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Homoacyclovir analogues of unnatural bases and their activity against Hepatitis B virus

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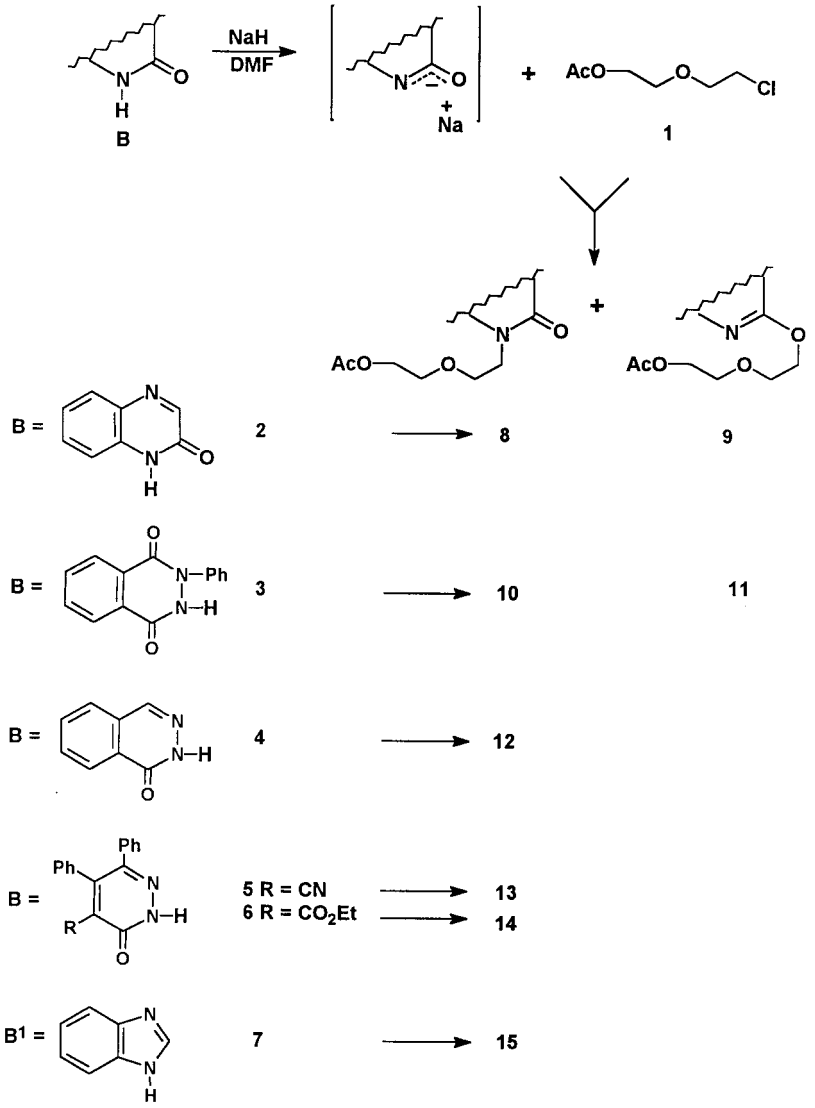
The ambident nucleophilic nature of the sodium salts of 2(1 *H*)-qunioxalinone (**2**) and the phthalazinedione (**3**) has been realized from their alkylation with 2-(2-chloroethoxy)ethylacetate (**1**) to afford 1-[2-(2-acetoxyethoxy)ethyl]-2(1 *H*)-quinoxalinone (**8**) and 2-[2-(2-acetoxyethoxy)ethoxy]qunioxaline (**9**) as well as **10** and **11**, respectively. The corresponding derivatives **12**–**15** were similary prepared by the alkylation of the unnatural bases **4**–**7** with **1**. Treatment of the alkylated derivatives **8**–**15** with methanolic ammonia solution (1 : 1) at room temperature gave the corresponding hydroxyalkyl derviatives **16**–**23**. The site of the alkylation was deduced from the spectral characteristics of the products. The activity of compounds **16**–**22** against Hepatitis B virus (HBV) in $HepG_2$ cell has been tested. Some of the compounds showed high viral replication inhibition with low cytotoxicity.

1. Introduction

The discovery that 9-[(2-hydroxyethoxy)methyl] guanine (acyclovir, ACV) possesses potent, selective, antiherpes activity [1–6], and it is essentially nontoxic to uninfected host cells [7, 8], has led to extensive efforts to synthesize other acyclic analogues of the natural nucleosides [1–5, 9–17] and to evaluate their biological activity. In this re-

Scheme 1



spect, different approaches have been explored. Thus modification of the acylic side chain has led to nucleosides displaying strong antiviral activity such as ganciclovir [18–20], penciclovir [21, 22], (*S*)-9-(2,3-dihydroxypropyl)-adenine [23], (*R*)-9-(3,4-dihydroxybutyl)guanine [24]. On the other hand, modification of the base moiety led to compounds possessing interesting biological activities. Among these nucleosides is 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) [25, 26] which showed a selective antiviral activity against human immunodeficiency virus type 1 (HIV-1).

Various HEPT anologues [25–29] as well as other acyclovir analogues with modified bases [1–6] have been of continued interest. The acyclo *C*-nucloside analogues are also interesting targets [1–3]. Although many nucleoside analogues have been synthesized and their antiviral activity were evaluated, an urgent need for new classes of compounds is still required.

2. Investigations, results and discussion

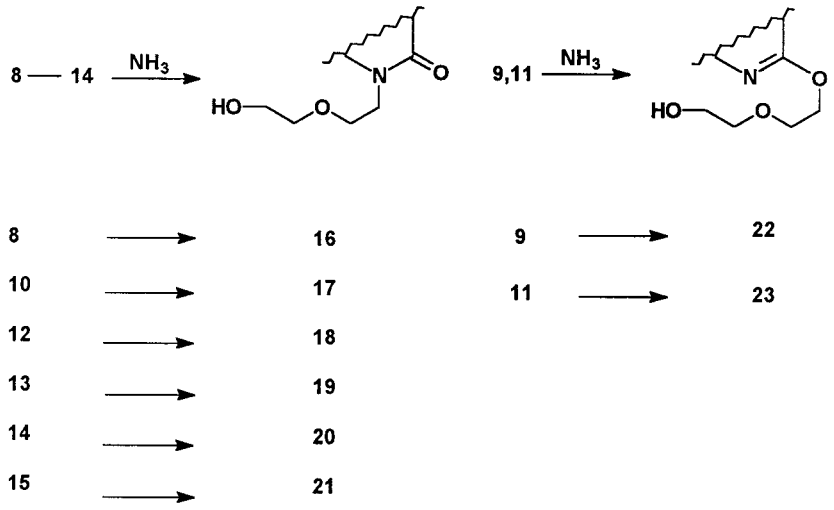
For a number of years, we have been engaged in a research program on acyclic nucleosides [30–32]. Recently, El Ashry et al. reviewed the literature on acyclic nucleosides and its analogues. [1–3]. The extensive literature in this area led to the development of a methods for their classification based on the number of missing bonds of the respective acyclic moiety. Thus, extending the term seco progressively for nucleosides with more missing bonds to be di, tri, tetra and pentaseco nucleosides has been used. Thus, acyclovir and HEPT are classified as triseconucleosides. According, the 1-[(2-hydroxyethoxy)ethyl] uracils that were prepared as antiviral agents [33] can be classified as either diseco or homotriseco nucleosides. In the present work, the homoacyclovir analogues of some unnatural bases have been synthesized and their activity against Hepatitis B virus (HBV) was evaluated. Alkylation of the sodium salt of the 2(1 *H*)-quinoxalinone (**2**) with 2-(2-chloroethoxy)ethylacetate (**1**) gave a mixture of two products which could by separated by column chromatography to give 28% and 36% yield of **8** and **9**, respectively. Both compounds showed in their FAB MS a peak at m/z 277 corresponding to a protonated molecular ion. Matching of their molecular ion peaks agreed with the molecular weight 276.2950 which has the formula $C_{14}H_{16}N_2O_4$. This led to the conclusion that both **8** and **9** are isomeric alkylated derivatives of **2**.

Their ^{1}H NMR spectra confirmed the presence of one acetyl and four methylene groups in addition to the aromatic protons of the quinoxaline ring (Table 1). The site of alkylation can be readily deduced from the carbon chemical shifts of the methylene groups in their ^{13}C NMR spectra (Table 2). Thus, C-3 and C-4 are located at the same range of chemical shifts whereas C-1 (δ 40.96) in **8** is highly shielded compared to that carbon (δ 65.22) in **9**. The shielding effect on that carbon in **8** can be attributed to its attachment to the N-1 of the quinoxalinone ring, whereas the respective carbon in **9** is linked to the O-2 of the quinoxaline ring i.e. an ether linkage causes a shift of the methylene carbon to a lower field.

Table 1: ^{1}H NMR Spectra (250 MHz, DMSO-d_6/TMS) of compounds 8–23

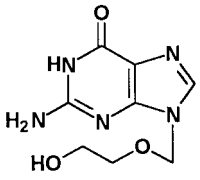
Compd.	δ (multiplicity, assignment)
8	1.91 (s, 3 H, $COCH_3$), 3.61 (t, 2 H, J = 4.5 Hz, CH_2), 3.76 (t, 2 H, J = 5.7 Hz, CH_2), 4.05 (t, 2 H, J = 4.8 Hz, CH_2), 4.42 (t, 2 H, J = 5.7 Hz, CH_2), 7.37 (t, 1 H, J = 8.0 Hz, Ar–H), 7.67 (m, 2 H, Ar–H), 7.81 (d, 1 H, J = 7.9 Hz, Ar–H), 8.24 (s, 1 H, Ar–H)
9	2.02 (s, 3 H, $COCH_3$), 3.74 (t, 2 H, J = 4.4 Hz, CH_2), 3.89 (t, 2 H, J = 4.4 Hz, CH_2), 4.18 (m, 2 H, CH_2), 4.60 (m, 2 H, CH_2), 7.65 (m, 1 H, Ar–H), 7.82 (m, 2 H, Ar–H), 8.00 (d, 1 H, J = 7.8 Hz, Ar–H), 8.61 (s, 1 H, Ar–H)
10	2.02 (s, 3 H, $COCH_3$), 3.79 (m, 2 H, CH_2), 3.91 (m, 2 H, CH_2), 4.21 (m, 2 H, CH_2), 4.61 (m, 2 H, CH_2), 7.40 (m, 1 H, Ar–H), 7.55 (t, 2 H, J = 7.5 Hz, Ar–H), 7.74 (d, 2 H, J = 7.6 Hz, Ar–H), 7.95 (m, 3 H, Ar–H), 8.32 (d, 1 H, J = 6.8 Hz, Ar–H)
11	2.00 (s, 3 H, $COCH_3$), 3.74 (m, 2 H, CH_2), 3.80 (m, 2 H, CH_2), 4.18 (m, 2 H, CH_2), 4.32 (m, 2 H, CH_2), 7.38 (m, 1 H, Ar–H), 7.51 (t, 2 H, J = 7.6 Hz, Ar–H), 7.75 (d, 2 H, J = 7.7 Hz, Ar–H), 7.96 (m, 3 H, Ar–H), 8.31 (d, 1 H, J = 6.9 Hz, Ar–H)
12	1.97 (s, 3 H, $COCH_3$), 3.71 (t, 2 H, J = 4.6 Hz, CH_2), 3.95 (t, 2 H, J = 5.8 Hz, CH_2), 4.18 (t, 2 H, J = 4.9 Hz, CH_2), 4.44 (t, 2 H, J = 5.8 Hz, CH_2), 7.69 (m, 3 H, Ar–H), 8.16 (s, 1 H, Ar–H), 8.39 (d, 1 H, J = 7.1 Hz, Ar–H)
13	2.04 (s, 3 H, $COCH_3$), 3.74 (t, 2 H, J = 4.5 Hz, CH_2), 4.00 (t, 2 H, J = 5.4 Hz, CH_2), 4.21 (t, 2 H, J = 4.4 Hz, CH_2), 4.52 (t, 2 H, J = 5.5 Hz, CH_2), 7.07 (m, 2 H, Ar–H), 7.19 (m, 4 H, Ar–H), 7.35 (m, 4 H, Ar–H)
14	1.99 (t, 3 H, J = 7.1 Hz, CH_2CH_3), 2.00 (s, 3 H, $COCH_3$), 3.72 (m, 2 H, CH_2), 3.97 (t, 2 H, J = 5.6 Hz, CH_2), 4.07 (q, 2 H, J = 7.0 Hz, CH_2CH_3), 4.19 (t, 2 H, J = 4.4 Hz, CH_2), 4.47 (t, 2 H, J = 5.4 Hz, CH_2), 7.12 (m, 4 H, Ar–H), 7.22 (m, 4 H, Ar–H), 7.40 (s, 2 H, Ar–H)
15	2.00 (s, 3 H, $COCH_3$), 3.56 (t, 2 H, J = 4.5 Hz, CH_2), 3.78 (t, 2 H, J = 5.2 Hz, CH_2), 4.13 (t, 2 H, J = 4.6 Hz, CH_2), 4.30 (t, 2 H, J = 5.1 Hz, CH_2), 7.27 (m, 2 H, Ar–H), 7.37 (m, 1 H, Ar–H), 7.78 (m, 1 H, Ar–H), 7.96 (s, 1 H, Ar–H)
16	3.46 (s, 4 H, 2 CH_2), 3.74 (t, 2 H, J = 5.9 Hz, CH_2), 4.34 (brs, 1 H, OH), 4.42 (t, 2 H, J = 5.9 Hz, CH_2), 7.38 (t, 1 H, J = 6.7 Hz, Ar–H), 7.69 (m, 2 H, Ar–H), 7.81 (d, 1 H, J = 7.1 Hz, Ar–H), 8.24 (s, 1 H, Ar–H)
17	3.58 (s, 2 H, CH_2), 3.91 (s, 4 H, 2 CH_2), 4.50 (s, 2 H, CH_2), 4.60 (brs, 1 H, OH), 7.32 (m, 1 H, Ar–H), 7.53 (m, 2 H, Ar–H), 7.70 (m, 2 H, Ar–H), 7.92 (m, 3 H, Ar–H), 8.30 (m, 1 H, Ar–H)
18	3.09 (brs, 1 H, OH), 3.66 (d, 4 H, J = 6.1 Hz, 2 CH_2), 3.95 (t, 2 H, J = 5.1 Hz, CH_2), 4.45 (t, 2 H, J = 3.9 Hz, CH_2), 7.73 (m, 3 H, Ar–H), 8.18 (s, 1 H, Ar–H), 8.40 (d, 1 H, J = 7.0 Hz, Ar–H)
19	3.23 (s, 1 H, OH), 3.67 (m, 4 H, 2 CH_2), 3.99 (t, 2 H, J = 5.2 Hz, CH_2), 4.51 (t, 2 H, J = 5.3 Hz, CH_2), 7.11 (m, 2 H, Ar–H), 7.23 (m, 4 H, Ar–H), 7.32 (m, 4 H, Ar–H)
20	0.96 (m, 3 H, CH_3), 3.64 (m, 4 H, 2 CH_2), 3.90 (m, 4 H, 2 CH_2), 4.33–4.48 (m, 3 H, CH_2, OH), 7.19 (m, 10 H, Ar–H).
21	3.41 (t, 2 H, J = 4.3 Hz, CH_2), 3.66 (m, 4 H, 2 CH_2), 4.14 (t, 2 H, J = 4.9 Hz, CH_2), 4.82 (s, 1 H, OH), 7.19 (m, 2 H, Ar–H), 7.21 (m, 1 H, Ar–H), 7.72 (m, 1 H, Ar–H), 7.96 (s, 1 H, Ar–H)
22	3.57 (s, 4 H, 2 CH_2), 3.88 (t, 2 H, J = 4.0 Hz, CH_2), 4.60 (t, 2 H, J = 5.0 Hz, CH_2), 4.67 (brs, 1 H, OH), 7.65 (t, 1 H, J = 4.1 Hz, Ar–H), 7.82 (m, 2 H, Ar–H), 8.00 (d, 1 H, J = 7.5 Hz, Ar–H), 8.61 (s, 1 H, Ar–H)
23	3.56 (s, 4 H, 2 CH_2), 3.87 (s, 2 H, CH_2), 4.44 (s, 2 H, CH_2), 4.64 (brs, 1 H, OH), 7.39 (m, 1 H, Ar–H), 7.51 (m, 2 H, Ar–H), 7.72 (m, 2 H, Ar–H), 7.99 (m, 3 H, Ar–H), 8.32 (m, 1 H, Ar–H)

Scheme 2

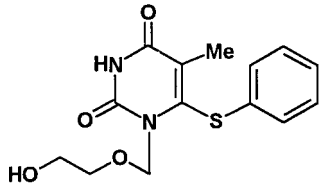


The ether linkage extends its effect to the β-carbon (C-2) which caused its shift to a lower field (δ 68.31) in **9** compared to that carbon (δ 66.88) in **8**. The rest of the ^{13}C NMR spectra are in agreement with the assigned structures. These results indicated that the sodium salt of the quinoxalinone **2** behaved as an ambident nucleophile in the nucleophilic displacement of the chlorine atom to give **8** and **9**. Similary the sodium salt of **3** was alkylated by **1** to give **10** and **11**. Alkylation of the sodium salts of the bases **4**–**7** with **1** gave the homotriseco nucleoside analogues **12**–**15**.

Deacetylation of **8**–**15** with methanolic ammonia solution gave **16**–**23**, respectively. Their MS agreed with molecular formulas which indicated hydrolysis of the acetyl groups.



ACV



HEPT

Compounds **16**–**22** were tested for their activity against Hepatitis B Virus (HBV) in $HepG_2$ 2.2.15 cells. The concentrations for the tested compounds were 10 μM. Compounds **16**, **18** and **19** showed high inhibition activity (Table 3) and low cytotoxicity. Compounds **21** and **22**

Table 2: ^{13}C NMR spectra of compounds 8–23

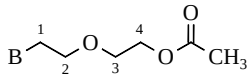
Compd.	Heterocyclic ring carbons						
		C-1	C-2	C-3	C-4	CO	CH_3
8	115.12, 123.24, 129.54, 130.70, 132.61, 149.95, 154.20	40.96	66.88	68.13	62.71	170.05	20.36
9	126.63, 128.54, 130.24, 138.24, 139.58, 139.67, 156.74	65.22	68.31	68.31	63.01	170.17	20.41
10	113.18, 123.71, 125.22, 126.78, 128.75, 132.41, 133.51, 141.71, 149.10, 157.20	50.33	66.10	68.30	63.04	170.18	20.47
11	123.16, 123.78, 125.21, 126.77, 128.27, 128.74, 132.44, 133.49, 141.73, 149.09, 157.18	66.08	68.15	68.32	63.02	170.17	20.46
12	125.46, 125.96, 129.05, 130.98, 132.49, 137.14, 158.82	49.55	67.67	68.00	62.80	170.15	20.19
13	113.10 (CN), 128.20, 128.62, 128.76, 128.88, 129.03, 130.28, 132.38, 134.10, 145.56, 150.84, 156.69	51.45	67.60	68.50	63.24	170.09	20.74
14	13.02 ($\underline{CH_3}CH_2O$), 61.02 ($CH_3\underline{CH_2}O$), 127.24, 127.61, 127.85, 128.06, 128.34, 128.57, 133.01, 134.40, 140.88, 145.35, 156.18, 163.27	50.33	67.14	68.25	62.76	170.00	20.11
15	109.29, 120.04, 121.77, 122.55, 133.58, 143.40	44.64	68.92	69.09	62.83	170.62	20.55
16	115.11, 123.33, 129.61, 130.85, 132.57, 149.88, 154.23	41.09	66.91	72.30	60.16		
17	123.32, 123.82, 125.32, 126.81, 128.30, 128.60, 132.51, 133.61, 141.52, 149.25, 157.20	50.18	66.61	72.31	60.26		
18	125.87, 126.52, 131.57, 133.00, 137.88, 159.59	50.19	68.55	72.19	60.50		
19	112.97 (CN), 127.78, 128.14, 128.35, 128.53, 128.53, 128.68, 128.81, 130.08, 133.75, 145.64, 150.67, 156.77	51.46	67.63	72.02	61.11		
20	13.03 ($\underline{CH_3}CH_2O$), 60.71 ($CH_3\underline{CH_2}O$), 127.34, 127.68, 128.08, 128.46, 128.60, 129.58, 132.88, 134.24, 141.20, 145.94, 156.60, 163.41	50.65	67.48	71.77	61.23		
21	109.14, 118.94, 121.42, 122.17, 132.94, 142.21, 143.11	44.05	68.24	72.01	60.14		
22	126.66, 126.77, 128.57, 130.29, 138.25, 139.44, 139.64, 156.82	65.52	68.28	72.37	60.19		
23	123.31, 123.88, 125.31, 126.86, 128.33, 128.80, 132.58, 133.67, 141.57, 149.20, 157.25	66.31	68.26	72.44	60.21		

Table 3: Inhibition of HBV replication by selected compounds

Compd. (10 μM)	% Inhibition			Cytotoxicity
	1 week	2 weeks	3 weeks	
16	79.9	79.0	71.3	6.8
17	33.5	30.1	27.6	7.8
18	84.7	82.9	75.9	6.9
19	81.5	80.1	78.6	7.2
20	27.1	26.2	25.1	5.7
21	81.7	79.6	70.7	14.8
22	82.5	80.3	70.9	15.5

showed high inhibition activity with moderate cytotoxicity while compounds **17** and **20** showed moderate viral replication inhibition and low cytotoxicity.

3. Experimental

The NMR spectra were recorded on Bruker AC 250 MHz and 200 MHz spectrometers. Chemical shifts were reported in δ scale (ppm) relative to TMS as internal, and described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or bs (broad singlet). EI and high resolution MS were recorded on a Varian MAT 311A spectrometer. FAB on a Kratos MS 50 spectrometer. Silica gel TLC was performed on 60 F-254 precoated plates (Merck). CC was performed on silica gel (Merck) (0.040–0.063). All solvents were distilled and dried before use.

3.1. 2-(Acetoxyethoxy)ethyl hetaryls 8–15

A mixture of the base **2–7** (14 mmol) and NaH (60% dispersed in mineral oil (0.56 g, 14 mmol) in dry DMF (20 ml) was stirred at 100 °C for 1 h, and then cooled to room temperature. 2-(2-Chloroethoxy)ethylacetate (2.66 g, 14 mmol) was added and the mixture was stirred at 100 C for 5–8 h. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel column with $CHCl_3$/MeOH (99 : 1) as eluent to give the products as gums.

3.1.1. 1-[2-(2-Acetoxyethyoxy)ethyl]-2(1 H)-quinoxalinone (**8**)

Reaction time: 5 h; yield: 28%; ms: (FAB) m/z (%) = 277 ($M^+ + 1$, 74), 235 (12), 173 (100). Anal. Calcd. for $C_{14}H_{16}N_2O_4$: M, 276.2950. Found: m/z 276.2944

3.1.2. 2-[2-(2-Acetoxyethoxy)ethoxy]quinoxaline (**9**)

Reaction time: 5 h; yield: 36%; ms: (FAB) m/z (%) = 277 ($M^+ + 1$, 100), 173 (20), 154 (56). Anal. Calcd. for $C_{14}H_{16}N_2O_4$: M, 276.2950. Found: m/z 276.2945

3.1.3. 3-[2-(2-Acetoxyethyoxy)ethyl]-2-phenyl-2,3-dihydrophthalazine-1,4-dione (**10**)

Reaction time: 6 h; yield: 71%; ms: (EI) m/z (%) = 368 (M^+, 17), 238 (100). Anal. Calcd. for $C_{20}H_{20}N_2O_5$: M, 368.3920. Found: m/z 368.3924

3.1.4. 4-[2-(2-Acetoxyethoxy)ethoxy]-2-phenyl-1(2 H)-phthalazinone (**11**)

Reaction time: 6 h; yield: 10%; ms: (EI) m/z (%) = 368 (M^+, 12), 265 (2), 238 (100). Anal. Calcd. for $C_{20}H_{20}N_2O_5$: M, 368.3920. Found: m/z 368.3913

3.1.5. 2-[2-(2-Acetoxyethoxy)ethyl]-1(2 H)-phthalzinone (**12**)

Reaction time: 5 h; yield: 73%; ms: (EI) m/z (%) = 276 (M^+, 5), 189 (49), 173 (42), 159 (61), 146 (100), Anal. Calcd. for $C_{14}H_{16}N_2O_4$: M, 276.2950. Found: m/z 276.2955

3.1.6. 1-[2-(2-Acetoxyethoxy)ethyl]-5-cyano-3,4-diphenyl-6(1 H)-pyridazinone (**13**)

Reaction time: 8 h; yield: 65%; ms: (FAB) m/z (%) = 404 ($M^+ + 1$, 76), 362 (68), 300 (100). Anal. Calcd. for $C_{23}H_{21}N_3O_4$: M, 403.4410. Found: m/z 403.4406

3.1.7. 1-[2-(2-Acetoxyethoxy)ethyl]-5-carbethoxy-3,4-diphenyl-6(1 H)-pyridazinone (**14**)

Reaction time: 8 h; yield: 59%; ms: (FAB) m/z (%) = 451 ($M^+ + 1$, 100), 363 (22). Anal. Calcd. for $C_{25}H_{26}N_2O_6$: M, 450.4950. Found: m/z 450.4954

3.1.8. 1-[2-(2-Acetoxyethoxy)ethyl]-(1 H)-benzimidazole (**15**)

Reaction time: 5 h; yield: 77%; ms: (EI) m/z (%) = 248 (M^+, 36), 206 (8), 132 (40), 131 (100). Anal. Calcd. for $C_{13}H_{16}N_2O_3$: M, 248.2840. Found: m/z 248.2835

3.2. Deprotection of the acetyl derivatives

Compounds **8–15** (0.3 g) were dissolved in a mixture of methanol (20 ml) and 25% ammonia solution (20 ml). The reaction mixture was stirred at room temperature for 3 h, and the resulting solution was evaporated. The residue was chromatographed on silica gel column with $CHCl_3$/MeOH (95 : 5) as eluent to give the products as gums.

3.2.1. 1-[2-(2-Hydroxyethoxy)ethyl]-2(1 H)-quinoxalinone (**16**)

Yield: 77%; ms: (EI) m/z (%) = 234 (M^+, 36), 173 (32), 146 (100). Anal. Calcd. for $C_{12}H_{14}N_2O_3$: M, 234.2570. Found: m/z 234.2575

3.2.2. 3-[2-(2-Hydroxyethoxy)ethyl]-2-phenyl-2,3-dihydrophthalazine-1,4-dione (**17**)

Yield: 79%; ms: (EI) m/z (%) = 326 (M^+, 12), 265 (3), 239 (12), 238 (100). Anal. Calcd. for $C_{18}H_{18}N_2O_4$: M, 326.3550. Found: m/z 326.3553

3.2.3. 2-[2-(2-Hydroxyethoxy)ethyl]-1(2 H)-phthalazinone (**18**)

Yield: 80%; ms: (EI) m/z (%): 234 (M^+, 5), 189 (20), 159 (58), 147 (100). Anal. Calcd. for $C_{12}H_{14}N_2O_3$: M, 234.4796. Found: m/z 234.3791

3.2.4. 1-[2-(2-Hydroxyethoxy)ethyl]-5-cyano-3,4-diphenyl-6(1 H)-pyridazinone (**19**)

Yield: 61%; ms: (FAB) m/z (%) = 362 ($M^+ + 1$, 100), 300 (88), 274 (24). Anal. Calcd. for $C_{21}H_{19}N_3O_3$: M, 361.4030. Found: m/z 361.4035

3.2.5. 1-[2-(2-Hydroxyethoxy)ethyl]-5-carbethoxy-3,4-diphenyl-6(1 H)-pyridazinone (**20**)

Yield: 69%; ms: (FAB) m/z (%) = 409 ($M^+ + 1$, 100), 319 (20). Anal. Calcd. for $C_{23}H_{24}N_2O_5$: M, 408.457. Found: m/z 408.4575

3.2.6. 1-[2-(2-Hydroxyethoxy)ethyl]-(1 H)-benzimidazole (**21**)

Yield: 75%; ms: (EI) m/z (%): 206 (M^+, 44), 175 (7), 131 (100). Anal. Calcd. for $C_{11}H_{14}N_2O_2$: M, 206.2470. Found: m/z 206.2476

3.2.7. 2-[2-(2-Hydroxyethoxy)ethyoxy]quinoxaline (**22**)

Yield: 73%; ms: (EI) m/z (%) = 234 (M^+, 6), 173 (7), 146 (100). Anal. Calcd. for $C_{12}H_{14}N_2O_3$: M, 234.2570. Found: m/z 234.2573

3.2.8. 4-[2-(2-Hydroxyethoxy)ethyoxy]-2-phenyl-1 (2 H)-phthalazinone (**23**)

Yield: 71%; ms: (EI) m/z (%) = 326 (M^+, 10), 239 (3), 238 (100). Anal. Calcd. for $C_{18}H_{18}N_2O_4$: M, 326.3550. Found: m/z 326.3548

3.3. Biological activity studies

Maintenance media (RPMI/Glutamax, 93%; Pencilin + streptomycin, 1%; Gentamycin, 1%; Fetal/Calf serum, 5% and Geneticin 4 ml/100 ml media) were added to the cell culture (Hep)G_2 [2.2.15] together with the tested compounds (concentration = 10 μM). The supernatant liquid was collected after one, two and three weeks. The DNA replication was estimated by the PCR (Polymerase chain reaction) technique which was carried out in three steps: extraction of DNA from supernatant, amplification of DNA by using a thermal cycler and finally detection by gel electrophoresis. The percentage inhibition could be calculated by the relation between the blank experiment (containing maintenance media without the tested compounds) and the results obtained after the above mentioned periods. The percentage cytotoxicity could be estimated by the relation between the number of the living and dead cells after three weeks counted by the hemocytometer.

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