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PEPTIDE METHYL KETONES AS REVERSIBLE INHIBITORS OF CYSTEINE PROTEINASES

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Peptide methyl ketones represent a new class of reversible, competitive cysteine proteinase inhibitor with little or no effect on serine proteinases. The affinity of the inhibitors to papain (EC 3.4.22.3), cathepsin B (EC 3.4.22.1) and cathepsin L (EC 3.4.22.15) depends on the peptide chain length and on side-chain effects. Variations in the P_1 and P_4 positions (terminology of Schechter and Berger[1]) and their influence on the efficiency of the inhibitors have been investigated. The most effective inhibitors display inhibition constants in the micromolar range. In contrast to the endopeptidases papain and the cathepsins B and L, the aminoendopeptidase cathepsin H (EC 3.4.22.16) is not inhibited by *N*-acylated peptide methyl ketones but only by amino methyl ketones containing a free α-amino group. The endopeptidases are not affected by amino methyl ketones.

KEY WORDS: Papain, cathepsin B, cathepsin L, cathepsin H, peptide methyl ketones, competitive inhibitors.

INTRODUCTION

Substituted methyl ketones (halomethyl and diazomethyl) and aldehyde derivatives of amino acids and peptides belong to the extensively investigated synthetic protease inhibitors. Whilst the irreversible halomethyl ketones and the reversibly acting peptide aldehydes inhibit both serine and cysteine proteinases,[2,3] the diazomethyl ketones are considered until now as specific inhibitors of cysteine proteinases.[3] Therapeutically, chloromethyl ketones and aldehydes are of limited interest because of their ability to alkylate various cellular nucleophiles or in case of aldehydes to react with other alcohol, thiol or amino residues. In contrast simple unsubstituted methyl ketones should be less reactive with regard to side reaction.

Peptide methyl ketones have been studied extensively so far only for the reversible inhibition of thermitase, a microbial serine proteinase.[4,5] The most potent inhibitors Z–Ala–Ala–Phe–CH_3 and Z–Ala–Ala–Ala–Phe–CH_3 display K_i – values of 0.46 μM and 0.63 μM, respectively. However, peptide methyl ketones designed for other serine proteinases are much less effective. The closely related serine proteinase subtilisin BPN′, characterized by a high homology in the primary structure to thermitase[6] and a very similar substrate specificity,[7,8] is inhibited only in the millimolar range by Z–Ala–Ala–Phe–CH_3^9. Also α-chymotrypsin,[9] trypsin[10] and pancreatic elastase[11] are

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Abbreviations: Ac-, acetyl; Z-, benzyloxycarbonyl; DMF, dimethylformamide; -MCA, 4–methyl–7–coumarylamide; -CH_3, methyl ketone; E–64, L–3-carboxy-trans-2,3-epoxypropyl-leucylamido-(4-guanidino)butane; pAB, p-amino benzoic acid; all amino used were of L-configuration.

only weakly inhibited by peptide methyl ketone inhibitors. Since thermitase possesses a cysteine residue close to its active site serine residue,[6] which is missing in the subtilisins, elastases and α-chymotrypsin, an interaction of this cysteine residue with the methyl ketone moiety of the inhibitor is possible and is currently being examined in our laboratory. The reaction of a ketone with a thiol is considerably facilitated by comparison with the reaction with an alcohol since thiols display a stronger tendency for the formation of a nucleophile than alcohols.[12] In cysteine proteinases, in contrast to serine proteinases, this nucleophil is already generated in the ground state of the enzyme.[13] This paper describes the efficiency of amino and peptide methyl ketones to inhibit the cysteine proteinases papain and the cathepsins B, L and H.

MATERIALS AND METHODS

Enzymes

Crystallized papain was purchased from SERVA (Heidelberg, F.R.G.) and used without further purification. Rat liver cathepsins B, L and H in electrophoretically homogenous form were prepared as described by Kirschke *et al.*[14] and Barrett and Kirschke.[15] The molarity of enzyme stock solutions was determined by active site titration with E-64.[16]

Substrates and Inhibitors

Preparation of Z–Phe–Arg–MCA The fluorescent substrate was synthesized by coupling Z–Phe–OH with HCl·Arg(HCl)MCA using the mixed anhydride method. The substrate was crystallized from dimethylformamide/ether in h.p.l.c. homogenous form with a yield of 74% (m.p. 165–168°C; $[\alpha_D^{25}]$ −18.8°, c = 0.5 in dimethylformamide). HCl·Arg(HCl)MCA, used as a substrate of cathepsin H was available from Z-Arg(HCl)MCA after treatment with HBr in glacial acetic acid followed by reprecipitation in 1.5 M HCl in methanol and recrystallization from water/acetone (m.p. 261–264°C; $[\alpha]_D^{25}$ + 90.2°, c = 0.5 in 1 M HCl). Z-Arg(HCl)MCA was synthesized from Z-Arg–OH and 4-methyl-7-aminocoumarin (synthesized according to Zimmerman *et al.*[17]) via the dicyclohexylcarbodiimide method in dimethylformamide (m.p. 204–207°C; $[\alpha]_D^{25}$ − 17.8°, c = 0.75 in dimethylformamide).

Preparation of Peptide Methyl Ketones Z–Ala_2-CH_3, Z–Ala_3-CH_3, Z–Ala_4-CH_3, Z–Ala_2Phe–CH_3, HCl·Ala-CH_3, HCl·Leu-CH_3 and HCl·Phe-CH_3 were available from previous studies.[5] HCl·Val-CH_3 [m.p. 140–143°C; $[\alpha]_D^{25}$ + 152.8°, c = 1 in acetic acid; ^{1}H n.m.r. ($CDCl_3$)δ1.05 (3H, d, $CHCH_3$), 1.21 (3H, d, $CHCH_3$), 2.29 (3H, s, $COCH_3$), 2.46 (1H, m, $CH(CH_3)_2$), 4.25 (1H, d, CHCO), 8.46 (3H, s, NH_3)] and HCl· Lys(ε-Z)-CH_3 [m.p. 109–112°C; $[\alpha]_D^{25}$ + 211.2°, c = 0.5 in acetic acid; ^{1}H n.m.r. ($CDCl_3$) δ 1.41–1.96 (6H, m, $(CH_2)_3$), 2.17 (3H, s, $COCH_3$), 3.14 (2H, m, NH-CH_2), 4.27 (1H, d, CHCO), 5.03 (2H, s, OCH_2), 7.28 (5H, s, C_6H_5), 8.39 (3H, d, NH_3)] via the N_α-Boc-derivatives were synthesized according to the method described previously.[18] For the synthesis of amino methyl ketones Z-or Boc-protected amino acids were converted into the chloromethyl ketones via the diazomethyl ketones. The halogen was removed by reductive elimination with Zn in glacial acetic acid giving well defined crystalline *N*-protected amino methyl ketones.

TABLE I
Peptide methyl ketones: analytical parameters

nd	no.	m.p. (°C)	$[\alpha]_D^{25}$ (°)	M_r		C %	H %	N %
Phe-CH_3	IV	167–171	−15.4 c1, CH_3COOH	444.48	found:	71.72	6.22	6.
					requ.:	72.96	6.35	6.
Lys(Z)-CH_3	V	150–154	−16.1 c1, DMF	559.57	found:	67.61	6.62	7.
					requ.:	68.68	6.66	7.
Phe-Ala-CH_3	VI	177–179	−23.3 c1, CH_3COOH	439.44	found:	65.35	6.64	9.
					requ.:	65.60	6.65	9.
Val-Phe-CH_3	VIII	199–202	−26.8 c0.5, DMF	495.56	found:	67.56	7.53	8.
					requ.:	67.86	7.52	8.
Ala-Phe-Ala-CH_3	IX	221–225	−33.4 c0.5, DMF	510.51	found:	62.32	6.61	10.
					requ.:	63.52	6.71	10.
Ala-Phe-Val-CH_3	X	223–225	+39.3 c1, DMF	538.57	found:	63.69	7.06	10.
					requ.:	64.66	7.11	10.
Ala-Phe-Leu-CH_3	XI	210–214	−81.3 c1, DMF	552.59	found:	63.45	7.26	9.
					requ.:	65.19	7.29	10.
Ala-Phe-Phe-CH_3	XII	209–213	−58.3 c1, DMF	586.62	found:	65.08	6.78	10.
					requ.:	67.98	6.53	9.
Ala-Phe-Lys(Z)-CH_3	XIII	200–203	−29.2 c1, DMF	701.71	found:	68.36	6.66	9.
					requ.:	71.95	6.59	9.
Ala-Phe-Ala-CH_3	XIV	220–224	−18.3 c0.5, CH_3COOH	586.62	found:	67.30	6.55	9.
					requ.:	67.55	6.52	9.
-Ala-Phe-Ala-CH_3	XV	260–265	+16.8 c0.5, CH_3COOH	466.32	found:	61.31	6.66	11.
					requ.:	64.36	6.48	12.

With the exception of Z–Phe–Ala–Phe–Ala–CH_3 and Ac–pAB–Ala–Phe–Ala–CH_3 all other *N*-protected methyl ketones were synthesized by an X + 1 fragment condensation via the mixed anhydride method (X = Z-protected amino acid or Z-protected peptide acid; 1 = amino methyl ketone derivative of alanine, phenylalanine, valine, leucine or N_ε-Z-lysine). Z–Phe–Ala–Phe–Ala–CH_3 and Ac–pAB–Ala–Phe–Ala–CH_3 were prepared by mixed anhydride coupling of Z–Phe–OH or Ac–pAB–OH with HCl·Ala–Phe–Ala–CH_3. HCl·Ala–Phe–Ala–CH_3 was available from the Z-protected peptide methyl ketone by hydrogenation (Pd-black) in methanol/HCl.

Amino acids were purchased from REANAL (Budapest, Hungary); chloroformic acid benzylester, di.-tert.-butylcarbonate from SERVA (Heidelberg, F.R.G.); isobutylchloroformate and dicyclohexylcarbodiimide from RIEDEL-DE HAEN (Hannover, F.R.G.). All other chemicals used were research grade from Laborchemie (Apolda, G.D.R.). The purity of all synthesized methyl ketones was checked by t.l.c. on silica sheets (Kavalier, Czechoslovakia) in various developing systems and by h.p.l.c. on a RP-8 column (LiChrospher 100 RP-8, 10 μm; HIBAR-Merck, F.R.G.) using isocratic or gradient elution with acetonitrile/water, containing 0.7% trifluoroacetic acid. Characteristic analytical data of the synthesized peptide methyl ketones are listed in Table I.

Kinetic methods

Initial rates of the hydrolysis of Z–Phe–Arg(HCl)MCA and HCl·Arg(HCl)MCA, respectively, were monitored in 1 cm cuvettes in a Shimadzu Spectrophotometer UV 300 with a fluorimetric attachment at an excitation wavelength of 383 nm and with an emission filter of 450 nm at 25°C. The substrate concentration (Z–Phe–Arg(HCl)MCA) was in the range of 2.5–40 μM for papain, 5–50 μM for cathepsin B, 1.25–10 μM for cathepsin L and 5–25 μM HCl·Arg(HCl)MCA for cathepsin H. The kinetic experiments were carried out using a constant enzyme concentration in 50 mM Tris-HCl buffer, pH 7.5 for papain, 50 mM acetate buffer, pH 5.5 for cathepsin L, 50 mM phosphate buffer, pH 6.0 for cathepsin B and 50 mM phosphate buffer, pH 6.5 for cathepsin H. The reaction mixtures were activated for 5 min at 25°C with 2.5 mM dithioerythritol (SERVA, Heidelberg, F.R.G.), 2.5 mM EDTA·Na_2 and 0.005% Brij-35 before addition of substrate. K_m and k_{cat} values were determined from linear Hanes plots with correlation coefficients greater than 0.995. The kinetic constants of the proteinases used were: papain, K_m 98 μM, k_{cat} 87 s^{-1}, k_{cat}/K_m 0.888 $s^{-1}\,\mu M^{-1}$ (Z–Phe–ArgMCA); cathepsin B, K_m 184 μM, k_{cat} 207 s^{-1}, k_{cat}/K_m 1.125 $s^{-1}\,\mu M^{-1}$ (Z–Phe–ArgMCA); cathepsin L, K_m 7 μM, k_{cat} 33 s^{-1}, k_{cat}/K_m 4.714 $s^{-1}\,\mu M^{-1}$ (Z–Phe–ArgMCA); cathepsin H, K_m 160 μM, k_{cat} 65 s^{-1}, k_{cat}/K_m 0.406 $s^{-1}\,\mu M^{-1}$ (ArgMCA).

The inhibition experiments with the reversibly acting methyl ketone derivatives were performed at 6 inhibitor concentrations for 4 substrate concentrations in 1 or 6.6% dimethylformamide in duplicate. The inhibitor was added to the reaction mixture after the enzyme activation for 5 min and before addition of substrate. The inhibition constants were determined from Dixon plots.

RESULTS AND DISCUSSION

Peptide methyl ketones have been seldom used as inhibitors for cysteine proteinases

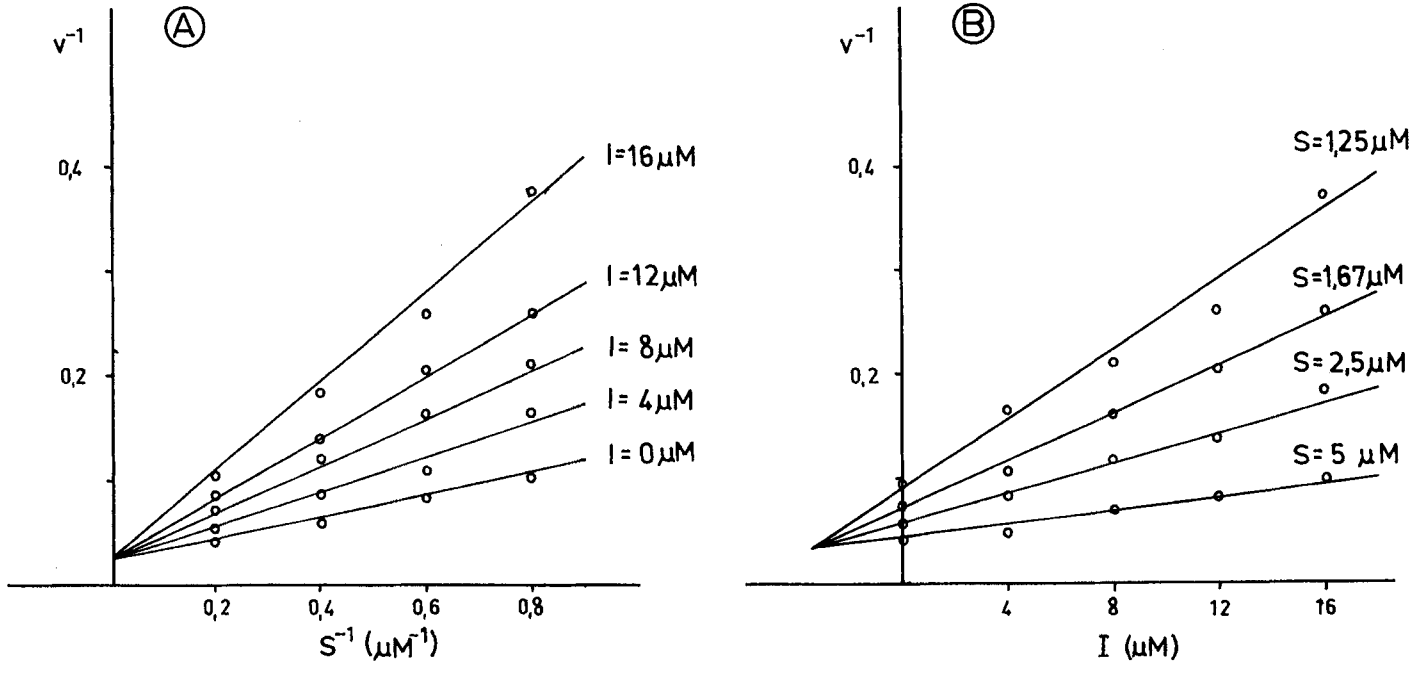

FIGURE 1 Lineweaver-Burk (A) and Dixon (B) plots for the inhibition of cathepsin L catalyzed hydrolysis of Z–Phe–Arg–(HCl)MCA by Ac–pAB–Ala–Phe–Ala–CH_3 (XV).

TABLE II
Inhibition constants (K_i) of peptide and amino methyl ketones

P_5– P_4– P_3– P_2– P_1	no.	K_i (μM)			
		Papain	Cathepsin B	Cathepsin L	Cathepsin H
Z-Ala-Ala-CH_3	I	370 ± 50	800 ± 50	80 $IC_{50}^{\wedge}$	≫ 1000
Z-Ala-Ala-Ala-CH_3	II	220 ± 30	220 ± 40	240 ± 40	≫ 1000
Z-Ala-Ala-Ala-Ala-CH_3	III	29 ± 14	12 ± 2	58 ± 6	≫ 500[§]
Z-Phe-Phe-CH_3	IV	> 30[§†]	58 ± 6	9.8 ± 1.2	≫ 30[§]
Z-Phe-Lys(Z)-CH_3	V	11 ± 1	26 ± 1	3.0 ± 0.1	n.e.
Z-Ala-Phe-Ala-CH_3	VI	51 ± 11	610 ± 10	45 ± 8	n.e.
Z-Ala-Ala-Phe-CH_3	VII	15 ± 2	n.e.	n.e.	n.e.
Z-Val-Val-Phe-CH_3	VIII	30 ± 6	n.e.	n.e.	n.e.
Z-Ala-Ala-Phe-Ala-CH_3	IX	330 ± 10	370 ± 10	400 ± 30	≫ 200[§]
Z-Ala-Ala-Phe-Val-CH_3	X	> 200[§†]	≫ 200[§‡]	> 200[§†]	n.e.
Z-Ala-Ala-Phe-Leu-CH_3	XI	24 ± 9	160 ± 80	18 ± 4	n.e.
Z-Ala-Ala-Phe-Phe-CH_3	XII	17 ± 1	16 ± 2	1.2 ± 0.3	≫ 20[§]
Z-Ala-Ala-Phe-Lys(Z)-CH_3	XIII	17 ± 3	33 ± 5	29 ± 5	n.e.
Z-Phe-Ala-Phe-Ala-CH_3	XIV	4.8 ± 0.4	6.6, $IC_{50}^{\wedge}$	18 ± 3	≫ 20[§]
Ac-pAB-Ala-Phe-Ala-CH_3	XV	11 ± 3	8.9 ± 2.4	3.2 ± 0.1	n.e.
HCl·Ala-CH_3	XVI	≫ 1000	≫ 1000	≫ 1000	> 1000
HCl·Leu-CH_3	XVII	≫ 1000	≫ 1000	≫ 1000	67 ± 8
HCl·Phe-CH_3	XVIII	≫ 1000	≫ 1000	≫ 1000	45 ± 5
HCl·Lys(Z)-CH_3	XIX	≫ 1000	≫ 1000	160 ± 40	13 ± 4

n.e., not estimated; [§]maximal solubility; [‡]≫ 200, no inhibition; [†] > 200, up to 20% inhibition; ^mixed type of inhibition; Mean K_i values determined by averaging the K_i values found from the Dixon plot of two separate experiments.

and little is known about their mechanism of inhibition. From n.m.r. studies the existence of a tetrahedral intermediate generated by the peptide aldehyde Ac–Phe–Gly–H in papain could be demonstrated.[19] Interestingly, with the appropriate dipeptide methyl ketone similar n.m.r. signals were also detected, an indication that peptide methyl ketones may react with papain via a hemithioketal adduct by analogy to the hemithioacetal formed with aldehyde inhibitors.

In this study peptide methyl ketones have been shown to be effective reversible inhibitors of cysteine proteinases in the micromolar range. Care was taken to exclude any contamination of the methyl ketone preparation with chloromethyl ketones, detectable by h.p.l.c. since traces of the very reactive chloromethyl ketones may falsify the kinetic data for the inhibitors. Early preliminary experiments,[20] were characterized by chloromethyl ketone contamination of the compounds Z–Ala_2–Phe–Ala–CH_3 and Z–Phe–Phe–CH_3 and kinetic data reported differ widely from the present results.

Chloromethyl ketones react with dithioerythritol added to the medium as activators of cysteine proteinases[21] so the effect of dithioerythritol on the stability of two methyl ketones (compound VI and VII) in aqueous solutions was studied. The inhibitory potency of the methyl ketone derivatives was not affected by preincubation for up to 30 min with 2.5 mM dithioerythritol at pH 5.5 and pH 7.5 so that a partial inactivation of the inhibitors under the conditions used in the inhibition experiments can be excluded. With two exceptions all synthesized amino- or peptide methyl ketones were competitive inhibitors. For example, Ac–pAB–Ala–Phe–Ala–CH_3 showed a typical double reciprocal and Dixon plot for competitive inhibition with cathepsin L (Figure 1). Only compounds I and XIV showed a mixed type of inhibition with cathepsin L and cathepsin B, respectively and IC_{50}-values were determined (see Table II).

The Effect of Peptide Chain Length on the Potency of the Methyl Ketone Derivatives

An elongation of the peptide chain with alanine residues up to the tetrapeptide enhances the affinity to papain, cathepsin B and with some differences to cathepsin L, too (compounds I–III in Table II). Tetrapeptide methyl ketones are in the range of one order of magnitude more affine inhibitors of papain and the cathepsins B and L than the tripeptide analogues. This is not surprising since papain,[1] cathepsin B[22] and cathepsin L[23] are proteinases with an extended subsite region. The reaction for the mixed type of inhibition of cathepsin L by Z–Ala–Ala–CH_3 is not clear. It may be possible that besides binding in S_3 the Z-residue also binds in S_2. Both subsites apparently favour the binding of bulky aromatic residues.[24] This may also explain the effective inhibition of cathepsin L by this dipeptide analogue in comparison to the tri- and tetrapeptide derivatives. Although at least four subsites are described in the *N*-terminal direction of the binding region in both cathepsin B and cathepsin H,[22] cathepsin H is not affected by all the *N*-acylated peptide methyl ketones studied. However, cathepsin H, a cysteine proteinase which exhibits besides its endopeptidase activity an aminopeptidase activity,[15] is inhibited by *N*-terminal unprotected amino methyl ketones. The more hydrophobic derivatives of phenylalanine and N_ε–Z–lysine are preferred and have K_i values in the range 10^{-5} M. Alternatively amino methyl ketones show, in general, no measureable effect on the endopeptidases papain and the cathepsins B and L.

The Effect of Variation of P_1 and P_4 on the Potency of the Methyl Ketones

In a series of tetrapeptide methyl ketones with the general structure Z–Ala–Ala–Phe–X–CH_3 the P_1 position was varied (compounds IX–XIII in Table II). The P_2 phenylalanine residue was chosen because an aromatic hydrophobic residue is favoured in this position for papain, cathepsin B and cathepsin L.[15] The decrease in K_i values is correlated for the three endopeptidases with an increase in size and hydrophobicity of the P_1 residues. An insertion of valine in P_1 (compound X) results in a very poor inhibitor for all tested proteinases. This discrimination by the valine residue is reflected in the behaviour of several serine proteinases to valine derivatives in P_1. Peptide chloromethyl ketones with a valine residue in P_1 have K_i-values only in the millimolar range for α-chymotrypsin, proteinase K, thermitase and various subtilisins.[25] Obviously, the β-methyl branching in valine hinders the binding. The effect of P_1 variations is different for the investigated proteinases. Whilst papain shows relatively little or no change in the K_i values the most distinct change is observed with cathepsin L. The K_i values decrease from the alanine via the leucine to the phenylalanine derivative in one order of magnitude in each case up to 10^{-6} M. The influence of P_1 variation on cathepsin B inhibition is less expressed and in general one order of magnitude lower than for cathepsin L. This may reflect the known restriction in binding of larger residues in S_1 of cathepsin B which are well accommodated in cathepsin L.[24,26] As expected Z–Phe–Phe–CH_3 and Z–Phe–Lys (ε Z)–CH_3 (compounds IV and V in Table II) are better inhibitors for cathepsin L than for cathepsin B.

Substitution of the small alanine residue in P_4 by the bulky phenylalanine or *p*-amino benzoic acid leads to a stronger inhibition of papain and the cathepsins B and L. These inhibitors are the most effective peptide methyl ketones used in this study and reveal an interaction of the aromatic moiety in the inhibitors with subsite S_4 of the proteinases. This observation is somewhat in contrast to X-ray diffraction studies of chloromethyl ketone substrate analogues bound to papain[27] where it seems likely that a P_4 Z-residue is not bound tightly to the protein surface of papain.

Although S_2P_2 enzyme interactions in papain with a clear preference for bulky hydrophobic residues in P_2 are described as the most important ones for subsite specificity,[15] a change of the –Phe–Ala–CH_3 sequence into –Ala–Phe–CH_3 of the tripeptide inhibitors VI and VII does not result in a pronounced effect on K_i (Table II). Also substitution of the dialanine sequence (P_2P_3) in peptide VII by a divaline in peptide VIII does not alter the efficiency of the methyl ketone inhibitor. It seems likely that papain prefers phenylalanine in P_1 as well as in P_2 position. This is confirmed by the recently isolated microbial pentapeptide aldehyde inhibitor Val–Val–Val–Val–Phe–H,[28] which inhibits papain by a factor of 20 better than antipain. However, insertion of a double phenylalanine in the P_1P_2 position does not display an additive effect on the inhibitory power of the methyl ketone (compound IV and XII in Table II) towards papain. This may be one reason why P_1 variations in the tetrapeptide series have only a little influence on the K_i values determined for papain, when the P_2 position is optimally occupied.

We can conclude from our results that amino and peptide methyl ketones are suitable competitive inhibitors of cysteine proteinases. The affinity of this new class of inhibitor may be enhanced by a well fitted peptide part to give K_i values in the micromolar range. Stronger inhibition within the class to be unlikely due to the reduced electrophilicity of the carbonyl carbon in comparison to an aldehyde moiety. This disadvantage may be offset by the lack of side reactions and the higher protease class specificity. Methyl ketone derivatives of amino acids[29] or peptides are therefore

considered to be suitable compounds as ligands in the affinity chromatography of cysteine proteinases.

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