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Original article

Synthesis and evaluation of new tripeptide phosphonate inhibitors of MMP-8 and MMP-2

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Abstract

The phosphotryptophan derivative L-Pro-L-Leu-L-(P)Trp(OH)$_2$ (**2b**) was reported as the first example of left-hand-side[1] phosphonate inhibitor of MMP-8. Its uncommon mode of binding to MMP-8 was mainly ascribed to the presence of the proline residue in P_3. Ten new analogues of **2b** were obtained by replacement of the aminoterminal L-Pro with aminoacid residues bearing small side chains. Most of the new analogues show an increase of affinity for MMP-2 and MMP-8, and different profiles of selectivity. Computer simulations were performed to explain the effects of substitutions on the preferred mode of binding. They reveal that most of the new analogues are probably accommodated in the right, rather than left-hand side of MMP-8 active site.

Keywords: MMP; Matrix metalloproteinases inhibitors; Tripeptide phosphonates; Right-hand inhibitors; Left-hand inhibitors

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that can degrade virtually all the constituents of the extracellular matrix. These enzymes are necessary for normal tissue remodelling and are particularly implicated in some processes such as ovulation, embryogenic growth, angiogenesis, differentiation and healing [1,2]. Overexpression of MMPs activity, however, or inadequate levels of the natural MMPs tissue inhibitors, can contribute to the pathophysiology of a variety of disease states and conditions such as psoriasis [3], multiple sclerosis [4,5] rheumatoid arthritis [6,7], and osteoporosis [8,9]. In addition, the angiogenetic process favoured by MMPs [10,11] is essential for vascolarisation and growth of tumours beyond the size of approximately 2 mm in diameter.

Thus, inhibitors of MMPs are actively studied for their utility in slowing or halting degradation of extracellular matrix and progression of tumour invasion and metastasis. A great variety of synthetic, low molecular weight, MMPs inhibitors have been prepared and tested [12,13]; some of them, such as marimastat, prinomastat and neovastat [14] are presently in advanced clinical trials. Their structures include a peptide or a peptidomimetic chain, which is generally accommodated

Abbreviations: BSA, bistrimethylsilyl acetamide; DCC, 1,3-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; QF24, McaPro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$; QF41, McaPro-Cha-Gly-Nva-His-Ala-Dpa-NH$_2$; TMIS, trimethylsilyl iodide.

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[1] Left-hand-side inhibitors: inhibitors that bind in the unprime region of the enzyme active site, in reference to the convention of drawing the unprimed residues of a peptide substrate on the left side. [R.P. Beckett et al., Drug Discov. Today 1 (1996) 16–26]. The opposite applies to right-hand-side inhibitors.

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in the S′ region of the active site, and a zinc-binding function, capable of coordinating the catalytic zinc ion of the enzyme.

Hydroxamate is, by far, the most effective zinc-binding group. Direct replacement of this function with carboxylate or phosphonate, for example, causes a 100–2000-fold decrease in potency [15,16]. Hydroxamate inhibitors, however, are generally affected by lack of specificity [17] due to the overwhelming contribution to binding of the hydroxamic group relative to that of the peptidomimetic moiety. In addition, they show, poor pharmacokinetic properties [18] and may cause toxic effects, in long-term treatments, due to the release of hydroxylamine, a well known carcinogenic compound [19]. Therefore, MMP inhibitors based on less potent zinc-binding functions, such as carboxylate, phosphonate and thiolate, are also currently investigated.

The pyroglutamate-containing tripeptide **1a** (Fig. 1), an endogenous MMP inhibitor isolated from snake venom [20], and its synthetic analogue **1b** were reported as carboxylate inhibitors of adamalysin II [21], a snake venom peptidyl-endopeptidase with an active site structure closely related to that of MMPs. We have recently studied tripeptide phosphonate MMP inhibitors **2a** [22] and **2b** [23] (Fig. 2), structurally related to the carboxylate inhibitors **1b** and **1a**.

The behaviour of phosphonates **2a** and **2b** was particularly interesting with respect to their binding mode in the enzyme active site. The phosphonate inhibitor **2a**, as well as its carboxylate analogue **1b**, were found to bind in the S′ region of the active site of adamalysin II, adopting a retrobinding[2] mode [24]. However, when L-Pro was introduced as the aminoterminal residue in a series of phosphonate analogues, the resulting inhibitor **2b** was found to bind in the S region of the MMP-8 active site, adopting a substrate-like binding mode [23].

The S_3 and S_3' subsites, obviously play an important role in the accommodation of **2a** and **2b** phosphonates, and their orientating effects appear to exceed even that of the S_1' primary specificity site, in the formation of **2b**:MMP-8 complex. Neither structural studies [23], nor molecular dynamic simulations [25], were able to identify a dominant interaction responsible for this change of binding mode. The unusual prevalence of the orientating effects of the S_3 and S_3' subsites over those of the S_1' subsite, can be explained on the basis of a concurrence of circumstances: i) ideal profile of the S_3 subsite for accommodation of the proline methylenes, ii) additional cation–π bonding interaction in S_3, probably involving the protonated proline NH, iii) non-bonding interactions that disfavour the arrangement of the proline residue in S_3', and iv) non-ideal alignment of the large indolyl group in the S_1' hydrophobic pocket.

In order to further study the behaviour of tripeptide phosphonates that may bind both in the S and S′ region of MMPs active site, a new series (Fig. 3) of analogues (**2c**–**2f** and **3b**–**3f**), differing in the aminoterminal residue, was synthesised and tested.

[2] Mode of binding antiparallel to that adopted by the substrate in the enzyme active site.

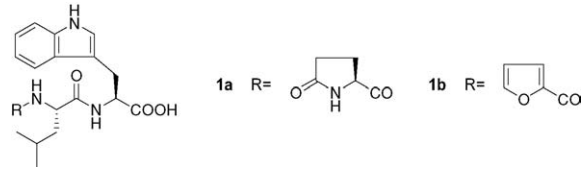


Fig. 1. Natural (**1a**) and synthetic (**1b**) carboxylate inhibitors of adamalysin II and MMPs.

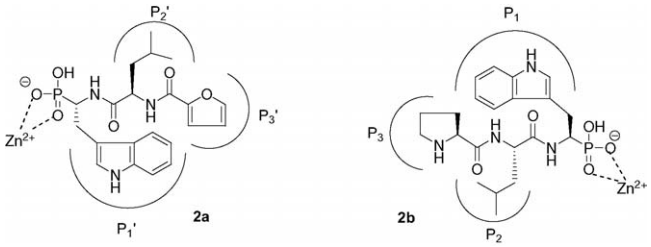
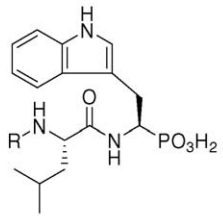


Fig. 2. Structure of the phosphonate inhibitors **2a** and **2b** and their mode of binding in the active site of adamalysin II and MMP-8, respectively.



N°	R	N°	R
2b	L-Pro	**3b**	N-Ac-L-Pro
2c	D-Pro	**3c**	N-Ac-D-Pro
2d	L-Ala	**3d**	N-Ac-L-Ala
2e	D-Ala	**3e**	N-Ac-D-Ala
2f	β-Ala	**3f**	N-Ac-β-Ala
2g	C_5H_9CO		

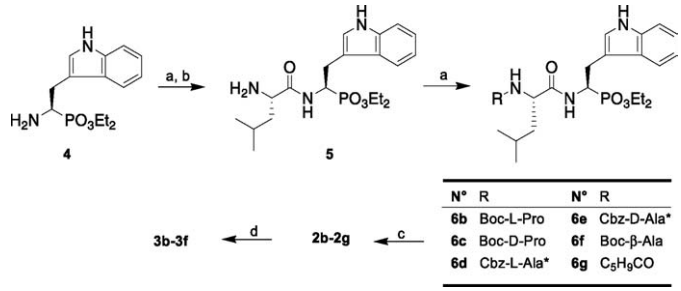
Fig. 3. New tripeptide phosphonate inhibitors of MMPs based on L-leucyl-L-phosphotryptophan.

Thus, the aminoterminal L-Pro of **2b** was replaced by Ala and β-Ala residues, as representatives of non-cyclic α and β aminoacids. Considering that the inhibitor could be accommodated either in the substrate-like orientation or in a retrobinding mode, the aminoterminal residues have been introduced both in the (*S*) and in the (*R*) configurations. The (*R*) configuration of the aminoterminal residue, in fact, was expected to decrease the ligand affinity in the S region, owing to the inversion of the substrate side chain topology in P_3, and to increase its affinity in the S′ region, due to the topology maintenance in P_3'.

Both the free aminoterminal (**2b**–**2f**) and the *N*-acetylated phosphonates (**3b**–**3f**) were prepared to evaluate the contribution of possible cation–π interactions in the S_3 subsite.

2. Chemistry

Inhibitors of the present study were prepared according to Scheme 1. Cbz-L-leucyl-L-phosphotryptophan diethylester was obtained by coupling L-phosphotryptophan diethylester (**4**) [26], with Cbz-L-leucine, in the presence of DCC and HOBt [27]. Removal of the Cbz protecting group, by Pd/C ammonium formate hydrogenolysis, gave L-leucyl-L-phospho-



Scheme 1. Synthesis of the tripeptide phosphonate inhibitors **2c**–**2f** and **3b**–**3f**, based on L-leucyl-L-phosphotryptophan. Reaction conditions: (a) Cbz-L-Leu-OH, DCC, HOBt, THF, 80–90%; (b) 10% Pd/C, $HCOONH_4$, CH_3OH, 92%; (c) TMIS, BSA, CH_2Cl_2, 49–92%; (d) Acetic anhydride, pyridine, 70–90%; *Phosphonates **6d** and **6e** required previous hydrogenolysis according to b.

tryptophan diethylester (**5**), common intermediate for preparation of the final phosphonates. The required N-protected aminoacids and the cyclopentane carboxylic acid were coupled (DCC, HOBt) with **5** to give phosphonate diethylesters **6b**–**6g**. Conversion into phosphonic acids **2b**, **2c**, **2f** and **2g** was performed by treatment with TMIS, in the presence of BSA [28], that also allows effective removal of the Boc protective group. Under these relatively mild conditions, required by the presence of the acid sensitive indolyl group, the Cbz protective group, present in the **6d** and **6e** intermediates, was partially retained. These Cbz derivatives were therefore deprotected by Pd/C ammonium formate hydrogenolysis, before treatment with TMIS. Free aminoterminal phosphonates **2b**–**2f** were finally converted into the corresponding *N*-acetyl derivatives, by acylation with acetic anhydride in pyridine. Internal salts **2b**–**2f** were purified by ion-exchange and crystallisation. Phosphonic acids **3b**–**3f** and **2g**, after purification by ion-exchange, were crystallised as cyclohexylamine salts.

3. Biochemistry

The ability of the phosphonates **2b**–**2g** and **3b**–**3f** to inhibit the enzymatic activity of MMP-2 and MMP-8, was measured by continuous fluorimetric assay, evaluating the residual enzyme activity in the presence of the fluorescent substrates QF24 and QF41 [29]. The inhibitory constants were determined according to the procedure reported in Section 6 and are expressed as K_i values (Table 1).

4. Results and discussion

4.1. Computational studies

All the inhibitors were minimised into the MMP-8 active site both as left- and right-side inhibitors. The crystallographic pose of **2b** in complex with MMP-8 was used as template to simulate the left-side inhibition, while the conformation of **2a**, as found in the crystal complex with adamalysin II, was employed as template for the right-side inhibition. The resulting complexes were qualitatively inspected, analysing how the N-terminal aminoacid replacement influences the interactions at the active site. Moreover, the inhibitors **2b** and **2c** were subjected to conformational search, with the aim of evaluating the energetic difference between the global minimum geometry of the two inhibitors and the respective poses found in the complex with MMP-8.

4.2. Discussion

Most of the new analogues, obtained by replacement of the N-terminal L-Pro in the reference phosphonate **2b** and by acetylation of amino group of the aminoterminal residue, show increased affinity for MMP-8, although none of them attained K_i lower than 260 μM.

In order to discuss the effects of the structural variations on the affinity of the new phosphonates, it would be desirable to know on which side of the active site they bind.

The mode of binding of the new inhibitors **2c**–**3f** was therefore briefly evaluated by means of computer simulations, start-

Table 1
Inhibition constants of phosphonates R-L-Leu-L-(P)Trp$(OH)_2$ against MMP-2 and MMP-8

Phosphonate		MMP-2	MMP-8	Phosphonate		MMP-2	MMP-8
Number	R	K_i (mM)[a]	K_i (mM)[a]	Number	R	K_i (mM)[a]	K_i (mM)[a]
2b	L-Pro	1.53[b]	3.94[c]	**3b**	*N*-Ac-L-Pro	1.76	0.34
2c	D-Pro	N.I.[d]	N.I.[d]	**3c**	*N*-Ac-D-Pro	N.I.[d]	N.I.[d]
2d	L-Ala	0.40	0.50	**3d**	*N*-Ac-L-Ala	0.55	0.26
2e	D-Ala	N.I.[d]	0.41	**3e**	*N*-Ac-D-Ala	3.77	2.95
2f	β-Ala	N.I.[d]	0.34	**3f**	*N*-Ac-β-Ala	0.56	0.49
2g	C_5H_9CO	0.40	1.36				

[a] Replicate determinations indicate standard deviations for the kinetic parameters less than 20%.
[b] Eleven micromolar of IC_{50} was erroneously reported previously [30].
[c] 3.2 μM IC_{50} was erroneously reported previously [30].
[d] No inhibition observed up to 1.0 mM, upper solubility limit of the inhibitor.

ing from the experimental evidence that the reference phosphonate **2b** binds in the S side of MMP-8 active site [23]. As previously reported [23,25], this orientation of the inhibitor can be explained by favourable accommodation of the terminal L-Pro pyrrolidine ring into the small hydrophobic pocket formed by the side chains of His162 and Phe 164. A possible cation–π interaction, involving the protonated pyrrolidine nitrogen and the aromatic ring of Phe164, has been also proposed for further stabilisation of the complex. In addition, the alternative binding of **2b** in the S′ side, burying the hydrophobic indolyl group of the (P)Trp residue in the large S_1' lipophilic pocket, is disfavoured by serious steric clashes in S_3', between the proline pyrrolidine ring and Tyr189 (Fig. 4). Alternative orientations of the Leu and Pro residues would prevent this unfavourable interaction, but would also increase the conformational strain of the ligand and/or reduce the favourable interactions with the protein.

Modelling results confirm these observations and suggest different preferences by replacement of the L-Pro unit and by acetylation of the N-terminal residue.

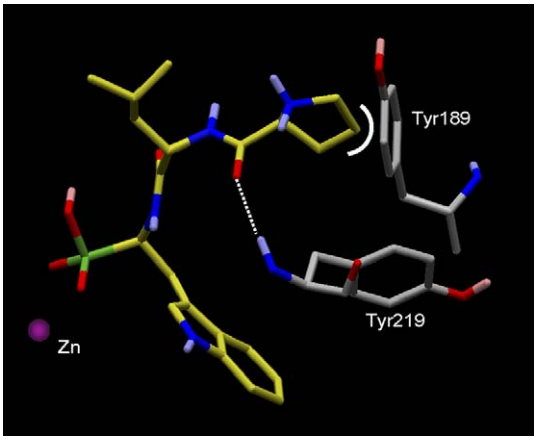


Fig. 4. Representation of the right-side MMP-8 active site complexed with **2b** (yellow) in its minimised conformation. Tyr189 and Tyr219 side chains and the catalytic zinc ion are depicted. Steric bump between proline ring and Tyr189 is highlighted. Hydrogen atoms on carbon are omitted for sake of clarity and H-bonds are represented by white dotted lines.

Acetylation of the N-terminal L-Pro of **2b** causes an about 10-fold increase of the affinity for MMP-8. The modelling of the *N*-acetyl derivative **3b** in the MMP-8 active site suggests that the orientation of the pyrrolidine ring, in the MMP-8 complex, should not be affected by *N*-acetylation and that the cation–π interaction in the **2b**:MMP-8 complex could be replaced by π–π interactions between the phenyl ring of the Phe164 and the amide group of the *N*-acetyl-proline of **3b**. The observed increase of binding affinity by L-Pro acetylation seems to indicate that the cation–π interaction, in the **2b**:MMP-8 complex, is not an important contributor to the overall binding affinity. Probably, the aromatic *N*-acetylamide π–π interaction of **3b** in S_3, due to acetylation of the N-terminal Pro residue, largely exceeds the consequent loss of the proposed cation–π interaction.

The slight increase of the binding affinity of **2g**, with respect to **2b**, is also in agreement with this hypothesis, assuming that the cyclopentyl ring could be easily accommodated in the S_3 subsite of the enzyme.

Replacement of the N-terminal L-Pro by D-Pro led, instead, to complete loss of observable inhibition. This result can be explained with the unfavourable accommodation of the D-Pro pyrrolidine ring both in S_3' and in S_3. Proper alignment of the D-Pro pyrrolidine ring, to maintain both favourable hydrophobic and cation–π interactions in the S_3 subsite, could only be achieved when the D-Pro NH is rotated toward the Ala163 (Fig. 5). In this arrangement, an additional hydrogen bond between D-Pro NH and Ala163 CO would further improve the stability of the complex, contrary to the experimental activity data. However, the internal energy of the two inhibitors **2b** and **2c** in their bound conformations was, respectively, 26 and 44 kJ mol^{-1} higher, respectively, than the energy of the global minimum geometry obtained from the conformational searches of the two compounds. The larger internal energy cost paid by **2c** to bind to the enzyme, can explain the decrease of binding affinity of **2c** with respect to **2b**.

Replacement of the N-terminal L-Pro by L-Ala, D-Ala or β-Ala leads to molecules that could preferentially be accom-

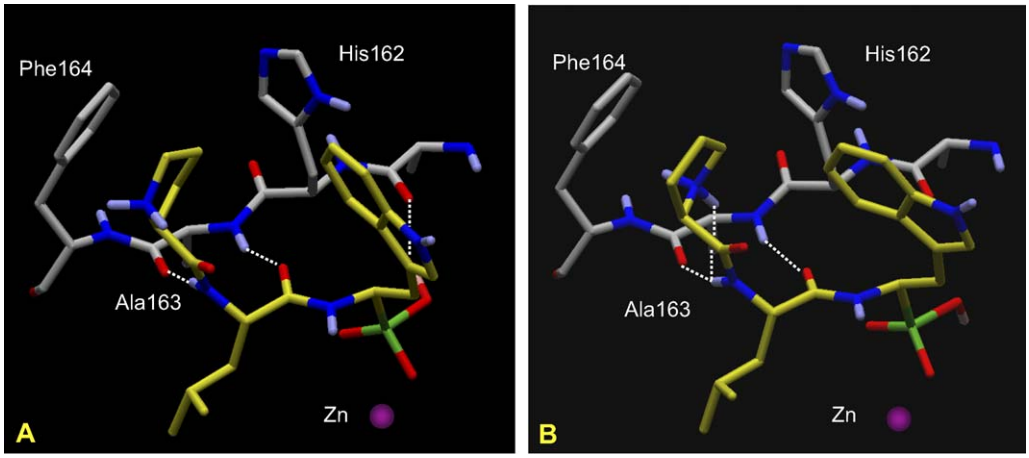


Fig. 5. Representation of the left-side MMP-8 active site complexed with **2b** (A) and **2c** (B) in their minimised conformations. Side chains of His162, Ala163, Phe164 and the catalytic zinc are depicted; π interactions between pyrrolidine ring of the ligands (yellow) and Phe164 are evident. Hydrogen atoms on carbon are omitted for sake of clarity and H-bonds are represented by white dotted lines.

modated in the S′ side of the MMP-8 active site. The smaller size, the lower hydrophobic character and the greater flexibility of the side chains of these residues, should decrease their affinity for the MMP-8 S_3 subsite and allow easier accommodation in the MMP-8 S_3' subsite [31].

Phosphonates **2d** and **3d**, containing L-Ala or *N*-acetyl-L-Ala as the N-terminal residue, show increased affinity both for MMP-2 and MMP-8. Introduction of D-Ala or β-Ala in P_3' causes an analogous increase of affinity for MMP-8, but strongly decreases MMP-2 inhibition and suggests a way to achieve selectivity between MMP-2 and MMP-8.

5. Conclusions

The present study indicates that modification of the N-terminal residue in the tripeptide phosphonate **2b** can slightly increase the affinity of the inhibitor for MMP-8 and MMP-2, and probably affects their mode of binding in the active site of MMP-8. The majority of the new analogues, containing L-Ala, D-Ala and β-Ala show increased affinity mainly for MMP-8 and probably bind in the S′ side of the MMP-8 active site, in agreement with the general remark [12] that the most potent MMP inhibitors are right-hand inhibitors. Some selectivity with respect to MMP-2 has also been attained by introduction of D-Ala or β-Ala in P3′.

6. Experimental protocols

6.1. Chemistry

Reagent grade materials from Fluka Gmbh or Aldrich Chemical and Co. were used without further purification. Silica gel 60 (230–400 mesh) for column chromatography and TLC silica gel 60 F254 plates were from Merck AG (Darmstadt, Germany). Ion-exchange resins (Dowex 50W and Amberlite IRA-68) were from Sigma-Aldrich. Melting points (Büchi B-540 melting point apparatus) are uncorrected. $[\alpha]_D$ were determined with a Perkin–Elmer 241 digital polarimeter. IR spectra were obtained with a Perkin–Elmer 1600 FT-IR spectrometer. ^{1}H-, ^{13}C- and ^{31}P-NMR spectra were recorded on a Varian VXR 300 spectrometer. Chemical shifts (δ) for ^{13}C and ^{1}H are given in ppm relative to TMS as the internal standard and the coupling constants (J) are reported in Hz. Chemical shifts (δ) for ^{31}P are given in ppm relative to phosphoric acid as the internal standard. Elemental microanalyses (C, H, N) were within ±0.4% of the calculated values.

*6.1.1. General procedure for compounds **6b–6g** and N-benzyloxycarbonyl-L-leucyl-L-phosphotryptophan diethylester*

To a solution of phosphonate diethylester **4** or **5** and the appropriate N-protected aminoacid (1 mmol) in anhydrous THF (7 ml), a solution of DCC (1.2 mmol) and HOBt (2 mmol) was added dropwise, under stirring, at 0 °C. The reaction mixture was further stirred for 1 h at 0 °C and stored for 20 h at r.t. After removal of *N*,*N*′-dicyclohexylurea by filtration, the solution was diluted with EtOAc and sequentially washed with 2 N HCl, 2 N NaOH and brine. The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to give the crude product as a slight yellow syrup, that was purified by silica gel column chromatography ($CHCl_3$/*i*PrOH 99:1). According to this procedure the following compounds were prepared.

6.1.1.1. N-*Benzyloxycarbonyl-L-leucyl-L-phosphotryptophan diethylester.* Colourless oil (92%); $[\alpha]_D = -45°$ (*c* 1; $CHCl_3$); IR (KBr) ν_{max} cm^{-1} 3286, 2960, 1695, 1450, 1236, 1036, 737, 534; ^{1}H-NMR ($CDCl_3$) δ 0.83 (6H, m, two CH_3 Leu); 1.10 (1H, m, γCH Leu); 1.27 (6H, m, two CH_3 diethylester); 1.48 (2H, m, βCH_2 Leu); 3.10 e 3.34 (2H, 2m, βCH_2 (P)Trp); 4.10 (4H, m, two CH_2 diethylester); 4.78 (2H, m, αCH Leu, αCH (P)Trp); 5.01 (2H, m, CH_2 phenyl); 6.32 (1H, d, J = 9.9 Hz, NH Leu); 6.99 (1H, d, J = 2.6 Hz, indole); 7.12 (2H, m, indole); 7.32 (7H, m, phenyl and indole); 7.60 (1H, d, J = 7.7 Hz, NH (P)Trp); 7.77 (1H, s, NH indole).

6.1.1.2. N-tert-*Butoxycarbonyl-L-prolyl-L-leucyl-L-phosphotryptophan diethylester (**6b**).* Colourless oil (88%); $[\alpha]_D = -78°$ (*c* 1; $CHCl_3$); IR (KBr) ν_{max} cm^{-1} 3294, 2974, 1673, 1395, 1232, 1023, 737, 534; ^{1}H-NMR ($CDCl_3$) δ 0.83 and 0.85 (6H, m, two CH_3 Leu); 1.63 (24H, m, two CH_3 diethylester, γCH Leu, βCH_2 Leu; $C(CH_3)_3$, three CH_2 Pro); 3.11 and 3.36 (3H, two m, βCH_2Trp, αCHPro); 4.11 (4H, m, two CH_2 diethylester); 4.32 (1H, m, αCH Leu,); 4.78 (1H, m, αCH (P)Trp); 6.59 (1H, s, NH Leu); 6.87 (1H, s, NH (P)Trp); 7.31 (5H, m, indole); 8.47 (1H, s, NH indole); ^{31}P-NMR ($CDCl_3$) δ 25.08. Anal. $C_{30}H_{47}N_4O_7P.H_2O$ (C, H, N).

6.1.1.3. N-tert-*Butoxycarbonyl-D-prolyl-L-leucyl-L-phosphotryptophan diethylester (**6c**).* Colourless oil (92%); $[\alpha]_D = -18°$ (*c* 1; $CHCl_3$); IR (KBr) ν_{max} cm^{-1} 3286, 2965, 1673, 1412, 1235, 1023, 737, 534; ^{1}H-NMR ($CDCl_3$) δ 0.86 (6H, m, two CH_3 Leu); 1.55 (24H, m, two CH_3 diethylester, $C(CH_3)_3$, γCH Leu, βCH_2 Leu, three CH_2 Pro); 3.08 and 3.35 (3H, two m, βCH_2 (P)Trp, αCH Pro); 4.11 (4H, m, two CH_2 diethylester); 4.37 (1H, m, αCH Leu); 4.73 (1H, m, αCH (P)Trp); 6.31 and 6.45 (2H, 2s, NH Leu and NH (P)Trp); 7.33 (5H, m, indole); 8.72 (1H, s, NH indole) ^{31}P-NMR ($CDCl_3$) δ 25.11; Anal. $C_{30}H_{47}N_4O_7P$ (C, H, N).

6.1.1.4. N-*Benzyloxycarbonyl-L-alanyl-L-leucyl-L-phosphotryptophan diethylester (**6d**).* Colourless oil (92%); $[\alpha]_D = -6°$ (*c* 1; $CHCl_3$); IR(KBr) ν_{max} cm^{-1} 3285, 3062, 2951, 1697, 1654, 1448, 1234, 1036, 967, 736; ^{1}H-NMR ($CDCl_3$) δ 0.81 and 0.84 (6H, two d, J = 5.3 Hz, two CH_3 Leu; 1.25 (11H, m, two CH_3 diethylester, βCH_2 Leu, CH_3 Ala); 1.52 (1H, m, γCH Leu); 3.22 (2H, m, βCH_2 (P)Trp), 4.10 (5H, m, two CH_2 diethylester and αCH Leu), 4.41 (1H, m, αCH Ala), 4.77 (1H, m, αCH (P)Trp); 5.1 (2H, two d, J = 11.9 Hz, CH_2

Ph); 5.31 (1H, d, *J* = 7.0 Hz, NH Ala); 6.29 (1H, d, *J* = 7.5 Hz, NH Leu); 6.7 (1H, d, *J* = 10.1 Hz, NH (P)Trp); 6.99 and 7.11 (2H, two s, indole); 7.33 (7H, m, phenyl and indole); 7.57 (1H, d, *J* = 7.0 Hz, indole); 8.34 (1H, s, NH indole). Anal. $C_{31}H_{43}N_4O_7P.H_2O$ (C, H, N).

6.1.1.5. N-*Benzyloxycarbonyl-D-alanyl-L-leucyl-L-phosphotryptophan diethylester (**6e**).* White solid (85%); $[\alpha]_D = -67°$ (*c* 1; $CHCl_3$); IR (KBr) ν_{max} cm^{-1} 3277, 3058, 2957, 1699, 1657, 1446, 1235, 1032, 965, 737; ^{1}H-NMR ($CDCl_3$) δ 0.82 and 0.86 (6H, two d, *J* = 6.6 Hz, two CH_3 Leu); 1.21 (11H, m, βCH_2 Leu, two CH_3 diethylester, CH_3 Ala); 1.54 (1H, m, γCH Leu); 3.06 (2H, m, βCH_2 (P)Trp); 4.08 (5H, m, two CH_2 diethylester, αCH Leu); 4.57 (1H, m, αCH Ala); 4.81 (1H, m, αCH (P)Trp); 5.12 (2H, two d, *J* = 11.9 Hz, CH_2Ph); 6.06 (1H, d, *J* = 9.2 Hz, NH Ala); 6.89 (1H, s, indole); 6.96 (1H, s, NH Leu); 7.09 (2H, m, indole); 7.30 (6H, m, phenyl and indole); 7.51 (1H, d, indole); 7.73 (1H, s, NH (P)Trp); 9.05 (1H, s, NH indole); Anal. $C_{31}H_{43}N_4O_7P$ (C, H, N).

6.1.1.6. N-tert-*Butoxycarbonyl-β-alanyl-L-leucyl-L-phosphotryptophan diethylester (**6f**).* Colourless oil (80%); $[\alpha]_D = -75°$ (*c* 1; $CHCl_3$); IR (KBr) ν_{max} cm^{-1} 3284, 2958, 1640, 1236, 1142, 1041, 737; ^{1}H-NMR ($CDCl_3$) δ 0.83 (6H, m, two CH_3 Leu); 1.37 (18H, m, two CH_3 diethylester, $(CH_3)_3C$, γCH Leu, βCH_2 Leu) 2.19 (2H, t, *J* = 6.0 Hz, αCH_2 β-Ala); 3.11 and 3.35 (4H, two m, βCH_2 (P)Trp, βCH_2 β-Ala); 4.18 (5H, m, two CH_2 diethylester and αCH Leu); 4.75 (1H, m, αCH (P)Trp); 5.48 (1H, s, NH β-Ala) 5.61 (1H, d, *J* = 8.7 Hz, NH Leu); 6.37 (1H, d, *J* = 10.2 Hz, NH (P)Trp); 7.34 (5H, m, indole); 8.84(1H, s, NH indole). Anal. $C_{28}H_{45}N_4O_7P$ (C, H, N).

*6.1.1.7. Cyclopentylcarbonyl-L-leucyl-L-phosphotryptophan diethylester (**6g**).* Pale yellow oil (64%); $[\alpha]_D = -53°$ (*c* 1; $CHCl_3$); IR (KBr) ν_{max} cm^{-1} 3282, 3060, 2951, 1647, 1229, 1024, 735; ^{1}H-NMR ($CDCl_3$) δ 0.86 (6H, m, two CH_3 Leu); 1.27 (8H, m, two CH_3 diethylester, βCH_2 Leu); 1.57 (9H, m, γCH Leu, four CH_2 cyclopentyl); 2.3 (1H, m, αCH cyclopentyl); 3.13 and 3.35 (2H, two m, βCH_2 (P)Trp); 4.08 (4H, m, two CH_2 diethylester); 4.39 (1H, m, αCH Leu); 4.77 (1H, m, αCH (P)Trp); 5.55 (1H, d, *J* = 8.4 Hz, NH Leu); 6.50 (1H, d, *J* = 9.9 Hz, NH (P)Trp); 7.14 (3H, m, indole); 7.32 (1H, d, *J* = 8.3 Hz, indole); 7.61 (1H, d, *J* = 7.9 Hz, indole); 8.27 (1H, s, NH indole); ^{31}P-NMR ($CDCl_3$) δ 25.0; Anal. $C_{26}H_{40}N_3O_5P \cdot 1/2H_2O$ (C,H,N).

*6.1.2. General procedure for Cbz deprotection of compounds **6d**, **6e** and* N-*benzyloxycarbonyl-L-leucyl-L-phosphotryptophan diethylester*

To a solution of the benzyloxycarbonyl derivative (1 mmol) in MeOH (10 ml) ammonium formate (4 mmol) and 10% Pd/C (100 mg) were added. After stirring for 3 h at r.t., the reaction mixture was filtered and evaporated under reduced pressure. A solution of the residue in EtOAc was washed with 2 N Na_2CO_3 and brine, dried over Na_2SO_4 and evaporated to give the expected amine, which was employed without further purification. According to this procedure the following compounds were prepared.

*6.1.2.1. L-Leucyl-L-phosphotryptophan diethylester (**5**).* Pale yellow oil (90%); $[\alpha]_D = -12°$ (*c* 1; $CHCl_3$); IR (KBr) ν_{max} cm^{-1} 3294, 3025, 1657, 1226, 1023, 737, 534; ^{1}H-NMR ($CDCl_3$) δ 0.77 (6H, m, two CH_3 Leu); 1.28 (9H, m, βCH_2 Leu, γCH Leu, two CH_3 diethylester); 1.58 (2H, s, NH_2 Leu); 3.24 (3H, m, αCH Leu, βCH_2 (P)Trp), 4.15 (4H, m, two CH_2 diethylester); 4.77 (1H, m, αCH (P)Trp), 7.11 (3H, m, indole); 7.33 (1H, d, *J* = 7.5 Hz, indole); 7.55 (1H, s, NH (P)Trp); 7.6 (1H, d, *J* = 7.8 Hz, indole); 8.21 (1H, s, NH indole); ^{31}P-NMR ($CDCl_3$) δ 25.72. Anal. $C_{20}H_{32}N_3O_4P$ (C, H, N).

6.1.2.2. L-Alanyl-L-leucyl-L-phosphotryptophan diethylester. Pale yellow oil (88%); $[\alpha]_D = -61°$ (*c* 1; MeOH); IR (KBr) ν_{max} cm^{-1} 3286, 3058, 2957, 1657, 1530, 1446, 1226, 1032, 965, 737; ^{1}H-NMR ($CDCl_3$) δ 0.82 and 0.85 (6H, two d, *J* = 6.4 Hz, two CH_3 Leu); 1.15 (3H, d, *J* = 7.0 Hz, CH_3 Ala); 1.41 (11H, m, two CH_3 diethylester, γCH Leu, βCH_2 Leu and NH_2 Ala); 3.04 (1H, q, *J* = 7.0 Hz, αCH Ala); 3.23 (2H, m, βCH_2 (P)Trp); 4.04 (4H, m, two CH_2 diethylester); 4.29 (1H, m, αCH Leu); 4.80 (1H, m, αCH (P)Trp); 6.67 (1H, d, *J* = 10.0 Hz, NH (P)Trp); 7.13 (3H, m, indole); 7.23 (1H, d, *J* = 8.2 Hz, NH Leu); 7.31 (1H, d, *J* = 7.0 Hz, indole); 7.61 (1H, d, *J* = 7.0 Hz, indole); 8.3 (1H, s, NH indole).

6.1.2.3. D-Alanyl-L-leucyl-L-phosphotryptophan diethylester. Pale yellow solid (95%); m.p. 129.9–131.7 °C; $[\alpha]_D = -74°$ (*c* 1; MeOH); IR (KBr) ν_{max} cm^{-1} 3269, 3067, 2957, 1649, 1547, 1230, 1047, 1026, 966, 741; ^{1}H-NMR (DMSO-d_6) δ 0.79 and 0.83 (6H, two d, *J* = 6.6 Hz, two CH_3 Leu); 1.07 (3H, d, *J* = 7.0 Hz, CH_3 Ala); 1.2 (6H, m, two CH_3 diethylester) 1.31 (2H, m, βCH_2 Leu); 1.46 (1H, m, γCH Leu) 2.9 and 3.21 (5H, m, βCH_2 (P)Trp, γCH Leu, NH_2 Ala); 3.99 (4H, m, two CH_2 diethylester); 4.34 (2H, m, αCH Leu, αCH (P)Trp); 7.00 (3H, m, indole); 7.29 (1H, d, *J* = 8.1 Hz, indole); 7.46 (1H, d, *J* = 7.7 Hz, indole); 7.87 (1H, d, *J* = 8.1 Hz, NH Leu); 8.34 (1H, d, *J* = 9.5 Hz, NH (P)Trp); 10.83 (1H, s, NH indole).

*6.1.3. General procedure for compounds **2b–2g***

A solution of the phosphonate diethylester (1 mmol) in anhydrous CH_2Cl_2 (5 ml) and bistrimethylsilylacetamide (BSA; 12 mmol) was stored under nitrogen, for 1 h at r.t. After cooling at –20 °C, trimethylsilyl iodide (8 mmol) was added dropwise, via syringe, under stirring. After 20 min at 0 °C and 1 h at r.t., the solvent was removed under reduced pressure to give a brown oily residue of the phosphonate trimethylsilylester that was hydrolysed by treatment with 7:3 CH_3CN/H_2O (5 ml). After evaporation of the solvents under reduced pressure, the residue was dissolved in water and decolourised by addition of solid Na_2SO_3 and 2 N HCl. The solution was passed through a column containing

10 equiv. of a Dowex 50W sulphonic resin and the phosphonic acids recovered by elution with 2 N NH_4OH. Evaporation of the ammonia solutions and recrystallisation yielded phosphonates **2b**–**2f** as analytically pure compounds. According to this procedure the following compounds were prepared.

*6.1.3.1. L-Prolyl-L-leucyl-L-phosphotryptophan (**2b**).* White solid (77%); m.p. 258.1–259.7 °C (MeOH/Et_2O) $[\alpha]_D = -114°$ (*c* 1; NH_4OH 2 N); IR (KBr) ν_{max} cm^{-1} 3335, 3257, 2955, 1645, 1552, 1133, 1071, 737, 551, ^{1}H-NMR (D_2O) δ 0.60 and 0.64 (6H, two d, *J* = 6.4 Hz, two CH_3 Leu); 1.17 (3H, m, βCH_2 Leu, γCH Leu); 1.46, 1.75 and 2.05 (4H, three m, β and γ CH_2 Pro); 2.77 and 3.17 (4H, two m, βCH_2 (P)Trp and δCH_2 Pro); 4.03 (2H, m, αCH Leu, αCH Pro); 4.15 (1H, m, αCH (P)Trp); 7.25 (5H, m, indole). Anal. $C_{21}H_{31}N_4O_5P$ (C, H, N).

*6.1.3.2. D-Prolyl-L-leucyl-L-phosphotryptophan (**2c**).* White solid (88.5%); m.p. 230.4–235.6 °C (dec.) (MeOH/Et_2O); $[\alpha]_D = -82°$ (*c* 1; NH_3 2 N); IR (KBr) ν_{max} cm^{-1} 3291, 2953, 1657, 1544, 1152, 1062, 738, 557; ^{1}H-NMR (DMSO-d_6) δ 0.71 and 0.78 (6H, two d, *J* = 6.4 Hz, two CH_3 Leu); 1.30 (3H, m, βCH_2 Leu, γCH Leu); 1.95 and 2.35 (4H, two m, β and γ CH_2Pro); 3.37 (4H, m, βCH_2 (P)Trp, δCH_2 Pro); 4.05 (1H, m, αCH Pro); 4.32 (1H, tl, αCH Leu); 4.48 (1H, m, αCH (P)Trp); 7.32 (5H, m, indole). Anal. $C_{21}H_{31}N_4O_5P$ (C, H, N).

*6.1.3.3. L-Alanyl-L-leucyl-L-phosphotryptophan (**2d**).* White solid (58%); m.p. 226.5–227.2 °C (H_2O/MeOH); $[\alpha]_D = -89°$ (*c* 1; NH_3 2 N); IR (KBr) ν_{max} cm^{-1} 3316, 3240, 3079, 2957, 1648, 1541, 1150, 913, 745, 538; ^{1}H-NMR (D_2O) δ 0.59 and 0.64 (6H, two d, *J* = 6.6 Hz, two CH_3 Leu); 1.07 (3H, m, γCH Leu, βCH_2 Leu); 1.19 (3H, d, *J* = 7.1 Hz, CH_3 Ala); 2.99 (2H, m, βCH_2 (P)Trp); 3.8 (1H, m, αCH Ala); 4.03 (1H, m, αCH Leu); 4.2 (1H, m, αCH (P)Trp); 7.0 (3H, m, indole); 7.3 (1H, d, *J* = 7.9 Hz, indole); 7.5 (1H, d, *J* = 7.5 Hz, indole); Anal. $C_{19}H_{29}N_4O_5P{\cdot}5/2H_2O$ (C, H, N).

*6.1.3.4. D-Alanyl-L-leucyl-L-phosphotryptophan (**2e**).* White solid (54%); m.p. 225.7–228.0 °C (MeOH/EtOAc); $[\alpha]_D = -84°$ (*c* 1; NH_3 2 N); IR (KBr) ν_{max} cm^{-1} 3395, 3176, 2957, 1648, 1395, 1150, 914, 737, 551, 526; ^{1}H-NMR (D_2O) δ 0.54 and 0.6 (6H, two d, *J* = 6.3 Hz, two CH_3 Leu); 0.79 (2H, tl, βCH_2 Leu) 1.14 (1H, m, γCH Leu); 1.30 (3H, d, *J* = 7.2 Hz, CH_3 Ala); 2.83 and 3.22 (2H, two m, βCH_2 (P)Trp); 3.92 (2H, m, αCH Ala, αCH Leu); 4.24 (1H, m, αCH (P)Trp); 7.27 (5H, m, indole); Anal. $C_{19}H_{29}N_4O_5P$ (C, H, N).

*6.1.3.5. β-Alanyl-L-leucyl-L-phosphotryptophan (**2f**).* White solid (77%); m.p. 226.2–227.7 °C (dec.) (MeOH/EtOAc); $[\alpha]_D = -103°$ (*c* 1; NH_3 2 N); IR (KBr) ν_{max} cm^{-1} 3404, 3286, 2957, 1640, 1539, 1142, 1041, 737, 551; ^{1}H-NMR (D_2O) δ 0.54 and 0.6 (6H, two d, *J* = 6.6 Hz, two CH_3 Leu); 0.84 (2H, m, βCH_2 Leu); 1.13 (1H, m, γCH Leu); 2.46 (2H, m, αCH_2 β-Ala); 2.82 and 3.21 (2H, two m, βCH_2 (P)Trp); 3.02 (2H, m, βCH_2 β-Ala); 3.90 (1H, m, αCH Leu); 4.23 (1H, m, αCH (P)Trp); 7.26 (5H, m, indole); Anal. $C_{19}H_{29}N_4O_5P{\cdot}2H_2O$ (C, H, N).

*6.1.3.6. Cyclopentylcarbonyl-L-leucyl-L-phosphotryptophan cyclohexylamine salt (**2g**).* The general procedure was modified as follows: the residue, recovered from the CH_3CN/H_2O solution, was dissolved in EtOAc, and sequentially washed with a 1:1 mixture of 3% Na_2SO_3/2 N HCl and brine and dried over Na_2SO_4. After removal of the solvent under reduced pressure the phosphonate was crystallised as cyclohexylamine salt (93%); m.p. 248.1–251.5 °C (MeOH/EtOAc); $[\alpha]_D = -69°$ (*c* 1; MeOH); IR (KBr) ν_{max} cm^{-1} 3289, 2937, 2858, 1636, 1539, 1451, 1035; ^{1}H-NMR (DMSO-d_6) δ 0.73 and 0.77 (6H, two d, *J* = 6.6 Hz, two CH_3 Leu); 1.49 (21H, m, four CH_2 cyclopentyl, five CH_2 cyclohexylamine, γCH Leu βCH_2 Leu); 2.61 (1H, t, *J* = 7.5 Hz, αCH cyclohexylamine); 2.72 and 3.21 (3H, two m, βCH_2 (P)Trp, αCH cyclopentyl); 3.97 (1H, m, αCH Leu); 4.21 (1H, m, αCH (P)Trp); 6.97 (3H, m, indole); 7.25 (1H, d, *J* = 7.5 Hz, indole); 7.31 (1H, d, *J* = 8.1 Hz, NH (P)Trp); 7.47 (1H, d, *J* = 7.5 Hz, indole); 8.16 (1H, d, *J* = 8.1 Hz, NH Leu); 10.6 (1H, s, NH indole); Anal. $C_{28}H_{45}N_4O_5P$ (C, H, N).

*6.1.4. General procedure for compounds **3b**–**3f***

To a stirred solution of tripeptide phosphonate (1 mmol) in pyridine (10 ml), acetic anhydride (10 ml) was added dropwise, at 0 °C, under stirring. After 20 h at r.t., the volatiles were removed under reduced pressure and the residue taken up in a 1:1 MeOH/H_2O mixture. The solution was passed through a column containing 10 equiv. of Amberlite IRA-68 weakly basic resin. The phosphonic acid retained by the resin was recovered by elution with 2 N NH_4OH and evaporation of the ammonia solution. Addition of cyclohexylamine to a solution of the crude residue in MeOH and evaporation of the solvent gave the pure cyclohexylamine phosphonates **3b**–**3f**, that were recrystallised from MeOH/EtOAc. According to this procedure the following compounds were prepared.

6.1.4.1. N-*Acetyl-L-prolyl-L-leucyl-L-phosphotryptophan cyclohexylamine salt (**3b**).* White solid (41%); m.p. 264.1–264.7° (dec.) (EtOAc); $[\alpha]_D = -33°$ (*c* 0.69; MeOH); IR (KBr) ν_{max} cm^{-1} 3291, 2940, 1637, 1544, 1042, 727, 556; ^{1}H-NMR (CD_3OD) δ 0.84 (6H, m, two CH_3 Leu); 1.64 (17H, m, γCH Leu, βCH_2 Leu, five CH_2 cyclohexylamine, β and γCH_2 Pro); 2.05 (3H, s, CH_3CO); 3.01 and 3.45 (5H, two m, βCH_2 (P)Trp, CH cyclohexylamine, δCH_2 Pro); 4.34 (2H, m, αCH Leu, αCH Pro); 5.00 (1H, m, αCH (P)Trp); 7.29 (5H, m, indole); ^{31}P-NMR (CD_3OD) δ 18.85. Anal. $C_{29}H_{46}N_5O_6P$ (C, H, N).

6.1.4.2. N-*Acetyl-D-prolyl-L-leucyl-L-phosphotryptophan cyclohexylamine salt (**3c**).* White solid (90%); m.p. 239.3–244.0 °C (dec.) (EtOAc); $[\alpha]_D = -16°$ (*c* 0.6; MeOH); IR (KBr) ν_{max} cm^{-1} 3284, 2940, 1637, 1551, 1400, 1042; ^{1}H-NMR (CD_3OD) δ 0.82 (6H, m, two CH_3 Leu); 1.59 (17H,

m, γCH Leu, βCH$_2$ Leu, five CH$_2$ cyclohexylamine, two CH$_2$ Pro); 2.11 (3H, s, CH$_3$CO) 3.02 and 3.51 (5H, two m, βCH$_2$ (P)Trp, CH cyclohexylamine, δCH$_2$ Pro); 4.34 (3H, m, αCH Leu, αCH Pro, αCH (P)Trp); 7.29 (5H, m, indole). Anal. $C_{29}H_{46}N_5O_6P$ (C, H, N).

6.1.4.3. N-*Acetyl-L-alanyl-L-leucyl-L-phosphotryptophan cyclohexylamine salt (**3d**).* White solid (98%); m.p. 246.3–247.2 °C (MeOH/EtOAc); $[\alpha]_D = -78°$ (*c* 1; MeOH); IR (KBr) ν_{max} cm^{-1} 3286, 2931, 1640, 1539, 1142, 1041, 914, 737, 551; ^{1}H-NMR (D$_2$O) δ 0.62 (6H, two d, J = 6.6 Hz, two CH$_3$ Leu); 1.31 (16H, m, five CH$_2$ cyclohexylamine, CH$_3$ Ala, γCH Leu, βCH$_2$ Leu), 1.8 (3H, s, CH$_3$CO); 2.80 (1H, m, αCH cyclohexylamine); 2.97 and 3.22 (2H, two m, βCH$_2$ (P)Trp); 4.00 (2H, m, αCH Ala, αCH Leu); 4.18 (1H, m, αCH (P)Trp); 7.02 (3H, m, indole); 7.29 (1H, d, J = 7.9 Hz, indole); 7.56 (1H, d, J = 7.5 Hz, indole); Anal. $C_{27}H_{44}N_5O_6P{\cdot}2H_2O$ (C, H, N).

6.1.4.4. N-*Acetyl-D-alanyl-L-leucyl-L-phosphotryptophan cyclohexylamine salt (**3e**).* White solid (82%); m.p. 210–216.3 °C (dec.) (EtOAc); $[\alpha]_D = -25°$ (*c* 1; MeOH); IR (KBr) ν_{max} cm^{-1} 3277, 2940, 1632, 1539, 1146, 1040, 720; ^{1}H-NMR (CD$_3$OD) δ 0.78 and 0.83 (6H, two d, J = 6.6 Hz, two CH$_3$ Leu); 1.54 (16H, m, γCH Leu, βCH$_2$ Leu, CH$_3$ Ala, five CH$_2$ cyclohexylamine); 2.00 (3H, s, CH$_3$CO); 3.05 (3H, m, βCH$_2$ (P)Trp, αCH cyclohexylamine); 3.4 (1H, m, αCH Ala); 4.24 (1H, m, αCH Leu); 4.37 (1H, m, αCH (P)Trp); 7.00 (3H, m, indole); 7.27 (1H, d, J = 7.5 Hz, indole); 7.6 (1H, d, J = 7.2 Hz, indole); ^{31}P-NMR (CD$_3$OD) δ 18.49; Anal. $C_{27}H_{44}N_5O_6P{\cdot}3H_2O$ (C, H, N).

6.1.4.5. N-*Acetyl-β-alanyl-L-leucyl-L-phosphotryptophan cyclohexylamine salt (**3f**).* White solid (81%); m.p. 238.2–244.2 °C (dec.) (MeOH/EtOAc); $[\alpha]_D = -53°$ (*c* 1; MeOH); IR (KBr) ν_{max} cm^{-1} 3285, 2939, 1631, 1547, 1040, 720, 534; ^{1}H-NMR (CD$_3$OD) δ 0.79 and 0.83 (6H, two d, J = 6.6 Hz, two CH$_3$ Leu); 1.60 (16H, m, five CH$_2$ cyclohexylamine, γCH Leu, βCH$_2$ Leu, CH$_3$CO) 2.37 (2H, t, J = 6.4 Hz, αCH$_2$ β-Ala); 3.01 and 3.33 (5H, two m, βCH$_2$ (P)Trp, βCH$_2$ β-Ala, CH cyclohexylamine); 4.22 (1H, m, αCH Leu); 4.41 (1H, m, αCH (P)Trp); 7.28 (5H, m, indole); Anal. $C_{27}H_{44}N_5O_6P{\cdot}H_2O$ (C, H, N).

6.2. Molecular modelling

All molecular modelling calculations were performed using the software packages MacroModel 7.1 [32], running on Silicon Graphics Octane R12000 workstation. The Amber* [33] united atom force field was used for all the calculations.

Models of MMP-8 complexed with the compounds **2c**–**3f** as left-hand inhibitors were constructed based on the crystal structure of the related complex **2b**:MMP-8 (entry 1i73 in the Protein Data Bank). The L-Pro aminoacid of the crystallographic inhibitor was replaced by the corresponding residues of the synthesised analogues.

To simulate the right-hand-side inhibition, the previously cited crystal structure of the complex **2b**:MMP-8 (entry 1i73 in the Protein Data Bank), was considered. As a template for the binding geometry to the enzyme, the Fur-L-Leu-L-(P)Trp(OH)$_2$ (**2a**) was used as found in the crystallographic complex with the adamalysin II (entry 4aig in the Protein Data Bank); to obtain a correct position of the **2a** inhibitor into the MMP-8 active site, the two complexes **2b**:MMP-8 and **2a**:adamalysin were aligned by superimposing the zinc ion and the three His residues. As for the left-side simulation, the furane ring was replaced to obtain the inhibitors tested in this article.

All the crystallographic water molecules were removed from the MMP-8 crystal structure. Hydrogen atoms were added on the heteroatoms and on the aromatic rings, as suggested by the MacroModel Batchmin User Manual. Charges on the zinc ion were corrected as suggested by Guida et al. [34]. All ligands were used in their deprotonated forms with negatively charged phosphonates.

The enzyme structure was relaxed without inhibitors before complexes evaluations, with a 10,000 iterations of Polak–Ribière Conjugate Gradient minimisations until the derivative convergence was 0.01 kcal Å^{-1} mol. An energy constraint of 400 kJ Å^{-2} mol was applied to the main chain to avoid large structural modifications.

The phosphonate function was kept in the crystallographically observed position, by fixing the distances between the phosphonate oxygen atoms and the zinc ion and applying an energy constraint of 500 kcal Å^{-2} mol, to avoid rearrangement of the zinc coordination.

Minimisations were performed using the truncated Newton conjugate gradient (TNCG) algorithm until the derivative convergence was 0.001 kJ Å^{-1} mol, the extended non-bonded treatment and the GB/SA water solvation model implemented in MacroModel. The inhibitors were free to minimise, while all the residues in a 10 Å range from the inhibitor were fixed in the 3D space with an energy restraint of 200 kcal Å^{-2} mol, even if their non-bonded interactions with all the relaxing atoms were calculated. The same protocol was applied for all the simulations.

The Batchmin Monte Carlo multiple minimum (MCMM) methodology was chosen to carry out conformational searches on **2b** and **2c**. The conformational space of the two structures was explored by random changes in the torsional angles of all the rotatables bonds, as given by the TORS command.

All the conformations obtained were subsequently minimised with the TNCG algorithm to a derivative convergence of 0.01 kJ Å^{-1} mol.

6.3. Enzyme assay and determination of the inhibition constants

Inhibition of MMP-2 and MMP-8 has been determined at 25 °C in Tris–HCl 50 mM pH 7.5, CaCl$_2$ 5 mM, Brij 0.05%, NaN$_3$ 0.02% continuously monitoring the hydrolysis of the fluorogenic substrates QF24 (0.20 μM) and QF41 (5.00 μM)

[19] by MMP-2 and MMP-8, respectively, after 5 min incubation in the presence of the inhibitors. Reactions were started by addition of the substrates in the cuvette, under continuous stirring. The hydrolysis was followed by measuring the increase in relative fluorescence at 393 nm (excitation at 328 nm) due to the formation of product. The highest enzyme concentration used in the assays was always lower than 3.8×10^{-10} M. Under these conditions, low enzyme concentration and substrate concentration lower than the K_m values, the substrate consumption is negligible but well measurable, and the reaction proceeds linearly for at least 1500 s. The rate of enzymatic substrate hydrolysis was determined in the 200–1400 s time interval. Data analysis has been performed assuming a reversible inhibition with rapid binding of the enzyme to the inhibitor.

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