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SYNTHESIS, ENZYMATIC PHOSPHORYLATION AND ANTIVIRAL ACTIVITY OF ACYCLIC DIENYL PHOSPHONATE DERIVATIVES OF GUANINE

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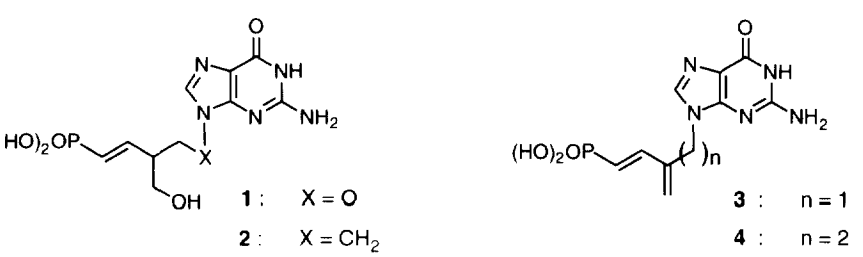
Abstract : The synthesis, phosphorylation by guanylate kinase, anti HIV-1 and anti-herpesvirus activity of two acyclic dienyl phosphonate derivatives of guanine are described. (E)-9-(5-phosphono-3-methylene-4-pentenyl)guanine (**4**) was identified as an excellent substrate of guanylate kinase and a significant inhibitor of HIV-1 replication.

In recent years, numerous acyclonucleotide analogues have been prepared as potential antiviral agents[1-6]. These compounds bear a chemically stable phosphonate-containing moiety in place of the labile phosphate monoester group and are structural mimics of nucleoside monophosphates. It has been shown that various acyclonucleotide analogues exert their antiviral effect following sequential activation by cellular kinases to their diphosphate derivatives[7,8] (nucleoside triphosphate analogs) which act as inhibitors of viral DNA polymerases[1,7,8]. As mimics of the phosphate monoester group, the phosphonomethyl and especially the phosphonomethoxy groups have been used. This has led to the discovery of promising antiviral agents such as 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA, Adefovir)[9] and (S)-1-(3-hydroxy-2-phosphonomethoxypropyl)-cytosine ((S)-HPMPC, Cidofovir)[9]. Very recently, acyclonucleotide analogues bearing an α,β-unsaturated phosphonate moiety as mimic of the phosphate group were found to exhibit significant antiviral activity[10,11]. Interestingly, we noticed that unlike (E)-9-[(2-hydroxymethyl-4-phosphonobut-3-enyl)oxy]guanine (**1**) (Scheme 1), the carba analogue **2** had low cellular toxicity while retaining anti-HIV activity. Moreover, compound **2** was found to be a good substrate of guanylate kinase[11], an enzyme held responsible for the intracellular phosphorylation of the monophosphates of acyclovir and ganciclovir[12,13] and more generally of various acyclic phosphonate derivatives of guanine[1,4,11]. These results suggested that the dienyl phosphonate **4** (scheme 1)

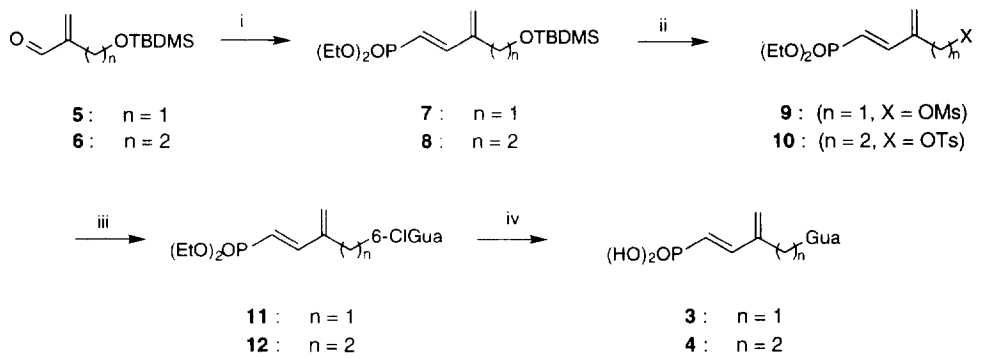
Scheme 1



could also be an efficient substrate of guanylate kinase. If so, and provided that the product of this first step is further phosphorylated by known cellular kinases[1,14] of broad specificities, the diphosphate of **4** could be formed intracellularly and perhaps behave as a good inhibitor of viral DNA polymerases. We report here the synthesis of **4** and of its shorter chain analogue **3**, their substrate properties for guanylate kinase as well as their antiviral activity profile. While this work was in progress, Harnden and collaborators[10] reported the synthesis of a series of phosphonoalkenyl and phosphonoalkenyloxy derivatives of purines. During that work, the dienyl phosphonate **3** was obtained incidentally and found to be inactive both against HIV-1 and herpes viruses[10].

Syntheses of (E)-9-(4-phosphono-2-methylene-3-butenyl)guanine **3** and (E)-9-(5-phosphono-3-methylene-4-pentenyl)guanine **4** were performed as outlined in scheme 2, starting from 3-*t*-butyldimethylsilyloxy-2-methylene-propanal **5** and 4-*t*-butyldimethylsilyloxy-3-methylene-butanal **6**, respectively. Condensation of these aldehydes with the lithium salt of tetraethyl methylene-bis-phosphonate[15] gave the corresponding dienylphosphonates **7** and **8**. The *t*-butyldimethylsilyl groups were removed to give the corresponding hydroxy derivatives which were converted to the sulfonates **9** and **10**. The purine moiety was then introduced by alkylation of **9** or **10** with the sodium salt of 6-chloro-2-aminopurine in acetonitrile to give the corresponding protected nucleotides **11** and **12**. These products were obtained with a high regioselectivity (N9/N7 > 90% as determined by ^{1}H NMR spectroscopy). The desired acyclonucleotides **3**[16] and **4**[17] were obtained by treatment of **11** and **12** with bromotrimethylsilane followed by alkaline or acidic hydrolysis, respectively.

Scheme 2

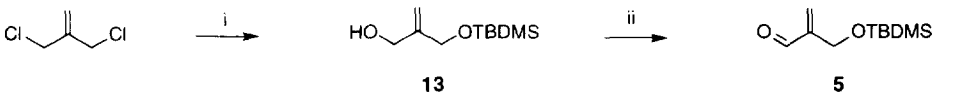


i: $((EtO)_2OP)_2CHLi$, THF, -78°C (n=1, 55%; n=2, 80%). ii: 1) TBAF; 2) MsCl (n=1) or TsCl (n=2), NEt_3. iii: 6-ClGua, NaH (3 steps: n=1, 25%; n=2, 52%). iv: 1) TMSBr, CH_3CN; 2) NaOH 1N (n=1, 2steps: 42%) or 0.5N HCl (n=2, 2steps: 25%).

Synthesis of the unsaturated aldehydes **5** and **6** is depicted in scheme 3 starting respectively from commercially available 2-methylene-1,3-dichloropropane and 2-bromo-ethanol. On one hand, the dichloride was transformed into the alcohol derivative **13** in three steps by known methods. Then, the remaining hydroxy function of **13** was oxidized using N-methylmorpholine N-oxyde and a catalytic amount of tetrapropylammonium per-ruthenate (NMNO/TPAP)[18] to afford the aldehyde **5**. On the other hand, 2-bromo-ethanol was first protected as a TBDMS-ether and then alkylated with the sodium salt of diethylmalonate to give the malonate **14.** The

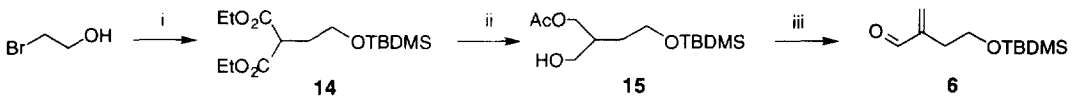
reduction of the ester functions of **14** gave the dihydroxy derivative which, by a controlled monoacetylation reaction, afforded the monohydroxy derivative **15**. The remaining hydroxy function of **15** was then oxidized using the NMNO/TPAP method. Interestingly, during the oxidation, the elimination of acetic acid occurred, affording the α,β-unsaturated aldehyde **6**.

Scheme 3



13 **5**

i: 1) Et_4NOAc; 2) MeOH, K_2CO_3; 3) TBDMSCl, NEt_3 (3 steps: 35%). ii:1.5eq. NMNO, 0.05eq.TPAP, CH_2Cl_2 (83%).



14 **15** **6**

i: 1) TBDMSCl, NEt_3; 2) $(EtO_2C)_2CH_2$, NaH (2 steps: 44%). ii: 1) LAH, ether (40%); 2) Ac_2O, NEt_3, CH_2Cl_2 (61%).

iii:1.5eq. NMNO, 0.05eq. TPAP, CH_2Cl_2 (77%).

The dienyl phosphonates **3** and **4** were evaluated as potential substrates of guanylate kinase[19]. Compound **4** proved to be an excellent substrate of the enzyme, being phosphorylated as efficiently[19] as the natural substrate GMP (Table 1). The Vmax value of **4** is 3.3-fold lower than that of GMP. However, since the Km of **4** is also 3.4 lower than that of GMP, the efficiency[19] of phosphorylation calculated for **4** is 102%. To our knowledge, compound **4** is the most efficient non-natural substrate ever reported for guanylate kinase. In contrast, its shorter chain analog **3** is a relatively poor substrate of guanylate kinase, being a 57-fold less efficient substrate than **4**.

Table 1. Comparison of kinetic parameters of GMP and dienyl phosphonates **3** and **4** for guanylate kinase

Substrate [a]	Km [b] (μM)	Vmax [c] (% rel. to GMP)	Vmax / Km (% rel. to GMP)
GMP	17 ± 2	100	100
3	57 ± 5	6.1	1.8
4	5 ± 2	30	102

[a] The kinetic parameters of **3** and **4** were determined at enzyme concentrations 20 and 5 times higher than that used for GMP, respectively.

[b] For GMP, **3** and **4**, Km and Vmax values are means of 5, 2 and 3 separate determinations, respectively.

[c] Vmax of GMP = 13 μmol . min^{-1}. $(mg\ protein)^{-1}$. Standard deviations on mean Vmax values of **3** and **4** were 2 and 13%, respectively. Vmax are expressed in % of Vmax of GMP.

The anti-HIV-1 activity of dienyl phosphonates **3** and **4** was evaluated in the human T-lymphoid cell lines MT-4 and C-8166[20] infected with the HIV-1-RF strain (Table 2). The nucleoside analogues 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (ddI) and the acyclonucleotide analogue 9-[2-phosphonomethoxy)ethyl]adenine (PMEA) which are selective inhibitors of HIV replication were used as references[21]. In MT-4 and C-8166 cells, compound **4** inhibited HIV-1 replication with IC_{50} values of 49 and 8 μM, respectively, and was therefore about 2.5- and 5-fold less potent than PMEA (Table 2). However, compound **4** was more than 5 times less cytotoxic to MT-4 cells than PMEA, thus showing an interesting selectivity index. In MT-4 cells, compound **3** did not inhibit HIV-1 replication at 100 μM.

Table 2. Anti-HIV-1 activity of acyclic dienyl phosphonate derivatives of guanine

Compound	EC_{50} (μM) [a,b]		CC_{50} (μM) [c]
	MT-4 cells	C-8166 cells	MT-4 cells
AZT	0.04 (± 0.01)	0.04 (± 0.03)	113 (± 36)
ddI	13 (± 10)	0.9 (± 0.4)	> 1000 *
PMEA	20 (± 10)	1.5 (± 1.0)	187 (±135)
3	> 100 *	n.d.	> 1000 *
4	49 (± 13)	8 (± 2)	> 1000 **

Except for data maked with * or ** (from one or two experiments, respectively), data are mean values of at least three independent experiments (± SD); n.d., not determined.

[a] Concentration required to inhibit HIV-1-induced cythopathic effect by 50% in MT-4 cells.

[b] Concentration required to inhibit p24 viral antigen production by 50% in C-8166 cells.

[c] Concentration required to reduce the viability of MT-4 cells by 50%.

Compounds **3** and **4** were also tested for activity against herpes simplex virus of type 1 (HSV-1, HF strain) and human cytomegalovirus (HCMV, AD 169 strain) grown in Vero and MRC-5 cells, respectively, by measuring inhibition of the virus-induced cytopathic effect as previously described[11]. Neither compound **3** nor **4** inhibited HCMV replication up to 250 μM. In contrast, compound **3** inhibited HSV-1 replication with an IC_{50} value of 119 ± 14 μM (n = 2), i.e. similar to that of PMEA (117 ± 38 μM (n = 2)). Acyclovir, used as reference, had an IC_{50} value of 0.5 ± 0.3 μM (n = 2). Compound **4** was inactive against HSV-1 at 125 μM. Compounds **3** and **4** were not toxic to Vero cells at least up to 250 μM. In contrast to our observation, Harnden *et al.*[10] found that **3** showed no significant anti-HSV-1 (SC 16 strain) activity at 300 μM. This discrepancy may be due to differences in uptake and/or intracellular activation (phosphorylation) of **3** between Vero and MRC-5 cells, the host cell system used by these authors.

In conclusion, we have shown that acyclic dienyl phosphonate derivatives of guanine can act as substrates of guanylate kinase. Compound **4** was found to be an excellent substrate of guanylate kinase and a significant inhibitor of HIV replication. In contrast, the shorter chain analogue **3** was a modest substrate of guanylate kinase and showed some activity only against HSV-1. These examples illustrate the potential of the dienyl phosphonate moiety as a surrogate of the phosphate group. In addition, the antiviral activity of such compounds could be related, at least in part, to their ability to serve as substrates for guanylate kinase.

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16. The ^1H NMR (360 MHz, D_2O) spectrum of compound **3** was in good agreement with that reported by Harnden *et al.*[10]

17. Compound **4**: Analysis of $C_{11}H_{14}N_5O_4P$, HCl: calc. C: 38.00, H: 4.35, N: 20.14; found: C: 38.10, H: 4.31, N: 20.12. ^1H NMR (360 MHz, D_2O): δ 7.70 (s, 1H, H-8); 6.68 (dd, J_{HH} = 16 Hz, J_{HP} = 20 Hz, 1H, CH-4'); 6.09 (dd, J_{HH} = 16 Hz, J_{HP} = 14 Hz, 1H, CH-5'); 5.10 (s, 1H, H_A of $C{=}CH_2$); 4.78 (s, 1H, H_B of $C{=}CH_2$); 4.25 (t, J = 4 Hz, 2H, CH_2-1'); 2.75 (t, J = 4 Hz, 2H, CH_2-2').

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20. Anti-HIV-1 activity in MT-4 and C-8166 cells was determined by the MTT reduction dye method and p24 viral core antigen assay, respectively. For details, see reference 11.

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