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Synthesis of Phosphonate Derivatives of Uridine, Cytidine, and Cytosine Arabinoside

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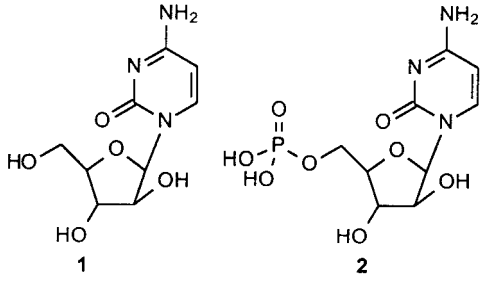
Abstract—The vinyl phosphonate derivatives of uridine, cytidine, and cytosine arabinoside (ara-C) have been prepared through oxidation of appropriately protected nucleosides to the 5′ aldehydes and Wittig condensation with [(diethoxyphosphinyl)methylidine]triphenylphosphorane. Dihydroxylation of these vinyl phosphonates with an AD-mix reagent generated the new 5′,6′-dihydroxy-6′-phosphonates. After hydrolysis of the phosphonate esters and the various protecting groups, the six phosphonic acids were tested for their ability to serve as substrates for the enzyme nucleotide monophosphate kinase and for their toxicity to K562 cells.

Introduction

Cytosine arabinoside (ara-C, **1**) is commonly used in clinical treatment of both chronic and acute myelogenous leukemia.[1] While the molecular basis of its activity is not yet entirely clear, to interfere directly with DNA synthesis this nucleoside must be metabolically converted to its triphosphate derivative ara-CTP.[2] The first step in this process is phosphorylation of the nucleoside to produce ara-CMP (**2**), a reaction that is catalyzed by the enzyme deoxycytidine kinase (dCK). This initial phosphorylation is the key step in activation of ara-C, because (a) it is the rate limiting step in formation of ara-CTP and (b) mutational loss of dCK activity is a principal cause of resistance to ara-C.[3] As a potential strategy to develop new anti-leukemia agents, especially those which might circumvent the resistance of dCK- cells, we have become interested in preparation of new phosphonate derivatives of ara-C that might function as ara-CMP analogues. While there are many precedents for preparation of phosphonate derivatives of other nucleosides[4] and nucleoside analogues,[5] at this time there are no reports describing phosphonate derivatives of ara-C. In this paper, we describe the preparation of several new ara-C phosphonates along with the corresponding uridine and cytidine derivatives.



Chemical synthesis

To develop synthetic procedures that could be used to modify commercially available ara-C, and to obtain model compounds for the bioassays, we chose to begin this investigation with preparation of vinyl phosphonate derivatives of the relatively inexpensive pyrimidine nucleosides uridine (**3**) and cytidine (**8**). After preparation of the acetonide **4**,[6] the uridine derivative **6** was obtained by a variation on the classic Moffatt strategy[7] that involved oxidation with CrO_3 and immediate condensation of the intermediate 5′ aldehyde with phosphonate **5**.[8] Hydrolysis of both the phosphonate esters and the acetonide was readily achieved upon treatment with TMSBr under standard conditions to provide the corresponding phosphonic acid **7**. In the cytidine series, preparation of the acetonide **9** proved relatively straightforward but attempts to prepare the

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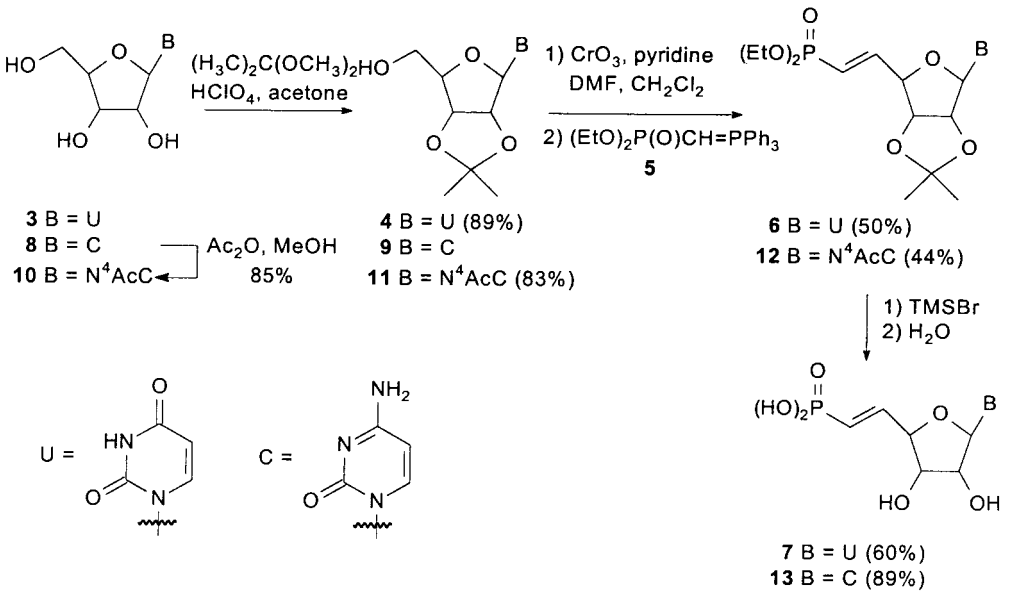
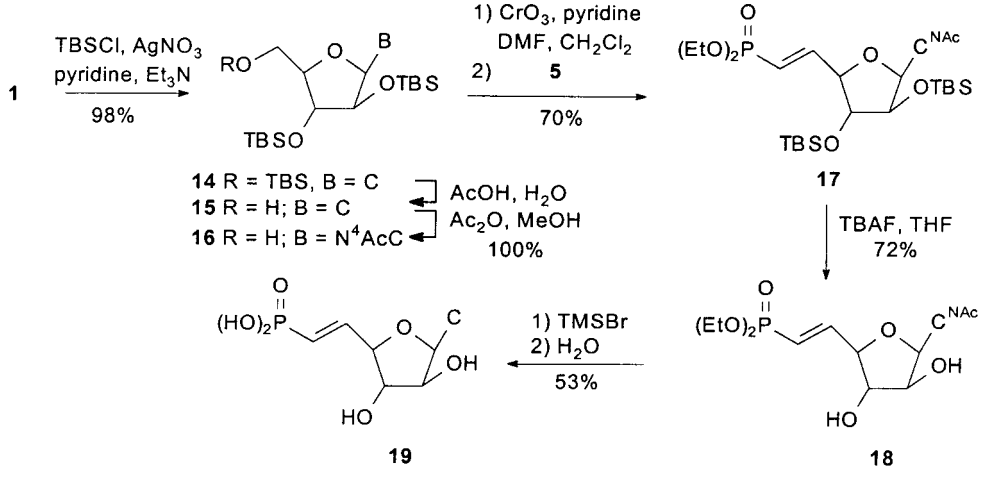
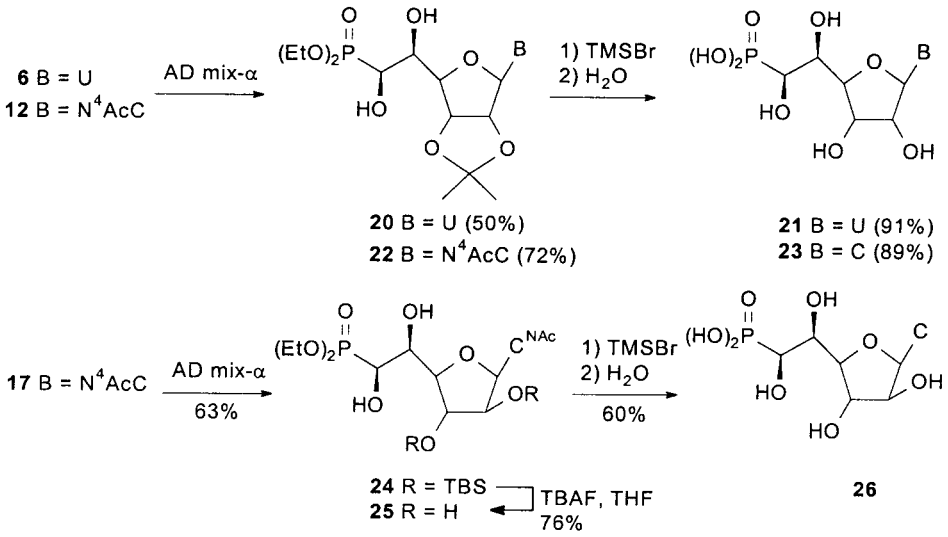


corresponding vinyl phosphonate through this oxidation–condensation strategy were frustrated by low yields. However, when the amino group of cytidine was first protected as the acetamide derivative (**10**) prior to formation of the acetonide (**11**), oxidation to the corresponding aldehyde proceeded smoothly and the aldehyde could be isolated in good yield (83%). Because this aldehyde may not be stable it normally was treated with phosphonate **5** after minimal purification to give the vinyl phosphonate **12**. Final hydrolysis via reaction with TMSBr cleaved the phosphonate esters, the acetonide, and the acetamide protecting groups, and gave the desired phosphonic acid **13**.2

Extension of this strategy to allow preparation of the ara-C phosphonates required introduction of a different protecting group for the 2′ and 3′ hydroxyl groups because this *trans* diol would not readily undergo formation of an acetonide. While TBS-protected ara-C derivatives have been reported,[9] the promise of a modest yield in preparation of the 2′,3′,5′-TBS derivative through these conditions was not attractive. Instead a nearly quantitative yield of compound **14** was obtained through combination of conditions employed with different ribosides,[10] and selective hydrolysis[9] of the 5′ protecting group gave the necessary intermediate **15**. After protection of the amino group through reaction with acetic anhydride in methanol,[11] the ara-C derivative **16** was subjected to the oxidation–condensation sequence described above. The new vinyl phosphonate **17** was isolated in 70% yield as a single olefin isomer. To convert this phosphonate ester to the parent phosphonic acid, compound **17** was first treated with TBAF to remove the silyl protecting groups. The resulting diol (**18**) was then treated with TMSBr to bring about hydrolysis of the phosphonate esters and the acetamide and to afford compound **19**.

While little is known about the suitability of vinyl phosphonic acids as substrates for enzymes that process phosphates, it is known that simple replacement of a phosphate ester oxygen by a methylene group generally may not afford a good enzyme substrate.[12] Addition of oxygen[13] or fluorine[14] substituents to the carbon α to the phosphorus can lower the pK_a of the phosphonic acid so that it more closely resembles that of the corresponding

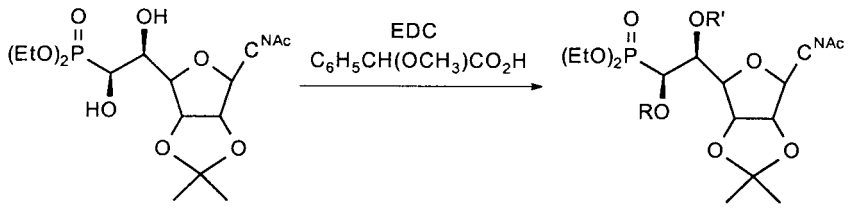




phosphate, and introduces non-bonding electrons that may be important for enzyme binding, but such functional groups can also introduce a new stereogenic center. To simplify studies of biological activity, stereocontrolled introduction of these substituents is highly desirable. Of the many methods known for stereocontrolled synthesis of non-racemic phosphonates,[15] we chose to elaborate the vinyl phosphonates **6**, **12** and **17** through reaction with the AD-mix reagents.[16] While the available precedent suggests that AD-mix oxidations of prochiral vinyl phosphonates proceed with modest enantiomeric excess when alkyl substituents are present at the β position,[17] the opportunity to reintroduce the 5′ hydroxyl group along with a hydroxyl group at the 6′ position drew our attention.

The AD-mix α oxidation of the uridine derivative **6** proceeded smoothly and gave a single diastereomer of the α,β-dihydroxylated product (tentatively shown as structure **20**) in reasonable yield. Oxidation of the *N*-acetylcytidine derivative **12** with AD-mix α also gave a single diastereomer (**22**) and proceeded in somewhat better yield (72%). In both cases, hydrolysis of the phosphonate esters was accomplished by standard treatment with TMSBr and based on the single resonance observed in each ^{31}P NMR spectra each reaction gave a single diastereomer (**21** and **23**). Oxidation of the ara-C derivative **17** proceeded in a similar fashion to give a single diastereomer (**24**) and in this case preparation of the phosphonic acid **26** was complicated only by the necessity of removing the TBS groups (**25**) through reaction with TBAF prior to hydrolysis of the phosphonate esters and acetamide group. Unfortunately, when compound **12** was treated with AD-mix β a mixture of diastereomers was obtained in a ratio of 2:1 to 1:1 based on integration of the ^{31}P NMR resonances. The resonance of the major diastereomer was identical to that of the only product obtained from the AD-mix α reaction. Thus it seems likely that the stereochemistry of the substrate has as much or more effect on the course of the oxidation as the stereochemistry of the reagent.

Assigning the stereochemistry of the new α,β-dihydroxy phosphonates is not a trivial task. The fundamental assumption that the osmium-mediated dihydroxylation proceeds with exclusively *cis* addition of the two hydroxyl groups allows only formation of either the 5′*R*,6′*R*-diastereomer or the 5′*S*,6′*S*-diastereomer. In an effort to distinguish between these two possibilities, compound **22** was treated with *S*-O-methylmandelic acid and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC). The major product of this reaction was the 6′-ester (**27**), accompanied by some of the 5′,6′-diester (**28**). While mandelate esters have been used to establish the absolute stereochemistry of several α-hydroxy phosphonates,[18] in most previous cases[19] the esters contained only two stereogenic centers (one from the mandelate reagent and one from the α-hydroxy phosphonate), and comparisons of NMR data could be used to assign stereochemistry after preparation of both possible



22

27 R = *S*-*O*-methylmandelyl; R' = H
28 R = R ' = *S*-*O*-methylmandelyl
29 R = *R*-O-methylmandelyl; R' = H

diastereomers. In the present case, this approach is complicated by the additional stereochemistry of the ribose, and the resulting difficulty in obtaining a full set of diastereomers. Nevertheless, once preparation of ester **27** demonstrated that it was possible to obtain the α ester with some regioselectivity, the diastereomeric ester **29** was prepared through reaction of compound **22** with *R*-*O*-methylmandelic acid and EDC. Analysis of the extended Newman projections (Fig. 1) suggests that in the 5′*S*,6′*S* series the *S*-mandelate would display a relatively deshielded phosphorus resonance and a relatively shielded 5′-H resonance, while the *R*-mandelate would be expected to display a relatively shielded phosphorus resonance and a relatively deshielded 5′-H resonance. A reversed pattern would be expected for the 5′*R*,6′*R* series, with the *S*-mandelate showing the more shielded phosphorus resonance and the more deshielded 5′-H resonance. The observed data (19.3 ppm and δ 4.43 for the *S*-mandelate and 17.7 ppm and δ 4.52 for the *R*-mandelate) would seem to indicate that the AD-mix α oxidation had given the *S,S*-diastereomer, and this analysis served as the basis for the tentative structure assignment. However, because this analysis discounts the additional stereocenters in the ribose ring, definitive determination of this issue will require a diffraction analysis and appropriate crystals have not yet been obtained.

Biological assays

Both the vinyl phosphonic acids (compounds **7**, **13** and **19**) and the dihydroxyphosphonic acids (compounds **21**, **23** and **26**) were tested for their ability to serve as substrate for nucleotide monophosphate (NMP) kinase. In brief, the in vitro enzyme assays used NMP kinase with adenosine triphosphate (ATP) as cofactor and linkage to pyruvate kinase and lactic dehydrogenase, as shown in Figure 2.[20] The degree of phosphorylation of the test phosphonate correlates with the reduced nicotinamide adenine dinucleotide (NADH) conversion to oxidized nicotinamide adenine dinucleotide (NAD^+), and can be monitored spectrophotometrically by a reduction in absorbance at 340 nm. As shown in Table 1, compound **13** is most able to serve as substrate for NMP kinase with approximately 6% of the activity of uridine monophosphate. While the other compounds may be phosphorylated very slowly, the observed rates vary little from the controls.

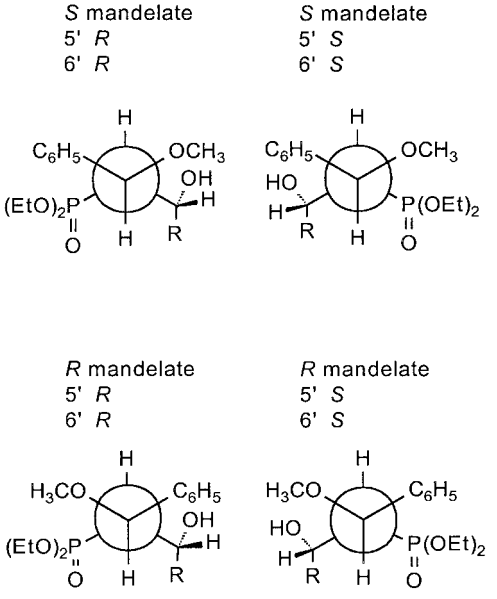


Figure 1. Extended Newman projections for diastereomeric 6′-esters.

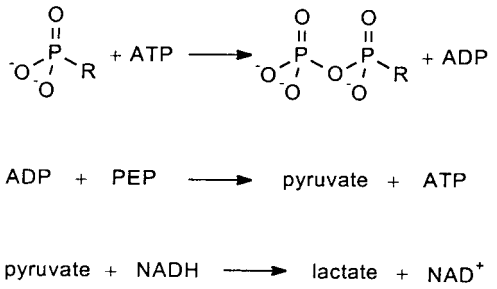


Figure 2. Enzymatic phosphorylation of nucleoside phosphonates.

All six of these phosphonic acids also were tested for their effect on the human-derived leukemia cell line K562 by measuring [^{3}H] thymidine incorporation into DNA of cells treated with various concentrations of the phosphonic acids.[21] In this assay, four of the tested compounds did not show LC_{50} values up to concentrations of 10 μM. However, compounds **19** and **23** had LC_{50} values of 1 and ∼4 μM respectively (Table 2). While ara-C itself gave an LC_{50} value of ∼5 nM in the same assay, it should be noted that the phosphonic acids would exist as dianions at the pH of these assays while ara-C is not acidic. Thus it would be reasonable to expect substantial differences in their ability to diffuse through the cell membrane.

Table 1. Relative phosphorylation of nucleoside phosphonates upon treatment with ATP and NMP kinase[a]

Compound	Rate of phosphorylation
7	1.9%
13	6.4%
19	1.2%
21	1.2%
23	1.2%
26	1.3%

[a]Relative phosphorylation of nucleoside phosphonates expressed as a percentage relative to UMP utilization.

Table 2. Toxicity of nucleoside phosphonates to K562 cells as measured by [^{3}H] thymidine incorporation[21]

Compound	LC_{50}
19	1 μM
23	∼4 μM
1 (ara-C)	∼5 nM

The surprising result of these bioassays is that compound **13** is more readily phosphorylated than compounds **19** and **23**, although the latter compounds, and not compound **13**, impair thymidine incorporation into DNA. These results support the premise that ara-C cytotoxicity is not entirely dependent upon its phosphorylation to ara-CTP. Several other mechanisms of ara-C cytotoxicity are known, including inhibition of DNA polymerases, endoreduplication, generation of reactive oxygen species, and alterations in membrane lipids and glycoproteins.[2] Alternatively, compounds **19** and **23** may inhibit the first phosphorylation of other pyrimidine nucleosides. This would explain the ability of compounds **19** and **23** to impair thymidine incorporation into DNA without requiring that they undergo phosphorylation by NMP kinase.

This investigation has led to the preparation of new phosphonate analogues of the important anti-leukemia drug ara-C as well as some related cytidine and uridine derivatives. While the initial bioassays on these compounds have uncovered only modest biological activity, the observations that some of these compounds are even weak substrates for the enzyme nucleotide monophosphate kinase, or display toxicity to a human-derived leukemia cell line at ~1 μM concentrations, encourage preparation of related compounds that may prove more biologically active.

Experimental

Tetrahydrofuran (THF) was distilled from sodium/benzophenone immediately prior to use. Commercially available anhydrous DMF was used and pyridine, CH_2Cl_2, and Et_3N were distilled from CaH_2. All nonaqueous reactions were conducted in oven-dried or flame-dried glassware, under an atmosphere of argon, with magnetic stirring. NMR spectra were recorded at 300 MHz for ^1H (75 MHz for ^{13}C) with $CDCl_3$ as solvent and $(CH_3)_4Si$ (^1H) or $CDCl_3$ (^{13}C, 77.0 ppm) as internal standards unless otherwise noted. All ^{31}P NMR chemical shifts are reported in ppm relative to 85% H_3PO_4 (external standard). High resolution FAB mass spectra were obtained at the University of Iowa Mass Spectrometry Facility.

Diethyl [1′-(5′,6′-dideoxy-2′,3′-*O*-isopropylidene-β-D-ribo-5′-hexenofuranosyl)uracil]-6′-phosphonate (6). A round bottomed flask was charged with CrO_3 (1.4 g, 14.1 mmol), CH_2Cl_2 (16 mL), and DMF (4 mL). To this solution was added pyridine (2.3 mL, 28.2 mmol). After 15 min at rt, alcohol **4** (1.0 g, 3.5 mmol) in a solution of CH_2Cl_2 (16 mL) and DMF (4 mL) was added, followed by addition of Ac_2O (1.3 mL, 14.1 mmol). After 7 min, the reaction was quenched by addition of ethanol (3 mL) and diluted with ethyl acetate (200 mL). The resulting mixture was filtered through a silica column topped with a layer of anhydrous Na_2SO_4, and the solvent was removed in vacuo to give the desired aldehyde. Without further purification, this aldehyde was immediately dissolved in anhydrous DMSO (20 mL), the Wittig reagent **5**[8] (1.6 g, 4.0 mmol) was added, and the solution was stirred for 6 h at rt. After the reaction was diluted with water (20 mL) and stirred for 1 h, the mixture was extracted with CH_2Cl_2. The extracts were washed with water and saturated brine. The organic layer was dried over anhydrous Na_2SO_4, the filtrate was concentrated in vacuo, and the residue was purified by column chromatography using a gradient solvent system (0 to 2% CH_3OH in CH_2Cl_2) to give vinyl phosphonate **6** (730 mg, 50%): ^1H NMR δ 9.33 (s, 1H), 7.23 (d, 1H, $J=8.1$ Hz), 6.87 (ddd, 1H, $J=22.2$, 17.1, 5.1 Hz), 5.94 (ddd, 1H, $J=19.1$, 17.1, 1.5 Hz), 5.76 (d, 1H, $J=7.8$ Hz), 5.67 (d, 1H, $J=1.8$ Hz), 5.10 (dd, 1H, $J=6.3$, 1.8 Hz), 4.88 (dd, 1H, $J=6.6$, 4.5 Hz), 4.66 (m, 1H), 4.11 (m, 4H), 1.59 (s, 3H), 1.36 (m, 9H); ^{13}C NMR δ 163.1, 149.8, 147.8 (d, $J=6.2$ Hz), 142.5, 119.8 (d, $J=187.9$ Hz), 114.7, 102.9, 95.0, 87.6 (d, $J=23.0$ Hz), 84.3, 84.0 (d, $J=0.8$ Hz), 62.0 (d, 2C, $J=5.3$ Hz), 27.0, 25.2, 16.3 (d, 2C, $J=6.0$ Hz); ^{31}P δ 16.7; HRFABMS calcd for $C_{17}H_{25}N_2O_8P$ $(M+H)^+$ 417.1427, found 417.1430.

[1′-(5′,6′-Dideoxy-β-D-ribo-5′-hexenofuranosyl)uracil]-6′-phosphonic acid (7). To a solution of vinyl phosphonate **6** (70 mg, 0.168 mmol) in CH_2Cl_2 (5 mL) was added TMSBr (0.23 mL, 1.68 mmol) at rt. After 8 h, the volatiles were removed in vacuo, the residue was diluted with wet CH_3OH, and the solution was concentrated. The residue was washed with ether and ethyl acetate several times to give a light yellow solid. This solid was washed with a mixture of 10% CH_3OH in CH_2Cl_2 to give pure product **7** (32 mg, 60%) as a white solid: ^1H NMR (D_2O) δ 7.73 (d, 1H, $J=7.8$ Hz), 6.53 (ddd, 1H, $J=23.1$, 17.1, 5.7 Hz), 6.19 (ddd, 1H, $J=18.3$, 17.1, 1.2 Hz), 5.96 (d, 1H, $J=8.1$ Hz), 5.94 (d, 1H, $J=3.0$ Hz), 4.60 (m, 1H), 4.43 (dd, 1H, $J=5.1$, 3.9 Hz), 4.22 (dd, 1H, $J=5.7$, 5.7 Hz); HRFABMS calcd for $C_{10}H_{13}N_2O_8P$ $(M-H)^-$ 319.0313, found 319.0331.

2′,3′-*O*-Isopropylidene-4-*N*-acetylcytidine (11). N^4-Acetylcytidine[11] (6.7 g, 23.50 mmol) was dissolved in acetone (300 mL). To this suspension was added $HClO_4$ dropwise until the reaction mixture just turned clear. Excess 2,2-dimethoxypropane (75 mL) was added, and the solution was stirred ~28 h at rt. After concentration in vacuo to one third of the original reaction volume, the product solidified. Ether was added to induce further precipitation, and the resulting solid was collected by filtration, washed with ether and dried to give the desired product **11** (6.3 g, 83%): ^1H NMR δ 9.94 (s, 1H), 7.94 (d, 1H, $J=7.5$ Hz), 7.48 (d, 1H, $J=7.2$ Hz), 5.67 (d, 1H, $J=2.4$ Hz), 5.15 (dd, 1H, $J=6.6$, 2.4 Hz), 5.03 (dd, 1H, $J=6.6$, 3.0 Hz), 4.42 (d, 1H, $J=2.4$ Hz), 3.99 (m, 3H), 2.30 (s, 3H), 1.61 (s, 3H), 1.39 (s, 3H); ^{13}C NMR δ 171.1, 162.8, 155.2, 147.6, 113.9, 97.9, 96.9, 88.2, 84.4, 80.6, 62.5, 27.2, 25.2, 24.9.

Diethyl [1′-(5′,6′-dideoxy-2′,3′-*O*-isopropylidene-β-D-ribo-5′-hexenofuranosyl)-4-*N*-acetylcytosyl]-6′-phosphonate (12). To a mixture of CrO_3 (1.1 g, 11.07 mmol) in CH_2Cl_2 (16 mL) and DMF (4 mL) was added pyridine (1.8 mL, 22.15 mmol). After 15 min at rt, alcohol **11** (900 mg, 2.76 mmol) was added as a solution in CH_2Cl_2 (8 mL) and DMF (2 mL), followed by addition of Ac_2O

(1.0 mL, 11.07 mmol). After 7 min, the reaction was quenched by addition of ethanol (3 mL) and diluted with ethyl acetate (200 mL). The resulting mixture was filtered through a silica column topped with a layer of anhydrous Na_2SO_4, and the filtrate was concentrated in vacuo to give the desired aldehyde. Normally this material was used in the subsequent reaction without further purification, but after purification by column chromatography (0 to 1% CH_3OH in CH_2Cl_2) the aldehyde could be obtained in 83% yield.

Immediately after its preparation, a solution of the unpurified aldehyde in anhydrous DMSO (20 mL) was treated with the Wittig reagent **5** (950 mg, 2.76 mmol) and the resulting solution was stirred ~13 h at rt. After the reaction was diluted with distilled water (20 mL) and stirred for 1 h, the mixture was extracted with CH_2Cl_2 several times. The extracts were washed with water and then with saturated brine. The organic layer was dried over anhydrous Na_2SO_4, concentrated in vacuo, and the residue was purified by column chromatography using a gradient solvent system (0 to 2% CH_3OH in CH_2Cl_2) to give vinyl phosphonate **12** (580 mg, 1.21 mmol, 44% yield over two steps): ^{1}H NMR δ 10.11 (s, 1H), 7.72 (d, 1H, J = 7.5 Hz), 7.48 (d, 1H, J = 7.5 Hz), 6.95 (ddd, 1H, J = 23.4, 17.1, 6.3 Hz), 5.98 (ddd, 1H, J = 19.2, 17.4, 1.5 Hz), 5.72 (d, 1H, J = 0.9 Hz), 5.17 (dd, 1H, J = 6.3, 1.2 Hz), 4.95 (dd, 1H, J = 6.3, 3.8 Hz), 4.76 (m, 1H), 4.11 (m, 4H), 2.31 (s, 3H), 1.60 (s, 3H), 1.38 (m, 9H); ^{13}C NMR δ 171.2, 163.5, 148.0, 146.2, 119.5 (d, J = 186.4 Hz), 114.3, 96.7 (d, J = 17.1 Hz), 88.8, 88.5, 85.2, 84.1, 62.1, 62.0 (d, J = 6.4 Hz), 61.9 (d, J = 7.1 Hz), 27.0, 25.2, 24.9, 16.3 (d, J = 6.5 Hz), 16.2 (d, J = 5.9 Hz); ^{31}P NMR δ 17.4; HRFABMS calcd for $C_{19}H_{28}N_3O_8P$ $(M+Na)^+$ 480.1508, found 480.1512.

[1′-(5′,6′-Dideoxy-β-D-ribo-5′-hexenofuranosyl)cytosyl]-6′-phosphonic acid (13). To a solution of vinyl phosphonate **12** (85 mg, 0.18 mmol) in CH_2Cl_2 (3 mL) was added TMSBr (0.3 mL, 2.2 mmol) at rt. After 26 h at rt, the solvent was removed, the residue was diluted with CH_3OH, and the resulting solution was concentrated in vacuo. The residue was washed with ether and ethyl acetate to give a dark yellow solid. The solid was dissolved in a minimum amount of MeOH and precipitated by addition of EtOAc. The resulting solid was washed with 10% CH_3OH in CH_2Cl_2 to give pure phosphonic acid **13** (50 mg, 89%) as a white solid: ^{1}H NMR (D_2O) δ 7.82 (d, 1H, J = 7.8 Hz), 6.64 (ddd, 1H, J = 21.9, 16.5, 5.1 Hz), 6.19 (m, 2H), 5.83 (d, 1H, J = 3.0 Hz), 4.63 (m, 1H), 4.35 (m, 1H), 4.11 (m, 1H); ^{31}P NMR δ 12.8; HRFABMS calcd for $C_{10}H_{14}N_3O_7P$ $(M-H)^-$ 318.0485, found 318.0491.

1′-(2′,3′,5′-Tri-*O*-*tert*-butyldimethylsilyl-β-D-arabinofuranosyl)-cytosine (14). In a variation on previous procedures,[10] commercially available Ara-C (3 g, 12.3 mmol) was dissolved in THF (120 mL) followed by addition of pyridine (4.73 mL, 58.5 mmol). Solid $AgNO_3$ (6.95 g, 40.8 mmol) was added. After 5 min at rt, TBSCl (6.15 g, 40.80 mmol) was added and stirring was continued. After 2 h, Et_3N (4.73 mL, 46.8 mmol) and TBSCl (6.15 g, 40.8 mmol) were added. After an additional 26 h at rt, the mixture was filtered, the filtrate was added to 10% $NaHCO_3$, and the product was extracted into CH_2Cl_2. After concentration of the extracts in vacuo, further purification of the residue by column chromatography using a gradient solvent system (0 to 2% CH_3OH in CH_2Cl_2) gave the protected Ara-C derivative **14** (7.1 g, 98%).[9]

1′-(2′,3′-Di-*O*-*tert*-butyldimethylsilyl-β-D-arabinofuranosyl)-4-*N*-acetylcytosine (16). Compound **15**[9] (900 mg, 1.91 mmol) was suspended in MeOH (40 mL), the solution was heated at reflux, and Ac_2O (3.2 mL) was added dropwise to the hot solution. After 35 min, the solution was first concentrated in vacuo and then diluted with distilled water. After extraction with EtOAc, the organic extract was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give a colorless oil. This oil was purified by column chromatography using a gradient solvent system (0 to 2% CH_3OH in CH_2Cl_2) to give compound **16** (990 mg, 100%). ^{1}H NMR δ 9.75 (br s, 1H), 8.05 (d, 1H, J = 7.5 Hz), 7.44 (d, 1H, J = 7.5 Hz), 6.22 (d, 1H, J = 2.7 Hz), 4.28 (dd, 1H, J = 3.0, 0.6 Hz), 4.09 (m, 2H), 3.84 (d, 2H, J = 5.1 Hz), 2.75 (br s, 1H), 2.26 (s, 3H), 0.89 (s, 9H), 0.77 (s, 9H), 0.12 (s, 3H), 0.11 (s, 3H), 0.02 (s, 3H), −0.21 (s, 3H); ^{13}C NMR δ 170.8, 162.7, 154.9, 146.8, 95.7, 88.4, 88.1, 79.2, 76.1, 62.8, 25.8 (3C), 25.7 (3C), 25.0, 18.0, 17.8, −4.5, −4.6, −5.1, −5.3; HRFABMS calcd for $C_{23}H_{43}N_3O_6Si_2$ $(M+H)^+$ 514.2769, found 514.2778.

Diethyl [1′-(5′,6′-dideoxy-2′,3′-di-*O*-*tert*-butyldimethylsilyl-β-D-arabino-5′-hexenofuranosyl)-4-*N*-acetylcytosyl]-6′-phosphonate (17). Pyridine (1.0 mL, 12.0 mmol) was added to a flask charged with CrO_3 (600 mg, 6.0 mmol) in CH_2Cl_2 (15 mL). After 15 min at rt, alcohol **6** (770 mg, 1.5 mmol) in CH_2Cl_2 (15 mL) was added followed by addition of Ac_2O (0.6 mL, 6.0 mmol). After 7 min, the reaction was quenched by addition of ethanol (3 mL) and the solution was diluted by addition of ethyl acetate (200 mL). The resulting mixture was filtered through a silica column topped with a layer of anhydrous Na_2SO_4, and the filtrate was concentrated in vacuo.

Without further purification, the aldehyde in anhydrous CH_2Cl_2 (15 mL) was quickly treated with the previously prepared ylide **5** (927 mg, 2.25 mmol), and then stirred for 6 h at rt. The reaction was diluted by addition of distilled water (30 mL) and stirred for 1 h. The mixture was extracted with CH_2Cl_2, and the extracts were washed with distilled water and saturated brine. The organic layer was dried over anhydrous Na_2SO_4, concentrated in vacuo, and the residue was purified by column chromatography using a gradient solvent system (0 to 2.5% CH_3OH in CH_2Cl_2) to give vinyl phosphonate **17** (680 mg, 70%). ^{1}H NMR δ 9.69 (br s, 1H), 7.94 (d, 1H, J = 7.8 Hz), 7.42 (d, 1H, J = 7.8 Hz), 6.83 (ddd, 1H, J = 23.7, 17.1, 6.6 Hz), 6.27 (d, 1H, J = 2.7 Hz), 5.92 (ddd, 1H, J = 18.8, 17.1, 1.5 Hz), 4.47 (dm, 1H, J = 6.3 Hz), 4.26 (dd, 1H, J = 2.4, 0.9 Hz), 4.08 (m, 4H), 4.03 (br s, 1H), 2.26 (s, 3H), 1.34 (dt, 6H, J = 7.1, 2.7 Hz), 0.90 (s, 9H), 0.77 (s, 9H), 0.12 (s, 3H), 0.11 (s, 3H), −0.01 (s, 3H), −0.23 (s, 3H); ^{13}C NMR δ 170.3, 162.4,

148.4, 147.1, 119.5 (d, $J=188.2$ Hz), 95.5, 89.4, 88.4, 82.5, 76.3, 62.5, 62.4 (d, $J=6.8$ Hz), 62.3 (d, $J=6.9$ Hz), 26.1 (3C), 26.0 (3C), 25.5, 18.3, 18.1, 16.6 (d, $J=6.8$ Hz), 16.5 (d, $J=6.4$ Hz), −4.7 (2C), −5.0 (2C); ^{31}P δ 17.2; HRFABMS calcd for $C_{28}H_{52}N_3O_8Si_2P$ $(M+Na)^+$ 668.2923, found 668.2928.

Diethyl [1′-(5′,6′-dideoxy-β-D-arabino-5′-hexenofuranosyl)-4-*N*-acetylcytosyl]-6′-phosphonate (18). Vinyl phosphonate **17** (120 mg, 0.18 mmol) was dissolved in THF (2 mL) and Bu_4NF (0.39 mL of 1M solution, 0.39 mmol) was added at rt. After 30 min the reaction mixture was diluted by addition of H_2O (5 mL). The aqueous solution was extracted with CH_2Cl_2 several times, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Final purification by column chromatography using a gradient solvent system (3 to 10% CH_3OH in CH_2Cl_2) gave pure diol **18** (58.6 mg, 72%). ^{1}H NMR δ 10.06 (br s, 1H), 8.01 (d, 1H, $J=7.2$ Hz), 7.44 (d, 1H, $J=7.2$ Hz), 7.03 (ddd, 1H, $J=23.4$, 17.4, 6.6 Hz), 6.28 (d, 1H, $J=3.6$ Hz), 6.01 (ddd, 1H, $J=19.5$, 17.1, 1.2 Hz), 5.83 (br s, 1H), 5.73 (br s, 1H), 4.61 (br s, 1H), 4.54 (br s, 1H), 4.26 (br s, 1H), 4.13 (m, 4H), 2.20 (s, 3H), 1.36 (q, 6H, $J=6.9$ Hz); ^{31}P NMR δ 18.3.

[1′-(5′,6′-Dideoxy-β-D-arabino-5′-hexenofuranosyl)cytosyl]-6′-phosphonic acid (19). To a solution of phosphonate **18** (110 mg, 0.25 mmol) in CH_2Cl_2 (2.5 mL) was added TMSBr (1.0 mL, 7.6 mmol) at rt. After 18 h, the volatile materials were removed in vacuo, the residue was diluted by addition of CH_3OH, and the solution was concentrated in vacuo. The residue was washed with ether and ethyl acetate to give a dark yellow solid. The solid was dissolved in a minimum amount of MeOH and precipitated by addition of EtOAc. The precipitate was washed with 10% CH_3OH in CH_2Cl_2 to give pure phosphonic acid **19** (42 mg, 53%). ^{1}H NMR (D_2O) δ 7.92 (d, 1H, $J=7.8$ Hz), 6.62 (ddd, 1H, $J=21.2$, 17.1, 6.0 Hz), 6.19 (m, 3H), 4.50 (m, 1H), 4.45 (dd, 1H, $J=4.8$, 4.5 Hz), 4.13 (dd, 1H, $J=4.8$, 4.5 Hz); ^{31}P NMR (D_2O) δ 13.0; HRFABMS calcd for C_{10} $H_{14}N_3O_7P$ $(M-H)^-$ 318.0491, found 318.0491.

Diethyl [1′-(5′,6′-dihydroxy-2′,3′-*O*-isopropylidene-β-D-ribo-hexofuranosyl)uracil]-6′-phosphonate (20). To a stirred suspension of AD-mix-α (336 mg) in 50% aqueous *tert*-BuOH (2.4 mL) was added $K_2OsO_4{\cdot}2H_2O$ (72 mg, 0.8 mol%) at rt. The yellow mixture was stirred until two phases were observed, and then $CH_3SO_2NH_2$ (22.8 mg, 0.24 mmol) was added. The resulting mixture was cooled to 0 °C and vinyl phosphonate **6** (100 mg, 0.24 mmol) was added. After 2 h, the reaction mixture was allowed to warm to rt and then stirred for 2 days. Sodium sulfite (720 mg, 5.71 mmol) was added to quench the reaction and the reaction mixture was stirred for 1 h. The brown solution was extracted several times with ethyl acetate, the extract was washed with water, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. Further purification by column chromatography (3 to 10% CH_3OH in CH_2Cl_2) gave pure phosphonate **20** (52 mg, 50%). ^{1}H NMR δ 10.02 (br s, 1H), 7.74 (d, 1H, $J=8.1$ Hz), 5.75 (d, 1H, $J=7.8$ Hz), 5.57 (d, 1H, $J=1.8$ Hz), 5.16 (dd, 1H, $J=6.6$, 2.9 Hz), 5.11 (dd, 1H, $J=6.6$, 1.5 Hz), 4.55 (m, 1H), 4.21 (m, 6H), 3.99 (m, 1H), 3.75 (m, 1H), 1.58 (s, 3H), 1.35 (m, 9H); ^{13}C NMR δ 163.2, 150.8, 140.1, 114.2, 102.9, 96.0, 87.2 (d, $J=14.1$ Hz), 84.4, 81.8, 69.5 (d, $J=2.4$ Hz), 67.5 (d, $J=160.2$ Hz), 63.6 (d, $J=5.8$ Hz), 62.8 (d, $J=7.3$ Hz), 27.1, 25.2, 16.4 (d, $J=6.2$ Hz), 16.3 (d, $J=4.7$ Hz); ^{31}P NMR δ 23.0; HRFABMS calcd for $C_{17}H_{27}N_2O_{10}P$ $(M+Na)^+$ 473.1300, found 473.1301.

[1′-(5′,6′-Dihydroxy-β-D-ribo-hexofuranosyl)uracil]-6′-phosphonic acid (21). To a solution of the α,β-dihydroxy phosphonate **20** (100 mg, 0.22 mmol) in CH_2Cl_2 (5 mL) was added TMSBr (0.3 mL, 2.2 mmol) at rt. After 26 h, the volatiles were removed in vacuo, the residue was diluted with CH_3OH, and the solution was concentrated. The residue was washed with ether and ethyl acetate several times to give a light yellow oil, which solidified upon treatment with MeOH and EtOAc (1:1). The resulting solid was washed with 10% CH_3OH in CH_2Cl_2 to give the pure phosphonic acid **21** (70 mg, 91%) as a white solid. ^{1}H NMR (D_2O) δ 7.72 (d, 1H, $J=6.3$ Hz), 5.85 (m, 2H), 4.38 (m, 2H), 4.11 (m, 3H); ^{13}C NMR($CDCl_3$ and CH_3OH) δ 168.8, 154.5, 144.6, 105.3, 91.0, 86.6 (d, $J=13.0$ Hz), 75.6, 73.0, 72.8, 69.5 (d, $J=158.6$ Hz); ^{31}P NMR δ 23.6; HRFABMS calcd for $C_{10}H_{15}N_2O_{10}P$ $(M-H)^-$ 353.0386, found 353.0386.

Diethyl [1′-(5′,6′-Dihydroxy-2′,3′-*O*-isopropylidene-β-D-ribo-hexofuranosyl)-4-*N*-acetylcytosyl]-6′-phosphonate (22). To a stirred suspension of AD-mix-α (918 mg) in 50% aqueous *tert*-BuOH (6.6 mL) was added $K_2OSO_4{\cdot}2H_2O$ (1.98 mg, 0.8 mol%) at rt. The yellow solution was stirred until two phases were observed and then $CH_3SO_2NH_2$ (63.0 mg, 0.63 mmol) was added to the light yellow mixture. The mixture was cooled to 0 °C and vinyl phosphonate **12** (300 mg, 0.65 mmol) was added. After 2 h, the reaction mixture was allowed to warm to rt and then stirred for 2 days. Sodium sulfite (1.9 g, 15.1 mmol) was added to quench the reaction and the resulting mixture was stirred for 1 h. The dark brown solution was extracted several times with ethyl acetate, the combined extracts were washed with water and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give the impure product. Final purification by column chromatography (3 to 10% CH_3OH in CH_2Cl_2) gave pure phosphonate **22** (230 mg, 72%). ^{1}H NMR δ 10.16 (s, 1H), 7.78 (d, 1H, $J=7.8$ Hz), 7.43 (d, 1H, $J=7.5$ Hz), 5.59 (d, 1H, $J=1.2$ Hz), 5.33 (dd, $J=6.6$, 2.7 Hz, 1H), 5.21 (dd, $J=6.6$, 1.2 Hz, 1H), 5.10 (br d, 1H, $J=4.5$ Hz), 4.38 (m, 2H), 4.24 (m, 2H), 4.18 (m, 2H), 4.05 (m 2H), 2.26 (s, 3H), 1.57 (s, 3H), 1.34 (m, 9H); ^{13}C NMR δ 171.1, 163.2, 155.1, 147.3, 113.8, 97.2, 96.7, 88.3 (d, $J=14.2$ Hz), 84.6, 81.1, 69.4 (d, $J=2.3$ Hz), 69.2 (d, $J=160.2$ Hz), 63.3, 63.2 (d, $J=6.8$ Hz), 62.8 (d, $J=7.5$ Hz), 27.2, 25.4, 24.9, 16.5 (d, $J=5.9$ Hz), 16.4 (d, $J=5.0$ Hz); ^{31}P NMR δ 23.7; HRFABMS calcd for $C_{19}H_{30}N_3O_{10}P$ $(M+H)^+$ 492.1743, found 492.1747.

[1′-(5′,6′-Dihydroxy-β-D-ribo-5′-hexofuranosyl)cytosyl]-6′-phosphonic acid (23). To a solution of the phosphonate **22** (70 mg, 0.14 mmol) in CH_2Cl_2 (5 mL) was added TMSBr (0.5 mL, 3.8 mmol) at rt. After 23 h at rt,

the volatiles were removed in vacuo, the residue was diluted with CH_3OH, and the solution was concentrated to dryness. The residue was washed with ether and ethyl acetate to give a dark yellow solid. The solid was dissolved in a minimum amount of MeOH and precipitated by addition of EtOAc. The precipitate was washed with 10% CH_3OH in CH_2Cl_2 to give pure phosphonic acid **23** (45 mg, 89%). ^1H NMR (D_2O) δ 8.03 (d, 1H, $J=7.8$ Hz), 6.25 (d, $J=8.1$ Hz), 5.90 (d, 1H, $J=4.2$ Hz), 4.41 (m, 2H), 4.21 (m, 2H), 3.99 (br d, 1H, $J=10.5$ Hz); ^{13}C NMR (D_2O and CH_3OH) δ 161.8, 151.1, 147.2, 98.0, 92.0, 87.0 (d, $J=11.3$ Hz), 76.3, 72.8, 72.7, 71.0 (d, $J=156.8$ Hz); ^{31}P NMR δ 20.9; HRFABMS calcd for $C_{10}H_{16}N_3O_9P$ $(M-H)^-$ 352.0531, found 352.0545.

Diethyl [1′-(5′,6′-dihydroxy-2′,3′-di-*O*-*tert*-butyldimethylsilyl-β-D-arabino-hexofuranosyl)-4-*N*-acetylcytosyl]-6′-phosphonate (24). To a stirred suspension of AD-mix-α (1.84 g) in 50% aqueous *tert*-BuOH (13.2 mL) was added $K_2OsO_4{\cdot}2H_2O$ (3.94 mg, 0.8 mol%) at rt. The yellow mixture was stirred until two phases were observed and then $CH_3SO_2NH_2$ (129.6 mg, 1.36 mmol) was added. The mixture was cooled to 0 °C and vinyl phosphonate **17** (600 mg, 0.93 mmol) was added. After 2 h, the reaction mixture was allowed to warm to rt and then stirred for an additional 22 h. Solid Na_2SO_3 (3.84 g, 30.1 mmol) was added to quench the reaction and the resulting mixture was stirred for 1 h. The dark brown reaction mixture was extracted with ethyl acetate, the combined extracts were washed with water and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (3 to 10% CH_3OH in CH_2Cl_2) to give pure phosphonate **24** (400 mg, 63%). ^1H NMR δ 9.63 (br s, 1H), 7.76 (d, 1H, $J=7.5$ Hz), 7.39 (d, 1H, $J=7.5$ Hz), 6.25 (d, 1H, $J=3.0$ Hz), 5.55 (br s, 1H), 4.43 (br s, 1H), 4.17 (m, 9H), 2.23 (s, 3H), 1.37 (q, 6H, $J=7.2$ Hz), 0.91 (s, 9H), 0.78 (s, 9H), 0.12 (s, 6H), 0.02 (s, 3H), −0.24 (s, 3H); ^{13}C NMR δ 170.7, 162.2, 154.9, 146.5, 95.2, 88.7, 85.1 (d, $J=11.6$ Hz), 78.4, 76.0, 69.8, 67.5 (d, $J=160.3$ Hz), 63.7 (d, $J=7.4$ Hz), 62.7 (d, $J=7.2$ Hz), 25.8 (3C), 25.5 (3C), 24.8, 17.8, 17.7, 16.5 (d, $J=5.0$ Hz), 16.4 (d, $J=4.1$ Hz), −4.6, −4.7, −5.2, −5.5; ^{31}P NMR δ 24.8; HRFABMS calcd for $C_{28}H_{54}N_3O_{10}Si_2P$ $(M+H)^+$ 680.3157, found 680.3163.

Diethyl [1′-(5′,6′-dihydroxy-β-D-arabino-hexofuranosyl)-cytosyl]-6′-phosphonate (25). Phosphonate **24** (300 mg, 0.44 mmol) was dissolved in THF (4.5 mL) at rt and Bu_4NF (1.76 mL of 1M solution, 1.76 mmol) was added. After 2 h, the solvent was removed in vacuo and the residue was purified by column chromatography using a gradient solvent system (5 to 15% CH_3OH in CH_2Cl_2) to obtain pure phosphonate **25** (151 mg, 76%). ^1H NMR (DMSO) δ 10.86 (s, 1H), 7.82 (d, 1H, $J=7.5$ Hz), 7.17 (d, 1H, $J=7.5$ Hz), 6.07 (d, 1H, $J=0.3$ Hz), 5.57 (d, 1H, $J=4.8$ Hz), 5.48 (d, 1H, $J=3.6$ Hz), 5.43 (d, 1H, $J=7.5$ Hz), 5.06 (d, 1H, $J=8.1$ Hz), 4.22 (d, 1H, $J=3.3$ Hz), 4.07 (m, 7H), 3.84 (d, 1H, $J=9.9$ Hz), 2.09 (s, 3H), 1.23(dt, 6H, $J=7.2$, 1.8 Hz); ^{13}C NMR (DMSO) δ 171.3, 162.4, 154.7, 146.7, 94.5, 87.8, 84.5 (d, $J=12.3$ Hz), 76.8, 74.5, 69.0, 66.5 (d, $J=161.2$ Hz), 62.3 (d, $J=6.5$ Hz), 61.5 (d, $J=6.8$ Hz), 24.6, 16.7 (d, $J=5.3$ Hz), 16.5 (d, $J=5.5$ Hz); ^{31}P NMR (DMSO) δ 27.6; HRFABMS calcd for $C_{16}H_{26}N_3O_{10}P$ $(M+H)^+$ 452.1426, found 452.1434.

[1′-(5′,6′-Dihydroxy-β-D-arabino-hexofuranosyl)cytosyl]-6′-phosphonic acid (26). To a solution of phosphonate **25** (90 mg, 0.20 mmol) in CH_2Cl_2 (2.0 mL) was added TMSBr (1.0 mL, 7.6 mmol) at rt. After 4 days at rt, the volatile materials were removed in vacuo, the residue was diluted with CH_3OH, and the resulting solution was concentrated in vacuo. The residue was washed with ether and ethyl acetate to give a dark yellow solid. The solid was dissolved in a minimum amount of MeOH and precipitated by addition of EtOAc. The resulting solid was washed with 10% CH_3OH in CH_2Cl_2 to give pure phosphonic acid **26** (41 mg, 60%). ^1H NMR (D_2O) δ 7.96 (d, 1H, $J=7.8$ Hz), 6.15 (m, 2H), 4.34 (m, 2H), 4.14 (m, 1H), 4.02 (m, 1H); ^{13}C NMR (D_2O and MeOH) 161.7, 150.7, 148.3, 96.6, 89.2, 86.0 (d, $J=11.8$ Hz), 78.8, 77.7, 72.5, 69.9 (d, $J=156.5$ Hz) ^{31}P NMR (DMSO) δ 19.8; HRFABMS calcd for $C_{10}H_{16}N_3O_9P$ $(M-H)^-$ 352.0531, found 352.0545.

(*S*)-Methoxyphenylacetate esters 27 and 28. To a solution of α,β-dihydroxyphosphonate **22** (40 mg, 0.08 mmol) in CH_2Cl_2 were added EDC (23.4 mg, 1.12 mmol) and DMAP (5 mg, 0.04 mmol) at rt. (*S*)-(+)-*O*-Methylmandelic acid (16.1 mg, 0.09 mmol) was added to this mixture and the reaction was monitored by TLC. After 5 min at rt, water was added and reaction mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous $CaSO_4$, filtered, and concentrated in vacuo. Purification of the residue by column chromatography (0 to 8% CH_3OH in CH_2Cl_2) gave the ester **27** (21 mg, 0.03 mmol), along with the corresponding diester (13 mg, 0.02 mmol) and some fractions that were a mixture of both products (∼9 mg). For ester **27**: ^1H NMR (400 MHz): δ 9.58 (br s, 1H), 7.63 (d, 1H, $J=7.6$ Hz), 7.52 (m, 2H), 7.39 (d, 1H, $J=8.0$ Hz), 7.37 (m, 3H), 5.37 (dd, 1H, $J=11.2$, 2.4 Hz), 5.33 (s, 1H), 5.05 (m, 2H), 4.90 (s, 3H), 4.43 (m, 1H), 4.27 (br s, 1H), 4.15 (m, 4H), 3.74 (dd, 1H, $J=7.2$, 3.2 Hz), 3.45 (s, 3H), 2.28 (s, 3H), 1.47 (s, 3H), 1.30 (m, 9H); ^{31}P NMR δ 19.3. For the diester **28**: ^1H NMR (400 MHz): δ 9.02 (br s, 1H), 7.51 (br d, 1H, $J=7.6$ Hz), 7.36 (m, 10H), 5.81 (ddd, 1H, $J=8.0$, 3.2, 2.4 Hz), 5.41 (dd, 1H, $J=13.2$, 2.4 Hz), 5.17 (d, 1H, $J=2.0$ Hz), 4.88 (s, 1H), 4.87 (dd, 1H, $J=6.4$, 1.6 Hz), 4.81 (s, 1H), 4.74 (dd, 1H, 6.4, 4.0 Hz), 3.96 (m, 3H), 3.81 (m, 1H), 3.60 (dd, 1H, $J=8.0$, 4.0 Hz), 3.50 (s, 3H), 3.44 (s, 3H), 2.26 (s, 3H), 1.43 (s, 3H), 1.18 (s, 3H), 1.19 (t, 3H, $J=7.2$ Hz), 1.11 (t, 3H, $J=7.2$ Hz); ^{31}P NMR δ 16.0.

(*R*)-Methoxyphenylacetate ester 29. As described for compound **27**, the α,β-dihydroxyphosphonate **22** (110 mg, 0.22 mmol) was treated with EDC (64.4 mg, 0.33 mmol), DMAP (13.7 mg, 0.11 mmol), and (*R*)-(−)-*O*-methylmandelic acid (44.4 mg, 0.26 mmol). Parallel work up and purification by column chromatography (0 to 8% CH_3OH in CH_2Cl_2) gave the ester **29** (117 mg, 82%). ^1H NMR (400 MHz): δ 9.53 (s, 1H), 7.90 (d, 1H, $J=7.6$ Hz), 7.40 (m, 5H), 7.36 (d, 1H, $J=7.6$ Hz), 5.69

(d, 1H, $J=1.6$ Hz), 5.48 (dd, 1H, $J=12.4$, 2.0 Hz), 5.30 (dd, 1H, $J=6.4$, 4.0 Hz), 5.03 (dd, 1H, $J=6.4$, 1.6 Hz), 4.90 (s, 3H), 4.63 (d, 1H, $J=2.4$ Hz), 4.52 (m, 1H), 4.21 (dd, 1H, $J=4.0$, 4.0 Hz), 3.91 (m, 2H), 3.79 (m, 1H), 3.49 (m, 1H), 3.45 (s, 3H), 2.29 (s, 3H), 1.56 (s, 3H), 1.38 (s, 3H), 1.15 (t, 3H, $J=7.2$ Hz), 1.03 (t, 3H, $J=7.2$ Hz); ^{31}P NMR δ 17.7.

Assays with NMP kinase

The conversion of phosphonates to monophosphate-phosphonates was measured utilizing a variation of a previously published method.[20] The test phosphonate was added to a mixture of NMP kinase (0.015 units, Sigma), pyruvate kinase (35 units, Sigma) and lactic dehydrogenase (50 units, Sigma) in 1 mL of 0.07 M triethanolamine buffer (pH = 7.6), containing KCl (65 mM), $MgSO_4$ (16 mM), ATP (1.7 mM), phosphoenolpyruvate (PEP, 1.2 mM), and NADH (0.36 μM). Control reactions contained UMP (0.87 mM) rather than the synthetic phosphonates as substrate. After mixture equilibration, reactions were started by addition of either the test phosphonates or UMP, and the decrease in absorbance at 340 nm was followed in a diode array spectrophotometer. The oxidation of NADH, and the decline in absorbance at 340 nm, reflected the rate of phosphorylation of either the synthetic phosphonate or of UMP.

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