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Synthesis and anti-HIV evaluation of some phosphoramidate derivatives of AZT: studies on the effect of chain elongation on biological activity

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Summary

A series of phosphoramidate derivatives of the anti-HIV drug AZT has been prepared as membrane soluble pro-drugs of the bio-active nucleotide forms and evaluated in vitro against HIV-1. Terminal substituted alkyl amines have a pronounced anti-HIV effect: this effect declines upon increasing the length of the methylene spacer. The results are consistent with a mechanism of action involving intracellular cleavage of the phosphoramidate bond, and release of the nucleotide, or a derivative thereof. Full spectroscopic data are included on the products and their phosphorochloridate precursors.

HIV; Phosphoramidate derivative; AZT

Introduction

Therapies for the acquired immunodeficiency syndrome (AIDS) have been dominated by strategies directed against the human immunodeficiency virus HIV-1. In particular, nucleoside analogues such as 3′-azido-3′-deoxythymidine (AZT) (1) are in clinical use (Yarchoan et al., 1986). Other nucleoside analogues, notably the

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2′,3′-dideoxy nucleosides, are also undergoing clinical evaluation (Merigan et al., 1989). However, all of these compounds share a number of problems: in particular, nucleoside analogues have a deleterious dependence on kinase mediated activation because the bio-active form is the nucleotide (Cooney et al., 1986). For AZT the triphosphate acts either by inhibition of the viral reverse transcriptase (RT), or by incorporation into the viral DNA chain (hence blocking further extension of the DNA) (Furman et al., 1986). It is well established that this dependence of chemotherapeutic nucleoside analogues on kinases may lead to poor activity, and the emergence of clinical resistance (Furman et al., 1979). Another common problem of nucleoside analogues is drug toxicity; for AZT this is manifested as neutropoenia and anaemia (Pinching et al., 1989).

As we have noted for the anti-herpes drug araA (McGuigan et al., 1989), and the anti-cancer drug araC (Jones et al., 1989), it is possible that the limiting kinase dependence of chemotherapeutic nucleoside analogues may be overcome by the synthesis of appropriate pro-drug forms; not of the nucleoside, but of the nucleotide. We have recently noted that simple dialkyl phosphate triester derivatives of AZT are inactive as anti-HIV agents (McGuigan et al., 1990a), whereas carboxy-protected amino-linked phosphoramidate derivatives (such as compound 2a) are potent anti-HIV agents (Devine et al., 1990). In particular, it was found that minor modifications in the amino acid side chain lead to marked changes in anti-HIV activity (McGuigan et al., 1990b). In this paper we report the synthesis and anti-HIV evaluation of several novel derivatives, which indicates a marked effect on anti-viral activity of increasing the methylene spacer length between the phosphoramidate moiety and the carboxyl ester group.

Materials and Methods

All reactions were carried out under scrupulously dry conditions unless otherwise indicated. THF was dried by distillation at atmospheric pressure from lithium aluminium hydride onto activated 4A molecular sieves. Dichloromethane was distilled from calcium hydride and stored over 4A molecular sieves. Analytical grade carbon tetrachloride was dried over 4A molecular sieves. *N*-Methylimidazole and triethylamine were distilled from calcium hydride. For tlc, Merck 60 F_{254} pre-coated silica plates were employed. For flash column chromatography either Merck Kieselgel 60 or Woelm silica was used. AZT and ethyl phosphorodichloridate were supplied by Aldrich Chemical Company. For the nucleoside derivatives: proton nmr spectra were recorded on a Varian VXR400 spectrometer operating at 400 MHz, ^{13}C spectra were obtained on this instrument, operating at 100 MHz, and ^{31}P spectra on a Varian XL200 instrument operating at 82 MHz. For the phosphorochloridates, all nmr spectra were recorded on the latter instrument (200 MHz for ^{1}H, 50 MHz for ^{13}C and 82 MHz for ^{31}P). Proton and carbon spectra were referenced to TMS, and phosphorus spectra to 85% phosphoric acid; positive shifts are downfield of the reference. All nmr spectra were run in $CDCl_3$. Mass spectra were recorded on a VG7070H spectrometer, courtesy of Dr M. Mruzek (EIMS). HPLC analyses were

conducted on all samples submitted for biological evaluation, using an ACS system with a 50+250 × 4.6 mm Spherisorb ODS2 5 μM column. Gradient elution was employed, using water (A), and acetonitrile (B) mixed as follows: 18% B for 0–10 min, then a linear gradient to 80% B at 30 min. Detection was by UV at 254 nm, with a flow rate of 1 ml/min. In every case the bulk purity exceeded 99%, with levels of AZT below detectable limits (<0.01%).

Ethyl methoxyglycinyl phosphorochloridate

Triethylamine (3.15 ml, 2.29 g, 22.6 mmol) in anhydrous dichloromethane (30 ml) was added dropwise with vigorous stirring over a period of 5.5 h to a mixture of glycine methyl ester hydrochloride (1.42 g, 11.3 mmol) and ethyl phosphorodichloridate (1.34 ml, 1.84 g, 11.30 mmol) in dichloromethane (30 ml) at −78°C. The mixture was allowed to warm to room temperature with stirring over 1.5 h. The solvent was then removed under reduced pressure, benzene (50 ml) added, and the mixture filtered. The filtrate was concentrated under reduced pressure, carbon tetrachloride (10 ml) added, and the mixture filtered once again. The filtrate was concentrated to dryness under reduced pressure, to yield the product as an oil (2.00 g, 82%). δ_P +12.48; δ_H 4.23(2H, m, CH_2OP), 3.84(1H, s, NH), 3.79(3H, s, OMe), 3.71(2H, s, CH_2N), 1.40(3H, m, CH_3CH_2); δ_C 170.37(d, C=0, J=10.4 Hz), 64.75(d, $POCH_2$, J=6.0 Hz), 52.61(OMe), 42.84(CH_2N), 15.92(d, CH_2CH_3, J=8.0 Hz); EIMS m/e 215.0126(M^+, $C_5H_{11}ClNO_4P$ requires 215.0114, 0.1%), 170(M^+-OEt, 2), 158(M^+-MeOCO, ^{37}Cl, 32), 156(M^+-MeOCO, base peak), 130(M^+-$MeOCOCH_2N$, ^{37}Cl, 31), 128(M^+-$MeOCOCH_2N$, 98).

3′-Azido-3′-deoxythymidine-5′-(ethyl methoxyglycinyl) phosphoramidate (2a)

3′-Azido-3′-deoxythymidine (1) (0.20 g, 0.75 mmol) and ethyl methoxyglycinyl phosphorochloridate (0.81 g, 3.75 mmol) were stirred together in anhydrous tetrahydrofuran (THF) (5 ml) containing *N*-methylimidazole (0.60 ml, 7.48 mmol) for 16 h, at ambient temperature. The solvent was removed in vacuo, and the residue dissolved in chloroform (30 ml), and extracted with saturated sodium bicarbonate solution (15 ml), and water (2 × 15 ml). The organic phase was dried ($MgSO_4$) and evaporated in vacuo. The residue was re-dissolved in chloroform (10 ml), and precipitated with petroleum (bp 30–40°C) (500 ml). The precipitate was purified by chromatography on silica gel (30 g), using 4% methanol in chloroform as eluent. Pooling and evaporation of appropriate fractions gave the pure product (0.28 g, 84%). δ_P +6.60; δ_H (starred peaks are duplicated due to diastereoisomers) 8.45*(1H, s, N^3H), 7.35*(1H, s, H6), 6.15*(1H, t, H1′), 4.35*(1H, m, H3′), 4.20(2H, m, H5′), 4.10(2H, m, CH_2OP), 4.00(1H, m, H4′), 3.70(5H, m, glycine OMe, CH_2), 3.30(1H, m, glycine NH), 2.40(1H, m, H2′), 2.20(1H, m, H2′), 1.90(3H, s, 5-Me), 1.30(3H, m, CH_3CH_2); EIMS m/e 447.1348(MH^+, $C_{15}H_{24}N_6O_8P$ requires 447.1393, 1%), 81($C_5H_5O^+$, base peak); HPLC retention times 14.45, 14.79 min (1:1). Carbon-13 nmr data for this compound, and for the other nucleoside phosphoramidates, are in Table 1.

TABLE 1

Carbon-13 nmr data for novel phosphoramidates

	2a	2b	2c	2d	2e	3
Base						
C2	164.03/163.91	163.78/163.76	164.03/164.01	163.99	163.89/163.86	164.10/164.07
C4	150.55/150.38	150.35/150.30	150.51/150.44	150.45/150.37	150.40/150.32	150.50/150.39
C5	111.37/111.28	111.52/111.42	111.39/111.24	111.27/111.11	111.39/111.23	111.24/111.07
C6	134.78/134.69	135.40	135.37	135.25	135.34	135.11
Me	12.42/12.39	12.56	12.47	12.43/12.40	12.54/12.50	12.37/12.33
Sugar						
C1'	84.81/84.75	85.22/84.91	85.20/84.80	85.04/84.60	85.17/84.74	84.84/84.41
C2'	37.43/37.38	37.58	37.50	37.41	37.56	37.41/37.38
C3'	60.36/60.27	60.47/60.42	60.47/60.45	60.35	60.45	60.37
C4'	82.36/82.28[a]	82.59[f]	82.60/82.53[k]	82.50/82.42[p]	82.61[u]	82.41[z]
C5'	65.26/65.18[b]	65.28/65.02[g]	65.15/64.94[l]	65.00/64.78[q]	65.09/64.86[v]	64.92/64.66[a']
Alkyl						
POC	63.12/63.09[c]	63.10/63.04[h]	62.91/62.84[m]	62.74/62.66[r]	62.84/62.76[w]	62.58/62.50[b']
POCC	16.22/16.18[d]	16.36[i]	16.24[n]	16.15[s]	16.30[x]	16.11/16.04[c']
Amine						
PNC	42.63/42.59	37.15/37.13	40.74/40.71	40.89/40.85	41.18/41.14	43.01/42.95
PNCC	–	35.80/35.75[j]	26.78/26.72[o]	31.02/30.96[t]	31.41/31.35[y]	24.75/24.68[d']
PNCCC	–	–	30.95	21.72	24.39	11.00
PNCCCC	–	–	–	33.28	26.05	–
PNCCCCC	–	–	–	–	33.79	–
C=0	171.52/171.35[e]	172.59/172.54	173.51/173.47	173.68/173.66	173.97/173.95	–
Ome	52.39	51.99	51.74	51.54	51.59	–

Carbon-13 nmr data for compounds (2a–e) and (3), recorded in $CDCl_3$ at 100 MHz. Many peaks are split due to diastereomeric and coupling effects. Coupling constants to phosphorus, in Hz: a 4.5/5.2, b 8.2/7.9, c 5.4/6.2, d 4.8/5.1, e 3.1/4.2, f 7.5, g 5.0/5.1, h 5.4/5.6, i 1.5, j 2.0/2.1, k 3.9/3.7, l 4.9/5.1, m 5.4/5.9, n 7.0, o 3.7/3.6, p 3.5/3.3, q 4.9/4.7, r 5.1/4.8, s 7.1, t 5.9/5.7, u 7.5, v 5.0/4.6, w 5.2/5.0, x 6.9, y 4.4/4.4, z 7.9, a' 5.0/4.5, b' 5.0/4.8, c' 2.0/2.0, d' 6.7/7.2.

Ethyl methoxy-β-alaninyl phosphorochloridate

This was prepared by a method entirely analogous to the glycinyl analogue above, except that the isolation procedure was modified slightly. Thus, the benzene treatment was omitted, and the volume of carbon tetrachloride was increased to 20 ml. Further trituration with hexane (20 ml) and carbon tetrachloride (10 ml) yielded the product as an oil. From 0.79 g of ethyl phosphorodichloridate was isolated 0.49 g (44%) of the title compound. δ_P +12.8; δ_H 4.16(2H, m, $POCH_2$), 3.65(3H, s, OMe), 3.64(1H, s, NH), 3.23(2H, m, NCH_2), 2.55(2H, m, NCH_2CH_2), 1.37(3H, m, CH_3CH_2); δ_C 172.30(C=0), 64.34(d, $POCH_2$, J=6.2 Hz), 51.81(OMe), 37.31(NCH_2), 34.84(d, NCH_2CH_2, J=6.6 Hz), 15.84(d, CH_3CH_2, J=8.0 Hz).

3′ - Azido - 3′ - deoxythymidine - 5′ - (ethyl methoxy - β - alaninyl) phosphoramidate (2b)

This was prepared by an entirely analogous method to compound (2a) above, except that the reaction was stirred for 24 h, and the chromatographic purification was achieved on two successive columns, using eluents of 2% methanol in chloroform, followed by 5% methanol in ethyl acetate. Thus, from 0.1 g of (1) was isolated 0.06 g, (35%) of (2b). δ_P +7.48; δ_H (starred peaks are duplicated due to diastereoisomers) 9.30*(1H, s, N^3H), 7.45*(1H, s, H6), 6.23*(1H, t, H1′), 4.41(1H, m, H3′), 4.03–4.27(5H, m, $POCH_2$, H5′, H4′), 3.71*(3H, s, OMe), 3.53(1H, bs, NH), 3.22(2H, m, NCH_2), 2.55(2H, m, NCH_2CH_2), 2.45(1H, m, H2′), 2.33(1H, m, H2′), 1.94(3H, s, 5-Me), 1.37(3H, t, CH_3CH_2, J=7.0 Hz); EIMS m/e 460.1515(M^+, $C_{16}H_{25}N_6O_8P$ requires 460.1472, 1%), 81($C_5H_5O^+$, base peak); HPLC retention times 23.08, 23.27 min (1:1).

Ethyl methoxy-4-aminobutyryl phosphorochloridate

This was prepared by a method entirely analogous to the glycinyl analogue above, except that the benzene treatment was omitted, and the volume of carbon tetrachloride was increased to 20 ml. Thus from 1.24 g of ethyl phosphorodichloridate was isolated 1.52 g (82%) of the title compound. δ_P +13.8; δ_C 173.48(C=0), 64.04(d, $POCH_2$, J=6.1 Hz), 51.44(OMe), 40.89(NCH_2), 30.75(CH_2CO), 25.69(d, NCH_2CH_2, J=7.9 Hz), 15.71(d, CH_3CH_2, J=8.0 Hz); EIMS m/e 246(MH^+, ^{37}Cl, 21%), 244.0505(MH^+, $C_7H_{16}ClNO_4P$ requires 244.0506, 64), 212(M^+-OMe, 73), 208(M^+-Cl, 23).

3′ - Azido - 3′ - deoxythymidine-5′ - (ethyl methoxy - 4 - aminobutyryl) phosphoramidate (2c)

This was prepared by an entirely analogous method to compound (2b) above, except that the reaction was stirred for 21 h, and the second chromatographic column was eluted with 3% methanol in ethyl acetate. Thus, from 0.11 g of (1) was isolated 0.13 g (66%) of (2c). δ_P +7.75; δ_H (starred peaks are duplicated due to

diastereoisomers) 9.90*(1H, s, N^3H), 7.41*(1H, s, H6), 6.21*(1H, t, H1′), 4.40(1H, m, H3′), 4.00–4.25(5H, m, $POCH_2$, H5′, H4′), 3.65(3H, s, OMe), 3.53(1H, bs, NH), 2.94(2H, m, NCH_2), 2.28–2.42(4H, m, CH_2CO, H2′), 1.90*(3H, d, 5-Me, J=1.0 Hz), 1.82(2H, m, NCH_2CH_2), 1.34(3H, t, CH_3CH_2, J=7.1 Hz); EIMS m/e 474.1566(M^+, $C_{17}H_{27}N_6O_8P$ requires 474.1628, 1%), 81($C_5H_5O^+$, base peak); HPLC retention time 21.39 min.

Ethyl methoxy-5-aminovaleryl phosphorochloridate

This was prepared by a method entirely analogous to the 4-aminobutyryl analogue above. Thus from 0.82 g of ethyl phosphorodichloridate was isolated 1.20 g (92%) of the title compound. δ_P +13.79; δ_H 4.5(1H, bs, NH), 4.16(2H, m, $POCH_2$), 3.60(3H, s, OMe), 2.92(2H, m, NCH_2), 2.29(2H, m, CH_2CO), 1.49–1.63(4H, m, CH_2CH_2CO, NCH_2CH_2), 1.36(3H, t, CH_3CH_2, J=7.0 Hz); δ_C 173.73(C=0), 64.03(d, $POCH_2$, J=6.1 Hz), 51.43(OMe), 41.28(NCH_2), 33.32(CH_2CO), 30.03(d, NCH_2CH_2, J=7.5 Hz), 21.70(CH_2CH_2CO), 15.80(d, CH_3CH_2, J=8.0 Hz); EIMS m/e 260(MH^+, ^{37}Cl, 45%), 258.0662(MH^+, $C_8H_{18}ClNO_4P$ requires 258.0662, 70), 226(M^+-OMe, 67), 222(M^+-Cl, 6).

3′ - Azido - 3′ - deoxythymidine-5′ - (ethyl methoxy - 5 - aminovaleryl) phosphoramidate (2d)

This was prepared by an entirely analogous method to compound (2a) above, except that the reaction was stirred for 19 h, and the chromatographic column was eluted with 2% methanol in chloroform. Thus, from 0.1 g of (1) was isolated 0.13 g (70%) of (2d). Analytical and biological data were obtained following further chromatographic purification on silica, using 2% methanol in ethyl acetate as eluent. δ_P +7.84; δ_H (starred peaks are duplicated due to diastereoisomers) 10.0(1H, bs, N^3H), 7.38*(1H, s, H6), 6.18*(1H, t, H1′, J=6.7 Hz), 4.36(1H, m, H3′), 3.99–4.20(5H, m, $POCH_2$, H5′, H4′), 3.61(3H, s, OMe), 3.42(1H, m, NH), 2.85(2H, m, NCH_2), 2.37(1H, m, H2′), 2.23–2.29(3H, m, CH_2CO, H2′), 1.86(3H, s, 5-Me), 1.61(2H, m, CH_2CH_2CO), 1.48(2H, m, NCH_2CH_2), 1.31(3H, t, CH_3CH_2, J=7.0 Hz); EIMS m/e 488.1733(M^+, $C_{18}H_{29}N_6O_8P$ requires 488.1784, 1%), 81($C_5H_5O^+$, base peak); HPLC retention times 22.83, 22.99 min (3:2).

Ethyl methoxy-6-aminocaproyl phosphorochloridate

This was prepared by a method entirely analogous to the 4-aminobutyryl analogue above. Thus from 1.05 g of ethyl phosphorodichloridate was isolated 1.74 g (99%) of the title compound. δ_P +13.85; δ_H 5.1(1H, bs, NH), 4.17(2H, m, $POCH_2$), 3.60(3H, s, OMe), 2.91(2H, m, NCH_2), 2.26(2H, m, CH_2CO), 1.50(4H, m, CH_2CH_2CO, NCH_2CH_2), 1.30(5H, m, CH_3CH_2, $PNHCH_2CH_2CH_2$); δ_C 173.79(C=0), 63.83(d, $POCH_2$, J=6.1 Hz), 51.22(OMe), 41.37(NCH_2), 33.61(CH_2CO), 30.06(d, NCH_2CH_2, J=7.4 Hz), 25.79($NCH_2CH_2CH_2$), 24.20(CH_2CH_2CO), 15.60(d, CH_3CH_2, J=8.0 Hz); EIMS

m/e 272.0779(MH^+, $C_9H_{20}ClNO_4P$ requires 272.0818, 1), 243(M^+-OMe, ^{37}Cl, 4), 241(M^+-OMe, 16), 129($MeOCO^+[CH_2]_5$, base peak).

3′-Azido-3′-deoxythymidine-5′-(ethyl methoxy-6-aminocaproyl) phosphoramidate (2e)

This was prepared by an entirely analogous method to compound (2c) above, except that the second chromatographic column was eluted with 2% methanol in ethyl acetate. Thus, from 0.094 g of (1) was isolated 0.067 g (38%) of (2e). Analytical and biological data were obtained following further chromatographic purification on silica, using 3.5% ethanol in chloroform as eluent. δ_P +7.83; δ_H (starred peaks are duplicated due to diastereoisomers) 9.61*(1H, s, N^3H), 7.45*(1H, s, H6), 6.24*(1H, t, H1′, J=6.6 Hz), 4.42(1H, m, H3′), 4.04–4.26(5H, m, $POCH_2$, H5′, H4′), 3.67(3H, s, OMe), 3.17(1H, m, NH), 2.91(2H, m, NCH_2), 2.43(1H, m, H2′), 2.28–2.35(3H, m, CH_2CO, H2′), 1.94*(3H, d, 5-Me, J=1.0 Hz), 1.63(2H, m, CH_2CH_2CO), 1.53(2H, m, NCH_2CH_2), 1.31–1.39(5H, m, CH_3CH_2, $CH_2CH_2CH_2NH$); EIMS m/e 502.1893(M^+, $C_{19}H_{31}N_6O_8P$ requires 502.1941, 1%), 81($C_5H_5O^+$, base peak); HPLC retention times 23.21, 23.32 min (1:1).

Ethyl propylamino phosphorochloridate

n-Propylamine (1.30 g, 22 mmol) in anhydrous diethyl ether (40 ml) was added dropwise with vigorous stirring to a solution of ethyl phosphorodichloridate (1.79 g, 11 mmol) in diethyl ether (40 ml) over a period of 3 h at −78°C. The mixture was allowed to warm to room temperature with stirring over 1 h, and was then filtered. The filtrate was concentrated to dryness under reduced pressure, to yield the product as an oil (1.94 g, 95%). δ_P +14.07; δ_H 4.9(1H, bs, NH), 4.14(2H, m, CH_2OP), 2.86(2H, m, CH_2N), 1.51(2H, m, CH_2CH_2N), 1.31(3H, m, CH_3CH_2O), 0.86(3H, m, $CH_3CH_2CH_2$); δ_C 64.05(d, $POCH_2$, J=6.0 Hz), 43.69(CH_2N), 24.02(d, CH_2CH_2N, J=7.8 Hz), 15.86(d, CH_3CH_2O, J=8.0 Hz), 11.19($CH_3CH_2CH_2$); EIMS m/e 187(M^+, ^{37}Cl, 1%), 185.0383(M^+, $C_5H_{13}ClNO_2P$ requires 185.0372, 4), 158(M^+-Et, ^{37}Cl, 32), 156(M^+-Et, 82), 150(M^+-Cl, 2), 130(MH^+-PrNH, ^{37}Cl, 53), 128(MH^+-PrNH, base peak).

3′-Azido-3′-deoxythymidine-5′-(ethyl propylamino) phosphoramidate (3)

This was prepared by an entirely analogous method to compound (2a) above. Thus, from 0.20 g of (1) was isolated 0.21 g, (68%) of (3). δ_P +9.81; δ_H (starred peaks are duplicated due to diastereoisomers) 9.10*(1H, s, N^3H), 7.35*(1H, s, H6), 6.15*(1H, t, H1′), 4.35(1H, m, H3′), 4.20(2H, m, H5′), 4.00–4.10(3H, m, $POCH_2$, H4′), 3.40(1H, m, NH), 2.80(2H, m, NCH_2), 2.40(1H, m, H2′), 2.30(1H, m, H2′), 1.90(3H, s, 5-Me), 1.50(2H, m, NCH_2CH_2), 1.40(3H, t, CH_3CH_2), 0.80(3H, t, $NCH_2CH_2CH_3$); EIMS m/e 416.1521(M^+ $C_{15}H_{25}N_6O_6P$ requires 416.1573, 1%), 81($C_5H_5O^+$, base peak); HPLC retention times 22.39, 22.59 min (3:2).

Biological evaluation

High titre virus stocks of the human immunodeficiency virus HIV-1 (RF strain) were grown in H9 cells with RPMI 1640 (Flow laboratories) supplemented with 10% fetal calf serum, penicillin (100 IU/l) and streptomycin (100 μg/ml). Cell debris was removed by low speed centrifugation, and the supernatant stored at −70°C until required. The target cell used in these assays was the C8166 CD4+ lymphoblastoid cell line. In a typical assay C8166 cells were incubated with 10 TCID50 HIV-1 at 37°C for 90 min and then washed three times with phosphate buffered saline (PBSA, Dulbecco A). Cell aliquots (2×10^5) were resuspended in 1.5 ml growth medium in 6 ml tubes, and compounds in half log dilutions [100 μM to 0.1 μM; 200 μM to 0.1 μM in the case of compound (3)] were added immediately. The nucleoside phosphate triesters were sparingly soluble in aqueous solution, and 10 mM stock solutions of each compound were made up in DMSO. The final DMSO concentration in the tissue culture medium was 1%. The cells were then incubated at 37°C in a 95% air/5% CO_2 incubator. At 72 h post infection 200 μl of supernatant was taken from each culture and assayed for HIV (Kinchington et al., 1989) using an antigen capture ELISA (Coulter, Luton, U.K.). The following controls were used: supernatants taken from uninfected, and infected cells, infected cells treated with AZT (Roche Products U.K. Ltd.), and ddCyd (Roche). The activities of AZT and ddCyd on infected cells consistently gave an ED_{50} of 0.005 and 0.2 μM respectively. The ELISA plates were read with a BioRad spectrophotometer. Compounds were tested in duplicate at each concentration, and each compound was tested on at least two different occasions. To test for compound toxicity, 2×10^5 aliquots of uninfected cells were cultured with the compounds in the same half log dilutions for 72 h. The cells were then washed with PBSA and resuspended in 200 μl of growth medium containing ^{14}C protein hydrolysate. After 12 h the cells were harvested and the ^{14}C incorporation measured. Uninfected, untreated cells were used as controls. The compounds (2a–e, 3) showed a range of activities (Table 2), but none showed toxicity at 100 μM in this system.

TABLE 2
Anti-viral data on novel phosphoramidates

Compound	n[a]	$EC_{50}/\mu M$
2a	1	10
2b	2	20
2c	3	100
2d	4	100
2e	5	100
3	–	50

Shows the anti viral activity (EC_{50} values; the concentration required to reduce HIV antigen production by 50%) for compounds (2a–e) and (3) in the assay system used. None of the compounds showed significant toxicity at 100 μM (14C protein hydrolysate uptake study) except (2c), which showed slight toxicity at 100 μM. The EC_{50} values shown for the phosphoramidates are the average of three different assays; the values for AZT and ddCyd are the average of >10 assays.
[a]Number of methylene groups separating methyl ester from phosphoramidate in structure (2).

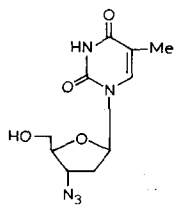
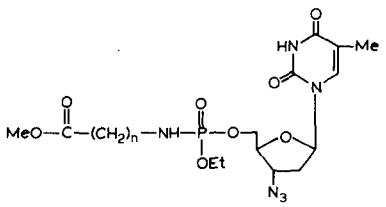


Fig. 1.



(2a) n = 1
(2b) n = 2
(2c) n = 3
(2d) n = 4
(2e) n = 5

Fig. 2.

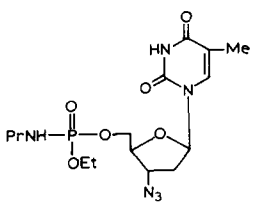


Fig. 3.

Results

A common, 2-stage synthetic procedure was adopted for each of the phosphoramidate compounds (2a–e). In the first step, ethyl phosphorodichloridate was reacted with the methyl ester of the appropriate *w*-amino acid, in the presence of triethylamine. The resulting phosphorochloridates were fully characterised by spectroscopic methods (see experimental section). These materials reacted with AZT in anhydrous THF, containing an excess of *N*-methylimidazole (Van Boom et al., 1975). The products (2a–e) were isolated by precipitation from petrol and column chromatography. In each case, a number of successive column chromatographic purification steps were necessary to produce homogeneous material, using different eluants in each step. In every case, spectral data clearly indicate the presence of two diastereoisomers in the products (2a–e); resulting from mixed stereochemistry at the phosphorus centre. In this study, the crude mixtures of diastereoisomers were

tested for their anti-viral effect; it is quite possible that the separate isomers may differ markedly in their activities. In each case, the products were characterised by ^{31}P nmr, chemical shift values of ca. δ 7.5–7.8 being noted. This compares closely to literature values for similar species (for example $OP(NEt_2)(OEt)_2$ δ_P +9; Mark et al., 1969). In each case, the ^{31}P nmr signals for the two diastereoisomers were coincident. However, in the ^{13}C nmr spectra, many signals for the base, sugar, alkoxy, and amino moieties appeared as two peaks, whose ratios reflected the ratio of the diastereoisomers, which was usually close to 1:1. In some cases, for the resonances of carbon atoms within three bonds of the phosphorus, further splitting was noted, due to phosphorus coupling. Some resonances displayed both diastereomeric splitting, and phosphorus coupling. Carbon-13 nmr data for the products (2a–e) are listed in Table 1. Proton nmr spectra and mass spectra further support the structures of the products (2a–e), the former also confirming the presence, and ratio of, diastereoisomers. The purity of the products was confirmed by HPLC; in particular, AZT levels were below detectable limits. This is important given the high activity of AZT in the biological assay used.

Discussion

The anti-HIV-1 activity of the phosphoramidates (2a–e) was measured in vitro, by methods described previously (Kinchington et al., 1989), the results being summarised in Table 2. Thus, compound (2a) was the most active of the series, causing 50% inhibition of viral replication at a concentration of 10 μM. Compound (2b) was less active, and compounds (2c–e) were active only at high concentrations. The latter three compounds were in fact equi-active in the test system employed, each causing 50% inhibition only at the highest concentration studied (100 μM). It is clear that the anti-HIV activity of the phosphoramidate derivatives declines with increasing separation of the phosphoramidate and carboxyl ester moieties. This is consistent with a mechanism of action involving intracellular cleavage of the P-N bond: increased separation of the carboxyl ester might be expected to stabilise this bond, and thus reduce activity. This cleavage would release AZT ethyl phosphate, which might either act as such, or undergo further hydrolysis to AZT or AZT monophosphate. Both of the latter species at least would be potent anti-HIV agents if released in an intracellular environment.

Given the apparent plateau in the activity of the later members of the series (2c–e) it was considered possible that the presence of the distant ester group was no longer making a contribution to the activity of the phosphoramidates. To test this hypothesis, the unsubstituted propylamino compound (3) was prepared by analogous methodology, and evaluated in vitro. As noted in Table 2 this compound displays anti-HIV activity intermediate in magnitude between that of (2b) and (2c–e). The surprisingly high activity of (3) clearly suggests that the remote ester group in (2c–e) does not contribute to their activity. Indeed, the activity is higher in the absence of the ester group. The reasons for this are unclear; however, it is interesting to note that a simple alkylamino group attached to the phosphorus

(as in [3]) does confer anti-HIV activity, whereas a simple alkyloxy group in this position (as in a nucleoside dialkyl phosphate) does not (McGuigan et al., 1990a). Lastly, the activity of, particularly (2b), indicates that a natural alpha-amino acid moiety is not required for activity. This indicates that specific hydrolysis by HIV aspartate proteinase (Navia et al., 1989), the original rationale for preparing amino acid derivatives, is unlikely to be the sole mode of activation of these pro-drugs. Further studies are underway to probe the mechanism of action of these compounds.

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