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Inhibition of α-chymotrypsin by 5-substituted -3-phenyl-2-thioxo-4-imidazolidinones and derivatives

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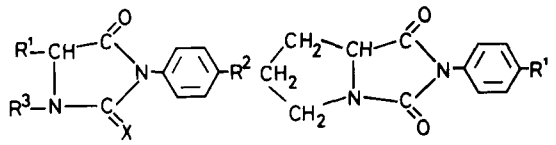
After an initial finding that 5-(2′-methylpropyl)-3-phenyl-2,4-imidazolidinedione (I; PTH-leucine) was an irreversible inhibitor of α-chymotrypsin we have examined the structure-activity relationships within a group of PTH-amino acids and certain derivatives. This study, in conjunction with the known topography of the active site of the enzyme (Birktoft et al 1970; Birktoft & Blow 1972), could lead to the rational design of inhibitors of elastase and cathepsin G released from PMNs which are considered to be responsible for the diseases emphysema, glomerulonephritis and rheumatoidal arthritis (Powers 1976; Davies et al 1978; Baici et al 1980).

Materials, methods and results

α-Chymotrypsin (BDH), thrice recrystallized, from bovine pancreas was used. PTH-amino acids were obtained from Sigma Chemical Co., or synthesized (Edman 1950). The ^{1}H nmr spectra were recorded at 90 MHz for solutions in $DMSO_{d6}$ with TMS as internal standard.



(I) X = S, $R^1 = (CH_3)_2CH{\cdot}CH{\cdot}_2$, $R^2 = R^3 = H$
(II) X = O, $R^1 = (CH_3)_2CH{\cdot}CH_2$, $R^2 = R^3 = H$
(III) X = O, $R^1 = (CH_3)_2CH{\cdot}CH{\cdot}_2$, $R^2 = R^3 = NO_2$
(IV) (a) $R^1 = H$ (b) $R^1 = NO_2$
(V) $(CH_3)_2CH{\cdot}CH_2{\cdot}CH(CO_2H){\cdot}NH{\cdot}CS{\cdot}NH{\cdot}C_6H_5$

PTH-dehydroserine was slowly deposited in the acidic mother liquors from the cyclization of the phenylthiohydantoic acid on attempted preparation of PTH-serine by the method of Ingram (1953) and had m.p. 269–271 °C (d). It readily polymerizes (cf. Fraenkel-Conrat & Harris, 1954) and the elemental analysis of our material was not satisfactory. ν_{max} (KBr) 3213 (NH), 1718 (C=O) 1660 (C=C), cm^{-1}. ^{1}H nmr, τ-2·5(s, 1H, NH), 2·63 (m, 5H, aromatic-H), 4·7 (d, 1H, J=3Hz, =CH), 4·88 (d, 1H, J = 3Hz, = CH). 5-(2′-methylpropyl)-3-phenyl-2,4-imidazolidinedione (II); PH-leucine was prepared by the method of Aberhalden & Brockmann (1930) and this general method was used for *hexahydro-2-phenyl-*1H*-pyrrolo (1,2-c) imidazol-1,3-dione* (IVa) m.p. 114–5 °C. Found: C, 66·0; H, 5·7; N, 12·6. $C_{12}H_{12}N_2O_2$ requires C, 66·7; H, 5·6; N, 13·0%.

* Correspondence.

The imidazolidinedione (1 g) in a mixture of acetic acid (15 ml), sulphuric acid (20 ml) and nitric acid (6 ml) was kept at 0 °C for 48 h. The mixture was then poured into ice-water and the yellow precipitate removed and crystallized from benzene-light-petroleum (80–100 °C) and then ethanol–water mixtures. *5-(2′-methypropyl-1-nitro-3(4′-nitrophenyl)-2-4-imidazolidinedione* (III) had m.p. 146–7 °C. Found: C, 48·5; H, 4·4; N, 17·2. $C_{13}H_{14}N_4O_6$ requires C, 48·5; H, 4·4; N, 17·4% ν_{max} (KBr), 1810 (w, C = O), 1745 (s, C = O), cm^{-1}. ^{1}H nmr, τ, 1·61 (d, 2H, J = 9Hz, aromatic-H), 2·29 (d, 2H, J = 9Hz, aromatic-H), 4·9 (t, 1H. J = 4·5 Hz, ·CH), 8·06 (m, 3H, ·CH_2·CH·) 9·05 (d, 6H, J = 6Hz, $(CH_3)_2$·). *Hexahydro-2(4′-nitrophenyl)*1H*-pyrrolo (1,2-c) imidazol-1,3-dione* (IVb) had m.p. 121–3 °C. Found: C, 54·7; H, 4·2; N, 16·1. $C_{12}N_{11}N_3O_4$ requires C, 55·2; H, 4·2; N, 16·1% ν_{max} (KBr), 1780 (w, C = O) 1720 (s, C = O), cm^{-1} ^{1}H nmr, τ, 1·67 (d, 2H, J = 9Hz, aromatic-H), 2·3 (d, 2H, J = 9Hz aromatic-H), 5·63 (t, 1H, J = 8Hz, ·CH.), 6·43 (m, 2H, ·CH_2·N), 7·93 (m, 4H, ·CH_2·CH_2·).

Inhibition of α-chymotrypsin. 22 PTH-amino acids, including those in Table 1, and their derivatives were examined. A solution of α-chymotrypsin ($3·2 \times 10^{-7}$ M) and PTH-amino acid ($1·6 \times 10^{-4}$ M) in acetonitrile (12%) - phosphate buffer (0·02 M), pH 7·4, was incubated at 25 °C and initially and at pre-determined intervals an aliquot (2 ml) was assayed for enzyme activity in the pH - Stat using *N*-acetyl tyrosine ethyl ester (ATEE) as substrate (Al Shabibi & Smith 1974). A control experiment in the absence of PTH-amino acid was also conducted. The residual activity (as a percentage of the activity of the control) versus time curve is

Table 1. Stability of PTH-amino acids, PH-amino acids and their nitrated derivatives in acetonitrile (12%) - phosphate buffer (0·02 M), pH 7·4.

Amino acid residue	PTH-amino acid k_{obs} h^{-1} ($X10^{-2}$)† (± s.e.)	PH-amino acid k_{obs} $days^{-1}$‡ (± s.e.)	Nitrated PH-amino acid k_{obs} h^{-1} ($X10^{-2}$)† (± s.e.)
Dehydroserine	73·30 (4·6)	—	—
Serine	14·28 (0·88)	—	—
Hydroxyproline	10·29 (0·99)	—	—
Arginine	8·01 (0·16)	—	—
Histidine	8·13 (0·76)	—	—
Leucine	4·03 (0·55)	$0·42 \times 10^{-2}$ (0·02)	506·7 (18·0); 13·82 (0·00)
Methionine	3·94 (0·50)	—	—
Alanine	2·40 (0·12)	—	—
Proline	1·57 (0·28)	$2·19 \times 10^{-2}$ (0·07)	1·70 (0·07)
Valine	—	—	—

† 25 °C. ‡ Room temperature, 20 °C.

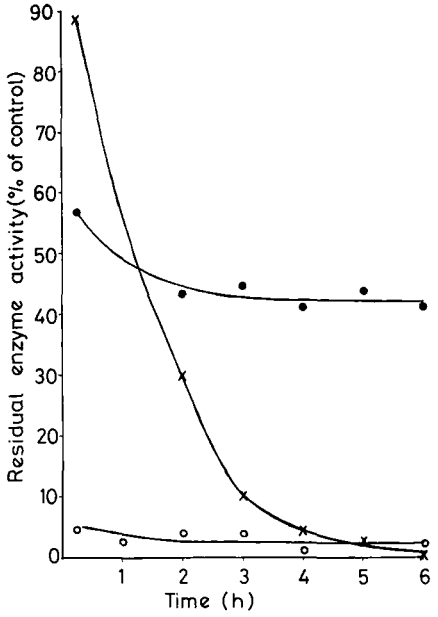


FIG. 1. Inhibition of α-chymotrypsin ($3{\cdot}2 \times 10^{-7}$ M) by PTH-arginine (●), PTH-dehydroserine (○) ($1{\cdot}6 \times 10^{-4}$ M) in acetonitrile (12%) phosphate buffer (0·02 M) at pH 7·4 and PTH-histidine (×) in phosphate buffer (0·02 M) at pH 7·4.

shown for the most active inhibitors in Fig 1.

The progress curves for the inhibition of α-chymotrypsin by PTH-histidine, PTH-serine and PTH-dehydroserine showed a progressive decrease in enzyme activity to low or zero activity, the order of potency being PTH-dehydroserine >> PTH-histidine > PTH-serine. The curves for the reaction with PTH-arginine, PTH-proline and PTH-hydroxyproline showed an initial rapid decrease in activity of the enzyme but then levelled off at a residual enzyme activity of about 40%. Of the remaining PTH-amino acids, PTH-leucine slowly inhibited the enzyme over 6 h to a residual enzyme activity level of about 50% whereas the others were less active or inactive. (III) rapidly decreased the enzyme activity to a low level (15% residual activity within 1 h), whereas (II), (IVa) and (IVb) did not inhibit the enzyme.

Stability of the inhibited enzyme. An inhibited enzyme solution (zero activity) (5 ml) prepared from α-chymotrypsin ($6{\cdot}4 \times 10^{-6}$ M) and PTH-dehydroserine (4×10^{-3} M) was placed on a column ($1{\cdot}5 \times 30$ cm) of Sephadex G-25 gel and eluted with phosphate buffer (0·02 M) pH 7·4. The fractions containing the protein (λ 282 nm) were pooled and incubated at 25 °C for 6 h. Aliquots containing 24–27 μg protein (Lowry et al 1951) were withdrawn at hourly intervals and showed zero activity with ATEE. A control experiment retained 85% of its activity in this procedure.

Table 2. Nociceptive response of mice as assessed by the tail immersion test 80 min after doses of compounds administered intraperitoneally.

Treatment	Dose mg kg^{-1} i.p.	Nociceptive latency (S ± s.e.m.)	Statistical significance compared with vehicle controls
Vehicle control	—	2·7 ± 0·4	P_m
Morphine	5·0	9·0 ± 1·2	<0·001
PTH-hydroxy-proline	100	5·1 ± 1·5	<0·01
PTH-arginine	100	9·3 ± 1·9	<0·001
PTH-histidine	100	9·1 ± 1·7	<0·001

Stability of PTH-amino acids and derivatives in phosphate buffer, pH 7.4. The spectrum of a solution of the PTH-amino acid (c. 1×10^{-4} M) in acetonitrile (12%) - phosphate buffer (0·02 M), pH 7·4 was measured on a Unicam SP800 recording spectrophotometer with the cell compartment maintained at 25 °C, over 6 h. The solution was then stored at room temperature (20 °C) until the absorbance reading was constant. The absorption at the maxima (c. λ 260–270 nm) decreased and a new maxima (c. λ 252–7 nm) corresponding to the phenylthiohydantoic acid appeared (Faden & Smith 1982) except for PTH-dehydroserine (315 to 272 nm). A first order plot of log (A_t–A_∞) versus t gave a straight line from which the apparent first order rate constant, k_{obs} for the hydrolysis was calculated (Table 1). The addition of α-chymotrypsin ($3{\cdot}2 \times 10^{-7}$ M) did not affect the rate of degradation.

(IVb) showed a slow decrease in absorption at λ_{max} 281 nm and a simultaneous increase in absorption at λ_{max} 335 nm. (III) showed a two step change in absorption; a very fast decrease in absorption with a shift to λ_{max} 282 nm and then a much slower decrease in absorption associated with an increase in absorption at a new λ_{max} 328 nm. (II) and (IVa) were relatively stable. The absorption, λ_{max} 218–225 nm, decreased very slowly and shifted with an increase in absorption to λ_{max} 240–242 nm.

Pharmacological studies (with R. Sewell). Nociceptive sensitivity was determined using the tail immersion test described by Sewell & Spencer (1976).

Most compounds were inactive as antinociceptive agents though PTH-arginine, PTH-histidine and PTH-hydroxyproline significantly increased nociceptive latencies to a peak at 80 min the overall response lasting up to 2 h after administration. In contrast, the time course of effects of morphine were different since peak effects occurred much earlier at 40 min. Even though at 80 min the nociceptive latencies of morphine were comparable to those of the active PTH derivatives, the opiate was much more potent on the basis of dose (see Table 2).

Discussion

Certain 5-substituted-3-phenyl-2-thioxo-4-imidazolidinones (PTH-amino acids) progressively inhibited α-chymotrypsin in slightly alkaline media, the more active

inhibitors falling into two groups: (1) PTH-dehydroserine, PTH-histidine and PTH-serine which reduced the enzyme level to low or zero titres. (2) PTH-arginine, PTH-proline and PTH-hydroxyproline which reduced the enzyme level after several hours to a constant level (ca. 40% residual activity) (Fig 1). The inhibition of the enzyme by dehydroserine was irreversible as shown by the stability of the inhibited enzyme after removal of excess inhibitor. The reaction between one of the PTH-amino acids (PTH-arginine) and α-chymotrypsin occurred at the active site since the reaction was slowed by a reversible competitive inhibitor of the enzyme, 2-(2′-methylpropyl)-5-phenylthiohydantoic acid (V).

PTH-amino acids contain a phenylamino residue which could slot into the hydrophobic cavity at the active site of the enzyme (Birktoft et al 1970; Birktoft & Blow 1972), and suitably position one of the electrophilic centres, C-4 (carbonyl) or C-2 (thiocarbonyl) for reaction with a nucleophile in the vicinity. Since an acylation reaction is most likely involved here a suitable nucleophile is the Ser-195 hydroxyl group. It was previously proposed (Faden & Smith 1982) that the α-chymotrypsin-catalysed hydrolysis of certain phenylamino thiazolinones proceeded by formation of a Ser-195 ester (acyl ester) of the 2-substituted-5-phenylthiohydantoic acid followed by a de-acylation step where attack of the phenylamino-*N*-atom on the ester gave PTH-amino-acid as the product of the reaction. Consequently reaction at C-4 of the PTH-amino acid does not give a stable acyl ester and reaction is considered to occur at C-2 to give the thiocarbamyl ester of Ser-195. The bond between C-2 and N-3 would be expected to break in preference to the C-2–N-1 bond since the conjugate acid of the acylanilino residue leaving group would be the stronger acid (c.f. Lowry & Richardson 1976). Studies on the rate of hydroxyl ion attack on PTH-amino acids at C-4 to give the phenylthiohydantoic acid showed that potent inhibitors of α-chymotrypsin, (except PTH-proline) were those which were more readily ring opened (Table 1). This correlation requires that the effect of the 5-substituent on the reactivity of the C-4 centre parallels a lesser effect on the C-2 atom through the interlinking nitrogen atom.

The reason for the obvious levelling off of the inhibition reaction with PTH-arginine, PTH-proline and PTH-hydroxyproline with incomplete inhibition of the enzyme is not clear. A possible explanation is that the PTH-amino acid binds non-covalently in two alternative modes, only one of which leads to irreversible inhibition.

(III) prepared in this work was a good irreversible inhibitor of the enzyme and was very labile to hydroxide ion, mainly due to the electron withdrawing effect of the $N_{(1)}$ - NO_2 group since (IVb) where nitration only occurs in the phenyl ring, was relatively stable. Consequently it would be expected that ring opening would occur by hydroxyl ion attack on the more electrophilic C-2 centre and that, by analogy with the PTH-amino acids, a stable carbamyl-enzyme would be formed by reaction with Ser-195.

Many of the PTH-amino acids and derivatives were screened for analgesic activity but only PTH-arginine, PTH-histidine and PTH-hydroxyproline showed weak activity (Table 2). These compounds are among the most potent PTH-amino acid inhibitors of α-chymotrypsin which together suggests that there may be a correlation between this property and the ability to acylate a nucleophilic group on a molecule participating directly or indirectly in the analgesic response. (III), which lacks the lipophilic sulphur atom, was inactive in the test.

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