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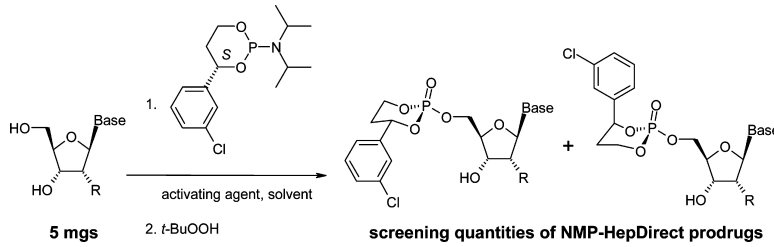

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High-Throughput Synthesis of HepDirect Prodrugs of Nucleoside Monophosphates

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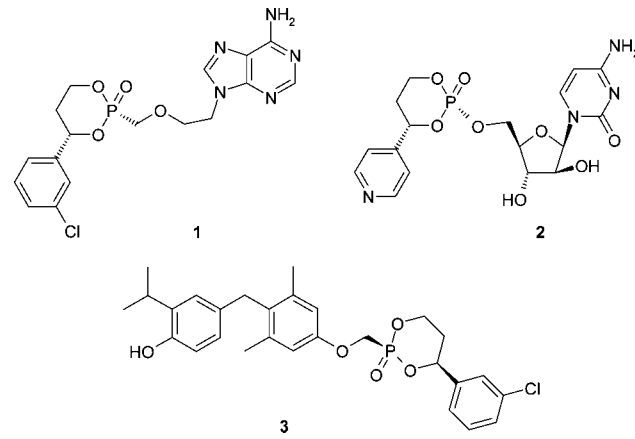
A high-throughput phosphoramidite method for HepDirect prodrug synthesis was optimized on seven representative nucleosides, adenosine, inosine, guanosine, uridine, cytidine, AICA-riboside, and thymidine, each on a 5 mg scale. The variables optimized included (1) reaction time, (2) reaction temperature, (3) activating agent, (4) solvent, (5) purification method, and (6) stoichiometry. Preparative HPLC with mass-based fraction collection and yield determination from an ELSD standard curve enabled high-throughput. The optimized conditions for the representative nucleosides required 6 mol equiv of phosphoramidite to nucleoside and resulted in an average HPLC determined yield of 31 ± 14% and HPLC purity of 93 ± 3%.

Introduction

Nucleoside research began over 100 years ago with the structural investigations of Emil Fischer.[1,2] The importance of these molecules to the viability of the cell was quickly recognized, and pharmaceutical applications made rapid progress, at first with nucleoside bases and then with nucleosides themselves beginning in the 1940s. In the past 40 years, research has been especially intense in seeking to identify therapeutic agents for the treatment of a variety of diseases, including viral infections, cancer, and cardiovascular and CNS diseases. However, few drugs have resulted from those efforts,[3] which were focused in areas other than viral[4] diseases and leukemias.[5] When used as an antiviral or an oncolytic agent, the nucleoside is often functioning as a prodrug of the active agent, its 5′-*O*-triphosphorylated derivative or nucleoside triphosphate (NTP). A sequence of kinases convert the nucleoside into its NTP, which then disrupts gene replication intracellularly to control progression of the disease. Because many nucleosides are poor substrates for the first kinase in the sequence, their efficacy potential goes unnoticed during the discovery stage.

Several nucleoside monophosphate (NMP) esters and amides have been developed as prodrugs to bypass this limitation.[6] These prodrugs are most often activated by esterase enzymes. Because esterases are broadly expressed throughout the gut, small intestine, plasma, muscle, and other tissues, the NMP formation is not localized. The resulting ionic phosphate has limited or no membrane penetration properties, and thus its distribution is limited to organs and tissues attained by the prodrug prior to metabolism. The lack of tissue selectivity can result in toxicity in off-target organs and thus limits the drug's potential.

The HepDirect phosphate/phosphonate prodrug[7] is not esterase activated but rather is activated by the cytochrome P_{450} enzyme CYP3A4, and this occurs predominantly in the liver. Thus, the HepDirect prodrug offers the advantage of tissue specific unmasking of the NMP in hepatocytes.[7] Three HepDirect prodrugs, pradefovir (**1**)[8] for hepatitis B, MB07133 (**2**)[9] for hepatocellular carcinoma, and MB07811 (**3**)[10] for hyperlipidemia are currently being evaluated in human clinical trials and have been found to be advantageous for delivering the active drug species specifically to the liver.



A general method for high-throughput synthesis of HepDirect prodrugs was desirable to biologically evaluate a wide collection of nucleosides[11] as their monophosphate prodrugs. However, available quantities of custom nucleosides in commercial and private collections are often limited. Consequently, a practical high-throughput synthesis of NMP HepDirect prodrugs would require no more than 5 mg of nucleosides. This scale has proven to be sufficient for generation of screening samples because only a small quantity (0.5−2 mg) of HepDirect prodrug is required for many biological assays.

Chemistry

There are two primary methods for HepDirect prodrug synthesis[7] as illustrated by Schemes 1 and 2.[12] The phosphoramidite method in Scheme 1 produces a mixture (most

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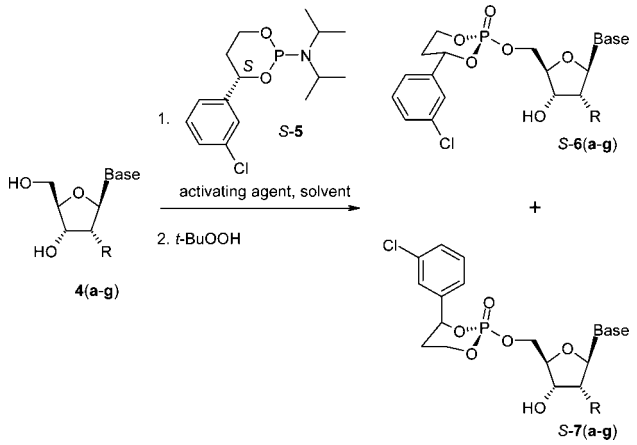
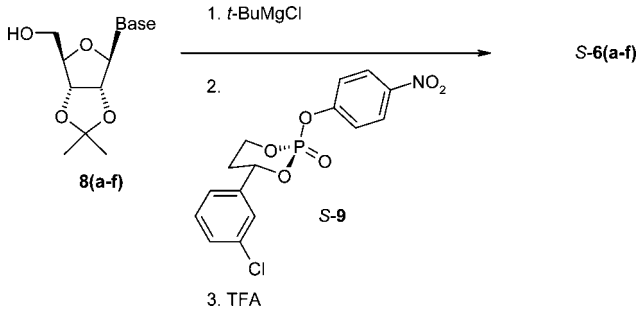
Scheme 1. Phosphoramidite Method



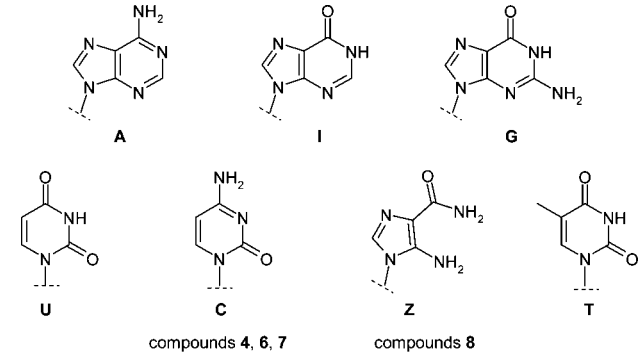
Scheme 2. *p*-Nitrophenylphosphate Method



often inseparable) of *cis*- and *trans*-phosphate cyclic diesters **6** and **7**, respectively; which is a disadvantage because only the cis form **6** is efficiently activated by CYP3A4 to form NMP.[7a] However, this reaction can be performed successfully on unprotected nucleosides making it the method of choice for production of screening quantities of HepDirect prodrugs from 5 mg quantities of nucleosides. Higher yields of cis HepDirect prodrugs **6** can be prepared from 2′,3′-bis-*O*-protected nucleosides using the stereospecific method of Scheme 2. This is the preferred method when nucleoside quantities are sufficient to allow for protection of competing reactive functionalities.

The phosphoramidite reaction was optimized on a 5 mg scale with seven representative nucleosides (see Chart 1): adenosine (**4a**), inosine (**4b**), guanosine (**4c**), uridine (**4d**), cytidine (**4e**), AICA riboside (**4f**), and thymidine (**4g**). To monitor product distribution, individual reference samples of *R*/*S*-**6**(**a**−**g**) and *R*/*S*-**7**(**a**−**g**) were prepared as **6**/**7** (cis/trans) mixtures.[12] The cis and trans isomers **6**(**a**−**g**) and **7**(**a**−**g**), respectively, were chromatographically inseparable using either normal or reverse-phase silica gel column chromatography and so, as a group, will be represented as **6**/**7**(**a**−**g**). To confirm that the Scheme 1 phosphorylation as applied to unprotected nucleosides occurs at the 5′-*O*-position, these standards (*R*/*S*-**6**/**7**(**a**−**f**)) were made by application of this reaction to 2′,3′-*O*-isopropylidenyl protected nucleosides (**8**(**a**−**f**)), except for the case of **6**/**7**(**g**). This was followed by a standard TFA deprotection to provide the *R*/*S*-**6**/**7**(**a**−**f**) reference compound mixtures (see Supporting Information). The reference compounds *R*/*S*-**6g**/**7g** were prepared from unprotected nucleoside **4g** directly using the method of Scheme 1 (Supporting Information). In

Chart 1. Intermediates and Products Used in Schemes *1* and *2* for Standard Synthesis and Reaction Study



compounds **4**, **6**, **7**

a, Base = **A**, R = OH
b, Base = **I**, R = OH
c, Base = **G**, R = OH
d, Base = **U**, R = OH
e, Base = **C**, R = OH
f, Base = **Z**, R = OH
g, Base = **T**, R = H

compounds **8**

a, Base = **A**
b, Base = **I**
c, Base = **G**
d, Base = **U**
e, Base = **C**
f, Base = **Z**

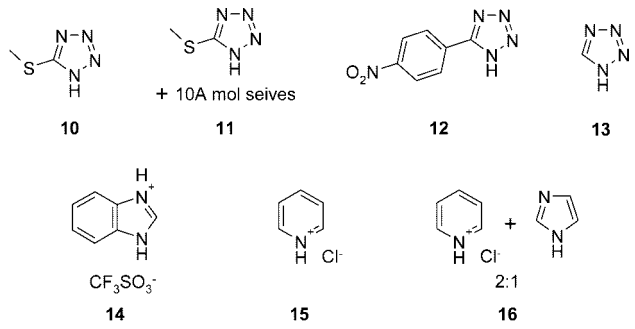
addition, samples of *R*/*S*-**6**(**a**−**f**) (two diastereomers each), were prepared using the method of Scheme 2 on 2′,3′-*O*-isopropylidene protected nucleosides (**8**(**a**−**f**)). Finally, the preparation of *S*-**6**/**7**(**a**−**f**) during the reaction optimization stages permitted the exact assignment of HPLC retention times to each of the four diastereomers of each *R*/*S*-**6**/**7**(**a**−**f**) standard, which permitted the detailed tracking of each isomeric product. Note that *R*/*S*-**6g** was not discreetly prepared, which prevented exact assignment of each diastereomer of *R*/*S*-**6**/**7**(**g**).

As a group, the standards served to represent the challenges of performing the reaction on a broad set of nucleosides because each contain secondary hydroxyls and NH or NH_2 groups to potentially complicate the product pool as a result of side reactions. The main side reaction is a second phosphorylation that likely occurs on the 2′- or 3′-hydroxy group after the 5′-hydroxy has been phosphorylated. Although not isolated, this side-product of double phosphorylation was evidenced by mass spectral characterization of the crude products that always contained a mass signal for a doubly phosphorylated product.

Optimization of the phosphoramidite method (Scheme 1) on 5 mg quantities of nucleosides was challenging because at a 0.02 mmol scale, only 0.36 mg of water would be necessary to quench 1 equiv of phosphoramidite **5**. Thus reactions were conducted in predried 1.5 mL HPLC vials in the presence of bead-form 4 Å molecular sieves. The method was optimized with respect to (1) reaction time, (2) reaction temperature, (3) activating agent, (4) solvent, (5) purification method, and (6) stoichiometry. For parameters 1, 2, 4, and 5, the reaction conditions of Scheme 1 used 2 equiv each of *R*/*S*-**5** and 5-methylthiotetrazole (**10**) as the activating agent. The reactions were performed in DMF, except during the solvent optimization. The final optimization of the stoichiometry was performed with *S*-**5**.

To study the reaction time, individual reactions with **4**(**a**−**f**) were conducted at 23 °C and quenched with *t*-butylhydroperoxide at time points of 30, 60, 180, and 240 min. Analysis of crude product mixtures by HPLC indicated that formation of products **6**/**7**(**a**−**f**) was maximized after 30−60 min. The optimal and most convenient temperature

Chart 2. Activating Agents



was determined to be 23 °C because reactions conducted on **4b** had not yet achieved maximal yield after being conducted at 5 °C and −20 °C for 7 h. When the reaction was conducted on **4**(**a**−**f**) for 24 h at 5 °C, the yields and product distribution did not differ from those observed when conducted at 23 °C for 1−2 h. Studies at higher temperatures were not investigated because the reaction rapidly plateaued within 1 h at 23 °C (see Supporting Information for a graph of time vs reactants and products).

The activating agents **10**,[13] **11**,[14] **12**,[13] **13**,[13] **14**,[15] **15**,[16] and **16**[16] (Chart 2) were studied in reactions conducted at 23 °C with **4b** and resulted in HPLC determined yields of **6b/7b** (combined within the crude product mixture) of 52%, 14%, 57%, 28%, 56%, 28%, and 11%, respectively. Although the use of reagents **10**, **12**, and **14** gave essentially equivalent yields (HPLC), reagent **10** was chosen for the general reaction conditions because of its ease of separation from products during purification.

When studied with the nucleosides **4**(**a**−**f**), the optimal reaction solvents were determined to be either DMF or DMSO. The reaction failed to proceed in 1-methyl-2-pyrrolidinone (NMP) and produced low yields of **6** and **7** when conducted in *N,N′*-dimethylpropyleneurea (DMPU) or 1,1,3,3-tetramethylurea (TMU). Less polar solvents were unsuitable because many polar nucleosides are only soluble in solvents such as DMF or DMSO. For the general reaction, DMF was chosen over DMSO as the solvent because of its favorable physical characteristics (lower mp and bp). For nucleosides that were not readily soluble in DMF such as **4c**, DMSO was used as a cosolvent.

For purification, preparative reverse-phase HPLC with mass-based fraction collection[17] proved to be the most efficient method because of its automation capabilities and ease of operation. With this method, crude filtered reaction mixtures could be injected directly for HPLC purification. Normal-phase silica gel cartridge-based purification was also effective but was less efficient because several sample preparation steps were needed prior to chromatography.

Optimization of the stoichiometric ratio of phosphoramidite *S*-**5** relative to nucleosides **4**(**a**−**g**) was performed with **10** as the activating agent and DMF as the solvent (DMSO as a cosolvent for **4c**). The results are described in Figure 1. The use of 2 equiv of *S*-**5** generally resulted in a <20% yield of phosphorylated products *S*-**6/7**(**a**−**g**). The production of *S*-**6/7** trended toward the highest yields when 6 equiv of *S*-**5** were used. This optimized ratio, 1:6:6 of **4**/*S*-**5**/**10**, was performed in quadruplicate, and the products were isolated

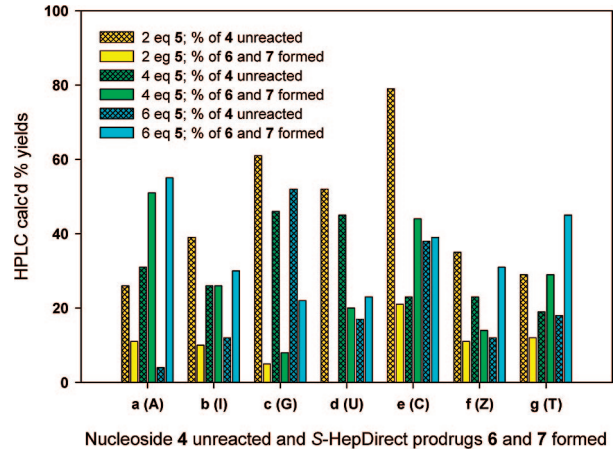


Figure 1. Phosphoramidite reaction trials with 2, 4, and 6 equiv of *S*-**5**.

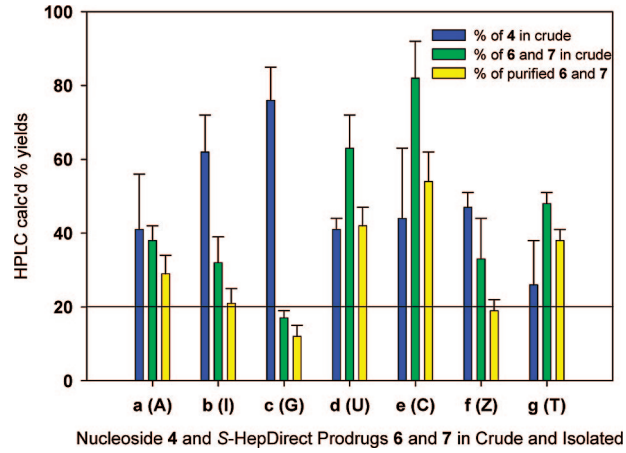


Figure 2. Yield comparison of products *S*-**6/7**(**a**−**g**) from reactions of **4**(**a**−**g**) with 6 equiv of *S*-**5**.

by preparative HPLC with mass-based fraction collection. The results with error bars to represent standard deviations are represented in Figure 2. Overall for the group **4**(**a**−**g**), the HPLC-determined average yield of the products *S*-**6/7**(**a**−**g**) was 31 ± 14% and the average HPLC purity was 93 ± 3%. The ratio of products *S*-**6**(**a**−**f**) to *S*-**7**(**a**−**f**) (cis to trans ratio) in the crude and purified products was not significantly different and slightly favored the products *S*-**6**(**a**−**f**). For purified products, the overall average ratio was 1.18 ± 0.13 (see Supporting Information for a graphical representation).

Application of these optimized conditions for HepDirect prodrug synthesis across a broad collection of nucleosides, each on a 5 mg scale, required an analytical method to quantitate the products. Evaporative light scattering detection (ELSD) is relatively universal for nonvolatile compounds because the signal is dependent only on the weight of the sample.[18] Thus, it was chosen to quantitate novel HepDirect prodrugs. For this study, the ELSD standard curve of log (mg/mL) vs log (ELSD peak area) was prepared from data generated from analysis of the nucleosides **4**(**a**−**f**) (see Supporting Information). In Figure 3, the yields determined for the purified reaction products *S*-**6/7**(**a**−**g**) from HPLC standard curves are compared with those determined from the ELSD standard curve. The comparison indicates that ELSD based quantification may provide a result slightly

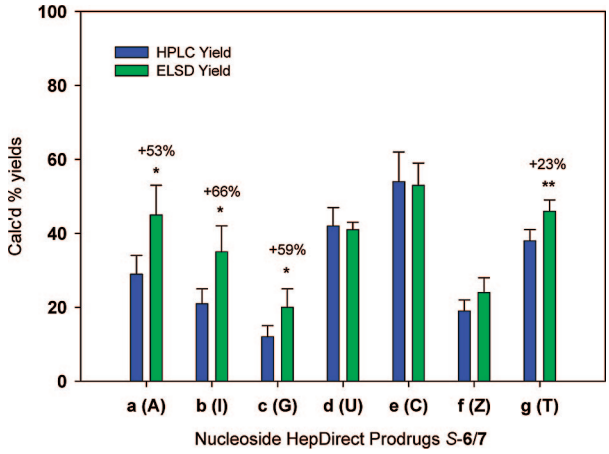


Figure 3. Yields of products *S*-**6/7**(**a**−**g**) as determined from HPLC standard curves compared to those determined by the ELSD universal curve. The asterisk (*) represents values that are significantly different from the HPLC determination according to the student's unpaired *t* test with $p = 0.05$. The double asterisk (**) indicates values that are significantly different with $p = 0.01$.

higher (23−66% higher in four of seven examples), but for the purposes of screening sample preparation, it was found to be adequate.[19]

Conclusions

A high-throughput synthesis of HepDirect prodrugs has been developed and optimized on a set of representative nucleosides **4**(**a**−**g**). A 1:6:6 ratio of nucleoside, *S*-**5** and **10** in DMF for 1 h at 23 °C, coupled with isolation by automated preparative HPLC with a mass-based fraction collection strategy, was found to be the best set of conditions for this group. Moreover, it is important to note that when this stoichiometry was applied to an expanded set of nucleosides, it resulted in a larger percentage of those nucleosides undergoing double phosphorylation as evidenced by mass spectroscopic analysis of crude mixtures. This resulted in only slightly more than half of those examples providing isolable monophosphorylated products (of the type *S*-**6/7**).[19] However, the change of the stoichiometric ratio to 1:2:2 of nucleoside, *S*-**5**, and **10** resulted in a substantial decrease in double phosphorylation and hence more isolable desired products *S*-**6/7,** although in decreased overall average yield.[19] ELSD proved to be useful for quantitation of the small amounts obtained. Overall the method was sufficient for high-throughput synthesis of small quantities of pure HepDirect prodrugs for screening from only 5 mg quantities of nucleosides.

Experimental Section

General Methods. Reactions were performed in oven-dried glassware. Reagent additions were performed with either Eppendorf pipettes or oven-dried glass syringes. Anhydrous solvents were purchased from commercial vendors and stored over 4 Å molecular sieves. Nucleosides **4**(**a**−**g**) were obtained from commercial vendors. Methods for the preparation of *S*-**5**, the reference standards *R*/*S*-**6**/**7**(**a**−**g**), and the reference standards *R*/*S*-**6** (**a**−**f**) are included in the Supporting Information. For the reactions, room temperature (rt) was 23 °C. ^{1}H NMR were obtained in DMSO-d_6 at 300 MHz and spectra were recorded in units δ with $CD_2HS(O)CD_3$ (δ 2.504) as the reference line internal standard. HPLC, LCMS, and ELSD conditions are described below.

General Analytical Method for HPLC Standard Curve Determinations and Compound Purity Assessment. The column consisted of three serially connected Chromolith SpeedRODs RP-18e, 100 × 4.6 mm with solvent A = HPLC grade acetonitrile and solvent B = 20 mM ammonium phosphate buffer (pH 6.1, 0.018 M $NH_4H_2PO_4$/0.002 M $(NH_4)_2HPO_4$) with 5% acetonitrile. The UV detector was set to 255 nm, and the flow rate was 4 mL/min. The gradient program was as follows: min (% B), 0 (100), 10 (60), 10.1 (100), 12 (100). For standard curves of **4**(**a**−**g**) and their HepDirect prodrug mixtures of **6/7**(**a**−**g**), 20 μL each of solutions of 0.1, 0.5, 1 and 2 mg/mL concentration were analyzed (see Supporting Information for standard curve details). The same volume was injected for crude and purified products. Corrections were made for volume differences and the standard curves were used to calculate the concentration of the analyte in the solution. The determined analyte concentrations were used to calculate the crude and purified product yields. For novel nucleoside *S*-**6/7** prodrugs, the 2 major peaks, if resolved, were summed to determine the HPLC purity.

General Analytical Method for ELSD Standard Curve Determination and Compound Quantity Determinations. Samples were analyzed as part of an LCMS system containing an Applied Biosystems API150EX MS operating in a positive ion mode receiving 0.1 mL/min flowrate and an Alltech ELSD 2000 connected inline with a Shimadzu UV detector receiving 0.1 mL/min flow rate. The ELSD column temperature was 80 °C, and the N_2 gas flow rate was 2 L/min. The LC conditions were solvent A = 0.03% TFA in acetonitrile; solvent B = 0.05% TFA in water; flowrate = 10 mL/min; the column was a Chromolith Performance RP-18e, 100 × 4.6 mm; gradient program: min (%B) 0 (95), 1.6 (5), 2.6 (5), 2.7 (95), 3.0 (95). For the ELSD standard curve, 50 μL each of solutions of 0.1, 0.5, 1, 3 and 10 mg/mL concentrations of **4**(**a**−**f**) were analyzed (see Supporting Information for standard curve details). The same volume was injected for purified products. Corrections were made for volume differences and the ELSD standard curve was used to calculate the concentration of the analyte in solution.

General Method for LCMS Controlled Preparative HPLC with Mass-Based Fraction Collection. The crude reaction solutions in DMF were filtered through a 0.45 μm PTFE filter into a Sun brand 1.5 mL point-bottomed HPLC vial. The filter was rinsed with 250 μL DMF into the vial. The LCMS system was an API150EX MS operating in the positive ion mode with dual Shimadzu pumps and a Gilson 215 liquid handler. The PC software controlling the fraction collection was Applied Biosystems Prep Express for 1.3 module of Analyst 1.3.1. The column was a YMC ODS-AQ (120 A) S-15/30 (15−30 μm), 10 × 10 mm guard cartridge, followed by a YMC Guardpack ODS-AQ (120A)

S-10P (5−15 μm), 30 × 70 mm. The LC conditions were solvent A = 0.03% TFA in acetonitrile, solvent B = 0.05% TFA in water; flowrate = 15 mL/min; gradient program: min (%B) 0 (80), 0.7 (80), 5.7 (60), 10.7 (40), 12.7 (5), 14.7 (5), 15 (80), 16 (80). Fractions, each 7 mL in volume, were collected based on a mass signal exceeding 100 000 cps, when a range of [M] to [M + 2] was monitored for the desired product. Fractions were individually analyzed by the general analytical HPLC method for product purity, and fractions that were >80% pure were combined and evaporated. The residues were dissolved in 0.5−2 mL DMF and evaluated for target mass [M + 1] by LCMS, HPLC purity, and quantified by an HPLC standard curve (for **6/7**(**a**−**g**)) or the ELSD standard curve for novel nucleosides.

General Method for Synthesis of cis/trans *S*-HepDirect Prodrugs of 4(a−g) on a 5 mg Scale (*S*-6/7(a−g)). In 1.5 mL HPLC vials, each containing 100 mg of bead-form 4 Å molecular sieves, 5 mg of nucleoside **4**(**a**−**g**) (ca. 0.02 mmol) was dissolved in 0.4 mL of DMF. (Note: if the nucleoside was not soluble even after mild heating, then 0.4 mL of DMSO was added to dissolve the nucleoside. This was necessary for **4c**). To this mixture, 0.12 mL of a 1 M solution of **10** (0.12 mmol) in DMF was added via pipette, and the mixture was shaken for 2 h at rt. A freshly prepared 0.5 M solution of the *S*-phosphoramidite (*S*-**5**) in DMF (predried over 4 Å molecular sieves for 2 h) was prepared, and 0.240 mL of this 0.5 M solution of *S*-**5** (0.12 mmol) was added to each vial via a glass syringe through a septum cap. The mixture was shaken at rt for 2 h and then 0.050 mL of 70% aqueous *t*-BuO_2H (0.36 mmol) was added to each vial via pipette. After the mixture was shaken for an additional 30 min at rt, 0.050 mL was removed from each and diluted with 0.2 mL of DMSO for HPLC analysis of the crude product ratio (see General Method of HPLC Analysis section). The remainder of each solution was subjected to the general method of preparative HPLC with mass-based fraction collection as described above. The isolated fractions of *S*-**6/7**(**a**−**g**) were evaluated by HPLC, and yield determinations were made based on the HPLC standard curve data (Table S2). See Supporting Information Figures S6−S12 for ^{1}H NMR of example isolations as compared to the cis/trans *R/S*-HepDirect prodrug standards (*R/S*-**6/7**(**a**−**g**)).

General Method for Synthesis of *S*-HepDirect Prodrugs of Novel Nucleosides on a 5 mg Scale. This method is essentially identical to that as described for the 5 mg scale synthesis of the *S*-HepDirect prodrugs (*S*-**6/7**(**a**−**g**)), but either 6 or 2 equiv each of *S*-**5** and **10** were used per nucleoside. Volumes of reagents were adjusted to match the required stoichiometry for each respective quantity of nucleoside. Final products were characterized by analytical HPLC for purity, MS for product identity, and ELSD for product quantity.

Acknowledgment. This work was supported in part by NIH SBIR Grant AI050278.

Supporting Information Available. Methods for the preparation of *S*-**5**, the reference standards *R*/*S*-**6/7**(**a**−**g**), and the reference standards *R*/*S*-**6**(**a**−**f**), analytical data for HepDirect prodrugs of adenosine, inosine, guanosine, uridine, cytidine, AICA riboside, and thymidine (**6/7**(**a**−**g**)), HPLC standard curve data for **4**(**a**−**g**) and *R/S*-**6/7**(**a**−**g**), a graph of time versus % HPLC determined nucleoside remaining, **4**(**a**−**f**), and product formed, *R/S*-**6/7**(**a**−**f**)), a bar graph of the ratio of products *S*-**6**(**a**−**f**) to *S*-**7**(**a**−**f**), an ELSD standard curve based on analysis of **4**(**a**−**f**), and ^{1}H NMR spectra and HPLC chromatograms for the *R/S*- and *S*-phosphoramidite (**5**) and (**6/7**(**a**−**g**)). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) These schemes represent compounds **5**, **6**, **7**, and **9** with an S-configuration at the chlorophenylmethine stereocenter for clarity. The reference standards for HPLC standard curves

were *R*/*S*-**6/7**(**a**−**g**) (four diastereomers each) because they were prepared with racemic mixtures of reagent **5**. As indicated in the text, the final reaction optimization was performed with *S*-**5** giving products with only an *S*-configuration at this position.

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(19) This procedure was applied to 363 nucleosides that were composed of a great variety of heteroaromatic bases linked to ribofuranosyl sugars with various 2′- and 3′-substituents. In practice, the stoichiometry of 1:6:6 nucleoside/*S*-**5/10** often resulted in excessive production of doubly phosphorylated products instead of the desired mono-phosphorylated derivatives *S*-**6/7** (148 examples with only 77, 52%, providing the desired product, each of which was analyzed by LCMS, HPLC and ELSD). Decreasing the stoichiometry to 1:2:2 nucleoside/*S*-**5/10** resulted in a substantially better success rate (215 examples with 171, 80%, providing the desired product) albeit in lower overall average yield (11 ± 9%) compared to the reference products **6/7**(**a**−**g**) but with acceptable average HPLC purity of 90 ± 7%. The stoichiometry 1:2:2 nucleoside/*S*-**5/10** was adopted for general use with nucleoside libraries.

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