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Design, modelling, synthesis and biological evaluation of peptidomimetic phosphinates as inhibitors of matrix metalloproteinases MMP-2 and MMP-8

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Abstract—Three novel peptidomimetic phosphinate inhibitors have been synthesized and evaluated as inhibitors of matrix metalloproteinases MMP-2 and MMP-8. Their IC_{50} values are in the micromolar range, and one of them showed to be the most effective inhibitor of MMP-2. The differences in binding affinities for MMP-2 and MMP-8 of the three phosphinates have been rationalized by means of modelling studies and molecular dynamics simulations.

1. Introduction

Matrix metalloproteinases (MMPs) form a growing family of zinc endopeptidases that play important roles in physiological processes such as ovulation, embryogenic growth, angiogenesis, and differentiation.[1–4] Imbalances in the expression of MMPs and their endogenous specific inhibitors (TIMPs)[5] are implicated in a number of pathological states such as tumour growth and metastasis,[6,7] multiple sclerosis,[8] rheumatoid arthritis,[9] Alzheimer's disease [10a] and heart failure.[10b] Although TIMPs have been successfully tested in animal experiments,[11] they could not be developed as therapeutic agents, owing to inadequate pharmacological stability.[12] Therefore, design and synthesis of potent, small molecule MMP inhibitors (MMPIs) endowed with adequate pharmacological and selectivity profile are actively investigated.[13]

General requirements for MMPIs are a peptidomimetic backbone mimicking the substrate moiety recognized in the enzyme active site and a zinc-binding function (ZBF) capable of coordinating the catalytic zinc ion. The hydroxamate group is considered the most powerful ZBF for MMPIs,[14] since its simple replacement by other zinc-binding groups causes a 100- to 2000-fold decrease in potency.[15] Hydroxamate inhibitors, however, are generally affected by lack of specificity,[16] owing to the overwhelming chelating power of the hydroxamate, which often prevails over the contribution of the peptidomimetic moiety. They show, in addition, poor pharmacokinetic properties[17] and can entail toxicity in long-term therapy, due to the release of hydroxylamine, a known carcinogenic agent.[18] Novel MMPIs containing different ZBF such as carboxylate, thiolate, phosphonate and phosphinate are therefore currently investigated.

2. Inhibitor design

We have previously studied some tripeptide phosphonate MMPIs structurally related to endogenous inhibitors isolated from snake venom.[19] Their mode of binding in the active site of zinc endopeptidases has been determined by X-ray structure resolution of their complexes. While the phosphonate Pro-Leu-L-Trp(P)-

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$(OH)_2$ binds in the S region of the MMP-8 active site, the analogue containing a pyroglutamate in place of the Pro residue is accommodated in the S′ region of the active site of adamalysin,[19c] a snake venom endopeptidase structurally related to human MMPs.

Superposition of these two crystal structures shows that the $[PO_2^-]$ anion, ligating the catalytic zinc ion, maintains exactly the same position relative to the metal, in both the complexes accommodating the peptidomimetic chains in opposite directions. This finding prompted us to start the design of novel MMP-8 phosphinic inhibitors from the coordinates of the $[PO_2^-]$ chelating group. Our efforts pointed to select peptidomimetic chains that, extending from the phosphinate group, could establish useful binding interactions in both the S and S′ region of the enzyme active site. While occupation of the S′ region usually guarantees adequate potency of the inhibitor, binding interactions in the S region could be chiefly exploited to improve selectivity.[20] Within the class of phosphinic acid-based pseudopeptide inhibitors, two main typologies of frameworks have been so far synthetized (**A** and **B**, Fig. 1), following receptor-based drug design strategies.[20c,21]

Our first approach towards phosphinate-based inhibitors pointed to the synthesis and evaluation of the three analogues **1a**–**1c** (Fig. 2). They are based on the Pro-Leu-NH-CH_2-PO_2^- sequence, in accordance with previous structural studies on both phosphonate[19c] and hydroxamate[22] inhibitors, complexed in the S region of the MMP-8 active site. The second chain bound to the central phosphorus atom was limited to a highly lipophilic biphenyl group, useful for increasing the affinity of the ligands by insertion into the deep S_1' hydrophobic pocket of the enzyme. The biphenyl group was connected to phosphorus by means of a methylene group in the phosphinates **1a** and **1b**. An ethylene bridge has been inserted in **1c**, to evaluate the effect of chain elongation, reorientation of the rigid biphenyl group, in view of higher flexibility, and deeper insertion into the S_1' pocket.

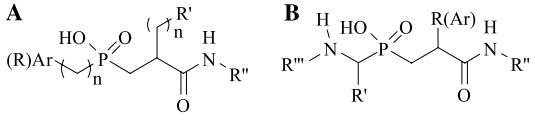
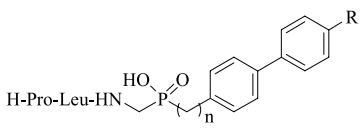


Figure 1. Selected frameworks of phosphinic acid-based pseudopeptide MMPIs. (A) Reiter, L. A., et al.[20c]; Hagmann, W. K., et al.[21c]. (B) Goulet, J. L., et al.[21d]; Yiotakis, A., et al.[21a]; Schiodt, C. B., et al.[21b].



No	n	R
1a	1	H
1b	1	OH
1c	2	H

Figure 2. Peptidyl biphenylalkylphosphinates designed on the basis of the mode of binding of phosphonate inhibitors complexed in both S and S′ subsites of MMP-8.

3. Chemistry

Despite the interesting biological properties of peptidyl phosphinates,[23] difficulties often encountered in the phosphorous alkylation steps, during their synthesis, have greatly limited the study of this kind of inhibitors.

Synthesis of the peptidyl phosphinates **1a**–**1c** required preparation of the biphenylalkyl iodides **4a**–**4c** (Scheme 1), powerful alkylating reagents necessary for an efficient phosphorous alkylation. The sequential double alkylation of the phosphorus atom, to give the protected intermediates **8a**–**8c** containing the unsymmetrically disubstituted phosphinate core, followed by coupling with the Pro-Leu dipeptide chain and protecting groups removal, is summarized in Scheme 2.

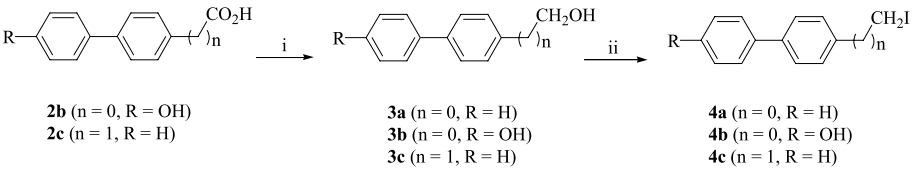
Alkylation of the phosphorus atom of ammonium ipophosphite has been performed by a modification of the Regan–Boyd[24] procedure. In the first alkylation step, pyrophoric bistrimethylsilylphosphonite (BTSP)[25] (**5**) was generated in situ by treatment of ammonium ipophosphite with hexamethyldisilazane (HMDS), and reacted with the appropriate biphenylalkyl iodides **4a**–**4c**, to give the expected monoalkylphosphinic acids **6a–6c** in 58–70% yields. These, in turn, were converted into the bistrimethylsilyl phosphinates **7a–7c** by treatment with HMDS and reacted overnight in CH_2Cl_2, under reflux, with commercially available phthalimidomethyl bromide, to perform the second alkylation step at the phosphorus atom. After conventional workup, the expected dialkylphosphinic acids, without further purification, were converted by treatment with diazomethane into the corresponding methyl esters **8a**–**8c** that can be easily purified and completely characterized.

Removal of the phthalimido protecting group was accomplished by treatment of an ethanol solution of methyl esters **8a**–**8c** with hydrazine, at room temperature, for 18 h. Finally, the α-aminophosphinates **9a–9c** were coupled with Z-Pro-Leu-OH by reaction with dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBT)[26] to afford the expected peptidomimetics **10a–10c**. Conversion into the final phosphinates **1a**–**1c** was carried out by treatment with bistrimethylsilylacetamide (BTSA) and trimethylsilyl iodide (TMSI), which caused contemporary removal of the carbobenzyloxy group from the terminal proline residue and of the methyl group from the phosphinate ester. Phosphinates **1a**–**1c** were isolated, purified and fully characterized by ^1H, ^{13}C and ^{31}P NMR, ESI-MS, MALDI-TOF-MS and elemental analyses.

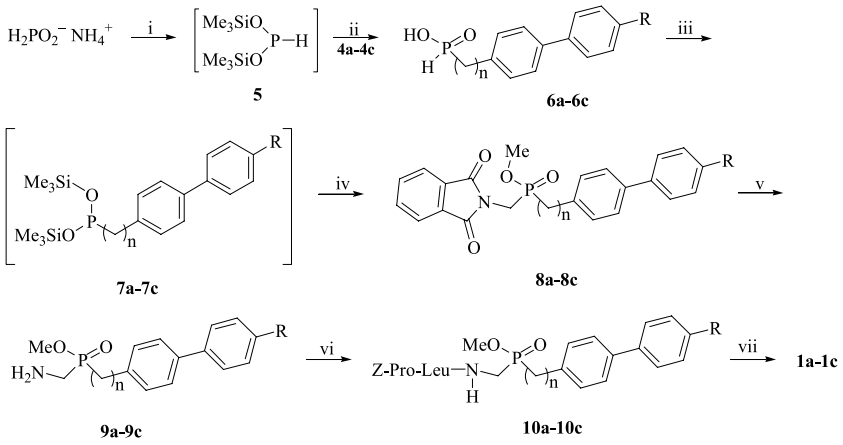
4. Results and discussion

4.1. In vitro enzyme inhibition assays

The inhibiting activities of the new compounds were evaluated against MMP-2 and MMP-8, measuring the



Scheme 1. Synthesis of the biphenylalkyl iodides **4a**–**4c**. Reagents and conditions: (i) $LiAlH_4$, ΔT, THF, 12 h, 87% yield for **3b**; 5 h, 99% yield for **3c**; (ii) HI, $CHCl_3$, rt, 4 h, 82% yield for **4a**; HI, rt, acetone, 12 h, 99% yield for **4b**; I_2, PPh_3, imidazole, CH_2Cl_2, rt, 3 h, 93% yield for **4c**.



Scheme 2. Synthesis of peptidyl phosphinates **1a**–**1c**. Reagents and conditions: (i) $(Me_3Si)_2NH$, 110 °C, 1–2 h; (ii) CH_2Cl_2 reflux, 12 h, 58–70% yield; (iii) $(Me_3Si)_2NH$, CH_2Cl_2, 0 °C, 1 h; (iv) phthalimidomethyl bromide, CH_2Cl_2, reflux, 12 h, and phosphinate methylation after quenching: CH_2N_2, 0 °C, toluene/MeOH, 49–57% yield; (v) H_2N–NH_2, EtOH, rt, 18 h, 70–78% yield; (vi) Z-Pro-Leu-OH, DCC, HOBT, THF, 0 °C to rt, 15 h, 67–80% yield; (vii) BTSA, CH_2Cl_2, rt, 1 h; TMSI, CH_2Cl_2, −20 °C to rt, 3 h, 72–83% yield.

residual enzyme activity by means of continuous fluorimetric assays in the presence of the fluorescent substrate QF 24.[27] The resulting IC_{50} values (Table 1) are in the micromolar range, and represent a validation of our design of phosphinate inhibitors extending in both the S and S′ region of the MMP active site.

Both phosphinates **1a** and **1b**, characterized by a methylene bridge between the phosphorus atom and the biphenyl group, are more potent than **1c**, which contains an ethylene bridge, against MMP-2 and MMP-8. Furthermore, while **1a** and **1b** present similar affinity towards MMP-2, **1a** is about three times more potent than **1b** against MMP-8. An attempt to rationalize the above results by means of computational simulations is described in the following paragraphs.

4.2. Molecular modelling study

The comparison of the four putative complexes formed by the inhibitors **1a** and **1b** in the active site of MMP-2 and MMP-8 can offer an interpretation at molecular level of their behaviour. The two ligands **1a** and **1b**, differing only in the terminal hydroxyl group, show similar binding with the two enzymes. However, the following different interactions emerge at the S_3 and S_1' subsites of the putative complexes formed with the two enzymes. The aromatic side chains of Tyr 155, His 166 and Phe 168 surrounding the S_3 subsite of MMP-2 form a hydrophobic cavity where favourable contacts with the Pro ring of each inhibitor can take place. At the S_3 subsite of MMP-8, the shorter and hydrophilic side chain of Ser 151, replacing the Tyr 155 present in MMP-2, prevents similar interactions probably forming H-bonds with water molecules. Moreover, the S_1' pocket of MMP-2 has the form of a tunnel open to the solvent, whereas in MMP-8 it is bottom panelled by the side chains of Arg 222 and Tyr 227. As a consequence, the biphenyl groups of both ligands can be easily inserted in the S_1' pocket of MMP-2, whereas the terminal hydroxyl of **1b** can give rise to steric hindrance with the side chains of the residues paving the S_1' pocket of MMP-8. The better interactions of both ligands at S_3 and S_1' subsites of MMP-2 can explain their better activities shown on this enzyme. The steric hindrance occurring between the terminal hydroxyl of **1b** with the bottom of the S_1' pocket in MMP-8 can give reason for the lower activity of **1b** compared to **1a**. The similar activities shown by the two ligands on MMP-2 can be interpreted by lack of ligand–protein H-bond through the additional hydroxyl group, probably solvated in the open tunnel of MMP-2.

Table 1. In vitro inhibition of MMP-2 and MMP-8 by phosphinates **1a**–**1c**

Inhibitor	IC_{50} (μM)	
	MMP-2	MMP-8
1a	1.7	5.1
1b	1.4	17.6
1c	48	44.5

4.3. Molecular dynamics simulation

The additional methylene group of **1c** was unexpectedly found to cause a decrease of inhibition (see Table 1), relative to other two ligands, of more than one order of magnitude on both enzymes.

To better ascertain the physicochemical reasons for this behaviour, we carried out molecular dynamics (MD) simulations. Our strategy was to compare the free energy values of the conformations adopted by **1c** in aqueous solution and in the putative binding with the MMP active site. To overcome the multidimensional problem due to the large number of internal degrees of freedom, we decided to analyze the simulated trajectories by essential dynamics.[28] This procedure greatly facilitates the description of conformational behaviour of the ligand, since it allows us to define a new set of coordinates where large amplitude motions (essential motions) responsible for the conformational changes can be extracted. Two essential coordinates were found to be sufficient for describing conformational fluctuations of ligand **1c**. This result allowed us to define a plane (essential plane) where Helmholtz free energy minima can be easily located as a function of only two conformational (essential) coordinates.[29] Five energy minima, denoted as **I–V**, populate the free energy map of **1c** reported in Figure 3, where contour lines are drawn every 2 kJ/mol. The binding conformation, indicated by a cross, is practically never sampled during the simulation, being characterized by a high value of free energy. The analysis of the conformations sampled in the free energy minima reveals that all of them present at least one intramolecular H-bond.

Moreover, the torsion angles involving the ethylenic bridge undergo an extent of fluctuations around the equilibrium positions, much larger than those around the other rotational degrees of the ligand (Table 2). Consequently, even though a conclusive assessment can be drawn only in the presence of the complete picture (i.e., the dynamical behaviour of **1c** within the enzyme) our results suggest that the inclusion of a further methylene group renders the confinement of **1c** into the active lock-and-key conformation, a high energy process.

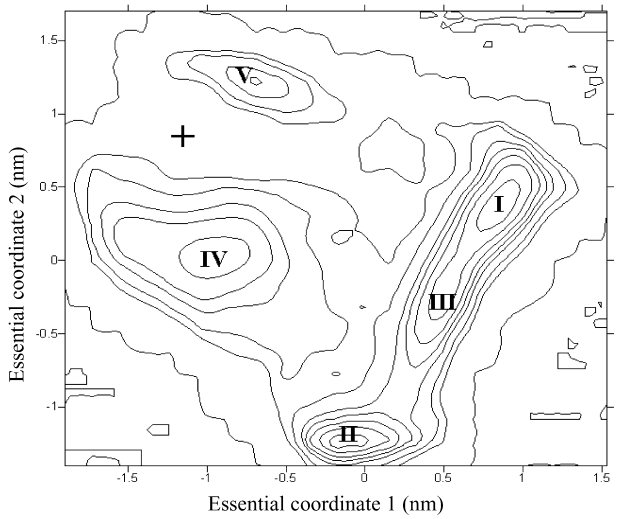


Figure 3. Three hundred kelvin free energy plot for **1c** on the plane of the first two essential motions.

5. Conclusion

Three novel peptidyl phosphinates **1a**–**1c** have been designed and synthesized as inhibitors of MMPs on the basis of the structures of phosphonate inhibitors complexed with MMPs. These ligands, extending in both the S and S′ subsites of MMPs, are based on the common phosphinic tripeptide moiety Pro-Leu-Gly(P)-(OH) linked to a biphenyl group through a methylene or ethylene bridge. Inhibition of MMP-2 and MMP-8, in the micromolar range, was confirmed by kinetic measurements and validates our structure-based design of this model of phosphinate inhibitors. Molecular modelling and molecular dynamics studies offer an interpretation at molecular level of their different behaviour and give reason for the lower activity shown by the ligand containing the longer ethylenic bridge **1c**. These results can be regarded as a useful starting point for future improvements in the development of more potent and selective phosphinate inhibitors of MMPs.

6. Experimental

6.1. Molecular dynamics

For the MD simulation of structure **1c**, we have adopted the gromos96 force field implemented in a modified version of the Gromacs package.[30] The point charges were calculated using the CHelpG protocol on the Gamess package.[31]

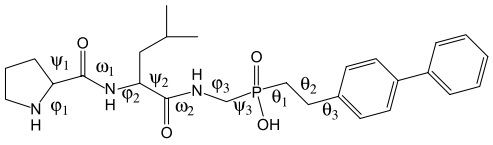
The structure in its 'active' configuration, after energy minimization, was put at the centre of a cubic box of 3.5 nm length filled with 868 water single point charge molecules,[32] and with one Cl^- counterion to ensure electroneutrality of the system.

After a short solvent relaxation at the desired density, the overall system was gently heated to 300 K and a simulation of 20 ns was finally carried out under isothermal/isochoric conditions (canonical ensemble) with a standard protocol.[33]

The simulation and the analysis of the trajectory were performed using the Gromacs software package and our own programs.

For checking the actual equilibration of the simulated system, the root mean square deviation (RMSD) with respect to the initial structures of **1c** was evaluated. On the equilibrated portion of the trajectory we finally carried out essential dynamics analysis for evaluating the conformationally relevant coordinates. This analysis, due to the limited dimensions of the investigated systems, was performed including all the atoms.

Table 2. Free energy minima, their values and relevant backbone torsion angles together with their RMSFs (°) for **1c**



Ligand **1c**

Energy minima	ΔA^0 (kJ/mol)	φ_1	ψ_1	φ_2	ψ_2	φ_3	ψ_3	θ_1	θ_2	θ_3
I	0	127 ± 23	150 ± 20	79 ± 20	126 ± 15	79 ± 14	114 ± 7	165 ± 12	69 ± 21	174 ± 90
II	0	124 ± 21	133 ± 19	87 ± 19	102 ± 14	97 ± 19	116 ± 7	119 ± 16	87 ± 11	93 ± 66
III	+1	127 ± 22	148 ± 19	90 ± 27	116 ± 18	77 ± 16	113 ± 8	166 ± 46	103 ± 59	162 ± 92
IV	+2	127 ± 23	145 ± 21	101 ± 24	115 ± 15	76 ± 14	114 ± 7	176 ± 59	178 ± 11	163 ± 91
V	+3	134 ± 25	143 ± 24	111 ± 26	52 ± 16	77 ± 12	118 ± 7	122 ± 67	172 ± 14	91 ± 18

6.2. Molecular modelling

The ligands herein described have been designed following structure-based drug-design criteria maximizing their putative structural affinity within the enzyme active site, using the program SYBYL on a Silicon Graphics workstation O_2. The coordinates of the enzymes have been taken from PDB (entries 1QIB and 1I73 for MMP-2 and MMP-8, respectively). The same program has been used for the minimization of the putative complexes formed by **1a** and **1b** with MMP-2 and MMP-8.

The following constraints have been imposed on the starting position of each ligand in the enzyme active site: the two phosphinic oxygens have been positioned at 2.1 and 2.5 Å from the catalytic zinc ion; moreover, the 2.5 Å Zn-coordinated oxygen atom has been H-bound (2.8 Å) to Glu 202 (MMP-2) and Glu 198 (MMP-8). The Leu NH and CO groups of inhibitor have been H-bound (2.8 Å) to CO and NH groups of Ala 167 (MMP-2) and Ala 163 (MMP-8), respectively. The biphenyl substituent has been inserted in the S_1' pocket. Free rotation has been left to all torsion angles of the inhibitors.

6.3. Chemistry

All reagents were purchased from commercial suppliers and used without further purification. Dry tetrahydrofuran (THF) was freshly distilled from sodium and benzophenone; dry dichloromethane was distilled from P_2O_5. In all other cases, commercially available reagent-grade solvents were employed without purification. Reactions performed in dry solvents were carried out in nitrogen atmosphere. Melting points are uncorrected and were obtained on a capillary apparatus (Büchi 510). Analytical thin-layer chromatography (TLC) was routinely used to monitor reactions: plates precoated with E. Merck silica gel 60 F_{254} or RP-8 F_{254S} of 0.25 mm thickness were used. Merck silica gel 60 (230–400 ASTM mesh) or Aldrich RP-18 silica gel (Cat. No. 377635) was employed for column flash-chromatography (FC). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Varian VXR 300 or a Bruker AC 200 spectrometer. Chemical shifts (δ) for ^{13}C and ^{1}H are given in parts per million relative to $(CH_3)_4Si$ (TMS) as the internal standard. Chemical shifts (δ) for ^{31}P are given in parts per million relative to phosphoric acid as the internal standard. Peak multiplicities are abbreviated: singlet, s; doublet, d; triplet, t; quartet, q; multiplet, m; broad, br. $[\alpha]_D$ values were determined with a Perkin-Elmer 241 polarimeter. Mass spectra were performed on an Applied Biosystems Voyager DE-PRO MALDI-TOF and on a Micromass Quattro triple-quadruple ESI-MS instrument. MALDI-TOF-MS analyses were performed on a matrix of α-cyano-4-hydroxycinnamic acid in acetonitrile/water 50:50, while for ESI-MS analyses samples were dissolved in acetonitrile solution containing 0.1% of trifluoroacetic acid (TFA). Elemental microanalyses (C, H, N) were within ±0.4% of the calculated values.

6.4. 2-(1,1′-Biphenyl-4-yl)ethanol (3c)

To a cooled (0 °C) 250 mL two-necked flask containing a solution of **2c** (3.0 g, 14.1 mmol) in 70 mL of anhydrous THF, 1.6 g (42.4 mmol) of $LiAlH_4$ was added portionwise while stirring, under nitrogen. When effervescence ceased, the mixture was heated at reflux for 5 h until disappearance of starting material. The mixture was diluted with ethyl acetate, washed with 1.0 M aqueous solution of HCl, water, and brine, and the organic layer dried over anhydrous sodium sulfate. Evaporation of the solvent afforded 2.76 g (99% yield) of product **3c**.

Compound **3c**: mp ($CHCl_3$) = 95–96 °C; ^{1}H NMR (acetone-d_6): δ 7.55–7.15 (m, 9H), 3.65 (t, J = 7 Hz, 2H), 2.80 (br signal, 1H), 2.72 (t, J = 7 Hz, 2H).

6.5. 4′-(Hydroxymethyl)-1,1′-biphenyl-4-ol (3b)

Compound **3b** (87% yield) was prepared similarly to **3c**.

Compound **3b**: mp ($CHCl_3$) = 181–183 °C; ^{1}H NMR (acetone-d_6): δ 8.46 (s, 1H), 7.60–7.35 (m, 6H), 6.92 (d, J = 8 Hz, 2H), 4.64 (s, 2H), 4.18 (br s, 1H).

6.6. 4-(Iodomethyl)-1,1′-biphenyl (4a)

To a solution containing **3a** (2 g, 10.9 mmol) in 10 mL of chloroform, 14 mL of an aqueous solution of HI (57%) was added dropwise, under stirring. The mixture was allowed to react at room temperature (rt) for 4 h and later worked-up as follows: the mixture was extracted with

chloroform, the separated organic layers were washed with a (10%) aqueous solution of $NaHCO_3$ and dried over anhydrous sodium sulfate. Evaporation of the solvent afforded 2.63 g (82% yield) of product **4a**.

Compound **4a**: mp (*n*-hexane/EtOAc) = 104–106 °C; ^{1}H NMR ($CDCl_3$): δ 7.60–7.38 (m, 9H), 4.51 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 140.75, 140.41, 138.27, 129.16, 128.79, 127.53, 127.00, 5.65.

6.7. 4-(2-Iodoethyl)-1,1′-biphenyl (4c)

To a solution containing PPh_3 (2.42 g, 9.23 mmol), imidazole (681 mg, 12 mol), and iodine (2.34 g, 9.23 mmol) dissolved in 50 mL of dry dichloromethane, 1.52 g (7.69 mmol) of alcohol **3c** was added portionwise with stirring, under nitrogen. The reaction was stirred for 3 h at rt, and then crude oil was purified by FC (*n*-hexane/EtOAc 80:20) affording 2.20 g (93% yield) of product **4c**.

Compound **4c**: mp (*n*-hexane/EtOAc) = 58–59 °C; ^{1}H NMR ($CDCl_3$): δ 7.60–7.20 (m, 9H), 3.45–3.15 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 140.64, 139.64, 139.54, 128.67, 127.26, 127.17, 126.91, 39.88, 5.50.

6.8. 4′-(Iodomethyl)-1,1′-biphenyl-4-ol (4b)

Compound **4b** (99% yield) was prepared similarly to **4a**.

Compound **3c**: mp (Et_2O) = 153–155 °C; ^{1}H NMR ($CDCl_3$): δ 7.52–7.40 (m, 7H), 6.90 (d, J = 8.8 Hz, 2H), 4.51 (s, 2H). ^{13}C NMR (acetone-d_6): δ 158.14, 141.22, 138.85, 132.41, 130.17, 128.78, 127.40, 116.56, 6.84.

6.9. 1,1′-Biphenyl-4-yl-methylphosphinic acid (6a)

A two-necked flask containing ammonium hypophosphite[24b] (564 mg, 6.8 mmol) was added with 2.13 mL (10.2 mmol) HMDS and the mixture was heated at 105 °C (temperature value controlled with Vertex) for 2 h, under nitrogen in order to synthesize BTSP **5**.[25] The system was cooled at rt, added via syringe with 2 g (6.8 mmol) of **4a** previously dissolved in 5 mL of anhydrous dichloromethane and refluxed under nitrogen for 12 h. The reaction mixture was filtered, and the solvent was removed to yield an oil, which was dissolved in dichloromethane and added with a stoichiometric amount (6.8 mmol) of methanol (to achieve desilylation and solution). The mixture was newly filtered and the solution was washed with 2.0 M aqueous solution of HCl, dried over anhydrous sodium sulfate, followed by removal of the solvent affording 1.03 g (70% yield) of product **6a** as a white solid; the latter was carefully dried over P_2O_5 overnight, under vacuum. The product was used without further purification.

Compound **6a**: R_f = 0.39 (EtOAc/AcOH 8:2); mp (CH_2Cl_2) = >220 °C; ^{1}H NMR (CD_3OD): δ 7.62–7.25 (m, 9H), 6.8 (d, J = 666.6 Hz, 1H), 3.16 (d, J = 21.7 Hz, 2H). ^{13}C NMR (CD_3OD): δ 142.14 (d, J = 1.5 Hz), 140.8 (d, J = 3.8 Hz), 133.47 (d, J = 9.4 Hz), 131.4 (d, J = 6.4 Hz), 129.84, 128.24, 127.95 (d, J = 3.2 Hz), 127.84 (d, J = 0.6 Hz), 35.48 (d, J = 134.8 Hz). ^{31}P NMR (CD_3OD): δ 33.25. MS-ESI$^+$: m/z = 233 [M+H]$^+$.

6.10. 2-(1,1′-Biphenyl-4-yl)ethylphosphinic acid (6c) and (4′-hydroxy-1,1′-biphenyl-4-yl)methylphosphinic acid (6b)

Compounds **6c** (58% yield) and **6b** (60% yield) were prepared starting, respectively, from **4c** and **4b**, analogously as described for **6a**.

Compound **6c**: R_f = 0.29 (EtOAc/AcOH 8:2); mp (CH_2Cl_2) = 203–205 °C; ^{1}H NMR ($CDCl_3$): δ 9.75 (br s, 1H), 7.60–7.22 (m, 9H), 7.15 (d, J = 548 Hz, 1H), 3.05–2.88 (m, 2H), 2.25–2.05 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 140.74, 139.53, 139.18 (d, J = 15.8 Hz), 128.80, 128.57, 127.43, 127.24, 127.01, 30.94 (d, J = 92.5 Hz), 26.48. ^{31}P NMR (CD_3OD): δ 37.90. MS-ESI$^+$: m/z = 247 [M+H]$^+$.

Compound **6b**: R_f = 0.26 (EtOAc/AcOH 8:2); mp ($CHCl_3$) = 211–213 °C; ^{1}H NMR (CD_3OD): δ 7.55–7.28 (m, 6H), 6.84 (d, J = 8.6 Hz, 2H), 6.98 (d, J = 550 Hz, 1H), 3.20 (d, J = 18.5 Hz, 2H). ^{13}C NMR (CD_3OD): δ 158.20, 141.01 (d, J = 3.9 Hz), 133.20, 131.25, (d, J = 6.1 Hz), 130.61 (d, J = 7.5 Hz), 128.90, 127.68 (d, J = 3.1 Hz), 116.63, 38.77 (d, J = 86.3 Hz). ^{31}P NMR (CD_3OD): δ 32.52. MALDI-TOF-MS: m/z = 249 [M+H$^+$].

6.11. Methyl-1,1′-biphenyl-4-yl-methyl](1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phosphinate (8a)

A two-necked flask containing a suspension of **6a** (500 mg, 2.17 mmol) in 6 mL of dry dichloromethane was added with 0.5 mL (2.4 mmol) HMDS, under nitrogen. The resulting solution was stirred for 1 h at rt, added with phthalimidomethyl bromide (521 mg, 2.17 mmol) and refluxed overnight. After TLC control (*n*-hexane/ethyl acetate 7:3) showed the disappearance of reagent **6a**, solvent was evaporated in vacuo and the residue, dissolved in 5 mL of a 1:1 methanol/toluene mixture, was cooled to 0 °C and added with an excess of an ethereal solution of diazomethane under stirring. After 1 h, TLC control (toluene/acetone 1:1) showed the end of reaction: solvent was evaporated in a rotavapor containing some drops of acetic acid in the receiver flask and crude oil was purified by FC (toluene/acetone 70:30 as eluent mixture) affording 500 mg (57% yield) of product **8a** as a white solid.

Compound **8a**: R_f = 0.38 (toluene/acetone 6:4); mp (EtOAc) = 146–148 °C; ^{1}H NMR ($CDCl_3$): δ 7.91–7.82 (m, 2H), 7.79–7.71 (m, 2H), 7.59–7.30 (m, 9H), 4.1 (d, J = 7.9 Hz, 2H), 3.78 (d, J = 10.7 Hz, 3H), 3.36 (ABX dd, J = 15.65 and 23.86 Hz, 1H), 3.28 (ABX dd, J = 15.65 and 23.86 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 167.39, 140.62, 139.96, 134.35, 131.85, 130.47 (d, J = 5.8 Hz), 129.32 (d, J = 9.3 Hz), 128.74, 127.38 (d, J = 2.7 Hz), 127.29, 127.02, 123.64, 52.23 (d, J = 6.9 Hz), 35.93 (d, J = 89.7 Hz), 34.73 (d, J = 95.5 Hz). ^{31}P NMR ($CDCl_3$): δ 45.03. MS-ESI$^+$: m/z = 406 [M+H]$^+$.

6.12. Methyl-2-(1,1′-biphenyl-4-yl)ethyl[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phosphinate (8c) and methyl (1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl-[(4′-hydroxy-1,1′-biphenyl-4-yl)methyl]phosphinate (8b)

Compounds **8c** (55% yield) and **8b** (49% yield) were prepared starting, respectively, from **6c** and **6b**, analogously as described for **8a**.

Compound **8c**: $R_f = 0.27$ (toluene/acetone 7:3); sticky oil; ^{1}H NMR ($CDCl_3$): δ 7.88–7.81 (m, 2H), 7.76–7.69 (m, 2H), 7.57–7.26 (m, 9H), 4.10 (d, $J = 8.2$ Hz, 2H), 3.83 (d, $J = 10.7$ Hz, 3H), 3.14–2.93 (m, 2H), 2.22–2.04 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 167.25, 140.78, 139.99, 139.66, 139.30, 134.34, 131.75, 128.67 (d, $J = 4.25$ Hz), 127.29, 127.14, 126.94, 123.62, 51.83 (d, $J = 6.7$ Hz), 34.77 (d, $J = 92.9$ Hz), 29.90 (d, $J = 91.8$ Hz), 26.85 (d, $J = 3.5$ Hz). ^{31}P NMR ($CDCl_3$): δ 49.84. MS-ESI$^+$: $m/z = 420$ [M+H]$^+$.

Compound **8b**: $R_f = 0.4$ (toluene/acetone 1:1); mp = 215–217 °C; ^{1}H NMR ($CDCl_3$): δ 7.91–7.87 (m, 2H), 7.78–7.74 (m, 2H), 7.44–7.33 (m, 6H), 6.86 (d, $J = 8.7$ Hz, 2H), 4.10 (d, $J = 7.7$ Hz, 2H), 3.77 (d, $J = 10.8$ Hz, 3H), 3.38–3.26 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 167.59, 156.59, 140.05, 134.58, 132.07, 131.81, 130.44 (d, $J = 5.9$ Hz), 128.10, 127.85, 126.88 (d, $J = 2.7$ Hz), 123.75, 115.71, 52.46 (d, $J = 7.1$ Hz), 35.73 (d, $J = 89.3$ Hz), 34.62 (d, $J = 95.5$ Hz). ^{31}P NMR ($CDCl_3$): δ 51.88. MALDI-TOF-MS: $m/z = 422$ [M+H$^+$].

6.13. Methyl-aminomethyl(1,1′-biphenyl-4-yl-methyl)phosphinate (9a)

To a flask containing **8a** (225 mg, 0.56 mmol) dissolved in 3 mL of absolute ethanol, 556 μL (2.80 mmol) of hydrazine monohydrate was added under stirring and the reaction mixture was allowed to stir, at room temperature, for 18 h. After filtration, the solvent was evaporated and crude oil was purified by FC (AcOEt/MeOH, 60:40) affording 120 mg of product **9a** (78% yield).

Compound **9a**: $R_f = 0.21$ (EtOAc/MeOH 7:3); sticky oil; ^{1}H NMR ($CDCl_3$): δ 7.51–7.25 (m, 9H), 3.66 (d, $J = 10.3$ Hz, 3H), 3.22 (d, $J = 16.7$ Hz, 2H), 3.10–2.75 (m, 2H), 1.64 (br s, 2H). ^{13}C NMR ($CDCl_3$): δ 140.57, 139.98, 130.37 (d, $J = 7.4$ Hz), 130.07 (d, $J = 5.5$ Hz), 128.82, 127.58 (d, $J = 2.8$ Hz), 127.38, 127.01, 51.75 (d, $J = 7$ Hz), 38.69 (d, $J = 101.2$ Hz), 33.51 (d, $J = 82.5$ Hz). ^{31}P NMR ($CDCl_3$): δ 52.51.

6.14. Methyl-aminomethyl[2-(1,1′-biphenyl-4-yl)ethyl]-phosphinate (9c) and methyl-aminomethyl[(4′-hydroxy-1,1′-biphenyl-4-yl)methyl]phosphinate (9b)

Compounds **9c** (70% yield) and **9b** (74% yield) were prepared starting, respectively, from **8c** and **8b**, analogously as described for **9a**.

Compound **9c**: $R_f = 0.35$ (EtOAc/MeOH 1:1); sticky oil; ^{1}H NMR ($CDCl_3$): δ 7.60–7.26 (m, 9H), 3.76 (d, $J = 10.3$ Hz, 3H), 3.05–2.92 (m, 4H), 2.25–2.10 (m, 2H), 1.58 (br s, 2H). ^{13}C NMR ($CDCl_3$): δ 140.82, 139.87, 139.75, 128.97, 128.71, 127.58, 127.46, 127.17, 51.75 (d, $J = 6.9$ Hz), 39.05 (d, $J = 97.3$ Hz), 27.45 (d, $J = 87.1$ Hz), 27.45 (d, $J = 2.3$ Hz). ^{31}P NMR ($CDCl_3$): δ 57.38.

Compound **9b:** $R_f = 0.34$ (EtOAc/MeOH 6:4); sticky oil; ^{1}H NMR (CD_3OD): δ 7.54–7.41 (m, 6H), 6.83 (d, $J = 8.6$, 2H), 3.85 (d, $J = 8.2$ Hz, 2H), 3.74 (d, $J = 10.2$ Hz, 3H), 3.44 (d, $J = 17.6$ Hz, 2H). ^{13}C NMR (CD_3OD): δ 158.36, 141.48, 133.0, 131.42 (d, $J = 5.7$ Hz), 129.85, 128.92, 127.81, 116.68, 52.91 (d, $J = 7.1$ Hz), 37.28 (d, $J = 99.3$ Hz), 34.28 (d, $J = 86.9$ Hz). ^{31}P NMR (CD_3OD): δ 55.67.

6.15. 1-[(Benzyloxy)carbonyl]prolyl-N^1-{[(1,1′-biphenyl-4-yl-methyl)(methoxy)phosphoryl] methyl}leucinamide (10a)

To a cooled (0 °C) funnel-equipped flask containing a dry THF (4 mL) solution of **9a** (118 mg, 0.43 mmol) and *Z*-Pro-Leu-OH (155 mg, 0.43 mmol), a solution of DCC (97 mg, 0.47 mmol) and HOBT (64 mg, 0.47 mmol) dissolved in 2 mL of the same solvent was added dropwise under stirring. After standing at rt overnight, *N*,*N*′-dicyclohexylurea was filtered off and the solution was evaporated under reduced pressure. The residue, dissolved in EtOAc, was washed with saturated solution of $NaHCO_3$ and brine, and organic layer dried over anhydrous Na_2SO_4. Crude oil was purified by FC (EtOAc/MeOH 95:5) affording 212 mg (80% yield) of product **10a** as 1:1 mixture of diastereomers.

Compound **10a**: $R_f = 0.60$ (EtOAc/MeOH 9:1); ^{1}H NMR ($CDCl_3$): δ 7.60–7.20 (m, 14H), 7.05 (br signal, 1H), 6.80 (br signal, 1H), 5.15 (s, 2H), 4.58–4.40 (m, 1H), 4.40–4.28 (m, 1H), 3.85–3.35 (br m, 7H), 3.26 (d, $J = 17.4$ Hz, 2H), 2.25–1.10 (br m, 7H), 1.05–0.75 (br signal, 6H). ^{13}C NMR ($CDCl_3$): δ 172.31, 172.02, 156.41, 140.64, 139.95, 136.23, 130.39, 129.63, 128.76, 128.60, 128.26, 127.87, 127.47, 127.30, 127.00, 67.62, 60.97, 52.03, 47.24, 40.20, 36.3 (d, $J = 94$ Hz), 34.42 (d, $J = 78$ Hz), 29.00, 25.02, 24.70, 22.99, 21.65. ^{31}P NMR ($CDCl_3$): δ 48.95, 48.62. MS-ESI$^+$: $m/z = 620$ [M+H$^+$], 642 [M+Na$^+$].

6.16. 1-[(Benzyloxy)carbonyl]prolyl-N^1-{[[2-(1,1′-biphenyl-4-yl)ethyl](methoxy)phosphoryl] methyl}leucinamide (10c) and 1-[(benzyloxy)carbonyl]prolyl-N^1-{[[(4′-hydroxy-1,1′-biphenyl-4-yl)methyl](methoxy)phosphoryl]methyl}leucinamide (10b)

Tripeptides **10c** (67% yield) and **10b** (71% yield) were prepared starting, respectively, from **9c** and **9b**, analogously as described for **10a**.

Compound **10c**: $R_f = 0.69$ (EtOAc/MeOH 8:2); ^{1}H NMR ($CDCl_3$): δ 7.56–7.26 (m, 14H), 7.05 (br signal, 1H), 6.70 (br signal, 1H), 5.15 (s, 2H), 4.42–4.36 (m, 1H), 4.32–4.26 (m, 1H), 3.76–3.48 (m, 7H), 3.00–2.92 (m, 2H), 2.16–2.05 (m, 3H), 1.96–1.90 (m, 3H), 1.73–1.66 (m, 3H), 0.91–0.86 (br signal, 6H). ^{13}C NMR ($CDCl_3$): δ 172.09, 157.12, 141.09, 140.23 (d, $J = 15.5$ Hz), 139.54, 136.41, 128.96, 128.83, 128.79, 128.53, 128.12, 128.04, 127.53, 127.37, 127.21, 67.86, 61.22, 52.28, 47.47, 40.28, 36.6 (d,

J = 98 Hz), 29.56 (d, J = 53 Hz), 27.22 (d, J = 3.7 Hz), 25.28, 24.91, 23.19, 21.82. ^{31}P NMR ($CDCl_3$): δ 53.31, 53.02. ESI$^+$-MS: m/z = 634.8 [M+H$^+$], 656.7 [M+Na$^+$].

Compound **10b**: R_f = 0.33 (EtOAc/MeOH 9:1); ^{1}H NMR (CD_3OD): δ 7.53–7.25 (m, 11H), 6.81 (d, J = 8.9 Hz, 2H), 5.14–5.06 (m, 2H), 4.43–4.25 (m, 2H), 3.69–3.43 (m, 9H), 2.32–1.48 (m, 7H), 0.96–0.80 (m, 6H). ^{13}C NMR (CD_3OD): δ 174.89, 174.54, 157.11, 156.12, 141.61, 141.07, 137.56, 131.49 (d, J = 5.4 Hz), 130.55 (d, J = 8.9 Hz), 129.66, 129.34, 128.94, 128.79, 128.65, 128.19, 128.04 (d, J = 2.5 Hz), 127.70, 68.31, 61.92, 60.71, 53.05, 41.45, 40.92, 36.70 (d, J = 98.5 Hz), 31.81 (d, J = 54 Hz), 25.30, 24.37, 23.45, 21.72. ^{31}P NMR ($CDCl_3$): δ 51.12, 50.10. MALDI-TOF-MS: m/z = 636 [M+H$^+$], 658 [M+Na$^+$].

6.17. Prolyl-N^1-{[(1,1′-biphenyl-4-yl-methyl)(hydroxy)-phosphoryl]methyl}leucinamide (1a)

To a solution containing tripeptide **10a** (190 mg, 0.307 mmol) dissolved in 4 mL of dry dichloromethane was added an excess of BTSA (0.825 mL, 3.37 mmol) while stirring, under nitrogen. After 1 h at rt, the reaction mixture was cooled (−20 °C) and an excess of TMSI (0.349 mL, 2.45 mmol) was added dropwise. The solution was allowed to warm at rt within 1 h and after 2 h solvent was evaporated under reduced pressure. Crude oil was taken up with 3.0 mL of a 7:3 CH_3CN/H_2O mixture. After removal of the solvents, the residue was purified by RP-18 FC (CH_3CN/H_2O, from 60/40 to 80/20) affording 109 mg (75% yield) of free pseudo-tripeptide **1a** as white solid.[38]

Compound **1a**: $[\alpha]_D^{20}$ −69 (c 0.29, DMSO); ^{1}H NMR (DMSO-d_6, 600 MHz): δ 9.0 (br signal, 1H), 8.38 (br s, 1H), 7.61 (d, J = 8 Hz, 2H), 7.53 (d, J = 8 Hz, 2H), 7.48–7.30 (m, 5H), 4.42 (br signal, 1H), 4.12 (br signal, 1H), 3.38–3.05 (m, 4H), 2.93 (d, 2H), 2.23–2.18 (m, 1H), 1.90–1.83 (m, 1H), 1.82–1.75 (m, 2H), 1.65–1.43 (m, 3H), 0.91–0.83 (m, 6H). ^{13}C NMR (DMSO-d_6): δ 171.48, 167.97, 140.03, 137.78, 130.44, 130.33, 128.95, 127.27, 126.5, 126.33, 58.91, 51.65, 45.69, 29.35, 24.24, 23.40, 23.09, 21.54. ^{31}P NMR (DMSO-d_6): δ 67.86. ESI$^+$-MS: m/z = 472 [M+H$^+$], 494 [M+Na$^+$]. Calcd for $C_{25}H_{34}N_3O_4P{\cdot}2H_2O$: C, 59.16; H, 7.55; N, 8.28. Found: C, 58.76; H, 7.38; N, 8.14.

6.18. Prolyl-N^1-{[[2-(1,1′-biphenyl-4-yl)ethyl](hydroxy)-phosphoryl]methyl}leucinamide (1c) and prolyl-N^1-({hydroxy[(4′-hydroxy-1,1′- biphenyl-4-yl)methyl]phosphoryl} methyl)leucinamide (1b)

Final tripeptides **1c** (83% yield) and **1b** (72% yield) were prepared starting, respectively, from **10c** and **10b**, analogously as described for **1a**.

Compound **1c**: $[\alpha]_D^{20}$ −54.3 (c 0.35, CH_3OH); ^{1}H NMR (CD_3OD): δ 7.59–7.30 (m, 9H), 4.49–4.42 (m, 1H), 4.35–4.28 (m, 1H), 3.38–3.31 (m, 4H), 3.01–2.87 (m, 2H), 2.51–2.37 (m, 1H), 2.17–2.00 (m, 5H), 1.71–1.68 (m, 3H), 0.99–0.93 (m, 6H). ^{13}C NMR (CD_3OD): δ 175.20, 169.66, 141.95, 140.96, 140.54, 129.67, 129.54, 129.37, 128.07, 127.71, 60.72, 52.33, 41.11, 32.33 (d, J = 85.8 Hz), 30.85, 29.28 (d, J = 35.5 Hz), 27.59, 25.90, 24.79, 23.33, 21.61. ^{31}P NMR (CD_3OD): δ 64.01. ESI$^+$-MS: m/z = 486.7 [M+H$^+$], 508.7 [M+Na$^+$]. Calcd for $C_{26}H_{36}N_3O_4P{\cdot}H_2O$: C, 62.01; H, 7.61; N, 8.34. Found: C, 62.38; H, 7.42; N, 8.60.

Compound **1b**: $[\alpha]_D^{20}$ −59.2 (c 1.35, CH_3OH); ^{1}H NMR (CD_3OD): δ 7.51–7.32 (m, 6H), 6.83 (d, J = 8.6 Hz, 2H), 4.48–4.31 (m, 2H), 3.59 (d, J = 9.2 Hz, 2H), 3.34–3.31 (m, 2H), 3.18 (d, J = 16 Hz, 2H), 2.53–2.36 (m, 1H), 2.16–1.97 (m, 3H), 1.68–1.61 (m, 3H), 0.99–0.94 (m, 6H). ^{13}C NMR (CD_3OD): δ 174.51, 169.95, 158.23, 148.32, 134.02, 131.60, 128.87, 127.49, 126.54, 116.64, 60.89, 53.93, 41.60, 38.82 (d, J = 98.6 Hz), 35.54 (d, J = 82.3 Hz), 31.10, 25.91, 24.96, 23.41, 21.88. ^{31}P NMR (CD_3OD): δ 62.86. MALDI-TOF-MS: m/z = 488 [M+H$^+$]. Calcd for $C_{25}H_{34}N_3O_5P{\cdot}2H_2O$: C, 57.35; H, 7.32; N, 8.03. Found: C, 57.02; H, 7.52; N, 8.23.

6.19. MMPs activation and inhibition assays

The proenzymes[27] (pro-MMP-2 and -8) were activated immediately before their use with p-aminophenylmercuric acetate (APMA, 1 mM) for 1 h at 37 °C.[39] For assay measurements, 10^{-2} mM solutions of the inhibitors (in MeOH for **1a**, **1b** and in a 90:10 mixture of MeOH/DMSO for **1c**) were further diluted as required in the assay buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, 0.02% NaN_3, 0.05% Brij 35, pH 7.4, for MMP-2, and 50 mM Tris–HCl, 10 mM $CaCl_2$, 150 mM NaCl, pH 7.5, for MMP-8). The activated enzyme added with inhibitor solution was incubated in the assay buffer for 3 h, the temperature being maintained at 25 °C for MMP-2 and at 37 °C for MMP-8. After addition of a 0.054 mM DMSO solution of fluorogenic substrate QF24, hydrolysis was monitored by recording the increase in fluorescence (λ_{ex} 328 nm, λ_{em} 393 nm), during 30 min, using a Perkin-Elmer spectrofluorimeter LS 50B. The IC_{50} values were calculated from control reactions without the inhibitor. The mean IC_{50} was obtained from at least three independent measurements, and the standard deviation of the mean was less than 10%.

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References and notes

1. (a) Coussens, L. M.; Fingleton, B.; Matrisian, L. M. *Science* **2002**, *295*, 2387–2392; (b) Foda, H. D.; Zucker, S. *Drug Discovery Today* **2001**, *6*, 478–482; (c) Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305–341.
2. Woessner, J. F., Jr. *FASEB J.* **1991**, *5*, 2145–2154.
3. Murphy, G.; Docherty, A. J. P. *J. Res. Cell Mol. Biol.* **1992**, *7*, 120–125.

4. Birkedal-Hansen, H.; Moore, W. G. I.; Bodden, M. K.; Windsor, L. J.; Birkedal-Hansen, B.; De Carlo, A.; Engler, J. A. *Crit. Rev. Oral Biol. Med.* **1993**, *4*, 197–250.
5. (a) Woessner, J. F.; Nagase, H. *Matrix Metalloproteinases and TIMPs*; Oxford University Press: New York, 2000; (b) Denhardt, D. T.; Feng, B.; Edwards, D. R.; Cocuzzi, E. T.; Malyankar, U. M. *Pharmacol. Ther.* **1993**, *59*, 329–341.
6. Pyke, C.; Ralfkiaer, E.; Huhtala, P.; Hurskainen, T.; Dano, K.; Tryggvason, K. *Cancer Res.* **1992**, *52*, 1336–1341.
7. Stetler-Stevenson, W. G.; Aznavoorian, S.; Liotta, L. A. *Annu. Rev. Cell Biol.* **1993**, *9*, 541–573.
8. Gijbels, K.; Masure, S.; Carton, H.; Opdenakker, G. *J. Neuroimmunol.* **1992**, *41*, 29–34.
9. Murphy, G.; Hembry, R. M. *J. Rheumatol.* **1992**, *19*, 61–64.
10. (a) Peress, N.; Perillo, E.; Zucher, S. *J. Neuropathol. Exp. Neurol.* **1995**, *54*, 16–22; (b) Lopez, B.; Gonzalez, A.; Diez, J. *Curr. Opin. Nephrol. Hypertens.* **2004**, *13*, 197–204.
11. (a) Martin, D. C.; Sanchez-Sweatman, O. H.; Inderdeo, D. S.; Tsao, M. S.; Khokha, R. *Lab. Invest.* **1999**, *79*, 225–234; (b) Krüger, A.; Fata, J. E.; Khokha, R. *Blood* **1997**, *90*, 1993–2000.
12. Overall, C. M.; López-Otin, C. *Nat. Rev. Cancer* **2002**, *2*, 657–672.
13. (a) Peterson, J. T. *Heart Fail. Rev.* **2004**, *9*, 63–79; (b) Skiles, J. W.; Gonnella, N. C.; Jeng, A. Y. *Curr. Med. Chem.* **2004**, *11*, 2911–2977; (c) Michaelides, M. R.; Curtin, M. L. *Curr. Pharm. Design* **1999**, *5*, 787–819; (d) Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H. *Chem. Rev.* **1999**, *99*, 2735–2776.
14. Muri, E. M. F.; Nieto, M. J.; Sindelar, R. D.; Williamson, J. S. *Curr. Med. Chem.* **2002**, *9*, 1631–1653.
15. Babine, E. R.; Bender, S. L. *Chem. Rev.* **1997**, *97*, 1359–1472.
16. Freskos, J. N.; McDonald, J. J.; Mischke, B. V.; Mullins, P. B.; Shieh, H. S.; Stegeman, R. A.; Stevens, A. M. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1757–1760.
17. Tamura, Y.; Watanabe, F.; Nakatani, T.; Yasui, K.; Fuji, M.; Komurasaki, T.; Tsuzuki, H.; Maekawa, R.; Yoshioka, T.; Kawada, K.; Sugita, K.; Ohtani, M. *J. Med. Chem.* **1998**, *41*, 640–649.
18. Hodgson, J. *Biotechnology* **1995**, *13*, 554–557.
19. (a) D'Alessio, S.; Gallina, C.; Gavuzzo, E.; Giordano, C.; Gorini, B.; Mazza, F.; Paglialunga Paradisi, M.; Panini, G.; Pochetti, G.; Sella, A. *Bioorg. Med. Chem.* **1999**, *7*, 389–394; (b) Cirilli, M.; Gallina, C.; Gavuzzo, E.; Giordano, C.; Gomis-Ruth, F. X.; Gorini, B.; Kress, L. F.; Mazza, F.; Paglialunga Paradisi, M.; Pochetti, G.; Politi, V. *FEBS Lett.* **1997**, *418*, 319–322; (c) Gavuzzo, E.; Pochetti, G.; Mazza, F.; Gallina, C.; Gorini, B.; D'Alessio, S.; Pieper, M.; Tschesche, H.; Tucker, P. A. *J. Med. Chem.* **2000**, *43*, 3377–3385; (d) Aschi, M.; Roccatano, D.; Di Nola, A.; Gallina, C.; Gavuzzo, E.; Pochetti, G.; Pieper, M.; Tschesche, H.; Mazza, F. *J. Comput.-Aided Mol. Des.* **2002**, *16*, 213–225.
20. (a) Finzel, B. C.; Baldwin, E. T.; Bryant, G. L., Jr.; Hess, G. F.; Wilks, J. W.; Trepod, C. M.; Mott, J. E.; Marshall, V. P.; Petzold, G. L.; Poorman, R. A.; O'Sullivan, T. J.; Schostarez, H. J.; Mitchell, M. A. *Protein Sci.* **1998**, *7*, 2118–2126; (b) Yeh, L. A.; Chen, J.; Baculi, F.; Gingrich, D. E.; Shen, T. Y. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1637–1642; (c) Reiter, L. A.; Mitchell, P. G.; Martinelli, G. J.; Lopresti-Morrow, L. L.; Yocum, S. A.; Eskra, J. D. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2331–2336.
21. The following references are cited as selected examples: (a) Matziari, M.; Beau, F.; Cuniasse, P.; Dive, V.; Yiotakis, A. *J. Med. Chem.* **2004**, *47*, 325–336; (b) Schiødt, C. B.; Buchardt, J.; Terp, G. E.; Christensen, U.; Brink, M.; Larsen, Y. B.; Meldal, M.; Foged, N. T. *Curr. Med. Chem.* **2001**, *8*, 967; (c) Caldwell, C. G.; Sahoo, S. P.; Polo, S. A.; Eversole, R. R.; Lanza, T. J.; Mills, S. G.; Niedzwiecki, L. M.; Izquierdo-Martin, M.; Chang, B. C.; Harrison, R. K.; Kuo, D. W.; Lin, T.-Y.; Stein, R. L.; Durette, P. L.; Hagmann, W. K. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 323–328; (d) Goulet, J. L.; Kinneary, J. F.; Durette, P. L.; Stein, R. L.; Harrison, R. K.; Izquierdo-Martin, M.; Kuo, D. W.; Lin, T.-Y.; Hagmann, W. K. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1221–1224.
22. (a) Netzel-Arnett, S.; Fields, G.; Birkedal-Hansen, H.; Van Wart, H. E. *J. Biol. Chem.* **1991**, *266*, 1; (b) Netzel-Arnett, S.; Sang, Q.-X.; Moore, W. G. I.; Navre, M.; Birkedal-Hansen, H.; Van Wart, H. E. *Biochemistry* **1993**, *32*, 6427; (c) Bode, W.; Reinemer, P.; Huber, R.; Kleine, T.; Schnierer, S.; Tschesche, H. *EMBO J.* **1994**, *13*, 1263; (d) Grams, F.; Reinemer, P.; Powers, J. C.; Kleine, T.; Pieper, M.; Tschesche, H.; Huber, R.; Bode, W. *Eur. J. Biochem.* **1995**, *228*, 830.
23. For an excellent discussion concerning the chemistry and biological activity of aminophosphonic and aminophosphinic acids see: *Aminophosphonic and Aminophosphinic Acids*; Kukhar, V. P., Ed.; Wiley: Chichester, 2000.
24. (a) Boyd, E. A.; Regan, A. C.; James, K. *Tetrahedron Lett.* **1994**, *35*, 4223–4226; (b) Boyd, E. A.; Regan, A. C.; James, K. *Tetrahedron Lett.* **1992**, *33*, 813–816.
25. CAUTION: due to its pyrophoric character, BTSP was prepared in situ and directly used in the reaction with alkyl iodides.
26. (a) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788–798; (b) Solas, D.; Hale, R. L.; Patel, D. V. *J. Org. Chem.* **1996**, *61*, 1537–1539.
27. Recombinant Human pro-MMP-2 and -8 were purchased from R&D Systems, catalogue numbers, respectively, 902-MP and 908-MP; the fluorogenic peptide QF24, used as a very sensitive substrate for in situ determination of MMP activity, was purchased from Bachem, Cat. No. M-1895.
28. Amadei, A.; Linssen, A. B. M.; Berendsen, H. J. C. *Proteins* **1993**, *17*, 412–425.
29. The free energy calculations were carried out by preventively dividing the essential plane in 2500 intervals. The trajectory was then projected on this plane and the probability (ρ_n) of finding the trajectory within each *n*th interval was evaluated. The Helmholtz free energy (ΔA^0_{i-j}) for the transition from an interval '*i*' to an interval '*j*' can be simply calculated using the standard relation $\Delta A^0_{i-j} = -kT \ln(\rho_j/\rho_i)$ where k is the Boltzmann constant.
30. Van der Spoel, D.; van Drunen R.; Berendsen, H.J.C., "GRoningen MAchine for Chemical Simulations," Dept. of Biophysical Chemistry, BIOSON Research Institute, Nijenborgh 4 NL-9717 AG Groningen, 1994, e-mail to gromacs@chem.rug.nl.
31. Schmidt, M. W.; Baldridge, K. K.; Boatz, J. A.; Elbert, S. T.; Gordon, M. S.; Jensen, J. J.; Koseki, S.; Matsunaga, N.; Nguyen, K. A.; Su, S.; Windus, T. L.; Dupuis, M.; Montgomery, J. *J. Comput. Chem.* **1993**, *14*, 1347.
32. Feenstra, K. A.; Hess, B.; Berendsen, H. J. C. *J. Comput. Chem.* **1999**, *20*, 786.
33. The temperature was kept constant by the isogaussian algorithm.[34] Periodic boundary conditions (pbc) were systematically applied and a cut-off of 1.10 nm was considered when Lennard-Jones (LJ) forces were computed. The long range electrostatics was calculated using the particle mesh ewald method (PME),[35] and the algorithm LINCS[36] was used to constrain all bond lengths. We also used roto-translational constraints to stop the solute in the simulated box.[37]
34. Evans, D. J.; Morriss, G. P. In *Statistical Mechanics of Non-Equilibrium Liquids*; Academic Press: London, 1990.

35. Darden, T. A.; York, D. M.; Pedersen, L. G. *J. Chem. Phys.* **1993**, *98*, 10089.
36. Bekker, B.; Berendsen, H. J. C.; Fraije, J. G. E. M. *J. Comput. Chem.* **1997**, *18*, 1463.
37. Amadei, A.; Chillemi, G.; Ceruso, M. A.; Grottesi, A.; Di Nola, A. *J. Chem. Phys.* **2000**, *112*, 9.
38. Occasionally free pseudo tripeptides **1a** and **1c** showed a dark-red colour probably due to the formation of a charge-transfer complex with elemental iodine. This complex can be destroyed by the following work-up: a water suspension of product was slowly added with the smallest amount of a 5% aqueous solution of $Na_2S_2O_3$ while stirring, until a white solid appeared; then, after centrifugation and water washing, carefully drying over P_2O_5 afforded pure product as a white solid.
39. (a) Murphy, G.; Crabbe, T. *Methods Enzymol.* **1995**, *248*, 470; (b) Tschesche, H. *Methods Enzymol.* **1995**, *248*, 431.