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# Synthesis and the biological evaluation of 2-benzenesulfonylalkyl-5-substituted-sulfanyl-[1,3,4]-oxadiazoles as potential anti-hepatitis B virus agents

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**Abstract**

Current treatments for chronic hepatitis B virus (HBV) infection include the use of interferon-α and of nucleoside analogs lamivudine, adefovir and entecavir. However, the use of interferon-α has many side effects while that of nucleosidic inhibitors can lead to the emergence of resistant viruses. Hence, new drugs for the treatment of HBV infection are still highly desired. Oxadiazoles have been observed to exhibit antiviral activities against RNA viruses. In this study, a facile synthesis of 2-benzenesulfonylalkyl-5-substituted-sulfanyl-[1,3,4]-oxadiazoles is reported. The compounds were then evaluated for their anti-HBV activity. 1-{2-[5-(1-Benzenesulfonyl-propyl)-[1,3,4]oxadiazol-2-yl-sulfanyl]-ethyl}-4-(2-methoxy-phenyl)-piperazine (**1i**) was able to inhibit the expression of the viral antigens, HBsAg and HBeAg in a concentration-dependent manner with no cytotoxic effects and without any effects on the expression of viral transcripts. Concentration- and time-dependent reductions in virion production were also observed. The inhibition of virion production was comparable to that of lamivudine and $EC_{50}$ values of 1.63 and 2.96 μM were obtained for compound **1i** and lamivudine, respectively. Thus, in addition to the antiviral effects on RNA viruses, oxadiazoles also have anti-HBV activities.


*Keywords:* Hepatitis B virus; Hepatitis B virus-s antigen; Hepatitis B virus-e antigen; Antiviral activity; Oxadiazoles

## 1. Introduction

Hepatitis B virus (HBV) is the major cause of acute and chronic hepatitis which could lead to hepatocellular carcinoma. Chronic hepatitis B infection affects more than 400 million people worldwide and 1–2% of them die each year from virus related complications (Lin and Kirchner, 2004). Currently, interferon-α and nucleosidic inhibitors of HBV reverse transcriptase/polymerase, lamivudine (3TC), adefovir and entecavir are the only drugs approved for the treatment of chronic HBV infection (Ocama et al., 2005; Mailliard and Gollan, 2006). However, interferon-α produces many side effects and its efficacy is partial with less than 30% of the chronic carriers responding to treatment. In addition, approximately 50% of the patients who respond positively to interferon-α treatments are known to suffer a recurrence of the viremia after cessation of the treatment (Thomas, 1998; Fattovich et al., 1998). In contrast, 3TC has more universal applicability and is able to reduce the viral load very rapidly. However, this initial rapid response is often followed by a slow elimination of the residual virus. Emergence of drug resistance commonly occurs during this slower phase thus preventing long-term treatment (Fischer et al., 2001). Adefovir is effective against 3TC-resistant viruses but long-term monotherapy can also result in resistance leading to reduced response to adefovir (Angus et al., 2003; Villeneuve et al., 2003; Fung et al., 2005). Resistance to entecavir has also been reported in lamivudine-resistant patients (Tenney et al., 2004). The wide prevalence of chronic HBV infection together with the lack of an effective drug available for the treatment warrants the search for novel therapeutic agents against the virus.

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Oxadiazoles are useful targets in the search for antivirals as they have been associated with a wide variety of interesting properties. Members of this class of compound are known to possess tyrosine kinase inhibitory (Vu et al., 1999), anti-inflammatory (Nicolaides et al., 1998), monoamine oxidase inhibitory (Matsumoto et al., 1994) and antitumor activities (Chimirri et al., 1996). More recently, 2,5-disubstituted-[1,3,4]-oxadiazoles have also gained considerable attention because of their uses as organic light-emitting devices (Zhang et al., 2005), orally active COX-2 inhibitors (Song et al., 1999) and heterocyclic amide and ester isosteres (Luthman et al., 1999).

Earlier works have shown that oxadiazoles possess antiviral activities against RNA viruses and are able to inhibit HIV replication (El-Emam et al., 2004). Antiviral activity towards picornaviruses has been described and to date, the best characterized is pleconaril (Diana et al., 1994; Fendrick, 2003; Webster, 2005) which binds to the hydrophobic pockets in the base of canyons on the surface of picornaviruses (Badger et al., 1988). This inhibits virus attachment to cells (Pevear et al., 1989; Shepard et al., 1993) and also inhibits the uncoating of the viral capsid (Fox et al., 1986; Shepard et al., 1993). Herein, we describe the synthesis of a new class of 2,5-disubstituted-[1,3,4]-oxadiazoles, the 2-benzenesulfonylalkyl-5-substituted-sulfanyl-[1,3,4]-oxadiazoles, and the biological evaluation of their anti-HBV activity.

## 2. Materials and methods

### 2.1. Synthesis of oxadiazoles

All chemicals were obtained from commercial suppliers and used without purification. The three 1-(2-chloroethyl)-4-substituted-piperazines used for the preparation of **1f–1i** were prepared according to a modified reported procedure (Modica et al., 2001). Analytical TLC was carried out on pre-coated plates (Merck Silica Gel 60, F254) and visualized with UV light. Flash column chromatograph was performed with silica (Merck, 230–400 mesh). NMR spectra ($^1$H and $^{13}$C) were recorded at 298 K on the Bruker DPX300 or AMX500 Fourier Transform spectrometer. Chemical shifts are expressed in $\delta$ (ppm), relative to the internal standard of tetramethylsilane (TMS). Mass spectra were performed on VG Micromass 7035 spectrometer under EI or ESI.

#### 2.1.1. Preparation of α-sulfonylesters (*3*)

Sodium benzenesulfinate **2** (0.80 g, 5 mmol) was dissolved in DMF (30 mL) in which $NBu_4I$ (0.18 g, 0.5 mmol), KI (1.0 g, 6 mmol) and the α-bromoester (6 mmol) were added. The mixture was stirred at room temperature for 4 h. DMF was then removed by freezing–drying and the residual yellow oil obtained was purified by flash column chromatography to give **3**. **3a**: Yield = 98%. IR: 1747, 1327, 1153 cm$^{-1}$. $^1$H NMR ($CDCl_3$): $\delta$ 3.70 (s, 3H, $OCH_3$), 4.12 (s, 2H, $CH_2$), 7.58 (t, 2H, $J$ = 7.8 Hz, $2H_{arom}$), 7.69 (t, 1H, $J$ = 7.8 Hz, $H_{arom}$), 7.94 (d, 2H, $J$ = 7.8 Hz, $2H_{arom}$). $^{13}$C NMR ($CDCl_3$): $\delta$ 52.9, 60.7, 128.4, 129.1, 134.2, 138.6, 162.7. **3b**: Yield = 97%. IR: 1741, 1323, 1149 cm$^{-1}$. $^1$H NMR ($CDCl_3$): $\delta$ 1.56 (d, 3H, $J$ = 7.2 Hz, $CH_3$-3), 3.68 (s, 3H, $OCH_3$), 4.06 (q, 1H, $J$ = 7.2 Hz, CH), 7.58 (t, 2H, $J$ = 7.8 Hz, $2H_{arom}$), 7.70 (t, 1H, $J$ = 7.8 Hz, $H_{arom}$), 7.89 (d, 2H, $J$ = 7.8 Hz, $2H_{arom}$). $^{13}$C NMR ($CDCl_3$): $\delta$ 11.8, 52.9, 65.3, 128.9, 129.2, 134.2, 136.8, 166.6. **3c**: Yield = 94%. IR: 1737, 1326, 1149 cm$^{-1}$. $^1$H NMR ($CDCl_3$): $\delta$ 0.97 (t, 3H, $J$ = 7.2 Hz, $CH_3$-4), 1.16 (t, 3H, $J$ = 7.2 Hz, $OCH_2CH_3$), 1.91–2.14 (m, 2H, $CH_2$-3), 3.86 (dd, 1H, $J$ = 4.0 Hz, 11.2 Hz, CH-2), 4.12 (q, 2H, $J$ = 7.2 Hz, $OCH_2$), 7.56 (t, 2H, $J$ = 7.2 Hz, $2H_{arom}$), 7.66 (t, 1H, $J$ = 7.2 Hz, $H_{arom}$), 7.88 (d, 2H, $J$ = 7.2 Hz, $2H_{arom}$). $^{13}$C NMR ($CDCl_3$): $\delta$ 11.3, 13.8, 20.4, 62.0, 72.2, 128.8, 129.2, 134.0, 137.2, 165.7.

#### 2.1.2. Preparation of α-sulfonylcarboxylic hydrazides (*4*)

Hydrazine hydrate (1.2 g, 20 mmol) was added to a solution of α-sulfonylester (2 mmol) in ethanol (50 mL). The resulting solution was refluxed for 16 h. The solvent was then evaporated and the residue was purified by flash column chromatography (50% EtOAc in hexane) to give **4**. **4a**: Yield = 92%. $^1$H NMR (DMSO-$d_6$): $\delta$ 4.25 (s, 2H, $CH_2$), 4.39 (b, 2H, $NH_2$), 7.69 (t, 2H, $J$ = 7.2 Hz, $2H_{arom}$), 7.79 (t, 1H, $J$ = 7.2 Hz, $H_{arom}$), 7.92 (d, 2H, $J$ = 7.2 Hz, $2H_{arom}$), 9.34 (b, 1H, NH). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 59.3, 127.9, 129.0, 133.8, 139.5, 160.0. HRMS (EI) calcd. for $C_8H_{10}N_2O_3S$, 214.0412; found, 214.0414. **4b**: Yield = 87%. $^1$H NMR (DMSO-$d_6$): $\delta$ 1.29 (d, 3H, $J$ = 7.2 Hz, $CH_3$), 4.03 (q, 1H, $J$ = 7.2 Hz, CH), 4.35 (b, 2H, $NH_2$), 7.66 (t, 2H, $J$ = 7.2 Hz, $2H_{arom}$), 7.75–7.83 (m, 3H, $3H_{arom}$), 9.33 (b, 1H, NH). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 11.9, 62.9, 128.8, 129.0, 134.0, 136.8, 163.6. HRMS (EI) calcd. for $C_9H_{12}N_2O_3S$, 228.0569; found, 228.0570. **4c**: Yield = 90%. $^1$H NMR (DMSO-$d_6$): $\delta$ 0.78 (t, 3H, $J$ = 7.2 Hz, $CH_3$), 1.68–1.79 (m, 2H, $CH_2$), 3.34 (b, 2H, $NH_2$), 3.83 (dd, 1H, $J$ = 6.0, 9.0 Hz, CH), 7.64 (t, 2H, $J$ = 7.5 Hz, $2H_{arom}$), 7.74–7.81 (m, 3H, $2H_{arom}$), 9.40 (b, 1H, NH). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 10.9, 19.9, 69.5, 128.8, 129.0, 134.0, 137.2, 162.9. HRMS (EI) calcd. for $C_{10}H_{14}N_2O_3S$, 242.0725; found, 242.0726.

#### 2.1.3. Preparation of 5-(1-benzenesulfonyl-alkyl)-3H-[1,3,4]-oxadiazole-2-thione (*5*)

Carbon disulfide (0.38 g, 5 mmol) and potassium hydroxide (86 mg, 1 mmol) were added to a solution of **4** (1 mmol) in ethanol (10 mL) at 0 °C. The mixture was then refluxed for 12 h. The solvent was evaporated and the residue obtained was dissolved in water and acidified with dilute HCl. **5** precipitated from the solution was pure enough to be used in the next step of the reaction. **5a**: Yield = 86%. IR: 1610, 1338, 1229, 1150 cm$^{-1}$. $^1$H NMR ($CD_3OD$): $\delta$ 4.82 (s, 2H, $CH_2$), 7.62–7.68 (m, 2H, $2H_{arom}$), 7.75–7.79 (m, 1H, $H_{arom}$), 7.90 (d, 2H, $J$ = 7.8 Hz, $2H_{arom}$). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 51.6, 128.0, 129.5, 134.6, 137.6, 154.2, 178.0. HRMS (ESI, $M$ + H) calcd. for $C_9H_9N_2O_3S_2$, 257.0055; found, 257.0054. **5b**: Yield = 80%. IR: 1606, 1363, 1241, 1165 cm$^{-1}$. $^1$H NMR ($CDCl_3$): $\delta$ 1.75 (d, 3H, $J$ = 7.2 Hz, $CH_3$), 4.41 (q, 1H, $J$ = 7.2 Hz, CH), 7.59–7.64 (m, 2H, $2H_{arom}$), 7.71–7.76 (m, 1H, $H_{arom}$), 7.83 (d, 2H, $J$ = 7.5 Hz, $2H_{arom}$). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 11.0, 57.0, 128.7, 129.5, 134.9, 135.7, 157.4, 177.8. HRMS (ESI, $M$ + H) calcd. for $C_{10}H_{11}N_2O_3S_2$, 271.0211; found, 271.0219. **5c**: Yield = 84%. IR: 1604, 1332, 1202, 1153 cm$^{-1}$. $^1$H NMR ($CDCl_3$): $\delta$ 1.01 (t, 3H, $J$ = 7.2 Hz,

$CH_3$), 2.06–2.22 (m, 1H, $CH_2$), 2.28–2.39 (m, 1H, $CH_2$), 4.32 (dd, 1H, $J$ = 3.9 Hz, 11.4 Hz, CH), 7.58–7.63 (m, 2H, $2H_{arom}$), 7.71–7.76 (m, 1H, $H_{arom}$), 7.83 (d, 2H, $J$ = 7.5 Hz, $2H_{arom}$). $^{13}C$ NMR (DMSO-$d_6$): $\delta$ 10.5, 19.5, 62.5, 128.6, 129.5, 134.8, 136.1, 156.5, 178.0. HRMS (EI) calcd. for $C_{11}H_{12}N_2O_3S_2$, 284.0289; found, 284.0297.

### 2.1.4. Preparation of substituted 2-benzenesulfonylalkyl-5-substituted-sulfanyl-[1,3,4]-oxadiazoles (**1a**–**1e**)

Potassium carbonate (83 mg, 0.6 mmol) was added to a solution of **5** in anhydrous THF (10 mL). The mixture was stirred at room temperature for 1 h. The corresponding halide (0.6 mmol) was then added and the mixture was stirred for an additional 24 h. The solvent was removed and the residue obtained was purified by flash column chromatography (20% EtOAc in hexane) to give **1**. **1a**: Yield = 62%. $^1H$ NMR ($CDCl_3$): $\delta$ 0.89–1.01 (m, 6H, $2CH_3$), 1.35–1.45 (m, 4H, $2CH_2$), 1.73–1.83 (m, 2H, $CH_2$), 2.11–2.26 (m, 1H, $CH_2$), 2.33–2.45 (m, 1H, $CH_2$), 3.21 (t, $J$ = 7.2 Hz, 2H, $SCH_2$), 4.39–4.44 (dd, 1H, $J$ = 3.9, 11.4 Hz, $CHSO_2$), 7.51–7.56 (m, 2H, $2H_{arom}$), 7.64–7.72 (m, 3H, $3H_{arom}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 11.3, 13.7, 20.2, 22.0, 28.7, 30.5, 32.4, 64.6, 128.9, 129.2, 134.5, 136.2, 160.7, 166.6. HRMS (ESI, $M$ + H) calcd. for $C_{16}H_{23}N_2O_3S_2$, 355.1150; found, 355.1150. **1b**: Yield = 70%. $^1H$ NMR ($CDCl_3$): $\delta$ 0.96 (t, 3H, $J$ = 7.2 Hz, $CH_3$), 1.41–1.53 (m, 2H, $CH_2$), 1.71–1.81 (m, 5H, $CH_2$ and $CH_3$), 3.22 (t, $J$ = 7.2 Hz, 2H, $SCH_2$), 4.56–4.64 (q, 1H, $J$ = 7.2 Hz, $CHSO_2$), 7.52–7.57 (m, 2H, $2H_{arom}$), 7.66–7.75 (m, 3H, $3H_{arom}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 12.0, 13.3, 21.6, 31.0, 32.1, 57.9, 129.1, 129.2, 134.5, 135.7, 161.5, 166.6. HRMS (EI) calcd. for $C_{14}H_{18}N_2O_3S_2$, 326.0759; found, 326.0759. **1c**: Yield = 77%. $^1H$ NMR ($CDCl_3$): $\delta$ 4.62 (s, 2H, $CH_2CO$), 4.86 (s, 2H, $CH_2SO_2$), 7.50 (d, 2H, $J$ = 8.4 Hz, $2H_{arom}$), 7.56–7.61 (m, 2H, $2H_{arom}$), 7.69–7.73 (m, 1H, $H_{arom}$), 7.83 (d, 2H, $J$ = 8.4 Hz, $2H_{arom}$), 7.96 (d, 2H, $J$ = 9.0 Hz, $2H_{arom}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 41.1, 52.8, 128.3, 129.3, 129.5, 129.8, 133.0, 134.7, 137.3, 140.9, 157.9, 165.9, 190.4. HRMS (EI) calcd. for $C_{17}H_{13}ClN_2O_4S_2$, 408.0005; found, 408.0006. **1d**: Yield = 72%. $^1H$ NMR ($CDCl_3$): $\delta$ 3.79 (s, 3H, $OCH_3$), 4.05 (s, 2H, $SCH_2$), 4.62 (s, 2H, $CH_2SO_2$), 7.55–7.60 (m, 2H, $2H_{arom}$), 7.68–7.73 (m, 1H, $H_{arom}$), 7.82 (d, 2H, $J$ = 7.5 Hz, $2H_{arom}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 33.9, 52.8, 53.1, 128.3, 129.4, 134.7, 137.2, 157.9, 165.3, 167.5. HRMS (EI) calcd. for $C_{12}H_{12}N_2O_5S_2$, 328.0188; found, 328.0188. **1e**: Yield = 74%. $^1H$ NMR ($CDCl_3$): $\delta$ 0.95 (t, 3H, $J$ = 7.2 Hz, $CH_3$), 1.41–1.53 (m, 2H, $CH_2$), 1.71–1.80 (m, 2H, $CH_2$), 3.22 (t, $J$ = 7.2 Hz, 2H, $SCH_2$), 4.62 (s, 2H, $CH_2SO_2$), 7.55–7.59 (m, 2H, $2H_{arom}$), 7.68–7.73 (m, 1H, $H_{arom}$), 7.83 (d, 2H, $J$ = 7.5 Hz, $2H_{arom}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 13.3, 21.6, 31.0, 32.1, 52.8, 128.4, 129.4, 134.7, 140.1, 157.4, 167.0. HRMS (EI) calcd. for $C_{13}H_{16}N_2O_3S_2$, 312.0602; found, 312.0598.

### 2.1.5. Preparation of substituted 1-[2-(5-benzenesulfonylalkyl-[1,3,4]-oxadiazol-2-ylsulfanyl)-ethyl]-4-substituted-piperazine (**1f**–**1i**)

Potassium carbonate (83 mg, 0.6 mmol) was added to a solution of **5** in anhydrous THF (10 mL). The mixture was stirred at room temperature for 1 h. The corresponding halide (0.6 mmol) and $NBu_4I$ (0.22 g, 0.6 mmol) were then added and the mixture was refluxed for a further 24 h. The solvent was removed and the residue obtained was purified by flash column chromatography (20% EtOAc in hexane) to give **1**. **1f**: Yield = 60%. $^1H$ NMR ($CDCl_3$): $\delta$ 0.97 (t, 3H, $J$ = 7.3 Hz, $CH_3$), 2.11–2.25 (m, 1H, $CH_2$), 2.31–2.40 (m, 1H, $CH_2$), 2.68–2.70 (t, 4H, $J$ = 4.8 Hz, $2NCH_2$ of piperazine), 2.85–2.89 (t, 2H, $J$ = 6.9 Hz, $NCH_2$), 3.46–3.50 (t, 2H, $J$ = 6.9 Hz, $SCH_2$), 3.58–3.61 (t, 4H, $J$ = 4.9 Hz, $2NCH_2$ of piperazine), 4.39–4.44 (dd, 1H, $J$ = 3.9, 11.4 Hz, $CHSO_2$), 6.61–6.66 (m, 2H, $H_{pyridine}$), 7.45–7.56 (m, 3H, $H_{arom}$), 7.65–7.70 (m, 3H, $2H_{arom}$ and $H_{pyridine}$), 8.17 (d, 1H, $J$ = 1.1 Hz, $H_{pyridine}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 11.4, 20.5, 29.9, 44.9, 52.5, 56.3, 64.7, 107.2, 113.5, 129.1, 129.3, 134.5, 136.4, 137.7, 147.7, 159.1, 160.9, 166.7. HRMS (EI) calcd. for $C_{22}H_{27}N_5O_3S_2$, 473.1555; found, 473.1549. **1g**: Yield = 57%. $^1H$ NMR ($CDCl_3$): $\delta$ 0.97 (t, 3H, $J$ = 7.2 Hz, $CH_3$), 2.11–2.24 (m, 1H, $CH_2$), 2.31–2.42 (m, 1H, $CH_2$), 2.58 (t, 4H, $J$ = 5.1 Hz, $2NCH_2$ of piperazine), 2.81 (t, 2H, $J$ = 6.9 Hz, $NCH_2$), 3.44 (t, 2H, $J$ = 6.9 Hz, $SCH_2$), 3.83 (t, 4H, $J$ = 4.8 Hz, $2NCH_2$ of piperazine), 4.39–4.44 (dd, 1H, $J$ = 3.9, 11.4 Hz, $CHSO_2$), 6.48 (t, 1H, $J$ = 4.8 Hz, $H_{pyrimidine}$), 7.50–7.55 (m, 2H, $2H_{arom}$), 7.64–7.73 (m, 3H, $3H_{arom}$), 8.29 (d, 2H, $J$ = 4.8 Hz, $2H_{pyrimidine}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 11.3, 20.3, 30.1, 43.4, 52.6, 56.2, 64.6, 109.9, 129.0, 129.1, 134.5, 136.2, 157.6, 160.8, 161.5, 166.8. HRMS (EI) calcd. for $C_{21}H_{26}N_6O_3S_2$, 474.1508; found, 474.1506. **1h**: Yield = 65%. $^1H$ NMR ($CDCl_3$): $\delta$ 2.55–2.58 (t, 4H, $J$ = 5.2 Hz, $2NCH_2$ of piperazine), 2.73–2.78 (t, 2H, $J$ = 6.8 Hz, $NCH_2$), 3.36–3.41 (t, 2H, $J$ = 6.8 Hz, $SCH_2$), 3.48–3.51 (t, 4H, $2NCH_2$), 4.61 (s, 2H, $SCH_2$), 6.55–6.61 (m, 2H, $H_{pyridine}$), 7.45–7.39 (m, 1H, $1H_{arom}$), 7.51–7.54 (m, 2H, $2H_{arom}$), 7.61–7.67 (m, 1H, $1H_{arom}$), 7.77–7.81 (m, 2H, $1H_{arom}$ + $1H_{pyridine}$), 8.12 (d, 1H, $J$ = 0.8 Hz, $1H_{pyridine}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 30.1, 44.8, 52.3, 52.5, 56.0, 106.8, 113.1, 128.2, 129.3, 134.5, 137.2, 147.6, 157.3, 159.1, 166.9. HRMS (EI) calcd. for $C_{20}H_{23}N_5O_3S_2$, 445.1242; found, 445.1228. **1i**: Yield = 62%. $^1H$ NMR ($CDCl_3$): $\delta$ 0.95 (t, 3H, $J$ = 7.2 Hz, $CH_3$), 2.07–2.22 (m, 1H, $CH_2$), 2.29–2.41 (m, 1H, $CH_2$), 2.70 (s, 4H, $2NCH_2$ of piperazine), 2.84 (t, 2H, $J$ = 6.9 Hz, $NCH_2$), 3.06 (s, 4H, $2NCH_2$ of piperazine), 3.41 (t, 2H, $J$ = 6.9 Hz, $SCH_2$), 3.82 (s, 3H, $OCH_3$), 4.38–4.43 (dd, 1H, $J$ = 3.9, 10.8 Hz, $CHSO_2$), 6.81–6.97 (m, 4H, $4H_{arom}$), 7.50–7.55 (m, 2H, $2H_{arom}$), 7.63–7.73 (m, 3H, $3H_{arom}$). $^{13}C$ NMR ($CDCl_3$): $\delta$ 11.3, 20.3, 30.2, 50.4, 52.9, 55.3, 56.2, 64.6, 111.2, 118.1, 120.9, 122.8, 129.0, 129.2, 134.5, 136.2, 141.1, 154.2, 160.8, 166.9. HRMS (EI) calcd. for $C_{24}H_{30}N_4O_4S_2$, 502.1708; found, 502.1707.

### 2.2. Reagents for cell culture and quantification of albumin, viral DNA, transcripts and antigens

Dulbeccco's modified Eagle medium (DMEM) and G418 were purchased from Sigma (St. Louis, MO, USA). All other cell culture reagents were obtained from Gibco (Grandisland, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine ethosulfate (MTS/PES) reagent (supplied as Cell Titer 96 Aqueous One Solution Cell Proliferation Assay) and the AMV reverse transcriptase were purchased from

Promega (Madison, WI, USA) and QIAamp DNA Blood Mini Kit was from Qiagen (Hilden, Germany). DNase I was purchased from Invitrogen (Carlsbad, CA, USA). The Murex HBsAg Version 3 Kit and Murex HBeAg Version 3 Kit were obtained from Murex Biotech Limited (Dartford, UK). Affinity-purified HBsAg and recombinant HBeAg were from Chemicon International (Temecula, CA, USA). TaqMan Universal PCR Mastermix and SYBR green PCR Mastermix were obtained from Applied Biosystems (Foster City, CA, USA). Biotin-16-dUTP and fluorescein-12-dUTP were purchased from Roche (Mannheim, Germany). Primers and probes for real-time PCR were synthesized by Proligo (Singapore). PeliCheck HBV-DNA-99 reference panel was purchased from VQC International (Alkmaar, The Netherlands). Lamivudine (3TC) was bought from Moravek Biochemicals (Brea, CA, USA). Human Albumin ELISA Quantitation Kit was purchase from Bethyl Laboratories, Montgomery, TX, USA.

### 2.3. Cell culture

The 2.2.15 human liver cells were maintained in DMEM, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids and 150 μg/mL G418 at 37 °C under 5% $CO_2$.

### 2.4. Cell viability assay

Cells were seeded in 96-well plate at the plating density of $3 \times 10^4$ in triplicates and allowed to recover for 24 h. Culture medium was replaced by assay medium containing the compound to be tested. All compounds used in this study were dissolved in dimethyl sulfoxide (DMSO) and controls were grown in medium containing 0.2% DMSO. The assay medium was changed every 24 h. After incubation for 2–5 days, MTS/PES reagent was added to each well. The absorbance at 490 nm was measured after incubation at 37 °C for 1 h.

### 2.5. Quantitative assay of HBsAg and HBeAg

Cells were seeded in 24-well plate at the plating density of $2 \times 10^5$ in triplicates and allowed to recover for 24 h. Culture medium was replaced by assay medium containing the compound that is being tested or 3TC. The assay medium was collected every 24 h and fresh assay medium was added. The amount of HBV-s antigen (HBsAg) or e antigen (HBeAg) secreted into the assay medium was determined using the Murex HBsAg Version 3 Kit or Murex HBeAg Version 3 Kit with the protocol described in the manufacturer's manual. Affinity-purified HBsAg and recombinant HBeAg were used to generate the standard curves. Results were expressed as percent of that observed for untreated cells which was considered as 100%.

### 2.6. HBV viral DNA quantitation

Extraction of HBV DNA from the medium of 2.2.15 cell cultures was performed using QIAamp DNA Blood Mini Kit. HBV DNA was extracted from 100 μL of medium following the protocol described by the manufacturer. The DNA was eluted with 50 μL nuclease-free water and the quantitation was carried out as previously described using real-time PCR (Li et al., 2004).

To assess the effect of compound **li** on HBV replication in 2.2.15 cells, intracellular core-associated HBV DNA was also extracted and quantified. This was carried out using a previously described method with some modifications (Nakazono et al., 1996; Li et al., 2004). Cells were seeded in 6-well plate at the plating density of $1 \times 10^6$ and allowed to recover for 24 h. Culture medium was replaced by assay medium containing 40 μM of the compound **li** or 0.2% DMSO. Assay medium was removed and fresh assay medium was added every 24 h. Five days after the initial treatment, treated and control cells were washed twice and lysed in 20 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 0.5% Nonidet P40, 1 mmol/L EDTA. The lysate was centrifuged at 50,000 × *g* for 45 min at 20 °C. The supernatant was collected and incubated with 10 U/mL DNase I for 30 min to digest non-core-associated DNA. The solution was layered on a 30% sucrose cushion and centrifuged at 178,000 × *g* for 3 h at 20 °C. The pellet (core-particles) was suspended in 200 μL nuclease-free water. HBV DNA was extracted using QIAamp DNA Blood Mini Kit and quantified by real-time PCR as previously described (Li et al., 2004).

### 2.7. Detection of viral transcripts

Total RNA was extracted from the cells using the RNeasy Mini Kit. The extraction was carried out according to the manufacturer's instructions.

In order to quantify the changes in transcript levels, reverse transcription followed by real-time PCR was carried out. Three sets of primers were used for reverse transcription–real-time PCR for viral transcripts. The primers had been previously described and were designed to transcribe and amplify the 3.5-kbp transcripts, or both the 3.5-kbp transcripts and the 2.4 kbp transcripts or all viral transcripts which include the 3.5, 2.4 and 2.1-kbp transcripts (Li et al., 2004).

The isolated RNA was first subjected to reverse transcription. A typical 25 μL reverse transcription reaction contained 0.5 μg of RNA, 0.1 mM dNTPs, 0.5 μM of the reverse gene-specific primers, 0.5 mM of $MgSO_4$ and 0.05 U/μL of AMV reverse transcriptase. The reverse transcription was carried out at 42 °C for 90 min. Five microliters of suitably diluted cDNA was then mixed with 900 nM of each corresponding PCR primers, 12.5 μL SYBR green PCR Mastermix in a total volume of 25 μL. The reaction mixture was first incubated at 50 °C for 2 min and 95 °C for 10 min. Following this, the PCR reaction was performed at 95 °C for 15 s and 60 °C for 1 min for 45 cycles. The levels of PCR products were examined with an ABI PRISM 7000 sequencing detection system and analyzed with ABI PRISM 7000SDS software (Applied Biosystems). Cycle numbers were determined at a reading of 0.2 fluorescence unit. To determine the relative quantity of HBV RNA transcripts, cycle numbers were normalized to that of GAPDH. The relative amount of each mRNA to GAPDH RNA was described using the equation—$2^{-\Delta CT}$, where $\Delta CT = CT_{RNA} - CT_{GAPDH}$. Relative

gene expression was multiplied by $10^3$ in order to simplify the presentation of the data.

### 2.8. Quantitation of albumin in culture medium

Albumin secreted by control and treated 2.2.15 cells was determined by using the Human Albumin ELISA Quantitation Kit. The determination was carried out according to the protocol described by the manufacturer.

## 3. Results

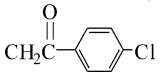
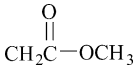
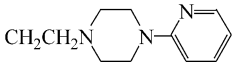
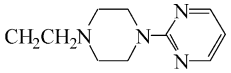
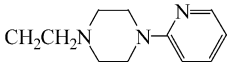
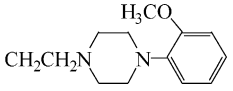
### 3.1. Synthesis of compounds

The general strategy employed in our synthesis is illustrated in Fig. 1. α-Sulfonylesters **3** were obtained in quantitative yields via the sulfinate → sulfone alkylation of sodium benzenesulfinate **2** with different α-bromoesters in the presence of KI/$NBu_4I$. **3** was subsequently treated with hydrazine hydrate under refluxing conditions for 16 h to give α-sulfonylcarboxylic hydrazides **4** in 87–92% yields. Reaction of **4** with $CS_2$/KOH in refluxing ethanol afforded the corresponding substituted 5-benzenesulfonylmethyl-3*H*-[1,3,4]-oxadiazole-2-thiones **5** (Polański et al., 2000) which were subsequently alkylated with various bromo- or iodo-substituted reagents in the presence of $K_2CO_3$ at room temperature to give the required compounds **1a–1e**. Attempts to alkylate **5** with 1-(2-chloroethyl)-4-substituted-piperazines using the same alkylating condition as the bromo- and iodo-reagents did not provide the desired product. However, when $NBu_4I$ was introduced and refluxing condition was applied, the desired 1-[2-(5-benzenesulfonylpropyl-[1,3,4]-oxadiazol-2-yl-sulfanyl)-ethyl]-4-substituted-piperazines, **1f–1i**, were obtained in 57–65% yield.

### 3.2. Cytotoxicity analysis

In this study, the cytotoxicity assays were carried out using the 2.2.15 cell line. Initial treatments were carried out for 2 days. At concentrations >1.5 μM for compounds **1a–1e**, >7.5 μM for compounds **5a–5c**, >20 μM for compound **1f** and >10 μM for compound **1g**, significant cytotoxicity was observed. In addition, treatment with compounds **1h** and **1i** at concentrations up to 40 μM also did not result in any adverse effect on cell growth and viability (Table 1).

Table 1
Cytotoxicity and HBsAg expression in 2.2.15 cells after treatment with compounds **1a–1i** and **5a–5c** for 2 days

| Code[a] | $R^1$ | $R^2$ | $CC_{50}$[b] (μM) | HBsAg[c] (%) |
|---|---|---|---|---|
| **1a** | $CH_2CH_3$ | $CH_2CH_2CH_2CH_2CH_3$ | >1.5 | 118.1 |
| **1b** | $CH_3$ | $CH_2CH_2CH_2CH_3$ | >1.5 | 109.0 |
| **1c** | H |  | >1.5 | 157.4 |
| **1d** | H |  | >1.5 | 110.7 |
| **1e** | H | $CH_2CH_2CH_2CH_3$ | >1.5 | 148.6 |
| **1f** | $CH_2CH_3$ |  | >20 | 97.8 |
| **1g** | $CH_2CH_3$ |  | >10 | 86.8 |
| **1h** | H |  | >40 | 105.4 |
| **1i** | $CH_2CH_3$ |  | >40 | 63.7 |
| **5a** | H | – | >7.5 | 108.5 |
| **5b** | $CH_3$ | – | >7.5 | 103.4 |
| **5c** | $CH_2CH_3$ | – | >7.5 | 106.7 |

[a] Purities of >95% as evaluated by NMR.
[b] Compounds were dissolved in DMSO. The 50% cytotoxic concentration ($CC_{50}$) is greater than highest concentration tested.
[c] HBsAg levels are expressed as a percentage of HBsAg concentration of the control. Control cells were grown in medium containing 0.2% DMSO.

### 3.3. Inhibition of viral-s antigen production

The 2.2.15 cell line has an integrated HBV genome and is capable of producing viral particles, HBeAg, as well as spheri-

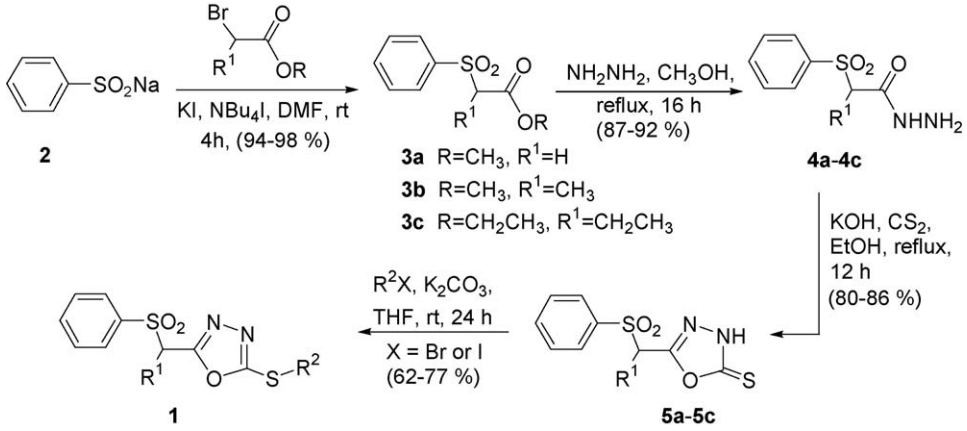



Fig. 1. Synthesis of 2-benzenesulfonylmethyl-5-substituted-sulfanyl-[1,3,4]-oxadiazoles.

cal and tubular particles comprising of only HBsAg without any virus DNA (Sells et al., 1987). Initial analysis following treatment for 2 days showed that none of the compounds tested had an effect on the production of HBsAg except **1i** which was effective in reducing HBsAg production (Table 1). Thus, all further experiments were carried out with compound **1i**.

### 3.4. Inhibition of viral antigens and virion production by *1i*

Subsequent analysis showed that treatment with 1–40 μM of compound **1i** led to significant inhibition of virion secreted after 3 days of treatment (Fig. 2A). Reduction of viral DNA content in the medium was evident following treatment with 1 μM and a maximal reduction of 55.2 ± 9.7% was observed at 40 μM. Further reductions in viral DNA in the medium ($EC_{50}$ = 1.63 μM) were observed after 5 days of treatment. Corresponding reductions in the viral antigens, HBsAg and HBeAg were also observed (Fig. 2B and C). The nucleoside analog, 3TC was used as a positive control and an $EC_{50}$ value of 2.96 μM was obtained after 5 days of treatment (Fig. 2A). Both **1i** and 3TC did not affect cell viability. After 5 days of treatment, viability of the treated cells at the highest concentration used was comparable to the control cells (>90% of control).

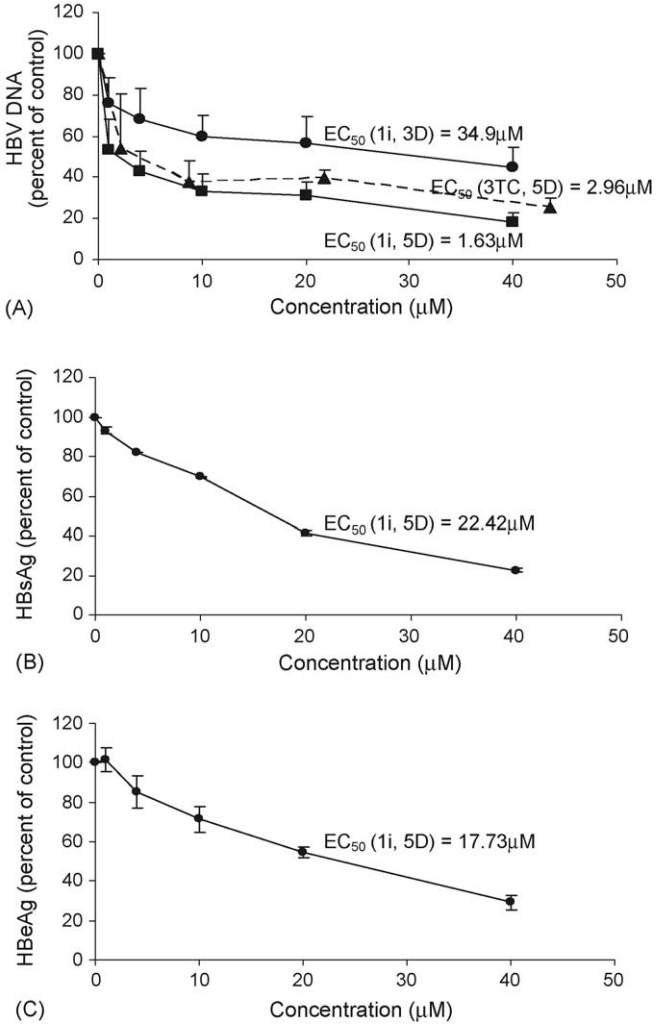



Fig. 2. Analysis of (A) HBV DNA, (B) HBsAg and (C) HBeAg levels in the culture medium of 2.2.15 cells. (A) HBV DNA levels were determined after cells were treated with 3TC for 5 days (triangle symbols) or with compound **1i** for 3 days (round symbols) or 5 days (square symbols). (B and C) HBsAg and HBeAg levels in the culture medium were determined after cells were treated for 5 days with compound **1i**. The HBsAg concentration in the control was 6.1 ± 0.1 ng/well while that for HBeAg was 163.3 ± 6.0 ng/well. These were set as 100% for HBsAg and HBeAg, respectively. The control cells for 3TC treatment were untreated and grown in culture medium while the control cells for compound **1i** treatment were grown in medium containing 0.2% dimethyl sulfoxide. Data shown represent the mean ± S.D. ($n$ = 3). All data points were significantly different from the corresponding controls ($p < 0.05$, ANOVA analysis).

Table 2
Expression of viral transcripts after treatment with 40 μM of compound **1i** for 5 days

| Viral transcripts | Relative gene expression ($\times 10^3$) | | |
|---|---|---|---|
| | Control | Treatment | $p$-Value |
| 2.1, 2.4 and 3.5-kbp transcripts | 309 ± 25.6 | 310 ± 52.8 | 0.98 |
| 2.4 and 3.5-kbp transcripts | 70.6 ± 13.4 | 67.7 ± 14.3 | 0.80 |
| 3.5-kbp transcripts | 0.70 ± 0.05 | 0.83 ± 0.16 | 0.24 |

The control cells were grown in medium containing 0.2% dimethyl sulfoxide while the treated cells were grown in medium containing 40 mM of **1i**. Total RNA was isolated from the control and treated cells. This was followed by reverse transcription–real-time PCR to quantify the expression of HBV RNAs. Data shown represent the mean ± S.D. ($n$ = 3). All data points were not significantly different from the corresponding controls as determined by ANOVA analysis.

No significant differences were observed for albumin secreted by control (2.88 ± 0.12 μg/mL) and treated cells (2.69 ± 0.17 μg/mL), following treatment with 40 μM of **1i** for 5 days. In contrast, viral antigens HBsAg and HBeAg were significantly reduced although there were no significant changes in the four HBV transcripts after the 5-day treatment period (Table 2). Viral DNA was also reduced by >80% in the medium (Fig. 2A). Intracellular encapsidated HBV DNA was determine and was also found to be reduced from $9.9 \pm 1.1 \times 10^7$ copies per well for the control to $4.6 \pm 0.9 \times 10^7$ copies per well for the treated cells.

## 4. Discussion

To date, the treatment for chronic hepatitis B infection is based on suppression of viral replication (Ferenci, 2004). This is achieved through the use of either interferon-α or nucleoside analogs. Interferon-α acts as an immunomodulatory agent while nucleoside analogs or their metabolites function as inhibitors of the viral reverse transcriptase/polymerase. The first nucleoside analog that was licensed for chronic hepatitis B treatment was 3TC. Although this has good clinical efficacy, the emergence of 3TC-resistant virus has led to the need for new drugs for treating chronic hepatitis B (Fischer et al., 2001). Emergence of resistance to the newer nucleosidic antivirals, adefovir and entecavir, has also been reported (Angus et al., 2003; Villeneuve et al., 2003; Fung et al., 2005; Tenney et al., 2004).

Current approaches to the development of therapy for HBV infection are centered mainly on nucleoside analogs which target the HBV reverse transcriptase/polymerase (Ying et al., 2003). This may be due to the successful use of nucleoside analogs

as inhibitors of viral reverse transcriptases including that of the human immunodeficiency virus (HIV) (Ying et al., 2003). As mentioned above, one major problem with the use of nucleoside analogs as therapeutic agents is the emergence of resistant viruses. However, it is possible that when used in combination, a series of different nucleoside analogs may prove to be effective therapy for chronic hepatitis B (Ferenci, 2004).

Various non-nucleoside analogs such as phenylpropenamide derivatives (King et al., 1998; Delaney et al., 2002; Perni et al., 2000), iminosugars (Lu et al., 1997; Block et al., 1998; Mehta et al., 2001) and a class of heteroarylpyrimidines (Weber et al., 2002; Deres et al., 2003; Stray et al., 2005) have all been shown to be selective inhibitors of hepatitis B virus replication. These inhibitors do not target HBV reverse transcriptase but work by preventing the encapsidation of viral pregenomic RNA (King et al., 1998), or the secretion of viruses (Mehta et al., 2001), or the assembly of the capsid (Deres et al., 2003; Stray et al., 2005).

In this study, we present the synthesis of a series of 2-benzenesulfonylalkyl-5-substituted-sulfanyl-[1,3,4]-oxadiazoles and examine their anti-HBV activity. Among the oxadizoles, compound **1i** showed the most promising antiviral activity with no cytotoxic effects. Treatment with compound **1i** did not lead to changes in the expression of viral transcripts but there was significant reduction in the amount of virus secreted by the cells as well as the amount of intracellular virion particles suggesting that there is an overall inhibition of virion replication. The reduced virion production also corresponded with markedly reduced levels of the HBV antigens; namely HBsAg and HBeAg. In contrast, the production and secretion of albumin by the 2.2.15 cells were not affected. Inhibition of virion production by compound **1i** was comparable to that of the nucleoside analog 3TC with $EC_{50}$ values of 1.63 and 2.96 μM, respectively. Having observed no changes in the expression of viral transcripts, it is evident that transcription was not affected by compound **1i**. Thus, compound **1i** is likely to affect events downstream of transcription in the viral replication process. Further investigation is necessary to fully evaluate and to understand the mechanism of action of oxadiazoles as anti-HBV agents.

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