Synthetic tools to illuminate matrix metalloproteinase and proteasome activities
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3.1 Introduction

As outlined in Chapter 2 MMPs and ADAMs are an important class of proteases that fulfil a significant role in (extra-cellular) physiological processes\textsuperscript{1-10} and, as a consequence, deregulation of metalloprotease catalytic activity can lead to several inflammatory processes\textsuperscript{6,11-15}. Conventional proteomics approaches to determine the relation of metalloproteases to disease states are limited by the fact that they take the total protein amount into account, whereas in many cases the functionality, that is the catalytic activity, is the relevant parameter. Several elegant methods to determine proteolytic activity in biological samples have been developed, such as zymography and activity-based ELISA\textsuperscript{16,17}. Although these approaches visualise and quantify active proteases, application to a family-wide proteomics approach is difficult. Substrate specificity in zymography (for instance, gelatinases in gelatin zymography) and antibody specificity in ELISA make that both techniques are inherently limited to specific enzymes. Due to these limitations, there is a growing interest in the development of family-wide functional proteomics probes. Considerable progress have been made in the development and application of activity-based probes targeting cysteine proteases\textsuperscript{18-21}, serine hydrolases\textsuperscript{22-25} and proteasome subunits\textsuperscript{26-28}. In these proteases a side chain residue (serine, cysteine or threonine) acts as the nucleophilic species involved in amide bond cleavage that is amenable to covalent and irreversible modification by instalment of an appropriate electrophilic trap in the activity-based probes. MMPs and ADAMs employ a water molecule as the nucleophile in their active site, which precludes the use
of such an electrophilic trap. Photoaffinity labeling represents an alternative way to introduce tags into the active site of metalloproteases. In this context, peptide hydroxamate 1 featuring both a biotin and a trifluoromethyl-diazirine moiety (Figure 1B) was developed (Chapter 2). Upon incubation of purified recombinant ADAM-10 and subsequent irradiation with UV light (366 nm) the metalloprotease was covalently and irreversibly modified, as was evidenced by SDS PAGE of the denatured protein followed by streptavidin blotting. The efficiency of the photoaffinity labeling however proved rather modest. This raised the question whether the photoactivatable group would be better directed towards the P1' pocket, rather than the P2' pocket (see Figure 1A for a general picture of the binding mode of N-terminal peptide hydroxamate-based metalloprotease inhibitors). Examination of the available 3-dimensional structures of metalloprotease-inhibitor complexes indicates that the P1' pocket in general should be able to accommodate rather bulky hydrophobic groups at this position. It was decided to address this issue by the synthesis of peptide hydroxamate 2a (Figure 1C) with the photoactivatable group at the P1' position, and compare its MMP/ADAM labeling efficiency to that of probe 1 having the photoactivatable group at the P2' position. This chapter describes an efficient synthesis of the required diazirine-modified succinyl hydroxamate building block and its application in the synthesis of activity-based probe 2a along with a pair of fluorescent analogues 2b and 2c. It is further demonstrated that 2a indeed is the more efficient photoactivatable MMP/ADAM activity-based probe compared to 1 in a head to head comparison towards a range of recombinant, purified metalloproteases.

![Figure 1](image_url)

**Figure 1.** (A) Generic binding mode of N-terminal peptide hydroxamates to metalloprotease active sites. (B) Peptide hydroxamate-based activity-based metalloprotease probe from previous studies with the photoactivatable group at the P2' position. (C) Compounds targeted in this study and featuring the photoactivatable group at the P1' site.
### 3.2 Results and Discussion

In the work described in Chapter 2 on the synthesis of ABP 1, a chiral, L-leucine mimetic succinyl hydroxamate was employed in which the hydroxamic acid moiety was protected with acid-labile protective groups (O(TBS)-N(Boc)-R) and the carboxylate activated as the pentafluorophenyl ester. This building block can be readily incorporated in Fmoc-based solid-phase peptide synthesis protocols as the final building block after which the immobilized peptide hydroxamate is in one step cleaved from the resin and concomitantly deprotected. This strategy appeared of general use as was demonstrated by the construction of a peptide hydroxamate library (Chapter 2) and it was therefore decided to apply a similar strategy for the construction of the target compounds (2a-c). This required the synthesis of trifluoromethylphenyldiazirine functionalised analogue of compound 2 in Chapter 2. The synthesis of this compound (24, Scheme 2) starts with the construction of the P1'-trifluoromethylphenyldiazirine side chain, shown in Scheme 1, which was prepared as follows. Reduction of 4-bromophenylpropionic acid 3 with LiAlH₄ provided alcohol 4 that was transformed into TBS ether 5. At this stage the trifluoroacetyl moiety was introduced by first lithiation and subsequent addition of 1-trifluoroacetyl piperidine. Refluxing the resulting ketone 6 and hydroxylamine in pyridine afforded oxime 7 as an E/Z mixture (~3:1). The hydroxyl in 7 was converted into the tosylate 8 after which reaction with liquid ammonia under 8 bar at room temperature led to the formation of diaziridine 9.

**Scheme 1.** Synthesis of chirally pure diazirine 15.

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**Reagents and conditions:** (a) LiAlH₄, Et₂O, 0 °C, quant.; (b) TBSCI, imidazole, DMF, quant.; (c) nBuLi, 1-trifluoroacetyl piperidine, Et₂O, −78 °C → RT, 76%; (d) HONH₂·HCl, pyridine, Δ, 93%; (e) TsCl, Et₃N, DMAP, DCM, 97%; (f) NH₃·H₂O, Et₂O, 8 bar, 95%; (g) I₂, Et₂N, MeOH, 94%; (h) HCl, H₂O, MeOH, 97%; (i) TEMPO, BAIB, DCM, H₂O, 96%; (j) i) (ClCO)₂, DMF, DCM; ii) 13, nBuLi, THF, 0 °C, 82%; (k) LiHMDS, tert-butyl bromoacetate, THF, −78 °C, 70%.
Oxidation of the diaziridine using iodine provided diazirine 10. Acidolysis of the TBS ether followed by biphase TEMPO/BAIB oxidation of the resulting alcohol 11 provided carboxylic acid 12 in 57% overall yield over the nine steps. The route of synthesis continued by condensation of 12, via its acyl chloride, with the lithium salt of chiral auxiliary 13. Deprotonation of the resulting intermediate 14 followed by enantioselective alkylation with tert-butyl bromoacetate afforded succinate 15 as the single observed diastereomer in 57% yield over the two steps.

In line with previous studies the Evans template was substituted with lithium benzyl alcoholate to give diester 16 (see Scheme 2). Selective acidic removal of the tert-butyl group gave carboxylic acid 17 which was transformed into fully protected succinyl hydroxamate 18 by first transformation into the corresponding acyl chloride and next reaction with the lithiate of N-Boc-O-TBS-hydroxylamine. It was previously found that condensation of N-Boc-O-TBS-hydroxylamine with related acyl chlorides proceeded well under the agency of two equivalents of 4-dimethylaminopyridine (DMAP) as the base. However, this procedure proved less efficient in the transformation aimed for here, and optimal results were obtained by adding dropwise the lithiate of N-Boc-O-TBS-hydroxylamine to a THF solution of the acyl chloride. Unfortunately deprotection of the benzyl ester to carboxylic acid 19 failed under the conditions attempted (Pd/C, H₂ or Pd(OAc)₂, Et₃SiH, Et₃N), either because the hydrogenation of the benzyl proceeded sluggishly or the diazirine was reduced concomitantly to the diaziridine.

Scheme 2. Construction of photocrosslinker containing building block 24.

Reagents and conditions: (a) BnOH, nBuLi, THF, 0 °C, 82%; (b) TFA, DCM, 97%; (c) i) (ClCO)₂, DMF, DCM; ii) TBSONHBoc, nBuLi, THF, 27% for 18 and 77% for 22; (d) several conditions, no yield; (e) PFPOH, EDC, DCM, 88% over 2 steps; (f) allyl alcohol, nBuLi, THF, 0 °C, 65%; (g) 23, Pd(PPh)₄, THF.

Rather than searching for a protocol in which the diaziridine is oxidised back to the diazirine after hydrogenation, it was opted to adapt the protective group scheme, as follows. Reaction of compound 15 with the lithium salt of allyl alcohol afforded allyl
ester 20. Partial deprotection and condensation with the protected hydroxylamine as described before gave succinyl hydroxamate 22. Now, the allyl ester was removed by reaction with tetrakis(triphenylphosphine)palladium in the presence of N,N'-dimethylbarbaturic acid 23 as the allyl scavenger, providing carboxylate 19. The latter was converted into the key intermediate, pentafluorophenyl ester 24, in 88% yield over the last two steps. The choice for chiral alkylation by means of Evans template chemistry was based on previous work in which it was shown that alkylation and substitution of the Evans template, both of which entail strong basic conditions, gave optically pure products. Indeed, in the here presented synthesis no signs of any kind of epimerization were observed. The chiral outcome of these steps however can vary with the type of side chain used and the use of a different kind of chiral auxiliary, for example the thiazolidinethione derivatives which are known to display a better leaving group ability, may then prove necessary.

The construction of peptide hydroxamates incorporating the chiral succinic acid derivative is depicted in Scheme 3. In the first instance pentafluorophenyl ester 24 was reacted with L-phenylalanine methylamide 25 in DMF to give bisamide 26, which was finally deprotected (TFA/H2O, 95:5 (v:v)) to the free hydroxamic acid 27 in 30% yield over the two steps. This experiment at once established that building block 24 is compatible with the peptide coupling/global deprotection conditions envisioned and delivered a tag-free analogue of the target compounds for control experiments (vide infra). The target compound 2a was prepared by Fmoc-based solid-phase peptide synthesis starting with RINK amide-bound Fmoc-biocytin 28. Standard Fmoc-based solid-phase peptide synthesis afforded immobilized tripeptide 29, which was transformed in three steps (first Fmoc removal, then condensation with pentafluorophenyl ester 24 and finally removal from the solid support with concomitant global deprotection) into peptide hydroxamate 2a in 30% overall yield after RP-HPLC purification and based on 28. In a similar fashion, but employing FmocLys(Boc) instead of Fmoc-biocytin, peptide hydroxamate 30 was prepared. Treatment of 30 with either Bodipy(Tmr)-OSu 50 or Bodipy(FL)-OSu 51 gave compounds 2b and 2c in a yield of 31% and 55% respectively.

Next, the labeling efficiencies of probes 1 and 2a against a panel of MMPs and ADAMs were compared. In a first experiment the inhibitory potency of the two probes against MMP-9, MMP-12, ADAM-10 and ADAM-17 were assessed (Table 1). Although the four enzymes are inhibited in the nanomolar range by both compounds, there are some differences in potency. The values differ especially for ADAM-17, for which hydroxamate 1 appeared about 25 fold more potent compared to 2a. Interestingly, both compounds appear equally efficient in labeling ADAM-17, as is evidenced from Figure 2. In this experiment, recombinant and purified ADAM-17 was exposed to either 1 or 2a and UV light prior to denaturation, SDS PAGE and streptavidin blotting. It seems that inhibitory efficiency is not directly correlated to photoaffinity labelling, a phenomenon that may be explained by the mechanism by which the trifluoromethyldiazirine dissociates and reacts upon irradiation. Upon photoexcitation nitrogen is expelled with concomitant formation of a highly reactive carbene that will insert in the first available X-H (where X
In case an (active site) amino acid is nearby effective photoaffinity labeling is the expected result, whereas poor labelling will occur in case the photoreactive group is solvent (water) exposed.

Scheme 3. Application of building block 24 in both solution-phase and solid-phase peptide chemistry. The construction of target compounds 2a-c.

Reagents and conditions: (a) DMF, 45%; (b) TFA/H2O, 95:5 (v:v), RP-HPLC, 67%; (c) i) 20% piperidine/DMF; ii) Fmoc-Ahx-OH, HCTU, DiPEA, NMP; iii) 20% piperidine/DMF; iv) Fmoc-Phe-OH, HCTU, DiPEA, NMP; (d) i) 20% piperidine/DMF; ii) 24, DiPEA, NMP; iii) TFA/H2O/TIS 95:2.5:2.5 (v:v:v), 30% from 28; (e) Bodipy(Tmr)-OSu, DiPEA, DMF, RP-HPLC, 31%; (f) Bodipy(FL)-OSu, DiPEA, DMF, RP-HPLC, 55%.

Table 1. IC_{50} values (in nM) of compounds 1 and 2a.

<table>
<thead>
<tr>
<th></th>
<th>MMP-9</th>
<th>MMP-12</th>
<th>ADAM-10</th>
<th>ADAM-17</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.1</td>
<td>3.60(a)</td>
<td>114(a)</td>
<td>20.6(a)</td>
</tr>
<tr>
<td>2a</td>
<td>24.2</td>
<td>12.5</td>
<td>54.1</td>
<td>490</td>
</tr>
</tbody>
</table>

\(a\) From reference.\(^{37}\)

A plausible hypothesis may be that the diazirine moiety in compound 2a is bound more tightly to ADAM-17 than the one in compound 1, even though the latter compound is the more potent inhibitor. Perusal of a panel of ten recombinant and purified MMPs and three more ADAMs reveals that, in general, peptide hydroxamate 2a is the more effective affinity label (Figure 2). In each case compound 2a is at least as effective (compare the data obtained for ADAM-9 and ADAM-10) and often in fact provides a signal where compound 1 does not (see for instance the results obtained for
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MMP-8 and MMP-13). Interestingly, multiple bands appear for some of the MMP labeling experiments (see for instance, MMP-1 and MMP-8 treated with 2a), with the band corresponding to the highest molecular weight in each case corresponding to the molecular weight of the full-length MMP at hand. These bands in all likelihood are the result of auto-degradation, in which unmodified MMP processes its photoaffinity labeled counterpart. From these experiments it can be concluded that positioning the photoactivatable group at P1’ as in 2a indeed gives a comparatively more potent MMP/ADAM photoactivatable activity-based probe.

In order to prove that labeling is activity-dependent, aliquots of MMP-9 and MMP-12 were incubated with either the natural inhibitor, TIMP-153 or the non-biotinylated inhibitor 27 (see Figure 3). In the presence of equimolar amounts (relative to 2a) of TIMP-1 neither MMP-9 nor MMP-12 were detectably labeled. Preincubation of MMP-9 or MMP-12 with two-fold molar excess, relative to 2a, of the non-biotinylated inhibitor 27 also effectively abolished labeling. Taken together, these data provide strong evidence that photoaffinity labeling is activity-dependent and that labelling occurs most likely in the active site of the enzymes.

3.3 Conclusion

In summary, the development of an efficient photoactivatable activity-based probe with which a broad panel of MMPs and ADAMs can be covalently and irreversibly modified in an activity-dependent fashion has been described. This work demonstrates that the enantioselective synthesis strategy previously reported37,42 for the preparation of an enantiomerically pure, alkylated succinyl hydroxamate is also effective, in adapted form, for the synthesis of the functionally more challenging key building block 24. Further, the hypothesis that placing the photoactivatable trifluoromethyl diazirine in the P1’ position would lead to more effective activity-based probes proved to be valid. The
next challenge would be to detect MMPs and ADAMs in their natural environment and at natural abundance levels in a photoactivatable activity-based proteomics profiling experimental setting. The ability to prepare, with relative ease, peptide hydroxamates analogous to compounds 1 and 2a such as Bodipy derivatives 2b,c may well be indispensable in reaching this research objective.

![Figure 3](image)

**Figure 3.** Activity-dependence of photoaffinity labeling of MMP-9 and MMP-12 (4 pmol each) with probe 2a (200 nM final concentration) as shown by competition with (A) an equimolar amount of the endogenous MMP inhibitor TIMP-1 (200 nM final concentration) and with (B) a twofold molar excess of compound 27 (400 nM final concentration).

**Experimental section**

**General**

Tetrahydrofuran was distilled over LiAlH₄ prior to 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 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-(1H-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 4 Å molecular sieves, except methanol and acetonitrile which were stored over 3 Å 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 Screening Devices b.v. Silica Gel, with a particle size of 40-63 µm and pore diameter of 60 Å. The eluents toluene, EtOAc and PE (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F₂₅₄). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid, a solution of KMnO₄ (20 g/L) and K₂CO₃ (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. ¹H- and ¹³C-NMR spectra were recorded on a Bruker AV-400 (400 MHz), a Bruker AV-500 (500 MHz) or a Bruker DMX-600 (600 MHz) spectrometer. ¹⁹F NMR spectra were recorded on a Bruker AV-200 (200 MHz) or a Bruker DMX-400 (400 MHz). Chemical shifts are given in ppm (δ) relative to tetramethysilane, CD₃OD, DMSO-d₆, CDCl₃ or CFCl₃ as internal standard. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile 50/50 (v/v))
and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at m/z = 400 (mass range m/z = 150-2000) and dioctylphthalate (m/z = 391.28428) as a “lock mass”. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations [α]D23 were recorded on a Propol automatic polarimeter. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 nm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H2O, ACN and 1.0% aq. TFA. RP-HPLC purifications were performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 µm 250 × 10 mm column and a GX281 fraction collector. The applied buffers were: 0.1% aq. TFA and ACN. Appropriate fractions were pooled, and concentrated in a Christ rotary vacuum concentrator overnight at room temperature at 0.1 mbar.

**General procedure A: solid-phase peptide synthesis**

Fmoc Rink Amide MBHA resin (0.64 mmol/g) was used as received. Prior to first use, it was washed twice with DMF, twice with MeOH and twice with DCM. Fmoc deprotection was performed by shaking the resin in a 20% piperidine/DMF (v/v) 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 4 equivalents of HCTU, 4 equivalents of amino acid and 8 equivalents 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 the 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 5 times alternately with DCM and MeOH. Cleavage from the solid support was done by shaking the resin in a TFA:H2O:TIS, 95:2.5:2.5 (v/v/v) solution for 2 h, followed by filtration and rinsing the resin with a small portion of TFA:H2O:TIS, 95:2.5:2.5 (v/v/v). The resulting filtrate was concentrated under reduced pressure and the product was purified by RP-HPLC.

**3-(4-bromophenyl)propanol (4)**

Commercially available 3-(4-bromophenyl)propanoic acid (3, 10.8 g, 46.22 mmol) was dissolved in Et2O (250 mL) and cooled to 0 °C. To this solution was carefully added LiAlH4 (1.3 eq., 60 mmol, 2.28 g) in portions. The reaction was slowly warmed to room temperature in 1 h after which TLC analysis indicated a completed reaction. 1M aq. HCl (200 mL) was slowly added and the layers were separated. The organic layer was extracted with 1M aq. HCl (200 mL), saturated aq. NaHCO3 (2 × 200 mL) and brine (200 mL), dried over MgSO4 and concentrated under reduced pressure. The product was obtained as a colourless oil (yield: 9.9 g, 46.2 mmol, quant.). The spectroscopic data correspond with those reported in literature.54

**3-(4-bromophenyl)propoxy)(tert-butyl)dimethylsilane (5)**

To a solution of alcohol 4 (9.9 g, 46.2 mmol) in DMF (80 mL) were added imidazole (4.77 g, 69.3 mmol) and tert-butylchlorodimethylsilane (TBS-Cl) (7.82 g, 50.8 mmol). The reaction was stirred for 2 h after which TLC analysis indicated complete conversion. Deionised H2O (300 mL) was added and the mixture was extracted 3 times with PE (200 mL). The combined organic layers were extracted with deionised H2O (4 × 200 mL) and brine.
(200 mL), dried over MgSO₄ and concentrated under reduced pressure. The product was obtained as a colourless oil (yield: 15.2 g, 46.2 mmol, quant.) and subjected to the next step without further purification. The spectroscopic data correspond with those reported in literature.\(^{55}\)

**1-(4-(3-(tert-butyldimethylsilyloxy)propyl)phenyl)-2,2,2-trifluoroethanone (6)**

Bromide 5 (7.03 g, 21.36 mmol) was dissolved in Et₂O (100 mL) and cooled to \(-78^\circ C\). \(\text{nBuLi (26.7 mmol, 16.7 mL, 1.6 M in THF)}\) was added dropwise and the solution was slowly warmed up to room temperature at which it was stirred for 1 h. Then the mixture was cooled again to \(-78^\circ C\) and a solution of 1-trifluoroacetyl piperidine (4.2 g, 23.2 mmol) in Et₂O (5 mL) was added dropwise. The solution was slowly warmed to 0 \(^\circ C\) in 2 h after which TLC analysis indicated a complete conversion. The reaction was quenched with saturated aq. NH₄Cl (100 mL) and the layers were separated. The organic layer was extracted with saturated aq. NH₄Cl, deionised water and brine (100 mL each), dried over MgSO₄ and concentrated under reduced pressure. The crude material was purified by column chromatography (10% → 30% toluene/PE) and the product was obtained as a colourless oil (yield: 5.63 g, 16.3 mmol, 76%).

\(^1\)H NMR (400 MHz, CDCl₃): \(\delta = 8.00 \text{ (d, } J = 8.0 \text{ Hz, 2H)}, 7.37 \text{ (d, } J = 8.0 \text{ Hz, 2H)}, 3.64 \text{ (t, } J = 6.4 \text{ Hz, 2H)}, 2.79 \text{ (t, } J = 7.6 \text{ Hz, 2H)}\), 1.89-1.84 (m, 2H), 0.91 (s, 9H), 0.06 (s, 6H) ppm. \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta = 151.3, 130.2, 129.3, 127.7, 116.8\) (q, \(J = 290 \text{ Hz}\)), 61.4, 33.9, 32.4, 25.9, 18.2, -5.4 ppm. HRMS: calcd. for C₁₇H₂₅F₃O₂Si \([M + H]^+\) 347.16487; found 347.16505.

**1-(4-(3-(tert-butyldimethylsilyloxy)propyl)phenyl)-2,2,2-trifluoroethanone oxime (7)**

Ketone 6 (9.43 g, 27.2 mmol) was dissolved in pyridine (30 mL) and hydroxylamine hydrochloride (5.68 mmol, 81.7 mmol) was added. The mixture was stirred at reflux for 2 h after which TLC analysis indicated a complete conversion. The mixture was concentrated under reduced pressure and dissolved in EtOAc (100 mL) and 0.2 M aq. citric acid (100 mL). The layers were separated and the organic layer was extracted with 0.2 M aq. citric acid, deionised water and brine (100 mL each), dried over MgSO₄ and concentrated under reduced pressure. The crude product was obtained as a colourless oil (yield: 9.13 g, 25.3 mmol, 93%) as a Z/E mixture. A small amount was purified by column chromatography (5% → 10% EtOAc/PE) for characterization.

\(^1\)H NMR (400 MHz, CDCl₃): \(\delta = 8.51 \text{ (bs, 1H)}, 8.25 \text{ (bs, 1H)}\), 7.44-7.39 (m, 2H), 7.31-7.23 (m, 2H), 3.67-3.62 (m, 2H), 2.74-2.69 (m, 2H), 1.89-1.82 (m, 2H), 0.91 (s, 9H), 0.06 (s, 6H) ppm. \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta = 147.4-146.5 \text{ (m)}, 144.9, 144.5, 129.2, 128.9, 128.9, 128.7, 128.6, 128.4, 128.3, 128.2, 127.9, 123.7, 120.9 \text{ (q, } J = 273 \text{ Hz)}, 118.6 \text{ (q, } J = 281 \text{ Hz)}, 63.0, 62.8, 34.0, 33.9, 32.0, 31.9, 25.9, 18.4, -5.3, -5.5 ppm. HRMS: calcd. for C₁₇H₂₆F₃NO₂Si \([M + H]^+\) 362.17577; found 362.17583.

**1-(4-(3-(tert-butyldimethylsilyloxy)propyl)phenyl)-2,2,2-trifluoroethanone O-tosyl oxime (8)**

Oxime 7 (9.13 g, 25.3 mmol) was dissolved in DCM (20 mL). To this solution were added Et₃N (5.26 mL, 37.95 mmol) and DMAP (60 mg, 0.5 mmol) after which TsCl (4.84 g, 25.3 mmol) in DCM (20 mL) was added dropwise over 1 h. After stirring the mixture at room temperature for 30 min. TLC analysis indicated a complete conversion. Aqueous citric acid (0.2 M, 100 mL) was added and the layers were separated. The organic layer was extracted with 0.2 M aq. citric acid, deionised water and brine (100 mL each), dried over MgSO₄ and concentrated under reduced pressure yielding the crude product (Z/E mixture) as a colourless oil (yield: 12.59 g, 24.4 mmol, 97%). A small amount was purified by column chromatography (1.5% → 7.5% EtOAc/PE) for characterization. \(^1\)H NMR (400 MHz, CDCl₃): \(\delta = 7.91-7.88 \text{ (m, 2H)}, 7.39-7.25 \text{ (m, 6H)}, 3.66-3.62 \text{ (m, 2H)}, 2.73 \text{ (t, } J = 7.8 \text{ Hz, 2H)}, 2.48 \text{ (s, 3H)}, 2.46 \text{ (s, 3H)}, 1.88-1.83 \text{ (m, 2H)}, 0.91 \text{ (s, 9H)}, 0.90 \text{ (s, 9H)}, 0.06 \text{ (s, 6H)}, 0.05 \text{ (s, 6H)}\) ppm. \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta = 153.8 \text{ (q, } J = 34.4 \text{ Hz)}, 146.8,
Photoreactive probes targeting MMPs and ADAMs

146.6, 146.0, 145.9, 131.5, 131.2, 129.7, 129.1, 128.9, 128.7, 128.4, 121.8, 119.7 (q, J = 276 Hz), 61.9, 61.8, 33.8, 33.8, 32.0, 31.9, 25.8, 21.5, 18.1, –5.5 ppm. HRMS: calculated for C_{24}H_{32}F_{3}NO_{4}SSi [M + H]^+ 516.18462, found 516.18443.

3-(4-(3-(tert-butyldimethylsilyloxy)propyl)phenyl)-3-(trifluoromethyl)diaziridine (9)

Tosylate 8 (12.6 g, 24.4 mmol) was dissolved in Et_{2}O (30 mL) in an autoclave and cooled to –78 °C. Freshly condensed ammonia (~5 mL) was added and the autoclave was closed and warmed to room temperature. The pressure inside increased to 8 bar. After 5 h the autoclave was cooled to –78 °C and opened. Deionised water (40 mL) was carefully added and the mixture was warmed to room temperature. The layers were separated and the organic layer was extracted with deionised water (3 × 50 mL)) and brine, dried over MgSO_{4} and concentrated under reduced pressure. The crude diaziridine was obtained as a colourless oil (yield: 8.32 g, 23.1 mmol, 95%). A small amount was purified by column chromatography (2% → 11% EtOAc/PE) for characterization. ^{1}H NMR (400 MHz, CDCl_{3}): δ = 7.51 (d, J = 8.0 Hz, 2H), 7.24 (d, J = 8.0 Hz, 2H), 3.63 (t, J = 6.2 Hz, 2H), 2.77 (d, J = 8.8 Hz, 1H), 2.71 (t, J = 7.8 Hz, 2H), 2.20 (d, J = 8.8 Hz, 1H), 1.86-1.81 (m, 2H), 0.90 (s, 9H), 0.05 (s, 6H) ppm. ^{13}C NMR (100 MHz, CDCl_{3}): δ = 144.3, 129.1, 128.8, 127.9, 123.6 (q, J = 277 Hz), 61.9, 57.7 (q, J = 35.7 Hz), 34.0, 31.7, 25.9, 18.3, –5.6 ppm. HRMS: calcd. for C_{17}H_{27}F_{3}N_{2}OSi [M + H]^+ 361.19175; found 361.19183.

3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)propan-1-ol (11)

Compound 10 (7.78 g, 21.7 mmol) was dissolved in MeOH (50 mL) and concentrated HCl (37% (w/v), 3 mL) was added. The reaction mixture was stirred at room temperature until TLC analysis revealed a completed reaction after 2 h. The solvent was evaporated under reduced pressure and the residue was dissolved in Et_{2}O (200 mL). The resulting solution was extracted twice with a saturated aq. NaHCO_{3} solution (150 mL) and brine (100 mL), dried over MgSO_{4} and concentrated under reduced pressure. The crude alcohol 11 was obtained as a colourless oil (yield: 5.13 g, 21.0 mmol, 97%). ^{1}H NMR (400 MHz, CDCl_{3}): δ = 7.25 (d, J = 8.0 Hz, 2H), 7.12 (d, J = 8.0 Hz, 2H), 3.66 (t, J = 6.4 Hz, 2H), 2.72 (d, J = 7.8 Hz, 2H), 1.90-1.83 (m, 2H), 1.43 (bs, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_{3}): δ = 143.7, 129.1, 126.5, 126.4, 122.2 (q, J = 263 Hz), 61.4, 33.7, 31.6, 28.2 (q, J = 40.2 Hz) ppm. HRMS: calcd. for C_{11}H_{11}F_{3}N_{2}O [M + H]^+ 359.16710; found 359.16716.
3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)propanoic acid (12)

Alcohol 11 (2.1 g, 8.6 mmol) was dissolved in a mixture of DCM (25 mL) and deionised H₂O (12.5 mL). To this mixture were added 2,2,6,6-tetramethylpiperidinoxy (TEMPO) (0.1 eq., 0.86 mmol, 134 mg) and (bis(acetoxy)iodo)benzene (BAIB) (2.5 eq., 21.5 mmol, 6.92 g). The reaction was stirred overnight at room temperature after which TLC analysis revealed a completed reaction. A saturated aq. Na₂S₂O₃ solution (100 mL) was added and the mixture was vigorously stirred for 5 min. EtOAc (150 mL) was added and the layers were separated. The organic layer was extracted with 1 M aq. HCl and brine (100 mL), dried over MgSO₄ and concentrated under reduced pressure. The crude material was purified by column chromatography (10% → 30% EtOAc/PE) and the product was obtained as a colourless solid (yield: 2.13 g, 8.23 mmol, 96%). 

1H NMR (600 MHz, CDCl₃): δ = 11.81 (bs, 1H), 7.22 (d, J = 9.0 Hz, 2H), 7.11 (d, J = 9.6 Hz, 2H), 2.94 (t, J = 8.1 Hz, 2H), 2.65 (t, J = 8.1 Hz, 2H) ppm. 13C NMR (150 MHz, CDCl₃): δ = 179.4, 142.0, 128.8, 127.2, 126.6, 122.2 (q, J = 273 Hz), 35.2, 30.1, 28.4 (q, J = 40.5 Hz) ppm. HRMS: calcd. for C₁₁H₉F₃N₂O₂ [M + H]⁺ 259.06889; found 259.06909.

(S)-4-benzyl-3-(3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)propanoyl)oxazolidin-2-one (14)

Carboxylic acid 12 (1.04 g, 4.03 mmol) was dissolved in DCM (25 mL) and DMF (1 drop) was added. The mixture was cooled to 0 °C and oxalyl chloride (1.7 mL, 20 mmol) was added dropwise. The reaction was warmed to room temperature and stirred for 30 min. after which gas formation ceased. Toluene was added and the mixture was concentrated in vacuo followed by coevaporation with toluene (twice). The crude product was subjected to the next step without further purification.

nBuLi (2.75 mL of a 1.6 M solution in THF, 4.4 mmol) was put into a flask under an argon atmosphere and cooled to 0 °C. To this was added dropwise a solution of (S)-4-benzyloxazolidin-2-one (13, 0.78 g, 4.43 mmol) in THF (15 mL) and the mixture was stirred at 0 °C for 15 min., thereby forming a white precipitant. Then the freshly prepared acyl chloride in THF (10 mL) was added to the reaction mixture dropwise and the mixture was slowly warmed to room temperature. After 2 h at room temperature, TLC analysis showed complete consumption of the starting material. To the mixture was added deionised water (50 mL) and it was extracted twice with EtOAc (50 mL) and it was extracted twice with EtOAc (50 mL). The combined organic layers were extracted with a saturated aq. solution of NaHCO₃ (2 × 50 mL) and brine (50 mL), dried over MgSO₄ and concentrated under reduced pressure. The obtained material was purified by column chromatography (10% → 30% EtOAc/PE) and the product was obtained as a colourless solid (yield: 1.38 g, 3.31 mmol, 82%). 

1H NMR (600 MHz, CDCl₃): δ = 7.32-7.25 (m, 5H), 7.16 (d, J = 7.2 Hz, 2H), 7.13 (d, J = 8.4 Hz, 2H), 4.67-4.63 (m, 1H), 4.18-4.14 (m, 2H), 3.32-3.19 (m, 3H), 3.04-3.01 (m, 2H), 2.75 (dd, J = 13.8, 9.6 Hz, 1H) ppm. 13C NMR (150 MHz, CDCl₃): δ = 171.9, 153.4, 142.4, 135.0, 129.3, 129.1, 128.9, 127.3, 127.0, 126.6, 122.1 (q, J = 273 Hz), 66.2, 55.0, 37.7, 36.7, 29.7, 28.3 (q, J = 40.0 Hz) ppm. [α] D 23 = +48.5 (c = 1, CHCl₃). HRMS: calcd. for C₂₁H₁₈F₃N₃O₃ [M + H]⁺ 418.13730; found 418.13743.

(R)-tert-butyl 4-((S)-4-benzyl-2-oxooxazolidin-3-yl)-4-oxo-3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)butanoate (15)

LiHMDS (5.5 mL of a 1 M solution, 5.5 mmol) was put into a flask under an argon atmosphere and cooled to –78 °C. To this was added a solution of compound 14 (2.0 g, 5.0 mmol) in THF (25 mL) over 15 min. The reaction was stirred for 1 h at –78 °C after which tert-butyl bromoacetate (2.2 mL, 15 mmol) was added. Then the mixture was slowly warmed to –10 °C in 4 h after which TLC analysis showed a completed reaction. A saturated aq. NH₄Cl solution (50 mL) was added and the mixture was extracted with EtOAc (3 × 30 mL). The combined organic layers were dried over MgSO₄ and concentrated under reduced pressure. The resulting crude mixture was purified by column chromatography (10% → 20% EtOAc/PE) and the
product was obtained as a colourless solid (yield: 1.86 g, 3.50 mmol, 70%). ¹H NMR (600 MHz, CDCl₃): δ = 7.30-7.25 (m, 4H), 7.21-7.18 (m, 3H), 7.05 (d, J = 9.0 Hz, 2H), 4.55-4.52 (m, 1H), 4.42-4.38 (m, 1H), 4.05 (dd, J = 9.0, 1.8 Hz, 2H), 2.79-2.70 (m, 2H), 2.57 (dd, J = 13.2, 9.6 Hz, 1H), 2.25 (dd, J = 16.8, 4.2 Hz, 1H), 1.34 (s, 9H) ppm. ¹³C NMR (150 MHz, CDCl₃): δ = 174.5, 170.8, 152.8, 139.9, 135.3, 129.6, 129.3, 128.7, 127.3, 127.1, 126.4, 121.9 (q, J = 273 Hz), 80.6, 65.7, 55.2, 41.0, 37.3, 36.2, 28.1 (q, J = 39.8 Hz), 27.2 ppm. [α]D = +72.8 (c = 1, CHCl₃). HRMS: calcd. for C₂₇H₂₈F₃N₃O₅ [M + H]+ 532.20538; found 532.20512.

(R)-1-benzyloxy-4-oxo-3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)butanoic acid (17)

Tert-butyl ester 16 (550 mg, 1.19 mmol) was dissolved in DCM (4 mL). To this was added TFA (3 mL) and the reaction was stirred at room temperature. After 1 h TLC analysis indicated a completed reaction. Toluene was added and the mixture was concentrated under reduced pressure. Coevaporating the mixture twice with toluene resulted in a yellowish oil. This crude product was purified by column chromatography (10% → 60% EtOAc/PE) and the free carboxylic acid was obtained as a colourless solid (yield: 558 mg, 1.21 mmol, 82%). ¹H NMR (600 MHz, CDCl₃): δ = 11.15 (bs, 1H), 7.35 (t, J = 7.2 Hz, 1H), 7.32 (d, J = 4.8 Hz, 2H), 7.14 (d, J = 7.8 Hz, 2H), 7.05 (d, J = 7.8 Hz, 2H), 5.06 (dd, J = 36.6, 12.0 Hz, 2H), 3.13-3.10 (m, 1H), 3.00 (dd, J = 13.8, 7.2 Hz, 1H), 2.81 (dd, J = 13.8, 7.8 Hz, 1H), 2.74 (dd, J = 10.8, 9.0 Hz, 1H), 1.40 (s, 9H) ppm. ¹³C NMR (150 MHz, CDCl₃): δ = 177.7, 173.4, 139.6, 135.3, 129.4, 128.5, 128.4, 127.5, 126.5, 122.0 (q, J = 273 Hz), 66.7, 42.4, 37.0, 34.9, 28.2 (q, J = 39 Hz) ppm.
protected hydroxylamine \(44\) (1.2 eq., 0.19 mmol, 48 mg) was dissolved in THF (2 mL) and the solution was cooled to 0 °C. nBuLi (1.05 eq., 0.185 mmol, 0.11 mL of a 1.6 M solution in hexane) was added dropwise and the reaction mixture was stirred at 0 °C for 30 min. In a separate flask the freshly prepared crude acyl chloride was dissolved in THF (2 mL) and cooled to 0 °C. To this the lithiated hydroxylamine mixture was added dropwise and the reaction was stirred at 0 °C for 2.5 h after which TLC analysis indicated complete conversion of compound 17. A 0.1 M aq. HCl solution (10 mL) was added and the mixture was extracted twice with EtOAc (10 mL). The combined organic layers were extracted with 0.1 M aq. HCl and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The crude mixture was purified by column chromatography (toluene → 3% EtOAc/toluene) and the product was obtained as a colourless oil (yield: 30 mg, 47 \(\mu\)mol, 27%). 

\[ \text{1H NMR (600 MHz, CDCl}_3\] } : \(\delta = 7.34-7.31 (m, 3H), 7.20 (t, J = 3.6 Hz, 2H), 7.15 (d, J = 8.4 Hz, 2H), 7.04 (d, J = 7.8 Hz, 2H), 5.06-5.01 (m, 2H), 3.27-3.21 (m, 2H), 2.98 (dd, J = 13.2, 6.6 Hz, 1H), 2.90-2.84 (m, 2H), 1.53 (s, 9H), 0.98 (s, 9H), 0.12 (s, 3H), 0.085 (s, 3H) ppm. 

\[ \text{13C NMR (150 MHz, CDCl}_3\] } : \(\delta = 174.1, 170.3, 151.8, 140.0, 135.5, 129.4, 128.4, 128.2, 128.1, 127.3, 126.4, 122.1 (q, J = 273 Hz), 84.6, 66.4, 42.7, 38.8, 37.4, 28.2 (q, J = 40 Hz), 27.9, 25.6, 18.0, -5.1, -5.1 ppm. 

\(\text{(R)-1-allyl 4-tert-butyl 2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)succinate (20)}\)

Allyl alcohol (190 \(\mu\)L, 2.75 mmol) was dissolved in THF (5 mL) and cooled to 0 °C. To this mixture was added nBuLi (812 \(\mu\)L of a 1.6 M solution, 1.3 mmol) and the reaction was stirred at 0 °C for 30 min. Then a solution of compound 15 (586 mg, 1.10 mmol) in THF (4 mL) was added and the reaction mixture was stirred for 1 h at 0 °C and then warmed to room temperature. After 2.5 h TLC analysis indicated complete conversion of the starting material. A saturated aq. NH\(_4\)Cl solution (20 mL) was added and the mixture was extracted twice with EtOAc (20 mL). The combined organic layers were dried over MgSO\(_4\) and concentrated under reduced pressure. After purification of the crude mixture by column chromatography (2.5% → 5% EtOAc/PE) the product was obtained as a colourless oil (yield: 293 mg, 0.71 mmol, 65%). 

\[ \text{1H NMR (600 MHz, CDCl}_3\] } : \(\delta = 7.21 (d, J = 8.4 Hz, 2H), 7.11 (d, J = 7.8 Hz, 2H), 5.83-5.77 (m, 1H), 5.22 (d, J = 16.8 Hz, 1H), 5.18 (d, J = 10.8 Hz, 1H), 4.57-4.50 (m, 2H), 3.11-3.07 (m, 1H), 3.02 (dd, J = 13.8, 7.2 Hz, 1H), 2.82 (dd, J = 13.8, 7.8 Hz, 1H), 2.59 (dd, J = 16.2, 8.4 Hz, 1H), 2.33 (dd, J = 16.8, 5.4 Hz, 1H), 1.42 (s, 9H) ppm. 

\[ \text{13C NMR (150 MHz, CDCl}_3\] } : \(\delta = 173.5, 170.6, 140.2, 131.7, 129.5, 127.4, 126.5, 122.1 (q, J = 273 Hz), 84.6, 66.4, 42.7, 38.8, 37.4, 28.2 (q, J = 40 Hz), 27.9, 25.6, 18.0, -5.1, -5.1 ppm. 

\[ \text{HRMS: calcd. for C}_{20}\text{H}_{23}\text{F}_3\text{N}_2\text{O}_4 \text{[M + H]}^+ 413.16827; found 413.16826. \]

\((\text{R})-4-(allyloxy)-4-oxo-3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)butanoic acid (21)\)

Tert-butyl ester 20 (1.31 g, 3.17 mmol) was dissolved in DCM (10 mL). To this was added TFA (10 mL) and the reaction was stirred at room temperature. After 1 h TLC analysis indicated a completed reaction. Toluene was added and the mixture was concentrated under reduced pressure. After purification of the crude mixture by column chromatography (10% → 50% EtOAc/PE) the crude product was purified by column chromatography (10% → 50% EtOAc/PE) and the free acid was obtained as a colourless solid (yield: 1.09 g, 3.07 mmol, 97%). 

\[ \text{1H NMR (600 MHz, CDCl}_3\] } : \(\delta = 10.80 (bs, 1H), 7.20 (d, J = 8.4 Hz, 2H), 7.12 (d, J = 8.4 Hz, 2H), 5.82-5.76 (m, 1H), 5.22 (dd, J = 15.6, 1.8 Hz, 1H), 5.19 (dd, J = 10.8, 1.2 Hz, 1H), 4.57-4.52 (m, 2H), 3.15-3.10 (m, 1H), 3.05 (dd, J = 13.8, 7.2 Hz, 1H), 2.83 (dd, J = 13.8, 7.8 Hz, 1H), 2.73 (dd, J = 17.4, 9.0 Hz, 1H), 2.44 (dd, J = 17.4, 5.4 Hz, 1H) ppm. 

\[ \text{13C NMR (150 MHz, CDCl}_3\] } : \(\delta = 177.7, 173.3, 139.8, 131.5, 129.5, 127.6, 126.6, 122.0 (q, J = 273 Hz), 118.5, 65.5, 42.5, 37.1, 34.8, 28.2 (q, J = 40 Hz) ppm. 

\[ \{\alpha\}_D^{23} = +13.2 (c = 1, CHCl}_3\] } . HRMS: calcd. for C\(_{10}\)H\(_{12}\)F\(_3\)N\(_2\)O\(_4\) [M + H\]^+ 357.10567; found 357.10576.
(R)-allyl 4-((tert-butoxycarbonyl(tert-butyldimethylsilyloxy)amino)-4-oxo-2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)butanoate (22)

Carboxylic acid 21 (1.22 g, 3.42 mmol) was dissolved in DCM (15 mL) and DMF (2 drops) were added. This mixture was cooled to 0 °C and oxalyl chloride (4 eq., 13.68 mmol, 1.20 mL) was added dropwise. The reaction was warmed to room temperature and stirred for 30 min. after which gas formation ceased. Toluene was added and the mixture was concentrated in vacuo followed by coevaporation with toluene (2×). The crude product was subjected to the next step without further purification. OTBS-MBoc protected hydroxylamine44 (1.2 eq., 4.1 mmol, 1.01 g) was dissolved in THF (10 mL) and a solution of nBuLi (1.05 eq., 3.59 mmol, 2.25 mL of a 1.6 M solution in hexane) was added dropwise and the reaction mixture was stirred at 0 °C for 30 min. In a separate flask the freshly prepared crude acyl chloride was dissolved in THF (15 mL) and cooled to 0 °C. To this the lithiated hydroxylamine mixture was added dropwise and the reaction was stirred at 0 °C for 2.5 h after which TLC analysis indicated complete conversion of compound 21. A 0.1 M aq. HCl solution (30 mL) was added and the mixture was extracted twice with ETOAc (30 mL). The combined organic layers were extracted with 0.1 M aq. HCl (30 mL) and brine, dried over MgSO4 and concentrated under reduced pressure. The crude mixture was purified by column chromatography (100% toluene → 2.5% EtOAc/toluene) and the product was obtained as a yellowish oil (yield: 1.54 g, 2.63 mmol, 77%). 1H NMR (600 MHz, CDCl3); δ = 7.22 (d, J = 8.4 Hz, 2H), 7.11 (d, J = 8.4 Hz, 2H), 5.80-5.75 (m, 1H), 5.21 (d, J = 17.4 Hz, 1H), 5.16 (d, J = 10.2 Hz, 1H), 4.51 (d, J = 6.0 Hz, 2H), 3.24-3.20 (m, 2H), 3.03 (dd, J = 13.8, 6.6 Hz, 1H), 2.89-2.85 (m, 2H), 1.53 (s, 9H), 0.99 (s, 9H), 0.13 (s, 3H), 0.10 (s, 3H) ppm. 13C NMR (150 MHz, CDCl3); δ = 173.7, 170.2, 151.8, 140.3, 131.8, 129.4, 127.3, 126.5, 122.0 (q, J = 273 Hz), 118.1, 84.5, 65.4, 42.7, 38.6, 37.3, 28.2 (q, J = 40.5 Hz), 27.8, 25.6, 18.0, −5.2 ppm. [α]D23 = +7.91 (c = 1, CHCl3). HRMS: calcd. for C27H38F3N3O6Si [M + H]+ 86.25562; found 86.25562.

(R)-pentfluorophenyl 4-((tert-butoxycarbonyl(tert-butyldimethylsilyloxy)amino)-4-oxo-2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)butanoate (24)

Allyl ester 22 (1.53 g, 2.63 mmol) was dissolved in THF (15 mL). To this solution were added N,N'-dimethylbarbaturic acid (23, 0.5 eq., 1.32 mmol, 210 mg) and tetrakis(triphenylphosphine)palladium (cat.). The reaction was stirred for 1 h at RT after which TLC analysis indicated complete consumption of the starting compound. The mixture was concentrated under reduced pressure and dissolved again in DCM (15 mL) without further purification. To this mixture were added pentfluorophenol (2 eq., 5.26 mmol, 556 μL) and EDC (2 eq., 5.26 mmol, 1.00 g) and the reaction was stirred for 12 h at RT. Et2O (50 mL) was added and the mixture was extracted twice with 0.1 M aq. HCl (50 mL) and brine, dried over MgSO4 and concentrated under reduced pressure. The resulting mixture was purified by column chromatography (1.5% ETOAc/PE) and the product was obtained as a yellow solid (yield: 1.65 g, 2.31 mmol, 88%). 1H NMR (500 MHz, CDCl3); δ = 7.30 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 8.0 Hz, 2H), 3.62-3.56 (m, 1H), 3.33 (dd, J = 18.5, 9.5 Hz, 1H), 2.23 (dd, J = 13.5, 6.5 Hz, 1H), 3.07-2.99 (m, 2H), 1.54 (s, 9H), 0.99 (s, 9H), 0.14 (s, 3H), 0.12 (s, 3H) ppm. 13C NMR (125 MHz, CDCl3); δ = 170.5, 169.6, 151.9, 141.1 (dd, J = 250, 9.0 Hz), 139.5 (dt, J = 264, 13.5 Hz), 139.3, 137.8 (dt, J = 249, 13.5 Hz), 129.5, 127.9, 126.8, 125.0 (t, J = 12.8 Hz), 122.1 (q, J = 273 Hz), 84.9, 42.4, 38.7, 37.1, 28.3 (q, J = 40.1 Hz), 27.8, 25.6, 18.1, −5.2, −5.3 ppm. 19F NMR (188 MHz, CDCl3); δ = −65.8 (s, 3F), −152.4 (d, J = 18.6 Hz, 2F), −158.6 (t, J = 21.1 Hz, 1F), −163.0 (t, J = 21.4 Hz, 2F) ppm. [α]D23 = +8.8 (c = 1, CHCl3). HRMS: calcd. for C27H38F5N3O6Si [M + Na]+ 734.19031; found 734.19061.
**Chapter 3**

**tert-butyl tert-butyldimethylsilyloxy((R)-4-((S)-1-(methylamino)-1-oxo-3-phenylpropan-2-ylamino)-4-oxo-3-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)butanoyl)carbamate (26)**

L-phenylalanine methylamide\(^8\) (25, 21 mg, 120 \(\mu\)mol) was added to a solution of compound 24 (85 mg, 120 \(\mu\)mol) in DMF (2 mL). The reaction was stirred at RT for 24 h after which no more starting material was consumed (followed by LC-MS analysis). Et\(_2\)O (10 mL) and 0.1 M aq. HCl solution (10 mL) were added and the layers were separated. The aqueous layer was extracted with Et\(_2\)O (10 mL) and the combined organic layers were extracted with a 0.1 M aq. HCl solution (2 × 10 mL) and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The resulting mixture was purified by column chromatography (10% \(\rightarrow\) 50% EtOAc/PE) and the product was obtained as a colourless oil (yield: 38 mg, 54.0 \(\mu\)mol, 45%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) = 7.29-7.17 (m, 7H), 7.06 (d, \(J = 8.0\) Hz, 2H), 6.14 (d, \(J = 8.0\) Hz, 1H), 5.29-5.28 (m, 1H), 4.50 (q, \(J = 6.9\) Hz, 1H), 3.21-3.11 (m, 2H), 2.97-2.85 (m, 4H), 2.77-2.69 (m, 1H), 2.57 (d, \(J = 4.8\) Hz, 3H), 1.54 (s, 9H), 0.99 (s, 9H), 0.14 (s, 3H), 0.11 (s, 3H) ppm. \(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) = 173.6, 170.8, 170.7, 151.8, 140.7, 136.7, 129.5, 128.6, 127.5, 126.9, 126.5, 122.1 (q, \(J = 273\) Hz), 84.8, 54.4, 44.5, 39.9, 37.9, 37.6, 29.7, 28.3 (q, \(J = 40.1\) Hz) ppm. \(\alpha\) = +5.6 (c = 1, CHCl\(_3\)). LC-MS: gradient 50% \(\rightarrow\) 90% ACN/(0.1% TFA/H\(_2\)O): Rt (min): 9.81 (ESI-MS (m/z): 705.87 (M + H\(^+\)). HRMS: calcd. for C\(_{34}\)H\(_{46}\)F\(_3\)N\(_5\)O\(_6\)Si [M + H\(^+\)] 706.32422; found 706.32419.

**TFA (1 mL) and deionised H\(_2\)O (50 \(\mu\)L) were added to a solution of compound 26 (21 mg, 30 \(\mu\)mol) in DCM (1 mL). After 1 h LC-MS and TLC analysis indicated complete conversion of the starting material. Toluene was added and the mixture was concentrated under reduced pressure. In order to remove excess TFA the mixture was coevaporated twice with toluene. The resulting mixture was purified by RP-HPLC (gradient 40% \(\rightarrow\) 65% ACN/0.1% TFA/H\(_2\)O). The title compound was obtained as a colourless solid (yield: 10.2 mg, 21 \(\mu\)mol, 69%). \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) = 10.44 (s, 1H), 8.78 (bs, 1H), 8.17 (d, \(J = 8.0\) Hz, 1H), 7.65-7.58 (m, 1H), 7.30-7.21 (m, 7H), 7.10 (t, \(J = 7.74\) Hz, 2H), 4.35-4.32 (m, 1H), 3.09 (dd, \(J = 12.83, 6.04\) Hz, 1H), 3.03-2.87 (m, 6H), 2.82 (bs, 1H), 2.78-2.72 (m, 1H), 2.68-2.66 (m, 1H), 2.60 (d, \(J = 12.4\) Hz, 2H), 2.07 (t, \(J = 7.60\) Hz, 2H), 2.02-1.96 (m, 1H) ppm. LC-MS: gradient 10% \(\rightarrow\) 90% ACN/0.1% TFA/H\(_2\)O: 

**HA-succ(Tmd)-Phe-Ahx-Lys(Biotin)-NH\(_2\) (2a)**

This compound was synthesized on solid support on a 20 \(\mu\)mol scale (based on the loading of Fmoc-Lys(Biotin)) following the General procedure A. The final coupling step involved the addition of compound 24 (70 \(\mu\)mol, 50 mg) and DiPEA (40 \(\mu\)mol, 90 \(\mu\)L 0.45 M in NMP) in NMP (0.40 mL) to the resin and shaking for 2 h. The compound was purified by RP-HPLC (gradient 10% \(\rightarrow\) 90% ACN/0.1% TFA/H\(_2\)O) and was obtained as a colourless solid (yield: 4.3 mg, 6.0 \(\mu\)mol, 30% after 3 coupling steps). \(^1\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) = 10.38 (s, 1H), 8.25 (bs, 1H), 8.13 (t, \(J = 8.72\) Hz, 1H), 7.80-7.72 (m, 4H), 7.65 (t, \(J = 5.58\) Hz, 1H), 7.37-7.13 (m, 8H), 7.10 (t, \(J = 7.74\) Hz, 2H), 6.94 (bs, 1H), 6.42 (bs, 1H), 6.36 (bs, 1H), 4.35-4.32 (m, 2H), 4.18-4.09 (m, 2H), 3.09 (dd, \(J = 12.83, 6.04\) Hz, 1H), 3.03-2.87 (m, 6H), 2.82 (dd, \(J = 12.4\) Hz, 1H), 2.78-2.72 (m, 1H), 2.68-2.66 (m, 1H), 2.64-2.60 (m, 1H), 2.60 (d, \(J = 12.4\) Hz, 2H), 2.13 (t, \(J = 7.35\) Hz, 2H), 2.07 (t, \(J = 7.60\) Hz, 2H), 2.02-1.96 (m, 1H) ppm. LC-MS: gradient 10% \(\rightarrow\) 90% ACN/0.1% TFA/H\(_2\)O:
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(R)-N1-((S)-1-(6-((S)-1,6-diamino-1-oxohexan-2-ylamino)-6-oxohexylamino)-1-oxo-3-phenylpropan-2-yl)-N4-hydroxy-2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)succinamide or (HA-succ(Tmd)-Phe-Ahx-Lys-NH₂ (30)

This compound was synthesized on solid support on a 175 μmol scale (based on the loading of Fmoc-Lys(Boc)) following the General procedure A. The final coupling step involved the addition of compound 24 (1.12 mmol, 800 mg) and DiPEA (750 μmol, 124 μL 0.45 M in NMP) in NMP (10 mL) to the resin and shaking for 2 h. The compound was purified by RP-HPLC (gradient 10% → 90% ACN/0.1% TFA/H₂O) and was obtained as a colourless solid (yield: 45 mg, 54 μmol, 30% after 3 coupling steps).  

1H NMR (400 MHz, CD₃OD): δ = 7.69 (t, J = 5.62 Hz, 1H), 7.38-7.34 (m, 1H), 7.29-7.16 (m, 9H), 7.10 (d, J = 8.03 Hz, 2H), 4.43 (t, J = 7.49 Hz, 1H), 4.35 (dd, J = 8.89, 5.32 Hz, 1H), 3.15-3.05 (m, 3H), 3.04-2.99 (m, 1H), 2.98-2.83 (m, 4H), 2.70 (dd, J = 13.62, 6.17 Hz, 1H), 2.33-2.24 (m, 3H), 2.11 (dd, J = 14.82, 6.37 Hz, 1H), 1.87 (ddd, J = 14.60, 7.64, 4.70 Hz, 1H), 1.78-1.68 (m, 3H), 1.57 (dd, J = 14.95, 7.45 Hz, 2H), 1.55-1.45 (m, 2H), 1.44-1.36 (m, 2H), 1.30-1.18 (m, 2H) ppm.  

13C NMR (100 MHz, CD₃OD): δ = 177.0, 176.5, 173.8, 173.7, 171.2, 143.4, 139.5, 131.8, 131.3, 128.9, 128.6, 128.4, 57.2, 54.9, 46.5, 41.4, 41.0, 39.4, 37.4, 37.3, 36.5, 33.4, 30.7, 28.9, 28.2, 27.2, 24.7 ppm. LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H₂O): Rt (min): 5.92 (ESI-MS (m/z): 719.27 (M + H⁺)). HRMS: calcd. for C₃₄H₄₅F₃N₈O₆ [M + H]⁺ 719.34869; found 719.34848.

HA-succ(Tmd)-Phe-Ahx-Lys(Bodipy(Tmr))-NH₂ (2b)

Compound 30 (9.1 mg, 11 μmol) and Bodipy(Tmr)-OSu (4.6 mg, 12 μmol) were dissolved in DMF (0.5 mL). DiPEA (4.5 μL, 27 μmol) was added and the reaction was stirred for 24 h after which the solvent was evaporated under reduced pressure. The resulting mixture was purified by RP-HPLC (gradient 10% → 90% ACN/(0.1% TFA/H₂O)) and the title compound was obtained as a brown solid (yield: 3.4 mg, 3.4 μmol, 31%).  

1H NMR (400 MHz, DMSO-d₆): δ = 10.38 (s, 1H), 8.73 (s, 1H), 8.15 (d, J = 8.0 Hz, 1H), 7.86 (d, J = 8.87 Hz, 2H), 7.85 (t, J = 5.20 Hz, 1H), 7.81 (d, J = 8.40 Hz, 1H), 7.67 (s, 1H), 7.68 (t, J = 5.49 Hz, 1H), 7.32 (s, 1H), 7.30-7.13 (m, 10H), 7.02 (d, J = 8.91 Hz, 2H), 6.93 (s, 1H), 6.70 (d, J = 4.02 Hz, 1H), 4.41-4.30 (m, 1H), 4.13 (dt, J = 8.80, 5.79 Hz, 1H), 3.82 (s, 3H), 3.04-2.83 (m, 6H), 2.82-2.70 (m, 2H), 2.64-2.58 (m, 3H), 2.48 (s, 3H), 2.24 (s, 3H), 2.23 (t, J = 6.8 Hz, 2H), 2.14-2.07 (m, 3H), 1.88 (dd, J = 15.01, 7.43 Hz, 1H), 1.68-1.58 (m, 1H), 1.54-1.42 (m, 3H), 1.39-1.11 (m, 8H) ppm.  

19F NMR (376 MHz, DMSO-d₆): δ = -64.09 (s, 3F), -136.82 (q, J = 32.8 Hz, 2F) ppm. LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H₂O): Rt (min): 1099.40 (M + H⁺)). HRMS: calcd. for C₅₅H₆₄BF₅N₁₀O₈ [M + H]⁺ 1099.49946; found 1099.50039.

HA-succ(Tmd)-Phe-Ahx-Lys(Bodipy(FL))-NH₂ (2c)

Compound 30 (9.1 mg, 11 μmol) and Bodipy(FL)-OSu (4.6 mg, 12 μmol) were dissolved in DMF (0.5 mL). DiPEA (4.5 μL, 27 μmol) was added and the reaction was stirred for 24 h after which the solvent was evaporated under reduced pressure. The resulting mixture was purified by RP-HPLC (gradient 10% → 90% ACN/(0.1% TFA/H₂O)) and the title compound was obtained as an orange solid (yield: 5.9 mg, 6.0 μmol, 55%).  

1H NMR (400 MHz, DMSO-d₆): δ = 10.38 (s, 1H), 8.73 (s, 1H), 8.12 (d, J = 8.23 Hz, 1H), 7.91 (t, J = 5.35 Hz, 1H), 7.80 (d, J = 8.15 Hz, 1H), 7.68 (s, 1H), 7.65 (t, J = 5.49 Hz, 1H), 7.31 (s, 1H), 7.27-7.13 (m, 7H), 7.11-7.07 (m, 3H), 6.95 (s, 1H), 6.34 (d, J = 3.99 Hz, 1H), 6.29 (s, 1H), 4.34 (dd, J = 14.65, 8.31 Hz, 1H), 4.15 (dt, J = 8.29, 5.47 Hz, 1H), 3.15-3.03 (m, 4H), 3.01-2.90 (m, 4H), 2.83-2.77 (m, 2H), 2.62 (dd, J = 13.6, 6.0 Hz, 1H), 2.50 (s, 3H), 2.49 (signal under DMSO signal, 3H), 2.29 (s, 3H), 2.18-2.06 (m, 3H), 1.88 (dd, J = 15.00, 7.51 Hz, 1H), 1.70-1.60 (m, 1H), 1.57-1.38 (m, 5H), 1.37-1.23 (m, 4H), 1.22-1.11 (m, 2H) ppm.  

19F NMR (376 MHz, DMSO-d₆): δ = -64.09 (s, 3F), -142.77 (q, J = 33.2 Hz, 2F) ppm. LC-MS: gradient 10% →
90% ACN/(0.1% TFA/H2O); Rt (min): 8.13 (ESI-MS (m/z): 993.33 (M + H+)). HRMS: calcd. for C48H58BF5N10O7 [M + H]+ 993.45759; found 993.45864.

Biological evaluation

Inhibition and labeling studies

Recombinant ADAM-8 (Catalog Number 1031-AD, catalytic domain of human ADAM-8 Glu158-Pro497, Murine myeloma cell line, NS0 derived), ADAM-9 (Catalog Number 939-AD, catalytic domain of human ADAM-9 Ala206-Asp697, Murine myeloma cell line, NS0 derived), ADAM-10 (Catalog Number 936-AD, catalytic domain of human ADAM-10 Thr214-Glu672, Spodoptera frugiperda, Sf 21 (baculovirus) derived) and ADAM-17 (ectodomain, Catalog Number 930-ADB, catalytic domain of human ADAM-17 Arg215-Asn671, Spodoptera frugiperda, Sf 21 (baculovirus) derived) were purchased from R&D systems (Minneapolis, MN, USA). ADAM-8 was autocatalytically activated by incubation at 37 °C for 5 days according to the manufacturer’s instructions. Recombinant catalytic domains (CD) of human MMP-1 (Catalog Number BML-SE180-0010, catalytic domain of human MMP-1 Phe100-Gln268), MMP-2 (Catalog Number BML-SE237-0010, catalytic domain of human MMP-2 Tyr110-Asp452), MMP-3 (Catalog Number BML-SE109-0010, catalytic domain of human MMP-3 Phe100-Thr272), MMP-7 (Catalog Number BML-SE181-0010, catalytic domain of human MMP-7 Tyr95-Lys267), MMP-8 (Catalog Number BML-SE181-0010, catalytic domain of human MMP-8 Phe99-Gln269), MMP-10 (Catalog Number BML-SE329-0010, catalytic domain of human MMP-10 Phe99-Glu271), MMP-11 (Catalog Number BML-SE282-0010, catalytic domain of human MMP-11 Phe98-Ser266) and MMP-13 (Catalog Number BML-SE246-0010, catalytic domain of human MMP-13 Tyr104-Asn274) were from Biomol International (Butler Pike, PA, USA). All hrMMPs were expressed in E. coli. Recombinant human MMP-12 CD and recombinant human MMP-9 CD without fibronectin type II inserts (expressed in E. coli as described56,57) were a kind gift from AstraZeneca R&D (Lund & Moelndal, Sweden). TIMP-1 from human neutrophil granulocytes was from Calbiochem (La Jolla, CA, USA). Alkaline phosphatase conjugated streptavidin was from Sigma-Aldrich (Zwijndrecht, The Netherlands). 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were from Duchefa (Haarlem, The Netherlands). Unless mentioned otherwise all other biochemicals were purchased from Sigma-Aldrich.

Determination of IC50 values

The affinity of the photoactivatable probes for ADAM and MMP proteases was determined in a competitive enzyme activity assay monitoring conversion of the fluorogenic substrate Mca-PLAQAV-Dpa-RSSSR-NH2 (R&D Systems) by recombinant ADAM-9, -10 and -17 in the presence of increasing concentrations photoactivatable probe. For MMP-9 and MMP-12 inhibition of the conversion of fluorogenic substrate Mca-PLGL-Dpa-AR-NH2 (Bachem, Bubendorf, Switzerland) was determined. Measurements were performed in Costar White 96-well plates (Corning, Schiphol-Rijk, The Netherlands), where each well contained either 10 ng ADAM-17, 100 ng ADAM-10 or 200 ng ADAM-9 and a final concentration of 10 μM substrate in a final volume of 100 μL ADAM assay buffer (25 mM Tris pH 9.0, 2.5 μM ZnCl2, 0.005% w/v Brij-35). Inhibition of MMP proteolytic activity was determined with 10 ng of Mmp-9 or MMP-12 per well with a final concentration of 2 μM substrate in 100 μL MMP assay buffer (50 mM Tris pH 7.4, 0.2 M NaCl, 10 mM CaCl2, 2.5 μM ZnCl2, 0.05 % (v/v) Brij-35). Proteolysis rates were followed by measuring fluorescence (λex,em = 320, 440 nm) increase using a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) at 37 °C. Six-point inhibition curves (0-10 μM) were plotted in Origin 7.0 (Micronal) and IC50 values were determined by sigmoidal fitting.

Labeling of active recombinant metalloproteases

Recombinant MMP catalytic domains and recombinant ADAM ectodomains were incubated with photoactivatable inhibitor probes in 96-well plates (Costar White). Each well (final volume 30 μL)
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contained 4 pmol enzyme and a final concentration of 1 µM inhibitor probe in MMP or ADAM assay buffer. The plate was irradiated at 366 nm using a Camag universal UV lamp (20W, distance to plate 4 cm) for 30 min. For subsequent analysis by Western blotting, the reaction was stopped by adding 10 µL 5× non-reducing SDS-PAGE sample buffer.

**Western blotting**

Samples were analyzed by SDS-PAGE on 0.75 mm thick 12.5% polyacrylamide gels. Electrophoresis was carried out at 20 mA per gel using a mini-Protein III electrophoresis system (Bio-Rad, Veenendaal, The Netherlands). The proteins were transferred to an Immun-Blot PVDF membrane by wet Western blotting in a mini Trans-blot cell at 350 mA for 60 min. in 25 mM Tris, 190 mM glycine with 20% (v/v) methanol (BioRad). Membranes were blocked overnight at 4 °C in TBST (25 mM Tris buffer pH 7.5 containing 150 mM NaCl, 0.05% (v/v) Tween-20) supplemented with 5% (w/v) non-fat dried milk (Protifar Plus, Nutricia, Zoetermeer, The Netherlands) and incubated for 1 h in a 1:1500 dilution of streptavidin-alkaline phosphatase (0.67 µg/mL) in TBST supplemented with 1% non-fat dried milk. Biotinylated proteins were visualized by staining with an NBT/BCIP substrate solution (0.1 M Tris buffer, pH 9.5 containing 5 mM MgCl2, 0.15 mg/mL BCIP and 0.30 mg/mL NBT).

**Competition experiments with TIMPs and compound 27**

Aliquots of 4 pmol of MMP-9 and MMP-12 were incubated overnight with equimolar equivalents of TIMP-1. Control aliquots were kept at 4 °C overnight without TIMPs. Photoactivatable inhibitor 2a was added to a final concentration of 200 nM. Labeling and analysis were performed as described above.

Aliquots of 4 pmol MMP-9 and MMP-12 (both catalytic domains) in assay buffer were preincubated for 15 min. with 400 nM control inhibitor 27 and irradiated with UV light. Positive controls were treated the same, but without control inhibitor 27 added to the solution. Next, photoactivatable probe 2a was added to a final concentration of 200 nM. Labeling and analysis were performed as described above.

**References**


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(43) The route of synthesis employed is essentially the same as reported, however in this work no characterization of the final product and intermediates leading to this are given. See: K. Hashimoto, T. Yoshioka, C. Morita, M. Sakai, T. Okuna, H. Shirahama, *Chem. Lett.* **1998**, *3*, 302-304.


(45) In a similar procedure both the methyl and trichloroethyl esters were readily prepared. However these proved less suitable precursors towards the preparation to key intermediate 24.


(51) Bodipy(FL)-OSu was prepared in a procedure analogous to that for Bodipy(Tmr)-OSu.


