Journal of Enzyme Inhibition, Vol. 16, pp. 339–350
Reprints available directly from the publisher
Photocopying permitted by license only

© 2001 OPA (Overseas Publishers Association) N.V.
Published by license under
the Harwood Academic Publishers imprint,
part of Gordon and Breach Publishing
a member of the Taylor & Francis Group.
All rights reserved.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

A New Class of Potent Reversible Inhibitors of Metallo-proteinases: C-terminal Thiol-peptides as Zinc-coordinating Ligands

KLAUS PETERS*, GÜNTHER JAHREIS and EVA-MARIA KÖTTERS

Faculty of Medicine, Institute of Physiological Chemistry, Martin Luther University, Halle-Wittenberg, Hollystraße 1, D-06097 Halle (Saale), Germany

(Received 28 February 2001)

A number of substrate analogous peptides containing a phosphoramidate, phosphonate ester, hydroxamate, carboxylate or sulfhydryl group are known to be inhibitors of thermolysin and other metalloproteinases. According to the specificity, most of the inhibitors mimic the prime site of the active center. Hitherto, peptidyl derivatives with a thiol group at the C-terminus have not been described. We have synthesized the protected cysteamides Ac-Ala-Ala-CA-SH and Z-Aa_1-Aa_2-CA-SH (Aa_1: Ala, Pro; Aa_2: Ala, Leu). The binding of these thiol peptide inhibitors to the metalloproteinases is characterized first by the coordination of the thiolate group of the inhibitor to the catalytic zinc ion and second by the subsite interaction of the peptide ligand in the active site of the enzyme. All peptide derivatives were competitive inhibitors of the zinc metalloproteinase thermolysin. The strongest inhibition was found with Z-Pro-Leu-CA-SH (K_i = 30 μM). Substitution of the N-protecting benzyloxycarbonyl residue towards the acetyl group in the peptide inhibitor, the inhibition constant decreased about 25 times.

Keywords: Metalloproteinases; Thermolysin; Thiol-peptides; Inhibitor

Abbreviations: Ac, acetyl; ACE, angiotensin converting enzyme; Agly, azaglycyl; CA, 2-mercaptoethylamine (cysteamine); CP(A,B), carboxypeptidase (A,B); DMF, dimethylformamide; EAC, ethylamidocarbonyl; FA, furylacryloyl; HNC, human neutrophil elastase (MMP-8); ibm, isobutylmalonyl; MMP, matrix metalloprotease; NEP, neutral endopeptidase (neprilysin); TLN, thermolysin; Z, benzyloxycarbonyl

INTRODUCTION

Metalloproteases are involved in different physiological and pathophysiological processes including tissue development, tissue remodeling and turnover, in degradation of extracellular matrix and basement membranes, regulation of blood pressure, wound healing, in degradation of proteins and biologically active peptides, processing of proenzymes as well as in tumor invasion and metastasis, rheumatoid and osteoarthritis, demyelating neuropathies and probable other autoimmune diseases.[1,2] Because of these

*Corresponding author. Tel.: +49-345-5525693. Fax: +49-345-5527030. E-mail: peters@chemie.uni-halle.de

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

aspects, investigations of the interactions of mammalian and bacterial metalloproteases[3] with substrate-analogous inhibitors at the molecular level are of considerable pathophysiological and pharmacological interest.

The superfamily of metalloendopeptidases type 1, termed as "Metzincins", can be subdivided into four families: matrixins, astacins, serralysins and reprolysins.[1,4,5] Metalloproteases of the groups of matrixins (collagenases, gelatinases, stromelysins, matrilysin, metalloelastase, enamelysin, membrane-type matrix metalloproteinases[2,6,7]) and other non-type 1-metallopeptidases like meprins,[1] thermolysin,[8] aminopeptidases,[8–11] carboxypeptidases[8,12] as well as angiotensin converting enzyme (ACE)[13] are characterized by the presence of an essential zinc ion.

Several types of low molecular weight inhibitors are known for these enzymes: amino acids,[14] synthetic amino acid and peptidyl derivatives like phosphonoamidates,[15–20] hydroxamates,[14,16,19,21–27] thiol-containing compounds,[8,16,23,24,28–33] carboxylates[34–36] and chelating agents. The most potent inhibitors of metalloproteases with inhibition constants in the nano- and picomolar range were found in the classes of peptidyl hydroxamates[21–27] and phosphonoamidate derivatives.[16,18,20,37] Many investigations for inactivation of metalloproteases were done with N-(HS-acyl) peptides for matrix metalloproteinases (MMPs),[23,24,30] neprilysin,[28,29] thermolysin[8,16,31,33] and ACE.[28,29] Further inhibition studies with thiol-containing compounds were described with cysteamine on thermolysin[14] and with non-peptidic cysteine derivatives[38] and thiol-diketopiperazines on matrix metalloproteinases.[39]

In contrast to the substrate specificity for serine[40] and cysteine proteases,[41,42] which is determined by the interactions at the S-subsites of the active center, the specificity of metalloproteases mainly depends on the affinity of the prime subsites, especially S1′. Therefore, the most effective peptide hydroxamates, thiols and carboxylates have their functional group at the N-terminus of the peptide chain and are bound to the S′-site of the active site.[16,31] Nevertheless, peptidyl derivatives with a hydroxamate group at the C-terminus of the peptide chain are also bound by metalloproteinases with a large inhibitory effect.[23] However, analogous peptidyl thiol inhibitors for thermolysin with the thiol group at the C-terminus have not as yet been described. On the other hand, thiol derivatives of leucine, lysine[9,10] and phenylalanine[43] were found to be very potent inhibitors of exopeptidases containing zinc.

An often used model enzyme for metalloproteases is thermolysin (EC 3.4.24.4), a thermostable peptide hydrolase from *Bacillus thermoproteolyticus*.[8] Thermolysin contains one zinc ion required for activity[44] and four calcium ions, which are necessary for thermostability.[45] The enzyme preferably hydrolyzes peptides with hydrophobic amino acids such as leucine, isoleucine and phenylalanine in the P1′-position.[46]

Therefore, we have synthesized substrate-like peptidyl inhibitors with a C-terminal thiol group, which would be directed to the catalytic site as well as to the binding site of metalloproteinases. Here, we report the synthesis of the N-protected dipeptidyl cysteamides Ac-Ala-Ala-CA-SH and Z-Aa_1-Aa_2-CA-SH (Aa_1: Ala, Pro; Aa_2: Ala, Leu) and their inhibitory activities towards thermolysin.

MATERIALS AND METHODS

Thermolysin with a protein content of 52% ($A_{280}^{1\%} = 17.6$)[47] was obtained from Sigma (Sigma/Aldrich, Deisenhofen, Germany) as a crystallized and lyophilized powder. The substrate FA-Gly-Leu-NH_2 as well as the peptides Ac-Ala_2-OH, Z-Ala_2-OH, Z-Pro-Ala-OH, Z-Pro-Leu-OH and Z-Ala-Leu-OH were purchased from Bachem (Heidelberg, Germany), cysteamine hydrochloride and ethyl isocyanate were obtained from Fluka (Sigma/Aldrich, Deisenhofen, Germany).

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

Synthesis

Analytical Instruments

The purity of the synthesized products was checked by (a) thin-layer chromatography (TLC aluminium sheets, silica gel 60, from Merck, Darmstadt, Germany), using the following solvent systems: system 1, sec. butanol:3% ammonia (100:44); system 2, n-butanol:acetic acid:water (4:1:1); system 3, that is $CHCl_3$:CH_3OH (7:3); system 4, $CHCl_3$:CH_3OH (9:1) and system 5, $CHCl_3$:CH_3OH (19:1), and (b) HPLC (gradient system, from Bischoff, Leonberg, Germany), using a Nucleosil RP-18 column (5 μm, 250 × 4 mm) and the solvent system acetonitrile/water/0.1% trifluoroacetic acid. Only products with a purity of ≥ 98% were used for further synthesis and for inhibition studies, respectively. Semipreparative HPLC was carried out with an Impaq RP-18 column (10 μm, 250 × 8 mm) using the solvent system acetonitrile/water. Silica gel 60 (from Merck, Darmstadt, Germany) in a 300 x 20 mm glass column and a mixture of chloroform and methanol were used for preparative silica gel chromatography.

Mass spectra were obtained on an ESI-mass spectrometer LCQ (from Finnigan Mat, USA). Elemental analysis were performed on a LECO-932 (CHNS; from Leco, USA) and a VARIO EL (CHN; from Elementar, Hanau, Germany), respectively. ^{1}H-NMR spectra were recorded on a Gemini 2000 spectrometer (400 MHz; from Varian, USA). DMSO-d_6 was used as solvent for all compounds, tetramethyl silane as internal standard. Questionable assignments of signals were decided by aid of H,H-COSY spectra. The kinetic measurements were conducted on a Beckman spectrophotometer DU 7500 using a sixfold cuvette holder at a constant temperature of 22°C.

S-Ethylamidocarbonyl Cysteamine Hydrochloride [CA-S-EAC. HCl, 1]

Following the synthesis of Cys(EAC)-OH,[48] to dried cysteamine hydrochloride (12.53 g, 110 mmol) dissolved in 100 ml DMF, ethyl isocyanate (10 ml, 127 mmol) was added with stirring and cooling in an ice bath over 10 min. After stirring for 70 h at room temperature, the reaction mixture was concentrated *in vacuo*. The pale yellow oil was digested with ether to give a crystalline product, which was dissolved in 120 ml water (pH 5.0) and three times extracted with ether. The aqueous phase was evaporated *in vacuo*, and the solid white product crystallized from ethanol/ether to give white crystals. Yield: 13.5 g, 66%; m.p.: 125–130°C; TLC: system 1, $R_f = 0.61$; system 2, $R_f = 0.57$. Calcd. for $C_5H_{12}N_2SO$ (CA-S-EAC): mol.wt. 148.22. Found (ESI-MS): m/e 149.0 $(M+H)^+$. Calcd. for $C_5H_{13}SOCl$ (H-CA-EAC. HCl): C, 32.52; H, 7.10; N, 15.17; S, 17.36. Found: C, 32.48; H, 7.40; N, 14.82; S, 17.56%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 8.30 (s, 1H, EAC-NH); 8.01 (s, 3H, CA-NH_3^+); 3.06-3.17 (m, 2H, EAC-CH_2); 2.98 (t, 2H, CA-CH_2N); 2.91 (t, 2H, CA-CH_2S); 1.01 (t, 3H, EAC-CH_3).

Compound **1** was coupled equimolar (in amounts of 1 mmol) with the N-terminal protected dipeptides Ac-Ala_2-OH, Z-Ala_2-OH, Z-Pro-Ala-OH, Z-Pro-Leu-OH, and Z-Ala-Leu-OH, respectively, using the mixed anhydride method, to give the products **2–6**.

Acetyl-dialanyl-S-ethylamidocarbonyl Cysteamide [Ac-Ala(2)-Ala(1)-CA-S-EAC, 2]

The crude product was crystallized from ethanol/water to give small white needles. Yield: 278 mg, 82%; m.p.: 240–250°C; TLC: system 3, $R_f = 0.85$; system 4, $R_f = 0.27$. Calcd. for $C_{13}H_{24}N_4SO_4$: mol.wt. 332.43. Found (ESI-MS): m/e 355.3 $(M+Na)^+$. Calcd. for $C_{13}H_{24}N_4SO_4$: C, 46.97; H, 7.28; N, 16.85; S, 9.65. Found: C, 47.01; H, 7.28; N, 16.55; S, 9.72%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 8.12 (t, 1H, EAC-NH); 8.02 (d, 1H, Ala(**2**)-NH); 7.95 (t, 1H, CA-NH); 7.88 (d, 1H, Ala(**1**)-NH); 4.16–4.24 (m, 2H, Ala(**2**)-CH+Ala(**1**)-CH); 3.09–3.25 (m, [4H]*), CA-CH_2N+EAC-CH_2); 2.78–2.88 (m, 2H, CA-CH_2S); 1.82 (s, 3H,

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

Ac-CH_3); 1.19/1.17 (d/d, 6H, Ala(**2**)-CH_3+ Ala(**1**)-CH_3); 1.01 (t, 3H, EAC-CH_3).

Benzyloxycarbonyl-dialanyl-S-ethylamidocarbonyl Cysteamide [Z-Ala(2)-Ala(1)-CA-S-EAC, 3]

The crude product was dissolved in DMF and precipitated with water to give a white powder. Yield: 293 mg, 69%; m.p.: 172–178°C; TLC: system 4, $R_f = 0.62$; system 5, $R_f = 0.31$. Calcd. for $C_{19}H_{28}N_4SO_5$: mol.wt. 424.52. Found (ESI-MS): m/e 447.2 $(M+Na)^+$. Calcd. for $C_{19}H_{28}N_4SO_5$: C, 53.76; H, 6.65; N, 13.20; S, 7.55. Found: C, 54.04; H, 6.69; N, 12.94; S, 7.55%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 8.13 (t, 1H, EAC-NH); 8.00 (t, 1H, CA-NH); 7.88 (d, 1H, Ala(**1**)-NH); 7.44 (d, 1H, Ala(**2**)-NH); 7.30–7.38 (m, 5H, Z-C_6H_5); 4.95–5.07 (m, 2H, Z-CH_2); 4.19 (m, 1H, Ala(**1**)-CH); 4.03 (m, 1H, Ala(**2**)-CH); 3.07–3.30 (m, [4H]*), CA-CH_2N+EAC-CH_2); 2.78–2.88 (m, 2H, CA-CH_2S); 1.18/1.17 (d/d, 6H, Ala(**1**)-CH_3+Ala(**2**)-CH_3); 1.00 (t, 3H, EAC-CH_3).

Benzyloxycarbonyl-prolyl-alanyl-S-ethylamidocarbonyl Cysteamide [Z-Pro-Ala-CA-S-EAC, 4]

The crude product was twice recrystallized from ethanol/water to give small white needles. Yield: 251 mg, 66%; m.p.: 100–104°C; TLC: system 4, $R_f = 0.67$; system 5, $R_f = 0.39$. Calcd. for $C_{21}H_{30}N_4SO_5$: mol.wt. 450.56. Found (ESI-MS): m/e 473.3 $(M+Na)^+$. Calcd. for $C_{21}H_{30}N_4SO_5$: C, 55.98; H, 6.71; N, 12.43; S, 7.12. Found: C, 55.79; H, 6.80; N, 11.83; S, 7.23%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 8.12 (t, 1H, EAC-NH); 8.03/7.98/7.89 (dd/t/t, 2H, Ala-NH_a+Ala-NH_b+CA-NH_a+CA-NH_b†); 7.20–7.40 (m, 5H, Z-C_6H_5); 4.90–5.15 (m, 2H, Z-CH_2); 4.13–4.30 (m, 2H, Pro-CH+Ala-CH); 3.04–3.52 (m, [6H]*, CA-CH_2N+EAC-CH_2+Pro-δCH_2); 2.78–2.90 (m, 2H, CA-CH_2S); 1.68–2.20 (m, 4H, Pro-βCH_2+Pro-γCH_2); 1.19/1.06/1.00 (d/d/t, 6H, Ala-CH_{3a}+AlaCH_{3b}†+EAC-CH_3).

Benzyloxycarbonyl-prolyl-leucyl-S-ethylamidocarbonyl Cysteamide [Z-Pro-Leu-CA-S-EAC, 5]

The crude product was dissolved in a small amount of ethanol and precipitated with water, followed by recrystallisation from ethanol/water to give small white needles. Yield: 374 mg, 76%; m.p.: 135–138°C; TLC: system 5, $R_f = 0.53$; system 6, $R_f = 0.31$. Calcd. for $C_{24}H_{36}N_4SO_5$: mol.wt. 492.64. Found (ESI-MS): m/e 515.4 $(M+Na)^+$.Calcd. for $C_{24}H_{36}N_4SO_5$: C, 58.51; H, 7.37; N, 11.37; S, 6.51. Found: C, 58.68; H, 7.37; N, 11.21; S, 6.51%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 8.12 (t, 1H, EAC-NH); 8.03/7.98/7.96/7.90 (t/d/d/t, 2H, CA-NH_a+Leu-NH_a+Leu-NH_b+CA-NH_b†); 7.20–7.40 (m, 5H, Z-C_6H_5); 4.87–5.15 (m, 2H, Z-CH_2); 4.15–4.33 (m, 2H, Pro-CH+Leu-αCH); 3.02–3.54 (m, [6H]*), CA-CH_2N+EAC-CH_2+Pro-δCH_2); 2.75–2.90 (m, 2H, CA-CH_2S); 1.72–2.20 (m, 4H, Pro-βCH_2+Pro-γCH); 1.26–1.68 (m, 3H, Leu-βCH_2+Leu-γCH); 1.00 (t, 3H, EAC-CH_3); 0.86/0.81/ 0.76/0.72 (d/d/d/d, 6H, Leu-CH_{3a}+Leu-CH_{3b}†).

Benzyloxycarbonyl-alanyl-leucyl-S-ethylamidocarbonyl Cysteamide [Z-Ala-Leu-CA-S-EAC, 6]

The crude product was crystallized from ethanol/water, passed down a semipreparative HPLC column ($CH_3CN : H_2O = 35 : 65$) and crystallized from acetonitrile/water to give small white needles. Yield: 243 mg, 52%; m.p.:

*Signals between 3.37 and 3.47 ppm are overlayed by the water signal.

†The indices a and b are indicating different conformeres due to the cis/trans isomerie of proline. According to the integrals of separate signals, the ratio of the isomers is about 45:55.

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

177–179°C; TLC: system 4, $R_f = 0.81$; system 5, $R_f = 0.47$. Calcd. for $C_{22}H_{34}N_4SO_5$: mol. wt. 466.60. Found (ESI-MS): m/e 489.3 $(M+Na)^+$. Calcd. for $C_{22}H_{34}N_4SO_5$: C, 56.63; H, 7.34; N, 12.01; S, 6.87. Found: C, 56.68; H, 6,97; N, 11.50; S, 6.78%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 8.12 (t, 1H, EAC-NH); 8.04 (t, 1H, CA-NH); 7.78 (d, 1H, Leu-NH); 7.43 (d, 1H, Ala-NH); 7.25–7.38 (m, 5H, Z-C_6H_5); 4.95–5.05 (m, 2H, Z-CH_2); 4.22 (m, 1H, Leu-αCH); 4.04 (m, 1H, Ala-CH); 3.05–3.37 (m, [4H]*, CA-CH_2N+EAC-CH_2); 2,78–2.88 (m, 2H, CA-CH_2S); 1.32–1.67 (m, 3H, Leu-βCH_2+ Leu-γCH); 1.18 (d, 3H, Ala-CH_3); 1.01 (t, 3H, EAC-CH_3); 0.86/0.82 (d/d, 6H, Leu-CH_3).

The S-protected peptidyl cysteamides **2–6** were treated as follows to give the free thiol-peptides **7–11**:

To 0.5 mmol EAC-compound dissolved in 100 ml isopropanol (in the case of **2** in 20% isopropanol in water), 100 ml 1N NaOH was added. The reaction mixture was stirred under argon atmosphere at room temperature. Free thiol groups could be identified with sodium-pentacyanonitrosyl-ferrat (nitroprusside sodium).[49] After 30 min, the pH was adjusted to 4.5–5.0 with 2.5 N HCl and the solvent was evaporated *in vacuo* to give a crude product. The thiol-peptides were strictly handled under inert gas during the purification procedures.

Acetyl-dialanyl Cysteamide [Ac-Ala(2)-Ala(1)-CA-SH, 7]

The crude product was extracted three times with dry ethanol. The ethanol was removed *in vacuo* and the product was put in succession down a silica gel column ($CHCl_3$: CH_3OH = 7 : 3) and a semipreparative HPLC column (CH_3CN : H_2O = 5 : 95). The solvent was evaporated and the solid residue was treated with ether to yield a white powder. Yield: 44 mg, 34%; m.p.: 250–255°C; TLC: system 3, $R_f = 0.87$; system 4, $R_f = 0,32$. Calcd. for $C_{10}H_{19}N_3SO_3$: mol.wt. 261.35. Found (ESI-MS): m/e 284.3 $(M+Na)^+$. Calcd. for $C_{10}H_{19}N_3SO_3$: C, 45.96; H, 7.33; N, 16.08; S, 12.27. Found: C, 45.58; H, 7.03; N, 15.32; S, 11.99%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 8.04/7.93/7.88 (d/d/t, 3H, Ala(**2**)-NH+Ala(**1**)-NH+CA-NH); 4.16–4.24 (m, 2H, Ala(**2**)-CH+Ala(**1**)-CH); 3.25–3.38 (m, [2H]*, CA-CH_2N); 3.17–3.22 (m, 2H, CA-CH_2S); 2.34 (t, 1H, CA-SH); 1.82 (s, 3H, Ac-CH_3); 1.19/1.17 (d/d, 6H, Ala(**2**)-CH_3+Ala(**1**)-CH_3).

Benzyloxycarbonyl-dialanyl Cysteamide [Z-Ala(2)-Ala(1)-CA-SH, 8]

The crude product suspended in water was three times extracted with ethyl acetate and the solvent was evaporated. The product was twice passed down a semipreparative HPLC column (CH_3CN : H_2O = 25 : 75) and finally lyophilized to give a white powder. Yield: 21.2 mg, 12%; m.p.: 210–215°C; TLC: system 4, $R_f = 0.86$; system 5, $R_f = 0.61$; Calcd. for $C_{16}H_{23}N_3SO_4$: mol.wt. 353.44. Found (ESI-MS): m/e 376.2 $(M+Na)^+$. Calcd. for $C_{16}H_{23}N_3SO_4$: C, 54.37; H, 6.56; N, 11.89; S, 9.07. Found: C, 53.97; H, 6.56; N, 11.25; S, 9.11%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 7.98–8.00 (m, 2H, CA-NH+Ala(**1**)-NH); 7.45 (d, 1H, Ala(**2**)-NH); 7.30–7.35 (m, 5H, Z-C_6H_5); 4.95–5.07 (m, 2H, Z-CH_2); 4.20 (m, 1H, Ala(**1**)-CH); 4.03 (m, 2H, Ala(**2**)-CH); 3.25–3.35 (m, [2H]*, CA-CH_2N); 3.17–3.22 (m, 2H, CA-CH_2S); 2.34 (t, 1H, CA-SH); 1.19 (d/d, 6H, Ala(**1**)-CH_3+Ala(**2**)-CH_3).

Benzyloxycarbonyl-prolyl-alanyl Cysteamide [Z-Pro-Ala-CA-SH, 9]

The crude product was distributed between water and ethyl acetate. Then, the ethyl acetate layer was washed with water to neutral pH. After evaporation of the ethyl acetate *in vacuo*, **9** was passed down a semipreparative HPLC column (CH_3CN : H_2O = 28 : 72). After evaporation of the acetonitrile, the product was lyophilized and than treated with ether to give

RIGHTSLINK

a white, hygroscopic powder. Yield: 131 mg, 69%; m.p.: 138–141°C; TLC: system 4, $R_f = 0.74$; system 5, $R_f = 0.53$. Calcd. for $C_{18}H_{25}N_3SO_4$: mol.wt. 379.48. Found (ESI-MS): m/e 402.4 $(M+Na)^+$, $I = 60$; 780.8 $(M_2+Na)^+$, $I = 100$. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 8.07 (d/d, 1H, Ala-NH_a+Ala-NH_b†); 7.94/7.83 (t/t, 1H, CA-NH_a+CA-NH_b†); 7.24–7.40 (m, 5H, Z-C_6H_5); 4.90–5.15 (m, 2H, Z-CH_2); 4.15–4.30 (m, 2H, Pro-CH+Ala-CH); 3.10–3.50 (m, [6H]*, CA-CH_2N+CA-CH_2S+Pro-δCH_2); 2.28–2.38 (m, 1H, CA-SH_a+CA-SH_b†); 1.70–2.20 (m, 4H, Pro-βCH_2+Pro-γCH_2); 1.21/1.08 (d/d, 3H, Ala-CH_{3a}+Ala-CH_{3b}†).

Benzyloxycarbonyl-prolyl-leucyl Cysteamide [Z-Pro-Leu-CA-SH, 10]

The crude product was distributed between water and ethyl acetate. Then, the ethyl acetate layer was washed with water to neutral pH. After evaporation of the ethyl acetate *in vacuo*, **10** was crystallized from ethanol/water and then passed down a semipreparative HPLC column ($CH_3CN : H_2O = 34 : 66$). Finally, **10** was crystallized from acetonitrile/water to give small white needles. Yield: 101 mg, 48%; m.p.: 91–93°C; TLC: system 5, $R_f = 0.74$; system 6, $R_f = 0.54$. Calcd. for $C_{21}H_{31}N_3SO_4$: mol.wt. 421.56. Found (ESI-MS): m/e 444.4 $(M+Na)^+$, $I = 60$; 864.9 $(M_2+Na)^+$, $I = 100$. Calcd. for $C_{21}H_{31}N_3SO_4$: C, 59.83; H, 7.41; N, 9.97; S, 7.61. Found: C, 59.32; H, 7.30; N, 9.54; S, 7.62%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 7.95–8.08/7.85 (m/t, 2H, CA-NH_a+Leu-NH_a+Leu-NH_b+CA-NH_b†); 7.20–7.42 (m, 5H, Z-C_6H_5); 4.87–5.15 (m, 2H, Z-CH_2); 4.15–4.33 (m, 2H, Pro-CH+Leu-αCH); 3.02–3.54 (m, [6H]*, CA-CH_2N+CA-CH_2S+Pro-δCH_2); 2.25–2.38 (m, 1H, CA-SH_a+CA-SH_b†); 1.72–2.22 (m, 4H, Pro-βCH_2+Pro-γCH_2); 1.25–1.68 (m, 3H, Leu-βCH_2+Leu-γCH); 0.87/0.82/0.77/0.73 (d/d/d/d, 6H, Leu-CH_{3a}+LeuCH$_{3b}$†).

Benzyloxycarbonyl-alanyl-leucyl Cysteamide [Z-Ala-Leu-CA-SH, 11]

The crude product was treated as described for **10** (semipreparative HPLC with solvent $CH_3CN : H_2O = 28 : 72$). Yield: 111 mg (small white needles), 56%; m.p.: 167–170°C; TLC: system 4, $R_f = 0.89$; system 5, $R_f = 0.66$. Calcd. for $C_{19}H_{29}N_3SO_4$: mol.wt. 395.52. Found (ESI-MS): m/e 418.3 $(M+Na)^+$, $I = 70$; 812.9 $(M_2+Na)^+$, $I = 100$. Calcd. for $C_{19}H_{29}N_3SO_4$: C, 57.70; H, 7.39; N, 10.02; S, 8.11. Found: C, 57.33; H, 6.92; N, 10.13; S, 7.92%. ^{1}H-NMR (DMSO-d_6): δ(ppm) = 7.98 (t, 1H, CA-NH); 7.83 (d, 1H, Leu-NH); 7.44 (d, 1H, Ala-NH); 7.25–7.40 (m, 5H, Z-C_6H_5); 4.93–5.07 (m, 2H, Z-CH_2); 4.24 (m, 1H, Leu-αCH); 4.03 (m, 1H, Ala-CH); 3.10–3.45 (m, [4H]*, CA-CH_2N+CA-CH_2S); 2.32 (t, 1H, CA-SH); 1.38–1.65 (m, 3H, Leu-βCH_2+Leu-γCH); 1.18 (d, 3H, Ala-CH_3); 0.86/0.82 (d/d, 6H, Leu-CH_3).

Kinetic Measurements

Stock solutions of enzyme, substrate and inhibitors were made as follows. The commercial enzyme preparation was dissolved in Tris/HCl buffer (0.1 M, pH 7.5) containing NaCl (0.3 M) and $CaCl_2$ (10 mM) with a concentration of about 1.0 mg protein/ml. The substrate FA-Gly-Leu-NH_2 was dissolved in DMF and diluted with the buffer to a concentration of 3.2 mM (DMF concentration, 2%). The inhibitors were dissolved in pure DMF. The determination of the inhibition constant was based on a set of 20 assays for each inhibitor. The enzymatic hydrolysis of the substrate was monitored for 5 min by following the decrease in absorbance at $\lambda =$ 345 nm for four different substrate concentrations (0.4, 0.6, 0.8, 1.0 mM) in combination with five different inhibitor concentrations. The enzyme concentration was about 10 µg protein/ml ([S]≫[E]) for each assay. Each assay was performed three times; the average value was used for graphic analysis. Because of the more or

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

RIGHTSLINK

TABLE I Peptidyl cysteamides as inhibitors of thermolysin

Inhibitor no.	Inhibitor	K_i (μM)	*DMF* (%)*
10	Z-Pro-Leu-CA-SH	30±6	2.2
9	Z-Pro-Ala-CA-SH	70±13	2.2
8	Z-Ala-Ala-CA-SH	150±29	3.75
11	Z-Ala-Leu-CA-SH	2300±690	8.12
7	Ac-Ala-Ala-CA-SH	4000±590	2.2
	Cysteamine	650±115	2.2
5	Z-Pro-Leu-CA-S-EAC	No inhibition	2.2
2	Ac-Ala-Ala-CA-S-EAC	No inhibition	2.2

* Nessesary concentration of the organic solvent for solubility of the inhibitor in the enzymatic assay.

less limited solubility of the inhibitors, different concentrations of DMF (2.2 to 8.12 %) in the assays was required (Table I). The data were analyzed by Dixon plots and, to confirm the competitive nature of the inhibition, by Lineweaver–Burk plots. The slopes of the straight lines obtained in the Dixon plot were replotted against inhibitor concentration to obtain the inhibition constant (K_i) from the horizontal intercept. In the case of the Lineweaver–Burk plots, $1/K_i$ could be obtained from the point of intersection of the straight lines. Each K_i value was determined twice; the average value is given in Table I.

RESULTS AND DISCUSSION

Zinc metalloproteases are, beside other proteases, responsible for protein degradation in different physiological and pathophysiological processes. In the most cases, these enzymes are not commercially available in large amounts and are also expensive. Therefore, thermolysin was often used as a model enzyme for inhibition studies although the binding of substrate-analogous inhibitors in thermolysin may be something different from the binding in other metalloproteinases.[35]

Thermolysin (EC 3.4.24.4) is a thermostable and a catalytic Zn^{2+}-containing endopeptidase[44] secreted from *Bacillus thermoprotolyticus*[8] and has a well-known three-dimensional structure.[16] Its primary specificity is governed by the S1′ subsite (nomenclature according to Schechter and Berger[50]), which prefers to bind large hydrophobic amino acid residues,[51] tolerates small D-amino acids[23] but excludes proline residues.[46] S2′ shows also a significant preference for hydrophobic amino acid residues but not to such an extent as S1′.[51] Proline residues, equally whether in the D- or L-configuration, are not accepted by S2′.[52] S3′ has a low but slightly hydrophobic specificity leading to only weak interactions between S3′ and P3′; for instance D-amino acids are well accepted.[51] S1 also favors hydrophobic interactions, but the restrictions are not so strong as for S1′. However, D-amino acids in P1 lead to competitive inhibition. The strength of influence of S2 to the substrate specificity is comparable to that of S2′. Small hydrophobic residues are bound weaker than large residues, but the differences are smaller as in the case of S1. S3 seems to be situated near the surface of the protein and has a very weak specificity, which includes nearly no stereospecificity.[51]

For thermolysin, a large number of inhibitors have been found and enzyme–ligand complexes characterized.[16] Thiol-peptides with the SH-group at the C-terminus of the peptide chain are not among them.

Inhibitors of metalloproteases like thermolysin have to fullfill two conditions: (a) a functional group, which is able to coordinate the zinc ion, and (b) a peptide sequence, which corresponds to the subsite specificity. Therefore, we have synthesized N-protected peptidyl cysteamides by coupling of S-protected cysteamine with

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

RIGHTSLINK

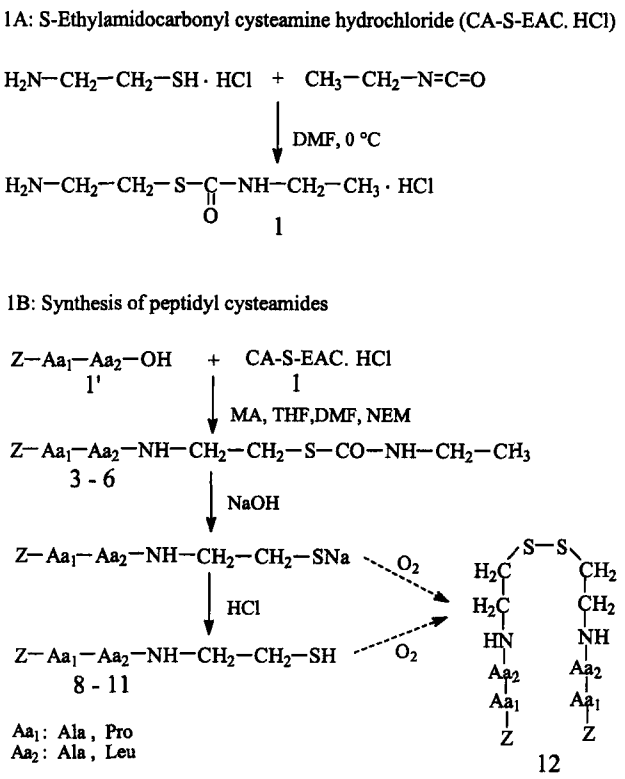

FIGURE 1 Synthesis of thiol-peptide inhibitors. (1A) S-Ethylamidocarbonyl cysteamine hydrochloride (CA-S-EAC HCl) (1B) Synthesis of peptidyl cysteamides.

different benzyloxycarbonyl and acetyl dipeptide acids using the mixed anhydride method, followed by deprotection of the C-terminal thiol group (Fig. 1). We decided on the ethylamidocarbonyl group as thiol protecting group, since it has proved to be a very effective one.[48] S-Ethylamidocarbonyl cysteamine hydrochloride (CA-S-EAC. HCl, **1**) was synthesized by reaction of cysteamine hydrochloride with ethyl isocyanate[48] (Fig. 1A). Then, CA-S-EAC. HCl was coupled to the Z- or Ac-dipeptide acids, using the mixed anhydride method again, to yield the S-EAC-protected thiol ethyl amide dipeptides **2–6** (Fig. 1B). In the following step, the EAC group was removed with NaOH to yield the peptidyl thiolate sodium salts, which could be converted with HCl into the C-terminal free thiol-peptides **7–11**. To suppress oxidation of the thiol-peptides to the more stable disulfides **12**, the peptide derivatives **7–11** were strictly handled in an argon atmosphere during the deprotection and purification procedures.

The S-EAC-protected peptides **2–6** do not act as inhibitors of thermolysin indicating, that an inhibitor needs a free thiol/thiolate group for interacting with the catalytic zinc ion of the metalloprotease.

The thiol-containing peptide inhibitors as well as their inhibition constants for thermolysin in comparison to the K_i value of the pure cysteamine are summarized in Table I. At pH 7.5 used in the measurements the SH group of the inhibitors appears in the thiolate form. It is assumed that the thiolate group is bound to the catalytic zinc ion and the peptidyl chain to the S-subsites of the active center in accord with the crystal structure studies with S′-oriented thiol-ligands.[31] For our inhibitors, the interactions of

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

the cysteamine in P1 of the peptide ligands with the thermolysin subsite S1 should be, because of the lacking side chain in cysteamine, negligible. Therefore, the binding of the inhibitor to thermolysin should mainly be governed by the P2/S2, followed by the P3/S3, and possibly the P4/S4 interactions.

Z-Pro-Leu-CA-SH (**10**) with a K_i of 30 μM was found to be the most effective inhibitor (Table I). When the amino acid leucine in P2 position was substituted with alanine, the K_i was increased to 70 μM. Replacing the proline by alanine led to Z-Ala-Leu-CA-SH (**11**) with an inhibition potency about 75 times lower. This means that both subsites, S2 and S3, prefer more hydrophobic amino acids as ligands. Comparing the inhibition constants of Ac-Ala_2-CA-SH (**7**) and Z-Ala_2-CA-SH (**8**), it is additionally shown, that the voluminous hydrophobic benzyloxycarbonyl residue in P4 is about 25 times more tightly bound than the small acetyl residue. Our results agree with those of other authors[51,53] resulting from investigations with different synthetic substrates. However, the differences of the K_i values for **7** and **8** as well as for **10** and **11** are somewhat surprising.

In comparison to the thiol-peptides, the small molecule cysteamine is already able to inactivate thermolysin in a substrate-unspecifical manner with a reasonable K_i value of 650 μM (Table I) comparable with a K_i of 700 μM[14] found earlier. All our results are also in good agreement with the micromolar inactivation range of the thiol inhibitors described for collagenases.[24]

The ranking of the inhibition constants found seems to support our thesis that the thiol-peptides are bound to the S-site of the active site of thermolysin. However, certainty can only be provided by X-ray investigations and "unusual" binding of the inhibitors like that described by Gaucher *et al.*[31] and Roedern *et al.*[36] or inverse binding at the prime site mentioned by Nishino and Powers[21] for Z-Gly-Gly-Leu-NHOH cannot be excluded at this point.

In comparison to the thiol-peptides synthesized, N-terminal thiol-containing peptide derivatives exhibit a stronger inhibitory effect if the peptide chain is adapted to the specificity of the prime site subsites. Gaucher *et al.*[31] have reviewed a number of HS-acyl compounds for their K_i values on thermolysin and their recognition mode of the enzyme. From this paper, it could be very clearly derived that the recognition mode decisively influences inhibitory potency. If the amino acid residues of the peptide inhibitor in P1′ and P2′ have strong hydrophobic properties, then P1′ interacts with subsite S1 of thermolysin and the side chain of P2′ with S1′ according to the pronounced hydrophobic specificity of both enzyme subsites (Fig. 2). In this case, the HS-acyl inhibitor develops a bidentate coordination to the zinc ion (the sulfur and the oxygen of the HS-acyl function being coordinated to the metal ion). This kind of "unusual" binding is the reason for lower K_i values (on thermolysin in the range of 10^{-8} M) in comparison to "usually"-bound HS-acyl peptides behaving as monodentate ligands (K_i values in the range of 10^{-6} M).[31] Figure 2 shows the enzyme-inhibitor interactions assumed for our C-terminal thiol-peptide inhibitors in comparison to those for the N-terminal thiol peptides considering the different recognition modes.

Tight-binding thiol-peptide inhibitors fitting to the prime site of the active center with inhibition constants in the nanomolar range were also found for metalloproteases other than thermolysin. Some examples are given in Table II. HS-(3-Phenyl)-propanoyl-Phe-Ala-OH, one of the strongest thiol-inhibitors of thermolysin known, interacts with the subsites S1, S1′, and S2′ of the active site and inhibits the enzyme with a K_i of 19 nM,[31] whereas the most potent monodentate-coordinating inhibitor ever described, HS-(2-benzyl)-propanoyl-Ala-Gly-NH_2, has a K_i of 750 nM.[19]

Except for the thiol group, other effective Zn^{2+}-coordinating groups are hydroxamates

RIGHTSLINK

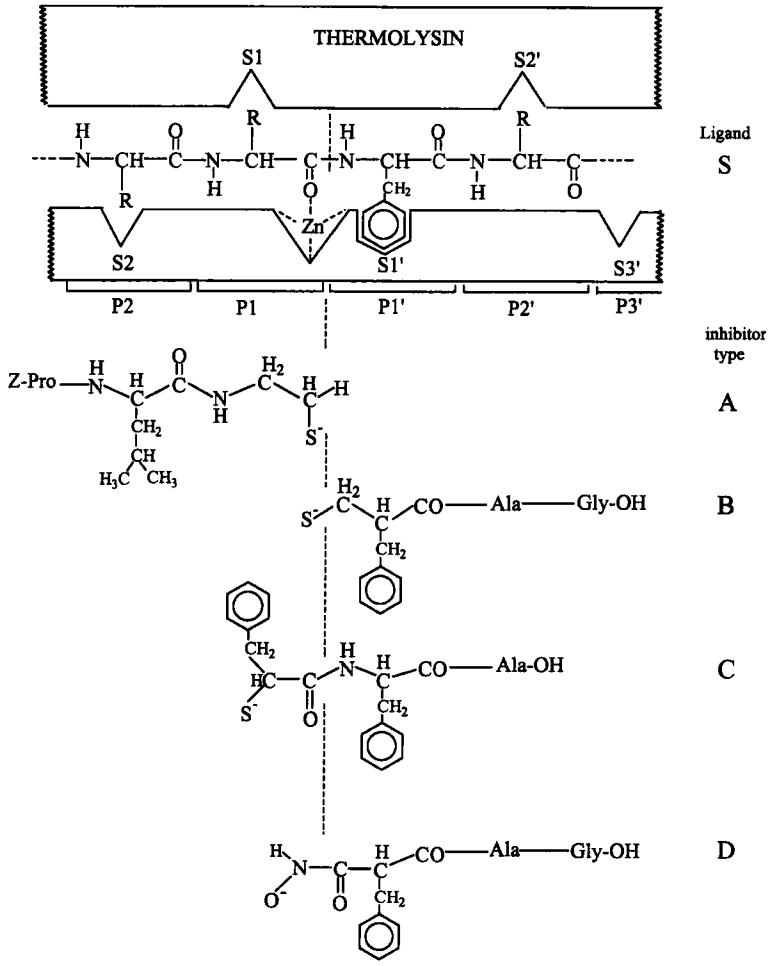

FIGURE 2 Schematic illustration demonstrating the enzyme-ligand interactions of different thermolysin inhibitors with substrate-analogous structure. S-peptidyl chain of the ligand; inhibitor typs: A, Z-Pro-Leu-CA-SH (**10**, this paper); B, HS-(2-benzyl)-propanoyl-Ala-Gly-NH_2;[19] C, HS-(3-phenyl)-propanoyl-Phe-Ala-OH;[31] D, HONH-benzylmalonyl-Ala-Gly-OH.[21]

and phosphoramidates. Quantum-chemical calculations[54] have proved that only the hydroxamate group exhibits a stronger binding to the metal ion than the thiolate group. However, the most potent among all reported inhibitors of thermolysin is the phosphoramidate derivative Z-Phe^{P}-Leu-Ala-OH, a slow binding transition state analogue inhibitor with a K_i of 68 pM and a first order rate constant k_{inact} of 6.8×10^{-8}/s.[16,18,55] Peptide-coupled hydroxamates[21,56] inhibit thermolysin in both situations, whether the functional group is bound at the C- or at the N-terminus of the peptide chain, with K_i values in the micromolar range. In general, according to the importance of the different subsites for the binding of ligands to metalloproteases, N-terminal hydroxamates are somewhat stronger inhibitors than C-terminal ones. The most effective hydroxamate inhibitor of thermolysin is with an N-terminal orientation of the functional group, HO-NH-ibm-Ala-Gly-NH_2 ($K_i = 0.48\,\mu M$) and with the hydroxamate in the C-terminal position, Z-Agly-Leu-NH-OH ($K_i = 2.7\,\mu M$).[19,21] It should be noted, that at pH 7–8, the optimal pH for catalytic activity of

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

RIGHTSLINK

TABLE II Comparison of the inactivation of zinc metalloproteases by thiol-inhibitors

Inhibitor	Enzyme	K_i (μM)	Reference
cysteamine	TLN	700	14
mercaptoethanol-2	TLN	1000	19
(S)-thiorphan	TLN	1.6	31
HS-(2-benzyl)-propanoyl-Ala-Gly-NH_2	TLN	0.75	19
HS-(3-phenyl)-propanoyl-Phe-Ala-OH	TLN	0.019	31
HS-(3-phenyl)-propanoyl-Phe-Tyr-OH	NEP	0.0029	28
HS-(3-phenyl)-propanoyl-Phe-Ala-OH	NEP	0.0023	31
HS-(3-phenyl)-propanoyl-(5′-Ph)-Pro-OH	NEP	0.0016	29
HS-(2-benzyl)-propanoyl-Ala-Gly-NH_2	MMP-8 (HNC)	1.2	23
HS-(2-ibu)-propanoyl-Leu-Gly-OEt	MMP-1	1.3*	54
HS-diketopiperazine	MMP-1	0.03*	39
HS-diketopiperazine	MMP-3	3.8*	39
HS-diketopiperazine	MMP-9	0.079*	39
HS-(3-phenyl)-propanoyl-Ala-Pro-OH	ACE	0.0003	31
HS-propionate-3	CPA	18	17
HS-propionate-3	CPB	71	17

*IC_{50} value

thermolysin, the reactive hydroxamate group of the inhibitor is deprotonated and in the cis-configuration. This is a precondition for hydroxamates to be able to act as bidentate donors to the metal ion like phosphoramidates and thiolates in "unusual" binding.

In conclusion, a new class of C-terminal-acting thermolysin inhibitors, which are able to coordinate the catalytic zinc ion by a thiolate group have been synthesized and characterized in this work. The described compounds should open the way for design of new pharmacologically active compounds inhibiting inflammation, degradation of extracellular matrix and other pathological processes.

Acknowledgements

We wish to thank Dr. Mohr as well as Dr. Kluge and Dr. Ströhl (Institute of Organic Chemistry, Department of Chemistry, Martin Luther University Halle) for analytical determinations (elementary analysis, mass spectra, ^1H NMR spectra) and discussions.

References

[1] Bond, J.S. and Jiang, W. (1997) In: Ansorge, S. and Langner, J., eds, Cellular Peptidases in Immune Functions and Diseases (Plenum Press, New York), pp 1–6.
[2] Tschesche, H. (1997) In: Ansorge, S. and Langner, J., eds, Cellular Peptidases in Immune Functions and Diseases (Plenum Press, New York), pp 290–301.
[3] Supuran, C.T., Scozzafava, A. and Mastrolorenzo, A. (2001), *Exp. Opin. Therap. Patents* **11**, 221–259.
[4] Stöckler, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Rüth, F.X., McKay, D.B. and Bode, W. (1995), *Protein Sci.* **4**, 823–840.
[5] Bode, W., Gomis-Rüth, F.X. and Stöckler, W. (1993), *FEBS Lett.* **331**, 134–140.
[6] Nagase, H. (1997), *Biol. Chem.* **378**, 151–160.
[7] Nagase, H. and Woessner, Jr., J.F. (1999), *J. Biol. Chem.* **274**, 21491–21494.
[8] Monzingo, A.F. and Matthews, B.W. (1984), *Biochemistry* **23**, 5724–5729.
[9] Chan, W.W.C. (1983), *Biochem. Biophys. Res. Commun.* **116**, 297–302.
[10] Ocain, T.D. and Rich, H.D. (1987), *Biochem. Biophys. Res. Commun.* **145**, 1038–1042.
[11] David-Basei, C., Fournie-Zaluski, M.C. and Roques, B.P. (2001), *Exp. Opin. Therap. Patents* **11**, 431–444.
[12] Kester, W.R. and Matthews, B.W. (1977), *J. Biol. Chem.* **252**, 7704–7710.
[13] Cushman, D.W., Cheung, H.S., Sabo, E.F. and Ondetti, M.A. (1977), *Biochemistry* **16**, 5484–5491.
[14] Pfuetzner, R.A. and Chan, W.W.C. (1993), *Eur. J. Biochem.* **218**, 523–528.
[15] Komiyama, T., Suda, H., Aoyagi, T., Takeuchi, T., Umezawa, H., Fugjimoto, K. and Umezawa, S. (1975), *Arch. Biochem. Biophys.* **171**, 727–731.
[16] Matthews, B.W. (1988), *Acc. Chem. Res.* **21**, 333–340.
[17] Chan, W.W.C. and Pfuetzner, R.A. (1993), *Eur. J. Biochem.* **218**, 529–534.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

RIGHTSLINK

[18] Bartlett, P.A. and Marlowe, C.K. (1987), *Biochemistry* **26**, 8553–8561.
[19] Nishino, N. and Powers, J.C. (1979), *Biochemistry* **18**, 4340–4347.
[20] Holden, H.M., Tronrud, D.E., Monzingo, A.F., Weaver, L.H. and Matthews, B.W. (1987), *Biochemistry* **26**, 8542–8553.
[21] Nishino, N. and Powers, J.C. (1978), *Biochemistry* **17**, 2846–2850.
[22] Bode, W., Reinemer, P., Huber, R., Kleine, T., Schnierer, S. and Tschesche, H. (1994), *EMBO J.* **13**, 1263–1269.
[23] Grams, F., Reinemer, P., Powers, J.C., Kleine, T., Pieper, M., Tschesche, H., Huber, R. and Bode, W. (1995), *Eur. J. Biochem.* **228**, 830–841.
[24] Johnson, W.H., Roberts, N.A. and Borkakoti, N. (1987), *J. Enzyme Inhib.* **2**, 1–22.
[25] Scozzafava, A. and Supuran, C.T. (2000), *J. Med. Chem.* **43**, 1858–1865.
[26] Scozzafava, A. and Supuran, C.T. (2000), *J. Med. Chem.* **43**, 3677–3687.
[27] Scozzafava, A. and Supuran, C.T. (2000), *Bioorg. Med. Chem. Lett.* **10**, 499–502.
[28] Coric, P., Turcaud, S., Meudal, H., Roques, B.P. and Fournie-Zaluski, M.C. (1996), *J. Med. Chem.* **39**, 1210–1219.
[29] Fournie-Zaluski, M.C., Coric, P., Thery, V., Gonzalez, W., Meudal, H., Turcaud, S., Michel, J.-P. and Roques, B.P. (1996), *J. Med. Chem.* **39**, 2594–2608.
[30] Fink, C.A., Carlson, J.E., Boehm, C., McTaggart, P., Qiao, Y., Doughty, J., Ganu, V., Melton, R. and Goldberg, R. (1999), *Bioorg. Med. Chem. Lett.* **9**, 195–200.
[31] Gaucher, J.F., Selkti, M., Tiraboschi, G., Prange, T., Roques, B.P., Tomas, A. and Fournie-Zaluski, M.C. (1999), *Biochemistry* **38**, 12569–12576.
[32] Gray, R.D., Saneii, H.H. and Spatola, A.F. (1981), *Biochem. Biophys. Res. Commun.* **101**, 1251–1257.
[33] Monzingo, A.F. and Matthews, B.W. (1982), *Biochemistry* **21**, 3390–3394.
[34] Byers, L.D. and Wolfenden, R. (1973), *Biochemistry* **12**, 2070–2078.
[35] Brandstetter, H., Engh, R.A., Roedern, E.G.v., Moroder, L., Huber, R., Bode, W. and Grams, F. (1998), *Protein Sci.* **7**, 1303–1309.
[36] Roedern, E.G.v., Grams, F., Brandstetter, H. and Moroder, L. (1998), *J. Med. Chem.* **41**, 339–345.
[37] Kam, C.M., Nishino, N. and Powers, J.C. (1979), *Biochemistry* **18**, 3032–3038.
[38] Müller, J.C.D., Roeder, E.G.v., Grams, F., Nagase, H. and Moroder, L. (1997), *Biol. Chem.* **378**, 1475–1480.
[39] Szardenings, A.K., Antonenko, V., Campbell, D.A., DeFrancisco, N., Ida, S., Shi, L., Sharkov, N., Tien, D., Wang, Y. and Navre, M. (1999), *J. Med. Chem.* **42**, 1348–1357.
[40] Peters, K., Brömme, D., Jahreis, G. and Fittkau, S. (1996), *Adv. Exp. Med. Biol.* **379**, 133–140.
[41] Barrett, A.J. and Kirschke, H. (1981), *Meth. Enzymol.* **80**, 535–561.
[42] Taralp, A., Kaplan, H., Sybwu, I.I., Vlattas, I., Bohacek, R., Knap, A.K., Hirama, T., Huber, C.P. and Hasnain, S. (1995), *J. Biol. Chem.* **270**, 18036–18043.
[43] Chan, W.W.C., Dennis, P., Demmer, W. and Brand, K. (1982), *J. Biol. Chem.* **257**, 7955–7957.
[44] Latt, S.A., Holmquist, B. and Vallee, B.L. (1969), *Biochem. Biophys. Res. Com.* **37**, 333–339.
[45] Feder, J., Garrett, L.R. and Wildi, B.S. (1971), *Biochemistry* **10**, 4552–4556.
[46] Morihara, K., Tsuzuki, H. and Oka, T. (1968), *Arch. Biochem. Biophys.* **123**, 572–588.
[47] Walsh, K.A., Burstein, Y. and Pangburn, M.K. (1974), *Meth. Enzymol.* **34**, 435–440.
[48] Wünsch, E. (1974) Houben-Weyl, Methoden der organischen Chemie XV/1 (Springer, Stuttgart), p 786.
[49] Toennies, G. and Kolb, J.J. (1951), *J. Analyt. Chem.* **23**, 823–826.
[50] Schechter, T. and Berger, A. (1967), *Biochem. Biophys. Res. Commun.* **27**, 157–162.
[51] Morihara, K. and Tsuzuki, H. (1970), *Eur. J. Biochem.* **15**, 374–380.
[52] Matsubara, H., Singer, A. and Sasaki, R. (1969), *Biochem. Biophys. Res. Commun.* **34**, 719–724.
[53] Pozgay, M., Michaud, C., Liebman, M. and Orlowski, M. (1986), *Biochemistry* **25**, 1292–1299.
[54] Garmer, D.R., Gresh, N. and Roques, B.P. (1998), *Proteins* **31**, 42–46.
[55] Shen, J. (1997), *J. Med. Chem.* **40**, 2953–2958.
[56] Blumberg, S., Vogel, Z. and Altstein, M. (1981), *Life Sci.* **28**, 301–306.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/19/11
For personal use only.

RIGHTSLINK