DI- AND TRIPEPTIDE N-ALKYL- AND N-ARALKYL AMIDES AS CHYMOTRYPSIN INHIBITORS*

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Dedicated to the memory of Dr Karel Bláha.

Two competitive inhibitors of chymotrypsin, Glt-Ala-Ala-Leu-EtPh and Glt-Ala-Ala-Pro-NH--EtPh, were synthesized and their inhibition constants K_i were determined. The K_i-determination was carried also with a set of peptides of type X-$(Ala)_n$NH-Y, where X is 3-carboxypropionyl- or 4-carboxybutyryl-, *n* is 2 or 3 and Y is methyl, ethyl, diethyl, isopropyl, propyl, butyl, isobutyl and 2-phenylethyl. Chymotrypsin inhibition was observed only with peptides containing an aralkyl residue whereas peptides with an alkyl are without any effect. Glt-Ala-Ala-Leu-NH-Et-Ph shows the highest K_i-value (80 μmol l^{-1})

Trypsin[1], elastase[2] and chymotrypsin[3] inhibitors are the most studied inhibitors of proteolytic enzymes. Chymotrypsin inhibitors have been examined predominantly from the viewpoint of cell transformation, such as, e.g. degranulation[4], of allergic inflammations[5] and fertililization[6].

This study deals with the synthesis and in vitro evaluation of competitive chymotrypsin inhibitors derived from specific synthetic chymotrypsin substrates[7-10]. The substrate specificity of chymotrypsin is dependent on the participation of a hydrophobic amino acid in the scissile bond, i.e. in P_1, preferably of a phenylalanine residue, or alternatively of a tyrosine or tryptophan residue**.

The design of chymotrypsin inhibitors of the type described here was made in a manner analogous to that described for pancreatic elastase inhibitors[13]. We incorporated into site P_1, i.e. into the site of primary interaction, a 2-phenylethylamide residue (decarboxylated phenylalanine) and thus synthesized inhibitors of general

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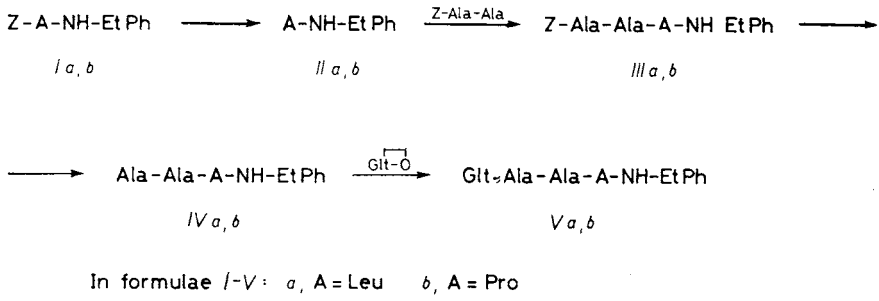
** The symbols and names of amino acids and peptides are according to the suggestions of the IUPAC-IUB Commission of Biochemical Nomenclature[11,12]. All amino acids are of L-configuration (with the exception of glycine). Nan = *p*-nitroanilide, Glt = 4-carboxybutanoyl.

formula Glt-Ala-Ala-X-NH-EtPh, where X in P_2 is a leucine or a proline. The choice of these residues was based on the known substrate specificity of chymotrypsin which prefers leucine and proline in P_2 (ref.[14]). We have also taken into account the fact that a naturally occurring chymotrypsin inhibitor of microbial origin, chymostatin A, has leucine[15,16] in P_2. By contrast a proline residue is located in P_2 of a synthetic chymostatin analog[17].

The synthesis of the chymotrypsin inhibitors was carried out according to Scheme 1. The starting material was either benzyloxycarbonylleucine or benzyloxycarbonylproline 2-phenylethylamide which were prepared from 2-phenylethylamine



SCHEME 1

and the corresponding protected amino acids by the mixed anhydride coupling method. The benzyloxycarbony group was removed by hydrogenolysis under pressure (2 MPa) over 5% Pd/C. The bases obtained were condensed with benzyloxycarbonylalanyl-alanine by the carbodiimide method via the active ester with N-hydroxysuccinimide[18] into the corresponding protected tripeptides. The bases obtained by hydrogenolysis under pressure (2 MPa) were acylated by glutaric anhydride to the final products, i.e. glutarylalanyl-alanyl-leucine and glutarylalanyl-alanyl-proline resp., 2-phenylethylamides. The yields and analytical constants of the intermediates and final products are listed in Table I, the inhibition constants of final products in Table II. In the discussion which follows the inhibition characteristics of newly prepared compounds *Va* and *Vb* are compared with the properties of peptides *VI*–*IX* prepared earlier and assayed with pancreatic elastase[13].

The results obtained show that the decisive role in the competitive inhibition by chymotrypsin inhibitors of our type (cf. Table II) play aralkylamide (2-phenylethyl) groups. Competitive inhibition show peptides containing a 2-phenylethyl residue. By contrast, the amides of tripeptide alanyl-alanyl-alanine (free or acylated by a succinyl residue), substituted by a methyl (Me), diethyl (Et_2), propyl (Pr), isopropyl (iPr), butyl (Bu) or isobutyl (iBu) do not inhibit chymotrypsin at all, cf. *VIIIa*–*e* or *IXa*–*d*.

$$X - (Ala)_n - N - Y$$

	n	X	Y		n	X	Y
VIa	2	Suc	HetPh	*VIIId*	3	H	HBu
VIb	2	Glt	HEtPh	*VIIIe*	3	H	HiBu
VIIa	3	Suc	HEtPh	*IXa*	3	H	HMe
VIIb	3	Glt	HEtPh	*IXb*	3	H	HPr
VIIIa	3	Suc	HMe	*IXc*	3	H	HiBu
VIIIb	3	Suc	Et_2	*IXd*	3	H	HBu
VIIIc	3	Suc	HiPr				

TABLE I

Derivatives of 2-phenylethylamides

Compound Solvent[b]	m.p., °C (yield, %)	$[\alpha]_D^{20}$[a]	Formula (mol. wt.)	Calculated/found % C	% H	% N
Ia	123–125	−31·6°	$C_{22}H_{28}N_2O_3$	71·71	7·66	7·60
A	(76)		(368·5)	71·33	7·55	7·53
Ib	84–86	−59·0°	$C_{21}H_{24}N_2O_3$	71·57	6·86	7·95
A	(87)		(352·4)	71·42	7·14	7·92
IIb	201–204	−42·2°	$C_{13}H_{18}N_2O$	71·52	8·31	12·83
B	(90)		(218·3)	71·54	8·56	12·82
IIIa	207–210	−15·6°	$C_{28}H_{38}N_4O_5$	65·86	7·50	10·97
C	(81)		(510·6)	65·57	7·57	10·86
IIIb	112–115	−43·8°	$C_{27}H_{34}N_4O_5$	65·57	6·93	11·33
D	(60)		(494·6)	65·89	7·57	11·35
IVa	179–182	−13·9°	$C_{20}H_{32}N_4O_3$	63·85	8·57	14·88
E	(90)		(376·5)	63·63	8·63	14·67
IVb	133–135	−107·1°	$C_{19}H_{28}N_4O_7$	63·31	7·83	15·54
F	(80)		(360·5)	63·34	8·17	14·89
Va	206–208	−50·9°	$C_{25}H_{38}N_4O_6$	61·20	7·81	11·42
F	(73)		(490·6)	60·90	7·80	11·54
Vb	150–151	−140·2°	$H_{24}H_{34}N_4O_{10}$	60·74	7·22	11·81
D	(42)		(474·6)	60·38	7·07	12·53

[a] In methanol; [b] A ethyl acetate–light petroleum, B toluene–light petroleum, C N,N-dimethylformamide–2-propanol, D 2-propanol–light petroleum, E ethyl acetate, F 2-propanol.

The most effective inhibitor of our set is glutaryl-alanyl-alanyl-leucine 2-phenylethylamide (*Va*), $K_i = 8{\cdot}0$ μmol l^{-1}, whereas inhibitor *Vb* containing a proline residue in position P_2 is less effective ($K_i = 45$ μmol l^{-1}). By contrast comparable inhibitors containing an alanine residue in position P_2 show considerably lower K_i-values: *VIIa* 170 μmol l^{-1}, *VIIb* 130 μmol l^{-1}, *VIa* 240 μmol l^{-1}, *VIb* 220 μmol . l^{-1}. These results are in good agreement with the optimalization of position P_2 of the chymotrypsin substrate in which the suitability of amino acids decreases in the order leucine > proline > alanine; the preference for the leucine residue in position P_2 is given by its interaction with Ile-99 of chymotrypsin[19]. The favorable effect of a hydrophobic residue in position P_2 has been also demonstrated by X-ray analysis[20] and experimentally confirmed in experiments with irreversible peptide inhibitors (chloromethyl ketones[21]).

The inhibition of chymotrypsin by derivatives containing a 2-phenylethylamide moiety has been reported with other inhibitor types such as, e.g. N-bromoacetylaryl amides[22] and a series of 2-phenylethylamides of N-acetylated amino acids and dipeptides[23]. It has been demonstrated that the 2-phenylethyl residue interacts in position $P_1 - S_1$ by a contribution of 8·4 kJ/mol; nevertheless these derivatives show relatively low K_i-values (of the order of mmol l^{-1}). Likewise, Ito et al. synthesized peptide Ac-Leu-Leu-NH-EtPh, which had practically no inhibitory activity when assayed with the natural substrate (casein)[24]. This discrepancy can be accounted for by the shorter peptide chain of the above dipeptides since the inhibition of chymotrypsin like the chymotryptic cleavage[8] of substrates is affected by the length of the peptide chain: tripeptide *VIIb* (130 μmol l^{-1}) is a more efficient inhibitor than comparable dipeptide *VIb* (220 μmol l^{-1}). The inhibition of chymotrypsin is also affected by the N-terminal anionic residue: the glutaryl residue of *VIIb* is more advantageous (130 μmol l^{-1}) than the comparable succinyl residue of *VIIa* (170 μmol. . l^{-1}).

TABLE II

Inhibition constants (K_i) of 2-phenylethylamides of ω-carboxyalkanoyl peptides

	Inhibitor[a]	K_i (μmol l^{-1})
Va	Glt-Ala-Ala-Leu-NH-EtPh	8·0
Vb	Glt-Ala-Ala-Pro-NH-EtPh	45
VIa	Suc-Ala-Ala-NH-EtPh	240
VIb	Glt-Ala-Ala-NH-EtPh	220
VIIa	Suc-Ala-Ala-Ala-NH-EtPh	170
VIIb	Glt-Ala-Ala-Ala-NH-EtPh	130

[a] Synthetic substrate: Suc-Gly-Gly-Phe-NAn.

The activity of serine proteinases of the chymotrypsin type manifests itself during allergic inflammatory reactions; efficient and nontoxic competitive inhibitors of these enzymes could therefore play the role of important therapeutics[25].

EXPERIMENTAL

Determination of Inhibition Constants K_i

Stock chymotrypsin solution: Bovine pancreatic alpha-chymotrypsin (Boehringer) was dissolved in 5 mM HCl containing 0·5 mM $CaCl_2.H_2O$. The concentration of the enzyme was 4·0M. Stock substrate solution: $2 . 10^{-2}$M succinyl-glycyl-glycyl-phenylalanine *p*-nitroanilide in dimethylformamide. Composition of incubation mixtures: 50 mM Tris. HCl buffer, pH 7·8, 50 mM $CaCl_2$, initial substrate concentration 250—750 μmol l^{-1}, inhibitor concentration 25—250 μmol l^{-1}, chymotrypsin concentration 0·2 μmol l^{-1}, dimethylformamide concentration 5 vol. %. The final volume of the incubation mixture was 2·00 ml. The inhibition of the enzyme activity was determined kinetically at 25°C (SPECOL, K 200 recorder, Zeiss, Jena). The absorbance changes corresponding to the liberated *p*-nitroaniline were recorded at 405 nm. The inhibition constants K_i were determined by the method of Dixon.

Synthetic Inhibitors

The melting points were determined in a Koffler block and were not corrected. The samples for analysis were dried in vacuo at 70 Pa over phosphorus pentoxide at 105°C. Compounds with a melting point below 102°C were dried at room temperature. The optical rotations were determined in a Perkin–Elmer photoelectric polarimeter; the concentration of the samples was 0·2 to 0·3. The evaporation of the samples was effected in a rotary evaporator at reduced pressure. A standard treatment of the compound is represented by its dissolving in an organic solvent and stepwise extraction with 1M HCl, water, 5% sodium bicarbonate, water, drying by anhydrous sodium sulfate and evaporation. Thin layer chromatography was carried out on silica gel plates (Kieselgel G, Merck) in systems 1-butanol–acetic acid–water, 4 : 1 : 1 (S_1) and 1-butanol–acetic acid–pyridine–water, 15 : 3 : 10 : 6 (S_2).

Benzyloxycarbonyl-L-proline 2-Phenylethylamide (*Ib*)

N-Ethylpiperidine (14 ml) and then ethyl chloroformate (10 ml) were added to a solution of benzyloxycarbonyl-L-proline (15 g; 100 mmol) in methylene chloride (200 ml) precooled at −10°C. After 15 min of stirring at 0°C a solution was added of 2-phenylethylamine in methylene chloride (150 ml) which had been released from the corresponding hydrochloride (15·8 g; 100 mmol) by the addition of N-ethylpiperidine (14 ml). The reaction mixture was then stirred 1 h at 0°C, set aside for 12 h at room temperature and taken to dryness. The dry residue was dissolved in ethyl acetate and treated by the standard procedure. The dry residue obtained was crystallized from ethyl acetate (35 ml) and light petroleum (200 ml). The yield was 30·5 g (87%) of a product of m.p. 82–85°C. Benzyloxycarbonyl-L-leucine 2-phenylethylamide (*Ia*) was prepared by the same procedure. The results of the analyses are given in Table I.

L-Proline 2-Phenylethylamide (*IIb*)

Pd/C (5%, 1·5 g, Fluka) in toluene (15 ml) was added to a solution of *Ib* (16·5 g, 47 mmol) in methanol (80 ml). The flask was flushed with nitrogen and hydrogenolysis was carried out at

2 MPa. The catalyst was filtered off on a Seitz EK filter after 20 min, was washed with methanol and toluene and the filtrate was evaporated. The residue was crystallized from toluene and light petroleum. The yield was 9·2 g (90%) of a product of m.p. 202—203°C. The results of the analyses are in Table I. L-Leucine 2-phenylethylamide (*IIa*) was prepared as a noncrystalline product by the same procedure. R_F 0·17 (S_1), 0·42 (S_2).

Benzyloxycarbonyl-L-alanyl-L-alanyl-L-proline 2-Phenylethylamide (*IIIb*)

N,N'-Dicyclohexylcarbodiimide (7·95 g) was added to a solution of benzyloxycarbonyl-L-alanyl-L-alanine (10·6 g; 36 mmol), N-hydroxysuccinimide (4·15 g) in dimethylformamide (200 ml) and tetrahydrofuran (100 ml) cooled down to 0°C. *IIb* (7·9 g) in dimethylformamide (50 ml) was added after 1 h of stirring and cooling (0°C). After 1 h of stirring at 0°C and 12 h of standing at room temperature the N,N'-dicyclohexylurea which had separated was filtered off, the filtrate was evaporated, the residue dissolved in 1-butanol and treated according to the standard procedure. The residue was crystallized from 2-propanol (50 ml) and light petroleum (1 500 ml). The yield was 10·7 g (60%) of a product of m.p. 110—113°C. Benzyloxycarbonyl-L-alanyl-L-alanyl-L-leucine 2-phenylethylamide (*IIIa*) was obtained by the same procedure. The results of the analyses are given in Table I.

L-Alanyl-L-alanyl-L-proline 2-Phenylethylamide (*IVb*)

A solution of *IIIb* (17·7 g; 35·8 mmol) in methanol (100 ml) was hydrogenated under pressure (2 MPa; 40 min) using 5% Pd/C (1·2 g) by a procedure similar to that used with *IIb*. Crystallization from ethyl acetate (250 ml) and light petroleum (1 000 ml) afforded 10·3 g of a product of m.p. 130—132°C. L-Alanyl-L-alanyl-L-leucine 2-phenylethylamide (*IVa*) was synthesized in the same manner. The results of the analyses are given in Table I.

Glutaryl-L-alanyl-L-alanyl-L-leucine 2-Phenylethylamide (*Va*)

A solution of *IVa* (2·3 g; 6 mmol) in dimethylformamide (10 ml) was treated with glutaric anhydride (820 mg; 7·2 mmol) in dimethylformamide (3 ml) and the mixture was heated 2 h at 80°C. It was taken to dryness afterwards and the product was precipitated by ethyl acetate (20 ml). Crystallization from a mixture of water (30 ml) and 2-propanol (5 ml) afforded 2·15 g (73%) of a product of m.p. 201—203°C. The sample for analysis was crystallized from 2-propanol. *Vb* was prepared in an analogous manner. The results of the analyses are given in Table I.

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