a foundation for means of estimation of the risk/benefit ratio of its use compared to that of 8-MOP in the phototherapy of psoriasis.

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4-Substituted-2-anilinothiazolin-5-ones as substrates of α-chymotrypsin

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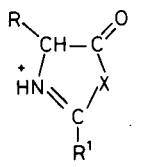
The 2-anilinothiazolin-5-one derivatives (ATZ; 1a) of leucine, alanine, valine and tryptophan are substrates of α-chymotrypsin, the order of reactivity being ATZ-Try >> ATZ-Ala ≃ ATZ-Leu >> ATZ-Val. Deacylation of the acyl enzyme gives the 5-substituted-3-phenyl-2-thioxo-4-imidazolidinone (PTH-amino acid; II) by intramolecular participation of the anilino-*N*-atom, except for ATZ-Try which may be alternatively bound at the active site since the 2-substituted-5-phenylhydantoic acid (III) is formed.

The oxazolinones, 2-phenyloxazolin-5-one (PO; Ib, R=H,) and 2-phenyl-4,4-dimethyloxazolin-5-one (PDMO; Ib, R=$(CH_3)_2$·)

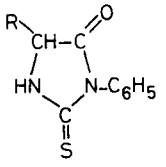
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{\text{acyl-enzyme}}{ES^1} \xrightarrow{k_3} E + P \quad (1)$$

are substrates of α-chymotrypsin (de Jersey & Zerner 1969; de Jersey et al 1966) the reaction proceeding through the acyl-enzyme intermediate where the rate controlling step is governed by k_3 (Bender 1962) (eqn 1). The related, 4-methyl-2-phenylthiazolin-5-one (Ic) is a poor substrate of the enzyme (Coletti-Previero et al 1973) and on the premise that poor substrates which give a relatively stable acyl enzyme (low value for k_3) would be satisfactory inhibitors in vivo we have studied the reaction between several 4-substituted-2-anilinothiazolin-5-ones (ATZ-amino acids; Ia) and the enzyme.

* Correspondence.



(I)



(II)

C_6H_5·NH·CS·NH·CH(R)COOH

(III)

(Ia): $R^1 = -NH.C_6H_5$, X = S
(Ib): $R^1 = -C_6H_5$, X = O
(Ic): R = Me, $R^1 = C_6H_5$, X = S

Materials and methods

α-Chymotrypsin (BDH) was thrice recrystallized. The 5-substituted-3-phenyl-2-thioxo-4-imidazolidinones (PTH-amino acids) were purchased from Sigma and the corresponding sodium phenylhydantoates were prepared by the general method of Edman (1956). Spectra were measured in acetonitrile (12%) - phosphate buffer (0·01 M), pH 7·4. All melting points are corrected.

4-(2'-Methylpropyl)-2-anilinothiazolin-5-one hydrochloride (ATZ-leucine, I(a), R = $(CH_3)_2CH.CH_2$). This was prepared by Edman's method (1956) except that the syrup containing the sodium phenylhydantoate was repeatedly extracted with acetone and ether before

cyclization to give crystals, m.p. 144–146 °C (insert at 140 °C; Edman, 1956, cites m.p. 128–131 °C), λ_{max} (acetonitrile) 250 nm (ε, 16 730). The sodium phenylhydantoate had λ_{max} 246 nm (ε, 15 000).

4-Methyl-2-anilinothiazolin-5-one (ATZ-alanine I(a), R = CH_3). White crystals, m.p. 159–160 °C (35% yield) were obtained. (Found: C, 49·6; H, 4·6; N, 11·6; S, 13·4; Cl, 14·7. $C_{10}H_{10}ON_2S.HCl$ requires, C, 49·5; H, 4·6; N, 11·5; S, 13·2; Cl, 14·6%). ν_{max} (KBr) 3190, 3060 (NH), 2700 (NH), 1755 (C = O), 1650 (C = N), cm^{-1}. λ_{max} (acetonitrile) 250 nm (ε, 16 500). The sodium phenylhydantoate had λ_{max} 246 nm (ε, 14 800).

4-(1'-methylethyl)-2-anilinothiazolin-5-one (ATZ-valine, I(a), R =$(CH_3)_2.CH$). White crystals, m.p. 150–152 °C (46% yield) were obtained. (Found: C, 53·2; H, 5·5; N, 10·2; S, 11·9, Cl, 13·2. $C_{12}H_{14}ON_2S.HCl$ requires C, 53·2; H, 5·6; N, 10·3; S, 11·8; Cl, 13·1%). ν_{max} (KBr) 3010 (NH), 2760 (NH) 1750 (C = O), 1640 (C = N^+), cm^{-1}. λ_{max} (acetonitrile) 250 nm (ε, 16 600). The sodium phenylhydantoate had λ_{max} 246 nm (ε, 14 900).

4'-(3'-Indolylmethyl)-2-anilinothiazolin-5-one (ATZ-tryptophan, I(a), R = $C_8H_6N.CH_2$). Yellow crystals m.p. 171–3 (d) (57% yield) were obtained. (Found: C, 60·3; H, 4·5; N, 11·4; S, 8·9; Cl, 9·7. $C_{18}H_{14}ON_3S.HCl$ requires, C, 60·6; H, 4·2; N, 11·8; S, 9·0; Cl, 9·9%). ν_{max} (KBr), 3350 (indole-NH), 3060, 2700 (NH) 1759 (C = O), 1630 (C = N), cm^{-1}. λ_{max} (acetonitrile) 253 nm (ε, 13 800). The sodium phenylhydantoate had λ_{max} 256 nm (ε, 16 800).

T.l.c. of PTH-amino acids, ATZ-amino acids and their sodium phenylhydantoates. The compounds (0·01%, 10 μl) were applied in acetonitrile (20%) - phosphate buffer (0·01 M) pH 7·4 on pre-coated silica gel 60 plates (E Merck, Darnstadt), developed with heptane-pyridine (70:30) and sprayed with iodine (5%) in methanol. The R_F values for the PTH-amino acid, ATZ-amino acid and their sodium phenylhydantoates were 0·23, 0·35, 0·067 (leucine) 0·2, 0·28, 0·05 (alanine) 0·31, 0·5, 0·09 (valine) and 0·09, 0·17, 0·03 (tryptophan) respectively.

Spontaneous hydrolysis of ATZ-amino acids in buffer at pH 7·4 and pH 5·0. The absorption (A_t) at λ_{max} of a solution of the ATZ-amino acid hydrochloride ($1·7 \times 10^{-5}$–$2·2 \times 10^{-4}$ M) in either phosphate buffer (0·01 M) pH 7·4 or acetate buffer (0·06 M) pH 5·0 containing acetonitrile (12%) was measured on a unicam SP 1800 recording spectrophotometer with the cell compartment maintained at 25 °C, at time (t) until the absorption had decreased to a constant value (A_∞). The apparent first order rate constant, k_{obs}, for the hydrolysis was calculated from the linear plot of log (A_t–A_∞) versus t (Table 1).

In separate experiments it was shown by t.l.c. (pH 7·4) and the change in the absorption curves (pH 5 and 7·4) that ATZ-tryptophan gave mainly the sodium phenylhydantoate (λ_{max} 252–257 nm) and some PTH-amino acid whereas the other compounds gave mainly the PTH-amino acid (λ_{max} 265–266 nm) and some phenylhydantoate.

α-Chymotrypsin-catalysed hydrolysis of ATZ-amino acids. Studies at pH 7·4: The initial rate (v) of the hydrolysis was obtained from the change in absorption (λ_{max} 250–256 nm)-time curves for different concentrations of substrate (4–12 $\times 10^{-5}$ M) at a fixed enzyme concentration ($0·08$–$2·6 \times 10^{-6}$ M) in acetonitrile (10%) - phosphate buffer (0·01 M) pH 7·4 as measured at 25 °C. K_m and k_{cat} values (Table 1) were calculated from a non-linear least squares fit directly to the Michaelis-Menten equation. The program used the Marquardt method and forms part of S.P.S.S. at University of London computing centre. T.l.c. and a scan of the λ 200–400 nm region after the reaction was complete confirmed that ATZ-tryptophan gave the sodium phenylhydantoate whereas the other ATZ-amino acids gave the PTH-amino acid.

Studies at pH 5: Similar studies where $[S] >> [E_o]$, ($[S] = 4 - 12 \times 10^{-5}$ M, $[E_o] = 0·26 - 10 \times 10^{-6}$ M) were carried out in acetonitrile (10%)-acetate buffer (0·06 M) pH 5 (Table 1). Studies were also conducted where $[S] \simeq [E_o]$ and $[S] < [E_o]$, ($[S] = 2·5 \times 10^{-5}$ M, $[E_o] = 2·6 - 3·2 \times 10^{-5}$ M) to determine whether k_2 (acylation) > k_3 (deacylation) (Kézdy et al 1964). The Δ A-time curves for the reactions with ATZ-leucine and ATZ-alanine showed a rapid decline to a minimum value (A_{min}, acyl enzyme) and then slowly increased to a

Table 1. Kinetic constants for the spontaneous hydrolysis and α-chymotrypsin-catalysed hydrolysis of ATZ-amino acid hydrochlorides*.

	pH 5			pH 7·4		
ATZ-amino acid hydrochloride	k_{cat} min^{-1} (± s.e.)	k_m (M) (± s.e.)	k_{obs} min^{-1} (± s.e.)	k_{cat} min^{-1} (± s.e.)	k_m (M) (± s.e.)	k_{obs} min^{-1} (± s.e.)
Enzyme catalysed						
Alanine	76·9 (13·0)	$3·94 \times 10^{-4}$ (0·78)	—	138·3 (5·1)	$1·17 \times 10^{-4}$ (0·06)	
Leucine	67·0 (4·8)	$2·11 \times 10^{-4}$ (0·18)	—	116·8 (4·5)	$1·13 \times 10^{-4}$ (0·05)	
Valine	—	—	—	23·1 (1·3)	$1·75 \times 10^{-4}$ (0·12)	
Tryptophan	172·9 (5·8)	$1·13 \times 10^{-4}$ (0·04)	—	1054·1 (119·4)	$2·29 \times 10^{-4}$ (0·29)	
Spontaneous						
Alanine	—	—	very slow	—	—	0·0154 (0·0002)
Leucine	—	—	0·0117 (0·0007)	—	—	0·0219 (0·0016)‡
Valine	—	—	very slow	—	—	0·0037 (0·0002)
Tryptophan	—	—	0·032 (0·0018)	—	—	†

* In acetonitrile (10%)—phosphate buffer (0·01 M), pH 7·4 or acetate buffer (0·06 M) pH 5·0 at 25 °C.
† —precipitate formed. ‡ Approximative value due to curved plot as cloudiness developed.

constant value (A_∞, PTH-amino acid). The overall calculated ΔA corresponded to the change in absorption noted for the same concentration of substrate at pH 7·4.

The Δ A-time curve for the reaction with ATZ-valine showed a steady decline to a constant value (PTH-valine) and acyl enzyme was not observable. The reaction with ATZ-tryptophan was too fast to observe using this technique.

Discussion

Specific synthetic substrates of α-chymotrypsin are ester or amide derivatives of *N*-acylated aromatic amino-acids. The aryl function slots into a hydrophobic pocket at the active site so that hydrogen bonding of Ser-214 carbonyl group to the acyl-NH group correctly positions the labile group to form the acyl enzyme with Ser-195 hydroxyl function which is activated by being part of the electron relay system Asp-102, His-57, Ser-195. Hydrolysis of the acyl enzyme is the reverse procedure where the attacking nucleophile is water (Steitz et al 1969; Blow et al 1969).

The ATZ-derivatives of alanine, leucine, valine and tryptophan are substrates of α-chymotrypsin, the relative rates of enzyme-catalysed hydrolysis being ATZ-tryptophan >> ATZ-alanine ≃ ATZ-leucine >> ATZ-valine at either pH 7·4 or pH 5 (Table 1) which parallels the relative rate of spontaneous hydrolysis.

The α-chymotrypsin-catalysed hydrolysis of the ATZ-amino acids (except ATZ-tryptophan) is considered to initially follow the same mechanistic pathway as for the related 2-phenyloxazolin-5-ones (de Jersey et al 1966). The aryl function is considered to bind in the hydrophobic cavity and the acyl enzyme is formed by attack of Ser-195 hydroxyl group on the C-5 carbonyl function. The product of the hydrolysis is the PTH-amino acid which is probably formed by intramolecular nucleophilic attack by the anilino-*N*-atom on the serine ester since it is not formed from the sodium phenylhydantoate in near neutral media. This reaction apparently occurs more readily than the alternative enzyme-catalysed attack by water on the acyl enzyme and is analogous to the formation of PTH-amino acid from the 2-substituted phenyl hydantoic acid in strongly acid media (Edman 1956) where the carboxyl group is unionized.

ATZ-tryptophan gave the sodium phenylhydantoate as the main product of the enzyme-catalysed reaction and this difference in product and the relative faster rate may be due to alternative binding of the substrate at the active site with the indole nucleus in the hydrophobic cavity. The C-5 carbonyl function may then be more correctly positioned by comparison with specific substrates for reaction with Ser-195 and subsequent enzyme-catalysed deacylation of the acyl-enzyme by water. However, it is difficult to explain why spontaneous hydrolysis gave the same product and there may be an alternative reason.

Studies on the enzyme-catalysed reaction at pH 5 showed that for ATZ-leucine and ATZ-alanine, $k_2 > k_3$ so that at pH 7·4 the rate controlling step was de-acylation, as it is for PO and PDMO (de Jersey et al 1966). With the poorest substrate, ATZ-valine it was not possible to discern the acylation step at pH 5. This may be because k_2 approaches k_3 at pH 7·4 and since the deacylation step is pH-independent over the range pH 5–7·4 (cf. pKa 16·60 for N(5) of 3-methyl-5-phenylhydantoic acid with expected slight lowering due to high polarizability of sulphur; Bergon & Calmon 1978) the acylation step alone was slowed at pH 5.

4-Methyl-2-phenylthiazolin-5-one (k_{cat}/Km, 210 M^{-1} min^{-1}) is a poor substrate of α-chymotrypsin compared with the structurally related ATZ-alanine ($11{\cdot}8 \times 10^5$ M^{-1} min^{-1}) and this difference is due to promotion of the rate controlling deacylation step by participation of the anilino residue in the intramolecular cyclization reaction.

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