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A NEW CLASS OF SYNTHETIC ELASTASE INHIBITOR

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Summary

Trifluoroacetyl-dialinine-*p*-nitroanilide is a highly potent inhibitor of pancreatic elastase (EC 3.4.21.11) ($K_i = 1.2 \cdot 10^{-7}$ M) and a moderately potent inhibitor of leukocyte elastase ($K_i = 2.5 \cdot 10^{-5}$ M) *meta*-Trifluoromethylbenzoyl-dialanine-*p*-nitroanilide is a potent inhibitor of leukocyte elastase ($K_i = 4 \cdot 10^{-6}$ M) while being completely inactive on pancreatic elastase. The interaction between leukocyte elastase and this inhibitor is considerably stereospecific, since the *ortho* derivative is 65 times less active and the *para* derivative is completely inactive.

Elastases (EC 3.4.21.11) have given rise to more and more interest in recent years because of their involvement in connective tissue diseases [1]. Elastase inhibitors may thus be considered as potential therapeutic agents. Several types of synthetic inhibitors have been designed, including peptide aldehydes [2], peptide carbazates [3], peptides chloromethylketones [4] and trifluoroacetyl-peptides [5]. In this paper we wish to report on a new class of potent reversible elastase inhibitors namely trifluoroacetyl- and trifluoromethylbenzoyl-dipeptide-*p*-nitroanilides.

Porcine pancreatic and human leukocyte elastases were isolated by published methods [6,7] Succinyl-trialanine-*p*-nitroanilide was synthesized as

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Abbreviation CF_3CO, trifluoroacetyl

described earlier [8]. Compounds 1 to 6 of Table II were prepared by acylation of the corresponding alanine, dialanine and trialanine *p*-nitroanilides which were synthesized according to methods published earlier [8] or by the repetitive mixed anhydride procedure [9,10] The acylation step was performed by methods A (compounds 1—3, table II) and B (compounds 4—6, Table II) described below All the compounds were checked by thin-layer chromatography and NMR and gave correct microanalysis values. Melting points are given in Table II The synthesis of compound 2 by method A was performed in the following way Dissolve 15 ml of dry triethylamine in a mixture of 2 5 g (0 0079 mol) of L-alanyl-L-alanine-*p*-nitroanilide hydrochloride and 70 ml of dry methylene chloride. Remove the solvent and the excess of triethylamine under reduced pressure. The residue is dissolved in methylene chloride and the solvent is again removed by evaporation. Then a solution of 2.2 ml (0.0158 mol) of triethylamine in 100 ml methylene chloride is added, followed by a dropwise addition of 3.3 g (0 0158 mol) of trifluoroacetic anhydride in 20 ml of methylene chloride. The mixture is stirred overnight at room temperature and then evaporated to dryness. The residue is triturated with water, the precipitate is collected by filtration and recrystallised twice in a mixture of water and acetic acid

The synthesis of compound 6 by method B will now be described. Cool to −15°C a solution of 1.7 g (0.009 mol) *p*-trifluoromethylbenzoïc acid and 0.91 g (0.009 mol) *N*-methylmorpholine in 15 ml of dimethylformamide. Then add 1 14 g (0.0084 mol) of isobutylchloroformiate and stir the mixture during 2—3 min. Add then in one time a solution of 1.9 g (0.006 mol) of L-alanyl-L-alanine-*p*-nitranilide hydrochloride and 0.6 g (0.006 mol) of *N*-methylmorpholine in 20 ml of dimethylformamide. After 2—3 h of stirring at −15°C, allow to warm up to 0°C and make alkaline with a 2 M solution of CO_3KH After 30 min of stirring add 100 ml of saturated NaCl solution, collect the precipitate and recrystallise in an acetic acid/water mixture.

The rate of *p*-nitroaniline release was measured spectrophotometrically at 410 nm. The elastase concentration was 0.2—0.3 μM. The kinetic constants were derived from double-reciprocal plots Inhibition was assayed by using two different concentrations ($0.5\,K_m$ and $3.0\,K_m$) of the succinyltrialinine-*p*-nitroanilide and 3—5 concentrations of inhibitor. Whenever possible, the range of inhibitor concentration was such that the extent of inhibition was at least up to 50% for each substrate concentration. Pancreatic and leukocyte elastase concentrations were 10 and 100 nM, respectively. The inhibition constants K_i were determined from Dixon plots [11]. Cornish-Bowden's [12] graphical method was also used to ascertain whether inhibition is purely competitive or is mixed

We have previously shown that CF_3CO-peptide-chloromethylketones have lower K_i values and alkylation rate constants for elastase than acetylated inhibitors [13,14]. In the case of pancreatic elastase, NMR spectroscopy demonstrated that this is due to the presence of tightly bound non-productive complexes [15]. In order to confirm this effect with true substrates we have compared the reactivities of trifluoroacetylated and succinylated di- and trialanine-*p*-nitroanilides. From Table I it can be seen that the former compounds have lower K_m and k_{cat} values than the latter ones The effect of trifluoroacetylation (i.e. better binding → lower reactivity), therefore, is similar whether the peptides

TABLE I

KINETIC PARAMETERS FOR THE ELASTASE-CATALYZED HYDROLYSIS OF *N*-ACYL-DI- AND TRIALANINE-*p*-NITROANILIDES AT pH 8 0 and 25°C

The data for the *N*-succinylated substrates [8,18] were obtained in the absence of organic solvent The other compounds were tested in the presence of 1% (v/v) and 7 4% (v/v) of dimethylformamide in the case of leukocyte and pancreatic elastase, respectively Suc = *N*-succinyl, n h , not hydrolyzed for E° = 0 3 μM

Substrates	Porcine pancreatic elastase		Human leukocyte elastase	
	k_{cat} (s^{-1})	K_m (M)	k_{cat} (s^{-1})	K_m (M)
Suc-Ala_2-*p*-nitroanilide	7 0 10^{-2}	1 4 10^{-2}	1 5 10^{-2}	1 3 10^{-3}
CF_3-CO-Ala_2-*p*-nitroanilide	n h	n h	7 1 10^{-4}	3 7 10^{-5}
Suc-Ala_3-*p*-nitroanilide	1 6 10	1 1 10^{-3}	1 5 10^{-1}	5 0 10^{-4}
CF_3CO-Ala_3-*p*-nitroanilide	5 7 10^{-2}	5 1 10^{-5}	1 4 10^{-2}	8 3 10^{-5}

are true substrates (*p*-nitroanilides) or irreversible inhibitors (chloromethylketones). The most likely explanation for this behavior is that a great proportion of CF_3CO-peptide-anilides form tightly bound non-productive complexes which are exclusive from the productive ones. The observed k_{cat} values would then correspond to the small fraction of productive complexes.

Since the CF_3CO-peptide-anilides had rather low K_m values, it was found to be of interest to measure their inhibitory capacity of the elastase/succinyl-trialanine-*p*-nitroanilide system The enzyme concentration used in this system is low enough to prevent significant hydrolysis of the CF_3CO-peptide-anilides. The results are shown in Table II The dialanine derivative is a potent inhibitor of both pancreatic and leukocyte elastase. Shorter or longer peptides are less efficient. The values of K_m (Table I) and K_i (Table II) are very similar. This con-

TABLE II

INHIBITION CONSTANTS AT pH 8 0 AND 25°C AND MELTING POINTS OF *N*-TRIFLUOROACETYL- AND *N*-TRIFLUOROMETHYLBENZOYL-PEPTIDE-*p*-NITROANILIDES

n i , no inhibition

Compound	Inhibitors		K_i (M)	
	Structure	Melting point (°C)	Porcine pancreatic elastase	Human leukocyte elastase
1	CF_3CO-Ala-*p*-nitroanilide	167	1 1 10^{-3} ***	1 0 10^{-4} **
2	CF_3CO-Ala_2-*p*-nitroanilide	218	1 2 10^{-7} *	2 5 10^{-5} **
			1 2 10^{-6} ***	
3	CF_3CO-Ala_3-*p*-nitroanilide	279	5 5 10^{-5} ***	1 0 10^{-4} **
4	*ortho*-CF_3-C_6H_4-CO-Ala_2-*p*-nitroanilide	248	n i	1 7 10^{-3} ***
5	*meta*-CF_3-C_6H_4-CO-Ala_2-*p*-nitroanilide	217	n i	4 0 10^{-6} **
				2 6 10^{-5} ***
6	*para*-CF_3-C_6H_4-CO-Ala_2-*p*-nitroanilide	261	n i	n i

* No organic solvent, ** 1% (v/v) dimethylformamide, *** 7 4% (v/v) dimethylformamide

firms that the productive and the non-productive binding modes of the CF_3CO-peptide-anilides are mutually exclusive

Replacement of the CF_3CO group by a trifluoromethylbenzoyl substituant leads to inhibitors which are highly specific for leukocyte elastase On the other hand, they exhibit a considerable degree of stereospecificity for this enzyme

Cornish-Bowden plots [12] showed that in all cases the inhibition is purely competitive This indicates that at least one subsite of the active centers of the elastases must be occupied by both substrate and inhibitor As shown in Table II, dimethylformamide decreases strongly the affinity of the inhibitors Hydrophobic interactions, therefore, must contribute to a large extent to the binding energy.

Compound 2 is among the most potent synthetic inhibitor of pancreatic elastase reported thus far [2,3,5]. It has about the same efficiency as elastatinal, the natural low molecular weight inhibitor produced by *Actinomycetes* [16] Compound 5 is the most potent synthetic inhibitor of leukocyte elastase reported thus far [3] (The hydrolysis of compound 5 by leukocyte elastase is very small ($k_{cat} \approx 10^{-3}$ s^{-1}).) It has the same K_i value as elasnin, a compound secreted by *Streptomyces* [17]. In addition, it is highly specific for leukocyte elastase This is not the case for other synthetic or natural inhibitors which usually act on both elastases [3,5,16,17].

It is hoped that compounds derived from this new class of elastase inhibitors will prove to be useful therapeutic agents for the treatment of connective tissue diseases

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