Introduction

Pyrimidines are an important class of compounds which have widespread applications from pharmaceuticals to materials (Brown, 1996). They have been recognized as important heterocyclic compounds due to their diverse biological activities, such as Tie-2 kinase inhibitory (Malibu and Kappe, 2007), HIV-1 inhibitory (Gadhachanda et al., 2007), antimalarial (Agarwal et al., 2005), secretive adenosine A1 receptor antagonistic (Chang et al., 2004), anticancer (Capdeville et al., 2002), analgesic (Zaki et al., 2006), cardiovascular (Atwal, 1987), and antiallergic (Ozeki et al., 1989) activities. Furthermore, it has been found that 3,4-dihydropyrimidin-2(1H)-ones (DHPMs) have an interesting multifaceted pharmacological profile as hepatitis B virus (HBV) replication inhibitors (Kappe, 2000; Deres et al., 2003; Lengar and Kappe, 2004). A number of synthetic pharmacophores with antibacterial (Hegab et al., 2007), antifungal (Atul et al., 2008), and antymycotic activities (Keutzberger and Gillesen, 1985) are based on the pyrimidyl moiety. Also, pyrimidines are present in numerous natural products and, significantly, in the pyrimidine and purine bases ribo- and deoxyribonucleosides (Lister, 1971; Hoffmann, 1996; Hurst, 1980; Brown, 1984). On the other hand, nucleoside analogues are structurally, metabolically, and pharmacodynamically related agents that have diverse biological actions and therapeutic effects including antiviral (Remy and Secrist, 1985; Abdel-Aal et al., 2006; Rashad et al., 2008) and antitumour (Chu et al., 1984; Park et al., 1986; Lin and Liu, 1985) activities. Furthermore, the acyclic nucleoside (Mereyala and Gurijala, 1993; El Ashry et al., 2005, 2006; El Ashry and El Kilany, 1996, 1997) analogues including modifications of both the acyclic glycon and aglycon parts are biological inhibitors and have stimulated extensive research as (El Ashry et al., 2000, 2007). The importance of the substituted hydroxyalkyl chain conformation in the interaction of acyclic nucleosides with enzymes has been demonstrated (Alvarez et al., 1994). Moreover, the 5-substituted derivatives of pyrimidine acyclonucleoside analogues have exhibited pronounced inhibitory properties with respect to uridine phosphorylase and have enhanced antitumour action (Park et al., 1986; Lin and Liu, 1985). Owing to the above significance and our interest in the synthesis of new and potent antiviral nucleoside analogues (El-Sayed et al., 2008; El-Zayate et al., 2009; Ali et al., 2007), herein we report the synthesis and anti-HBV activity of new substituted pyrimidine acyclic nucleosides.
Results and Discussion

Chemistry

The coupling reaction at N-1 of the substituted pyrimidine derivatives with acyclic and cyclic oxygenated halides is the most efficient route to our target acyclic nucleosides. Thus, the reaction of the substituted pyrimidin-2,4-dione derivatives 1a–c with 2-(2-chloroethoxy)ethyl acetate (2) in N,N-dimethylformamide (DMF) and in the presence of sodium hydride at room temperature (r.t.) afforded the corresponding substituted 2-[2-[2,4-dioxo-3-phenyl-3,4-dihydropyrimidin-(2H)-1-yl]ethoxy]ethyl acetate derivatives 3a–c in

![Chemical diagram]

Fig. 1. Synthesis of new pyrimidine acyclic nucleosides.
75–80% yields (Fig. 1). The chemical structures of 3a–c were confirmed by means of their spectral and analytical data. Thus, their IR spectra showed characteristic absorption bands for the C=O groups at 1665–1785 and 1735–1740 cm⁻¹ in addition to an absorption band at 2205 cm⁻¹ for the CN group of compound 3a. The ³H NMR spectra of 3a–c showed characteristic absorption bands for the C=O groups at 1665–1785 and 1735–1740 cm⁻¹ in addition to an absorption band at 2205 cm⁻¹ for the CN group of compound 3a. The ¹H NMR spectra of 3a–c showed four CH₂ groups, each as a triplet at δ 3.82–4.92 ppm, the methyl group at δ 2.38–2.44 ppm in addition to the signals of aromatic protons at δ 6.78–7.92 ppm. When the (2,4-dioxo-3,4-dihydropyrimidin-(2H)-1-yl)ethoxymethyl acetate derivatives 3a–c were treated with methanolic ammonia solution at room temperature, the corresponding substituted 1-[2-(hydroxymethoxy)ethyl]-2,4-dioxo-1,2,3,4-tetrahydropyrimidine derivatives 4a–c were produced in 70–72% yields. The structures of the deprotected acyclic nucleoside analogues 4a–c were established on the basis of their IR, ¹H NMR, and elemental analyses data which all agreed with the assigned structures. The IR spectra showed an absorption band at 3420–3440 cm⁻¹ for the OH group and revealed the absence of the carbonyl ester group. Furthermore, the ¹H NMR spectra revealed the disappearance of the peak corresponding to the methyl group, and instead a signal corresponding to the OH group appeared at δ 4.35 ppm.

The reaction of the substituted pyrimidin-2,4-dione derivatives 1a–c with (2,2-dimethyl-1,3-dioxolan-4-yl)methyl 4-methylbenzenesulfonate (5) in DMF and in the presence of sodium hydride at room temperature afforded the corresponding substituted ethyl 1-{[2,2-dimethyl-1,3-dioxolan-4-yl]methyl}-2,4-dioxo-1,2,3,4-tetrahydropyrimidines 6a–c in 80–82% yields. The ¹H NMR spectrum of 6a as a representative example showed δ 1.88, 1.92 (s, 6H, 2CH₃), 4.58 (d, 2H, CH₂), 4.96 (d, 2H, CH₂), 5.08 (m, 1H, CH), 6.88 (m, 4H, Ar–H), 7.40 (m, 3H, Ar–H), 7.85 (m, 3H, Ar–3H). Treatment of 6a–c with 70% acetic acid at reflux temperature afforded the corresponding deprotected acyclic nucleoside analogues, 1-(2,3-dihydroxypropyl)-2,4-dioxo-3,6-diphenyl-1,2,3,4-tetrahydropyrimidine derivatives 7a–c, in 75–80% yields. Their chemical structures were proved by means of IR, ¹H NMR and elemental analyses data which were in full agreement with the assigned structures. The ¹H NMR spectra revealed the absence of the signals corresponding to the two methyl groups and instead the signals corresponding to the two hydroxy groups appeared at δ 4.15–4.65 ppm.

Antiviral activity

The synthesized compounds were tested for their antiviral activity against HBV using the HepG2.2–2.15 cell line, a human hepatoplasma cell line producing hepatitis B viral particles (Korba and Gerin, 1992). The results of our preliminary screening indicated that compounds 4a–c and 7a showed the highest effect at 10 μg/10⁵ cells, while compounds 7b and 7c showed moderate activity (Tables I and II).

In conclusion, new acyclic nucleosides were synthesized in order to increase the number of compounds screened for antiviral activity. Some of them displayed promising activities.

**Experimental**

**General**

Melting points were determined using a Büchi apparatus. IR spectra (KBr) were recorded with a Bruker-Vector22 instrument (Bruker, Bremen, Germany) and the ¹H NMR spectra were recorded with a Bruker-DRX500 instrument (Bruker, Bremen, Germany). The chemical shifts were recorded in ppm (δ) with respect to SiMe₄ for ¹H NMR spectra and KBr for IR spectra.

**Table I. Inhibitory concentration (IC₅₀) and selectivity index (SI) of the synthesized compounds.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ [µM]</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamivudine</td>
<td>0.1</td>
<td>1000</td>
</tr>
<tr>
<td>4a</td>
<td>0.2</td>
<td>500.0</td>
</tr>
<tr>
<td>4b</td>
<td>0.3</td>
<td>333.3</td>
</tr>
<tr>
<td>4c</td>
<td>0.5</td>
<td>200.0</td>
</tr>
<tr>
<td>7a</td>
<td>0.6</td>
<td>166.6</td>
</tr>
<tr>
<td>7b</td>
<td>0.7</td>
<td>142.8</td>
</tr>
<tr>
<td>7c</td>
<td>0.7</td>
<td>142.8</td>
</tr>
</tbody>
</table>

**Table II. Inhibition of HBV replication by the synthesized compounds.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration [µM]</th>
<th>HBV DNA in supernatant</th>
<th>HepG2 viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamivudine</td>
<td>0.1</td>
<td>0.25</td>
<td>1.00</td>
</tr>
<tr>
<td>4a</td>
<td>0.1</td>
<td>0.83</td>
<td>0.90</td>
</tr>
<tr>
<td>4b</td>
<td>0.1</td>
<td>0.21</td>
<td>0.63</td>
</tr>
<tr>
<td>4c</td>
<td>0.1</td>
<td>0.13</td>
<td>0.19</td>
</tr>
<tr>
<td>7a</td>
<td>0.1</td>
<td>0.17</td>
<td>0.56</td>
</tr>
<tr>
<td>7b</td>
<td>0.1</td>
<td>0.28</td>
<td>0.35</td>
</tr>
<tr>
<td>7c</td>
<td>0.1</td>
<td>0.28</td>
<td>0.35</td>
</tr>
</tbody>
</table>
Germany). 1H NMR spectra were recorded with a Varian Gemini spectrometer at 300 MHz and 200 MHz with TMS as internal standard. Chemical shifts are reported in δ scale (ppm) relative to TMS as a standard, and the coupling constants (J values) are given in Hz. The progress of the reactions was monitored by TLC using aluminum silica gel plates 60 F245. EI-mass spectra were recorded with a HP D5988 A 1000 MHz instrument (Hewlett Packard, Palo Alto, CA, USA). Antiviral activity against HBV was tested at the Liver Institute, Menoufia University, Shebin El-Koam, Egypt.

Antiviral activity

The synthesized compounds were tested for their antiviral activity against hepatitis B virus (HBV) using the HepG2.2–2.15 cell line (Sells et al., 1987), a human hepatoplasma cell line producing HBV particles (Korba and Gerin, 1992).

The cell line was maintained in RPMI-1640 (Glutamax) (Gibco BRL Life Technologies, Paisly, Scotland) culture medium containing 100 IU/mL nystatin (Gibco BRL Life Technologies), 380 μg/mL G418 (genetecin) (Gibco BRL Life Technologies), and 10% fetal calf serum (FCS) (Gibco BRL Life Technologies). The transferred HepG2.2–2.15 cells were kept in a tissue culture flask at 37 ºC and 5% CO2. Subcultures were set up after one week by trypsination [10% versin/trypsin (Biochrome KG, Berlin, Germany)] and transferred to a 96-well tissue culture plate. Serial dilutions of the test compounds were added to the cell suspension and incubated for 6 d at 37 ºC and 5% CO2. The antiviral activity and cytotoxic effect of the test compounds was estimated by comparing the DNA content in the culture supernatant and the viability of the cells containing the test compounds with those of HepG2.2–2.15 cells containing no compounds added to their supernatant (blank cells). The drug lamivudine (4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one, GlaxoSmithKline, Uxbridge, UK) which is a potent selective inhibitor of HBV replication has been used as a standard positive control. Each compound was tested in triplicate.

DNA extraction

DNA extraction was done by incubating 10 μL of diluted supernatant with 10 μL of 0.2 m NaOH at 37 ºC for 1 h, then carefully adding 9.6 μL of 0.2 m HCl, followed by addition of 90 μL of Tris-EDTA [2-amino-2-(hydroxymethyl)-1,3-propanediol-EDTA (TE) (Gibco BRL Life Technologies)] buffer.

PCR-ELISA detection of HBV DNA

The DNA content in the cell culture supernatant was determined by PCR amplification of the HBV DNA using 1 μmol/L of each of the following primers: HCID-1 (5'-GGAAAAG AAGTCAGAAGGCA-3') and HCID-2 (5'-TT- GGGGGAGGAGATTAGGGTT-3'), in a reaction mixture containing 14 μL extracted supernatant, 4 mmol/L MgCl2, 10 μmol/L DIG-11-dUTP (Roche, Munich, Germany), 190 μmol/L dTTP, 200 μmol/L dATP, dGTP, dCTP (Roche), 1.5 U Taq polymerase (Roche), in a total volume of 50 μL. PCR reaction conditions were: 32 cycles of 10 min at 94 ºC, 30 s at 58 ºC, and 30 s at 72 ºC, with a 3-s increment for each cycle, in a Perkin Elmer 480 thermal cycler (Perkin Elmer, Waltham, MA, USA). The PCR product was detected by the DIG-ELISA assay (Roche). The optical density (OD) of DNA of the test compounds was compared to that of the blank culture.

Cytotoxicity assay

3-(3,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) is a colourless substrate that is transferred to a coloured product by any living cell but not by dead cells. The assay utilizes MTT to test the viability of the cells in the presence of the test compound compared to the viability of the blank cells (Fouad et al., 1998).

Calculation of IC50, CC50 and SI values

The 50% inhibitory concentration (IC50) of an antiviral drug was determined by plotting the DNA content of the serial dilutions of the tested compound versus the concentration of this compound. The 50% cytotoxic effect (CC50) was calculated from the average viability of the cells in proportion to the concentration of the drug. The selective index (SI) was calculated as CC50/IC50.


