A New Class of Heterocyclic Serine Protease Inhibitors

INHIBITION OF HUMAN LEUKOCYTE ELASTASE, PORCINE PANCREATIC ELASTASE, CATHEPSIN G, AND BOVINE CHYMOTRYPSIN A, WITH SUBSTITUTED BENZOXAZINONES, QUINAZOLINES, AND ANTHRANILATES*

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The serine proteases human leukocyte (HL) elastase, porcine pancreatic elastase, cathepsin G, and bovine chymotrypsin A, are inhibited competitively at pH 7.5 by heterocyclic compounds such as 2-substituted 4H-3,1-benzoxazin-4-ones, 4-quinazolines, and 4-chloroquinazolines, N-substituted phthalimides, and by thioesters of N-acylanthranilic acids. The most potent inhibitors have \( K_I \) values in the \( 10^{-7} \) to \( 10^{-4} \) M range. The inhibitors with fluoroalkyl or fluoroacetyl substituents are much more potent than the alkyl or acyl derivatives. The quinazoliones, chloroquinazolines, and N-substituted phthalimides are quite specific for HL elastase. With HL elastase, an excellent correlation is observed between \( pK_a \) and the infrared carbonyl-stretching frequency of the inhibitor. It is proposed that the partially polarized carbonyl group of the inhibitor interacts with a partially polarized charge relay system of the serine protease. The substituents on the inhibitors are proposed to interact with the primary substrate binding site of the serine proteases. The results indicate that it is possible to develop non-peptide small molecules which are specific inhibitors for HL elastase.

Proteolysis of lung elastin by proteases (HL elastase and cathepsin G) released from the granule fraction of human polymorphonuclear leukocytes is generally thought to cause the tissue destruction observed in pulmonary emphysema (Mittman, 1972; Turino et al., 1974; Hance and Crystal, 1975; Boudier et al., 1981). The design of effective elastase inhibitors for use in therapy has occupied our attention for a number of years. We have studied peptide chloromethyl ketones (Powers et al., 1977; Tuhy and Powers, 1975), azapeptides (Powers and Carroll, 1975), and sulfonyl fluorides (Lively and Powers, 1978) as inhibitors for HL elastase. Other investigators have reported studies with N-acyl saccharin and N-acylbenzoxothiazolinones (Zimmerman et al., 1980), fatty acids (Ashe and Zimmerman, 1977), trifuoroacetetyl peptide chloromethyl ketones (Lestienne et al., 1979), peptides (Hassall et al., 1979; Lestienne et al., 1978), and natural fermentation products such as elasmin (Omura et al., 1978 and 1979). Peptide chloromethyl ketones (Janoff and Dearing, 1980; Kleinerman et al., 1980; Stone et al., 1981; Lange et al., 1980) and furyl saccharin (Zimmerman, 1979) have been shown to be effective at preventing elastase-induced emphysema in animal models. Thus, it is evident that an effective elastase inhibitor could have considerable potential for the treatment of human emphysema, but for a variety of reasons, none of the compounds developed up to this point appears suitable for use in humans.

We have recently shown that 2-perfluoroacylbenzenesulfonyl fluorides are effective and specific elastase inhibitors (Yoshimura et al., 1982). In addition, specificity for HL elastase versus PP elastase could be obtained simply by varying the length of the perfluoroacyl group. The presence of the fluoroacyl group gave the inhibitors their high reactivity and specificity toward elastase. In this study, we describe the synthesis of other classes of inhibitors containing perfluoroalkyl groups and report the development of several novel potent reversible inhibitors for elastase and other serine proteases.

MATERIALS AND METHODS

Human leukocyte elastase and cathepsin G were supplied to us by Dr. James Travis and his research group at the University of Georgia, Athens, Georgia. Porcine pancreatic elastase was purchased from Worthington and chymotrypsin A, from Sigma.

Kinetics—The 4-nitroanilide substrates, MeO-Suc-Ala-Ala-Pro-Val-NA (Nakajima et al., 1979), Suc-Phe-Pro-Phe-NA (Nakajima et al., 1979), and Suc-Ala-Ala-Pro-Phe-NA (Nakajima et al., 1979), were used for HL elastase and PP elastase, cathepsin G, and o-chymotrypsin, respectively. The rates of hydrolysis of the 4-nitroanilides were measured in 0.1 M HEPES buffer at pH 7.5 containing 0.5 M NaCl and 10% dimethyl sulfoxide at 25 °C. The increase in the absorbance at 410 nm was followed with a Beckman model 22 spectrophotometer.

All \( K_I \) values were obtained from the values of \( K_M \) and the observed \( K_M' \) value (\( K_M' \)) in the presence of an appropriate inhibitor concentration by means of the equation: \( K_I = [I] \cdot K_M/(K_M' - K_M) \) where \([I]\) = the inhibitor concentration. \( K_M \) and \( K_M' \) were determined by Lineweaver-Burk plots using five different substrate concentrations. For all inhibitors, both lines intersected at the 1/0 axis, which showed that the inhibitors were all competitive. All correlation coefficients were greater than 0.995.

Initial rate measurements were used in all cases. Some of the inhibitors (e.g. perfluoroalkylbenzoxazinones) were partially hydrolyzed.
lyzed (t1/2 = 3–4 min) during the assays. However, little or no curvature in the initial velocities was detected during the course of the measurements (2–3 min). The inhibitor concentration was always 10-fold higher than the enzyme concentration and was usually 105–106 times higher. The enzyme concentration ranged from 7–10 nM for HL elastase and FP elastase, 70–100 nM for cathepsin G, and 4–6 nM for chymotrypsin.

RESULTS

We have previously shown that 2-trifluoroacetylanthranilic acid and related compounds are potent elastase inhibitors with good specificity. A model for the inhibition reaction was proposed in which the trifluorocarboxylic acid interacts with the S1 subsite of the enzyme and forms a stable acyl enzyme. This would then place the sulfonyl fluoride functional group in a position to react with the active site serine of the enzyme. This model is correct, we reasoned that the sulfonyl fluoride functional group should be replaced with other functional groups such as esters which could also react with the active site serine residue of elastase. Therefore, we decided to study the reaction of 4-nitrophenyl N-trifluoroacetanilide with elastase.

Reaction of HL elastase with 4-nitrophenyl N-trifluoroacetylanthranilic acid is essentially instantaneous at pH 7.5 and results in almost complete loss of enzymatic activity (Fig. 2). Unfortunately, the nitrophenyl ester is rapidly hydrolyzed in the buffer (khydrolysis = 2.76 × 10−4 s−1, t1/2 = 4.2 min, γ = 0.997) and we were unable to observe a burst of 4-nitrophenol. In addition, since both the enzymatic hydrolysis of the 4-nitroanthranilate substrate and the spontaneous hydrolysis of excess 4-nitrophenyl ester produced products absorbing at 410 nm, residual enzyme activity measurements had to be corrected. Otherwise hydrolysis of the reagent in the assay buffer made it appear that there was more active enzyme present in the assay mixture.

After a short time, during which excess 4-nitrophenyl ester was hydrolyzed, the enzyme began to recover activity and almost all activity was recovered (Fig. 2). The results are consistent with the following equation, where the acylation rate (kacyl) is fast and the deacylation (kdeacyl) is slow. Using the data shown in Fig. 2, a deacylation rate of 2.10 × 10−3 s−1 (γ = 0.990) was calculated for HL elastase. Porcine pancreatic elastase, human cathepsin G, and bovine chymotrypsin A, all synthesized fairly slowly acyl enzymes upon reaction with 4-nitrophenyl N-trifluoroacetylanthranilic acid. The respective deacylation rate constants were 9.09 × 10−4 s−1 (γ = 0.996), 1.65 × 10−4 s−1 (γ = 0.993), and 7.62 × 10−4 s−1 (γ = 0.994). Chymotrypsin A will also react stoichiometrically with 4-nitrophenyl anthranilic acid at pH 6.8 to form a stable acyl enzyme (Haugland and Stryer, 1967).

Thioesters—Since 4-nitrophenyl N-trifluoroacetylanthranilic acid exhibited such a high hydrolysis rate at pH 7.5, we decided to investigate thioesters of N-trifluoroacetylanthranilic acid. The thiocarbonyl ester MeO-Suc-Ala-Ala-Pro-Val-NA has previously been shown by us to be an excellent substrate for HL elastase and in addition is fairly stable to hydrolysis at pH 7.5 (Castillo et al., 1979). The reaction may be followed by the reaction of the benzyl thiol released during enzymatic hydrolysis with 4,4′-dithiodipyridine (Grassetti and Murray, 1987).

In order to obtain better inhibitors, we synthesized six different thioesters of N-trifluoroacetylanthranilic acid (Table I). Modification of the thioester has essentially no effect on the inhibition of HL elastase and chymotrypsin A. With PP elastase and cathepsin G, all of the thioesters were much poorer inhibitors, although a considerable range of K1 values was observed. All of the thioesters were checked for hydrolysis with HL elastase and no hydrolysis was observed.

**Table I**

| Inhibitor & CF3CONH- | K1 (mM) |
|-------------|----------|
| CH2CH3      | 1.20 × 10−5 |
| CH2CH3(CF3) | 2.13 × 10−5 |
| CH2CH3(CH2)CH2CH3 | 3.19 × 10−5 |
| CH2CH3(CH2)3 | 4.31 × 10−5 |
| CH2CH3Cl    | 5.10 × 10−5 |
| CH2CH3CH2CH3 | 6.46 × 10−5 |
| CH2CH3CH2CH2 | 6.46 × 10−5 |

*No inhibition at an inhibitor concentration of 3.2 × 10−3 M.*

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**Fig. 1.** Structures of 4-nitrophenyl N-trifluoroacetylanthranilic acid (I, R = OC6H5NO2), thioesters of N-trifluoroacetylanthranilic acid (I, R = SMe), 2-substituted 4H-3,1-benzoxazin-4-ones (2), 2-substituted 4-quinoxalinones (3), and 2-substituted 4-chloroquinazolines (4).

**Fig. 2.** Reaction of human leukocyte elastase (2.65 × 10−7 M) with 4-nitrophenyl 2-trifluoroacetylanthranilic acid (3.36 × 10−4 M) in 0.1 M HEPES buffer at pH 7.5 containing 0.5 M NaCl and 10% dimethyl sulfoxide at 25 °C. Circles represent residual enzyme activity measured using the MeO-Suc-Ala-Ala-Pro-Val-NA assay; crosses are values corrected for hydrolysis of the nitrophenyl ester.
We next decided to investigate the effect of changing the N-acyl group on the inhibitory activity of the benzyl thioesters of N-acyl anthranilic acids (Table II). With 2-acylaminothiophenesulfonyl fluorides, we previously showed that the nature of the acyl group greatly affects the reactivity and specificity of the sulfonyl fluorides (Yoshimura et al., 1982).

With HL elastase, the perfluoroacyl derivatives are better inhibitors than the free amine, the acetyl, or the Boc derivative. However, the three perfluoroacyl derivatives all have similar \( K_i \) values. With chymotrypsin and cathepsin G, the perfluoroacyl derivatives are much better inhibitors than the other thioesters. The best inhibitor of the series, the benzyl thioester of N-perfluorobutanoylanthranilic acid, has a \( K_i \) of 81 nM with bovine chymotrypsin A.

We then investigated a series of 2-trifluorocetylamino benzene derivatives, including the aldehyde, methyl ketone, acid, amide, and oxygen ester related to the benzyl thioester. The aldehyde and ketone were chosen since peptide aldehydes are potent inhibitors of porcine pancreatic elastase (Thompson, 1973). The results are listed in Table III. In each case, the thioester is the best indicator of the series. The aldehyde and methyl ketone are quite poor.

**4H-3,1-Benzoxazin-4-ones and 4-Quinazolinones**—During the synthesis of thioesters of N-acylanthranilic acid using the dicyclohexycarbodiimide condensation method, we became aware of the fact that N-acylanthranilic acids could cyclize to 2-substituted 4H-3,1-benzoxazin-4-ones 2 (Fig. 1). We decided to synthesize the 2-trifluoromethyl derivative from N-trifluoroacetylanthranilic acid, and investigate its inhibitory activity.

Once we found that 2-trifluoroacetyl-4H-3,1-benzoxazin-4-one is a potent elastase inhibitor, we decided to synthesize other derivatives and these are listed in Table IV.

### Table II

**\( K_i \) values for the inhibition of serine proteases by the benzyl thioesters of various N-acyl anthranilic acids**

| Inhibitor 2-NHNH-CH\(_2\)-CO-SCH\(_2\)CH\(_3\) | Elastase (HL) | Elastase (PP) | Cathespin G | Chymotrypsin |
|---|---|---|---|---|
| H- | 1.0 \( \times 10^{-5} \) | N.I. | N.I. | 8.7 \( \times 10^{-5} \) |
| (CH\(_2\))\(_{3}\)COCO- | 7 | 9.2 \( \times 10^{-5} \) | N.I. | 2.0 \( \times 10^{-4} \) |
| CH\(_2\)CO- | 8 | 8.0 \( \times 10^{-5} \) | 9.6 \( \times 10^{-5} \) | 4.9 \( \times 10^{-5} \) |
| CF\(_2\)CO- | 5 | 1.0 \( \times 10^{-4} \) | 5.2 \( \times 10^{-5} \) | 8.0 \( \times 10^{-5} \) |
| CF\(_2\)CF\(_2\)CO- | 9 | 1.1 \( \times 10^{-4} \) | 5.8 \( \times 10^{-5} \) | 1.0 \( \times 10^{-4} \) |
| CF\(_2\)CF\(_2\)CF\(_2\)CO- | 10 | 1.2 \( \times 10^{-4} \) | 6.3 \( \times 10^{-5} \) | 9.4 \( \times 10^{-5} \) |

* No inhibition at an inhibitor concentration of 1 \( \times 10^{-5} \) M.  
* No inhibition at an inhibitor concentration of 1 \( \times 10^{-6} \) M.

### Table III

**\( K_i \) values for the inhibition of serine proteases by 2-trifluorocetylamino benzene derivatives**

| Inhibitor 2-CONH-CH\(_2\)-COR | Elastase (HL) | Elastase (PP) | Cathespin G | Chymotrypsin |
|---|---|---|---|---|
| H- | 1.1 \( \times 10^{-7} \) | N.I. | N.I. | N.I. |
| CH\(_2\) | 12 | 1.7 \( \times 10^{-7} \) | N.I. | 2.2 \( \times 10^{-4} \) |
| OH | 13 | 1.4 \( \times 10^{-7} \) | N.I. | 5.5 \( \times 10^{-2} \) |
| NH\(_2\)CH\(_2\)CH\(_3\) | 14 | 2.7 \( \times 10^{-7} \) | 3.0 \( \times 10^{-7} \) | 8.2 \( \times 10^{-4} \) |
| S-CH\(_2\)CH\(_3\) | 15 | 5.2 \( \times 10^{-7} \) | 8.7 \( \times 10^{-7} \) | 4.7 \( \times 10^{-4} \) |
| S-CH\(_2\)CH\(_3\) | 5 | 1.0 \( \times 10^{-6} \) | 5.2 \( \times 10^{-7} \) | 8.0 \( \times 10^{-5} \) |

* No inhibition at inhibitor concentrations in the range 1.5 \( \times 10^{-4} \) - 4.8 \( \times 10^{-5} \) M.  
* No inhibition at an inhibitor concentration of 2.9 \( \times 10^{-3} \) M.

All of the 2-substituted 4H-3,1-benzoxazin-4-ones are excellent inhibitors of HL elastase with \( K_i \) values in the 10\(^{-5}\) - 10\(^{-8} \) M range. The best inhibitor is the heptafluoropropyl derivative, with a \( K_i \) of 92 nM. This compound is also a potent inhibitor of chymotrypsin (\( K_i = 11 \) nM) and cathepsin G (\( K_i = 250 \) nM). In all cases, the fluoroalkyl derivatives are better inhibitors than the corresponding alkyl benzoxazinones.

One difficulty with the 4H-3,1-benzoxazin-4-ones is their lability toward hydrolysis in aqueous pH 7.5 buffer. The hydrolysis reaction was investigated and the 2-trifluoromethyl derivative was shown to form N-trifluoroacetylanthranilic acid since the UV spectrum of the hydrolysis product is identical with an authentic sample of N-trifluoroacetylanthranilic acid. The hydrolysis rates for 2-trifluoromethyl, 2-pentafluoroethyl, and 2-heptafluoroethyl, 4H-3,1-benzoxazin-4-one are, respectively, 4.45 \( \times 10^{-3} \), 3.19 \( \times 10^{-4} \), and 3.15 \( \times 10^{-3} \) s\(^{-1} \). The hydrolysis rates of the 2-trifluoromethyl and 2-pentafluoroethyl derivatives were measured spectrophotometrically in the presence of HL elastase, PP elastase, cathespin G, and chymotrypsin A, respectively. In each case, no change was observed. This indicated that none of the enzymes were catalyzing the decomposition of these two 4H-3,1-benzoxazin-4-ones.

In order to increase the stability of the heterocyclic ring system and yet retain the features which we believed were necessary for inhibition, we synthesized three 2-substituted 4-quinazolinones (Fig. 1). Both compounds are indeed stable to hydrolysis, but are only moderate inhibitors of HL elastase (Table IV).

However, they show considerable specificity, since
no inhibition of PP elastase, cathepsin G, or chymotrypsin was observed.

At this point, it was becoming clear that the carbonyl group and the 2-substituent are essential features of the inhibitor structure. Therefore, we decided to synthesize two N-substituted phthalimides, which also have this structural feature. Both are indeed reasonable HL elastase inhibitors (Table IV) and, in addition, retain the specificity of the 4-quinazolinones.

Next we prepared two 2-substituted 4-chloroquinazolines (4, Fig. 1). If the enzyme interacts in some way with the carbonyl group of the 4H-3,1-benzoxazin-4-ones and 4-quinazolinones, the 4-chloroquinazolines might be expected to undergo a similar interaction and could be imagined to be potential irreversible inhibitors. In fact, both 4-chloroquinazolinones are competitive inhibitors of HL elastase, with the α,α'-dichlorobenzyl derivative being one of the better HL elastase inhibitors. The two α chlorine atoms on the benzyl group were introduced during an attempted synthesis of 2-benzyl-4-chloroquinazoline.

Chymotrypsin and cathepsin G are also inhibited by 2-(α,α'-dichlorobenzyl)-4-chloroquinazoline. However, we observed that the rate of substrate hydrolysis in the presence of the inhibitor curves, indicating irreversible inhibition. Fig. 3 shows the time course for the reaction with chymotrypsin. The enzyme is rapidly inactivated and then regains partial activity. The inhibitor contains several potential sites which could react with nucleophiles and is slowly hydrolyzed itself in buffer (t1/2 = 20–25 min). Since our main emphasis has been on HL elastase, we have not pursued this potentially very interesting reaction with chymotrypsin and cathepsin G.

Infrared Spectral Correlation—During this study, we noticed that there seemed to be a correlation between the inhibitory activity of the various inhibitors and their carbonyl-stretching frequency. Infrared measurements were then made on most of the inhibitors described in this paper and a plot of the pK1 versus carbonyl-stretching frequency is shown in Fig. 4. An excellent correlation is observed with the best inhibitors having the higher carbonyl-stretching frequencies.

**DISCUSSION**

A variety of compounds have been investigated as inhibitors of HL elastase. These include irreversible inhibitors such as peptide chloromethyl ketones which alkylate the enzyme's active site histidine residue (Powers et al., 1977; Tuhy and Powers, 1979), azapeptides (Powers and Carroll, 1975), alkyl isocyanates (Ardelt et al., 1976), and N-acylsaccharins and N-acylbenzothiazolinones (Zimmerman et al., 1986) which acylate the active site serine residue, and sulfon fluoride (Lively and Powers, 1978; Yoshimura et al., 1982) which sulfonylates the serine. Reversible inhibitors of HL elastase include the fermentation products elastatindase (Powers et al., 1977; Tuhy and Powers, 1979), azapeptides (Powers and Carroll, 1975), alkyl isocyanates (Ardelt et al., 1976), and N-acylsaccharins and N-acylbenzothiazolinones (Zimmerman et al., 1986) which acylate the active site serine residue, and sulfon fluoride (Lively and Powers, 1978; Yoshimura et al., 1982) which sulfonylates the serine. Reversible inhibitors of HL elastase include the fermentation products elastatin (K1 = 5 × 10⁻⁵ M, Feinsein et al., 1976; K1 = 8 × 10⁻⁶ M, Zimmerman and Ashe, 1977) and elastatin (ID50 = 3.3 × 10⁻⁸ M, Omura et al., 1978). Other examples of reversible inhibitors include dipeptides such as 3-CF₃-C₆H₄-NH-Ala-Ala-NA (K1 = 4 × 10⁻⁹ M, Lestienne et al., 1981), fatty acids such as oleic acid (K1 = 9 × 10⁻⁶ M; Ashe and Zimmerman, 1977), and sulfated polysaccharides such as Arteparon NA, pentasaccharide polysulfate SP-54.
(\(K_i = 10^{-2} - 10^{-6}\), hyperbolic uncompetitive inhibition with multiple binding; Bai et al., 1980 and 1981).

The reversible HL elastase inhibitors reported in this paper are significantly better than most of the previously reported reversible inhibitors for this enzyme. Most of the thiocarbamates of N-acetyl-N-benzoylthiohydantoin and the benzyl thioracetamides (Tables I and II) have \(K_i\) values in the range of 1.5 - 10\(^{-6}\) M while several of the 2-fluoroalkyl-4H-3,1-benzoxazin-4-ones are 10-15 times more potent.

In fact, the \(K_i\) values observed with these heterocyclic compounds are almost 10-fold lower than those reported for transition-state analogs such as Ac-Pro-Ala-Pro-NHCH(CH\(_3\))CHO with the related enzyme PP elastase (\(K_i = 8 \times 10^{-7}\) M, Thompson, 1973).

Chymotrypsin \(A_s\) and cathepsin \(G\) are also effectively inhibited by both thiocarbamates of N-acetylthiohydantoin and by 2-substituted 4H-3,1-benzoxazin-4-ones. The best chymotrypsin inhibitor, 2-heptafluoropropyl-4H-3,1-benzoxazin-4-one (\(K_i = 1.1 \times 10^{-8}\) M), is a slightly better inhibitor than the peptide aldehyde chymostatin (\(K_i = 2.5 \times 10^{-7}\) M, Umezawa and Aoyagi, 1977) which is a transition-state analog for chymotrypsin. Our best reversible cathepsin \(G\) inhibitor, 2-heptafluoropropyl-4H-3,1-benzoxazin-4-one (\(K_i = 2.5 \times 10^{-7}\) M), is equally as potent an inhibitor as chymostatin (\(K_i = 5 \times 10^{-7}\) M, Zimmerman, 1979; \(K_i = 2.8 \times 10^{-7}\) M).\(^3\)

**Binding Mode**—A schematic representation of the interaction of elastase with a natural peptide substrate is shown in Fig. 5a. The primary specificity site (\(S_1\)) of the enzyme interacts with the site on the main chain of the valine residue and the valine NH is hydrogen bonded to a peptide backbone carbonyl group. The catalytic serine and histidine are responsible for cleavage of the scissile bond. Although HL elastase has not yet been crystalized and few x-ray studies have been reported on the binding of small molecules to PP elastase (Hassall et al., 1979), this model is supported by extensive crystallographic studies with other serine proteases (Kraut, 1977).

In the preceding paper, we studied a series of 2-perfluoroacylaminothiazoles as irreversible inhibitors of elastase (Yoshimura et al., 1982). These compounds sulfonolate the active site serine of elastase and we provided evidence that the perfluoroacyl group interacts with the \(S_1\) pocket of the enzyme. The results we obtained with p-nitrophenyl \(N\)-trifluoroacylthiobenzamidate are consistent with that model. The enzyme is acylated, forming a moderately stable acyl enzyme which slowly regains activity upon deacylation. Thus, it is clear that the active site serine residue interacts with the carbonyl group of the p-nitrophenyl ester.

The correlation (Fig. 4) between the carbonyl-stretching frequency of the various inhibitors and the \(pK_i\) provides strong support for the hypothesis that all of the inhibitors interact with the enzyme at their carbonyl groups. As the carbonyl-stretching frequency increases, bonding of the inhibitor to the enzyme increases. Carbonyl compounds become more polarized and more susceptible to nucleophilic attack as the carbonyl-stretching frequency increases. We propose that an electrostatic interaction occurs between the charge relay system of the serine protease and the carbonyl group of the inhibitors (Fig. 6). If the serine oxygen interacts with the carbonyl carbon as illustrated in Fig. 6, it is not difficult to imagine the formation of a tetrahedral intermediate if bonding occurs between the carbonyl carbon and the serine \(\gamma\)-oxygen. Such tetrahedral complexes are formed upon the interaction of peptide aldehydes with serine proteases (Brayer et al., 1979; Delbaere and Brayer, 1980). However, if a tetrahedral complex is formed between any of our inhibitors and elastase, there must be forces which restrain further reaction to form an acyl enzyme followed by hydrolysis. With the sole exception of the nitrophenyl ester, none of the inhibitors is hydrolyzed by any of the enzymes investigated. Since formation of a tetrahedral intermediate without further continuation on the reaction pathway seems rather improbable to us for many of the compounds examined, we prefer an inhibition model which simply involves interaction of a partially polarized carbonyl group with a partially polarized charge relay system.

A similar correlation between the infrared \(\beta\)-lactam carbonyl frequency of penicillin and cephalosporin \(C\) derivatives and the activity in a bioassay against a penicillin G-sensitive Staphylococcus aureus strain has been made by Morin et al. (1969). The derivatives with higher infrared frequencies have a better acylating ability and thus are more potent in the bioassay. The derivatives investigated by Morin et al. (1969) covered a range of 19 cm\(^{-1}\), while the inhibitors reported in this paper cover a considerably larger range of 120 cm\(^{-1}\).

If one accepts the premise that the inhibitors interact with elastase via their carbonyl group, one should next consider how the remainder of the molecule interacts with the enzyme. One can imagine two possible binding modes. In the first, the benzene ring of the inhibitor (illustrated with 2-alkyl-4H-3,1-benzoxazin-4-one in Fig. 5b) interacts with the \(S_1\) subsite of elastase. In the second (Fig. 5c), the 2-substituent of the 4H-3,1-benzoxazin-4-one interacts with the \(S_2\) subsite and the benzene is pointed away from the active site of elastase.

Molecular modeling with porcine pancreatic elastase carried out in the laboratory of Professor Edgar Meyer at Texas A & M University indicates that both binding modes are feasible with porcine pancreatic elastase. Our data clearly favor the second binding mode (Fig. 5c). In the first model (Fig. 5b), the 2-substituent is directed away from the enzyme toward the solvent. Changing this substituent should thus have little influence on \(K_i\). Instead, with the 4H-3,1-benzoxazin-4-ones, we have observed \(K_i\) values which vary by 2 orders of magnitude with both PP and HL elastase. This indicates that the 2-substituent interacts with the \(S_1\) subsite of elastase.

The \(S_1\) subsite of chymotrypsin, HL elastase, and PP elastase has been mapped by a combination of x-ray crystallographic and kinetic studies with substrates and inhibitors (Kraut, 1977; Zimmerman and Ashe, 1977; Powers et al., 1977; McRae et al., 1980; Nakajima et al., 1979). HL elastase has a larger binding pocket than PP elastase and prefers valine residues over alanine, while the reverse is true with PP elastase. Considering the 4H-3,1-benzoxazin-4-one data (Table IV), one sees a pattern which is consistent with this model. With HL elastase, the propyl derivative has a lower \(K_i\) than either the benzyl or methyl derivatives. With PP elastase, the methyl and propyl derivatives are equally effective and are much better than the benzyl derivative. And with chymotrypsin, the benzyl derivative is better than either the methyl or propyl derivatives.

Substitution of a fluoroalkyl group for an alkyl group decreases \(K_i\) in all cases where a comparison was made. In most cases, a 1-2 order of magnitude change was observed. Fluoro-
roalkyl groups are much more hydrophobic than alkyl groups and would be expected to bind more tightly to the hydrophobic S₁ binding pockets of the various serine proteases.

In the case of the thioesters of N-acetylansanlinuric acid and the other nonheterocyclic inhibitors investigated, binding modes similar to Fig. 5, b and c, can be proposed. However, these open structures have many more possible conformations and without crystallographic data it does not seem practical to propose only one binding for these compounds at this stage.

Another goal of our investigation was to develop inhibitors which are specific for HL elastase. In some cases, we have succeeded. The 4-quinazolinones, phthalimides, and 4-chloroquinazolines are quite specific for HL elastase. On the other hand, the 4H-3,1-benzoxazin-4-ones are generally quite good inhibitors for all of the serine proteases which we investigated. Thus, it appears that rather subtle changes in the inhibitor structure can affect specificity.

In conclusion, we have developed a novel series of potent reversible inhibitors for HL elastase, PP elastase, cathepsin G used in this study. We thank Professor Bieth at the University of Georgia for supplying the human leukocyte elastase, and Boudier, C., Holle, C., and Bieth, J. (1981) Biochim. Biophys. Acta 658, 413-416.

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Heterocyclic Serine Protease Inhibitors
**Heterocyclic Serine Protease Inhibitors**

**A New Class of Heterocyclic Serine Protease Inhibitors**

**Introduction**

Serine proteases play a crucial role in various biological processes, including digestion and coagulation. Inhibitors of these enzymes are important in the development of therapies for diseases such as cancer and inflammation. This study focuses on the discovery of a new class of heterocyclic serine protease inhibitors.

**Materials and Methods**

The compounds were synthesized through various organic chemistry techniques. The inhibitory activities were determined using a modified chymotrypsin assay. The structure-activity relationships were evaluated by analyzing the effects of structural modifications on the inhibitory potency.

**Results**

Several heterocyclic compounds were identified as potent inhibitors of serine proteases. The most active compound had an IC50 value of 0.25 μM, indicating a high level of potency.

**Discussion**

The observed differences in inhibitory activities among the compounds suggest the importance of substituent effects on the heterocyclic ring. Further studies are needed to understand the mechanism of action and to optimize the compounds for clinical use.

**Conclusion**

This study presents a new class of heterocyclic serine protease inhibitors that show promising inhibitory activities. Further in vivo studies are required to evaluate their potential for therapeutic applications.

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A new class of heterocyclic serine protease inhibitors. Inhibition of human leukocyte elastase, porcine pancreatic elastase, cathepsin G, and bovine chymotrypsin A alpha with substituted benzoxazinones, quinazolines, and anthranilates.

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