Synthesis of Sulfur Analogues of Alkyl Lysophospholipid and Neoplastic Cell Growth Inhibitory Properties

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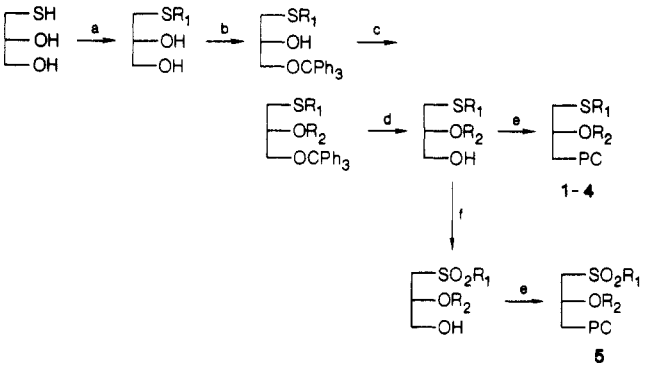
Five sulfur-containing phospholipid analogues (compounds **1–5**) of alkyl lysophospholipid (1-*O*-alkyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, ALP) were synthesized and tested for inhibition of neoplastic cell proliferation with two human ovarian carcinoma cell lines in a clonogenic assay and with the HL-60 promyelocytic leukemia cell line. Compared with 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OMe), the most active reference analogue, these thio analogues are at least as active against HL-60 cells, and the 1-*S*-hexadecyl-2-*O*-ethyl analogue (**2**) is twice as active in the clonogenic assays.

Alkyl lysophospholipid (1-*O*-alkyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, ALP)[1] is a lipid structurally related to platelet-activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, PAF).[2,3] While PAF is a naturally occurring substance found in many mammalian tissues,[4] ALP is a synthetic compound not found in biological systems. The focus of interest in PAF and its analogues has been on their aggregation and degranulation properties[4,5] and hypotensive effects,[6] whereas interest in ALP and its analogues has focused on their antineoplastic properties,[7] but the activities of these two sets of analogues are not mutually exclusive.[8] Structure–activity relationship studies have shown that both sets of analogues should contain a 1-*O*-alkyl ether at the 1-position and a phosphocholine moiety at the 3-position for maximal bioactivity.[1,4,9-12] What appears to differentiate these two classes of lipids chemically is the presence of a small, easily hydrolyzable moiety (acetate ester) at the *sn*-2-position for PAF and a small, relatively nonhydrolyzable moiety (methyl ether) at the 2-position for ALP (Figure 1). All of the biological activity of PAF and its analogues resides in the *sn*-1-*O*-alkyl isomer[13] (implying a stereospecific fit to a putative biological receptor[14]), but little else is known at this time about its mechanism of action. The mechanism of antitumor action of ALP and its analogues has been attributed to the generation of tumoricidal macrophages,[15-17] reduced alkyl cleavage enzyme activity in tumors,[1,18-20] membrane interactions,[21] malignant cell differentiation,[11] direct cytotoxicity,[7] and most recently the inhibition of a phospholipid cofactor of a phospholipid-sensitive Ca^{2+}-dependent protein kinase.[22] Storme et al. related the antiinvasive effect of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OMe) in a mouse fibrosarcoma cell line to cellular membrane alteration,[23] and Glasser et al. reported that this compound can successfully purge murine leukemic bone marrow, eliminating leukemic blasts and sparing sufficient normal stem cells to allow hematopoietic reconstitution.[24] Unlike the majority of antitumor agents currently available, these lipid analogues of ALP do not appear to have a direct effect on DNA synthesis or function and are nonmutagenic,[7,11] thereby offering the possibility of an alternate approach to standard cancer chemotherapy.

We became interested in sulfur-containing analogues of ALP since these compounds tend to be more lipophilic than their oxygen counterparts. Thus they may more easily insert into membranes and disrupt membrane function, if this is indeed the ALP mechanism of action. Support for this hypothesis has appeared in the literature where Berdel and co-workers[12] have shown that a 1-*S*-alkyl ALP analogue (1-*S*-hexadecyl-2-deoxymethoxymethyl-

Scheme I.[a,b] Synthesis of 1-Thio ALP Analogues 1–5



1: R_1 = hexadecyl, R_2 = methyl
2: R_1 = hexadecyl, R_2 = ethyl
3: R_1 = octadecyl, R_2 = methyl
4: R_1 = octadecyl, R_2 = ethyl
5: R_1 = hexadecyl, R_2 = ethyl

[a] a, alkyl bromide, alcoholic KOH; b, trityl chloride, pyridine; c, NaH, alkyl iodide; d, BF_3·2MeOH, CH_2Cl_2; e, $POCl_3$, Et_3N, choline tosylate, pyridine, $CHCl_3$; f, $KHSO_5$. [b] All final products gave 250-MHz ^1H NMR spectra and elemental analyses consistent with the proposed structures.

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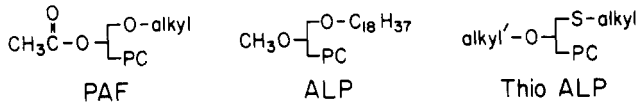

Figure 1.

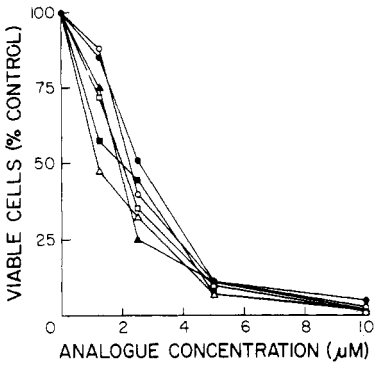

Figure 2. Growth inhibition of HL-60 cells by 1-thio phospholipid analogues. HL-60 cells, 5×10^5/mL, were incubated in RPMI 1640 medium containing 10% fetal bovine serum and the indicated amounts of phospholipid analogues for 48 h. The viable cell number was determined by hemocytometer counting of trypan blue treated cells. Phospholipid analogues were added in a small volume of ethanol (<0.5% final concentration). Cell numbers are presented as percent of cells in control cultures (0.5% ethanol) that were 1.2×10^6 cells/mL. The results presented are from an experiment that was representative of four separate experiments. (▲) ET-16*S*-OMe, (△) ET-16*S*-OEt, (■) ET-18*S*-OMe, (□) ET-18*S*-OEt, (●) ET-16SO_2-OEt, (○) ET-18-OMe.

rac-glycero-3-phosphocholine) was an active in vitro antineoplastic agent with reduced PAF activity as measured by neutrophil degranulation.

Results and Discussion

We report the synthesis of 1-thio ALP analogues **1–5** as shown in Scheme I. The general synthetic route used to prepare the 1-*S*(or SO_2)-alkyl-2-*O*-alkyl-*rac*-thioglycero-3-phosphocholines is as follows: (1) alkylation of the mercaptan of 3-thioglycerol with hexadecyl or octadecyl bromide and alcoholic potassium hydroxide;[25,26] (2) protection of the primary alcohol as the trityl ether;[26] (3) alkylation of the secondary alcohol with methyl or ethyl iodide and sodium hydride; (4) removal of the trityl group with boron trifluoride–methanol complex;[27] (5) oxidation of the sulfide to the sulfone with potassium hydrogen persulfate;[28] (6) treatment with phosphorus oxychloride and choline tosylate to form the phosphocholine.[29]

Table I. Effect of Compound **2** (ET-16*S*-OEt) and ET-18-OMe on Colony Growth of Human Ovarian Adenocarcinoma Cell Lines BG-1 and BG-3[a]

	colony survival, % of control			
	BG-1		BG-3	
concn, μg/mL	ET-16*S*-OEt	ET-18-OMe	ET-16*S*-OEt	ET-18-OMe
0.0	100	100	100	100
0.03	65	104	56	91
0.1	64	107	54	98
0.3	53	86	56	56
1.0	8	3	17	12
3.0	2	4	2	5
10.0	2	2	3	6
30.0	2	4	2	12

[a] Cells were initiated in soft agarose clonogenic culture, followed by continuous exposure to the phospholipid analogues over a three log range of concentrations.[32] Untreated cultures served as controls. After incubation for 7 days at 37 °C/7.5% CO_2, colony formation from the single cell suspension was evaluated by inverted microscopy and automated image analysis. All experiments were carried out in triplicate.

These thio analogues were found to be active growth inhibitors in several human malignant cell lines: the HL-60 promyelocytic leukemic cell line and two ovarian carcinoma cell lines.[30] In Figure 2 the activity of compounds **1–5** in the HL-60 system is compared with that of the previously studied analogue, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OMe). ET-18-OMe is generally regarded as the most active reference analogue in the literature.[31] The thio analogues are at least as active as ET-18-OMe in this test system. Table I shows the evaluation of compound **2** (1-*S*-hexadecyl-2-*O*-ethyl-*rac*-thioglycero-3-phosphocholine) and ET-18-OMe in a soft agarose clonogenic assay against the BG-1 and BG-3 human ovarian carcinoma cell lines,[32] derived from primary explants at the Bowman Gray School of Medicine and now carried in continuous cell culture. In these assays compound **2** is approximately twice as active as ET-18-OMe at very low inhibitor concentrations of 0.03 and 0.1 μg/mL. These data represent a preliminary screening for efficacy only and further studies are in progress. Compounds **2** and **3** have also been tested against MethA sarcoma cell cultures.[37] The 1-thio ALP derivatives (**1–5**) have shown reduced PAF effects (as measured by their neutrophil aggregation and degranulation) when compared to their

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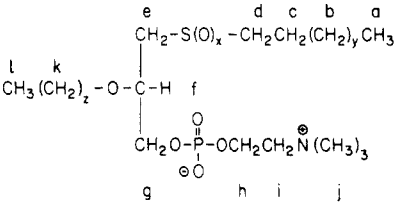
Table II. 250-MHz NMR Spectral Data for Compounds 1–5



1: $x = 0, y = 13, z = 0$
2: $x = 0, y = 13, z = 1$
3: $x = 0, y = 15, z = 0$
4: $x = 0, y = 15, z = 1$
5: $x = 2, y = 13, z = 1$

		ppm (no. H)				
assignment	mult[a]	1	2	3	4	5
a	t	0.90 (3)	0.86 (3)	0.90 (3)	0.90 (3)	0.87 (3)
b	m	1.34 (26)	1.26 (26)	1.26 (30)	1.26 (30)	1.26 (26)
c	p	1.56 (2)	1.56 (2)	1.56 (2)	1.56 (2)	1.80 (2)
d	t	2.53 (2)	2.53 (2)	2.53 (2)	2.53 (2)	3.05 (2)
e	d of d	2.68 (2)	2.68 (2)	2.68 (2)	2.68 (2)	3.28 (2)
f	m	3.49 (1)	3.56 (1)	3.49 (1)	3.60 (1)	3.53 (1)
g	m	3.95 (2)	3.90 (2)	3.95 (2)	3.90 (2)	3.95 (2)
h	m	4.34 (2)	4.26 (2)	4.34 (2)	4.34 (2)	4.28 (2)
i	m	3.86 (2)	3.79 (2)	3.80 (2)	3.82 (2)	3.75 (2)
j	s	3.38 (9)	3.38 (9)	3.34 (9)	3.38 (9)	3.28 (9)
k	q		3.56 (2)		3.60 (2)	3.75 (2)
l[b]	s or t	3.41 (3)	1.19 (3)	3.41 (3)	1.15 (3)	1.15 (3)

[a] Singlet, s; doublet, d; triplet, t; multiplet, m; pentet, p. [b] Signal l is a singlet for 1 and 3 and a triplet for 2, 4, and 5.

oxygen counterparts.[33] These data tend to support a nonspecific membrane perturbation[21] mechanism of action for ALP and its analogues since there appears to be a greater tolerance for differing chemical moieties at the 1-position (-*S*-alkyl, SO_2, chain length) and the 2-position (MeO, EtO, CH_3OCH_2) than is the case for PAF, which appears to bind to a specific receptor.[14] Substitution of sulfur,[34] methylene,[35] or ester functionality[2,36] for the *sn*-1 ether leads to diminished PAF properties, as does increasing the *sn*-2 ester moiety to more than three carbons.[2,36] Confirmation of this hypothesis would require the synthesis and testing of the two enantiomers of ALP. These preliminary data are encouraging, and further details of the testing of these and other promising thio analogues will be reported later in greater detail.

Experimental Section

All chemicals were used as provided by the supplier without further purification unless otherwise indicated. Oxone is the trade name for a 2:1:1 mixture of $KHSO_5$, K_2SO_4, and $KHSO_4$ available from Aldrich Chemicals. Column chromatography was performed with use of silica gel 60 (230–400 mesh). All melting points were obtained on a Hoover Meltemp apparatus and are uncorrected. Proton nuclear magnetic resonance spectra were obtained on either a JEOLCO 60-MHz or a Bruker 250-MHz spectrometer as solutions in $CDCl_3$ with internal Me_4Si as standard. Elemental analyses of final products were performed by Atlantic Microlabs, Inc.

Growth Inhibition of HL-60 Cells. HL-60 cells were obtained from Dr. Robert Capizzi, Bowman Gray School of Medicine, and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL streptomycin, 0.22% $NaHCO_3$, and 2 mM glutamine. The cells were subcultured at 2–3-day intervals and maintained in an atmosphere of 5% CO_2. Cell numbers in the stock cultures were maintained in the range 5×10^5 to 1.5×10^6. To determine the effects of inhibitors on cell proliferation, the cells were incubated at 5×10^5 cells/mL with various concentrations of the inhibitors, which were added in a small volume of ethanol. Stock solutions of the lipid inhibitors were made at 1 mg/mL in ethanol and diluted into cell culture medium immediately prior to use. The concentrations of ethanol used were found to have no effect on cell proliferation. After 48 h, cell numbers were determined by hemocytometer counting.

1-*S*-Hexadecyl-3-*O*-trityl-*rac*-thioglycerol. 1-*S*-Hexadecyl-*rac*-thioglycerol (12.0 g, 0.036 mol), mp 68–70 °C (lit.[25] mp 76–77 °C), was dissolved in pyridine (50 mL) and allowed to react with trityl chloride (15.0 g, 0.054 mol, recrystallized from 1:2 benzene/petroleum ether) for 5 h at 70 °C under nitrogen. The solution was cooled, diluted with water (100 mL), and extracted with ether (1 × 100 mL, 2 × 50 mL). The organic fractions were combined, washed with cold 2 N sulfuric acid and water, dried over sodium sulfate, filtered, and concentrated. The residue was dissolved in 75 mL of hot hexane and allowed to cool at room temperature overnight. The precipitate (mostly trityl alcohol) was filtered and discarded and the filtrate cooled to −15 °C. The resulting solid was filtered and recrystallized from hexane a second time, giving 12.8 g (61%) of product melting at 60.5–61.5 °C. A second crop weighed 2.0 g with a melting point of 59–60 °C. NMR ($CDCl_3$): δ 0.7–1.7 (m, 31 H, $(CH_2)_{14}CH_3$), 2.3–2.8 (m, 4 H, CH_2SCH_2), 3.25 (d, 2 H, CH_2OTr), 3.6–4.0 (m, 1 H, CH), 7–7.6 (m, 15 H, $(C_6H_5)_3$).

1-*S*-Octadecyl-3-*O*-trityl-*rac*-thioglycerol. This product was synthesized from 1-*S*-octadecyl-*rac*-thioglycerol, mp 75–76 °C (lit.[25,26] mp 74–75 °C, 75–76 °C), in a manner analogous to that for the *S*-hexadecyl compound in a 60% yield with a melting point of 64–66 °C (lit.[26] mp 64–65 °C).

1-*S*-Hexadecyl-2-*O*-methyl-3-*O*-trityl-*rac*-thioglycerol. 1-*S*-Hexadecyl-3-*O*-trityl-*rac*-thioglycerol (5.7 g, 0.010 mol) was dissolved in 20 mL of dry THF and added dropwise to a suspension of sodium hydride (1.0 g, 0.020 mol, 50% in oil, washed with hexane) in 20 mL of THF under nitrogen. The reaction mixture was stirred at room temperature for 30 min, 1.3 mL of methyl iodide was added, and stirring was continued overnight. Water (20 mL) was added dropwise to decompose excess sodium hydride. The organic fraction was separated, washed successively with 20 mL of 15% $Na_2S_2O_3$, water, and NaCl solutions, and dried over sodium sulfate. After filtration and concentration, the resulting oil was chromatographed on silica gel eluting with 4:1 petroleum ether/ether. Gas chromatography and NMR spectroscopy were used to identify pure fractions, giving 2.0 g (34%) of clear oil. NMR ($CDCl_3$): δ 0.7–1.6 (m, 31 H, $(CH_2)_{14}CH_3$), 2.3–2.8 (m, 4 H, CH_2SCH_2), 3.1–3.5 (m, 3 H, CH_2O and OCH), 3.4 (s, 3 H, OCH_3), 7.1–7.6 (m, 15 H, $(C_6H_5)_3$). 1-*S*-Hexadecyl-2-*O*-ethyl-3-*O*-trityl-*rac*-thioglycerol and 1-*S*-octadecyl-2-*O*-methyl- and 1-*S*-octadecyl-2-*O*-ethyl-3-*O*-trityl-*rac*-thioglycerol were

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prepared by using an analogous procedure. Purification by column chromatography gave yields of approximately 40%; however, overall yields were higher and purity of the final product was unaffected if this intermediate was not purified before the detritylation.

1-*S*-Hexadecyl-2-*O*-methyl-*rac*-thioglycerol. 1-*S*-Hexadecyl-2-*O*-methyl-3-*O*-trityl-*rac*-thioglycerol (2.0 g, 0.0031 mol) was dissolved in 125 mL of CH_2Cl_2 and cooled to 0 °C under nitrogen. Boron trifluoride–methanol complex (50%, 0.4 mL) was added in one portion. The yellow solution was stirred for 1 h, an additional 0.4-mL portion of BF_3·2MeOH was added, and stirring was continued for 1 h. Water (50 mL) was added and the organic fraction separated and washed with two additional 50-mL portions of water. The CH_2Cl_2 fraction was dried over Na_2SO_4, filtered, and concentrated. The residue was dissolved in 10 mL of petroleum ether, and a small amount of insoluble material (TrOH) was filtered and discarded. Chromatography on silica gel with 9:1 petroleum ether/ether gave pure alcohol (750 mg, 63%) as a waxy solid. NMR ($CDCl_3$): δ 0.87 (t, 3 H, CH_3), 1.2–1.4 (m, 26 H, $(CH_2)_{13}$), 1.58 (p, 2 H, SCH_2CH_2), 2.0 (br s, 1 H, OH), 2.54 (t, 2 H, SCH_2CH_2), 2.62 (d of d, 2 H, $CHCH_2S$), 3.40 (m, 1 H, CH), 3.44 (s, 3 H, OCH_3), 3.75 (d of d, 2 H, CH_2OH). The 1-*S*-hexadecyl-2-*O*-ethyl and the 1-*S*-octadecyl-2-*O*-methyl and -ethyl ethers (1-*S*-alkyl-2-*O*-alkyl-*rac*-thioglycerols) were prepared in a similar manner. Use of crude tritylated starting material gave yields of approximately 50% in two steps from the 1-*S*-alkyl-3-*O*-trityl-*rac*-thioglycerols.

1-*SO*$_2$-Hexadecyl-2-*O*-ethyl-*rac*-sulfonylglycerol. 1-*S*-Hexadecyl-2-*O*-ethyl-*rac*-thioglycerol (0.9 g, 2.5 mmol) was dissolved in methanol (15 mL). Oxone (2.8 g, 9 mmol) in 15 mL of water was added dropwise. The cloudy solution was stirred overnight at room temperature. Water (30 mL) was added and the solution extracted with chloroform (3 × 30 mL). The organic fractions were combined, washed with NaCl solution (20 mL), dried over Na_2SO_4, filtered, and concentrated. The resulting solid (800 mg, 2.0 mmol, 80% yield) was used without further purification. NMR ($CDCl_3$): δ 0.7–2.0 (m, 34 H $(CH_2)_{14}CH_3$ and CH_3), 2.8–3.4 (overlapping m, 4 H, $CH_2SO_2CH_2$), 3.4–4.0 (overlapping multiplets, 3 H, OCH_2 and OCH).

1-*S*-Hexadecyl-2-*O*-methyl-*rac*-thioglycero-3-phosphocholine (1). 1-*S*-Hexadecyl-2-*O*-methyl-*rac*-thioglycerol (3.4 g, 0.0094 mol, dried under high vacuum over P_2O_5) and triethylamine (1.21 g, 0.012 mol, freshly distilled) were dissolved in ethanol-free $CHCl_3$ (100 mL) and added dropwise to $POCl_3$ (1.1 mL, 0.012 mol) under nitrogen. The solution was stirred for 30 min at 60 °C. After cooling, pyridine (4.9 mL, freshly distilled over KOH) was added in one portion followed by solid choline tosylate (4.54 g, 0.0165 mol, dried under high vacuum over P_2O_5). The reaction mixture was stirred at room temperature overnight. Water (3 mL) was added and stirring continued for 30 min. The solution was then extracted with solutions of 3% Na_2CO_3 (3 × 70 mL), 5% HCl (2 × 60 mL), and water (2 × 60 mL) with the addition of methanol to break the emulsions that formed. After drying over Na_2SO_4 and filtration, the chloroform was removed on a rotary evaporator. The resulting semisolid was dissolved in hot chloroform (15 mL) and cooled to room temperature. Acetone (30 mL) was added and the solution cooled to −15 °C. The precipitate that formed was filtered and purified by column chromatography on silica gel with use of $CHCl_3$/MeOH/HOAc/H_2O (50:25:8:4) as eluant. Pure fractions were evaporated to an oil, which required a reprecipitation from $CHCl_3$/acetone to give a solid product (3.4 g, 67%) melting with decomposition at 248–251 °C. The 1-*S*-hexadecyl-2-*O*-ethyl-*rac*-thioglycero-3-phosphocholine (**2**) (semisolid, no mp, lit.[12] mp 238–243 °C), 1-*S*-octadecyl-2-*O*-methyl-*rac*-thioglycero-3-phosphocholine (**3**) (mp 246–249 °C, lit.[12] mp 251–252 °C), 1-*S*-octadecyl-2-*O*-ethyl-*rac*-thioglycero-3-phosphocholine (**4**) (mp 242–245 °C), and the 1-*SO*$_2$-hexadecyl-2-*O*-ethyl-*rac*-sulfonylglycero-3-phosphocholine (**5**) (mp 247–250 °C) were prepared by the same procedure in 40, 69, 58, and 40% yields, respectively.

NMR spectral data for each new phosphocholine (1–5) are given in Table II.

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Registry No. 1, 103304-63-8; 2, 103304-64-9; 3, 103304-65-0; 4, 103321-05-7; 5, 103304-73-0; (±)-Me$(CH_2)_{15}SCH_2CH(OH)CH_2OH$, 25666-00-6; (±)-Me$(CH_2)_{17}SCH_2CH(OH)CH_2OH$, 25666-01-7; (±)-Me$(CH_2)_{15}SCH_2CH(OH)CH_2OTr$, 103321-06-8; (±)-Me$(CH_2)_{17}SCH_2CH(OH)CH_2OTr$, 91274-06-5; (±)-Me$(CH_2)_{15}SCH_2CH(OMe)CH_2OTr$, 103304-66-1; (±)-Me$(CH_2)_{15}SCH_2CH(OEt)CH_2OTr$, 103304-67-2; (±)-Me$(CH_2)_{17}SCH_2CH(OMe)CH_2OTr$, 103321-07-9; (±)-Me$(CH_2)_{17}SCH_2CH(OEt)CH_2OTr$, 103321-08-0; (±)-Me$(CH_2)_{15}SCH_2CH(OMe)CH_2OH$, 103304-68-3; (±)-Me$(CH_2)_{15}SCH_2CH(OEt)CH_2OH$, 103304-69-4; (±)-Me$(CH_2)_{17}SCH_2CH(OMe)CH_2OH$, 103304-70-7; (±)-Me$(CH_2)_{17}SCH_2CH(OEt)CH_2OH$, 103304-71-8; (±)-Me$(CH_2)_{15}SO_2CH_2CH(OEt)CH_2OH$, 103304-72-9; choline tosylate, 55357-38-5.

Synthesis of 10-Acetyl-5,8-dideazafolic Acid: A Potent Inhibitor of Glycinamide Ribonucleotide Transformylase[1]

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10-Acetyl-5,8-dideazafolic acid has been synthesized in good yield from the parent compound, 5,8-dideazafolic acid. This quinazoline folate analogue showed no activity as a substrate for the folate-requiring de novo purine biosynthetic enzyme glycinamide ribonucleotide transformylase isolated from the murine lymphoma cell line L5178Y, but proved to be a potent competitive inhibitor, K_i = 1.3 μM, of the purified enzyme.

Recently, it has been amply demonstrated that quinazoline (5,8-dideaza) analogues of reduced folate cofactors can serve as substrate or inhibitors for many of the enzymes that require folate cofactors. This has led to an increased interest in these compounds as potential chemotherapeutic agents. The chemical stability of the quinazolines, relative to the oxidatively labile reduced folate,[2] presents an additional advantage.

In addition to the numerous examples of the interaction of these analogues with dihydrofolate reductase (DHFR)[3] and thymidylate synthase (TS)[4] isolated from a variety of

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