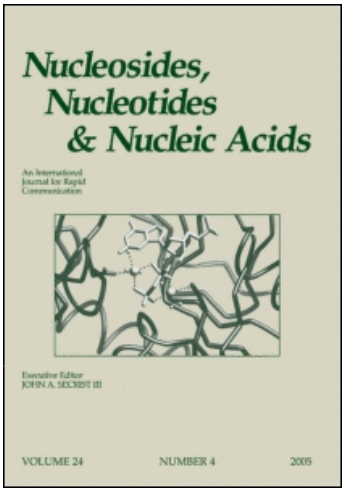


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Phosphoralaninate Pronucleotides of Pyrimidine Methylenecyclopropane Analogues of Nucleosides: Synthesis and Antiviral Activity

Amalraj Ambrose[a]; Jiri Zemlicka[a]; Earl R. Kern[b]; John C. Drach[c]; Elizabeth Gullen[d]; Yung-Chi Cheng[d]
[a] Department of Chemistry, Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan, USA [b] Department of Pediatrics, The University of Alabama School of Medicine, Birmingham, Alabama, USA [c] Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan, USA [d] Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut, USA

To cite this Article Ambrose, Amalraj , Zemlicka, Jiri , Kern, Earl R. , Drach, John C. , Gullen, Elizabeth and Cheng, Yung-Chi(2005) 'Phosphoralaninate Pronucleotides of Pyrimidine Methylenecyclopropane Analogues of Nucleosides: Synthesis and Antiviral Activity', Nucleosides, Nucleotides and Nucleic Acids, 24: 10, 1763 — 1774
To link to this Article: DOI: 10.1080/15257770500266867
URL: http://dx.doi.org/10.1080/15257770500266867

Nucleosides, Nucleotides, and Nucleic Acids, 24:1763–1774, 2005

ISSN: 1525-7770 print/1532-2335 online
DOI: 10.1080/15257770500266867

Taylor & Francis
Taylor & Francis Group

PHOSPHORALANINATE PRONUCLEOTIDES OF PYRIMIDINE METHYLENECYCLOPROPANE ANALOGUES OF NUCLEOSIDES: Synthesis and Antiviral Activity

Amalraj Ambrose and Jiri Zemlicka □ *Department of Chemistry, Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan, USA*

Earl R. Kern □ *Department of Pediatrics, The University of Alabama School of Medicine, Birmingham, Alabama, USA*

John C. Drach □ *Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan, USA*

Elizabeth Gullen and Yung-Chi Cheng □ *Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut, USA*

□ *The Z- and E-thymine and cytosine pronucleotides* **3d**, **4d**, **3e**, *and* **4e** *of methylenecyclopropane nucleosides analogues were synthesized, evaluated for their antiviral activity against human cytomegalovirus (HCMV), herpes simplex virus 1 and 2 (HSV-1 and HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human immunodeficiency virus type 1 (HSV-1), and hepatitis B virus (HBV) and their potency was compared with the parent compounds* **1d**, **2d**, **1e**, *and* **2e**. *Prodrugs* **3d** *and* **4d** *were obtained by phosphorylation of parent analogues* **1d** *or* **2d** *with reagent* **8**. *A similar phosphorylation of* N^4*-benzoylcytosine methylenecyclopropanes* **9a** *and* **9b** *gave intermediates* **11a** *and* **11b**. *Deprotection with hydrazine in pyridine–acetic acid gave pronucleotides* **3e** *and* **4e**. *The Z-cytosine analogue* **3e** *was active against HCMV and EBV. The cytosine E-isomer* **4e** *was moderately effective against EBV.*

Keywords Antivirals; HCMV; EBV; VZV; Methylenecyclopropane analogues; Pronucleotides; Phenyl phosphoralaninates; Prodrugs

This paper is dedicated to the memory of John A. Montgomery.

Received 28 December 2004; accepted 28 April 2005.

We thank L.M. Hrihorczuk from the Central Instrumentation Facility, Department of Chemistry, Wayne State University (D. M. Coleman, Director) for mass spectra and Katherine Z. Borysko for the HCMV (Towne) and cytotoxicity assays. The work described herein was supported by program project PO1-AI46390 (J.C.D.) and contracts NO1-AI85347 and NO1-30049 (E.R.K.) from the National Institute of Allergy and Infectious Diseases, and U.S. Public Health Service grant RO1-CA44358 (Y.-C.C.) from the National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

Address correspondence to Jiri Zemlicka, Department of Chemistry, Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan 48201-1379. Fax: (313) 832-7294; E-mail: zemlicka@kci.wayne.edu

INTRODUCTION

In recent years, much of our attention has been focused on methylenecyclopropane analogues of nucleosides as antiviral agents.[1,2] In this group of compounds, the biological effects are mostly displayed by the purine *Z*-isomers **1**, whereas *E*-isomers **2** or pyrimidine derivatives **1** and **2** are generally less effective or inactive. In several cases, the antiviral efficacy of purine analogues was increased by transformation to phenyl phosphoralaninate pronucleotides.[3,4] This effect was most striking in adenine and 2,6-diaminopurine analogues **3a** and **3b** where a 300–500 times increase of anti-HIV potency relative to parent analogues **1a** and **1b** was noted. A significant potentiation of antiviral effects of the *E*-isomer **2a** following a transformation to phenyl phosphoralaninate **4a** was also observed.[5,6] In addition, pronucleotides **3a** and **3b** have favorable cross-resistance patterns[7] with anti-HIV nucleoside analogues and in case of 2-amino-6-methoxypurine analogue **3c** in vivo effect against murine cytomegalovirus (MCMV) was noted.[8]

As indicated above, the pyrimidine methylenecyclopropanes **1** and **2** were largely devoid of antiviral effects but some exceptions were observed.[9] Thus, thymine analogue **1d** was effective against herpes simplex virus type 1 (HSV-1/BSC-1 ELISA) with EC_{50}/CC_{50} 2.0/>100 μM, Epstein-Barr virus (EBV/H-1 and varicella zoster virus [VZV/HFF, Table 1]). Cytosine analogues **1e** and **2e** were equally potent against EBV in Daudi culture. The *Z*-isomer **1e** was also active against EBV/H-1, varicella zoster virus (VZV/HFF), and it displayed some potency against human cytomegalovirus (HCMV). Also, transformation of pyrimidine anti-HIV agents, thymidine analogues zidovudine (AZT) and stavudine (d4T), into the corresponding phenyl phosphoralaninates provided effective and non-cytotoxic antivirals independent on the first phosphorylation step ("kinase bypass").[10] Investigation of the pyrimidine phosphoralaninate analogues **3** and **4** is then of interest. In this communication, synthesis and antiviral activity of thymine and cytosine pronucleotide analogues **3d**, **3e**, **4d**, and **4e** are described.

RESULTS AND DISCUSSION

Synthesis

Synthymol (**1d)** and the *E*-isomer **2d** served as convenient starting materials for pronucleotides **3d** and **4d**. Previously,[9,11] both isomers were obtained by an alkylation of 2,4-*bis*-O-trimethylsilyl-5-methylpyrimidine (**5)** with ethyl 2-bromo-2-bromomethylcyclopropropane carboxylate **(6a)** followed by β-elimination and reduction (Scheme 1). We have now simplified this protocol using acetate **6b** as an alkylating agent.[12,13] Intermediate **7** was obtained in 66% yield. The reduction step was eliminated and base-

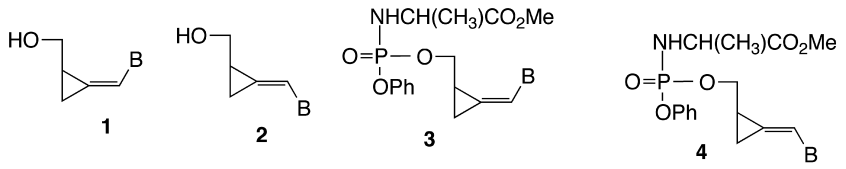
TABLE 1 Inhibition of HCMV, EBV, and VZV Replication by Phosphoralaninate Pronucleotides of Pyrimidine Methylenecyclopropane Analogues **3d**, **3e**, **4d**, **4e** and Parent Analogues **1d**, **1e**, **2d**, and **2e**

	EC_{50}/CC_{50} (μM)				
	HCMV/HFF		EBV		
Compound[a]	Towne[b,c]	AD169[d,e]	Daudi[f]	H-1[g]	EC_{50} VZV/HFF[b,h]
3d	>100/>100	>300/>300	>100/>100	>20/45	69.3
1d	>100/>100	>480/437	1.3/>240[i]	>10/>50[j]	3.6
4d	>100/>100	243/>300	>100/>100	>20/>100	>300[d]
2d	>100/>100	>96/>480	>240/>240[i]	>10/>50[j]	>100
3e	1.5/100	29.8/>300	0.65/>100	16/74	56.2
1e	28.5/>100	3.4/>518	<0.41/>259[i]	2.5/>50[j]	3.6
4e	>100/>100	>60/>300	14.4/>100	12/>100	92.4
2e	>100/>100	>518/>518	<0.41/>259[i]	>50/>50[j]	>518
Control	1.7/>100[k]	0.22/40[k]	0.73[l]	5[k]	0.22[l]

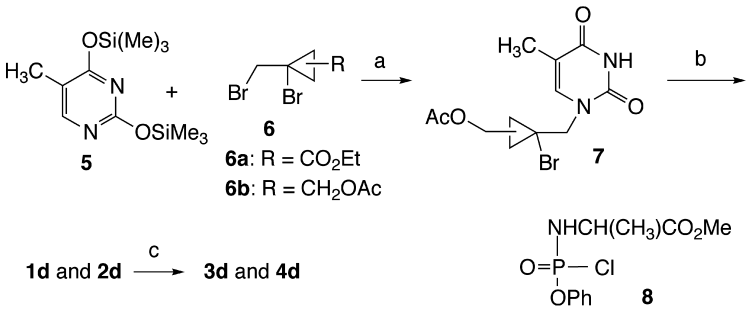
[a]Most of the results obtained with parent analogues **1d**, **1e**, **2d**, and **2e** were taken from Qiu et al.;[9] the rest are new data.
[b]Plaque reduction assay.
[c]Visual cytotoxicity.
[d]Cytopathic effect (CPE) inhibition assay.
[e]Stationary HFF cells. Cytotoxicity was determined by neutral red uptake.
[f]Viral capsid antigen (VCA) ELISA.
[g]DNA hybridization assay. Cytotoxicity was determined in CEM cells unless stated otherwise.
[h]For CC_{50} values see HCMV(AD169)/HFF.
[i]Viral capsid antigen immunofluorescence (VCA-IF) assay.
[j]Cytotoxicity was determined in H-1 cells.
[k]Ganciclovir. EC_{50} only.
[l]Acyclovir. EC_{50} only.

catalyzed elimination of elements of HBr from **7** combined with deacetylation afforded after chromatographic separation[9] *Z*- and *E*-isomers **1d** and **2d** in 33 and 19% yield, respectively. Phosphorylation with reagent **8** then afforded pronucleotides **3d** and **4d** in 54 and 42% yield, respectively.

Although cytidine analogue lamivudine (3TC) was directly phosphorylated using tert-butylmagnesium chloride and reagent **8** in THF[14] it was more convenient to start with N^4-benzoylcytosine analogues **9a** and **9b** (Scheme 2). Both compounds were intermediates in synthesis[9] of the *Z*- and *E*-isomers **1e** and **2e** and, unlike a mixture of **1e** + **2e**, they were separated by chromatography. Thus, alkylation-elimination of N^4-acetylcytosine (**10**) with acetate **6b** afforded, after deacetylation, a mixture of the (*Z,E*)-isomers **1e** + **2e** in the ratio of 1:2 and 53% yield. The N-benzoylation and following chromatographic separation provided isomers **9a** and **9b** in 21 and 41% yield, respectively. Phosphorylation with reagent **8** gave N^4-benzoyl pronucleotides **11a** and **11b** in 65 and 52% yield, respectively. The N-debenzoylation was effected with hydrazine in pyridine–acetic acid[15] to afford pronucleotides **3e** and **4e** in 29 and 27% yield.



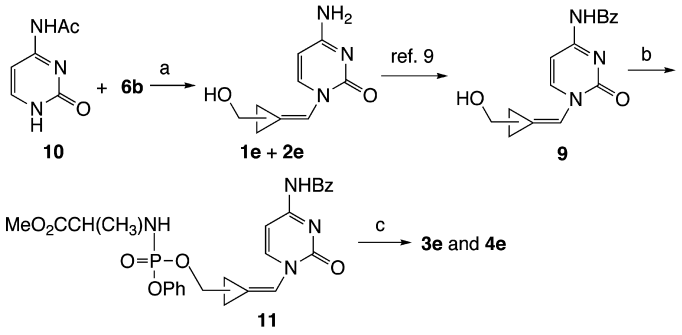
Series a: B = Ade, series b: B = 2,6-diaminopurine, series c: B = 2-amino-6-methoxypurine, series d: B = Thy, series e: B = Cyt



a. MeCN, Δ. b. K_2CO_3, DMF, Δ. c. **8**, 1-methylimidazole, pyridine.

SCHEME 1

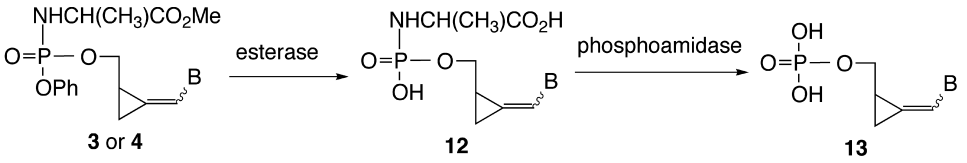
The ^{31}P NMR spectra indicated the expected presence of four diastereoisomers in pronucleotides **3d**, **4d**, **3e**, and **11a** whereas in the *E*-isomers **4e** and **11b** only three signals were observed due to overlapping peaks. This stereoisomerism was also reflected in numerous signals of ^{1}H and ^{13}C NMR spectra.



9a, **11a**: *Z*-isomer; **9b**, **11b**: *E*-isomer

a. 1. K_2CO_3, DMF, Δ. 2. MeOH, Δ. 3. Separation of isomers.
b. **8**, 1-methylimidazole, pyridine.
c. $N_2H_4.H_2O$, pyridine - AcOH (4 : 1).

SCHEME 2



SCHEME 3

Antiviral Activity

The antiviral activity of pyrimidine pronucleotides was restricted to cytosine analogues **3e** and **4e** (Table 1). Thus, the *Z*-cytosine analogue **3e** was effective against HCMV. It was more effective than analogue **1e** and as potent as ganciclovir in Towne virus assay. The activity pattern was reversed in AD169 strain where **1e** was superior to pronucleotide **3e**. The *E*-isomer **4e** was devoid of anti-HCMV potency. Pronucleotide **3e** was as potent against EBV in Daudi cells as the parent compound **1e** but less so in H-1 culture. All tested analogues were ineffective against HSV-1, HSV-2, HIV-1 and HBV. Pronucleotides **3d** and **3e** were significantly less active against VZV than the parent compounds **1d** and **1e**. The *E*-isomer **4e** was moderately active against EBV in both cell cultures. It was more effective in H-1 culture but less active in Daudi cells than the parent *E*-isomer **2e**.

Taken together, strong potentiating effects of phenyl phosphoralaninate group observed in the purine series[5,6] are absent in pronucleotides **3e** and **4e**. It has been established[3,4] that two key enzymes, esterase and phosphoamidase, are important for intracellular activation of phenyl phosphoralaninate pronucleotides (Scheme 3). The esterase action leads to phosphoralaninate **12** which is then converted to the respective phosphate **13** by phosphoamidase. All pronucleotides reported herein including the N^4-benzoyl derivatives **11a** and **11b** were substrates for porcine liver esterase (PLE) that is considered as a good model of intracellular esterases.[3,4] The substrate activity followed the lipophilicity pattern: **11a**, **11b** > **2d**, **3d** > **2e**, **3e**. It is then interesting that in several cases the pronucleotides were less potent than the parent analogues (Table 1). Possibly, a limited affinity of intermediates **12** toward phosphoamidase enzyme may be responsible for a decrease of activation effect of pyrimidine methylenecyclopropane pronucleotides.

EXPERIMENTAL SECTION

General Methods

The ^1H, ^{13}C, and ^{31}P NMR spectra were determined at 400, 100 and 162 MHz, respectively, in CD_3SOCD_3 as a solvent unless stated otherwise.

The ^{13}C NMR assignments were verified by DEPT spectra. The UV spectra were measured in ethanol. Mass spectrometry (MS) was perfomed in an electron-impact (EI) or electrospray ionization mode (ESI) on MICROMASS QUATTRO LC-MS instrument in MeOH-KOAc or NaCl. Porcine liver esterase (PLE) was a product of Sigma, St. Louis, Missouri. The (*Z,E*)-1-acetoxymethyl-2-bromo-2-bromoethylcyclopropane (**6b**) was prepared as described.[12,13]

(*Z,E*)-1-{[(Acetoxymethyl)-2-bromocyclopropyl]methyl}thymine (7). A mixture of 2,4-*bis*-O-(trimethylsilyloxy)-5-methylpyrimidine[16] (**5**, 4.62 g, 17 mmol), acetate **6b** (4.89 g, 17 mmol) was refluxed in acetonitrile (40 mL) under N_2 for 144 h. After cooling, ethanol (40 mL) was added and solvents were evaporated. The residue was triturated with CH_2Cl_2 (250 mL), the insoluble portion was filtered off using a Celite bed and it was washed with the same solvent (3 × 20 mL). The combined filtrate and washings were evaporated and the crude product was chromatographed on silica gel using CH_2Cl_2-MeOH (100:0 to 98:2) to give compound **7** as a white solid (3.7 g, 66%), mp 95–99°C. UV λ_{max} 269 nm (ε 10,600), 210 (ε 9,700). ^1H NMR ($CDCl_3$) δ 1.04 (s) and 1.41 (d, 2H, $H_{3'}$), 1.52 (bs, 1H, $H_{4'}$), 1.92 (s, 3H, 5-CH_3), 2.02, 2.06 (2s, 3H, CH_3 of Ac), 3.87–4.05 (m, 2H, $H_{5'}$), 4.27–4.47 (m, 2H, $H_{1'}$), 7.21, 7.27 (2s, 1H, H_6), 10.08 (bs, 1H, NH); ^{13}C NMR 12.6 (5-CH_3), 19.9, 21.0 ($C_{3'}$), 21.1 (CH_3 of Ac), 22.7, 27.4 ($C_{4'}$), 35.0, 38.4 ($C_{2'}$), 53.6, 57.6 ($C_{1'}$), 63.48, 66.49 ($C_{5'}$), 110.4, 110.5 (C_5), 141.0, 141.1 (C_6), 151.8, 151.9 (C_2), 164.8, 164.9 (C_4), 170.9, 171.1 (CO of Ac). EI-MS 332 and 330 (M, 9.2 and 9.2), 272 (M-OAc, 66.3), 250 (M-Br, 43.9), 191 (M-Br-OAc, 100.0). EI-HRMS Calcd. for $C_{12}H_{15}{}^{79}BrN_2O_4$: 330.0215; found 330.0220. Anal. Calcd. for $C_{12}H_{15}BrN_2O_4$; C, 43.52; H, 4.57; N, 8.46. Found: C, 43.70; H, 4.70; N, 8.49.

(*Z*)- and (*E*)-1-({[2-Hydroxymethyl)cyclopropylidene]methyl}thymine (1d) and (2d). Compounds **1d** and **2d** were prepared by a modification of the described procedure.[9] A mixture of compound **7** (1.30 g, 3.92 mmol) and K_2CO_3 (1.62 g, 11.76 mmol) in DMF (100 mL) was stirred at 100–110°C under N_2 for 7 h. After cooling, methanol-water (9:1, 25 mL) was added with stirring continued at room temperature for 1 h. The insoluble portion was filtered off and it was washed with DMF (2 × 20 mL). The filtrate was evaporated and the residue was chromatographed on a silica gel column with hexane-ethyl acetate mixture (2:3 to 3:2) to give the *Z*-isomer **1d** (269 mg, 33%) and *E*-isomer **2d** (155 mg, 19%) as white solids. The ^1H NMR and UV spectra corresponded to those reported previously.[9]

(*Z*)-1-{[(Hydroxymethyl)cyclopropylidene]methyl}thymine(methylphenylphosphoryl)-P→N-L-alaninate (3d). A suspension of the *Z*-isomer **1d** (288

mg, 1.384 mmol) in pyridine (35 mL) was sonicated for 5 min. Phosphorochloridate **8** in THF (0.184 M, 38.38 mL, 6.92 mmol) was then added dropwise with stirring at room temperature. After addition of 1-methylimidazole (1.10 mL, 13.84 mmol) the mixture was stirred for 2 h. The solvents were evaporated at room temperature and the residue was dried in vacuo overnight. Chromatography on silica gel using CH_2Cl_2-MeOH (98.5:1.5) gave a colorless syrup, which slowly solidified. Hexane (10 mL) was added, white solid **3d** (338 mg, 54%) was filtered off, and it was dried in vacuo. UV λ_{max} 287 nm (ε 13,100), 234 nm (ε 14,400), 205 nm (ε 17,800); ^{1}H NMR δ 1.13–1.27 (m, 4H, CH_3 of Ala, $H_{3'}$) and 1.44–1.50 (m, 1H, $H_{3'}$), 1.76, 1.77, 1.78 (3 poorly resolved d, 3H, 5-CH_3), 2.30–2.39 (m, 1H, $H_{4'}$), 3.54, 3.55, 3.57 (3s, 3H, OCH_3), 3.70–3.91 (m, 2H, $H_{5'}$), 4.08–4.27 (m, 1H, CH, Ala), 5.96–6.05 (m, 1H, NH, Ala), 7.10–7.16, 7.20–7.22, and 7.30–7.35 (3m, 6H, Ph, $H_{1'}$), 7.78–7.82 (m, 1H, H_6), 11.47, 11.48, 11.50 (3s, 1H, NH, Thy); ^{13}C NMR 6.2, 6.3, 6.4 ($C_{3'}$), 12.70. 12.73 (CH_3, Thy), 16.9, 17.0, 17.1 ($C_{4'}$), 20.28, 20.34 (CH_3, Ala), 50.2, 50.35, 50.4, 50.5 (CH, Ala), 52.52, 52.53, 52.6 (OCH_3), 68.3, 68.6 ($C_{5'}$), 110.93, 110.96, 111.01, 111.05 and 111.21, 111.24, 111.3 ($C_{2'}$ and C_5), 114.87, 114.93, 115.01, 115.04 ($C_{1'}$), 120.55, 120.6, 120.7, 120.8 (Ph, C_{meta}), 125.1, 125.2 (Ph, C_{ortho}), 130.2, 130.3 (Ph, C_{para}), 136.1, 136.3 (C_6), 150.1 (C_2), 151.27, 151.30 (Ph, C_{ipso}), 164.3 (C_4), 174.5 (CO, Ala); ^{31}P NMR 4.18, 4.30, 4.41, 4.60; ESI-MS 488 (M + K, 100.0), 450 (M + H, 18.5). Anal. Calcd. for $C_{20}H_{24}N_3O_7P$: C, 53.45; H, 5.38; N, 9.35. Found: C, 53.41; H, 5.66; N, 9.42.

(*E*)-1-{[(2-Hydroxymethyl)cyclopropylidene]methyl}thymine(methylphenylphosphoryl)-P→(N-L-alaninate (4d). The experiment was performed as described for the *Z*-isomer **1d** with *E*-isomer **2d** (187 mg, 0.9 mmol), pyridine (22 mL), phosphorochloridate **8** in THF (0.184 M, 24.9 mL, 4.5 mmol) and 1-methylimidazole (0.72 mL, 8.99 mmol). Chromatography afforded a colorless syrup **4d** which solidified during drying in vacuo (170 mg, 42%). UV λ_{max} 286 nm (ε 13,100), 233 nm (ε 13,500), 206 nm (ε 16,500); ^{1}H NMR δ 1.21 (t, 3H, CH_3 of Ala), 1.48–1.53 (m, 1H) and 1.76–1.79 (m, 1H, $H_{3'}$), 1.82 (s, 3H, 5-CH_3), 1.97–2.04 (m, 1H, $H_{4'}$), 3.57, 3.586, 3.590 (3s, 3H, OCH_3), 3.80–3.87 (m, 1H, CH of Ala), 3.90–4.02 (m, 2H, $H_{5'}$), 5.93–6.03 (m, 1H, NH of Ala), 7.13–7.20 (m), 7.29–7.37 (m, 6H, Ph, $H_{1'}$), 7.82 (poorly resolved d, 1H, H_6), 11.50 (s, 1H, NH, Thy); ^{13}C NMR 10.1, 10.2 ($C_{3'}$), 12.8 (5-CH_3), 14.0 ($C_{4'}$), 20.3, 20.4 (CH_3), 50.3, 50.5 (CH), 52.5 (OCH_3), 68.9 ($C_{5'}$), 111.0, 111.6, 111.7 ($C_{2'}$, C_5), 115.1 ($C_{1'}$), 120.8, 120.85, 120.9 (Ph, C_{meta}), 125.1 (Ph, C_{ortho}), 130.3 (Ph, C_{para}), 136.2 (C_6), 150.2 (C_2), 151.5 (Ph, C_{ipso}), 164.4 (C_4), 174.5 (CO, Ala); ^{31}P NMR 4.31, 4.33, 4.66, 4.69; EI-MS 449 (M, 2.1), 191 (M-O(PO)(OPh)NHCH(CH_3)CO_2CH_3, 100.0), 126 (Thy, 26.9); EI-HRMS calcd for $C_{20}H_{24}N_3O_7P$ 449.1352, found 449.1347. Anal. Calcd.

for $C_{20}H_{24}N_3O_7P$: C, 53.45; H, 5.38; N, 9.35. Found: C, 52.63; H, 5.70; N, 9.14.

(*Z,E*)-1-{[(2-Hydroxymethyl)cyclopropylidene]methyl}cytosine (1e + 2e). A mixture of N^4-acetylcytosine (**10**, 1.72 g, 11.2 mmol), acetate **6b** (3.21 g, 11.2 mmol) and K_2CO_3 (9.27 g, 67.2 mmol) in DMF (170 mL) was heated at 100–110°C (bath temperature) with stirring under N_2 for 13 h. The reaction mixture was cooled to 50°C and methanol (12 mL) was added with stirring continued for 7 h. After cooling, the insoluble portion was filtered off and it was washed with DMF (3 × 20 mL). The filtrate was evaporated in vacuo and the residue was chromatographed on a silica gel column using CH_2Cl_2-MeOH (9:1) to give the title compound **1d** + **2d** (1.13 g, 53%). The ^{1}H NMR spectrum (except the isomeric ratio *Z/E* = 1:2) corresponded to that of the product obtained by another method.[9]

(*Z*)- and (*E*)-N^4-Benzoyl-1-{[(2-hydroxymethyl)cyclopropylidene]methyl}cytosine (9a) and (9b). Both isomers were prepared as described.[9]

(*Z*)-N^4-Benzoyl-1-{[(2-hydroxymethyl)cyclopropylidene]methyl}cytosine (Methylphenylphosphoryl)-P→N-L-alaninate (11a). A suspension of *Z*-isomer **9a** (280 mg, 0.94 mmol) in pyridine (30 mL) was sonicated for 5 min. Phosphorochloridate **8** in THF (0.184 M, 26.1 mL, 4.71 mmol) was then added dropwise with stirring at room temperature. After addition of 1-methylimidazole (0.91 mL, 11.4 mmol) the stirring was continued for 2 h. The solvents were evaporated at room temperature, the oily residue was dried in vacuo overnight whereupon it was partitioned between ethyl acetate (250 mL) and water (100 mL). The aqueous phase was extracted with ethyl acetate (100 mL), the combined organic phase was washed with water (4 × 120 mL) and brine (2 × 80 mL), It was dried over Na_2SO_4 and the solvent was evaporated. Chromatography on silica gel with CH_2Cl_2-MeOH (98.5:1.5) gave pronucleotide **11a** as a colorless syrup, which solidified during drying in vacuo. Hexane (10 mL) was added, the white solid was filtered off and dried in vacuo (330 mg, 65%). UV λ_{max} 330 nm (ε 14,000), 270 (ε 19,900), 205 (ε 29,100); ^{1}H NMR δ 1.15–1.21 (m, 3H, CH_3 of Ala), 1.33–1.37 (m, 1H) and 1.54–1.58 (m, 1H, $H_{3'}$), 2.38–2.46 (m, 1H, $H_{4'}$), 3.53, 3.54, 3.55, 3.57 (4s, 3H, OCH_3), 3.70–3.90 (m, 1H, CH of Ala), 3.92–4.05 (m, 1H) and 4.06–4.19 (m, 1H, $H_{5'}$), 5.93–6.03 (m, 1H, NH of Ala), 7.14 (dd), 7.30 (dd), 7.41 (bs), 7.50 (t), 7.61 (t) and 7.99 (m, total 11H, Ph, Bz, $H_{1'}$ and H_5), 8.36–8.40 (m, 1H, H_6), 11.30 and 11.32 (2bs, 1H, BzNH); ^{13}C NMR 6.6, 6.7, 6.8 ($C_{3'}$), 16.9, 17.0 ($C_{4'}$), 20.2, 20.3 (CH_3, Ala), 50.2, 50.35, 50.41, 50.5 (CH, Ala), 52.5, 52.6 (OCH_3), 68.2 ($C_{5'}$), 97.7 (C_5), 116.0 ($C_{2'}$), 117.0 ($C_{1'}$), 120.69, 120.74, 120.8 (Ph, C_{meta}), 125.1 (Ph, C_{ortho}), 128.1, 128.9, 129.2, 130.2 (Ph, C_{para}, Bz, C_{meta}, C_{ortho}), 133.5, 133.8 (Bz, C_{para}, C_{ipso}),

145.3 (C_6), 151.4 (Ph, C_{ipso}), 154.1 (C_4), 163.8 (C_2), 168.1 (CO, Bz), 174.4 (CO, Ala); ^{31}P NMR 4.20, 4.34, 4.46, 4.62; ESI-MS 577 (M + K, 100.0), 539 (M + H, 12.8). Anal. Calcd. for $C_{26}H_{27}N_4O_7P$: C, 57.99; H, 5.05; N, 10.40. Found: C, 58.12; H, 5.17; N, 10.42.

(*E*)-N^4-Benzoyl-1-{[(2-hydroxymethyl)cyclopropylidene]methyl}cytosine (Methylphenylphosphoryl)-P→N-L-alaninate (11b). The experiment was performed as described for the *Z*-isomer **11a** with *E*-isomer **9b** (339 mg, 1.14 mmol), phosphorochloridate **8** (0.184 M, 32 mL, 5.7 mmol) and 1-methylimidazole (0.91 mL, 11.4 mmol), reaction time 7 h. Chromatography afforded product **11b** as a colorless syrup, which was converted to a white solid (332 mg, 52%) by trituration with ether (10 mL). UV λ_{max} 330 nm (ε 14,700), 270 nm (ε 20,400), 205 nm (ε 28,200); ^{1}H NMR δ 1.20–1.24 (m, 3H, CH_3 of Ala), 1.55 (m, 1H) and 1.78–1.82 (m, 1H, $H_{3'}$), 2.04–2.12 (m, 1H, $H_{4'}$), 3.58, 3.59 (2s, 3H, OCH_3), 3.82–3.88 (m, 1H, CH of Ala), 3.95–4.02 (m, 2H, $H_{5'}$), 5.96–6.06 (m, 1H, NH of Ala), 7.14–7.15 (m), 7.20–7.36 (m), 7.49 (t), 7.61 (t) and 8.00 (d, total 11H, Ph, Bz, $H_{1'}$ and H_5, 8.46 (poorly resolved d, 1H, H_6), 11.33 (s, 1H, BzNH); ^{13}C NMR 9.9, 10.0 ($C_{3'}$), 14.3 ($C_{4'}$), 20.3, 20.4 (CH_3, Ala), 50.4, 50.5 (CH, Ala), 52.5 (CH_3O), 68.7 ($C_{5'}$), 97.8 (C_5), 116.0 ($C_{2'}$), 116.8 ($C_{1'}$), 120.87, 120.91 (Ph, C_{meta}), 125.1 (Ph, C_{ortho}), 129.1, 129.2, 130.3 (Ph, C_{para}, Bz, C_{meta}, C_{ortho}), 133.4, 133.8 (Bz, C_{para}, C_{ipso},), 145.1 (C_6), 151.4, 151.5 (Ph, C_{ipso}), 154.1 (C_4), 163.8 (C_2), 168.2 (CO); ^{31}P NMR 4.34, 4.37, 4.73; ESI-MS 577 (M + K, 22.2), 539 (M + H, 100.0). Anal. Calcd. For $C_{26}H_{27}N_4O_7P$: C, 57.99; H, 5.05; N, 10.40. Found: C, 57.78; H, 5.21; N, 10.28.

(*Z*)-1-{[(2-Hydroxymethyl)cyclopropylidene]methyl}cytosine (Methylphenylphosphoryl)-P→N-L-alaninate (3e). A mixture of **11a** (330 mg, 0.61 mmol) and hydrazine hydrate (0.35 mL, 4.88 mmol) in pyridine–acetic acid (10 mL, 4:1 v/v) was stirred at room temperature for 27 h. The solvents were removed at room temperature in vacuo at room temperature and the oily residue was partitioned between CH_2Cl_2 (250 mL) and water (200 mL). The aqueous phase was extracted with the same solvent (2 × 25 mL). The combined organic phase was washed with brine (2 × 100 mL) and water (2 × 100 mL), it was dried over Na_2SO_4 and evaporated to a yellow syrup. Chromatography on silica gel with CH_2Cl_2-MeOH (94:6) gave product **3e** as a pale yellow solid (78 mg, 29%) after washing with hexane-ethyl acetate (99:1, 10 mL) and drying in vacuo. UV λ_{max} 297 nm (ε 13,100), 228 nm (ε 14,300), 205 nm (ε 26,400); ^{1}H NMR δ 1.15–1.21 (m, 4H, CH_3 of Ala, $H_{3'}$), 1.40–1.47 (m, 1H, $H_{3'}$), 2.21–2.28 (m, 1H, $H_{4'}$), 3.55, 3.57, 3.58 (3s, 3H, OCH_3), 3.74–3.86 (m, 1H, CH of Ala), 3.88–4.10 (m, 2H, $H_{5'}$), 5.76–5.80 (m, 1H, H_5), 5.95–6.05 (m, 1H, NH of Ala), 7.12–7.18 and 7.32–7.40 (2m, 8H, Ph, NH_2, $H_{1'}$), 7.88–7.94 (m, 1H, H_6); ^{13}C NMR 6.2, 6.4 ($C_{3'}$), 16.75,

16.81 ($C_{4'}$), 20.26, 20.32, 20.4 (CH_3, Ala), 50.2, 50.4, 50.46, 50.50 (CH, Ala), 52.6 (OCH_3), 68.4, 68.6 ($C_{5'}$), 95.8, 95.9 (C_5), 109.7, 109.89, 109.93 ($C_{2'}$), 117.2, 117.3 ($C_{1'}$), 120.8, 120.9 (Ph, C_{meta}), 125.2 (Ph, C_{ortho}, 130.3 (Ph, C_{para}), 140.7, 140.8, 140.9 (C_6), 151.4 (Ph, C_{ipso}), 154.5 (C_4), 166.1 (C_2), 174.3, 174.5 (CO, Ala); ^{31}P NMR 4.20, 4.34, 4.55, 4.68; ESI-MS 891 (2M + Na, 41.1), 869 (2M + H, 100.0), 457 (M + Na, 74.4), 435 (M + H, 100.0). Anal. Calcd. for $C_{19}H_{23}N_4O_6P$: C, 52.54; H, 5.34; N, 12.90. Found: C, 52.60; H, 5.51; N, 12.97.

(*E*)-1-{[(2-Hydroxymethyl)cyclopropylidene]methyl}cytosine (Methylphenylphosphoryl)-P→N-L-alaninate (4e). A mixture of **11b** (337 mg, 0.625 mmol), hydrazine hydrate (0.4 mL, 6.4 mmol) in pyridine–acetic acid (18 mL, 4:1 v/v) was stirred at room temperature for 17 h. The reaction mixture was worked up as described for the *Z*-isomer **3e**. Chromatography on silica gel with CH_2Cl_2-MeOH (92:8) gave product **4e** as a pale yellow solid (74 mg, 27%). UV λ_{max} 296 nm (ε 13,000), 229 nm (ε 13,700), 204 nm (ε 27,400); ^{1}H NMR δ 1.19–1.23 (m, 3H, CH_3 of Ala), 1.42–1.46 (m, 1H), 1.69–1.73 (m, 1H, $H_{3'}$), 1.93–1.99 (m, 1H, $H_{4'}$), 3.57, 3.58 (2s, 3H, OCH_3), 3.80–3.88 (m, 1H, CH of Ala), 3.90–4.02 (m, 2H, $H_{5'}$), 5.83 (d, 1H, $^3J_{5,6}$ 7.6 Hz, H_5), 5.95–6.05 (m, 1H, NH of Ala), 7.12–7.20 (m), 7.33–7.39 (m) and 7.45 (s, total 8H, Ph, NH_2, $H_{1'}$), 7.95–7.98 (m, 1H, H_6); ^{13}C NMR 9.8, 9.9 ($C_{3'}$), 13.7 ($C_{4'}$), 20.3, 20.4 (CH_3), 50.4, 50.5 (CH, Ala), 52.5 (OCH_3), 69.1 ($C_{5'}$), 95.9 (C_5), 110.4 ($C_{2'}$), 117.1 ($C_{1'}$), 120.9 (Ph, C_{meta}), 125.1 (Ph, C_{ortho}), 130.3 (Ph, C_{para}), 140.8 (C_6), 151.5 (Ph, C_{ipso}), 154.6 (C_4), 166.1 (C_2), 174.5 (CO, Ala); ^{31}P NMR 4.32, 4.37, 4.70; ESI-MS 869 (2M + H, 23.5), 457 (M + Na, 42.9), 435 (M + H, 100.0). Anal. Calcd. for $C_{19}H_{23}N_4O_6P$: C, 52.54; H, 5.34; N, 12.90. Found: C, 52.39; H, 5.39; N, 12.72.

Hydrolysis of Phosphoralaninate Pronucleotides with Porcine Liver Esterase (PLE)

Compounds **3d**, **3e**, **4d**, **4e**, **11a**, and **11b** (1.6 μmol each) were stirred with PLE (200 U, 5 mg) in 0.01 M Na_2HPO_4 (pH 7.5, 1 mL) at room temperature. Aliquots were periodically withdrawn and checked by TLC in CH_2Cl_2-MeOH (19:1 or 9:1). The N^4-benzoyl pronucleotides **11a**, **11b** were hydrolyzed within 1 h, thymine analogues **3d**, **4d** within 16–20 h and cytosine derivatives **3e**, **4e** within 36–40 h.

Antiviral Assays

The antiviral assays were described in details in the previous communications.[9,17,18] The HCMV (Towne and AD169 strains) and VZV assays were performed in HFF culture using a plaque reduction or cytopathic effect

(CPE) inhibition assay. The EBV assays were performed in Daudi cells by viral capsid antigen (VCA) ELISA and in H-1 cells by DNA hybridization assay. The cytotoxicity assays were performed in HFF and CEM cells. For further details see Table 1. For comparison, antiviral data for the parent analogues **1d**, **2d**, **1e**, and **2e** are also given.

REFERENCES

1. Zemlicka, J. Unusual analogues of nucleosides: Chemistry and biological activity. In *Recent Advances in Nucleosides: Chemistry and Chemotherapy*; Chu, C.K., Ed.; Elsevier Science, 2002; 327–357.
2. Zemlicka, J.; Chen, X. Methylenecyclopropane analogues of nucleosides as antiviral agents. In *Frontiers in Nucleosides and Nucleic Acids*; Schinazi, R.F.; Liotta, D.C., Eds.; IHL Press: Tucker, GA, 2004; 267–307.
3. Zemlicka, J. Lipophilic phosphoramidates as antiviral pronucleotides. Biochim. Biophys. Acta **2002**, 1587, 276–286.
4. Cahard, D.; McGuigan, C.; Balzarini, J. Aryloxy phosphoramidate triesters as pro-tides. Mini-Rev. Med. Chem. **2004**, 4, 371–382.
5. Qiu, Y.-L.; Ptak, R.G.; Breitenbach, J.M.; Lin, J.-S.; Cheng, Y.-C.; Drach, J.C.; Kern, E.R.; Zemlicka, J. Synthesis and antiviral activity of phosphoralaninate derivatives of methylenecyclopropane analogues of nucleosides. Antiviral Res. **1999**, 43, 37–53.
6. Uchida, H.; Kodama, E.N.; Yoshimura, K.; Maeda, Y.; Kosalaraksa, P.; Maroun, V.; Qiu, Y.-L.; Zemlicka, J.; Mitsuya, H. In vitro anti-human immunodeficiency virus activities of *Z*- and *E*-methylenecyclopropane nucleoside analogues and their phosphoro-L-alaninate diesters. Antimicrob. Agents Chemother. **1999**, 43, 1487–1490.
7. Yoshimura, K.; Feldman, R.; Kodama, E.; Kavlick, M.F.; Qiu, Y.-L.; Zemlicka, J.; Mitsuya, H. In vitro induction of Human Immunodeficiency Virus Type 1 variants resistant to phosphoralaninate prodrugs of *Z*-methylenecyclopropane nucleoside analogues. Antimicrob. Agents Chemother. **1999**, 43, 2479–2483.
8. Rybak, R.J.; Zemlicka, J.; Qiu, Y.-L.; Hartline, C.B.; Kern, E.R. Effective treatment of murine cytomegalovirus infections with methylenecyclopropane analogues of nucleosides. Antiviral Res. **1999**, 43, 175–188.
9. Qiu, Y.-L.; Ptak, R.G.; Breitenbach, J.M.; Lin, J.-S.; Cheng, Y.-C.; Kern, E.R.; Drach, J.C.; Zemlicka, J. (*Z*)- and (*E*)-2-(hydroxymethylcyclopropylidene)methyl-purines and -pyrimidines as antiviral agents. Antiviral Chem. Chemother. **1998**, 9, 341–352.
10. McGuigan, C.; Cahard, D.; Sheeka, H.M.; De Clercq, E.; Balzarini, J. Phosphoramidate derivatives of d4T with improved anti-HIV efficacy retain full activity in thymidine kinase-deficient cells. Bioorg. Med. Chem. Lett. **1996**, 6, 1183–1186.
11. Zhou, S.; Kern, E.R.; Gullen, E.; Cheng, Y.-C.; Drach, J.C.; Matsumi, S.; Mitsuya, H.; Zemlicka, J. (*Z*)- and (*E*)-[2-Fluoro-2-(hydroxymethyl)cyclopropylidene]methyl-purines and -pyrimidines, a new class of methylenecyclopropane analogues of nucleosides: Synthesis and antiviral activity. J. Med. Chem. **2004**, 47, 6964–6972.
12. Qiu, Y.-L.; Zemlicka, J. A new efficient synthesis of antiviral methylenecyclopropane analogs of purine nucleosides. Synthesis **1998**, 1447–1452.
13. Chen, X.; Zemlicka, J. Revision of absolute configuration of enantiomeric (methylenecyclopropyl)carbinols obtained from (*R*)-(−)-and (*S*)-(+)-epichlorohydrin and methylenetriphenylphosphorane. Implications for reaction mechanism and improved synthesis of methylenecyclopropane analogues of nucleosides. J. Org. Chem. **2002**, 67, 286–289.
14. Balzarini, J.; Wedgwood, O.; Kruining, J.; Pelemans, H.; Heijtink, R.; De Clercq, E.; McGuigan, C. Anti-HIV and anti-HBV activity and resistance profile of 2′,3′-dideoxy-3′-thiacytidine (3TC) and its arylphosphoramidate derivative CF 1109. Biochem. Biophys. Res. Commun. **1996**, 225, 363–369.
15. Letsinger, R.L.; Miller, P.S.; Grams, G.W. Selective N-debenzoylation of N,O-polybenzoylnucleosides. Tetrahedron Lett. **1968**, 2621–2624.

16. Iwai, I.; Nishimura, T.; Shimizu, B. Anomeric pentofuranosyluracils and pentofuranosylthymines. In *Synthetic Procedures in Nucleic Acid Chemistry*; Zorbach, W.W., Tipson, R.S., Eds.; Wiley: New York, 1968; Vol. 1, 388–394.
17. Qiu, Y.-L.; Ksebati, M.B.; Ptak, R.G.; Fan, B.Y.; Breitenbach, J.M.; Lin, J.-S.; Cheng, Y.-C.; Kern, E.R.; Drach, J.C.; Zemlicka, J. (*Z*)- and (*E*)-2-(hydroxymethyl)cyclopropylidenemethyladenine and -guanine. New nucleoside analogues with a broad spectrum of antiviral activity. J. Med. Chem. **1998**, 41, 10–23.
18. Kushner, N.L.; Williams, S.L.; Hartline, C.B.; Harden, E.A.; Bidanset, D.J.; Chen, X.; Zemlicka, J.; Kern, E.R. Efficacy of methylenecyclopropane analogs of nucleosides against herpesvirus replication in vitro. Nucleosides, Nucleotides & Nucleic Acids **2003**, 22, 2105–2119.