Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 43 (2008) 1008–1014
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

Peptidyl 3-substituted 1-hydroxyureas as isosteric analogues of succinylhydroxamate MMP inhibitors

Cristina Campestre [a], Paolo Tortorella [b], Mariangela Agamennone [a], Serena Preziuso [a], Alessandro Biasone [a], Elisa Nuti [c], Armando Rossello [c], Carlo Gallina [a,*]

[a] *Dipartimento di Scienze del Farmaco, Università ''G. d'Annunzio'', Via dei Vestini 31, 66013 Chieti, Italy*
[b] *Dipartimento Farmaco-Chimico, Università degli Studi di Bari, Via Orabona 4, 70125 Bari, Italy*
[c] *Dipartimento di Scienze Farmaceutiche, Università degli Studi di Pisa, Via Bonanno 6, 56126 Pisa, Italy*

Received 15 April 2007; received in revised form 31 May 2007; accepted 2 July 2007
Available online 15 July 2007

Abstract

To evaluate *N*-hydroxyurea as zinc binding group in the design of MMP inhibitors, two peptidyl 1-hydroxyureas were prepared by *N*-hydroxycarbamoylation of the diastereomeric dipeptides H-Leu-Phe-NHMe and H-D-Leu-Phe-NHMe. Peptidyl 1-hydroxyureas were more potent than the parent peptides, but dramatically weaker (4–5 orders of magnitude) than the isosteric (*R*)-succinylhydroxamate analogue, which displays IC_{50} in the range of nM *vs* MMP-1, -3, -7 and sub-nM *vs* MMP-2, -8, and -9. The peptidyl 1-hydroxyurea **1a** attained an IC_{50} of 20 μM *vs* MMP-9, and substantially approaches inhibition of known *N*-hydroxyureas based on aminoacids or peptides against other zinc metalloenzymes and non-peptidic *N*-hydroxyureas against MMPs. Strong preference of the O–N1–C=O unit for the antiperiplanar amide bond conformation seems to be the major limit for more effective zinc chelation. Methylation of a peptidyl 1-hydroxyurea at N3, to promote the synperiplanar O–N1–C=O conformation required for zinc chelation and improve affinity, resulted in release of a methylimidazolidine-2,4-dione through an undesired intramolecular reaction reminiscent of the Edman peptide degradation.

Keywords: MMP inhibition; Peptidyl *N*-hydroxyureas; Succinyl hydroxamates

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-endopeptidases that can degrade components of the extracellular matrix for homeostatic regulation of the extracellular environment [1]. Overexpression and/or misregulation of these enzymes, however, may cause undesired breakdown of extracellular matrix and have been implicated in pathologies such as cardiovascular disease, cancer and arthritis [2]. Appropriate inhibition of MMPs has been therefore recognized as an important therapeutic target since 1980s [3], and some MMPs inhibitors (MPIs) are being tested in clinical trials [3c,4].

MMP active site contains an essential zinc ion which catalyzes the nucleophilic attack of a structured water molecule to the carbonyl of the scissile peptide bond [5]. MPIs are therefore designed by assembling a zinc binding group (ZBG) with a substrate like peptide or peptidomimetic chain which is most frequently accommodated in the S′ subsites of the enzyme. It has been estimated [6] that more than 90% of MPIs contain the hydroxamate function that is, by far, the most effective ZBG. Its potency is accounted for by an ideal accommodation [7] in the MMP catalytic site (Fig. 1a), where it not only forms five member chelates with optimal Zn–oxygen distances, but even establishes two strong H-bonds with the protein. Interest in non-hydroxamate MPIs, however, is recently increased owing to poor clinical efficacy of hydroxamate based MPIs [6,8].

* Corresponding author. Tel.: +39 0871 3555373; fax: +39 0871 3555267.
E-mail address: cgallina@unich.it (C. Gallina).

0223-5234/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2007.07.002

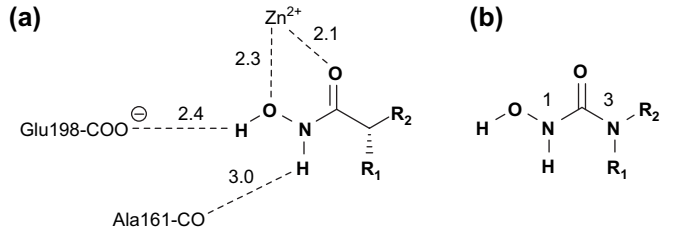
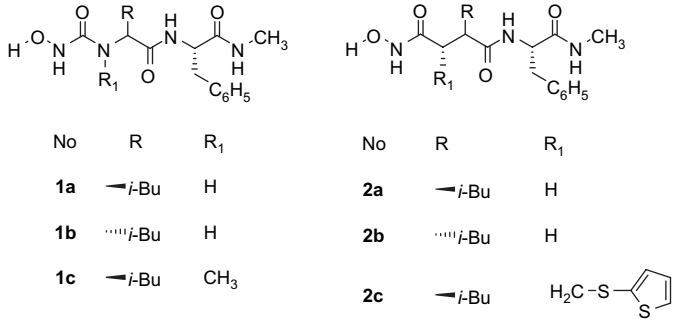


Fig. 1. Key bonding interactions of hydroxamate function (a) as found in the complex of batimastat with MMP-8 (distances in Å) [7], and 3-substituted 1-hydroxyureas (b) as potential alternatives.

The *N*-hydroxyurea function seems particularly suited as hydroxamate alternative in MPIs since it incorporates the CO–NH–OH group (Fig. 1b) that is necessary to establish the same ideal network of bonding interactions in the MMP active site, and also presents a more favorable pharmacokinetic profile. *N*-Hydroxyurea itself, used since 1960s as antineoplastic drug, is characterized by optimal oral bioavailability (80–100%) and *in vivo* half-live of 3.5–4.5 h [9]. In addition, 3-substituted 1-hydroxyureas have been reported as 5-lipoxygenase inhibitors with improved oral bioavailability and slower *in vitro* metabolism with respect to analogous hydroxamates [10]. Several 3-substituted 1-hydroxyureas have been also evaluated as cytostatic and antiviral agents [11], as inhibitors of ribonuclease [12], and of enzymes that incorporate a catalytic zinc ion, including carboxypeptidase A [13], bacterial peptide deformylase [14], carbonic anhydrase [15], histone deacetylases [16], and MMPs [17]. We have recently shown [18] that non-peptidic 3-substituted 1-hydroxyureas are weak inhibitors of MMPs, binding the catalytic zinc ion as monodentate ligands.

As an extension of our program aimed to evaluate 3-substituted 1-hydroxyureas in the design of MPIs, we planned to synthesize the peptidyl 1-hydroxyurea **1a** (Fig. 2) as isosteric analogue of the potent succinylhydroxamate MMP inhibitor **2a** [19] which is a simplified version of batimastat (**2c**) [20].

The 1-hydroxyurea **1a** contains the dipeptide H-Leu-Phe-NHMe with the Leu residue in the L configuration, so that its side chain retains the same orientation of the *iso*-butyl chain of the (*R*)-succinyl hydroxamate **2a**. Replacement of the sp^3 carbon of the succinylhydroxamate **2a** with the sp^2



No	R	R_1
1a	*i*-Bu	H
1b	*i*-Bu	H
1c	*i*-Bu	CH_3

No	R	R_1
2a	*i*-Bu	H
2b	*i*-Bu	H
2c	*i*-Bu	H_2C–S–

Fig. 2. Peptidyl 3-substituted 1-hydroxyureas (**1**) and analogous succinyl hydroxamic acids (**2**).

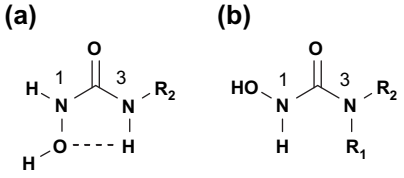


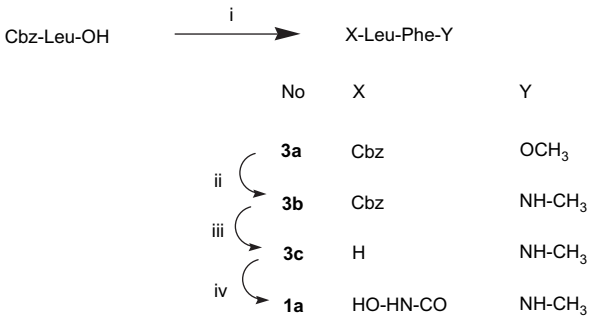
Fig. 3. (a) Antiperiplanar O–N1–C=O conformation in the solid state [21], favoured when N3 is monosubstituted; (b) expected synperiplanar O–N1–C=O conformation favoured when N3 is disubstituted; O–H hydrogen bond represented as a dotted line.

NH of the 1-hydroxyurea **1a**, however, changes substantially the geometry of the molecule in view of its accommodation in the MMPs' active site. To further evaluate and compare the effects of stereochemistry variations in the area of the ZBG, we synthesized also the diastereomeric 1-hydroxyurea **1b**, containing the Leu residue in the D configuration.

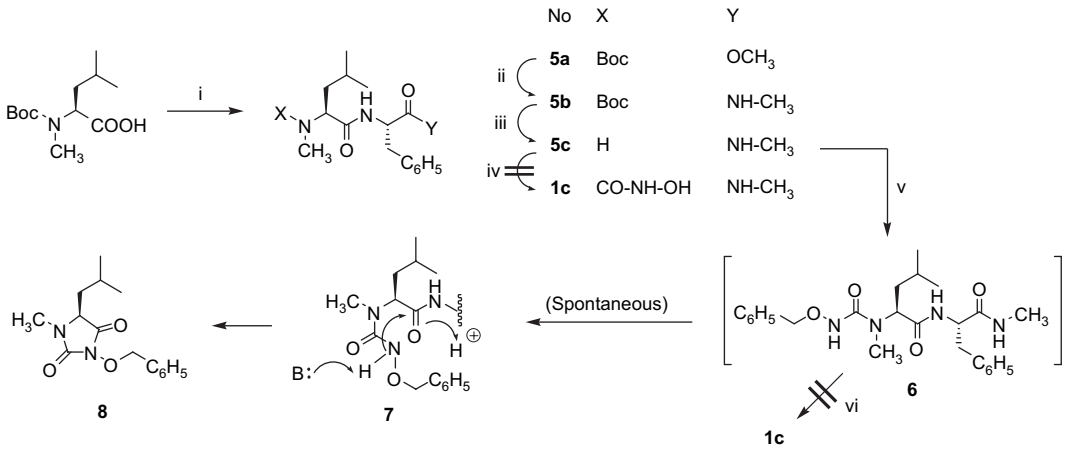
As *N*-hydroxyureas bearing a hydrogen atom on N3 (Fig. 3a) adopt, in the solid state [21], a roughly antiperiplanar O–N1–C=O conformation unproductive for zinc chelation, it was also planned to synthesize the *N*3-methyl derivative **1c**, where the synperiplanar O–N1–C=O conformation (Fig. 3b) would be favoured. In addition, *N*-methylation of the amide nitrogen is reported to favour pyramidalization of nitrogen, as observed in a *N*3-methylated 1-hydroxyurea:MMP-8 complex [18] and in *N*-methylated azapeptides [22]. Owing to the consequent increase in flexibility, the *N*3-methylated 1-hydroxyurea **3c** was expected to approach its conformational behaviour to that of analogous hydroxamates like **2a** or **2c** that are potent MMP inhibitors.

2. Chemistry

Synthesis of the required 1-hydroxyureas **1a** and **1b** was achieved through Scheme 1 that also provides dipeptides **3c** and **4c** for evaluation of their potency as MPIs. Coupling of Z-Leu-OH with H-Phe-OMe by treatment with DCC in the presence of 1-hydroxybenzotriazole [23] gave the dipeptide methyl ester **3a** that was converted into the corresponding *N*-methylamide **3b** by treatment with *N*-methylamine in MeOH. Removal of the Z group by hydrogenolysis gave the free dipeptide *N*-methylamide **3c**. Conversion into the desired



Scheme 1. Reagents: (i) H-Phe-OMe, 4-methylmorpholine DCC, HOBt, THF; (ii) CH_3NH_2, CH_3OH, 50 °C; (iii) H_2, Pd/C, CH_3OH; (iv) *p*-nitrophenyl-*N*-hydroxycarbamate, CH_3CN. Compound **1b** was prepared accordingly through intermediates **4a**, **4b**, and **4c**, starting from Cbz-D-Leu-OH.



Scheme 2. Reagents: (i) H-Phe-OMe, 4-methylmorpholine DCC, HOBt, THF; (ii) CH_3NH_2, CH_3OH, 50 °C; (iii) TFA; (iv) *p*-nitrophenyl-*N*-hydroxycarbamate, CH_3CN; (v) *O*-benzyl-hydroxylamine·HCl, Et_3N, CDI, CH_2Cl_2; (vi) H_2, Pd/C, CH_3OH.

1-hydroxyurea **1a** was achieved by treatment with *p*-nitrophenyl-*N*-hydroxycarbamate [24] in CH_3CN, while the milder acylating reagent phenyl-*N*-hydroxycarbamate [25], commonly used for *N*-hydroxyureas preparation, proved to be completely ineffective for acylation of the amino group of α-aminoacid amides. The diastereomeric dipeptide *N*-methylamide H-D-Leu-Phe-NHMe (**4c**) and the 1-hydroxyurea **1b** were obtained starting from D-Leu-OMe, by following the same synthetic sequence of Scheme 1. The two diastereomeric succinyl hydroxamates **2a** and **2b**, necessary to compare their inhibition constants with that of the 1-hydroxyureas **1a** and **1b**, were also prepared following methods previously reported [19].

Synthesis of the *N*-methyl-hydroxyurea **1c** (Scheme 2) was attempted starting from Boc-*N*-methyl-L-leucine, in a procedure analogous to Scheme 1. When the dipeptide **5c** (Scheme 2) was subjected to *N*-acylation with *p*-nitrophenyl-*N*-hydroxycarbamate we failed to obtain the expected *N*-hydroxyurea **1c**. To overcome this difficulty, we tried an alternative route progressing through an *O*-benzyl-*N*-hydroxyurea (**6**) [24], a less polar and easier to handle intermediate, in the expectations that it could be converted into the target compound **1c** by catalytic hydrogenolysis. When the dipeptide **5c** was coupled with *O*-benzylhydroxylamine in the presence of carbonyldiimidazole in CH_2Cl_2 [22] at room temperature, for 24 h, the imidazolidine-2,4-dione **8** was isolated in 64%yield. The structure is in accordance with ^1H and ^{13}C NMR spectra, and stereochemistry was assigned on the basis of the (*S*)-configuration of the *N*-methyl-leucine residue of **5c**.

The expected *O*-benzyl-*N*-hydroxyurea **6** probably underwent a facile intramolecular reaction reminiscent of the Edman peptide degradation [27]. While the original Edman method requires strong acid catalysis (HCl in nitromethane), the modified Edman procedure [28], applied to peptides containing an *N*-alkylated amino terminal residue, proceeds with release of a phenylthiohydantoin under mild basic conditions. We believe that the presence of an additional alkyl group on the amide nitrogen, both in *N*-terminal *O*-benzyl-*N*-hydroxyureas and in phenyl-thiocarbamyl derivatives of the modified Edman procedure, strongly increases flexibility of the chain and pyramidalization of nitrogen. These circumstances probably favour bended conformations (**7**) required by cyclization and increase nitrogen nucleophilicity for intramolecular attack and cleavage of the Leu–Phe peptide bond. Analogous effects probably intervene in the target *N*-hydroxyurea **1c**, when formed from **5c**, and favour the undesired Leu–Phe peptide bond cleavage.

3. Results and discussion

The (*R*)-succinyl hydroxamate **2a** was selected as a typical broad spectrum [19] peptidomimetic MMP inhibitor, in order

Table 1
In vitro inhibiting activity of 3-substituted 1-hydroxyureas (**1a**,**b**) of analogous succinyl hydroxamates (**2a**,**b**) and related dipeptides (**3c**,**4c**)

No.	MMPs IC_{50} (μM)[a]					
	1	2	3	7	8	9
1a	270	60	>300	>300	70	20
1b	>300	>300	>300	>300	150	200
2a	0.025	0.0007	0.043	0.0083	0.000046	0.00038
2b	0.64	0.17	20	4.1	0.048	0.15
3c	ND[b]	>300	ND	>300	ND	>300
4c	>300	>300	>300	>300	>300	>300

[a] Errors in the range of 5–10% of the reported value (from three different assays).
[b] Not determined.

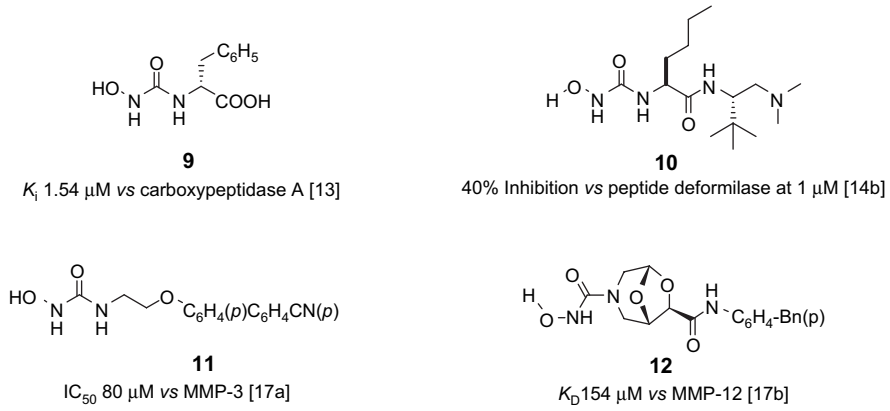


Fig. 4. Inhibition potencies of known *N*-hydroxyurea inhibitors against different zinc metalloenzymes.

to compare the target *N*-hydroxyurea **1a** with a hydroxamate analogue that displays nM potencies against several different MMPs with relevant differences in the sequence and flexibility of the active site loop [29]. As expected, the hydroxamate **2a** proved to be the most potent inhibitor (Table 1) against MMP-2, -3, -8, and -9, as previously reported [19], and against MMP-1 and -7. The diastereomeric (*S*)-succinyl hydroxamate **2b** showed binding affinities at least 100-fold lower, confirming that this model of ligand attains maximum potency when the *iso*-butylsuccinyl unit presents the (*R*)-configuration.

Results reported in Table 1 show that conversion of the substrate analogue dipeptide **3a** ($IC_{50} > 300$ μM) into the *N*-hydroxyurea **1a** causes a measurable increase of binding affinity. These values, however, are in the μM range, with a dramatic decrease (4–5 orders of magnitude) in binding affinities with respect to the reference hydroxamate **2a**. A decrease in binding affinity of 2–3 orders of magnitude is also observed with respect to the (*S*)-hydroxamate **2b**. Thus, replacement of the CH_2–CO–NH–OH chain of the hydroxamate with the NH–CO–NH–OH group causes a decrease in potencies even higher than that observed for inversion of configuration at the *iso*-butylsuccinyl unit. As previously observed for a non-peptidyl MMP inhibitor [18], the dramatic decrease in affinity of the *N*-hydroxyurea with respect to closely structural related hydroxamate can be related to the rigidity of –NH–CO–NHOH sequence and to the high energy penalty required to promote the synperiplanar O–N1–C=O amide bond conformation productive for zinc chelation. These troubles probably represent a general limit for *N*-hydroxyurea inhibitors, since the value of 20 μM attained by **1a** *vs* MMP-9, substantially approaches inhibition of *N*-hydroxyureas (Fig. 4) based on aminoacids (**9**) or peptides (**10**) against other zinc metalloenzymes and non-peptidic *N*-hydroxyureas (**11** and **12**) against MMPs.

Methylation of the N3 atom was attempted to favour pyramidalization of N3 and flexibility of the chain with improved resemblance to hydroxamate and increased affinity for MMPs. This modification of the *N*-hydroxyurea group and its *O*-benzyl derivative **6**, however, strongly increases tendency to cyclization and appears to prevent any access to *N*3-methylated peptidyl *N*-hydroxyureas.

4. Conclusions

Both peptidyl (**1a**, **1b**, and **10**) and non-peptidyl [17,18] 3-substituted 1-hydroxyurea inhibitors attain inhibition constants that are 4–5 order of magnitude higher than that of analogous hydroxamates. Potency improvements could probably be achieved by use of peptidomimetic backbones that favour zinc chelation by the NH–CO–NHOH in its planar conformation. The energy penalty for promotion of the synperiplanar O–N1–C=O amide bond conformation, however, seems to be the major limit for more effective zinc chelation. Experimental measurements for zinc complexation constants in solution could be useful to disclose *N*-hydroxyurea attitude for zinc chelation.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer 241 digital polarimeter at 20 °C; concentrations are expressed as g/100 mL. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR 300 instrument; chemical shifts are expressed in δ units, using TMS as an internal standard. Microanalyses indicated by the symbols of the elements are within ±0.4% of the theoretical values and were performed on a Carlo Erba mod. 1106 analyzer. All solvents and reagents were obtained from commercial sources and used without further purification.

5.1.1. *N*-(Benzyloxycarbonyl)-L-leucyl-L-phenylalanine methyl ester (**3a**)

To a solution of L-phenylalanine methyl ester hydrochloride (3.11 g, 14.4 mmol), 4-methylmorpholine (1.6 mL, 14.4 mmol) and *N*-(benzyloxycarbonyl)-L-leucine (3.82 g, 14.4 mmol) in anhydrous THF (35 mL), a solution of DCC (3.56 g, 17.3 mmol) and HOBt (3.28 g, 28.8 mmol) in anhydrous THF (30 mL) was added dropwise at 0 °C. The reaction mixture was stirred for 1 h at 0 °C, and for additional 20 h at room temperature. After removal of *N*,*N*′-dicyclohexylurea by

filtration, the mixture was diluted with EtOAc (150 mL) and washed with 2 N HCl (2 × 70 mL), $NaHCO_3$ (2 × 70 mL) and brine. The organic layer was dried (Na_2SO_4) and concentrated to give a residue that was triturated with hexane to provide ester **3a** as a white solid (5.96 g, 97%). Mp 80.0–81.5 °C; $[\alpha]_D^{20}$ −22.8° (*c* 1.0, CH_3OH); ^1H NMR ($CDCl_3$) δ 0.91 (d, 6H, *J* = 6.3 Hz), 1.42–1.63 (m, 3H), 3.09 (dd, 1H, *J* = 13.7 and 5.7 Hz), 3.12 (dd, 1H, *J* = 13.7 and 5.7 Hz), 3.72 (s, 3H), 4.12–4.18 (m, 1H), 4.84 (dd, 1H, *J* = 13.7 and 5.7 Hz), 5.04–5.17 (m, 2H), 6.41 (d, 1H, *J* = 7.0 Hz), 7.08 (d, 1H, *J* = 6.9 Hz), 7.19–7.29 (m, 5H), 7.31–7.35 (m, 5H).

5.1.2. N-(Benzyloxycarbonyl)-L-leucyl-L-phenylalanine methylamide (**3b**)

To a solution of methyl ester **3a** (6.12 g, 14.4 mmol) in CH_3OH (40 mL), a solution (33% in EtOH) of methylamine (17.0 mL, 143.5 mmol) was added. After 5 h at 50 °C, the volatiles were evaporated. Crystallization from EtOAc/*n*-hexane gave 5.49 g (90%) of **3b** as a white solid. Mp 180.7–181.0 °C; $[\alpha]_D^{20}$ −33.0° (*c* 1.0, CH_3OH); ^1H NMR ($CDCl_3$) δ 0.89 (d, 3H, *J* = 6.0 Hz), 0.90 (d, 3H, *J* = 6.0 Hz), 1.34–1.59 (m, 3H), 2.69 (d, 3H, *J* = 5.2 Hz), 3.00–3.10 (m, 2H), 4.00–4.18 (m, 1H), 4.57 (dd, 2H, *J* = 14.5 and 8.0 Hz), 5.00–5.11 (m, 2H), 5.30 (d, 1H, *J* = 5.2 Hz), 5.93 (s, 1H), 6.81 (d, 1H, *J* = 9.0 Hz), 7.15–7.41 (m, 10H).

5.1.3. L-Leucyl-L-phenylalanine methylamide (**3c**)

A solution of the methylamide **3b** (3.00 g, 7.0 mmol) in CH_3OH (100 mL) was stirred under hydrogen, for 5 h, at room pressure and temperature, in the presence of 5% Pd/C (300 mg). The reaction mixture was filtered and evaporated under reduced pressure to give the crude amine. Trituration with Et_2O/hexane 1:1 gave 2.02 g (98%) of **3c** as a white solid. Mp 118.4–120.3 °C; $[\alpha]_D^{20}$ +4.1° (*c* 1.0, CH_3OH); ^1H NMR ($CDCl_3$) δ 0.86 (d, 3H, *J* = 9.3 Hz), 0.88 (d, 3H, *J* = 9.3 Hz), 1.08–1.49 (m, 3H), 1.47 (br s, 2H), 2.70 (d, 3H, *J* = 4.8 Hz), 3.01 (dd, 1H, *J* = 13.5 and 7.5 Hz), 3.13 (dd, 1H, *J* = 13.8 and 6.9 Hz), 3.28–3.36 (m, 1H), 4.50–4.56 (m, 1H), 6.19 (s, 1H), 7.18–7.30 (m, 5H), 7.84 (d, 1H, *J* = 8.4 Hz). Anal. $C_{16}H_{25}N_3O_2$ (C, H, N).

5.1.4. N-[(Hydroxyamino)carbonyl]-L-leucyl-L-phenylalanine methylamide (**1a**)

To a solution of L-leucyl-L-phenylalanine methylamide **3c** (716 mg, 2.46 mmol) in CH_3CN (30 mL), *p*-nitrophenyl-*N*-hydroxycarbamate (730 mg, 3.68 mmol) was added portionwise under N_2. After 24 h under stirring, the reaction mixture was concentrated *in vacuo*. Purification by silica gel column chromatography (MeOH/EtOAc 1:9), followed by crystallization from EtOAc afforded 712 mg (83%) of the title compound **1a** as a white solid. Mp 145.2–145.5 °C; $[\alpha]_D^{20}$ −35.7° (*c* 1.0, CH_3OH); ^1H NMR (DMSO-d_6) δ 0.80 (t, 6H, *J* = 6.3 Hz), 1.21–1.46 (m, 3H), 2.52 (d, 3H, *J* = 4.8 Hz), 2.77 (dd, 1H, *J* = 13.5 and 9.0 Hz), 2.92 (dd, 1H, *J* = 14.1 and 6.0 Hz), 4.08–4.16 (m, 1H), 4.35–4.46 (m, 1H), 6.57 (d, 1H, *J* = 8.7 Hz), 7.15–7.25 (m, 5H), 7.90 (d, 1H, *J* = 4.5 Hz), 8.07 (d, 1H, *J* = 8.7 Hz), 8.50 (s, 1H), 8.71 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 20.1, 21.4, 22.4, 23.8, 36.1, 40.0, 49.8, 52.1, 124.6, 126.4, 127.5, 136.1, 159.1, 169.5, 170.6. Anal. $C_{17}H_{26}N_4O_4$ (C, H, N).

5.1.5. N-(Benzyloxycarbonyl)-D-leucyl-L-phenylalanine methyl ester (**4a**)

Prepared as described for **3a**, starting from D-leucine. Crystallization from EtOAc/*n*-hexane gave 16.0 g (98%) of **4a** as a white solid: mp 114.1–114.5 °C; $[\alpha]_D^{20}$ +50.1° (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (d, 6H, *J* = 6.3 Hz), 1.39–1.59 (m, 3H), 3.04 (dd, 1H, *J* = 13.8 and 6.9 Hz), 3.14 (dd, 1H, *J* = 15.0 and 5.4 Hz), 3.69 (s, 3H), 4.16–4.24 (m, 1H), 4.83–4.89 (m, 1H), 5.04–5.12 (m, 2H), 5.25 (d, 1H, *J* = 8.1 Hz), 6.60 (d, 1H, *J* = 7.5 Hz), 7.18–7.25 (m, 5H), 7.29–7.35 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 22.1, 23.1, 24.9, 38.1, 41.8, 52.6, 53.2, 53.7, 67.3, 127.4, 128.3, 128.4, 128.7, 128.7, 129.5, 136.0, 136.4, 156.3, 172.0, 172.1.

5.1.6. N-(Benzyloxycarbonyl)-D-leucyl-L-phenylalanine methylamide (**4b**)

Prepared as described for **3b**, starting from methyl ester **4a**. Crystallization from CH_3OH/Et_2O gave 14.3 g (92%) of **4b** as a white solid. Mp 182.9–183.9 °C; $[\alpha]_D^{20}$ −3.0° (*c* 1.0, CH_3OH); ^1H NMR ($CDCl_3$) δ 0.81 (d, 3H, *J* = 6.0 Hz), 0.83 (d, 3H, *J* = 6.0 Hz), 1.26–1.52 (m, 3H), 2.70 (d, 3H, *J* = 5.4 Hz), 3.07 (dd, 1H, *J* = 14.1 and 7.2 Hz), 3.17 (dd, 1H, *J* = 14.7 and 7.2 Hz), 3.89–3.96 (m, 1H), 4.65–4.70 (m, 1H), 5.04–5.12 (m, 2H), 5.18 (d, 1H, *J* = 6.3 Hz), 6.27 (d, 1H, *J* = 9.3 Hz), 6.44 (br s, 1H), 7.16–7.35 (m, 10H); ^{13}C NMR ($CDCl_3$) δ 22.6, 22.8, 24.7, 26.5, 37.9, 41.1, 51.6, 54.5, 67.5, 127.3, 128.2, 128.6, 128.8, 128.9, 129.4, 136.1, 136.9, 156.7, 171.1, 172.4.

5.1.7. D-Leucyl-L-phenylalanine methylamide (**4c**)

Prepared as described for **3c**, starting from methylamide **4b**. Crystallization from CH_2Cl_2/*n*-hexane afforded 1.86 g (98%) of **4c** as a white solid. Mp 130.9–131.5 °C; $[\alpha]_D^{20}$ +16.7° (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.88 (d, 3H, *J* = 9.0 Hz), 0.90 (d, 3H, *J* = 9.0 Hz), 1.20–1.63 (m, 3H), 1.95 (s, 2H), 2.71 (d, 3H, *J* = 5.1 Hz), 3.10 (dd, 1H, *J* = 13.5 and 7.8 Hz), 3.13 (dd, 1H, *J* = 13.5 and 7.2 Hz), 3.28–3.36 (m, 1H), 4.50–4.56 (m, 1H), 6.17 (s, 1H), 7.20–7.30 (m, 5H), 7.77 (d, 1H, *J* = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 21.7, 23.5, 25.0, 26.4, 38.1, 44.1, 53.3, 54.8, 127.1, 128.8, 129.5, 147.2, 171.8, 176.0. Anal. $C_{16}H_{25}N_3O_2$ (C, H, N).

5.1.8. N-[(Hydroxyamino)carbonyl]-D-leucyl-L-phenylalanine methylamide (**1b**)

Prepared as described for **1a**, starting from *N*-methylamide **4c**. Yield 75%, white solid; mp 195.0–196.0 °C; $[\alpha]_D^{20}$ −10.4° (*c* 1.0, CH_3OH). ^1H NMR (DMSO-d_6) δ 0.70 (t, 6H, *J* = 6.3 Hz), 1.04–1.19 (m, 3H), 2.61 (d, 3H, *J* = 4.8 Hz), 2.68 (dd, 1H, *J* = 13.5 and 9.0 Hz), 3.05 (dd, 1H, *J* = 14.1 and 7.5 Hz), 4.04–4.11 (m, 1H), 4.32–4.40 (m, 1H), 6.37 (d, 1H, *J* = 7.2 Hz), 7.15–7.22 (m, 5H), 7.77 (d, 1H, *J* = 4.8 Hz), 8.44 (d, 1H, *J* = 8.7 Hz), 8.53 (s, 1H), 8.70 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 22.9, 23.3, 24.5, 26.3, 37.9,

42.0, 52.0, 54.9, 126.8, 128.6, 129.8, 138.8, 161.6, 172.0, 176.0. Anal. $C_{17}H_{26}N_4O_4$ (C, H, N).

5.1.9. N-(tert-Butoxycarbonyl)-N-methyl-L-leucyl-L-phenylalanine methyl ester (**5a**)

Prepared as described for **3a**, starting from *N*-*tert*-butoxycarbonyl-*N*-methyl-L-leucine and L-phenylalanine methyl ester. Purification by silica gel chromatography ($CHCl_3$/hexane 2:8) gave 2.27 g (68%) of **5a** as colourless liquid. ^{1}H NMR ($CDCl_3$) δ 0.86 (d, 3H, $J = 6.5$ Hz), 0.90 (d, 3H, $J = 6.5$ Hz), 1.42 (s, 9H), 1.50–1.80 (m, 3H), 2.51 (s, 3H), 3.14 (d, 1H, $J = 5.1$ Hz), 3.18 (d, 1H, $J = 6.0$ Hz), 3.72 (s, 3H), 4.56–4.65 (m, 1H), 4.81–4.85 (m, 1H), 6.38 (d, 1H, $J = 7.0$ Hz), 7.06–7.29 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 21.7, 23.4, 24.7, 28.5, 29.6, 36.4, 38.2, 53.4, 56.2, 57.3, 81.0, 127.3, 128.8, 129.3, 136.2, 156.7, 171.2, 172.0.

5.1.10. N-(tert-Butoxycarbonyl)-N-methyl-L-leucyl-L-phenylalanine methylamide (**5b**)

Prepared as described for **3b**, starting from methyl ester **5a**. Purification by silica gel column chromatography (2% *iso*-propanol in $CHCl_3$) provided 1.75 g (88%) of **5b** as a white solid. Mp 98.8–100.5 °C; $[\alpha]_D^{20}$ −57.4° (*c* 1.0, CH_3OH); ^{1}H NMR ($CDCl_3$) δ 0.88 (d, 3H, $J = 8.1$ Hz), 0.91 (d, 3H, $J = 8.1$ Hz), 1.46 (s, 9H), 1.53–1.63 (m, 3H), 2.47 (s, 3H), 2.73 (d, 3H, $J = 4.8$ Hz), 3.04 (d, 2H, $J = 6.9$ Hz), 4.21–4.58 (m, 2H), 5.93 (s, 1H), 6.47 (br s, 1H), 7.15–7.31 (m, 5H); ^{13}C NMR (CD_3OD) δ 20.7, 22.4, 24.7, 25.1, 27.5, 29.2, 36.7, 37.9, 54.5, 56.4, 80.0, 126.6, 128.4, 129.0, 137.1, 155.6, 172.2, 172.7.

5.1.11. N-Methyl-L-leucyl-L-phenylalanine methylamide (**5c**)

Compound **5b** (1.54 g, 3.8 mmol) was dissolved in TFA (20 mL). After 4 h at room temperature, the reaction mixture was concentrated, diluted with EtOAc (200 mL), and washed with $NaHCO_3$ (3 × 100 mL) and brine. Evaporation of the solvents, followed by crystallization from EtOAc/*n*-hexane gave 894 mg (77%) of **5c** as a white solid. Mp 134.3–135.3 °C; $[\alpha]_D^{20}$ −1.99° (*c* 1.0, CH_3OH); ^{1}H NMR ($CDCl_3$) δ 0.84 (d, 3H, $J = 6.6$ Hz), 0.86 (d, 3H, $J = 6.6$ Hz), 1.09–1.55 (m, 3H), 2.27 (s, 3H), 2.73 (d, 3H, $J = 5.2$ Hz), 2.90–3.18 (m, 3H), 4.53–4.61 (m, 1H), 6.10 (br s, 1H), 7.19–7.30 (m, 5H), 7.66 (d, 1H, $J = 7.5$ Hz); ^{13}C NMR ($CDCl_3$) δ 22.2, 23.3, 25.2, 26.4, 35.6, 38.0, 42.8, 54.3, 63.6, 127.0, 128.8, 129.4, 137.3, 171.9, 175.6.

5.1.12. (5S)-3-(Benzyloxy)-5-isobutyl-1-methylimidazolidine-2,4-dione (**8**)

To a solution of *O*-benzylhydroxylamine hydrochloride (159 mg, 1.0 mmol) and Et_3N (112 μL, 0.8 mmol) in anhydrous CH_2Cl_2 (7 mL), 1,1′-carbonyldiimidazole (133 mg, 0.8 mmol) was added [26]. After 1 h under stirring, compound **5c** (205 mg, 0.7 mmol) was added, and the resulting mixture was stirred for 24 h. The reaction mixture was then diluted with CH_2Cl_2 (10 mL) and washed with 1 N HCl (2 × 10 mL), $NaHCO_3$ (2 × 10 mL) and brine. The organic layer was concentrated to dryness and purified by column chromatography (EtOAc/*n*-hexane 2:8), to provide **8** (82 mg, 64%) as a yellow oil. $[\alpha]_D^{20}$ −15.4° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.89 (d, 3H, $J = 6.6$ Hz), 0.91 (d, 3H, $J = 6.6$ Hz), 1.55–1.80 (m, 3H), 2.92 (d, 3H, $J = 1.3$ Hz), 3.74–3.78 (m, 1H), 5.14 (s, 2H), 7.26–7.51 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 22.9, 23.2, 24.3, 28.6, 38.1, 58.4, 79.4, 128.7, 129.5, 130.2, 133.8, 153.3, 167.7. Anal. $C_{15}H_{20}N_2O_3$ (C, H, N).

5.2. MMP inhibition assays [30]

Recombinant human progelatinase A (pro-MMP-2) and B (pro-MMP-9) from transfected mouse myeloma cells, were supplied by Prof. Gillian Murphy (Department of Oncology, University of Cambridge, UK); pro-MMP-1, pro-MMP-3, pro-MMP-7 and pro-MMP-8 were purchased from Calbiochem. Proenzymes were activated immediately prior to use with *p*-aminophenylmercuric acetate (APMA 2 mM for 1 h at 37 °C for MMP-2, MMP-1 and MMP-8, 1 mM for 1 h at 37 °C for MMP-9 and MMP-7). Pro-MMP-3 was activated with trypsin 5 μg/mL for 15 min at 37 °C followed by soybean trypsin inhibitor (SBTI) 62 μg/mL.

For assay measurements, the inhibitor stock solutions (DMSO, 100 mM) were further diluted, at seven different concentrations (0.01 nM–300 μM) for each MMP in the fluorimetric assay buffer (FAB: Tris 50 mM, pH = 7.5, NaCl 150 mM, $CaCl_2$ 10 mM, Brij 35 0.05% and DMSO 1%). Activated enzyme (final concentration 2.9 nM for MMP-2, 2.7 nM for MMP-9, 2.4 nM for MMP-7, 1 nM for MMP-3, 1.5 nM for MMP-8 and 0.20 nM for MMP-1) and inhibitor solutions were incubated in the assay buffer for 4 h at 25 °C. After the addition of 200 μM solution of the fluorogenic substrate Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 (Sigma) for MMP-3 and Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Bachem) for all the other enzymes in DMSO (final concentration 2 μM), the hydrolysis was monitored every 15 s for 20 min recording the increase in fluorescence ($\lambda_{ex} = 325$ nm, $\lambda_{em} = 400$ nm) using a Molecular Device SpectraMax Gemini XS plate reader. The assays were performed in triplicate in a total volume of 200 μL per well in 96-well microtitre plates (Corning, black, NBS). Control wells lack inhibitor. The MMP inhibition activity was expressed in relative fluorescent units (RFU). Percent of inhibition was calculated from control reactions without the inhibitor. IC_{50} was determined using the formula: $V_i/V_o = 1/(1 + [I]/IC_{50})$, where V_i is the initial velocity of substrate cleavage in the presence of the inhibitor at concentration [I] and V_o is the initial velocity in the absence of the inhibitor. Results were analyzed using SoftMax Pro software and GraFit software [31].

Acknowledgments

Financial support by Italian MIUR is gratefully acknowledged.

References

[1] J.F. Woessner, H. Nagase (Eds.), Matrix Metalloproteinases and TIMPS, Oxford University Press, New York, NY, USA; 2000.

[2] (a) M.L. Lindsey, Heart Fail. Rev. 9 (2004) 7–19;
(b) L.M. Coussens, B. Fingleton, M.L. Matrisian, Science 295 (2002) 2387–2392;
(c) C. Overall, O. Kleifeld, Br. J. Cancer 94 (2006) 941–946.
[3] (a) M. Whittaker, C.D. Floyd, P. Brown, A.J.H. Gearing, Chem. Rev. 99 (1999) 2735–2376;
(b) J.W. Skiles, N.C. Gonnella, A.Y. Jeng, Curr. Med. Chem. 11 (2004) 2911–2977;
(c) H. Matter, M. Schudok, Curr. Opin. Drug Discov. Devel. 7 (2004) 513–535;
(d) B.G. Rao, Curr. Pharm. Des. 11 (2005) 295–322.
[4] G. Abbenante, D.P. Fairlie, J. Med. Chem. 1 (2005) 71–104.
[5] B. Lovejoy, A.M. Hassel, M.A. Luther, D. Weigl, S.R. Jordan, Biochemistry 33 (1994) 8207–8217.
[6] D.T. Puerta, M.O. Griffin, J.A. Lewis, D. Romero-Perez, R. Garcia, F.J. Villareal, S.M. Cohen, J. Biol. Inorg. Chem. 11 (2006) 131–138.
[7] F. Grams, M. Crimmin, L. Hinnes, P. Huxley, M. Pieper, H. Tschesche, W. Bode, Biochemistry 34 (1995) 14012–14020.
[8] E. Breuer, J. Frant, R. Reuven, Expert Opin. Ther. Pat. 15 (2005) 253–269.
[9] R.C. Donehower, in: B. Chabner (Ed.), Pharmacological Principles of Cancer Treatment, W.B. Saunders Co., Philadelphia, 1982, pp. 269–275.
[10] A.O. Stewart, P.A. Bhatia, J.G. Martin, J.B. Summers, K.E. Rodriques, M.B. Martin, J.H. Holms, J.L. Moore, R.A. Craig, T. Kolasa, J.D. Ratajczyk, H. Mazdiyasni, F.A.J. Kerdesky, S.L. DeNinno, R.G. Maki, J.B. Bouska, P.R. Young, C. Lanni, R.L. Bell, G.W. Carter, C.D.W. Brooks, J. Med. Chem. 40 (1997) 1955–1968.
[11] N. Opacic, M. Barbaric, B. Zorc, M. Cetina, A. Nagl, D. Frkovic, M. Kralj, K. Pavelic, J. Balzarini, G. Andrei, R. Snoeck, E. De Clercq, S. Raic-Malic, M. Mintas, J. Med. Chem. 48 (2005) 475–482.
[12] J.J. Higgin, G.I. Yakovlev, V.A. Mitchevich, A.A. Makarov, R.T. Raines, Bioorg. Med. Chem. Lett. 13 (2003) 409–412.
[13] S.J. Chung, D.H. Kim, Bioorg. Med. Chem. 9 (2001) 185–189.
[14] (a) A. Thorarensen, M.R. Douglas Jr., D.C. Rohrer, A.F. Vosters, A.W. Yem, V.D. Marshall, J.C. Lynn, M.J. Bohanon, P.K. Tomich, G.E. Zurenko, M.T. Sweeney, R.M. Jensen, J.W. Nielsen, E.P. Seest, L.A. Dolak, Bioorg. Med. Chem. Lett. 11 (2001) 1355–1358;
(b) H.K. Smith, R.P. Beckett, J.M. Clements, S. Doel, S.P. East, S.B. Launchbury, L.M. Pratt, Z.M. Spavold, W. Thomas, R.S. Todd, M. Whittaker, Bioorg. Med. Chem. Lett. 12 (2002) 3595–3599.
[15] C. Temperini, A. Innocenti, A.T. Scozzafava, C. Supuran, Bioorg. Med. Chem. Lett. 16 (2006) 4316–4320.
[16] S. Hanessian, V. Vinci, L. Auzzas, M. Marzi, G. Giannini, Bioorg. Med. Chem. Lett. 16 (2006) 4784–4787.
[17] (a) M.R. Michaelides, J.F. Dellaria, J. Gong, J.H. Holms, J.J. Bouska, J. Stacey, C.K. Wada, H.R. Heyman, M.L. Curtin, Y. Guo, C.L. Goodfellow, I.B. Elmore, D.H. Albert, T.J. Magoc, P.A. Marcotte, D.W. Morgan, S.K. Davidsen, Bioorg. Med. Chem. Lett. 11 (2001) 1553–1556;
(b) C. Mannino, M. Nievo, F. Machetti, A. Papakyriakou, V. Calderone, M. Fragaid, A. Guarna, Bioorg. Med. Chem. 14 (2006) 7392–7403.
[18] C. Campestre, M. Agamennone, P. Tortorella, S. Preziuso, A. Biasone, E. Gavuzzo, G. Pochetti, F. Mazza, O. Hiller, H. Tschesche, V. Consalvi, C. Gallina, Bioorg. Med. Chem. Lett. 16 (2006) 20–24.
[19] (a) J.P. Dickens, D.K. Donald, G. Kneen, W.R. McKey, Eur. Pat. Appl. EP0214639, 1987;
(b) D.A. Levy, F. Lapierre, W. Liang, W. Ye, C.W. Lange, X. Li, D. Grobelny, M. Casabonne, D. Tyrrell, K. Holme, A. Nadzan, R.E. Galardy, J. Med. Chem. 41 (1998) 199–233.
[20] C. Campion, A.H. Davidson, A.E.H. Gearing, M.J. Crimmin, PCT Int. Appl. WO9005719, British Bio-Technology Ltd, U.K., 1990.
[21] B.B. Nielsen, K. Frydenvang, I.K. Larsen, Acta Crystallogr. C49 (1993) 1018–1022.
[22] H.J. Lee, J.W. Song, Y.S. Choi, H.M. Park, K.B. Lee, J. Am. Chem. Soc. 124 (2002) 11881–11893 and literature therein.
[23] B.M. Schiavi, D.J. Richard, M.M. Joullie, J. Org. Chem. 67 (2002) 620–624.
[24] P. Beier, J. Mindl, V. Sterba, J. Hanusek, Org. Biomol. Chem. 2 (2004) 562–569.
[25] A. Defoin, A. Brouillard-Poichet, J. Streith, Helv. Chim. Acta 74 (1991) 103–109.
[26] H.A. Staab, W. Benz, Angew. Chem. 73 (1961) 657.
[27] J.P. Greenstein, M. Winitz, Chemistry of the Aminoacids, vol. 2, Wiley and Sons Inc., New York, 1961, pp 1566–1568.
[28] P. Rydberg, B. Luning, C.A. Wachtmeister, L. Eriksson, M. Tornqvist, Chem. Res. Toxicol. 15 (2002) 570–581.
[29] (a) G.E. Terp, G. Cruciani, I.T. Christensen, F.S. Jørgensen, J. Med. Chem. 45 (2002) 2675–2684;
(b) K. Maskos, Biochimie 87 (2005) 249–263;
(c) P. Cuniasse, L. Devel, A. Makaritis, F. Beau, D. Georgiadis, M. Matziari, A. Yiotakis, V. Dive, Biochimie 87 (2005) 393–402.
[30] (a) G. Murphy, T. Crabbe, Methods Enzymol. 248 (1995) 470–484;
(b) T. Tuccinardi, A. Martinelli, E. Nuti, P. Carelli, F. Balzano, G. Uccello-Barretta, G. Murphy, A. Rossello, Bioorg. Med. Chem. 14 (2006) 4260–4276.
[31] (a) SoftMax Pro 4.7.1 by Molecular Device;
(b) GraFit version 4 by Erithecus Software.