Competitive inhibition of human skin collagenase by *N*-benzyloxycarbonyl-L-prolyl-L-alanyl-3-amino-2-oxopropyl-L-leucyl-L-alanylglycine ethyl ester

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A 'ketomethylene' peptide, *N*-benzyloxycarbonyl-L-prolyl-L-alanyl-3-amino-2-oxopropyl-L-leucyl-L-alanylglycine ethyl ester, was synthesized and shown to be a fairly potent competitive inhibitor of human skin collagenase.

INTRODUCTION

Natarajan *et al.* (1984) have synthesized a series of 'ketomethylene' peptides as potential inhibitors of angiotensin-converting enzyme. *N*-Benzyl-L-3-amino-4-phenyl-2-oxobutylglycyl-L-proline (II) was marginally the most potent ($IC_{50} = 4$ nM). Other potent inhibitors of angiotensin-converting enzyme include 1-carboxyalkyl-dipeptides and *N*-phosphonodipeptides. Although the replacement of a peptide bond by a secondary amine in all but the last class might suggest that these inhibitors function by acting as ligands for Zn, this seems not to be the case (Patchett *et al.*, 1980; Galardy, 1982; Natarajan *et al.*, 1984). It has been proposed that the methylene bridge occupies the S_1 site in a conformation that is energetically close to that of a putative tetrahedral intermediate.

We decided to synthesize a 'ketomethylene' hexapeptide (I) and test it as a potential reversible inhibitor of another metalloproteinase, human collagenase. This structure was chosen because the analogous peptide, Z-Pro-Ala-Gly-Ile-Ala-Gly-OEt, is a substrate for mammalian collagenases (Kay, 1984).

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, penicillin, streptomycin and amphotericin B were purchased from

Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Other materials were purchased as follows: foetal-calf serum (Randox Laboratories, Crumlin, Antrim, N. Ireland, U.K.), disposable tissue-culture flasks (Gibco, Paisley, Strathclyde, Scotland, U.K.), bovine insulin, prostaglandin E_2 , L-thyroxine, biotin, L-phosphatidylcholine (bovine brain), protamine sulphate (salmon), fluorescamine and 23 lauryl ether (Brij 35) (Sigma Chemical Co., Poole, Dorset, U.K.), Cytodex 1 and Sephacryl 200 (Pharmacia Fine Chemicals, Milton Keynes, Bucks., U.K.), micro carrier spinner flasks and magnetic stirrer (A. R. Horwell, London N.W.6, U.K.). All other materials purchased were of the best commercial grade. Neutral-salt-soluble collagen was isolated from young rat skin by a modification (Jackson & Cleary, 1967) of the method of Gross *et al.* (1955).

Cell culture

Primary cultures were established from fresh human skin obtained at surgery, grown in Dulbecco's modified Eagle's medium containing 20% (v/v) foetal-calf serum, streptomycin (200 μ g/ml), penicillin (200 units/ml) and amphotericin B (2.5 μ g/ml) in plastic tissue-culture flasks incubated at 37 °C in an atmosphere of air/CO₂ (19:1). The cells were cultured at a split ratio of 1:2, and the concentrations of foetal-calf serum and antibiotics were halved. A micro carrier culture (Cytodex 1, 2.0 g) was initiated by the addition of 8.0×10^6 cells. The culture was incubated in medium (1000 ml) as described above until 75% confluence was achieved. The serum-containing medium was then removed and the culture was washed with Dulbecco's phosphate saline buffer (Dulbecco & Vogt, 1954) (3×1000 ml). Collagenase was harvested over a 48 h period in serum-free medium (1000 ml) supplemented with insulin (5.0 μ g/ml) and protamine sulphate (20 μ g/ml), biotin (2.0 ng/ml), L-thyroxine (1.94 μ g/ml) and prostaglandin E_2 (0.1 μ g/ml). The medium was changed after the first 24 h.

Isolation of collagenase

The pooled spent medium was concentrated to about 10 ml, diafiltered with 20 mM-Tris/HCl buffer (500 ml), pH 7.6, containing 170 mM-NaCl, 10 mM-CaCl₂ and 0.05% (w/v) Brij 35 and then further concentrated to

(I)

(II)

Abbreviations used: Z-, benzyloxycarbonyl-; Bz-, benzyl-; -OEt, ethyl ester; -OSu, succinimido-oxy-.

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approx. 5 ml. The latent collagenase was activated with 4-aminophenylmercuric acetate (Sellers & Reynolds, 1977). The activated collagenase was purified by gel filtration on a column (90 cm × 1.5 cm) of Sephacryl 200 that had previously been equilibrated with the sample buffer. The column was irrigated with the same buffer, and fractions containing collagenase activity were concentrated and split into 0.5 ml portions. The enzyme solution was rapidly frozen in liquid N₂ and stored at -70 °C. The purified enzyme gave one major band and two minor bands of lower *M_r* on electrophoresis in an SDS/polyacrylamide gel with a 12–18% linear gradient. The purified collagenase gave the characteristic TC^A and TC^B fragments with neutral-salt-soluble collagen.

Kinetic measurements

The kinetics of the hydrolysis of neutral-salt-soluble collagen (0.2%, w/v) by human skin collagenase (5.2 µg/ml) were followed viscosimetrically at 27 °C (Macartney *et al.*, 1983). First-order kinetics were obeyed under these conditions, where $[S] \ll K_m$. The pseudo-first-order rate constant was evaluated by application of the non-linear least-squares regression method to the equation:

$$[S]_t = [S]_0 \cdot e^{-k_1 t}$$

Values of the pseudo-first-order rate constant were determined by using a range (12.5–62.5 µM) of inhibitor concentrations. If the pseudo-first-order rate constant in the absence of inhibitor is k_0 and in the presence of inhibitor is k_1 , then application of the weighted linear least-squares regression method to the equation:

$$k_0/k_1 = 1 + [I]/K_i$$

permits the evaluation of K_i from the reciprocal of the slope of the fitted line.

Synthesis of *N*-benzyloxycarbonyl-L-prolyl-L-alanyl-glycine

Z-Ala-OSu was coupled to glycine in aqueous *NN*-dimethylformamide, the protecting group was removed by hydrogenolysis over Pd/charcoal and the resultant dipeptide was coupled to Z-Pro-OSu. The product (72%) was used without further purification.

Synthesis of L-leucyl-L-alanylglycine ethyl ester

Z-Ala-OSu was coupled to Gly-OEt, the benzyloxycarbonyl group was removed by hydrogenolysis over Pd/charcoal, the resulting Ala-Gly-OEt was coupled to Z-Leu-OSu and the benzyloxycarbonyl group was removed as before to give the tripeptide ester (80%).

Synthesis of *N*-benzyloxycarbonyl-L-prolyl-L-alanyl-3-amino-1-chloro-2-oxopropane

Z-Pro-Ala-Gly-OH was converted into the chloromethyl ketone (91%), m.p. 127–130 °C, by the standard method of Kettner & Shaw (1978) (Found: C, 55.9; H, 6.1; N, 10.5. C₁₉H₂₄ClN₃O₅ requires C, 55.7; H, 5.9; N, 10.3%).

Synthesis of *N*-benzyloxycarbonyl-L-prolyl-L-alanyl-3-amino-2-oxopropyl-L-leucyl-L-alanylglycine ethyl ester (I)

L-Leucyl-L-alanylglycine ethyl ester hydrochloride (3.2 mmol, 1.04 g) was dissolved in *NN*-dimethylformamide (50 ml). The solution was added to a

Table 1. Inhibition of human skin collagenase by *N*-benzyloxycarbonyl-L-prolyl-L-alanyl-3-amino-2-oxopropyl-L-leucyl-L-alanylglycine

Pseudo-first-order rate constants for the hydrolysis of neutral-salt-soluble collagen (0.2%, w/v) by human skin collagenase at pH 7.6 and 27 °C in the absence and in the presence of inhibitor were determined viscosimetrically (Macartney *et al.* 1983).

Concn. of inhibitor (µM)	10 ³ × <i>k</i> (min ⁻¹)
0.0	8.9 ± 0.3
12.5	7.8 ± 0.5
25.0	6.9 ± 0.3
37.5	5.8 ± 0.3
50.0	4.9 ± 0.5
62.5	4.0 ± 0.5

suspension containing *N*-benzyloxycarbonyl-L-prolyl-L-alanyl-3-amino-1-chloro-2-oxopropane (2.7 mmol, 1.10 g), NaHCO₃ (5.4 mmol, 0.45 g) and NaI (0.1 g) in *NN*-dimethylformamide (50 ml) and the mixture was stirred overnight at room temperature. Solvent was removed under reduced pressure and the residue was taken up in aq. 8% (w/v) NaHCO₃. The solution was extracted with ethyl acetate and the organic layer was dried (over MgSO₄). Removal of solvent gave a yellow oil, which crystallized from ethyl acetate/light petroleum (b.p. 40–60 °C). The product (65%) had m.p. 136–138 °C (Found: C, 58.0; H, 7.5; N, 12.5. C₃₂H₄₈N₆O₉ requires C, 58.2; H, 7.3; N, 12.7%).

RESULTS AND DISCUSSION

First-order rate constants determined in the absence and in the presence of the inhibitor (I) are given in Table 1. Application of the weighted least-squares method to the regression of k_0/k_1 on (I) gave a value of 60 ± 16 µM for K_i calculated from the reciprocal of the slope and an ordinal intercept of 1.04 ± 0.17 , which is not statistically significantly different from unity. Few synthetic inhibitors of vertebrate collagenase have been described. Most have contained a thiol group (Yankeelov *et al.*, 1978; Sundeen & Dejneka, 1981; Gray *et al.*, 1981), but the most effective inhibitor ($K_i = 0.3$ µM) reported is *N*-[3-*N*-(benzyloxycarbonyl)amino-1-(*R*)-carboxypropyl]-L-leucyl-L-*O*-methyltyrosine *N*-methylamide (Delaisse *et al.*, 1985). Design of synthetic collagenase inhibitors is difficult because knowledge of the three-dimensional structure of the enzyme is lacking and we do not know if the interaction between collagen and collagenase induces conformation changes in substrate and/or enzyme. Synthetic substrates designed for collagenase to date have had K_m values about three orders of magnitude greater than the K_m for collagen (Vaes, 1972; Harris & Vater, 1980; Gray *et al.*, 1981; Welgus *et al.*, 1981; Weingarten *et al.*, 1985). This suggests that simple peptides, whether intended as substrates or inhibitors, may not have a conformation that is complementary to the enzyme binding site. Additionally, a short peptide may not be able to adopt the most favourable conformation for catalysis. Nevertheless, the inhibitor described in the present paper has a structure that is

novel for collagenase, and a study of further examples is probably merited.

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