120 Å, Part No r15.aq.s1504, Dr. Maisch HPLC GmbH (Germany), in a linear gradient of acetonitrile in deionized water from 2 to 12% for 10 min at the flow rate of 1 ml/min with UV detection at wavelength 260 nm. Retention times of the starting nucleosides were as follows: **1b** – 8.6 min; **1i** – 8.6 min; **1j** – 13.5 min; **1k** – 10.3 min; **1l** – 9.7 min; **1m** – 10.3 min; **1n** – 8.6 min. Retention times of the products were as follows: **2b** – 8.9 min; **2i** – 8.8 min; **2j** – 14.9 min; **2k** – 10.9 min; **2l** – 10.1 min; **2m** – 10.9 min; **2n** – 10.1 min.

In this work, we used recombinant *E. coli* purine nucleoside phosphorylase (PNP, EC 2.4.2.1, Sigma) with the concentration of 1mg/ml and activity 35 units/mg (1 unit corresponds the phosphorolysis of 1 μmol of inosine to hypoxanthine and ribose 1-phosphate per minute at pH 7.4 at 25°C).

The following nucleosides were obtained according to the previously published protocols: *N*^{6}-benzyladenosine [9], *N*^{6}-isopentenyladenosine, *N*^{6}-geranyladenosine [10], *N*^{6}-phenyladenosine, *N*^{6}-(3-phenylpropan-1-yl)adenosine, *N*^{6}-(4-phenylbutane-1-yl)-adenosine, *N*^{6}-(3-phenyl-2-propin-1-yl)adenosine, O^6-(2-phenylethyl)inosine [11], O^6-benzylinosine [24], *N*^{6}-(*β*-naphthylmethyl)adenosine [25], 6-chloro-2-amino-2',3',5'-tri-*O*-acetyladenosine [26].

Acid hydrolysis

N^6-Phenyladenine (2a)

The solution of *N*^{6}-phenyladenosine (71 mg, 0.206 mmol) in 0.5M HCl (2.9 ml, 1.44 mmol) was stirred at 100°C. The reaction was monitored by TLC (acetonitrile – 25% ammonia, 9:1). After 20 min when the traces of initial nucleoside had disappeared, the reaction was cooled and neutralized to pH 7 with concentrated NH$_3$. The reaction mixture was kept at 0°C for 16 h. The resulting precipitate was filtered, washed with cold water (3×5 ml) and dried in vacuum over P$_2$O$_5$. The yield of *N*^{6}-phenyladenine (**2a**) was 37 mg (85%) as white powder. MP = 243-245°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.02 (t, 1H, J_{p-m} = 7.3 Hz, Hp-Ph), 7.32 (dd, 2H, J_{m-o} = 7.9 Hz, J_{m-p} = 7.3 Hz, Hm-Ph), 7.95 (d, 2H, J_{o-m} = 7.9 Hz, Ho-Ph), 8.27 (s, 1H, H2), 8.37 (s, 1H, H8), 9.69 (s, 1H, *N*^{6}H), 13.03 (br s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 119.50 (C5), 120.55 (Ph), 122.33 (Ph), 128.35 (Ph), 139.84 (Ph, C8), 150.44 (C4), 151.81 (C2, C6). HRMS: *m/z* [M−H]$^-$ calculated C$_{11}$H$_8$N$_5^-$ 210.0774, found 210.0782; *m/z* [M+Cl]$^-$ calculated C$_{11}$H$_9$N$_5$Cl$^-$ 246.0541, found 246.0550.

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N^6-Benzyladenine (2b)

Following the typical procedure for preparation of N^6-phenyladenine (**2a**), heating N^6-benzyladenosine (100 mg, 0.279 mmol) in 3.9 ml of 0.5M HCl (1.95 mmol) during 3 h at 100°C gave N^6-benzyladenine (**2b**) (58 mg, 92%) as white powder. R_f = 0.50 (acetonitrile – 25% ammonia, 9:1). MP =231–233°C (Lit. [27] 229-231°C) ^{1}H NMR and ^{13}C NMR spectra were identical to those reported earlier [9].

N^6-(3-Phenylpropan-1-yl)-adenine (2c)

Following the typical procedure for preparation of N^6-phenyladenine (**2a**), heating N^6-(3-phenylpropan-1-yl)adenosine (**1c**) (208 mg, 0.539 mmol) in 7.6 ml of 0.5M HCl (3.77 mmol) during 2 h at 100°C gave N^6-(3-phenylpropan-1-yl)adenine (**2c**) (115 mg, 84%) as white powder. R_f = 0.40 (acetonitrile – 25% ammonia, 9:1). MP = 193–194°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.91 (p, 2H, $J_{CH2-CH2}$= 7.4Hz, CH$_2$C**H$_2$**CH$_2$), 2.65 (t, 2H, $J_{CH2-CH2}$= 7.4Hz, CH$_2$Ph), 3.52 (br s, 1H, N^6CH$_2$), 7.13-7.31 (m, 5H, Ph), 7.64 (br s, 1H, N^6H), 8.06 (H2), 8.16 (H8), 12.84 (br s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 30.89, 32.63 (**C**H$_2$CH$_2$Ph), 38.8-40.3 (N^6**C**H$_2$-overlapping with DMSO-d_6), 118.67 (C5), 125.62, 128.22, 138.49 (C8), 141.84 (Ph), 149.46 (C4), 152.34 (C2), 154.51 (C6). HRMS: m/z [M−H]$^-$ calculated C$_{14}$H$_{14}$N$_5$$^-$ 252.1244, found 252.1245; m/z [M+Cl]$^-$ calculated C$_{14}$H$_{14}$N$_5$Cl$^-$ 288.1010, found 288.1015; m/z [M+HCO$_3$]$^-$ calculated C$_{15}$H$_{15}$N$_5$O$_3$$^-$.

N^6-(4-phenylbutan-1-yl)adenine (2d)

Following the typical procedure for preparation of N^6-phenyladenine (**2a**), heating N^6-(4-phenylbutane-1-yl)adenosine (**1d**) (222 mg, 0.55 mmol) in 7.9 ml of 0.5M HCl (3.89 mmol) during 2 h at 100°C gave N^6-(4-phenylbutane-1-yl)adenine (**2d**) (115 mg, 78%) as white powder. R_f = 0.51 (acetonitrile - 25% ammonia, 9:1). MP = 162–164°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.58-1.67 (m, 4H, CH$_2$, CH$_2$), 2.61 (t, 2H, $J_{CH2-CH2}$=6.8Hz, CH$_2$CH$_2$C**H$_2$**CH$_2$Ph), 3.53 (br s, 2H, N^6CH$_2$), 7.12-7.29 (m, 5H, Ph), 7.68 (br s, 1H, N^6H), 8.09 (s, 1H, H2), 8.19 (s, 1H, H8), 12.88 (br s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 28.41, 28.85, 34.85 (**C**H$_2$**C**H$_2$CH$_2$Ph), 38.8-40.3 (N^6CH$_2$-overlapping with DMSO-d_6), 117.49 (C5), 125.55, 128.14, 128.24 (Ph), 139.06 (C8), 142.16 (Ph), 150.16 (C4), 151.93 (C2), 153.88 (C6). HRMS: m/z [M−H]$^-$ calculated C$_{15}$H$_{16}$N$_5$$^-$ 266.1400, found 266.1402; m/z [M+Cl]$^-$ calculated C$_{15}$H$_{17}$N$_5$Cl$^-$ 302.1167, found 302.1164.

N^6-(β-Naphthylmethyl)adenine (2e)

Following the typical procedure for preparation of N^6-phenyladenine (**2a**), heating N^6-(β-naphthylmethyl)adenosine (**1e**) (101 mg, 0.248 mmol) in 3.5 ml of 0.5M HCl (1.77 mmol) during 2 h at 100°C gave N^6-(β-naphtylmethyl)adenine (**2e**) (65 mg, 94%) as white powder. R_f = 0.40 (acetonitrile – 25% ammonia, 9:1). MP = 245–247°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 4.96 (br s, 2H, N^6CH$_2$), 7.43-7.58 (m, 3H, naphthalene), 7.82-7.92 (m, 5H, N^6H, naphthalene), 8.31 (s, 1H, H2), 8.38 (s, 1H, H8), 9.00 (br s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 43.67 (N^6CH$_2$), 115.97 (C5), 125.37, 125.62, 125.88, 126.12, 127.49, 127.89, 132.12, 132.84, 136.83 (naphthalene), 140.65

(C8), 149.59 (C4), 150.44 (C2), 153.04 (C6). HRMS: m/z [M−H]$^-$ calculated C$_{16}$H$_{12}$N$_5$$^-$ 274.1087, found 274.1089; m/z [M+Cl]$^-$ calculated C$_{16}$H$_{13}$N$_5$Cl$^-$ 310.0854, found 310.0848.

N^6-Propargyladenine (2f)

Following the typical procedure for preparation of N^6-phenyladenine (**2a**), heating N^6-acetyl-N^6-propargyl-2′,3′,5′-tri-O-acetyladenosine (**1f**) (100 mg, 0.211 mmol) in 2.5 ml of 0.5M HCl (1.26 mmol) during 8 h at 100°C gave N^6-propargyladenine (**2f**) (35 mg, 94%) as white powder. R_f = 0.67 (acetonitrile – 25% ammonia, 9:1). MP = 206-207°C. ^{1}H NMR (DMSO-d_6): 3.01 (s, 1H, ≡CH), 4.30 (s, 2H, N^6CH$_2$), 7.91 (br s, 1H, N^6H), 8.13 (s, 1H, H-2), 8.24 (s, 1H, H-8), 12.94 (br s, 1H, 9NH). ^{13}C NMR (DMSO-d_6): δ = 29.26 (N^6CH$_2$), 72.38 (HC≡), 82.01 (-C≡), 117.95 (C-5), 139.51 (C-8), 152.16 (C-2), 153.23 (C-6). HRMS: m/z [M−H]$^-$ calculated C$_8$H$_6$N$_5$$^-$ 172.0618, found 172.0611; m/z [M+Cl]$^-$ calculated C$_8$H$_7$N$_5$Cl$^-$ 208.0384, found 208.0384.

N^6-Allyladenine (2g)

Following the typical procedure for preparation of N^6-phenyladenine (**2a**), heating N^6-acetyl-N^6-allyl-2′,3′,5′-tri-O-acetyladenosine (**1g**) (100 mg, 0.210 mmol) in 2.5 ml of 0.5M HCl (1.26 mmol) during 8 h at 100°C gave N^6-allyladenine (**2g**) (26 mg, 70%) as white powder. R_f = 0.45 (acetonitrile – 25% ammonia, 9:1). MP = 224-226°C. ^{1}H NMR (DMSO-d_6): δ = 4.16 (br s, 2H, N^6CH$_2$), 5.06 (d, 1H, J = 9.5 C=CH-cis), 5.17 (d, 1H, J = 17 C=CH-$trans$), 5.93-6.0 (m, 2H, CH$_2$), 7.69 (br s, 1H, N^6H), 8.09 (s, 1H, H-2), 8.17 (s, 1H, H-8), 12.82 (br s, 1H, 9NH). ^{13}C NMR (DMSO-d_6): δ = 42.00 (N^6CH$_2$), 114.96 (=CH$_2$), 135.91 (CH), 138.73 (C-8), 149.83 (C-4), 152.30 (C-2), 154.26 (C-6). HRMS: m/z [M−H]$^-$ calculated C$_8$H$_8$N$_5$$^-$ 174.0774, found 174.0773; m/z [M+Cl]$^-$ calculated C$_8$H$_9$N$_5$Cl$^-$ 210.0541, found 210.0542.

N^6-(2-Phenylethyl)adenine (2h)

Following the typical procedure for preparation of N^6-phenyladenine (**2a**), heating N^6-acetyl-N^6-(2-phenylethyl)-2′,3′,5′-tri-O-acetyladenosine (**1h**) (330 mg, 0.694 mmol) in 8.3 ml of 0.5M HCl (4.16 mmol) during 8 h at 100°C gave N^6-(2-phenylethyl)adenine (**2h**) (102 mg, 84%) as white powder. R_f = 0.57 (acetonitrile – 25% ammonia, 9:1). MP = 248-250°C. ^{1}H NMR (DMSO-d_6): δ = 2.93 (t, J = 7.5, 2H, N^6CH$_2$), 3.73 (br s, 2H, CH$_2$) 7.15-7.35 (m, 5H, H-Ph), 7.59 (br s, 1H, N^6H), 8.07 (s, 1H, H-2), 8.19 (s, 1H, H-8), 12.85 (br s, 1H, 9NH). ^{13}C NMR (DMSO-d_6): δ = 35.15 (**C**H$_2$Ph), 41.29 (N^6CH$_2$), 118.68 (C5), 125.97, 128.26, 128.62 (Ph), 138.78 (C8), 139.57 (Ph), 149.53 (C4), 152.34 (C2), 154.27 (C6). HRMS: m/z [M−H]$^-$ calculated C$_{13}$H$_{12}$N$_5$$^-$ 238.1087, found 238.1093; m/z [M+Cl]$^-$ calculated C$_{13}$H$_{13}$N$_5$Cl$^-$ 274.0854, found 274.0854.

Enzymatic arsenolysis

Analytical arsenolysis reaction of adenosine

The reaction solution contained 0.05 ml of 4 mM adenosine stock solution (0.2 μmol), 0.025 ml of 4 mM Na$_2$HAsO$_4$×7H$_2$O stock solution (0.1 μmol) (1:0.5 ratio) in 0.925ml of 50mM Tris-HCl buffer, pH 7.5 (total volume 1 ml). The reaction was

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triggered by the addition of 0.002 ml of 1 mg/ml solution of PNP *E. coli* (0.07 activity units). The reaction was incubated at 20°C, and was followed by HPLC (Figure 3).

Preparative arsenolysis reaction of N^6-substituted adenosines

N^6-Benzyladenine (2b)

To the mixture of N^6-benzyladenosine (**1b**) (94 mg, 0.263 mmol) and $Na_2HAsO_4 \times 7H_2O$ (41 mg, 0.131 mmol) in 50 mM Tris-HCl buffer pH 7.5 (10 ml) PNP, (0.143 ml, 5 activity units) was added and the mixture was kept at 50°C for 16 h. During the reaction, the initial nucleoside dissolved and the product crystallized. The precipitate was filtered, washed with H_2O (5×5 ml) and dried in vacuum dessicator over P_2O_5 to give N^6-benzyladenine (**2b**) (48 mg, 81%) as white powder. R$_f$ = 0.53 (CH_2Cl_2 – EtOH, 95:5). MP = 232–234°C (Lit. [27] 229-231°C). ^{1}H NMR and ^{13}C NMR spectra were identical to those reported earlier [9]. HRMS: *m/z* [M−H]$^-$ calculated $C_{12}H_{10}N_5^-$ 224.0931, found 224.0941; *m/z* [M+Cl]$^-$ calculated $C_{12}H_{11}N_5Cl^-$ 260.0697, found 260.0708.

N^6-Isopentenyladenine (2i)

Following the procedure for preparation of N^6-benzyladenine (**2b**), exposure of N^6-isopentenyladenosine (**1i**) (100 mg, 0.298 mmol) with $Na_2HAsO_4 \times 7H_2O$ (46 mg, 0.149 mmol) and PNP (0.143 ml, 5 activity units) in 50 mM Tris-HCl buffer pH 7.5 (10 ml) during 16 h at 50°C gave N^6-isopentenyladenine (**2i**) (48 mg, 79%) as white powder. R$_f$ = 0.43 (CH_2Cl_2 – EtOH, 95:5). MP = 210-212°C (Lit. [28] 216-218°C). ^{1}H NMR and ^{13}C NMR spectra were identical to those reported earlier [9]. HRMS: *m/z* [M−H]$^-$ calculated $C_{10}H_{12}N_5^-$ 202.1087, found 202.1085; *m/z* [M+Cl]$^-$ calculated $C_{10}H_{13}N_5Cl^-$ 238.0854, found 238.0851.

N^6-Geranyladenine (2j)

Following the procedure for preparation of N^6-benzyladenine (**2b**), exposure of N^6-geranyladenosine (**1j**) (100 mg, 0.248 mmol) with $Na_2HAsO_4 \times 7H_2O$ (39 mg, 0.124 mmol) and PNP (0.143 ml, 5 activity units) in 50 mM Tris-HCl buffer pH 7.5 (10 ml) during 16 h at 50°C gave N^6-geranyladenine (**2j**) (61 mg, 90%) as white powder. R$_f$ = 0.56 (CH_2Cl_2 – EtOH, 95:5). MP = 162–163°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.54 (s, 3H, Me), 1.60 (s, 3H, Me), 1.70 (s, 3H, Me), 1.96 (ddd, 2H, J_{CH2-CH} = 6.9 Hz, J_{CH2CH2} = 7.8 Hz, $J_{CH2-CH2}$ = 7.2 Hz, CH_2CH_2CH=CMe$_2$), 2.04 (dd, 2H, J_{CH2CH2} = 7.8 Hz, $J_{CH2-CH2}$ = 7.2 Hz, CH_2CH$_2$CH=CMe$_2$), 4.09 (br s, 1H, N^6CH$_2$), 5.06 (tt, 1H, J_{CH-CH2} = 6.9 Hz, J_{CH-CH2} = 1.2 Hz, CH=CMe$_2$), 5.32 (dd, 1H, J_{CH-CH2} = 5.8 Hz, J_{CH-CH2} = 6.4 Hz, N^6CH$_2CH$=), 7.58 (br s, 1H, N^6H), 8.05 (s, 1H, H-8), 8.16 (s, 1H, H-2), 12.84 (s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 16.10, 17.47 (=C**Me$_2$**-geranyl), 25.37 (Me-geranyl), 25.93 (CH$_2$-geranyl), 37.65, 37.79 (N^6CH$_2$, CH$_2$-geranyl), 118.69 (C5), 122.06 (CH=), 123.92 (CH=), 130.73 (=C(Me)CH$_2$-geranyl), 136.34 (=C**Me$_2$**-geranyl), 138.49 (C8), 149.43 (C4), 152.31 (C2), 154.18 (C6). HRMS: *m/z* [M−H]$^-$ calculated $C_{15}H_{20}N_5^-$ 270.1713, found 270.1719; *m/z* [M+Cl]$^-$ calculated $C_{15}H_{21}N_5Cl^-$ 306.1480, found 306.1481.

N^6-(3-Phenyl-2-propin-1-yl)adenine (2k)

Following the procedure for preparation of N^6-benzyladenine (**2b**), exposure of N^6-(3-phenyl-2-propin-1-yl)adenosine (**1k**) (91 mg, 0.238 mmol) with $Na_2HAsO_4 \times 7H_2O$ (37 mg, 0.119 mmol) and PNP (0.143 ml, 5 activity units) in 50 mM Tris-HCl buffer pH 7.5 (10 ml) during 16 h at 50°C gave N^6-(3-phenyl-2-propin-1-yl)adenine (**2k**) (54 mg, 91%) as white powder. R$_f$ = 0.37 (CH_2Cl_2 – EtOH, 95:5). MP = 227-229°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 4.57 (br s, 2H, N^6CH$_2$), 7.31-7.41 (m, 5H, Ph), 8.06 (br s, 1H, N^6H), 8.13 (s, 1H, H8), 8.26 (s, 1H, H2), 12.97 (s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 30.01 (N^6CH$_2$), 81.04, 88.01 (**C≡C**), 118.93 (C5), 122.43, 128.33, 128.56, 131.28 (Ph), 139.16 (C8), 150.01 (C4), 152.20 (C2), 153.68 (C6). HRMS: *m/z* [M−H]$^-$ calculated $C_{14}H_{10}N_5^-$ 248.0931, found 248.0933; *m/z* [M+Cl]$^-$ calculated $C_{14}H_{11}N_5Cl^-$ 284.0697, found 284.0704.

O-6-Benzylhypoxanthine (2l)

Following the procedure for preparation of N^6-benzyladenine (**2b**), exposure of O-6-benzylinosine (**1l**) (124 mg, 0.346 mmol) with $Na_2HAsO_4 \times 7H_2O$ (54 mg, 0.170 mmol) and PNP (0.143 ml, 5 activity units) in 50 mM Tris-HCl buffer pH 7.5 (10 ml) during 16 h at 50°C gave O-6-benzylhypoxanthine (**2l**) (67 mg, 85%) as white powder. R$_f$ = 0.23 (CH_2Cl_2 – EtOH, 95:5). MP = 174-176°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.62 (s, 2H, O^{6}CH$_2$), 7.32-7.56 (m, 5H, Ph), 8.38 (s, 1H, H-2), 8.52 (s, 1H, H-8), 13.43 (s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 67.56 (OCH$_2$), 128.08, 128.28, 128.41, 136.36 (Ph), 142.77 (C8), 151.17 (C2) HRMS: *m/z* [M−H]$^-$ calculated $C_{12}H_9N_4O^-$ 225.0771, found 225.0775; *m/z* [M+Cl]$^-$ calculated $C_{12}H_{10}N_4OCl^-$ 261.0538, found 261.0543.

O-6-(2-Phenylethyl)hypoxanthine (2m)

Following the procedure for preparation of N^6-benzyladenine (**2b**), exposure of O-6-(2-phenylethyl)inosine (**1m**) (100 mg, 0.268 mmol) with $Na_2HAsO_4 \times 7H_2O$ (42 mg, 0.134 mmol) and PNP (0.143 ml, 5 activity units) in 50 mM Tris-HCl buffer pH 7.5 (10 ml) during 16 h at 50°C gave O-6-(2-phenylethyl)hypoxanthine (**2m**) (58 mg, 90%) as white powder. R$_f$ = 0.30 (CH_2Cl_2 – EtOH, 95:5). MP = 204-206°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.14 (t, 2H, $J_{CH2-CH2}$ = 6.9 Hz, CH$_2$Ph), 4.75 (t, 2H, $J_{CH2-CH2}$ = 6.9 Hz, O^{6}CH$_2$), 7.22 (tt, 1H, J_{p-m} = 7.0 Hz, J_{p-o} = 1.4 Hz, Hp-Ph), 7.30 (t, 2H, J_{m-o} = 7.3 Hz, Hm-Ph), 7.34 (dd, 2H, J_{o-m} = 7.3 Hz, Ho-Ph), 8.34 (s, 1H, H-2), 8.48 (s, 1H, H-8), 13.37 (s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 34.55 (**C**H$_2$Ph), 66.77 (O**C**H$_2$), 120.02 (C5), 126.32, 128.32, 128.89, 138.02 (Ph), 142.05 (C8), 151.25 (C2), 153.15 (C4), 159.75 (C6). HRMS: *m/z* [M−H]$^-$ calculated $C_{13}H_{11}N_4O^-$ 239.0927, found 239.0931; *m/z* [M+Cl]$^-$ calculated $C_{13}H_{12}N_4OCl^-$ 275.0694, found 275.0698.

2-Amino-N^6-benzyladenine (2n)

Following the procedure for preparation of N^6-benzyladenine (**2b**), exposure of 2-amino-N^6-benzyladenosine (**1n**) (100 mg, 0.268 mmol) with $Na_2HAsO_4 \times 7H_2O$ (42 mg, 0.134 mmol) and PNP (0.143 ml, 5 activity units) in 50 mM Tris-HCl buffer pH 7.5 (10 ml) during 16 h at 50°C gave 2-amino-N^6-benzyladenine (**2n**) (48 mg, 75%) as yellow powder. R$_f$ = 0.40 (CH_2Cl_2 – EtOH, 95:5). MP = 232-233°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 4.67

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(br s, 2H, N^6CH$_2$), 5.65 (2H, s, 2-NH$_2$), 7.19 (t, 1H, J_{p-m} = 7.0 Hz, Hp-Ph), 7.28 (t, 2H, J_{m-o} = 7.4 Hz, Hm-Ph), 7.34 (dd, 2H, J_{o-m} = 7.4 Hz, Ho-Ph), 7.58 (br s, 1H, N^6H), 7.64 (s, 1H, H-8), 12.05 (s, 1H, 9NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 42.70 (N^6CH$_2$), 113.04 (C5), 126.42 (Ph), 127.24 (Ph), 128.06 (Ph), 135.26 (Ph), 140.68 (C8), 152.03 (C4), 154.65 (C6), 160.15 (C2). HRMS: m/z [M−H]$^-$ calculated C$_{12}$H$_{11}$N$_6^-$ 239.1040, found 239.1046; m/z [M+Cl]$^-$ calculated C$_{12}$H$_{12}$N$_6$Cl$^-$ 275.0806, found 275.0816.

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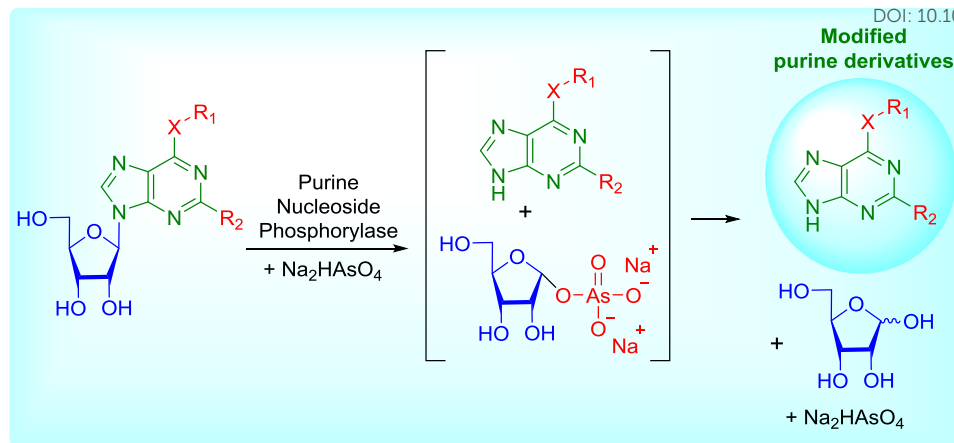
Cytokinins synthesis based on the irreversible enzymatic cleavage by Purine Nucleoside Phosphorylase in the presence of Na_2HAsO_4 has been developed.