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STEREOCHEMISTRY OF THE ACTIVE SITE OF α-CHYMOTRYPSIN

THE EFFECT OF SOME TRICYCLIC BROMOMETHYL KETONES ON α-CHYMOTRYPSIN

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SUMMARY

4:5-Benzindanyl-1-bromomethyl ketone, 4:5-benzindanyl-2-bromomethyl ketone (II), 4:5-benzindanyl-3-bromomethyl ketone, 5:6-benzindanyl-2-bromomethyl ketone, 1,2-dihydronaphtho[2,1-*b*]-furanyl-2-bromomethyl ketone (V), and 2,3-dihydronaphtho[1,2-*b*]-furanyl-2-bromomethyl ketone were tested as inhibitors for α-chymotrypsin (EC 3.4.4.5). Compounds II and V inactivated the enzyme rapidly and their actions were stereospecific, confirming that the binding site of α-chymotrypsin is planar, elongated, and curved. While Compound V alkylated a histidine residue Compound II did not.

INTRODUCTION

SCHOELLMANN AND SHAW[1,2] first reported the active-site-directed inhibition of α-chymotrypsin (EC 3.4.4.5) by L-(1-tosylamido-2-phenylethyl)chloromethyl ketone, a typical substrate analogue. WALLACE *et al.*[3] observed that benzo(*f*)quinoline is a powerful reversible inhibitor of this enzyme and suggested that the binding site "has greater length than breadth and with reference to its longest dimension is not straight but curved". Studies with space-filling, locked substrates in this laboratory[4,5] confirmed this thesis. The present communication deals with preliminary studies on the effect of tricyclic bromomethyl ketones on α-chymotrypsin. The results provide additional evidence for the conclusions[4,5] in regard to the geometry of the binding site and its relative positioning with respect to the catalytic region.

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MATERIALS AND METHODS

Melting points are uncorrected. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y., or by G. I. Robertson, Jr., Florham Park, N.J. Three-times crystallized α-chymotrypsin was obtained from Worthington Biochemical Corp., Freehold, N.J. Anhydrous dimethyl sulfoxide was purchased from Matheson, Coleman and Bell, East Rutherford, N.J. and was distilled under vacuum before use.

The bromomethyl ketones were synthesized commencing with the acids, whose synthesis is described elsewhere[4,5]. The acids (3 mM) were converted to the acid chlorides by treating with 15–20 molar excess of thionyl chloride at 35–40° for 3 h. The crude acid chloride in diethyl ether was treated with 2–3 molar excess of diazomethane solution in diethyl ether for 3 h and the solution was evaporated to dryness. The resulting diazoketone was shaken with 15-20 ml of an ice-cold solution of 20% LiBr in 80% acetic acid and kept in ice for 10 min. The temperature of the solution was raised to 50° and the solution was shaken with 25–30 ml of water. The bromomethyl ketone that separated was extracted with diethyl ether. The extract was washed with water, 5% $KHCO_3$, and saturated NaCl, treated with charcoal, dried over Na_2SO_4, and evaporated. The product was crystallized as indicated under the individual cases.

4:5-Benzindanyl-1-bromomethyl ketone (I)

Crystallized as long white needles from diethyl ether–light petroleum m.p. 72–73° (yield 60%). Elemental analysis. Found: C, 62.09; H, 4.61; Br, 27.55. $C_{15}H_{13}OBr$ requires C, 62.30; H, 4.53; Br, 27.64%.

4:5-Benzindanyl-2-bromomethyl ketone (II)

Obtained as white plates on recrystallization from methanol m.p. 85.0–85.5° (yield 55%). Elemental analysis. Found: C, 62.58; H, 4.33; Br, 27.59. $C_{15}H_{13}OBr$ requires C, 62.30; H, 5.43; Br, 27.64%.

(−)-4:5-Benzindanyl-2-bromomethyl ketone (−)-(II)

Crystallized as white needles from diethyl ether–light petroleum (m.p. 99–100°) (yield 54%, and $[\alpha]_D^{25}$ − 78.7° (*c*, 0.68, in ethanol)). Elemental analysis. Found: C, 62.23; H, 4.72; Br, 27.4. $C_{15}H_{13}OBr$ requires C, 62.30; H, 4.53; Br, 27.64%.

(+)-4:5-Benzindanyl-2-bromomethyl ketone (+)-(II)

Obtained as white needles from diethyl ether–light petroleum (m.p. 98.5–100.0°) (yield 47% and $[\alpha]_D^{25}$ + 79.7° (*c*, 0.82, in ethanol)). Elemental analysis. Found: C, 62.34; H, 4.62; Br, 27.39. $C_{15}H_{13}OBr$ requires C, 62.30; H, 4.53; Br, 27.64%.

4:5-Benzindanyl-3-bromomethyl ketone (III)

Crystallized as colorless cubes from diethyl ether–light petroleum (m.p. 72–73°) (yield 40%). Elemental analysis. Found: C, 62.20; H, 4.39; Br, 27.44. $C_{15}H_{13}OBr$ requires C, 62.30; H, 4.53; Br, 27.64%.

5:6-Benzindanyl-2-bromomethyl ketone (IV)

Crystallized as pale yellow needles from diethyl ether–light petroleum (m.p.

108.0–109.5°) (yield 57%). Elemental analysis. Found: C, 62.31; H, 4.25; Br, 27.36. $C_{15}H_{13}OBr$ requires C, 62.30; H, 4.43; Br, 27.64%.

1,2-Dihydronaphtho[2,1-b]furanyl-2-bromomethyl ketone (V)

Obtained as white needles from diethyl ether–light petroleum (m.p. 88.5–90.0°) (yield 58%). Elemental analysis. Found: Br, 27.43. $C_{14}H_{11}O_2Br$ requires Br, 27.45%.

D(−)-*1,2-Dihydronaphtho[2,1-b]furanyl-2-bromomethyl ketone (D-V)*

Crystallized as white glistening plates from diethyl ether–light petroleum (m.p. 117.0–118.5°) (yield 44% and $[\alpha]_D^{25}$ − 89.6° (*c*, 0.82, in ethanol)). Elemental analysis. Found: Br, 27.16. $C_{14}H_{11}O_2Br$ requires Br, 27.45%.

L(+)-*1,2-Dihydronaphtho[2,1-b]furanyl-2-bromomethyl ketone (L-V)*

Obtained as white needles from diethyl ether–light petroleum (m.p. 116–118°) (yield 57% and $[\alpha]_D^{25}$ + 94.5° (*c*, 0.88, in ethanol)). Elemental analysis. Found: Br, 27.48. $C_{14}H_{11}O_2Br$ requires Br, 27.45%.

2,3-Dihydronaphtho[1,2-b]furanyl-2-bromomethyl ketone (VI)

Obtained as white granular crystals from diethyl ether–light petroleum (m.p. 123–125°) (yield 50%). Elemental analysis. Found: Br, 27.32. $C_{14}H_{11}O_2Br$ requires Br, 27.45%.

Determination of inhibition of enzyme activity

The inhibitor solution (5 ml) in 60% dimethyl sulfoxide was added to 7 ml of a solution of α-chymotrypsin (inhibitor concentration, 0.25–1.0 mM; enzyme concentration, 0.05 mM) in 0.143 M Tris–HCl buffer (pH 7.0) or in 0.143 M acetate buffer (pH 5.0), at 25°. Aliquots (25 μl) were removed at definite intervals over a 6-h period and added to 3 ml of ethyl *N*-acetyl L-tyrosinate solution (1 mM) in 0.1 M phosphate buffer (pH 6.5) and residual chymotryptic activity was measured as described[6]. Time for 50% inhibition was obtained from first-order plots of enzyme activity *versus* time.

Amino acid analysis

α-Chymotrypsin was incubated at pH 7.0 with the bromomethyl ketones as described above. With Compound II in 5 molar excess, complete inhibition of enzyme activity was observed in 30 min. Complete inactivation of chymotrypsin occurred in 24 h with Compounds I and V, when treated with 2 doses of the inhibitors in 10 molar excess. Compound III inactivated the enzyme 72%, on treating in 4 repeated doses of 10 molar excess, after 6 days. The solutions were extracted with diethyl ether and the aqueous layer containing the inactivated enzymes was dialyzed against several changes of 1 mM HCl at 4° and lyophilized. Samples (4–5 mg) of native and modified enzyme were mixed with constant-boiling HCl, deaerated and hydrolyzed at 105° for 21 h. Some samples were oxidized with performic acid[7] at 4°. Amino acid analyses were performed according to the method of SPACKMAN *et al.*[8].

RESULTS AND DISCUSSION

The bromomethyl ketones (Fig. 1) were prepared by reaction of the corresponding diazoketones with a 20% solution of LiBr (LiCl may be used for α-chloro ketones)

in 80% acetic acid. This convenient method was developed[9] for the preparation of acid-sensitive α-halo ketones, but is generally applicable where HCl or HBr gases are usually used[1,2].

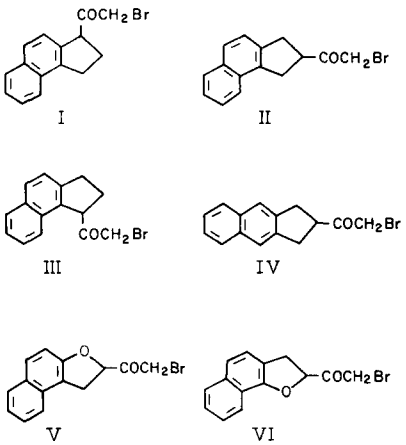

Fig. 1. Structure of compounds.

In Table I, the $t_{1/2}$ values for the inhibitors are listed. Of the four benzindanyl bromomethyl ketones tested, the most powerful inhibitor was Compound II, with a $t_{1/2}$ of 2.2 min. Compound I is 35 times less reactive. Compound III was far less effective, and no detectable inhibition was observed with Compound IV. These data correspond to the relative efficacies of different methyl benzindancarboxylates as substrates for chymotrypsin[5]. Methyl benzindan-2-carboxylate was found to be a reactive substrate, whereas the other positional isomers were hydrolyzed at very slow rates. HAYASHI AND LAWSON[4] showed that methyl 1,2-dihydronaphtho[2,1-*b*]furan-2-carboxylate is hydrolyzed much faster by chymotrypsin than methyl 2,3-dihydronaphtho[1,2-*b*]furan-2-carboxylate. The data in Table I show that Compound V (corresponding to the former ester) is a powerful inhibitor for chymotrypsin. No detectable inhibition was observed with Compound VI*.

Methyl benzindan-2-carboxylate and methyl 1,2-dihydronaphtho[2,1-*b*]-2-carboxylate were shown to be hydrolyzed stereospecifically by chymotrypsin[4,5]. The inhibitory actions of Compounds II and V were also found to be stereoselective. Compound (−)-II was 620 times more reactive than its optical antipode. While Compound D(−)-V inactivated chymotrypsin rapidly, no inhibition was observed with its enantiomer.

The rate of inhibition in all cases was found to be pH dependent. None of the compounds except Compound II showed any inhibition at pH 5.0. Compound II showed a $t_{1/2}$ of 238 min at pH 5.0 compared to 2.2 min at pH 7.0.

* Compounds V and VI were found to be extremely unstable at room temperature. Additionally, the rate of inhibition of α-chymotrypsin by D-V and V was very fast during the first minute of incubation, falling very sharply thereafter. This does not appear to be due to nonspecific interaction with peptide contaminants in the enzyme preparations, since the same phenomenon was observed with the enzyme purified according to the method of YAPEL *et al.*[10] (see KÉZDY *et al.*[11]).

TABLE I

INHIBITION OF α-CHYMOTRYPSIN BY BROMOMETHYL KETONES AT pH 7.0 IN 25% DIMETHYL SULFOXIDE

Inhibitor	*Molar excess over enzyme*	*Time for 50% inhibition (min)*
I	5	78
II	5	2.2
III	15	555
IV	10	—
V	10	4.0
VI	10	—
(−)-II	5	1.0
(+)-II	5	620
D(−)-V	10	2.5
L(+)-V	20	—

Amino acid analyses[1,2,12–17] of the enzyme inhibited with Compounds I, II, and III, showed no loss of methionine or histidine residues, suggesting that the site of alkylation of these inhibitors is presumably Ser-195 (*cf.* ref. 17). In contrast, Compound V, whose geometry is similar to that of Compound II except that it has a polar oxygen adjacent to the α-carbon, alkylated a histidine residue. Analyses of the inhibited enzyme showed a loss of 0.8 residue of histidine. The reason for the differential behaviour of Compounds II and V is not known at present. Further studies are under way for a better understanding of this point.

The present data indicate and confirm the earlier suggestion[3–5] that the binding site of α-chymotrypsin is planar, elongated, and curved (L-shaped). The relative geometry of this region and the catalytic site was such that they are complementary to the hydrophobic and alkylating groups of Compounds (−)-II and D(−)-V. Finally, space-filling models of the latter compounds fit nicely into a space-filling model of the active site of α-chymotrypsin, built according to the X-ray data[18].

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