Anti-Hepatitis B Virus Activity of New 1,2,4-Triazol-2-yl- and 1,3,4-Oxadiazol-2-yl-2-pyridinone Derivatives

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A number of 1,3,4-oxadiazole, **3**–**9**, and 1,2,4-triazole derivatives, **13**–**15**, were synthesized starting form the acid hydrazide **1**. The 1,3,4-thiadiazole derivative **12** was prepared from the substituted phenylthiosemicarbazide derivative **11** by treatment with sulfuric acid. The aryl hydrazone derivatives **10a**–**c** were synthesized by reaction of the hydrazide **1** with the corresponding ketones. The thioalkyl derivatives **16a**–**e** were prepared by akylation of the thiol derivatives **3** and **13** with different alkylating agents. The newly synthesized compounds were tested for their anti-HBV activity and some of these compounds showed high antiviral activity.

Key words: 1,2,4-Triazoles, 1,3,4-Oxadiazoles, Anti-Hepatitis B Virus

Introduction

Because the resistance to antiviral drugs is widespread, there is an increasing need for the synthesis and identification of novel structure leads that may be of use in designing new, potent and less toxic antiviral agents. Many 1,3,4-oxadiazole, 1,2,4-triazole and 1,3,4-thiadiazole derivatives are associated with a broad spectrum of pharmacological activities (Colanceska-Ragenovic *et al*., 2001; Labanauskas *et al.*, 2004; Al-Soud *et al*., 2004; Foroumadi *et al*., 2001; Awad and El Ashry, 1998; Varvarasou *et al*., 1998; Palaska *et al*., 2002; Holla *et al*., 2002; Amir and Shikha, 2004; Demirbas *et al*., 2004). Ribavirin, fluconazole and cefazolin are antiviral, antifungal and antibacterial drugs which contain 1,2,4-triazole and 1,3,4-thiadiazole units. Furthermore, the 1,3,4-oxadiazole ring system has been found in the skeleton of fungicidal and bactericidal, analgesic, antipyretic, antiphlogestic, anticompulsive, paralytic hypnotic and sedative agents (Zareen *et al*., 2004; El-Azzouny *et al*., 2003; Loetchutinat *et al*., 2003; Grover and Kini, 2003) in addition to antiviral (Ali *et al*., 2004), antitumour (Lokanatha Rai and Linganna, 2000) and tyrosinase inhibiting agents (Hassan Khan *et al*., 2005). Moreover, various substituted 1,2,4-triazolo[3,4-*b*]-1,3,4-thiadiazoles and their dihydro analogues have been shown to possess antimicrobial (Swamy *et al*., 2006), antibacterial (Karabasanagouda *et al*., 2007), anti-inflammatory (Vinod *et al*., 2007; Birsen *et al*., 2007), antifungal, CNS-depressant and antiviral (Zhang and Sun, 1998) activities. On the other hand, the use of non-nucleosides as antiviral chemotherapeutic agents has stimulated extensive research on the synthesis of this class of compounds (Larson *et al*., 1983; Bernan *et al*., 1995; Miyasaka *et al*., 1989). This class of antiviral agents exhibits action by binding to a specific allosteric site, thereby resulting in non-competitive inhibition of this enzyme (Venkatachalam *et al*., 2004). Examples of such non-nucleoside inhibitors are 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) (Miyasaka *et al.*, 1989) and trovirdine-HCl (Uberla *et al.*, 1995) which contain substituted thiurea in the pyridine backbone (Fig. 1). Owing to the above significance and the existing antiviral activity of many of these ring system-containing compounds (Ali *et al*., 2004; Sidwell *et al*., 1972), it is of interest to synthesize new 1,3,4-oxadiazoles, 1,2,4-triazoles and 1,3,4-thiadiazoles linked to a 1,2-dihydropyridine backbone as well as their acyclic analogues in order to investigate the effect of such structural variation on the anticipated antiviral activities.

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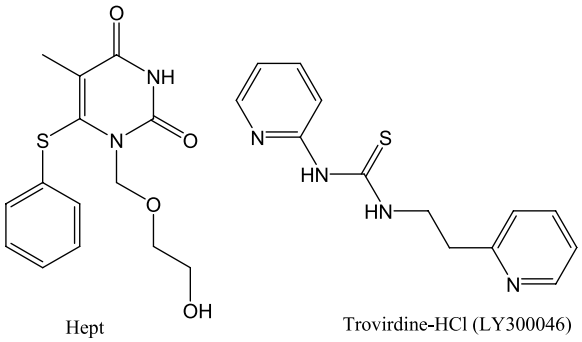

Fig. 1. Chemical structures of Hept and trovirdine-HCl (LY300046). Trovirdine-hydrochloride (LY300046.HCl) is currently in phase one clinical trials for potential use in the treatment of AIDS. Registries: ChemSpider; InChI-Key HOCFDYZWQYGULA-CUNFQGHECV; PubChem CID 3000870; PubChem ID 197155.

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 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 F_{245}. EIMS 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, 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) and 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 tissue culture flasks at 37 °C and 5% CO_2. Subcultures were set up after a week by trypsination with 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 was estimated by comparing the DNA content in the culture supernatant and the viability of the cells with the test compounds to those of HepG2.2.2.15 cells with no compounds added to their supernatant (blank cells). The drug lamivudine 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 adding 90 μl of Tris-EDTA (TE) buffer (Gibco BRL Life Technologies).

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′-GGAAAGAAGTCAGAAGGCA-3′) and HCID-2 primer (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 °C, 30 s at 58 °C and 30 s at 72 °C with a 3 s increment for each cycle in a Perkin Elmer 480 thermal cycler (Perkin Elmer, USA). The PCR product was detected by DIG-ELISA assay (Roche). The optical density (OD) of the 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, USA) is a colourless substrate that is transferred to a coloured product by any living cell but not by dead cells. The assay utilized this compound to test for the

viability of the cells with the test compounds added compared to the viability of the blank cells (Fouad *et al.*, 1998).

Calculation of IC_{50}, CC_{50} and SI

The 50% inhibitory concentration of antiviral drugs (IC_{50}) 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 (CC_{50}) was calculated from the average viability of the cells with added drugs. The selectivity index (SI) could be calculated as CC_{50}/IC_{50}.

Results and Discussion

Chemistry

In the present investigation, the hydrazide derivative **2** was prepared by the reaction of ethyl-[3-cyano-4,6-dimethyl-2-oxopyridin-1(*2H*)-yl]acetate (**1**) with hydrazine hydrate in ethanol (El-Essawy and Khattab, 2004). The structure of the produced acid hydrazide was proved by its spectral data; IR, ^{1}H NMR and Mass spectra were in agreement with the assigned structure. The IR spectrum showed a characteristic absorption band at 1685 cm^{-1} for the carbonyl group. Reaction of the acid hydrazide **2** with carbon disulfide in ethanolic potassium hydroxide at the reflux temperature afforded the corresponding oxadiazole derivative 1-[(5-mercapto-1,3,4-oxadiazol-2-yl)methyl]-4,6-dimethyl-2-oxo-1,2-dihydro-pyridine-3-carbonitrile (**3**) in 77% yield. Reaction of **3** with acrylonitrile in the presence of triethylamine (TEA) afforded the *N*-substituted oxadiazole derivative **4** in 82% yield. The structures of these oxadiazole derivatives were confirmed by IR, ^{1}H NMR and mass spectra which were in agreement with the assigned structures. The ^{1}H NMR spectrum of **3**, as representative example, revealed signals at (δ in ppm): 2.41 (s, CH_3), 2.48 (s, CH_3), 5.31 (s, CH_2), 6.42 (s, H-5), 14.5 (s, NH). The ^{1}H NMR spectrum of the *N*-substituted oxadiazole derivative **4** showed two triplet signals at δ 2.99 and 4.27 ppm for the two CH_2 groups. When the 1,3,4-oxadiazole derivative **3** was allowed to react with hydrazine hydrate in absolute ethanol under reflux it afforded 1-[(4-amino-5-mercapto-*4H*-1,2,4-triazol-3-yl)methyl]-4,6-dimethyl-2-oxo-1,2-dihydro-pyridine-3-carbonitrile (**6**). On the other hand, reaction of the acid hydrazide **2** with carbon disulfide in ethanolic potassium hydroxide at room temperature (RT) afforded the intermediate potassium salt **5** in good yield. It was interesting that the 1,2,4-triazole derivative **6** has also been synthesized from the potassium salt of the thiosemicarbazide derivative **5** by refluxing it with hydrazine hydrate. The structure of the 1,2,4-triazole derivative **6** obtained by the two previously mentioned methods was confirmed by IR, ^{1}H NMR and mass spectra which were in agreement with the assigned structure. The ^{1}H NMR spectrum showed signals at (δ, ppm): 2.41 (s, CH_3), 2.49 (s, CH_3), 5.26 (brs, NH_2), 5.64 (s, *N*-CH_2), 6.39 (s, H-5), 13.58 (brs, SH). When the aminotriazole derivative **6** was reacted with benzoic acid in $POCl_3$ at the reflux temperature, it afforded the corresponding bicyclic derivative 4,6-dimethyl-2-oxo-1-[(6-phenyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-3-yl)-methyl]-1,2-dihydro-pyridine-3-carbonitrile (**7**) in 74% yield. Alkylation of the aminotriazole derivative **6** with methyl iodide gave the corresponding *S*-methyl-1-aminotriazole derivative **9**. On the other hand, reaction of potassium salt **5** with methyl iodide at room temperature afforded the dimethylthiohydrazone derivative **7**. Its ^{1}H NMR spectrum showed a singlet at δ 2.58 ppm corresponding to protons of the two *S*-Me groups. We also succeeded to synthesize the *S*-methyl-1-aminotriazole derivative **9** by reaction of the dimethylthiohydrazone derivative **7** with hydrazine hydrate at the reflux temperature. The structure of compound **8** obtained by the two previously mentioned methods was proved by the spectral data. Thus, its IR spectrum exhibited a characteristic band for NH_2 at 3471 cm^{-1}. The ^{1}H NMR spectrum showed signals at (δ, ppm): 2.48 (s, CH_3), 2.49 (s, CH_3), 2.54 (s, SCH_3), 5.31 (s, *N*-CH_2), 6.04 (brs, NH_2), 6.41 (s, H-5) (Fig. 2).

Reaction of the acid hydrazide **2** with benzaldehyde, acetophenone or *p*-nitrobenzaldehyde gave the corresponding arylhydrazone derivatives **10a**–**c**. The structures of the produced arylhydrazone derivatives were confirmed by the spectral data. On the other hand, when the acid hydrazide **2** was allowed to react with phenylisothiocyanate in absolute ethanol at the reflux temperature it afforded the corresponding phenyl thiosemicarbazide derivative **11** in 88% yield. Its IR spectrum showed a characteristic carbonyl band at 1675 cm^{-1} corresponding to the *N*-CO group and its ^{1}H NMR spectrum agreed with the assigned structure.

It is well known that thiosemicarbazide derivatives are versatile intermediates for the synthesis

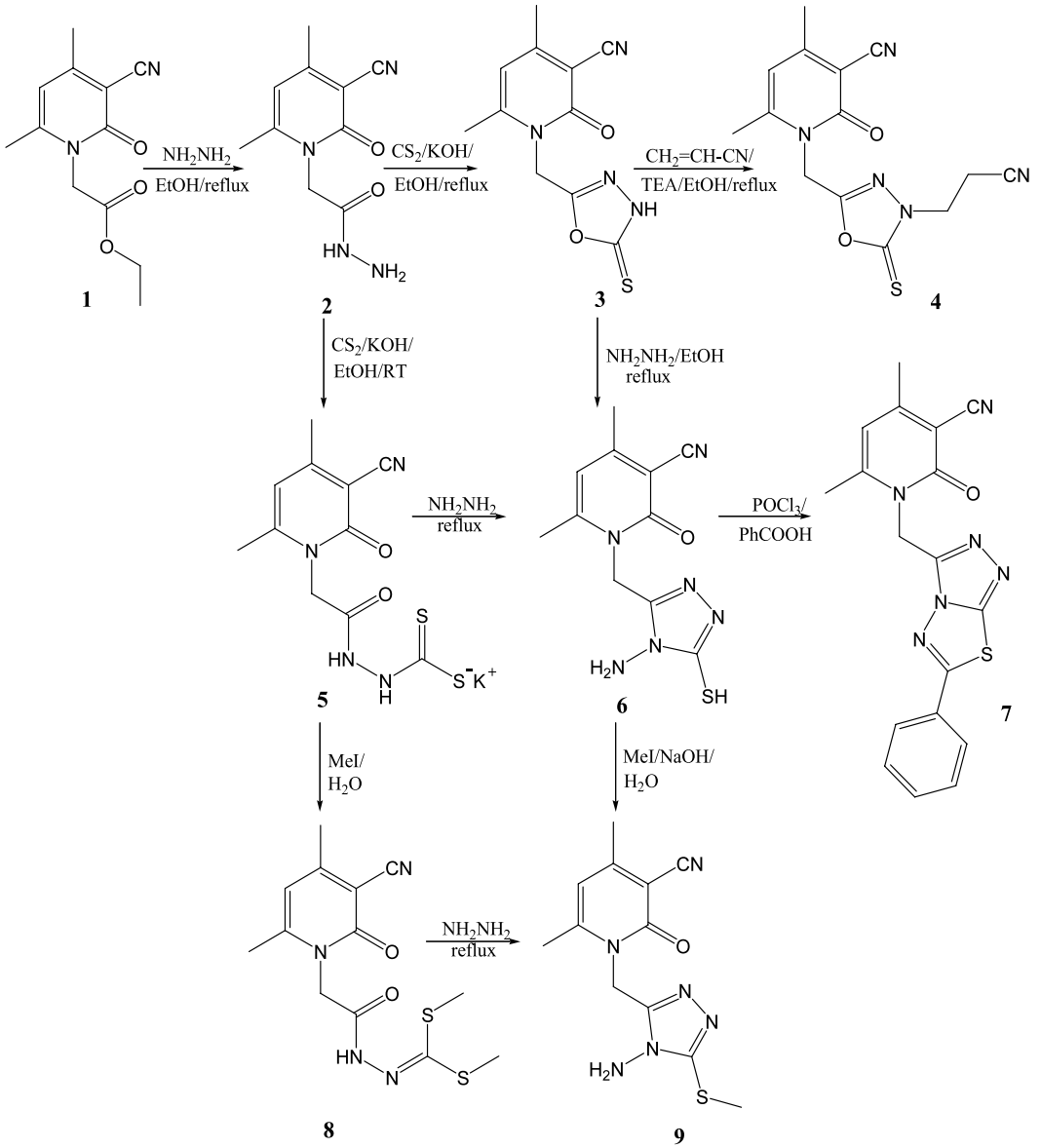

Fig. 2. Synthesis of 5-mercapto-1,3,4-oxadiazole and 1,2,4-triazoles.

of a variety of five-membered heterocyclic systems, and the product obtained depends on the applied conditions for the cyclization process (Abdel Aal *et al.*, 2003). Thus, when the thiosemicarbazide derivative **11** was treated with sulfuric acid, the corresponding arylamino-1,3,4-thiadiazole derivative **12** was obtained in 84% yield. The preferred formation of the thiadiazole ring under such acidic conditions can be explained by the loss of nucleophilicity of the nitrogen atom as a result of its protonation leading to an increase in the nucleophilicity of the sulfur atom towards attack of the carbonyl carbon atom. On the other hand, when the cyclization of **11** was carried out under alkaline conditions, the nucleophilicity of the nitrogen atomis enhanced and leads to cyclization with the carbonyl carbon atom to give the *N*-phenyl-1,2,4-triazole-5-thione derivative **13** in 76% yield. When the cyclization was performed by mercuric oxide, the 1,3,4-oxadiazole derivative **14** was

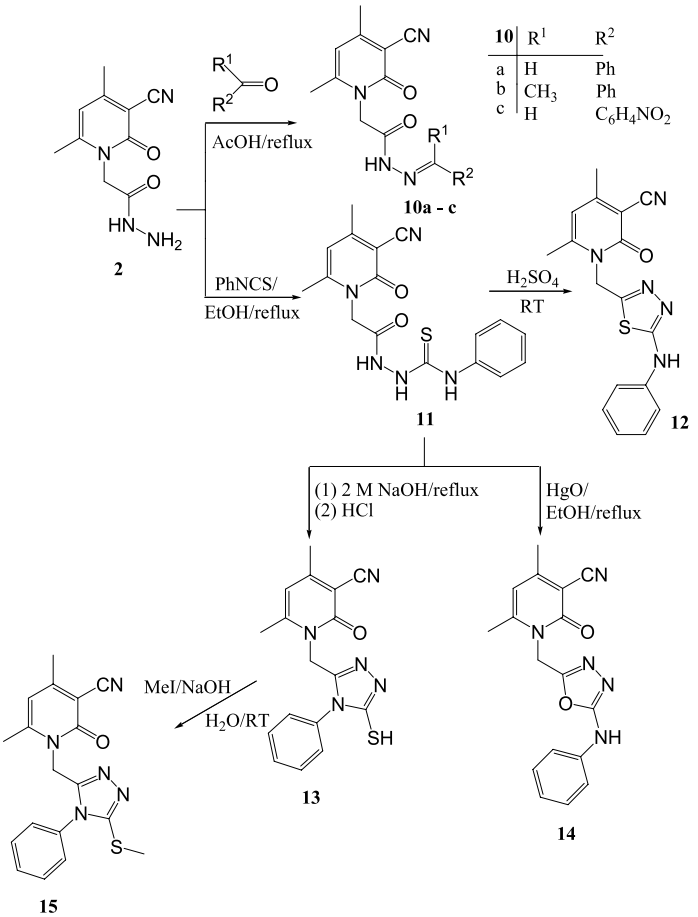

Fig. 3. Synthesis of thiadiazole, oxadiazole, and triazole derivatives.

formed in 83% yield. The mode of cyclization leading to the 1,3,4-oxadiazole derivative includes desulfurization by mercuric oxide, which introduces the oxygen atom in the cyclization process. When the *N*-phenyl-1,2,4-triazole-5-thione derivative **13** was alkylated with methyl iodide it produced the methylthio-1,2,4-triazole derivative **15** in 74% yield. The structures of these five-membered heterocycles were proven on the basis of their IR, ^{1}H NMR and mass spectra which were in agreement with the assigned structure. The ^{1}H NMR spectrum of compound **15**, as representative example, showed signals at (δ, ppm) in 2.54 (s, CH_3), 2.58 (s, CH_3), 2.88 (s, SCH_3), 5.24 (s, CH_2), 6.59 (m, Ar), 7.58–7.61 (m, Ar) (Fig. 3).

In order to synthesize a number of non-nucleoside analogues using the 1,2,4-triazole and 1,3,4-oxadiazole ring systems as the heterocyclic bases, we selected the two derivatives **3** and **13** to be alkylated with a number of alkylating agents substituted either with oxygen or sulfur atoms (chloromethyl methyl sulfide, chloromethyl ethyl ether, chloroethyl methyl ether, ethyl chloroacetate, chloroacetonitrile, allyl bromide, 2-bromoethyl acetate, methyl iodide and ethyl iodide) to afford the corresponding alkylthio derivatives **16a**–**k**. The alkylation reaction was carried out in *N,N*-dimethylformamide (DMF) in the presence of anhydrous potassium carbonate. The structures of the produced alkylthio derivatives were proven on the basis of their IR, ^{1}H NMR and mass spectra which were in full agreement with the assigned structures. Deacetylation of **16i** with methanolic ammonia afforded the corresponding hydroxyethylthio-1,3,4-oxadiazole derivative **17** in 65% yield. The IR spectrum showed the characteristic band for

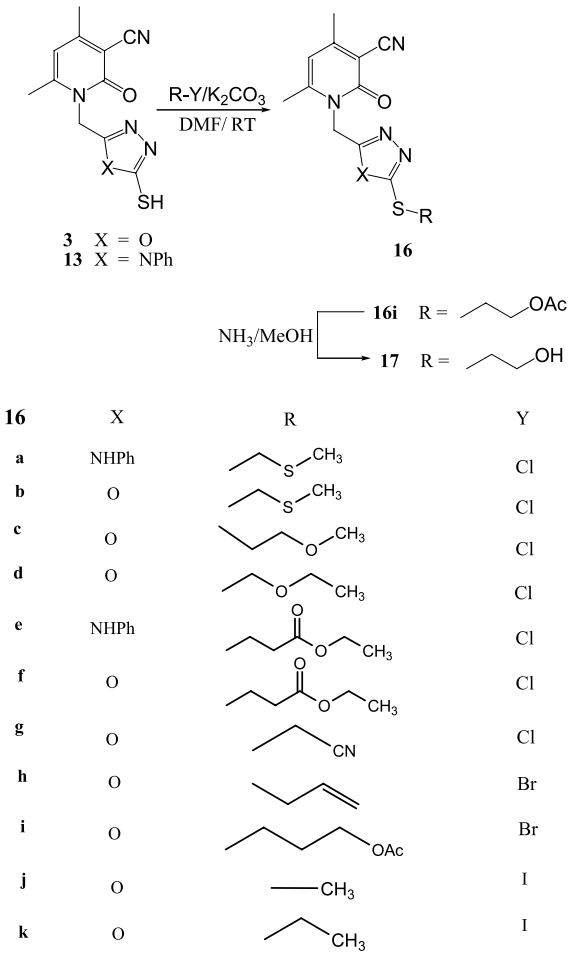

Fig. 4. Synthesis of non-nucleoside analogues using the 1,2,4-triazole and 1,3,4-oxadiazole ring system.

OH at 3436 cm^{-1} and its ^1H NMR spectrum the absence of the methyl-acetyl group and the presence of the free hydroxy group (Fig. 4).

Antiviral testing

Preliminary screening indicated that compound **16b** showed the highest inhibitory activity against HBV among this series of tested compounds with low cytotoxicity and a selectivity index of 2500.0 followed by compounds **7**, **8** and **12**. Compounds **3**, **6**, **9**, **13** and **16c** showed moderate inhibition with moderate cytotoxicity while the other tested compounds exhibited less activity against HBV (Table I).

From the results of the viral inhibition activity test we can conclude that substitution of the oxygen atom in the acyclonucleoside analogue by a sulfur atom (compound **16b**) increases the viral inhibition. It is also obvious that compounds containing the 1,3,4-thiadiazole or [1,2,4]triazolo[3,4-*b*][1,3,4]-thiadiazole ring system show higher activity than other ring system-containing compounds.

Table I. Cytotoxic effect (CC_{50})[a] and inhibitory concentration (IC_{50}) of newly synthesized compounds.

Compound	HBV DNA IC_{50} [μM]	HepG2.2.2.15 CC_{50} [μM]
Lamivudine	0.1	1000.0
2	0.9	111.1
3	0.4	250.0
4	1.3	76.9
5	1.4	71.4
6	0.5	200.0
7	0.3	333.3
8	0.3	333.3
9	0.5	200.0
10a	1.7	58.8
10b	1.4	71.4
10c	3.0	33.3
11	1.3	76.9
12	0.3	333.3
13	0.5	200.0
14	4.0	25.0
15	3.4	29.4
16a	1.8	55.5
16b	0.2	500.0
16c	0.5	200.0
16d	1.2	383.3
16e	0.9	111.1
16f	0.8	125.0
16g	1.1	90.1
16h	0.8	125.0
16i	0.8	156.2
17	1.2	83.3

[a] Cytotoxic effect (CC_{50}) of all tested compounds is 100 μM.

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