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The interaction of alkynyl carboxylates with serine enzymes

A potent new class of serine enzyme inhibitors

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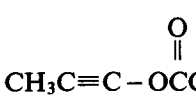
The recently reported alkynyl esters, propynyl benzoate and propynyl *p*-methoxybenzoate, were found to interact with a variety of serine enzymes. α-Chymotrypsin was inhibited very rapidly by an equivalent amount of the esters. Trypsin, elastase and pronase were also inhibited by the esters. On the other hand, liver esterase started to hydrolyze the alkynyl esters rapidly, but the enzyme became inhibited during the course of reaction. The inhibited enzymes exhibited slow reactivation which could be considerably enhanced by hydroxylamine.

Alkynyl carboxylate; Serine enzyme; Covalent inhibitor

1. INTRODUCTION

The considerable research interest in protease and esterase inhibition prompted us to study the interaction of the recently synthesized alkynyl carboxylates [1,2] with a variety of enzymes. Here we report the results of the reaction of propynyl benzoate (**I**) and propynyl *p*-methoxybenzoate (**II**), members of highly reactive alkynyl esters [3], with (bovine) α-chymotrypsin, a prototypical serine enzyme, as well as other serine enzymes from mammalian and microorganism origin.



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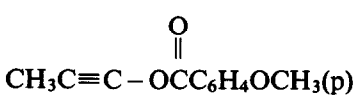

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The alkynyl esters inhibited chymotrypsin with a rate constant much greater than that observed with other powerful inhibitors. The esters also effectively inhibited trypsin and pronase as well as thrombin and plasmin, but they served as substrates for subtilisin and proteinase K. Incubation of the alkynyl esters with (pig liver) esterase resulted in fast turnover which decayed during the reaction due to inhibition of the enzyme. The inhibited enzymes underwent slow spontaneous reactivation, the rate of which was markedly increased by hydroxylamine. These novel alkynyl carboxylates represent a potent new class of inhibitors for a broad range of serine enzymes.

2. MATERIALS AND METHODS

Enzymes were purchased from Sigma and Worthington. Acetyltyrosine ethyl ester (ATEE), benzoyl arginine ethyl ester (BAEE), ethyl butyrate, propyl benzoate, carbobenzoxy-L-alanine *p*-nitrophenyl ester (Z-Ala-pNA) and proflavine hydrochloride were the products of Sigma. Propynyl benzoate and propynyl *p*-methoxybenzoate were prepared according to Stang et al. [1,2].

2.1. *Inhibition of enzymes*

A stock solution (10 mM) of alkynyl ester in acetonitrile was prepared and a measured amount of the solution was added to

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an enzyme solution (1–5 mg/ml) at pH 7–8, 25°C. The final reaction mixture contained 5% (v/v) acetonitrile and 0.1 N NaCl. 2 min after addition of the ester, 10–50 μl of the reaction mixture was removed and added to a substrate solution (1–5 mM, containing 0.1 N NaCl), and the enzymic activity was generally followed in the pH-stat at pH 8, 25°C. Hydrolysis of *p*-nitrophenyl ester substrate (0.1 mM) was followed spectrophotometrically at 400 nm. From the course of the alkali uptake in the pH-stat, or from the absorbance change in the spectrophotometer, the hydrolysis rate of the enzymic reaction was calculated.

The activities of chymotrypsin, subtilisin and proteinase K were determined with ATEE as a substrate, and those of trypsin, pronase, thrombin and plasmin were estimated with BAEE. The activity of elastase was assayed with Z-Ala-pNP and that of esterase with ethyl butyrate.

The interaction of chymotrypsin with the propynyl esters was also followed spectrophotometrically. Aliquots of the ester dissolved in acetonitrile were added to the enzyme solution at pH 7 and the absorbance at 270 or 250 nm was recorded. The absorbance change was instantaneous and in order to estimate the rate constant of the reaction between the propynyl ester and chymotrypsin we used the method of competitive covalent inhibition of the enzyme in the presence of a substrate [4].

3. RESULTS AND DISCUSSION

3.1. *Inhibition of chymotrypsin with propynyl esters*

When propynyl benzoate (**I**) or propynyl *p*-methoxybenzoate (**II**) was added to an α-chymotrypsin solution, the activity of the latter decreased proportionally to the amount of ester added. The quantity of ester required to completely inhibit the enzymic activity was equal to the active site concentration which was also determined by other titration methods [5,6]. However, the inhibited enzyme slowly regained activity according to a first order reaction. The enzyme inhibited with **I**, at pH 8, underwent reactivation with $t_{0.5}$ = 25 min, and that inhibited with **II** regained activity with $t_{0.5}$ = 130 min. Hydroxylamine considerably enhanced the reactivation rate. Thus, chymotrypsin inhibited with **I** was reactivated at 0.5 M hydroxylamine with $t_{0.5}$ = 1.5 min. Reactivation was also followed spectrophotometrically by the proflavine displacement method [7] which gave the same result as that obtained using enzyme activity measurements.

These results are interpreted by the two-step mechanism of hydrolysis catalyzed by serine enzymes. In the first step (acylation) chymotrypsin (CT-OH) and alkynyl benzoate react rapidly forming a benzoyl chymotrypsin intermediate (eqn 1) which is devoid of enzymic activity. This is followed by a slow deacylation step in which the enzyme is regenerated (eqn 2).

$$\text{CT-OH} + \text{CH}_3\text{C}{\equiv}\text{COBz} \longrightarrow \text{CT-OBz} + \text{CH}_3\text{C}{\equiv}\text{COH} \quad (1)$$

$$\text{CT-OBz} + \text{CH}_3\text{C}{\equiv}\text{COH} \xrightarrow{2\text{H}_2\text{O}} \text{CT-OH} + \text{BzO}^- + \text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}^+ \quad (2)$$

The reactivation rate constants found by us concur with those values reported in the literature for deacylation of benzoyl- and *p*-methoxybenzoyl-chymotrypsin [8–10]. As deacylation in the above cases is a slow rate-determining step, the esters studied actually inhibit chymotrypsin.

The acylation step could be detected spectrophotometrically since the sum of the absorbances of the enzyme and alkynyl benzoate is larger than that of the products formed by their interaction. When **I** or **II** was added to an enzyme solution there was a sizable instantaneous change in absorbance at 250 and 270 nm, respectively. When subequivalent amounts of **I** or **II** were added to chymotrypsin solution, there was a linear increase in absorbance at 250 or 270 nm up to a point where additional aliquots of the ester caused a change in the slope of the line (fig.1). The break in the line in fig.1 occurs at an ester concentration equivalent to that of the enzyme active site. Thus, alkynyl benzoates can be used for convenient titration of the active site of chymotrypsin.

The rate of the formation of benzoyl-chymotrypsin was too fast to follow by conventional methods. In order to estimate the rate constant of the reaction we added compound **II** to a solution of chymotrypsin and ATEE. The reaction rate steadily declined until it was completely inhibited due to the formation of inactive *p*-methoxybenzoyl-chymotrypsin. By analyzing the kinetics of the enzyme catalyzed hydrolysis of ATEE in the absence and presence of **II** we found that the second order specificity constant k_i/K_I for acylation is 900000 $M^{-1} \cdot min^{-1}$. (K_I is the dissociation equilibrium constant between chymotrypsin and **II**, and k_i is the first order rate constant for acylation of the enzyme in the enzyme-inhibitor complex [4].) This constant is 20–50-fold greater than the values obtained with conventional power-

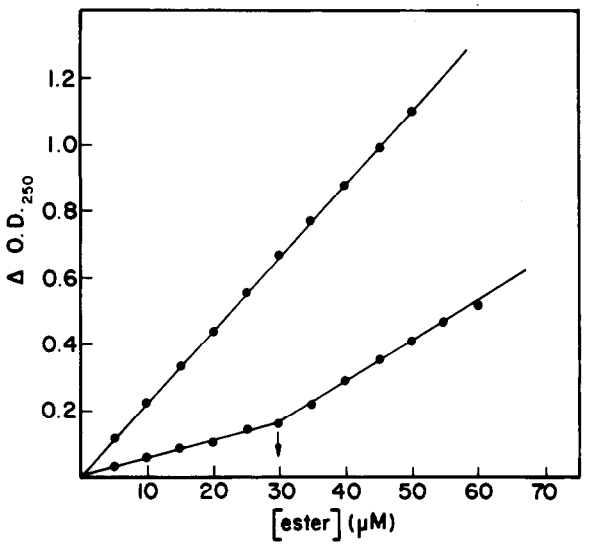

Fig.1. Change of the absorbance at 205 nm caused by the addition of propynyl benzoate to a solution of 0.1 M phosphate buffer, pH 7, at 25°C in the presence of 30 μM α-chymotrypsin (lower line) and in the absence of enzyme (upper line).

ful inhibitors of chymotrypsin. Diphenylcarbamoyl chloride reacts with chymotrypsin with a rate constant of 37000 $M^{-1} \cdot min^{-1}$ [6]. Phenylmethanesulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) react with the enzyme with rate constants of 18000 and 20000 $M^{-1} \cdot min^{-1}$, respectively [11]. On the other hand, the alkyl analogue of **I**, propyl benzoate, failed to react with chymotrypsin. It was neither a substrate nor an inhibitor. The triple bond of the alkynyl carboxylates renders the esters highly susceptible to attack by enzymes.

When alkynyl esters were added to inactive forms of chymotrypsin-like chymotrypsinogen or diphenylcarbamoyl enzyme, there was no sharp instantaneous change in the absorbance of the added ester, indicating that only active enzyme reacts specifically and rapidly with these esters.

The pancreatic proteases trypsin and elastase were also inhibited by the alkynyl esters in a similar manner to chymotrypsin. Moreover, the trypsin-like activity of pronase was very effectively inhibited by **I**, and like other inhibited enzymes underwent reactivation which was enhanced by hydroxylamine. Thrombin and plasmin, too, were inhibited by the esters. They slowly reactivated ($t_{0.5}$ ≃ 3 h), but 1 N hydroxylamine enhanced the reactivation rate by 4–6-fold.

In contrast to the reaction with chymotrypsin, propynyl benzoate served as a substrate for subtilisin Carlsberg, subtilisin Novo and proteinase K with k_{cat} of 18, 6 and 15 min^{-1}, respectively. Porcine liver esterase displayed fast hydrolysis at 0.1 N of **I**, with an initial rate constant of 1800 min^{-1}. However, the rate slowed down during the course of the reaction. At higher concentrations of ester, its inhibitory effect became more pronounced. The enzyme activity recovered slowly ($t_{0.5}$ = 160 min) and under treatment with 0.5 N hydroxylamine the reactivation became faster ($t_{0.5}$ = 9 min). These results indicate that the acyl-enzyme formed during the reaction between esterase and **I** turns over very rapidly, but groups other than the active serine are probably affected by the substrate, or by the product formed, resulting in enzyme inactivation.

These discriminative reactivities of the ester towards different enzymes reflect differences in the structure of the active sites of the enzymes concerned.

In conclusion we have established that despite the fact that benzoate esters are generally not specific substrates of the enzymes investigated, due to the unique alkynyl moiety these new, novel alkynyl esters became activated and highly susceptible to enzymic attack. Specifically, propynyl esters **I** and **II** represent a new class of potent enzyme inhibitors for a wide variety of serine enzymes. Hence, appropriately functionalized alkynyl esters with groups that satisfy the specificity of a particular enzyme are expected to be excellent specific inhibitors. Studies along these lines will be the subject of future reports.

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