3-Substituted Thieno[2,3-*b*][1,4]thiazine-6-sulfonamides. A Novel Class of Topically Active Carbonic Anhydrase Inhibitors

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3-Aminoalkyl derivatives of thieno[2,3-*b*][1,4]thiazine-6-sulfonamide were prepared for evaluation as topically active ocular hypotensive agents. The compounds described were found to be excellent in vitro inhibitors of carbonic anhydrase II and in vivo to lower intraocular pressure in three rabbit models of ocular hypertension. Compounds **20A**, **20B**, and **20C** met the requirement of formulation as a 1% solution at pH 5.2, but none of the compounds described exhibited greater activity in the normotensive albino rabbit, the α-chymotrypsin-treated albino rabbit, or the normotensive pigmented rabbit than MK-927 or MK-507, the present clinical candidates.

Introduction

Elevated intraocular pressure is a characteristic feature of glaucoma, an ocular disease which when left untreated can lead to diminished vision or blindness. Intraocular pressure (IOP) is controlled by the rate at which aqueous humor enters the eye through the posterior chamber or leaves the eye from the anterior chamber. The systemic administration of inhibitors of carbonic anhydrase II (CA II), an enzyme present in secretory nonpigmented epithelial cells of the ciliary process, slows the rate at which aqueous humor forms and thus lowers IOP.[1,2] The orally active agents acetazolamide (**1**), methazolamide (**2**), dichlorophenamide (**3**), and ethoxzolamide (**4**) have clinical utility in the treatment of glaucoma in man. However, oral administration of these carbonic anhydrase inhibitors (CAIs) at effective doses for lowering intraocular pressure also inhibits carbonic anhydrase in extraocular tissue and causes side effects serious enough to prevent their use or diminish patient compliance.[2,3] The development of a topically effective CAI which should obviate the undesired systemic side effects of orally administered CAIs has been a goal of these laboratories for several years. Earlier investigations of varied classes of compounds narrowed the criteria for a successful topical CAI.[4-7] The breakthrough in design was the incorporation of a weakly basic amine into the structural motif.[8-12] The addition of an alkylamino substituent facilitated aqueous solubility such that the compounds could be formulated in a topically applicable solution at a nonirritating pH. Examples include the 4-(alkylamino)thienothiopyran-2-sulfonamides **5A,B,C**,[12-15] the isomeric thieno[2,3-*b*]thiophene-2-sulfonamide **6**[8] and thieno[3,2-*b*]thiophene-2-sulfonamide **7**,[8] the 4- and 5-(aminoalkyl)-4,5-dihydrothieno[2,3-*b*]-thiophene-2-sulfonamides **8**,[9] and the 4- and 5-(arylsulfonyl)thiophene-2-sulfonamides **9**.[10,11] The clinical success of the 4-(alkylamino)thienothiopyran-2-sulfonamides **5A,B,C** as topically effective carbonic anhydrase inhibitors suggested investigation of a series of compounds having an additional heteroatom in the A-ring. One such construct is the substituted thieno[2,3-*b*][1,4]thiazine-6-sulfonamides **10**, an unexplored ring system.[16] The 6-substituted 4-(alkylamino)thienothiopyrans such as **5C** contain two chiral centers. Incorporation of nitrogen into the thienothiazine ring reduces the number of chiral centers to one and still affords opportunities for attaching an amine-containing side chain at the 1-, 2-, or 3-position of the thiazine ring. Work from these laboratories[8,15] and modeling experiments involving the active site of human carbonic anhydrase II suggested that the target enzyme could accommodate 3-substituted thienothiazine-6-sulfonamides. Described here are novel heterocycles with side-chain attachments in the 3-position.

Chart 1

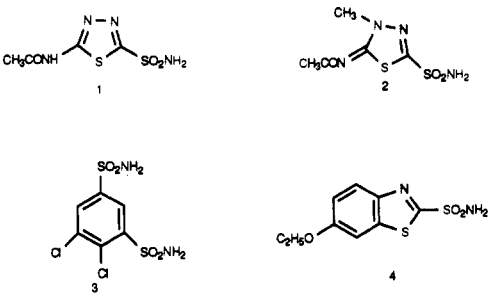
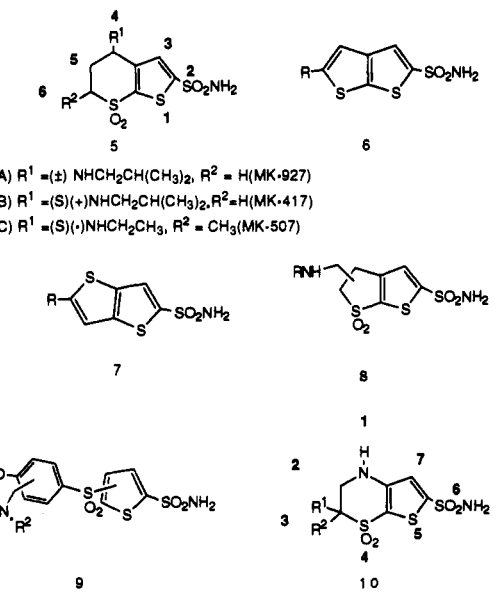


Chart 2



Chemistry

Two strategies were investigated for the synthesis and elaboration of the 3-substituted thienothiazinesulfon-

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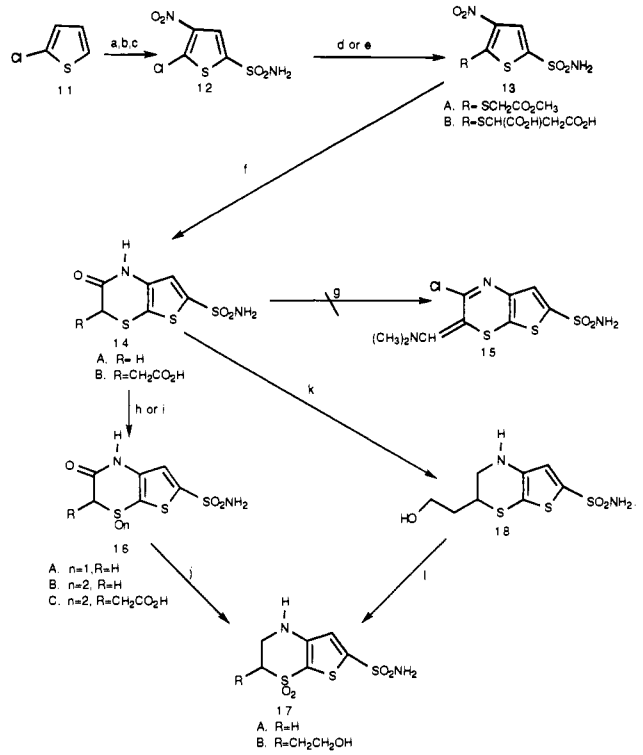
amides. The first strategy employed chemistry analagous to that reported by Chandramohan[17] for the preparation of the benzothiazine nucleus and was an unsuccessful attempt to functionalize the "active methylene" at the 3-position of 2-oxothienothiazinesulfonamide **14A**. The successful approach involved ring closure to form the thienothiazinesulfonamide with functionality in the 3-position already in place. Chlorothiophene **11** was chlorosulfonated, nitrated, and treated with ammonium hydroxide to give thienosulfonamide **12**.[18] Displacement of chloride with methyl thioglycolate gave nitro ester **13A**.[19] Analogously, displacement of chloride in **12** with 2-mercaptosuccinic acid gave diacid **13B**. A key step in the formation of both the parent and the functionalized thieno[2,3-*b*][1,4]thiazinesulfonamides was the facile reductive cyclization of **13A** and **13B** to lactams **14A** and **14B** with titanium trichloride. Reduction of nitro ester **13A** with titanium trichloride in aqueous acetic acid afforded an amine which spontaneously cyclized to lactam **14A**, a product isolated in good yield and purity. Attempts to cyclize the dessulfamoyl analog of **13A** with titanium trichloride resulted in intractable tars. Using conditions reported by Chandramohan[17] for the benzothiazine ring system, attempts to functionalize the 3-position of **14A** by reaction with phosphorus oxychloride in DMF under Vilsmaier–Haack conditions to form (dimethylamino)methylene iminochloride **15** were unsuccessful. Oxidation of **14A** with *m*-chloroperbenzoic acid in EtOAc/EtOH slowly produced sulfoxide **16A**. The sulfoxide was essentially insoluble in this medium, and no further oxidation occurred even with excess oxidant. However, when the oxidation of **14A** was carried out with *m*-chloroperbenzoic acid in DMF, sulfone **16B** was formed quickly via the sulfoxide as indicated by TLC examination of the reaction mixture. Reduction of the amide functionality of **16B** with borane–dimethylsulfide gave thieno[2,3-*b*][1,4]-thiazine-4,4-dioxo-6-sulfonamide **17A**.

To circumvent the problem in functionalizing **14A** and **17A**, a strategy of incorporating the necessary solubilizing functionality prior to reductive cyclization was pursued. Reaction of **12** with 2-mercaptosuccinic acid yielded dicarboxylic acid **13B**. Reduction and spontaneous ring closure of **13B** occurred in the acidic medium to give sulfide **14B**. Oxidation of **14B** with *m*-chloroperbenzoic acid to **16C** followed by reduction with borane–dimethylsulfide gave **17B**. Alternatively, reduction of **14B** with borane–tetrahydrofuran to **18** followed by oxidation with *m*-chloroperbenzoic acid also gave **17B**. Activation of the hydroxy group of **17B** (Scheme 2) by conversion to tosylate **19** and in situ displacement of the tosylate with isobutylamine and bis(2-methoxyethyl)amine gave **20A** and **20B**, respectively. Tosylate **19** was isolated and treated with the appropriate amine[8] in DME to give **20C**. In cases where the displacing amine was a weak nucleophile, competition between the displacing amine and the ring nitrogen was observed. Reaction of **19** with morpholine in DMSO gave a mixture of **20D** and **21**. Reaction of **19** with diisopropylamine to form **20E** yielded only **21**.

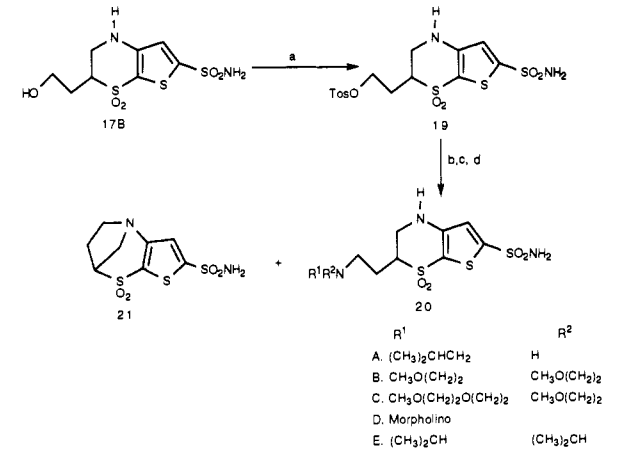
The competitive ring closure encountered in the displacement of the tosyl group of **19** by non-nucleophilic amines prompted investigation of alternate synthetic routes. An approach which exploited the non-nucleophilic character of the lactam nitrogen of **14B** and **16C** was investigated (Scheme 3). Lactam amides **22A** and **22B** were synthesized from **14B** by activation of the carboxy group followed by displacement with isobutylamine and (methoxyethyl)amine, respectively. Attempted preparation of the dimethyl and diisopropyl amides utilizing this methodology failed. Oxidation of **22A** with *m*-chloroperbenzoic acid to **23** followed by reduction with borane–dimethylsulfide gave a mixture of **20A** and **24** which could not be readily separated by chromatography. Oxidation of **22B** with *m*-chloroperbenzoic acid failed. Lactam **22C** was synthesized from **16C** using the hydroxybenzotriazole ester followed by displacement with the appropriate amine.

Scheme 1



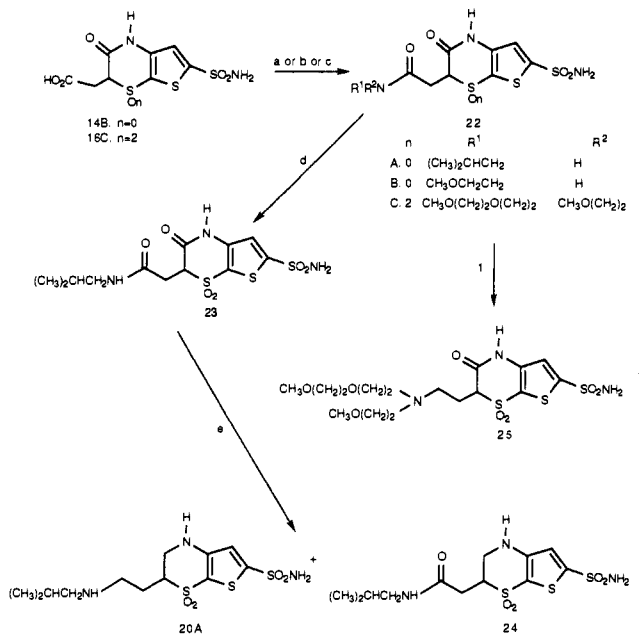
[a] (a) PCl_5, $ClSO_3H$;[18a] (b) 90% HNO_3;[18b] (c) concentrated NH_4OH; (d) methyl thioglycolate, MeOH, Et_3N; (e) mercaptosuccinic acid, MeOH, Et_3N; (f) $TiCl_3$, AcOH, H_2O; (g) $POCl_3$, DMF; (h) *m*-CPBA, EtOAc, EtOH yields **16A**; (i) *m*-CPBA, DMF yields **16B,C**; (j) BH_3·DMS, THF or DME; (k) BH_3·THF, THF; (l) *m*-CPBA, 50% THF/H_2O.

Scheme 2



[a] (a) Tosyl chloride, pyridine; (b) tosylate **19** was generated in situ and treated with isobutylamine and bis(2-methoxyethyl)amine in pyridine at 80 °C for 18 h to give **20A** and **20B**; (c) ref 8 for preparation of *N*-[(methoxyethoxy)ethyl]-*N*-(methoxyethyl)amine; (d) tosylate **19** was isolated and then treated with appropriate amine in a separate step to give **20C** and **20D**.

Scheme 3



[a] (a) CDI, isobutylamine, DMF; (b) HOBT, EDC, (methoxyethyl)amine, DMF; (c) HOBT, EDC, *N*-[(methoxyethoxy)ethyl]-*N*-(methoxyethyl)amine,[8] DMF; (d) *m*-CPBA, DMF, **22A**; (e) BH_3·DMS, DME; (f) LAH, DME, **22C**.

Reduction of **22C** with $LiAlH_4$ gave lactam **25** which was resistant to further reduction with borane.

Results and Discussion

Candidates for development as topical ocular hypotensive agents were evaluated in a sequence of in vitro, ex vivo, and in vivo studies. The in vitro interaction of compounds with HCA II was assessed by two methods. The first method measured a compound's ability to inhibit human erythrocyte carbonic-anhydrase-II-catalyzed carbon dioxide hydration (I_{50}).[12] The second method assessed a compound's ability to compete with dansylamide for binding to HCA II (K_i).[12] All sulfone thienothiazine-sulfonamides submitted were found to be potent, tightly bound inhibitors of HCA II displaying nanomolar I_{50} and K_i values (Table 1). Modeling studies of the interaction of HCA II and selected thienothiazines **20B** and **20D** indicate the potential for good interaction with CA II. The thienothiazines synthesized were racemic, but for reasons of illustration, we modeled enantiomers of **20B** and **20D** which have the same stereochemistry as MK-507 (**5C**) at the chiral center α to the sulfone. X-ray crystallographic studies describing MK-417 (**5B**) and MK-507 (**5C**) ligand complexes with HCA II have been reported, and the structure of the bound comformer has been established.[13,20] Using the bound conformer of MK-507 as a template, models of **20B** and **20D** were constructed from standard bond lengths and angles using Quanta (available from MSI). The models were then hand-docked onto the X-ray structure of MK-507 (Figure 1) and the side chains rotated to search for low-energy conformations which fit into the active site. All decisions about the quality of the fit were made on the basis of distance and angle measurements; no energy calculations were performed. We were encouraged that the bicyclic ring systems of the thienothiazines and the thienothiopyrans were superimposable and that the 3-alkylamino side chains added to impart aqueous solubility did not form undesirable contacts with the enzyme.

An ex vivo assay[11,13] was used to determine if test compounds could penetrate the albino rabbit eye and reach the iris–ciliary body in sufficient quantity to inhibit carbonic anhydrase II. To effect a decrease in IOP, the target ocular enzyme must be essentially totally inhibited.[21,22] A topically effective CAI must, therefore, efficiently penetrate ocular tissue and be localized in the iris–ciliary body. One hour after topical administration of the test compound, the iris–ciliary body was excised from the albino rabbit. The ocular tissue was homogenized and CA activity measured. As shown in Table 2, the 3-substituted thienothiazines showed some ex vivo inhibitory activity; however, the best compounds (**20A**, **20B**, **20C**, and **20D**) did not approach the inhibitory potency exhibited by clinical candidates **5A** (MK-927), **5B** (MK-417), and **5C** (MK-507). The thienothiopyrans under clinical investigation (**5A,B,C**) showed 93%–100% inhibition of CA II activity at 0.1% and 0.5%, whereas the thienothiazines reported here exhibited CA II inhibition in the 3%–79% range at these concentrations.

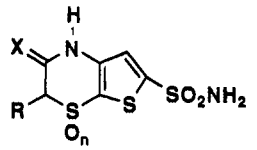
In vivo evaluation of intraocular pressure reduction was carried out on three animal models: the normotensive albino rabbit,[11,12] the α-chymotrypsin-treated albino rabbit,[4b,6] and the normotensive pigmented rabbit.[4b,13] In the normotensive albino rabbit, thienothiazines **20A**, **20B**, **20C**, and **20D** showed good activity as defined by the ability to decrease IOP after topical dosing of a 2% solution in 0.5% aqueous hydroxyethylcellulose (HEC) vehicle with no signs of irritation. For the α-chymotrypsin model, ocular hypertension was induced by the injection of α-chymotrypsin into the right eye of the albino rabbit and the ability of test compounds to reduce the chronically elevated IOP in that eye was measured. In this model, thienothiazines **20A**, **20B**, **20C**, and **20D** display IOP lowering properties comparable to the best thienothiopyrans (Table 2). Additional in vivo evaluation was carried out by a head-to-head comparison with MK-927, measuring IOP lowering ability in normotensive pigmented rabbits. As shown in Table 2, the thienothiazines showed reasonable activity with **20B** and **20C**, showing similar activity to MK-927.

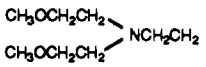
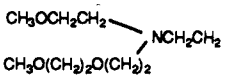
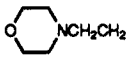
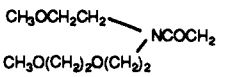
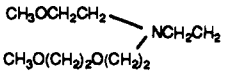
The 3-(aminoalkyl)-thieno[2,3-*b*][1,4]thiazine-6-sulfonamides described here are new compounds from a relatively unexplored ring system that exhibited effectiveness as topical ocular hypotensive agents in animal models. They display excellent in vitro IOP lowering activity, ex vivo activity, and good in vivo activity when tested as a 2% solution in 0.5% HEC, a pharmacological formulation. The solubility requirement for a successful clinical candidate was the ability to be formulated as a 1%–2% solution at pH 5.2. Compounds **20A**, **20B**, and **20C** could be formulated as 1% solutions at pH 5.2, but only **20A**, the isobutylamino derivative, displayed a pK_a basic enough to make a 2% solution at pH 5.2 (Table 1). Although the thienothiazines described are a novel class of topically active carbonic anhydrase inhibitors, the compounds show no obvious advantages over the present clinical candidates, MK-927 and MK-507.

Experimental Section

Starting materials were obtained from commercial suppliers and were used without further purification. Dimethylformamide (DMF) was distilled over CaH_2 and degassed just prior to use.

Table 1.



compd	R	n	X	formula	mp (°C)	anal.	solubility (mg/mL) pH 5.2 buffer	pK_a[a]	I_{50} (10^{-9} M)[b]	K_I (10^{-9} M)[c]
14A	H	0	O	$C_6H_6N_2O_3S_3$	>240	C,H,N			12.8	24
14B	HO_2CCH_2	0	O	$C_6H_6N_2O_5S_3$	240	C,H,N	2.4	4.3 9.30		47.5
16A	H	1	O	$C_6H_6N_2O_4S_3$	200	C,H,N			31.6	18
16B	H	2	O	$C_6H_6N_2O_5S_3$	259–260	C,H,N	0.6	7.43 9.00	3.7	2.9
17A	H	2	H,H	$C_6H_8N_2O_4S_3$	250–252	C,H,N	0.19	8.80	2.46	3.36
17B	$HOCH_2CH_2$	2	H,H	$C_8H_{12}N_2O_5S_3$	158–160	C,H,N	4.5	8.45	1.9	1.19
18	$HOCH_2CH_2$	0	H,H	$C_8H_{12}N_2O_3S_3$	149–150	C,H,N	0.64	9.62	3.18	109
20A	$(CH_3)_2CHCH_2NHCH_2CH_2$	2	H,H	$C_{12}H_{21}N_3O_4S_3$·HCl	263–265	C,H,N	24.0	8.18 9.50	2.9	4.05
20B		2	H,H	$C_{14}H_{25}N_3O_6S_3$·HCl	140–142	C,H,N	>6.7	6.40 8.55	2.14	0.97
20C		2	H,H	$C_{16}H_{29}N_3O_7S_3$·HCl	75–100	C,H,N	11.8	6.55 9.00	1.45	4.14
20D		2	H,H	$C_{12}H_{19}N_3O_5S_3$·HCl	280–282	C,H,N	2.1	3.23 6.05 8.70	0.58	4.46
21	$-CH_2CH_2-$	2	H,H	$C_8H_{10}N_2O_4S_3$	240–245	C,H,N	0.52	8.29	2.0	10.93
22A	$(CH_3)_2CHCH_2NHCOCH_2$	0	O	$C_{12}H_{17}N_3O_4S_3$	>250	C,H,N	0.03	3.65 8.85	4.6	7.95
22C		2	O	$C_{16}H_{25}N_3O_9S_3$	156–158	C,H,N			2.18	3.60
25		2	O	$C_{16}H_{27}N_3O_6S_3$·HCl	170–175	C,H,N			5.15	4.14
5A MK-927							33		1.2	0.7
5B MK-417							>16		0.54	0.6
5C MK-507							61.0		0.18	0.28

[a] pK_a determination in 30% EtOH/H_2O. [b] Inhibition of HCA-II-catalyzed hydration of CO_2.[12] [c] Competitive binding study between the test compound and dansylamide to bind to HCA II.[12]

Tetrahydrofuran (THF) was distilled over sodium. Moisture-sensitive reactions were carried out under nitrogen. Solvent evaporation was carried out on a Büchi rotary evaporator. Chromatographic separations were carried out with 230–400 mesh silica (Silica 60-EM Science) under 5–8 psi. Melting points were determined in open capillary tubes on a Thomas-Hoover apparatus and are uncorrected. NMR spectra were determined on a 300-MHz Varian XL-300. Chemical shift data are reported in ppm downfield from $(CH_3)_4Si$ as the internal standard. Elemental analyses are within 0.4% of the theoretical value.

5-Chloro-4-nitrothiophene-2-sulfonamide (12). Phosphorus pentachloride (100 g) was added portionwise to chlorosulfonic acid (80 mL). The mixture was stirred until gas evolution ceased (15 min) and then cooled to 0 °C. 2-Chlorothiophene (22 mL, 24 mol) was added dropwise with cooling. The reaction mixture was stirred at ambient temperature for 5 h and then added dropwise with stirring to 2 L of ice/H_2O. After stirring overnight, the aqueous solution was extracted with ether (3 × 1000 mL). The ether extract was washed (H_2O, brine), dried (Na_2SO_4), filtered, and concentrated in vacuo to yield 5-chlorothiophene-2-sulfonyl chloride (45 g).[18a] The crude sulfonyl chloride was added dropwise to fuming HNO_3 (250 mL) while maintaining the temperature below 60 °C and then stirred at ambient temperature for 4 h. The reaction mixture was added slowly with stirring to 2.5 L of ice/H_2O. The 5-chloro-4-nitrothiophene-2-sulfonyl chloride (47 g) was collected by filtration, washed (H_2O), and air-dried.[18b] The sulfonyl chloride (47 g) was added to 180 mL of concentrated NH_4OH at 0 °C. When the addition was complete, the reaction mixture was stirred at 25 °C until the solid dissolved. The clear orange solution was cooled in ice, diluted with H_2O (800 mL), and acidified with concentrated HCl to precipitate solid. The product was collected by filtration, washed (H_2O), and air-dried; yield 39 g, mp 150–153 °C.

Methyl [(3-Nitro-5-sulfamoylthien-2-yl)thio]acetate (13A). Triethylamine (3.06 mL, 22 mmol) was added to a stirred mixture of methyl thioglycolate (2.12 g, 26 mmol) and 5-chloro-4-nitrothiophene-2-sulfonamide (12) (4.90 g, 30 mmol) in MeOH (30 mL).[19] After a slight exotherm, the reaction mixture became very thick and difficult to stir. The mixture was stirred for an additional 16 h and then added to water (250 mL). The yellowish solid was collected, washed (H_2O), and air-dried to yield 5.2 g, mp 154–155 °C. Recrystallization was accomplished from CH_2Cl_2. Anal. ($C_7H_6N_2O_6S_3$) C, H, N. ^{1}H NMR (DMSO-d_6): δ 3.75 (3H, s), 4.35 (2H, s), 7.95 (1H, s), 8.05 (2H, s).

2,3-Dihydro-2-oxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]-thiazine (14A). A solution of titanium trichloride (200 mL, 15 wt % in 20–30 wt % HCl, 1.16 M, 240 mL) was added dropwise to a stirred suspension of 13A (12.5 g, 40 mmol) in 50% aqueous HOAc (250 mL). After 2 h, a dark purple solution was formed. The reaction mixture was stirred overnight at ambient temperature. The solid that precipitated was collected, washed (H_2O), and air-dried to yield 7.1 g, mp 243–245 °C. Recrystallization was accomplished from EtOH. Anal. ($C_6H_6N_2O_3S_3$) C, H, N. ^{1}H NMR (DMSO-d_6): δ 3.61 (2H, s), 7.14 (1H, s), 7.74 (2H, s).

2,3-Dihydro-2,4-dioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]-thiazine (16A). To a solution of 14A (250 mg, 1 mmol) in EtOAc (8 mL) and EtOH (8 mL) was added *m*-chloroperbenzoic acid (*m*-CPBA) (430 mg, 80–85%, 2 mmol). The reaction mixture

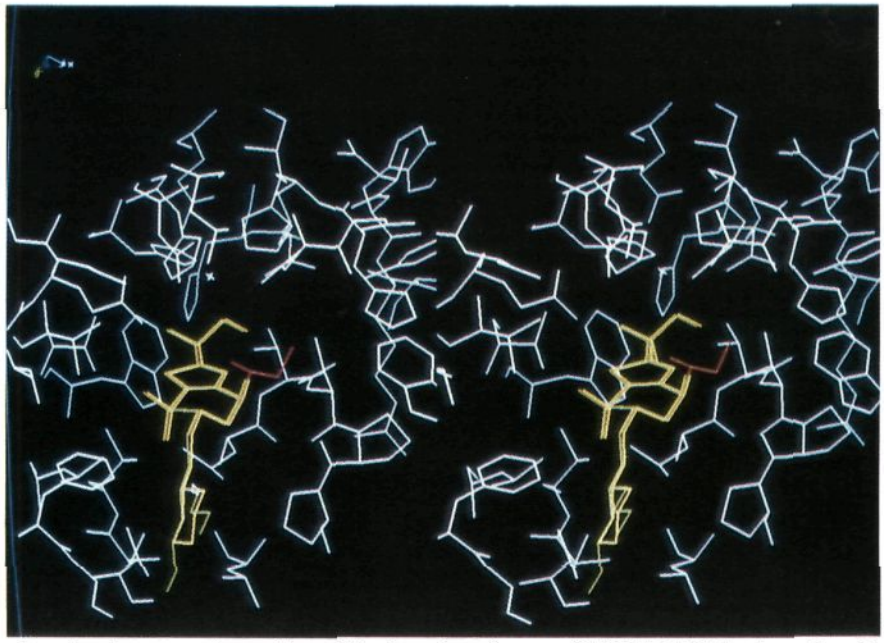


Figure 1. Stereodrawing showing a superimposition of the bound conformer of MK-507 (**5C**) in the active site of HCA II. MK-507 is in red, and the amino acid residues lining the cavity are grey. For experimental details of the cocrystallization of MK-507 with HCA II, see refs 13 and 20. Compounds **20B** (in green) and **20D** (in yellow) are superimposed on the bound conformer of MK-507. The bicyclic ring systems of **20B** and **20D** overlay the bicyclic system of MK-507. The alkyl side chains in the 3-position of **20B** and **20D** fall into the open space of the cavity making no undesirable contacts.

Table 2. Biological Properties of Selected 3-(Alkylamino)thieno[2,3-*b*][1,4]thiazine-6-sulfonamides

	ex vivo CA inhibn in albino rabbit[a]		Δ IOP in α-CT-treated albino rabbit[b]		Δ IOP in normotensive pigmented rabbit[c] 2% vs 2% MK-927	
compd	(%)	concn (%)	mmHg	significant time points	compound (mmHg)	MK-927 (mmHg)
20A	53	0.1	−4.5	4	−2.1	−6.2
	54	0.5				
20B	41	0.1	−5.0	4	−4.1	−5.1
	79	0.5				
20C	14	0.1	−6.5	6	−1.6	−5.3
	78	0.5				
20D	3	0.1	−4.2	5	−2.8	−4.4
	59	0.5				
5A (MK-927)	67	0.02	−6.5	5		
	95	0.1				
	99	0.5				
5B (MK-417)	77	0.02	−4.3	4	−4.1	−4.9
	93	0.1				
	100	0.5				
5C (MK-507)	87	0.02	−4.7	5	−3.9	−3.5
	100	0.1				
	100	0.5				

[a] One hour after topical administration (1 drop, 50 μL) of the test compound at 0.02% and/or 0.1%, 0.5% of the iris–ciliary body was excised from albino rabbits and the tissue homogenized in Tris–HCl buffer (20 mM, pH 8.5). CA activity was determined using a pH stat assay.[11,13] [b] Ocular hypertension was induced in the right eye of albino rabbits by the intraocular injection of α-chymotrypsin.[4b,6] Test compound at 0.5% in 0.5% aqueous hydroxyethylcellulose was instilled (1 drop, 50 μL) and the IOP of six rabbits measured just before (t_0) and at 0.5, 1, 2, 3, 4, and 5 h after treatment. Results are expressed as the maximum fall (mean ± SE) in IOP (mmHg) from the t_0 value, and values in parentheses refer to the number of time points at which the IOP was significantly reduced ($P \leq 0.05$). [c] Compound or MK-927 was bilaterally instilled (1 drop, 50 μL) at 2% in 0.5% aqueous hydroxyethylcellulose three times with 10 min between each dose. IOP was measured at 0.5, 1, 2, 3, and 4 h after the third instillation. Results were expressed as the difference (mmHg) from the measurement taken just prior to treatment (t_0).

was stirred overnight at ambient temperature, and the insoluble sulfoxide precipitated from the reaction. The solid was collected, triturated with hot EtOAc, and air-dried to give 250 mg, mp 222–223 °C. Anal. ($C_6H_6N_2O_4S_3$) C, H, N. ^{1}H NMR (DMSO-d_6): δ 4.1, 4.3 (2H, dd), 7.3 (1H, s), 8.05 (2H, s).

2,3-Dihydro-6-sulfamoyl-2,4,4-trioxo-1*H*-thieno[2,3-*b*][1,4]-thiazine (16B). *m*-CPBA (16 g, 74 mmol) was added to a solution of **14A** (5.4 g, 21.6 mmol) in DMF (100 mL). The resulting solution generated a slight exotherm and was allowed to stir with no external temperature control for 2 h. A TLC probe (fl. silica, 90 $CHCl_3$:8 MeOH:2 HOAc) indicated complete conversion to product **16B** which had an R_f between starting material **14A** and sulfoxide **16A**. DMF was removed in vacuo and the residue crystallized from water to give 3.85 g, mp 259–260 °C. Anal. ($C_6N_6N_2O_5S_3$) C, H, N. ^{1}H NMR (DMSO-d_6): δ 4.9 (2H, s), 7.4 (1H, s), 8.2 (2H, s).

2,3-Dihydro-4,4-dioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]-thiazine (17A). Borane–dimethylsulfide in THF (4.4 mL, 10 M, 44 mmol) was added dropwise to a solution of **16B** (2.5 g, 8.9 mmol) in THF (10 mL) at 0 °C. The reaction mixture was warmed to ambient temperature and stirred for 1 h. Excess borane was destroyed by the addition of 6 N HCl and the borane adduct cleaved by refluxing for 1 h. The solvent was removed in vacuo, and the residue was partitioned between water and EtOAc. The

EtOAc was washed (H_2O, brine), dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by chromatography eluting with 80 $CHCl_3$:20 MeOH:2 NH_4OH followed by trituration with EtOAc to yield 0.36 g, mp 250–252 °C. Anal. ($C_6H_8N_2O_4S_3$) C, H, N. ^1H NMR (DMSO-d_6): δ 3.40 (2H, m), 3.78 (2H, m), 7.0 (1H, s), 7.5 (1H, s br), 7.95 (2H, s).

5-[(1,2-Dicarboxyethyl)thio]-4-nitrothiophene-2-sulfonamide (13B). Triethylamine (40.6 mL, 0.29 mol) was added to a stirred solution of mercaptosuccinic acid (14.7 g, 0.097 mol) and **12** (25 g, 0.10 mol) in MeOH (500 mL). After a slight exotherm, the reaction mixture was stirred at ambient temperature for 18 h. The solvent was removed under reduced pressure and the residue partitioned between 5% aqueous Na_2CO_3 and EtOAc. The basic fraction was acidified with 3 N HCl and then extracted with EtOAc. The EtOAc was washed (H_2O, brine), dried (Na_2SO_4), filtered, and concentrated in vacuo to yield 23.7 g (66% yield). ^1H NMR (DMSO-d_6): δ 3.0 (2H, dd), 4.2 (1H, t), 7.9 (1H, s), 8.1 (2H, s).

3-(Carboxymethyl)-2,3-dihydro-2-oxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazine (14B). Titanium trichloride (256 mL, 15 wt % in 20–30 wt % HCl) was added to a stirred suspension of **13B** (21.8 g, 61 mmol) in acetic acid (128 mL) and water (128 mL). The resultant purple mixture was stirred at ambient temperature for 18 h. The solid that precipitated was filtered, washed (H_2O), and air-dried to give 17.6 g (92% yield). Chromatography eluting with 60 $CHCl_3$:30 MeOH:10 concentrated NH_4OH followed by recrystallization from water gave the pure compound, mp 240 °C. Anal. ($C_6H_8N_2O_5S_3$) C, H, N. ^1H NMR (DMSO-d_6): δ 2.58 (1H, dd), 2.92 (1H, dd), 4.0 (1H, dd), 7.2 (1H, s), 7.8 (2H, s), 11 (1H, s), 12.6 (1H, s br).

(2,3-Dihydro-2,4,4-trioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazin-3-yl)acetic Acid (16C). *m*-Chloroperbenzoic acid (30 g, 80%–85% pure) was added to a stirred solution of **14B** (12.3 g, 39.9 mmol) in DMF (200 mL) at 10–20 °C. The mixture was stirred at ambient temperature for 24 h and the solvent removed in vacuo. The residue was triturated with CH_2Cl_2, and the resulting solid was collected and air-dried to yield 12 g. Recrystallization from water followed by trituration with EtOAc gave the pure compound. Anal. ($C_6H_8N_2O_7S_3$) C, H, N. ^1H NMR (DMSO-d_6): δ 2.50 (2H, m), 5.2 (1H, t), 7.3 (1H, s), 8.2 (2H, s), 11.8 (1H, s).

3-(2-Hydroxyethyl)-2,3-dihydro-4,4-dioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazine (17B). A. Borane–dimethylsulfide (9.5 mL, 10 M, 95 mmol) was added at ambient temperature to a solution of **16C** (3.25 g, 9.5 mmol) in dimethoxyethane (DME) (325 mL) and the resulting solution refluxed for 24 h. MeOH (100 mL) was added to the cooled reaction mixture (0 °C). The mixture was stirred for an additional 24 h and then concentrated in vacuo to an oil. The oil was partitioned between water and EtOAc. The EtOAc was washed (brine), dried (Na_2SO_4), and concentrated in vacuo. Crystallization of the residue from water gave 1.5 g. Chromatographic purification eluting with 70 $CHCl_3$:30 MeOH:3 NH_4OH yielded the pure product **17B**, mp 158–160 °C. Anal. ($C_8H_{12}N_2O_5S_3$) C, H, N. ^1H NMR (DMSO-d_6): δ 1.5 (1H, m), 1.9 (1H, m), 3.3 (1H, m), 3.6 (2H, m), 3.7 (1H, m), 3.9 (1H, dd), 5.0 (1H, t), 7.1 (1H, s), 7.6 (1H, s), 8.0 (2H, s).

B. Borane–tetrahydrofuran solution (200 mL, 1 M, 200 mmol) was added dropwise at ambient temperature to a solution of **14B** (12.3 g, 40 mmol) in THF (200 mL). The mixture was stirred until gas evolution ceased and the resulting clear solution refluxed overnight. Methanol (200 mL) was added to the cooled reaction mixture (0 °C). When gas evaluation ceased, the mixture was concentrated in vacuo and the residue swished with CH_3OH and concentrated two times. Recrystallization from water yielded **18**, 7.6 g, mp 145–147 °C. Anal. ($C_8H_{12}N_2O_3S_3$) C, H, N. ^1H NMR (DMSO-d_6): δ 1.6 (1H, m), 1.8 (1H, m), 3.2 (1H, m), 3.3 (1H, m), 3.5 (1H, m), 3.6 (1H, m), 4.6 (1H, t), 6.0 (1H, t), 7.0 (1H, s), 7.5 (2H, s).

A solution of *m*-chloroperbenzoic acid (11.5 g, 53 mmol, 80%–85% pure) in THF (45 mL) was added to a solution of **18** (5 g, 18 mmol) in 50% THF/H_2O (50 mL) with cooling to maintain the temperature at 25 °C. The reaction mixture was stirred at ambient temperature overnight and then concentrated in vacuo to remove THF. The aqueous suspension was diluted with H_2O and solid $NaHSO_3$ added to destroy excess peroxide. After concentration in vacuo, the residue was chromatographed eluting with 80 $CHCl_3$:1 MeOH:1 HOAc. Fractions containing product by TLC were combined, concentrated in vacuo, and treated with EtOAc. The EtOAc was filtered to remove white solid (no UV activity) and the filtrate concentrated in vacuo. The residue was crystallized from water to yield 1.5 g, mp 143–146 °C. The NMR was identical to that of **17B** isolated from the reduction of **16C** with borane–dimethylsulfide.

3-[2-[[(4-Methylphenyl)sulfonyl]oxy]ethyl]-2,3-dihydro-4,4-dioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazine (19). *p*-Toluenesulfonyl chloride (1.9 g, 9.9 mmol) was added dropwise at 0 °C to a solution of **17B** (2.9 g, 9.0 mmol) in pyridine (30 mL). After being stirred at 0 °C for 3 h and 25 °C for 1 h, the reaction mixture was added dropwise to 1 N HCl (35 mL) at 0 °C. The acid solution was decanted from the sticky solid which formed. Repeated triturations with ether yielded 4.6 g of solid. Anal. ($C_{15}H_{18}N_2O_7S_4$) C, H, N. ^1H NMR (DMSO-d_6): δ 1.8 (1H, m), 2.0 (1H, m), 2.4 (3H, s), 3.3 (2H, m br), 3.6 (2H, dd), 3.8 (2H, d), 4.2 (1H, t), 7.1 (1H, s), 7.5 (2H, d), 7.8 (2H, d), 8.0 (2H, s).

3-[2-(Isobutylamino)ethyl]-2,3-dihydro-4,4-dioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazine Hydrochloride (20A). *p*-Toluenesulfonyl chloride (1.91 g, 10 mmol) was added dropwise at 10 °C to a solution of **17B** (1.56 g, 5 mmol) in pyridine (10 mL). The solution was stirred for 0.5 h, and then isobutylamine (10 mL) was added at 0 °C. The reaction mixture was heated at 100 °C overnight and concentrated in vacuo to dryness and the residue treated with $CHCl_3$. The resulting white solid (1.7 g) was dissolved in a minimum of water, the pH was adjusted to 7.5 with concentrated NH_4OH, and the solution was extracted with EtOAc. The EtOAc was washed (brine), dried (Na_2SO_4), and concentrated in vacuo. The residue was dissolved in EtOH and treated with excess ethanolic HCl. Ether was added to the cloud point. The resulting hydrochloride salt was collected and dried to yield 1.2 g, mp 263–265 °C. Anal. ($C_{12}H_{21}N_3O_4S_3$·HCl) C, H, N. ^1H NMR (DMSO-d_6): δ 0.93 (6H, d), 1.93 (1H, m), 1.98 (1H, m), 2.10 (1H, m), 2.72 (2H, m), 3.13 (2H, m), 3.6 (1H, m), 3.7 (1H, m), 3.8 (1H, dd), 7.1 (1H, s), 7.7 (1H, s), 8.0 (2H, s), 8.63 (1H, s br).

3-[2-[Bis(2-methoxyethyl)amino]ethyl]-2,3-dihydro-4,4-dioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazine Hydrochloride (20B). *p*-Toluenesulfonyl chloride (1.91 g, 10 mmol) was added dropwise at 0 °C to a solution of **17B** (1.56 g, 5 mmol) in pyridine (10 mL) to generate the tosylate. The solution was stirred at 0 °C for 10 min, and then bis(2-methoxyethyl)amine (10 mL) was added. The reaction mixture was heated at 80 °C overnight and then concentrated in vacuo. The residue was chromatographed eluting with 90 $CHCL_3$:10 CH_3OH:1 NH_4OH to give the product **20B**. Treatment with ethanolic HCl yielded the hydrochloride, 393 mg, mp 140–142 °C. Anal. ($C_{14}H_{25}N_3O_6S_3$·HCl) C, H, N. ^1H NMR (DMSO-d_6): δ 1.98 (1H, m), 2.10 (1H, m), 3.37 (6H, s), 3.40 (3H, m), 3.5 (2H, m), 3.67 (6H, m), 3.8 (1H, dd), 7.1 (1H, s), 7.75 (1H, s), 8.0 (2H, s), 10 (1H, m).

3-[2-[*N*-[(Methoxyethoxy)ethyl]-*N*-(methoxyethyl)amino]ethyl]-2,3-dihydro-4,4-dioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazine Hydrochloride (20C). A solution of **19** (2.96 g, 6 mmol) and *N*-[(methoxyethoxy)ethyl]-*N*- (methoxyethyl)-amine[8] (5.32 g, 30 mmol) in DME (20 mL) was refluxed for 24 h. The reaction mixture was concentrated in vacuo and the residue partitioned between 0.5 N HCl and EtOAc. The acid fraction was adjusted to pH 7.4 with NH_4OH and extracted with EtOAc. The EtOAc fraction was washed (brine), dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by chromatography eluting with 60 $CHCl_3$:20 MeOH:1 HOAc to give 843 mg which was converted to the HCl salt by the addition of ethanolic HCl, mp 75–100 °C. Anal. ($C_{16}H_{29}N_3O_7S_3$·HCl) C, H, N. ^1H NMR (DMSO-d_6): δ 1.86 (1H, m), 2.18 (1H, m), 3.21 (3H, s), 3.30 (3H, s), 3.40 (4H, m), 3.48 (3H, m), 3.52 (3H, m), 3.68 (3H, m), 3.75 (3H, t), 7.09 (1H, s), 7.75 (1H, s br), 8.0 (2H, s), 10 (1H, s br).

3-(2-Morpholinoethyl)-2,3-dihydro-4,4-dioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazine Hydrochloride (20D). A solution of **19** (2.36 g, 5 mmol) and morpholine (4.4 mL, 50 mmol) in DMSO (15 mL) was heated at 80 °C for 16 h. The reaction mixture was diluted with ice/H_2O and extracted with EtOAc. The EtOAc extract was washed (H_2O, brine), dried (Na_2SO_4), filtered, and concentrated to yield 1.2 g which HPLC indicated was a mixture containing 45% of what was later ientified as **20D**

and 38% of what was later identified as 21. The mixture was partitioned between EtOAc and 1 N HCl. The EtOAc fraction was concentrated to yield 325 mg of 21, confirmed by NMR and mass spectroscopy (see below for spectral data). The acid fraction was adjusted to pH 7.5 with NH_4OH and extracted with EtOAc. The EtOAc was washed (brine), dried (Na_2SO_4), filtered, and concentrated to yield 523 mg of **20D**. Final purification was effected by chromatography eluting with 90 $CHCl_3$:10 CH_3OH:1 NH_4OH and conversion to the HCl salt with ethanolic HCl, 390 mg, mp 280–282 °C. Anal. ($C_{12}H_{19}N_3O_5S_3$·HCl) C, H, N. ^1H NMR (DMSO-d_6): δ 1.9 (1H, m), 2.18 (1H, m), 3.18 (3H, m), 3.4 (5H, m), 3.69 (1H, m), 3.75 (3H, m), 3.95 (2H, d), 7.1 (1H, s), 7.7 (1H, s), 8.0 (2H, s), 10.8 (1H, s br).

1,3-Ethano-2,3-dihydro-4,4-dioxothieno[2,3-*b*][1,4]thiazine-6-sulfonamide (21). Anal. ($C_8H_{10}N_2O_4S_3$) C, H, N. ^1H NMR (DMSO-d_6): δ 2.19 (1H, m), 2.45 (1H, m), 3.1 (1H, m), 3.2 (1H, d), 3.38 (1H, dd), 3.82 (1H, d), 4.21 (1H, t), 7.0 (1H, s), 8.07 (2H, s). Mass spectroscopy.

***N*-Isobutyl(2,3-dihydro-2-oxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazin-3-yl)acetamide (22A).** 1,1-Carbonyldiimidazole (3.2 g, 19 mmol) was added to a solution of **14B** (5 g, 16 mmol) in DMF (65 mL) and the solution stirred at ambient temperature for 5 h. Isobutylamine (1.9 mL, 19 mmol) was added, and stirring was continued for 18 h. The reaction mixture was concentrated in vacuo and the residue triturated with 0.5 N HCl to form a brown solid (5.8 g). Final purification was effected by chromatography eluting with 90 $CHCl_3$:10 MeOH:1 NH_4OH and recrystallization from MeOH, mp >250 °C. Anal. ($C_{12}H_{17}N_3O_4S_3$) C, H, N. ^1H NMR (DMSO-d_6): δ 0.93 (6H, d), 1.7 (1H, m), 2.41 (1H, dd), 2.72 (1H, dd), 2.9 (2H, m), 3.95 (1H, dd), 7.8 (1H, s), 7.85 (2H, s), 8.0 (1H, t), 10.9 (1H, s).

***N*-(Methoxyethyl)(2,3-dihydro-2-oxo-6-sulfomoyl-1*H*-thieno[2,3-*b*][1,4]thiazin-3-yl)acetamide (22B).** To a solution of **14B** (308 mg, 1 mmol) dissolved in DMF (5 mL) were added 1-hydroxybenzotriazole hydrate (HOBT) (135 mg, 1 mmol), (methoxyethyl)amine (0.2 mL, 2 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC) (288 mg, 1.5 mmol). The reaction mixture was stirred at ambient temperature for 4 h and the reaction quenched with water (5 mL). The mixture was concentrated in vacuo. The residue was partitioned between 0.5 N HCl and EtOAc. The EtOAc was washed (brine), dried (Na_2SO_4), and concentrated in vacuo. Final purification was accomplished by chromatography eluting with 80 $CHCl_3$:20 MeOH:2 NH_4OH to give 209 mg. ^1H NMR (DMSO-d_6): δ 2.42 (1H, dd), 2.7 (1H, dd), 3.21 (2H, m), 3.28 (3H, s), 3.3 (2H, m), 3.95 (1H, dd), 7.15 (1H, s), 7.78 (2H, s), 8.15 (1H, t), 10.86 (1H, s).

***N*-[(Methoxyethoxy)ethyl]-*N*-(methoxyethyl)(2,3-dihydro-2,4,4-trioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazin-3-yl)acetamide (22C).** Via the procedure for **22B** described above, **16C** (3.0 g, 9 mmol), HOBT (1.22 g, 9 mmol), *N*-[(methoxyethoxy)ethyl]-*N*-(methoxyethyl)amine[8] (3.19 g, 18 mmol), and EDC (2.59 g, 14 mmol) were reacted in DMF (25 mL); yield 1.9 g, mp 156–158 °C. Anal. ($C_{16}H_{25}N_3O_9S_3$) C, H, N. ^1H NMR (DMSO-d_6): δ 3.15 (1H, m), 3.2–3.7 (19H, m complex), 5.2 (1H, t), 7.3 (1H, s), 8.2 (2H, s), 11.9 (1H, s).

***N*-Isobutyl(2,3-dihydro-2,4,4-trioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazin-3-yl)acetamide (23).** *m*-CPBA (3.3 g, 18.9 mmol) was added in portions to a solution of **22A** (2.3 g, 6.3 mmol) in DMF (35 mL) while maintaining the temperature at 20 °C with cooling. The reaction mixture was stirred at ambient temperature overnight and then concentrated in vacuo. The residue was triturated with hot ether to dissolve *m*-CPBA and the suspension filtered to collect crude **23**. Recrystallization from water yielded 970 mg. ^1H NMR (DMSO-d_6): δ 0.94 (6H, dd), 1.7 (1H, m), 3.0 (1H, dd), 3.1 (3H, m), 5.1 (1H, m), 7.3 (1H, s), 8.2 (3H, m), 11.7 (1H, s).

Reduction of **23** (970 mg, 2.45 mmol) was carried out with borane–dimethlysulfide in THF (2.45 mL, 10 M, 24.5 mmol) in 15 mL of DME. The reaction mixture was refluxed for 8 h and the reaction quenched with 6 N HCl (15 mL). The solution was extracted with EtOAc. The EtOAc was washed (brine), dried (Na_2SO_4), filtered, and concentrated in vacuo to yield 365 mg. HPLC and TLC showed two components with close retention times and R_f's. The mixture was chromatographed eluting with 85 $CHCl_3$:15 CH_3OH:1.5 NH_4OH, resulting in isolation of a pure sample of each component. NMR analysis identified the components as **20A** and **24**.

3-[2-[*N*-[(Methoxyethoxy)ethyl]-*N*-(methoxyethyl)amino]ethyl]-2,3-dihydro-2,4,4-trioxo-6-sulfamoyl-1*H*-thieno[2,3-*b*][1,4]thiazine Hydrochloride (25). A solution of **22C** (500 mg, 1 mmol) in DME (5 mL) was added dropwise to a suspension of $LiAlH_4$ (152 mg, 4 mmol) in DME (5 mL) at such a rate that the reaction mixture refluxed gently. After the mixture refluxed two additional hours, the reaction was quenched with a saturated solution of Rochelle salt. The reaction mixture was filtered to remove granular lithuim salts and the filtrate concentrated in vacuo. The residue was partitioned between 0.5 N HCl and EtOAc. The acid fraction was treated with NH_4OH to pH 8 and extracted with EtOAc. The EtOAc was washed (brine), dried (Na_2SO_4), filtered, and concentrated. The residue was purified by chromatography eluting with 80 $CHCl_3$:20 MeOH:2 NH_4OH and, after treatment with ethanolic HCl, isolated as the HCl salt, mp 170–175 °C. Anal. ($C_{18}H_{27}N_3O_6S_3$·HCl) C, H, N. Mass spectroscopy. IR. ^1H NMR (DMSO-d_6): δ 2.44 (2H, m), 3.25 (3H, s), 3.30 (3H, s), 3.4 (4H, m), 3.48 (5H, m), 3.58 (2H, m), 3.66 (2H, m), 3.76 (2H, m), 7.4 (1H, s), 8.25 (2H, s), 10.0 (1H, s br).

Solubility in pH 5.2 Buffer. A standard solution was prepared by dissolving 1 mg of sample in 10 mL of CH_3OH. The standard solution was scanned by UV (Beckman ACTA M-VI spectrophotometer) to determine the wavelength of maximum absorbance, diluting as necessary. A saturated solution was prepared by magnetically stirring a small volume of pH 5.2 citrate buffer (750 μL) in the presence of excess compound. The solution was checked periodically and additional compound added if necessary to maintain saturation. After 4 h, the saturated solution was filtered through a Gelman Acrodisc LC13 PVDF 0.45-μm filter. The standard and saturated solutions were analyzed by HPLC using a Hewlett-Packard HP 1090 with a Vydac Protein and Peptide C-18 column (acetonitrile/phosphoric acid, 5%–95% gradient over 10 min). The integrated areas for analyzed peaks were used to calculate total solubility, determined by the relationship $C' = A'C/A$ where C = concentration of the saturated solution in mg/mL, A = peak area of the standard solution, A' = peak area of the saturated solution (correcting for any dilutions), and C' = concentration of the saturated solution in mg/mL.

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 a cold 0.9% NaCl solution 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 0.1–3.9 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.[12]

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. A fluorescence cuvette containing 1×10^{-7} M human CA II (HCA II) and 2×10^{-6} M dansylamide in pH 7.4, 0.1 ionic strength phosphate buffer was place 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.[12]

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