Synthesis, crystallization and properties of acetyl phenylalanyl lysine chloromethyl ketone: A potential inhibitor of serine proteases

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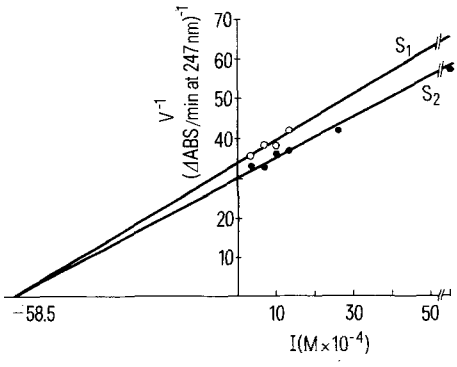
Summary. A procedure for the synthesis of acetyl phenylalanyl lysine chloromethyl ketone is described. The chloromethyl ketone derivative is a noncompetitive inhibitor of trypsin ($K_I = 5.9 \times 10^{-3}$ M).

Serine proteases contain homologous sequences in their active center regions and hence possess a common active site conformation[2]. The availability of inhibitors which can discriminate among these trypsin like enzymes, would be very useful in labelling the active centers of such enzymes. Irreversible inactivation by small molecules like the chloromethyl ketones have been useful in the study of proteolytic enzymes whose normal physiological substrates may have relatively high affinity[3]. Some of these homologous enzymes are refractory to tosyl lysine chloromethyl ketone. It was therefore desirable to synthesize chloromethyl ketone derivatives, which took fuller advantage of enzyme subsites now recognized to be important for the catalytic activity of proteolytic enzymes like trypsin[4]. We have succeeded in synthesizing the acetyl phenylalanyl lysine chloromethyl ketone and characterizing it as a noncompetitive inhibitor of trypsin.

Materials and methods. N-acetyl phenylalanine (Ac-Phe), N-ε-cbz-lysine methyl ester hydrochloride (Z-lys-OMe, HCl) and tosylarginine methyl ester (Tos-Arg-OMe) were from Sigma. Crystalline trypsin was purchased from Worthington Biochemicals. All other chemicals were obtained commercially.

Results and discussion. Synthesis of the acetyl phenylalanyl lysine chloromethyl ketone (Ac-Phe-Lys-CH_2Cl) was accomplished using the following procedure. A solution of Z-Lys-OMe, HCl (0.01 mole), an equivalent amount of Ac-Phe and triethylamine (1.4 ml) in tetrahydrofuran (50 ml) was cooled to about 15 °C in a flask fitted with a $CaCl_2$ drying tube. After 10 min, $POCl_3$ (0.92 ml) and triethylamine (2.8 ml) were added and stirred for 1 h. 20 ml water was then added to destroy any unused $POCl_3$ and the solvent was removed by evaporation under reduced pressure at 25 °C. The residue was extracted with ethylacetate (3 × 20 ml). The combined ethylacetate fraction was washed 3 times with 5 ml each of water, 5% $NaHCO_3$ and water respectively, dried over $MgSO_4$ and evaporated under reduced pressure at 25 °C, to yield the acetyl phenylalanyl-cbz-lysine methyl ester (Ac-Phe-Z-Lys-OMe). The methyl ester was dissolved in p-dioxane (10 ml) and hydrolyzed by adding 10 ml of 1 M NaOH and stirring for 1 h at 0 °C. The p-dioxane was removed by extracting with ethyl ether (20 ml) and the aqueous fraction was acidified to pH 4.0 using 1.2 M HCl. The resulting acetyl phenylalanyl-Z-lysine (Ac-Phe-Z-lysine) was extracted with ethylacetate (20 ml). The ethylacetate fraction was dried and evaporated as before. Ac-Phe-Z-Lys was converted to a mixed anhydride intermediate according to the procedure of Birch et al.[5]. Ac-Phe-Z-Lys was dissolved in tetrahydrofuran (10 ml) in a flask fitted with a $CaCl_2$ drying tube. Triethylamine (1.4 ml) and ethyl chloroformate (0.95 ml) were added and the mixture stirred for 30 min at 0 °C, followed by addition of 75 ml of 0.5 M diazomethane. After 2 h, the mixture was treated with cold HCl saturated ethanol (15 ml) for 10 min and extracted with ethylacetate. The extract was washed with H_2O and 0.1 M HCl, dried ($MgSO_4$) and evaporated under reduced pressure. This resulted in the formation of Ac-Phe-Z-Lys-CH_2Cl. This conversion of Ac-Phe-Z-Lys to Ac-Phe-Z-Lys-CH_2Cl is analogous to the synthesis of Z-Lys-CH_2Cl described by Coggins et al.[6]. The removal of the benzyloxycarbonyl protecting group was achieved by employing trifluoracetic acid at 50 °C, as described by Shaw and Glover[7]. The end product, Ac-Phe-Lys-CH_2Cl was obtained as an oil. The oil was dissolved in a minimum volume of trifluoroacetic acid and HCl saturated ether (10 ml) was added at 0 °C. Crystallization was induced by scatching the wall of the container with a glass rod. The crystals were collected by centrifugation and washed with anhydrous ether. The crystalline derivative was stored in a desiccator at −15 °C. The synthetic product gave a single spot after TLC on silica gel G plates (Brinkmann) developed with chloroform-ethylacetate (8:2 V/V). The crystalline Ac-Phe-Lys-CH_2Cl was found to melt in the range of 135–136 °C with decomposition. Infrared spectral analysis of the compound confirmed the presence of methylene and keto groups (bands in the wavelength region of 3.4–3.6 μm and 5.8–6.01 μm) and the absence of carboxyl group (no broad band in region of 3.3–4.0 μm) indicating that the chloromethylketone was intact during the deblocking of the ε-amino group of the lysine moiety. A further check of the purity of the compound was made with reference to its chlorine content. The observed and calculated values were in good agreement (calculated for $C_{18}H_{26}N_3O_3Cl$: 9.54%; found: 9.68%). The overall yield was about 5% (190 mg). Recently we have been successful in employing this procedure to synthesize the corresponding alanyl and valyl derivatives.

A stock solution of 10 mM Ac-Phe-Lys-CH_2Cl in 1 mM HCl was used for the inhibition studies with trypsin. The inhibitory potential was estimated by following the rate of hydrolysis of Tos-Arg-OMe in the absence and presence of varying concentrations of the compound, in a standard assay mixture at 25 °C according to the procedure of Hummel[8]. Under these conditions, the initial rate of increase in absorbance at 247 nm was a direct measure of the enzyme activity. The inhibition constant, K_I was calculated using the Dixon plot[9] as shown in the figure. The synthetic chlo-



Inhibition of trypsin by Ac-Phe-Lys-CH_2Cl. Initial velocities (V) of the trypsin catalyzed hydrolysis of Tos-Arg-OMe was measured at 25 °C, in the presence of 10 mM Tris buffer (pH 8.1) employing 2 different substrate concentrations of 3.3×10^{-4} M (S_1) and 6.6×10^{-4} M (S_2) respectively, in the presence of varying concentrations of the synthetic inhibitor (I). The amount of trypsin present in the reaction mixture (3 ml) was 2 μg. The point where the 2 lines intersect the X-axis represents the value equal to $-K_I$.

romethylketone exhibited noncompetitive inhibition with an estimated K_I-value of 5.9×10^{-3} M.

Earlier studies with Tos-Lys-CH_2Cl and Tos-Phe-CH_2Cl have demonstrated the utility of these agents as active site inhibitors of trypsin and chymotrypsin respectively[10,11]. However, proteases like subtilisin and plasma kallikrein, which possess in common, the esterase activity on lysine derivatives exhibited by trypsin, are not affected by Tos-Lys-CH_2Cl[6]. The synthetic Ac-Phe-Lys-CH_2Cl described here should prove very useful in such studies with subtilisin, in view of the fact that the peptide structure derived from the acetyl aminoacid and lysine affords better structural similarity to its physiological substrate.

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Genotype-isopropanol interaction in the *Adh* locus of *Drosophila buzzatii*[1]

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Summary. Drosophila buzzatii, when reared in a medium with isopropanol, shows a significant band interconversion in ADH zymograms and a general lowering of ADH activity. Changes in activity are greater in Adh^F homozygotes than in Adh^S homozygotes and generate a significant genotype-isopropanol interaction. These mechanisms are relevant to an explanation of the high natural *Adh* polymorphism of this species.

In recent years much effort has been devoted to demonstrating selection in individual allozyme loci. The alcohol dehydrogenase *(Adh)* locus has been the one used most successfully in this search, since now a general agreement exists that this locus seems to be adaptive in at least 2 species of *Drosophila*[2-9]. Here we show evidence that the *Adh* locus of *Drosophila buzzatii* may be also a likely candidate for the operation of natural selection.

We have sampled 16 different natural populations of *D. buzzatii* from a large area which includes localities in the Mediterranean (Egypt, Balearic islands, and Iberian peninsula) and in the Atlantic regions (Canary islands, Madeira island and Cotonou). The *Adh* polymorphism has been found to be high in all but 1 of the sampled populations, with 2 electromorphs in high frequencies (Adh^F and Adh^S) and 1 electromorph very rare and found only in 1 population. Figure 1 shows the standard electrophoretic bands of the 2 common alleles. Adh^F has frequencies ranging from 0.35 to 1.00. We have performed segregation tests (F_2 and backcrosses) which showed that Adh^F and Adh^S allozyme variants segregated in a Mendelian way. All this information is in accordance with data reported by Barker and Mulley[10] for Australian populations.

We observed that the intensity of the electrophoretic bands was highly variable and faded away during a time of culturing in the laboratory. In order to understand this variability in activity, we tested changes in staining intensity when larvae were reared on media containing several kinds of alcohols, since we know that these are likely substrates for the ADH enzyme. Tests were performed with 3 short chain alcohols (methanol, ethanol and isopropanol), which were added to the culture medium at initial concentrations of 1 and 2% in volume. Staining of zymograms was carried out with each of 4 alcohol substrates individually: methanol, ethanol, isopropanol and isobutanol, and revealed that only the medium with isopropanol induces significant changes in the standard pattern of bands. These changes are of 2 kinds: 1st, the most electropositive bands disappear or lose much of their activity; and 2nd, there is an increase of staining in the electronegative bands. This enhancement of activity, although general, is much stronger when the substrate is isopropanol, in which case new, more electronegative, bands may appear (figure 2).

This qualitative experiment showed that important changes in ADH activity may be induced by isopropanol in *D. buzzatii.* However, we wanted to know how these changes may be quantified and assigned to individual *Adh* genotypes. For this purpose, we isolated 5 independent homozygous strains for each of the 2 alleles Adh^F and Adh^S. These strains were cultured separately in standard laboratory medium either with 1% isopropanol or without alcohol, and their 'in vitro' specific activity (SA) measured spectrophotometrically. Previous experiments indicated that activity is highest in early pupae, so we chose this stage for the experimental check.

Table 1 shows that isopropanol induces an overall significant decrease of SA. This decrease is much stronger for the Adh^F homozygote than for the Adh^S homozygote. The analysis of variance of table 2 shows a significant genotype-isopropanol interaction, which confirms the non-proportional changes of activity between genotypes. The analysis of variance depicts also significant differences between genotypes, which are due exclusively to the differential effect of isopropanol on the genotypes. Thus, differences in SA between genotypes are only statistically significant when isopropanol is added to the medium ($t = 7.25$; $p < 0.001$).

David et al.[11] have advanced the hypothesis that the high toxicity shown by secondary alcohols on *Drosophila* may be explained not as a direct toxic effect of the alcohol, but as