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An AZT Analog with Strongly Pairing Ethynylpyridone Nucleobase and Its Antiviral Activity against HSV1

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In memory of *Enrique Pedroso*

Challenges resulting from novel viruses or new strains of known viruses call for new antiviral agents. Nucleoside analogs that act as inhibitors of viral polymerases are an attractive class of antivirals. For nucleosides containing thymine, base pairing is weak, making it desirable to identify nucleobase analogs that pair more strongly with adenine, in order to compete successfully with the natural substrate. We have recently described a new class of strongly binding thymidine analogs that contain an ethynylmethylpyridone as base and a *C*-nucleosidic linkage to the deoxyribose. Here we report the synthesis of the 3′-azido-2′,3′-deoxyribose derivative of this compound, dubbed AZW, both as free nucleoside and as ProTide phosphoramidate. As a proof of principle, we studied the activity against Herpes simplex virus type 1 (HSV1). Whereas the ProTide phosphoramidate suffered from low solubility, the free nucleoside showed a stronger inhibitory effect than that of AZT in a plaque reduction assay. This suggests that strongly pairing *C*-nucleoside analogs of pyrimidines have the potential to become active pharmaceutical ingredients with antiviral activity.

Keywords: antiviral agents, *C*-nucleosides, HSV1, nucleosides, phosphoramidates, polymerase inhibitors.

Introduction

Nucleoside analogs with the ability to be incorporated in the growing strand during viral replication are attractive as active pharmaceutical ingredients. Therapeutic applications for such nucleoside analogs have a rich history.[1] Today, they are widely used in the treatment of human viral diseases caused by human immunodeficiency virus (HIV),[2] hepatitis C virus (HCV),[3,4] or herpes simplex virus (HSV).[5] Among the best-known compounds for the treatment of HIV and HCV are AZT and ribavirin, respectively (*Figure 1*).[2,4] Both are prodrugs that, once delivered into the cell, are converted to their corresponding mono-, di-, and triphosphate forms by kinases and transferases.[6,7] The triphosphate form is the active species. It inhibits viral replication by interfering with chain extension in the host cell.[6]

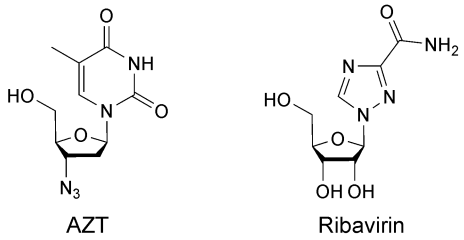

Figure 1. Structures of two known nucleoside inhibitors of viral replication.[2,4]

Some of the antiviral nucleoside analogs are obligate inhibitors that lack a hydroxy group at the 3′-

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position of their sugar, so that strand growth is terminated after their incorporation. Other antiviral nucleosides possess a 3′-hydroxy group and act as non-obligate inhibitors. They induce termination through other means, usually by causing a steric block that hinders extension after one or several subsequent extension steps. This favors the bypassing of excision of the modified nucleoside, as the exonuclease activity used for proofreading usually acts on the newly incorporated residue and does not excise longer stretches of sequence. This is also true for the replicase complex of the SARS virus.[8] Other issues affecting the antiviral activity of nucleosides is their limited ability to diffuse across membranes and slow rates in the first phosphorylation step of their intracellular metabolism. To address these issues, nucleoside phosphoramidate prodrugs (ProTides) were developed and are successfully being employed in the clinic.[9,10] Other prodrug strategies also use derivatives of nucleoside phosphates.[11]

Most nucleoside analogs are *N*-nucleosides, making their glycosidic linkage labile toward enzymatic and chemical hydrolysis. Some *C*-nucleoside analogs, though, including analogs of favipiravir ribose,[12] have their nucleobase linked to the sugar through a carbon–carbon bond. This glycosidic linkage is much less susceptible to hydrolysis or phosphorolysis. Besides natural *C*-nucleosides, such as pseudouridine, there is a large group of synthetic *C*-nucleosidic compounds that are used in chemical biology and medicinal chemistry.[13] For antivirals with a substituent at the 1′-position, a *C*-glycosidic bond is particularly attractive, as the substituent usually stabilizes the oxocarbenium ion formed upon loss of the nucleobases, and thus favors the breaking of the glycosidic bond. Remdesivir is one of the compounds for which this is avoided by a *C*-nucleosidic bond. Here, the base is a pyrrolotriazine, which ensures strong pairing and thus high activity.[14,15]

Among the canonical nucleobases, thymine and uracil pair most weakly with their complementary base.[16] Either forms only two hydrogen bonds with their Watson-Crick pairing partner, experiences weak stacking interactions and causes a modest hydrophobic effect upon binding. The binding strength can be increased by placing an ethynyl substituent at the 2-position, but the resulting *N*-nucleoside (**1**) suffers from a positively charged base, resulting in a labile glycosidic bond (*Figure 2*). To avoid this, the *C*-nucleosides dubbed 'E' and 'W' were designed. Both were shown to pair more strongly with A than T, when incorporated in oligodeoxynucleotides.[17,18] In fact,

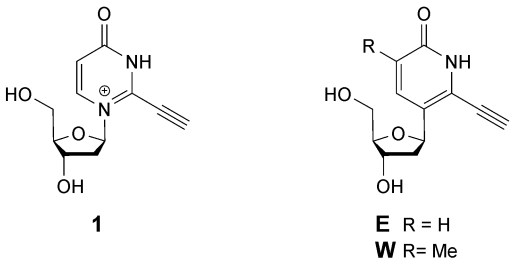


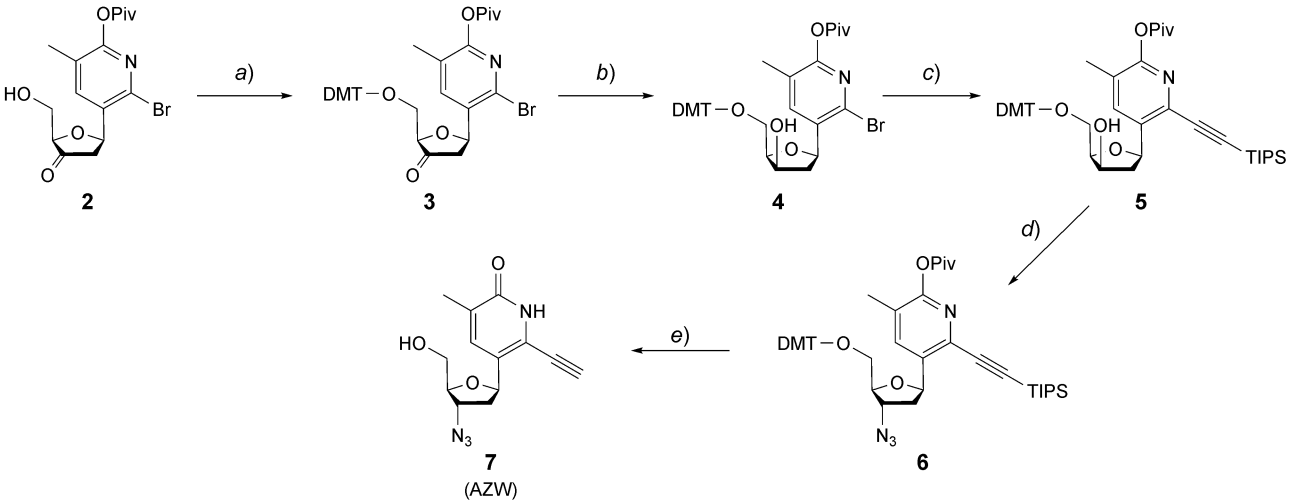
Figure 2. Structures of hypothetical 2-deoxy-2-ethynylthymidine and of two ethynylpyridine analogs known to pair strongly with adenosine.[17,18]

W:A base pairs were found to be approximately as stable as C:G base pairs in several duplex contexts. Such an increase in pairing strength should also help with the incorporation of inhibitory nucleosides in polymerase-catalyzed reactions, prompting us to explore this effect. Here, we report the synthesis of an azido-*C*-nucleoside derivative of W abbreviated 'AZW' and a phosphoramidate ProTide form of it, together with its inhibitory effect against HSV1.

Results and Discussion

The synthesis of *C*-nucleosides is more challenging than that of most *N*-nucleosides, as carbon atoms of nucleobase analogs seldom possess a lone pair that makes them highly nucleophilic centers. Unless the aglycone is constructed on a carbohydrate moiety, or a sugar is formed on the aglycone unit, special techniques are required to link the base to the sugar.[19,20]

The synthesis of azido-W **7** is shown in *Scheme 1*. It starts from ketone **2**, which was prepared in an overall yield of 29% over seven steps following protocols that were published previously.[17,18] First, ketone **2** was DMT protected to set it up for subsequent steps. Protected nucleoside **3** then underwent diastereoselective reduction at the 3′-position to give **4**, with the borohydride preferentially attacking from the sterically less hindered α-face. Under the reaction conditions chosen, the diastereomeric ratio between xylofuranoside **4** and its undesired ribo isomer was 6:1. The two diastereomers were separated by column chromatography, providing **4** in 59% yield over two steps. The introduction of a TIPS-protected ethynyl substituent to obtain **5** used a Sonogashira coupling and was followed by a Mitsunobu reaction to introduce the azido group with inversion of configuration at the 3′-



Scheme 1. Synthesis of azido-*C*-nucleoside **7**, starting from ketone **2**.[17,18] *a*) DMT–Cl, DMAP, pyridine. *b*) $NaBH_4$, THF/EtOH, −60 °C, 59% over two steps. *c*) Triisopropylsilylacetylene, $Pd(PPh_3)_2Cl_2$, CuI, NEt_3, DMF, 80 °C, 75%. *d*) PPh_3, DIAD, DPPA, THF, 74%. *e*) 1. NH_4OH, THF/EtOH; 2. TCA, CH_2Cl_2; 3. TBAF (1.0 M), THF, 92% over three steps. Abbreviations: Piv = pivaloyl, DMT = 4,4′-dimethoxytrityl, DMAP = 4-dimethylaminopyridine, TIPS = triisopropylsilyl, DIAD = diisopropyl azodicarboxylate, DPPA = diphenylphosphoryl azide, TBAF = tetra-*n*-butylammonium fluoride.

position. Different reaction conditions were tested for the following step to obtain azide **6** in high yield. With NaN_3, the yield did not exceed 40%, most probably due to low solubility of the salt. This problem was overcome by using diphenylphosphoryl azide (DPPA), which allowed the isolation of compound **6** in 74% yield. Subsequently, the protecting groups (Piv, DMT and TIPS) were removed in 92% yield over three steps to afford deprotected azido-*C*-nucleoside **7** (AZW). The ethynylpyridone derivative of AZT was thus obtained in an overall yield of 30% over seven steps. The NMR and UV/Vis spectra of the new azidonucleoside are shown in *Figure 3*.

Besides the free nucleoside, we also prepared a first nucleotide prodrug version of AZW. For this, we chose the ProTide approach of McGuigan and colleagues that has successfully been applied for compounds like sofosbuvir,[21] tenofovir alafenamide,[22] and remdesivir.[23,24] The active nucleoside phosphate metabolites are free of stereochemistry in the phosphate substituent, and we opted for a non-diastereoselective version of the phosphorylation for our exploratory study (*Scheme 2*). The nitrophenyl-phosphoramidate reagent **8** was prepared in two steps, following published protocols.[15,25] The yield of the formation of prodrug **9** was 24%. Optimization is expected to increase this yield, as no more than 60% conversion was achieved under the current conditions. The unreacted nucleoside and the product were

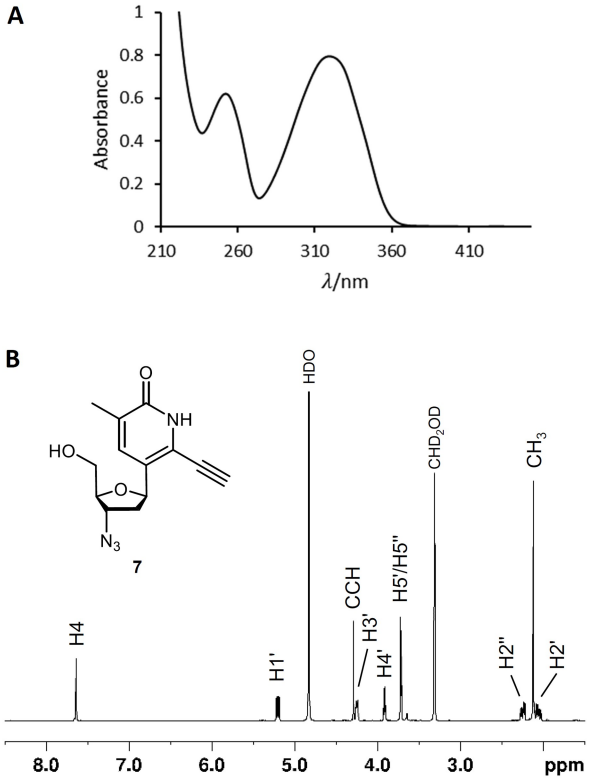
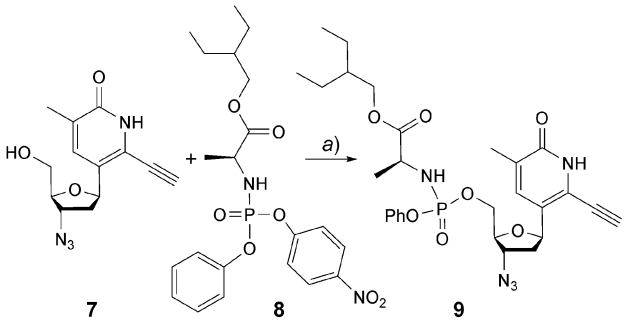


Figure 3. Spectra of AZW (**7**). A) UV-Vis spectrum in MeOH, and B) ^{1}H-NMR spectrum (CD_3OD, 400 MHz) with assignment of peaks given above the signals, including those of residual proton signals from the solvent.



Scheme 2. Synthesis of the AZW phosphoramidate prodrug **9** through phosphorylation with **8**. *a*) $MgCl_2$, $(^iPr)_2NEt$, CH_3CN, 50 °C, 24 %.

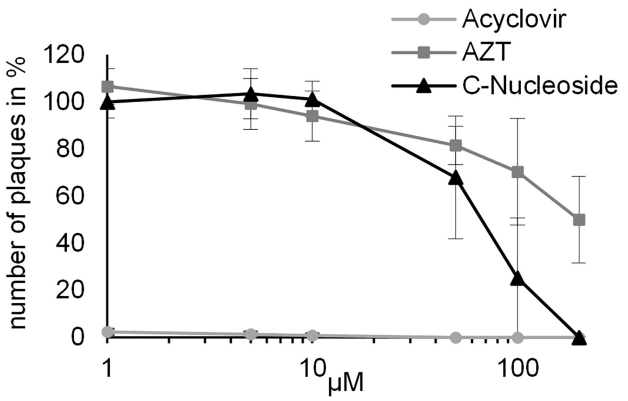


Figure 4. Effect of AZT and AZW on HSV1 plaque formation in Vero cells, compared to ACV as positive control and carrier only (DMSO) as negative control, set to 100 % viability. Plaque reduction upon exposure to 1 μM – 200 μM of antiviral compound is plotted, with standard deviations of mean values of technical triplicates of three independent experiments.

readily separated by column chromatography, though, and excess starting material was recovered. The ^{31}P-NMR spectrum of **9** is shown in *Figure S12* of the *Supporting Information*.

To obtain proof-of-principle data on the antiviral activity of AZW and its phosphoramidate, we performed a plaque reduction assay with HSV1 on Vero cells.[26] HSV1 is one of the most common human pathogens with a prevalence of 67 % among those under the age of fifty.[27] Besides harmless inflammations in oral regions, HSV1 can cause severe diseases like keratitis or encephalitis. Since 1981 the nucleoside analogue acyclovir is mainly used to treat HSV1 infections,[28,29] either locally or systemically. However, resistance to acyclovir arises especially in immunocompromised patients indicating the need for new antiviral therapies.[30]

We tested AZW against AZT and acyclovir (ACV) as positive control. Carrier alone (DMSO) served as negative control. The choice of test system was affected by availability during the current pandemic. We note that neither AZT nor AZW were specifically designed to be used against HSV1, and that the target polymerases of HSV1 and HIV are not closely related.[31] Rather, the current tests were meant to demonstrate whether AZW has antiviral activity against a DNA virus at all, and whether this activity is greater than that of AZT.

A plot of results from the antiviral assays is shown in *Figure 4*. After infection, the cells were exposed to methylcellulose medium containing the antiviral compound or DMSO as carrier, and plaques were counted after 3 d. The solubility of **9** was found to be 35 μM in H_2O, and 58 μM in H_2O/DMSO (99 : 1). This precluded performing the plaque reduction assay in the concentration range chosen, but exploratory experiments indicated a reduction of the plaque number at 5 μM of the phosphoramidate. For nucleoside **7**, full inhibition of viral replication was observed at 200 μM, whereas the same concentration of AZT reduced the number of plaques to just 50 % of the control value. Because AZT and AZW differ by the structure of the nucleobases only, this indicates that the ethynylpyridone *C*-nucleoside has the potential to be used as antiviral agent.

The need to explore nucleoside analogs, and particularly *C*-nucleosides,[32] more systematically as antivirals has been emphasized.[19,33] The *C*-glycosidic bond renders them resistant to phosphorolysis by phosphorylase enzymes, a common degradation pathway for *N*-nucleoside analogs. The ethynylpyridones are not among the *C*-nucleosides considered as antivirals previously.[1,32] They combine increased stability with high affinity for adenine as base pairing partner,[17,18,34] as found in the template of polymerase reactions. Establishing that they are active is important, as, among other structural features, they lack a hydrogen acceptor at what is the O(2)-position in their pyrimidine counterparts. This hydrogen bond acceptor can play a role in the recognition of base pairs in the active site of enzymes,[35] including polymerases.[36] We note that the pyridone ring offers several sites for structural modification and thus optimization through structure-activity studies, and so does the deoxyribose moiety, opening the door for the exploration of a number of potential new obligate or non-obligate inhibitors of polymerases.

Conclusions

Our synthesis shows how an azido group can be introduced to the ribose of ethynylpyridone *C*-nucleosides at the 3′-position without resorting to anhydronucleosides as intermediates. The results presented here establish, for the first time, that ethynyl pyridone *C*-nucleosides are active antivirals. While the structural details of the ProTide construct will have to be adapted to compensate for the increase in lipophilicity of W over thymidine, it is now clear that strongly pairing analogs of thymidine can inhibit viral replication *in vitro*. These results thus pave the way for a more systematic exploration of the biological activity of pyridone *C*-nucleosides with improved base pairing strength and their nucleotide prodrugs. Broader antiviral testing of such nucleoside analogs is needed to determine their full therapeutic potential.

Experimental Section

General

Chemicals and solvents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Carbosynth (Compton, Berksire, UK), Acros Organics (Geel, Belgium) or TCI (Eschborn, Germany), and were used without further purification. The reactions were carried out under argon atmosphere, unless stated otherwise. Thin layer chromatography (TLC) was performed on Macherey Nagel pre-coated TLC sheets ALUGRAM Xtra SIL G/UV_{254} and visualized with UV light (254 nm) staining with an aqueous solution of phosphomolybdic acid, cerium (IV) sulfate and sulfuric acid. Column chromatography used silica 60 M (0.040–0.063 mm, Macherey Nagel). The NMR spectra were recorded on Bruker Avance 300 MHz, 400 MHz, 500 MHz or 700 MHz spectrometers. Mass spectra were measured on a Bruker micro TOF–Q spectrometer in positive mode.

1-*β*-(2-Bromo-5-methyl-6-pivaloyloxypyridin-3-yl)-5-*O*-(dimethoxytrityl)-3-oxo-1,2,3-trideoxy-D-ribofuranose (**3**). Ketone **2** (0.95 g, 2.46 mmol, 1.0 equiv.) was co-evaporated twice from dry pyridine (30 mL), then dissolved again in dry pyridine (30 mL). To the stirred solution, 4-dimethylaminopyridine (30 mg, 0.25 mmol, 0.1 equiv.) was added, and the mixture was stirred for 30 min at room temperature. DMT–Cl (2.50 g, 7.38 mmol, 3.0 equiv.) previously dried by two co-evaporations from dry pyridine (30 mL) was dissolved in dry pyridine (20 mL) and added into the reaction flask. The resulting mixture was stirred for 18 h at room temperature. After TLC monitoring indicated full conversion, the solvent was removed *in vacuo*. The residue was taken up in ethyl acetate (5 mL) and filtered through silica gel, using petroleum ether/ethyl acetate (7:3, *v/v*) as eluent. The filtrate was concentrated *in vacuo* to obtain the crude product of compound **3** as a light-yellow foam. This crude product was directly used in the next step without further purification. An analytical sample was purified by silica chromatography, eluting with petroleum ether/ethyl acetate (9:1 to 7:3, *v/v*). R_f (petroleum ether/ethyl acetate 9:1) 0.10. ^{1}H-NMR (500 MHz, CD_3CN): 8.13 (s, 1 H), 7.41–7.22 (m, 9 H), 6.88 (d, J = 8.8 Hz, 4 H), 5.54 (dd, J = 10.0 Hz, J = 6.3 Hz, 1 H), 4.18 (t, J = 2.7 Hz, 1 H), 3.76 (s, 6 H), 3.44 (d, J = 10.5 Hz, 1 H), 3.36 (d, J = 10.5 Hz, 1 H), 3.18 (dd, J = 18.0 Hz, J = 6.7 Hz, 1 H), 2.48 (dd, J = 18.0 Hz, J = 10.1 Hz, 1 H), 1.91 (s, 3 H), 1.36 (s, 9 H). ^{13}C-NMR (125 MHz, CD_3CN): 213.8, 176.9, 159.6, 156.2, 145.8, 141.4, 138.2, 136.7, 136.5, 135.4, 130.92, 130.89, 130.87, 130.84, 128.87, 128.82, 127.8, 126.8, 114.0, 87.0, 81.7, 75.6, 63.6, 55.8, 44.5, 39.8, 27.1, 15.1. HR-ESI-MS: 710.172 ($C_{37}H_{38}BrNO_7^+$, [M + Na]$^+$; calc. 710.172).

1-*β*-(2-Bromo-5-methyl-6-pivaloyloxypyridin-3-yl)-1,2-dideoxy-5-*O*-(dimethoxytrityl)-D-xylofuranose (**4**). Compound **3** (1.69 g, 2.46 mmol, 1.0 equiv.) was dissolved in a mixture of THF (12 mL) and ethanol (12 mL) and cooled to −60 °C. Sodium borohydride (140 mg, 3.69 mmol, 1.5 equiv.) was ground and added. The mixture was stirred for 2 h at a temperature of −60 °C. After TLC monitoring indicated full conversion, cold acetone (10 mL) was added, and the cold mixture was poured into ethyl acetate (200 mL). Saturated aqueous $NaHCO_3$ (200 mL) was added and the two phases were separated. The organic layer was washed with H_2O (100 mL) and brine (10% NaCl in H_2O, 100 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography, using petroleum ether/ethyl acetate (9:1 to 7:3, *v/v*) as eluent. After removal of the solvent, xylonucleoside **4** (1.00 g, 1.45 mmol, 59% over two steps) was obtained as an off-white foam. R_f (petroleum ether/ethyl acetate 7:3, *v/v*) 0.65. ^{1}H-NMR (500 MHz, CD_3CN): 7.88 (s, 1 H), 7.52–7.23 (m, 9 H), 6.88 (d, J = 8.8 Hz, 4 H), 5.12 (dd, J = 8.9 Hz, J = 4.7 Hz, 1 H), 4.35–4.30 (m, 1 H), 4.12–4.10 (m, 1 H), 3.77 (s, 6 H), 3.48 (d, J = 12.0 Hz, 1 H), 3.33 (d, J = 12.0 Hz, 1 H), 2.84 (d, J = 4.4 Hz, 1 H), 2.80–2.75 (m, 1 H), 2.06 (s, 3 H), 1.80–1.74 (m, 1 H), 1.36 (s, 9 H). ^{13}C-NMR (175 MHz, CD_3CN): 177.0, 159.6, 155.6, 146.2, 141.5, 140.2, 137.1, 137.0,

135.0, 131.0, 130.9, 129.9, 129.0, 128.7, 128.5, 127.7, 126.0, 114.0, 87.0, 83.9, 77.8, 72.9, 64.1, 55.8, 43.2, 39.8, 27.1, 15.3. HR-ESI-MS: 712.188 ($C_{37}H_{40}BrNO_7^+$, $[M+Na]^+$; calc. 712.188).

1,2-Dideoxy-5-*O*-(dimethoxytrityl)-1-β-[3-methyl-2-pivaloyloxy-6-(triisopropylsilylethynyl)pyridin-5-yl]-D-xylofuranose (**5**). In a pressure-stable reaction vessel, xylo-nucleoside **4** (831 mg, 1.20 mmol, 1.0 equiv.) was dissolved in dry dimethylformamide (2.2 mL). Then, bis(triphenylphosphine) palladium (II) dichloride (84 mg, 0.12 mmol, 0.1 equiv.), copper (I) iodide (46 mg, 0.24 mmol, 0.2 equiv.), triethylamine (333 μL, 2.40 mmol, 2.0 equiv.) and triisopropylsilylacetylene (673 μL, 3.00 mmol, 2.5 equiv.) were added. The reaction vessel was sealed, and the mixture was stirred for 2 h at 80 °C. The black suspension was then filtered through *Celite*, eluting with ethyl acetate. The solution was concentrated *in vacuo*, and the residue was purified by column chromatography, eluting with petroleum ether/ethyl acetate (9:1 to 7:3, *v/v*). Compound **5** (712 mg, 0.90 mmol, 75%) was obtained as a light-yellow foam. TLC: R_f (petroleum ether/ethyl acetate 7:3, *v/v*) 0.78. ^{1}H-NMR (300 MHz, CD_3CN): 7.88 (s, 1 H), 7.53–7.21 (m, 9 H), 6.91–6.85 (m, 4 H), 5.35 (dd, J=8.8 Hz, J=5.4 Hz, 1 H), 4.35–4.30 (m, 1 H), 4.10–4.06 (m, 1 H), 3.77 (s, 6 H), 3.47 (dd, J=10.5 Hz, J=7.1 Hz, 1 H), 3.33 (dd, J=10.5 Hz, J=3.8 Hz, 1 H), 2.83 (d, J=4.6 Hz, 1 H), 2.79–2.70 (m, 1 H), 2.10 (s, 3 H), 1.82–1.75 (m, 1 H), 1.37 (s, 9 H), 1.23–1.10 (m, 21 H). ^{13}C-NMR (175 MHz, CD_3CN): 176.9, 159.2, 156.0, 145.8, 142.4, 139.0, 136.8, 136.7, 136.1, 130.64, 130.61, 128.7, 128.4, 127.4, 126.9, 113.6, 103.5, 96.9, 86.6, 83.3, 76.4, 72.6, 63.8, 55.5, 43.4, 39.4, 26.87, 26.85, 18.60, 18.58, 16.0, 11.7. HR-ESI-MS: 792.429 ($C_{48}H_{61}NO_7Si^+$, $[M+H]^+$; calc. 792.429); 814.411 ($[M+Na]^+$; calc. 814.411).

3-Azido-5-*O*-(dimethoxytrityl)-1-β-[3-methyl-2-pivaloyloxy-6-(triisopropylsilylethynyl)pyridin-5-yl]-1,2,3-trideoxy-D-ribofuranose (**6**). In a Schlenk reaction flask, triphenylphosphine (353 mg, 1.35 mmol, 1.5 equiv.) was dissolved in dry THF (1.4 mL) and cooled to 0 °C. A solution of diisopropyl azodicarboxylate (264 μL, 1.35 mmol, 1.5 equiv.) in dry THF (1.4 mL) was prepared in another flask and added into the Schlenk flask slowly. The mixture was stirred for 15 min at 0 °C until a suspension formed. Compound **5** (712 mg, 0.90 mmol, 1.0 equiv.) was dissolved in dry THF (1.4 mL) and added into the reaction flask, followed by the addition of diphenylphosphoryl azide (290 μL, 1.35 mmol, 1.5 equiv.) at 0 °C. The resulting mixture was allowed to warm up to room temperature by stirring and stirred for 16 h at room temperature. After TLC monitoring indicated full conversion, water (2.0 mL) was added and the mixture was poured into ethyl acetate (200 mL). The organic phase was washed with H_2O (100 mL), then with brine (10% NaCl in H_2O, 100 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography using petroleum ether/ethyl acetate (9:1 to 8:2, *v/v*) as eluent to give azide **6** (541 mg, 0.66 mmol, 74%) as an off-white foam. R_f (petroleum ether/ethyl acetate 9:1, *v/v*) 0.47. ^{1}H-NMR (300 MHz, CD_3CN): 7.92 (s, 1 H), 7.48–7.21 (m, 9 H), 6.89-6.85 (m, 4 H), 5.45 (dd, J=9.4 Hz, J=6.0 Hz, 1 H), 4.32–4.28 (m, 1 H), 4.01–3.97 (m, 1 H), 3.77 (s, 6 H), 3.36–3.26 (m, 2 H), 2.56–2.49 (m, 1 H), 2.17–2.08 (m, 1 H), 2.01 (s, 3 H), 1.37 (s, 9 H), 1.19–1.13 (m, 21 H). ^{13}C-NMR (175 MHz, CD_3CN): 176.8, 159.3, 156.4, 145.5, 139.9, 138.6, 136.48, 136.44, 136.43, 130.6, 130.5, 128.6, 128.5, 127.5, 127.4, 113.7, 103.1, 96.9, 86.9, 84.1, 77.0, 64.4, 63.8, 55.5, 40.1, 39.5, 26.8, 26.6, 18.58, 18.57, 15.9, 11.6. HR-ESI-MS: 817.436 ($C_{48}H_{60}N_4O_7Si^+$, $[M+H]^+$; calc. 817.436).

3-Azido-1-β-[6-ethynyl-3-methyl-2-pyridon-5-yl]-1,2,3-trideoxy-D-ribofuranose (**7**). Azide **6** (541 mg, 0.66 mmol, 1.0 equiv.) was dissolved in THF (10 mL) in a round bottom flask. Then ammonia solution (25%, 10 mL) was added, followed by ethanol (20 mL) to give a clear solution. The mixture was stirred for 1 h at room temperature, then diluted with ethyl acetate (250 mL). The organic phase was washed twice with brine (10% NaCl in H_2O, 100 mL), dried over Na_2SO_4, and evaporated to dryness *in vacuo* to get the crude product of pivaloyl-deprotected azide. This crude product was dissolved in trichloroacetic acid contained CH_2Cl_2 (3%, 30 mL), and stirred for 30 min at room temperature. After TLC monitoring indicated full conversion, the solution was poured into ethyl acetate (200 mL), washed twice with saturated $NaHCO_3$ solution (100 mL) and once with brine (10% NaCl in H_2O, 100 mL). After drying over Na_2SO_4, solvent was removed *in vacuo*. The residue was dissolved in dry THF (20 mL), followed by the addition of TBAF solution (1.0 M in THF, 660 μL, 0.66 mmol, 1.0 equiv.). The mixture was stirred for 15 min at room temperature to give full conversion. Then the reaction was quenched with H_2O (30 mL). CH_2Cl_2 (60 mL) was added, and the two phases were separated. The aqueous phase was extracted with CH_2Cl_2 (30 mL). The combined organic layer was washed twice with H_2O (30 mL), twice with brine (10% NaCl in H_2O, 30 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified

by column chromatography using CH_2Cl_2/methanol (99:1 to 95:5, *v/v*) as eluent to obtain the deprotected azido-nucleoside **7** (167 mg, 0.61 mmol, 92%) as a white solid. R_f (CH_2Cl_2/methanol = 10:1, *v/v*) 0.44. ^{1}H-NMR (400 MHz, CD_3OD): 7.63 (s, 1 H), 5.20 (dd, J = 10.2 Hz, J = 5.6 Hz, 1 H), 4.29 (s, 1 H), 4.26–4.23 (m, 1 H), 3.93–3.90 (m, 1 H), 3.71 (d, J = 4.5 Hz, 2 H), 2.27–2.22 (m, 1 H), 2.12 (s, 3 H), 2.08–2.02 (m, 1 H). ^{13}C-NMR (100 MHz, CD_3OD): 163.5, 136.7, 131.6, 124.0, 122.9, 87.3, 85.0, 76.0, 71.6, 62.9, 62.1, 38.5, 15.4. HR-ESI-MS: 275.113 ($C_{13}H_{14}N_4O_3^+$, $[M+H]^+$; calc. 275.113), 297.096 ($[M+Na]^+$; calc. 297.096).

Azido-W phosphoramidate (**9**). In a round bottom flask, azido-nucleoside **7** (30 mg, 0.11 mmol, 1.0 equiv.), nitrophenyl-phosphoramidate **8** (60 mg, 0.13 mmol, 1.2 equiv.) and magnesium chloride (10 mg, 0.11 mmol, 1.0 equiv.) were suspended in dry CH_3CN (1.0 mL). The mixture was warmed to 50 °C and stirred for 10 min at this temperature under nitrogen atmosphere. *N*,*N*-Diisopropylethylamine (48 μL, 0.28 mmol, 2.5 equiv.) was added to the flask, and the mixture was stirred further for 30 min at 50 °C. After cooling to room temperature, the reaction mixture was diluted with ethyl acetate (10 mL), washed with aqueous citric acid solution (5%, 5 mL), saturated aqueous ammonium chloride solution (5 mL), twice with potassium carbonate solution (5%, 5 mL), and brine (5 mL). The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography using CH_2Cl_2/methanol (99:1 to 95:5, *v/v*) as eluent to afford compound **9** (15 mg, 0.026 mmol, 24%) as a diastereomeric mixture. R_f (CH_2Cl_2/methanol 100:5, *v/v*) 0.37. ^{1}H-NMR (400 MHz, CD_3CN): 9.79 (s, 1 H), 7.42–7.17 (m, 6 H), 5.13 (dd, J = 10.3 Hz, J = 5.7 Hz, 1 H), 4.28–4.17 (m, 4 H), 4.07–3.95 (m, 4 H), 3.94 (s, 1 H), 2.23–2.09 (m, 2 H), 2.03 and 2.00 (2s, 3 H), 1.51–1.44 (m, 1 H), 1.37–1.27 (m, 7 H), 0.86 (t, J = 7.4 Hz, 6 H). ^{13}C-NMR (100 MHz, CD_3CN): 174.8, 163.0, 151.9, 136.3, 133.6, 132.4, 130.6, 125.8, 123.4, 121.2, 87.8, 83.3, 77.2, 67.7, 67.6, 63.7, 58.8, 51.4, 41.1, 39.3, 23.8, 21.0, 16.9, 11.2. ^{31}P-NMR (162 MHz, CD_3CN): 3.02, 2.88. HR-ESI-MS: 586.244 ($C_{28}H_{36}N_5O_7P^+$, $[M+H]^+$; calc. 586.243); 608.225 ($[M+Na]^+$; calc. 608.225).

Cells, Viruses and Antiviral Compounds

Vero cells (ATCC® CCL-81™) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 1% gentamicin and 1% sodium pyruvate under humidified atmosphere of 5% CO_2 and 37 °C. HSV1 strain F (B. Roizman, University of Chicago, USA) was used for the experiments. Propagation and titration of HSV1 strain F was done as previously described.[37] Acyclovir (CAS Number 59277-89-3, Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to give a 22.2 mM stock solution. Both AZT and AZW were also dissolved in DMSO and stored as 40 mM stock solutions.

Plaque Reduction Assay

To test the efficacy of antiviral compounds against HSV1, a plaque reduction assay was applied. In the event, $1.6 \cdot 10^5$ Vero cells per 12 wells were seeded the day before infection and incubated under humidified atmosphere of 5% CO_2 and 37 °C. Each well was inoculated with approximately 100 plaque forming units (PFU) and incubated under standard conditions for 1 h. The inoculum was removed, and the cells were overlaid with methylcellulose medium (supplemented DMEM with 0.75% (*w/v*) carboxymethylcellulose) containing different concentrations of one of the antiviral compounds or DMSO as control. After 3 d, cells were fixed and stained with crystal violet solution (0.2% (*w/v*) crystal violet, 13% (*v/v*) formaldehyde (37% solution), 2% (*v/v*) EtOH) to count plaques formed. Each concentration of an antiviral compound was titrated as technical triplicates in three independent experiments performed at different days. The mean number of plaques of the control (DMSO) was set as 100% in each experiment.

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Author Contribution Statement

All authors planned the project and experiments, J. H. synthesized the compounds, C. F. and S. B. performed and analyzed the biological assays, C. R., J. H., C. F.,

and J. E. contributed to writing and correcting the manuscript.

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