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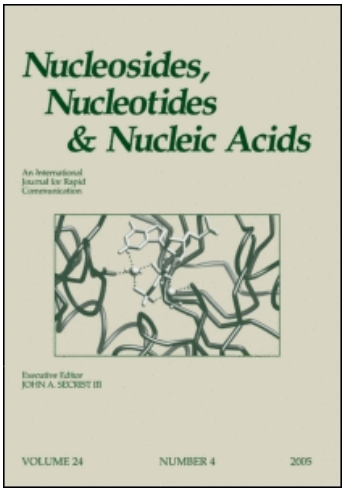

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SYNTHESIS AND ENANTIOSELECTIVITY OF CYCLOPROPAVIR PHOSPHATES FOR CELLULAR GMP KINASE

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□ *Enantiomeric cyclopropavir phosphates (+)-**9** and (−)-**9** were synthesized and investigated as substrates for GMP kinase. N^2-Isobutyryl-di-O-acetylcyclopropavir (**11**) was converted to (+)-monoacetate **12** using hydrolysis catalyzed by porcine liver esterase. Phosphorylation via phosphite **13** gave after deacylation, phosphate (+)-**9**. Acid-catalyzed tetrahydropyranylation of (+)-monoacetate **12** gave, after deacylation, tetrahydropyranyl derivative **14**. Phosphorylation via phosphite **15** furnished, after deprotection, enantiomeric phosphate (-)-**9**. Racemic diphosphate **16** was also synthesized. The phosphate (+)-**9** is a relatively good substrate for GMP kinase with a K_M value of 57 μM that is similar to that of the natural substrates GMP (61 μM) and dGMP (82 μM). In contrast, the enantiomer (−)-**9** is not a good substrate (K_M 1200 μM) indicating a significant enantioselectivity for the GMP kinase catalyzed reaction of monophosphate to diphosphate.*

Keywords Methylenecyclopropanes; nucleoside analogues; cyclopropavir; phosphates; enantiomers; enantioselectivity; antivirals; GMP kinase

INTRODUCTION

Methylenecyclopropane analogues of nucleosides are established antiviral agents that are particularly effective against herpesviruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpes virus 6 and 8 (HHV-6 and HHV-8).[1] The most potent analogues

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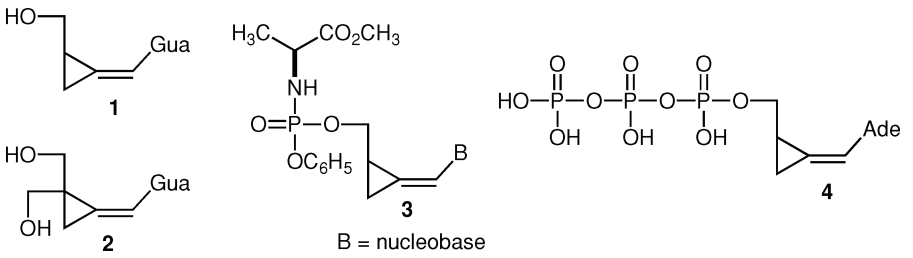

CHART 1

against CMV are the purine *Z*-isomers. In the first generation group, the 2-aminopurines like synguanol (**1**) have gained particular prominence (Chart 1). The anti-CMV potency of synguanol and other 2-aminopurine methylenecyclopropanes is strictly enantioselective; the *S*-(+)-enantiomers are effective and *R*-(-)-enantiomers are inactive.[2,3] In the second generation series, cyclopropavir (**2**) has emerged as the most potent anti-CMV analogue presently in preclinical development as a therapeutic agent against human cytomegalovirus (HCMV) infections.[4–7] Mechanism of action of methylenecyclopropanes, such as **1** and **2**, undoubtedly includes the phosphorylation cascade observed for other nucleoside analogues: monophosphate–diphosphate–triphosphate. In the first generation series, potent antiviral activity of lipophilic phosphorylated prodrugs[8,9] **3** as well as inhibition of HIV-1 reverse transcriptase with synadenol triphosphate[10] (**4**) provided strong evidence for the above activation pathway. Resistance studies have supported involvement of HCMV-encoded UL97 phosphotransferase in the first phosphorylation step of synguanol[11] (**1**) and cyclopropavir[12–15] (**2**).

Cyclopropavir (**2**) can be considered a rigid analogue of ganciclovir (Cytovene, **5**), an approved drug against HCMV infections (Scheme 1). Ganciclovir is also achiral but phosphorylation studies revealed that chirality is critically involved in its activation. Unlike methylenecyclopropane analogues, such as **1** and **2**, ganciclovir (**5**) is also effective against herpes simplex 1 (HSV-1) infections. It was established that the first phosphorylation step is catalyzed by HSV-1 thymidine kinase to give the phosphates **6** in an enantioselective process.[16] It has to be stated that HCMV lacks thymidine kinase but it employs UL97 phosphotransferase[17,18] of unknown enantioselectivity in the first phosphorylation step of ganciclovir (**5**). It was also established that the formation of a diphosphate **7** is catalyzed by guanylate (GMP) kinase in an enantioselective process.[16] This enzyme is also involved in activation of other guanine nucleoside analogues.[19,20] In the last step, diphosphate 7 is converted to the respective triphosphate **8** by cellular kinases.

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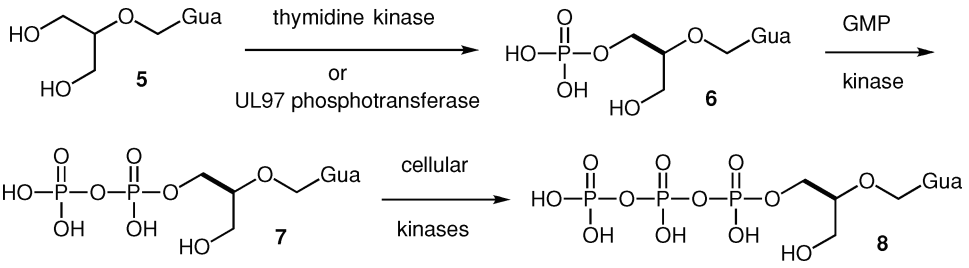

SCHEME 1 Phosphorylation cascade of ganciclovir (**5**).

The purpose of this study was two-fold: (i) synthesis of enantiomeric phosphates of cyclopropavir (+)-**9** and (−)-**9**; and (ii) to determine their substrate affinity for GMP kinase.

RESULTS AND DISCUSSION

Synthesis

Acetylation of N^2-isobutyrylganciclovir catalyzed by porcine pancreatic lipase (PPL) was employed for synthesis of protected enantiomers of ganciclovir.[21] We then assumed that N^2-isobutyrylcyclopropavir **(10)** could be used as a starting point for both enantiomeric phosphates (+)-**9** and (−)-**9**. Therefore, cyclopropavir (**2**) was transformed to compound **10** in 85% yield by acylation-deacylation procedure[21] (Scheme 2). Nevertheless, under the conditions described for N^2-isobutyrylganciclovir[21]/porcine pancreatic lipase (PPL) in pyridine- benzene/ no acetylation was observed. For this reason, N^2-isobutyrylcyclopropavir **(10)** was transformed to diacetate **11** (84%). The PLE-catalyzed hydrolysis afforded (+)-acetate **12** in 64% yield and 95% ee. Unreacted diacetate **11** (18%) and diol **10** (16%) were also isolated. Phosphorylation of (+)-**12** was performed in analogy to the corresponding racemic[22] compound *rac*-**12** (*i*-But = H). Reaction of (+)-**12** with diphenyl phosphite in pyridine followed by triethylamine gave phosphite **13** (83%). The latter was converted to phosphate **(+)-9** by oxidation of trimethylsilyl ester intermediate with iodine in pyridine. Deprotection in 80% acetic acid followed by ammonolysis gave (+)-**9** in 82% yield. Synthesis of enantiomeric phosphate (−)-**9** made use of a simple interchange of the protecting groups (Scheme 3). Starting material, tetrahydropyranyl derivative **14**, was synthesized in 97% yield from acetate (+)-**12** by a procedure described[22] for compound **14** obtained from acetate *rac*-**12** (*i*-But = H). Phosphorylation followed the procedure employed for enantiomeric phosphate (+)-**9**. Intermediary phosphite **15** obtained in 93% yield was oxidized and the respective intermediate was deprotected by a treatment with 80% acetic acid to give phosphate (−)-**9** (69%). Tentatively, the *S* configuration can be proposed for phosphate (+)-**9** as based on the antiviral

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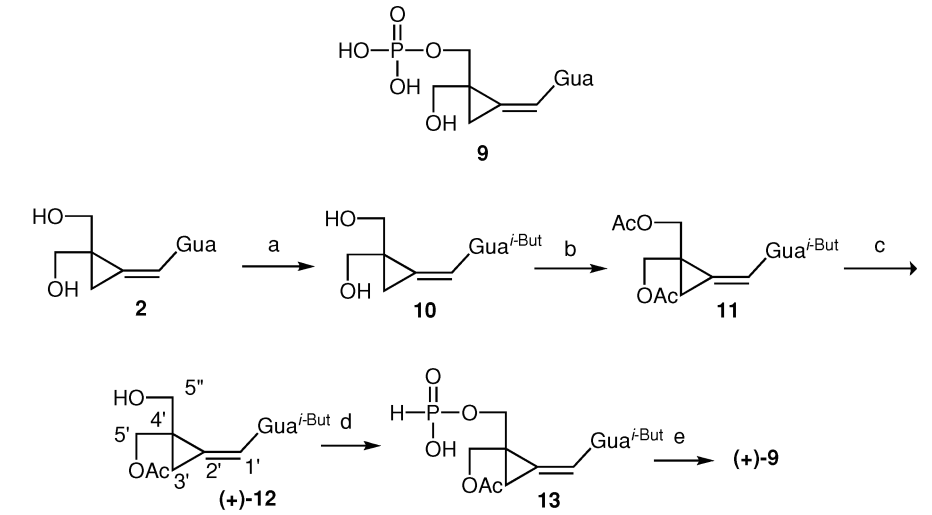

SCHEME 2 Synthesis of cyclopropavir phosphate (+)-**9** (*i*-But, isobutyryl; THP, tetrahydropyranyl; $COIm_2$, N,N′-carbonyldiimidazole).

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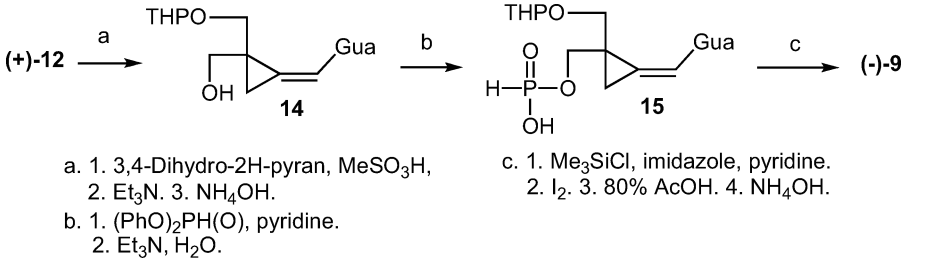
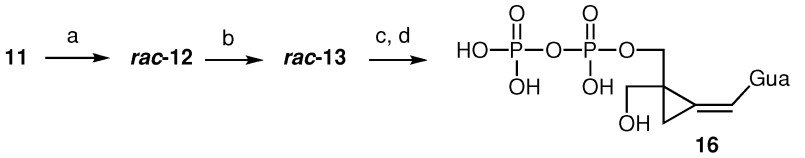
activity of *S*-(+)-synguanol.[2] We also prepared racemic diphosphate **16** to confirm identity of the product of GMP kinase catalyzed phosphorylation (Scheme 4). Compound **11** was transformed to racemic acetate *rac*-**12** (93%) by a procedure previously used[22] for unprotected racemic compound *rac*-**12** (*i*-But = H). Compound *rac*-**12** was converted to phosphite *rac*-**13** in 80% yield as described above for enantiomeric compound **13**. Oxidation followed by activation with N,N-carbonyldiimidazole[23] and reaction with inorganic phosphate followed by deacylation gave the racemic diphosphate **16** in 62% yield.



SCHEME 3 Synthesis of cyclopropavir phosphate (−)-**9** (*i*-But, isobutyryl; THP, tetrahydropyranyl; $COIm_2$, N,N′-carbonyldiimidazole).



a. $MeC(OMe)_3$, TsOH, CH_2Cl_2.
b. 1. $(PhO)_2P(O)H$, pyridine. 2. NEt_3-H_2O (3 : 2)..
c. 1. Me_3SiCl, imidazole, pyridine. 2. I_2.
d. 1. Bu_3N, $COIm_2$, 2. MeOH.
3. $(Bu_3NH)_2HPO_4$, MeOH. 4. NH_4OH.

SCHEME 4 Synthesis of racemic cyclopropavir diphosphate **16** (*i*-But, isobutyryl; THP, tetrahydropyranyl; $COIm_2$, N,N′-carbonyldiimidazole).

Guanylate Kinase Assays

Experiments to determine whether or not phosphate (+)-**9** and/or (−)-**9** are substrates for GMP kinase were performed under the conditions described[21] for enantiomeric ganciclovir phosphates **6** and *ent*-**6** at 34°C. A time course (Figure 1) demonstrated that cyclopropavir diphosphate **16** formed from phosphate (+)-**9** reached equilibrium after 3 hours with approximately 80% of diphosphate **16**, whereas a maximum of only 10% of enantiomeric diphosphate **16** was formed from phosphate (−)-**9** during the course of the experiment. This established a significant enantioselectivity for the reaction of (−)-**9** to diphosphate **16** with GMP kinase.

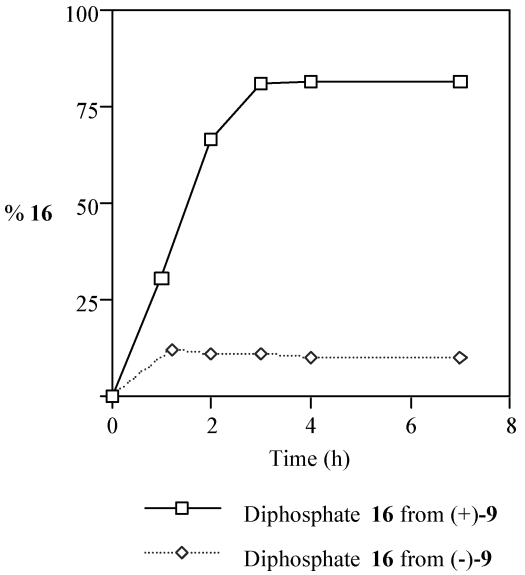


FIGURE 1 Time course of phosphorylation of enantiomeric cyclopropavir phosphates (+)-**9** and (−)-**9** catalyzed by GMP kinase.

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TABLE 1 Kinetic values for enantiomeric cyclopropavir phosphates and natural substrates

Compound	K_M (μM)	V_{max} (nmol/min)	V_{max}/K_M
Phosphate (+)-**9**	57	0.16	2.8×10^{-3}
Phosphate (−)-**9**	1200	0.23	1.9×10^{-4}
GMP	61	0.33	5.4×10^{-3}
dGMP	82	0.54	6.6×10^{-3}

Kinetic values of K_M and V_{max} for (+)-**9** and (−)-**9** were determined using the Lineweaver-Burk double reciprocal plot methodology (Table 1). A K_M value of 57 μM was found for (+)-**9** compared to 1200 μM for the (−)-**9** enantiomer. The value for the (+)-enantiomer was similar to that found for the natural substrates GMP (61 μM) and dGMP (82 μM). Although the V_{max} for (−)-**9** (0.23 nmol/min) was somewhat greater than that of (+)-**9** (0.16 nmol/min), the difference was not enough to make up for the relatively high K_M resulting in the inability of GMP kinase to effectively phosphorylate (−)-**9** to diphosphate **16** (Figure 1).

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EXPERIMENTAL SECTION

General Methods

The NMR spectra were determined at 300 or 400 MHz (^{1}H), 75 or 100 MHz (^{13}C) and 121 or 162 MHz (^{31}P) in DMSO-d_6, UV spectra were measured in ethanol and mass spectra were run in electrospray ionization (ESI) mode (methanol-NaOAc) unless stated otherwise. High performance liquid chromatography (HPLC) was performed on Hamilton PRP-1 column, 150 × 4.1 mm, 10 μ (Hamilton Co., Reno, NV, USA; column 1) and Brownlee Anion Aquapore AX-300 column, 250 × 4.6 mm, 10 μ (Anspec, Inc., Ann Arbor, MI, USA; column 2). Chiral HPLC was run on Chirobiotic T column, 250 × 4.6 mm, 5 μ (Advanced Separation Technologies, Inc., Whippany, NJ, USA) unless stated otherwise.

Starting Materials and Enzymes

Cyclopropavir (**2**) was obtained from Microbiotix, Inc. (Worcester, MA, USA). Porcine liver esterase (PLE), phosphoenol pyruvate, lactate dehydrogenase, pyruvate kinase, bovine serum albumin (BSA), and bovine guanylate (GMP) kinase were products of Sigma-Aldrich Corp. (St. Louis, MO, USA).

(Z)-9-{[2,2-Bis(hydroxymethyl)cyclopropylidene]methyl}-N^2-isobutyrylguanine (10): Isobutyryl chloride (0.5 mL, 4.5 mmol) was added dropwise to a suspension of cyclopropavir (**2**, 220 mg, 0.84 mmol) in pyridine (20 mL) at 0°C. The mixture was stirred at room temperature for 16 h. The reaction

was quenched with methanol (5 mL) and the solvents were evaporated. The residue was dissolved in (pyridine-methanol (2: 1, 30 mL) and the pH was carefully adjusted to 12.5 with 2M NaOH and the mixture was kept at room temperature for 10 minutes. The pH was then adjusted to 7.0 with 2M HCl and the solvents were evaporated. The residue was chromatographed on a silica gel column using dichloromethane-methanol (10 : 1) to give compound **10** (236 mg, 85%) as a white solid, m.p. 214–216°C. UV λ_{max} 294 nm (ε 11,900), 240 nm (ε 26,800). ^{1}H NMR δ 12.08, 11.70 (2bs, 2H, NH), 8.68 (s, 1H, H_8), 7.16 (s, 1H, $H_{1'}$), 5.02 (2 overlapped t, 2H, OH), 3.67, 3.49 and 3.66, 3.48 (2AB, 4H, J = 11.2 and 11.0 Hz, $H_{5'}$), 2.75 (m, 1H, CH of isobutyryl), 1.35 (s, 2H, $H_{3'}$), 1.10 (d, 6H, J = 7.2 Hz, CH_3). ^{13}C NMR 180.8 (CO), 155.5, 149.0, 147.7, 137.3, 120.4, 120.2 (purine, $C_{2'}$), 110.6 ($C_{1'}$), 62.7 ($C_{5'}$), 35.4 (CH of isobutyryl), 31.5 ($C_{4'}$), 19.5 (CH_3), 11.7 ($C_{3'}$). ESI-MS 334 (M + H, 6.0), 356 (100.0, M + Na), 689 (42.0, 2M + Na). Anal. Calcd for $C_{15}H_{19}N_5O_4 \times 2\ H_2O$: C, 48.78; H, H, 6.28; N, 18.96. Found: C, 48.84; H, 6.22; N, 18.75.

(Z)-9-{[2,2-Bis(acetoxymethyl)cyclopropylidene]methyl}-N^2-isobutyrylguanine (11): A mixture of isobutyryl derivative **10** (200 mg, 0.6 mmol), 4-(N-dimethylamino)pyridine (DMAP, 10 mg, 0.08 mmol) in DMF (20 mL) and acetic anhydride (0.4 mL, 4.2 mmol) was stirred for 1 hour at room temperature. Solvent was evaporated and the crude product was chromatographed on a silica gel column using dichloromethane-methanol (50 : 1 to 25 : 1) to give diacetate **11** (202 mg, 84%) as a white solid, m.p. 124–126°C. UV λ_{max} 292 nm (ε 12,600), 236 nm (ε 26,100). ^{1}H NMR δ 12.10, 11.69 (2bs, 2H, NH), 8.22 (s, 1H, H_8), 7.25 (s, 1H, $H_{1'}$), 4.34, 4.07 (AB, 4H, J = 11.6 Hz, $H_{5'}$), 2.75 (m, 1H, CH of isobutyryl), 1.95 (s, 6H, CH_3 of acetate), 1.69 (s, 2H, $H_{3'}$), 1.10 (d, J = 6.5 Hz, 6H, CH_3 of isobutyryl). ^{13}C NMR 180.9, 170.7 (CO), 155.4, 149.2, 148.1, 137.0, 120.5 (purine), 118.6 ($C_{2'}$), 112.6 ($C_{1'}$), 66.0 ($C_{5'}$), 35.5 (CH of isobutyryl), 25.3 ($C_{4'}$), 21.1 (CH_3 of acetate), 19.5 (CH_3 of isobutyryl), 13.4 ($C_{3'}$). ESI-MS 418 (M + H, 3.6), 440 (100.0, M + Na). Anal. Calcd for $C_{19}H_{23}N_5O_6 \times 1.2\ H_2O$: C, 51.98; H, 5.79; N, 15.95. Found: C, 51.91; H, 5.74; N, 15.82.

(+)-(R,Z)-9-{[2-(Acetoxymethyl)-2-(hydroxymethyl)cyclopropylidene]methyl}-N^2-isobutyrylguanine (12): A mixture of diacetate **11** (100 mg, 0.25 mmol) and porcine liver esterase (PLE, 210 mg, 8,610 units) in DMF (15 mL) and 0.02 M Na_2HPO_4 (pH 7.0, 100 mL) was stirred for 45 minutes at room temperature, then lyophilized. The residue was sonicated with dichloromethane-methanol (3 : 1, 3 × 100 mL) and the insoluble portion was filtered off. The solvents were evaporated and the crude product was chromatographed on a silica gel column using dichloromethane-methanol (10: 1) to give monoacetate **12** (60 mg, 64%) as a white solid. Starting diacetate **11** (18 mg, 18%) and diol **10** (13 mg, 16%) were also obtained.

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(+)-Enantiomer **12**: m.p. 192–195°C, $[\alpha]_D^{25}$ 21.7° (c 1.0, DMSO), chiral HPLC in methanol as an eluent, flow rate 1 mL/min, detection at 290 nm, retention time (RT) 5.5 minutes, 97.6%, ee 95.2%)/ and 6.6 minutes /(-)-enantiomer **12**, 2.4%/. UV λ_{max} 291 nm (ε 12,800), 236 nm (ε 27,400). ^{1}H NMR δ 12.07, 11.71 (2bs, 2H, NH), 8.47 (s, 1H, H_8), 7.20 (s, 1H, $H_{1'}$), 5.29 (bs, 1H, OH), 4.21, 4.10 (AB, 2H, J = 11.6 Hz, CH_2OAc), 3.76, 3.44 (AB, 2H, J = 10.8 Hz, CH_2OH), 2.75 (m, 1H, CH of isobutyryl), 1.92 (s, 3H, CH_3 of acetate) 1.52, 1.50 (AB, 2H, J = 9.8 Hz, $H_{3'}$), 1.10 (d, J = 7.3 Hz, 6H, CH_3 of isobutyryl). ^{13}C NMR 180.9, 170.7 (CO), 155.4, 149.1, 147.9, 137.1, 120.5, 119.2 (), 111.6 (purine, $C_{2'}$, C_{1v}), 65.5, 63.2 ($C_{5'}$), 35.5 (CH of isobutyryl), 28.2 ($C_{4'}$), 21.2 (CH_3 of acetate), 19.5 (CH_3 of isobutyryl), 12.5 ($C_{3'}$). ESI-M 376 (M + H, 7.2), 398 (100.0, M + Na). Anal. Calcd for $C_{17}H_{21}N_5O_5$: C, 54.38; H, 5.64; N, 18.66. Found: C, 54.18; H, 5.71; N, 18.37.

The racemic compound (*rac*-**12**) was prepared using a modified procedure for unprotected acetate (*rac*-**12**, *i*-But = H).[22] A mixture of compound **10** (27 mg, 0.08 mmol), trimethyl orthoacetate (16 μl, 0.12 mmol) and p-toluenesulfonic acid (trace) in CH_2Cl_2 (2 mL) was stirred at room temperature for 2 hours. Triethylamine (0.1 mL) was added, the solvent was evaporated, and the solution of residue in acetic acid (80%, 2 mL) was stirred at room temperature for 30 minutes. The solvent was removed in vacuo and the crude product was chromatographed on a silica gel column in CH_2Cl_2-MeOH (15 : 1) to give *rac*-**12** (27 mg, 93%).

(Z)-9-{[2-(Acetoxymethyl)-2-(hydroxymethyl)cyclopropylidene]methyl}-N^2-isobutyrylguanine Phosphite (13): Acetate (+)-**12** (110 mg, 0.3 mmol) was added dropwise to diphenyl phosphite (1 mL, 5 mmol) in pyridine (5 mL). The mixture was stirred for 16 hours at room temperature, triethylamine-water (3 : 2, 5 mL) was added and the stirring was continued for 2 hours. Volatile components were evaporated and the residue was chromatographed on a silica gel column using chloroform-methanol-triethylamine (75 : 4 : 2). The crude product was passed through Dowex-50W (H^+) column (elution with water) to give 112 mg (83%) of phosphite **13** as a white solid. ^{1}H and ^{31}P NMR indicated the presence of about 15% of deacetylated compound. UV λ_{max} 274 nm, 230 nm. ^{1}H NMR δ 12.11, 11.71 (2s, 2H, NH), 8.18 (s, 1H, H_8), 7.27 (s, 1H, $H_{1'}$), 6.71 (d, J = 667.3 Hz, 1H, P-H), 4.35, 4.02 (AB, 2H, J = 11.4 Hz, CH_2OAc), 4.18–4.05 (m, 2H, CH_2OP), 2.76 (m, 1H, CH of isobutyryl), 1.91 (s, 3H, acetate), 1.68 (collapsed AB, 2H, J = 11.0 Hz, $H_{3'}$), 1.10 (d, 6H, J = 7.3 Hz, CH_3 of isobutyryl). ^{13}C NMR 180.9, 170.6 (CO), 155.4, 149.1, 148.1, 137.0, 120.5, 118.2, 112.7 (purine, $C_{2'}$, $C_{1'}$), 65.9 (d, J = 4.5 Hz, CH_2OP), 65.5 (CH_2OAc), 35.4 (CH of isobutyryl), 26.4 (d, J = 8.1 Hz, $C_{4'}$), 21.1 (CH_3 of OAc), 19.5 (CH_3 of isobutyryl), 13.1 ($C_{3'}$). ^{31}P NMR 5.96, 5.81. Negative ESI-MS 438 (M – H, 100.0).

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The racemic compound *rac*-**13** was obtained from racemic acetate rac-**12** (25 mg, 0.7 mmol), diphenyl phosphite (0.2 mL, 1 mmol), in pyridine (1 mL), reaction time 23 hours following the procedure described above to give *rac*-**13** (33 mg, 80%).

(+)-(Z-9-{[2,2-Bis(hydroxymethyl)cyclopropylidene]methyl}guanine Phosphate/(+)-9: A mixture of phosphite **13** (87.8 mg, 0.2 mmol) and imidazole (67 mg, 8 mmol) in pyridine (5 mL) was sonicated for 20 minutes. Trimethylsilyl chloride (0.5 mL, 4.0 mmol) was then added dropwise with stirring at room temperature. After 20 minutes, iodine (103 mg, 0.4 mmol) in pyridine (1 mL) was added and the stirring was continued for 16 hours. The solvent was evaporated and the residue was partitioned between water (30 mL) and dichloromethane (3 × 30 mL). Aqueous ammonia (15 mL) was added to the aqueous portion and the mixture was heated at 50–60°C for 16 hours. The solution was lyophilized after removing ammonia in vacuo (aspirator). The product was passed through the Dowex-50W X2–200 (H^+) column with water as an eluent. The appropriate fractions were combined and they were lyophilized to give phosphate (+)-**9** (51 mg, 82%), which was converted to ammonium salt by adding aqueous ammonia (30%) and lyophilization. HPLC (column 1, 150 × 4.1 mm, 10 μ, 0.05 M KH_2PO_4 in 5% methanol as eluent, flow rate 1 mL/min, retention time 6.38 minutes, purity >99%). UV (H_2O) λ_{max} 269 nm (ε 11,400), 230 nm (ε 27,800). $[\alpha]_D^{27}$ 27.6° (c 0.5, H_2O). ^1H and ^{13}C NMR (D_2O) corresponded to those of racemic sodium salt.[22] ^{31}P NMR 1.36. Negative ESI-MS (MeOH) 342 (M – H, 100.0).

(-)-(Z)-9-{[2-(Hydroxymethyl)-2-(2-tetrahydropyranyloxymethyl) cyclopropylidene]-methyl}guanine (14): A mixture of (+)-acetate **12** (21.2 mg, 0.06 mmol), 3,4-dihydro-2H-pyran (86 μL, 0.95 mmol) and methanesulfonic acid (4 μL, 0.06 mmol) was stirred for 16 hours at room temperature. The reaction was quenched with triethylamine (0.1 mL) and the volatile components were evaporated. The residue was dissolved in aqueous ammonia (30%, 10 mL) and the solution was heated at 50–60°C for 4 hours. Ammonia was evaporated in vacuo (aspirator) and the solution was lyophilized. The crude product was chromatographed on a silica gel column using dichloromethane-methanol (10 : 1) to give 19.9 mg (97%) of compound **14** as a white solid, m.p. 268–271°C, $[\alpha]_D^{27}$ -3.2° (c 1.0, DMSO). UV, ^1H, ^{13}C NMR and mass spectra corresponded to the known racemic compound[22] and differing only by a diastereoisomeric composition.

(Z)-9-{[2-(2-Tetrahydropyranyloxymethyl)-2-(hydroxymethyl) cyclopropylidene]-methyl}guanine phosphite (15): A solution of compound **14** (16.6 mg, 0.05 mmol) in pyridine (1 mL) was added dropwise to diphenyl phosphite (85–90%, 0.2 mL, 0.89 mmol) in pyridine (1 mL) with stirring at room temperature. The stirring was continued for 16 hours, triethylamine-water (3 : 2, 1 mL) was added and the mixture was stirred for 2 hours. The volatile

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components were evaporated and the crude product was chromatographed on a silica gel column using dichloromethane-methanol (4 : 1 to 1.5 : 1) to give phosphite **15** (19 mg, 93%) as a white solid. UV λ_{max} 274 nm (ε 10,100), 230 nm (ε 24,300). ^{1}H NMR δ 11.17 (bs, 1H, NH), 8.25, 8.18 (2s, 1H, H_8), 7.13 (s, 1H, $H_{1'}$), 6.83 (bs, 2H, NH_2), 6.59 (d, 1H, J = 600.5 Hz, P-H), 4.58, 4.48 (2s, 1H, CHO of THP), 4.00 (bs, 1H, OH), 3.76–3.24 (2AB, overlapped with H_2O, $H_{5'}$, $H_{5''}$, CH_2O of THP), 1.10–1.70 (m, 8H, CH_2 of THP, $H_{3'}$). ^{13}C NMR 157.6, 154.9, 150.4, 134.6, 116.8, 111.9 (purine, $C_{2'}$, $C_{1'}$), 98.5, 98.3 (CHO of THP), 69.2, 68.7 (CH_2O of THP), 64.3, 61.7, 61.3 ($C_{5'}$, $C_{5''}$), 30.5, 27.6, 25.6. 25.5, 19.5, 19.2 (3xCH_2 of THP, $C_{4'}$), 12.5 ($C_{3'}$). Negative ESI-MS (MeOH) 410 (M – H, 100.0).

(-) - (Z) - 9 - {[2,2-Bis(hydroxymethyl)cyclopropylidene]methyl}guanine phosphate/(-)-9/: A mixture of phosphite **15** (19 mg, 0.045 mmol) and of imidazole (12.1 mg, 0.18 mmol) in pyridine (2 mL) was sonicated for 20 minutes at room temperature. Trimethylsilyl chloride (0.15 mL, 1.2 mmol) was then added dropwise and the mixture was stirred for 20 minutes. Iodine (30 mg, 0.09 mmol) in pyridine (1 mL) was added dropwise and the mixture was stirred for 16 hours. The solvent was removed in vacuo and the residue was stirred in acetic acid (80%, 10 mL) at room temperature for 24 hours. The solution was lyophilized and the residue was stirred in aqueous ammonia (30%, 50 mL) at room temperature for 3 hours. The volatile components were evaporated and the crude product was chromatographed on Dowex-50 WX2–200 (H^+) column using water as an eluent. The appropriate fractions were collected and they were lyophilized to give phosphate (−)-**9** (10.5 mg, 69%) as a white powder. Ammonium salt was obtained by adding aqueous ammonia and subsequent lyophilization. HPLC /see enantiomer (+)-**9**/ retention time 6.40 minutes, purity 99%, $[\alpha]^{27}_D$ -29.6° (c 0.5, H_2O). UV (H_2O) λ_{max} 268 (ε 10,400), 229 (ε 24,600). ^{1}H NMR corresponded to that of the racemic sodium salt.[22] ^{31}P NMR 1.30. Negative ESI-MS (MeOH) 342 (M – H, 100.0).

(Z)-9-{[2,2-Bis(hydroxymethyl)cyclopropylidene]methyl}guanine Diphosphate (16): A mixture of racemic phosphite *rac*-**13** (23 mg, 0.052 mmol) and imidazole (18 mg, 0.26 mmol) was stirred at room temperature for 30 minutes. Me_3SiCl (0.2 mL 1.6 mmol) was added and the stirring was continued for 20 minutes. After addition of iodine (27 mg, 0.08 mmol) and stirring for another 20 minutes the volatile components were evaporated. The residue was partitioned between water (20 mL) and CH_2Cl_2 (2 × 20 mL). The aqueous portion was lyophilized, crude product was put on the column of Dowex 50 /$H^{(+)}$/, which was eluted with water. The appropriate fractions were lyophilized to give phosphate (18 mg, 75%). This product (16 mg, 0.035 mmol) and tributylamine (8.4 μL, 0.035 mmol) in DMF (0.5 mL) was stirred for 30 minutes at room temperature. N,N′-Carbonyldiimidazole (28.6 mg, 0.175 mmol) was added and the stirring was continued for 3

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hours. The reaction was quenched with methanol (11.4 μL, 0.28 mmol) and, after 30 minutes, bis(tributylammonium) phosphate (200 mg, 0.71 mmol) was added and the mixture was stirred for 16 h. The solvents were removed in vacuo, the residue was dissolved in aqueous ammonia (30%, 20 mL) and the solution was stirred for 60 hours at room temperature. The volatiles were evaporated and the crude product was chromatographed on DEAE Sephadex A-25 /$HCO_3^{(-)}$, 20 × 1 cm/ column using a discontinuous gradient of NH_4HCO_3: 0.1, 0.15, 0.2, 0.25, and 0.3 M NH_4HCO_3 (20 mL each). Appropriate fractions were combined and lyophilized to give product **16** (10 mg, 62%) as an ammonium salt. HPLC (see phosphate **13**) retention time 3.20 minutes, purity 87.5%. ^{1}H NMR (D_2O) δ 8.29 (s, 1H, H_8), 7.08 (s, 1H, $H_{1'}$), 4.12 (m, 1H), 4.12, 3.89 (2m, 2H, CH_2OP), 3.71, 3.58 (AB, 2H, J = 11.9 Hz, CH_2OH), 1.46 (m, 2H, $H_{3'}$). ^{31}P NMR -7.49 (d, J = 21.4 Hz, P_β), -9.97 (d, J = 21.4 Hz, P_α). Negative ESI-MS 422 (100.0, M – H).

Guanylate Kinase Assay

Method A

The procedure for phosphorylation of enantiomeric ganciclovir phosphates **6** and *ent*-**6** was followed.[21] A stock GMP kinase assay buffer (0.1 M Tris-acetate, pH 7.6; 0.1 M KCl; 10 mM $MgCl_2$; 4 mM ATP; 1.5 mM phosphoenol pyruvate; 0.1 mg/mL BSA; 11 units/mL lactate dehydrogenase; and 5 units/mL pyruvate kinase) was prepared. Phosphate (+)-**9** or (−)-**9** (0.14 mg, 0.4 μmol), NADH (1.5 mM, 4 μL) was preincubated in stock buffer (0.5 mL) at 34°C for 5 minutes. GMP kinase from bovine liver (6 μg, 0.08 unit) was then added and the reaction was allowed to proceed at 34°C. At specific time intervals, a 70 μL aliquot was removed, filtered through a 0.45 μ filter, and analyzed by HPLC for the conversion to diphosphate **16**. The HPLC was run as described for phosphates (+)-**9** and (−)-**9**. The reaction times are as follows: ATP and ADP 1.71 minutes, diphosphates **16** 3.19 minutes, and phosphates **9** 6.40 minutes. The mobility of diphosphates formed was identical with that of racemic compound **16**. The results are shown in Figure 1.

In a control experiment, conversion of GMP to GDP was followed under the conditions described above. The HPLC was performed using column 2 and 0.6 M KH_2PO_4 (pH 4.5) as a mobile phase. The eluate was monitored at 265 nm and flow rate was 2.0 mL/min. The reaction times are as follows: GMP 3.28 minutes, ADP 5.04 minutes, GDP 8.80 minutes, ATP 18.50 minutes. After 0.5 hours, >95% conversion to GDP was observed.

Method B

The GMP kinase assay was adopted from Marshalko et al.[21] Briefly, GMP kinase buffer (0.05 M Tris, pH 7.6; 0.05 M KCl; 5.0 mM $MgCl_2$), ATP

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(2.0 mM), BSA (0.1 mg/mL) (all final concentrations), and substrate (GMP, dGMP, phosphates (+)-**9** and (−)-**9**; 5.0–2000 μM) were incubated at 37°C for 20 minutes prior to introduction of enzyme. At time zero, bovine GMP kinase was added to the solution to give a final concentration ranging from 0.01 to 0.2 units/mL (unit is defined as the amount of enzyme necessary to convert 1.0 μmol of GMP to GDP in 1.0 min at pH 7.5 at 30°C). At the designated times, an aliquot was removed, placed on ice, and proteins precipitated with 0.04 volumes of 10 M perchloric acid. The samples were centrifuged, the supernatants neutralized with KOH and the samples were stored at −20°C until analyzed by HPLC.

Phosphates (+)-**9**, (−)-**9** and **16** were separated and quantified by reversed phase HPLC (Beckman Coulter, Fullerton, CA, USA; System Gold Programmable Solvent Module 125 and System Gold Programmable Detector Module 166 controlled by 32 Karat Software (ver. 7.0). Before injection, each sample was centrifuged at 14,000 rpm for 5 minutes to remove any remaining particulate matter. Samples were loaded onto a 10 μm Alphabond C18 300 × 3.9 mm reversed phase column (Alltech, Deerfield, IL, USA) at a flow rate of 2.0 mLs/min and phosphorylated cyclopropavir derivatives were eluted using 300 mM potassium phosphate (pH 3.0) and 100% methanol (5% methanol linear gradient over 30 minutes). Phosphorylated cyclopropavir derivatives were quantified by comparing their peak area with that of a known amount of the appropriate standard at a wavelength of 254 nm.

In experiments with GMP and dGMP as substrates, nucleotides were separated and quantified by strong anion-exchange HPLC using a Beckman Coulter (Fullerton, CA, USA) Proteome Lab PF 2D Protein Fractionation System controlled by 32 Karat Software (ver. 7.0). Before injection, each sample was centrifuged as above to remove any remaining particulate matter. Samples were then loaded onto a Partisil 5 SAX analytical 4.6 × 250 mm column (Whatman, Clifton, NJ, USA) at a flow rate of 2.0 mL/min and phosphorylated nucleotides were eluted using a 60 minute linear gradient of 10 mM ammonium phosphate (pH 3.0) to 500 mM ammonium phosphate (pH 3.0) followed by 30 minutes of 500 mM ammonium phosphate (pH 3.0). Nucleotides were quantified by comparing their peak area with that of a known amount of the appropriate standard at a wavelength of 254 nm.

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