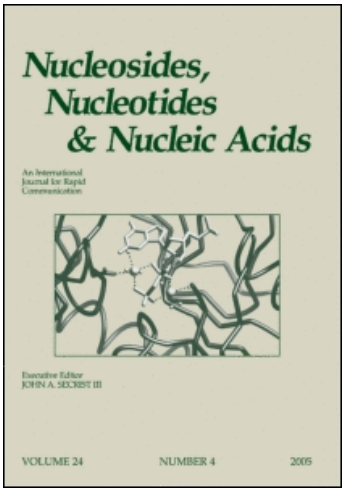

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Synthesis of 6-Arylthio Analogs of 2',3'-Dideoxy-3'-Fluoroguanosine and Their Effect against Hepatitis B Virus Replication

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SYNTHESIS OF 6-ARYLTHIO ANALOGS OF 2′,3′-DIDEOXY-3′-FLUOROGUANOSINE AND THEIR EFFECT AGAINST HEPATITIS B VIRUS REPLICATION

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□ *A key compound, 2-amino-6-chloro-9-(2,3-dideoxy-3-fluoro-β-D-erythro-pentofuranosyl)purine, was prepared from 2-amino-6-chloropurine riboside in 5 steps, then subjected to the nucleophilic displacement with benzenethiols to afford 6-arylthio congeners. These compounds showed a similar anti-HBV effect to that of 2′,3′-dideoxy-3′-fluoroguanosine.*

Keywords FddG analogs; Benzenethiol; Anti-HBV activity

INTRODUCTION

The hepatitis B virus (HBV), an hepadnavirus, was identified in 1963 by Blumberg et al.[22] as the causative agent of hepatitis B. Today, there are an estimated 300 million chronic carriers worldwide. There is a particular high prevalence in Asia and Africa. Treatment of hepatitis B infection is a challenge of modern medicine. There is an effective vaccine available against HBV.[1] Antiviral agents are, however, needed for those patients already infected with HBV. Although interferon α is approved for

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This paper is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.

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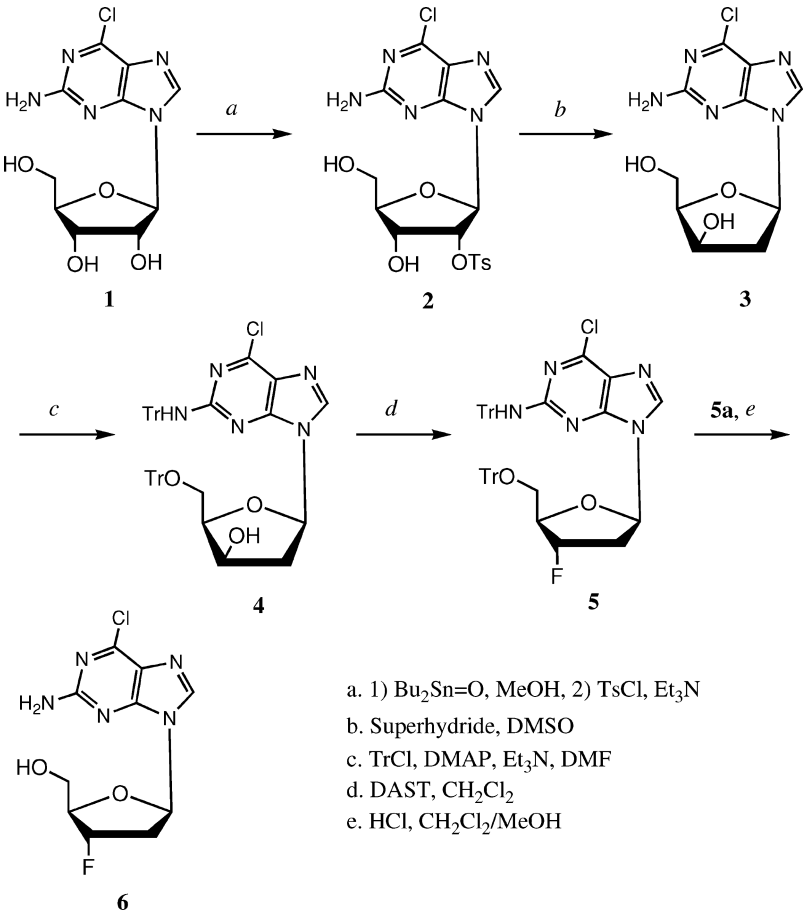
the treatment of chronic HBV infection, it is effective in only 10–30% of treated patients.[2,3] Several nucleoside reverse transcriptase inhibitors are effective against both HIV and HBV. Lamivudine acts as an inhibitor of HBV DNA polymerase as well as HIV reverse transcriptase after it has been converted to the 5′-triphosphate.[4,5] A class of phosphonomethoxyethylpurine analogs such as 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) and 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP) show a broad spectrum of activity against several viruses including HBV.[6] Sekiya et al. developed a new type of phosphonomethoxyethylpurine analogs, 2-amino-6-arylthio-9-[2-(phosphonomethoxy)ethyl]purine bis (2,2,2-trifluoroethyl) esters, as HBV-specific antiviral agents.[7] Recently, 2′,3′-dideoxy-3′-fluoroguanosine (FddG) has been proved to be a strong inhibitor of duck hepatitis B virus.[8] We have already reported practical synthetic approaches to lodenosine (FddA) starting from inosine, in which fluorination of 6-chloropurine nucleosides has been involved as a key step.[9] In this perspective, our current attention was focussed on the synthesis of FddG via 2-amino-6-chloropurine riboside. Also reported here is the antiviral effect of 6-arylthio analogs of FddG against HBV.

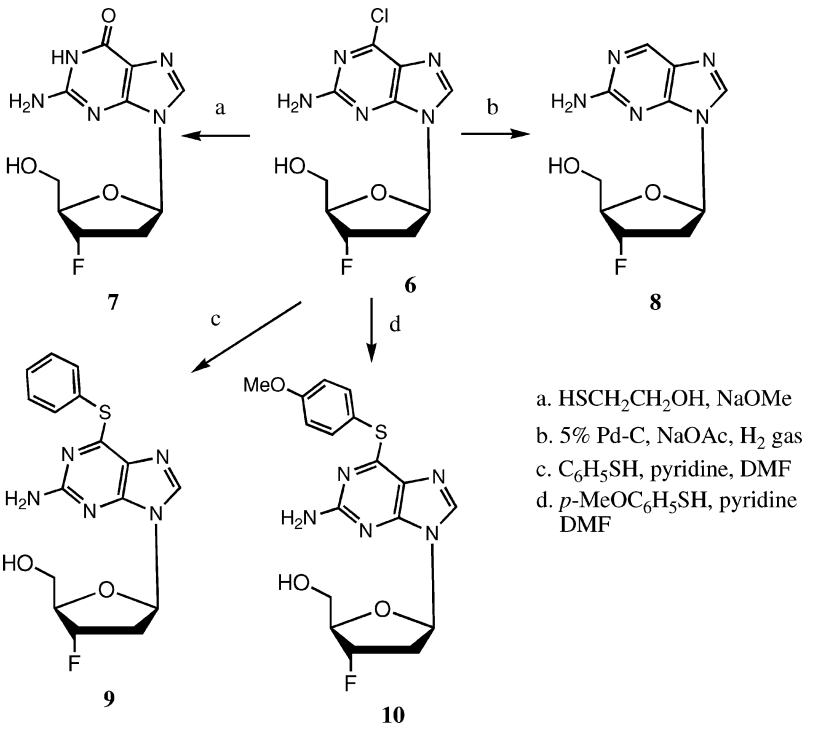
SYNTHESIS

Introduction of fluorine at the sugar moiety of nucleosides has been recognized as important since many fluoronucleosides are biologically active and stable by chemical and PNP catalyzed hydrolysis. Three synthetic methods are classified as follows: (1) Epoxide ring-opening using HF; (2) nucleophilic displacement by fluoride ion; (3) opening of the anhydro bond formed between sugar and pyrimidine.[10] However, vigorous conditions are needed for the reaction due to the relatively poor nucleophilicity of the fluoride ion. Recently, diethylaminosulfur trifluoride (DAST) has been extensively used for the introduction of the fluorine into carbohydrate.[11] For the synthesis of FddG, several approaches have been reported.[12] In 1988, Herdewijn et al. reported the conversion of guanosine to FddG, in which reaction of 9-(2-deoxy-β-D-*threo*-pentofuranosyl)guanine with DAST was involved as a key step.[13] However, the yield was not satisfactory. In our research to prepare FddA,[9a] introduction of 6-chloro on purine proved to be efficient to prevent intramolecular nucleophilic attack by 3-nitrogen. Therefore, 2-amino-6-chloropurine riboside (**1**)[14] was selected as a starting material for the synthesis of 2-amino-6-chloro-9-(2,3-dideoxy-3-fluoro-β-D-*erythro*-pentofuranosyl)purine (**6**),[15] an intermediary compound for the 6-substituted derivative (Scheme 1). At first, 2′-*O*-tosyl compound was prepared as follows. The 2′,3′-*O*-di-*n*-butylstannylene complex is useful intermediate for introducing only one tosyl group at the 2′-OH.[16] Thus, compound **1** has been successively treated with di-*n*-butyltin (IV) oxide and excess tosyl



SCHEME 1

chloride in the presence of triethylamine (Et_3N) in MeOH to give the 2′-*O*-tosyl congener[17] (**2**) as white prisms in 85% yield. This product was subjected to a deoxygenative [1,2]-hydride shift rearrangement which converts *cis*-diol monotosylates to inverted secondary alcohols.[18] A solution of **2** in anhydrous dimethyl sulfoxide (DMSO) under nitrogen was treated with 1 M lithium triethylborohydride (LTBH) in THF. Since 6-chloro group is susceptible to alkaline condition, the reaction was quenched with acetic acid. After usual workup and separation using silica gel chromatography, the 2-deoxy-β-D-*threo*-pentofuranose **3** was obtained as a colorless solid in 83% yield. To protect the 5′-OH and 2-NH_2 group,[19] **3** was treated with trityl chloride in the presence of triethylamine and 4-dimethylaminopyridine (DMAP) to give the ditrityl congener (**4**) as a foam in 96% yield. Then, **4** was treated with DAST in CH_2Cl_2 at 40°C for 30 min. After work-up of the solution, 3′-fluoro derivative (**5**) was isolated as colorless prisms in 60% yield. The yield of this step was superior to that of guanine nucleoside (35%).[13] Next,



SCHEME 2

the trityl group was removed by acid treatment to give a key intermediary compound (**6**) in 69% yield. Several 6-modified derivatives were prepared by nucleophilic displacement or hydrogenation as follows (Scheme 2): at first **6** was converted to FddG (**7**)[13] by treatment with 2-mercaptethanol and NaOMe in MeOH[20] in 67% yield. Next, catalytic hydrogenation of **6** using 5% Pd-C was achieved to give the 2-aminopurine nucleoside (**8**)[21] in 63% yield. When **6** was treated with benzenethiol[7] in the presence of pyridine in DMF, 6-phenylthio derivative (**9**) was obtained in 90% yield. 6-(4-Methoxyphenyl)thio congener (**10**) was also synthesized in a similar manner to that of **9** using *p*-methoxybenzenethiol as a nucleophile.

ANTI-HBV ACTIVITY

The antiviral effects against HBV of the 2′,3′-dideoxy-3′-fluororibosides **6-10** were assayed according to the procedures described in the experimental section and the results are presented in Table 1. Compound **7** (FddG) exhibited moderate anti-HBV activity as was expected from earlier reports.[8,9] 6-Chloro derivative (**6**) also showed anti-HBV activity to almost the same degree as that of **7**, suggesting 6-chloro had been converted to 6-oxo before it acts as an inhibitor of HBV DNA polymerase. It is interesting that the

TABLE 1 Anti-HBV Activity of FddG and Its 6-Substituted Analogs Prepared by Nucleophilic Displacement or Catalytic Hydrogenation of **6**

Compound	EC_{50} (μM)	CC_{50} (μM)
6	10.4	>93
7	9	>87
8	>99	>99
9	9.7	>69
10	3.6	>64
PMEA	0.26	>92

activity of 6-(4-methoxy)phenylthio (**10**) derivative was superior to that of **7**. Also, 6-phenylthio derivative (**9**) maintained inhibitory effect against HBV comparable to that of **7**. These results indicate that 2-amino-6-arylthiopurine is also changeable to guanine in the assay system. One exception to this rule is 2-aminopurine derivative (**8**), which showed no anti-HBV activity even at the concentration of 99 μM, suggesting that **10** had not been converted to an active form. However, antiviral effect of all compounds including FddG against HBV was weak compared to that of the positive control, PMEA. Therefore, we will attempt to optimize in further studies the activity of compound **7**.

EXPERIMENTAL

Melting points (mp) were determined using a Yanagimoto micro-melting point apparatus (hot stage type) and are uncorrected. UV spectra were recorded with a Shimadzu UV-190 digital spectrometer. Low-resolution mass spectra were obtained on a FAB-JMS-700 mass spectrometer in the direct-inlet mode. High-resolution mass spectra were obtained on a JMS-T100-CSI spectrometer in the ESI mode. ^{1}H-NMR spectra were recorded on JEOL JMM-ECX400 (400 MHz) in $CDCl_3$ (or dimethyl sulfoxide (DMSO)-d_6) with tetramethylsilane as an internal standard. Merck Art 5554 plates pre-coated with silica gel 60 containing fluorescent indicator F_{254} were used for thin-layer chromatography and silica gel 60 (Merck 7734, 60–200 mesh) was employed for column chromatography.

*2-Amino-6-chloro-9-(2-O-tosyl-β-D-ribofuranosyl)purine (***2***).* Dibutyltin (IV) oxide (2.7 g, 11.6 mmol) was added to the solution of **1** (3.5 g, 11.6 mmol) in MeOH (200 ml), and refluxed for 1 h. After being cooled to 0°C, Et_3N (24 ml, 174 mmol) and TsCl (32.5 g, 174 mmol) were added to a solution and stirred at 4°C for 12 h, and the solvent was removed. The residue was diluted with H_2O and extracted with EtOAc, and dried over $MgSO_4$. Removal of the organic layer afforded an oily residue, which was purified by column

chromatography on silica gel (66% EtOAc in hexane) and recrystallization from EtOH to give **2** (4.5 g, 85%) as colorless crystals: mp 194–196°C. ^{1}H NMR (DMSO-d_6, RT) δ 8.11 (1H, s, H8), 7.43 (2H, d, J = 8.1 Hz, Ph), 7.05 (2H, d, J = 8.1 Hz, Ph), 6.96 (2H, brs, 2-NH_2), 6.04 (1H, brs, 3′-OH), 5.96 (1H, d, J = 7.3 Hz, CH1′), 5.45 (1H, dd, J = 5.0, 7.3 Hz, CH2′), 5.18 (1H, brs, 5′-OH), 4.34-4.28 (1H, m, CH3′), 3.98–3.92 (1H, m, CH4′), 3.70–3.50 (2H, m, $CH_2$5′), 2.28 (3H, s, CH_3). ^{13}C NMR (DMSO-d_6, RT) δ 159.90, 153.32, 149.76, 145.21, 141.32, 132.00, 129.79, 127.21, 123.73, 87.17, 84.02, 79.69, 70.16, 61.46, 21.42. ESI-MS *m/z*: 456 $[M+H]^+$.

*2-Amino-6-chloro-9-(2-deoxy-β-D-threo-pentofuranosyl)purine (**3**).* To a solution of **2** (3.42 g, 7.5 mmol) in DMSO (75 ml) was added 1 M lithium triethylborohydride in THF solution (75 ml) and stirred at 35°C for 14 h. The solution was diluted with H_2O (35 ml) at 0°C and neutralized with CH_3COOH. Removal of the solvent afforded an oily residue, which was dissolved EtOH (50 ml) and filtered through Celite. After removal of the filtrate, the residue was purified by column chromatography on silica gel (15% EtOH in EtOAc) and recrystallization from EtOH-H_2O to give **3** (1.77 g, 83%) as colorless crystals: mp 126–127°C; ^{1}H NMR (DMSO-d_6, RT): δ 8.28 (1H, s, H8), 6.91 (2H, brs, 2-NH_2), 6.12 (1H, d, J = 7.8 Hz, 3′-OH), 5.34 (1H, d, J = 4.2 Hz, CH1′), 4.64 (1H, t, J = 5.8 Hz, 5′-OH), 4.32–4.31 (1H, m, CH3′), 3.89–3.88 (1H, m, CH4′), 3.69 (1H, ddd, J = 5.5, 11.2, 17.0 Hz, $CH_2$5′-one), 3.58 (1H, ddd, J = 6.0, 11.7, 17.5 Hz, $CH_2$5′-one), 2.66 (1H, ddd, J = 5.1, 8.3, 14.2 Hz, $CH_2$2′-one), 2.21 (1H, d, J = 14.7 Hz, $CH_2$2′-one); UV λ_{max} (MeOH) nm: 248, 310; ESI-MS *m/z*: 308 $[M + Na]^+$; elemental analysis calcd (%) for $C_{10}H_{12}ClN_5O_3 \cdot H_2O$: C 39.55, H 4.65, N 23.06; found C 39.57, H 4.34, N 23.05.

*6-Chloro-9-(5-O-trityl-2-deoxy-β-D-threo-pentofuranosyl)-2-tritylaminopurine (**4**).* Et_3N (0.95 ml, 6.8 mmol), DMAP (85 mg, 0.7 mmol), and TrCl (1.67 g, 6.0 mmol) were added to a solution of **3** (500 mg, 1.8 mmol) in DMF (15 ml) and stirred at 45°C for 24 h. MeOH (5 ml) was added to the solution and stirred at RT for 30 min and the solvent was removed. The residue was diluted with toluene and washed with H_2O, and dried over $MgSO_4$. Removal of the organic layer afforded an oily residue, which was purified by column chromatography on silica gel (50% EtOAc in hexane) to give **4** (1.30 g, 96%) as a colorless foam: mp 140–142°C; ^{1}H NMR ($CDCl_3$, RT, TMS): δ 8.00 (1H, s, H8), 7.42–7.21 (30H, m, Ph), 6.51 (1H, brs, 2-NH), 5.69–5.61 (1H, m, CH1′), 4.39–4.38 (1H, m, CH3′), 4.05 (1H, ddd, J = 3.2, 5.9, 9.1 Hz, CH4′), 3.58 (1H, dd, J = 5.9, 10.1 Hz, $CH_2$5′-one), 3.35 (1H, dd, J = 6.4, 9.6 Hz, $CH_2$5′-one), 2.28-2.20 (1H, m, $CH_2$2′-one), 2.00–1.95 (1H, m, $CH_2$2′-one); UV λ_{max} (MeOH) nm: 312; ESI-MS *m/z*: 792 $[M+Na]^+$; elemental analysis calcd (%) for $C_{48}H_{40}ClN_5O_3 \cdot 0.5\ H_2O$: C 73.79, H 5.16, N 8.96; found C 73.74, H 5.36, N 8.65.

*6-Chloro-9-(5-O-trityl-2,3-didexy-3-fluoro-β-D-erythro-pentofuranosyl)-2-tritylaminopurine (**5**).* Diethylaminosulfur trifluoride (DAST) (14 μl, 0.104

mmol) was added to a solution of **4** (50 mg, 0.065 mmol) in CH_2Cl_2 (2ml) at 0°C. After being stirred at 40°C for 30 min, the solution was added to 5% aqueous $NaHCO_3$ at 0°C and extracted with CH_2Cl_2, and dried over $MgSO_4$. Removal of the organic layer afforded a white solid residue, which was purified by column chromatography on silica gel (33% EtOAc in hexane) and recrystallization from EtOAc-hexane gave **5** (30 mg, 60%) as colorless crystals: mp 232–234°C; ^{1}H NMR ($CDCl_3$, RT, TMS): δ 7.79 (1H, s, H8), 7.33–7.19 (30 H, m, Ph), 6.62 (1H, brs, 2-NH), 5.72–5.62 (1H, m, CH1′), 4.98 (1H, d, J = 54.5 Hz, CH3′), 4.28 (1H, d, J = 25.6 Hz, CH4′), 3.27 (2H, d, J = 4.2 Hz, $CH_2$5′), 2.20–2.15 (2H, m, $CH_2$2′); UV λ_{max} (MeOH) nm: 313, 259; ESI-MS *m/z*: 794 $[M+Na]^+$; elemental analysis calcd (%) for $C_{48}H_{39}ClFN_5O_2 \cdot 0.5\ H_2O$: C 73.79, H 5.16, N 8.96; found C 74.04, H 5.05, N 8.93.

*2-Amino-6-chloro-9-(2,3-didexy-3-fluoro-β-D-erythro-pentofuranosyl)-purine (***6***).* To a solution of **5** (250 mg, 0.32 mmol) in CH_2Cl_2 (15 ml) and MeOH (15 ml) was added 35% aqueous HCl (0.1 ml) dropwise. After being stirred at 30°C for 1.5 h, Et_3N (0.2 ml) was added to the solution at 0°C, and the solvent was removed. The residue was diluted with H_2O and extracted with CH_2Cl_2, and dried over $MgSO_4$. Removal of the organic layer afforded an oily residue, which was purified by column chromatography on silica gel (EtOAc) and recrystallization from EtOH gave **6** (63 mg, 69%) as colorless crystals: mp 169–171°C. ^{1}H NMR (DMSO-d_6, RT) δ 8.36 (1H, s, H8), 7.00 (2H, brs, 2-NH_2), 6.27 (1H, dd, J = 5.8, 9.0 Hz, CH1′), 5.42 (1H, dd, J = 4.4, 53.6 Hz, CH3′), 5.13 (1H, t, J = 5.4 Hz, OH), 4.20 (1H, dt, J = 5.0, 26.5 Hz, CH4′), 4.16 (2H, t, J = 5.4 Hz, $CH_2$5′), 2.90 (1H, ddd, J = 5.0, 9.3, 39.2 Hz, $CH_2$2′-one), 2.71–2.60 (1H, m, $CH_2$2′-one). ^{13}C NMR (DMSO-d_6, RT) δ 159.38, 153.37, 149.18, 140.66, 123.14, 94.40 (J = 173 Hz), 84.97 (J = 22 Hz), 82.65, 60.45 (J = 11 Hz), 36.12 (J = 20 Hz). ESI-MS *m/z*: 288 $[M+H]^+$.

9-(2,3-Didexy-3-fluoro-β-D-erythro-pentofuranosyl)guanine (FddG, **7***).* To a solution of **6** (50 mg, 0.174 mmol) in MeOH (5 ml) was added 2-mercaptoethanol (0.03 ml) and 28% NaOMe (0.058 ml) and the solution was kept at 50°C for 2 days, then neutralized by addition of acetic acid. After concentration of the solvent, the residue was applied to a column of silica gel. The main fraction was evaporated to give a solid, which was crystallized from EtOH to give **7** as a pale yellowish powder (31.6 mg, 67%). ^{1}H NMR (DMSO-d_6, RT) δ 10.54 (1H, brs, NH), 7.92 (1H, s, H8), 6.49 (2 H, brs, 2-NH_2), 6.15 (1H, dd, J = 5.6, 9.4 Hz, CH1′), 5.38 (1H, dd, J = 4.3, 53.6 Hz, CH3′), 5.15 (1H, t, J = 5.4 Hz, OH), 4.16 (1H, dt, J = 4.9, 27.0 Hz, CH4′), 3.60–3.50 (2 H, m, $CH_2$5′), 2.90–2.70 (1H, m, $CH_2$2′-one), 2.67–2.50 (1H, m, $CH_2$2′-one). ^{13}C NMR (DMSO-d_6, RT) δ 156.26, 153.30, 150.60, 134.88, 116.34, 94.94 (J = 173 Hz), 85.17 (J = 22 Hz), 82.64, 60.98 (J = 11 Hz), 36.87 (J = 20 Hz). ESI-MS *m/z*: 270 $[M+H]^+$.

*2-Amino-9-(2,3-didexy-3-fluoro-β-D-erythro-pentofuranosyl) purine (***8***).* A mixture of **6** (10 mg, 0.035 mmol), CH_3COONa (3.0 mg, 0.035 mmol) and 5%

Pd-C (5 mg) in MeOH (2 ml) was vigorously stirred under an H_2 atmosphere for 6 h. The Pd-catalyst was filtered off, and the filtrate was concentrated in vacuo to leave a white solid residue, which was purified by column chromatography on silica gel (15% EtOH in EtOAc) and recrystallization from EtOH to give **8** (5.5 mg, 63%) as colorless crystals: mp 148–149°C; ^{1}H NMR (CD_3OD, RT):δ 8.57 (1H, s, H6), 8.27 (1H, s, H8), 6.41 (1H, dd, J=5.6, 9.2 Hz, CH1′), 5.41 (1H, dd, J=4.8, 53.6 Hz, CH3′), 4.34 (1H, dt, J=3.6, 26.8 Hz, CH4′), 3.85-3.77 (2H, m, $CH_2$5′), 2.96 (1H, dddd, J=4.8, 9.2, 14.4, 39.6 Hz, $CH_2$2′-one), 2.67 (1H, ddd, J=6.0, 14.4, 21.2 Hz, $CH_2$2′-one); ^{13}C NMR (CD_3OD, RT): δ 161.7, 154.0, 150.4, 143.5, 128.7, 96.2 (J=174 Hz), 87.5 (J=23 Hz), 86.3, 63.1 (J=11 Hz), 38.9 (J=21 Hz); UV λ_{max} (MeOH) nm: 310, 221; FAB-MS *m/z*: 254 $[M+H]^+$.

*2-Amino-9-(2,3-didexy-3-fluoro-β-D-erythro-pentofuranosyl)-6-phenyl-thiopurine (***9***).* Benzenethiol (27 μl, 0.26 mmol) and pyridine (69 μl, 0.85 mmol) were added to a solution of **6** (50 mg, 0.17 mmol) in 1.5 ml of DMF and stirred at 80°C for 12 h. Removal of the solvent afforded an oily residue, which was purified by column chromatography on silica gel (30% EtOAc in hexane) and recrystallization from EtOH to give **9** (55 mg, 90%) as colorless crystals: mp 192–193°C; ^{1}H NMR (DMSO-d_6, RT): δ 8.19 (1H, s, H8), 7.58–7.56 (2H, dd, J=3.0, 6.6 Hz, Ph), 7.42 (3H, t, J=3.2 Hz, Ph), 6.39 (2H, brs, 2-NH_2), 6.22 (1H, dd, J=6.0, 9.2 Hz, CH1′), 5.38 (1H, dd, J=4.1, 53.6 Hz, CH3′), 5.11 (1H, t, J=4.6 Hz, OH), 4.20–4.11 (1H, dt, J=5.0, 27.1 Hz, CH4′), 3.53 (2H, t, J=5.1 Hz, $CH_2$5′), 2.89 (1H, dddd, J=4.6, 8.7, 14.2, 39.4 Hz, $CH_2$2′-one), 2.59 (1H, ddd, J=5.5, 14.6, 20.6 Hz, $CH_2$2′-one); UV λ_{max} (MeOH) nm: 312, 246; ESI-MS *m/z*: 384 $[M+Na]^+$; elemental analysis calcd (%) for $C_{16}H_{16}FN_5O_2S$: C, 53.17; H, 4.46; N, 19.38; found C 53.22, H 4.33, N 19.25.

*2-Amino-9-(2,3-didexy-3-fluoro-β-D-erythro-pentofuranosyl)-6-(4-methoxyphenyl)-thiopurine (***10***).* Compound **9** was prepared from *p*-methoxybenzenethiol and **6** in a manner similar to that described for the preparation of **9**: mp 184–185°C; ^{1}H NMR (DMSO-d_6, RT): δ 8.17 (1H, s, H8), 7.47 (2H, d, J=8.7 Hz, Ph), 6.98 (2H, d, J=9.1 Hz, Ph), 6.33 (2H, brs, 2-NH_2), 6.21 (1H, dd, J=5.5, 9.2 Hz, CH1′), 5.38 (1H, dd, J=4.4, 53.6 Hz, CH3′), 5.12 (1H, t, J=5.5 Hz, 5′-OH), 4.15 (dt, J=5.0, 26.9 Hz, CH4′), 3.77 (3H, s, *p*-OCH_3), 3.53 (2H, t, J=5.3 Hz, $CH_2$5′), 2.87 (1H, dddd, J=5.0, 9.1, 14.2, 39.8 Hz, $CH_2$2′-one), 2.58 (1H, ddd, J=6.0, 14.7, 21.1 Hz, $CH_2$2′-one); UV λ_{max} (MeOH) nm: 314; ESI-MS *m/z*: 414 $[M+Na]^+$; elemental analysis calcd (%) for $C_{17}H_{18}FN_5O_3S0.2H_2O$: C 51.69, H 4.69, N 17.73; found C 51.37, H 4.32, N 17.46.

Anti-HBV Assay

The tetracycline-responsive cell lines HepAD38 was used. These are hepatoma cells that have been stably transfected with a cDNA copy of the

pregenomic RNA of wild-type virus. Withdrawal of tetracycline from the culture medium results in the initiation of viral replication. Cells were cultured at 37°C in a humidified 5% CO_2/air atmosphere in seeding medium, DMEM/Ham's F12 (50/50) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin/50 μg/ml streptomycin mix, 400 μg/ml G418, and 0.3 μg/ml tetracycline. When the assay was started, the cells were seeded in 48-well plates at a density of 1.5×10^5/well. After 2–3 days the cultures were induced for viral production by washing with pre-warmed PBS and were fed with 200-μl assay medium (seeding medium without tetracycline and G418) with or without the antiviral compounds. The medium was changed after 3 days. The antiviral effect was quantified by measuring levels of viral DNA [isolated (Qiagen) from the cell cultures] at day 4 post-induction, by a real-time quantitative PCR (Q-PCR). The Q-PCR was performed in a reaction volume of 25 μl using the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ) with forward primer (5′-CCG TCT GTG CCT TCT CAT CTG-3′; final concentration: 600 nM), reversed primer (5′-AGT CCA AGA CTY CTC TTA TRY AAG ACC TT-3′; final concentration 600 nM), and Taqman probe (6-FAM-CCG TGT GCA CTT CGC TTC ACC TCT GC-TAMRA; final concentration 150 nM). The reaction was analyzed using a SDS 7000 (Applied Biosystems, Foster City, CA). A plasmid containing the full-length insert of the HBV genome was used to prepare the standard curve. The amount of viral DNA produced in treated cultures was expressed as a percentage of the mock treated samples. The cytostatic effect of the various compounds was assessed employing the parent hepatoma cell line HepG2. The effect of the compounds on exponentially growing HepG2 cells was evaluated by means of the MTS method (Promega, Leiden, The Netherlands). Briefly, cells were seeded at a density of 3000/well (96 well plate) and were allowed to proliferate for 3 days in the absence or presence of compounds after which time cell density was determined.

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