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Synthesis and biological evaluation of some phosphate triester derivatives of the anti-viral drug AraA

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ABSTRACT

A number of novel phosphate triester derivatives of the anti-viral nucleoside analogue araA have been prepared by a rapid 2-step procedure, not necessitating prior sugar protection. Spectroscopic and lipophilicity data have been collected on these compounds, and they have been assayed with a range of hydrolytic enzymes. The compounds have been found to be highly resistant to hydrolysis at physiological pH, enzymatic or otherwise. An in vitro assay indicated inhibition of DNA synthesis by mammalian cells, by each of these compounds, in the range 3−300μM. Moreover, the degree of inhibition showed a close correlation to chemical structure; in particular, there was a direct relationship between inhibition of thymidine incorporation and lipophilicity. These results suggest cellular penetration by the phosphate triesters and intracellular hydrolysis, by an unspecified mechanism, to the free nucleotide or nucleoside.

INTRODUCTION

The nucleoside analogue araA[1], 9-β-D-arabinofuranosyladenine (1) has found widespread use in the treatment of a number of viral infections, including herpes simplex encephalitis and disseminated herpes zoster[2]. However, as with most nucleoside analogues, araA suffers from a number of limitations. Firstly, it is subject to rapid enzymatic deactivation; this corresponds to adenosine deaminase mediated conversion to arahypoxanthine[3] (araHx). In an effort to overcome this major problem, potent inhibitors of adenosine deaminase have been sought, and found[4]. Indeed, co-administration of araA and an adenosine deaminase inhibitor greatly increases the plasma half-life of the anti-viral drug, and may increase its therapeutic effect[5]. However, the long term inhibition of crucial metabolic enzymes, such as adenosine deaminase, may have toxic side-effects[6]. A better alternative might be to search for deamination resistant derivatives of araA. Since the major substrate requirement of adenosine deaminase is a free 5′-hydroxyl group[7], it is not surprising in this context that many 5′-modified adenosine nucleosides have been prepared[8]. Indeed, 5′-esters of araA seem promising as deamination resistant transport forms of araA[9]. However, they do not solve the second problem of araA, one which it shares with almost all nucleoside analogues. This is a dependence on kinase mediated activation to the 5′-phosphate forms. Thus, not only is araA known to act as its nucleotide(s)[10], but so are the clinical anti-viral agents acycloguanosine[11], 5-iodo-2′-deoxyuridine (IDU)[12], and 3′-azido-2′,3′-dideoxythymidine (AZT)[13], and the anti-cancer agents 5-fluorouracil (5-FU)[14], 5-fluoro-2′-deoxyuridine (FUDR)[15], 6-mercaptopurine (6-MP)[16], and 6-thioguanine (6-TG)[17], and numerous other clinical and experimental drugs[18]. In some cases a dependence on (viral coded) kinases is

advantageous, since it leads to enhanced anti-viral selectivity[11], but in most cases it is deleterious. There are now many reports of the absence, low activity, or deletion, of the appropriate kinase being correlated with poor clinical response[19]. This has prompted the investigation of the bio-active nucleotides as chemotherapeutic agents in their own right. However, little, if any, clinical benefit arises from the use of the pre-formed nucleotide, by comparison to the nucleoside[20]. This is commonly attributed to the poor membrane penetration of the (charged) nucleotide, and the rapid extracellular cleavage to the corresponding nucleoside[21]. There have now been a number of reports detailing the use of uncharged, phosphate triester derivatives of araA[22], and other chemotherapeutic nucleoside analogues[23], as possible membrane soluble pro-drugs of the nucleotides. In the present study a number of simple dialkyl phosphate triester derivatives of araA have been prepared by a rapid 2-step procedure, and have been evaluated by enzymatic and biological methods. In particular a correlation was sought between phosphate structure and biological activity.

RESULTS AND DISCUSSION

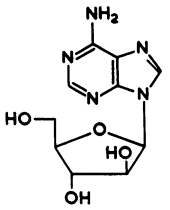
Diethyl, dipropyl, dibutyl, and dipentyl phosphorochloridates were prepared by the reaction of the appropriate alcohol and phosphoryl chloride, in the presence of triethylamine[24]. These phosphorylating agents were fully characterised by analytical and spectroscopic data. Diethyl phosphorochloridate reacted with adenosine in dry pyridine to give the model compound adenosine-5′ diethyl phosphate (2)[25] in moderate yield after chromatographic purification. This was fully characterised by microanalysis and spectroscopy. The ^{13}C nmr was of particular interest; phosphorus coupling was observed to the methylene and methyl carbons of the ethyl moiety, and to the 5′- and 4′-carbons of the sugar; no longer range phosphorus coupling was observed. The target araA analogue (3) was prepared by an identical method, and displayed similar spectra to (2), but there was a number of differences. For example, electron impact mass spectrometry gave MH^+ and M^+ for (2), but only fragments such as M^+-H_2O for (3). The structures of each of these fragments were confirmed by exact mass measurement. The molecular ion of (3) was observed using fast atom bombardment mass spectrometry (FAB MS). Another marked difference between (2) and (3) was in the multiplicity of the ^{13}C nmr signals at approximately δ65.6, assigned to the methylene carbons of the ethyl groups. In (2) a simple doublet was observed, the splitting corresponding to 2-bond phosphorus coupling (J=6Hz). A similar situation had prevailed for the phosphorylating reagent, diethyl phosphorochloridate. However, in (3) this signal appeared as a pair of doublets at 100MHz. This is attributed to the non-equivalence of the two ethyl chains, which are diastereotopic; presumably their resonances being coincident in (2).

In an entirely analogous manner, dipropyl phosphorochloridate was reacted with adenosine to give (4), and with araA to give (5). For both of these nucleotide triesters, and for the phosphorylating agent, ^{13}C nmr revealed 2- and 3-bond phosphorus coupling, but no longer range coupling. Other spectral and analytical data fully confirmed the structure and purity of these materials. Again, the ^{13}C nmr data showed the non-equivalence of the two alkyl chains for the araA compound (5).

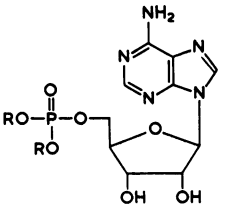
The butyl compounds (6) and (7), and the pentyl analogues (8) and (9), were similarly prepared and characterised.

A comparison of the various nucleotide triesters reveals several interesting trends in the ^{31}P nmr chemical shifts. Thus, the propyl, butyl, and pentyl adenosines (4), (6), and

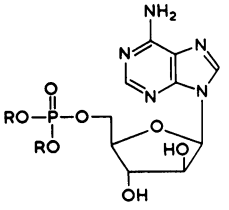
Scheme



(1)



(2) R = Et
(4) R = n-Pr
(6) R = n-Bu
(8) R = n-Pent



(3) R = Et
(5) R = n-Pr
(7) R = n-Bu
(9) R = n-Pent

(8) all resonate at approximately −0.66 ppm, whilst the analogous araA compounds (5), (7), and (9) all occur at −0.54 ppm. No dependence on alkyl chain length is noted here, although a dependence on sugar (2′-OH configuration) is evident. However, in each series the ethyl compound is anomalous; (2) resonates at −0.8 ppm, and (3) at −0.7 ppm. It is notable that in every case, the araA analogue occurs 0.1 ppm downfield of the adenosine compound.

In several of the reactions of phosphorylating agents with nucleosides, chromatographically faster running (more hydrophobic) by-products were isolated during purification procedures. In the case of the reaction to produce (4), this by-product was characterised as consisting of mixed diphosphorylated adenosines [e.g. adenosine-3′,5′-bis(dipropyl phosphate)], by ^{31}P nmr and microanalysis. In all cases these were minor products, and they were easily separable from the intended, 5′-monophosphorylated materials.

It was envisaged that the triester derivatives would increase in lipophilicity with lengthening alkyl chain and, moreover, that some correlation might exist between lipophilicity (P) and biological activity. Therefore, octanol/water partition coefficients were measured for each of the araA derivatives (3), (5), (7) and (9), and for araA (1), by uv analysis of the separated layers; each analysis being run in triplicate. The ethyl derivative (3) is some 8 times more lipophilic than araA, this value increasing to almost 250 for the pentyl compound (9)[26]. Thus, passive diffusion of the nucleotide triesters through cell membranes should be greatly facilitated.

As anticipated, the phosphate triesters were all entirely resistant to adenosine deaminase, as followed by uv spectroscopy under standard conditions[27]. Moreover, unlike free 5′-phosphates such as araAMP, they were entirely resistant to adenylic acid deaminase.

Thus, lipophilic, deactivation-resistant derivatives of araA had been successfully prepared. However, it was unclear as to whether these could act as intracellular sources of araA or, preferably, araAMP. Being simple phosphate triesters, it was not expected that they would undergo rapid chemical hydrolysis at physiological pH and temperature. Indeed, no decomposition of (5) was detected by tlc or ^{31}P nmr after 1 week at 37 °C, pH 7.4. To test the possibility of enzyme mediated hydrolysis of the phosphate moiety, the compounds were incubated under standard conditions with phosphodiesterase I (crude snake venom), alkaline phosphatase, and lipase. In no case was any enzyme mediated hydrolysis

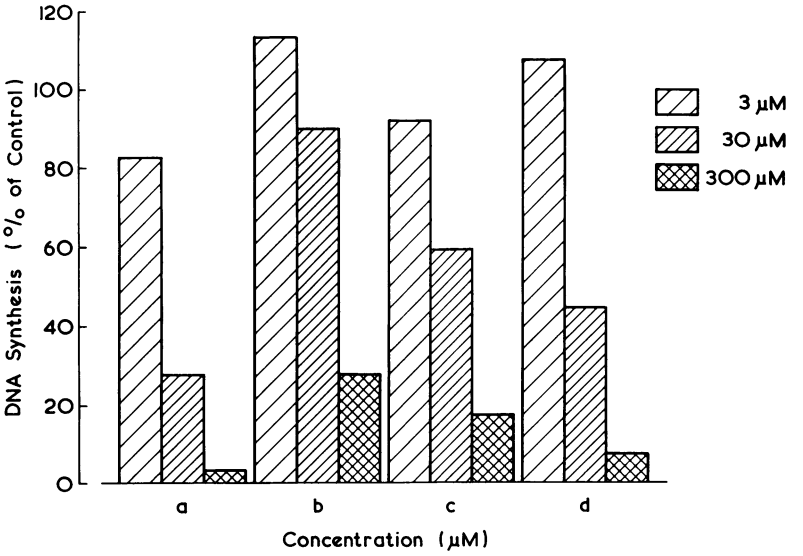


Figure 1. Inhibition of DNA synthesis by 1 (a), 3 (b), 5 (c), and 7 (d), with 1 h incubation of cells and compounds; for full details see experimental.

noted, by tlc or ^{31}P nmr. In fact *no* hydrolysis was noted in any case, except with alkaline phosphatase. Here, the alkaline pH of the medium (10.4) led to a slow hydrolysis of the triesters. However, no enhancement in rate was noted in the presence of enzyme as compared to the control. Thus, the means by which the triesters might be 'activated' within the cell remained uncertain, and indeed, it seemed likely that they would exhibit only low biological activity.

The biological effects of the araA derivatives were tested on a mammalian epithelial cell line (CNCM I221) grown in layer culture, employing a thymidine incorporation assay. The incorporation of extracellular radiolabelled thymidine into acid-insoluble material was estimated in replicate cultures of cells exposed to various concentrations of compounds 1, 3, 5, 7, and 9. Each experiment was performed at least three times, araA being included as a positive control in every experiment. The results are presented in Figure (1).

Thus, it is clear that the triesters are, by some means, exerting a biological effect. Moreover, the degree of inhibition of thymidine incorporation is profoundly dependent on the structure of the phosphate moiety. In particular, there is a clear correlation between degree of inhibition and lipophilicity; at each concentration studied activity rises with increasing P value, (Figure 2). This is strongly indicative of membrane penetration being an important step in the biological action of these triesters, and is taken as evidence against extracellular cleavage of the triesters to the free nucleoside. Of course, such data do not distinguish between intracellular release of the nucleotide, or nucleoside, or direct action by the triesters; further experiments to probe this important question are in progress.

Finally, since the primary use of araA is as an anti-viral drug, it was of interest to investigate whether the triesters had enhanced anti-viral activity and/or selectivity, by comparison with araA. Each of the compounds (2) to (9) was tested against a range of viruses[28] in tissue culture, but in no case was any anti-viral activity (or toxicity) noted

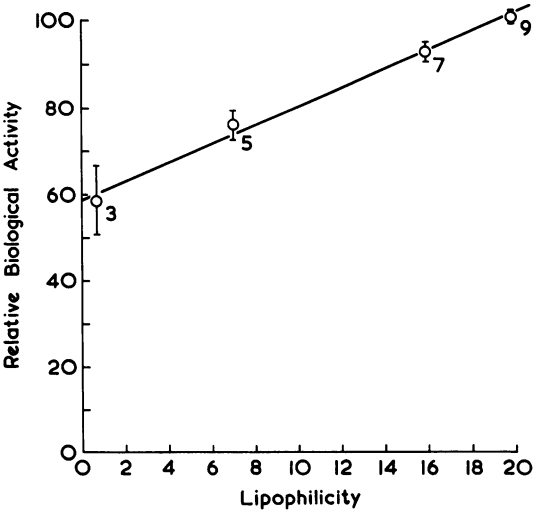


Figure 2. A plot of inhibition of DNA synthesis by compounds 3, 5, 7, and 9 (compound 1 = 100%) at 150μM and 30 min incubation, against octanol−water partition coefficients for the derivatives.

at concentrations up to 100μg/ml. It is surprising that no cytotoxicity was found in these latter tests, given our earlier observations using tritiated thymidine incorporation. This may be due to the lower sensitivity of the plaque reduction assay used in the anti-viral tests.

In conclusion, simple phosphate triesters of araA appear to inhibit DNA synthesis *in vitro*, their efficacy increasing with their lipophilicity. This finding may be of great importance in the chemotherapy of viral and other infections, if the phenomenon is a general one, and if intracellular delivery of the nucleotides is being achieved. These are amongst the questions we are currently addressing.

EXPERIMENTAL

All reactions were carried out under scrupulously dry conditions unless otherwise indicated. Pyridine was dried by distillation at atmospheric pressure from calcium hydride onto activated 4Å molecular sieves. Triethylamine was dried by distillation at atmospheric pressure from calcium hydride. Phosphoryl chloride was purified by distillation at atmospheric pressure. 'Anhydrous' diethyl ether was further dried over activated 4Å molecular sieves. Ethanol was dried over 3Å molecular sieves, and higher alcohols over 4Å sieves.

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.

Proton nmr spectra were recorded on a Varian XL200 spectrometer operating at 200MHz. ^{13}C nmr spectra were obtained on this instrument, operating at 50MHz, or on a Varian VXR400 operating at 100 MHz. ^{31}P spectra were obtained on the former instrument, at 82MHz. Proton spectra were referenced to TMS or sodium 3-(trimethylsilyl)-1-propanesulphonic acid, phosphorus spectra to 85% phosphoric acid, and carbon spectra to methanol; positive shifts are downfield of the reference. Mass spectra were recorded on a VG7070H spectrometer, courtesy of Dr.M.Mruzek (EIMS), or on a VG Zab1F, courtesy of the University of London Mass Spectrometry Service (FAB MS).

UV spectra were recorded on a Perkin Elmer 554 spectrophotometer, fitted with a Perkin Elmer temperature controller. Microanalyses were performed at UCL, courtesy of Mr.A.T.T.Stones. Nucleotide triesters were noted to be hygroscopic, and correct analytical data were frequently not obtained for anhydrous formulae.

Adenosine-5'-diethyl phosphate (2).[25]

Adenosine (2.00g, 7.48mmol) was dissolved in pyridine (120ml), and diethyl phosphorochloridate[29] (2.16ml, 2.58g, 15.0mmol) added dropwise with vigorous stirring. After stirring for 17h at ambient temperature, the solvent was removed under reduced pressure, last traces being co-evaporated with toluene (3×20ml). The resulting oil was treated with diethyl ether (3×50ml), dried in vacuum, and purified by flash column chromatography on silica. Elution with 20% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave a product which was pure by spectroscopy (1.08g, 36%). Analytical data were obtained on a small sample subjected to further silica column chromatography (eluant: 10% methanol in chloroform). ^{1}H nmr δ(D_2O) 8.31(1H, s, H8), 8.25(1H, s, H2), 6.13(1H, d, H1', J=4Hz), 4.90(1H, t, H2'), 4.62(1H, t, H3'), 4.32−4.41(3H, m, H4', 2H5'), 4.02(4H, m, CH_3CH_2), 1.22(6H, m, CH_3); ^{31}P nmr δ(CH_3OD) −0.797; ^{13}C nmr δ(CD_3OD) 153.96(s, C2), 150.63(s, C4), 141.06(s, C8), 90.24(s, C1'), 84.06(d, C4', J=7.6Hz), 75.22(s, C2'), 71.44(s, C3'), 68.02(d, C5', J=5.7Hz), 65.63(d, CH_2OP, J=6Hz), 16.34(d, CH_3, J=6.5Hz); EIMS m/e 404.1306 (MH^+, 0.03%, calc. for $C_{14}H_{23}N_5O_7P$ 404.1335), 403.1329(M^+, 0.01, calc. for $C_{14}H_{22}N_5O_7P$ 403.1257); Found C 41.56%, H 5.49, N, 17.15, P 7.71; $C_{14}H_{22}N_5O_7P$ requires C 41.69, H 5.50, N 17.36, P 7.68.

9-β-D-Arabinofuranosyladenine-5'-diethyl phosphate (3).

9-β-D-Arabinofuranosyladenine (0.5g, 1.87mmol) was dissolved in pyridine (60ml), and diethyl phosphorochloridate (0.54ml, 0.645g, 3.74mmol) added dropwise with vigorous stirring. After stirring for 2h at ambient temperature, the solvent was removed under reduced pressure and the residue triturated with diethyl ether (4×20ml). The resulting oil was dried in vacuum, and purified by flash column chromatography on silica. Elution with 10% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave the product (0.43g, 57%). ^{1}H nmr δ(CD_3OD) 8.34(1H, s, H2), 8.22(1H, s, H8), 6.46(1H, d, H1', J=4Hz), 3.80−4.50(5H, m, H2', H3', H4', H5'), 4.13(2H, quintet, CH_3CH_2, J=7Hz),4.12(2H, quintet, CH_3CH_2, J=7Hz), 1.31(6H, t, CH_3, J=7Hz); ^{31}P nmr δ(CH_3OD) −0.703; ^{13}C nmr δ(CD_3OD) 156.80(s, C6), 153.18(s, C2), 150.42(s, C4), 142.66(s, C8), 119.57(s, C5), 86.48(s, C1'), 84.04(d, C4', J=7.3Hz), 77.22(s, C2'or C3'), 76.92(s, C3'or C2'), 68.18(d, C5', J=5.5Hz), 65.58(m, CH_2OP), 16.38(d, CH_3, J=6.7Hz); EIMS m/e 385.1227($M^+ - H_2O$, calc. for $C_{14}H_{20}N_5O_6P$ 385.1151); FAB MS m/e 405(M^+H_2, 17%), 404(MH^+, 72); Found (from water/methanol) C 39.35%, H 5.64, N 16.26, P 7.31; $C_{14}H_{22}N_5O_7P[H_2O]_{1.5}$ requires C 39.07, H 5.86, N 16.27, P 7.20.

Adenosine-5'-dipropyl phosphate (4).

Adenosine (1.00g, 3.74mmol) was dissolved in pyridine (50ml), and dipropyl phosphorochloridate[30] (1.5g, 7.48mmol) added dropwise with vigorous stirring. After stirring for 17h at ambient temperature, water was added (135μl, 7.48mmol), and the solvent was removed under reduced pressure. The resulting oil was treated with diethyl ether (3×50ml), dried in vacuum, and purified by flash column chromatography on silica. Elution with 20% methanol in dichloromethane, followed by pooling and evaporation of appropriate fractions gave two separate products: Product 1: ^{31}P nmr δ(CH_3OD) −1.4, −1.5, −1.8, −1.9; Found H 6.51%, N 11.82, P 10.78; $C_{22}H_{39}N_5O_{10}P_2$ requires H 6.60, N 11.76,

P 10.40. Product 2: Adenosine 5′-dipropyl phosphate (4) (0.5g, 31%) ^{1}H nmr δ(D_2O) 8.46(1H, s, H2 or H8), 8.41(1H, s, H8 or H2), 6.18(1H, d, H1′, J=4Hz), 4.00−4.85(5H, m, H2′, H3′, H4′, H5′), 3.97(4H, q, CH_2OP, J=6.6Hz), 1.52−1.69(4H, m, CH_3CH_2), 0.86(3H, t, CH_3, J=7.3Hz), 0.85(3, t, CH_3, J=7.3Hz); ^{31}P nmr δ(CH_3OD) −0.650; EIMS m/e 431.1589 (M^+, calc. for $C_{16}H_{26}N_5O_7P$ 431.1570); Found C 44.07%, H 6.25, N 15.68, P 7.12; $C_{16}H_{26}N_5O_7P[H_2O]_{0.4}$ requires C 43.82, H 6.16, N 15.97, P 7.06.

9-β-D-Arabinofuranosyladenine-5′-dipropyl phosphate (5).

This was prepared in an entirely analogous manner to (4) above. Thus, from 1.0g araA, was isolated 1.10g, (68%) of (5). ^{1}H nmr δ(CD_3OD) 8.32(1H, s, H2), 8.21(1H, s, H8), 6.46(1H, d, H1′, J=4Hz), 4.08−4.50(5H, m, H2′, H3′, H4′, H5′), 4.02(2H, q, CH_2OP, J=7Hz), 4.01(2H, q, CH_2OP, J=7Hz), 1.68(4H, sextet, CH_3CH_2, J=7Hz), 0.94(3H, t, CH_3, J=7Hz), 0.93(3H, t, CH_3, J=7Hz); ^{31}P nmr δ(CH_3OD) −0.543; ^{13}C nmr δ(CD_3OD) 157.22(s, C6), 153.80(s, C2), 150.49(s, C4), 142.53(s, C8), 119.58(s, C5), 86.39(s, C1′), 84.00(d, C4′, J=7Hz), 77.25(s, C2′or C3′), 76.95(s, C3′or C2′), 70.97(m, CH_2OP), 68.26(d, C5′, J=5.7Hz), 24.62(d, CH_3CH_2, J=7.1Hz), 10.29(s, CH_3); EIMS m/e 432.1616(MH^+, 0.4%, calc. for $C_{16}H_{27}N_5O_7P$ 432.1648), 431.1572(M^+, 0.04, calc. for $C_{16}H_{26}N_5O_7P$ 431.1570); Found C 43.70%, H 6.22, N 15.75, P 7.03; $C_{16}H_{26}N_5O_7P[H_2O]_{0.5}$ requires C 43.64, H 6.18, N 15.90, P 7.03.

Adenosine-5′-dibutyl phosphate (6).

Adenosine (1.00g, 3.74mmol) was dissolved in pyridine (60ml), and dibutyl phosphorochloridate[31] (1.71g, 7.48mmol) added dropwise with vigorous stirring. After stirring for 2h at ambient temperature, the solvent was removed under reduced pressure, last traces being co-evaporated with toluene (3×20ml). The resulting oil was treated with diethyl ether (2×30ml), dried in vacuum, and purified by flash column chromatography on silica. Elution with 20% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave material which was pure by spectroscopic analysis (1.02g, 59%). Analytical data were obtained on a small sample subjected to a second chromatographic column (eluant 5% methanol in chloroform). ^{1}H nmr δ(D_2O) 8.34(1H, s, H2 or H8), 8.29(1H, s, H8 or H2), 6.13(1H, d, H1′, J=4Hz), 4.92(1H, t, H2′, J=5Hz), 4.61(1H, t, H3′, J=5Hz), 4.30−4.40(3H, m, H4′, H5′), 3.88−4.00(4H, m, CH_2OP), 1.37−1.72(4H, m, CH_2CH_2OP), 1.20−1.37(4H, m, CH_3CH_2), 0.81(6H, t, CH_3, J=7Hz); ^{31}P nmr δ(CH_3OD) −0.663; ^{13}C nmr δ(CH_3OD) 155.79(s, C6), 152.16(s, C2), 149.56(s, C4), 140.41(s, C8), 119.54(s, C5), 89.33(s, C1′), 83.17(d, C4′, J=8.1Hz), 74.35(s, C2′), 70.53(s, C3′), 68.27(d, CH_2OP, J=6.1Hz), 67.21(d, C5′, J=5.5Hz), 32.34(d, CH_2CH_2OP, J=6.7Hz), 18.72(s, CH_3CH_2), 12.99(s, CH_3); EIMS m/e 460.1975 (MH^+, calc. for $C_{18}H_{31}N_5O_7P$ 460.1961, 0.2%), 459.2041(M^+, calc. for $C_{18}H_{30}N_5O_7P$ 459.1883, 0.24); Found C 46.44%, H 6.53, N 14.79, P 6.61; $C_{18}H_{30}N_5O_7P[H_2O]_{0.5}$ requires C 46.15, H 6.67, N 14.95, P 6.61.

9-β-D-Arabinofuranosyladenine-5′-dibutyl phosphate (7).

This was prepared in an entirely analogous manner to (6) above. Thus, from 1.0g araA, was isolated 1.15g, (67%) of (7); in this case only one chromatographic column was required. ^{1}H nmr δ(D_2O) 8.45(1H, s, H8 or H2), 8.40(1H, s, H2 or H8), 6.50(1H, d, H1′), 4.70(1H, m, H2′), 4.20−4.50(8H, m, H3′, H4′, H5′, CH_2OP), 1.60(4H, m,CH_2CH_2OP), 1.30(4H, m, CH_3CH_2), 0.80(6H, t, CH_3); ^{31}P nmr δ(CH_3OD) −0.536; ^{13}C nmr δ(CH_3OD) 154.59(s, C6), 150.27(s, C2), 149.46(s, C4), 142.52(s, C8), 118.76(s, C5), 85.71(s, C1′), 83.29(d, C4′, J=6.7Hz), 76.35(s, C2′or C3′), 76.13(s, C3′or C2′), 68.32(m, CH_2OP, J=4.7Hz), 67.41(d, C5′, J=5.4Hz), 32.44(d,

CH_2CH_2OP, J=6.1 Hz), 18.83(s, CH_3CH_2), 13.00(s, CH_3); FAB MS m/e 461(M^+H_2, 16%), 460(MH^+, 57); Found (from water) C 47.27%, H 6.48, N 15.34, P 6.75; $C_{18}H_{30}N_5O_7P$ requires C 47.06, H 6.58, N 15.24, P 6.74.

Adenosine-5'-dipentyl phosphate (8). This was prepared by an entirely analogous method to (4) above, except using dipentyl phosphorochloridate[32], and that a column eluant of 5% methanol in chloroform was used in the purification step. From 1.0g of adenosine, was isolated 1.37g (75%) of (8).

^1H nmr δ($CDCl_3$) 8.16(2H, s, H2, H8), 6.62(2H, sb, NH_2), 6.12(1H, d, H1', J=5Hz), 4.72(1H, t, H2'), 4.50(1H, t, H3'), 4.39(1H, m, H4'), 4.30(2H, m, H5'), 3.99(4H, quintet, CH_2OP, J=7Hz), 2.5(2H, sb, OH), 1.56−1.67(4H, m, CH_2CH_2OP), 1.19−1.34(8H, m, $CH_3CH_2CH_2$), 0.87(3H, t, CH_3, J=7Hz), 0.81(3H, t, CH_3, J=7Hz); ^{31}P nmr δ(CH_3OD) −0.670; ^{13}C nmr δ(CH_3OD) 156.36(s, C6), 152.92(s, C2), 149.73(s, C4), 140.29(s, C8), 119.63(s, C5), 89.37(s, C1'), 83.22(d, C4', J=7.8Hz), 74.28(s, C2'), 70.55(s, C3'), 68.63(d, CH_2OP, J=4.1Hz), 67.27(d, C5', J=5.7Hz), 30.09(d, CH_2CH_2OP, J=6.7Hz), 27.80(s, $CH_3CH_2CH_2$), 22.35(s, CH_3CH_2), 13.42(s, CH_3); FAB MS m/e 489 (MH_2^+, 13%), 488(MH^+, 40); Found C 48.67%, H 7.05, N 13.98, P 6.51; $C_{20}H_{34}N_5O_7P[H_2O]_{0.5}$ requires C 48.38, H 7.11, N 14.11, P 6.24.

9-β-D-Arabinofuranosyladenine-5'-dipentyl phosphate (9).

This was prepared in an entirely analogous manner to (8) above. Thus, from 1.0g araA, was isolated 1.11g, (61%) of (9). ^1H nmr δ($CDCl_3$) 8.27(1H, s, H8 or H2), 8.08(1H, s, H2 or H8), 6.42(1H, d, H1', J=5Hz), 6.35(2H, sb, NH_2), 4.3−4.6(3H, m, H2', H3', H4'), 3.93−4.13(6H, m, H5', CH_2OP), 1.70(2H, m, CH_2CH_2OP), 1.58(2H, m, CH_2CH_2OP), 1.36(4H, m, $CH_3CH_2CH_2$), 1.22(4H, m, CH_3CH_2), 0.91(3H, t, CH_3), 0.78(3H, t, CH_3); ^{31}P nmr δ(CH_3OD) −0.543; ^{13}C nmr δ(CH_3OD) 156.08(s, C6), 152.60(s, C2), 149.56(s, C4), 141.77(s, C8), 118.66(s, C5), 85.36(s, C1'), 82.98(d, C4', J=7.2Hz), 76.28(s, C2'or C3'), 76.04(s, C3'or C2'), 68.59(m, CH_2OP), 67.38(d, C5', J=5.6Hz), 30.05(d, CH_2CH_2OP, J=6.8 Hz), 27.78(s, $CH_3CH_2CH_2$), 22.31(s, CH_3CH_2), 13.44(s, CH_3); FAB MS m/e 489 (MH_2^+, 16%), 488(MH^+, 54); Found C 49.21%, H 7.06, N 14.07, P 6.59; $C_{20}H_{34}N_5O_7P$ requires C 49.28, H 7.03, N 14.37, P 6.35.

Adenosine deaminase assay.

A stock solution of adenosine deaminase (type II, from calf intestinal mucosa, Sigma, London) was prepared by dissolving 0.50 mg of solid in 1.0ml of pH 7.5, 0.05M phosphate buffer. Stock solutions of 1, 3, 5, 7, and 9, were similarly prepared; each of 44μM concentration. For each assay 2.90ml of sample solution was placed in a clean, dry 1 cm UV cell, thermostatted at 25°C, and a UV spectrum recorded over the range 300 to 220nm. A portion of enzyme solution (100μl) was added, and spectra recorded after 1 min, and at 1 min intervals thereafter, for 10−20 min. Under these conditions, complete deamination of (1) was noted in ca. 20 min. In cases of no apparent reaction, a further portion of enzyme solution (500μl) was added, and spectra recorded for a further 20 min. Compounds (3), (5), (7) and (9) were not deaminated.

Adenylic acid deaminase assay.

The procedure used was similar to that detailed above for adenosine deaminase. Adenylic acid deaminase (from rabbit muscle, Sigma, London) was diluted with 1M potassium chloride solution to give a final enzyme concentration of 2.5 enzyme units/ml. For each assay, a portion of this solution (10μl) was added to a sample (2.5ml) of nucleoside solution (45.2μM) in pH 6.5, 0.01M citrate buffer at 25°C. Spectra were recorded as above, and a further portion of enzyme solution (100μl) added as required. Under these conditions,

adenosine-5′-monophosphate was completely deaminated in ca. 25 min; compounds (3), (5), (7) and (9) were not deaminated.

Phosphodiesterase I assay.

Phosphodiesterase I (*Crotalus atrox*, crude snake venom, Sigma, London) was dissolved in 0.1M, pH 8.8 glycine buffer, to give a final enzyme concentration of 2.7 units/ml. A control assay was performed by adding a portion of this solution (10μl) to a sample of bis(p-nitrophenyl)phosphate (0.42mg), dissolved in glycine buffer (2.0ml), at 37°C and recording uv spectra every 3 min, over the wavelength range 500−350nm. For compounds 1, 3, 5, 7, and 9, similar solutions were used, but the reaction was followed by tlc. In cases where no reaction could be discerned after 43h, a further portion of enzyme solution (100μl) was added.

Alkaline phosphatase assay.

Each of the compounds 1, 3, 5, 7, and 9 was dissolved in water (1.25ml) to give a concentration of 5.066mM. To each sample was added D_2O (0.25ml), and pH 10.4 glycine (0.1M) / $MgCl_2$ (1mM) buffer (1.5ml). The samples were equilibrated at 37°C, and proton decoupled ^{31}P nmr spectra recorded. Alkaline phosphatase (from *E.coli*, type III-N, 127units/ml, Sigma, London) was added (5μl), and ^{31}P nmr spectra recorded at suitable intervals, over a period of 2d.

Lipase assay.

Lipase (type VII, from *Candida cylindracea*, Sigma, London) (10mg) was dissolved in water (0.5ml) to give an enzyme concentation of 10000units/ml.

Samples of olive oil and compounds 3, 5, 7, and 9 were dissolved in pH 7.7, 0.2 M tris(hydroxymethyl)aminomethane/HCl buffer (2ml), to give a final concentration of 0.85mM, and to each was added a portion of enzyme solution (50μl), and the solutions incubated at 37°C. Aliquots were removed at appropriate intervals over a period of 4d, and examined by tlc.

Tritiated Thymidine Incorporation Assay.

The cells employed for the testing of the compounds were a line of mammalian epithelial cells, CNCM I221. These cells were routinely cultivated in polystyrene flasks (Falcon) in growth medium consisting of minimum essential medium (Eagle with Earle's salts, Flow Labs, Ltd.), supplemented with foetal bovine serum (10%, Imperial Labs. Ltd.), penicillin (100 units per ml) and streptomycin (100μg per ml). The medium was buffered with 20mM HEPES, and cells were grown in a 37°C incubator, with a 2% CO_2 atmosphere. Cells were routinely subcultured at weekly intervals using trypsin, and with a seeding density of 2×10^3 cells per ml. All experiments were carried out on passage numbers 23 to 30, which were screened for mycoplasma contamination, both by autoradiography and broth culture. Experiments were made in multiwell trays, each well being seeded with 5×10^4 cells in 1ml of growth medium. Only the inner eight wells of the 24 were used; the outer 16 wells being filled with 1ml of serum-free medium. The cells were incubated for 48 h before testing. Solutions of the compounds to be tested were made up at appropriate concentrations, filter sterilised and added to the cells. Mixing was achieved by gentle rocking of the multiwell plate. At the end of the incubation time with the agent (30 or 60 min.) a solution of [methyl^{3}H]thymidine (50μl, 20μCi per ml) was added to each well. The radiothymidine (specific activity 925GBq per mmol, Amersham International plc) was diluted in sterile distilled water prior to use. Mixing was achieved by gentle rocking of the dish. After the incubation time of 30 min the cells were washed with phosphate buffered saline, fixed in 5% trichloroacetic acid (1ml per well) for 30 min at 4°C, washed with

phosphate buffered saline, drained and dried under a stream of warm air. The acid insoluble material was dissolved in sodium hydroxide (1M, 250μl per well) by overnight digestion at 37 °C. Aliquots (100μl) of the digests were placed in BBOT scintillator solution (5ml), composed of 2,5-bis(5′-t-butylbenzoxazolyl-[2′])thiophene (BBOT, 4g/l), 2-methoxyethanol (400ml/l), toluene (sulphur free, 600ml/l, and napthalene (80g/l), and counted in an Intertechnique ABAC SL 40 scintillation counter. Aliquots (100μl) of the residues of the digests were pooled for each set of 4 wells and the absorbance at 280nm read in a UV spectrophotometer. This was used as an estimate of the cell numbers in each experiment. The results of the assay were expressed as mean cpm, for each set of 4 replicate wells. From these data the mean percentage inhibition of thymidine incorporation into acid insoluble material was expressed, with respect to the water control.

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REFERENCES

1. W.W. Lee, A. Benitez, L. Goodman, and B.R. Baker, *J.Am.Chem.Soc.*, 1960, 82, 2648.
2. T.W. North, and S.S. Cohen, *J.Pharmacol.Therap.*, 1979, 69, 1.
3. Y.J. Bryson, and J.D. Connor, *Antimicrob.Agents Chemother.*, 1976, 9, 540; T.H. Haskell, *Ann.N.Y.Acad.Sci.*, 1977, 284, 81.
4. S. Cha, *Biochem.Pharmacol.*, 1976, 25, 2695; H.J. Schaeffer and D.F. Schwender, *J.Med.Chem.*, 1974, 17, 6.
5. R.P. Agarwal, S. Cha, G.W. Crabtree, and R.E. Parks, in Chemistry and Biology of Nucleosides and Nucleotides, ed. R.E. Harmon, R.K. Robins, and L.B. Townsend, Academic press, New York and London, 1978; B.J. Sloan, J.K. Kielty, and F.A. Miller, *Ann.N.Y.Acad.Sci.*, 1977, 284, 60.
6. T.W. North, and S.S. Cohen, *Pharmacol.Ther.*, 1979, 4, 81.
7. A. Bloch, M.J. Robins, and J.R. McCarthy Jr., *J.Med.Chem.*, 1967, 10, 908.
8. E. DeClercq, J. Descamps, E. Krajewska, and D. Shugar, *Biochem.Pharmacol.*, 1977, 26, 794.
9. A.J. Repta, B.J. Rawson, R.D. Shaffer, K.B. Sloan, N. Bodor, and T. Higuchi, *J.Pharm.Sci.*, 1975, 64, 392.
10. J.L. York, and G.A. LePage, *Can.J.Biochem.Physiol.*, 1966, 44, 19.
11. P.A. Furman, M.H. St. Clair, J.A. Fyfe, J.L. Rideout, P.M. Keller, and G.B. Elion, *J.Virol.*, 1979, 32, 72.
12. W.H. Prusoff, and B. Goz, *Fed.Proc.Fed.Am.Soc.Exp.Biol.*, 1973, 32, 1679.
13. P.A. Furman, J.A. Fyfe, M.H. St. Clair, K. Weinhold, J.L. Rideout, G.A. Freeman, S.N. Lehrman, D.P. Bolognesi, S. Broder, H. Mitsuya, and D.W. Barry, *Proc.Nat.Acad.Sci.USA*, 1986, 83, 8333.
14. C.E. Myers, *Pharm.Rev.*, 1981, 33, 1.
15. S.S. Cohen, J.G. Flaks, H.D. Barner, M.R. Loeb, and J. Lichtenstein, *Proc.Nat.Acad.Sci.USA*, 1958, 44, 1004.
16. G.B. Elion, *Fed.Proc.*, 1967, 26, 898.
17. G.A. LePage, *Cancer Res.*, 1963, 23, 1202.
18. E. DeClercq, *Biochem.J.*, 1982, 205, 1.
19. P. Reichard, O. Skold, G. Klein, L. Revesz, and P.-H. Magnusson, *Cancer Res.*, 1962, 22, 235; P.A. Morse, and V.R. Potter, *ibid*, 1965, 25, 499; A.R. Bapat, C. Zarow, and P.V. Danenberg, *J.Biol.Chem.*, 1983, 258, 4130.
20. C. Heidelberger, A.V. Sunthankar, L. Griesbach, and S. Randerson, *Proc.Soc.Exp.Biol.Med.*, 1960, 104, 127.
21. Th. Posternak, Th. *Ann.Rev.Pharmacol.*, 1974, 14, 23; J. Lichtenstein, H.D. Barner, and S.S. Cohen, *J.Biol.Chem.*, 1960, 235, 457; K.C. Liebman, and C. Heidelberger, *ibid*, 1955, 216, 823.
22. D. Farquhar, and R. Smith, *J.Med.Chem.*, 1985, 28, 1358.
23. D. Farquhar, N.J. Kuttesch, M.G. Wilkerson, and T. Winkler, *J.Med.Chem.*, 1983, 26, 1153; R.N. Hunston, A.S. Jones, C. McGuigan, R.T. Walker, J. Balzarini, and E. DeClercq, *ibid*, 1984, 27, 440; R.R. Chawla, J.J. Freed, and A. Hampton, *ibid*, 1984, 27, 1733.

24. G.M. Kosolapoff, Organophosphorus Compounds, Wiley, New York, 1950, 216.
25. R.G. Brennan, N.S. Kondo, and M. Sundaralingam, *J.Am.Chem.Soc.*, 1984, 106, 5671.
26. P values are given as; compound number, P value: 1, 0.08; 3, 0.64; 5, 7.0; 7, 16; 9, 20.
27. J.G. Cory, *Biochemistry*, 1975, 4, 1729, 1733; A. Coddington, *Biochem.Biophys.Acta*, 1965, 99, 442.
28. Plaque reduction assays were conducted by Beecham Pharmaceuticals, Great Burgh (U.K.) against HSV1 (SC16) in MRC5 (human fibroblast) cells, HSV1 (HFEM) in Vero (African green monkey kidney) cells, HSV2 (MS) in MRC5 cells, VZV (Ellen) in MRC5 cells, Influenza A (A/HK/I/68) in MDCK cells, Parainfluenza type I (Sendai) in MDCK cells; No indications of activity were noted for compounds 2−9 at concentrations of up to 100μg/ml.
29. ^{31}P nmr $\delta(CDCl_3)$ +2.50; ^{13}C nmr $\delta(CDCl_3)$ 65.88(d, CH_2OP, J=6.9Hz), 15.75(d, CH_3, J=7.7Hz); Found C 27.58%, H 5.88, P 18.41, Cl 20.70; $C_4H_{10}ClO_3P$ requires C 27.84, H 5.84, P 17.95, Cl 20.55.
30. ^{31}P nmr $\delta(CDCl_3)$ +2.61; ^{13}C nmr $\delta(CDCl_3)$ 71.2(d, CH_2OP, J=7.3Hz), 23.3(d, CH_3CH_2, J=7.9Hz), 9.9(s, CH_3); Found C 36.21%, H 6.89, P 15.39, Cl 17.45; $C_6H_{14}ClO_3P$ requires C 35.92, H 7.03, P 15.44, Cl 17.67.
31. ^{31}P nmr $\delta(CDCl_3)$ +2.60; ^{13}C nmr $\delta(CDCl_3)$ 69.23(d, CH_2OP, J=7.1Hz), 31.56(d, CH_2CH_2OP, J=7.8Hz), 18.35(s, CH_3CH_2), 13.2(s, CH_3); Found Cl 15.73%; $C_8H_{18}ClO_3P$ requires Cl 15.50.
32. ^{31}P nmr $\delta(CDCl_3)$ +2.13; ^{13}C nmr $\delta(CDCl_3)$ 69.7(d, CH_2OP, J=7.4Hz), 29.4(d, CH_2CH_2OP, J=7.8Hz), 27.4(s, $CH_3CH_2CH_2$), 22.1(s, CH_3CH_2), 13.8(s, CH_3); Found P 12.00%, Cl 14.57; $C_{10}H_{22}ClO_3P$ requires P 12.07, Cl 13.81.

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