7156 J. Med. Chem. 2010, 53, 7156–7166
DOI: 10.1021/jm100803c

Journal of Medicinal Chemistry Article

Antiviral Activity of Various 1-(2′-Deoxy-β-D-lyxofuranosyl), 1-(2′-Fluoro-β-D-xylofuranosyl), 1-(3′-Fluoro-β-D-arabinofuranosyl), and 2′-Fluoro-2′,3′-didehydro-2′,3′-dideoxyribose Pyrimidine Nucleoside Analogues against Duck Hepatitis B Virus (DHBV) and Human Hepatitis B Virus (HBV) Replication

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Received June 29, 2010

Despite the existence of successful vaccine and antiviral therapies, infection with hepatitis B virus (HBV) continues to be a major global cause of acute and chronic liver disease and high mortality. We synthesized and evaluated several lyxofuranosyl, 2′-fluoroxylofuranosyl, 3′-fluoroarabinofuranosyl, and 2′-fluoro-2′,3′-didehydro-2′,3′-dideoxyribose pyrimidine nucleoside analogues for antiviral activities against hepatitis B virus. Among the compounds examined, 1-(2-deoxy-β-D-lyxofuranosyl)thymine (**23**), 1-(2-deoxy-β-D-lyxofuranosyl)-5-trifluoromethyluracil (**25**), 1-(2-deoxy-2-fluoro-β-D-xylofuranosyl)uracil (**38**), 1-(2-deoxy-2-fluoro-β-D-xylofuranosyl)thymine (**39**), 2′,3′-dideoxy-2′,3′-didehydro-2′-fluorothymidine (**48**), and 2′,3′-dideoxy-2′,3′-didehydro-2′-fluoro-5-ethyluridine (**49**) were found to possess significant anti-HBV activity against DHBV in primary duck hepatocytes with EC_{50} values of 4.1, 3.3, 40.6, 3.8, 0.2, and 39.0 μM, respectively. Compounds **23**, **25**, **39**, **48**, and **49** (EC_{50} = 41.3, 33.7, 19.2, 2.0−4.1, and 39.0 μM, respectively) exhibited significant activity against wild-type human HBV in 2.2.15 cells. Intriguingly, **25**, **39**, **48**, and **49** retained sensitivity against lamivudine-resistant HBV containing a single mutation (M204I) and **48** emerged as an effective inhibitor of drug-resistant HBV with an EC_{50} of 4.1 μM. In contrast, 50% inhibition could not be achieved by lamivudine at 44 μM concentration in the drug-resistant strain. The compounds investigated did not show cytotoxicity to host cells up to the highest concentrations tested.

Introduction

Globally, hepatitis B virus (HBV[a]) is one of the most prevalent blood-borne viruses causing infectious disease. An estimated 2 billion people worldwide have been infected with HBV with ∼400 million people (∼6% of the world's population) being chronic carriers.[1] HBV is transmitted through mucosal or parenteral routes through infected blood or other body fluids such as semen and saliva. The most common modes of transmission are sexual contact, mother to child transmission during birth, and needle exposures.[2] There is an inverse correlation between the age of HBV acquisition and development of chronic infection. Approximately 90% of the perinatally transmitted infections, 25−50% of infections during the age 1−5 yrs, and 5% of adult infections will progress to chronic HBV. Most adults acutely infected with HBV enter an immune active phase, followed by resolution of viral replication and resolution of hepatitis. HBV infections cause a wide range of liver diseases from subclinical infection to acute self-limiting hepatitis and fulminant hepatitis. Importantly, the chronic carriers not only develop long-term progression to liver diseases but also represent a significant source of infection to others. Around 4.5 million new HBV infections and 500000−700000 deaths occur every year worldwide due to HBV infections.[2] Of the people infected with HBV, 15−40% people develop end-stage liver diseases, cirrhosis, liver failure, and hepatocellular carcinoma.[3] In North America, ∼300000 new cases of HBV infection occur every year, resulting in ∼43000 chronic infections and 3000 deaths.[2]

HBV is a small, enveloped, partially double-stranded circular DNA virus of the hepadnaviridae family. Eight genotypes of HBV, denoted A−H, have been identified.[1] The genotypes are associated with a particular geographic distribution. Genotype A is most common in North America and Northern Europe. Different genotypes of HBV have been associated with different clinical outcomes and response to interferon therapy.

Recombinant yeast derived HBsAg became available as a commercial vaccine for HBV in 1986. The full vaccination course involves three intramuscular immunizations at 0, 1, and 6 months. The full course of immunization generates protective levels of anti-HBsAg in 95% of the immunized people, including infants.[3] The vaccine is highly protective and generates long-term memory to provide protection from new acute and chronic HBV infection against all eight genotypes. However, the vaccine is not useful for ∼400 million chronic carriers worldwide.[3]

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[a] Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; 3-TC, β-L-(−)-2′,3′-dideoxy-3′-thiacytidine; DHBV, duck hepatitis B virus; EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration.

 Published on Web 09/21/2010

Antiviral therapy with nucleoside/nucleotide agents and immunomodulatory agents (such as interferon α) is the only option to control and prevent the progression of diseases in chronic HBV infected patients.[4] The DNA polymerase of the HBV is significantly different than human nuclear and mitochondrial DNA polymerases regarding the specificity of substrates and/or inhibitors and therefore is an excellent target for antiviral drug design using nucleosides and nucleotides. Nucleosides and nucleotides, after conversion to triphosphate derivatives by cellular kinases, can act as selective substrates and/or inhibitors of HBV DNA polymerase/reverse transcriptase. Currently, there are three categories of nucleosides available as anti-HBV antiviral agents:[5] (i) L-nucleosides (lamivudine and telbivudine), (ii) acyclic phosphonate derivatives adefovir and tenofovir, and (iii) the cyclopentane deoxyguanosine analogue entecavir.

Lamivudine, β-L-(−)-2′,3′-dideoxy-3′-thiacytidine (3-TC), was the first anti-HBV drug approved by the FDA.[5] Lamivudine is an efficient, well tolerated, safe, and orally bioavailable nucleoside anti-HBV agent and provides the primary clinical benefits of reduction in viremia and improvement in liver pathology. However, upon interruption of treatment, the majority of people relapse and viral rebound occurs accompanied with severe worsening of liver disease. In addition, long-term monotherapy results in development of drug-resistance in 24% and 70% patients upon treatment for 1 and 4 years, respectively.[6] Adefovir dipivoxil, (9-[2-[bis(pivaloyloxy)methoxy] phosphenyl] ethoxy]-ethyladenine), is an oral prodrug of adefovir.[5] Treatment with adefovir dipivoxil leads to reduced HBV viremia and improved liver pathology. It is effective against lamivudine-resistant HBV strains and the frequency of developing resistance against it is lower: 3−28% after 2−5 years of treatment.[7] However, dose-related side effects such as nephrotoxicity, lactic acidosis, and severe hepatomegaly with steatosis are frequent. In addition, severe hepatitis develops after cessation of therapy in 25% of patients.[8] Tenofovir, approved in 2008, is structurally related to adefovir and has a similar mode of action and similar side effects and antiviral resistance profile.[5] Entecavir, a carbocyclic analogue of 2-deoxyguanosine, has potent and selective anti-HBV activity.[5] In contrast to lamivudine, entecavir shows slower development of resistance and slower rebound of viremia after cessation of therapy. Entecavir is also effective against lamivudine- and adefovir-resistant HBV strains. Severe lactic acidosis has been observed in patients with impaired liver function and chronic hepatitis B when treated with entecavir.[9] Telbivudine, β-L-2′-deoxythymidine, approved in 2006, is a potent inhibitor of HBV replication, has good oral bioavailability, and is tolerated well. The resistance profile of telbivudine is similar to lamivudine. The most common side effects of telbivudine are upper respiratory tract infection, fatigue, malaise, headache, nasopharyngitis, and abdominal pain.[10]

Therefore, the current antiviral therapy for chronic HBV remains inadequate due to limited efficacy, side effects, rapid development of resistance, and most importantly, viral rebound with exacerbation of liver pathology after cessation of therapy. Thus, there is an urgent need to investigate and develop new antiviral agents against HBV which could be used alone, in sequential therapy, and/or in combination therapy.

Among several other different classes of nucleoside analogues, 1-(2-fluoro-5-methyl-β-L-arabinofuranosyl)uracil (L-FMAU), 3′-fluoro-3′-deoxythymidine (FLT), 3′-fluoro-2′,3′- dideoxyguanosine (FLG), 2′,3′-dideoxycytidine (DDC), and 2′,3′-dideoxy-2′,3′-didehydro-β-L-(−)-5-fluorocytidine (β-L-Fd4C) are particularly promising as potent inhibitors of HBV DNA polymerase and the production of HBV in HepG2 cells transfected with HBV DNA.[4,8,11−14] A common feature of these compounds is the absence of a 3′-hydroxyl group except L-FMAU. In addition, presence of a fluorine atom at 2′- or 3′- or a double bond between 2′- and 3′-position in the carbohydrate moiety seems to be important. L-FMAU and FLT possess a common 5-methyl substituent at the C-5 position of the base. These compounds, after converting into the corresponding 5′-triphosphates by cellular enzymes, selectively inhibit HBV DNA polymerase and/or are incorporated into the growing viral DNA chain, halting further DNA synthesis because they lack a 3′-OH group except L-FMAU.

While the 5′-hydroxyl group must be retained in order to allow phosphorylations to the 5′-triphosphates, the 3′-hydroxyl may be replaced by other groups which mimic it functionally. Alternatively, we postulate that it can be incorrectly oriented in a lyxo configuration so that it may not form a 3′,5′-phosphodiester bond, resulting in chain termination.

There has been considerable interest in nucleosides modified on the sugar moiety as potential anti-HBV agents. In earlier studies, lyxofuranosyl analogues of potent antiherpes agents (5-bromovinyl (or methoxymethyl)-2′-deoxyuridines) have been investigated, but they resulted in loss of activity.[15] However, the lyxofuranonucleosides have not been explored for their anti-HBV properties. Earlier work by Veres et al.[16] indicated that introduction of a 3′-OH substituent in the "up" position stabilized the glycosidic bond to enzymatic phosphorolysis relative to the natural 2′-deoxyribose structure that possess a 3′-OH in a proper position.

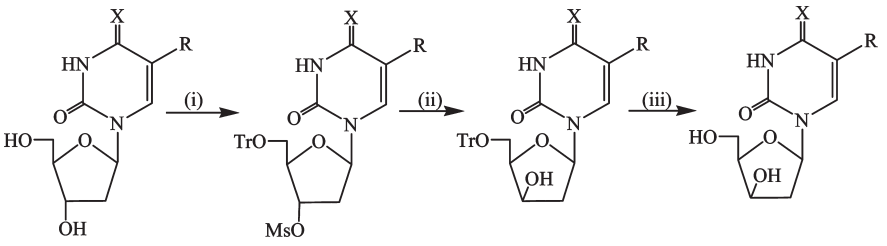
As a part of our investigation in the search of new anti-HBV agents, we became interested in examining the role of 3′-OH "up" as a function of "dideoxy-like" activity. In this paper, we report the antiviral activities of various 1-(2-deoxy-β-D-lyxofuranosyl) nucleosides containing uracil, 5-alkyluracil, and 5-halouracil bases against duck hepatitis B virus (DHBV) and human hepatitis B virus. A fluorine atom has been the most favored replacement for hydrogen in carbohydrates because it has approximately the same van der Waals radius as hydrogen[17] but is strongly electronegative and sterically undemanding. Therefore, we also prepared and tested related 2′- and 3′-fluoro analogues in the hope that such nucleosides may exert even more potent anti-HBV activity. Among different nucleoside analogues structurally modified on the sugar moiety, introduction of a 2′,3′-double bond has provided compounds that hold characteristics of viral DNA chain termination after selective phosphorylation. On the basis of these considerations, we also investigated 2′-fluoro olefinic dideoxy nucleosides for their anti-HBV activity.

In these studies, for the first time, we note that thymine (or trifluorothymine or 5-ethyluracil) analogues of 1-(2-deoxy-β-D-lyxofuranosyl) (**23**, **25**), 1-(2-deoxy-2-fluoro-β-D-xylofuranosyl) (**39**), and 2′-fluoro-2′,3′-didehydro-2′,3′-dideoxyribose (**48**, **49**) glycosyl moieties in particular display potent and selective inhibition against duck HBV, wild-type human HBV, and drug-resistant HBV in vitro.

Chemistry

5-Substituted-2′-deoxyuridines (**1**−**7**) were converted to 3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridines (**8**−**14**) in 19−99% yields by the reaction of trityl chloride in dry pyridine in the presence of catalytic 4-(dimethylamino)pyridine (DMAP)

Scheme 1[a]



1, R = H, X = O
2, R = CH_3, X = O
3, R = CH_3, X = S
4, R = CF_3, X = O
5, R = C_2H_5, X = O
6, R = F, X= O
7, R= I, X = O

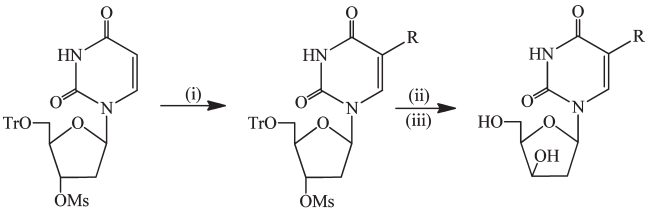
8, R = H, X = O
9, R = CH_3, X = O
10, R = CH_3, X = S
11, R = CF_3, X = O
12, R = C_2H_5, X = O
13, R = F, X= O
14, R= I, X = O

15, R = H, X = O
16, R = CH_3, X = O
17, R = CH_3, X = S
18, R = CF_3, X = O
19, R = C_2H_5, X = O
20, R = F, X= O
21, R= I, X = O

22, R = H, X = O
23, R = CH_3, X = O
24, R = CH_3, X = S
25, R = CF_3, X = O
26, R = C_2H_5, X = O
27, R = F, X= O
28, R= I, X = O

[a] Reagents and conditions: (i) trityl chloride, 4-(dimethylamino)pyridine, dry pyridine, 80 °C, 5−8 h; mesyl chloride, 0 °C, overnight; (ii) NaOH, 90% aq EtOH, reflux, 2 h; (iii) 80% aq AcOH, 90 °C, 0.5 h.

Scheme 2[a]



8

29, R = Cl
30, R = Br

31, R = Cl
32, R = Br

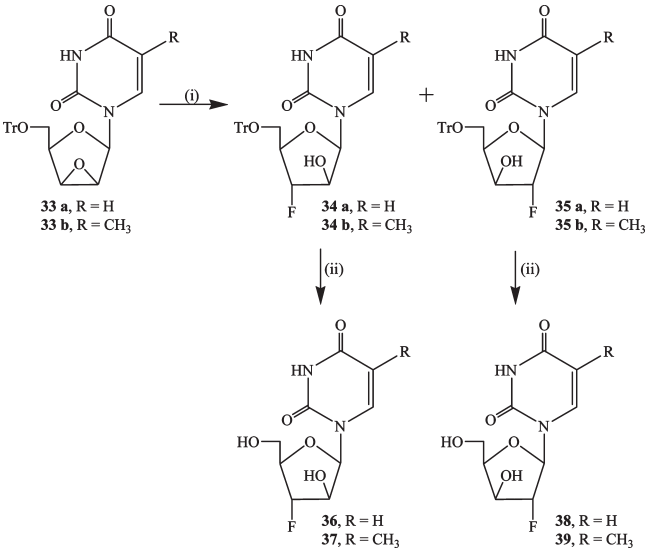
[a] Reagents and conditions: (i) *N*-chlorosuccinimide (**29**), dry pyridine, 100 °C, 1.5 h or *N*-bromosuccinimide (**30**), dry DMF, 0 °C to room temperature, 2.5 h; (ii) NaOH, 90% aq EtOH, reflux, 2 h; (iii) 80% aq AcOH, 90 °C, 0.5 h.

followed by addition of mesyl chloride at 0 °C. Basic hydrolysis of **8**−**14** with NaOH in 90% aqueous ethanol under reflux yielded 1-(5-*O*-trityl-2-deoxy-β-D-lyxofuranosyl)pyrimidines (**15**−**21**) in 19−97% yields. Detritylation of **15**−**21** with 80% aqueous AcOH at 90 °C afforded the target 1-(2-deoxy-β-D-lyxofuranosyl)pyrimidines (**22**−**28**) in 49−84% yields (Scheme 1). Chlorination of **8** at C-5 position with *N*-chlorosuccinimide in dry pyridine at 100 °C gave 5-chloro-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (**29**) in 94% yield. Similarly, bromination of **8** with *N*-bromosuccinimide in dry DMF at room temperature yielded 5-bromo-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (**30**) in 69% yield. Hydrolysis of the obtained compounds **29**, **30** with NaOH followed by their detritylation with 80% aqueous AcOH provided the final 5-halo pyrimidine nucleosides **31**, **32** in 43% and 33% yields, respectively (Scheme 2).

The ^{1}H NMR of the 1-(2-deoxy-β-D-lyxofuranosyl) pyrimidines (**22**−**28**, **31**, and **32**) showed marked differences from their respective 2′-deoxyuridine nucleosides. The major differences were: splitting of H-2′ protons in two groups, downfield shift in H-5′ protons, upfield shift in 5′-OH protons, and splitting in H-1′ protons. Also, these *lyxo* nucleosides were less polar on the TLC in MeOH/$CHCl_3$ solvent system in comparison to their 2′-deoxyuridine analogues.

To synthesize the compounds **36**−**39**, the 5′-tritylated 2′,3′-*lyxo*-epoxides **33a** and **33b** were refluxed with KHF_2 and NaF in anhydrous 2-ethoxyethanol to yield the respective

Scheme 3[a]



33 a, R = H
33 b, R = CH_3

34 a, R = H
34 b, R = CH_3

35 a, R = H
35 b, R = CH_3

36, R = H
37, R = CH_3

38, R = H
39, R = CH_3

[a] Reagents and conditions: (i) KHF_2, NaF, dry 2-ethoxyethanol, reflux, 20−26 h; (ii) 80% aq AcOH, 90 °C, 0.5 h.

fluorohydrins **34a**, **34b**, and **35a**, **35b** (Scheme 3). During the course of this reaction, attack of the fluoride ion occurred predominantly from α-face either at C-2′ or at C-3′. The *lyxo* configuration of the epoxide hinders and prevents attack from the β-face of the sugar moiety (Figure 1). The regioselectivity with the fluorine atom at 3′-postion predominates, which may be due to certain factors, e.g.: (a) the electron-withdrawing anomeric center is closer to C-2′, which retains the oxygen atom intact at C-2′and makes facile opening of the epoxide at C-3′,[18] and (b) the favorable conformation of the sugar rings facilitating nucleophilic attack, predominantly at C-3′. These parameters render the formation of the 3′-deoxy-3′-fluoroarabino compounds **34a** and **34b** as major products, while their 2′-fluoroxylo analogues form **35a** and **35b** as minor products (Figure 1). The compounds **34a**, **34b**, **35a**, and **35b** were deprotected using 80% aqueous (v/v) acetic acid to afford the target compounds **36**, **37**, **38**, and **39**, respectively (Scheme 3). The 2′-fluoro analogues, 2′,3′-dideoxy-2′,3′-didehydro-2′-fluorothymidine (**48**) and 2′,3′-dideoxy-2′,3′-didehydro-2′-fluoro-5-ethyluridine (**49**), were

prepared as illustrated in Scheme 4. Reaction of 2′-ribofluoro-2′-deoxythymidine (**40**) and 1-(2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**41**) with trityl chloride in dry pyridine gave the 5′-*O*-tritylated derivatives **42** and **43**, which upon treatment with mesyl chloride in dry pyridine provided 3′-*O*-mesyl-5′-*O*-trityl derivatives **44** and **45** in 94% and 97% yields, respectively. Reaction of **44** and **45** with NaOH in aqueous EtOH at reflux temperature gave the 2′,3′-olefinic nucleosides **46** and **47** in 20% and 34% yields, respectively. Detritylation of **46** and **47** with 80% aqueous AcOH provided the desired products **48** and **49** in 63% and 65% yields, respectively.

Results and Discussion

The anti-HBV activities of the synthesized pyrimidine nucleosides (**22**−**28**, **31**, **32**, **36**−**39**, **48**−**50**) were evaluated against duck hepatitis B virus (DHBV) in confluent cultures of primary duck hepatocytes obtained from chronically infected Pekin ducks.[19,20] DHBV is a member of the family hepadnaviridae and has a virion structure and genome organization similar to that of human HBV. Thus, DHBV has been used extensively to screen potential drugs to control chronic HBV infection. The anti-HBV activity of the compounds **22**−**28**, **31**, **32**, **36**−**39**, and **48**−**50** were also assessed in confluent cultures of the human hepatoma cell line 2.2.15 that chronically produces infectious HBV. Cell line 2.2.15 is a stable human HBV-producing human hepatoblastoma cell line which carries HBV DNA stably integrated into the genome of HepG2 cells. To analyze the antiviral effects of the selected compounds against the drug-resistant HBV, we used human hepatoma cell line HepG2 transfected with mutated HBV genome. The cell line contains single mutant HBV (B1 cell line transfected with M204I).[21] This resistant cell line has a mutation that is clinically relevant. In all of the cell lines used, the antiviral activity was determined by analysis of intracellular viral DNA using dot-blot hybridization. The concentrations required to inhibit 50% of HBV DNA (EC_{50}) and 50% cytotoxic concentration (CC_{50}) on Huh-7 cells, are shown in Table 1. Lamivudine (3-TC) and abacavir were used in these assays as reference antiviral drugs.

Among the 1-(2-deoxy-β-D-lyxofuranosyl) pyrimidine nucleosides (**22**−**28**, **31**, **32**) investigated, 5-methyl- (**23**) (65% inhibition at 10 μg/mL) and 5-trifluoromethyl- (**25**) (70% inhibition at 10 μg/mL) analogues exhibited the most promising anti-HBV activity against DHBV. The EC_{50} of **23** and **25** was obtained at 4.13 and 3.37 μM concentrations, respectively. At a concentration of 10 μg/mL, 5-unsubstituted (**22**), 4-thio-5-methyl (**24**), 5-ethyl (**26**), and 5-halogenated (**27**, **28**, **31**, **32**) analogues of 1-(2-deoxy-β-D-lyxofuranosyl)-uracil showed no inhibition of DHBV replication, except the 5-chloro derivative (**31**), which provided 25% inhibition of viral replication. The 5-unsubstituted (**22**) and 5-halogenated (**27**, **28**, **31**, **32**) derivatives were almost devoid of anti-DHBV activity, suggesting that in this series of compounds, only 5-alkyl substituents at the C-5 position of pyrimidine contribute to the

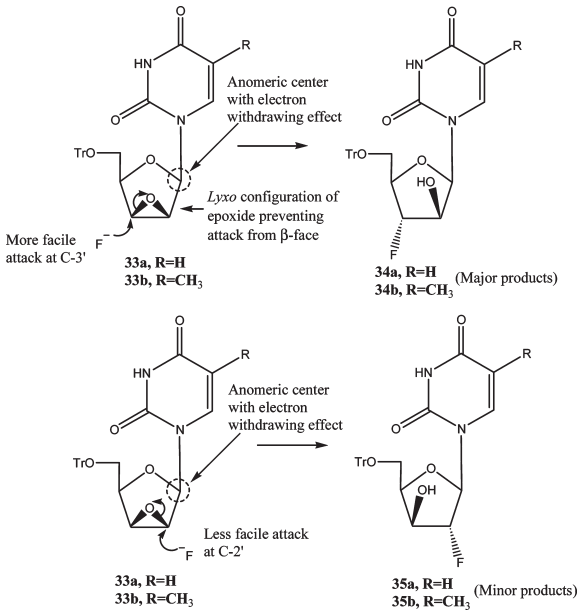
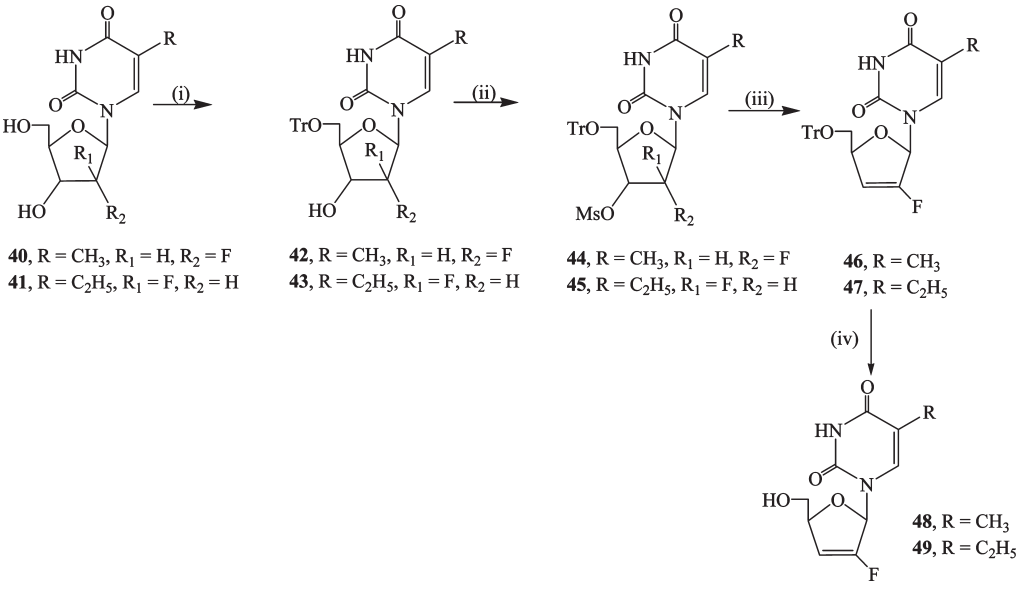


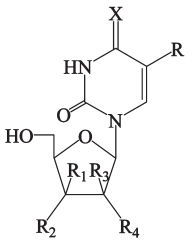
Figure 1

Scheme 4[a]

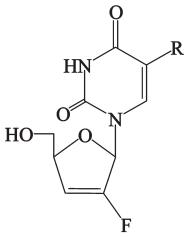


[a] Reagents and conditions: (i) trityl chloride, 4-(dimethylamino)pyridine, dry pyridine, 80 °C, 5−8 h; (ii) mesyl chloride, 0 °C, 48 h; (iii) NaOH, 90% aq EtOH, reflux, 2 h; (iv) 80% aq AcOH, 90 °C, 0.5 h.

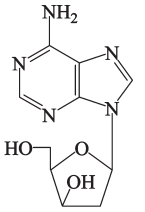
Table 1. In Vitro Antiviral Activity of Pyrimidine Nucleosides Against Wild-Type (Duck HBV and Human HBV) and Drug-Resistant HBV



22-28, 31,32, 36-39



48, 49



50

compd	X	R	R_1	R_2	R_3	R_4	DHBV primary duck hepatocytes % inhibition (conc μg/mL)[a]	[EC_{50} (μM)][b]	2.2.15 wild-type HBV % inhibition (conc μg/mL)[a]	[EC_{50} (μM)][b]	B1 cell line M204I mutant HBV % inhibition (conc μg/mL)[a]	[EC_{50} (μM)][b]	cytotoxicity Huh-7 cells CC_{50} (μM)
22	O	H	OH	H	H	H	0		0		ND[c]		> 877
23	O	CH_3	OH	H	H	H	65 (10), 50 (1)	[4.13]	50 (10)	[41.3]	45 (10)		> 826
24	S	CH_3	OH	H	H	H	0		0		ND		> 775
25	O	CF_3	OH	H	H	H	70 (10), 50 (1)	[3.37]	55 (10)	[33.7]	50 (10)	[33.7]	> 675
26	O	C_2H_5	OH	H	H	H	0		0		ND		> 781
27	O	F	OH	H	H	H	0		0		ND		> 813
28	O	I	OH	H	H	H	0		0		ND		> 282
31	O	Cl	OH	H	H	H	25 (10)		15 (10)		ND		> 763
32	O	Br	OH	H	H	H	0		0		ND		> 651
36	O	H	H	F	OH	H	25 (10)		15 (10)		ND		> 813
37	O	CH_3	H	F	OH	H	0		0		ND		> 769
38	O	H	OH	H	H	F	50 (10)	[40.65]	40 (10)		ND		> 813
39	O	CH_3	OH	H	H	F	72 (10), 50 (1)	[3.84]	60 (10), 50 (5)	[19.23]	50 (5)	[19.23]	> 769
48		CH_3					94(10), 67(1), 50 (0.05)	[0.2]	85 (10), 58 (1), 50 (0.5)	[2.0−4.1]	50 (1)	[4.1]	> 413
49		C_2H_5					50 (10)	[39.06]	50 (10)	[39.06]	50 (10)	[39.06]	> 781
50							40 (10)		36 (10)		ND		ND
3-TC[e]							95 (0.01−0.05)	[< 0.044]	85 (0.5−1)	[0.22−0.44]	40 (10)	[> 44][d]	> 873
Abv[f]											80−90 (1−5)	[< 2.6]	ND

[a] The data are expressed as percent inhibition of viral DNA in the presence of the test compounds at different concentrations as compared to untreated infected controls. [b] The drug concentration (μM) required to reduce the viral DNA in infected cells to 50% of untreated infected controls. [c] Not determined. [d] (>) sign indicates that 50% inhibition was not reached at the stated (highest) concentration tested. [e] (−)−β-L-2′,3′-dideoxy-3′-thiacytidine (Lamivudine, 3-TC). [f] Abv = Abacavir.

antiviral activity. However, the length of the alkyl chain appears to play an important role because **23** and **25** have potent activity, whereas 5-ethyl (**26**) analogue was found to be inactive, indicating that only a single carbon atom substituent is tolerated at the 5-position of the base. In marked contrast with natural 2′-deoxythymidine, it appears that an inverted configuration of the 3′-OH (as in compound **23**) contributes to activity as an inhibitor or substrate of HBV DNA polymerase, leading to viral DNA chain termination. Surprisingly, the alteration of the 4-position by replacement of the oxygen with a sulfur atom (**24**) resulted in a loss of antiviral activity. Palomino et al.[22] reported that a 4-thiated analogue of 3′-azidothymidine had significantly poorer susceptibilities to thymidine kinase phosphorylation compared to 3′-azidothymidine. Therefore, lack of anti-DHBV activity of compound **24** could be due to its inefficient 5′-monophosphorylation and/or further triphosphorylation. It was interesting to note that incorporation of a fluorine atom at the C-2′ position of the carbohydrate portion (as in **38** and **39**) increased the inhibitory activity of compounds **22** and **23**. The 1-(2-deoxy-2-fluoro-β-D-xylofuranosyl)uracil (**38**), exhibited an EC_{50} value of 40.65 μM, whereas parent compound **22** was virtually inactive against DHBV. Similarly, 1-(2-deoxy-2-fluoro-β-D-xylofuranosyl)thymine (**39**) also showed improved inhibition of viral replication (72% inhibition at 10 μg/mL) compared to that of **23**. This reversal and upgrading of the anti-HBV activity of **22** and **23** in the form of **38** and **39** demonstrates that substituents at the 2′-position of the sugar moiety also significantly influence the anti-HBV activity in addition to the nature of substituents present at the 5-position of the pyrimidine ring. In contrast to **23**, **38**, and **39**, unexpectedly, 1-(3-deoxy-3-fluoro-β-D-arabinofuranosyl) derivatives **36** and **37** did not provide appreciable inhibition of DHBV. The marked differences in the activity between compounds **23**, **38**, **39**, and **36**, **37** indicate that a 3′-OH in a *threo* configuration is well tolerated, but a 2′-OH in the same configuration is not. This fact was further supported by the purine analogue **50** possessing an inverted 3′-OH, which also showed modest anti-HBV activity.

In an attempt to develop compounds with improved in vitro anti-HBV activity, olefinic compounds 2′,3′-dideoxy-2′,3′-didehydro-2′-fluorothymidine (**48**) and 2′,3′-dideoxy-2′,3′-didehydro-2′-fluoro-5-ethyluridine (**49**) were also prepared and evaluated. Removal of the 3′-OH group and introduction of a 2′,3′-double bond in the carbohydrate moiety of potent nucleoside **39** dramatically increased the anti-DHBV activity. The results show that 2′-fluoro olefinic analogue (**48**) (EC_{50} = 0.2 μM) has 19 times higher activity than **39** (EC_{50} = 3.84 μM). These results are in agreement with previous observations where pyrimidine nucleoside (β-L-Fd4C) possessing a 2′,3′-double bond provided potent anti-HBV activity.[4] Thus compound **48** holds characteristics of an HBV DNA chain terminator after selective phosphorylation. Substitution of the 5-methyl group in **48** by a longer saturated alkyl group (C_2H_5)

reduced the inhibitory activity (**49**, EC_{50} = 39.0 μM). However, the activity of **49** was increased as compared to its 1-(2-deoxy-β-D-lyxofuranosyl) derivative **26**, which was devoid of antiviral activity. These results further suggest that 2′-fluoro-2′,3′-didehydro-2′,3′-dideoxy sugar is an important determinant of anti-DHBV activity.

The antiviral activity of compounds **22**−**28**, **31**, **32**, **36**−**39**, and **48**−**50** was also examined against wild-type human hepatitis B virus in 2.2.15 cells. Most of the compounds active against DHBV retained anti-HBV activity in 2.2.15 cells. Similar SARs as to anti-DHBV activity were observed except that inhibition of HBV in 2.2.15 cells was slightly diminished. The most active compounds **23**, **25**, **39**, **48**, and **49** exhibited EC_{50} values in the 2.0−41.3 μM range. Although their anti-HBV activity was reduced compared to their anti-DHBV activity, it was encouraging to note that compound **48** showed potent anti-HBV activity (EC_{50} = 2.0−4.1 μM) compared to the reference drug lamivudine (EC_{50} 0.22−0.44 μM) in 2.2.15 cells. The differential activity between DHBV and HBV could be attributable to inherent differences in human vs duck hepatitis B virus, metabolic differences between the two cells, and/or genome organization of hepadnavirus (i.e., integrated in 2.2.15 cells and not integrated in duck hepatocytes).

The discovery of potent anti-HBV activity exhibited by compound **48** is a surprising finding. Compound **48** had been subjected to anti-HIV activity evaluation in earlier studies[23] where it showed no activity. The lack of its efficacy against HIV indicated that triphosphate derivative of **48** is not a good substrate for HIV-RT, however, from our studies, it appears that it has high affinity toward HBV DNA polymerase/RT.

Compounds **23**, **25**, **39**, **48**, and **49** that displayed efficient inhibition of HBV in both primary duck hepatocytes and stably transfected 2.2.15 cells were also evaluated against a drug-resistant strain. A cell-based DNA replication assay was used to measure the antiviral potency against lamivudine-resistant HBV in the B1 cell with a single mutant HBV (M204I) (Table 1). It was encouraging to note that all of these compounds retained activity against single mutant HBV. The anti-HBV activity of **23** was slightly reduced (50% vs 45% inhibition at 10 μg/mL against wild-type and single mutant, respectively), but compounds **25**, **39**, **48**, and **49** exhibited EC_{50} values of 33.7, 19.2, 4.1, and 39.0 μM, respectively, which was comparable to that against wild-type HBV (Table 1). In contrast, single mutant M204I was much less susceptible to lamivudine than was wild-type HBV, and EC_{50} was not achieved up to 44 μM. These results suggest that compounds **25**, **39**, **48**, and **49** are still sensitive to the lamivudine resistant HBV strain.

The cytotoxicity of compounds **22**−**28**, **31**, **32**, **36**−**39**, and **48**−**50** was determined using the XTT assay in vitro against a human hepatoma cell line (Huh-7). No toxicity was observed for these derivatives (CC_{50} > 282−877 μM) (Table 1). It appears therefore that the active compounds can inhibit a crucial step during the life cycle of the HBV. There are no known HBV-encoded enzymes, such as thymidine kinase of herpes viruses, which could initiate the phosphorylation of anti-HBV nucleosides. The fact that compounds **23**, **25**, **31**, **36**, **38**, **39**, **48**, and **49** show selective anti-HBV activity and low toxicity to host cells suggests that by analogy with other antiviral nucleosides, these compounds, after their phosphorylation by cellular kinases to triphosphates, may selectively inhibit HBV DNA polymerase or act as substrates of HBV DNA polymerase.

Summary

In this work, we have identified new classes of anti-HBV agents possessing lyxofuranosyl, 2′-fluoroxylofuranosyl, and 2′-fluoro-2′,3′-dideoxyribose glycosidic moieties. The results obtained here support our strategy for the investigation of new anti-HBV agents based on modifications in the sugar moieties of pyrimidine nucleosides. In this study, compounds **23**, **25**, **39**, **48**, and **49** were found to be very good inhibitors of HBV replication against DHBV, human HBV, and drug-resistant HBV. The most active compound, **48** exhibited an EC_{50} of 0.2 and 2.0−4.1 μM compared to reference drug lamivudine (EC_{50} < 0.044 and 0.22−0.44 μM) in DHBV and wild-type 2.2.15 cells, respectively, and retained sensitivity (EC_{50} = 4.1 μM) against lamivudine-resistant strain (M204I). Of various compounds examined, **48** appears sufficiently promising to warrant further investigations.

Experimental Section

Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were determined for samples in Me_2SO-d_6, $CDCl_3$, or CD_3OD on a Bruker AM 300 spectrometer. ^{13}C NMR (*J* modulated spin echo) spectra were determined for selected compounds where methyl and methyne carbon resonances appear as positive peaks and where methylene and quaternary carbon resonances appear as negative peaks. Chemical shifts are given in ppm relative to TMS as an internal standard, and signals are described as s (singlet), d (doublet), t (triplet), br (broad signal), q (quartet), m (multiplet), dm (doublet of multiplet), and dd (doublet of doublets). The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of D_2O. All of the final compounds had >95% purity, determined by microanalysis. Microanalysis results were within ±0.4% of theoretical values for all elements listed unless otherwise indicated. Silica gel column chromatography was carried out using Merck 7734 silica gel (100−200 μM particle size). Thin-layer chromatography (TLC) was performed with Machery-Nagel Alugram SiL G/UV silica gel slides (20 μM thickness). 2′-Deoxyuridine (**1**), thymidine (**2**), trifluorothymidine (**4**), 5-ethyl-2′-deoxyuridine (**5**), 5-fluoro-2′-deoxyuridine (**6**), 5-iodo-2′-deoxyuridine (**7**), and 2′-fluoro-2′-deoxythymidine (**40**) were purchased from Aldrich. 4-Thio-thymidine (**3**) and 1-(2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**41**) were synthesized using reported procedure.[24,25]

Synthesis of 3′-*O*-(Methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridines (8−14). A full modified procedure is provided for 3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (**8**). For other analogues, only yield and spectroscopic data are presented.

3′-*O*-(Methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (8). A dried mixture of 2′-deoxyuridine (**1**, 3.0 g, 13.15 mmol), trityl chloride (5.86 g, 21.02 mmol), and 4-(dimethylamino)pyridine (0.1 g, 0.82 mmol) in dry pyridine (75 mL) was heated at 80 °C for 8 h. The progress of the reaction was monitored by TLC in MeOH/$CHCl_3$ (10:90, v/v). The reaction mixture was then cooled to 0 °C in an ice bath, and methanesulphonyl chloride (2.03 mL, 26.19 mmol) was added dropwise with constant stirring. The reaction mixture was stirred at 0 °C for 1 h and then kept in a refrigerator overnight. The solvent was removed in vacuo, and then ice−water mixture was added to the residue. The product was extracted with EtOAc (3 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated to give the crude product, which was purified on a silica gel column using MeOH/$CHCl_3$ (2:98, v/v) as eluent to give **8** (7.15 g, 99%) as a syrup.[26] ^{1}H NMR ($CDCl_3$): δ 2.40−2.47 (m, 1H, H-2′), 2.69−2.76 (m, 1H, H-2″), 3.03 (s, 3H, CH_3SO_2), 3.53 (m, 2H, H-5′), 4.33 (m, 1H, H-4′), 5.37−5.42 (m, 2H, H-3′ and H-5), 6.36 (m, 1H, H-1′), 7.29−7.43 (m, 15 H, 5′-*O*-trityl), 7.69 (d, *J* = 7.93 Hz, 1H, H-6), 8.69 (s, 1H, NH).

3′-*O*-(Methylsulfonyl)-5′-*O*-trityl-2′-deoxythymidine (9). Yield 83%, solid,[27] mp 120−122 °C. ^{1}H NMR ($CDCl_3$): δ 1.47 (s, 3H, CH_3), 2.43−2.53 (m, 1H, H-2′), 2.65−2.72 (m, 1H, H-2″), 3.03 (s, 3H, CH_3SO_2), 3.50 (m, 2H, H-5′), 4.32 (m, 1H, H-4′), 5.41 (m, 1H, H-3′), 6.43 (m, 1H, H-1′), 7.29−7.41 (m, 15 H, 5′-*O*-trityl), 7.54 (s, 1H, H-6), 8.52 (s, 1H, NH).

4-Thio-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxythymidine (10). Yield 19%, syrup. ^{1}H NMR ($CDCl_3$): δ 1.65 (s, 3H, CH_3), 2.49−2.54 (m, 1H, H-2′), 2.71−2.73 (m, 1H, H-2″), 3.02 (s, 3H, CH_3SO_2), 3.53 (m, 2H, H-5′), 4.36 (m, 1H, H-4′), 5.40 (m, 1H, H-3′), 6.39 (m, 1H, H-1′), 7.28−7.41 (m, 15 H, 5′-*O*-trityl), 7.61 (s, 1H, H-6), 10.12 (s, 1H, NH), ES-MS (+ve mode) = 601.1 $(M + Na)^+$; ES-MS (−ve mode) = 577.1 $(M - 1)^+$.

5-Trifluoromethyl-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (11). Yield 79%, syrup. ^{1}H NMR (DMSO-d_6): δ 2.54−2.73 (m, 2H, H-2′), 3.20 (s, 3H, CH_3SO_2), 3.24−3.36 (m, 2H, H-5′), 4.25 (m, 1H, H-4′), 5.23 (m, 1H, H-3′), 6.07 (t, J = 6.71 Hz, 1H, H-1′), 7.24−7.41 (m, 15 H, 5′-*O*-trityl), 8.13 (s, 1H, H-6), 12.0 (s, 1H, NH). ES-MS (+ve mode) = 639.1 $(M + Na)^+$; ES-MS (−ve mode) = 615.1 $(M - 1)^+$.

5-Fluoro-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (13). Yield 99%, solid,[27] mp 96−98 °C. ^{1}H NMR ($CDCl_3$): δ 2.41 (m, 1H, H-2′), 2.77 (m, 1H, H-2″), 3.04 (s, 3H, CH_3SO_2), 3.52 (m, 2H, H-5′), 4.36 (m, 1H, H-4′), 5.36 (m, 1H, H-3′), 6.32 (m, 1H, H-1′), 7.24−7.42 (m, 15 H, 5′-*O*-trityl), 7.78 (d, J = 6.10 Hz, 1H, H-6), 8.71 (br s, 1H, NH).

Synthesis of 1-(5-*O*-Trityl-2-deoxy-β-D-lyxofuranosyl)pyrimidines (15−21). A full procedure is provided for 1-(5-*O*-trityl-2-deoxy-β-D-threo-pentofuranosyl)uracil (**15**). For other analogues, only yield and spectroscopic data are presented.

1-(5-*O*-Trityl-2-deoxy-β-D-lyxofuranosyl)uracil (15). NaOH (1 g, 25 mmol) was added to a solution of compound **8** (1.29 g, 2.35 mmol) in 90% aqueous ethanol (50 mL), and the reaction mixture was refluxed for 2 h. The reaction mixture was cooled in an ice bath and neutralized with 80% acetic acid. Solvent was removed in vacuo, and the crude product thus obtained was purified on silica gel column using MeOH/$CHCl_3$ (4:96, v/v) as eluent to give **15**[28,29] (0.82 g, 74%) as a solid; mp 237−239 °C. ^{1}H NMR (DMSO-d_6): δ 1.85 (d, J = 14.65 Hz, 1H, H-2′), 2.47−2.60 (m, 1H, H-2″), 3.16−3.39 (m, 2H, H-5′), 4.07 (m, 1H, H-4′), 4.18 (m, 1H, H-3′), 5.26 (d, J = 3.66 Hz, 1H, 3′−OH), 5.55 (d, J = 7.93 Hz, 1H, H-5), 6.11 (m, 1H, H-1′), 7.24−7.44 (m, 15 H, 5′-*O*-trityl), 7.73 (d, J = 7.93 Hz, 1H, H-6), 11.29 (s, 1H, NH).

1-(5-*O*-Trityl-2-deoxy-β-D-lyxofuranosyl)thymine (16). Yield 67%, solid,[27] mp 110−112 °C. ^{1}H NMR (DMSO-d_6): δ 1.64 (s, 3H, CH_3), 1.84 (d, J = 14.65 Hz, 1H, H-2′), 2.49−2.58 (m, 1H, H-2″), 3.16−3.42 (m, 2H, H-5′), 4.09 (m, 1H, H-4′), 4.18 (m, 1H, H-3′), 5.21 (d, J = 3.36 Hz, 1H, 3′−OH), 6.11 (m, 1H, H-1′), 7.25−7.44 (m, 15 H, 5′-*O*-trityl), 7.59 (s, 1H, H-6), 11.29 (s, 1H, NH).

1-(5-*O*-Trityl-2-deoxy-β-D-lyxofuranosyl)-4-thiothymine (17). Yield 76%, solid,[22] mp 136−138 °C. ^{1}H NMR (DMSO-d_6): δ 1.81 (s, 3H, CH_3), 1.95 (d, J = 18.31 Hz, 1H, H-2′), 2.44−2.56 (m, 1H, H-2″), 3.15−3.45 (m, 2H, H-5′), 4.17 (m, 2H, H-3′ and H-4′), 5.15 (br s, 1H, 3′-OH), 6.05 (d, J = 6.71 Hz, 1H, H-1′), 7.25−7.46 (m, 15 H, 5′-*O*-trityl), 7.60 (s, 1H, H-6), 12.62 (br s, 1H, NH).

1-(5-*O*-Trityl-2-deoxy-β-D-lyxofuranosyl)-5-trifluoromethyluracil (18). Yield 19%, syrup. ^{1}H NMR (DMSO-d_6): δ 1.97 (m, 1H, H-2′), 2.47−2.58 (m, 1H, H-2″), 3.20−3.52 (m, 2H, H-5′), 4.14−4.22 (m, 2H, H-3′ and H-4′), 5.22 (brs, 1H, 3′-OH), 6.12 (m, 1H, H-1′), 7.22−7.44 (m, 15 H, 5′-*O*-trityl), 8.69 (s, 1H, H-6), 11.53 (brs, 1H, NH). ES-MS (+ve mode) = 561.2 $(M + Na)^+$; ES-MS (−ve mode) = 537.2 $(M - 1)^+$.

1-(5-*O*-Trityl-2-deoxy-β-D-lyxofuranosyl)-5-ethyluracil (19). This compound was synthesized from 5-ethyl-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (**12**)[26] in 97% yield as a syrup. ^{1}H NMR (DMSO-d_6): δ 0.83 (t, J = 7.63 Hz, 3H, CH_3), 1.89 (m, 1H, H-2′), 2.08 (q, J = 7.32 Hz, 2H, CH_2), 2.53−2.62 (m, 1H, H-2″), 3.21 and 3.42 (2 m, 2H, H-5′), 4.10−4.24 (m, 2H, H-3′ and H-4′), 5.26 (m, 1H, 3′−OH), 6.17 (dt, J = 8.24 Hz, 6.1 Hz, 1H, H-1′), 7.23−7.50 (m, 15H, 5′-*O*-trityl), 7.60 (s, 1H, H-6), 11.24 (s, 1H, NH). ES-MS (+ve mode) = 521.2 $(M + Na)^+$; ES-MS (−ve mode) = 497.2 $(M - 1)^+$.

1-(5-*O*-trityl-2-deoxy-β-D-lyxofuranosyl)-5-fluorouracil (20). Yield 76%, solid,[27] mp 226−229 °C. ^{1}H NMR (DMSO-d_6): δ 1.90 (d, J = 14.65 Hz, 1H, H-2′), 2.44−2.59 (m, 1H, H-2″), 3.15−3.41 (m, 2H, H-5′), 4.09 (m, 1H, H-4′), 4.18 (m, 1H, H-3′), 5.36 (d, J = 3.66 Hz, 1H, 3′−OH), 6.13 (d, J = 8.54 Hz, 1H, H-1′), 7.25−7.46 (m, 15 H, 5′-*O*-trityl), 7.92 (d, J = 7.32 Hz, 1H, H-6), 11.85 (s, 1H, NH).

1-(5-*O*-Trityl-2-deoxy-β-D-lyxofuranosyl)-5-iodoracil (21). This compound was synthesized from 5-iodo-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (**14**) in 41% yield as a syrup.[30] ^{1}H NMR (DMSO-d_6): δ 1.9 (m, 1H, H-2′), 2.47−2.60 (m, 1H, H-2″), 3.16−3.25 and 3.39−3.58 (2 m, 2H, H-5′), 4.09−4.25 (m, 2H, H-3′ and H-4′), 5.29 (d, J = 3.36 Hz, 1H, 3′−OH), 6.12 (d, J = 7.02 Hz, 1H, H-1′), 7.22−7.55 (m, 15H, 5′-*O*-trityl), 8.18 (s, 1H, H-6), 11.70 (s, 1H, NH). ES-MS (+ve mode) = 619.0 $(M + Na)^+$; ES-MS (−ve mode) = 595.1 $(M - 1)^+$.

Synthesis of 1-(2-Deoxy-β-D-lyxofuranosyl)pyrimidines (22−28). A full procedure is provided for 1-(2-deoxy-β-D-lyxofuranosyl)-uracil (**22**). For other analogues, only yield and spectroscopic data are presented.

1-(2-Deoxy-β-D-lyxofuranosyl)uracil (22). The compound **15** (0.82 g, 1.74 mmol) was dissolved in 80% aqueous acetic acid (25 mL) and heated at 90 °C for 0.5 h. Solvent was removed in vacuo, and the crude product thus obtained was purified on silica gel column using MeOH/$CHCl_3$ (8:92, v/v) as eluent to give **26** (0.323 g, 81%) as a solid; mp 168−170 °C; $[\alpha]_D$ −27.19 (c 0.30, DMSO). ^{1}H NMR (DMSO-d_6): δ 1.86 (m, 1H, H-2′), 2.55 (m, 1H, H-2″), 3.58−3.75 (m, 2H, H-5′), 3.78−3.83 (m, 1H, H-4′), 4.22 (m, 1H, H-3′), 4.69 (t, J = 5.49 Hz, 1H, 5′-OH), 5.26 (d, J = 3.36 Hz, 1H, 3′−OH), 5.65 (d, J = 8.24 Hz, 1H, H-5), 6.02 (dd, J = 8.5 Hz, 2.14 Hz, 1H, H-1′), 7.92 (d, J = 8.24 Hz, 1H, H-6), 11.24 (s, 1H, NH). Anal. ($C_9H_{12}N_2O_5$) C, H, N.

1-(2-Deoxy-β-D-lyxofuranosyl)thymine (23). Yield 81%, solid,[27] mp 172−174 °C; $[\alpha]_D$ +14.0 (c 0.36, H_2O); UV (H_2O) λ_{max} 268 nm (ε 8818). ^{1}H NMR (DMSO-d_6): δ 1.76 (s, 3H, CH_3), 1.84 (dd, J = 14.95 Hz, 2.14 Hz, 1H, H-2′), 2.55−2.59 (m, 1H, H-2″), 3.58−3.82 (m, 3H, H-4′ and H-5′), 4.22 (m, 1H, H-3′), 4.69 (t, J = 5.49 Hz, 1H, 5′-OH), 5.25 (d, J = 3.35 Hz, 1H, 3′-OH), 6.06 (dd, J = 8.54 Hz, 2.44 Hz, 1H, H-1′), 7.79 (s, 1H, H-6), 11.25 (s, 1H, NH). Anal. ($C_{10}H_{14}N_2O_5$) C, H, N.

1-(2-Deoxy-β-D-lyxofuranosyl)-4-thiothymine (24). Yield 49%, solid, mp 69−70 °C; $[\alpha]_D$ +67.52 (c 0.52, MeOH); UV (MeOH) λ_{max} 336 nm (ε 12871). ^{1}H NMR (DMSO-d_6): δ 1.93−1.98 (m, 1H, H-2′), 1.96 (s, 3H, CH_3), 2.52−2.59 (m, 1H, H-2″), 3.63−3.78 (m, 2H, H-5′), 3.84−3.89 (m, 1H, H-4′), 4.24 (m, 1H, H-3′), 4.74 (t, J = 5.49 Hz, 1H, 5′-OH), 5.21 (d, J = 3.36 Hz, 1H, 3′-OH), 5.99 (dd, J = 7.94 Hz, 1.83 Hz, 1H, H-1′), 7.87 (s, 1H, H-6), 12.67 (s, 1H, NH). ^{13}C NMR (CD_3OD): δ 19.92 (CH_3), 44.99 (C-2′), 64.03 (C-5′), 73.40 (C-3′), 89.05 (C-4′), 89.30 (C-1′), 122.18 (C-5), 137.34 (C-6), 152.54 (C-2), 194.88 (C-4). Anal. ($C_{10}H_{14}N_2O_4S$) C, H, N, S.

1-(2-Deoxy-β-D-lyxofuranosyl)-5-trifluoromethyluracil (25). Yield 67%, solid, mp 78−80 °C; $[\alpha]_D$ −5.63 (c 0.43, MeOH); UV (MeOH) λ_{max} 276 nm (ε 7338). ^{1}H NMR (DMSO-d_6): δ 2.01 (d, J = 14.35 Hz, 1H, H-2′), 2.45−2.50 (m, 1H, H-2″), 3.66−3.81 (m, 2H, H-5′), 3.91−3.93 (m, 1H, H-4′), 4.22 (m, 1H, H-3′), 4.79 (m, 1H, 5′-OH), 5.18 (m, 1H, 3′-OH), 5.99 (d, J = 7.63 Hz, 1H, H-1′), 8.80 (s, 1H, H-6), 11.50 (s, 1H, NH). Anal. ($C_{10}H_{11}F_3N_2O_5$) C, H, N.

1-(2-Deoxy-β-D-lyxofuranosyl)-5-ethyluracil (26). Yield 72%, solid,[16] mp 148−151 °C; $[\alpha]_D$ −12.12 (c 0.50, MeOH); UV (MeOH) λ_{max} 267 nm (ε 10970). ^{1}H NMR (DMSO-d_6): δ 1.02 (t, J = 7.33 Hz, 3H, 5-CH_3), 1.85 (dd, J = 14.65 and 2.44 Hz, 1H, H-2′), 2.21 (q, J = 6.71 Hz, 2H, 5-CH_2), 2.53−2.64 (m, 1H, H-2″), 3.68 (m, 2H, H-5′), 3.81 (m, 1H, H-4′), 4.25 (m, 1H, H-3′), 4.70 (t, J = 5.50 Hz, 1H, 5′-OH), 5.28 (d, J = 3.35 Hz, 1H,

3′-OH), 6.09 (dd, J = 8.24 Hz, 2.14 Hz, 1H, H-1′), 7.82 (s, 1H, H-6), 11.22 (s, 1H, NH). ^{13}C NMR (CD_3OD): δ 13.03 (CH_3), 20.98 (5-CH_2), 42.41 (C-2′), 61.64 (C-5′), 70.95 (C-3′), 86.21 (C-1′, C-4′), 116.56 (C-5), 138.66 (C-6), 152.37 (C-2), 166.09 (C-4). Anal. ($C_{11}H_{16}N_2O_5$), C, H, N.

1-(2-Deoxy-β-D-lyxofuranosyl)-5-fluorouracil (27). Yield 50%, solid,[27] mp 202−204 °C; $[\alpha]_D$ +39.66 (c 0.30, H_2O); UV (H_2O) λ_{max} 270 nm (ε 7786). ^{1}H NMR (DMSO-d_6): δ 1.91 (m, 1H, H-2′), 2.52−2.60 (m, 1H, H-2″), 3.58−3.75 (m, 2H, H-5′), 3.78−3.83 (m, 1H, H-4′), 4.23 (m, 1H, H-3′), 4.73 (t, J = 5.50 Hz, 1H, 5′-OH), 5.39 (d, J = 3.36 Hz, 1H, 3′-OH), 6.06 (m, 1H, H-1′), 8.17 (d, J = 7.63 Hz, 1H, H-6), 11.79 (s, 1H, NH). Anal. ($C_9H_{11}FN_2O_5$) C, H, N.

1-(2-Deoxy-β-D-lyxofuranosyl)-5-iodoracil (28). Yield 84%, solid, mp 190−192 °C; $[\alpha]_D$ −20.0 (c 0.35, MeOH); UV (MeOH) λ_{max} 287 nm (ε 4457). ^{1}H NMR (DMSO-d_6): δ 1.91 and 2.56 (2 m, 2H, H-2′), 3.69 (m, 2H, H-5′), 3.80 - 3.88 (m, 1H, H-4′), 4.23 (m, 1H, H-3′), 4.76 (t, J = 5.49 Hz, 1H, 5′-OH), 5.33 (d, J = 3.36 Hz, 1H, 3′-OH), 6.02 (dd, J = 8.24 Hz, 1.83 Hz, 1H, H-1′), 8.38 (s, 1H, H-6), 11.67 (s, 1H, NH). Anal. ($C_9H_{11}IN_2O_5$) C, H, N.

5-Chloro-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (29). Dry pyridine (30 mL) was added to a dried mixture of **8** (0.87 g, 1.57 mmol) and *N*-chlorosuccinimide (0.254 g, 1.90 mmol). The reaction mixture was heated at 100 °C for 2.5 h. Solvent was removed in vacuo, and the crude product thus obtained was purified on silica gel column using EtOAc/hexane (50:50, v/v) as eluent to give **29** (0.87 g, 94%) as syrup. ^{1}H NMR ($CDCl_3$): δ 2.42−2.48 (m, 1H, H-2′), 2.73−2.80 (m, 1H, H-2″), 3.04 (s, 3H, CH_3SO_2), 3.43−3.56 (m, 2H, H-5′), 4.37 (m, 1H, H-4′), 5.37 (m, 1H, H-3′), 6.33 (m, 1H, H-1′), 7.27−7.43 (m, 15 H, 5′-*O*-trityl), 7.96 (s, 1H, H-6), 8.73 (br s, 1H, NH). ES-MS (+ve mode) = 605.1 $(M + Na)^+$, 607.1 $(M + 2 + Na)^+$; ES-MS (−ve mode) = 581.1 $(M - 1)^+$, 583.1 $(M + 1)^+$.

5-Bromo-3′-*O*-(methylsulfonyl)-5′-*O*-trityl-2′-deoxyuridine (30). Dry dimethylformamide (30 mL) was added to a dried mixture of **8** (1.4 g, 2.55 mmol) and *N*-bromosuccinimide (0.681 g, 3.83 mmol). The reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 1.5 h. Solvent was removed in vacuo, and the crude product thus obtained was purified on silica gel column using MeOH/$CHCl_3$ (3:97, v/v) as eluent to give **30** (1.10 g, 69%) as syrup. ^{1}H NMR ($CDCl_3$): δ 2.42−2.48 (m, 1H, H-2′), 2.74−2.80 (m, 1H, H-2″), 3.04 (s, 3H, CH_3SO_2), 3.43−3.57 (m, 2H, H-5′), 4.36 (m, 1H, H-4′), 5.37 (m, 1H, H-3′), 6.33 (m, 1H, H-1′), 7.28−7.44 (m, 15 H, 5′-*O*-trityl), 8.07 (s, 1H, H-6), 8.80 (br s, 1H, NH). ES-MS (+ve mode) = 649.1 $(M + Na)^+$, 651.1 $(M + 2 + Na)^+$; ES-MS (−ve mode) = 625.1 $(M - 1)^+$, 627.1 $(M + 1)^+$.

Compounds **31** and **32** were prepared by basic hydrolysis of **29** and **30**, respectively, followed by their detritylation using procedures as described for **22**.

1-(2-Deoxy-β-D-lyxofuranosyl)-5-chlorouracil (31). Yield 43% based on **29**, solid, mp 173−175 °C; $[\alpha]_D$ −0.92 (c 0.50, MeOH); UV (MeOH) λ_{max} 278 nm (ε 6732), ^{1}H NMR (DMSO-d_6): δ 1.93 (d, J = 14.95 Hz, 1H, H-2′), 2.54−2.59 (m, 1H, H-2″), 3.58−3.75 (m, 2H, H-5′), 3.81−3.86 (m, 1H, H-4′), 4.23 (m, 1H, H-3′), 4.76 (t, J = 5.50 Hz, 1H, 5′-OH), 5.36 (d, J = 3.05 Hz, 1H, 3′-OH), 6.05 (dd, J = 7.93 Hz, 1.52 Hz, 1H, H-1′), 8.24 (s, 1H, H-6), 11.83 (s, 1H, NH). ^{13}C NMR (CD_3OD): δ 42.51 (C-2′), 61.52 (C-5′), 70.72 (C-3′), 86.79 (C-4′), 87.04 (C-1′), 108.54 (C-5), 140.39 (C-6), 151.41 (C-2), 161.64 (C-4). Anal. ($C_9H_{11}ClN_2O_5$) C, H, N.

1-(2-Deoxy-β-D-lyxofuranosyl)-5-bromouracil (32). Yield 33% based on **30**, solid, mp 175−176 °C; $[\alpha]_D$ −10.64 (c 0.50, MeOH); UV (MeOH) λ_{max} 280 nm (ε 6076). ^{1}H NMR (DMSO-d_6): δ 1.93 (d, J = 15.26 Hz, 1H, H-2′), 2.54−2.59 (m, 1H, H-2″), 3.60−3.75 (m, 2H, H-5′), 3.81−3.86 (m, 1H, H-4′), 4.23 (m, 1H, H-3′), 4.76 (t, J = 5.49 Hz, 1H, 5′-OH), 5.36 (d, J = 3.35 Hz, 1H, 3′-OH), 6.04 (dd, J = 8.24 Hz, 1.83 Hz, 1H, H-1′), 8.32 (s, 1H, H-6), 11.79 (s, 1H, NH). Anal. ($C_9H_{11}BrN_2O_5$) C, H, N.

1-(3-Deoxy-3-fluoro-5-*O*-trityl-β-D-arabinofuranosyl)uracil (34a) and 1-(2-Deoxy-2-fluoro-5-*O*-trityl-β-D-xylofuranosyl)uracil (35a). A mixture of 2′,3′-*lyxo*-epoxide (33a)[31] (2.20 g, 4.7 mmol), KHF_2 (2.6 g, 33.7 mmol), and NaF (2.6 g, 61.9 mmol) in dry 2-ethoxyethanol (20 mL) was refluxed for 26 h. 2-Ethoxyethanol was removed in vacuo. $CHCl_3$ (100 mL) was added to the reaction mixture, and the organic layer was washed with water (1 × 25 mL), followed by drying over Na_2SO_4 and concentrating in vacuo. The residue obtained was purified by silica gel column chromatography using MeOH/CH_2Cl_2 (2.5:97.5, v/v) as eluent to yield **35a** (0.095 g, 4%) as a syrup, which was used as such in the next step.

Subsequent elution with the same eluent afforded **34a** (0.52 g, 23%) as a syrup. ^{1}H NMR (DMSO-d_6): δ 3.30 (m, 2H, H-5′), 4.15−4.37 (m, 2H, H-2′, H-4′), 4.97 (dm, $J_{H3'\text{-}3'F}$ = 51.88 Hz, 1H, H-3′), 5.45 (d, J = 7.93 Hz, 1H, H-5), 6.02 (d, J = 4.27 Hz, 2H, H-1′ and 2′-OH), 7.23−7.47 (m, 16H, H-6 and trityl), 11.38 (s, 1H, NH). ES-MS (+ve mode) = 511.1 $(M + Na)^+$; ES-MS (−ve mode) = 487.0 $(M - 1)^+$.

1-(3-Deoxy-3-fluoro-5-*O*-trityl-β-D-arabinofuranosyl)thymine (34b), 1-(2-deoxy-2-fluoro-5-*O*-trityl-β-D-xylofuranosyl)thymine (35b). The title compounds were synthesized from 2′,3′-*lyxo*-epoxide (**33b**)[32] using the same method as described above. The compound **34b** was obtained in 20% yield as a syrup. ^{1}H NMR (DMSO-d_6): δ 1.61 (s, 3H, CH_3), 3.29 (m, 2H, H-5′), 4.10−4.40 (m, 2H, H-2′, H-4′), 5.02 (dm, $J_{H3'\text{-}3'F}$ = 52.849 Hz, 1H, H-3′), 6.00 (d, 1H, J = 4.88 Hz, 2′-OH), 6.05 (d, J = 4.88 Hz, 1H, H-1′), 7.21−7.47 (m, 16H, H-6 and trityl), 11.37 (s, 1H, NH). ES-MS (+ve mode) = 525.1 $(M + Na)^+$; ES-MS (−ve mode) = 501.2 $(M - 1)^+$.

The compound **35b** was obtained in 7% yield as a syrup. ^{1}H NMR (DMSO-d_6): δ 1.61 (s, 3H, CH_3), 3.44−3.57 (m, 2H, H-5′), 4.21 (m, 1H, H-3′), 4.34 (m, 1H, H-4′), 4.97 (d, $J_{H2'\text{-}2'F}$ = 49.44 Hz, 1H, H-2′), 5.76 (d, J = 4.27 Hz, 1H, 3′-OH), 5.99 (d, J = 21.36 Hz, 1H, H-1′), 7.23−7.25 (m, 16H, H-6 and trityl), 11.45 (s, 1H, NH). ES-MS (+ve mode) = 503.2 $(M + 1)^+$, 525.1 $(M + Na)^+$; ES-MS (−ve mode) = 501.2 $(M - 1)^+$.

1-(3-Deoxy-3-fluoro-β-D-arabinofuranosyl)uracil (36). A solution of **34a** (0.15 g, 0.31 mmol) in 80% aqueous acetic acid (10 mL; v/v) was heated at 90 °C for 30 min. The solvent was evaporated in vacuo. The residue obtained was purified on a silica gel column using MeOH/$CHCl_3$ (9:91; v/v) as eluent to yield **36** (0.04 g, 53%) as a white solid; mp 179−180 °C; $[\alpha]_D$ +105.94 (c 0.50, MeOH); UV (MeOH) λ_{max} 262 nm (ε 4913). The NMR spectrum of **36** was identical to that reported in the literaure.[33] ^{1}H NMR (DMSO-d_6): δ 3.61 (t, J = 5.49 Hz, 2H, H-5′), 4.08 (dm, $J_{H4'\text{-}3'F}$ = 26.2 Hz, 1H, H-4′), 4.32 (dm, $J_{H2'\text{-}3'F}$ = 15.2 Hz, 1H, H-2′), 4.94 (dt, $J_{H3'\text{-}3'F}$ = 51.87 Hz, 3.05 Hz, 1H, H-3′), 5.20 (t, J = 5.49 Hz, 1H, 5′-OH), 5.58 (d, J = 8.54 Hz, 1H, H-5), 6.00 (d, J = 3.05 Hz, 1H, H-1′,), 6.05 (d, 1H, J = 4.88 Hz, 2′-OH), 7.56 (d, J = 7.93 Hz, 1H, H-6), 11.36 (s, 1H, NH). Anal. ($C_9H_{11}FN_2O_5$) C, H, N.

1-(3-Deoxy-3-fluoro-β-D-arabinofuranosyl)thymine (37). Detritylation of **34b** as described above afforded **37** in 58% yield as a syrup; $[\alpha]_D$ +76.56 (c 0.21, MeOH); UV (MeOH) λ_{max} 267 nm (ε 7880). ^{1}H NMR (DMSO-d_6): δ 1.75 (s, 3H, CH_3), 3.63 (d, J = 5.49 Hz, 2H, H-5′), 4.02 (dm, $J_{H4'\text{-}3'F}$ = 25.02 Hz, 1H, H-4′), 4.33 (dm, $J_{H2'\text{-}3'F}$ = 17.70 Hz, 1H, H-2′), 5.05 (dt, $J_{H3'\text{-}3'F}$ = 52.49 Hz, 3.05 Hz, 1H, H-3′), 5.22 (br s, 1H, 5′-OH), 5.98 (br s, 1H, 2′-OH), 6.00 (d, J = 4.88 Hz, 1H, H-1′), 7.42 (s, 1H, H-6), 11.34 (s, 1H, NH). Anal. ($C_{10}H_{13}FN_2O_5$) C, H, N.

1-(2-Deoxy-2-fluoro-β-D-xylofuranosyl)uracil (38). The compound **35a** was deprotected using the same method as described for **34a** to give **38** in 51% yield as a solid; mp 159−160 °C; $[\alpha]_D$ +7.13 (c 0.39, MeOH); UV (MeOH) λ_{max} 260 nm (ε 7333). The NMR spectrum of **38** was identical to that reported in the literaure.[23] ^{1}H NMR (DMSO-d_6): δ 3.74 (m, 2H, H-5′), 4.12 (m, 1H, H-4′), 4.25 (d, $J_{H3'\text{-}2'F}$ = 10.38 Hz, 1H, H-3′), 4.89 (m, 1H, 5′-OH), 5.01 (d, $J_{H2'\text{-}2'F}$ = 48.82 Hz, 1H, H-2′), 5.67 (d, J = 7.93 Hz, 1H, H-5), 5.82 (m, 1H, 3′-OH), 5.94 (d, $J_{H1'\text{-}2'F}$ = 21.9 Hz, 1H, H-1′), 7.66 (d, J = 8.5 Hz, 1H, H-6), 11.40 (s, 1H, NH). Anal. ($C_9H_{11}FN_2O_5$) C, H, N.

1-(2-Deoxy-2-fluoro-β-D-xylofuranosyl)thymine (39). This compound was obtained in 52% yield as a syrup after detritylation of

35b using 80% aqueous acetic acid as described for the deprotection of **34a**. ^{1}H NMR (DMSO-d_6): δ 1.74 (s, 3H, CH_3), 3.72 (m, 2H, H-5′), 4.07 (m, 1H, H-4′), 4.24 (dd, $J_{H3'-2'F}$ = 11.60 and 3.05 Hz, 1H, H-3′), 4.90 (br s, 1H, 5′-OH), 4.99 (d, 1H, $J_{H2'-2'F}$ = 49.45 Hz, H-2′), 5.86 (br s, 1H, 3′-OH), 5.92 (d, $J_{H1'-2'F}$ = 22.58 Hz, 1H, H-1′), 7.53 (d, J = 1.22 Hz, 1H, H-6), 11.32 (br s, 1H, NH). Anal. ($C_{10}H_{13}FN_2O_5$) C, H, N.

1-(2-Deoxy-2-fluoro-5-*O*-trityl-β-D-ribofuranosyl)-5-methyluracil (42). Anhydrous pyridine (50 mL) was added to a dried mixture of **40** (2.0 g, 7.68 mmol), trityl chloride (3.21 g, 11.51 mmol), and 4-(dimethylamino)pyridine (0.05 g, 0.41 mmol), and the reaction mixture was heated at 80 °C for 5 h. The solvent was removed in vacuo, and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (2:98, v/v) as eluent to give **42** (3.45 g, 89%) as a solid; mp 115−117 °C. ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 3H, CH_3), 3.53 (m, 2H, H-5′), 4.13 (m, 1H, H-4′), 4.59 (m, 1H, H-3′), 5.17 (dm, $J_{H-2',2'-F}$ = 50.05 Hz, 1H, H-2′), 6.09 (dd, $J_{H-1',2'-F}$ = 16.78 Hz, 2.44 Hz, 1H, H-1′), 7.23−7.49 (m, 15H, 5′-*O*-trityl), 7.54 (s, 1H, H-6), 8.44 (br s, 1H, NH). ES-MS (+ve mode) = 525.2 $(M + Na)^+$; ES-MS (−ve mode) = 501.2 $(M - 1)^+$.

1-(2-Deoxy-2-fluoro-5-*O*-trityl-β-D-arabinofuranosyl)-5-ethyluracil (43). Compound **43** was prepared using the procedure as described for **42**. Yield 89%, mp 212−214 °C. ^{1}H NMR (DMSO-d_6): δ 0.83 (t, J = 7.32 Hz, 3H, CH_3), 2.03 (q, J = 7.32 Hz, 2H, CH_2), 3.17−3.35 (m, 2H, H-5′), 3.97 (m, 1H, H-4′), 4.24−4.39 (m, 1H, H-3′), 5.04 (dm, $J_{H-2',2'-F}$ = 52.49 Hz, 1H, H-2′), 5.95 (d, J = 4.88 Hz, 1H, 3′-OH), 6.17 (dd, $J_{H-1',2'-F}$ = 17.70 Hz, 4.27 Hz, 1H, H-1′), 7.25−7.45 (m, 16H, 5′-*O*-trityl and H-6), 11.48 (s, 1H, NH). ES-MS (+ve mode) = 539.2 $(M + Na)^+$; ES-MS (−ve mode) = 515.2 $(M - 1)^+$.

1-(2-Deoxy-2-fluoro-3-*O*-mesyl-5-*O*-trityl-β-D-ribofuranosyl)-5-methyluracil (44). To an ice cooled solution of **42** (3.40 g, 6.77 mmol) in anhydrous pyridine (50 mL) was added mesyl chloride (1.05 mL, 13.62 mmol) dropwise with stirring. The reaction mixture was then kept in the refrigerator for 48 h. After the addition of water (2 mL), the solvent was evaporated and the resulting residue was dissolved in $CHCl_3$ (100 mL), washed with water (2 × 30 mL) and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo, and the residue was purified on a silica gel column using MeOH/$CHCl_3$ (1:99, v/v) as eluent to give **44** (3.70 g, 94%) as a solid; mp 110−112 °C. ^{1}H NMR ($CDCl_3$): δ 1.50 (s, 3H, CH_3), 3.04 (s, 3H, CH_3SO_2), 3.54 (m, 2H, H-5′), 4.37 (m, 1H, H-4′), 5.28−5.47 (m, 2H, H-2′ and H-3′), 6.07 (dd, $J_{H-1',2'-F}$ = 16.48 Hz, 3.05 Hz, 1H, H-1′), 7.29−7.42 (m, 15H, 5′-*O*-trityl), 7.46 (s, 1H, H-6), 8.46 (s, 1H, NH). ES-MS (+ve mode) = 603.2 $(M + Na)^+$; ES-MS (−ve mode) = 579.2 $(M - 1)^+$.

1-(2-Deoxy-2-fluoro-3-*O*-mesyl-5-*O*-trityl-β-D-arabinofuranosyl)-5-ethyluracil (45). Compound **45** was synthesized using the procedure as described for **44**. Yield 97%, mp 90−92 °C. ^{1}H NMR (DMSO-d_6): δ 0.86 (t, J = 7.32 Hz, 3H, CH_3), 2.04 (q, J = 7.32 Hz, 2H, CH_2), 3.24 (s, 3H, CH_3SO_2), 3.30−3.47 (m, 2H, H-5′), 4.25 (m, 1H, H-4′), 5.41 (m, 1H, H-3′), 5.55 (dm, $J_{H-2',2'-F}$ = 56.15 Hz, 1H, H-2′), 6.26 (dd, $J_{H-1',2'-F}$ = 16.48 Hz, 4.27 Hz, 1H, H-1′), 7.25−7.45 ((m, 16H, 5′-*O*-trityl and H-6), 11.55 (s, 1H, NH). ES-MS (+ve mode) = 617.2 $(M + Na)^+$; ES-MS (−ve mode) = 593.2 $(M - 1)^+$.

2′,3′-Dideoxy-2′,3′-didehydro-2′-fluoro-5′-*O*-tritylthymidine (46). NaOH (1 g, 25 mmol) was added to a solution of compound **44** (1.0 g, 1.72 mmol) in 90% aqueous ethanol (50 mL), and the reaction mixture was refluxed for 2 h. The reaction mixture was cooled on an ice bath and neutralized with 80% acetic acid. Solvent was removed in vacuo, and the crude product thus obtained was purified on silica gel column using MeOH/$CHCl_3$ (1:99, v/v) as eluent to give **46** (0.17 g, 20%) as a syrup. The compound was used as such in the next step.

2′,3′-Dideoxy-2′,3′-didehydro-2′-fluoro-5′-*O*-trityl-5-ethyluridine (47). Compound **47** was prepared using the procedure as described for **46**. Yield 34%, mp 88−90 °C. ^{1}H NMR ($CDCl_3$): δ 0.70 (t, J = 7.32 Hz, 3H, CH_3), 1.76 (q, J = 7.32 Hz, 2H, CH_2), 3.36 (d, J = 4.27 Hz, 2H, H-5′), 4.98 (m, 1H, H-4′), 5.77 (s, 1H, H-1′), 6.98 (m, 1H, H-3′), 7.23−7.50 (m, 16H, 5′-*O*-trityl and H-6), 8.23 (br s, 1H, NH). ES-MS (+ve mode) = 521.2 $(M + Na)^+$.

2′,3′-Dideoxy-2′,3′-didehydro-2′-fluorothymidine (48). Compound **46** (0.16 g, 0.33 mmol) was dissolved in 80% acetic acid (10 mL) and heated at 90 °C for 0.5 h. Solvent was removed in vacuo, and the crude product thus obtained was purified on silica gel column using MeOH/$CHCl_3$ (3:97, v/v) as eluent to give **48** (0.05 g, 63%) as a solid; mp 156−158 °C; $[\alpha]_D$ +45.43 (*c* 0.50, MeOH); UV (MeOH) λ_{max} 263 nm (ε 5753). The ^{1}H NMR spectrum obtained for **48** was identical to that reported in the literaure.[32] ^{1}H NMR (DMSO-d_6): δ 1.76 (s, 3H, CH_3), 3.60 (br s, 2H, H-5′), 4.79 (br s, 1H, H-4′), 5.17 (br s, 1H, 5′-OH), 5.99 (m, 1H, H-1′), 6.76 (m, 1H, H-3′), 7.89 (s, 1H, H-6), 11.47 (s, 1H, NH). Anal. ($C_{10}H_{11}FN_2O_4$) C, H, N.

2′,3′-Dideoxy-2′,3′-didehydro-2′-fluoro-5-ethyluridine (49). Compound **49** was prepared using the procedure as described for **48**. Yield 65%; mp 145−147 °C; $[\alpha]_D$ +45.79 (*c* 0.50, MeOH); UV (MeOH) λ_{max} 262 nm (ε 8486). ^{1}H NMR (DMSO-d_6): δ 1.01 (t, J = 7.32 Hz, 3H, CH_3), 2.20 (q, J = 7.32 Hz, 2H, CH_2), 3.63 (m, 2H, H-5′), 4.82 (m, 1H, H-4′), 5.19 (t, J = 5.49 Hz, 1H, 5′-OH), 6.00 (m, 1H, H-1′), 6.79 (m, 1H, H-3′), 7.86 (s, 1H, H-6), 11.46 (br s, 1H, NH). Anal. ($C_{11}H_{13}FN_2O_4$) C, H, N.

In Vitro Antiviral Assay (Duck Hepatitis B Virus, DHBV). Primary hepatocyte cultures obtained from congenitally infected ducks were used to determine the anti-DHBV activity of test compounds, as reported previously.[19,20,34] Pekin duck eggs were obtained from a duck colony maintained at the University of Alberta farm and were stored in a 37 °C egg incubator until hatching occurred. Livers from congenitally DHBV infected ducks were used. Persistently infected ducks were identified by detection of DHBV DNA in sera by dot hybridization.[19,34]

Primary cultures of duck hepatocytes were prepared from 9−14 day old DHBV-infected ducklings using a modified method.[20] Cells were cultured in 6-well plates in 3 mL of L-15 medium containing 5% fetal bovine serum, penicillin G sodium (10 IU/mL), and streptomycin sulfate (10 μg/mL). The test compounds were added in triplicate to the hepatocyte cultures on day 2 and were maintained in culture with media changed every second day until day 16. Cells were harvested at day 17. Initially, the test compounds were screened at a 10 μg/mL final concentration. Inhibition in DHBV replication at 10 μg/mL was calculated as the average of triplicate wells. Standard deviations were within 10% of the average values. After initial testing, the compounds were serially diluted to determine more precise anti-DHBV EC_{50} values. The hepatocytes were lysed with 1.0 mL of lysis buffer containing 10 mM Tris-HCl and 1% SDS. The lysate was digested with 0.2 mg of proteinase K and extracted with an equal volume of phenol saturated with Tris-HCl EDTA and 0.1% 8-hydroxyquinoline, followed by extraction with chloroform. Concentrated NaCl (5M) was added to the aqueous phase to yield a final concentration of 0.5 M NaCl, and the DNA was precipitated with two volumes of 95% ethanol. The DNA pellet was washed with 70% ethanol and dried. The dried DNA was dissolved in 50 μL of a solution containing Tris-HCl EDTA.

DNA samples were applied to a nylon filter (Hybond-N, Amersham) using a Bio-Dot (Bio-Rad Laboratories) microfiltration apparatus. DNA samples from one experiment including positive and negative controls were applied on the same filter to maintain standardization of exposure. Also, 100 pg^{-1} ng/μL of DHBV DNA standards were applied on the same blot as DNA control. DNA on the filter was denatured with NaOH/NaCl at room temperature for 30 min and neutralized in Tris-HCl/NaCl. The filters were exposed to ultraviolet irradiation for 3 min. DNA hybridization was initiated by adding a recently prepared DHBV (^{32}P) DNA probe at 10^6 CPM/mL and incubating overnight. Filters were washed twice in 1× SSC (20× SSC = 3 M NaCl plus 0.3 M sodium citrate, pH 7.0)−0.1% SDS at 65 °C for 30 min and 0.1 SSC, 0.15 DS at room temperature for 30 min. After an autoradiographic image was obtained, the filters were exposed in a phosphoimaging screen for 1−2 h, and samples were quantitated by a Fujix BAS1000 and the percentage density of phosphoimaging units were calculated.[20] Lamivudine was used as the reference

compound. Tests were repeated 2−3 times, and the data for each test compound were compared with a positive and negative control performed at the same time under identical conditions. For the compounds where the EC_{50} obtained from 2−3 experiments was within 10% standard deviation, average values are shown, otherwise a range of EC_{50} values are shown. Percent inhibition was calculated by using the formula:

$$\%\text{inhibition} = ((\text{untreated positive control} - \text{treated test sample})/\text{untreated positive control}) \times 100$$

In Vitro Antiviral Assay (Human Hepatitis B Virus, 2.2.15 Cells). The human HBV transfected 2.2.15 cells were obtained from Dr. M. A. Sells and were used to determine the anti-HBV activity of test compounds, as reported previously.[20,35,36] These cells were derived from HepG2 cells that were transfected with a plasmid vector containing G418-resistant sequences and two head-to-tail dimers of the HBV genome. The cell culture medium consisted of MEM (Sigma), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), Geneticin/G148 sulfate (380 μg/mL), 5−7 mL of 7.5% sodium bicarbonate, and L-glutamine (2 mM). Initially, the test compounds were screened at a 10 μg/mL final concentration. Inhibition of HBV replication at 10 μg/mL was calculated as the average of triplicate wells. Standard deviations were within 10% of the average values. After initial testing, the compounds were serially diluted to determine more precise anti-HBV EC_{50} values.

The 2.2.15 cells were grown in MEM media in a humidified 37 °C incubator with 5% CO_2 atmosphere and seeded into 6-well plates overnight to obtain a confluent monolayer. The compounds were tested in triplicate for each concentration. The drug solutions were added at a volume of 3 mL per well, replacing the prior media. The media containing test compounds was replaced every second day for 5−6 treatments in total. On the day subsequent to the last treatments, the cells were harvested for the analysis of intracellular HBV DNA. The method for isolated intracellular DNA and dot-blot hybridization were essentially similar to that for DHBV, except a human HBV specific radioactive probe was used. Percent inhibition was calculated by using the formula: (untreated positive control − treated test sample) × 100/untreated positive control. Tests were repeated 2−3 times, and the data for each test compound were compared with a positive and negative control performed at the same time under identical conditions. For the compounds where the EC_{50} obtained from three experiments was within 10% standard deviation, average values are shown, otherwise a range of EC_{50} values are shown.

In Vitro Antiviral Assay against Lamivudine-Resistant Human Hepatitis B Virus [(B1, M204I) Cell Line]. The 3TC resistant cell line B1 was previously constructed by Tyrrell et al. and grown from liquid nitrogen frozen stocks.[21] The B1 cell line features a single mutation (rtM204I). The cell culture, treatment with compounds, and dot-blot hybridization procedures were identical to those as described previously for 2.2.15 cells.[21,35,36] With this cell line as well, percent inhibition was calculated using the formula shown in the previous section.

Cell Cytotoxicity. Human hepatoma cell line (Huh-7) was used to determine the effect of compounds **22**−**28**, **31**, **32**, **36**−**39**, and **48**−**50** on human cell cytotoxicity using the XTT assay. Cell viability was measured using the cell proliferation kit II (XTT; Roche) as per manufacturer's instructions. Briefly, a 96-well plate was seeded with Huh-7 cells at a density of 1×10^5 cells per well. Cells were allowed to attach for 6−8 h when the medium was replaced with medium containing compounds at concentrations of 200, 100, 50, 10, and 1 μg/mL. DMSO was also included as control. Plates were incubated for 2 days at 37 °C. The color reaction involved adding 50 μL XTT reagents per well and incubating for 4 h at 37 °C. Plates were read on an ELISA plate reader (Abs 450−500 nm).

Acknowledgment. We are grateful to the Canadian Institutes of Health Research (CIHR) for an operating grant (MOP-36393) for the financial support of this research. B.A. is thankful to the Alberta Innovates-Health Solutions (formerly Alberta Heritage Foundation for Medical Research, AHFMR) for a Senior Scholar Award.

Supporting Information Available: Elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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