F·2.5H_2O) C, H, F (dried at 50 °C under vacuum); N: calcd, 22.79; found, 21.24.

3′-Fluoroaminopterin (9). This isomer was obtained in similar yield to 8 by condensing 1 and 7 in an identical reaction and purification scheme as described above for obtaining 8 from 6. The product, a pale yellow fluffy solid, was identified as follows: NMR of disodium salt, pH ~11 (D_2O, DHO at δ 4.8), δ 2.22 (m, 4 H), 4.32 (broad, 2 H), 4.38 (broad, α-H, 1H), 6.60 (t, arom, 1 H), 7.32–7.52 (arom, 2 H), 8.54 (s, 1 H); UV (0.1 M phosphate, pH 7.2) λ_{max} 260 nm (ϵ_M 28400), 279 (27800), 371 (7950); ^{19}F NMR (0.05 M phosphate, pH 7.2, 25% D_2O) sharp multiplet centered at 58.57 ppm upfield of trifluoroacetate. Anal. ($C_{18}H_{19}N_8O_5$F·2.5H_2O) C, H, F (after drying for 16 h at 50 °C); C: calcd, 44.15; found, 45.43; N: calcd, 22.70; found, 21.40.

Enzyme Assays. *L. casei* dihydrofolate reductase (DHFR) was purified to homogeneity from an MTX-resistant overproducer strain by the procedure of Dann et al.[20] Enzyme was assayed spectrophotometrically at pH 7.2, 400 mM KCl in the presence of 22 μM NADPH. For dihydrofolate, K_m = 0.36 μM under these conditions.[20] *E. coli* (MB 1428) MTX-resistant overproducer DHFR was purified to homogeneity according to the method of Poe[21] as modified by Williams[22] using MTX affinity chromatography. The enzyme was assayed spectrophotometrically at pH 7.2 in the presence of 40 μM NADPH, under conditions described by Poe et al., where the K_m for dihydrofolate is equal to 0.44 μM.[21] The concentrations of the above enzymes were established by MTX titration.[12] Rates (OD/min) were taken in linear regions from 0.5 to 2 min where less than 10% of the dihydrofolate was consumed. I_{50} is the inhibitor concentration that is found to reduce the observed rate to one-half its control value. K_i was calculated by the equation of Cha:[14]

$$I_{50} = K_i(1 + S/K_m) + 0.5E_t$$

HeLa cell DHFR was obtained from an overproducer (500-fold) strain of HeLa S-3 cells which are resistant to MTX and are grown in the presence of the drug.[23] These cells (HeLa S-3-500) were then grown for 2 generations in MTX-free, Dulbecco's modified Eagle's medium. Harvested cells were sonicated at 0 °C in phosphate buffer, and cell debris was removed by centrifugation at 40000*g*. The supernatant was assayed utilizing tritiated folic acid as substrate according to the method of Rothenberg[24] as modified by Alt et al.[25]

Biological Studies. Cytotoxicity studies were carried out on L1210 cells obtained from EG and G Mason Research Institute and on human stomach cancer line HuTu 80. Testing was carried out by standard methods:[26] Cells (Ca. 1×10^5) were suspended in 1 mL of their original media containing specified inhibitors and incubated at 37 °C for 72 h. Cell number was determined on a Coulter counter ZB_1 and maximally increased 10- to 20-fold over that inoculated. EC_{50} values refer to the concentration of inhibitor necessary to inhibit cell growth by 50% compared to controls grown in the absence of inhibitor.

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Registry No. 1, 52853-40-4; 2, 403-24-7; 3, 403-21-4; 4, 85803-25-4; 5, 85803-26-5; 6, 85803-27-6; 7, 85803-28-7; 8, 85803-29-8; 8 di-*tert*-butyl ester, 85803-30-1; 8·2Na, 85803-32-3; 9, 85803-34-5; 9 di-*tert*-butyl ester, 85803-31-2; 9·2Na, 85803-33-4; 2-fluoro-4-nitrotoluene, 1427-07-2; 3-fluoro-4-nitrotoluene, 446-34-4; di-*tert*-butyl L-glutamate hydrochloride, 32677-01-3; dihydrofolate reductase, 9002-03-3.

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Inhibitors of Glycolic Acid Oxidase. 4-Substituted 2,4-Dioxobutanoic Acid Derivatives

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Fourteen new 4-substituted 2,4-dioxobutanoic acids have been synthesized. These compounds, all of which contain lipophilic 4-substituents, are potent inhibitors in vitro of porcine liver glycolic acid oxidase. The I_{50} value of the two most potent representatives, 4-(4′-bromo[1,1′-biphenyl]-4-yl)-2,4-dioxobutanoic acid (8) and 4-[4′-[[(3,4-dihydro-3-hydroxy-2*H*-1,5-benzodioxepin-3-yl)methyl]thio][1,1′-biphenyl]-4-yl]-2,4-dioxobutanoic acid (13) is 6×10^{-8} M.

Recently we described[1] a series of 4-substituted 3-hydroxy-1*H*-pyrrole-2,5-dione derivatives which were shown to be potent inhibitors of the enzyme glycolic acid oxidase (glycolate:O_2 oxidoreductase, EC 1.1.3.1) (GAO). This enzyme, which catalyzes the oxidation of glycolate to glyoxylate along with the conversion of the latter to oxalate, is considered to play an important role in oxalate production in plants and animals.[2] Earlier studies by Schuman and Massey[2a,3] had suggested that the active site of porcine liver GAO contained two cationic groups in close proximity along with a hydrophobic bonding region. On

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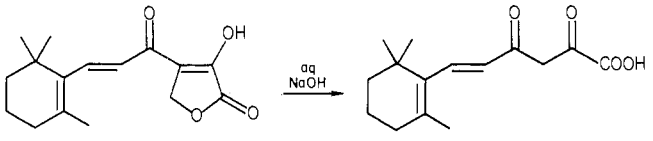
the basis of this information, we have investigated a number of mono- and diacidic systems containing lipophilic substituents in a search for novel, potent inhibitors of GAO. Such compounds may be of use in the treatment of clinical disorders in which oxalate is involved, such as the primary hyperoxalurias, and calcium oxalate renal lithiasis. In this report we describe a series of 4-substituted 2,4-dioxobutanoic acid derivatives which are highly active inhibitors of the porcine enzyme in vitro.

Chemistry. Syntheses of 4-substituted 2,4-dioxobutanoic acids are readily accomplished by the Claisen condensation of appropriate methyl ketone intermediates with dimethyl (or diethyl) oxalate and base, followed by saponification and acidification. These compounds in the free acid form are stable and exhibit pK_a values in the range of 3–4 and 8–10, corresponding to the dissociation of the carboxyl and enolic functions, respectively.[4] Yields, plus physical and biological data for the 2,4-dioxobutanoic acid derivatives studied in this work, are presented in Table I. Syntheses of previously unknown methyl ketone starting materials are reported under Experimental Section, along with the standard procedure by which the majority of 4-substituted 2,4-dioxobutanoic acid derivatives were synthesized. Compounds **10–14** were prepared by S-alkylation of ethyl 4-(4′-mercapto[1,1′-biphenyl]-4-yl)-2,4-dioxobutanoate (**23**), followed by saponification of the ethyl esters. Procedures for these alkylation steps are presented under Experimental Section. Because of solubility problems, dimethylformamide was utilized as the solvent for the preparation of **23**.

Results and Discussion

The initial lead into the 2,4-dioxobutanoic acid series stemmed from the testing of the tetronic acid derivative **1**[5] as a potential inhibitor of porcine GAO. It was determined that **1** was active in our standard in vitro assay of

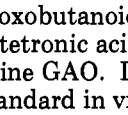


1 **2**

GAO inhibition[1,10] only after warming with excess aqueous sodium hydroxide solution. Adopting the hypothesis that the base treatment resulted in ring opening followed by a reverse aldol reaction to yield **2**, we synthesized the latter and showed it to be a potent inhibitor of GAO with an I_{50} value of 1.4×10^{-6} M. This finding led to the synthesis and testing of the other 4-substituted 2,4-dioxobutanoic acid derivatives listed in Table I.

Compound **3**, in which a phenyl substituent replaces the 2,6,6-trimethylcyclohexen-1-yl portion of **2**, was less than one-tenth as potent as **2** as an inhibitor of porcine GAO. In an effort to determine if the conjugated diene of **2** was an important feature for inhibitory activity, we tested the saturated *n*-alkyl derivative, **4**,[12] and showed it to be comparable to **2** in inhibitory potency. These results suggested that, as was found with the 4-substituted 3-hydroxy-1*H*-pyrrole-2,5-dione series of inhibitors,[1] high lipophilicity would probably be the most important com-

Table I. Title Compounds



compd	R	mp, °C, Me ester	mp, °C	recrystn solvent	yield,[a] %	formula	anal.	pK_a[b]	I_{50}, M × 10^{-6}, for inhibn of porcine GAO in vitro
2	(*E*)-2,6,6-Me$_3$-Δ^1-c-C$_6$H$_6$CH=CH	84–86	viscous oil		50	$C_{15}H_{20}O_4$[c]			1.4 ± 0.7
3	(*E*)-C$_6$H$_5$CH=CH		135–136 dec	benzene	20	$C_{12}H_{10}O_4$	C, H		19 ± 2
4	n-C$_9$H$_{19}$[h]							3.85, 9.60	1.4 ± 0.1
5	4-(*n*-C$_9$H$_{19}$)C$_6$H$_4$	55.5–57	75–77	benzene	19	$C_{19}H_{26}O_4$	C, H		0.6 ± 0.1
6	4-(c-C$_6$H$_{11}$)C$_6$H$_4$	96–98	140–142	benzene	15	$C_{16}H_{18}O_4$	H[d]		3.0 ± 0.7
7	4-(C$_6$H$_5$)C$_6$H$_4$	142–144	206–207 dec	EtOAc	11	$C_{16}H_{12}O_4$	C, H	3.34, 8.35	1.1 ± 0.2
8	4-(4-BrC$_6$H$_4$)C$_6$H$_4$	133–136	201–202 dec	MeCN	17.5	$C_{16}H_{11}BrO_4$	C, H, Br	3.60, 8.35	0.063 ± 0.006
9	4-(4-HSC$_6$H$_4$)C$_6$H$_4$	*e*	185.5–187 dec	EtOAc	26.5	$C_{16}H_{12}O_4S$	C, H, S		0.13 ± 0.03
10	4-(4-MeSC$_6$H$_4$)C$_6$H$_4$	144–146	191–195	MeCN	32.5	$C_{17}H_{14}O_4S$	C, H, S		0.16 ± 0.03
11	4-(4-C$_6$H$_5$CH$_2$SC$_6$H$_4$)C$_6$H$_4$	*e*	208–210 dec	MeCN	55[f]	$C_{23}H_{18}O_4S$	C, H		0.40 ± 0.08
12	4-[4-[(4-C$_5$H$_4$N)CH$_2$CH$_2$SC$_6$H$_4$]C$_6$H$_4$]	*e*	221–222.5 dec	DMF	56[f]	$C_{23}H_{19}NO_4S$	C, H, N		0.09 ± 0.02
13	4-(4-HBDM-SC$_6$H$_4$)C$_6$H$_4$[g]	*e*	218–219 dec	THF–MeCN	73[f]	$C_{26}H_{22}O_7S$	C, H, S		0.06 ± 0.02
14	4-(1,2,3,4-H$_4$-naphth-1-yl)C$_6$H$_4$	103–105	117–120	benzene	23	$C_{20}H_{18}O_4 \cdot 0.5H_2O$	C, H		2.1 ± 0.1
15	4-(3,4-Cl$_2$C$_6$H$_3$CH$_2$)C$_6$H$_4$	106–107	142–144	CCl_4	34	$C_{17}H_{12}Cl_2O_4$	C, H, Cl	3.38, 9.22	0.44 ± 0.04
16	4-(2-C$_6$H$_5$-indol-1-yl)C$_6$H$_4$	125–127	189–190 dec	MeCN	43	$C_{24}H_{17}NO_4$	C, H, N		0.18 ± 0.06

[a] Overall yield for two steps from methyl ketone intermediate. [b] Determined in 50% aqueous EtOH. [c] This compound, which did not crystallize, gave the expected ^{1}H NMR spectrum. [d] C: calcd, 70.05; found, 69.60. [e] Ethyl ester prepared (see Experimental Section). [f] Yield is for saponification of ethyl ester. [g] HBDM = 3,4-dihydro-3-hydroxy-2*H*-1,5-benzodioxepin-3-yl)methyl. [h] Reference 12.

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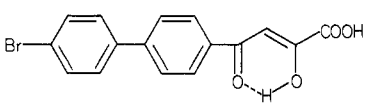
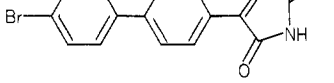
mon feature to be found in 4-substituents which would confer potent GAO inhibitory properties to the 2,4-dioxobutanoic acids. At this point the decision was made to direct the effort to a series of substituted phenyl and biphenyl analogues, since these were types of substituents that had resulted in high inhibitory potencies in the pyrrole-2,5-dione series of inhibitors.[1] Derivatives of 4-phenyl-2,4-dioxobutanoic acid with *n*-nonyl (**5**), 3,4-dichlorobenzyl (**15**), and 2-phenylindol-1-yl (**16**) substituents in the 4-position of the aromatic ring were more potent than **2**, whereas those with cyclohexyl (**6**) and 1,2,3,4-tetrahydro-1-naphthyl (**14**) substituents were slightly less active than **2**. While the 2,4-dioxobutanoic acid derivative (**7**) with a 4-biphenyl substituent was comparable to **2**, introduction of bromine into the 4′-position of the biphenyl residue gave **8** with an I_{50} of 6.3×10^{-8} M. (This enhancement in potency by a bromine substituent was also seen with the corresponding pyrrole-2,5-dione derivative **17**.) Testing of **8** against enzyme from human liver[2a] gave an I_{50} value of 8×10^{-8} M. In order to explore further the question of whether there are limits to the size of groups which can be accommodated in the hydrophobic bonding region of the enzyme active site, we elected to investigate a small but varied selection of substituents attached through a sulfide link to the 4′-position of the biphenyl residue. Whereas the mercapto (**9**) and methylthio (**10**) compounds were at most tenfold more potent as inhibitors than the parent biphenyl (**7**), the larger 4-pyridylethylthio (**12**) and 3,4-dihydro-3-hydroxy-2*H*-1,5-benzodioxepin-3-yl (**13**) substituents produced potencies in the 10^{-8} M range. (It is noteworthy that the terminal substitutents in **12** and **13** both possess some hydrophilic character.) The lipophilic benzylthio-substituted **11**, on the other hand, showed decreased potency relative to **9**, **10**, **12**, and **13**.

A comparison of the absolute potencies of the five aromatic ring substituted derivatives **6–8**, **14**, and **16** with their counterparts in the 4-substituted 3-hydroxy-1*H*-pyrrole-2,5-dione series of inhibitors[1] indicates a close parallel. The values for the two bromobiphenyl derivatives are typical ($I_{50} = 8.7 \times 10^{-8}$ M for the pyrrole-2,5-dione **17** compared



8



17

with 6.3×10^{-8} M for **8**). While this suggests that compounds in the two series with identical 4-substituents probably interact at the enzyme active site in a similar manner, a comparison of the structure of **8**, in its enolic hydrogen-bonded form,[6] with that of **17** indicates that even though each has two adjacent acidic functions, the two molecules are not exactly superimposable.

Compounds in the 4-substituted 2,4-dioxobutanoic acid series have not yet been subjected to extensive biological investigation.[7] The acute toxicity determination in mice for **7** suggests that oral absorption of this compound may be limited (approximate LD_{50} = 302 mg/kg ip, >1856 mg/kg po). On the other hand, oral absorption in the 4-substituted 3-hydroxy-1*H*-pyrrole-2,5-dione series does not appear to be a problem.[1]

Conclusion

A series of 2,4-dioxobutanoic acid derivatives with lipophilic 4-substituents has been synthesized. These compounds are potent inhibitors in vitro of porcine liver GAO, with I_{50} values in the 10^{-8} M range for the most active representatives. Their structures conform to what appears to be emerging as a general design for potent inhibitors of this enzyme—namely, the presence of two acidic groups in close proximity to which is attached a relatively large lipophilic substituent.

Experimental Section

Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained in $CDCl_3$ or Me_2SO-d_6 with a Varian Associates EM-360 instrument employing tetramethylsilane as an internal standard. ^{13}C NMR spectra were recorded in Me_2SO-d_6 with a Varian CFT-20 instrument. Infrared spectra were determined with a Perkin-Elmer 257 spectrophotometer. Elemental analyses were performed by Dr. C. Daessle, Organic Microanalysis, Montreal, Quebec, and are within ±0.4% of theory unless otherwise noted.

4-(4′-Bromo[1,1′-biphenyl]-4-yl)-2,4-dioxobutanoic Acid (8) (Standard Procedure). To a stirred suspension of sodium methoxide (1.4 g, 0.025 mol) in benzene (20 mL) was added dropwise at about 5 °C a solution of 4-acetyl-4′-bromo[1,1-biphenyl] (5.5 g, 0.02 mol) and dimethyl oxalate (2.36 g, 0.02 mol) in benzene (90 mL). The thick yellow reaction mixture was stirred at ice-bath temperature for 0.5 h and at room temperature for 2 h. Following the addition of 2 N HCl (16 mL) and EtOAc (100 mL), the organic layer was separated, washed with water, dried ($MgSO_4$), and evaporated. The crude solid was recrystallized from MeCN to give methyl 4-(4′-bromo[1,1′-biphenyl]-4-yl)-2,4-dioxobutanoate (**18**; 3.42 g, 47.3%), mp 130–133 °C. Anal. ($C_{17}H_{13}BrO_4$) C, H, Br.

To **18** (1.44 g, 0.004 mol) suspended in a mixture of EtOH (20 mL) and dioxane (2.4 mL) was added 2 N NaOH (4 mL). After the mixture was stirred at room temperature overnight, the organic solvents were removed by evaporation. The solid residue was suspended in H_2O, and the mixture made acidic to pH 1 with 6 N HCl. Extraction with $MeCl_2$, followed by evaporation, gave **8** as a light yellow solid: ^{1}H NMR (Me_2SO-d_6) δ 7.11 (s, 1 H), 7.71 (s, 4 H), 7.87 (d, J = 8.4 Hz, 2 H), 8.17 (d, J = 8.4 Hz, 2 H).

The same procedure was followed for the other 2,4-dioxobutanoic acid derivatives, which were prepared by way of their methyl ester intermediates. However, for compounds **9** and **11–13**, prepared from their ethyl esters, different methods were used for the syntheses of the ethyl ester intermediates, as indicated subsequently in this section. Saponification of these ethyl ester intermediates was carried out as described above.

4-Acetyl-4′-mercapto[1,1′-biphenyl] (19). To a solution of 4-acetyl-4′-hydroxy[1,1′-biphenyl] (15.9 g, 0.075 mol) in DMF (110 mL) cooled in an ice bath was added sodium hydride (1.98 g, 0.082 mol) in portions. The mixture was warmed briefly to achieve complete reaction. To the mixture, cooled in an ice bath, was added dropwise *N,N*-dimethylthiocarbamoyl chloride (11.6 g, 0.094 mol). The mixture was stirred for 15 min at ice-bath temperature and then overnight at room temperature. After the mixture was poured onto ice and extracted with $MeCl_2$, the separated solvent layer was washed with dilute NaOH and water and dried ($MgSO_4$). Evaporation, followed by recrystallization of the residue, gave *O*-(4′-acetyl[1,1′-biphenyl]-4-yl) *N,N*-dimethylthiocarbamate (**20**; 13.8 g, 61.5%), mp 154–156 °C. Anal. ($C_{17}H_{17}NO_2S$) C, H, N, S.

(6) Kurkovskaya, L. N.; Shapet'ko, N. N.; Andreichikov, Yu. S.; Saraeva, R. F. *Zh. Strukt. Khim.* **1972**, *13*, 1026. In our work, both ^{1}H NMR ($CDCl_3$) and ^{13}C NMR (Me_2SO-d_6) indicated that the 2,4-dioxobutanoic acids exist in the enolized form in solution.

(7) See Kawamatsu, Y.; Saraie, T.; Imamiya, E.; Nishikawa, K.; Hamuro, Y. *Arzneim.-Forsch.* **1980**, *30*, 454, for a report of hypoglycemic activity with the related 4-[4-(4′-chlorophenoxy)phenyl]-2,4-dioxobutanoic acid.

This intermediate (**20**; 12.4 g, 0.041 mol) was heated neat under nitrogen for 2 h at 255–260 °C.[8] After cooling, the residue was recrystallized from MeCN to give *S*-(4′-acetyl[1,1′-biphenyl]-4-yl) *N,N*-dimethylthiocarbamate (**21**; 11.2 g, 90.3%), mp 178–180 °C. Anal. ($C_{17}H_{17}NO_2S$) C, H, N, S.

A mixture of **21** (0.90 g, 0.003 mol) and 2 N NaOH (3.75 mL) in MeOH (45 mL) was heated at reflux under nitrogen for 6 h. Following acidification with 10% HCl (3 mL) and dilution with ice–water (50 mL), the product was extracted into $MeCl_2$. After the extract was dried ($MgSO_4$) and evaporated, the residue was dissolved in hot toluene (5 mL). Following filtration to clarify, isopropyl ether (10 mL) and then petroleum ether (10 mL) were added, and the product was allowed to crystallize overnight. There was obtained **19** (0.32 g, 49.4%), mp 126–128 °C. Anal. ($C_{14}H_{12}OS$) C, H, S.

4-Acetyl-4′-(methylthio)[1,1′-biphenyl] (22). A mixture of **21** (2.7 g, 0.009 mol) in MeOH (135 mL) containing 2 N NaOH (11.25 mL) was refluxed under nitrogen overnight. While heating was continued there were added simultaneously 10 N NaOH (10.9 mL) and dimethyl sulfate (0.86 mL, 0.009 mol). Refluxing was continued for 2 h. After the addition of 10 N NaOH (0.8 mL), heating was continued for another 1 h. After cooling, the mixture was poured over ice. Filtration and drying gave **22** as a light brown solid (1.80 g, 82.5%), mp 182–183.5 °C. Anal. ($C_{15}H_{14}OS$) C, H, S.

Ethyl 4-(4′-Mercapto[1,1′-biphenyl]-4-yl)-2,4-dioxobutanoate (23). To a suspension of sodium hydride (0.81 g, 0.034 mol) in DMF (6 mL) was added dropwise EtOH (1.93 g, 0.042 mol) with ice cooling. When hydrogen evolution had ceased, a solution of **19** (1.93 g, 0.042 mol) and diethyl oxalate (3.07 g, 0.021 mol) in DMF (18 mL) was added slowly over 1 h while cooling was continued. After standing at room temperature for 3 h, the mixture was poured into ice–water containing 2 N HCl (21 mL). The crude product, after filtration and drying, was recrystallized from benzene–petroleum ether to give **23** (3.3 g, 83.8%). Anal. ($C_{18}H_{16}O_4S$) C, H, S. Saponification of **23** by the standard procedure gave **9**.

Ethyl 4-[4′-(Benzylthio)[1,1′-biphenyl]-4-yl]-2,4-dioxobutanoate (24). To a mixture of **23** (3.82 g, 0.01 mol) and diisopropylethylamine (2.7 g, 0.021 mol) in THF (35 mL) at ice-bath temperature was added a solution of benzyl bromide (1.88 g, 0.011 mol) in THF (10 mL). The mixture was stirred at room temperature for 5 h. After filtration to clarify, the volume was reduced by vacuum evaporation to approximately one-third. Filtration of the slurry and recrystallization of the solids from MeCN gave **24** (1.55 g, 37%), mp 131–133 °C. An additional 0.36 g (8.6%) of **24** was obtained on workup of mother liquor material. Anal. ($C_{25}H_{22}O_4S$) C, H, S.

Ethyl 4-[4′-[[2-(4-Pyridyl)ethyl]thio][1,1′-biphenyl]-4-yl]-2,4-dioxobutanoate (25). A mixture of **23** (0.33 g, 0.001 mol), and 4-vinylpyridine (0.105 g, 0.001 mol) in THF (5 mL) was allowed to react at room temperature overnight under a nitrogen atmosphere. Following evaporation of the solvent, the residue was recrystallized from MeCN to give **25** (0.21 g, 48.5%), mp 108–110 °C. Anal. ($C_{25}H_{23}NO_4S$) C, H, N.

Ethyl 4-[4′-[[(3,4-Dihydro-3-hydroxy-2*H*-1,5-benzodioxepin-3-yl)methyl]thio][1,1′-biphenyl]-4-yl]-2,4-dioxobutanoate (26). To a filtered solution of **23** (1.87 g, 0.006 mol) and 3,4-dihydro-2*H*-1,5-benzodioxepin-3-spirooxirane[9] (1.12 g, 0.0063 mol) in dry THF (24 mL) under argon was added triethylamine (1.2 mL). After the mixture was stirred overnight at room temperature, the solvent was evaporated under vacuum. Recrystallization of the residue from MeCN gave **26** (2.29 g, 75.6%), mp 113–117 °C. Anal. ($C_{28}H_{26}O_7S$) C, H, S.

1-Acetyl-4-cyclohexylbenzene (27). Acetyl chloride (39.3 g, 0.5 mol) was added dropwise to a slurry of $AlCl_3$ (41.7 g, 0.31 mol) in $MeCl_2$ (250 mL) cooled in an ice bath. To the resulting red mixture was slowly added cyclohexylbenzene (40.0 g, 0.25 mol) in $MeCl_2$ (100 mL). After an additional 0.5 h at ice-bath temperature, the reaction was quenched with ice (25 g) and water (25 mL). The organic phase was separated and washed well with water and then brine. After the solution was dried ($MgSO_4$) and evaporated, the residue was crystallized from petroleum ether (60–100 °C) to give **27** (31.2 g, 61%), mp 65–67 °C. Anal. ($C_{14}H_{18}O$) C, H.

Prepared in a similar manner in 68.9% yield was 1-acetyl-4-(3,4-dichlorobenzyl)benzene, mp 37.5–39 °C. Anal. ($C_{15}H_{12}Cl_2O$) C, H, Cl.

1-(4-Acetylphenyl)-2-phenylindole (28). A mixture of *p*-bromoacetophenone (9.96 g, 0.15 mol), 2-phenylindole (10.13 g, 0.15 mol), K_2CO_3 (6.91 g, 0.15 mol), and cupric oxide (0.25 g) in pyridine (10 mL) was heated in a sealed glass ampule at 175 °C for 24 h. On cooling, the mixture was diluted with EtOAc and filtered. The residue from evaporation of the filtrate was redissolved in EtOAc (150 mL), and the solution was washed successively with 1 N HCl, water, 1 N NaOH, and water and then dried ($MgSO_4$). Evaporation gave a semisolid residue, which was recrystallized from MeCN to give **28** (6.8 g, 43.5%), mp 153–155 °C. Anal. ($C_{22}H_{17}NO$) C, H, N.

In Vitro Enzyme Assay Procedure for Inhibition of GAO. The standard assay of enzyme inhibition has been described previously.[1,10] Briefly, this employs the spectrophotometric determination (600 nm) of the enzyme-mediated reduction of 2,6-dichlorophenolindophenol (DCIP) by glycolate at 25 °C. Initial substrate concentrations of DCIP and glycolate were 5×10^{-5} and 2×10^{-4} M, respectively. Three inhibitor concentrations were tested concurrently with a control containing no inhibitor, and the resulting rates were expressed as a fraction of the control.

The determination of enzyme inhibition patterns for a selected set of compounds was done at the same DCIP concentrations as above. For these determinations, the initial velocity of the reaction was determined as a function of the initial glycolate concentration at a series of fixed inhibitor concentrations.

Methods. Data were fitted to equations by the method of nonlinear least squares.[11] This method provides both the values of the adjustable parameters which would best fit an equation to experimental data as well as estimates of the standard deviations of these parameters.

Values of I_{50}, the inhibitor concentration in molar units required to reduce the enzyme activity to one-half the control value, were determined from the following equation:

$$f_I = \frac{1}{1 + I_{50}/[I]_t}$$

In this equation, f_I is the fractional inhibition (= R_z/R_c where R_z and R_c are the initial rates in the presence and absence of inhibitor, respectively) and $[I]_t$ is the total inhibitor concentration.

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Registry No. **1**, 85763-11-7; **2**, 85763-12-8; **2** (methyl ester), 85763-13-9; **3**, 85763-14-0; **4**, 85763-15-1; **5**, 82760-75-6; **5** (methyl ester), 82760-74-5; **6**, 82760-73-4; **6** (methyl ester), 82760-72-3; **7**, 85763-16-2; **7** (methyl ester), 63656-27-9; **8**, 82760-77-8; **9**, 83085-93-2; **10**, 83085-96-5; **10** (methyl ester), 83085-95-4; **11**, 83085-85-2; **12**, 83085-87-4; **13**, 83085-97-6; **14**, 82760-79-0; **14** (methyl ester), 82760-78-9; **15**, 82760-81-4; **15** (methyl ester), 82760-80-3; **16**, 85763-17-3; **16** (methyl ester), 85763-18-4; **18**, 82760-76-7; **19**, 83085-91-0; **20**, 83085-89-6; **21**, 85763-19-5; **22**, 83085-94-3; **23**, 83085-92-1; **24**, 83085-86-3; **25**, 83085-88-5; **26**, 83085-98-7; **27**, 18594-05-3; **28**, 84864-21-1; 4-acetyl-4′-bromo[1,1′-biphenyl], 5731-01-1; 4-acetyl-4′-hydroxy[1,1′-biphenyl], 13021-17-5; *N,N*-dimethylthiocarbamoyl chloride, 16420-13-6; diethyl oxalate, 95-92-1; 4-vinylpyridine, 100-43-6; 3,4-dihydro-2*H*-1,5-benzodioxepin-3-spirooxirane, 27612-42-6; cyclohexyl-

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benzene, 827-52-1; 1-acetyl-4-(3,4-dichlorobenzyl)benzene, 77529-35-2; *p*-bromoacetophenone, 99-90-1; 2-phenylindole, 948-65-2; (*E*)-4-phenyl-3-buten-2-one, 1896-62-4; 2-undecanone, 112-12-9; 4′-nonylacetophenone, 37593-05-8; (*E*)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one, 79-77-6; 4′-phenylacetophenone, 92-91-1; 4-acetyl-4′-(benzylthio)[1,1′-biphenyl], 85763-20-8; 4-acetyl-4′-[[2-(4-pyridyl)ethyl]thio][1,1′-biphenyl], 85763-21-9; 4-acetyl-4′-[[(3,4-dihydro-3-hydroxy-2*H*-1,5-benzodioxepin-3-yl)methyl]thio][1,1′-biphenyl], 85763-22-0; 4′-(1,2,3,4-tetrahydronaphth-1-yl)acetophenone, 40845-96-3.

1,3-Dipalmitoylglycerol Ester of Chlorambucil as a Lymphotropic, Orally Administrable Antineoplastic Agent

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A glyceride derivative of chlorambucil (**2**), 1,3-dipalmitoyl-2-[4-[bis(2-chloroethyl)amino]benzenebutanoyl]glycerol (**1**), was synthesized and tested as an orally administrable antineoplastic drug endowed with lymphotropic properties. A significantly higher efficacy (increased life span) and a reduced toxicity of **1**, relative to **2**, were apparent when both compounds given per os were evaluated against P388 leukemia subcutaneously implanted in mice, a situation where the tumor cells disseminate along the lymphatic route. In order to assess the selective absorption of **1** by the intestinal lymphatic system after oral administration, we determined plasma and intestinal lymphatic concentrations and compared them with that obtained with **2**. The results clearly demonstrate that the esterification of **2** to a diacylglycerol moiety brings about considerably higher levels in the lymph and reduces plasma levels. Moreover, pharmacokinetic and biological data suggest that **1** is most probably acting by itself rather than as a prodrug of chlorambucil.

In recent years, there has been increasing interest in the design of lymphotropic delivery systems for anticancer agents.[1] Indeed, a selective lymphatic administration of antineoplastic drugs can be of major significance for cancer chemotherapy in the frequent situation where tumor cells spread along the lymphatic pathways and metastasize in the lymph nodes. Various types of emulsions and liposomes administered via the parenteral route have been used to deliver drugs into lymphatics for the treatment of metastasis.[2-8] Similarly, selective absorption of bleomycin from intestinal loops has been observed in rat by using a bifunctional delivery system that combines the lymphotropic properties of dextran sulfate with the absorption-inducing properties of lipid-surfactant mixed micelles.[9,10]

As an alternative to these supramolecular vectorized forms of antineoplastic drugs, we are presently interested in the design of synthetic acylglycerols incorporating a chemotherapeutic moiety in the 2-position of glycerol and fatty acids in the 1- and 3-positions, which might be absorbed via enteral route, preferably through the intestinal lymphatic system. The rationale behind this monomolecular approach is based on the well-known essential role played by this system in the physiological process of lipid absorption. Moreover, the use of a glyceride-like structure as a lymphotropic carrier of anticancer agents currently administered per os is expected to reduce the gastrointestinal toxicity of these compounds. As a matter of fact, such an effect has been previously reported in the group of antiinflammatory agents with a dipalmitoyl derivative of acetylsalicylic acid.[11]

In order to explore this possibility, we synthesized 1,3-dipalmitoyl-2-[4-[bis(2-chloroethyl)amino]benzenebutanoyl]glycerol (**1**), a glyceride derivative of chlorambucil (**2**). Chlorambucil was selected as a model on the basis of the following considerations: (a) it is one of the most effective agents in the treatment of Hodgkin's disease and other lymphomas, (b) it is a standard orally administered antineoplastic drug, and (c) it can be easily attached to glycerol via an ester linkage, with its alkylating bis(2-chloroethyl)amino head remaining free; consequently, the resulting derivative **1** might be expected to have by itself carcinostatic activity.

In the present paper, we describe the preparation of **1** and the comparative study of the antitumor activity of **1** and **2**, determined against P388 leukemia in mice. This tumor disseminates lymphatically when it is transplanted subcutaneously (sc).[12] Experimental results reveal a highly significant increase in activity of **1** compared with **2** when both compounds are orally administered. Additional evidence of the lymphotropic properties of **1** is presented, resulting from a pharmacokinetic experiment designed to study the intestinal lymphatic uptake of the radiolabeled compound after oral administration.

Chemistry. Compound **1** was synthesized as outlined in the Scheme I. Initially, chlorambucil (**2**) was allowed to react with 1,3-dipalmitin (**3**) in solution in CH_2Cl_2 in

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