Design and Synthesis of Novel 5-Substituted Acyclic Pyrimidine Nucleosides as Potent and Selective Inhibitors of Hepatitis B Virus

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A novel class of 5-substituted acyclic pyrimidine nucleosides, 1-[(2-hydroxyethoxy)methyl]-5-(1-azidovinyl)uracil (**9a**), 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-(1-azidovinyl)uracil (**9b**), and 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-(1-azidovinyl)uracil (**9c**), were synthesized by regiospecific addition of bromine azide to the 5-vinyl substituent of the respective 5-vinyluracils (**2a**−**c**) followed by treatment of the obtained 5-(1-azido-2-bromoethyl) compounds (**3a**−**c**) with *t*-BuOK, to affect the base-catalyzed elimination of HBr. Thermal decomposition of **9b** and **9c** at 110 °C in dioxane yielded corresponding 5-[2-(1-azirinyl)]uracil analogues (**10b**,**c**). The 5-(1-azidovinyl)uracil derivatives **9a**−**c** were found to exhibit potent and selective in vitro anti-HBV activity against duck hepatitis B virus (DHBV) infected primary duck hepatocytes at low concentrations (EC_{50} = 0.01−0.1 μg/mL range). The most active anti-DHBV agent (**9c**), possessing a [4-hydroxy-3-(hydroxymethyl)-1-butyl] substituent at N-1, exhibited an activity (EC_{50} of 0.01−0.05 μg/ mL) comparable to that of reference compound (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3-TC) (EC_{50} = 0.01−0.05 μg/mL). In contrast, related 5-[2-(1-azirinyl)]-uracil analogues (**10b**,**c**) were devoid of anti-DHBV activity, indicating that an acyclic side chain at C-5 position of the pyrimidine ring is essential for anti-HBV activity. The pyrimidine nucleosides (**9a**−**c**, **10b**,**c**) exhibited no cytotoxic activity against a panel of 60 human cancer cell lines. All of the compounds investigated did not show any detectable toxicity to several stationary and proliferating host cell lines or to mitogen stimulated proliferating human T lymphocytes, up to the highest concentration tested.

Introduction

Hepatitis B virus (HBV) is a causative agent of acute and chronic hepatitis. Hepatitis B virus infection is the world's ninth leading cause of death.[1−3] There are approximately 400 million people with chronic HBV infection.[4] Chronic HBV infection leads to liver damage, cirrhosis, and hepatocellular carcinoma.[4,5] HBV is probably the major cause of liver cancer worldwide. According to the World Health Organization report, each day ∼1500 people die of hepatocellular carcinoma and ∼3000 people die of liver cirrhosis, both of which are secondary to HBV infections. HBV infections are increasingly serious problems among homo- and heterosexual populations, intravenous drug users, organ transplant recipients, patients on renal dialysis, and cancer patients receiving chemotherapy.[6] HBV infections are present in people in all parts of the world. HBV infections are also common among human immunodeficiency virus (HIV) infected individuals because it has a similar mode of transmission.

New infection with HBV can be prevented by vaccination. However, the present vaccination is not effective for ∼400 million chronic carriers worldwide. An HBV infected mother can pass the infection to her nonvaccinated infant at the time of birth.[7] It has been observed that suppression of the replication of HBV in the liver leads to improved liver pathology and decreased progression to liver cirrhosis and hepatocellular carcinoma. Antiviral therapy can be aimed to clear the virus load in body fluids of the infected people and therefore reduce the risk of viral transmission to others.

The major therapeutic option for HBV carriers is α-interferon. However, use of α-interferon is limited, success rate is low, and serious side effects are observed.[8,9] Nucleoside analogues have gained increasing importance as antiviral agents. The mechanism of action of nucleoside analogues is suggested to be through the interaction of their triphosphate derivatives, formed after cellular metabolic transformation, with HBV DNA polymerase or reverse transcriptase as substrates and/or inhibitors. HBV is an incomplete double-stranded DNA virus. Its DNA replication is unique and includes a reverse transcriptase catalyzed reverse transcription step.[10] The fact that HBV DNA polymerase is quite different than human DNA polymerases suggests that compounds that selectively inhibit HBV replication can be identified.[11]

Lamivudine [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine, 3-TC, **1a**, Chart 1], a pyrimidine nucleoside analogue, has been approved for the treatment of HBV infection. The administration of lamivudine induces significant drop in the amount of virus in the serum of most HBV carriers and causes no significant side effects in most patients. Unfortunately, emergence of drug resistant HBV may begin after 6−12 months of therapy.[12] In addition, long-term remission after completion of treatment with lamivudine is not commonly observed, and most patients experience a rebound in viremia once the

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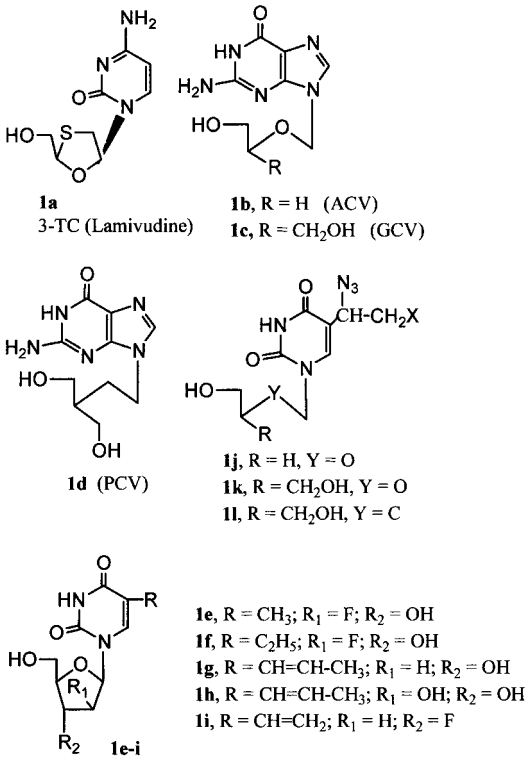
Chart 1



use of drug is stopped.[13,14] The rebound in viremia has been suggested to be due to either unaffected virus infected cells against which nucleoside was ineffective[15] and/or intranuclear covalently closed circular DNA (CCC DNA) species whose replicative intermediates are nonresponsive to lamivudine.[16,17]

A promising class of nucleoside analogues for antiviral chemotherapy belongs to a group with open chain "acyclic" sugar moieties.[18] The effectiveness of acyclic nucleoside analogues as a substrate or inhibitor of viral enzymes is likely dependent on the ability of the acyclic side chain to mimic the interaction of the glycosyl portion of the natural substrate with the enzyme. The flexibility of the acyclic chain may allow it to adopt a conformation favorable for enzyme interaction as substrates or inhibitors.[18]

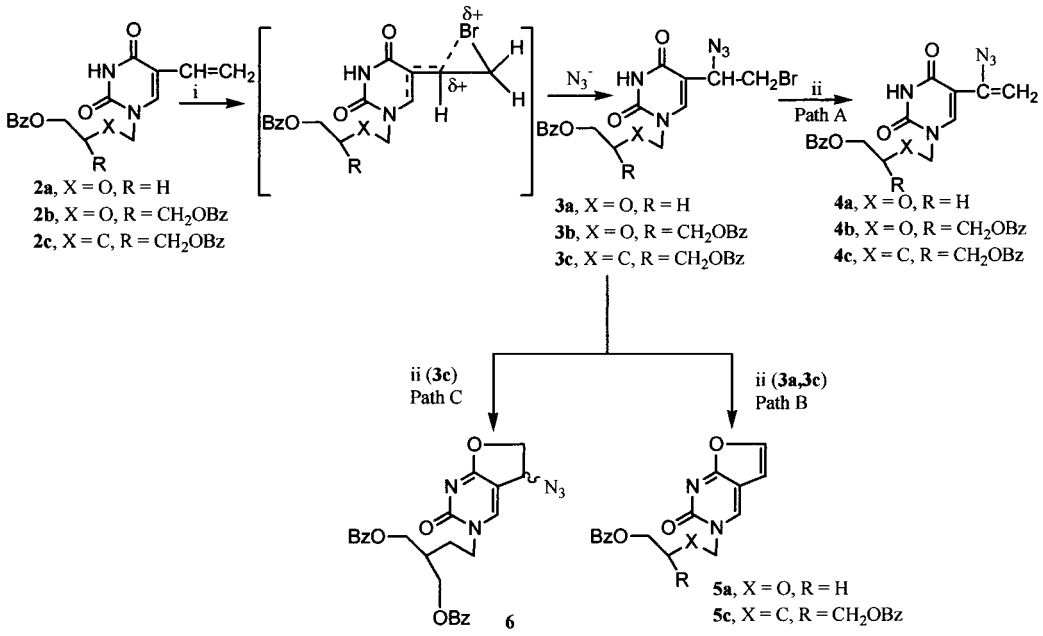
Among acyclic purine nucleosides, Acyclovir (ACV, **1b**), 9-[(2-hydroxyethoxy)methyl]guanine has been shown to be a modest inhibitor of HBV DNA polymerase in triphosphate form, exhibited both in vitro and in vivo activity in animal models, but demonstrated disappointing anti-HBV efficacy in human clinical trials.[19] Ganciclovir (GCV, **1c**), 9-[(2-hydroxy-1-(hydroxymethyl)-ethoxy)methyl]guanine, structurally similar to ACV but differs in having the functional equivalent of a 3′-OH group, is a potent inhibitor of HBV, both in cell culture and in vivo in animal models.[20,21] However, long-term clinical use of GCV is limited due to its severe dose-related toxicity.[22] Penciclovir (PCV, **1d**), 9-[4-hydroxy-3-(hydroxymethyl)-1-butyl]guanine, is an acyclic nucleoside closely related to GCV with the exception that the ether oxygen in the acyclic side chain is replaced with a methylene bridge. PCV has shown anti-HBV activity in vitro and in vivo.[23] The basis of potent and selective anti-HBV activity of PCV is at the HBV DNA polymerase level. The triphosphate derivative of PCV has a very high affinity for HBV DNA polymerase and a low affinity for the cellular DNA polymerase and is a potent inhibitor of HBV DNA polymerase in vitro.[23,24] Thus, acyclic moieties contribute significantly to potent anti-HBV activity and selectivity. Despite excellent anti-HBV activity, all of these purine analogues (ACV, GCV, PCV) are less water-soluble and are poorly absorbed when given orally to rodents and humans.[25] In contrast, homologous acyclic derivatives of pyrimidine nucleosides were found to have excellent water solubility, which could increase their bioavailabilty as well as facilitate formulation.[26] To increase oral bioavailability, famciclovir, 9-[4-acetoxy-3-(acetoxymethyl)-1-butyl]-guanine, a prodrug of penciclovir has been investigated. Unfortunately, in a placebo-controlled phase III trial, famciclovir did not exhibit sufficient efficacy and has been stopped from further development.[27]

It has been shown that chronic infection of hepatocytes is maintained by the presence of 30−40 copies of viral covalently closed circular (ccc) DNA in the hepatocyte nucleus. Viral cccDNA is the template for virus transcription and is dependent upon viral DNA synthesis.[28] Therefore, it can be envisioned that continuous and complete suppression of HBV DNA replication may deplete cccDNA. It has been shown in in vitro culture that the combination of 3-TC and PCV act synergistically as anti-HBV agents and can substantially reduce cccDNA.[29] However, short-term therapy with HBV DNA synthesis inhibitors cannot deplete the pool of cccDNA, and this could be one reason for rapid rebound of viral replication after cessation of therapy.[30] Continuous long term therapy with an array of potent nontoxic individual anti-HBV agents or combination of anti-HBV agents may lead to continuous inhibition of viral DNA synthesis resulting eventually in the depletion of the cccDNA pool. In addition, continuous suppression of viral DNA synthesis may lead to restoration of T cell immune responses,[31] which in turn could lead to elimination of HBV infected cells from chronically infected patients. Thus, there is a tremendous clinical need to investigate novel classes of antiviral agents for the chemotherapy of HBV infections.

Among many pyrimidine nucleosides that have been studied, C-5 alkyl substituted pyrimidine nucleosides with 2′-fluoro substituted arabinosyl analogues, FMAU (**1e**) [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil] and FEAU (**1f**) [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil], have been found to exhibit potent in vitro and in vivo anti-HBV activity.[32] The triphosphate derivatives of these analogues inhibited viral DNA polymerase of HBV in vitro.[33] However, all of these have been shown to be toxic in vivo.[32] In a study of 5′-triphosphates of 2′-deoxyuridine and 2′-arabinouridine analogues of 5-substituted pyrimidine nucleosides, 5-propenyl derivatives (**1h**,**i**) were the most potent inhibitors of DHBV DNA polymerase with high selectivity relative to cellular DNA polymerase-α.[34] In a group of 5-substituted 3′-fluoro-2′,3′-dideoxyuridine analogues in the triphosphate form, structure−activity correlation studies indicated that substitution at the C-5 position of the pyrimidine by an alkyl group including a double bond (**1i**) increases the inhibitory activity against human HBV DNA polymerase.[35]

Therefore, several strategies based on nucleoside analogues have evolved for the treatment of HBV infection

Scheme 1[a]



[a] Reagents: (i) *N*-bromosuccinimide, sodium azide, DME, 0 °C; (ii) *t*-BuOK, THF, 0 °C.

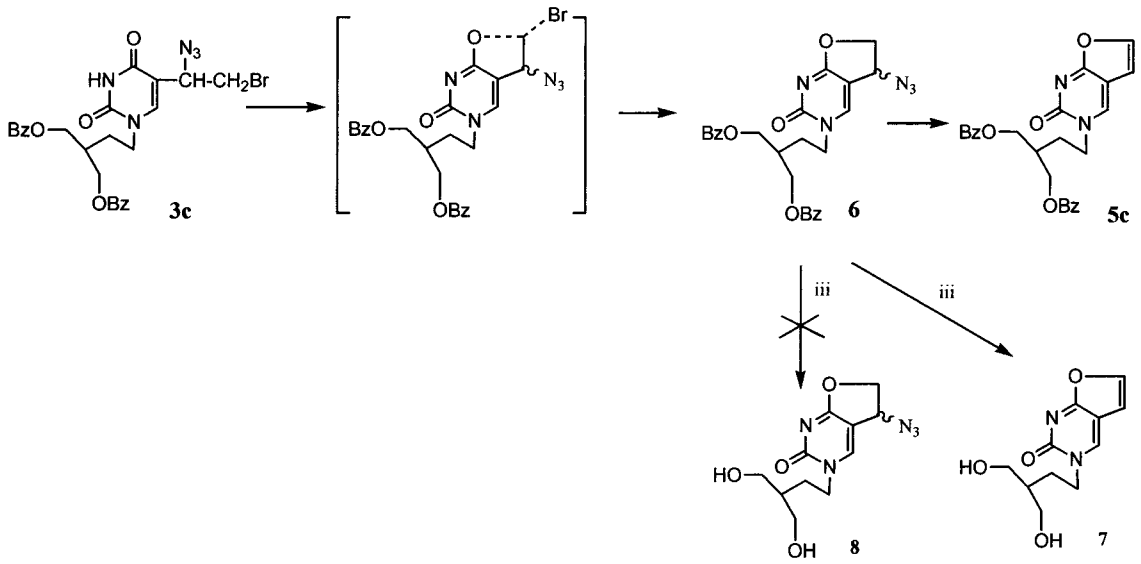
which include (1) substitution of a C-atom in the sugar ring by a heteroatom,[12] (2) β-L-stereochemistry,[11] (3) acyclic purine nucleosides and their derivatives,[19−23] and (4) 5-substituted pyrimidine nucleosides.[32] However, 5-substituted acyclic pyrimidine nucleosides have not been studied for anti-HBV activity.[16,17,36,37] Recently, we reported the synthesis of 5-(1-azidoethyl) analogues (**1j**−**l**) containing acyclic moieties at the N-1 position. The acyclic pyrimidine nucleosides **1k**,**l** exhibited appreciable in vitro anti-DHBV activity (EC_{50} = 0.1−5 μg/mL), whereas related homologues (**1j**) were inactive or moderately active (EC_{50} = >10 μg/mL).[38] To expand the structure−activity relationship and to improve anti-HBV activity, we now report the synthesis, anti-DHBV activity, and toxicity of a novel class of 5-substituted acyclic pyrimidine nucleosides (**9a**−**c**, **10b**,**c**). The 5-(1-azidovinyl) analogues (**9a**−**c**) can be considered to be hybrids of 5-(1-azidoethyl) (**1j**−**l**) and 5-vinyl substituent (**1i**). 1-[(2-Hydroxyethoxy)methyl]-5-(1-azidovinyl)uracil (**9a**), 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-(1-azidovinyl)uracil (**9b**), and 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-(1-azidovinyl)uracil (**9c**) were found to exhibit highly potent and selective anti-DHBV activity in vitro. In contrast, related analogues of **9b**,**c** in which the 5-substituent had undergone intramolecular cyclization to provide 5-[2-(1-azirinyl) analogues of 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)]uracil (**10b**) and 1-(4-hydroxy-3-(hydroxy methyl butyl)uracil (**10c**), respectively, were devoid of anti-DHBV activity.

Chemistry

The regiospecific addition of bromine azide to the 5-vinyl substituent of the uracil derivatives (**2a**−**c**) yielded 1-[(2-benzoyloxyethoxy)methyl]-5-(1-azido-2-bromoethyl)uracil (**3a**, 45.5%), 1-[(2-benzoyloxy-1-(benzoyloxymethyl)ethoxy]methyl)]-5-(1-azido-2-bromoethyl)uracil (**3b**, 84.8%), and 1-(4-benzoyloxy-3-benzoyloxymethyl-but-1-yl)-5-(1-azido-2-bromoethyl)uracil (**3c**, 81.5%), respectively. The ^{13}C NMR (*J* modulation) spectrum of **3a** provided conclusive evidence for the regiospecific addition of bromine azide across the 5-vinyl substituent of **2a**. For example, the bromine atom in **3a** is attached to a methylene carbon that exhibited resonance at δ 34.0, whereas the azido substituent is attached to a chiral methine carbon that exhibited resonance at δ 58.2. This regiospecific addition is consistent with reports that unsymmetrical olefins, capable of halonium ion formation, were found to favor an unsymmetrical bridged intermediate of the type illustrated in Scheme 1, even in solvents having a high dipole moment.[39] Reaction of 1-[(2-benzoyloxyethoxy)methyl]-5-(1-azido-2-bromoethyl)uracil (**3a**) with *t*-BuOK in THF yielded the 5-(1-azidovinyl) analogue **4a** (38.7%) and bicyclic compound **5a** (9.8%). A similar reaction employing 1-[(2-benzoyloxy-1-(benzoyloxymethyl)ethoxy)methyl]-5-(1-azido-2-bromoethyl)uracil (**3b**) yielded one major compound, 5-(1-azidovinyl) analogue **4b** (66%). In contrast, reaction of 1-(4-benzoyloxy-3-benzoyloxymethyl-1-butyl)-5-(1-azido-2-bromoethyl)uracil (**3c**) with *t*-BuOK in THF gave rise to three products. Thus, the direct elimination of HBr yielded 1-(4-benzoyloxy-3-benzoyloxymethyl-1-butyl)-5-(1-azidovinyl)uracil (**4c**, 51.6%, path A), whereas intramolecular cyclization reactions gave rise to the bicyclic products 5-(4-benzoyloxy-3-benzoyloxymethyl-1-butyl)furano-[2,3-*d*] pyrimidin-6-(5*H*)-one (**5c**, 15%, path B) and 3-azido-2,3-dihydro-5-(4-benzoyloxy-3-benzoyloxymethyl-1-butyl)furano-[2,3-*d*] pyrimidin-6-(5*H*)-one (**6**, 1.1%, path C), respectively.

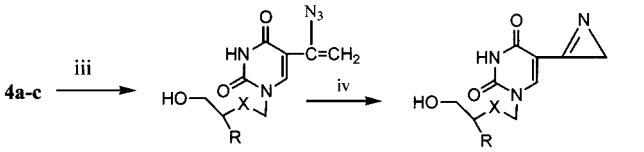
The base-catalyzed intramolecular cyclization reactions of **3c** to **6**, as illustrated in Scheme 2, is analogous to the reported conversion of 5-[2-[(methylsulfonyl)oxy]-ethyl]uracil to 2,3-dihydrofurano[2,3-*d*]pyrimidin-6(5*H*)-one using *t*-BuOK in DMSO.[40] The mechanism responsible for the formation of the bicyclic compound **5c** proceeds via an elimination reaction involving expulsion of HN_3 from **6** as shown in Scheme 2, since treatment of **6** with a saturated solution of ammonia in methanol gave the bicyclic derivative **7**, rather than the azido compound **8** (Scheme 2). Deprotection of **4a**−**c** with a saturated solution of NH_3 in MeOH yielded target 5-(1-

Scheme 2[a]



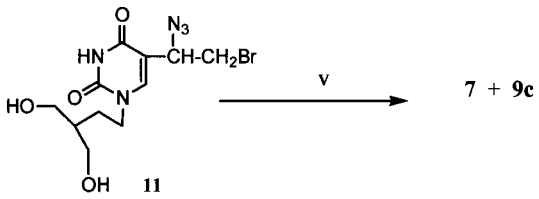
[a] Reagents: (iii) NH_3, MeOH, 25 °C.

Scheme 3[a]



[a] Reagents: (iii) NH_3, MeOH, 25 °C; (iv) dioxane, 110 °C.

Scheme 4[a]



[a] Reagents: (v) NaOH, or *t*-BuOK, DMSO or DME, 25 °C.

azidovinyl) analogues of 1-[(2-hydroxyethoxy)methyl]-uracil (**9a**, 60.8%), 1-[(2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl)]uracil (**9b**, 58.4%), and 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]uracil (**9c**, 79%) (Scheme 3). Thermal decomposition, using a procedure similar to that reported for the conversion of α-azidostyrene to phenylazirine,[41] of the 5-(1-azidovinyl) compounds **9b** and **9c** in dry dioxane at 110 °C yielded the corresponding 5-[2-(1-azirinyl)] analogues **10b** and **10c** in 35.7 and 26% yield, respectively (Scheme 3). In contrast, the above reaction employing **9a** produced the desired product (**10a**) as indicated by the thin-layer chromatography, but could not be eluted out from the silica gel column due to decomposition.

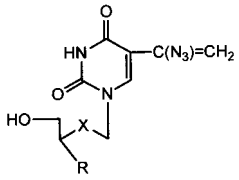
The synthesis of target compound 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-(1-azidovinyl)uracil (**9c**) was also attempted by the reaction of unprotected 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-(1-azido-2-bromoethyl)uracil (**11**) with either *t*-BuOK or NaOH using DMSO or DME as solvent, which produced bicyclic compound **7** (56%) and a low yield of **9c** (11%) (Scheme 4).

Results and Discussion

The anti-DHBV activity for the new class of acyclic pyrimidine nucleosides, 5-(1-azidovinyl)-(**9a**−**c**) and related 5-[2-(1-azirinyl)-(**10b**,**c**) analogues, along with the reference antiviral drug 3-TC was assessed in confluent cultures of primary duck hepatocytes obtained from chronically infected Pekin ducks. These cells chronically produce HBV DNA, and therefore antiviral activity was determined by analysis of viral DNA, using dot blot hybridization. Duck hepatitis B virus (DHBV), a member of hepadnaviridae, shares properties of hepatotropism, virion structure, genome organization, replication, and epidemiology with human HBV.[42] DHBV-based in vitro and in vivo systems have been used extensively to screen drugs for potential anti-HBV activity.[43] It has been shown that compounds such as 3-TC and PCV, both potent inhibitors of DHBV, are also potent inhibitors of HBV in chimpanzees as well as humans.[44] The concentrations required to inhibit 50% of DHBV DNA (EC_{50}), 50% cytotoxic concentration (CC_{50}) on stationary cells, and 50% inhibitory concentration (IC_{50}) on proliferating cells, are shown in Table 1. Purine acyclic nucleosides (GCV and PCV) are also included in Table 1 for comparison of their anti-DHBV potency.

Among the new class of acyclic pyrimidine nucleosides, 5-(1-azidovinyl)-(**9a**−**c**) analogues exhibited potent in vitro activity against DHBV (EC_{50} = 0.01−0.1 μg/mL). The anti-DHBV activity exhibited by analogues **9a**−**c** compares favorably to that of reference drug 3-TC (EC_{50} = 0.01−0.05 μg/mL). Surprisingly, compounds **9a**−**c** were found to be significantly more potent inhibitors of DHBV replication compared to the corresponding potent acyclic purine nucleosides (**1b**−**d**), as measured previously in the DHBV infected primary duck hepatocyte cultures.[45] The 5-(1-azidovinyl)uracil (**9b**) with 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] substituents at N-1 possessed higher activity (EC_{50} = 0.01−0.1 μg/mL) than GCV (EC_{50} = 1.5 μg/mL). Similarly, 5-(1-azidovinyl)uracil (**9c**) containing 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl] moiety demonstrated superior activity (EC_{50} = 0.01−0.05 μg/mL) than PCV (EC_{50} = 0.3 μg/mL). This study reveals an unexpected difference be-

Table 1. In Vitro Antiviral Activity against Hepatitis B Virus in Primary Duck Hepatocyte Cultures (DHBV) and Toxicity in Stationary and Proliferating Cells for 5-Substituted Uracils (**9a**−**c**) and Reference Compounds



9a-c

			% inhibition at 10 μg/mL[a] [EC_{50} (μg/mL)][b]	toxicity CC_{50} (μg/mL)		cell proliferation IC_{50} (μg/mL)		
no.	X	R	DHBV primary duck hepatocytes	HFF[c]	Vero[e]	HFF[f]	Daudi[g]	human T cells[h]
9a	O	H	84 [0.01−0.1]	>100[d]	>100	>100	>50	>50
9b	O	CH_2OH	86 [0.01−0.1]	>100	>200	100	>50	>50
9c	C	CH_2OH	93 [0.01−0.05]	>100	>200	>100	>50	>50
3-TC[i]	−	−	96 [0.01−0.05]	ND	>100	ND	ND	>50
GCV[j,k]			[1.5]					
PCV[j,l]			[0.3]					

[a] The data are expressed as percent inhibition of viral DNA in the presence of 10 μg/mL of the test compounds as compared to untreated infected controls. [b] The drug concentration (μg/mL) required to reduce the viral DNA in infected cells to 50% of untreated infected controls. [c] The drug concentration (μg/mL) required to reduce the uptake of neutral red stain by uninfected cell monolayers to 50% of untreated uninfected human foreskin fibroblast (HFF) cell controls after 7 days. [d] ">" sign indicates that 50% inhibition was not reached at the stated (highest) concentration tested. [e] The drug concentration required to reduce the viability of Vero cells as determined by MTT assay, by 50% of untreated control after 3 days. [f] The drug concentration (μg/mL) required to reduce the proliferation of HFF cells to 50% of untreated controls. [g] The drug concentration (μg/mL) required to reduce the proliferation of Daudi cells to 50% of untreated controls. [h] The drug concentration (μg/mL) required to reduce the proliferation of PHA stimulated human peripheral blood T lymphocytes to 50% of untreated PHA stimulated controls. [i] (−)-β-L-2′,3′-dideoxy-3′-thiacytidine. [j] Data taken from Shaw et al.[45] [k] Ganciclovir. [l] Penciclovir.

tween novel N-alkoxy derivatives of pyrimidine and purine in their ability to inhibit DHBV replication and emphasizes the influential effect of the novel 5-substituted pyrimidine base.

Comparison of anti-DHBV activity of **9a**−**c** with their related 5-(1-azidoethyl) analogues (**1j**−**l**) revealed that anti-DHBV activity of **1j** (EC_{50} = >10 μg/mL)[38] was remarkably improved by alteration of the 5-(1-azidoethyl) substituent to the 5-(1-azidovinyl) moiety (**9a**, EC_{50} = 0.01−0.1 μg/mL). Similar observations were also obtained with novel compounds **9b** (EC_{50} = 0.01−0.1 μg/mL) and **9c** (EC_{50} = 0.01−0.05 μg/mL), when compared with their respective 5-(1-azidoethyl) analogues (**1k,** EC_{50} = 1−10 μg/mL; **1l**, EC_{50} = 0.1−10 μg/mL).[38] These results suggest that the 1-azidovinyl moiety at the 5-position of the pyrimidine ring of **9a**−**c** is an important determinant for potent anti-HBV activity. Compounds **9a**−**c** inhibited DHBV replication at concentrations that were significantly lower than the highest concentration range tested to determine the toxicity to several host cells (Table 1). Up to a concentration of 50 μg/mL, compounds **9a**−**c** did not have any visual effect on morphology and viability of duck hepatocytes.

The precise mechanism of action of these compounds is currently unknown. However, by analogy with other antiviral nucleosides, the potent and selective anti-DHBV activity of compounds **9a**−**c** is likely due to their phosphorylation by cellular kinases followed by selective inhibition of HBV DNA synthesis by acting as substrate and/or as inhibitor of HBV DNA polymerase by their triphosphate derivatives.[36,37] The derivative **9c**, with 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl] moiety demonstrated remarkable anti-DHBV activity with a 50% effective concentration (EC_{50}) of 0.01−0.05 μg/mL, while compounds **9a** and **9b** with 1-[(2-hydroxyethoxy)methyl] and 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] moieties, respectively, were slightly less inhibitory to DHBV replication (EC_{50} = 0.01−0.1 μg/mL) (Table 1). The observation that 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-(1-azidovinyl)uracil (**9c**) showed better activity than 1-[(2-hydroxyethoxy)methyl]-5-(1-azidovinyl)-uracil (**9a**) and 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)-methyl]-5-(1-azidovinyl)uracil (**9b**) is consistent with previous observations reported in the case of purine acyclic nucleosides that 9-[4-hydroxy-3-(hydroxymethyl)-1-butyl]guanine (PCV) exhibited higher anti-DHBV activity than 9-[(2-hydroxyethoxy)methyl]guanine (ACV) and 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-guanine (GCV).[45] It has been suggested that greater antiviral potency of PCV relative to that of GCV is probably partly due to the increased intracellular stability of its nucleotides, as well as other factors such as phosphorylation efficiency or recognition by the viral polymerase.[45]

Interestingly, modification of the 5-(1-azidovinyl) substituent in the novel pyrimidine acyclic nucleosides (**9b**,**c**) to the corresponding 5-[2-(1-azirinyl)] analogues (**10b**,**c**) provided compounds that were devoid of any anti-DHBV activity in the in vitro assay at 10 μg/mL. These results suggested that an acyclic substituent at the C-5 position of the pyrimidine ring is essential for anti-HBV activity.

The cytotoxic activities of compounds **9a**−**c** and **10b**,**c** were also determined by the National Cancer Institute (NCI, USA) using an in vitro assay[46] in which compounds were evaluated against approximately 60 human tumor cell lines (e.g., melanoma, leukemia, non-small cell lung, small cell lung, colon, central nervous system, ovarian, prostate, and renal cancers). None of the compounds showed significant activity or selectivity in these assays at the highest concentration tested (30 μg/mL) (data not shown), indicating that novel compounds **9a**−**c** and **10b**,**c** do not have cytotoxicity against various human cell lines nor any anticancer activity.

The potent anti-HBV compounds **9a**−**c** were tested in vitro for their toxicity against several other cell lines (Table 1). None of these compounds exhibited in vitro

toxicity against stationary phase cells [Vero cells (CC_{50} > 100 or > 200 μg/mL), HFF cells (CC_{50} > 100 μg/mL)] and proliferating cell lines [HFF (IC_{50} ≥ 100 μg/mL), Daudi (IC_{50} > 50 μg/mL)]. In rapidly proliferating fresh human T lymphocyte cell culture, cellular DNA synthesis, as monitored by the incorporation of [methyl-^{3}H]-thymidine into DNA, was also not affected by compounds **9a**−**c** in concentrations up to 50 μg/mL (highest concentration tested).

Summary

In conclusion, we present the synthesis and in vitro biological evaluation of a novel class of hitherto unknown 5-substituted acyclic pyrimidine nucleosides (**9a**−**c**, **10b**−**c**). Compounds possessing a 5-(1-azidovinyl) substituent were found to be strong and selective inhibitors of DHBV replication in vitro at concentrations several orders of magnitude lower than their toxicity thresholds in a variety of cell lines. The fact that the compounds **9a**−**c** show potent and selective anti-DHBV activity suggests that they have higher affinity for the virus specific DNA polymerase than the host cell enzymes. Among the compounds investigated, **9c** emerged as the most potent and selective anti-DHBV agent. The spectrum of anti-DHBV activity and cytotoxicity of compounds **9a**−**c** in culture is similar or better to that of established anti-HBV agents that are currently used clinically for HBV infections. Thus, compounds **9a**−**c** have emerged as a new series of compounds with potent anti-DHBV activity and merit further studies to develop as potential chemotherapeutic agents for anti-HBV therapy. Additional in vitro and in vivo tests as well as studies of structure−activity relationships and mechanism of action of this new family of compounds are ongoing in our laboratories.

Experimental Section

Melting points were determined with a Buchi capillary apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were determined for solutions in $CDCl_3$, DMSO-d_6, or CD_3OD on a Bruker AM 300 spectrometer using Me_4Si as an internal standard (^{1}H NMR). The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of the D_2O. ^{13}C NMR spectra were acquired using the *J* modulated spin−echo technique where methyl and methine carbon resonances appear as positive peaks, and methylene and quaternary carbons appear as negative peaks. Microanalyses were within ±0.4% of theoretical values for all elements listed, unless otherwise indicated. Preparative thin-layer chromatography (PTLC) was performed using Whatman PLK 5F plates, 1.0 mm in thickness, and silica gel column chromatography was carried out using Merck 7734 silica gel (100−200 μM particle size). ***Warning***: Halogenated solvents such as dichloromethane must not be used in certain reactions, such as those described for the preparation of products **3a**−**c**, since its reaction with sodium azide may produce potentially explosive polyazidomethane.

1-[(2-Benzoyloxyethoxy)methyl]-5-vinyluracil (2a). Benzoyl chloride (410 mg, 2.9 mmol) was added to a solution of 1-[(2-hydroxyethoxy)methyl]-5-vinyluracil[38] (614 mg, 2.89 mmol) in dry pyridine (30 mL), and the reaction was allowed to proceed at 25 °C with stirring for 6 h. Removal of the solvent in vacuo and purification of the product by elution from a silica gel column using chloroform−methanol (99:1, v/v) as eluent gave **2a** as a viscous oil (615 mg, 67.3%) which was used directly in the next reaction step.

1-[(2-Benzoyloxy-1-(benzoyloxymethyl)ethoxy)methyl]-5-vinyluracil (2b). A mixture of palladium(II) acetate (26 mg, 0.11 mmol), triphenylphosphine (57 mg, 0.22 mmol), and triethylamine (1.5 mL) (dried over calcium hydroxide) in dry DMF (50 mL) was maintained at 70 °C with stirring until an intense red color appeared. 1-[(2-Benzoyloxy-1-(benzoyloxymethyl)ethoxy)methyl]-5-iodouracil[47] (1.0 g, 1.81 mmol) and vinyl acetate (10 mL, 110 mmol) were then added, and the reaction was allowed to proceed at 70 °C for 6 h with stirring. The solvent was removed in vacuo, and the residue obtained was purified by elution from a silica gel column starting with chloroform and then changing to chloroform−methanol (98:2, v/v). Removal of the solvent from the combined fractions containing the desired products yielded **2b** as a viscous oil (480 mg, 59%). ^{1}H NMR ($CDCl_3$) δ: 4.4−4.65 (m, 5H, C*H*, OCH_2), 5.20 (dd, J = 11 Hz, J = 2.1 Hz, 1H, −CH=C*H* H′), 5.38 (m, 2H, NCH_2), 5.86 (dd, J = 18.0 Hz, J = 2.1 Hz, 1H, −CH=CH*H*′), 6.28 (dd, J = 18 Hz, J = 11.0 Hz, 1H, −C*H*=CHH′), 7.30−8.05 (m, 11H, H-6, benzoyl hydrogens), 8.26 (s, 1H, NH). Anal. ($C_{24}H_{22}N_2O_7$) C, H, N.

1-[4-Benzoyloxy-3-(benzoyloxymethyl)-1-butyl]-5-vinyluracil (2c). Benzoyl chloride (600 mg, 4.2 mmol) was added to a solution of 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-vinyluracil[38] (450 mg, 1.87 mmol) in dry pyridine (50 mL), and the reaction was allowed to proceed at 25 °C with stirring for 48 h. The reaction mixture was poured onto ice water (50 mL) and extracted with dichloromethane (3 × 50 mL). The dichloromethane extract was dried over sodium sulfate, the solvent was removed in vacuo, and the residue obtained was purified by elution from a silica gel column using chloroform−methanol (97:3, v/v) as eluent to yield **2c** as a viscous oil (550 mg, 65.4%), which was used directly in the next reaction step.

1-[(2-Benzoyloxyethoxy)methyl]-5-(1-azido-2-bromoethyl)uracil (3a). *N*-Bromosuccinimide (NBS, 350 mg, 1.96 mmol) was added in aliquots to a precooled (−5 °C) suspension prepared by mixing a solution of **2a** (61 mg, 1.95 mmol) in 1,2-dimethoxyethane (25 mL) with a solution of sodium azide (510 mg, 7.84 mmol) in water (1.2 mL). The initial yellow color faded away after each addition. When all the NBS was consumed, the reaction mixture was allowed to stand at 0 °C for 30 min, prior to pouring onto ice−water (25 mL) and extraction with dichloromethane (3 × 50 mL). The dichloromethane extract was washed with cold water (25 mL) and dried (Na_2SO_4), and the solvent was removed in vacuo to yield a residue which was purified by elution from a silica gel column using chloroform:methanol (98:2, v/v) as eluent to yield **3a** (388 mg, 45.5%) as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 3.58 (m, 1H, C*H*H′Br), 3.78 (m, 1H, CH*H*′Br), 3.97 (m, 2H, OCH_2-CH_2OBz), 4.48 (m, 2H, OCH$_2$CH_2OBz), 4.83 (m, 1H, C*H*N$_3$), 5.28 (s, 2H, NCH_2), 7.42−8.06 (m, 6H, H-6, benzoyl hydrogens), 9.90 (s, 1H, NH, exchanges with deuterium oxide). ^{13}C NMR ($CDCl_3$) δ: 34.0 (*C*H$_2$Br), 58.2(*C*HN$_3$), 63.3 (O*C*H$_2$*C*H$_2$), 68.0 (N*C*H$_2$), 111.5 (C-5), 128.4, 129.6, 129.7, 133.2 (Ar−C), 141.8 (C-6), 150.6 (C-2 *C*=O), 162.0 (C-4 *C*=O), 166.3 (Ar *C*=O). Anal. ($C_{16}H_{16}N_5O_5Br$) C, H, N.

1-[(2-Benzoyloxy-1-(benzoyloxymethyl)ethoxy)methyl]-5-(1-azido-2-bromoethyl)uracil (3b). Reaction of **2b** (30 mg, 0.066 mmol) with *N*-bromosuccinimide (13 mg, 0.073 mmol), using the procedure described for the preparation of **3a**, and purification of the product by silica gel column chromatography using dichloromethane−methanol (97:3, v/v) as eluent afforded **3b** as a viscous oil (32 mg, 84.8%). ^{1}H NMR ($CDCl_3$) δ: 3.45 (m, 1H, C*H*H′Br), 3.65 (m, 1H, CH*H*′Br), 4.40−4.65 (complex m, 6H, *C*H, OCH_2, C*H*N$_3$), 5.35 (m, 2H, NCH_2), 7.38−8.04 (m, 11H, H-6, benzoyl hydrogens), 8.30 (s, 1H, NH). Anal. ($C_{24}H_{22}N_5O_7Br$) C, H, N.

1-[4-Benzoyloxy-3-(benzoyloxymethyl)-1-butyl]-5-(1-azido-2-bromoethyl)uracil (3c). A mixture of **2c** (510 mg, 1.14 mmol), saturated solution of sodium azide in water (300 mg, 4.6 mmol), *N*-bromosuccinimide (223 mg, 1.25 mmol), and 1,2-dimethoxyethane (50 mL) was stirred at 0 °C for 30 min. Removal of the solvent in vacuo gave a viscous oil, which was purified by silica gel column chromatography. Elution with chloroform−methanol (96:4, v/v) yielded **3c** (530 mg, 81.5%) as a syrup. ^{1}H NMR ($CDCl_3$) δ: 1.92 (m, 2H, NCH$_2$CH_2), 2.36 (m, 1H, C*H*), 3.52−3.80 (m, 2H, NCH_2), 4.0 (m, 2H, CH_2Br), 4.48 (m, 4H, OCH_2), 4.82 (m, 1H, C*H*N$_3$), 7.36−8.0 (m, 11H,

H-6, benzoyl hydrogens), 9.38 (s, 1H, NH). Anal. ($C_{25}H_{24}N_5O_6$-Br) C, H, N.

[(2-Benzoyloxyethoxy)methyl]-5-(1-azidovinyl)uracil (4a) and 5-[(2-Benzoyloxyethoxy)methyl]furano-[2,3-*d*]-pyrimidin-6-(5*H*)-one (5a). Potassium *tert*-butoxide (425 mg, 1.87 mmol) was added to a suspension of **3a** (832 mg, 2.58 mmol) in dry THF (100 mL) at −5 °C with stirring. The cooling bath was removed, and the reaction mixture was stirred at 0 °C for 3 h. Removal of the solvent in vacuo gave a residue which was dissolved in dichloromethane (50 mL) and washed with cold water (25 mL), the dichloromethane fraction was dried (Na_2SO_4), and the solvent was removed in vacuo. The reaction mixture was separated by silica gel column chromatography using chloroform−methanol (95:5, v/v) as eluent to give two products, which eluted in the following order:

Fraction 1 (**4a**): 358 mg, 38.7%; mp 129 °C. ^{1}H NMR ($CDCl_3$) δ: 3.98 (m, 2H, OCH_2CH_2), 4.50 (m, 2H, OCH$_2$CH_2), 5.08 [d, J = 2 Hz, 1H, C(N$_3$)=CHH′], 5.30 (s, 2H, NCH_2), 6.33 [d, J = 2 Hz, 1H, C(N$_3$)=CHH′], 7.40−8.10 (m, 6H, H-6, benzoyl hydrogens), 8.65 (s, 1H, NH). Anal. ($C_{16}H_{15}N_5O_5$) C, H, N.

Fraction 2 (**5a**): 80 mg, 9.8%; mp 180 °C. ^{1}H NMR ($CDCl_3$) δ: 4.08 and 4.50 (2m, 4H total, OCH_2CH_2), 5.52 (s, 2H, NCH_2), 6.40 (d, J = 2.5 Hz, 1H, OCH =CH), 7.32 (d, J = 2.5 Hz, 1H, OCH=CH), 7.40−8.02 (m, 5H, benzoyl hydrogens), 8.10 (s, 1H, H6). Anal. ($C_{16}H_{14}N_2O_5$) C, H, N.

1-[(2-Benzoyloxy-1-(benzoyloxymethyl)ethoxy)methyl]-5-(1-azidovinyl)uracil (4b). Potassium *tert*-butoxide (243 mg, 2.16 mmol) was added to a suspension of **3b** (620 mg, 1.08 mmol) in dry THF (250 mL) at −5 °C with stirring. The cooling bath was removed, and the reaction mixture was stirred at 5 °C for 3 h. Removal of the solvent in vacuo gave a residue, which was dissolved in dichloromethane and washed with cold water (25 mL). The dichloromethane fraction was dried (Na_2SO_4), and the solvent was removed in vacuo. The purification of the residue by elution from a silica gel column using dichloromethane−methanol (96:4, v/v) as eluent yielded **4b** as a viscous oil. ^{1}H NMR ($CDCl_3$) δ: 4.40−4.65 (m, 5H, CH, OCH_2), 5.0 [d, J = 2 Hz, 1H, C(N$_3$)=CHH′], 5.38 (s, 2H, NCH_2), 6.25 [d, J = 2 Hz, 1H, C(N$_3$)=CHH′], 7.40−8.08 (m, 11H, H-6, benzoyl hydrogens), 8.18 (s, 1H, NH). Anal. ($C_{24}H_{21}N_5O_7$) C, H, N.

1-[4-Benzoyloxy-3-(benzoyloxymethyl)-1-butyl]-5-(1-azidovinyl)uracil (4c), 5-[4-Benzoyloxy-3-(benzoyloxymethyl)-1-butyl]furano-[2,3-*d*]pyrimidin-6-(5*H*)-one (5c), and 3-Azido-2,3-dihydro-5-[4-benzoyloxy-3-(benzoyloxymethyl)-1-butyl]furano-[2,3-*d*]pyrimidin-6-(5*H*)-one (6). Reaction of potassium *tert*-butoxide (208 mg, 1.84 mmol) with **3c** (530 mg, 0.93 mmol), using the procedure described for the preparation of **4a** and purification of the product using chloroform−methanol (97:3, v/v) as eluent yielded three products which eluted in the following order:

Fraction 1 (**4c**): 235 mg, 51.6%; mp 111 °C dec. ^{1}H NMR ($CDCl_3$) δ: 1.95 (m, 2H, NCH$_2$CH_2), 2.40 (m, 1H, CH), 4.0 (t, J = 7.2 Hz, 2H, NCH_2), 4.50 (m, 4H, OCH_2), 5.05 [d, J = 2.0 Hz, 1H, C(N$_3$)=CHH′], 6.32 [d, J = 2.0 Hz, 1H, C(N$_3$)=CHH′], 7.40−8.08 (m, 11H, H-6, benzoyl hydrogens), 8.26 (s, 1H, NH). Anal. ($C_{25}H_{23}N_5O_6$) C, H, N.

Fraction 2 (**5c**): 63 mg, 15%. ^{1}H NMR ($CDCl_3$) δ: 2.08 (m, 2H, NCH$_2$CH_2), 2.38 (m, 1H, CH), 4.26 (t, J = 7.2 Hz, 2H, NCH_2), 4.48 (m, 4H, OCH_2), 6.50 [d, J = 2.6 Hz, 1H, OCH=CH], 7.32 [d, J = 2.5 Hz, 1H, OCH=CH], 7.38−8.08 (m, 11H, H-6, benzoyl hydrogens). Anal. ($C_{25}H_{22}N_2O_6$) C, H, N.

Fraction 3 (**6**): 5 mg, 1.1%. ^{1}H NMR ($CDCl_3$) δ: 1.92 (m, 2H, NCH$_2$CH_2), 2.38 (m, 1H, CH), 4.26 (m, 2H, NCH_2), 4.40 (m, 4H, OCH_2), 4.62−4.80 [m, 2H, furanyl CH_2], 4.90 [m, 1H, furanyl CHN$_3$], 7.38−8.08 (m, 11H, H-6, benzoyl hydrogens), Anal. ($C_{25}H_{22}N_2O_6\cdot{}^1/_4H_2O$) C, H, N.

1-[(2-Hydroxyethoxy)methyl]-5-(1-azidovinyl)uracil (9a). A solution of **4a** (357 mg, 1.0 mmol) in a saturated solution of ammonia in methanol (50 mL) was stirred at 25 °C for 84 h. Removal of solvent in vacuo yielded a residue which was purified by silica gel column chromatography using chloroform−methanol (92:8, v/v) as eluent to yield **9a** (154 mg, 60.8%) as a solid: mp 141−142 °C. ^{1}H NMR (DMSO-d_6) δ: 3.50 (m, 4H, OCH_2CH_2), 4.72 (m, 1H, OH), 5.03 [d, J = 2 Hz, 1H, C(N$_3$)=CHH′], 5.18 (s, 2H, NCH_2), 5.95 [d, J = 2 Hz, 1H, C(N$_3$)=CHH′], 7.98 (s, 1H, H-6), 11.68 (br, 1H, NH). ^{13}C NMR (CD_3OD) δ: 61.9 (OCH$_2$CH_2), 72.1 (OCH_2CH$_2$), 78.6 (NCH_2), 101.8 [C(N$_3$)=CH_2], 109.6 (C-5), 138.6 [C(N$_3$)], 144.0 (C-6), 152.0 (C-2 C=O), 163.2 (C-4 C=O). Anal. ($C_9H_{11}N_5O_4$) C, H, N.

1-[(2-Hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-(1-azidovinyl)uracil (9b). A solution of **4b** (350 mg, 0.71 mmol) in a saturated solution of ammonia in methanol (25 mL) was stirred at 25 °C for 48 h. Removal of solvent in vacuo yielded a residue which was purified by silica gel column chromatography using chloroform−methanol (90:10, v/v) as eluent to yield **9b** (120 mg, 58.4%) as a solid: mp 129−130 °C. ^{1}H NMR (DMSO-d_6) δ: 3.30−3.50 (m, 4H, OCH_2), 3.55 (m, 1H, CH), 4.65 (m, 2H, OH), 5.02 [d, J = 2 Hz, 1H, C(N$_3$)=CHH′], 5.25 (s, 2H, NCH_2), 5.92 [d, J = 2 Hz, 1H, C(N$_3$)=CHH′], 7.98 (s, 1H, H-6), 11.60 (s, 1H, NH);^{13}C NMR (CD_3OD) δ: 62.7 (OCH_2), 78.1 (NCH_2), 82.2 (CH), 101.7 [C(N$_3$)=CH_2], 109.5 (C-5), 138.7 [C(N$_3$)], 144.4 (C-6). Anal. ($C_{10}H_{13}N_5O_5$) C, H, N.

1-[4-Hydroxy-3-(hydroxymethyl)-1-butyl]-5-(1-azidovinyl)uracil (9c). A solution of **4c** (220 mg, 0.45 mmol) in a saturated solution of ammonia and methanol (30 mL) was stirred at 25 °C for 5 days. Removal of the solvent in vacuo yielded a residue that was purified by silica gel column chromatography using chloroform−methanol (92:8, v/v) as eluent to yield **9c** (100 mg, 79%) as a solid: mp 126−127 °C. ^{1}H NMR (CD_3OD) δ: 1.6−1.8 (m, 3H, CH, NCH$_2$CH_2), 3.6 (m, 4H, OCH_2), 3.90 (t, J = 7.2 Hz, 2H, NCH_2), 5.02 (d, J = 2 Hz, 1H, C(N$_3$)=CHH′), 6.10 (d, J = 2 Hz, 1H, C(N$_3$)=CHH′), 7.90 (s, 1H, H-6);^{13}C NMR (CD_3OD) δ: 29.0 (NCH$_2$CH_2), 41.9 (CH), 48.2 (NCH_2), 63.4 (OCH_2), 101.3 [C(N$_3$)=CH_2], 109.0 (C-5), 138.6 [C(N$_3$)], 145.0 (C-6), 151.9 (C-2, C=O), 163.4 (C-4, C=O). Anal. ($C_{11}H_{15}N_5O_4$) C, H, N.

1-[(2-Hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-[2-(1-azirinyl)]uracil (10b). A solution of **9b** (82 mg, 0.28 mmol) in dry dioxane (45 mL) was heated at 110 °C for 3 h. Removal of the solvent in vacuo gave a viscous oil, which was purified by silica gel column chromatography. Elution with chloroform−methanol (82:18, v/v) gave **10b** as a dark brown oil (26 mg, 35.7%). ^{1}H NMR (DMSO-d_6) δ: 1.30 (d, 2H, azirinyl hydrogens), 3.30−3.55 (m, 4H, OCH_2), 3.62 (m, 1H, CH), 4.70 (br, 2H, OH), 5.30 (s, 2H, NCH_2), 8.45 (s, 1H, H-6). ^{13}C NMR (CD_3OD) δ: 16.35 (azirinyl CH_2), 62.4 (OCH_2), 78.0 (NCH_2), 82.5 (CH), 102.7 (C-5), 154.0 (C-6), 161.6 (azirinyl C-2). Anal. ($C_{10}H_{13}N_3O_5\cdot{}^1/_2H_2O$) C, H, N.

1-[4-Hydroxy-3-(hydroxymethyl)-1-butyl]-5-[2-(1-azirinyl)]uracil (10c). A solution of **9c** (26 mg, 0.09 mmol) in dry dioxane (10 mL) was stirred at 110 °C for 4 h. Removal of the solvent in vacuo gave a dark brown residue, which was purified by PTLC using chloroform−methanol (80:20, v/v) as development solvent to yield **10c** (6 mg, 26%) as a dark yellow oil. ^{1}H NMR (CD_3OD) δ: 1.30−1.50 (m, 2H, azirinyl hydrogens), 1.65−1.85 (m, 3H, CH, NCH$_2$CH_2), 3.60 (m, 4H, OCH_2), 4.0 (t, J = 7.2 Hz, 2H, NCH_2), 8.42 (s, 1H, H-6). ^{13}C NMR (CD_3OD) δ: 16.15 (azirinyl CH_2), 29.0 (NCH$_2$CH_2), 42.2 (CH), 48.0 (NCH_2), 63.0 (OCH_2), 102.2 (C-5), 154.5 (C-6), 161.5 (azirinyl C-2). Anal. ($C_{11}H_{15}N_3O_5\cdot{}^1/_2H_2O$) C,H,N.

Reaction of 1-[4-Hydroxy-3-(hydroxymethyl)-1-butyl]-5-(1-azido-2-bromoethyl)uracil (11) with Sodium Hydroxide. A solution of sodium hydroxide (5 mg, 0.12 mmol) in water 12 μL) was added to a solution of **11**[38] (32.6. mg, 0.09 mmol) in DMSO (5 mL) at ice-bath temperature with stirring. Removal of the solvent under high vacuum gave a residue that was purified by silica gel column chromatography using chloroform−methanol (88:12, v/v) as eluent to yield a mixture of **9c** (3 mg, 11%) and **7** (12 mg, 56%). The ^{1}H NMR spectrum for this mixture of **9c** and **7** was identical to that of **9c** and **7** prepared by other methods described in this study.

Reaction of 3-Azido-2,3-dihydro-5-[4-benzoyloxy-3-(benzoyloxymethyl)-1-butyl]furano-[2,3-*d*]pyrimidin-6-(5*H*)-one (6) with Methanolic Ammonia. A solution of **6** (15 mg, 0.3 mmol) in a saturated solution of ammonia in methanol (1 mL) was stirred at 25 °C for 5 days. Removal of the solvent in vacuo and purification of the residue obtained

on PTLC using chloroform−methanol (90:10 v/v) as a development solvent afforded **7** (4.3 mg, 60%) as a syrup. ^{1}H NMR (CD_3OD) δ: 1.68−1.84 (m, 3H, C*H*, NCH_2CH_2), 3.58−3.64 (m, 4H, OCH_2), 2.75 (t, J = 7.2 Hz, 2H, NCH_2), 6.75 (d, J = 2.5 Hz, 1H, OC*H*═CH), 7.58 (d, J = 2.5 Hz, 1H, OCH═C*H*), 8.58 (s, 1H, H-6). ^{13}C NMR (CD_3OD) δ: 29.3 (NCH_2CH_2), 42.13 (*C*H), 51.6 (NCH_2), 63.5 (OCH_2), 105.8 (C-3), 107.9 (C-3a), 145.0 (C-4), 146.3 (C-2), 157.5 (C-6), 173.1 (C-7a). Anal. ($C_{11}H_{14}N_2O_4$), C, H, N.

In Vitro Antiviral Assay (Duck Hepatitis B Virus, DHBV). 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. Newly hatched ducklings were infected with a 50 μL intravenous injection of duck serum containing DHBV. Persistently infected ducks were identified by detection of DHBV DNA in sera by dot hybridization.[48]

Primary cultures of duck hepatocytes were prepared from 9−14 day old DHBV infected ducklings using a modified method.[43] Cells were cultured in 60 mm cell culture dishes in 4 mL of L-15 medium containing 5% fetal bovine serum, penicillin G sodium (10 IU/mL), streptomycin sulfate (10 μg/mL), and nystatin (25 U/mL). The test compounds were always added in triplicate to the hepatocyte cultures on day 2 and were maintained in culture with media changed every second day until day 12. Cells were harvested at day 14. 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 0.5 mg/mL 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 (5 M) 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 100 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 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 1xSSC (20xSSC in 3 M NaCl plus 0.3 M sodium citrate, pH 7.0)−0.1% SDS at 65 °C for 2 h and 1xSSC at room temperature for 30 min. After an autoradiographic image was obtained, the filters were exposed in a phosphoimaging screen for 1−2 h, samples were quantitated by a Fujix BAS1000, and the percentage density of phosphoimaging units were calculated.[43] 3-TC 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 three 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 following formula: (untreated positive control − treated test sample) × 100/untreated positive control.

Cell Cytotoxicity: Neutral Red Uptake Assay. Cytotoxicity of test compounds on human foreskin fibroblast (HFF) cells was determined using neutral red uptake assay as reported previously.[49]

MTT Assay. Cell viability was measured using the cell proliferation kit 1 (MTT; Boehringer Mannheim), as per manufacturer's instructions. Briefly, a 96-well plate was seeded with Vero cells at a density of 2.5×10^4 cells per well. Cells were allowed to attach for 6−8 h, the media was replaced with media containing drugs at concentrations of 200, 100, 50, 25, 12.5, 6.3, and 1.5 μg/mL. DMSO was also included as control. Plates were incubated for 3 days at 37 °C. The color reaction involved adding 10 μL of MTT reagent per well, incubating 4 h at 37 °C, and then adding 100 μL of solubilization reagent. Plates were read on an ELISA plate reader (Abs 560−650 nm) following an overnight incubation at 37 °C.

Cell Proliferation Assay: HFF and Daudi Cells. The effect of test compounds on proliferation of HFF cells was determined according to previously reported procedure.[49] The procedure for determining the effect of test compounds on proliferating Daudi cells was essentially the same as that for HFF cells except that the trypsin treatment to detach the cells from the wells was not required because Daudi wells grow in cell suspensions.

Human T Cells. Enriched T cell populations were purified from buffy coats obtained from normal Canadian Blood Service donors, by using nylon wool columns according to published procedures.[50] Test compounds dissolved in DMSO at 10 mg/mL were prepared as stock solutions. In 96-well flat bottom plates, test compounds were plated in triplicate wells starting from 50 μg/mL concentrations. Serial dilutions of compounds were prepared at 1:2, 1:5, or 1:10 dilutions for up to eight dilutions. All of the dilutions were made with serum free AIM V media for lymphocytes (Life Technologies, ON). Control wells with the same DMSO concentration as those with test compounds, were also prepared in triplicate wells. A total of 2×10^5 T cells in AIM V media were added to each of these prepared wells. This was followed by the addition of phytohemagglutinin (PHA, Sigma Chemical Company) to make up a final concentration of 1 μg/mL of PHA. Positive control wells with PHA and T cells and negative control wells with T cells in media alone, were also prepared on each plate. The cultures were incubated for 3−4 days in 5% CO_2 and 95% humidity at 37 °C. After 3−4 days, [^{3}H]thymidine (1 μCi/well) was added to each well. Eighteen hours after pulsing with radioactive thymidine, the cells were harvested onto nylon filters, and [^{3}H]-thymidine incorporation into the DNA of proliferating cells was measured by liquid scintillation counting. CPM of wells containing test compounds were always compared with CPM of the wells containing stimulated T cells and corresponding DMSO concentration.

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References

(1) Dusheiko, G. Hepatitis B. In *Oxford Textbook of Clinical Hepatology*; McIntyre, N., Eds.; Oxford University Press: New York, 1991, pp 571−592.
(2) Purcell, R. H. The Discovery of the Hepatitis Viruses. *Gastroenterology* **1993**, *104*, 955−963.
(3) Mast, E. E.; Alter, M. J.; Margolis, H. S. Strategies to Prevent and Control Hepatitis B and C Virus Infections: A Global Perspective. *Vaccine* **1999**, 1730−1733.
(4) Regev, A.; Schiff, E. R. Drug Therapy for Hepatitis B. *Adv. Intern. Med.* **2001**, *46*, 107−135.
(5) Maddrey, W. C. Hepatitis B-An Important Public Health Issue. *Clin. Lab.* **2001**, *47*, 51−55.
(6) Dushieko, G. M. Treatment and Prevention of Chronic Viral Hepatitis. *Pharmacol. Ther.* **1995**, *65*, 47−73.
(7) Mims, C. A. Ed. In *Medical Microbiology*; Mosby: London, 1993; Chapter 38.
(8) Perrillo, R. P.; Schiff, E. R.; Davis, G. L.; Bodenheimer, H. C., Jr.; Lindsay, K.; Payne, J.; Dienstag, J. L.; O'Brien, C.; Tamburro, C.; Jacobson, I. M. A Randomized, Controlled Trial of Interferon Alfa-2b Alone and After Prednisone Withdrawal for the Treatment of Chronic Hepatitis B. The Hepatitis Interventional Therapy Group. *N. Engl. J. Med.* **1990**, *323*, 295−301.

(9) Wong, D. K.; Cheung, A. M.; O'Rourke, K.; Naylor, C. D.; Detsky, A. S.; Heathcote, J. Effect of Alpha-interferon Treatment in Patients with Hepatitis B e Antigen-positive Chronic Hepatitis B. A Meta-analysis. *Ann. Intern. Med*. **1993**, *119*. 312−323.
(10) Summers, J.; Mason, W. S. Replication of the Genome of a Hepatitis B-like Virus by Reverse Transcription of an RNA Intermediate. *Cell* **1982**, *29*, 403−415.
(11) Liu, S.-H.; Grove, K. L.; Cheng, Y. C. Unique Metabolism of a Novel Antiviral L-Nucleoside Analog, 2′-Fluoro-5-methyl-β-L-arabinofuranosyluracil: a Substrate for both Thymidine Kinase and Deoxycytidine Kinase. *Antimicrob. Agents Chemother*. **1998**, *42*, 833−839.
(12) Tipples, G. A.; Ma, M. M.; Fischer, K. P.; Bain, V. G.; Kneteman, N. M.; Tyrrell, D. L. J. Mutation in HBV DNA-dependent DNA Polymerase Confers Resistance to Lamivudine in vivo. *Hepatology* **1996**, *24*, 714−717.
(13) Dienstag, J. L.; Perillo, R. P.; Schiff, E. R.; Bortholomew, M.; Vicary, C.; Rubin, M. A Preliminary Trial of Lamivudine for Chronic Hepatitis B Virus Infection. *N. Engl. J. Med*. **1995**, *333*, 1657−1661.
(14) Main, J.; Brown, J.; Howells, C.; Galassini, R.; Crossey, M.; Karayiannis, P.; Georgiou, P.; Atkinson, G.; Thomas, H. A Double blind, Placebo Controlled Study to Assess the Effect of Famciclovir on Virus Replication in Patients with Chronic Hepatitis B Virus Infection. *J. Viral Hepatitis* **1996**, *3*, 211−215.
(15) Luscombe, C. A.; Pederson, J.; Bowden, S.; Locarini, S. Alterations in Intrahepatic Expression of Duck Hepatitis B Viral Markers with Ganciclovir Chemotherapy. *Liver* **1994**, *14*, 182−192.
(16) Torresi, J.; Locarnini, S. Antiviral Chemotherapy for the Treatment of Hepatitis B Virus Infections. *Gastroenterology* **2000**, *118*, S83−S103.
(17) Delaney, W. E.; Edwards, R.; Colledge, D.; Shaw, T.; Torresi, J.; Miller, T. G.; Isom, H. C.; Bock, T.; Manns, M. P.; Trautwein, C.; Locarnini, S. Cross-resistance Testing of Antihepadnaviral Compounds Using Novel Recombinant Baculoviruses Which Encode Drug-resistant Strains of Hepatitis B Virus. *Antimicrob. Agents Chemother*. **2001**, *45*, 1705−1713.
(18) Chu, C. K.; Cutler, S. J. Chemistry and Antiviral Activities of Acyclonucleosides. *J. Heterocycl. Chem*. **1986**, *23*, 289−319.
(19) Minuk, G. Y.; German, G. B.; Bernstein, C.; Benarroch, A.; Gauthiar, T.; Sekla, L. A Pilot Study of Steroid Withdrawal Followed by Oral Acyclovir in the Treatment of Chronic Type B Hepatitis. *Clin. Invest. Med*. **1992**, *15*, 506−512.
(20) Luscombe, C.; Pedersen, J.; Uren, E.; Locarnini, S. Long-term Ganciclovir Chemotherapy for Congenital Duck Hepatitis B Virus Infection in vivo. Effect on Intrahepatic-viral DNA, RNA and Protein Expression. *Hepatology* **1996**, *24*, 766−773.
(21) Kruining, J.; Heijtink, R. A.; Schalm, S. W. Antiviral Agents in Hepatitis B Virus Transfected Cell Lines: Inhibitory and Cytotoxic Effects Related to Time and Treatment. *J. Hepatol*. **1995**, *22*, 263−267.
(22) Chachoua, A.; Dieterich, D.; Krasinski, K.; Greene, J.; Laubenstein, L.; Wernz, J.; Buhles, W.; Koretz, S. 9-(1,3-Dihydroxy-2-propoxymethyl)guanine (Ganciclovir) in the Treatment of Cytomegalovirus Gastrointestinal Disease with the Acquired Immunodeficiency Syndrome. *Ann. Int. Med*. **1987**, *107*, 133−137.
(23) Korba, B. E.; Boyd, M. R. Penciclovir is a Selective Inhibitor of Hepatitis B Virus Replication in Cultured Human Hepatoblastoma Cells. *Antimicrob. Agents. Chemother*. **1996**, *40*, 1282−1284.
(24) Shaw, T.; Mok, S. S.; Locarnini S. A. Inhibition of Hepatitis B Virus DNA Polymerase by Enantiomers of Penciclovir Triphosphate and Metabolic Basis for Selective Inhibition of HBV Replication by Penciclovir. *Hepatology* **1996**, *24*, 996−1002.
(25) Filer, C. W.; Allen, G. D.; Brown, T. A.; Fowles, S. E.; Hollis, F. J.; Mort, E. E.; Prince, W. T.; Ramji, J. V. Metabolic and Pharmacokinetic Studies Following Oral Administration of 14C−Famciclovir to Healthy Subjects. *Xenobiotica* **1994**, *24*, 357−368.
(26) Lin, T. S.; Liu, M. C. Synthesis of 1-[[2-Hydroxy-1-(hydroxymethyl)ethoxy] methyl]-5-benzyluracil and its Amino Analogue, New Potent Uridine Phosphorylase Inhibitors with High water Solubility. *J. Med. Chem*. **1985**, *28*, 971−973.
(27) Zoulim, F. Detection of Hepatitis B Virus Resistance to Antivirals. *J. Clin. Virol*. **2001**, *21*, 243−253.
(28) Dean, J.; Bowden, S.; Locarnini, S. Reversion of Duck Hepatitis B Virus DNA Replication in vivo Following Cessation of Treatment with the Nucleoside Analog Ganciclovir. *Antiviral Res*. **1995**, *27*, 171−178.
(29) Colledge, D.; Locarnini, S.; Shaw, T. Synergistic Inhibition of Hepadnaviral Replication by Lamivudine in Combination with Penciclovir *in vitro*. *Hepatology* **1997**, *26*, 216−25.
(30) Seeger, C. Hepadnavirus Replication and Approaches to Antiviral Therapy. In *Virus Strategies*; Doerfler, W., Bohm. P., Eds.; VCH: Weinheim, Germany, 1993; Vol. 4, pp 77−93.
(31) Boni, C.; Bertoletti, A.; Penna, A.; Cavalli, A.; Pilli, M.; Urbani, S.; Scognamiglio, P.; Boehme, R.; Panebianco, R.; Fiaccadori, F.; Ferrari, C. Lamivudine Treatment can Restore T Cell Responsiveness in Chronic Hepatitis B. *J. Clin. Invest*. **1998**, *102*, 968−75.
(32) Fourel, I..; Hantz, O.; Watanbe, K. A.; Jacquet, C.; Chomel, B.; Fox, J. J.; Trepo, C. Inhibitory Effects of 2′-Fluorinated Arabinosyl-pyrimidine Nucleoside on Woodchuck Virus Replication in Chronically Infected Woodchucks. *Antimicrob. Agents. Chemother*. **1990**, *34*, 473−475.
(33) Hantz, O.; Allauden, H. S.; Ooka, T.; De Clercq, E.; Trepo, C. Inhibition of Human and Woodchuck Hepatitis Virus DNA Polymerase by the Tri-phosphates of Acyclovir, 1-(2′-Deoxy-2′-fluoro-b-D-arabinosyl)-5-iodocytosine and E-5-(2-Bromovinyl)-2′-deoxyuridine. *Antiviral Res*. **1984**, *4*, 187−199.
(34) Tao, P.-Z.; Lofgren, B.; Lake-Bakaar, D.; Johansson, N. G.; Datema, R.; Oberg, B. Inhibition of Human Hepatitis B Virus DNA Polymerase and Duck Hepatitis B Virus DNA Polymerase by Tri-Phosphates of Thymidine Analogs and Pharmacokinetic Properties of the Corresponding Nucleosides. *J. Med. Virol*. **1988**, *26*, 353−362.
(35) Reimer, K.; Matthes, E.; Janta-Lipinski, M. V.; Meisel, H. Inhibition of Hepatitis B Virus DNA Polymerase by Thymidine Phosphate Analogues in vitro. *Antiviral Chem. Chemother*. **1991**, *2*, 249−253.
(36) Hong, J. H.; Choi, Y.; Chun, B. K.; Lee, K.; Chu C. K. Current Status of Anti-HBV Chemotherapy. *Arch. Pharm. Res*. **1998**, *21*, 89−105.
(37) Colacino, J. M.; Staschke, K. A. The Identification and Development of Antiviral Agents for the Treatment of Chronic Hepatitis B Virus Infections. In *Progress in Drug Research*; Jucker, E., Ed.; Birkhauser Verlag: Basel, Switzerland, 1998; 50, pp 263−310.
(38) Kumar, R.; Sharma, N.; Nath, M.; Saffran, H. A.; Tyrrell, D. L. J. Synthesis and Antiviral Activity of Novel Acyclic Nucleoside Analogues of 5-(1-Azido-2-haloethyl)uracils. *J. Med. Chem*. **2001**, *44*, 4225−4229.
(39) Dalton, D. R.; Dutta, V. P.; Johns, D. C. Bromohydrin Formation in Dimethyl sulfoxide. *J. Am. Chem. Soc*. **1968**, *90*, 5498−5501.
(40) Fissekis, J. D.; Sweet, F. The Chemistry of Some 5-(2-Hydroxyalkyl)uracil Derivatives and a Synthesis of 5-Vinyluracil. *J. Org. Chem*. **1973**, *38*, 264−269.
(41) Jordan, D. Solvent and Aryl-substituent Effects on the Rates of Thermal Decomposition of α-Azidostyrenes. *J. Org. Chem*. **1987,** *54*, 3584−3587.
(42) Mason, W. S.; Seal, G.; Summer, J. Virus of Pekin Ducks with Structural and Biological Relatedness to Human Hepatitis B Virus. *J. Virol*. **1980**, *36*, 829−836.
(43) Tuttleman, J. S.; Pugh, J. C.; Summers, J. W. In vitro Experimental Infection of Primary Duck Hepatocyte Cultures with Duck Hepatitis B Virus. *J. Virol*. **1986**, *58*, 17−25.
(44) Tyrrell, D. L. J.; et al. Treatment of Chimpanzee and Ducks with Lamivudine, 2′3′-Dideoxy-3′-thiacytidine Results in a Rapid Suppression of Hepadnaviral DNA in Serum. *Clin. Invest. Med*. **1993**, *16(S)*, B77.
(45) Shaw, T.; Amor, P.; Civitico, G.; Boyd, M.; Locarnini, S. In vitro Antiviral Activity of Penciclovir, A Novel Purine Nucleoside, Against Duck Hepatitis B Virus. *Antimicrob. Agents Chemother*. **1994**, *38*, 719−723.
(46) Boyd, M. R.; De Vita, V. T.; Hellman, S.; Rosenberg. S. A., Eds., Cancer: In *Principles and practice of oncology update*; Lippincott: Philadelphia, PA, 1989; Vol. 3, pp 1−12.
(47) Beauchamp, L. M.; Serling, B. L.; Kelsey, J. E.; Biron, K. K.; Collins, P.; Selway, J.; Lin, J.-C.; Schaeffer, H. J. Effect of Acyclic Pyrimidines Related to 9-[(1,3-Dihydroxy-2-propoxy)methyl]-guanine on Herpes Viruses. *J. Med. Chem*. **1988**, *31*, 144−149.
(48) Lee, B.; Luo, W.; Suzuki, S.; Robins, M.. J.; Tyrrell, D. L. J. *In vitro* and *in vivo* Comparison of the Abilities of Purine and Pyrimidine 2′,3′-Dideoxynucleosides to Inhibit Duck Hepadnavirus. *Antimicrob. Agents Chemother*. **1989**, *33*, 336−339.
(49) Kumar, R.; Wiebe, L. I.; Knaus, E. E. Synthesis and Antiviral Activity of Novel 5-(1-Azido-2-haloethyl) and 5-(1-Azido-, amino-, or methoxyethyl) analogs of 2′-deoxyuridine. *J. Med. Chem*. **1993**, *36*, 2470−2474.
(50) Agrawal, B.; Reddish, M. A.; Longenecker, B. M. CD30 Expression on Human CD8+ T cells Isolated from Peripheral Blood Lymphocytes of Normal Donors, *J. Immunol*. **1996,** *157*, 3229−3234.

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