benzoxazinone (2, R = H, R_5 = H). First, 2-alkylamino substitution can greatly increase acylation rates. Secondly, branched or bulky R in 2-NHR significantly slow deacylation, and branched R block deacylation by N-cyclization (k_2). Finally, alkyl substitution of R_5 dramatically slows all modes of deacylation when R is larger than methyl. For example, the combination of these effects in 2-(isopropylamino)-5-ethylbenzoxazinone results in an 86-fold increase in k_1 and a 770-fold decrease in k_{off}, for an overall 67 000-fold decrease in K_i vs. the lead compound, thereby demonstrating the utility of the mechanistic approach in guiding the design of enzyme inhibitors.

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Articles

Thienothiopyran-2-sulfonamides: A Novel Class of Water-Soluble Carbonic Anhydrase Inhibitors

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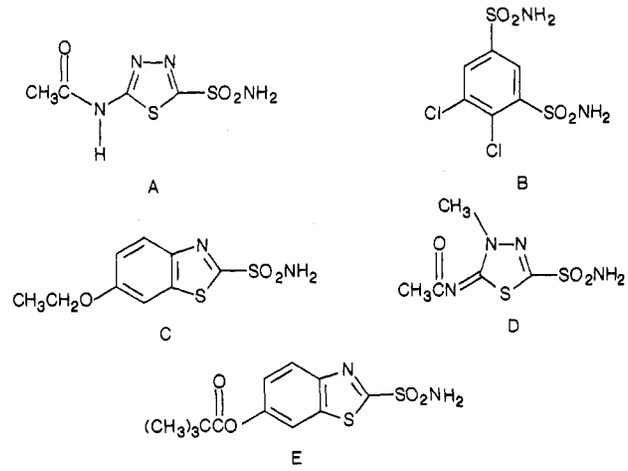
An attempt to develop a water-soluble carbonic anhydrase inhibitor focused on exploring structure–activity relationships in the thienothiopyransulfonamide class. The strategy to influence water solubility while retaining carbonic anhydrase activity involved the introduction of a hydroxyl moiety and adjusting the oxidation state of the sulfur on the thiopyran portion of the molecule. Compounds 4 and 17 best fit the criteria of aqueous solubility and inhibitory potency vs. human carbonic anhydrase II and are candidates for evaluation as topically effective antiglaucoma agents.

Since the discovery of carbonic anhydrase (CA) by Meldrum and Roughton in 1932,[1a] various aryl and heteroaryl sulfonamides have been synthesized and evaluated as inhibitors (CAI)[1b,c] for possible therapeutic use as diuretics,[2] cerebral vasodilators,[3] anticonvulsants,[4] and antiglaucoma agents.[5,6] The CAIs in current use[1] include acetazolamide (A), dichlorophenamide (B), ethoxzolamide (C), and methazolamide (D); these compounds, when administered systemically, lower intraocular pressure (IOP) by reducing aqueous humor formation.[6] However, their use is limited by side effects, which include fatigue, depression, gastrointestinal disturbances, metabolic acidosis, and anorexia. In order to circumvent these problems, attempts have been made to develop compounds that are effective when applied topically to the eye. Such an approach would permit therapeutically useful concentrations to be achieved locally, i.e., at the level of the ciliary process,[1] while reducing the systemic presentation of the drug



to a no-effect level. Recently, 2-sulfamoyl-6-benzothiazolyl 2,2-dimethylpropanoate (E)[7] and other ethoxzolamide derivatives[8] have been reported to be topically effective ocular hypotensive agents in rabbits.

To date, all of the CAIs studied as antiglaucoma agents have lacked water solubility in the 1–2% range and, therefore, have been administered to patients either systemically or topically as suspensions or gels. In this paper we wish to report on a novel class of CAIs, the thienothiopyran-2-sulfonamides, which exhibit water solubility

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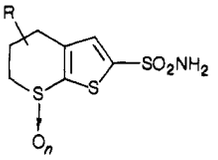
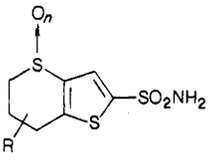
Table I



compd	R	*n*	analysis	mp, °C	recrystn solvent	% yield	total sol,[a] mg/mL	partitn coeff (1-octanol, pH 7.4 buffer)	pK_a[b]	CAI:[cd] $I_{50} \times 10^{-8}$, M	CA binding: $K_i \times 10^{-8}$, M
2	4-(=O)	0	$C_7H_7NO_3S_3$ (C, H, N)	222–223	CH_3CN	64	0.07	5.5	8.6	0.85	0.363 ± 0.084
3	4-OH	0	$C_7H_9NO_3S_3$ (C, H, N)	168–170	CH_3CN	95	0.64	4.8	9.4	3.0	2.8 ± 1.2
4	4-OH	2	$C_7H_9NO_5S_3$ (C, H, N)	167–168	CH_3CN–Et_2O	84	12.5	0.45	8.35	1.3	0.84 ± 0.09
***R*-4**	4-OH	2	$C_7H_9NO_5S_3$ (C, H, N)	170–171	CH_3CN	79	6.3	0.42	8.35	2.2	1.54 ± 0.13
***S*-4**	4-OH	2	$C_7H_9NO_5S_3$ (C, H, N)	170–171	CH_3CN	73	6.4	0.42	8.3	0.75	0.67 ± 0.3
5	4-(=O)	2	$C_7H_7NO_5S_3$ (C, H, N)	242–243	CH_3CN	67	0.27	0.92	7.92	0.5	
6	4-OH	1	$C_7H_9NO_4S_3$ (C, H, N)	155–175	$(CH_3)_2CHOH$–CH_3OH	53	12.0	0.12	8.68	10.0	10.4 ± 1.7
8	4-H	2	$C_7H_9NO_4S_3$ (C, H, N)	199.5–200.5	CH_3NO_2	76	0.56	0.69	8.35	0.45	0.33 ± 0.11
13	5-OH	2	$C_7H_9NO_5S_3$ (C, H, N)	172–176	CH_3OH–$CHCl_3$	12	8.2	0.36	8.6	1.0	0.711 ± 0.024
ethoxzolamide (C)							0.024			0.04	0.049 ± 0.0009
2-sulfamoyl-6-benzothiazolyl 2,2-dimethylpropanoate (E)							0.058			0.4	

[a] Total solubility measured in pH 7.4 buffer at 25 °C. [b] pK_a values were determined in 30% EtOH–H_2O. [c] Acetozolamide was utilized as a control for each determination shown and had an I_{50} value that ranged between 0.9 and 1.1 × 10^{-8} M. [d] Inhibitor and CA were preincubated for 5 min.

Table II



compd	R	*n*	analysis	mp, °C	recrystn solvent	% yield	total sol,[a] mg/mL	partitn coeff (1-octanol, pH 7.4 buffer)	pK_a[b]	CAI:[c,d] $I_{50} \times 10^{-8}$, M	CA binding: $K_i \times 10^{-8}$, M
15	7-(=O)	0	$C_7H_7NO_3S_3$ (C,H,N)	213–214	CH_3CN	31	0.08	8.1	8.45	0.35	0.115 ± 0.006
16	7-OH	0	$C_7H_9NO_3S_3$ (C,H,N)	157–158	CH_3CN	98	1.4	3.0	9.0	2.3	1.42 ± 0.17
17	7-OH	2	$C_7H_9NO_5S_3$ (C,H,N)	163–165	CH_3OH–$CHCl_3$	49	15.0	0.26	8.34	4.0	3.05 ± 0.30
18	7-(=O)	2	$C_7H_7NO_5S_3$ (C,H,N)	265–268	CH_3OH–CH_3CN	41	0.06	0.63	7.32	2.2	1.41 ± 0.06
19	7-OH	1	$C_7H_9NO_4S_3$ (C,H,N)	190–200	$(CH_3)_2CHOH$–CH_3OH	89	7.2	0.098	8.82	25	30.9 ± 1.2
20	6-OH	2	$C_7H_9NO_5S_3$ (C,H,N)	186–188	CH_3OH	46	7.3	0.2	8.52	4	1.82 ± 0.56

[a] Total solubility measured in pH 7.4 buffer at 25 °C. [b] pK_a values were determined in 30% EtOH–H_2O. [c] Acetozolamide was utilized as a control for each determination shown and had an I_{50} value that ranged between 0.9 and 1.1 × 10^{-8} M. [d] Inhibitor and CA were preincubated for 5 min.

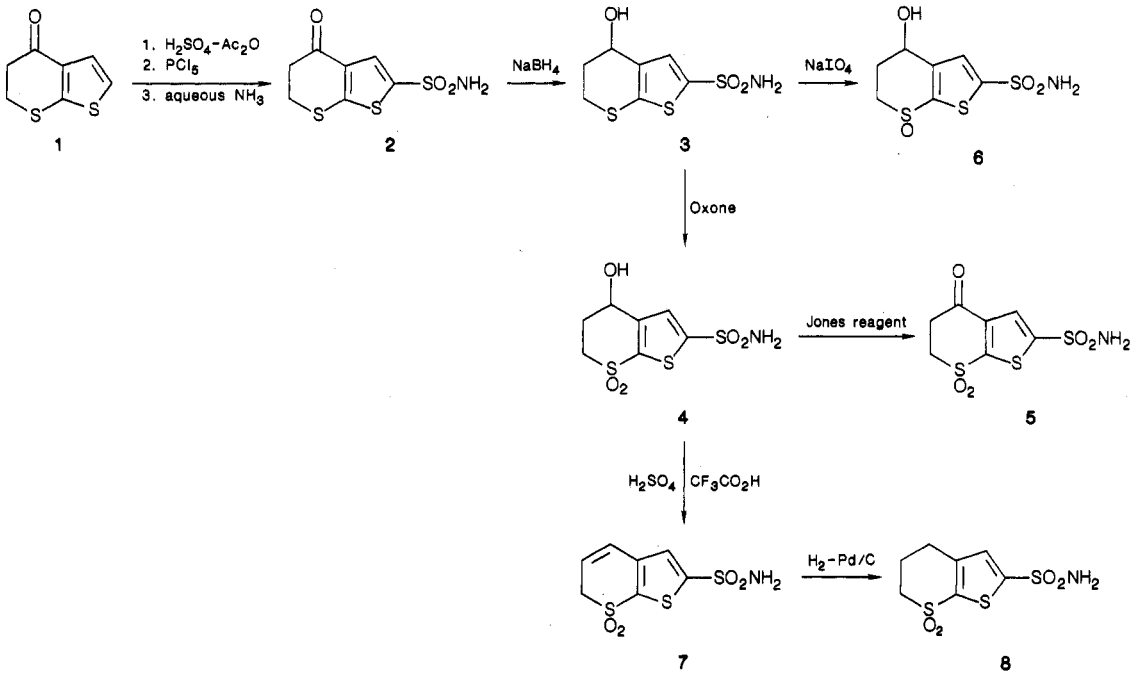
in the range of 1% and are capable of being formulated in aqueous solution for topical administration.

Chemistry. The compounds prepared in this study, **2–20**, have been divided into two structural groups, the thieno[2,3-*b*]thiopyrans and the thieno[3,2-*b*]thiopyrans. The particular examples within these two classes are summarized in Tables I and II.

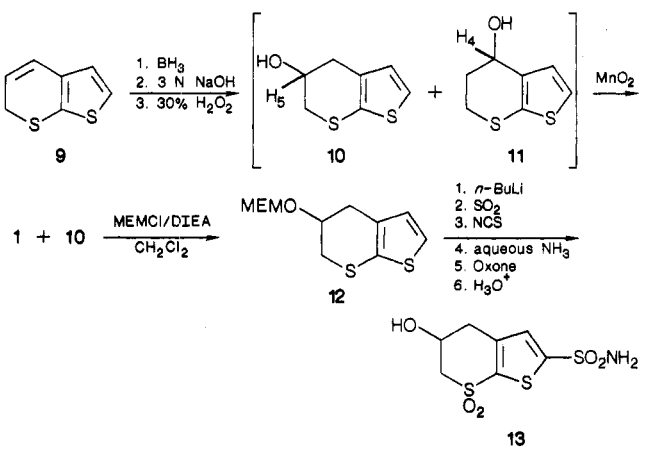
The examples in Table I are members of the 5,6-dihydro-4*H*-thieno[2,3-*b*]thiopyran class. The synthesis of the key intermediate, ketone **1**, has been described by Degani et al.[9] and Cagniant and Cagniant.[10] Reaction of ketone **1** with H_2SO_4–Ac_2O in CH_2Cl_2 provided the sulfonic acid derivative in nearly quantitative yield. Conversion of the sulfonic acid to the intermediate sulfonyl chloride

(9) Degani, I.; Fochi, R.; Spunta, G. *Ann. Chim. (Rome)* **1968**, *58*, 263.
(10) Cagniant, P.; Cagniant, D. *Bull. Soc. Chim. Fr.* **1966**, 2172.

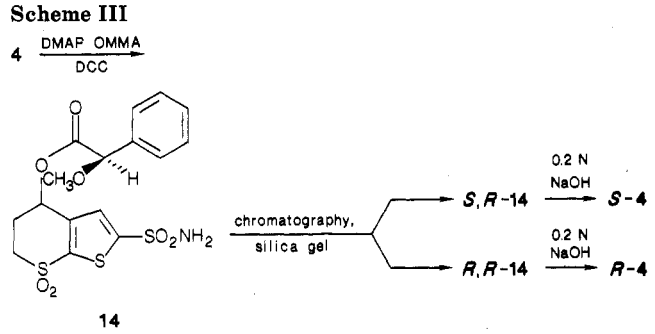
Scheme I



Scheme II



Scheme III



was accomplished by using PCl_5 in CH_2Cl_2. Subsequent treatment of the intermediate with aqueous NH_3 gave **2** in modest yield. Reduction of **2** with $NaBH_4$ in absolute EtOH provided the alcohol **3**. Oxidation of **3** with Oxone (2 $KHSO_5$–$KHSO_4$–K_2SO_4) gave the alcohol sulfone **4**. Alternate procedures involving protection of ketone **1** or alcohol **11** as the ethylene ketal or MEM ether, respectively, proved inferior vis-à-vis the approach described vide supra. Oxidation of **4** with Jones reagent and **3** with $NaIO_4$ yielded the ketone sulfone **5** and the hydroxy sulfoxide **6**, respectively. In the latter case, the sulfoxide **6** was obtained as a mixture of diastereomers, as evidenced by HPLC analysis. Dehydration of **4** with CF_3CO_2H–H_2SO_4 yielded **7**, which was hydrogenated over 10% Pd/C as catalyst to yield the saturated compound **8**.

The 4-hydroxy moiety of **4** was transposed to the 5-position, as illustrated in Scheme II. Hydroboration of **9** followed by oxidation with H_2O_2 provided a mixture of alcohols **10** and **11**, which were inseparable by TLC. The formation of the 5-hydroxy isomer was evidenced in the NMR by a triplet at δ 4.7 for H_4 of alcohol **11** and by a multiplet at δ 4.33 for H_5 of alcohol **10**, respectively. Further oxidation of the mixture with MnO_2 in $CHCl_3$ selectively oxidized the benzylic alcohol of **11** to yield the ketone **1**, leaving **10** essentially untouched, as evidenced by both TLC and ^1H NMR. The separation of **1** and **10** was easily achieved by chromatography. Protection of **10** as the MEM ether[11] **12** was accomplished by using (methoxyethoxy)methyl chloride with diisopropylethylamine in CH_2Cl_2. Sequential treatment of **12** with *n*-BuLi yielded the 2-lithio derivative, which on reaction with SO_2, then *N*-chlorosuccinimide followed by aqueous NH_3, gave the 2-sulfonamide intermediate. Without isolation, the crude sulfonamide was treated with Oxone and then hydrolyzed in acid to yield **13** in overall 12% yield.

Since compound **4**, which was one of the most potent carbonic anhydrase inhibitors, contained a chiral center, it was of interest to resolve this compound. A successful approach is outlined in Scheme III. Treatment of **4** with (*R*)-*O*-methylmandelic acid, (dimethylamino)pyridine (DMAP), and dicyclohexylcarbodiimide (DCC) in THF gave the mixture of diastereomers ***S*,*R*-14** and ***R*,*R*-14**, which were separated on a preparative scale by careful silica gel chromatography to yield first ***S*,*R*-14** followed by ***R*,*R*-14**. Mild base hydrolysis then provided pure samples of ***S*-4** and ***R*-4**, respectively. The absolute stereochemistry was assigned on the basis of ^1H NMR and X-ray crystallographic analysis of ***R*,*R*-14**.[12] Alternatively, use of (−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid

(11) Corey, E. J.; Gras, J. L.; Ulrich, P. *Tetrahedron Lett.* **1976**, 809.

(12) Trost, B. M.; Belletire, J. L.; Godleski, S.; McDougal, P.; Balkovec, J. M.; Baldwin, J. J.; Christy, M. E.; Ponticello, G. S.; Varga, S. L.; Springer, J. M. *J. Org. Chem.* **1986**, *51*, 2370.

as a resolving agent failed to provide a diastereomeric mixture that could be conveniently separated by chromatography.

When the points of ring fusion between the thiophene and thiopyran are reversed as in the thieno[3,2-*b*]thiopyrans,[9,10] the examples **16–20** were prepared from the ketone **15** in essentially an analogous manner to the compounds in the thieno[2,3-*b*]thiopyran class. This key intermediate **15** was obtained from the ethylene ketal derivative of 6,7-dihydro-5*H*-7-oxothieno[3,2-*b*]thiopyran[9] via the *n*-BuLi procedure described vide supra.

Biochemistry. The in vitro evaluation of compounds **2–20** and ethoxzolamide (C) is summarized in Tables I and II. The various examples were evaluated by the pH state assay using purified human erythrocyte carbonic anhydrase II as described in the Experimental Section. Results are expressed as I_{50} values and are obtained from semilog plots of percent inhibition of enzyme against log concentration of test compound. K_i values were determined by a fluorescence competition assay employing the fluorescent CA inhibitor dansylamide.[13] The data were fitted by a nonlinear least-squares program, and all determinations were performed in triplicate.

Results and Discussion

It has been reported that the CA inhibitor 2-sulfamoyl-6-benzothiazolyl 2,2-dimethylpropanoate (E)[7] is capable of lowering intraocular pressure in rabbits when administered as an aqueous suspension. Because of the potential disadvantages of suspensions as pharmaceutical vehicles, such as changes in particle size with time, variability in the applied dose, lower pharmaceutical acceptability, and in some cases poorer bioavailability, an investigation was initiated to discover a CA inhibitor with increased water solubility vis-à-vis known drugs. This effort has concentrated on an evaluation of the thienothiopyran sulfonamides **2–20** found in Tables I and II.

The interaction of these compounds with CA was determined by two methods. The first of these was a measure of the inhibition of carbonic anhydrase II (CA II) and is recorded as an I_{50} value. The second method is a competition study based on an interaction of the test compound and dansylamide for binding to CA II. The comparison of in vitro inhibition (I_{50}) and binding (K_i) values of the examples studied (Table I and II) demonstrates a close agreement between the two assays, with a correlation coefficient of 0.9945.

For CA inhibitory activity within these series, the thieno[2,3-*b*]thiopyrans were slightly more potent inhibitors than the corresponding thieno[3,2-*b*]thiopyrans. The only exceptions are compounds **3** and **16**, which exhibit essentially the same level of inhibitory potency. In both ring systems, the sulfoxides **6** and **19** show markedly reduced activity from their corresponding sulfides **2**, **3**, **15**, and **16** and sulfones **4**, **5**, **17**, and **18**. The oxidation state of the C-4 or C-7 oxygen is also important for CA activity, with the ketone possessing an I_{50} half that of the alcohol (**5** vs. **4** and **18** vs. **17**, respectively). In contrast, little or no difference between the transposed alcohols, **4** vs. **13**, **17** vs. **20**, respectively, was observed in these series.

The water solubility was influenced by several factors: (a) presence and position of a hydroxyl group, (b) oxidation state of the sulfur atom, and (c) chirality of the hydroxyl substituent. Replacement of the 4-hydrogen of **8** by a hydroxyl moiety as in **4** has a significant effect on water solubility; for example, a solubility of 0.56 mg/mL for **8** compares with 12.5 mg/mL for **4**, respectively. Transposition of the hydroxyl group from the 4- to the 5-position and from the 7- to the 6-position attenuates water solubility (**4** vs. **13** and **17** vs. **20**, respectively). In compounds containing a hydroxyl substituent, the sulfone derivative **4** and **17** have higher aqueous solubility than the corresponding sulfides **3** and **16** and the sulfoxides **6** and **19**. The des-hydroxyl derivative **8** and the ketones **2**, **5**, **15**, and **16** are among the least soluble members of these series; however, they are among the most potent inhibitors of CA.

For the first time, a chiral preference for CA was demonstrated by a comparison of the enantiomers ***R*-4** and ***S*-4**. The results showed that the *S* isomer was favored over the *R* enantiomer by a factor of 3. Interestingly, the water solubility of the enantiomers ***R*-4** and ***S*-4** was half that of the racemate **4**.

The application of quantitative structure–activity relationships to these classes of carbonic anhydrase inhibitors is certainly desirable in view of the successful applications of this technique to benzenesulfonamides.[14–16] The restricted size of the set of compounds in the present study, however, precludes more than very preliminary analyses of this sort. Multiple regression was used to fit log *P* and pK_a to equations of the form

$$pY = A(\log P) + BpK_a + C$$

In this equation pY represents $-\log (I_{50})$ or $-\log (K_i)$ and the concentration units are 1×10^{-8} M. The two enantiomers of compound **4** were not included in this study.

For both the inhibition and binding data, log *P* was found to enter the above equation in a statistically significant manner, $p < 0.02$, while the contribution of pK_a was marginal, $0.05 < p < 0.10$. The resulting equations are

$$pI_{50} = (0.61 \pm 0.17) \log P - (0.49 \pm 0.22)pK_a + 3.928$$
$$n = 13,\ r = 0.775,\ s = 0.469,\ F_{2,10} = 7.52$$
$$pK_i = (0.76 \pm 0.21) \log P - (0.56 \pm 0.28)pK_a + 4.742$$
$$n = 12,\ r = 0.792,\ s = 0.447,\ F_{2,9} = 7.57$$

These results suggest that an increase in log *P* and a decrease in pK_a promote the binding and inhibitory potency of these compounds.

In summary, compounds **4** and **17** appear to adequately combine both water solubility and CAI activity. These features render **4** and **17** worthy candidates for further evaluation as antiglaucoma agents.

Experimental Section

^{1}H NMR spectra were determined in the indicated solvent on a Varian T-60, XL-300 or a GE-NMR NT360 spectrometer with tetramethylsilane as an internal standard. Optical rotation measurements were obtained on a Perkin-Elmer 141 polarimeter. Melting points were determined on a Thomas-Hoover apparatus, in open capillary tubes, and are uncorrected. Microanalyses are within 0.4% of theoretical values when indicated by symbols of the elements. Silica gel 60 (E. Merck, Darmstadt) was used for column chromatography, 230–430 mesh for Still columns and 70–230 mesh for gravity columns. Organic solutions were dried over $MgSO_4$ and filtered, and the filtrates were concentrated to dryness on a Büchi rotary evaporator under water-aspirator pressure (20 mm).

5,6-Dihydro-4*H*-4-oxothieno[2,3-*b*]thiopyran-2-sulfonamide (2). To a stirred solution of 1 (85.1 g, 0.5 mol) in CH_2Cl_2 (800 mL) at −10 °C was added in one portion Ac_2O (143.8 mL, 1.5 mol) followed by concentrated H_2SO_4 (30.7 mL, 0.55 mol)

(13) Chen, R. F.; Kernohan, J. C. *J. Biol. Chem.* **1967**, *242*, 5813.

(14) Kakeya, N.; Yata, N.; Kamada, A.; Aoki, M. *Chem. Pharm. Bull.* **1969**, *17*, 2000.

(15) Kakeya, N.; Yata, N.; Kamada, A.; Masaru, A. *Chem. Pharm. Bull.* **1969**, *17*, 2558.

(16) Hansch, C.; McClarin, J.; Klein, T.; Langridge, R. *Mol. Pharmacol.* **1985**, *27*, 493.

added dropwise over 10 min. The mixture was stirred at 10–15 °C for 0.5 h and then filtered. The olive green, hydroscopic solid was washed with Et_2O and dried immediately in vacuo to yield 120.9 g (96.6%) of the sulfonic acid intermediate. The compound (120.9 g, 0.483 mol) was suspended in CH_2Cl_2 (800 mL) and stirred at 0–4 °C while PCl_5 (140.8 g, 0.68 mol) was added in one portion. The cooling bath was removed, and the mixture was stirred for 1.5 h as the temperature rose to ambient temperature. The resulting dark maroon solution was added to ice (400 mL). The CH_2Cl_2 layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3×). The combined organic layers were washed with saturated NaCl and filtered through activated carbon on top of a pad of Super-Cel, and the amber filtrate was dried, filtered, and concentrated to dryness to yield 129 g (99%) of the sulfonyl chloride intermediate as a pale grayish-green solid. This compound (129 g, 0.48 mol) was dissolved in acetone (400 mL) with warming and added to concentrated NH_4OH (300 mL) at −30 °C with rapid stirring over a 20-min period. The mixture was stirred at 0 °C for an additional 15 min and was concentrated to remove the acetone. The remaining yellow suspension was filtered, and the solid was washed with cold H_2O and dried overnight at 100 °C to yield 99 g of **2**.

5,6-Dihydro-4*H*-4-hydroxythieno[2,3-*b*]thiopyran-2-sulfonamide (3). To a mixture of **2** (50 g, 0.2 mol) in absolute EtOH (2 L) was added with stirring $NaBH_4$ (10 g, 0.26 mol). After the reaction mixture was heated at reflux for 20 min, the suspension was cooled and the solvent was removed under reduced pressure. Water was added to the residue and the pH adjusted to 8.5 with 6 N HCl. The mixture was extracted with EtOAc (3×), and the organic extracts were dried, filtered, and concentrated to dryness to yield 47.9 g of **3**.

5,6-Dihydro-4*H*-4-hydroxythieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-Dioxide (4). To a suspension of **3** (50 g, 0.19 mol) in CH_3OH (650 mL) was added dropwise a solution of Oxone (184 g, 0.3 mol) in H_2O (1 L). After a slight exotherm (25–45 °C), the reaction was stirred at room temperature for 3 h, and then the CH_3OH was removed under reduced pressure. The aqueous layer was extracted with EtOAc (6×). The organic extracts were dried, filtered, and concentrated to dryness. The residue was triturated with Et_2O, and the solid was filtered to yield 47.3 g of **4**.

5,6-Dihydro-4*H*-4-[2-methoxy-2-phenylacetoxy]thieno-[2,3-*b*]thiopyran-2-sulfonamide 7,7-Dioxide (*S*,*R*-14 and *R*,*R*-14). (Dimethylamino)pyridine (320 mg, 2.6 mmol), **4** (4.95 g, 17.5 mmol), and (*R*)-*O*-methylmandelic acid (4.4 g, 26.3 mmol) were dissolved in THF (100 mL). Dicyclohexylcarbodiimide (5.4 g, 26.3 mmol) was added, and the mixture was stirred at ambient temperature overnight. Dicyclohexylurea was removed by filtration and washed with THF. The filtrate and wash were combined and concentrated to dryness, and the residue was chromatographed on silica gel 60. The products were eluted with EtOAc–hexane (1:1) to yield 1.7 g of ***S*,*R*-14** and 1.4 g of ***R*,*R*-14**, respectively. Recrystallization of these products from THF–hexane yielded ***S*,*R*-14** (mp 160–161 °C) and ***R*,*R*-14** (mp 173–174 °C), respectively: $[\alpha]^{20}_D$ −33.1 (*c* 1, THF) for ***S*,*R*-14** and +6.4 (*c* 1, THF) for ***R*,*R*-14**. The full absolute stereochemistry of ***R*,*R*-14** was determined by X-ray crystallography.[12]

(*R*)-5,6-Dihydro-4*H*-4-hydroxythieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-Dioxide (4). A mixture of ***R*,*R*-14** (6.0 g, 13.9 mmol) and 0.2 N NaOH (290 mL) was stirred at ambient temperature for 1 h. The pH of the solution was adjusted to 7.0 with 3 N HCl, and the mixture was extracted with EtOAc (3×). The extracts were combined and concentrated to dryness, and the residue was crystallized from CH_3CN to yield 3.1 g of ***R*-4**: $[\alpha]^{20}_D$ −16.0 (*c* 1, CH_3OH).

(*S*)-5,6-Dihydro-4*H*-4-hydroxythieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-Dioxide (4). The *S* isomer was prepared in an analogous manner from ***S*,*R*-14**: $[\alpha]^{20}_D$ +16.0 (*c* 1, CH_3OH).

5,6-Dihydro-4*H*-4-oxothieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-Dioxide (5). To a solution of **4** (0.6 g, 2.1 mmol) in acetone (20 mL) was added Jones reagent (1 mL), and the mixture was stirred at ambient temperature for 10 min. After the mixture was poured into H_2O, the aqueous phase was extracted with EtOAc (3×), and the organic extracts were washed with saturated $NaHCO_3$, dried, filtered, and concentrated to dryness to yield 0.4 g of **5**.

5,6-Dihydro-4*H*-4-hydroxythieno[2,3-*b*]thiopyran-2-sulfonamide 7-Oxide (6). To a solution of $NaIO_4$ (1.6 g, 7.7 mmol) in H_2O (40 mL) was added dropwise at ambient temperature a solution of **4** (1.6 g, 6.4 mmol) in CH_3OH (100 mL). After the mixture was stirred overnight at ambient temperature, it was filtered, the solid residue was washed with CH_3OH, and the combined organic extracts were concentrated to dryness. The residue was treated with silica gel 60 and dry packed on a column of silica gel 60. The column was eluted with 20% CH_3OH–$CHCl_3$ to yield 0.9 g of **6** as a mixture of diastereomers by HPLC (64% α and 34% β).

5,6-Dihydro-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-Dioxide (8). Preparation of 6*H*-Thieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-Dioxide (7). A mixture of **4** (0.9 g, 0.003 mol), concentrated H_2SO_4 (3 mL), and CF_3CO_2H (20 mL) was heated with stirring at 50 °C for 20 h. After removal of the CF_3CO_2H under reduced pressure, the residue was dissolved in EtOAc (25 mL) and the solution was washed with H_2O (2×), saturated $NaHCO_3$ (2×), and H_2O (2×). The organic extracts were dried, filtered, and concentrated to dryness to yield 0.74 g (90%) of **7**: mp 214.5–216 °C (CH_3NO_2). Anal. ($C_7H_7NO_4S_3$) C, H, N.

A solution of **7** (3.05 g, 0.011 mol) in absolute EtOH (130 mL) and CH_3OH (20 mL) was hydrogenated on a Parr apparatus at 40 psi with 5% Pd/C (250 mg) at ambient temperature. After 15 min, 10% Pd/C was added, followed by another addition after 30 min. After 16 h, the catalyst was filtered under N_2 and the filtrate concentrated to dryness. The residue was dissolved in absolute EtOH (100 mL) and CH_3OH (50 mL) and hydrogenated at 50 psi with 10% Pd/C (500 mg) for 16 h. The catalyst was filtered under N_2, and the filtrate was concentrated to dryness. The residue was chromatographed on silica gel, and the product was eluted with 10% CH_3OH–$CHCl_3$ to yield 2.3 g of **8**.

5,6-Dihydro-4*H*-5-hydroxythieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-Dioxide (13). Preparation of 5,6-Dihydro-4*H*-thieno[2,3-*b*]thiopyran-5-ol (10). To a solution of **9** (4.35 g, 0.028 mol) in THF (90 mL) under N_2 was added at room temperature with stirring a solution of 1 M BH_3 in THF (60 mL, 60 mmol). After 18 h, H_2O (4.35 mL) was added dropwise, then 3 N NaOH (11 mL, 0.033 mol) and 30% H_2O_2 (3.2 mL, 0.028 mol). After 2 h, the mixture was poured into H_2O and extracted with EtOAc (3×). The organic extracts were washed with saturated NaCl, dried, filtered, and concentrated to dryness to yield a mixture of **10** and **11**. The residue was treated with $CHCl_3$ (150 mL) and MnO_2 (26 g), and the mixture was stirred at room temperature overnight. The dark suspension was then filtered through a Super-Cel pad, and the filtrate was concentrated to dryness. The residue was chromatographed on silica gel eluting with 15% EtOAc–hexane (v/v) to yield 1.0 g of **1** and then 1.5 g of **10** (31%).

Preparation of 5,6-Dihydro-4*H*-5-[(2-methoxyethoxy)-methoxy]thieno[2,3-*b*]thiopyran (12). To a solution of **10** (4.0 g, 0.023 mol), CH_2Cl_2 (55 mL), and diisopropylethylamine (4.6 g, 0.036 mol) was added, under N_2, dropwise, (2-methoxyethoxy)methyl chloride (4.3 g, 0.034 mol). After 3 days, the reaction mixture was poured into CH_2Cl_2 (200 mL) and the mixture was washed with cold 1 N HCl (2×). The aqueous phase was backwashed with CH_2Cl_2. The organic extracts were washed with saturated $NaHCO_3$ solution, dried, filtered, and concentrated to dryness to yield 5.85 g (97.5%) of **12**.

To a solution of **12** (5.85 g, 0.023 mol) in THF (100 mL) under N_2 was added 1.6 M *n*-butyllithium in hexane (17 mL, 0.027 mol) at −78 °C. After the mixture was stirred for 0.5 h, SO_2 gas was passed over the surface for 45 min at −78 °C, and then Et_2O (800 mL) was added and the mixture was stirred for 1 h at ambient temperature. The suspension was concentrated to dryness, and the residue was treated with saturated $NaHCO_3$ solution (100 mL), cooled in an ice bath with stirring, and treated with *N*-chlorosuccinimide (3.3 g, 0.025 mol). After the solution was stirred at ambient temperature for 15 min, it was extracted with EtOAc (2×). The organic extracts were dried, filtered, and concentrated to dryness. The residue was dissolved in acetone (50 mL) and added to concentrated aqueous NH_3 (50 mL). The acetone was removed under reduced pressure, and the aqueous layer was extracted with EtOAc (2×). The organic extracts were dried, filtered, and concentrated to dryness to yield 8.0 g (100%) of crude sulfonamide intermediate.

To a solution of sulfonamide intermediate (8.0 g, 0.0225 mol) in CH_3OH (150 mL) was added with stirring at room temperature a solution of Oxone (18 g, 0.029 mol) in H_2O (150 mL). After 2 days, the suspension was filtered and the solid washed with CH_3OH. The CH_3OH was removed under reduced pressure from the filtrate, and the aqueous layer was extracted with EtOAc (3×). The organic extracts were dried, filtered, and concentrated to dryness to yield 8.6 g of crude product. The residue was dry packed on silica gel, chromatographed on silica gel, and eluted with 5% CH_3OH–$CHCl_3$ (v/v) to yield 2.8 g of crude (2-methoxyethoxy)methyl ether of the product. The ether (2.8 g) was dissolved in CH_3OH (50 mL) and treated with a cooled solution of H_2SO_4 (50 mL) and H_2O (50 mL). After the solution was stirred for 0.5 h at room temperature, it was poured into H_2O and extracted with EtOAc (5×). The organic extracts were washed with saturated $NaHCO_3$ solution and saturated NaCl solution, dried, filtered, and concentrated to dryness. A total of 3.0 g of crude product was obtained. This material was then treated with MnO_2 (10 g), Oxone (5.0 g), and acetone (100 mL), and the mixture was stirred at room temperature overnight. After 18 h, the mixture was filtered through Super-Cel, the pad was washed with acetone, and the filtrate was concentrated to dryness. The residue was dry packed on silica gel and chromatographed on silica gel by eluting with 5% CH_3OH–$CHCl_3$ (v/v) to yield 0.75 g of **13**.

6,7-Dihydro-5*H*-7-oxothieno[3,2-*b*]thiopyran-2-sulfonamide (15). A solution of 6,7-dihydro-5*H*-7-oxothieno[3,2-*b*]-thiopyran (5.0 g, 0.029 mol), benzene (75 mL), ethylene glycol (7.5 mL), and *p*-toluenesulfonic acid (0.3 g) was heated at reflux with a Dean–Stark trap for removal of H_2O. After 4 days, the solution was cooled, washed with saturated $NaHCO_3$, and separated. The aqueous phase was further extracted with CH_2Cl_2 (2×), and the combined organic extracts were dried, filtered, and concentrated to dryness. The residue was chromatographed on activity 2 alumina and the product eluted with 10% EtOAc–hexane to yield 3.95 g (63%) of ketal. Utilizing the same procedure described for the preparation of **13** and using the ketal (vide supra) in place of **12** gave the ketal sulfonamide, which on hydrolysis in acetone 1 N HCl provided **15**.

6,7-Dihydro-5*H*-7-hydroxythieno[3,2-*b*]thiopyran-2-sulfonamide (16). Utilizing the same procedure described for the preparation of **3** and using **15** in place of **2** provided **16**.

6,7-Dihydro-5*H*-7-hydroxythieno[3,2-*b*]thiopyran-2-sulfonamide 4,4-Dioxide (17). A solution of **16** (2.65 g, 0.01 mol), AcOH (27 mL), and 30% H_2O_2 (2.7 mL, 0.24 mol) was heated at 100 °C for 1 h. After cooling, the solution was dry packed on silica gel and placed on a column of silica gel, and the product was eluted with 7.5% CH_3OH–$CHCl_3$ to yield 1.4 g of **17**.

6,7-Dihydro-5*H*-7-oxothieno[3,2-*b*]thiopyran-2-sulfonamide 4,4-Dioxide (18). To a solution of **16** (2.0 g, 8 mmol) in CH_3OH (100 mL) was added dropwise at ambient temperature a solution of 3-chloroperbenzoic acid (4.0 g, 20 mmol) in $CHCl_3$ (100 mL). After the mixture was stirred overnight, it was concentrated to dryness. The residue was treated with acetone (75 mL) and Jones reagent (6 mL). After 15 min, H_2O was added and the mixture was extracted with EtOAc (4×). The organic extracts were dried, filtered, and concentrated to dryness. The residue was dry packed on activity 5 alumina and placed on a column of activity 2 alumina, and the product was eluted with 20–70% CH_3OH–$CHCl_3$ to yield 0.9 g of **18**.

6,7-Dihydro-5*H*-7-hydroxythieno[3,2-*b*]thiopyran-2-sulfonamide 4-Oxide (19). Utilizing the same procedure described for the preparation of **6** and using **16** in place of **3** provided **19**.

6,7-Dihydro-5*H*-6-hydroxythieno[3,2-*b*]thiopyran-2-sulfonamide 4,4-Dioxide (20). Utilizing the same procedure described for the preparation of **13** and using 5*H*-thieno[3,2-*b*]-thiopyran[9] in place of **9** provided **20**.

H_2O Solubility. A standard solution was prepared by dissolving 1 mg of sample in 10 mL of CH_3OH. The standard solution was scanned by UV (Acta M VI Beckman spectrophotometer) to determine the wavelength of maximum absorbance, diluting as necessary. A saturated solution was prepared by stirring magnetically a small volume of pH 7.4 phosphate buffer 0.039 M (~500 μL) in the presence of excess compound. The saturated solution was checked every 30 min and additional compound added if necessary to maintain saturation. After 4 h, the solution was filtered to remove excess compound, using HA 0.45 μm Millipore filters. The saturated solution was diluted to at least 3 mL and then scanned by UV at the wavelength of maximum absorbance. Total solubility was then determined by the relationship $C' = A'C/A$ where C = concentration of saturated solution in milligrams/milliliter, A = absorbance of the standard solution (correcting for any dilutions), A' = absorbance of the saturated solution (correcting for any dilutions), and C' = concentration of saturated solution in milligrams/milliliter.

pK_a. The half-neutralization point was measured by titrating the organic acids and bases with 0.5 N NaOH and 0.5 N HCl in H_2O and mixed solvents, using a glass-columned electrode system. All of the compounds were run in 30% EtOH–H_2O.

Partition Coefficients. Partition coefficients were obtained by equilibrating the test compound between octanol and 0.1-ionic-strength pH 7.4 phosphate buffer. The concentration in each phase was determined by UV spectrophotometry.

In Vitro Inhibition of Human Carbonic Anhydrase II. Human erythrocyte CA II was isolated from lysed red blood cells by the following affinity chromatography procedure. Citrated human blood (500 mL) was centrifuged at 5000*g* for 10 min at 4 °C and the resultant plasma decanted. Red blood cells were washed with cold 0.9% NaCl and then centrifuged. The supernatant was discarded and the process of washing and centrifugation repeated. Cell lysis was achieved at 4 °C by adding an equal volume of cold water, and cellular debris was removed by centrifugation. Lysed human red blood cells (80 mL) were diluted 5-fold with 0.05 M Tris-sulfate buffer, pH 8.8, and poured onto a 0.9 × 8 cm (4-(aminomethyl)benzenesulfonamide–CM agarose) affinity chromatography gel column. Chromatography was carried out at 4 °C, and fractions were monitored by determining optical density at 280 nm with an LKB Uvicord III.

The column was eluted with 0.2 M sodium sulfate in 0.1 M Tris-sulfate buffered at pH 8.8 to remove all hemoglobin and other proteins not specifically bound. Low-activity carbonic anhydrase I was eluted as a single peak with 0.6 M potassium chloride in 0.1 M potassium phosphate buffer (pH 7.2). Elution was continued until the optical density at 280 nm was less than 0.1. Highly purified carbonic anhydrase II was eluted with 0.6 M potassium chloride in 0.1 M potassium phosphate buffer (pH 5.2). Carbonic anhydrase II purity was assessed by disc gel and starch gel electrophoresis. The gels were stained for protein with Coomassie Blue, and carbonic anhydrase II bands were visualized by fluorescein diacetate staining. The enzyme solution was desalted and concentrated to 1 mg of protein/mL of 0.1 M phosphate, pH 7.2, on an Amicon UM-10 Ultrafiltration membrane and stored at 2–5 °C.

Inhibition of the purified human erythrocyte carbonic anhydrase II was assessed by using a pH stat assay. This assay measures the rate of hydration of CO_2[17] by determining the rate at which a standard solution of NaOH has to be added to a lightly buffered solution to maintain a constant pH as CO_2 is bubbled into the buffer. Enzymatic activity is proportional to the volume of a standard NaOH solution that is required to maintain the pH at a given value, e.g., 8.3. To 4 mL of 0.02 M Tris-chloride buffer, pH 8.6, in a 5-mL Radiometer V531 jacketed assay vessel equilibrated at 2 °C was added buffer-diluted enzyme (25 μL). CO_2–air (5:95) was bubbled into the assay vessel at a rate of 150 mL/min. The pH stat end point was set at pH 8.3, and the volume of 0.025 N NaOH added over a 3-min period in order to maintain pH 8.3 was measured. Enzyme inhibition was measured by the addition of an inhibitor in 0.1 mL to 3.9 mL of buffer followed by the addition of enzyme and titration with NaOH. Results were expressed as the I_{50} values, which were obtained from semilog plots of percent inhibition against log concentration.

In Vitro Binding for Human Carbonic Anhydrase II. The binding of test compounds to purified human erythrocyte carbonic anhydrase II was determined by a fluorescence competition assay employing the fluorescent CA inhibitor dansylamide. This compound has been shown to produce a large increase in fluorescence upon binding to the active site of carbonic anhydrase.[13] A fluorescence cuvette containing 1×10^{-7} M human CA II (HCA

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II) and 2×10^{-5} M dansylamide in pH 7.4, 0.1 ionic strength phosphate buffer was placed in the thermostated cell holder of a Perkin-Elmer MPF-44B fluorescence spectrophotometer. The temperature was maintained at 37 °C by using a constant-temperature water circulator. The excitation and emission wavelengths were set at 280 and 460 nm, respectively. Fluorescence intensities were recorded following addition, with stirring, of small, measured aliquots of a solution of the test compound in pH 7.4 buffer. The resulting data were converted to fluorescence intensity vs. compound concentration, corrected for dilution by the titrant, and fitted by nonlinear least squares to a model in which the compound and dansylamide compete for a single binding site on HCA II. The dissociation constant of the dansylamide–HCA II complex, which is needed for these calculations, was found to be 1.98×10^{-6} M under these conditions. It was found in all cases that the data fitted well to a single-site model. There was no evidence for additional, lower-affinity binding sites. All binding determinations were done a minimum of three times.

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Registry No. 1, 7675-04-9; 2, 105951-31-3; 2 (acid), 106319-47-5; 2 (acid chloride), 106319-48-6; (±)-3, 106319-38-4; ±-4, 106400-04-8; (*R*)-4, 105951-84-6; (*S*)-4, 105951-48-2; 5, 105951-35-7; (±)-6α, 106335-79-9; (±)-6β, 106319-49-7; 7, 105951-67-5; 8, 105951-71-1; 9, 21339-38-8; (±)-10, 106319-39-5; (±)-11, 106319-40-8; (±)-12, 106319-41-9; (±)-12 (sulfonamide), 106319-50-0; (±)-13, 106319-42-0; (±)-13 (MEM ether), 106335-80-2; (*S*,*R*)-14, 106319-43-1; (*R*,*R*)-14, 101859-94-3; 15, 105951-32-4; (±)-16, 106319-44-2; (±)-17, 106319-45-3; 18, 105951-39-1; 19, 105951-36-8; (±)-20, 106319-46-4; MEMCl, 3970-21-6; (*R*)-$C_6H_5CH(OCH_3)CO_2H$, 3966-32-3; 6,7-dihydro-5*H*-7-oxothieno[3,2-*b*]thiopyran, 7677-33-0; 6,7-dihydro-5*H*-7-oxothieno[3,2-*b*]thiopyran ketal, 106319-51-1; 6,7-dihydro-5*H*-7-oxothieno[3,2-*b*]thiopyran ketal sulfonamide, 106319-52-2; 5*H*-thieno[3,2-*b*]thiopyran, 10558-81-3; carbonic anhydrase, 9001-03-0.

Linear Free Energy Relationships and Cytotoxicities of Para-Substituted 2-Haloethyl Aryl Selenides and Bis(2-chloroethyl) Selenides

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Examples of a new class of alkylating agents, selenium mustards, were prepared for study of their chemical kinetic properties and cytotoxicities against human lymphoblastoid CCRF-CEM cells. In a series of para-substituted aryl 2-chloroethyl selenides, a linear free energy relationship between the first-order rate constant, k'_{nbp} and σ_p gave a ρ value of −1.3, indicating that formation of a cyclic ethylene selenonium ion is the rate-controlling step for alkylation of 4-(4-nitrobenzyl)pyridine (NBP). Consistent with the ethyleneselenonium ion pathway, rates of solvolyses were extremely sensitive to increasing water content, and a positive correlation was found between reactivity with NBP and nucleophilic selectivity (Swain–Scott *s* constant). The *s* constant, which predicts for variation in intracellular product spread, varied from 0.53 up to 0.95, equal to aliphatic nitrogen mustards. Alkylating activities based on extent of NBP alkylation, however, showed relatively low values, 8–23% of that of mechlorethamine, possibly due to hydrolysis occurring by a separate pathway from nucleophilic substitution. Reactivities and nucleophilic selectivities both showed positive correlations with cytotoxicities, suggesting that the rate and extent of alkylation of relatively strong nucleophilic centers mediate the biologic effects of these compounds. Two bifunctional selenium mustards were substantially more cytotoxic than monofunctional aromatic selenides. No additional cytotoxicity due to the selenium atom was observed, with the exception of diselenide (–SeSe–) compounds. Thus, selenium alkylating agents kinetically and biologically resemble classical, mustard-type alkylating agents.

Discovery of the antitumor properties of mechlorethamine hydrochloride led to the synthesis of thousands[1] of (2-haloethyl)imonium, aziridine, (2-haloethyl)sulfonium, and oxygen analogues by the early 1960s and to the development of alkylating agents as an established class of cancer chemotherapy agents. Classical alkylating agents may be defined as compounds that in protic media undergo aliphatic nucleophilic substitution reactions at saturated, sp^3 carbon bearing an acidic leaving group. Ligand substitution reactions of platinum salts follow a similar nucleophilic reactivity order.[2] Although the synthesis of bis(2-chloroethyl) selenide was first described in 1920,[3] the antitumor potential of 2-haloethyl selenides has not been discussed in the literature until the present paper. This is surprising, given the antitumor activities of selenium antimetabolites,[4] the anticarcinogenic effect of dietary selenium,[5] and the important role of selenium in glutathione metabolism.[6]

Our own interest in 2-haloethyl selenides was prompted by theoretical considerations of alkylating agent nucleophilic selectivity. High nucleophilic selectivity in an alkylating agent, represented by the *s* constant of Swain and Scott,[7] should increase alkylation of the N-7 position of guanine of DNA[8] and other moderately strong intracellular

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