INHIBITORS OF PANCREATIC AND LEUKOCYTE ELASTASE

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The following alkylamides of ω-carboxyalkanoyldi- and tripeptides of the Ala-Pro or Ala-Ala-Pro sequence have been prepared: ethylamide-, propylamide-, and isobutylamide of 3-carboxypropionylalanyl-proline, ethylamide of 3-carboxypropionylalanyl-alanyl-proline and ethylamide-, propylamide, and isobutylamide of 4-carboxybutyrylalanyl-alanyl-proline. The inhibitors were synthesized by fragment condensation in solution or by gradual construction. The inhibition constants K_i were determined by means of pancreatic elastase (substrates — *p*-nitroanilides of 4-carboxybutyrylalanyl-alanyl-alanyl-alanine and 3-carboxypropionylalanyl-alanyl-alanyl-alanine) and leukocyte elastase (substrate — *p*-nitroanilide of 4-carboxybutyrylalanyl-alanyl-alanyl-valine). The strongest inhibitions (K_i) were recorded in ethylamide of 4-carboxybutyrylalanyl-alanyl-proline, 2·0 and 1·6 μmol l^{-1} for pancreatic elastase, and in propylamide of 4-carboxybutyryl-alanyl-alanyl-proline, 0·4 mmol l^{-1} for leukocyte elastase.

The ethiology of acute pancreatitis[1,2], pulmonary emphysema[3-5], some forms of joint inflammations[6-8], aneurysm[9], kidney insufficiency[10] is connected with the activity of pancreatic elastase (PE) or leukocyte elastase (LE). Therefore the discovery of specific and non-toxic inhibitors of PE and LE enables their application in pharmacotherapy.

In our preceding studies on inhibitors of PE and LE we investigated the oligomeric series of alkylamides of alanine peptides and we followed the importance of the size of the alkylamide residue, the length of the peptide chain and the length of the N-terminal anionic residue[13-16].

In this paper we have extended and completed a similar series of anionic inhibitors of PE and LE and we synthesized the mentioned inhibitors with an in-built C-terminal proline; the reason for this substitution was to find the advantage of the proline residue in position P_2 in the substrate for PE[17] and for LE[18]. Thus, we prepared ethylamide of succinylalanyl-proline (*Ia*), propylamide of succinylalanyl-proline (*Ib*), isobutylamide of succinylalanyl-proline (*Ic*), ethylamide of alanyl-alanyl-proline

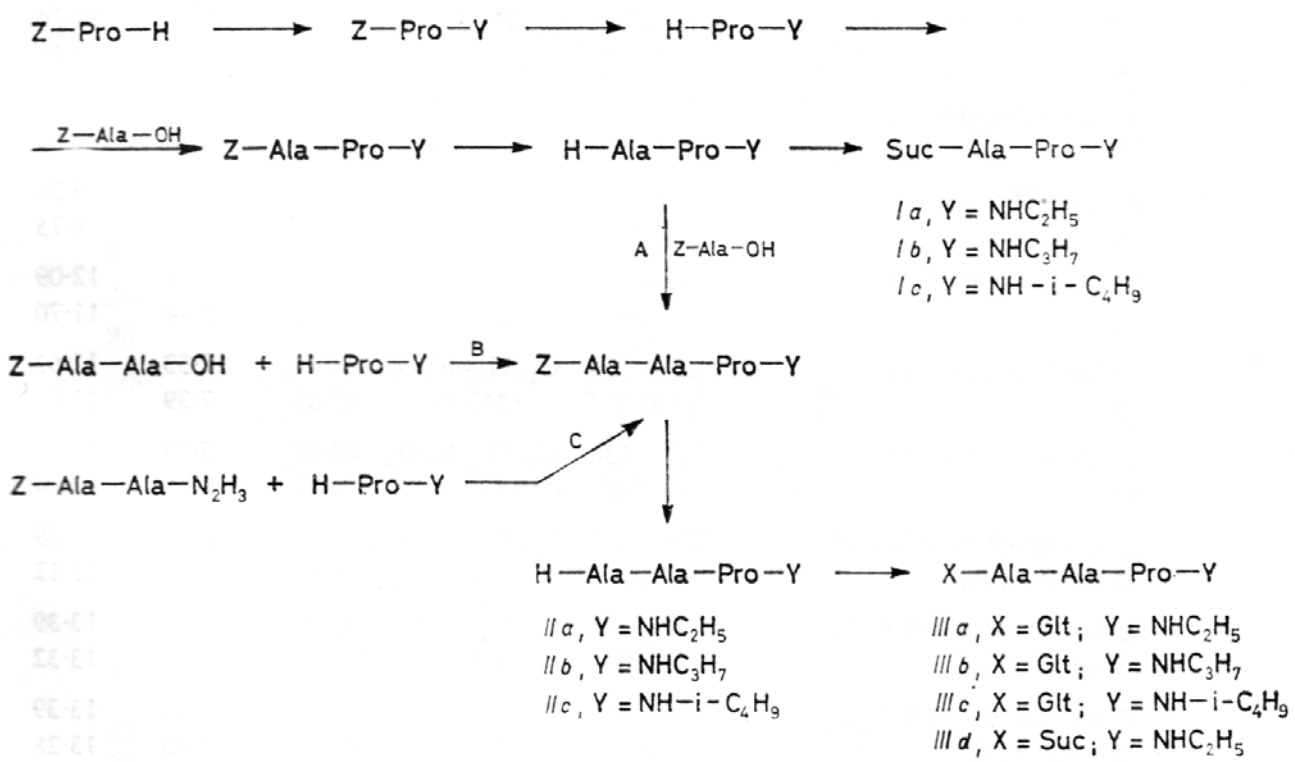
* The symbols and names of amino acids and peptides agree with the IUPAC-IUB Commission on Biochemical Nomenclature[11,12]. All amino acids are of L-configuration. Suc = = succinyl, *i.e.* 3-carboxypropionyl, Glt = glutaryl, *i.e.* 4-carboxybutyryl, NAn = 4-nitroaniline.

(*IIa*), propylamide of alanyl-alanyl-proline (*IIb*), isobutylamide of alanyl-alanyl-proline (*IIc*), ethylamide of glutarylalanyl-alanyl-proline (*IIIa*), propylamide of glutarylalanyl-alanyl-proline (*IIIb*), isobutylamide of glutarylalanyl-alanyl-proline (*IIIc*), and ethylamide of succinylalanyl-alanyl-proline (*IIId*).

The synthesis of alkylamides of benzyloxycarbonylproline and of dipeptides was carried out using the method of mixed anhydrides with ethyl chloroformate.

The synthesis of protected tripeptides was carried out in three variants: the method of gradual construction (method A), the carbodiimide method (2 + 1), from benzyloxycarbonylalanyl-alanine[19] in the presence of N-hydroxybenzotriazole[20] (method *B*), and the azide method from hydrazide of benzyloxycarbonylalanyl-alanine[16] (method *C*). All three methods afforded comparably optically pure products, while method *A* gave the highest optical purity (see ethylamide of benzyloxycarbonylalanyl-alanyl-proline, *IIIa*); method *C* was less suitable and it also gave a lower yield.

A survey of the preparative methods used is given in Scheme 1. The elimination of the protecting benzyloxycarbonyl group was carried out with hydrogen bromide



SCHEME 1

in glacial acetic acid and the hydrobromides obtained were liberated using a basic anion exchanger (Zerolite FF/OH^- form) or ammonia in chloroform. Acylation of free alkylamides of di- and tripeptides was carried out in dimethylformamide with succinic or glutaric anhydride at 80°C. The analytical data and the yields of alkyl-

amides of benzyloxycarbonyl derivatives are given in Table I, and of free ω-carboxyalkanoylpeptides in Table II.

On the basis of our results of the optimalisation of elastolytic inhibitors[16] we used the N-terminal glutaryl residue in the series of tripeptides, and only in one case (*IIId*) a succinyl residue, for the sake of comparison. In the series of dipeptides the succinyl residue was used exclusively.

We have determined the inhibition constants K_i (at two inhibitor concentrations) according to Lineweaver and Burk[21] and Dixon[22]; the K_i values are given in Table III.

TABLE I

Alkylamides of benzyloxycarbonyl-derivatives

Compound yield, %	M.p., °C $[\alpha]_D^{20}$ [a]	Formula (mol. wt.)	Calculated/found		
			% C	% H	% N
Z-Pro-NH-C_2H_5	103—104	$C_{15}H_{20}N_2O_3$	65·20	7·30	10·14
83	−29·4°	(276·3)	64·89	7·54	10·29
Z-Pro-NH-C_3H_7	74—75	$C_{16}H_{22}N_2O_3$	66·19	7·64	9·65
83	−30·4°	(290·4)	66·05	7·41	9·52
Z-Pro-NH-C_4H_9	86—87	$C_{17}H_{24}N_2O_3$	67·08	7·95	9·20
84	−29·0°	(304·4)	67·82	8·18	8·75
Z-Ala-Pro-NH-C_2H_5	99—101	$C_{18}H_{25}N_3O_4$	62·23	7·25	12·09
81	−93·4°	(347·4)	62·13	7·44	11·70
Z-Ala-Pro-NH-C_3H_7	123—124	$C_{19}H_{27}N_5O_4$	63·14	7·53	11·63
73	−97·9° [b]	(361·4)	63·03	7·39	11·28
Z-Ala-Pro-NH-C_4H_9	137—138	$C_{20}H_{29}N_3O_4$	63·98	7·79	11·19
87	−57·5°	(418·6)	64·17	8·06	10·94
Z-Ala-Ala-Pro-NH-C_2H_5	138—139	$C_{21}H_{30}N_4O_5$	60·27	7·23	13·39
43 (method *A*)	−47·0°	(418·5)	59·97	7·21	13·12
Z-Ala-Ala-Pro-NH-C_2H_5	139—140	$C_{21}H_{30}N_4O_5$	60·27	7·23	13·39
51 (method *B*)	−46·5°	(418·5)	60·08	7·55	13·32
Z-Ala-Ala-Pro-NH-C_2H_5	134—136	$C_{21}H_{30}N_4O_5$	60·27	7·23	13·39
15 (method *C*)	−44·8°	(418·5)	60·25	7·03	13·28
Z-Ala-Ala-Pro-NH-C_3H_7	101—103	$C_{22}H_{32}N_4O_5$	61·09	7·46	12·95
81 (method *A*)	−47·1°	(432·5)	61·11	7·69	12·98
Z-Ala-Ala-Pro-NH-C_4H_9	102—103	$C_{23}H_{34}NgO_5$	61·86	7·67	12·55
44 (method *A*)	−48·2°	(446·6)	62·47	7·94	12·01

[a] In N,N-dimethylformamide; [b] in methanol.

The advantages of the N-terminal ω-carboxyl residue (negative charge) in the inhibition of PE are evident from the Table III when compared with the peptides with a free α-amino group (unsubstituted); the differences of K_i are sometimes higher by one order of magnitude: *IIa*/*IIIa* 0·05/0·002, or *IIc*/*IIIc* 0·87/0·085. The proline residue in the C-terminal position is distinctly better than the alanine residue, for example, *IIa*/Ala-Ala-Ala-NH- (ref.[16]) 0·05/0·1 or 0·06/0·2. This is similar in the series of tripeptides *IIIa*/Suc-Ala-Ala-Ala-NH- (ref.[16]) 0·002/0·02 or 0·0015/ /0·02.

The substitution in P_2 with a proline residue in serine substrates is the requirement

TABLE II

Alkylamides of free and ω-carboxyalkanoyl-peptides

Compound yield, %	M.p., °C $[\alpha]_D^{20}$ [a]	Crystn. solvent	Formula (mol. wt.)	Calculated/found		
				% C	% H	% N
Ia	144—146	A	$C_{15}H_{26}N_4O_6$	53·53	7·38	13·38
62	−129·2° [b]		(359·2)	52·90	7·45	12·88
Ib	138—140	A	$C_{15}H_{25}N_2O_5$	54·91	7·68	12·81
66	−122·0°		(328·1)	54·71	7·86	12·98
Ic	156—158	A	$C_{20}H_{29}N_3O_3$	56·17	7·95	12·28
62	−122·5°		(375·5)	55·78	7·97	12·09
IIa	109—111	B	$C_{13}H_{24}N_4O_3$	54·91	8·51	19·70
90	−126·1°		(284·4)	54·43	8·61	19·34
IIb	103—105	B	$C_{14}H_{26}N_4O_3$	56·35	8·78	18·78
71	−135·3°		(298·4)	55·67	8·93	18·78
IIc	120—123	B	$C_{15}H_{27}N_4O_3$	57·67	9·03	17·93
87	−120·2°		(312·4)	57·66	9·14	18·07
IIIa	154—156	C	$C_{18}H_{30}N_4O_4$	54·26	7·59	14·06
91	−137·3° [b]		(398·5)	53·77	7·82	14·61
IIIb	130—134	C	$C_{19}H_{32}N_4O_4$	55·32	7·82	13·58
77	−147·7° [b]		(412·5)	55·25	7·99	13·75
IIIc	101—106	D	$C_{20}H_{34}N_4O_5$	56·32	8·04	13·14
79	−146·2° [b]		(426·5)	56·33	8·19	12·60
IIId	185—186	E	$C_{17}H_{28}N_4O_6$	53·01	7·33	14·55
34	−69·3° [c]		(358·2)	52·74	7·45	14·44

[a] In methanol; [b] in methanol–water 1 : 1; [c] in N,N-dimethylformamide. A methanol/diethyl ether, B benzene/light petroleum, C dimethylformamide/ethyl acetate, D ethyl acetate, E 2-propanol/ /light petroleum.

of a productive bond[23]. In our type of inhibitors we realized a productive bond by the presence of a negative charge in the N-terminal position and the minimal length of the peptide chain, *i.e.* by three amino acid residues[16]. It is possible that the proline residue will also increase the affinity toward elastase, for example by *cis–trans* isomerism, as for example in chymotrypsin substrates[24].

The size of the alkylamide residue and K_i has the same sequence as found in the alanine series[16]: propyl < ethyl < isobutyl, *i.e.* *IIIb* < *IIIa* < *IIIc*.

In contrast to this the design of the inhibitor LE differs to a certain extent. While the electrostatic interaction is dominant in PE, this is not so in LE; this fact was also observed in synthetic substrates. Free alkylamides of tripeptides are less active inhibitors than the corresponding ω-carboxyalkanoyl derivatives, but the differences are not as distinct, for example *IIa*/*IIIa* 4·8/1·1.

The structural proposals of both competitive inhibitors for both enzymes, *i.e.* PE and LE, were derived from their substrate models[25], with the aim of preparing substances with a therapeutic effect. The design of our inhibitors was proposed for the first time in 1982 (ref.[13]); this concept of the modelling of competitive inhibitors for elastase is now accepted[26].

EXPERIMENTAL

The melting points were determined on a Kofler block and they are not corrected. The samples for analysis were dried in a vacuum at 70 Pa over phosphorus pentoxide at 105°C. Substances with a melting point lower than 120°C were dried at room temperature. Optical rotations were

TABLE III

Inhibition constants (K_i) of alkylamides of free and ω-carboxyalkanoyl-peptides. Synthetic substrates: A Glt-$(Ala)_4$-NAn; B Suc-$(Ala)_4$-NAn; C Glt-$(Ala)_3$-Val-NAn.

Inhibitor	K_i, μmol Pancreatic elastase		K_i, mmol Leucocyte elastase
	A	B	C
Ia	0·08	0·11	1·8
Ib	0·02	0·03	2·3
Ic	14·4	2·0	1·8
IIa	0·05	0·06	4·8
IIb	0·05	0·05	2·0
IIc	0·87	1·0	1·7
IIIa	0·002	0·0015	1·1
IIIb	0·0013	0·0012	0·4
IIIc	0·085	0·07	0·9
IIId	0·004	0·006	1·4

measured with a photoelectric polarimeter Perkin–Elmer; the concentration of the solution was 0·2—0·3. The evaporation of the solutions was carried out on a rotational evaporator, under reduced pressure. The standard method of working up the substance consisted in the dissolution in ethyl acetate and gradual extraction with 1M hydrochloric acid, water, 5% sodium hydrogen carbonate and water, drying over sodium sulfate and evaporation. Thin-layer chromatography was carried out on silica gel plates (Kieselgel G, Merck) in the systems 1-butanol–acetic acid–water 4 : 1 : 1 (S_1) and 1-butanol–acetic acid–pyridine–water 15 : 3 : 10 : 6 (S_2).

Benzyloxycarbonylproline Ethylamide

Ethyl chloroformate (40 ml) was added to a solution of benzyloxycarbonylproline (100 g; 400 mmol) in methylene chloride (500 ml) and N-ethylpiperidine (56 ml), under stirring and cooling at —20°C. After 20 min stirring at 0°C the mixture was cooled to —20°C and a 4·26M solution of ethylamine in tetrahydrofuran (110 ml) was then added. After 1 h stirring at 0°C and 12 h standing at room temperature the solution was worked up by the standard method. The residue was crystallized from boiling ethyl acetate (220 ml) and light petroleum (600 ml). Yield 92 g.

Ethylamide of benzyloxycarbonylproline was prepared earlier (ref.[27]) by the carbodiimide method *via* the active ester of N-hydroxy-5-norbornene-2,3-dicarboximide, $[\alpha]_D^{25}$ —38·6° (*c* 0·94; ethanol); our procedure gave a product with $[\alpha]_D^{20}$ —40·7° (*c* 1; ethanol).

Propylamide and isobutylamide of benzyloxycarbonylproline were prepared in a similar manner.

Proline Ethylamide

Hydrogen bromide (36%) in acetic acid (40 ml) was added to a solution of ethylamide of benzyloxycarbonylproline (11 g) in acetic acid (10 ml) and after 1 h diethyl ether (300 ml) was added. The separated non-crystalline hydrobromide was dried in a desiccator over phosphorus pentoxide and sodium hydroxide for 2 h, then suspended in a saturated ammonia solution in chloroform (0°C) (60 ml) and the suspension allowed to stand for 20 min at 3°C. It was filtered and the filtrate evaporated. Yield, 5·7 g of a non-crystalline product; R_F 0·25 (S_1); 0·56 (S_2). Propylamide of proline was prepared in a similar manner, R_F 0·21 (S_1); 0·61 (S_2), as was also isobutylamide of proline, R_F 0·32 (S_1); 0·68 (S_2).

Benzyloxycarbonylalanyl-proline Ethylamide

N,N′-Dicyclohexylcarbodiimide (9·0 g) was added to a solution of ethylamide of proline (5·6 g) and benzyloxycarbonylalanine (9·0 g) in dimethylformamide (5 ml) and tetrahydofuran (60 ml), cooled at —5°C. After 1 h stirring of the mixture the suspension was allowed to stand at room temperature for 12 h. The separated N,N′-dicyclohexylurea was filtered off, the filtrate evaporated and worked up using the standard method. Crystallization from diethyl ether (30 ml) and light petroleum (30 ml) gave 5·4 g of a product melting at 99—101°C. Corresponding propyl- and isobutyl derivatives were prepared in a similar manner.

Alanyl-proline Ethylamide

This was prepared in a similar manner as ethylamide of proline, in quantitative yield as a non-crystalline substance; R_F 0·13 (S_1); 0·57 (S_2). Propylamide of alanyl-proline was prepared analogously, R_F 0·14 (S_1); 0·49 (S_2), as also was isobutylamide of alanyl-proline, R_F 0·23 (S_1); 0·56 (S_2).

3-Carboxypropionylalanyl-proline Ethylamide (*Ia*)

Succinic anhydride (500 mg) was added to a solution of ethylamide of alanyl-proline (490 mg; 2·3 mmol) in dioxane (10 ml) and dimethylformamide (0·25 ml) and the mixture was heated at 80°C for 30 min. After evaporation of the solvent the residue was crystallized from acetone (10 ml) and diethyl ether (30 ml). Yield, 510 mg of a product with m.p. 139—141°C. *Ib* and *Ic* were prepared in a similar manner.

Benzyloxycarbonylalanyl-alanyl-proline Ethylamide

A) N,N′-Dicyclohexylcarbodiimide (5·6 g) was added to a solution of ethylamide of alanyl-proline (prepared from corresponding benzyloxycarbonyl derivative (7·0 g; 25 mmol) and subsequent liberation of the hydrobromide obtained with Zerolite FF in OH-form in methanol) and benzyloxycarbonylalanine (5·6 g; 25 mmol) in methylene chloride (60 ml) cooled at 0°C, and the mixture was allowed to react at this temperature for 2 h and then at room temperature for 12 h. The separated N,N′-dicyclohexylurea was filtered off, the filtrate was evaporated and the residue dissolved in ethyl acetate (50 ml). After 12 h standing at 0°C the separated product was filtered off; yield, 7·1 g of a product melting at 124—127°C. Crystallization from ethyl acetate gave 4·35 g (43%) of a product, m.p. 130—132°C. A sample for analysis was crystallized analogously.

B) N,N′-Dicyclohexylcarbodiimide (11 g) was added to a solution of benzyloxycarbonyl-alanyl-alanine (14·7 g; 50 mmol), N-hydroxybenzotriazole (6·8 g; 50 mmol), and ethylamide of proline (set free from its hydrobromide with a solution of ammonia in chloroform, prepared from corresponding benzyloxycarbonyl derivative (7·6 g) in dimethylformamide (100 ml), cooled at 0°C, and the mixture was further treated as under *A*. Yield, 10·6 g (51%) of a product melting at 128—131°C. Crystallization from ethyl acetate gave 8·95 g of a product, m.p. 131—134°C. Benzyloxycarbonyl derivatives of propylamide and isobutylamide of alanyl-alanyl-proline were prepared analogously. The analytical data and the yields are given in Table I.

C) A solution of sodium nitrite (1·4 g) in water (5·6 ml) was added to hydrazide of benzyloxycarbonylalanyl-alanine (6·17 g; 20 mmol) dissolved in tetrahydrofuran (200 ml) and azeotropic HCl (8 ml) cooled at −10°C, and the mixture was stirred and cooled at −8°C for 8 min. Then precooled ethyl acetate (−20°C) (200 ml) was added and after 2 min stirring at −10°C the organic phase was separated, washed with a cooled saline and sodium hydrogen carbonate solution and dried over anhydrous sodium sulfate. The organic solution was added to a solution of ethylamide of proline (20 mmol) prepared as under *B* in dimethylformamide (20 ml). After 12 h standing at +3°C the reaction mixture was evaporated and the residue crystallized from ethyl acetate to yield 1·25 g (15%) of product, m.p. 128—133°C.

Enzymes. Porcine pancreatic elastase of Serva (cat. No. 20929) was dissolved in 1 mmol l^{-1} of acetic acid; the concentration of the enzyme was 15·5 nmol l^{-1}. Leukocyte elastase was isolated from heparinized human blood[16]; elastolytic activity was 1·35 % ml^{-1}.

Substrates. Suc-$(Ala)_4$-NAn, K_m = 0·356 mmol l^{-1} (ref.[25]); Glt-$(Ala)_4$-NAn, K_m = = 0·314 mmol l^{-1}; Glt-$(Ala)_3$-Val-NAn, K_m = 0·216 mmol l^{-1}; the synthesis of the latter two substrates will be published in the next communication in this Journal.

Inhibition constants. K_i. The incubation medium contained 0·1 ml of a substrate solution (in dimethyl sulfoxide), 1·3 ml of Tris buffer pH 8·0, 0·05 ml of inhibitor in the same buffer, and 0·05 ml of a solution of elastase. The enzymatic activity was determined kinetically (spectrophotometer Unicam SP 800B) by continuous measurement of the 4-nitroaniline set free (25°C, 410 nm). The K_m values of Suc-$(Ala)_4$-NAn, Glt-$(Ala)_4$-NAn, and Glt-$(Ala)_3$-Val-NAn were

determined from three concentrations of substrate (0·3125—0·625—1·25 mmol l^{-1}) by plotting according to Lineweaver–Burk. The inhibition constants K_i were determined at two concentrations of the inhibitor, using the Lineweaver–Burk or Dixon plot.

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