6.65 (d, 2, J = 8.64 Hz, Ar H ortho to -OR), 6.76 (d, 2, J = 8.64 Hz, Ar H meta to -OR), 7.11-7.37 (m, 10, Ar H); mass spectrum (10 eV), m/e (relative intensity) 399 (M^+, 5). Anal. ($C_{28}H_{34}ClNO$) C, H, N.

(Z)-1,2-Diphenyl-1-[4-[2-(N-methylpiperazino)ethoxy]-phenyl]-1-butene (12). This compound was prepared in 94% yield according to the procedure used for the synthesis of 8, using bromo compound 2[14] and N-methylpiperazine. The product was obtained as flocculent white needles from hexane: mp 109-110.5 °C; ^1H NMR ($CDCl_3$) δ 0.92 (t, 3, J = 7.56 Hz, CH_2CH_3), 2.27 (s, 3, NCH_3), 2.39-2.70 (br m, 8, ring CH_2), 2.45 (q, 2, J = 7.56 Hz, CH_2CH_3), 2.73 (t, 2, J = 5.76 Hz, OCH_2CH_2N), 3.96 (t, 2, J = 5.76 Hz, OCH_2CH_2N), 6.54 (d, 2, J = 8.64 Hz, Ar H ortho to -OR), 6.76 (d, 2, J = 8.64 Hz, Ar H meta to -OR), 7.10-7.36 (m, 10, Ar H); mass spectrum (10 eV), m/e (relative intensity) 426 (M^+, 7). Anal. ($C_{29}H_{34}N_2O$) C, H, N.

(Z)-1,2-Diphenyl-1-[4-[2-(N-morpholino)ethoxy]-phenyl]-1-butene (13). This compound was prepared in 83% yield according to the procedure used for the synthesis of 8, using bromo compound 2[14] and morpholine. The product was obtained as colorless plates from hexane containing a trace of THF: mp 131.5-133 °C (lit.[23] mp 130-132 °C); ^1H NMR ($CDCl_3$) δ 0.92 (t, 3, J = 7.56 Hz, CH_2CH_3), 2.45 (q, 2, J = 7.56 Hz, CH_2CH_3), 2.52 (t, 4, J = 4.32 Hz, ring CH_2 adjacent to N), 2.71 (t, 2, J = 6.48 Hz, OCH_2CH_2N), 3.70 (t, 4, J = 4.32 Hz, ring CH_2 adjacent to O), 3.97 (t, 2 J = 6.48 Hz, OCH_2CH_2N), 6.54 (d, 2, J = 8.64 Hz, Ar H ortho to -OR), 6.76 (d, 2, J = 8.64 Hz, Ar H meta to -OR), 7.11-7.34 (m, 10, Ar H); mass spectrum (10 eV), m/e (relative intensity) 413 (M^+, 28). Anal. ($C_{28}H_{31}NO_2$) C, H, N.

(Z)-1,2-Diphenyl-1-[4-[2-(N-pyrrolo)ethoxy]phenyl]-1-butene (14). n-Butyllithium (3 mL of a 2.44 M solution in hexane, 7.32 mmol) was added dropwise to a stirred solution of pyrrole (600 mg, 8.94 mmol) in 15 mL of THF at 0 °C. After 15 min, 1 mL of HMPA was added, the solution was cooled to -78 °C, and bromo compound 2[14] (200 mg, 0.491 mmol) in 8 mL of THF was slowly added. The reaction was allowed to warm slowly to room temperature, and after 3 h, product isolation (ether, water, brine, sodium sulfate) and chromatography (one 20 × 20 cm preparative TLC plate; 10% ether in hexane) gave two bands, which were isolated and crystallized from hexane.

The more mobile product gave large prisms (36 mg) with mp 110-111.5 °C whose spectral characteristics identified it as the vinyl ether (4) resulting from dehydrohalogenation of the starting material: IR (CCl_4) 1648 cm^{-1} ($OCH{=}CH_2$); ^1H NMR ($CDCl_3$) δ 0.92 (t, 3, CH_2CH_3), 2.45 (q, 2, CH_2CH_3), 4.15-4.90 (m, 3, vinyl H), 6.56-7.50 (m, 14, Ar H); mass spectrum (70 eV) m/e (relative intensity) 326 (M^+, 100). Anal. ($C_{24}H_{22}O$) C, H.

The less mobile product gave white flocculent crystals (65 mg, 33%), mp 97.5-98 °C, whose spectral characteristics identified it as the pyrrole analogue of tamoxifen (14): IR (CCl_4) 722 cm^{-1} (pyrrole CH); ^1H NMR ($CDCl_3$) δ 0.92 (t, 3, J = 7.56 Hz, CH_2CH_3), 2.45 (q, 2, J = 7.56 Hz, CH_2CH_3), 4.06 and 4.18 (each t, each 2, J = 5.4 Hz, OCH_2CH_2N), 6.12 (m, 2, pyrrole β-H), 6.51 (d, 2, J = 8.64 Hz, Ar H ortho to -OR), 6.70 (m, 2, pyrrole α-H), 6.76 (d, 2, J = 8.64 Hz, Ar H meta to -OR), 7.10-7.34 (m, 10 Ar H); mass spectrum (10 eV), m/e (relative intensity) 393 (M^+, 100). Anal. ($C_{28}H_{27}NO$) C, H, N.

Biochemical and Biological Methods. Complete experimental details for the relative binding affinity and uterotrophic and antiuterotrophic activity determinations can be found in ref 19. A synopsis of these methods is given in the legend to the figure and the table footnotes.

Acknowledgment. We thank Dr. A. Todd (ICI Ltd., Macclesfield, United Kingdom) and Dr. L. Trench (Stuart Pharmaceuticals, Division of ICI America, Wilmington, DE) for supplying *trans*-tamoxifen and Dr. C. D. Jones (Eli Lilly and Co.) for a gift of LY117018. Support of this research was provided by grants from the National Institutes of Health (HHS AM 15556 to J.A.K. and HHS CA18119 and HD06726 to B.S.K). D.W.R. was supported by fellowships from the University of Illinois and the Lubrizol Corp.

Lipophilic 5'-(Alkyl phosphate) Esters of 1-β-D-Arabinofuranosylcytosine and Its N^4-Acyl and 2,2'-Anhydro-3'-O-acyl Derivatives as Potential Prodrugs

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Lipophilic 5'-(alkyl phosphate) esters of 1-β-D-arabinofuranosylcytosine (*ara*-C) and several N^4-acyl and 3'-O-acyl-2,2'-anhydro derivatives of *ara*-C were synthesized as potential prodrugs of *ara*-C 5'-monophosphate (*ara*-CMP). Alkylphosphorylation of *ara*-C, N^4-palmitoyl-*ara*-C, and N^4-stearoyl-*ara*-C was achieved in a single continuous operation by allowing the nucleoside to react with $POCl_3$ in trimethyl or triethyl phosphate and adding the appropriate anhydrous alcohol directly to the intermediate phosphorodichloridate without isolation. Similar reaction of cytidine yielded cytidine 5'-(alkyl phosphate) esters, which on treatment with myristoyl or palmitoyl chloride in the presence of boron trifluoride gave 3'-O-acyl-2,2'-anhydro-*ara*-C 5'-(alkyl phosphate) esters. *Ara*-C 5'-(n-butyl phosphate) (**1b**), N^4-palmitoyl-*ara*-C 5'-(n-butyl phosphate) (**1h**), and 2,2'-anhydro-3'-O-palmitoyl-*ara*-C 5'-(n-butyl phosphate) (**2h**) were tested against L1210/*ara*-C leukemia in mice in the hope that this kinase-deficient tumor would respond to treatment with these "prephosphorylated" derivatives, but no activity was observed. Of the simple 5'-(alkyl phosphate) esters tested in culture against L1210 leukemic cells, only *ara*-C 5'-(glyceryl phosphate) (**1g**) showed toxicity comparable to *ara*-CMP (ID_{50} = 0.35 and 0.65 μM, respectively), suggesting that β-hydroxyalkyl phosphate esters may be worthwhile to examine further as prodrugs of *ara*-CMP.

Although 1-β-D-arabinofuranosylcytosine (*ara*-C) enjoys widespread use in cancer chemotherapy and is the drug of choice for the treatment of adult myelogenous leukemia,[1] its short plasma half-life and marked schedule dependence require that it be given either in precisely adjusted cycles of intermittent therapy[2] or by continuous infusion[3,4] for maximum benefit. The schedule dependence of *ara*-C results from the fact that its biochemical effect on DNA synthesis is highly S-phase specific,[5] whereas its

(1) Clarkson, B.; Dowling, B. D.; Gee, T. S.; Cunningham, I. B.; Burchenal, J. H. *Cancer* **1975**, *36*, 775.

(2) Skipper, H. E.; Schabel, F. M., Jr.; Wilcox, W. S. *Cancer Chemother. Rep.* **1967**, *51*, 125.

(3) Ho, D. H. W.; Frei III, E. *Clin. Pharmacol. Ther.* **1971**, *12*, 944.

(4) Momparler, R. L. *Cancer Res.* **1974**, *34*, 1775.

(5) Bhuyan, B. K.; Scheidt, L. G.; Fraser, T. G. *Cancer Res.* **1972**, *32*, 398.

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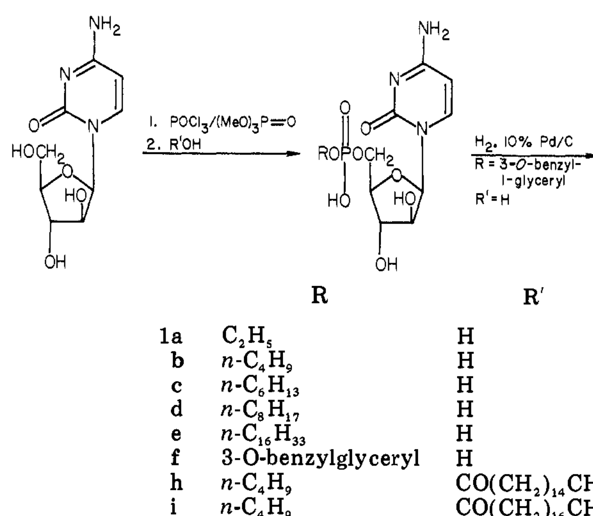
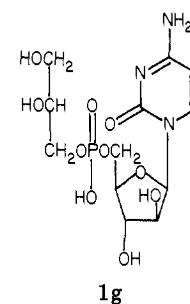
short half-life is due to rapid cleavage to the inactive metabolite 1-β-D-arabinofuranosyluracil (*ara*-U) by cytidine deaminase.[4,6,7] In order to circumvent these practical limitations, major efforts have been made to develop *ara*-C prodrugs that combine deaminase resistance with prolonged tissue retention. Prominent examples of this approach include lipophilic alkyl esters[8] and a variety of N^4-acyl derivatives.[9-11] Another important prodrug of *ara*-C is the 2,2′-anhydro derivative (cyclo-C),[12,13] which has been shown to have positive therapeutic value both in experimental animal models[14] and in man.[15] Sustained levels of free *ara*-C are observed in plasma following treatment with cyclo-C[16] because the 4-imino group in this molecule is deaminase stable and because cleavage of the 2,2′-anhydro bond to form *ara*-C occurs relatively slowly, by chemical as opposed to enzymatic cleavage.[17,18] More extensive latentiation has been achieved also with long-chain 5′-*O*-acyl- and 3′,5′-di-*O*-acyl-2,2′-anhydro derivatives[19] and more recently with 5′-phosphorylated 3′-*O*-acyl-2,2′-anhydro analogues, which were prepared with a view to increasing water solubility and ease of formulation.[10,20] A number of these "multiply latentiated" prodrug derivatives possess greater antitumor activity against L1210 leukemia than either *ara*-C or cyclo-C and are essentially schedule independent.

A more serious and thus far unresolved problem in the clinical use of *ara*-C is that tumors may become resistant because of decreased deoxycytidine kinase activity, which results in the inability of *ara*-C to undergo intracellular conversion to *ara*-C 5′-triphosphate (*ara*-CTP) in the tumor.[21] Additionally, certain tumors that are refractory

Scheme I

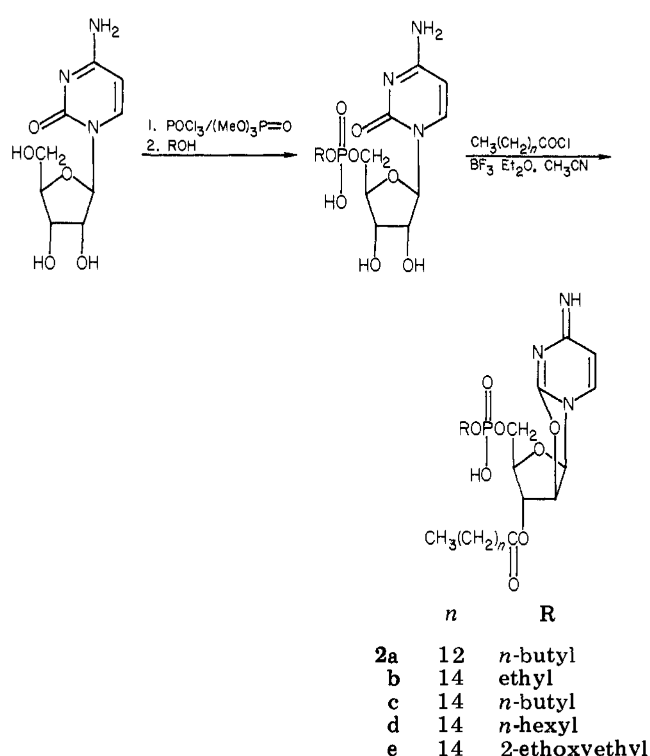


	R	R′
1a	C_2H_5	H
b	n-C_4H_9	H
c	n-C_6H_{13}	H
d	n-C_8H_{17}	H
e	n-$C_{16}H_{33}$	H
f	3-O-benzylglyceryl	H
h	n-C_4H_9	$CO(CH_2)_{14}CH_3$
i	n-C_4H_9	$CO(CH_2)_{16}CH_3$



1g

Scheme II



	n	R
2a	12	n-butyl
b	14	ethyl
c	14	n-butyl
d	14	n-hexyl
e	14	2-ethoxyethyl

(6) Camiener, G. W.; Smith, C. G. *Biochem. Pharmacol.* **1965**, *14*, 1405.
(7) Creasey, W. A.; Papac, R. J.; Markin, M. E.; Calabresi, P.; Welch, A. D. *Biochem. Pharmacol.* **1966**, *15*, 1417.
(8) Neil, G. L.; Wiley, P. F.; Manak, R. C.; Moxley, T. E. *Cancer Res.* **1970**, *30*, 1047. Gish, D. T.; Kelly, R. C.; Camiener, G. W.; Wechter, W. J. *J. Med. Chem.* **1971**, *14*, 1159. Neil, G. L.; Buskirk, H. H.; Moxley, T. E.; Manak, R. C.; Kuentzel, S. L.; Bhuyan, B. K. *Biochem. Pharmacol.* **1971**, *20*, 3295. Gray, G. D.; Nichol, F. R.; Mickelson, M. M.; Camiener, G. W.; Gish, D. T.; Kelly, R. C.; Wechter, W. J.; Moxley, T. E.; Neil, G. L. *Biochem. Pharmacol.* **1972**, *21*, 465. Warner, D. T.; Neil, G. L.; Taylor, A. J.; Wechter, W. J. *J. Med. Chem.* **1972**, *15*, 790. Wechter, W. J.; Johnson, M. A.; Hall, C. M., Warner, D. T.; Berger, A. E.; Wenzel, A. H.; Gish, D. T.; Neil, G. L. *J. Med. Chem.* **1975**, *18*, 339. Ho, D. H. W.; Neil, G. L. *Cancer Res.* **1977**, *37*, 1640.
(9) Wechter, W. J.; Gish, D. T.; Greig, M. E.; Gray, G. D.; Moxley, T. E.; Kuentzel, S. L.; Gray, L. G.; Gibbons, A. J.; Griffin, R. L.; Neil, G. L. *J. Med. Chem.* **1976**, *19*, 1013.
(10) Aoshima, M.; Tsukagoshi, S.; Sakurai, Y.; Oh-Ishi, J.; Ishida, T.; Kobayashi, H. *Cancer Res.* **1976**, *36*, 2726. Tsuruo, T.; Iida, H.; Tsukagoshi, S.; Sakurai, Y. *Cancer Res.* **1979**, *39*, 1063.
(11) Akiyama, M.; Oh-Ishi, J.; Shirai, T.; Akashi, K.; Yoshida, K.; Nishikido, J.; Hayashi, H.; Usubuchi, Y.; Nishimura, D.; Itoh, H.; Shibuya, C.; Ishida, T. *Chem. Pharm. Bull.* **1978**, *26*, 981.
(12) Hoshi, A.; Kanzawa, F.; Kuretani, K. *Gann* **1972**, *279*, 353.
(13) Ho, D. H. W. *Biochem. Pharmacol.* **1974**, *23*, 1235.
(14) Venditti, J. M.; Baratta, M. C.; Greenberg, N.; Abbott, B. J.; Kline, I. *Cancer Chemother. Rep.* **1972**, *56*, 483.
(15) Lokich, J. J.; Chwala, P. L.; Jaffe, N.; Frei III, E. *Cancer Treat. Rep.* **1975**, *59*, 389.
(16) Ho, D. H. W.; Rodriguez, V.; Loo, T. L.; Bodey, G. P.; Freireich, E. J. *Clin. Pharmacol. Ther.* **1975**, *17*, 66.
(17) Wang, M. C.; Sharma, R. A.; Bloch, A. *Cancer Res.* **1973**, *33*, 1265.
(18) Kessel, D. *Biochem. Pharmacol.* **1974**, *23*, 2657.
(19) Moffatt, J. G.; Hamamura, E. K.; Russell, A. F.; Prystasz, M.; Verheyden, J. H. P.; Yamaguchi, K.; Uchida, N.; Sato, K.; Nishiyama, S.; Shiratori, O.; Katagiri, K. *Cancer Chemother. Rep. Part 1* **1974**, *58*, 451. Hamamura, E. K.; Prystasz, M.; Verheyden, J. H. P.; Moffatt, J. G.; Yamaguchi, K.; Uchida, N.; Sato, K.; Nomura, A.; Shiratori, O.; Takase, S.; Katagiri, K. *J. Med. Chem.* **1976**, *19*, 654, 663, 667.
(20) Kondo, K.; Inoue, I. *J. Org. Chem.* **1977**, *42*, 2809. Kondo, K.; Nagura, T.; Arai, Y.; Inoue, I. *J. Med. Chem.* **1979**, *22*, 639.
(21) Chou, T.-C.; Arlin, Z.; Clarkson, B. D.; Philips, F. *Cancer Res.* **1977**, *37*, 3561. Rustum, Y. *Cancer Res.* **1978**, *38*, 543. Rustum, Y.; Preisler, H. D. *Cancer Res.* **1979**, *39*, 42.

Table I. Physical Constants of 1-β-D-Arabinofuranosylcytosine Derivatives

no.[a]	method	% yield[b]	mp,[c] °C	TLC data R_f	TLC data system[d]	mol formula	anal.[e]
1d	A	44	156-163	0.54	1	$C_{17}H_{30}N_3O_8P{\cdot}HCOOH{\cdot}0.5H_2O$	C, H, N
1e	A	20	160 (120)	0.20	2	$C_{25}H_{46}N_3O_8P{\cdot}0.5H_2O$	C, H, N
1f	A	18	125 (75)	0.79	3	$C_{19}H_{26}N_3O_{10}P{\cdot}2.5H_2O$	C, H, N
1g	*f*	82	125 (85)	0.14	3	$C_{12}H_{20}N_3O_{10}P{\cdot}H_2O$	C, H, N
1h	B	29	175-180 (160)	0.44	4	$C_{29}H_{52}N_3O_9P{\cdot}H_2O$	C, H, N, P
1i	B	16	165-185 (150)	0.52	4	$C_{31}H_{56}N_3O_9P$	C, H, N, P
2a	C	30	233 dec (210)	0.25	2	$C_{27}H_{46}N_3O_8P{\cdot}0.5H_2O$	C, H, N
2b	C	46	217 dec (210)	0.27	2	$C_{27}H_{46}N_3O_8P{\cdot}0.25C_3H_7OH$	C, H, N
2c	C	56	210 dec (190)	0.28	2	$C_{29}H_{50}N_3O_8P{\cdot}C_3H_7OH$	C, H, N
2d	C	55	212 dec (165)	0.30	2	$C_{31}H_{54}N_3O_8P{\cdot}0.5H_2O$	C, H, N
2e	C	55	215 dec (195)	0.30	2	$C_{29}H_{50}N_3O_9P{\cdot}0.5C_3H_7OH$	C, H, N

[a] Compounds 1a–c were described previously; see ref 30. [b] Values given are for chromatographed material; yields were not optimized. [c] Temperatures in parentheses are those at which softening or sintering occurred. [d] TLC systems: 1 = cellulose, 80:15:5 saturated $(NH_4)_2SO_4$–1 M NaOAc–*i*-PrOH; 2 = silica gel, 9:1 *i*-PrOH–0.4 M ammonium formate; 3 = cellulose, 25:15:2:4 $CHCl_3$–MeOH–AcOH–H_2O; 4 = silica gel, 1:1 $CHCl_3$–EtOH. [e] Found values for the elements listed were within ±0.4% of theoretical values. [f] Catalytic hydrogenolysis of 1f (see Experimental Section).

to *ara*-C may suffer from an unfavorable ratio of kinase activity to deaminase activity.[22] For these reasons, a desirable goal in designing improved *ara*-C analogues would be the preparation of compounds that not only exhibit deaminase resistance and prolonged duration of action in tissues but also are toxic to kinase-deficient cells. One obvious approach to overcoming kinase-based resistance might be to use *ara*-C 5'-monophosphate (*ara*-CMP). This would, at the same time, serve to prevent cleavage by cytidine deaminase, since this enzyme requires the nucleoside substrate to contain a free 5'-OH group.[6,23] Unfortunately there are two negative factors that conspire to defeat this "prephosphorylation" strategy. First, since there is no active-transport pathway for pyrimidine nucleotides into cells,[24] *ara*-CMP has to cross the cell membrane by passive diffusion, a process retarded by the fact that the molecule carries a double negative charge on the phosphate group and is therefore poorly lipid soluble. Secondly, since nucleoside 5'-phosphates are cleaved rapidly to nucleosides by serum phosphatases, *ara*-CMP tends to have about the same in vivo effect as *ara*-C itself (i.e., tumors that are resistant to *ara*-C are cross-resistant to *ara*-CMP).[25] Similar arguments apply to the in vivo use of "pre-phosphorylated" derivatives of cyclo-C, which likewise undergo cleavage by serum phosphatases before reaching the target tumor.[26]

We reasoned that it might be possible to enhance cellular uptake of *ara*-CMP or cyclo-C 5'-monophosphate (cyclo-CMP) by administering them in the form of lipophilic long-chain 5'-(alkyl phosphate) derivatives. The decreased ionic charge and increased lipid solubility of such esters should favor passive diffusion across the cell membrane. On entry into tumor cells, phosphodiesterase hydrolysis would be expected to occur,[27] and depending on the rate and regioselectivity of cleavage, the intracellular product might be nucleoside alone (*ara*-C or cyclo-C), nucleotide alone (*ara*-CMP or cyclo-CMP), or a mixture of both. Provided that phosphodiesterase cleavage yields at least some *ara*-CMP or cyclo-CMP intracellularly, the use of these blocked esters against tumors consisting of a heterogeneous population of kinase-containing and kinase-lacking cells[28] might result in a higher cell kill, and thus a better therapeutic response, than is obtained with either *ara*-C or cyclo-C itself.

Chemistry. Two types of masked *ara*-CMP derivatives were prepared, as indicated in Schemes I and II. The simple *ara*-C 5'-(alkyl phosphate) esters **1a–e** were obtained conveniently from *ara*-C in a single, essentially continuous operation involving reaction with $POCl_3$ in trimethyl or triethyl phosphate,[29] followed 2 h later by quenching with the appopriate anhydrous alcohol and storing at 0–5 °C overnight.[30] After aqueous workup, purification consisted of column chromatography on Dowex 1-X2 (H^+). Unchanged *ara*-C was eluted with water, and the desired alkyl phosphate ester was eluted from the column with an aqueous formic acid gradient. The final concentration of the gradient was such that any *ara*-CMP would remain on the column. For the preparation of the 5'-(glyceryl phosphate) analogue **1g**, phosphorylation was quenched by the addition of L-1-*O*-benzylglycerol. Following ion-exchange chromatography, the *O*-benzylglyceryl phosphate ester **1f** was hydrogenolyzed to **1g** over 10% Pd/C. Reaction was assumed to occur only on the primary OH group of L-1-*O*-benzylglycerol, and there was no evidence of more than one product. In the synthesis of the N^4-acyl derivatives **1h** and **1i**, alkylphosphorylation of the known N^4-palmitoyl and N^4-stearoyl derivatives of *ara*-C[11] was performed in the usual manner, but the products, which were sparingly water-soluble, could not be purified by ion-exchange chromatography on a preparative scale. Analytically pure material was obtained, however, by repeated washing with water and hot EtOAc. In the synthesis of the 3'-*O*-acyl-2,2'-anhydro derivatives **2a–e** (Scheme II), cytidine was alkylphosphorylated, and after ion-exchange chromatography, the purified CMP esters were condensed with myristoyl or palmitoyl chloride in refluxing acetonitrile containing 3 equiv of $BF_3{\cdot}Et_2O$.[20] The cyclic products **2a–e** were purified by column chromatography on silica gel with 9:1 *i*-PrOH–ammonium formate (pH 4) as the eluent. Typical yields in the com-

(22) Hart, J. S.; Ho, D. H. W.; George, S. L.; Salem, P.; Gottlieb, J. A.; Frei III, E. *Cancer Res.*, **1972**, *32*, 2711. Coleman, C. N.; Johns, D. G.; Chabner, B. A. *Ann. N.Y. Acad. Sci.* **1975**, *255*, 247.
(23) Bloch, A.; Robins, M. J.; McCarthy, J. R., Jr. *J. Med. Chem.* **1967**, *10*, 908.
(24) Leibman, K.; Heidelberger, C. *J. Biol. Chem.* **1955**, *216*, 823.
(25) Smith, C. G.; Buskirk, H. H.; Lummis, W. L. *J. Med. Chem.* **1967**, *10*, 774.
(26) Hayashikawa, R. H.; Nagyvary, J. *Biochem. Pharmacol.* **1973**, *22*, 609. Focke III, J. H.; Broussard, W. J.; Nagyvary, J. *Biochem. Pharmacol.* **1973**, *22*, 703.
(27) Zeleznick, L. D. *Biochem. Pharmacol.* **1969**, *18*, 855.
(28) Barranco, S. C.; Ho, D. H. W.; Drewinko, B.; Romsdahl, M. M.; Humphrey, R. M. *Cancer Res.* **1972**, *32*, 2733.
(29) Yoshikawa, M.; Kato, T.; Takenishi, T. *Bull. Chem. Soc. Jpn.* **1969**, *42*, 3505.
(30) Kim, S.-H.; Rosowsky, A. *J. Carbohydr. Nucleosides Nucleotides.* **1979**, *6*, 229.

Table II. Growth Inhibition of Cultured L1210 Leukemia and B16 Melanoma Cells by 1-β-D-Arabinofuranosylcytosine Derivatives

compd	ID_{50},[a] μM	
	L1210 cells	B16 cells
ara-CMP	0.35	1.5
1a	4.0	70
1b	7.5	130
1c	7.8	*b*
1d	11	360
1e	11	*b*
1f	1.0	25
1g	0.65	26

[a] ±10%. [b] Not determined.

bined acylation and ring-closure step were 50–60%. Physical constants for compounds **1a–g** and **2a–e** are given in Table I.[31]

Biological Activity and Discussion

Compounds **1a–g** were tested as inhibitors of the growth of L1210 mouse leukemia and B16 mouse melanoma cells in culture, and their activity was compared with that of *ara*-CMP. Compounds **1h**, **1i**, and **2a–e** were insufficiently water soluble to be tested in vitro and were therefore assayed only in vivo (see below). The results of the cell culture experiments are shown in Table II. The cytotoxicity of the simple 5′-(alkyl phosphate) esters **1a–g** toward L1210 cells appeared to obey an inverse structure–activity relationship with respect to alkyl chain length, with the *n*-butyl (C_4) and *n*-hexyl (C_6) derivatives being about half as active on a molar basis as the ethyl (C_2) ester. The *n*-octyl (C_8) analogue was approximately fivefold less active, but extending the length of the alkyl chain still further, as in **1i** (C_{16}), led to no additional decrease in activity. The severalfold higher activity of the glyceryl (**1g**) and *O*-benzylglyceryl (**1f**) esters relative to **1a–e** suggested that 5′-(β-hydroxyalkyl phosphate) esters may be superior to ordinary 5′-(alkyl phosphate) esters as prodrugs of *ara*-CMP, especially if the β-hydroxy substituent is combined with a lipophilic moiety (e.g., an *O*-benzyl group) on the terminal carbon of the ester chain. A plausible role for the β-hydroxy group in the mechanism of activation of the prodrug might be to facilitate hydrolysis of the phosphate bond via intramolecular nucleophilic catalysis. The cytotoxicity of the 5′-(alkyl phosphate) and 5′-(β-hydroxyalkyl phosphate) esters was 20- to 40-fold lower against B16 than L1210 cells and was qualitatively consistent with the lower response of B16 cells to *ara*-CMP itself. However, 5′-*O*-alkylphosphorylation appeared to decrease activity more markedly in the B16 cells. Thus, the ID_{50} ratio for *ara*-CMP and the 5′-(*n*-butyl phosphate) ester **1b** was 0.05 in L1210 cells but only 0.01 in B16 cells. Similarly, for *ara*-CMP and the 5′-(glyceryl phosphate) ester **1g** these ratios were 0.35 and 0.06, respectively. The relatively greater loss of activity observed in B16 cells on alkylation of the phosphate group may be indicative of a low rate of intracellular hydrolysis to *ara*-CMP and/or *ara*-C, although slow uptake cannot be ruled out.

The ethyl, *n*-butyl, and *n*-hexyl phosphate esters **1a–c** were also tested in vitro against L1210/*ara*-C cells, a kinase-deficient mutant that cannot convert *ara*-C to *ara*-CMP.[32] No appreciable inhibition of cell growth was observed after 48 h of exposure to 10^{-4} M, the highest concentration that could be tested in culture because of solubility limitations. The esters were thus 10–100 times less active against the kinase-deficient cells than against the parent line. In view of the activity of **1a–c** against wild-type L1210 cells, it seems improbable that their lack of toxicity to L1210/*ara*-C cells was due to slow uptake. A more likely explanation is that the esters were degraded extracellularly by enzymes in the incubation medium, which in order to simulate in vivo conditions was supplemented with fetal calf serum that was not dialyzed or heat inactivated. It is also possible that some intact ester penetrated the cells but was cleaved regioselectively to *ara*-C rather than *ara*-CMP. However, since it was evident that simple 5′-*O*-alkylphosphorylation did not fulfill our hope of overcoming kinase-based resistance, further work to distinguish between these possibilities was not carried out.

The 5′-(*n*-butyl phosphate) ester **1b**, which was the most cytotoxic of the simple masked *ara*-CMP derivatives in culture, was subjected also to in vivo antitumor assay against L1210 and L1210/*ara*-C leukemia in mice. The results are given in Table III. At 320 mg/kg (equivalent to 200 mg/kg of *ara*-C), **1b** produced an increase in life span (ILS) of +87% on the qd×5 schedule, with no weight loss or other evidence of host toxicity. *Ara*-C at 200 mg/kg gave a +112% ILS with slight weight loss. Although **1b** was still well tolerated at 640 mg/kg (equivalent to 400 mg/kg of *ara*-C), there was no further prolongation of survival beyond +87%. A 400 mg/kg dose of *ara*-C cannot be given on this schedule without significant toxicity (data not shown), and we have observed that even 200 mg/kg can produce 15–25% weight loss in some experiments. When **1b** was tested against the L1210/*ara*-C tumor, no therapeutic effect was seen at up to 400 mg/kg. Thus, even though **1b** had activity comparable to *ara*-C against the parent tumor and seemed to be slightly better tolerated at the upper end of the dose range, there was no effect on the kinase-deficient tumor, in agreement with the negative cell culture results.

In vivo antitumor assays against L1210 and L1210/*ara*-C leukemia were performed also with the N^4-acyl derivatives **1h** and **1i** (Table III). Because of their very poor solubility in aqueous pH 7.4 buffer, these compounds had to be given as fine suspensions in sterile saline containing 0.4% Tween 80, 0.5% low-density carboxymethylcellulose, and 0.9% benzyl alcohol (control experiments showed this vehicle to be well tolerated). On the q4d(2,6) schedule, a dose of the N^4-stearoyl derivative **1i** of 160 mg/kg (equivalent to 60 mg/kg of *ara*-C) gave an ILS of +55%, with negligible weight loss. A comparable increase in the ILS of +62% was obtained with *ara*-C only at 250 mg/kg; i.e., there was approximately a fourfold difference in the equiactive molar dose on this schedule in favor of the prodrug. A similar effect was observed on the qd×5 schedule. Whereas a dose of **1i** of 120 mg/kg (equivalent to 45 mg/kg of *ara*-C) produced a +111% ILS, the dose of *ara*-C to achieve a comparable +100% ILS was 100 mg/kg; i.e., the equiactive

(31) We thank one of the reviewers for calling our attention to recent Japanese work describing the preparation, for possible oral or topical use, of a series of 5′-(alkyl phosphate) esters of *ara*-C with saturated and unsaturated alkyl groups ranging in chain-length from C_{14} to C_{23}. These compounds were synthesized from 2,3,N^4-triacetyl-*ara*-CMP by tosyl chloride mediated esterification of the phosphate group, followed by removal of the acetyl blocking groups. See, for example, Kodama, K.; Kuninaka, A.; Saneyoshi, M. Ger. Offen. 2924691 (1980) [*Chem. Abstr.* **1980**, *92*, 203586*t*] and Kodama, K.; Morozumi, M.; Saneyoshi, M. Ger. Offen. 2924692 (1980) [*Chem. Abstr.* **1980**, *93*, 47113*z*].

(32) Schrecker, A. W.; Urshel, M. J. *Cancer Res.* **1968**, *28*, 793. Schrecker, A. W. *Cancer Res.* **1970**, *30*, 632.

Table III. Antitumor Activity of 1-β-D-Arabinofuranosylcytosine Derivatives against L1210 and L1210/*ara*-C Leukemia in Mice

				L1210					L1210/*ara*-C				
				av wt on	7-day wt change[b]		median		av wt on	7-day wt change[b]		median	
compd	dose,[a] mg/kg	schedule	no. of mice	day 1, g/mouse	g/mouse	%	survival: T/C, days	ILS,[c] %	day 1, g/mouse	g/mouse	%	survival: T/C, days	ILS, %
1b	80 (50)	qd× 5	9	22.0	+2.6	+10	11/8	+37	23.4	+3.0	+13	8/8	0
	160 (100)		9	23.6	+1.0	+4	12/8	+50	22.2	+3.6	+16	8/8	0
	320 (200)		9	22.4	+0.6	+3	15/8	+87	23.4	+1.0	+5	8/8	0
	640 (400)		9	22.8	+0.2	+1	15/8	+87	22.6	+0.2	0	7/8	−13
1h	80 (30)	q4d(2,6)	5	20.8	+2.8	+18	10/8	+25	19.6	+2.2	+11	7/8	−13
	160 (60)		5	20.6	+1.0	+5	11/8	+37	21.8	+1.8	+8	8/8	0
	80 (30)	q4d(2,6)	5	23.6	+1.2	+5	12/9	+33					
	120 (45)		5	24.4	+0.4	+2	13/9	+44					
	40 (15)	qd× 5	5	23.4	+2.4	+11	12/9	+33					
	80 (30)		5	23.2	0	0	14/9	+55					
	120 (45)		5	22.6	−0.8	−2	16/9	+77					
1i	40 (15)	q4d(2,6)	5	23.0	+3.6	+16	11/9	+22					
	80 (30)		5	19.8	+3.4	+17	12/9	+33					
	120 (45)		5	23.8	+1.2	+5	13/9	+44					
	160 (60)		5	21.6	+0.4	+2	14/9	+55					
	20 (7.5)	qd× 5	5	17.8	+4.0	+22	12/9	+33					
	40 (15)		5	17.4	+3.0	+17	13/9	+44					
	80 (30)		5	17.4	+2.4	+14	14/9	+55					
	120 (45)		5	17.4	+1.4	+8	19/9	+111					
2a	180 (75)	qd× 5	7	23.2	−2.6	−11	16/8	+100 (1)					
	240 (100)		7	23.4	−4.0	−17	17/8	+112 (1)					
2b	117 (50)	qd× 5	9	24.4	−1.6	−7	14/8	+75	22.4	+2.0	+9	8/8	0
	234 (100)		9	24.2	−2.6	−11	19/8	+138 (3)	21.4	+0.2	+1	8/8	0
	468 (200)		9	23.6	−3.8	−16	16/8	+100 (2)	22.0	−2.4	−20	7/8	−13
2c	130 (50)	qd× 5	6	23.0	+0.9	+4	13/8	+62					
	260 (100)		6	22.8	+0.9	+4	13/8	+62					
	390 (200)		6	24.5	−1.6	−7	14/8	+74					
2d	115 (50)	qd× 5	7	20.6	+2.0	+10	10/8	+25					
	230 (100)		7	22.2	+1.8	+8	11/8	+37					
	460 (200)		7	19.8	+2.8	+14	14/8	+75 (1)					
2e	133 (50)	qd× 5	7	24.0	−2.6	−11	17/8	+112 (1)					
	200 (75)		7	24.4	−4.0	−16	19/8	+137 (2)					
	267 (100)		7	24.0	−3.6	−16	20/8	+150 (4)					
ara-C	250	q4d(2,6)	5	21.4	+1.4	+7	13/8	+62	19.4	+4.0	+20	9/8	+12
	500		5	22.6	+0.4	+2	13/8	+62	20.2	+3.8	+19	9/8	+12
	1000		5	24.0	+0.8	+3	15/8	+87	22.0	+2.4	+11	8/8	0
	50	qd× 5	9	24.0	+0.2	0	14/8	+75	24.0	+0.8	+3	8/8	0
	100		9	22.4	−1.0	−5	16/8	+100	24.0	−1.1	−4	7/8	−13
	200		9	22.6	−1.6	−7	17/8	+112	22.4	−3.1	−14	7/8	−13

[a] Highest doses listed are the maximum nontoxic levels that were found for each drug on the indicated schedule. Numbers in parentheses represent approximate *ara*-C equivalent doses. [b] The average weight per mouse on day 1 for the multiple groups of untreated controls in these experiments was 23.8 g (range 21.0–26.8 g). The average 7-day weight gain for the controls was 3.1 g (+13%), and there was no difference between the untreated mice with L1210 leukemia and those with the L1210/*ara*-C tumor. [c] T = treated; C = control; ILS = increase in life span. Numbers in parentheses represent mice surviving 20 or more days (ILS ≥ 150%). There were no 30-day survivors.

molar dose of **1i** was 2.5-fold lower than that of *ara*-C. The N^4-palmitoyl derivative **1h** was less active than **1i**, and once again activity against L1210/*ara*-C leukemia could not be demonstrated even though **1h** was active against the parent tumor.

All five of the cyclo-CMP esters **2a–e** were tested in vivo against L1210 leukemia, and one of them (**2b**) was tested against the L1210/*ara*-C tumor in order to compare it with **1b** and **1h**. The effect of varying the nature of the 5′-(alkyl phosphate) group was examined by comparing the ILS of the homologous esters **2b–d** at equimolar doses. At "*ara*-C equivalent doses" of 100 mg/kg, for example, the median ILS for these compounds was +138, +62, and +37%, respectively. Changing the alkyl chain length from C_2 to C_6 thus caused a progressive decrease in activity which was reminiscent of the effect noted in culture with the analogous *ara*-CMP esters **1a–c**. Not surprisingly, compounds **2b–d** also exhibited an inverse correlation between host toxicity and alkyl chain length, with the C_2 ester **2b** causing the greatest weight loss and the C_6 ester **2d** causing the least. Shortening of the 3′-*O*-acyl chain by two carbons while retaining the 5′-(*n*-butyl phosphate) moiety, as in the pair of compounds **2a** and **2c**, had a favorable effect, with *ara*-C equivalent doses of 100 mg/kg producing life span increases of +112 and +62%, respectively. Since the 3′-*O*-myristoyl ester **2a** would be expected to be less lipophilic than the 3′-*O*-palmitoyl ester **2c**, which contains two more CH_2 groups, we reasoned that other structural changes leading to a more favorable water–lipid partition coefficient might likewise improve activity. This was borne out by the results with the 5′-(ethoxyethyl phosphate) ester **2e**, which was the most water soluble and also the most active of the five compounds in the series, with four out of seven animals showing a greater than +150% ILS at an *ara*-C equivalent dose of 100 mg/kg.

In summary, while simple 5′-*O*-alkylphosphorylation has not thus far provided a means to overcome *ara*-C resistance based on kinase deficiency, it does represent a feasible method of "multiple latentiation" that gives rise to products whose in vivo antileukemic activity on the qd×5 and q4d(2,6) schedule is comparable to or in some instances greater than that of *ara*-C. A substantial reduction in optimal dose was seen with several compounds, including especially the N^4-stearoyl 5′-(*n*-butyl phosphate) and 3′-*O*-palmitoyl-2,2′-anhydro 5′-(ethoxyethyl phosphate) derivatives **1i** and **2e**. Even though this type of dose-sparing effect would undoubtedly have been possible with *ara*-C itself by increasing the frequency of administration or by the use of continuous infusion, there are obvious practical advantages to once-daily treatment that militate in favor of the prodrug approach. Structure–activity comparisons in vitro and in vivo indicated that the nature of the 5′-(alkyl phosphate) group can markedly affect activity, probably by influencing the water–lipid partition coefficient. From the data obtained with the limited number of compounds in this study, we conclude that with highly lipophilic groups already in place in the molecule, as in 3′-*O*-acyl-2,2′-anhydro or N^4-acyl derivatives, the introduction of additional 5′-(alkyl phosphate) substitution is effective only when it does not cause the partition coefficient to shift too far in the direction of lipid solubility. The favorable results obtained with the glyceryl phosphate esters **1f** and **1g** in vitro and with the ethoxyethyl phosphate ester **2e** in vivo support this interpretation and suggest that other kinds of hydroxyalkyl and alkoxyalkyl substitution on the 5′-phosphate group may be worthwhile to pursue. The glyceryl phosphate ester **1g** is of interest because of its relationship to 2,3-diacyl-1-glyceryl phosphate esters of *ara*-C, which have been reported to possess in vivo activity against a kinase-deficient P388 mouse leukemia.[33] Recently described esters of *ara*-CMP in which the 5′-phosphate group is joined to corticosteroids[34] represent more complex examples of such hydroxyalkyl substitution. The lack of activity of **1b**, **1h**, and **2b** against the L1210/*ara*-C tumor in vivo suggests that metabolic degradation of these prodrugs in the host circulation occurs so rapidly that the amount of intact phosphodiester reaching the tumor is too small to be therapeutically significant. Hong and co-workers[34] have shown with purified enzymes that steroid conjugates of *ara*-CMP yield *ara*-CMP rather than *ara*-C on incubation with phosphodiesterase but that the further action of serum phosphatases can rapidly convert *ara*-CMP to *ara*-C, which thereupon becomes vulnerable to cleavage by cytidine deaminase. We similarly observed that when **1b** was incubated in fresh mouse serum, which contains both phosphodiesterase and phosphatase activity,[27] no *ara*-CMP was detectable by TLC analysis. If cleavage of *ara*-CMP to *ara*-C by serum phosphatases were occurring very rapidly in vivo, the L1210 tumor would be expected to respond to prodrugs such as **1b**, **1h**, and **2b**, whereas the kinase-lacking L1210/*ara*-C tumor would not. The alternative possibility that *ara*-CMP was formed intracellularly but failed to be further converted to the biologically active triester *ara*-CMP is improbable, since nucleotide kinase activity in L1210 and L1210/*ara*-C cells is known to be approximately the same.[32]

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 137B double-beam recording spectrophotometer, and ultraviolet spectra were recorded on a Cary Model 15 instrument. NMR spectra were determined by means of a Varian T60A instrument with Me_4Si as the reference. TLC was performed on Eastman 13181 silica gel or Eastman 13254 cellulose sheets containing a fluorescent indicator, and spots were visualized under UV light at 254 nm. Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) and are not corrected. Microchemical analyses were performed by Galbraith Laboratories, Knoxville, TN, and were within ±0.4% of theoretical values.

Preparation of *Ara*-C 5′-(Alkyl phosphate) Esters. Method A. 1-β-D-Arabinofuranosylcytosine 5′-(*n*-Octyl phosphate) (1d). A stirred mixture of redistilled trimethyl phosphate (10 mL) and $POCl_3$ (0.7 mL) was cooled to 0 °C, and *ara*-C (1.0 g, 4.1 mmol) was added. Stirring was continued at 0–5 °C for 2 h, anhydrous *n*-octyl alcohol (3 mL, dried over Linde type 4Å molecular sieves) was added, the mixture was kept at 0–5 °C overnight, and the reaction was terminated by pouring the solution into a mixture of Et_2O (200 mL) and ice-cold 10% $NaHCO_3$ (10 mL). The aqueous layer was separated, adjusted to pH with dilute HCl, and applied onto an ion-exchange column (Dowex 1-X2, formate, 3 × 12 cm) which was eluted first with deionized water (300 mL) and then with a linear formic acid gradient (0–0.3 M, 500 mL total). Individual 5-mL fractions were collected, analyzed by TLC, pooled as appropriate, and freeze-dried: yield 0.88 g; NMR (Me_2SO-d_6) δ 7.86 (d, 1 H, J = 8 Hz, H_6), 5.8–6.0 (m, 2 H, H_5 nd $H_{1'}$), 0.6–1.7 (m, 15 H, alkyl chain).

1-β-D-Arabinofuranosylcytosine 5′-(*n*-Hexadecyl phosphate) (1e). *Ara*-C (1.0 g, 4.1 mmol) was added with stirring to an ice-cold mixture of $POCl_3$ (0.38 mL) and redistilled trimethyl phosphate (20 mL). After 2 h of stirring at 0–5 °C, a cold (10

(33) Turcotte, J. G; Srivastava, S. P.; Meresak, W.; Rizkella, B.; Louzon, F.; Wunz, T. P. *Biochim. Biophys. Acta* **1980**, *619*, 604. Turcotte, J. G., Srivastava, S. P.; Steim, J. M.; Calabresi, P.; Tibbetts, L. M.; Chu, M. Y. *Biochim. Biophys. Acta* **1980**, *619*, 619.

(34) Hong, C. I.; Nechaev, A.; West, C. R. *J. Med. Chem.* **1979**, *22*, 1428. Hong, C. I.; Nechaev, A.; Kirisits, A. J.; Buchheit, D. J.; West, C. R. *J. Med. Chem.* **1980**, *23*, 1343.

°C) solution of n-hexadecyl alcohol (1 g) in $CHCl_3$ was added dropwise, and the mixture was allowed to come to room temperature overnight. The homogeneous solution was then poured slowly into ice-cold 10% $NaHCO_3$ (10 mL), and the product was extracted into $CHCl_3$ (2 × 100 mL). The organic layer was washed with H_2O (3 × 50 mL) and evaporated to dryness under reduced pressure. The residue was triturated with Et_2O, and the insoluble material was purified by column chromatography on silica gel (2.5 × 28.5 cm). The column was eluted first with $CHCl_3$ (100 mL) and then with 9:1 *i*-PrOH–ammonium formate (0.4 M). Appropriate TLC-homogeneous fractions were pooled and lyophilized: yield 0.63 g. The analytical sample was obtained by repeating the chromatography on a column of cellulose (2.5 × 26 cm) with 7:2:1 *i*-PrOH–H_2O–concentrated NH_4OH as the eluent. Appropriate fractions were combined and freeze-dried: yield 0.47 g. Since the elemental analysis showed the product to be the free acid rather than the ammonium salt, freeze-drying apparently was accompanied by loss of ammonia.

1-β-D-Arabinofuranosylcytosine 5'-(1-L-Glyceryl phosphate) (1g). *Ara*-C (1.0 g, 4.1 mmol) was added with stirring to an ice-cold mixture of $POCl_3$ (0.45 mL) and redistilled trimethyl phosphate (10 mL). After 2 h of stirring at 0–5 °C, a cold solution of L-1-*O*-benzylglycerol (1.0 g, 5.5 mmol) in trimethyl phosphate (3 mL) was added dropwise, and the mixture was allowed to come gradually to room temperature and left to stir for 2 days. The mixture was then poured slowly into ice-cold 10% $NaHCO_3$ (10 mL) and the pH was adjusted to 6.0. After being washed with $CHCl_3$ (3 × 20 mL), the aqueous layer was readjusted to pH 6.0 and applied onto an ion-exchange column (Dowex 1-X2-formate, 2.8 × 11 cm) which was eluted successively with deionized water (300 mL) and a linear formic acid gradient (0 to 0.1 M, 500 mL total). TLC-homogeneous fractions of 5 mL each were pooled as appropriate and freeze-dried to obtain the intermediate **1f** as a fluffy colorless solid (0.31 g): NMR (Me_2SO-d_6) δ 7.88 (d, 1 H, J = 8 Hz, H_6), 7.28 (s, 5 H, aromatic protons), 5.7–6.1 (m, 2 H, H_5 and $H_{1'}$), 4.46 (s, 2 H, benzylic CH_2). To a solution of this material (120 mg, 0.25 mmol) in 80% EtOH (20 mL) in a 500-mL Parr hydrogenation bottle was added a slurry of 10% Pd/C (20 mg) in H_2O and the mixture was shaken under H_2 (1 atm) at room temperature for 4 h. The catalyst was removed by filtration through Celite and washed with aqueous EtOH, and the combined filtrate and wash solution were concentrated by rotary evporation until all the EtOH was removed. Lyophilization of the remaining aqueous solution afforded a foam which on trituration with Et_2O formed a colorless powder (85 mg): NMR (Me_2SO-d_6) δ 7.85 (d, 1 H, J = 8 Hz), 5.7–6.2 (m, 2 H, H_5 and $H_{1'}$).

Preparation of 2,2'-Anhydro-*ara*-C 5'-(Alkyl phosphate) Esters. Method B. 2,2'-Anhydro-1-β-D-(3'-*O*-palmitoylarabinofuranosyl)cytosine 5'-(2-Ethoxyethyl phosphate) (2e). Cytidine (2 g, 8.2 mmol) was added with stirring to an ice-cold mixture of $POCl_3$ (1.4 mL) and trimethyl phosphate (20 mL). After 2 h at 0–5 °C, dry 2-ethoxyethanol (5 mL) was added dropwise and stirring was continued in the cold overnight. The reaction was terminated by being poured slowly into ice–water containing 1.5 g of $NaHCO_3$. After extraction with $CHCl_3$ (3 × 50 mL), the aqueous layer was adjusted to pH 4 and applied onto a column of activated carbon (60 g) which was eluted with 10:10:1 EtOH–H_2O–concentrated NH_4OH. Most of the alcohol and ammonia were removed from the eluate under reduced pressure, and the concentrate was lyophilized. The colorless residue was redissolved in H_2O (20 mL), and the solution was applied onto an ion-exchange column (Dowex 1-X2, formate, 3 × 29 cm) which was eluted successively with deionized H_2O (100 mL) and a linear gradient of 0 to 0.1 M formic acid (total volume 500 mL). Individual volumes of 5 mL were monitored by TLC, pooled as appropriate, and freeze-dried to obtain cytidine 5'-(2-ethoxyethyl phosphate) as a colorless solid (1.3 g, 40%): NMR (D_2O) δ 8.13 (d, J = 8 Hz, H_6), 6.23 (d, J = 8 Hz, H_5), 5.85 (d, J = 3 Hz, $H_{1'}$), 1.13 (t, J = 7 Hz, CH_3). Palmitoyl chloride (2.1 g) was added dropwise to a refluxing solution of the above cytidine ester (1 g) in dry acetonitrile (40 mL) containing $BF_3 \cdot Et_2O$ (0.94 mL), and refluxing was continued for 25 min. Vacuum evaporation of the solvent and $BF_3 \cdot Et_2O$ left a residue, which was taken up in EtOH (100 mL). After treatment of the solution with Dowex 1-X2 (Cl^- form, 5 g), filtration, and solvent removal, the residue was applied onto a silica gel column (2.5 × 26 cm) which was eluted successively with $CHCl_3$ (200 mL) and 9:1 *i*-PrOH–ammonium formate (0.4 M, pH 4). Appropriate TLC-homogeneous fractions (5 mL each) were pooled, most of the *i*-PrOH was removed by rotary evaporation, and the remaining aqueous solution was freeze-dried: yield 0.89 g.

Preparation of N^4-Acyl-*ara*-C 5'-(Alkyl phosphate) Esters. Method C. N^4-Stearoyl-1-β-D-arabinofuranosylcytosine 5'-(*n*-Butyl phosphate) (1i). *Ara*-C (2.0 g, 8.2 mmol) was dissolved in H_2O (15 mL) and a solution of stearic anhydride (8.8 g, 16 mmol) in dioxane (200 mL) was added dropwise at room temperature. The mixture was then heated with stirring at 90 °C (bath temperature) for 5 h, allowed to cool to room temperature overnight, and concentrated to dryness on the rotary evaporator. Washing of the residue with benzene (to remove unreacted stearic anhydride), followed by recrystallization from EtOAc (900 mL), gave N^4-stearoyl-1-β-D-arabinofuranosylcytosine as a colorless powder (3.3 g, 80%): mp 149–153 °C (lit.[11] mp 147–151 °C). A slurry of this product (1.1 g, 2.6 mmol) in triethyl phosphate (5 mL) was added in portions with stirring to a mixture of $POCl_3$ (0.4 mL) and triethyl phosphate (10 mL) at 0 °C. After 3 h at 0 °C, *n*-butyl alcohol (1.6 mL) was added to the clear solution, and stirring was continued in the cold overnight. The reaction was terminated by being poured into ice–H_2O (250 mL), and the precipitated solid was filtered, washed with H_2O (3 × 20 mL), and air-dried. Washing with hot EtOAc (50 mL), in portions, yielded analytically pure white solid (0.28 g).

Cytotoxicity Assays. The use of L1210 mouse leukemia and B16 mouse melanoma cells for in vitro bioassay has been described.[35,36] The L1210 cells were grown in suspension in Eagle's minimal essential medium containing 15% whole fetal calf serum, streptomycin (100 μg/mL), penicillin (100 units/mL), and 0.05 mM 2-mercaptoethanol. The B16 cells were grown as monolayers in McCoy's Medium 5A containing serum and antibiotics as specified above, except that the concentration of serum was 10%; 2-mercaptoethanol was omitted. Cells were incubated in both instances in a 5% CO_2 humidified atmosphere at 37 °C. Cultures were treated with regularly spaced doses of each drug, generally ranging from 10^{-7} to 10^{-3} M. Compounds were dissolved in pH 7.4 potassium phosphate buffer (0.05 M) or in Me_2SO prior to being added to the medium. Cells were counted after 48 h with the aid of a Coulter Model F hemocytometer, survival was plotted to give a dose–response curve, and the 50% inhibitory dose (ID_{50}) relative to untreated controls was determined. Assays were performed in triplicate and have a standard deviation of ±10%. The results are given in Table II.

Antitumor Assays against L1210 Leukemia in Mice. Standard NCI protocols were employed.[37] Groups of five to nine male B6D2F$_1$J mice (Jackson Laboratory, Bar Harbor, ME) were inoculated intraperitoneally with 10^5 L1210 or L1210/*ara*-C cells on day 0, and drug treatment was begun on day 1 on either the qd×5 or q4d(2,6) schedule. *Ara*-C was administered in sterile water, **1b** in pH 7.4 potassium phosphate buffer (0.05 M), and the other compounds in a vehicle consisting of 0.4% Tween 80, 0.5% low-density carboxymethylcellulose, 0.9% benzyl alcohol, and 0.9% saline. The increase in survival (ILS) was calculated according to the following formula: % ILS = (T/C − 1) × 100. In this formula, T and C are the median survival times in days for the treated and control groups, respectively. All animals were weighed on days 1 and 7, and 7-day weight changes were calculated as a percentage. The results are given in Table III.

TLC Analysis of Serum Hydrolysis of 1b. A 20-μL aliquot of a stock aqueous solution of **1b** (6 mg/mL) was added to fresh mouse serum (180 μL) from several B6D2F$_1$J mice, and the mixture was placed in a shaking water bath at 37 °C. At various times, aliquots of the incubation mixture were spotted on cellulose TLC sheets which were developed immediately with (A) 16:3:1 saturated $(NH_4)_2SO_4$–1 N NaOAc–*i*-PrOH or (B) plain distilled H_2O. Authentic specimens of *ara*-CMP, *ara*-C, and *ara*-U were spotted as controls. The R_f values for **1b**, *ara*-C, and *ara*-CMP in system A were 0.40, 0.55, and 0.68, respectively. *Ara*-U could

(35) Wick, M. M. *Cancer Treat. Rep.* **1979**, *63*, 991.
(36) Wick, M. M. *Cancer Res.* **1980**, *40*, 1414.
(37) Geran, R. I.; Greenberg, N. H.; Macdonald, M. M.; Schumacher, A. M.; Abbott, B. J. *Cancer Chemother. Rep. Part 3*, 1972, *3*(3), 1.

not be separated from *ara*-C in system A but was readily distinguishable in system B, which produced R_f values of 0.72 (*ara*-U) and 0.83 (*ara*-C). Unchanged starting material was observed in the incubation of **1b** even after 24 h, along with an approximately equal amount of a second compound which had the same R_f as *ara*-U. However, there was never more than a trace of *ara*-CMP (control experiments indicated that the TLC assay would be sensitive down to ~5% conversion of **1b** to *ara*-CMP). When *ara*-CMP itself was used in place of **1b**, the product comigrating with authentic *ara*-U was again observed, but no unchanged *ara*-CMP could be seen. It thus appeared that formation of *ara*-CMP from **1b** proceeds much more slowly than subsequent cleavage of the *ara*-CMP to *ara*-C and ultimately *ara*-U. As a result, the amount of free *ara*-CMP present at any one time when **1b** is exposed to the enzymes in mouse serum must be extremely small.

Acknowledgment. This work was supported in part by Grants CA 23151 and CA 22738 from the National Cancer Institute (NIH). The authors are indebted to Gerda Swedowsky and Linda Anderson for their technical assistance in obtaining the cytotoxicity and in vivo antitumor data, respectively, and to Dr. Frank M. Schabel, Jr., Southern Research Institute, Birmingham, AL, for providing the L1210/*ara*-C cells for these studies.

Activated *N*-Nitrosocarbamates for Regioselective Synthesis of *N*-Nitrosoureas

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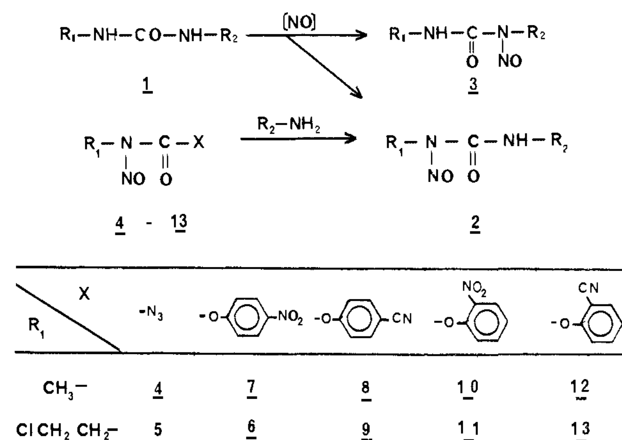
A practical and convenient method for synthesizing antitumor compounds, *N*-alkyl-*N*-nitrosoureas, regioselectively nitrosated on the nitrogen atom bearing the alkyl group is proposed. *N*-Alkyl-*N*-nitrosocarbamates are interesting intermediates in these syntheses and yield, by reaction with amino compounds, the regioselectively nitrosated *N*-alkyl-*N*-nitrosoureas. As an interesting example, *N,N'*-bis[(2-chloroethyl)nitrosocarbamoyl]cystamine, a new attractive oncostatic derivative, has been prepared. The cytotoxic activity of these various compounds were tested on L1210 leukemia.

Among significant compounds, nitrosoureas are an extremely active class of antitumor agents that are effective against solid tumors, as well as leukemias. In particular, 2-haloethyl derivatives and some of their metabolites show great promise as effective antitumor agents.[1-3] For the treatment of a number of experimental and clinical tumors, several *N*-(2-chloroethyl)-*N*-nitrosoureas have successfully been applied as chemotherapeutic agents.[2] Not only do these drugs show the ability to inhibit the growth and spread of many forms of solid tumors in men and animals,[2,4,5] but some of them, such as *N,N'*-bis(2-chloroethyl)-*N'*-nitrosourea (BCNU) and *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (CCNU), also have been found to rapidly enter the cerebrospinal fluid and control meningeal tumor implants.[6] All of these compounds are undergoing intense clinical trials, and some of them have recently been made commercially available.

N-Nitrosoureas are usually and readily obtained by the conventional route of preparing the urea structure first then subsequently nitrosating it with a variety of nitrosating agents, e.g., sodium nitrite in acidic medium, nitrous anhydride, nitrosyl chloride, dinitrogen tetroxide, or nitrosium tetrafluoroborate. However, it is known that when nitrosoureas are synthesized by one of the above-mentioned procedures, difficulties in achieving selective nitrosation of the urea 1 at the required position are encountered and both isomers 2 and 3 are sometimes produced.[7] Selective nitrosation at the nitrogen bearing the methyl or the 2-chloroethyl group is critical in syntheses of unsymmetrical N,N'-disubstituted 2-chloroethyl-*N*-nitrosoureas.[7] Nitrosation by those conventional methods have been shown to favor formation of *N*-(2-chloroethyl)-*N*-nitroso compound 2 in those cases where the geometry of the substituent at position N' provides steric control and directs the nitroso group into the required position. Selective nitrosation fails, however, when there is no such steric control. Moreover, *N*-nitrosoureas of type 3 have been shown in many cases to present no antitumor activity.[8] Furthemore, separation of the unwanted byproduct 3 from the desired compound 2 requires complex

Scheme I



(1) H. H. Hansen, O. S. Selawry, F. M. Muggia, and M. D. Walker, *Cancer Res.*, **31**, 223 (1971).
(2) R. C. Young, M. D. Walker, G. P. Canellos, P. S. Schein, B. A. Chabner, and V. T. de Vita, *Cancer*, 31, 1164 (1973).
(3) J. A. Montgomery, T. P. Johnston, and Y. F. Shealy, "Burger's Medicinal Chemistry", 4th ed., Part II, M. E. Wolfe, Ed., Wiley, New York, 1979, p 595.
(4) K. Sigiura, *Proc. Am. Assoc. Cancer Res.*, **6**, 62 (1965).
(5) G. R. Gale, *Biochem. Pharmacol.*, 14, 1707 (1965).
(6) F. M. Schabel Jr., T. P. Johnston, G. S. McCaleb, J. A. Montgomery, W. R. Laster, and H. E. Skipper, *Cancer Res.*, **23**, 725 (1963). T. H. Wasserman, M. Slanik, and S. K. Carter, *Cancer*, **36**, 1258 (1975).
(7) J. A. Montgomery, H. J. Thomas, R. W. Brockman, and G. P. Wheeler, *J. Med. Chem.*, 24, 184, (1981).
(8) J. Oiry, J. Martinez, J. L. Imbach, and F. Winternitz, *Eur. J. Med. Chem.*, in press.

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