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Synthesis and Evaluation of α -Hydroxythiol Esters as Antitumor Agents and Glyoxalase I Inhibitors¹

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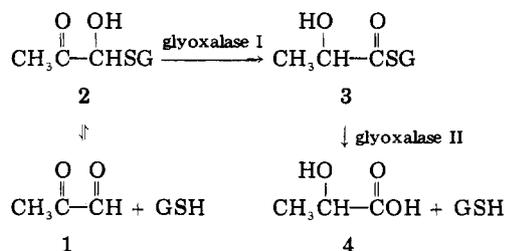
Synthesis of a series of α -hydroxythiol esters made available, for the first time, product-like molecules that were evaluated as inhibitors of the enzyme glyoxalase I and as potential antitumor agents. All the α -hydroxythiol esters tested were competitive inhibitors of the enzyme, albeit weak; however, the relative $[I]_{50}$ values suggested information about the active site. Antileukemic activity in L1210 lymphoid leukemia indicated no significant activity by these α -hydroxythiol esters.

The glyoxalase system, which consists of glyoxalase I [*S*-lactoylglutathione methylglyoxal lyase (isomerizing); E.C. 4.4.1.5], the coenzyme glutathione (GSH), and glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase; E.C. 3.1.2.6), converts methylglyoxal (**1**) to lactic acid (**4**) (see Scheme I).

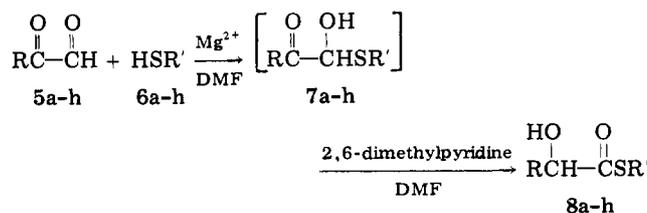
It has been suggested that the glyoxalase enzyme system, which is found widely distributed in cells of all forms of life,^{2,3} may be involved in the regulation of cell growth by maintaining a proper concentration of one of the substrate components methylglyoxal (**1**).^{4,5} The deficiency of this ketoaldehyde **1**⁶ and the unusually high concentration of lactic acid⁷ in cancer cells imply that such cells, having lost the ability to maintain a proper concentration of methylglyoxal, continue to proliferate at an unchecked rate. Selective inhibition of glyoxalase I that may result in a buildup of methylglyoxal (**1**) again in these cells might provide an effective means of chemotherapy.

Numerous studies, testing this hypothesis, have been carried out using substrate-related molecules. The carcinostatic activity of α -ketoaldehydes, including methylglyoxal (**1**), is known⁸ but not effective since these agents are rapidly metabolized to the corresponding α -hydroxy acids by the glyoxalase enzyme system.⁴ *S*-Alkyl derivatives and related compounds of the coenzyme glutathione (GSH) cause potent competitive inhibition of glyoxalase I^{4,9-11} and cytotoxic activity against L1210 leukemia and KB cells in tissue culture.¹² However, the rapid degradation of *S*-substituted glutathione derivatives by glu-

Scheme I



Scheme II



tathionase and cysteinylglycinase renders many of these inhibitors ineffective when tested in vivo.^{13,14} Consequently attempts have been made to find degradation resistant analogues.¹³

The discovery in this laboratory of a convenient method to prepare α -hydroxythiol esters¹⁵ made available a unique series of compounds that should be competitive inhibitors of the glyoxalase system and might be effective anticancer

Table I. Synthesis and Inhibition Studies of α -Hydroxythiol Esters

$\begin{array}{c} \text{HO} \quad \text{O} \\ \quad \\ \text{R}-\text{CH}-\text{C}-\text{SR}' \end{array}$					
No.	R	R'	% yield ^a	K_i^b	$[I]_{50}^c$
8a	CH ₃	(CH ₂) ₃ CH ₃	41	12.9	16.5
8b	CH ₃	CH ₂ C ₆ H ₅	56	5.5	7.1
8c	C ₆ H ₅	(CH ₂) ₃ CH ₃	79	29.6	37.8
8d	C ₆ H ₅	CH ₂ C ₆ H ₅	63	6.3	8.1
8e	C ₆ H ₅	CH ₂ COOCH ₃	42	8.2	10.5
8f	C ₆ H ₅	CH ₂ COO(CH ₂) ₃ - CH ₃	80	34.7	44.5
8g ^d	C ₆ H ₅	CH ₂ COOC ₂ H ₅	76	27.3	35.0
8h	C ₆ H ₅	(CH ₂) ₂ COOCH ₃	67	38.6	49.6

^a Isolated pure by column chromatography. ^b K_i values (mM) were determined from a Lineweaver-Burke treatment of inhibition data at 16.36 mM methylglyoxal and 1.42–7.09 mM GSH. ^c The $[I]_{50}$ is defined as the millimolar concentration of inhibitor required to inhibit the enzymatic reaction 50% and was calculated using the expression $[I]_{50} = K_i(K_m + [S]_0)/K_m$ for the substrate component concentrations of 16.36 mM methylglyoxal and 2.84 mM GSH. ^d The thiol was prepared from an isomeric mixture of octanol and isooctyl alcohol.

agents. This new synthetic procedure provided the opportunity to evaluate, for the first time, product-like structures.

Synthesis. A series of α -hydroxythiol esters was prepared as shown in Scheme II. Equilibration of the glyoxal 5 with the thiol 6 was carried out in the presence of Mg²⁺ (0.5 equiv) in DMF solvent. The resulting α -keto-hemithiol acetal 7, which is presumably chelated to the Mg²⁺, is subsequently converted to the corresponding α -hydroxythiol ester 8 by the addition of 2,6-dimethylpyridine (1 equiv). The mechanism of this reaction has been previously discussed.^{1,15} Increased isolated yields were realized by the presence of trace amounts of hydroquinone that retarded oxidation of the thiols to dimers and by the use of the sterically hindered base, 2,6-dimethylpyridine, rather than pyridine or triethylamine, which catalyzes the hydrolysis of the α -hydroxythiol esters during work-up. The isolated yields of the α -hydroxythiol esters synthesized by this procedure are listed in Table I.

Enzyme Inhibition Studies. The usual assay procedure¹⁶ for glyoxalase I that monitors α -hydroxythiol ester 3 concentration at 240 nm could not be used for this study because of the interference from the α -hydroxythiol ester inhibitor at this wavelength. Consequently, the assay method chosen for this study, which was originally designed for crude cellular extracts,¹⁷ measures the concentration of unreacted methylglyoxal as a monitor of enzyme activity. Aliquots of the reaction mixture were quenched in known volumes of semicarbazide solutions that resulted in the quantitative conversion of methylglyoxal to the corresponding bis(semicarbazone). The optical density of these solutions at 286 nm afforded the concentration of the bis(semicarbazone). To prevent precipitation of the α -hydroxythiol esters, all assay solutions contained DMF (3.6%) as a cosolvent. Initial velocities of the glyoxalase I reaction were measured in the presence and absence of a suitable concentration of α -hydroxythiol esters 8a–h for a series of substrate concentrations. The dissociation constants of the enzyme inhibitor complex (K_i) obtained from double reciprocal plots¹⁸ are tabulated in Table I. The concentration of inhibitor required to inhibit the enzymatic reaction 50% was then calculated for the substrate component concentrations of 16.36 mM methylglyoxal and 2.84 mM

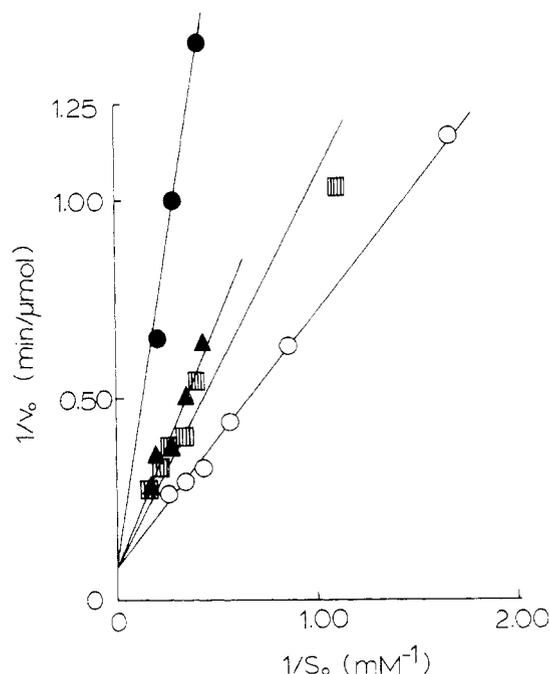


Figure 1. Representative double reciprocal plots showing competitive inhibition of glyoxalase I. V_0 is the initial velocity and S_0 is the initial substrate concentration of 2: (○—○) no inhibitor present; (▲—▲) 5.6 mM 8b; (■—■) 16.7 mM 8c; (●—●) 8.4 mM 8e.

glutathione (Table I). In addition, double reciprocal plots also established that each α -hydroxythiol ester 8a–h was a competitive inhibitor of glyoxalase I (Figure 1). The K_m and V_{max} values for glyoxalase I were determined to be 8.26 mM and 12.8 μ mol/min, respectively.

These competitive inhibitors presumably bind at the active site of glyoxalase I and of these the most effective were 8b and 8d. Since both methyl- and phenylglyoxal are substrates for the enzyme, the significant common structural feature of 8b and 8d is the benzyl R' group. The effective binding of the benzyl group suggests that there is an important hydrophobic binding region at the glyoxalase I active site normally occupied by glutathione that accommodates the benzyl group exceedingly well.

Biological Results. The α -hydroxythiol esters 8a–f and 8h were evaluated in the L1210 lymphoid leukemia and assays were performed according to the specifications of Drug Evaluation Branch, National Cancer Institute, Bethesda, Md. 20014. The tests were made on BDF₁ mice. The compounds, as emulsions in saline, saline with Tween 80, or Klucel (hydroxypropylcellulose, HPC), were given as intraperitoneal injections 24 or 48 h after tumor implantation using multiple dose assays and a three- or six-mouse assay procedure. When injections were repeated the interval was every fourth day to a maximum of three injections. Control animals were similarly treated except the injections contained no test compound. Antitumor activity was evaluated with respect to the mean survival time in days of the treated animals over that of the controls. These results are summarized in Table II. No significant antileukemic activity was found in these α -hydroxythiol esters. Failure of these compounds in the in vivo studies, in spite of their rather effective competitive inhibition of glyoxalase I, may be primarily due to the ease of hydrolysis of α -hydroxythiol esters.

Experimental Section

Infrared spectra were determined in CCl₄ with a Beckman Model IR-10 infrared recording spectrophotometer. Ultraviolet

Table II. L1210^a Screening Data on α -Hydroxythiol Esters

No.	Vehicle ^b	Day of first inj ^c	No. of inj ^d	O.D. ^e	Toxicity ^f	Tumor evaln. ^g test/control	L1210 (T/C) % ^h
8a	Tween 80	2	2	100	3/3	10.0/10.1	99
8b	Tween 80	2	2	200	3/3	10.3/10.5	98
8c	Saline	1	1	100	6/6	9.8/9.3	105
8d	Saline	1	3	200	6/6	10.7/10.6	100
8e	Klucel	1	3	200	6/6	10.5/10.1	103
8f	Tween 80	1	3	100	6/6	10.2/9.7	105
8h ⁱ	Klucel	1	1	400	6/6	9.8/9.3	105

^a Standard inoculum of 10⁵ L1210 lymphoid leukemia cells implanted intraperitoneally (ip) on day 0 in BDF₁ mice.

^b Compounds injected ip as suspensions in saline, saline with Tween 80, or Klucel (hydroxypropylcellulose, HPC). ^c Day of first injection after cell implantation. ^d Number of injections every fourth day at same dosage level. ^e Optimum dose in mg/kg for ip drug administration that provided maximum (T/C) %. The multiple dose levels assayed were 400, 200, and 100 mg/kg. ^f Number of survivors of total number of animals started on test. Recorded on day 5 in survival systems as a measure of drug toxicity. ^g Mean survival time of animals in days. ^h (T/C) % is the ratio of mean survival time of treated animals to control animals expressed as percent. In general, an increase in survival of treated animals as compared to control animals resulting in a (T/C) % of 125 or more is required for further testing. ⁱ 8g was not tested.

spectra were determined in ethanol with a Cary Model 14 recording spectrophotometer equipped with a thermostated cell compartment. The ¹H NMR spectra were determined in CCl₄ at 60 MHz using a Varian Associates Model A-60 NMR spectrometer. The chemical shifts are expressed in δ values (parts per million) relative to a Me₄Si internal standard. The mass spectra were obtained with a Consolidated Electronics Corp. Model 110-21B mass spectrometer. All pH measurements were determined with a Radiometer/Copenhagen Model PHM62 pH meter equipped with a Radiometer combination Model GK2301C single electrode. Analyses of the elements indicated by the symbols were within $\pm 0.4\%$ of the calculated values for all new compounds. All syntheses were performed under a static pre-purified nitrogen atmosphere. Purification of the product by column chromatography was accomplished on 60–200 mesh silica gel (Grace, grade 62) by elution with petroleum ether, benzene, and diethyl ether and appropriate intermediate mixtures thereof. *N,N*-Dimethylformamide (DMF) and 2,6-dimethylpyridine were distilled just prior to use. The thiols (Evans Chemetics, Inc.) were used without further purification.

Phenylglyoxal monohydrate (Aldrich Chemical Co., 97%) was purified by three recrystallizations from CH₂Cl₂-acetone (4:1) yielding colorless needles: mp 88–90 °C.¹⁹ Methylglyoxal hydrate (25 mL, Aldrich Chemical Co., 40% aqueous solution), used in the syntheses, was partitioned between 50 mL of diethyl ether-methylene chloride (1:1) and 25 mL of water saturated with NaCl, KCl, and Na₂CO₃. After separation, the aqueous phase was extracted with three 25-mL portions of Et₂O-CH₂Cl₂ (1:1). The combined organic phase was dried (Na₂SO₄), filtered, and stored at 0 °C. As needed, the solvent of an aliquot of this solution was removed in vacuo yielding a pale yellow viscous oil shown to be a 1:1 mixture of the mono- and dihydrate of methylglyoxal.¹

Glyoxalase I (grade IV from yeast, solution in 50% glycerol-0.4 M (NH₄)₂SO₄-0.002 M KH₂PO₄, pH 6.5) is available from Sigma Chemical Co. The activity of this solution was 1000 units/mL (1.59 mg of protein/mL and 630 units/mg of protein). One unit will convert 1.0 μ mol of substrate to product/min at pH 6.6 at 25 °C. A fresh GSH solution (Fisher Scientific Co.) was prepared daily using distilled water.

General Synthesis. S-Butyl Thiollactate (8a). To a solution of 1.48 g (15 mmol) of methylglyoxal hydrate and 1.61 g (7.5 mmol) of Mg(OAc)₂·4H₂O, in 16 mL of DMF that had stirred for 10 min, was added a solution of 2.70 g (30 mmol) of *n*-butylthiol and 55 mg (0.5 mmol) of hydroquinone in 2 mL of DMF. After 1 h at 25 °C, a solution of 1.60 g (15 mmol) of 2,6-dimethylpyridine in 2 mL of DMF was added to the reaction mixture. Then after an additional 1 h of stirring, the reaction mixture was partitioned between a cold mixture of 50 mL of diethyl ether and 25 mL of water saturated with NaCl and KCl. After separation, the aqueous phase was extracted with two 25-mL portions of ether. The combined organic phase was dried (Na₂SO₄), filtered, and concentrated in vacuo at water aspiration pressure and then at vacuum pump pressure (lyophilized) to remove the DMF. Filtration column chromatography yielded 0.99 g (41%) of 8a as a pale yellow viscous oil: IR 3625, 3550, 1680 cm⁻¹; UV λ max 235 nm (ϵ 3310); NMR δ 0.97 (3 H, perturbed t), 1.44 (3 H, d, J =

6 Hz) superimposed on 1.2–1.8 (4 H, m), 2.89 (2 H, t, J = 7 Hz), 3.70 (1 H, br, exchangeable with D₂O), 4.30 (1 H, q, J = 7 Hz); MS m/e (rel intensity) 162 (M⁺, 5), 145 (4), 134 (36), 117 (16), 105 (14), 90 (39), 57 (28), 56 (84), 45 (100). Anal. (C₇H₁₄O₂S) C, H.

S-Benzyl thiollactate (8b): IR 3625, 3550, 1675 cm⁻¹; UV λ max 233 nm (ϵ 2225); NMR δ 1.08 (3 H, d, J = 6 Hz), 3.74 (2 H, s), 4.05 (1 H, q, J = 7 Hz), 4.37 (1 H, br, exchangeable with D₂O), 6.96 (5 H, apparent s); MS m/e (rel intensity) 196 (M⁺, 21), 124 (11), 91 (100), 45 (71). Anal. (C₁₀H₁₂O₂S) C, H.

S-Butyl thiolmandelate (8c): IR 3520, 1670 cm⁻¹; UV λ max 238 nm (ϵ 5240); NMR δ 0.75 (3 H, perturbed t), 1.0–1.6 (4 H, m), 2.65 (2 H, t, J = 7 Hz), 4.00 (1 H, d, J = 5 Hz, exchangeable with D₂O), 4.92 (1 H, d, J = 5 Hz, collapses to a singlet with D₂O), 7.17 (5 H, apparent s); MS m/e (rel intensity) 224 (M⁺, 0.2), 196 (3), 107 (100), 79 (31), 77 (16). Anal. (C₁₂H₁₆O₂S) C, H.

S-Benzyl thiolmandelate (8d): IR 3530, 1670 cm⁻¹; UV λ max 238 nm (ϵ 827); NMR δ 3.48 (1 H, br, exchangeable with D₂O), 3.94 (2 H, s), 4.96 (1 H, s), 7.07 (5 H, apparent s), 7.19 (5 H, apparent s); MS m/e (rel intensity) 167 (38), 124 (50), 107 (62), 91 (100), 79 (88), 77 (55). Anal. (C₁₅H₁₄O₂S) C, H.

Methyl mercaptoacetate mandelate (8e): IR 3520, 1745, 1690 cm⁻¹; UV λ sh 234 nm (ϵ 1770); NMR δ 3.52 (2 H, s), 3.59 (3 H, s), 4.3 (1 H, br, exchangeable with D₂O), 5.17 (1 H, s), 7.36 (5 H, m); MS m/e (rel intensity) 209 (7), 181 (4), 135 (14), 107 (57), 105 (100), 77 (86). Anal. (C₁₁H₁₂O₄S) C, H.

Butyl mercaptoacetate mandelate (8f): IR 3520, 1740, 1690 cm⁻¹; UV λ sh 233 nm (ϵ 6330); NMR δ 0.91 (3 H, perturbed t), 1.1–1.8 (4 H, m), 3.55 (2 H, s), 4.08 (2 H, t, J = 7 Hz), 4.72 (1 H, br, exchangeable with D₂O), 5.22 (1 H, s), 7.41 (5 H, m); MS m/e (rel intensity) 254 (3), 209 (6), 148 (26), 107 (100), 79 (33), 77 (21), 57 (17). Anal. (C₁₄H₁₈O₄S) C, H.

Octyl mercaptoacetate mandelate (8g): IR 3530, 1740, 1690 cm⁻¹; UV λ sh 233 nm (ϵ 2160); NMR δ 0.75–1.0 (9 H, complex m), 1.0–1.8 (6 H, br m), 3.58 (2 H, s), 3.8–4.2 (2 H, br m), 4.3 (1 H, br, exchangeable with D₂O), 5.22 (1 H, br s, sharpens with D₂O), 7.4 (5 H, m); MS m/e (rel intensity) 204 (12), 181 (5), 107 (100), 79 (39), 77 (26), 71 (42), 57 (64), 43 (37). Anal. (C₁₈H₂₆O₄S) C, H.

Methyl 3-mercaptopropionate mandelate (8h): IR 3525, 1745, 1675 cm⁻¹; UV λ sh 236 (ϵ 4454); NMR δ 2.48 (2 H, t, J = 6 Hz), 2.98 (2 H, t, J = 6 Hz), 3.58 (3 H, s), 4.25 (1 H, br, exchangeable with D₂O), 5.14 (1 H, s), 7.38 (5 H, m); MS m/e (rel intensity) 119 (13), 107 (36), 105 (20), 89 (8), 79 (73), 77 (100), 59 (47). Anal. (C₁₂H₁₄O₄S) C, H.

Enzyme Inhibition. A commercial 40% methylglyoxal solution was distilled at 90–100 °C (760 Torr) to remove polymeric material, the acidic material removed by passing the diluted distillate through Dowex 1 (hydroxide form), and the filtrate solution standardized using a semicarbazide method.¹⁷ Glyoxalase I was diluted to 20 units/mL with a solution of 30% glycerol containing 0.1% bovine serum albumin. All enzymatic reactions were performed at 30 °C in 0.1 M phosphate buffer at pH 6.6. For each assay the reaction contained a total volume of 11.0 mL that was 16.36 mM with respect to methylglyoxal and 1.42–7.09 mM with respect to GSH. Sufficient glyoxalase I (200 μ L of the

20 units/mL solution) was employed to give an easily measurable rate which was followed by removing 65- μ L aliquots at 30-s intervals for the first 5 min and immediately quenching each aliquot in 10.0 mL of 0.1 M phosphate buffer at pH 7.0 containing 0.15 M semicarbazide hydrochloride. After complete conversion of methylglyoxal to the bis(semicarbazone) (ca. 10 h), the initial rate of the enzymatic reaction could be determined by measuring the absorption of the bis(semicarbazone) in these solutions at 286 nm (ϵ 3.2×10^4 M⁻¹ cm⁻¹). Methylglyoxal (16.36 mM), GSH (1.42–7.09 mM), inhibitor (none or 5.6–16.7 mM) in 400 μ L of DMF, and buffer (10.3–9.9 mL) were allowed to equilibrate at 30 °C for 5 min to allow time for the formation of the substrate before addition of the enzyme. The initial substrate concentration [S]₀ was varied by increasing the GSH concentration from 1.42 to 7.09 mM and calculated at equilibrium from a quadratic equation using the dissociation constant of 3.0 mM.²⁰ A computer program was used to determine the line of best fit by the method of least squares. The dissociation constants of the enzyme inhibitor complex (K_i) were obtained from double reciprocal plots¹⁸ of $1/V_0$ vs. $1/[S]_0$ and $1/V_i$ vs. $1/[S]_0$. V_0 = initial velocity of the uninhibited enzymatic reaction and V_i = initial velocity of the inhibited reaction at a suitable inhibitor concentration (5.6–16.7 mM). The concentration of inhibitor required to inhibit the enzymatic reaction 50% was calculated using the expression $[I]_{50} = K_i(K_m + [S]_0)/K_m$ for the substrate component concentrations of 16.36 mM methylglyoxal and 2.84 mM glutathione.

Biological Testing. 10⁵ L1210 lymphoid leukemia were implanted intraperitoneally into BDF₁ mice on day 0. Ip drug treatment was administered on day 1; day 1, 5, and 9; or day 2 and 6; and evaluated by the mean survival time in days of the treated animals over that of the control. Multiple dose assay levels included 400, 200, and 100 mg/kg and used a three- or six-mouse assay procedure.

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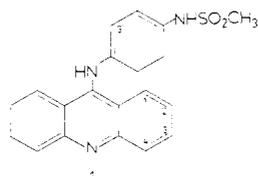
Potential Antitumor Agents. 25. Azalogues of the 4'-(9-Acridinylamino)methanesulfonanilides

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Tumor inhibition produced by isomeric, nitro-substituted 4'-(9-acridinylamino)methanesulfonanilide analogues of low base strength ($pK_a = 4.79$ – 5.72) might result from in vivo reduction to the corresponding, higher pK_a (7.15–9.80), tumor active amines. The aza analogues, -N= in place of -C(NO₂)=, have been prepared as nonclassical bioisosteres and screened in the L1210 system. Significant L1210 inhibition produced by isomeric 3- and 4-azalogues, of similar base strength to the corresponding nitro-substituted derivatives, demonstrates that weakly basic analogues can provide biologic activity when there is no prospect of in vivo reduction to more strongly basic products. Obligatory reduction of nitro function, for biologic activity, need not be postulated in this drug series.

Certain nitro group substituted 4'-(9-acridinylamino)-methanesulfonanilide (AMSA, **1**) congeners are significantly tumor inhibitory in L1210 screening assays^{1,2} with the 3-nitro isomer (**13**, Table I) proving particularly ef-



fective. The powerfully electron-withdrawing nitro function markedly lowers acridine base strength and from the pK_a values of the L1210 active isomers (**12**, **13**) it can be calculated that less than 2% of these compounds would be ionized at physiologic pH (7.4). Additionally, the 3,6-dinitro-substituted AMSA agent ($pK_a = 3.68$), which would be less than 0.03% ionized at pH 7.4, is also convincingly L1210 active.³ It has usually been considered that the cation of chemotherapeutic acridines provides observed biologic activity.⁴ More highly ionized acridines normally prove either more efficacious or more dose po-