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Novel 5-Vinyl Pyrimidine Nucleosides with Potent Anti-Hepatitis B Virus Activity

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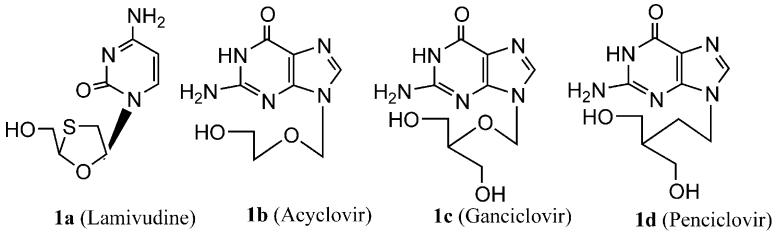
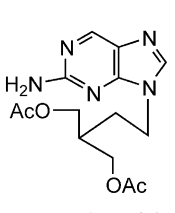
Abstract—Synthesis and antiviral activities of novel *N*-1 alkyl substituted pyrimidines, 1-[(2-hydroxyethoxy)methyl]-5-vinyluracil (**5**), 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-vinyluracil (**6**), and 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-vinyluracil (**7**) are reported. Compounds **6** and **7** were potent inhibitors of DHBV in cell culture, in contrast, all of the compounds described were devoid of activity against TK^+ HSV-1 and TK^- HSV-1.

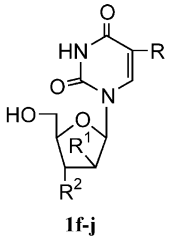
Hepatitis B virus (HBV) is a causative agent of acute and chronic hepatitis. Hepatitis B virus infection is the world's 9th leading cause of death.[1–4] There are approximately 350 million people with chronic HBV infection. Chronic HBV infection leads to liver damage, cirrhosis, and hepatocellular carcinoma.[3,4] New infection with HBV can be prevented by vaccination. However, the present vaccination is not effective for chronic carriers worldwide. The major therapeutic option for HBV carriers is α-interferon. However, use of α-interferon is limited, the success rate is low, and serious side effects are observed.[5,6] Lamivudine, [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine, (−)-3-TC, **1a**], a pyrimidine nucleoside analogue has been approved for the treatment of HBV infection. The administration of lamivudine induces a 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.[7] 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 therapy is stopped.[8,9] Thus, there is a tremendous clinical need to investigate novel classes of antiviral agents for the chemotherapy of HBV infections.



1a (Lamivudine) **1b** (Acyclovir) **1c** (Ganciclovir) **1d** (Penciclovir)



1e (Famciclovir)



1f-j

1f, R = CH_3; R^1 = F; R^2 = OH
1g, R = C_2H_5; R^1 = F; R^2 = OH
1h, R = CH=CH-CH_3; R^1 = H; R^2 = OH
1i, R = CH=CH-CH_3; R^1 = OH; R^2 = OH
1j, R = CH=CH_2; R^1 = H; R^2 = F

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One promising class of nucleoside analogues for antiviral chemotherapy belongs to a group with open-chain 'acyclic' sugar moieties.[10] Among acyclic purine nucleosides, Acyclovir (ACV, **1b**), 9-[(2-hydroxyethoxy)methyl]guanine, and Ganciclovir (GCV, **1c**), 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine, an analogue structurally similar to ACV, have been shown to be active against HBV in tissue culture and in vivo studies in animal models.[11–14] Penciclovir (PCV, **1d**), 9-[4-hydroxy-3-(hydroxymethyl)-1-butyl]guanine, is an acyclic nucleoside closely related to GCV, has also shown anti-HBV activity in vitro and in vivo.[15–17] The basis of potent and selective anti-HBV activity of PCV is at the HBV DNA polymerase level. The tri-phosphate derivative of PCV has a very high affinity for HBV DNA polymerase and a very low affinity for the cellular DNA polymerase.[17,18] Thus, acyclic moieties contribute significantly to potent anti-HBV activity and selectivity. Famciclovir (**1e**), a prodrug of PCV, has been investigated, in order to increase oral bioavailability and has shown promise as an anti-HBV agent in clinical studies.[19]

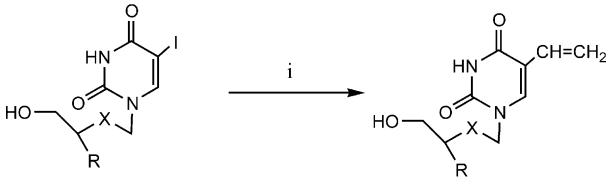
Among many pyrimidine nucleosides that have been studied, C-5 substituted pyrimidine nucleosides with 2′-fluoro substituted arabinosyl analogues FMAU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil] (**1f**) and FEAU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil] (**1g**) have been found to exhibit potent in vitro and in vivo anti-HBV activity.[20] However, they have been shown to be toxic in vivo.[20] In a study of 5′-tri-phosphates 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.[21] The tri-phosphate derivative of the 5-propenyl-2′-deoxyuridine was also found to efficiently inhibit herpes simplex virus encoded DNA polymerase together with DHBV DNA polymerase.[21] In a group of tri-phosphate derivatives of 5-substituted 3′-fluoro-2′,3′-dideoxyuridine analogues, structure–activity correlation studies indicated that substitution at the 5-position by an alkyl group, including a double bond (**1j**), increases the inhibitory activity against human HBV DNA polymerase.[22] The strong inhibition of HBV DNA polymerase activity by 5-vinyl substituted pyrimidine nucleosides[21,22] and the contribution of acyclic moieties in the purine analogues (**1b–d**) with highly selective antiviral activity, encouraged us to explore the effect of acyclic chain analogues of 5-vinyl pyrimidine nucleosides on enhancing anti-HBV activity while minimizing toxicity.

In this communication, we now report the synthesis, in vitro anti-DHBV and anti-herpes activity of 5-vinyl pyrimidine nucleosides (**5–7**) in which the furanose moiety is replaced with various acyclic substituents. To our knowledge, 5-substituted acyclic pyrimidine nucleosides have not previously reported to possess anti-HBV activity.

The desired compounds 1-[(2-hydroxyethoxy)methyl]-5-vinyluracil (**5**), 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)-methyl]-5-vinyluracil (**6**), and 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-vinyluracil (**7**) were synthesized (Scheme 1) by the palladium acetate-triphenyl phosphine-catalyzed reactions of corresponding 5-iodo derivatives[23,24] (**2–4**) with vinyl acetate.[25–27] The products were purified by silica gel column chromatography and structural assignments of the synthesized derivatives were made on the basis of the ^{1}H NMR and elemental analysis studies.

The anti-DHBV activity of newly synthesized analogues **5–7**, along with the reference antiviral compound (−) 3-TC (**1a**) was assessed in confluent cultures of primary duck hepatocytes obtained from chronically infected Pekin ducks according to the procedure reported by us.[28] Duck hepatitis B virus (DHBV), a member of hepadnaviridae, shares properties of hepatotropism, virion structure, genome organization and replication with human HBV.[29] DHBV-based in vitro and in vivo systems have been used extensively to screen drugs for potential anti-HBV activity.[30] It has been shown that compounds like 3-TC and PCV, both potent inhibitors of DHBV, are also potent inhibitors of HBV in chimpanzees as well as humans.[31] Table 1 shows the concentrations required to inhibit 50% of DHBV DNA (EC_{50}), 50% cytotoxic concentration (CC_{50}) on stationary Vero cells and the 50% inhibitory concentration (IC_{50}) on proliferating human T cells. Related purine acyclic nucleosides ganciclovir (**1c**) and penciclovir (**1d**) are also included in Table 1 for comparison of their anti-DHBV potency.[32]

In our studies, 5-vinyl derivative **6** (EC_{50} = 0.3–1.0 μg/mL), with a (2-hydroxy-1-(hydroxymethyl)ethoxy)-methyl moiety, emerged as the most active analogue. The analogue containing a 4-hydroxy-3-(hydroxymethyl)-1-butyl chain (**7**) was approximately 3-fold less potent (EC_{50} = 1.0–3.0 μg/mL) than **6**. In contrast, a 5-vinyl pyrimidine, with (2-hydroxyethoxy)methyl substituents (**5**), was weakly inhibitory to DHBV replication (EC_{50} = > 10 μg/mL). These results suggest that acyclic substituents at the *N*-1 position can be determinants of anti-DHBV activity in this series of compounds. Also, a hydroxyl group at a position mimicking the 3′-OH group of a deoxyribose ring of nucleosides, contributes significantly to the anti-DHBV activity. The anti-DHBV activity exhibited by analogue **6** compares favorably to that of corresponding guanine acyclic nucleoside analogue (**1c**, EC_{50} = 1.5 μg/mL), as measured in the DHBV infected primary duck hepatocyte cultures.[32] The anti-DHBV activity of compound **7** was

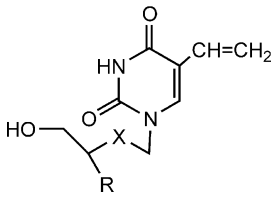


2, X = O, R = H
3, X = O, R = CH_2OH
4, X = C, R = CH_2OH

5, X = O, R = H
6, X = O, R = CH_2OH
7, X = C, R = CH_2OH

Scheme 1. Reagents: (i) $Pd(OAc)_2$, Ph_3P, triethylamine, vinyl acetate, 70 °C.

Table 1. In vitro antiviral activity and cytotoxicity of 5-vinyl acyclic pyrimidine nucleosides



Compd	X	R	% Inhibition @10 μg/mL	EC_{50} (μg/mL)	EC_{50} (μg/mL)		CC_{50} (μg/mL)	IC_{50} (μg/mL)
			DHBV[a]	DHBV[b]	HSV-1[c]	HSV-1[c]	Cytotoxicity	Cell proliferation
			Primary duck hepatocytes	Primary duck hepatocytes	KOSSB (TK^-)	KOS (TK^+)	Vero[d,e]	Human T Cells[f]
5	O	H	40.6	>10.0	>50	>25	>100	>50
6	O	CH_2OH	81.0	0.3–1.0	>50	>25	>100	>50
7	C	CH_2OH	77.5	1.0–3.0	>50	>25	>100	>50
1a			96.0	0.01–0.05	ND	ND	>100	>50
1b			ND	ND	40	0.1–0.5	>100	ND
1c			ND	1.5[g]	ND[h]	ND	ND	ND
1d			ND	0.3[g]	ND	ND	ND	ND

[a]The data are expressed as percent inhibition of viral DNA in the presence of 10 μg/mL of 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. The experiment was repeated three times and range of EC_{50} obtained in different experiments is shown.
[c]The concentration (μg/mL) required to inhibit plaque formation in monolayers of Vero cells by 50%.
[d]The drug concentration required to reduce the viability of Vero cells as determined by MTT assay, by 50% of untreated control after 3 days.
[e]The sign (>) indicates that 50% inhibition was not reached at the stated (highest) concentration tested.
[f]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.
[g]Data taken from Shaw et al.[32]
[h]ND, Not determined.

3- to 10-fold less than that of related guanine analogue (**1d**, $EC_{50}=0.3$ μg/mL). It is interesting to note that unlike the corresponding acyclic guanine analogues (**1c**,**d**), the (2-hydroxy-1-(hydroxymethyl)ethoxy)methyl analogue of 5-vinyl pyrimidine (**6**) was more active than its 4-hydroxy-3-(hydroxymethyl)-1-butyl analogue (**7**).

The compounds **5**–**7** were also evaluated for their anti-herpes activity against TK^+ HSV-1 (strain KOS) and TK^- HSV-1 (strain KOSSB).[33] However, none of these agents exhibited notable inhibition at concentrations up to 25 and 50 μg/mL, respectively. The absence of anti-herpes action could be attributed to their lack and/or insufficient phosphorylation to the requisite tri-phosphate form and/or their inability to interact with herpes simplex virus DNA polymerase.

The contrast between the potent activity of **6** and **7** against DHBV and the lack of anti-herpes activity, is unusual since related acyclic purine nucleosides (**1c**,**d**) exhibit both potent antiherpes and anti-HBV activities.[32]

The compounds **5**–**7** exhibited no in vitro cytotoxicity against stationary Vero cells (CC_{50} >100 μg/mL) (Table 1). In rapidly proliferating fresh human T lymphocyte cell culture,[34] cellular DNA synthesis, as monitored by the incorporation of [methyl-^{3}H]-deoxyribosylthymidine into DNA, was also not affected by compounds **5**–**7** up to a concentration of 50 μg/mL.

In conclusion, we present the synthesis and in vitro biological evaluation of a new class of 5-substituted acyclic pyrimidine nucleosides (**5**–**7**). The fact that the compounds **6** and **7** show potent and selective anti-DHBV activity and low toxicity to host cells suggests that they have higher affinity for the virus specific DNA polymerase than the host cell enzymes. The anti-DHBV activity of compounds **6** and **7** could likely be attributed to their phosphorylation by cellular kinases followed by selective inhibition of HBV DNA polymerase by their triphosphate derivatives, as suggested for other antiviral nucleosides.[35,36] The unprecedented anti-DHBV properties of 5-substituted acyclic pyrimidine nucleosides observed in this report delineate a new approach for discovery of new and effective anti-HBV agents, which is based on modifications in the sugar moiety with alkyl chains at *N*-1 position in the pyrimidine nucleoside analogues. Further biological and structure activity relationship studies of this new class of anti-HBV agents are ongoing.

Acknowledgements

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25. Experimental synthesis of 1-[(2-hydroxyethoxy)methyl]-5-vinyluracil **(5)**. A mixture of palladium(II) acetate (26 mg, 0.11 mmol), triphenylphosphine (57 mg, 0.21 mmol) and triethylamine (1.5 mL), (dried over calcium hydroxide) in dry DMF (25 mL) was maintained at 70 °C with stirring until an intense red color appeared. 1-[(2-Hydroxyethoxy)methyl]-5-iodouracil (**2**) (683 mg, 2.19 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 extracted with water (2×50 mL). The water layer was washed with dichloromethane (3×20 mL), evaporated in vacuo, and the residue obtained was purified by elution from a silica gel column using ethyl acetate–methanol (95:5, v/v) as eluent to give **5** as a viscous oil (250 mg, 54%). ^{1}H NMR (DMSO-d_6) δ 3.50 (m, 4H, OCH_2CH_2), 4.70 (br, 1H, OH), 5.15 (m, 3H, NCH_2, CH=CHH′), 5.98 (dd, J=18, 2 Hz, 1H, CH=CHH′), 6.4 (dd, J=18, J=11 Hz, 1H, CH=CHH′), 7.95 (s, 1H, H-6), 11.48 (s, 1H, NH, exchanges with D_2O). Anal. ($C_9H_{12}N_2O_4$) C, H, N.
26. Experimental synthesis of 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-vinyluracil (**6**). This was obtained as a syrup (250 mg, 47%) from 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-iodouracil (**3**) (750 mg, 2.19 mmol) by using the procedure as described for **5**. ^{1}H NMR (DMSO-d_6) δ 3.40 (m, 4H, OCH_2), 3.55 (m, 1H, CH), 4.65 (m, 2H, OH), 5.10 (dd, J=11, 2.1 Hz, 1H, CH=CH H′), 5.20 (s, 2H, NCH_2), 5.95 (dd, J=18, 2 Hz, 1H, CH=CHH′), 6.35 (dd, J=18, 11 Hz, 1H, CH=CHH′), 7.90 (s, 1H, H-6), 11.40 (br s, 1H, NH, exchanges with D_2O). Anal. ($C_{10}H_{14}N_2O_5$) C, H, N.
27. Experimental synthesis of 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-vinyluracil (**7**) This was obtained as a viscous oil (800 mg, 48%) from 1-[4-hydroxy-3-(hydroxymethyl)-1-butyl]-5-iodouracil (**4**) (2.36 g, 6.95 mmol) by using the procedure as described for **5**. ^{1}H NMR (DMSO-d_6) δ 1.42–1.62 (m, 3H, CH, NCH$_2$CH_2), 3.32–3.45 (m, 4H, OCH_2 + DMSO), 3.72 (t, J=7 Hz, 2H, NCH_2), 4.42 (t, J=5 Hz, 2H, OH), 5.10 (dd, J=11, 2 Hz, 1H, CH=CHH′), 5.94 (dd, J=18, 2 Hz, 1H, CH=CHH′), 6.35 (dd, J=18, 11 Hz, 1H, CH=CHH′), 7.88 (s, 1H, H-6), 11.34 (s, 1H, NH, exchanges with D_2O). Anal. ($C_{11}H_{16}N_2O_4$) C, H, N.
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33. In vitro antiviral assays [HSV-1 (KOS, TK$^+$), (KOSSB, TK$^-$)]. African green monkey kidney (Vero) cells were grown in DMEM supplemented with 5% FBS. Cells were seeded into 24-well plates one day prior to the assay. Wild-type HSV-1, KOS, and a thymidine kinase deficient mutant, KOSSB, were used to infect at ~100 plaque forming units per well for 1 h at 37 °C. After infection, the inoculum was replaced with serial dilutions of media containing diluted drug. All drug dilutions were done in duplicate. Once an activity range was determined, compounds were again tested at 1:2 serial dilutions to obtain a more precise EC_{50}. Controls included infected wells that were not treated with drugs, as well as infected wells treated with acyclovir at 5 and 1 μg/mL (for TK$^+$ virus) and 50, 25, 10 and 1 μg/mL (for TK$^-$ virus). Plates were incubated for 48 h at 37 °C. To visualize plaques, wells were fixed by incubation with methanol for 10 minutes at 25 °C, and added with 1× Giemsa stain (Sigma) for 1 h at 25 °C. Plaques were counted and compared to the number of plaques in the no-drug controls in order to calculate EC_{50}.
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