# Anti-Hepatitis B Virus Activity of New $N^4$-*β*-D-Glycoside Pyrazolo[3,4-*d*]pyrimidine Derivatives

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The reaction of 6-hydrazinyl-1,3-dimethylpyrimidine-2,4-(1*H*,3*H*)-dione (**1**) with ethoxymethylenemalononitrile afforded 5-amino-1-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-6-yl)-1*H*-pyrazole-4-carbonitrile (**2**). The latter was reacted with formamide and urea affording the corresponding 4-aminopyrazolo[3,4-*d*]pyrimidines **3** and **4**. The reaction of monosaccharide aldoses with **3** and **4** gave stereoselectively the *β*-*N*-glycosides **5a**–**d** and **6a**–**d** which were treated with acetic anhydride in pyridine to afford the corresponding acetylated derivatives **7a**–**d** and **8a**–**d**. The prepared compounds were tested for their antiviral activity against hepatitis B virus (HBV) and showed moderate to high activities.

*Key words:* Pyrazolo[3,4-*d*]pyrimidines, *N*-Glycosides, Anti-Hepatitis B Virus

## Introduction

Pyrazole and pyrimidine derivatives attracted organic chemists very much due to their biological and chemotherapeutic importance. Pyrazolopyrimidines and related fused heterocycles are of interest as potential bioactive molecules. They are known to exhibit pharmacological activities such as CNS-depressant (Abdou *et al.*, 2004; Julino and Stevens, 1998), antiviral (Gadhachanda *et al.*, 2007) anticancer (Capdeville *et al.*, 2002; Taylor *et al.*, 1992; Al-Saadi *et al.*, 2008) and tuberculostatic (Ghorab *et al.*, 2004) activities. Pyrazolo[3,4-*d*]pyrimidines were identified as a general class of adenosine receptors (Davies *et al.*, 1983, 1984). Furthermore, some pyrazolo[3,4-*d*] pyrimidine derivatives demonstrated significant antiviral activity (Anderson *et al.*, 1990; Rashad *et al.*, 2008). There is not much difference in the basic structures of pyrazolopyrimidines and purines (Holla *et al.*, 2006). On the other hand, glycosyl amines (Ellis and Honeyman, 1955; Kuhn, 1957; Durette *et al.*, 1978; Katzen, 1979; Cascieri *et al.*, 1979; Danishefsky and Allen, 2000) are important because these occur as junctures in glycoproteins (Kent, 1967; Hughes, 1973). The chemical and structural nature of the derivatives formed by the reaction of a monosaccharide and different nitrogen bases depends upon the reaction conditions and the base used (Ojala *et al.*, 2000). *N*-Glycosyl amines (Fig. 1) have advantage over their saccharide counterparts in binding to metal ions, as these compounds provide additional binding centres. In view of such important aspects of *N*-glycosyl amines (Ali *et al.*, 2007a) and our interest in the synthesis of biologically active glycosides (El-Sayed *et al.*, 2008; Ali *et al.*, 2007b) the aim of the present work was the synthesis and anti-hepatitis B virus evaluation of different *N*-glycosyl amines of simple saccharides using 4-aminopyrazolo[3,4-*d*]-pyrimidines as amines.

## Experimental

### General

Melting points were determined using a Büchi apparatus. IR spectra (KBr) were recorded with a Bruker-Vector22 instrument (Bruker, Bremen, Germany). $^1$H NMR spectra were recorded with a Varian Gemini spectrometer at 300 MHz and 200 MHz with TMS as internal standard. Chemical shifts were reported in $\delta$ 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 $F_{245}$. EI-mass spectra were

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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.

*6-(4-Amino-1H-pyrazolo[3,4-d]pyrimidin-1-yl)-1,3-dimethylpyrimidine-2,4(1H,3H)-dione (**3**)*

5 g of the carbonitrile **2** and 10 mL formamide were heated at 180–190 °C for 30 min. The cooled solution was diluted with 100 mL ice-cold water, and the precipitate obtained was filtered to yield 3.1 g of the crude product **3**. The product was purified by dissolving it in 10% HCl, the insoluble part was removed by filtration. The filtrate was taken and the pH value was adjusted to 8 with concentrated ammonium hydroxide. This solution was allowed to cool to 10–15 °C. Then it was filtered, washed with water to afford **3** in a pure form. Yield: 2.5 g (45%).

*6-(4-Amino-6-oxo-6,7-dihydropyrazolo[3,4-d]-pyrimidin-1-yl)-1,3-dimethylpyrimidine-2,4-(1H,3H)-dione (**4**)*

5 g of the carbonitrile **2** and 10 g urea were heated at 180–185 °C for 10 min. The resulting solid was dissolved in dilute sodium hydroxide and then carefully acidified with acetic acid to obtain 3.9 g of crude product **4**. Further purification was accomplished by reprecipitation from dilute sodium hydroxide with acetic acid. Yield: 3.0 g (51%).

*General procedure for the synthesis of the $N^4$-β-D-glycosides **5a–d** and **6a–d***

A solution of the respective sugar (5 mmol) in ethanol (5 mL) was treated with **3** or **4** (5 mmol) in ethanol (40 mL) and glacial acetic acid (0.1 mL). The mixture was heated under reflux for 2 h (control by TLC). The solid, which separated out on cooling, was filtered, washed with ethanol, and crystallized from ethanol to afford **5a–d** in 80–85% and **6a–d** in 78–83% yields.

*General procedure for the synthesis of the $N^4$-β-D-glycosides **7a–d** and **8a–d***

A mixture of **5a–d** or **6a–d** (0.2 g), dry pyridine (3 mL), and acetic anhydride (3 mL) was stirred for 15 min at 0 °C, then kept overnight at room temperature (r.t.) with stirring. The mixture was poured onto crushed ice (30 g); and the precipitate was collected by filtration, washed repeatedly with water, dried, and recrystallized from ethanol/water (2:8, v/v) to afford **7a–d** in 95–98% and **8a–d** in 96–97% yields.

*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 hepatoplastoma cell line producing HBV viral 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 °C and 5% $CO_2$. Subcultures were set up after a 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 °C and 5% $CO_2$. The antiviral activity and cytotoxic effect of the test compounds were estimated by comparing the DNA content in the culture supernatant and the viability of the cells with the test compounds added to those of HepG2.2.2.15 cells with no compounds added to their supernatant (blank cells). The drug lamivudine [4-amino-1-[(2*R*,5*S*)-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 °C for 1 h, then carefully adding 9.6 μL of 0.2 M HCl followed by addition of 90 μL of Tris-EDTA (TE) buffer [2-amino-2-(hydroxymethyl)-1,3-propanediol-EDTA (Gibco BRL Life Technologies)].

*PCR-ELISA detection of HBV DNA*

The DNA content of the cell culture supernatant was determined by PCR amplification of the HBV DNA using 1 μmol/L of each of the primers HCID-1 (5′-GGAAAGAAGTCAGAAGGCA-3′) and HCID-2 (5′-TTGGGGGAGGAGATTAGGTT-3′), in a reaction mixture containing 14 μL extracted supernatant, 4 mmol/L $MgCl_2$, 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 DIG-ELISA assay (Roche). The optical density (OD) of DNA of the test compound 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 this compound to test for the viability of the cells with the test compound added compared to the viability of the blank cells (Fouad *et al.*, 1998).

*Calculation of $IC_{50}$, $CC_{50}$, and SI values*

The 50% inhibitory concentration ($IC_{50}$) of an antiviral drug is determined by plotting the DNA content of the serial dilutions of the tested compound versus the concentration of this compound. The 50% cytotoxic effect ($CC_{50}$) is calculated from

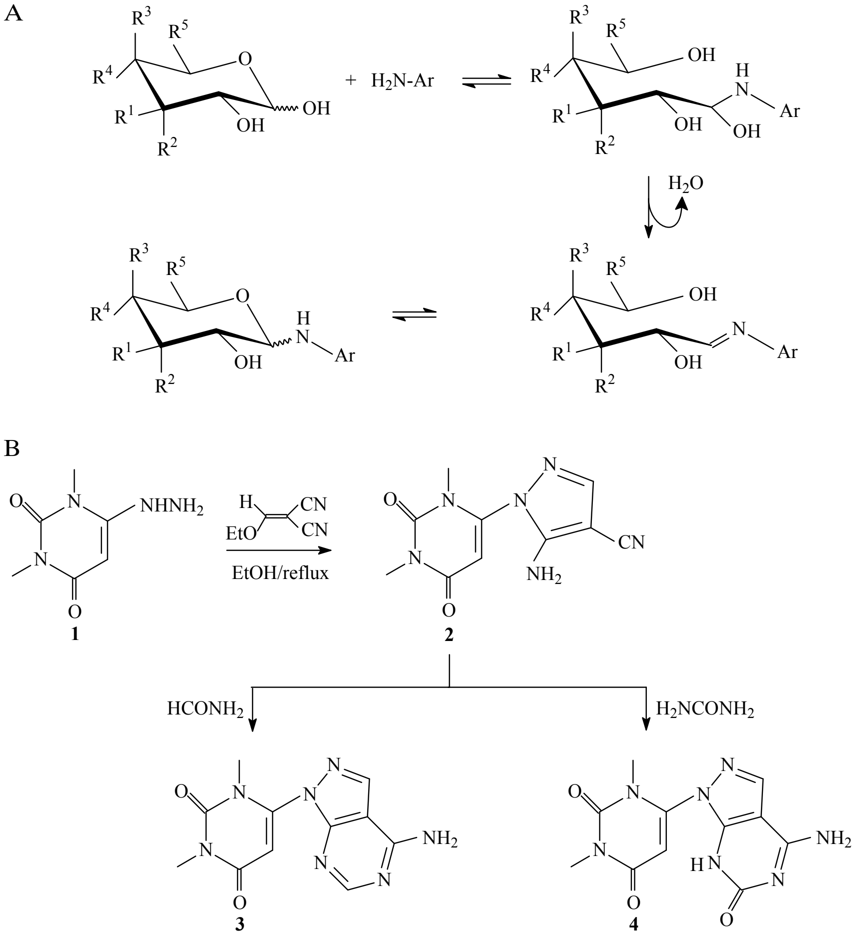


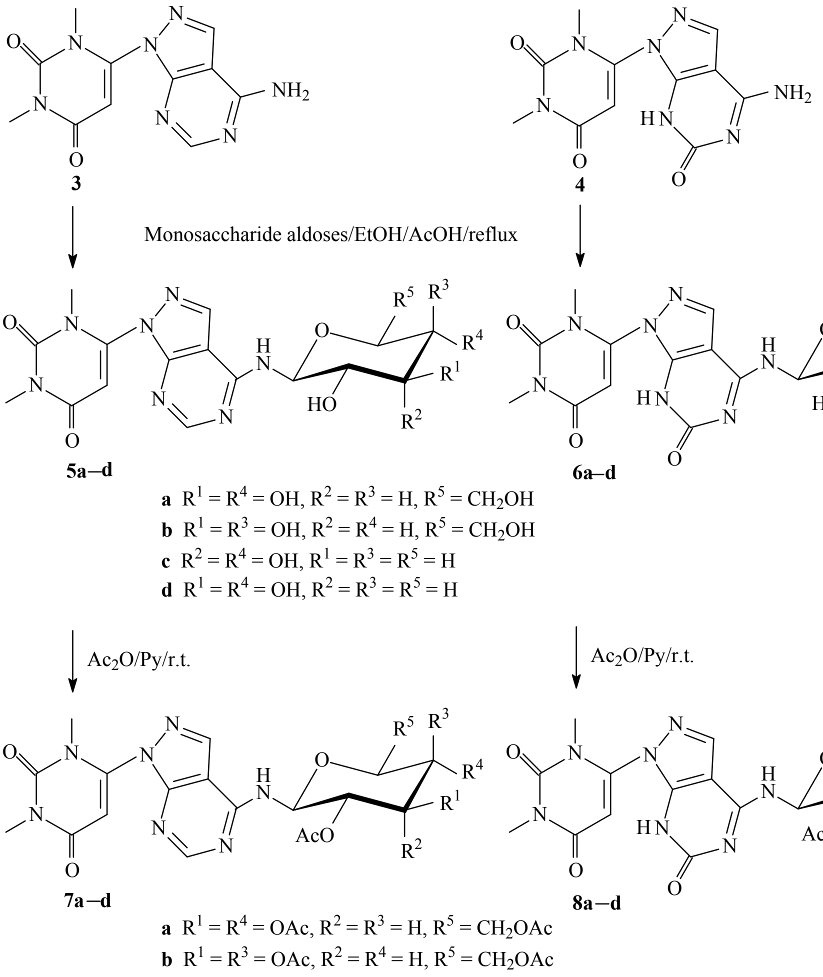
Fig. 1. (A) Mechanism of glycosyl amines formation and (B) synthesis of **3** and **4**.

the average viability of the cells in proportion to the concentration of the drug. The selective index (SI) can be calculated as $CC_{50}/IC_{50}$.

## Results and Discussion

### *Chemistry*

In the present investigation, 6-hydrazinyl-1,3-dimethylpyrimidine-2,4-(1*H*,3*H*)-dione (**1**) (Pfleiderer and Schündehütte, 1958) was condensed with ethoxymethylenemalononitrile in ethanol at reflux temperature to afford 5-amino-1-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydropyrimidin-6-yl)-1*H*-pyrazole-4-carbonitrile (**2**) in 65% yield. The structure of the produced carbonitrile was proved by its spectral data; IR, $^1$H NMR and mass spectra were in agreement with the assigned structure. The IR spectrum showed characteristic absorption bands at 3450 cm$^{-1}$ ($NH_2$) and 2225 cm$^{-1}$ (CN). The $^1$H NMR spectrum showed a signal at $\delta$ 6.99 ppm, a broad singlet, integrating for two protons and indicating the presence of an $NH_2$ group in the pyrazole ring (which disappeared in $D_2O$); all aromatic proton signals exactly matched the structure. The formation of carbonitrile **2** was also confirmed by recording its mass spectrum. The molecular ion peak appeared at *m/z* 247 ($[MH]^+$,



**a** $R^1 = R^4 = OH, R^2 = R^3 = H, R^5 = CH_2OH$
**b** $R^1 = R^3 = OH, R^2 = R^4 = H, R^5 = CH_2OH$
**c** $R^2 = R^4 = OH, R^1 = R^3 = R^5 = H$
**d** $R^1 = R^4 = OH, R^2 = R^3 = R^5 = H$

**a** $R^1 = R^4 = OAc, R^2 = R^3 = H, R^5 = CH_2OAc$
**b** $R^1 = R^3 = OAc, R^2 = R^4 = H, R^5 = CH_2OAc$
**c** $R^2 = R^4 = OAc, R^1 = R^3 = R^5 = H$
**d** $R^1 = R^4 = OAc, R^2 = R^3 = R^5 = H$

Fig. 2. Synthesis of glycosyl amines from pyrazolo[3,4-*d*]pyrimidine derivatives.

66%), which is in accordance with its molecular formula.

Compound **2** was reacted with formamide and urea to afford 6-(4-amino-1*H*-pyrazolo[3,4-*d*]-pyrimidin-1-yl)-1,3-dimethylpyrimidine-2,4-(1*H*,3*H*)-dione (**3**) and 6-(4-amino-6-oxo-6,7-dihydropyrazolo[3,4-*d*]pyrimidin-1-yl)-1,3-dimethylpyrimidine-2,4(1*H*,3*H*)-dione (**4**), respectively (Fig. 1). The formation of the pyrimidines was confirmed by recording their spectra. Thus, the $^1$H NMR spectrum of **3** showed a signal at $\delta$ 7.37 ppm corresponding to the $NH_2$ peak, and all other peaks exactly matched the structure. In the IR spectrum, the absence of a CN peak indicated that the cyclization was completed. The mass spectrum of **3** also confirmed the formation of the molecule. The structure of compound **4** was also confirmed by recording its spectra. Condensation of **3** and **4** with a number of monosaccharides (D-glucose, D-galactose, D-ribose, and D-xylose) in boiling ethanol and in the presence of a catalytic amount of glacial acetic acid gave stereoselectively 4-($\beta$-D-glycopyranosylamino)pyrazolo[3,4-*d*]pyrimidines **5a–d** in 80–85% yields and **6a–d** in yields of 78–83% (Fig. 2). In the $^1$H NMR spectra of **5a–d** and **6a–d** the anomeric protons appeared as doublet in the range $\delta$ 7.10–7.15 ppm with $J_{(1,2)}$ = 8.0 Hz. This value is consistent with the $\beta$-configuration of $^4C_1$ (D) conformation (Gorin and Mazurek, 1975; Abdel-Megeed *et al.*, 1995). The reaction of **5a–d** and **6a–d** with acetic anhydride in pyridine at room temperature (r.t.) led to the acetylation of sugar hydroxy groups, without affecting the NH group, to give the tri- and tetraacetoxy-$\beta$-D-glycosides **7a–d** in yields of 95–98% and **8a–d** in 96–97% yields. The IR spectra of **7a–d** and **8a–d** are characterized by two absorption bands at 3380–3395 and 1745–1755 cm$^{-1}$ indicating the presence of OAc and NH groups. In addition, the $^1$H NMR spectra showed triplets at low field at $\delta$ 5.10–5.20 ppm assigned as H-1', with $J_{(1,2)}$ = 9.0 Hz, which confirmed the expected $\beta$-configuration of $^4C_1$ (D) conformation (Fig. 2).

### *Antiviral testing*

The synthesized compounds were tested for their antiviral activity against hepatitis B virus (HBV) using the HepG2.2.2.15 cell line, a human hepatoplastoma cell line producing HBV viral particles (Korba and Gerin, 1992). The results of our preliminary screening indicated that compounds **5a**, **5b**, **6a**, and **6b** showed the highest inhibitory activity among this series of tested compounds with an effective inhibitory concentration of 0.2 $\mu$M and a selectivity index > 500.0, followed by compounds **5c**, **5 d**, **6c**, and **6 d**. Compounds **7a–d** and **8a–d** showed moderate inhibitory activity with moderate cytotoxicity, while the other tested compounds **2–4** exhibited less activity against HBV (Table I).

From the results of the viral inhibition tests and structure-activity relationship, we conclude that the glycosyl amine residues attached to the pyrazolo[3,4-*d*]pyrimidine derivatives increase the antiviral activity against HBV. Moreover, the free hydroxy *N*-glycosyl amine derivatives of pyrazolo[3,4-*d*]pyrimidines showed higher inhibitory activity than the corresponding acetylated derivatives.

Table I. Inhibitory concentration ($IC_{50}$) and selectivity index (SI) of the newly synthesized compounds **2–8**. Cytotoxic effect ($CC_{50}$) of all tested compounds is > 100 $\mu$M.

| Compound | $IC_{50}$ [$\mu$M] | SI |
|---|---|---|
| Lamivudine | <0.1 | > 1000.0 |
| **2** | 1.4 | > 71.4 |
| **3** | 0.9 | > 111.1 |
| **4** | 1.3 | > 76.9 |
| **5a** | 0.2 | > 500.0 |
| **5b** | 0.2 | > 500.0 |
| **5c** | 1.2 | > 383.3 |
| **5d** | 0.3 | > 333.3 |
| **6a** | 0.2 | > 500.0 |
| **6b** | 0.2 | > 500.0 |
| **6c** | 0.3 | > 333.3 |
| **6d** | 0.3 | > 333.3 |
| **7a** | 0.4 | > 250.0 |
| **7b** | 0.5 | > 200.0 |
| **7c** | 0.5 | > 200.0 |
| **7d** | 0.5 | > 200.0 |
| **8a** | 4.0 | > 250.0 |
| **8b** | 0.5 | > 200.0 |
| **8c** | 0.5 | > 200.0 |
| **8d** | 0.5 | > 200.0 |

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