Synthesis, antiviral activity, and computational study of β-D-xylofuranosyl nucleoside phosphonates

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Abstract

Molecular dynamics (MD) simulations provided insights into the favorable interactions between xylose nucleosides bearing a phosphonate moiety at their 3'-position and specific residues at the active site of the archetypal RNA-dependent RNA-polymerase (RdRp) of Enterovirus 71. Therefore, a series of xylosyl nucleoside phosphonates with adenine, uracil, cytosine, guanosine, and hypoxanthine as nucleobases were synthesized through multistep sequences starting from a single common precursor. Following antiviral activity evaluation, the adenine containing analogue was found to possess good antiviral activity against RNA viruses displaying an EC_{50} of 12 and 16 μM against measles virus (MeV) and enterovirus-68 (EV-68), respectively, whereas lacking cytotoxicity.

Highlights:

- Molecular dynamics (MD) simulations were performed.
- The synthesis of five xylosyl nucleoside phosphonates was carried out.
- The adenine analogue displayed good activity against measles and enterovius-68.
- The synthesized xylosyl nucleoside phosphonates lacked cytotoxicity.

Keywords: nucleoside phosphonates, xylofuranosyl nucleosides, antiviral activity, molecular dynamics

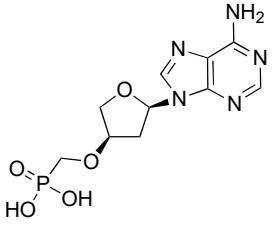
1. Introduction

Chemical modifications of canonical nucleosides and nucleotides have been extensively investigated, often providing analogues with remarkable biological properties that in some cases entered and underwent successful clinical development and approval as either anticancer or antiviral drugs.[1, 2] One important class of such therapeutic agents are acyclic nucleoside phosphonates (ANPs), which constitute the mainstay of DNA virus and retrovirus therapies, some in their prodrug form to enhance oral bioavailability.[3] These nucleotide analogues are distinguished by an aliphatic chain and oxygen-carbon-phosphorus (O-C-P) bond in place of the natural furanose ring and carbon-oxygen-phosphorus (C-O-P) ester bond,

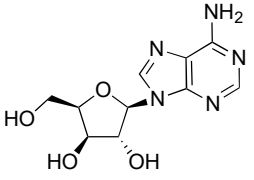
respectively. The utility of the phosphonate motif as metabolically stable phosphate mimic has also been exploited in combination with a range of alternative sugar structures.[4] Our recent efforts in this area involved the synthesis of cyclic nucleoside phosphonates based on an unnatural α-L-2'-deoxythreose sugar, as exemplified by phosphonomethoxydeoxythreosyl adenine (PMDTA, Figure 1) that emerged as a potent dual-activity inhibitor of human immunodeficiency virus 1 and 2 (HIV-1, EC_{50} = 4.69 μM ; HIV-2, EC_{50} = 5.23 μM) as well as hepatitis B virus (HBV, EC_{50} = 0.50 μM).[5, 6] Further prodrug derivatization of PMDTA delivered a preclinical candidate with antiviral activities in the low nanomolar range and exceptionally high selectivity index.[6, 7]

As a logical extension of these findings, herein we have undertaken molecular modeling, chemical, and biological studies to define the potential effect of the introduction of a 3'-phosphonate moiety on the biological properties of β-D-xylofuranosyl nucleosides. Structurally, both L-threose and D-xylose are in fact 3'-epimers of ribose, which differ by the absence and presence of a 4'-hydroxymethyl moiety, respectively. D-xylose is the second most abundant monosaccharide on the planet after glucose and of special interest because of its potential prebiotic relevance.[8] The majority of reports to date have focused on the antitumor activity of β-D-xylofuranosyl nucleosides carrying either natural [e.g., 9-β-D-xylofuranosyladenine (XyloA),[9] Figure 1] or modified nucleobases, including 6-thioguanine,[10] 5-fluorocytosine,[11] and pyrrolo[2,1-f] [1,2,4]triazin-4-amine.[12] In contrast, little to none is known regarding the ability of these analogues or that of their phosphonated counterparts to inhibit viral replication, which in the latest case can be primarily inferred to their challenging synthetic preparation.

By exploiting the same metabolic pathways of endogenous nucleosides, nucleoside phosphonates undergo intracellular bisphosphorylation at the phosphonate moiety to their active diphosphate forms, which can mimic the function of triphosphates by being incorporated into nucleic acids, thus preventing chain elongation.[1] Therefore, we initially performed a computational study to ascertain whether a xylofuranosyl nucleotide could be accommodated by the active site of a selected viral RNA polymerase. Promising modeling results were followed by the development of suitable synthetic routes that could provide access to different members of this series of sugar-modified nucleotide analogues, which were subsequently subjected to broad biological profiling.



PMDTA



XyloA

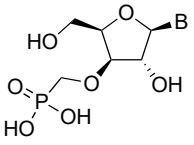


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Figure 1. Examples of biologically active α-L-2'deoxythreosyl and β-D-xylofuranosyl nucleosides along with the structure of planned β-D-xylofuranosyl nucleoside phosphonates (**1**).

2. Results and Discussion

2.1 Molecular Modeling

The terminal residue at the 3'-end of the primer in the crystal structure of the elongation complex of the extensively characterized RNA-dependent RNA polymerase of Enterovirus 71 (pdb:5f8j)[13] was replaced with a 3'- phosphonate xylose nucleoside (Figure 2). An adenine nucleobase was selected for the modified residue based on previous biological results on its phosphonomethoxydeoxythreosyl congeners. After an initial energy minimization, molecular dynamics (MD) was performed. Throughout the MD simulation, base pairing was maintained between the adenosyl xylose phosphonate and its uracil is puckered in the C3'-endo conformation despite of the diaxial orientation of vicinal 2'- and 3'-hydroxyl groups, which is known to be sterically less favorable.[14] Its 2'-OH is involved in stable hydrogen bonding to Ser268, while the bulky 4'-hydromethyl moiety is positioned in a pocket that was originally occupied by a water molecule with the 5'-OH switching hydrogen bonding with the backbone nitrogen of Asp329 of the polymerase and the 2'-OH of the preceding nucleoside in the RNA strand (Figure 2, 2.A). In comparison with the crystal structure, the ribose of this penultimate residue is slightly repositioned with its 2'-OH within hydrogen bonding distance to the carbonyl of Tyr327. Stacking of nucleobases in the double stranded nucleic acid at the active site is maintained but is less efficient with the nucleobase of a terminal xylose phosphonate relative to the ribose nucleosides in the crystal structure (Figure 2, 3.A).

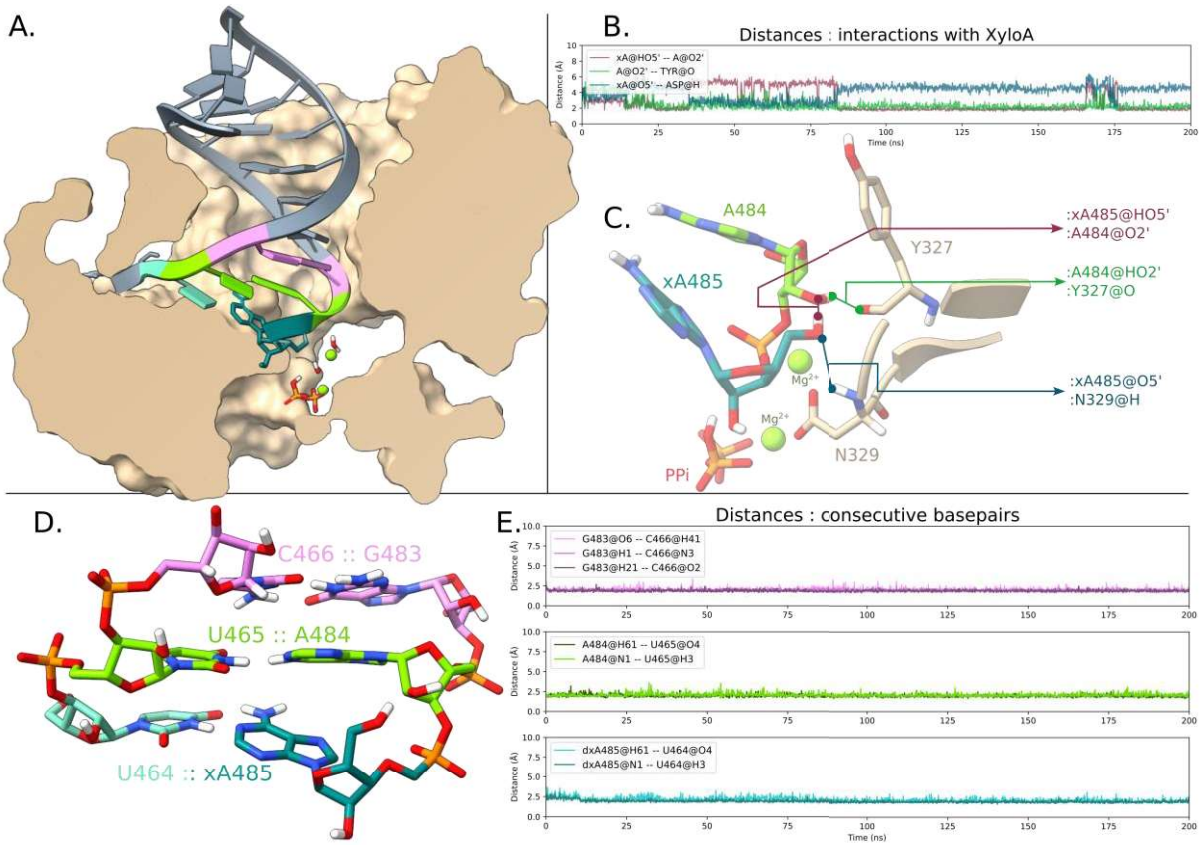


Figure 2. (1) Full-scale image of the RdRp complex including Mg^{2+} ions (lime green) coordinated by a pyrophosphate, RNA (light blue), and the 3'-phosphonate xylo-adenine (pink). The polymerase has been clipped through to visualise the core of the complex. (2.A) Distances between interacting residues of the protein and the modified residue (xA485) and the neighbouring ribo adenosine (A484). (2.B) Interactions that are quantified on 2.A. (3.A, 3.B) Quantitative data on base pairing stability within the (xA-rA-rG::rC-rU-rU), located in the inner core of the protein-nucleic acid complex.

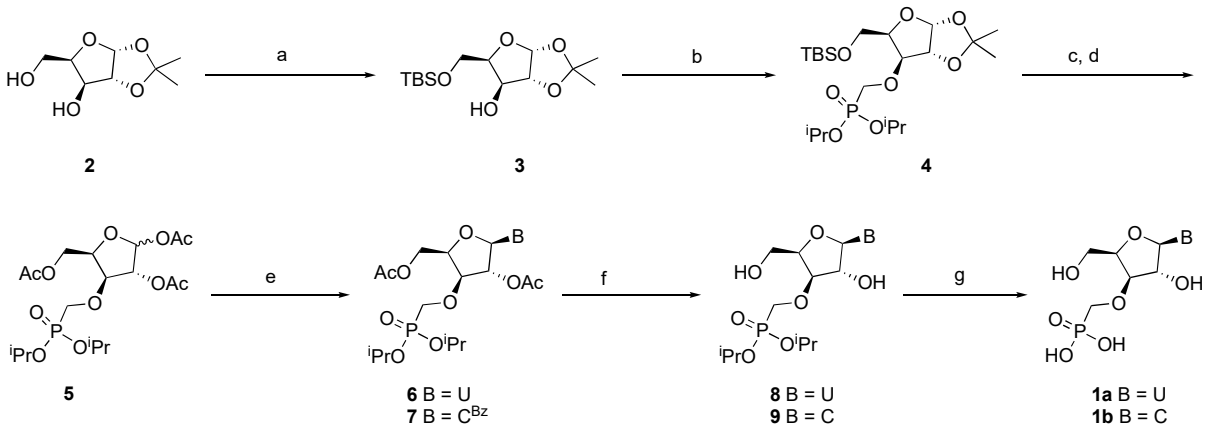
2.2 Synthesis of xylosyl nucleoside phosphonates with pyrimidine and purine bases

On the basis of the above considerations, we decided to prepare a set of xylofuranosyl nucleoside phosphonates (**1**, Figure 1) bearing natural purine and pyrimidine nucleobases. Over the last decades, numerous synthetic strategies have been established to access variously modified acyclic and cyclic nucleoside phosphonates.[15-17] Typically, a nucleobase is introduced on either a glycone or pseudosugar moiety followed by phosphonomethylation of the resulting nucleoside via a glycosylation or S_N2 nucleophilic substitution reaction. The reverse order of steps represents an equally practicable route, which was initially pursued in this study as shown in Scheme 1.

The free primary hydroxyl group of commercially available 1,2-*O*-isopropylidene-α-D-xylofuranose **2** was regioselectively protected by treatment with *tert*-butyldimethylsilyl chloride to afford 5-TBS sugar intermediate **3** in 86% yield. A phosphonate functionality was then

introduced at the 3 position by reacting **3** with diisopropylphosphonomethyl triflate, which was freshly prepared according to a literature procedure,[18] in the presence of NaH to furnish 3-*O*-phosphonomethylated sugar **4** in 81% yield. The subsequent steps included a trifluoroacetic acid-catalyzed hydrolysis of both the 1,2-acetonide and 5-TBS groups and subsequent peracetylation to afford fully protected phosphonated glycosyl donor **5** as an anomeric mixture. Subsequently, compound **5** was subjected to a glycosylation reaction using either uracil or N^4-benzoylcytosine as coupling partner, which led to the formation of pyrimidine xylonucleotides **6** and **7** in 40 and 45% yield, respectively. However, when N^6-benzoyladenine and N^2-acetyl-O^6-diphenylcarbamoyl protected guanine derivative ($G^{DPC}{}_{Ac}$), which were prepared as described in the literature,[19] were reacted with **5** under the same reaction conditions, the glycosylation did not proceed to furnish the desired purine nucleotides, most likely due to the steric hindrance imposed by the 3'-β-phosphonate moiety on the anomeric position.

Compounds **6** and **7** were then treated under basic hydrolysis conditions with K_2CO_3 in methanol to afford **8** and **9**, respectively, both in 70% yield upon acetyl and benzoyl protecting group cleavage. Final hydrolysis of the phosphonate isopropyl ester groups was carried out with TMSBr in the presence of 2,6-lutidine at room temperature to provide uracil and cytosine derivates **1a** and **1b** in 43 and 46% yield, respectively.

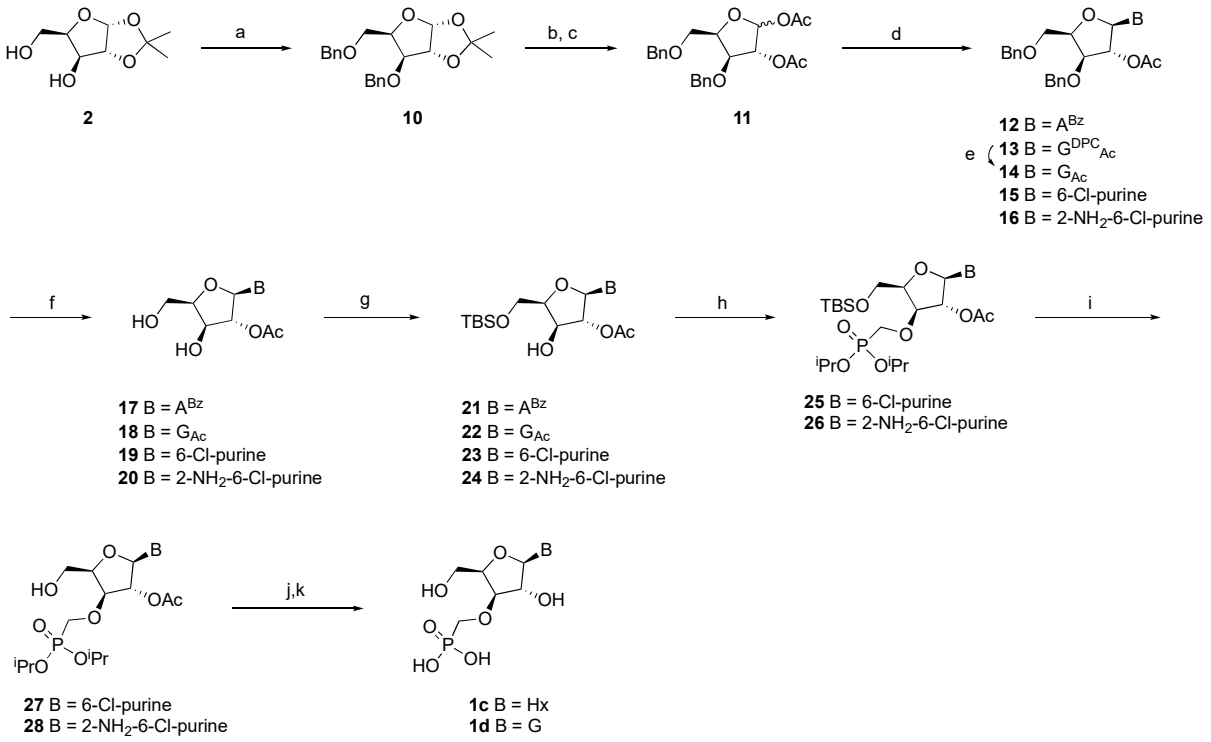


Scheme 1. *Reagents and conditions:* (a) TBSCl, DMAP, imidazole, CH_3CN, 0 °C to rt, overnight, 86%; (b) $(^iPrO)_2POCH_2OTf$, NaH, THF, -5 °C, 15 min, 81%; (c) 60% TFA in H_2O, 0 °C to rt, 4 h; (d) Ac_2O, dry pyridine, 0 °C to rt, 4 h, 90% from **4**; (e) for **6**: uracil, BSA, CH_3CN, $SnCl_4$, rt, 7 h, 45%; for **7**: N^4-benzoylcytosine, BSA, CH_3CN, $SnCl_4$, rt, 7 h, 40%; (f) K_2CO_3, MeOH, rt, overnight, 70% for both **8** and **9**; (g) TMSBr, 2,6-lutidine, CH_3CN, rt, overnight, 43% for **1a** and 46% for **1b**.

To overcome the difficulties encountered in the synthesis of purine containing analogues, we explored an alternative route (Scheme 2). To this end, both 3- and 5-hydroxyl groups of 1,2-*O*-isopropylidene-α-D-xylofuranose **2** were benzylated to afford compound **10**, which was then subjected to acid-catalyzed acetonide deprotection and acetylation to furnish protected glycosyl donor **11**. N^6-benzoyladenine and N^2-acetyl-O^6-diphenylcarbamoyl protected guanine

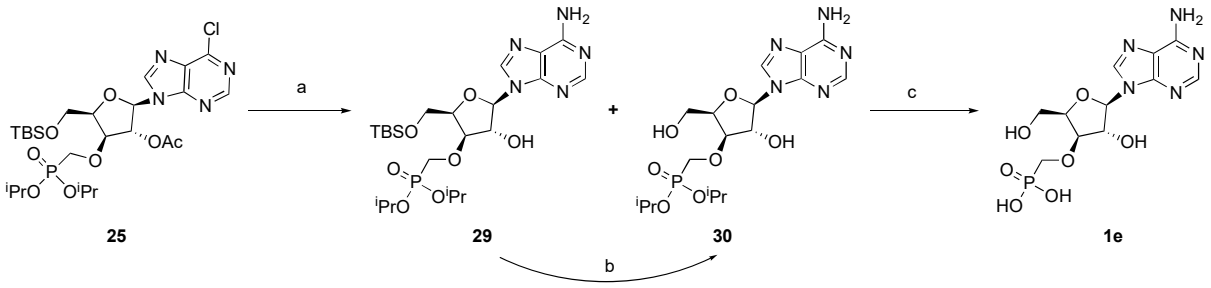
derivative ($G^{DPC}{}_{Ac}$) were reacted with **11** under Vorbruggen conditions. In this case, the glycosylation reactions proceeded smoothly and purine nucleosides **12** and **13** were isolated in 41 and 52% yield, respectively. The DPC protecting group of **13** was then removed by treatment with 90% aq. TFA to give N^2-acetyl-guanine xylonucleoside **14**. Compounds **12** and **14** underwent further debenzylation by either treatment with BCl_3 or catalytic hydrogenation to provide compounds **17** and **18**, respectively. The free 5'-hydroxyl group of **17** and **18** was then protected with a TBS group to afford protected adenine and guanine derivates **21** and **22**, respectively.

However, when compounds **21** and **22** were subjected to a phosphonomethylation reaction with diisopropylphosphonomethyl triflate, the desired phosphonated products were not formed. Therefore, we decided to switch to 6-chloropurine and 2-amino-6-chloropurine in the glycosylation reaction as relatively less bulky coupling partners, which resulted in the formation of xylonucleoside analogues **15** and **16** in 81 and 47% yield, respectively. Next, debenzylation afforded intermediates **19** and **20**, whose free 5'-hydroxyl group was further protected with a TBS group to deliver compounds **23** and **24** in good yields. The phosphonate function could then be introduced at the 3'-position of compounds **23** and **24** using diisopropylphosphonomethyl triflate to afford **25** and **26** in 55 and 43% yield, respectively. Deprotection of the TBS group was achieved by treatment with $Et_3N{\cdot}3HF$, followed by transformation of the 6-chloro group of **27** and **28** to a hydroxyl group upon refluxing with 2-mercaptoethanol and NaOMe in methanol, which further underwent keto-enol tautomerism to a carboxyl group. Finally, hydrolysis of the phosphonate ester function furnished hypoxanthine and guanosine derivatives **1c** and **1d** in 35 and 28% yield, respectively.



Scheme 2. *Reagents and conditions:* (a) BnBr, NaH, dry THF, 0 °C to rt, 16 h, 97%; (b) 60% TFA in H_2O, 0 °C to rt, 4 h; (c) Ac_2O, dry pyridine, 0 °C to rt, 4 h, 70%; (d) for **12**: N^6-benzoyladenine, BSA, CH_3CN, 80 °C, 2 h, 41%; for **13**: N^2-acetyl-O^6-DPC guanine, BSA, TMSOTf, DCE, 80 °C, 1.5 h, 52%; for **15**: 6-chloropurine, DBU, TMSOTf, CH_3CN, 70 °C, 1.5 h, 81%; for **16**: 2-amino-6-chloropurine, DBU, TMSOTf, CH_3CN, 70 °C, 1.5 h, 77%; (e) 90% TFA, rt, 4 h, 90%; (f) for **17**, **19**, and **20**: BCl_3, DCM, -78 °C, 44% for **17**, 57% for **19**, and 46% for **20**; for **18**: Pd/C 10%, $Pd(OH)_2$/C 20%, H_2O, MeOH, H_2, 50 °C, overnight, 49%; (g) TBSCl, DMAP, imidazole, CH_3CN, 0 °C to rt, overnight, 89% for **21**, 82% for **22**, 85% for **23**, and 65% for **24**; (h) $(^iPrO)_2POCH_2OTf$, NaH, THF, -5 °C, 15 min, 55% for **25** and 43% for **26**; (i) $Et_3N{\cdot}3HF$, THF, rt, 24 h, 62% for **27** and 55% for **28**; (j) 2-mercaptoethanol, NaOMe, MeOH, reflux, 19 h; (k) TMSBr, 2,6-lutidine, CH_3CN, rt, overnight, 35% for **1c**, 28% for **1d**.

Adenine containing β-D-xylonucleotide **1e** was obtained from intermediate compound **25**, as shown in Scheme 3. Replacement of the chlorine atom of **25** by an amino group was achieved via treatment with a 7 N NH_3 solution in methanol. The amination at the 6-position of the purine moiety led to a mixture of 5'-TBS protected and unprotected adenine analogues **29** and **30**, however compound **29** could be readily converted to **30** upon treatment with $Et_3N{\cdot}3HF$ to remove the residual TBS group. Hydrolysis of the phosphonate ester groups was carried out with TMSBr in the presence of 2,6-lutidine at room temperature to afford **1e** in 52% yield.



Scheme 3. *Reagents and conditions:* (a) 7 N NH_3 MeOH, 120 °C for 16 h; (b) $Et_3N{\cdot}3HF$, THF, rt, 24 h, 57% from **25**; (c) TMSBr, 2,6-lutidine, CH_3CN, rt, overnight, 52%.

2.3 Biological evaluation

All synthesized β-D-xylofuranosyl nucleoside phosphonates **1a-e** were evaluated for their potential antiviral activity against a selection of RNA viruses with pandemic potential including dengue virus 2 (DENV-2), enterovirus-68 (EV-68), influenza A (H1N1), measles virus (MeV), MERS coronavirus (MERS-CoV), respiratory syncytial virus (RSV), and Tacaribe virus (TCRV). Cytopathic effect (CPE) assays were performed, using Infergen (for DENV-2), enviroxime (for EV-68), ribavirin (for influenza A, RSV, and TCRV), 2'-fluoro-2'-deoxycytidine (MeV), and M128533 (for MERS-CoV) as positive controls. The resulting antiviral activity is summarized in Table 1 and Table S1 (Supporting Information).

No activity against DENV-2, Influenza A (H1N1), MERS-CoV, RSV, and TCRV was observed for all tested compounds. On the other hand, hypoxanthine, guanosine, and adenine derivatives containing analogues **1c-e** exhibited moderate activity against enterovirus-68 with EC_{50} values between 16 and 89 μM. In contrast, pyrimidine analogues **1a** and **1b** were completely devoid of activity against EV-68.

Adenine derivative **1e** also exhibited significant activity against measles virus with an EC_{50} of 12 μM, while other purine derivatives **1c-d** lacked activity. Pyrimidine analogues **1a-b** exhibited moderate activity against measles with EC_{50} values between of 49 and 82 μM.

All compounds were also found to be inactive against HSV and HBV (Tables S2 and S3, Supporting Information). Their cytostatic activity was also evaluated against a panel of cancer cell lines, but no obvious cytotoxicity was observed (Table S4, Supporting Information).

Table 1. In vitro antiviral activity and cytotoxicity of compounds 1a-e.

Compound	Assay	DENV-2			EV-68			MeV		
		EC_{50}	CC_{50}	SI_{50}	EC_{50}	CC_{50}	SI_{50}	EC_{50}	CC_{50}	SI_{50}
1a	A	>100	>100	0	>100	>100	0	72	>100	>1.4
	B	>84	>84	0	>100	>100	0	82	>100	>1.2
1b	A	>100	>100	0	>100	>100	0	72	>100	>1.4
	B	>100	>100	0	>100	>100	0	49	>100	>2
1c	A	>100	>100	0	46	>100	>2.2	>100	>100	0
	B	>100	>100	0	89	>100	>1.1	>100	>100	0
1d	A	>100	>100	0	47	73	1.6	>100	>100	0
	B	>100	>100	0	40	>100	>2.5	>100	>100	0
1e	A	>100	>100	0	16	>100	>6.3	12	>100	>8.3
	B	>100	>100	0	20	>100	>5	14	>100	>7.1
Infergen	A	<0.00001	>0.01	>1000						
	B	<0.00001	>0.01	>1000						
Enviroxime	A				0.19	10	53			
	B				0.16	5.5	34			
2'-Fluoro-2'-deoxycytidine	A							0.7	>100	>140
	B							0.95	>100	>110

EC_{50}: compound concentration (μM) that reduces viral replication by 50%; CC_{50}: compound concentration (μM) that reduces cell viability by 50%; selectivity index (SI_{50}): CC_{50}/EC_{50}. EC_{50} and CC_{50} values are calculated by a single independent experiment in which replicate wells were used for each dilution of a given compound. Assay: (A) Visual, (B) Neutral Red.

4. Conclusion

By means of MD simulation, the replacement of the final ribose cytosine residue in the elongation complex of a well characterized enteroviral RNA-dependent RNA polymerase with a xylo adenine phosphonate showed that base pairing was maintained between the modified residue and its uracil complement, although less efficiently than a regular ribose nucleotide. Therefore, a series of xylosyl nucleoside phosphonates containing nucleobases (adenine, uracil, cytosine, guanosine, and hypoxanthine) were synthesized and their antiviral and anticancer activity was evaluated using cell-based assays. The synthetic path relied on two key steps, i.e., the introduction of a phosphonate functionality and nucleobase moiety. Steric hindrance resulted to be a major obstacle for the installation of both groups on the sugar moiety. As a result, two diverse synthetic strategies were developed for accessing purine and pyrimidine nucleosides. Among those compounds, the xylo-modified adenine nucleotide showed significant activity against measles virus, while other purine derivatives showed limited activity against enterovirus-68. Pyrimidine containing analogues completely lacked antiviral activity, while no cytotoxicity was observed for all five compounds.

5. Experimental Section

5.1 Molecular Modeling

The PDB model [5F8J][14] was used as initial model. The final residue ribose cytosine (702.C) was replaced with a xylofuranosyl adenine phosphonate and its complement (600.B) was altered to a ribose uracil for ensuring correct base pairing conditions. The MD simulations were run using the AMBER18[20, 21] software package with AMBERTools19, making use of the Particle Mesh Ewald MD (PMEMD)[22] in the simulation engine. Computational chemistry was applied to construct a potential energy surface of the xylose adenosine that served for parametrization of the modified residue in AMBER (Supporting Information). Both the phosphonate moiety and the xylose adenosine nucleoside were subjected to a charge derivation following the Merz-Kollmann population analysis scheme[23] and charges were further optimised using Restrained ElectroStatic Potential (RESP)[24, 25] to properly fragment and fit the point charges of the multiple conformers per molecule. Equivalent atoms were equivalenced, and degenerate hydrogens were refitted in the second stage to better represent methyl and methylene moieties. Bond, angle, torsions, and non-bonded terms for the nucleoside were applied from the RNA.OL3 Forcefield (FF)[26, 27] and validated to the QM data. For the phosphonate moiety, data were gathered from Generalised Amber FF (GAFF) and validated as well. The pyrophosphate parameters were directly imported from a previous report[28] and the pyrophosphate molecule was protonated once, resulting in charge (-3).

The ff14SB protein FF[29] was employed to apply parameters to standard amino acids, while

the TIP3P water model[30] together with the accompanied ion parameters were used for the solvent. A truncated octahedron box was virtualised around the complex at 13 Å. A general cut-off distance of 10 Å was used throughout the MD simulations. Step-size was set to 2 fs, using the SHAKE algorithm.[31] Minimisation was carried for a total of 50.000 cycles, with 20.000 cycles using the steepest descent method and the remaining cycles in conjugate gradient method. Heating was performed in two steps, with heating from 0 to 100 K for 50 ps and 100 to 300 K for an additional 50 ps. Equilibration was run for 1 ns with a density set at 1 kg/L. The Langevin thermostat[32] and Berendsen barostat[33] were utilised for heating, density, and equilibration steps. Production runs were set up for a total of 200 ns by restarting the random seed of the MD simulation every 5 ns. All preparatory and production simulations up to the first 10 ns were run with cartesians restraints on the Mg^{2+} ions at 5.0 kcal/mol·Å^2, as well as weak restraints on the base-pairing of the XyloA phosphonate and its uracil complement; distance restraints were run at 5.0 kcal/mol·Å^2 and planarity restraints at 20.0 kcal /mol·rad^2. Data from the MD simulations was extracted using Cpptraj[34] and analysed using UCSF Chimera[35] for visual data, while the Matplotlib library of Python3 was employed for numerical data. All data transformations made use of NumPy and NumPy-derived libraries.[36] All MD simulations were performed on an NVIDIA GTX 2070 GPU by using the cuda-accelerated computation.[37, 38]

5.2 Chemistry

All reagents and solvents were obtained from commercial sources and used as received. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a 300, 500, or 600 MHz Bruker Avance spectrometer either by using tetramethylsilane as an internal standard or referencing to the residual solvent signal and 85% H_3PO_4 for ^{31}P NMR. Two-dimensional NMR (H-COSY, NOESY, HSQC, and HMBC) was used for the assignment of both intermediates and final compounds. High resolution mass spectra (HRMS) were measured on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3 μL/min, and spectra were obtained in positive (or negative) ionization mode with a resolution of 15000 FWHM using leucine enkephalin as a lock mass. Pre-coated aluminum sheets (254 nm) were used for thin layer chromatography (TLC). Column chromatography was carried out on silica gel 60 Å, 0.060-0.200 mm (Acros Organics). Preparative high performance liquid chromatography (HPLC) purification was performed on a Phenomenex Gemini 110A column (C18, 10 μm, 21,2 mm × 250 mm) using CH_3CN/0.05 M TEAB buffer or H_2O/CH_3CN as eluent. Purities of all the tested compounds were above 95% by HPLC analysis.

*5.2.1 5-O-Tert-butyldimethylsilyl-1,2-O-isopropylidene-α-D-xylofuranose (**3**).*

To a solution of commercially available 1,2-*O*-isopropylidene-α-D-xylofuranose **2** (4.40 g, 23.13 mmol) in dry acetonitrile were added DMAP (31.3 mg, 0.26 mmol) and imidazole (2.67

g, 39.21 mmol) at 0 °C. A solution of *tert*-butyldimethylchlorosilane (3.8 g, 25.44 mmol) in anhydrous acetonitrile was then added dropwise to the above mixture, and the reaction mixture was stirred at room temperature overnight. After removal of all the volatiles under reduce pressure, the resulting residue was partitioned between EtOAc and saturated aq. $NaHCO_3$, the organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by column chromatography (8:1 to 2:1 Heptane/EtOAc) to afford compound **3** (6.05 g, 86% yield) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.96 (d, *J* = 3.6 Hz, 1H, H1), 4.51 (d, *J* = 3.6 Hz, 1H, H3), 4.37 (d, *J* = 2.8 Hz, 1H, H4), 4.33 (s, 1H, OH), 4.12 (m, 3H, H2 and H5), 1.48 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 0.90 (s, 9H, $C(CH_3)_3$), 0.11 (d, 6H, $Si(CH_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 111.5 ($O\underline{C}(CH_3)_2$), 105.0 (C1), 85.6 (C2), 78.1 (C4), 77.2 (C3), 62.4 (C5), 26.8, 26.2 ($COCH_3$), 25.7 ($C(\underline{C}H_3)_3$), 18.1 ($\underline{C}(CH_3)_3$), -5.51, -5.63 ($Si(CH_3)_2$) ppm. HRMS: $[M+H]^+$ calcd for $C_{14}H_{29}O_5Si$, 305.1779; found 305.1772.

*5.2.2 3-O-Diisopropylphosphonomethyl-5-O-tert-butyldimethylsilyl-1,2-O-isopropylidene-α-D-xylofuranose (**4**).*

To a solution of compound **3** (4.0 g, 13.15 mmol) in anhydrous THF (60 mL) was added sodium hydride (60% in mineral oil, 0.63 g, 26.30 mmol) at -78 °C. Then, a solution of diisopropylphosphonomethanol triflate (8.63 g, 26.30 mmol) in dry THF was added dropwise and the reaction mixture was slowly warmed to room temperature. It was then quenched with saturated aq. $NaHCO_3$ and concentrated under reduced pressure. The residue was partitioned between water and EtOAc, and the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by column chromatography (6:1 to 2:1 Heptane/EtOAc) to afford compound **4** (5.14 g, 81% yield) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.88 (d, *J* = 3.8 Hz, 1H, H1), 4.81–4.68 (m, 2H, $C\underline{H}(CH_3)_2$), 4.63 (d, *J* = 3.8 Hz, 1H, H2), 4.27–4.18 (m, 1H, H4), 3.97 (d, *J* = 3.1 Hz, 1H, H3), 3.93–3.76 (m, 4H, PCH_2 and H5), 1.50 (s, 3H, CH_3), 1.35–1.31 (m, 15H, CH_3 and $CH(C\underline{H}_3)_2$), 0.90 (s, 9H, $C(CH_3)_3$), 0.08 (s, 6H, $Si(CH_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 112.0 ($O\underline{C}(CH_3)_2$), 105.3 (C1), 84.8 (d, $^3J_{P,C}$ = 10.4 Hz, C3'), 82.8 (C2), 81.1 (C4), 71.5, 71.4, 71.3 ($\underline{C}H(CH_3)_2$), 71.2 ($CH(\underline{C}H_3)_2$), 65.9 (d, $^1J_{P,C}$ = 167.9 Hz, PCH_2), 60.4 (C5), 27.1, 26.6 (CH_3), 26.2 ($C(\underline{C}H_3)_3$), 24.4, 24.3, 24.3 ($CH(\underline{C}H_3)_2$), 18.5 ($\underline{C}(CH_3)_3$), -5.04, -5.15 ($Si(CH_3)_2$) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 19.1 ppm. HRMS: $[M+H]^+$ calcd for $C_{21}H_{44}O_8PSi$, 483.2537; found 483.2551.

*5.2.3 1,2-Di-O-acetyl-3-O-diisopropylphosphonomethyl-5-O-tert-butyldimethylsilyl-α,β-D-xylofuranose (**5**).*

To a stirred suspension of **4** (5.0 g, 10.37 mmol) in H_2O (20 mL) was added TFA (30 mL) at 0 °C and the solution was stirred at room temperature for 4 h. After removal of all the volatiles in vacuo, the resulting residue was coevaporated with toluene (3×) to remove the residual TFA. The obtained yellow viscous oil was then dissolved in dry pyridine (40 mL), and acetic anhydride (20 mL, 196 mmol) was added dropwise at 0 °C, and the reaction mixture was stirred at room temperature for 4 h. It was then concentrated to dryness and the residue was coevaporated with toluene (3×). The crude product was purified by column chromatography

on silica gel (6:1 to 1:1 Heptane/EtOAc) to afford compound **5** (4.24 g, 90% yield) as a light-yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.25–5.68 (m, 1H, H1), 5.04–4.88 (m, 2H, H2 and H4), 4.80–4.65 (m, 2H, C<u>H</u>$(CH_3)_2$), 4.21–3.80 (m, 3H, H3 and H5), 3.76–3.46 (m, 2H, PCH_2), 2.17–2.06 (m, 9H, $COCH_3$), 1.36–1.29 (m, 12H, CH(C<u>H</u>$_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.84, 169.64, 169.22, 168.93 (<u>C</u>OCH_3), 91.91, 89.60 (C1), 78.71, 78.52, 78.34 (C3), 71.40, 71.31, 71.22, 70.80, 70.23, 69.16, 69.07, 68.32, 67.33, 66.08, 65.09 (PCH_2), 61.93, 60.93 (C5), 24.06, 23.99, 23.91 (CH(<u>C</u>$H_3)_2$), 20.93, 20.87, 20.85, 20.73 (CO<u>C</u>H_3) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 18.4, 18.3 ppm. HRMS (ESI+), calcd for $C_{18}H_{32}O_{11}P$, $[M+H]^+$ 455.1677; found 455.1645.

*5.2.4 1'β-(Uracil-1-yl)-2',5'-O-acetyl-3'-O-diisopropylphosphonomethyl-D-xylofuranose (**6**).*

To a solution of uracil (112 mg, 1.00 mmol) and **5** (454 mg, 1.00 mmol) in dry acetonitrile (6 mL) was added *N*,*O*-bis(trimethylsilyl)acetamide (0.24 mL, 1.00 mmol). Next, $SnCl_4$ (1 M solution in CH_2Cl_2, 1.5 mL, 1.5 mmol) was added dropwise, and the reaction mixture was stirred for 7 h. It was then quenched with saturated aq. $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with saturated aq. $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by column chromatography on silica gel (100:1 to 100:5 CH_2Cl_2/MeOH) to afford **6** (227 mg, 45% yield) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.00 (s, 1H, NH), 7.32 (d, *J* = 8.3 Hz, 1H, H6), 5.78 (d, *J* = 8.3 Hz, 1H, H1'), 5.67 (d, *J* = 9.4 Hz, 1H, H5), 5.11–4.93 (m, 2H, H2' and H3'), 4.79–4.65 (m, 2H, C<u>H</u>$(CH_3)_2$), 4.23–4.14 (m, 1H, H4'), 3.95 (t, *J* = 9.2 Hz, 2H, H5'), 3.80–3.38 (m, 2H, PCH_2), 2.14 (s, 3H, $COCH_3$), 2.08 (s, 3H, $COCH_3$), 1.36–1.29 (m, 12H, CH(C<u>H</u>$_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.7, 169.5 (<u>C</u>OCH_3), 162.4 (C4), 150.4 (C2), 139.4 (C6), 103.5 (C5), 81.9 (d, $^{3}J_{P,C}$ = 12.8 Hz, C3'), 81.0, 71.4, 71.3, 70.8, 70.4 (<u>C</u>H$(CH_3)_2$), 67.5 (d, $^{1}J_{P,C}$ = 168.3 Hz, PCH_2), 65.3 (C5'), 24.1, 24.0, 23.9 (CH(<u>C</u>$H_3)_2$), 20.9, 20.6 (CO<u>C</u>H_3) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 17.4 ppm. HRMS: $[M+H]^+$ calcd for $C_{20}H_{32}N_2O_{11}P$, 507.1738; found 507.1739.

*5.2.5 1'β-(N^4-Benzoylcytosin-1-yl)-2',5'-O-acetyl-3'-O-diisopropylphosphonomethyl-D-xylofuranose (**7**).*

To a solution of N^4-benzoylcytosine (215 mg, 1.00 mmol) and **5** (454 mg, 1 mmol) in dry acetonitrile (6 mL) was added *N*,*O*-bis(trimethylsilyl)acetamide (0.24 mL, 1 mmol). Next, $SnCl_4$ (1 M solution in CH_2Cl_2, 1.5 mL, 1.5 mmol) was added dropwise, and the reaction mixture was stirred for 7 h. The solution was quenched with saturated aq. $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with saturated aq. $NaHCO_3$ solution, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by column chromatography on silica gel (100:1 to 100:5 CH_2Cl_2/MeOH) to afford **7** (202 mg, 40% yield) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.79 (s, 1H, NH), 7.90 (d, *J* = 7.6 Hz, 2H, Ar-H), 7.79 (d, *J* = 7.6 Hz, 1H, H6), 7.62 (t, *J* = 7.3 Hz, 1H, Ar-H), 7.51 (t, *J* = 7.6 Hz, 2H, Ar-H), 5.94 (d, *J* = 9.4 Hz, 1H, H1'), 5.14–4.97 (m, 2H, H2' and H4'), 4.81–4.66 (m, 2H, C<u>H</u>$(CH_3)_2$), 4.29–4.19 (m, 1H, H3'), 4.01–3.92 (m, 2H, H5'), 3.90–3.42 (m, 2H, PCH_2), 2.14 (s, 3H, $COCH_3$), 2.06 (s, 3H, $COCH_3$), 1.37–1.28 (m, 12H, CH(C<u>H</u>$_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.8 (<u>C</u>OCH_3), 162.5 (C4), 154.8 (C2), 144.6 (C6), 133.3, 133.0, 129.0, 127.6 (Ar-C), 97.7 (C5), 81.9, 81.8

(C3'), 72.3, 71.3, 71.2 (<u>C</u>H(CH_3)$_2$), 70.3, 67.5 (d, $^1J_{P,C}$ = 168.0 Hz, PCH_2), 65.6 (C5'), 24.0, 24.0, 23.9 (CH(<u>C</u>H_3)$_2$), 20.9, 20.7 (CO<u>C</u>H_3) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 17.5 ppm. HRMS: $[M+H]^+$ calcd for $C_{27}H_{37}N_3O_{11}P$, 610.2160; found 610.2166.

*5.2.6 1'β-(Uracil-1-yl)-3'-O-diisopropylphosphonomethyl-D-xylofuranose (**8**).*

To a solution of **6** (80 mg, 0.16 mmol) in MeOH/H_2O (10:1) was added K_2CO_3 (345 mg, 2.5 mmol), and the solution was stirred at room temperature overnight. After removal of all the volatiles under reduced pressure, the residue was partitioned between water and EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (100:1 to 100:10 CH_2Cl_2/MeOH) to afford **8** (47 mg, 70% yield) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.1 (s, 1H, NH), 7.77 (d, *J* = 8.2 Hz, 1H, H6), 5.80 (d, *J* = 1.9 Hz, 1H, H1'), 5.70 (d, *J* = 8.1 Hz, 1H, H5), 4.80–4.67 (m, 2H, C<u>H</u>(CH_3)$_2$), 4.46 (q, *J* = 4.9 Hz, 1H, H4'), 4.39 (d, *J* = 2.0 Hz, 1H, OH), 4.22–4.18 (m, 1H, H3'), 3.97–3.72 (m, 4H, H5' and PCH_2), 1.37–1.31 (m, 12H, CH(C<u>H</u>$_3$)$_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.8 (C4), 151.2 (C2), 140.4 (C6), 101.8 (C5), 91.9 (C1'), 85.5 (C3'), 82.5 (C4'), 78.8 (C2'), 72.0, 71.9, 71.8 (<u>C</u>H(CH_3)$_2$), 65.0 (d, $^1J_{P,C}$ = 166.7 Hz, PCH_2), 59.8 (C5'), 24.1 (CH(<u>C</u>H_3)$_2$) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 19.6 ppm. HRMS: $[M+H]^+$ calcd for $C_{16}H_{28}N_2O_9P$, 423.1527; found 423.1534.

*5.2.7 1'β-(Cytosin-1-yl)-3'-O-diisopropylphosphonomethyl-D-xylofuranose (**9**).*

To a solution of **7** (300 mg, 0.49 mmol) in MeOH/H_2O (10/1) was added K_2CO_3 (345 mg, 2.5 mmol), and the solution was stirred at room temperature overnight. After removal of all the volatiles under reduced pressure, the residue was partitioned between water and EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (100:1 to 100:10 CH_2Cl_2/MeOH) to afford **9** (152 mg, 70% yield) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (d, *J* = 7.4 Hz, 1H, H6), 5.84–5.71 (m, 2H, H5 and H1'), 4.69 (m, 6.2 Hz, 2H, C<u>H</u>(CH_3)$_2$), 4.48 (q, *J* = 5.0 Hz, 1H, H4'), 4.39 (d, *J* = 2.5 Hz, 1H, H2'), 4.07 (dd, *J* = 4.5, 2.5 Hz, 1H, H3'), 3.93 (dd, *J* = 13.7, 9.1 Hz, 1H), 3.82–3.58 (m, 4H, H5' and PCH_2), 1.35–1.26 (m, 12H, CH(C<u>H</u>$_3$)$_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.8 (C4), 156.6 (C2), 141.4 (C6), 94.2 (C5), 93.2 (C1'), 85.7 (d, $^3J_{P,C}$ = 12.0 Hz, C3'), 81.3 (C4'), 79.6 (C2'), 72.2, 71.3, 71.2, 71.1 (<u>C</u>H(CH_3)$_2$), 65.8 (PCH_2), 63.6 (C5'), 24.0 (CH(<u>C</u>H_3)$_2$) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 18.7 ppm. HRMS: $[M+H]^+$ calcd for $C_{16}H_{29}N_3O_8P$, 442.1687; found 422.1680.

*5.2.8 1'β-(Uracil-1-yl)-3'-O-phosphonomethyl-D-xylofuranose triethylammonium salt (**1a**).*

To a solution of **8** (32 mg, 0.075 mmol) and 2,6-lutidine (0.14 mL, 1.20 mmol) in dry CH_3CN (3 mL) was added bromotrimethylsilane (0.08 mL, 0.6 mmol) at 0 °C. The reaction mixture was stirred at rt overnight and quenched with a 1.0 M TEAB solution (1 mL). After removal of all the volatiles under reduced pressure, the residue was partitioned between water and EtOAc/ether (1:1), the water layer was lyophilized, and the crude residue was first purified by column chromatography on silica gel (8:1:1 Acetone/H_2O/Et_3N), followed by further purification using preparative reverse phase HPLC using a gradient of CH_3CN in 0.05 M TEAB from 2 to

30%. Compound **1a** (14 mg, 43 % yield) was isolated as a white foam. ^{1}H NMR (600 MHz, D_2O) δ 7.92 (d, *J* = 8.1 Hz, 1H, H6), 5.93 (d, *J* = 2.5 Hz, 1H, H1'), 5.87 (d, *J* = 8.1 Hz, 1H, H2), 4.47 (t, *J* = 2.3 Hz, 1H, H2'), 4.43–4.38 (m, 1H, H4'), 4.10 (dd, *J* = 4.5, 2.1 Hz, 1H, H3'), 4.03–3.90 (m, 2H, H5'), 3.67–3.48 (m, 2H, PCH_2) ppm. ^{13}C NMR (151 MHz, D_2O) δ 166.3 (C4), 151.7 (C2), 142.6 (C6), 102.1 (C5), 90.1 (C1'), 84.2 (d, $^{3}J_{P,C}$ = 10.0 Hz, C3'), 82.2 (C4'), 77.2 (C2'), 67.2 (d, $^{1}J_{P,C}$ = 152.9 Hz, PCH_2), 59.0 (C5') ppm. ^{31}P NMR (121 MHz, D_2O) δ 14.6 ppm. HRMS: $[M-H]^-$ calcd for $C_{10}H_{14}N_2O_9P$, 337.0442; found 337.0450.

*5.2.9 1'β-(Cytosin-1-yl)-3'-O-phosphonomethyl-D-xylofuranose triethylammonium salt (**1b**).*

To a solution of **9** (32 mg, 0.075 mmol) and 2,6-lutidine (0.14 mL, 1.20 mmol) in dry CH_3CN (3 mL) was added bromotrimethylsilane (0.08 mL, 0.6 mmol) at 0 °C. The reaction mixture was stirred at rt overnight and quenched with a 1.0 M TEAB solution (1 mL). After removal of all the volatiles under reduced pressure, the residue was partitioned between water and EtOAc/ether (1:1), the water layer was lyophilized, and the crude residue was first purified by column chromatography on silica gel (8:1:1 Acetone/H_2O/Et_3N), followed by further purification using preparative reverse phase HPLC with a gradient of CH_3CN in 0.05 M TEAB from 2 to 30%. Compound **1a** (15 mg, 46% yield) was obtained as a white foam. ^{1}H NMR (600 MHz, D_2O) δ 7.86 (d, *J* = 7.6 Hz, 1H, H6), 6.02 (d, *J* = 7.6 Hz, 1H, H5), 5.93 (d, *J* = 2.0 Hz, 1H, H1'), 4.44–4.40 (m, 2H, H2' and H4'), 4.06 (dd, *J* = 4.1, 1.7 Hz, 1H, H3'), 4.05–3.92 (m, 2H, H5'), 3.64–3.46 (m, 2H, PCH_2) ppm. ^{13}C NMR (151 MHz, D_2O) δ 166.2 (C4), 157.6 (C2), 142.4 (C6), 96.0 (C5), 91.2 (C1'), 84.4 (d, $^{3}J_{P,C}$ = 10.6 Hz, C3'), 82.4 (C4'), 77.2 (C2'), 66.5 (d, $^{1}J_{P,C}$ = 154.5 Hz, PCH_2), 59.0 (C5') ppm. ^{31}P NMR (121 MHz, D_2O) δ 14.9 ppm. HRMS: $[M-H]^-$ calcd for $C_{10}H_{15}N_3O_8P$, 336.0602; found 336.0609.

*5.2.10 3,5-Di-O-benzyl-1,2-O-isopropylidene-α-D-xylofuranose (**10**).*

To a solution of commercially available 1,2-*O*-isopropylidene-α-D-xylofuranose **2** (5.00 g, 26.3 mmol) in dry THF (120 mL) was added NaH (60% in mineral oil, 4.2 g, 105.2 mmol) at 0 °C and the reaction mixture was stirred at room temperature for 1 h. Then, benzyl bromide (19.0 mL, 159.9 mmol) was added and the reaction mixture was further stirred at room temperature overnight. It was then quenched on ice by careful addition of water. The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL) and the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (20:1 to 8:1 Heptane /EtOAc) to afford compound **10** as a light-yellow oil (9.5 g, 97% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.20 (m, 10H, Ar-H×2 Bn), 5.92 (d, *J* = 3.8 Hz, 1H, H1), 4.66–4.55 (m, 3H, 2 CH_2-Ph), 4.53–4.45 (m, 2H, CH_2-Ph and H2), 4.43 – 4.36 (m, 1H, H4), 3.96 (d, *J* = 3.3 Hz, 1H, H3), 3.82–3.69 (m, 2H, H5), 1.47 (s, 3H, CH_3), 1.30 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 138., 137.9 (*ipso* C×2 Bn), 128.8, 128.7, 128.2, 128.1, 127.9, 127.9 (Ar-C), 112.0 ($\underline{C}(CH_3)_2$), 105.4 (C1), 82.8 (C2), 82.2 (C3), 79.6 (C4), 73.9, 72.4 (CH_2-Ph), 67.9 (C5), 27.2 (CH_3), 26.7 (CH_3) ppm. HRMS: $[M+Na]^+$ calcd for $C_{22}H_{26}O_5Na$, 393.1673; found 393.1675.

*5.2.11 1,2-Di-O-acetyl-3,5-di-O-benzyl-α,β-D-xylofuranose (**11**).*

To a stirred suspension of **10** (7.4 g, 20 mmol) in H_2O (20 mL) was added TFA (30 mL) at 0 °C and the solution was stirred at room temperature for 4 h. After removal of all the volatiles under reduced pressure, the resulting residue was coevaporated with toluene (3×) to remove any residual TFA. The obtained yellow viscous oil was then dissolved in dry pyridine (40 mL) and acetic anhydride (20 mL, 212 mmol) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 4 h, it was then concentrated to dryness, and the residue was coevaporated with toluene (3×). The crude product was purified by column chromatography on silica gel (10:1 to 4:1 Heptane /EtOAc) to afford a mixture of anomers **11** as a light-yellow oil (5.8 g, 70 % yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34–7.26 (m, 20H, Ar-H×4 Bn), 6.44–6.13 (m, 2H, H1), 5.35–5.23 (m, 2H, H2), 4.81–4.54 (m, 8H, 4 CH_2-Ph), 4.52–4.45 (m, 2H, H4), 4.28–3.98 (m, 2H, H3), 3.86–3.61 (m, 5H, H5), 2.13–1.99 (m, 12H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.9, 169.8, 169.6 ($\underline{C}OCH_3$), 138.4, 138.3, 137.9, 137.8, 128.7, 128.6, 128.6, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.8 (Ar-C), 99.9, 96.4, 94.3 (C1), 82.6 (C4), 80.5, 80.3, 80.3, 79.7, 78.8, 77.7, 77.3, 76.9, 76.8, 73.9, 73.7, 72.8, 72.2, 68.8, 68.5 (C5), 21.4, 21.1, 21.0, 20.8 ($CO\underline{C}H_3$) ppm. HRMS: $[M+Na]^+$ calcd for $C_{23}H_{26}O_7Na$, 437.1571; found 437.1572.

*5.2.12 1'β-(6-Benzoyladenine-9-yl)-2'-O-acetyl-3',5'-di-O-benzyl-D-xylofuranose (**12**).*

To a solution of N^6-benzoyladenine (1.40 g, 5.9 mmol) in dry acetonitrile (12 mL), N,O-bis(trimethylsilyl)acetamide (4.8 mL, 19.6 mmol) was added under a N_2 atmosphere, followed by a solution of **11** (2.0 g, 4.8 mmol) in dry acetonitrile (3 × 4 mL). Next, $SnCl_4$ (1 M solution in CH_2Cl_2, 10 mL, 10 mmol) was added dropwise, and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with EtOAc and washed by saturated aq. $NaHCO_3$. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (20:1 to 1:1 Hexane/EtOAc) to afford compound **12** (1.17 g, 41% yield) as a white foam. ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.16 (s, 1H, NH), 8.79 (s, 1H, H2), 8.35 (s, 1H, H8), 8.05–8.00 (m, 2H, Ar-H), 7.62–7.57 (m, 1H, Ar-H), 7.51 (t, *J* = 7.8 Hz, 2H, Ar-H), 7.36–7.28 (m, 8H, Ar-H), 7.24–7.20 (m, 2H, Ar-H), 6.40 (d, *J* = 1.3 Hz, 1H, H1'), 5.49 (t, *J* = 1.1 Hz, 1H, H2'), 4.73–4.54 (m, 4H, CH_2Ph), 4.52–4.47 (m, 1H, H4'), 4.10 (dd, 1H, H3'), 3.88 (d, *J* = 5.8 Hz, 2H, H5'), 2.16 (s, 3H, $COCH_3$) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.6 ($\underline{C}OCH_3$), 164.6 ($\underline{C}$OPh), 152.7 (C2), 151.4 (C6), 149.3 (C4), 141.8, 137.5, 136.4, 133.6, 132.7, 128.8, 128.5, 128.5, 128.3, 128.2, 127.9, 127.9, 127.8 (Ar-C), 122.7 (C5), 87.3 (C1'), 82.1 (C4'), 80.0 (C3'), 79.7 (C2'), 73.7, 72.2 ($\underline{C}H_2$Ph), 67.4 (C5'), 20.8 ($CO\underline{C}H_3$) ppm. HRMS (ESI+), calcd for $C_{33}H_{31}N_5O_6$ $[M+H]^+$ 594.2347; found 594.2360.

*5.2.13 1'β-(-2-N-Acetyl-6-O-(diphenylcarbamoyl)guanine-9-yl)-2'-O-acetyl-3',5'-di-O-benzyl-D-xylofuranose (**13**).*

To a solution of *N*^{2}-acetyl-*O*^{6}-(diphenylcarbamoyl)guanine (3.1 g, 8.0 mmol) in dry 1,2-dichloroethane (12 mL), *N,O*-bis(trimethylsilyl)acetamide (6.0 mL, 24.5 mmol) was added, and

the solution was refluxed at 80 °C for 30 min under a N_2 atmosphere. The solution was cooled to room temperature and a solution of **11** (3.0 g, 7.2 mmol) in dry toluene (3 × 7 mL) was added. Trimethylsilyl trifluoromethanesulfonate (2.5 mL, 7.2 mmol) was then added dropwise, and the reaction mixture was stirred at 80 °C for 1 h. The reaction mixture was cooled, diluted with EtOAc, and washed with saturated aq. $NaHCO_3$. The organic layer was purified by column chromatography (4:1 to 1:1 Heptane/EtOAc) to afford compound **13** (2.9 g, 52% yield) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.26 (d, *J* = 1.3 Hz, 1H, , NH), 8.01 (s, 1H, H8), 7.46–7.16 (m, 27H, Ar-H), 6.19 (d, *J* = 1.2 Hz, 1H, H1'), 5.41 (s, 1H, H2'), 4.70–4.52 (m, 4H, CH_2Ph), 4.50–4.43 (m, 1H, H4'), 4.06 (d, *J* = 3.8 Hz, 1H, H3'), 3.85 (d, *J* = 5.7 Hz, 2H, H5'), 2.53 (s, 3H, $COCH_3$), 2.14 (s, 3H, $COCH_3$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.3 ($\underline{C}OCH_3$), 169.6 ($\underline{C}OCH_3$), 156.4 (C6), 154.7 ($\underline{C}O$), 150.6 (C2), 142.7, 142.1, 137.9 (C8), 136.8, 129.7, 129.5, 128.8, 128.8, 128.5, 128.2, 128.2, 128.1, 127.2, 126.2, 120.9 (C5), 88.0 (C1'), 82.6 (C4'), 80.5, 79.9, 74.0 (CH_2Ph), 72.8 (C3'), 67.7 (C5'), 25.3 ($CO\underline{C}H_3$), 20.9 ($CO\underline{C}H_3$) ppm. HRMS (ESI+), calcd for $C_{41}H_{39}N_6O_8$ $[M+H]^+$ 743.2824; found 743.2827.

*5.2.14 1'β-(-2-N-Acetyl-guanine-9-yl)-2'-O-acetyl-3′,5′-di-O-benzyl-D-xylofuranose (**14**).*

Compound **13** (2.36 g, 3.2 mmol) was dissolved in aq. trifluoroacetic acid (90% TFA, 20 mL) at 0 °C and the resulting mixture was stirred at room temperature for 2 h. After removal of all the volatiles, the resulting residue was coevaporated with toluene (3×). The crude product was purified by flash column chromatography (4:1 to 1:4 Heptane/EtOAc) to afford compound **14** (1.57 g, 90% yield) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.10 (s, 1H, NH), 7.96 (s, 1H, H8), 7.35–7.27 (m, 9H, Ar-H), 7.25–7.21 (m, 2H, Ar-H), 5.89 (d, *J* = 1.3 Hz, 1H, H1', H1'), 5.35 (s, 1H, H2'), 4.75–4.50 (m, 4H, CH_2Ph), 4.40 (q, *J* = 5.1 Hz, 1H, H4'), 3.99 (d, *J* = 3.6 Hz, 1H, H3'), 3.89–3.76 (m, 2H, H5'), 2.16 (s, 3H, $COCH_3$), 2.03 (d, *J* = 4.3 Hz, 3H, $COCH_3$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7 ($\underline{C}OCH_3$), 169.9 ($\underline{C}OCH_3$), 156.3 (C6), 148.5 (C4), 148.0 (C2), 138.1 (C8), 137.7, 136.8, 128.9, 128.8, 128.6, 128.3, 128.3, 128.1 (Ar-C), 120.9 (C5), 87.3 (C1'), 82.3 (C4'), 80.4, 80.1 (CH_2Ph), 74.0 (C3'), 72.4 (C2'), 68.2 (C5'), 24.5 ($CO\underline{C}H_3$), 21.0 ($CO\underline{C}H_3$) ppm. HRMS (ESI+), calcd for $C_{28}H_{30}N_5O_7$ $[M+H]^+$ 548.2140; found 548.2142.

*5.2.15 1'β-(6-Benzoyladenine-9-yl)-2'-O-acetyl-D-xylofuranose (**17**).*

Compound **12** (2.25 g, 3.8 mmol) was dissolved in dry CH_2Cl_2 (30 mL), the solution was cooled to -78 °C and BCl_3 (1 M in CH_2Cl_2, 12 mL, 12 mmol) was added dropwise. The reaction mixture was stirred at this temperature for 1 h and then slowly warmed to -10 °C over 1.5 h and stirred at this temperature for another 30 min. The solution was then cooled to -78 °C and MeOH (15.5 mL) was added dropwise, and the reactions mixture was slowly warmed to room temperature over 30 min upon stirring. After removal of all the volatiles under reduced pressure, the resulting crude product was purified by column chromatography (100:1 to 100:6 CH_2Cl_2/MeOH) to afford compound **17** (0.69 g, 44% yield) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.30 (s, 1H, NH), 8.73 (s, 1H, H2), 8.23 (s, 1H, H8), 8.07–7.96 (, *J* = 7.0 Hz, 2H, Ar-H), 7.65–7.46 (m, 3H, Ar-H), 5.95 (d, *J* = 2.5 Hz, 1H, H1'), 5.47 (t, *J* = 2.2 Hz, 1H, H2'), 4.49 (s, 1H, H4'), 4.29 (q, *J* = 4.3 Hz, 1H, H3'), 4.14–3.98 (m, 2H, H5'), 2.13 (s, 3H, $COCH_3$) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1 ($\underline{C}OCH_3$), 164.8 ($\underline{C}OPh$), 152.2 (C2), 150.1 (C6), 142.9 (C8), 132.9, 128.9, 128.0 (Ar-C), 123.5 (C5), 89.1 (C1'), 83.0 (C4'), 82.6 (C2'), 74.9 (C3'), 60.9 (C5'), 20.7 ($CO\underline{C}H_3$) ppm. HRMS (ESI+), calcd for $C_{19}H_{20}N_5O_6$ $[M+H]^+$ 414.1408; found 414.1406.

*5.2.16 1'β-(-2-N-Acetyl-guanine-9-yl)-2'-O-acetyl-D-xylofuranose (**18**).*

Compound **14** (1.58 g, 2.9 mmol) was dissolved in MeOH (100 mL), and 10% Pd/C (0.8 g), 20% $Pd(OH)_2/C$ (0.8 g), and H_2O (10 drops) were then added to the above solution. The reaction mixture was then hydrogenated at room temperature under atmospheric pressure for 3 days using a balloon filled with H_2. The reaction mixture was filtered through a Celite pad and washed with MeOH (400 mL). The filtrate was concentrated under reduced pressure, and the resulting crude product was purified by column chromatography using 0-10% MeOH in CH_2Cl_2. Compound **18** (0.52 g, 49% yield) was isolated as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.1 (s, 1H, NH), 11.72 (s, 1H, CONH), 8.16 (s, 1H, H8), 6.00 (d, *J* = 4.1 Hz, 1H, H1'), 5.89 (s, 1H, OH), 5.24 (s, 1H, OH), 4.87 (t, *J* = 5.5 Hz, 1H, H3'), 4.26 (d, *J* = 4.5 Hz, 1H, H2'), 4.13 (t, *J* = 4.9 Hz, 1H, H4'), 3.80–3.62 (m, 2H, H5'), 2.18 (s, 3H, $COCH_3$), 2.11 (s, 3H, $COCH_3$) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.7 ($\underline{C}OCH_3$), 169.6 ($\underline{C}OCH_3$), 154.9 (C6), 148.5 (C4), 148.3 (C2), 138.0 (C8), 119.8 (C5), 86.1 (C1'), 84.2 (C4'), 82.3 (C2'), 72.3 (C3'), 59.1 (C5'), 24.0 ($CO\underline{C}H_3$), 20.7 ($CO\underline{C}H_3$) ppm. HRMS (ESI+), calcd for $C_{14}H_{18}N_5O_7$ $[M+H]^+$ 368.1201; found 368.1190.

*5.2.17 1'β-(6-Benzoyladenine-9-yl)-2'-O-acetyl-5'-O-tert-butyldimethylsilyl-D-xylofuranose (**21**).*

To a solution of **17** (0.72 g, 1.75 mmol), DMAP (2.34 mg, 0.018 mmol), and imidazole (0.20 g, 3.01 mmol) in pyridine at 0 °C was added dropwise a solution of *tert*-butyldimethylchlorosilane (0.29 g, 1.93 mmol) in anhydrous acetonitrile. The reaction mixture was stirred at room temperature overnight. After removal of all the volatiles under reduce pressure, the resulting residue was partitioned between EtOAc and saturated aq. $NaHCO_3$, the organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by column chromatography (8:1 to 2:1 Heptane/EtOAc) to afford compound **21** (0.82 g, 89% yield) as a crude white solid. HRMS (ESI+), calcd for $C_{25}H_{34}N_5O_6Si$ $[M+H]^+$ 528.2278; found 528.2285.

*5.2.18 1'β-(-2-N-Acetyl-guanine-9-yl)-2'-O-acetyl-5'-O-tert-butyldimethylsilyl-D-xylofuranose (**22**).*

To a solution of **18** (0.50 g, 1.75 mmol), DMAP (2.34 mg, 0.018 mmol), and imidazole (0.20 g, 3.01 mmol) in pyridine at 0 ˚C, a solution of *tert*-butyldimethylchlorosilane (0.29 g, 1.93 mmol) in anhydrous acetonitrile was added dropwise. The reaction mixture was stirred at room temperature overnight. After removal of all the volatiles under reduce pressure, the resulting residue was partitioned between EtOAc and saturated aq. $NaHCO_3$, the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by column chromatography using 0-5% MeOH in CH_2Cl_2 to afford **22** (0.69 g, 82%

yield) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.2 (s, 1H, NH), 9.61 (s, 1H, CONH), 8.09 (s, 1H, H8), 5.82 (d, *J* = 1.6 Hz, 1H, H1'), 5.33 (s, 1H, H2'), 4.41 (s, 1H, H3'), 4.19 (q, *J* = 4.3 Hz, 1H, H4'), 4.13–3.98 (m, 2H, H5'), 2.28 (s, 3H, $COCH_3$), 2.09 (s, 3H, $COCH_3$), 0.88 (s, 9H, $C(CH_3)_3$), 0.06 (d, 6H, $Si(CH_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.4, 170.0 ($\underline{C}OCH_3$), 155.9 (C6), 147.9 (C4), 139.4 (C8), 121.4 (C5), 88.4 (C1'), 83.2 (C2'), 83.1 (C4'), 74.9 (C3'), 62.0 (C5'), 26.1 ($C(\underline{C}H_3)_3$), 24.7, 21.0 ($CO\underline{C}H_3$), 18.6 ($\underline{C}(CH_3)_3$), -5.12, -5.15 ($Si(CH_3)_2$) ppm. HRMS (ESI+), calcd for $C_{21}H_{32}N_5O_7Si$ $[M+H]^+$ 482.2065; found 482.2060.

*5.2.19 1'β-(6-Chloropurin-9-yl)-2'-O-acetyl-3',5'-di-O-benzyl-D-xylofuranose (**15**).*

To a solution of **11** (0.38 g, 0.92 mmol), 6-chloropurine (0.17 g, 1.1 mmol), and DBU (0.41 mL, 2.76 mmol) in dry MeCN (9 mL) was added dropwise TMSOTf (0.66 mL, 3.68 mmol) at 0 °C. The resulting clear-brown solution was stirred for 1.5 h at 70 °C. The reaction mixture was cooled, diluted with EtOAc, and washed with saturated aq. $NaHCO_3$. The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by column chromatography on silica gel (10:1 to 4:1 Heptane/EtOAc) to afford compound **15** as a white solid (0.38 g, 81% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.72 (s, 1H, H2), 8.44 (s, 1H, H8), 7.36–7.27 (m, 8H, Ar-H), 7.20–7.17 (m, 2H, Ar-H), 6.35 (d, *J* = 1.1 Hz, 1H, H1'), 5.46 (t, *J* = 1.1 Hz, 1H, H2'), 4.68–4.55 (m, 5H, CH_2-Ph), 4.51 (td, *J* = 5.8, 3.7 Hz, 1H, H4'), 4.11 (dd, *J* = 3.7, 1.0 Hz, 1H, H3'), 3.88 (d, *J* = 5.7 Hz, 2H, H5'), 2.17 (s, 3H, $COCH_3$) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.5 ($\underline{C}OCH_3$), 152.0 (C2), 151.1, 150.9, 144.0 (C8), 137.4, 136.3 (*ipso* C × 2 Bn), 131.6 (C6), 128.6, 128.5, 128.3, 127.9, 127.9, 127.8 (Ar-C × 2 Bn), 87.7 (C1'), 82.3 (C4'), 80.0 (C3'), 79.5 (C2'), 73.7, 72.5 (CH_2-Ph), 67.3 (C5'), 20.8 ($CO\underline{C}H_3$) ppm. HRMS: $[M+H]^+$ calcd for $C_{26}H_{26}ClN_4O_5$, 509.1586; found 509.1576.

*5.2.20 1'β-(2-Amino-6-chloropurin-9-yl)-2'-O-acetyl-3',5'-di-O-benzyl-D-xylofuranose (**16**).*

To a solution of **11** (0.38g, 0.92 mmol), 2-amino-6-chloro-9H-purine (0.17 g, 1.01 mmol), and DBU (0.41 mL, 2.76 mmol) in dry MeCN (9 mL) was added dropwise TMSOTf (0.66 mL, 3.68 mmol) at 0 °C. The resulting clear-brown solution was stirred for 1.5 h at 70 °C. The reaction mixture was cooled, diluted with EtOAc, and washed by saturated aq. $NaHCO_3$. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by column chromatography on silica gel (4:1 to 1:2 Heptane/EtOAc) to afford compound **16** (0.37 g, 77% yield) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.37 (s, 1H, H8), 7.36–7.12 (m, 10H, Ar-H×2 Bn), 6.45 (s, 1H, H1'), 5.35 (s, 1H, H2'), 5.15 (s, 2H, H4'), 4.65–4.46 (m, 6H, CH_2-Ph), 4.01 (d, *J* = 3.6 Hz, 1H, H3'), 3.93–3.83 (m, 2H, H5'), 2.15 (s, 3H, ($COCH_3$)) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.3 ($\underline{C}OCH_3$), 164.9, 159.4, 147.1, 142.6, 137.5, 136.5, 128.5, 128.3, 128.2, 128.1, 128.0, 127.9, 127.6, 115.5, 90.0 (C1'), 82.8 (C4'), 80.1 (C2'), 79.8 (C3'), 73.8, 72.3 (CH_2-Ph), 67.2 (C5'), 20.7 ($CO\underline{C}H_3$) ppm. HRMS: $[M+H]^+$ calcd for $C_{26}H_{25}ClN_5O_5$, 524.1695; found 524.1705.

*5.2.21 1'β-(6-Chloropurin-9-yl)-2'-O-acetyl-D-xylofuranose (**19**).*

Compound **15** (1.93 g, 3.8 mmol) was dissolved in dry CH_2Cl_2 (30 mL). The solution was cooled to -78 °C and BCl_3 (1 M in CH_2Cl_2, 12 mL, 12 mmol) was then added dropwise. The

reaction was stirred at this temperature for 1 h and then slowly warmed to -10 °C over 1.5 h, and further stirred at this temperature for another 30 min. The solution was then cooled to -78 °C and a mixture of EtOAc (19 mL) and TEA (15.5 mL) was added dropwise. The reaction mixture was slowly warmed to room temperature over 30 min upon stirring. TAfter removal of all the volatiles under reduced pressure, the residue was coevaporated with MeOH (2 x). The crude product was purified by column chromatography using 0-10% MeOH in CH_2Cl_2 to give **19** as a white solid (0.73 g, 57% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.76 (s, 1H, H2), 8.45 (s, 1H, H8), 6.02 (d, *J* = 2.7 Hz, 1H, H1'), 5.44 (t, *J* = 2.3 Hz, 1H, H2'), 4.53 (t, *J* = 3.0 Hz, 1H, H3'), 4.34 (q, *J* = 4.1 Hz, 1H, H4'), 4.18–4.04 (m, 2H, H5'), 2.16 (s, 3H, $COCH_3$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2 ($\underline{C}OCH_3$), 151.9 (C4), 151.7 (C2), 150.4 (C6), 145.1 (C8), 132.5 (C5), 89.0 (C1'), 83.0 (C4'), 82.4 (C2'), 75.0 (C3'), 61.1 (C5'), 20.7 ($COCH_3$) ppm. HRMS: $[M+Na]^+$ calcd for $C_{12}H_{13}ClN_4O_5Na$, 351.0467; found 351.0465.

*5.2.22 1'β-(2-Amino-6-chloropurin-9-yl)-2'-O-acetyl-D-xylofuranose (**20**).*

Compound **20** was prepared as described for compound **19** starting from compound **16** (1.99 g, 3.8 mmol) and BCl_3 (1 M in CH_2Cl_2, 12 mL, 12 mmol), and obtained as a white solid (0.6 g, 46% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.46 (s, 1H, H8), 6.73 (s, 2H, NH_2), 6.30 (s, 1H, H1'), 5.81 (d, *J* = 3.6 Hz, 1H, H2'), 5.25 (s, 1H, OH), 4.88 (t, *J* = 5.5 Hz, 1H, H4'), 4.26–4.16 (m, 2H, OH and H3'), 3.84–3.71 (m, 2H, H5'), 2.13 (s, 3H, $COCH_3$) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.8 ($\underline{C}OCH_3$), 165.0 (C2), 160.5 (C4), 147.5 (C8), 142.4, 141.8, 114.7 (C5), 89.5 (C1'), 85.3 (C4'), 82.4 (C2'), 72.6 (C3'), 59.3 (C5'), 21.0 ($CO\underline{C}H_3$) ppm. HRMS: $[M+H]^+$ calcd for $C_{12}H_{15}ClN_5O_5$, 344.0756; found 344.0766.

*5.2.23 1'β-(6-Chloropurin-9-yl)-2'-O-acetyl-5'-O-tert-butyldimethylsilyl-D-xylofuranose (**23**).*

To a solution of **19** (0.57 g, 1.75 mmol), DMAP (2.34 mg, 0.018 mmol), and imidazole (0.20 g, 3.01 mmol) in dry DCM at 0 °C, a solution of *tert*-butyldimethylchlorosilane (0.29 g, 1.93 mmol) in anhydrous acetonitrile was added dropwise. The reaction mixture was stirred at room temperature overnight. After removal of all the volatiles under reduce pressure, the residue partitioned between EtOAc and saturated aq. $NaHCO_3$, the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by column chromatography (8:1 to 2:1 Heptane/EtOAc) to afford compound **23** as a white solid (0.66 g, 85% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.77 (s, 1H, H2), 8.47 (s, 1H, H8), 6.08 (d, *J* = 1.7 Hz, 1H, H1'), 5.61 (d, *J* = 6.9 Hz, 1H, H2'), 5.31 (s, 1H, OH), 4.44 (dd, *J* = 6.9, 3.1 Hz, 1H, H4'), 4.24 (q, *J* = 4.1 Hz, 1H, H3'), 4.19–4.04 (m, 2H, H5'), 2.17 (s, 3H, $COCH_3$), 0.89 (s, 9H, $C(CH_3)_3$), 0.08 (d, 6H, $Si(CH_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2 ($\underline{C}OCH_3$), 152.1 (C2), 152.9 (C4), 150.9 (C6), 145.2 (C8), 132.6 (C5), 89.3 (C1'), 83.7 (C4'), 83.0 (C2'), 75.3 (C3'), 61.9 (C5'), 26.1 ($C(\underline{C}H_3)_3$), 21.0 ($CO\underline{C}H_3$), 18.6 ($\underline{C}(CH_3)_3$), -5.14, -5.20 ($Si(CH_3)_2$) ppm. HRMS: $[M+H]^+$ calcd for $C_{18}H_{28}ClN_4O_5Si$, 443.1512; found 443.1514.

*5.2.24 1'β-(2-Amino-6-chloropurin-9-yl)-2'-O-acetyl-5'-O-tert-butyldimethylsilyl-D-xylofuranose (**24**).*

Compound **24** was prepared as described for compound **23** from compound **20** (0.60 g, 1.75 mmol), DMAP (2.34 mg, 0.018 mmol), imidazole (0.20 g, 3.01 mmol), and *tert*-butyldimethylchlorosilane (0.29 g, 1.93 mmol), and obtained as a white solid (0.52 mg, 65% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.43 (s, 1H, H8), 6.72 (s, 2H, NH_2), 6.31 (s, 1H, H1'), 5.87 (d, *J* = 3.3 Hz, 1H, H2'), 5.27 (s, 1H, OH), 4.28–4.19 (m, 2H, H3' and H4'), 4.04–3.87 (m, 2H, H5'), 2.13 (s, 3H, $COCH_3$), 0.88 (s, 9H, $C(CH_3)_3$), 0.07 (s, 6H, $Si(CH_3)_2$) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.8 ($\underline{C}OCH_3$), 165.0 (C2), 160.5 (C4), 147.3 (C8), 142.5 (C6), 114.7 (C5), 89.5 (C1'), 85.1 (C4'), 82.1 (C2'), 72.5 (C3'), 61.7 (C5'), 26.3 ($C(\underline{C}H_3)_3$), 21.0 ($CO\underline{C}H_3$), 18.5 ($\underline{C}(CH_3)_3$), -4.82, -4.91 ($Si(CH_3)_2$) ppm. HRMS: $[M+H]^+$ calcd for $C_{18}H_{29}ClN_5O_5Si$, 458.1621; found 458.1618.

*5.2.25 1'β-(6-Chloropurin-9-yl)-2'-O-acetyl-3'-O-diisopropylphosphonomethyl-5'-O-tert-butyldimethylsilyl-D-xylofuranose (**25**).*

To a solution of compound **23** (2.21 g, 5 mmol) and triflate diisopropylphosphonomethanol (2.46 g, 7.5 mmol) in anhydrous THF (30 mL) was added sodium hydride (60% in mineral oil, 0.24 g, 6 mmol) at 0 °C, and the reaction mixture was stirred for 20 min at room temperature. It was then quenched with saturated aq. NH_4Cl and concentrated in vacuo. The resulting residue was partitioned between H_2O and EtOAc, and the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by column chromatography using 0-5% MeOH in CH_2Cl_2 to afford compound **25** as a white solid (1.71 g, 55% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.75 (s, 1H, H2), 8.58 (s, 1H, H8), 6.36 (d, *J* = 1.3 Hz, 1H, H1'), 5.41 (s, 1H, H2'), 4.81–4.68 (m, 2H, $C\underline{H}(CH_3)_2$), 4.41–4.32 (m, 1H, H4'), 4.21 (d, *J* = 3.5 Hz, 1H, H3'), 4.12–3.87 (m, 4H, H5' and PCH_2), 2.19 (s, 3H, $COCH_3$), 1.37–1.25 (m, 12H, $CH(C\underline{H}_3)_2$), 0.91 (s, 9H, $C(CH_3)_3$), 0.10 (s, 6H, $Si(CH_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.8 ($\underline{C}OCH_3$), 152.4 (C2), 151.7 (C4), 144.7 (C8), 143.5 (C6), 131.9 (C5), 87.7 (C1'), 84.0 (C4'), 83.2 (d, $^{3}J_{P,C}$ = 9.7 Hz, C3'), 80.4 (C2'), 71.8, 71.7, 71.7, 71.6 ($\underline{C}H(CH_3)_2$), 65.8 (d, $^{1}J_{P,C}$ = 166.9 Hz, PCH_2), 60.8 (C5'), 26.2 ($C(\underline{C}H_3)_3$), 24.4, 24.3, 24.2 ($CH(\underline{C}H_3)_2$), 21.1 ($CO\underline{C}H_3$), 18.6 ($\underline{C}(CH_3)_3$), -5.04, -5.08 ($Si(CH_3)_2$) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 18.4 ppm. HRMS: $[M+H]^+$ calcd for $C_{25}H_{43}ClN_4O_8PSi$, 621.2271; found 621.2269.

*5.2.26 1'β-(2-Amino-6-chloropurin-9-yl)-2'-O-acetyl-3'-O-diisopropylphosphonomethyl-5'-O-tert-butyldimethylsilyl-D-xylofuranose (**26**).*

Compound **26** was prepared as described for compound **25** from compound **24** (1.14 g, 2.5 mmol), triflate diisopropylphosphonomethanol (1.23 g, 3.7 mmol), and sodium hydride (60% in mineral oil, 0.12 g, 3 mmol), and obtained as a white solid (0.68 mg, 43% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (s, 1H, H8), 6.49 (d, *J* = 1.0 Hz, 1H, H1'), 5.33 (s, 1H, H2'), 5.15 (s, 2H, NH_2), 4.81–4.64 (m, 2H, $C\underline{H}(CH_3)_2$), 4.39–4.32 (m, 1H, H4'), 4.17 (d, *J* = 3.4 Hz, 1H, H3'), 4.12–3.99 (m, 2H, H5'), 3.86 (d, *J* = 8.3 Hz, 2H, PCH_2), 2.18 (s, 3H, $COCH_3$), 1.37–1.22 (m, 12H, $CH(C\underline{H}_3)_2$), 0.92 (s, 9H, $C(C\underline{H}_3)_3$), 0.11 (s, 6H, $Si(CH_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$)

δ 169.7 ($\underline{C}OCH_3$), 165.0 (C2), 159.6 (C4), 147.7 (C8), 143.0 (C6), 115.9 (C5), 89.9 (C1'), 84.5 (C4'), 83.3 (d, $^3J_{P,C}$ = 8.4 Hz, C3'), 80.5 (C2'), 71.7, 71.7, 71.6, 71.5 ($\underline{C}H(CH_3)_2$), 65.4 (d, $^1J_{P,C}$ = 166.8 Hz, PCH_2), 60.9 (C5'), 26.2 ($C(\underline{C}H_3)_3$), 24.4, 24.3, 24.3, 24.2 ($CH(\underline{C}H_3)_2$), 21.0 ($CO\underline{C}H_3$), 18.6 ($\underline{C}(CH_3)_3$), -5.05 ($Si(CH_3)_2$) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 18.4 ppm. HRMS: $[M+H]^+$ calcd for $C_{25}H_{44}ClN_5O_8PSi$, 636.2380; found 636.2350.

*5.2.27 1'β-(6-Chloropurin-9-yl)-2'-O-acetyl-3'-O-diisopropylphosphonomethyl-D-xylofuranose (**27**).*

To a solution of compound **25** (0.43 g, 0.7 mmol) in 5.0 mL of THF was added TBAF (1 M in THF; 1.4 mL, 1.4 mmol), and the reaction mixture was stirred at rt for 1 h. It was then diluted with EtOAc, washed sequentially with H_2O and saturated aq. NaCl, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by column chromatography using 0-10% MeOH in CH_2Cl_2 to afford compound **27** as a white solid (0.2 g, 62% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.73 (s, 1H, H2), 8.47 (s, 1H, H8), 6.07 (d, *J* = 4.4 Hz, 1H, H1'), 5.72 (s, 1H, OH), 4.85–4.83 (m, 1H, H2'), 4.74–4.67 (m, 2H, $C\underline{H}(CH_3)_2$), 4.55–4.50 (m, 1H, H4'), 4.42 (t, *J* = 6.7 Hz, 1H, OH), 4.34 (dd, *J* = 6.2, 4.6 Hz, 1H, H3'), 4.00–3.89 (m, 4H, H5' and PCH_2), 1.34–1.29 (m, 12H, $CH(C\underline{H}_3)_2$) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ 151.7 (C2), 151.1 (C4), 151.0 (C6), 144.6 (C8), 132.1 (C5), 89.6 (C1'), 85.9 (d, $^3J_{P,C}$ = 5.9 Hz, C3'), 81.3 (C4'), 78.2 (C2'), 72.3, 72.2, 72.0, 72.0 ($\underline{C}H(CH_3)_2$), 65.9 (d, $^1J_{P,C}$ = 167.6 Hz, PCH_2), 60.2 (C5'), 24.0, 23.9, 23.9, 23.8 ($CH(\underline{C}H_3)_2$) ppm.^{31}P NMR (121 MHz, $CDCl_3$) δ 20.1 ppm. HRMS: $[M+Na]^+$ calcd for $C_{17}H_{26}ClN_4O_7PNa$, 487.1120; found 487.1118.

*5.2.28 1'β-(2-Amino-6-chloropurin-9-yl)-2'-O-acetyl-3'-O-diisopropylphosphonomethyl-D-xylofuranose (**28**).*

Compound **28** was prepared as described for compound **27** from compound **26** (0.45 g, 0.7 mmol), and TBAF (1 M in THF; 1.4 mL, 1.4 mmol), and obtained as a white solid (0.2 g, 55% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.35 (s, 1H, H8), 6.30 (d, *J* = 2.2 Hz, 1H, H1'), 5.51 (s, 2H, NH_2), 4.79 (s, 1H, H2'), 4.75–4.69 (m, 1H, $C\underline{H}(CH_3)_2$), 4.68–4.61 (m, 2H, $C\underline{H}(CH_3)_2$ and H4'), 4.55–4.41 (m, 2H, H5'), 4.30 (dd, *J* = 4.4, 1.8 Hz, 1H, H3'), 3.95–3.84 (m, 2H, PCH_2), 2.13 (s, 3H, $COCH_3$), 1.35–1.21 (m, 14H $CH(C\underline{H}_3)_2$) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.6 ($\underline{C}OCH_3$), 163.9 (C2), 159.4 (C4), 146.8 (C8), 143.3 (C6), 115.4 (C5), 91.9 (C1'), 86.2 (d, $^3J_{P,C}$ = 7.2 Hz, C3'), 80.1 (C4'), 78.9 (C2'), 71.8, 71.77, 71.7 ($\underline{C}H(CH_3)_2$), 65.1 (d, $^1J_{P,C}$ = 167.5 Hz, PCH_2), 62.3 (C5'), 24.0, 24.0, 23.9, 23.9, 23.8 ($CH(\underline{C}H_3)_2$), 20.9 ($CO\underline{C}H_3$) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 19.0 ppm. HRMS: $[M+H]^+$ calcd for $C_{19}H_{30}ClN_5O_8P$, 522.1515; found 522.1517.

*5.2.29 1'β-(Hypoxanthin-9-yl)-3'-O-phosphonomethyl-D-xylofuranose triethylammonium salt (**1c**).*

To a solution of **27** (34.8 mg, 0.075 mmol) in anhydrous MeOH, were added 2-mercaptoethanol (0.027 mL, 0.375 mmol) and NaOMe (5.4 M in MeOH, 0.054 mL, 0.375 mmol), and the reaction mixture was refluxed for 19 h. It was then cooled to rt and concentrated under reduced pressure. The crude residue was purified by column chromatography (20: 1 to 10: 1, CH_2Cl_2/MeOH) to give a colorless oil, which was dissolved in

dry CH_3CN (3 mL), followed by the addition of 2,6-lutidine (0.14 mL, 1.20 mmol) and bromotrimethylsilane (0.08 mL, 0.6 mmol) at 0 °C. The reaction mixture was stirred at rt overnight. It was then quenched by adding 2 mL of 1 M TEAB and concentrated in vacuo. The resulting residue was partitioned between water and EtOAc, the aqueous phase was lyophilized, and the crude residue was first purified by column chromatography on silica gel (8:1:1 Acetone/H_2O/Et_3N), followed by further purification using preparative reverse phase HPLC with a gradient of CH_3CN in 0.05 M TEAB from 2 to 30%. Compound **1c** (12 mg, 35% yield) was isolated as a white foam. ^{1}H NMR (600 MHz, D_2O) δ 8.39 (s, 1H, H2), 8.21 (s, 1H, H8), 6.11 (d, *J* = 3.1 Hz, 1H, H1'), 4.83 (t, *J* = 3.1 Hz, 1H, H2'), 4.55 (q, *J* = 5.1 Hz, 1H, H4'), 4.26 (dd, *J* = 5.0, 3.0 Hz, 1H, H3'), 4.02–3.93 (m, 2H, H5'), 3.73–3.60 (m, 2H, PCH_2) ppm. ^{13}C NMR (151 MHz, D_2O) δ 158.6 (C6), 148.5 (C4), 146.1 (C8), 140.5 (C2), 123.5 (C5), 88.7 (C1'), 84.6 (d, $^3J_{P,C}$ = 10.7 Hz, C3'), 82.2 (C4'), 77.4 (C2'), 67.2 (d, $^1J_{P,C}$ = 154.8 Hz, PCH_2), 59.4 (C5') ppm. ^{31}P NMR (121 MHz, D_2O) δ 14.3 ppm. HRMS: $[M-H]^-$ calcd for $C_{11}H_{14}N_4O_8P$, 361.0555; found 361.0524.

*5.2.30 1'β-(Guanin-9-yl)-3'-O-phosphonomethyl-D-xylofuranose triethylammonium salt (**1d**).*

Compound **1d** was prepared as described for compound **28** from compound **1c** (39 mg, 0.075 mmol), 2-mercaptoethanol (0.027 mL, 0.375 mmol), NaOMe (5.4 M in MeOH, 0.054 mL, 0.375 mmol), 2,6-lutidine (0.14 mL, 1.20 mmol), and bromotrimethylsilane (0.08 mL, 0.6 mmol), and obtained as a white solid (10 mg, 28% yield). ^{1}H NMR (600 MHz, D_2O) δ 8.30 (s, 1H, H8), 6.22 (d, *J* = 3.2 Hz, 1H, H1'), 4.74 (t, *J* = 3.2 Hz, 1H, H2'), 4.49 (q, *J* = 5.3 Hz, 1H, H4'), 4.24 (dd, *J* = 5.2, 3.1 Hz, 1H, H3'), 4.02–3.87 (m, 2H, H5'), 3.59–3.46 (m, 2H, PCH_2) ppm. ^{13}C NMR (151 MHz, D_2O) δ 159.2 (C6), 157.0 (C2), 154.43 (C4), 142.7 (C8), 108.0 (C5), 90.2 (C1'), 84.4 (d, $^3J_{P,C}$ = 8.5 Hz, C3'), 82.0 (C4'), 77.8 (C2'), 68.4 (d, $^1J_{P,C}$ = 150.1 Hz, PCH_2), 59.2 (C5') ppm. ^{31}P NMR (121 MHz, D_2O) δ 14.4 ppm. HRMS: $[M-H]^-$ calcd for $C_{11}H_{15}N_5O_8P$, 376.0664; found 376.0676.

*5.2.31 1'β-(Adenin-9-yl)-3'-O-diisopropylphosphonomethyl-5'-O-tert-butyldimethylsilyl-D-xylofuranose (**29**) and 1'β-(adenin-9-yl)-3'-O-diisopropylphosphonomethyl-D-xylofuranose (**30**).*

A solution of compound **25** (0.43 g, 0.7 mmol) in methanolic ammonia was transferred to a screw-top pressure tube, which was sealed and heated at 120 °C for 16 h. After cooling to rt, all the volatiles were evaporated under reduced pressure to afford a mixture of compounds **29** and **30** in an approximate 1:1 ratio. The crude residue was purified by column chromatography on silica gel using 0-10% MeOH in CH_2Cl_2. Compound **30** was further dissolved in 5.0 mL of THF, and TBAF (1 M in THF; 1.4 mL, 1.4 mmol) was then added. The reaction mixture was stirred at rt for 1 h, it was then diluted with EtOAc and washed sequentially with H_2O and saturated aq. NaCl. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by column chromatography using 0-10% MeOH in CH_2Cl_2 to afford compound **30** as a white solid (0.18 g, 57% yield over two steps).

Data for compound **29**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (s, 1H, H8), 8.12 (s, 1H, H2), 6.09 (s, 2H, NH_2), 6.01 (d, *J* = 3.2 Hz, 1H, H1'), 4.75–4.63 (m, 3H, H2' and C<u>H</u>$(CH_3)_2$), 4.47 (q, *J* = 5.3 Hz, 1H, H4'), 4.24 (t, *J* = 4.3 Hz, 1H, H3'), 4.02–3.83 (m, 4H, PCH_2 and H5'), 1.33–1.23 (m, 12H, CH(C<u>H</u>$_3)_2$), 0.86 (s, 9H, C$(CH_3)_3$), 0.05 (s, 6H, Si$(CH_3)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.5 (C6), 152.6 (C2), 149.2 (C4), 139.0 (C8), 119.7 (C5), 90.3 (C1'), 85.4 (d, $^{3}J_{P,C}$ = 8.5 Hz, C3'), 82.4 (C4'), 79.7 (C2'), 71.4, 71.3, 71.2 (<u>C</u>H$(CH_3)_2$), 65.9 (d, $^{1}J_{P,C}$ = 166.5 Hz, PCH_2), 61.1 (C5'), 25.9 (C(<u>C</u>H$_3)_3$), 24.0, 24.0, 23.90 (CH(<u>C</u>H$_3)_2$), 22.7 (<u>C</u>$(CH_3)_3$), -5.35, -5.47 (Si$(CH_3)_2$) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 18.7 ppm. HRMS: $[M+H]^+$ calcd for $C_{23}H_{41}N_5O_7PSi$, 560.2664; found 560.2664.

Data for compound **30**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.25 (s, 1H, H8), 7.95 (s, 1H, H2), 5.96 (s, 2H, NH_2), 5.80 (d, *J* = 5.9 Hz, 1H, H1'), 4.94 (t, *J* = 5.9 Hz, 1H, H2'), 4.85–4.66 (m, 2H, C<u>H</u>$(CH_3)_2$), 4.50–4.43 (m, 1H, H4'), 4.33 (t, *J* = 6.7 Hz, 1H, H3'), 4.09–3.84 (m, 4H, PCH_2 and H5'), 1.37–1.30 (m, 12H, CH(C<u>H</u>$_3)_2$) ppm. ^{13}C NMR (75 MHz, CDCl3) δ 155.6 (C6), 152.5 (C2), 148.8 (C4), 140.0 (C8), 120.3 (C5), 89.6 (C1'), 86.3 (d, $^{3}J_{P,C}$ = 5.4 Hz, C3'), 80.7 (C4'), 77.3 (C2'), 72.4, 72.4, 71.8, 71.7 (<u>C</u>H$(CH_3)_2$), 66.3 (d, $^{1}J_{P,C}$ = 166.3 Hz, PCH_2), 60.8 (C5'), 24.0 (CH(<u>C</u>H$_3)_2$) ppm. ^{31}P NMR (121 MHz, $CDCl_3$) δ 19.7 ppm. HRMS: $[M+H]^+$calcd for $C_{17}H_{29}N_5O_7P$, 446.1799; found 446.1803.

*5.2.32 1'β-(Adenin-9-yl)-3'-O-phosphonomethyl-D-xylofuranose triethylammonium salt (**1e**)*.

To a solution of **30** (33 mg, 0.075 mmol) and 2,6-lutidine (0.14 mL, 1.20 mmol) in dry CH_3CN (3 mL) was added bromotrimethylsilane (0.08 mL, 0.6 mmol) at 0 °C. The reaction mixture was stirred at rt overnight and quenched with 1.0 M TEAB (1 mL). After removal of all the volatiles under reduced pressure, the resulting residue was partitioned between water and EtOAc/ether (1:1), the water layer was lyophilized, and the crude residue was first purified by column chromatography on silica gel (8:1:1 Acetone/H_2O/Et_3N), followed by further purification using preparative reverse phase HPLC with a gradient of CH_3CN in 0.05 M TEAB from 2 to 30%. Compound **1e** (18 mg, 52% yield) was isolated as a white foam. ^{1}H NMR (600 MHz, D_2O) δ 8.37 (s, 1H, H8), 8.21 (s, 1H, H2), 6.08 (d, *J* = 2.9 Hz, 1H, H1'), 4.79 (t, *J* = 2.9 Hz, 1H, H2'), 4.57–4.53 (m, 1H, H4'), 4.23 (dd, *J* = 4.9, 2.7 Hz, 1H, H3'), 4.03–3.94 (m, 2H, H5'), 3.77–3.62 (m, 2H, PCH_2) ppm. ^{13}C NMR (151 MHz, D_2O) δ 155.5 (C6), 152.6 (C2), 148.6 (C4), 140.7 (C8), 118.5 (C5), 88.7 (C1'), 84.7 (d, $^{3}J_{P,C}$ = 11.8 Hz, C3'), 82.2 (C4'), 77.2 (C2'), 66.6 (d, $^{1}J_{P,C}$ = 156.9 Hz, PCH_2), 59.5 (C5') ppm. ^{31}P NMR (121 MHz, D_2O) δ 14.9 ppm. HRMS: $[M-H]^-$ calcd for $C_{11}H_{15}N_5O_7P$, 360.0714; found 360.0710.

5.3 In vitro antiviral assays

Compounds **1a-e** were evaluated according to a procedure adapted and slightly modified from a previous report[1] against the following viruses: dengue virus 2 (DENV2, New Guinea C; in Huh7 cells; positive control, Infergen; Incub. Days, 6), enterovirus-68 (EV68, US/KY/14-18953; in RD cells; positive control, Enviroxime; Incub. Days, 3), influenza A (H1N1) (California/07/2009; in MDCK cells; positive control, Ribavirin; Incub. Days, 6), measles (MeV,

CC strain; in Vero 76 cells; positive control, 2'-Fluoro-2'-deoxycytidine; Incub. Days, 6), Middle East respiratory syndrome coronavirus (MERS-CoV, EMC strain; in Vero 76 cells; positive control, M128533; Incub. Days, 5), respiratory syncytial virus (RSV, A2 strain; in MA-104 cells; positive control, Ribavirin; Incub. Days, 6) and Tacaribe virus (TCRV, TRVL 11573 strain; in Vero 76 cells; positive control, Ribavirin; Incub. Days, 7).

Confluent or near-confluent cell culture monolayers of an appropriate cell line were prepared in 96-well disposable microplates the day before testing. Cells were maintained in MEM supplemented with 5% FBS. For antiviral assays the same medium was used but with FBS reduced to 2% and supplemented with 50-µg/mL gentamicin. Compounds **1a-e** were dissolved in DMSO, and prepared at four serial $\log_{10}$ concentrations, 100, 10, 1.0, and 0.1 µM. Five microwells were used per dilution: three for infected cultures and two for uninfected toxicity cultures. Controls for the experiment consisted of six microwells that were infected and not treated (virus controls) and six that were untreated and uninfected (cell controls) on every plate. A known active drug was tested in parallel as a positive control drug using the same method as that applied for the test compounds. The positive control was tested with every test run.

On the testing day, the growth media was removed from the cells and each test compound was applied in 0.1 mL volume to wells at 2X concentration. Virus, normally at a titer that will cause >80% CPE (usually an MOI <0.003), in 0.1 mL volume was added to the wells designated for virus infection. Medium devoid of virus was placed in toxicity control wells and cell control wells. Plates were incubated at 37 °C with 5% CO_2 until marked CPE (>80% CPE for most virus strains) was observed in virus control wells. The plates were then stained with 0.011% neutral red for approximately 2 hours at 37 °C in a 5% CO_2 incubator. The neutral red medium was removed by complete aspiration, and the cells were rinsed 1X with phosphate buffered solution (PBS) to remove the residual dye. The PBS was completely removed, and the incorporated neutral red was eluted with 50% Sorensen's citrate buffer/50% ethanol for at least 30 min. The neutral red dye penetrates living cells, thus, the more intense the red color, the larger the number of viable cells present in the wells. The dye content in each well was quantified using a spectrophotometer at 540 nm wavelength. The dye content in each set of wells was converted to a percentage of dye present in untreated control wells using a Microsoft Excel-based spreadsheet and normalized based on the virus control. The 50% effective (EC_{50}, virus-inhibitory) concentrations and 50% cytotoxic (CC_{50}, cell-inhibitory) concentrations were then calculated by regression analysis. The quotient of CC_{50} divided by EC_{50} gave the selectivity index (SI) value. Compounds showing SI values $\geq$10 are considered active.

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