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Original article

Microwave-assisted synthesis of C-8 aryl and heteroaryl inosines and determination of their inhibitory activities against *Plasmodium falciparum* purine nucleoside phosphorylase

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ABSTRACT

8-Arylinosines have been scarcely studied for therapeutic purposes, probably due to difficulties in their synthesis. The recently described direct arylation reaction at position 8 of purine nucleosides has been employed to synthesize a series of 8-aryl and 8-pyridylinosines. These compounds have been studied for hydrolytic stability and subjected to biological evaluation. Three compounds have shown a pronounced specific inhibition of *Plasmodium falciparum*-encoded purine nucleoside phosphorylase, an important target for antimalarial chemotherapy.

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1. Introduction

C-8 Arylpurine nucleosides are interesting chemical entities due to their potential as therapeutic agents and as tools for biomolecular applications, as recently reviewed [1]. These compounds are mostly synthesized by Suzuki or Stille cross-coupling processes starting from the corresponding 8-bromonucleosides. More recently a direct arylation reaction at position 8 of purine nucleosides has been described by two independent groups [2,3] mediated by a Pd–Cu catalyst system with aryl iodides using an appropriate base. This reaction has been applied to the unprotected nucleosides to obtain C-8 aryl nucleosides in a single reaction step [2,3]. Moreover the reaction works well with either electron-donating or electron-withdrawing aryl iodides allowing the introduction of different aryl substituents. This interesting synthetic procedure has been applied mostly to adenosine derivatives [2,3] and has been scarcely explored for guanosine and inosine [4].

Our research group has been involved in the synthesis and the biological evaluation of inosine nucleosides, and we have reported their behaviour as inhibitors of nucleoside-processing enzymes [5] as well as their capacity to inhibit new blood vessel formation [6–8]. Moreover it is well-known that inosine derivatives are interesting entities for evaluation against purine nucleoside phosphorylase, a key enzyme in the purine salvage pathway of humans and different pathogens [9,10]. Among the latest, PNP from *Plasmodium falciparum* is probably the best studied target [11–14]. Thus, the recently described direct arylation reaction sounds appealing to our interests to access C-8 aryl inosine nucleosides (Fig. 1) and to explore their biological potential as chemotherapeutics.

So our initial approach was to apply the reaction conditions set up by Fairlamb for adenosines [4] and to introduce a variety of differently substituted aryl groups at position 8 of inosine. However, our attempts to apply this described procedure to inosine did not meet a successful outcome. Only a very low conversion to the

Abbreviations: MAOS, microwave-assisted organic synthesis; *Pf*PNP, *Plasmodium falciparum* purine nucleoside phosphorylase.

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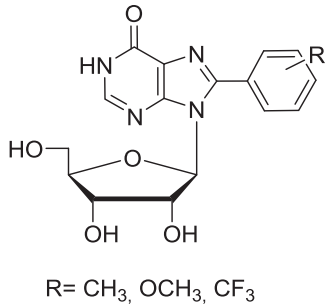


Fig. 1. Proposed 8-arylinosines.

desired 8-aryl compound was detected accompanied by serious difficulties to isolate a pure compound for biological testing. This different behaviour of inosine compared to adenosine could be partially ascribed to low solubility, a problem already described in other synthetic approaches. Therefore, our working strategy has been to increase the solubility of inosine by protection of the nucleoside OHs, prior to performing the arylation reaction. Very recently other authors have also used a protection/deprotection procedure while performing C–C cross coupling reactions in inosines [15]. The reported examples in adenosine require, in general, long reaction times, typically overnight [4] or even up to 60 h [2]. We have found that application of microwave-assisted synthesis has allowed performing the reaction on protected inosine in one hour. The synthesized inosines have been evaluated against purine nucleoside phosphorylases of different origins. In parallel their antiviral activity has also been determined.

2. Results and discussion

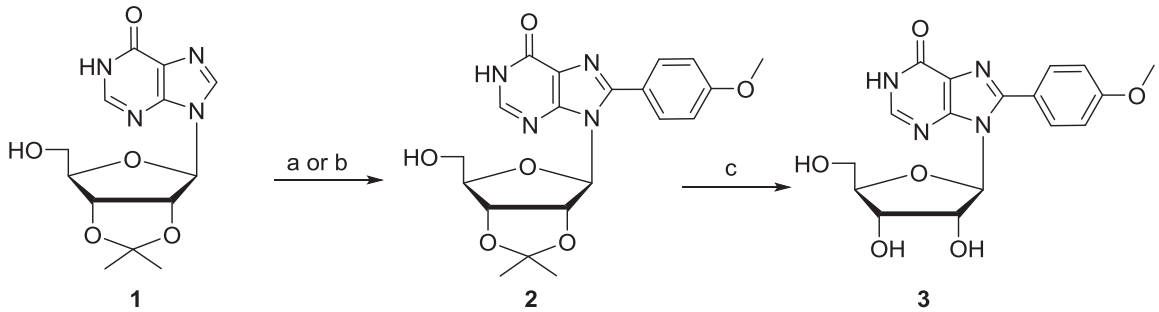
2.1. Chemistry

The described procedures for the direct C-8 arylation of adenosines [2–4] require the participation of an appropriate base (mostly Cs_2CO_3 and/or piperidine) whose role has been well-studied by Fairlamb and co. [3,4]. Therefore the protection of the sugar OHs had to be performed with a basic-stable protecting group, and this made us to consider the commercially available 2′,3′-*O*-isopropylideninosine (**1**) as a convenient starting material. Reaction of **1** with 4-iodoanisole in the presence of Cs_2CO_3 (2.5 eq), CuI (3.0 eq), piperidine (0.4 eq), $Pd(OAc)_2$ (0.05 eq) and 4-iodoanisole (2.0 eq) in dry and degassed DMF (5 mL/mmol) in a vacuum-dried Schlenk tube at 80 °C overnight afforded the C-8 arylated compound (**2**) in approximately 22% yield (Scheme 1). HPLC-MS analysis of the reaction mixture indicated that the only accompanying nucleoside product was the starting material. Attempts to increase the yield by prolonging the reaction time and/or by increasing the quantities of the reactives were almost unsuccessful; only increasing the temperature slightly improved the conversion. At this stage, the use of microwave (MW) heating was considered. Indeed Alami [16] had previously successfully applied microwave irradiation for the direct C-8 arylation of non-ribose adenines employing high temperatures (160 °C). However when Farirlamb's group applied these conditions to the arylation reaction of 2′-deoxyadenosine [4], extensive breaking of the glycosidic bond was detected ascribed to the sensitivity of this bond to such high temperatures. Based on the higher stability of ribonucleosides compared to their 2′-deoxynucleoside analogues, we decided to assay microwave-irradiation for the synthesis of **2** applying lower temperatures. We were glad to see that the microwaved-reaction of **1** with 4-iodoanisole in DMF in the presence of Cs_2CO_3, CuI, $Pd(OAc)_2$ and piperidine at 120 °C for 1 h showed a clear conversion to the coupling product **2** with no detection of glycosidic bond cleavage.

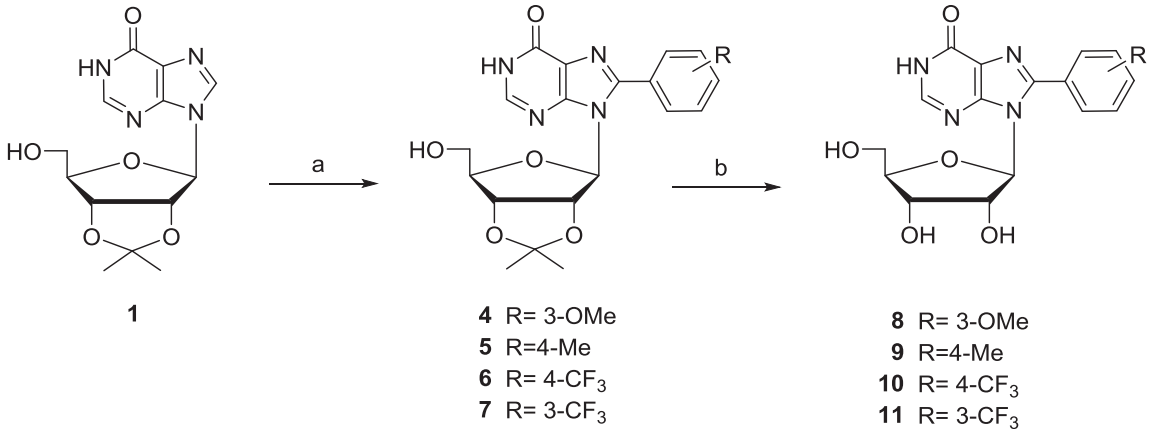
However, the work-up procedure was found problematic. Removal of the metal salts was partially performed by filtration, but attempts to completely eliminate these salts by acid treatment as described by other authors led to extensive decomposition through breakage of the glycosidic bond. In our hands, the solid isolated after filtration and subsequent chromatography was deeply coloured, probably indicating the concomitant presence of some metal salts. We were seriously concerned about the coexistence of metal containing by-products that might be a source of influencing and misinterpretation of the biological results. Indeed the use of copper salts, extensively applied in click chemistry, is being reconsidered when applied to the synthesis of compounds meant for biological screening, since such salts may have a clear impact on cellular events masking the real properties of the compounds [17–19]. Therefore, to assure the removal of any metal contaminants, the compound obtained after chromatography was extensively treated with Quadrasil MP in DMF overnight. Filtration of the resin afforded the 8-arylnucleoside **2** as a white solid in 52% overall yield.

The next step involved the removal of the isopropylidene group but the acid conditions required provoked partial breaking of the glycosidic bond generating a variable proportion of 8-(4-methoxyphenyl)hypoxanthine. The cleanest deprotection was observed by time-controlled treatment with a mixture of TFA/dioxane/H_2O (4:1:1) at rt, and quenching with NH_4HCO_3. In this way, compound **3** was isolated in 98% yield.

The pathway step-up for the synthesis of **3** as a model compound was applied to the reaction of **1** with different aryl iodides. The results obtained are shown in Scheme 2. Thus reaction of 2′,3′-*O*-isopropylideneinosine (**1**) with a variety of aryl iodides (3-iodoanisole, 4-iodotoluene, 4(3)-trifluoromethylphenyl iodides) under microwave conditions (120 °C, 1 h) using Cs_2CO_3, CuI, $Pd(OAc)_2$ and piperidine afforded the 8-arylinosines (**4**–**7**) in good



Scheme 1. *Reagents and conditions*: (a) 4-Iodoanisole; Cs_2CO_3, $Pd(OAc)_2$, CuI, piperidine; DMF; 80 °C, 16 h (22% yield); (b) 4-Iodoanisole; Cs_2CO_3, $Pd(OAc)_2$, CuI, piperidine; DMF; MW 120 °C, 1 h (52% yield); (c) TFA 60%, H_2O:1,4-dioxane, 15 min, and then quenching with NH_4HCO_3 (98% yield).



Scheme 2. *Reagents and conditions*: (a) Ar–I; Cs_2CO_3, $Pd(OAc)_2$, CuI, piperidine; DMF; MW 120 °C, 1 h (46–61%); (b) TFA 60%, H_2O:1,4-dioxane, 15 min, and then quenching with NH_4HCO_3 (60–87% yield).

yields. No significant differences were observed when employing electron-donating or electron-withdrawing groups at the aryl moiety, in agreement with that reported by other authors [4,20]. The reaction also took place when an aryl bromide was used (i.e., 4-bromoanisole) but the yield was significantly lower than that obtained with the corresponding aryl iodide. In all cases, Quadrasil MP treatment was crucial to obtain a pure compound. Removal of the isopropylidene group in compounds **4**–**7** afforded the deprotected ribonucleosides **8**–**11** in good to excellent yields.

Based on these results, the reaction was extended to the synthesis of 4-pyridyl or 3-pyridyl 8-substituted inosines (Scheme 3). Thus reaction of 2′,3′-*O*-isopropylideneinosine (**1**) with 4-iodopyridine or 3-iodopyridine afforded the corresponding coupling products at position 8, **12** and **13**, in 55 and 54% yields, respectively. Deprotection of the isopropylidene group provided the 8-pyridylinosines **14** and **15**, in 78 and 69% yields, respectively.

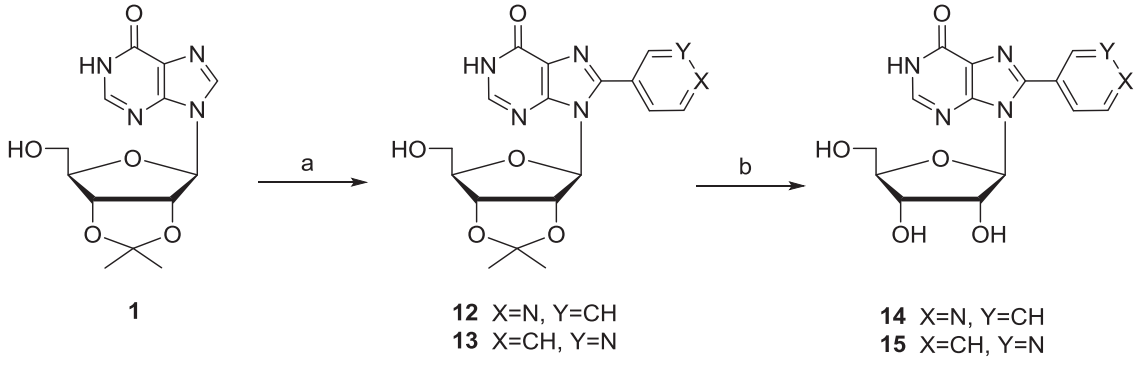
2.2. Hydrolytic stability studies

It has been described that 8-substituted purine nucleosides are more sensitive to acidic conditions than their non-substituted analogues [1,21] and indeed we have experienced serious difficulties to remove the isopropylidene group by acid treatment without affecting the glycosidic bond. In this scenario, we considered relevant to determine the hydrolytic stability of the synthesised 8-substituted inosines at different pH values. The compounds were incubated at 37 °C in buffered solutions at three different pHs (1.2, 5.0 and 7.4) and the evolution was followed by HPLC at different time points (0, 1 h, 4 h and 24 h). Compounds **3**, **8**–**11**, **14** and **15** were found to be completely stable in the buffer solutions at pH 5.0 and 7.4 up to 24 h (data not shown). Only at pH 1.2, the compounds suffer from the breakage of the glycosidic bond with half-lifes around 5 h (Fig. S1 in Supplementary data). Interestingly the two pyridyl derivatives **14** and **15** showed a higher stability with larger half-lifes (between 10 and 15 h).

2.3. Biological evaluation

The synthesized compounds **3**, **8**–**11**, **14** and **15** were evaluated for their inhibitory activity against a wide variety of DNA and RNA viruses (including, among others, herpes simplex virus type 1, herpes simplex virus type 2, vaccinia virus, vesicular stomatitis virus, cytomegalovirus, human immunodeficiency virus and influenza virus). Compound **9** could not be tested due to solubility problems. Unfortunately, no significant antiviral activity was observed at 100 μM. It should be mentioned that no cytotoxicity was detected against the host cell lines (i.e. CEM, HEL, HeLa, Vero and MDCK) at concentrations up to 100 μM suggesting that the compounds are free of any toxic contaminant.

Compounds **3**, **8**–**11**, **14** and **15** were also evaluated against purine nucleoside phosphorylase of *P. falciparum* (*Pf*PNP). This enzyme, that preferentially uses inosine as substrate, has a central role in purine salvage for the parasite and is a promising validated target for antimalarial intervention [12,14]. Immucillins have been deeply investigated as transition state analogues inhibiting *Pf*PNP and compounds such as 5′-methylthio-immucillin-H kills



Scheme 3. *Reagents and conditions*: (a) 4-iodo- or 3-iodopyridine, Cs_2CO_3, $Pd(OAc)_2$, CuI, piperidine; DMF; MW 120 °C; 1 h (**12**: 55%; **13**: 54%); (b) TFA 60%, H_2O:1,4-dioxane, 15 min, and then quenching with NH_4HCO_3 (**14**: 78%; **15**: 69%yield).

P. falciparum in culture [22]. More recently, another immucillin, DADMe-Immucillin-G, has been shown to kill the parasite in a primate animal model [14]. When tested against *Pf*PNP using inosine as the natural substrate, compounds **8**, **10** and **11** showed significant inhibitory activity against this enzyme in the low μM range (Ki = 8.1, 3.7 and 5.3 μM, respectively), with values quite comparable to the Km for the natural substrate inosine (Km = 5.9 μM) (Table 1). Compounds **8**, **10** and **11** were also investigated as potential inhibitors or substrates against human and prokaryotic (i.e. *Escherichia coli*, *Mycoplasma hyorhinis*) PNP. When the compounds were evaluated as potential inhibitors of the conversion of 100 μM inosine to hypoxanthine, no signs of direct inhibitory activity were observed for both enzymes. Also, no substrate activity was found under conditions where 100 μM inosine was converted to hypoxanthine by 96–98% within 1 h. These findings point to a significant degree of selectivity of these compounds as specific anti-*Pf*PNP agents. Unfortunately the compounds were devoid of significant inhibitory activity of growth of intraerythrocytic forms of the *P. falciparum* chloroquine-sensitive strain 3D7 when tested at 25 μM, while chloroquine in the same assay gave an IC_{50} value of 5 nM. The lack of cellular activity may be due to inefficient uptake by the erythrocytes and/or the parasites in addition to limited intracellular efficacy. Specific uptake studies might provide more insights on this issue.

3. Conclusions

A number of 8-aryl and pyridyl-substituted inosines have been synthesized making use of the recently reported direct arylation reaction mediated by Pd–Cu complexes. Special attention has been paid to the removal of metal contaminants that has been accomplished including an extensive Quadrasil MP treatment. The 8-substituted inosines obtained proved to be stable at pH values of 7.4 and 5.0, while at pH = 1.2 their half-lives varied between 5 and 10 h. Concerning the biological evaluation, no significant toxicity was detected in different types of cell cultures supporting the absence of metal-contaminants. Moreover compounds **8**, **10** and **11** were able to inhibit *P. falciparum* PNP at low micromolar Ki values similar to that of the Km of inosine. The lack of inhibitory or substrate properties of these inosine derivatives **8** against human and prokaryotic PNP let us conclude that these 8-arylinosines are highly selective inhibitors of *Pf*PNP and therefore represent interesting starting points for further development of specific inhibitors of the enzyme.

4. Experimental

4.1. Synthesis

Melting points were obtained on a Reichert-Jung Kofler apparatus and are uncorrected. The elemental analysis was performed with a Heraeus CHN–O-RAPID instrument. The elemental compositions of the compounds fell within ±0.4% of the calculated values. Electrospray mass spectra were measured on a quadrupole mass spectrometer equipped with an electrospray source (Hewlett–Packard, LC/MS HP 1100). ^1H and ^{13}C NMR spectra were recorded on a Varian INNOVA 300 operating at 299 MHz (^1H) and 75 MHz (^{13}C), respectively, and Varian INNOVA-400 operating at 399 MHZ (^1H) and 99 MHz (^{13}C), respectively.

Analytical TLC was performed on silica gel 60 F_{254} (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or charring with ninhydrin. Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron^R (Kiesegel 60 PF_{254} gipshaltig (Merck)), with layer thickness of 1 and 2 mm and flow rate of 4 or 8 mL/min, respectively. Flash column chromatography was performed in a Biotage Horizon instrument.

Microwave reactions were performed using the Biotage Initiator 2.0 single-mode cavity instrument from Biotage (Uppsala). Experiments were carried out in sealed microwave process vials utilizing the standard absorbance level (400 W maximum power). The temperature was measured with an IR sensor on the outside of the reaction vessel.

High-performance liquid chromatography (HPLC) analysis was performed using an Agilent Technologies 1120 Compact LC and an ACE5 C18-300 column (150 × 4.6 mm, 300 Å). UV absorption was monitored at diode array.

Table 1
Inhibition of *P. falciparum* PNP by compounds **3**, **8**–**11**, **14** and **15**.

Compound	*Pf*PNP Ki (μM)
3	–[a]
8	8.1
9	–[b]
10	3.69
11	5.33
14	>80
15	–[a]

[a] No inhibition at 200 μM for *Pf*PNP.
[b] Poor solubility.

4.1.1. 2′,3′-O-isopropylidene-8-(4″-methoxyphenyl)inosine (**2**)

To a microwave vial containing oven-dried 2′,3′-*O*-isopropilideneinosine (**1**) (200 mg, 0.65 mmol) and Cs_2CO_3 (528 mg, 1.62 mmol), CuI (371 mg, 1.95 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol) and 4-iodoanisole (304 mg, 1.30 mmol) in dry DMF (5 mL) were added. The vial was sealed and deoxygenated. Then dried and deoxygenated piperidine (26 μL, 0.26 mmol) was added. The mixture was microwaved irradiated at 120 °C for 1 h. The reaction mixture was diluted with 150 mL of dichloromethane:methanol (1:1) and filtered. The filtrate was evaporated to dryness and the residue obtained was purified by flash chromatography (dichloromethane:methanol, 10:1). The purified solid was solved in DMF, treated with Quadrasil MP overnight, filtered and coevaporated with diethyl ether to yield 140 mg (52%) of **2** as a white amorphous solid. EM (ES, positive mode): *m/z* 415 $(M+H)^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 1.27, 1.44 (s, 6H, $C(CH_3)_2$), 3.57 (m, 2H, H-5′), 3.84 (s, 3H, OCH_3), 4.13 (m, 1H, H-4′), 5.02 (m, 2H, H-3′, OH), 5.51 (dd, *J* = 5.9, 2.2 Hz, 1H, H-2′), 5.86 (d, *J* = 2.1 Hz, 1H, H-1′), 7.16 (d, *J* = 8.6 Hz, 2H, H-3″), 7.65 (d, *J* = 8.5 Hz, 2H, H-2″), 8.11 (s, 1H, H-2), 12.52 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.2, 27.1 ($C(\underline{C}H_3)_2$), 55.4 (OCH_3), 61.6 (C-5′), 81.7 (C-3′), 82.4 (C-2′), 87.2 (C-4′), 90.1 (C-1′), 113.2 ($\underline{C}(CH_3)_2$), 114.4 (C-2″), 121.1 (C-1″), 124.4 (C-5), 130.9 (C-3″), 145.6 (C-2), 148.4 (C-4), 149.6 (C-8), 156.4 (C-6), 160.8 (C-4″). Anal. Calc. for $C_{20}H_{22}N_4O_6$ (%): C, 57.97; H, 5.35; N, 13.52. Found: C, 57.69; H, 5.64; N, 13.49.

4.1.2. 8-(4″-Methoxyphenyl)inosine (**3**)

A solution of **2** (100 mg, 0.24 mmol) in a mixture of TFA/H_2O/dioxano (3:1:1) (2.4 mL) was stirred at rt for 15 min. The reaction was quenched by addition of a saturated solution of NH_4HCO_3 and extracted with EtOAc (3 × 15 mL). The organic extracts were dried on anhydrous $MgSO_4$, filtered and evaporated. The residue was purified by CCTLC using as eluent dichloromethane/methanol (10:1 to 5:1) to yield 88 mg (98%) of **3** as a white solid. Mp: 136–137 °C. EM (ES, positive mode): *m/z* 375 $(M+H)^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 3.53, 3.67 (m, 2H, H-5′), 3.84 (s, 3H, OCH_3), 3.90 (m, 1H, H-4′), 4.14 (dd, *J* = 2.1, 4.8 Hz, 1H, H-3′), 5.10 (dd, *J* = 6.0, 6.0 Hz, 1H, H-2′), 5.11 (br s, 2H, OH), 5.47 (br s, 1H, OH), 5.72 (d, *J* = 6.8 Hz, 1H,

H-1′), 7.12 (d, J = 8.7 Hz, 2H, H-3″), 7.65 (d, J = 8.7 Hz, 2H, H-2″), 8.06 (s, 1H, H-2); ^{13}C NMR (100 MHz, DMSO-d_6): δ 56.0 (OCH_3), 62.8 (C-5′), 71.5 (C-3′), 71.9 (C-2′), 87.0 (C-4′), 89.9 (C-1′), 114.9 (C-3″), 122.4 (C-1″), 125.2 (C-5), 131.6 (C-2″), 147.9 (C-2), 149.9 (C-4), 150.6 (C-8), 158.3 (C-6), 161.2 (C-4″). Anal. Calc. for $C_{17}H_{18}N_4O_6$ (%): C, 54.54; H, 4.85; N, 14.97. Found: C, 54.36; H, 5.08; N, 14.69.

4.1.3. 2′,3′-O-isopropylidene-8-(3″-methoxyphenyl)inosine (**4**)

Following the conditions described for the synthesis of compound **2**, 2′,3′-*O*-isopropylideninosine (**1**) (200 mg, 0.65 mmol) reacted with 3-iodoanisole (152 μL, 1.30 mmol) in the presence Cs_2CO_3 (528 mg, 1.62 mmol), CuI (371 mg, 1.95 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol) and piperidine (26 μL, 0.26 mmol) in anhydrous DMF (3.2 mL). After work-up, chromatography purification and Quadrasil® MP treatment as described for **2**, 148 mg (55%) of **4** were obtained as a white amorphous solid. MS (ES, positive mode): m/z 415 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.19, 1.41 (s, 6H, $C(CH_3)_2$), 3.56 (m, 2H, H-5′), 3.81 (s, 3H, OCH_3), 4.09 (m, 1H, H-4′), 4.98 (m, 2H, H-3′, OH), 5.50 (dd, J = 6.2, 2.8 Hz, 1H, H-2′), 5.86 (d, J = 2.6 Hz, 1H, H-1′), 7.22 (m, 3H, H-4″, H-5″, H-6″), 7.49 (t, J = 7.9 Hz, 1H, H-2″), 8.09 (s, 1H, H-2), 12.53 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.2, 27.0 ($C(\underline{C}H_3)_2$), 55.3 (OCH_3), 61.5 (C-5′), 81.6 (C-3′), 82.5 (C-2′), 87.2 (C-4′), 90.1 (C-1′), 113.3 ($\underline{C}(CH_3)_2$), 114.8, 116.0, 121.4 (C-4″, C-5″, C-6″), 124.5 (C-5), 130.0 (C-2″), 130.1 (C-1″), 145.9 (C-2), 148.5 (C-4), 149.4 (C-8), 156.4 (C-6), 159.3 (C-3″). Anal. Calc. for $C_{20}H_{22}N_4O_6$ (%): C, 57.97; H, 5.35; N, 13.52. Found: C, 57.71; H, 5.44; N, 13.39.

4.1.4. 2′,3′-O-isopropylidene-8-(4″-toly)inosine (**5**)

Following the conditions described for the synthesis of compound **2**, 2′,3′-*O*-isopropylideninosine (**1**) (200 mg, 0.65 mmol) reacted under microwave conditions in anhydrous DMF (3.2 mL) with 4-iodotoluene (284 mg, 1.30 mmol) in the presence of Cs_2CO_3 (528 mg, 1.62 mmol), CuI (371 mg, 1.95 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol) and piperidine (26 μL, 0.26 mmol). After work-up, chromatography purification and Quadrasil® MP treatment as described for **2**, 158 mg (61%) of **5** were obtained as a white amorphous solid. MS (ES, positive mode): m/z 399 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.27, 1.43 (s, 6H, $C(CH_3)_2$), 2.40 (s, 3H, CH_3), 3.57 (m, 2H, H-5′), 4.12 (m, 1H, H-4′), 5.01 (m, 2H, H-3′, OH), 5.51 (dd, J = 6.2, 2.7 Hz, 1H, H-2′), 5.86 (d, J = 2.7 Hz, 1H, H-1′), 7.41 (d, J = 7.9 Hz, 2H, H-3″), 7.59 (d, J = 8.0 Hz, 2H, H-2″), 8.12 (s, 1H, H-2), 12.52 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.7 (CH_3), 25.9, 27.7 ($C(\underline{C}H_3)_2$), 62.2 (C-5′), 82.3 (C-3′), 83.1 (C-2′), 87.8 (C-4′), 90.8 (C-1′), 113.9 ($\underline{C}(CH_3)_2$), 125.2 (C-5), 126.7 (C-1″), 130.0 (C-2″), 130.2 (C-3″), 140.8 (C-4″), 146.4 (C-2), 149.1 (C-4), 150.4 (C-8), 157.0 (C-6). Anal. Calc. for $C_{20}H_{22}N_4O_5$ (%): C, 60.29; H, 5.57; N, 14.06. Found: C, 60.05; H, 5.68; N, 14.22.

4.1.5. 2′,3′-O-isopropylidene-8-(4″-trifluoromethylphenyl)inosine (**6**)

Following the conditions described for the synthesis of compound **2**, 2′,3′-*O*-isopropylideninosine (**1**) (200 mg, 0.65 mmol) in dry DMF (3.2 mL) reacted with 4-trifluoromethylphenyl iodide (191 μL, 1.30 mmol) in the presence of Cs_2CO_3 (528 mg, 1.62 mmol), CuI (371 mg, 1.95 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol) and piperidine (26 μL, 0.26 mmol). After work-up, chromatography purification and Quadrasil® MP treatment as described for **2**, 135 mg (46%) of **6** were obtained as a white amorphous solid. MS (ES, positive mode): m/z 453 $(M+H)^+$, 475 $(M+Na)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.27, 1.43 (s, 6H, $C(CH_3)_2$), 3.56 (m, 2H, H-5′), 4.15 (m, 1H, H-4′), 5.30 (m, 2H, H-3′, OH), 5.53 (dd, J = 6.2, 2.3 Hz, 1H, H-2′), 5.87 (d, J = 2.3 Hz, 1H, H-1′), 7.97 (m, 4H, H-2″, H-3″), 8.17 (s, 1H, H-2), 12.60 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.8, 27.6 ($C(\underline{C}H_3)_2$), 62.2 (C-5′), 82.3 (C-3′), 83.3 (C-2′), 88.2 (C-4′), 90.8 (C-1′), 113.9 ($\underline{C}(CH_3)_2$), 124.6 (q ap, J = 272.2 Hz, CF_3), 125.4 (C-5), 126.6 (d, J = 3.1 Hz, C-3″), 130.9 (d, J = 31.9 Hz, C-4″), 130.9 (C-2″), 133.6 (C-1″), 146.7 (C-2), 148.8, 149.3 (C-8, C-4), 157.0 (C-6). Anal. Calc. for $C_{20}H_{19}F_3N_4O_5$ (%): C, 53.10; H, 4.23; N, 12.38. Found: C, 53.00; H, 4.35; N, 12.42.

4.1.6. 2′,3′-O-isopropylidene-8-(3″-trifluoromethylphenyl)inosine (**7**)

Following the conditions described for the synthesis of compound **2**, 2′,3′-*O*-isopropylideninosine (**1**) (200 mg, 0.65 mmol) in dry DMF (3.2 mL) reacted with 3-trifluoromethylphenyl iodide (187 μL, 1.30 mmol) in the presence of Cs_2CO_3 (528 mg, 1.62 mmol), CuI (371 mg, 1.95 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol) and piperidine (26 μL, 0.26 mmol). After work-up, chromatography purification and Quadrasil® MP treatment as described for **2**, 162 mg (55%) of **7** were obtained as a white amorphous solid. MS (ES, positive mode): m/z 453 $(M+1)^+$, 475 $(M+Na)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.26, 1.40 (s, 6H, $C(CH_3)_2$), 3.52 (m, 2H, H-5′), 4.12 (m, 1H, H-4′), 4.99 (m, 2H, H-3′, OH), 5.53 (dd, J = 6.3, 2.6 Hz, 1H, H-2′), 5.82 (d, J = 2.6 Hz, 1H, H-1′), 7.83 (t, J = 8.0 Hz, 1H, H-5″), 7.95 (d, J = 7.9 Hz, 1H, H-4″), 8.00 (m, 2H, H-2″, H-6″), 8.14 (s, 1H, H-2), 12.57 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.8, 27.6 ($C(\underline{C}H_3)_2$), 62.1 (C-5′), 82.3 (C-3′), 83.1 (C-2′), 88.1 (C-4′), 91.0 (C-1′), 113.9 ($\underline{C}(CH_3)_2$), 124.5 (q ap, J = 272.7 Hz, CF_3), 125.3 (C-5), 126.7 (d, J = 4.3 Hz, C-2″), 127.6 (d, J = 4.1 Hz, C-4″), 130.3 (d, J = 32.4 Hz, C-3″), 130.7 (C-1″), 130.9 (C-5″), 133.9 (C-6″), 146.8 (C-2), 148.9 (C-4), 149.3 (C-8), 157.0 (C-6). Anal. Calc. for $C_{20}H_{19}F_3N_4O_5$ (%): C, 53.10; H, 4.23; N, 12.38. Found: C, 52.95; H, 4.41; N, 12.35.

4.1.7. 8-(3″-Methoxyphenyl)inosine (**8**)

Following the conditions described for the synthesis of compound **3**, compound **4** (100 mg, 0.24 mmol) was treated a mixture of TFA/H_2O/dioxane (3:1:1) (2.4 mL) at rt for 15 min. The reaction was quenched by addition of a saturated solution of NH_4HCO_3. After work-up and CCTLC purification, 76 mg (85%) of **8** were obtained as a white solid. Mp: 205–207 °C. MS (ES, positive mode): m/z 375 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.54, 3.67 (m, 2H, H-5′), 3.83 (s, 3H, OCH_3), 3.88 (m, 1H, H-4′), 4.15 (m, 1H, H-3′), 5.03 (s, 1H, OH), 5.10 (m, 1H, H-2′), 5.15 (s, 1H, OH), 5.47 (s, 1H, OH), 5.76 (d, J = 6.7 Hz, 1H, H-1′), 7.15 (d, J = 8.3 Hz, 1H, H-4″), 7.29 (m, 2H, H-2″, H-6″), 7.49 (t, J = 7.9 Hz, 1H, H-5″), 8.12 (s, 1H, H-2), 12.35 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.6 (OCH_3), 62.4 (C-5′), 71.1 (C-3′), 71.7 (C-2′), 86.6 (C-4′), 89.7 (C-1′), 115.2 (C-2″), 116.4 (C-4″), 122.1 (C-6″), 125.2 (C-5), 130.5 (C-5″), 130.8 (C-1″), 146.0 (C-2), 149.4 (C-4), 150.7 (C-8), 156.8 (C-6), 159.7 (C-3″). Anal. Calc. for ($C_{17}H_{18}N_4O_6$) (%): C, 54.54; H, 4.85; N, 14.97. Found: C, 54.36; H, 5.08; N, 14.69.

4.1.8. 8-(4″-Methylphenyl)inosine (**9**)

Following the conditions described for the synthesis of compound **3**, compound **5** (100 mg, 0.25 mmol) was treated a mixture of TFA/H_2O/dioxane (3:1:1) (2.5 mL) at rt for 15 min, and quenched by addition of a saturated solution of NH_4HCO_3. After work-up and CCTLC purification, 78 mg (87%) of **9** were obtained as a white solid. Mp: 296–297 °C. MS (ES, positive mode): m/z 359 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3), 3.60 (m, 2H, H-5′), 3.87 (m, 1H, H-4′), 4.13 (m, 1H, H-3′), 5.07 (m, 3H, H-2′, 2 OH), 5.44 (s, 1H, OH), 5.71 (d, J = 6.8 Hz, 1H, H-1′), 7.38 (d, J = 7.4 Hz, 2H, H-3″), 7.60 (d, J = 7.5 Hz, 2H, H-2″), 8.10 (s, 1H, H-2), 12.08 (br s, 1H, NH); (^{1}H NMR values are in agreement with those partially previously described [23]). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.7 (CH_3), 62.7 (C-5′), 71.3 (C-3′), 71.8 (C-2′), 86.8 (C-4′), 90.0 (C-1′), 125.4 (C-5), 127.1 (C-1″), 130.0, 130.1 (C-2″, C-3″), 140.6 (C-4″), 146.2 (C-2), 149.6 (C-4), 151.2 (C-8), 157.3 (C-6). Anal. Calc for $C_{17}H_{18}N_4O_5$ (%): C, 56.98; H, 5.06; N, 15.63. Found: C, 56.75; H, 5.35; N, 15.49.

*4.1.9. 8-(4″-Trifluoromethylphenyl)inosine (**10**)*

Following the conditions described for the synthesis of compound **3**, compound **6** (100 mg, 0.22 mmol) was treated a mixture of TFA/H_2O/dioxane (3:1:1) (2.2 mL) at rt for 15 min and quenched by addition of a saturated solution of NH_4HCO_3. After work-up and CCTLC purification, 64 mg (70%) of **10** were obtained as a white solid. Mp > 170 °C (decomp). MS (ES, positive mode): *m/z* 413 $(M+H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.54, 367 (m, 2H, H-5′), 3.91 (dd, *J* = 7.4, 4.6 Hz, 1H, H-4′), 4.14 (m, 1H, H-3′), 5.08 (m, 2H, H-2′, OH), 5.19 (s, 1H, OH), 5.46 (s, 1H, OH), 5.70 (d, *J* = 6.8 Hz, 1H, H-1′), 7.95 (s, 4H, H-2″, H-3″), 8.14 (s, 1H, H-2). ^{13}C NMR (125 MHz, DMSO-d_6): δ 62.3 (C-5′), 71.0 (C-3′), 71.7 (C-2′), 86.9 (C-4′), 89.8 (C-1′), 124.4 (q ap, *J* = 272.4 Hz, CF_3), 125.4 (C-5), 126.6 (d, *J* = 4.1 Hz, C-3″), 130.6 (d, *J* = 32.1 Hz, C-4″), 130.8 (C-2″), 133.7 (C-1″), 146.7 (C-2), 149.3 (C-4), 149.6 (C-8), 157.2 (C-6). Anal. Calc for $C_{17}H_{15}F_3N_4O_5$ (%): C, 49.52; H, 3.67; N, 13.59. Found: C, 49.31; H, 3.88; N, 13.70.

*4.1.10. 8-(3″-Trifluoromethylphenyl)inosine (**11**)*

Following the conditions described for the synthesis of compound **3**, compound **7** (100 mg, 0.22 mmol) was treated a mixture of TFA/H_2O/dioxane (3:1:1) (2.2 mL) at rt for 15 min and quenched by addition of a saturated solution of NH_4HCO_3. After work-up and CCTLC purification, 55 mg (60%) of **11** were obtained as a white solid. Mp: 194–195 °C. MS (ES, positive mode): *m/z* 413 $(M+H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.54, 3.66 (m, 2H, H-5′), 3.90 (dd, *J* = 7.6, 4.7 Hz, 1H, H-4′), 4.14 (dd, *J* = 7.6, 4.8 Hz, 1H, H-3′), 5.06 (m, 2H, H-2′, OH), 5.17 (d, *J* = 4.8 Hz, 1H, OH), 5.48 (d, *J* = 6.2 Hz, 1H, OH), 5.69 (d, *J* = 6.7 Hz, 1H, H-1′), 7.84 (t, *J* = 7.8 Hz, 1H, H-5″), 7.97 (d, *J* = 7.8 Hz, 1H, H-4″), 8.05 (m, 2H, H-2″, H-6″), 8.16 (s, 1H, H-2). ^{13}C NMR (125 MHz, DMSO-d_6): δ 61.9 (C-5′), 70.5 (C-3′), 71.2 (C-2′), 86.3 (C-4′), 89.3 (C-1′), 123.8 (q ap, *J* = 272.3 Hz, CF_3), 125.0 (C-5), 126.5 (d, *J* = 4.5 Hz, C-2″), 126.8 (d, *J* = 4.5 Hz, C-4″), 130.1 (d, *J* = 32.1 Hz, C-3″), 130.2, 130.3 (C-5″, C-1″), 133.3 (C-6″), 146.0 (C-2), 148.9 (C-8), 149.1 (C-4), 156.4 (C-6). Anal. Calc for $C_{17}H_{15}F_3N_4O_5$ (%): C, 49.52; H, 3.67; N, 13.59. Found: C, 49.25; H, 3.90; N, 13.42.

*4.1.11. 2′,3′-O-isopropylidene-8-(4″-pyridyl)inosine (**12**)*

Following the conditions described for the synthesis of compound **2**, 2′,3′-*O*-isopropylideninosine (**1**) (200 mg, 0.65 mmol) in dry DMF (3.2 mL) reacted with 4-iodopyridine (266 mg, 1.30 mmol) in the presence of Cs_2CO_3 (528 mg, 1.62 mmol), CuI (371 mg, 1.95 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol) and piperidine (26 μL, 0.26 mmol) in the microwave at 120 °C for 1 h. The reaction was diluted with $CHCl_3$:MeOH (1:1) (150 mL) and filtered. The filtrate was evaporated to dryness and the residue obtained was purified by flash chromatography (dichloromethane:methanol, 10:1). The purified solid was solved in DMF, treated with Quadrasil MP overnight, filtered and coevaporated with diethyl ether (2 × 5 mL) to yield 138 mg (55%) of **12** as a white amorphous solid. MS (ES, positive mode): *m/z* 386 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.27, 1.44 (s, 6H, $C(CH_3)_2$), 3.55 (m, 2H, H-5′), 4.15 (m, 1H, H-4′), 5.02 (m, 2H, H-3′, OH), 5.53 (dd, *J* = 6.3, 2.6 Hz, 1H, H-2′), 5.90 (d, *J* = 2.5 Hz, 1H, H-1′), 7.74 (m, 2H, H-2″), 8.17 (s, 1H, H-2), 8.85 (m, 2H, H-3″), 12.68 (br s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.9, 27.7 ($C(\underline{C}H_3)_2$), 62.1 (C-5′), 82.3 (C-3′), 83.2 (C-2′), 88.3 (C-4′), 90.8 (C-1′), 113.9 ($\underline{C}(CH_3)_2$), 124.2 (C-2″), 125.5 (C-5), 137.0 (C-1″), 147.1 (C-2), 147.9 (C-3″), 149.5 (C-4), 151.0 (C-8), 157.0 (C-6). Anal. Calc for $C_{18}H_{19}N_5O_5$ (%): C, 56.10; H, 4.97; N, 18.17. Found: C, 55.93; H, 5.13; N, 18.25.

*4.1.12. 2′,3′-O-isopropylidene-8-(3″-pyridyl)inosine (**13**)*

Following the conditions described for the synthesis of compound **2**, 2′,3′-*O*-isopropylideninosine (**1**) (200 mg, 0.65 mmol) in dry DMF (3.3 mL) reacted under MW conditions with 3-iodopyridine (266 mg, 1.30 mmol) in the presence Cs_2CO_3 (528 mg, 1.62 mmol), CuI (371 mg, 1.95 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol) and piperidine (26 μL, 0.26 mmol). Following the work-up described for the synthesis of **12**, compound **13** was obtained (135 mg, 54%). EM (ES, positive mode): *m/z* 386 $(M+H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.27, 1.43 (s, 6H, $C(CH_3)_2$), 3.53 (m, 2H, H-5′), 4.13 (dd, *J* = 9.0, 5.9 Hz, 1H, H-4′), 5.00 (m, 2H, H-3′, OH), 5.53 (dd, *J* = 6.2, 2.1 Hz, 1H, H-2′), 5.82 (d, *J* = 2.0 Hz, 1H, H-1′), 7.79 (m, 1H, H-5″), 8.15 (s, 1H, H-2), 8.17 (m, 1H, H-6″), 8.81 (m, 1H, H-4″), 9.29 (s, 1H, H-2″), 12.57 (s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 25.6, 27.5 ($C(\underline{C}H_3)_2$), 61.9 (C-5′), 82.1 (C-3′), 83.0 (C-2′), 87.89 (C-4′), 90.6 (C-1′),113.7 ($\underline{C}(CH_3)_2$), 124.0 (C-5″), 125.2 (C-5), 126.1 (C-1″), 137.0 (C-6″), 146.6 (C8), 147.9 (C-2), 149.1 (C-4), 150.2 (C-2″), 151.4 (C-4″), 156.8 (C-6). Anal. Calc for $C_{18}H_{19}N_5O_5$ (%): C, 56.10; H, 4.97; N, 18.17. Found: C, 55.99; H, 5.10; N, 18.28.

*4.1.13. 8-(4″-Pyridyl)inosine (**14**)*

Following the conditions described for the synthesis of compound **3**, compound **12** (100 mg, 0.26 mmol) was treated a mixture of TFA/H_2O/dioxane (3:1:1) (2.6 mL) at rt for 15 min and quenched by addition of a saturated solution of NH_4HCO_3. After work-up and CCTLC purification, 63 mg (78%) of **14** were obtained as a white solid. Mp > 200 °C (decomp). MS (ES, positive mode): *m/z* 346 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.55, 3.68 (m, 2H, H-5′), 3.92 (dd, *J* = 7.4, 4.4 Hz, 1H, H-4′), 4.15 (m, 1H, H-3′), 4.95 (dd, *J* = 5.1, 5.1 Hz, 1H, OH), 5.05 (dd, *J* = 6.0, 6.0 Hz, 1H, H-2′), 5.11 (d, *J* = 4.8 Hz, 1H, OH), 5.40 (d, *J* = 6.3 Hz, 1H, OH), 5.74 (d, *J* = 6.6 Hz, 1H, H-1′), 7.73 (d, *J* = 5.9 Hz, 2H, H-2″), 8.13 (s 1H, H-2), 8.79 (d, *J* = 5.8 Hz, 1H, H-3″), 12.52 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 61.7 (C-5′), 70.3 (C-3′), 71.2 (C-2′), 86.3 (C-4′), 89.2 (C-1′), 123.4 (C-2″), 125.0 (C-5), 136.5 (C-1″), 146.0 (C-2), 147.8 (C-8), 149.2 (C-4), 150.1 (C-3″), 156.2 (C-6). Anal. Calc. for $C_{15}H_{15}N_5O_5$ (%): C, 52.17; H, 4.38; N, 20.38. Found: C, 52.03; H, 4.44; N, 20.25.

*4.1.14. 8-(3-Pyridyl)inosine (**15**)*

Following the conditions described for the synthesis of compound **3**, compound **13** (100 mg, 0.26 mmol) was treated a mixture of TFA/H_2O/dioxane (3:1:1) (2.6 mL) at rt for 15 min and it was quenched by addition of a saturated solution of NH_4HCO_3. After work-up and CCTLC purification, 55 mg (69%) of **15** were obtained as a white solid. Mp > 200 °C (decomp). MS (ES, positive mode): *m/z* 346 $(M+H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.25 (br s, 2H, 2 OH), 3.53 (dd, *J* = 11.9, 4.2 Hz, 1H, H-5′), 3.66 (dd, *J* = 11.9, 4.0 Hz, 1H, H-5′), 3.91 (m, 1H, H-4′), 4.13 (m, 1H, H-3′), 5.06 (dd, *J* = 5.9, 5.9 Hz, 1H, H-2′), 5.36 (br s, 1H, OH), 5.68 (d, *J* = 6.7 Hz, 1H, H-1′), 7.60 (dd, *J* = 7.5, 4.9 Hz, 1H, H-5″), 8.06 (s 1H, H-2), 8.11 (d, *J* = 7.8 Hz, 1H, H-6″), 8.74 (d, *J* = 3.6 Hz, 1H, H-4″), 8.89 (s, 1H, H-2″). ^{13}C NMR (125 MHz, DMSO-d_6): δ 62.5 (C-5′), 71.2 (C-3′), 71.9 (C-2′), 87.0 (C-4′), 89.8 (C-1′), 124.1 (C-5″), 125.5 (C-5), 126.3 (C-1″), 137.4 (C-6″), 147.5, 147.7 (C-8, C-2), 149.7 (C-4), 150.1 (C-2″), 151.1 (C-4″), 158.8 (C-6). Anal Calc for $C_{15}H_{15}N_5O_5$ (%): C, 52.17; H, 4.38; N, 20.28. Found: C, 52.10; H, 4.42; N, 20.31.

4.1.15. Hydrolytic stability studies

Solutions were prepared using 10 μL of a 5 mM stock solution of each compound in DMSO and 990 μL of phosphate buffer (pH 7.4) or citrate buffer (pH 5.0) or trifluoroacetate buffer (pH 1.2) to obtain a final concentration of 50 μM. The samples were incubated at 37 °C. Aliquots were taken at different time points (*t* = 0, 0.5, 1, 4 and 24 h) and analyzed by HPLC. Injection volume: 20 μL. Flow rate: 1.0 mL/min. Bottle A: Acetonitrile; Bottle B: water containing 0.05% trifluoroacetic acid. Gradient employed: 10% of bottle A to 100% bottle A in 20 min.

4.2. Biological assays

4.2.1. Antiviral assays

The compounds were evaluated against the following viruses: herpes simplex virus type 1 (HSV-1) strain KOS, thymidine kinase-deficient (TK^-) HSV-1 KOS strain resistant to ACV (ACV^r), herpes simplex virus type 2 (HSV-2) strains Lyons and G, vaccinia virus Lederle strain, respiratory syncytial virus (RSV) strain Long, vesicular stomatitis virus (VSV), Coxsackie B4, Parainfluenza 3, Influenza virus A (subtypes H1N1, H3N2), influenza virus B, Reovirus-1, Sindbis and Punta Toro. The antiviral assays were based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) fibroblasts, African green monkey cells (Vero), human epithelial cervix carcinoma cells (HeLa) or Madin–Darby canine kidney cells (MDCK). Confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures) in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the EC_{50} or compound concentration required to reduce virus-induced cytopathicity by 50%.

4.2.2. Human and prokaryotic PNP assays

The separation of inosine from hypoxanthine was performed by HPLC on a reverse phase column RP-18 (Merck, Darmstadt, Germany) using a linear gradient [from 100% buffer A (50 mM NaH_2PO_4 (Acros Organics, Geel, Belgium) and 5 mM heptane sulfonic acid; pH 3.2) to 75% buffer A and 25% acetonitrile] as follows: 10 min linear gradient of 100% buffer A to 98% buffer A + 2% acetonitrile; 10 min linear gradient to 90% buffer A + 10% acetonitrile; 5 min linear gradient to 75% buffer A + 25% acetonitrile; 5 min linear gradient to 100% buffer A followed by equilibration at 100% buffer A for 10 min.

To study the phosphorolysis of the different compounds and inosine by PNPHyor, PNP_{Ecoli} and human PNP (ProSpec, Rehovot, Israel) the compounds (100 μM) were exposed to the enzyme (20 nM PNPHyor and PNP_{Ecoli} or 6 nM human PNP) and incubated at 37 °C in PNP buffer (50 mM MOPS; 0.2 mM EDTA; 200 mM potassium phosphate; pH = 6.5) in a total volume of 500 μL. After 60 min, a 100 μL-fraction was withdrawn and subjected to HPLC analysis. For analysis of the inhibitory activity of the test compounds against PNP-catalysed hydrolysis of 100 μM inosine, the test compounds were added to PNP at 250 μM and hydrolysis of inosine to hypoxanthine was measured after 60 min incubation by the above-described HPLC analysis.

4.2.3. PfPNP inhibition assays

The coding sequence of *Pf*PNP was previously cloned in pET28a and expression was performed in *E. coli* BL21 (DE3) cells, by induction with 1 mM IPTG at 37 °C for 4 h. The purification was performed as previously described [13]. To monitor the reaction, the activity of the enzyme was coupled to the xanthine oxidase, that catalyze the oxidation of hypoxanthine to uric acid, whose appearance can be followed at 293 nm (molar extinction coefficient for uric acid: 12.9 mM^{-1} cm^{-1}). The reaction mixture to determine the activity contained 50 mM KH_2PO_4, pH 7.5, 60 mU xanthine oxidase, 25 nM (0.73 μg) *Pf*PNP, and 25 μM inosine at 25 °C in a final volume of 1 mL. For inhibition studies, the reaction rates were measured with variable inhibitor concentrations and inosine as substrate (25 μM) and at least five concentrations were tested.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2014.05.073. These data include MOL files and InChiKeys of the most important compounds described in this article.

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