ions and conditions is sometimes necessary to obtain satisfactory crystalline end products.

Necessary intermediates not listed in Table I or described in the literature are listed below; methods of synthesis are obvious from the preamble.

4-[p-(p-Amino-o-nitrobenzamido)anilino]pyridine: bronze plates; EtOH; mp 259–260°. *Anal.* ($C_{18}H_{15}N_5O_3$) C, H, N.

4-(p-Acetamido-o-nitroanilino)pyridine: red needles; n-BuOH; mp 265.5–266.5°. *Anal.* ($C_{13}H_{12}N_4O_3$) C, H, N.

4-(p-Amino-o-nitroanilino)pyridine: deep red, almost black needles; EtOH; mp 167.5–168°. *Anal.* ($C_{11}H_{10}N_4O_2$) C, H, N.

4-[p-(p-Aminobenzamido)-o-nitroanilino]pyridine: scarlet plates; DMF–H_2O; mp 244–245°. *Anal.* ($C_{18}H_{15}N_5O_3$) C, H, N.

4-[p-(p-Amino-o-nitrobenzamido)-o-nitroanilino]pyridine: orange plates; DMF–H_2O; mp 265.5–277°. *Anal.* ($C_{18}H_{14}N_6O_5$) C, H, N.

2,4-Diamino-5-[p-(p-amino-o-nitrobenzamido)phenyl]pyrimidine: yellow needles; DMF–H_2O; mp 247–249°. *Anal.* ($C_{17}H_{15}N_7O_3$) C, H, N.

p-(p-Acetamido-o-nitrophenylcarbamoyl)aniline: orange-red prisms; DMF–EtOH; mp 283.5–284°. *Anal.* ($C_{15}H_{14}N_4O_4$) C, H, N.

4-[p-(p-Amino-o-nitrophenylcarbamoyl)anilino]pyridine: red prisms; EtOH; mp 269–270°. *Anal.* ($C_{18}H_{15}N_5O_3$) C, H, N.

p-(p-Acetamido-o-nitrobenzamido)acetophenone: cream prisms; EtOH–H_2O; mp 237–238°. *Anal.* ($C_{17}H_{15}N_3O_5$) C, H, N.

p-(p-Amino-o-nitrobenzamido)acetophenone: yellow plates; EtOH–H_2O; mp 239–240°. *Anal.* ($C_{15}H_{13}N_3O_4$) C, H, N.

p-(p-Amino-o-nitrobenzamido)acetanilide: yellow prisms; DMF–EtOH–H_2O; mp 300–302°. *Anal.* ($C_{15}H_{14}N_4O_4$) C, H, N.

4-[p-(p-Acetamidophenylcarbamoyl)-m-nitroanilino]pyridine: yellow plates; EtOH; mp 298–299°. *Anal.* ($C_{20}H_{17}N_5O_4$) C, H, N.

4-[p-(p-Aminophenylcarbamoyl)-m-nitroanilino]pyridine: orange needles; EtOH–H_2O; mp 258–259°. *Anal.* ($C_{18}H_{15}N_5O_3$) C, H, N.

Biological Testing. The routine test consists of intraperitoneal inoculation of 10^5 L1210 cells into 18.5–22.5-g C_3H/DBA_2 F_1 hybrids on day 1; drug treatment was initiated 24 hr later and continued for 5 days. Average survivals were calculated in the usual way. An attempt was made to test all drugs from a level which was frankly toxic, giving either toxic deaths before control deaths or marked weight loss; serial twofold dilutions were then tested until an obviously nontoxic dose was reached; this usually required a total of three tests. Compounds which under these test conditions were not given T/C values greater than 125% have been classified as negative and this is recorded in the requisite column in Table I. On retesting positives doses have been arranged at 0.18 log dose intervals, the levels ensuring tests from toxic levels to those which give less than 40% increase in life span. All dosage has been in 0.2 ml of H_2O. Groups of six animals per dose level were used and one control group for every five tests.

In the subcutaneous tests 10^5 L1210 cells were implanted subcutaneously above the right axilla. Drugs were administered by the intraperitoneal route on days 2–6 as for tests against intraperitoneally implanted tumor.

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Supplementary Material Available. Full details of L1210 screening data will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JMED-74-930.

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Potential Antitumor Agents. 11. Inhibitors of Alkaline Phosphatase, an Enzyme Involved in the Resistance of Neoplastic Cells to 6-Thiopurines†

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A series of 4′-substituted derivatives of 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP) has been synthesized and evaluated as inhibitors of alkaline phosphatase partially purified from a murine ascitic cell line of Sarcoma 180 resistant to the antileukemic agents 6-mercaptopurine and 6-thioguanine. These agents were also tested as inhibitors of ribonucleoside diphosphate reductase from rat Novikoff hepatoma and for antineoplastic activity in mice bearing either 6-thiopurine-sensitive or -resistant cells of Sarcoma 180. Structure–activity relationship studies have delineated the bulk requirement for a five-membered ring at the 4′ position for optimum phosphatase-inhibitor interaction. Similar bulk produced loss of activity by α-(N)-heterocyclic carboxaldehyde thiosemicarbazones as inhibitors of ribonucleoside diphosphate reductase. Some of these agents were found to possess potent tumor-inhibitory potential.

Investigation of potential mechanisms by which leukemic cells of man acquire resistance to the antileukemic 6-thiopurines (*i.e.*, 6-mercaptopurine and 6-thioguanine) has been pursued in this laboratory in both an experimental animal model tumor system[1,2] and in leukocytes from patients with leukemia.[3] Initial studies with the animal model system (Sarcoma 180/TG ascites cells) have indicated that the mechanism of acquired insensitivity to the 6-thiopurines exhibited by this variant was not the result of impaired uptake or increased catabolism of the 6-thiopurine *per se*, nor of a decreased capacity to synthesize the inhibitory nucleotide form.[1] Since an increased rate of loss of the active analog nucleotide form occurred in resistant cells as compared with the parent subline, in-

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creased catabolism of the nucleotide forms of these antileukemic agents by the neoplastic cells was suggested as the possible mechanism of acquired resistance to these agents.[1,4] Corroborating this hypothesis was the finding that particulate bound alkaline phosphatase activity was markedly increased in the resistant variant (Sarcoma 180/TG).[2] Henderson, *et al.*,[5] and Bennett, *et al.*,[6] have also proposed a similar mechanism of resistance to 6-methylmercaptopurine ribonucleoside and 4-aminopyrazolo[3,4-*d*]pyrimidine, respectively. More recent studies have suggested that a similar mechanism may be operative in the acquisition of insensitivity to 6-thiopurines by acute lymphocytic leukocytes of man.[3] The development of an effective inhibitor of the enzyme alkaline phosphatase could conceivably restore sensitivity to the 6-thiopurines in neoplasms developing resistance by this mechanism and thereby be of clinical significance.

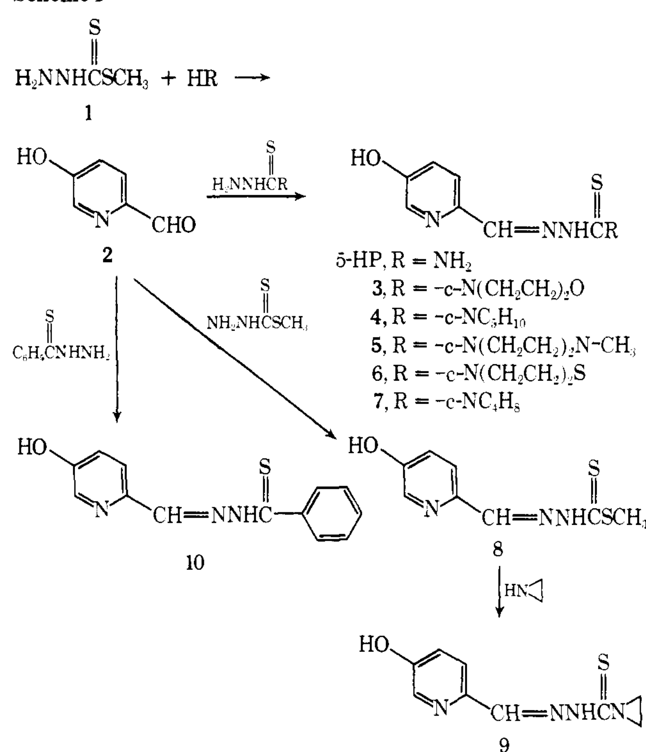
Effective inhibitors of alkaline phosphatase are not abundant to date. Fishman and coworkers[7,8] have reported three examples of amino acids (*i.e.*, L-phenylalanine, L-tryptophan, and L-homoarginine) that have relatively weak organ-specific inhibitory properties toward isoenzymes of alkaline phosphatase from human placenta, intestine, liver, and bone tissue. These organ-specific inhibitors of alkaline phosphatase have been explored in the area of serum enzymology applied to the differential diagnosis of disease. Fishman, *et al.*,[9] also have reported the presence of high concentrations of serum alkaline phosphatase in a patient with bronchogenic carcinoma, indicating the possibility of the neoplastic origin of this enzyme, a factor which is conceivably of significance in the relative resistance of this disease to purine and pyrimidine antimetabolites which require conversion to the nucleotide level to exert cytotoxicity.

More efficacious inhibitors of alkaline phosphatase from mammalian tissues have been reported recently by Brunel and Cathala[10] and Van Belle.[11] The former workers reported that imidazole was inhibitory to L-phenylalanine-insensitive alkaline phosphatases of tissues other than intestine and placenta, while Van Belle[11] found that anthelmintic compounds of the imidazo[2,1-*b*]thiazole series were inhibitory toward alkaline phosphatase enzymes from kidney, bone, liver, placenta, and mammary tumor but were ineffective as inhibitors of the intestinal form of the enzyme.

An initial attempt from this laboratory to design new inhibitors of alkaline phosphatase of Sarcoma 180/TG ascites cells has been described.[12] The rationale on which these agents were designed was based upon evidence that zinc is an essential cofactor for the active form of alkaline phosphatases from both microbial and mammalian sources; for this reason a number of α-(N)-heterocyclic carboxaldehyde thiosemicarbazones with metal chelating ability were evaluated as inhibitors of a particulate-bound alkaline phosphatase purified about 20-fold from Sarcoma 180/TG.[12] The results of this study indicated that the introduction of a morpholine ring system onto the side chain of these compounds, in place of the terminal NH_2 group, greatly increased inhibitory potency toward alkaline phosphatase, whereas substitution of only one terminal proton by a hydroxyethyl group resulted in an agent with about six times less potency as an inhibitor of this enzyme. Therefore, in the present investigation we have synthesized and studied various derivatives for structure–activity relationships by (a) isosteric replacement of O in the morpholine ring by C, N, or S, and (b) substitution of smaller ring systems for morpholine in an effort to delineate the bulk tolerance of the enzyme for hydrophobic interaction at the terminal 4′ position of the α-(N)-heterocyclic carboxaldehyde thiosemicarbazones. In addition, the requirement for the terminal N atom in the thiosemicarbazone side chain was ascertained by replacing it with a phenyl ring to give a thiobenzhydrazone derivative.

Chemistry. 4′-Substituted thiosemicarbazides, the intermediates employed for the syntheses of corresponding thiosemicarbazone derivatives, were prepared in general by the procedure of McElhinney[13] with some modifications in a few instances that are described in the Experimental Section. The various amines used for the nucleophilic substitution of methyl dithiocarbazate (**1**, Scheme I) were obtained commercially except for thiomorpholine, which was made by $LiAlH_4$ reduction of 3-thiomorpholinone.[14] The reaction of ethylenimine with **1** was not successful under various conditions; therefore, 5-hydroxy-2-formylpyridine 4′-dimethylenethiosemicarbazone (**9**) was synthesized by first forming the corresponding methyl dithiocarbazate ester **8** and then reacting it with ethylenimine. Reaction of **2** with thiobenzhydrazide[15] yielded the desired compound **10**.

Scheme I



Biological Results and Discussion. The concentrations of 4′-substituted thiosemicarbazones required to produce 50% inhibition of the activity of a particulate-bound alkaline phosphatase from Sarcoma 180/TG ascites cells are shown in Table I. The parent unsubstituted compound, 5-hydroxy-2-formylpyridine thiosemicarbazone (5-HP), a potent antineoplastic agent against various transplanted neoplasms,[16,17] required 250 μM to inhibit phosphatase activity by 50%. Replacement of the two terminal protons of the 4′-NH_2 group of the thiosemicarbazone side chain to produce a six-membered ring, containing either a C, N, O, or S atom para to the 4′-N, resulted in compounds **4**, **5**, **3**, and **6**, respectively, which were about 10- to 20-fold more active than 5-HP. No major differences as inhibitors of the activity of alkaline phosphatase appeared to exist between compounds containing the various isosteric elements. However, when the ring size was reduced from a six- to a five-membered (*i.e.*, pyrrolidine) ring, an agent (**7**) was obtained that required a concentration of only 4 μM to inhibit alkaline phosphatase activity by 50%. This was 62-fold more potent than 5-HP. Further reduc-

Table I. Concentrations of 4′-Substituted 5-Hydroxy-2-formylpyridine Thiosemicarbazones Required for 50% Inhibition of Alkaline Phosphatase Activity[a]

Compd	ID_{50},[a] μM	Ratio ID_{50} (5-HP)/ID_{50}
5-HP	250	1.0
3	17	14.7
4	17	14.7
5	24	10.4
6	12	20.8
7	4	62.5
8	80	3.1
9	100	2.5
10	110	2.3

[a]The ID_{50} is the concentration of drug required to reduce by 50% the observed activity of the enzyme. The values were estimated from graphs summarizing the results of at least two experiments with four concentrations of each compound.

Table II. Concentrations of 4′-Substituted 5-Hydroxy-2-formylpyridine Thiosemicarbazones Required for 50% Inhibition of Ribonucleoside Diphosphate Reductase Activity[a]

Compd	ID_{50},[a] μM	Ratio ID_{50}/ID_{50} (5-HP)
5-HP	3	1.0
3	69	23.0
4	42	14.0
5	72	24.0
6	82	27.3
7	66	22.0
8	11	3.7
9	74	24.7
10	49	16.3

[a]The ID_{50} is the concentration of drug required to reduce by 50% the observed activity of the enzyme. The values were estimated from graphs summarizing the results of at least two experiments with four concentrations of each compound.

tion of the ring size to a three-membered ethylenimine (9) resulted in a derivative that was 25-fold less active than **7**. The intermediate methyl dithioester 8 and compound **10**, a derivative in which the terminal group was completely replaced by a phenyl group, were also about 25-fold less active than 7. Thus, these findings seem to have delineated the bulk requirement for a five-membered ring at the 4′ position of 5-HP for optimum phosphatase-inhibitor interaction.

Previous work from this laboratory has demonstrated that α-(N)-heterocyclic carboxaldehyde thiosemicarbazones are primarily inhibitors of DNA synthesis, due to their inhibitory effect on the enzyme ribonucleoside diphosphate reductase,[18-20] an enzyme that catalyzes the conversion of ribonucleotides to deoxyribonucleotides. Therefore, we have also tested these 4′-substituted derivatives of 5-HP for inhibition of the activity of this latter enzyme from the Novikoff rat tumor. The results shown in Table II indicate that while 5-HP, the parent compound, needed only 3 μM for 50% inhibition of the activity of ribonucleoside diphosphate reductase, the 4′-substituted derivatives required approximately 4-27 times more drug to achieve a similar degree of inhibition. These results suggest that the ribonucleoside diphosphate reductase enzyme is intolerant of bulk at the terminal $-NH_2$ group and delineates a second area of low bulk tolerance by this enzyme,[21] whereas bulk, of optimal size, can increase the potency of these derivatives for inhibition of alkaline phosphatase.

The 4′-substituted derivatives of 5-HP were also tested for antineoplastic activity in mice bearing the ascites cell forms of Sarcoma 180 and Sarcoma 180/TG, the variant subline resistant to 6-thiopurines. The results obtained are shown in Table III. The data indicate that the newly synthesized agents possess significant tumor-inhibitory potency in these neoplastic model systems. Compounds 4 and 7 produced about a 100% increase in survival time over controls in mice bearing either Sarcoma 180 or Sarcoma 180/TG ascites cells. Compound **3**, which contained a morpholine ring, was most interesting, in that its growth inhibitory effect on Sarcoma 180 was marginal (*i.e.*, it increased the average survival time from 13.4 days for untreated control tumor-bearing animals to 19.0 days at its maximum effective daily dosage of 40 mg/kg), but in the variant subline, Sarcoma 180/TG, the increase in the average survival time produced by this agent was much more dramatic, being from 14.2 days for untreated controls to a maximum extension of the life of drug-treated animals to 37.2 days, with 40% of the mice surviving at least 50 days. The reason for this differential in antineoplastic potency is unclear. The precise biochemical locus of action of these agents responsible for inhibition of cell growth is currently under investigation in our laboratory. However, it does not seem hypothetically possible that the potent activity of compound **3** against Sarcoma 180/TG is primarily due to inhibition of alkaline phosphatase activity. Compounds other than **3**, **4**, and **7** were either weaker inhibitors of these experimental tumors or inactive.

Preliminary experiments were also carried out to determine if these inhibitors of alkaline phosphatase would restore the sensitivity of the variant subline (Sarcoma 180/TG) to 6-thiopurines. Combination treatments using azaserine (0.4 mg/kg/day), to enhance the synthesis of 6-thiopurine nucleotides,[22,23] in combination with thioguanine (2 mg/kg/day) and an inhibitor of alkaline phosphatase administered simultaneously once daily for 6 consecutive days were carried out in mice bearing Sarcoma 180/TG ascites cells. No restoration of sensitivity to 6-thioguanine was achieved under these conditions. Thus, either the concentrations of the 4′-substituted thiosemicarbazones employed in these studies were not sufficient to achieve and maintain adequate inhibition of alkaline phosphatase activity *in situ* for a reasonable period of time or other mechanisms are also involved in the expression of insensitivity to the 6-thiopurines by this subline. These two alternatives are presently under investigation in our laboratory. However, the significant antineoplastic activity of some of these new compounds when employed alone, even with relatively low affinity for ribonucleoside diphosphate reductase, an apparent determinant of tumor susceptibility to the related parent α-(N)-heterocyclic carboxaldehyde derivatives, suggests that a new site(s) of action may in part be involved in the cytotoxicity of this series and encourages further exploration.

Experimental Section

Melting points were determined in capillary tubes using a Thomas-Hoover stirred liquid apparatus and are corrected. The ir absorption spectra were obtained with a Perkin-Elmer Model 257 spectrophotometer with KBr pellets of solids and were consistent with the proposed structures. Elemental analyses were performed by the Baron Consulting Co., Orange, Conn. Where analyses are indicated only by symbols of the elements, the analytical results for those elements were within ±0.4% of the theoretical values.

Inhibition of Alkaline Phosphatase. Alkaline phosphatase from murine ascites cells of Sarcoma 180/TG was partially purified by a procedure described by Lee and Sartorelli[24] involving solubilization of particulate bound enzyme (alkaline phosphatase A), ethanol fractionation, DEAE column chromatography, and gel filtration. The purity of the enzyme thus obtained was approximately 5000-8000-fold. The assay procedure was the same as

Table III. Effect of 4′-Substituted 5-Hydroxy-2-formylpyridine Thiosemicarbazones on the Survival Time of Mice Bearing Sarcoma 180 and Sarcoma 180/TG Ascites Cells

	Sarcoma 180				Sarcoma 180/TG			
Compd	Optimal daily dose, mg/kg[a]	Av Δ wt, %[b]	Av survival time, days ± S.E.	No. of 50-day survivors[c]	Optimal daily dose, mg/kg[a]	Av Δ wt, %[b]	Av survival time, days ± S.E.	No. of 50-day survivors[c]
None		+17.4	13.4 ± 0.5	0/55		+21.9	14.2 ± 0.4	0/25
5-HP	60	−3.3	31.4 ± 2.0	3/15	30	+9.3	26.2 ± 6.1	1/5
3	40	−19.3	19.0 ± 3.0	1/20	40	−12.7	37.2 ± 4.9	4/10
4	40	−12.0	26.6 ± 6.1	1/5	40	−6.2	29.4 ± 4.9	1/9
5	10	+10.0	17.4 ± 1.6	0/5	20	+21.0	17.4 ± 2.1	0/10
6	30	−12.6	21.8 ± 7.4	1/5	30	−18.1	18.0 ± 4.4	0/5
7	10	+0.1	28.1 ± 3.7	1/10	40	−2.5	27.2 ± 1.5	0/15
8	20	+9.7	15.2 ± 6.3	0/5	20	+6.8	16.6 ± 1.5	0/5
9	40	+6.3	15.8 ± 1.2	0/5	20	+17.7	16.6 ± 2.9	0/5
10	20	+16.4	14.0 ± 0.5	0/5				

[a]Administered once daily for 6 consecutive days, beginning 24 hr after tumor transplantation; each value represents the results obtained with 5–20 animals. [b]Average weight change from onset to termination of drug treatment. [c]Number of animals that survived at least 50 days; these mice were calculated as 50-day survivors in determination of the average survival time.

Table IV

Compd	Crystn solvent	Mp, °C dec	Yield, %	Formula	Analyses
$NH_2NHC(=S)$-c-$N(CH_2CH_2)_2N$-CH_3	EtOAc	156–157	26	$C_6H_{14}N_4S$	C, H, N
$NH_2NHC(=S)$-c-$N(CH_2CH_2)_2S$	EtOH–H_2O	159–160	30	$C_5H_{11}N_3S_2$	C, H, N
4	EtOH–H_2O	159–160	76	$C_{12}H_{16}N_4OS$	C, H, N
5	EtOAc	138–140	58	$C_{12}H_{17}N_5OS$	C, H, N
6	EtOH–H_2O	175–176	78	$C_{11}H_{14}N_4OS_2$	C, H, N, S
7	EtOH–H_2O	210–212	90	$C_{11}H_{14}N_4OS$	C, H, N
8	EtOH	198–199	70	$C_8H_9N_3OS_2$	C, H, N
9	EtOH–H_2O	251–253	52	$C_9H_{10}N_4OS$	C, H, N
10	EtOAc–C_6H_6	140–141	72	$C_{13}H_{11}N_3OS$	C, H, N

described earlier.[12] Inhibitor stock solutions were made at 10^{-3} *M* in 20–50% DMSO and varying amounts of inhibitor were added to the reaction mixture. The concentration of DMSO was less than 5% in the assay mixture and an appropriate control containing DMSO was included. The enzyme was incubated at 25° for 15 min in 0.8 *M* Tris-HCl (pH 9.2) with various concentrations of inhibitors; substrate was then added to initiate the reaction. Enzyme activity was measured by determination of the initial rate of hydrolysis of *p*-nitrophenyl phosphate using the change in absorbancy at 410 nm produced by the formation of *p*-nitrophenol with a Gilford thermostated spectrophotometer.

Inhibition of Ribonucleoside Diphosphate Reductase. The ribonucleoside diphosphate reductase of the rat Novikoff ascites hepatoma was partially purified as described earlier.[25] Reduction of CDP was assayed as described,[21,25] except that $Fe(NH_4)_2(SO_4)_2$ was used instead of $FeCl_3$. The assay system contained 8.3 m*M* phosphate buffer (pH 7), 2.1 m*M* ATP, 4.2 m*M* MgOAc, 0.04 m*M* $Fe(NH_4)_2(SO_4)_2$, 0.17 m*M* CDP-^{32}P (1.6 × 10^6 cpm/μmol), 6.3 m*M* dithioerythritol, and about 0.15 mg of partially purified ribonucleoside diphosphate reductase in 0.12 ml of incubation mixture. The inhibitors were dissolved in DMSO; the final concentration of DMSO in the final enzyme assay was not over 1% and was not inhibitory. The reaction was begun by adding enzyme to the mixture of substrates and inhibitors at ice temperature and immediately warming to 37°. Incubation time was 30 min and the reaction was terminated by addition of 1 ml of 1 *M* perchloric acid.

Antitumor Activity. Experiments were performed on 9–11-week-old female CD-1 mice. Transplantation of Sarcoma 180 and Sarcoma 180/TG ascites cells was carried out using a donor mouse from each line bearing a 7-day tumor growth. The experimental details have been described earlier.[26] Mice were weighed during the course of the experiments, and the percentage change in body weight from onset to termination of therapy was used as an indication of drug toxicity. Determination of the sensitivity of ascitic neoplasms to these agents was based on the prolongation of survival time afforded by the drug treatments. Each compound was tested in at least three appropriate dose levels varying between 10 and 60 mg/kg/day, with the exact dose levels employed being influenced by host toxicity; only the maximum effective daily dosage level for each compound is included in Table III.

4′-Substituted Thiosemicarbazides. Methyl dithiocarbazate (1.22 g, 0.01 mol), made according to Audrieth, *et al.*,[27] was treated with the appropriate amine (0.03 mol) and 10 ml of water. The mixture was heated for 6 hr at the reflux temperature of the solvent (CH_3SH was generated during the reaction). In the case of *N*-methylpiperazine, the reaction was carried out in absolute ethanol, and refluxing time was increased to 12 hr. The solution was then cooled and neutralized with AcOH. Further cooling yielded the desired thiosemicarbazides, which were filtered and recrystallized.

4′-Substituted Thiosemicarbazones. The 4′-substituted thiosemicarbazones, except for **9**, were prepared by heating a solution of 5-hydroxy-2-formylpyridine[16] containing a molar equivalent quantity of 4′-substituted thiosemicarbazide in EtOH–H_2O. A few drops of diluted AcOH were added, and the solution was heated for 5–10 min. On cooling, the thiosemicarbazone derivatives crystallized out; they were filtered and recrystallized from appropriate solvents as listed in Table IV. The synthesis of compound **3** has been reported earlier.[28]

5-Hydroxy-2-formylpyridine 5′-Dimethylenethiosemicarbazone (9). Ethylenimine (1 ml) was added slowly with stirring to a solution of **8** (0.454 g, 2 mmol) in 5 ml of DMF cooled in an ice bath. The solution was stirred for 2 hr at room temperature, at which time the reaction was complete as shown by tlc (silica gel using EtOAc as solvent). DMF was removed under vacuum, and the residue crystallized from EtOH–H_2O.

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Studies on the Conformational Requirements of Substrate and Inhibitor on Acetylcholinesterase

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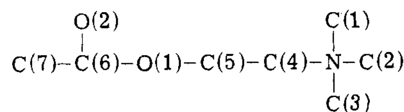
The substrate and inhibitory activity of 2-, 4-, and 6-methyl-3-trimethylammonium phenols and their acetates, propionates, and methyl ethers on AChE (*E. Electricus*) shows that there is a preferred conformation for hydrolysis of the ester group and that the mode of binding for inhibition of AChE by these compounds is not identical with that for substrate activity. A comparison of inhibitory activity of the 2- and 6-methyl-3-trimethylammonium phenols and acetates on substrates such as acetylcholine, acetylthiocholine, and phenyl acetate was used to indicate that these compounds acted on the free enzyme. The data and its interpretation are consistent with an earlier proposal that the imidazole group of histidine is closer than the serine hydroxyl to the anionic site in the AChE active center.

The most probable explanation for the hydrolysis of acetylcholine (ACh, 1) by acetylcholinesterase (AChE) at the esteratic site is by a base-catalyzed mechanism[1] and involves an imidazole group of histidine activating the OH group of serine. The activated serine residue then initiates the hydrolytic mechanism by nucleophilic attack on the carbonyl group of ACh. While considerable effort has been made to study the $N^+ \rightarrow O$ steric parameters[2-6] of the choline portion of ACh and the kinetic aspects of the reaction,[7-16] not as much attention has been given to the conformation of the acetyl group in ACh required for hydrolysis. Recently Belveridge, *et al.*,[17,18] have calculated conformation energy profiles for ACh and a number of analogous molecules using INDO molecular orbital calculations. ACh, (*R*)- and (*S*)-acetyl-α-methylcholine, and *erythro*-acetyl-$\alpha(S),\beta(R)$-dimethylcholine have low conformational energies when the torsion angle that corresponds to C(6)–O(1)–C(5)–C(4) in **1** for these molecules is 100, 120, 120, and 120°, respectively. The rates of hydrolysis of these molecules by AChE are not the same. ACh and the acetyl-α-methylcholine enantiomers have relatively high rates of hydrolysis. However, *erythro*-acetyl-$\alpha(S),\beta(R)$-dimethylcholine is not hydrolyzed by the enzyme while (*S*)-acetyl-β-methylcholine is hydrolyzed 54% as fast as ACh but has an energy minimum when the torsion angle has a value of 30°. Crystallographic studies[5,6] have also shown that the torsion angle that corresponds to C(7)–C(6)–O(1)–C(5) in ACh (1) and many of its derivatives, *e.g.*, (*R*)-(+)-acetyl-α-methylcholine, (*S*)-(+)-acetyl-β-methylcholine, lactoylcholine, and *trans*-(1*S*,2*S*)-(+)-acetoxycyclopropyltrimethylammonium iodide, is approximately 180° for all molecules. However, the torsion angle that corresponds to C(6)–O(1)–C(5)–C(4) in 1 varies and has been measured as +79° in ACh bromide, −147° for (*S*)-(+)-acetyl-β-methylcholine iodide, and −170° for one of the crystal forms of (*R*)-(+)-acetyl-α-methylcholine iodide. These differences in molecular electronic structure and crystal structure only emphasize the dangers of attempting to extend certain parameters to the *in vivo* or *in vitro* or solution conditions.

Wilson and Quan[19] have studied the molecular comple-



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