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Quaternary ammonium substituted thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxides: Potential membrane-impermeable inhibitors of carbonic anhydrase

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Abstract—Thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxides, which have a quaternary ammonium moiety incorporated into their structures, were synthesized. All of the quaternary ammonium salts prepared in the present study are potent inhibitors of both human carbonic anhydrase-II and recombinant human carbonic anhydrase-IV; they are significantly more potent as inhibitors of these carbonic anhydrase isozymes than the previously reported inhibitor quaternary ammonium homosulfanilamide. By virtue of the permanent cationic charge on these compounds they are anticipated to be membrane-impermeable inhibitors of carbonic anhydrase. Spiro quaternary ammonium compounds, such as **15** and **16**, when formed by intracellular cyclization following transport of a suitable precursor molecule, such as **14**, may be selective prolonged inhibitors of cytosolic carbonic anhydrase due to intracellular entrapment.

1. Introduction

Multiple membrane-bound isozymes of carbonic anhydrase (CA), a zinc-containing metalloprotein that catalyzes the reversible hydration of carbon dioxide, have been identified. Isozymes IV, IX, XII, and XIV are catalytically active with an extracellular active site. Additionally, a membrane-bound non-catalytic carbonic anhydrase-like domain has been identified in the extracellular region of receptor-like protein tyrosine phosphatase (RPTP) β and γ, wherein the active site residues of the CA sequences have been modified.[1,2] The specific physiological role for the various membrane-bound isozymes has yet to be determined. Isozyme IV has been suggested to be involved in the secretion of aqueous humor.[3–5] More recently, it has been shown that expression of isozyme XII is elevated in human glaucomatous eyes and this is the isozyme most likely responsible for an overproduction of aqueous humor.[6] Selective inhibition of these isozymes would result in a reduction of aqueous humor production and thereby be useful in the treatment of glaucoma. Isozymes IX and XII are highly expressed in certain tumors and inhibition of these membrane-bound isozymes has been postulated to be beneficial for reducing the invasiveness of tumors.[2,7–9] The presence of isozyme XIV on neuronal membranes and axons in human brain has led to the proposal that this isozyme has an important role in modulating excitatory synaptic transmission in the brain.[10,11] It would, therefore, be advantageous to have available potent isozyme selective inhibitors to assist in further delineating the functional role of these membrane-bound isozymes or for use as therapeutic agents.

Membrane-impermeable inhibitors are of considerable interest in view of their potential for selectively inhibiting membrane-bound isozymes, while not inhibiting intracellular carbonic anhydrase. Three general approaches have been explored in the effort to prevent CA inhibitors from crossing cell membranes: (1)

Keywords: Carbonic anhydrase; Enzyme inhibitor; Membrane-bound; Quaternary ammonium; Sulfonamide.
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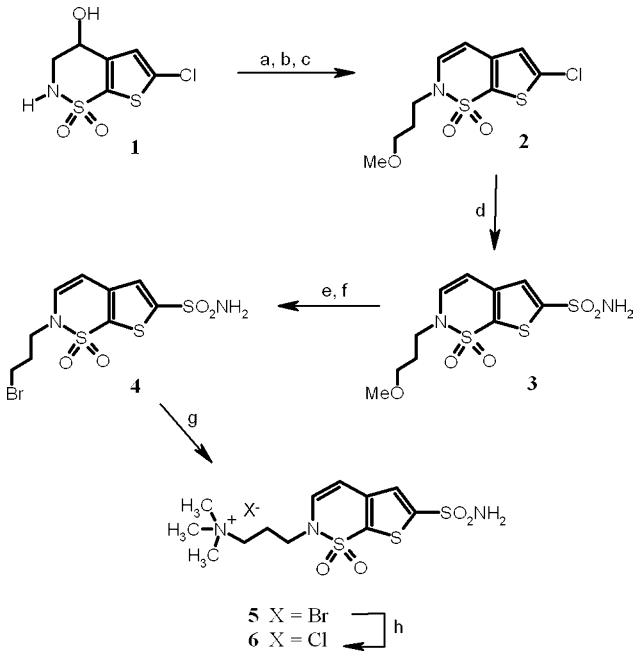
attachment of an inhibitor to a high molecular weight polymeric matrix,[12–14] (2) the use of molecules that are permanently charged, such as quaternary ammonium[15] or pyridinium[16,17] salts, and (3) compounds, which exist as charged species under physiologic conditions, such as benzolamide.[18,19] Of these approaches, attachment of an inhibitor to a polymeric matrix has not been particularly useful due to the high molecular weight and general toxicity associated with the dextran matrix; however, compounds attached to a polyethylene glycol matrix might reduce the toxicity concerns.[13] The use of compounds that are ionized under physiologic conditions requires careful maintenance of pH to eliminate the possibility of penetration of non-ionized molecules. Benzolamide, a frequently used compound of this type, was shown to cross the cell membrane of erythrocytes to an extent comparable to that of acetazolamide.[20] Furthermore, benzolamide has been implicated as an inhibitor of low threshold calcium currents in central neurons, suggesting that the use of benzolamide solely to inhibit extracellular carbonic anhydrase in these tissues should be done with caution.[21] At the present time the most appealing approach for achieving selective inhibition of CA isozymes with an extracellular active site appears to be the use of inhibitors that contain a permanent cationic charge on the molecule. Though such molecules would likely present challenges with regard to effective in vivo delivery, they are envisioned as potentially useful tools to assist in identifying the physiological role of the various membrane-bound CA isozymes and possibly lead the way for the design of other molecules more suitable for therapeutic use. The success of this approach will be enhanced by the identification of unique and accessible secondary binding sites outside the sulfonamide binding pocket of the enzyme active site.[22] A better understanding of the structural differences in the extracellular domain of the membrane-bound isozymes will assist in the development of isozyme-specific inhibitors.

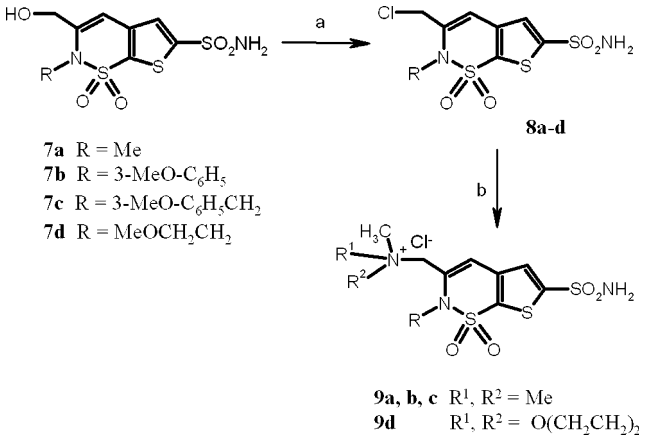
Substituted thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxides have been shown to be potent high affinity inhibitors of both hCA-II and rhCA-IV.[23,24] The thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxide structure is particularly well suited for the incorporation of a quaternary ammonium moiety into one of several different regions of the molecule, allowing multiple presentations of the cationic moiety for the investigation of possible selectivity among the various membrane-associated carbonic anhydrase isozymes. Therefore, it was of interest to initially prepare representative compounds based on this template and to evaluate the effect that such permanently charged quaternary ammonium groups might have on the inhibition of CA-II and CA-IV.

2. Chemistry

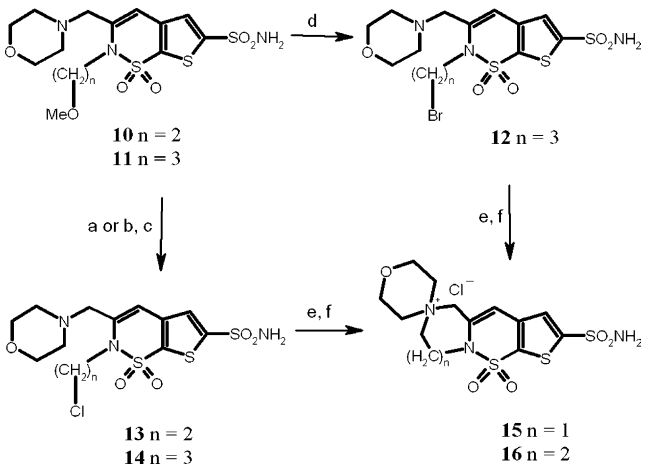
The preparation of the quaternary ammonium compounds was readily accomplished under Menshutkin quaternarization conditions. Reaction of the appropriate haloalkyl substituted thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxide with the desired tertiary amine provides the quaternary ammonium compounds. Incorporation of the quaternary ammonium group at ring position two was accomplished according to Scheme 1. Alkylation of **1**[25] with 3-methoxypropyliodide provided 6-chloro-3,4-dihydro-2-(3-methoxypropyl)-1*H*-thieno[3,2-*e*]-1,2-thiazine-4-ol 1,1-dioxide, which was dehydrated via pyrolysis of the intermediate thiocarbonate to give **2**. Reaction of **2** with *n*-butyllithium, sulfur dioxide, and hydroxylamine-*O*-sulfonic acid provided the sulfonamide **3**. Cleavage of the methoxy ether of **3** with boron tribromide followed by bromination of the alcohol with phosphorus tribromide gave the requisite 3-bromopropyl compound **4**. Treatment of **4** with trimethylamine in a sealed reactor gave the quaternary ammonium bromide salt **5**, which was converted to the chloride salt **6** using Amberlyst-A27 anion exchange resin. Incorporation of the quaternary ammonium group at ring position three was accomplished as shown in Scheme 2. Chlorination of 2-substituted-3-hydroxymethyl compounds **7** using *p*-toluenesulfonyl chloride in the presence of 4-dimethylaminopyridine provided the desired 3-chloromethyl derivatives **8**, which were reacted with the appropriate tertiary amine to give the quaternary ammonium chloride derivatives **9a**–**d**. Spiro quaternary ammonium compounds were prepared by following a procedure generally similar to that discussed above, however, the tertiary amine was attached to ring position C3 and the alkyl halide substituent was incorporated at the N2 ring position. The juxtaposition of these two groups facilitated intramolecular cyclization to provide the desired spiro quaternary ammonium compounds **15** and **16** as shown in Scheme 3.



Scheme 1. Reagents: (a) $MeO(CH_2)_3Br$, NaH, DMF; (b) $ClC(S)OC_6H_5$, CH_2Cl_2; (c) 200 °C, vacuum; (d) *n*-BuLi, SO_2, HOSA, THF; (e) BBr_3, CH_2Cl_2; (f) PBr_3, CH_2Cl_2; (g) NMe_3; (h) Amberlyst-A27.



Scheme 2. Reagents: (a) *p*-toluenesulfonyl chloride, DMAP, THF; (b) NMe_3, THF or *N*-methylmorpholine, DMSO.



Scheme 3. Reagents: (a) 48% HBr; (b) $BrBMe_2$, CH_2Cl_2; (c) $SOCl_2$, CH_2Cl_2; (d) 48% HBr, H_2O; (e) $NaHCO_3$, H_2O; (f) DMF, 80 °C.

3. Results and discussion

The in vitro enzyme assay data demonstrate that the quaternary ammonium compounds of the present study are potent inhibitors of both human CA-II (hCA-II) and recombinant human CA-IV (rhCA-IV) (Table 1). Inhibition of rhCA-IV by these compounds was, with one exception, 2- to 10-fold weaker than that observed for hCA-II. Inhibition of hCA-II was comparable to or greater than that of acetazolamide for all compounds, except for **9d**, which was approximately 10-fold less potent than acetazolamide. The compound with the highest affinity for and the greatest level of inhibition of hCA-II was **6** (K_i = 0.57 nM; IC_{50} = 2.39 nM), where the quaternary ammonium group was incorporated into the substituent at the N2 ring position. This compound also showed favorable inhibition of rhCA-IV (IC_{50} = 24.8 nM). All of the compounds were significantly more potent as inhibitors of rhCA-IV (100- to 650-fold) than quaternary ammonium sulfanilamide (QAS). The most potent inhibitor of rhCA-IV was **16** (IC_{50} = 11.6 nM).

Table 1. In vitro enzyme data and distribution coefficients for quaternary ammonium substituted thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxides and reference compounds

Compound	hCA-II		rhCA-IV	$DC_{7.4}$[c]
	K_i nM[a]	IC_{50}nM[b]	IC_{50} nM[b]	
6	0.57 (0.33)	2.39	24.8	0
9a	0.67 (0.20)	7.82	44.0	0
9b	0.66 (0.22)	4.31	19.5	0
9c	4.56 (1.15)	12.4	25.6	0
9d	1.49 (0.37)	77.9	81.3	0
10[d]	0.22	5.58	39.9	3.85
14	0.19 (0.06)	2.40	—	93.9
15	1.13 (0.14)	4.58	51.7	0
16	1.51 (0.07)	6.33	11.6	0
Brinzolamide[d]	0.13	3.19	44.9	6.56
Acetazolamide	15.3[d]	8.88[d]	17.5[d]	0.230
QAS	—	2700	7610	0

[a] Determined using a fluorescence competition assay measuring the displacement of dansylamide from hCA-II at 37 °C, mean of at least two determinations (±SD).
[b] Determined using a pH stat assay measuring the rate of CO_2 hydration at 4 °C; value calculated from the regression analysis of six data points.
[c] Mean of duplicate determinations.
[d] Data from Ref. 24.

Introduction of a 3-methoxyphenyl moiety at the N2 ring position, **9b**, when the quaternary ammonium group was at the C3 ring position, resulted in an approximately 2-fold improvement in the inhibition of rhCA-IV compared to **9a**, where the substituent at ring position N2 is methyl; that is, **9b** and **6** have comparable potency. Substitution of the ring nitrogen with 3-methoxybenzyl (**9c**) resulted in a 7-fold decrease in affinity for hCA-II along with a 3-fold decrease in the IC_{50} for this isozyme compared to **9b**. Comparable inhibition of rhCA-IV was observed for these two compounds. Incorporation of a 2-methoxyethyl group at N2 and increasing the bulk of the quaternary ammonium group at C3 by the incorporation of a methyl morpholinium group rather than the trimethylammonium group resulted in a 6- to 10-fold decrease in hCA-II inhibition for **9d** compared to **9a–c**. It is clear that quaternarization of the morpholine nitrogen by methylation (**9d**) has a detrimental affect on enzyme inhibition, since the non-quaternarized molecule **10** is a more potent inhibitor of both isozymes (see Table 1).[24] That is, a 14-fold decrease in hCA-II inhibition and a 2-fold decrease in inhibition of rhCA-IV were observed.

In order to assess the impact that reduced flexibility of the morpholinium moiety of **9d** might have on enzyme affinity and inhibition, molecules were synthesized with an ethyl or propyl bridge between the nitrogen of the morpholine moiety and the nitrogen atom at ring position two to give the spiro quaternary morpholinium compounds **15** and **16**, respectively. Both **15** and **16** were more potent inhibitors of hCA-II than **9d**. Their level of inhibition of hCA-II was comparable to that observed for the non-quaternarized molecule **10**. While the level of inhibition of rhCA-IV by **15** was marginally increased compared

to that of **9d**, it is of interest to note that **16** showed a substantial 7-fold greater inhibition of rhCA-IV than **9d** and 3-fold greater inhibition than **10**. It appears, therefore, that for rhCA-IV the more favorable binding orientation of the morpholinium moiety is the restrained conformation found in **16**.

The inability of the quaternary ammonium compounds to partition into 1-octanol (i.e., $DC_{7.4} = 0$) suggests they will not readily cross cellular membranes by passive diffusion. Due to the increased potency of these compounds compared to that of QAS and the absence of an equilibrium with a neutral species, as occurs with benzolamide, quaternary ammonium substituted thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxides are anticipated to have utility as selective inhibitors of membrane-bound CA isozymes.

Another potential utility for the spiro quaternary ammonium compounds is suggested by their high affinity for and potent inhibition of hCA-II. During the course of conducting certain studies, it would be advantageous to selectively inhibit cytosolic CA-II for extended periods of time while maintaining the activity of membrane-bound isozymes.[5,26] Such a profile would require compounds that have an extended cytosolic residence time. As previously noted, the spiro quaternary compounds **15** and **16** would be unlikely to inhibit cytosolic CA-II due to their anticipated inability to traverse cellular membranes. However, entrapment of such compounds within cells would be anticipated to provide prolonged inhibition of cytosolic CA-II due to the inability of the inhibitor to readily exit the cell. The entrapment of an unrelated class of quaternary ammonium compounds formed by intracellular cyclization of 2-[(3-haloalkyl)(methyl)amino]-*N*-(2,6-dimethylphenyl)acetamides has been proposed to explain the long-lasting anesthesia observed for members of this class of compounds.[27,28]

We investigated the rate of cyclization at physiologic pH for alkyl halides **12**–**14** to form spiro quaternary ammonium compounds in order to assess the suitability of this transformation for potential entrapment of compounds **15** or **16** within cells. Cyclization of the chloroethyl intermediate **13** to give **15** under these conditions was observed to be quite rapid with an estimated $t_{1/2}$ of less than 5 min. The cyclization of the chloropropyl intermediate **14** to give the seven-membered ring spiro compound **16** was found to be considerably slower with an estimated $t_{1/2}$ of 10.7 h. However, formation of **16** from the more reactive bromopropyl intermediate **12** was significantly more rapid with an estimated $t_{1/2}$ of 17.4 min under the same conditions. These results suggest that quaternization can occur within a time frame suitable for intracellular entrapment. Since the alkyl halides are neutral lipophilic molecules ($DC_{7.4} = 93.3$ for **14**), they should readily penetrate cellular membranes. Validation of this entrapment concept with 2-(haloalkyl)-3-[(dialkylamino) methyl]-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxides has not been pursued; however, compounds with which to conduct such studies are now available.

4. Conclusion

In conclusion, thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxides, which bear a quaternary ammonium substituent, have been prepared as potential membrane-impermeable inhibitors of extracellular carbonic anhydrase isozymes. These compounds are potent inhibitors of both hCA-II and rhCA-IV, and are significantly more potent as inhibitors of these isozymes than quaternary ammonium sulfanilamide (QAS), a membrane-impermeable CA inhibitor. The permeability of the present compounds remains to be determined; however, their inability to partition into 1-octanol suggests that they will not readily penetrate cell membranes.

5. Experimental protocols

5.1. Chemistry

5.1.1. General procedures. Melting points were determined in open capillaries with a Thomas–Hoover Uni-Melt Apparatus and are uncorrected. Organic extracts were dried over $MgSO_4$ (unless otherwise noted), which was removed by filtration and washed with the appropriate dry solvent. Chromatography refers to low pressure column chromatography conducted on 230–400 mesh silica gel from E. Merck. Evaporations were performed under reduced pressure on a rotary evaporator at 40 °C. ^{1}H NMR spectra were determined at 200 MHz with a Varian Model VXR-200 spectrometer and ^{13}C NMR spectra were determined at 50.3 MHz with the Varian instrument. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane as internal standard. Isobutane chemical ionization mass spectra were obtained with a Finnigan TSQ 46 triple-quadrupole mass spectrometer. Elemental analyses were performed by Atlantic Microlabs, Norcross, Georgia, and are within ±0.4% of the theoretical values unless otherwise noted.

5.1.2. 2-(3-Methoxypropyl)-2*H*-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamine 1,1-dioxide sodium salt (3). Sodium hydride (1.1 g of a 60% dispersion in mineral oil, 27 mmol) was added in portions to a solution of 6-chloro-3,4-dihydro-4-hydroxy-2*H*-thieno[3,2-*e*]-1,2-thiazine 1,1-dioxide (**1**)[25] (6.0 g, 25 mmol) in DMF (100 ml) under nitrogen. The mixture was stirred for 1 h, cooled to 0 °C in an ice bath, and 3-bromopropyl methyl ether (3.8 g, 25 mmol) was added. The mixture was stirred at room temperature for 18 h, evaporated to a residue that was mixed with water (100 ml), and extracted with EtOAc (5 × 20 ml). The extract was washed with brine, dried, and evaporated to give the N-alkylated intermediate as an oil [MS CI *m*/*z* 312 (M+1)]. A solution of this oil and DMAP (4.6 g, 38 mmol) in 1,2-dichloroethane (100 ml) was cooled to 4 °C and phenyl chlorothiono formate (4.2 ml, 38 mmol) was added slowly. The cooling bath was removed and the reaction mixture was stirred at room temperature for 18 h, diluted with a mixture of 25% EtOAc in hexane (200 ml), and filtered through silica. The filtrate was evaporated to a residue, which was purified by chromatography (hexane to 25% EtOAc

in hexane) to give the thionocarbonate intermediate as an oil (5.1 g, 46%) [MS CI *m/z* 448 (M+1)]. This oil was heated under vacuum (200 °C/0.5 mmHg) for 5 min. The flask was cooled and the residue was purified by chromatography (hexane to 25% EtOAc in hexane) to give the elimination product **2** as an oil (2.33 g, 70%): ^{1}H NMR ($CDCl_3$) δ 6.85 (s, 1H), 6.62 (d, J = 7.7 Hz, 1 H), 6.13 (d, J = 7.7 Hz, 1H), 3.87 (t, J = 6.8 Hz, 2H), 3.42 (t, J = 5.7 Hz, 2H), 3.33 (s, 3H), 2.02 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 141.1, 133.3, 129.6, 123.9, 120.8, 101.9, 68.3, 58.5, 45.8, 30.3.

To a solution of **2** in THF (35 ml) at −78 °C was added *n*-BuLi (4.1 ml of a 2.1 M solution in hexane, 8.7 mmol) and the mixture was stirred for 45 min. Sulfur dioxide gas was passed over the reaction mixture for 5 min followed by evaporation to a residue, which was dissolved in water (40 ml). Sodium acetate trihydrate (5.35 g, 39.3 mmol) and hydroxylamine-*O*-sulfonic acid (2.67 g, 23.6 mmol) were added and the mixture was stirred at room temperature for 4 h and extracted with EtOAc (5 × 8 ml). The extract was washed with brine, dried, and evaporated to a residue, which was purified by chromatography (gradient, 25% EtOAc in hexane to 30% MeOH in CH_2Cl_2) to give the sulfamoyl product as an oil (0.91 g, 34%). To a solution of this oil in ethanol (1.5 ml) was added 2 N NaOH (1.3 ml). Ethyl ether was added to form a precipitate, which was isolated and dried to give **3** as a colorless solid (0.81 g, 90%): mp 90–92 °C; ^{1}H NMR (DMSO-d_6) δ 7.16 (s, 1H), 6.93 (d, J = 7.7 Hz, 1H), 6.32 (d, J = 7.7 Hz, 1H), 3.76 (t, J = 7.0 Hz, 2H), 3.30 (t, J = 6 Hz, 2H), 3.20 (s, 3H), 1.86 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 161.2, 141.5, 133.2, 122.9, 122.1, 102.3, 68.4, 57.9, 44.5, 29.9; MS CI *m/z* 339 (M+1). Anal. ($C_{10}H_{13}N_2O_5S_3Na \cdot 2H_2O$) C, H, N.

5.1.3. 6-(Aminosulfonyl)-*N*,*N*,*N*-trimethyl-1,1-dioxo-2*H*-thieno[3,2-*e*]-1,2-thiazine-2-propanaminium bromide (5). A solution of **3** (1.85 g, 5.47 mmol) in dichloromethane (80 ml) was cooled (0 °C) and boron tribromide (10.30 g, 60.45 mmol) was added; this mixture was stirred for 1 h at 0 °C and then allowed to warm to room temperature and stirred for an additional hour. Water (50 ml) was added and the organic layer was washed with brine (40 ml), dried, and evaporated to a residue, which was purified by chromatography (hexane/acetone, 1:1) to give the hydroxypropyl intermediate as an oil (1.0 g, 56%) [^{1}H NMR ($CDCl_3$) δ 7.52 (s, 1H), 6.75 (d, J = 7.6 Hz, 1H), 6.30 (d, J = 7.6 Hz, 1H), 5.15 (br s, 2H), 3.96 (s, 2H), 3.70 (m, 2H), 1.90 (m, 2H)]. A solution of this oil (1.0 g, 3.1 mmol) in dichloromethane (20 ml) was cooled (0 °C) and phosphorus tribromide (30 ml of a 1.0 M dichloromethane solution, 30 mmol) was added; this mixture was allowed to warm to room temperature and stirred for 4 h. After evaporating the solvent, the residue was dissolved in EtOAc (50 ml) and the solution was washed with water (50 ml) and brine (50 ml), dried, and evaporated to a residue, which was purified by chromatography (acetone/hexane, 1:1) to give **4** as an oil (0.40 g, 32%): ^{1}H NMR ($CDCl_3$) δ 7.52 (s, 1H), 6.88 (d, J = 7.6 Hz, 1H), 6.30 (d, J = 7.7 Hz, 1H), 5.65 (br s, 2H), 3.90 (t, J = 5.9 Hz, 2H), 3.55 (t, J = 6.1 Hz, 2H), 2.33 (m, 2H).

To a solution of **4** (0.40 g, 1.0 mmol) in THF (5 ml) at −30 °C in a glass pressure tube was added an excess of trimethylamine (1 ml). The sealed tube was allowed to warm to room temperature (1 h) and then heated at 50 °C for 3 h. After cooling, the solid was collected by filtration and dried to give **5** (0.40 g, 89%): mp >255 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.13 (s, 2H), 7.69 (s, 1H), 7.19 (d, J = 7.6 Hz, 1H), 6.55 (d, J = 7.6 Hz, 1H), 3.88 (t, J = 6.0 Hz, 2H), 3.35 (t, J = 6.0 Hz, 2H), 3.05 (s, 9H). Anal. ($C_{12}H_{20}BrN_3O_4S_3$) C, H, N.

5.1.4. 6-(Aminosulfonyl)-*N*,*N*,*N*-trimethyl-1,1-dioxo-2*H*-thieno[3,2-*e*]-1,2-thiazine-2-propanaminium chloride (6). A solution of **5** (3 g, 0.67 mmol) in water (20 ml) was passed through a column of Amberlyst-A27 resin (20 g). Evaporation of the water provided a syrup which, when scratched under ethanol, provided **6** as a white solid (0.25 g, 92%): mp 255 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.23 (s, 2H), 7.71 (s, 1H), 7.24 (d, J = 7.6 Hz, 1H), 6.55 (d, J = 7.6 Hz, 1H), 3.86 (t, J = 7.2 Hz, 2H), 3.35 (t, 2H), 3.05 (s, 9H), 2.12 (m, 2H). Anal. ($C_{12}H_{20}ClN_3O_4S_3$) C, H, N.

5.1.5. 6-(Aminosulfonyl)-*N*,*N*,*N*-2-tetramethyl-1,1-dioxo-2*H*-thieno[3,2-*e*]-1,2-thiazine-3-methanaminium chloride (9a). A solution of **7a**[24] (1.00 g, 3.22 mmol) in THF (20 ml) was cooled (0 °C) and DMAP (0.78 g, 6.5 mmol) was added followed by *p*-toluenesulfonyl chloride (1.2 g, 6.5 mmol). This mixture was stirred for 2 h at 0 °C and then at room temperature for an additional 2 h. Ethyl acetate (50 ml) was added and the mixture was washed with water (30 ml). The organic layer was dried and the solvent was evaporated to give a residue, which was purified by chromatography (20% acetone in hexane) to give **8a** as an oil (0.50 g, 50%): ^{1}H NMR (DMSO-d_6) δ 8.12 (s, 2H), 7.72 (s, 1H), 6.89 (s, 1H), 4.81 (s, 2H), 3.55 (s, 3H).

A solution of **8a** (0.40 g, 1.2 mmol) in THF (5.0 ml) was added to a saturated solution of trimethylamine in THF (10 ml) at −78 °C and this mixture was heated at 60 °C for 12 h in a sealed tube. After cooling to room temperature, the solid that formed was collected, washed with ether, and dried to give **9a** as a white powder (0.25 g, 53%): mp >190 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.45 (br s, 2H), 7.95 (s, 1H), 7.40 (s, 1H), 4.81 (s, 2H), 3.55 (m, 12H). Anal. ($C_{11}H_{18}ClN_3O_4S_3 \cdot 0.25$-$C_4H_8O$) C, H, N.

5.1.6. 6-(Aminosulfonyl)-*N*,*N*,*N*-trimethyl-2-(3-methoxyphenyl)-1,1-dioxo-2*H*-thieno[3,2-*e*]-1,2-thiazine-3-methanaminium chloride (9b). A solution of **7b**[24] (1.00 g, 2.48 mmol) in THF (20 ml) was treated as described for the preparation of **8a** to give, following chromatography (20% acetone in hexane), **8b** as an oil (0.60 g, 60%): ^{1}H NMR (DMSO-d_6) δ 8.13 (s, 2H), 7.81 (s, 1H), 7.35 (t, J = 8 Hz, 1H), 7.15 (s, 1H), 7.10 (dd, J = 6 Hz, 1H), 6.77 (m, 2H), 4.32 (s, 2H), 3.75 (s, 3H).

A solution of **8b** (0.30 g, 0.70 mmol) in THF (5.0 ml) was treated as described for the preparation of **9a** to give **9b** as a white solid (0.20 g, 50%): mp > 208 °C dec.; ^{1}H

NMR (DMSO-d_6) δ 8.23 (s, 2H), 7.97 (s, 1H), 7.59 (s, 1H), 7.31 (t, J = 8 Hz, 1H), 7.06 (dd, J = 6 Hz, 1H), 6.79 (s, 1H), 6.65 (s, 1H), 4.24 (s, 2H), 3.75 (s, 3H). Anal. ($C_{17}H_{22}ClN_3O_5S_3 \cdot 0.75H_2O$) C, H, N.

5.1.7. 6-(Aminosulfonyl)-*N*,*N*,*N*-trimethyl-2-(3-methoxyphenylmethyl)-1,1-dioxo-2*H*-thieno[3,2-*e*]-1,2-thiazine-3-methanaminium chloride (9c). A solution of **7c**[24] (0.10 g, 0.48 mmol) in THF (5 ml) was treated as described for the preparation of **8a** to give, following chromatography (20% acetone in hexane), **8c** as an oil (0.05 g, 50%): ^{1}H NMR (DMSO-d_6) δ 8.13 (s, 2H), 7.19 (s, 1H), 6.99 (s, 1H), 6.80 (d, J = 7.8, 2H), 6.70 (d, J = 7.8 Hz, 1H), 6.52 (s, 1H), 5.03 (s, 2H), 4.62 (d, 2H), 3.53 (s, 3H).

To a saturated solution of trimethylamine in THF (10 ml) at −78 °C in a sealed tube was added a solution of **8c** (0.30 g, 0.70 mmol) in THF (5 ml). The mixture was allowed to warm to 60 °C with stirring and held at this temperature for 12 h. The reaction mixture was cooled to room temperature and filtered. The solid was washed with ether and dried to give **9c** as a colorless solid (0.2 g, 50%): mp dec >190 °C; ^{1}H NMR (DMSO-d_6) δ 8.18 (s, 2H), 7.70 (s, 1H), 7.20 (s, 1H), 7.06 (t, 1H), 6.75 (d, 1H), 6.64 (d, 1H), 6.47 (t, 1H), 5.09 (s, 2H), 4.62 (d, 2H), 3.49 (s, 3H), 3.19 (s, 9H). Anal. ($C_{18}H_{24}ClN_3O_5S_3$) C, H, N.

5.1.8. 4-[[6-(Aminosulfonyl)-2-(2-methoxyethyl)-1,1-dioxo-2*H*-thieno[3,2-*e*]-1,2-thiazin-3-yl]methyl]-4-methylmorpholinium chloride (9d). A solution of **7d**[24] (5.02 g, 14.1 mmol) in anhydrous THF (50 ml) was treated as described for the preparation of **8a** to give, following chromatography (gradient: 50% ethyl acetate/hexane to ethyl acetate), starting material **7d** (2.0 g, 40%) and **8d** as a viscous syrup (1.58 g, 30%): ^{1}H NMR (DMSO-d_6) δ 8.07 (s, 2H), 7.70 (s, 1H), 6.97 (s, 1H), 4.76 (s, 2H), 3.92–3.97 (m, 2H), 3.25–3.30 (m, 2H), 2.97 (s, 3H).

To a solution of **8d** (1.5 g, 4.1 mmol) in DMSO (1.5 ml) at ambient temperature was added *N*-methylmorpholine (0.89 ml, 8.1 mmol) and this mixture was stirred for 18 h. After evaporation of the solvent, the residue was dissolved in methanol (4 ml) and this solution was added to 2-propanol (200 ml). The solvent was removed and the residue was dissolved in methanol (3 ml) and 2-propanol was added to the cloud point. This mixture was heated to reflux and allowed to cool; the solid (0.26 g) was collected. The filtrate was evaporated and the residue was treated in a similar manner two more times to give additional **9d** (1.0 g, 52%): mp 230–231 °C; ^{1}H NMR (DMSO-d_6) δ 8.22 (s, 2H), 7.85 (s, 1H), 7.46 (s, 1H), 4.84 (s, 2H), 4.08–4.12 (m, 2H), 3.99 (br s, 4H), 3.65–3.75 (m, 2H), 3.45–3.50 (m, 2H), 3.29 (s, 3H), 3.08–3.12 (m, 2H), 2.80 (s, 3H); MS (*m*/*z*) 438 (M−Cl) [MALDI]. Anal. ($C_{15}H_{24}ClN_3O_6S_3$) C, H, N.

5.1.9. 2-(3-Bromopropyl)-3-(4-morpholinomethyl)-2*H*-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxide hydrochloride (12). A solution of **11**[24] (1.00 g, 2.25 mmol) in 48% hydrobromic acid (20 ml) was heated at reflux temperature for 3 h, cooled to room temperature, and slowly added to an ice/water mixture (100 ml). The organic material was extracted into EtOAc (3 × 50 ml), the extract was dried, and evaporated to an oil, which was purified by chromatography (silica, 10% acetone in hexane) to give an oil (0.6 g, 58%) that was converted to the hydrochloride salt by treatment with ethanolic HCl: mp 188–190 °C; ^{1}H NMR (DMSO-d_6) δ 8.16 (s, 2H), 7.84 (s, 1H), 7.30 (s, 1H), 4.36 (s, 2H), 3.80–4.20 (m, 6H), 3.50–3.80 (m, 6H), 1.69 (m, 2H). Anal. ($C_{14}H_{20}BrN_3O_5S_3 \cdot HCl$) C, H, N.

5.1.10. 2-(2-Chloroethyl)-3-(4-morpholinomethyl)-2*H*-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide-1,1-dioxide hydrochloride (13). A mixture of **10**[24] (1.56 g, 3.68 mmol), 48% hydrobromic acid (16 ml), and water (4 ml) was heated at reflux temperature for 18 h, evaporated to dryness, mixed with 5% aqueous sodium bicarbonate (60 ml), and extracted with EtOAc (2 × 80 ml). The extract was dried and evaporated to a residue, which was purified by chromatography (5% methanol in CH_2Cl_2) to give the 2-(2-hydroxyethyl) intermediate as an amorphous solid (0.85 g, 56%): mp 104–108 °C; ^{1}H NMR (DMSO-d_6) δ 8.07 (s, 2H), 7.65 (s, 1H), 6.69 (s, 1H), 4.98 (m, 1H), 4.00 (t, J = 6.4 Hz, 2H), 3.60 (m, 4H), 3.46 (m, 4H), 2.41 (m, 4H). Anal. Calcd for $C_{13}H_{19}N_3O_6S_3 \cdot 0.3H_2O$: C, 37.63; H, 4.76; N, 10.13. Found: C, 37.61; H, 4.60; N, 10.00. A solution of this solid (1.00 g, 2.18 mmol) in a mixture of DMF (2 ml) and THF (20 ml) was combined with thionyl chloride (0.60 ml, 8.7 mmol) and the mixture was heated at reflux temperature for 3 h. Evaporation of the reaction mixture provided a residue that was dissolved in 6 N HCl (20 ml) and stirred for 12 h followed by neutralization with 5% aqueous sodium bicarbonate. The organic material was extracted into EtOAc (3 × 10 ml) and the combined extracts were dried and evaporated to an oil, which was dissolved in ether (50 ml) and treated with a 1 N solution of HCl in ether. The solid, which formed, was purified by multiple recrystallizations (EtOAc–hexane) to give **13** (0.2 g, 20%): mp >215 °C; ^{1}H NMR (DMSO-d_6) δ 8.22 (s, 2H), 7.81 (s, 1H), 6.97 (s, 1H), 4.16 (s, 2H), 4.0–4.12 (m, 6H), 3.96 (m, 4H), 3.49 (s, 2H). Anal. ($C_{13}H_{18}ClN_3O_5S_3 \cdot HCl \cdot 0.5H_2O$) C, H, N.

5.1.11. 2-(3-Chloropropyl)-3-(4-morpholinomethyl)-2*H*-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide 1,1-dioxide hydrochloride (14). Bromodimethylborane (0.82 g, 6.75 mmol) was added to a solution of **11**[24] (1.00 g, 2.25 mmol) in CH_2Cl_2 (20 ml) at 0 °C. The mixture was allowed to warm to room temperature and after 3 h was diluted with EtOAc (100 ml) and water (100 ml). The organic layer was separated, dried, and evaporated to an oil that was dissolved in a mixture of DMF (2 ml) and THF (20 ml). Thionyl chloride (0.60 ml, 8.7 mmol) was added and the mixture was stirred at reflux temperature for 3 h. Evaporation provided a residue that was dissolved in 6 N HCl (20 ml); this solution was stirred for 12 h, neutralized with 5% aqueous sodium bicarbonate, and extracted with EtOAc (3 × 50 ml). The extract was dried and evaporated to give a residue, which was purified by chromatography (10% acetone in hexane) to give an oil that was dissolved in ether (50 ml) and converted to the hydrochloride salt by adding a 1 N solution of HCl in

ether. The solid that formed was collected by filtration and dried (0.54 g, 54%): mp 205–207 °C; ^{1}H NMR (DMSO-d_6) δ 8.30 (s, 2H), 7.90 (s, 1H), 7.30 (s, 1H), 4.6 (s, 2H), 3.80–4.20 (m, 6H), 3.50–3.80 (m, 6H), 1.69 (m, 2H). Anal. ($C_{14}H_{20}ClN_3O_5S_3 \cdot HCl \cdot 0.2EtOAc$) C, H, N.

5.1.12. 2-(Aminosulfonyl)-7,8-dihydro-spiro[morpholine-4,6′(5*H*)-pyrazino[1,2-*b*]thieno[3,2-*e*][1,2]thiazinium]10,10-dioxide chloride (15). A solution of **13** (0.50 g, 0.10 mmol) in water (20 ml) was neutralized by the addition of a 5% aqueous sodium bicarbonate, and the organic material was extracted into EtOAc (3 × 10 ml). The extract was dried and evaporation of the solvent at 60 °C under vacuum gave a yellow solid (0.4 g, 89%): mp 220–222 °C; ^{1}H NMR (DMSO-d_6) δ 8.22 (s, 2H), 7.81 (s, 1H), 6.97 (s, 1H), 4.16 (s, 2H), 4.00–4.12 (m, 6H), 3.96 (m, 4H), 3.49 (s, 2H). Anal. ($C_{13}H_{18}ClN_3O_5S_3 \cdot H_2O$) C, H, N.

5.1.13. 2-(Aminosulfonyl)-8,9-dihydro-spiro[morpholine-4,6′(7*H*)-5*H*-thieno[3′,2′:5,6][1,2]thiazine[2,3-*a*][1,4]diazepinium]11,11-dioxide chloride (16). A solution of **14** (0.24 g, 0.042 mmol) in water (20 ml) was neutralized by the addition of 5% aqueous sodium bicarbonate. The organic material was extracted into EtOAc (3 × 20 ml) and the extract was dried and evaporated to a residue, which was dissolved in DMF (20 ml). This solution was heated at 80 °C for 12 h, cooled to room temperature, and the solvent was evaporated to give a yellow solid (0.17 g, 80%): mp 210–212 °C; ^{1}H NMR (DMSO-d_6) δ 8.30 (s, 2H), 7.78 (s, 1H), 7.22 (s, 1H), 5.04 (m, 2H), 4.20 (m, 2H), 3.88–3.97 (m, 6H), 3.32–3.54 (m, 4H), 2.20 (m, 2H). Anal. ($C_{14}H_{20}ClN_3O_5S_3 \cdot 0.5H_2O$) C, H, N.

Compound **16** was also prepared in a similar manner from intermediate **12**.

5.2. In vitro enzyme assays

5.2.1. General procedure. Inhibition constants (IC_{50}) of inhibitors for hCA-II and rhCA-IV were determined by using a pH stat assay which measured the rate of hydration of carbon dioxide at 4 °C as previously described; these values were calculated from a regression analysis of six data points.[24] Inhibitor binding constants (K_i) for hCA-II were determined using a fluorescence competition assay measuring the displacement of dansylamide from enzyme at 37 °C as previously described.[24]

5.3. Determination of distribution coefficients

5.3.1. General procedure. The compound of interest was partitioned between 1-octanol and aqueous buffer (pH 7.4, 0.1 M phosphate). The initial concentration (C_1) of compound in buffer and the buffer concentration following extraction with 1-octanol (C_2) were determined by RP-HPLC analysis against concentration standards for the specific compound. The distribution coefficient (DC) of a compound at a given pH was calculated using the equation $DC_{pH} = (C_1 - C_2)/C_2$. Each determination was run in duplicate.

5.4. Determination of cyclization rates for the formation of 15 and 16

The alkyl halide (**12**, **13**, or **14**) of interest was dissolved (8 μg/mL) in sodium phosphate buffer (pH 7.4, 0.1 M) and the solution was heated at 37.5 °C. Periodically an aliquot was removed from this solution and analyzed using a previously established RP-HPLC method to determine the concentration (C) of the alkyl halide remaining at each time point. A preliminary evaluation was conducted for each compound to establish an appropriate sampling schedule. A plot of $\log C$ for each time point versus time gave a linear curve indicating the cyclization was a first-order reaction. The half-life ($t_{1/2}$) of each compound was calculated from the curve.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.10.054.

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