Journal of Enzyme Inhibition and Medicinal Chemistry, June 2003 Vol. 18 (3), pp. 233–242

Taylor & Francis
healthsciences

Protease Inhibitors: Synthesis of Bacterial Collagenase and Matrix Metalloproteinase Inhibitors Incorporating Succinyl Hydroxamate and Iminodiacetic Acid Hydroxamate Moieties

M. AMÉLIA SANTOS[a], SÉRGIO MARQUES[a], MARCO GIL[a], MATTEO TEGONI[b], ANDREA SCOZZAFAVA[c] and CLAUDIU T. SUPURAN[c,*]

[a]*Centro de Química Estrutural, Complexo I, Instituto Superior Técnico, 1049-001 Lisboa, Portugal;* [b]*Dipartimento di Chimica Generale ed Inorganica, Chimica Analitica, Chimica Fisica, Università di Parma, 43100 Parma, Italy;* [c]*Università degli Studi di Firenze, Laboratorio di Chimica Inorganica e Bioinorganica, Via della Lastruccia 3, Rm 188, Polo Scientifico, 50019-Sesto Fiorentino (Firenze), Italy*

(Received 18 November 2002)

A series of succinyl hydroxamates/bishydroxamates as well as a new structural type of matrix metalloproteinase (MMP)/bacterial protease (BP) inhibitors, incorporating iminodiacetic (IDA) hydroxamate/bishydroxamate moieties, has been synthesized and tested for interaction with four vertebrate proteases, MMP-1, MMP-2, MMP-8 and MMP-9, and a BP, the collagenase isolated from *Clostridium histolyticum* (ChC). The new derivatives generally showed inhibition constants in the range of 8–62 nM against the five proteases mentioned above.

Keywords: MMPs; Bacterial proteases; Hydroxamate; Enzyme inhibitor

INTRODUCTION

Proteases, such as the matrix metalloproteinases (MMPs)[1–7] or the bacterial proteases (BPs)[7,8] have recently become interesting targets for drug design, in the search of novel types of anticancer, anti-arthritis, antibacterial or other pharmacological agents useful in the management of inflammatory processes.[1–8] All these conditions are generally associated with enhanced activity of several zinc endopeptidases, of which the different MMPs actually known (more than 20 such enzymes have been reported at the present time)[1–8] and the large number of BPs[8] isolated in many pathogenic bacterial species, are responsible for the efficient degradation of all components of the extracellular matrix (ECM).[3,4] ECM turnover is involved in crucial physiological and physiopathological events, such as embryonic development, blastocyst implantation, nerve growth, ovulation, morphogenesis, angiogenesis, tissue resorption and remodeling (such as in the case of wound healing), bone remodeling, apoptosis, cancer invasion and metastasis, arthritis, atherosclerosis, aneurysm, breakdown of the blood–brain barrier, periodontal disease, skin and corneal ulceration, gastric ulcer, or liver fibrosis in the case of the vertebrate enzymes mentioned above.[1–12] In bacteria, proteases are involved in critical processes such as colonization and evasion of host immune defenses, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection.[8]

As for other metallo-enzymes, inhibition of MMPs/BPs is correlated with binding of the inhibitor molecule to the catalytic metal ion, with or without substitution of the zinc-bound water molecule (the nucleophile attacking the scissile peptide bond).[1–8] Thus, MMP inhibitors (MMPIs) must contain a zinc-binding function attached to a scaffold that assures good interactions with the binding regions of the protease.[1–8] Depending on the zinc-binding function contained in their molecule, MMPIs belong to several chemical classes, such as carboxylates, hydroxamates, thiols, phosphorus-based compounds, sulfodiimines, etc.[1–8] The strongest inhibitors are generally the hydroxamates; 5–10 fold less potent are the "reverse hydroxamates"

*Corresponding author. Tel.: +39-055-4573005. Fax: +39-055-4573385. E-mail: claudiu.supuran@unifi.it

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/1475636031000081134

(of the type HCON(OH)-R), while thiols are 20–50 times less potent, and carboxylates/phosphonates 100–2000 times less potent than hydroxamates. Many of these inhibitors were derived by replacing the scissile peptide bond with such a zinc-binding function (eventually followed by a methylene moiety) in such a way that the zinc-binding moiety is available for coordination to the catalytic Zn(II) ion.[1–8] Hydroxamates are by far the most investigated class of MMPIs.[3,4] Thousands of structural variants containing the CONHOH moiety have been synthesized and assayed as inhibitors of MMPs and other types of metallo-enzymes (BPs for example).[3–8] Three main classes of such inhibitors have been reported: (1) the succinyl hydroxamates (and their derivatives), (2) the malonic/oxalic acid-based inhibitors and, (3) the sulfonamide-based inhibitors.[3,4] Here we report the synthesis and MMP/BP inhibitory activity of a small library of succinyl hydroxamates/bishydroxamates as well as a new structural type of MMP/BP inhibitor, incorporating iminodiacetic (IDA) hydroxamate moieties in its molecule.

MATERIALS AND METHODS

Chemicals and Equipment

Analytical grade reagents were purchased from Aldrich, Sigma and Fluka and were used as supplied. Solvents were dried according to standard methods.[13] The chemical reactions were monitored by TLC using alumina plates coated with silica gel 60 F_{254} (Merck). Column flash chromatography separations were performed on silica gel Merck 230–400 mesh ASTM. Melting points were measured with a Leica Galen III hot stage apparatus and are uncorrected. IR spectra were recorded on a Bio-Rad Merlin, FTS 3000 MX. The ^{1}H NMR spectra were recorded on a Varian Unity 300 spectrometer at 25°C. Chemical shifts are reported in ppm (δ) with tetramethylsilane (TMS) as internal reference in organic solvents and sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]-propionate (DSS) in D_2O solutions. The following abbreviations are used: d = duplet; s = singlet; t = triplet; q = quartet; br = broad. Mass spectra (FAB) were performed on a VG TRIO-2000 GC/MS instrument and ESI spectra on a Quattro LC mass spectrometer (Micromass, Manchester). The High Resolution Mass Spectra (HRMS) were obtained with a High Resolution Fourier transform ion cyclotron resonance (FTICR) instrument, Finnigan FT/MS 2001-DT, equipped with a 3.0-T superconducting magnet, by electron impact (EI), typically with 15-eV electron beam energies, 5-micro emission currents and 150°C sample temperatures. Elemental analyses were performed on a Fisons EA1108 CHNF/O instrument.

Synthesis

(S)-N-(3-Carboxy-propanoyl)-phenylalanine Benzyl Ester (1a)

Triethylamine (1.30 mL, 9.38 mmol) was added to a solution of *L*-phenylalanine-*O*-benzyl ester *p*-toluenesulfonate salt (2.00 g, 4.68 mmol) in dry CH_3CN (40 mL), keeping the temperature at 0°C, and the solution was left stirring for 15 min, under nitrogen. A solution of succinic anhydride (0.47 g, 4.69 mmol) in dry CH_3CN (20 mL) was then added dropwise with stirring and the reaction mixture was stirred for 2 h, keeping the temperature at 0°C. The solvent was then removed under vacuum and the crude material was dissolved in ethyl acetate (30 mL), and washed with 0.5 M citric acid solution (15 mL) and brine (2 × 25 mL). The organic phase was dried with anhydrous Na_2SO_4 and the solvent removed in vacuum. Recrystallization of the solid residue from ethyl ether afforded the final product (1.33 g, 80%), mp 110°–112°C. ^{1}H NMR ($CDCl_3$) δ 2.48 (2H, t, CH_2CONHR), 2.65 (2H, t, CH_2COOH), 3.11 (2H, t, C$_\beta H_2$Ph), 4.91 (m, 1H, C$_\alpha$H), 5.13 (m, 2H, OCH_2Ph), 6.21 (1H, d, NH), 7.00–7.37 (10H, m, 2 × Ph); IR (KBr, cm^{-1}) 1784 (C=O, ester), 1730 (C=O, acid), 1643 (C=O, amide); MS (FAB), *m/z* 356 (M + 1).

(S)-N-(3-Benzyloxycarbamoyl-propanoyl)-phenylalanine Benzyl Ester (1b)

O-benzylhydroxylamine was obtained by adding a solution of KOH (0.21 g, 3.74 mmol) in dry methanol (5 mL) to a solution of *O*-benzylhydroxylamine hydrochloride (0.60 g, 3.76 mmol) in the same solvent (10 mL), keeping the temperature at 0°C. The mixture was allowed to stir for 15 min and then the KCl formed was filtered off. Meanwhile, a solution of **1a** (1.34 g, 3.77 mmol) in dry THF (25 mL) was cooled in an ice–water bath and *N*-methylmorpholine (0.41 ml, 3.77 mmol) and ethylchloroformate (0.36 mL, 3.77 mmol) were added under nitrogen with stirring. The reaction mixture was stirred for 15 min and then the solid *N*-methylmorpholine hydrochloride formed was filtered and discarded. The *O*-benzylhydroxylamine solution was added dropwise to the solution of **1a** under stirring and the mixture stirred for other 2 h at 0°C. The solution was then filtered, the solvent removed in vacuum, and the crude material dissolved in CH_2Cl_2 (25 mL) and extracted with a solution of $NaHCO_3$ (0.5 M). The product was re-extracted from the aqueous solution with CH_2Cl_2 (5 × 25 mL), the organic layer dried over anhydrous Na_2SO_4 and the solvent removed under vacuum. The product was purified *via* column flash chromatography (eluent CH_2Cl_2/MeOH, 100:5). The solvent was removed in vacuum and the solid washed twice with a small amount of

diethylether, yielding 1.45 g of the pure product (84%), mp = 133°–135 °C; ^{1}H NMR ($CDCl_3$) δ 2.34 (2H, t, CH_2CONHR), 2.50 (2H, t, CH_2CONHOR), 3.08 (2H, br, C$_\beta H_2$Ph), 4.87 (3H, br, C$_\alpha$H + NHOCH_2Ph), 5.13 (2H, m, COOCH_2Ph), 6.21 (1H, br, C$_\alpha$HN*H*), 7.00–7.37 (15H, m, 3 × Ph), 8.75 (1H, br, CONH); IR (KBr, cm^{-1}) 1760 (C=O, ester), 1637 (C=O, amide); MS (FAB), *m*/*z* 461 (M + 1).

(S)-N-(3-Hydroxycarbamoyl-propanoyl)-phenylalanine (1)

To a solution of **1b** (1.45 g, 3.15 mmol) in dry methanol (50 mL) was added 0.07 g 10% Pd/C and the mixture was shaken under H_2 (1 atm) for 4 h at room temperature. The solvent was removed in vacuum and the solid product was further purified by washing it four times with CH_2Cl_2, to give the pure final product in 99% yield, mp = 182°–184°C. ^{1}H NMR (CD_3OD) δ 2.27 (2H, t, CH_2CONHR), 2.47 (2H, t, CH_2CONHOH), 2.94–3.18 (2H, m, C$_\beta H_2$Ph), 4.63 (1H, q, C$_\alpha$H), 7.25 (5H, m, Ph); IR (KBr, cm^{-1}) 1725 (C=O, acid), 1651, (C=O, amide); MS (FAB), *m*/*z* 281 (M + 1). Anal. Calcd for $C_{13}H_{16}N_2O_5$: C, 55.71; H, 5.75; N, 9.99. Found: C, 55.8; H, 5.7; N, 9.9%.

(S)-N-(3-Carboxy-propanoyl)-proline Benzyl Ester (2a)

This was prepared from *L*-proline-*O*-benzylester hydrochloride and succinic anhydride, following the procedure described for compound **1a**. The product was obtained as a colourless solid, from recrystallization in CH_2Cl_2/ethyl ether, yield 89%, mp 119°–121°C; ^{1}H NMR (CD_3CN) δ 1.96 (2H, m, C$_\gamma H_2$,), 2.17 (2H, m, C$_\beta H_2$), 2.51 (2H, m, CH_2CONR_2), 2.57 (2H, m, CH_2COOH,), 3.55 (2H, m, C$_\delta H_2$), 4.40 (1H, q, C$_\alpha$H), 5.12 (2H, s, OCH_2Ph), 7.36 (5H, m, Ph); IR (KBr, cm^{-1}) 1732 (C=O, acid), 1610 (C=O, amide); MS (FAB), *m*/*z* 306 (M + 1).

(S)-N-(3-Benzyloxycarbamoyl-propanoyl)-proline Benzyl Ester (2b)

This was prepared from the carboxylic acid derivative (**2a**), following the procedure described for compound **1b**, to yield a pale oil (76%). ^{1}H NMR (CD_3CN) δ 1.98 (2H, m, C$_\gamma H_2$), 2.20–2.15 (4H, m, C$_\beta H_2$ + CH_2CONR_2), 2.58 (2H, m, CH_2CONHOR), 3.58 (2H, m, C$_\delta H_2$), 4.37 (1H, q, C$_\alpha$H), 4.80 (2H, s, NHOCH_2Ph), 5.10 (2H, s, COOCH_2Ph), 7.37 (10H, m, Ph), 9.26 (1H, br, NH); IR (neat, cm^{-1}) 1741 (C=O, ester), 1643 (C=O, amide); MS (FAB), *m*/*z* 411 (M + 1).

(S)-N-(3-Hydroxycarbamoyl-propanoyl)-proline (2)

The hydroxamic acid **2** was prepared from **2b**, according to the general process of hydrogenolysis described in the synthesis of **1**, but under H_2 (1.5 atm) for 5 h. It was obtained as a hygroscopic pale foam (>99%). ^{1}H NMR (CD_3OD) δ 1.86 (2H, m, C$_\gamma H_2$), 2.08 (2H, m, C$_\beta H_2$), 2.25 (2H, m, CH_2CONR_2), 2.53 (2H, m, CH_2CONHOH), 3.49 (2H, m, C$_\delta H_2$), 4.25 (1H, q, C$_\alpha$H); IR (KBr, cm^{-1}) 1724 (C=O, acid), 1624 (C=O, amide); MS (FAB), *m*/*z* 231 (M + 1). Anal. Calcd for $C_9H_{14}N_2O_5$: C, 46.95; H, 6.13; N, 12.17. Found: C, 47.0; H, 6.2; N, 12.2%.

(S)-N-(3-Carboxy-propanoyl)-proline (3a)

Triethylamine (4.71 mL; 0.034 mol) was added under stirring to a suspension of *L*-proline (2 g, 0.017 mol) in dry acetonitrile (40 mL) keeping the temperature at 0°C. The solution was stirred for 15 min. A solution of succinic anhydride (1.70 g, 0.013 mol) in dry acetonitrile (25 mL) was then added dropwise with stirring and the stirring continued for 2 h at 0°C. The solvent was then removed under vacuum and the crude material dissolved in ethyl acetate (25 mL) and extracted with a pH 2 HCl solution (25 mL). The reaction product was re-extracted from the aqueous solution with ethyl acetate (6 × 25 mL), the organic phase dried (Na_2SO_4) and the solvent removed under vacuum. The glue obtained was dried by treatment with absolute ethanol (4 × 20 mL) then evaporated in vacuum and treated again with CH_2Cl_2 (5 × 20 mL), to obtain a white hygroscopic foam, 78% yield. ^{1}H NMR (CD_3OD) δ 1.98–2.13 (2H, m, C$_\gamma H_2$), 2.18 (2H, t, C$_\beta H_2$), 2.50–2.85 (4H, m, 2 × CH_2CO), 3.52–3.70 (2H, m, C$_\delta H_2$), 4.51 (1H, t, C$_\alpha$H), 8.52 (2H, br, 2 × COOH); IR (CH_2Cl_2, cm^{-1}) 1720 (C=O, acid), 1616 (C=O, amide); MS (FAB), *m*/*z* 216 (M + 1).

(S)-N-(3-Benzyloxycarbamoyl-propanoyl)-proline-benzyloxyamide (3b)

This was prepared from the corresponding dicarboxylic acid (**3a**), following the same procedure described for **1b**, but using two equivalents of both *O*-benzylhydroxylamine and coupling agent. The final reaction mixture was filtered and the solvent removed in vacuum. The crude material was dissolved in 0.5 M $NaHCO_3$ (30 mL) and the product was re-extracted from the aqueous solution with ethyl acetate (5 × 30 mL). The organic phase was washed with brine (2 × 30 mL), dried (Na_2SO_4), the solvent removed under vacuum and the product purified via column flash chromatography (eluent CH_2Cl_2/MeOH 100:5). The solvent was removed under vacuum, to yield the pure final product as a white hygroscopic foam (42%). ^{1}H NMR (CD_3CN) δ 1.90 (2H, m, C$_\gamma H_2$), 1.99 (2H, t, C$_\beta H_2$), 2.29 (2H, m, CH_2CONR_2), 2.51 (2H, m, CH_2CONHOR), 3.41–3.55 (2H, m, C$_\delta H_2$), 4.23 (1H, t, C$_\alpha$H), 4.81 (4H, s, 2 × CH_2Ph), 7.38 (10H, m, 2 × Ph), 9.30

(1H, br C_αHCON*H*), 9.75 (1H, br, CH_2CON*H*); IR (CH_2Cl_2, cm^{-1}) 1658, 1631 (2 × C=O, amide); MS (FAB), m/z 426 (M + 1).

(S)-N-(3-Hydroxycarbamoyl-propanoyl)-proline Hydroxamic Acid (3)

This was prepared from the corresponding *O*-benzyl derivative (**3b**), using the same procedure described for the preparation of **1**. It was obtained as white crystals, 97% yield, mp = 200°–202°C. ^{1}H NMR (CD_3OD) δ 1.95–2.10 (4H, m, $C_\gamma H_2 + C_\beta H_2$), 2.33 (2H, t, CH_2CONR_2), 2.59 (2H, t, C*H*$_2$CONHOH), 3.51–3.60 (2H, m, $C_\delta H_2$), 4.27 (1H, q, C_αH); IR (KBr, cm^{-1}) 1630 (C=O, amide); MS (FAB), m/z 246 (M + 1). Anal. Calcd for $C_9H_{15}N_3O_5$: C, 44.08; H, 6.17; N, 17.13 Found: C, 44.0; H, 6.1; N, 17.3%.

(S)-N-(3-Carboxy-propanoyl)-phenylalanine (4a)

The dicarboxylic acid (**4a**) was prepared from *L*-phenylalanine and succinic anhydride, following the procedure described for compound **3a**. The product was obtained as a white solid, 60% yield. ^{1}H NMR (CD_3CN) δ 2.40–2.45 (4H, m, 2 × CH_2CO), 2.91–3.17 (2H, m, $C_\beta H_2$Ph), 4.59 (1H, m, C_αH), 6.73 (1H, br, NH), 7.21–7.30 (5H, m, Ph); IR (KBr, cm^{-1}) 1720 (C=O, acid), 1651 (C=O, amide); MS (ESI), m/z 266 (M + 1).

(S)-N-(3-Benzyloxycarbamoyl-propanoyl)-phenylalanine Benzyloxy-amide (4b)

The di-(*O*-benzylhydroxamic) acid **4b** was prepared from the corresponding dicarboxylic acid (**4a**) and *O*-benzylhydroxylamine, following the same procedure used for **3b**. The final pure product was obtained as a white solid, (41% yield). ^{1}H NMR (d_6-DMSO) δ 2.12 (2H, t, C*H*$_2$CONHR), 2.27–2.36 (2H, m, C*H*$_2$CONHOR), 2.74–2.95 (2H, m, $C_\beta H_2$Ph), 4.32 (1H, q, C_αH), 4.63–4.73 (2H, q, C_αHCONHOC*H*$_2$Ph), 4.75 (2H, s, CH_2CONHOC*H*$_2$Ph), 7.18–7.38 (15H, m, 3 × Ph), 8.24 (1H, d, NH); IR (KBr, cm^{-1}) 1665–1639 (C=O, amide); MS (ESI), m/z 476 (M + 1).

(S)-N-(3-Hydroxycarbamoyl-propanoyl)-phenylalanine Hydroxamic Acid (4)

The hydroxamic acid **4** was prepared from the corresponding *O*-benzyl derivative **4b** by standard hydrogenolysis, as described for **1**, but the reaction mixture was shaken overnight under H_2 (1 atm) at room temperature. Recrystallization of the final product from acetonitrile afforded the pure final product as a hygroscopic white solid (99% yield). ^{1}H NMR (d_6-DMSO) δ 2.25 (2H, m, C*H*$_2$CONHOH), 2.45 (2H, m, C*H*$_2$CONHR), 2.90–3.12 (2H, m, $C_\beta H_2$Ph), 4.52 (1H, q, C_αH), 7.33–7.45 (5H, m, Ph), 8.35 (1H, d, NH), 8.80 (1H, s, CH_2CON*H*), 8.97 (1H, s, C_αHCON*H*), 10.52 (1H, s, CH_2CONHO*H*), 10.78 (1H, s, C_αHCONHO*H*); IR (KBr, cm^{-1}) 1646, 1622 (C=O, amide); MS (ESI) m/z 296 (M + 1). Anal. Calcd for $C_{13}H_{17}N_3O_5$: C, 52.88; H, 5.80; N, 14.23. Found: C, 52.8; H, 5.8; N, 14.3%.

(S)-N-(3-Carboxy-propanoyl)-phenylalanine Methyl Ester (5a)

This was prepared by reaction of *L*-phenylalanine-methyl ester with succinic anhydride, following the procedure described for compound **1a**. The final product was obtained as a colorless glue (88%). ^{1}H NMR (d_6-DMSO) δ 2.31–2.35 (4H, m, C*H*$_2$CO), 2.85–3.04 (2H, m, $C_\beta H_2$Ph), 3.58 (3H, s, CH_3), 4.61 (1H, q, C_αH), 7.19–7.30 (5H, m, Ph), 8.32 (1H, d, NH); IR (CH_2Cl_2, cm^{-1}) 1743 (C=O, ester), 1715 (C=O, acid), 1680 (C=O, amide); MS (ESI), m/z 280 (M + 1).

(S)-N-(3-Benzyloxycarbamoyl-propanoyl)-phenylalanine Methyl Ester (5b)

This was prepared by reacting **5a** with *O*-benzyl-hydroxylamine, following the procedure described for **1b**. After removal of the solvent from the reaction mixture, the crude product was dissolved in 0.5 M $NaHCO_3$ (30 mL), which was then extracted with ethyl acetate (4 × 30 mL). The organic solution was washed with a concentrated NaCl solution (2 × 30 mL), dried (Na_2SO_4) and the solvent removed under vacuum. The white solid was washed with diethyl ether, affording the pure final product as a white solid (63%). ^{1}H NMR (CD_3CN) δ 2.20 (2H, t, C*H*$_2$CONHR), 2.40 (2H, m, C*H*$_2$CONHOH), 2.91–3.11 (2H, m, $C_\beta H_2$Ph), 3.62 (3H, s, CH_3), 4.61 (1H, q, C_αH), 4.79 (2H, s, NHOC*H*$_2$Ph), 6.86 (1H, NH, d), 7.17–7.38 (10H, m, Ph), 9.32 (1H, s, CH_2CON*H*); IR (KBr, cm^{-1}) 1740 (C=O, ester), 1646 (C=O, br, amide); MS (ESI), m/z 400 (M + 1).

(S)-N-(3-Hydroxycarbamoyl-propanoyl)-phenylalanine Methyl Ester (5)

The hydroxamic acid **5** was prepared from the corresponding *O*-benzyl derivative **5b** using standard hydrogenolysis, as described for **1**, but the reaction mixture was stirred overnight under H_2 (1 atm) at room temperature. Recrystallization of the final product from acetonitrile afforded the pure final product as a white solid (99% yield), mp = 44°–45°C. ^{1}H NMR (d_6-DMSO) δ 2.07–2.12 (2H, t, C*H*$_2$CONHOH), 2.28–2.34 (2H, t, C*H*$_2$CONHR), 2.83–3.03 (2H, m, $C_\beta H_2$Ph), 3.58 (3H, s, CH_3), 4.44 (1H, q, C_αH), 7.19–7.30 (5H, m, Ph), 8.35 (1H, d, NH), 8.66 (1H, s, CH_2CON*H*), 10.34 (1H, s, OH); IR (KBr, cm^{-1}) 1736 (C=O, ester), 1658

(C=O, amide); MS (ESI), m/z 310 (M + 1). Anal. Calcd for $C_{14}H_{19}N_3O_5$: C, 54.36; H, 6.19; N, 13.58. Found: C, 54.3; H, 6.2; N, 13.5%.

*(S)-N-(3-hydroxy-*N*-methyl-carbamoyl-propanoyl)-phenylalanine-*N*-methylhydroxamic Acid (6)*

The di-(*N*-methylhydroxamic) acid **6** was prepared from the corresponding dicarboxylic acid (**4a**) and *N*-methylhydroxylamine hydrochloride, following the same procedure used for **3b**. The final product was recrystallized from acetonitrile/diethyl ether, affording the pure product as a colourless hygroscopic solid (73% yield). ^{1}H NMR ($CDCl_3$): 2.52–2.65 (4H, m, 2 × CH_2CO), 2.94 (2H, m, $C_\beta H_2Ph$), 3.21 (6H, s, 2 × CH_3), 5.21 (1H, q, $C_\alpha H$), 7.26 (5H, m, Ph);. IR (KBr, cm^{-1}) 1633 (C=O, amide). HRMS m/z calculated for $C_{15}H_{21}N_3O_5$: 323.14812; found: 323.14836.

N-benzyl-N-carboxymethyl-iminoacetohydroxamic Acid (7)

To an ice-cold solution of *N*-benzyliminodiacetic acid (1.00 g, 4.48 mmol) in THF (10 ml) under nitrogen, was added dropwise a solution of ethylchloroformate (0.43 mL, 4.48 mmol) and *N*-methylmorpholine (0.49 mL, 4.48 mmol) both in dry THF. After reacting for 30 min at 0°C, the precipitate of *N*-methylmorpholinium chloride was filtered off. Meanwhile the free hydroxylamine was prepared by adding a solution of KOH (0.25 g, 4.48 mmol) in dry methanol (10 mL) to a suspension of NH_2OH HCl (0.31 g, 4.48 mmol) in the same solvent (10 mL). The mixture was stirred for 30 min at 0°C, and then the solid KCl formed was filtered off. The hydroxylamine solution was then added dropwise to the first solution containing the activated acid, and the mixture was stirred for 4 h at 0°C. The solid residue obtained from solvent evaporation was first washed with ethyl acetate and then acetonitrile, and recrystallized from dry methanol to afford the pure product (0.367 g, yield of 34%); mp 146°–147°C; ^{1}H NMR (D_2O): 3.88 (2H, s, CH_2COOH), 4.02 (2H, s, CH_2CONHOH), 4.52 (2H, s, CH_2Ph), 7.51 (5H, m, Ph); IR (KBr, cm^{-1}) 1688 (C=O, acid), 1588 (C=O, amide); HRMS (MH^+), m/z calculated for $C_{11}H_{14}N_2O_4$: 239.102634; found: 239.102629. Analysis. Calcd for $C_{11}H_{14}N_2O_4$: C, 55.46; H 5.92; N, 11.76. Found: C, 55.54; H, 6.40; N, 11.33%.

N-benzyl-N′-hydroxy-piperazine-2,6-dione (8)

This was prepared from *N*-benzyl-iminodiacetic acid and hydroxylamine, using the same procedure described for **7**, but with two equivalents of ethylchloroformate and hydroxylamine. The final reaction mixture was roto-evaporated and the white solid residue washed with acetonitrile and dried under vacuum. Then a small amount of cold water was added, to remove water soluble products, and the solid residue was filtered off and recrystallized from methanol to give the pure compound (41% yield); mp 170°–172°C; ^{1}H NMR (D_2O): 3.75 (4H, s, 2 × CH_2CO), 3.81 (2H, s, CH_2Ph), 7.43 (5H, m, Ph); IR (KBr, cm^{-1}) 1693 (C=O, amide); HRMS (MH^+), m/z calculated for $C_{11}H_{13}N_2O_3$: 239.102634; found: 239.103250. Analysis. Calcd. for $C_{11}H_{12}N_2O_3$: C, 59.99; H 5.49; N, 12.72. Found: C, 59.57; H, 5.83; N, 12.34%.

N-carboxymethyl-iminoacetohydroxamic Acid (9)

To a solution of **7** (100 mg, 0.42 mmol) in dry methanol (15 mL) were added 13 mg of 10% Pd/C and the mixture was stirred under H_2 (1 atm) for 4 h at room temperature. The solvent was removed under vacuum and the solid residue was recrystallized from methanol/acetonitrile to give the pure product (53 mg, 85%); mp 112°–114°C (lit. 113°–115°C.[14] ^{1}H NMR (D_2O): 3.69 (2H, s, CH_2COOH), 3.85 (2H, s, CH_2CONHOH); IR (KBr, cm^{-1}) 1662 (C=O, acid), 1623 (C=O, amide); HRMS (MH^+), m/z calculated for $C_4H_9N_2O_4$: 149.056232; found: 149.056408.

Enzyme Preparations and Assay

Human purified MMP-s (MMP-1, MMP-2, MMP-8 and MMP-9) were purchased from Calbiochem (Inalco, Milano, Italy). They were activated[15] in the assay buffer by adding bovine trypsin (50 μL, 0.6 mg/mL) to the proenzyme, followed by incubation at 37°C for 10 min. The trypsin was then inactivated with aprotinin (50 μL, 1.2 mg/mL). Initial rates for the hydrolysis of the thioester substrate AcProLeuGly-S-LeuLeuGlyOEt, coupled to the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid), were used for assessing the catalytic activity and inhibition of the four MMP-s mentioned above by the spectrophotometric method of Powers and Kam,[16] as modified by Johnson *et al.*[17] The change of absorbance ($\epsilon = 19.800\,M^{-1}\,cm^{-1}$)[16] at 405 nm was monitored continuously at room temperature, using a Cary 3 spectrophotometer interfaced with a PC. A typical 100 μL reaction contained 50 mM MES, pH 6.0, 10 mM $CaCl_2$, 100 μM substrate, 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 5 nM MMP. For the K_I determinations, DMSO solutions of the inhibitor were included in the assay, resulting in a final concentration of 2% DMSO in the reaction mixture. K_I-s were then determined by using Easson-Stedman[18] plots and a linear regression

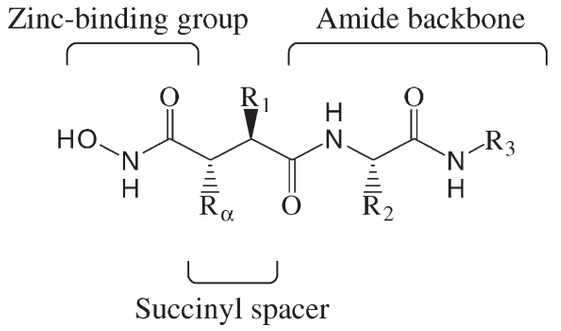
program. Under these conditions, K_I values varied from 5 to 10% in replicate experiments.

Clostridium histolyticum highly purified collagenase and its substrate, FALGPA (furanacryloyl-leucyl-glycyl-prolyl-alanine) were purchased from Sigma-Aldrich (Milano, Italy), and their concentrations were determined from the absorbance at 280 nm and the extinction coefficients furnished by the supplier. The activity of such preparations was in the range of 10 NIH units/mg solid. The potency of standard and newly obtained inhibitors was determined from the inhibition of the enzymatic (amidolytic) activity of the collagenase, at 25°C, using FALGPA as substrate, by the method of van Wart and Steinbrink.[19] The substrate was reconstituted as 5 mM stock in 50 mM Tricine buffer, 0.4 M NaCl, 10 mM $CaCl_2$, pH 7.5. The rate of hydrolysis was determined from the change in absorbance at 324 nm using an extinction coefficient for FALGPA of $\epsilon_{305} = 24.700\,M^{-1}\,cm^{-1}$ in the above-mentioned reaction buffer.[19] Measurements were made using a Cary 3 spectrophotometer interfaced with a PC. Initial velocities were thus estimated using the direct linear plot-based procedure reported by van Wart and Steinbrink.[19] K_I-s were then determined according to Easson-Stedman[16] plots and a linear regression program.

RESULTS AND DISCUSSION

Molecular Design

The first approach for the identification of new synthetic MMPs inhibitors was a substrate-based design of peptide derivatives on the basis of information from the peptide sequence of the cleavage site of the substrates.[1–4] More recently, several non-peptide inhibitors have been identified based on the X-ray structure of some adducts of MMPs with different types of inhibitors.[2–6] However, the most important structural requirements of a MMP inhibitor are: a zinc binding group (ZBG) capable of chelating the active-site zinc(II) ion, at least one functional group which provides a hydrogen bond interaction with the enzyme backbone, and one or more side chains for van der Waals interactions with the enzyme subsites.[2–6,20] A large number of succinyl hydroxamate derivatives possessing good metalloproteinase inhibition activity have been described so far, such as batimastat.[20] They generally incorporate a large variety of substituents (see Scheme 1), for the sake of improving selectivity for particular MMPs.[2–6,20] The exploitation of hydrophobic R_1 residues, to interact with the specific subsites of the protein, has ultimately been a major goal in the design of MMPs inhibitors.[2–6,20] The effect of R_2 and R_3 residues has been explored thoroughly, although it has been suggested that the latter is mostly solvent exposed and contributes less to efficient binding of the inhibitor to the enzyme.[2–6,20] Accordingly, much effort has been devoted to the synthesis of a large variety of potential MMP inhibitors. Most of them involved quite complex synthetic procedures with a large number of complicated steps.[2–6,20]

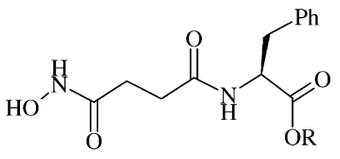


SCHEME 1 Succinyl hydroxamate-based MMP inhibitors: the ZBG assures coordination to the Zn(II) ion; R_α and R_1 may be H or bulkier alkyl/hetaryl moieties; R_2 and R_3 must assure favourable interactions with the primed site of the protease (subsites $S_{2'}$ and $S_{3'}$, respectively).[2–6]

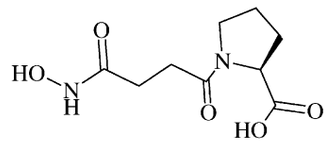
However, broad-spectrum enzyme inhibitors have been proved to possess higher efficacy in animal models of cancer.[1,2] Therefore, the strategy for the design of the set of MMP inhibitors described here (of types **1**–**9**) was to incorporate in a readily made peptide, or pseudopeptide skeleton with the basic surrogate functionality, a minimum set of groups considered to be critical for binding the zinc ion of the enzyme active site as well as some of the most important molecular residues included therein. As structural "backbones" we used succinylated amino acids, as well as iminodiacetic acid skeletons, to test the effect of the peptide bond truncation. This option was aimed at increasing the half-life of the inhibitors, due to the susceptibility of peptides to enzymatic hydrolysis *in vivo*.[2–6,20] As ZBG to be attached to these scaffolds we have included a primary hydroxamate group, as it is well-known that the *N*-proton participates in a hydrogen bonding interaction with the protein.[2–6,20] A hydrophobic residue (provided by the benzyl group or the cyclic moiety of proline) was also incorporated in order to provide van der Waals interactions with the lipophilic moieties of the enzyme active site residues, whereas a carboxylic group was thought to be necessary for interaction with positively charged residues within the active site of the enzyme. Some further variations on those groups were also included on this basic model to test the actual importance of each specific residue.

Chemistry

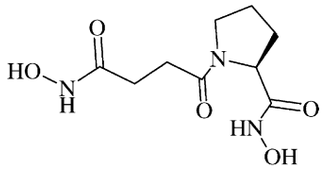
The compounds discussed below were prepared according to synthetic routes shown in Schemes 2–4. Unless indicated, all the reactions were performed in



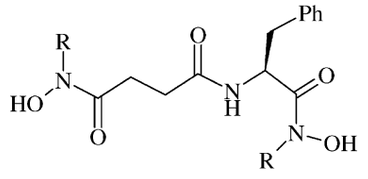
1 (R = H)
5 (R = Me)



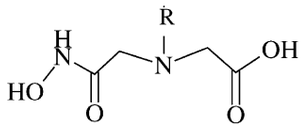
2



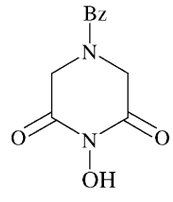
3



4 (R = H)
6 (R = Me)

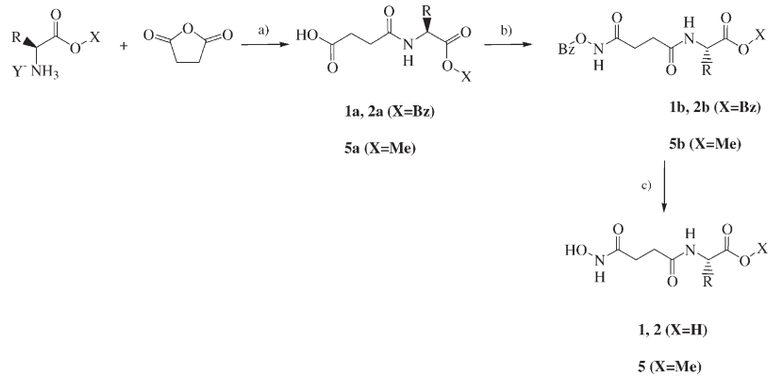


7 (R = Bz)
9 (R = H)

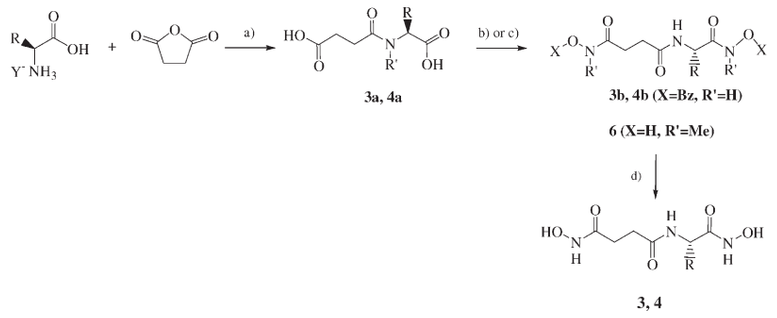


8

dry solvents and under a nitrogen atmosphere to avoid moisture. Succinic anhydride was used as the starting material for the synthesis of all the succinylhydroxamate derivatives described here. In the first reaction step, the option for the amino acid esters (Scheme 2) or just the aminoacids (*L*-phenylalanine or *L*-proline) (Scheme 3), to be coupled with the anhydride (through the free amino groups) was determined by the target compound, a monohydroxamate (**1**, **2**, **5**) or dihydroxamate (**3**, **4**, **6**) derivative, respectively. In these syntheses, the selection of the benzyl or methyl ester was based on the assumption that the tailored final compound has one free carboxylic moiety (**1**, **2**) or one ester moiety (**5**) included therein. In fact, the benzyl group is easily removed by mild hydrogenolysis conditions used in the last (third) step, while this does not apply for the methyl group. The second step consisted in the conversion of the carboxylic groups of these acids (**1a**–**5a**) into the hydroxamic acid functionality (**1b**–**5b**, **3b**, **4b**, **6**). This was performed in THF, through a one-pot reaction, where the carboxylic groups had previously been activated by reacting with ethylchloroformate (ECF), followed by reaction with the corresponding hydroxylamine derivatives (commercially available *O*-benzylhydroxylamine or *N*-methylhydroxylamine), in basic methanol and THF. The last step involved



SCHEME 2 (a) TEA/CH_3CN, 0°C; (b) (ECF, *N*-Me-Morpholine)/THF, NH_2OBz/CH_3OH, 0°C; (c) H_2 (1 atm), Pd/C 10%, CH_3OH, r.t.



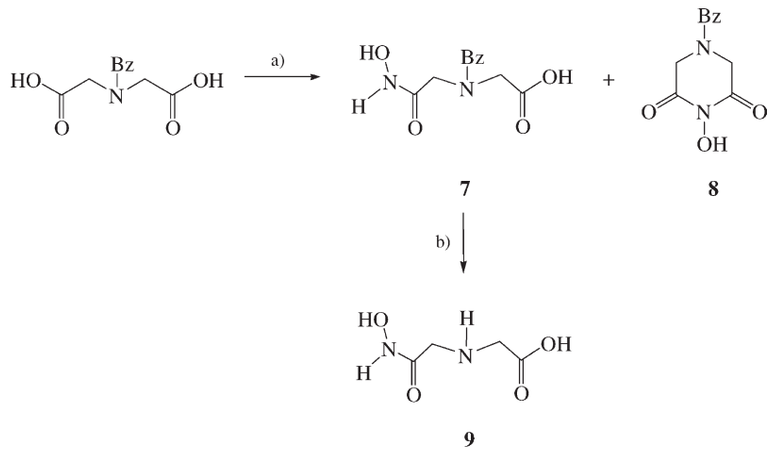
SCHEME 3 (a) TEA/CH_3CN, 0°C; (b) (ECF, *N*-Me-Morpholine)/THF, NH_2Obz/CH_3OH, 0°C; (c) (ECF, *N*-Me-Morpholine)/THF, CH_3NHOH/CH_3OH, 0°C; (d) H_2 (1 atm), Pd/C 10%, CH_3OH, r.t.

a quantitative *O*-benzyl deprotection of the hydroxamic groups (and esters) and led to the target hydroxamic acids in good overall yield.

Regarding the preparation of the *N*-benzyl-iminodiacetic derivatives (Scheme 4), the main target was the mono-hydroxamic compound **7**. Synthesis involved an one-pot reaction for the interconversion of one of the two carboxylic groups into a hydroxamic acid functionality, using the same coupling agent (ECF) and the same procedure described above. Using a two-fold excess of both the coupling agent and the hydroxylamine, the reaction interestingly led to the formation of a major cyclic *N*-hydroxy-diimide (**8**) instead of the di-hydroxamic acid. This can be rationalized in terms of a clear competition of the internal nucleophilic addition to the activated carboxylic group over the external one, due to entropic and stereochemical effects, since a 6-membered ring is formed. Standard hydrogenolysis of (**7**) led to the non-benzylated derivative (**9**) in quantitative yield.

Protease Inhibition

As seen from the data of Table I, except for **6** and **9**, which behave as very weak protease inhibitors, the other compounds synthesized in the present work act as effective inhibitors against four MMPs, i.e., MMP-1, -2, -8 and -9, as well as against BP for ChC. The inactivity of **6** may be due to its *N*-methylation at the hydroxamate moiety. This feature could be rationalized in terms of some hindrance of the methyl group on the hydroxamate binding to the Zn(II) ion of the enzyme, although it is known that the *N*-methyl-hydroxamic acids are better chelators than the corresponding primary derivatives,[21] due to the counterpart electron donation effect of this alkyl group. Such a big decrease in activity of this secondary hydroxamate, as compared with the corresponding primary derivative (**4**), can probably result from the decrease of the adduct enzyme-inhibitor binding interaction, due to the absence of the hydrogen-bond in the active site. Against MMP-1, the other compounds investigated herein showed inhibition constants in the range of 9–26 nM; against MMP-2 in the range of 8–20 nM; against MMP-8 in the range of 11–15 nM; against MMP-9 in the range of 8–24 nm, whereas against ChC in the range of 9–62 nM. No significant differences emerged between the compounds having, as the second functional group (besides the hydroxamate one), either a carboxylic acid or



SCHEME 4 (a) (ECF, *N*-Me-Morpholine)/THF, NH_2OH/CH_3OH, 0°C; (b) H_2 (1 atm), Pd/C 10%, CH_3OH, r.t.

TABLE I Inhibition of MMP-s and BP with the newly synthesized compounds of types **1**–**9**

	K_I[a] (nM)				
Compound	MMP-1[b]	MMP-2[b]	MMP-8[b]	MMP-9[b]	ChC[c]
1	21	13	15	18	46
2	26	20	12	14	13
3	20	10	13	24	9
4	20	8	12	16	32
5	23	10	14	15	41
6	>200	>200	>200	>200	>200
7	9	10	14	10	62
8	10	9	11	8	36
9	>200	>200	>200	>200	>200

[a] K_I-s values were obtained from Easson-Stedman[18] plots using a linear regression program, from at least three different assays. Standard errors were 5–10%. [b] With the thioester substrate Ac-ProLeuGly-S-LeuLeuGlyOEt, spectrophotometrically.[15] [c] With FALGPA as substrate, spectrophotometrically.[19]

a methyl carboxylic ester or even a hydroxamic acid (see for example **1**, **5** and **4**, respectively). This suggested that, at least for the enzymes tested in the present study, the interactions involving these groups do not seem to be critically important. Pro or Phe as P2′ residues seemed also to be equally efficient in inducing good MMP/BP inhibitory activity in these compounds, although the inhibition constants against ChC showed a consistently higher efficiency in the more rigid Pro derivatives than in the Phe derivatives (compare **1** and **2** as well as **4** and **3**, respectively). Remarkable was also the fact that the easy-to-make IDA derivatives (**7**, **8**)—two new structural variants never tested before for MMP/BP inhibition—proved to be potent broad spectrum MMP and ChC inhibitors. A larger amount of these compounds can be convenient prepared in order to get supporting conclusions about the mechanism involved in their inhibitory activity against these enzymes. However, these results suggest that the existence of a peptide segment in the scaffold of the inhibitor does not seem to be a determinant factor for achieving high affinity for the enzymes investigated here. On the other hand, in the IDA derivatives, the *N*-benzyl lipophilic group seems to be very important (compare **7** and **9**), as shown for some succinyl hydroxamate MMP inhibitors investigated earlier,[2–6,20] and it is expected to improve the enzyme-inhibitor binding interaction. It is also probable that removal of benzylic group from the imino nitrogen of **9** is detrimental for the binding to the zinc ion within the protease active site, and in consequence, **9** is a much weaker inhibitor as compared to **7**. The similarity between the inhibitory constants of the acyclic (**7**) and the cyclic derivative (**8**) prompted us, at a first stage, to hypothesize that this cyclic *N*-hydroxy-diimide could have been hydrolysed to give **7**. On the other hand, each of these biological assays was performed at neutral pH and each experiment lasted less then 1 h (time between the preparation of the compound solution and the end of the biological assay measurements). However, such a hypothesis seems to be out of the question under our experimental conditions (pH and time). In fact, a stability study performed at c.pH 7 for the cyclic compound **8** (M.A. Santos *et al.*, manuscript in preparation), showed that this compound is completely stable for several hours in solution. Only after three days was one fourth of compound **8** hydrolysed and transformed into **7** and after one week the hydrolysis was complete. Probably a different binding interaction can be admitted for this cyclic derivative. Some further studies are under way which might bring some light on its mechanism of MMP/BP inhibition.

In conclusion, we report here the synthesis and MMP/ChC inhibitory properties of a series of succinyl hydroxamate/IDA hydroxamate derivatives which showed promising *in vitro* protease inhibitory action.

Acknowledgements

The authors thank the Portuguese Foundation for Science and Technology (FCT) and to the COST-D21-001 project program for financial support, as well as a financing from the Italian CNRS, target project Biotechnologies.

References

[1] Smith, H.J., Simons, C., eds (2002) *Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development* (Taylor & Francis, London & New York), and references cited therein.
[2] Woessner, Jr, J.F. and Nagase, H. (2000) *Matrix Metalloproteinases and TIMPS* (Oxford University Press, Oxford), pp 1–223.
[3] Supuran, C.T. and Scozzafava, A. (2002) "Matrix metalloproteinases (MMPs)", In: Smith, H.J. and Simons, C., eds, *Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development* (Taylor & Francis, London & New York), pp 35–61.
[4] Casini, A., Scozzafava, A. and Supuran, C.T. (2002), *Exp. Opin. Ther. Patents* **12**, 1307–1327.
[5] Leung, D., Abbenante, G. and Fairlie, D.P. (2000), *J. Med. Chem.* **43**, 305–341.

[6] Whittaker, M., Floyd, C.D., Brown, P. and Gearing, A.J.H. (1999) "Design and therapeutic application of matrix metalloproteinase inhibitors", *Chem. Rev.* **99**, 2735–2776.
[7] Supuran, C.T., Scozzafava, A. and Mastrolorenzo, A. (2001), *Exp. Opin. Ther. Patents* **11**, 221–259.
[8] Supuran, C.T., Scozzafava, A. and Clare, B.W. (2002), *Med. Res. Rev.* **22**, 329–372.
[9] Giavazzi, R. and Taraboletti, G. (2001), *Crit. Rev. Oncol. Hematol.* **37**, 53–60.
[10] Hidalgo, M. and Eckhardt, S.G. (2001), *J. Natl Cancer* **93**, 178–193.
[11] Gearing, A.J.H., Adams, S.E., Clements, J.C. and Miller, K.M. (1999) "Matrix metalloproteinases in neuroinflammatory disease", In: Bottomley, K.M.K., Bradshaw, D. and Nixon, J.S., eds, *Metalloproteinases as Targets for Anti-Inflammatory Drugs* (Birkhäuser Verlag, Basel–Boston–Berlin), pp. 85–98.
[12] Hooper, N.M. (1999) "Overview of the biological roles of metalloproteinases in health and disease", In: Bottomley, K.M.K., Bradshaw, D. and Nixon, J.S., eds, *Metalloproteinases as Targets for Anti-Inflammatory Drugs* (Birkhäuser Verlag, Basel–Boston–Berlin), pp 145–162.
[13] Armarego, W.L.F. and Perring, D.D. (1999) *Purification of Laboratory Chemicals*, 4th edn. (Butterworth-Heinemann Press, Oxford).
[14] Karlicek, R. and Polasek, M. (1987), *Collect. Czech. Chem. Commun.* **53**, 592.
[15] Nagase, H. (1997), *Biol. Chem.* **378**, 151–160.
[16] Powers, J.C. and Kam, C.M. (1995), *Meth. Enzymol.* **248**, 3–18.
[17] Johnson, L.L., Bornemeier, D.A., Janowicz, J.A., Chen, J., Pavlovsky, A.G. and Ortwine, D.F. (1999), *J. Biol. Chem.* **274**, 24881–24887.
[18] Bieth, J.G. (1995), *Meth. Enzymol.* **248**, 59–84.
[19] Van Wart, H.E. and Steinbrink, D.R. (1981), *Anal. Biochem.* **113**, 156–165.
[20] Hanessian, S., Mackay, D.B. and Moitessier, N. (2001), *J. Med. Chem.* **44**, 3074–3082.
[21] Smith, R.M. and Martell, A.E. (1989) *Critical Stability Constants* (Plenum Press, New York) Vol. **6**, p 418.