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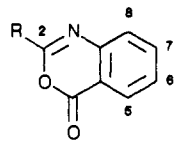
Communications to the Editor

Design of Alternate Substrate Inhibitors of Serine Proteases. Synergistic Use of Alkyl Substitution To Impede Enzyme-Catalyzed Deacylation[1]

Sir:

There is considerable interest in the design of stable small molecule inhibitors of serine proteases and in devising tactics to enhance the specificity and potency of such reagents. A particular challenge is to design specific inhibitors of high potency without resorting to peptide functionality as a means of delivering covalent labels to the target enzyme active site.[2] *4H*-3,1-Benzoxazin-4-one (**1**) represents an attractive lead structure for accomplishing this task[3-6] provided that electron-donating groups are present at C_2 to counter the lability of the oxazinone ring to nucleophiles.[7]



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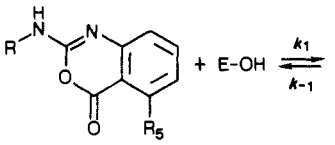
Recently, 2-aminobenzoxazinone **2** (R = H, R_5 = H) has been reported to be rapidly isomerized to the 2,4-(1*H*,3*H*)-quinazolinedione **4** by chymotrypsin.[5] Apparently, deacylation to regenerate free enzyme with concomitant formation of **4** occurs via cyclization of the acyl enzyme **3** and is considerably faster than the "normal" enzyme-catalyzed hydrolysis observed for 2-oxa and 2-carba benzoxazinones.[5] We have observed similar results with **2** (R = H, R_5 = H) and human leukocyte elastase (HLE), an enzyme of potential clinical interest.[8,9] We now



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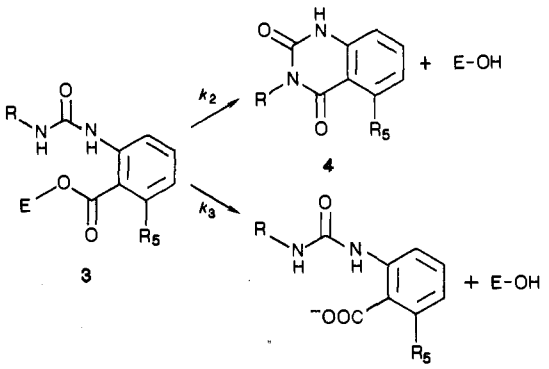

3, **4**, **5**

report that the lifetime of acyl elastases derived from benzoxazinones can be enormously increased by using tactics that are intended to obstruct deacylation pathways.

Experimental Section

2-(Alkylamino)-4*H*-3,1-benzoxazin-4-ones were prepared by published procedures.[10-13] HL elastase was obtained from human leukocytes[6,14-16] and assayed by the progress curve method[6,14,17] as follows. HL elastase (20 μL of 0.3 μM) and a fluorogenic substrate (5 μL of 1 mM 7-(methoxysuccinylalanylalanyl-prolylvalinamido)-4-(trifluoromethyl)coumarin (Enzyme Systems

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(7) Alkaline hydrolysis serves as a measure of lability. For example, a potent 2-carba benzoxazinone (**1**, R = C_3F_7) has k_{OH^-} = 1900 M^{-1} s^{-1} (25 °C, aqueous).[6] A 2-(alkylamino)benzoxazinone with comparable K_i (2-*n*-butylamino) has k_{OH^-} = 12 M^{-1} s^{-1}.
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Table I. HL Elastase Inhibition by Benzoxazinones **2**

R	R_5	K_i, μM	k_1, M^{-1} s^{-1}	k_{off},[a] s^{-1}	% 4[b]
H	H	63	810[c]	0.051[c]	91 ± 1
	Me			≈0.47	≈100[d]
	Et	5.1	<15		
Me	H	6.9	5500[c]	0.038[c]	98 ± 2
	Me	31	4800[c]	0.15[c]	95 ± 5
Et	H	0.051	730000	0.037	81 ± 1
	Me	0.034	52000	0.0018	
n-Pr	H	0.091	450000	0.041	94 ± 3
	Me	0.0060	56000	0.00034	
i-Pr	H	0.052	59000	0.0030	28 ± 9
	Me	0.0015	11800	0.000018	
	Et	0.00094	70000	0.000066	
n-Bu	H	0.084	66000	0.0055	81 ± 1
	Me	0.023	7600	0.00017	
	Et	0.0042	130000	0.00053	
sec-Bu	H	0.043	210000	0.0091	18 ± 9
n-Hex	H	3.6	1500	0.0053	

[a] Except as noted,[c] $k_{off} = k_1K_i$. [b] Equals 100 [4]/([4] + [5]), by analysis of fluorescence emission spectra after exhaustive incubation of HLE and **2**. Fluorescence standards used were authentic **4** and **5** (R = *n*-Bu and *i*-Pr, R_5 = H). [c] k_1 calculated as k_{off}/K_i, with k_{off} = measured rate of recovery of enzyme activity from the isolated acyl enzyme.[14,19] [d] From a comparison of ultraviolet difference spectra to standards.

Products, Livermore, CA) are added to 2 mL of buffer (25 mM K^+ *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid, 1 M NaCl, 0.1% Brij 35, pH 7.8, 25 °C) and catalysis monitored by coumarin fluorescence (400-nm excitation, 505-nm emission). At this substrate concentration ([S] ≈ 0.04 K_m), turnover of the substrate serves simply as a probe for the concentration of free enzyme. Upon addition of benzoxazinone (0.5–20 μL of a stock solution in Me_2SO), a first-order approach to a new steady-state rate is observed. Linear regression of the rate constants of this approach vs. [benzoxazinone] gives k_1; no saturation of k_1 is observed. Regression of the steady-state rates vs. [benzoxazinone] with program VKKI (a special case of the program COMP[18] for [S] ≪ K_m) gives K_i, and k_{off} is calculated as k_1K_i.[19]

Results and Discussion

The results of treating HLE with various 2-(alkylamino)benzoxazinones are displayed in Table I. The K_i is minimal for R = ethyl, propyl, and butyl and in these cases differs from the parent (R = H, R_5 = H) by 3 orders of magnitude. The large increases in potency are due primarily to enhancements of acylation rates (k_1). Deacylation rates ($k_{off} = k_{-1} + k_2 + k_3$) are also significantly reduced for R groups larger than *n*-propyl when R_5 = H and for R groups larger than methyl when R_5 = methyl.

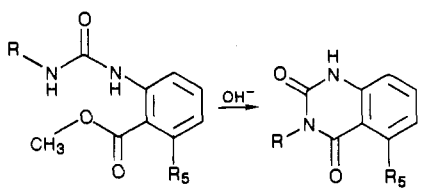
In addition, Table I shows how the ultimate[20] product composition varies with the structure of R. The nature of the alkyl group is clearly a determinant of product as 2-(alkylamino)benzoxazinones with linear R give predominantly quinazolinediones, and those with branched R form primarily[20] 2-ureidobenzoates **5**.

We have also observed significant reductions in the rate of a model reaction, the base-catalyzed cyclization of 2-ureidobenzoic acid esters **6** to quinazolinediones **4**, by increasing bulk at the terminal ureido nitrogen (Table II).

Table II. Substituent Effects on the Cyclization of 6 to 4[a]

R	R_5	k_{OH^-}, M^{-1} s^{-1}
Me	H	630
n-Bu	H	84
n-Bu	Et	52
i-Pr	H	0.98
i-Pr	Et	0.34

[a] Aqueous, 25 °C.



6 **4**

Compared to the cyclization rate of the *N*-methyl derivative (**6**, R = Me, R_5 = H), the values corresponding to the linear (R = *n*-Bu) and branched (R = *i*-Pr) groups are lower by factors of 8 and 640, respectively. It seems reasonable to assume that steric interactions that develop in the transition state for cyclization (between R and vicinal oxy functions) are bound to intensify as R increases in bulk and that such steric effects are at least partly responsible for the diminished deacylation rates and altered product partition observed in the enzyme-catalyzed reaction.

5-Alkyl-substituted benzoxazinones transform to diortho-substituted benzoic acid esters (**3**, $R_5 \neq$ H) upon treatment with HLE. Bulk at C_5 should have a profound effect on the hydrolytic rate (k_3) at the very least, since it is known that $A_{AC}2$ and $B_{AC}2$ hydrolyses of benzoic acid esters are blocked by flanking ortho substituents that shield the carbonyl from nucleophilic attack.[21]

Table I presents the impact of alkyl substituents at C_5 on K_i and the constituent rate constants k_1 and k_{off}. For the 2-*n*-butylamino system, K_i is lowered fourfold by replacing the C_5 hydrogen with a methyl group and by a factor of 20 on changing hydrogen to ethyl. The major effect of these changes is on k_{off}, which is reduced 32-fold for the methyl substitution and 10-fold for the ethyl. Essentially the same story unfolds for all R larger than methyl, suggesting an important synergy between the sizes of R and R_5. When R is methyl or hydrogen, R_5 substitution does not slow deacylation, and the products in these cases (R = H, Me; R_5 = Me) are 85–100% quinazolinedione. When R is larger, R_5 substitution slows deacylation dramatically through a combination of substituent effects.[22] Note that this synergy is absent in the model reaction (Table II).

It is especially noteworthy that k_{off} is diminished when either linear or branched alkylamino functions reside at C_2. Since the predominant mode of deacylation is N-cyclization (k_2) for R = *n*-Bu and *O*-cyclization[20] (k_{-1}) or hydrolysis (k_3) for R = *i*-Pr, 5-alkyl substitution must significantly depress all component rates of the enzyme-catalyzed deacylation.

In summary, we have identified three tactics that improve HLE inhibition by the lead compound, 2-amino-

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(19) In separate experiments, the acyl enzymes for R = *i*-Pr, *n*-Bu, and *sec*-Bu (all R_5 = H) were prepared and isolated (Sephadex G-25) at low pH. Their deacylation was monitored by the reappearance of enzyme activity upon dilution into assay buffer containing fluorogenic substrate.[14] The rate constants obtained (0.0046, 0.0051, and 0.0088 s^{-1}, respectively) are in good agreement with those calculated as k_1K_i (Table I).
(20) The results shown are after exhaustive enzyme turnover of **2**. Preliminary experiments in which free enzyme is trapped after a single deacylation show that cyclization of **3** to regenerate **2** is a significant pathway for deacylation when R = *i*-Pr or *sec*-Bu, but minor (≤5%) when R = *n*-Bu.
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(22) The detailed effects of 5-substitution, i.e., the more rapid acylation and deacylation shown by R_5 = ethyl vs. R_5 = methyl, are under continuing enzymatic and model system investigation.

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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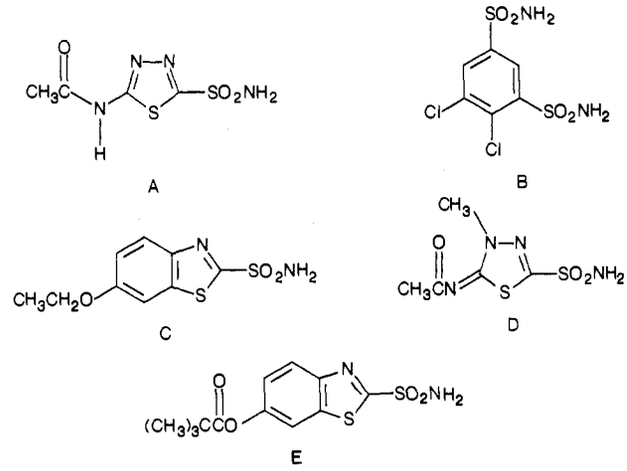
An attempt to develop a water-soluble carbonic anhydrase inhibitor focused on exploring structure–activity relationships in the thienothiopyransulfonamide class. The strategy to influence water solubility while retaining carbonic anhydrase activity involved the introduction of a hydroxyl moiety and adjusting 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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