# A peptide hydroxamate library for enrichment of metalloproteinases: towards an affinity-based metalloproteinase profiling protocol†

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*Received 28th November 2007, Accepted 18th January 2008*
*First published as an Advance Article on the web 22nd February 2008*
**DOI: 10.1039/b718352f**

A compound library of 96 enantiopure N-terminal succinyl hydroxamate functionalized peptides was synthesized on solid phase. All compounds were tested for their inhibitory potential towards MMP-9, MMP-12 and ADAM-17, which led to the identification of both broad spectrum inhibitors and metalloproteinase-selective ones. Eight potent and less potent inhibitors were immobilized on Sepharose beads and evaluated in solid-phase enrichment of active MMP-9, MMP-12 and ADAM-17. In addition, one of these inhibitors was used for solid-phase enrichment of endogenous ADAM-17 from a complex proteome (a lysate prepared from cultured A549 cells).

## 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.[1,6–11] 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, thereby enhancing its reactivity towards nucleophilic attack of the water molecule that is present in the active site.[7,12] As a result, a requirement for potent MMP or ADAM inhibitors is that they contain a good zinc binding group. 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. In these structures the oligopeptide portion ensures recognition by the metalloproteinases, whereas the hydroxamate acts as a zinc chelator.

C-terminal peptide hydroxamic acids are readily available through modified solid-phase peptide synthesis (SPPS) protocols.[13–18] In contrast, there are very few synthetic procedures towards N-terminal peptide hydroxamates,[19–21] which obviate a non-SPPS step during synthesis.[22–26] The preparation of compound libraries containing N-terminal peptide hydroxamates would be greatly facilitated by the existence of suitable, complete SPPS methods. With this aim in mind, we recently reported the synthesis of an enantiomerically pure *N*,*O*-diprotected succinyl hydroxamate building block **1** (Fig. 1) and demonstrated that *N*-terminal peptide hydroxamates can be prepared by SPPS using compound **1** in the penultimate step, prior to acid cleavage and deprotection.[27] Here we report the application of building block **1** in the preparation of a library containing 96 enantiopure peptide hydroxamates **2** (Fig. 1). We further demonstrate the use of several members of our library in the solid-phase extraction of active metalloproteinases from complex biological samples.[28,29]

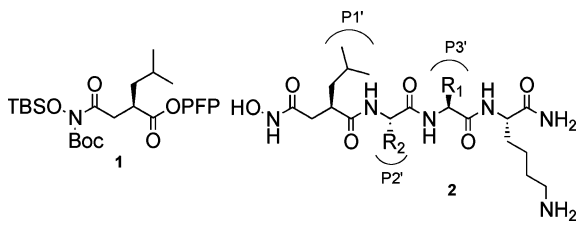


**Fig. 1** Structure of the chiral succinylhydroxamate building block **1** for SPPS of a library of 96 compounds with the general structure **2**. P1′, P2′ and P3′ refer to the binding pockets in the metalloproteinase. Boc: *tert*-butyloxycarbonyl, TBS: *tert*-butyldimethylsilyl, PFP: pentafluorophenyl. $R_1$ and $R_2$ represent amino acid side chains.

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† Electronic supplementary information (ESI) available: Experimental details for building block **1** and compound library **2** (including spectroscopic data) and all biological experiments. See DOI: 10.1039/b718352f

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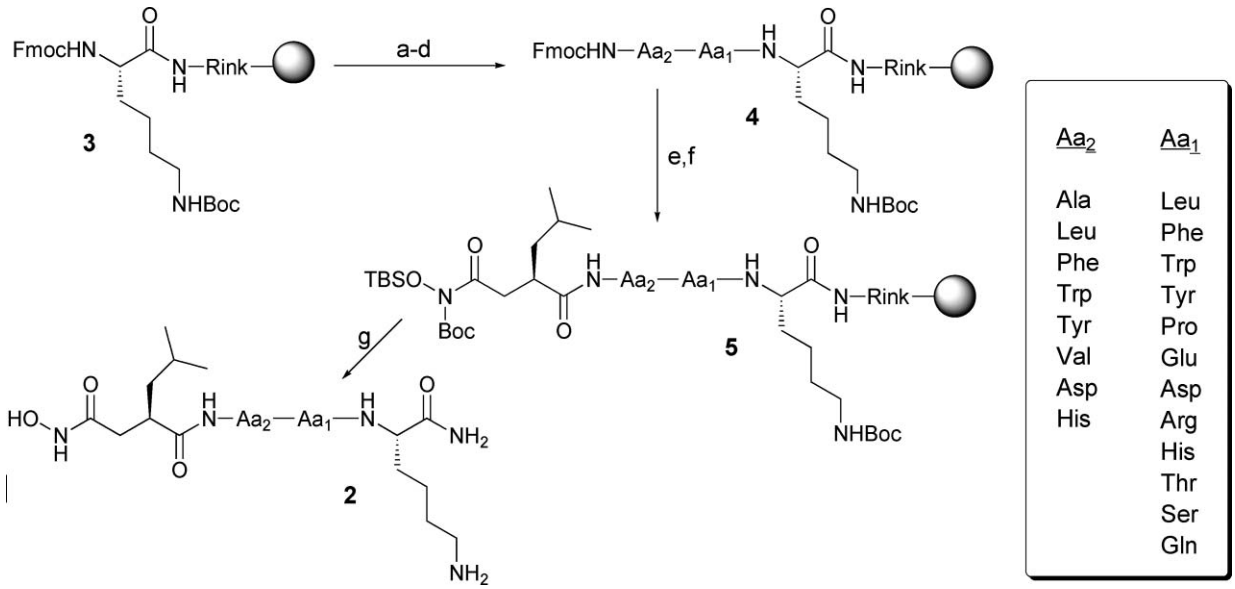


**Scheme 1** Solid-phase synthesis of a succinylhydroxamate library containing 96 compounds. *Reagents and conditions*: (a) 20% piperidine–DMF, 15 min.; (b) Fmoc-$Aa_1$-OH (5 equiv.), HCTU (5 equiv.), DIPEA (10 equiv.), NMP, 1 h; (c) 20% piperidine–DMF, 15 min.; (d) Fmoc-$Aa_2$-OH (5 equiv.), HCTU (5 equiv.), DIPEA (10 equiv.), NMP, 1 h; (e) 20% piperidine–DMF, 15 min.; (f) compound **1** (5 equiv.), DIPEA (2 equiv.), NMP, 2 h; (g) 95% TFA–$H_2O$, 1 h (2.5 h for $Aa_1$ = Arg(Pmc)). $Aa_1$ = D, E, F, H, L, P, Q, R, S, T, W or Y; $Aa_2$ = A, D, F, H, L, V, W or Y.

## Results and discussion

The preparation of the target compound library (see Scheme 1) commenced with α-NHFmoc-, ε-NHBoc-protected lysine on Rink amide resin **3**. 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 next Fmoc group and coupling of the second amino acid ($Aa_2$) gave 96 immobilized peptides with the general structure **4**. Final Fmoc deprotection and condensation with building block **1** (see Fig. 1) in the presence of 2 equivalents of DIPEA resulted in the immobilized and fully protected peptide hydroxamates **5**. Acidic cleavage from the resin and concomitant deprotection of the TBS and Boc protecting groups resulted in a 96-membered library of crude compounds **2**, which were purified by HPLC. The yields of the pure peptides based on **3** (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. Representative LC-MS analyses of crude peptides with and without high levels of side products together with their HPLC-purified counterparts are shown in the supporting information.†

The results of the inhibitory potential of the 96 compounds against MMP-9, MMP-12 and ADAM-17 are depicted in Fig. 2 (heat map representation, the compounds were screened at 100 nM). We performed this initial screen to obtain qualitative insight into the difference in inhibitory potential of the 96 peptide

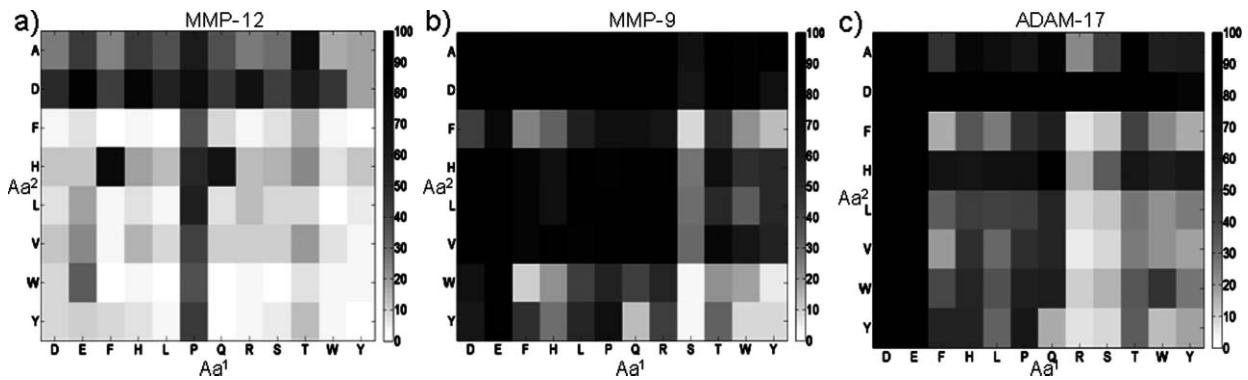


**Fig. 2** Remaining enzymatic activity of three recombinant metalloproteinases (5 ng) at 100 nM of inhibitor (black: no inhibition; white: complete inhibition). Each value is the average of three individual experiments: (a) MMP-12 (0.25 pmol); (b) MMP-9 (0.25 pmol); (c) ADAM-17 (66 fmol).

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.[30,31] 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 extent 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.[31]

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.[32] 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, our 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 we observe a detrimental effect for both residues at this position. 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.[32] 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.[7,33]

To assess the applicability of the novel inhibitors for activity-based extraction[34] eight inhibitors were selected for further experiments. The $IC_{50}$ values (see Table 1) of these inhibitors for the target enzymes span the entire range from sub-nanomolar to over 10 μM (see 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). Activity-based extraction of the three metalloproteinases was performed at both 5 and 0.5 nM enzyme concentrations.[35] Especially at lower concentrations of active enzyme, highly efficient interaction of the immobilized ligand with metalloproteinases in the sample is likely to be critical for efficient extraction. Next to achieving high affinity, it is important to minimize non-specific binding to the carrier material, especially at low enzyme concentrations. Since finding a carrier material that exhibits no non-specific interaction with proteins in the sample is practically impossible, good controls are required. For these experiments two control materials were used: (a) NHS-Sepharose that was reacted with ethanolamine instead of the inhibitors to study non-specific interaction with the Sepharose itself, and (b) a low-affinity inhibitor (PD) was immobilized to assess the importance of a good fit with the enzyme's active site.

**Table 1** $IC_{50}$ values (nM) of eight selected inhibitors for MMP-9, MMP-12 and ADAM-17. Each value represents the mean of three independent inhibition curves (standard deviation in parentheses)

| | MMP-9 | MMP-12 | ADAM-17 |
|---|---|---|---|
| DV | 905 (221) | 10.5 (4.0) | 2241 (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[a] | 2788 (392) | 5998 (2555) |
| PL | 3624 (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] Activity of enzyme greater than 50% at 10 μM inhibitor.

Table 2 shows the results of extraction studies with the eight selected inhibitors. Quantitative extraction of ADAM-17 proves much more challenging than extraction of the MMPs, which may be caused by the fact that the recombinant ADAM-17 ectodomain is substantially larger in size than the catalytic domains of the MMPs used in this study, possibly giving rise to steric effects that decrease extraction yield. MMP-9 and MMP-12 can be extracted by a number of inhibitors with yields above 99% at both 5 and 0.5 nM. Inhibitors QY, SF, FF and YW show enrichment of ADAM-17 at 5 nM enzyme concentration (extraction yields of 70% or higher). The efficiency drops significantly at 0.5 nM for inhibitors QY, SF and YW. The negative control with immobilized ethanolamine shows no detectable non-specific binding to ADAM-17 at both concentrations which is also the case for the low-affinity inhibitor PD. The results (summarized in Table 2) show that it is difficult to classify immobilized inhibitors according to their inhibition efficacy by affinity-SPE (see Tables 1 and 2) and that it may be misleading to assess inhibitor selectivity in this manner.[28] One interesting inhibitor in this respect is DV, which gives extraction yields of >99% for MMP-12 at both concentrations while its $IC_{50}$ value is 10.5 nM. This value is higher than, for example, inhibitor YW, which extracts between 96% and 99%. Inhibitor DV also loses its selectivity towards MMP-12 after immobilization, since both MMP-12 and MMP-9 were almost completely extracted even at a low concentration. In contrast ADAM-17 is not extracted at all although the $IC_{50}$ values for MMP-9 and ADAM-17 are in the same range.

Fig. 3 shows activity-dependent enrichment of ADAM-17 from a complex proteome (a lysate prepared from cultured A549 cells) using immobilized inhibitor FF. The extraction was almost complete, with no loss of mature 70 kDa ADAM-17 in the flow-through fraction (FT) and minor loss in the washing fractions. Active ADAM-17 could be eluted with a competitive inhibitor with a similar $IC_{50}$ value (SF; see Table 1), further confirming that the interaction was inhibitor-mediated. We have shown in previous work that inhibitor–metalloproteinase interactions are strictly dependent on a functional active site and that

 

**Table 2** Extraction yield (%) of three recombinant active metalloproteinases by using the immobilized inhibitors as affinity ligand (standard deviation in parentheses)

| | | Inhibitor | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Enzyme | Conc./nM | DV | FF | FW | PD | PL | QY | SF | YW | Control EA[a] |
| MMP-9 | 5.0 | 98.7 (1.2) | 98.9 (0.85) | 99.0 (0.78) | 7.2 (9.4) | 98.9 (1.1) | 98.4 (0.57) | >99 | >99 | <1 |
| | 0.5 | >99 | 98.8 (0.99) | >99 | 31.7 (24) | 96.9 (0.92) | 98.6 (1.3) | 98.5 (0.21) | >99 | <1 |
| MMP-12 | 5.0 | >99 | 98.9 (0.92) | 95.1 (0.14) | 96.6 (0.071) | 97.8 (0.49) | >99 | 98.9 (0.92) | 97.1 (1.3) | <1 |
| | 0.5 | >99 | >99 | >99 | 75.7 (11) | 80.8 (20) | >99 | >99 | 96.0 (5.0) | <1 |
| ADAM-17 | 5.0 | <1 | 72.9 (1.8) | 35.7 (24) | <1 | 65.8 (0.85) | 88.2 (12) | 85.7 (1.3) | 70.0 (6.4) | <1 |
| | 0.5 | <1 | 73.7 (9.8) | 43.3 (10) | <1 | <1 | 4.60 (5.8) | 49.6 (22) | 2.15 (2.3) | <1 |

[a] EA: ethanolamine.

enzyme–inhibitor complexes or pro-enzymes are not bound.[29] The minimal losses in the flow-through and washing fractions indicate that there is little inactive ADAM-17 in non-stimulated A549 cells and that the interaction is relatively tight. We anticipate that enrichment of active ADAM-17 from larger sample volumes will be possible allowing detection of low levels of active ADAM-17. Extraction of the same sample on a control material that was derivatized with ethanolamine (EA cartridge) did not result in any ADAM-17 enrichment.

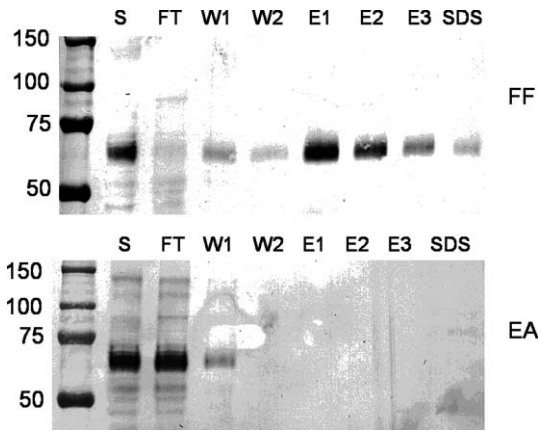


**Fig. 3** Activity-dependent enrichment of ADAM-17 from a cell lysate of non-stimulated cultured A549 cells using immobilized inhibitor FF and control ethanolamine–Sepharose (EA) cartridges. S: original lysate, FT: flow-through, W: washing fractions, E: elution with 100 μM competitive inhibitor SF, SDS: final elution with 1% sodium dodecyl sulfate. Fractions were analyzed by electrophoresis on 8% polyacrylamide gels and transferred to PVDF membranes. ADAM-17 was detected by Western blotting.

Phorbol esters have been described to enhance ADAM-mediated shedding of membrane anchored compounds.[36] It is therefore assumed that ADAM-17 becomes activated upon PMA (phorbol-12-myristate-13-acetate) stimulation. Fig. 4 shows the effect of PMA on A549 cells with respect to extraction of active ADAM-17. Interestingly, short-term exposure (30 min) to PMA results in the appearance of a non-active form of ADAM-17 with an apparent molecular weight corresponding to the 93 kDa pro-ADAM-17 zymogen (marked with *). Extraction efficiency decreases, as shown by the recovery of a substantial fraction of ADAM-17 in the flow-through and washing fractions, although the major portion still binds the immobilized inhibitor. Decreased extraction might be explained by mobilization of endogenous inhibitors or by inactivation of ADAM-17 by another, unknown mechanism. After two hours of exposure the overall level of ADAM-17 has decreased significantly (Fig. 4, lower panel, lane S). Down-regulation of ADAM-17 after cell stimulation has been described.[37] Our results show, however, that there is still a small amount of active ADAM-17.

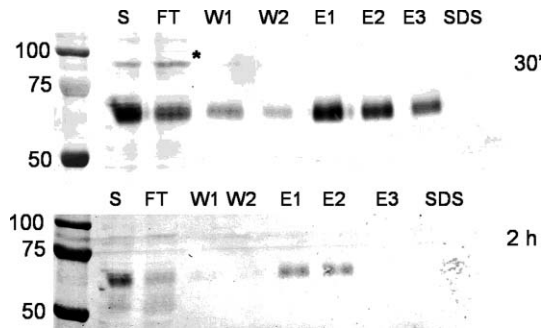


**Fig. 4** Activity-dependent enrichment of ADAM-17 from a cell lysate of PMA-stimulated cultured A549 cells using immobilized inhibitor FF. S: original lysate, FT: flow-through, W: wash fractions, E: elution with 100 μM competitive inhibitor SF, SDS: final elution step with 1% sodium dodecyl sulfate. Fractions were analyzed by electrophoresis on 8% polyacrylamide gels and transferred to PVDF membrane. ADAM-17 was detected by Western blotting.

## Conclusion

In summary we prepared a library of 96 enantiopure peptide hydroxamates which were tested with respect to their inhibitory efficacy towards three metalloproteinases.[38] Our results show that different amino acids at positions $Aa_1$ and $Aa_2$ (see Scheme 1) have a substantial influence on inhibitory capacity. This is in contrast to a report by Whittaker *et al.* stating that amino acids at position $Aa_2$ do not enter the active site of the enzyme and therefore have 'a modest effect' on potency.[7] Several potent inhibitors were immobilized on Sepharose beads and evaluated in solid-phase enrichment of active metalloproteinases. Experiments showed complete enrichment of recombinant MMP-9 and MMP-12 even with lower-affinity inhibitors. Enrichment of active ADAM-17 was more challenging, but two of the tested inhibitors proved to be efficient. Indeed, one of the inhibitors, FF, could be used for enrichment of endogenous ADAM-17 from a complex biological sample. Our data are complementary to the results

recently published by Cravatt and co-workers on ADAM-17 enrichment from biological samples.[39] The strategy employed by Cravatt and co-workers makes use of a photo-activatable probe that is biotinylated in a two-step bioorthogonal fashion after irreversible enzyme modification, allowing streptavidin-mediated enrichment. Our approach is based on a straightforward affinity-based enrichment protocol. Of interest is our finding that the correlation between affinity (*i.e.* $IC_{50}$ value) of free inhibitor and suitability for activity-based enrichment is not as obvious as we originally expected. We are currently evaluating the suitability of our library, in combination with solid-phase enrichment, as a tool to monitor MMP/ADAM activity in patient-derived biological samples in relation to aberrant MMP/ADAM activity. With respect to the enrichment protocol we foresee that our current constructs can possibly be optimized, for instance by variation of the linker connecting the bait to the matrix.

## Experimental

### General

Tetrahydrofuran was distilled over $LiAlH_4$ before use. Acetonitrile (MeCN), 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 $g^{-1}$) was purchased at Novabiochem, as well as all appropriately protected amino acids (FmocAlaOH, FmocArg(Pmc)OH, FmocAsp(*t*Bu)OH, FmocGln(Trt)OH, FmocGlu(*t*Bu)OH, FmocHis(Trt)OH, FmocLeuOH, FmocLys(Boc)OH, FmocPheOH, FmocProOH, FmocSer(*t*Bu)OH, FmocThr(*t*Bu)OH, FmocTrp(Boc)OH, FmocTyr(*t*Bu)OH and FmocValOH). *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 co-evaporation 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 or nitrogen atmosphere. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 μm C18 50 × 4.60 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% acetonitrile in 0.1% TFA–water. 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% acetonitrile 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.

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 from R & D Systems. The fluorogenic MMP substrate Mca-PLGL-Dpa-AR-$NH_2$ (where Mca = (7-methoxycoumarin-4-yl)acetyl and Dpa = *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl) was from Bachem, the ADAM substrate Mca-PLAQAV-Dpa-RSSSR-$NH_2$ was from R & D Systems. *N*-Hydroxysuccinimide (NHS)-Sepharose was from Amersham Bioscience. Acetonitrile (gradient grade) was from Biosolve, ultra-pure water was produced in-house by an Elga water purifying system and used for all mobile phase and buffer preparations. Other chemicals were purchased from Sigma.

### Preparation of the library

Rink amide resin was rinsed with DCM, MeOH and DMF (2× each), then deprotected by shaking with 20% piperidine in DMF for 10 minutes (2×), and rinsed with DMF and DCM (2× each). Loading of the resin was effected by shaking with FmocLys(Boc)OH (5 equiv. relative to the stated loading), HCTU (5 equiv.) and 0.45 M DIPEA (10 equiv.) 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 equiv.) in 0.45 M DIPEA (10 equiv.) in NMP for 5 minutes. 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 $g^{-1}$.

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 were added compound **1** (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% TFA–water (0.5 mL) for 1 h (with the exception of resins containing Arg(Pmc), these were reacted for 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 at −20 °C overnight. The tubes were then centrifuged, and the filtrates were decanted. The crude products were analysed by LC-MS, then purified by semi-preparative HPLC. The yields of the pure peptides based on **3** (purity >95% as determined by LC-MS analysis) varied between 3% and 40%

### Screening of inhibitor efficacy

Efficacy of the inhibitors was tested by evaluating their ability to inhibit proteolytic conversion of a fluorogenic substrate by recombinant metalloproteinases. 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 (see supporting information†) and plotted in heat maps generated in Matlab.

### Determination of $IC_{50}$ values

The $IC_{50}$ values of eight selected novel inhibitors were determined in a competitive enzyme activity assay monitoring conversion of the same fluorogenic substrates by recombinant metalloproteinases in presence of increasing concentrations of 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. Seven-point inhibition curves (0–10 μM) were plotted in Origin 7.0 (Micronal) and $IC_{50}$ values were determined by sigmoidal fitting.

### Immobilization of inhibitors on Sepharose beads

The eight selected inhibitors were linked to Sepharose as described earlier.[28] Briefly, NHS-activated Sepharose beads were washed at 4 °C with several volumes of 1 mM HCl and several volumes of coupling buffer (0.1 M $K_2HPO_4$, pH 7.5). The washed beads were then incubated with an equal volume of a 5 mM solution of inhibitor in coupling buffer for 2 h in a shaking incubator (Eppendorf thermomixer, 1200 rpm, 25 °C). After removal of the supernatant the unreacted NHS-groups were quenched by incubation with 10 bead volumes of blocking buffer (0.5 M ethanolamine in coupling buffer). The immobilization was monitored by HPLC analysis of each inhibitor-containing coupling buffer before and after the procedure. Analysis was performed on a Merck-Hitachi system fitted with a UV detector at 214 nm. Samples were separated on a Zorbax Eclipse XDB-C8 column (4.6 × 150 mm, 5 μm particles, Agilent Technologies). Mobile phase A was 0.1% v/v TFA in water, mobile phase B was 0.1% TFA in acetonitrile. Samples were diluted by ten times in mobile phase A and 10 μL was injected. A linear gradient separation was performed by increasing the percentage B from 0 to 60% in 30 minutes. The theoretical ligand density for each immobilized inhibitor was calculated by evaluation of the peak area for the free inhibitor before and after the immobilization (see supporting information†).

### Determination of extraction yield

The efficacy of the immobilized inhibitors for use as activity-based affinity ligands in solid-phase extraction (SPE) of MMPs and ADAMs was determined by performing column-based extractions on each material. Each batch of inhibitor-beads was slurry packed into Prospekt SPE cartridges fitted on one side with a 0.2 μm stainless steel frit (Spark Holland, 2 mm ID × 10 mm) in MMP assay buffer. The packed cartridges were placed in a clamp and attached to a syringe pump (KD Scientific). Extractions were performed in the assay buffers described above. Before extraction the cartridges were conditioned by flushing two times with 250 μL of the appropriate assay buffer. All steps were performed at 50 μL $min^{-1}$. Samples containing recombinant enzyme (200 μL of 0.5 or 5 nM solution) were applied to the cartridges and the cartridges were washed four times with 200 μL assay buffer to determine breakthrough. All eluting fractions were collected separately and immediately placed on ice. Between extractions the cartridges were regenerated by eluting bound enzyme with 10 mM ethylenediaminetetraacetic acid, disodium salt (EDTA) in assay buffer and reconditioning two times with 200 μL assay buffer. Enzyme activity in the collected fractions was determined by the activity assay described above and related to the activity in the original sample.

### Extraction of active ADAM-17 from a lung carcinoma cell line

Human alveolar carcinoma cell line A549 (ATCC nr. CCL-185) was grown to 90% confluency in RPMI-1640 with l-glutamine (Cambrex) supplemented with 10% (v/v) fetal bovine serum (Cambrex) and 20 μg $mL^{-1}$ gentamycin (Centafarm) in 25 $cm^2$ culture plates at 37 °C, 5% $CO_2$. The cells were serum starved for 24 h and stimulated with 100 nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) for different time periods. After stimulation, the cells were harvested in 1 mL ice-cold lysis buffer (25 mM Tris pH 9.0, 2.5 μM $ZnCl_2$, 1% v/v Triton X-100). After cell lysis (1 h on ice) the lysates were centrifuged in a cooled (4 °C) Eppendorf centrifuge at 20 000 × g for 15 minutes to remove cell debris. The supernatant was kept on ice until the extraction. Spark cartridges (2 mm ID × 10 mm) packed with either Sepharose immobilized inhibitor FF or control ethanolamine–Sepharose were equilibrated with lysis buffer (2 × 250 μL pumped at 50 μL $min^{-1}$). The lysates were applied to the cartridge (250 μL, 25 μL $min^{-1}$) and the flowthrough was collected. The cartridges were washed once with lysis buffer (250 μL, 50 μL $min^{-1}$) and three times with wash buffer (25 mM Tris pH 9.0, 2.5 μM $ZnCl_2$, 0.005% v/v Brij-35). Elution was carried out by elution with a competitive inhibitor in wash buffer (100 μM inhibitor SF, 3 × 60 μL, 10 μL $min^{-1}$) and a non-specific elution step with 1% sodium dodecyl sulfate (SDS) in wash buffer (100 μL, 20 μL $min^{-1}$). 60 μL of each sample was diluted with 20 μL non-reducing sample buffer (Bio-Rad) and, after heating (5 min at 95°C), separated by discontinuous SDS-PAGE according to Laemmli[40] on 0.75 mm thick slabs (8% polyacrylamide). Electrophoresis was carried out in a Mini-Protean III cell (Bio-Rad). The proteins were transferred to Immun-blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad) by wet Western blotting in a mini Trans-blot cell (Bio-Rad) at 350 mA for 60 minutes in 25 mM Tris, 190 mM glycine with 20% v/v methanol. The membranes were blocked overnight in TBS-T (10 mM Tris pH 7.4, 137 mM NaCl, 0.05% (v/v) Tween-20) supplemented with 5% w/v non-fat dried milk (Protifar, Nutricia) at 4 °C. Incubation with the primary anti-ADAM17 ectodomain monoclonal antibody (R & D Systems, clone 111636) was performed at room temperature for 2 h at 0.5 μg $mL^{-1}$ in

TBS-T with 1% w/v non-fat milk, followed by 1.5 h exposure to a secondary rabbit anti-mouse antibody conjugated with alkaline phosphatase (Sigma-Aldrich) at a 1 : 15 000 dilution. Bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (Duchefa).

## Acknowledgements

We thank the Dutch Technology Foundation STW (project GPC 6150) and The Netherlands Proteomics Centre (NPC) for financial support of this work. We thank Dr Peter Horvatovich for assistance in preparing the heat maps (Fig. 2) in Matlab.

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