New modified nucleoside 5′-triphosphates: synthesis, properties towards DNA polymerases, stability in blood serum and antiviral activity

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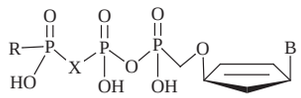
A series of new nucleoside 5′-triphosphate mimetics, **2**, **3**, **5**, **6**, **8**–**10**, modified at the glycone and all three phosphate residues, have been synthesised and studied. These compounds only bear the enzymatically labile anhydride bond between the α and β phosphorus atoms. The preparative chemistry involved the preparation of phosphonic salts **30**, **31** and **32** and coupling of these species to the morpholidate **33**. The mechanism of formation of some of the intermediates '*en route*' to **27** and **28** is discussed. All of the target compounds demonstrated high stability in human blood serum with half lives towards hydrolysis of up to 4.5 days. Some of these nucleoside triphosphonates have been shown to be selective inhibitors of DNA synthesis catalysed by retroviral reverse transcriptases and terminal deoxynucleotidyl transferases. They inhibited replication of the artificial virus containing Moloney murine leukemia virus reverse transcriptase in infected cell culture, probably due to the inhibition of a reverse transcription step of a genomic RNA. Compared to the triphosphonates, the corresponding monophosphonates demonstrated decreased antiviral activity by 1–2 orders of magnitude. This implies that the triphosphonates inhibit virus replication directly, rather than by a two-step mechanism based on their hydrolysis to the monophosphonates and subsequent intracellular diphosphorylation. Being totally independent of the enzymatic phosphorylation pathways of the host cell, the compounds under study may also be able to inhibit retrovirus reproduction both in kinase deficient cell lines and in the intercellular blood media.

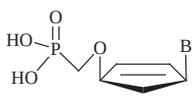
Introduction

The study of DNA and RNA metabolism often employs deoxynucleoside 5′-triphosphates (dNTP), ribonucleoside 5′-triphosphates (rNTP) and their analogues as substrates or inhibitors. However, the usage of these compounds in cell biology is limited due to their rapid dephosphorylation in intercellular media and during their diffusion into cells. For the same reason, modified dNTP and rNTP cannot be used as antiviral agents, hence the need for more stable derivatives. The design of dNTP analogues with increased stability *in vivo* could also produce a new group of highly effective inhibitors of HIV reproduction. The advantages of such compounds would be as follows: (i) Independence of the nucleoside phosphorylation pathways, catalysed by intracellular kinases, and direct inhibition of proviral DNA synthesis. (ii) The lack of the cell cycle effect on their activity. (iii) The ability to inhibit reverse transcription in blood plasma or seminal fluids [in the case of human immunodeficiency virus (HIV)].[1] It is also desirable that such dNTP/rNTP analogues act as substrates only towards viral enzymes and be hydrophobic enough to penetrate into the cell or be able to bind to membrane proteins which could facilitate their transportation into cells.

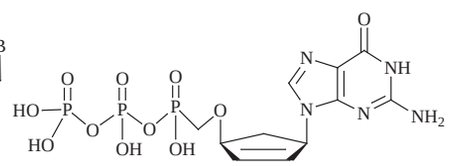
We recently reported that the replacement of the γ-phosphate moiety by a methyl- or phenylphosphonate unit in both ring modified and unmodified dNTP analogues enhances their selectivity whilst preserving their substrate properties towards the reverse transcriptases of HIV (HIV-rt) and avian myeloblastosis virus (AMV-rt).[2] Similar results were obtained when the α-phosphate residue in dNTPs were substituted by the methylphosphonate, phenylphosphonate,[3,4] or α-methylphosphonate units,[5,6] the β,γ-pyrophosphate residue by diphosphonate units[7–11] and all the three phosphate groups by phosphonates.[12,13] Some of these modified dNTP analogues displayed enhanced stability towards dephosphorylating enzymes in human blood serum as well as towards alkaline phosphatase.[2,12,13] They also showed a significant degree of hydrophobicity.[2] These properties suggest that such compounds have promising potential as substrates or inhibitors of polymerase-dependent DNA synthesis in cell cultures. We present herein the preparation of the carbocyclic L-dideoxyNTP analogues (ddNTP) **1**–**10** together with the D-stereoisomer of compound **4**, *i.e.* (*ent*)-**4**, and the biological evaluation of these compounds in cell free systems with HIV-rt, AMV-rt, terminal deoxynucleotidyl transferase (TDT) and DNA polymerases α and β. The efficacy of these compounds as inhibitors of viral replication in Rat1 cell culture infected by artificial retrovirus containing Moloney murine leukemia virus (Mu-MLV) and their stability in human blood serum is also reported. The monophosphonates **11** and **12** were used as reference compounds in the experiments with cell cultures.



1 B=Adenine, X=O, R=OH
2 B=Adenine, X=CBr_2, R=OH
3 B=Adenine, X=CF_2, R=OH
4 B=Guanine, X=O, R=OH
5 B=Guanine, X=CBr_2, R=OH
6 B=Guanine, X=CF_2, R=OH
7 B=Adenine, X=O, R=Ph
8 B=Adenine, X=CH_2, R=Me
9 B=Guanine, X=CF_2, R=Me
10 B=Guanine, X=CF_2, R=Ph



11 B=Adenine
12 B=Guanine



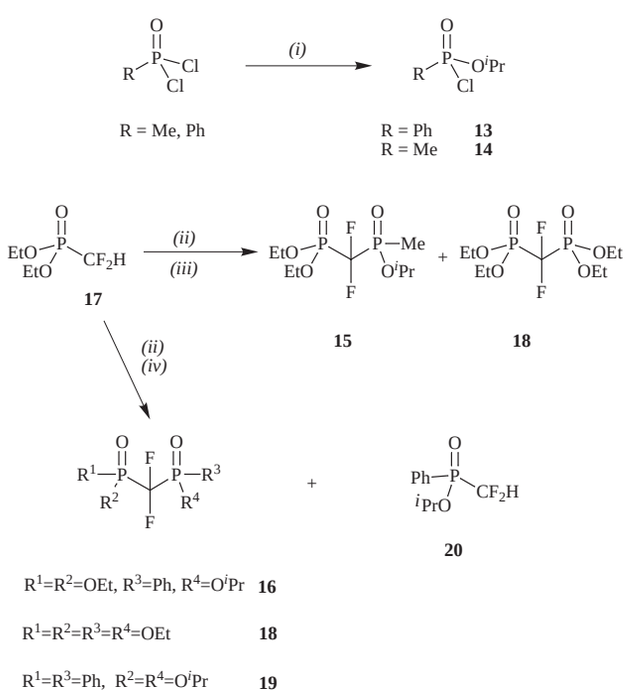
(*ent*)-**4**

Results

Chemistry: Synthesis and mechanistic considerations

The syntheses of compounds **1**,[4] **2**,[13] **4**,[5] **5**,[13] **6**,[15] **11**[14] and **12**[5] have been reported elsewhere. The major new elements of chemistry investigated in connection with this paper involved the preparation of the requisite phosphorylphosphonate moieties which were to be appended to the morpholidate derivative of the known carbocyclic monophosphonate **12**.

The phosphorylphosphonate triesters **15** and **16** were key intermediates and these compounds were prepared from the chlorophosphates **13** and **14**, respectively, as outlined in Scheme 1. Thus treatment of diethyl difluoromethylphosphonate **17**

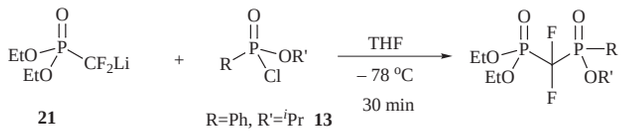


Scheme 1 (*i*) ^{i}PrOH (1 equiv.), Et_3N, PhMe, 0–20 °C, 90 min; (*ii*) LDA, THF, −78 °C, 30 min; (*iii*) (**14**), THF, −78 °C, 90 min; (*iv*) (**13**), THF, −78 °C, 30 min.

with LDA followed by the addition of isopropyl methylphosphonochloridate **14** gave the phosphorylphosphonate triester **15** in low yields. A small amount of tetraethyl difluoromethylenediphosphonate **18** was isolated as a by-product.[16]

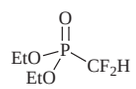
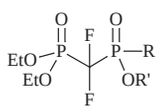
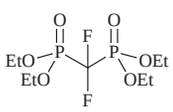
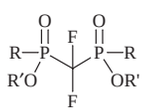
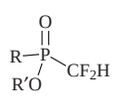
Treatment of the phenylchlorophosphonate **13** under the same reaction conditions gave a more complex reaction mixture. In addition to the desired phenylphosphoryl phosphonate **16**, a number of other by-products were also isolated and characterised. These were the bisphosphonate **18**, the difluoromethylenebis[isopropyl(phenyl)phosphonate] **19** and the (difluoromethyl)phenylphosphonate **20**; a small amount of the starting material **17** was also recovered (Scheme 1). A detailed study of these two reactions was carried out, by ^{19}F NMR analysis of the crude reaction mixtures, in order to compare the influence of the methyl/phenyl substituent on the product ratios. The results are shown in Table 1.

Table 1 Study of the effects of phenyl and methyl functionalised phosphonochloridates on product distribution when reacting with **21**



21

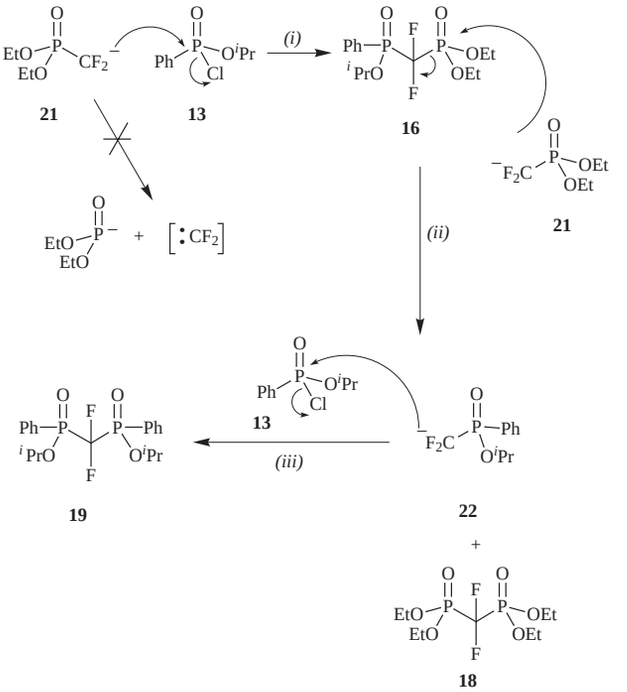
R=Ph, R'=^{i}Pr **13**
R=Me, R'=^{i}Pr **14**

Reaction products	Electrophile **13**[a]	**14**[a]
	**17** (8%)	**17** (45%)
	**16** (37%)	**15** (53%)
	**18** (30%)	**18** (2%)
	**19** (12%)	—[b]
	**20** (13%)	—[b]

[a] Product ratios as determined by ^{19}F NMR. [b] Not detectable.

The most notable difference between the reactions of **21** with either methyl or phenyl phosphonochloridates is the extent of by-product formation. When the phenylphosphonochloridate **13** is used, significant by-product formation is observed but in the same reaction with the methylphosphonochloridate **14**, the degree of side product formation is much lower. In the former transformation there are side-reactions taking place which account for the poorer yields of the desired product **16** (Scheme 2).

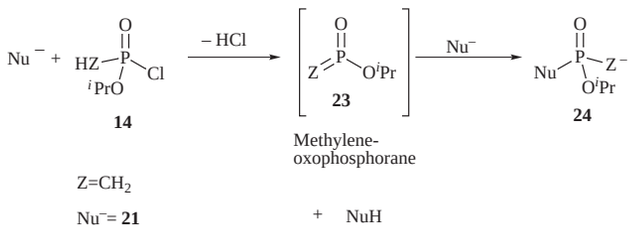
Thus initially, nucleophilic substitution of **21** onto the phosphonochloridate **13** gives the desired product **16**, step (i). Following initial product formation, some of the unreacted anion **21** can further react with **16** at P_α to give the bisphosphonate **18** and the phosphoryl carbanion **22** which is evidently a good leaving group in the presence of such hard nucleophiles, step (ii). The phosphoryl carbanion **22** can also react with some of the unconsumed phosphonochloridate **13** to give **19** as a 1:1 mixture of the racemic and *meso* products (by ^{19}F NMR), step (iii). Unreacted carbanions, **21** and **22** may be protonated (to form **17** and **20**) following an aqueous work up. Reactions were carried out at −78 °C in order to avoid the degradation of **21** *via* the loss of difluorocarbene.



Scheme 2

As by-product formation is initiated by further reaction of the phosphorylphosphonate product with unconsumed nucleophile **21** at P_α, the increased susceptibility of the phenylphosphorylphosphonate **16** to nucleophilic attack, by **21**, could account for the greater proportion of by-products arising from the reaction of **21** with **13**.[17] Alternatively, the differences in the reaction profiles for the methyl- and phenyl-phosphonochloridates may be due to significant differences in the mechanisms of their reactions with nucleophiles such as **21**. Thus the reaction of **21** with **13** probably proceeds *via* an associative addition–elimination (AE) mechanism whereby nucleophilic displacement proceeds through a trigonal bipyramidal (TBP) intermediate.[18] The same mechanism is also possible for the reactions involving the methylphosphonochloridate **14** but the presence of the relatively acidic α-methyl protons allows the possibility of an alternative elimination–addition (EA) reaction pathway. This unimolecular process proceeds through a high energy three-coordinate P(v) intermediate; the formation of which is strongly dependent on the acidity of ligand HZ—relative to the basicity *versus* nucleophilicity of the attacking nucleophile. The generation of such phosphorane type intermediates in nucleophilic reactions at phosphoryl centres is well documented for substrates with strongly acidic HZ-ligands where Z = oxygen,[19] sulfur,[20] or nitrogen[21] but in nucleophilic substitution reactions there is very little evidence for the formation of methylene(oxo)phosphorane intermediates such as **23** where Z is an unsaturated carbon atom.[22] If the nucleophile **21** is basic enough to deprotonate **14**, eliminating HCl, to give the methylene(oxo)phosphorane **23** then any unreacted carbanion **21** could then attack this electrophilic intermediate to give the product anion **24** (Scheme 3).

To differentiate between the two possible mechanisms, the reaction of **21** with **14** was repeated and quenched, after 30 minutes, with deuterium oxide. If the reaction proceeds through the AE mechanism then any unreacted carbanion **21** would be deuteriated upon quenching. Conversely, if the EA mechanism predominates then the product would be mono-deuteriated and the recovered starting material **17** protonated (the *in situ* proton source for **21** being the deprotonation of **14**). Partial deuteriation of both **17** and **15** would indicate that both reaction pathways were operating. The results of this experiment surprisingly showed both **17** and **15** to be fully protonated and the



Scheme 3

unreacted phosphonochloridate **14** was isolated crude (from the aqueous layer during work-up) as the deuteriated phosphonic acid **26** (Scheme 4).

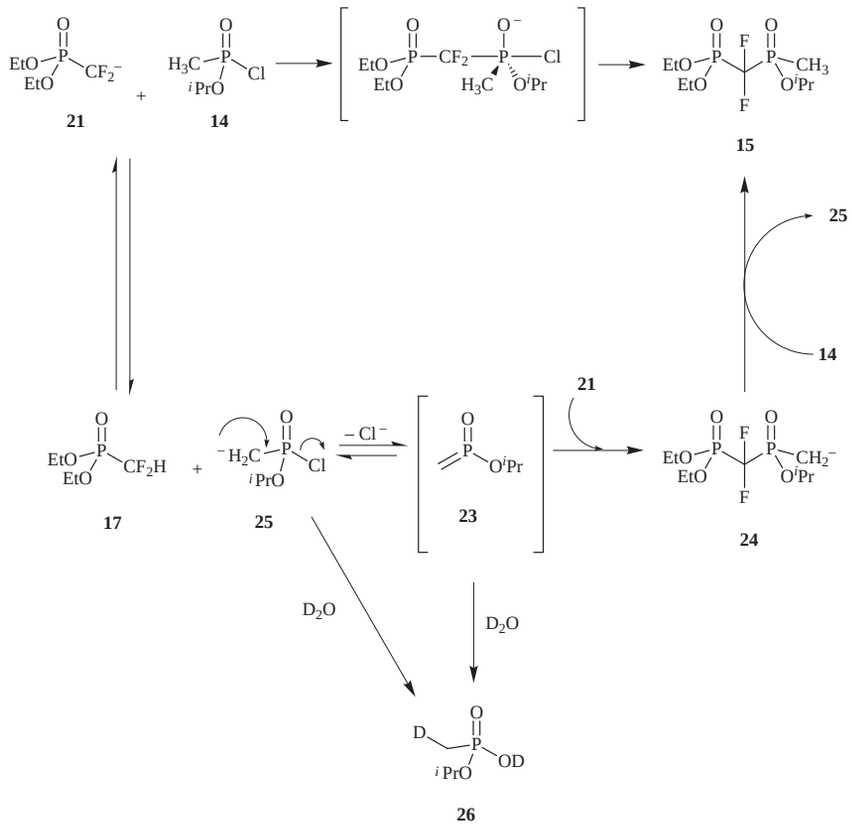
Thus one possibility, considered initially, was that the formation of **23** is energetically disfavoured and simple deprotonation of the methyl phosphonochloridate **14** forms the anion **25**. This process would be reversible but if the equilibrium should favour the conjugate acid **17** only a low concentration of **21** would be maintained *in situ* (Scheme 4). Hence **17** would be isolated in its protonated form following a deuterium quench. Likewise, if **21** can only react, in this manner, through the AE pathway then the isolated product **15** would also be fully protonated and the only deuteriated product would be the methylphosphonic acid **26**, as is observed.

This mechanistic proposition certainly fits in with the results of the deuteriation study but there are several reasons why one should seek an alternative explanation. Firstly, if this was the predominant mechanism then it would be reasonable to expect the reaction to be driven further towards completion as none of **21** is irreversibly consumed unless it reacts as a nucleophile with **14** to give the substitution product **15**. This is not what is observed as half of **21** is still unconsumed after 30 minutes. Secondly, it is also unlikely that the acid–base equilibria of **21** with its conjugate acid **17** would strongly favour the formation of the latter in the presence of **14** as the α-methyl group of **14** will be significantly less acidic than the difluoromethyl group of **17**. This means that the equilibrium (Scheme 4) will favour the presence of **21** and **14** and, on quenching with deuterium oxide, most of the unconsumed nucleophile **21** would be isolated as the deuteriated species and the phosphonomethyl group of any unconsumed electrophile **14** would be undeuteriated, contrary to our observations. Therefore, notwithstanding the lack of literature precedent, we were forced to the conclusion that **23** was indeed a viable intermediate. Hence we believe it is feasible that formation of **23** from **14** and reaction with **21** furnishes **24** which deprotonates unreacted **14** to generate the fully protonated product and additional **23**; hydrolysis of residual **23** with deuterium oxide would yield the deuteriated phosphonic acid **26** as observed.

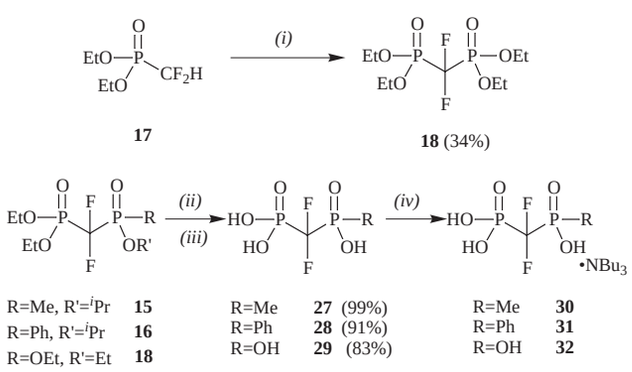
Although it is difficult to deduce the exact mechanistic details of this reaction, from the information available, it is possible that the elimination of HCl from the phosphonochloridate **14** leads to a reduction in the proportion of reactive anion **21** which is present in the reaction mixture at any one time. Hence only small traces of by-products are evident from the reaction with the methylphosphonochloridate **14**. In contrast, the phenylphosphonochloridate **13** has no acidic protons and hence, as stated earlier, the reaction can only proceed by the simple AE mechanism (Scheme 2). When the first traces of the initial condensation product **16** are formed, there will be a large excess of unreacted nucleophile available to initiate the side product formation that is observed.

Tetraethyl difluoromethylenediphosphonate **18** was prepared from **17** as shown in Scheme 5.[23] Deprotection of the phosphoric/phosphonic esters **15**, **16** and **18** was accomplished with bromotrimethylsilane to give the free acids **27**, **28** and **29** (Scheme 5) which were subsequently converted to their respective tributylammonium salts **30**, **31** and **32**.

The final coupling steps for the preparation of the new NTP



Scheme 4



Scheme 5 (*i*) LDA, diethyl chlorophosphate, THF, −78 °C, 1 h; (*ii*) TMSBr, 3 d; (*iii*) H_2O; (*iv*) NBu_3, $EtOH_{(aq)}$.

analogues are summarised in Scheme 6. The morpholidate **33** was prepared from compound **12** in standard fashion[5] and coupled to **30** to give the NTP analogue **9** which was isolated as its ammonium salt. Following a recent report of improved reaction yields being achieved when using 1*H*-tetrazole as a catalyst in pyrophosphate coupling reactions between 5′-monophosphomorpholidates and sugar-1-phosphates,[24] this procedure was employed in the synthesis of **10**. Disappointingly the yield of **10** was still modest. Compounds **3** and **7** were prepared, using the carbonyl diimidazole coupling reagent, in similar yields. The Pγ-substituted *pseudo*-triphosphate **8** was prepared from **11**, *via* the phosphorylphosphonate **34**, using a 1,2,4-triazole mediated coupling procedure.

Biological evaluations

All the compounds were evaluated as terminating substrates for HIV-rt, AMV-rt, human placenta DNA polymerases α and β and calf thymus TDT. HIV-rt was chosen because of its continued importance, AMV-rt was under investigation as a second example of this group of enzymes and human DNA polymerases α and β are known as the most significant enzymes of

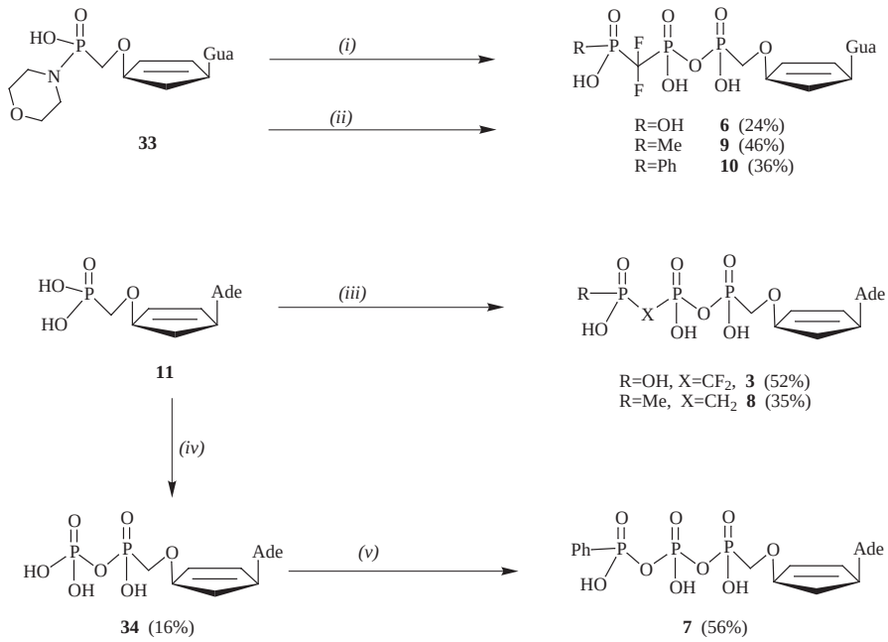
Table 2 Semiquantitative data on the terminating activity of the newly synthesised compounds towards different DNA polymerases

	Enzymes[a]				
Compound	HIV-rt	AMV-rt	α	β	TDT
1	<0.002	<0.02	>600	<200	<0.2
2	>600	<2.0	>600	>600	<0.02
3	<0.02	<0.2	>600	>600	<0.2
ddATP	<0.02	<0.02	>200	<20	<0.02
4	<0.002	<0.002	>600	<200	<0.02
5	>2.0	<2.0	>600	>600	<0.2
6	<0.02	<0.2	>600	>600	<0.2
ddGTP	<0.02	<0.02	>200	<2	<0.02
7		<2.0	>200	>200	>2.0
8		>2.0	>200	>200	>2.0
9	<0.2	<2.0			
10	<0.02	<2.0			>2.0
ent-**4**	<0.02	<0.02			>2.0

[a] Concentration required (μM) for 50% incorporation of the terminating substrate in the template–primer complex.

DNA replication and repairing, respectively. TDT is also of interest as a unique example of a template independent DNA polymerase. Table 2, and the complementary data in Figs. 1–3, summarise the terminating activity of the compounds under study, based on the analysis of electrophoretic data. It can be seen that most compounds did not terminate DNA synthesis catalysed by DNA polymerases α and β but were active in the case of TDT, though this was one order of magnitude weaker than their terminating activity with HIV-rt.

The termination pattern of substrates **1**–**3** in the HIV-rt-catalysed elongation of the tetradecadeoxynucleotide primer–RNA template complex (complex 4) was assessed. As can be seen, the effectivity of the pyrophosphoryl phosphonate **1** was comparable with that of dATP and could be observed at concentrations as low as 0.002 μM. The introduction of a Pβ,Pγ-difluoromethylenediphosphonate linkage, in compound



Scheme 6 (*i*) **30** or **32**, DMSO 5–13 h room temp.; (*ii*) **31**, DMSO, 1*H*-tetrazole (2.0 equiv.), 14 h at room temp.; (*iii*) *N*,*N*′-carbonyldiimidazole, DMF, 12 h, then add **32** or (methylphosphinyl)methylene phosphonate, 12 h at 37 °C; (*iv*) $(Me)_2NCH(OMe)_2$, DMF, 20 h, then *N*,*N*′-carbonyldiimidazole, DMF, 12 h, then $H_3PO_4 \cdot NBu_3$, 3 h at room temp.; (*v*) 1,2,4-triazole, phenylphosphonic dichloride, DMF, 5 min, 3 °C.

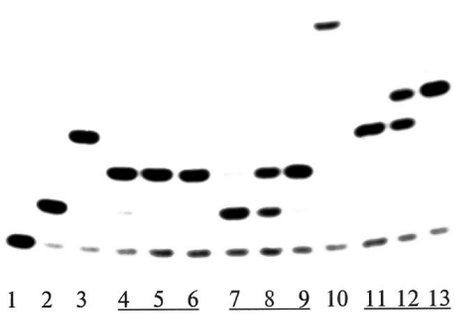


Fig. 1 Primer extension catalysed by AMV-rt in the presence of **4**, **5** and **2**: *Lane 1*, template–primer (complex 1) + enzyme; *lane 2*, template–primer (complex 1) + enzyme + dTTP (2 μM); *lane 3*, as *lane 2* + dGTP (2 μM); *lanes 4–6,* as *lane 2* + **4** [0.01 μM, (*4*)], [0.1 μM, (*5*)] and [1 μM (*6*)]; *lanes 7–9*, as *lane 2* + **5** [0.2 μM, (*7*)], [2 μM, (*8*)] and [20 μM, (*9*)]; *lane 10*, as *lane 2* + dGTP and dATP (2 μM each); *lanes 11–13*, as *lane 2* + dGTP (2 μM) + **2** [0.2 μM, (*11*)], [2 μM, (*12*)] and [20 μM, (*13*)].

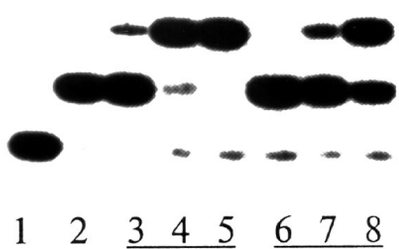


Fig. 2 Primer extension catalysed by AMV-rt in the presence of **6**: *Lane 1*, template–primer (complex 1) + enzyme; *lane 2*, template-primer (complex 1) + enzyme + dTTP (2 μM); *lanes 3–5*, as *lane 2* + ddGTP [0.02 μM, (*3*)], [0.2 μM, (*4*)] and [2 μM, (*5*)]; *lanes 6–8*, as *lane 2* + **6** [0.02 μM, (*6*)], [0.2 μM, (*7*)] and [2 μM, (*8*)].

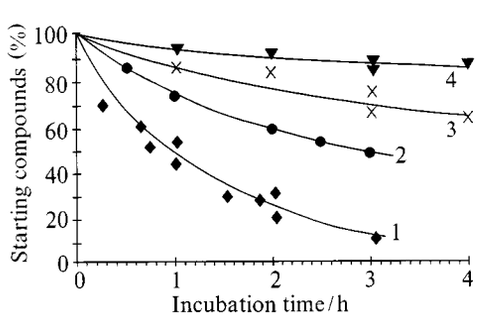


Fig. 3 The effect of **2** and **3** on the dephosphorylation rate of **7** in human blood serum. *Curves: (1)* **7** (0.5 mM); (*2*) **7** (2.0 mM); (*3*) **7** (0.33 mM) + **3** (1.65 mM); (*4*) **7** (0.33 mM) + **2** (1.65 mM).

3, reduced the activity with HIV-rt by at least an order of magnitude when compared with the parent pyrophosphoryl phosphonate **1**. The dibromomethylene analogue **2** was not utilised when either the RNA template (complex 4) or the DNA template (complex 2, data not shown) were used, even at concentrations of 600 μM. Similar results were obtained for compounds **4**–**6** in chain elongation experiments (with complex 1) catalysed by HIV-rt. The activity of the γ-methyl analogue **9** (with HIV-rt and complex 1) was approximately one tenth that of **10** and triphosphonates **6** and **10** were 10–50 times less active than their parent pyrophosphoryl phosphonate **4**. The relative efficacies of compounds **4**, **6** and **10**, determined from these experiments, are in close agreement with their IC_{50} values (relative to AZTTP) which have been determined in an independent colourimetric assay (Table 3).[25]

Figs. 1 and 2 demonstrate the terminating substrate properties of compounds **2** and **4**–**6** in the process of AMV-rt catalysed DNA synthesis. Incorporation of triphosphonates **2** and **5** at the 3′-position of the primer was 2000-fold weaker than that of **4** (compare lanes 13 and 9 with lane 4, Fig. 1). The incorporation of ATP into the 3′-terminus of the growing primer chain (complex 1) in the presence of **2** was inhibited by not more than 10% when the molar ratio of **2** to ATP was 100 : 1 (data not shown). It is evident from Fig. 2 that the utilisation of **6** (lanes 7, 8) is more than 10 times less effective than that of ddATP (lanes 3, 4). The difluoromethylenediphosphonate derivative **6** proved to be at least 200 times weaker as a terminating substrate than its pyrophosphate counterpart **4** (compare lanes 6–8, Fig. 2, and lanes 4–6, Fig. 1). Triphosphonates **7**, **9** and **10** showed weak terminating substrate properties with AMV-rt and compound **8** was even less effectively utilised by this enzyme (Table 2).

In the experiments with DNA polymerase β, the ddGTP analogue **4** was a rather poor substrate for this enzyme and the

dihalomethylenediphosphonate derivatives **5** and **6** showed no substrate properties. Similar results were obtained for the adenine derivatives **1**–**3**. None of the analogues **1**–**6** were utilised by DNA polymerase α up to concentrations of 600 μM (Table 2). Since DNA polymerases α and β did not recognise compounds **6–8**, we did not evaluate the α,β,γ-phosphate modified analogues **9** and **10** with these enzymes.

The results of the experiments with TDT demonstrate that the replacement of the β,γ-phosphate by a dibromo- or difluoromethylenediphosphonate unit decreases the terminating substrate properties by about 10-fold as compared with ddATP and ddGTP (Table 2). Modified triphosphonates **7**, **8** and **10** are more than 100 times less effective as terminating substrates for TDT than the triphosphonates **2**, **3**, **5** and **6**. The terminating substrate properties of the pyrophosphoryl phosphonate **1** and its D-stereoisomer under catalysis by reverse transcriptases and TDT have been demonstrated previously.[26]

The kinetic parameters for compounds **1**–**6** in the reactions catalysed by HIV-rt and AMV-rt are given in Table 4. The K_m values for **2** and **5** in the reaction catalysed by HIV-rt are not presented since their substrate activity was not evident, even at concentrations of 600 μM (Table 2). A comparison of the K_m values for **3** and **6** relative to **1** and **4**, respectively, demonstrates the 8–10-fold decrease in the affinity of the former compounds for HIV-rt. It should be noted that the K_m values for compounds **1** and **4** were 2–3 times higher than those of ddATP and ddGTP, respectively, whereas their reaction rates were close to V_{max} of their respective triphosphonate derivatives, **2**, **3** and **5**, **6**. In the case of AMV-rt the K_m and V_{max} values of the compounds under investigation were close to those obtained for the reactions catalysed by HIV-rt and compounds **2** and **5** also revealed terminating substrate properties towards AMV-rt.

Table 5 contains data on the enzymatic stability of the test compounds in human blood serum. Blood serum is an appropriate medium in which to perform this assay as it contains numerous dephosphorylating enzymes and so provides a good model system of the extracellular environment *in vivo*. The half-lives of **3**, **5** and **6** were shown to be 70–100 times higher than those of the corresponding dNTP; the value for **2** was 200-fold greater. Surprisingly, the half-lives of triphosphonates **9** and **10**, bearing an additional modification at the γ-phosphate residue, were only 20 times greater than that of dGTP. As illustrated by the reversed-phase-HPLC retention times (Table 5), all of the test compounds showed a marked increase in hydrophobicity when compared to the 'natural' dNTP. This is particularly significant for the Pγ-phenyl substituted derivatives **7** and **10**. The HPLC retention time of **10** lies between those of the monophosphonate and the *pseudo*-triphosphate (*ent*)-**4**.

Fig. 3 illustrates the enzymatic stability of diphosphonate phosphate **7** in human blood serum at two concentrations and in the presence of triphosphonates **2** and **3**. The dephosphorylation rate of **7** decreases by seven-fold in the presence of **2** and four-fold in the presence of **3** (compare curve 4 with curves 1 and 2, respectively); in these experiments the molar ratio of either **2** or **3** to **7** is 5 : 1. Increasing the concentration of **7** to 2 mM (which corresponds to the combined concentrations of **7** and **2/3** in the experiments in the presence of triphosphonates) decreases the hydrolysis rate of **7** only by two-fold (curve 3). When the concentration of **2** was about 20 times higher than that of **7** the extent of hydrolysis of the latter was not more than 5% after 3.5 h (data not shown).

The data on the inhibition of pSG1 virus replication in Rat 1 fibroblasts are presented in Table 6. Monophosphonates **11** and **12** were used as controls and AZT, FLT and d_4T were used as reference standards. Both the triphosphonates **2** and **6** inhibited virus reproduction with IC_{50} values of 0.12 μM and 12.67 μM and the values for compounds **11** and **12** were 7.46 μM and >100 μM, respectively. Triphosphonates **9** and **10** were inactive up to the maximum concentrations tested (100 μM).

To evaluate the effects of the compounds on transcription of the *lac*Z gene compounds **2**, **6**, **11** and **12**, at concentrations of 100 and 200 μM, were added to Rat1 cells, infected by pSG1 virus 24 h earlier. By this time reverse transcription was com-

Table 3 The relative efficacy of compounds **4**, **6** and **10** as inhibitors of HIV-rt[a]

Compound	IC_{50}/μM
4	1.3
6	16.5
10	49.4
AZTTP	1.65

[a] Testing was carried out using the DuPont RT-Detect™ Reverse Transcriptase Assay (DuPont, NEK-070A) and recombinant HIV-1-rt (Dupont, NEI-490).

Table 5 Half-life of test compounds in blood serum and retention time on reversed-phase HPLC

Compound	Half-life time	Retention time/min
2	110 h	17.7
3	30 h	17.5
5	31 h	16.4
6	45 h	16.3
7	50 min	22.3
9	10 h	15.8
10	12 h	19.5
(*ent*)-**4**	65 min	16.5
dATP	<30 min	12.6
dGTP	<30 min	11.6
dTTP	5 min[a2]	
11		23.4
12		21.9

[a] Conditions were identical to those used above.

Table 4 Kinetic constants for **1**–**6** in the one-step primer extension reaction catalysed by HIV-rt and AMV-rt

	HIV-rt		AMV-rt	
Compound	K_m/μM	$V_{max}/V_{max(ddNTP)}$	K_m/μM	$V_{max}/V_{max(ddNTP)}$
ddATP[a]	0.105 ± 0.001	1	0.095 ± 0.023	1
1[a]	0.008 ± 0.0008	1.15	0.026 ± 0.004	1.52
2[a]	—	—	0.537 ± 0.075	0.41
3[a]	0.016 ± 0.001	1.05	0.091 ± 0.014	1.01
ddGTP[b]	0.0517 ± 0.0048	1	0.0139 ± 0.0016	1
4[b]	0.00107 ± 0.0002	1.10	0.0077 ± 0.0001	1.50
5[b]	—	—	0.1831 ± 0.059	0.27
6[b]	0.0078 ± 0.0001	1.02	0.0249 ± 0.0026	1.01
ent-**4**[b]	0.0147 ± 0.0001	1.20	0.0377 ± 0.0002	1.62

[a] With the template–primer complex 2. [b] With the template–primer complex 3.

Table 6 Inhibition of Mu-MLV by modified nucleoside triphosphate analogues

Compound	IC_{50}/μM
5	0.12 ± 0.05
6	12.67 ± 1.86
8	>10
9	>100
10	>100
11	7.46 ± 1.16
12	>100
AZT[a]	0.003 ± 0.0003
FLT[b]	0.011 ± 0.001
d_4T[c]	0.845 ± 0.101

[a] AZT = 3′-azido-3′-deoxythymidine. [b] FLT = 3′-fluoro-3′-deoxythymidine. [c] d4T = 2′,3′-dideoxy-2′,3′-didehydrothymidine.

pleted (half-life of reverse transcription for pSG1 was determined as 6 h when 0.1 μM AZT was used to inhibit this process).[27] After an additional incubation for 24 h the cells were stained with Xgal. The number of blue colonies did not differ from the control at test compound concentrations of up to 200 μM indicating that transcription of *lac*Z gene was not altered by these compounds. Cytotoxic effects were not observed with any of the above compounds at concentrations up to 1 mM.

Discussion

To date, there have been very few reports on the properties of natural and modified nucleoside triphosphonates. In 1995 AZT-α,β,γ-(bismethylene)triphosphonate was synthesised and shown to inhibit HIV-rt catalysed oligodeoxythymidine synthesis 1000 times less efficiently than AZTTP.[28] This can be ascribed to the fact that the compounds bearing the hydrolytically stable α,β-methylenediphosphonate linkage are unable to be incorporated at the 3′-terminus of a polydeoxythymidine chain and thereby terminate further chain elongation.

The experiments reported herein demonstrate the terminating substrate properties of compounds **1**–**6** for a range of DNA polymerases. The introduction of the β,γ-dibromomethylenediphoshonate moiety, in compounds **2** and **5**, results in a sharp decrease in affinity to HIV-rt on both DNA and RNA templates. The difluoromethylene triphosphonate **3** is a more powerful terminating substrate for AMV-rt than its dibromomethylene analogue **2**. The affinity of compounds **3** and **6** towards DNA-synthesising complexes is, as a rule, one order of magnitude lower than those of the natural dNTP (dATP and dGTP) and the parent pyrophosphoryl phosphonates **1** and **4**, respectively. DNA polymerases α and β do not recognise the adenine and guanine derivatives **2**, **3**, **5**, **6** and **8** all of which bear a diphosphonate residue instead of the terminal pyrophosphate moiety. In the case of TDT the replacement of the pyrophosphate unit by either a dibromo- or difluoromethylenediphosphonate unit decreases the terminating substrate properties of the compounds by ten-fold. Compounds **7**, **8** and **10** are more than 100 times weaker than the triphosphonates **2**, **3**, **5** and **6** which do not possess an alkyl/aryl substituent at Pγ. Thus triphosphonates **2**, **3**, **5** and **6** selectively inhibit DNA synthesis catalysed by AMV-rt and template independent TDT but do not significantly affect the process catalysed by DNA polymerases α and β. The activity of the dibromo-derivatives **2** and **5** towards HIV-rt is 1000-fold lower than that of the parent pyrophosphoryl phosphonates **1** and **4** whereas the difluoro-derivatives **3** and **6** only show a ten-fold reduction in activity. This significant loss of activity, with compounds **2** and **5**, may be a result of greater steric and electronic dissimilarities between the dibromomethylenediphosphonate group and the pyrophosphate unit which it replaces; the difluoromethylene group is evidently a superior bioisostere.[29] The enhanced hydrophobicity of the triphosphonate **10** (by HPLC, Table 5) makes it a promising candidate for further development as an antiviral agent. Although compound **10** proved to be a good inhibitor of retroviral DNA polymerases in cell free assays, its activity as an inhibitor of pSG1 virus replication in cell culture was less significant. Thus further modification of this compound is warranted in order to increase its lipophilicity, to further facilitate cell penetration, without compromising its potency as a terminating substrate.

The stability studies of the triphosphonates **2**, **3**, **5** and **6** in human blood serum testifies that, in these compounds, the phosphate bond between Pα and Pβ is essentially more stable towards enzymatic hydrolysis than that of the natural triphosphates. This could be attributed to these analogues being poorer substrates for those dephosphorylating enzymes which function to hydrolyse NTPs at this position. The markedly increased stability of **2** is noteworthy. The half-life of the diphosphonate phosphate **7**, bearing the β-phosphate group, is close to that of dATP. A decrease in the rate of hydrolysis of **7** in the presence of either **2** or **3** indicates that **2** and **3** may be bound by these enzymes as a result of a catalytic process or by specific adsorption.

Inhibition of pSG1 virus replication in Rat1 cells by the compounds under study is clearly evident. Triphosphonate **2** is about 30 times more potent than the corresponding monophosphonate **11**. Triphosphonate **6** (with the guanine base) exhibits an effect similar to monophosphonate **11** (with the adenine base) whilst monophosphonate **12** (with the guanine base) shows no activity up to concentrations of 100 μM. These data support the hypothesis of the direct action of these triphosphonates as inhibitors of viral replication in whole cell systems and indicate that a two-step molecular mechanism of inhibition, involving hydrolysis of **2** and **6** to the corresponding monophosphonates **11** and **12** followed by intracellular diphosphorylation, is unlikely. The results obtained are promising with respect to the development of a new type of active inhibitor of virus reproduction which does not require intracellular phosphorylation. The 50% inhibition of virus reproduction by **2** in this system is 40 times lower (and **6** 4000 times lower) than that of AZT but 7 times higher than that of d_4T. This decrease in activity could be rationalised by one or more of the following reasons: (i) Cell membrane permeability is insufficient for the penetration of **2** and **6** into the cell. (ii) Partial dephosphorylation of the triphosphonates during the process of diffusion across the cell membrane may take place. (iii) The triphosphonates may show decreased affinity towards retroviral reverse transcriptases compared to AZTTP.[15] We do not know at present whether modified triphosphonates inhibit reverse transcription of the virus genome in the intercellular media, prior to the virus penetrating the cell (as has been shown for AZTTP),[1] or whether they do so in the infected cells during the process of ordinary reverse transcription. To answer this question will require another model system since the pSG1 virus loses the infectivity after 20 h of incubation with dNTP at 37 °C.[30]

Conclusion

Taking an overview of the data obtained, it is important to emphasise the complementary combination of useful properties of the new compounds. These compounds are examples of a new generation of dNTP analogues, modified at both the triphosphate side-chain and the glycone, that are able to inhibit the replication of retroviruses in cell cultures and show high stability in human blood serum. Not only do these compounds point the way to potential antiviral chemotherapeutics but also the use of such compounds in cell biology studies is worthy of consideration. For these purposes, slowly dephosphorylating substrates (or terminating substrates) must bear a ligand or a reporter group in the nucleic base or another part of the molecule. The synthesis and biological evaluation (in cell free

systems) of the first compounds in this series is already in progress.[31]

Experimental

General

Petroleum ether had bp 40–60 °C (referred to as petrol). TLC was performed on pre-coated silica gel glass plates (Merck silica gel 60F 254). The plates were visualised using UV light (254 nm), alkaline potassium permanganate, or ethanolic bromocresol green. The TLC assay of reactions involving the highly polar phosphate and triphosphate analogues were carried out as above eluting with either 1 M NH_4HCO_3(aq)–EtOH–NH_4OH(dilute) (20 : 50 : 1) or dioxane–ammonium–water (6 : 4 : 5). Flash chromatography was performed over silica (Merck silica gel 60 40–63 μm). Anion exchange chromatography was performed on a column of Fractogel EMD DEAE-650 (M) (Merck, Art. 16886) (40 × 100 mm) eluting with a linear gradient of aqueous ammonium hydrogen carbonate at a flow rate of *ca*. 200 ml h^{-1}. The column was connected to a UV detector (254 nm, LKB 2238, Uvicord SII) and a fraction collector. NMR spectra were recorded on a Bruker AM300 and a Bruker Avance DRX 400 spectrometer at the following frequencies: 300 or 400 MHz for ^{1}H NMR, 75 or 100 MHz for ^{13}C NMR, 162 MHz for ^{31}P NMR (H_3PO_4 external reference) and for ^{19}F NMR either 376 MHz (C_6F_6 external reference) or 188 MHz (CF_3CO_2H external reference); *J* values are given in Hz. Infrared spectra were recorded on a Perkin-Elmer 881 Fourier Transform Spectrophotometer. UV spectra were recorded on a UV4-200 Unicam UV–VIS spectrometer. Mass spectra were obtained on either a Kratos Profile HV3, Kratos MS 50TC or a VG7070E/DEC VAX 4000.60 spectrometer.

Enzymes and DNA. HIV1-rt was purchased from Worthington Corp. (USA); DNA polymerases α and β were isolated from human placenta.[32,33] AMV-rt and TDT were from Omutninsk Chemicals (Russia) and Amersham Corp., respectively. M13mp10 DNA was isolated from the culture medium of the recipient *E. coli* K12XL1 strain.[34] Heteropolymeric RNA was synthesised by run-off transcription of *Sal*GI-digested plasmid pPV-19 with T7 RNA polymerase.[35] The plasmid containing a fragment of pBR322 DNA between the *Sal*GI and *Sph*I sites was a gift from Dr S. Kochetkov. The oligonucleotide primers were labelled at the 5′-terminus using [γ-^{32}P]ATP (Radioizotop, Russia) and T4 polynucleotide kinase.[34] The DNA (0.5 mM) was hybridised with 0.75 mM [5′-^{32}P]-labelled primer in the following buffers: 10 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$, 40 mM KCl and 1 mM dithiothreitol (for reverse transcriptases); 10 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$ and 0.4 mM dithiothreitol (for DNA polymerase α); 10 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$ and 1 mM dithiothreitol (for DNA polymerase β).

Methods

(1′*S*,4′*R*)-9-[4′-(Hydroxy){[(difluoro)(phosphono)methyl]-hydroxyphosphoryloxy}phosphorylmethoxy)cyclopent-2′-enyl]-adenine (tris-ammonium salt) (3). To a solution of **11** (156 mg, 0.5 mmol) in absolute DMF (5 cm^3) was added *N*,*N*′-carbonyldiimidazole (162 mg, 1.0 mmol) under an inert atmosphere and the mixture was stirred for 12 h at 20 °C. A solution of **32** (0.5 M in DMF, 2.5 cm^3) was then added and the reaction mixture was stirred at 37 °C for 12 h. The reaction mixture was diluted with water (80 cm^3) before purification by anion exchange chromatography eluting with a linear gradient of $NH_4HCO_{3(aq)}$ (0–0.4 M, 600 cm^3). Product fractions were combined and coevaporated several times with water (3 × 10 cm^3) and ethanol (1 × 5 cm^3) before further purification by reversed-phase chromatography, eluting with water. Product fractions were lyophilised to give **3** as a white amorphous solid (130 mg, 52%). ν_{max}(KBr)/cm^{-1} 1269 (P=O); λ_{max}(H_2O)/nm 263 (14800); δ_H(400 MHz, D_2O) 1.96 (1H, m, 5′-βH), 2.92 (1H, m, 5′-αH), 3.61 (2H, d, *J* 9.0, CH_2P), 4.66 (1H, s, 4′-H), 5.39 (1H, m, 1′-H), 5.92 (1H, m, 2′-H), 6.13 (1H, m, 3′-H), 8.16 (1H, s, 8-H), 8.18 (1H, s, 2-H); δ_P(162 MHz, D_2O) −2.08 (m, P_β), 6.37 (m, *J* 65.3, P_γ), 11.34 (d, *J* 32.4, P_α); δ_F(188 MHz, D_2O) −44.1 (dd, *J* 95.1, 81.0); *m*/*z* (FAB) 523 ($MH^+ + NH_3$), 540 ($MH^+ + 2NH_3$).

(1′*S*,4′*R*)-9-[4′-(Hydroxy{[(difluoro)(phosphono)methyl]-hydroxyphosphoryloxy}phosphorylmethoxy)cyclopent-2′-enyl]-guanine (tris-ammonium salt) (6). A solution of the morpholidate **33** (0.081 mmol) in DMSO (1 cm^3) was added dropwise to a solution of **32** (0.4 mmol) in DMSO (2 cm^3) and left to stir at room temperature for 13 h. The reaction mixture was diluted with water (200 cm^3) before purification by anion exchange chromatography eluting with a linear gradient of $NH_4HCO_{3(aq)}$ (0–0.4 M, 800 cm^3). The product eluted at 0.22 M. Fractions were combined and coevaporated several times with water before further purification by reverse-phase chromatography, eluting with water. Product fractions were lyophilised to give **6** as a fluffy white lyophilate (11 mg, 24%). ν_{max}(KBr)/cm^{-1} 1269 (P=O); λ_{max}(H_2O)/nm 252.0; δ_H(400 MHz, D_2O) 1.89 (1H, dt, *J* 15.6, 4.4, 5′-βH), 2.97 (1H, dt, *J* 14.0, 7.2, 5′-αH), 3.84 (2H, d, *J* 9.2, PCH_2), 4.76–4.81 (1H, br, 4′-H), 5.0–5.28 (1H, br, 1′-H), 6.07–6.11 (1H, m, 2′-H), 6.33–6.38 (1H, m, 3′-H), 7.75–7.87 (1H, br, 8-H); δ_F(376 MHz, D_2O) 42.40–42.90 (br, CF_2); δ_P(162 MHz, D_2O) −1.69 (br, P_β), 3.90 (br, P_γ), 11.44 (d, *J* 31.0, P_α); *m*/*z* (ES) 522 (6%, MH^+), 328 (15, M − $CH_2F_2O_4P_2$).

(1′*S*,4′*R*)-9-[4′-(Hydroxy{[(phenyl)hydroxyphosphoryloxy]-hydroxyphosphoryloxy}phosphorylmethoxy)cyclopent-2′-enyl]-adenine (tris-ammonium salt) (7). a) To a solution of **11** (120 mg, 0.39 mmol) in DMF (15 cm^3) was added dimethylformamide dimethyl acetal (0.2 cm^3) and the reaction was kept for 20 h at 20 °C. The solvent was removed *in vacuo*, the residue diluted in DMF (10 cm^3) and *N*,*N*′-carbonyldiimidazole (137 mg, 1.15 mmol) was added. After stirring for 12 h at 20 °C a solution of the tributylammonium salt of phosphoric acid (0.5 M in DMF, 0.6 cm^3) was added; the suspension was stirred for 3 h. The reaction mixture was quenched with 15% aqueous ammonium hydroxide (to pH 10) and the mixture was stirred for 1 h. The product was isolated and purified as described for **3** to give **34** (52 mg, 16%). δ_P(162 MHz, D_2O) −8.05 (d, *J* 25.5), 8.8 (d, *J* 25.5).

b) A solution of 1,2,4-triazole (20.7 mg, 0.3 mmol) and triethylamine (30.3 mg, 0.3 mmol) in acetonitrile (10 cm^3) was cooled to 3 °C and phenylphosphonic dichloride (29.3 mg, 0.15 mmol) was added. The reaction mixture was kept for 30 min at 3 °C and then centrifuged. The supernatant was added to a solution of the tributylammonium salt of **34** (0.1 mmol) in DMF (2 cm^3). The reaction mixture was stirred for 5 min and then evaporated. The residue was quenched with water (30 cm^3) and then purified, as described for **3**, to afford **7** (29.7 mg, 56%). δ_P(162 MHz, D_2O) −19.9 (m, P_β), 7.1 (d, *J* 24, P_γ), 8.9 (d, *J* 26, P_α); *m*/*z* (FAB) 549 ($MH^+ + NH_3$), 566 ($MH^+ + 2NH_3$).

(1′*S*,4′*R*)-9-[4′-(Hydroxy{[(methylhydroxyphosphoryl)-methyl]hydroxyphosphoryloxy}phosphorylmethoxy)cyclopent-2′-enyl]adenine (tris-ammonium salt) (8). This was synthesised, as described for **3**, from **11** and the bis(tributylammonium) salt of (methylphosphoryl)methylenephosphonate (35% yield). δ_H(400 MHz, D_2O) 1.32 (3H, d, *J* 14.5, PCH_3), 1.86 (1H, m, 5′-βH), 2.16 (2H, dd, *J* 20, 17, PCH_2P), 2.98 (1H, m, 5′-αH), 3.78 (2H, d, *J* 8.5, PCH_2O), 4.78 (1H, s, 4′-H), 5.45 (1H, m, 1′-H), 6.10 (1H, m, 2′-H), 6.40 (1H, m, 3′-H), 8.10 (1H, s, 8-H), 8.20 (1H, s, 2-H); δ_P(162 MHz, D_2O) 9.3 (Pα + Pβ), 35.9 (P_α); *m*/*z* (FAB) 486 ($MH^+ + NH_3$), 503 ($MH^+ + 2NH_3$).

(1′*S*,4′*R*)-9-[4′-(Hydroxy{[(difluoro)(methylhydroxyphosphoryl)methyl]hydroxyphosphoryloxy}phosphorylmethoxy)-cyclopent-2′-enyl]guanine (tris-ammonium salt) (9). A solution of the morpholidate **33** (0.068 mmol) in anhydrous DMSO (1.5 cm^3) was added dropwise to a solution of **30** (0.306 mmol) in DMSO (1.5 cm^3). This was left to stir for 5 h until all of **33** was consumed (by TLC). The reaction mixture was then diluted in water (400 cm^3) before purification by anion exchange chromatography eluting with a linear gradient of $NH_4HCO_{3(aq)}$ (0–0.3 M, 800 cm^3). The product eluted at 0.19 M. Product fractions were coevaporated several times with water before finally being lyophilised to give **9** as a fluffy white lyophilate (18 mg, 46%). ν_{max}(KBr)/cm^{-1} 3600–2600 (br, NH_2, OH), 1690, 1624 (C=N, C=O, C=C), 1247, 1195 (P=O); λ_{max}(H_2O)/nm 252.4; δ_H(400 MHz, D_2O) 1.40 (3H, d, *J* 14.8, CH_3P), 1.86 (1H, dt, *J* 14.4, 4.4, 5′-βH), 2.97 (1H, dt, *J* 14.5, 7.1, 5′-αH), 3.83 (2H, d, *J* 9.1, PCH_2), 4.76–4.80 (1H, m, 4′-H), 5.25–5.31 (1H, m, 1′-H), 6.07–6.11 (1H, m, 2′-H), 6.36 (1H, dt, *J* 5.6, 1.9, 3′-H), 7.70–8.95 (1H, br, 8-H); δ_C(100 MHz, D_2O) 13.31 (d, *J* 98.9, PCH_3), 37.65 (C-5′), 57.38 (C-1′), 65.16 (d, *J* 162.9, PCH_2), 84.61 (d, *J* 11.6, C-4′), 132.81 (C-2′), 136.07 (C-3′), 153.66, 158.95 (C); δ_F(376 MHz, D_2O) 39.56 (dd, *J* 85.1, 75.4); δ_P(162 MHz, D_2O) −4.44 (br, P_β), 10.57 (d, *J* 32.1, P_α), 30.96 (br, P_γ); *m*/*z* (ES) 520 (100%, MH^+), 328 (88, M − $C_2H_4F_2O_4P_2$).

(1′*S*,4′*R*)-9-[4′-(Hydroxy{[(difluoro)(phenylhydroxyphosphoryl)methyl]hydroxyphosphoryloxy}phosphorylmethoxy)-cyclopent-2′-enyl]guanine (tris-ammonium salt) (10). A solution of the morpholidate **33** (0.044 mmol) in anhydrous DMSO (1.5 cm^3) was added dropwise to a solution of **31** (0.250 mmol) in DMSO (1.5 cm^3) followed by 1*H*-tetrazole (6.2 mg, 0.088 mmol) in DMSO (0.5 cm^3). This was left to stir overnight by which time all of **33** had been consumed (by TLC). The reaction mixture was then dissolved in water (400 cm^3) before purification by anion exchange chromatography eluting with a linear gradient of $NH_4HCO_{3(aq)}$ (0–0.3 M, 800 cm^3). The product was eluted at 0.21 M. The product fractions were coevaporated several times with water before finally being lyophilised to give **10** as a fluffy white lyophilate (10 mg, 36%). ν_{max}(KBr)/cm^{-1} 3600–2700 (br, NH_2, NH_3), 1685, 1632 (C=N, C=O, C=C), 1245, 1208 (P=O); λ_{max}(H_2O)/nm 252.0; δ_H(400 MHz, D_2O) 1.72 (1H, dt, *J* 14.5, 4.0, 5′-βH), 2.85 (1H, dt, *J* 14.5, 7.2, 5′-αH), 3.65 (2H, d, *J* 9.0, PCH_2), 5.20–5.26 (1H, m, 1′-H), 6.01–6.06 (1H, m, 2′-H), 6.27 (1H, m, 3′-H), 7.36–7.50 (3H, m, 3 × Ar-H), 7.70–7.85 (3H, m, 2 × Ar-H + 8-H); δ_C(100 MHz, D_2O) 37.50 (C-5′), 57.28 (C-1′), 64.80 (d, *J* 164.4, PCH_2), 84.52 (d, *J* 12.5, C-4′), 128.11 (d, *J* 12.5, Ar-CH), 132.13 (C-2′), 132.85 (d, *J* 9.6, Ar-CH), 132.93 (Ar-CH), 135.93 (C-3′), 154.10, 159.48 (C); δ_F(376 MHz, D_2O) 43.33 (t, *J* 80.8); δ_P(162 MHz, D_2O) −4.82 (br, P_β), 10.86 (d, *J* 32.9, P_α), 19.59 (m, $J_{P_\beta P_\gamma}$ 49.1, P_γ); *m*/*z* (ES) 582 (100%, MH^+), 28 (47, M − $C_7H_6F_2O_4P_2$), 260 (65).

Isopropyl phenylphosphonochloridate (13). To a three necked round bottomed flask fitted with an overhead mechanical stirrer was added a solution of phenylphosphonic dichloride (25.64 g, 0.131 mol) in anhydrous toluene (75 cm^3). This was cooled to 0 °C and a mixture of triethylamine (18.4 cm^3, 0.131 mol) and anhydrous propan-2-ol (10.1 cm^3, 0.131 mol) was slowly added dropwise whilst stirring vigorously. This reagent addition gave an exothermic reaction with the formation of a white precipitate. The thick slurry was allowed to warm to room temperature and left to stir for 90 min. The solids were filtered and washed with toluene. Solvents were then removed to give a crude yellow oil which was purified by vacuum distillation to give **13** as a colourless oil (12.17 g, 42%). Boiling point 102–108 °C (1.5 mmHg); ν_{max}(neat)/cm^{-1} 1453 (Ph–P), 1275 (P=O); δ_H(300 MHz, $CDCl_3$) 1.44 (3H, d, *J* 6.2, CH_3), 1.48 (3H, d, *J* 6.3, CH_3), 5.02–5.15 (1H, m, CH), 7.46–7.54 (3H, m, Ar-H), 7.83–7.93 (2H, m, Ar-H); δ_C(100 MHz, $CDCl_3$) 23.53 (d, *J* 5.2, CH_3), 24.02 (d, *J* 4.3, CH_3), 74.11 (d, *J* 7.7, CH), 128.63 (d, *J* 16.7, CH), 130.91 (d, *J* 11.8, CH), 131.38 (d, *J* 180.7, C), 133.32 (d, *J* 3.4, CH); δ_P(162 MHz, $CDCl_3$) 28.16 [Found: (EI) M^+, 218.02642. $C_9H_{12}Cl^{35}O_2P$ requires 218.02635]; *m*/*z* 220 (0.3%, M^+), 218 (1.3, M^+), 141 (65.7, M–Ph), 77 (100, Ph^+).

Diethyl difluoro[isopropoxy(phenyl)phosphoryl]methylphosphonate (16). To a solution of LDA (6.87 mmol) in THF (8 cm^3) at −78 °C was added a solution of diethyl difluoromethylphosphonate (1.09 g, 5.79 mmol) in THF (2 cm^3) dropwise over 10 min. This gave a yellow solution which was stirred at −78 °C for 30 min. A solution of **13** (1.45 g, 6.63 mmol) in THF (2 cm^3), pre-cooled to −78 °C, was then added *via* a cannula and was left to stir for another 30 min at −78 °C. The reaction was warmed to 0 °C before quenching with a saturated solution of aqueous KH_2PO_4 (20 cm^3). The aqueous layer was then extracted with ethyl acetate (3 × 25 cm^3). The combined organics were dried ($MgSO_4$) and solvents were then removed to give a crude oil which was purified by chromatography eluting with EtOAc–petrol (1 : 1) to give **16** as a colourless oil (364 mg, 17%). ν_{max}(neat)/cm^{-1} 1435 (Ph–P), 1275 (α-P=O), 1256 (β-P=O); δ_H(300 MHz, $CDCl_3$) 1.28 (3H, dt, *J* 7.1, 0.5, CH_3), 1.36 (3H, dt, *J* 7.1, 0.5, CH_3), 1.37 (3H, d, *J* 6.0, CH_3), 1.48 (3H, d, *J* 6.0, CH_3), 5.01–5.12 (1H, m, CH), 7.47–7.55 (2H, m, 2 × ArH), 7.59–7.67 (1H, m, ArH), 7.86–7.97 (2H, m, 2 × ArH); δ_C(100 MHz, $CDCl_3$) 16.23 (d, *J* 5.6, CH_3), 16.32 (d, *J* 5.7, CH_3), 23.84, (d, *J* 5.1, CH_3), 24.58 (d, *J* 3.0, CH_3), 64.96 (d, *J* 6.5, CH_2), 65.12 (d, *J* 6.2, CH_2), 73.65 (d, *J* 6.9, CH), 126.42 (d, *J* 141, C), 128.40 (d, *J* 13.6, CH), 133.25 (d, *J* 9.9, CH), 133.62 (d, *J* 2.9, CH); δ_P(162 MHz, $CDCl_3$) MX of an ABMX system, 4.80 (ddd, *J* 88.4, 86.7, 50.3, P_α), 24.10 (ddd, *J* 84.6, 74.3, 56.2, P_β); δ_F(376 MHz, $CDCl_3$) AB of an ABMX system, 42.00 (ddd, *J* 370.3, 88.5, 74.4), 43.86 (dt, *J* 370.1, 85.8) [Found: (EI) M^+, 370.09240. $C_{14}H_{22}F_2O_5P_2$ requires 370.09106]; *m*/*z* 370 (2.7%, M^+), 141 (96.3, $PhP(O)OH^+$), 77 (100, $C_6H_5^+$), 51 (34.8, CF_2H^+).

The following side products were also characterised following isolation from the appropriate fractions of the chromatographic separation described above. Any additional purification procedures are as detailed below:

Isopropyl methylphosphonochloridate (14). This was prepared from methylphosphonic dichloride (4.87 g, 0.037 mmol) as described for **13** and was isolated as a colourless oil (2.58 g, 45%). Boiling point 68–74 °C (17 mmHg); ν_{max}(neat)/cm^{-1} 1315 (CH_3–P), 1269 (P=O), 1012 (P–O^{i}Pr); δ_H(300 MHz, $CDCl_3$) 1.39 (3H, d, *J* 6.1, CH_3), 1.40 (3H, d, *J* 6.1, CH_3), 1.94 (3H, d, *J* 17.4, CH_3), 4.87–5.01 (1H, m, CH); δ_C(100 MHz, $CDCl_3$) 20.69 (d, *J* 129.9, PCH_3), 23.35 (d, *J* 5.0, CH_3), 23.97 (d, *J* 4.7, CH_3), 73.32 (d, *J* 8.0, CH); δ_P(162 MHz, $CDCl_3$) 39.24 [Found: (EI) M^+, 156.01107. $C_4H_{10}Cl^{35}O_2P$ requires 156.010696]; *m*/*z* 159 (27.9%, MH^+), 157 (89.7, MH^+).

Diethyl difluoro[isopropoxy(methyl)phosphoryl]methylphosphonate (15). To a solution of LDA (3.20 mmol) in THF (4 cm^3) at −78 °C was added a solution of diethyl difluoromethylphosphonate **17** (506 mg, 2.69 mmol) in THF (2 cm^3) dropwise over 10 min. This was stirred at −78 °C for 30 min. A solution of **14** (485 mg, 3.09 mmol) in THF (2 cm^3) was then pre-cooled to −78 °C before addition *via* a cannula; the resultant solution was left to stir for 30 min at −78 °C. The reaction was warmed to 0 °C before quenching with a saturated solution of aqueous KH_2PO_4 (10 cm^3). This mixture was extracted with ethyl acetate (3 × 15 cm^3), the combined organics were dried ($MgSO_4$) and solvents were then removed to give a crude oil which was purified by chromatography, eluting with EtOAc–petrol (3 : 2), to give **15** as a colourless oil (209 mg, 25%). ν_{max}(neat)/cm^{-1} 1274 (α-P=O), 1251 (β-P=O); δ_H(400 MHz, $CDCl_3$) 1.34 (3H, d, *J* 7.0, CH_3), 1.37 (3H, d, *J* 7.0, CH_3), 1.38 (6H, dt, *J* 7.5, 0.6, 2 × CH_3), 1.68 (3H, ddd, *J* 15.6, 2.0, 1.1, PCH_3), 4.28–4.39 (4H, m, 2 × CH_2), 4.96 (1H, dseptet, *J* 7.1, 5.5, CH); δ_C(100 MHz,

$CDCl_3$) 12.36 (d, *J* 100.4, PCH_3), 16.33 (d, *J* 5.5, CH_3), 23.42 (d, *J* 5.5, CH_3), 24.81 (d, *J* 2.2, CH_3), 65.20 (d, *J* 6.7, CH_2), 65.42 (d, *J* 6.5, CH_2), 72.98 (d, *J* 7.0, CH); δ_P(162 MHz, $CDCl_3$) MR of an ABMR system, 4.64 (dt, *J* 85.5, 60.3, P_α), 38.12 (ddd, *J* 80.0, 71.5, 60.2, P_β); δ_F(376 MHz, $CDCl_3$) AB of an $ABMRX_3$ system, 37.99 (ddd, *J* 369.7, 84.5, 71.6), 41.72 (dddq, *J* 369.7, 85.8, 79.9, 1.9) [Found: (EI) M^+, 308.07557. $C_9H_{20}F_2O_5P_2$ requires 308.07539]; *m*/*z* 308 (0.7%, M^+), 210 (100), 171 (7.4, $M - (EtO)_2P(O)$), 121 (13.0, $M - (EtO)_2P(O)CF_2$), 51 (1.9, CF_2H^+).

Difluoromethylenebis[isopropyl(phenyl)phosphonate] (19). The crude fractions of the isomeric mixture of **19** were further purified by chromatography using EtOAc–petrol (2 : 5) as eluent to give a racemic mixture of **19** (43 mg). The mesomeric isomers were not isolated although they were evident in the crude mixture. δ_H(300 MHz, $CDCl_3$) 1.33 (6H, d, *J* 6.6, 2 × CH_3), 1.44 (6H, d, *J* 6.6, 2 × CH_3), 5.05–5.19 (2H, m, 2 × CH), 7.40–7.49 (4H, m, 4 × ArH), 7.53–7.60 (2H, m, 2 × ArH), 7.80–7.90 (4H, m, 4 × ArH); δ_C(100 MHz, $CDCl_3$) 23.77 (CH_3), 24.57 (CH_3), 73.57 (d, *J* 6.6, CH), 126.80 (d, *J* 150.6, C), 128.33 (d, *J* 13.6, CH), 133.07 (d, *J* 10.1, CH), 133.41 (CH); δ_P(162 MHz, $CDCl_3$) 24.74 (t, *J* 82.0); δ_F(376 MHz, $CDCl_3$) 44.82 (t, *J* 82.0).

The ^{19}F NMR data for the meso isomer of **19** is as follows: δ_F(376 MHz, $CDCl_3$) AB of an ABX spectra, 41.70 (dt, *J* 367.0, 75.2), 45.28 (dt, *J* 367.1, 85.3).

Isopropoxydifluoromethylphenylphosphine oxide (20). This was obtained as a crude mixture with **17** (130 mg, purity 85% *w*/*w*). Attempts to remove the contaminant **17** by Kugelrohr distillation were not successful. ν_{max}(neat)/cm^{-1} 1440 (Ph–P), 1249 (P=O); δ_H(300 MHz, $CDCl_3$) 1.37 (3H, d, *J* 6.1, CH_3), 1.46 (3H, d, *J* 6.1, CH_3), 4.84–4.96 (1H, m, CH), 5.97 (1H, dt, *J* 50.7, 24.3, PCF_2H), 7.50–7.58 (2H, m, 2 × ArH), 7.60–7.68 (1H, m, ArH), 7.82–7.95 (2H, m, 2 × ArH); δ_C(100 MHz, $CDCl_3$) 24.13 (d, *J* 4.0, CH_3), 24.32 (d, *J* 4.1, CH_3), 72.71 (d, *J* 6.6, CH), 113.27 (dt, *J* 259.9, 147.4, PCF_2H), 125.68 (d, *J* 135.5, C), 128.76 (d, *J* 13.3, CH), 132.80 (d, *J* 10.0, CH), 133.78 (d, *J* 2.9, CH); δ_P(162 MHz, $CDCl_3$) X of an ABX system, 23.43 (dd, *J* 83.8, 76.9); δ_F(376 MHz, $CDCl_3$) AB of an ABX system, 28.13 (ddd, *J* 346.2, 83.8, 49.0), 30.18 (ddd, *J* 346.2, 76.9, 49.3) [Found: (EI) M^+, 234.06228. $C_{10}H_{13}F_2O_2P$ requires 234.06213]; *m*/*z* 234 (0.1%, M^+), 183 (37.3, $M - CF_2H$), 141 (100, PhP(O)OH^+), 77 (86, $C_6H_5^+$), 51 (39.3, CF_2H^+).

Tetraethyl difluoromethylenediphosphonate (18).[23] To a solution of LDA (1.27 mmol) in THF (1.5 cm^3) at −78 °C was added a solution of diethyl difluoromethylphosphonate **17** (218 mg, 1.16 mmol) in THF (1.5 cm^3). This was left to stir at −78 °C for 30 min before the addition of diethyl chlorophosphate (0.17 cm^3, 1.16 mmol) in THF (1.5 cm^3). The reaction was then stirred at −78 °C for 60 min before warming to 0 °C and quenching with a saturated solution of aqueous potassium dihydrogen phosphate. This was extracted with EtOAc (3 × 8 cm^3), dried ($MgSO_4$) and the solvents were then removed to give a crude yellow oil. Purification by chromatography, using EtOAc–petrol (3 : 2) as eluent gave a colourless oil which was further purified by Kugelrohr distillation (impurities and some of the product distilled over at 130 °C, 2 mmHg) to leave the clean product residue **18** as a colourless oil (128 mg, 34%). ν_{max}(neat)/cm^{-1} 1282 (P=O), 1038 (P–OEt); δ_H(300 MHz, $CDCl_3$) 1.39 (12H, t, *J* 7.1, 4 × CH_3), 4.25–4.42 (8H, m, 4 × CH_2); δ_C(100 MHz, $CDCl_3$) 16.34 (t, *J* 2.7, CH_3), 65.33 (t, *J* 3.1, CH_2); δ_P(162 MHz, $CDCl_3$) 4.29 (t, *J* 86.3); δ_F(376 MHz, $CDCl_3$) 42.40 (t, *J* 86.3) [Found: (EI) M^+, 324.07061. $C_9H_{20}F_2O_6P_2$ requires 324.07032]; *m*/*z* 324 (2.5%, M^+), 212 (100), 188 (36.3, $(EtO)_2P(O)CF_2H^+$).

Difluoro[hydroxy(methyl)phosphoryl]methylphosphonic acid (tris-ammonium salt) (27). Bromotrimethylsilane (0.4 cm^3, 3.03 mmol) was added dropwise to a neat sample of the triester **15** (77 mg, 0.27 mmol) under an inert atmosphere and was left to stir at room temperature for three days. The excess bromosilane was removed *in vacuo* before the addition of water (2 cm^3) which was then left to stir for 30 min after which the cloudy emulsion had cleared. The reaction mixture was washed with ether (4 × 1 cm^3). Aqueous ammonium bicarbonate solution (1 M, 2 cm^3) was added to the aqueous layer and excess volatile salts were removed by repeated coevaporation with water before the sample was finally lyophilised to give the product ammonium salt **27** as a glassy white solid in quantitative yield (73 mg). ν_{max}(KBr)/cm^{-1} 3600–2600 (br, NH_3), 2300–2100, 2050–1800 (br, POH), 1183 (P=O), 1091 and 1124 (d, P=O); δ_H(300 MHz, D_2O) 1.51 (3H, d, *J* 15.0, PCH_3); δ_C(100 MHz, D_2O) 13.58 (d, *J* 96.0, PCH_3); δ_F(376 MHz, D_2O) 41.36 (t, *J* 76.6); δ_P(162 MHz, D_2O) 32.28 (dt, *J* 78.4, 48.6, P_β), 5.00 (dt, *J* 73.6, 47.3, P_α) [Found: (FAB$^-$) $M - H^-$, 208.95768. $C_2H_5F_2O_5P_2$ requires 208.95803]; *m*/*z* 209 (100%, $M - H^-$).

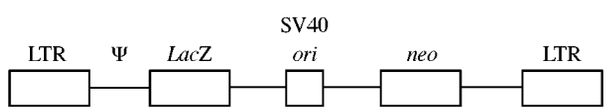
Difluoro[hydroxy(phenyl)phosphoryl]methylphosphonic acid (28). Bromotrimethylsilane (2.4 cm^3, 18.18 mmol) was added dropwise to a neat sample of **16** (210 mg, 0.57 mmol) under nitrogen and left to stir at room temperature for 3 days. The mixture was cooled to 0 °C and quenched with water (3 cm^3). This solution was left to stir for 2 h before extracting with ethyl acetate (4 × 4 cm^3). Decolourising charcoal (100 mg) was then added to the aqueous layer which was agitated at 50 °C for 2 h. The charcoal was filtered and solvents removed to give a colourless oil. Residual impurities and hydrobromic acid were removed by reverse-phase chromatography eluting with H_2O. Product fractions were evaporated to give **28** as a white solid (137 mg, 91%). This was characterised as the free acid, but resolution of the infra-red spectra was markedly improved by first converting a sample into the tris-ammonium salt. ν_{max}(neat)/cm^{-1} 2500–3500 (NH_3), 2100–2300, 1800–2050 (br, P–OH), 1703 (P–OH), 1485, 1457 (PhP), 1186 (α-P=O), 1124 and 1085 (β-P=O); δ_H(300 MHz, D_2O) 7.60–7.68 (2H, m, 2 × ArH), 7.70–7.77 (1H, m, ArH), 7.88–7.98 (2H, m, 2 × ArH); δ_C(100 MHz, D_2O) signals too broad to resolve; δ_P(162 MHz, D_2O) 4.46 (br, P_α), 21.57 (dt, *J* 76.6, 50.0, P_β); δ_F(376 MHz, D_2O) 41.34 (dd, *J* 85.2, 78.6) [Found: (FAB) M^+, 272.98911. $C_7H_8F_2O_5P_2$ requires 272.98933]; *m*/*z* 273 (46%, M^+), 77 (46, $C_6H_5^+$), 51 (24, CF_2H^+).

General procedure for the preparation of the tributylammonium salts

All the tributylammonium salts of the pyrophosphate analogues were prepared as follows: To a solution of **29**[36] (1.32 mmol) in 50% aqueous ethanol (2 cm^3) was added tributylamine (1.98 mmol). This was stirred at room temperature for 30 min before removing the solvents to give the tributylammonium salt **32** as a viscous brown oil. This was stored as a solution in DMF (0.6 M). Before being used in the subsequent phosphorylation reaction, solvents were removed from an appropriately sized aliquot and residual water was removed *in vacuo* as an azeotropic mixture (3 × 1 cm^3 of pyridine followed by 1 cm^3 of benzene).

Recombinant retroviral vector and virus production

The recombinant retrovirus encoding β-galactosidase (pSG1) used for measuring antiviral activity has been described previously.[27] The pSG1 vector was constructed by introducing a full size 3.4 kilobase fragment containing the *E. coli lac*Z gene from pWB310 plasmid[37] to the cloning site of pPS-3-neo eukaryotic vector derived from the Mu-MLV.[38] The structure of the resulting vector pSG1 is as follows:



The vector containing *cis*-acting regulatory elements of Mu-MLV genome [long terminal repeats (LTR) and ψ-region] which are necessary for viral transcription, packaging and integration into the host genome. The *lac*Z gene is transcribed from 5′-LTR. The transmitting G418 resistance transposon 5 neomycin phosphotransferase (*neo*) gene is located downstream from the *lac*Z gene and transcribed from the internal simian virus 40 (SV40) early promoter. The β-lactamase (Amp^r) gene and the origin of replication are derived from the bacterial pSP64/65 plasmid.[38] The vector is replication defective and allows the analysis of a single cycle of infection, since infectious progeny is not produced and subsequent cycles of viral replication do not occur. The pSG1 construct was transferred into the PA317 amphotropic packaging cell line by the calcium–phosphate-DNA coprecipitation.[39] After 48 h the culture medium was harvested and used to infect the ψ2 ecotropic packaging cell line in the presence of polybrene (8 μg ml^{-1}).[40] Individual ψ2 clones expressing the *lac*Z gene were isolated after the selection in the G418 (0.5 mg ml^{-1}) medium for 20 days. Titres of *lac*Z transducing retroviruses were determined by infecting Rat1 fibroblasts with a series of dilutions of the culture medium from each clone. After 48 h cells were incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal; Sigma) at 37 °C overnight to reveal β-galactosidase activity[41] and blue cell colonies were counted. When assayed on Rat1 cells, the filtered supernatant from ψ-PA-pSG1 clone contained approximately 10^4 β-galactosidase focus-forming units (ffu) per cm^3. With murine Balb/3T3 fibroblasts used as targets for the infection, the apparent titre of pSG1 virus was about ten-fold higher than that with Rat1 cells. The virus was used immediately or stored at −70 °C.

Evaluation of activity

For measuring antiviral activity, Rat1 cells were seeded in 2 cm wells of 24-well tissue culture plates in 1 cm^3 of Dubelco modified essential medium (ICN Biomedicals. Inc.) with 10% foetal bovine serum (ICN Biomedicals. Inc.), 2 mM glutamine and penicillin–streptomycin (Pen/STrep; ICN Biomedicals. Inc.). After 2 days the medium was replaced with 1 cm^3 of 1 : 10 fresh viral supernatant containing Polybrene (8 μg ml^{-1}), and the compounds under study were added (as a series of ten-fold dilutions in the medium) at the time of infection. After incubating for 24 h (37 °C), the old media was exchanged with 1 cm^3 of a fresh one (without test compounds), and after a day of additional incubation the cells were fixed and stained with Xgal. The blue cell colonies expressing β-galactosidase were counted. The results were expressed as the percentage of β-gal-positive cells in the presence of inhibitor compared to the proportion of β-gal-positive cells in the control. The results were averaged from data taken from 3 parallels in 2 independent experiments.

The estimation of the dephosphorylation rate

The assay mixture, containing 2.5 μl of 10 mM solution of the compound under study and 47.5 μl of 100% foetal blood serum, was incubated at 37 °C for 2.5, 5, 10, 20, 30, 60 min, 2, 3, 4, 5, 8, 12 h, 2, 4 and 7 days. The sampled aliquots (9 μl) were stirred with an aqueous suspension of phosphocellulose (2 mg per 50 μl) and then kept for 2 h at 20 °C. The samples were then centrifuged for 6 min (12000 rpm) and the supernatants were analysed by HPLC on a Silasorb C18 column (4 × 250 mm, 10 μ), eluting with a linear gradient of methanol (0–7.5%) in aqueous potassium phosphate buffer (0.01 M, pH 6.5) over 25 min at a flow rate of 0.7 ml min^{-1}. The extent of hydrolysis was assessed by measuring the accumulation of the phosphonate **11** or **12**. The experiments on inhibition of the phosphate hydrolysis of **7** by triphosphonates **2** and **3** were carried out, in a similar manner, using either 2.5 μl of **7** (8 mM, final concentration 0.33 mM), 5 μl of **2** or **3** (20 mM, final concentration 1.65 mM) and 52.5 μl of human blood serum or 2.0 μl of **7** (8 mM, final concentration 0.32 mM), 4 μl of **2** or **3** (40 mM, final concentration 3.2 mM) and 44 μl of human blood serum. Control assays were performed for **7** at a concentration of 2 mM.

Primer extension assays

For the template-dependent DNA polymerases, the assay mixture (volume 6 μl) contained [5′-^{32}P]-labelled-template-primer (0.01 μM), the compounds under study or dNTPs, the enzyme (2 activity units of RTs or 1 unit of DNA polymerases α and β) and the corresponding buffer. For TDT, the assay mixture (volume 5 μl) contained the primer (0.1 μM), the compound under study, 2 units of the enzyme, 100 mM sodium cacodylate (pH 7.2), 10 mM $MgCl_2$, 1 mM $CoCl_2$ and 1 mM dithiothreitol. The reaction was carried out for 30 min at 37 °C and terminated by adding deionised formamide (3 μl containing 0.5 mM EDTA), 2% bromophenol blue and xylene cyanol. The reaction products were separated by electrophoresis in 20% polyacrylamide gel, and the gels obtained were radioautographed.

Kinetic measurements

These were performed within the linear region of the product formation, as previously described.[26] The reaction time was 2 min.

Cytotoxic assay

To assess cytotoxicity of the compounds under study, a lactate dehydrogenase (LDH)-release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega, USA) was utilised. Rat1 cells were incubated for 24 h with the compounds under study and the colourimetric LDH-release assay was performed according to the instructions of the manufacturer. Cells incubated in the absence of the NTP analogues were used as a control. Cytotoxic effects were not observed with any of the test compounds at concentrations up to 1 mM (data not shown).

Template–primer complexes used in the chain elongation experiments:

Complex 1:

dGGGTCAGTGCTGCAACATTTTGCTGCCGGTCAC
GGTTC . . . .
[5′-^{32}P] dCCCAGTCACGACGT ⟶

Complex 2:

dCTGCAACATTTTGCTGCCGGTCACGGTTCGAAC
CCGA . . . .
[5′-^{32}P] dGACGTTGTAAAACG ⟶

Complex 3:

dCATTTTGCTGCCGGTCACGGTTCGAACCCGACG
TCCA . . . .
[5′-^{32}P] dGTAAAACGACGGCCAGT ⟶

Complex 4:

rUACGCUGAGGACGUAAUCCUUCGUCGGGUC
AU . . . .
[5′-^{32}P] dATGCGACTCCTGC ⟶

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References

1 H. Zhang, G. Dornadula and R. Pomerantz, *J. Virol.*, 1996, **70**, 2809.

2 A. A. Arzumanov, D. G. Semizarov, L. S. Victorova, N. B. Dyatkina and A. A. Krayevsky, *J. Biol. Chem.*, 1996, **271**, 24389.
3 L. S. Victorova, N. B. Dyatkina, D. J. Mozzherin, A. M. Atrazhev, A. A. Krayevsky and M. K. Kukhanova, *Nucleic Acids Res.*, 1992, **20**, 783.
4 N. B. Dyatkina, A. A. Arzumanov, L. S. Victorova, M. K. Kukhanova and A. A. Krayevsky, *Nucleosides Nucleotides*, 1995, **14**, 91.
5 V. Merlo, S. M. Roberts, R. Storer and R. C. Bethell, *J. Chem. Soc.*, *Perkin Trans. 1*, 1994, 1477.
6 D. Coe, S. M. Roberts and R. Storer, *J. Chem Soc.*, *Perkin Trans. 1*, 1992, 2695.
7 J. Balzarini, P. Herdewijn, R. Pauwels, S. Broder and E. De Clercq, *Biochem. Pharmacol.*, 1988, **37**, 2395.
8 T. J. Kilesso, N. B. Tarussova, E. D. Atrazheva, M. K. Kukhanova, S. V. Shulenin, A. F. Bobkov, M. M. Garayev, G. A. Galegov and A. A. Krayevsky, *Bioorg. Khim.*, 1990, **16**, 531.
9 T. A. Rozovskaya, A. V. Tischenko, N. B. Tarussova, M. K. Kukhanova, A. A. Krayevsky and R. Sh. Beabealashvilli, *Mol. Biol. Russian*, 1993, **27**, 1051.
10 L. Arabshahi, N. N. Khan, M. Butler, T. Noothy, N. C. Brown and G. E. Wright, *Biochemistry*, 1990, **29**, 6820.
11 B. I. Martynov, E. A. Shirokova, M. V. Jasko, L. S. Victorova and A. A. Krayevsky, *FEBS Lett.*, 1997, **410**, 423.
12 E. A. Shirokova and N. B. Dyatkina, *Collect. Czech. Chem. Commun.*, 1996, **61**, S158.
13 N. Dyatkina, E. Shirokova, F. Theil, S. M. Roberts and A. Krayevsky, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 2639.
14 N. Dyatkina, F. Theil and M. von Janta Lipinski, *Tetrahedron*, 1995, **51**, 761.
15 C. J. Hamilton, S. M. Roberts and A. Shipitsin, *Chem. Commun.*, 1998, 1087.
16 D. Burton and R. Flynn, *J. Fluorine Chem.*, 1980, **15**, 263.
17 The high electrophilic reactivity of the acylation products of **21**, leading to the rapid formation of secondary products, has previously been reported; G. M. Blackburn, D. Brown, S. Martin and M. Parrat, *J. Chem. Soc.*, *Perkin Trans. 1*, 1987, 181.
18 F. Westheimer, *Acc. Chem. Res.* 1968, **1**, 70.
19 (*a*) E. Lightcap and P. Frey, *J. Am. Chem. Soc.*, 1992, **114**, 9750; (*b*) J. Friedman, S. Freeman and J. Knowles, *J. Am. Chem. Soc.*, 1988, **110**, 1268.
20 (*a*) P. Cullis, R. Misra and D. Wilkins, *J. Chem. Soc.*, *Chem. Commun.*, 1987, 1594; (*b*) P. Cullis and R. Misra, *J. Am. Chem. Soc.*, 1991, **113**, 9679; (*c*) S. Hamett and G. Lowe, *J. Chem. Soc.*, *Chem. Commun.*, 1987, 1416.
21 (*a*) M. Harger, *J. Chem. Soc.*, *Perkin Trans. 2*, 1991, 1057; (*b*) A. Gerrard and N. Hamer, *J. Chem. Soc.* (*B*), 1968, 539; (*c*) S. Freeman and M. Harger, *J. Chem. Soc.*, *Perkin Trans. 2*, 1988, 82; (*d*) S. Freeman and M. Harger, *J. Chem. Soc.*, *Perkin Trans. 1*, 1988, 2737.
22 M. Harger and B. Hurman, *J. Chem. Soc.*, *Chem. Commun.*, 1995, 1701.
23 The synthesis of **18** using this procedure has previously been noted in the following paper: M. Obayashi, E. Ito, K. Matsui and K. Kondo, *Tetrahedron Lett.*, 1982, **23**, 2323. Full experimental details are reported herein as the experimental procedure was not described in the original paper and to our knowledge has not been published since. Characterisation data are available for **18** from reports of alternative preparations.[16,35,41]
24 V. Wittmann and C. H. Wong, *J. Org. Chem.*, 1997, **62**, 2144.
25 C. J. Hamilton, PhD Thesis, University of Exeter, 1998.
26 D. G. Semizarov, L. S. Victorova, N. B. Dyatkina, M. von Janta Lipinski and A. Krayevsky, *FEBS Lett.*, 1994, **354**, 187.
27 A. Y. Shevelev, O. A. Deyanova, L. E. Goryunova, A. Y. Zhukova and R. Sh. Beabealashivilli, *Mol. Biol. Russian*, 1995, **29**, 658.
28 P. Labataille, P. Pelicano, J.-P. Imbach and G. Gosselin, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 2315.
29 G. R. J. Thatcher and A. S. Campbell, *J. Org. Chem.*, 1993, **58**, 2272.
30 A. A. Krayevsky, unpublished work.
31 L. A. Alexandrova, A. J. Scoblov, M. V. Jasko, L. S. Victorova and A. A. Krayevsky, *Nucleic Acids Res.*, 1998, **26**, 778.
32 D. Y. Mozzherin, A. M. Atrazhev and M. K. Kukhanova, *Mol. Biol. Russian*, 1992, **26**, 999.
33 T. A. Kolocheva and G. A. Nevinsky, *Mol. Biol. Russian*, 1993, **27**, 1368.
34 A. V. Krayev, *Mol. Biol. Russian*, 1988, **22**, 1164.
35 J. R. Sampson and O. Uhlenbeck, *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 1033.
36 C. McKenna and P. Shen, *J. Org. Chem.*, 1981, **46**, 4573.
37 N. Lehming, J. Sartorius, M. Niemoller, G. Genenger, B. von Wilken-Bergmann and B. Muller Hill, *EMBO J.*, 1987, **6**, 3145.
38 V. S. Prasolov and P. M. Chumakov, *Mol. Biol. Russian*, 1988, **22**, 1371.
39 A. D. Miller and C. Buttimore, *Mol. Cell Biol.*, 1986, **6**, 2895.
40 R. Mann, R. C. Milligan and D. Baltimore, *Cell*, 1983, **33**, 153.
41 J. R. Sanes, J. L. R. Rubenstein and J. F. Nicolas, *EMBO J.*, 1986, **5**, 3133.

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