Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 9, pp 1789–1797, 1997

PII: S0968-0896(97)00105-3
0968-0896/97 $17.00 + 0.00

E-64 Analogues as Inhibitors of Cathepsin B. On the Role of the Absolute Configuration of the Epoxysuccinyl Group

Norbert Schaschke,[a] Irmgard Assfalg-Machleidt,[b] Werner Machleidt,[b] Dushan Turk[c] and Luis Moroder[a,*]

[a]*Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany*
[b]*Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie, Ludwig-Maximilians-Universität, 80336 München, Germany*
[c]*Department of Biochemistry and Molecular Biology, Josef Stefan Institute, 61111 Ljubljana, Slovenia*

Abstract—A series of *trans*-epoxysuccinyl-peptide derivatives based on the natural inhibitor E-64 were synthesized in the (2*R*,3*R*) and (2*S*,3*S*) configuration in order to analyze the role of the stereochemistry of this residue in dictating inhibitory potency and selectivity for cysteine proteases. We confirmed that binding of E-64 like *trans*-epoxysuccinyl compounds is remarkably favored by the (2*S*,3*S*) configuration, but we also found that CA030-type compounds are stronger inhibitors in the (2*R*,3*R*) configuration than the related diastereomers. Consequently, the structural requirements for exploiting both the S and S′ subsites are not additive and a structure-based design of bis-peptidyl derivatives of *trans*-epoxysuccinic acid to increase selective inhibition becomes even more difficult. Additional contrasting effects were observed for the pH optima required in the electrostatic interactions at the S and S′ subsites. © 1997 Elsevier Science Ltd.

Introduction

Specific inhibition of cysteine proteases of the papain superfamily has become one of the major targets of drug design as several of these enzymes, particularly the cathepsins are directly involved in a variety of diseases. Thereby the natural product (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucyl-agmatine (E-64) (compound **1** of Fig. 1A), isolated fom *Aspergillus japonicus*,[1,2] has often been used as lead structure. In fact, it represents a very powerful irreversible inhibitor of cysteine proteases although of limited selectivity.[3]

The crystal structures of papain inactivated by E-64[4] and the synthetic analogue (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucyl-isoamylamide (E-64c)[5,6] showed that, in contrast to earlier predictions,[3,7] the E-64 type inhibitors bind to the S subsites of the enzyme in a manner similar to that observed for peptide chloromethane inhibitors,[8] but with a peptide backbone orientation opposite to that of the substrate. Moreover, the X-ray analysis[4] confirmed that the epoxysuccinyl moiety represents the thiol-alkylating entity of E-64 since in support of the NMR data[9] it was shown that the nucleophilic attack of the active site-thiol group of the enzyme on the oxirane ring is taking place at the C-2 position (Fig. 1A) with the formation of the thioether bond.

A screening of synthetic E-64 analogues led to the discovery of the two epoxysuccinyl-peptides EtO-(2*S*,3*S*)-*t*Eps-Ile-Pro-OH (CA030) (compound **2** in Fig. 1B) and *n*PrNH-(2*S*,3*S*)-*t*Eps-Ile-Pro-OH (CA074)[10,11] of high selectivity for cathepsin B. The selectivity of these inhibitors with a free C-terminal carboxyl function was hypothetically attributed to a binding to the S′ subsites of the enzyme.[12,13] Recently, this different mode of binding has been experimentally proved with the crystal structure of the cathepsin B/CA030 adduct[14] whereby the nucleophilic attack by the cysteine-thiol group occurs at the C-3 of the epoxysuccinyl moiety, as shown in Fig. 1B.

The stereochemical requirements for optimal inhibition of cysteine proteases by E-64 has been analyzed with synthetic (2*S*,3*S*)- and (2*R*,3*R*)-E-64 and related

*Address for correspondence: Prof. L. Moroder, Max-Planck-Institut für Biochemie, AG Bioorganische Chemie, Am Klopferspitz 18A, D-82152 Martinsried, Germany; Tel.: 49-89-8578 3905; Fax: 49-89-8578 2847; E-mail: moroder@biochem.mpg.de

Abbreviations: Standard abbreviations as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature are used for amino acids and related derivatives; all amino acids used are of the L-configuration; Agm, agmatine (1-amino-4-guanidino-butane); Adoc, adamantyloxycarbonyl; *t*Eps, *trans*-epoxysuccinyl; DMF, dimethylformamide; MeOH, methanol, BuOH, *n*-butanol; TEA, trietylamine; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; $PrNH_2$, *n*-propylamine; AcOH, acetic acid; AcOEt, ethyl acetate; DMSO, dimethylsulfoxide; DBSI, dibenzosulfimide (bis-phenylsulfonylamine); DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; EDC, *N*-ethyl-*N*′-(3-dimethylamino-propyl)-carbodiimide hydrochloride; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; CE, capillary zone electrophoresis; FABMS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance.

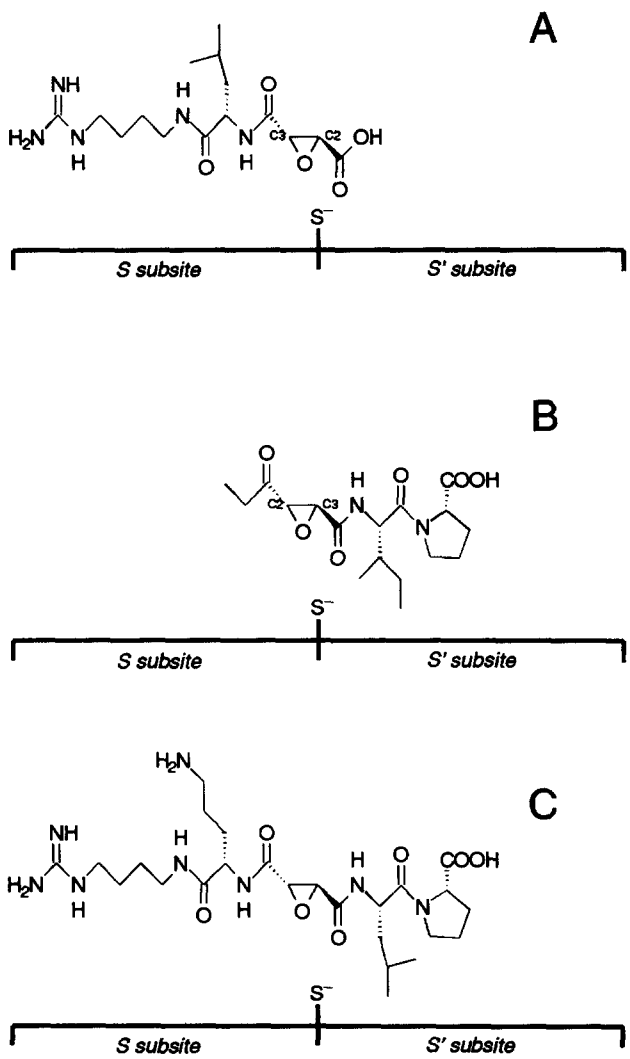


Figure 1. Schematic presentation of the binding mode of epoxysuccinyl-peptides to cathepsin B as predicted by modelling and X-ray crystallography (S^- represents the thiolate function of the active site Cys-29). (**A**) HO-(2*S*,3*S*)-*t*Eps-Leu-Agm (**1**, E-64); (**B**) EtO-(2*S*,3*S*)-*t*Eps-Ile-Pro-OH (**2**, CA030); (**C**) Agm-Orn-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**10b**).

analogues.[3,15,16] In all cases, compounds in the (2*S*,3*S*) configuration, as present in E-64, were found to exhibit a significantly higher inhibitory activity than the corresponding (2*R*,3*R*) diastereomers. Consequently, this configuration has been choosen in the past for the design of new selective inhibitors of cysteine proteases of the papain superfamily.

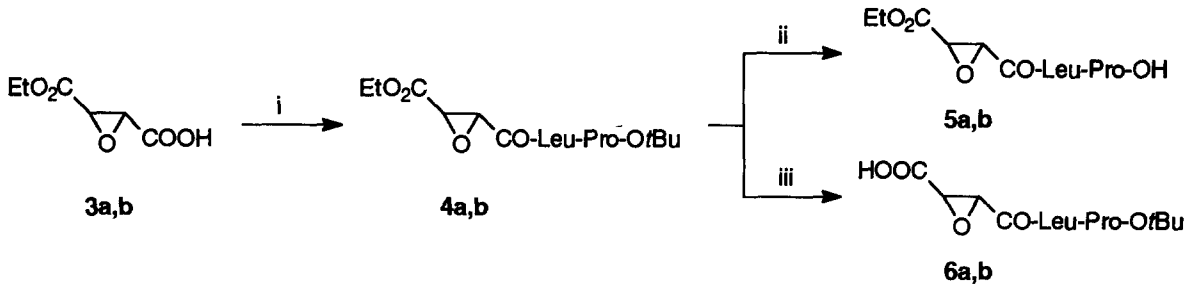
Docking experiments of E-64 to the S subsites of the X-ray structure of cathepsin B[17] in analogy to the binding mode of E-64 to papain[3] clearly revealed that the S2 subsite may easily accomodate the Leu residue.[14] Replacement of leucine with large hydrophobic residues like phenylalanine, tyrosine and related iodinated derivatives is accepted but does not significantly improve cathepsin B selectivity.[16] However, substitution with arginine, also a preferred P2 residue of cathepsin B substrates, leads to collision with the side chain of Glu-245 of cathepsin B which occludes the bottom of the S2 pocket. This observation fully agrees with the experimental results obtained by Gour-Salin et al.[18] with a series of E-64 analogues. In fact, by replacing the P2 residue leucine in HO-*t*Eps-AA-OBzl or HO-*t*Eps-AA-NHBzl with Arg, no improvement of the cathepsin B selectivity could be achieved. In the modelling experiments, even a lysine residue as electrostatic partner for the Glu-245 side-chain carboxylate appeared to be too large, whereas an ornithine residue should possibly be of the correct size to exploit such a buried salt bridge.[14] Moreover, an electrostatic interaction could be derived from the positively charged agmatine that becomes anchored between the carboxylic group of Asp-69 and Tyr-75 of cathepsin B.

The stereochemical requirements of the epoxysuccinyl moiety have only been assessed for optimal binding of related peptides to the S subsites but not to the S' subsites of cysteine proteases. We have therefore analyzed this aspect with a series of epoxysuccinyl-peptides in the (2*S*,3*S*) and (2*R*,3*R*) configuration to gain the information required for the design of bis-peptidyl derivatives of *trans*-epoxysuccinic acid with the potential ability to interact with both the S and S' subsites of cathepsin B (Fig. 1C). With these E-64 analogues we have attempted to disclose the structural requirements that dictate optimal selectivity for this enzyme at the level of molecular recognition.

Results and Discussion

Chemistry

The key intermediates for the synthesis of both the (2*R*,3*R*)- and (2*S*,3*S*)-*trans*-epoxysuccinyl-peptides are the related monoalkyl epoxysuccinates in high configurational purity. The enantiomeric dialkyl esters were synthesised from diethyl L-(+)- and D-(−)-tartrate, respectively, essentially following the stereoselective procedure of Mori and Iwasawa[19] as described previously.[20] These esters were then converted to the related monoethyl (2*R*,3*R*)- and (2*S*,3*S*)-*trans*-epoxysuccinate (**3a,b**). For the synthesis of both the mono- and bis-amidated epoxysuccinyl compounds a maximum acid-labile protection on *tert*-butanol and 1-adamantol basis was applied (Schemes 1 and 2) in order to allow for optimization of the coupling steps. Epoxysuccinyl derivatives have previously been amidated by activation of the carboxyl function with diethylphosphorylcyanide,[2] DCC/HOBt[13,16,21] and various active esters[15,20] whereby yields were not satisfactory in all cases. In the present syntheses we succeeded in generally improving the yields using the EDC/HOBt procedure in the coupling steps. Since standard deprotection protocols with trifluoroacetic acid, as generally applied in peptide chemistry, led to partial hydrolysis of the oxirane ring, the deprotection conditions also had to be optimized. Exposure of the fully protected epoxysuccinyl-peptides to 20% trifluoroacetic acid in dichloromethane at room temperature for 24 h led to the desired (2*R*,3*R*)- and (2*S*,3*S*)-*trans*-epoxysuccinyl-peptides as homogeneous



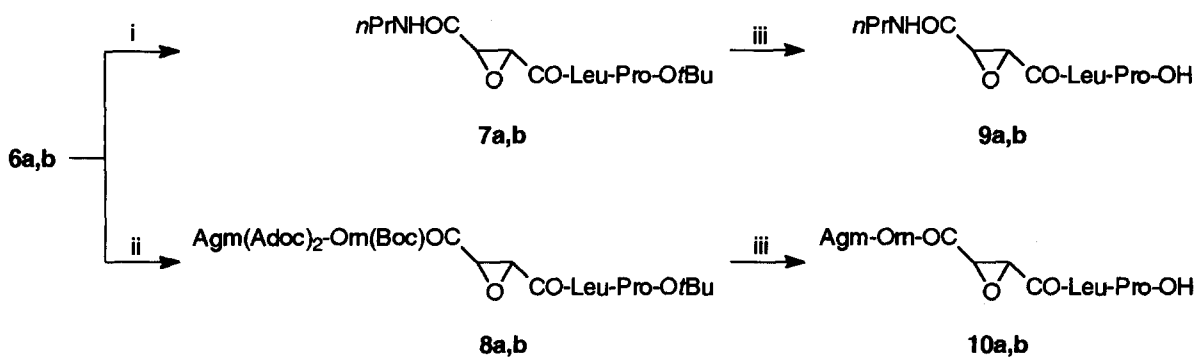
Scheme 1. Synthesis of monoamidated epoxysuccinyl-peptides. Conditions: (i) H-Leu-Pro-O*t*Bu·DBSI/NMM/EDC/HOBt/$CHCl_3$; (ii) 20% TFA/CH_2Cl_2; (iii) KOH/EtOH; (**a**) (2*R*,3*R*)-compounds, (**b**) (2*S*,3*S*)-compounds.

compounds. Applying the new synthetic strategy, compounds **5a** and **5b** were obtained in significantly better yields than previously reported.[13,20] The synthesis of EtO-(2*R*,3*R*)-*t*Eps-Leu-Arg-OH (**11**), EtO-(2*R*,3*R*)-*t*Eps-Leu-Agm·0.5H_2SO_4 (**12**), and of HO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**13**), HO-(2*R*,3*R*)-*t*Eps-Leu-Arg-OH (**14**), HO-(2*R*,3*R*)-*t*Eps-Leu-Agm (**15**) as potassium salts were described elsewhere.[20]

(2*R*,3*R*) vs. (2*S*,3*S*) configuration in CA030-type inhibitors

The second order rate constants (k_2/K_i) for the irreversible inhibition of papain, human cathepsin B and L by E-64 analogues in both the (2*R*,3*R*)- and (2*S*,3*S*)-*t*Eps configuration are reported in Table 1. To allow for direct comparison with literature data the rate constants of EtO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**5b**) were also determined in our cathepsin B assay system. The resulting values of 44,400 M^{-1} s^{-1} at pH 5.5 and 22,300 M^{-1} s^{-1} at pH 6.0 (150 mM NaCl) compare well to the known values of 13,800 M^{-1} s^{-1} at pH 6.0.[13] Similarly, *n*PrNH-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**9b**) exhibited rate constants (153,000 M^{-1} s^{-1} at pH 5.5, 97,500 M^{-1} s^{-1} at pH 6.0, 150 mM NaCl) consistent with those reported for the structurally related *i*BuNH-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (52,000 M^{-1} s^{-1} at pH 6.0).[13] Surprisingly, the diastereomer EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**5a**) was found to exhibit a 13-fold enhanced inhibitory activity and an improved selectivity for cathepsin B relative to papain (1400-fold) and cathepsin L (84-fold). However, in the case of the corresponding *n*-propylamide in the (2*R*,3*R*) configuration (**9a**) the opposite effect was observed. With a k_2/K_i of 29,400 M^{-1} s^{-1} (at pH 5.5) it inhibits cathepsin B with a fivefold reduced efficiency in comparison to the diastereomeric amide (**9b**); at the same time the selectivity for cathepsin B vs. L is reduced by a factor of eleven, whereas relative to papain it is slightly enhanced. Because of the structural similarity of the two compounds these experimental differences are difficult to rationalize.

In the X-ray structure of human cathepsin B inactivated by CA030[14] (Entry 1CSB of Brookhaven Protein Data Bank) the inhibitor binds to the S′ subsites and the resulting 3-thio-2-hydroxysuccinyl moiety is in the (2*S*,3*S*) configuration that can only result from the nucleophilic attack of the active-site thiol group at the C-3 atom of a (2*R*,3*R*)-CA030, although this synthetic *trans*-epoxysuccinyl derivative has been described as the (2*S*,3*S*) diastereomer.[10,11] Correspondingly, both the esters **5** and amides **9** are expected to bind to the S′ subsites in order to exploit the electrostatic interaction of the C-terminal carboxyl group with the two histidine residues (His-110 and His-111) located on the occluding loop (106–124) of cathepsin B.[14] A comparison of the kinetic data of the two pairs of diastereomers (**5a,b** and **9a,b**) clearly reveals a preference of cathepsin B for the (2*R*,3*R*) configuration in the case of the ester **5** and for the (2*S*,3*S*) configuration of the amide **9**. This could possibly derive from an enhanced interaction of compound **9** with the S subsites, for example, of the *n*-propyl moiety with the hydrophobic S2 subsite as well as via hydrogen bonding of the amide proton to the Gly-198 carbonyl.



Scheme 2. Synthesis of bisamidated epoxysuccinyl-peptides. Conditions: (i) *n*$PrNH_2$/EDC/HOBt/$CHCl_3$; (ii) H-Orn(Boc)-Agm(Adoc)$_2$/EDC/HOBt/$CHCl_3$; (iii) 20% TFA/CH_2Cl_2; (**a**) (2*R*,3*R*)-compounds, (**b**) (2*S*,3*S*)-compounds.

Table 1. Second-order rate constants of inactivation of cysteine proteinases by (2*R*,3*R*)- and (2*S*,3*S*)-epoxysuccinyl-peptides

Inhibitor		Papain k_2/K_i ($M^{-1}s^{-1}$)	Cathepsin B k_2/K_i ($M^{-1}s^{-1}$)	Cathepsin L k_2/K_i ($M^{-1}s^{-1}$)	Ratio CB/PA	Ratio CB/CL
HO-(2*S*,3*S*)-*t*Eps-Leu-Agm (E-64)	(1)	869,000 ± 23,500	81,400 ± 2760	43,800 ± 4390	0.09	1.9
EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH	(5a)	56[a]	567,000 ± 21,500	26[a]	10,100	21,800
EtO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH	(5b)	6130 ± 55	44,400 ± 1690	170 ± 17	7.2	260
*n*PrNH-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH	(9a)	5[a]	29,400 ± 1320	46[a]	5900	640
*n*PrNH-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH	(9b)	103 ± 4	153,000 ± 7590	22[a]	1490	6960
Agm-Orn-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH	(10a)	225[a]	63,300 ± 3870	26[a]	281	2440
Agm-Orn-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH	(10b)	6220 ± 535	197,000 ± 8250	250[a]	32	790
EtO-(2*R*,3*R*)-*t*Eps-Leu-Arg-OH	(11)	92 ± 9	291,000 ± 15,800	81 ± 17	3160	3590
EtO-(2*R*,3*R*)-*t*Eps-Leu-Agm	(12)	1030 ± 31	73 ± 5	93[a]	0.07	0.8
HO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH	(13)	3270 ± 108	270 ± 6	74 ± 6	0.08	3.6
HO-(2*R*,3*R*)-*t*Eps-Leu-Arg-OH	(14)	3890 ± 220	520 ± 18	680 ± 76	0.13	0.8
HO-(2*R*,3*R*)-*t*Eps-Leu-Agm	(15)	86,000 ± 3260	1170 ± 40	4930 ± 183	0.01	0.2

k_2/K_i determined at pH 5.5 in 250 mM sodium acetate buffer without NaCl, ± standard error of mean from 6 to 14 single experiments, [a]calculated from k_2 and K_i which were obtained as explained in Experimental. CB, cathepsin B; CL, cathepsin L; PA, papain.

Design and synthesis of cathepsin B inhibitors in the (2*R*,3*R*) configuration

On the basis of these findings the E-64 analogue EtO-(2*R*,3*R*)-*t*Eps-Leu-Arg-OH (**11**) was synthesized to analyze the effect of both the configuration and a C-terminal carboxyl group. This compound was found to inhibit cathepsin B with a surprisingly high selectivity and with a potency only slightly inferior to that of EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**5a**) (Table 1). The high specificity for cathepsin B in comparison to EtO-(2*R*,3*R*)-*t*Eps-Leu-Agm (**12**) and HO-(2*R*,3*R*)-*t*Eps-Leu-Agm (**15**) may be derived from an inverted binding mode, that is to the S′ subsites: the leucine side-chain could insert into the hydrophobic S1′ pocket and the carboxyl function of the Arg residue interact in a salt-bridge manner with the His residues of the occluding loop. Thereby, according to modelling experiments, the guanido function would be exposed to the bulk water environment. This putative binding mode is further supported by the observation that saponification of the ester group of EtO-(2*R*,3*R*)-*t*Eps-Leu-Arg-OH (**11**) is accompanied by loss of cathepsin B inhibition at extents comparable to those resulting from the saponification of EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**5a**) (Table 1).

In order to improve the selectivity of epoxysuccinyl-based inhibitors for cathepsin B by exploiting both the S and S′ subsites, bis-peptidyl derivatives of *trans*-epoxysuccinic acid were designed in which the ethyl ester group of EtO-*t*Eps-Leu-Pro-OH in both the (2*R*,3*R*) and (2*S*,3*S*) configuration was replaced by the pseudo-dipeptide moiety ornithyl-agmatine in order to induce S subsite interactions as suggested by modelling experiments (Fig. 1C). The ornithine residue was expected to interact electrostatically with the Glu-245 on the bottom of the S2 pocket of cathepsin B and the guanido function of agmatine with the side chain carboxylate of Asp-64 and the phenolic group of Tyr-75. The rate constants listed in Table 1 show that the 'chimeric' inhibitor in the (2*S*,3*S*) configuration (**10b**) is threefold more potent against cathepsin B than the (2*R*,3*R*) compound (**10a**), but less selective by a factor of three in respect to cathepsin L. A direct comparison of the bis-peptidyl compounds **10a,b** with the CA030 analogues **5a,b** reveals that in the (2*R*,3*R*) configuration the contribution of the *t*Eps-Leu-Pro-OH moiety to the overall inhibition potency of **10** is lowered by a factor of nine, whereas in the (2*S*,3*S*)-configuration a fivefold enhancement of the rate constant for cathepsin B is observed. Thereby the potential salt bridge between the ornithine side-chain and Glu-245, as suggested from the modelling experiments, seems not to contribute decisively in the inhibitor design as made evident by a comparison of the inhibition potency of *n*PrNH-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**9b**) (153,000 M^{-1} s^{-1}) with that of Agm-Orn-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**10b**) (197,000 M^{-1} s^{-1}).

pH and salt dependence of cathepsin B inhibition

The rates of inhibition of cathepsin B were found to be strongly affected by the pH (Fig. 2 A) in agreement with previous results on the pH dependence of cathepsin B substrate binding.[22,23] The strong pH-effect on all derivatives that are supposed to bind in a CA030-like manner can be explained by electrostatic interaction of the C-terminal carboxylate group of the inhibitor with the two protonated histidine residues (110 and 111) of the occluding loop of cathepsin B. The observed pH profile is consistent with pK_a values similar to those postulated earlier.[23] In contrast, the pH effect on the binding of E-64 is much smaller and could be due to interaction of the carboxylic group of the epoxysuccinyl moiety with His-199 of cathepsin B in analogy to the interaction with His-159 in the case of papain.[4]

A significant salt dependence in the range of 50–150 mM NaCl was observed for the 'chimeric' inhibitor Agm-Orn-*t*Eps-Leu-Pro-OH (**10a,b**) (Fig. 2B) and may be explained by the shielding effect of the salt on electrostatic interactions.

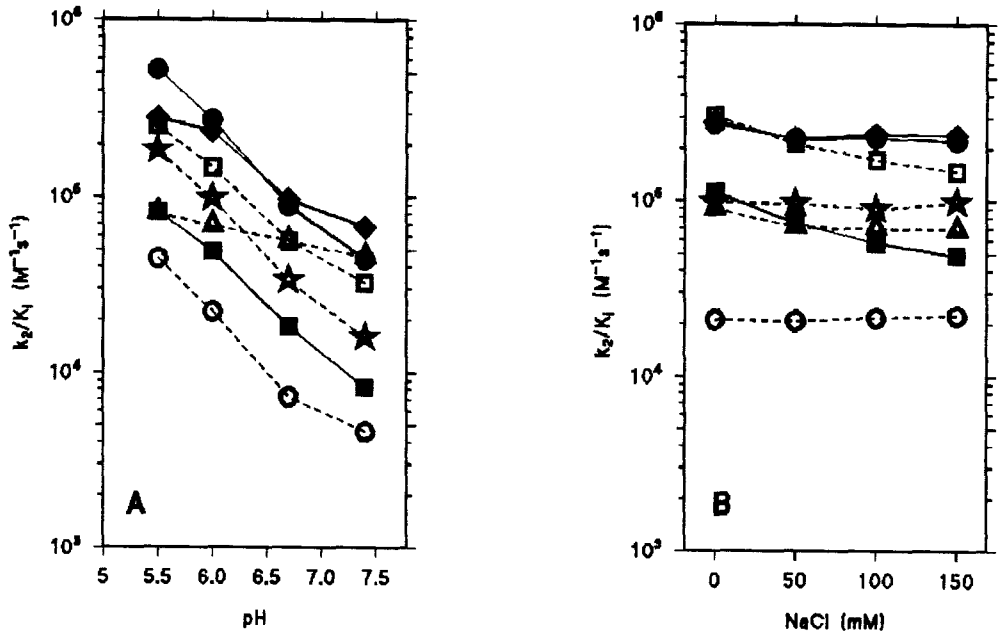


Figure 2. (A) Second-order rate constants measured at different pH values (buffer each 50 mM, for detail see methods) in the presence of 150 mM NaCl. —●— EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**5a**), - - -○- - - EtO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**5b**), —♦— EtO-(2*R*,3*R*)-*t*Eps-Leu-Arg-OH (**11**), - - -△- - - HO-(2*S*,3*S*)-*t*Eps-Leu-Agm (E-64, **1**), - - -☆- - - *n*PrNH-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**9b**), —■— Agm-Orn-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (**10a**), - - -□- - - Agm-Orn-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**10b**). (B) Second order rate constants measured at pH 6.0 (50 mM potassium/sodium phosphate) without and with additional different concentrations of NaCl. For symbols see A.

The strong pH dependence of cathepsin B inhibition by epoxysuccinyl peptides should be considered in the future design of inhibitors for biomedical applications. At the extracellular pH of 7.4 the reactivity of all newly designed cathepsin B inhibitors is comparable to or even lower than that of E-64 (see Fig. 2A). The main advantage of the E-64 analogues seems to be their selectivity (see above). Selectivity for cathepsin B vs. cathepsin L, however, has not been studied at pH 7.4 because of the high instability of the latter enzyme at this pH.[24] Moreover, neglecting pH dependence may be the reason for unexpected differences in the kinetic constants as reported for the same epoxysuccinyl peptides by different laboratories.

Conclusion

The main goal in the design of new cysteine proteinase inhibitors based on the oxirane moiety is certainly their selectivity among the cathepsins. Besides *n*PrNH-*t*Eps-Leu-Pro-OH in the (2*S*,3*S*) configuration (**9b**), EtO-*t*Eps-Leu-Arg-OH, EtO-*t*Eps-Leu-Pro-OH and the 'chimeric' Agm-Orn-*t*Eps-Leu-Pro-OH, all in (2*R*,3*R*) configuration (**11**, **5a**, **10a**), appear to be the most promising lead compounds in terms of selectivity for cathepsin B vs. cathepsin L. From our attempts to combine the structural characteristics of E-64- and CA030-type inhibitors in 'chimeric' constructs we learned that the requirements for optimal interaction with the S and S′ subsites are not simply additive and thus a fully de novo design is needed to further increase the efficiency and selectivity of inhibition. Moreover, the potency of selective cathepsin B inhibitors in the physiological pH range is limited by the marked pH-dependence of their specific interaction with the enzyme.

Experimental

Materials and methods

Solvents and reagents for the synthesis were of the highest quality commercially available. Papain (EC 3.4.22.2) from Boehringer (Mannheim) was repurified on S-Sepharose as described.[25] Human Cathepsin B (EC 3.4.22.1) and Cathepsin L (EC 3.4.22.15) were purchased from Calbiochem (Bad Soden/ Ts.), Z-Phe-Arg-NH-Mec from Bachem (Heidelberg), E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane and all other chemicals from Sigma. CE was performed on a Spectra Phoresis 1000 capillary electrophoresis apparatus (TSP, Darmstadt) at 25 kV using an underivatized fused silica capillary (67 cm × 75 μm; length × ID) and 50 mM sodium borate buffer (pH 8.5). For general methods see ref. 20.

Synthesis of *trans*-epoxysuccinyl-peptides

H-Leu-Pro-O*t*Bu·DBSI. H-Pro-O*t*Bu hydrochloride (11.42 g, 55.0 mmol) was reacted in 50 mL DMF with NMM (6.06 mL; 55.0 mmol) and Z-Leu-OSu (18.12 g; 50.0 mmol) overnight at room temperature. The reaction mixture was worked up by standard procedures of peptide chemistry and the resulting Z-Leu-Pro-O*t*Bu (oil) was hydrogenated over Pd/C in

95% aqueous MeOH (100 mL). The catalyst was filtered off and the solution evaporated to dryness. The product was isolated from MeOH as DBSI salt by precipitation with ether; yield: 16.9 g (55%); homogeneous on TLC (BuOH:AcOH:H_2O:AcOEt, 3:1:1:5); mp 177–178 °C; $[\alpha]_D^{24}$ −48.1° (*c* 1, MeOH); FABMS: *m/z* 285.3 $[M+H]^+$; calcd for $C_{15}H_{28}N_2O_3$: 284.2.

Z-Orn(Boc)-Agm(Adoc)$_2$. To agmatine sulfate (2.00 g; 8.80 mmol) in water:CH_3CN (1:1, 130 mL) Z-Orn(Boc)-OSu (3.71 g, 8.00 mmol; prepared by standard procedures in 98% yield; mp 81–83 °C; $[\alpha]_D^{24}$ −9.6° (*c* 1, $CHCl_3$)) and TEA (1.22 mL; 8.80 mmol) were added. Some precipitate was filtered off and the aqueous solution was extracted with BuOH (5 ×). The combined organic layers were evaporated to a small volume and Z-Orn(Boc)-Agm·0.5H_2SO_4 was precipitated with ether; yield: 2.17 g (51%); homogeneous on TLC (BuOH:AcOH:H_2O:AcOEt, 3:1:1:5).

To an ice-cold solution of Z-Orn(Boc)-Agm·0.5H_2SO_4 (0.65 g, 1.2 mmol) in 10 mL dioxane 0.1 N NaOH (1.3 mL) was added followed by adamantyl fluoroformate[26] (1 g, 5.0 mmol) and 0.1 N NaOH (5 mL). After 16 h, equal amounts of adamantyl fluoroformate and 0.1 N NaOH were added and finally again after 8 h. The reaction mixture was stirred for additional 16 h, then the reaction mixture was diluted with water and extracted with AcOEt. The combined organic layers were evaporated and the residue was chromatographed on silica gel 60 (eluent: cyclohexane:$CHCl_3$:CH_3CN, 10:25:10); yield: 0.69 g (69%); homogeneous on TLC (cyclohexane:$CHCl_3$:CH_3CN, 10:25:10); ^{1}H NMR (DMSO-d_6) δ 1.36 (s, 9H, OC(C$\underline{H}_3$)$_3$), 1.61, 1.63 (2 br. s, 12H, Adoc), 1.30–1.70 (br. m, 8H, βCH_2 Agm, γCH_2 Agm, βCH_2 Orn, γCH_2 Orn), 2.06, 2.11, 2.16 (3 br. s, 18H, Adoc), 2.88 (m, 2H, δCH_2 Orn), 3.04 (m, 2H, αCH_2 Agm), 3.77 (m, 2H, δCH_2 Agm), 3.91 (m, 1H, αCH Orn), 4.99, 5.02 (2 d, 2H, *J* = 12.6 Hz, C$\underline{H}_2$-C$_6$H$_5$), 6.71 (m, 1H, δNH Orn), 7.25 (d, 1H, *J* = 8.1 Hz, NH Orn), 7.27–7.37 (m, 5H, CH$_2$-C$_6\underline{H}_5$), 7.81 (m, 1H, αNH Agm), 9.03 (br. s, 2H, Adoc-N$\underline{H}$-(C=N$\underline{H}$)); FABMS: *m/z* 835.7 $[M+H]^+$; calcd for $C_{45}H_{66}N_6O_9$: 834.48.

H-Orn(Boc)-Agm(Adoc)$_2$. Z-Orn(Boc)-Agm(Adoc)$_2$ (0.5 g; 0.6 mmol) was hydrogenated in 40 mL MeOH:H_2O (95:5) over Pd/C. The catalyst was filtered off, and the solution was evaporated to dryness. Yield: 0.42 g (quantitative); homogeneous on TLC ($CHCl_3$:MeOH, 9:1); ^{1}H NMR (DMSO-d_6) δ 1.37 (s, 9H, OC(C$\underline{H}_3$)$_3$), 1.61, 1.63 (2 br. s, 12H, Adoc), 1.35–1.70 (br. m, 8H, βCH_2 Agm, γCH_2 Agm, βCH_2 Orn, γCH_2 Orn), 2.06, 2.11, 2.17 (3 br. s, 18H, Adoc), 2.90 (m, 2H, δCH_2 Orn), 3.08, 3.18 (2 m, 2H, αCH_2 Agm), 3.67 (m, 1H, αCH Orn), 3.77 (m, 2H, δCH_2 Agm), 6.84 (m, 1H, δNH Orn), 8.16 (br. s, 2H, αNH_2 Orn), 8.30 (br. s, 1H, αNH Agm), 9.08 (br. s, 2H, Adoc-N$\underline{H}$-(C=N$\underline{H}$)); FABMS: *m/z* 701.6 $[M+H]^+$; calcd for $C_{37}H_{60}N_6O_7$: 700.45.

Coupling with EDC/HOBt. To a solution of the mono-ethyl ester or mono-amidated epoxysuccinate (1 mmol) and the amino component (1 mmol) in 5 mL $CHCl_3$ NMM (1 mmol) was added when neutralization of the amino component was required, followed by HOBt (1 mmol). After cooling in an ice-bath EDC (1.1 mmol) was added and the reaction mixture was stirred for 2 h at room temperature. The solvent was removed and the residue distributed between AcOEt and water. The organic phase was washed with a 5% $KHSO_4$, 5% $NaHCO_3$ and brine and then dried over Na_2SO_4. The solution was evaporated and the residue was chromatographed on silica gel 60 using appropriate eluents.

Saponification of epoxysuccinyl-peptides monoethyl esters. The ethyl esters (1 mmol) were dissolved in 10 mL EtOH and then reacted in an ice-bath under vigorous stirring with KOH (1 mmol) dissolved in 5 mL EtOH. After 3 h the solvent was evaporated and the residue distributed betwen water and AcOEt. The aqueous phase was acidified with 5% $KHSO_4$ and immediately extracted with AcOEt (3 ×). The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated to dryness.

Acidolytic deprotection. The fully protected epoxysuccinyl-peptides (1 mmol) were dissolved in dichloromethane (2.4 mL) and reacted at room temperature with trifluoroacetic acid (0.6 mL) for 24 h. The solvent was evaporated and the product precipitated with methyl *tert*-butyl ether.

EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-O*t*Bu (4a). Isolated by silica gel chromatography (eluent: AcOEt:petroleum ether, 1:1) in 67% yield; homogeneous on HPLC (t_R = 22.83 min); ^{1}H NMR ($CDCl_3$) δ 0.95 (d, 3H, *J* = 6.5 Hz, δ$_2$CH_3 Leu), 1.00 (d, 3H, *J* = 6.3 Hz, δ$_1$CH_3 Leu), 1.28 (t, 3H, *J* = 7.1 Hz, OCH_2-C$\underline{H}_3$) 1.44 (s, 9H, OC(C$\underline{H}_3$)$_3$), 1.55 (m, 2H, βCH_2 Leu), 1.70 (m, 1H, γCH Leu), 1.87–2.11 (m, 3H, β$_2$CH_2 Pro, γCH_2 Pro) 2.18 (m, 1H, β$_1$CH_2 Pro), 3.54 (d, 1H, *J* = 1.8 Hz, *t*Eps CH), 3.57 (m, 1H, δ$_2$CH_2 Pro), 3.65 (d, 1H, *J* = 1.8 Hz, *t*Eps CH), 3.72 (m, 1H, δ$_1$CH_2 Pro), 4.23 (m, 2H, OC$\underline{H}_2$-CH$_3$), 4.36 (m, 1H, αCH Pro), 4.76 (m, 1H, αC$\underline{H}$ Leu), 6.59 (d, 1H, *J* = 9.0 Hz, NH Leu); FABMS: *m/z* 427.5 $[M+H]^+$; calcd for $C_{21}H_{34}N_2O_7$: 426.23.

EtO-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (5a). Yield: 90% (28%)[20]; homogeneous on HPLC (t_R = 14.07 min); ^{1}H NMR (DMSO-d_6) δ 0.89 (2 d, 6H, *J* = 7.1 Hz, δ$_1$CH_3 Leu, δ$_2$CH_3 Leu), 1.22 (t, 3H, *J* = 7.0 Hz, OCH_2-C$\underline{H}_3$), 1.36–1.56 (m, 2H, βCH_2 Leu), 1.63 (m, 1H, γCH Leu), 1.76–1.99 (m, 3H, β$_2$CH_2 Pro, γCH_2 Pro), 2.16 (m, 1H, β$_1$CH_2 Pro), 3.51 (m, 1H, δ$_2$CH_2 Pro), 3.58 (d, 1H, *J* = 1.4 Hz, *t*Eps CH), 3.70 (m, 1H, δ$_1$CH_2 Pro), 3.71 (d, 1H, *J* = 1.4 Hz *t*Eps CH), 4.18 (m, 2H, OC$\underline{H}_2$-CH$_3$), 4.26 (m, 1H, αCH Pro), 4.57 (m, 1H, αCH Leu), 8.75 (d, 1H, *J* = 8.0 Hz, NH Leu); FABMS: *m/z* 371.4 $[M+H]^+$; calcd for $C_{17}H_{26}N_2O_7$: 370.17.

HO-(2*R*,3*R*)-*t*Eps-Leu-Pro-O*t*Bu (6a). Yield 83%; homogeneous on HPLC (t_R = 19.70 min); ^{1}H NMR ($CDCl_3$) δ 0.97 (2 d, 6H, J = 6.5 Hz, δ_2CH_3 Leu, δ_1CH_3 Leu), 1.43 (s, 9H, $OC(C\underline{H}_3)_3$), 1.53 (m, 1H, β_2CH_2 Leu), 1.64 (m, 1H, $\beta_1C\underline{H}_2$ Leu), 1.72 (m, 1H, γCH Leu), 1.90–2.11 (m, 3H, β_2CH_2 Pro, γCH_2 Pro) 2.21 (m, 1H, β_1CH_2 Pro), 3.58 (d, 1H, J = 1.8 Hz, *t*Eps CH), 3.59 (m, 1H, δ_2CH_2 Pro), 3.67 (d, 1H, J = 1.8 Hz, *t*Eps CH), 3.82 (m, 1H, δ_1CH_2 Pro), 4.39 (m, 1H, αCH Pro), 4.78 (m, 1H, αCH Leu), 7.08 (d, 1H, J = 8.7 Hz, NH Leu); FABMS: *m/z* 399.4 $[M+H]^+$; calcd for $C_{19}H_{30}N_2O_7$: 398.20.

***n*PrNH-(2*R*,3*R*)-*t*Eps-Leu-Pro-O*t*Bu (7a)**. Isolated by silica gel chromatography (eluent: AcOEt:petroleum ether 4:1) in 51% yield; homogeneous on HPLC (t_R = 18.70 min); ^{1}H NMR ($CDCl_3$) δ 0.91 (t, 3H, J = 7.5 Hz, NH-CH_2-CH_2-$C\underline{H}_3$), 0.95 (d, 3H, J = 6.6 Hz, δ_2CH_3 Leu), 1.00 (d, 3H, J = 6.6 Hz, δ_1CH_3 Leu), 1.44 (s, 9H, $OC(C\underline{H}_3)_3$), 1.48–1.65 (m, 4H, βCH_2 Leu, NH-CH_2-$C\underline{H}_2$-CH_3), 1.71 (m. 1H, γCH Leu), 1.86–2.12 (m, 3H, $\beta_2C\underline{H}_2$ Pro, γCH_2 Pro), 2.21 (m, 1H, β_1CH_2 Pro), 3.22 (m, 2H, NH-$C\underline{H}_2$-CH_2-CH_3), 3.46 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.52 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.57 (m, 1H, δ_2CH_2 Pro), 3.72 (m, 1H, δ_1CH_2 Pro), 4.36 (m, 1H, αCH Pro), 4.78 (m, 1H, αCH Leu), 6.00 (m, 1H, N$\underline{H}$-CH_2-CH_2-CH_3), 6.62 (d, 1H, J = 9.0 Hz, NH Leu); FABMS: *m/z* 440.5 $[M+H]^+$; calcd for $C_{22}H_{37}N_3O_6$: 439.26.

Agm(Adoc)$_2$-Orn(Boc)-(2*R*,3*R*)-*t*Eps-Leu-Pro-O*t*Bu (8a). Isolated by silica gel chromatography (eluent: $CHCl_3$:CH_3CN, 10:4) in 33% yield; homogeneous on HPLC (linear gradient of CH_3CN:2% H_3PO_4 from 5:95 to 80:20 in 30 min, then isocratic CH_3CN:2% H_3PO_4 80:20 10 min; t_R = 31.29 min); ^{1}H NMR (DMSO-d_6) δ 0.89 (d, 3H, J = 6.4 Hz, δ_2CH_3 Leu), 0.90 (d, 3H, J = 6.4 Hz, δ_1CH_3 Leu), 1.36, 1.37 (2 s, 18H, $OC(C\underline{H}_3)_3$), 1.61, 1.63 (2 br. s, 12H, Adoc), 1.30–1.70 (br. m, 11H, βCH_2 Agm, γCH_2 Agm, βCH_2 Orn, γCH_2 Orn, βCH_2 Leu, γCH Leu), 1.79 (m, 1H, β_2CH_2 Pro), 1.93 (m, 2H, γCH_2 Pro), 2.06, 2.11, 2.16 (3 br. s, 19H, Adoc, β_1CH_2 Pro (below overlapping singlets)), 2.88 (m, 2H, δCH_2 Orn), 3.05 (m, 2H, αCH_2 Agm), 3.49 (m, 1H, δ_2CH_2 Pro), 3.60 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.63 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.70 (m, 1H, δ_1CH_2 Pro), 3.77 (m, 2H, δCH_2 Agm), 4.18 (m, 2H, αCH Pro, αCH Orn), 4.57 (m, 1H, αCH Leu), 6.72 (m, 1H, δNH Orn), 7.95 (m, 1H, αNH Agm), 8.48, 8.67 (2 d, 2H, J = 8.1 Hz, J = 8.0 Hz, NH Orn, NH Leu), 9.03 (br. s, 2H, Adoc-N$\underline{H}$-(C=N$\underline{H}$)); FABMS: *m/z* 1081.6 $[M+H]^+$; calcd for $C_{56}H_{88}N_8O_{13}$: 1080.64.

***n*PrNH-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH (9a)**. Yield: 68%; homogeneous on HPLC (t_R = 12.90 min); ^{1}H NMR ($CDCl_3$) δ 0.88–1.01 (m, 9H, δ_2CH_3 Leu, δ_1CH_3 Leu, NH-CH_2-CH_2-$C\underline{H}_3$), 1.46–1.76 (m, 5H, βCH_2 Leu, γCH Leu, NH-$C\underline{H}_2$-$C\underline{H}_2$-CH_3), 2.00–2.32 (m, 4H, βCH_2 Pro, γCH_2 Pro), 3.23 (m, 2H, NH-$C\underline{H}_2$-CH_2-CH_3), 3.53 (br. s, 1H, *t*Eps CH), 3.57 (br. s, 1H, *t*Eps CH), 3.60 (m, 1H, δ_2CH_2 Pro), 3.82 (m, 1H, δ_1CH_2 Pro), 4.53 (m, 1H, αCH Pro), 4.79 (m, 1H, αCH Leu), 6.30 (m, 1H, N$\underline{H}$-CH_2-CH_2-CH_3), 6.97 (d, 1H, J = 8.1 Hz, NH Leu); FABMS: *m/z* 440.5 $[M+H]^+$; calcd for $C_{22}H_{37}N_3O_6$: 439.26.

Agm-Orn-(2*R*,3*R*)-*t*Eps-Leu-Pro-OH trifluoroacetate (10a). Yield: 50%; homogeneous on HPLC (linear gradient of CH_3CN:2% H_3PO_4 from 5:95 to 12.5: 87.5 in 30 min; t_R = 10.15 min) and CE (t_m = 3.17 min); ^{1}H NMR (DMSO-d_6) δ 0.90 (2 d, 6H, J = 6.4 Hz, δ_2CH_3 Leu, δ_1CH_3 Leu), 1.35–1.77 (br. m, 11H, βCH_2 Agm, γCH_2 Agm, βCH_2 Orn, γCH_2 Orn, βCH_2 Leu, γCH Leu), 1.85 (m, 1H, β_2CH_2 Pro), 1.94 (m, 2H, γCH_2 Pro), 2.16 (m, 1H, β_1CH_2 Pro), 2.77 (m, 2H, δCH_2 Orn), 2.98–3.16 (br. m, 4H, αCH_2 Agm, δCH_2 Agm), 3.52 (m, 1H, δ_2CH_2 Pro), 3.62 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.66 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.70 (m, 1H, δ_1CH_2 Pro), 4.20–4.27 (m, 2H, αCH Pro, αCH Orn), 4.57 (m, 1H, αCH Leu), 7.03 (br. s, 3H, $\underline{H}_2$N-(C=N$\underline{H}$) Agm), 7.50 (m, 1H, δNH Agm), 7.66 (br. s, 2H, δN$\underline{H}_2$ Orn), 8.06 (m, 1H, αNH Agm), 8.60, 8.63 (2 d, 2H, J = 8.1 Hz, NH Orn, NH Leu); FABMS: *m/z* 569.4 $[M+H]^+$; calcd for $C_{25}H_{44}N_8O_7$: 568.33.

EtO-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu (4b). Isolated by silica gel chromatography (eluent: AcOEt:petroleum ether, 1:1) in 69% yield; homogeneous on HPLC (t_R = 23.3 min); ^{1}H NMR ($CDCl_3$) δ 0.94 (d, 3H, J = 6.3 Hz, δ_2CH_3 Leu), 0.99 (d, 3H, J = 6.3 Hz, δ_1CH_3 Leu), 1.31 (t, 3H, J = 7.2 Hz, OCH_2-$C\underline{H}_3$) 1.45 (s, 9H, $OC(C\underline{H}_3)_3$), 1.52–1.66 (m, 3H, βCH_2 Leu, γCH Leu), 1.88–2.12 (m, 3H, β_2CH_2 Pro, γCH_2 Pro) 2.20 (m, 1H, β_1CH_2 Pro), 3.45 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.58 (m, 1H, δ_2CH_2 Pro), 3.63 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.72 (m, 1H, δ_1CH_2 Pro), 4.19–4.32 (m, 2H, $OC\underline{H}_2$-CH_3), 4.39 (m, 1H, αCH Pro), 4.79 (m, 1H, αC$\underline{H}$ Leu), 6.76 (d, 1H, J = 8.8 Hz, NH Leu); FABMS: *m/z* 427.5 $[M+H]^+$ calcd for $C_{21}H_{34}N_2O_7$: 426.23.

EtO-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (5b). Yield: 71% (51.3%)[13]; homogeneous on HPLC (t_R = 13.97 min); ^{1}H NMR (DMSO-d_6) δ 0.90 (2 d, 6H, J = 6.5 Hz, δ_1CH_3 Leu, δ_2CH_3 Leu), 1.22 (t, 3H, J = 7.0 Hz, OCH_2-$C\underline{H}_3$), 1.47 (m, 2H, βCH_2 Leu), 1.65 (m, 1H, γCH Leu), 1.84 (m, 1H, β_2CH_2 Pro), 1.93 (m, 2H, γCH_2 Pro), 2.15 (m, 1H, β_1CH_2 Pro), 3.51 (m, 1H, δ_2CH_2 Pro), 3.58 (br. s, 1H, *t*Eps CH), 3.68 (m, 1H, δ_1CH_2 Pro), 3.71 (br. s, 1H, *t*Eps CH), 4.19 (m, 2H, $OC\underline{H}_2$-CH_3), 4.25 (m, 1H, αC$\underline{H}$ Pro), 4.61 (m, 1H, αC$\underline{H}$ Leu), 8.70 (d, 1H, J = 8.0 Hz, NH Leu); FABMS: *m/z* 371.4 $[M+H]^+$; calcd for $C_{17}H_{26}N_2O_7$: 370.17.

HO-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu (6b). Yield: 84%; homogeneous on HPLC (t_R = 16.91 min); ^{1}H NMR ($CDCl_3$) δ 0.96 (d, 3H, J = 6.5 Hz, δ_2CH_3 Leu), 0.97 (d, 3H, J = 6.5 Hz, δ_1CH_3 Leu), 1.44 (s, 9H, $OC(C\underline{H}_3)_3$), 1.52 (m, 1H, β_2CH_2 Leu), 1.69 (m, 1H, $\beta_1C\underline{H}_2$ Leu), 1.78 (m, 1H, γCH Leu), 1.93–2.14 (m, 3H, β_2CH_2 Pro, γCH_2 Pro) 2.24 (m, 1H, β_1CH_2 Pro), 3.47 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.58 (d, 1H, J = 2.0

Hz, *t*Eps CH), 3.60 (m, 1H, δ_2CH_2 Pro), 3.82 (m, 1H, δ_1CH_2 Pro), 4.37 (m, 1H, αCH Pro), 4.83 (m, 1H, αCH Leu), 8.23 (d, 1H, J = 8.7 Hz, NH Leu); FABMS: m/z 399.2 $[M+H]^+$; calcd for $C_{19}H_{30}N_2O_7$: 398.20.

***n*PrNH-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu (7b)**. Isolated by silica gel chromatography (eluent: CH_3CN) in 67% yield; homogeneous on HPLC (t_R = 21.08 min); ^1H NMR ($CDCl_3$) δ 0.90 (t, 3H, J = 7.5 Hz, NH-CH_2-CH_2-CH_3), 0.93 (d, 3H, J = 6.5 Hz, δ_2CH_3 Leu), 0.98 (d, 3H, J = 6.5 Hz, δ_1CH_3 Leu), 1.44 (s, 9H, $OC(CH_3)_3$), 1.40–1.68 (m, 5H, βCH$_2$ Leu, γCH Leu, NH-CH_2-CH_2-CH_3), 1.88–2.10 (m, 3H, β_2CH_2 Pro, γCH$_2$ Pro) 2.20 (m, 1H, β_1CH_2 Pro), 3.21 (m, 2H, NH-CH_2-CH_2-CH_3), 3.46 (d, 1H, J = 2.3 Hz, *t*Eps CH), 3.49 (d, 1H, J = 2.3 Hz, *t*Eps CH), 3.58 (m, 1H, δ_2CH_2 Pro), 3.74 (m, 1H, δ_1CH_2 Pro), 4.36 (m, 1H, αCH Pro), 4.80 (m, 1H, αCH Leu), 6.30 (m, 1H, NH-CH_2-CH_2-CH_3), 6.93 (d, 1H, J = 8.7 Hz, NH Leu); FABMS: m/z 440.5 $[M+H]^+$; calcd for $C_{22}H_{37}N_3O_6$: 439.26.

Agm(Adoc)$_2$-Orn(Boc)-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu (8b). Isolated by silica gel chromatography (eluent: $CHCl_3$:CH_3CN, 10:4) in 45% yield; homogeneous on HPLC (linear gradient of CH_3CN:2% H_3PO_4 from 5:95 to 80:20 in 30 min, then isocratic CH_3CN:2% H_3PO_4 80:20 10 min; t_R = 31.25 min); ^1H NMR (DMSO-d_6) δ 0.91 (d, 3H, J = 6.6 Hz, δ_2CH_3 Leu), 0.92 (d, 3H, J = 6.6 Hz, δ_1CH_3 Leu), 1.37 (s, 18H, $OC(CH_3)_3$), 1.61, 1.63 (2 br. s, 12H, Adoc), 1.30–1.70 (br. m, 11H, βCH$_2$ Agm, γCH$_2$ Agm, βCH$_2$ Orn, γCH$_2$ Orn, βCH$_2$ Leu, γCH Leu), 1.79 (m, 1H, β_2CH_2 Pro), 1.93 (m, 2H, γCH$_2$ Pro), 2.06, 2.11, 2.17 (3 br. s, 19H, Adoc, β_1CH_2 Pro (below overlapping singlets)), 2.89 (m, 2H, δCH$_2$ Orn), 3.05 (m, 2H, αCH$_2$ Agm), 3.49 (m, 1H, δ_2CH_2 Pro), 3.60 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.61 (d, 1H, J = 2.0 Hz, *t*Eps CH), 3.68 (m, 1H, δ_1CH_2 Pro), 3.77 (m, 2H, δCH$_2$ Agm), 4.18 (m, 1H, αCH Pro), 4.23 (m, 1H, αCH Orn), 4.61 (m, 1H, αCH Leu), 6.73 (m, 1H, δNH Orn), 7.99 (m, 1H, αNH Agm), 8.48, 8.64 (2 d, 2H, J = 8.0 Hz (each), NH Orn, NH Leu), 9.03 (br. s, 2H, Adoc-NH-(C=NH)); FABMS: m/z 1081.5 $[M+H]^+$; calcd for $C_{56}H_{88}N_8O_{13}$: 1080.64.

***n*PrNH-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (9b)**. Yield: 79%; homogeneous on HPLC (t_R = 13.85 min); ^1H NMR ($CDCl_3$) δ 0.87–1.00 (m, 9H, δ_2CH_3 Leu, NH-CH_2-CH_2-CH_3, δ_1CH_3 Leu), 1.41–1.65 (m, 5H, βCH$_2$ Leu, γCH Leu, NH-CH_2-CH_2-CH_3), 2.00–2.36 (m, 4H, βCH$_2$ Pro, γCH$_2$ Pro), 3.22 (m, 2H, NH-CH_2-CH_2-CH_3), 3.49 (d, 1H, J = 1.9 Hz, *t*Eps CH), 3.54 (d, 1H, J = 1.9 Hz, *t*Eps CH), 3.60 (m, 1H, δ_2CH_2 Pro), 3.79 (m, 1H, δ_1CH_2 Pro), 4.53 (m, 1H, αCH Pro), 4.80 (m, 1H, αCH Leu), 6.18 (m, 1H, NH-CH_2-CH_2-CH_3), 7.11 (d, 1H, J = 8.5 Hz, NH Leu); FABMS: m/z 384.5 $[M+H]^+$; calcd for $C_{18}H_{29}N_3O_6$: 383.20.

Agm-Orn-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH trifluoroacetate (10b). Yield: 77%; homogeneous on HPLC (linear gradient of CH_3CN:2% H_3PO_4 from 5:95 to 12.5: 87.5 in 30 min; t_R = 10.04 min); ^1H NMR (DMSO-d_6) δ 0.90 (2 d, 6H, J = 7.1 Hz, δ_2CH_3 Leu, δ_1CH_3 Leu), 1.37–1.76 (br. m, 11H, βCH$_2$ Agm, γCH$_2$ Agm, βCH$_2$ Orn, γCH$_2$ Orn, βCH$_2$ Leu, γCH Leu), 1.85 (m, 1H, β_2CH_2 Pro), 1.93 (m, 2H, γCH$_2$ Pro), 2.14 (m, 1H, β_1CH_2 Pro), 2.77 (m, 2H, δCH$_2$ Orn), 3.01–3.12 (br. m, 4H, αCH$_2$ Agm, δCH$_2$ Agm), 3.52 (m, 1H, δ_2CH_2 Pro), 3.62 (d, 1H, J = 1.7 Hz, *t*Eps CH), 3.66 (d, 1H, J = 1.7 Hz, *t*Eps CH), 3.68 (m, 1H, δ_1CH_2 Pro), 4.24 (m, 1H, αCH Pro), 4.28 (m, 1H, αCH Orn), 4.60 (m, 1H, αCH Leu), 6.60–7.30 (br. s, 3 H, H_2N-(C=NH) Agm), 7.50 (br. s, 1H, δNH Agm), 7.66 (br. s, 2H, δNH_2 Orn), 8.13 (t, 1H, J = 5.5 Hz αNH Agm) , 8.64 (d, 1H, J = 7.9 Hz, NH Orn), 8.70 (d, 1H, J = 8.2 Hz, NH Leu); FABMS: m/z 569.4 $[M+H]^+$; calcd for $C_{25}H_{44}N_8O_7$: 568.33.

Enzyme inhibitory acitivity

Inhibition assays. Continuous fluorometric inhibition assays were performed and evaluated as described in detail elsewhere.[25,27] Inhibition of papain (10 pM), cathepsin B (27 pM) and cathepsin L (5 pM) were assayed at 30 °C with the substrate Z-Phe-Arg-NH-Mec (for cathepsin B 10 μM, K_m = 220 μM; for papain 10 μM, K_m = 52 μM; for cathepsin L 4 μM, K_m = 2.9 μM) in 1.2 mL of 0.25 M sodium acetate buffer pH 5.5, containing 2 mM EDTA, 0.015% Brij-35, 1 mM dithiothreitol (added freshly), 1% DMSO (from added substrate solution). The inhibitors were dissolved in DMSO (30–100 mM), diluted in buffer or DMSO and added in 1–10 μL to the preactivated enzymes after a constant substrate release was observed. The final inhibitor concentrations in the assay varied from 2 nM for the fast reacting compounds up to 1 mM for the slow reacting ones in order to achieve almost complete inhibition within 15–90 min. For analysis of pH and salt dependence different buffers were used: 50 mM sodium acetate pH 5.5, 50 mM potassium/sodium phosphate pH 6.0, 6.7, and 7.4, each containing 0, 50, 100, and 150 mM NaCl; all other conditions were the same as above.

Determination of kinetic constants. The apparent pseudo-first-order rate constant k_{obs} was obtained from the presteady-state phase of the progress curve as described by Morrison[28] and Knight[29] by nonlinear regression analysis of the collected data using the commercial program FigP (Biosoft, Cambridge). The apparent second-order rate constants k_2/K_i were then calculated as $k_2/K_i = k_{obs}/[I]$ when k_{obs} increased linearly with increasing inhibitor concentration ($[I] \ll K_i$). For some slow reacting epoxides the experimental condition $[I] \approx K_i$ was fulfilled and k_{obs} saturated with increasing inhibitor concentrations. In these cases, the values of both K_i and k_2 were obtained by nonlinear regression analysis from $k_{obs} = k_2 \cdot [I]/([I]+K_i)$.[29] Substrate depletion was <5% within the duration of the experiments; a correction was made for competition of the inhibitors with the substrate.

Acknowledgements

The authors thank Prof. W. Schäfer (Max-Planck-Institut) for the FABMS spectra and Prof. Sonnenbichler (Max-Planck-Institut) for the NMR spectra. The skillful technical assistance of Mrs Rita Zauner in the kinetic measurements is gratefully acknowledged. The study was partly supported by the SFB 207 of the Ludwig-Maximilians-Universität München (grant H-9/ Machleidt).

References

1. Hanada, K.; Tamai M.; Yamagishi, M.; Ohmura, S.; Sawada, J.; Tanaka, I. *Agric. Biol. Chem.* **1978**, *42*, 523.

2. Hanada, K.; Tamai, M.; Ohmura, S.; Sawada, J.; Seki, T.; Tanaka, I. *Agric. Biol. Chem.* **1978**, *42*, 529.

3. Barrett, A. J.; Kembahvi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. *Biochem. J.* **1982**, *201*, 189.

4. Varughese, K. I.; Ahmed, F. R.; Carey, P. R.; Hasnain, S.; Huber, C. P.; Storer A. C. *Biochemistry* **1989**, *28*, 1330.

5. Yamamoto, D.; Matsumoto, K.; Ohishi, H.; Ishida, T.; Inoue, M.; Kitamura, K.; Mizuno, H. *J. Biol. Chem.* **1991**, *266*, 14771.

6. Kim, M.; Yamamoto, D.; Matsumoto, K.; Inoue, M.; Ishida, T.; Mizuno, H.; Sumiya, S.; Kitamura, K. *Biochem. J.* **1992**, *287*, 797.

7. Rich, D. H. In *Proteinase Inhibitors*; Barrett A. J.; Salvesen, G. Eds.; Elsevier: Amsterdam, 1986; pp 153–178.

8. Drenth, J.; Kalk, K. H.; Swen, H. M. *Biochemistry* **1976**, *15*, 3731.

9. Yabe, Y.; Guillaume, D.; Rich, D. H. *J. Am. Chem. Soc.* **1988**, *110*, 4043.

10. Murata, M.; Miyashita, S.; Yokoo, C.; Tamai, M.; Hanada, K.; Hatayama, K.; Towatari, T.; Nikawa, T.; Katunuma N. *FEBS Lett.* **1991**, *280*, 307.

11. Towatari, T.; Nikawa, T.; Murata, M.; Yokoo, C.; Tamai, M.; Hanada, K. *FEBS Lett.* **1991**, *280*, 311.

12. Buttle, D. J.; Murata, M.; Knight, G. C.; Barrett, A. *Arch. Biochem. Biophys.* **1992**, *299*, 377.

13. Gour-Salin, B. J.; Lachance, P.; Plouffe, C.; Storer A. C.; Menard R. *J. Med. Chem.* **1993**, *36*, 720.

14. Turk, D.; Podobnik, M.; Popovic, T.; Katunuma, N.; Bode, W.; Huber, R.; Turk, V. *Biochemistry* **1995**, *34*, 4791.

15. Tamai, M.; Yokoo, C.; Murata, M.; Oguma, K.; Sota, K.; Sato, E.; Kanaoka, Y. *Chem. Pharm. Bull.* **1987**, *35*, 1098.

16. Giordano, C.; Calabretta, R.; Gallina C.; Consalvi, V.; Scandurra, R.; Noya, F. C.; Franchini, C. *Eur. J. Med. Chem.* **1993**, *28*, 917.

17. Musil, D.; Zucic, D.; Turk, D.; Engh, R. A.; Mayr, I.; Huber, R.; Popovic, T.; Turk, V.; Towatari T.; Katunuma, N.; Bode, W. *EMBO J.* **1991**, *10*, 2321.

18. Gour-Salin, B. J.; Lachance, P.; Magny, M.-C.; Plouffe, C.; Menard R.; Storer A. C. *Biochem. J.* **1994**, *299*, 389.

19. Mori K.; Iwasawa, H. *Tetrahedron* **1980**, *36*, 87.

20. Korn, A.; Rudolph-Böhner, S; Moroder, L. *Tetrahedron* **1994**, *50*, 8381.

21. Gour-Salin, B. J.; Lachance, P.; Bonneou, P. R.; Storer, A. C.; Kirschke, H.; Broemme, D. *Bioorg. Chem.* **1994**, *22*, 227.

22. Khouri, H. E., Plouffe, C.; Hasnian, S.; Hirama, T.; Storer, A. C.; Menard, R. *Biochem. J.* **1991**, *275*, 751.

23. Hasnian, S.; Huber, C. P.; Muir, A.; Rowan, A. D.; Mort, J. S. *Biol. Chem. Hoppe-Seyler* **1992**, *373*, 413.

24. Turk, B.; Dolenc, I.; Turk, V.; Bieth, J. G. *Biochemistry* **1993**, *32*, 375.

25. Machleidt, W.; Nägler, D. K.; Assfalg-Machleidt, I.; Stubbs, M.; Fritz, H.; Auerswald, E. A. *FEBS Lett.* **1995**, *361*, 185.

26. Moroder, L.; Wackerle, L.; Wünsch, E. *Biol. Chem. Hoppe-Seyler* **1976**, *357*, 1647.

27. Machleidt, W.; Assfalg-Machleidt, I.; Auerswald, E. A. In *Monographs, innovation on proteases and inhibitors;* Avilés, F. X., Ed:; Walter de Gruyter: Berlin, 1993; pp 179–196.

28. Morrison, J. F. *Trends Biochem. Sci.* **1982**, *7*, 102.

29. Knight C. G. In *Proteinase Inhibitors*; Barrett A. J.; Salvesen, G. Eds.; Elsevier: Amsterdam, 1986; pp 23–51.

(*Received in U.S.A.* 21 *January* 1997; *accepted* 28 *March* 1997)