

## Synthesis of nucleotide analogues, EFdA, EdA and EdAP, and the effect of EdAP on hepatitis B virus replication

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### ABSTRACT

4'-Ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) and 4'-ethynyl-2'-deoxyadenosine (EdA) are nucleoside analogues which inhibit human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. EdAP, a cyclosaligenyl (cycloSal) phosphate derivative of EdA, inhibits the replication of the influenza A virus. The common structural feature of these compounds is the ethynyl group at the 4'-position. In this study, these nucleoside analogues were prepared by a common synthetic strategy starting from the known 1,2-di-*O*-acetyl-D-ribofuranose. Biological evaluation of EdAP revealed that this compound reduced hepatitis B virus (HBV) replication dose-dependently without cytotoxicity against host cells tested in this study.

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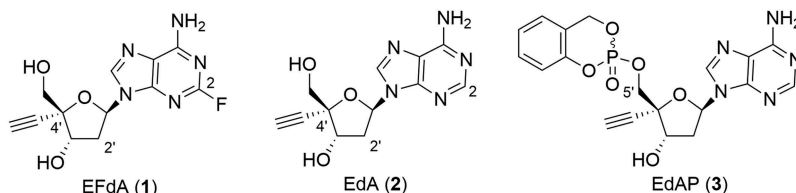
### KEYWORDS

Nucleotide analogue; EdAP; synthesis; hepatitis B virus

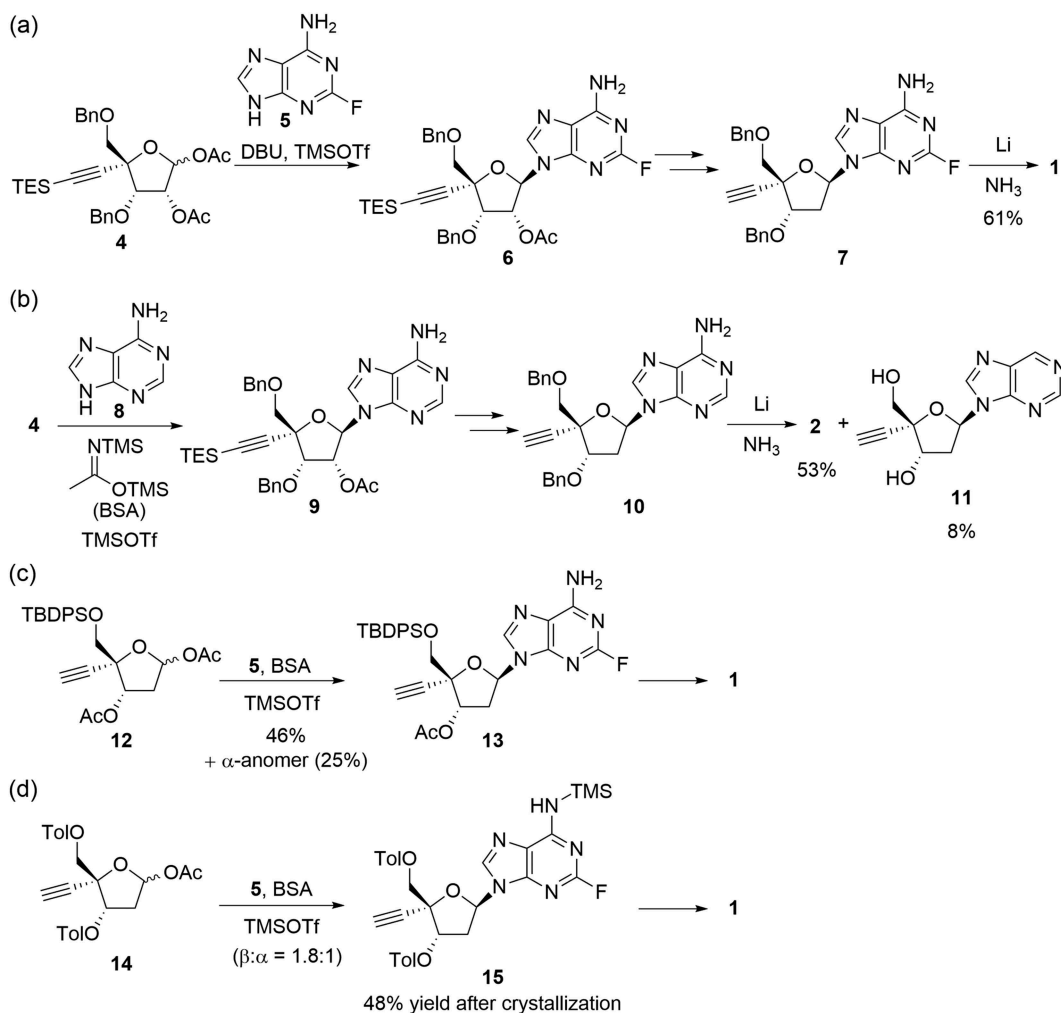
4'-Ethynyl-2-fluoro-2'-deoxyadenosine (EFdA, **1**) inhibits human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (Figure 1) [1–9]. This compound inhibits the replication of a wide spectrum of HIV-1 strains resistant to nucleoside reverse transcriptase inhibitors (NRTIs) [10–14]. Furthermore, EFdA has a long half-life *in vivo* and is currently in human clinical trials [15–20]. 4'-Ethynyl-2'-deoxyadenosine (EdA, **2**) also inhibits HIV-1 reverse transcriptase [9,21]. However, this compound is susceptible to degradation by adenosine deaminase (ADA) [22–24]. To overcome the sensitivity against ADA, the fluorine atom was introduced at the 2-position in **2** to generate **1** [3,4,7,20,22]. The susceptibility of **1** to ADA was drastically decreased compared to that of **2**. EdAP (**3**) is a cyclosaligenyl (cycloSal) phosphate derivative of EdA [25,26]. EdAP inhibits the replication of the influenza virus A both *in vivo* and *in vitro*. The cycloSal moiety generally serves as a lipophilic masking unit to increase membrane-permeability of the nucleoside or nucleoside 5'-monophosphate [27–29]. The cycloSal moiety is hydrolyzed in cells to release the nucleoside 5'-monophosphate.

EFdA (**1**) was designed and first synthesized beginning with 2-amino-2'-deoxyadenosine via 4'-ethynyl-2-amino-2'-deoxyadenosine by Ohru [1,2]. Other previous syntheses of **1** and **2** included the *N*-glycosylation between an electrophilic sugar and

nucleophilic base (Scheme 1). Kuwahara and Ohru synthesized **1** through a direct *N*-glycosylation between **4** and 2-fluoro-adenine (**5**) (Scheme 1(a)) [30]. Ohru also used **4** as the glycosyl donor for the synthesis of **2** (Scheme 1(b)) [21]. The silyl-Hilbert-Johnson reaction [31,32] between **4** and adenine (**8**) afforded **9**. The *N*-glycosylation reactions between **4** and the bases proceeded selectively. The high  $\beta$ -selectivity is explained by the neighboring group effect of the acetyl group at the C-2 position [31]. However, the syntheses of **1** and **2** included the deprotection of the benzyl groups using hazardous ammonia and highly reactive lithium metal. In the synthesis of **2**, the deprotection of the benzyl groups in **10** under the Birch reduction conditions gave **1**, together with the deaminated product **11** as a byproduct [21]. The use of 2-deoxyribose derivatives as glycosyl donors decreased the diastereoselectivity of the *N*-glycosylations due to the lack of neighboring group participation at the C-2 position (Scheme 1(c,d)). Kuwahara and Ohru reported that the silyl-Hilbert-Johnson reaction between 2-deoxyribose derivative **12** and **5** gave the desired  $\beta$ -anomer **13** and undesired  $\alpha$ -anomer in 46% and 25% yields, respectively (Scheme 1(c)) [33,34]. After separation of **12** by silica gel chromatography, this compound was converted into **1**. MacLaughlin used 3,5-di-*O*-(*p*-toluoyl)-2-deoxyribose derivative **14** as the glycosyl donor [35]. The coupling between **14** and **5** gave a 1.8:1 mixture of  $\beta$ - and  $\alpha$ -anomers



**Figure 1.** Structures of EFdA (**1**), EdA (**2**), and EdAP (**3**).



**Scheme 1.** Previous syntheses of EFdA (**1**) and EdA (**2**). (a) synthesis of **1** reported by Kuwahara and Ohruï [30]. (b) synthesis of **2** reported by Ohruï [21]. (c) alternative synthesis of **1** reported by Kuwahara and Ohruï [33,34]. (d) synthesis of **1** reported by MacLaughlin [35].

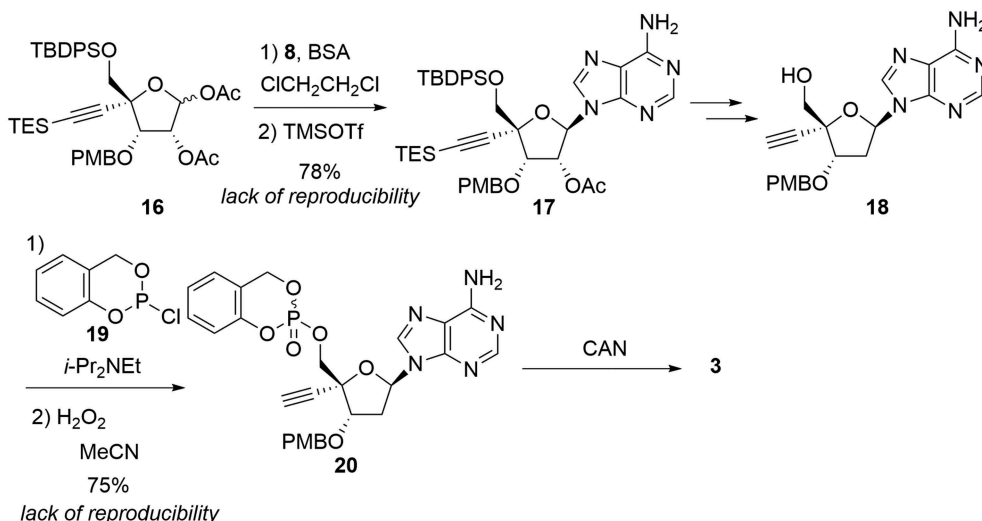
(Scheme 1(d)). After separation of the desired  $\beta$ -anomer **15** by crystallization, deprotection of the protective groups in **15** afforded **1**.

Takeuchi, Sugawara and Ohruï designed **16** as a glycosyl donor and used this compound for the synthesis of EdAP (**3**) (Scheme 2) [25,26]. The silyl-Hilbert-Johnson reaction between **16** and **8** afforded **17** in 78% yield. After conversion of **17** into **18**, the introduction of the cycloSal group into the hydroxy group in **18**, followed by deprotection of the *p*-methoxybenzyl (PMB) group gave **3**. However, we suffered from the lack of the reproducibility of the silyl-Hilbert-Johnson reaction between **16** and **8**, and the introduction of the cycloSal group into **18**. The yields were

not reproducible, and the reactions were often accompanied by the formations of unidentified byproducts.

Chronic infection of hepatitis B virus (HBV) affects approximately 257 million people worldwide and is a risk factor for developing liver cirrhosis and hepatocellular carcinoma [36]. To solve this public health problem, antiviral agents that eliminate the infection are needed. Because EFdA (**2**) shows anti-HBV activity with IC<sub>50</sub> value of 0.16  $\mu$ M [37], we have been interested in the anti-HBV activity of EdA and EdAP.

In this paper, we report the syntheses of EFdA (**1**), EdA (**2**), and EdAP (**3**) starting from the 1,2-di-*O*-acetyl-D-ribofuranose **16** and the anti-HBV activity of EdAP. We optimized the conditions of the silyl-Hilbert-Johnson



**Scheme 2.** Synthesis of EdAP (**3**) reported by Takeuchi, Sugawara and Ohru.

reaction and the cycloSal-introduction. The utility of **16** as a glycosyl donor has been demonstrated by the present synthesis of these nucleoside analogues.

## Materials and methods

### General information

Melting point (Mp) data were determined using a Micro Melting Point Determination Apparatus Type MM-2 instrument (Shimadzu Seisakusyo, Ltd) and were uncorrected. IR spectra were recorded on a Horiba FT-720 spectrometer using KBr pellets.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker Avance 400 (400 and 100 MHz, respectively) spectrometer, using chloroform- $d$  ( $\text{CDCl}_3$ ), methanol- $d_4$  ( $\text{CD}_3\text{OD}$ ) or dimethyl sulfoxide- $d_6$  ( $\text{DMSO}-d_6$ ) as a solvent. Chemical shift values are expressed in  $\delta$  (ppm) relative to tetramethylsilane (TMS,  $\delta$  0.00 ppm) or the solvent resonance ( $\text{CDCl}_3$ ,  $\delta$  7.26 ppm for  $^1\text{H}$  NMR and  $\delta$  77.0 ppm for  $^{13}\text{C}$  NMR;  $\text{CD}_3\text{OD}$ ,  $\delta$  3.30 ppm for  $^1\text{H}$  NMR and  $\delta$  49.0 ppm for  $^{13}\text{C}$  NMR;  $\text{DMSO}-d_6$ ,  $\delta$  2.49 ppm for  $^1\text{H}$  NMR). Data are reported as follows: chemical shift, multiplicity ( $s$  = singlet,  $d$  = doublet,  $t$  = triplet,  $q$  = quartet,  $br$  = broad,  $m$  = multiplet), coupling constants ( $J$ ; Hz), and integration. Mass spectra were obtained by on a JEOL high-resolution double-focusing mass spectrometer (JMS-700, "MStation") using fast atom bombardment (FAB). Specific rotations were recorded on a JASCO polarimeter (P-1010), and recorded as  $[\alpha]_D$  values (concentration in g/100 mL).

### Synthesis

(2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-9*H*-purin-9-yl)-5-(tert-butylidiphenylsilyloxymethyl)-4-(4-methoxybenzyloxy)-

5-[(triethylsilyl)ethynyl]tetrahydrofuran-3-yl acetate (**17**) and (2*R*,3*R*,4*S*,5*R*)-2-(6-amino-3*H*-purin-3-yl)-5-(tert-butylidiphenylsilyloxymethyl)-4-(4-methoxybenzyloxy)-5-[(triethylsilyl)ethynyl]tetrahydrofuran-3-yl acetate (**21**)

*N*,*O*-Bis(trimethylsilyl)acetamide (BSA) (198  $\mu\text{L}$ , 0.810 mmol) was added to a solution of adenine (**8**, 54.7 mg, 0.405 mmol) in 1,2-dichloroethane (810  $\mu\text{L}$ ). The mixture was stirred under an argon atmosphere at 70°C for 5.5 h. A solution of **16** [25,26] (98.8 mg, 0.135 mmol) in 1,2-dichloroethane (1.9 mL) was added to the mixture at room temperature. Trimethylsilyl triflate (TMSOTf) (73.3  $\mu\text{L}$ , 0.405 mmol) was added to the resultant mixture at 0°C. The mixture was stirred under an argon atmosphere at 90°C for 19.5 h. The reaction was quenched by the addition of saturated aqueous  $\text{NaHCO}_3$  solution. The mixture was diluted with  $\text{CHCl}_3$  at 0°C to give a biphasic solution. The aqueous layer was extracted with  $\text{CHCl}_3$ . The combined organic layer was dried over  $\text{Na}_2\text{SO}_4$ , and concentrated to give a residue. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/1, then MeOH) to afford **17** (49.9 mg, 46%) as a pale yellow oil and **21** (38.2 mg, 35%) as a pale yellow oil. The NMR spectroscopic data for **17** were identical with the reported data [25,26]. Compound **21**:  $[\alpha]_D^{23}$  = -17.1 ( $c$  0.34,  $\text{CHCl}_3$ ). IR (KBr)  $\nu_{\text{max}}$  = 3438, 3363, 3072, 3049, 2956, 2933, 2873, 2858, 2171, 1749, 1658, 1614, 1589, 1556, 1514, 1471  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ; relative to TMS)  $\delta$  8.43 (s, 1H), 7.95 (s, 1H), 7.64 (m, 4H), 7.46–7.40 (m, 2H), 7.38–7.32 (m, 4H), 7.22 (d,  $J$  = 8.6 Hz, 2H), 6.83 (d,  $J$  = 8.6 Hz, 2H), 6.50 (d,  $J$  = 4.0 Hz, 1H), 5.85 (dd,  $J$  = 6.0, 4.0 Hz, 1H), 4.75 (d,  $J$  = 6.0 Hz, 1H), 4.60 (d,  $J$  = 11.0 Hz, 1H), 4.52 (d,  $J$  = 11.0 Hz, 1H), 4.14 (d,  $J$  = 11.4 Hz, 1H), 3.85 (d,  $J$  = 11.4 Hz, 1H), 3.80 (s, 3H), 2.05 (s, 3H), 1.07 (s, 9H), 0.94 (t,  $J$  = 7.9 Hz, 9H), 0.58 (q,  $J$  = 7.9 Hz, 6H).

$^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ; relative to the solvent resonance)  $\delta$  169.9, 159.2, 154.4, 154.2, 149.5, 140.4, 135.7 (2C), 135.5 (2C), 132.4, 132.3, 130.0, 129.9, 129.7, 129.2 (2C), 127.9 (2C), 127.8 (2C), 120.7, 113.5 (2C), 101.2, 92.1, 91.4, 84.2, 76.3, 74.4, 73.3, 66.5, 55.2, 26.9 (3C), 20.7, 19.2, 7.4 (3C), 4.1 (3C). HRMS (FAB)  $m/z$  calcd. for  $\text{C}_{44}\text{H}_{56}\text{N}_5\text{O}_6\text{Si}_2$  ( $[\text{M} + \text{H}]^+$ ) 806.3769, found 806.3768.

#### Isomerization of **21** to **17**.

*p*-Toluenesulfonic acid monohydrate (*p*-TsOH·H<sub>2</sub>O) (2.75 mg, 14.5  $\mu\text{mol}$ ) was added to a solution of **21** (38.9 mg, 48.3  $\mu\text{mol}$ ) in chlorobenzene (1.0 mL). The reaction mixture was refluxed for 50 min. The mixture was diluted with  $\text{CHCl}_3$ , and concentrated to a residue. The residue was purified by silica gel column chromatography (hexane/EtOAc = 2/1) to afford **17** (32.5 mg, 84%).

(2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-9*H*-purin-9-yl)-5-[[*tert*-butyldiphenylsilyloxy]methyl]-4-(4-methoxybenzyloxy)-5-[[triethylsilyl]ethynyl]tetrahydrofuran-3-ol (**22**)

A solution of **17** (1.32 g, 1.64 mmol) in a 2:5 mixture of  $\text{Et}_3\text{N}$  and MeOH (6.3 mL) was stirred at 45°C for 21 h. The mixture was diluted with EtOAc, and concentrated to a residue. The residue was purified by silica gel column chromatography (EtOAc only) to afford **22** (1.23 g, 98%) as a pale yellow oil. The NMR spectroscopic data for **22** were identical with the reported data [25,26].

*O*-((2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-9*H*-purin-9-yl)-5-(*tert*-butyldiphenylsilyloxymethyl)-4-(4-methoxybenzyloxy)-5-[[triethylsilyl]ethynyl]tetrahydrofuran-3-yl)-*O*-phenyl carbonothioate (**23**)

Phenyl chlorothionoformate (37.3  $\mu\text{L}$ , 0.276 mmol) was added to a solution of **22** (0.211 mg, 0.276 mmol) and 4-dimethylaminopyridine (DMAP) (67.4 mg, 0.552 mmol) in  $\text{CH}_2\text{Cl}_2$  (1.8 mL) at 0°C. The reaction mixture was stirred under an argon atmosphere at room temperature for 5 h. The mixture was diluted with EtOAc, and concentrated to a residue. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/1) to afford **23** (0.215 g, 87%) as a colorless oil. The NMR spectroscopic data for **23** were identical with the reported data [25,26].

9-((2*R*,4*S*,5*R*)-5-(*tert*-Butyldiphenylsilyloxymethyl)-4-(4-methoxybenzyloxy)-5-[[triethylsilyl]ethynyl]tetrahydrofuran-2-yl)-9*H*-purin-6-amine (**24**)

Azobisisobutyronitrile (AIBN) (9.84 mg, 59.9  $\mu\text{mol}$ ) was added to a solution of **23** (0.216 g, 0.236 mmol) and tri-(*n*-butyl)tin hydride (387  $\mu\text{L}$ , 1.44 mmol) in toluene (6.0 mL). The reaction mixture was refluxed under an argon atmosphere for 3 h. The mixture was diluted with EtOAc, and concentrated to a residue. The residue was purified by column chromatography using silica gel containing 10% w/w anhydrous potassium carbonate (hexane/EtOAc = 1/2) to afford **24** (0.163 g, 91%) as a pale yellow oil. The NMR

spectroscopic data for **24** were identical with the reported data [25,26].

[(2*R*,3*S*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-2-ethynyl-3-(4-methoxybenzyloxy)tetrahydrofuran-2-yl]methanol (**18**)

A 1.0 M solution of tetra-*n*-butylammonium fluoride (TBAF) in THF (1.2 mL, 1.20 mmol) was added to a solution of **24** (0.41 g, 0.548 mmol) in THF (5.5 mL). The mixture was stirred under an argon atmosphere at room temperature for 2 h. The reaction was quenched by the addition of water. The mixture was diluted with EtOAc to give a biphasic solution. The layers were separated. The aqueous layer was extracted with EtOAc. The combined organic layer was dried over  $\text{Na}_2\text{SO}_4$ , and concentrated to a residue. The residue was purified by silica gel column chromatography (EtOAc/MeOH = 15/1) to afford **18** (0.196 g, 95%) as a white solid. Mp = 135–136°C. The NMR spectroscopic data for **18** were identical with the reported data [25,26].

EdA, (2*R*,3*S*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol (**2**)

Cerium ammonium nitrate (CAN) (122.7 mg, 0.224 mmol) was added to a solution of **18** (35.4 mg, 89.5  $\mu\text{mol}$ ) in a 5:1 mixture of MeCN and water (0.9 mL) at 0°C. The mixture was stirred at 0°C for 5.5 h. The mixture was diluted with MeOH, and concentrated to a residue. The residue was purified by silica gel column chromatography (EtOAc/MeOH = 10/1) to afford EdA (**2**) (20.3 mg, 82%) as a white solid. Mp = 154–155°C.  $[\alpha]_{\text{D}}^{23} + 13.7$  (*c* 0.14, MeOH). IR (KBr)  $\nu_{\text{max}} = 3392, 3273, 2927, 2112, 1649, 1604, 1506, 1479, 1421 \text{ cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ; relative to the solvent resonance)  $\delta$  8.31 (s, 1H), 8.12 (s, 1H), 7.32 (br s, 2H), 6.34 (t,  $J = 6.3$  Hz, 1H), 5.58–5.55 (2H, overlapped), 4.57 (q,  $J = 6.1$  Hz, 1H), 3.66 (dd,  $J = 12.0, 5.3$  Hz, 1H), 3.55 (dd,  $J = 11.8, 7.4$  Hz, 1H), 3.51 (s, 1H), 2.78–2.72 (m, 1H), 2.43–2.38 (m, 1H).  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ ; relative to the solvent resonance)  $\delta$  8.51 (s, 1H), 8.34 (s, 1H), 6.49 (dd,  $J = 7.2, 4.5$  Hz, 1H), 4.75 (t,  $J = 7.1$  Hz, 1H), 3.85 (d,  $J = 12.2$  Hz, 1H), 3.77 (d,  $J = 12.2$  Hz, 1H), 3.11 (s, 1H), 2.84–2.78 (m, 1H), 2.70–2.63 (m, 1H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{OD}$ ; relative to the solvent resonance)  $\delta$  153.3, 149.6, 147.6, 143.3, 120.4, 87.0, 85.0, 80.6, 78.9, 71.5, 65.7, 40.1. HRMS (FAB)  $m/z$  calcd. for  $\text{C}_{12}\text{H}_{14}\text{N}_5\text{O}_3$  ( $[\text{M} + \text{H}]^+$ ) 276.1097, found 276.1095.

2-[[[(2*R*,3*S*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-2-ethynyl-3-(4-methoxybenzyloxy)tetrahydrofuran-2-yl]methoxy]-4*H*-benzo[*d*] [1,3,2]dioxaphosphinine 2-oxide (**20**)

*N,N*-Diisopropylethylamine (61.5  $\mu\text{L}$ , 0.353 mmol) and **19** (66.5  $\mu\text{L}$ , 0.353 mmol) were added to a solution of **18** (69.7 mg, 0.176 mmol) in THF (2.7 mL). The mixture was stirred under an argon atmosphere at –40°C for 1 h. An aqueous 30% hydrogen peroxide solution (39.9  $\mu\text{L}$ , 0.353 mmol) was added at –40°C. The mixture was stirred at room temperature for 50

min. The mixture was diluted with EtOAc, and concentrated to a residue. The residue was purified by silica gel column chromatography (EtOAc/MeOH = 15/1) to afford a crude product. This purification was repeated three times to afford pure **20** (40.4 mg, 41%) as a 1.43:1 diastereomeric mixture as a pale yellow oil. The NMR spectroscopic data for **20** were identical with the reported data [25,26].

*EdAP*, 2-[[[(2*R*,3*S*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl]methoxy]-4*H*-benzo[*d*] [1,3,2]dioxaphosphinine 2-oxide (**3**)

CAN (80.7 mg, 0.108 mmol) was added to a solution of **20** (30.4 mg, 53.9  $\mu$ mol) in a 5:1 mixture of MeCN and water (0.9 mL) at 0°C. The mixture was stirred at 0°C for 4.5 h. The mixture was diluted with MeOH, and concentrated to a residue. The residue was purified by silica gel column chromatography (CHCl<sub>3</sub>/MeOH = 10/1) to afford **3** (15.5 mg, 65%) as a 1.25:1 diastereomeric mixture as a pale yellow solid. Mp = 128–130°C (dec.).  $[\alpha]_{\text{D}}^{22}$  – 2.67 (*c* 0.54, MeOH). The NMR spectroscopic data for **3** were identical with the reported data [25,26].

(2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-fluoro-9*H*-purin-9-yl)-5-(*tert*-butyldiphenylsilyloxymethyl)-4-(4-methoxybenzyloxy)-5-[(triethylsilyl)ethynyl]tetrahydrofuran-3-yl acetate (**25**)

BSA (128  $\mu$ L, 0.524 mmol) was added to a solution of 2-fluoro-adenine (**5**, 40.1 mg, 0.262 mmol) in 1,2-dichloroethane (550  $\mu$ L). The mixture was stirred under an argon atmosphere at 70°C for 7 h. A solution of **16** (63.9 mg, 87.4  $\mu$ mol) in 1,2-dichloroethane (1.2 mL) was added to the mixture at room temperature. TMSOTf (47.5  $\mu$ L, 0.262 mmol) was added to the resultant mixture at 0°C, and the resultant mixture was stirred under an argon atmosphere at 90°C for 13 h. The reaction was quenched by the addition of saturated aqueous NaHCO<sub>3</sub> solution. The mixture was diluted with CHCl<sub>3</sub> to give a biphasic solution. The layers were separated. The aqueous layer was extracted with CHCl<sub>3</sub>. The combined organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated to a residue. The residue was purified by silica gel column chromatography (hexane/EtOAc = 2/1) to afford **25** (61.6 mg, 86%) as a white solid. Mp = 144–146°C.  $[\alpha]_{\text{D}}^{15}$  – 33.3 (*c* 0.28, CHCl<sub>3</sub>). IR (KBr)  $\nu_{\text{max}}$  = 3324, 3176, 3072, 2955, 2933, 2873, 2859, 1747, 1647, 1612, 1587, 1559, 1515, 1472 cm<sup>-1</sup>. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>; relative to TMS)  $\delta$  7.86 (s, 1H), 7.64–7.60 (m, 4H), 7.43–7.39 (m, 2H), 7.36–7.31 (m, 4H), 7.27 (d, *J* = 8.6 Hz, 2H), 6.85 (d, *J* = 8.6 Hz, 2H), 6.22 (d, *J* = 5.0 Hz, 1H), 5.97 (br s, 2H), 5.74 (dd, *J* = 6.1, 5.0 Hz, 1H), 4.74 (d, *J* = 6.1 Hz, 1H), 4.70 (d, *J* = 11.2 Hz, 1H), 4.53 (d, *J* = 11.2 Hz, 1H), 3.97 (d, *J* = 11.2 Hz, 1H), 3.802 (d, *J* = 11.2 Hz, 1H), 3.797 (s, 3H), 2.04 (s, 3H), 1.03 (s, 9H), 0.96 (t, *J* = 7.9 Hz, 9H), 0.59 (q, *J* = 7.9 Hz, 6H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>; relative to the solvent resonance)  $\delta$  169.9, 159.2, 159.1 (d, *J*<sub>C-F</sub> = 209.9 Hz), 157.1 (d, *J*<sub>C-F</sub>

= 19.9 Hz), 151.1 (d, *J*<sub>C-F</sub> = 19.5 Hz), 139.5 (d, *J*<sub>C-F</sub> = 2.3 Hz), 135.6 (2C), 135.5 (2C), 132.6, 132.4, 129.9, 129.8, 129.7, 129.4 (2C), 127.8 (4C), 118.1 (d, *J*<sub>CF</sub> = 4.0 Hz), 113.6 (2C), 101.6, 91.8, 86.3, 83.5, 76.3, 73.8, 73.3, 66.7, 55.2, 26.7 (3C), 20.6, 19.2, 7.41 (3C), 4.13 (3C). HRMS (FAB) *m/z* calcd. for C<sub>44</sub>H<sub>55</sub>FN<sub>5</sub>O<sub>6</sub>Si<sub>2</sub> ([M + H]<sup>+</sup>) 824.3675, found 824.3676.

(2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-fluoro-9*H*-purin-9-yl)-5-(*tert*-butyldiphenylsilyloxymethyl)-4-(4-methoxybenzyloxy)-5-[(triethylsilyl)ethynyl]tetrahydrofuran-3-ol (**26**)

A solution of **25** (61.6 mg, 74.8  $\mu$ mol) in a 2:5 mixture of Et<sub>3</sub>N and MeOH (1.4 mL) was stirred at 45°C for 19 h. The mixture was diluted with EtOAc, and concentrated to a residue. The residue was purified by silica gel column chromatography (EtOAc only) to afford **26** (53.5 mg, 91%) as a pale yellow oil.  $[\alpha]_{\text{D}}^{21}$  – 2.1 (*c* 0.27, CHCl<sub>3</sub>). IR (KBr)  $\nu_{\text{max}}$  = 3333, 3183, 3072, 3049, 2998, 2955, 2933, 2912, 2874, 2858, 2169, 1648, 1611, 1588, 1559, 1515, 1490 cm<sup>-1</sup>. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>; relative to TMS)  $\delta$  7.81 (s, 1H), 7.62–7.60 (m, 4H), 7.44–7.39 (m, 2H), 7.36–7.32 (m, 6H), 6.88 (d, *J* = 8.7 Hz, 2H), 6.22 (br s, 2H), 6.00 (d, *J* = 5.0 Hz, 1H), 4.89 (d, *J* = 11.2 Hz, 1H), 4.75 (ddd, *J* = 8.2, 6.0, 5.0 Hz, 1H), 4.63 (d, *J* = 11.2 Hz, 1H), 4.55 (d, *J* = 6.0 Hz, 1H), 3.97 (d, *J* = 11.0 Hz, 1H), 3.83 (d, *J* = 11.0 Hz, 1H), 3.80 (s, 3H), 3.45 (br s, 1H), 1.03 (s, 9H), 0.97 (t, *J* = 7.8 Hz, 9H), 0.62 (q, *J* = 7.8 Hz, 6H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>; relative to the solvent resonance)  $\delta$  159.6, 158.9 (d, *J*<sub>C-F</sub> = 209.9 Hz), 156.9 (d, *J*<sub>C-F</sub> = 20.0 Hz), 151.1 (d, *J*<sub>C-F</sub> = 19.5 Hz), 139.9, 135.6 (2C), 135.4 (2C), 132.6, 132.4, 129.93, 129.90, 129.8 (2C), 128.9, 127.8 (4C), 118.3, 113.9 (2C), 102.2, 91.2, 89.4, 83.0, 77.2, 73.1, 72.9, 66.8, 55.3, 26.7 (3C), 19.2, 7.42 (3C), 4.10 (3C). HRMS (FAB) *m/z* calcd. for C<sub>42</sub>H<sub>53</sub>FN<sub>5</sub>O<sub>5</sub>Si<sub>2</sub> ([M + H]<sup>+</sup>) 782.3569, found 782.3568.

*O*-((2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-fluoro-9*H*-purin-9-yl)-5-(*tert*-butyldiphenylsilyloxymethyl)-4-(4-methoxybenzyloxy)-5-[(triethylsilyl)ethynyl]tetrahydrofuran-3-yl} *O*-phenyl carbonothioate (**27**)

Phenyl chlorothionoformate (9.3  $\mu$ L, 68.4  $\mu$ mol) was added to a solution of **26** (53.5 mg, 68.4  $\mu$ mol) and DMAP (16.7 mg, 0.137 mmol) in dichloromethane (1.0 mL) at 0°C. The reaction mixture was stirred under an argon atmosphere at room temperature for 2 h. The mixture was diluted with CHCl<sub>3</sub>, and concentrated to a residue. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/1) to afford **27** (56.9 mg, 91%) as a pale yellow oil.  $[\alpha]_{\text{D}}^{21}$  – 37.5 (*c* 0.30, CHCl<sub>3</sub>). IR (KBr)  $\nu_{\text{max}}$  = 3327, 3176, 3070, 2999, 2955, 2932, 2873, 2858, 2171, 1722, 1649, 1610, 1590, 1514, 1490 cm<sup>-1</sup>. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>; relative to TMS)  $\delta$  7.87 (s, 1H), 7.64–7.61 (m, 4H), 7.42–7.27 (m, 11H), 6.93 (d, *J* = 8.7 Hz, 2H), 6.87 (d, *J* = 8.7 Hz, 2H), 6.35 (d, *J* = 4.9 Hz, 1H), 6.22 (dd, *J* = 6.0, 4.9 Hz, 1H), 6.13 (br s, 2H), 5.01

(d,  $J = 6.0$  Hz, 1H), 4.83 (d,  $J = 11.1$  Hz, 1H), 4.62 (d,  $J = 11.1$  Hz, 1H), 4.02 (d,  $J = 11.2$  Hz, 1H), 3.86 (d,  $J = 11.2$  Hz, 1H), 3.80 (s, 3H), 1.03 (s, 9H), 0.97 (t,  $J = 7.9$  Hz, 9H), 0.61 (q,  $J = 7.9$  Hz, 6H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ; relative to the solvent resonance)  $\delta$  194.4, 159.3, 159.0 (d,  $J_{\text{C-F}} = 210.5$  Hz), 157.0 (d,  $J_{\text{C-F}} = 19.8$  Hz), 153.4, 151.2 (d,  $J_{\text{C-F}} = 19.5$  Hz), 140.0 (d,  $J_{\text{C-F}} = 2.7$  Hz), 135.6 (2C), 135.5 (2C), 132.5 (2C), 129.9, 129.8, 129.7 (2C), 129.6, 129.5 (2C), 127.8 (2C), 127.7 (2C), 126.7, 121.7 (2C), 118.3 (d,  $J_{\text{C-F}} = 4.0$  Hz), 113.7 (2C), 101.3, 92.4, 86.1, 83.6, 80.8, 76.0, 73.8, 66.5, 55.3, 26.7 (3C), 19.2, 7.44 (3C), 4.10 (3C). HRMS (FAB)  $m/z$  calcd. for  $\text{C}_{49}\text{H}_{57}\text{FN}_5\text{O}_6\text{SSi}_2$  ( $[\text{M} + \text{H}]^+$ ) 918.3552, found 918.3552.

*9-((2R,4S,5R)-5-(tert-Butyldiphenylsilyloxymethyl)-4-(4-methoxybenzyloxy)-5-[(triethylsilyl)ethynyl]tetrahydrofuran-2-yl)-2-fluoro-9H-purin-6-amine (28)*

AIBN (4.38 mg, 26.7  $\mu\text{mol}$ ) was added to a solution of **27** (98.1 mg, 0.107 mmol) and tri-(*n*-butyl)tin hydride (172  $\mu\text{L}$ , 0.641 mmol) in toluene (2.7 mL). The reaction mixture was refluxed under an argon atmosphere for 6 h. The mixture was diluted with EtOAc, and concentrated to a residue. The residue was purified by column chromatography using silica gel containing 10% w/w anhydrous potassium carbonate (hexane/EtOAc = 2/1) to afford **28** (65.4 mg, 80%) as a white solid. Mp = 163–165°C.  $[\alpha]_{\text{D}}^{19} = 21.4$  ( $c$  0.13,  $\text{CHCl}_3$ ). IR (KBr)  $\nu_{\text{max}} = 3437, 3317, 3166, 3072, 2954, 2932, 2873, 2858, 2165, 1674, 1648, 1613, 1585, 1514, 1498$   $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ; relative to TMS)  $\delta$  7.94 (s, 1H), 7.64–7.60 (m, 4H), 7.43–7.39 (m, 2H), 7.36–7.31 (m, 4H), 7.28 (d,  $J = 8.6$  Hz, 2H), 6.86 (d,  $J = 8.6$  Hz, 2H), 6.36 (dd,  $J = 6.7, 5.2$  Hz, 1H), 6.00 (br s, 2H), 4.68 (d,  $J = 11.6$  Hz, 1H), 4.61 (d,  $J = 6.7$  Hz, 1H), 4.55 (d,  $J = 11.6$  Hz, 1H), 4.00 (d,  $J = 11.1$  Hz, 1H), 3.87 (d,  $J = 11.1$  Hz, 1H), 3.81 (s, 3H), 2.73–2.61 (m, 2H), 1.04 (s, 9H), 0.97 (t,  $J = 7.8$  Hz, 9H), 0.61 (q,  $J = 7.8$  Hz, 6H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ; relative to the solvent resonance)  $\delta$  159.2, 158.9 (d,  $J_{\text{C-F}} = 209.3$  Hz), 156.9 (d,  $J_{\text{C-F}} = 20.1$  Hz), 150.7 (d,  $J_{\text{C-F}} = 19.6$  Hz), 139.5 (d,  $J_{\text{C-F}} = 2.6$  Hz), 135.6 (2C), 135.5 (2C), 132.7, 132.5, 129.90, 129.86 (2C), 129.1 (2C), 127.8 (4C), 118.2, 113.7 (2C), 102.3, 91.2, 84.6, 83.4, 77.1, 72.1, 66.3, 55.3, 37.3, 26.8 (3C), 19.2, 7.45 (3C), 4.21 (3C). HRMS (FAB)  $m/z$  calcd. for  $\text{C}_{42}\text{H}_{53}\text{FN}_5\text{O}_4\text{Si}_2$  ( $[\text{M} + \text{H}]^+$ ) 766.3620, found 766.3619.

*[(2R,3S,5R)-5-(6-Amino-2-fluoro-9H-purin-9-yl)-2-ethynyl-3-(4-methoxybenzyloxy)tetrahydrofuran-2-yl]methanol (29)*

A 1.0 M solution of TBAF in THF (377  $\mu\text{L}$ , 0.377 mmol) was added to a solution of **28** (0.131 g, 0.171 mmol) in THF (1.7 mL). The mixture was stirred under an argon atmosphere at room temperature for 2 h. The reaction was quenched by the addition of water. The mixture was diluted with EtOAc to give a biphasic solution. The layers were separated. The aqueous layer was extracted with EtOAc. The

combined organic layer was dried over  $\text{Na}_2\text{SO}_4$ , and concentrated to a residue. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/3) to afford **29** (66.4 mg, 94%) as a pale yellow amorphous.  $[\alpha]_{\text{D}}^{23} = 24.8$  ( $c$  0.49,  $\text{CHCl}_3$ ). IR (KBr)  $\nu_{\text{max}} = 3294, 3180, 3012, 2935, 2836, 2114, 1685, 1614, 1514, 1442$   $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ ; relative to the solvent resonance)  $\delta$  8.20 (s, 1H), 7.33 (d,  $J = 8.7$  Hz, 2H), 6.88 (d,  $J = 8.7$  Hz, 2H), 6.33 (dd,  $J = 7.0, 5.6$  Hz, 1H), 4.69 (d,  $J = 11.3$  Hz, 1H), 4.59 (d,  $J = 6.4$  Hz, 1H), 4.58 (d,  $J = 11.3$  Hz, 1H), 3.85 (d,  $J = 13.3$  Hz, 1H), 3.76 (s, 3H), 3.74 (d,  $J = 13.3$  Hz, 1H), 3.11 (s, 1H), 2.80–2.74 (m, 1H), 2.63–2.57 (m, 1H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ; relative to the solvent resonance)  $\delta$  159.4, 158.5 (d,  $J_{\text{C-F}} = 212.3$  Hz), 157.3 (d,  $J_{\text{C-F}} = 20.0$  Hz), 149.9, 140.3, 129.53, 129.49 (2C), 119.1, 113.9 (2C), 86.4, 86.1, 79.6, 79.5, 77.2, 72.6, 67.9, 55.3, 38.3. HRMS (FAB)  $m/z$  calcd. for  $\text{C}_{20}\text{H}_{21}\text{FN}_5\text{O}_4$  ( $[\text{M} + \text{H}]^+$ ) 414.1578, found 414.1576.

*EFdA, (2R,3S,5R)-5-(6-Amino-2-fluoro-9H-purin-9-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol (1)*

CAN (96.3 mg, 0.176 mmol) was added to a solution of **29** (24.2 mg, 58.5  $\mu\text{mol}$ ) in a 5:1 mixture of MeCN and water (0.9 mL) at 0°C. The mixture was stirred at 0°C for 3 h. The mixture was diluted with MeOH, and concentrated to a residue. The residue was purified by silica gel column chromatography ( $\text{CHCl}_3/\text{MeOH} = 8/1$ ) to afford EFdA (**1**) (10.0 mg, 58%) as a white solid. Mp = 219–220°C (dec.).  $[\alpha]_{\text{D}}^{19} = 12.7$  ( $c$  0.50, MeOH). IR (KBr)  $\nu_{\text{max}} = 3321, 3167, 3001, 2116, 1695, 1618, 1496$   $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ ; relative to the solvent resonance)  $\delta$  8.25 (s, 1H), 6.34 (dd,  $J = 7.2, 4.9$  Hz, 1H), 4.73 (t,  $J = 6.9$  Hz, 1H), 3.85 (d,  $J = 12.2$  Hz, 1H), 3.76 (d,  $J = 12.2$  Hz, 1H), 3.08 (s, 1H), 2.81–2.74 (m, 1H), 2.63–2.56 (m, 1H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{OD}$ ; relative to the solvent resonance)  $\delta$  160.4 (d,  $J_{\text{C-F}} = 207.7$  Hz), 159.1 (d,  $J_{\text{C-F}} = 19.9$  Hz), 151.5 (d,  $J_{\text{C-F}} = 19.3$  Hz), 141.4, 118.7, 86.8, 84.8, 80.7, 78.8, 71.7, 65.9, 39.9. HRMS (FAB)  $m/z$  calcd. for  $\text{C}_{12}\text{H}_{13}\text{FN}_5\text{O}_3$  ( $[\text{M} + \text{H}]^+$ ) 294.1002, found 294.1000.

### Cell culture

Hep38.7-Tet cells were cultured at 37°C, 5%  $\text{CO}_2$  as described previously [38,39]. The Hep38.7-Tet cells produce HBV replication under depletion of tetracycline from medium.

### Quantification of cell viability

At 3 days after seeding, Hep38.7-Tet cells were incubated in the absence of tetracycline to induce HBV replication and treated with or without the compound for 6 days. 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide salt (XTT) cell

viability assays were performed with XTT cell proliferation kit II (Roche), according to the manufacturer's instructions [40]. The absorbance values were then measured at 450 nm with a 96-well plate reader. Cell growth inhibition was evaluated as the ratio of the absorbance of the sample to that of the control.

### HBV replication assay

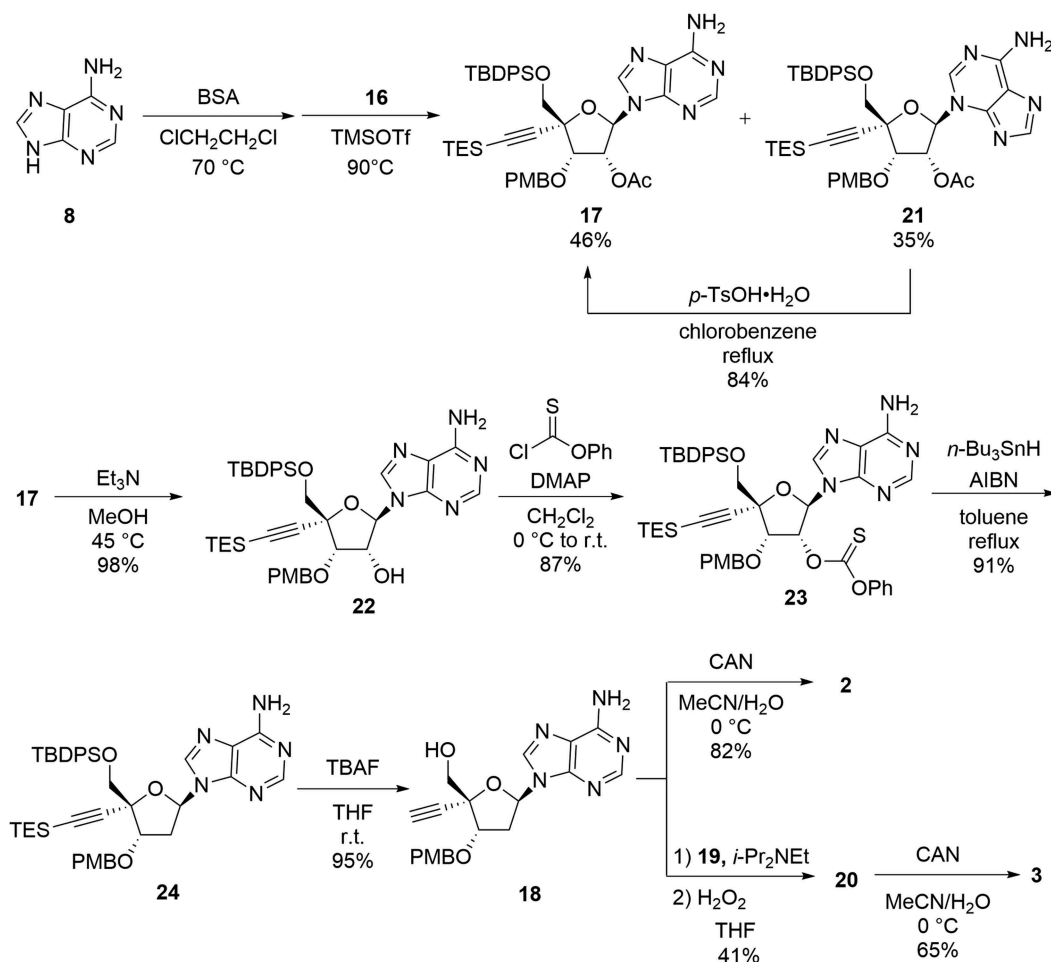
At 3 days after seeding, Hep38.7-Tet cells were incubated in the absence of tetracycline to induce HBV replication and treated with or without the compound for 6 days. HBV DNA in the culture supernatant were recovered and quantified by real-time PCR using 5'-AAGGTAGGAGCTGGAGCATTCG-3' (Forward) and 5'-AGGCGGATTTGCTGGCAAAG-3' (Reverse) as a primer set and 5'-AGCCCTCAGGCTCAGGGC ATAC-3' as a probe [41].

## Results

### Synthesis of EdA and EdAP

The syntheses of EdA (**2**) and EdAP (**3**) are depicted in Scheme 3. The silyl-Hilbert-Johnson reaction consists of

the following two reactions: silylation of a base and glycosylation between the silylated base and an electrophilic sugar. In the previous protocol [25,26], the silylation of adenine (**8**) with BSA was performed in the presence of 1,2-di-*O*-acetyl-D-ribofuranose **16**. Following this protocol, we frequently observed decomposition of **16** during the silylation step. Thus, we considered that the silylation of **8** with BSA in the absence of **16** will improve the reproducibility of the results. Actually, we obtained reproducible results according to the following procedure. First, **8** was reacted with an excess amount of BSA in 1,2-dichloroethane at 70°C. Then, a mixture of the silylated adenine and **16** ( $\alpha:\beta = 7.7:1$ ) was treated with TMSOTf and heated at 90°C. This reaction afforded the desired *N*-glycoside **17** in 46% yield, together with isadenine derivative **21** in 35% yield [42,43]. The structure of **21** was confirmed by HMBC correlation from H-2 ( $\delta$  8.42) at the adenine moiety to C-1' ( $\delta$  91.5) at the ribose moiety (Figure 2 and Figure S10 in Supplementary material). Although compound **21** was obtained, this compound easily isomerized to **17** by heating with a catalytic amount of *p*-TsOH·H<sub>2</sub>O in refluxing chlorobenzene [44]. Deprotection of the acetyl group in **17** under basic conditions gave alcohol **22**. The hydroxy group in **22** was replaced with a hydrogen atom by a Barton-McCombie



Scheme 3. Synthesis of EdA (**2**) and EdAP (**3**).

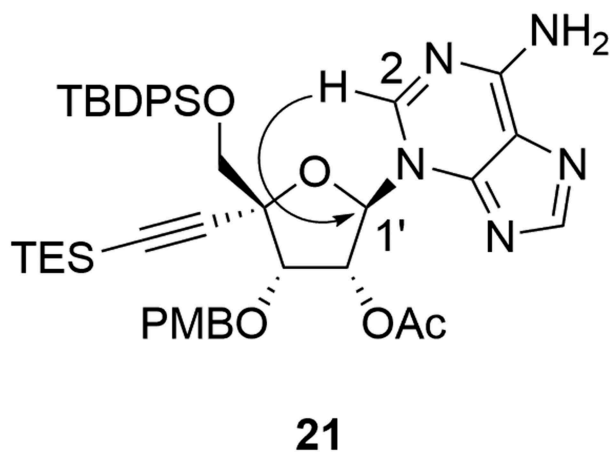


Figure 2. A selected HMBC correlation in compound **21**.

deoxygenation reaction [45,46]. The alcohol **22** was converted into phenyl thionocarbonate **23**, which was heated with tri-*n*-butyltin hydride and AIBN to give **24**. Removal of the *tert*-butyldiphenylsilyl (TBDPS) and triethylsilyl (TES) groups in **24** with TBAF gave **18** in 95% yield. Deprotection of the PMB group in **18** with CAN gave EdA (**2**) in 82% yield. We found that the lack of reproducibility in the introduction of the cycloSal into **18** was due to the low solubility of **18** in acetonitrile. The use of THF as a solvent instead of acetonitrile improved the reproducibility of the cycloSal-introduction. Coupling of **18** with chlorophosphite **19** [27] in THF, followed by oxidation of the resultant phosphite ester with 30% hydrogen peroxide gave **20** as a 1.43:1 diastereomeric mixture in 41% yield. The difficulty in removal of trace impurities from the product by silica gel chromatography caused the low-isolated yield. Deprotection of the PMB group in **20** with CAN gave **3** as a 1.25:1 diastereomeric mixture in 65% yield.

### Synthesis of EFdA

The glycosyl donor **16** was also used for the synthesis of EFdA (**1**) (Scheme 4). The silyl-Hilbert-Johnson reaction between **5** and **16** afforded the desired  $\beta$ -anomer **25** as a sole product in 86% yield. In the synthesis of EdA, the *N*-glycoside **17** and isoadenine derivative **21** were obtained in the glycosylation step. Because the nucleophilicity of the nitrogen at the three-position in 2-fluoro-adenine will be suppressed by the electron withdrawing fluorine at the C-2 position, the silylated 2-fluoro-adenine will selectively react with **16** at the nitrogen at the nine-position (Figure 3). According to the same protocol for the synthesis of **2**, compound **25** was converted into compound **29**. Deprotection of the PMB group in **29** with CAN gave **1** in 58% yield.

### Effect of EdAP on hepatitis B virus replication

The inhibitory effect of EdAP on the replication of HBV is depicted in Figure 4. EdAP did not show significant cytotoxic activity against Hep38.7-Tet

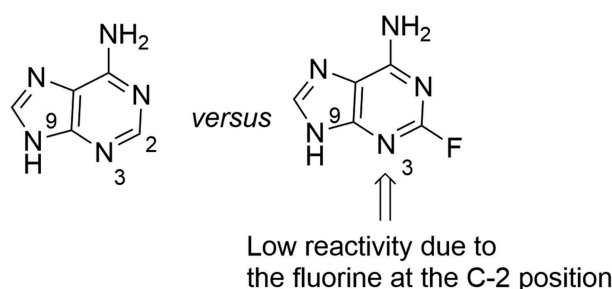
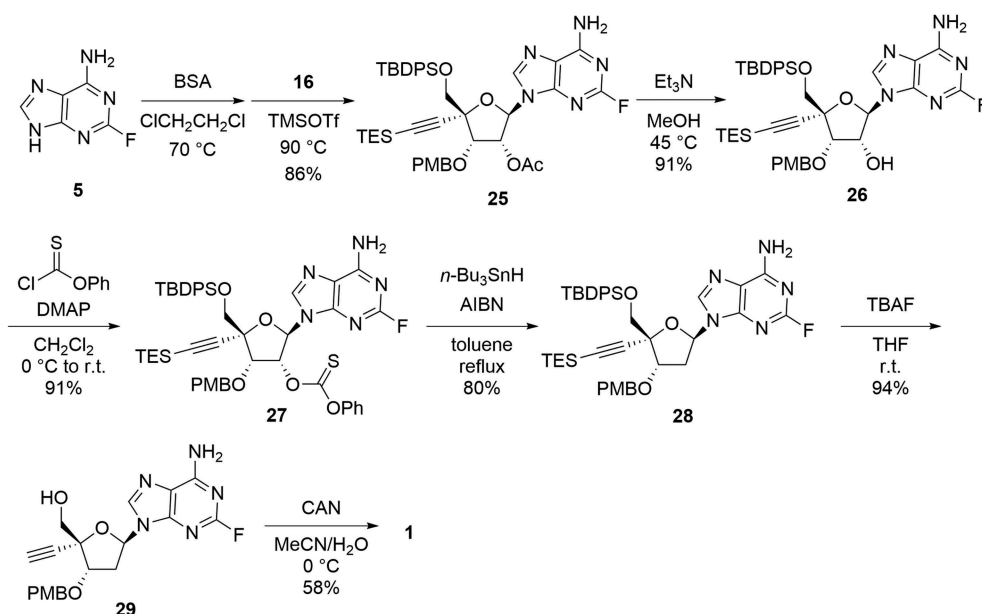
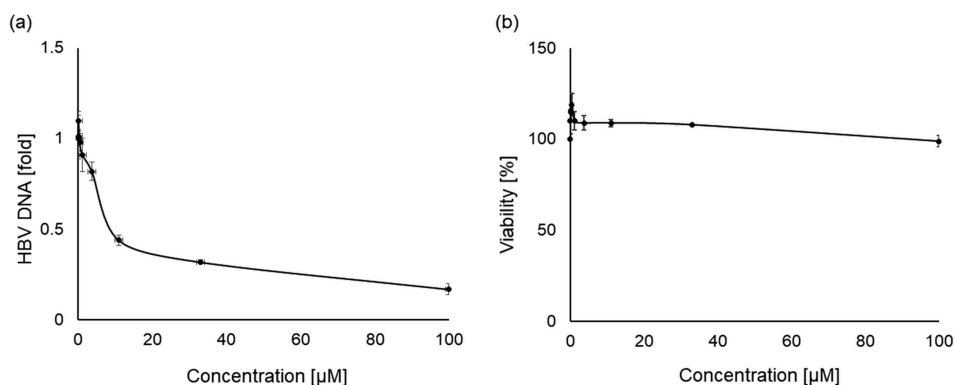


Figure 3. Comparison of the reactivity between adenine and 2-fluoro-adenine.



Scheme 4. Synthesis of EFdA (**1**).





**Figure 4.** Effect of EdAP on the replication of HBV. (a) Cytotoxicity of EdAP against Hep38.7-Tet cells after 6 days was determined by XTT assay [40]. (b) Anti-HBV activity of EdAP was evaluated by real-time PCR quantifying HBV DNA in the culture supernatant of Hep38.7-Tet cells treated with or without the compound for 6 days [41]. The data indicate the means  $\pm$  SD of five samples from an experiment.

cells at a concentration below 100  $\mu$ M (Figure 4(a)). EdAP reduced the HBV DNA secreted from the cells in a dose-dependent manner (Figure 4(b)). The half-maximal inhibitory concentration ( $IC_{50}$ ) value of EdAP was determined to be 14.5  $\mu$ M. On the other hand, the effect of EdA on the replication of HBV is weak (Figure S21 in Supplementary material). EdA at 30  $\mu$ M reduced HBV DNA to only 78%. These results suggest that the cycloSal moiety installed in EdAP improved the anti-HBV activity of EdA.

## Conclusion

The syntheses of EFdA, EdA, and EdAP were achieved using a common synthetic strategy starting from known 1,2-diacetylribose derivative **16**. The present method provides easy and reproducible access to these nucleoside analogues. The present biological studies indicate that EdAP reduced HBV replication with an  $IC_{50}$  value of 14.5  $\mu$ M, whereas this compound did not influence the proliferation of the host cells. The present findings provide valuable information for the design and development of novel nucleoside/nucleotide drugs against HBV.

## Author contributions

S.Kamisuki., H.O., F.S., and K.K. designed the research; M. K., T.T., E.H., K.N., M.O., M.I., and K.N. and K.K. performed the research. S.Kamo., S.T., K.W., S.Kamisuki., and K. K. analyzed the data; S.T. and K.K. wrote the article.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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