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A library of novel hydroxamic acids targeting the metallo-protease family: Design, parallel synthesis and screening

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Abstract—We report here the design and parallel synthesis of 217 compounds based on a malonic–hydroxamic acid template. These compounds are obtained via a two-step solution-phase procedure. The set of diverse building-blocks used makes this strategy suitable for the search of inhibitors of various metallo-proteases and for the investigation of the biological role of new metallo-proteases. As a proof of concept, we screened this library on Neutral Aminopeptidase (APN; EC 3.4.11.2), the prototypal enzyme of the M1 family. Several submicromolar inhibitors were identified.

1. Introduction

Proteases are important pharmacological targets for a wide variety of therapeutic applications.[1–3] Their drug ability is illustrated by the development of potent inhibitors for the four classes of proteases (cysteine-, aspartyl-, serine- and metallo-proteases). The most successful examples are inhibitors of ACE (Angiotensin-converting enzyme), as antihypertensive drugs, and inhibitors of the aspartyl-protease of HIV.[4,5] More recently, numerous teams have focused on metallo-proteases and closely related metallo-hydrolases for the treatment of cancer or arthritis with inhibitors of TACE (TNF-α-converting enzyme), HDAC (histone desacetylase) and MMPs (matrix-metallo-proteases).[6–9] More generally, metallo-proteases represent about 35% of the proteases identified in the sequenced human genome.[10] They thus deserve the development of inhibitors for the investigation of their biological function.[11] Nevertheless, the design of such inhibitors using computational methods remains challenging because of the key role of the metal ion in the binding of inhibitors, as recently demonstrated by Irwin et al.[12]

In this context, we decided to design and screen a targeted library of hydroxamates. Indeed, hydroxamate is a recognized and well-characterized Zn^{2+} binding group.[13,14] Diversely substituted hydroxamates should therefore be useful to clarify functions of metallo-proteases by providing specific inhibitors. Furthermore, they could represent potential starting points for the development of novel therapeutic agents, as exemplified by current clinical trials.[15] To our knowledge, only one group reported the design of a chemical library explicitly designed to interact with multiple members of the metallo-protease family.[16] Here, we describe the preparation and first screening results of a library of malonylhydroxamic acids, based on a simple convergent solution synthesis using diverse amines and *O*-*tert*-butyl hydroxamic acids bearing a free carboxylic acid function.

As a proof of concept, we have screened our library on APN (an enkephalin-processing enzyme also called APN/CD13), the prototypal metallo-protease of the M1 family.

2. Library design

2.1. Selection of hydroxamic acid as Zn-chelating moiety

Many metallo-enzyme inhibitors currently investigated contain an hydroxamate to chelate the zinc ion present

Keywords: Metallo-protease inhibitors; Hydroxamate; Malonic; Zn^{2+} binding group; Targeted library; Chemical biology.
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in the catalytic site of hydrolases (Fig. 1).[17,18] When targeting metallo-proteases, an hydroxamate is convenient to achieve high affinity at the screening stage. More specifically, *N*-acylamino-acid or *N*-succinylamino-acid derived hydroxamates and naphthylthioacetic acid hydroxamate were independently reported as APN inhibitors.[19–22] Hydroxamates have been reported to have poor pharmacokinetic properties due to the hydrolysis of the hydroxamic acid to the corresponding shorter and inactive carboxylic acids.[23,24] Strategies have been developed to eliminate the metabolic liability of hydroxamate-containing inhibitors, which may limit their usefulness as clinical agent, such as the incorporation of modifications at or near the hydroxamate moiety in an attempt to decrease the rate of hydrolysis or biliary excretion in vivo.[9,25,26] Indeed, many successes, not yet materialized with the launch of a new drug on the market, are reported.[27]

2.2. Selection of the malonic template

In order to introduce diversity in our structures, we wanted our template to possess both a carboxylic function that can be transformed into a hydroxamic acid, and at least one other readily diversifiable function (COOH or NH_2). A survey of bioactive hydroxamates in MDDR database (Table 1) showed that out of 1679 structures, hydroxamic acids derived from succinic acid (substituted or not) have already been studied comprehensively (Entry 1). Indeed, due to a good flexibility, succinyl hydroxamic acids display a conformational space that overlaps significantly with the conformational space of their amino-acid homologues, allowing a substrate-like binding mode. In particular, this substructure is found in many MMPs inhibitors.[28] Amino-acid derived hydroxamates are the second most represented subfamily (Entry 2). Thus, in order to explore a more virgin chemical space and to get out of the peptide field, we chose as a core template of our library the malonic template which has only been marginally studied (Entry 7). Compared to succinyl hydroxamic acids, malonic compounds are more constrained and less susceptible to bind in a substrate-like binding mode to the targets.[29] Moreover, various substituents can easily be introduced at the malonic carbon. We have previously shown that non-peptidic quinoline-based malonic hydroxamic acids could inhibit proteases of the M1 family.[30] A small series of malonic-based hydroxamates were also described as inhibitors of MMP-8 and ECE, two metallo-proteases belonging to the M10 and M13 family, respectively.[29,31]

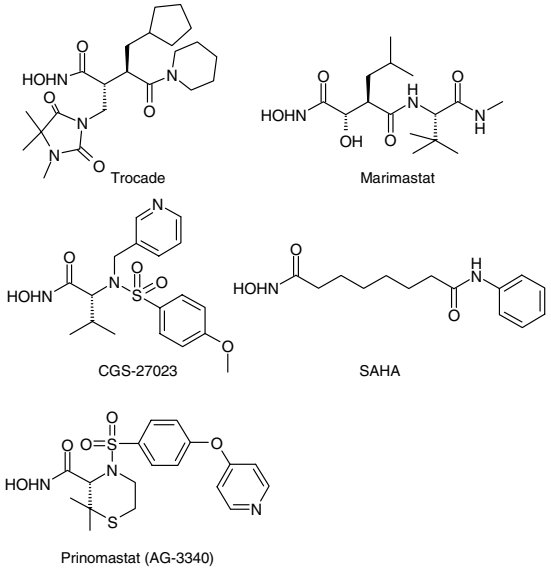
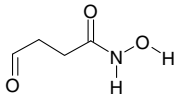
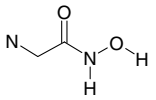
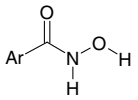
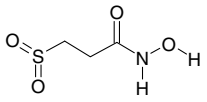
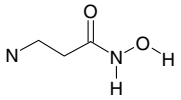
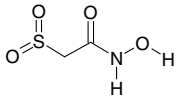
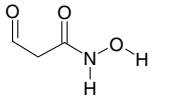


Figure 1. Hydroxamates in the clinic.

Table 1. Chemical structures of hydroxamic acids in bioactive compounds[a]

Entry	Substructure search	% of total
		
1		32.6[b]
2		19.2
3		7.1
4		6.9[b]
5		5.5
6		5.1
7		0.9

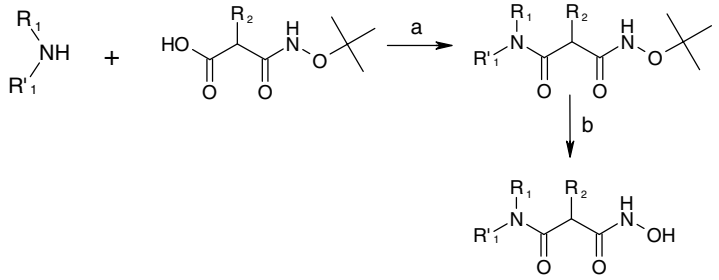
Atoms with no explicit hydrogens were allowed to bear any substituents.

[a] Search updated in March 2006 within MDL Drug Data Report from Prous Science Publisher.

[b] Queries 4 and 1 show some overlap.

2.3. Selection of inputs

Scheme 1 shows the general solution-phase method for the parallel synthesis of library members, using the malonic acids and amines presented in Figures 2 and 3. The malonic acids (**1**–**7**) were synthesized through different procedures to display a variety of substituents on the malonic carbon: hydrogen, a polar group: amino group, different hydrophobic groups: an isobutyl group and four different aromatic groups, to target diverse metallo-proteases.



Scheme 1. Reagents and conditions: (a) i—acid 0.5 M/DMF (1 equiv), DIEA (1.1 equiv), CDI (1.1 equiv) 0.25 M/THF, 2 h, rt; ii—amine 0.1 M/DMF (1 equiv), DIEA (1 equiv), rt, 2 h; (b) BTFA 0.5 M/TFA, 0.4% H_2O (30 equiv), 0 °C, 2 h to overnight.

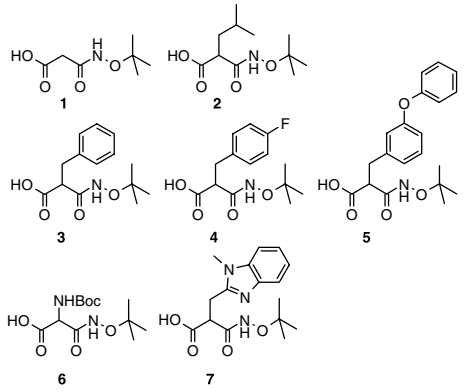


Figure 2. Carboxylic acids inputs.

Amines were chosen in order to generate a diversity of pharmacophore properties and geometries. Amines with linear backbones of various lengths were selected (Series A–E). Cyclic amines were also incorporated into the library (Series F), as well as amines displaying two phenyl rings (Series G).

3. Synthesis[32]

Commercially available amines were purchased, whereas amine **22** was synthesized from commercially available 4-phenyl-butylamine via a reductive amination (Scheme 2).

Spiropiperidine **33**, a privileged structure, was synthesized by condensation of *ortho* hydroxy-acetophenone and *N*-benzyl-piperidone.[33] Reductive ring extension was performed on the corresponding oxime with diisobutylaluminium hydride (DIBAL-H). Finally, the benzyl protection was removed to give **33** (Scheme 3).

The synthesis of the malonic acids inputs proceeds in three steps when the corresponding diethyl malonic ester (**45a**–**c**) is commercially available (Scheme 4): mono-saponification of the diester, coupling of H_2N–O–*t*-Bu·HCl and saponification of the remaining ethyl ester.[29] Other substituted malonic acids were obtained from *N*-*tert*-butoxy-malonamic acid ethyl ester (**49**) and aldehydes. (Scheme 5). Malonic acid **1** was prepared from **49** by saponification.

According to Scheme 1, the first step of the library synthesis consists in coupling an amine to a malonic acid derivative using *N*,*N*′-carbonyldiimidazole (CDI). All protections, *tert*-butyl based, were cleaved in acidic conditions (boron tris(trifluoroacetate) (BTFA) in TFA).[34,35] Though BTFA is not easily synthesized and handled, it was preferred over TFA/DCM solutions since it yielded the desired deprotected products in shorter times (2 h vs 72 h).

The 217 (7×31) library members were synthesized in deepwell plates at a 5 μmol scale. The library was analyzed by HPLC (detection at 215 nm) and MS. Out of the 217 library members, 70% displayed purity above 80%, which fulfils our specifications for screening.[36]

4. Screening

The whole library was screened against mammalian Neutral Aminopeptidase (microsomal), in order to prove that our library could deliver hits on a given zinc metallo-enzyme and be a useful tool for the study of other metallo-enzymes. Screening was performed at a classical concentration of 10 μM.[37] Enzyme activity was assessed at 405 nm by the release of the chromogenic *p*-nitro-aniline resulting from the cleavage of the substrate Leu-*p*-nitro-anilide. A Z' factor of 0.75 was obtained using these conditions, which allowed us to screen singlicate.[38] Tables of inhibition percentages are given in the Supporting Information Section. As expected from an array of hydroxamates, our library yielded a significant number of hits on APN.

5. Statistical results

Tables 2 and 3 display statistics on screening results sorted by class of inputs. The contrast of activities observed when results are sorted by malonic acid is consistent with (1) the design of the library and (2) the binding site of APN.

Binding of the hydroxamate to the Zn^{2+} ion is probably the critical anchoring event and it is expected that the enzyme discriminates more dramatically amongst substituents close to the hydroxamate than amongst those remotely bound by a flexible chain. Carboxylic acid **2** bearing the isobutyl moiety gave the highest number

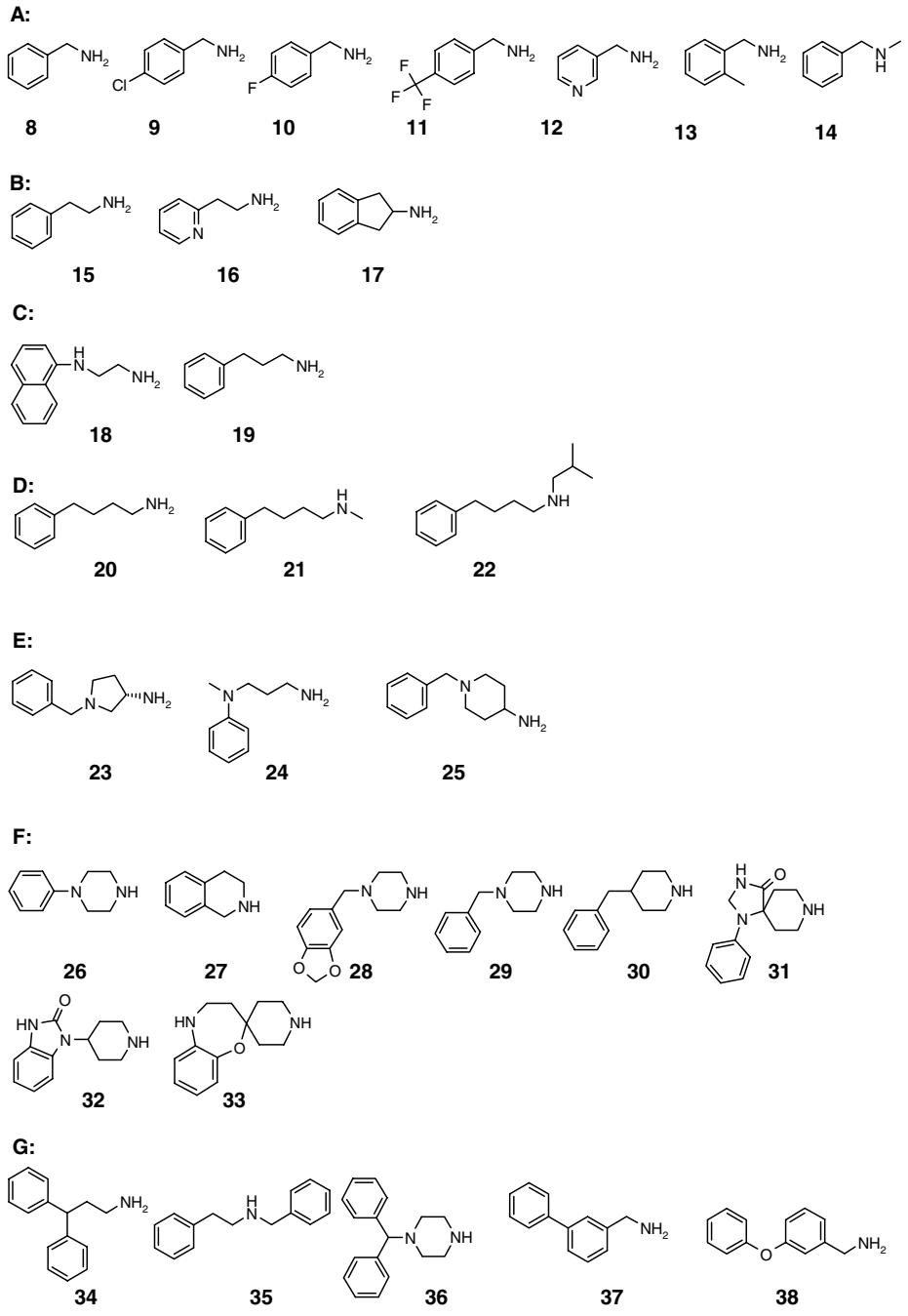
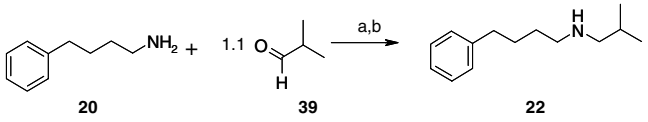


Figure 3. Amines inputs.



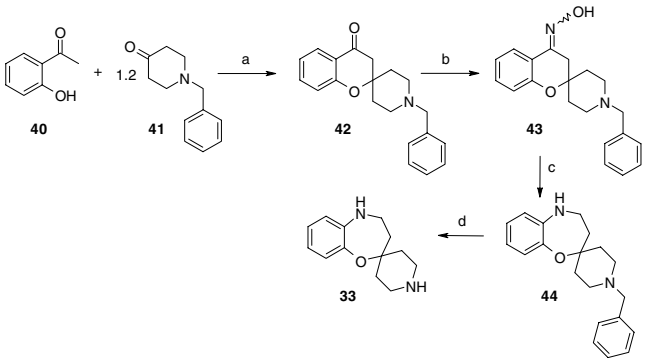
Scheme 2. Reagents and conditions: (a) 0.4 equiv TEA, DCM, molecular sieves 4 Å, 3 h, rt; (b) 5 equiv $NaBH_4$, overnight, rt.

of hits above 80% of inhibition. This is consistent with known substrate preferences for the pockets surrounding the Zn^{2+} ion in APN.[19] Interestingly, an aromatic substituent is also accepted.

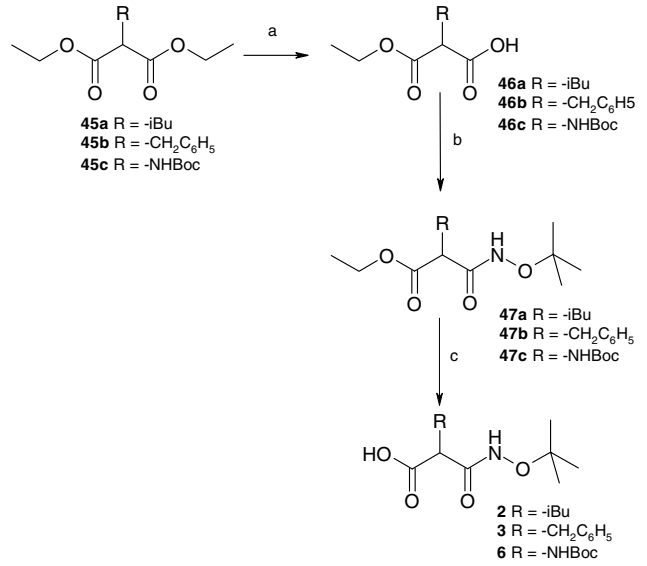
The amine substituents are supposed to bind to more remote pockets relative to the Zn^{2+} ion. Only constrained amines (series F) gave lower hit rates than the other series. In order to confirm the overall behaviour of the library, we resynthesized a limited number of compounds to establish primary structure–activity relationships as described in the next section.

6. Re-synthesis of selected hits and analogues

Library members displaying a percentage of inhibition above 80% as well as some structurally similar, but less



Scheme 3. Reagents and conditions: (a) 0.5 equiv pyrrolidine, MeOH, reflux, overnight; (b) 2 equiv $NH_2OH{\cdot}HCl$, 2 equiv pyridine, EtOH, reflux, 2 h; (c) 5.8 equiv DIBAL-H, anhyd DCM, 0 °C, 3 h then MeOH, H_2O, 20% H_2SO_4, 20 min, then 30% NaOH to pH 9; (d) H_2, Pd/C (10%), MeOH, 18 h, rt.



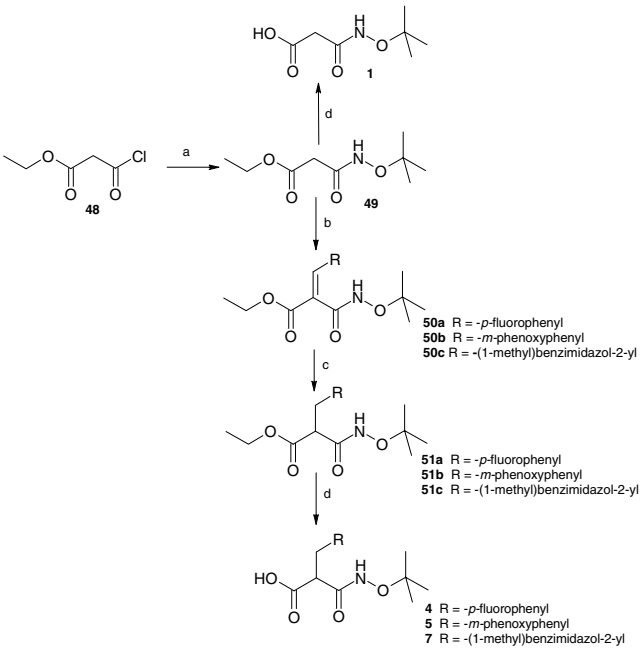
Scheme 4. Reagents and conditions: (a) 1 equiv KOH, EtOH, cat. H_2O, 2–3 h, rt; (b) 3 equiv oxalylchloride, DCM, cat. DMF, 1 h, 0 °C then rt, 1 equiv *O*-*t*-Bu-hydroxylamine·HCl, 3 equiv DIEA, DCM, 2 h, rt; or 1.1 equiv EDCI, 1.1 equiv HOBt, 1 equiv *O*-*t*-Bu-hydroxylamine·HCl, 2 equiv DIEA, THF, rt, overnight; (c) 3 equiv KOH, EtOH, cat. H_2O, overnight, rt.

active library members were selected for resynthesis and further biological characterization (IC_{50}). For some compounds, *O*-tritylhydroxamate precursors were used instead of *O*-*tert*-butylhydroxamates to avoid the use of BTFA at a larger scale (Scheme 6).

Trityl is easily removed using TFA 2% in DCM in the presence of triisopropylsilane at rt, in less than 5 min. All re-synthesized compounds were controlled by TLC, RP-HPLC, mass spectrometry and NMR.

7. Structure–activity relationships

IC_{50}s obtained on resynthesized samples are given in Table 4.



Scheme 5. Reagents and conditions: (a) 1 equiv *O*-*t*-Bu-hydroxylamine·HCl, 2.1 equiv DIEA, DCM, 4 h, rt; (b) 1.1 equiv malonic derivative, 1 equiv aldehyde, cat. piperidine, EtOH, 3–4 h, reflux; (c) 3.5–6 equiv $NaBH_3CN$, acetic acid, acetonitrile, 3–24 h, rt; (d) 3 equiv KOH, EtOH, cat. H_2O, overnight, rt.

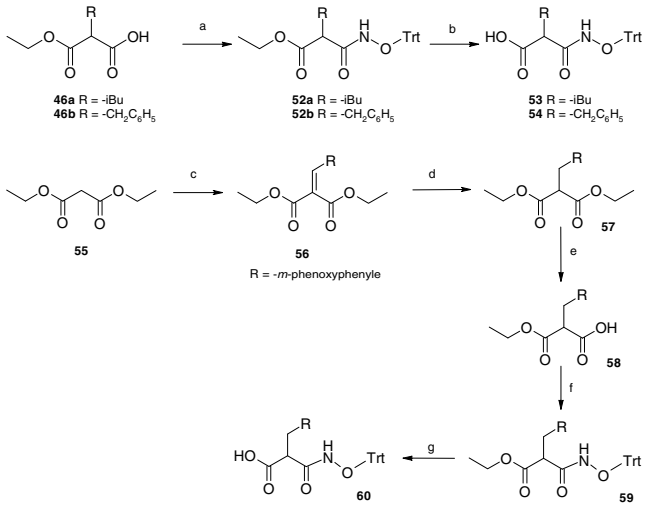
Table 2. Percentage of library members derived from carboxylic acids in the corresponding range of inhibition

Range of % inhibition at 10 μM	Carboxylic acid inputs						
	1	**2**	**3**	**4**	**5**	**6**	**7**
>90%	0	42	6	0	3	0	0
80–90%	3	13	13	3	42	0	0
50–80%	6	13	29	39	42	16	45
<50%	91	32	52	58	13	84	55

Table 3. Percentage of library members derived from class of amine inputs in the corresponding range of inhibition

Range of % inhibition at 10 μM	Classes of amine inputs						
	A	**B**	**C**	**D**	**E**	**F**	**G**
>90%	6	10	15	10	5	4	10
80–90%	12	14	7	10	19	6	10
50–80%	37	33	21	29	19	14	32
<50%	45	43	57	51	57	76	48

We first analyzed data from compounds synthesized from malonic acid **2** (R_2 = isobutyl) among which most of the hits were identified. In this class of compounds, activity depends on the choice of the amine input. A four carbon chain between the amide NH and the terminal aromatic ring gives the best activity (compounds **64** and **65**, IC_{50} = 82 and 452 nM, respectively). Compounds **65** and **66** were useful to study the influence of flexibility on the activity. Both compounds are less active than **64**. Interestingly, **66**, the most constrained compound, also a direct analogue of **61**, is seven times less active than **65**. This suggests that the chain between the nitrogen atom and the aryl moiety needs to be



Scheme 6. Reagents and conditions: (a) i—1 equiv carboxylic acid, 2 equiv oxalyl chloride, cat. DMF, DCM, 1 h, rt; ii—1.2 equiv *O-tert*-hydroxylamine, 1.2 equiv DIEA, DCM, 3 h, rt; (b) EtOH/DCM, 4 equiv KOH, cat. H_2O, 24 h, rt; (c) 1 equiv diethyl malonate, 1 equiv 3-phenoxybenzaldehyde, cat. piperidine, EtOH, 3 h 30, reflux; (d) 4 equiv $NaBH_3CN$, acetonitrile, 16 h, rt; (e) 1 equiv KOH, 1 equiv H_2O, EtOH, 4 h, rt; (f) i—1 equiv carboxylic acid, 1.2 equiv oxalyl chloride, cat. DMF, DCM, 1 h, rt; ii—3 equiv DIEA, 0.85 equiv *O-tert*-hydroxylamine, DCM, 4 h, rt; (g) 4 equiv KOH, 4 equiv H_2O, EtOH, 24 h, rt.

extended to provide the required distance. The partial loss of activity of compound **65** cannot be unambiguously attributed to the conformational effect of the piperidine ring since it has lost a NH functionality compared to **64**. Introduction of a chlorine or fluorine atom in the para-position of the phenyl ring of **61**, (compounds **68** and **67**) leads to a 3- or 5-fold loss of activity. Interestingly, introduction of a phenoxy substituent in the meta position of the phenyl ring of **61** (compound **69**) improves activity that is now comparable to **65**. This confirms the need for a 4- to 5-atom chain between the terminal aromatic ring and the amide NH.

As suggested by the screening results, compounds where R_2 is benzyl or 3-phenoxy-benzyle are less active, independently of the chain length of the amine input (**70** and **73** vs **61**, and **71** and **74** vs **64**). Interestingly, compound **72**, derived from (1-methyl-1*H*-benzo-imidazol-2-ylmethyl)-malonamic acid and benzylamine, displays a submicromolar IC_{50}.

8. Conclusion

We designed and prepared via parallel synthesis a library of 217 hydroxamic acids, targeting metallo-proteases. As a proof of concept, these compounds were tested for their ability to inhibit microsomal APN, a prototypal aminopeptidase of the M1 family. We successfully identified new inhibitors of this enzyme demonstrating the usefulness of the library. Furthermore, compound **64**, displaying an IC_{50} of 82 nM, is a reliable starting point for further optimization. As suggested by our preliminary SAR analysis, a way to increase its activity could be to rigidify the carbon backbone between the hydroxamate and the aromatic ring. More generally, we expect our library to be a useful tool in our current attempt to elucidate the function of recently identified metallo-proteases of the M1 family. Finally, the diversity of the library, already revealed by our screening results, suggests that it could be useful for the study of other classes of Zn metallo-proteases.

9. Experimental

9.1. Biology

Microsomal neutral aminopeptidase (APN) from porcine kidney was purchased from Sigma. Inc. as an ammonium sulfate suspension (3.5 M $(NH_4)_2SO_4$ solution containing 10 mM $MgCl_2$) 10–40 U/mg protein. The enzyme suspension was diluted 600-fold in Tris–HCl buffer (25 mM, pH 7.4) before use. The assays were performed in 96-well plates. The compounds were tested at the concentration of 10 μM. Thirty-three microliters of purified APN was pre-incubated for 10 min at rt with 33 μL of the inhibitor (30 μM in Tris–HCl buffer, 0.3% DMSO). Thirty-three microlitres of the substrate Leucine–para-nitroanilide (Leu-pNA) (K_m = 0.099 mM) 0.3 mM in Tris–HCl buffer was then added. The reaction kinetics was followed on a UV-microplate reader MultiskanRC (Labsystems, Finland) at 405 nm. The control activity was determined by incubating the enzyme in the same conditions without inhibitor. Bestatin was used as the reference inhibitor (IC_{50} = 2.7 μM). The statistical Z' factor for the test was 0.75, allowing activities to be determined with a single point with a 95% confidence. Initial velocities are expressed in μmol min^{-1}. Data were normalized to the controls that represent V^{max}. For the determination of IC_{50}s, initial velocities were plotted as a function of inhibitor concentration, using XLfit software from IDBS™.

9.2. Chemistry

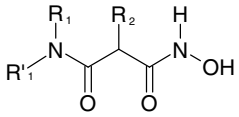
9.2.1. General information. NMR spectra were recorded on a Bruker DRX-300 spectrometer. Chemical shifts are in parts per million (ppm). The assignments were made using one-dimensional (1D) ^1H and ^{13}C spectra and two-dimensional (2D) HSQC and COSY spectra.

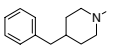
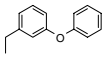
Mass spectra were recorded on a MALDI-TOF Voyager-DE-STR spectrometer, or with a LC/MS/MS Varian 1200ws.

HPLC analysis was performed using a C18 TSK-GEL Super ODS 2 μm particle size column, dimensions 50× 4.6 mm. A gradient starting from 100% H_2O/0.05% TFA and reaching 20% H_2O/80% CH_3CN/0.05% TFA within 10 min at a flow rate of 1 mL/min was used. LCMS gradient starting from 100% H_2O/0.1% formic acid and reaching 20% H_2O/80% CH_3CN/0.08% formic acid within 10 min at a flow rate of 1 mL/min was used.

Melting points were determined on a Büchi B-540 apparatus.

Table 4. Inhibition of APN by compounds **61**–**74** (IC_{50} nM)



Compound	$R_1R'_1N$–	*n*	R_2–	IC_{50}[a] (nM)
61	C_6H_5–$(CH_2)_n$–NH–	1		590
62	C_6H_5–$(CH_2)_n$–NH–	2		984
63	C_6H_5–$(CH_2)_n$–NH–	3		528
64	C_6H_5–$(CH_2)_n$–NH–	4		82
65		—		452
66		—		3040
67	4-F–C_6H_4–$(CH_2)_n$–NH–	1		3000
68	4-Cl–C_6H_4–$(CH_2)_n$–NH–	1		2520
69	3-C_6H_5–*O*-C_6H_4–$(CH_2)_n$–NH–	1		443
70	C_6H_5–$(CH_2)_n$–NH–	1		>10,000
71	C_6H_5–$(CH_2)_n$–NH–	4		>10,000
72	C_6H_5–$(CH_2)_n$–NH–	1		718
73	C_6H_5–$(CH_2)_n$–NH–	1		>10,000
74	C_6H_5–$(CH_2)_n$–NH–	4		>10,000

[a] Bestatin IC_{50} (nM): 2700.

All commercial reagents and solvents were used without further purification. Organic layers obtained after extraction of aqueous solutions were dried over $MgSO_4$ and filtered before evaporation in vacuo. Purification yields were not optimized. Bond-Elut SAX-columns were purchased from Varian.Inc. All the 5 μmol-scale reactions were performed in 96-well (round-bottomed) polypropylene plates (1200 μL).

9.2.2. *N*-Isobutyl-(4-phenylbutylamine) (22). In a round-bottomed flask, 3.97 mL of 4-phenylbutylamine (**20**) (1 equiv), 2.50 mL of isopropyl-aldehyde (**39**) (1.1 equiv) and 1.39 mL of triethylamine (0.4 equiv) were dissolved in 15 mL DCM. Molecular sieves (4 Å) were added, and the reaction mixture was stirred for 3 h at rt. The flask was cooled in an ice-bath and 4.75 g $NaBH_4$ (5 equiv) was added slowly. The reaction mixture was stirred overnight at rt and then quenched with 20 mL of water. The reaction mixture was filtered and the aqueous phase was washed three times with DCM. Organic phases were joined, dried over $MgSO_4$ and evaporated under reduced pressure. The desired product was purified via silica gel chromatography (95 DCm, 4 MeOH, 1 NH_4OH). Compound **22** was obtained as a yellow liquid. Yield 46%, purity 100%, NMR ^1H (DMSO-d_6) δ ppm: 0.83 (d, J = 6.6 Hz, 6H), 1.35–1.43 (m, 2H), 1.52–1.64 (m, 3H), 2.26 (d, J = 6.7 Hz, 2H), 2.47 (t, J = 7.1 Hz, 2H), 2.55 (t, J = 7.5 Hz, 2H), 7.10–7.27 (m, 5H); MS $(MH)^+$ *m*/*z* 206.

9.2.3. 4,5-Dihydro-3*H*-spiro[1,5-benzoxazepine-2,4′-piperidine] (33). *N*-Benzyl-4-piperidone (**41**) (6.8 g, 36 mmol, 1.2 equiv) and 2-hydroxyacetophenone (**40**) (4.1 g, 30 mmol, 1 equiv) were dissolved in MeOH (30 mL) under stirring, followed by addition of pyrrolidine (1.25 mL, 15 mmol, 0.5 equiv). The mixture was heated overnight under refluxing conditions. The reaction mixture was concentrated and dissolved in AcOEt (200 mL). The solution was washed with Na_2CO_3 1 M (3× 50 mL), water (50 mL), brine (50 mL), dried over $MgSO_4$ and concentrated to give a yellow solid. The residue was recrystallized in methanol to give (**42**) as light yellow crystals (10.5 g). Yield 98%, mp 99.6–100.9 °C; NMR ^1H ($CDCl_3$) ppm: 1.75 (m, 2H), 2.03 (d, J = 17.5 Hz, 2H), 2.46 (td, J = 6.6 Hz, J = 2.7 Hz, 2H), 2.65 (dt, J = 11.9, 3.8 Hz, 2H), 2.7 (s, 2H), 3.5 (s, 2H),

6.8 (ddd, J = 8.2, 6.2, 1.2 Hz, 2H), 7.3 (m, 5H), 7.47 (m, 1H), 7.84 (dd, J = 8.2, 1.8 Hz, 1H); MS $(MH)^+$ m/z 308. Compound **42** (8.33 g, 27.1 mmol, 1 equiv), hydroxylamine hydrochloride (3.77 g, 54.2 mmol, 2 equiv) and pyridine (4.38 mL, 54.2 mmol, 2 equiv) were mixed in EtOH (25 mL). The mixture was then stirred under refluxing conditions. After 2 h, the reaction mixture was filtered and the filtrate was washed with water. The residue was recrystallized in MeOH to give oxime (**43**) as a white powder (5.6 g). Yield 64%, mp 229–231 °C; NMR ^1H (DMSO-d_6) δ ppm: 1.6 (ddd, J = 13.9, 13.0, 3.9 Hz, 2H), 1.73 (dm, J = 13.9 Hz, 2H), 2.3 (ddm, J = 13.0, 12.36 Hz, 2H), 2.5 (m, 2H), 2.75 (s, 2H), 3.45 (s, 2H), 6.85 (m, 2H), 7.2–7.3 (m, 6H), 7.75 (dd, J = 7.8, 1.6 Hz, 1H), NMR ^{13}C (DMSO-d_6) δ ppm: 32.1, 35.3, 40.5, 49.6, 63.2, 75.8, 119.0, 122.3, 124.6, 128.7, 129.2, 130.1, 132.2, 146.2, 155.7, 164.1; MS $(MH)^+$ m/z 323. Oxime (**43**) (3 g, 9.3 mmol, 1 equiv) was dissolved in anhydrous DCM (20 mL). The mixture was stirred at 0 °C for 30 min, and 1 N diisobutylaluminium hydride in DCM (54 mL, 54 mmol, 5.8 equiv) was added dropwise over 1 h. The mixture was stirred for 3 h under nitrogen at 0 °C. The reaction was quenched by slowly adding MeOH (9 mL), followed by distilled water (9 mL) and 20% sulfuric acid (50 mL). The solution was stirred for a further 20 min. The solution was basified to pH 9 using a 30% sodium hydroxide solution. The resulting mixture was extracted with AcOEt (2× 100 mL). The organic layer was dried over $MgSO_4$ and concentrated to give a yellow oil. The residue was purified by chromatography on alumina with cyclohexane/AcOEt (1:1, v/v) to give 1.5 g of (**44**) a yellow oil, which crystallized. Yield 52%, mp 99–101 °C; NMR ^1H ($CDCl_3$) δ ppm: 1.54 (ddd, J = 12.8, 12.6, and 4.6 Hz, 2H), 1.82 (t, J = 5.2 Hz, 2H), 1.86 (dm, J = 12.6 Hz, 2H), 2.40 (ddm, J = 12.6, 11.4 Hz, 2H), 2.51 (dm, J = 11.4 Hz, 2H), 3.18 (t, J = 5.2 Hz, 2H), 3.47 (s, 2H), 6.53 (dd, J = 7.7, 1.6 Hz, 1H), 6.63 (td, J = 75, 1.6 Hz, 1H), 6.77 (td, J = 7.5, 1.5 Hz, 1H), 6.87 (dd, J = 7.8, 1.5 Hz, 1H), 7.15–7.30 (m, 5H). NMR ^{13}C ($CDCl_3$) δ ppm: 34.3, 40.2, 40.9, 48.7, 62.6, 117.7, 119.1, 123.0, 123.8, 126.2, 127.4, 127.4, 128.5, 137.7, 141.8; MS $(MH)^+$ m/z 309. Protected amine (**44**) (1 g, 3.1 mmol, 1 equiv) was dissolved in MeOH (5 mL). (10%) Pd/C (0.1 g) was added and the mixture was stirred for 18 h at rt under hydrogen. The mixture was filtered on Celite, washed with MeOH (20 mL) and the filtrate was dried over $MgSO_4$ and concentrated to give 0.68 g of the free amine (**33**) as a white powder. Yield 100%, purity 97%, NMR ^1H (DMSO-d_6) δ ppm: 1.38–1.48 (m, 4H), 1.79–1.84 (m, 4H), 3.15–3.19 (m, 2H), 3.64–3.69 (m, 2H), 5.38–5.41 (m, 1H), 6.55 (td, J = 7.8, 1.8 Hz, 1H), 6.67 (dd, J = 7.8, 1.5 Hz, 1H), 6.78 (td, J = 7.5, 1.5 Hz, 1H), 6.84 (dd, J = 7.8, 1.2 Hz, 1H); MS $(MH)^+$ m/z 219.

9.2.4. *N*-*tert*-Butoxy-malonamic acid (1). In a round-bottomed flask, 5 g of *O*-*tert*-butyl-hydroxyl-amine·HCl (40 mmol, 1 equiv) was dissolved in DCM (200 mL) and DIEA (14.6 mL, 2.1 equiv). The reaction mixture was cooled in an ice bath and the acid chloride (**48**) (40 mmol, 5.06 mL) was added slowly. After stirring for 4 h at rt, the reaction was quenched with $NaHCO_3$ 5%. The organic phase was extracted, dried and evaporated under reduced pressure to give (**49**) as a brown oil. Yield 95%, NMR ^1H ($CDCl_3$) δ ppm: 1.26–1.31 (m, 12H), 3.36 (s, 2H), 4.21 (q, J = 7.1 Hz, 2H), 9.05 (s, NH), NMR ^{13}C ($CDCl_3$) δ ppm: 14.4, 26.6, 40.5, 60.8, 82.8, 164.2, 169.0; MS $(MH)^+$ m/z 204.2, $(MNa)^+$ m/z 226.1.

In a 100 mL round-bottomed flask was dissolved 60 mmol KOH (3 equiv) in ethanol (50 mL) and water (1 mL). Compound **49** was then added (20 mmol, 1 equiv) and the reaction mixture was stirred overnight at rt. The solvent was removed under reduced pressure and the crude product was dissolved in water. The aqueous phase was acidified with concd HCl and the product was extracted (6×) with AcOEt. The organic phases were joined, dried over $MgSO_4$ and evaporated under reduced pressure to give (**1**) as a beige powder. Yield 77%, NMR ^1H (DMSO-d_6) δ ppm: 1.14 (s, 9H), 3.03 (br s, 2H), 10.46 (s, 1H, NH), 12.46 (br s, 1H, COOH), NMR ^{13}C (DMSO-d_6) δ ppm: 26.5, 38.3, 82.9, 166.3, 169.4.

9.2.5. 2-*tert*-Butoxycarbamoyl-4-methyl-pentanoic acid (2). In a 50 mL flask containing a solution of KOH (898 mg, 1 equiv) in absolute ethanol (20 mL) was added 2-isobutyl-malonic diethyl ester (**45a**) (3.57 mL, 16 mmol, 1 equiv). The reaction mixture was stirred at r t for 2 h. Ninety percent of the solvent was then evaporated under reduced pressure. A solution of $NaHCO_3$ 5% (20 mL) was added and the remaining diethyl ester was extracted with AcOEt. The aqueous phase was acidified with $KHSO_4$ (powder), at 0 °C. The expected product was extracted with AcOEt. The organic phases were joined, dried over $MgSO_4$ and evaporated under reduced pressure (max temp 25 °C) to give 2-isobutyl-malonic acid monoethyl ester (**46a**) as a colourless oil. Yield 75%, NMR ^1H ($CDCl_3$) δ ppm: 0.93 (d, J = 6.5 Hz, 6H), 1.28 (t, J = 7.1 Hz, 3H), 1.57–1.65 (m, 1H), 1.80–1.89 (m, 2H), 3.46 (t, J = 7.6 Hz, 1H), 4.22 (q, J = 7.1 Hz, 2H), 10.7 (br s, 1H, COOH). NMR ^{13}C ($CDCl_3$) δ ppm: 14.1, 22.2, 22.3, 26.2, 37.7, 50.2, 61.6, 169.7, 174.8. Compound **46a** (8 mmol, 1 equiv) was then added with *N*-ethyl-3-(3-dimethyaminopropyl)carbodiimide (EDCI) (1.684 g, 8.8 mmol, 1.1 equiv) and *N*-hydroxybenzotriazole (HOBt) (1.187 g, 8.8 mmol, 1.1 equiv) to a solution of *O*-*tert*-butyl-hydroxyl-amine·HCl (1 g, 8 mmol, 1 equiv) in THF (20 mL) and DIEA (2.77 mL, 16 mmol, 2 equiv). The reaction mixture was stirred overnight at rt. The solvent was removed under reduced pressure and the crude product was solubilized in AcOEt. The organic phase was washed with $KHSO_4$ 5% (5×), $NaHCO_3$ 1 M (5×). The organic phases were dried and evaporated under reduced pressure to give 2-*tert*-butoxycarbamoyl-4-methyl-pentanoic acid ethyl ester (**47a**) as a white powder. Yield 71%, purity 97%, NMR ^1H ($CDCl_3$) δ ppm: 1.07 (d, J = 6.5 Hz, 6H), 1.38–1.42 (m, 12H), 1.70–1.80 (m, 1H), 1.92–1.98 (m, 2H), 3.52 (t, J = 7.5 Hz, 1H), 4.32 (q, J = 7.1 Hz, 2H). In a 50 mL flask containing a solution of KOH (840 mg, 15 mmol, 3 equiv) in absolute ethanol (15 mL) was added (**47a**) (5 mmol, 1 equiv). The reaction mixture was stirred at rt overnight. Ninety

percent of the solvent was then evaporated under reduced pressure. A solution of $NaHCO_3$ 1 M (20 mL) was added and the remaining ester was extracted with AcOEt. The aqueous phase was acidified with $KHSO_4$ (powder), at 0 °C. The expected product was extracted with AcOEt. The organic phases were joined, dried and evaporated under reduced pressure (max temp 25 °C) to give (**2**) as a white powder. Yield 70%, purity 97%, mp 129–132 °C, NMR ^1H ($CDCl_3$) δ ppm: 0.93–0.96 (m, 6H), 1.26 (s, 9H), 1.63–1.69 (m, 1H), 1.79–1.83 (m, 2H), 3.24 (t, $J = 7.0$ Hz, 0.73H), 3.80 (t, $J = 7.0$ Hz, 0.27H), 8.65 (s, 1H, NH).

9.2.6. 2-Benzyl-*N*-*tert*-butoxy-malonamic acid (3). In a round-bottomed flask, containing a solution of KOH (1.68 g, 30 mmol, 1 equiv) dissolved in absolute ethanol (45 mL), was added benzylmalonate diethyl ester (**45b**) (7.5 g, 30 mmol, 1 equiv). The reaction mixture was stirred for 3 h at rt. The solvent was removed under reduced pressure and the residue was partitioned between $NaHCO_3$ 5% and AcOEt. The aqueous phase was then acidified to pH 1 and the expected product extracted with AcOEt. The organic phase was dried and evaporated under reduced pressure to give (**46b**) as a colourless oil. Yield 62%, purity 100%, NMR ^1H (DMSO-d_6) δ ppm: 1.08 (t, $J = 7.0$ Hz, 3H), 3.05 (dd, $J = 4.0$, 7.2 Hz, 2H), 3.68 (dd, $J = 7.4$, 8.7 Hz, 1H), 4.05 (q, $J = 7.1$ Hz, 2H), 7.16–7.29 (m, 5H), 12.9 (s, 1H, COOH). Compound **46b** (4.1 g, 1 equiv, 18.4 mmol) was dissolved in DCM (50 mL) with catalytic DMF (100 μL). The mixture was cooled at 0 °C (ice bath) and oxalyl chloride (4.8 mL, 3 equiv) was added dropwise. The reaction mixture was stirred for 1 h at rt and then evaporated under reduced pressure. The residue was dissolved in DCM and *O*-*tert*-butyl-hydroxylamine·HCl (18.4 mmol, 1 equiv) in suspension in DCM with DIEA (3 equiv) was added. The reaction mixture was stirred for 2 h at r t. The organic phase was washed with $NaHCO_3$ 5% (3× 50 mL), with brine, dried and evaporated under reduced pressure. Trituration with Et_2O/pentane allowed to obtain (**47b**) as a beige powder. Yield 81%, purity 100%, NMR ^1H ($CDCl_3$) δ ppm: 1.09–1.14 (m, 12H), 3.10–3.27 (m, 2H), 3.36 (t, $J = 7.5$ Hz, 1H), 4.06 (q, $J = 7.1$ Hz, 2H), 7.11–7.23 (m, 5H). In a round-bottomed flask was added a solution of KOH (2.150 g, 38.4 mmol, 3 equiv) in ethanol (100 mL), compound **47b** (3.741 g, 12.8 mmol, 1 equiv). The reaction mixture was stirred at rt overnight. The suspension obtained was filtered and washed with AcOEt. The residue was dissolved in a small amount of water and the solution was acidified with concd HCl at pH 1, and extracted with AcOEt. The organic layers were joined, dried and evaporated under reduced pressure to give (**3**) as a white powder. Yield 59%, purity 95%, NMR ^1H (DMSO-d_6) δ ppm: 0.98 (s, 9H), 2.92–3.05 (m, 2H), 3.44 (dd, $J = 5.6$, 9.9 Hz, 1H), 7.12–7.27 (m, 5H), 10.45 (s, 1H, NH), 12.50 (br s, 1H, COOH), NMR ^{13}C (DMSO-d_6) δ ppm: 26.9, 34.5, 51.5, 81.7, 127.0, 128.9, 129.7, 139.5, 166.6, 171.5.

9.2.7. *N*-*tert*-Butoxy-2-(4-fluoro-benzyl)-malonamic acid (4). To a solution of *N*-*tert*-butoxy-malonamic acid ethyl ester (**49**) (7.163 g, 35.2 mmol, 1.1 equiv) and piperidine (1 mL) in absolute ethanol (150 mL) was added 4-fluorobenzaldehyde (32.0 mmol, 1 equiv). The reaction mixture was refluxed for 3h30. Purification over silica gel (DCM/MeOh, 98/2) allowed 7.05 g of (**50a**) as a light yellow solid. Yield 71%, purity 85%, NMR ^1H ($CDCl_3$) δ ppm: 1.21–1.29 (m, 12H), 4.24 (q, $J = 7.1$ Hz, 2H), 7.01 (t, $J = 8.6$ Hz, 2H), 7.51–7.55 (m, 2H), 7.69 (s, 1H), 7.86 (s, NH); MS $(MH)^+$ *m*/*z* 310.2 and $(MNa)^+$ *m*/*z* 332.2. (**50a**) (6.94 mmol) was dissolved in CH_3CN (25 mL). $NaBH_3CN$ (41.6 mmol, 6 equiv) and AcOH (3 mL) were added and the reaction mixture was stirred at rt for 24 h. The solvent was removed in vacuo, the residue dissolved in AcOEt and washed twice with 5% $NaHCO_3$ and twice with water. The organic layer was dried over $MgSO_4$, concentrated to give (**51a**) as a yellow oil. Yield 93%, purity 75%, NMR ^1H ($CDCl_3$) δ ppm: 1.09 (s, 9H), 1.15–1.23 (m, 3H), 3.15 (d, $J = 7.8$ Hz, 2H), 3.52 (t, $J = 7.8$ Hz, 1H), 4.02–4.11 (m, 2H), 6.91 (t, $J = 8.6$ Hz, 2H), 7.08–7.13 (m, 2H); MS $(MNa)^+$ *m*/*z* 334.2. (**51a**) (6.40 mmol) was dissolved in absolute ethanol (65 mL) and KOH (19.2 mmol, 3 equiv) was added to the mixture. After stirring overnight at r t, the solvent was removed and the mixture acidified to pH 1 with HCl 1 N. The aqueous layer was extracted four times with AcOEt. The organic layer was dried over $MgSO_4$ and concentrated to give (**4**) (1.50 g) as a white solid. Yield 83%, purity 92%, mp 146–149 °C, NMR ^1H (DMSO-d_6) δ ppm: 0.99 (s, 9H), 2.92–3.01 (m, 2H), 3.38–3.45 (m, 1H), 7.04–7.11 (m, 2H), 7.18–7.23 (m, 2H), 10.47 (s, NH); MS $(MH)^+$ *m*/*z* 284.1.

9.2.8. *N*-*tert*-Butoxy-2-(3-phenoxy-benzyl)-malonamic acid (5). To a solution of *N*-*tert*-butoxy-malonamic acid ethyl ester (**49**) (31.7 mmol, 1.1 equiv) and piperidine (1 mL) in absolute ethanol (140 mL) was added 3-phenoxybenzaldehyde (28.9 mmol, 1 equiv). The reaction mixture was refluxed for 3 h. The solvent was removed in vacuo and the residue was dissolved in AcOEt, washed with HCl 1 N, $NaHCO_3$ 5% and NaCl, dried over $MgSO_4$ and evaporated. Purification over silica gel (cyclohexane/AcOEt, 80:20) allowed 5.49 g of (**50b**) as a yellow oil. Yield 50%, purity 85%, NMR ^1H (DMSO-d_6) δ ppm: 1.17 (s, 9H), 1.25 (t, $J = 7.1$ Hz, 3H), 4.22 (q, $J = 7.1$ Hz, 2H), 6.99–7.06 (m, 3H), 7.13–7.18 (m, 1H), 7.34–7.47 (m, 5H), 7.66 (s, 1H); MS $(MH)^+$ *m*/*z* 384.2 and $(MNa)^+$ 406.2, (**50b**) (14.3 mmol) was dissolved in CH_3CN (60 mL). $NaBH_3CN$ (85.8 mmol, 6 equiv) and AcOH (6 mL) were added and the reaction mixture was stirred at rt for 24 h. The solvent was removed in vacuo, the residue dissolved in AcOEt and washed twice with 5% $NaHCO_3$ and twice with water. The organic layer was dried over $MgSO_4$, concentrated to give (**51b**) as a yellow oil. Yield 100%, purity 70%, NMR ^1H (DMSO-d_6) δ ppm: 1.03 (s, 9H), 1.17 (t, $J = 7.1$ Hz, 3H), 2.92–3.00 (m, 1H), 3.07 (dd, $J = 5.8$, 13.9 Hz, 1H), 3.54 (dd, $J = 5.9$, 9.7 Hz, 1H), 3.99–4.10 (m, 2H), 6.79–6.82 (m, 1H), 6.88–6.89 (m, 1H), 6.94–7.00 (m, 3H), 7.09–7.15 (m, 1H), 7.24–7.29 (m, 1H), 7.33–7.42 (m, 2H), 10.57 (s, NH); MS $(MH)^+$ *m*/*z* 386.2 $(MNa)^+$ *m*/*z* 408.2, (**51b**) (14 mmol) was dissolved in ethanol (150 mL) and KOH (42 mmol, 3 equiv) was added to the mixture. After stirring overnight at rt, the solvent was removed and the mixture acidified to pH 1 with 1 N HCl. The aqueous layer was extracted

three times with AcOEt. The organic layer was dried over $MgSO_4$ and concentrated to give pure (**5**) as a white solid, yield 90%, purity 95%, NMR ^1H (DMSO-d_6) δ ppm: 1.01 (s, 9H), 2.90–3.07 (m, 2H), 3.37–3.47 (m, 1H), 6.78–6.81 (m, 1H), 6.88–6.89 (m, 1H), 6.95–6.98 (m, 3H), 7.09–7.15 (m, 1H), 7.24–7.29 (m, 1H), 7.34–7.41 (m, 2H), 10.6 (s, NH); MS $(MH)^+$ *m/z* 358.2, $(MNa)^+$ *m/z* 380.2, $(MK)^+$ *m/z* 396.2.

9.2.9. *N*-*tert*-Butoxy-2-*tert*-butoxycarbonylamino-malonamic acid (6). In a 50 mL flask containing a solution of KOH (440 mg, 1 equiv) in absolute ethanol (10 mL) was added 2-*tert*-butoxycaronylamino-malonic acid diethyl ester (**45c**) (2 mL, 7.84 mmol, 1 equiv). The reaction mixture was stirred at rt for 2 h 30 min. Ninety percent of the solvent was then evaporated under reduced pressure. A solution of $NaHCO_3$ 1 M (20 mL) was added and the remaining diethyl ester was extracted with AcOEt. The aqueous phase was acidified with $KHSO_4$ (powder), at 0 °C. The expected product was extracted with AcOEt. The organic phases were joined, dried and evaporated under reduced pressure (max temp 25 °C) to give 2-*tert*-butoxycarbonylamino-malonic acid monoethyl ester (**46c**) as a white powder. Yield 87%, purity 100%, NMR ^1H ($CDCl_3$) δ ppm: 1.32 (t, J = 7.1 Hz, 3H), 1.44 (s, 9H), 4.24–4.33 (m, 2H), 4.78 (d, J = 4.8 Hz, 0.6H), 4.99 (d, J = 7.3 Hz, 0.4H), 5.67 (d, J = 6.9 Hz, 0.4H, NH), 6.65 (d, J = 4.4 Hz, 0.6H, NH), 9.09 (br s, 1H, COOH). To a solution of *O*-*tert*-butyl-hydroxylamine·HCl (780 mg, 6.2 mmol, 1 equiv) in THF (40 mL) and DIEA (2.1 mL, 12.4 mmol, 2 equiv) were added (**46c**) (6.2 mmol, 1 equiv), EDCI (1.31 g, 6.8 mmol, 1.1 equiv) and HOBt (920 mg, 6.8 mmol, 1.1 equiv). The reaction mixture was stirred overnight at r t. The solvent was removed under reduced pressure and the crude product was dissolved in AcOEt and the organic phase was washed with $KHSO_4$ 5% (twice), $NaHCO_3$ 1 M (5×). The organic phases were dried and evaporated under reduced pressure to give (**47c**) as a yellow oil. Yield 75%, purity 100%, NMR ^1H ($CDCl_3$) δ ppm: 1.28 (s, 9H), 1.29 (t, J = 7.2 Hz, 3H), 1.45 (s, 9H), 4.18–4.32 (m, 2H), 4.80 (d, J = 7.5 Hz, 1H), 5.82 (d, J = 6.9 Hz, 1H, NH), 8.95 (br s, 1H, CONHO). In a 50 mL flask containing a solution of KOH (521 mg, 9.3 mmol, 3 equiv) in absolute ethanol (10 mL) was added (**47c**) (990 mg, 3.1 mmol, 1 equiv). The reaction mixture was stirred at rt overnight. Ninety percent of the solvent was then evaporated under reduced pressure. A solution of $NaHCO_3$ 1 M (20 mL) was added and the remaining ester was extracted with AcOEt. The aqueous phase was acidified with $KHSO_4$ (powder), at 0 °C. The expected product was extracted with AcOEt. The organic phases were joined, dried and evaporated under reduced pressure (max temp 25 °C) to give (**6**) as a white powder. Yield 40%, purity 100%, mp 128–132 °C; NMR ^1H ($CDCl_3$) δ ppm: 1.21 (d, J = 5.1 Hz, 9H), 1.39 (d, J = 4.3 Hz, 9H), 4.74 (d, J = 5.9 Hz, 1H), 5.75 (d, J = 5.8 Hz, NH), 9.0 (1H, CONHO).

9.2.10. *N*-*tert*-Butoxy-2-(1-methyl-1*H*-benzoimidazol-2-yl methyl)-malonamic acid (7). To a solution of *N*-*tert*-butoxy-malonamic acid ethyl ester (**49**) (350 mg, 1.72 mmol, 1.1 equiv) and piperidine (54 μL) in absolute ethanol (12 mL) was added 1-methyl-2-formylbenzimidazole (1.56 mmol, 1 equiv). The reaction mixture was refluxed for 4 h. The solvent was removed in vacuo and (**50c**) as a crude oil was directly used without any purification. (**50c**) was dissolved in CH_3CN (6.5 mL). $NaBH_3CN$ (343 mg, 5.46 mmol, 3.5 equiv) and AcOH (0.65 mL) were added and the reaction mixture was stirred at rt for 3 h. The solvent was removed in vacuo, the residue dissolved in AcOEt and washed with $NaHCO_3$ 5% and water. The organic layer was dried over $MgSO_4$, concentrated and the residue was purified by thick layer chromatography on silica gel (DCM/MeOH, 95:5) to give (**51c**). Compound **51c** was dissolved in ethanol (12 mL) and H_2O (0.11 mL, 6.2 mmol) and KOH (348 mg, 6.2 mmol) was added to the mixture. After stirring overnight at rt, the solvent was removed and the mixture acidified to pH 1 with 1 N HCl. The aqueous layer was extracted by DCM. The organic layer was dried over $MgSO_4$ and concentrated to give pure (**7**) as a white solid (280 mg). Overall yield 56 %, NMR ^1H (DMSO-d_6) δ ppm: 1.00 (s, 9H), 3.66–3.38 (m, 2H), 4.01 (s, 3H), 4.07 (dd, J = 6.3 Hz and J = 9.0 Hz, 1H), 7.58–7.54 (m, 2H), 7.78 (d, J = 8.9 Hz, 1H), 7.93 (d, J = 8.9 Hz, 1H), 11.04 (s, 1H, NH), NMR ^{13}C (DMSO-d_6) δ ppm: 26.6, 26.9, 31.9, 47.5, 81.9, 114.8, 126.0, 126.5, 133.2, 152.0, 165.3, 169.9; MS $(MH)^+$ *m/z* 320.0.

9.2.11. Parallel synthesis at the scale of 5 μmol. On a solution of carboxylic acid (0.5 M/DMF; 1 equiv) and DIEA (1.1 equiv) was added *N*,*N*′-carbonyl-diimidazole (0.25 M/THF; 1.1 equiv). The mixture was stirred for 2 h at rt. Then the appropriate amine (0.1 M/DMF; 1 equiv) and DIEA (1 equiv + 1 equiv if the amine was a hydrochloride) were added and the mixture stirred 2 h at r t. Solvents were removed under reduced pressure in a Genevac™ EZ-2. The residue was washed once with HCl 10^{-2} M and solvents were reduced under pressure. A suspension of BTFA (0.5 M/TFA; 30 equiv) with 0.4% H_2O was added and the reaction mixture was stirred for 2 h to overnight at 0 °C and then at rt. The reaction mixture was evaporated under reduced pressure in a Genevac™ EZ-2 and the residue was washed with cyclohexane (3×) and dried in Genevac™ EZ-2.

9.2.12. 4-Methyl-2-trityloxycarbamoyl-pentanoic acid (53). 2-Isobutyl-malonic acid monoethyl ester (**46a**) (7.91 g, 42 mmol, 1 equiv) was dissolved in DCM (140 mL) with DMF (280 μL). To this solution cooled to 0 °C was added dropwise oxalyl chloride (84 mmol, 7.2 mL, 2 equiv). The mixture was stirred at rt for 1 h and evaporated under reduced pressure (max temp 30 °C). The crude product was dissolved in DCM (100 mL). A solution of *O*-tritylhydroxylamine (50 mmol, 13.88 g, 1.2 equiv) in DCM containing DIEA (50 mmol, 8.3 mL, 1.2 equiv), was added dropwise. The reaction mixture was stirred at rt for 3 h. The solution was washed with $NaHCO_3$ 5% and the organic layer was dried and evaporated under reduced pressure. The crude product was dissolved in AcOEt and washed three times with water. The organic layer was dried over $MgSO_4$ and evaporated to give a yellow solid. The solid

was washed with diethyl ether and filtered to provide 3.67 g of (**52a**) as a white powder. Yield 20%, purity 90%, mixture of 2 isomers (67:33) NMR ^{1}H ($CDCl_3$) δ ppm: 0.53–0.56 (m, 2H), 0.69 (d, J = 6.4 Hz, 4H), 1.08 (t, J = 7.1 Hz, 2H), 1.15–1.23 (m, 2H), 1.43 (t, J = 7.6 Hz, 2H), 2.53–2.58 (m, 0.33H), 2.97 (t, J = 7.6 Hz, 0.67H), 3.96 (q, J = 7.1 Hz, 1.33H), 4.06–4.13 (m, 0.67H), 7.21–7.25 (m, 15H); MS $(MH)^+$ m/z 444. Compound **52a** (8.2 mmol, 3.67 g) was dissolved in a mixture of absolute EtOH (70 mL) and DCM (50 mL). KOH (32.8 mmol, 1.85 g, 4 equiv) and water (250 μL) were added. The reaction mixture was stirred at rt for 24 h and evaporated. The crude product was dissolved in water and washed three times with AcOEt. The aqueous phase was acidified with $KHSO_4$ to pH 6 and extracted three times with AcOEt. The organic layers were dried over $MgSO_4$, filtered and evaporated. The product precipitated in Et_2O to give 2.89 g of (**53**) as a white powder. Yield 85%, purity 100%, NMR ^{1}H (DMSO-d_6) δ ppm: 0.62–0.66 (m, 6H), 0.84–0.91 (m, 1H), 1.12–1.27 (m, 2H), 3.08 (t, J = 7.3 Hz, 1H), 7.33 (s, 15H), 10.69 (br s, OH); MS $(MH)^+$ m/z 416.

9.2.13. 2-Benzyl-3-oxo-3-[(trityloxy)amino]-propanoic acid (54). Three grams (13.5 mmol) of 2-benzyl-malonic acid monoethyl ester (**46b**) was solubilized in 25 mL DCM containing 200 μL of DMF. The flask was immerged in an ice bath. A solution of oxalyl chloride (2.3 mL, 2 equiv) in 10 mL DCM was then added dropwise. The reaction mixture was stirred at rt for 1 h. Solvents were removed under reduced pressure and the crude product was dissolved in 25 mL DCM. A solution of *O*-tritylhydroxylamine (1.2 equiv, 4.46 g) in 10 mL DCM and 2.36 mL DIEA (1.2 equiv) was then added. The reaction mixture was stirred at rt for 3 h. Solvents were removed under reduced pressure and the crude product was dissolved in 50 mL AcOEt. The organic layer was washed with NH_4Cl aq (pH 5), K_2CO_3 aq (pH 11) and water, dried over $MgSO_4$ and evaporated under reduced pressure. The product was purified by column chromatography (BPSUP 70 g Silice Flash-smart) with DCM as the eluent. The product obtained was then crystallized in a mixture of diethyl ether and *n*-pentane to give (**52b**) as white powder. Yield 26%, purity 96%, NMR ^{1}H (DMSO-d_6) δ ppm: 0.99 (t, J = 7.2 Hz, 3H), 2.5 (m, 1H), 2.81 (dd, J = 14.4, 8.7 Hz, 1H), 3.50 (dd, J = 8.1, 6.3 Hz, 1H), 3.80–3.97 (m, 2H), 7.00–7.03 (m, 2H), 7.16–7.18 (m, 3H), 7.31 (s, 15H), 10.50 (s, 1H, NH); MS $(MH)^+$ m/z 480. 1.7 g (3.5 mmol, 1 equiv) of (**52b**) was dissolved in 50 mL of a mixture EtOH abs/DCM (4:1). Eight hundred milligrams of KOH (14 mmol, 4 equiv) and 71 μL of water (1 equiv) were added and the mixture was stirred for 24 h at rt. The solvents were evaporated under reduced pressure, the crude product was dissolved in water (7.5 mL), and washed twice with 30 mL AcOEt. The aqueous layer was acidified to pH 6 with $KHSO_4$ and the product was extracted with AcOEt. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure to give an oil that precipitated in pentane to give (**54**) as a white powder. Yield 56%, purity 97%, mp 149–151 °C; NMR ^{1}H (DMSO-d_6) δ ppm: 2.50 (m, 1H), 2.80 (dd, J = 13.8, 7.2 Hz, 1H), 3.21 (m, 1H), 6.93–6.96 (m, 2H), 7.11–7.12 (m, 3H), 7.29 (m, 15H), 10.48 (br, 1H, NH); MS $(MH)^-$ m/z 450.

9.2.14. 2-(3-Phenoxy-benzyl)-*N*-trityloxy-malonamic acid (60). To a solution of diethyl malonate (**55**) (50 mmol, 7.59 mL) in 100 mL of ethyl alcohol absolute were added 3-phenoxybenzaldehyde (50 mmol, 8.62 mL) and piperidine (1 mL). The mixture was heated at reflux for 3h30 and evaporated. The crude product was dissolved in ethyl acetate, washed twice with $NaHCO_3$ 5% and once with NaCl, dried over $MgSO_4$, filtered and evaporated to give (**56**) as a yellow oil. This product was used in the next step without further purification. (**56**) (50 mmol) was dissolved in acetonitrile (100 mL). To this solution cooled to 0 °C was slowly added $NaBH_3CN$ (200 mmol, 12.6 g, 4 equiv). The mixture was stirred at rt for 16 h and evaporated. The crude product was dissolved in AcOEt, and the organic layer was washed once with aq $NaHCO_3$ 5%, 10 times with water and once with NaCl aq, dried over $MgSO_4$, filtered and evaporated. Purification over silica gel (cyclohexane/ethyl acetate, 100:0–98:2) provided 5.14 g of (**57**) as a yellow oil. Overall yield 30%, purity 100%, NMR ^{1}H (DMSO-d_6) δ ppm: 1.09 (t, J = 7.0 Hz, 6H), 3.06 (d, J = 8.1 Hz, 2H), 3.81 (t, J = 8.1 Hz, 1H), 4.05 (q, J = 7.0 Hz, 4H), 6.83–6.84 (m, 1H), 6.86–6.88 (m, 1H), 6.94–7.01 (m, 3H), 7.10–7.15 (m, 1H), 7.28 (t, J = 7.8 Hz, 1H), 7.35–7.40 (m, 2H); MS $(MH)^+$ m/z 343. Six hundred and seventy milligrams of KOH (12 mmol, 1 equiv) was dissolved in ethyl alcohol absolute (50 mL) with water (12 mmol, 220 μL). Compound **57** (12 mmol, 1 equiv) was added, the mixture was stirred at rt for 4 h and evaporated. The residue was dissolved in aq $NaHCO_3$ 5% and washed once with AcOEt to get rid of the diethyl ester. The aqueous phase was acidified with concn HCl to pH 1 and extracted four times with AcOEt. The organic layers were dried over $MgSO_4$, filtered and evaporated to give 3.39 g of (**58**) as a yellow oil. Yield 90%, purity 95%; MS $(MH)^+$ m/z 315. Compound **58** (10 mmol) was dissolved in DCM (30 mL) with DMF (70 μL). To this solution cooled to 0 °C was added dropwise oxalyl chloride (1.0 mL, 12 mmol, 1.2 equiv). The mixture was stirred at rt for 1 h and evaporated under reduced pressure (max temp 30 °C). The residue was dissolved in DCM (30 mL). To this solution cooled to 0 °C was added dropwise DIEA (30 mmol, 4.96 mL, 3 equiv). Then, *O*-tritylhydroxylamine (8.5 mmol, 2.34 g, 0.85 equiv) was added and the mixture was stirred at rt for 4 h. The mixture was washed once with aq $NaHCO_3$ 5% and the organic layer was separated, dried over $MgSO_4$ and evaporated. The oil was precipitated in petroleum ether to give 3.88 g of (**59**) as a beige powder. Yield: 80 %, purity 93%; MS $(MH)^+$ m/z 570. 1.8 g KOH (32 mmol, 4 equiv) was dissolved in absolute EtOH (35 mL) with water (32 mmol, 580 μL). Compound **59** (8 mmol) was added, the mixture was stirred at rt for 24 h and evaporated. The residue was dissolved in water and washed once with AcOEt. The aqueous phase was acidified with $KHSO_4$ to pH 6 and extracted three times with AcOEt. The organic layers were dried over $MgSO_4$, filtered and evaporated to give 3.90 g of (**60**) as a white powder. Yield 90%, purity 95%, NMR ^{1}H (DMSO-d_6) δ ppm:

2.42 (dd, $J = 13.6$ Hz, $J = 5.2$ Hz, 1H), 2.81 (dd, $J = 13.5$, 7.5 Hz, 1H), 3.15–3.19 (m, 1H), 6.72–6.76 (m, 2H), 6.95 (d, $J = 8.4$ Hz, 2H), 7.10–7.14 (m, 2H), 7.25–7.40 (m, 18H); MS $(M-H)^-$ *m/z* 542.

9.2.15. Method A: general procedure for the synthesis of selected compounds using tertbutyle building-blocks. Carboxylic acid (0.7–0.8 mmol, 0.5 M/DMF, 1 equiv), DIEA (1.2 equiv) and bromo-tris-pyrrolidinophosphonium-hexa-fluorophosphate (PyBrop) (0.2 M/DCm, 1.2 equiv) were stirred for 30 s at rt. Then the appropriate amine (0.1 M/DMF, 1 equiv) and DIEA (2 equiv + 1 equiv if the amine is a hydrochloride) were added, the mixture was stirred overnight at rt and then solvents were removed under reduced pressure. The residue was dissolved in DCM and the organic phase was washed with aqueous HCl 1 N (3×), aq $NaHCO_3$ 5% (3×) and water (once). The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by thick layer chromatography on silica gel (DCM/MeOH, 95:5). *tert*-Butyl intermediate was dissolved in a suspension of BTFA (0.75 M/TFA, 25 equiv) with 0.4% H_2O. The reaction mixture was stirred for 4 h to 5 h 30 min at rt. Then, solvents were removed under reduced pressure and the residue was dissolved in water. The aqueous phase was basified with NaOH 1 M to pH 7 and extracted three times with AcOEt. The organic layers were dried over $MgSO_4$, filtered and evaporated. The residue was precipitated in ether–pentane. Yields given are those of the deprotection step.

9.2.16. Method B: general procedure for the synthesis of selected compounds using trityle building-blocks. Carboxylic acid (0.3–0.4 mmol, 0.5 M/DMF, 1 equiv), DIEA (1.1 equiv) and *N*,*N*′-carbonyldiimidazole (0.25 M/THF, 1.1 equiv) were stirred 2 h at rt. Then the appropriate amine (0.1 M/DMF, 1 equiv) and DIEA (2 equiv + 1 equiv if the amine is a hydrochloride) were added, the mixture was stirred for 2 h at rt and then solvents were removed under reduced pressure. The residue was dissolved in the minimum AcOEt and the organic phase was washed with aqueous $KHSO_4$ (pH 3) and water (3×). The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified on Bond-Elut SAX column using DCM, to remove residual carboxylic acid. The powder obtained was triturated in ether–pentane. Tritiyle intermediate was dissolved in TFA 2%/DCM (0.03 M) and triisopropylsilane was added drop by drop until the yellow colour disappeared. The reaction mixture was stirred for 5 min at rt, solvents were removed under reduced pressure and the residue was washed with ether–pentane (20:80). Yields given are those of the deprotection step.

9.2.17. Compound 61 method B. White powder; 23 mg, yield 75%, purity 99%, NMR ^{1}H DMSO-d_6 δ ppm: 0.85 (d, $J = 6.0$ Hz, 6H), 1.39–1.52 (m, 1H), 1.53–1.70 (m, 2H), 3.04 (t, $J = 7.5$ Hz, 1H), 4.26 (d, $J = 5.7$ Hz, 2H), 7.21–7.34 (m, 5H), 8.15 (t, $J = 5.7$ Hz, NHCO), 8.97 (s, OH), 10.53 (s, CONHO). NMR ^{13}C DMSO-d_6 δ ppm: 22.6, 23.0, 26.0, 38.9, 42.7, 49.4, 127.2, 127.5, 128.7, 139.8, 167.2, 169.5. $t_{R\ LCMS}$ 3.90 min; MS $(MH)^+$ *m/z* 265 and $(MNa)^+$ *m/z* 287, mp 187–188 °C.

9.2.18. Compound 62 method B. White powder; 18 mg, yield 74%, purity 99%, NMR ^{1}H DMSO-d_6 δ ppm: 0.82 (d, $J = 6.6$ Hz, 6H), 1.30–1.43 (m, 1H), 1.45–1.58 (m, 2H), 2.69 (t, $J = 7.2$ Hz, 2H), 2.94 (t, $J = 7.5$ Hz, 1H), 3.27 (q, $J = 6.9$ Hz, 2H), 7.18–7.31 (m, 5H), 7.68 (t, $J = 5.7$ Hz, NHCO), 8.94 (s, OH), 10.47 (s, CONHO), NMR ^{13}C DMSO-d_6 δ ppm: 22.6, 22.9, 25.9, 35.5, 39.2, 41.0, 49.4, 126.5, 128.7, 129.1, 139.8, 167.3, 169.3. $t_{R\ LCMS}$ 4.11 min; MS $(MH)^+$ *m/z* 279 and $(MNa)^+$ *m/z* 301, mp 154–155 °C.

9.2.19. Compound 63 method B. White powder; 20 mg, yield 75%, purity 99%, NMR ^{1}H DMSO-d_6 δ ppm: 0.85 (d, $J = 6.0$ Hz, 6H), 1.38–1.51 (m, 1H), 1.55–1.73 (m, 4H), 2.48–2.52 (m, 2H), 2.98 (t, $J = 7.5$ Hz, 1H), 3.00–3.07 (m, 2H), 7.18–7.31 (m, 5H), 7.69 (br s, NHCO), 8.93 (s, OH), 10.47 (s, CONHO), NMR ^{13}C DMSO-d_6 δ ppm: 22.6, 22.9, 26.1, 31.3, 32.9, 38.7, 38.9, 49.5, 126.2, 128.7, 128.8, 142.1, 167.3, 169.4. $t_{R\ LCMS}$ 4.63 min; MS $(MH)^+$ *m/z* 293, mp 158–160 °C.

9.2.20. Compound 64 method A. The compound was deprotected using TFA 80%/DCM, rt, 72 h. It was purified by Reverse-phase Preparative HPLC and lyophilized, white powder; 10 mg, yield 7%, purity 99%, NMR DMSO-d_6 δ ppm: 0.75 (dd, $J = 1.2$, 6.5 Hz, 6H), 1.28–1.35 (m, 3H), 1.40–1.50 (m, 4H), 2.48 (t, $J = 7.3$ Hz, 2H), 2.87 (t, $J = 7.5$ Hz, 1H), 2.95–3.00 (m, 2H), 7.07–7.12 (m, 3H), 7.16–7.20 (m, 2H), 7.54 (t, $J = 5.7$ Hz, NHCO), 8.82 (br s, OH), 9.30 (br s, CONHO). $t_{R\ LCMS}$ 5.10 min; MS $(MH)^+$ *m/z* 307.

9.2.21. Compound 65 method B. White powder; 15 mg, yield 30%, purity 99%, NMR ^{1}H CD_2Cl_2 δ ppm: 0.91–0.98 (m, 6H), 1.11–1.22 (m, 2H), 1.55–1.59 (m, 1H), 1.61–1.82 (m, 5H), 2.52–2.59 (m, 3H), 3.03 (t, $J = 12.9$ Hz, 1H), 3.73 (t, $J = 7.0$ Hz, 1H), 3.98 (d, $J = 13.2$ Hz, 1H), 4.58 (d, $J = 12.9$ Hz, 1H), 7.16–7.24 (m, 3H), 7.29–7.34 (m, 2H). $t_{R\ LCMS}$ 5.30 min; MS $(MH)^+$ *m/z* 333.

9.2.22. Compound 66 method B. White powder; 10 mg, yield 24%, purity 99%, mixture of the *cis* and *trans* form of the amide function; NMR ^{1}H DMSO-d_6 δ ppm: 0.86 (d, $J = 6.3$ Hz, 6H), 1.42–1.86 (m, 7H), 2.64–2.82 (m, 2H), 3.01–3.08 (m, 1H), 4.91–4.94 (m, 1H), 7.09–7.17 (m, 4H), 7.95 (d, $J = 8.4$ Hz, 0.6H, NHCO), 7.99 (d, $J = 8.4$ Hz, 0.4H, NHCO), 8.96 (s, OH), 10.42 (s, CONHO). $t_{R\ LCMS}$ 4.71 min; MS $(MH)^+$ *m/z* 305.

9.2.23. Compound 67 method A. Beige powder; 7 mg, yield 15%, purity 95%, NMR ^{1}H DMSO-d_6 δ ppm: 0.85 (d, $J = 5.4$ Hz, 6H), 1.39–1.67 (m, 3H), 3.03 (t, $J = 7.5$ Hz, 0.7H), 3.19 (t, $J = 6.9$ Hz, 0.3H), 4.24 (d, $J = 5.7$ Hz, 2H), 7.12–7.17 (m, 2H), 7.25–7.29 (m, 2H), 8.17 (t, $J = 5.7$ Hz, 0.7H, NHCO), 8.40 (s, 0.3H, NHCO), 8.96 (s, OH), 10.54 (s, CONHO). $t_{R\ LCMS}$ 4.13 min; MS $(MH)^+$ *m/z* 283, mp 140–152 °C.

9.2.24. Compound 68 method A. White powder; 10 mg, yield 30%, purity 95 %, NMR ^{1}H DMSO-d_6 δ ppm: 0.85 (d, $J = 6.3$ Hz, 6H), 1.42-1.64 (m, 3H), 3.03 (t, $J = 7.5$ Hz, 0.5H), 3.20 (t, $J = 6.3$ Hz, 0.5H), 4.24 (d,

J = 6 Hz, 2H), 7.23–7.39 (m, 4H), 8.20 (t, J = 6.0 Hz, 0.5H, NHCO), 8.43 (m, 0.5H, NHCO), 8.96 (s, 1H, OH), 10.55 (s, 1H, CONHO). $t_{R\ LCMS}$ 4.65 min; MS $(MH)^+$ m/z 299.

9.2.25. Compound 69 method B. White powder; 11 mg, yield 52%, purity 99%, NMR ^{1}H DMSO-d_6 δ ppm: 0.82 (d, J = 6.3 Hz, 6H), 1.35–1.44 (m, 1H), 1.51–1.63 (m, 2H), 3.02 (t, J = 7.5 Hz, 1H), 4.24–4.27 (m, 2H), 6.85–6.90 (m, 2H), 6.98–7.01 (m, 3H), 7.14 (t, J = 7.2 Hz, 1H), 7.32 (t, J = 7.8 Hz, 1H), 7.39 (t, J = 8.1 Hz, 2H), 8.19 (t, J = 5.4 Hz, NHCO), 8.95 (s, 1H, OH), 10.52 (s, 1H, CONHO). $t_{R\ LCMS}$ 5.32 min; MS $(MH)^+$ m/z 357, mp 171–173 °C.

9.2.26. Compound 70 method A. White powder; 30 mg, yield 46%, purity 99%, NMR ^{1}H DMSO-d_6 δ ppm: 3.00–3.08 (m, 2H), 3.30 (t, J = 6.75 Hz, 1H), 4.20 (dd, J = 5.1, 15 Hz, 1H), 4.30 (dd, J = 5,4, 15 Hz, 1H), 7.10–7.23 (m, 10H), 8.20–8.23 (m, NHCO), 10.44 (s, CONHO), NMR ^{13}C DMSO-d_6 δ ppm: 35.1, 42.7, 52.7, 126.6, 127.1, 127.5, 128.6, 128.7, 129.3, 139.4, 139.6, 166.2, 168.6. $t_{R\ LCMS}$ 4.25 min; MS $(MH)^+$ m/z 299, mp 167–169 °C.

9.2.27. Compound 71 method B. White powder; 13 mg, yield 18%, purity 99%, NMR ^{1}H DMSO-d_6 δ ppm: 1.29–1.39 (m, 2H), 1.43–1.53 (m, 2H), 2.53 (t, J = 7.5 Hz, 2H), 2.98–3.06 (m, 4H), 3.19 (t, J = 7.8 Hz, 1H), 7.16–7.25 (m, 10H), 7.69 (t, J = 5.4 Hz, 1H, NHCO), 8.91 (s, 1H, OH), 10.4 (br s, 1H, CONHO). $t_{R\ LCMS}$ 5.20 min; MS $(MH)^+$ m/z 341.

9.2.28. Compound 72 method A. Beige powder; 16 mg, yield 44%, purity 98%, ^{1}H NMR DMSO-d_6 δ ppm: 3.24 (dd, J = 15.9, 6.6 Hz, 1H), 3.40 (dd, J = 16.2, 8.1 Hz, 1H), 3.76 (s, 3H), 3.94 (t, J = 6.6 Hz, 1H), 4.18 (dd, J = 15.3, 5.4 Hz, 1H), 4.37 (dd, J = 15.6, 6.3 Hz, 1H), 7.27–7.13 (m, 7H), 7.56–7.50 (m, 2H), 8.33 (t, J = 5.7 Hz, NHCO), 9.03 (s, 1H, OH), 10.54 (br s, 1H, CONHO). $t_{R\ LCMS}$ 3.02 min; MS $(MH)^+$ m/z 353, mp 142–145 °C.

9.2.29. Compound 73 method B. Beige powder; 17 mg, yield 89%, purity 99%, NMR ^{1}H DMSO-d_6 δ ppm: 2.96–3.10 (m, 2H), 3.29–3.32 (m, 1H), 4.17 (dd, J = 15.1, 5.2 Hz, 1H), 4.29 (dd, J = 15.1, 6.1 Hz, 1H), 6.81–7.39 (m, 14H), 8.26 (t, J = 5.7 Hz, NHCO), 8.95 (s, OH), 10.47 (s, CONHO). $t_{R\ LCMS}$ 5.47 min; MS $(MH)^+$ m/z 391, mp 164–167 °C.

9.2.30. Compound 74 method B. White powder; 34 mg, yield 88%, purity 99%, NMR ^{1}H DMSO-d_6 δ ppm: 1.31–1.38 (m, 2H), 1.43–1.50 (m, 2H), 2.52–2.55 (m, 2H), 2.96–3.04 (m, 4H), 3.19 (t, J = 7.6 Hz, 1H), 6.78–6.97 (m, 5H), 7.10–7.39 (m, 9H), 7.69 (t, J = 5.4 Hz, NHCO), 8.92 (s, OH), 10.39 (s, CONHO), NMR ^{13}C DMSO-d_6 δ ppm: 28.6, 29.0, 34.9, 35.2, 39.2, 52.6, 117.0, 118.8, 119.7, 123.7, 124.6, 126.1, 128.7, 128.8, 130.1, 130.4, 141.7, 142.5, 156.7, 157.2, 166.2, 168.3. $t_{R\ LCMS}$ 6.26 min; MS $(MH)^+$ m/z 433, mp 131–133 °C.

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Supplementary data

Screening results are available via http://www.sciencedirect.com. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.10.010.

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