Volume 310, number 2, 171–174 FEBS 11578 September 1992

Phosphate derivatives of AZT display enhanced selectivity of action against HIV1 by comparison to the parent nucleoside

Christopher McGuigan[a], Caleb Nickson[b], Juraj Petrik[c] and Abraham Karpas[c]

[a]*Department of Chemistry, University of Southampton, Highfield, Southampton, SO9 5NH, UK,* [b]*Department of Chemistry, University College London, London, WC1H 0AJ, UK* and [c]*Department of Haematology, Clinical School, University of Cambridge, Hills Road, Cambridge, CB2 2QL, UK*

Received 3 August 1992

Novel phosphate derivatives of the anti-HIV nucleoside analogue AZT have been prepared by phosphorochloridate chemistry. In particular, phosphates carrying ester-containing side-chains are described. These materials are designed to act as membrane-soluble pro-drugs of the bio-active free nucleotides. In vitro evaluation revealed the compounds to have a pronounced, selective antiviral activity. In several cases the phosphate derivatives are more selective in their action than the parent nucleoside AZT. In particular, this arises from the low toxicity of the phosphate pro-drugs by comparison to AZT. These data support the suggestion that the phosphate derivatives exert their biological effects via intracellular release of the nucleotide forms, and suggests that such pro-drug forms may be worthy of further study.

HIV; AZT; Nucleotide; Pro-drug

1. INTRODUCTION

The nucleoside analogue 3′-azido-3′-deoxythymidine (AZT, **1**) is a potent inhibitor of human immunodeficiency virus (HIV1) proliferation [1], and is now established as a useful treatment for acquired immunodeficiency syndrome (AIDS). As with other chemotherapeutic nucleoside analogues, AZT acts only after metabolic activation to its 5′-phosphate forms. This dependence on (kinase mediated) phosphorylation may be a limitation; especially in cellular environments low in particular nucleoside kinase activities.

We have previously outlined the potential advantages of utilising masked phosphate derivatives of anti-cancer and anti-herpetic nucleoside analogues [2,3]. We have found that simple dialkyl phosphate derivatives of AZT, and other nucleoside analogues, are inactive as anti-HIV agents [4], whereas substituted dialkyl phosphates (**2a–b**) are active [5]. In this paper we report the preparation of novel ester-containing phosphate derivatives of AZT, which selectively inhibit the replication of HIV. In particular, in contrast to AZT, the agents are selective, less toxic, antiviral agents in vitro. One of the products herein reported (**3a**) was recently described by us [6]. However, its activity is now described in a new assay, where it is included alongside new agents described here for the first time.

Correspondence address: C. McGuigan, Dept. of Chemistry, University of Southampton, Highfield, Southampton, SO9 5NH, UK. Fax: (44) (703) 59 3781.

2. MATERIALS AND METHODS

All reactions were carried out under scrupulously dry conditions, using general procedures we have described [7]. P-31 NMR spectra were recorded on a Varian XL-200 spectrometer (82 MHz) or a Jeol FX90Q (36.2 MHz) and are reported in units of δ relative to 85% phosphoric acid as external standard, positive shifts are downfield. Carbon-13 NMR spectra were recorded on a Varian XL-200 spectrometer (50 MHz), a Varian VXR-400 (100 MHz) or a Bruker AM360 (90.6 MHz) in units of δ relative to $CDCl_3$ at 77,000 ppm. Both phosphorus-31 and carbon-13 NMR spectra were proton noise decoupled and all signals were singlets unless otherwise stated. H-1 NMR spectra were recorded on a Varian XL-200 spectrometer (200 MHz), a Varian VXR-400 (400 MHz), or a Bruker AM360 (360 MHz) and are reported in units of δ relative to internal $CHCl_3$ at 7,240 ppm unless otherwise stated. All NMR spectra were recorded in $CDCl_3$. HPLC data were recorded as described [7]; all samples for biological testing were free of AZT (<0.01%).

2.1. *3′-Azido-3′-deoxythymidine-5′-dodecyl (ethyl glycolyl) phosphate [3a]*

This was prepared entirely as recently described by us [6].

2.2. *3′-Azido-3′-deoxythymidine-5′-(ethyl glycolyl) (methyl lactyl) phosphate [3b]*

Ethyl glycolyl methyl lactyl phosphorochloridate (1.62 g, 1.5 ml, 0.006 mol) was added to AZT (0.25 g, 0.94 mmol) and *N*-methylimidazole (0.61 g, 0.55 ml, 0.007 mol) in tetrahydrofuran (5 ml) with stirring at room temperature. After stirring for two days the mixture was concentrated to dryness under reduced pressure, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (15 ml) and water (10 ml). The aqueous layers were extracted with chloroform (15 ml) and the combined organic layers dried over magnesium sulphate (ca. 5 g). Filtration and concentration to a small volume (ca. 3 ml) followed by precipitation from petroleum ether (bp 30–40°C) (500 ml) overnight at −20°C gave an amber gum. Flash column chromatography on silica eluted with 0.5% methanol in chloroform gave a colourless gum. This was dissolved in 2-propanol, filtered and concentrated under reduced pressure to yield the product

as a glass (0.42 g 86%); δ_c:170.1 (m, C(O)CH), 167.9 (m, C(O)CH_2), 163.6 (C-2), 150.2(C-4), 135.5(C-6, A), 135.2(C-6, B), 111.5(C-5, A), 111.6(C-5, B), 84.6(C-1′), 82.3(d, C-4′, A, J=8.4 Hz), 82.2(d, C4′, B, J=8.3 Hz), 72.75(d, CHO, A, J=5.4 Hz), 72.70(d, CHO, B, J=5.8 Hz), 67.0(d, C-5′, A , J=5.8 Hz), 66.9(d, C-5′, B, J=5.3 Hz), 64.1(d, C(O)CH2, B, J=5.1 Hz), 63.8(d, C(O)CH_2, A, J=5.0 Hz), 61.9(CH_2OC, A), 61.8(CH_2OC, B), 60.2(C-3′, A), 60.15(C-3′, B), 52.7(MeO), 37.53(C-2′, A), 37.49(C-2′, B), 19.07(d, CH_3CH, B, J=6.6 Hz), 18.96(d, CH_3CH, A, J=6.9 Hz), 14.1(CH_3CH_2), 12.41(5-Me, B), 12.37(5-Me, A); δ_p −1.605, −2.369, A:B 3:1; δ_H 8.81, 8.83 (1H, s, NH, B, A), 7.49, 7.46(1H, d, H6, A, B, J=1.3, 1.2 Hz), 6.0(1H, m, H-1′), 5.0(1H, m, CHO), 4.7(1H, m, H-3′), 4.5(4H, m, H-5′, C(O)CH_2), 4.25(2H, q, CH_2OC, J=7.1 Hz), 4.1(1H, m, H-4′), 3.87, 3.79(3H, s, MeO, A, B), 2.5(2H, m, H-2′), 1.94, 1.93(3H, d, 5-Me, A, B, J=1.17, 1.19 Hz), 1.6(m, CH_3CH, B, A), 1.3(3H, m, CH_3CH_2); E.I.M.S. m/e: 519(M^+, 2%), 435(0.1), 416(M^+-$C_4H_7O_3$, 1), 393(0.4), 386(MH^+-$C_4H_7O_3$-CH_3, 30), 373(MH^+-$C_4H_7O_3$-N_3H, 5), 351(MH^+-thymine - N_3H, 30), 313(M^+-$C_4H_7O_3$-$C_4H_7O_3$, 1), 271(7), 250(AZT.H^+-H_2O, 18), 185($C_4H_7O_3PO_3H^+$, 3), 167 (45), 149(16), 126(thymine.H^+, 25), 87 (10), 81($C_5H_5O^+$, 100); Found: C 41.45%, H 5.03, N 12.86, P 6.25, $C_{18}H_{26}N_5O_{11}P$ requires C 41.62%, H 5.05, N 13.48, P 5.96.

2.3. *3′-Azido-3′-deoxythymidine-5′-bis(methyl lactyl) phosphate [3c]*

Bis(methyl lactyl) phosphorochloridate was added to AZT (0.10 g 0.31 mmol) and *N*-methylimidazole (0.25 g, 0.24 ml, 0.003 mol) in tetrahydrofuran (3 ml) with stirring at room temperature. After stirring for a further 48 h. the mixture was concentrated to dryness under reduced pressure, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (15 ml) and then water (10 ml). The aqueous layers were extracted with chloroform (ca.15 ml) and the combined organic layers dried over magnesium sulphate (ca. 3 g). Filtration and concentration to small volume (ca. 3 ml) under reduced pressure followed by precipitation of the product from petroleum ether (bp 30–40°C) (500 ml) overnight at −20°C gave an amber gum. Further purification was achieved with flash column chromatography, (silica ca. 60 g) eluted with chloroform containing 0.5% methanol. Pooling and evaporation of the appropriate fractions gave a gum. (0.17 g, 88%); δ_c 171.1(d, C(O)CH, J=3.3 Hz), 170.7(d, C(O)CH, J=3.9 Hz), 163.9(C-2), 150.5(C-4), 135.3(C-6), 111.5(C-5), 84.5(C-1′), 82.3(d, C-4′, J=8.4 Hz), 72.7(d, CHO J=5.2 Hz), 72.5(d, CHO J=5.1 Hz), 66.6(d, C-5′, J=5.9 Hz), 60.3(C-3′), 52.7(MeO), 37.5(C-2′), 19.1(d, $\underline{C}H_3CH$, J=6.9 Hz), 18.9(d, $\underline{C}H_3CH$, J=7.1 Hz), 12.4(5-Me); δ_p −2.709; δ_H 9.46(1H, s, NH), 7.48(1H, s, H-6), 6.31(1H, t, H-1′, J=6.7 Hz), 5.1(1H, m, CHO), 4.9(1H, m, CHO), 4.5(2H, m, H-5′), 4.5(1H, m, H-3′), 4.1(1H, m, H-4′), 3.79(3H, s, MeO), 3.77(3H, s, MeO), 2.4(1H, m, H-2′), 2.4(1H, m, H-2′), 1.94 (3H, d, 5-Me, J=1.2 Hz), 1.60(3H, dd, $C\underline{H}_3CH$, J= 7.0, 1.0 Hz), 1.58(3H, dd, $C\underline{H}_3CH$, J= 6.9, 1.0 Hz); E.I.M.S. m/e 519 (M^+, 0.9%), 518(0.4), 460(<0.1), 416(5), 394(M^+-thymine, 0.3), 373(M^+-$C_4H_7O_3$-N_3H, 9), 351(MH^+-thymine-N_3H, 31), 313(M^+-$C_4H_7O_3$, 1), 271(2), 250(AZT.H^+-H_2O, 14), 185($C_4H_7O_3PO_3H^+$, 3), 167(79), 126(thymine.H^+, 12), 99(17), 87(25), 81($C_5H_5O^+$, 100); Found C 41.12%, H 5.10, N 13.14, P 6.00, $C_{18}H_{26}N_5O_{11}P$ requires C 41.62%, H 5.05, N 13.48, P 5.96.

2.4. *3′-Azido-3′-deoxythymidine-5′-[(2,2,2,-trichloroethyl) (methyl lactyl)] phosphate [3d]*

2,2,2,-Trichloroethyl (methyl lactyl) phosphorochloridate (1.16 g, 0.88 ml, 0.003 mol) was added to AZT (0.25 g, 0.94 mmol) and *N*-methylimidazole (0.61 g, 0.60 ml, 0.007 mol) in tetrahydrofuran (5 ml) with stirring at room temperature. After stirring for two days the reaction mixture was concentrated to dryness under reduced pressure, dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (15 ml) and water (10 ml). The aqueous layers were back extracted with chloroform (15 ml) and the combined organic layers dried over magnesium sulphate (ca. 3 g). Filtration and concentration to small volume (ca. 3 ml) followed by precipitation from petroleum ether (500 ml) overnight at −20°C gave an amber gum. Flash column chromatography on silica eluted with 0.5% methanol in chloroform afforded the product as a glass, on pooling and evaporation of the appropriate fractions. This was dissolved in 2-propanol and filtered under reduced pressure. Concentration to dryness yielded the product as a glass. (0.38 g, 79%); δ_c 170.5(d, C(O)CH, A, J=3.9 Hz), 170.2(d, C(O)CH, B, J=4.2 Hz), 163.45(C-2, A), 163.42(C-2, B), 150.11(C-4, A), 150.08(C-4, B), 135.2(C-6), 111.68(C-5, B), 111.55(C-5, A), 94.8(m, CCl_3), 84.84(C-1′, B), 84.79(C-1′, A), 82.1(d, C-4′, A, J=8.4 Hz), 82.0(d, C4′, B, J=7.9 Hz), ca. 77($CCl_3\underline{C}H_2$), 73.1(CHO, B, J=5.5 Hz), 72.1(d, CHO, A, J=5.2 Hz), 67.4(d, C-5′, A, J=6.1 Hz), 67.1(d, C-5′, B, J=6.0 Hz), 60.1(C-3′, A), 60.0(C-3′, B), 52.9(MeO), 37.5(C-2′, A), 37.3(C-2′, B), 18.9(m, $\underline{C}H_3CH$), 12.5(5-Me); δ_p −1.118, −1.462, A:B 1:3; δ_H 8.78, 8.72(1H, bs, NH, B, A), 7.38, 7.36(1H, d, H-6, A, B, J=1.2, 1.2 Hz), 6.2(1H, m, H-1′), 5.0(1H, m, CHO), 4.7, 4.6(2H, m, $CCl_3C\underline{H}_2$, B, A), 4.3(3H, m, H-3′, H-5′), 4.0(1H, m, H-4′), 3.773, 3.770(3H, s, MeO, B, A), 2.4(1H, m, H-2′), 2.3(1H, m, H-2′), 1.92, 1.91(3H, d, 5-Me, B, A, J=1.3, 1.2 Hz), 1.59, 1.57(3H, dd, $C\underline{H}_3CH$, J=1.2/7.0, 1.2/7.0 Hz).

2.5. *Materials and experimental procedures: virology*

2.5.1. Anti-HIV-1 assay

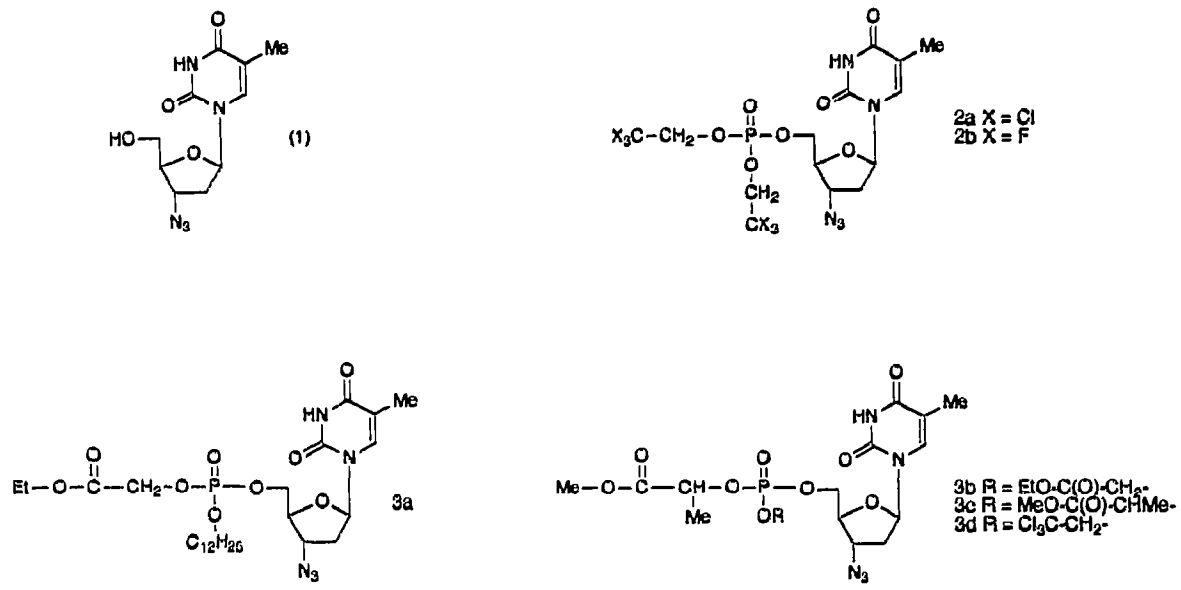
We have tested the cytotoxicity and anti-HIV-1 activity of the various compounds in an anti-HIV assay system which has been described in detail previously [8]. In this assay system, we have used Molt4 as the target cells which were pre-incubated overnight with the compounds being tested, before infection at a multiplicity of 1 infectious unit of HIV-1 per cell on the following day. This enabled us to determine the anti-viral activity and cytotoxicity of the compounds. The effects on virus inhibition and cell division are compared to that of AZT at the same time.

3. RESULTS AND DISCUSSION

3.1. *Chemistry*

Whereas simple dialkyl phosphorochloridates react relatively rapidly with nucleosides in pyridine at ambient temperature [4], the analogous reactions with highly substituted phosphorochloridates are rather slow. However, a strategy similar to that we have previously employed for phosphoramidates [9,10] was found to be successful in the case of substituted dialkyl phosphates. Thus, as we have recently noted [6] dodecyl (ethyl glycolyl) phosphorochloridate reacts relatively rapidly with AZT (1) in THF at ambient temperature in the presence of *N*-methylimidazole [11]. The target product (3a) was isolated in moderate yield and fully characterised by a range of spectroscopic methods. The sample was pure by HPLC, and entirely free of any contaminating AZT; this is particularly important given the high activity of AZT in the biological assay used.

We have noted the major effect on antiviral activity of proceeding from simple dialkyl phosphate derivatives of AZT to halogen-substituted dialkyl phosphates [5], and very recently we have noted the activity of ester-substituted alkyl phosphates [6]. However, in the former report both alkyl groups were substituted, whilst in the latter only one group was ester-containing, the other group being a simple alkyl moiety. Thus, it was now of interest to prepare the corresponding compounds with two ester-containing groups. By an analogous route to that used for (3a) above, the novel compound with one ethyl glycolyl group and one methyl lactyl group (3b)



Scheme

was prepared from AZT in high yield. As with (**3a**) this displayed two signals in the ^{31}P NMR spectrum, corresponding to the two diastereoisomers which result from mixed stereochemistry at the (chiral) phosphorus centre. As previously, these isomers are not produced in a 1:1 ratio; (**3a**) displayed a 2:1 preponderance of the down-field isomer, whilst the ratio is 3:1 for (**3b**). The magnitude of the splitting is also greater in the case of (**3b**) ($\Delta\delta$ 0.8 ppm), and the position of the peaks is slightly further up-field. The latter might be expected on the basis of the increased substitution at the phosphate centre [12]. Compound (**3b**) was fully characterised by ^{13}C and ^{1}H NMR, mass spectrometry and microanalysis. The presence of diastereoisomers was also evident from the ^{13}C NMR; indeed the 3:1 ratio of isomers allowed an un-equivocal assignment of many of the signals to each of the two structures. As we have noted [9,14], it may well be that the individual diastereoisomers in a particular case may differ in their biological activities; however, in this study the mixtures of isomers were not separated, and were tested as such.

We have recently noted the apparent preference for ethyl glycolyl over methyl lactyl side-chains in the case of simple phosphate derivatives of AZT; compounds with only one ester-containing group [6]. It was of interest to determine whether the same preference applied to more substituted structures such as (**3b**). Thus, the symmetrical bis(methyl lactyl) compound (**3c**) was prepared using analogous chemistry. The ^{31}P NMR of this sample clearly revealed the presence of only one isomer; entirely as would be expected for such a (symmetrical) phosphate. The position of the ^{31}P resonance was rather similar to that noted for (**3b**). Other data were also similar to the previous compounds, except that again they revealed only one compound to be present.

Finally, it was of interest to examine whether other substituted groups would be equally effective, besides ester-containing groups. We have already reported the activity of bis(trihaloethyl) phosphate derivatives of AZT and other nucleosides [5]. It was therefore reasonable to examine the effects of such a group in combination with an ester-containing group. Thus, the trichloroethyl (methyl lactyl) phosphate of AZT (**3d**) was prepared following our established synthetic route. Again, two isomers were noted in the ^{31}P NMR; though the up-field isomer now predominated. Carbon-13 NMR data also confirmed the structure, purity, and isomeric constitution of (**3d**).

3.2. *Antiviral activity*

The effect of various concentrations of (**1**) and (**3a-d**) on HIV-1 replication are outlined in Table I. Interestingly, despite the differences between this assay and that previously used [6,13] compound (**3a**) was of rather similar activity in both assay systems. Moreover, comparing (**3a**) to (**3b–c**) it appears that the presence of two ester-substituted groups enhances activity approximately 100-fold, relative to having only one substituted group; although this cannot be precisely defined given the different structures of the esters. However, the identical activity of (**3b**) and (**3c**) indicates no difference between ethyl glycolyl and methyl lactyl groups in this series; unlike the case with one ester-containing group [6]. Lastly, the intermediate activity of (**3d**), being ap-

Table 1

Cytotoxicity and anti-HIV1 activity

Compound	Dosage (μg/ml)	Estimated cell growth in %*	Estimated anti-HIV activity in %+
1	10	10	100
	1	40	100
	0.1	80	100
	0.01	100	90
3a	100	50	100
	10	70	90
	1	90	60
3b	100	80	100
	10	100	100
	1	100	100
	0.1	100	90
3c	500	40	100
	100	60	100
	10	90	100
	1	100	100
	0.1	100	90
3d	100	20	100
	10	80	100
	1	100	90

*% of cell growth compared to cells grown in the absence of drug counted on day 10.
+% of cells not stained for HIV antigen by immunoperoxidase method at day 10.

proximately 10-fold less active than (**3b–c**), suggests that a trihaloethyl group may substitute for an ester-containing group, but with reduced potency.

Compound (**3a**) is of low antiviral selectivity; being toxic to uninfected cells even at high dilution. On the other hand, compounds (**3b–c**) are very selective inhibitors of viral proliferation. Both are at least 100-times less toxic than the parent nucleoside. Since they are only 10-times less active than the nucleoside, the therapeutic index of (**3b–c**) is at least 10-times that of AZT in this assay system. The origins of this reduced toxicity are uncertain, but this may be consistent with a mode of action involving slow intracellular release of phosphate derivatives of AZT, rather than the free nucleoside.

In conclusion, we have reported the synthesis and anti-HIV evaluation of a series of novel ester-containing phosphate derivatives of AZT. The compounds show a range of activities, but are generally less active than AZT, but also less toxic. They display much greater antiviral selectivity than the parent nucleoside. The data are consistent with a mode of action involving intracellular release of the bio-active free nucleotides. If these in vitro findings could be translated into a demonstrable in vivo advantage, such phosphate pro-drugs could have merit as candidates for clinical development.

Acknowledgements: C.N. thanks the SERC for a studentship and Mr. S. Corker for HPLC analyses.

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., and Broder, S. (1985) Proc. Natl. Acad. Sci. USA 82, 7096–7100.
[2] Jones, B.C.N.M., McGuigan, C. and Riley, P.A. (1989) Nucleic Acids Res. 17, 7195–7201.
[3] McGuigan, C., Tollerfield, S.M. and Riley, P.A. (1989) Nucleic Acids Res. 17, 6065–6075.
[4] McGuigan, C., Nicholls, S.R., O'Connor, T.J. and Kinchington, D. (1990) Antiviral Chem. Chemother. 1, 25–33.
[5] McGuigan, C., O'Connor, T.J., Nicholls, S.R., Nickson, C. and Kinchington, D. (1990) Antiviral Chem. Chemother. 1, 355–360.
[6] McGuigan, C., Nickson, C. O'Connor, T.J. and Kinchington, D. (1992) Antiviral Res. 17, 197–212.
[7] McGuigan, C., Devine, K.G., O'Connor, T.J. and Kinchington, D. (1991) Antiviral Res. 15, 255–263.
[8] Karpas, A., Fleet, G.W.J., Dwek, R.A., Petursson,S., Namgoong, S.K., Ramsden, N.G., Jacob, G.S. and Rademacher, T.W. (1988) Proc. Natl. Acad. Sci. USA 85, 9229–9233.
[9] McGuigan, C., Devine, K.G., O'Connor, T.J., Galpin, S.A., Jeffries, D.J. and Kinchington, D. (1990) Antiviral Chem. Chemother. 1, 107–113.
[10] Curley, D., McGuigan, C., Devine, K.G., O'Connor, T.J., Jeffries, D.J. and Kinchington, D. (1990) Antiviral Res. 14, 345–356.
[11] Van Boom, J.H., Burgers, P.M.J., Crea, R., Luyten, W.C.M.M. and Reese, C.B. (1975) Tetrahedron 31, 2953–2959.
[12] Mark, V., Dungan, C.H., Crutchfield, M.M. and Van Wazer, J.R. (1969) in: Topics in Phosphorus Chemistry, vol. 5 (Grayson, M. and Griffith, E.J. eds.) Wiley, New York.
[13] Holmes, H.C., Mahmood, N., Karpas, A., Petrik, J., Kinchington, D., O'Connor, T.J., Jeffries, D.J., Desmyter, J., DeClercq, E., Pauwels, R. and Hay, J. (1991) Antiviral Chem. Chemother. 2, 287–293.
[14] Devine, K.G., McGuigan, C., O'Connor, T.J., Nicholls, S.R. and Kinchington, D. (1990) AIDS 4, 371–372.