Synthetic tools to illuminate matrix metalloproteinase and proteasome activities

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2 A Straightforward Synthesis of Peptide Hydroxamate MMP/ADAM Inhibitors

Synthesis and biological evaluation of an inhibitor library

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2.1 Introduction

Matrix metalloproteinases (MMPs) are involved in numerous biological processes such as cell migration, wound repair and tissue remodeling. MMPs exert their role by the processing of extracellular matrix proteins including gelatin, elastin, and collagen and the release of growth factors. ADAMs (a disintegrin and metalloproteinase) are metalloproteinases that contain a membrane-spanning and a disintegrin (integrin-binding) domain. These membrane-bound enzymes are involved in membrane fusion, cytokine and growth factor shedding, cell migration, muscle development, fertilization, cellular differentiation, cell-cell interactions and cell-matrix interactions.[1-3] The best known ADAM is ADAM-17, also known as TACE or tumor necrosis factor α (TNFα) converting enzyme, which was discovered based on its sheddase activity with respect to membrane-bound TNFα.[4,5] The expression of MMPs and ADAMs is regulated by transcription factors and activity is controlled by natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Disturbances in these regulatory mechanisms are believed to cause, or be involved in, a wide range of pathological states. These include cancer metastasis, rheumatoid arthritis and autoimmune diseases. Deregulation of ADAM expression or activity has also been linked to asthma, Alzheimer's disease, bacterial lung infections and allergies of the airways.[1,6-12]

MMPs and ADAMs contain a Zn^{2+} ion in their active site, which forms a complex with the carbonyl group of the scissile amide bond. This complexation enhances the reactivity of the carbonyl towards nucleophilic attack of the water molecule, that is present in the active site and also coordinated by the Zn^{2+} ion (see Figure 1A).[7,13] As a result, a requirement for potent MMP or ADAM inhibitors is that they contain a good zinc binding group (ZBG). A large number of MMP and ADAM inhibitors that have appeared in the literature consist of an oligopeptide sequence that is equipped with a hydroxamate moiety at either the C- or the N-terminus. Notably, commercially available members of this type are marimastat, batimastat and TAPI-2 (see Figure 1B), each displaying sub- to low-nanomolar, broad MMP/ADAM inhibitory activity. In these structures the oligopeptide portion ensures recognition by the metalloproteinases by directing the substituents to the corresponding enzyme's binding pockets. The bidentate Zn^{2+} chelating properties of the hydroxamic acid has the dual effect of a strong zinc coordination as well as expulsion of the nucleophilic water molecule from the active site, thereby preventing hydrolysis to occur (Figure 1C).

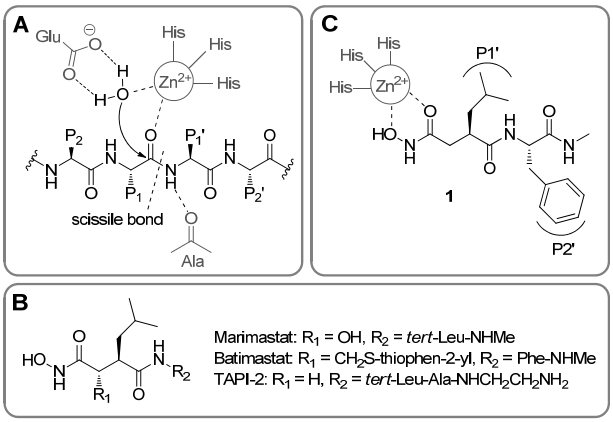


Figure 1. (A) Schematic representation of the position of a substrate in an MMP active site and mechanism of proteolysis. (B) Structures of some commercially available MMP/ADAM inhibitors. (C) Schematic representation of a potent MMP inhibitor **1**[14] containing the hydroxamic acid zinc binding group in an MMP active site. P1' and P2' refer to the enzyme's binding pockets.

C-terminal peptide hydroxamic acids are readily available through modified solid phase peptide synthesis (SPPS) protocols.[15-20] In contrast, there are very few synthetic procedures towards N-terminal peptide hydroxamates,[21-23] which obviate a non-SPPS step during synthesis.[14,24-27] The preparation of compound libraries containing N-terminal peptide hydroxamates would be greatly facilitated by the existence of suitable, complete SPPS methods. For this reason, a building block was devised that can be used in a linear SPPS strategy, immediately leading to products as depicted in Figure 1B with R_1 = H. The first part of this chapter describes the synthesis of this enantiomerically pure *N,O*-diprotected succinyl hydroxamate building block **2** (Figure 2) and its use in the synthesis of inhibitors and functionalized probes for the study of MMP and ADAM

activities. The second part deals with the preparation of a library containing 96 enantiopure peptide hydroxamates with general structure **3** (Figure 2) by SPPS using building block **2**. This compound library was used to study the inhibitor preference of three metalloproteases (MMP-9, MMP-12 and ADAM-17) with respect to the substituents at the P2' and P3' positions (R_2 and R_1 respectively).

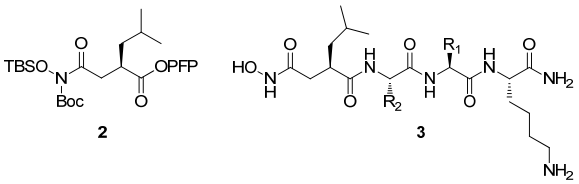


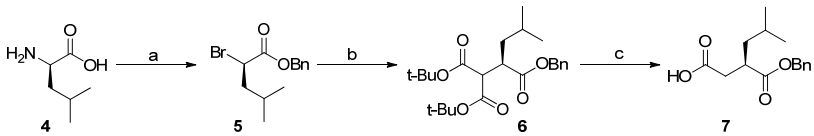
Figure 2. Structure of chiral succinylhydroxamate building block **2** for SPPS of peptide hydroxamate MMP/ADAM inhibitors. General structure **3** represents the library containing 96 compounds made via SPPS using building block **2**. R_1 and R_2 are amino acid side chains.

2.2 Results and Discussion

2.2.1 Synthesis and application of building block 2

Retro-synthetically, compound **2** can be prepared from chiral succinate **7** (Scheme 1) by condensation of the carboxylate with an appropriately protected hydroxylamine derivative, followed by transesterification of the benzyl ester. In the first instance, compound **7** was synthesized from D-leucine **4** following a modified literature procedure[28] as depicted in Scheme 1. In short, D-leucine **4** was converted into the corresponding bromide, followed by esterification with benzyl alcohol to give compound **5**. Alkylation of di-*tert*-butyl malonate with bromide **5** gave **6**, which was converted into **7** in a two step procedure (removal of the *tert*-butyl esters followed by thermal decarboxylation). Chiral HPLC measurements (see Figure 4 in the Experimental section) indicated that compound **7** was formed as a 3.3:1 mixture of stereoisomers. Incubation of this compound in TFA for 16 hours did not reduce the enantiomeric excess, proving that scrambling of chirality takes place before the deprotection step.

Scheme 1. Synthesis of succinate **7** from D-leucine.[28]

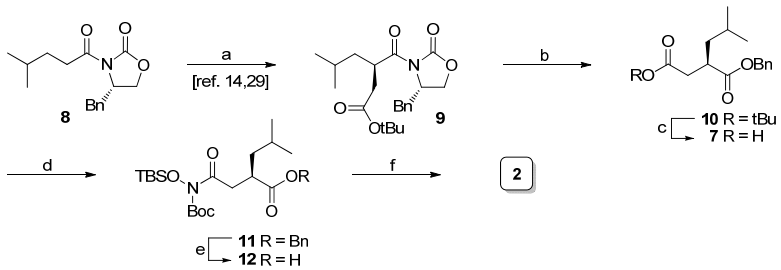


Reagents and conditions: (a) i) $NaNO_2$, HBr, H_2O, 90%; ii) BnOH, TsOH, toluene, reflux, 79%; (b) di-*tert*-butyl malonate, KO*t*Bu, DMF, 92%; (c) i) TFA, DCM; ii) toluene, reflux, 83%.

In order to obtain building block **2** in optically pure form, a second method for the synthesis of **7** was investigated (see Scheme 2). Chiral alkylation of known compound **8** gave enantiopure *tert*-butyl ester **9**.[14,29] Removal of the chiral auxiliary using lithium benzyl alcoholate gave benzyl ester **10**. Partial deprotection led to monoester **7**, which

was analyzed again by chiral HPLC (see Figure 4 in the Experimental section) and now the e.e. was determined to be >99%. Next, the carboxylic acid was converted into its acyl chloride derivative and reacted with *N*-Boc-*O*-TBS-hydroxylamine[30] giving fully protected succinyl hydroxamate **11**. The benzyl ester was removed by catalytic hydrogenation to obtain free acid **12**. It was soon discovered that **12** is not only labile during storage (even at –20 °C), but also extremely base-sensitive. Attempts to precipitate it as several different alkylammonium salts led to complete degradation. Also, standard peptide coupling conditions (HCTU/DiPEA) led to complicated reaction mixtures, presumably due to cyclisation of **12** to the anhydride. Therefore, in order to minimize the amount of base encountered by compound **12**, it was transformed into an active ester derivative, which can, in theory, be coupled without additional base. The PFP ester **2**, obtained by reaction of **12** with pentafluorophenol under the influence of EDC, proved to be far more stable during storage than acid **12**.

Scheme 2. Synthesis of building block **2**.

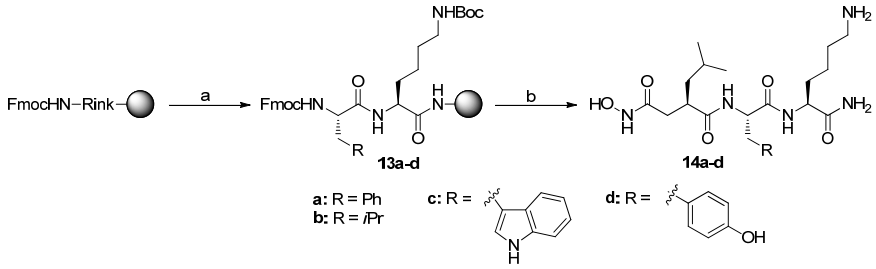


Reagents and conditions: (a) LiHMDS, *tert*-butyl bromoacetate, THF, –78 °C; (b) BnOH, *n*BuLi, THF, 0 °C, 92%; (c) TFA, DCM, 94%; (d) i) $(ClCO)_2$, cat. DMF, DCM; ii) TBSONHBoc, DMAP, ACN, 85%; (e) H_2, Pd/C, MeOH; (f) PFPOH, EDC, DCM, 55% from **11**.

Next, the potential of PFP-ester **2** in SPPS was evaluated (see Scheme 3). Dipeptide **13a** was synthesized on Rink amide resin using standard SPPS protocols. After removal of the Fmoc group, several conditions for the coupling of **2** were investigated.[31] The optimal conditions proved to be shaking the resin for 2 hours with 5 equivalents of **2** and 2 equivalents of DiPEA relative to the resin-bound peptide in NMP. The resulting product was cleaved from the resin and concomitantly deprotected using 95% aqueous TFA, cleanly yielding peptide **14a** in 64% after RP-HPLC purification. In the same fashion, the analogous peptides in which phenylalanine is replaced by leucine (**14b**, 56% yield), tryptophan (**14c**, 42%) and tyrosine (**14d**, 71%) were synthesized.

Enzyme inhibition tests using a fluorogenic substrate revealed IC_{50} values in the low nanomolar range for the four compounds against both MMP-12 (catalytic domain) and ADAM-17 (ectodomain), as is shown in Table 1, entries 1–4. The observation that the aromatic amino acid containing compounds **14acd** are more potent inhibitors than the aliphatic **14b** corroborates earlier findings.[7]

Scheme 3. Solid-phase peptide synthesis of succinylhydroxamate peptides using **2**.



Reagents and conditions: (a) i) 20% piperidine/DMF; ii) FmocLys(Boc)OH, HCTU, DiPEA, NMP; iii) 20% piperidine/DMF; iv) Fmoc<u>AA</u>OH, HCTU, DiPEA, NMP; (b) i) 20% piperidine/DMF; ii) compound **2**, DiPEA, NMP; iii) 95% TFA/H_2O, RP-HPLC, **14a**: 64%, **14b**: 56%, **14c**: 42%, **14d**: 71%. <u>AA</u> = **a**: Phe, **b**: Leu, **c**: Trp, **d**: Tyr.

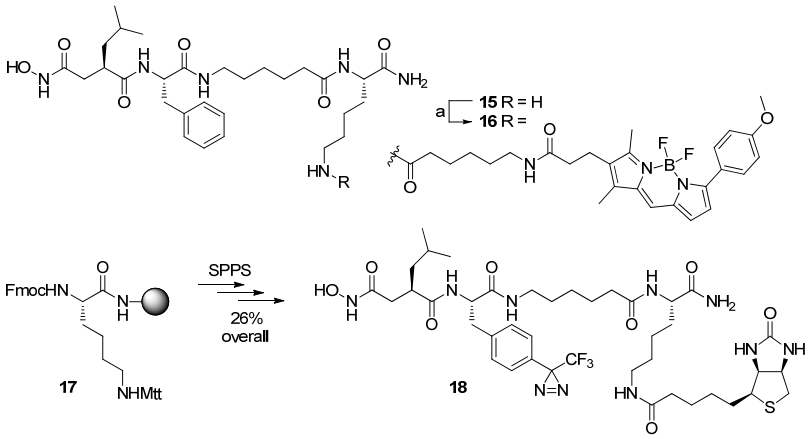
Table 1. Inhibitory activities of the synthesized MMP/ADAM inhibitors (IC_{50} values in nM).

Entry	Compound	MMP-12	ADAM-17
1	**14a**	5.3	15.1
2	**14b**	41.8	58.2
3	**14c**	7.8	26.1
4	**14d**	4.8	10.7
5	**15**	4.1	23.6
6	**16**	13.1	42.7
7	**18**	3.6	20.6

Functionalization of the free amine in the prepared inhibitors with a fluorescent label can readily be accomplished, as is demonstrated in Scheme 4. Compound **15**, a modified version of **14a** with an additional spacer, was obtained via the presented method in 53% overall isolated yield. It was established that this spacer does not significantly influence the inhibitory activity (see Table 1, entry 5). Reaction of **15** in DMF with 1 equivalent of Bodipy(TMR)-Ahx-OSu and DiPEA cleanly furnished labeled compound **16** in 77% isolated yield. Incorporation of the fluorescent label led only to a slight drop in inhibitory potency (Table 1, entry 6).

The usefulness of building block **2** is further demonstrated in the on-resin synthesis of the biotinylated inhibitor **18** (Scheme 4), containing a photoactivatable group. This compound is designed to bind to the active site of MMPs and ADAMs, after which it can be covalently locked by irradiating the photocrosslinker at 366 nm.[15,32] The biotin moiety can then be used as a handle for affinity purification of the tagged enzymes, as well as for visualization by streptavidin after blotting. Rink amide-bound FmocLys(Mtt)OH (**17**) was side-chain deprotected and then coupled to biotin. Next, the 6-aminohexanoic (Ahx) spacer and the photocrosslinker amino acid FmocPhe(Tmd) were coupled. Finally, after removal of the Fmoc group and coupling of **2**, the entire construct was removed from the resin and concomitantly deprotected, to give **18** in an overall yield of 26% after RP-HPLC purification.

Scheme 4. Synthesis of fluorescent probe **16** and photocrosllinker containing biotinylated probe **18**.



Reagents and conditions: (a) Bodipy(Tmr)-Ahx-OSu, DiPEA, DMF, 77%.

Testing of compound **18** on MMP-12 and ADAM-17 revealed that it is a highly potent inhibitor of both enzymes (see Table 1, entry 7), as well as ADAM-10 (IC_{50} = 114 nM). In order to validate it as a potential activity-based probe, **18** was incubated with recombinant ADAM-10, followed by irradiation at 366 nm, SDS-PAGE and detection with streptavidin-alkaline phosphatase. The results of this experiment are shown in Figure 3. Clearly, compound **18** is able to irreversibly bind ADAM-10 in an activity-based manner, as evidenced by the weak staining after preheating the enzyme (lane 1) in comparison with active ADAM-10 (lane 2). The binding efficiency of **18** is shown in lane 3, where a 40-fold excess of the generic MMP/ADAM inhibitor TAPI-2 only slightly decreases the observed staining. Figure 3 also shows that the covalent labeling is light-dependent, since no labeling is observed without irradiation (lane 8) and the maximum labeling occurs after approximately 30 minutes of irradiation. These facts make compound **18** a promising candidate for activity-based profiling of MMPs and ADAMs.

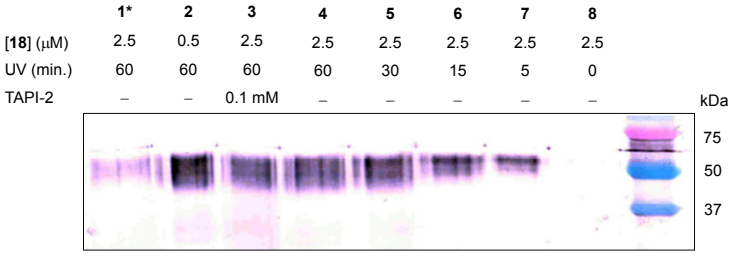
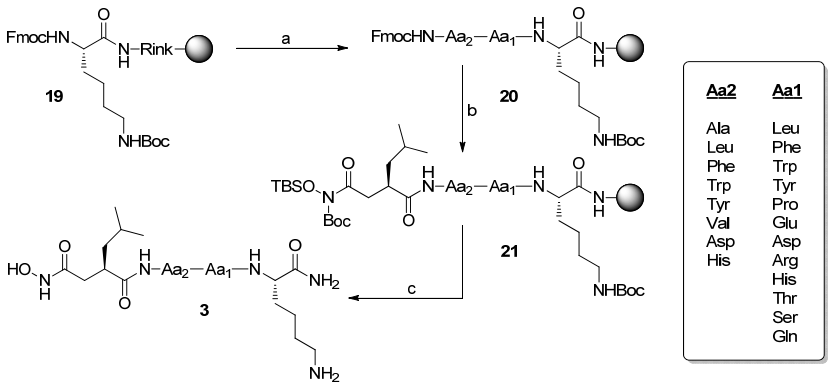


Figure 3. Labeling of ADAM-10 with probe **18**. Recombinant ADAM-10 (100 ng) was incubated with probe **18**, followed by irradiation with UV light (366 nm) for 0-60 min., as indicated. The mixtures were denatured, resolved by 10% SDS-PAGE, stained with streptavidin-AP and visualized by Western blotting. *Denatured ADAM-10 (preheated with 2% SDS).

2.2.2 Synthesis and evaluation of a peptide hydroxamate library

The preparation of the target compound library (see Scheme 5) commenced with α-NHFmoc-, ε-NHBoc-protected lysine on Rink amide resin **19**. After removal of the Fmoc protecting group the first set of amino acids (Aa_1) was coupled in a parallel fashion under standard SPPS coupling conditions giving 12 different peptides. These resin bound peptides were divided into 8 equal portions. Removal of the Fmoc group and coupling of the second amino acid (Aa_2) gave 96 immobilized peptides with the general structure **20**. Final Fmoc deprotection and condensation with building block **2** (see Figure 2) in the presence of 2 equivalents of DiPEA resulted in the immobilized and fully protected peptide hydroxamates **21**. Acidic cleavage from the resin and concomitant removal of all acid labile protecting groups resulted in a 96-membered library of crude compounds **3**, which were purified by RP-HPLC. The yields of the pure peptides based on **19** (purity >95% as determined by LC-MS analysis) varied between 3% and 40%. The amount of side products formed differed considerably between the compounds. Hydrolysis of the hydroxamic acid to the carboxylic acid in the final step appeared in most cases to be the major side reaction. The formation of this side product was apparent from the LC-MS analyses of the crude mixtures by a 15 Da decrease in molecular weight. In some cases condensation with the activated hydroxamate ester was incomplete. In general, the best results in terms of yield and side product formation were obtained for compounds containing an amino acid with an aliphatic side chain at the Aa_2 position. All compounds were given a unique two-letter code, which resembles the order of coupling of amino acids Aa_1 and Aa_2 (for instance PA stands for compound **3** with Aa_1 = proline and Aa_2 = alanine).

Scheme 5. Solid-phase synthesis of the peptide succinyl hydroxamate library.



Reagents and conditions: (a) i) 20% piperidine/DMF; ii) FmocAa_1OH, HCTU, DiPEA, NMP; iii) 20% piperidine/DMF; iv) FmocAa_2OH, HCTU, DiPEA, NMP; (b) i) 20% piperidine/DMF; ii) compound **2**, DiPEA, NMP; (c) 95% TFA/H_2O, RP-HPLC.

The results of the single-point (100 nM) inhibitory potential of the 96 compounds against MMP-9, MMP-12 and ADAM-17 are depicted in Table 2. This initial screen was

performed in order to obtain qualitative insight in the difference in inhibitory potential of the 96 peptide hydroxamates. It is apparent that the efficacy of the inhibitors towards MMP-12 is generally higher than for the other two enzymes. Introduction of a proline residue at the Aa_1-position greatly decreases the activity of the inhibitor with respect to both MMPs. This effect appears to be strongest for MMP-12 but inhibition of ADAM-17 appears to be less affected. This observation can be explained by the fact that MMPs contain a straight horizontal cleft and therefore a proline would result in a large steric hindrance within the active site. ADAMs, however, do not contain such a rigid cleft and the inhibitor activity is thus less affected by proline.[33,34]

Table 2. Remaining enzymatic activity (%) after inhibition.[a]

Inhibitor	MMP9	MMP12	ADAM17	Inhibitor	MMP9	MMP12	ADAM17	Inhibitor	MMP9	MMP12	ADAM17
DA	97.3	25.3	90.0	LA	98.7	36.2	73.5	SA	69.2	29.1	42.2
DD	102.3	51.4	105.7	LD	98.2	55.2	92.1	SD	66.2	42.5	96.2
DF	55.8	4.3	92.5	LF	57.0	2.1	25.4	SF	8.6	7.2	11.2
DH	90.8	11.6	97.6	LH	92.5	13.7	69.0	SH	27.9	14.7	33.6
DL	95.2	7.4	98.7	LL	98.3	4.3	41.6	SL	28.4	9.0	11.8
DV	106.0	11.0	100.6	LV	85.6	8.6	30.0	SV	30.1	9.6	8.4
DW	70.4	8.5	108.0	LW	42.6	2.3	36.5	SW	3.4	3.1	14.1
DY	65.3	9.0	101.8	LY	53.7	3.6	32.8	SY	3.7	5.0	8.9
EA	101.2	45.0	92.3	PA	101.8	83.9	64.8	TA	99.3	74.2	86.0
ED	103.2	85.5	91.4	PD	97.8	101.2	94.0	TD	95.4	62.1	94.8
EF	77.8	7.6	86.8	PF	72.9	63.2	49.7	TF	50.8	15.9	41.2
EH	98.2	11.4	99.5	PH	84.9	68.2	50.3	TH	73.2	22.9	66.0
EL	106.2	18.1	92.2	PL	100.2	77.5	43.0	TL	52.7	8.8	26.7
EV	106.8	23.4	96.7	PV	97.4	50.3	48.5	TV	70.0	19.4	26.0
EW	103.3	33.0	103.3	PW	73.1	36.9	63.7	TW	21.2	7.1	35.8
EY	86.8	10.2	99.7	PY	72.5	45.3	63.3	TY	32.7	13.5	33.7
FA	96.7	24.4	47.6	QA	100.0	36.0	68.0	WA	84.6	16.3	56.3
FD	91.8	43.3	92.8	QD	96.6	59.0	90.7	WD	85.0	45.6	96.7
FF	24.6	0.6	16.4	QF	73.3	8.7	57.8	WF	21.6	4.3	23.0
FH	81.4	61.1	67.9	QH	90.8	61.4	87.0	WH	48.5	7.2	58.6
FL	79.7	3.7	33.3	QL	90.0	7.7	53.9	WL	33.6	2.9	21.2
FV	82.6	3.4	19.4	QV	97.1	9.5	53.9	WV	64.6	6.3	20.4
FW	10.4	0.4	40.2	QW	42.7	2.8	58.8	WW	18.5	4.6	48.3
FY	49.8	9.0	55.6	QY	12.8	1.9	16.1	WY	8.1	4.2	13.3
HA	95.1	44.6	78.8	RA	97.6	25.8	23.2	YA	88.4	17.9	60.8
HD	93.8	82.7	89.8	RD	96.2	81.5	87.6	YD	70.5	32.6	81.3
HF	31.3	4.1	35.4	RF	66.5	4.6	6.5	YF	13.2	1.8	16.3
HH	72.2	17.4	67.0	RH	88.1	14.0	14.7	YH	52.7	12.2	64.0
HL	72.1	7.4	43.7	RL	93.8	12.8	8.8	YL	52.1	5.2	25.0
HV	86.4	15.0	49.6	RV	99.5	9.5	5.0	YV	54.8	4.3	17.4
HW	21.0	3.2	53.4	RW	54.1	4.4	9.5	YW	4.9	3.2	28.1
HY	29.2	7.6	56.1	RY	42.8	4.0	7.0	YY	8.1	1.1	18.3

[a] Determined by evaluation of their ability to inhibit proteolytic conversion of a fluorogenic substrate. MMP-12 catalytic domain, MMP-9 catalytic domain and ADAM-17 ectodomain (5 ng) were incubated with 100 nM final concentration of inhibitor. The appropriate fluorogenic substrate was added (2 μM final concentration) and proteolysis rates were determined by measuring fluorescence (λ_{ex} 320 nm, λ_{em} 440 nm) increase. The remaining catalytic activity was calculated by comparison with proteolysis rates of equal amounts of uninhibited enzyme. Each value is the average of three individual experiments. Each inhibitor is identified by its two-letter code, which resembles the order of the coupling of amino acids Aa_1–Aa_2. For example: compound PA means that first proline (Aa_1) was coupled, followed by the coupling of alanine (Aa_2).

It is also obvious that the presence of acidic residues (D and E) in either position greatly reduces the efficacy of the inhibitors for MMP-9 and ADAM-17 and to a somewhat lesser extend for MMP-12. The inhibitors with the highest efficacy towards MMP-12 are those with the aromatic amino acids phenylalanine, tryptophan or tyrosine in either position. These results are in line with earlier observations by Lang and co-workers.[34]

The beneficial effect of incorporating aromatic moieties also holds true for MMP-9, especially if both positions are occupied by phenylalanine, tryptophan or tyrosine. Interestingly, a serine residue in the Aa_1 position yields very active MMP-9 inhibitors, whereas threonine at Aa_1 has a much weaker beneficial effect. MMP-12 and ADAM-17 show a similar, albeit not so strong tendency. The presence of an aliphatic amino acid (A, L or V) in the Aa_2 position decreases the efficacy against MMP-9 in a more pronounced way than for the other two enzymes. Netzel-Arnett and co-workers reported an extensive study on the substrate preference of MMP-9 towards a set of oligopeptides.[35] Their findings corroborate our results with respect to a positive effect of aromatic moieties in both positions (Aa_1 and Aa_2) or a serine residue at Aa_1 on inhibitory potential towards MMP-9. Interestingly, the here presented results are in disagreement with their findings that leucine, and to a lesser extent alanine, at Aa_2 have a beneficial effect on inhibitory potential, since a detrimental effect for both residues at this position is observed. Incorporation of arginine in position Aa_1 improves efficacy towards MMP-12 and ADAM-17 but highly reduces the efficacy towards MMP-9, as was also shown by Netzel-Arnett and co-workers.[35] The positive effect of aromatic amino acids also holds true for ADAM-17 but to a lesser extent than for the tested MMPs. In addition it is found that heteroaryl moieties (His and Trp) or a serine at the Aa_1 position improves the potency towards ADAM-17. These observations are consistent with reports in the literature.[7,36]

An interesting feature of these inhibitors is that they can be used for solid-phase extraction (SPE) of active metalloproteases.[37-39] The key element in this is the free amine functionality these compounds contain, with which they can be immobilized onto matrix material (Sepharose beads). By flushing a biological sample through the modified beads, the target active metalloenzymes are captured, which subsequently leads to the chemical enrichment of active MMPs and ADAMs. Eight inhibitors (selected on the basis of their different potency towards the three enzymes) were studied in more detail. The IC_{50} values of these inhibitors for the target enzymes were determined (see Table 3). These values span the entire range from sub-nanomolar to over 10 μM (compare for instance FF and PD) and some of them show considerable selectivity towards one or two of the three tested enzymes (for example YW towards both MMPs and PL towards ADAM-17). As can be seen, all values nicely corroborate the results from the single-point determinations, depicted in Table 2. All eight inhibitors were immobilized on Sepharose beads and the extraction efficiency of the three recombinant acitve enzymes was determined.[40] Experiments showed complete enrichment of MMP-9 and MMP-12, however enrichment of ADAM-17 was much more challenging. Of interest is the finding that correlation between affinity (IC_{50} value) of free inhibitor and suitability for SPE is not

as obvious as one might expect. One inhibitor in particular (FF) was found to be very powerful for SPE and it was therefore only natural to use it for the efficient activity-based chemical enrichment of ADAM-17 from a complex biological sample (a cell lysate from cultured human alveolar carcinoma cell line A549).[40] The result from this straightforward affinity-based enrichment protocol nicely complements the results from Cravatt and co-workers on ADAM-17 enrichment from biological samples in a two-step bioorthogonal fashion using a photo-activatable probe and streptavidin-mediated pull-down.[41]

Table 3. IC_{50} values (in nM) of eight selected inhibitors[a]

	MMP-9	MMP-12	ADAM-17
DV	905 (221)	10.5 (4.0)	2,241 (250)
FF	23.2 (3.9)	0.92 (0.22)	16.0 (6.4)
FW	6.69 (0.66)	2.57 (0.80)	29.6 (9.1)
PD	>10,000[b]	2,788 (392)	5,998 (2,555)
PL	3,624 (328)	147 (12)	92.1 (28)
QY	9.92 (0.79)	0.85 (0.020)	18.9 (2.0)
SF	9.93 (1.3)	7.70 (1.3)	11.1 (2.3)
YW	6.71 (0.96)	4.03 (0.95)	36.0 (3.4)

[a] Each value represents the mean of three independent inhibition curves. The standard deviation is given in parentheses. [b] Activity of enzyme >50% at 10 μM inhibitor.

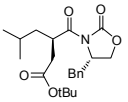
2.3 Conclusion

In summary, a straightforward solid phase synthesis of succinylhydroxamate peptides using the readily accessible building block **2** is presented. Potent, free amine containing MMP and ADAM inhibitors were conveniently synthesized and could be functionalized with a fluorescent label. Photoactivatable inhibitor **18** was efficiently synthesized entirely on solid phase and shown to be suited for activity-based covalent labeling of a model ADAM. Compound **16** may be used for fluorescent staining of active MMPs and ADAMs. Compound **12** nicely complements reported activity-based probes for MMPs.[15,32] In addition a library of 96 enantiopure peptide hydroxamates was prepared and tested with respect to their inhibitory efficacy towards three metalloproteases.[42] The results show that different amino acids at the P2′ (Aa_2) and P3′ (Aa_1) positions (see Scheme 5) have a substantial influence on inhibitory capacity. This is in contrast to reported findings,[7] which state that amino acids at the P2′ position have 'a modest effect' on potency.

Experimental section

General

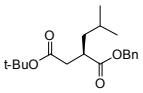
Tetrahydrofuran (THF) was distilled over $LiAlH_4$ before use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), methanol (MeOH), piperidine, diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma) were used as received. Rink amide MBHA resin (0.64 mmol/g) was purchased at Novabiochem, as well as all appropriately protected amino acids. *O*-(1*H*-6-Chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased at Iris Biotech (Marktrewitz, Germany). Traces of water were removed from reagents used in reactions that require anhydrous conditions by coevaporation with toluene. Solvents that were used in reactions were stored over 4Å molecular sieves, except methanol and acetonitrile which were stored over 3Å molecular sieves. Molecular sieves were flame dried before use. Unless noted otherwise all reactions were performed under an argon atmosphere. Column chromatography was performed on Silicycle Silia-P Flash Silica Gel, with a particle size of 40-63 μm. The eluents toluene, ethyl acetate and petroleum ether (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^{1}H-, ^{13}C- and ^{19}F-NMR spectra were recorded on a Jeol JNM-FX-200 (200MHz), a Bruker DMX-400 (400 MHz) or a Bruker DMX-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane (^{1}H-NMR), $CDCl_3$ (^{13}C-NMR) or TFA (^{19}F NMR) as internal standard. Mass spectra were recorded on a PE/Sciex API 165 instrument equipped with an Electrospray Interface (ESI) (Perkin-Elmer). High resolution MS (HRMS) spectra were recorded with a Finnigan LTQ-FT (Thermo Electron). IR spectra were recorded on a Shimadzu FTIR-8300 and absorptions are given in cm^{-1}. Optical rotations $[\alpha]_D^{23}$ were recorded on a Propol automatic polarimeter at room temperature. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 μm C18 50 × 4.6 mm column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI. HPLC gradients were 10 → 90%, 0 → 50% or 10 → 50% ACN in 0.1% TFA/H_2O. Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 × 4.6 mm). The compounds were purified on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 × 10 mm column and a GX281 fraction collector. The used gradients were either 0 → 30% or 10 → 40% ACN in 0.1% TFA/water, depending on the lipophilicity of the product. Appropriate fractions were pooled, and concentrated in a Christ rotary vacuum concentrator overnight at room temperature at 0.1 mbar.



(R)-*tert*-butyl 3-((S)-4-benzyl-2-oxooxazolidine-3-carbonyl)-5-methylhexanoate (9)[14,29]

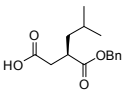
To a cooled solution (–78 °C) of LiHMDS (1.18 mmol, 1.18 mL of a 1M solution in THF) in THF (5 mL) was added (S)-4-benzyl-3-(4-methylpentanoyl)oxazolidin-2-one (**8**, 0.39 g, 1.07 mmol) in THF (5 mL) and the mixture was stirred at –78 °C for 30 min. *tert*-butyl bromoacetate (0.58 g, 2.97 mmol) was added and the mixture was allowed to warm to RT. After 3 h TLC analysis indicated a complete reaction. EtOAc was added and the mixture was extracted with sat. aq. NH_4Cl. The aqueous layer was extracted with EtOAc (2×) and the combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The title compound was obtained as a colourless solid (yield: 0.32 g, 0.85 mmol, 79%). ^{1}H NMR (200 MHz, $CDCl_3$) δ = 7.35-7.24 (m, 5H), 4.72-4.57 (m, 1H), 4.33-4.20 (m, 1H), 4.15 (d, *J* = 4.61 Hz, 2H), 3.34 (dd, *J* = 13.45,

3.21 Hz, 1H), 2.82-2.66 (m, 2H), 2.48 (dd, *J* = 16.60, 4.67 Hz, 1H), 1.62-1.28 (m, 3H), 1.43 (s, 9H), 0.93 (dd, *J* = 6.26, 3.56 Hz, 6H) ppm. $[\alpha]_D^{23} = +1.5$ (*c* = 1, $CHCl_3$).



tert-Butyl 3-(R)-benzyloxycarbonyl-5-methylhexanoate (10)

Benzyl alcohol (1.68 mL, 16.36 mmol) was dissolved in THF (40 mL) and cooled to 0 °C. *n*BuLi (6.14 mL, 1.6M in THF, 9.80 mmol) was added dropwise and the reaction mixture was stirred for 20 min. A solution of compound **9** (3.10 g, 8.18 mmol) in THF (5 mL) was added and the reaction was stirred at 0 °C until TLC analysis (10% EtOAc/PE) revealed a complete reaction (about 4 h). The reaction mixture was quenched with sat. aq. NH_4Cl and extracted three times with EtOAc. The combined organic layers were dried over $MgSO_4$ and concentrated. The crude material was purified by column chromatography (3% → 10% EtOAc/PE) giving the product as a colourless oil (yield: 2.4 g, 7.5 mmol, 92%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 7.40-7.25 (m, 5H), 5.13 (dd, *J* = 36.6, 12.0 Hz, 2H), 2.98-2.83 (m, 1H), 2.62 (dd, *J* = 16.4, 9.1 Hz, 1H), 2.35 (dd, *J* = 16.1, 5.2 Hz, 1H), 1.63-1.15 (m, 3H), 1.40 (s, 9H), 0.92-0.84 (m, 6H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 174.92, 170.80, 135.92, 128.31, 127.95, 80.49, 66.05, 40.97, 39.61, 37.70, 27.84, 25.63, 22.51, 22.05 ppm. $[\alpha]_D^{23} = +1.5$ (*c* = 1, $CHCl_3$). IR (thin film): 2962.5, 1728.1, 1365.5, 1249.8, 1141.8. HRMS: calcd. for $C_{19}H_{28}O_4$ $[M + H]^+$: 321.2060; found: 321.2064.



3-(R)-Benzyloxycarbonyl-5-methylhexanoic acid (7)

To a stirred solution of **10** (2.4 g, 7.5 mmol) in DCM (20 mL) was added TFA (20 mL) and the reaction was stirred until TLC analysis indicated complete consumption of starting material (after 30 min.). Toluene was added and the mixture was concentrated under reduced pressure. In order to remove all traces of TFA the product was coevaporated with toluene three times. The title compound was obtained as a colourless oil (yield: 1.98 g, 7.5 mmol, quant.) ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 7.36-7.29 (m, 5H), 5.14 (s, 2H), 3.00-2.85 (m, 1H), 2.76 (dd, *J* = 16.8, 9.12 Hz, 1H), 2.48 (dd, *J* = 16.8, 4.9 Hz, 1H), 1.65-1.29 (m, 3H), 0.93-0.86 (2×d, 6H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 178.04, 174.86, 135.71, 128.37, 128.01, 127.95, 66.36, 40.91, 39.12, 36.03, 25.60, 22.35, 22.05 ppm. $[\alpha]_D^{23} = +10.6$ (*c* = 1, $CHCl_3$). IR (thin film): 2954.7, 1705.0, 1450.4, 1388.7, 1157.2. HRMS: calcd. for $C_{15}H_{20}O_4$ $[M + H]^+$: 265.1434; found: 265.1437.

Synthesis from D-leucine **4**: Compounds **5** and **6** were prepared according to a procedure described in literature.[28] Compound **6** (15.5 g, 36.9 mmol) was treated with a 1:1 mixture of DCM/TFA (100 mL) for 2 h, after which TLC analysis indicated complete consumption of starting material. Toluene was added and the mixture was concentrated under reduced pressure, followed by coevaporating the mixture with toluene twice. The resulting material was dissolved in toluene (100 mL) and refluxed for 2 h, after which the mixture was concentrated to dryness under reduced pressure. The product was obtained as a colourless oil (yield: 8.09 g, 30.6 mmol, 83%). NMR data corresponded to the data shown above. $[\alpha]_D^{23} = +6.4$ (*c* = 1, $CHCl_3$).

Verification of the chiral purity of compound 7

As a reference compound, the racemic form of **7** was synthesized.[43] NMR data and purities were identical for **7** and its racemate. Chiral HPLC chromatograms are depicted in Figure 4. Based on the relative intensities of the peaks (depicted under the peaks) in chromatogram B, the e.e. of **7** was determined to be >99%. Chromatogram D shows the chiral HPLC run for compound **7** synthesized from D-leucine as depicted in Scheme 1. Based on the relative intensities of the peaks this compound was determined to be a ~3.3:1 mixture of stereoisomers.

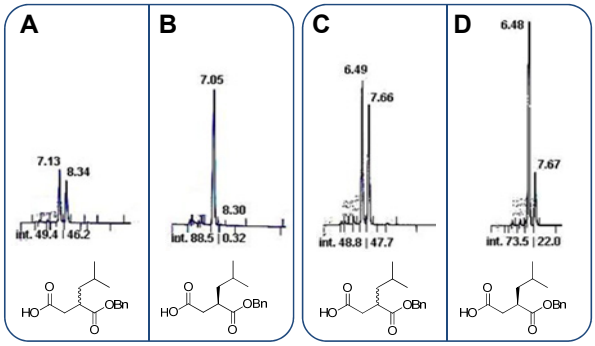
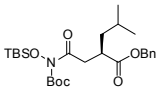
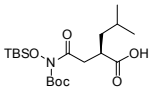


Figure 4. Results from the chiral HPLC analyses of (R)-2-isobutylmonobenzylsuccinate **7** and the racemate synthesized by the method of Chatterjee *et al.*[43] Measurements were done with a Chiral OD column; eluent: hexane:isopropanol 95:5 (v/v) + 0,25% AcOH; flow: 1 mL/min.; UV detection at $\lambda = 254$ nm. Retention times and relative integrals are given. (A) racemate; (B) compound **7**; (C) racemate; (D) compound **7** synthesized from D-leucine as depicted in Scheme 1.



Benzyl 2-(R)-isobutyl-3-(*N-tert*-butoxycarbonyl-*N*-(*tert*-butyldimethylsilyloxy)carbamoyl)-propionate (11)

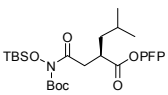
Carboxylic acid **7** (2.11 g, 8.0 mmol) was dissolved in DCM (30 mL) and DMF (2 drops) was added. The solution was cooled to 0 °C and oxalyl chloride (40 mmol, 3.4 mL) was added dropwise. The solution was warmed to room temperature and stirred until gas evolution ceased (about 30 min.). Toluene (30 mL) was added and the mixture was concentrated. Residual reagent was removed by repeated coevaporation with toluene. The intermediate acid choride was obtained as a yellowish oil and used without further purification. The crude acid chloride was redissolved in ACN (15 mL) and cooled to 0 °C. To this, a mixture of *N*-Boc-*O*-TBS-hydroxylamine[30] (8.8 mmol, 2.17 g) and DMAP (16 mmol, 1.95 g) in ACN (15 mL) was added. The reaction mixture was slowly warmed to room temperature and stirred until TLC (10% EtOAc/PE) revealed a complete reaction (approx. 2 h). The mixture was diluted with Et_2O (200 mL) and extracted subsequently with 1M aq. HCl and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (5% EtOAc/PE), and the product was obtained as a colourless oil (yield: 3.36 g, 6.8 mmol, 85%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 7.35-7.29 (m, 5H), 5.18-5.03 (dd, 2H, *J* = 36.5, 12.8 Hz), 3.24 (dd, *J* = 17.1, 9.7 Hz, 1H), 3.10-3.00 (m, 1H), 2.88 (dd, *J* = 17.1, 3.6 Hz, 1H), 1.60-1.10 (m, 3H), 1.53 (s, 9H), 0.98 (s, 9H), 0.92-0.83 (m, 6H), 0.12 (s, 3H), 0.10 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 175.28, 170.43, 151.93, 136.04, 128.22, 127.79, 84.13, 65.93, 41.06, 39.67, 39.15, 27.78, 25.60, 22.29, 17.96, -5.21 ppm. $[\alpha]_D^{23} = +16.5$ (*c* = 1, $CHCl_3$). IR (thin film): 2954.7, 1728.1, 1465.8, 1303.8, 1141.8. HRMS: calcd. for $C_{26}H_{43}NO_6Si$ $[M+H]^+$: 494.2932; found: 494.2933.



2-(R)-Isobutyl-3-(*N-tert*-butoxycarbonyl-*N*-(*tert*-butyldimethylsilyloxy)carbamoyl)propionic acid (12)

To a solution of compound **11** (3.16 g, 6.40 mmol) in methanol (50 mL) Pd/C (10% w/w, 150 mg) was added. Hydrogen gas was then bubbled through the mixture until TLC analysis (10% EtOAc/PE) revealed a complete reaction (approx. 1.5 h). The reaction mixture was filtered over Celite and concentrated to obtain the product as a sticky syrup (2.6 g, 6.40 mmol, quant.). The compound was used without further purification. ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 10.47 (bs, 1H), 3.12 (dd, *J* = 16.8, 8.7 Hz, 1H), 3.00-2.70 (m, 2H), 1.70-1.15 (m, 3H), 1.47 (s, 9H), 0.91 (s, 9H), 0.88-0.81 (m, 6H), 0.06 (s, 3H), 0.04 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 181.62, 170.49, 151.96, 84.28, 40.85, 39.46, 39.06, 27.81, 25.57, 22.20, 17.96, -5.27

ppm. $[\alpha]_D^{23} = +12.4$ ($c = 1$, $CHCl_3$). IR (thin film): 2954.7, 1705.0, 1465.8, 1249.8, 1145.5. ESI-MS (*m/z*): 404.2 (M + H^+), 426.1 (M + Na^+), 442.4 (M + K^+), 829.5 (2M + Na^+), 304.1 (M – Boc + H^+), 326.0 (M – Boc + Na^+).



Pentafluorophenyl 2-(R)-isobutyl-3-(*N*-*tert*-butoxycarbonyl-*N*-(*tert*-butyl-dimethylsilyloxy)-carbamoyl)propionate (2)

A mixture of crude compound **12** (1.15 g, ~2.85 mmol) and pentafluorophenol (5.6 mmol, 1.03 g) in DCM (15 mL) was treated with EDC (5.6 mmol, 1.07 g). After stirring the mixture at room temperature overnight, Et_2O (100 mL) was added and the mixture was extracted with 1M aq. HCl and brine, dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (2% EtOAc/PE) and the product was obtained as a colourless oil (yield: 0.87 g, 1.53 mmol, 55%). ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 3.33-3.28 (m, 2H), 3.09-3.04 (m, 1H), 1.80-1.71 (m, 2H), 1.56 (s, 9H), 1.52-1.47 (m, 1H), 1.00 (d, 3H, J = 6.6 Hz), 0.99 (s, 9H), 0.95 (d, 3H, J = 6.6 Hz), 0.13, (s, 3H), 0.12 (s, 3H) ppm. ^{13}C-NMR (150 MHz, $CDCl_3$): δ = 171.82, 169.85, 152.03, 141.16 (dd, J = 250, 9.0 Hz), 139.31 (dt, J = 264, 13.5 Hz), 137.71 (dt, J = 249, 13.5 Hz), 125.16 (t, J = 12.8 Hz), 84.49, 41.02, 39.91, 39.01, 27.75, 25.67, 25.55, 22.24, 22.01, 18.03, –5.31, –5.39. ^{19}F NMR (376 MHz, $CDCl_3$): δ = –155.72 (d, J = 17.6 Hz, 2F), –162.46 (t, J = 21.6 Hz, 1F), –166.77 (dd, J = 21.4, 17.3 Hz, 2F). $[\alpha]_D^{23} = +10.9$ ($c = 1$, $CHCl_3$). IR (thin film): 2962.5, 2360.7, 1735.8, 1519.8, 1311.5. HRMS: calcd. for $C_{25}H_{36}F_5NO_6Si$ $[M + Na]^+$: 592.2124; found: 592.2140.

General procedure A: solid phase peptide synthesis

Prior to first use, the Fmoc Rink Amide MBHA resin was washed twice with DMF, twice with methanol and twice with DCM. Fmoc deprotection was performed by shaking the resin in a 20% piperidine/DMF stock solution for 20 min. The resin was washed twice with DMF and twice with DCM after every coupling and deprotection step. The first amino acid was loaded by reacting the resin with 5 eq. of HCTU, 5 eq. of amino acid and 10 eq. DiPEA (0.45 M stock solution in NMP). The amino acid was pre-activated in solution (5 min.) before adding it to the resin and shaking the resin for 1 h (standard coupling protocol). Loading was determined by UV spectroscopy at 300 nm of a freshly prepared Fmoc-deprotected resin sample. A capping step was performed by shaking the resin for 10 min. with 0.45 M acetic anhydride and 0.45 M DiPEA/NMP solution. Mtt deprotection was done by shaking repeatedly in a 1% TFA/DCM solution until the characteristic yellow colour of Mtt cation did no longer appear (7-12 times, 2 min. each). After Mtt-cleavage, immediately before the following coupling step, the resin was washed with a 0.45 M DiPEA stock solution in NMP. The coupling and deprotection reactions were checked on the presence of free amines by performing a Kaiser test. Before cleaving the peptide from the resin, it was washed 5 times alternatingly with DCM and MeOH. Cleavage from the solid support was done by shaking the resin in a 95% v/v TFA/H_2O solution for two hours, followed by filtration and rinsing the resin with a small portion of 95% v/v TFA/H_2O. The filtrates were immediately poured into cold Et_2O/PE (1:1 approx. 20 volume eq.) and stored overnight at –20 °C. Centrifugation, followed by decantation of the supernatant afforded the crude product.

Hydroxamic acid Phe-Lys-NH_2 (14a)

This compound was synthesized on solid support on 25 μmol scale (based on the loading of Fmoc-Lys(Boc)) following the General procedure A. Coupling of Fmoc-Phe-OH gave the precursor **13a**. The final coupling step involved the addition of compound 2 (125 μmol, 71 mg) and DiPEA (50.0 μmol, 0.11 mL 0.45 M in NMP) with NMP (0.40 mL) to the resin and shaking for 2 hours. Cleavage from the resin, precipitation (see General Procedure) and purification by RP-HPLC gave pure **14a** (yield: 7.4 mg, 16.0 μmol, 64%). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 4.54. HRMS: calcd. for $C_{23}H_{37}N_5O_5$ $[M + H]^+$: 464.28675; found: 464.28674.

Hydroxamic acid Leu-Lys-NH_2 (14b)
Synthesized in the same way as described for **14a**, using Fmoc-Leu-OH in the second coupling (yield: 6.1 mg, 14.0 μmol, 56%). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 4.09. HRMS: calcd. for $C_{20}H_{39}N_5O_5$ $[M + H]^+$: 430.30240; found: 430.30239.

Hydroxamic acid Trp-Lys-NH_2 (14c)
Synthesized in the same way as described for **14a**, using Fmoc-Trp(Boc)-OH in the second coupling (yield: 5.3 mg, 11.0 μmol, 42%). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 3.94. HRMS: calcd. for $C_{25}H_{38}N_6O_5$ $[M + H]^+$: 503.29764; found: 503.29768.

Hydroxamic acid Tyr-Lys-NH_2 (14d)
Synthesized in the same way as described for **14a**, using Fmoc-Tyr(*t*Bu)-OH in the second coupling (yield: 8.5 mg, 18.0 μmol, 71%). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 3.57. HRMS: calcd. for $C_{23}H_{37}N_5O_6$ $[M + H]^+$: 480.28166; found: 480.28163.

Hydroxamic acid Phe-Ahx-Lys-NH_2 (15)
This compound was prepared as described in General procedure A on 50 μmol scale (based on the loading of Fmoc-Lys(Boc)). Sequential coupling of Fmoc-Ahx-OH, Fmoc-Phe-OH and compound **2** (see for the last coupling, compound **14a**). Cleavage from the resin followed by RP-HPLC purification afforded pure **15** (yield: 13.6 mg, 23.6 μmol, 47%). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 4.67. HRMS: calcd. for $C_{29}H_{48}N_6O_6$ $[M + H]^+$: 577.37081; found: 577.37088.

Hydroxamic acid Phe-Ahx-Lys(Ahx-BODIPY(TMR))-NH_2 (16)
To a solution of **15** (2.2 mg, 3.8 μmol) in DMF (250 μL) were added DiPEA (7.6 μmol, 1.3 μL) and BODIPY(TMR)-Ahx-OSu (3.8 μmol, 2.3 mg). The solution was agitated overnight in the dark. Evaporation of the solvent, followed by RP-HPLC purification yielded **16** as a purple solid (3.2 mg, 2.9 μmol, 77%). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.33. HRMS: calcd. for $C_{56}H_{78}BF_2N_9O_9$ $[M + H]^+$: 1070.60564; found: 1070.60518.

Hydroxamic acid Phe(Tmd)-Ahx-Lys(Biotin)-NH_2 (18)
This compound was synthesized on 50 μmol scale (based on the loading of Fmoc-Lys(Mtt)) following General procedure A. A slight modification was made for the coupling of Fmoc-Phe(Tmd)-OH. Because of its high price, only 1 eq. of Fmoc-Phe(Tmd)-OH was used, along with HCTU (1 eq.) and DIPEA (2 eq.) in NMP and shaking for 16 h. After rinsing the resin, this step was repeated. The final coupling included the addition of compound **2** (5 eq.) and DiPEA (2 eq.) in NMP to the resin and shaking for 2 h. After cleavage and purification by RP-HPLC the product was obtained as white solid (yield: 11.7 mg, 12.9 μmol, 26%). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 6.60. HRMS: calcd. for $C_{41}H_{61}F_3N_{10}O_8S$ $[M + H]^+$: 911.4419; found: 911.4391.

Synthesis of the compound library (3)

Rink amide resin was rinsed with DCM, MeOH and DMF (2× each), then deprotected by shaking with 20% piperidine in DMF for 10 min. (2×) and rinsed with DMF and DCM (2× each). Loading of the resin was effected by shaking with FmocLys(Boc)OH (5 eq. relative to the stated loading), HCTU (5 eq.) and 0.45 M DIPEA (10 eq.) in NMP for 2 h. The resin was filtered, then rinsed with DMF and DCM (2× each). Any non-reacted amines were capped by shaking the resin with Ac_2O (5 eq.) in 0.45 M DIPEA (10 eq.) in NMP for 5 min. After rinsing the resin with DMF, DCM and Et_2O (2× each) and drying *in vacuo*, Fmoc determination (UV measurement at 300 nm) gave a loading of 0.47 mmol/g. Twelve portions of 80 μmol (170 mg resin) were rinsed with DCM and DMF (2× each), deprotected using 20% piperidine in DMF (10 min) and shaken with preactivated solutions of the appropriate Fmoc-amino acids Aa_1 (400 μmol), HCTU (400 μmol, 165 mg) and DIPEA (800 μmol,

1.8 mL 0.45 M in NMP) for 1 h. After rinsing with DMF, DCM and Et_2O (2× each), and drying *in vacuo*, each portion was in turn divided into eight equal portions. Every portion (~ 10 μmol) was respectively reacted with preactivated solutions of the eight amino acids Aa_2 (50 μmol each), HCTU (50 μmol) and DIPEA (100 μmol as a 0.45 M solution in NMP) and shaken for 1 h. After rinsing the resins with DMF and DCM (2× each), all portions were deprotected using 20% piperidine in DMF for 10 min., then filtered and rinsed with DMF and DCM (2× each). Finally, to all 96 resins was added compound **2** (250 μL 0.2 M in NMP) and DIPEA (44 μL 0.45 M in NMP). After shaking for 2 h, the resins were filtered, rinsed with DMF, MeOH and DCM (2× each) and treated with 95% v/v TFA/water (0.5 mL) for 1 h (with the exception of resins containing Arg(Pmc), these were reacted 2.5 h). The filtrates, as well as small portions of 95% TFA/water used for rinsing the resins, were collected in tubes containing chilled Et_2O/petroleum ether (1/1, ~5 mL) and left overnight at –20 °C. The tubes were then centrifuged, and the filtrates were decanted. The crude products were analysed by LC-MS, then purified by semi-preparative RP-HPLC.

Biological evaluation

Materials

Recombinant human MMP-12 catalytic domain and MMP-9 catalytic domain (without fibronectin type II inserts) were a gift from AstraZeneca R&D (Lund & Moelndal, Sweden) and were produced in *E. coli* (Parkar 2000, Shipley 1996). Recombinant human ADAM-17 ectodomain was obtained from R&D systems. The fluorogenic MMP substrate Mca-PLGL-Dpa-AR-NH_2 (where Mca = (7-methoxycoumarin-4-yl)acetyl and Dpa = dinitrophenyl)-L-2,3-diaminopropionyl) was obtained from Bachem, the ADAM substrate Mca-PLAQAV-Dpa-RSSSR-NH_2 was obtained from R&D systems. Ultra-pure water was produced in-house by an Elga water purifying system and used for all mobile phase and buffer preparations.

Determination of IC_{50} values shown in Table 1

The compounds were tested for efficacy by determining their ability to inhibit conversion of a fluorogenic peptide substrate by recombinant metalloproteinases. IC_{50} values for MMP-12 were determined using recombinant catalytic domain (a kind gift by AstraZeneca R&D, Lund and Moelndal, Sweden) and substrate Mca-PLGL-Dpa-AR-NH_2 (Bachem). IC_{50} values for ADAM-17 were determined using recombinant ectodomain (R&D Systems) and substrate Mca-PLAQAV-Dpa-RSSSR-NH_2 (R&D Systems). Proteolysis rates of the substrates were determined at 25 °C by measuring increase in fluorescence ($\lambda_{ex,em}$ = 320, 440 nm) for 15 min. in 96-well plates (Costar White) using a Fluorostar Optima plate reader (BMG Labtech). Each well (final volume 100 μL) contained 10 ng enzyme and a final concentration of 4 μmol/L substrate in assay buffer (MMP-12: 50 mM Tris pH 7.4, 0.1 M NaCl, 10 mM $CaCl_2$, 0.05% w/v Brij-35. ADAM-17: 25 mM Tris pH 9.0, 2.5 μM $ZnCl_2$, 0.005% w/v Brij-35). Inhibition curves were plotted in Origin 7.0 (Micronal) and IC_{50} values were determined by sigmoidal fitting.

Determination of covalent labeling of active ADAM-10 by probe 18 (Figure 3)

Recombinant ADAM-10 ectodomain (R&D systems) was incubated with probe **18** in 96-well plates (Costar White). Each well (final volume 50 μL) contained 100 ng protein and 2.5 μM inhibitor in assay buffer (25 mM Tris pH 9.0, 2.5 μM $ZnCl_2$, 0.005% w/v Brij-35). One well also contained 100 μM ADAM inhibitor TAPI-2 (Calbiochem). In one well ADAM-10 was denatured prior to incubation by boiling for 5 min. in assay buffer with 2% w/v SDS. The plate was irradiated by UV light under a Camag universal UV lamp with a 366 nm filter for different times. The reaction was stopped by adding 10 μL 5× non-reducing SDS-PAGE sample buffer (Pierce). Samples were analyzed by SDS-PAGE by loading 20 μl of each sample on an 8% polyacrylamide Precise Protein gel (Pierce) and running in a mini-Protean III electrophoresis system (Bio-Rad) at 100 V for 45 min. The protein was transferred to an Immun-Blot PVDF membrane (Bio-Rad) by wet Western blotting in a mini Trans-

blot cell (Bio-Rad) at 350 mA for 90 min. in 25 mM Tris, 190 mM glycine with 20% v/v methanol. The membrane was blocked for 30 min. in TBS-Tween supplemented with 5% w/v non-fat dried milk and incubated for 1 h in TBS-Tween with 1:1500 streptavidin-alkaline phosphatase (Sigma-Aldrich). Staining was performed with NBT/BCIP (Duchefa).

Determination of inhibitor efficacy (Table 2)

The efficacy of the inhibitors was tested by evaluating their ability to inhibit proteolytic conversion of a fluorogenic substrate by recombinant metalloproteases. A fixed concentration of each inhibitor (final concentration 100 nM) was incubated with 5 ng of either MMP-9 catalytic domain, MMP-12 catalytic domain or ADAM-17 ectodomain in assay buffer (for MMP-9 and -12: 50 mM Tris pH 7.4, 0,1 M NaCl, 10 mM $CaCl_2$, 0.05 % w/v Brij-35; for ADAM-17: 25 mM Tris pH 9.0, 2.5 μM $ZnCl_2$, 0.005% w/v Brij-35) in 96-well plates (Costar White). The appropriate fluorogenic substrate was added to a final concentration of 2 μM and proteolysis rates were determined by measuring fluorescence ($\lambda_{ex,em}$ = 320, 440 nm) increase using a Fluostar Optima plate reader (BMG Labtech) at 28 °C. The remaining catalytic activity was calculated by comparing with proteolysis rates of 5 ng uninhibited enzyme.

Determination of IC_{50} values shown in Table 3

The IC_{50} values of eight selected inhibitors were determined in a competitive enzyme activity assay monitoring conversion of the same fluorogenic substrates by recombinant metalloproteinases in presence of increasing concentrations inhibitor. Measurements were performed in 96-well plates (Costar white), where each well contained 5 ng of either MMP-9 catalytic domain, MMP-12 catalytic domain or ADAM-17 and a final concentration of 2 μM of the appropriate substrate in a final volume of 100 μL assay buffer. Proteolysis rates were determined by measuring fluorescence increase like above. Seven-point inhibition curves (0-10 μM) were plotted in Origin 7.0 (Micronal) and IC_{50} values were determined by sigmoidal fitting.

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