Zwitterionic 3′-*O*-Acyl Derivatives of Thymidine 5′-Phosphate as Potential Sources of Intracellular Thymidine 5′-Phosphate in Cells in Culture

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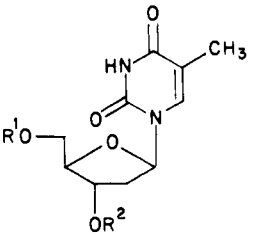
A convenient route is described for attachment of acyl groups (1) $CO(CH_2)_nN(Et)_2(CH_2)_mNH(Et)_2$ (n = 3, m = 2; n = 4, m = 2–4), (2) $CO(CH_2)_nN(Et)_2(CH_2)_mNEt_3$ (n = 4, m = 2–4), or (3) $CO(CH_2)_4N(CH_2CH_2)_3N(CH_2)_nCH_3$ (n = 1 or 9) to O-3′ of thymidine 5′-phosphate (TMP). The compounds are prototypes of 5′-nucleotide derivatives in which the two anionic charges could become partially masked in intramolecular anionic–cationic interactions and which might be able to diffuse into mammalian cells to furnish intracellular antimetabolite 5′-nucleotides by hydrolytic loss of a dicationic 3′-*O*-acyl group. At pH 7.6, 37 °C, hydrolyses of the 3′-ester linkages were pseudo first order with $t_{1/2}$ values in the range 28–85 h. Paper chromatography in n-PrOH–H_2O at pH 7.6 showed that type 1 or 2 derivatives were equally or slightly less hydrophobic than TMP (R_f 0.24), whereas the n-decyl type 3 compound (R_f 0.66) was markedly more hydrophobic, apparently because chain branching in group 3 is less than in 1 or 2. A sensitive and specific assay was developed for liberation of intracellular TMP in cultured mouse L fibroblasts in which synthesis of TMP and, hence, of DNA was suppressed by a combination of aminopterin and 5′-amino-5′-deoxythymidine (5′-NH_2-dT). The TMP derivatives (100 μM) stimulated DNA synthesis, but omission of 5′-NH_2-dT increased stimulation 6.5-fold, suggesting that stimulation occurred via degradation of the derivatives to dT. In confirmation, derivatives of type 2 (n = 4, m = 2 or 4) (100 μM) or type 3 (n = 9) (200 μM), in the presence of aminopterin, did not stimulate DNA synthesis in the LM(TK$^-$) strain of L cells, which is genetically deficient in dT kinase. TMP (1 mM) stimulated DNA synthesis 2–3-fold and appeared to enter LM(TK$^-$) cells without dephosphorylation, because dT (1 mM) gave no stimulation. If TMP is assumed to enter solely by passive diffusion, the inactivity of the TMP derivatives can be ascribed in part to their 2-fold higher molecular weight which can be expected to reduce flux through natural membranes ca. 16-fold.

Growth inhibition by most antineoplastic purine and pyrimidine base and nucleoside analogues requires their intracellular anabolism to 5′-mononucleotides. In drug-resistant neoplasms the level of these nucleotides is usually low.[1] The nucleotides themselves have been chemotherapeutically ineffective against drug-resistant neoplasms, most likely because they are dephosphorylated by plasma enzymes[2-4] and are present at physiological pH principally in the form of poorly membrane-permeable dianions.[5-7] In light of this, various potential nucleotide prodrugs have been studied as inhibitors of resistant neoplasms, though only partial successes have as yet been reported.[8] In all cases, nucleotide derivatives were studied in which one or both anionic oxygens were masked by covalent bond formation with a group expected to enhance hydrophobicity and, thereby, membrane permeability. The present work initiates exploration of a new approach in which partial masking of the nucleotide anionic charges is sought via their involvement in intramolecular ionic interactions with two amino groups that are protonated at physiological pH. The amino groups are incorporated into an acyl substituent that is attached to *O*-3′ of the furanose ring of the nucleotide (as in **6**, **7**, and **10**) to produce an ester that can undergo hydrolysis at physiological pH to regenerate the free nucleotide (**1**). Derivatives of the 2′-deoxy nucleotide, thymidine 5′-phosphate (TMP, **1**), were employed as models for candidate prodrugs of antimetabolite 5′-nucleotides in the present work in order to simplify syntheses and to preclude the 2′,3′ acyl migrations that tend to occur with ribonucleoside derivatives under physiological conditions. Since tri- and tetraethylammonium salts of 5′-nucleotides are normally sufficiently hydrophobic to be soluble in relatively polar organic solvents (e.g., the lower alcohols), the present studies employed acyl groups containing either one tertiary and one quaternary diethylammonium residue (structures **6**) or two such quaternary residues (**7**, **10**). An *N*,*N*′-di-*n*-alkyl-1,4-diazabicyclo[2.2.2]octane residue was incorporated into several of the 3′-*O*-acyl TMP derivatives (**10**: m = 1 or 9) because ionic complexes of certain nucleotides with *N*,*N*′-di-*n*-octadecyl-1,4-diazabicyclo[2.2.2]octane possess a high $CHCl_3$–H_2O (pH 8) partition coefficient.[9]



1: $R^1 = PO_3^{2-}$, $R^2 = H$

2: $R^1 = (Ph)_3C$, $R^2 = H$

3: $R^1 = H$, $R^2 = C(O)(CH_2)_nCl$

4: $R^1 = H$, $R^2 = C(O)(CH_2)_nN^+(Et)_2(CH_2)_mNH(Et)_2 \cdot 2Cl^-$

5: $R^1 = H$, $R^2 = C(O)(CH_2)_nN^+(Et)_2(CH_2)_mNEt_3 \cdot 2Cl^-$

6: $R^1 = PO_3^{2-}$, $R^2 = C(O)(CH_2)_nN^+(Et)_2(CH_2)_mNH(Et)_2$

7: $R^1 = PO_3^{2-}$, $R^2 = C(O)(CH_2)_nN^+(Et)_2(CH_2)_mNEt_3$

8: $R^1 = H$, $R^2 = C(O)(CH_2)_4N^+$⟨pyridinium⟩$N \cdot Cl^-$

9: $R^1 = H$, $R^2 = C(O)(CH_2)_4N^+$⟨pyridinium⟩$N^+ \cdot (CH_2)_mCH_3 \cdot 2Cl^-$

10: $R^1 = PO_3^{2-}$, $R^2 = C(O)(CH_2)_4N^+$⟨pyridinium⟩$N^+ \cdot (CH_2)_mCH_3$

We describe here a convenient synthetic route to the 3′-*O*-acyl TMP derivatives **6** (n = 3, m = 2; n = 4, m = 2–4), **7** (n = 4, m = 2–4), and **10** (m = 1 or 9), together with studies of the influence of the structure of the acyl groups upon the hydrolysis rates of the 3′-ester bonds and upon the hydrophobicities of the molecules as indicated by R_f

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values from paper partition chromatography carried out at pH 7.6. The prodrug potential of several of these TMP derivatives was evaluated by assay of the incorporation of radioactive 2′-deoxycytidine into deoxyribonucleic acid (DNA) in cultured cells in which biosynthesis of TMP, and thus of DNA, was blocked genetically and/or by enzyme inhibitors. Under these conditions, labeling of DNA would be expected only as a result of intracellular formation of TMP by hydrolysis of the 3′-ester bonds.

Syntheses. Treatment of 5′-*O*-tritylthymidine (**2**) with 1.5 equiv of the appropriate ω-chloroacyl chloride in tetrahydrofuran in the presence of pyridine, followed by removal of the trityl group from the product with aqueous 80% acetic acid, gave the 3′-*O*-(ω-chloroacyl)thymidines **3** (n = 3 or 4) in 75–80% yields. Treatment of **3** with NaI–acetone gave the corresponding iodoacyl derivatives quantitatively. These were treated in *N*,*N*-dimethylformamide (DMF) with 20 equiv of the appropriate *N*,*N*,*N*′,*N*′-tetraethyl-α,ω-diaminoalkane, after which the desired mono-N-alkylated derivatives **4** (n = 3, m = 2; n = 4, m = 2–4) of those bases were obtained in 45–55% yields as tertiary ammonium hydrochlorides of quaternary ammonium chlorides by elution with HCl from a carboxylic acid type cation-exchange resin. Prior to cation-exchange chromatography the reaction mixtures contained ca. 15% as much of a second UV-absorbing product that was more polar on paper chromatograms and was inert toward ethyl iodide and was concluded to be an α,ω-bis(alkyldiethylammonio) alkane arising from alkylation of **4** by unreacted 3′-*O*-(ω-iodoacyl)thymidine. Mixtures of this byproduct with the nonprotonated tertiary base forms of compounds **4** were isolated following the foregoing reactions of 3′-*O*-(ω-iodoacyl)thymidines with tetraethyl-α,ω-diaminoalkanes and were treated with 5 equiv of EtI in DMF at 40 °C, leading to quantitative conversion of compounds **4** to the diquaternary ammonio derivatives **5**. These were purified by cation-exchange chromatography and isolated in ca. 80% yields as homogeneous dichlorides. Phosphorylation of **4** or **5** was readily effected with $POCl_3$ in trimethyl phosphate.[10] The resulting 3′-*O*-acylthymidine 5′-phosphorodichloridates were hydrolyzed to **6** or **7** at 0 °C in aqueous tetrahydrofuran in reactions conveniently followed from the progressive lowering in pH that occurred. Inorganic phosphate was removed by anion-exchange chromatography on Dowex-1 (Cl^-) resin, after which **6** (n = 3, m = 2; n = 4, m = 2–4) and **7** (n = 4, m = 2–4) were purified by paper chromatography and isolated in 60–65% yields as tri- or tetrahydrates. Compounds **6** and **7** were homogeneous in the paper chromatographic and electrophoretic systems of Table I. Their structure assignment is supported by elemental analyses, by UV spectral properties identical with those of **1**, by electrophoretic characteristics at pH 3.6 and 7.6, and by their conversion to **1** under mildly basic conditions (see below). In confirmation of their zwitterionic character, they contained no chloride ions.

The 3′-*O*-acylthymidine derivative **8** was obtained in 88% yield by reaction of **3** (n = 4) in DMF solution with 1,4-diazabicyclo[2.2.2]octane. With this more reactive base, prior conversion of **3** to its iodo analogue was unnecessary. In addition. the reaction produced little or no diquaternary ammonium byproduct, possibly due to the low basicity of the tertiary amino group of **8** compared with the tertiary amino groups of compounds **4**, as evidenced by the electrophoretic mobilities of **4** and **8** (Table I). Conversions of **8** to its *N*-(*n*-alkyl) derivatives **9** and of **9** to the corresponding TMP derivatives **10** (m = 1 or 9) were carried out by the methods employed in the syntheses of **5** and **7**, respectively.

Table I. Electrophoretic and Paper Chromatographic Properties

compd	n	m	electrophoresis[a] pH 3.6	electrophoresis[a] pH 7.6	R_f values[b] B	C	D	E
thymidine					0.77	0.73	0.70	0.78
TMP (1)			−9.5	−16.5	0.26	0.36	0.23	0.24
3	3				0.86	0.87		
3	4				0.87	0.88		
4	3	2	+15.6				0.37	
4	4	2	+15.1	+9.2	0.74	0.71	0.39	
4	4	3	+15.3	+11.7	0.75	0.73	0.41	
4	4	4	+15.0	+13.2	0.75	0.73	0.43	
6	3	2	+7.5	−4.3	0.24	0.25	0.18	0.28
6	4	2	+7.9	−4.3	0.16	0.12	0.20	
6	4	3	+8.5	+1.6	0.17	0.13	0.21	0.24
6	4	4	+8.1	+1.8	0.18	0.14	0.21	
5	4	2	+15.6	+13.6	0.52	0.43	0.25	
5	4	3	+15.5	+14.2	0.53	0.44	0.29	
5	4	4	+15.2	+14.0	0.54	0.45	0.31	
7	4	2	+8.6	+2.5	0.07	0.12	0.10	0.08
7	4	3	+8.7	+2.4	0.09	0.12	0.12	0.10
7	4	4	+8.3	+2.2	0.10	0.13	0.14	0.11
8			+11.5	+9.5		0.60		
9		10	+13.3	+10.8	0.74	0.88	0.84	0.86
10		1						0.05
10		9	+6.3	+1.6	0.45	0.76		0.66

[a] Mobilities (centimeters toward the cathode) under conditions given under the Experimental Section. [b] For compositions of solvent systems B–E, see the Experimental Section.

Table II. Hydrolysis Rates of 3′-Ester Groups of Thymidine and Thymidine 5′-Phosphate Derivatives in Aqueous 50 mM Potassium Phosphate, pH 7.6, 37 °C

compd	$t_{1/2}$,[a] h	compd	$t_{1/2}$,[a] h
4 (n = 3, m = 2)	28	**5** (n = 4, m = 4)	87
6 (n = 3, m = 2)	28	**7** (n = 4, m = 4)	85
6 (n = 4, m = 3)	74	3′-*O*-acetylthymidine	135
7 (n = 4, m = 3)	72		

[a] Time for hydrolysis of 50% of the ester.

Hydrolysis Rates of 3′-Substituents. Table II lists hydrolysis rates in phosphate buffer at pH 7.6 of the carboxylic ester bonds in representative 3′-*O*-acylthymidine 5′-phosphates **6** and **7** and in 3′-*O*-acylthymidines **4** and **5**. All hydrolyses followed pseudo-first-order kinetics. Rates were unaffected by substituting the relatively poorly nucleophilic sulfonic acid buffer Hepes for the phosphate buffer, indicating lack of participation of phosphate anions in hydrolytic events. The number and position of methylene groups in the acyl moiety were found to yield a variety of hydrolysis rates falling within a range (28–87 h) that appears desirable in hydrolyzable masked TMP derivatives that might act as extracellular sources of intracellular TMP in mammalian cells. Rates rapidly diminished with increasing numbers of methylene groups, in accord with the diminished electron-withdrawing effects of the two ammonium groups, although even when two groups of four methylenes were present, hydrolyses remained faster than that of 3′-*O*-acetylthymidine. No significant differences were detected between the hydrolytic stabilities of analogous nucleosides and nucleotides or of nucleotides with either one or two quaternary ammonium groups in the 3′-substituent.

Hydrophobic Properties of TMP Derivatives. Paper partition chromatography in *n*-propanol–aqueous buffer, pH 7.6 (system E, Table I), and, to a lesser extent, in propanol–water systems (B, C; Table I) was used as a gauge of hydrophobic character in structures **6**, **7**, and **10**. Introduction of 3′-*O*-acyl substituents with one quaternary

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and one tertiary amine function (giving **4** and **6**) had little influence on the R_f values of thymidine (dT) or TMP, suggesting that intramolecular ionic interactions, if occurring in the TMP derivatives **6** under the conditions used for paper chromatography, had little effect on overall hydrophobicity. When the substituent possessed two quaternary amino groups (**5**, **7**, **9**, **10**), a significant decrease in hydrophobicity was seen except for **9** and **10** (m = 9), which were markedly more hydrophobic than dT and TMP, respectively. Among TMP derivatives of structures **6** and **7**, R_f values in solvent E, with one exception noted below, increased in small increments with increasing numbers of methylenes in the acyl group. Notably, hydrophobicity was not influenced in a marked manner by the number of methylene spacer groups between nitrogens in the acyl group, a factor that could have an important bearing on the tendency for intramolecular anionic–cationic bonds to form in **6** and **7**. Compound **6** (n = 3, m = 2) was exceptional in that it was slightly more hydrophobic than its homologues while possessing the least number of methylenes; this effect might stem from reduced basicity of the terminal amine and hence reduced polarity of the acyl group brought about by combined electron withdrawal by the acyl carbonyl and the quaternary ammonium group.

Compound **10** (m = 9) has only two more methylenes with **7** (n = m = 4) yet has a 6-fold higher R_f value, a property that could reflect the higher oil/water partition coefficients that are in general associated with reduced alkyl branching in amines.[11] The important contribution to hydrophobicity of the terminal decyl chain of **10** (m = 9) is further indicated by the large (13-fold) reduction in R_f in solvent E that results when the decyl group is replaced by an ethyl group to give the homologue **10** (m = 1).

Studies with Cells in Culture. To determine the ability of test compounds to enter mammalian cells and serve as precursors of TMP in the synthesis of DNA, a sensitive assay method was developed, based on the ability of the compounds to relieve a block of TMP synthetase, and hence to DNA synthesis, induced by aminopterin in mouse L929 fibroblasts. The blocking medium was a variant of a medium used in cell culture genetics[12] that contains hypoxanthine, aminopterin, and thymidine (dT). In the present studies, dT was omitted and 5'-amino-5'-deoxythymidine was added as an inhibitor of the only alternate route for TMP synthesis, i.e. from dT via dT kinase.[13] Glycine and hypoxanthine were added as exogenous sources of amino acids and purines, respectively. Under these conditions DNA synthesis can resume without delay if an intracellular supply of TMP becomes available. The system could detect significant recovery of DNA synthesis upon the addition of as little as 1 μM dT to the medium (Table III) (see the Experimental Section for further details of the assay).

Compounds representative of structures in the present series [**6** (n = 4, m = 3); **10**] were found to be stable in the culture medium for at least 6 h, 37 °C, except for minor hydrolysis to TMP expected from the rate studies at pH 7.6 (Table II). Furthermore, the rate of DNA synthesis following addition to the blocked system of 50 μM dT was unaffected by 100 μM of any of the present series of TMP derivatives, showing that these compounds did not inhibit the assay system for intracellular TMP at that level. DNA synthesis in cells exposed for 2 h to 10^{-4} M **6** (n = 4, m = 3 or 4), **7** (n = 4, m = 2 or 4), or **10** (m = 9) was enhanced 2- to 6-fold, and to a similar degree by 10^{-6} M dT (Table III). The level of enhancement by **6** and **7** is consistent with the kinetic data of Table II, which show that 1–2% of **6** and **7** is hydrolyzed to TMP under the above conditions. However, the observed DNA synthesis could have arisen via reutilization of dT formed from dephosphorylation of TMP. In the absence of the dT kinase inhibitor, 5'-NH_2-dT, the stimulation of DNA synthesis by **7** (n = 4, m = 2 or 4) increased almost 7-fold (Table IV), suggesting that the stimulation by compounds **7** might in fact have resulted from their chemical and/or enzymatic hydrolysis to dT. This was confirmed by studies under the same conditions with the LM(TK^-) variant of mouse fibroblasts which lacks the predominant (cytoplasmic) form of TK.[14,15] DNA synthesis in LM(TK^-) cells was not stimulated during 4 h by 1 mM dT but was significantly stimulated by 1 mM TMP or 1 mM TTP (Table V), indicating that the cells retained ability to synthesize DNA

Table III. TMP Derivatives **6**, **7**, and **10** as Enhancers of DNA Synthesis in Mouse L929 Cells Blocked in DNA Synthesis with Aminopterin and 5'-Amino-5'-deoxythymidine[a]

compd	concn, M	rate of incorp of [^{3}H]-dC into DNA, cpm h^- (5×10^5 cells)$^{-1}$	enhancement factor[b]
none		174[c]	
thymidine	10^{-6}	465	1.7
	10^{-5}	2937	10.6
	10^{-4}	28200	102.0
6 (n = 4, m = 3)	10^{-4}	605	1.9
	10^{-4}	504	2.2
6 (n = m = 4)	10^{-4}	782	3.5
	10^{-4}	821	3.5
7 (n = 4, m = 2)	10^{-4}	673	5.6
	10^{-4}	1103	4.7
7 (n = m = 4)	10^{-4}	1356	5.7
	10^{-4}	1299	5.6
10 (m = 9)	10^{-5}	163	1.4
	10^{-4}	253	2.3
	10^{-4}	303	1.3

[a] Cells were blocked for 3 h, exposed in blocking medium to test compound for 2 h, then labeled with 1 μCi/mL of [^{3}H]deoxycytidine. All media contained 1.0 mM 5'-amino-5'-deoxythymidine. [b] Ratio of incorporation rate with test compound to that in control cultures from the same experiment. [c] Mean value from 34 individual control plates from various experiments.

Table IV. Effect of 5'-Amino-5'-deoxythymidine (5'-NH_2-dT) on the Stimulation of DNA Synthesis in L929 Cells by TMP Derivatives[a]

compd	5'-NH_2-dT	incorpn rate[b]	enhancement in absence of 5'-NH_2-dT
none	+	185	
	−	426	2.3
7 (n = m = 4)	+	1135	
	−	7462	6.6
7 (n = 4, m = 2)	+	808	
	−	5462	6.8

[a] Experiments were conducted as described in Table III, but 5'-NH_2-dT was either present at 1.0 mM in the recovery medium with the test compound or was omitted. [b] Units: cpm h^{-1} (5×10^5 cells).

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Table V. Effect of TMP Derivatives 7 and 10 on DNA Synthesis in Mouse LM(TK⁻) Cells Blocked in DNA Synthesis with Aminopterin[a]

compd	concn, M	rate of incorpn of [³H]-dC into DNA, cpm h^{-1} $(5 \times 10^5$ cells$)^{-1}$	enhancement factor[b]
none		198[c]	
dT	10^{-6}	185	1.3
	10^{-5}	204	1.5
	10^{-4}	228	1.6
	10^{-4}	251	0.9
	10^{-3}	213	0.8
TMP	10^{-5}	323	1.3
	10^{-5}	256	1.1
	10^{-4}	381	1.5
	10^{-4}	295	1.3
	10^{-3}	596	2.3
	10^{-3}	668	2.9
	10^{-3}	837	3.0
TTP	10^{-3}	671	2.4
7 ($n = m = 4$)	10^{-4}	204	0.8
	10^{-4}	147	1.1
7 ($n = 4$, $m = 2$)	10^{-4}	171	0.6
	10^{-4}	171	1.3
10 ($m = 9$)	2×10^{-4}	389	1.1

[a] Experiments were conducted as described in Table III except that 5′-NH_2-dT was omitted from all media. [b] Ratio of incorporation rate with test compound to that in control cultures from the same experiment. [c] Mean value from 12 control plates from various experiments.

under the test conditions and that a small fraction of the TMP and TTP can enter the cells either in intact form or in a not totally dephosphorylated form and can generate therein the TMP, TDP, or TTP required as sequential intermediates for DNA synthesis. The TMP derivatives (**7**, $n = 4$, $m = 2$ or 4) (0.1 mM) and **10** ($m = 9$) (0.2 mM) appeared to be ineffective in enhancing DNA synthesis in LM(TK⁻) cells (Table V), indicating that they did not furnish detectable amounts of intracellular TMP that was utilizable in DNA synthesis. The ability of TMP, at the same or a somewhat higher level, to enhance DNA synthesis may be associated in part with its 2-fold lower molecular weight. Thus, if it is assumed that TMP crosses the membrane solely by unmediated diffusion, compounds **7** and **10** ($m = 9$) can be expected, by virtue of their higher molecular weights, to permeate about 16 times more slowly than TMP if the mass selectivity index of the membrane is taken to be about 4, as is commonly the case.[16] The slightly higher polarity of **7** will tend further to reduce its permeation rate relative to TMP. On the other hand, the lower polarity of **10** ($m = 9$) should tend to increase its permeation rate relative to TMP; however, the contribution appears, from the results in Table V, to be insufficient to redress the unfavorable effect of the higher molecular weight of **10** ($m = 9$).

Conclusions. The present findings indicate (1) that zwitterionic 3′-*O*-acyl derivatives of TMP that contain one quaternary and one tertiary ammonium group or two quaternary groups are synthetically relatively easy of access, (2) that hydrolysis rates of such derivatives fall within a range that appears suitable in potential prodrugs of intracellular 5′ nucleotides, (3) that R_f values obtained from paper partition chromatography at pH 7.6 suggest that structures involving intramolecular salt bonds, if present under those conditions, were about equally as polar in the present series of TMP derivatives as structures not involving such bonds, and (4) that by minimizing chain branching in the acyl group, it is possible to generate zwitterionic 3′-*O*-acyl TMP derivatives that are considerably more hydrophobic than TMP. If TMP itself enters cells solely by a process of passive diffusion, such derivatives could, in principle, possess relatively good permeability if the unfavorable influence of increased molecular weight was sufficiently offset by enhanced hydrophobicity. Finally, our studies have shown that mouse fibroblasts genetically and/or chemically blocked in TMP synthesis provide a sensitive and specific assay for intracellular TMP and that TMP derivatives are useful models for 5′-nucleotide derivatives that might be able to act as extracellular sources of intracellular antimetabolite 5′-nucleotides.

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Experimental Section

Chemical Synthesis. General Procedures. ω-Chloroacyl chlorides, 1-iododecane, and 1,4-diazabicyclo[2.2.2]octane were purchased from Aldrich Chemical Co., and α,ω-diaminoalkanes, from the Ames Laboratories, Milford, CT. TLC employed Merck F-254 silica gel plates in (A) $CHCl_3$–MeOH (19:1). Paper chromatography (descending technique) employed Whatman No. 1 or 3 MM papers in (B) *i*-PrOH–H_2O (8:2), (C) *n*-PrOH–H_2O (7:3), (D) *n*-BuOH–AcOH–H_2O (4:1:5, upper layer), or (E) *n*-PrOH–aqueous 50 mM potassium phosphate pH 7.6 (7:3). Column chromatography employed Merck 60 silica gel, 70–230 mesh. CG-50 cation-exchange resin was obtained from Sigma Chemical Co. UV spectra were obtained on a Varian Model 635 spectrophotometer. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN, and unless otherwise indicated were within ±0.4% of the theoretical values. Melting points were determined in capillary tubes and are uncorrected. Electrophoresis was performed on Whatman No. 1 paper at 80 V/cm for 30 min at pH 3.6 (0.05 M citrate) and pH 7.6 (0.05 M triethylammonium bicarbonate). Radioactivity was determined with a Beckman Model LS230 liquid scintillation spectrometer.

3′-*O*-(ω-Chloroacyl)thymidines (3). To a stirred solution at 0 °C of 5′-*O*-tritylthymidine (968 mg, 2 mmol) in dry THF (20 mL) and pyridine (1 mL) was added dropwise the appropriate freshly distilled ω-chloroacyl chloride (3 mmol). The mixture was stored at 0–5 °C, and the reaction was monitored by TLC in solvent A. After 4 h, the mixture was filtered and the filtrate was added to an ice–water mixture (200 mL). The white precipitate was collected by filtration, dried in vacuo, and then heated on a steam bath for 1 h with aqueous 80% acetic acid (20 mL). The cooled solution was filtered from insoluble material and was evaporated to dryness in vacuo. The residue was dissolved in MeOH (2 mL) and chromatographed in solvent A on silica gel (50 g). The major product was crystallized from EtOH to yield **3** ($n = 3$) (75%; mp 125–126 °C) or **3** ($n = 4$) (80%; mp 121–122 °C). The compounds were homogeneous in the systems of Table I and in solvent A: R_f 0.21 for $n = 3$, R_f 0.24 for $n = 4$; UV $\epsilon_{267}^{max}(H_2O)$ 9400 ($n = 3$), 9500 ($n = 4$). Anal. [$C_{14}H_{19}N_2O_6Cl$ ($n = 3$) and $C_{15}H_{21}N_2O_6Cl$ ($n = 4$)] C, H, Cl, N.

3′-*O*-[ω-[*N*-[ω-(Diethylamino)alkyl]diethylammonio]-acyl]thymidine Chloride Hydrochlorides (4). To a solution of **3** (1 mmol) in dry acetone (3 mL) was added NaI (1.5 mmol). The solution was stored at 50 °C for 16 h, then filtered from NaCl, and evaporated to dryness under reduced pressure. A solution of the residue in dry DMF (5 mL) was added to the appropriate *N,N,N′,N′*-tetraethyl-α,ω-diaminoalkane (20 mmol), and the mixture was stored at 50 °C for 48 h. Volatiles were removed at 0.05 mm (20 °C), and the syrupy residue was dissolved in EtOH (2 mL) and added dropwise to Et_2O–light petroleum (1:1, 50 mL). The gum was dissolved in EtOH and reprecipitated. A solution of the gum in EtOH (5 mL) was adjusted to pH 4 by addition of 1 N HCl. Volatiles were removed under reduced pressure, and a solution of the residue in water (2 mL) was applied to a column (2.5 × 15 cm) of CG-50 (Na^+) cation-exchange resin. The column was eluted with water until the eluate had $A_{267} < 0.05$ and then with a linear gradient of 0–0.4 N HCl (3 L), when two UV-absorbing components of λ_{max} 267 nm were successively eluted in a ratio of 1:4. Fractions containing the second of these were

combined and evaporated to dryness. The residue was dissolved in MeOH and precipitated by addition of Et_2O to give **4** as pale yellow hygroscopic powders. Yields, determined spectroscopically, were 45–60%. Apparent values of $\epsilon_{267}^{max}(H_2O)$ calculated for unsolvated materials were in the range (8.0–8.5) × 10^3 to indicate purities of not less than 85%. Compounds **4** showed only one UV-absorbing component in the paper electrophoretic and chromatographic systems of Table I.

3'-O-[ω-[N-[ω-(Triethylammonio)alkyl]diethylammonio]acyl]thymidine Dichlorides (5). To a solution of **4** (1 mmol of the reprecipitated free-base form) in dry DMF (5 mL) was added EtI (5 mmol). The mixture was stored at 40 °C for 16 h. The reaction was followed by electrophoresis at pH 7.6 or by paper chromatography in solvent C. The volatiles were removed in vacuo at 20 °C, and the residue was dissolved in water (2 mL) and applied to a column (2.5 × 15 cm) of CG-50 (Na^+) resin. The column was eluted with water until the eluant had $A_{267} < 0.05$ and then with a linear gradient of 0–0.4 N HCl (3 L). Two peaks of UV-absorbing material were seen in the elution diagram. Material in the second peak was obtained by removal of volatiles under reduced pressure. It was dissolved in MeOH and the solution added dropwise to Et_2O to yield **5** as pale yellow powders. Yields, determined spectrophotometrically, were 75–85%. The products were homogeneous when analyzed in the systems of Table I and were at least 90% pure as judged by apparent $\epsilon_{267}^{max}(H_2O)$ values of (8.5–8.7) × 10^3 that were calculated from the assumption that the compounds were unsolvated.

General Method for Synthesis of the Thymidine 5'-Phosphate Derivatives 6 and 7. To a suspension of 0.25 mmol of **4** or **5** in freshly distilled trimethylphosphate (1.0 mL) at 0 °C was added freshly distilled $POCl_3$ (150 μL). The solid dissolved after 1 h. The reaction was monitored by electrophoresis at pH 3.6. The mixture was stored at 0–5 °C for 3 h and poured slowly into anhydrous Et_2O (25 mL). The precipitated gum was dissolved in THF (1 mL), and the solution was added to a mixture of ice and water (5 mL). After 30 min at 2–5 °C the pH, as determined with a glass electrode, had ceased to decrease. The solution was applied to a column (2.5 × 5 cm) of Dowex-1 (Cl^- form) ion-exchange resin. The column was washed with water (5 bed volumes). The eluant, which contained all the UV-absorbing material applied to the column, was evaporated to dryness. The residue was dissolved in DMF (2 mL), and the solution was evaporated to dryness under reduced pressure (bath at 30 °C) to remove final traces of HCl. The residue was dissolved in MeOH and chromatographed on Whatman 3 MM paper in solvent B. The major band was eluted at 5 °C with water, and the solution was evaporated to dryness. The residue was dissolved in MeOH, and the solution was added dropwise to Et_2O to give **6** or **7** in 60–65% yield as white powders (dried at 56 °C). The compounds were homogeneous when analyzed in the systems of Table I. **6** (n = 3, m = 2; n = 4, m = 2–4): $\epsilon_{267}^{max}(H_2O)$ 9200–9300 (calculated for trihydrated compounds). Anal. [$C_{26}H_{47}N_4O_9P \cdot 3H_2O$ (**6**, n = 4, m = 3)] C, H, N, P. **7** (n = 4, m = 2–4): $\epsilon_{267}^{max}(H_2O)$ 9300–9400 (calculated as trihydrates). Anal. [$C_{27}H_{49}N_4O_9P \cdot 3H_2O$ (**7**: n = 4, m = 2)] C, H, P, N: calcd, 8.51; found, 7.95. Anal. [$C_{29}H_{53}N_4O_9P \cdot 4H_2O$ (**7**: $n = m$ = 4)] C, H, N, P.

Synthesis of the Thymidine 5'-Phosphate Derivative 10 (m = 9). To a solution of 540 mg (1.5 mmol) of **3** (n = 4) in DMF (3 mL) was added 1,4-diazabicyclo[2.2.2]octane (840 mg, 7.5 mmol). The mixture was stored at 50 °C for 16 h. The reaction was followed by TLC in solvent A and paper electrophoresis at pH 3.6. The mixture was added dropwise to Et_2O (200 mL). The precipitate was collected by centrifugation and redissolved in DMF (2 mL). The solution was added dropwise to anhydrous Et_2O (100 mL). The precipitate was dried under vacuum to yield 625 mg (88%) of **8** as a white powder. This intermediate (0.25 mmol) was dissolved in DMF (1 mL), and 1-iododecane (110 μL, 1 mmol) was added. The mixture was stored at 50 °C. TLC in solvent C indicated the reaction was complete after 3 h. Volatiles were removed at 22 °C under reduced pressure. The residue was dissolved in water (2 mL) and applied to a column (2.5 × 4 cm) of Dowex-1 [Cl^-] ion-exchange resin. The column was eluted with water (5 bed volumes), and the eluant was evaporated to dryness. The residue was dissolved in MeOH (0.5 mL), and the solution was added dropwise to Et_2O to yield **9** (m = 9) (145 mg; 90% yield by spectrophotometric analysis). The apparent ϵ_{267}^{max} value of 8500 calculated for unsolvated material indicated a purity of at least 90%. Compound **9** (75 mg) was phosphorylated by the method described for **4** and **5**. Purification of the product as described for **6** and **7** gave **10** (m = 9) (28 mg, 40% yield) as a white powder, $\epsilon_{267}^{max}(H_2O)$ 9200, that appeared homogeneous in the systems of Table I. Compound **10** (m = 1) was prepared as above by substituting EtI for $C_{10}H_{21}I$.

Rates of Hydrolysis of 4–7. Solutions (5 mM) of the esters in aqueous 50 mM potassium phosphate, pH 7.6, were kept at 37 °C. The formation of thymidine from **4** and **5** was followed by silica gel TLC in $CHCl_3$–CH_3OH (8:2) with visual inspection of plates in light of ca. 255 nm and the formation of **1** from **6** and **7** by electrophoresis at pH 3.6. The half-life ($t_{1/2}$) was determined graphically after plotting log (residual **4–7**) vs. time. All plots were linear, indicating pseudo-first-order kinetics.

Solutions (5 mM) of **6** (n = 4, m = 3) or **10** (m = 9) in the tissue culture medium were maintained at 37 °C in a 5% CO_2–95% air atmosphere and were analyzed by thin-layer cellulose chromatography (Merck No. 5503; solvents B and D) and paper electrophoresis at pH 3.6. Both compounds appeared stable for at least 6 h.

Cell Culture Assays. Mouse L-strain fibroblasts, wild-type L929 and thymidine kinase deficient LM(TK^-), were propagated as described previously.[13] Assay cultures were prepared in 6-cm plastic Petri dishes with 5 × 10^5 or 1 × 10^6 cells in 5 mL of growth medium: Dulbecco's modified Eagle medium (GIBCO 430–2100, glucose 4.5 g/L) supplemented with 10% fetal bovine serum (Rehatuin, Armour Pharmaceutical), penicillin (50 U/mL), streptomycin (50 μg/mL) and kanamycin (100 μg/mL). These cultures were incubated overnight at 37 °C under 5% CO_2.

Blocking medium was prepared as above, but the serum was inactivated for 1 h at 55 °C to reduce the activities of enzymes that might alter the test compounds. Aminopterin was added at 1 μg/mL, glycine at 15 μg/mL, and hypoxanthine at 15 μg/mL. When required for wild-type cells, 5'-NH_2-dT was added at 1.0 mM.[13] With the addition of 20 μM dT, this medium lacking 5'-NH_2-dT supported the normal proliferation of wild-type cells.

To estimate DNA synthesis, the cultures were labeled by addition of 1 μCi/mL of [1',2',5'-^{3}H]-2'-deoxycytidine (Amersham, 62 Ci/mmol); dC specifically labeles DNA in acid-insoluble cell fractions.[17] After an incorporation period of 1–3 h, the cells were collected on membrane filters and extracted with cold 5% trichloroacetic acid and acid-insoluble radioactivity was determined as described previously.[13] Duplicate samples were assayed for each time point. The number of cells per culture was estimated by using an electronic cell counter as previously described.[13]

During exposure to blocking medium, wild-type cells in the presence of 5'-NH_2-dT or LM(TK^-) cells in the presence or absence of 5'-NH_2-dT showed a rapid decline in the extent of [^{3}H]-dC incorporation. Inhibition was >97% at 2 h and approached 99% at 5 h. A blocking time of 3 h was used routinely in the present experiments. 5'-NH_2-dT was omitted from experiments with LM(TK^-) cells because it did not affect the results.

To assay for reversal of the block, test compounds were diluted into fresh blocking medium from 10 mM solutions in 50% ethanol–water. Control medium received an equal amount of 50% ethanol–water. The medium on the test cultures was then replaced with 2-mL portions of these preparations. The time course for recovery of DNA synthesis was studied in wild-type cultures in which the recovery medium contained 50 μM dT and no 5'-NH_2-dT. [^{3}H]-dC incorporation rose immediately and after 2 h reached a peak with a value nearly 100 times greater than that for the blocked cells. A recovery time of 2 h was used routinely in further experiments after which the [^{3}H]-dC was added.

The ability of the blocked cells to respond to intracellular TMP was assessed by addition of precursors: with wild-type cells, addition of 1 μM dT, even in the presence of 1 mM of the dT kinase inhibitor 5'-NH_2-dT, gave a detectable enhancement (Table III); with the LM(TK^-) cells, 1 mM of TMP stimulated ^{3}H incorporation to a significant extent (Table V).

Assay results are reported as the enhancement of [^{3}H]-dC incorporation due to the presence of test compound. Incorporation (cpm/h for 5 × 10^5 cells) in the presence of the compound is

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divided by the incorporation measured in parallel control cultures. Generally, enhancements of 2-fold or greater were reproducible and linear with incorporation time.

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Synthesis and Biological Properties of Chitin Synthetase Inhibitors Resistant to Cellular Peptidases

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The synthesis and biological properties of seven polyoxins (**4–10**) designed to avoid peptidase hydrolysis in *Candida albicans* are presented. Five dipeptidyl and two tripeptidyl polyoxin analogues were synthesized by coupling an amino acid active ester or azlactone to uracil polyoxin C (**2**) or polyoxin D (**1**), subsequent removal of the protecting group, and purification by preparative HPLC. A new and novel route for introducing an *n*-propyl group onto the α-amino group of peptides is reported. With the exception of a carboxamide derivative, **8**, all analogues were resistant to hydrolysis by a cell extract or permeabilized cells of *Candida*. Chitin synthetase inhibition constants were determined for **4–10** and the K_I values ranged from 7.15×10^{-6} M for octanoyl-phenylalanyl-polyoxin D (**10**) to 1.06×10^{-3} M for D-tryptophanyl-uracil polyoxin C (**6**). These novel polyoxins do not compete with the transport of either peptides or uridine into the cell. Millimolar concentrations of compounds **4–10** are required to inhibit growth, cause morphological alterations, or reduce the viability of *C. albicans*.

The polyoxins are a group of closely related peptidyl nucleoside antibiotics produced by species of *Streptomyces*. Previous studies have demonstrated that these compounds are toxic to phytopathogenic fungi.[1,2] The biochemical target for the polyoxins within the cell is chitin synthetase. This membrane-bound enzyme catalyzes the synthesis of chitin, a polysaccharide of *N*-acetylglucosamine (GlcNAc) and an important structural component of yeast and fungal cell walls.[3] Our laboratory has demonstrated that polyoxin D (**1**), one of the most active natural polyoxins, is toxic to the zoopathogenic fungi *Candida albicans* and *Cryptococcus neoformans*. However, this toxicity is manifested only at millimolar concentrations.[4]

The need for development of an antifungal agent for *C. albicans* is clearly indicated by the large number of opportunistic infections caused by *C. albicans* in compromised hosts. Accordingly, we have directed our research efforts toward the synthesis of novel polyoxin compounds that will ultimately find application in the clinical treatment of candidiasis.

In a previous report we showed that a series of dipeptidyl antibiotic analogues were strongly inhibitory to chitin synthetase and that some of these peptidyl antibiotics at millimolar concentrations were effective in killing the yeast *C. albicans*.[5] We also reported that two tripeptidyl polyoxins, leucyl-norleucyl-uracil polyoxin C and leucyl polyoxin D, which were inactive when assayed against chitin synthetase in a membrane preparation, generated toxic compounds within *Candida*.[6] We believe that these tripeptidyl polyoxins represent prodrugs that are activated by intracellular peptidases.

In the above studies we examined the role of cellular peptidases in the intracellular degradation of the polyoxins by *C. albicans*. These hydrolysis studies have shown us that the major difference between polyoxin D and the synthetic di- and tripeptidyl compounds is that polyoxin D is stable to peptidases whereas the synthetic analogues are hydrolyzed inside the yeast.[5,6] Recently polyoxin analogues with a modified peptide bond were found to lose inhibitory activity. However, no attempt to measure intracellular stability was reported in this study.[7]

In order to increase the stability of synthetic polyoxins toward intracellular hydrolysis we modified the backbone and chain ends of the dipeptidyl nucleoside. The chemical modifications we have chosen to circumvent the peptidase hydrolysis problem include the utilization of D-amino acids, N-alkylation of the terminal amine with methyl or propyl groups or replacement of this amine with an aminooxy group (NH_2O), inclusion of a terminal amide in place of the unprotected carboxyl group, utilization of dehydro amino acids in the peptide portion of the polyoxin, and acylation of tripeptidyl polyoxins with a lipophilic group.

Studies have indicated that the above modifications are often tolerated by target molecules yet render the peptide stable to proteolysis.[8,9] In this paper we report on the

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