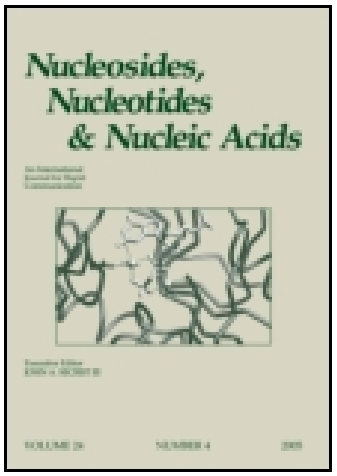

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Design, Synthesis, and Evaluation of Anti-HBV Activity of Hybrid Molecules of Entecavir and Adefovir: Exomethylene Acycloguanine Nucleosides and Their Monophosphate Derivatives

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DESIGN, SYNTHESIS, AND EVALUATION OF ANTI-HBV ACTIVITY OF HYBRID MOLECULES OF ENTECAVIR AND ADEFOVIR: EXOMETHYLENE ACYCLOGUANINE NUCLEOSIDES AND THEIR MONOPHOSPHATE DERIVATIVES

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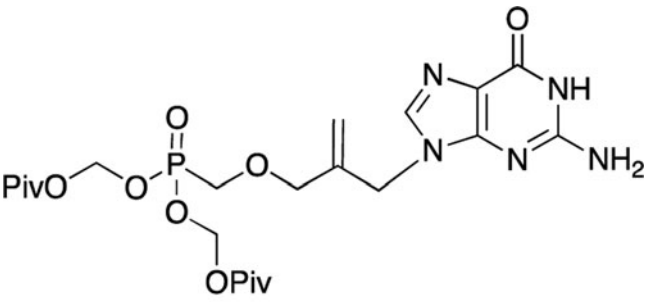

Piv-P-MEP-G

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*Exomethylene acycloguanine nucleosides **4**, **6** and its monophosphate derivatives **5**, **7**, and **8** have been synthesized. Mitsunobu-type coupling of 2-N-acetyl-6-O-diphenylcarbamoylguanine (**11**) with primary alcohols proceeded regioselectively to furnish the desired N^9-substituted products in moderate yield. Evaluation of **4-8** for anti-HBV activity in HepG2 cells revealed that the phosphonate derivative **8** was found to exhibit moderated activity (EC_{50} value of 0.29 μM), but cytotoxicity (CC_{50} value of 39 μM) against the host cells was also observed.*

Keywords Nucleoside; acyclonucleoside; nucleoside phosphonate; pro-drug; hybrid; entecavir; adefovir; anti-HBV activity

INTRODUCTION

Hepatitis B is one of the most prevalent viral diseases in the world and is known to be a major cause of chronic disease, leading to cirrhosis/hepatocellular carcinoma.[1] Among the most frequently used drugs for treatment of the disease[2] are the nucleoside analogue entecavir (**1**)[3a,b] and the nucleotide analogue adefovir (2)[4] (Figure 1). Entecavir **1** is especially considered as one of the best choices for chronic patients due to its lack of significant adverse effects.[5] Entecavir is structurally a carbocyclic analogue of 2′-deoxyguanosine. The exomethylene functionality at the 5′-position of **1** would appear to be an important pharmacophore for significant antiviral activity because the potency of carbocyclic dG that truncates the double bond is 10 times less than that of **1**.[3a] Meanwhile, adefovir **2** is the phosphonate analogue of monophosphate of acycloadenine nucleoside. The feature of this class of nucleotide analogues is that the requisite first phosphorylation, which is a crucial step for the activation of biologically active nucleoside derivatives, has been bypassed.

Further studies on the structure–activity relationship of these classes of nucleosides should increase our knowledge of structural requirements for developing novel antiviral agents for HBV, and will aid in the search for better anti-HBV agents. In this context, we have envisioned combining the above two structural features in one molecule and designed the exomethylene acyclic guanine nucleosides and its monophosphate derivatives as shown in Figure 2. The initial target molecules are exomethylene

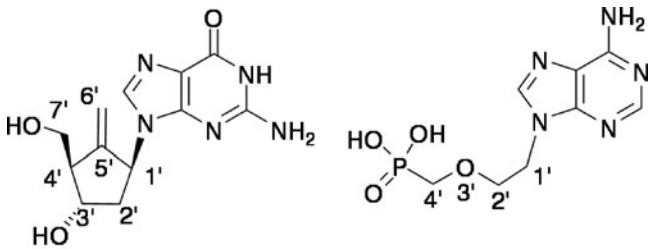


FIGURE 1 Structure of entecavir (**1**) and adefovir (**2**).

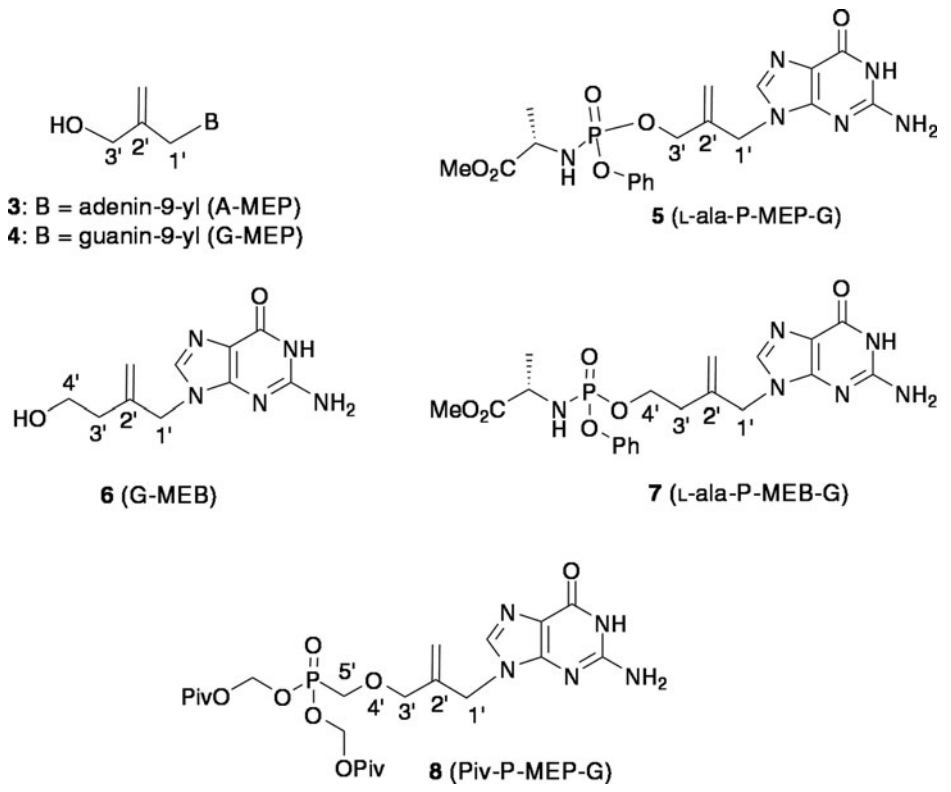


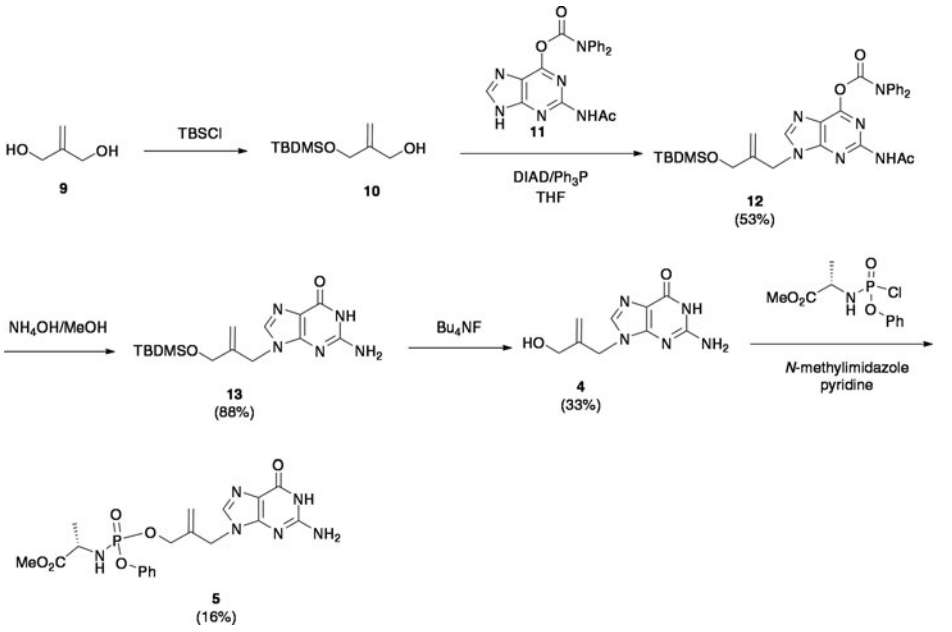
FIGURE 2 Structure of A-MEP (**3**) and the target molecules (**4–8**).

propyl-(**4**, MEP-G) and butyl-(**6**, MEB-G) guanine nucleosides. The number of constituted atoms (1′ to 4′-position) in the acyclic side chain of MEB-G **6** corresponds to the structure comprising C1′, C5′, C4′, and C7′ in entecavir **1,** whereas MEP-G truncates one-carbon atom in acyclic moiety. L-ala-P-MEP-G **5** and L-ala-P-MEB-G **7** are the respective phosphoalaninate pro-drugs of the monophophates of **4** and **6**. Moreover, the phosphonate analogue Piv-P-MEP-G **8** of **5** was also designed. The phosphonate **8** has a one-carbon elongated side chain (C1′ to C5′) compared with that of adefovir **2**. Herein, we describe the results of the synthesis of **4-8** and evaluation of their anti-HBV activity.

RESULTS AND DISCUSSION

Chemistry

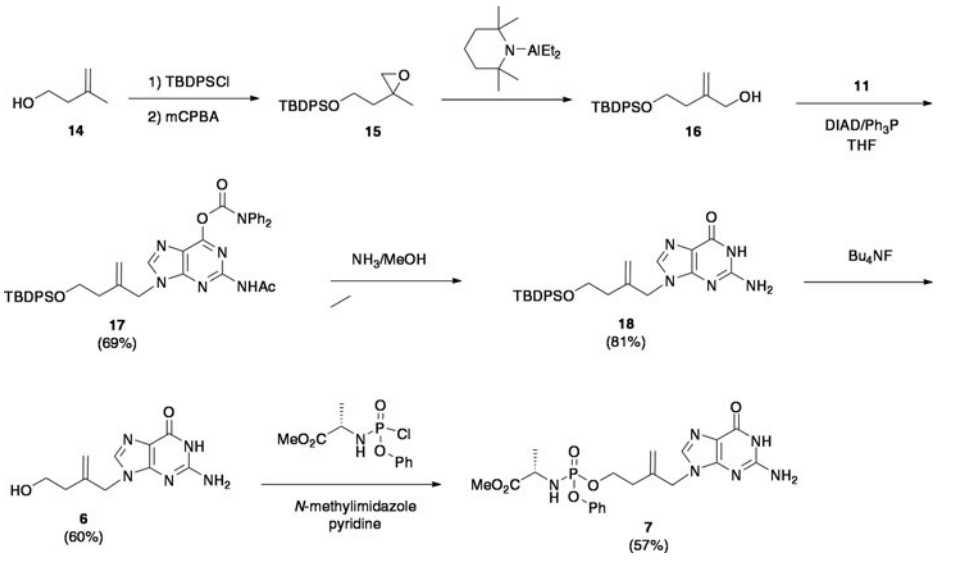
Initially, synthesis of G-MEP (**4**) was carried out (Scheme 1). Synthesis of the adenine counterpart **3** (A-MEP) of the target molecule **4** has been reported.[6] Therefore, according to the procedure given in literature,



SCHEME 1 Synthesis of G-MEP (**4**) and its monophosphate pro-drug L-ala-P-MEP-G (**5**).

2-methylenepropane-1,3-diol (**9**) was utilized as a starting material. Compound **9** was converted into 2-*O*-(*tert*-butyldimethylsilyloxymethyl)prop-2-en-1-ol (**10**). The literature procedure for the coupling of adenine with acyclic moiety involved the mesylation of **10** followed by nucleophilic substitution of respective mesylate with nucleobase under basic reaction conditions. To reduce synthetic steps to the target G-MEP **4**, Mitsunobu-type reaction of **10** with 2-*N*-acetyl-6-*O*-diphenylcarbamoylguanine (**11**)[7] was examined. Thus, when **10** was reacted with **11** in the presence of DIAD/Ph_3P in THF at 70°C, the desired protected acyclopurine nucleoside **12** could be obtained in 53% yield. Removal of protecting groups in the base moiety was carried out by the treatment of **12** with ammonium hydroxide in methanol to give guanine derivative **13** in 88% isolated yield. In the Heteronuclear Multiple-Bond Correlation (HMBC) spectra of **13**, the correlation between C$\underline{H_2}$-1′/$\underline{C}$-4 and C$\underline{H_2}$-1′/$\underline{C}$-8 was observed, by which **13** was assigned as N^9-isomer. Compound **13** was converted to MEP-G **4** in 33% yield by treating with Bu_4NF. Finally, **4** was transformed into the phosphoalaninate pro-drug **5** (16%) by reaction with methyl chlorophenylphosphoryl $P\rightarrow N$-L-alaninate and *N*-mehtylimidazole in pyridine.[8]

Next, synthesis of G-MEB (**6**) was performed (Scheme 2). Initially, 4-(*tert*-butyldiphenylsilyloxy)-2-methylenebutan-1-ol (**16**) was prepared from **14** in the following three steps: (1) silylation of **14**, (2) epoxidation of the resulting silylated alkene, (3) β-elimination of the obtained epoxide **15** with diethylaluminium 2,2,6,6-tetramethylpiperidide.[9] When **16** was reacted with **11**



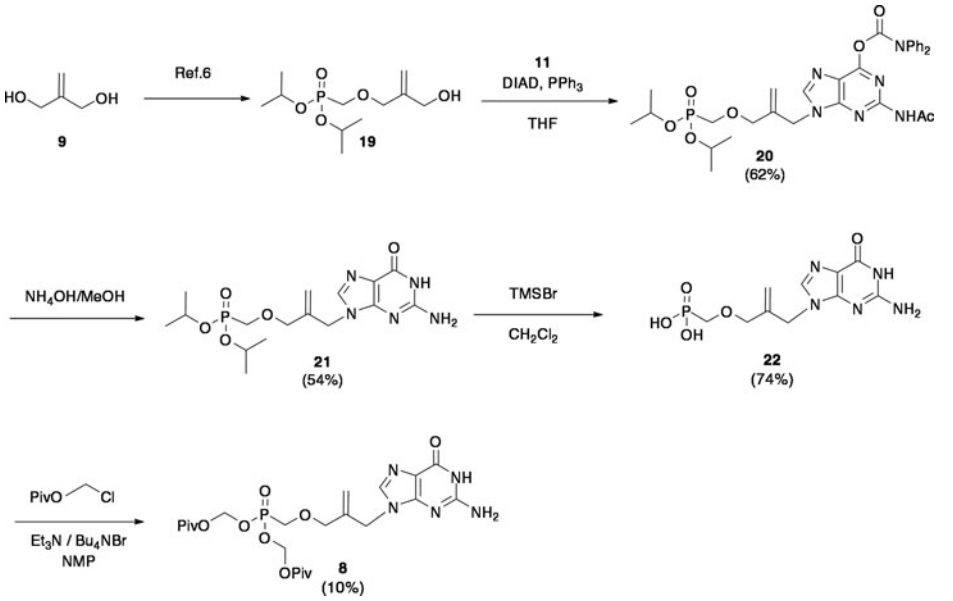
SCHEME 2 Synthesis of G-MEB (**6**) and $_{L}$-ala-P-MEB-G (**7**).

under the above-mentioned reaction conditions, the desired N^9-substituted **17** was obtained in 69% isolated yield as a single regio-isomer. Compound **17** was converted into **18** in 81% yield by ammonolysis in methanol, and its HMBC spectra revealed the correlation between C$\underline{H_2}$-1′/$\underline{C}$-4 and C$\underline{H_2}$-1′/$\underline{C}$-8. Desilylation of **18** gave G-MEB (**6**) in 60% yield. As described above for **4**, MEB-G **6** was transformed into phosphoralaninate pro-drug **7** in 57% yield.

Finally, synthesis of the phosphonate analogue **8** of $_{L}$-ala-P-MEB-G **7** was accomplished as illustrated in Scheme 3. Phosphonate alcohol **19** was prepared from **9** according to the published procedure.[6] Reaction of the alcohol **19** with **11** under the identical conditions for the synthesis of **17** gave the desired **20** in 62% isolated yield. Treatment of **20** with aqueous ammonia in methanol provided acycloguanine phosphonate derivative **21** in 54% yield, and at this stage, the regiochemistry was confirmed on the basis of HMBC spectrum, in which the same correlations as for **4** and **6** were observed. The phosphonate **21** was transformed into phosphonic acid (**22**) in 74% yield by treatment with TMSBr in CH_2Cl_2, and the resulting phosphonic acid **22** was converted into its pivaloyloxymethyl (POM) ester (**8**; Piv-P-MEP-G) in 10% yield.[10]

Biological Evaluation

Evaluation of anti-HBV activity of the novel acyclonucleosides synthesized in this study was conducted with HepG2 2.2.15 cells transfected with the HBV genome.[11] As shown in Table 1, G-MEP **4** and G-MEB **6** neither



SCHEME 3 Synthesis of Piv-P-MEP-G (**8**).

exhibit anti-HBV activity nor display any cytotoxicity toward HepG2 cells (entries 1 and 2). These results revealed that the conformationally rigid cyclopentane ring of entecavir is an essential structure for antiviral activity. Moreover, inactivity of phosphoalaninates **5** and **7** suggested that the initial phosphorylation step of **4** and **6** is not responsible for the lack of anti-HBV activity.

In contrast with the above results, the POM-protected phosphonate derivative **8** was found to possess antiviral activity with an EC_{50} of 0.29 μM, which is 1500 times less potent than that of entecavir. However, cytotoxicity toward HepG2 cells was also observed. With POM-protected prodrugs, it has been pointed out that the toxicity could be attributed to pivalic acid generated during the release of parent compounds.[10b] Therefore, we have evaluated the toxicity of pivalic acid in this assay system and found that the

TABLE 1 Anti-HBV activity and cytotoxicity of **4-8**

Compound	EC_{50} (μM)	CC_{50} (μM) (MT-4)
4	>1	>100
5	>1	>100
6	>1	>100
7	>1	>100
8	0.29	39
Entecavir	0.00077	ND[a]

[a]ND: not determined.

acid did not show any cytotoxicity up to 100 μM, which most likely ruled out this issue.

CONCLUSIONS

We have synthesized the novel exomethylene acycloguanine nucleosides MEP-G (**4**) and MEB-G (**6**). The respective monophosphate derivatives, that is, phosphoalaninate pro-drugs L-ala-P-MEP-**5** and L-ala-P-MEB-G **7** of the monophosphate of **4** and **6** were also synthesized. Furthermore, the phosphonate analog Piv-P-MEP-G (**8**) of **7** was also evaluated for anti-HBV activity. Mitsunobu-type alkylation of 2-*N*-acetyl-6-*O*-diphenylcarbamoylguanine (**11**) with respective primary alcohols proceeded regioselectively at its N^9-position and this synthetic strategy enabled us to shorten the synthetic route for the target molecules compared with the previously reported method. Evaluation of anti-HBV activity and its cytotoxicity toward HepG2 cells revealed that the rigidity of aglycon moiety is an important requirement for the inhibition of HBV replication. The phosphonate derivative **8** showed moderate activity with an EC_{50} of 0.29 μM, and the selectivity index (SI) was 137, although some cytotoxicity (CC_{50} of 39 μM) was observed.

EXPERIMENTAL

General

Melting points are uncorrected. Reagents and solvents were used without any further purification unless otherwise noted. Thin layer chromatography (TLC) was performed using precoated TLC plates (Merck, Silica gel 60 F254, 0.25 mm). ^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA 500 spectrometer operating at room temperature. Chemical shifts are reported in parts per million (δ) relative to the residual solvent peak. DSS served as internal standard for ^{13}C NMR measurements in D_2O. Mass spectra analysis was performed on a JEOL JMS-T100LP.

2-Acetyl -9-[2-(*tert*-butyldimethylsilyloxymethyl)allyl]-6-*O*-diphenylcarbamoyl (12)

To a solution of **10** (989 mg, 5.68 mmol), triphenylphosphine (1.49 g, 5.68 mmol) and **11** (2.10 g, 5.41 mmol) in THF (50 mL) was added DIAD (1.15 g, 5.68 mmol). The reaction mixture was heated to 70°C and stirred for 2 h. After cooling, the mixture was filtered through a celite pad. The filtrate was evaporated, and the residue was purified by silica gel column chromatography (Hexane-EtOAc 3:2 to 2:3, v/v) to provide **12** (1.71 g, 2.99 mmol, 53% yield) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (brs, 1H), 7.95 (s, 1H), 7.43-7.25 (m, 10H), 5.23 (s, 1H), 4.96 (s, 1H), 4.80 (s, 2H), 4.10 (s,

2H), 2.56 (s, 3H), 0.89 (s, 9H), 0.05 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.08, 156.09, 155.13, 152.13, 150.36, 144.18, 142.43, 141.69, 129.15, 127.08, 125.86, 120.52, 114.27, 64.08, 45.50, 25.77, 25.13, 18.22, −5.46. MS (ESI) m/z $(M+Na)^+$ calcd. 595.2465, found 595.2485.

9-[2-(*tert*-Butyldimethylsilyloxymethyl)allyl]guanine (13)

To a solution of **12** (868 mg, 1.51 mmol) in methanol (6 mL) was added 28% ammonia solution (3 mL) and heated to 60°C in a sealed tube. After 2 h, the reaction mixture was concentrated and purified by silica gel column chromatography (CH_2Cl_2-MeOH 92:8 to 84:16, v/v) to provide **13** (446 mg, 1.33 mmol, 88% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.58 (brs, 1H), 7.60 (s, 1H), 6.42 (brs, 2H), 5.12 (s, 1H), 4.65 (s, 1H), 4.54 (s, 2H), 4.11 (s, 2H), 0.86 (s, 9H), 0.03 (s, 6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.05, 153.80, 151.52, 144.56, 137.67, 116.57, 110.84, 63.69, 44.20, 25.96, 18.15, –5.31; MS (ESI) m/z $(M+Na)^+$ calcd. 358.1675, found 358.1714.

9-[(Hydroxymethyl)allyl]guanine (4)

To a suspension of **13** (421 mg, 1.26 mmol) in THF (15 mL) was added 1 M TBAF in THF solution (3.14 mL, 3.14 mmol). After 2 h, the reaction mixture was concentrated. The residue was recrystalized from MeOH to provide **3** (91 mg, 0.41 mmol, 33% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.57 (brs, 1H), 7.63 (s, 1H), 6.45 (brs, 2H), 5.07 (s, 1H), 5.05 (t, $J = 5.4$ Hz, 1H), 4.56 (s, 3H), 3.91 (d, $J = 5.7$ Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.01, 153.78, 151.51, 145.86, 137.82, 116.53, 110.25, 62.19, 44.44; MS (ESI) m/z $(M+Na)^+$ calcd. 244.0810, found 244.0827.

Synthesis of $_L$-ala-P-MEP-G (5)

To a solution of **4** (100 mg, 0.452 mmol) in pyridine (15 mL) was added a solution of methyl chlorophenylphosphoryl $P\rightarrow N$-$_L$-alaninate in THF (0.2 M, 11.3 mL, 2.26 mmol). After addition of *N*-methylimidazole (0.36 mL, 4.52 mmol), the mixture was stirred for 16 h. The solvents were evaporated and the residue was re-dissolved into AcOH (6 mL) and stirred at room temperature overnight. The solution was evaporated, and the residue was purified by silica gel column chromatography (EtOAc-MeOH 9:1 to 7:3, v/v) to provide **5** (33 mg, 0.072 mmol, 16%). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.62 (brs, 1H), 7.63 and 7.61 (2s, 1H), 7.41-7.35 (m, 2H), 7.22-7.16 (m, 3H), 6.48 (brs, 2H), 6.16-6.05 (m, 1H), 5.22 and 5.18 (2s, 1H), 4.70 (s, 1H), 4.61 and 4.58 (2s, 2H), 4.54 (d, $J = 6.3$ Hz, 2H) and 4.49 (*d*, J = 5.7 Hz, 2H), 3.91-3.81 (m, 1H), 3.61 and 3.59 (2s, 3H), 1.26-1.19 (m, 3H); ^{13}C NMR (DMSO-d_6, 126 MHz) δ 173.98 and 173.90 (2d, $J = 4.8$ Hz), 157.06, 153.91, 151.56, 150.86 and 150.83, 140.63 and 140.53, 137.76, 129.91, 124.90, 120.50

and 120.47, 116.61, 114.00 and 113.74, 66.56 and 66.40 (2d, $J = 4.8$ Hz), 52.16, 50.03 and 49.90, 44.12 and 44.07, 19.87; MS (ESI) m/z $(M+H)^+$ calcd. 463.1495; found 463.1540.

2-Acetylamino-9-[4-(*tert*-butyldiphenylsilyloxy)-2-methylenebutyl]-9*H*-purin-6-yl diphenylcarbamate (17)

To a solution of **16** (1.50 g, 4.40 mmol), triphenylphosphine (1.38 g, 5.28 mmol) and **11** (2.05 g, 5.28 mmol) in THF (35 mL) was added DIAD (1.07 g, 5.28 mmol) in THF (7 mL). The reaction mixture was heated to 70°C and stirred for 2 h. After cooling, the mixture was filtered through a celite pad. The filtrate was evaporated, and the residue was purified by silica gel column chromatography (Hexane-EtOAc 6:4 to 3:7, v/v) to provide compound **17** (2.17 g, 3.05 mmol, 69% yield) as a white foam. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.92 (brs, 1H), 7.82 (s, 1H), 7.65-7.62 (m, 4H), 7.47-7.34 (m, 16H), 5.04 (s, 1H), 4.84 (s, 1H), 4.70 (s, 2H), 3.80 (t, $J = 6.3$ Hz, 2H), 2.50 (s, 3H), 2.24 (t, $J = 6.3$ Hz, 2H), 1.05 (s, 9H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 171.11, 156.12, 155.10, 152.16, 150.38, 143.98, 141.72, 140.85, 135.49, 133.31, 129.82, 129.17, 127.73, 125.92, 120.48, 115.42, 62.58, 48.48, 36.48, 26.82, 25.12, 19.14; MS (ESI) m/z $(M+Na)^+$ calcd. 733.2935; found 733.2924.

2-Amino-9-[4-((*tert*-butyldiphenylsilyloxy)-2-methylenebutyl)-1*H*-purin-6(9*H*)-one (18)

A solution of compound **17** (1.00 g, 1.41 mmol) in 2 M NH_3 (MeOH sol) (8 mL) was stirred at 70°C for 2 h. The solution was evaporated, and the residue was purified by silica gel column chromatography (CH_2Cl_2-MeOH 97:3 to 94:6, v/v) to provide **18** (540 mg, 1.14 mmol, 81% yield) as a white foam. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.56 (brs, 1H), 7.64-7.58 (m, 4H), 7.54 (s, 1H), 7.50-7.41 (m, 6H), 6.39 (brs, 2H), 4.90 (s, 1H), 4.60 (s, 1H), 4.50 (s, 2H), 3.74 (t, $J = 6.7$ Hz, 2H), 2.26 (t, $J = 6.7$ Hz, 2H), 0.98 (s, 9H); ^{13}C NMR (DMSO-d_6, 126 MHz) δ 157.01, 153.78, 151.46, 142.15, 137.58, 135.22, 133.25, 130.08, 128.12, 116.56, 113.03, 63.23, 47.08, 36.51, 26.84, 18.94; MS (ESI) m/z $(M+Na)^+$ calcd. 496.2145; found 496.2187.

2-Amino-9-(4-hydroxy-2-methylenebutyl)-1*H*-purin-6(9*H*)-one (6)

To a solution of **18** (437 mg, 0.923 mmol) in THF (35 mL) was added 1 M TBAF in THF (1.11 mL, 1.11 mmol). The mixture was stirred at room temperature for 1 h. The solvent was removed, and the residue was purified by silica gel column chromatography (CH_2Cl_2-MeOH 9:1 to 2:8, v/v) to provide **6** (130 mg, 0.55 mmol, 60% yield) as a white foam. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.58 (brs, 1H), 7.61 (s, 1H), 6.45 (brs, 2H), 4.88 (s, 1H),

4.60 (t, $J = 5.2$ Hz, 1H), 4.53 (s, 2H), 4.50 (s, 1H), 3.52 (dt, $J = 6.8$, 5.2 Hz, 2H), 2.14 (t, $J = 6.8$ Hz, 2H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 157.08, 153.82, 151.54, 143.07, 137.85, 116.51, 111.97, 59.64, 47.15, 36.99; MS (ESI) m/z $(M+Na)^+$ calcd. 258.0967; found 258.0966.

Synthesis of L-ala-P-MEB-G (7)

To a solution of **6** (50 mg, 0.213 mmol) in pyridine (7.5 mL) was added a solution of methyl chlorophenylphosphoryl $P\rightarrow N$-L-alaninate in THF (0.2 M, 5.3 mL, 1.06 mmol). After addition of *N*-methylimidazole (0.17 mL, 2.13 mmol), the mixture was stirred for 16 h. The solvents were evaporated and the residue was re-dissolved into AcOH (6 mL) and stirred at room temperature overnight. The solution was evaporated, and the residue was purified by silica gel column chromatography (CH_2Cl_2-MeOH 97:3 to 91:9, v/v) to provide **7** (58 mg, 0.12 mmol, 57%).^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.59 (brs, 1H), 7.61 and 7.59 (2s, 1H), 7.44-7.28 (m, 2H), 7.25-7.08 (m, 3H), 6.45 (s, 2H), 6.09-5.91 (m, 1H), 4.95 and 4.93 (2s, 1H), 4.58 and 4.57 (2s, 1H), 4.55 and 4.53 (2s, 2H), 4.21-4.07 (m, 2H), 3.89-3.79 (m, 1H), 3.59 and 3.58 (2s, 3H), 2.39-2.30 (m, 2H), 1.26-1.18 (m, 3H); ^{13}C NMR (DMSO-d_6, 126 MHz) δ 174.02 and 173.90 (2d, $J = 4.8$ Hz), 157.06, 153.85, 151.55, 150.94, 141.38 and 141.30, 137.77, 129.82, 124.74, 120.41, 116.54, 113.07 and 113.01, 64.29 and 64.23 (2d, $J = 4.8$ Hz), 52.10, 50.04 and 49.87, 46.82 and 46.80, 34.02 and 33.98, 19.88; MS (ESI) m/z $(M+H)^+$ calcd. 477.1651; found 477.1700.

2-Acetylamino-9-[(diisopropoxyphosphorylmethoxy)allyl]-6-diphenylcarbamoylpurine (20)

To a solution of (2-hydroxymethylallyloxymethyl)phosphonic acid diisopropyl ester **19** (2.13 g, 8.00 mmol), triphenylphosphine (2.73 g, 10.4 mmol) and **11** (3.73 g, 9.60 mmol) in THF (40 mL) was added DIAD (1.94 g, 9.60 mmol). The reaction mixture was heated to 70°C and stirred for 2 h. After cooling, the mixture was filtered through a celite pad. The filtrate was evaporated, and the residue was purified by silica gel column chromatography (EtOAc-MeOH 100:0 to 92:8, v/v) to provide **20** (3.14 g, 4.93 mmol, 62% yield) as a pale yellow foam. ^{1}H NMR (500 MHz, CD_3OD) δ 8.31 (s, 1H), 7.49-7.27 (m, 10H), 5.31 (s, 1H), 5.10 (s, 1H), 4.97 (s, 2H), 4.79-4.65 (m, 2H), 4.10 (s, 2H), 3.74 (d, $J = 8.6$ Hz, 2H), 2.31 (s, 3H), 1.36 (dd, $J = 8.0$, 6.3 Hz, 12H); ^{13}C NMR (126 MHz, CD_3OD) δ 172.63, 157.21, 156.76, 154.14, 152.58, 147.49, 143.55, 141.76, 130.61, 128.61, 121.78, 117.98, 72.24 (d, $J = 12.0$ Hz), 73.46 (d, $J = 6.0$ Hz), 65.53 (d, $J = 167.9$ Hz), 47.06, 25.06, 24.62 (d, $J = 3.6$ Hz), 24.58 (d, $J = 4.8$ Hz); MS (ESI) m/z $(M+H)^+$ calcd. 637.2540, found 637.2569.

9-[(Diisopropoxyphosphorylmethoxy)allyl]guanine (21)

Compound **20** (361 mg, 0.567 mmol) was dissolved in ca. 9 M ammonia in methanol (5 mL) and heated to 70°C in a sealed tube. After 2 h, the reaction mixture was concentrated and purified by silica gel column chromatography (CH_2Cl_2-MeOH 95:5 to 88:12, v/v) to provide **21** (123 mg, 54% yield) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.81 (brs, 1H), 7.60 (s, 1H), 7.00 (brs, 2H), 5.22 (s, 1H), 4.86-4.79 (m, 3H), 4.62 (s, 2H), 4.12 (s, 2H), 3.78 (d, J = 9.7 Hz, 2H), 1.36 (dd, J = 8.0 and 6.3 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.40, 154.49, 151.16, 139.67, 137.52, 117.12, 116.53, 74.41 (d, J = 14.4 Hz), 71.85 (d, J = 6.0 Hz), 64.78 (d, J = 171.5 Hz), 44.69, 24.07 (d, J = 3.6 Hz), 24.03(d. J = 4.8 Hz); MS (ESI) m/z $(M+Na)^+$ calcd. 422.1569, found 422.1581.

9-[2-(Hydroxymethyl)allyloxymethylphosphonic acid]guanine (22)

To a solution of **21** (475 mg, 1.19 mmol) in CH_2Cl_2 (10 mL) was added TMSBr (910 mg, 5.95 mmol). The reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with CH_2Cl_2 and extracted with water. The aqueous layer was lyophilized and the residue was purified by ODS column chromatography (H_2O-MeOH 95:5 to 7:3, v/v) to provide **22** (278 mg, 74% yield) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 8.72 (s, 1H), 5.38 (s, 1H), 5.11 (s, 1H), 4.88 (s, 2H), 4.10 (s, 2H), 3.58 (d, J = 9.2 Hz, 2H); ^{13}C NMR (126 MHz, D_2O) δ 158.52, 157.93, 152.92, 141.08, 140.82, 121.33, 111.85, 76.00 (d, J = 13.2 Hz), 68.34 (d, J = 158.4 Hz), 49.37; MS (ESI) m/z $(M-H)^-$ calcd. 314.0654 found 314.0690.

9-[Bis-*O*-(pivaloyoxymethyl)phosphorylmethoxy]allyl]guanine (8)

To a solution of **22** (101 mg, 0.320 mmol), triethylamine (179 μL, 1.28 mmol) and tetrabutylammonium bromide (103 mg, 0.320 mmol) in *N*-methylpyrrolidone (3 mL) was added chloromethyl pivalate (241 mg, 1.60 mmol). The reaction mixture was heated to 50°C and stirred for 20 h. The reaction was quenched with MeOH and directly applied to silica gel column chromatography (CH_2Cl_2-MeOH 98:2 to 9:1, v/v). Appropriate fractions were evaporated, triturated from acetonitrile/water = 1/1 to provide **8** (17.0 mg, 10% yield) as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ 7.72 (s, 1H), 5.74-5.69 (m, 4H), 5.26 (s, 1H), 4.94 (s, 1H), 4.71 (s, 2H), 4.11 (s, 2H), 3.93 (d, J = 8.6 Hz, 2H), 1.22 (s, 18H); ^{13}C NMR (126 MHz, CD_3OD) δ 178.42, 159.69, 155.64, 153.53, 142.19, 140.25, 117.75, 117.37, 83.52 (d, J = 6.0 Hz), 75.22 (d, J = 14.4 Hz), 64.81 (d, J = 166.74 Hz), 46.04, 40.03, 27.51; MS (ESI) m/z $(M+Na)^+$ calcd. 566.1992 found 566.1963.

Cell Culture, Anti-HBV Assay, and Cytotoxicity Assay

HepG2 2.2.15 human hepatoblastoma cell line, which can stably produce HBV particles,[11] was kindly gifted from B. Korba of Georgetown University. The HepG2 2.2.15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) and MT-4 cells were grown in RPMI 1640-based culture medium, which supplemented with 10% (v/v) fetal calf serum (FCS; Gibco, USA) and 50 U/mL penicillin and 50 μg/mL streptomycin.

The HepG2 2.2.15 cells were plated at 1×10^5/mL in the presence or absence of various concentrations of a test compound in 96-well microtiter culture plates, then followed by incubation at 37°C for six days. After incubation, the concentration of HBV DNA in the supernatant was determined.

Cytotoxicity of a compound in MT-4 cells was also determined. Cells were plated in a 96-well plate at a density of 1×10^5/mL and cultured in the absence or the presence of various concentrations of a compound at 37°C for seven days. After 100 mL of the medium was removed from each well, 10 mL of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Nacalai Tesque, Kyoto, Japan) was added to each well in the plate, followed by incubation at 37°C for 2 h. After incubation to dissolve formazan crystals, 100 mL of acidified isopropanol containing 4% (v/v) Triton X-100 was added to each well, and the optical density was measured in a kinetic microplate reader (Vmax; Molecular Devices, Sunnyvale, CA).

Detection of HBV DNA by Real-Time Quantitative Polymerase Chain Reaction

Viral DNA in the supernatants was extracted using QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA) according to the manufacture's instruction. HBV DNA was quantified by real-time PCR relative to an external plasmid DNA standard on a Light Cycler instrument using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche, Mannheim, Germany) and primers HBV-RT-F (5′-GAGTCTAGACTCGTGGTGGA-3′) and HBV-RT-R (5′ -TGAGGCATAGCAGCAGGATG- 3′), which amplified a 184-bp fragment in the RT region of the HBV genome. The PCR conditions used were an initial 3 min at 95°C, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s.

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