Scalable Synthesis, In Vitro cccDNA Reduction, and In Vivo Antihepatitis B Virus Activity of a Phosphonmethoxydeoxythreosyl Adenine Prodrug

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Cite This: <https://dx.doi.org/10.1021/acs.jmedchem.0c01381>

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ABSTRACT: Standard literature procedures for the chemical synthesis of L-threose nucleosides generally employ L-ascorbic acid as starting material. Herein, we have explored two alternative routes that start from either L-arabitol or L-diethyl tartrate, both affording 2-O-methyl-L-threofuranose as a key building block for nucleobase incorporation. The access to multigram quantities of this glycosyl donor in a reproducible fashion allows for the preparation of 2'-deoxy- α -L-threofuranosyl phosphonate nucleosides on a large scale. This methodology was applied to the gram scale synthesis of an aryloxy amidate prodrug of phosphonmethoxydeoxythreosyl adenine. This prodrug exerted potent activity against an entecavir-resistant hepatitis B virus (HBV) strain, while leading to a significant reduction in the levels of HBV covalently closed circular DNA in a cellular assay. Furthermore, its remarkable anti-HBV efficacy was also confirmed in vivo using a hydrodynamic injection-based HBV mouse model, without relevant toxicity and systemic exposure occurring.

INTRODUCTION

Chronic infection due to hepatitis B virus (HBV) is a global health problem affecting approximately 257 million people, which is likely to continue increasing in the coming decades, particularly in developing countries.^{1,2} This contagious liver disease often correlates with a high risk of developing life-threatening complications such as cirrhosis and hepatocellular carcinoma, sometimes leaving liver transplantation as the only treatment option for infected individuals.³ Presently approved treatments for HBV infection include interferon-based immunotherapy as well as the use of antiviral nucleoside analogues [lamivudine, entecavir (ETV), and telbivudine] and acyclic nucleoside phosphonate prodrugs [adefovir dipivoxil, tenofovir disoproxil, and tenofovir alafenamide (TAF, Figure 1)].⁴ However, none of the available drugs allow for HBV eradication, mainly because of the unique replication mechanism of this virus.⁵ Upon hepatocyte infection, the partially double-stranded viral DNA genome matures into a covalently closed circular DNA (cccDNA) located in the nucleus, which serves as a template for the reverse transcription of an RNA intermediate occurring in the cytoplasm. Therefore, in the continuing search for novel antiviral drug candidates that could inhibit HBV with improved efficacy and safety, it is important to identify compounds able not only to inhibit the viral reverse transcriptase but also affect the pool of

Figure 1. Structure of TAF and phosphonmethoxydeoxythreosyl adenine prodrug **1** investigated in this study.

nuclear cccDNA molecules, which are responsible for HBV persistence and reactivation.^{6,7}

A particularly interesting class of nucleoside analogues are built on a 2-deoxythreose sugar ring,⁸ especially those

Received: August 6, 2020

derivatized at the 3'-OH position with a phosphonomethoxy functionality. Among these analogues, phosphonomethoxy-deoxythreosyl adenine (PMDTA) and phosphonomethoxy-deoxythreosyl thymine emerged as potent inhibitors of the replication of human immunodeficiency virus (HIV) and HBV without concomitant cytotoxicity.^{9,10} A number of aryloxy phosphonoamidate and phosphonobisamidate PMDT prodrugs were also synthesized, leading to a significant enhancement in antiviral activity against HIV and HBV. Specifically, compound **1** (Figure 1), which is the L-aspartic acid diisoamylester prodrug of PMDTA, was identified as a promising anti-HBV candidate with EC₅₀ and EC₉₀ values of 0.01 and 0.98 μ M,¹⁰ respectively, thus warranting further in-depth biological investigation both in vitro and in vivo. These preclinical efficacy and toxicity studies require large amounts of compound **1**. Preferentially, such a compound should be obtained in a few high yielding steps, without the need of cumbersome chromatographic purification procedures. Therefore, we decided to compare different synthetic routes that could be amenable to the large-scale synthesis of 2'-deoxythreose nucleosides and especially expand access to the desired prodrug **1**. In particular, we elaborated two distinct synthetic schemes starting from inexpensive and commercially available L-arabitol and L-diethyl tartrate toward a versatile L-threonolactone synthon as alternatives to the previously and routinely used L-ascorbic acid approach.¹⁰ Given the above-mentioned broad array of applications of threose nucleoside derivatives as both antiviral drugs and constituents of oligonucleotides, such synthetic optimization constitutes a generally useful endeavor. With compound **1** in hand, a follow-up study of its anti-HBV profile was then undertaken, the details of which are reported below.

RESULTS AND DISCUSSION

Chemical Synthesis Studies. The first synthesis of an L-threose nucleoside was described in 1967 by Murry and Prokop beginning with L-arabitol.¹¹ However, the most popular starting compound for the preparation of the L-threonolactone (and its lactol) sugar that serves as a key precursor to L-threose nucleoside analogues is L-ascorbic acid.^{12–15} This pathway was first optimized for large scale production by Eschenmoser and coworkers,^{16,17} and later by the Chaput group¹⁸ (Scheme 1). Owing to our interest in the phosphonate analogues of threose nucleosides, we previously reported the regioselective protection of the 2-OH group of **3** to furnish 2-O-benzoylated lactone **5** as a precursor of glycosyl acetate **6**, which was used for the synthesis of PMDT analogues including compound **1**.¹⁹

Scheme 1. Reported Synthesis of Glycosyl Donors **4 and **6** from L-ascorbic Acid **2****

A variation of this regioselective reaction was also employed by the Chaput group for the synthesis of threose nucleic acid monomers.¹⁸

In this study, we developed additional synthetic approaches toward methyl L-threoside **9** as a convenient key intermediate for subsequent glycosylation reactions (Scheme 2). First, we

Scheme 2. Synthesis of 1-O-Methyl-L-threofuranose **9 from L-arabitol **7** and L-diethyl Tartrate **10**^a**

^aReagents and conditions: (a) (i) NaIO₄, aq MeOH, 1 h, 0 °C and (ii) 1.25 M HCl/MeOH, rt, 12 h, 62% over 2 steps and (b) 1.25 M HCl/MeOH, rt, 12 h, 50%.

revisited the synthetic route from L-arabitol **7**, which has been used previously as the starting material for the synthesis of the four-carbon L-threose sugar²⁰ as well as an adenine containing L-threose nucleoside.¹¹ Treatment of L-arabitol **7** with benzaldehyde in the presence of HCl gas yielded a mixture of isomers, from which enantiomerically pure 1,3-O-benzylidene arabitol **8** could be isolated upon recrystallization from 2-propanol in 60% yield and >95% optical purity.²¹ Oxidative cleavage of the diol moiety of compound **8** with sodium periodate was followed by deprotection of the benzylidene moiety under acidic conditions, which occurred with concomitant cyclization, thus leading to the desired compound **9** in a 62% yield over two steps. The three-step route proceeded in an overall 37% yield and was reproducible on a multigram scale. Alternatively, compound **9** was accessible starting from L-diethyl tartrate **10**, which was converted into aldehyde **11** following a reported method.^{22–24} Simultaneous removal of the *tert*-butyldimethylsilyl (TBS) and isopropylidene-protecting groups by treatment with 1.25 M HCl/MeOH led to the formation of methyl threoside **9** in 35% yield over five steps. Such a synthetic sequence can also be easily performed on a large scale.

Subsequent efforts were directed toward the synthesis of L-threose nucleoside **17**, as illustrated in Scheme 3. The regioselective benzoylation of L-threonolactone **3** to the corresponding 2-O-benzoyl analogue has been described previously.^{18,19} However, when similar reaction conditions were applied to achieve the protection of the 2-hydroxyl group of 1-O-methyl-L-threose **9**, a mixture of regioisomers was formed instead along with 2,3-di-O-benzoylated product **12**. Therefore, we decided to continue our synthesis with dibenzoylated compound **12**, which was first prepared in 85% yield from **9** and then converted to 1-O-acetyl-2,3-O-dibenzoyl-L-threofuranose **13**. This intermediate served as the glycosyl donor in a Vorbrüggen reaction with N⁶-benzoyladenine in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf), furnishing benzoyl-protected L-threose nucleoside **14** in 77% yield. The regioselective cleavage of the 2'-O-

Scheme 3. Synthesis of 2'-Deoxy- α -L-threofuranosyl Nucleoside 17^a

^aReagents and conditions: (a) BzCl, Pyr, 0 °C to rt, 12 h, 85%; (b) acetic anhydride, H₂SO₄, CH₂Cl₂, 0 °C to rt, 5 h, 80%; (c) N⁶-benzoyladenine, BSA, TMSOTf, toluene, 2.5 h, 95 °C, 77%; (d) KO^tBu, THF, 0 °C, 5 min, 81%; (e) (i) TCDI, DMAP, CH₂Cl₂, rt, 12 h, (ii) Bu₃SnH, AIBN, toluene, reflux, 30 min, 80% over 2 steps; and (f) 1 N NaOH, THF/MeOH/H₂O, 0 °C, 2 h, 71%.

Scheme 4. Synthesis of Phosphonomethoxydeoxythreosyl Adenine Prodrug 1^a

^aReagents and conditions: (a) diisopropylphosphonemethyl tosylate, NaH, DMF, 0 °C to rt, 12 h; (b) (i) NaH, DMF, 0 °C to rt, 12 h; (ii) NH₃/MeOH, rt, 12 h, 80% over 2 steps; (c) TMSBr, 2,6-lutidine, CH₃CN, 0 °C to rt, 12 h, 70%; and (d) L-aspartic acid isoamyl ester HCl, PhOH, 2,2'-dithiodipyridine, PPh₃, Et₃N, Pyr, 60 °C, 12 h, 66%.

benzoyl group of 2',3'-di-O-benzoyl threose nucleoside **14** with 0.5 M KO^tBu in tetrahydrofuran (THF) at 0 °C successfully led to 3'-O-benzoyl threose nucleoside **15**, in complete agreement with the literature data.²⁵

Reductive removal of the 2'-hydroxyl group under standard Barton-McCombie deoxygenation conditions gave nucleoside **16**. Finally, removal of the 3'-O-benzoyl group under alkaline conditions furnished L-2'-deoxythreose nucleoside **17**.

The most convenient method to introduce a phosphonomethoxy functionality on the sugar moiety of threose nucleosides is a phosphorylation reaction in the presence of diisopropylphosphonemethyl triflate and a strong base (e.g., NaH or BuLi).⁹ However, treatment of compound **17** under these reaction conditions resulted exclusively in the formation of undesired side products. The use of diisopropylphosphonomethyl tosylate in place of diisopropylphosphonomethyl

triflate led only to a partial conversion of compound **17** to **18**, resulting in a difficult chromatographic separation and unsatisfactory yield. Therefore, we decided to use a less sterically crowded monoester reagent, and for this purpose, we selected (*p*-toluenesulfonyloxymethyl)phosphonic acid monoethyl ester **19**, which was synthesized from the corresponding diethyl ester by alkaline hydrolysis. The introduction of the phosphonate group proceeded efficiently, followed by debenzoylation to give compound **20** in 80% yield over two steps (Scheme 4). Lewis-acid-assisted removal of the monoethyl ester using trimethylsilylbromide afforded L-2'-deoxythreose nucleoside phosphonate **21**. Finally, the free phosphonic acid **21** was converted to the corresponding aryloxy phosphonoamidate prodrug **1** using an established protocol.¹⁰ Protide **1** was isolated as a mixture of diastereomers

and this mixture was used for all subsequent biological evaluations.

In Vitro Evaluation against an Entecavir-Resistant HBV Strain. As abovementioned, prodrug **1** was previously tested for its ability to inhibit the replication of HBV in the human hepatoblastoma HepG2 2.2.15 subclone cell line and reported to cause a reduction of both extracellular and intracellular HBV DNA copy numbers with low nanomolar potency.¹⁰ To further evaluate the anti-HBV activity, compound **1** was retested in HepG2 cells using wild-type and ETV-resistant HBV. To this end, HepG2 cells were infected with adenoviruses AdH294-WT and AdH294-7LR harboring wild-type HBV and ETV-resistant genomes (Figure 2A), respectively, followed by treatment with **1** for 5 days.

Figure 2. Inhibition of the replication of both HBV wild-type and ETV-resistant viruses in the presence of prodrug **1**. (A) Diagrams of adenoviruses AdH294-WT and AdH294-7LR. ETV-resistant strain AdH294-7LR was generated by mutagenesis of four amino acids in the HBV reverse transcriptase region (red arrows). (B) Adenovirus-infected HepG2 cells were treated with serially diluted ETV, TAF, and prodrug **1** for 5 days. HBV replicative intermediate DNAs were extracted and detected with Southern blot using a P32-labeled HBV probe. The radioactive signal beneath each lane was quantified with ImageJ software proportioned to DMSO treatment.

When the assay was conducted using wild-type HBV in the presence of compound **1**, the levels of viral replicative intermediates in the cell were significantly suppressed. In particular, the EC_{50} of **1** was found to be lower than 4 nM, suggesting a potency comparable to that of ETV, while surpassing that of TAF (Figure 2B, upper panel). As expected, in HepG2 cells containing the ETV-resistant mutant, entecavir was unable to block the viral replication even at high

concentrations up to 10 μ M. In contrast, both **1** and TAF were able to reduce the viral DNA levels significantly at submicromolar concentrations. In the case of the ETV-resistant virus, prodrug **1** seemed to be less effective than TAF (Figure 2B, lower panel).

Effect on HBV cccDNA in a Cell-Based Assay. HBV persists in the nucleus of the infected cells in the form of cccDNA. cccDNA is thought to play a crucial role in the persistence and replication of HBV.^{26,27} Elimination of the HBV cccDNA from infected cells is thought to be important as a HBV cure strategy.²⁷ To evaluate the effect of compound **1** on the HBV cccDNA levels in the cells, a cell-based assay using HepDE19 cells was used.²⁸ HepDE19 cells can be induced to allow HBV replication and produce cccDNA in response to tetracycline withdrawal (doxycycline in the experiments described here). HBV cccDNA synthesis was induced in HepDE-19 cells (doxycycline withdrawal) in the presence of various concentrations of **1** (6-point titration in triplicates ranging from 1 μ M to 1×10^{-5} μ M). TAF was used as a positive control in the assay. HBV cccDNA levels were measured at the end of 9 days after T5 exonuclease digestion using HBV cccDNA specific quantitative PCR (qPCR). Beta actin (β -actin) DNA levels before T5 exonuclease treatment were measured by qPCR to evaluate the compound cytotoxicity. Although currently available nucleoside analogues are not shown to target cccDNA, it is worth noting that most of the published literature for cccDNA activity for nucleoside analogues is based upon Southern blotting, which is less sensitive (requiring 10^5 to 10^6 copies/gel lane). The assay system used in the current study is based upon a more sensitive HBV cccDNA detection system (cccDNA-specific qPCR, sensitivity up to 100 copies/reaction) in the inducible HepDE19 cccDNA assay system. As shown in Table 1,

Table 1. Activity of Prodrug **1** in a HBV cccDNA Assay

compound	IC_{50} (μ M) ^a	CC_{50} (μ M) ^b	IC_{90} (μ M)	SI = CC_{50}/IC_{50}
1	0.006	0.23	0.07	37
TAF ^c	0.04	0.50	>0.1	13

^aAntiviral activity against HBV cccDNA measured by cccDNA-specific qPCR in HepDE19 cells. ^bCytotoxicity measured by β -actin qPCR. ^cPositive control.

compound **1** exhibited profound activity in reducing HBV cccDNA in this assay, with an EC_{50} of 0.006 μ M and a selectivity index of 37. Notably, compound **1** was more effective in reducing HBV cccDNA compared to the positive control TAF (EC_{50} of 0.04 μ M and selectivity index of 13) (Table 1).

In Vivo Plasma Pharmacokinetic Study. In order to determine its plasma pharmacokinetics, compound **1** was administered to mice via a single oral dose of 6 mg/kg and plasma samples were taken at different time points after administration (0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after dosing). The plasma concentrations of compound **1** were not quantifiable (with a lower limit of quantification of 1.01 ng/mL) across these time points. Similarly, the parent phosphonate **21** was also undetectable in these different plasma samples. Previously, we demonstrated that **1** is highly metabolically unstable in the presence of liver S9 fraction and liver microsomes with a half-life of approximately 10 min.¹⁰ We, therefore, speculated that, upon oral administration of compound **1**, first-pass metabolism will quickly generate high

levels of nucleoside phosphonate **21** (which is the biologically active species after its intracellular conversion to the diphospho metabolite) in hepatocytes, ultimately resulting in high drug exposure in the liver, making it a promising drug candidate for the treatment of HBV infection. The absence of detectable levels of prodrug **1** and phosphonate **21** in plasma strongly suggests that these compounds accumulate in the liver. This tissue targeted drug delivery and lack of systemic exposure is especially advantageous for treating a liver disease such as the HBV infection.

In Vivo Anti-HBV Study. Compound **1** was investigated for its in vivo efficacy against HBV infection in a hydrodynamic injection (HDI) mouse model. An HBV 1.3 mer plasmid was injected hydrodynamically to produce HBV replication in hepatocytes. One day later, the mice were treated with either ETV (at a dose of 0.1 mg/kg/day) or TAF (at doses of 0.6, 6 and 60 mg/kg/day) as positive controls. Similar to TAF, compound **1** was given orally at three different doses (0.6, 6, and 60 mg/kg/day). During the whole time of treatment, no body weight change was observed (Figure S1, Supporting Information). Blood samples were collected at four different time points and serum HBV DNA was measured by qPCR. In comparison to the vehicle control at each time point after treatment, a clear dose-dependent reduction in HBV DNA copy numbers was observed for both positive controls ETV and TAF (Figure 3). After 2 days, TAF and compound **1**

Figure 3. Antiviral activity of compound **1** in a HBV hydrodynamic mouse model. One day after hydrodynamic injection of HBV 1.3mer plasmid (day 0), six-week-old male C57BL/6 mice were treated with TAF or compound **1** at doses of 0.6, 6, and 60 mg/kg or the vehicle via oral gavage once daily. ETV, which serves as positive control, was also administered orally once daily at a single dose of 0.1 mg/kg. Ten mice were included in each group. Blood was collected at 0.2, 4, and 6 days post-treatment and serum HBV DNA was extracted and analyzed by a real-time PCR assay. Mean values $\pm$ SD are plotted for each group.

performed similarly. After 4 and 6 days, compound **1** performed somewhat better than TAF except at the lowest concentration of 0.6 mg/kg/day (Figures S2–S4, Supporting Information). Altogether, these data demonstrate that the oral administration of compound **1** resulted in potent anti-HBV activity.

In Vivo Toxicity Study. As no signs of toxicity were observed when compound **1** was evaluated in the HDI mouse model, a repeated dose toxicity study in mice was performed. Compound **1** was orally administered once daily at a dose of 60 mg/kg during 14 consecutive days. During the treatment, no animals died, and the clinical observations did not reveal any signs of functional deficits in male or female mice. A loss of body weight (4%) and decreased food consumption in male mice relative to those of the controls were only observed after

11 days. Hematology evaluation showed that the number of neutrophils increased, whereas the number of erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, and lymphocytes decreased in male mice but not in female mice. Clinical chemistry results were statistically comparable to control animals of both sexes, except for increases in the low-density lipoprotein cholesterol concentration in males. Organ weights of all treated animals were comparable to those of control animals of both sexes, except for increased thymus weights observed in females.

Ames Test. Compound **1** was tested at 1 μ M in an Ames test and no mutagenic effect was observed. In particular, the number of colonies of auxotrophic *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537, and TA1537) did not significantly increase in the presence of compound **1** as compared with the spontaneous revertants counted when the experiment was conducted without adding the compound.

CONCLUSIONS

In summary, the synthesis of a protected L-threofuranose sugar synthon is described starting from either L-arabitol or L-diethyl tartrate as an alternative to the commonly used route from L-ascorbic acid. This L-threose intermediate was employed as a glycosyl donor for the large-scale preparation of the aryloxy phosphonoamidate prodrug **1** that was previously shown to be a very potent inhibitor of HBV replication in vitro. In this study, it was shown that compound **1** displayed potent in vitro antiviral activity against ETV-resistant HBV and caused a significant reduction of HBV cccDNA in a cellular assay. Furthermore, the easy access to larger amounts of compound **1** allowed us to demonstrate an in vivo antiviral efficacy of prodrug **1** comparable to TAF. In addition, a 14 day toxicity study did not reveal any signs of toxicity and an in vivo pharmacokinetic study strongly suggested that prodrug **1** was predominantly metabolized in the liver. Overall, these data suggest that prodrugs of PDMTA are promising lead compounds for anti-HBV drug discovery.

EXPERIMENTAL SECTION

Chemistry. ^1H , ^{13}C , and ^{31}P NMR spectra were recorded with a 300, 500, or 600 MHz Bruker AVANCE spectrometer using tetramethylsilane or the residual solvent signal as the internal standard. 2D NMR (H-COSY, HSQC, and HMBC) spectroscopic techniques were used for the assignment of the proton and carbon signals of intermediates and final compounds. All reagents and solvents were purchased from commercial sources and used as received without further purification. All moisture-sensitive reactions were performed in oven-dried glassware under a nitrogen or argon atmosphere. High-resolution mass spectra (HRMS) were obtained on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3 $\mu\text{L}/\text{min}$, and spectra were obtained in a positive (or negative) ionization mode with a resolution of 15 000 FWHM using leucine enkephalin as lock mass. Column chromatography was performed using silica gel 60 $\text{\AA}$, 0.035–0.070 mm (Acros Organics). Precoated aluminum sheets (silica gel matrix, 254 nm) were used for thin layer chromatography. The purity of compound **1** was determined to be > 95% by analytical HPLC analysis. The following HPLC equipment and conditions were employed: Shimadzu HPLC equipped with a LC-20AT pump, a DGU20A5 degasser, and a SPD-20A UV detector; Inertsil ODS-3 (4 μm , 4.6 mm $\times$ 100 mm) or Symmetry C18 column (5 μm , 4.6 mm $\times$ 150 mm); gradient elution of $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ from 95/5 to 5/95 in 15 min; flow rate, 1 mL/min; and UV wavelength, 254 nm.

(3*R*,4*S*)-2-Methoxytetrahydrofuran-3,4-diol (**9**). *Method A*. An aqueous solution of NaIO₄ (41.8 g, 196 mmol) in water (100 mL) was added dropwise to a solution of compound **8**²¹ (47 g, 196 mmol) in methanol (250 mL) at 0 °C over 10 min. The resulting mixture was stirred for 1 h. The white solid was filtered off, and the filtrate was concentrated affording a crude residue that was redissolved in ethyl acetate (200 mL). This solution was washed with brine (100 mL). The organic layer was separated, dried over Na₂SO₄, filtered, and concentrated in vacuo to afford crude 2,4-*O*-benzylidene-L-threose as a white foam that was used in the next step without further purification. The crude 2,4-*O*-benzylidene-L-threose was dissolved in a 1.25 M methanolic HCl solution (200 mL), which was stirred at room temperature for 12 h. The reaction mixture was quenched with solid NaHCO₃ and filtered. The filtrate was evaporated to give a crude residue, which was purified by silica gel chromatography (gradient DCM/MeOH, 25:1, v/v; 20:1, v/v; 15:1, v/v) to afford compound **9** as a colorless oil (16.3 g, 62% over two steps). Compound **9** was isolated as an anomeric mixture, consisting of diastereoisomers **9α** and **9β**. At this stage, the assignment of compound **9α** or **9β** was not performed, and their mixture was used as such in the next step. ¹H NMR (600 MHz, CD₃OD): δ 4.83–4.82 (m, H-1, α or β), 4.75–4.74 (m, H-1, α or β), 4.19–4.15 (m, H-3, H-4, α or β), 4.09–4.05 (m, H-4, H-3, α or β), 3.94–3.92 (m, H-2, α + β), 3.73 (dd, *J* = 9.3, 4.6 Hz, H-4, α or β), 3.57 (dd, *J* = 9.2, 4.3 Hz, H-4, α or β), 3.40 (s, OCH₃, α or β), 3.34 (s, OCH₃, α or β); ¹³C NMR (125 MHz, CD₃OD): δ 111.0, 104.7 (C-1), 82.7, 79.5 (C-2), 77.8, 76.5 (C-3), 74.1, 72.2 (C-4), 55.7, 55.2 (OCH₃).

Method B. A solution of compound **11**^{22–24} (1.0 g, 3.64 mmol) in 1.25 M methanolic HCl (100 mL) was stirred at room temperature for 12 h. The reaction mixture was quenched with solid NaHCO₃ and filtered. The filtrate was evaporated to give a crude residue, which was purified by silica gel chromatography (gradient DCM/MeOH, 25:1, v/v; 20:1, v/v; 15:1, v/v) to afford compound **9** (244 mg, 50%), as a mixture of anomers **9α** and **9β**.

1-*O*-Methyl-2,3-di-*O*-benzoyl-L-threose (**12**). To a solution of compound **9** (16.0 g, 12 mmol) in dry pyridine (200 mL) was added BzCl (48.0 mL, 42.0 mmol) dropwise at 0 °C. The reaction mixture was warmed to room temperature and stirred for 12 h. It was then evaporated to give a crude residue that was redissolved in DCM (300 mL). This solution was washed with saturated aq NaHCO₃ (200 mL) and brine (100 mL). The organic layer was separated, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (gradient hexane/ethyl acetate, 30:1, v/v; 20:1, v/v) to afford compound **12** as a colorless oil (37.5 g, 85%) consisting of two diastereoisomers **12α** and **12β**. At this stage, **12α** or **12β** was not separated, and their mixture was used as such in the next step. ¹H NMR (300 MHz, CDCl₃): δ 8.11–8.04 (m, ArH), 7.57–7.39 (m, ArH), 5.79–5.75 (m, H-1, α or β), 5.55–5.36 (m, H-1, H-2, H-3, α or β), 5.11 (s, H-3, α or β), 4.64 (dd, *J* = 10.0, 6.8 Hz, H-4, α or β), 4.48 (dd, *J* = 10.5, 6.6 Hz, H-4, α or β), 4.02 (dd, *J* = 10.0, 5.1 Hz, H-4, α or β), 3.94 (dd, *J* = 10.5, 3.3 Hz, H-4, α or β), 3.45 (s, OCH₃, α or β), 3.38 (s, OCH₃, α or β); ¹³C NMR (75 MHz, CDCl₃): δ 166.6, 166.2, 165.7 (COPh), 138.3, 133.7, 130.2, 130.1, 129.6, 129.5, 128.8, 128.7 (Ar-C), 107.1, 101.6 (C-1), 81.9, 78.6 (C-2), 77.3, 77.1 (C-3), 71.4, 69.4 (C-4), 55.6, 55.1 (OCH₃); HRMS for C₁₉H₁₈O₆Na [M + Na]⁺ calcd, 365.0996; found, 365.0994.

1-*O*-Acetyl-2,3-di-*O*-benzoyl-L-threose (**13**).¹⁷ To a solution of compound **12** (12.0 g, 34.8 mmol) in anhydrous DCM (200 mL) was added acetic anhydride (13.2 mL, 140.0 mmol) and a catalytic amount of sulfuric acid (10 drops) at 0 °C. After stirring at room temperature for 5 h, the reaction mixture was neutralized by addition of Et₃N at 0 °C and concentrated under reduced pressure. The crude product was dissolved in EtOAc (200 mL), washed with brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (gradient hexane/ethyl acetate, 15:1, v/v; 10:1, v/v; 7:1, v/v) to afford compound **13** as a colorless oil (10.4 g, 80%). Compound **13** consisted of a mixture of two diastereoisomers **13α** and **13β**. At this stage, **13α** or **13β** was not separated, and their mixture was used as such in the next step. ¹H

NMR (300 MHz, CDCl₃): δ 8.10–8.02 (m, ArH), 7.61–7.39 (m, ArH), 6.65 (d, *J* = 4.5 Hz, H-1, α or β), 6.39 (s, H-1, α or β), 5.76–5.68 (m, H-3, H-2, α or β), 5.64 (s, H-2, α or β), 5.58–5.55 (m, H-3, α or β), 4.65 (dd, *J* = 10.5, 6.0 Hz, H-4, α or β), 4.61–4.57 (m, H-4, α or β), 4.21 (ddd, *J* = 10.5, 3.7, 1.2 Hz, H-4, α or β), 4.04 (dd, *J* = 10.6, 3.4 Hz, H-4, α or β), 2.13 (OCH₃, α or β), 2.01 (OCH₃, α or β); ¹H NMR (75 MHz, CDCl₃): δ 169.2, 166.0, 165.5, 165.3, 165.0 (COPh, COMe), 133.6, 133.5, 129.9, 129.8, 128.5, 128.4 (Ar-C), 99.4, 94.3 (C-1), 80.4, 76.7 (C-2), 76.0 (C-3), 73.2, 70.9 (C-4), 20.9, 20.8 (OCH₃); HRMS for C₂₀H₁₈O₇Na [M + Na]⁺ calcd, 393.0945; found, 393.0949.

1'α-(*N*⁶-Benzoyladenine-9-yl)-2',3'-di-*O*-benzoyl-L-threose (**14**).¹⁷ A mixture of compound **13** (8.5 g, 23.0 mmol) and *N*⁶-benzoyladenine (5.5 g, 23.0 mmol) was coevaporated with anhydrous toluene (100 mL) and then suspended in anhydrous toluene (200 mL). To this suspension was added *N,O*-bis(trimethylsilyl)acetamide (11.2 mL), and the mixture was stirred for 30 min at 95 °C until a clear solution was formed. Then, TMSOTf (6.23 mL) was added dropwise. After heating at 95 °C for 2 h, the mixture was cooled to room temperature and poured into an ice-cold solution of saturated aq NaHCO₃/EtOAc (300 mL, 1:1). The organic layer was separated and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (gradient hexane/ethyl acetate, 8:1, v/v; 7:1, v/v; 6:1, v/v) to afford compound **14** (10 g, 77%) as a white foam. ¹H NMR (600 MHz, CDCl₃): δ 9.05 (s, 1H, NH), 8.76 (s, 1H, H-2), 8.32 (s, 1H, H-8), 8.10–8.08 (m, 2H, ArH), 8.03–8.01 (m, 2H, ArH), 7.88–7.86 (m, 2H, ArH), 7.65–7.43 (m, 9H, ArH), 6.46 (d, *J* = 2.0 Hz, 1H, H-1'), 6.30 (d, *J* = 1.8 Hz, 1H, H-2'), 5.77–5.76 (m, 1H, H-3'), 4.67 (dd, *J* = 11.0, 4.8 Hz, 1H, H-4'a), 4.56 (dd, *J* = 11.0, 2.2 Hz, 1H, H-4'b); ¹³C NMR (125 MHz, CDCl₃): δ 165.5, 165.1, 164.7 (COPh), 153.1 (C-2), 151.8 (C-4), 149.7 (C-6), 141.2 (C-8), 134.2, 134.0, 132.9, 130.2, 129.8, 129.0, 128.9, 128.6, 128.5, 128.0 (Ar-C), 123.5 (C-5), 89.2 (C-1'), 80.2 (C-2'), 76.6 (C-3'), 73.7 (C-4'); HRMS for C₃₀H₂₄N₅O₆ [M + H]⁺ calcd, 550.1721; found, 550.1733.

1'α-(*N*⁶-Benzoyladenine-9-yl)-3'-*O*-benzoyl-L-threose (**15**).²⁵ To a solution of compound **14** (10.0 g, 18.2 mmol) in dry THF (300 mL) was added a freshly prepared 0.5 M KO^tBu solution in THF (91 mL, 45.5 mmol) dropwise at 0 °C. After stirring for 5 min, the mixture was quenched by addition of a 1 N HCl solution (45.5 mL, 45.5 mmol). The resulting solution was diluted with ethyl acetate (250 mL), washed with brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure to give a crude residue, which was recrystallized from ethanol to afford compound **15** as a white power (6.5 g, 81%). ¹H NMR (600 MHz, CDCl₃): δ 9.03 (s, 1H, NH), 8.72 (s, 1H, H-8), 8.27 (s, 1H, H-2), 8.01 (d, *J* = 7.7 Hz, 2H, ArH), 7.83 (d, *J* = 7.8 Hz, 2H, ArH), 7.61 (t, *J* = 7.3 Hz, 1H, ArH), 7.55–7.51 (m, 3H, ArH), 7.42–7.39 (m, 3H, ArH), 6.16 (d, *J* = 2.9 Hz, 1H, H-1'), 5.50–5.49 (m, 1H, H-3'), 5.06 (d, *J* = 2.8 Hz, 1H, H-2'), 4.63 (dd, *J* = 10.6, 5.2 Hz, 1H, H-4'a), 4.49 (dd, *J* = 10.7, 2.9 Hz, 1H, H-4'b); ¹³C NMR (125 MHz, CDCl₃): δ 166.2, 164.7 (COPh), 152.7 (C-2), 151.3 (C-4), 149.7 (C-6), 141.2 (C-8), 133.9, 133.6, 133.1, 129.8, 129.5, 129.1, 128.8, 128.0 (Ar-C), 123.5 (C-5), 91.7 (C-1'), 79.6 (C-3'), 79.0 (C-2'), 73.0 (C-4'); HRMS for C₂₃H₂₀N₅O₅ [M + H]⁺ calcd, 446.1459; found, 446.1456.

1'α-(*N*⁶-Benzoyladenine-9-yl)-2'-deoxy-3'-*O*-benzoyl-L-threose (**16**). To a solution of compound **15** (6.5 g, 14.6 mmol) and DMAP (550 mg, 4.38 mmol) in anhydrous CH₂Cl₂ (300 mL) was added 1,1'-thiocarbonyldiimidazole (TCDI) (3.9 g, 21.9 mmol) at room temperature. The reaction mixture was stirred at room temperature for 12 h. Then, the solution was washed with brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure to give a crude residue that was redissolved in toluene (400 mL). Azobis(isobutyronitrile) (AIBN) (1.2 g, 7.30 mmol) and tributyltin hydride (15.7 mL, 53.4 mmol) were then added, and the reaction mixture was refluxed for 30 min. The solvent was removed under reduced pressure, and the residue was purified by silica gel chromatography (gradient DCM/acetone, 8:1, v/v; 7:1, v/v; 6:1, v/v; 6:1, v/v) to

afford compound **16** as a white foam (5.0 g, 80%). ^1H NMR (300 MHz, CDCl_3): δ 9.52 (s, 1H, NH), 8.62 (s, 1H, H-8), 8.28 (s, 1H, H-2), 7.96–7.93 (m, 2H, ArH), 7.70–7.67 (m, 2H, ArH), 7.53–7.29 (m, 6H, ArH), 6.49 (dd, J = 7.0, 2.1 Hz, 1H, H-1'), 5.69–5.65 (m, 1H, H-3'), 4.41–4.30 (m, 2H, H-4'), 2.98–2.80 (m, 2H, H-2'); ^{13}C NMR (75 MHz, CDCl_3): δ 165.5, 164.9 (COPh), 152.4 (C-2), 151.5 (C-4), 149.6 (C-6), 141.0 (C-8), 133.7, 133.5, 132.6, 129.3, 128.9, 128.7, 128.5, 127.9 (Ar-C), 123.6 (C-5), 85.1 (C-1'), 80.0 (C-4'), 73.8 (C-3'), 38.5 (C-2'); HRMS for $\text{C}_{23}\text{H}_{20}\text{N}_5\text{O}_4$ $[\text{M} + \text{H}]^+$ calcd, 430.1510; found, 430.1515.

1'- α -(*N*⁶-Benzoyladenine-9-yl)-2'-deoxy-L-threose (17).²⁹ To a suspension of compound **16** (4.5 g, 10.5 mmol) in THF/MeOH/ H_2O (280 mL, 5:4:1) at 0 °C was added an ice-cold 1 N aq NaOH solution (21 mL, 21.0 mmol). The reaction was stirred at 0 °C for another 2 h, and then the solution was neutralized with a 1 N HCl solution. The mixture was concentrated under reduced pressure. The residue was purified by silica gel chromatography (gradient DCM/MeOH, 50:1, v/v; 30:1, v/v; 25:1, v/v) to afford compound **17** as a white solid (2.4 g, 71%). ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 8.73 (s, 1H, H-8), 8.66 (s, 1H, H-2), 8.07–8.03 (m, 2H, ArH), 7.66–7.51 (m, 2H, ArH), 6.45 (dd, J = 7.9, 2.2 Hz, 1H, H-1'), 4.53–4.51 (m, 1H, H-3'), 4.05–3.96 (m, 2H, H-4'), 2.75–2.66 (m, 1H, H-2'a), 2.41–2.36 (m, 1H, H-2'b); ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 165.7 (COPh), 151.7 (C-2), 151.3 (C-4), 150.6 (C-6), 142.9 (C-8), 133.8, 132.1, 128.3 (Ar-C), 125.5 (C-5), 85.1 (C-1'), 76.5 (C-4'), 69.1 (C-3'), 40.1 (C-2'); HRMS for $\text{C}_{16}\text{H}_{16}\text{N}_5\text{O}_3$ $[\text{M} + \text{H}]^+$ calcd, 326.1248; found, 326.1250.

(*p*-Toluenesulfonyloxymethyl)phosphonic Acid Monoethyl Ester (19). Diethyl (*p*-toluenesulfonyloxymethyl)phosphonate (3.00 g, 9.31 mmol) was dissolved in 1 M NaOH/THF (v/v = 1/1, 30 mL) and stirred for 2 h at room temperature. The reaction mixture was extracted with ethyl acetate (2 $\times$ 50 mL). The layers were separated and the pH of the aqueous layer was adjusted to 1 with 2 M HCl. The aqueous layer was extracted three times with ethyl acetate (3 $\times$ 100 mL). The combined organic phases were dried over NaSO_4 and concentrated under reduced pressure to give a crude residue, which was recrystallized from ethyl acetate/hexane to afford compound **19** as a colorless oil (2.10 g, 80%). ^1H NMR (300 MHz, CD_3OD): δ 7.82 (d, J = 8.4 Hz, 2H, ArH), 7.46 (d, J = 8.0 Hz, 2H, ArH), 4.17 (d, J = 10.0 Hz, 2H, PCH_2), 4.00–4.10 (m, 2H, CH_2CH_3), 2.46 (s, 3H, CH_3Ph), 1.26 (t, J = 7.0 Hz, 3H, CH_2CH_3); ^{13}C NMR (75 MHz, CD_3OD): δ 147.1 (Ar-C), 131.2 (Ar-C), 129.3 (Ar-C), 63.2 (d, $^1J_{\text{P-C}}$ = 165.0 Hz, PCH_2), 64.0 (d, $^2J_{\text{P-C}}$ = 6.1 Hz, CH_2CH_3), 21.6 (CH_3Ph), 16.7 (d, $^3J_{\text{P-C}}$ = 6.1 Hz, CH_2CH_3); ^{31}P NMR (121 MHz, CD_3OD): 13.9; HRMS for $\text{C}_{10}\text{H}_{14}\text{O}_6\text{PS}$ $[\text{M} - \text{H}]^-$ calcd, 293.0254; found, 293.0293.

1'- α -(Adenine-9-yl)-2'-deoxy-3'-O-monoethylphosphonomethyl-L-threose (20). To a solution of compound **17** (500 mg, 1.54 mmol) and compound **19** (900 mg, 3.07 mmol) in anhydrous DMF (50 mL) was added NaH (60% in mineral oil, 290 mg, 7.68 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 12 h. The reaction was quenched with a 0.1 M HCl solution, and was subsequently concentrated under reduced pressure. The residue was dissolved in a 30% methanolic ammonia solution (100 mL) and stirred at room temperature for 12 h. After removal of all the volatiles, the residue was purified by silica gel chromatography (gradient acetone/ H_2O / Et_3N , 20:1:1, v/v/v; 15:1:1, v/v/v; 10:1:1, v/v/v; 8:1:1, v/v/v) to afford compound **20** (420 mg, 80%) as a colorless foam. ^1H NMR (300 MHz, D_2O): δ 8.37 (s, 1H, H-8), 8.13 (s, 1H, H-2), 6.33 (dd, J = 7.3, 2.2 Hz, 1H, H-1'), 4.43–4.33 (m, 2H, H-3', H-4'a), 4.07 (dd, J = 10.5, 3.9 Hz, 1H, H-4'b), 3.80–3.52 (m, 4H, PCH_2 , CH_2CH_3), 2.77–2.62 (m, 2H, H-2'), 1.08 (t, J = 7.1 Hz, 3H, CH_2CH_3); ^{13}C NMR (75 MHz, D_2O): δ 155.2 (C-6), 152.2 (C-2), 148.3 (C-4), 141.2 (C-5), 140.6 (C-8), 118.3 (C-5), 83.9 (C-1), 80.0 (d, $^3J_{\text{P-C}}$ = 13.1 Hz, C-3'), 73.8 (C-4'), 63.7 (d, $^1J_{\text{P-C}}$ = 158.8 Hz, PCH_2), 61.1, 61.0 (CH_2CH_3), 37.1 (C-2'), 15.8, 15.7 (CH_2CH_3); ^{31}P NMR (121 MHz, D_2O): 17.3; HRMS for $\text{C}_{12}\text{H}_{17}\text{N}_5\text{O}_5\text{P}$ $[\text{M} - \text{H}]^-$ calcd, 342.0973; found, 342.0970.

1'- α -(Adenine-9-yl)-2'-deoxy-3'-O-phosphonomethyl-L-threose Triethylammonium Salt (PMDTA, 21). To a solution of compound

20 (540 mg, 1.57 mmol) and 2,6-lutidine (2.90 mL, 25.2 mmol) in dry CH_3CN (20 mL) was added bromotrimethylsilane (1.70 mL, 12.6 mmol) at 0 °C. The reaction mixture was stirred at room temperature overnight and quenched with a 1.0 M TEAB solution. After removal of all the volatiles under reduced pressure, the remaining residue was purified by silica gel column chromatography (gradient acetone/ H_2O / Et_3N , 8:1:1, v/v/v; 5:1:1, v/v/v; 4:1:1, v/v/v) to afford compound **21** (500 mg, 70%) as a white foam. Spectroscopic data were in agreement with a previous report.⁹

1'- α -(Adenine-9-yl)-2'-deoxy-3'-O-[[*N*-(diisoamyl-L-aspartate)]-(phenoxy)methylphosphonamidate]-L-threose (1). Compound **21** (540 mg, 1.71 mmol) was mixed with L-aspartic acid isoamyl diester HCl salt (800 mg, 2.57 mmol) and phenol (710 mg, 7.54 mmol) in anhydrous pyridine (30 mL). Then, Et_3N (2.2 mL) was added and the mixture was stirred at 60 °C under a nitrogen atmosphere for 15–20 min. 2,2'-Dithiodipyridine (1.90 g, 8.57 mmol) was mixed in a separate flask with PPh_3 (2.25 g, 8.57 mmol) in anhydrous pyridine (10 mL) and the resulting mixture was stirred for 10–15 min to give a clear light-yellow solution. This solution was then added to the above solution and the combined mixture was stirred at 60 °C for 12 h. The mixture was then concentrated under reduced pressure to give a residue that was redissolved in EtOAc . This solution was washed with saturated aq NaHCO_3 and brine, the organic layer was separated, dried over Na_2SO_4 , filtered, and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (gradient DCM/MeOH, 60:1, v/v; 30:1, v/v) to give the desired phosphonamidate **1** in pure form (750 mg, 66%) as a mixture of P (S) and P (R) isomers. Spectroscopic data were in agreement with a previous report.¹⁰

In Vitro Evaluation against an Entecavir Drug-Resistant HBV Strain. Adenovectors AdH294-WT and AdH294-7LR harboring HBV replicating wild-type (genotype D, accession# V01460) and ETV-resistant variant, respectively, were generated using Viral-Power System (Invitrogen, cat# K4930). Briefly, HBV replicons under the drive of the tet-CMV promoter were cloned into a pENTRY4 shuttle plasmid expressing tetracycline transactivator (tTA). ETV-resistant mutations (rtL180M + rtT184G + S202I + M204V) were introduced into HBV wild-type replicon by point mutagenesis.³⁰ Cloned shuttle plasmids were verified with DNA sequencing and recombined with the pDESTINY vector. Adenoviruses were then packaged in 293A cells and viral titers were determined with qPCR.

HepG2 cells (ATCC, cat# HB8065) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum and 100 U/mL penicillin plus 100 $\mu\text{g}/\text{mL}$ streptomycin. When cells reached 95% confluence, adenoviruses were inoculated into the culture medium at a multiplicity of infection of 500. Antiviral treatment with ETV, TAF, and compound **1** was initiated 8 h post adenovirus infection. Medium was changed every two days and cells were harvested 5 days after drug treatment. HBV replicative intermediate DNA was extracted as previously described.³¹ Briefly, infected HepG2 cells were lysed in core DNA extraction buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% NP40, and 8% sucrose. Cell debris and nuclei were removed by centrifugation. The supernatant was mixed with PEG-8000 to a final concentration of 9% and placed on ice for 2 h. Capsidated DNA was pelleted by centrifugation at 10,000 rpm for 4 min at 4 °C, followed by Proteinase K digestion, phenol/chloroform extraction, and isopropanol precipitation. Purified viral DNA was resolved in 1.5% agarose gel, blotted onto Hybond N+ membrane (GE, cat# RPN303B), and hybridized with a P32-labeled HBV probe.

Quantification of HBV cccDNA Using qPCR. The study was conducted at Southern Research under NIAID screening task order, using a previously published method³² and using HepDE19 cells.²⁸ 50,000 HepDE19 cells were seeded in collagen-coated 24-well plates in the DMEM-F12 medium with 10% fetal bovine serum (FBS) and 0.1 $\mu\text{g}/\text{mL}$ of doxycycline and incubated at a 37 °C incubator with 5% CO_2 . After 24 h, the cell culture medium was replaced with serial dilutions of the compounds (6-point titration, triplicates per compound concentration) prepared in the DMEM-F12 medium with 10% FBS without doxycycline (for induction of cccDNA

synthesis). TAF was used as the positive control, while media alone was added to cells as a negative control (virus control, VC). Three and six days later, the culture medium was replaced with a fresh medium containing the appropriately diluted test compounds. Cells were harvested after 9 days and nuclear DNA was extracted using the NucleoSpin kit (cat# 740952.250, Takara Bio), according to the kit protocol with couple of modifications as published previously.³² Extracted DNA was subjected to T5 exonuclease treatment (cat# M0363, New England Biolabs, Ipswich, MA) for 60 min at 37 °C, followed by inactivation at 70 °C for 20 min. The resulting product was subjected to HBV cccDNA-specific qPCR using the primers HBV1523 5'GGGGCGACCTCTCTTTA3' and HBV1874 5'AGG-CACAGCTTGGA-GGC3' and probe HBV1562 5'VIC-156 TTCTCATCTGCCGGA CCGTG-TAMRA3' labeled with VIC. PCR was performed using a QuantStudio 6 Real-Time PCR (qPCR) instrument (Thermo Fisher Scientific) with the following conditions; denaturing at 95 °C for 10 min, 45 cycles with denaturing at 95 °C for 10 s, annealing at 56 °C for 30 s, and extension at 60 °C for 1 min. A standard curve generated by using log dilutions of a gel-purified DNA standard was used to extrapolate and quantify the copy numbers of cccDNA in the test samples. Test samples without the T5 exonuclease treatment were subjected to β -actin qPCR using primers and a probe described previously³³ as a measure of cytotoxicity of the compounds. qPCR was performed using the primers β -actin F 5'CCCAGC-CATGTACGTTGCTA3', β -actin R 5'TCACCG-GAGTCCATCACGAT3', and β -actin probes Fam-5'ACGCCTCTGGCCGTAC-CACTGG3'-Tamra. β -Actin PCR was performed using the following conditions; denaturing at 95 °C for 10 min, 45 cycles with denaturing at 95 °C for 10 s, annealing and extension at 60 °C for 1 min. Data analysis was performed by calculating the cccDNA and β -actin levels as a percentage of vehicle controls. The selectivity index (SI_{50}) is calculated as CC_{50}/EC_{50} .

Determination of Plasma Pharmacokinetics. Nine male C57BL/6 mice were administered orally with a solution (10% DMSO, 10% Cremophor EL and 80% saline) of compound **1** at a dose of 6 mg/kg. Blood samples (approximately 60 μ L) were collected under light isoflurane anesthesia from retro orbital plexus from a set of three mice 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h, postadministration. Plasma samples were separated by centrifugation of whole blood and stored below -70 ± 10 °C until bioanalysis. All samples were processed for analysis by protein precipitation using acetonitrile and analyzed with the fit-for-purpose LC/MS/MS method (lower limit of quantification: 1.01 ng/mL).

Antiviral Efficacy in a HBV Hydrodynamic Injection Mouse Model. Compound **1** was tested in an HBV DNA HDI mouse model as described by Wang et al.³⁴ Briefly, one day after HDI of HBV 1.3 mer plasmid (day 0), six-week-old male C57BL/6 mice were administered orally once daily for 6 days with compound **1** and TAF (60, 6, and 0.6 mg/kg/day, $n = 10$ animals per group). The vehicle-treated mice were gavaged with solution containing 10% DMSO, 10% Cremophor, and 80% saline. ETV was also administered orally once daily at a single dose of 0.1 mg/kg as a positive control. The body weight measurements and blood collections from retro orbital plexus were conducted before compound treatment (day 0), and 2, 4, and 6 days post-treatment. Serum HBV DNA was extracted and its levels were then measured using a qPCR assay kit (Hunan Shengxiang Biotechnology Co., Ltd, China).

Toxicity Evaluation. The study was conducted at Sai Life Sciences Limited, Pune, India. In a 14 day repeated-dose toxicity study, 12 male and 12 female healthy C57BL/6 mice were randomly divided into eight groups (three per sex per group). Compound **1** was administered orally at 60 mg/kg once daily to C57BL/6 mice. Mice from the control group received 10% DMSO, 10% Cremophor EL, and 80% saline as vehicle. Treatment-related clinical signs of animals were observed daily. At day 15, blood samples were collected for analysis of hematology, pathology, and chemical parameters. In addition, organs such as liver, spleen, kidneys, heart, thymus, brain, testicles, epididymis, ovaries, and uterus were collected and weighted.

Ames Test. To test the mutagenicity of compound **1**, an Ames test was carried out as described by Mortelmans and Zeiger.³⁵ Briefly, the

histidine-auxotrophic *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1537 were grown overnight at 37 °C in the LB medium with the appropriate antibiotic. To test the compound, both an overlay assay and droplet test were performed. For the overlay assay, 1.9 mL melted top agar (0.6% agar, 0.6% NaCl, 0.05 mM histidine, and 0.05 mM biotin) was mixed with 100 μ L of compound **1** or control solution and $\sim 5 \times 10^6$ bacteria, and this mixture was poured on glucose minimal agar plates.³⁵ In the case of the droplet assay, 100 μ L of compound **1** or control solution was put upon solidified top agar with embedded bacteria. After incubation for 48–60 h at 37 °C, colonies were counted..

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01381>.

NMR spectra of compounds **9**, **12**, **13**, **14**, **15**, **16**, **17**, **19**, and **20**; additional in vivo anti-HBV data and in vivo toxicity data; and molecular formula strings (PDF)

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All authors have given approval to the final version of the manuscript.

Funding

HBV cccDNA assays were supported by the Division of Microbiology and Infectious Diseases (DMID), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) under contract number 75N93019D00015 "In Vitro Assessment for Antimicrobial Activity" Task Order B01 "Comprehensive Viral Screening Panel", under the direction of Dr. Mindy Davis as NIAID's Contracting Officer's Representative (COR), Ms. Amanda Ulloa as the Alternate COR, Dr. Roger Ptak as the Principal Investigator, and Dr. Raj Kalkeri as the Coinvestigator. Synthetic work was supported by the KU Leuven Research Fund via a C3 project (C32/17/045).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Sai Life Sciences Limited for plasma pharmacokinetic and toxicity evaluation. We are grateful to Prof. Jef Rozenski for recording the HRMS spectra and Luc Baudempretz for running the 2D NMR experiments. S.H. and Y.L. are grateful for financial support through The Drug Innovation Major Project (2018ZX09101003-003-003). R.G.P. and R.K. thank Ellen Nordgren for support in data processing and reporting for NIAID contract 75N93019D00015.

■ ABBREVIATIONS

cccDNA, covalently closed circular DNA; ETV, entecavir; HBV, hepatitis B virus; HDI, hydrodynamic injection; PMDTA, phosphonomethoxydeoxythreosyl adenine; TAF, tenofovir alafenamide

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