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Simultaneous presence of unsaturation and long alkyl chain at P_1' of Ilomastat confers selectivity for gelatinase A (MMP-2) over gelatinase B (MMP-9) inhibition as shown by molecular modelling studies

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Abstract—Structural analogues of Ilomastat (Galardin®), containing unsaturation(s) and chain extension carrying bulky phenyl group or alkyl moieties at P_1' were synthesized and purified by centrifugal partition chromatography. They were analyzed for their inhibitory capacity towards MMP-1, MMP-2, MMP-3, MMP-9 and MMP-14, main endopeptidases involved in tumour progression. Presence of unsaturation(s) decreased the inhibitory potency of compounds but, in turn increased their selectivity for gelatinases. **2b** and **2d** derivatives with a phenyl group inhibited preferentially MMP-9 with IC_{50} equal to 45 and 38 nM, respectively, but also display activity against MMP-2 (IC_{50} equal to 280 and 120 nM, respectively). Molecular docking computations confirmed affinity of these substances for both gelatinases. With aims to obtain a specific gelatinase A (MMP-2) inhibitor, P_1' of Ilomastat was modified to carry one unsaturation coupled to an alkyl chain with pentylidene group. Docking studies indicated that MMP-2, but not MMP-9, could accommodate such substitution; indeed **2a** proved to inhibit MMP-2 (IC_{50} = 123 nM), while displaying no inhibitory capacity towards MMP-9.
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1. Introduction

Matrix metalloproteinases (MMPs), a family of zinc endopeptidases, exhibit a wide spectrum of physiological and pathological activities. They are involved in connective tissue remodelling but their action is not solely directed towards matrix constituents since recent investigations pin-pointed their pivotal function in the processing of bioactive molecules on cytokines and growth factors.[1] MMPs, overexpression in most solid tumours has been correlated with high grade status and is often associated with poor prognosis; thus, a myriad of MMP inhibitors has been synthesized and then used as potential therapeutic agents to limit tumour progression. In most instances, results from clinical trials were rather disappointing. Such pharmacological failure might have several origins. It might be due to (i) delay in compound administration since recent data revealed that MMPs were mainly involved in early steps of cancer progression,[2] (ii) important side effects,[3] and (iii) lack of selectivity of inhibitors[4] in keeping with the recent classification of MMPs as being either target or antitarget in tumours.[3a] Since decades, gelatinase A (MMP-2) has been considered as one main MMP directing the invasiveness of breast, lung or colon carcinoma as well as melanoma.[5]

Keywords: Matrix metalloproteinases; Hydroxamate inhibitors; Centrifugal partition chromatography; Enzyme inhibition; Molecular modelling; Docking.
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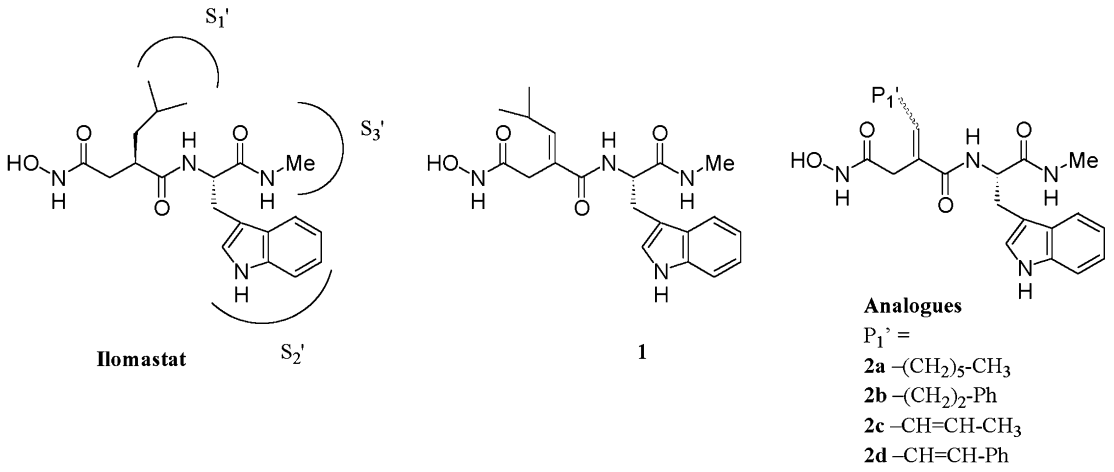


Figure 1. Modification of P'_1 substituted Ilomastat; analogue **1** with isobutylene group at P'_1 and analogues **2a–d** with one or two *E*-unsaturations, flexible alkyl chain or bulky block-like phenyl groups.

The S'_1 subsite, also designated as the MMP specificity pocket, is the most prominent subsite within MMP catalytic domain and differences in its size and shape among MMPs suggest that it acts as a main determinant in driving inhibitor selectivity.[6–8]

In a previous preliminary investigation we synthesized Ilomastat (Galardin®) analogues featuring an isobutylene group at P'_1; although such derivative was less potent as compared to its unmodified counterpart, it proved to display greater selectivity for MMP-2 inhibition as compared to MMP-3 (Fig. 1).[9]

This work was here pursued with aims to increase the selectivity of such compounds for gelatinase A (MMP-2). For that purpose, P'_1 site of Ilomastat was modified to contain one or two *E*-unsaturations, flexible alkyl chain or bulky block-like phenyl groups. All substances were purified by centrifugal partition chromatography (CPC) and analyzed in parallel by docking studies and for inhibitory capacity towards several MMPs.

2. Results and discussion

2.1. Chemistry and purification of compounds

The access to the targeted molecules with different P'_1 substitutions required the preparation of alkylidenesuccinic monoesters (Scheme 1).

The strategy as described by Marcq et al.[9] has been used with some modifications. A Wittig reaction, using the process developed by Mc Combie et al.,[10] was carried out to prepare in one-pot different alkyl/arylidenesuccinate esters. The phosphorane generated in situ by reaction between the dimethyl maleate **3** and tri-*n*-butyl phosphine was reacted with different aldehydes **4** to yield the dimethyl alkylidene succinates **5** as an *E*/*Z* mixture. Structures of alkylidene succinates and ratios of *E* and *Z* isomers were determined by ^{1}H NMR spectroscopy (Table 1).[11]

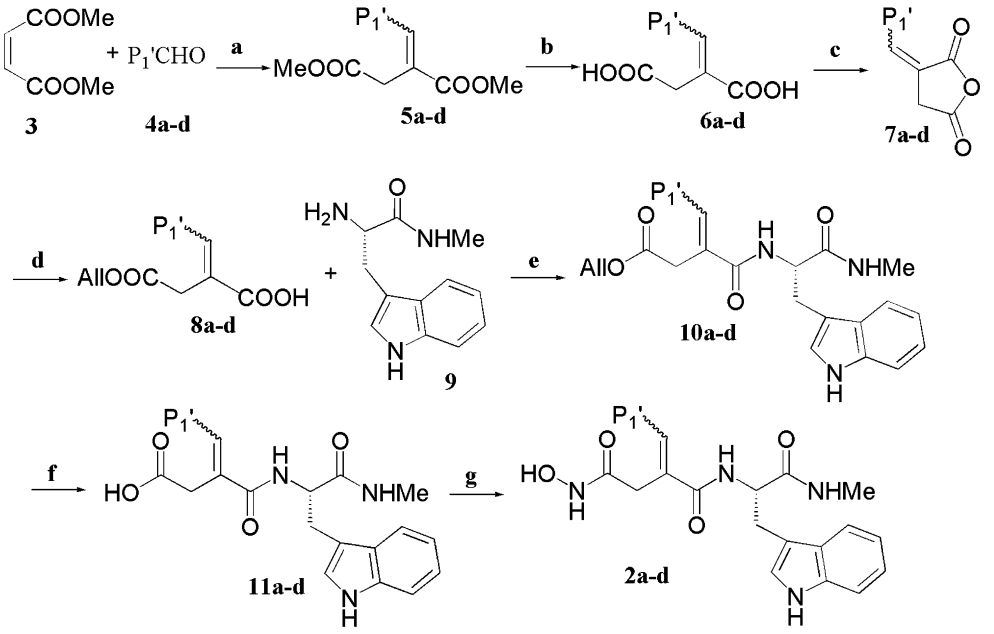
The *E*/*Z* ratios calculated from data provided by NMR spectra integrations show that major compounds consisted of *E*-isomers. This stereoselectivity could be explained by the stabilization of the *erythro* intermediate by the attractive group which can be isomerized to the *threo* intermediate, the latter leading to less steric hindrance (Scheme 2a).

Diesters **5** were saponified and the resulting diacids **6** were dehydrated, thus leading to the anhydrides **7** (*E*-isomer as major compound). Finally, refluxing in allyl alcohol provided the monoallyl esters **8** as the single *E*-isomer.[12]

Coupling of (*L*)-tryptophan methylamide[13] with monoallyl esters **8** mediated by DMTMM[14] gave the succinyltryptophanamides **10**. After deprotection of the allyl esters **10** with tetrakis (triphenylphosphine) palladium (0), the free acids **11** were reacted with hydroxylamine hydrochloride in the presence of DMTMM as coupling reagent to convert them directly into the pure *E*-hydroxamic acids **2**. The coupling reaction between hydroxylamine hydrochloride and acid compounds **11** was not as successful as attempted. We obtained very low yield of hydroxamic acids **2** (4–13%), a cyclisation reaction occurred during this step to give cyclic compounds **12** (Scheme 2b). These compounds seemed to be the most stable form of this kind of molecules.

These compounds were purified by centrifugal partition chromatography (CPC).[15] CPC is a non-solid-support, liquid–liquid chromatography that uses two immiscible solvents (or solutions) prepared from an equilibrated liquid–liquid biphasic system. One phase is kept stationary by a constant centrifugal field, whereas the other liquid is pumped through it, thus playing the role of a mobile phase. The analytes are separated according to their partition coefficient.

This type of chromatography is now widely used for preparative separations of fragile biomolecules. Indeed, it avoids problems related to interaction and/or irreversible adsorption of the injected material to a solid



Scheme 1. Synthesis of hydroxamic acids **2a**–**d**. Reagents and conditions: (a) aldehyde, $(n\text{-Bu})_3P$, THF, 25 °C, 5 days, 85–97%; (b) NaOH (10%), 25 °C, 4 days, 56–92%; (c) AcCl, 52 °C, 3 h, 100%; (d) AllOH, 110 °C, 16 h, 30–97%; (e) DMTMM, THF, 25 °C, 16 h, 52–69%; (f) $Pd(PPh_3)_4$, morpholine, THF, 25 °C, 30 min., 53–74%; (g) DMTMM, NMM, $NH_2OH{\cdot}HCl$, THF, 25 °C, 16 h, 4–13%.

Table 1. Reaction conditions of alkylidene succinate **5a**–**d** formation and the ratio of the *E*/*Z* mixture

Compound	P_1'	Time (days)	Yield (%)	*E*/*Z* (%)[a]
5a	$-(CH_2)_5-Me$	7	97	80/20
5b	$-(CH_2)_2-Ph$	12	97	92/8
5c	–CH=CH–Me	4	85	*E* > 95
5d	–CH=CH–Ph	12	97	*E* > 95

[a] For the compounds with different P_1' groups, the ratio of the *E*/*Z* mixture of the alkylidene succinates **5a**–**d** is expressed in %.

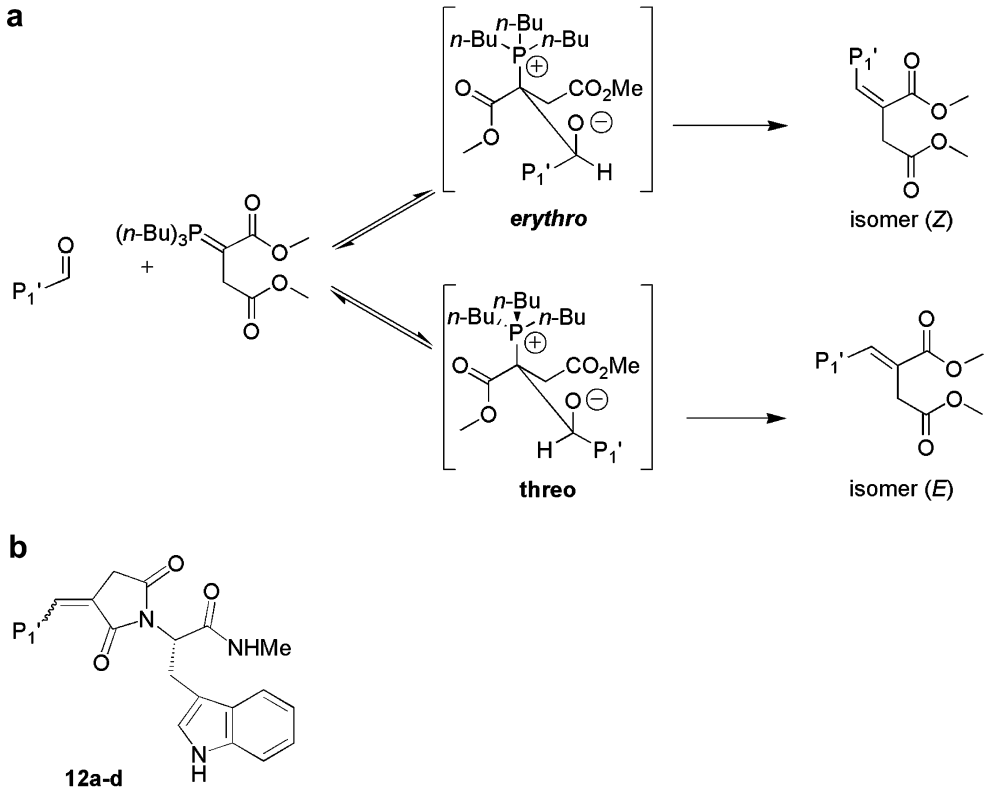
support as it might occur with the hydroxamic acid moiety of the target molecules. Moreover, it avoids any problem due to saturation of the stationary phase, allowing CPC to be efficient in preparative separation. The biphasic systems have been selected according the homogeneous scale approach using the ARIZONA solvent scale.[16] The biphasic systems composing this scale are prepared by mixing heptane, ethyl acetate, methanol and water in suitable proportions (see below Section 4).

The synthesized compounds were structurally studied using Electronic Circular Dichroism (ECD) spectroscopy. A CD spectrum is the summation of the optical activities of all chiral groups composing the molecule. ECD spectra are usually divided into 'near' and 'far' UV regions. The near UV in the 250–300 nm wavelength range is often described as the aromatic region. The far UV region (<250 nm) is dominated by the electronic transitions of the peptidic bonds. Our studied compounds are peptide-like backbone and all of them possess the same aromatic amino acid residue, that is, tryptophan. Thus, ECD spectra were recorded in the wavelength range 200–300 nm (Fig. 2). The main obtained result is that the CD spectra obtained from the five studied compounds can be divided into two well-defined groups. On the one hand, the first group corresponds to analogues **1**, **2a** and **2b**. Their spectra are characterized by a broad positive band with a maximum at 232 nm corresponding to the $\pi\pi^*$ peptidic bond electronic transition. On the other hand, the second group corresponds to analogues **2c** and **2d**. Their spectra present a shifted narrower band at 228 nm corresponding to the $\pi\pi^*$ peptidic bond electronic transition. Two additional negative bands can also be observed at 239 and 215 nm. ECD spectra below 250 nm are generally difficult to be interpreted, as being the result of both overlapped optically active group transitions and conformational lability.

So, the interpretation of these ECD spectra is rather complicated due to the presence of several chromophores (peptidic bond, tryptophan amino acid, diene group, etc.). Moreover, the lack of CD bibliography data for these compounds makes hazardous the trying to explain all the observed bands.

2.2. MMP inhibition and molecular docking studies

S_1' subsites of MMPs present several structural and chemical differences, properties which were taken into considerations with aims to design inhibitors with improved selectivity. In a previous study, we synthesized an Ilomastat analogue featuring an isobutylidene group at this subsite which proved to display greater selectivity for MMP-2 over MMP-3.[9] This was here confirmed (Table 2). However, this compound (**1**) was shown to inhibit MMP-1, with shallow S_1' pocket, and MMP-9 and MMP-14, with deep S_1' pocket, to



Scheme 2. (a) Stereoselectivity of the alkylidene succinates **5a**–**d**. Both transition states are described to provide isomer *Z* or *E*. (b) Structure of the cyclic compounds **12a**–**d**.

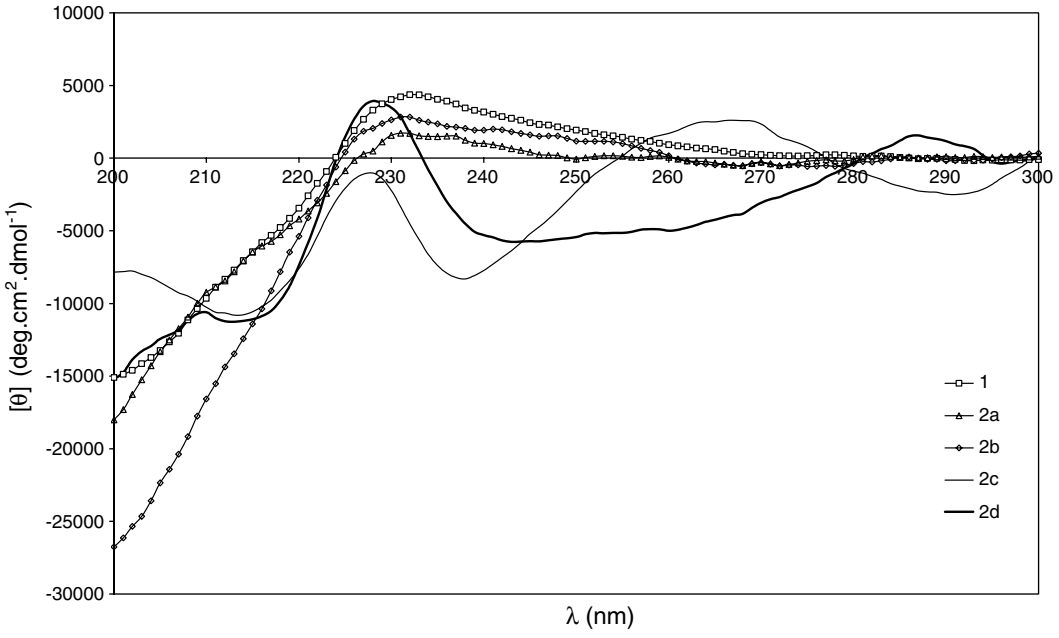


Figure 2. ECD spectra, in the UV wavelength range 200–400 nm, of Ilomastat analogue (compound **1**) and of hydroxamic acids **2a**–**d** in aqueous solution (at 25 °C). The data are expressed in terms of molecular ellipticity [θ] (deg cm^2 dmol^{-1}).

similar extent as MMP-2. Also, introducing a second unsaturation decreased the inhibitory potential of the corresponding compound (**2c**) towards all MMPs (Table 2).

S_1' pockets of MMP-2 and MMP-9 have relatively good affinity with the aromatic amino acid located at P_1' position. As described in the literature, increasing the length of the chain to phenylpropyl derivative leads to com-

Table 2. Tests in vitro of hydroxamic acids **1**, **2a–d** for inhibition of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-14 using specific quenched fluorescent substrates

Compound	P'_1	MMP-1	MMP-2	MMP-3	MMP-9	MMP-14
Ilomastat		1.5	1.1	1.9	0.5	13.4
1	–*i*-Pr	18	9.2	179	17	10
2a	$-(CH_2)_5-Me$	32,000	123	$>10^4$	$>10^4$	2660
2b	$-(CH_2)_2-Ph$	9130	280	1920	45	53,100
2c	–CH=CH–Me	984	78,500	8610	974	913
2d	–CH=CH–Ph	1240	120	21,000	38	2490

IC_{50} values are expressed in nM.

pounds with improved activity for MMP-2 and MMP-9.[17,18]

We first analyzed the influence of aromatic group at P'_1 in the presence of one ($(CH_2)_2$–Ph) or two (CH=CH–Ph) unsaturations by docking computations with MMP-2 and MMP-9. In a general sense, lowest energy models obtained by molecular docking computations between inhibitors and these two MMPs are characterized by:

(i) the zinc chelation by hydroxamic acid group,
(ii) four H-bonds implicating the inhibitor backbone and the MMP main chain,
(iii) favourable steric interactions with the S'_1 pocket of MMP.

Molecular docking shows all the same H-bonds: the P'_1 carbonyl H-bonds with the NH of Leu181, the Trp (P'_2) NH with the carbonyl of Pro238, the Trp (P'_2) carbonyl with the NH of Tyr240 and the terminal NH with the carbonyl of Gly179. The H-bond distances between acceptor and donor atoms are close to those observed in the MMP-9/reverse hydroxamate inhibitor complex.[19] Thus the studied inhibitors strongly interact with the second half of the upper rim (Gly179 → Leu181) and the lower rim (Pro238 → Tyr240) of the active site. In fact, the inhibitors form a short β-strand complementary to the active site upper rim in an anti-parallel way and in a parallel way with the active site lower rim. This kind of interaction is similar to the one observed experimentally with reverse MMP hydroxamate inhibitors[19–21] and with TIMP–MMP complexes.[22]

The introduction of an unsaturation at P'_1 leads to a decrease of inhibitor's flexibility and imposes a favourable three-dimensional direction to penetrate into the S'_1 site of gelatinases but not MMP-1.

The analogue **2d** is only different from the analogue **2b** by the presence of a second unsaturation in P'_1 chain. These two analogues have a good affinity for both MMP-2 and MMP-9. The shape and the depth of their S'_1 pockets are sufficient to allow the entrance of the P'_1 chain with aromatic group (Fig. 3). Surprisingly, no π-stacking interaction is observed between aromatic groups of analogues and aromatic amino acids of MMPs. The second unsaturation in the P'_1 chain (analogue **2d**) seems to have no influences on the affinity of interaction. The positions of aromatic and alkyl groups (i.e., the alkyl groups linking aromatic groups and the main chain) of these two analogues in the MMPs' S'_1 pocket are not the same ones. No common structural conformation, at atomic level, of the P'_1 chain can be highlighted by our molecular docking computations. The interactions between P'_1 chain and S'_1 pocket in these analogue–MMP complexes seem to be mainly due to hydrophobic forces.

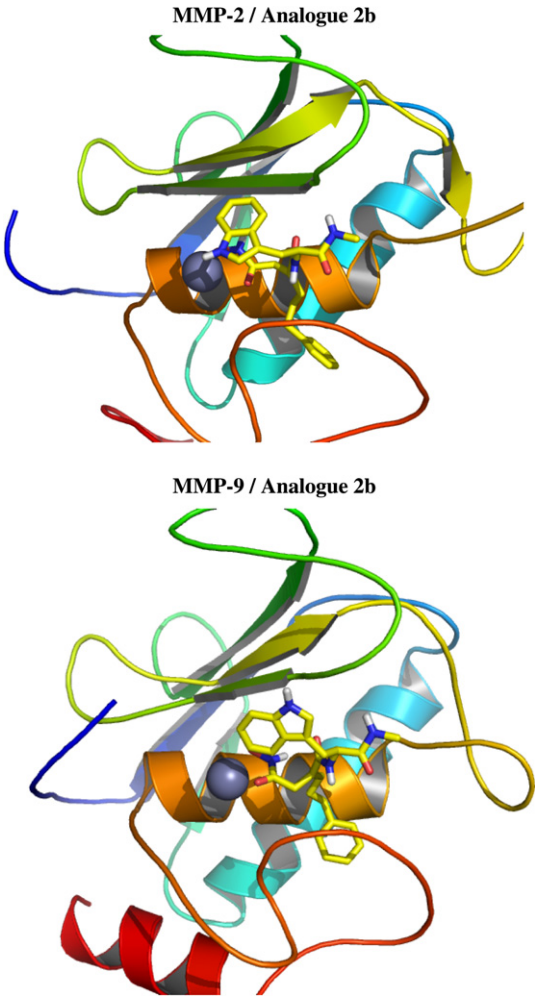


Figure 3. Molecular dockings of analogue **2b** into MMP-2 and MMP-9. The P'_1 chain is inserted in the P'_1 pocket of MMP-2 and MMP-9. MMPs are drawn in cartoon representation, with rainbow colours and the analogue **2b** in sticks.

Such favourable docking between phenyl containing side chain and gelatinases has been validated by kinetic studies (Table 2). Interestingly, compounds **2b** and/or **2d**

inhibit gelatinase B (MMP-9) with IC_{50} values much lower (2–3 log orders of difference) than those determined for MMP-1, MMP-3 or MMP-14. However, differences in inhibitory potency towards MMP-2 and MMP-9 are far less important. Therefore, either **2b** or **2d** compounds would probably be unable to discriminate between these two gelatinases in any in vivo situation.

Although S'_1 pockets of MMP-2 and MMP-9 are both described as a tunnel more or less accessible to solvents, their conformations are different. The S'_1 pocket of MMP-9, at the end of the tunnel, is restrained, like a funnel, by the residues Glu233, Arg241, Thr246 and Pro247.

Data from the literature also indicated that MMP-2 at S'_1 could accommodate long alkyl chain, with major effect observed with heptyl chain.[23–25] Accordingly, we synthesized compound **2a**.

Analysis of docking studies clearly shows that compound **2a** is unable to bind favourably MMP-1 and MMP-9. All the models obtained for these MMPs show unrealistic structures and/or wrong locations at their MMP surface, also associated with higher energies than those obtained for the computations with analogues **2b** and **2d**. The molecular docking computations for the analogue **2a** are energetically favourable for MMP-2. The lowest energy model obtained is reported in Figure 4. The hydroxamic acid group chelates the zinc atom in the MMP-2 active site. The interaction model is stabilized by four H-bonds linking the analogue **2a** and the MMP-2 through the following amino acids: Gly179, Leu181, Pro238 and Tyr240 (Fig. 5). The P'_1 chain is seen inserted in the S'_1 pocket of the MMP-2. This model is shown very similar to those obtained from the theoretical docking calculations with the analogues **2d** and **2b** and also close to the experimental crystallographic structures published previously.[19–22] The P'_1 chain for

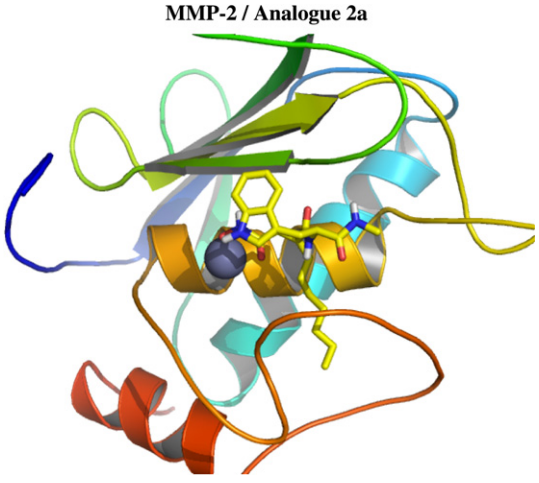


Figure 4. Molecular docking of analogue **2a** into MMP-2. The P'_1 chain is inserted in the P'_1 pocket of MMP-2. On the left: cartoon representation of the analogue **2a** (yellow) and the MMP (in cyan). On the right: solvent accessible surface showing the residues (red) implicated in hydrogen bond with the analogue **2a** (yellow).

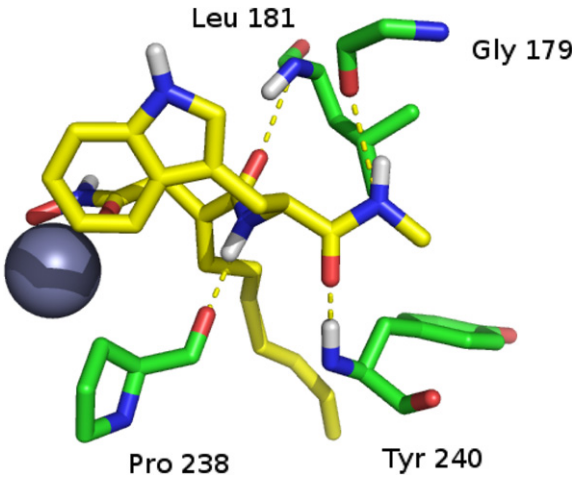


Figure 5. Molecular docking of analogue **2a** into MMP-2: analogue **2a** is coloured in yellow, MMP residues forming hydrogen bonds with inhibitors in green, hydrogen bonds in yellow dashes and the zinc atom in grey.

the analogue **2a** is the longest one among the inhibitors tested in this study. The S'_1 pocket of MMP-2 is the only one sufficiently deep to accept this kind of P'_1 chain. The alkyl groups of the P'_1 chain do not show specific interactions with the residues constituting the S'_1 pocket of MMP-2. Such as the analogues **2b** and **2d**, the analogue **2a** binds to MMP-2 with hydrophobic interactions between its P'_1 chain and the S'_1 pocket of MMP-2.

Parallely, **2a** displays selective inhibitory capacity towards MMP-2 with IC_{50} = 123 nM. At a concentration as high as 5 μM this compound has no influence, to any extent, on the activity of either MMP-1, MMP-3, MMP-14 or importantly MMP-9 (Table 2); however, it has to be delineated that **2a** could inhibit MMP-8 and MMP-13 with 101 and 122 nM IC_{50} values, respectively. Such data are consistent with molecular modelling showing that S'_1 pocket of those MMPs could accommodate, similarly as MMP-2, P'_1 chain of **2a** (not shown).

3. Conclusion

The presence of one unsaturation and long alkyl chain at P'_1 of Ilomastat leads to a substance that preferentially inhibits gelatinase A (MMP-2), one pivotal target enzyme in cancer invasiveness.

Potency and selectivity of this inhibitor might be improved by further modification of alkyl chain at P'_1 or/and substitutions of the indole group at P'_2.

4. Experimental

4.1. General synthetic methods and materials

Solvents were dried by standard procedures and redistilled under N_2 prior to use. All organometallic reactions were run under N_2, and pure products were obtained after flash chromatography using Merck Geduran SI

silica gel 60 (70–230 mesh ASTM). Mass spectra were recorded with a MSQ (mass spectroscopy quadrupolar) spectrometer (ESI (electrospray ionization) or APCI (atmospheric pressure chimic ionization)). NIR-FT spectra (NaCl film or powder in KBr pellet) were acquired using a Perkin Elmer BX spectrometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker AC 300 spectrometer in $CDCl_3$ with TMS as internal standard, or in DMSO-d_6, and chemical shifts are expressed in parts per million (δ). Melting points were determined with a Reichert Thermovar hot-stage apparatus and are uncorrected.

4.1.1. General procedure for the preparation of dimethyl alkylidene succinates (5a–d). Tri-*n*-butylphosphine (1.4 equiv) was added slowly by syringe to a stirred solution of methyl maleate **3** (1 equiv) and aldehyde **4** (1 equiv) in dry THF (1 mL per 3 mmol of aldehyde) at room temperature under nitrogen and stirring was continued for 5 days. The mixture was diluted with CH_2Cl_2 (15 mL) and stirred for 0.5 h with the addition of H_2O_2 (35% aqueous) to oxidise any remaining *n*-Bu_3P. The mixture was stirred for 0.5 h. The organic phase was washed with water, a solution of $NaHCO_3$ (1 N), dried over $MgSO_4$ and evaporated. The crude product was purified by flash chromatography (CH_2Cl_2) to yield dimethyl alkylidene succinate **5** as a yellow oil.

4.1.1.1. (2*E*) Dimethyl-2-heptylidenesuccinate (5a). Ratio: *Z*/*E*: 20/80; yield = 97%. ^{1}H NMR ($CDCl_3$) δ ppm; *Z* isomer: 0.81 (t, *J* = 6.40 Hz, 3H, CH_3), 1.22 (m, 6H, CH_2), 1.37 (m, 2H, CH_2CH_2CH), 2.12 (dt, *J* = 7.16 Hz, *J* = 14.69 Hz, 2H, CH_2CH), 3.29 (s, 2H, CH_2COO), 3.66–3.67 (2s, 6H, CH_3OCO), 6.00 (t, *J* = 7.53 Hz, 1H, *CH*=); *E* isomer: 0.81 (t, *J* = 6.40 Hz, 3H, CH_3), 1.22 (m, 6H, CH_2), 1.37 (m, 2H, CH_2CH_2CH), 2.12 (dt, *J* = 7.16 Hz, *J* = 14.69 Hz, 2H, CH_2CH), 3.29 (s, 2H, CH_2COO), 3.66–3.67 (2s, 6H, CH_3OCO), 6.90 (t, *J* = 7.53 Hz, 1H, *CH*=). ^{13}C NMR ($CDCl_3$) δ ppm; *Z* isomer: 13.9; 22.3; 28.2; 28.7; 29.7; 31.4; 39.8; 51.2; 125.0; 148.0; 167.2; 171.1; *E* isomer: 13.9; 22.3; 28.2; 28.7; 29.7; 31.4; 31.9; 51.2; 125.0; 145.9; 167.2; 171.1. IR (film): *v* (cm^{-1}): 2960, 2927, 2861, 1750, 1717, 1437, 1280, 1206, 1173, 1009, 778. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 260 (80) $[M+18]^+$, 219 (100).

4.1.1.2. (2*E*) Dimethyl-2-(3-phenylpropylidene)succinate (5b). Ratio: *Z*/*E*: 8/92; yield = 97%. ^{1}H NMR ($CDCl_3$) δ ppm; *Z* isomer: 2.50 (q, *J* = 7.72 Hz, 2H, $PhCH_2CH_2CH$), 2.75 (t, *J* = 8.04 Hz, 2H, $PhCH_2CH_2$), 3.27 (s, 2H, CH_2COOMe), 3.65–3.73 (2s, 6H, MeOCO), 6.10 (t, *J* = 7.53 Hz, 1H, CH=), 7.17–7.31 (m, 5H, H aromatic); *E* isomer: 2.50 (q, *J* = 7.72 Hz, 2H, $PhCH_2CH_2CH$), 2.75 (t, *J* = 8.04 Hz, 2H, $PhCH_2CH_2$), 3.27 (s, 2H, CH_2COOMe), 3.65–3.73 (2s, 6H, MeOCO), 7.01 (t, *J* = 7.53 Hz, 1H, CH=), 7.17–7.31 (m, 5H, H aromatic). ^{13}C NMR ($CDCl_3$) δ ppm; *Z* isomer: 30.7; 34.4; 39.8; 51.8; 51.9; 125.7–128.4 (6C); 140.6; 146.4; 167.1; 171.0; *E* isomer: 30.7; 31.9; 34.4; 51.8; 51.9; 125.7–128.4 (6C); 140.6; 144.6; 167.1; 171.0. IR (film): *v* (cm^{-1}): 3024, 2958, 2859, 1748, 1723, 1443, 1287, 1204, 1180, 1114, 1064, 1023, 751, 702. MS APCI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 260 (27) $[M-2H]^+$, 235 (100), 219 (80).

4.1.1.3. (2*E*) Dimethyl-2-[(2*E*)-but-2-en-1-ylidene]succinate (5c). Ratio: *Z*/*E*: 5/95; yield = 85%. ^{1}H NMR ($CDCl_3$) δ ppm; 1.88 (d, *J* = 5.65 Hz, 3H, CH_3), 3.44 (s, 2H, CH_2), 3.69–3.76 (2s, 6H, CH_3OCO), 6.22 (dd, 1H, $CHCHCH$), 6.26 (dq, 1H, CH_3CH), 7.33 (d, *J* = 10.55 Hz, 1H, CH=). ^{13}C NMR ($CDCl_3$) δ ppm; 18.9; 39.8; 51.7; 121.4; 123.8; 126.5; 140.6; 167.8; 171.2. IR (film): *v* (cm^{-1}): 2954, 1736, 1439, 1250, 1209. MS APCI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% formic acid): 219 (100) $[M+23-2]^+$.

4.1.1.4. (2*E*) Dimethyl-2-[(2*E*)-3-phenylprop-2-en-1-ylidene]succinate (5d). Ratio: *Z*/*E*: 5/95; yield = 97%. ^{1}H NMR ($CDCl_3$) δ ppm; 3.55 (s, 2H, CH_2COOMe), 3.68–3.76 (2s, 6H, MeOCO), 6.96 (m, 1H, $PhCHCHCH$), 7.25–7.35 (m, 5H, H aromatic), 7.46 (d, *J* = 6.64 Hz, 1H, $PhCHCHCH$), 7.51 (d, *J* = 8.82 Hz, 2H, $PhCHCHCH$). ^{13}C NMR ($CDCl_3$) δ ppm; 32.4; 52.0; 52.1; 122.7–128.7 (6C); 129.1; 135.9; 141.2; 141.3; 167.6; 171.1. IR (film): *v* (cm^{-1}): 3408, 2963, 2387, 1728, 1670, 1456, 1226, 1020, 748. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 260 (52) $[M]^+$.

4.1.2. General procedure for the preparation of alkylidene succinic acids (6a–d). Dimethyl alkylidene succinate **5** was saponified with a solution of NaOH (10%, 1 mL per mmol of compound) for 4 days. The reaction mixture was cooled to 0 °C and acidified with HCl to pH 1. A white solid appeared; it was collected by suction, washed with hexane and dried to give the diacid compound **6**.

4.1.2.1. (2*E*)-2-Heptylidenesuccinic acid (6a). Ratio: *Z*/*E*: 9/91; yield = 92%. ^{1}H NMR (CD_3OD) δ ppm; *Z* isomer: 1.00 (t, *J* = 6.89 Hz, 3H, CH_3), 1.41 (m, 6H, CH_2), 2.54 (m, 2H, CH_2CH_2CH), 2.59 (q, *J* = 7.30 Hz, 2H, CH_2CH), 3.30 (s, 2H, CH_2COO), 6.09 (t, *J* = 7.53 Hz, 1H, CH=); *E* isomer: 1.00 (t, *J* = 6.89 Hz, 3H, CH_3), 1.41 (m, 6H, CH_2), 2.31 (m, 2H, CH_2CH_2CH), 2.20 (q, *J* = 7.54 Hz, 2H, CH_2CH), 3.41 (s, 2H, CH_2COO), 6.90 (t, *J* = 7.53 Hz, 1H, CH=). ^{13}C NMR (CD_3OD) δ ppm; *Z* isomer: 14.4; 23.6; 29.5; 30.1; 30.2; 32.9; 42.2; 128.1; 146.0; 171.6; 175.5; *E* isomer: 14.4; 23.6; 29.5; 30.1; 30.6; 32.7; 32.9; 128.1; 145.0; 171.6; 175.5. IR (film): *v* (cm^{-1}): 2923, 2858, 1705, 1639, 1573, 1433, 1301, 1235, 939.

4.1.2.2. (2*E*)-2-(3-Phenylpropylidene)succinic acid (6b). Ratio: *Z*/*E*: 8/92; yield = 92%. ^{1}H NMR (CD_3OD) δ ppm; *Z* isomer: 2.49 (q, *J* = 7.56 Hz, 2H, $PhCH_2CH_2$), 2.76 (t, *J* = 7.37 Hz, 2H, $PhCH_2CH_2$), 3.22 (s, 2H, CH_2COOMe), 6.09 (t, *J* = 7.15 Hz, 1H, CH=), 7.15–7.20 (m, 5H, H aromatic); *E* isomer: 2.49 (q, *J* = 7.56 Hz, 2H, $PhCH_2CH_2$), 2.76 (t, *J* = 7.37 Hz, 2H, $PhCH_2CH_2$), 3.22 (s, 2H, CH_2COOMe), 6.96 (t, *J* = 7.53 Hz, 1H, CH=), 7.15–7.20 (m, 5H, H aromatic). ^{13}C NMR (CD_3OD) δ ppm; *Z* isomer: 31.8; 35.5; 40.1; 126.9-129.4 (6C); 142.3; 146.3; 170.7; 175.0; *E* isomer: 31.8; 33.0; 35.5; 126.9–129.4 (6C); 142.3; 145.5; 170.7; 175.0. IR (film): *v* (cm^{-1}): 3020, 2543, 1695, 1653, 1423, 1299, 1225, 937, 698. MS APCI(−)

(*m*/*z*), 50/50 H_2O/CH_3CN (0.1% imidazole): 233 (45) [M−H], 189 (100), 112 (96).

4.1.2.3. (2*E*)-2-[(2*E*)-But-2-en-1-ylidene]succinic acid (6c). Ratio: *Z*/*E*: 5/95; yield = 56%. ^{1}H NMR (CD_3OD) δ ppm; 1.86 (d, *J* = 7.91 Hz, 3H, CH_3), 3.40 (s, 2H, CH_2), 6.24 (m, 1H, CH*CH*CH), 6.40 (m, 1H, CH_3*CH*), 7.33 (d, *J* = 10.55 Hz, 1H, CH=). ^{13}C NMR (CD_3OD) δ ppm; 19.0; 32.9; 123.7; 128.0; 141.2; 142.7; 171.0; 174.9. IR (film): *v* (cm^{-1}): 3490, 3012, 2592, 1711, 1678, 1637, 1431, 1316, 1233, 954, 937, 764, 616.

4.1.2.4. (2*E*)-2-[(2*E*)-3-Phenylprop-2-en-1-ylidene]succinic acid (6d). Ratio: *Z*/*E*: 5/95; yield = 86%. ^{1}H NMR (CD_3OD) δ ppm; 3.55 (s, 2H, *CH*$_2$COOH), 6.97 (m, 1H, PhCH*CH*CH), 7.25–7.35 (m, 5H, H aromatic), 7.49 (d, *J* = 6.64 Hz, 1H, Ph*CH*CHCH), 7.55 (d, *J* = 7.61 Hz, 1H, PhCHCH*CH*). ^{13}C NMR (CD_3OD) δ ppm; 33.2; 124.3–129.8 (6C); 130.1; 137.6; 142.3; 142.6; 170.8; 175.0. IR (film): *v* (cm^{-1}): 3032, 2563, 1707, 1665, 1624, 1435, 1287, 1237, 974, 933, 751, 686. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 231 (100) $[M-H]^+$, 220 (27), 110 (20).

4.1.3. General procedure for the preparation of alkylidene succinic anhydrides (7a–d). Alkylidene succinic acid **6** was stirred with freshly distilled acetyl chloride (2 mL per mmol of diacid) under reflux for 3 h. Acetic acid and the excess of acetyl chloride were distilled under vacuo. The anhydride **7** was obtained as yellow oil and used directly for the next step without any other purification.

4.1.3.1. (2*E*)-2-Heptylidenesuccinic anhydride (7a). Ratio: *Z*/*E*: 9/91; yield = 100%. ^{1}H NMR ($CDCl_3$) δ ppm; *Z* isomer: 0.89 (t, *J* = 6.78 Hz, 3H, CH_3), 1.30 (m, 6H, CH_2), 1.44 (m, 2H, *CH*$_2$CH$_2$CH), 2.23 (dt, *J* = 7.16 Hz, *J* = 14.69 Hz, 2H, *CH*$_2$CH), 3.50 (s, 2H, CH_2COO), 6.49 (t, *J* = 7.53 Hz, 1H, CH=); *E* isomer: 0.89 (t, *J* = 6.78 Hz, 3H, CH_3), 1.30 (m, 6H, CH_2), 1.44 (m, 2H, *CH*$_2$CH$_2$CH), 2.23 (dt, *J* = 7.16 Hz, *J* = 14.69 Hz, 2H, *CH*$_2$CH), 3.50 (s, 2H, CH_2COO), 7.05 (t, *J* = 7.53 Hz, 1H, CH=). ^{13}C NMR ($CDCl_3$) δ ppm; *Z* isomer: 13.9; 22.4; 27.6; 28.5; 29.1; 31.4; 34.5; 122.4; 150.3; 164.9; 168.5; *E* isomer: 13.9; 22.4; 27.6; 28.5; 29.1; 31.4; 31.7; 122.4; 146.1; 164.9; 168.5. IR (film): *v* (cm^{-1}): 2925, 2854, 1841, 1775, 1705, 1458, 1423, 1401, 1269, 1229, 1154, 1110, 956, 899, 776, 718, 661.

4.1.3.2. (2*E*)-2-(3-Phenylpropylidene)succinic anhydride (7b). Ratio: *Z*/*E*: 8/92; yield = 100%. ^{1}H NMR (CD_3OD) δ ppm; *Z* isomer: 2.54 (q, *J* = 7.38 Hz, 2H, PhCH$_2$*CH*$_2$), 2.84 (t, *J* = 7.37 Hz, 2H, Ph*CH*$_2$CH$_2$), 3.17 (s, 2H, *CH*$_2$COOMe), 6.45 (t, *J* = 7.9 Hz, 1H, CH=), 7.15–7.20 (m, 5H, H aromatic); *E* isomer: 2.54 (q, *J* = 7.38 Hz, 2H, PhCH$_2$*CH*$_2$), 2.84 (t, *J* = 7.37 Hz, 2H, Ph*CH*$_2$CH$_2$), 3.17 (s, 2H, *CH*$_2$COOMe), 7.05 (t, *J* = 7.9 Hz, 1H, CH=), 7.15–7.20 (m, 5H, H aromatic). ^{13}C NMR (CD_3OD) δ ppm; *Z* isomer: 31.5; 33.7; 39.8; 123.5–128.6 (6C); 139.7; 146.7; 164.6; 168.3; *E* isomer: 31.5; 33.0; 33.7; 123.5–128.6 (6C); 139.7; 144.1; 164.6; 168.3. IR (film): *v* (cm^{-1}): 2942, 2933, 2863, 1832, 1779, 1669, 1493, 1454, 1388, 1269, 1229, 1128, 956, 903, 758, 723, 701.

4.1.3.3. (2*E*)-2-[(2*E*)-But-2-en-1-ylidene]succinic anhydride (7c). Ratio: *Z*/*E*: 5/95; yield = 100%. ^{1}H NMR ($CDCl_3$) δ ppm; 1.97 (d, *J* = 6.90 Hz, 3H, CH_3), 3.58 (s, 2H, CH_2), 6.15 (m, 1H, CH*CH*CH), 6.43 (m, 1H, CH_3*CH*), 7.30 (d, *J* = 9.12 Hz, 1H, CH=). ^{13}C NMR ($CDCl_3$) δ ppm; 19.2; 32.0; 117.8; 126.7; 139.9; 145.9; 165.8; 168.8. IR (film): *v* (cm^{-1}): 2925, 2854, 1832, 1771, 1643, 1445, 1401, 1260, 1233, 1176, 1132, 969, 899, 732, 666.

4.1.3.4. (2*E*)-2-[(2*E*)-3-Phenylprop-2-en-1-ylidene]succinic anhydride (7d). Ratio: *Z*/*E*: 5/95; yield = 100%. ^{1}H NMR ($CDCl_3$) δ ppm; 3.67 (s, 2H, CH_2COO), 7.12 (m, 1H, PhCH*CH*CH), 7.25–7.35 (m, 5H, H aromatic), 7.48 (d, *J* = 6.64 Hz, 1H, Ph*CH*CHCH), 7.52 (d, *J* = 7.61 Hz, 1H, PhCH*CH*CH). ^{13}C NMR ($CDCl_3$) δ ppm; 33.2; 124.3–129.8 (6C); 130.1; 137.6; 142.3; 142.6; 165.4; 167.2.

4.1.4. General procedure for the preparation of allylic esters (8a–d). Alkylidene succinic anhydride **7** was stirred with allyl alcohol (1 mL per mmol of anhydride) under reflux overnight. The excess of alcohol was evaporated under vacuo. The allylic ester **8** was obtained as orange oil and used directly for the next step without any other purification.

4.1.4.1. (2*E*)-4-Allyloxy-2-heptylidenesuccinic acid (8a). Yield = 97%. ^{1}H NMR ($CDCl_3$) δ ppm; 0.88 (t, *J* = 6.95 Hz, 3H, CH_3), 1.28–1.51 (m, 8H, CH_2), 2.17–2.24 (m, 2H, CH_2–*CH*$_2$=CH), 3.38 (s, 2H, CH_2–CO), 4.59 (d, *J* = 5.6 Hz, 2H, CH_2O), 5.20–5.34 (m, 2H, *CH*$_2$=CH), 5.82–5.96 (m, 1H, CH_2=*CH*), 7.12 (t, *J* = 7.58 Hz, 1H, CH_2–*CH*=), 10.30 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ ppm; 14.0; 22.4; 28.2; 28.9; 29.1; 31.5; 31.8; 65.4; 118.1; 124.7; 131.9; 148.7; 170.3; 172.3. IR (film): *v* (cm^{-1}): 2929, 2854, 2660, 1735, 1687, 1643, 1423, 1370, 1317, 1282, 1172, 987, 930, 767. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 255 (100) $[M+H]^+$, 237 (85).

4.1.4.2. (2*E*)-4-Allyloxy-2-(3-phenylpropylidene)succinic acid (8b). Yield = 97%. ^{1}H NMR ($CDCl_3$) δ ppm; 2.52–2.54 (m, 2H, *CH*$_2$–CH=), 2.78 (t, *J* = 7.05 Hz, 2H, CH_2–Ph), 3.30 (s, 2H, CH_2–CO), 4.57 (d, *J* = 4.57 Hz, 2H, CH_2O), 5.20–5.32 (m, 2H, *CH*$_2$=CH), 5.84–5.93 (m, 1H, CH_2=*CH*), 7.18–7.29 (m, 6H, H aromatic, CH=), 9.03 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ ppm; 31.0; 31.8; 34.3; 65.5; 118.3; 125.4; 126.2; 128.3; 128.3; 128.5; 128.5; 131.8; 140.5; 147.2; 170.1; 172.1. IR (film): *v* (cm^{-1}): 3030, 2933, 2572, 1731, 1691, 1643, 1498, 1449, 1423, 1322, 1291, 1167, 983, 930, 749, 696. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 297 (100) $[M+23]^+$, 275 (95) $[M+H]^+$, 257 (48), 132 (20).

4.1.4.3. (2*E*)-4-Allyloxy-2-[(2*E*)-but-2-en-1-ylidene]-succinic acid (8c). Yield = 97%. ^{1}H NMR ($CDCl_3$) δ ppm; 1.90 (d, *J* = 5.38 Hz, 3H, CH_3–*CH*=), 3.46 (s, 2H, CH_2–CO), 4.60 (d, *J* = 5.59 Hz, 2H, CH_2O), 5.20–5.34 (m, 2H, *CH*$_2$=CH), 5.83–5.96 (m, 1H, CH_2=*CH*), 6.22–6.37 (m, 2H, CH_3–*CH*=*CH*), 7.44 (d, *J* = 10.12 Hz, 1H, CH=C–CO), 9.98 (br s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ ppm; 19.0; 31.9; 65.5; 118.1;

120.8; 126.5; 131.8; 142.0; 143.7; 170.4; 172.9. IR (film): ν (cm^{-1}): 3418, 2925, 2590, 1735, 1674, 1643, 1432, 1414, 1374, 1308, 1269, 1176, 1093, 983, 930, 776. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 211 (91) $[M+H]^+$, 193 (39), 130 (100).

4.1.4.4. (2*E*)-4-Allyloxy-2-[(2*E*)-3-phenylprop-2-en-1-ylidene]succinic acid (8d). Yield = 30%. ^1H NMR ($CDCl_3$) δ ppm; 3.59 (s, 2H, CH_2–CO), 4.62 (d, J = 5.32 Hz, 2H, CH_2O), 5.21–5.35 (m, 2H, CH_2=CH), 5.85–5.98 (m, 1H, CH_2=CH), 7.00–7.02 (m, 2H, *CH*=*CH*), 7.35–7.67 (m, 6H, H aromatic, CH=). ^{13}C NMR ($CDCl_3$) δ ppm; 32.3; 65.6; 118.3; 122.7; 123.0; 127.4; 127.4; 128.8; 128.8; 129.4; 131.8; 135.9; 142.5; 143.4; 170.2; 172.5. IR (film): ν (cm^{-1}): 2916, 2845, 2563, 1718, 1661, 1616, 1436, 1291, 1185, 1159, 978, 934, 749, 683. MS APCI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% formic acid): 273 (15) $[M+H]^+$, 235 (19), 219 (20), 189 (100), 144 (33), 130 (46).

4.1.5. General procedure for condensation of carboxylic acids and (*L*)-tryptophan methylamide (9). A mixture of acid **8** and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (1.5 equiv) in dry THF (2 mL per 1 mmol of acid compound) was stirred at room temperature for 30 min. (*L*)-tryptophan methylamide (**9**) (1 equiv) was added to the precedent mixture. The reaction mixture was stirred overnight at room temperature. The organic phase was washed successively with a solution of citric acid (5%), water, a solution of $NaHCO_3$ (5%) and dried over $MgSO_4$. The organic phase was filtered and the filtrate was concentrated in vacuo. The crude product (orange oil) was purified by silica gel column chromatography using $[CH_2Cl_2]/[MeOH]$ = 98/2 as solvent, to give compound **10** as yellow foam.

4.1.5.1. (2*E*)-3-(Allyloxycarbonyl)-2-heptylidenepropionyl-*L*-tryptophan-*N*-methylamide (10a). Yield = 53%. ^1H NMR ($CDCl_3$) δ ppm; 0.88 (t, J = 7.10 Hz, 3H, CH_3–CH_2), 1.24–1.45 (m, 8H, CH_2), 2.01–2.06 (m, 2H, CH_2=CH), 2.66 (d, J = 4.54 Hz, 3H, CH_3–NH), 3.16–3.47 (m, 2H, CH_2–CO, CH_2–CH), 3.70–4.04 (m, 2H, CH_2–CO, CH_2–CH), 4.52 (d, J = 5.64 Hz, 2H, CH_2O), 4.73–4.80 (m, 1H, CH_2–*CH*), 5.19–5.33 (m, 2H, CH_2=CH–), 5.78–5.95 (m, 1H, CH_2=*CH*–), 6.14 (t, J = 7.23 Hz, 1H, CH=), 6.27 (q, J = 4.43 Hz, 1H, CH_3–*NH*), 6.60 (d, J = 7.95 Hz, 1H, NHTrp), 7.02–7.38 (m, 4H, H aromatic), 7.67 (d, J = 7.62 Hz, 1H, H aromatic), 8.42 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 14.0; 22.4; 26.2; 27.1; 28.4; 28.4; 28.8; 31.5; 32.6; 54.2; 65.6; 110.4; 111.2; 118.5; 118.7; 119.6; 122.1; 123.2; 127.5; 129.3; 131.6; 136.0; 138.5; 168.8; 171.2; 171.8. IR (film): ν (cm^{-1}): 3303, 2925, 2854, 1722, 1656, 1581, 1537, 1458, 1370, 1269, 1172, 987, 925, 815. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 476 (38) $[M+23]^+$, 454 (100) $[M+H]^+$, 423 (35), 357 (18).

4.1.5.2. (2*E*)-3-(Allyloxycarbonyl)-2-(3-phenylpropylidene)propionyl-*L*-tryptophan-*N*-methylamide (10b). Yield = 69%. ^1H NMR ($CDCl_3$) δ ppm; 2.32–2.41 (m, 2H, CH_2–CH_2–Ph), 2.57–2.72 (m, 2H, CH_2–CH_2–Ph), 2.67 (d, J = 4.72 Hz, 3H, CH_3–NH), 3.17–3.50 (m, 4H, CH_2–CO, CH_2–CH), 4.49 (d, J = 5.75 Hz, 2H, CH_2O), 4.71–4.78 (m, 1H, CH_2–*CH*), 5.18–5.29 (m, 2H, CH_2=CH–), 5.77–5.93 (m, 1H, CH_2=*CH*–), 6.11 (t, J = 7.24 Hz, 1H, CH=), 6.28 (q, J = 4.52 Hz, 1H, CH_3–*NH*), 6.47 (d, J = 7.95 Hz, 1H, NHTrp), 6.98–7.28 (m, 8H, H aromatic), 7.37 (d, J = 8.11 Hz, 1H, H aromatic), 7.67 (d, J = 7.77 Hz, 1H, H aromatic), 8.36 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 26.2; 27.0; 30.1; 32.5; 34.5; 54.0; 65.7; 110.4; 111.3; 118.6; 118.6; 119.7; 122.2; 123.2; 126.1; 127.5; 128.2; 128.2; 128.4; 128.4; 130.3; 131.6; 136.1; 136.6; 140.6; 168.7; 171.0; 171.6. IR (film): ν (cm^{-1}): 3392, 3303, 3057, 2933, 1727, 1656, 1533, 1449, 1361, 1322, 1264, 1172, 1097, 983, 925, 745, 701. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 496 (72) $[M+23]^+$, 474 (100) $[M+H]^+$, 443 (32), 357 (40).

4.1.5.3. (2*E*)-3-(Allyloxycarbonyl)-2-[(2*E*)-but-2-en-1-ylidene]propionyl-*L*-tryptophan-*N*-methylamide (10c). Yield = 52%. ^1H NMR ($CDCl_3$) δ ppm; 1.83 (d, J = 6.68 Hz, 3H, CH_3–CH=), 2.65 (d, J = 4.49 Hz, 3H, CH_3–NH), 3.17–3.54 (m, 2H, CH_2–CO, CH_2–CH), 3.89–4.03 (m, 2H, CH_2–CO, CH_2–CH), 4.52 (d, J = 5.60 Hz, 2H, CH_2O), 4.74–4.81 (m, 1H, CH_2–*CH*), 5.18–5.30 (m, 2H, CH_2=CH–), 5.78–5.99 (m, 1H, CH_2=*CH*–), 6.12–6.24 (m, 2H, CH=, NH), 6.60–6.67 (m, 2H, CH=, NH), 7.01–7.38 (m, 5H, H aromatic), 7.67 (d, J = 7.43 Hz, 1H, H aromatic), 8.53 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 18.8; 26.2; 27.2; 32.7; 54.3; 65.7; 110.4; 111.3; 118.4; 118.6; 119.6; 122.1; 123.2; 125.3; 125.9; 127.6; 131.6; 135.6; 136.1; 138.8; 168.6; 171.0; 171.8. IR (film): ν (cm^{-1}): 2960, 2916, 2845, 1731, 1647, 1581, 1462, 1361, 1260, 1167, 974, 925, 736. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 432 (100) $[M+23]^+$, 410 (44) $[M+H]^+$, 379 (23).

4.1.5.4. (2*E*)-3-(Allyloxycarbonyl)-2-[(2*E*)-3-phenylprop-2-en-1-ylidene]propionyl-*L*-tryptophan-*N*-methylamide (10d). Yield = 53%. ^1H NMR (DMSO-d_6) δ ppm; 2.60 (d, J = 3.52 Hz, 3H, CH_3–NH), 3.02–3.20 (m, 2H, CH_2–CH), 3.63–3.98 (m, 2H, CH_2–CO), 4.50–4.58 (m, 3H, CH_2–*CH*, CH_2O), 5.13–5.35 (m, 2H, CH_2=CH–), 5.79–5.96 (m, 1H, CH_2=*CH*–), 6.86 (d, J = 15.13 Hz, 1H, CH=), 6.98–7.67 (m, 12H, H aromatic, CH=), 7.86 (q, J = 4.58 Hz, 1H, CH_3–*NH*), 8.12 (d, J = 7.96 Hz, 1H, NH), 10.83 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm; 26.1; 27.7; 32.5; 54.4; 64.7; 110.2; 111.6; 117.5; 118.4; 118.5; 121.1; 123.4; 124.6; 125.5; 125.9; 127.2; 127.3; 127.3; 128.8; 128.8; 132.3; 136.2; 140.5; 140.6; 141.4; 168.5; 169.7; 170.5. IR (film): ν (cm^{-1}): 3356, 2925, 1696, 1634, 1383, 1277, 1172, 969, 925, 736, 683. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 494 (23) $[M+23]^+$, 472 (100) $[M+H]^+$, 441 (49), 255 (14), 141 (13).

4.1.6. General procedure for the preparation of carboxylic acids (11a–d). The allyl ester **10** was dissolved in THF (10 mL per mmol of allyl ester) in a nitrogen atmosphere before tetrakis(triphenylphosphine)palladium-(0) ($Pd(PPh_3)_4$, 0.1 equiv) and morpholine (3.1 equiv) were added in the dark. After 30 min, the solvent was evaporated. The mixture was diluted with CH_2Cl_2 (15 mL). The organic phase was washed successively with a

solution of citric acid (5%), a solution of NaCl and dried over $MgSO_4$. The organic phase was filtered and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography using $[CH_2Cl_2]/[MeOH] = 98/2$ as solvent, to give acid compound **11** as yellow foam.

4.1.6.1. (2*E*)-3-(Hydroxycarbonyl)-2-heptylidenepropionyl-*L*-tryptophan-*N*-methylamide (11a). Yield = 74%. ^{1}H NMR ($CDCl_3$) δ ppm; 0.87 (t, J = 6.68 Hz, 3H, CH_3–CH_2), 1.20–1.27 (m, 8H, CH_2), 2.10–2.15 (m, 2H, CH_2=CH), 2.62 (d, J = 4.56 Hz, 3H, CH_3–NH), 3.12–3.36 (m, 4H, CH_2–CO, CH_2–CH), 4.67–4.74 (m, 1H, CH_2–CH), 6.00 (q, J = 3.88 Hz, 1H, CH_3–NH), 6.22 (t, J = 7.17 Hz, 1H, CH=), 7.02–7.65 (m, 5H, H aromatic), 8.40 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 14.0; 22.4; 26.2; 27.6; 28.4; 28.4; 28.9; 31.5; 33.7; 54.5; 109.7; 111.4; 118.3; 119.4; 121.9; 123.4; 127.3; 127.9; 136.1; 140.5; 170.2; 172.3; 173.5. IR (film): ν (cm^{-1}): 3321, 3057, 2925, 2854, 1713, 1625, 1533, 1458, 1432, 1410, 1198, 1097, 908, 736. MS ESI(+) (m/z), 50/50 H_2O/CH_3CN (0.1% formic acid): 414 (100) $[M+H]^+$, 383 (20).

4.1.6.2. (2*E*)-3-(Hydroxycarbonyl)-2-(3-phenylpropylidene)propionyl-*L*-tryptophan-*N*-methylamide (11b). Yield = 65%. ^{1}H NMR ($CDCl_3$) δ ppm; 2.34–2.63 (m, 7H, CH_2–CH_2–Ph, CH_3–NH), 3.08–3.30 (m, 3H, CH_2–CO, CH_2–CH), 3.65–3.68 (m, 1H, CH_2CO), 4.67–4.73 (m, 1H, CH_2–CH), 6.20 (t, J = 6.70 Hz, 1H, CH=), 6.58 (s, 1H, NH), 6.91–7.28 (m, 9H, H aromatic), 7.54 (d, J = 9.05 Hz, 1H, H aromatic), 8.76 (br s, 2H, OH, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 26.2; 27.5; 30.0; 34.5; 51.4; 54.3; 109.8; 111.4; 118.3; 119.4; 121.9; 123.4; 126.1; 127.3; 128.2; 128.2; 128.4; 128.4; 129.7; 136.0; 138.2; 140.7; 169.8; 171.0; 172.2. IR (film): ν (cm^{-1}): 3286, 3048, 2916, 2854, 1656, 1625, 1537, 1436, 1357, 1264, 1176, 1115, 740, 692. MS ESI(+) (m/z), 50/50 H_2O/CH_3CN (0.1% formic acid): 434 (100) $[M+H]^+$, 403 (81), 375 (20), 357 (18).

4.1.6.3. (2*E*)-3-(Hydroxycarbonyl)-2-[(2*E*)-but-2-en-1-ylidene]propionyl-*L*-tryptophan-*N*-methylamide (11c). Yield = 53%. ^{1}H NMR ($CDCl_3$) δ ppm; 1.80 (d, J = 6.51 Hz, 3H, CH_3–CH=), 2.59 (d, J = 4.41 Hz, 3H, CH_3–NH), 3.14–3.39 (m, 4H, CH_2–CO, CH_2–CH), 4.71–4.77 (m, 1H, CH_2–CH), 5.91–6.02 (m, 1H, CH=), 6.24–6.34 (m, 2H, CH=, NH), 6.68 (d, J = 10.88 Hz, 1H, CH=), 6.99–7.32 (m, 4H, H aromatic), 7.60 (d, J = 7.69 Hz, 1H, H aromatic), 8.62 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 18.8; 26.2; 27.7; 34.5; 54.5; 110.1; 111.4; 118.5; 119.5; 122.0; 123.3; 124.5; 126.0; 127.3; 136.1; 136.7; 139.4; 170.3; 172.1; 173.4. IR (film): ν (cm^{-1}): 3303, 3048, 2916, 2854, 1713, 1647, 1528, 1458, 1379, 1260, 1172, 1097, 974, 908, 736, 701. MS ESI(+) (m/z), 50/50 H_2O/CH_3CN (0.1% TFA): 392 (100) $[M+23]^+$, 370 (100) $[M+H]^+$, 339 (63), 311 (8), 278 (9), 159 (23), 128 (50).

4.1.6.4. (2*E*)-3-(Hydroxycarbonyl)-2-[(2*E*)-3-phenylprop-2-en-1-ylidene]propionyl-*L*-tryptophan-*N*-methylamide (11d). Yield = 74%. ^{1}H NMR (MeOD) δ ppm; 2.58 (d, J = 3.74 Hz, 3H, CH_3–NH), 3.07–3.32 (m, 2H, CH_2–CO, CH_2–CH), 3.43–3.46 (m, 2H, CH_2–CO, CH_2–CH), 4.54–4.58 (m, 1H, CH_2–CH), 6.66-6.74 (m, 2H, CH=), 6.92-7.70 (m, 11H, H aromatic). ^{13}C NMR (MeOD) δ ppm; 26.5; 28.5; 33.4; 54.8; 111.1; 112.3; 119.0; 119.8; 122.4; 124.1; 128.9; 129.0; 130.0; 130.5; 133.0; 136.2; 137.9; 138.0; 142.0; 171.5; 174.5; 174.6; 175.0. IR (film): ν (cm^{-1}): 3330, 3048, 2925, 1696, 1634, 1533, 1414, 1260, 1189, 974, 740, 688. MS ESI(+) (m/z), 50/50 H_2O/CH_3CN (0.1% TFA): 454 (72) $[M+23]^+$, 390 (100), 277 (37), 224 (10), 141 (8).

4.1.7. General procedure for the preparation of hydroxamic acids (2a–d). A mixture of acid **11** and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 1.1 equiv) in dry THF (4 mL per 1 mmol of acid compound) were stirred at room temperature for 10 min. Hydroxylamine hydrochloride (5 equiv) and *N*-methylmorpholine (7 equiv) in dry THF (2 mL) was stirred at room temperature for 10 min, this solution was added to the precedent mixture. The reaction mixture was stirred overnight at room temperature and was cooled to 0 °C. The organic phase was washed successively with a solution of citric acid (5%), a solution of $NaHCO_3$ (5%) and dried over $MgSO_4$. The organic phase was filtered and the filtrate was concentrated in vacuo. The crude product was purified by CPC (centrifugal partition chromatography) to give the hydroxamic acid **2** as a foam.

4.1.7.1. (2*E*)-3-(*N*-Hydroxycarbamoyl)-2-heptylidenepropionyl-*L*-tryptophan-*N*-methylamide (2a). CPC (Arizona system L, t_R = 54–60 min, λ = 272 nm). The hydroxamic acid was obtained as yellow foam (yield = 4%). ^{1}H NMR (DMSO-d_6) δ ppm; 0.88 (t, J = 7 Hz, 3H, CH_3–CH_2–), 1–1.40 (m, 8H, CH_2), 2.05–2.12 (m, 2H, CH_2–CH=), 2.60 (d, J = 4.19 Hz, 3H, CH_3–NH), 2.96–3.24 (m, 4H, CH_2–CO, CH_2–CH), 4.39–4.49 (m, 1H, CH_2–CH), 6.22 (t, J = 7.18 Hz, 1H, CH=), 6.22 (t, J = 7.22 Hz, 1H, H indole), 6.97 (t, J = 7.25 Hz, 1H, H indole), 7.03 (s, 1H, H indole), 7.33 (d, J = 7.96 Hz, 1H, H indole), 7.59 (d, J = 7.68 Hz, 1H, H indole), 7.92 (q, J = 4.10 Hz, 1H, CH_3–NH), 8.17 (d, J = 7.94 Hz, 1H, NHTrp), 8.87 (s, 1H, NH), 10.57 (s, 1H, OH), 10.81 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm; 14.2; 22.2; 24.0; 25.9; 27.8; 28.5; 29.2; 31.3; 54.4; 110.8; 111.5; 112.9; 118.4; 118.5; 121.0; 123.6; 127.5; 130.1; 136.2; 137.8; 163.9; 168.4; 172.1. IR (KBr): ν (cm^{-1}): 3289, 2921, 2842, 1646, 1533, 1459, 1371, 1258, 1097, 1023, 796, 735. MS ESI(+) (m/z), 50/50 H_2O/CH_3CN (0.1% TFA): 429 (10) $[M+H]^+$, 396 (38) $[M-(NH–OH)]^+$, 357 (100), 326 (15), 298 (36).

4.1.7.2. (2*E*)-3-(*N*-Hydroxycarbamoyl)-2-(3-phenylpropylidene)propionyl-*L*-tryptophan-*N*-methylamide (2b). CPC (Arizona system K, t_R = 30–44 min, λ = 280 nm). The hydroxamic acid was obtained as yellow foam (yield = 13%). ^{1}H NMR (DMSO-d_6) δ ppm; 2.28–2.69 (m, 4H, CH_2–CH_2–Ph), 2.60 (d, J = 4.38 Hz, 3H, CH_3–NH), 2.93–3.24 (m, 4H, CH_2–CO, CH_2–CH), 4.41–4.49 (m, 1H, CH_2–CH), 6.30 (t, J = 7.13 Hz, 1H, CH=), 6.96–7.35 (m, 12H, H aromatic), 7.60 (d, J = 7.74 Hz, 1H, H aromatic), 7.93–7.96 (q,

J = 4.36 Hz, 1H, CH_3–*NH*), 8.22 (d, J = 8 Hz, 1H, *NH*Trp), 8.93 (br s, 1H, NH), 10.64 (br s, 1H, OH), 10.82 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm; 25.9; 27.5; 29.9; 31.0; 34.5; 54.3; 110.7; 111.5; 118.4; 118.5; 121.0; 123.6; 126.1; 127.5; 128.5; 130.5; 136.2; 137.0; 141.4; 167.0; 168.2; 172.0. IR (KBr): v (cm^{-1}): 3280, 2929, 2842, 1655, 1524, 1454, 1358, 1097, 743, 695. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 449 (12) $[M+H]^+$, 424 (39), 416 (44) $[M-(NH–OH)]^+$, 396 (95), 368 (100), 357 (8), 287 (9).

4.1.7.3. (2*E*)-3-(*N*-Hydroxycarbamoyl)-2-[(2*E*)-but-2-en-1-ylidene]propionyl-*L*-tryptophan-*N*-methylamide (2c). CPC (Arizona system G, t_R = 42–56 min, λ = 272 nm). The hydroxamic acid was obtained as yellow foam (yield = 11%). ^{1}H NMR (DMSO-d_6) δ ppm; 1.83 (d, J = 6.43 Hz, 3H, *CH*$_3$–CH=), 2.59 (d, J = 4.41 Hz, 3H, *CH*$_3$–NH), 2.94–3.24 (m, 4H, *CH*$_2$–CO, *CH*$_2$–CH), 4.44–4.52 (m, 1H, CH_2–*CH*), 6.03 (dq, J = 7.07 Hz, J = 21.8 Hz, 1H, CH_3–*CH*=), 6.45 (dd, J = 13.62 Hz, 1H, =CH–*CH*=CH=), 6.76 (d, J = 11.2 Hz, 1H, =CH–CH=*CH*=), 6.98 (t, J = 7.50 Hz, 1H, H indole), 7.06 (t, J = 7.01 Hz, 1H, H indole), 7.16 (s, 1H, H indole), 7.33 (d, J = 8.00 Hz, 1H, H indole), 7.60 (d, J = 7.72 Hz, 1H, H indole), 7.97 (q, J = 4.51 Hz, 1H, CH_3–*NH*), 8.39 (d, J = 7.67 Hz, 1H, *NH*Trp), 8.98 (s, 1H, NH), 10.83 (s, 2H, NH, OH). ^{13}C NMR (DMSO-d_6) δ ppm; 18.8; 25.9; 27.6; 31.1; 34.5; 54.5; 110.9; 111.5; 118.4; 118.5; 121.0; 123.6; 127.1; 127.2; 127.5; 135.3; 136.2; 136.8; 166.9; 168.2; 172.1. IR (KBr): v (cm^{-1}): 3271, 2921, 1646, 1533, 1354, 1258, 1166, 1088, 970, 743. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 385 (60) $[M+H]^+$, 352 (100) $[M-(NH–OH)]^+$, 293 (19).

4.1.7.4. (2*E*)-3-(*N*-Hydroxycarbamoyl)-2-[(2*E*)-3-phenylprop-2-en-1-ylidene]propionyl-*L*-tryptophan-*N*-methylamide (2d). CPC (Arizona system K, t_R = 27–42 min, λ = 290 nm). The hydroxamic acid was obtained as white foam (yield = 8%). ^{1}H NMR (DMSO-d_6) δ ppm; 2.61 (d, J = 4.37 Hz, 3H, *CH*$_3$–NH), 2.99–3.30 (m, 4H, *CH*$_2$–CO, *CH*$_2$–CH), 4.56–4.87 (m, 1H, CH_2–*CH*), 6.84–7.64 (m, 13H, H aromatic), 8.00 (q, J = 4.61 Hz, 1H, CH_3–*NH*), 8.45 (d, J = 7.92 Hz, 1H, *NH*Trp), 9.01 (s, 1H, NH), 10.79 (s, 1H, OH), 10.83 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm; 25.9; 27.6; 31.5; 54.5; 110.8; 111.5; 118.4; 118.6; 121.0; 123.7; 124.1; 126.6; 127.3; 127.5; 127.5; 128.9; 128.9; 129.9; 135.4; 136.2; 136.6; 138.2; 166.9; 168.0; 172.1. IR (KBr): v (cm^{-1}): 3228, 2929, 1655, 1620, 1546, 1485, 1376, 1284, 1249, 1188, 1153, 1097, 1040, 975, 739, 687. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 447 (75) $[M+H]^+$, 414 (100) $[M-(NH–OH)]^+$, 159 (12), 120 (34).

4.1.8. Cyclic compounds (12a–d)

4.1.8.1. (*S*,*E*)-2-(3-Heptylidene-2,5-dioxopyrrolidin-1-yl)-3-(*1H*-indol-3-yl)-*N*-methylpropanamide (12a). CPC (Arizona system L, t_R = 12 min, λ = 272 nm). The cyclic compound was obtained as yellow foam (yield = 23%). Mp 52–55 °C. ^{1}H NMR ($CDCl_3$) δ ppm; 0.87 (t, J = 6.33 Hz, 3H, *CH*$_3$–CH$_2$), 1.20–1.41 (m, 8H, CH_2), 2.00–2.07 (m, 2H, CH_2), 2.75 (d, J = 4.73 Hz, 3H, *CH*$_3$–NH), 2.90–3.10 (m, 2H, CH$_2$–CO), 3.52–3.69 (m, 2H, *CH*$_2$–CH), 5.15 (dd, J = 7.12 Hz, J = 8.90 Hz, 1H, CH_2–*CH*), 6.46 (q, J = 4.66 Hz, 1H, CH_3–*NH*), 6.68–6.73 (m, 1H, CH=), 7.00–7.16 (m, 5H, H indole), 8.60 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 13.9; 22.4; 24.6; 26.4; 27.8; 28.8; 29.7; 31.4; 31.6; 55.5; 110.5; 111.3; 118.2; 119.4; 122.0; 122.8; 124.7; 126.8; 136.1; 139.8; 169.1; 169.8; 174.0. IR (film): v (cm^{-1}): 3350, 3061, 2921, 2850, 1764, 1707, 1664, 1542, 1454, 1380, 1262, 1210, 1171, 1097, 1005, 914, 800, 739. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 396 (100) $[M+H]^+$, 365 (21), 337 (25).

4.1.8.2. (*S*,*E*)-2-(2,5-Dioxo-3-(3-phenylpropylidene)pyrrolidin-1-yl)-3-(*1H*-indol-3-yl)-*N*-methylpropanamide (12b). CPC (Arizona system K, t_R = 12 min, λ = 280 nm). The cyclic compound was obtained as yellow foam (yield = 7%). Mp 67–68 °C. ^{1}H NMR ($CDCl_3$) δ ppm; 2.29–2.41 (m, 2H, CH_2), 2.69–2.85 (m, 7H, CH$_2$–CO, CH_2, CH_3–NH (d, J = 4.80 Hz)), 3.51–3.67 (m, 2H, *CH*$_2$–CH), 5.13 (dd, J = 7.10 Hz, J = 9.00 Hz, 1H, CH_2–*CH*), 6.36 (q, J = 4.27 Hz, 1H, CH_3–*NH*), 6.71–6.76 (m, 1H, CH=), 6.71–7.69 (m, 10H, H aromatic), 8.40 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 24.5; 26.4; 31.6; 31.6; 34.0; 55.5; 110.7; 111.2; 118.4; 119.5; 122.1; 122.7; 125.6; 126.3; 126.9; 128.2; 128.4; 128.5; 136.1; 138.0; 140.2; 169.0; 169.6; 173.8. IR (film): v (cm^{-1}): 3342, 3061, 2921, 2850, 1768, 1703, 1672, 1546, 1494, 1380, 1258, 1162, 1114, 905, 735, 695. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 416 (61) $[M+H]^+$, 357 (24), 279 (100).

4.1.8.3. (*S*)-2-((*E*)-2,5-Dioxo-3-((*E*)-pent-2-enylidene)pyrrolidin-1-yl)-3-(*1H*-indol-3-yl)-*N*-methylpropanamide (12c). CPC (Arizona system G, t_R = 12 min, λ = 272 nm). The cyclic compound was obtained as yellow foam (yield = 22%). Mp 72–75 °C. ^{1}H NMR ($CDCl_3$) δ ppm; 1.86 (d, J = 6.90 Hz, 3H, CH_3), 2.74 (d, J = 5.10 Hz, 3H, *CH*$_3$–NH), 2.98–3.15 (m, 2H, CH$_2$–CO), 3.53–3.69 (m, 2H, *CH*$_2$–CH), 5.16 (dd, J = 7.24 Hz, J = 8.46 Hz, 1H, *CH*$_2$–CH), 5.98 (dd, J = 12.65 Hz, J = 15.1 Hz, 1H, CH=*CH*–CH=), 6.13–6.25 (m, 1H, CH_3–*CH*=), 6.51 (q, J = 4.57 Hz, 1H, CH_3–*NH*), 6.98–7.68 (m, 6H, H aromatic, CH=), 8.59 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 19.0; 24.6; 26.4; 31.9; 55.5; 110.6; 111.2; 118.3; 119.4; 121.2; 122.0; 122.8; 126.6; 126.8; 134.5; 136.1; 142.3; 169.1; 170.5; 173.9. IR (film): v (cm^{-1}): 3342, 3052, 2921, 1764, 1699, 1651, 1542, 1433, 1380, 1280, 1175, 1114, 970, 918, 796, 735, 691. MS ESI(+) (*m*/*z*), 50/50 H_2O/CH_3CN (0.1% TFA): 352 (100) $[M+H]^+$, 321 (27), 293 (81), 279 (94), 227 (19).

4.1.8.4. (*S*)-2-((*E*)-2,5-Dioxo-3-((*E*)-3-phenylallylidene)-pyrrolidin-1-yl)-3-(*1H*-indol-3-yl)-*N*-methylpropanamide (12d). CPC (Arizona system K, t_R = 12 min, λ = 290 nm). The cyclic compound was obtained as yellow foam (yield = 22%). Mp 62–64 °C. ^{1}H NMR ($CDCl_3$) δ ppm; 2.75 (d, J = 4.75 Hz, 3H, *CH*$_3$–NH), 3.10–3.28 (m, 2H, CH$_2$–CO, CH$_2$–CH), 3.57–3.72 (m, 2H, CH$_2$–CO, CH$_2$–CH), 5.18 (dd, J = 7.04 Hz, J = 9.08 Hz, 1H, CH$_2$–CH), 6.59–6.88 (m, 2H, CH=),

6.88–7.69 (m, 11H, H aromatic, CH=), 8.66 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ ppm; 24.4; 26.3; 32.0; 55.4; 110.5; 111.1; 118.2; 119.3; 121.9; 122.5; 122.6; 123.3; 126.7; 127.2; 128.2; 128.4; 128.6; 129.3; 134.0; 135.4; 136.0; 142.4; 168.9; 170.0; 173.5. IR (film): ν (cm^{-1}): 3268, 3066, 2907, 2845, 1762, 1700, 1665, 1638, 1546, 1436, 1383, 1269, 1172, 1119, 736, 723, 688. MS ESI(+) (*m/z*), 50/50 H_2O/CH_3CN (0.1% TFA): 414 (67) $[M+H]^+$, 320 (100).

4.2. Electronic circular dichroism (ECD) spectroscopy

ECD spectra of 0.1 mg mL^{-1} aqueous solution of Ilomastat analogue (compound **1**) and hydroxamic acids **2a–d** in a rectangular cell with a 1 mm optical path length were recorded on a JASCO J-810 CD spectropolarimeter using a Peltier type temperature control system. Time averaged spectra (9 scans) were acquired in the UV wavelength range 200–400 nm at a temperature of 25 °C by taking points every 1 nm, with a 20 nm min^{-1} scan rate, an integration time of 1 s and a 1 nm bandwidth. The data are expressed in terms of molecular ellipticity [θ] (deg cm^2 $dmol^{-1}$) (Fig. 2).

4.3. Centrifugal partition chromatography (CPC)

4.3.1. CPC apparatus. Separations of hydroxamic acids **2a–d** were performed on a FCPC Kromaton Technologies apparatus (Angers, France), using a rotor of 20 circular partition discs (1320 partition cells, column capacity: 200 mL). Rotation speed could be adjusted from 200 to 2000 rpm, producing a centrifugal force field in the partition cell of about 120*g* at 1000 rpm and 470*g* at 2000rpm. The solvents were pumped using a Dionex P580HPG 4-way binary high-pressure gradient pump (Sunnyvale, CA, USA). The samples were introduced into the CPC column via a low-pressure injection valve (Upchurch, CIL Cluzeau, Sainte-Foy-La-Grande, France) equipped with a 5 mL sample loop. The effluent was monitored with a Dionex UVD 170S detector equipped with a preparative flow cell (6 μL internal volume, path length of 2 mm). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). The experiments were conducted at room temperature (22 ± 1 °C).

4.3.2. Solvent systems' preparation. Biphasic systems were prepared by mixing heptane, ethyl acetate, methanol and water in suitable proportions (Table 3) in a separatory funnel, shaking them vigorously and allowing them to settle until the phases became limpid.

Table 3. Composition of the different biphasic systems (by mixing heptane, ethyl acetate, methanol and water in suitable proportions) of the ARIZONA scale selected for each hydroxamic acid **2** purification

ARIZONA systems	Composition heptane/ethyl acetate/methanol/water (v/v)	Isolated compounds
G	1:4:1:4	**2c**
K	1:2:1:2	**2b** and **2d**
L	2:3:2:3	**2a**

4.3.3. Injection and CPC operating procedure. The rotor was first entirely filled with the stationary phase while rotating at 300 rpm. The sample was then injected dissolved in 2.5 mL of organic phase and 2.5 mL of aqueous phase. The rotation speed was increased to 1300 rpm, and the organic mobile phase was pumped into the column in the ascending mode at a flow-rate of 6 mL/min for 74 min. The mobile phase displaced 25% of the stationary phase and therefore the retention of the stationary phase was 75%. The back-pressure was 37 bar. Detection was performed at 272, 280 or 290 nm and the fraction size was 1 min.

4.4. Materials and methods for inhibition studies

4.4.1. Materials. Quenched fluorogenic substrates DNP-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(*N*-Me-Abz)–NH_2 for MMP-1 or MMP-9 inhibition (where DNP, 2,4-dinitrophenyl; Cha, β-cyclohexylalanyl; Abz, 2-aminobenzoyl (antraniloyl) were purchased from Calbiochem (VWR, Strasbourg, France)), Mca L-Pro-Leu-Gly-Leu-Dap-Ala-Arg–NH_2 for MMP-2, MMP-8, MMP-13 or MMP-14 inhibition where Mca, (7-methoxycoumarin-4-yl); Dpa, [N-3-(2,4-Dinitrophenyl)-*L*-2,3-diaminopropionyl] and 6-(7-Nitro-benzo[1,2,5]oxodiazol-4-ylamino)-hexanoyl-Arg-Pro-Lys-Pro-Leu- Ala-Nva-Trp-Lys(7-dimethylaminocoumarin-4-yl)NH_2 for MMP-3 inhibition from Bachem (Weil am Rhein, Germany).

Human recombinant pro-MMP-1, pro-MMP-2, pro-MMP-8, pro-MMP-9, pro-MMP-13 and catalytic domains of MT1-MMP or of MMP-3 were obtained from Calbiochem. The pro-enzymes were freshly activated with 1 mM (pro-MMP-2) or 2 mM (pro-MMP-1 and pro-MMP-9) *p*-aminophenylmercuric acetate (Sigma–Aldrich, Saint Quentin Fallavier, France) at 37 °C in water bath for 2 h.

4.4.2. In vitro assay of synthetic compounds. Briefly, 1 nM of MMP-1, 0.95 nM of MMP-2, 3.8 nM of MMP-8, 0.6 nM of MMP-13, 5 nM of catalytic domain of MMP-3, 0.89 nM of MMP-9 and 3.1 nM of catalytic domain of MMP-14 were incubated with increasing concentrations of synthetic compounds (from 1 to 5000 nM). Assays were initiated by adding the appropriate fluorogenic substrate (1–10 nM). The fluorescence was monitored with a Perkin Elmer HT Soft 7000 plus spectrofluorimeter (Perkin Elmer, Courtaboeuf, France). Upon cleavage of fluorogenic peptide by the MMP, the initial rate of the peptide hydrolysis in absence (Vo) or presence (Vi) of synthetical molecule is determined. The IC_{50} were calculated after plotting Vi/Vo as function of synthetical molecule concentrations by fitting with a non-linear regression (Grafit Computer program, R. Leatherbarrow, Erithacus Software).

4.5. Molecular modelling study

We used the AutoDock 3.0.5 program[26] to perform the molecular docking simulations and calculations. The molecular graphics of the three-dimensional structures and their display have been done using PyMOL

program (Warren L. DeLano 'The PyMOL Molecular Graphics System'. DeLano Scientific LLC, San Carlos, CA, USA. http://www.pymol.org).

4.5.1. MMPs and ligands initial data. The three-dimensional coordinates of MMPs' structures were downloaded from the Protein Data Bank.[27] The PDB codes for the structures of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 were, respectively, 1HFC,[28] 1CK7,[29] 1KBC,[30] 1GKC[19] and 2E2D.[31]

All the elements including organic or inorganic cofactor, water molecules and the ligand were removed. Only the zinc ion located at the active site was retained. The zinc parameters are those[32] optimized for MMP docking studies using the AutoDock program (zinc radius: 0.87 Å; well depth: 0.35 kcal mol^{-1} and zinc charge: +0.95 e). Polar hydrogens were added and Kollman united atom charges were assigned. The desolvation parameter was assigned to protein using the AutoDock module ADDSOL.

Ligands were built using the module BIOPOLYMER of Insight98 software package (MSI, Inc., San Diego, Ca, USA). The partial atomic charges for ligands were carried out at AM1 level of theory as implemented in Insight98's Ampac/Mopac module. Rotatable dihedrals in the ligands were assigned using the auxiliary AutoTors program and were allowed to rotate freely.

4.5.2. Molecular docking simulations and calculations. The three-dimensional affinity and electrostatic potential grids were calculated for each type of atom in the MMP using AutoGrid. Grids were centred on the MMP active site. The grid size was set to provide enough space for the ligand translational and rotational walks. The number of grid points was then $124 \times 124 \times 124$ with a separation of 0.203 Å between grid points.

Four runs of docking were performed using the Lamarckian genetic algorithm (LGA). A maximum number of 250 LGA operations were generated on a single population of 200 individuals for each run of the 250 independent cycles that were set to stop after a maximum of either 5 000 000 energy evaluations or 500 000 generations. The default parameters for the LGA and Sollis and Wet local search were used. Our used protocol was first successfully checked for MMP-9 using the reverse hydroxamate inhibitor of MMP-9 as ligand[19]; both the conformation and the position resulting from the molecular docking calculations were very close to experimental structural data.

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