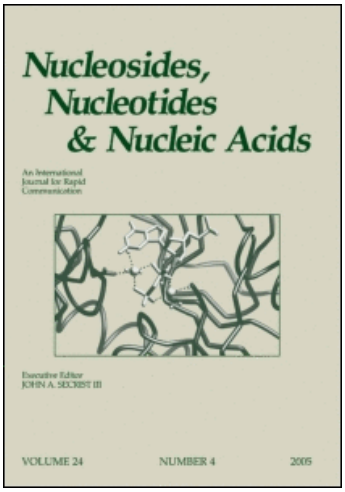

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N^4-HYDROXYCYTOSINE DIOXOLANE NUCLEOSIDES AND THEIR ACTIVITY AGAINST HEPATITIS B VIRUS

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▫ *Novel racemic, D- and L-β-dioxolane N^4-hydroxycytosine nucleosides have been synthesized and evaluated for their activity against hepatitis B virus. None of the synthesized nucleosides demonstrated selective anti-HBV activity.*

Keywords HBV, Dioxolane, Nucleoside

INTRODUCTION

Hepatitis B virus (HBV) is a causative agent of acute and chronic hepatitis. HBV infection is the world's ninth leading cause of death. The number of chronic carriers is estimated to be more than 400 million worldwide, with roughly 4 million deaths annually from the resulting cirrhosis and hepatocellular carcinoma. Only three FDA-approved drugs are currently available for the treatment of hepatitis B infections. The first therapeutic option involves immune stimulation by using interferon-α. However, IFN-α is only effective in 10–30% of treated patients, making its usefulness limited, and often serious side effects result in cessation of therapy.[1] A second therapeutic option involves nucleoside analogues. Two nucleosides are FDA approved, i.e., lamivudine, (−)-β-2′,3′-dideoxy-3′-thiacytidine, 3TC and

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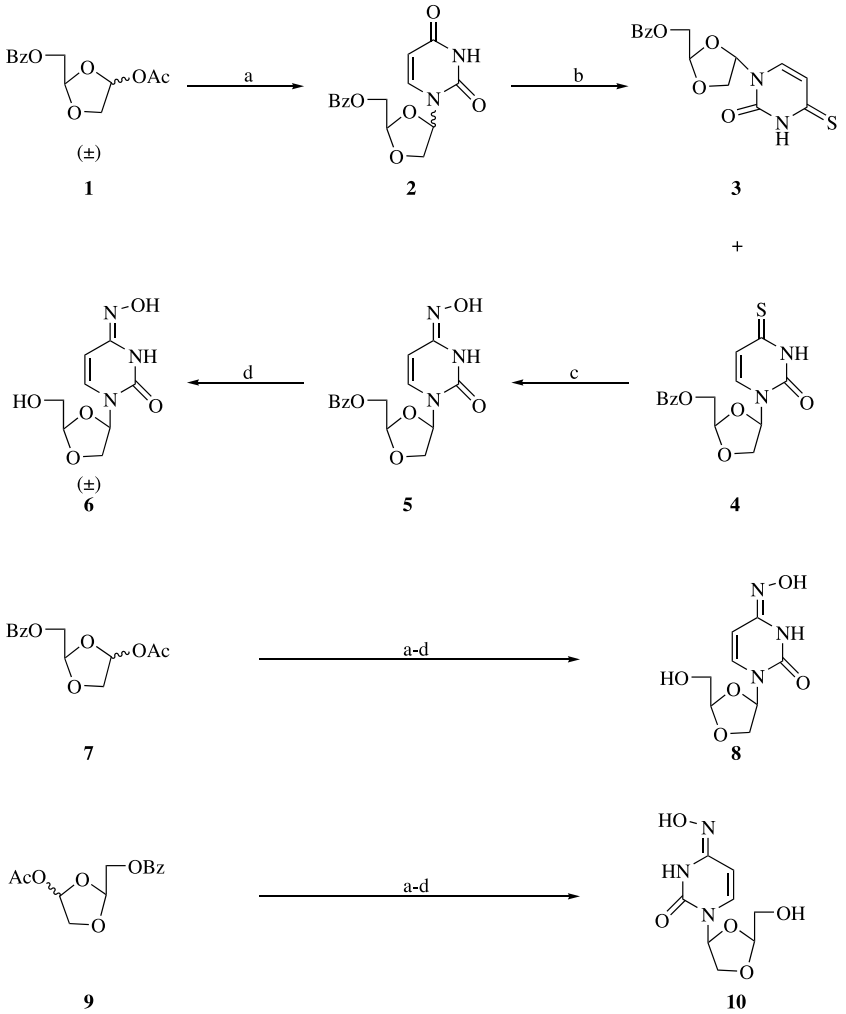
hepsera, 9-{2-[bis(pivaloyloxymethyl)phosphonylmethoxy]ethyl}adenine, adefovir dipivoxil. Both drugs are now entering widespread clinical use associated with effective suppression of viral replication, reduced disease activity and improved liver histology.

Long-term treatment with lamivudine results in the emergence of drug-resistant HBV, the first signs of which might appear within 6–12 months of therapy. Resistance to 3TC is associated to accumulation of mutations in the viral polymerase, with rtM204V and rtM204I as the key mutations for resistance. The latter ones are often accompanied with a compensatory mutation at rtL180M. In addition, long-term remission after completion of treatment with 3TC is not commonly



SCHEME 1 Reagents and conditions: a) i. uracil, HMDS, reflux, ii. TMSOTf, CH_2Cl_2; b) Lawesson's reagent, $ClCH_2CH_2Cl$, reflux; c) NH_2OH, EtOH, reflux; NH_3, MeOH, RT.

TABLE 1 Anti-HBV Activity and Toxicity of the Selected Compounds

	HepAD38 EC_{90}, μM	HepAD79 EC_{90}, μM
6	0.43	5.71
8	>10	>10
10	0.05	2.43
3TC	0.04	>10

ND: not determined; EC_{90}: effective concentration required for reducing the HBV DNA levels by 90% in 5 days in HepAD38 and 10 days in HepAD79.

observed, and most patients experience a rebound in viremia once the use of drug is stopped.[1]

Adefovir dipivoxil is a nucleotide analogue that has been shown to have potent anti-HBV activity similar to lamivudine. Several clinical studies concluded that prolonged suppression of HBV replication might be possible without the emergence of resistance. However, at least one case of resistance against adefovir dipivoxil therapy has now been described and is associated with the selection of a rtN236T mutation.[2]

Because of the ongoing epidemic and the rise of drug-resistant HBV, it remains an urgent task to develop additional safe and selective agents for the treatment of HBV infection. We herein report the synthesis and evaluation of antiviral activity of racemic and enantiomerically pure D- and L-β-1-[2-(hydroxymethyl)-1,3-dioxolan-4-yl]-N^4-hydroxycytosine against HBV. N^4-Hydroxycytosine nucleosides were selected since we recently determined that N^4-hydroxycytosine nucleoside can be substrate of cytidine deaminase[3–5] and dioxolane cytosine nucleoside showed potent anti-HBV activity but is toxic.

RESULTS AND DISCUSSION

The synthesis of racemic and enantiomerically pure D- and L-β-dioxolane N^4-hydroxycytosine is shown in Scheme 1. Condensation of (±)-4-acetoxy-2-(benzoyloxymethyl)-1,3-dioxolane (**1**) with silylated uracil in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) in methylene chloride afforded an inseparable mixture of anomers (**2**). Treatment of the mixture with Lawesson's reagent in 1,2-dichloroethane at reflux gave β-**4*** and α-**3**† after silica gel

*Compound **4**: foam, ^{1}H NMR ($CDCl_3$) δ 9.87 (s, 1H, NH, D_2O exchangeable), 8.06–7.48 (m, 5H, Bz), 7.45 (d, J = 8.0 Hz, 1H, 6-H), 6.34 (d, J = 4.4 Hz, 1H, 4′-H), 6.13 (dd, J = 1.6, 7.6 Hz, 1H, 5-H), 5.31 (t, J = 2.4 Hz, 1H, 2′-H), 4.70 (m, 2H, 5′-H), 4.25 (m, 2H, 6′-H). Anal Calcd for $C_{15}H_{14}N_2O_5S$ + 0.1H_2O: C, 53.54; H, 4.22; N, 8.33. Found: C, 53.45; H, 4.19; N, 8.26.

†Compound **3**: foam, ^{1}H NMR ($CDCl_3$) δ 9.86 (s, 1H, NH, D_2O exchangeable), 8.07–7.46 (m, 5H, Bz), 7.22 (d, J = 8 Hz, 1H, 6-H), 6.46 (dd, J = 1.6, 7.6 Hz, 1H, 5-H), 6.28 (dd, J = 2.4, 5.2 Hz, 1H, 4′-H), 5.81 (t, J = 4 Hz, 1H, 2′-H), 4.45 (m, 3H, 5′-H_A, 6′-H), 4.16 (m, 1H, 5′-H_B). Anal Calcd for $C_{15}H_{14}N_2O_5S$ + 0.1H_2O: C, 53.54; H, 4.22; N, 8.33. Found: C, 53.32; H, 4.18; N, 8.04.

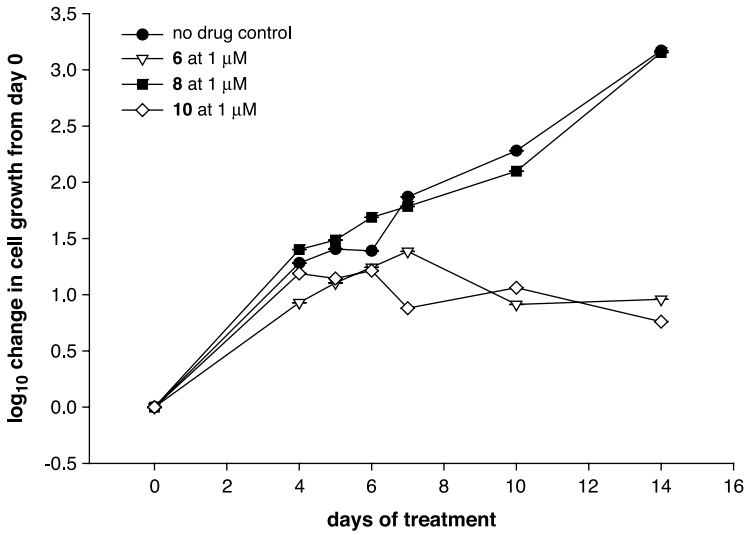


FIGURE 1 Effect of selected compounds on HepG2 cell proliferation. ●, cell proliferation in the absence of drug; ▽, cell proliferation in the presence of 1 μM of (±)-β-1-[2-(hydroxymethyl)-1,3-dioxolan-4-y1]-N^4-hydroxycytosine (**6**); ■, cell proliferation in the presence of 1 μM of D-β-1-[2-(hydroxymethyl)-1,3-dioxolan-4-y1]-N^4-hydroxycytosine (**8**); ◇, cell proliferation in the presence of 1 μM of L-β-1-[2-(hydroxymethyl)-1,3-dioxolan-4-y1]-N^4-hydroxycytosine (**10**).

chromatographic separation in excellent yields. The final nucleoside **6*** was obtained by treatment of β-4-thiocytosine analog **4** with aqueous hydroxylamine in ethanol at reflux followed by deprotection with methanolic ammonia in good yield. Similarly, coupling of enantiomerically pure (2*R*)- and (2*S*)-4-acetoxy-2-(benzoyloxymethyl)-1,3-dioxolane (**7** and **9**) with silylated uracil followed by reaction with Lawesson's reagent, α/β-isomers separation, replacement with hydroxylamine and deprotection afforded the corresponding (2*R*,4*R* or D)- and (2*S*,4*S* or L)-β-1-[2-(hydroxymethyl)-1,3-dioxolan-4-yl]-N^4-hydroxycytosine (**8** and **10**).† The enantiomerically pure **7** and **9** were prepared by the reported method.[6]

The assignment of the anomeric configuration of these nucleosides was established on the basis of the characteristics of the proton NMR spectra. The 2′-proton of the α-anomer **3** appeared at a lower field (5.81 ppm) than that (5.31 ppm) of the β-anomer **4**. However, the 5′-proton of the α-anomer **3** appeared at a higher field (4.30 ppm) than that (4.70 ppm) of the β-anomer **4**. Chemical shifts of specific protons were affected by their electronic environment. The proton signals shifted to lower field in NMR spectra when they were located on the same side as the

*Compound **6**: crystals, mp. 159–161°C. ^{1}H NMR (DMSO-d_6) δ 10.00, 9.55 (ss, 2H, NH, NOH, D_20 exchangeable), 6.94 (d, J = 8.4 Hz, 1H, 6-H), 6.20 (d, J = 4.8 Hz, 1H, 4′-H), 5.58 (d, J = 7.2 Hz, 1H, 5-H), 5.13 (t, J = 6.0 Hz, 1H, OH, D_2O exchangeable), 4.86 (t, J = 3.2 Hz, 1H, 2′-H), 4.05 m 2H, 5′-H), 3.58 (m, 2H, 6′-H). Anal Calcd for $C_8H_{11}N_3O_5$: C, 41.92; H, 4.84; N, 18.33. Found: C, 42.13; H, 4.84; N, 18.40. HRMS (FAB) obsd, *m/z* 230.0784, calcd for $C_8H_{11}N_3O_5$+ H, *m/z* 230.0777, $(M + H)^+$.

†^{1}H-NMR is identical to that of (±)-nucleoside.

pyrimidine base due to its deshielding effect. The assignment for these nucleosides is consistent with reports by others with similar pyrimidine nucleosides.[7]

The synthesized nucleosides **6**, **8**, and **10** were evaluated in vitro for their biological activity against HBV. These compounds were tested in an HBV-inducible system under control of tetracycline. In the presence of tetracycline, the HBV production is suppressed, but upon removal of tetracycline from the culture media, these cells produce HBV particles. The compounds were evaluated in two different cells lines; e.g., HepAD38 expressing the wild type virus, and HepAD79, expressing the lamivudine resistant virus.[8,9] The methodology for testing was essentially as described previously.[10]

The results of the antiviral testing are shown in Table 1. The racemic mixture **6** and the enantiomerically pure L-**10** showed potent antiviral activity against wild-type virus and a modest activity against the 3TC resistant virus. Compound D-**8** did not show any antiviral activity.

L-Dioxolane cytidine is a notoriously toxic compound.[11] Description of the antiviral effect as given in Table 1 is calculated from the amount of viral DNA in cell supernatant. These values do not take into account the possible toxic side effects that these compounds might have on cell proliferation. Therefore, a separate toxicity experiment was conducted. HepG2 cells were exposed for 14 days to 1 μM of the test compound, with the medium being renewed every 4 days. The results are shown in Figure 1. While the racemic mixture **6** and the L-enantiomer **10** show significant inhibition of cell proliferation, the D-enantiomer **8** is rather indistinguishable from the untreated control.

It is concluded from these toxicity results that the observed antiviral effect of **6** and **10** might be secondary to the inhibitory effect on cell proliferation. A favorable therapeutic window (antiviral efficacy/cellular toxicity) could not be established.

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