SYNTHESIS OF DIDEOXY-PEPSTATIN. MECHANISM OF INHIBITION OF PORCINE PEPSIN.

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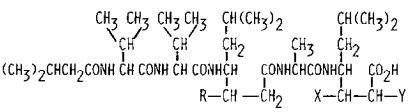
SUMMARY

Dideoxypepstatin has been synthesized and shown to be a competitive inhibitor of pepsin (K_i = 2.1 X 10^{-7} M).

INTRODUCTION

Pepstatin, 1, a pentapeptide isolated from various species of actinomycetes (1), strongly inhibits several acid proteases; the K_i for inhibition of pepsin (2) and human renin (3) is 10^{-10} M.



1 : R = OH; X = OH, Y = H
2 : R = OAc; X = Y = C = C
3 : R = H; X, Y = H

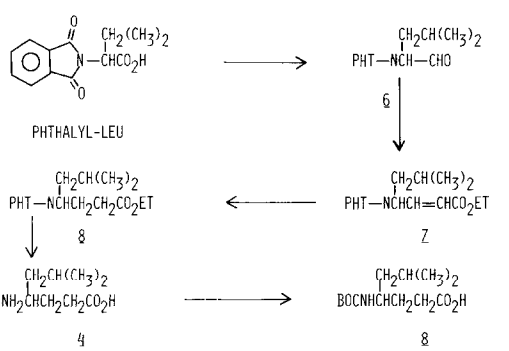
The unusually strong inhibition of this group of enzymes with similar active sites has led to the suggestion that pepstatin 1 is a transition-state analog (4) that inhibits acid proteases by a mechanism involving the hydroxyl group in the third residue. Supporting this hypothesis is the observation that 3(O-acetylstatin)-5-dehydropepstatin, 2, is a much less effective inhibitor of pepsin (K_i = 10^{-6} M) (1). However the low activity of the acetyl derivative 2 could be caused by steric hindrance between the acetyl group and the enzyme and does not prove that the hydroxyl group is essential for activity. This has been observed with the peptide hormone, oxytocin (5), where acetylation of the amino

group in the 1-position reduced activity by a thousand-fold while removal of the same amino group doubled activity. A transition-state analog hypothesis also requires that pepstatin bind to the active-site of the enzyme. However Gregermann et al. have reported that pepstatin is a non-competitive inhibitor of both renin and pepsin (3) which implies that pepstatin binds to the enzyme at a site other than the catalytic site.

In order to obtain a better understanding of the nature of the inhibition of pepsin by pepstatin, particularly with respect to the importance of the hydroxyl group in the 3-position, we have synthesized dideoxy-pepstatin, 3, and report herein its kinetics of inhibition of porcine pepsin. These results are analyzed in terms of the proposed mechanisms of inhibition of pepsin by pepstatin.

MATERIALS AND METHODS

Synthesis of 4-amino-6-methylheptanoic acid, 4, (deoxystatin) (Scheme 1). Phthalyl-leucine was converted by reaction with



SCHEME 1. SYNTHESIS OF DEOXYSTATIN. PHT = PHTHALYL.

thionyl chloride in benzene to the acid chloride 5 which was reduced with hydrogen using Palladium over barium sulfate to the aldehyde 6 (90%). The crude aldehyde 6 (20.280 g) was reacted with ethyl diethylphosphonoacetate (18.58 g/.0827 m) and sodium hydride (57% oil dispersion) (3.50 g/.0830 m) in dimethoxyethane at 50° C to give the olefin 7 (65% yield after chromatography). Catalytic hydrogenation (Pd/C in ethanol, 100% yield) followed by acid hydrolysis ($HCl/HCO_2H/H_2O$) gave deoxystatin 4 in 52% yield: (6) $[\alpha]_D = -18.7$ (C = 0.477%, H_2O); Lit.: $[\alpha]_D = -17.2$, C = 2,

H_2O. Reaction of 4 with tert-butyloxycarbonylazide and triethylamine in DMSO gave Boc-deoxystatin 8 in 69% yield: m.p. 91-92°; NMR (DMSO-d_6) δ 0.834 (d, 6H, J = 6 Hz), 1.367 (s, 9H), 1.085-1.646 (m,5H), 2.17 (t, 2H, J = 8), 6.5 ppm (d, 1H, J = 10) and 3.49 ppm (1H, m).

Synthesis of dideoxy-pepstatin, 3. Boc-DeoxySta-resin, 9, (0.371 mmol/g), prepared by esterification to 1% cross-linked Merrifield resin (7) using $CsHCO_3$ (8), was placed in a Beckman Model 990 peptide synthesizer and carried through the previously described synthetic schedule (9) for the introduction of Boc-Ala, Boc-Deoxy-Sta, Boc-Val and Boc-Val. The pentapeptide was acylated using isovaleric acid and DCC in CH_2Cl_2, removed from the resin by methanolysis (10), and purified by gel filtration over LH-20 in methanol (70% yield). Crystallization from methanol gave pure IVA-Val-Val-DeoxySta-Ala-DeoxySta-OMe, 10: R_f (15% CH_3OH in $CHCl_3$), 0.68. Mass spectrum, m/e 699, 424, 284, 185, 86. These peaks (and others) are formed by the normal cleavage of peptide bonds establishing the presence of the correct sequence of amino acids. The NMR spectrum showed absorptions which integrated correctly for the constituent residues. NMR (DMSO-d_6) δ 0.9-1.0 (30), 1.2-1.8 (16), 2.0-2.4 (6), 3.6-4.3 (5) and 3.5 (3, CH_3O) ppm.

Saponification (KOH/CH_3OH) of 10 followed by gel filtration (LH-20, CH_3OH) gave dideoxypepstatin 3 in 90% yield: m.p. 265° dec; R_f (15% CH_3OH in $CHCl_3$), 0.34. NMR (DMSO-d_6) δ 0.9-1.0 (30), 1.2-1.8 (16), 2.0-2.4 (6), and 3.6-4.3 (5) ppm. Amino acid analysis: Val, 1.9; DeoxySta, 1.9; Ala, 1.0.

Synthesis of Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe, 11. The heptapeptide substrate 11 was prepared by solid phase synthesis (7) from Boc-Phe-resin (0.32 mmol/g; 1% cross-linked) using Boc-amino acids following our standard procedure (9). The histidine imidazole group was protected with the 2,4-dinitrophenyl group (11). The finished peptide was removed from the resin by methanolysis (10) and purified by crystallization. Treatment with TFA gave the amine TFA salt 11 (12). Amino acid analysis: Phe, 3.01; Gly, 1.0; His, 0.93; Ala, 1.02; Phe(NO_2), 0.93.

Kinetic studies. Porcine pepsin was purchased from Sigma and used without further purification. The hydrolysis of heptapeptide 11 was determined at 25° C by continuous monitoring of the increase of absorbance at 310 Nm with a Gilford Model 240 recording spectrophotometer (12,2). The substrate was dissolved in 0.04 M formate buffer pH 4.0 and diluted to final concentration. The inhibitor was dissolved in methanol diluted with 0.04 M buffer. The enzyme reaction was started by addition of enzyme.

Reaction velocity data were processed on a digital computer according to the procedure described by Cleland (13). Reaction velocity data were first analyzed graphically using primary plots of reciprocal velocities and reciprocal substrate concentrations. Data from single plots were fitted to equations for linear competitive inhibition and for linear noncompetitive inhibition using the Fortran computer programs of Cleland (13).

RESULTS

Dideoxypepstatin 3 was found to strongly inhibit the porcine pepsin catalyzed hydrolysis of the heptapeptide substrate 11. Analysis of the Lineweaver-Burke plot of the initial velocities

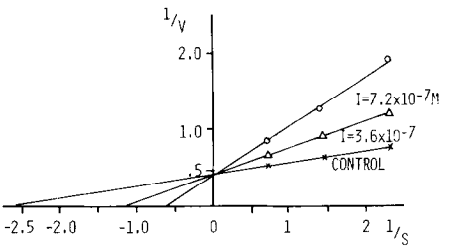


FIGURE 1.
INHIBITION PEPSIN BY DEOXYPEPSTATIN
K_I = 2.1 x 10^{-7} M S = PHEGLYHISPHE (NO_2) PHEALAPHEOME

indicated that the inhibition was competitive (Figure 1) with a K_i of 2.1 X 10^{-7} molar. This result was substantiated by fitting the data to computer programs for both competitive and noncompetitive inhibition. This procedure avoids the inherent errors of reciprocal analysis of enzyme kinetics described by Dowd and Riggs (14). In addition to calculating kinetic constants, the method can be employed to distinguish between possible mechanisms by providing a quantitative estimate of the precision of the fit of data to alternate rate equations (15). Analysis of our inhibition data according to a noncompetitive program showed that no intercept effect was detectable (K_{ii} = 7 $\pm$ 17 X 10^{-6} M), while K_{is} obtained from both computer analyses was 2.1 $\pm$ 0.05 X 10^{-7} molar. Thus our data establish that dideoxypepstatin is a competitive inhibitor of porcine pepsin when the substrate is heptapeptide 11.

DISCUSSION

The results reported here prove that the hydroxyl group in the 3-position of pepstatin is an important contributor to efficient inhibition of pepsin. Removal of this hydroxyl increases K_i by a factor of 2000 and this fact establishes that the low inhibition reported for [3-acetyl]pepstatin 2 ($K_i \sim 10^{-6}$ M) (1) was not caused by steric hindrance between the acetyl group and the enzyme.

In fact since both 2 and 3 have similar K_i values and therefore bind almost equally well, it would appear that the acetyl group encounters little if any steric hindrance when 2 is bound to pepsin.

It is surprising that dideoxypepstatin 3 is a competitive inhibitor of pepsin when pepstatin 1 has been reported to be a noncompetitive inhibitor (3). These inhibitors differ only with respect to the essential hydroxyl group in the 3-position. Earlier work had shown that the hydroxyl group in the 5-position of pepstatin did not contribute to inhibition (1). Thus, removal of a single hydroxyl group from the third residue not only increases the K_i by 2000 fold but also would appear to change the mechanism of inhibition from noncompetitive to competitive. It is difficult to rationalize how a single hydroxyl group could interact with the enzyme outside of the active-site to produce this change.

Analysis of the inhibition data reported (3) for pepstatin in terms of tight-binding-inhibition (16) shows that competitive inhibition of pepsin by pepstatin has not been excluded. Morrison (16) has shown that, in the presence of tight-binding-inhibitors (e.g., those with $K_i \simeq 10^{-10}$ M) plots of 1/V against the reciprocals of the substrate concentrations yield families of concave-down, non-rectangular hyperbolas whether inhibition is competitive or noncompetitive. The non-linearity of these plots can be seen only at high substrate concentrations (10 μM). If initial velocities are determined only at lower substrate concentrations (0.1 to 1.0 μM) then an inhibitor which combines with the same enzyme form as the variable substrate (i.e., competitive inhibitor), can appear to act in a noncompetitive manner. The conclusion that pepstatin inhibits pepsin in a noncompetitive manner was based on kinetics carried out at substrate concentrations (0.1-0.75 μM) too low to observe non-linearity between 1/V and 1/S and therefore cannot

establish whether or not there is an intercept effect. This plus the fact that dideoxypepstatin 3, a closely related structural analog of 1, is a potent competitive inhibitor of pepsin, suggests that pepstatin is a competitive inhibitor of pepsin.

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