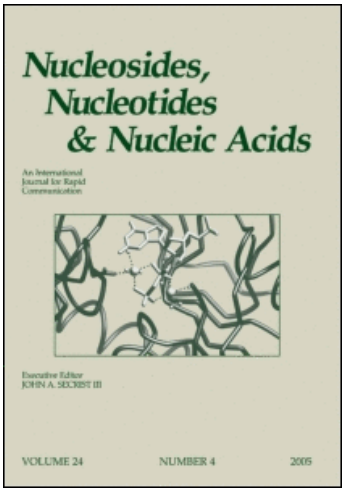


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthesis, Bioactivation and Anti-HIV Activity of 4-Acyloxybenzyl *bis*(Nucleosid-5'-yl) Phosphates

Anne Routledge[ab]; Ian Walker[a]; Sally Freeman[ac]; Alan Hay[d]; Naheed Mahmood[e]
[a] Department of Pharmaceutical and Biological Sciences, Aston University, Birmingham, U.K. [b] Department of Chemistry, University of Cambridge, Cambridge, U.K. [c] Department of Pharmacy, University of Manchester, Manchester, U.K. [d] National Institute for Medical Research, London, U.K. [e] MRC Collaborative Centre, London, U.K.

To cite this Article Routledge, Anne , Walker, Ian , Freeman, Sally , Hay, Alan and Mahmood, Naheed(1995) 'Synthesis, Bioactivation and Anti-HIV Activity of 4-Acyloxybenzyl *bis*(Nucleosid-5'-yl) Phosphates', Nucleosides, Nucleotides and Nucleic Acids, 14: 7, 1545 — 1558
To link to this Article: DOI: 10.1080/15257779508009491
URL: http://dx.doi.org/10.1080/15257779508009491

NUCLEOSIDES & NUCLEOTIDES, 14(7), 1545-1558 (1995)

SYNTHESIS, BIOACTIVATION AND ANTI-HIV ACTIVITY OF 4-ACYLOXYBENZYL *BIS*(NUCLEOSID-5'-YL) PHOSPHATES

Anne Routledge[a]*%, Ian Walker[a]+, Sally Freeman[a]$, Alan Hay[b] and Naheed Mahmood[c]

[a]Department of Pharmaceutical and Biological Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, U.K.
[b] National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, U.K.
[c] MRC Collaborative Centre, 1-3 Burtonhole Lane, Mill Hill, London, NW7 1AD, U.K.

Abstract: 4-Acyloxybenzyl *bis*(nucleosid-5'-yl) phosphates **7a-c** and **9a-c** were prepared as potential prodrugs of the anti-HIV nucleosides 3'-azido-3'-deoxythymidine (AZT) and 2',3'-dideoxyinosine (ddI) or their 5'-monophosphates.

The anti-HIV activities of these triesters were determined in two T-cell lines. In a C8166 cell line they displayed activities comparable to and in some cases superior to AZT, but they also exhibited an increase in cytotoxicity. In a thymidine kinase deficient JM T-cell line the activity was reduced but was still superior to AZT. In the presence of porcine liver carboxyesterase (PLCE), triester **7b** biodegrades to the diester **10** which, with phosphodiesterase, gives initially AZT monophosphate **3** and AZT.

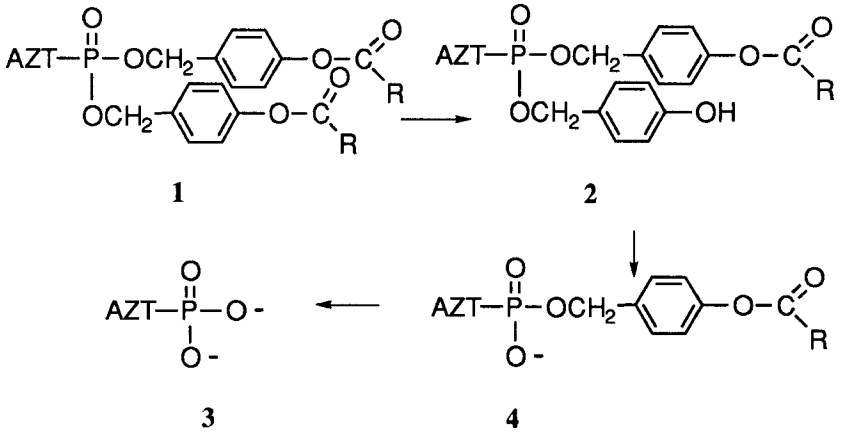
INTRODUCTION

The nucleoside analogue 3'-azido-3'-deoxythymidine (AZT) is, after *in vivo* phosphorylation, a potent inhibitor of reverse transcriptase in the human immunodeficiency virus (HIV)[1]. AZT has been licensed for the treatment of acquired immunodeficiency syndrome (AIDS) since 1987[2]. However, as a chemotherapeutic agent AZT has limitations in its use: it is rapidly eliminated from the body as 3'-azido-3'-deoxy-5'-glucuronylthymidine[3], drug resistant viruses appear after prolonged use[4] and it displays particularly toxic side-effects such as bone marrow suppression[5]. This

% Current address: Department of Chemistry, University of Cambridge, Lensfield Rd, Cambridge, CB2 1EW, U.K.
+ Deceased
$ Current address: Department of Pharmacy, University of Manchester, Manchester, M13 9PL, U.K.

encouraged the investigation of other nucleoside analogues such as 2',3'-dideoxyinosine (ddI)[6], 2',3'-dideoxycytidine (ddC)[6] and 2',3'-didehydro-3'-deoxythymidine (d4T)[7] as alternative drugs for the treatment of AIDS. In most cases to exert their biological activity, nucleoside therapeutics are converted by host cell kinases into triphosphate metabolites[8] which can act as terminators of the growing viral DNA chain or as inhibitors of viral polymerases[3,9]. The polar nature of nucleotides results in poor penetration across cell membranes[10], therefore they seldom achieve their therapeutic potential. The use of the 5'-monophosphate of the nucleoside could avoid the problem of glucuronidation[3] and eliminate the need for initial phosphorylation. However, the dianionic nature at physiological pH is a limiting factor in cell membrane penetration. Our group[11] and others[12-16] have attempted to circumvent this delivery problem by preparing lipophilic phosphate triester prodrugs that are designed to release the nucleotide monophosphate in a controlled manner. We have recently described the synthesis, enzymatic bioactivation and antiviral activity of the *bis*(4-acyloxybenzyl) esters of AZT monophosphate **1**[11]. In the presence of porcine liver carboxyesterase, triester **1**, R= CH_3 underwent initial hydrolysis at the 4-acyloxybenzyl ester function to give the 4-hydroxybenzyl derivative **2**, R= CH_3. It then further degraded by a cascade mechanism to the AZT monophosphate **3** *via* the diester **4**, R= CH_3 as shown in **SCHEME 1.**
Both the triesters **1** and diesters **4** displayed anti-HIV activity comparable to that of AZT but in some cases they showed greatly increased cytotoxicity. Other recently reported phosphate protecting groups requiring bio-activation include DTE [*S*-(2-hydroxyethylsulphidyl)-2-thioethyl][17], SATE (*S*-acetyl-2-thioethyl)[17,18] and POM (pivaloyloxymethyl)[19-21].

Combination therapy, the co-administration of two or more anti-HIV drugs is being evaluated in the treatment of AIDS. This includes combinations of drugs to treat opportunistic infections and HIV. One approach to combination therapy involves two nucleosides linked *via* a 5',5'-phosphate bridge[22-24]. As an extension to our previous study we have synthesised both symmetric **7a-c** and asymmetric 4-acyloxybenzyl *bis*(nucleosid-5'-yl) phosphates **9a-c** using the nucleosides AZT and ddI. These prodrugs may display superior anti-HIV activity and lower cytotoxicity compared to the *bis*(4-acyloxybenzyl) esters of AZT monophosphate **1** for the following three reasons. Firstly, the nucleotide dimer itself could display enhanced antiviral properties. Secondly, upon hydrolysis only one potentially toxic benzyl carbonium ion is released. Thirdly, after delivery through cellular membranes, enzymatic bioactivation could liberate either a nucleotide monophosphate and a nucleoside, or two nucleosides inside the cell.



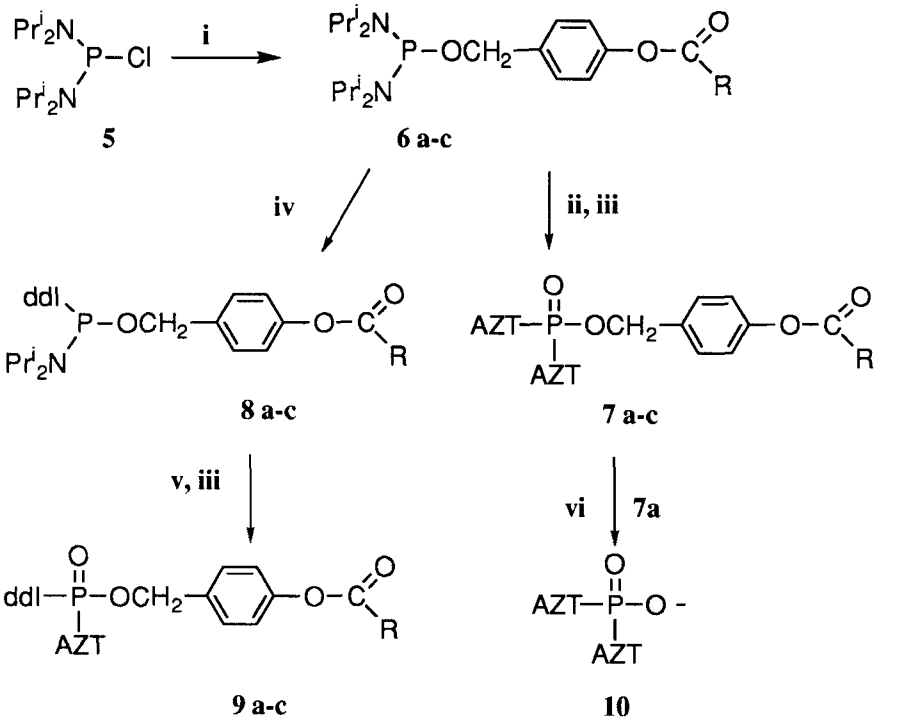
SCHEME 1

RESULTS AND DISCUSSION

Chemical Synthesis

The 4-acyloxybenzyl *bis*(nucleosid-5'-yl) phosphates **7a-c** and **9a-c** were synthesised from *bis*(diisopropylamino) phosphochloridite **5** as shown in **SCHEME 2**. The required 4-acyloxybenzyl *bis*(diisopropylamino)phosphoramidites **6a-c** were synthesised by reaction of **5** with the appropriate 4-acyloxybenzyl alcohol[25] in good yield. Reaction of **6a-c** with two equivalents of AZT in the presence of 1*H*-tetrazole, followed by *in situ* oxidation of the P^{III} compound with *m*CPBA[26] resulted in the 4-acyloxybenzyl *bis*(3'-azido-3'-deoxythymidin-5'-yl) phosphates **7a-c** in yields of 19-31%.

Alkyl *bis*(dialkylamino)phosphoramidites show strong p_π–d_π interactions[27] that make them susceptible to protonation with weak acids. This enables them to react with alcohols to form dialkyl (dialkylamino)phosphoramidites. The newly introduced alcohol function has the effect of reducing the p_π-d_π interaction and hence slightly more acidic conditions are required before further reaction with an alcohol can occur[28]. This phenomenon was utilised in the synthesis of the asymmetric 4-acyloxybenzyl *bis*(nucleosid-5'-yl) phosphates **9a-c**. Treatment of **6a-c** with ddI in the presence of catalytic diisopropylammonium tetrazolide gave the intermediate phosphoramidites **8a-c**. All attempts to isolate these compounds resulted in degradation so they were further reacted without isolation. Treatment of **8a-c** with AZT in the presence of 1-*H* tetrazole followed by *in situ* oxidation of the P^{III} phosphite gave the 4-acyloxybenzyl (3'-azido-3'-



a. R = CH_3, **b.** R = $CH_2CH_2CH_3$, **c.** R = $C(CH_3)_3$

Reagents: **i)** 4-acyloxybenzyl alcohol, diisopropylethylamine, **ii)** 2eq. AZT, 3eq. tetrazole. **iii)** *m*CPBA, **iv)** 1eq. ddI, 0.5eq. diisopropylammonium tetrazolide, **v)** 1eq. AZT, 3eq. 1*H*-tetrazole, **vi)** 1eq. LiI.

SCHEME 2

deoxythymidin-5'-yl) (2',3'-dideoxyinosin-5'-yl) phosphates **9a-c** as 1:1 mixtures of (*Rp*)- and (*Sp*)-diastereoisomers.

Bis(3'-azido-3'-deoxythymidin-5'-yl) phosphate **10**[29] was prepared in good yield by treatment of triester **7a** with lithium iodide[30]. Reaction of triester **9a** with lithium iodide resulted in an unstable compound which could not be characterised. All new compounds were fully characterised by ^1H, ^{13}C, ^{31}P n.m.r. and i.r. spectroscopy, together with high resolution mass spectrometry and / or elemental analysis.

Bioactivation of Triester 7b

For both chemical and enzymatic hydrolyses, the reactions were monitored over time by ^{31}P n.m.r. spectroscopy. The triester **7b** (δP −0.18), diester **10** (δP 0.5) and AZT

monophosphate **3** (δP 4.1) showed no evidence of hydrolysis when incubated in methylphosphonate buffer in D_2O [pD(31) 8.0, 0.1 mol dm^{-3}] - acetonitrile mixture (9:1 v/v) at 37°C over a 24 h period.

In cell culture medium (RPMI 1640 + 10% foetal calf serum) - acetonitrile mixture (9:1), hydrolysis of the triester **7b** to the diester **10** was observed at 37°C over 48 h. Further degradation of **10** could not be detected by ^{31}P n.m.r. spectroscopy because of the large peak for inorganic phosphate present in the culture medium. After 5 days, t.l.c. analysis of the reaction mixture indicated the presence of AZT, which suggested partial degradation of the diester **10** *via* the monophosphate **3** catalysed by serum phosphodiesterases and phosphatases.

A solution of the triester **7b** in methylphosphonate buffer in D_2O [pD 8.0, 0.1 mol dm^{-3}] - acetonitrile mixture (9:1 v/v), was treated with 5U of porcine liver carboxyesterase (PLCE) which resulted in rapid decomposition to the diester **10**. The reaction mixture was then treated with 0.2U of snake venom phosphodiesterase which gave the immediate formation of AZT monophosphate **3**. On incubation overnight, **3** had decomposed to inorganic phosphate (δP 2.6) and AZT, presumably by contaminant phosphatase present in the phosphodiesterase preparation(32,33).

Anti-HIV Activity

The triesters **7a-c** and **9a-c**, diester **10**, AZT, ddI and an equimolar mixture of AZT and ddI were evaluated for anti-HIV activity in two different cell lines. In cell line C8166, the effects of the compounds on HIV-1 IIIB were assessed over 4-6 days by the inhibition of syncytia formation, the production of gp120 and the protection of cell viability. Toxicity to uninfected cells was assessed in parallel in the latter MTT assay. All the compounds exhibited antiviral activities, in terms of EC_{50}, comparable or superior to that of AZT, however, the corresponding increase in toxicity reduced the selectivity indices. In the second assay, the effects of the compounds on HIV-1 U455 in the thymidine kinase deficient JM T-cell line were measured over 5 days. All the substrates tested showed a substantial decrease in activity compared to the results with the C8166 cells (**TABLE 1**).

Although the bioactivation studies with commercial enzyme preparations support the formation of nucleoside monophosphate **3** *via* diester **10**, work done on the stability of 5',5'-dinucleoside phosphodiesters in cell extract(34,35) suggested a lack of phosphodiesterase activity towards the unnatural *bis*(nucleotid-5'-yl) phosphates. In our hands, the published procedure(35) gave cell extract without phosphodiesterase activity as determined by an absence of cleavage of dinucleotidyl-5',3'-phosphodiester linkages in an oligonucleotide, therefore the decomposition of triester **7b** in cell extract could not be determined.

TABLE 1 Anti-HIV-1 activities (EC_{50} μmol dm^{-3}) and toxicities (TC_{50} μmol dm^{-3}) of 4-acyloxybenzyl *bis*(nucleosid-5'-yl) phosphates **7a-c** and **9a-c** and *bis*(3'-azido-3'-deoxythymidin-5'-yl) phosphate **10** with **A** the IIIB strain of HIV-1 in C8166 cells, and **B** the U445 strain of HIV-1 in thymidine kinase deficient JM T-cells.

	A	A	B	B
Compound	**EC_{50}**	**TC_{50}**	**EC_{50}**	**TC_{50}**
7a	0.003	100-150	4	50
7b	0.003	50-100	4	50
7c	0.003	20	2	40
9a	0.032	400	4	400
9b	0.032	80-100	1.5	100
9c	0.020	50-60	1	100
10[29]	0.016	1000	20	400
AZT	0.016	>1000	>100	>1000
ddI	0.8	>100	0.8	>100
AZT+ddI	0.016	>10	0.4	>10

From the results in **Table 1**, no increase in anti-HIV activity was achieved by combining AZT and ddI in the same triester, **9a-c**. The activity measured in thymidine kinase deficient JM T-cells was similar to that displayed by ddI or an equimolar mixture of AZT and ddI. The triesters **7a-c** were significantly more active than AZT in thymidine kinase deficient JM T-cells, although this activity was somewhat less than that found in C8166 cells. This suggested three possible decomposition routes for the triester. Firstly, some of the triester could undergo metabolism prior to transport through the cell membrane. Secondly, after transport and carboxyesterase activation the diester **10** is stable and only a small percentage degrades to AZT monophosphate **3**. Thirdly, AZT monophosphate **3** is degrading to AZT and inorganic phosphate rather than being phosphorylated to the triphosphate inside the cell.

EXPERIMENTAL

General: N.m.r. spectra were recorded on a Bruker AC-250 spectrometer operating at 250.1 MHz for ^{1}H, 62.9 MHz for ^{13}C and 101.3 MHz for ^{31}P. Spectra were recorded as dilute $CDCl_3$, D_2O or CD_3OD solutions. ^{1}H and ^{13}C spectra were referenced to tetramethylsilane and ^{31}P spectra were referenced to 85% phosphoric acid. Positive chemical shifts are down field from the reference. Mass spectra were recorded on either a

VG Micromass 12 instrument at 70 eV with a source temperature of 300°C and ammonia as a carrier gas for CI, or a VG 7070E instrument using positive ion FAB with a nitrobenzyl alcohol matrix. I.r. spectra were recorded on a Mattson 3000 FTIR or a Nicolet 205 FTIR spectrometer as KBr discs, Nujol mulls or thin films. Frequencies ($\upsilon_{max.}$) are recorded as cm^{-1}. Flash chromatography[36] was performed using Sorbsil C60 silica gel. Thin layer chromatography (t.l.c.) was performed on Kieselgel 60 silica gel plates containing a fluorescent indicator. Compounds were detected by either ultra-violet light (254 nm), iodine vapour or by spraying with a 10% ethanolic solution of phosphomolybdic acid and heating. Elemental analyses were performed by Butterworths Laboratories, Middlesex, U.K.

Reagents: All reagents were from commercial sources unless stated otherwise. 4-Acyloxybenzyl alcohols were synthesised by a literature method[25]. AZT was a gift from Wellcome Ltd. and ddI a gift from Bristol Meyers Squibb. The units of enzyme added to the hydrolyses were based on an assay defined by Sigma Chemical Company. For porcine liver carboxyesterase, one unit hydrolyses 1μmol of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 and 25°C. For snake venom phosphodiesterase, one unit hydrolyses 1μmol of *bis*(p-nitrophenyl) phosphate per min at pH 8.8 and 37°C.

4-Acetyloxybenzyl *bis*(N,N-diisopropyl)phosphoramidite 6a

Under an argon atmosphere at -78°C, a solution of diisopropylethylamine (0.71 g, 5.50 mmol) and 4-acetyloxybenzyl alcohol (0.83 g, 5.00 mmol) in anhydrous dichloromethane (10 ml) was added to a stirred solution of *bis*(N,N-diisopropylamino) phosphochloridite (1.33 g, 5.00 mmol). The mixture was allowed to warm to room temperature and stirring was continued for 1 h. The solvent was removed *in vacuo* to give a yellow oil. Flash chromatography (hexane-ethyl acetate-triethylamine, 8:2:1) gave **6a** as a white semi-solid (1.20 g, 61%). I.r.(film) $\upsilon_{max.}$ 1762(C=O ester); ^1H n.m.r.($CDCl_3$) 1.21(d, J_{HH} 6.8 Hz, 12H), 1.22(d, J_{HH} 6.8 Hz, 12H), 2.32(s, 3H), 3.61(d sept, J_{PH} 10.8, J_{HH} 6.8 Hz, 4H), 4.66(d, J_{PH} 7.3 Hz, 2H), 7.07(d, J_{HH} 8.6 Hz, 2H), 7.41(d, J_{HH} 8.6 Hz, 2H); ^{13}C n.m.r.($CDCl_3$) 21.2, 23.9(d, J_{PC} 5.8 Hz), 24.7(d, J_{PC} 8.0 Hz), 44.5(d, J_{PC} 12.4 Hz), 65.6(d, J_{PC} 23.8 Hz), 121.2, 127.8, 138.2(d, J_{PC} 10.5 Hz), 149.9, 169.6; ^{31}P n.m.r. ($CDCl_3$) 123.7(s); M.s.: *m/z* (CI) found 397.2620 ($M+H^+$) required for $C_{21}H_{38}N_2O_3P$ 397.2621; Anal. found C, 63.06; H, 8.94; N, 6.64%, expected for $C_{21}H_{37}N_2O_3P$ C, 63.61; H, 9.41; N, 7.06%.

The following compounds were prepared from the appropriate 4-acyloxybenzyl alcohol[25] using a method analogous to that described previously:

4-Butanoyloxybenzyl *bis*(N,N-diisopropyl)phosphoramidite 6b

4-Butanoyloxybenzyl alcohol (0.97 g, 5.00 mmol) gave **6b** (1.25 g, 56 %) as a pale yellow oil. I.r.(film) $\upsilon_{max.}$ 1763 (C=O ester); ^{1}H n.m.r.($CDCl_3$) 0.97(t, J_{HH} 7.4 Hz, 3H), 1.10 (d, J_{HH} 6.8 Hz, 12H), 1.11(d, J_{HH} 6.8 Hz, 12H), 1.71(sext, J_{HH} 7.4 Hz, 2H), 2.46(t, J_{HH} 7.4 Hz, 2H), 3.50(d sept, J_{PH} 10.8, J_{HH} 6.8 Hz, 4H), 4.56(d, J_{PH} 7.3 Hz, 2H), 6.96(d, J_{HH} 8.6 Hz, 2H), 7.30(d, J_{HH} 8.6 Hz, 2H); ^{13}C n.m.r.($CDCl_3$) 13.7, 18.5, 23.9(d, J_{PC} 5.8 Hz), 24.7(d, J_{PC} 8.0 Hz), 36.3, 44.5(d, J_{PC} 12.4 Hz), 65.6(d, J_{PC} 23.3 Hz), 121.2, 127.8, 138.0 (d, J_{PC} 10.9 Hz), 149.6, 172.0; ^{31}P n.m.r. ($CDCl_3$) 123.8(s); M.s.: *m/z* (CI) found 425.2933 ($M+H^+$) required for $C_{23}H_{42}N_2O_3P$ 425.2924; Anal. found C, 65.05; H, 9.74; N, 6.60%, expected for $C_{23}H_{41}N_2O_3P$ C, 65.34; H, 9.74; N, 7.01%.

4-Pivaloyloxybenzyl *bis*(N,N-diisopropyl)phosphoramidite 6c

4-Pivaloxybenzyl alcohol (0.84 g, 4.00 mmol) gave **6c** (1.20 g, 69 %) as a clear oil. I.r.(film) $\upsilon_{max.}$ 1766(C=O ester); ^{1}H n.m.r.($CDCl_3$) 1.18(d, J_{HH} 6.8 Hz, 12H), 1.19(d, J_{HH} 6.8 Hz, 12H), 1.35(s, 9H), 3.57(d sept, J_{PH} 10.5, J_{HH} 6.8 Hz, 4H), 4.63(d, J_{PC} 7.3 Hz, 2H), 7.01(d, J_{HH} 8.5 Hz, 2H), 7.37(d, J_{HH} 8.5 Hz, 2H); ^{13}C n.m.r.($CDCl_3$) 23.9(d, J_{PH} 5.7 Hz), 24.6(d, J_{PC} 8.0 Hz), 27.2, 39.1, 44.4, (d, J_{PC} 12.4 Hz), 65.6(d, J_{PC} 23.1 Hz), 121.1, 127.8, 137.9(d, J_{PC} 10.3 Hz), 149.9, 177.2; ^{31}P n.m.r. ($CDCl_3$) 123.8(s); M.s.: *m/z* (CI) found 439.3090 ($M+H^+$) required for $C_{24}H_{44}N_2O_3P$ 439.3090.

4-Acetyloxybenzyl *bis*(3'-azido-3'-deoxythymidin-5'-yl) phosphate 7a

1*H*-Tetrazole (0.29 g, 3.90 mmol) was added to a stirred solution of **6a** (0.49 g, 1.25 mmol) and AZT (0.70 g, 2.60 mmol) in anhydrous acetonitrile (3 ml) under argon. Stirring was continued for 30 min, after which time the mixture was cooled to -40°C and *m*CPBA (0.28 g) was added portionwise. The reaction mixture was allowed to warm to room temperature and stirring was continued for an additional 5 min. Dichloromethane (20 ml) was added and the solution washed with saturated $NaHCO_3$ (2x20 ml) and then water (20 ml). The organic phase was dried over $MgSO_4$ and the solvent removed *in vacuo* to give a yellow oil. Flash chromatography (ethyl acetate-methanol, 99:1) gave **7a** as a white foam (0.24 g, 26%). I.r.(Nujol) $\upsilon_{max.}$ 2108 (N_3), 1760 (C=O ester), 1694 (C=O thymine); ^{1}H n.m.r.($CDCl_3$) 1.88(s, 6H), 2.29(s, 3H), 2.3-2.4(m, 4H), 3.9-4.0(m, 2H), 4.1-4.2(m, 6H), 5.12(d, J_{PH} 10.5 Hz, 2H), 6.04(dd, J_{HH} 6.4, 6.4Hz, 1H), 6.06(dd, J_{HH} 6.4, 6.4 Hz, 1H), 7.09(d, J_{HH} 8.3 Hz, 2H), 7.20(s, 1H), 7.25(s, 1H), 7.42(d, J_{HH} 8.3 Hz, 2H), 9.91(br s, 2H); ^{13}C n.m.r.($CDCl_3$) 12.5, 21.1, 37.6, 60.2, 66.7(d, J_{PC} 5.7 Hz), 66.8(d, J_{PC} 5.7 Hz), 69.7(d, J_{PC} 5.2 Hz), 82.1(d, J_{PC} 5.7 Hz), 85.9, 111.3,

111.4, 122.2, 129.8, 132.8(d, J_{PC} 4.8 Hz), 136.0, 136.1, 150.4, 151.2, 164.1, 164.2, 169.5; ^{31}P n.m.r.($CDCl_3$) -0.4(s); M.s.: *m/z* (FAB) found 745($M+H^+$), 767 ($M+Na^+$); Anal. found C, 46.43; H, 4.41; N, 18.40%, expected for $C_{29}H_{33}N_{10}O_{12}P$ C, 46.78; H, 4.47; N, 18.80%.

The following compounds were prepared from the appropriate phosphoramidite using a method analogous to that described previously:

4-Butanoyloxybenzyl *bis*(3'-azido-3'-deoxythymidin-5'-yl) phosphate 7b
AZT (0.71 g, 2.60 mmol) gave **7b** as a white foam (0.18 g, 19%). I.r.(Nujol) $\upsilon_{max.}$ 2107(N_3), 1750(C=O ester), 1649(C=O thymine); ^1H n.m.r.($CDCl_3$) 1.02(t, J_{HH} 7.4 Hz, 3H), 1.76(sext, J_{HH} 7.4 Hz, 2H), 1.88(s, 6H), 2.3-2.4(m, 4H), 2.52(t, J_{HH} 7.4 Hz, 2H), 3.9-4.0(m, 2H), 4.1-4.2(m, 6H), 5.11(d, J_{PH} 10.8 Hz, 2H), 6.04(dd, J_{HH} 6.5, 6.5 Hz, 1H), 6.07(dd, J_{HH} 6.5, 6.5 Hz, 1H), 7.08(d, J_{HH} 8.5 Hz, 2H), 7.18(s, 1H), 7.22(s, 1H), 7.41(d, J_{HH} 8.5 Hz, 2H), 9.37(br s, 1H), 9.40(br s, 1H); ^{13}C n.m.r.($CDCl_3$) 13.1, 14.2, 19.0, 36.7, 37.6, 60.8, 67.2(d, J_{PC} 5.2 Hz), 67.3(d, J_{PC} 6.7 Hz), 70.3(d, J_{PC} 4.0 Hz), 82.1(d, J_{PC} 7.6 Hz), 86.0, 86.1, 111.4, 111.5, 122.9, 130.4, 132.7(d, J_{PC} 4.8 Hz), 136.6, 136.7, 151.0, 152.2, 164.3, 172.8; ^{31}P n.m.r.($CDCl_3$) -0.4(s); M.s.: *m/z* (FAB) found 773($M+H^+$), 795 ($M+Na^+$); Anal. found C, 47.88; H, 4.96; N, 17.53%, expected for $C_{31}H_{37}N_{10}O_{12}P$ C, 48.19; H, 4.83; N, 18.12%.

4-Pivaloyloxybenzyl *bis*(3'-azido-3'-deoxythymidin-5'-yl) phosphate 7c
AZT (0.21 g, 0.80 mmol) gave **7c** (0.09 g, 31 %) as a white foam. I.r.(KBr) $\upsilon_{max.}$ 2107(N_3), 1758(C=O ester), 1691(C=O thymine); ^1H n.m.r.($CDCl_3$) 1.32(s, 9H), 1.86(s, 6H), 2.3-2.5(m, 4H), 3.8-4.0(m, 2H), 4.1-4.3(m, 6H), 5.11(d, J_{PH} 10.7 Hz, 2H), 6.02(dd, J_{HH} 7.3, 7.3 Hz, 1H), 6.05(dd, J_{HH} 7.3, 7.3 Hz, 1H), 7.04(d, J_{HH} 8.2 Hz, 2H), 7.18(s, 1H), 7.23(s, 1H), 7.41(d, J_{HH} 8.2 Hz, 2H), 9.87(br s, 1H), 9.92(br s, 1H); ^{13}C n.m.r.($CDCl_3$) 12.4, 27.0, 36.9, 39.0, 60.2, 66.7(d, J_{PC} 7.2 Hz), 66.8(d, J_{PC} 5.7 Hz), 69.7(d, J_{PC} 5.7 Hz), 82.0(d, J_{PC} 7.6 Hz), 85.8, 111.3, 111.4, 122.1, 129.7, 132.6(d, J_{PC} 4.8 Hz), 136.0, 136.1, 150.4, 151.7, 164.1, 177.1; ^{31}P n.m.r. -0.4(s); M.s.: *m/z* (FAB) found 787.2561($M+H^+$), 809.2364($M+Na^+$), required for $C_{32}H_{40}N_{10}O_{12}P$ 787.2565, $C_{32}H_{39}NaN_{10}O_{12}P$ 809.2344.

(*R*p)- and (*S*p)-4-Acetyloxybenzyl (3'-azido-3'-deoxythymidin-5'-yl) (2',3'-dideoxyinosin-5'-yl) phosphate 9a
Diisopropylammonium tetrazolide (0.04 g, 0.25 mmol) was added to a stirred solution of **6a** (0.20 g, 0.50 mmol) and ddI (0.06 g, 0.25 mmol) in anhydrous dichloromethane (5

ml) under argon. After 18 h, AZT (0.07 g, 0.25 mmol) and 1*H*-tetrazole (0.11 g, 1.50 mmol) were added and stirring was continued for a further 2 h. The mixture was cooled to -40°C and *m*CPBA (0.10 g) in anhydrous dichloromethane (3 ml) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirring was continued for 30 min. Dichloromethane (20 ml) was added and the solution washed with saturated $NaHCO_3$ (2x20 ml). The organic phase was dried over $MgSO_4$ and the solvent removed *in vacuo* to give the crude product. Flash chromatography (ethyl acetate-methanol-triethylamine, 15:4:1) gave **9a** as a white foam (0.07g, 39%). I.r.(KBr) $\upsilon_{max.}$ 2110(N_3), 1759(C=O ester); ^1H n.m.r.(CD_3OD) 1.76(s, 3H), 2.27(s, 3H), 2.0-2.6(m, 6H), 3.8-4.0(m, 1H), 4.1-4.4(m, 6H), 5.02(d, J_{PH} 9.7 Hz, 1H), 5.04(d, J_{PH} 9.7 Hz, 1H), 6.1-6.2(m, 1H), 6.2-6.3(m, 1H), 7.04(d, J_{HH} 8.5 Hz, 2H), 7.3-7.5(m, 3H), 8.0(s, 1H), 8.17(s, 0.5H), 8.21(s, 0.5H); ^{13}C n.m.r.(CD_3OD) 12.5, 20.9, 26.7, 26.8, 32.7, 37.5, 61.5, 68.1(d, J_{PC} 3.9 Hz), 68.2(d, J_{PC} 5.7 Hz), 70.2(d, J_{PC} 6.6 Hz), 70.5(d, J_{PC} 5.8 Hz), 70.6(d, J_{PC} 5.6 Hz), 81.0(d, J_{PC} 7.5 Hz), 83.3(d, J_{PC} 7.7 Hz), 86.5, 86.9, 111.9, 123.1, 125.8, 130.5, 130.6, 134.5(d, J_{PC} 5.7 Hz), 137.8, 140.2, 146.7, 149.6, 152.1, 152.5, 158.8, 166.6, 171.1; ^{31}P n.m.r. (CD_3OD) 1.1(s) and 1.2(s); M.s: *m/z* (FAB) found 714.2066 ($M+H^+$), expected for $C_{29}H_{33}N_9O_{11}P$ 714.2037; Anal. found C, 48.68; H, 4.68%, expected for $C_{29}H_{32}N_9O_{11}P$ C, 48.81; H, 4.52%.

The following compounds were prepared from the appropriate phosphoramidite using a method analogous to that described previously:

(*Rp*)- and (*Sp*)-4-Butanoyloxybenzyl (3'-azido-3'-deoxythymidin-5'-yl) (2',3'-dideoxyinosin-5'-yl) phosphate 9b

ddI (0.12 g, 0.50 mmol) gave **9b** (0.18 g, 49%) as a white foam. I.r.(KBr) 2108 (N_3), 1755(C=O ester); ^1H n.m.r.(CD_3OD) 0.97(t, J_{HH} 7.3 Hz, 3H), 1.6-1.8(m, 2H), 1.70(s, 3H), 2.0-2.2(m, 2H), 2.2-2.4(m, 2H), 2.4-2.6(m, 4H), 3.8-3.9(m, 1H), 4.0-4.4(m, 6H), 5.01(d, J_{PH} 9.7 Hz, 1H), 5.03(d, J_{PH} 9.7 Hz, 1H), 6.0-6.1(m, 1H), 6.2-6.3(m, 1H), 7.02(d, J_{HH} 8.5 Hz, 2H), 7.3-7.4(m, 3H), 8.00(s, 1H), 8.17(s, 0.5H), 8.20(s, 0.5H); ^{13}C n.m.r.(CD_3OD) 12.5, 13.8, 19.2, 26.5, 26.6, 32.7, 36.7, 37.4, 61.4, 68.0(d, J_{PC} 4.8 Hz), 68.1(d, J_{PC} 4.7 Hz), 70.0(d, J_{PC} 6.5 Hz), 70.4(d, J_{PC} 5.7 Hz), 70.5(d, J_{PC} 7.2 Hz), 81.0(d, J_{PC} 7.3 Hz), 83.2(d, J_{PC} 7.7 Hz), 86.4, 86.8, 111.7, 123.0, 125.6, 130.5, 130.6, 134.4(d, J_{PC} 5.2 Hz), 137.6, 140.0, 146.6, 149.4, 151.9, 152.4, 158.7, 166.0, 173.5; ^{31}P n.m.r.(CD_3OD) 1.2(s) and 1.3(s); M.s: *m/z* (FAB) found 764.2160 ($M+Na^+$), expected for $C_{31}H_{36}N_9NaO_{11}P$ 764.2169; Anal. found C, 50.36; H, 5.05%, expected for $C_{31}H_{36}N_9O_{11}P$ C, 50.20; H, 4.89%.

(*Rp*)- and (*Sp*)-4-Pivaloyloxybenzyl (3'-azido-3'-deoxythymidin-5'-yl) (2',3'-dideoxyinosin-5'-yl) phosphate 9c

ddI (0.12 g, 0.50 mmol) gave **9c** (0.12 g, 32%) as a white foam. I.r.(KBr) $\upsilon_{max.}$ 2108(N_3), 1748(C=O ester); ^1H n.m.r.(CD_3OD) 1.23(s, 9H), 1.69(s, 3H), 2.0-2.2(m, 2H), 2.2-2.4(m, 2H), 2.4-2.6(m, 2H), 3.7-3.9(m, 1H), 4.0-4.4(m, 6H), 4.95(d, J_{PH} 9.4 Hz, 1H), 4.97(d, J_{PH} 10.2 Hz, 1H), 5.9-6.0(m, 1H), 6.1-6.2(m, 1H), 6.93(d, J_{HH} 8.4 Hz, 2H), 7.2-7.4(m, 3H), 7.91(s, 1H), 8.09(s, 0.5H), 8.13(s, 0.5H); ^{13}C n.m.r.(CD_3OD) 12.5, 12.6, 26.6, 26.7, 27.4, 32.8, 37.5, 40.0, 61.5, 68.1(d, J_{PC} 3.3 Hz), 68.3(d, J_{PC} 4.3 Hz), 70.1, 70.5(d, J_{PC} 5.7 Hz), 70.6(d, J_{PC} 6.2 Hz), 81.1(d, J_{PC} 5.2 Hz), 83.3(d, J_{PC} 7.6 Hz), 86.5, 86.9, 111.8, 123.0, 125.7, 130.7, 130.8, 134.5(d, J_{PC} 5.7 Hz), 137.8, 140.1, 146.8, 149.5, 152.0, 152.8, 158.9, 166.2, 178.5; ^{31}P n.m.r.(CD_3OD) 1.1(s) and 1.3(s); M.s: *m/z* (FAB) found 756 ($M+H^+$), 778 ($M+Na^+$); Anal. found C, 50.12; H, 5.20%, expected for $C_{32}H_{38}N_9O_{11}P$ C, 50.86; H, 5.07%.

Lithium *bis*(3'-azido-3'-deoxythymidin-5'-yl) phosphate 10

Lithium iodide (0.02 g, 0.15 mmol) was added to a stirred solution of **7a** (0.10g, 0.14 mmol) in anhydrous acetone (2 ml) under an argon atmosphere. The reaction was protected from light and stirring was continued for 20 h. The resultant white precipitate was collected by filtration, washed with anhydrous acetone (2x20 ml) and dried under vacuum (1 mBar, 40°C) for 48 h to give (**10**) as a pale yellow solid (0.06 g, 66%). I.r.(Nujol) $\upsilon_{max.}$ 2109(N_3), 1687(C=O thymine); ^1H n.m.r.(D_2O) 1.88(s, 6H), 2.4-2.6(m, 4H), 4.0-4.2(m, 6H), 4.4-4.5(m, 2H), 6.18(dd, J_{HH} 6.4, 6.4 Hz, 2H), 7.64(s, 2H); ^{13}C n.m.r.(D_2O) 14.6, 39.0, 63.1, 68.0(d, J_{PC} 5.0 Hz), 85.6(d, J_{PC} 8.6 Hz), 87.9, 114.4, 140.5, 154.3, 169.8; ^{31}P n.m.r. (D_2O) 2.6(s); M.s.: *m/z* (FAB) found 603.1647($M+H^+$) expected for $C_{20}H_{25}LiN_{10}O_{10}P$ 603.1653; Anal. found C, 39.34; H, 4.66%, expected for $C_{20}H_{24}LiN_{10}O_{10}P$ C, 39.88; H, 4.02%.

Chemical hydrolysis of triester 7b, diester 10 and AZT monophosphate 3

The appropriate compound was dissolved in a solution of potassium methylphosphonate buffer in D_2O [pD 8.0, 0.1 mol dm^{-3}] - acetonitrile (9:1 v/v) (0.5 ml) to give a final concentration of 5 mmol dm^{-3}. The mixture was incubated at 37°C and monitored by ^{31}P n.m.r. spectroscopy.

Incubation of the triester 7b in cell culture medium

A mixture of RPMI 1640 media and 10% foetal calf serum (0.45 ml) in a 5mm n.m.r. tube was pre-incubated at 37°C for 0.5 h. Triester **7b** in acetonitrile was added to give a final

concentration of 5 mmol dm^{-3}. A sealed capillary tube containing D_2O (0.07 ml) was inserted and the reaction was monitored by ^{31}P n.m.r. spectroscopy at regular time intervals. After 5 days t.l.c. analysis [**A**) ethyl acetate-methanol, 4:1 **B**) ethyl acetate-methanol, 3:2] revealed the presence of AZT (**A**, R_f 0.85), diester **10** (**B**, R_f 0.60) and AZT monophosphate **3** (**B**, R_f 0.21). These compounds were identified by running authentic samples in parallel on the t.l.c. plate.

Incubation of triester 7b with porcine liver carboxyesterase and snake venom phosphodiesterase

Triester **7b** was dissolved in a methylphosphonate buffer in D_2O [pD 8.0, 0.1 mol dm^{-3}] - acetonitrile mixture (9:1 v/v) (0.45 ml) to give a final concentration of 5 mmol dm^{-3}. The mixture was heated to 37°C, 5U of porcine liver carboxyesterase (PLCE) was added and the sample monitored by ^{31}P n.m.r. spectroscopy. After 0.5h, 0.2U of snake venom phosphodiesterase was added and monitoring by ^{31}P n.m.r. spectroscopy was continued at regular time intervals.

Antiviral assay

The anti HIV-1 activities and toxicities of the compounds were assessed in C8166 and JM T-cell lines infected with the IIIB and U445 strains of HIV-1 respectively. Cells were grown in RPMI 1640 media with 10% foetal calf serum. $4x10^4$ cells per microtitre plate well were mixed with 5-fold dilutions of compound prior to addition of 10 $CCID_{50}$ units of virus and incubated at 37°C for 4-6 days. Inhibition of syncytia was examined from 2 days post infection. Culture supernatants were collected at 4-6 days and gp120 antigen production measured by ELISA(37). Viability of infected and uninfected cell controls were assessed by the MTT-Formazan method(38).

ACKNOWLEDGEMENTS

We thank the MRC AIDS Directed programme for a project grant, the Lister Institute for a fellowship (to S.F.) and the SERC mass spectrometry service (Swansea) for recording the mass spectra. We would also like to thank Drs S. Akhtar, D.B. Clarke, W. Fraser, J.M. Gardiner and W.J. Irwin for helpful discussions and technical assistance.

REFERENCES

(1) Mitsuya, H; Weinhold, K.J.; Furman, P.A.; St Clair, M.H.; Nusinoff-Lehrman, S.; Gallo, R.C.; Bolognesi, D.; Barry, D.W.; Broder, S., *Proc. Natl. Acad. Sci., U.S.A.*, 1985, **82**, 7096.

(2) Yarchoan, R.; Mitsuya, H.; Broder, S., *Scientific American,* 1988, **259**, No.4, 88.

(3) Yarchoan, R.; Weinhold, K.J.; Lyerly, H.K.; Gelmann, E.; Blum, R.M.; Shearer, G.M.; Mitsuya, H.; Collins, J.M.; Myers, C.E.; Klecker, R.W.; Markham, P.D.; Durack, D.T.; Nusinoff-Lehrman, S.; Barry, D.W.; Fischl, M.A.; Gallo, R.C.; Bolognesi, D.P.; Broder, S., *Lancet*, 1986, **1**, 575.

(4) Larder, B.A.; Kemp, S.D., *Science*, 1989, **246**, 1155.

(5) Richman, D.D.; Fischl, M.A.; Grieco, M.H.; Gottlieb, M.S.; Volberding, P.A.; Laskin, O.L.; Leedom, J.M.; Groopman, J.E.; Mildvan, D.; Hirsch, M.S.; Jackson, G.G.; Durack, D.T.; Nusinoff-Lehrman, S., *N. Eng. J. Med.*, 1987, **317**, 192.

(6) Mitsuya, H.; Broder, S., *Proc. Natl. Acad. Sci. U.S.A.*, 1986, **83**, 1911.

(7) Hamamoto, Y.; Nakashima, H.; Matsui, T.; Matsuda, A.; Ueda, T.; Yamamoto, N., *Antimicrob. Agents Chemother.*, 1987, **31**, 907.

(8) Cooney, D.A.; Dalal, M.; Mitsuya, H.; McMahon, J.B.; Nadkarni, M.; Balzarini, J.; Broder, S.; Johns, D.G., *Biochem. Pharmacol.*, 1986, **35**, 2065.

(9) Heidenreich, O.; Eckstein, F., *Nucleosides Nucleotides*, 1991, **10**, 535.

(10) Bungaard, H., in *Design of Prodrugs*, ed. Bungaard, H., Elsevier, Amsterdam, 1985, pp70-74.

(11) Thomson, W.; Nicholls, D.; Irwin, W.J.; Al-Mushadani, J.S.; Freeman, S.; Karpas, A.; Petrik, J.; Mahmood, N.; Hay, A.J., *J. Chem. Soc. Perkin Trans. I*, 1993, 1239.

(12) Henin, Y.; Gouyette, C.; Schwartz, O.; Debouzy, J.-C.; Neumann, J.-M.; Huynh-Dinh, T., *J. Med. Chem.*, 1991, **34**, 1830.

(13) Le Bec, C.; Huynh-Dinh, T., *Tetrahedron Lett.*, 1991, **32**, 6553.

(14) McGuigan, C.; Nicholls, S.R.; O'Connor, T.J.; Kinchington, D., *Antiviral Chem. Chemother.*, 1990, **1**, 25.

(15) Jones, B.C.N.M.; McGuigan, C.; O'Connor, T.J.; Jeffries, D.J.; Kinchington, D., *Antiviral Chem. Chemother.*, 1991, **2**, 35.

(16) Meier, C., *Angew. Chem. Int. Ed. Engl.*, 1993, **32**, 1704.

(17) Périgaud, C.; Gosselin, G.; Lefebvre, I.; Girardet, J.-L.;Benzaria, S.; Barber, I.; Imbach, J.-L., *Bioorg. Med. Chem. Lett.*, 1993, **3**, 2521.

(18) Périgaud, C.; Aubertin, A.-M.; Benzaria, S.; Pelicano, H.; Girardet, J.-L.; Maury, G.; Gosselin, G.; Kirn, A.; Imbach, J.-L., *Biochem. Pharmacol.*, 1994, **48**, 11.

(19) Farquhar, D.; Khan, S.; Srivastva, D.N.; Saunders, P.P., *J. Med. Chem.*, 1994, **37**, 3902.

(20) Starrett Jr, J.E.; Tortolani, D.R.; Russell, J.; Hitchcock, J.M.; Whiterock, V.; Martin, J.C.; Mansuri, M.M., *J. Med. Chem.*, 1994, **37**, 1857.

(21) Farquhar, D.; Chen, R.; Khan, S., *J. Med. Chem.*, 1995, **38**, 488.

(22) Shimizu, S.I.; Balzarini, J.; De Clercq, E.; Walker, R.T., *Nucleosides Nucleotides*, 1992, **11**, 583.

(23) Hahn, E.F.; Busso, M.; Mian, A.M.; Resnick, L., *Nucleotide Analogues as Antiviral Agents*, ACS Symposium Series 401, ACS, Washington D.C., 1989, pp156-169.

(24) Meier, C.; Neumann, J.-M.; André, F.; Henin, Y.; Huynh-Dinh, T., *J. Org. Chem.*, 1992, **57**, 7300.

(25) Grice, R.; Owen, L.N., *J. Chem. Soc.*, 1963, 1947.

(26) Perich, J.W.; Johns, R.B., *Synthesis*, 1988, 142.

(27) Boudjebel, H.; Concalves, H.; Mathis, F., *Bull. Soc. Chim. Fr.*, 1975, 628.

(28) Bannwarth, W.; Trzeciak, A., *Helv. Chimica. Acta*, 1987, **70**, 175.

(29) Busso, M.; Mian, A.M.; Hahn, E.F.; Resnick, L., *AIDS Res. Hum. Retrov.*, 1988, **4**, 449.

(30) Zervas, L.; Dilaris, I., *J. Am. Chem. Soc.*, 1955, **77**, 5354.

(31) Fife, T.H.; Bruice, T.C., *J. Phys. Chem.*, 1961, **65**, 1079.

(32) Balzarini, J.; Herdewijn, P.; Pauwels, R.; Broder, S.; De Clercq, E., *Biochem. Pharmacol.*, 1988, **37**, 2395.

(33) Puech, F.; Gosselin, G.; Balzarini, J.; De Clercq, E.; Imbach, J.-L., *J. Med. Chem.*, 1988, **31**, 1897.

(34) Puech, F.; Pompon, A.; Lefebvre, I.; Gosselin, G.; Imbach, J.-L., *Bioorg. Med. Chem. Lett.*, 1992, **2**, 603.

(35) Pompon, A.; Lefebvre, I.; Imbach, J.-L.; Kahn, S.; Farquhar, D., *Antiviral Chem. Chemother.*, 1994, **5**, 91.

(36) Still, W.C.; Kahn, M.; Mitra, A., *J. Org. Chem.*, 1978, **43**, 2923.

(37) Mahmood, N.; Hay, A.J., *J. Immunol. Methods*, 1992, **151**, 9.

(38) Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn, P.; Desmyter, J.; De Clercq, E., *J. Virol. Methods*, 1988, **20**, 309.

Received November 17, 1994
Accepted April 26, 1995