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Synthesis and biological evaluation of some phosphate triester derivatives of the anti-cancer drug AraC

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ABSTRACT

A number of novel phosphate triester derivatives of the anti-cancer nucleoside analogue araC have been prepared by a rapid 2-step procedure, not necessitating prior sugar protection. Spectroscopic and lipophilicity data have been collected on these compounds. An in vitro assay indicated inhibition of thymidine incorporation by mammalian epithelial 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 clear relationship between inhibition of thymidine incorporation and log(P). These results are consistent with cellular penetration by the intact phosphate triesters and intracellular action by an unspecified mechanism. Triethyl phosphate is inactive under the conditions of the test.

INTRODUCTION

The nucleoside analogue araC, 9-β-D-arabinofuranosylcytosine (1) has found widespread use in the treatment of a number of cancers, in particular various leukaemias, including acute lymphoblastic, acute myeloid, and chronic myeloid (blast phase)[1]. As with many anti-cancer drugs, araC is especially useful in combination with other agents, in particular cyclophosphamide; for example, in the treatment of acute lymphoblastic leukaemia of childhood[2]. However, as with most nucleoside analogues, araC suffers from a number of limitations. Firstly, it is subject to rapid enzymatic deactivation; this corresponds to cytidine deaminase mediated conversion to arauracil[3] (araU). In an effort to overcome this major problem, potent inhibitors of cytidine deaminase have been sought, and found[4]. Indeed, co-administration of araC and a cytidine deaminase inhibitor greatly increases the plasma half-life of the drug, and may increase its therapeutic effect[5]. However, the long term inhibition of crucial metabolic enzymes may have toxic side-effects[6]. A better alternative might be to search for deamination resistant derivatives of araC. Since the major substrate requirement of cytidine deaminase is a free 5′-hydroxyl group[7], it is not surprising in this context that many 5′-modified cytidine nucleosides have been prepared[8]. Indeed, 5′-esters of araC seem promising as deamination resistant transport forms of araC[9]. However, they do not solve the second problem of araC, 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 araC 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′-dideoxythmidine (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 araC[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 araC have been prepared by a rapid 2-step procedure, and have been evaluated by biological methods. In particular a correlation was sought between phosphate structure and biological activity. We have recently noted just such a correlation for similar dialkyl phosphate triesters of the anti-viral drug araA[24].

DISCUSSION

The first nucleotide triester synthesised was the diethyl phosphate derivative (2) of araC, which was prepared in 2 steps from the parent nucleoside. In the first step diethyl phosphorochloridate was produced by the reaction of ethanol and phosphoryl chloride, in the presence of triethylamine[25]. This phosphorylating agent was reacted with araC in dry pyridine to give (2) 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.

In an entirely analogous manner, dipropyl phosphorochloridate was prepared and reacted with araC to give (3). Again, ^{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 this material.

Similarly prepared and characterised were the butyl (4), pentyl (5), and hexyl (6) analogues.

Comparing the various nucleotide triesters reveals several interesting trends, in the ^{31}P nmr chemical shifts. Thus, the propyl, butyl, pentyl and hexyl compounds (3), (4), (5), and (6) all resonate at approximately $+0.18$ ppm, whilst the ethyl compound is anomalous; (2) resonates at -0.02 ppm.

It was envisaged that the triester derivatives would increase in lipophilicity with lengthening alkyl chain and, moreover, that some correlation might exist between lipophilicity (log[P]) and biological activity. Therefore, octanol/water partition coefficients were measured for each of the araC derivatives (2)−(6) and for araC (1), by uv analysis of the separated layers; each analysis being run in duplicate. The results are presented in the table; the ethyl derivative (2) is twice as lipophilic as araC, this value increasing to 34 times for the hexyl compound (6). Thus, passive diffusion of the nucleotide triesters through cell membranes should be greatly facilitated.

Thus, lipophilic derivatives of araC had been successfully prepared. However, it was unclear as to whether these could act as intracellular sources of araC or, preferably, araCMP. The biological effects of the compounds were tested on a mammalian epithelial cell line (CNCM I221) grown in layer culture, employing a thymidine incorporation assay[24]. The incorporation of extracellular radiolabelled thymidine into acid-insoluble material was

Table

Compound	% Inhibition	SD	P	Log(P)
(1)	81	3	0.1	−1.0
(2)	11	4	0.2	−.68
(3)	25	2	0.6	−.19
(4)	37	2	1.4	.14
(5)	54	3	2.4	.39
(6)	62	4	3.4	.53

The inhibition of the incorporation of tritiated thymidine into DNA in the presence of compounds (1)−(6) at 30μM, relative to 10% DMSO control, and lipophilicity data on the compounds. For full details see experimental.

estimated in replicate cultures of cells exposed to various concentrations of compounds (2)−(6). Each experiment was performed at least twice, araC being included as a positive control in every experiment. Selected results are presented in the table.

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; activity rises with increasing log(P) value, (figure). 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. Under the conditions of the test, triethyl phosphate[26] was completely inactive, indicating the necessity of a nucleoside moiety for biological activity. 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.

In conclusion, simple phosphate triesters of araC appear to inhibit DNA synthesis *in vitro*, their efficacy increasing with their lipophilicity. This finding confirms the generality of our earlier observations[24], and may be of great importance in the chemotherapy of cancer, viral and other infections, if intracellular delivery of the nucleotides is being achieved. This is amongst the questions we are currently addressing.

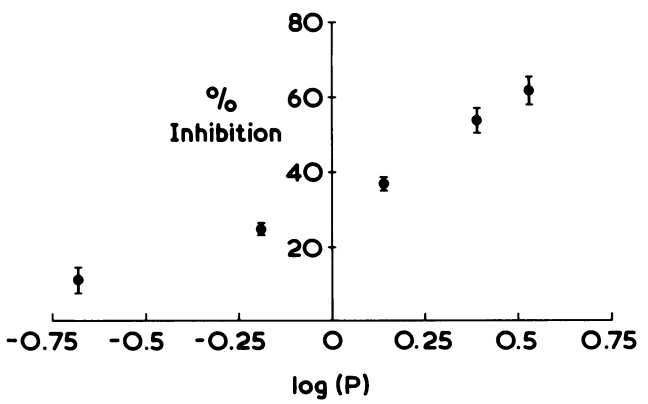
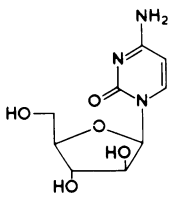
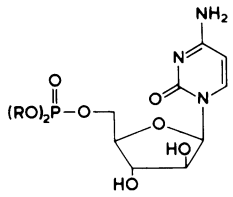


Figure. Inhibition of DNA synthesis by compounds 2−6 at 30μM, relative to 10% DMSO control, plotted against logarithm of octanol/water partition coefficients.



(1)



(2) R=Et
(3) R=nPr
(4) R=nBu
(5) R=nPentyl
(6) R=nHexyl

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. 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 a VXR400 instrument, operating at 100MHz, and ^{31}P spectra similarly, at 160MHz. Proton spectra were referenced to TMS, phosphorus spectra to 85% phosphoric acid, and carbon spectra to TMS; positive shifts are downfield of the reference. Mass spectra were recorded in FAB mode, on a VG Zab1F spectrometer, using m-nitrobenzyl alcohol as matrix, courtesy of the University of London Mass Spectrometry Service. 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; the phosphate triesters were frequently noted to be hygroscopic, and analytical data reflect this, and are calculated appropriately.

9-β-D-Arabinofuranosylcytosine-5'-diethyl phosphate (2).

araC (0.5g, 2.06mmol) was dissolved in pyridine (100ml), and diethyl phosphorochloridate (0.60ml, 0.716g, 4.15mmol) added dropwise with vigorous stirring at Ca. 4°C. After stirring for 5h at ambient temperature, the reaction was quenched with water (75μl, 4.14mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in vacuum, and purified by flash column chromatography on silica. Elution with 15% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave an impure product (0.62g). This was re-chromatographed, using first chloroform (150ml), and then 20% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions of the latter eluant gave the product as a white solid (0.38g, 49%).

^1H nmr δ(CD_3OD) 7.91(1H, d, H6), 6.26(1H, d, H1'), 5.99(1H, d, H5), 4.15(8H, m, H5', CH_2OP, 2×OH), 3.99(1H, m, H4'), 3.31(2H, m, H2', H3'), 1.35(6H, t, CH_3); ^{31}P nmr δ(CD_3OD) −0.019; ^{13}C nmr δ(CD_3OD) 166.38(C4), 156.81(C2), 145.19(C6), 94.91(C5), 88.92(C1'), 84.94(d, C4', J=6.7Hz), 77.85(C2'), 76.16(C3'), 68.26(d, C5', J=5.6Hz), 65.85(d, CH_2OP, J=5.5Hz), 16.50(d, CH_3, J=6.4Hz); FAB MS m/e 759(M_2H^+, 2%), 380(MH^+, 12), 112(cytosineH^+, base peak); Found C 39.33%, H 5.83, N 10.32, P 7.79; $C_{13}H_{22}N_3O_8P.H_2O$ requires C 39.30, H 6.09, N 10.58, P 7.80.

9-β-D-Arabinofuranosylcytosine-5'-dipropyl phosphate (3).
This was prepared in an entirely analogous manner to (2) above, except that the reaction mixture was stirred for longer (24h), and that purification was achieved using a single chromatographic column, eluted with 15% methanol in chloroform. Thus, from 0.5g araC, was isolated 0.49g, (58%) of (3).

^{1}H nmr δ(CD_3OD) 7.85(1H, d, H6), 6.26(1H, d, H1'), 5.91(1H, d, H5), 4.07(8H, m, H5', CH_2OP, 2×OH), 4.00(1H, m, H4'), 3.31(2H, m, H2', H3'), 1.74(4H, m, CH_3CH_2), 0.98(6H, m, CH_3); ^{31}P nmr δ(CD_3OD) 0.179; ^{13}C nmr δ(CD_3OD) 167.45(C4), 158.06(C2), 144.67(C6), 94.81(C5), 88.85(C1'), 84.71(d, C4', J=6.8Hz), 77.86(C2'), 76.16(C3'), 70.99(d, CH_2OP, J=4.4Hz), 68.34(d, C5', J=5.7Hz), 24.68(d, CH_3CH_2, J=5.2Hz), 10.36(CH_3); FAB MS m/e 815(M_2H^+, 5.5%), 408(MH^+, 27.9), 112(cytosineH^+, base peak); Found C 42.48%, H 6.54, N 9.62, P 7.45; $C_{15}H_{26}N_3O_8P.H_2O$ requires C 42.35, H 6.64, N 9.88, P 7.28.

9-β-D-Arabinofuranosylcytosine-5'-dibutyl phosphate (4).
This was prepared in an entirely analogous manner to (2) above, except that a slightly longer reaction time was employed (6h), and the first chromatographic column was eluted with a slightly more polar solvent mixture (20% methanol in chloroform). Thus, from 0.5g araC, was isolated 0.32g, (36%) of (4).

^{1}H nmr δ(CD_3OD) 7.84(1H, d, H6), 6.26(1H, d, H1'), 5.90(1H, d, H5), 4.11(8H, m, H5', CH_2OP, 2×OH), 3.99(1H, m, H4'), 3.31(2H, m, H2', H3'), 1.68(4H, m, CH_2CH_2OP), 1.43(4H, m, CH_3CH_2), 0.96(6H, m, CH_3); ^{31}P nmr δ(CD_3OD) 0.185; ^{13}C nmr δ(CD_3OD) 167.21(C4), 158.12(C2), 144.34(C6), 94.34(C5), 87.77(C1'), 83.67(d, C4', J=6.7Hz), 76.73(C2'), 75.67(C3'), 68.45(d, CH_2OP, J=5.3Hz), 67.33(d, C5', J=5.6Hz), 32.50(d, CH_2CH_2OP, J=6.8Hz), 18.87(CH_3CH_2), 13.61(CH_3); FAB MS m/e 871(M_2H^+, 1%), 436(MH^+, 9); Found C 45.75%, H 7.09, N 8.83, P 6.51; $C_{17}H_{30}N_3O_8P[H_2O]_{0.8}$ requires C 45.39, H 7.08, N 9.34, P 6.89.

9-β-D-Arabinofuranosylcytosine-5'-dipentyl phosphate (5).
This was prepared in an entirely analogous manner to (4) above, except that a longer reaction time was employed (17h), and (unsuccessful) recrystallisation attempts were made in place of the second column. Thus, from 0.5g araC, was isolated 0.06g, (6%) of (5).

^{1}H nmr δ(CD_3OD) 7.83(1H, d, H6), 6.26(1H, d, H1'), 5.90(1H, d, H5), 4.10(9H, m, H5', H4', CH_2OP, 2×OH), 3.31(2H, m, H2', H3'), 1.69(4H, m, CH_2CH_2OP), 1.36(8H, m, $CH_3CH_2CH_2$), 0.92(6H, m, CH_3); ^{31}P nmr δ(CD_3OD) 0.186; ^{13}C nmr δ(CD_3OD) 167.43(C4), 157.97(C2), 144.77(C6), 94.74(C5), 88.88(C1'), 84.73(d, C4', J=6.7Hz), 77.99(C2'), 76.15(C3'), 69.47(d, CH_2OP, J=6.5Hz), 68.48(d, C5', J=5.8Hz), 31.05(d, CH_2CH_2OP, J=2.2Hz), 28.74($CH_2CH_2CH_2OP$), 23.25(CH_3CH_2), 14.34(CH_3); FAB MS m/e 928($M_2H_2^+$, 3%), 464(MH^+, 10), 99($C_5H_7O_2^+$, base peak); Found C 47.70%, H 7.46, N 8.84, P 6.43; $C_{19}H_{34}N_3O_8P.H_2O$ requires C 47.40, H 7.54, N 8.73, P 6.43.

9-β-D-Arabinofuranosylcytosine-5'-dihexyl phosphate (6).
This was prepared in an entirely analogous manner to (5) above. Thus, from 0.38g araC, was isolated 0.24g, (31%) of (6).

^{1}H nmr δ(CD_3OD) 7.87(1H, d, H6), 6.27(1H, d, H1'), 5.92(1H, d, H5), 4.10(8H, m, H5', CH_2OP, 2×OH), 3.98(1H, m, H4'), 3.31(2H, m, H2', H3'), 1.70(4H, m, CH_2CH_2OP), 1.34(12H, m, 3×CH_2), 0.91(6H, m, CH_3); ^{31}P nmr δ(CD_3OD) 0.193; ^{13}C nmr δ(CD_3OD) 167.26(C4), 157.76(C2), 144.88(C6), 94.79(C5), 88.92(C1'), 84.82(d, C4', J=6.5Hz), 78.01(C2'), 76.14(C3'), 69.51(d, CH_2OP, J=6.5Hz), 68.45(d, C5',

J=5.5Hz), 32.46(d, CH_2CH_2OP, J=2.3Hz), 31.25($CH_2CH_2CH_2OP$), 26.27(CH_3CH_2-CH_2), 23.63(CH_3CH_2), 14.39(CH_3); FAB MS m/e 982($M_2H_2^+$, 0.5%), 491(MH^+, 3), 112(cytosineH^+, base peak); Found C 50.28%, H 7.83, N 8.26, P 6.38; $C_{21}H_{38}N_3O_8P$-$[H_2O]_{0.5}$ requires C 50.39, H 7.85, N 8.40, P 6.19.

Lipophilicity evaluation.

Samples (ca. 50μmol) of compounds 1–6 were partitioned between distilled water (25ml) and n-octanol (25ml) at ambient temperature. In some cases emulsion formation was noted; being overcome by the addition of solid NaCl. Two samples of each separate layer were taken in each case, and their uv spectra recorded. Each experiment was carried out twice. Absorbances were averaged to give lipophilicity values.

Tritiated Thymidine Incorporation Assay.

The procedure used was entirely as recently reported[24], except that a single incubation time only (30 min) was employed.

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26. Tested at the suggestion of a referee.

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