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N-(CARBOBENZYLOXY)ISATIN: A SLOW BINDING α-KETO LACTAM INHIBITOR OF α-CHYMOTRYPSIN

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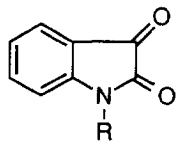
Abstract The N-carbobenzyloxy derivative of 2,3-dioxindole (N-Cbz-isatin) (**1**) has been synthesized and shown to be a slow binding inhibitor of chymotrypsin. Compound **1** does not inhibit porcine pancreatic elastase. The N-*tert*-butoxycarbonyl derivative of 2,3-dioxindole (**2**) is a weak inhibitor of chymotrypsin and does not inhibit porcine pancreatic elastase.

Serine proteases have been implicated in a number of pathological conditions, including emphysema, rheumatoid arthritis, tumor metastasis, and neurodegenerative diseases.[1-3] The apparent involvement of serine proteases in the uncontrolled proteolysis associated with these and other disease states has stimulated the investment of considerable effort in the design of synthetic inhibitors and in their evaluation as potential therapeutic agents.[3-5] Much of the search for inhibitors has focused on heterocycles that undergo nucleophilic attack on a carbonyl group by the active site serine hydroxyl to produce an acylated enzyme. The acylation, although reversible, can result in an enzyme-inhibitor complex that is sufficiently stable to afford substantial inhibition. Among the types of heterocycles that have been investigated as acylating inhibitors of serine proteases are benzoxazinones, pyrones, saccharins, and related agents.[4-7]

As part of an investigation of structurally modified benzolactams as serine protease inhibitors, compounds **1** and **2** were prepared and evaluated for their effects on α-chymotrypsin and porcine pancreatic elastase. For the preparation of **1**, 2,3-dioxindole (isatin) (2.0 mmol) was suspended and stirred in CH_3CN (4 mL) at 0 °C. Benzyl chloroformate (2.2 mmol) was added, followed by dimethylaminopyridine (DMAP) (0.2 mmol) and triethylamine (2.2 mmol). Recrystallization (ethyl acetate-hexane) of the product obtained on work-up of the reaction mixture afforded **1** (82%, mp 155-156 °C; lit.[8] mp 154-156 °C). For the synthesis of **2**, isatin (10 mmol) was suspended in CH_3CN (20 mL) to which di-*tert*-butyl dicarbonate (12 mmol) and DMAP (1 mmol) were added, and the mixture was stirred at room temperature for 3 h. Recrystallization (ethyl acetate-hexane) of the crude product afforded **2** (94%, mp 113-114 °C). The IR, NMR and elemental analytical data were consistent with structures **1** and **2**.



R = $COOCH_2Ph$ **1**
$COOC(CH_3)_3$ **2**

Incubation of either **1** or **2** with α-chymotrypsin resulted in inhibition of substrate hydrolysis, but neither compound inhibited porcine pancreatic elastase. The inhibition of α-chymotrypsin by **2** was competitive (K_i > 2mM), as determined from a Dixon plot. In contrast, the progress curves for α-chymotrypsin-catalyzed hydrolysis in the presence of various concentrations of **1** showed that **1** is a slow binding inhibitor (Fig. 1). The progress curves for slow binding inhibitor **1** are described by the integrated equation (1) developed by Cha.[9]

$$P = v_s t - (v_s - v_o)\,[1 - \exp(-k_{obs}t)] / k_{obs} \qquad (1)$$

In equation (1), P is the product concentration, v_s is the steady state velocity, v_o is the initial velocity, t is the time, and k_{obs} is the apparent first order rate constant. The k_{obs} value is related to k_{on} and k_{off} by equation (2).

$$k_{obs} = k_{on}\,[1 / (1 + S/K_m)]\,I + k_{off} \qquad (2)$$

Thus, a plot of k_{obs} vs [I] (Fig. 2) gives the value for k_{off} as the y-intercept, and k_{on} can be calculated from the slope. The K_i is k_{off}/k_{on}. For compound **1**, k_{on}, k_{off} and K_i were $1.52 \pm 0.3 \times 10^4$ $M^{-1}min^{-1}$, $5.69 \pm 1.8 \times 10^{-2}$ min^{-1} and 3.8 μM, respectively. Compound **1** also exhibited time-dependent inhibition of α-chymotrypsin (data not shown), and its inhibitory effect was of long duration. The enzyme regained approximately 50% of its activity within three hours after treatment with a sufficient concentration of **1** to cause 85% inhibition. The k_{on} and k_{off} values that were determined from the time dependent inhibition and reactivation data were 4.57×10^2 $M^{-1}min^{-1}$ and 2.4×10^{-3} min^{-1}, respectively. The latter values, which are more consistent with the observed reactivation time than the lower values obtained from the progress curves, may indicate that a steady state velocity was not achieved during the progress curve experiments.

The N-*tert*-butoxycarbonyl analogue **2** was a much less potent inhibitor of α-chymotrypsin than **1**. The superior potency of **1** may be due to the ability of the aromatic ring of the N-substituent to interact with the S_1 binding site of α-chymotrypsin, which preferentially binds substrates and inhibitors that contain aromatic residues in the P_1 position.[5] Similarly, the inability of either **1** or **2** to inhibit porcine

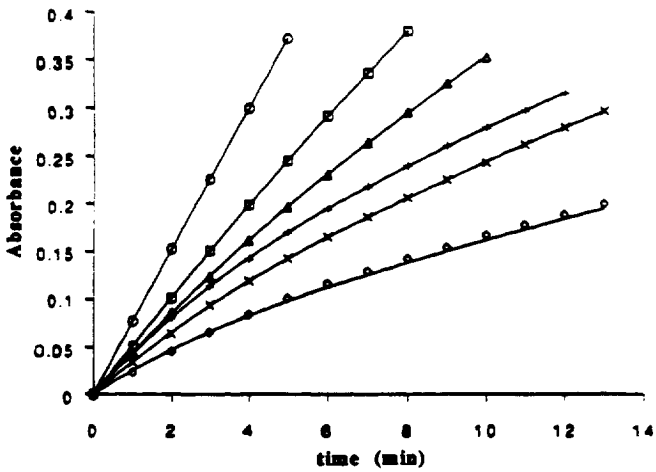


Fig 1. Inhibition of α-chymotrypsin by **1**. Progress curves of substrate hydrolysis by α-chymotrypsin in the presence of various concentrations of **1**. Compound **1** (O, 0 μM; □, 20 μM; Δ, 40 μM; +, 60 μM; x, 80 μM; ◊, 100 μM) was dissolved in 0.1 M sodium phosphate buffer, pH 7.5. The substrate, Succ-Ala_2-Pro-Phe-pNA (300 μM), was added, followed by α-chymotrypsin (5 nM). The release of *p*-nitroaniline was monitored at 410 nm for 15 min.

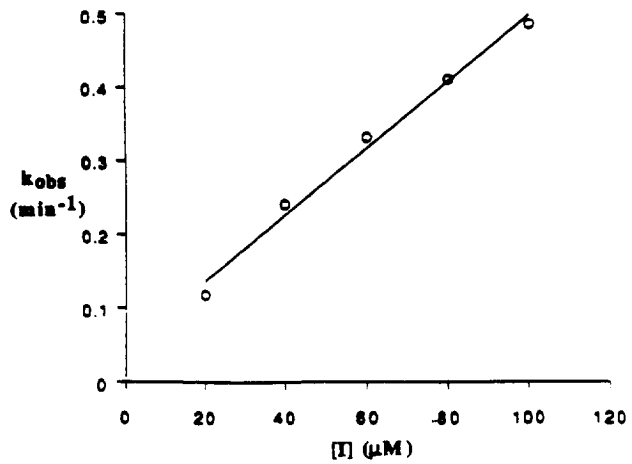


Fig 2. Plot of k_{obs} vs [I] (see text for explanation).

pancreatic elastase is consistent with that enzyme's selectivity for compounds with small aliphatic residues in the P_1 position.[5]

Although α-keto amides are well documented as inhibitors of proteases, compound **1** appears to be the first such benzolactam reported to exhibit either serine protease or cysteine protease inhibitory activity.[10] The mechanism by which **1** inhibits α-chymotrypsin has not been determined. Although the formation of a stable acyl enzyme complex is a possibility, the formation of a tetrahedral "transition state" adduct between one of the carbonyl groups of **1** and the active site serine hydroxyl cannot be excluded at present. It is unlikely that the inhibitory effect of **1** is due to acylation of the active site serine hydroxyl by the N-carbobenzyloxy group to form a benzylcarbonate adduct because addition of hydroxylamine to the inhibited enzyme preparation does not increase the rate of recovery of activity and because structure-activity studies show that the 3-keto group of isatin is essential for the activity of **1** (unpublished data). The low k_{off} value of **1** is consistent with the reversible, slow binding inhibition often exhibited by transition state inhibitors.[11] Regardless of the molecular mechanism responsible for the inhibitory action of **1**, it can be expected that suitable structural modifications of isatin may result in potentially useful protease-specific inhibitors.

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