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Synthetic tools to illuminate matrix metalloproteinase and proteasome activities

Geurink, P.P.

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Synthetic Tools to Illuminate Matrix Metalloproteinase and Proteasome Activities

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P. F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 6 oktober 2010
klokke 15.00 uur

door

Paulus Petrus Geurink

Geboren te Haarlem in 1983

Promotie commissie

Promotores	:	Prof. dr. H. S. Overkleeft
	:	Prof. dr. G. A. van der Marel
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		Dr. H. Ovaa

"Feiten, waarnemingen en proefnemingen zijn als de bouwmaterialen voor een groot bouwwerk; maar als men ze bij elkaar zoekt, moet men voorkomen dat ze een ongeordende en hinderlijke puinhoop in de wetenschap worden. In plaats daarvan moet men ernaar streven ze in categorieën in te delen, zodat elk deel van het bouwwerk nog te onderscheiden is."

Antoine Laurent Lavoisier, 1777

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List of Abbreviations

AA, Aa	amino acid
ABP	activity-based probe
ABPP	activity-based protein profiling
Ac	acetyl
Ac_2O	acetic anhydride
AcOH	acetic acid
ACN	acetonitrile
Ada	1-adamantyl acetyl
ADAM	a disintegrin and metalloprote(in)ase
Ahx	aminohexanoic acid
AMC	7-acetoxy-4-methylcoumarin
AP	alkaline phosphatase
APT	attached proton test
ATP	adenosine triphosphate
aq.	aqueous
BAIB	[bis(acetoxy)iodo]benzene
Bio	biotin
BM	biotinylated marker
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
Boc_2O	*tert*-butyloxycarbonic anhydride
Bodipy	boron-dipyrromethene, boradiazaindacene
bs	broad singlet
Bpa	4-benzoyl-L-phenylalanine
BPB	bromophenol blue
BSA	bovine serum albumin
Bu	butyl
calcd.	calculated
cat.	catalytic amount
Cbz	benzyloxycarbonyl
CL	cleavable linker
δ	chemical shift
Δ	heating (reflux)
d	doublet
Da	Dalton
DBU	diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
dd	double doublet
ddd	double double doublet
DIC	*N*,*N*'-diisopropyl carbodiimide
DiPEA	diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
dt	double triplet
DTT	dithiothreitol
dq	double quartet
EDC	1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide
EDTA	ethylenediaminetetraacetate
EL-4	murine lymphoid cell line
eq.	molar equivalent
ESI	electron spray ionization
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
Fmoc	(9*H*-fluoren-9-yl) methoxycarbonyl
h	hour(s)
HCTU	(2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HEK	human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMDS	hexamethyldisilazane
HOBt	*N*-hydroxybenzotriazole
HOSu	*N*-hydroxysuccinimide
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HRP	horse radish peroxydase
Hz	Hertz
IC_{50}	inhibition concentration resulting in 50% inhibition of enzyme activity
ICAT	isotope-coded affinity tagging
IR	infrared spectroscopy
J	coupling constant
LC-MS	liquid chromatography coupled to mass spectrometry
Lev	levulinoyl ester
m	multiplet
M	molar
MBHA	*para*-methylbenzhydryl amine
mCPBA	*meta*-chloroperoxybenzoic acid
MHC I	major histocompatibility complex class I
Me	methyl
MeOH	methanol
MI	McIlvaine's buffer

min.	minute(s)
MMP	matrix metalloprote(in)ase
Mtt	4-methyltrityl
m/z	mass-to-charge ratio
*n*Bu	*n*-butyl
NHS	*N*-hydroxysuccinimide
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
o/n	overnight
p	pentet
PAGE	polyacrylamide gel electrophoresis
PAL	photoaffinity labelling
PBS	phosphate buffered saline
PD	pull-down (buffer)
Pd/C	palladium on charcoal
PE	petroleum ether
PEG	polyethyleneglycol
PFP	pentafluorophenyl
Ph	phenyl
ppm	parts per million
PPTS	pyridinium *para*-toluenesulfonate
PS	pre-stained marker
q	quartet
quant.	quantitative
ref.	reference
RP	reverse phase
RT	room temperature
R_t	retention time
s	singlet
sat.	saturated
SB	sample buffer
SDS	sodium dodecyl sulphate
SPE	solid phase extraction
SPPS	solid phase peptide synthesis
Su	succinimidyl
t	triplet
t, *tert*	tertiary
T	temperature
TACE	TNFα converting enzyme
TBS	*tert*-butyldimethylsilyl
TBS	tris buffered saline
*t*Bu	*tert*-butyl
*t*BuONO	*tert*-butyl nitrite
*t*BuOH	*tert*-butanol
TBS	*tert*-butyl dimethylsilyl
td	triple doublet
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIMP	tussue inhibitor of metalloprote(in)ases
TIS	triisopropylsilane
TLC	thin layer chromatography
Tmd	trifluoromethyldiazirine
TMS	tetramethylsilane
TNFα	tumor necrosis factor α
Tol	toluene
Tr	trityl
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Ts	tosyl
UV	ultra violet
VE	vinyl ethyl ester
VS	vinyl methyl sulfone
ZBG	zinc binding group

Note: the one and three letter codes for the amino acids follow the recommendations of IUPAC. *J. Biol. Chem.* **1968**, *243*, 3557-3559 and *J. Biol. Chem.* **1972**, *247*, 977-983.

1 General Introduction

1.1 Introduction

Proteomics research focuses on the study of proteins, their functioning and interactions with other biomolecules in the context of complex biological samples. Rather than focusing on a single protein, proteomics research takes on large numbers of distinct proteins at the same time, in an ideal setting the whole pool of proteins (called the proteome) expressed at a given time by an organism or cell type.[1,2] In the overall study of biological systems proteomics is situated between genomics (study of the genome) and metabolomics (study of metabolites produced in cellular processes). The ultimate goal in proteomics is the complete understanding of each single protein in all physiological processes, which is of great importance in understanding pathological states. Since it is extremely difficult to study all proteins and their properties at once, proteomic research is usually performed by taking on one specific subset of proteins from two or more different biological systems (*e.g.* healthy cells and infected cells) in a comparative study. Traditionally, this is done by separation of the protein subset of choice, for example by 1D/2D gel-electrophoresis and immunoprecipitation, followed by determination of its level of abundance. An attractive alternative strategy developed for proteomics research is activity-based protein profiling (ABPP).[3,4] This strategy is based on the determination of an enzyme's activity rather than its levels of expression, which is important since an enzyme's abundance is not necessarily linked to its activity in biological processes. A reason for this is that a large number of enzymes is translated as inactive pro-enzymes and therefore need to be transformed into their active form. In

addition, the enzyme's state can be switched between active and inactive by post-translational modifications, such as phosphorylation, glycosylation, acetylation, ubiquitination and methylation. In the first part of this introduction the general idea of ABPP will be outlined. The major part of the introduction deals with an interesting aspect of this strategy in which a photophore is used to profile specific enzymes or enzyme families. The properties of the most commonly applied photophores and their use in chemical biology research are discussed. Finally, the aim and outline of this Thesis will be described at the end of the introduction.

1.2 Activity- and affinity-based protein profiling

Activity-based protein profiling makes use of relatively small organic molecules to label a specific enzyme (class) in their active state. These organic molecules are called 'activity-based probes' (ABPs) and comprise three major elements. The recognition unit directs the ABP to the target enzyme and is designed to resemble structural and functional motives of the natural substrate of the target enzyme. It can often be designed such that a number of related enzymes within a family can bind. Closely attached to the recognition part is a reactive group (also termed 'warhead'), which reacts in the enzyme's active site and thereby establishes a covalent bond between the ABP and enzyme. Depending on the type of enzyme and reactive group this interaction can be either reversible or irreversible. The recognition element and the warhead are linked, most often via a spacer, to a reporter group or 'tag', which allows visualization and/or purification of the bound enzyme. The most commonly used tags are fluorophores, radioactive labels and biotin, of which the latter can be used for both visualization and purification purposes (Figure 1, top).

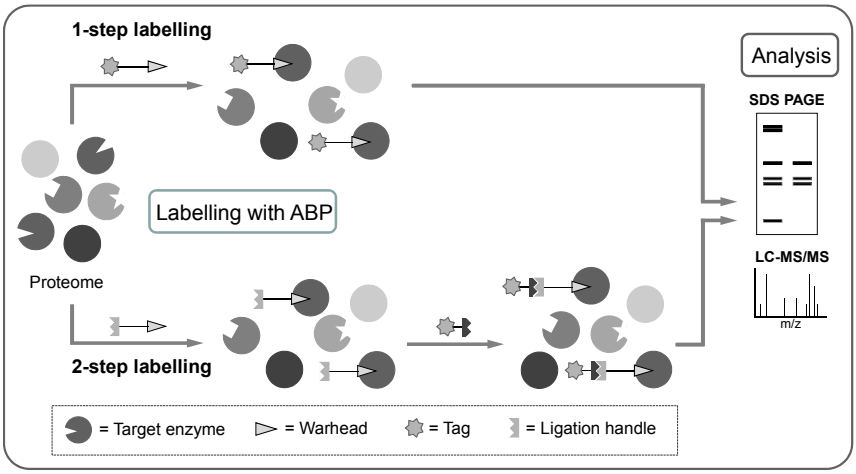


Figure 1. Schematic representation of an ABPP experiment. Both the 1-step (top) and the 2-step (bottom) labelling strategies are shown.

An ingenious extension to ABPP has been developed for those cases in which the reporter group hampers the interaction between ABP and enzyme or drastically lowers the ability to cross the cell's membrane, which is especially of interest for labelling in living cells. In this approach (referred to as two-step labelling) the ABP's tag is replaced by a ligation handle, which can be connected to the reporter group after the enzyme has been captured (Figure 1, bottom).[5] A requirement for this ligation handle is that it is unreactive towards all functionalities present in a biological sample (bioorthogonal) and the most popular ligation handles are the azide and the (terminal) alkyne. The ligation reactions used (shown in Figure 2) are the Staudinger-Bertozzi ligation (I)[6] and the Huisgen 2,3-dipolar cycloaddition or 'click' reaction, which can be divided into copper-catalyzed (II)[7,8] and strain-promoted (III).[9,10] Recently Boons and co-workers developed a new reagent, which allows for a strain-promoted click reaction after irradiation with light and is therefore termed the 'photoclick' reaction (IV).[11] In addition, the use of the Diels-Alder reaction as alternative ligation reaction is subject to growing attention[12] and of the different Diels-Alder type reactions, the inverse-electron-demand variation appears the most promising for *in vivo* labelling.[13,14]

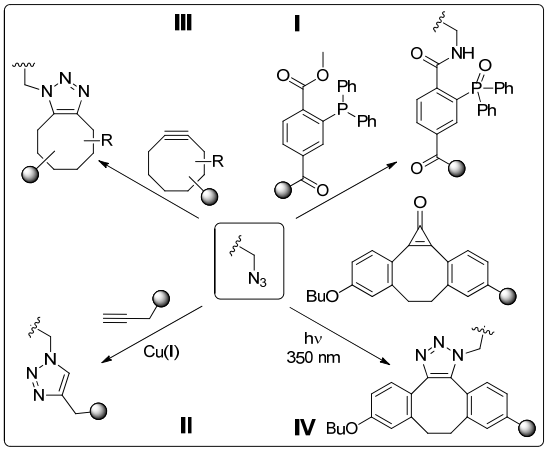


Figure 2. Ligation reactions used in 2-step ABPP using the azide functionality as ligation handle. I) Staudinger-Bertozzi ligation; II) Cu(I) catalyzed click reaction; III) Copper-free click reaction; IV) Photoclick reaction.

The ABPP strategy as shown in Figure 1 is especially well suited for those enzymes that contain a nucleophilic amino acid side chain residue in their active site (*e.g.* serine, cysteine, threonine), which is responsible for the enzyme's catalytic activity. The ABP reactive group which binds the target enzyme is designed such that it reacts with this nucleophilic residue to form a covalent bond and is therefore named 'electrophilic trap'. Examples of enzymes targeted with this strategy are cysteine proteases,[15-18] serine hydrolases[19-22] and proteasome subunits.[23-25] A difficulty arises for enzymes that do not rely on a nucleophilic residue in their active site, which precludes the use of an electrophilic trap. Among these are the metalloproteases, histone deacetylases (both of which employ a water molecule for their catalytic activity) and kinases. A good

alternative for the use of an electrophilic trap is the so-called photoaffinity labelling (PAL), in which the probe used is commonly referred to as 'affinity-based probe' (AfBP).[26] The basic principle is shown in Figure 3. In this approach the AfBP binds the target enzyme in a reversible manner, either via non-covalent interactions (electrostatic, hydrophobic) or via a reversible covalent bond. Although these interactions can be relatively strong, they can not withstand harsh denaturing conditions often applied in biochemical protocols. An additional feature of the AfBP is the introduction of a photoreactive group (also termed photophore or photocrosslinker), which forms an irreversible covalent bond between probe and enzyme upon activation by light. In principle, photoaffinity labelling probes do not necessarily label active enzymes, however the probe can be designed as such that it has to enter the active site of an enzyme prior to photocrosslinking. Therefore, ABPP and PAL often go hand-in-hand.

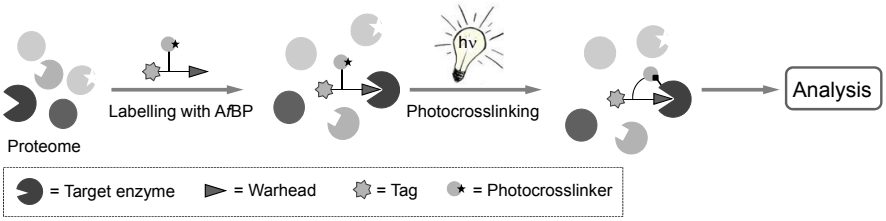


Figure 3. Basic principle of photoaffinity labelling (PAL) using an affinity-based probe (AfBP).

1.3 Photoaffinity labelling

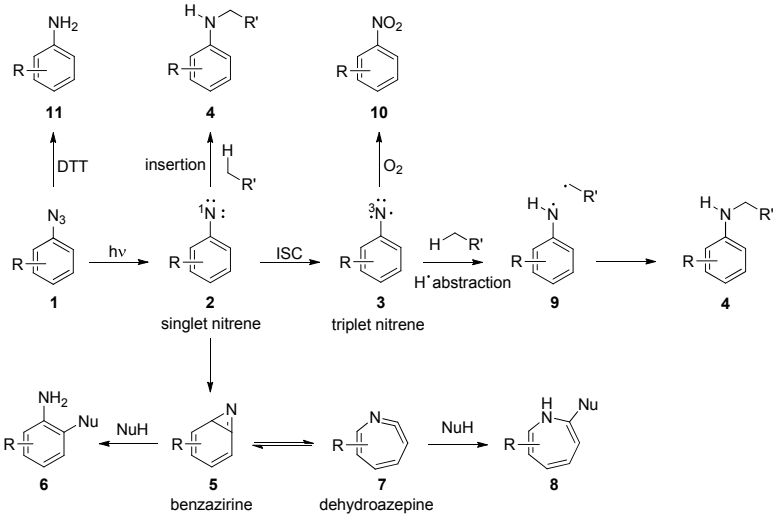
In order to use an AfBP for labelling of (active) enzymes in biological environments the photoreactive group must meet with certain criteria.[27,28] First, the photophore has to be stabile towards the various conditions a biological sample may be exposed to, as well as the intrinsic reactivity of the sample content, and must only be activated upon irradiation with light of a specific wavelength, which may not damage the biological system (λ_{act} > 300 nm). Second, the generated reactive species needs to have a shorter lifetime compared to the lifetime of the studied enzyme-substrate complex in order to limit non-specific labelling. It is important that the activated species reacts with any chemical entity in close proximity, regardless of its nature (including relatively unreactive C-H bonds), and forms a stabile covalent adduct. Finally, the photoreactive moiety must be relatively small, compared to the probe, so that it does not negatively influence the binding mode or activity of the AfBP towards the enzyme.

The use of PAL in enzyme modifications was first described in 1962, where Westheimer and co-workers reported on the use of a diazoacetyl group to inactivate chymotrypsin.[29] Considerable research on the development of new PAL reagents has taken place ever since,[27,28,30-36] but only a few number of photophores, which largely meet the above-mentioned requirements, are being used nowadays in AfBPs. These are aryl azides (first reported use in 1969)[37], diazirines (1973)[38] and benzophenones (1973).[39] The chemistry of these three photoreactive groups after photolysis as well as their use in recently reported AfBPs will be discussed.

1.3.1 Aryl azide

Upon activation of an aryl azide (**1**, see Scheme 1) by irradiation with light of the appropriate wavelength, molecular nitrogen (N_2) is expelled and a singlet nitrene (**2**) is formed initially. This high energetic, highly reactive species has a short lifetime ($\sim 10^{-4}$ s) and is quickly converted into other intermediates.[35,40] Intersystem crossing (ISC) leads to a triplet nitrene (**3**), which is about 20 kcal/mole lower in energy.[41] A major difference between the two nitrene states is their nature of reactivity. Singlet nitrenes behave like electrophiles and can readily undergo an insertion reaction with C-H bonds, whereas the triplet state can be seen as a diradical, which first abstracts a hydrogen radical from a nearby C-H followed by coupling to the formed carbon radical. Although they react via two different mechanisms the product is the same (**4**). Singlet nitrenes can also undergo a rapid rearrangement into the corresponding benzazirine (**5**), which can further rearrange into dehydroazepine (**7**). Both these species are long-lived electrophiles and can react with a nearby nucleophile, which results in compounds **6** and **8** respectively. Two observed side-reactions that are not to be ignored when the aryl azide is applied in PAL are aerobic oxidation of the triplet nitrene to the corresponding nitro species **10**[42] and reduction of the initial aryl azide to the amine **11** by dithiols, such as DTT.[43]

Scheme 1. Possible reaction mechanisms of the reactive intermediates formed after photolysis of aryl azides.

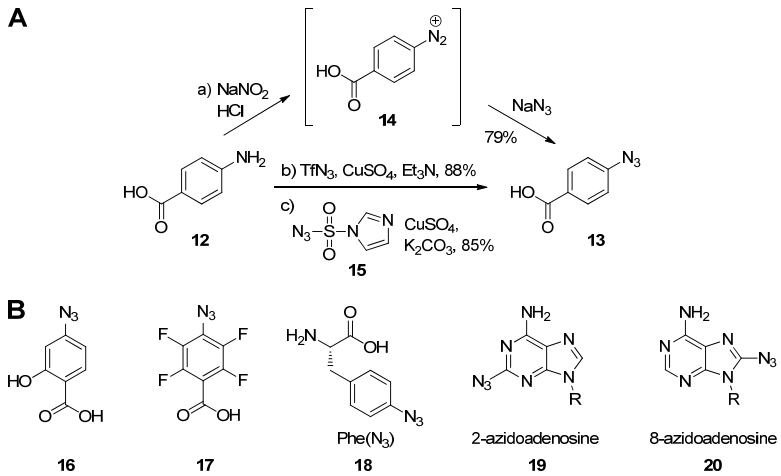


Aryl azides can be easily prepared from their corresponding amines in one or two steps. Three examples are given in Scheme 2A. The most common method is the diazotization of the amine with sodium nitrite under acidic conditions, followed by addition of sodium azide in an aqueous medium (route a).[44] In 2003 the synthetic method was improved by application of triflyl azide (TfN_3), which allowed a one-step conversion and higher yield (route b).[45] Recently, the development of imidazole-1-sulfonyl azide **15** was reported, which proved to be a more stabile reagent and allowed a conversion under mild conditions (route c).[46]

A major drawback of phenyl azides that their maximum absorption wavelength being below 300 nm, since electromagnetic irradiation at these wavelengths can substantially damage the biological system. Consequently, a large number of substituted aryl azides have been made and evaluated for their absorption properties. In general most substituents *ortho* to the azide are to be avoided, since they can lead to undesired cyclizations after photolysis.[30] It has been found that introduction of electron withdrawing substituents (*e.g.* nitro, hydroxyl and acyl groups, for example see compound **16**[47] in Scheme 2B) has the dual effect of increasing the molar absorptivity and red-shifting the maximum absorption wavelength, both of which positively influence the photoactivatable properties.[34,48] In addition, it has been found that (per)fluorinated aryl azides (such as **17**)[49] rearrange more slowly from the singlet nitrene species to the benzazirine and dehydroazepine, which leads to more efficient insertion reactions.[35] The main advantage of aryl azides is their relatively small size and the possibility to incorporate them into natural biological compounds, such as phenylalanine **18**[50] and adenosines **19**[51] and **20**,[52] without significant alteration of the original structure.

Due to the many possible reaction pathways after irradiation (including capturing of the reactive intermediates by the solvent) cross-linking yields are often low (<30%). Arguably, the popularity of the aryl azide moiety in PAL studies is based on its relative ease of preparation and incorporation rather than on its photochemical properties.

Scheme 2. Preparation and examples of aryl azides.

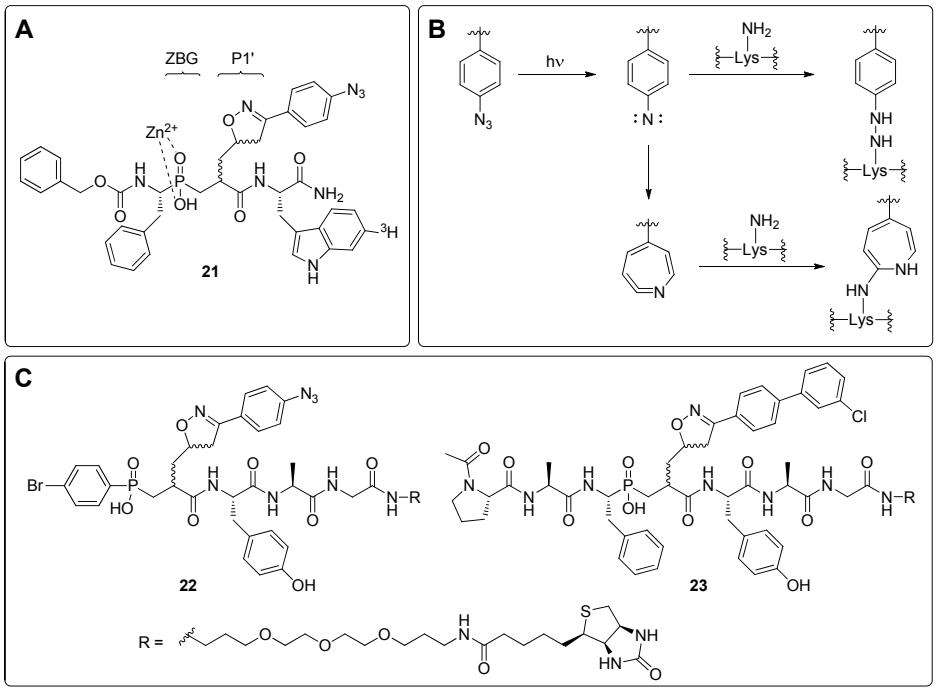


(A) Three possible routes for the conversion of an aryl amine into its aryl azide: (a) via diazotization,[44] (b) by the use of triflyl azide[45] and (c) with imidazole-1-sulfonyl azide **15**.[46] (B) Some examples of substituted aryl azides.

An extensive study towards matrix metalloproteinases (MMPs) with the use of an aryl azide modified A/BP was recently reported by Dive and co-workers.[53] MMPs are metallo-proteases which reside in the extracellular matrix and are responsible for degradation of extracellular matrix material. Their mode of action depends on a Zn^{2+} ion

in the active site which coordinates the scissile bond carbonyl of the substrate and a water molecule. As a result, the carbonyl becomes more electrophilic and is subsequently hydrolysed. The fact that there is no formation of a covalent bond between enzyme and substrate during the proteolysis makes this class of enzymes an interesting target for photo-affinity labelling. The authors describe the use of radio-labelled compound **21** (Scheme 3A), a potent, subnanomolar MMP inhibitor, to label and visualize purified human MMPs.[53] The aryl azide photoreactive group is located at the P1′ pocket, which leads to a tight interaction with the enzyme's cavity. A big difference in terms of labelling efficiency and sensitivity between several MMPs was found, with MMP-12 giving the best results. The estimated crosslinking yield was ~42% after two minutes of irradiation, based on silver staining and as little as 2.5 fmol MMP-12 could be detected. In a second study the specifics of photocrosslinking were further explored, using compound **21** in combination with mass spectrometry and site-directed mutagenesis.[54] Interestingly, the ε-amine side chain substituent of Lys^{241} in MMP-12 appeared to play a crucial role in the photocrosslinking. Two possible covalent constructs were proposed (Scheme 3B), but due to the fact that they have the same molecular weight it was impossible to distinguish between these using mass spectrometry. In theory, some other constructs are possible (see Scheme 1), however

Scheme 3. Affinity-based probes targeting MMPs from studies by Dive *et al.*



(A) Photoreactive A*f*BP containing a tritium label. (B) Possible constructs formed between hMMP-12 and **21** after photolysis proposed by Dive *et al.* (C) Structures of A*f*BPs with or without a photophore for pull-down of active MMPs.

they were not mentioned by the authors. The lysine at position 241 is not conserved throughout the MMP family and photocrosslinking of **21** to other MMPs was therefore further explored.[55] MMP-3 (containing His in position 241) and MMP-9 (Arg in position 241) could also be labelled, although with a lower overall efficiency. In addition, labelling performed at different pH values indicated that a more basic environment resulted in more efficient crosslinking. These results led to the conclusion that the nucleophilicity of the residue at position 241 plays a key role in the photoaffinity labelling. This conclusion was further substantiated by the finding that mutants of hMMP-12 (Ala^{241} and Thr^{241}) gave no labelling whatsoever.

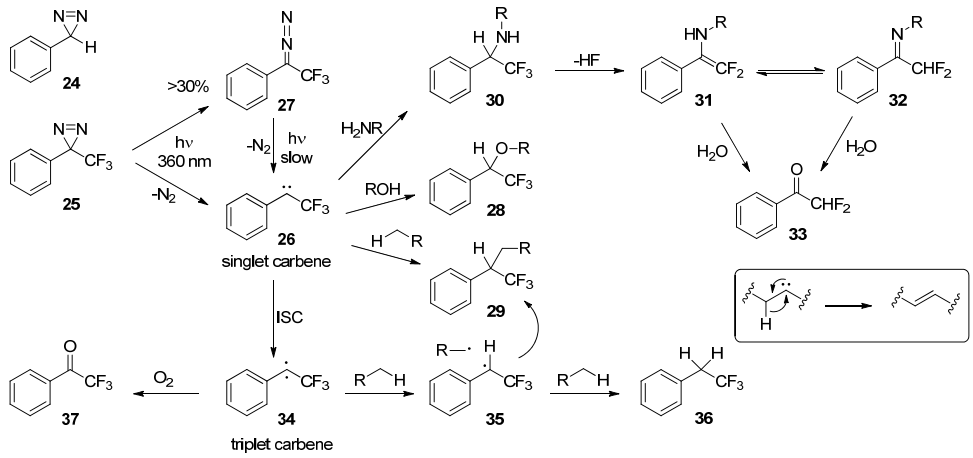
In addition to the attempts of unravelling the modification site, Dive and co-workers also constructed two biotinylated A*f*BPs, **22** and **23** (Scheme 3C) and used these to study the difference in affinity- and photo-affinity MMP enrichment from a complex proteome.[56] For this, tumor extracts were spiked with hMMP-12 and hMMP-8, after which compounds **22** and **23** were applied followed by streptavidin-coated magnetic beads for MMP pull-down. Affinity-based labelling with **23** appeared superior to photo-affinity-based labelling with **22** in terms of quantity of captured MMPs, although it should be noted that the compounds are structurally different and **23** is a 100 fold more potent MMP-8 and MMP-12 inhibitor.

1.3.2 Diazirine

One of the greatest advantages of the photolabile diazirine group over aryl azides is that all its members absorb most efficiently at a wavelength of 350-380 nm. This is well above the 300 nm limit (*vide supra*) and therefore no significant damage to the biological system will occur. The most important reactions that occur after photolysis of 3-aryl-3*H*-diazirines are shown in Scheme 4. When a diazirine (such as **24** or **25**) is irradiated molecular nitrogen is expelled and a singlet carbene is formed (**26**). Competitively, a substantial amount (>30%) of the diazirine is converted into diazoisomer **27**. This diazo compound can be converted into the singlet carbene under the influence of light, however at the wavelengths normally used (360 nm) this process is relatively slow. For this reason the diazo species is relatively long-lived and thus has time to diffuse resulting in either aspecific labelling or hydrolysis. This problem was largely tackled when Brunner and co-workers reported the development of 3-aryl-3-(trifluoromethyl)-3*H*-diazirine **25**.[57] The strong electron-withdrawing properties of the trifluoromethyl group stabilize the diazoisomer, which makes it almost completely resistant towards undesired 'dark' reactions. Singlet carbene **26** is a very short-lived species ($t_{1/2}$ ~1 ns) and can be transformed into triplet carbene **34** via intersystem crossing (ISC). Singlet and triplet carbenes display a similar behaviour compared to their corresponding nitrenes. A singlet carbene can react as an electrophile, nucleophile or ambiphile, depending on the nature of its substituents, whereas triplet carbenes behave like diradicals. The formed singlet carbenes can give fast insertion reactions, in which they do not discriminate much between different reaction sites. Insertions into hydroxyl groups (giving **28**) usually do give a higher yield compared to C-H insertions (**29**).[58] Insertion into a primary or secondary N-H bond (**30**) can lead to an undesired side

reaction. The formed construct easily expels HF, thereby giving enamine **31**, which is in equilibrium with imine **32**. In aqueous environments, such as a physiological sample, these species are subsequently hydrolysed into the corresponding ketone, with loss of the captured substrate as the result.[58] The triplet carbene can react with C-H bonds analogues to triplet nitrenes. Initial hydrogen abstraction leads to radical intermediate **35**, which either reacts with the formed carbon radical to give a netto C-H insertion (**29**) or abstracts a second hydrogen from another C-H bond, resulting in a reduction (**36**). Another undesired side reaction occurs when the triplet carbene is oxidized by molecular oxygen (a 'notorious scavenger of triplet states') to the corresponding ketone **37**.[34] In general, unsubstituted 3-alkyl-3*H*-diazirines should be avoided since their corresponding carbenes are prone to hydride shift, which results in an olefin (see the insert in Scheme 4).[30]

Scheme 4. Possible reactions of the intermediates formed after photolysis of 3-aryl-3*H*-diazirines.

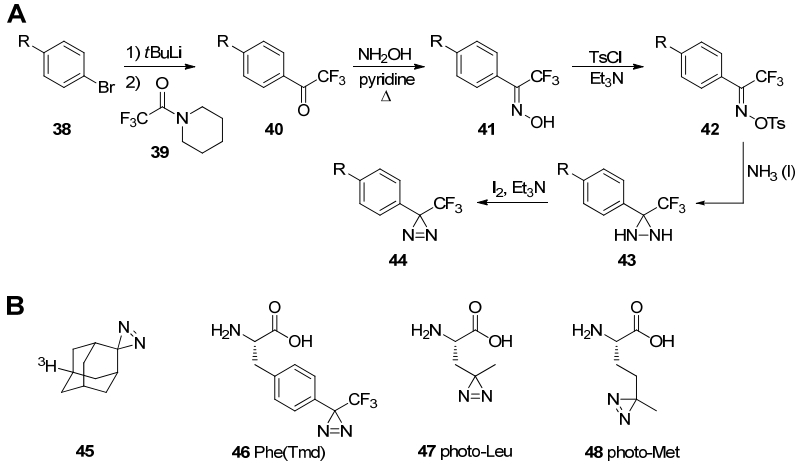


Although the diazirine group itself is relatively small, aryl diazirines are quite bulky, but they can be incorporated into molecules with a structure similar to naturally occurring compounds. Furthermore, they are quite stabile towards a wide variety of conditions, including strongly acidic, strongly basic, oxidative and several reducing agents, which is a big advantage of diazirines compared to aryl azides. Drawbacks of diazirines are the formation of substantial amounts (>30%) of the diazo species after photolysis and the intrinsic efficient reactivity of the singlet carbene with O-H bonds, which often leads to scavenging of the reactive species by water. Also, the synthesis of diazirines is somewhat complicated compared to aryl azides. The synthetic scheme often applied for the preparation of 3-aryl-3-(trifluoromethyl)-3*H*-diazirine nowadays is shown in Scheme 5A.[59] It starts by lithiation of an aryl bromide (**38**), which subsequently reacts with *N*-(trifluoroacetyl)piperidine **39** (easily prepared from trifluoroacetic anhydride and piperidine) under the formation of trifluoroacetophenone **40**. Next, the ketone is converted into the corresponding oxime **41**, after which the hydroxyl group is

converted into its tosylate (**42**). Reaction with liquid ammonia (usually under pressure) allows the instalment of the diaziridine group (**43**). Subsequent oxidation with iodine finally results in the diazirine (**44**). This five-step reaction sequence is especially well compatible with acid labile protective groups, which are often used to protect and/or install functionalities at the R position.

Some interesting examples of diazirines used in biologically relevant studies are shown in Scheme 5B. Tritium functionalized adamantyl diazirine **45** was used for selective labelling of intrinsic membrane proteins in human erythrocytes. Despite the presence of α-hydrogen atoms, the formed carbene is not prone to hydride shift (see Scheme 4) due to the constraints of this caged ring system. However, photolabelling of species is reported to be quite inefficient, probably due to its propensity to intramolecular C-H insertion reactions and reaction with water.[30,34] Among the diazirine functionalized amino acids developed, modified L-phenylalanine (Phe(Tmd), **46**) is the most popular one. Its first stereoselective preparation was reported by Nassal in 1984[60] and it has been used extensively ever since.[33,36] Recently, the synthesis of its D-phenylalanine analogue was reported and this compound was used to probe the sweet taste receptor.[61] In 2005 Thiele and co-workers reported the chemo-enzymatic synthesis of diazirinized leucine (**47**) and methionine (**48**), which were abbreviated as 'photo-Leu' and 'photo-Met'. It was shown that these unnatural amino acids could be incorporated into proteins by a eukaryotic cell with genetically unmodified translational machinery and this methodology was applied in the identification of protein-protein interactions in living cells.[62] The synthesis of photo-Met was optimized by Muir and co-workers in 2007, who circumvented the enzymatic resolution step and incorporated the unnatural amino acid into a protein using solid phase peptide synthesis (SPPS) and expressed protein ligation (EPL) strategies.[63]

Scheme 5. Preparation and examples of diazirines.



(A) Synthetic scheme for preparation of 3-aryl-3-(trifluoromethyl)-3*H*-diazirines. (B) Some examples of diazirine functionalized compounds.

Some interesting examples of A*f*BPs containing the diazirine moiety, which were used to target active metalloenzymes, are shown in Figure 4. Yao and co-workers reported a library of hydroxamate oligopeptides **49**, with varying types of amino acids at the P1 position.[64] The hydroxamate moiety is a potent zinc binding group (ZBG). The oligopeptides were modified with an N-terminal aryl diazirine for covalent modification of the target enzyme and a fluorescent label (Cy3) for visualization. They were able to selectively label and visualize thermolysin (a Zn^{2+} dependent metalloprotease found in gram-positive bacteria) spiked in a crude yeast extract after covalent modification by irradiation for twenty minutes. In addition, the library of compounds was incorporated in a large-scale profiling study, in which the 'fingerprint' labelling of twelve yeast metalloproteases towards probe library **49** was determined.

In two other studies photoaffinity labelling of metalloenzymes was combined with two-step modification and visualization using the Cu(I) catalyzed click reaction. Qiu *et al.* reported the use of succinylhydroxamate oligopeptide **50** containing an azide functionality in labelling MMP-2 (a secreted Zn^{2+} dependent matrix metalloproteinase) both as a purified enzyme and in a mouse melanoma B16-F10 cell culture medium.[65] Visualization of the photocaptured construct was achieved by a click reaction to biotin-propargylamide and subsequent streptavidin-HRP Western-blotting. The same group reported the development of one-step and two-step A*f*BPs **51a** and **51b**,[66] the design of which was based on parent compound **L288**. The latter is a potent inhibitor (IC_{50} 0.13 μM) of type I methionine aminopeptidase (MetAP1), a cobalt dependent metalloenzyme expressed by both prokaryotic and eukaryotic cells, and which removes N-terminal (initiator) methionine from polypeptides. The modifications made led to a slight decrease in inhibitory potency compared to **L288**. Incubation of overexpressed *E. coli* MetAP1 in *E. coli* cell lysate with compound **51b** followed by UV irradiation, click reaction and Western blotting revealed labelling of the target enzyme, which could be competed away by **L288**. Interestingly, incubation with one-step probe **51a** resulted in substantial non-selective labelling, which overwhelmed the MetAP1signal. Apparently, the two-step probe is much more selective in this case.

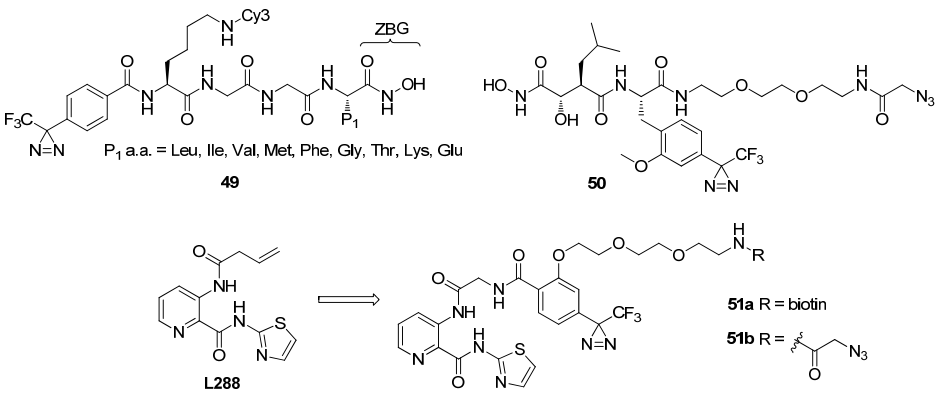
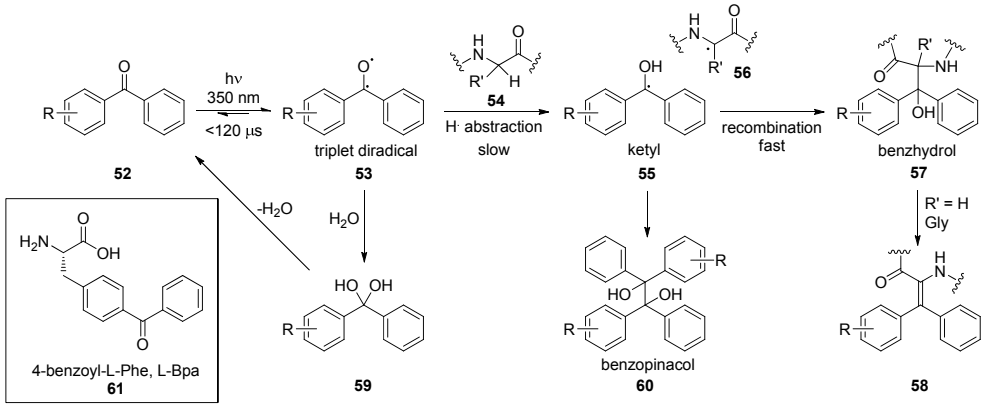


Figure 4. Examples of A*f*BPs containing the diazirine moiety used to study active metalloenzymes.

1.3.3 Benzophenone

A major advantage of benzophenones is that they can be excited at wavelengths of 350-360 nm, just like diazirines. The possible reaction pathways of benzophenones after photolysis are shown in Scheme 6. Absorption of a photon of the proper wavelength by a benzophenone (**52**) initially results in the formation of a triplet state benzhydril diradical (**53**). The formation of the triplet diradical is reversible and it can exist as long as 120 μs before relaxing back to its ground state in the case that it is unable to find a reaction partner. The first reaction step of the formed reactive species is abstraction of a hydrogen and the reaction rate is therefore dependent on the nature of a nearby X-H bond.[67] In general, the diradical is more reactive towards C-H bonds than O-H bonds. Especially those C-H bonds that form relatively stabile carbon radicals are prone to react and these include benzylic positions, amino acid α-positions, hydrogen atoms adjacent to heteroatoms and tertiary carbon centres. Reactions with aromatic and vinylic C-H bonds have not been reported. All amino acids can react, although it has been shown that there is a preference for the ε-H in methionine when the benzophenone moiety is mobile enough to choose.[68] Abstraction of a hydrogen from an amino acid α-centre (**54**) by **53** results in the formation of a ketyl (**55**) and an alkyl radical (**56**), which recombine fast to form a benzhydrol (**57**). In the case of glycine, there is a possibility of elimination of water under the formation of olefin **58**. A big advantage of the benzophenone group is that its photoactivated counterpart is more reactive towards C-H bonds compared to nitrenes and is less prone to intramolecular rearrangements than carbenes. Also, when the diradical inserts into water the corresponding hydrate (**59**) is formed. This species quickly dehydrates to form the ketone again, which can be recycled under irradiation to the diradical species. This ability of benzophenones to 'search' for a good reaction centre is a big advantage in terms of crosslink efficiency, however when the reactive species is not quenched in time there is a big chance of aspecific labelling, especially when a corresponding A*f*BP is not interacting with the target enzyme, but moves around freely in the medium. A possible

Scheme 6. Chemistry of benzophenones after photolysis.



side reaction which can take place is the homodimerization of ketyl **55** to form benzopinacol **60**, however due to the relatively big difference in reaction rates of hydrogen abstraction and recombination, normally only a very small amount of this is formed.

In contrast to aryl azides and diazirines, the most commonly used benzophenone building blocks are commercially available. Benzophenone substituted amino acid analogues were also created, similar to what was previously discussed for aryl azides and diazirines. Not surprisingly, the most studied amino acid derivative is the one derived from phenylalanine, commonly abbreviated as L-Bpa (**61** in Scheme 6).[69] Although the benzophenone group may seem like the ideal photocrosslinking reagent, it also suffers from some drawbacks. It is relatively bulky, which can negatively influence the interaction between enzyme and substrate. Also, the resulting steric hindrance can give rise to a discrimination between reaction sites and, as a result of that, can lead to a non-specific labelling. Finally, irradiation for prolonged times (>30 minutes) is often needed in order to obtain a reasonable crosslinking efficiency.[27]

In recent literature many examples of A*f*BPs containing benzophenones can be found. A first example concerns the study of histone deacetylases (HDACs). These enzymes catalyze the hydrolysis of acetylated lysine amine side chains in histones and are thus involved in the regulation of gene expression. There are approximately twenty human HDACs which are divided into three classes (I, II and III). Class I and II HDACs are zinc-dependent metallohydrolases that do not form a covalent bond with their substrates during their catalytic process, which is similar to MMPs. It has been found that hydroxamate **62** (SAHA, see Figure 5) is a potent reversible inhibitor of class I and II HDACs. In 2007 Cravatt and co-workers reported the transformation of SAHA into an A*f*BP, by instalment of a benzophenone and an alkyne moiety, which resulted in SAHA-BPyne (**63**).[70] They showed that the probe can be used for the covalent modification and enrichment of several class I and class II HDACs from complex proteomes in an activity-dependent manner. In addition, they identified several HDAC associated proteins,

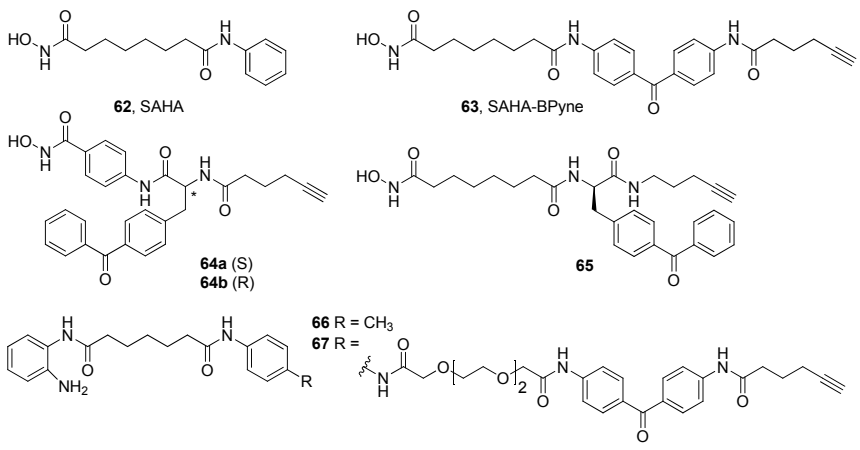


Figure 5. Examples of benzophenone modified two-step labelling probes to study HDACs.

possibly arising from the tight interaction with HDACs. Also, the probe was used to measure differences in HDAC content in human disease models. Later they reported the construction of a library of related probes and studied the differences in HDAC labelling.[71] Their most interesting finding was that the labelling efficiency is not directly linked to a compound's inhibitory potency. For example, compounds **64a**, **64b** and **65**, containing Bpa (Figure 5), showed a higher potency (in terms of inhibition of HDAC activities) than **63**, however the latter compound proved to be superior in HDAC labelling. A similar disparancy in inhibitory potential and photo-affinity labelling will be addressed in Chapter 4. A similar approach was reported by Xu *et al.* in 2009, in which potent HDAC class I (comprising HDACs 1, 2, 3 and 8) inhibitor **66** was modified with a benzophenone-spacer-alkyne moiety (**67**) to study its binding affinities in more detail.[72] Incubation of FRDA lymphoblast derived nuclear extract with **67** followed by photoaffinity crosslinking and a click reaction with either rhodamine azide or biotin azide, identified HDAC-3 as the single target of this inhibitor.

In addition to the aryl azide and diazirine groups, the benzophenone moiety was also incorporated in probes that target matrix metalloproteinases (see Figure 6). Potent, broad-spectrum succinyl-hydroxamate MMP inhibitor GM6001 (**68**) was converted into photoaffinity probe **69** by incorporation of Bpa (**61**) and a fluorophore, as reported by Cravatt and co-workers in 2004.[73] It was shown that this probe can be used to covalently label (through photocrosslinking) and visualize several active MMPs in complex proteomes. In addition, the authors were able to identify a number of other metalloproteases targeted by GM6001, which do not belong to the MMP family. In order to address this metalloenzyme's lack of selectivity towards a single inhibitor, this same group developed an alternative profiling strategy.[74] A two-step photoaffinity labelling MMP probe library was constructed, in which the Bpa moiety was incorporated at either the P3′ (**70a,b**) or P2′ (**70c**) position and the P1′ and P2′ substituents were varied (see Figure 6). The library compounds were applied as a 'cocktail' to proteomes and instead

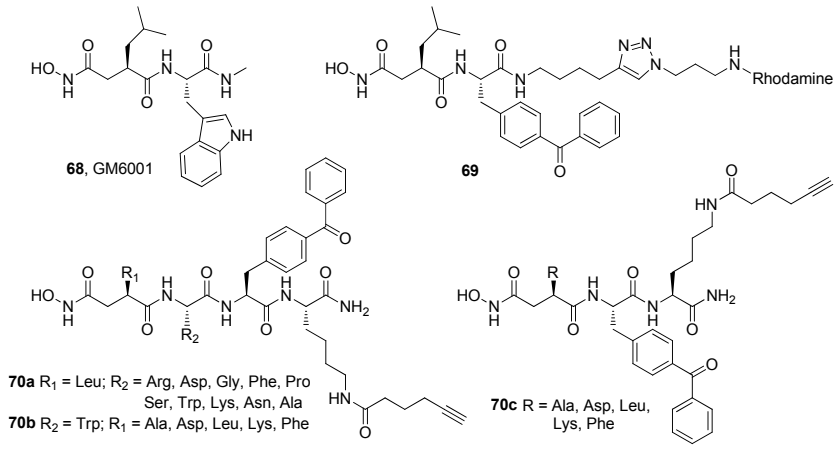


Figure 6. Examples of benzophenone modified probes to target matrix metalloproteinases. The amino acid three letter abbreviations in the R_1 (**70a,b**) and R (**70c**) substituents refer to their corresponding side chains.

of identifying the affinity of each metalloprotease towards a single probe the labelling profiles towards the entire library were analyzed collectively. This method proved to be powerful in that more than twenty metalloproteases (MMPs and others) could be identified from a complex biological mixture. In a later study they showed the use of some of the library compounds and related photoaffinity probes as competitive A*f*BPs to study the affinity of four MMP-13 inhibitors, for a large number of other metalloproteases.[75]

A powerful application of the use of benzophenone containing A*f*BPs to locate the active site within a protease complex has been reported for γ-secretase.[76] This multi-subunit, integral membrane protein complex is responsible for the proteolysis of transmembrane proteins. Together with β-secretase, it generates the amyloid β-protein, which is known as the central pathogenic feature in Alzheimer's disease. Although the nature of the γ-secretase catalytic activity was determined to be of aspartate protease type, the exact location of its active site within the complex was unknown. A potent inhibitor of γ-secretase activity is L-685,485 (**71**, Figure 7), containing a hydroxyethylene dipeptide-isostere. This compound mimics the transition state of the aspartate protease catalytic process and hence, forms a reversible, non-covalent adduct with the active enzyme (the binding of the inhibitor in the enzyme's active site is shown in Figure 7). Li and co-workers reported the modification of the inhibitor's P2 and P3' substituents with a benzophenone moiety, which led to A*f*BPs **72a** and **72b**.[76] The modifications did not result in a decrease in inhibitory potency towards γ-secretase activity inhibition compared to parent compound **71**. The interaction of both probes with γ-secretase was studied by addition of the probes to HeLa cell membranes containing solubilised γ-secretase and subsequent photocrosslinking. From the obtained results the authors were able to identify membrane-spanning protein presenilin 1 (PS1) as the γ-secretase active site bearing subunit. Four years later the same group reported the preparation of the synthetically more challenging P1' Bpa containing biotinylated analogue of **71** (structure not shown).[77] With this probe, they were able to label active γ-secretase in

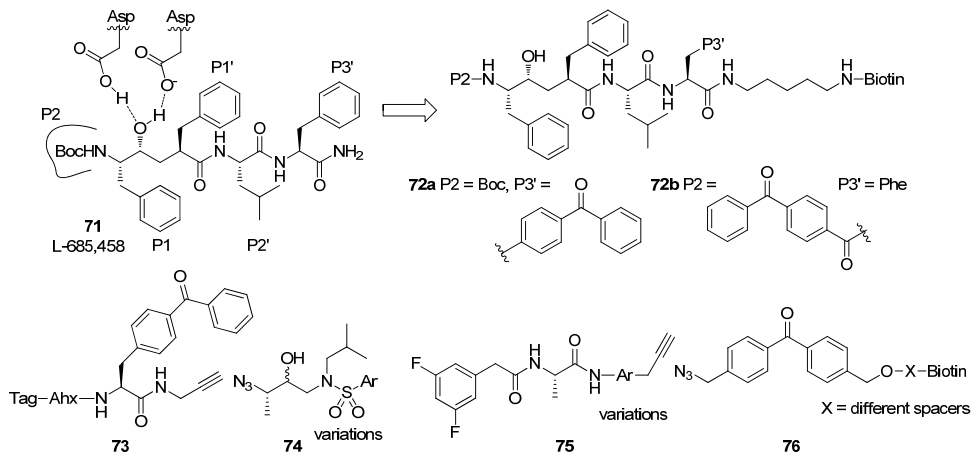


Figure 7. Examples of A*f*BPs modified with the benzophenone photophore that target γ-secretase.

living cells and their results demonstrated, for the first time, that active γ-secretase is presented on the cell surface.

The use of click chemistry has been applied to the synthesis of benzophenone modified γ-secretase probes as well. The group of Yao reported the preparation of a compound library built up from Bpa containing alkyne **73** and azide **74** (Figure 7).[78] The azide part contains a racemic hydroxyethylene moiety and variations were made in its aryl sulfonamide domain. The compound library was screened for its potency against γ-secretase inhibition and the most potent compounds were used to label active PS1 in a cell lysate. In addition, Fuwa and co-workers reported a divergent synthesis of γ-secretase A*f*BPs by means of click chemistry with alkyne **75** and azide **76**.[79] Variations were made in the aryl part of the alkyne (dibenzoazepine or benzodiazepine) and in the type of spacer between the benzophenone moiety and biotin in the azide. Photoaffinity labelling using these probes provided the authors with evidence that the molecular targets of this type of probes are the N-terminal fragment of PS1.

Another class of enzymes that is especially well suited for PAL is the kinase family. These enzymes catalyze the ATP dependent phosphorylation of several substrates, but do not form a covalent linkage with either reaction partner and can therefore not be caught by means of a suicide trap. Some examples of photoreactive A*f*BPs targeting kinases are shown in Figure 8. In 2003 the group of Sewald reported the preparation of fluorescently tagged A*f*BP **78**,[80] which was obtained after modification of the potent kinase inhibitor H-9 (**77**), a isoquinolinesulfonamide containing, competitive inhibitor, targeting a broad range of kinases by occupying their ATP binding site. The probe proved to able of labelling several kinases in a concentration dependent manner and could be eliminated by preincubation of the kinase with competing ligands. Furthermore, the authors were able to label, although not very selective, creatine kinase added to a mixture of isolated thylakoid proteins.

In 2006 Kawamura and co-workers reported a study towards the photoactivated labelling of kinases with compound **79**, with Aa being glycine (Figure 8).[81] In an initial screen towards six different kinases it was shown that this probe selectively labelled one

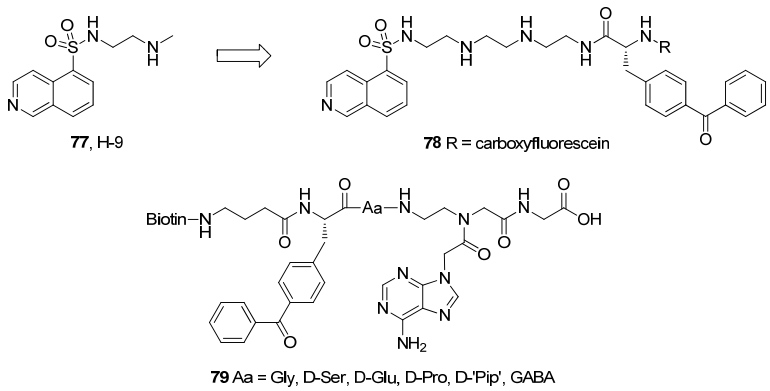


Figure 8. Examples of benzophenone containing A*f*BPs for labelling of kinases. D-'pip' = (R)-piperidine-2-carboxylic acid; GABA = γ-aminobutyric acid.

kinase, namely leukocyte-specific protein tyrosine kinase (Lck) and that labelling could be blocked by adenine. In addition, the probe selectively labelled Lck in an extract of Jurkat cells. With the aid of LC-MS/MS after tryptic digest, it was possible to identify the labelled fragment within the kinase. Photocrosslinking had taken place in the Ile^{379}-Arg^{386} tryptic fragment and, more precisely, to either Gly^{383} or Leu^{384}. In a later study, the influence of target-binding affinity and conformational flexibility on the photocrosslinking efficiency was assessed.[82] For this, a small library was prepared, in which the glycine moiety in **79** was replaced by other (non-proteinogenic) amino acids. Variations in length, flexibility and hydrogen bonding capability were made. The results corroborated the earlier mentioned finding (see the examples of HDAC inhibitors and Chapter 4) that the inhibitor potency does not necessarily correlate with the photocrosslinking efficiency. Interestingly, it was found that higher crosslinking yields were obtained for the more flexible compounds, while in other cases (for other enzymes) crosslinking efficiencies increased upon a more tightly bound probe (see for example Chapter 4), however this probably depends on the type of photocrosslinker used as well as the class of enzymes studied.

1.3.4 Comparing photocrosslinkers

As has been pointed out, different photophores can give rise to different labelling products. Therefore, the outcome of a photolabelling experiment substantially depends on the type of photocrosslinker applied. In order to select the ideal photophore for a specific experiment, one should carefully examine the different possibilities. Interestingly, there are only a few reports on the comparison of different photophores under otherwise identical conditions, especially where all three are taken into account or when they are applied in an A*f*BP. Some representative examples will be given here.

Weber and Beck-Sickinger reported a study towards the photochemical behaviour of the different photophores.[83] For this, the synthetic pentapeptide thymopentin, or TP5 (Arg-Lys-Asp-Val-Thr) was modified with each of the photocrosslinkers (C-terminal Tyr was replaced by Phe(Tmd) and Bpa or 4-azidobenzoic acid was coupled to the N-terminus), thereby obtaining three different photoreactive peptides. The conversion and product formation were followed by LC-MS analysis after photolysis in water, *n*-propanol and a water/*n*-propanol mixture (1:1, v/v). It was found that both the diazirine and aryl azide were quickly converted into a reactive species, but only the diazirine gave a relatively pure product formation resulting from insertion into the solvent, predominantly in the O-H bond. The aryl azide also gave a clean O-H insertion reaction in water, however when *n*-propanol was present numerous unidentified products were formed, probably arising from intramolecular rearrangements and insertion reactions. The benzophenone containing peptide gave clean C-H insertions in the presence of *n*-propanol, but the conversion yield was low after fifteen minutes of irradiation (68% and 82% starting material left in water/*n*-propanol and *n*-propanol respectively). In pure water the conversion was much higher (94% after fifteen minutes), however due to the poor reactivity towards O-H bonds many unidentified products were generated. This led to the conclusion that the use of diazirines and aryl azides is preferred over

benzophenones, however the latter should be used in case the crosslinking site contains many water molecules.

Another direct comparison between the photophores, however not in an activity-based setting, was reported by Tate and co-workers.[84] They synthesized dUTP analogues containing four different photoreactive moieties **80a-d** (Figure 9A) and incorporated them enzymatically into DNA constructs. With these, the DNA photoaffinity labelling of yeast RNA polymerase III transcription complexes was studied. It was shown that photolabelling with the diazirine construct rendered many protein-DNA contacts, whereas labelling with the other three photoreactive moieties proved only marginal.

In a recently reported study by Dalhoff and co-workers the aryl azide and diazirine photophores were directly compared in an activity-based labelling of methyltransferases (MTases).[85] This class of enzymes catalyzes the transfer of the activated methyl group in *S*-adenosyl-L-methionine (SAM) to a nucleophilic position in various substrates (DNA, RNA, proteins) under the formation of *S*-adenosyl-L-homocysteine (SAH) as shown in the insert in Figure 9B. SAH itself is a general competitive product inhibitor of MTases and because of this it was transformed into four A*f*BPs by attachment of a spacer moiety containing biotin and either an aryl azide or aryl diazirine (**81a,b**, Figure 9B). Since the optimal modification site was unknown, the linker moiety was attached to either the N6 or C8 position, as indicated in **81**. Initially, the compounds were used to label and capture (by means of streptavidin-coated magnetic beads) purified DNA adenine N6 MTase from *Thermus aquatis* (M.Taql). All four compounds could be used for M.Taql pull-down in a yield of 5-10%, however the capture efficiency of the diazirine-based probes was slightly higher compared to the aryl azides, as determined from the Western blots. Labelling proved to be activity-based since it could be competed with SAH and, in addition, was shown to be light dependent. With these results in hand, the authors also tested the N6-aryl azide-derived probe **81a** for pull-down of three other NTases in there purified form and two other SAH binding proteins in *E. coli* lysate, which were identified by LC-MS/MS analysis. Unfortunately, the diazirine derived probes were not further examined, because the authors argued that 'the small improvement in capture yield might not balance the much higher cost of preparation'.

Up to here it seems that the diazirine-group is the most effective photocrosslinker. Interestingly, there are two reports showing that the potency of the applied photoreactive group largely depends on the system it is used in. In a study towards photoreactive metalloprotease probes by Yao *et al.*,[64] as was outlined in paragraph 1.3.2, an additional, benzophenone containing, probe was synthesized. In order to compare the diazirine and benzophenone moieties, the authors replaced the diazirine moiety in compound **82a** (Figure 9C, this is the same compound as **49** with P1 = Leu (see Figure 4)) with the corresponding benzophenone counterpart (**82b**). This compound proved to label purified thermolysin as well, however no signs of labelling were observed when applying the compound to crude yeast extract, spiked with thermolysin. In this case the diazirine-based probe appeared superior compared to its benzophenone counterpart. In contrast, Fuwa and co-workers obtained the opposite results, although in a completely different system.[79] In their study towards the

development of benzophenone derived photoreactive γ-secretase probes (see paragraph 1.3.3 and Figure 7), they also incorporated the diazirine photophore into their probes (**83**, Figure 9D). These diazirine containing analogues of the compounds resulting from a click reaction between **75** and **76** (Figure 7), proved to be completely ineffective in the labelling of γ-secretase active subunits, despite the fact that they showed the same inhibitory potential compared to their benzophenone counterparts.

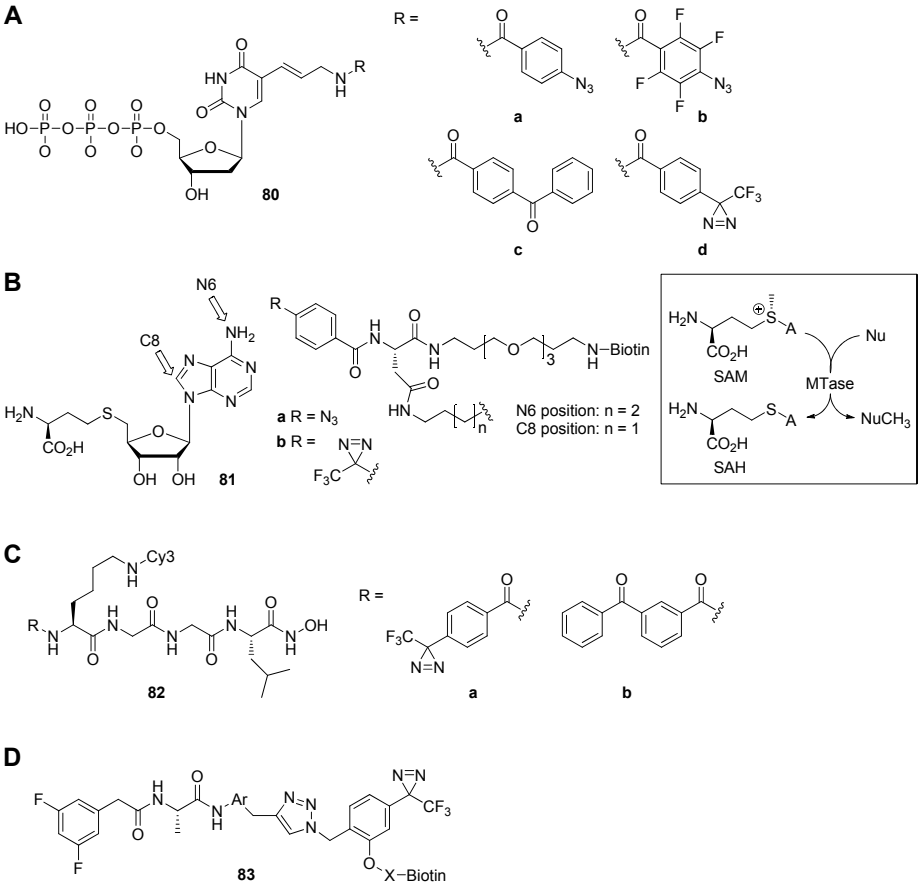


Figure 9. Structures of compounds used to compare the photoaffinity labelling of different photoreactive groups. (A) Photoreactive dUTP analogues **80a-d** reported by Tate *et al.*[84] (B) Photoreactive SAH analogues **81a,b** reported by Dalhoff *et al.*[85] The insert shows the transfer of the activated methyl group from SAM to nucleophilic positions (Nu) in various substrates by methyltransferases (MTase). A = adenosine. (C) Photoreactive metalloprotease probes **82a,b** reported by Yao *et al.*[64] (D) Diazirine containing analogue of clicked compound **75-76** for labelling of γ-secretase reported by Fuwa *et al.*[79]

These results show clearly that there is no such thing as the 'ideal photophore' (yet). Due to the differences in reaction site preferences and the nature of the reactive intermediates of each individual moiety, the optimal photophore type needed differs from system to system. In most cases it can be worthwhile to spend some time on the

optimization of the used photophore in a certain A*f*BP, in order to obtain the best results (an issue that will also be addressed in Chapter 6). In addition, interesting information can be obtained when the results derived from different photophores are combined.[34]

1.3.5 Identification of the photocrosslinked sites

As outlined in paragraph 1.2, the tag incorporated in an ABP or A*f*BP is used for visualization, both *in vivo* and as read-out after SDS-PAGE, of the bound enzyme. In addition, a bifunctional tag, such as biotin, can be used as a pull-down handle for purification of the covalently modified complex. Subsequent (tryptic) digestion and LC-MS/MS analysis allows the identification of the tryptic peptides and hence, the labelled enzyme(s). In the case a warhead containing ABP is used, the modified peptide fragments can in general be found easily in the LC-MS/MS analysis, since the chemistry of modification and hence, the nature of the final construct is known. In photoaffinity labelling, the identification of the photolabelled fragment is usually much more difficult. This is largely due to the fact that the site of modification is unknown (usually there are multiple modification sites) and the photocrosslinking reaction itself can lead to many possible products, because of the multiple reactive species involved. In some cases, the identification of the photolabelled site is possible (see for instance the labelling of hMMP-12 reported by Dive *et al.* (paragraph 1.3.1) or the labelling of Lck reported by Kawamura and colleagues (paragraph 1.3.3)), but this is generally difficult, because of complicated mixtures. A possible solution to this problem is the mixed isotope photoaffinity labelling strategy, which makes use of so-called 'MS-friendly' photoprobes, or target identification probes (TIPs).[86] The general idea of this strategy is schematically shown in Figure 10. A proteome is treated with a 1:1 mixture of two structurally identical A*f*BPs, which only differ in their absolute mass by incorporation of stable isotopes (*e.g.* deuterium, ^{13}C, ^{18}O or ^{15}N). Subsequent photocrosslinking, purification (using the tag) and tryptic digestion leads to a mixture of labelled and unlabelled tryptic peptides. It is

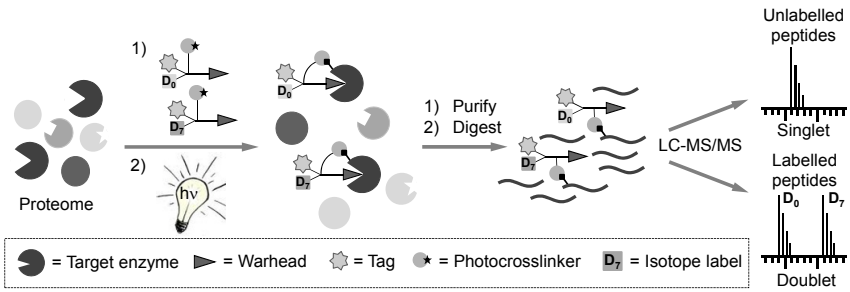


Figure 10. Mixed isotope photoaffinity labelling strategy for determination of the labelling site. After binding and photocrosslinking the target enzyme with a 1:1 mixture of the 'light' (D_0) and 'heavy' (D_7) isotopically labelled A*f*BPs, the construct is purified and tryptically digested. LC-MS/MS analysis of the peptide pool allows discrimination between the labelled and unlabelled peptides. The modified fragment can easily be retrieved and identified by searching for the isotope signature.

now possible to discriminate between these two types of peptides in a LC-MS/MS analysis because of the double peaks separated by the mass difference between the 'light' and 'heavy' isotopically labelled species. Thus, the modified peptide(s) can be identified by searching for the unique isotopic pattern, or 'isotope signature'.Although this concept is relatively new in the field of photoaffinity labelling, some examples have been recently reported. Lamos and colleagues reported the modification of cyclophilin A (CypA) binding immunosuppressive CsA with a benzophenone-D_{11} and a biotin moiety (compound **84**, Figure 11).[87] As a proof of principle, they used a 1:1 mixture of this TIP and its non-deuterated isoster for the selective photoaffinity labelling and pull-down of CypA among three other proteins. Subsequent tryptic digestion of the elutes and LC-MS/MS analysis allowed for the identification of eleven CypA characteristic peptides, two of which were modified with the probe as evidenced from the double, 11 Da separated, peaks in the mass spectra. The large 11-Da mass difference allowed easy visual recognition of labelled peptides in the mass spectra, which makes this a powerful method in determination of the modification site after photoaffinity labelling, however application in more complex systems still remains to be done.

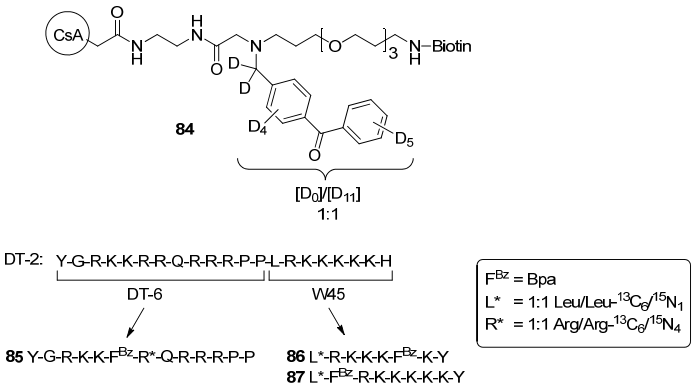


Figure 11. Examples of 'MS-friendly' photoreactive compounds used in mixed photoaffinity labelling.

A second, more extensive, study was recently reported by the group of Heck.[88] These authors describe the application of peptides containing stable isotopes and a photoreactive moiety in the localization of cGMP-dependent protein kinase (PKG) substrate binding sites. For this, they focussed on oligopeptide DT-2 (Figure 11), a potent and selective inhibitor of PKG, which is a construct of a PKG tight binding sequence (W45) and a membrane translocating sequence (DT-6). Both of these peptides were modified by incorporation of a benzophenone moiety (as Bpa) and an isotopically labelled amino acid Leu-$^{13}C_6/^{14}N_1$ or Arg-$^{13}C_6/^{15}N_4$ (peptides **85-87**, notice the difference of the Bpa position between the W45 derived peptides). LC-MS/MS analysis was performed on photocrosslinked complexes of PKG with each of the peptides. The power of the strategy was reflected in their results, which led to the identification of each peptide's binding site. For example, the binding site of compound **87** was located in tryptic peptide Gln^{195}-Arg^{203} and fragmentation of this sequence identified Met^{201} as the

single cross-linked amino acid. Interestingly, the binding site of the highly similar peptide **86** was located on a completely differently situated peptide in the PKG sequence, namely Phe^{359}-Glu^{374}. In this case the fragmentation did not result in the determination of the exact crosslinked amino acid, however it could be pinpointed to the residues Thr^{364}-Glu^{374}. With all the obtained results the authors were unfortunately unable to determine the exact binding site of DT-2, however they obtained the interesting results that both the DT-6 and W45 peptides are targeted to the same pocket in the PKG's catalytic domain and that therefore DT-2 is preferentially bound to dimeric PKG.

Altogether, the mixed isotope photoaffinity labelling strategy is still in development, but is believed to become a powerful tool in photoaffinity-based protein profiling strategies in the near future. With its aid the photocrosslinking site(s) of an A*f*BP can more easily be identified, which will lead to a better understanding of protein structure and function in general.

1.4 Aim and outline of this Thesis

The research described in this Thesis aims at the development of chemical biology research tools to study proteolytic activities, with the main focus on metalloproteases (MMPs and ADAMs) and proteasomes. The first part of **Chapter 2** describes the synthesis of the enantiomerically pure *N,O*-diprotected succinyl hydroxamate building block **88** (Figure 12) and its use in the construction of peptide hydroxamate-based MMP/ADAM inhibitors in a highly efficient, linear SPPS protocol. In addition, photoreactive probe **89** was synthesized and used for covalent modification of ADAM-10. The second part of this chapter deals with the preparation of a library containing 96 enantiopure peptide hydroxamates with general structure **90** by SPPS using building block **88**. This compound library was used to study the inhibitor preference of three metalloproteases (MMP-9, MMP-12 and ADAM-17) with respect to the substituents at the P2' and P3' positions (R_2 and R_1 respectively).

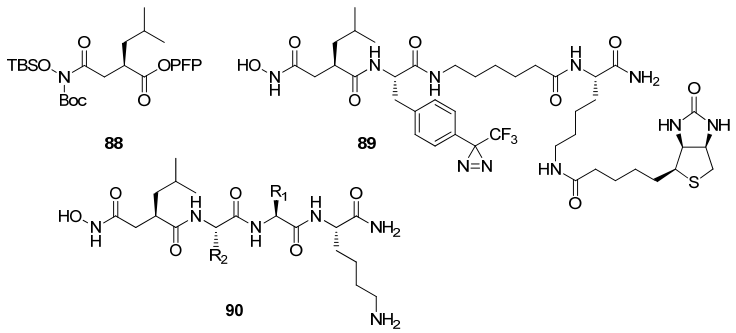


Figure 12. Structure of building block **88**, used for the construction of photoreactive metalloprotease probe **89** and a library containing 96 inhibitors with general structure **90**.

Although the covalent modification of ADAM-10 with compound **89** was shown to be possible, the efficiency proved rather modest. Therefore, the effect of moving the photophore to the more firmly binding P1' pocket was studied and is outlined in **Chapter 3**. The synthesis of photoreactive building block **91** (Figure 13) and its application in the preparation of activity-based probes **92a-c** is described. It is further demonstrated that **92a** is indeed the more efficient MMP/ADAM A*f*BP, compared to **89**, in a head to head comparison towards a range of recombinant, purified metalloproteases.

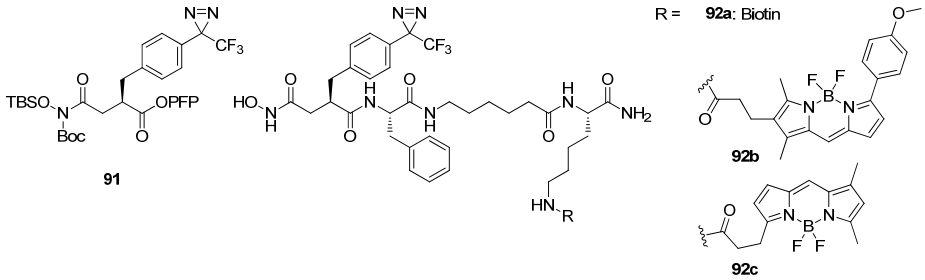


Figure 13. Structure of photoreactive building block **91**, used for the synthesis of MMP/ADAM A*f*BPs **92a-c**.

The research described in **Chapter 4** entails a study towards the effect of fluorine incorporation into proteasome inhibitors on their specificity towards the different proteasome subunits. It was found that fluorine incorporation led to a large decrease in potency towards the β1 and β2 subunits and, hence resulted in selectivity for β5. Tripeptide epoxyketone **93** (Figure 14) was identified as one of the most β5 selective inhibitors known to date.

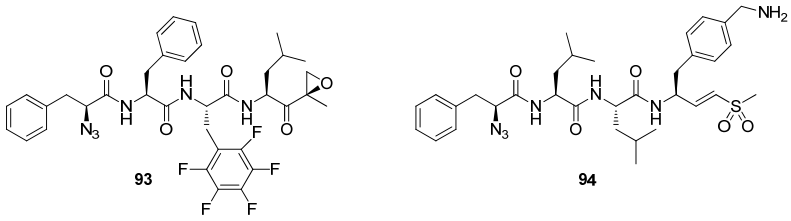


Figure 14. Structures of β5 selective (**93**) and β2 selective (**94**) proteasome inhibitors.

The development of selective inhibitors for the proteasome's trypsin-like sites (β2) is discussed in **Chapter 5**. Selectivity for β2 was achieved by instalment of basic side chain residues in the P1 position of tripeptide vinylsulfones. Incorporation of a 4-aminomethylene benzylamine substituent at this position resulted in compound **94** (Figure 14), which showed high β2 selectivity both in cell extracts and in living cells.

Chapter 6 describes the study towards the orientation of extended peptide vinylsulfones inside the proteasome's catalytically active cavity. This study combined activity-based modification by means of a warhead with photocrosslinking. A panel of

bifunctional two-step probes, comprising of an extended peptide vinylsulfone equipped with an N-terminal photophore, was prepared. Of these probes, compound **95** (Figure 15) was able to establish a crosslink between the active β5 subunit and a neighbouring β6 subunit which could be identified by subsequent SDS-PAGE and LC-MS/MS analysis.

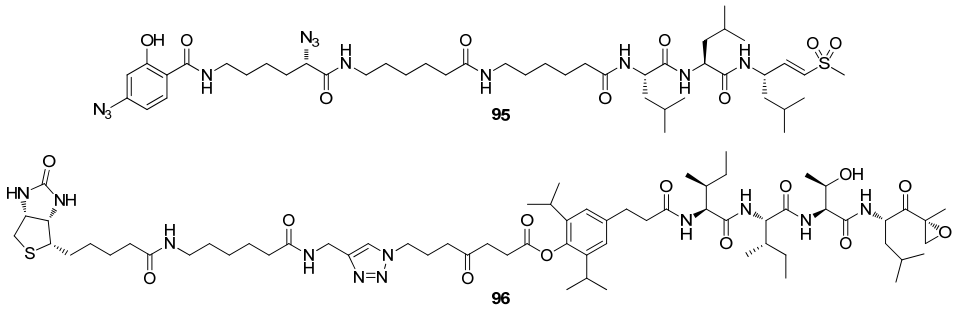


Figure 15. Structures of photoactivatable proteasome probe **95** and levulinoyl-based cleavable linker containing proteasome probe **96**.

The design and preparation of a novel levulinoyl ester-based cleavable linker is described in **Chapter 7**. The cleavable linker was incorporated into potent proteasome inhibitor epoxomicin and attachment of biotin resulted in ABP **96** (Figure 15). The optimal characteristics, in terms of stability and cleavability, of the cleavable linker were reflected by the use of **96** for pull-down of proteasome active subunits from a cell lysate. Subsequent treatment with hydrazine allowed a chemoselective release of **96**-derived bound proteins.

Finally, the research described in this Thesis is summarised in **Chapter 8**, and some future prospects based the presented results are discussed.

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2 A Straightforward Synthesis of Peptide Hydroxamate MMP/ADAM Inhibitors

Synthesis and biological evaluation of an inhibitor library

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2.1 Introduction

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

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

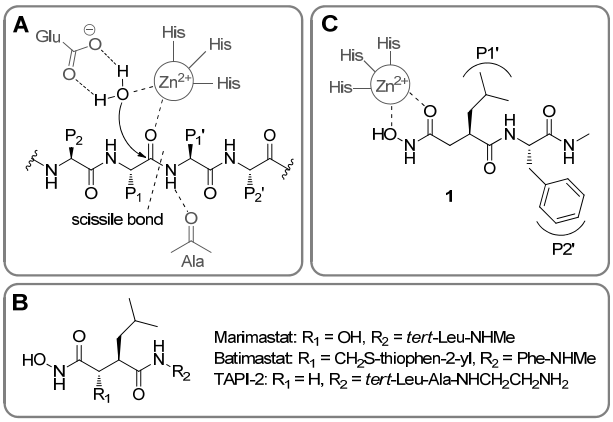


Figure 1. (A) Schematic representation of the position of a substrate in an MMP active site and mechanism of proteolysis. (B) Structures of some commercially available MMP/ADAM inhibitors. (C) Schematic representation of a potent MMP inhibitor **1**[14] containing the hydroxamic acid zinc binding group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

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

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

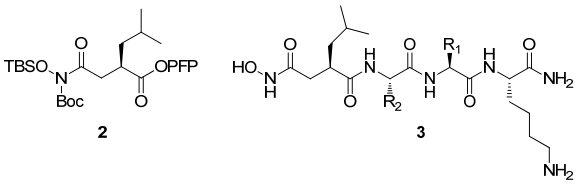


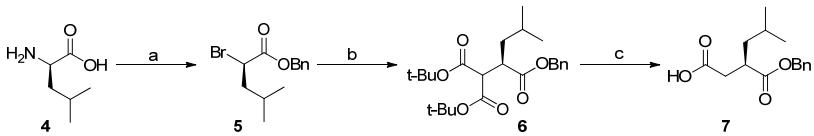
Figure 2. Structure of chiral succinylhydroxamate building block **2** for SPPS of peptide hydroxamate MMP/ADAM inhibitors. General structure **3** represents the library containing 96 compounds made via SPPS using building block **2**. R_1 and R_2 are amino acid side chains.

2.2 Results and Discussion

2.2.1 Synthesis and application of building block 2

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

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

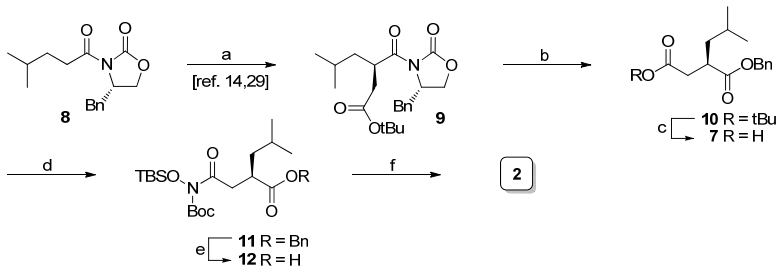


Reagents and conditions: (a) i) $NaNO_2$, HBr, H_2O, 90%; ii) BnOH, TsOH, toluene, reflux, 79%; (b) di-*tert*-butyl malonate, KO*t*Bu, DMF, 92%; (c) i) TFA, DCM; ii) toluene, reflux, 83%.

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

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

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

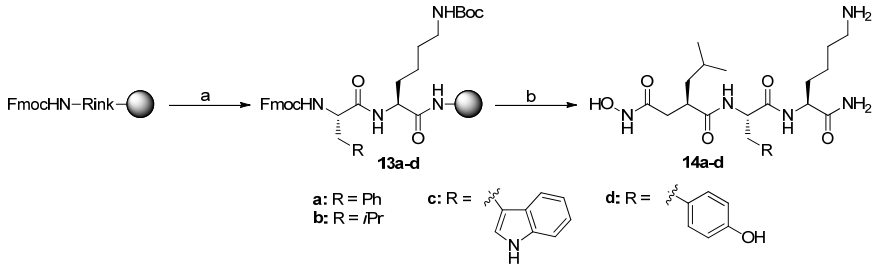


Reagents and conditions: (a) LiHMDS, *tert*-butyl bromoacetate, THF, –78 °C; (b) BnOH, *n*BuLi, THF, 0 °C, 92%; (c) TFA, DCM, 94%; (d) i) $(ClCO)_2$, cat. DMF, DCM; ii) TBSONHBoc, DMAP, ACN, 85%; (e) H_2, Pd/C, MeOH; (f) PFPOH, EDC, DCM, 55% from **11**.

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

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

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



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

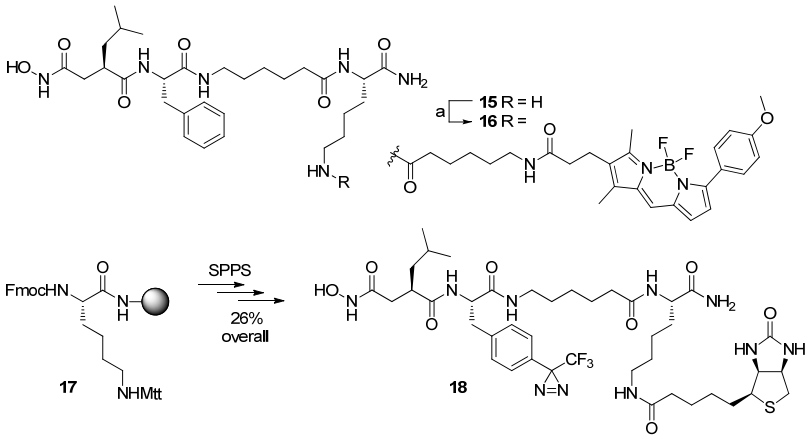
Table 1. Inhibitory activities of the synthesized MMP/ADAM inhibitors (IC_{50} values in nM).

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

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

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

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



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

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

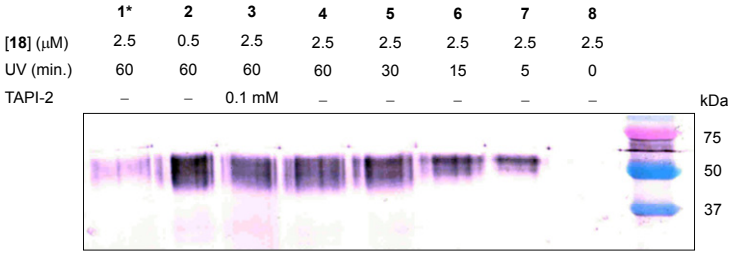
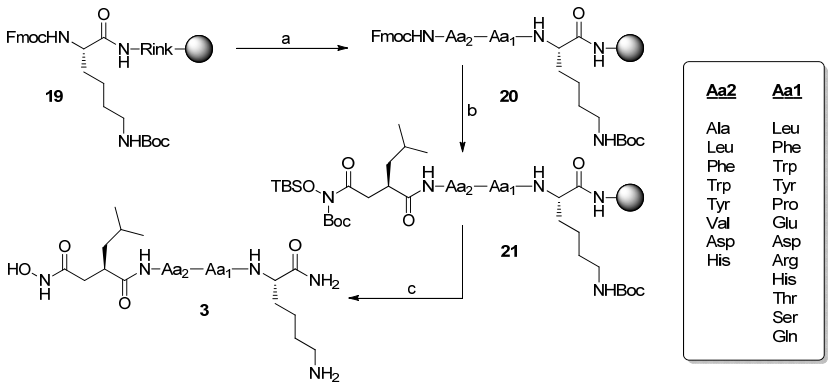


Figure 3. Labeling of ADAM-10 with probe **18**. Recombinant ADAM-10 (100 ng) was incubated with probe **18**, followed by irradiation with UV light (366 nm) for 0-60 min., as indicated. The mixtures were denatured, resolved by 10% SDS-PAGE, stained with streptavidin-AP and visualized by Western blotting.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

2.2.2 Synthesis and evaluation of a peptide hydroxamate library

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

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



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

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

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

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

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

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

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

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

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

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

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

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

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

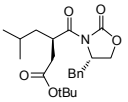
2.3 Conclusion

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

Experimental section

General

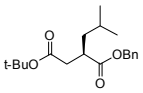
Tetrahydrofuran (THF) was distilled over $LiAlH_4$ before use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), methanol (MeOH), piperidine, diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased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



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

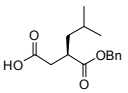
To a cooled solution (–78 °C) of LiHMDS (1.18 mmol, 1.18 mL of a 1M solution in THF) in THF (5 mL) was added (S)-4-benzyl-3-(4-methylpentanoyl)oxazolidin-2-one (**8**, 0.39 g, 1.07 mmol) in THF (5 mL) and the mixture was stirred at –78 °C for 30 min. *tert*-butyl bromoacetate (0.58 g, 2.97 mmol) was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

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



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

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



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

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

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

Verification of the chiral purity of compound 7

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

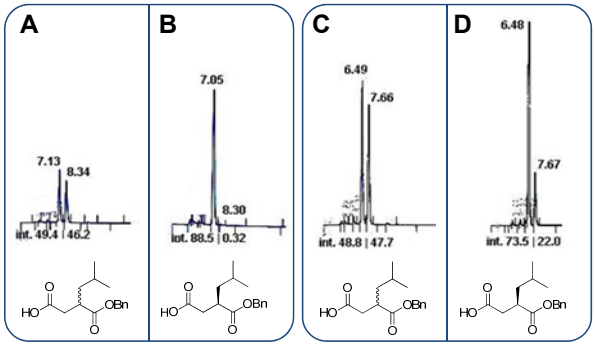
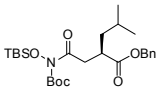
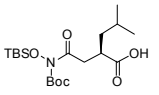


Figure 4. Results from the chiral HPLC analyses of (R)-2-isobutylmonobenzylsuccinate **7** and the racemate synthesized by the method of Chatterjee *et al.*[43] Measurements were done with a Chiral OD column; eluent: hexane:isopropanol 95:5 (v/v) + 0,25% AcOH; flow: 1 mL/min.; UV detection at $\lambda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



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

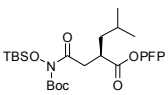
Carboxylic acid **7** (2.11 g, 8.0 mmol) was dissolved in DCM (30 mL) and DMF (2 drops) was added. The solution was cooled to 0 °C and oxalyl chloride (40 mmol, 3.4 mL) was added dropwise. The solution was warmed to room temperature and stirred until gas evolution ceased (about 30 min.). Toluene (30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



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

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

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



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

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

General procedure A: solid phase peptide synthesis

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

Hydroxamic acid Phe-Lys-NH_2 (14a)

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

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

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

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

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

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

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

Synthesis of the compound library (3)

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

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

Biological evaluation

Materials

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

Determination of IC_{50} values shown in Table 1

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

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

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

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

Determination of inhibitor efficacy (Table 2)

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

Determination of IC_{50} values shown in Table 3

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

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3 Peptide Hydroxamate-Based Photoreactive Probes of Zinc-Dependent Metalloproteases

Synthesis and biological evaluation

P. P. Geurink, T. Klein, L. Prèly, K. Paal, M. A. Leeuwenburgh, G. A. van der Marel, H. F. Kauffman, H. S. Overkleeft, R. Bischoff, *Eur. J. Org. Chem.* **2010**, 2100–2112.

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[1-10] and, as a consequence, deregulation of metalloprotease catalytic activity can lead to several inflammatory processes.[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.[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,[18-21] serine hydrolases[22-25] and proteasome subunits.[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.[29-36] In this context, peptide hydroxamate **1** featuring both a biotin and a trifluoromethyldiazirine moiety (Figure 1B) was developed (Chapter 2).[37] 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.[38-41] 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.

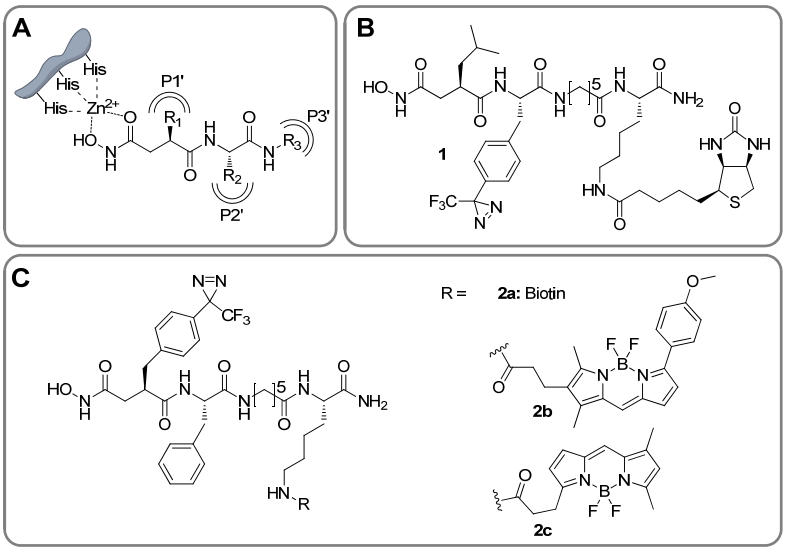
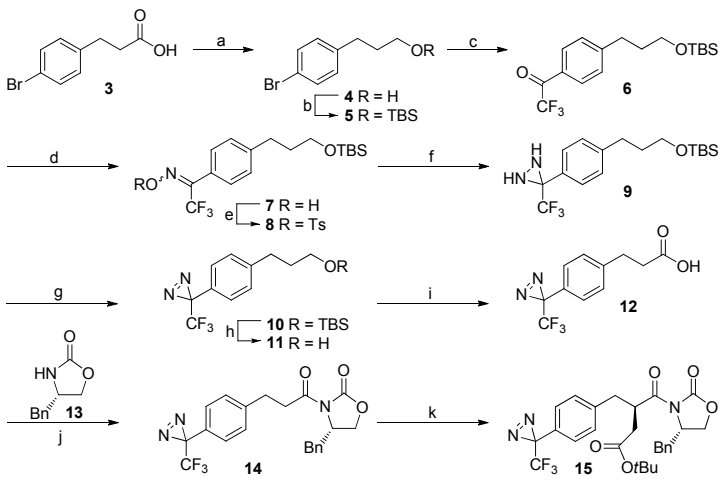


Figure 1. (A) Generic binding mode of N-terminal peptide hydroxamates to metalloprotease active sites. (B) Peptide hydroxamate-based activity-based metalloprotease probe from previous studies with the photoactivatable group at the P2' position.[37] (C) Compounds targeted in this study and featuring the photoactivatable group at the P1' site.

3.2 Results and Discussion

In the work described in Chapter 2[37] 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 buidling 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)[42] 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 $LiAlH_4$ 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**.

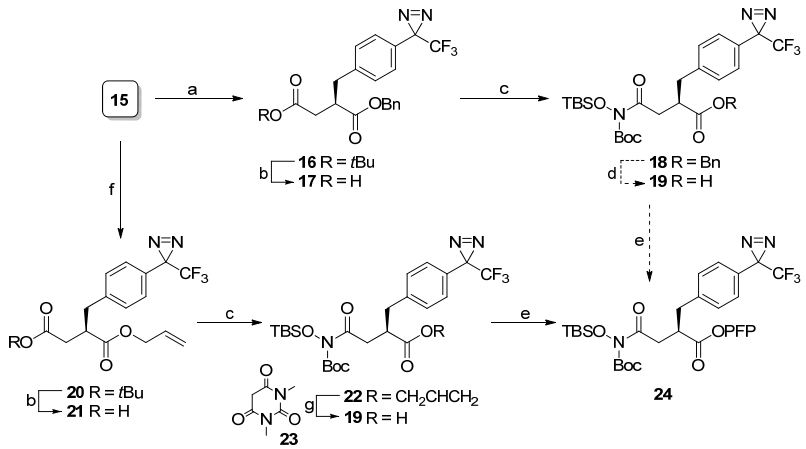


Reagents and conditions: (a) $LiAlH_4$, Et_2O, 0 °C, quant.; (b) TBSCl, imidazole, DMF, quant.; (c) *n*BuLi, 1-trifluoroacetyl piperidine, Et_2O, –78 °C → RT, 76%; (d) $HONH_2$·HCl, pyridine, Δ, 93%; (e) TsCl, Et_3N, DMAP, DCM, 97%; (f) NH_3, Et_2O, 8 bar, 95%; (g) I_2, Et_3N, MeOH, 94%; (h) HCl, H_2O, MeOH, 97%; (i) TEMPO, BAIB, DCM, H_2O, 96%; (j) i) $(ClCO)_2$, DMF, DCM; ii) **13**, *n*BuLi, 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 biphasic TEMPO/BAIB oxidation of the resulting alcohol **11**[43] 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 auxilliary **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[37] 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.[44] It was previously found (Chapter 2)[37] 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, H_2 or $Pd(OAc)_2$, Et_3SiH, Et_3N), 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, *n*BuLi, THF, 0 °C, 82%; (b) TFA, DCM, 97%; (c) i) $(ClCO)_2$, DMF, DCM; ii) TBSONHBoc, *n*BuLi, THF, 27% for **18** and 77% for **22**; (d) several conditions, no yield; (e) PFPOH, EDC, DCM, 88% over 2 steps; (f) allyl alcohol, *n*BuLi, 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

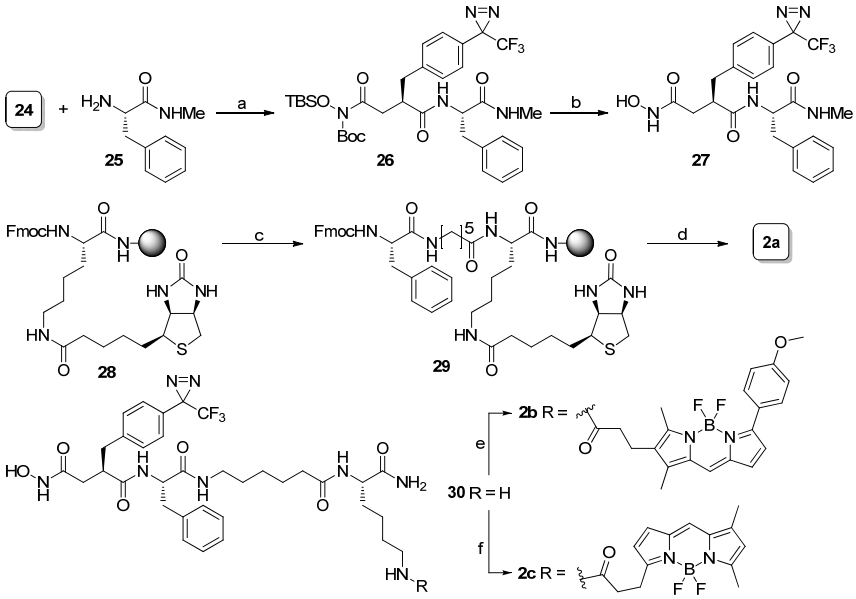
ester **20**.[45] Partial deprotection and condensation with the protected hydroxylamine as described before gave succinyl hydroxamate **22**. Now, the allyl ester was removed by reaction with tetrakis(triphenylphosphine)palladium in the presence of *N,N'*-dimethylbarbaturic acid **23** as the allyl 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.[37] 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,[46,47] 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**[48] in DMF to give bisamide **26**, which was finally deprotected (TFA/H_2O, 95:5 (v:v)) to the free hydroxamic acid **27** in 30% yield over the two steps.[49] 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 expulsed with concomitant formation of a highly reactive carbene that will insert in the first available X-H (where X

= C, N, O, S) bond (Chapter 1).[52] 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/H_2O, 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/H_2O/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**.

	MMP-9	MMP-12	ADAM-10	ADAM-17
1	25.1	3.60[a]	114[a]	20.6[a]
2a	24.2	12.5	54.1	490

[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

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.

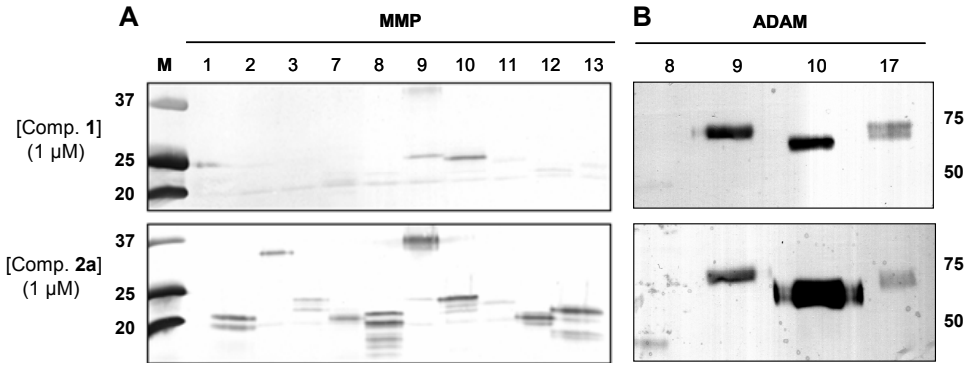


Figure 2. Photoaffinity labeling of MMPs (A) and ADAMs (B) using probe **1** (upper panels) and probe **2a** (lower panels) at 1 μM. The modified proteins were visualized by anti-biotin Western blots with streptavidin-alkaline phosphatase. Ten recombinant MMP and four recombinant ADAM proteases (4 pmol each; numbers above the lanes correspond to the respective protease) were studied. Multiple biotinylated bands in the recombinant MMPs indicate auto-degradation. M: molecular weight marker.

In order to prove that labeling is activity-dependent, aliquots of MMP-9 and MMP-12 were incubated with either the natural inhibitor, TIMP-1[53] 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 reported[37,42] 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 trifluoromethyldiazirine 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 analoguous to compounds **1** and **2a** such as Bodipy derivatives **2b**,**c** may well be indispensable in reaching this research objective.

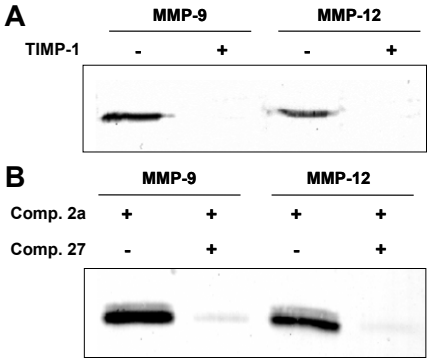


Figure 3. Activity-dependence of photoaffinity labeling of MMP-9 and MMP-12 (4 pmol each) with probe **2a** (200 nM final concentration) as shown by competition with (A) an equimolar amount of the endogenous MMP inhibitor TIMP-1 (200 nM final concentration) and with (B) a twofold molar excess of compound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

Experimental section

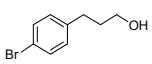
General

Tetrahydrofuran was distilled over $LiAlH_4$ 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*-(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 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 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^{1}H- and ^{13}C-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. ^{19}F 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 tetramethylsilane, CD_3OD, DMSO-d6, $CDCl_3$ or $CFCl_3$ 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 dioctylpthalate (*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 $[\alpha]_D^{23}$ 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 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H_2O, 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.

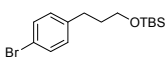
General procedure A: solid-phase peptide synthesis

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 with a 0.45 M DiPEA stock solution in NMP. The coupling and deprotection reactions were checked on the presence of free amines by performing a Kaiser test. Before cleaving the peptide from the resin, it was washed 5 times alternatingly with DCM and MeOH. Cleavage from the solid support was done by shaking the resin in a TFA:H_2O: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:H_2O: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)

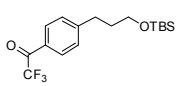
Commercially available 3-(4-bromophenyl)propanoic acid (**3**, 10.8 g, 46.22 mmol) was dissolved in Et_2O (250 mL) and cooled to 0 °C. To this solution was carefully added $LiAlH_4$ (1.3 eq., 60 mmol, 2.28 g) in portions. The reaction was slowly warmed to room temperature in 1 h after which TLC analysis 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. $NaHCO_3$ (2 × 200 mL) and brine (200 mL), dried over $MgSO_4$ 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)

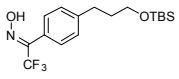
To a solution of alcohol **4** (9.9 g, 46.2 mmol) in DMF (80 mL) were added imidazole (4.77 g, 69.3 mmol) and *tert*-butylchlorodimethylsilane (TBS-Cl) (7.82 g, 50.8 mmol). The reaction was stirred for 2 h after which TLC analysis indicated complete conversion. Deionised H_2O (300 mL) was added and the mixture was extracted 3 times with PE (200 mL). The combined organic layers were extracted with deionised H_2O (4 × 200 mL) and brine

(200 mL), dried over $MgSO_4$ 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]



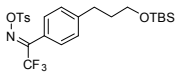
1-(4-(3-(*tert*-butyldimethylsilyloxy)propyl)phenyl)-2,2,2-trifluoroethanone (6)

Bromide **5** (7.03 g, 21.36 mmol) was dissolved in Et_2O (100 mL) and cooled to –78 °C. *n*BuLi (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 °C and a solution of 1-trifluoroacetyl piperidine (4.2 g, 23.2 mmol) in Et_2O (5 mL) was added dropwise. The solution was slowly warmed to 0 °C in 2 h after which TLC analysis indicated a complete conversion. The reaction was quenched with saturated aq. NH_4Cl (100 mL) and the layers were separated. The organic layer was extracted with saturated aq. NH_4Cl, deionised water and brine (100 mL each), dried over $MgSO_4$ 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, $CDCl_3$): δ = 8.00 (d, *J* = 8.0 Hz, 2H), 7.37 (d, *J* = 8.0 Hz, 2H), 3.64 (t, *J* = 6.4 Hz, 2H), 2.79 (t, *J* = 7.6 Hz, 2H), 1.89-1.84 (m, 2H), 0.91 (s, 9H), 0.06 (s, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 151.3, 130.2, 129.3, 127.7, 116.8 (q, *J* = 290 Hz), 61.4, 33.9, 32.4, 25.9, 18.2, –5.4 ppm. HRMS: calcd. for $C_{17}H_{25}F_3O_2Si$ $[M + H]^+$ 347.16487; found 347.16505.



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

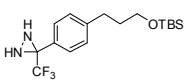
Ketone **6** (9.43 g, 27.2 mmol) was dissolved in pyridine (30 mL) and hydroxylamine hydrochloride (5.68 mmol, 81.7 mmol) was added. The mixture was stirred at reflux for 2 h after which TLC analysis indicated a complete conversion. The mixture was concentrated under reduced pressure and dissolved in 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 $MgSO_4$ 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, $CDCl_3$): δ = 8.51 (bs, 1H), 8.25 (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, $CDCl_3$): δ = 147.4-146.5 (m), 144.9, 144.5, 129.2, 128.9, 128.9, 128.7, 128.6, 128.6, 128.4, 128.3, 128.2, 127.9, 123.7, 120.9 (q, *J* = 273 Hz), 118.6 (q, *J* = 281 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 $C_{17}H_{26}F_3NO_2Si$ $[M + H]^+$ 362.17577; found 362.17583.



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

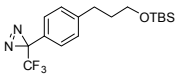
Oxime **7** (9.13 g, 25.3 mmol) was dissolved in DCM (20 mL). To this solution were added Et_3N (5.26 mL, 37.95 mmol) and DMAP (60 mg, 0.5 mmol) after which TsCl (4.84 g, 25.3 mmol) in DCM (20 mL) was added dropwise over 1 h. After stirring the mixture at room temperature for 30 min. TLC analysis indicated 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 (100 mL) and brine, dried over $MgSO_4$ 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, $CDCl_3$): δ = 7.91-7.88 (m, 2H), 7.39-7.25 (m, 6H), 3.66-3.62 (m, 2H), 2.73 (t, *J* = 7.8 Hz, 2H), 2.48 (s, 3H), 2.46 (s, 3H), 1.88-1.83 (m, 2H), 0.91 (s, 9H), 0.90 (s, 9H), 0.06 (s, 6H), 0.05 (s, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 153.8 (q, *J* = 34.4 Hz), 146.8,

146.6, 146.0, 145.9, 131.5, 131.2, 129.7, 129.1, 128.9, 129.8, 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 $C_{24}H_{32}F_3NO_4SSi$ $[M + H]^+$ 516.18462, found 516.18443.



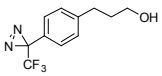
3-(4-(3-(*tert*-butyldimethylsilyloxy)propyl)phenyl)-3-(trifluoromethyl)diaziridine (9)

Tosylate **8** (12.6 g, 24.4 mmol) was dissolved in Et_2O (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, 25.7, 18.1, –5.6 ppm. HRMS: calcd. for $C_{17}H_{27}F_3N_2OSi$ $[M + H]^+$ 361.19175; found 361.19183.



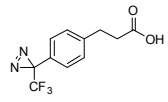
3-(4-(3-(*tert*-butyldimethylsilyloxy)propyl)phenyl)-3-(trifluoromethyl)-3*H*-diazirine (10)

Et_3N (46.2 mmol, 6.4 mL) was added to a solution of crude diaziridine **9** (8.32 g, 23.1 mmol) in MeOH (25 mL). Then iodine (23.1 mmol, 5.86 g) was added in portions, letting the mixture decolorize after every portion. Eventually the reaction mixture did not decolorize anymore and the mixture was stirred for another 30 min. TLC analysis indicated a completed reaction and a 10% w/w aq. citric acid solution (100 mL) was added. The mixture was extracted twice with Et_2O (150 mL) and the combined organic layers were extracted with 10% w/w aq. citric acid (100 mL), a saturated aq. $NaHSO_3$ solution (100 mL), deionised water (100 mL) and brine (100 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude diazirine was obtained as a colourless oil (yield: 7.77 g, 21.7 mmol, 94%). A small amount was purified by column chromatography (1% → 2.5% EtOAc/PE) for characterization. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.22 (d, J = 8.4 Hz, 2H), 7.10 (d, J = 7.8 Hz, 2H), 3.61 (t, J = 6.3 Hz, 2H), 2.69 (t, J = 7.5 Hz, 2H), 1.82-1.79 (m, 2H), 0.90 (s, 9H), 0.05 (s, 6H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 144.2, 129.0, 126.6, 126.5, 122.4 (q, J = 273 Hz), 61.9, 34.2, 31.8, 28.4 (q, J = 40 Hz), 25.9, 18.3, –5.5 ppm. HRMS: calcd. for $C_{17}H_{25}F_3N_2OSi$ $[M + H]^+$ 359.17610; found 359.17616.



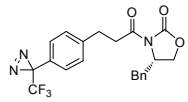
3-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenyl)propan-1-ol (11)

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_2O (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 mixture was purified by column chromatography (10% → 60% EtOAc/PE) and the pure 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 $C_{11}H_{11}F_3N_2O$ $[M + H]^+$ 245.08962; found 245.08978.



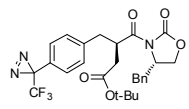
3-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenyl)propanoic acid (12)

Alcohol **11** (2.1 g, 8.6 mmol) was dissolved in a mixture of DCM (25 mL) and deionised H_2O (12.5 mL). To this mixture were added 2,2,6,6-tetramethylpiperidinooxy (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. $Na_2S_2O_3$ 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%). ^{1}H NMR (600 MHz, $CDCl_3$): 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. ^{13}C NMR (150 MHz, $CDCl_3$): 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 $C_{11}H_9F_3N_2O_2$ [M + H]$^+$ 259.06889; found 259.06909.



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

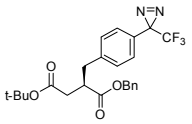
Carboxylic acid **12** (1.04 g, 4.03 mmol) was dissolved in DCM (25 mL) and DMF (1 drop) was added. The mixture was cooled to 0 °C and oxalyl chloride (1.7 mL, 20 mmol) was added dropwise. The reaction was warmed to room temperature and stirred for 30 min. after which gas formation ceased. Toluene was 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. *n*BuLi (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). The combined organic layers were extracted with a saturated aq. solution of $NaHCO_3$ (2 × 50 mL) and brine (50 mL), dried over $MgSO_4$ 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%). ^{1}H NMR (600 MHz, $CDCl_3$): 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. ^{13}C NMR (150 MHz, $CDCl_3$): 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. $[\alpha]_D^{23}$ = +48.5 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{21}H_{18}F_3N_3O_3$ [M + H]$^+$ 418.13730; found 418.13743.



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

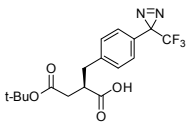
LiHMDS (5.5 mL of a 1 M solution, 5.5 mmol) was put into a flask under an argon atmosphere and cooled to –78 °C. To this was added a solution of compound **14** (2.0 g, 5.0 mmol) in THF (25 mL) over 15 min. The reaction was stirred for 1 h at –78 °C after which *tert*-butyl bromoacetate (2.2 mL, 15 mmol) 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. NH_4Cl solution (50 mL) was added and the mixture was extracted with EtOAc (3 × 30 mL). The combined organic layers were dried over $MgSO_4$ 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%). ^{1}H NMR (600 MHz, $CDCl_3$): 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, 1H), 3.92 (t, *J* = 8.4 Hz, 1H), 3.23 (dd, *J* = 13.5, 2.7 Hz, 1H), 3.00 (dd, *J* = 13.2, 6.0 Hz, 1H), 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. ^{13}C NMR (150 MHz, $CDCl_3$): 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. $[\alpha]_D^{23}$ = +72.8 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{27}H_{28}F_3N_3O_5$ $[M + H]^+$ 532.20538; found 532.20512.



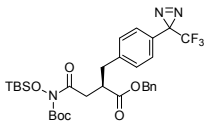
(R)-1-benzyl 4-*tert*-butyl 2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl)succinate (16)

Benzyl alcohol (350 μL, 3.40 mmol) was dissolved in THF (8 mL) and cooled to 0 °C. To this mixture was added *n*BuLi (1.10 mL of a 1.6 M solution, 1.76 mmol) and the reaction was stirred at 0 °C for 30 min. Then a solution of compound **15** (784 mg, 1.47 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 30 min. TLC analysis indicated complete conversion of the starting material. A saturated aq. NH_4Cl 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 (3% → 10% EtOAc/PE) the product was obtained as a colourless oil (yield: 558 mg, 1.21 mmol, 82%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.37 (t, *J* = 7.2 Hz, 1H), 7.33 (d, *J* = 4.8 Hz, 2H), 7.22 (t, *J* = 3.6 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), 2.99 (dd, *J* = 13.2, 7.2 Hz, 1H), 2.82 (dd, *J* =13.8, 7.8 Hz, 1H), 2.60 (dd, *J* = 16.2, 8.4 Hz, 1H), 2.34 (dd, *J* = 16.8, 5.4 Hz, 1H), 1.40 (s, 9H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 173.4, 170.3, 140.0, 135.5, 129.3, 128.1, 128.0, 127.1, 126.4, 126.3, 122.0 (q, *J* = 273 Hz), 80.7, 66.3, 42.8, 37.1, 36.4, 28.1 (q, *J* = 40.5 Hz), 27.70 ppm. $[\alpha]_D^{23}$ = +3.6 (*c* = 1, $CHCl_3$).



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

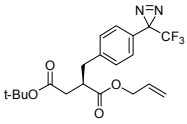
Tert-butyl ester **16** (550 mg, 1.19 mmol) was dissolved in DCM (4 mL). To this was added TFA (3 mL) and the reaction was stirred at room temperature. After 1 h TLC analysis indicated a completed reaction. Toluene was added and the mixture was concentrated under reduced pressure. Coevaporating the mixture 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: 1.09 g, 3.07 mmol, 97%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 11.15 (bs, 1H), 7.35 (t, *J* = 7.2 Hz, 1H), 7.32 (d, *J* = 4.8 Hz, 2H), 7.21 (t, *J* = 3.6 Hz, 2H), 7.11 (d, *J* = 7.8 Hz, 2H), 7.04 (d, *J* = 7.8 Hz, 2H), 5.06 (dd, *J* = 36.6, 12.0 Hz, 2H), 3.17-3.12 (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* = 17.4, 9.0 Hz, 1H), 2.43 (dd, *J* = 17.4, 5.4 Hz, 1H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 177.7, 173.4, 139.6, 135.3, 129.4, 128.5, 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.



(R)-benzyl 4-(*tert*-butoxycarbonyl(*tert*-butyldimethylsilyloxy)amino)-4-oxo-2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl)butanoate (18)

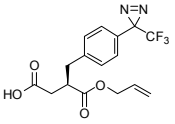
Carboxylic acid **17** (71 mg, 0.18 mmol) was dissolved in DCM (1.5 mL) and DMF (1 drop) was added. This mixture was cooled to 0 °C and oxalyl chloride (4 eq., 0.70 mmol, 60 μL) was added dropwise. The reaction was warmed to room temperature and stirred for 30 min. after which gas formation ceased. Toluene was added and the mixture was concentrated *in vacuo* followed by coevaporation with toluene (2×). *O*(TBS)-*N*(Boc)

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. *n*BuLi (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 (10 mL) 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 μmol, 27%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 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. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 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.



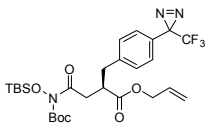
(R)-1-allyl 4-*tert*-butyl 2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl)succinate (20)

Allyl alcohol (190 μL, 2.75 mmol) was dissolved in THF (5 mL) and cooled to 0 °C. To this mixture was added *n*BuLi (812 μL 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_4Cl 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%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 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. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 173.5, 170.6, 140.2, 131.7, 129.5, 127.4, 126.5, 122.1 (q, *J* = 273 Hz), 118.3, 80.9, 65.3, 43.0, 37.2, 36.4, 28.2 (q, *J* = 40.5 Hz), 27.9 ppm. $[\alpha]_D^{23}$ = +9.9 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{20}H_{23}F_3N_2O_4$ $[M + H]^+$ 413.16827; found 413.16826.



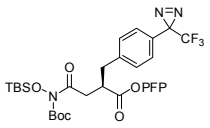
(R)-4-(allyloxy)-4-oxo-3-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl)butanoic acid (21)

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. Coevaporating the mixture twice with toluene resulted in yellowish oil. This 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%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 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. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 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* = 40.0 Hz) ppm. $[\alpha]_D^{23}$ = +13.2 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{16}H_{15}F_3N_2O_4$ $[M + H]^+$ 357.10567; found 357.10576.



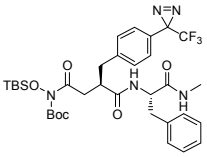
(R)-allyl 4-(*tert*-butoxycarbonyl(*tert*-butyldimethylsilyloxy)amino)-4-oxo-2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl)butanoate (22)

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. *O*(TBS)-*N*(Boc) protected hydroxylamine[44] (1.2 eq., 4.1 mmol, 1.01 g) was dissolved in THF (10 mL) and the solution was cooled to 0 °C. *n*BuLi (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 $MgSO_4$ 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%). ^{1}H NMR (600 MHz, $CDCl_3$): 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. ^{13}C NMR (150 MHz, $CDCl_3$): 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, 65.2, 42.7, 38.6, 37.3, 28.2 (q, *J* = 40.5 Hz), 27.8, 25.6, 18.0, –5.2 ppm. $[\alpha]_D^{23}$ = +7.91 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{27}H_{38}F_3N_3O_6Si$ [M + H]$^+$ 586.25547; found 586.25562.



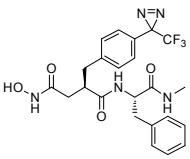
(R)-pentafluorophenyl 4-(*tert*-butoxycarbonyl(*tert*-butyldimethylsilyloxy)amino)-4-oxo-2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl)butanoate (24)

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 (**19**). To this mixture were added pentafluorophenol (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. Et_2O (50 mL) was added and the mixture was extracted twice with 0.1 M aq. HCl (50 mL) and brine, dried over $MgSO_4$ 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%). ^{1}H NMR (500 MHz, $CDCl_3$): 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. ^{13}C NMR (125 MHz, $CDCl_3$): 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. ^{19}F NMR (188 MHz, $CDCl_3$): 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. $[\alpha]_D^{23}$ = +8.8 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{30}H_{33}F_8N_3O_6Si$ [M + Na]$^+$ 734.19031; found 734.19061.



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

L-phenylalanine methylamide[48] (**25**, 21 mg, 120 μmol) was added to a solution of compound **24** (85 mg, 120 μmol) 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_2O (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_2O (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% → 50% EtOAc/PE) and the product was obtained as a colourless oil (yield: 38 mg, 54.0 μmol, 45%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 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. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 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), 28.0, 26.0, 25.7, 18.1, –4.9 ppm. $[\alpha]_D^{23}$ = +5.6 (*c* = 1, $CHCl_3$). LC-MS: gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.81 (ESI-MS (*m/z*): 705.87 ($M + H^+$)). HRMS: calcd. for $C_{34}H_{46}F_3N_5O_6Si$ $[M + H]^+$ 706.32422; found 706.32419.



(R)-_N_4-hydroxy-_N_1-((S)-1-(methylamino)-1-oxo-3-phenylpropan-2-yl)-2-(4-(3-(trifluoromethyl)-3_H_-diazirin-3-yl)benzyl)succinamide (27)

TFA (1 mL) and deionised H_2O (50 μL) were added to a solution of compound **26** (21 mg, 30 μmol) 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% → 65% ACN/(0.1% TFA/H_2O)). The title compound was obtained as a colourless solid (yield: 10.2 mg, 21 μmol, 69%). ^{1}H NMR (400 MHz, DMSO-d6): δ = 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), 7.14 (d, *J* = 8.0 Hz, 2H), 4.38-4.36 (m, 1H), 3.00 (dd, *J* = 13.6, 5.2 Hz, 1H), 2.98-2.92 (m, 1H), 2.82-2.75 (m, 2H), 2.62 (dd, *J* = 13.6, 5.6 Hz, 1H), 2.57 (d, 3H), 2.11 (dd, *J* = 14.8, 7.2 Hz, 1H), 1.94 (dd, *J* = 14.6, 7.4 Hz, 1H) ppm. ^{13}C NMR (100 MHz, DMSO-d6): δ = 173.7, 172.0, 168.3, 142.8, 139.0, 130.8, 130.0, 129.0, 127.1, 127.0, 126.2, 55.0, 44.1, 38.2, 37.8, 35.1, 26.4 ppm. $[\alpha]_D^{23}$ = –10.8 (*c* = 1, DMSO). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.20 (ESI-MS (*m/z*): 492.07 ($M + H^+$)). HRMS: calcd. for $C_{23}H_{24}F_3N_5O_4$ $[M + H]^+$ 492.18532; found 492.18494.

HA-succ(Tmd)-Phe-Ahx-Lys(Biotin)-NH_2 (2a)

This compound was synthesized on solid support on a 20 μmol 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 μmol, 50 mg) and DiPEA (40 μmol, 90 μL 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% → 90% ACN/(0.1% TFA/H_2O)) and was obtained as a colourless solid (yield: 4.3 mg, 6.0 μmol, 30% after 3 coupling steps). ^{1}H NMR (400 MHz, DMSO-d6): δ = 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* = 12.40, 5.07 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, 1H), 2.13 (t, *J* = 7.35 Hz, 2H), 2.07 (t, *J* = 7.60 Hz, 2H), 2.02-1.96 (m, 1H) 1.91 (dd, *J* = 13.98, 7.50 Hz, 1H), 1.65-1.56 (m, 1H), 1.52-1.40 (m, 9H), 1.40-1.22 (m, 6H), 1.20-1.12 (m, 2H) ppm. LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O):

R_t (min): 6.45 (ESI-MS (*m/z*): 945.53 (M + H^+)). HRMS: calcd. for $C_{44}H_{59}F_3N_{10}O_8S$ [M + H]$^+$ 945.42629; found 945.42682.

(R)-*N*1-((S)-1-(6-((S)-1,6-diamino-1-oxohexan-2-ylamino)-6-oxohexylamino)-1-oxo-3-phenylpropan-2-yl)-*N*4-hydroxy-2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl)succinamide or (HA-succ(Tmd)-Phe-Ahx-Lys-NH_2 (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/H_2O)) and was obtained as a colourless solid (yield: 45 mg, 54 μmol, 30% after 3 coupling steps). ^{1}H NMR (400 MHz, CD_3OD): 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. ^{13}C NMR (100 MHz, CD_3OD): δ = 177.0, 176.5, 173.8, 173.7, 171.2, 143.4, 139.5, 131.8, 131.3, 130.3, 128.9, 128.6, 128.4, 57.2, 54.9, 46.5, 41.4, 41.0, 39.4, 39.4, 37.4, 36.5, 33.4, 30.7, 28.9, 28.2, 27.2, 24.7 ppm. LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 5.92 (ESI-MS (*m/z*): 719.27 (M + H^+)). HRMS: calcd. for $C_{34}H_{45}F_3N_8O_6$ [M + H]$^+$ 719.34869; found 719.34848.

HA-succ(Tmd)-Phe-Ahx-Lys(Bodipy(Tmr))-NH_2 (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/H_2O)) and the title compound was obtained as a brown solid (yield: 3.4 mg, 3.4 μmol, 31%). ^{1}H 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), 7.30-7.11 (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. ^{19}F 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/H_2O): R_t (min): 8.84 (ESI-MS (*m/z*): 1099.40 (M + H^+)). HRMS: calcd. for $C_{55}H_{64}BF_5N_{10}O_8$ [M + H]$^+$ 1099.49946; found 1099.50039.

HA-succ(Tmd)-Phe-Ahx-Lys(Bodipy(FL))-NH_2 (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/H_2O)) and the title compound was obtained as an orange solid (yield: 5.9 mg, 6.0 μmol, 55%). ^{1}H 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. ^{19}F 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/H_2O): R_t (min): 8.13 (ESI-MS (*m/z*): 993.33 ($M + H^+$)). HRMS: calcd. for $C_{48}H_{58}BF_5N_{10}O_7$ $[M + H]^+$ 993.45759; found 993.45864.

Biological evaluation

Inhibition and labeling studies

Recombinant ADAM-8 (Catalog Number 1031-AD, catalytic domain of human ADAM-8 Glu^{158}-Pro^{497}, Murine myeloma cell line, NS0 derived), ADAM-9 (Catalog Number 939-AD, catalytic domain of human ADAM-9 Ala^{206}-Asp^{697}, Murine myeloma cell line, NS0 derived), ADAM-10 (Catalog Number 936-AD, catalytic domain of human ADAM-10 Thr^{214}-Glu^{672}, Spodoptera frugiperda, Sf 21 (baculovirus) derived) and ADAM-17 (ectodomain, Catalog Number 930-ADB, catalytic domain of human ADAM-17 Arg^{215}-Asn^{671}, 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 Phe^{100}-Gln^{268}), MMP-2 (Catalog Number BML-SE237-0010, catalytic domain of human MMP-2 Tyr^{110}-Asp^{452}), MMP-3 (Catalog Number BML-SE109-0010, catalytic domain of human MMP-3 Phe^{100}-Thr^{272}), MMP-7 (Catalog Number BML-SE181-0010, catalytic domain of human MMP-7 Tyr^{95}-Lys^{267}), MMP-8 (Catalog Number BML-SE255-0010, catalytic domain of human MMP-8 Phe^{99}-Gln^{269}), MMP-10 (Catalog Number BML-SE329-0010, catalytic domain of human MMP-10 Phe^{99}-Glu^{271}), MMP-11 (Catalog Number BML-SE282-0010, catalytic domain of human MMP-11 Phe^{98}-Ser^{266}) and MMP-13 (Catalog Number BML-SE246-0010, catalytic domain of human MMP-13 Tyr^{104}-Asn^{274}) 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 described[56,57]) 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 IC_{50} 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-NH_2 (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-NH_2 (Bachem, Bubendorf, Switserland) 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 $ZnCl_2$, 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 $CaCl_2$, 2.5 μM $ZnCl_2$, 0.05 % (v/v) Brij-35). Proteolysis rates were followed by measuring fluorescence ($\lambda_{ex,em}$ = 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 IC_{50} 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)

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-Protean III electrophoresis system (Bio-Rad, Veenendal, 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 $MgCl_2$, 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.

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4 Incorporation of Fluorinated Phenylalanine Generates Highly Specific Inhibitor of Proteasome's Chymotrypsin-like Sites

P. P. Geurink, N. Liu, M. P. Spaans, S. L. Downey, A. M. C. H. van den Nieuwendijk, G. A. van der Marel, A. F. Kisselev, B. I. Florea, H. S. Overkleeft, *J. Med. Chem.* **2010**, *53*, 2319–2323.

4.1 Introduction

The majority of all cytosolic and nuclear proteins in eukaryotic cells are degraded by the ubiquitin-proteasome pathway. In this system, proteins destined for degradation are modified with a poly-ubiquitin chain, which serves as a recognition tag for the 26S proteasome where proteolysis occurs. The 26S proteasome contains one or two 19S regulatory caps together with the proteolytically active, cylindrical 20S core (see Figure 1A). Within the mammalian constitutive 20S core three pairs of proteolytically active sites are present displaying different substrate specificity. Of these, the β1 subunits (caspase-like) cleave after acidic residues, the β2 subunits (trypsin-like) cleave after basic residues and the β5 subunits (chymotrypsin-like) cleave after bulky, hydrophobic residues.[1,2] Next to the constitutive proteasome some specific mammalian cells also express the so-called immunoproteasome, in which the active subunits are replaced by their immuno counterparts β1i, β2i and β5i. The peptidyl boronic acid proteasome inhibitor Bortezomib (also known as PS-341 or Velcade, see Figure 1B)[3] is used for the treatment of multiple myeloma and targets the β5(i) and β1(i) subunits. In order to study the role of the individual active subunits, inhibitors that specifically target one active subunit are imperative. Inhibitors with moderate to good selectivity for either one of the subunits have been developed.[4] There remains, however, room for improvements, for instance in the direction of inhibitors that can distinguish between a constitutive proteasome active site and its immunoproteasome counterpart.

The search for subunit selective inhibitors is predominantly conducted by either screening of natural products,[5] rational design[6] or compound library building.[4b,7] Interestingly, in these studies the effect of fluorine functionality in proteasome inhibitors is relatively uncharted.[8] In contrast, fluorine has found wide interest in bioorganic and structural chemistry over the past decade and has become an important feature in drug design.[9] This is predominantly due to the typical characteristics of fluorine (when bound to carbon), such as its comparable size to hydrogen, its electron withdrawing ability, superhydrophobicity of fluorocarbons and self-association between fluorinated moieties. In protein structure design introduction of fluorine can mimic functional groups, alter structural properties and thereby (de)stabilize protein structures or function as recognition motifs.[10] In addition, the beneficial ^{19}F nuclear magnetic characteristics have found their use in structure analysis by (solid state) ^{19}F NMR spectroscopy or ^{19}F MRI to study, for example, protein aggregation.[11]

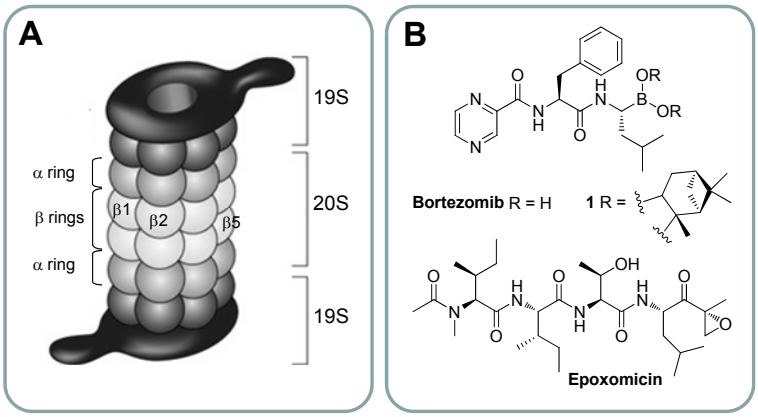


Figure 1. (A) Schematic representation of the 26S proteasome. (B) Structures of potent proteasome inhibitors.

The set of fluorinated proteasome inhibitors prepared in the context of the here presented studies are depicted in Figure 2. Compounds **2a** and **2b** containing *penta*-fluoroPhe (Phe(F_5)) and 3,5-bis(trifluoromethyl)Phe (Phe(m-CF_3)$_2$) respectively, are based on Bortezomib derivative **1** (having a comparable potency towards the β1 and β5 proteasome subunits with respect to Bortezomib)[12] and differ in that the phenylalanine in **1** is replaced by the corresponding fluorinated analogue. In addition, incorporation of fluorinated phenylalanines at different positions in tripeptide epoxyketones[2] led to compounds **3-6** in which systematically either one or both of the P2 and P3 positions were altered. Fluorinated amino acids Phe(m-CF_3)$_2$ and Phe(F_5) were used for the dual reason that these are readily available and that hydrophobic amino acids (that is the non-fluorinated analogues) are in principle accepted by all proteasome active sites. The epoxyketone electrophilic trap was selected based on the natural product epoxomicin (see Figure 1B). The epoxyketone warhead featured by epoxomicin displays a specific and selective reactivity towards the N-terminal threonine residue that makes up the

proteasome catalytic active sites.[2,5] For this reason synthetic peptide epoxyketones are now much studied leads in medicinal chemistry studies in which the proteasome plays a role.[13] The tripeptide epoxyketones **3-6** feature an azide moiety at the N-terminal end for future modifications (for instance, coupling to a fluorophore or biotin in either one- or two-step labeling experiments).[14]

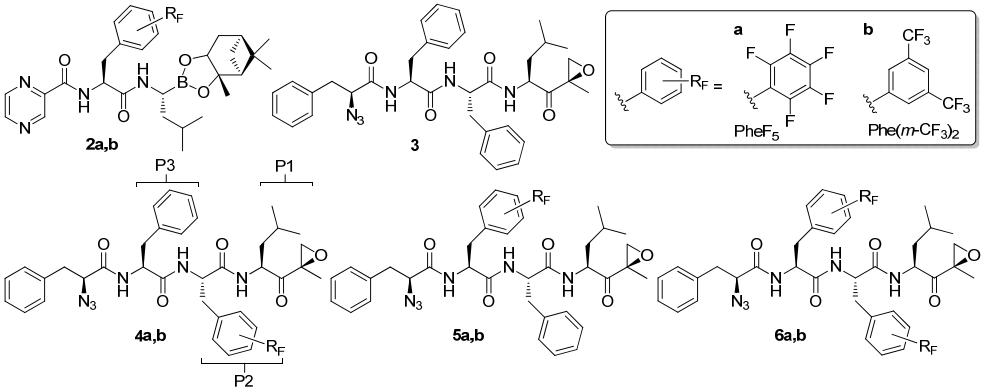
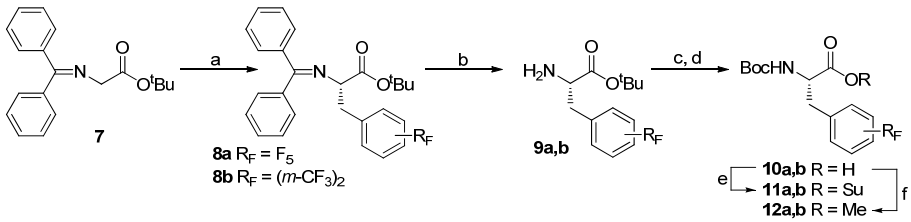


Figure 2. Synthesized fluorinated proteasome inhibitors. Indicated are the enzyme pockets (P1, P2, P3).

4.2 Results and Discussion

The C-terminally modified oligopeptides were produced via solution phase peptide chemistry following reported protocols.[7] The fluorinated amino acids used were prepared according to the procedure outlined in Scheme 1. In this procedure fully protected glycine **7** was alkylated with the appropriate fluorinated benzyl bromide and chirality was introduced by application of a chiral phase-transfer catalyst.[15] The alkylation products **8a,b** were obtained in high yields with an e.e. of >98% (as determined by chiral HPLC). Removal of the two acid labile protecting groups and introduction of a Boc protecting group on the amine led to fluorinated amino acids **10a,b**, which were now ready for use in peptide synthesis schemes.

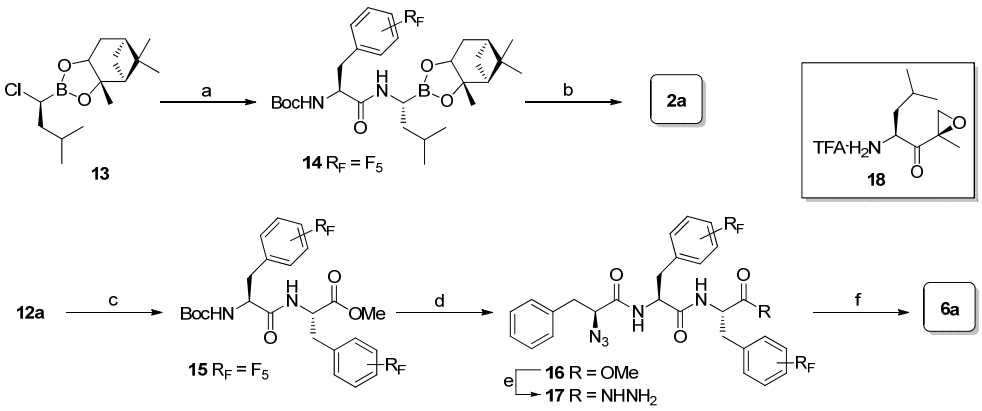
Scheme 1. Synthesis of fluorinated phenylalanine derivatives.



Reagents and conditions: (a) R_FBnBr, KOH, 2,7-bis[*O*(9)-allylhydrocinchonidinium-*N*-methyl]naphthalene dibromide, H_2O, $CHCl_3$, toluene, –20 °C, quant., e.e. >98%; (b) citric acid, H_2O, THF, 95%; (c) TFA, DCM; (d) Boc_2O, Na_2CO_3, H_2O, 1,4-dioxane, 96%; (e) HOSu, DIC, DCM; (f) $TMSCHN_2$, MeOH, toluene, quant.

Boronic ester **2a** was constructed from chloride **13** (see Scheme 2).[12] First, the chloride was substituted with LiHMDS, giving the corresponding doubly silylated amine. Acidic removal of the silyl groups resulted in the free amine, which was coupled to the *N*-hydroxysuccinimide ester **11a** (made from **10a**, Scheme 1). The resulting protected dipeptide **14** was debocylated and coupled to 2-pyrazine carboxylic acid using HCTU and DiPEA, which resulted in inhibitor **2a** (compound **2b** was created by the same strategy from **11b**). Peptide epoxyketone **6a** was constructed in three peptide couplings starting from methyl ester **12a** (obtained by esterification of **10a**) as shown in Scheme 2. After two peptide couplings (HCTU, DiPEA) leading to compound **16**, the methyl ester was converted into its hydrazide **17** and an 'azide-coupling' was performed in which amine **18**[16] was reacted to the C-terminus of the tripeptide, thereby facilitating an epimerization free product formation.[7] All other peptide epoxyketones were constructed in the same fashion.

Scheme 2. Synthesis of the fluorinated proteasome inhibitors.



Reagents and conditions: (a) i) LiHMDS, THF, –78 °C; ii) HCl; iii) **11a,b**, DiPEA; (b) i) TFA, DCM; ii) PyrOH, HCTU, DiPEA, DCM, 16% from **13** after RP-HPLC; (c) i) TFA, DCM; ii) **10a,b**, HCTU, DiPEA, DCM, 79-95%; (d) i) TFA, DCM; ii) N_3PheOH, HCTU, DiPEA, DCM, 48-93%; (e) $NH_2NH_2 \cdot H_2O$, MeOH, reflux, quant.; (f) i) *t*BuONO, HCl, DCM, DMF, –30 °C; ii) **18**, DiPEA, –30 °C → RT, 7-99%.

The inhibition potential of compounds **2a** and **2b**, in comparison with their non-fluorinated analogue boronic ester **1** (the pinanediol analogue of the clinical drug Bortezomib), was assessed in a competition assay employing cell lysates from human embryonic kidney cells (HEK-293T) and mouse lymphoma cells (EL4) in combination with the fluorescent broad spectrum proteasome probe MV151.[12] Cell lysates were incubated with each of the three compounds at 0.05, 0.1 and 1 μM final concentrations, prior to treatment with 0.5 μM final concentration of MV151. The samples were denatured, resolved by SDS-PAGE and the wet gel slabs were scanned on a fluorescence scanner. The gel images are shown in Figure 3. HEK-293T lysates treated with the fluorescent probe display three bands that correspond to the three active subunits (β1, β2 and β5) as depicted in Figure 3A lane 1. The ability of a compound to inhibit the

proteasome active sites is reflected by disappearance of the bands. As apparent from this image the fluorinated compounds **2a** and **2b** are at least as potent as their non-fluorinated counterpart **1**, since they show complete inhibition of the β1 and β5 subunits between 0.1 and 1 μM. In addition, incorporation of fluorinated Phe leaves the selectivity of β1 and β5 over β2 subunits for this type of inhibitor unchanged. The experiments in EL4 cell lysate (Figure 3B) show a similar selectivity for these three compounds towards the β1(i) and β5(i) subunits. This result is also apparent from the activity (IC_{50}) measurements of the inhibitors towards the different subunits in purified rabbit 26S proteasome as shown in Table 1.

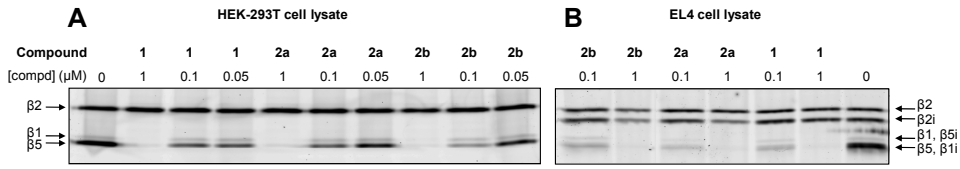


Figure 3. Characterization of the specificity of the fluorinated dipeptide boronates. Competition assay in (A) HEK-293T lysate and (B) EL4 lysate. Lysates were incubated for one hour with compounds **1**, **2a** and **2b** at the indicated final concentrations. Residual proteasome activity was labeled with 0.5 μM MV151 for one hour.

Table 1. Activity (IC_{50} in μM) of **2**-**5** against the three active constitutive 26S proteasome subunits.[a]

Compound	β1 (nLPnLD)	β2 (RLR)	β5 (LLVY)
2a	0.44	>15	0.031
2b	0.16	11	0.0030
3	>15	1.8	0.0010
4a	>15	>15	0.0020
4b	>15	>15	0.10
5a	>15	>15	0.20
5b	>15	>15	0.13

[a] Determined with the indicated subunit specific fluorogenic peptide substrates. All values are averages of two experiments.

Next, the inhibition properties of the seven epoxyketone containing compounds were determined in a similar competition assay against MV151 at 1 and 100 μM final concentrations employing HEK-293T cell lysates (Figure 4) and by measuring inhibition of purified proteasomes for the most active compounds (Table 1). When comparing non-fluorinated compound **3** to the fluorinated ones (**4-6**), it becomes apparent that none of these compounds inhibit the β1 subunit at concentrations up to 100 μM and that introducing fluorines (in either position) leads to a decrease in the inhibition of the β2 subunit and hence to an increase in β5 specificity. When comparing Table 1 with Figure 4 it appears that the results from the two independent assays deviate in places. For example, the β2-IC_{50} value for **4a** is >15 μM (Table 1), whereas the majority of the corresponding band in Figure 4 is gone at 1 μM. Intrinsic differences in both assays,

neither of which deliver k_i values, are at the basis of these small but distinct differences. The complementary assays however both show similar trends. For instance, in Figure 4 there appears to be almost no difference in inhibition potential of β5 between the non-fluorinated compound **3** and either P2 or P3 fluorinated compounds **4a**,**b** and **5a**,**b**. Although there is a big difference in IC_{50} values, all compounds are at least sub-micromolar inhibitors.

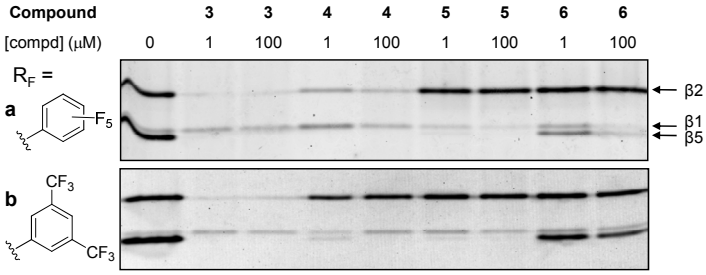


Figure 4. Characterization of the specificity of fluorinated peptide epoxyketones. Competition assay in HEK-293T lysate. Lysates were incubated for one hour with compounds **3**, **4a**,**b**, **5a**,**b** or **6a**,**b** at the indicated final concentrations. Residual proteasome activity was labeled with 0.5 μM MV151 for one hour.

Interestingly, the presence of fluorine substituents at both P2 and P3 positions (**6a**,**b**, Figure 4) has a dramatic effect on the inhibition. This effect is most pronounced for the Phe(*m*-CF_3)$_2$ analogues (**b** series). In general the Phe(F_5) compounds (**a** series) are more active against the β5 subunit than their hexafluoro-Phe analogues (**b** series). Thus, it appears that introduction of Phe(F_5) in the P2 position generates a highly specific inhibitor of the β5 site. This most potent and β5 selective inhibitor (compound **4a**) was further investigated. The inhibitory potential at much lower concentrations (1 nM to 1 μM) is shown in Figure 5 (competition assay against MV151). Inhibition of β5 is already apparent at 5 nm and between 100 and 250 nM all β5 subunits are saturated, while β1 and β2 are unaffected or even upregulated (a phenomenon which is not fully understood, but has been observed by others as well).[17] Having an IC_{50} value of 2 nM for the β5 subunit against >15 μM for β1 and β2, this compound is one of the most β5 selective inhibitors known to date. For instance, this compound compares well with NC005, a β5 selective inhibitor recently discovered.[6] Comparison of non-fluorinated compound **3** with **4a** reveals that both compounds are equally active towards the β5 subunit. Enhanced selectivity for β5 arises by the dramatic drop in activity for the β2 subunit when fluorine is introduced, as in **4a**.

For the direct labeling of β5 a new fluorescent probe was made by reacting compound **4a** with a Bodipy-alkyne[18] in a Cu(I) mediated Huisgen 1,3-dipolar cycloaddition giving green fluorescent probe **19** (Figure 6A). The potential of this probe to label the β5 subunit was explored in a competition assay against MV151 as explained before. The gel was scanned on a fluorescence scanner at two different wavelengths allowing visualization of one of the two fluorescent dyes at a time. Figure 6B shows the read-out at 520 nm visualizing the appearance of one band: labeling of the β5 subunit

by compound **19**. The labeling is already visible at a concentration of 1 nM and the subunit appears to be saturated (no more increase in the bands intensity) between 50 and 100 nM. At this point only a faint β2 band is visible. The fluorescence read-out at 560 nm in Figure 6C (displaying labeling with MV151 of remaining activities) reveals that the β5 band disappears while leaving the remaining subunit-bands intact. The IC_{50} values of compound **19** towards each active subunit were determined in the same manner as for the other compounds: β1 >10 μM, β2 > 10 μM, β5 = 0.30 μM.

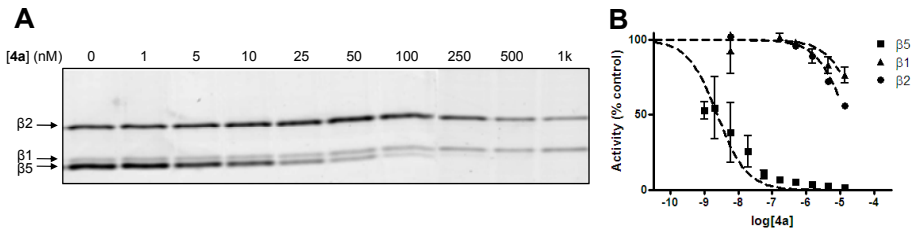


Figure 5. Characterization of the specificity of compound **4a**. (A) Competition assay in HEK-293T lysate. Lysates were incubated for one hour with compound **4a** at the indicated final concentrations. Residual proteasome activity was labeled with 0.5 μM MV151 for one hour. (B) Remaining subunit activity after inhibition with compound **4a** determined with fluorogenic peptides.

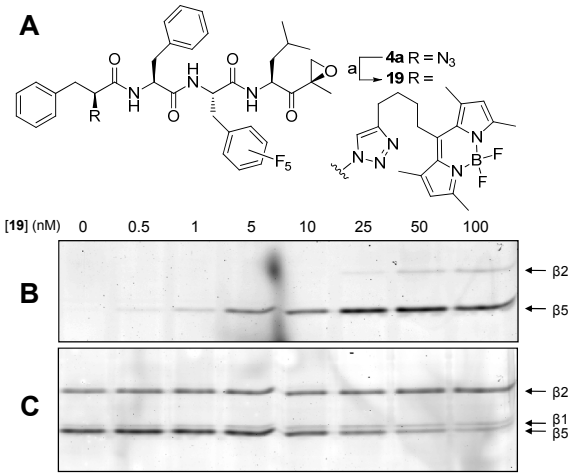


Figure 6. (A) Construction of a novel probe for the β5 site. Reagents and conditions: a) Bodipy-alkyne,[18] 10 mol% $CuSO_4$, 15 mol% sodium ascorbate, toluene/H_2O/*t*BuOH 1:1:1, 80 °C, 87%. (B,C) Competition assay in HEK-293T cell lysate. Lysates were incubated for one hour with compound **19** at the indicated final concentrations. Residual proteasome activity was labeled with 0.5 μM MV151 for one hour. Fluorescence read-out at (B) λ_{ex} 488 nm, λ_{em} 520 nm (compound **19**) and (C) λ_{ex} 532 nm, λ_{em} 560 nm (MV151).

4.3 Conclusion

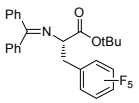
In summary, the effect of incorporation of fluorinated Phe in proteasome inhibitors was studied. It was found that substitution of non-fluorinated Phe in Bortezomib analogue **1** with fluorinated versions does not affect its selectivity for the different active subunits whereas the potency is slightly increased for the Phe(*m*-CF_3)$_2$ version. In addition, the effect of incorporation of fluorinated Phe in peptide epoxyketone proteasome inhibitors appeared to depend on the site of substitution. Fluorination of both the P2 and P3 sites decreases potency dramatically, however fluorinated Phe at the P2 position hardly affects the potency, but instead yields much more β5 selective inhibitors. Comparison of the results obtained with the boronic esters with those from the epoxyketone studies invites the tentative conclusion that β2 is most sensitive towards fluorine substituents. Compound **1** is ineffective towards β2 and introduction of fluorines into this sequence has no apparent effect. When comparing epoxyketone **3** with **4-6**, however, the major difference is that the latter, fluorinated analogues leave β2 largely intact. Further studies, for instance making use of different fluorine amino acids, are needed to substantiate this finding. Finally, compound **4a** was identified as one of the most β5 selective inhibitors known to date and was converted to a β5 selective fluorescent probe **19**, which can be used to label and visualize the β5 subunit selectively.

Experimental section

General

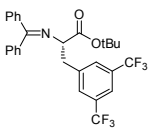
Tetrahydrofuran was distilled over $LiAlH_4$ before use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Fischer, Merck, Sigma-Aldrich) were used as received. *O*-(1*H*-6-Chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased at Iris Biotech (Marktrewitz, Germany). Traces of water were removed from reagents used in reactions that require anhydrous conditions by coevaporation with toluene. Solvents that were used in reactions were stored over 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 silicagel (Screening Devices b.v.) with a particle size of 40-63 μm and a pore size of 60 Å. The eluents toluene, ethyl acetate and petroleum ether (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 ml/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) or a Bruker DMX-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, $CDCl_3$ or CD_3OD as internal standard. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 1/1; 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* = 150-2,000) and dioctylpthalate (*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 $[\alpha]_D^{23}$ were recorded on a Propol automatic polarimeter at room temperature. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 μm C18 50 × 4.60 mm column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI (system A) or on a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI (system B). Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 × 4.6 mm). RP-HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 × 10 mm column and a GX281 fraction collector. Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 × 4.6 mm).



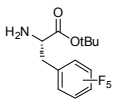
(S)-*tert*-butyl 2-(diphenylmethyleneamino)-3-(perfluorophenyl)propanoate (8a)

To a solution of *tert*-butyl 2-(diphenylmethyleneamino)acetate (**7**, 5.10 g, 17.3 mmol) in toluene/$CHCl_3$ (7/3 v/v, 80 mL) 2,3,4,5,6-pentafluorobenzylbromide (3.6 mL, 25.6 mmol, 1.5 eq.) and 2,7-bis[*O*(9)-allylhydrocinchonidinium-*N*-methyl]naphthalene dibromide (0.045 g, 0.045 mmol, 0.003 eq.) were added. The mixture was stirred at –20 °C for 30 min.. Hereafter, a precooled 50% aqueous solution of KOH (30 mL) was added and the resulting mixture was stirred at –20 °C for 3.5 days. Diethyl ether (150 mL) was added and the mixture was washed with H_2O (2×) and brine before being dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography (100% PE → 9% EtOAc/PE) to give the title compound (yield: 8.22 g, 17.3 mmol, quant.) as a yellowish oil. Using chiral HPLC the e.e. was determined >98%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.57 (d, *J* = 1.2 Hz, 2H), 7.42-7.28 (m, 6H), 6.88 (d, *J* = 6.4 Hz, 2H), 4.26 (dd, *J* = 8.8, 5.2 Hz, 1H), 3.37-3.31 (m, 1H), 3.27-3.22 (m, 1H), 1.46 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.41, 169.63, 146.53, 144.08 (t, *J* = 9.00 Hz), 140.96, 139.01, 138.51, 135.88, 135.65, 129.95, 128.18, 127.97, 127.76, 127.45, 111.76 (t, *J* = 24.0 Hz), 81.78, 64.33, 27.84, 26.10 ppm. $[\alpha]_D^{23}$ = –155.6 (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{26}H_{22}F_5NO_2$ [M + H]$^+$ 475.15707; found 475.15705.



(S)-*tert*-butyl 3-(3,5-bis(trifluoromethyl)phenyl)-2-(diphenylmethyleneamino)propanoate (8b)

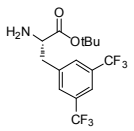
This compound was prepared by the same method described for compound **8a**, using 2,4-trifluoromethylbenzylbromide as alkylating agent. The product was obtained as a colourless oil (yield: 2.21 g, 4.23 mmol, quant.). Using chiral HPLC the e.e. was determined >98%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.70 (s, 1H), 7.58 (s, 2H), 7.54 (d, *J* = 7.22 Hz, 2H), 7.40-7.27 (m, 6H), 6.68 (d, *J* = 6.95 Hz, 2H), 4.16 (dd, *J* = 8.32, 4.87 Hz, 1H), 3.38-3.27 (m, 2H), 1.45 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.87, 140.94, 130.50, 130.20, 131.2 (q, *J* = 33.14 Hz), 128.67, 128.34, 127.99, 127.32, 123.31 (q, *J* = 272.51 Hz), 120.20, 81.82, 66.66, 39.11, 27.99 ppm. $[\alpha]_D^{23}$ = –165.7° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{28}H_{25}F_6NO_2$ [M + H]$^+$ 522.18622; found 522.18587.



(S)-*tert*-butyl 2-amino-3-(perfluorophenyl)propanoate (9a)

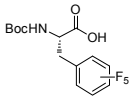
Aqueous citric acid (15% w/w, 110 mL) was added to a solution of compound **8a** (8.22 g, 17.3 mmol) in THF (90 mL) and the mixture was stirred overnight at RT. Aqueous K_2CO_3 (sat.) and EtOAc were added and the layers were separated before the aqueous layer was extracted twice with EtOAc. The combined organic layers were dried over $MgSO_4$ and concentrated. The product (yield: 4.92 g, 15.8 mmol, 91%) was obtained as a yellowish oil after column chromatography (100% PE → 50% EtOAc/PE). ^{1}H NMR (400 MHz, $CDCl_3$): δ =

3.58 (dd, *J* = 8.4, 6.4 Hz, 1H), 3.10 (dd, *J* = 13.6, 6.0 Hz, 1H), 2.90 (dd, *J* = 14.0, 8.8 Hz, 1H), 1.63 (bs, 1H), 1.45 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.39, 146.57-146.38 (m), 144.13-143.94 (m), 141.21-141.02 (m), 138.70-138.37 (m), 135.99 (t, *J* = 17.0 Hz), 111.70 (t, *J* = 20.0 Hz), 81.57, 54.38, 27.75, 27.60 ppm. $[\alpha]_D^{23}$ = +12.1 (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{13}H_{14}F_5NO_4$ [M + H]$^+$ 312.10175; found 312.10182.



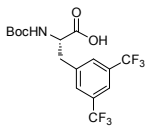
(S)-*tert*-butyl 2-amino-3-(3,5-bis(trifluoromethyl)phenyl)propanoate (9b)

This compound was prepared by the same method described for compound **9a**. The product was obtained as a colourless oil (yield: 1.37 g, 3.83 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (s, 1H), 7.73 (s, 2H), 3.66 (t, *J* = 6.61, 6.61 Hz, 1H), 3.07 (ddd, *J* = 20.92, 13.78, 6.62 Hz, 2H), 1.57 (s, 1H), 1.42 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.62, 140.34, 131.43 (q, *J* = 33.16 Hz), 129.58, 123.28 (q, *J* = 272.53 Hz), 120.55, 81.66, 55.75, 40.51, 27.72 ppm. $[\alpha]_D^{23}$ = +12.6° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{15}H_{17}F_6NO_2$ [M + H]$^+$ 358.12362; found 358.12364.



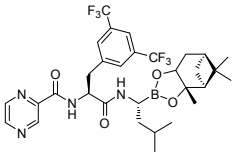
(S)-2-(*tert*-butoxycarbonylamino)-3-(perfluorophenyl)propanoic acid (10a)

Compound **9a** (1.25 g, 4.0 mmol) was treated with TFA (15 mL) for 30 min., after which the mixture was coevaporated three times with toluene. The resulting product was dissolved in water (40 mL) containing Na_2CO_3 (1.61 g, 15.2 mmol) and cooled to 0 °C. A solution of di-*tert*-butyldicarbonate (0.96 g, 4.43 mmol) in 1,4-dioxane (25 mL) was slowly added and the resulting mixture was stirred for 12 h slowly warming up to RT. Next, water was added followed by extracting twice with EtOAc. The aqueous layer was acidified with 4M aq. HCl to pH 2 and extracted with EtOAc (3×). The latter organic layers were combined, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was purified by column chromatography (30% EtOAc/PE → 100% EtOAc) and obtained as a white solid (yield: 1.35 g, 3.80 mmol, 95%). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 10.57 (bs, 1H), 5.14 (d, *J* = 8.4 Hz, 1H), 4.63-4.43 (m, 1H), 3.40-3.11 (m, 2H), 1.37 (s, 9H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 173.88, 156.68, 148.35, 143.16, 139.83, 134.88, 110.11, 82.43, 53.38, 27.94, 26.61 ppm. $[\alpha]_D^{23}$ = +22.3° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{14}H_{14}F_5NO_4$ [M + H]$^+$ 356.09158; found 356.09165.



(S)-3-(3,5-bis(trifluoromethyl)phenyl)-2-(*tert*-butoxycarbonylamino)propanoic acid (10b)

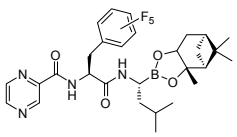
This compound was prepared by the same method described for compound **10a**. The product was obtained as a colourless solid (yield: 1.47 g, 3.67 mmol, 96%). Spectroscopic data are given for a mixture of rotamers. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.27 (s, 1H), 7.79 (s, 1H), 7.77 (s, 1H), 7.68 (s, 2H), 7.65 (s, 2H), 6.99 (d, *J* = 6.83 Hz, 1H), 5.13 (d, *J* = 7.38 Hz, 1H), 4.66 (d, *J* = 6.05 Hz, 1H), 4.43 (d, *J* = 4.43 Hz, 1H), 3.45-3.30 (m, 2H), 3.21-3.02 (m, 2H), 1.41 (s, 9H), 1.29 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.16, 174.64, 156.69, 155.18, 139.21, 138.70, 131.84-131.51 (m), 129.69, 123.23 (q, *J* = 272.57 Hz), 121.05, 121.02, 82.54, 80.85, 55.53, 53.91, 38.97, 37.55, 28.08, 27.86 ppm. HRMS: calcd. for $C_{16}H_{17}F_6NO_4$ [M + H]$^+$ 402.11345; found 402.1134.



Pyrazine-2-carboxylic acid (1-[3-methyl-1-(2,9,9-trimethyl-3,5-dioxa-4-boratri-cyclo[6.1.1.0^{2,6}]dec-4-yl)-butylcarbamoyl]-2-(3,5-bis(trifluoromethyl)phenyl) ethyl)-amide (2b)

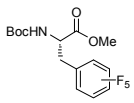
N,N-Diisopropylcarbodiimide (1.3 eq., 0.41 mmol, 64 μL) was added to a solution of BocPhe(*m*-$(CF_3)_2$)-OH (**10b**, 1 eq., 127 mg, 0.32 mmol) and *N*-hydroxysuccinimide (1.12 eq., 0.35 mmol, 41 mg) in DCM (5 mL) and the reaction mixture was stirred for 12 h yielding the crude BocPhe(*m*-$(CF_3)_2$)-OSu (**11b**) solution. Lithium hexamethyldisilazide (1.3 eq., 0.91 mmol, 0.75 mL 1M in THF) was added to a solution of (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane (**13**,

200 mg, 0.70 mmol) in THF (10 mL) at –78 °C. The reaction mixture was allowed to warm to RT and was stirred for 12 h before it was cooled to –78 °C. HCl (4.6 mmol, 1.15 mL 4M in 1,4-dioxane) was added and the reaction mixture was allowed to warm to RT, before being cooled to –78 °C. To the stirred solution DiPEA (8.0 mmol, 1.32 mL) and the crude BocPhe(*m*-$(CF_3)_2$)-OSu solution (0.32 mmol) were added and the reaction mixture was allowed to warm to RT. The reaction mixture was stirred for an additional 2 h before being filtered over Celite and the filtrate was concentrated under reduced pressure. Purification of the residue by column chromatography (10% → 20% EtOAc/PE) resulted in the dipeptide (yield: 40 mg, 61 μmol, 19%). This compound was dissolved in a 1/1 (v/v) mixture of DCM/TFA (2 mL) and stirred for 30 min. before being coevaporated three times with toluene. The crude TFA salt was dissolved again in DCM (2 mL) and HCTU (1.5 eq., 92 μmol, 38 mg), 2-pyrazine carboxylic acid (1.5 eq., 92 μmol, 115 mg) and DiPEA (4 eq., 0.25 mmol, 41 μL) were added and the mixture was stirred for 3 h before being concentrated under reduced pressure and purified by HPLC (linear gradient 80% → 100% ACN in H_2O, 0.1% TFA, 15 min). The product was obtained as a colourless solid (yield: 7.4 mg, 11 μmol, 18%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.34 (s, 1H), 8.78 (s, 1H), 8.55 (s, 1H), 8.38 (d, *J* = 8.47 Hz, 1H), 7.75 (s, 3H), 6.04 (d, *J* = 4.24 Hz, 1H), 4.85 (dd, *J* = 14.30, 6.92 Hz, 1H), 4.28 (d, *J* = 8.48 Hz, 1H), 3.41-3.20 (m, 3H), 2.34-2.28 (m, 1H), 2.23-2.14 (m, 1H), 2.01 (t, *J* = 5.19, 5.19 Hz, 1H), 1.94-1.88 (m, 1H), 1.81 (d, *J* = 14.60 Hz, 1H), 1.57-1.46 (m, 1H), 1.45-1.41 (m, 2H), 1.40 (s, 3H), 1.28 (s, 3H), 1.15 (d, *J* = 10.88 Hz, 1H), 0.89-0.81 (m, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.62, 163.01, 147.66, 144.19, 142.85, 138.99, 131.77 (q, *J* = 33.23 Hz), 129.83, 129.80, 123.19 (q, *J* = 272.80 Hz), 121.07, 86.23, 77.91, 53.60, 51.23, 39.87, 39.50, 37.97, 35.37, 28.38, 27.04, 26.26, 25.47, 24.00, 22.80, 22.05 ppm. $[\alpha]_D^{23}$ = –16.2° (*c* = 1 in $CHCl_3$). LC-MS: system B, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.33. HRMS: calcd. for $C_{31}H_{37}BF_6N_4O_4$ [M + H]$^+$ 655.28848; found 655.28839.



Pyrazine-2-carboxylic acid (1-[3-methyl-1-(2,9,9-trimethyl-3,5-dioxa-4-boratri-cyclo[6.1.1.0^{2,6}]dec-4-yl)-butylcarbamoyl]-2-(2,3,4,5,6-pentafluorophenyl)-ethyl)-amide (2a)

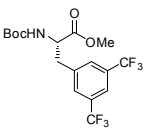
Prepared according to the procedure as described for compound **2b** using BocPhe(F_5)-OH. The product was obtained as a colourless solid (yield: 12 mg, 19.7 μmol, 14%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.31 (s, 1H), 8.77 (d, *J* = 2.20 Hz, 1H), 8.56 (d, *J* = 1.29 Hz, 1H), 8.40 (d, *J* = 8.63 Hz, 1H), 6.07 (d, *J* = 4.53 Hz, 1H), 4.87 (dd, *J* = 14.43, 8.57 Hz, 1H), 4.30 (d, *J* = 8.64 Hz, 1H), 3.38-3.31 (m, 2H), 3.23 (dd, *J* = 13.98, 8.84 Hz, 1H), 2.36-2.31 (m, 1H), 2.23-2.17 (m, 1H), 2.02 (t, *J* = 5.41, 5.41 Hz, 1H), 1.94-1.90 (m, 1H), 1.82 (d, *J* = 14.61 Hz, 1H), 1.65-1.54 (m, 3H), 1.40 (s, 3H), 1.29 (s, 3H), 1.16 (d, *J* = 10.87 Hz, 1H), 0.92-0.82 (m, 9H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 169.13, 163.14, 147.68, 144.24, 143.57, 142.85, 86.32, 78.08, 51.98, 51.17, 40.03, 39.43, 38.18, 35.35, 29.69, 28.43, 27.03, 26.24, 25.44, 24.01, 22.89, 22.09 ppm. $[\alpha]_D^{23}$ = –18.9° (*c* = 1 in $CHCl_3$). LC-MS: system B, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 12.15. HRMS: calcd. for $C_{29}H_{34}BF_5N_4O_4$ [M + H]$^+$ 609.26660; found 609.26664.



(S)-methyl 2-(*tert*-butoxycarbonylamino)-3-(perfluorophenyl)propanoate (12a)

TMS-diazomethane (3.0 mL as a 2M solution in hexanes, 6.0 mmol, 4 eq.) was added dropwise to a solution of BocPhe(F_5)-OH (**10a**, 0.52 g, 1.5 mmol) in MeOH/toluene (1/1 (v/v), 8 mL) and the mixture was stirred until TLC analysis revealed a completed reaction after 2 h. The mixture was concentrated to obtain the product (yield: 0.54 g, 1.5 mmol, quant.) as a white solid without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.27 (d, *J* = 7.6 Hz, 1H), 4.60 (d, *J* = 4.4 Hz, 1H), 3.79 (s, 3H), 3.34 (dd, *J* = 13.2, 3.6 Hz, 1H), 3.08 (dd, *J* = 13.6, 6.8 Hz, 1H), 1.40 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.19, 154.79, 146.75, 144.30, 141.70-141.3 (m), 139.10-138.21 (m), 136.10-1.35.91 (m), 110.05 (t, *J* = 18.0 Hz), 80.07,

52.54, 27.91, 25.94 ppm. $[\alpha]_D^{23} = +37.8°$ ($c = 1$ in $CHCl_3$). HRMS: calcd. for $C_{15}H_{16}F_5NO_4$ $[M + H]^+$ 370.10723; found 370.10718.

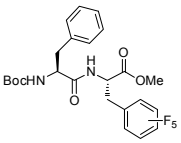


(S)-methyl 3-(3,5-bis(trifluoromethyl)phenyl)-2-(*tert*-butoxycarbonylamino)propanoate (12b)

This compound was prepared by the same method described for compound **12a**. The product was obtained as a colourless solid (yield: 623 mg, 1.5 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.77 (s, 1H), 7.59 (s, 2H), 5.14 (d, *J* = 7.09 Hz, 1H), 4.63 (d, *J* = 6.25 Hz, 1H), 3.75 (s, 3H), 3.26 (ddd, *J* = 72.60, 13.71, 5.68 Hz, 2H), 1.42 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.42, 154.82, 138.85, 131.63 (q, *J* = 32.67 Hz), 129.59, 123.22 (q, *J* = 272.66 Hz), 120.98, 80.33, 54.08, 52.46, 37.92, 28.11 ppm. $[\alpha]_D^{23} = +45.2°$ ($c = 1$ in $CHCl_3$). HRMS: calcd. for $C_{17}H_{19}F_6NO_4$ $[M + H]^+$ 416.12910; found 416.12899.

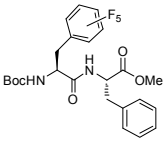
General procedure A: peptide coupling

The Boc-protected amine (1 eq.) was treated with TFA (5 mL/mmol) for 30 min. followed by coevaporation of the mixture three times with toluene. The resulting TFA salt of the deprotected amine was dissolved in DCM (5 mL/mmol) followed by addition of the carboxylic acid (1 eq.) and HCTU (1.2 eq.). The pH was set to pH 9 by addition of DiPEA (~3.5 eq.) and the mixture was stirred until TLC analysis revealed complete consumption of either of the starting compounds (usually after 1 h). Next, the DCM layer was washed with aq. 1M HCl (2×), saturated aq. Na_2CO_3 (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. Peptides were further purified by column chromatography using an EtOAc/PE or a MeOH/EtOAc eluent system.



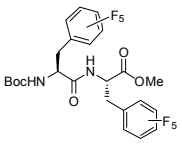
(S)-methyl 2-((S)-2-(*tert*-butoxycarbonylamino)-3-phenylpropanamido)-3-(perfluorophenyl)propanoate, BocPhePhe(F_5)OMe

Prepared via general procedure A using Boc-protected amine **12a** and BocPheOH. The product was obtained as a colourless solid (yield: 759 mg, 1.47 mmol, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.30-7.19 (m, 3H), 7.17 (d, *J* = 6.68 Hz, 2H), 6.62 (d, *J* = 7.58 Hz, 1H), 4.95 (d, *J* = 7.80 Hz, 1H), 4.79 (dd, *J* = 13.52, 7.00 Hz, 1H), 4.36-4.29 (m, 1H), 3.73 (s, 3H), 3.27 (dd, *J* = 14.06, 5.85 Hz, 1H), 3.06 (dd, *J* = 15.68, 6.89 Hz, 2H), 2.98-2.89 (m, 1H), 1.39 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.33, 170.45, 155.28, 145.44 (dd, *J* = 237, 11.0 Hz), 140.17 (dt, *J* = 251, 11.0 Hz), 137,30 (dt, *J* = 251, 11.0 Hz), 136.37, 129.08, 128.56, 126.87, 109.60 (t, *J* = 19.0 Hz), 80.29, 55.77, 52.73, 51.21, 38.06, 28.05, 25.55 ppm. $[\alpha]_D^{23} = +2.45°$ ($c = 1$ in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.68. HRMS: calcd. for $C_{24}H_{25}F_5N_2O_5$ $[M + H]^+$ 517.17564; found 517.17563.



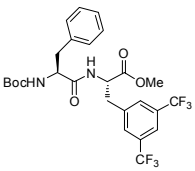
(S)-methyl 2-((S)-2-(*tert*-butoxycarbonylamino)-3-(perfluorophenyl)propanamido)-3-phenylpropanoate, BocPhe(F_5)PheOMe

Prepared via general procedure A using Boc-protected amine BocPheOMe and carboxylic acid **10a**. The product was obtained as a colourless solid (yield: 497 mg, 0.96 mmol, 91%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.30-7.20 (m, 3H), 7.10 (d, *J* = 6.62 Hz, 2H), 6.72 (d, *J* = 7.23 Hz, 1H), 5.24 (d, *J* = 8.58 Hz, 1H), 4.86 (td, *J* = 7.88, 6.08, 6.08 Hz, 1H), 4.47-4.39 (m, 1H), 3.71 (s, 3H), 3.19 (dd, *J* = 14.17, 5.27 Hz, 1H), 3.10 (dd, *J* = 7.87, 6.29 Hz, 2H), 2.95 (dd, *J* = 14.02, 8.91 Hz, 1H), 1.37 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.54, 169.72, 155.09, 145.44 (dd, *J* = 237, 11.0 Hz), 140.17 (dt, *J* = 251, 11.0 Hz), 137,30 (dt, *J* = 251, 11.0 Hz), 135.50, 129.15, 128.52, 127.13, 110.49 (t, *J* = 19.0 Hz), 80.27, 53.16, 52.30, 37.87, 27.97, 25.87 ppm. $[\alpha]_D^{23} = +28.5°$ ($c = 1$ in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.67. HRMS: calcd. for $C_{24}H_{25}F_5N_2O_5$ $[M + H]^+$ 517.17564; found 517.17542.



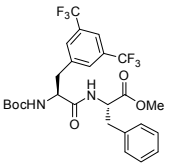
(S)-methyl 2-((S)-2-(*tert*-butoxycarbonylamino)-3-(perfluorophenyl)propanamido)-3-(perfluorophenyl)propanoate, BocPhe(F_5)Phe(F_5)OMe (15)

Prepared via general procedure A using Boc-protected amine **12a** and carboxylic acid **10a**. The product (yield: 1.18 g, 1.9 mmol, 79%) was obtained as a white solid after purification by column chromatography (1% - 13% EtOAc/toluene). ^{1}H NMR (400 MHz, MeOD): δ = 4.77 (dd, *J* = 9.6, 5.2 Hz, 1H), 4.37-4.25 (m, 1H), 3.79 (s, 3H), 3.43 (dd, *J* = 14.4, 5.2 Hz, 1H), 3.23 (dd, *J* = 14.0, 9.6 Hz, 1H), 3.15-3.08 (m, 1H), 2.98-2.91 (m, 1H), 1.29 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.63, 170.09, 155.15, 146.70 (m), 144.25 (m), 141.61 (m), 139.25-138.66 (m), 136.15 (m), 110.32, 80.68, 53.40, 52.98, 51.58, 27.90, 25.54, 25.07 ppm. $[\alpha]_D^{23}$ = +6.5° (*c* = 1 in $CHCl_3$). LC-MS analysis: R_t 10.43 min (linear gradient 10-90% ACN in H_2O, 0.1% TFA, 15 min). HRMS: calcd. for $C_{24}H_{20}F_{10}N_2O_5$ $[M+H]^+$ 607.12853; found 607.12834.



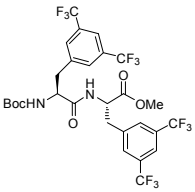
(S)-methyl 3-(3,5-bis(trifluoromethyl)phenyl)-2-((S)-2-(*tert*-butoxycarbonylamino)-3-phenylpropanamido)propanoate, BocPhePhe(F_6)OMe

Prepared via general procedure A using Boc-protected amine **12b** and BocPheOH. The product was obtained as a colourless solid (yield: 823 mg, 1.46 mmol, 96%). ^{1}H NMR (400 MHz, ($CDCl_3$): δ = 7.76 (s, 1H), 7.53 (s, 2H), 7.31-7.21 (m, 3H), 7.18 (d, *J* = 6.87 Hz, 2H), 6.64 (d, *J* = 7.24 Hz, 1H), 4.97 (d, *J* = 7.52 Hz, 1H), 4.82 (dd, *J* = 12.66, 5.98 Hz, 1H), 4.37-4.30 (m, 1H), 3.67 (s, 3H), 3.21 (dq, *J* = 13.89, 13.88, 13.88, 5.88 Hz, 2H), 3.10-2.97 (m, 2H), 1.38 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.30, 170.61, 155.37, 138.59, 136.25, 131.64 (q, *J* = 33.0 Hz), 129.54, 129.11, 128.66, 127.02, 123.14 (q, *J* = 271 Hz), 121.03, 80.35, 55.97, 52.95, 52.40, 37.63, 28.03 ppm. $[\alpha]_D^{23}$ = +26.1° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 10.41. HRMS: calcd. for $C_{26}H_{28}F_6N_2O_5$ $[M+H]^+$ 563.19752; found 563.19753.



(S)-methyl 2-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-2-(*tert*-butoxycarbonylamino)propanamido)-3-phenylpropanoate, BocPhe(F_6)PheOMe

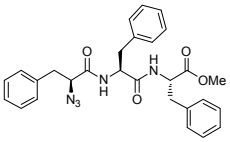
Prepared via general procedure A using Boc-protected amine BocPheOMe and carboxylic acid **10b**. The product was obtained as a colourless solid (yield: 551 mg, 0.98 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (s, 1H), 7.64 (s, 2H), 7.28-7.17 (m, 3H), 7.05 (d, *J* = 6.55 Hz, 2H), 6.70 (d, *J* = 7.15 Hz, 1H), 5.33 (d, *J* = 7.66 Hz, 1H), 4.83 (dd, *J* = 13.80, 6.14 Hz, 1H), 4.51-4.43 (m, 1H), 3.69 (s, 3H), 3.21 (dd, *J* = 13.92, 5.89 Hz, 1H), 3.13-2.99 (m, 3H), 1.38 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.53, 170.17, 155.18, 139.25, 135.47, 131.48 (q, *J* = 33 Hz), 129.69, 129.13, 128.56, 127.17, 123.24 (q, *J* = 271 Hz), 120.76, 80.34, 54.82, 53.19, 52.55, 37.77, 28.05 ppm. $[\alpha]_D^{23}$ = +28.2° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.88. HRMS: calcd. for $C_{26}H_{28}F_6N_2O_5$ $[M+H]^+$ 563.19752; found 563.19745.



(S)-methyl 3-(3,5-bis(trifluoromethyl)phenyl)-2-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-2-(*tert*-butoxycarbonylamino)propanamido)propanoate, BocPhe(F_6)Phe(F_6)OMe

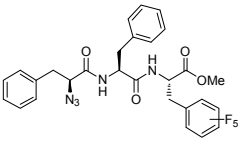
Prepared via general procedure A using Boc-protected amine **12b** and carboxylic acid **10b**. The product was obtained as a colourless solid (yield: 971 mg, 1.39 mmol, 93%). ^{1}H NMR (400 MHz,$CDCl_3$): δ = 7.77 (s, 1H), 7.76 (s, 1H), 7.65 (s, 2H), 7.54 (s, 2H), 6.67 (d, *J* = 7.28 Hz, 1H), 4.98 (d, *J* = 7.51 Hz, 1H), 4.87-4.78 (m, 1H), 4.37 (dd, *J* = 14.62, 7.09 Hz, 1H), 3.71 (s, 3H), 3.32-3.16 (m, 4H), 1.38 (s, 9H) ppm. ^{13}C NMR

(100 MHz, $CDCl_3$): δ = 170.610, 170.322, 138.987, 138.367, 131.79 (q, *J* = 33.31 Hz), 129.513, 123.15 (q, *J* = 267.69 Hz), 121.220, 80.940, 53.013, 52.634, 37.592, 27.985 ppm. LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 10.94. HRMS: calcd. for $C_{28}H_{26}F_{12}N_2O_5$ [M +H]$^+$ 699.17229; found 699.17228.



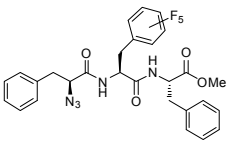
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-phenylpropanamido)-3-phenylpropanoate, N_3PhePhePheOMe

Prepared via general procedure A using Boc-protected amine BocPhePheOMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 217 mg, 0.43 mmol, 87%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.32-7.12 (m, 9H), 7.07-6.94 (m, 6H), 4.95 (dd, *J* = 14.23, 6.97 Hz, 1H), 4.81 (dd, *J* = 12.13, 5.88 Hz, 1H), 3.87 (dd, *J* = 4.76, 3.55 Hz, 1H), 3.64 (s, 3H), 3.17 (dd, *J* = 13.84, 2.01 Hz, 1H), 3.08-2.96 (m, 2H), 2.92 (d, *J* = 6.47 Hz, 2H), 2.81 (dd, *J* = 13.87, 8.70 Hz, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.04, 169.96, 168.22, 135.96, 135.89, 135.52, 129.32, 129.28, 129.06, 128.40, 128.26, 127.03, 126.90, 126.76, 64.80, 53.45, 53.16, 52.02, 38.43, 38.20, 37.77 ppm. $[\alpha]_D^{23}$ = +55.2° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.96. HRMS: calcd. for $C_{28}H_{29}N_5O_4$ [M + H]$^+$ 500.22923; found 500.22909.



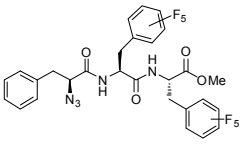
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-phenylpropanamido)-3-(perfluorophenyl)propanoate, N_3PhePhePhe(F_5)OMe

Prepared via general procedure A using Boc-protected amine BocPhePhe(F_5)OMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 205 mg, 0.35 mmol, 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.34-7.18 (m, 8H), 7.05 (dd, *J* = 7.64, 1.44 Hz, 2H), 6.92 (d, *J* = 8.00 Hz, 1H), 6.87 (d, *J* = 8.34 Hz, 1H), 4.77 (ddd, *J* = 19.50, 14.54, 7.08 Hz, 2H), 4.19 (dd, *J* = 8.47, 3.99 Hz, 1H), 3.72 (s, 3H), 3.28-3.19 (m, 2H), 3.03 (dd, *J* = 14.05, 7.07 Hz, 1H), 2.92-2.89 (m, 2H), 2.85 (dd, *J* = 14.12, 8.49 Hz, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.23, 170.17, 168.52, 145.44 (dd, *J* = 237, 11.0 Hz), 140.17 (dt, *J* = 251, 11.0 Hz), 137,30 (dt, *J* = 251, 11.0 Hz), 135.84, 135.75, 129.34, 129.12, 128.52, 128.41, 127.15, 126.89, 109.51 (dt, *J* = 18.60, 3.47 Hz), 65.07, 53.81, 52.66, 51.15, 38.33, 37.75, 25.37 ppm. $[\alpha]_D^{23}$ = +13.7° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.58. HRMS: calcd. for $C_{28}H_{24}F_5N_5O_4$ [M + H]$^+$ 590.18212; found 590.18204.



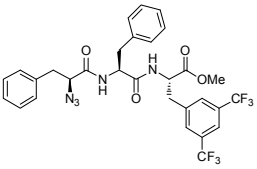
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-(perfluorophenyl)propanamido)-3-phenylpropanoate, N_3PhePhe(F_5)PheOMe

Prepared via general procedure A using Boc-protected amine BocPhe(F_5)PheOMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 117 mg, 0.20 mmol, 62%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.45 (d, *J* = 8.00 Hz, 1H), 7.30-7.20 (m, 8H), 7.11 (d, *J* = 8.80 Hz, 1H), 7.03 (d, *J* = 6.85 Hz, 2H), 5.09 (dd, *J* = 15.60, 7.20 Hz, 1H), 4.93 (dd, *J* = 12.66, 5.98 Hz, 1H), 3.72 (s, 3H), 3.55 (dd, *J* = 8.47, 3.99 Hz, 1H), 3.18 (dd, *J* = 14.00, 3.60 Hz, 1H), 3.13-3.08 (m, 3H), 2.93 (dd, *J* = 13.20, 6.00 Hz, 1H), 2.78 (dd, *J* = 13.80, 9.00 Hz, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.14, 169.18, 168.72, 145.44 (dd, *J* = 237, 11.0 Hz), 140.17 (dt, *J* = 251, 11.0 Hz), 137,30 (dt, *J* = 251, 11.0 Hz), 135.96, 135.62, 129.41, 129.26, 128.43, 128.39, 127.21, 127.10, 109.65 (dt, *J* = 18.57, 3.61 Hz), 64.74, 53.36, 52.27, 51.24, 38.31, 37.95, 25.86 ppm. $[\alpha]_D^{23}$ = +18.0° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.84. HRMS: calcd. for $C_{28}H_{24}F_5N_5O_4$ [M + H]$^+$ 590.18212; found 590.18212.



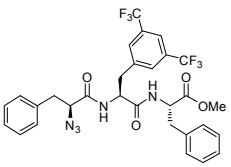
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-(perfluorophenyl)propanamido)-3-(perfluorophenyl)propanoate, N_3PhePhe(F_5)Phe(F_5)OMe (16)

Prepared via general procedure A using Boc-protected amine **15** and azidophenylalanine. The product was obtained as a colourless solid (yield: 0.22 g, 0.32 mmol, 61%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.31-7.21 (m, 5H), 4.90-4.85 (m, 1H), 4.83-4.77 (m, 1H), 4.21 (dd, *J* = 8.0, 3.2 Hz, 1H), 3.80 (s, 3H), 3.35-3.23 (m, 2H), 3.12-3.08 (m, 2H), 2.99-2.90 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.47, 169.22, 169.02, 146.69 (m), 144.17 (m), 141.69 (m), 138.60 (m), 136.08, 135.64, 129.39, 128.69, 126.01, 109.57 (m), 65.05, 52.93, 51.70, 51.38, 38.21, 25.58, 24.81 ppm. $[\alpha]_D^{23}$ = +12.6° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 10.59. HRMS: calcd. for $C_{24}H_{19}F_{10}N_5O_4$ [M + H]$^+$ 680.13501; found 680.13494.



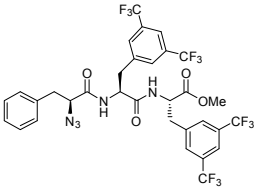
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-phenylpropanamido)-3-(3,5-bis(trifluoromethyl)phenyl)propanoate, N_3PhePhePhe(F_6)OMe

Prepared via general procedure A using Boc-protected amine BocPhePhe(F_6)OMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 149 mg, 0.23 mmol, 48%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74 (s, 1H), 7.52 (s, 2H), 7.34-7.19 (m, 8H), 7.05 (d, *J* = 6.25 Hz, 2H), 6.84 (d, *J* = 7.90 Hz, 1H), 6.67 (d, *J* = 7.37 Hz, 1H), 4.76 (dd, *J* = 13.42, 6.17 Hz, 1H), 4.67 (dd, *J* = 14.62, 7.20 Hz, 1H), 4.15 (dd, *J* = 8.15, 4.04 Hz, 1H), 3.66 (s, 3H), 3.22 (dd, *J* = 14.19, 3.92 Hz, 1H), 3.16 (dd, *J* = 14.08, 6.13 Hz, 2H), 3.00-2.84 (m, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.49, 170.15, 168.57, 138.52, 135.77, 135.67, 131.61 (q, *J* = 33.29, Hz), 129.44, 129.14, 128.60, 127.26, 127.13, 123.12 (q, *J* = 272.76 Hz), 121.04, 65.04, 54.24, 53.10, 52.42, 38.33, 38.04, 37.63 ppm. $[\alpha]_D^{23}$ = +37.5° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 10.14. HRMS: calcd. for $C_{30}H_{27}F_6N_5O_4$ [M + H]$^+$ 636.20400; found 636.20406.



(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-(3,5-bis(trifluoromethyl)phenyl)propanamido)-3-phenylpropanoate, N_3PhePhe(F_6)PheOMe

Prepared via general procedure A using Boc-protected amine BocPhe(F_6)PheOMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 193 mg, 0.30 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (s, 1H), 7.55 (s, 2H), 7.33-7.16 (m, 9H), 7.13 (d, *J* = 8.65 Hz, 1H), 6.99 (dd, *J* = 6.92, 2.33 Hz, 2H), 5.10 (dd, *J* = 15.02, 6.46 Hz, 1H), 4.85 (td, *J* = 7.62, 5.62, 5.62 Hz, 1H), 3.77 (dd, *J* = 8.95, 3.78 Hz, 1H), 3.72 (s, 3H), 3.24 (dd, *J* = 14.00, 3.68 Hz, 1H), 3.06 (dd, *J* = 14.03, 5.99 Hz, 4H), 2.80 (dd, *J* = 14.00, 8.99 Hz, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.14, 169.20, 168.77, 138.47, 135.89, 135.48, 131.47 (q, *J* = 33.21 Hz), 129.77 (d, *J* = 2.42 Hz), 129.37, 129.13, 128.58, 128.45, 127.25, 127.21, 123.18 (q, *J* = 272.74 Hz), 121.01, 64.89, 53.15, 52.84, 52.23, 38.53, 38.31, 37.78 ppm. $[\alpha]_D^{23}$ = +41.8° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 10.20. HRMS: calcd. for $C_{30}H_{27}F_6N_5O_4$ [M + H]$^+$ 636.20400; found 636.20406.



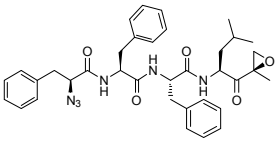
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-(3,5-bis(trifluoromethyl)phenyl)propanamido)-3-(3,5-bis(trifluoromethyl)phenyl)propanoate, N_3PhePhe(F_6)Phe(F_6)OMe

Prepared via general procedure A using Boc-protected amine BocPhe(F_6)Phe(F_6)OMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 0.34 g, 0.44 mmol, 88%). ^{1}H NMR

(400 MHz, $CDCl_3$): δ = 7.76 (s, 2H), 7.51 (s, 2H), 7.49 (s, 2H), 7.34-7.23 (m, 5H), 4.81-4.75 (m, 1H), 4.70 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.19 (dd, *J* = 8.0, 4.0 Hz, 1H), 3.70 (s, 3H), 3.30-3.24 (m, 2H), 3.20 (dd, *J* = 16.8, 5.9 Hz, 2H), 3.00-2.93 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.48, 169.18, 168.72, 138.26, 135.50, 131.81 (q, *J* = 11.0 Hz), 129.62, 129.51, 129.25, 128.73, 123.12 (q, *J* = 127 Hz), 121.27, 64.97, 53.56, 53.03, 52.66, 38.41, 37.59, 37.48 ppm. $[\alpha]_D^{23}$ = +2.2° (*c* = 1 in MeOH). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 11.66. HRMS: calcd. for $C_{32}H_{25}F_{12}N_5O_4$ $[M + H]^+$ 772.17877; found 772.17899.

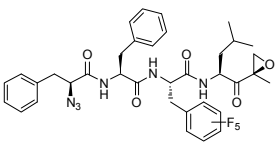
General procedure B: azide coupling of the peptidehydrazides to the epoxyketone warhead (18)

Hydrazine hydrate (20 eq.) was added to the peptide methyl ester (1 eq.) in MeOH (20 mL/mmol) and refluxed until TLC analysis revealed complete consumption of the starting material (usually after 3 h). Toluene was added and the mixture was concentrated under reduced pressure followed by coevaporation with toluene (2×). The resulting acylhydrazide (1 eq.) was dissolved in a 1:1 mixture of DCM/DMF (10 mL/mmol) and cooled to –35 °C. To this were added *tert*-butylnitrite (1.1 eq.) and HCl (2.8 eq. as a 4M solution in 1,4-dioxane) and the mixture was stirred for 3 h at –35 °C. Next, a mixture of the deprotected amine (**18**, 1.1 eq., as a TFA salt) and DiPEA (5 eq.) in DMF (1 mL) were added. The reaction was slowly warmed to RT and stirred for another 12 h before being diluted with DCM and washed with 1M HCl (2×), saturated Na_2CO_3 (2×) and brine. After drying ($MgSO_4$) and concentrating the obtained crude product was purified by column chromatography, applying a 1% → 15% MeOH/DCM eluent system, or, where indicated, by RP-HPLC.



(S)-2-azido-N-((S)-1-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)-3-phenylpropanamide, N_3PhePhePheLeu-EK (3)

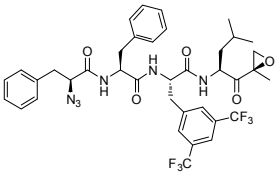
Prepared via general procedure B. The product was obtained as a colourless solid (yield: 157 mg, 0.25 mmol, 77%). ^1H NMR (400 MHz, $CDCl_3$): δ = 7.33-7.12 (m, 12H), 7.04 (d, *J* = 6.38 Hz, 1H), 7.00 (d, *J* = 7.69 Hz, 3H), 6.94 (d, *J* = 7.93 Hz, 1H), 6.63 (d, *J* = 8.15 Hz, 1H), 4.80 (dd, *J* = 14.46, 6.89 Hz, 1H), 4.74 (dd, *J* = 14.29, 6.80 Hz, 1H), 4.59-4.53 (m, 1H), 4.03 (dd, *J* = 8.63, 4.02 Hz, 1H), 3.21-3.15 (m, 2H), 3.03-2.87 (m, 4H), 2.87-2.79 (m, 2H), 1.56-1.50 (m, 2H), 1.49 (s, 3H), 1.22 (dd, *J* = 16.68, 7.16 Hz, 1H), 0.90 (dd, *J* = 9.10, 6.22 Hz, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 207.96, 170.12, 168.61, 135.96, 135.87, 129.35, 129.27, 129.19, 128.62, 128.56, 128.52, 128.39, 128.33, 127.18, 127.01, 126.82, 64.96, 58.80, 53.91, 53.86, 52.10, 49.74, 40.13, 38.25, 37.88, 25.01, 23.20, 21.38, 16.50 ppm. $[\alpha]_D^{23}$ = +47.5° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 5.82. HRMS: calcd. for $C_{36}H_{42}N_6O_5$ $[M + H]^+$ 639.32894; found 639.32906.



(S)-2-azido-N-((S)-1-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-(perfluorophenyl)propan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)-3-phenylpropanamide, N_3PhePhePhe(F_5)Leu-EK (4a)

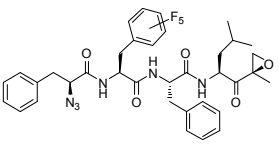
Prepared via general procedure B. The product was obtained as a colourless solid (yield: 234 mg, 0.32 mmol, quant.). ^1H NMR (400 MHz, $CDCl_3$): δ = 7.38 (d, *J* = 8.10 Hz, 1H), 7.33-7.13 (m, 10H), 7.03 (d, *J* = 6.67 Hz, 2H), 4.91 (dd, *J* = 12.63, 6.05 Hz, 2H), 4.67 (t, *J* = 7.71, 7.71 Hz, 1H), 4.21 (dd, *J* = 8.55, 3.74 Hz, 1H), 3.23 (dd, *J* = 13.85, 3.76 Hz, 2H), 3.12 (dd, *J* = 13.58, 5.98 Hz, 1H), 3.00-2.81 (m, 5H), 1.71-1.54 (m, 2H), 1.52 (s, 3H), 1.31-1.21 (m, 1H), 0.93 (dd, *J* = 10.64, 6.35 Hz, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 208.71, 170.74, 169.26, 168.84, 145.4 (dd, *J* = 246.15, 11.0 Hz), 140.32 (dt, *J* = 253.22, 11.0 Hz), 137.43 (dt, *J* = 246.40, 11.0 Hz), 136.20, 135.89, 129.50, 129.25, 128.65, 128.49, 127.24, 127.02, 109.81 (dt, *J* = 18.91,

3.28 Hz), 65.09, 58.88, 53.85, 52.13, 51.90, 50.02, 40.67, 38.33, 38.18, 26.03, 25.20, 23.31, 21.51, 16.50 ppm. $[\alpha]_D^{23} = +8.7°$ (c = 1 in $CHCl_3$). LC-MS: system B, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.73. HRMS: calcd. for $C_{36}H_{37}F_5N_6O_5$ $[M + H]^+$ 729.28184; found 729.28213.



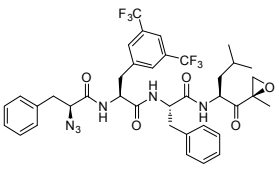
(S)-2-azido-N-((S)-1-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxopropan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)-3-phenylpropanamide, N_3PhePhePhe(F_6)Leu-EK (4b)

Prepared via general procedure B. The product was obtained as a colourless solid (yield: 156 mg, 0.20 mmol, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.71 (s, 1H), 7.53 (s, 2H), 7.34-7.16 (m, 8H), 7.05 (d, *J* = 7.63 Hz, 1H), 7.00 (d, *J* = 6.14 Hz, 3H), 6.93 (d, *J* = 7.75 Hz, 1H), 4.76 (dd, *J* = 13.13, 6.42 Hz, 1H), 4.68 (dd, *J* = 14.27, 7.10 Hz, 1H), 4.54 (dd, *J* = 13.00, 5.25 Hz, 1H), 4.15 (dd, *J* = 8.35, 3.84 Hz, 1H), 3.24-3.14 (m, 2H), 3.07 (dd, *J* = 13.92, 5.77 Hz, 2H), 2.92 (d, *J* = 6.64 Hz, 2H), 2.84 (d, *J* = 4.68 Hz, 2H), 1.67-1.57 (m, 1H), 1.53 (dd, *J* = 13.48, 2.57 Hz, 1H), 1.49 (s, 3H), 1.30-1.22 (m, 1H), 0.95-0.91 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 208.28, 170.34, 169.28, 168.61, 162.53, 138.62, 135.86, 135.66, 131.27 (q, *J* = 33.14 Hz), 129.80, 129.39, 129.03, 128.57, 128.54, 127.20, 127.06, 123.19 (q, *J* = 272.84 Hz), 120.84, 64.98, 59.02, 54.20, 53.43, 52.14, 50.21, 39.79, 38.23, 38.00, 37.84, 36.44, 31.36, 25.07, 23.24, 21.14, 16.39 ppm. $[\alpha]_D^{23} = +41.9°$ (c = 1 in $CHCl_3$). LC-MS: system B, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.73. HRMS: calcd. for $C_{38}H_{40}F_6N_6O_5$ $[M + H]^+$ 775.30371; found 775.30428.



(S)-2-azido-N-((S)-1-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-ylamino)-1-oxo-3-(perfluorophenyl)propan-2-yl)-3-phenylpropanamide, N_3PhePhe(F_5)PheLeu-EK (5a)

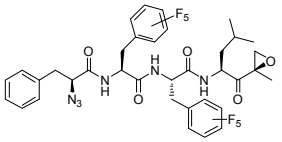
Prepared via general procedure B. The product was obtained as a colourless solid (yield: 135 mg, 0.18 mmol, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.69 (d, *J* = 6.88 Hz, 1H), 7.31-7.15 (m, 9H), 7.09 (d, *J* = 6.46 Hz, 2H), 7.04 (d, *J* = 6.34 Hz, 1H), 6.73 (d, *J* = 6.27 Hz, 1H), 5.03 (dd, *J* = 14.61, 7.19 Hz, 1H), 4.83 (dd, *J* = 13.94, 6.69 Hz, 1H), 4.59 (dd, *J* = 12.37, 5.10 Hz, 1H), 3.91 (dd, *J* = 8.90, 3.47 Hz, 1H), 3.22 (dd, *J* = 13.83, 3.23 Hz, 1H), 3.13-3.06 (m, 2H), 3.02-2.97 (m, 3H), 2.83 (dd, *J* = 13.94, 9.17 Hz, 1H), 2.76 (d, *J* = 4.71 Hz, 1H), 1.64-1.52 (m, 2H), 1.50 (s, 3H), 1.29-1.22 (m, 1H), 1.00-0.82 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 208.27, 169.95, 169.31, 168.94, 145.44 (dd, *J* = 237, 11.0 Hz), 140.17 (dt, *J* = 251, 11.0 Hz), 137,30 (dt, *J* = 251, 11.0 Hz), 135.98, 129.33, 128.46, 128.27, 127.09, 127.04, 126.91, 109.66 (dt, *J* = 19.00, 2.82 Hz), 64.79, 58.83, 54.07, 52.04, 51.48, 49.82, 40.22, 38.73, 38.21, 25.62, 25.06, 23.10, 21.30, 16.45 ppm. $[\alpha]_D^{23} = +27.0°$ (c = 1 in $CHCl_3$). LC-MS: system B, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.86. HRMS: calcd. for $C_{36}H_{37}F_5N_6O_5$ $[M + H]^+$ 729.28184; found 729.28211.



(S)-2-azido-N-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-1-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-ylamino)-1-oxopropan-2-yl)-3-phenylpropanamide, N_3PhePhe(F_6)PheLeu-EK (5b)

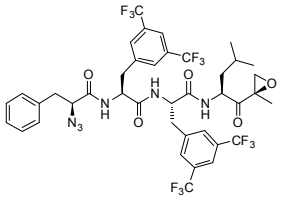
Prepared via general procedure B. The product was obtained as a colourless solid (yield: 220 mg, 0.28 mmol, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.85 (d, *J* = 7.85 Hz, 1H), 7.71 (s, 1H), 7.60 (s, 2H), 7.40 (d, *J* = 8.60 Hz, 1H), 7.32-7.08 (m, 9H), 7.05 (d, *J* = 6.92 Hz, 2H), 5.13 (dd, *J* = 13.66, 7.60 Hz, 1H), 4.87 (dd, *J* = 13.75, 6.59 Hz, 1H), 4.62 (dd, *J* = 12.45, 5.05 Hz, 1H), 4.04 (dd, *J* = 9.31, 3.45 Hz, 1H), 3.25-3.15 (m, 2H), 3.11-2.96 (m, 4H), 2.79 (d, *J* = 4.02 Hz, 1H), 2.76 (dd, *J* = 13.05, 8.88 Hz, 1H), 1.63-1.52 (m, 2H), 1.51 (s, 3H), 0.91 (dd, *J* = 8.50, 6.71 Hz, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 208.30, 170.16, 169.74, 168.88, 162.55, 138.90, 136.00, 131.24 (q, *J* = 33.13 Hz), 129.65, 129.23, 129.17, 129.01,

128.51, 128.16, 127.07, 126.72, 123.11 (q, *J* = 272.74 Hz), 120.75, 65.00, 58.77, 53.95, 52.89, 51.99, 49.81, 40.08, 38.53, 38.39, 37.86, 25.04, 23.01, 21.32, 16.37 ppm. $[\alpha]_D^{23} = +43.4°$ (*c* = 1 in $CHCl_3$). LC-MS: system B, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.73. HRMS: calcd. for $C_{38}H_{40}F_6N_6O_5$ $[M + H]^+$ 775.30371; found 775.30404.



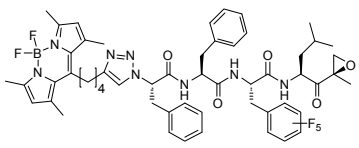
(S)-2-azido-N-((S)-1-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-(perfluorophenyl)propan-2-ylamino)-1-oxo-3-(perfluorophenyl)propan-2-yl)-3-phenylpropanamide, N_3PhePhe(F_5)Phe(F_5)Leu-EK (6a)

Prepared via general procedure B from **16**. Purification by HPLC (linear gradient 72 → 82% ACN in H_2O, 0.1% TFA, 15 min) furnished the title compound (yield: 16.4 mg, 20.1 μmol, 10%) as a white solid. ^{1}H NMR (400 MHz, MeOD): δ = 7.31-7.23 (m, 5H), 4.63 (dd, *J* = 14.4, 6.8 Hz, 2H), 4.54 (dd, *J* = 10.0, 3.2 Hz, 1H), 4.04 (dd, *J* = 8.8, 5.2 Hz, 1H), 3.28 (d, *J* = 5.2 Hz, 1H), 3.17-3.09 (m, 3H), 3.01-2.98 (m, 1H), 2.96-2.83 (m, 3H), 1.74-1.60 (m, 1H), 1.44 (s, 3H), 1.38-1.34 (m, 1H), 1.32-1.31 (m, 1H), 0.93 (t, *J* = 6.8 Hz, 6H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 209.55, 171.41, 171.12, 148.20, 145.78, 143.0-142.50 (m), 140.15-139.8 (m), 137.75, 137.68, 130.36, 129.60, 128.06, 111.54 (q, *J* = 18 Hz), 65.49, 59.95, 53.09, 52.77, 51.27, 40.46, 38.69, 26.33, 26.02, 23.70, 21.58, 16.73 ppm. $[\alpha]_D^{23} = +5.5°$ (*c* = 1 in MeOH). LC-MS: system A, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.63. HRMS: calcd. for $C_{36}H_{32}F_{10}N_6O_5$ $[M + H]^+$ 819.23473; found 819.23498.



(S)-2-azido-N-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-1-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxopropan-2-ylamino)-1-oxopropan-2-yl)-3-phenylpropanamide, N_3PhePhe(F_6)Phe(F_6)Leu-EK (6b)

Prepared via general procedure B. The product was obtained by HPLC purification (linear gradient 62 → 69% ACN in H_2O, 0.1% TFA, 15 min) as a yellowish oil (yield: 29.3 mg, 32.1 μmol, 7%). ^{1}H NMR (400 MHz, MeOD): δ = 7.84 (s, 2H), 7.79 (s, 4H), 7.25-7.14 (m, 5H), 4.76-4.59 (m, 2H), 4.49 (dd, *J* = 10.8, 2.8 Hz, 1H), 3.97 (dd, *J* = 8.8, 2.8 Hz, 1H), 3.28 (d, *J* = 5.7 Hz, 1H), 3.25 (d, *J* = 4.8 Hz, 1H), 3.21 (d, *J* = 5.2 Hz, 1H), 3.04-2.98 (m, 3H), 2.91 (d, *J* = 4.8 Hz, 1H), 2.74 (dd, *J* = 13.9, 9.1 Hz, 1H), 1.74-1.70 (m, 1H), 1.51-1.47 (m, 1H), 1.45 (s, 3H), 1.39-1.31 (m, 1H), 0.95 (d, *J* = 6.4 Hz, 3H), 0.91 (d, *J* = 6.4 Hz, 3H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 209.65, 172.28, 171.92, 171.25, 141.46, 137.81, 132.60 (q, *J* = 33.0 Hz), 131.20, 130.25, 129.57, 128.07, 124.90 (q, *J* = 332 Hz), 121.77, 65.51, 60.18, 55.05, 54.78, 52.98, 51.94, 40.08, 38.88, 38.33, 26.32, 23.73, 21.47, 16.95 ppm. $[\alpha]_D^{23} = +29.4°$ (*c* = 1 in MeOH). LC-MS: system A, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 10.09. HRMS: calcd. for $C_{40}H_{38}F_{12}N_6O_5$ $[M + H]^+$ 911.27848; found 911.27902.



Bodipy-triazole-Phe-Phe-Phe(F_5)-Leu-EK (19)

Compound **4a** (70.7 mg, 97 μmol) was dissolved in a mixture of toluene/*t*BuOH/H_2O (1/1/1 v/v/v, 2 mL) and to this were added Bodipy-alkyne[18] (1 eq., 97 μmol, 32 mg), $CuSO_4$ (10 mol%, 9.7 μmol, 9.7 μL; 1M in H_2O) and sodium ascorbate (15 mol%, 14.5 μmol, 14.5 μL; 1M in H_2O). The mixture was stirred at 80 °C for 2.5 h after which TLC analysis indicated complete consumption of the starting material. The mixture was concentrated under reduced pressure and the compound was purified by column chromatography (DCM → 2.5% MeOH/DCM) and obtained as a brownish solid (yield: 89.2 mg, 84.4 μmol, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.07 (d, *J* = 5.49 Hz, 1H), 7.62 (s, 1H), 7.52 (d, *J* = 8.17 Hz, 1H), 7.19 (d, *J* = 5.94 Hz, 1H), 7.08 (d, *J* = 4.92 Hz, 4H), 7.03-7.00 (m, 2H), 6.95 (d, *J* = 4.02 Hz, 4H), 6.84 (d, *J* = 4.15 Hz, 2H), 6.02 (s, 2H), 5.77 (t, *J* = 7.40,

7.40 Hz, 1H), 5.30-5.24 (m, 1H), 4.82 (dd, *J* = 13.07, 6.73 Hz, 1H), 4.75 (dd, *J* = 12.64, 5.94 Hz, 1H), 3.44-3.32 (m, 2H), 3.24 (d, *J* = 4.58 Hz, 1H), 3.05 (dd, *J* = 13.96, 6.43 Hz, 1H), 3.00-2.63 (m, 8H), 2.50 (s, 6H), 2.37 (s, 6H), 1.95-1.86 (m, 2H), 1.73-1.58 (m, 4H), 1.54 (s, 3H), 1.31 (dd, *J* = 6.89, 3.54 Hz, 1H), 0.92 (dd, *J* = 21.75, 6.16 Hz, 6H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$): δ = 208.40, 170.33, 169.33, 167.56, 153.79, 147.36, 145.92, 140.22, 135.57, 135.24, 131.33, 129.02, 128.72, 128.42, 128.23, 127.09, 126.60, 121.56, 120.54, 64.65, 58.79, 54.28, 52.03, 51.56, 49.92, 40.50, 38.83, 38.64, 31.29, 29.58, 28.06, 25.43, 25.13, 23.19, 21.44, 16.46, 16.27, 14.35 ppm. $[\alpha]_D^{23}$ = +27.5° (*c* = 1 in $CHCl_3$). LC-MS: system A, gradient 50% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.95. HRMS: calcd. for $C_{55}H_{60}BF_7N_8O_5$ $[M + H]^+$ 1057.47407; found 1057.47562.

Competition assays

Whole cell lysates of HEK-293T or EL4 were made by sonication in 3 volumes of lysis buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 250 mM sucrose, 2 mM ATP. Protein concentration was determined by the Bradford assay. Cell lysates (13.5 μg total protein for HEK-293T lysates and 9 μg total protein for EL4 lysates) were exposed to the inhibitors for 1 h prior to incubation with MV151 (0.5 μM) for 1 h at 37 °C. Reaction mixtures were boiled with Laemmli's buffer containing β-mercaptoethanol for 5 min. before being resolved by 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532nm, λ_{em} 560 nm) to detect MV151 and Cy2/Fam settings (λ_{ex} 488 nm, λ_{em} 520 nm) to detect compound **19**.

IC_{50} determinations

Purified 26S proteasome (~10 ng/mL) was incubated with various concentrations of inhibitors at 37 °C for 30 min. in the assay buffer (50 mM Tris-HCl, pH 7.5, 40 mM KCl, 2 mM EDTA, 1 mM DTT, 100 μM ATP, 50 μg/mL BSA). In the meantime, 100 μM solution of the fluorogenic peptide substrates (Suc-LLVY-7-amido-4-methyl-coumarin (amc) for the β5 site, Ac-nLPnLD-amc for the β1 site and Ac-RLR-amc or Ac-RQR-amc for the β2 site) in the assay buffer were pre-incubated at 37 °C. Immediately after the end of this incubation, an aliquot of the inhibitor-treated proteasome was mixed with the substrate, and fluorescence of released amc was measured continuously for 30 min. at 37 °C. The rate of reaction was determined from the slope of the reaction progress curves. Mock-treated proteasomes served as control. Residual activity in inhibitor treated samples were plotted against concentration of inhibitors and IC_{50} values were determined from these plots.

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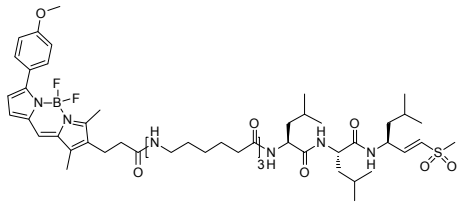

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5 Selective Inhibitors of Proteasome's Trypsin-like Sites
Synthesis and biological evaluation

5.1 Introduction

The mammalian 20S proteasome catalytic core contains two sets of three catalytically active β subunits, which display a different substrate specificity, namely β1 (caspase-like) cleaves after acidic residues, β2 (trypsin-like) cleaves after basic residues and β5 (chymotrypsin-like) cleaves after bulky, hydrophobic residues.[1-3] In specific cell types involved in the immune surveillance system four additional active subunits can be expressed. In the so-called immunoproteasome the β1i, β2i and β5i replace their corresponding constitutive counterparts[4] and, in addition to that, β5 is replaced by β5t in cortical thymic epithelial cells.[5] To study the role of each individual catalytic subunit in the generation of oligopeptides, the development of cell permeable inhibitors that target one specific subunit has become an important field of research. Peptide-based inhibitors targeting the β5 subunit can be created by introduction of hydrophobic amino acids,[6,7] as was, for example, shown in Chapter 4. In addition, Van Swieten *et al.*[8] reported on the development of a cell permeable β1 selective inhibitor containing hydrophobic amino acids as well. In contrast, the search for highly selective inhibitors for the β2 and/or β2i active sites with good cell permeability remains a challenging task. One reason for this might be that introduction of basic amino acids is often required to target the trypsin-like site more selectively. These basic amino acids (Arg, Lys) are positively charged at neutral pH, making it very difficult to cross the cell membrane. A second problem is the synthesis of inhibitors bearing an electrophilic trap (for instance the epoxy ketone) in combination with basic amino acids (especially at the P1 position, next to the warhead), for they are susceptible to cyclisation via nucleophilic attack of the free amine/guanidine on the electrophilic trap. Some modified peptides that target

the β2 subunit selectively have been described in the literature (see Figure 1A). In a P2-P4 side chain positional scanning study Bogyo *et al.*[9] found the β2 selective inhibitor Ac-YRLN-VS **1** and showed that the P3 substituent (Arg) is of considerable importance in selectivity enhancement. In addition, the group of Tomatis[10,11] reported on the vinyl ethyl ester tripeptide HMB-VSL-VE **2**, which was able to selectively target the β2 active site, both in purified proteasome and in living cells.

This chapter describes the development of inhibitors targeting the trypsin-like subunits (β2 and β2i) by modification of the P1 site, which plays a key role in subunit binding, with basic residues. The initial set of inhibitors synthesized and studied is shown in Figure 1C. The general structure is based on the tripeptide vinyl sulfone Z-L_3VS **3**, which targets all proteasome active sites (Figure 1B).[12] The P1 leucine side chain was replaced by either a panel of phenylalanine derivatives containing an amine with varying basicity (benzyl amine **4a**, aniline **5a**, pyridine **6**) or a lysine (**7**) side chain. In addition to the vinyl sulfone electrophilic trap, the epoxyketone featured by natural proteasome inhibitor epoxomicin was incorporated as well (**4b**, **5b**), since it displays a specific reactivity towards proteasome active sites (see also Chapter 4).[3,13] The N-terminal benzyloxycarbonyl group was replaced by the structurally related azidophenylalanine, which opens the possibility for additional modifications,[14-16] yet it does not significantly influence the inhibitory properties compared to the benzyloxycarbonyl group.[17]

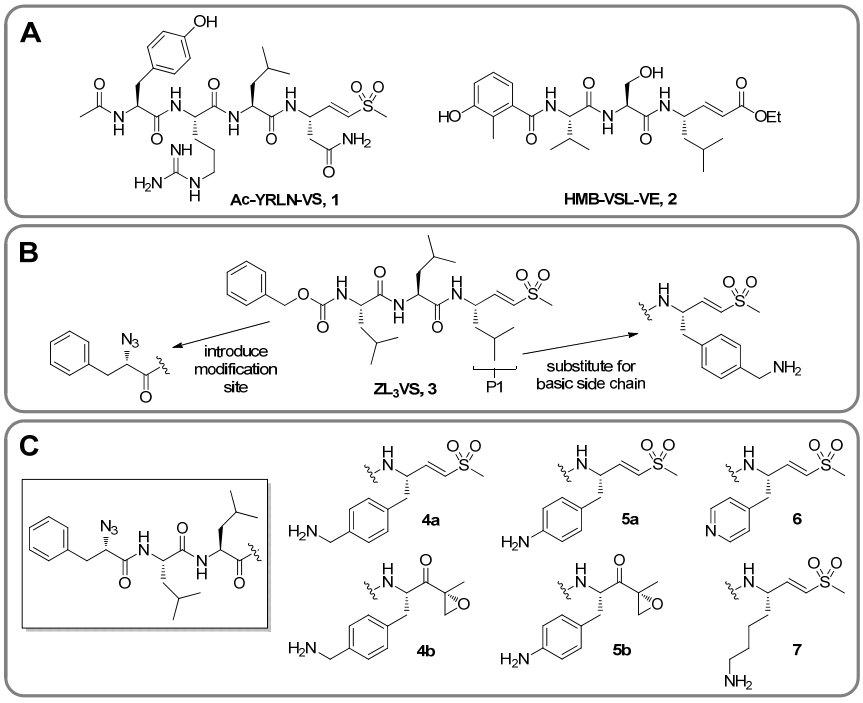
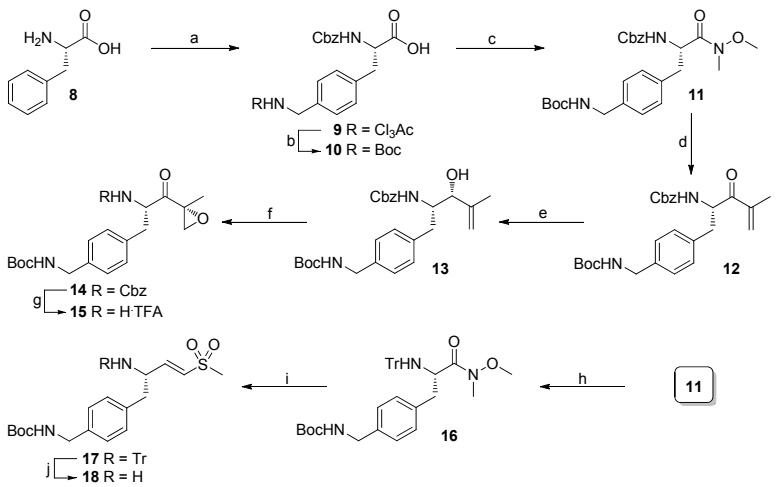


Figure 1. (A) Examples of two published β2 selective proteasome inhibitors. (B) Modifications of broad-spectrum proteasome inhibitor ZL_3VS at the basis of the here presented inhibitors. (C) Initial panel of inhibitors prepared and studied in this chapter.

5.2 Results and Discussion

Retro-synthetically, the modified oligopeptides can be prepared from tripeptide hydrazide N_3Phe-Leu-Leu-$NHNH_2$ and the properly protected warhead amines in an (epimerization free) azide coupling.[18] The synthesis of P1-benzyl amine containing vinyl sulfone and epoxyketone warheads leading to inhibitors **4a** and **4b** is shown in Scheme 1. The synthetic scheme commenced with the introduction of the aminomethylene substituent on L-phenylalanine **8**, by performing an electrophilic aromatic substitution with *N*-(hydroxymethyl)trichloroacetamide under acidic conditions.[19,20] In this reaction both the *ortho* and the *para* substituted isomers were formed, which could be separated by column chromatography. The desired *para* substituted isomer was obtained in 35% yield. After Cbz-protection of the α-amine compound **9** was obtained. Basic removal of the trichloroacetamide group followed by Boc protection of the formed amine gave **10**, which was coupled to *N,O*-dimethylhydroxylamine to give Weinreb-amide **11**. Upon a reaction with 2-lithiumpropene the α',β'-unsaturated ketone **12** was obtained. Stereoselective reduction to the allylic alcohol **13** and subsequent asymmetric epoxidation and Dess-Martin oxidation resulted in epoxyketone **14**.[21] This compound was α-amine deprotected by hydrogenation, which finalized the synthesis of compound **15**. The vinyl sulfone analogue was created by α-amine deprotection of compound **11**, followed by tritylation (**16**). Reduction of the Weinreb-amide, followed by a Horner-Wadworth-Emmons reaction and de-tritylation finally resulted in compound **18**.

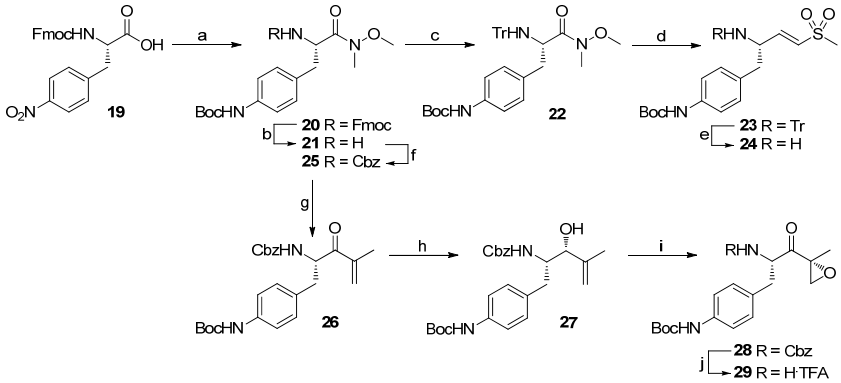
Scheme 1. Synthesis of warheads **15** and **18**.



Reagents and conditions: (a) i) *N*-(hydroxymethyl)trichloroacetamide, H_2SO_4, H_2O; ii) benzyl chloroformate, Na_2CO_3, H_2O, 1,4-dioxane, 35%; (b) i) 20% NaOH, EtOH/H_2O 1:1; ii) Boc_2O, Na_2CO_3, THF, H_2O, 75%; (c) NH(Me)OMe·HCl, HCTU, DiPEA, DCM, 98%; (d) 2-bromopropene, *t*BuLi, THF, –78 °C, 94%; (e) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, MeOH, 0 °C, 92%; (f) i) *t*BuOOH, $VO(Acac)_2$, DCM, 0 °C; ii) Dess-Martin periodinane, DCM, 56%; (g) H_2, Pd black, TFA, MeOH; (h) i) H_2, Pd/C, AcOH, EtOH; ii) TrCl, Et_3N, DMAP, DCM, 38%; (i) i) $LiAlH_4$, Et_2O, 0 °C; ii) diethyl ((methylsulfonyl)methyl)phosphonate, NaH, THF, 0 °C, 85%; (j) 1% TFA/DCM.

Aniline containing warheads **24** and **29** (Scheme 2) were made from Weinreb-amide **21** by following a similar reaction sequence as described for **18** and **15** respectively (see Scheme 1). Compound **21** was made from Fmoc protected *para*-nitrophenylalanine **19**. Reduction of the nitro group followed by Boc-protection of the formed amine and formation of the Weinreb-amide gave fully protected **20**, which was converted into free α-amine **21** by removal of the Fmoc group.

Scheme 2. Synthesis of warheads **24** and **29**.



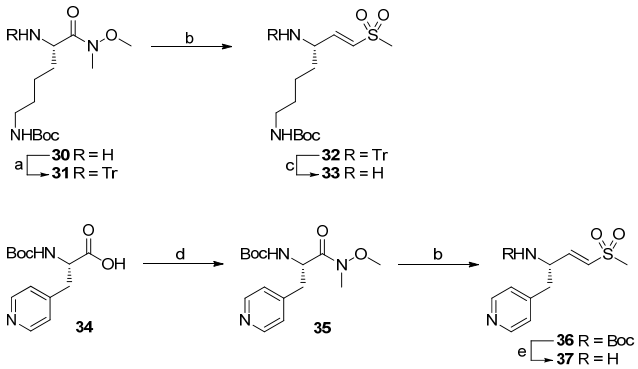
Reagents and conditions: (a) i) NH_4HCO_2H, Pd/C, MeOH; ii) Boc_2O, $NaHCO_3$, H_2O, 1,4-dioxane; iii) NH(Me)OMe·HCl, HCTU, DiPEA, DCM, 99%; (b) DBU, THF, 85%; (c) TrCl, Et_3N, DMAP, DCM, 96%; (d) i) $LiAlH_4$, Et_2O, 0 °C; ii) diethyl ((methylsulfonyl)methyl)phosphonate, NaH, THF, 0 °C, 85%; (e) 1% TFA/DCM; (f) benzyl chloroformate, DiPEA, THF, 85%; (g) 2-bromopropene, *t*BuLi, THF, –78 °C, 94%; (h) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, MeOH, 0 °C, 92%; (i) i) *t*BuOOH, $VO(Acac)_2$, DCM, 0 °C; ii) Dess-Martin periodinane, DCM, 56%; (j) H_2, Pd black, TFA, MeOH.

The synthesis of lysine (**33**) and pyridine (**37**) functionalized vinyl sulfones is depicted in Scheme 3. These compounds were produced in a procedure similar to that for **18**, from ε-amine-Boc-protected Weinreb-amide **30**[22] and commercially available Boc-β-(4-pyridyl)-L-alanine **34** respectively.

Scheme 4 shows the azide coupling of amine warheads **18** and **24** with tripeptide hydrazide **38**, giving, after TFA mediated deprotection and RP-HPLC purification, inhibitors **4a** and **5a**. The other inhibitors were made in a similar reaction from the appropriate amines in varying yields of 7-48% after RP-HPLC. LC-MS and NMR analysis showed for neither compound any sign of epimerization of the final products.

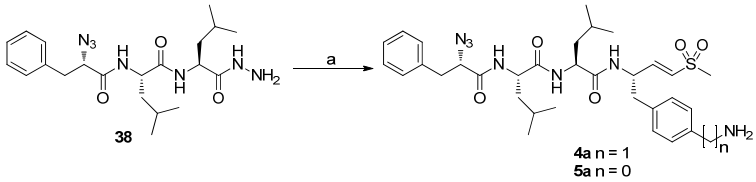
The inhibition potential of the inhibitors for each of the catalytically active subunits was assessed in competition assays employing extracts of human embryonic kidney cells (HEK-293T) and mouse lymphoma cells (EL-4) in combination with the fluorescent broad spectrum proteasome probe MV151[23] (see also Chapter 4). The gel images are shown in Figure 2. Competitive inhibition of a proteasome active site is reflected by the disappearance of the corresponding band.

Scheme 3. Synthesis of warheads **33** and **37**.



Reagents and conditions: (a) TrCl, DiPEA, DCM, 68%; (b) i) $LiAlH_4$, Et_2O, 0 °C; ii) diethyl ((methylsulfonyl)methyl)phosphonate, NaH, THF, 0 °C, 64%; (c) 1% TFA/DCM; (d) NH(Me)OMe·HCl, HCTU, DiPEA, DCM, quant.; (e) TFA, DCM.

Scheme 4. Azide coupling towards the target inhibitors.



Reagents and conditions: (a) i) *t*BuONO, HCl, DMF, DCM, –30 °C; ii) compound **18** or **24**, DiPEA; iii) TFA, DCM, then RP-HPLC, yields: 7-48%.

It is apparent from these results (Figure 2) that the selectivity for β2 decreases with decreasing basicity (compare compounds **4a** and **7** with **5a** and **6**). When the substituent becomes less basic the inhibitor targets both β5 and β2. This phenomenon can be explained by the fact that the nature of the substituent becomes more hydrophobic and is therefore more favoured by β5. In general, the β1 subunit is not affected by any compound and is even upregulated at higher concentrations (a related effect was also seen in Chapter 4 in case β5-specific inhibitors were employed).[24] There appears to be little difference between the experiments in HEK-293T and EL-4 lysate with respect to the potency towards β2, however the selectivity for β2 over β5 is difficult to determine since the β5(i) and β1(i) bands are overlapping. Apparently, the inhibitors do not distinguish between the constitutive subunits and their immuno counterparts. Interestingly, the vinyl sulfones seem to display better characteristics, in terms of selectivity and potency, compared to the epoxyketones (compare compounds **4a** and **5a** with **4b** and **5b**). This observation is remarkable since epoxyketones are generally more active than their vinyl sulfone counterparts (in a head-to-head comparison).[16] Benzyl amine derivative **4a** and lysine derivative **7** are the most β2 selective inhibitors in

this series, however in terms of potency, compound **4a** is about a 10 fold more potent than **7**. Capable of (almost) complete inhibition of β2(i) at a concentration of 0.5 μM, while leaving the other subunits untouched, compound **4a** is the most valuable compound derived from this series.

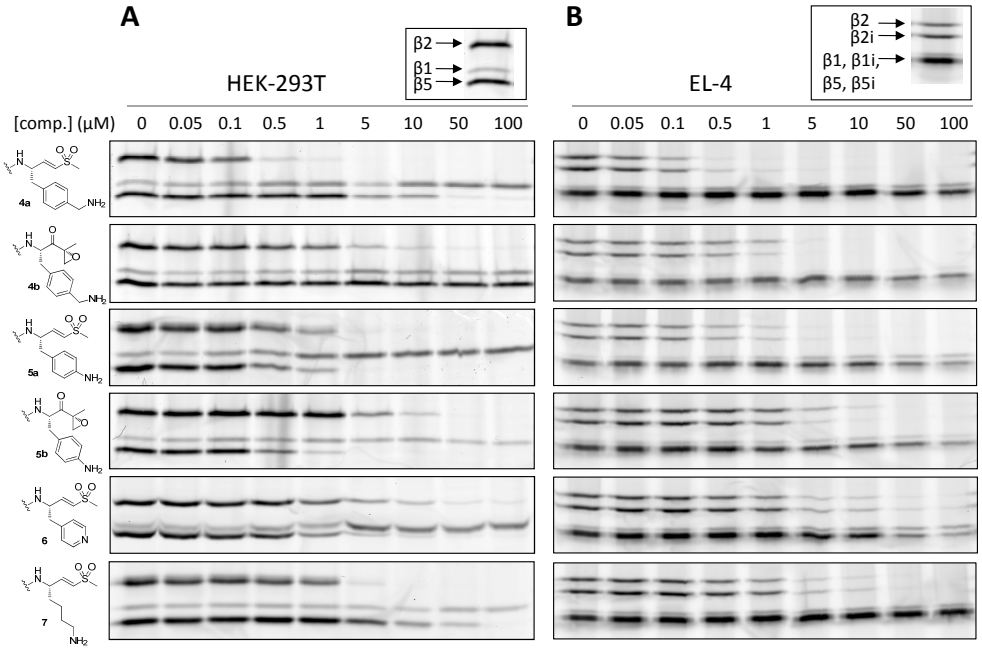


Figure 2. Characterization of the specificity of the inhibitors shown in Figure 1C. Competition assay in (A) HEK-293T cell lysate and (B) EL-4 cell lysate. Lysates were incubated with the inhibitors at the indicated final concentrations. Residual proteasome activity was labelled with 0.5 μM MV151.

Three inhibitors were selected from this panel and tested for their capability to cross the cell membrane. Primary amine containing compounds **4a** and **7** were selected because of their enhanced preference for β2 and compound **5a** was tested for its ability to target both β2 and β5. Living HEK-293T cells were incubated with each of the three inhibitors at 0.5, 5 and 50 μM final concentrations for 4 hours, after which all residual proteasome activity was labelled with cell permeable probe MV151. The cells were lysed, all proteins denatured and resolved by SDS-PAGE. As a control the broad-spectrum proteasome inhibitor $AdaAhx_3L_3VS$,[25] which is known to be able to cross the cell membrane, was used. From the results shown in Figure 3 it follows that the primary amine in compound **4a** does not result in inpermeability towards the cell membrane and is still able of inhibiting (almost) all β2 activity at 5 μM. Aniline containing compound **5a** was also able to cross the cell membrane, after which it targets both β2 and β5. Lysine derived inhibitor **7** appears to be unable of crossing the cell membrane as evidenced from Figure 3.

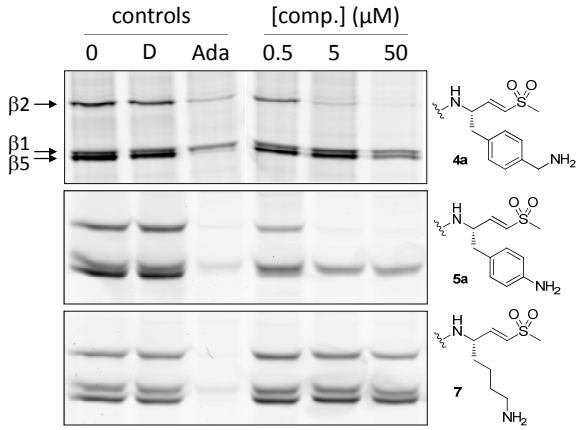


Figure 3. Competition assay in live HEK-293T cells. The cells were treated with compounds **4a**, **5a** and **7** at the indicated final concentrations for 4 hours, followed by incubation with MV151 (5 μM final concentration) for 2 hours. After cell lysis and denaturation the samples were resolved by SDS-PAGE and analyzed by fluorescence scanning. Controls used: 0 = no inhibitor, D = DMSO, Ada = $AdaAhx_3L_3VS$ (20 μM).

For direct labelling of β2 a new fluorescent probe was made by reacting compound **4a** with a green fluorescent Bodipy-alkyne[18] in a 'click' reaction (see Figure 4A). This reaction however was not as straightforward as could be expected from earlier results (see Chapter 4). Upon reaction of both compounds with $CuSO_4$ and sodium ascorbate in an aqueous medium compound **4a** was completely consumed, however the formed product had a mass of 1 Da less compared to the expected product mass and it was dramatically more hydrophobic compared to the starting material, as evidenced from LC-MS measurements. It was reasoned that the free benzylic amine was oxidized and hydrolyzed into its corresponding benzaldehyde (Figure 4A). This reaction has been previously observed by Srogl and Voltrova,[26] who describe a copper/ascorbic acid dyad catalytic system for the selective aerobic oxidation of amines (both benzylic and aliphatic). Indeed, upon addition of ammonium acetate and $NaCNBH_3$ a reductive amination took place, resulting in the desired product **39**.

The ability of compound **39** to label proteasome actives both in HEK-293T cell lysate and living cells was assessed in a competition assay as described above. A dual-wavelength fluorescence read-out was performed allowing visualisation of one of the two fluorescent dyes at a time. The results are shown in Figure 4B. From this it becomes clear that the introduction of the bulky, hydrophobic bodipy moiety has resulted in the loss of the inhibitor's selectivity for β2 over β5. Both subunits are inhibited equally well, leaving only β1 untouched. Probably the large hydrophobic moiety is too close to the active site and introduction of a spacer between tag and warhead may reinstall β2 selectivity. Interestingly, introduction of the bodipy has had a detrimental effect on cell permeability. At a concentration of 5 μM both subunits (β2 and β5) seem not to be competed away at all, although a faint band for each subunit is visible in the lower gel. Even at a concentration of 50 μM not all proteasome activity is silenced. This

observations must come from the cell penetrating properties of the probe, since all β2 and β5 proteasomal activity is inhibited at a 5 μM concentration in cell lysate.

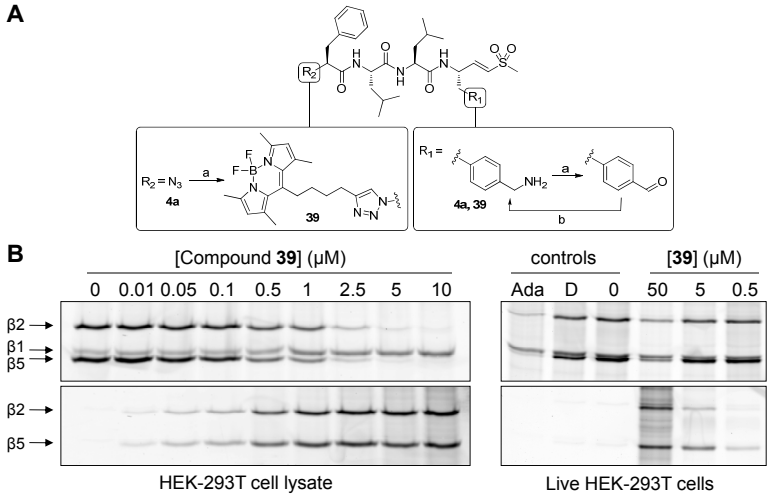


Figure 4. (A) Synthesis of fluorescent probe **39** in a 'click' reaction with inhibitor **4a** and Bodipy-alkyne. As a side reaction the benzylamine was converted to the corresponding benzaldehyde. Reagents and conditions: (a) Bodipy-alkyne, $CuSO_4$, sodium ascorbate, H_2O/*t*BuOH/toluene 1:1:1, 80 °C; (b) NH_4OAc, $NaCNBH_4$, MeOH, RP-HPLC, 29%. (B) Competition assay in HEK-293T cell lysate (left) and living cells (right) with compound **39** at the indicated final concentrations. Residual proteasome activity was labelled with MV151. Fluorescence read-out at λ_{ex} 532 nm, λ_{em} 560 nm (MV151, upper panels) and λ_{ex} 488 nm, λ_{em} 520 nm (compound **39**, lower panels). Controls used: Ada = $AdaAhx_3L_3VS$ (20 μM final concentration), D = DMSO, 0 = no inhibitor.

As discussed in the introduction of this Chapter vinyl ethyl ester tripeptide HMB-VSL-VE **2** was identified as a potent, cell permeable β2 selective inhibitor.[10,11] Other inhibitors containing the vinyl ethyl ester warhead have been made, which are known to target other subunits as well.[27] It is therefore likely that the majority of the β2 selectivity comes from the unique HMB-Val-Ser peptide sequence. For this reason, a combination of the HMB-Val-Ser peptide sequence and the P1-functionalized warheads discussed so far may result in inhibitors with an even enhanced preference for the β2(i) subunit. To this end compounds **40** and **41** (see Figure 5A) were synthesized via the method outlined above from HMB-Val-Ser(*t*Bu)-$NHNH_2$.[28] First, both compounds were tested for their inhibitory activity in HEK-293T cell lysate in a competition assay as discussed earlier. The results are depicted in Figure 5A. When comparing compound **40** and **4a** it becomes clear that substitution of the $N_3PheLeu_2$ for the HMB-Val-Ser motif the general potency is decreased by a factor two. In addition, the selectivity for β2 over β5 is substantially increased. Only a part of the β5 activity is inhibited at 50 μM by **40**, whereas compounds **4a** completely blocks β5 at this concentration. This difference is even more pronounced for the inhibition in living cells by **40** (Figure 5B). The β2 band has almost disappeared at a concentration of 5 μM and β5 is not affected at all at concentrations up to 50 μM. The most striking result from this assay is the apparent

selectivity of compound **40** for β2 over β2i in EL-4 lysate (Figure 5A). At a concentration of 0.5 μM β2 is almost completely blocked, whereas the compound starts to inhibit β2i only at 5 μM. The attachment of the HMB-Val-Ser peptide sequence to the aniline derived vinyl sulfone (**41**) only resulted in a drop of potency of the inhibitor compared to **5a**. The characteristics in terms of selectivity remain unchanged (it still targets both β2 and β5). These observations invite the conclusion that the HMB-Val-Ser sequence on its own is not enough to active β2 selectivity, but that by selection of a suitable P1 substituent this objective might be reached after all.

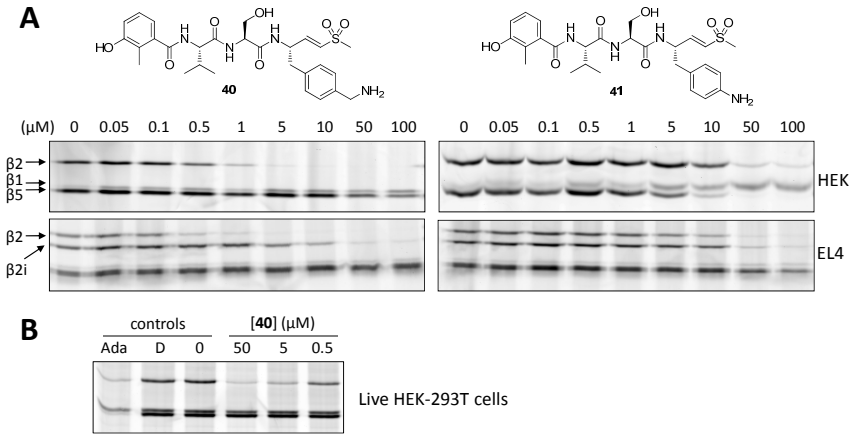


Figure 5. Competition assay of (A) compounds **40** and **41** in HEK-293T and EL-4 cell lysate and (B) compound **40** in HEK-293T living cells. Residual proteasome activity was labelled with MV151 as described above. Controls used: Ada = $AdaAhx_3L_3VS$ (20 μM final concentration), D = DMSO, 0 = no inhibitor.

5.3 Conclusion

In summary, the effect of introduction of different amines of varying basicity, at the P1 position in oligopeptide proteasome inhibitors with respect to the selectivity for proteasome's trypsin-like sites was studied. As expected, it was found that the β2 selectivity increases with increasing basicity of the side chain. All compounds were ineffective towards β1, but upon decreasing basic character of the substituent β5 was targeted. The most β2 selective compounds identified were lysine derived **7** and 4-aminomethylene phenylalanine derived **4a**, of which the latter one proved to be most potent. It was shown that **4a** was capable of inhibiting β2 selectively both in cell lysate and in living cells. This demonstrates that the nature of the side chain amine is such, that it is basic enough to direct the inhibitor towards β2, yet it allows the inhibitor to cross the cell membrane. Introduction of a hydrophobic fluorescent tag into **4a**, to label and visualize the β2 subunit selectively, resulted in a decreased selectivity for β2 over β5. A good alternative would be the use of two-step labelling, in which a biological sample is first treated with inhibitor **4a**, after which the construct is captured at the azide moiety via, for instance the Staudinger-Bertozzi ligation[29] or 'click' chemistry.[30] Preliminary results however, showed that the azide in **4a** is relatively inreactive towards

a biotin-phosphane reagent.[31] Therefore, improvements have to made, for instance by introduction of a more accessible azide or a spacer between warhead and modification site/tag.

In addition, the 4-aminomethylene phenylalanine vinyl sulfone warhead was coupled to the HMB-Val-Ser peptide, of which a preference for β2 has been reported.[10,11] This resulted in inhibitor **40**, which is completely ineffective towards β5 up to 50 μM *in vivo* and has a comparable potency towards β2. Interestingly, compound **40** was found to be able to distinguish between the constitutive active subunit β2 and its immunoproteasome counterpart β2i, showing a ten fold higher preference for β2 in EL-4 lysate.

Experimental section

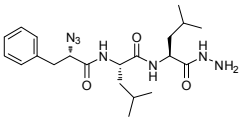
General Procedures:

Tetrahydrofuran was distilled over $LiAlH_4$ prior to use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), methanol (MeOH), 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. *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 4 Å molecular sieves, except methanol and acetonitrile which were stored over 3 Å molecular sieves. 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, ethyl acetate and petroleum ether (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CD_3OD or $CDCl_3$ 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* = 150-2,000) and dioctylpthalate (*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 $[\alpha]_D^{23}$ 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 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H_2O, ACN and 1.0% aq. TFA. 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.

General procedure I: azide coupling of N_3Phe-Leu-Leu-$NHNH_2$ or HMB-Val-Ser(*t*Bu)-$NHNH_2$ to an amine-warhead followed by acidic deprotection

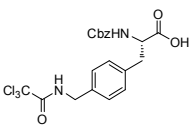
N_3Phe-Leu-Leu-$NHNH_2$ **38** or HMB-Val-Ser(tBu)-$NHNH_2$ (1 eq.) was dissolved in a 9:1 mixture of DCM/DMF (10 mL/mmol) and cooled to –35 °C. To this were added *tert*-butylnitrite (1.1 eq.) and

HCl (2.8 eq. as a 4 M solution in 1,4-dioxane) and the mixture was stirred for 3 h at –35 °C. Next, a mixture of the deprotected amine (1.1 eq.) and DiPEA (5 eq.) in DMF (1 mL) were added. The reaction was slowly warmed to room temperature and stirred for another 12 h before being diluted with DCM and extracted with 1M aq. HCl (2×), saturated aq. Na_2CO_3 (2×) and brine. After drying ($MgSO_4$) and concentrating the obtained crude product was dissolved in DCM (2.5 mL/mmol). TFA (2.5 mL/mmol) was added and the mixture was stirred for 30 min, after which it was concentrated under reduced pressure in the presence of toluene (3×). The obtained crude product was purified by RP-HPLC.



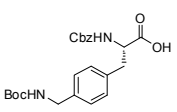
N_3-Phe-Leu-Leu-$NHNH_2$ (38)

This compound was synthesized via general Boc-based peptide coupling procedures using HCTU from H-Leu-OMe, Boc-Leu-H and N_3-Phe-H. The last step involved the introduction of the hydrazide by stirring of a mixture containing tripeptide N_3-Phe-Leu-Leu-OMe (1.51 g, 3.49 mmol) and hydrazine monohydrate (30 eq., 105 mmol, 5.1 mL) in MeOH (30 mL) for 15 h at RT. The title compound was obtained after coevaporation of the mixture with toluene (3×) as a colourless solid (yield: 1.51 g, 3.49 mmol, quant.). LC-MS: R_t (min): 6.87 (ESI-MS (*m/z*): 432.13 ($M + H^+$)).



(S)-2-(((benzyloxy)carbonyl)amino)-3-(4-((2,2,2-trichloroacetamido)methyl)phenyl)propanoic acid (9)

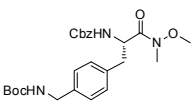
L-Phenylalanine (**8**, 8.26 g, 50.0 mmol) was added in portions to concentrated H_2SO_4 (35 mL) maintaining the temperature at 25 °C. *N*-(hydroxymethyl)trichloroacetamide (1.05 eq., 52.5 mmol, 10.1 g) was added in portions while maintaining the temperature at 20-25 °C. The cooling bath was removed and the light-brown cloudy solution was stirred at room temperature for 1 h. The reaction mixture was added to ice (500 mL) and the pH was adjusted to pH 5.5 with 8 M aq. NaOH solution while maintaining the quench temperature at 15-20 °C. The white solid was filtered off and washed with ice-cold H_2O. The residue was dissolved in a 1:1 mixture of H_2O/dioxane (100 mL) and the pH was adjusted to pH 9 by addition of Na_2CO_3. Next, benzyl chloroformate (7.32 mL, 50.0 mmol) was added and the mixture was stirred for 4 h. Concentrated aq. HCl was added until pH 1 and the mixture was extracted twice with EtOAc. The combined organic layers were extracted with brine, dried ($MgSO_4$) and concentrated under reduced pressure. The resulting crude product was purified by column chromatography (25% → 60% EtOAc/PE) and the title compound was obtained as a colourless solid (yield: 8.29 g, 17.5 mmol, 35%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.16 (s, 1H), 7.31-7.21 (m, 6H), 7.13 (d, *J* = 7.88 Hz, 2H), 7.09 (d, *J* = 7.97 Hz, 2H), 5.58 (d, *J* = 8.21 Hz, 1H), 5.05-4.97 (m, 2H), 4.60 (dd, *J* = 13.68, 6.42 Hz, 1H), 4.40 (d, *J* = 5.54 Hz, 2H), 3.14 (dd, *J* = 13.57, 4.65 Hz, 1H), 3.01 (dd, *J* = 13.81, 6.53 Hz, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.70, 162.06, 155.85, 135.68, 135.31, 135.15, 129.57, 128.31, 128.28, 127.76, 127.57, 92.30, 66.91, 54.41, 44.54, 36.99 ppm.



(S)-2-(((benzyloxy)carbonyl)amino)-3-(4-(((*tert*-butoxycarbonyl)amino)methyl)phenyl)propanoic acid (10)

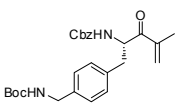
Compound **9** (2.82 g, 5.94 mmol) was treated with 20% w/w NaOH in H_2O/EtOH (1:1) for 1 h after which TLC analysis indicated complete conversion of starting material. Next, 3 M aq. HCl was added until pH 7 and the mixture was concentrated under reduced pressure. The resulting crude compound was dissolved in THF (40 mL) and cooled to 0 °C. Boc_2O (1.5 eq., 8.91 mmol, 2.0 g) was added and the solution was basified by addition of Na_2CO_3 until pH 9. The mixture was stirred at RT for 3 h, after which it was acidified with 10% w/v aq. HCl until pH 2 and extracted with EtOAc (3×). The combined organic layers were extracted with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting crude mixture was purified by column chromatography (20% → 100% EtOAc/PE) and the title compound

was obtained as a colourless solid (yield: 1.90 g, 4.45 mmol, 75%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.32 (s, 1H), 7.36-7.28 (m, 5H), 7.16-7.02 (m, 4H), 5.33 (d, *J* = 7.64 Hz, 1H), 5.09 (q, *J* = 12.32, 12.32, 12.29 Hz, 2H), 4.95 (s, 1H), 4.65 (d, *J* = 6.41 Hz, 1H), 4.26-4.19 (m, 2H), 3.20-3.04 (m, 2H), 1.45 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.79, 156.16, 155.77, 137.47, 136.15, 134.80, 129.61, 128.46, 128.14, 128.03, 127.69, 79.85, 66.99, 54.52, 44.31, 37.30, 28.36 ppm.



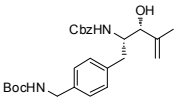
(S)-2-(((benzyloxy)carbonyl)amino)-3-(4-(((*tert*-butoxycarbonyl)amino)methyl)phenyl)-*N*-methoxy-*N*-methylpropionamide (11)

Carboxylic acid **10** (4.45 g, 10.4 mmol) was dissolved in DCM (75 mL). To this were added NH(Me)OMe·HCl (1.5 eq., 15.6 mmol, 1.55 g), HCTU (1.5 eq., 15.6 mmol, 6.45 g) and DiPEA (4.5 eq., 46.7 mmol, 7.72 mL) and the mixture was stirred for 2 h until TLC analysis indicated a completed reaction. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc. This was extracted with 1 M aq. HCl (2×), saturated aq. Na_2CO_3 (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The product was purified by column chromatography (10% → 75% EtOAc/PE) and obtained as colourless oil (yield: 4.81 g, 10.2 mmol, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.29-7.22 (m, 5H), 7.14 (d, *J* = 8.12 Hz, 2H), 7.09 (d, *J* = 8.17 Hz, 2H), 6.02 (d, *J* = 8.49 Hz, 1H), 5.35 (s, 1H), 5.00 (dd, *J* = 28.51, 12.34 Hz, 2H), 4.96-4.94 (m, 1H), 4.21 (d, *J* = 5.20 Hz, 2H), 3.62 (s, 3H), 3.10 (s, 3H), 3.02 (dd, *J* = 13.63, 5.63 Hz, 1H), 2.85 (dd, *J* = 13.27, 7.70 Hz, 1H), 1.43 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.54, 155.50, 137.23, 136.02, 135.04, 129.04, 127.92, 127.48, 127.42, 126.98, 78.64, 66.11, 61.01, 51.78, 43.76, 37.46, 31.52, 27.96 ppm. $[\alpha]_D^{23} = +10.1$ (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{25}H_{33}N_3O_6$ 472.24421 $[M+H]^+$; found 472.24402.



(S)-benzyl (1-(4-((*tert*-butyloxycarbonylamino)methyl)phenyl)-4-methyl-3-oxopent-4-en-2-yl)carbamate (12)

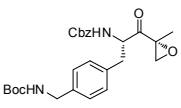
2-Bromopropene (3.5 eq., 14.0 mmol, 1.25 mL) was dissolved in THF (50 mL) and cooled to –78 °C. *t*BuLi (6.5 eq., 26.0 mmol, 16.3 mL; 1.6 M in hexane) was added slowly and the mixture was stirred for 1 h at –78 °C after which Weinreb amide **11** (1 eq., 4.0 mmol, 1.89 g) was added in THF (5 mL). The mixture was allowed to warm to –20 °C in 6 h after which TLC analysis indicated complete consumption of the Weinreb amide. A saturated aqueous NH_4Cl solution and EtOAc were added and the layers were separated. The organic layer was extracted with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after column chromatography (20% → 50% EtOAc/PE) as a colourless oil (yield: 1.71 g, 3.77 mmol, 94%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.33-7.24 (m, 5H), 7.11 (d, *J* = 7.87 Hz, 2H), 6.97 (d, *J* = 8.00 Hz, 2H), 6.03 (s, 1H), 5.85 (s, 1H), 5.77 (d, *J* = 8.18 Hz, 1H), 5.30 (dd, *J* = 14.10, 6.11 Hz, 1H), 5.12-5.08 (m, 1H), 5.04 (dd, *J* = 26.54, 12.35 Hz, 2H), 4.21 (d, *J* = 5.41 Hz, 2H), 3.09 (dd, *J* = 13.79, 5.88 Hz, 1H), 2.89 (dd, *J* = 13.76, 5.97 Hz, 1H), 1.84 (s, 3H), 1.44 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 199.28, 155.66, 155.34, 141.99, 137.40, 136.13, 134.60, 129.27, 128.17, 127.80, 127.71, 126.50, 79.01, 66.45, 55.13, 43.96, 38.76, 28.14, 17.44 ppm. HRMS: calcd. for $C_{26}H_{32}N_2O_5$ 453.23840 $[M+H]^+$; found 453.23818.



benzyl ((2S,3R)-1-(4-((*tert*-butyloxycarbonylamino)methyl)phenyl)-3-hydroxy-4-methylpent-4-en-2-yl)carbamate (13)

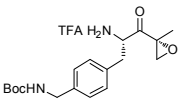
Ketone **12** (2.81 g, 4.30 mmol) was dissolved in MeOH (25 mL) and cooled to 0 °C. To this were added $CeCl_3 \cdot 7H_2O$ (1.5 eq., 6.45 mmol, 2.43 g) and $NaBH_4$ (1.4 eq., 6.0 mmol, 227 mg) portionwise and the mixture was stirred for 5 min. after which TLC analysis indicated a complete conversion. Glacial acetic acid (10 mL) was added and the mixture was concentrated under reduced pressure. The resulting residue was dissolved in EtOAc and extracted with half saturated aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The title compound was obtrained as a colourless oil (yield: 1.79 g, 3.94 mmol, 92%). ^{1}H

NMR (400 MHz, $CDCl_3$): δ = 7.31-7.01 (m, 9H), 5.30 (d, *J* = 9.18 Hz, 1H), 5.06 (s, 1H), 5.00 (d, *J* = 5.19 Hz, 1H), 4.96-4.91 (m, 3H), 4.21 (d, *J* = 4.41 Hz, 1H), 4.16-4.11 (m, 1H), 4.07-3.98 (m, 1H), 2.85 (d, *J* = 12.55 Hz, 1H), 2.65 (dd, *J* = 13.60, 10.41 Hz, 1H), 1.77 (s, 3H), 1.44 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.96, 155.85, 144.44, 137.34, 136.44, 129.34, 128.19, 127.77, 127.66, 127.25, 112.17, 79.25, 76.65, 66.27, 54.06, 44.15, 33.69, 28.22, 18.73 ppm. $[\alpha]_D^{23} = -18.7$ (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{26}H_{34}N_2O_5$ 455.25405 $[M+H]^+$; found 455.25392.



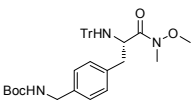
benzyl ((S)-3-(4-((*tert*-butyloxycarbonylamino)methyl)phenyl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)carbamate (14)

Allylic alcohol **13** (1.79 g, 3.94 mmol) was dissolved in DCM (25 mL) and cooled to 0 °C after which vanadyl acetylacetonate (0.1 eq., 0.4 mmol, 107 mg) and *t*BuOOH (3 eq., 12.0 mmol, 2.18 mL; 5.5 M in decane) were added and the mixture was stirred at 0 °C until TLC analysis indicated complete consumption of starting material after 2 h. The mixture was concentrated under reduced pressure, redissolved in EtOAc and extracted with half sat. aq. $NaHCO_3$, H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting product was quickly purified by column chromatography (20% → 60% EtOAc/PE) and immediately subjected to the next step because of the possible instability of the intermediate. The compound was dissolved in DCM (25 mL) and Dess-Martin periodinane (3 eq., 11.0 mmol, 4.50 g) was added. The mixture was stirred at RT for 12 h after which TLC analysis indicated complete conversion. Next, a 1:4 (v/v) mixture (150 mL) of $NaHCO_3$ (sat. aq.)/$Na_2S_2O_3$ (1 M aq.) and the resulting emulsion was stirred vigorously for 30 min. after which the layers were separated and the aqueous layer extracted with DCM. The combined organic layers were extracted with sat. aq. $NaHCO_3$, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after column chromatography (20% → 30% EtOAc/PE) as a colourless oil (yield: 1.03 g, 2.20 mmol, 56%). ^1H NMR (400 MHz, $CDCl_3$): δ = 7.33-7.22 (m, 5H), 7.16 (d, *J* = 7.94 Hz, 2H), 7.08 (d, *J* = 7.95 Hz, 2H), 5.51 (d, *J* = 8.19 Hz, 1H), 5.06-5.01 (m, 1H), 4.97 (d, *J* = 4.39 Hz, 2H), 4.60 (dd, *J* = 12.65, 7.86 Hz, 1H), 4.24 (d, *J* = 4.36 Hz, 2H), 3.26 (d, *J* = 4.62 Hz, 1H), 3.08 (dd, *J* = 13.96, 4.48 Hz, 1H), 2.87 (d, *J* = 4.53 Hz, 1H), 2.70 (dd, *J* = 13.88, 8.12 Hz, 1H), 1.49 (s, 3H), 1.44 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 207.77, 155.74, 155.66, 137.61, 135.97, 134.62, 129.32, 128.26, 127.91, 127.76, 127.44, 79.15, 66.62, 58.99, 54.07, 52.12, 44.07, 36.61, 28.21, 16.34 ppm. $[\alpha]_D^{23} = +82.2$ (*c* = 1, $CHCl_3$).



***tert*-butyl 4-((S)-2-amino-3-((R)-2-methyloxiran-2-yl)-3-oxopropyl)benzylcarbamate TFA salt (15)**

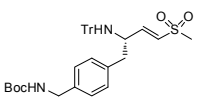
Cbz protected amine **14** (107 mg, 0.23 mmol) was dissolved in MeOH (5 mL) and to this was added TFA (1.2 eq., 0.27 mmol, 21 μL). Argon was bubbled through the solution for 15 min., after which Pd black (10 mg) was added and the flask was charged with hydrogen gas. After 10 min, TLC analysis indicated complete conversion of starting material and all solids were removed by filtration over Celite. Toluene (10 mL) was added and the mixture was concentrated under reduced pressure followed by coevaporation with toluene (2×) in order to remove excess TFA. The purity of the deprotected amine (as TFA salt) was confirmed by LC-MS analysis and the compound was subjected to the next step without further purification.



(S)-*tert*-butyl 4-(3-(methoxy(methyl)amino)-3-oxo-2-(tritylamino)propyl)benzylcarbamate (16)

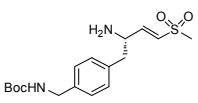
Compound **11** (1.43 g, 3.04 mmol) was dissolved in a 50:1 mixture EtOH/AcOH (25 mL) and argon was bubbled through this solution for 15 min. Next, Pd/C (10% w/w, 0.1 g) was added and hydrogen was bubbled through the mixture until TLC indicated complete consumption of starting material after 4 h. Argon was bubbled through for another 15 min. after which the mixture was filtered over Celite and the filtrate concentrated under reduced pressure. The deprotected amine (as AcOH salt) was obtained in a crude yield of

1.21 g (max. 3.04 mmol) and was subsequently dissolved in DCM (20 mL). To this were added Et_3N (2 eq., 6.08 mmol, 0.85 mL), DMAP (0.1 g) and tritylchloride (1.5 eq., 4.56 mmol, 1.30 g). The mixture was stirred for 6 h after which it was concentrated under reduced pressure, redissolved in EtOAc and extracted with 10 mM aq. HCl and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting mixture was purified by column chromatography (10% → 50% EtOAc/PE) and the title compound was obtained as colourless foam (yield: 0.68 g, 1.17 mmol, 38%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.47 (s, 1H), 7.34 (d, *J* = 7.33 Hz, 6H), 7.26-7.20 (m, 4H), 7.18-7.05 (m, 9H), 5.10 (s, 1H), 4.28 (s, 2H), 4.00 (t, *J* = 5.60, 5.60 Hz, 1H), 3.18 (s, 3H), 2.92 (dd, *J* = 13.24, 5.63 Hz, 1H), 2.77 (dd, *J* = 12.93, 7.51 Hz, 1H), 2.63 (s, 3H), 1.44 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.80, 155.74, 145.92, 137.18, 137.13, 130.25, 128.70, 127.33, 127.15, 125.86, 79.07, 70.59, 60.00, 54.09, 44.19, 41.86, 31.96, 28.20 ppm. $[\alpha]_D^{23} = +58.6$ (*c* = 1, $CHCl_3$).



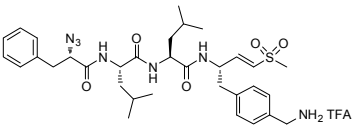
(S,E)-*tert*-butyl 4-(4-(methylsulfonyl)-2-(tritylamino)but-3-en-1-yl)benzylcarbamate (17)

Weinreb amide **16** (0.65 g, 1.12 mmol) was dissolved in Et_2O (15 mL), put under an argon atmosphere and cooled to 0 °C. $LiAlH_4$ (2 eq., 2.25 mmol, 0.56 mL of a 4 M solution in Et_2O) was added slowly and the mixture was stirred at 0 °C for 1 h after which TLC analysis indicated complete conversion of the starting compound. 0.1 M aq. HCl (15 mL) was slowly added and the layers were separated. The organic layer was extracted with 0.1 M aq. HCl and brine, dried over $MgSO_4$ and concentrated under reduced pressure. Diethyl ((methylsulfonyl)methyl)phosphonate (1.5 eq., 1.68 mmol, 0.39 g) was dissolved in THF (20 mL) and cooled to 0 °C under an argon atmosphere. NaH (1.5 eq., 1.68 mmol, 67.2 mg, 60% w/w in mineral oil) was slowly added and the mixture was stirred at 0 °C for 30 min. Next, the freshly obtained aldehyde (in THF (2 mL)) was slowly added and the mixture was stirred for 2 h while slowly warming it to RT. After this time TLC analysis indicated complete conversion of the aldehyde. EtOAc (20 mL) was added and the mixture was extracted with 10 mM aq. HCl (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after column chromatography (20% → 50% EtOAc/PE) as a colourless foam (yield: 0.57 g, 0.95 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.46 (d, *J* = 7.6 Hz, 6H), 7.28 (t, *J* = 7.20, 6.80 Hz, 6H), 7.20 (t, *J* = 7.20, 7.20 Hz, 3H), 7.13 (d, *J* = 7.60 Hz, 2H), 6.87 (d, *J* = 8.00 Hz, 2H), 6.57 (dd, *J* = 14.80, 7.00 Hz, 1H), 5.96 (d, *J* = 14.80 Hz, 1H), 4.80 (s, 1H), 4.24 (d, *J* = 5.60 Hz, 2H), 3.49 (q, *J* = 6.00 Hz, 1H), 2.61 (s, 3H), 2.54 (dd, *J* = 13.20, 5.20 Hz, 1H), 2.33 (dd, *J* = 13.20, 8.20 Hz, 1H), 1.44 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.59, 150.21, 145.74, 137.42, 135.28, 129.53, 128.35, 128.02, 127.70, 127.14, 126.44, 78.91, 71.05, 55.33, 43.79, 42.43, 41.86, 28.09 ppm. $[\alpha]_D^{23} = -21.3$ (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{36}H_{40}N_2O_4S$ 619.26010 $[M+ Na]^+$; found 619.26001.



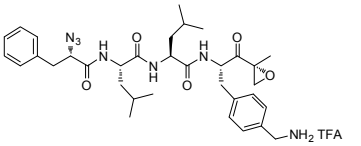
(S,E)-*tert*-butyl 4-(4-(methylsulfonyl)-2-aminobut-3-en-1-yl)benzylcarbamate (18)

Trityl protected amine **17** (0.54 g, 0.90 mmol) was treated with 1% v/v TFA/DCM (15 mL) at RT. To this yellow solution was added H_2O (1 mL) which resulted in a colourless suspension. After stirring the mixture for 30 min., 10 mM aq. HCl (20 mL) was added and DCM was removed under reduced pressure. The aqueous layer was extracted with Et_2O (3×) and basified with $NaHCO_3$ until pH 9, after which it was extracted with DCM (3×). The latter combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The resulting deprotected amine proved to be pure on LC-MS analysis and was subjected to the next step without further purification.



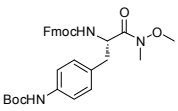
N_3Phe-Leu-Leu-Phe(4-CH_2NH_2)VS TFA salt (4a)

This compound was synthesized according to General procedure I on a 100 μmol scale by addition of amine **18**. The title compound was obtained after RP-HPLC purification (gradient: 20% → 60% MeOH/0.1% aq. TFA) as a colourless solid (yield: 15.4 mg, 20.1 μmol, 20%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.39-7.21 (m, 9H), 6.78 (dd, *J* = 15.20, 5.34 Hz, 1H), 6.55 (dd, *J* = 15.21, 1.52 Hz, 1H), 4.82-4.77 (m, 1H), 4.36-4.27 (m, 2H), 4.17 (dd, *J* = 8.61, 4.80 Hz, 1H), 4.07 (s, 2H), 3.19 (dd, *J* = 14.05, 4.75 Hz, 1H), 3.02-2.95 (m, 3H), 2.92 (s, 3H), 1.63-1.43 (m, 6H), 0.93 (t, *J* = 5.65, 5.65 Hz, 6H), 0.88 (d, *J* = 6.24 Hz, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 174.45, 174.27, 171.95, 146.65, 139.63, 137.85, 133.01, 131.90, 131.30, 130.47, 130.26, 129.67, 128.13, 65.56, 53.74, 53.49, 52.46, 44.11, 42.83, 41.80, 41.61, 40.29, 38.71, 25.95, 25.86, 23.47, 23.46, 21.96, 21.94 ppm. LC-MS: R_t (min): 6.99 (ESI-MS (*m/z*): 654.20 (M + H^+)). HRMS: calcd. for $C_{33}H_{47}N_7O_5S$ 654.34321 [M+ H]$^+$; found 654.34322.



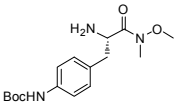
N_3Phe-Leu-Leu-Phe(4-CH_2NH_2)EK TFA salt (4b)

This compound was synthesized according to General procedure I on a 100 μmol scale by addition of amine **15**. The title compound was obtained after RP-HPLC purification (gradient: 20% → 60% MeOH/0.1% aq. TFA) as a colourless solid (yield: 17.6 mg, 23.5 μmol, 24%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.36-7.20 (m, 9H), 4.68 (dd, *J* = 9.34, 4.20 Hz, 1H), 4.38-4.28 (m, 2H), 4.12 (dd, *J* = 8.58, 4.79 Hz, 1H), 4.05 (s, 2H), 3.21 (d, *J* = 4.97 Hz, 1H), 3.15 (dd, *J* = 14.18, 4.69 Hz, 1H), 3.08 (dd, *J* = 13.84, 4.06 Hz, 1H), 2.95-2.87 (m, 2H), 2.72 (dd, *J* = 13.90, 9.34 Hz, 1H), 1.52-1.43 (m, 6H), 1.41 (s, 3H), 0.94-0.83 (m, 12H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 208.54, 174.48, 174.13, 171.69, 139.71, 137.87, 132.95, 131.15, 130.47, 130.14, 129.65, 128.10, 65.59, 60.25, 54.51, 53.40, 53.15, 52.79, 44.14, 42.13, 41.79, 38.73, 37.11, 25.83, 23.49, 22.03, 21.94, 16.81 ppm. LC-MS: R_t (min): 7.36 (ESI-MS (*m/z*): 634.20 (M + H^+)). HRMS: calcd. for $C_{34}H_{47}N_7O_5$ 634.37114 [M+ H]$^+$; found 634.37090.



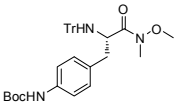
(S)-(9*H*-fluoren-9-yl)methyl (3-(4-(*tert*-butyloxycarbonylamino)phenyl)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (20)

To a suspension of Fmoc-Phe(4-NO_2)-OH (**19**, 1.27 g, 2.93 mmol) in MeOH (60 mL) was added ammonium formate (10 eq., 30.0 mmol, 1.95 g) which resulted in a clear solution. Pd/C (10% w/w, 0.5 g) was added and the mixture was stirred at RT for 14 h after which TLC analysis indicated complete consumption of starting material. All solids were removed by filtration over Celite and the filtrate was concentrated under reduced pressure. In order to remove excess ammonium formate the resulting product was coevaporated with a 3:1 (v/v) mixture of MeOH/H_2O (5×). Next, the residue was dissolved in H_2O (40 mL) containing $NaHCO_3$ (11.7 mmol, 0.99 g) and cooled to 0 °C. To this was added Boc_2O (4.40 mmol, 0.99 g) in 1,4-dioxane (20 mL) and the mixture was allowed to stir at RT for 14 h. The mixture was acidified with 10% w/v aq. HCl until pH 1 and extracted three times with EtOAc. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. Finally the Weinreb amide was created in a peptide couplings procedure similar to that for **11**. The title compound was obtained after column chromatography (10% → 50% EtOAc/PE) as a colourless oil (yield: 1.59 g, 2.91 mmol, 99%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.70-7.66 (m, 2H), 7.53 (dd, *J* = 12.68, 7.49 Hz, 2H), 7.33 (dd, *J* = 13.74, 6.75 Hz, 4H), 7.27-7.21 (m, 2H), 7.11 (d, *J* = 8.33 Hz, 2H), 6.17 (d, *J* = 8.85 Hz, 1H), 5.06-4.98 (m, 1H), 4.32 (dd, *J* = 10.25, 7.60 Hz, 1H), 4.25-4.19 (m, 1H), 4.14 (t, *J* = 7.25, 7.25 Hz, 1H), 3.60 (s, 3H), 3.13 (s, 3H), 3.05 (dd, *J* = 13.68, 5.45 Hz, 1H), 2.90 (dd, *J* = 13.46, 7.49 Hz, 1H), 1.47 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.76, 155.65, 152.62, 143.49, 143.42, 140.78, 137.22, 130.36, 129.47, 127.25, 126.67, 124.85, 124.81, 119.51, 118.14, 79.76, 66.61, 61.13, 51.93, 46.66, 37.40, 31.64, 27.96 ppm.



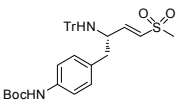
(S)-*tert*-butyl (4-(2-amino-3-(methoxy(methyl)amino)-3-oxopropyl)phenyl)carbamate (21)

To a solution of compound **20** (1.50 g, 2.75 mmol) in THF (20 mL) was added DBU (1.5 mmol, 229 μL). After 10 min. TLC analysis indicated complete consumption of starting material. 1 M aq. HCl (30 mL) and EtOAc (25 mL) were added and the layers were separated. The organic layer was extracted with 1 M aq. HCl and the combined aqueous layers were basified with Na_2CO_3 until pH 10. This layer was extracted with EtOAc (3×) and the latter combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained as a colourless oil (yield: 0.75 g, 2.34 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.62 (s, 1H), 7.33 (d, *J* = 8.14 Hz, 2H), 7.09 (d, *J* = 8.43 Hz, 2H), 4.02-3.95 (m, 1H), 3.57 (s, 3H), 3.16 (s, 3H), 2.98 (dd, *J* = 13.30, 5.56 Hz, 1H), 2.65 (dd, *J* = 13.25, 7.71 Hz, 1H), 1.93 (s, 2H), 1.49 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ = 175.13, 152.66, 137.04, 131.64, 129.29, 118.28, 79.54, 60.96, 52.38, 40.41, 31.82, 27.94 ppm. $[\alpha]_D^{23}$ = +19.6 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{16}H_{25}N_3O_4$ 324.19178 [M+ H]$^+$; found 324.19193.



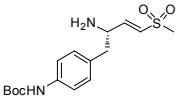
(S)-*tert*-butyl (4-(3-(methoxy(methyl)amino)-3-oxo-2-(tritylamino)propyl)phenyl)carbamate (22)

To a solution of amine **21** (0.38 g, 1.16 mmol) in DCM (15 mL) was added Et_3N (1.2 eq., 1.40 mmol, 195 μL), and tritylchloride (1.2 eq., 1.40 mmol, 0.40 g) and the mixture was stirred for 14 h. DCM was evaporated under reduced pressure and the residue was dissolved in EtOAc, extracted with 10 mM aq. HCl (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after column chromatography (10% → 50% EtOAc/PE) as a colourless foam (yield: 0.61 g, 1.11 mmol, 96%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.46 (s, 1H), 7.36 (d, *J* = 7.55 Hz, 6H), 7.22-7.02 (m, 13H), 6.95 (s, 1H), 4.01-3.95 (m, 1H), 3.10 (s, 3H), 2.88 (dd, *J* = 13.31, 5.95 Hz, 1H), 2.73 (dd, *J* = 13.03, 7.26 Hz, 1H), 2.61 (s, 3H), 1.49 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.86, 152.68, 145.93, 136.93, 132.49, 130.36, 128.66, 127.31, 125.82, 118.18, 79.91, 70.58, 59.93, 41.63, 31.92, 28.15 ppm. $[\alpha]_D^{23}$ = +75.8 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{35}H_{39}N_3O_4$ 566.30133 [M+ H]$^+$; found 566.30120.



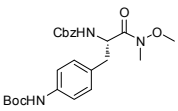
(S,E)-*tert*-butyl (4-(4-(methylsulfonyl)-2-(tritylamino)but-3-en-1-yl)phenyl)carbamate (23)

This compound was prepared from Weinreb amide **22** (0.61 g, 1.11 mmol) in a synthetic procedure similar to that for compound **17** and obtained after column chromatography (10% → 40% EtOAc/PE) as a colourless foam (yield: 0.24 g, 0.41 mmol, 37%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.45 (d, *J* = 7.49 Hz, 6H), 7.28-7.15 (m, 12H), 6.81 (d, *J* = 8.38 Hz, 2H), 6.58 (s, 1H), 6.56 (dd, *J* = 15.04, 6.90 Hz, 1H), 5.92 (d, *J* = 15.11 Hz, 1H), 3.45 (dd, *J* = 12.80, 6.98 Hz, 1H), 2.58 (s, 3H), 2.51 (dd, *J* = 13.36, 5.25 Hz, 1H), 2.28 (dd, *J* = 13.33, 7.86 Hz, 1H), 1.49 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.59, 150.70, 145.91, 137.06, 130.81, 130.06, 128.55, 127.91, 126.64, 118.38, 80.34, 71.26, 55.55, 42.73, 41.68, 28.22 ppm. $[\alpha]_D^{23}$ = −14.8 (*c* = 1, $CHCl_3$).



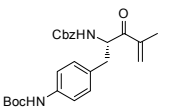
(S,E)-*tert*-butyl (4-(4-(methylsulfonyl)-2-aminobut-3-en-1-yl)phenyl)carbamate (24)

This compound was prepared from trityl protected amine **23** in a synthetic procedure similar to that for compound **18**. The purity was checked by LC-MS analysis and the amine was subjected to the next step without further purification.



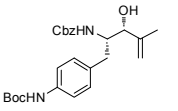
(S)-benzyl (3-(4-(*tert*-butyloxycarbonylamino)phenyl)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (25)

To a solution of amine **21** (0.79 g, 2.43 mmol) in THF (20 mL) was added DiPEA (1.2 eq., 2.91 mmol, 482 μL), and benzylchloroformate (1.1 eq., 2.67 mmol, 392 μL) and the mixture was stirred for 4 h in which a colourless solid precipitated. EtOAc was added and the mixture was extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after column chromatography (10% → 75% EtOAc/PE) as a colourless foam (yield: 0.95 g, 2.06 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.33-7.23 (m, 7H), 7.14 (s, 1H), 7.05 (d, *J* = 8.21 Hz, 2H), 5.80 (d, *J* = 8.71 Hz, 1H), 5.04 (dd, *J* = 26.77, 12.39 Hz, 2H), 4.98-4.94 (m, 1H), 3.62 (s, 3H), 3.13 (s, 3H), 3.01 (dd, *J* = 13.65, 5.91 Hz, 1H), 2.85 (dd, *J* = 13.58, 7.35 Hz, 1H), 1.49 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.72, 155.64, 152.64, 137.21, 136.07, 130.35, 129.52, 128.13, 127.69, 127.64, 118.18, 79.88, 66.41, 61.21, 51.95, 37.53, 31.73, 28.05 ppm. $[\alpha]_D^{23}$ = +8.1 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{24}H_{31}N_3O_6$ 458.22856 [M+ H]$^+$; found 458.22845.



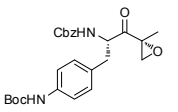
(S)-benzyl (1-(4-(*tert*-butyloxycarbonylamino)phenyl)-4-methyl-3-oxopent-4-en-2-yl)carbamate (26)

This compound was prepared from Weinreb amide **25** (0.90 g, 1.96 mmol) in a synthetic procedure similar to that for compound **12** and obtained after column chromatography (10% → 25% EtOAc/PE) as a colourless oil (yield: 0.70 g, 1.58 mmol, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.37-7.20 (m, 7H), 6.92 (d, *J* = 8.47 Hz, 2H), 6.81 (s, 1H), 6.00 (s, 1H), 5.86 (d, *J* = 1.20 Hz, 1H), 5.66 (d, *J* = 8.15 Hz, 1H), 5.30 (dd, *J* = 14.10, 6.04 Hz, 1H), 5.07 (q, *J* = 12.28, 12.28, 12.25 Hz, 2H), 3.07 (dd, *J* = 13.85, 6.11 Hz, 1H), 2.89 (dd, *J* = 13.86, 5.86 Hz, 1H), 1.84 (s, 3H), 1.49 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 199.48, 155.44, 152.61, 142.11, 137.24, 136.17, 129.91, 129.69, 128.31, 127.92, 127.84, 126.73, 118.22, 80.17, 66.63, 55.33, 38.83, 28.16, 17.54 ppm. $[\alpha]_D^{23}$ = +77.1 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{25}H_{30}N_2O_5$ 439.22275 [M+ H]$^+$; found 439.22276.



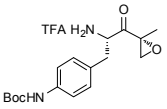
benzyl ((2S,3R)-1-(4-(*tert*-butyloxycarbonylamino)phenyl)-3-hydroxy-4-methylpent-4-en-2-yl)carbamate (27)

This compound was prepared from ketone **26** (0.70 g, 1.85 mmol) in a synthetic procedure similar to that for compound **13** and obtained after column chromatography (10% → 25% EtOAc/PE) as a colourless oil (yield: 0.71 g, 1.85 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.32-7.18 (m, 7H), 7.04 (d, *J* = 8.15 Hz, 2H), 6.77 (s, 1H), 5.17 (d, *J* = 9.01 Hz, 1H), 5.05-4.91 (m, 4H), 4.14-4.10 (m, 1H), 4.05-3.97 (m, 1H), 3.01 (s, 1H), 2.86-2.78 (m, 1H), 2.64 (dd, *J* = 14.18, 9.64 Hz, 1H), 1.76 (s, 3H), 1.50 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.03, 152.82, 144.45, 136.48, 136.33, 132.60, 129.65, 128.24, 127.79, 127.70, 118.49, 112.28, 80.17, 76.61, 66.37, 54.06, 33.46, 28.20, 18.71 ppm. $[\alpha]_D^{23}$ = −13.8 (*c* = 1, $CHCl_3$). HRMS: calcd. for $C_{25}H_{32}N_2O_5$ 441.23840 [M+ H]$^+$; found 441.23843.



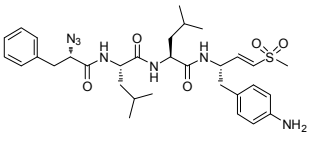
benzyl ((S)-3-(4-(*tert*-butyloxycarbonylamino)phenyl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)carbamate (28)

This compound was prepared from allylic alcohol **27** (0.70 g, 1.58 mmol) in a synthetic procedure similar to that for compound **14** and obtained after column chromatography (10% → 30% EtOAc/PE) as a colourless oil (yield: 0.35 g, 0.76 mmol, 48%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.28-7.17 (m, 7H), 7.10 (s, 1H), 6.96 (d, *J* = 8.42 Hz, 2H), 6.50 (s, 1H), 5.27 (d, *J* = 8.21 Hz, 1H), 5.00-4.87 (m, 2H), 4.51 (dt, *J* = 7.98, 7.96, 4.96 Hz, 1H), 3.19 (d, *J* = 4.85 Hz, 1H), 2.98 (dd, *J* = 14.06, 4.82 Hz, 1H), 2.82 (d, *J* = 4.81 Hz, 1H) 2.62 (dd, *J* = 14.04, 7.84 Hz, 1H), 1.47 (s, 3H), 1.43 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ =. 207.87, 155.70, 155.61, 137.34, 136.02, 129.93, 129.78, 128.40, 128.04, 127.91, 118.53, 112.31, 82.57, 66.80, 59.12, 54.15, 52.24, 36.62, 28.25, 16.47 ppm. $[\alpha]_D^{23}$ = +91.3 (*c* = 1, $CHCl_3$).



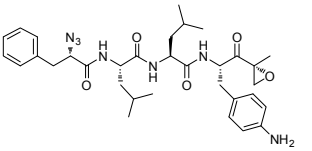
tert-butyl (4-((S)-2-amino-3-((R)-2-methyloxiran-2-yl)-3-oxopropyl)phenyl)carbamate TFA salt (29)

This compound was prepared from Cbz protected amine **28** in a synthetic procedure similar to that for compound **15**. The purity was checked by LC-MS analysis and the amine (as TFA salt) was subjected to the next step without further purification.



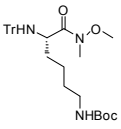
N_3Phe-Leu-Leu-Phe(4-NH_2)VS (5a)

This compound was synthesized according to General procedure I on a 100 μmol scale by addition of amine **24**. The title compound was obtained after RP-HPLC purification (gradient: 10% → 90% ACN/0.1% aq. TFA) as a colourless solid (yield: 8.2 mg, 10.8 μmol, 11%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.25 (d, *J* = 8.29 Hz, 1H), 7.93 (d, *J* = 7.42 Hz, 1H), 7.32 (d, *J* = 8.47 Hz, 2H), 7.26-7.19 (m, 8H), 6.77 (dd, *J* = 15.20, 5.35 Hz, 1H), 6.53 (dd, *J* = 15.21, 1.52 Hz, 1H), 4.81-4.73 (m, 1H), 4.33-4.27 (m, 1H), 4.27-4.22 (m, 1H), 4.14 (dd, *J* = 8.58, 4.82 Hz, 1H), 3.16 (dd, *J* = 14.07, 4.83 Hz, 1H), 2.99-2.90 (m, 3H), 2.89 (s, 3H), 1.59-1.38 (m, 6H), 0.89 (dd, *J* = 6.13, 3.43 Hz, 6H), 0.85 (d, *J* = 6.11 Hz, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 174.55, 174.47, 171.98, 146.53, 138.54, 137.85, 132.22, 132.01, 130.48, 129.68, 128.14, 123.43, 65.57, 53.73, 53.70, 52.53, 42.79, 41.83, 41.60, 39.97, 38.72, 25.94, 25.86, 23.48, 23.46, 21.94 ppm. LC-MS: R_t (min): 6.95 (ESI-MS (*m/z*): 640.0 (M + H^+)). HRMS: calcd. for $C_{32}H_{45}N_7O_5S$ [M+ H]$^+$ 640.32756; found 640.32756.



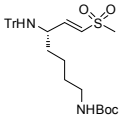
N_3Phe-Leu-Leu-Phe(4-NH_2)EK (5b)

This compound was synthesized according to General procedure I on a 100 μmol scale by addition of amine **29**. The title compound was obtained after RP-HPLC purification (gradient: 30% → 50% ACN/0.1% aq. TFA) as a colourless solid (yield: 4.1 mg, 6.60 μmol, 6.6%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.36 (d, *J* = 8.47 Hz, 2H), 7.28-7.16 (m, 9H), 4.66 (dd, *J* = 9.33, 4.29 Hz, 1H), 4.35-4.27 (m, 2H), 4.10 (dd, *J* = 8.46, 4.68 Hz, 1H), 3.19 (d, *J* = 4.88 Hz, 1H), 3.15-3.10 (m, 2H), 2.98-2.88 (m, 2H), 2.73 (dd, *J* = 8.77, 5.04 Hz, 1H), 1.56-1.42 (m, 8H), 1.38 (s, 3H), 0.90-0.82 (m, 12H) ppm. LC-MS: R_t (min): 7.37 (ESI-MS (*m/z*): 620.20 (M + H^+)). HRMS: calcd. for C33H45N7O5 [M+ H]+ 620.35549; found 620.35532.



((S)-5-(trityl-amino)-5-(methoxy-methyl-carbamoyl)-pentyl)-carbamic acid _tert_-butyl ester (31)

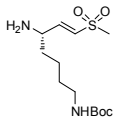
H-Lys(Boc)-N(Me)OMe (**30**) (2.72 g, 6.44 mmol) was dissolved in DCM (40 mL) and to this were added DiPEA (2 eq., 12.9 mmol, 2.13 mL) and tritylchloride (1.1 eq., 7.08 mmol, 1.97 g). The reaction mixture was stirred for 15 h, after which TLC analysis indicated complete consumption of starting material, extracted with H_2O (4×) and dried over $MgSO_4$. The title compound was obtained after purification by column chromatography (DCM → 3% MeOH/DCM) as a colourless foam (yield: 2.30 g, 4.32 mmol, 68%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.49 (d, *J* = 7.47 Hz, 6H), 7.23 (t, *J* = 7.60, 7.60 Hz, 6H), 7.14 (t, *J* = 7.25 Hz, 3H), 4.63 (s, 1H), 3.81 (s, 1H), 3.31 (s, 3H), 3.15-3.03 (m, 2H), 2.70 (s, 3H), 1.81-1.68 (m, 1H), 1.61-1.29 (m, 14H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.06, 155.58, 145.92, 128.52, 127.17, 125.77, 78.26, 70.68, 59.98, 51.52, 40.00, 34.85, 31.77, 29.92, 28.04, 21.55 ppm.



(S,E)-_tert_-butyl (5-(trityl-amino)-7-(methylsulfonyl)hept-6-en-1-yl)carbamate (32)

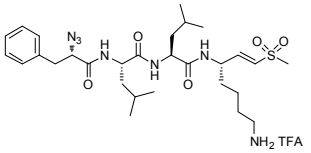
This compound was prepared from Weinreb amide **31** (0.81 g, 1.52 mmol) in a synthetic procedure similar to that for compound **17** and obtained after column chromatography (0% → 30% EtOAc/PE) as a colourless foam (yield: 0.53 g, 0.97 mmol, 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.51-7.46 (m, 6H), 7.29-7.23 (m, 6H), 7.20-7.14 (m,

3H), 6.62 (dd, *J* = 15.05, 6.98 Hz, 1H), 6.22 (d, *J* = 15.06 Hz, 1H), 4.68-4.61 (m, 1H), 3.28-3.20 (m, 1H), 2.98-2.89 (m, 2H), 2.71-2.67 (m, 3H), 1.42 (s, 9H), 1.30-0.81 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.64, 150.93, 145.89, 128.38, 128.01, 127.69, 126.42, 78.62, 71.06, 53.72, 42.56, 39.77, 35.29, 29.54, 28.14, 22.08 ppm.



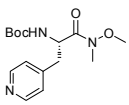
(S,E)-tert-butyl (5-amino-7-(methylsulfonyl)hept-6-en-1-yl)carbamate (33)

This compound was prepared from trityl protected amine **32** in a synthetic procedure similar to that for compound **18**. The purity was checked by LC-MS analysis and the amine was subjected to the next step without further purification.



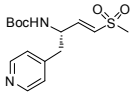
N_3Phe-Leu-Leu-LysVS TFA salt (7)

This compound was synthesized according to General procedure I on a 360 μmol scale by addition of amine **33**. The title compound was obtained after RP-HPLC purification (gradient: 20% → 65% ACN/0.1% aq. TFA) as a colourless solid (yield: 96.5 mg, 134 μmol, 37%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.16-7.04 (m, 5H), 6.65 (dd, *J* = 15.20, 5.10 Hz, 1H), 6.46 (dd, *J* = 15.21, 1.40 Hz, 1H), 4.44 (td, *J* = 9.60, 4.87, 4.87 Hz, 1H), 4.17 (td, *J* = 9.78, 4.95, 4.95 Hz, 2H), 4.04 (dd, *J* = 8.56, 4.80 Hz, 1H), 3.05 (dd, *J* = 14.05, 4.77 Hz, 1H), 2.82 (dd, *J* = 16.0, 8.00 Hz, 1H), 2.82 (s, 3H), 2.76 (t, *J* = 7.42, 7.42 Hz, 2H), 1.65-1.22 (m, 12H), 0.81 (d, *J* = 6.25 Hz, 3H), 0.76 (d, *J* = 6.04 Hz, 6H), 0.72 (d, *J* = 5.77 Hz, 3H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 174.72, 174.64, 171.90, 147.77, 137.79, 131.19, 130.46, 129.64, 128.10, 65.47, 53.82, 53.67, 50.53, 42.83, 41.56, 41.44, 40.54, 38.66, 33.76, 27.89, 25.97, 25.80, 23.77, 23.42, 21.99, 21.93 ppm. LC-MS: R_t (min): 5.73 (ESI-MS (*m/z*): 606.5 (M + H^+)). HRMS: calcd. for $C_{29}H_{47}N_7O_5S$ [M+ H]$^+$ 606.34321; found 606.34299.



(S)-*tert*-butyl (1-(methoxy(methyl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-yl)carbamate (35)

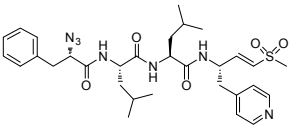
To a mixture of Boc-β-(4-pyridyl)-L-alanine (**34**, 300 mg, 1.08 mmol) and NH(Me)OMe·HCl (1.2 eq., 1.30 mmol, 129 mg) in DCM (10 mL) was added HCTU (1.2 eq., 1.30 mmol, 536 mg) and DiPEA (3.5 eq., 3.78 mmol, 625 μL) and the mixture was stirred for 1 h after which TLC analysis indicated complete conversion of starting material. The solvent was evaporated under reduced pressure en the residue was taken up in EtOAc, extracted with sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The title compound was obtained after purification by column chromatography (EtOAc → 2% MeOH/EtOAc) as a pale yellow solid (yield: 334 mg, 1.08 mmol, quant.). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.40 (d, *J* = 4.59 Hz, 2H), 7.29 (d, *J* = 4.88 Hz, 2H), 6.97 (d, *J* = 7.26 Hz, 1H), 4.77-4.68 (m, 1H), 3.76 (s, 3H), 3.15 (s, 3H), 3.02 (dd, *J* = 13.59, 4.85 Hz, 1H), 2.83-2.77 (m, 1H), 1.32 (s, 9H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 173.49, 157.58, 149.88, 149.50, 126.48, 80.55, 62.25, 52.71, 38.03, 32.49, 28.66 ppm. $[\alpha]_D^{23}$ = −2.9 (*c* = 1, MeOH). HRMS: calcd. for $C_{15}H_{43}N_3O_4$ [M+ H]$^+$ 310.17613; found 310.17615.



(S,E)-*tert*-butyl (4-(methylsulfonyl)-1-(pyridin-4-yl)but-3-en-2-yl)carbamate (36)

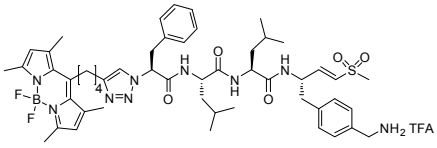
Weinreb amide **35** (0.33 g, 1.08 mmol) was dissolved in THF (15 mL), put under an argon atmosphere and cooled to 0 °C. $LiAlH_4$ (1.5 eq., 1.68 mmol, 1.68 mL of a 1 M solution in THF) was added slowly and the mixture was stirred at 0 °C for 1 h after which TLC analysis indicated complete conversion of the starting compound. 0.1 M aq. HCl (1 mL) was slowly added and the mixture was stirred vigorously for 5 min. The organic layer was extracted with sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. Diethyl ((methylsulfonyl)methyl)phosphonate (1.5 eq., 1.68 mmol, 0.39 g) was dissolved in THF (20 mL) and cooled to 0 °C under an argon atmosphere. NaH (1.3 eq., 1.46 mmol, 58.0 mg, 60% w/w in

mineral oil) was slowly added and the mixture was stirred at 0 °C for 30 min. Next, the freshly obtained aldehyde (in THF (2 mL)) was slowly added and the mixture was stirred for 2 h while slowly warming it to RT. After this time TLC analysis indicated complete conversion of the aldehyde. The reaction was quenched by addition of 1 M aq. HCl (1 mL) after which the mixture was diluted with EtOAc and extracted with sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after column chromatography (40% → 80% Acetone/PE) as a colourless solid (yield: 117 mg, 0.36 mmol, 32%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.50 (d, *J* = 5.28 Hz, 2H), 7.15 (d, *J* = 5.90 Hz, 2H), 6.92 (dd, *J* = 15.10, 4.76 Hz, 1H), 6.53 (d, *J* = 15.10 Hz, 1H), 5.56 (d, *J* = 8.99 Hz, 1H), 4.80-4.72 (m, 1H), 3.02-2.84 (m, 2H), 2.93 (s, 3H), 1.39 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 154.87, 149.59, 146.40, 145.41, 130.04, 124.51, 80.10, 51.10, 42.58, 39.48, 28.06 ppm. $[\alpha]_D^{23}$ = −6.0 (*c* = 1, MeOH). HRMS: calcd. for $C_{15}H_{22}N_2O_4S$ [M+ H]$^+$ 327.13730; found 327.13741.



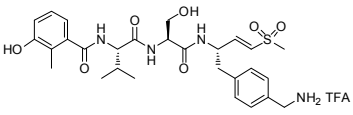
N_3Phe-Leu-Leu-Phe(4-N)VS (6)

This compound was synthesized according to General procedure I on a 300 μmol scale by addition of amine **36** (after Boc deprotection with TFA/DCM 1:1 v/v, **37**). The title compound was obtained after RP-HPLC purification (gradient: 25% → 45% ACN/0.1% aq. TFA) as a colourless syrup (yield: 89.0 mg, 142 μmol, 48%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.62 (d, *J* = 6.12 Hz, 2H), 7.85 (d, *J* = 6.16 Hz, 2H), 7.21-7.06 (m, 5H), 6.81 (dd, *J* = 15.13, 5.03 Hz, 1H), 6.62 (dd, *J* = 15.23, 1.46 Hz, 1H), 5.00-4.93 (m, 1H), 4.20-4.14 (m, 1H), 4.10-4.04 (m, 2H), 3.31 (dd, *J* = 13.99, 4.46 Hz, 1H), 3.11-3.03 (m, 2H), 2.88 (s, 3H), 2.86-2.82 (m, 1H), 1.51-1.08 (m, 6H), 0.80 (dd, *J* = 7.81, 6.26 Hz, 6H), 0.75 (d, *J* = 6.00 Hz, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 174.63, 174.48, 171.74, 160.53, 145.75, 142.38, 137.86, 132.60, 130.47, 129.63, 129.45, 128.07, 65.49, 53.64, 53.55, 50.74, 42.76, 41.57, 41.45, 40.32, 38.64, 25.92, 25.80, 23.49, 23.37, 21.86, 21.80 ppm. LC-MS: R_t (min): 6.86 (ESI-MS (*m/z*): 626.2 (M + H$^+$)). HRMS: calcd. for $C_{31}H_{43}N_7O_5S$ [M+ H]$^+$ 626.31191; found 626.31172.



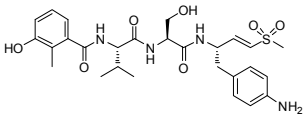
Bodipy-triazole-Phe-Leu-Leu-Phe(4-CH_2NH_2)VS TFA salt (39)

Compound **4a** (5.68 mg, 8.69 μmol) and Bodipy-alkyne (1.5 eq., 13.0 μmol, 4.28 mg) were dissolved in a 1:1:1 mixture of H_2O/*t*BuOH/Tol (1.5 mL) and to this were added $CuSO_4$ (0.1 eq., 0.87 μmol, 0.87 μL of a 1M solution in H_2O) and sodium ascorbate (0.15 eq., 1.3 μmol, 1.3 μL of a 1M solution in H_2O) and the reaction was stirred at 80 °C for 4 h. LC-MS analysis revealed complete consumption of the azide and formation of a single product (R_t (min.): 10.41 (ESI-MS (*m/z*): 981.20 (M + H$^+$))), which was assigned to be the corresponding benzaldehyde. The mixture was concentrated under reduced pressure and dissolved in MeOH (1.5 mL). To this were added NH_4OAc (10 eq., 70 μmol, 5.4 mg) and $NaCNBH_4$ (2 eq., 15 μmol, 1.0 mg) and the reaction was stirred for 15 h, after which LC-MS analysis indicated a complete disappearance of the aldehyde peak. The reaction was quenched by addition of aqueous HCl (100 μL, 1M) and the mixture was concentrated under reduced pressure. The title compound was obtained after RP-HPLC purification (gradient: 30% → 70% ACN/0.1% aq. TFA) as a red/brown solid (yield: 2.1 mg, 2.14 μmol, 29%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.77 (s, 1H), 7.31 (d, *J* = 7.91 Hz, 2H), 7.25 (d, *J* = 8.08 Hz, 2H), 7.03-6.97 (m, 5H), 6.75 (dd, *J* = 15.17, 5.40 Hz, 1H), 6.50 (dd, *J* = 15.26, 1.28 Hz, 1H), 6.08 (s, 2H), 5.52 (dd, *J* = 10.52, 5.15 Hz, 1H), 4.85-4.81 (m, 1H), 4.29-4.22 (m, 2H), 3.95 (s, 2H), 3.37-3.34 (m, 2H), 2.97-2.91 (m, 4H), 2.87 (s, 3H), 2.72-2.67 (m, 2H), 2.40 (s, 6H), 2.33 (s, 6H), 1.88-1.79 (m, 2H), 1.64-1.41 (m, 8H), 0.93-0.75 (m, 12H) ppm. LC-MS: R_t (min): 8.42 (ESI-MS (*m/z*): 982.40 (M + H$^+$)). HRMS: calcd. for $C_{52}H_{70}BF_2N_9O_5S$ [M+ H]$^+$ 982.53545; found 982.53653.



(Val-Ser-Phe(4-CH_2NH_2)-methyl vinyl sulfone)-3-hydroxy-2-methylbenzamide (40)

This compound was synthesized according to General procedure I on a 245 μmol scale by addition of amine **18**. The title compound was obtained after RP-HPLC purification (gradient: 10% → 25% ACN/0.1% aq. TFA) as a colourless solid (yield: 57.3 mg, 83.2 μmol, 34%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.22 (d, *J* = 8.48 Hz, 2H), 7.19 (d, *J* = 8.45 Hz, 2H), 6.95 (t, *J* = 7.80 Hz, 1H), 6.77-6.69 (m, 3H), 6.65 (dd, *J* = 15.17, 1.46 Hz, 1H), 4.81-4.76 (m, 1H), 4.29 (t, *J* = 5.53 Hz, 1H), 4.17 (d, *J* = 7.16 Hz, 1H), 3.90 (d, *J* = 5.45 Hz, 2H), 3.69 (dd, *J* = 10.78, 5.01 Hz, 1H), 3.60 (dd, *J* = 10.76, 6.19 Hz, 1H), 2.92 (dd, *J* = 13.83, 6.47 Hz, 1H), 2.84-2.80 (m, 1H), 2.82 (s, 3H), 2.10-2.01 (m, 1H), 2.06 (s, 3H), 0.89 (dd, *J* = 6.67, 4.95 Hz, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 174.00, 173.61, 171.83, 157.08, 146.95, 139.62, 139.11, 132.93, 131.78, 131.18, 130.18, 127.60, 123.26, 119.16, 117.19, 62.90, 61.62, 56.49, 52.60, 44.04, 42.84, 40.16, 31.40, 19.89, 19.06, 13.06 ppm. LC-MS: R_t (min): 4.19 (ESI-MS (*m/z*): 575.20 (M + H^+)). HRMS: calcd. for $C_{28}H_{38}N_4O_7S$ [M+ H]$^+$ 575.25340; found 575.25336.



(Val-Ser-Phe(4-NH_2)-methyl vinyl sulfone)-3-hydroxy-2-methylbenzamide (41)

This compound was synthesized according to General procedure I on a 100 μmol scale by addition of amine **24**. The title compound was obtained after RP-HPLC purification (gradient: 10% → 25% ACN/0.1% aq. TFA) as a colourless solid (yield: 14.2 mg, 21.0 μmol, 21%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.33 (d, *J* = 8.45 Hz, 2H), 7.19 (d, *J* = 8.46 Hz, 2H), 7.00 (t, *J* = 7.81 Hz, 1H), 6.84-6.70 (m, 4H), 4.88-4.80 (m, 1H), 4.33 (dd, *J* = 6.11, 5.13 Hz, 1H), 4.22 (d, *J* = 7.14 Hz, 1H), 3.72 (dd, *J* = 10.74, 5.01 Hz, 1H), 3.64 (dd, *J* = 10.75, 6.27 Hz, 1H), 3.03 (dd, *J* = 13.89, 5.94 Hz, 1H), 2.90-2.85 (m, 1H), 2.88 (s, 3H), 2.11 (s, 3H), 2.10-2.06 (m, 1H), 0.94 (t, *J* = 6.28 Hz, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 174.12, 173.55, 171.89, 157.13, 146.75, 139.97, 139.12, 132.23, 131.98, 130.63, 127.60, 124.19, 123.27, 119.14, 117.18, 62.90, 61.63, 56.46, 52.54, 42.80, 39.85, 31.40, 19.87, 19.04, 13.04 ppm. LC-MS: R_t (min): 3.99 (ESI-MS (*m/z*): 561.07 (M + H^+)). HRMS: calcd. for $C_{27}H_{36}N_4O_7S$ [M+ H]$^+$ 561.23775; found 561.23775.

Biological evaluation

Competition assays in cell-lysate

Whole cell lysates of HEK-293T or EL-4 were made by sonication in 3 volumes of lysis buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 250 mM sucrose, 2 mM ATP, 0.025% digitonin. Protein concentration was determined by the Bradford assay. Cell lysates (13.5 μg total protein for HEK lysates and 9 μg total protein for EL-4 lysates) were exposed to the inhibitors for 1 h at 37 °C prior to incubation with MV151 (0.5 μM) for 1 h at 37 °C. Reaction mixtures were boiled with Laemmli's buffer containing β-mercaptoethanol for 5 min. before being resolved by 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532nm, λ_{em} 560 nm) to detect MV151 and Cy2/Fam settings (λ_{ex} 488 nm, λ_{em} 520 nm) to detect compound **39**.

Competition assays in living cells

Human embryonic kidney cells (some 1 × 10^6) were cultured in 6-well plates in DMEM containing 10% fetal calf serum, 10 units/mL penicillin and 10 μg/mL streptomycin in a 7% CO_2 humidified incubator at 37 °C overnight. Part of the medium was taken and to this was added the appropriate inhibitor in DMSO (1 μL of a 1,000× stock solution), after which the medium was added to the cells. The cells were incubated with the inhibitors for 4 h at 37 °C and this was followed by

addition of MV151 (1 μL of a 5 mM stock solution in DMSO) and incubation for 2 h at 37 °C. Next, the medium was removed and the cells were washed with PBS and harvested. After flash freezing in liquid N_2, the cells were resuspended in 4 volumes of homogenation buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin) containing 10 μM AdaKBio, sonicated (12 W, 1 min.) and centrifuged at 16,000 rcf at 0 °C for 20 min. The supernatant was collected and the protein concentration was determined by the Bradford assay. All samples were normalized to the same protein concentration with lysis buffer. After boiling the samples with Laemmli's buffer containing β-mercaptoethanol for 5 min. and resolving by 12.5% SDS-PAGE the residual proteasome activity was detected as described above.

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6 Probing the 20S Proteasome Cavity with Photoreactive Peptide Vinyl Sulfones

6.1 Introduction

Inhibitors and activity-based probes have proven their value in the study of proteasomal functioning and the role of the individual catalytic subunits (β1, β2 and β5).[1] Some prominent proteasome inhibitors (Figure 1) are the peptide boronic acid Bortezomib (**1**, PS-341, Velcade),[2] the natural product epoxomicin (**2**)[3] and peptide vinyl sulfone ZL_3VS (**3**).[4] Other examples comprise the β5 and β2 subunit specific inhibitors discussed in Chapters 4 and 5 respectively. Somewhat surprising, given its involvement in the multitude of physiological processes, the proteasome has been found to be a valid drug target, and Bortezomib is now used in the clinic as a last resort treatment for multiple myeloma.[5] The small proteasome inhibitors **1-3** have in common that they are not selective for one specific catalytic subunit, but neither are capable of disabling all three subunits with equal efficiency. Kessler *et al.*[6] hypothesized that an extended version of the hydrophobic peptide vinyl sulfones would result in a better mimic of the natural substrates and an increased mean residence time of the inhibitor at the active centre. Indeed they found that $Ada(Ahx)_3L_3VS$ (**4**) and analogues thereof are much more potent and less selective proteasome inhibitors than their tri- or tetrapeptide vinyl sulfone counterparts. Arguably, extended peptide-like inhibitors such as **4** resemble the manner in which polypeptidic proteasome substrates are positioned in the inner proteasome cavity more closely, when compared to the tri- or tetrapeptide based inhibitors. This assumption invites a new application of extended proteasome activity-based probes, in which they are used to probe interactions of residues at positions

distal to the active site-reactive group. With this aim in mind, a panel of bifunctional ABPs was developed. These ABPs bind active proteasome subunits via their C-terminal vinyl sulfone warhead and can successively be crosslinked, via the N-terminal photocrosslinker, to residues it associates with (Figure 2A), providing information on the orientation the inhibitor adapts within the 20S cavity.

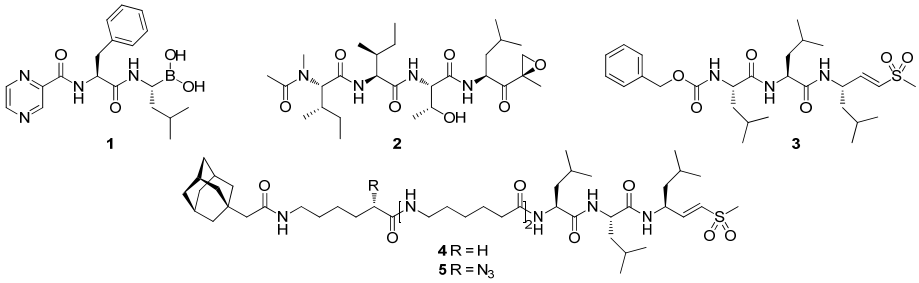


Figure 1. Some proteasome inhibitors known in literature.

The target extended peptide vinyl sulfones, equipped with three different photoreactive moieties (**6-8**) are shown in Figure 2B. They are analogues of proteasome probe **5** (Figure 1) described by Ovaa *et al.*,[7] which differs from **4** in the azide, introduced in the Ahx moiety closest to the N-terminus. This azide allows two-step labelling of proteasome activity (in living cells). For this, a biological sample is incubated with ABP **5** and subsequently treated with a biotin-phosphane reagent, which reacts with the azido moiety in a Staudinger-Bertozzi ligation.[8] Here, the synthesis of compounds **6-8** and the result of incubating purified human erythrocyte 20S proteasome with these, followed by photolysis of the photocrosslinking moiety and Staudinger-Bertozzi ligation are described.

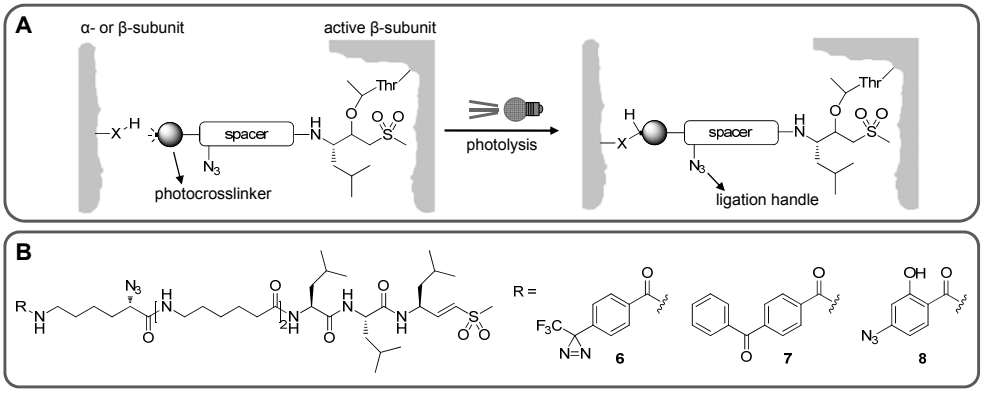


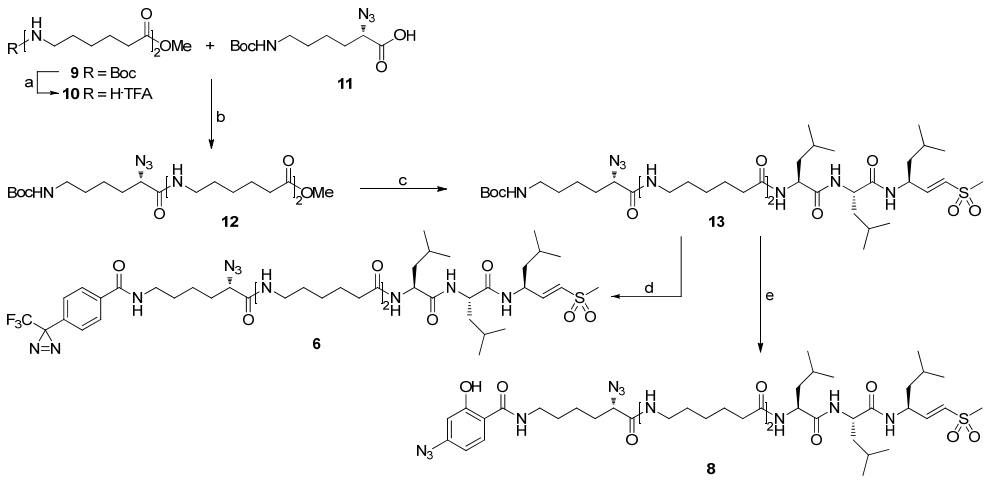
Figure 2. (A) The bifunctional ABP binds an active site subunit and is subsequently photo-crosslinked to another subunit. X = C, N, O, S. (B) Proteasome probes discussed in this chapter.

6.2 Results and Discussion

The target compounds were synthesized via a solution-phase Boc-based peptide synthesis protocol as shown in Scheme 1. The Boc protecting group in Boc-Ahx_2-OMe (**9**)[9] was removed and the resulting amine (**10**) was coupled to α-azido-ε-Boc-lysine (**11**),[10] yielding tripeptide **12**. Saponification of the methyl ester followed by coupling to tripeptide vinyl sulfone TFA·H-Leu_3-VS[4] gave hexapeptide **13**. Acidic removal of the N-terminal Boc protecting group followed by a final HCTU mediated peptide coupling with 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoic acid[11] resulted in proteasome probe **6** in 72% yield. Compound **7** was synthesized in a similar fashion, employing 4-benzoylbenzoic acid in the final coupling step, resulting in 77% yield.

The synthesis of compound **8** proved to be less straightforward. Boc- deprotection of compound **13** and coupling to 4-azido-2-hydroxybenzoic acid[12] gave only a small amount of the desired product. The main product formed was the N-terminal trifluoro-acetylated version of **13** (as evidenced from LC-MS analysis). In order to circumvent this undesired reaction, compound **13** was deprotected with dry HCl in 1,4-dioxane and the final coupling step was executed with 4-azido-2-hydroxybenzoic acid *N*-hydroxysuccinimide ester,[13] which allowed formation of target compound **8** in a yield of 53%. To prevent light initiated decomposition of the photocrosslinking moieties, all final compounds were stored in the dark.

Scheme 1. Synthesis of photoreactive proteasome probes **6** and **8**.



Reagents and conditions: (a) TFA, DCM, quant.; (b) HCTU, DiPEA, DCM, 81%; (c) i) LiOH, MeOH; ii) TFA·H-Leu_3-VS,[4] HCTU, DiPEA, DCM, 71%; (d) i) TFA, DCM; ii) 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoic acid,[11] HCTU, DiPEA, DCM, 72%; (e) i) HCl, 1,4-dioxane; ii) 4-azido-2-hydroxybenzoic acid *N*-hydroxysuccinimide ester,[13] DMF, 53%.

The ability of each probe to label the proteasome active sites was assessed in competition assays employing mouse lymphoma cell extracts (EL-4) in combination with

the fluorescent broad spectrum proteasome probe MV151[14] (see also Chapter 4). The three probes display approximately equal characteristics in terms of the overall potency and the lack of selectivity towards the individual active subunits (Figure 3). In general, they appear to be slightly less active compared to pan-reactive inhibitor **4**.[6,14] At a concentration of 10 μM (almost) all proteasome activity is blocked by each of the three compounds and for this reason all succeeding experiments were conducted at this concentration.

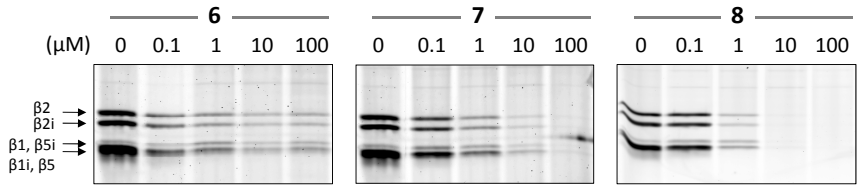


Figure 3. Competition studies of proteasome probes **6-8**. Extracts of EL-4 cells (10 μg) were incubated with increasing concentrations of the probes for 1 hour at 37 °C. Residual proteasome activity was labelled with MV151 (0.5 μM final concentration). After denaturation and resolving by 12.5% SDS-PAGE, the gels were analyzed by fluorescence scanning. Inhibition of a proteasome active site is reflected by disappearance of the corresponding band.

At this stage, three sets of three samples containing purified human 20S proteasome were treated with either one of the three probes for two hours at 37 °C in the dark. Next, the samples were either kept in the dark or exposed to UV light (365 nm) for 30 minutes or 60 minutes at 0 °C, prior to treatment with biotin-phosphane **14**[15] for one hour at 37 °C. After denaturation and separation of the protein contents by gel-electrophoresis, all biotinylated proteins were visualized by Western blotting. AdaK(biotin)Ahx$_3$L$_3$VS (Ada)[6] was used as a positive control for labelling of all proteasome active subunits. The results are shown in Figure 4. As expected from the previous results (Figure 3), all three peptide vinyl sulfones efficiently label the three catalytic activities (β1, β2 and β5) present in the constitutive 20S proteasome. An interesting observation is the difference in signal intensity for probe **8** compared to **6** and **7** (compare the non-exposed samples to the corresponding Ada labelled samples), which can be caused by several factors. An explanation might be a difference in potency between the probes, however, with the results from the competition assays in mind (Figure 3), this seems highly unlikely. Another possibility arises from the fact that compound **8** contains two azides, instead of one as in **6** and **7**. The difference in labelling intensity is especially substantial for the non-UV light-exposed samples, in which case the azide present in the photocrosslinker is still intact and is very well capable of participating in the Staudinger-Bertozzi ligation.[16] Therefore, either the aryl azide is much more reactive compared to the aliphatic azide, which results in a more efficient reaction and hence, a bigger signal intensity, or two biotin-phosphane reagents have reacted with this probe, which would result in a two-fold increase in intensity. A combination of these effects can not be excluded.

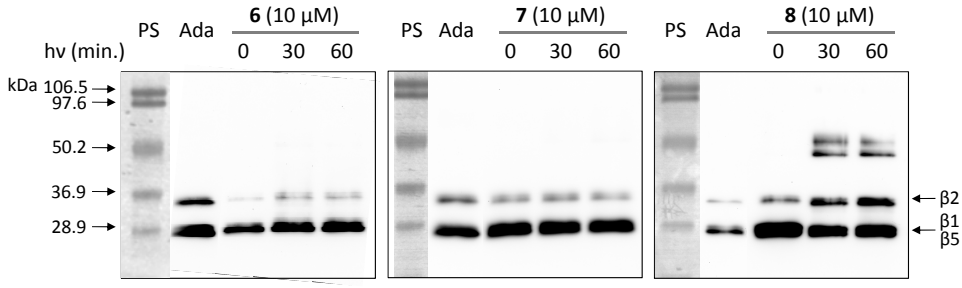


Figure 4. Labelling of purified 20S proteasome with probes **6-8**, followed by light activation of the photoreactive moiety. Purified 20S proteasome (200 ng) was incubated with compounds **6**, **7** or **8** (10 μM final concentration) for two hours at 37 °C, followed by irradiation of the samples with UV light (λ = 365 nm) at 0 °C for 0, 30 or 60 minutes. After denaturing and resolving by 12.5% SDS-PAGE all biotinylated proteins were visualized by Western blotting. PS: pre-stained marker low range (Bio-Rad). Ada: samples were incubated with AdaK(biotin)Ahx$_3$L$_3$VS (10 μM final concentration).

The formation of a new construct with a higher molecular weight is the expected result when effective photocrosslinking of the covalently bound probe to another proteasome subunit occurs. This would be reflected by the appearance of a new band, which corresponds to a higher molecular weight polypeptide. The samples labelled with probes **6** and **7** and exposed to UV light do not show any difference compared to the non-exposed samples, which indicates that photocrosslinking efficiency of these compounds to other proteasome subunits is at most marginal. Either the photocrosslinking moiety was not activated at all, or the reactive species (after UV light mediated activation) was unable to react with another proteasome subunit. Interestingly, the photocrosslinking moiety in **8** is able to crosslink other subunits, which is shown by the appearance of two new bands with a mass of approximately 50 kDa. Indeed, these bands are within the expected mass range corresponding to either β1 or β5 attached to another proteasome subunit.[17]

The successive labelling-photocrosslinking properties of compound **8** were further explored in an experiment in which the samples (obtained after incubation as described above) were irradiated at various durations (Figure 5A). Again, two new bands, corresponding to an increased molecular weight (~50 kDa), appear upon exposure to light. Remarkably, after five minutes of irradiation the bands are already slightly visible and maximal intensity is reached after ten minutes.

To assess the nature of the newly formed bands, four samples of purified 20S proteasome were incubated with compound **8** and irradiated for 0, 15, 30 or 60 minutes. Subsequent Staudinger-Bertozzi ligation, SDS-PAGE and silver staining allowed visualization of all proteins. The results are shown in Figure 5B. All 14 distinct 20S constitutive proteasome proteins ($\alpha_{1\text{-}7}$ and $\beta_{1\text{-}7}$) are now visible. Those samples that were exposed to UV light reveal one additional band (indicated by the arrows) corresponding to a higher molecular weight polypeptide. This is in contrast to the results shown in Figure 5A, in which two bands were visible, however it might be possible that other bands are present, but are invisible because of the high intensity background at higher

MW. Nonetheless, this one band was cut from the gel (in each of the three lanes) and analyzed by LC-MS/MS after in-gel tryptic digest. The results were identical for each of the three lanes and multiple characteristic peptides for both the β5 and β6 subunits were identified (Table 1). This can only be the result of the formation of a covalent linkage between these two subunits and the molecular weight of the construct (calculated MW is 49.8 kDa) correlates well with the expected mass of the, via compound **8**, crosslinked subunits.

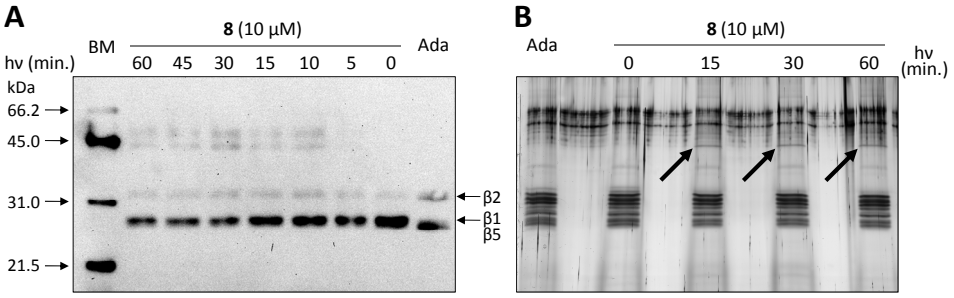


Figure 5. Labelling of purified 20S proteasome with probe **8**, followed by light initiated photocrosslinking. Purified 20S proteasome (200 ng) was incubated with **8** (10 μM final concentration) for two hours at 37 °C, after which the samples were irradiated with UV light (λ = 365 nm) for increasing amounts of time (0-60 minutes). The samples were denatured and resolved by 12.5% SDS-PAGE. Visualization of (A) all biotinylated proteins by Western blotting and (B) the total protein content by silver stain. The arrows in (B) indicate the excised bands analyzed by LC-MS/MS after in-gel tryptic digest. BM: biotinylated marker low range (Bio-Rad). Ada: samples were incubated with AdaK(biotin)Ahx$_3$L$_3$VS (10 μM final concentration).

Table 1. Proteasome subunits identified from the indicated bands in Figure 4B after in-gel tryptic digest and LC-MS/MS analysis.

Subunit	Accession number	Mass (kDa)	n_p[a]	Seq. coverage
β5	IPI00479306	22.4	6	36.3%
β6	IPI00025019	26.5	4	25.7%

[a] n_p = number of identified peptides.

This result raises the question in what way the crosslinked probe is orientated within the proteasome cavity. There are two possibilities: either a neighbouring β5 and β6 subunit, within the same β ring, were crosslinked or the subunits from two different β rings were covalently attached to each other. The calculated maximum length of compound **8** is approximately 38 Å.[18] In comparison, the distance between two active sites of neighbouring β subunits (within the same β ring) is 28 Å and that of the subunits from two separate β rings 65 Å (determined from X-ray analysis of the yeast proteasome).[19-21] It must be noted that the β6 subunit does not contain an active site, but photocrosslinking can take place at any subunit site, therefore the minimal distance

the probe needs to span is from the β5 active site to the edge of β6 and is probably shorter than the distance between two active sites. Although no solid conclusions can be drawn from this, it is unlikely that the probe spans the entire distance between β5 and β6 from two different β rings.

An interesting observation from the results presented here is the appearance of only two bands (of which only one could be further analyzed) of higher molecular weight, whereas many more are possible. An explanation might be that the overall photocrosslinking efficiency is low, so that only a small, undetectable, percentage of the subunits has been crosslinked. In addition to that, it might also be possible that the majority of the crosslinking takes place within the same subunit, which excludes the formation of higher molecular weight polypeptides. Finally, a third possibility might be that the probe is orientated relatively rigid in the 20S cavity and can therefore only be crosslinked to a limited number of subunits or is 'solvent exposed'.

6.3 Conclusion

In summary, the synthesis of three photoreactive peptide vinyl sulfones is described. They can be used to label the proteasome active sites in a two step fashion, however only 4-azidophenyl containing probe **8** was capable of crosslinking to other subunits. The labelling-photocrosslinking approach in combination with two step ligation and LC-MS/MS analysis may become an alternative for existing methods (for instance X-ray crystallography)[20-24] in proteasome research, although considerable optimizations are needed to extrapolate this methodology to a study towards an inhibitor's mode of action and orientation within the 20S core particle. For example, improvements can be made in the photocrosslinking efficiency, or visualization methods. Also, the introduction of elongated spacers between nucleophilic trap and photoactivatable moiety can lead to a more complete picture of a substrate's structural orientation.

Experimental section

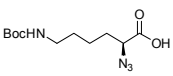
General

Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), methanol (MeOH), 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. *O*-(1*H*-6-Chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased at Iris Biotech (Marktrewitz, Germany). Traces of water were removed from reagents used in reactions that require anhydrous conditions by coevaporation with toluene. Solvents that were used in reactions were stored over 4 Å molecular sieves, except methanol and acetonitrile which were stored over 3 Å molecular sieves. 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, ethyl acetate and petroleum ether (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid

(12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to CD_3OD 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* = 150-2,000) and dioctylpthalate (*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 $[\alpha]_D^{23}$ 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 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The applied buffers were H_2O, ACN and 1.0% aq. TFA. 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.

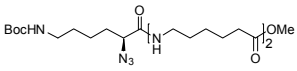
General procedure I: peptide coupling

The Boc-protected amine was treated with a 1:1 (v/v) mixture of DCM/TFA (5 mL/mmol) for 30 min. followed by addition of toluene and concentration of the mixture under reduced pressure. In order to remove excess TFA the mixture was coevaporated with toluene twice. The deprotected amine TFA salt (1 eq.) was dissolved in DCM (5 mL/mmol) and to this were added the carboxylic acid (1 eq.), HCTU (1.1 eq.) and DiPEA (3.5 eq.). The mixture was stirred until TLC analysis revealed a complete conversion (usually after 2 h). The organic layer was washed with 1M aq. HCl (2×), sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure.



(S)-2-azido-6-((*tert*-butoxycarbonyl)amino)hexanoic acid (11)

This compound was synthesized from H-Lys(Boc)-OH according to the procedure described by R. Stick *et al.*[25] In short: H-Lys(Boc)-OH (1.325 g, 5.38 mmol) was dissolved in MeOH (30 mL) and to this were added K_2CO_3 (3.2 eq., 17.21 mmol, 2.37 g), $CuSO_4 \cdot 5H_2O$ (1 mol%, 54.0 μmol, 13.0 mg) and imidazole-1-sulfonyl azide hydrochloride (1.2 eq., 6.46 mmol, 1.35 g). The mixture was stirred for 15 h after which TLC analysis indicated a complete conversion. The mixture was concentrated under reduced pressure, redissolved in EtOAc and washed with 1M aq. HCl. The aqueous layer was extracted twice with EtOAc followed by washing of the combined organic layers with brine, drying over $MgSO_4$ and concentration under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 50% EtOAc/PE) as a colourless oil (yield: 524 mg, 1.92 mmol, 36%). The spectroscopic data corresponded to those reported in literature.[10]



Boc-Ahx(α-N_3)-Ahx_2-OMe (12)

This compound was synthesized according to general procedure I from **11** (1.92 mmol) and Boc-Ahx_2-OMe **9**[9] (2.0 mmol). The title compound was purified by column chromatography (75% EtOAc/PE → 5% MeOH/EtOAc) and obtained as a colourless oil (yield: 0.80 g, 1.55 mmol, 81%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.14 (t, *J* = 5.58 Hz, 1H), 7.88 (t, *J* = 5.39 Hz, 1H), 3.79 (dd, *J* = 11.98, 4.97 Hz, 1H), 3.64 (s, 3H), 3.21 (dd, *J* = 13.06, 7.28 Hz, 2H), 3.16 (dd, *J* = 12.84, 7.02 Hz, 2H), 3.04 (t, *J* = 6.73 Hz, 2H), 2.32 (t, *J* = 7.40 Hz, 2H), 2.18 (t, *J* = 7.44 Hz, 2H), 1.87-1.70 (m, 2H), 1.62 (td, *J* = 15.35, 7.53 Hz, 4H), 1.57-1.47 (m, 6H), 1.43 (s, 9H), 1.39-1.27 (m, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 175.63, 175.54, 175.44, 172.03, 171.95, 158.22, 79.61, 64.17, 64.12, 51.98, 40.00, 40.31, 40.17, 40.04, 36.94, 34.59, 32.40, 30.45, 30.02, 29.98, 28.86, 27.42, 27.36, 26.57, 25.58, 23.91 ppm. $[\alpha]_D^{23}$ = +9.7° (*c* = 1 in MeOH). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.47. HRMS: calcd. for $C_{24}H_{44}N_6O_6$ $[M + H]^+$ 513.33951; found 513.33949.

Boc-Ahx(α-N_3)-Ahx_2-Leu_3-VS (13)

Compound **12** (0.80 g, 1.55 mmol) was dissolved in MeOH (8 mL) and cooled to 0 °C, after which LiOH (2 mL of a 1M aq. solution) was added slowly. The mixture was slowly warmed to RT and stirred for 15 h, after which TLC analysis indicated complete consumption of starting material. Next, 1M aq, HCl (3.6 mL) was added to neutralize the solution (~pH 5) and the mixture was concentrated under reduced pressure. The product was dissolved in DCM and dried over $MgSO_4$ to remove all traces of water. The resulting carboxylic acid was coupled to Boc-Leu_3-VS[4] (1.61 mmol) according to general procedure I and the title compound was obtained after purification by column chromatography (75% EtOAc/PE → 10% MeOH/EtOAc) as a colourless solid (yield: 0.99 g, 1.10 mmol, 71%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.15 (t, *J* = 5.61 Hz, 1H), 8.06 (d, *J* = 8.14 Hz, 2H), 7.88 (t, *J* = 5.40 Hz, 2H), 6.80 (dd, *J* = 15.16, 5.09 Hz, 1H), 6.63 (dd, *J* = 15.18, 1.19 Hz, 1H), 4.70-4.64 (m, 1H), 4.42-4.34 (m, 2H), 3.79 (t, *J* = 6.72 Hz, 1H), 3.21 (t, *J* = 6.98 Hz, 2H), 3.15 (t, *J* = 6.92 Hz, 2H), 3.04 (t, *J* = 6.73 Hz, 2H), 2.98 (s, 3H), 2.26 (dt, *J* = 7.12, 3.26 Hz, 2H), 2.18 (t, *J* = 7.44 Hz, 2H), 1.87-1.73 (m, 2H), 1.71-1.45 (m, 22H), 1.43 (s, 9H), 1.39-1.30 (m, 3H), 0.97-0.89 (m, 18H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 176.06, 175.62, 174.81, 174.04, 172.03, 158.30, 148.36, 130.77, 79.68, 79.23, 64.19, 53.37, 53.26, 49.03, 43.22, 42.90, 41.62, 41.46, 41.00, 40.22, 40.13, 36.91, 36.59, 32.42, 30.45, 30.08, 29.98, 28.86, 27.53, 27.43, 26.59, 26.50, 25.87, 25.82, 25.74, 23.90, 23.49, 23.46, 22.20, 22.11, 22.01 ppm. $[\alpha]_D^{23}$ = –29.5° (*c* = 1 in MeOH). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.50. HRMS: calcd. for $C_{43}H_{79}N_9O_9S$ [M + H]$^+$ 898.57942; found 898.58048.

4-(3-(Trifluoromethyl)-3*H*-diazirin-3-yl)benzamido-Ahx(α-N_3)-Ahx_2-Leu_3-VS (6)

This compound was synthesized according to general procedure I from compound **13** (0.21 mmol) and 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoic acid[11] (0.23 mmol). The title compound was purified by crystallization from MeOH/Et_2O and obtained as a colourless solid (yield: 155 mg, 0.15 mmol, 72%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.60 (t, *J* = 5.33 Hz, 1H), 8.17 (t, *J* = 5.41 Hz, 2H), 8.06 (t, *J* = 7.59 Hz, 3H), 7.90 (d, *J* = 8.48 Hz, 2H), 7.33 (d, *J* = 8.21 Hz, 2H), 6.79 (dd, *J* = 15.17, 5.08 Hz, 1H), 6.61 (dd, *J* = 15.19, 1.12 Hz, 1H), 4.72-4.62 (m, 1H), 4.42-4.30 (m, 2H), 3.81 (t, *J* = 6.69 Hz, 1H), 3.40 (dd, *J* = 12.09, 6.52 Hz, 2H), 3.23-3.12 (m, 4H), 2.97 (s, 3H), 2.25 (t, *J* = 7.18 Hz, 2H), 2.17 (t, *J* = 7.42 Hz, 2H), 1.93-1.75 (m, 2H), 1.72-1.40 (m, 22H), 1.39-1.27 (m, 3H), 0.93 (ddd, *J* = 9.86, 9.32, 5.12 Hz, 18H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 176.38, 175.88, 175.15, 174.39, 172.30, 168.78, 148.50, 137.35, 133.01, 130.85, 129.05, 127.67, 123.4 (q, *J* = 273.92 Hz), 64.41, 53.56, 49.29, 43.33, 42.86, 41.65, 41.60, 40.91, 40.79, 40.43, 40.31, 40.24, 36.98, 36.66, 32.50, 30.16, 30.07, 29.99, 27.63, 27.51, 26.67, 26.59, 25.98, 25.95, 25.85, 24.13, 23.46, 22.11, 22.02, 21.96 ppm. $[\alpha]_D^{23}$ = –28.2° (*c* = 1 in MeOH). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 9.16. HRMS: calcd. for $C_{47}H_{74}F_3N_{11}O_8S$ [M + H]$^+$ 1010.54674; found 1010.54811.

4-Benzoylbenzamido-Ahx(α-N_3)-Ahx_2-Leu_3VS (7)

This compound was synthesized according to general procedure I from compound **13** (0.22 mmol) and commercially available 4-benzoylbenzoic acid (0.24 mmol). The title compound was purified by crystallization from MeOH/Et_2O and obtained as a colourless solid (yield: 170 mg, 0.17 mmol, 77%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.97 (d, *J* = 8.30 Hz, 2H), 7.83 (d, *J* = 8.31 Hz, 2H), 7.78 (d, *J* = 7.17 Hz, 2H), 7.66 (t, *J* = 7.41 Hz, 1H), 7.54 (t, *J* = 7.66 Hz, 2H), 6.80 (dd, *J* = 15.17, 4.97 Hz, 1H), 6.62 (dd, *J* = 15.18, 1.26 Hz, 1H), 4.66 (td, *J* = 8.85, 4.85 Hz, 1H), 4.41-4.29 (m, 2H), 3.83 (t, *J* = 6.73 Hz, 1H), 3.48-3.41 (m, 2H), 3.20 (t, *J* = 7.03 Hz, 2H), 3.14 (t, *J* = 7.02 Hz, 2H), 2.98 (s, 3H), 2.29-2.24 (m, 2H), 2.17 (t, *J* = 7.42 Hz, 2H), 1.94-1.77 (m, 2H), 1.74-1.43 (m, 22H), 1.38-1.28 (m, 3H), 0.97-0.88 (m, 18H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 197.64, 176.49, 175.82, 175.24, 174.37, 172.20, 169.06, 148.50, 141.22, 139.33, 138.34, 134.18, 131.06, 130.98, 129.67, 128.43, 64.30, 53.81, 53.54, 49.90, 49.17, 43.22, 42.88, 41.57, 41.41, 40.80, 40.29, 40.21, 36.97, 36.67, 32.48, 30.13, 30.03, 29.98, 27.62, 27.50, 26.65, 26.56, 25.97, 25.93, 24.13, 23.48, 23.44, 22.08,

21.94 ppm. $[\alpha]_D^{23}$ = –26.8° (*c* = 1 in MeOH). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.69. HRMS: calcd. for $C_{52}H_{79}N_9O_9S$ $[M + H]^+$ 1006.57942; found 1006.58079.

4-Azido-2-hydroxybenzamido-Ahx(α-N_3)-Ahx_2-Leu_3-VS (8)

Compound **13** (0.23 g, 0.25 mmol) was Boc-deprotected with HCl (3 mL of a 4M solution in 1,4-dioxane), followed by coevaporation with toluene (3×) and the resulting amine HCl salt was dissolved in DMF (2 mL). To this were added DiPEA (1 eq., 0.25 mmol, 43.0 μL) and 4-azido-2-hydroxybenzoic acid *N*-hydroxysuccinimide ester[13] (1.3 eq., 91.0 mg, 0.33 mmol) and the reaction mixture was stirred for 15 h, after which LC-MS analysis indicated complete consumption of the amine. DCM (10 mL) was added and the mixture was washed with 1M aq. HCl (2×), sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (100% DCM → 6% MeOH/DCM) as a colourless solid (yield: 130 mg, 0.14 mmol, 53%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.75 (d, *J* = 8.32 Hz, 1H), 6.79 (dd, *J* = 15.17, 5.08 Hz, 1H), 6.61 (dd, *J* = 15.19, 1.27 Hz, 1H), 6.57-6.52 (m, 2H), 4.65 (td, *J* = 9.10, 4.93 Hz, 1H), 4.35 (ddd, *J* = 14.62, 8.85, 6.09 Hz, 2H), 3.80 (t, *J* = 6.69 Hz, 1H), 3.38 (t, *J* = 6.95 Hz, 2H), 3.20-3.12 (m, 4H), 2.97 (s, 3H), 2.24 (dd, *J* = 8.01, 6.18 Hz, 2H), 2.16 (t, *J* = 7.43 Hz, 2H), 1.89-1.74 (m, 2H), 1.71-1.39 (m, 22H), 1.38-1.25 (m, 3H), 0.97-0.86 (m, 18H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 176.27, 175.79, 174.96, 174.18, 172.11, 170.31, 162.90, 148.43, 146.49, 130.83, 130.43, 113.88, 110.89, 108.23, 79.29, 64.31, 53.52, 53.38, 49.15, 43.27, 42.88, 41.62, 41.51, 40.28, 40.21, 40.16, 36.96, 36.64, 32.44, 30.12, 30.00, 27.59, 27.47, 26.61, 26.53, 25.94, 25.90, 25.81, 24.07, 23.45, 22.14, 22.04, 21.98 ppm. $[\alpha]_D^{23}$ = –27.1° (*c* = 1 in MeOH). LC-MS: gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.95. HRMS: calcd. for $C_{45}H_{74}N_{12}O_9S$ $[M + H]^+$ 959.54952; found 959.55103.

Competition assays

Whole cell lysates of EL4 were made by sonication of cell pellets in 3 volumes of lysis buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 250 mM sucrose, 2 mM ATP. Protein concentration was determined by the Bradford assay. Cell lysate (9 μg total protein) was exposed to the inhibitors **6**, **7** or **8** for 1 hour prior to incubation with MV151[14] (0.5 μM) for 1 hour at 37 °C. Reaction mixtures were boiled with Laemmli's buffer containing β-mercaptoethanol for 5 min. before being resolved by 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532nm, λ_{em} 560 nm) to detect MV151.

Photocrosslinking in purified 20S proteasome

Purified 20S proteasome (human erythrocyte, 1 mg/mL, Enzo life sciences) was diluted with lysis buffer (50 mM Tris pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 250 mM sucrose, 2 mM ATP) to a concentration of 20 ng/μL. From this, 10 μL (200 ng 20S proteasome) was incubated with compound **6**, **7** or **8** (10 μM final concentration) for 2 h at 37 °C in the dark. The samples were irradiated with UV light (λ 365 nm) for the appropiate time at 0 °C, by placing the lamp (Spectroline® ENF-260C/FE, 6W) directly on top of the opened Eppendorff tubes (distance from light source to sample ~4 cm). After irradiation, the samples were stored at 4 °C in the dark. Next, the samples were treated with a Biotin-phospane reagent[15] (200 μM final concentration) for 1 h at 37 °C. After boiling the mixture with Laemmli's buffer containing β-mercaptoethanol for 5 min., the samples were resolved by 12.5% SDS-PAGE and all biotinylated proteins were visualized by Western blotting. The blots were blocked with 1% BSA in TBS-Tween 20 (0.1 % Tween 20) for 30 min. at RT, hybridized for 1 h with Streptavidin-HRP (1:10,000) in blocking buffer, washed and visualized using an ECL+ kit (Amersham Biosciences). Also, in a different gel, the total protein content was visualized by silverstain. The appropriate bands were cut from the gel and an in-gel digestion was performed according to the procedure described in literature.[26]

LC-MS/MS analysis

Tryptic peptides were analyzed on a Surveyor nanoLC system (Thermo) hyphenated to a LTQ-Orbitrap mass spectrometer (Thermo). Gold and carbon coated emitters (OD/ID = 360/25 μm tip ID = 5 μm), trap column (OD/ID = 360/100 μm packed with 25 mm robust Poros®10R2/15 mm BioSphere C18 5 μm 120 Å) and analytical columns (OD/ID = 360/75 μm packed with 20 cm BioSphere C18 5 μm 120 Å) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% formic acid/H_2O, B: 0.1% formic acid/ACN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column via a 15 mm long TFE teflon tubing sleeve (OD/ID 0.3 × 1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a nanosource base (Upchurch scientific, Idex, USA). General mass spectrometric conditions were: an electrospray voltage of 1.8 kV was applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150 °C, capillary voltage 41 V, tube lens voltage 150 V. Internal mass calibration was performed with air-borne protonated polydimethylcyclosiloxane (*m/z* = 445.12002) and the plasticizer protonated dioctyl phthalate ions (*m/z* = 391.28429) as lock mass.[27] For shotgun proteomics analysis, 10 μL of the samples was pressure loaded on the trap column with a 10 μL/min flow for 5 min. followed by peptide separation with a gradient of 35 min. 5 → 30% B, 15 min. 30 → 60% B, 5 min. A, at a flow of 300 μL/min. split to 250 nL/min. by the LTQ divert valve. For each data dependent cycle, one full MS scan (300-2000 *m/z*) acquired at high mass resolution (60,000 at 400 *m/z*, AGC target 1 × 106, maximum injection time 1,000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the LTQ linear ion trap (AGC target 5 × 103, max injection time 120 ms) from the three most abundant ions.[28] MS/MS settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 500 counts, activation q = 0.25 and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 60 s and ions with $z < 2$ or unassigned were not analyzed. Data from MS/MS was validated manually.

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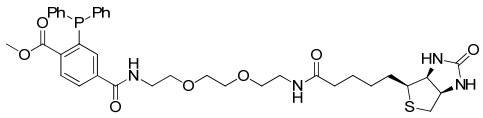

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7 A Levulinoyl Ester-Based Cleavable Linker for Activity-Based Protein Profiling

P. P. Geurink, B. I. Florea, N. Li, M. D. Witte, J. Verasdonck, C.-L. Kuo, G. A. van der Marel, H. S. Overkleeft, *Angew. Chem. Int. Ed.* **2010**, accepted for publication.

7.1 Introduction

Activity-based protein profiling has become an attractive strategy to report on enzyme activity in the context of complex biological samples (see Chapter 1).[1,2] An activity-based profiling probe (ABP) normally consists of a recognition element targeted at a specific enzyme (family), and is equipped with a reactive group through which the targeted enzymes are covalently and irreversibly modified. Enzymes that use an amino acid side-chain nucleophile in their catalytic process can be made to react with an electrophilic trap, or suicide substrate,[3,4] whereas activity-based profiling strategies of enzymes that do not employ such active site residues normally rely on the use of photo-activatable groups.[5-7] A third essential element of ABPs is the affinity/identification tag, which can be either incorporated into the ABP directly or alternatively installed via a bioorthogonal process in two-step activity-based protein profiling strategies.[8-10] Visualization/identification tags come in several flavours, including fluorophores and immune epitope tags. The most popular tag in this context is the biotin group. It is relatively small, easy to install in a wide variety of differently functionalized activity-based probes and has strong binding affinity to (strept)avidin. As a consequence a range of biochemical tools have become available over the decades for either the visualization of biotinylated biomolecules or their pull-down. As such, biotin is both an identification tag and visualization tag in one.

The strong affinity of streptavidin for biotin makes that the release of biotinylated proteins captured by, for instance, streptavidin-coated beads can be difficult. Most

effective procedures rely on relatively harsh conditions, such as boiling of the sample in denaturing conditions and often in the presence of unmodified biotin. As a consequence, next to (denatured) streptavidin, the eluted protein pool is often contaminated with endogenously biotinylated biomolecules, present in the initial biological sample.

The introduction of a specific linker between biotin and the ABP reactive group, which can be cleaved chemoselectively so that only the ABP bound material is released, is an attractive strategy to circumvent this problem (Figure 1A). Examples of such cleavable linkers (CL) are the disulfide linkage,[11] enzymatically (Tev) cleavable,[12] acid cleavable,[13,14] diazobenzene derived (cleavable with $Na_2S_2O_4$),[15] and hydrazone-based linkers.[16,17] An ideal cleavable linker is stabile towards the various conditions (acidic, basic, reductive, including generally applied buffer systems) to which the biological sample may be exposed, depending on the nature of the experiment, and can also withstand the reactive (nucleophilic) species that are present in a cell extract. At the same time, the linker should be susceptible to mild cleavage conditions which are selective with respect to functional groups inherent to the biological sample. With this reasoning in mind, attention was focussed on the levulinoyl ester, a versatile protective group often applied in synthetic organic chemistry. It is acid stabile and can be removed selectively with respect to other esters by treatment with hydrazine. The levulinoyl ester thus meets to considerable extend the demands outlined above. A shortcoming is its intrinsic base-lability and it was reasoned that this drawback can be rectified by choosing the alcohol, with which the levulinolate group is condensed, such that it is both electron-rich (to reduce its leaving group properties) and sterically congested (to minimize intermolecular nucleophilic attack). Altogether, epoxomicin based,[18] levulinolate modified activity-based proteasome probe **1** (Figure 1B) was designed. This chapter describes its synthesis and application in the activity-based enrichment and identification of the proteasome active subunits from cell lysate.

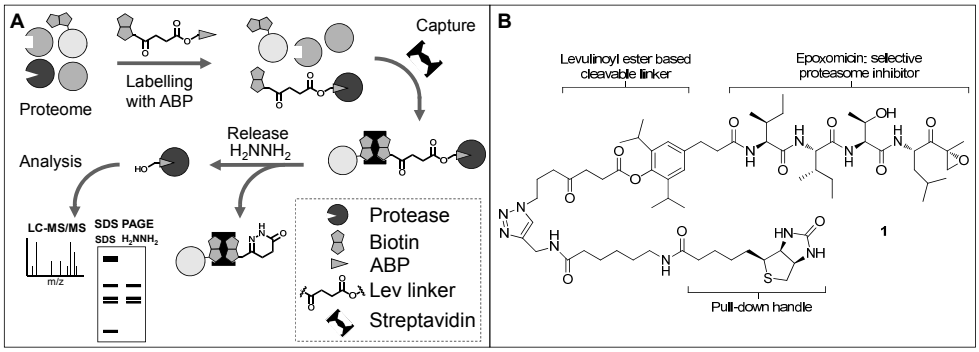
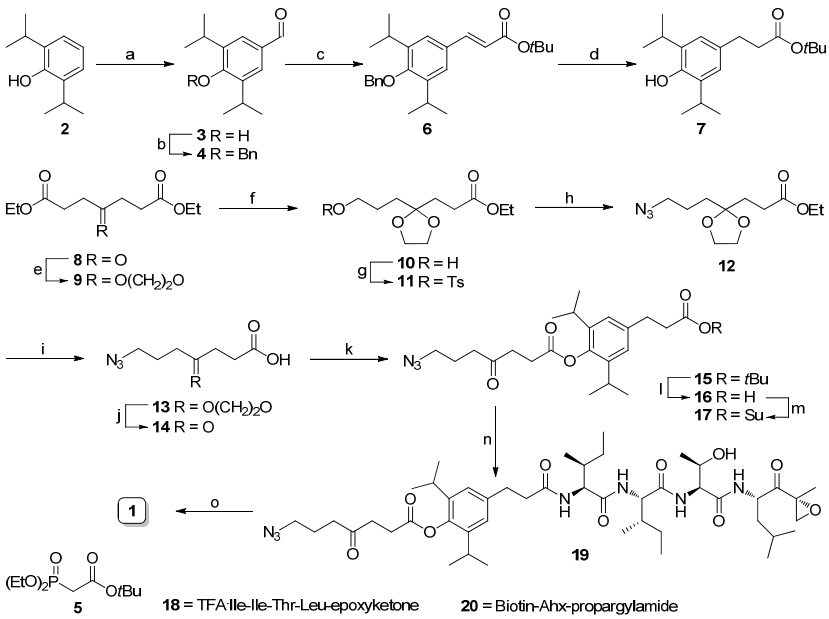


Figure 1. (A) Schematic representation of an activity-based enrichment experiment using the levulinoyl ester based cleavable linker. After labelling of the target enzyme and pull-down the linker is (chemo)selectively cleaved with hydrazine. (B) Structure of target proteasome probe **1** containing the levulinoyl ester based cleavable linker.

7.2 Results and Discussion

The synthesis of ABP **1** is shown in Scheme 1. Commercially available 2,6-diisopropylphenol **2** was converted into benzaldehyde **3** in a Duff reaction[19] and the alcohol was subsequently protected as the benzyl ether (**4**). The ensuing Horner-Wadsworth-Emmons reaction with phosphonate **5** proceeded smoothly to give **6**. Hydrogenation and concomitant benzyl deprotection led to alcohol **7**. Next, the ketone in diethyl 4-oxopimelate (**8**) was protected as ketal (**9**)[20] and one of the ethyl esters was selectively saponificated. The obtained carboxylate was reduced to the alcohol (**10**), which was converted into azide **12** via its tosylate (**11**). Saponification followed by acidic hydrolysis gave compound **14**, which was condensed with **7** to produce levulinoyl ester **15**. The *tert*-butyl ester was removed and the resulting carboxylic acid (**16**) was transformed into its activated NHS ester (**17**). Condensation with the epoxomicin peptide sequence **18** gave compound **19**, which was reacted with alkyne **20** to give target compound **1**.

Scheme 1. Synthesis of two-step labelling probe **19** followed by a 'click' reaction leading to target probe **1**.

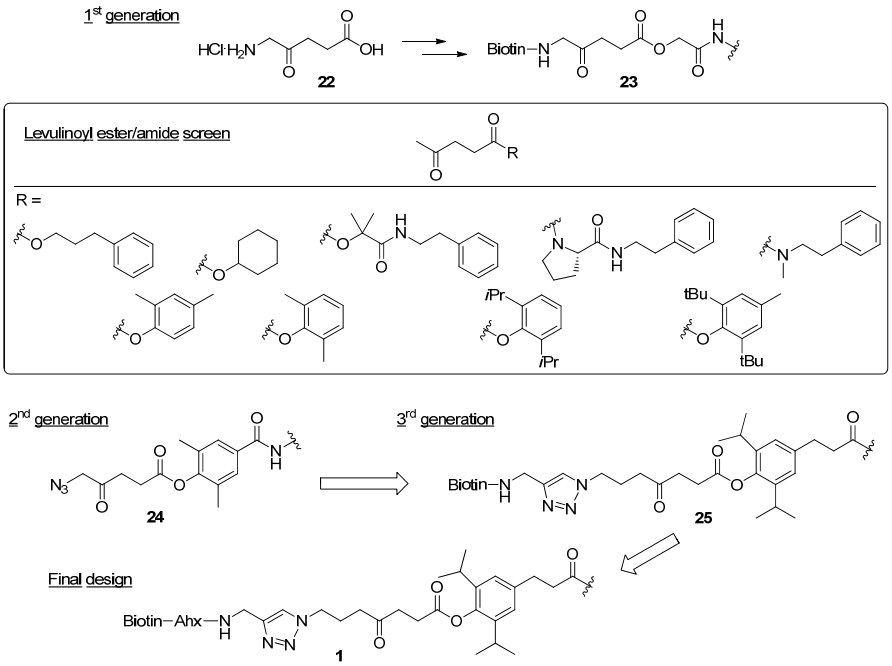


Reagents and conditions: (a) hexamine, AcOH, H_2O, reflux, 97%; (b) BnBr, K_2CO_3, acetone, 93%; (c) **5**, NaH, THF, 0 °C, quant.; (d) Pd/C, H_2, MeOH, 94%; (e) ethylene glycol, PPTS, toluene, reflux, quant.; (f) i) KOH, EtOH, 50 °C; ii) ethyl chloroformate, Et_3N, THF; iii) $NaBH_4$, H_2O, 37%; (g) TsCl, Et_3N, DMAP, DCM, 85%; (h) NaN_3, DMF, 75 °C, 99%; (i) NaOH, MeOH, 89%; (j) HCl, H_2O, THF, quant.; (k) **7**, DIC, DMAP, DCM, 73%; (l) TFA, DCM, quant.; (m) HOSu, EDC, DCM, 95%; (n) **18**, DiPEA, DMF, 63%; (o) **20**, $CuSO_4$, sodium ascorbate, DMF, 41%.

The linker system present in **1** was designed after conducting a number of studies (see Scheme 2). Initially, compound **23** was synthesized in order to test the possibility of hydrazine mediated cleavage in a Tris buffer in general. Cleavage of the ester

appeared to be fast (within 30 minutes at room temperature), however the ester linkage was also easily hydrolyzed in a Tris buffer system at pH 7.5. In addition, the synthesis from 5-aminolevulinic acid hydrochloric acid (**22**) is cumbersome due to its lability under basic conditions. Next, a panel of compounds derived from levulinic acid coupled to different alcohols (primary, secondary, tertiary alcohols and phenols) or secondary amines (Scheme 2) was studied for their synthetic viability, cleavage properties and stability towards hydrolysis. The primary and secondary alcohols derived esters proved to be substantially susceptible towards hydrolysis. Both the tertiary alcohol and 2,6-di-*tert*-butylphenol derived esters could not be constructed under the commonly applied condensation conditions. Furthermore, the secondary amides, although being stabile to hydrolysis, could not be cleaved with hydrazine. The optimal results, in terms of synthesis, cleavage and stability, were obtained for the 2,6-dimethyl- and 2,6-diisopropyl phenols.

Scheme 2. Design of the levulinoyl ester-based cleavable linker system.

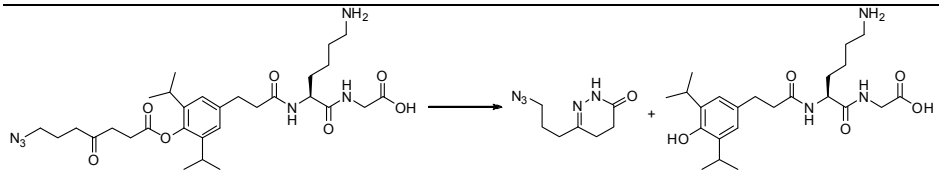


Based on these results compound **24** was created, in which the α-amine functionality (in **23**) was replaced by an azide. Although this compound showed improved properties, compared to **23**, it still suffered from two issues. The ester linkage was still a little prone to hydrolysis in Tris buffer and the α-azide moiety was too labile to allow for a clean 'click' reaction. The third generation designed was compound **25**. The isopropyl groups *ortho* to the ester appeared sterically sufficiently bulky to avoid hydrolysis under basic conditions. Yet they allow intramolecular cyclization of the

hydrazone, which is formed after condensation of the ketone with hydrazine. The 4-alkyl substituent proved to be favourable compared to a 4-carbonyl substituent in that this more electron rich phenol makes the ester less prone to hydrolysis. Also, the additional ethylene moiety introduced between azide and ketone allowed for a smooth click reaction with biotin propargyl amide. Although the ester linkage was completely optimized, a preliminary pull-down experiment showed that hydrazine mediated cleavage of the captured construct from streptavidin-coated beads was marginal. It was reasoned that the cleavable linker was in too close proximity to the streptavidin-bound biotin and hence inaccessible for hydrazine. Therefore in the final design (**1**) an additional Ahx spacer was placed between the cleavable linker and biotin, which allowed an easier release from the beads (*vide infra*).

The stability and cleavability of the linker system under several conditions was studied by LC-MS analysis of a test-substrate containing the linker. The results are shown in Table 1.

Table 1. Stability/cleavage of the indicated test-substrate (10 mM) under different conditions.[a]



Entry	Buffer (pH)	H_2NNH_2	Temp. (°C)	Time (h)	Additive	Cleavage
1	Tris (7.5)	–	23	15	–	–
2	Tris (7.5)	–	37	15	–	–
3	Tris (7.5)	+	23	15	–	+
4	Tris (7.5)	+	37	1	–	+
5	Tris (7.5)	+	37	15	–	+
6	Tris (7.5)	NH_2OH	37	15	–	–
7	Tris (7.5)	–	23	15	0.4% SDS	–
8	Tris (7.5)	–	100	5 min.	4×SB	–
9	HEPES (5.8)	–	23	15	–	–
10	PBS (7.4)	–	23	15	–	–
11	MI (3.0)	–	23	15	–	–
12	MI (4.0)	–	23	15	–	–
13	MI (5.0)	–	23	15	–	–
14	MI (6.0)	–	23	15	–	–

[a] Concentrations used: $[H_2NNH_2]$ = 100 mM; $[NH_2OH]$ = 100 mM; [Tris] = 100 mM; [HEPES] = 50 mM. PBS (phosphate buffered saline): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.14 M NaCl, 2.7 mM KCl. MI (McIlvaine's buffer): combination of 0.1 M citric acid and 0.2 M Na_2HPO_4. SB (sample buffer): 20 mM Tris, 1% SDS, 10% glycerol, 0.05% BPB, 0.25 M β-mercaptoethanol.

From this, it can be seen that the cleavable linker is stabile towards a wide variety of conditions to which a biological sample may be exposed, including different buffer systems, strong denaturing conditions and pH 3-7.5. Only upon addition of hydrazine the linker is cleaved (entries 3-5) into the two expected products. Hydroxyl amine was unable to cleave the linkage (entry 6).

The efficacy of compound **1** to select and identify proteasome catalytic activities from cell extracts in an activity-based labelling experiment followed by hydrazine mediated linker cleavage was investigated as follows. Incubation of HEK-293T cell lysate with **1** in a Tris buffer followed by treatment with or without hydrazine under different conditions and visualization by streptavidin Western blotting revealed that all three active subunits (β1, β2 and β5) are labelled (see Figure 2A). Cleavage of the linker appears to be hydrazine concentration dependent (lanes 3-7) and full cleavage was achieved after exposure to 50 mM hydrazine for 15 h. A second experiment, in which samples of HEK-293T cell lysate, incubated with **1**, were denatured and exposed to 100 mM hydrazine for different amounts of time, revealed that full cleavage was achieved within three to five hours (Figure 2B).

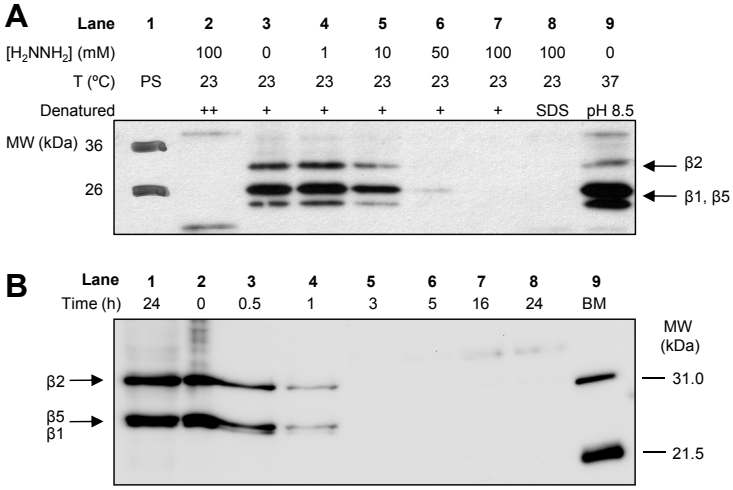


Figure 2. Optimal cleavage conditions in a biological environment. HEK-293T cell lysate was incubated with probe **1** (2.5 μM final concentration) for 1 h at 37 °C followed by denaturation. (A) Samples were treated with the indicated hydrazine concentration for 15 h, resolved by SDS-PAGE and all biotinylated proteins were visualized by anti-biotin Western blotting. Cleavage of the linker is shown by disappearance of the bands. PS: prestained marker low range. Lane 2: sample was pre-boiled with 1% SDS prior to incubation as a negative control. Lane 8: 20 mM SDS without denaturing. Lane 9: pH 8.5 is the pH value for a combination of 100 mM Tris and 100 mM hydrazine and was reached by addition of 1 μL 1 M Tris to the sample. (B) Samples were treated with 100 mM hydrazine for the indicated amounts of time and subsequently analyzed as described above. Lane 1: compound **21** (*vide infra*). BM: Biotinylated marker low range.

To evaluate the use of **1** for protein capture and release, HEK-293T cell lysate was treated with this compound followed by denaturation, cysteine bridge reduction (DTT) and capping (iodoacetamide), and capturing with streptavidin coated magnetic beads.

The beads were divided into equal aliquots and the elutes from each sample after treatment with or without hydrazine under different conditions were resolved by SDS-PAGE and visualized by silver stain (see Figure 3A). As a control experiment, biotinylated probe AdaLys(biotin)Ahx$_3$Leu$_3$VS (**21**)[21] was added to the lysate which was then treated with either standard (non-selective) elution conditions (1% SDS, 10 μM biotin, 100 °C, lanes 1 and 7) or hydrazine (lanes 2 and 8). These results show that the captured proteins from **1** can be released chemoselectively with hydrazine both at RT and at 37 °C (lanes 4 and 10), whereas the captured construct derived from **21** is resistant towards these conditions (lanes 2 and 8). The steptavidin blots of the same samples in Figure 3B show a complete absence of biotin for the with compound **1** captured proteins, which proves that the cleavable linker is indeed cleaved. In addition it appears that a small amount of SDS (0.05%) is necessary during cleavage (compare lanes 3 and 9 with 4 and 10), whereas SDS at this concentration solely does not sustain cleavage (lane 5 and 11). Cleavage in HEPES buffer at pH 5.8 occurs as well, but is accompanied by a higher release of undesired material (compare lane 10 with 12). The cleavage efficiency was determined by extensive washing of the beads from Figure 3, eluting all captured leftovers with SDS boiling and silver staining after SDS-PAGE (see Figure 4). For the samples of lanes 6, 10 and 12 (Figure 2) the cleavage efficiency appears to be (near) quantitative (no proteasome characteristic bands are present) and for that of lane 4 only a small amount of active proteasome subunits is visible.

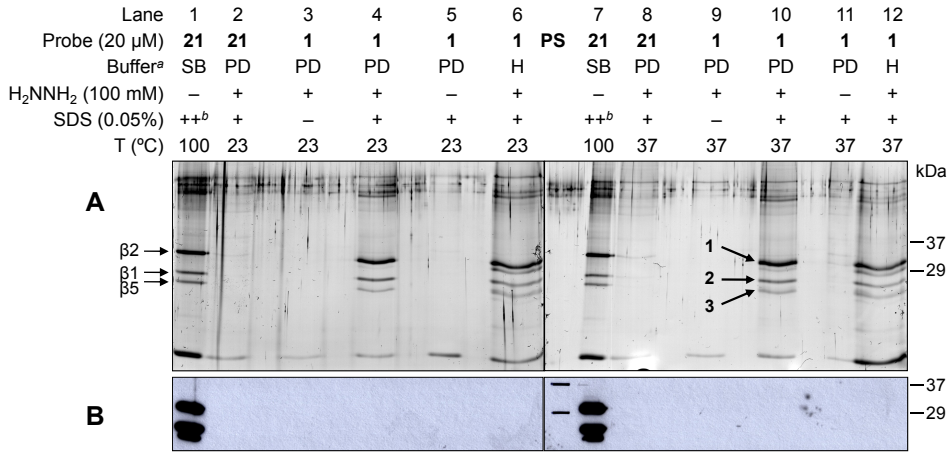


Figure 3. Enriched proteins after pull-down with ABPs **1** and **21** and cleavage from streptavidin beads under the indicated conditions for 15 h. (A) Silver stain. (B) Streptavidin Western blot from the same samples. PS = prestained marker low range. [a] The applied buffers were: SB (30 mM Tris·HCl pH 6.8, 1% SDS, 9% glycerol, bromophenolblue (BPB), 0.25 M β-mercaptoethanol, 10 μM biotin); PD (50 mM Tris·HCl pH 7.5, 150 mM NaCl); H (50 mM HEPES pH 5.8). [b] ++ refers to the 1% SDS present in SB. The arrows indicate the excised bands analyzed by LC-MS/MS after in-gel tryptic digest.

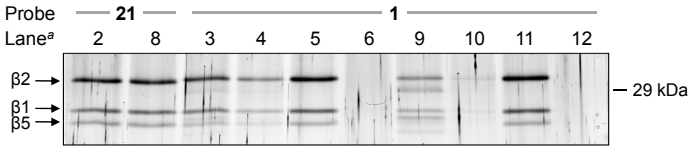


Figure 4. Determination of the cleavage efficiency. The beads from the samples in Figure 3 after hydrazine mediated cleavage were washed extensively and boiled for 5 min. with sample buffer (20 mM Tris, 1% SDS, 10% glycerol, 0.05% BPB, 0.25 M β-mercaptoethanol) containing 10 μM biotin. Samples were resolved by SDS-PAGE and all proteins were visualized by silver stain. [a] The numbers correspond to the same samples in the lanes in Figure 3.

To establish that the visualized bands indeed correspond to the targeted proteasome subunits the indicated bands (arrows in Figure 3) were cut from the gel and analyzed by LC-MS/MS after in-gel tryptic digest. In this analysis multiple characteristic peptides for each of the indicated subunits were identified (see Figure 5A). Moreover, the expected modified active site fragment peptides derived from β2 and β5 were identified (see Figure 5B showing the MS/MS spectrum for the modified β5 active site).

A

Band	Subunit	n_p	Seq. Coverage
1	β2	6	42.7%
2	β1	5	39.0%
3	β5	7	54.4%

B

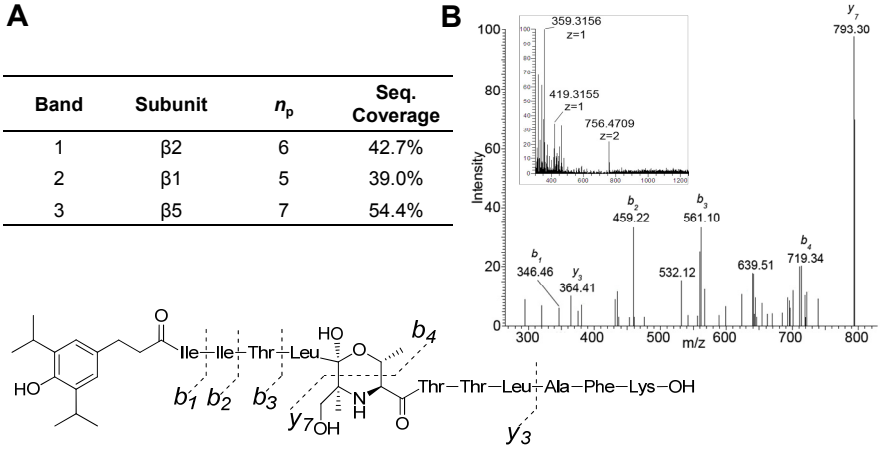


Figure 5. (A) Table showing the proteasome active subunits identified from the indicated bands in Figure 3 after in-gel tryptic digest and LC-MS/MS analysis. n_p= number of identified peptides. (B) MS/MS spectrum of the β5 active site fragment attached to the expected cleaved probe. Insert: part of the LC-MS run showing the parent ion *m/z* (calc. *m/z* = 756.4704 for *z* = 2) for the peptide-inhibitor construct as shown at the bottom.

Finally, the stability of compound **1** in serum and plasma was investigated. To this end, compound **1** was added to samples containing PBS, plasma or serum and incubated at 37 °C for 15 hours. LC-MS analysis (see Figure 6) indicated that compound **1** was completely stabile in these environments, as evidenced by the fact that no products resulting from ester cleavage (hydrolysis) could be detected. These last results demonstrate the viability of the levulinoyl linker for ABP profiling of enzymes *in vivo*.

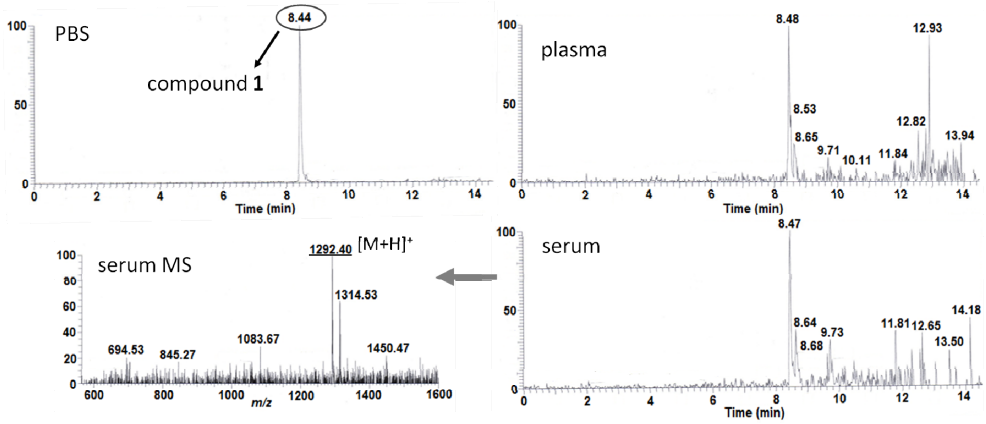


Figure 6. LC-MS traces of samples containing PBS, plasma or serum and treated with compound **1** for 15 hours at 37 °C. The MS spectrum is shown for the peak at 8.47 minutes in the serum sample, indicating that compound **1** is intact. No products resulting from hydrolysis of the ester linkage could be detected.

7.3 Conclusion

In summary, a levulinoyl ester-based linker system for use in activity-based protein profiling was developed. Tuning the nature of the levulinoyl ester has produced a linker system that is robust enough to survive conditions commonly applied to cell extracts in biochemical experiments. The linker withstands aqueous, acidic and basic media (including the widely used Tris buffer) and is resistant towards disulfide reducing conditions. It is thus believed that the linker has some important advantages over the reported[11-17] linker systems. The versatility of the linker in the ABP-mediated enrichment of the proteasome active sites was demonstrated and it was shown that the expected cleavage product is indeed found back on the active site peptides obtained after tryptic digestion. These results thus form the basis for the development of new activity-based profiling strategies, for instance two-step labelling strategies that include bio-orthogonal chemistry. From the synthetic scheme it is apparent that the linker is compatible with 'click' chemistry. In a preliminary experiment HEK-293T cell lysates were exposed to azide-containing probe **19**. Ensuing treatment with biotinylated Staudinger phosphane,[22] SDS-PAGE resolving of the protein contain by SDS-PAGE and streptavidin blotting revealed a pattern characteristic for the proteasome catalytic activities. This last result demonstrates the viability of the levulinoyl linker for two-step ABP profiling of enzymes in general.

Experimental section

General

Tetrahydrofuran was distilled over $LiAlH_4$ prior to use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), methanol (MeOH), 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. *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 4 Å molecular sieves, except methanol and acetonitrile which were stored over 3 Å molecular sieves. 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, ethyl acetate (EtOAc) and petroleum ether (PE) (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CD_3OD or $CDCl_3$ as internal standard. IR spectra were recorded on a Shimadzu FTIR-8300 and absorptions are given in cm^{-1}. 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 dioctylpthalate (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 $[\alpha]_D^{23}$ were recorded on a Propol automatic polarimeter. 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 (System A) or a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI (System B). The applied buffers were H_2O, ACN and 1.0% aq. TFA. Unless noted otherwise the gradient used was 10% → 90% ACN/0.1% aq. TFA. 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.

4-hydroxy-3,5-diisopropylbenzaldehyde (3)[19]

2,6-diisopropylphenol (**2**, 18.4 g, 100 mmol) was dissolved in AcOH (83 mL) and H_2O (17 mL). To this was added hexamine (2 eq., 200 mmol, 28.0 g) and the mixture was heated to reflux for 5 min. after which a distillation head was installed and ca. 9 mL distillate was collected at 110 °C. The distillation head was removed again and the mixture was refluxed for another 2.5 h after which TLC analysis indicated complete consumption of the phenol starting compound. Next, the mixture was cooled to RT and H_2O (20 mL) was added. Upon further cooling to 0 °C a pale yellow solid precipitated. The mixture was allowed to stand at 0 °C for 1 h followed by filtration of the solid. The residue was washed two times with ice-cold water and dried at 60 °C under reduced pressure. The title compound was obtained without further purification as a pale yellow solid (yield: 20.1 g, 97.6 mmol, 97%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.79 (s, 1H), 7.62 (s, 2H), 4.14 (bs, 1H), 3.30 (p, J = 6.80 Hz, 2H), 1.28 (d, J = 6.80 Hz, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 192.35, 157.16, 135.26, 128.67, 126.01, 26.47, 22.24 ppm.

4-(benzyloxy)-3,5-diisopropylbenzaldehyde (4)

Phenol **3** (4.14 g, 20.0 mmol) was dissolved in acetone (100 mL) and to this were added benzylbromide (1.01 eq., 20.2 mmol, 3.46 g) and K_2CO_3 (2 eq., 40.0 mmol, 5.53 g). The suspension was stirred vigorously for 14 h after which TLC analysis revealed a completed reaction. The mixture was concentrated under reduced pressure, redissolved in EtOAc (100 mL) and extracted with H_2O and brine. After drying ($MgSO_4$) and concentration *in vacuo* of the organic layer the title

compound was obtained as a colourless oil (yield: 5.50 g, 18.6 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.95 (s, 1H), 7.70 (s, 2H), 7.48 (d, *J* = 7.18 Hz, 2H), 7.41 (t, *J* = 7.30 Hz, 2H), 7.35 (t, *J* = 7.20 Hz, 1H), 4.86 (s, 2H), 3.41 (sept., *J* = 6.85 Hz, 2H), 1.27 (d, *J* = 6.96 Hz, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 191.68, 158.53, 143.13, 136.76, 133.13, 128.50, 128.07, 127.25, 126.13, 76.42, 26.66, 23.74 ppm.

(E)-*tert*-butyl 3-(4-(benzyloxy)-3,5-diisopropylphenyl)acrylate (6)

To a stirred solution of aldehyde **4** (0.23 g, 0.76 mmol) and *tert*-butyl 2-(diethoxyphosphoryl)acetate (**5**, 1.5 eq., 1.14 mmol, 0.29 g) in THF (10 mL) at 0 °C was added NaH (1.5 eq., 1.14 mmol, 46.0 mg). The reaction mixture was stirred for 1 h at RT after which TLC analysis indicated a completed reaction. EtOAc (10 mL) was added and the mixture was extracted with 0.1 M aq. HCl (2×), sat. aq. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (100% PE → 5% EtOAc/PE) as a colourless oil (yield: 0.31 g, 0.77 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.60 (d, *J* = 15.94 Hz, 1H), 7.47 (d, *J* = 7.08 Hz, 2H), 7.40 (t, *J* = 7.31 Hz, 2H), 7.33 (t, *J* = 7.24 Hz, 1H), 7.30 (s, 2H), 6.33 (d, *J* = 15.93 Hz, 1H), 4.81 (s, 2H), 3.38 (sept., *J* = 6.89 Hz, 2H), 1.54 (s, 9H), 1.24 (d, *J* = 6.91 Hz, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.38, 154.87, 143.78, 142.42, 137.20, 130.97, 128.47, 127.94, 127.26, 124.02, 118.73, 80.14, 76.36, 28.13, 26.54, 23.88 ppm. HRMS: calcd. for $C_{26}H_{34}O_3$ 395.25807 $[M + H]^+$; found 395.25797.

***Tert*-butyl 3-(4-hydroxy-3,5-diisopropylphenyl)propanoate (7)**

Compound **6** (0.30 g, 0.76 mmol) was dissolved in MeOH (10 mL) and the solution was bubbled through with argon for 15 min. before Pd/C 10% w/w (10 mg) was added. The flask was charged with hydrogen for 1 h, after which TLC analysis indicated complete reduction. Argon was bubbled through for another 15 min. and all solids were removed by filtration over Celite. The title compound was obtained after evaporation of the solvent under reduced pressure as a colourless oil (yield: 0.22 g, 0.71 mmol, 94%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.87 (s, 2H), 5.09 (s, 1H), 3.17 (sept., *J* = 6.80 Hz, 2H), 2.85 (t, *J* = 7.84 Hz, 2H), 2.52 (t, *J* = 7.86 Hz, 2H), 1.42 (s, 9H), 1.24 (d, *J* = 6.91 Hz, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.73, 148.26, 133.75, 132.36, 123.10, 80.17, 37.51, 30.83, 27.98, 27.00, 22.73 ppm. HRMS: calcd. for $C_{19}H_{30}O_3$ 329.20872 $[M + Na]^+$; found 329.20871.

Diethyl 3,3'-(1,3-dioxolane-2,2-diyl)dipropanoate (9)[20]

Diethyl-4-oxopimelate (**8**, 11.4 g, 48.4 mmol), ethylene glycol (436 mmol, 24.0 mL) and PPTS (7.26 mmol, 1.82 g) were dissolved in toluene (50 mL) and the mixture was heated to reflux under Dean-Stark conditions for 2 h, after which TLC analysis indicated complete consumption of the ketone. The mixture was cooled to RT and extracted twice with sat. aq. $NaHCO_3$ (50 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained as a colourless liquid (yield: 13.2 g, 48.4 mmol, quant.). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.13 (q, *J* = 7.13 Hz, 4H), 3.94 (s, 4H), 2.37 (t, *J* = 7.65 Hz, 4H), 1.98 (t, *J* = 7.64 Hz, 4H), 1.25 (t, *J* = 7.14 Hz, 6H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 173.24, 109.84, 65.02, 60.20, 32.07, 28.78, 14.07 ppm.

Ethyl 3-(2-(3-hydroxypropyl)-1,3-dioxolan-2-yl)propanoate (10)

A solution of KOH (1 eq., 47.7 mmol, 47.7 mL; 1 M in EtOH) was added dropwise to diethyl ester **9** (13.2 g, 47.7 mmol) at 50 °C in 4 h. The resulting mixture was stirred at 50 °C for 14 h after which all EtOH was evaporated under reduced pressure. The resulting residue was suspended in THF (250 mL) and Et_3N (0.5 eq., 23.8 mmol, 3.31 mL) and ethyl chloroformate (1.5 eq., 71.5 mmol, 6.84 mL) were added. After vigorously stirring for 2 h the mixture was added to a cooled (0 °C) solution of $NaBH_4$ (1.5 eq., 71.5 mmol, 2.71 g) in H_2O (250 mL) and the mixture was stirred at RT for 1 h.

The reaction was quenched by addition of 1 mM aq. HCl (100 mL) and the resulting mixture was extracted with Et_2O (3×). The combined organic layers were extracted with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 100% EtOAc/PE) as a colourless liquid (yield: 4.14 g, 17.8 mmol, 37%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.13 (q, *J* = 7.13 Hz, 2H), 3.96 (s, 4H), 3.64 (t, *J* = 5.83 Hz, 2H), 2.37 (t, *J* = 7.75 Hz, 2H), 2.01 (t, *J* = 7.65 Hz, 2H), 1.93 (bs, 1H), 1.76-1.62 (m, 4H), 1.26 (t, *J* = 7.13 Hz, 3H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 208.84, 110.72, 65.00, 62.84, 60.34, 33.79, 31.90, 28.92, 26.89, 14.15 ppm. HRMS: calcd. for $C_{11}H_{20}O_5$ 255.12029 $[M + Na]^+$; found 255.12036.

Ethyl 3-(2-(3-(tosyloxy)propyl)-1,3-dioxolan-2-yl)propanoate (11)
Alcohol **10** (4.14 g, 17.8 mmol) was dissolved in DCM (125 mL) and Et_3N (2.1 eq., 37.4 mmol, 5.18 mL), DMAP (0.25 eq., 4.45 mmol, 0.50 g) and TsCl (2.55 eq., 8.66 mmol, 45.4 g) were added. The mixture was stirred for 4 h after which TLC analysis indicated a complete consumption of the starting compound. DCM was evaporated under reduced pressure and the residue was dissolved in EtOAc, extracted with 1 mM aq. HCl (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 50% EtOAc/PE) as a colourless liquid (yield: 5.87 g, 15.2 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.79 (d, *J* = 8.0 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 2H), 4.12 (q, *J* = 7.1 Hz, 2H), 4.04 (t, *J* = 6.4 Hz, 2H), 3.9-3.8 (m, 4H), 2.45 (s, 3H), 2.31 (t, *J* = 7.6 Hz, 2H), 1.92 (t, *J* = 7.6 Hz, 2H), 1.8-1.7 (m, 2H), 1.7-1.6 (m, 2H), 1.26 (t, *J* = 6.8 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.38, 144.80, 133.30, 129.80, 127.86, 110.15, 70.53, 65.06, 60.36, 33.01, 32.07, 28.83, 23.39, 21.60, 14.19 ppm.

Ethyl 3-(2-(3-azidopropyl)-1,3-dioxolan-2-yl)propanoate (12)
A solution of tosylate **11** (5.87 g, 15.2 mmol) and NaN_3 (1.2 eq., 18.2 mmol, 1.19 g) in DMF (120 mL) was stirred at 75 °C for 14 h, after which TLC analysis indicated a complete conversion. The mixture was concentrated under reduced pressure and the residue was dissolved in EtOAc, extracted with sat. aq. $NaHCO_3$ (2×), H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained as a colourless liquid (yield: 3.86 g, 15.0 mmol, 99%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.13 (q, *J* = 7.14 Hz, 2H), 3.94 (s, 4H), 3.32-3.26 (m, 2H), 2.36 (t, *J* = 7.60 Hz, 2H), 1.99 (t, *J* = 7.60 Hz, 2H), 1.71-1.65 (m, 4H), 1.26 (t, *J* = 7.15 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.23, 110.20, 64.93, 60.17, 51.29, 34.07, 31.94, 28.73, 23.24, 14.04 ppm. HRMS: calcd. for $C_{11}H_{19}N_3O_4$ 258.14483 $[M + H]^+$; found 258.14491. IR film (cm^{-1}) 2954.7, 2885.3, 2090.7, 1728.1, 1450.4, 1257.5, 1180.3, 1134.1, 1033.8, 948.9, 910.3, 864.1.

3-(2-(3-azidopropyl)-1,3-dioxolan-2-yl)propanoic acid (13)
Ethyl ester **12** (3.86 g, 15.0 mmol) was dissolved in MeOH (80 mL) and to this was added NaOH (4 eq., 60.0 mmol, 30 mL; 2 M in H_2O) at 0 °C. The mixture was allowed to slowly warm to RT and was stirred for 14 h after which TLC analysis revealed complete conversion. The mixture was concentrated under reduced pressure and the residue was dissolved in sat. aq. $NaHCO_3/H_2O$ (3:1 v/v, 80 mL) and extracted twice with EtOAc. Next, the aqueous layer was acidified with 10% w/v aq. HCl until pH 2 and extracted again twice with EtOAc. The latter organic layers were combined, dried over $MgSO_4$ and concentrated under reduced pressure, which yielded the title compound as a colourless liquid (yield: 3.05 g, 13.3 mmol, 89%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.20 (bs, 1H), 3.96 (s, 4H), 3.34-3.25 (m, 2H), 2.41 (t, *J* = 7.50 Hz, 2H), 2.00 (t, *J* = 7.51 Hz, 2H), 1.71-1.65 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 179.31, 110.16, 64.95, 51.23, 34.07, 31.68, 28.46, 23.21 ppm.

7-azido-4-oxoheptanoic acid (14)

Concentrated aq. HCl (19 mL) was added to a solution of compound **13** (3.05 g, 13.3 mmol) in THF (60 mL) and the mixture was stirred for 3 h, after which TLC analysis showed complete consumption of starting material. Water (150 mL) was added carefully and the aqueous layer was extracted with EtOAc three times. The combined organic layers were extracted with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The title compound was obtained as a colourless oil (yield: 2.46 g, 13.3 mmol, quant.) without further purification necessary. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.26 (bs, 1H), 3.32 (t, *J* = 6.64 Hz, 2H), 2.73 (t, *J* = 6.20 Hz, 2H), 2.64 (t, *J* = 6.16 Hz, 2H), 2.57 (t, *J* = 7.06 Hz, 2H), 1.88 (p, *J* = 6.86 Hz, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 207.65, 178.28, 50.56, 39.10, 36.80, 27.68, 22.79 ppm.

4-(3-(*tert*-butoxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-azido-4-oxoheptanoate (15)

To a solution of alcohol **7** (1 eq., 13.3 mmol, 4.08 g), carboxylic acid **14** (1 eq., 13.3 mmol, 2.46 g) and DMAP (0.1 eq., 1.33 mmol, 0.16 g) in DCM (100 mL) was added DIC (1.2 eq., 16.0 mmol, 2.51 mL) and the mixture was stirred for 14 h. Next, the mixture was concentrated under reduced pressure, the residue dissolved in EtOAc (100 mL), extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (100% Tol → 10% EtOAc/Tol) as a colourless oil (yield: 4.41 g, 9.64 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.96 (s, 2H), 3.30 (t, *J* = 6.68 Hz, 2H), 2.93-2.80 (m, 8H), 2.58 (t, *J* = 7.06 Hz, 2H), 2.53 (t, *J* = 7.83 Hz, 2H), 1.87 (p, *J* = 6.88 Hz, 2H), 1.42 (s, 9H), 1.17 (d, *J* = 6.91 Hz, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 207.21, 172.21, 171.48, 143.67, 140.03, 138.61, 123.69, 80.15, 50.52, 39.19, 36.95, 36.88, 30.90, 27.95, 27.65, 27.32, 22.85 ppm. HRMS: calcd. for $C_{26}H_{39}N_3O_5$ 496.27819 $[M + Na]^+$; found 496.27775.

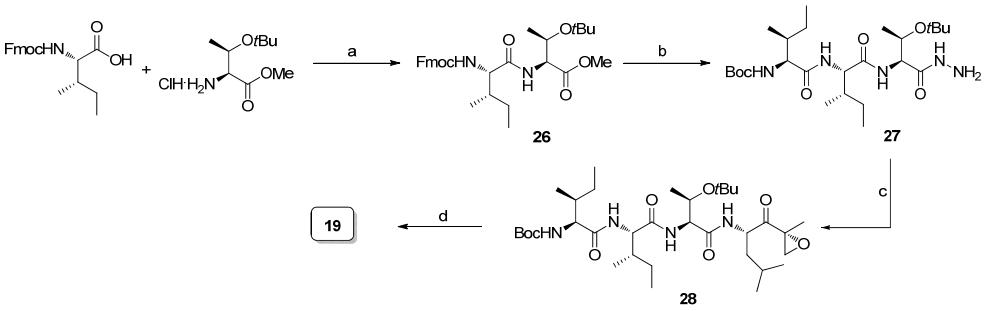
3-(4-((7-azido-4-oxoheptanoyl)oxy)-3,5-diisopropylphenyl)propanoic acid (16)

TFA (10 mL) was added to a solution of *tert*-butyl ester **15** (1.40 g, 3.06 mmol) in DCM (10 mL) and this mixture was stirred for 30 min. after which TLC analysis showed complete consumption of starting material. Toluene (25 mL) was added and the mixture was concentrated under reduced pressure. In order to remove traces of TFA the mixture was coevaporated with toluene three times. The title compound was obtained as a colourless oil (yield: 1.28 g, 3.06 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.40 (s, 1H), 6.98 (s, 2H), 3.29 (t, *J* = 6.69 Hz, 2H), 2.95-2.81 (m, 8H), 2.67 (t, *J* = 7.91 Hz, 2H), 2.59 (t, *J* = 7.06 Hz, 2H), 1.87 (p, *J* = 6.92 Hz, 2H), 1.17 (d, *J* = 6.91 Hz, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 207.43, 178.29, 171.58, 143.75, 140.18, 138.08, 123.64, 50.44, 39.14, 36.82, 35.59, 30.48, 27.59, 27.27, 22.79 ppm. HRMS: calcd. for $C_{22}H_{31}N_3O_5$ 440.21559 $[M + Na]^+$; found 440.21539.

4-(3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-azido-4-oxoheptanoate (17)

N-hydroxysuccinimide (1.5 eq., 1.30 mmol, 150 mg) and EDC (1.5 eq., 1.30 mmol, 249 mg) were added to a solution of carboxylic acid **16** (0.36 g, 0.87 mmol) in DCM (7 mL) and the mixture was stirred for 14 h, after which TLC analysis indicated complete consumption of starting material. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc (20 mL). This was extracted with 1 M aq. HCl (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (30% → 60% EtOAc/PE) as a colourless oil (yield: 0.43 g, 0.83 mmol, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.99 (s, 2H), 3.31 (t, *J* = 6.67 Hz, 2H), 3.06-3.00 (m, 2H), 2.95-2.81 (m, 12H), 2.60 (t, *J* = 7.06 Hz, 2H), 1.88 (p, *J* = 6.90 Hz, 2H), 1.18 (d, *J* = 6.89 Hz, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 207.26, 171.53, 169.03, 167.90, 144.16, 140.59, 137.15, 123.75, 50.60, 39.28, 36.95, 32.70, 30.35, 27.70, 27.44, 25.54, 22.91 ppm. LC-MS (System A): R_t (min): 9.38 (ESI-MS (*m/z*): 515.4 ($M + H^+$)). HRMS: calcd. for $C_{26}H_{34}N_4O_7$ 515.25003 $[M + H]^+$; found 515.24963.

Scheme 3. Synthesis of compound **19**.



Reagents and conditions: (a) HCTU, DiPEA, DCM, 89%; (b) i) DBU, DMF; ii) HOBt; iii) HCTU, DiPEA; iv) $NH_2NH_2 \cdot H_2O$, MeOH, 72%; (c) i) *t*BuONO, HCl, DCM, DMF, –30 °C; ii) TFA·H_2N-Leu-EK, DiPEA, 89%; (d) i) TFA, DCM; ii) **17**, DiPEA, DMF, 63%.

Fmoc-Ile-Thr(*t*Bu)-OMe (26)

Fmoc-Ile-OH (1.2 eq., 13.3 mmol, 4.70 g) was dissolved in DCM (60 mL) and to this were added HCTU (1.2 eq., 13.3 mmol, 5.50 g), DiPEA (3.3 eq., 36.0 mmol, 6.0 mL) and HCl·H-Thr(*t*Bu)-OMe (1 eq., 11.0 mmol, 2.50 g) successively. The mixture was stirred for 2 h after which TLC analysis indicated a completed reaction. DCM was evaporated under reduced pressure and the residue was dissolved in EtOAc, extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (10% → 50% EtOAc/PE) as a colourless solid (yield: 5.16 g, 9.83 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.48 Hz, 2H), 7.60 (d, *J* = 7.41 Hz, 2H), 7.39 (t, *J* = 7.46 Hz, 2H), 7.31 (dt, *J* = 7.43, 0.98 Hz, 2H), 6.48 (d, *J* = 8.84 Hz, 1H), 5.58 (d, *J* = 8.70 Hz, 1H), 4.49 (dd, *J* = 9.00, 1.68 Hz, 1H), 4.44-4.33 (m, 2H), 4.28-4.21 (m, 2H), 4.18 (dd, *J* = 8.53, 6.39 Hz, 1H), 3.71 (s, 3H), 1.94-1.83 (m, 1H), 1.65-1.53 (m, 1H), 1.33-1.21 (m, 1H), 1.17 (d, *J* = 6.27 Hz, 3H), 1.11 (s, 9H), 1.02-0.94 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.42, 170.86, 156.07, 143.78, 141.24, 127.63, 127.01, 125.07, 119.90, 74.21, 67.19, 66.96, 59.30, 57.83, 52.13, 47.17, 38.18, 28.27, 24.82, 21.04, 15.08, 11.52 ppm. LC-MS (System B): R_t (min): 10.87 (ESI-MS (*m/z*): 525.0 ($M + H^+$)).

Boc-Ile-Ile-Thr(*t*Bu)-$NHNH_2$ (27)

DBU (1.05 eq., 10.3 mmol, 1.57 mL) was added to a solution of Fmoc-Ile-Thr(*t*Bu)-OMe (**26**, 5.16 g, 9.83 mmol) in DMF (50 mL) and the mixture was stirred for 5 min. after which HOBt (1.5 eq., 14.7 mmol, 1.98 g) was added. After stirring the mixture for another 30 min. Boc-Ile-OH (1.2 eq., 11.8 mmol, 2.73 g), HCTU (1.2 eq., 11.8 mmol, 4.88 g) and DiPEA (3 eq., 29.5 mmol, 4.87 mL) were added. TLC analysis indicated sufficient product formation after 14 h and the mixture was concentrated under reduced pressure. The residue was redissolved in DCM and extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The intermediate was purified by column chromatography (10% → 50% EtOAc/PE) and the obtained product (3.69 g, 7.15 mmol) was dissolved in MeOH (50 mL). Hydrazine monohydrate (30 eq., 215 mmol, 10.4 mL) was added and the mixture was stirred for 14 h, after which TLC analysis indicated complete consumption of starting material. Toluene was added and the mixture was concentrated under reduced pressure. The title compound was obtained after coevaporation with toluene (3×) as a colourless solid (yield: 3.66 g, 7.10 mmol, 72%). ^{1}H NMR (400 MHz, CD_3OD): δ = 4.34 (d, *J* = 3.53 Hz, 1H), 4.29 (d, *J* = 8.12 Hz, 1H), 4.05-3.99 (m, 1H), 3.92 (d, *J* = 7.90 Hz, 1H), 1.90-1.70 (m, 2H), 1.61-1.47 (m, 2H), 1.42 (s, 3H), 1.22-1.10 (m, 1H), 1.17 (s, 9H), 1.08 (d, *J* = 6.32 Hz, 3H), 0.92-0.85 (m, 12H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 174.83, 173.39, 171.30, 157.91, 80.56, 75.84,

68.52, 60.62, 59.22, 58.56, 37.94, 37.85, 28.77, 28.66, 25.94, 19.78, 16.23, 15.95, 11.39, 11.32 ppm. LC-MS (System A): R_t (min): 6.08 (ESI-MS (*m/z*): 516.4 (M + H^+)). HRMS: calcd. for $C_{25}H_{49}N_5O_6$ 516.37556 $[M + H]^+$; found 516.37530.

Boc-Ile-Ile-Thr(*t*Bu)-Leu-EK (28)

Boc-Ile-Ile-Thr(*t*Bu)-$NHNH_2$ (**27**, 1 eq., 3.87 mmol, 2.0 g) was dissolved in a 9:1 v/v mixture of DCM/DMF (40 mL) and cooled to –30 °C. To this were added *t*BuONO (1.1 eq., 4.25 mmol, 0.57 mL) and HCl (2.8 eq., 10.8 mmol, 2.70 mL; 4 M in 1,4-dioxane). This mixture was stirred at –30 °C for 3 h, after which (S)-2-amino-4-methyl-1-((R)-2-methyloxiran-2-yl)pentan-1-one TFA salt[23,24] (1.1 eq., 4.25 mmol, 1.16 g) in DMF (5 mL) and DiPEA (5 eq., 20.0 mmol, 3.31 mL) were added. The reaction was allowed to warm to RT and stirred for 14 h. Next, DCM (15 mL) was added and the mixture was extracted with 0.1 M aq., HCl (2×) and H_2O, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (20% → 50% EtOAc/PE) as a colourless solid (yield: 2.25 g, 3.43 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.64 (d, *J* = 7.47 Hz, 1H), 6.99 (d, *J* = 5.63 Hz, 1H), 6.45 (d, *J* = 8.22 Hz, 1H), 5.22 (d, *J* = 7.84 Hz, 1H), 4.46 (ddd, *J* = 10.45, 7.55, 2.94 Hz, 1H), 4.41-4.32 (m, 2H), 4.14-4.07 (m, 1H), 3.94 (t, *J* = 7.34 Hz, 1H), 3.38 (d, *J* = 5.07 Hz, 1H), 2.89 (d, *J* = 5.06 Hz, 1H), 1.92-1.77 (m, 2H), 1.74-1.63 (m, 1H), 1.60-1.55 (m, 1H), 1.54-1.48 (m, 2H), 1.52 (s, 3H), 1.44 (s, 9H), 1.33-1.24 (m, 1H), 1.28 (s, 9H), 1.19-1.08 (m, 2H), 1.06 (d, *J* = 6.44 Hz, 3H), 0.96 (d, *J* = 6.54 Hz, 6H), 0.92-0.86 (m, 12H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 208.06, 171.59, 170.73, 169.51, 155.80, 79.76, 75.49, 66.14, 59.24, 57.68, 56.95, 52.39, 50.74, 39.80, 37.30, 36.97, 28.28, 28.08, 25.42, 24.88, 24.69, 23.35, 21.35, 16.75, 16.33, 15.53, 15.40, 11.28 ppm. LC-MS (System B): R_t (min): 11.33 (ESI-MS (*m/z*): 655.27 (M + H^+)). HRMS: calcd. for $C_{34}H_{62}N_4O_8$ 655.46404 $[M + H]^+$; found 655.46451.

N_3-Lev-phenol-epoxomicin (19)

Compound **24** (165 mg, 0.25 mmol) was treated with a 1:1 v/v mixture of DCM/TFA (2 mL) for 1 h and subsequently coevaporated with toluene (3×). The resulting intermediate was dissolved in DMF (2 mL) and to this were added compound **17** (1.1 eq., 0.258 mmol, 144 mg) and DiPEA (2 eq., 0.50 mmol, 83 μL). The reaction was stirred for 14 h before being concentrated under reduced pressure. The residue was dissolved in DCM (10 mL) and extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 100% EtOAc/PE) as a colourless solid (yield: 141 mg, 0.16 mmol, 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.75 (s, 1H), 8.28 (s, 1H), 8.24 (s, 1H), 7.46 (s, 1H), 6.97 (s, 2H), 4.94-4.76 (m, 3H), 4.63-4.55 (m, 1H), 4.34 (s, 1H), 4.09-4.01 (m, 1H), 3.31 (t, *J* = 6.64 Hz, 2H), 3.22 (s, 1H), 3.00-2.77 (m, 9H), 2.59 (t, *J* = 7.01 Hz, 4H), 1.88 (p, *J* = 6.89 Hz, 2H), 1.83-1.73 (m, 1H), 1.70-1.35 (m, 8H), 1.49 (s, 3H), 1.20-1.06 (m, 15H), 0.91-0.71 (m, 18H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 208.14, 207.22, 172.12, 171.40, 170.57, 143.75, 140.05, 138.75, 123.71, 67.38, 58.99, 57.47, 57.44, 57.16, 52.12, 50.53, 50.41, 39.22, 38.07, 37.77, 36.92, 31.56, 27.65, 27.37, 25.29, 25.14, 24.88, 23.12, 22.85, 21.09, 17.35, 16.65, 15.20, 15.16, 11.51, 11.35 ppm. $[\alpha]_D^{23}$ = –0.57 (*c* = 1 in $CHCl_3$). LC-MS (System B): R_t (min): 10.66 (ESI-MS (*m/z*): 898.40 (M + H^+)). HRMS: calcd. for $C_{47}H_{75}N_7O_{10}$ 898.56482 $[M + H]^+$; found 898.56539.

Biotin-Ahx-propargylamide (20)

Boc-Ahx-propargylamide[25] (275 mg, 1.03 mmol) was treated with a 1:1 v/v mixture of DCM/TFA (6 mL) for 1 h and subsequently coevaporated with toluene (3×). The resulting intermediate was dissolved in DMF (5 mL) and to this were added biotin-OSu (1.1 eq., 1.1 mmol, 375 mg) and DiPEA (1.5 eq., 1.50 mmol, 248 μL). The reaction was stirred for 14 h before being concentrated under reduced pressure. The title compound was obtained after crystallisation from MeOH/Et_2O as a colourless solid. The compound was sufficiently pure based on LC-MS analysis and subjected to

the next step without further purification. LC-MS (System B): R_t (min): 4.27 (ESI-MS (*m/z*): 395.13 ($M + H^+$)).

Biotin-Ahx-triazole-Lev-phenol-epoxomicin (1)

To a solution of compound **19** (45.0 mg, 50.1 μmol) and biotin-Ahx-propargylamide (**20**, 1.5 eq., 70.0 μmol, 30.0 mg) in DMF (1.5 mL) were added $CuSO_4$ (0.2 eq., 10.0 μmol, 10.0 μL; 1 M in H_2O) and sodium ascorbate (0.3 eq., 15.0 μmol, 15.0 μL; 1 M in H_2O) and the mixture was stirred for 14 h at RT after which LC-MS analysis indicated a complete conversion of the azide. The mixture was concentrated under reduced pressure and the title compound was obtained after RP-HPLC purification (40% → 70% ACN/0.1% aq. TFA) as a colourless solid (yield: 26.3 mg, 20.4 μmol, 41%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.76 (s, 1H), 6.92 (s, 2H), 4.45 (dd, *J* = 10.68, 3.02 Hz, 1H), 4.38 (dd, *J* = 7.43, 5.04 Hz, 1H), 4.34-4.26 (m, 4H), 4.22 (d, *J* = 5.04 Hz, 1H), 4.19 (dd, *J* = 7.87, 4.49 Hz, 1H), 4.15 (d, *J* = 7.99 Hz, 1H), 4.10 (d, *J* = 8.14 Hz, 1H), 3.93 (dd, *J* = 6.19, 5.28 Hz, 1H), 3.15 (d, *J* = 5.07 Hz, 1H), 3.12-3.08 (m, 1H), 3.05 (t, *J* = 7.07 Hz, 2H), 2.87-2.70 (m, 10H), 2.59 (d, *J* = 12.70 Hz, 1H), 2.51-2.39 (m, 4H), 2.15-2.01 (m, 6H), 1.81-1.70 (m, 1H), 1.69-1.37 (m, 12H), 1.36 (s, 3H), 1.35-1.17 (m, 6H), 1.10-1.01 (m, 16H), 1.00-0.88 (m, 1H), 0.86-0.77 (m, 12H), 0.76-0.69 (m, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 209.52, 209.43, 176.00, 175.27, 174.10, 173.71, 173.61, 172.25, 145.32, 141.72, 140.24, 124.93, 68.57, 63.45, 61.69, 60.14, 59.84, 59.41, 59.32, 57.06, 53.10, 51.85, 50.58, 41.09, 40.37, 40.23, 39.53, 38.73, 38.04, 37.88, 37.79, 36.85, 35.64, 32.80, 30.16, 29.83, 29.54, 28.68, 28.61, 27.58, 26.98, 26.54, 26.26, 26.05, 25.46, 23.82, 21.53, 20.06, 17.06, 16.01, 15.97, 11.49, 11.37 ppm. $[\alpha]_D^{23} = -1.28$ (*c* = 1 in MeOH). LC-MS (System B): R_t (min): 8.42 (ESI-MS (*m/z*): 1292.53 ($M + H^+$)). HRMS: calcd. for $C_{66}H_{105}N_{11}O_{13}S$ 1292.76868 $[M + H]^+$; found 1292.76980.

Stability/cleavage of the test-substrate

The test-substrate N_3-CL-Lys-Gly-OH was made from compound **17** and dipeptide Fmoc-Lys(Boc)-Gly-O*t*Bu via standard peptide chemistry. The test-substrate was dissolved (10 mM) in either 100 mM Tris pH 7.5, 100 mM HEPES pH 5.8, PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_3, 0.14 M NaCl, 2.7 mM KCl) or McIlvaine's buffers (combinations of 0.1 M citric acid and 0.2 M Na_2HPO_4, pH 3, 4, 5, 6). Hydrazine or hydroxylamine (100 mM final concentration) was added where appropriate. The samples were incubated for either 1 h or 15 h (o/n) at 23 °C or 37 °C. LC-MS samples were prepared by mixing 250 μL H_2O with 25 μL of the reaction mixture and 1 drop of TFA. The samples were analyzed by LC-MS by injection of 20 μL of the LC-MS sample and the applied gradient was 10% → 90% ACN/0.1% aq. TFA. LC-MS results: test-substrate: R_t (min.): 5.60 (ESI-MS (m/z): 603.7 ($M + H^+$)); cleavage product azide: R_t (min.): 2.71 (ESI-MS (m/z): 182.1 ($M + H^+$)); cleavage product phenol: R_t (min.): 4.24 (ESI-MS (m/z): 436.4 ($M + H^+$)). No signals for hydrolyzed substrate was detected in either sample.

Search for optimal cleavage conditions

HEK-293T cell lysate (13.5 μg protein) was incubated with compound **1** (2.5 μM final concentration) for 1 h at 37 °C, after which the samples were denatured by addition of 4 μL 4× SB and boiling for 5 min. The appropriate amount of hydrazine (0, 1, 10, 50, 100 mM final concentration) was added and the samples were incubated at 23 °C for 15 h (o/n). After resolving of the protein content by 12.5% SDS-PAGE all biotinylated proteins were visualized by Western blotting. The blots were blocked with 1% BSA in TBS-Tween 20 (0.1 % Tween 20) for 30 min. at RT, hybridized for 1 h with Streptavidin-HRP (1:10,000) in blocking buffer, washed and visualized using an ECL+ kit (Amersham Biosciences).

Pull-down experiments

HEK-293T cell lysate (containing some 1.3 mg of protein) was incubated with 20 μM ABPs **1** or **21** for 1 h at 37 °C, denatured by boiling for 5 min. with 1% SDS and precipitated with

chloroform/methanol (C/M).[26] The protein pellet was rehydrated in 180 μL 8 M urea/100 mM NH_4HCO_3, reduced with 10 μL 90 mM DTT for 30 min. at 37 °C, alkylated with 15 μL 200 mM iodoacetamide at RT in the dark, cleared by centrifugation at 13,000 g and desalted by C/M. The pellet was dispersed in 25 μL pull down (PD) buffer (50 mM Tris·HCl pH 7.5, 150 mM NaCl) with 2% SDS in a heated (37 °C) sonic bath. Stepwise (3 × 25 μL, 4 × 100 μL, 1 × 500 μL) addition of PD buffer afforded a clear solution that was incubated with 50 μL MyOne T1 Streptavidin grafted magnetic beads (Invitrogen) at RT with vigorous shaking for 1 h. The beads were stringently washed with 2 × 300 μL PD buffer with 0.1% SDS, 2 × 300 μL PD buffer, 2 × 300 μL wash buffer I (4 M urea/50 mM NH_4HCO_3), 2 × 300 μL wash buffer II (50 mM Tris·HCl pH 7.5, 10 mM NaCl) and 2 × 300 μL water. All 5 samples from ABP **1** were mixed and divided over 8 equal portions. The same was done for the 2 samples from ABP **21**, which were divided over 3 equal portions. To the samples was added 65 μL of the appropriate cleavage cocktail (see Figure 3) and the samples were shaken for 15 h at RT or at 37 °C. Next, the supernatant was removed, diluted with 20 μL 4× sample buffer and boiled at 100 °C for 5 min. One sample from ABP **21** was treated directly with 85 μL 1× sample buffer containing 10 μM biotin, boiled at 100 °C for 5 min. and stored o/n at 4 °C. The samples were resolved by 12.5% SDS-PAGE (25 μL of each sample was loaded) and all proteins were visualized by silverstain. The appropriate bands were cut from the gel and an in-gel digestion was performed according to the procedure described in literature.[27]
After removal of the supernatant all beads were stringently washed as described above, eluted with 85 μL 1× sample buffer containing 10 μM biotin at 100 °C for 5 min., resolved by SDS-PAGE and visualized by silverstain to determine the cleavage efficiency (see Figure 4).

LC-MS/MS analysis

Trytic peptides were analyzed on a Surveyor nanoLC system (Thermo) hyphenated to a LTQ-Orbitrap mass spectrometer (Thermo). Gold and carbon coated emitters (OD/ID = 360/25 μm tip ID = 5 μm), trap column (OD/ID = 360/100 μm packed with 25 mm robust Poros®10R2/15 mm BioSphere C18 5 μm 120 Å) and analytical columns (OD/ID = 360/75 μm packed with 20 cm BioSphere C18 5 μm 120 Å) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% formic acid/H_2O, B: 0.1% formic acid/ACN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column via a 15 mm long TFE teflon tubing sleeve (OD/ID 0.3 × 1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a nanosource base (Upchurch scientific, Idex, USA). General mass spectrometric conditions were: an electrospray voltage of 1.8 kV was applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150 °C, capillary voltage 41 V, tube lens voltage 150 V. Internal mass calibration was performed with air-borne protonated polydimethylcyclosiloxane (m/z = 445.12002) and the plasticizer protonated dioctyl phthalate ions (m/z = 391.28429) as lock mass.[28] For shotgun proteomics analysis, 10 μL of the samples was pressure loaded on the trap column with a 10 μL/min flow for 5 min. followed by peptide separation with a gradient of 35 min. 5 → 30% B, 15 min. 30 → 60% B, 5 min. A, at a flow of 300 μL/min. split to 250 nL/min. by the LTQ divert valve. For each data dependent cycle, one full MS scan (300-2000 m/z) acquired at high mass resolution (60,000 at 400 m/z, AGC target 1 × 106, maximum injection time 1,000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the LTQ linear ion trap (AGC target 5 × 103, max injection time 120 ms) from the three most abundant ions.[29] MS/MS settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 500 counts, activation q = 0.25 and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 60 s and ions with $z < 2$ or unassigned were not analyzed. Data from MS/MS was validated manually.

Stability of the cleavable linker in blood serum and plasma

Plasma and serum were collected from blood voluntarily donated by a healthy person. For plasma collection: blood was extracted in an EDTA tube and centrifuged for 15 min. at 4,000 g. For serum

collection no EDTA was used. All experiments were executed in duplo. A volume of 100 μL plasma or serum was incubated with compound **1** (10 μM final concentration) for 15 h at 37 °C. Next, 900 μL of cooled (–20 °C) acetone was added and the samples were kept at –20 °C for 2 h and an additional 1 h at –80 °C. This was followed by centrifuging of the samples for 10 min. at 14,000 g, after which all acetone was evaporated under reduced pressure. The resulting yellowish pellet was extracted with 100 μL ACN/H_2O/*t*BuOH (1:1:1) for 5 min. and the samples were analyzed by LC-MS (injection of 20 μL of the extract, system B, 10% → 90% ACN/0.1% aq. TFA).

References

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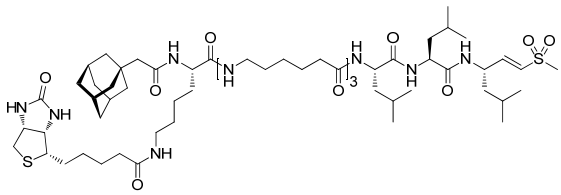

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8 Summary and Future Prospects

The research described in this Thesis aims at the development of chemical biology research tools to study proteolytic activities of metalloproteases (MMPs and ADAMs) and proteasomes. The common thread throughout the Thesis is the use of photocrosslinking in combination with activity-based protein profiling. **Chapter 1** describes the properties of the three most commonly applied photophores and examples of their use in chemical biology.

A potent class of MMP/ADAM inhibitors comprise the peptide succinyl hydroxamates. **Chapter 2** deals with the synthesis of *N,O*-diprotected succinyl hydroxamate building block **1** (Figure 1) and its use in the construction of enantiopure peptide hydroxamates in a highly efficient, linear solid-phase peptide synthesis (SPPS) protocol. With the aid of this building block, a biotinylated photoreactive probe was synthesized, which was applied in the covalent modification and visualization of ADAM-10. In addition, a library containing 96 inhibitors with general structure **2** was created and used to study the inhibitor preference of three metalloproteases (MMP-9, MMP-12 and ADAM-17) with respect to the substituents at the P2' and P3' positions. The presented highly efficient method for SPPS of peptide hydroxamates allows an easy extension of the inhibitor library by incorporation of other (both proteinogenic and non-proteinogenic) amino acids at all positions. Especially the modification of the P1' substituent will be of great interest, since this position has a big influence on the enzyme's substrate preference.[1,2] It should however be noted that modification of this site will lead to a change in building block structure and hence a greater synthetic

challenge. Extension of the inhibitor arsenal will hopefully lead to more potent, but especially more selective inhibitors for the MMP/ADAM family.

Another interesting modification is the change of the hydroxamic acid zinc binding group (ZBG) into a zinc coordinating electrophilic moiety, which would allow for the covalent capture of metalloproteases. This concept has proven to be useful in the covalent modification of histone deacetylases (HDACs), which represent another class of zinc dependent metalloenzymes. For example, it was shown that the epoxyketone moiety is able to coordinate the Zn^{2+} ion in HDACs and can subsequently trap a nearby nucleophile in the active site.[3,4] A possible mechanism of action for epoxyketone containing MMP inhibitors is shown in Figure 1 (insert).[5] As a first attempt diastereoisomers **3a** and **3b** were synthesized. These compounds are analogues of a known potent MMP inhibitor, which contains the hydroxamic acid ZBG.[6] Unfortunately, in a preliminary activity study it was shown that these compounds only display inhibitory activity in the millimolar range towards MMP-12. Therefore, variations in the epoxyketone moiety or the use of other electrophilic ZBGs will be necessary.

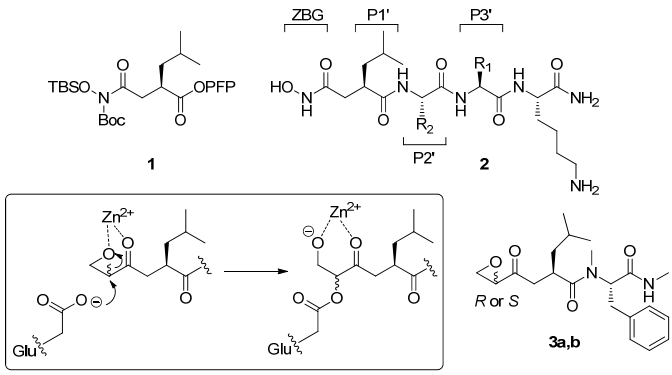


Figure 1. Structure of building block **1**, used for the construction of a library containing 96 metalloprotease inhibitors with general structure **2**. The epoxyketone ZBG containing MMP inhibitors **3a,b** are supposed to form a covalent adduct with the enzyme's active site via the mechanism shown in the insert.

The efficiency of photolabelling of ADAM-10 with the photoreactive probe described in Chapter 2 proved to be rather modest. Therefore, the effect of moving the photophore to the more tightly binding P1' pocket was studied and this is outlined in **Chapter 3**. The synthesis of building block **4** (Figure 2) and its application in the preparation of activity-based probe **5** is described herein. It was further demonstrated that **5** is indeed the more efficient MMP/ADAM probe in a head to head comparison towards a range of recombinant, purified metalloproteases. It has been shown that the use of other photophores in related MMP probes can also lead to efficient labelling.[7,8] However, the structural differences between these probes make mutual comparisons difficult. It is therefore of interest to synthesize the aryl azide (**6a**) and benzophenone (**6b**) analogues of **5** and study the differences with respect to inhibitory potency and labelling efficiency.

One of the most challenging tasks in photoaffinity labelling is the determination of the crosslinking site(s). Indeed, despite many efforts, locating the modified fragment after incubation and photocrosslinking MMPs with **5** has proven very difficult so far. A possible solution to this problem is the use of mixed isotope labelling (see Chapter 1). For this, the photoaffinity probe should be designed with incorporation of a stable isotope containing moiety. In this respect, a valuable set of stable isotope tags may be the ^{13}C-labelled PEG reagents **7a-g** (Figure 2),[9] which were initially designed as labelling reagents for the quantitative analysis of low molecular weight metabolites (intracellular amino acids and glutathione) by LC-MS. They can be easily incorporated into an ABP by means of a reaction between the activated PFP ester and an amine, both in solution-phase peptide chemistry and in SPPS. This would allow for the preparation of a set of structurally identical probes of different molecular weight, which can be used directly in mixed isotope labelling.

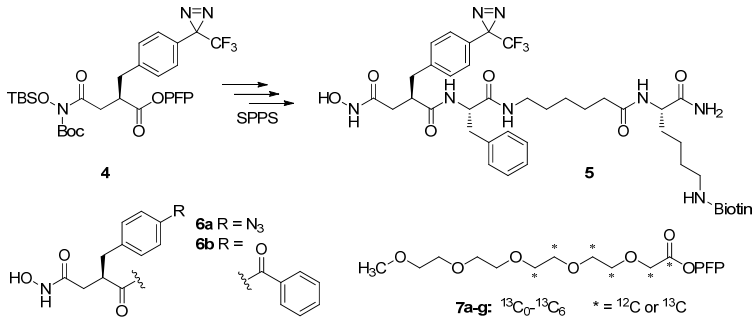


Figure 2. Structure of photoreactive building block **4**, used for the synthesis of MMP/ADAM probe **5**. Incorporation of the aryl azide or benzophenone photophores will lead to **6a** and **6b** respectively. The set of ^{13}C-labelled PEG derivatives **7a-g** was used for quantitative analysis of low molecular weight metabolites.[9]

The research described in **Chapter 4** entails the study towards the effect of fluorine incorporation into proteasome inhibitors on their specificity towards the different subunits. It was found that substitution of non-fluorinated Phe in peptide boronic ester **8** (Figure 3) with fluorinated versions (see insert) does not affect the selectivity. In addition, a set of peptide epoxyketones **9** was prepared, in which either one or both of the P2 and P3 positions is fluorinated. Interestingly, it was shown that fluorine incorporation, especially at the P2 site, led to a large decrease in potency towards the β1 and β2 subunits and hence resulted in selectivity for β5. Tripeptide epoxyketone **10** was identified as one of the most β5 selective inhibitors known to date and was subsequently converted into fluorescent probe **11**, which can be used for specific labelling and visualization of the β5 subunit.

It will be of interest to study the effect of fluorine incorporation at the P1 position, since this substituent is of great importance for substrate specificity. Also, the use of other fluorinated amino acids can give rise to improved (β5) selectivity. Especially fluorinated leucine (Hfl, Leu(F_6), Figure 3)[10,11] will be of interest, since it serves as a good,

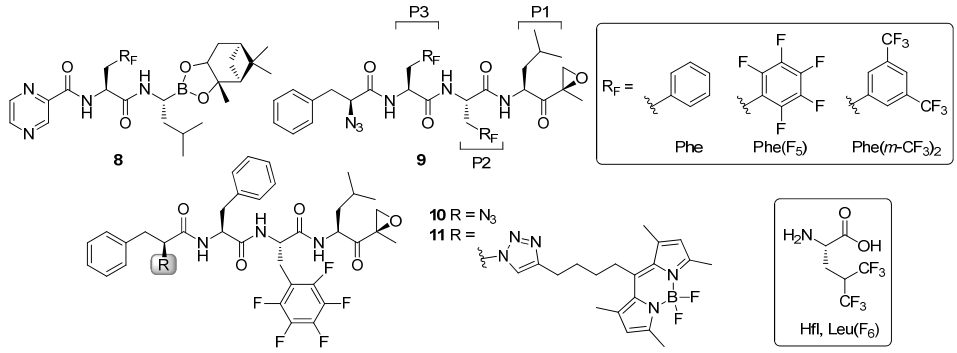


Figure 3. Structures of fluorinated proteasome inhibitors.

structurally similar, substitute for leucine, which is commonly used in proteasome inhibitors.

The development of selective inhibitors for the proteasome's trypsin-like sites (β2) with good cell penetrating properties is outlined in **Chapter 5**. Selectivity for β2 was achieved by instalment of basic side chain residues in the P1 position of tripeptide vinylsulfones and epoxyketones. Incorporation of a 4-aminomethylene phenylalanine analogue at this position resulted in compound **12** (Figure 4), which showed high inhibitory potency and selectivity for β2, both in cell extracts and in living cells. Attachment of this 4-aminomethylene-Phe-vinylsulfone warhead to the 3-hydroxy-2-methylbenzoyl-Val-Ser motif further increased β2 selectivity and even resulted in selectivity for β2 over its immuproteasome counterpart β2i.

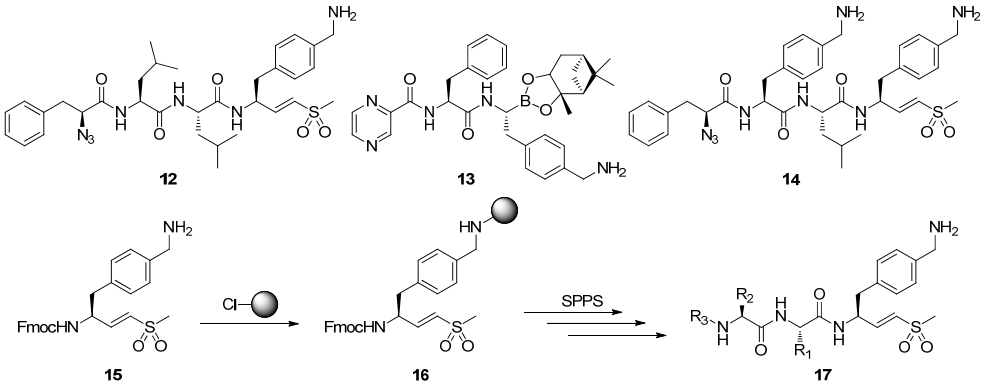


Figure 4. Structures of proteasome inhibitors functionalized with an amine at the P1 site and the use of this modification in SPPS.

It will be of interest to study the effect of incorporating the 4-aminobenzyl moiety in other peptide inhibitors. For example, incorporating the β2 directing substituent into β1,5 selective proteasome inhibitor Bortezomib (resulting in compound **13**) can lead to interesting insights into the selectivity determining part of the inhibitor. In addition, incorporation of 4-aminomethylene-Phe at the P3 position, resulting in **14**, can lead to

an even higher selectivity for β2, since it was shown that the substituent at the P3 position is also of great importance in subunit selectivity.[12] It should be noted that this added basic residue may interfere with cell permeability. An interesting feature of the side-chain amine functionality is that it allows for an easy, linear SPPS of inhibitor libraries. For example, vinyl sulfone **15** (Figure 4) can be attached to a solid support via its side-chain amine, giving **16**, after which a peptide chain can be build up at the N-terminus. A subsequent cleavage step will then result in inhibitors with general structure **17**.

Chapter 6 deals with the study towards the orientation of extended peptide vinylsulfones inside the proteasome's catalytically active cavity. The study combined activity-based modification, by means of a warhead, with photocrosslinking. A panel of bifunctional two-step probes, comprising of an extended peptide vinylsulfone equipped with a N-terminal photophore, was prepared. Of these probes, compound **18** (Figure 5) was able to establish a crosslink between the active β5 subunit and a neighbouring β6 subunit, which could be identified by subsequent SDS-PAGE and LC-MS/MS analysis. Although these results showed the applicability of this approach, it still needs improvements in order to be used in studies towards an inhibitor's orientation within the proteasome's active 20S core particle. The use of longer photoreactive probes, for example compounds **19** and **20**, may be of interest in this respect and can lead to a more complete picture of the interactions of an inhibitor with the proteasome's internal structure in general. Besides the spacer length, also a change of its nature, for instance by introduction of a more hydrophilic (PEG) or a more rigid spacer, can lead to interesting structural insights. This may well be combined with stable isotope incorporation, for example ^{13}C labelled PEG spacers (modified versions of the PEG compounds in Figure 2), which allows an easier identification of the photocross-linked adducts.

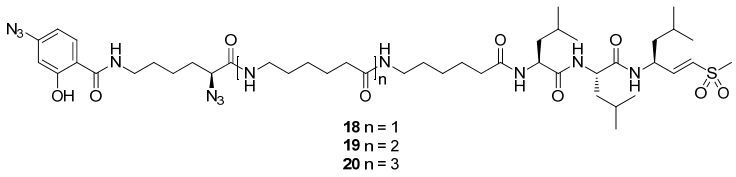


Figure 5. Structures of photoreactive proteasome probes.

The design and preparation of a novel cleavable linker system, based on the levulinoyl ester, is described in **Chapter 7**. The cleavable linker was incorporated into potent proteasome inhibitor epoxomicin and attachment of biotin resulted in ABP **21** (Figure 6). The optimal characteristics, in terms of stability and cleavability, of this linker system were reflected by the use of **21** for pull-down of proteasome active subunits from a cell lysate. Subsequent treatment with hydrazine allowed a chemoselective release of **21**-derived bound proteins. It was shown that the cleavable linker is well compatible with transformations often applied in chemical biology research, including click chemistry and Staudinger-Bertozzi ligation. In order to asses proteomics-wide

applicability of the system, it needs to be used for the enrichment of other enzymes as well. In this respect, compound **22** was synthesized, in which the cleavable linker moiety was incorporated into DCG-04, for the ABPP of cysteine proteases.[13] However, this compound remains to be tested.

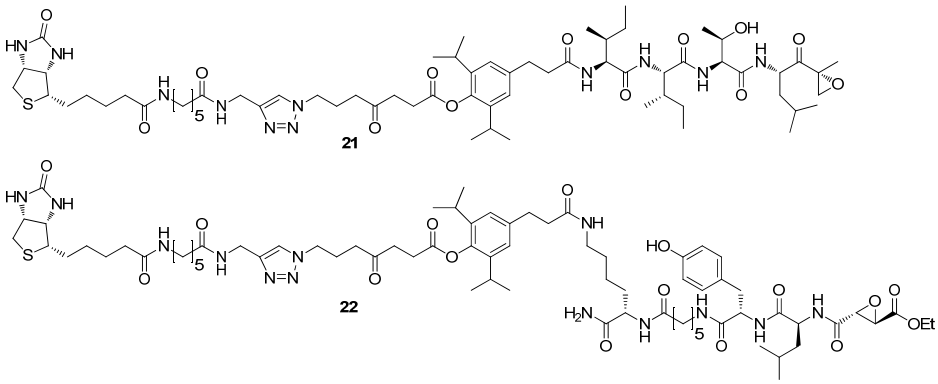
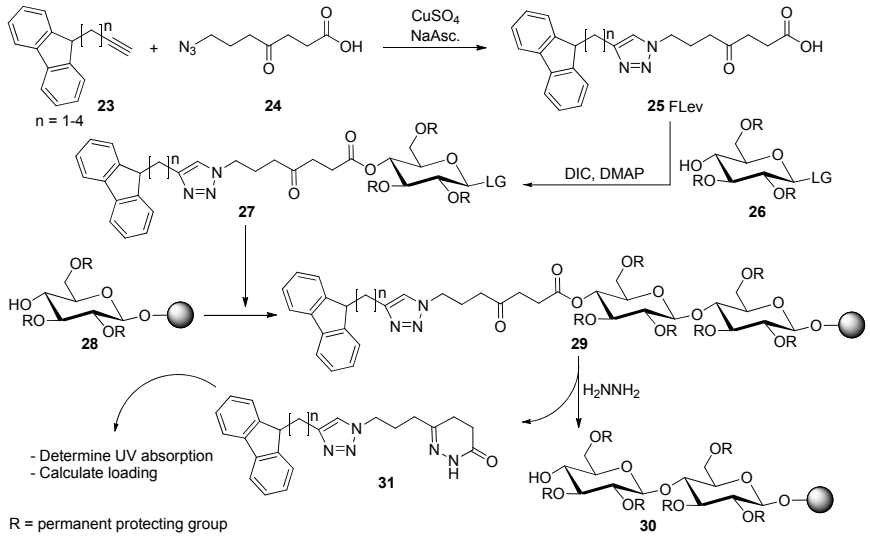


Figure 6. Structures of proteasome probe **21** and cysteine protease probe **22** containing the levulinoyl ester-based cleavable linker system.

The ease of cleavage of this linker system under relatively mild conditions and the fact that excess hydrazine can be easily removed by evaporation make that the use of this cleavable linker is not limited to activity-based enrichment of enzymes. For example, the linker system may find its use in affinity-based chromatography, or solid-phase extraction (SPE), of proteins. In addition, the synthetic strategy of the linker system allows for a straightforward modification of levulinic acid. A possible application of this can be in the field of (automated) solid-phase oligosaccharide synthesis.[14,15] This technique is used for the synthesis of oligosaccharides from their monomeric building blocks on a solid support, analogous to SPPS. The levulinoyl ester is often used as a temporal protecting group herein, because of the possibility to be cleaved orthogonally in the presence of other (permanent) protecting groups. A major difficulty in solid-phase oligosaccharide synthesis is the lack of straightforward methods to determine the loading of the resin, which indicates the coupling efficiency or yield. A possible solution for this is shown in Scheme 1. A click reaction of 9*H*-fluorene alkyne **23**, easily prepared from fluorene and the appropriate terminal alkyne halide,[16] with azido modified levulinic acid **24** (see Chapter 7) results in compound **25** (termed FLev). Subsequent condensation with **26** gives fully protected monomeric sugar building block **27**, which can be applied in solid-phase oligosaccharide synthesis. For example, a coupling between immobilized sugar **28** with donor **27** results in immobilized disaccharide **29**. Treatment of this construct with hydrazine results in a FLev deprotection and concomitant formation of fluorene containing compound **31**. Measuring the UV absorbance (λ = 301 nm) of the filtrate leads to the determination of the concentration of this species and hence, the loading on the solid support, which is analogous to Fmoc determination in SPPS.

Scheme 1. Suggested application of modified levulinic acid in solid-phase oligoaccharide synthesis.



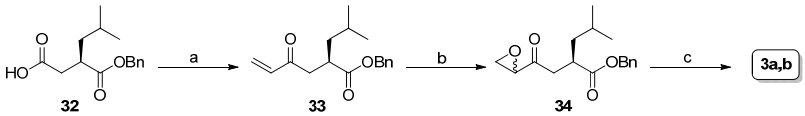
Experimental section

General

Tetrahydrofuran was distilled over $LiAlH_4$ before use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Fischer, Merck, Sigma-Aldrich) were used as received. *O*-(1*H*-6-Chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased at Iris Biotech (Marktrewitz, Germany). [1-$^{13}C_1$]acetic acid and [1,2-$^{13}C_2$]acetic acid were from Buchem b.v.. [1-$^{13}C_1$]bromoacetic acid and [1,2-$^{13}C_2$]bromoacetic acid were prepared from [1-$^{13}C_1$]acetic acid and [1,2-$^{13}C_2$]acetic acid via a reported procedure.[17] Amberlite IR120 H resin (Acros) was rinsed with MeOH (4×) and Et_2O (4×) before use. Traces of water were removed from reagents used in reactions that require anhydrous conditions by coevaporation with toluene. Solvents that were used in reactions were stored over 4 Å molecular sieves, except methanol and acetonitrile, which were stored over 3 Å molecular sieves. Column chromatography was performed on silicagel (Screening Devices b.v.) with a particle size of 40-63 μm and a pore size of 60 Å. Automated column chromatography was performed on a CombiFlash Companion with a Silicycle FLH-R10030B-ISO12 Sillaflash 12 g cartridge. The eluents toluene, ethyl acetate and petroleum ether (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 ml/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol JNM-FX-200 (200 MHz), a Bruker AV-400 (400 MHz) or a Bruker DMX-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, $CDCl_3$ or CD_3OD as internal standard. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 1/1; 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* = 150-2,000) and dioctylpthalate (*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 $[\alpha]_D^{23}$ were recorded on a Propol automatic polarimeter at room temperature. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 lm C18 50 × 4.60 mm column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI (system A) or on a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI (system B). Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 × 4.6 mm). RP-HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 × 10 mm column and a GX281 fraction collector. Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 × 4.6 mm).

Scheme 2. Synthesis of MMP inhibitors **3a,b** equipped with an epoxyketone ZBG.



Reagents and conditions: (a) i) $(ClCO)_2$, cat. DMF, DCM; ii) $Pd(PPh_3)_2Cl_2$, tributylvinyltin, toluene, 80°C, 81%; (b) H_2O_2, K_2CO_3, *t*BuOH, H_2O, 45%; (c) i) Pd/C, H_2, THF, 0°C; ii) TFA·MePheNHMe, HATU, DiPEA, DMF, 32%, 39%.

(R)-benzyl 2-isobutyl-4-oxohex-5-enoate (33)

Carboxylic acid **32** (2.65 g, 10 mmol) was dissolved in DCM (70 mL), put under an argon atmosphere and cooled to 0 °C. After addition of a few drops of DMF, oxalyl chloride (4 eq., 5.08 g, 40 mmol, 3.38 mL) was added to the solution. The mixture was stirred for 15 min. at 0 °C, then allowed to warm to room temperature and stirred until CO_2 formation seized after 1 h. Toluene was added and the solution was concentrated under reduced pressure followed by coevaporation three times with toluene. The resulting acyl chloride was dissolved in toluene (35 mL) before tributylvinyltin (1.05 eq, 3.07 mL, 3.33 g, 10.5 mmol) and $Pd(PPh_3)_2Cl_2$ (1 mol%, 70.2 mg, 0.1 mmol) were added. The mixture was then stirred for 20 h at 80 °C, a black colour was observed and TLC analysis indicated a complete conversion. The solution was allowed to cool to room temperature before being concentrated under reduced pressure. The product was obtained after purification by column chromatography (0% → 13% Et_2O/PE) as a clear yellow liquid (yield: 2.28 g, 8.32 mmol, 81%) ^1H NMR (400 MHz, $CDCl_3$): δ = 7.45-7.23 (m, 5H), 6.32 (dd, *J* = 17.7, 10.4 Hz, 1H), 6.20 (d, *J* = 17.6 Hz, 1H), 5.80 (d, *J* = 10.4 Hz, 1H), 5.11 (s, 2H), 3.07-2.98 (m, 2H), 2.71-2.62 (m, 1H), 1.69-1.49 (m, 2H), 1.39-1.23 (m, 1H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.86 (d, *J* = 6.3 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 198.3, 175.3, 136.1, 135.9, 128.3, 128.2, 127.9, 66.1, 41.5, 41.1, 38.2, 25.7, 22.4, 22.1 ppm. $[\alpha]_D^{23}$ = +14.6° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{17}H_{22}O_3$ $[M + H]^+$: 275.16417; found: 275.16432.

(R)-benzyl 4-methyl-2-(2-((S/R)oxiran-2-yl)-2-oxoethyl)pentanoate (34)

Compound **33** (1.1 g, 4.0 mmol) was dissolved in a mixture of *tert*-butanol (7.0 mL), hydrogen peroxide (2.5 eq, 1 mL, 10 mmol, 30% in H_2O) and H_2O (3.0 mL), before being cooled to 0 °C. An aqueous saturated solution of K_2CO_3 (344 μL) was added dropwise and the reaction was stirred for 1 h at 0 °C. The mixture was then allowed to warm to room temperature and stirred until TLC analysis indicated complete conversion after 2.5 h. Water was added to dilute the solution before being extracted three times with Et_2O. The organic layers were combined, dried over anhydrous

$MgSO_4$ and concentrated under reduced pressure. The crude mixture was then purified by column chromatography (3% → 8% EtOAc/PE) yielding 316 mg (1.09 mmol) of one diastereoisomer and 210 mg (0.72 mmol) of the other diastereoisomer. The total yield was 326 mg (1.81 mmol, 45.3%). Diastereoisomer A: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.42-7.28 (m, 5H), 5.16-4.99 (m, 2H), 3.42-3.33 (m, 1H), 3.09-2.96 (m, 1H), 2.95-2.88 (m, 2H), 2.69 (dd, *J* = 18.2, 10.2 Hz, 1H), 2.37 (dd, *J* = 18.2, 4.1 Hz, 1H), 1.63-1.49 (m, 2H), 1.35-1.21 (m, 1H), 0.91 (d, *J* = 6.3 Hz, 3H), 0.86 (d, *J* = 6.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 206.2, 175.2, 135.8, 128.4, 128.1, 128.0, 66.3, 53.6, 45.8, 41.0, 37.7, 37.6, 25.6, 22.3, 22.2 ppm. $[\alpha]_D^{23}$ = +56.6° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{17}H_{22}O_4$ $[M + H]^+$: 291.15909; found: 291.15917. Diastereoisomer B: ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.39-7.25 (m, 5H), 5.11(s, 2H), 3.42-3.38 (m, 1H), 3.02-2.81 (m, 3H), 2.79 (d, *J* = 5.7 Hz, 1H), 2.40 (dd, *J* = 17.9, 4.4 Hz, 1H), 1.63-1.47 (m, 2H), 1.33-1.21 (m, 1H), 0.90 (d, *J* = 5.8 Hz, 3H), 0.85 (d, *J* = 5.7 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 205.4, 174.9, 135.7, 128.3, 128.0, 127.9, 66.2, 53.1, 46.0, 40.8, 38.9, 37.7, 25.6, 22.3, 22.1 ppm. $[\alpha]_D^{23}$ = –12.6° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{17}H_{22}O_4$ $[M + H]^+$: 291.15909; found: 291.15921.

(S)-2-((R)-N,4-dimethyl-2-(2-((R/S)-oxiran-2-yl)-2-oxoethyl)pentanamido)-3-phenylpropanoic acid (3a)

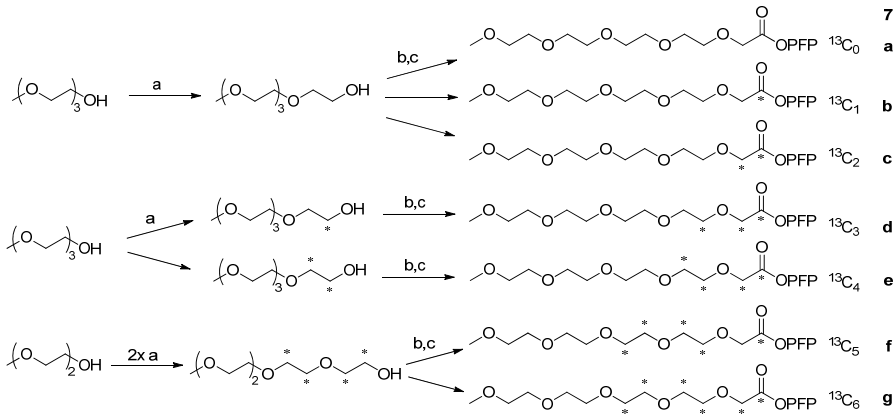
Compound **34** (156 mg, 0.54 mmol) was dissolved in THF (4 mL), put under an argon atmosphere and cooled to 0 °C. Next, 10% Pd/C was added and the mixture was subjected to hydrogen at 0 °C until TLC analysis indicated complete consumption of starting material after 4 h. The argon atmosphere was restored and the solution was diluted with 2 mL of DMF. HATU (1.2 eq., 246 mg, 0.65 mmol) and DiPEA (3.2 eq., 285 μL, 1.72 mmol) were added and the mixture was preactivated for 10 min. Next, a solution of Me-Phe-NHMe TFA salt (2 eq., 331 mg, 1.08 mmol) and DiPEA (2 eq., 178 μL, 1.08 mmol) in DMF (4 mL) was added and the mixture was stirred overnight while being allowed to slowly warm to room temperature. TLC analysis indicated the appearance of several spots, including one major spot at R_F 0.44 (50% EtOAc/PE). The mixture was filtrated over glassfiber patches and concentrated under reduced pressure. The residue was dissolved in DCM and washed with aqueous 1 M HCl, three times with a saturated aqueous $NaHCO_3$ solution and once with brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The product was isolated after purification by column chromatography (20% → 40% EtOAc/toluene) yielding 64.7 mg (0.17 mmol, 32%) of the title compound. ^{1}H NMR (400 MHz, $CDCl_3$, exists as a mixture of two rotamers): δ = 7.71 (d, J = 4.1 Hz, 0.5H), 7.35-7.08 (m, 5H), 6.08 (d, *J* = 4.1 Hz, 0.5H), 4.82 (t, *J* = 7.1 Hz, 0.5H), 4.46 (dd, *J* = 10.3, 3.6 Hz, 0.5H), 3.62 (dd, *J* = 14.5, 3.6 Hz, 0.5H), 3.41-3.28 (m, 1.5H), 3.15-3.04 (m, 1.5H), 3.01 (dd, *J* = 6.5, 3.9 Hz, 1H), 2.97 (s, 1.5H), 2.95 (d, *J* = 4.6 Hz, 2.5H), 2.86 (s, 1.5H), 2.86-2.83 (m, 0.5H), 2.73 (d, *J* = 4.9 Hz, 2H), 2.64 (dd, *J* = 18.4, 10.0 Hz, 0.5H), 2.34 (dd, *J* = 18.5, 3.4 Hz, 1H), 1.55-1.42 (m, 0.5H), 1.42-1.29 (m, 0.5H), 1.29-1.18 (m, 1H), 0.89 (d, *J* = 6.6 Hz, 1.5H), 0.86-0.76 (m, 2H), 0.61 (d, *J* = 6.6 Hz, 1.5H), 0.59 (d, *J* = 6.6 Hz, 1.5H), –0.19 (ddd, *J* = 14.2, 11.3, 1.5 Hz, 0.5H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, exists as a mixture of two rotamers): δ = 210.6, 207.6, 176.1, 175.8, 170.4, 169.9, 138.3, 137.7, 129.5, 129.1, 128.9, 128.4, 126.9, 126.4, 63.3, 60.6, 53.8, 53.6, 46.4, 46.0, 40.7, 38.7, 38.2, 37.8, 34.4, 33.9, 33.8, 33.4, 29.8, 26.7, 26.1, 25.5, 24.5, 23.3, 23.3, 21.6, 19.9 ppm. $[\alpha]_D^{23}$ –65.8° (*c* = 1 in $CHCl_3$). LC-MS: gradient 10% → 90% ACN(0.1% TFA/H_2O): R_t (min): 7.49. HRMS: calcd. for $C_{21}H_{30}N_2O_4$ $[M + H]^+$: 375.22783; found: 375.22784.

(S)-2-((R)-N,4-dimethyl-2-(2-((S/R)-oxiran-2-yl)-2-oxoethyl)pentanamido)-3-phenylpropanoic acid (3b)

Synthesized from its other diastereoisomer following the procedure described for compound **3a**. The compound was obtained in a yield of 114 mg (0.30 mmol, 39%). ^{1}H NMR (400 MHz, $CDCl_3$, exists as a mixture of rotamers): δ = 7.75 (d, J = 3.9 Hz, 0.5H, 7.33-7.12 (m, 5H), 6.16 (d, *J* = 4.1 Hz, 0.5H), 4.91 (t, *J* = 7.6 Hz, 0.5H), 4.46 (dd, *J* = 10.3, 3.5 Hz, 0.5H), 3.63 (dd, J = 14.5, 3.5 Hz, 0.5H), 3.43 (ddd, *J* = 25.1, 4.6, 2.4 Hz, 1H), 3.38-3.32 (m, 0.5H), 3.17-3.02 (m, 1.5H), 2.98 (d, *J* =

3.2 Hz, 1.5H), 3.01-2.94 (m, 1.5H), 2.93 (d, *J* = 4.7 Hz, 1.5H), 2.89 (d, *J* = 5.9 Hz, 1.5H), 2.85-2.79 (m, 0.5H), 2.79-2.75 (m, 1.5H), 2.74 (d, *J* = 4.9 Hz, 1.5H), 2.40 (ddd, *J* = 23.0, 18.8, 3.8 Hz, 1H), 1.53-1.40 (m, 0.5H), 1.40-1.29 (m, 0.5H), 1.29-1.19 (m, 1H), 0.94-0.86 (m, 2H), 0.83 (d, *J* = 6.5 Hz, 1.5H), 0.61 (d, *J* = 1.5 Hz, 1.5H), 0.59 (d, *J* = 1.5 Hz, 1.5H), –0.16 (ddd, *J* = 14.2, 11.3, 3.1 Hz, 0.5H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, exists as a mixture of rotamers) δ = 209.2, 176.1, 175.6, 170.4, 169.9, 138.3, 137.6, 129.5, 129.1, 128.9, 128.4, 126.9, 126.5, 63.4, 53.0, 47.0, 46.5, 40.7, 40.1, 39.9, 38.6, 34.5, 34.0, 33.9, 33.5, 29.9, 26.8, 26.1, 25.7, 24.6, 23.3, 23.2, 21.9, 19.9 ppm. $[\alpha]_D^{23}$ –100.9° (*c* = 1 in $CHCl_3$). LC-MS: gradient 10% → 90% ACN(0.1% TFA/H_2O): R_t (min): 7.28. HRMS: calcd. for $C_{21}H_{30}N_2O_4$ $[M + H]^+$: 375.22783; found: 375.22786.

Scheme 3. Synthesis of ^{13}C-labelled PEG-OPFP derivatives **7a-g**.



The asterisks (*) indicate the positions of ^{13}C atoms. Reagents and conditions: (a) i) $^{12}C_2$, 1-$^{13}C_1$, 1,2-$^{13}C_2$-BrAcOH, NaH, NaI, THF; ii) $LiAlH_4$, 15 minutes, then 2M NaOH (aq.), 75-95%; (b) i) $^{12}C_2$, 1-$^{13}C_1$, 1,2-$^{13}C_2$-BrAcOH, NaH, NaI, THF; ii) Amberlite H^+; (c) PFPOH, EDC, DCM, 55-60% over two steps.

General procedure A: extension of the PEG chain by one ethyleneglycol unit

To a solution of the alcohol (1 eq.), bromoacetic acid (either $^{12}C_2$, 1-$^{13}C_1$ or 1,2-$^{13}C_2$, 1.2 eq.) and NaI (0.01 eq.) in THF (5 mL/mmol) was carefully added NaH (60% in mineral oil, 4 eq.) in portions. After complete conversion of the alcohol (monitored by TLC analysis, usually after 2 h) $LiAlH_4$ (2.5 eq.) was carefully added in portions. The reaction was stirred for 30 min. after which NaOH (as a 2M aqueous solution, 2.5 eq.) was added dropwise. After 15 min. of stirring Et_2O (5 mL/mmol) and $MgSO_4$ were added. The reaction was stirred vigorously for another 5 min., after which the solid was filtrated over Celite. The filtrate was concentrated under reduced pressure and the resulting crude product was purified by column chromatography (100% EtOAc → 15% MeOH/EtOAc).

General procedure B: reaction of a PEG chain alcohol with bromoacetic acid followed by acidic work-up

To a solution of the alcohol (1 eq.), bromoacetic acid (either $^{12}C_2$, 1-$^{13}C_1$ or 1,2-$^{13}C_2$, 1.2 eq.) and NaI (0.01 eq.) in THF (5 mL/mmol) was carefully added NaH (60% in mineral oil, 4 eq.) in portions. After complete conversion of the alcohol (monitored by TLC analysis, usually after 2 h) Amberlyte IR120 H resin and Et_2O (2 mL/mmol) were added. After 30 min. of stirring all solids and the resin were filtered off over Celite and the filtrate was concentrated under reduced pressure. The crude product was subjected to the next step (general procedure C) without further purification.

General procedure C: conversion of the carboxylic acid to the pentafluorophenyl ester

The obtained (crude) carboxylic acid (obtained in general procedure B, 1 eq.) was dissolved in DCM (5 mL/mmol). To this solution were added pentafluorophenol (2 eq.) and EDC (2 eq.) and the reaction was stirred for 2 h. The mixture was then concentrated under reduced pressure and the product was purified by automated column chromatography (10% → 80% EtOAc/PE).

[13-^{13}C]2,5,8,11-tetraoxatridecan-13-ol

Prepared according to general procedure A, starting from 2-(2-(2-methoxyethoxy)ethoxy)ethanol (210 mg, 1.28 mmol). The product was obtained as a colourless liquid (yield: 199 mg, 0.95 mmol, 74%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 3.70 (dt, *J* = 150, 4.5 Hz, 2H), 3.70-3.53 (m, 14H), 3.39 (s, 3H), 2.61 (bs, 1H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 61.72 ppm.

[12,13-$^{13}C_2$]2,5,8,11-tetraoxatridecan-13-ol

Prepared according to general procedure A, starting from 2-(2-(2-methoxyethoxy)ethoxy)ethanol (210 mg, 1.28 mmol). The product was obtained as a colourless liquid (yield: 194 mg, 0.92 mmol, 72%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 3.70 (dt, *J* = 150, 4.5 Hz, 2H), 6.67 (dt, *J* = 150, 4.5 Hz, 2H), 3.70-3.53 (m, 12H), 3.40 (s, 3H), 2.72 (bs, 1H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 72.53 (d, *J* = 41 Hz), 61.70 (d, *J* = 41 Hz) ppm.

[1,2-$^{13}C_2$]2-(2-(2-methoxyethoxy)ethoxy)ethanol

Prepared according to general procedure A, starting from 2-(2-methoxyethoxy)ethanol (310 mg, 2.58 mmol). The product was obtained as a colourless liquid (yield: 398 mg, 2.39 mmol, 93%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 3.73 (dt, *J* = 142, 4.7 Hz, 2H), 3.62 (dm, *J* = 139 Hz, 2H), 3.69-3.53 (m, 8H), 3.38 (s, 3H), 2.68 (bs, 1H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 72.47 (d, *J* = 39 Hz), 61.61 (d, *J* = 41 Hz) ppm.

[9,10,12,13-$^{13}C_4$]2,5,8,11-tetraoxatridecan-13-ol

Prepared according to general procedure A, starting from [1,2-$^{13}C_2$]2-(2-(2-methoxyethoxy)ethoxy)ethanol (398 mg, 2.39 mmol). The product was obtained as a colourless liquid (yield: 444 mg, 2.09 mmol, 87%). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.11-3.96 (m, 4H), 3.67-3.53 (m, 8H), 3.40 (s, 3H), 3.38-3.24 (m, 4H), 2.61 (bs, 1H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 72.30 (d, *J* = 41 Hz), 70.18, 61.45 (d, *J* = 39 Hz) ppm.

[16-^{13}C]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from 2,5,8,11-tetraoxatridecan-13-ol (206 mg, 0.99 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.10 (d, *J* = 4.0 Hz, 2H), 3.74-3.55 (m, 16H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 173.83 ppm. LC-MS (system B): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 8.35 (ESI-MS (*m/z*): 268.1 ($M + H^+$)).

[15,16-$^{13}C_2$]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from 2,5,8,11-tetraoxatridecan-13-ol (201 mg, 0.97 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.19 (dd, *J* = 145, 4.5 Hz, 2H), 3.80-3.57 (m, 16H), 3.39 (s, 3H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 171.95 (d, *J* = 56 Hz), 68.78 (d, *J* = 56 Hz) ppm. LC-MS (system B): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 7.97 (ESI-MS (*m/z*): 269.3 ($M + H^+$)).

[13,15,16-$^{13}C_3$]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from [13-^{13}C]2,5,8,11-tetraoxatridecan-13-ol (199 mg, 0.95 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.15 (dt, *J* = 145, 4.4 Hz, 2H), 3.73 (dm, *J* = 145 Hz, 2H), 3.72-3.54 (m, 14H), 3.39 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 172.13 (d, *J* =

58 Hz), 70.78, 68.86 (d, J = 59 Hz) ppm. LC-MS (system B): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 8.37 (ESI-MS (*m/z*): 270.3 ($M + H^+$)).

[12,13,15,16-$^{13}C_4$]2,5,8,11,14-pentaoxahexadecan-16-oic acid
Prepared according to general procedure B, starting from [12,13-$^{13}C_2$]2,5,8,11-tetraoxatridecan-13-ol (194 mg, 0.92 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.21 (dt, J = 145, 4.4 Hz, 2H), 3.73 (dm, J = 144 Hz, 2H), 3.80-3.57 (m, 14H), 3.40 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 172.25 (d, J = 58 Hz), 71.63, 69.90, 69.00 (d, J = 56 Hz) ppm. LC-MS (system B): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 8.37 (ESI-MS (*m/z*): 271.3 ($M + H^+$)).

[9,10,12,13,16-$^{13}C_5$]2,5,8,11,14-pentaoxahexadecan-16-oic acid
Prepared according to general procedure B, starting from [9,10,12,13-$^{13}C_4$]2,5,8,11-tetraoxatridecan-13-ol (222 mg, 1.04 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.20-3.99 (m, 6H), 3.67-3.57 (m, 8H), 3.48-3.33 (m, 4H), 3.39 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 172.31, 71.88, 70.90, 70.33, 69.42 ppm. LC-MS (system A): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 7.59 (ESI-MS (*m/z*): 272.0 ($M + H^+$)).

[9,10,12,13,15,16-$^{13}C_6$]2,5,8,11,14-pentaoxahexadecan-16-oic acid
Prepared according to general procedure B, starting from [9,10,12,13-$^{13}C_4$]2,5,8,11-tetraoxatridecan-13-ol (222 mg, 1.04 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.18 (dt, J = 145 Hz, 4.4 Hz, 2H), 4.12-3.99 (m, 4H), 3.67-3.47 (m, 8H), 3.39 (s, 3H), 3.37-3.29 (m, 4H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 172.45 (d, J = 56 Hz), 71.88, 70.94, 70.36, 69.60 ppm. LC-MS (system A): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 7.63 (ESI-MS (*m/z*): 273.1 ($M + H^+$)).

Perfluorophenyl 2,5,8,11,14-pentaoxahexadecan-16-oate (7a)
Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 230 mg, 0.53 mmol, 58% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (s, 2H), 3.85-3.55 (m, 16H), 3.39 (s, 3H) ppm. ^{13}C-NMR (150 MHz, $CDCl_3$): δ = 166.30, 140.75 (dd, J = 243, 12.0 Hz), 139.28 (dt, J = 252, 13.5 Hz), 137.41 (dt, J = 256, 15.0 Hz), 124.15 (t, J = 14.0 Hz), 71.46, 70.76, 70.12, 70.11, 70.01, 67.33, 51.18 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.05 (ESI-MS (*m/z*): 433.1 ($M + H^+$)). HRMS: calcd. for $C_{17}H_{21}F_5O_7$ $[M + H]^+$: 433.12802; found: 433.12818.

Perfluorophenyl [16-^{13}C]2,5,8,11,14-pentaoxahexadecan-16-oate (7b)
Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 226 mg, 0.52 mmol, 54% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (d, J = 4.4 Hz, 2H), 3.84-3.52 (m, 16H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.67 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.32 (ESI-MS (*m/z*): 434.0 ($M + H^+$)). HRMS: calcd. for $C_{16}{}^{13}CH_{21}F_5O_7$ $[M + H]^+$: 434.13138; found: 434.13145.

Perfluorophenyl [15,16-$^{13}C_2$]2,5,8,11,14-pentaoxahexadecan-16-oate (7c)
Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 223 mg, 0.51 mmol, 52% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (dd, J = 145, 4.7 Hz, 2H), 3.86-3.52 (m, 16H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.64 (d, J = 64 Hz), 67.87 (d, J = 64 Hz) ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.33 (ESI-MS (*m/z*): 435.0 ($M + H^+$)). HRMS: calcd for $C_{15}{}^{13}C_2H_{21}F_5O_7$ $[M + H]^+$: 435.13473; found: 435.13482.

Perfluorophenyl [13,15,16-$^{13}C_3$]2,5,8,11,14-pentaoxahexadecan-16-oate (7d)
Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 67 mg, 0.15 mmol, 21% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (dt, J = 145, 4.8 Hz, 2H),

3.76-3.43 (m, 16H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.64 (d, *J* = 64 Hz), 71.27, 67.84 (d, *J* = 64 Hz) ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.05 (ESI-MS (*m/z*): 436.0 ($M + H^+$)). HRMS: calcd. for $C_{14}{}^{13}C_3H_{21}F_5O_7$ $[M + H]^+$: 436.13808; found: 436.13816.

Perfluorophenyl [12,13,15,16-$^{13}C_4$]2,5,8,11,14-pentaoxahexadecan-16-oate (7e)

Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 147 mg, 0.34 mmol, 37% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (dt, *J* = 146, 4.7 Hz, 2H), 3.79 (dm, *J* = 145 Hz, 2H), 3.67-3.50 (m, 14H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.67 (d, *J* = 63 Hz), 71.06, 70.84, 67.84 (d, *J* = 64 Hz) ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.03 (ESI-MS (*m/z*): 437.1 ($M + H^+$)). HRMS: calcd. for $C_{13}{}^{13}C_4H_{21}F_5O_7$ $[M + H]^+$: 437.14144; found: 437.14157.

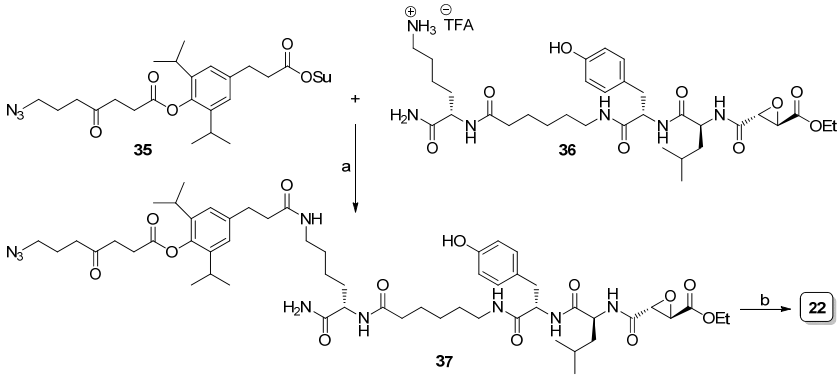
Perfluorophenyl [9,10,12,13,16-$^{13}C_5$]2,5,8,11,14-pentaoxahexadecan-16-oate (7f)

Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 230 mg, 0.53 mmol, 53% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (t, *J* = 4.7 Hz, 2H), 4.19-3.93 (m, 4H), 3.66-3.32 (m, 12H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.64, 71.06, 70.87, 70.60 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.05 (ESI-MS (*m/z*): 438.1 ($M + H^+$)). HRMS: calcd. for $C_{12}{}^{13}C_5H_{21}F_5O_7$ $[M + H]^+$: 438.14479; found: 438.14491.

Perfluorophenyl [9,10,12,13,15,16-$^{13}C_6$]2,5,8,11,14-pentaoxahexadecan-16-oate (7g)

Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 277 mg, 0.63 mmol, 63% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (dt, *J* = 146, 4.7 Hz, 2H), 4.16-4.01 (m, 4H), 3.66-3.32 (m, 12H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.67 (d, *J* = 64 Hz), 71.06, 70.87, 70.60, 67.87 (d, *J* = 64 Hz) ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.05 (ESI-MS (*m/z*): 439.1 ($M + H^+$)). HRMS: calcd. for $C_{11}{}^{13}C_6H_{21}F_5O_7$ $[M + H]^+$: 439.14815; found: 439.14827.

Scheme 3. Synthesis of cleavable linker containing cathepsin probe **22**.



Reagents and conditions: (a) DiPEA, DMF, 73%; (b) Biotin-Ahx-propargylamide, $CuSO_4$, sodium ascorbate, DMF, 34%.

(2R,3R)-Ethyl 3-((9S,19S,22S)-1-(4-(7-azido-4-oxoheptanoyloxy)-3,5-diisopropylphenyl)-9-carbamoyl-19-(4-hydroxybenzyl)-24-methyl-3,11,18,21-tetraoxo-4,10,17,20-tetraazapentacosan-22-ylcarbamoyl)oxirane-2-carboxylate (37)

DCG-04 amine **36**[18] (0.12 g, 0.15 mmol) was dissolved in DMF (3 mL). To this were added NHS activated ester **35**[19] (1.1 eq., 89.0 mg, 0.165 mmol) and DiPEA (1.5 eq., 0.225 mmol, 37 μL) and the mixture was stirred for 15 h. LC-MS analysis showed complete consumption of starting material. The mixture was concentrated *in vacuo*. The remaining crude oil was crystallized in MeOH/Et_2O, filtered off and washed with Et_2O to give the title compound as a colourless solid (yield: 118 mg, 0.11 mmol, 73%). ^{1}H NMR (400 MHz, MeOD): δ = 7.02 (d, *J* = 8.4 Hz, 2H), 7.00 (s, 2H), 6.69 (d, *J* = 8.4 Hz, 2H), 4.5-4.4 (m, 2H), 4.3-4.2 (m, 3H), 3.66 (d, *J* = 1.6 Hz, 1H), 3.58 (d, *J* = 1.6 Hz, 1H), 3.14 (t, *J* = 6.8 Hz, 2H), 3.1-2.8 (m, 12H), 2.61 (t, *J* = 6.8 Hz, 2H), 2.44 (t, *J* = 7.6 Hz, 2H), 2.23 (t, *J* = 7.4 Hz, 2H), 1.9-1.8 (m, 2H), 1.6-1.1 (m, 15H), 1.36 (t, *J* = 3.8 Hz, 3H), 1.55 (d, *J* = 6.8 Hz, 12H), 0.91 (dd, *J* = 16.0, 6.0 Hz, 6H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 175.3, 173.7, 157.3, 141.7, 140.3, 131.4, 128.9, 124.9, 116.2, 63.2, 56.5, 54.4, 54.3, 53.4, 53.2, 51.8, 41.6, 40.2, 40.0, 39.2, 38.2, 37.8, 36.6, 32.9, 30.0, 29.8, 28.7, 28.5, 27.4, 26.5, 25.9, 24.3, 24.1, 23.3, 22.0, 14.4 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.66 (ESI-MS (*m/z*): 1076.33 ($M + H^+$)).

(2S,3R)-Ethyl 3-((9S,19S,22S)-9-carbamoyl-1-(3,5-diisopropyl-4-(4-oxo-7-(4-((6-(5-((3aS,4S,6aR)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)pentanamido)-hexanamido)methyl)-1H-1,2,3-triazol-1-yl)heptanoyloxy)phenyl)-19-(4-hydroxybenzyl)-24-methyl-3,11,18,21-tetraoxo-4,10,17,20-tetraazapentacosan-22-ylcarbamoyl)oxirane-2-carboxylate (22)

Compound **37** (118 mg, 0.11 mmol) was dissolved in DMF (2 mL). To this were added Biotin-Ahx-propargylamide[19] (1.4 eq., 0.154 mmol, 61.0 mg), 1 M $CuSO_4$ in H_2O (22 μL) and 1 M sodium ascorbate in H_2O (33 μL) and the mixture was stirred for 15 h, after which LC-MS analysis showed complete consumption of starting material. The mixture was concentrated *in vacuo* and purified by RP-HPCL (10% → 90% ACN/0.1% aqueous TFA), which afforded the title compound as a colourless solid (yield: 55.0 mg, 38.5 μmol, 35%). ^{1}H NMR (400 MHz, MeOD): δ = 6.87 (d, *J* = 8.4 Hz, 2H), 6.85 (s, 2H), 6.55 (d, *J* = 8.4 Hz, 2H), 4.4-4.2 (m, 6H), 4.2-4.0 (m, 2H), 3.52 (d, *J* = 1.6 Hz, 1H), 3.43 (d, *J* = 2.0 Hz, 1H), 3.2-2.9 (m, 7H), 2.8-2.6 (m, 15H), 2.55 (d, *J* = 12.8 Hz, 1H), 2.41 (t, *J* = 6.8 Hz, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.7-1.1 (m, 28H), 1.16 (t, *J* = 7.0 Hz, 3H), 1.00 (d, *J* = 6.8 Hz, 12H), 0.76 (dd, *J* = 16.0, 6.0 Hz, 6H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 209.4, 177.2, 176.2, 176.1, 176.0, 175.2, 173.8, 173.7, 173.1, 168.8, 168.4, 166.1, 160.1, 159.7, 157.3, 145.2, 141.7, 140.3, 131.4, 128.9, 124.9, 124.5, 116.3, 63.4, 63.2, 61.7, 57.0, 56.5, 54.4, 53.4, 53.2, 50.6, 41.7, 41.0, 40.2, 39.5, 39.1, 38.2, 37.8, 36.8, 36.7, 35.5, 32.9, 32.8, 30.1, 30.0, 29.8, 29.8, 29.5, 28.7, 28.5, 27.5, 27.4, 26.9 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.08 (ESI-MS (*m/z*): 1470.47 ($M + H^+$)).

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(19) See Chapter 7.

Samenvatting

Moleculair gereedschap om de werking van matrix metalloproteinases en proteasomen te belichten

Het in dit proefschrift beschreven onderzoek richt zich op de ontwikkeling van moleculair gereedschap of "probes", waarmee de aanwezigheid en/of de activiteit van enzymen in biologische monsters kan worden vastgesteld. In het bijzonder is aandacht besteed aan het bestuderen van metalloproteinases (MMPs en ADAMs) en proteasomen. Een probe bestaat doorgaans uit drie belangrijke onderdelen: een herkennings element om de probe specifiek één enzymfamilie te laten herkennen, een label voor de visualisatie/analyse van het gebonden enzym en een reactieve groep voor het covalent binden van het enzym. Zogenaamde "activiteitsgebaseerde probes" bevatten een reactieve (electrofiele) groep die reageert met een nucleofiel in het actieve centrum van het enzym, waarbij een covalente binding ontstaat. Enzymen die voor hun katalytische werking onafhankelijk zijn van een nucleofiele groep kunnen niet met activiteitsgebaseerde probes worden bestudeerd, omdat er geen covalent gebonden constructie ontstaat. Voor dit type enzymen kunnen zongenaamde "affiniteits-gebaseerde probes" of "fotoreactieve probes" worden gebruikt. Deze probes bevatten een speciale groep die door middel van fotochemie een covalente binding kan bewerkstelligen, ook wel een fotofoor genoemd. **Hoofdstuk 1** beschrijft de eigenschappen van de drie meest gebruikte fotoforen alsmede enkele voorbeelden van het gebruik ervan in chemisch-biologisch onderzoek.

Een potente klasse van MMP/ADAM remmers wordt gevormd door de peptide succiniyl hydroxamaten. **Hoofdstuk 2** behandelt de synthese van een volledig beschermde succinyl hydroxamaat bevattende bouwsteen. Deze bouwsteen werd gebruikt voor de constructie van chiraal zuivere peptide hydroxamaten in een efficiënt, lineair vaste drager peptide synthese protocol. Hiermee werd onder anderen een biotine-gekoppelde, fotoreactieve probe (fotolabiele groep op de P2' positie) gesynthetiseerd, welke gebruikt werd voor het covalent modificeren en visualiseren van ADAM-10. Daarnaast werd er een bibliotheek, bestaande uit 96 peptide hydroxamaat

gebaseerde remmers, gemaakt waarbij de zijgroepen op de P2' en de P3' posities van de remmers zijn gevarieerd. Deze set van remmers werd vervolgens gebruikt voor het bestuderen van de voorkeur van MMP-9, MMP-12 en ADAM-17 voor de verschillende type remmers.

De efficiëntie van het lichtgeactiveerde koppelen van ADAM-10 met de fotoreactieve probe beschreven in Hoofdstuk 2 bleek zeer gering te zijn. Om die reden werd het verplaatsen van de fotolabiele groep naar de P1' positie bestudeerd, zoals uiteengezet wordt in **Hoofdstuk 3**. Hiervoor werd eerst een bouwsteen gemaakt, analoog aan die beschreven in Hoofdstuk 2, maar voorzien van een 3-aryl-3-(trifluoromethyl)-3*H*-diazirine groep. Vervolgens werd deze bouwsteen gebruikt voor de synthese van een biotine-gekoppelde, fotoreactieve probe (fotolabiele groep op de P1' positie). De hypothese dat het verplaatsen van de fotolabiele groep naar de P1' positie een efficiëntere labeling zou bewerkstelligen bleek juist te zijn. De beschreven probe bleek superieur in het covalent modificeren van verschillende MMPs en ADAMs in vergelijking met de eerder beschreven probe (Hoodstuk 2). Een interessante uitkomst van deze studie is dat de efficiëntie van het fotogeactiveerde koppelen van een probe niet altijd evenredig samenhangt met zijn enzymremmende capaciteiten.

Het onderzoek beschreven in **Hoofdstuk 4** omvat een studie naar het effect van het opnemen van fluor in proteasoom remmers op hun specificiteit voor de verschillende, katalytisch actieve subunits. Er is gebleken dat het vervangen van fenylalanine in een boorester bevattende, potente proteasoom remmer door gefluoreerde fenylalanine derivaten geen invloed had op de potentie en de selectiviteit van de remmer. Verder werd er een set van tripeptide epoxyketonen vervaardigd, waarbij één van beide of beide P2 en P3 posities werden voorzien van een gefluoreerd fenylalanine derivaat. Een interessante bevinding bij het analyseren van de enzymremmende capaciteiten van deze verbinding, was dat het invoeren van fluor, met name op de P2 positie, leidde tot een sterke daling van de affiniteit voor de 'caspase-achtige' (β1) en 'trypsine-achtige' (β2) subunits. Dit resulteerde uiteindelijke in een verhoogde selectiviteit voor de 'chymotrypsine-achtige (β5) subunit. Eén specifieke verbinding uit deze set kon worden aangemerkt als één van de meest β5 selectieve remmers die er vandaag de dag bekend zijn. Deze verbinding werd omgezet in een probe door middel van de 'click' reactie met een fluorescent bodipy-alkyn groep op het, in de verbinding aanwezige, N-terminale azide. Deze probe kon worden gebruikt voor het specifiek binden en visualiseren van de β5 subunit.

De ontwikkeling van selectieve remmers voor de trypsine-achtige site van het proteasoom (β2) met goede celpenetrerende eigenschappen staat beschreven in **Hoofdstuk 5**. De selectiviteit voor β2 werd bereikt door het installeren van basische zijketen residuen in de P1 positie van tripeptide vinylsulfonen en epoxyketonen. Het invoeren van een 4-aminomethyleen fenylalanine analoog op deze positie in een vinylsulfon resulteerde in een proteasoom remmer met een hoge selectiviteit voor β2, zowel in celextracten als in levende cellen. Het koppelen van dit 4-aminomethyleen fenylalanine vinylsulfon aan de β2 sturende peptide sequentie (3-hydroxy-2-methylbenzoyl)-valine-serine zorgde voor een verdere verhoging van de selectiviteit voor

β2 en resulteerde zelfs in selectiviteit voor β2 ten opzichte van zijn immunoproteasoom pendant β2i.

Hoofdstuk 6 behandelt een studie naar de oriëntatie van verlengde peptide vinylsulfonen binnen in de katalytisch actieve holte van het proteasoom. In deze studie is het activiteitsgebaseerde modificeren van een enzym met behulp van een reactive, electrofiele groep gecombineerd met fotochemisch koppelen. Hiervoor werden in eerste instantie drie probes, bestaande uit een verlengd peptide vinylsulfon gekoppeld aan een N-terminale fotofoor (diazirine, arylazide en benzofenon), gemaakt. Van deze drie verbindingen bleek de arylazide variant als enige in staat om een koppeling tussen de actieve β5 subunit en een naburige β6 subunit te bewerkstelligen. Het gehele construct kon geïdentificeerd worden door middel van gel electroforese gevolgd door LC-MS/MS analyse.

Het ontwerp en de synthese van een nieuwe selectief splitsbare linker, gebaseerd op de levuline ester, is beschreven in **Hoofdstuk 7**. Een speciaal ontworpen ester van levulinezuur en 2,6-diisopropylphenol werd ingebouwd in een activiteitsgebaseerde probe, gebaseerd op de potente proteasoom remmer epoxomicin en voorzien van biotine. De optimale eigenschappen van deze splitsbare linker, in termen van stabiliteit en splitsbaarheid, werden gedemonstreerd aan de hand van het gebruik ervan voor het selectief immobiliseren van proteasoom actieve subunits uit een celextract. Een directe behandeling van de vaste drager met hydrazine leidde vervolgens tot de selectieve elutie van de aan deze probe gebonden eiwitten. Verder bleek de nieuwe splitsbare linker zeer goed te combineren met transformaties die veelvuldig toegepast worden in chemisch biologisch onderzoek, zoals ‘click’ chemie en de Staudinger-Bertozzi ligatie.

List of Publications

Solid-phase synthesis of succinylhydroxamate peptides: Functionalized matrix metalloproteinase inhibitors
M. A. Leeuwenburgh, P. P. Geurink, T. Klein, H. F. Kauffman, G. A. van der Marel, R. Bischoff, H. S. Overkleeft.
Organic Letters, **2006**, *8*, 1705–1708.

A peptide hydroxamate library for enrichment of metalloproteinases: towards an affinity-based metalloproteinase profiling protocol
P. Geurink, T. Klein, M. Leeuwenburgh, G. van der Marel, H. Kauffman, R. Bischoff, H. Overkleeft.
Organic & Biomolecular Chemistry, **2008**, *6*, 1244–1250.

Poly(ethylene glycol)-based stable isotope labeling reagents for the quantitative analysis of low molecular weight metabolites by LC-MS
N. Abello, P. P. Geurink, M. van der Toorn, A. J. M van Oosterhout, J. Lugtenburg, G. A. van der Marel, H. A. M. Kerstjens, D. S. Postma, H. S. Overkleeft, R. Bischoff.
Analytical Chemistry, **2008**, *80*, 9171–9180.

Functional proteomics on zinc-dependent metalloproteinases using inhibitor probes
T. Klein, P. P. Geurink, H. S. Overkleeft, H. K. Kauffman, R. Bischoff.
ChemMedChem, **2009**, *4*, 164–170.

Incorporation of fluorinated phenylalanine generates highly specific inhibitors of proteasome's chymotrypsin-like sites
P. P. Geurink, N. Liu, M. P. Spaans, S. L. Downey, A. M. C. H. van den Nieuwendijk, G. A. van der Marel, A. F. Kisselev, B. I. Florea, H. S. Overkleeft.
Journal of Medicinal Chemistry, **2010**, *53*, 2319–2323.

Design of peptide hydroxamate-based photoreactive activity-based probes of zinc-dependent metalloproteases
P. P. Geurink, T. Klein, L. Prèly, K. Paal, M. A. Leeuwenburgh, G. A. van der Marel, H. F. Kauffman, H. S. Overkleeft, R. Bischoff.
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W. A. van der Linden, P. P. Geurink, C. Oskam, G. A. van der Marel, B. I. Florea, H. S. Overkleeft.
Organic & Biomolecular Chemistry, **2010**, *8*, 1885–1893.

Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit β5t
B. I. Florea, M. Verdoes, N. Li, W. A. van der Linden, P. P. Geurink, H. van den Elst, T. Hofmann, A. de Ru, P. A. van Veelen, K. Tanaka, K. Sasaki, S. Murata, H. den Dulk, J. Brouwer, F. A. Ossendorp, A. F. Kisselev, H. S. Overkleeft.
Chemistry & Biology, **2010**, accepted for publication.

A cleavable linker based on the levulinoyl ester for activity-based protein profiling
P. P. Geurink, B. I. Florea, N. Li, M. D. Witte, J. Verasdonck, C. -L Kuo, G. A. van der Marel, H. S. Overkleeft.
Angewandte Chemie International Edition, **2010**, accepted for publication.

The nature of pharmacophore influences active site specificity of proteasome inhibitors
M. Screen, M. Britton, S. L. Downey, M. Verdoes, M. J. Voges, A. E. M. Blom, P. P. Geurink, M. D. P. Risseeuw, B. I. Florea, W. A. van der Linden, A. A. Pletnev, H. S. Overkleeft, A. F. Kisselev.
Journal of Biological Chemistry, **2010**, accepted for publication.

Curriculum Vitae

Paulus Petrus Geurink werd op 18 maart 1983 geboren te Haarlem. Na het behalen van het VWO-diploma aan het Rijnlands Lyceum te Oegstgeest in 2001 werd in dat jaar begonnen met de studie Scheikunde aan de Universiteit Leiden. De propedeuse werd behaald in september 2002 (*cum laude*). In het kader van een bijvakstage werd van maart 2004 tot juni 2004 onderzoek verricht in de vakgroep Soft Condensed Matter (SCM) onder leiding van Prof. Dr. Ir. J. G. E. M. Fraaije en Dr. Ir. J. van Male. Dit onderzoek omvatte de ontwikkeling van een methode voor de synthese van monodisperse polyacrylamide gel deeltjes via een inverse emulsie verkregen met membraan-emulsificatie. Van september 2004 tot september 2005 werd onder leiding van Dr. M.A. Leeuwenburgh een hoofdvakstage gedaan in de vakgroep Bio-organische Synthese (BioSyn) van Prof. Dr. H. S. Overkleeft en Prof. Dr. G. A. van der Marel. Dit onderzoek was gericht op het ontwikkelen van een nieuwe methode voor de efficiënte (vaste drager) synthese van peptide succinyl-hydroxamaat-gebaseerde metalloprotease remmers. Deze hoofdvakstage werd bekroond met de Unilever Research Prize in 2006. Het doctoraal examen (Master of Science exam) werd *cum laude* behaald op 21 maart 2006.

Van april 2006 tot en met juni 2010 werd als assistent in opleiding het in dit proefschrift beschreven onderzoek uitgevoerd in de vakgroep Bio-organische Synthese (BioSyn) onder leiding van Prof. Dr. H. S. Overkleeft en Prof. Dr. G. A. van der Marel. Delen van het onderzoek zijn gepresenteerd op 'Dutch Peptide Symposium' (Utrecht, april 2007), 'International Symposium on Advances in Synthetic and Medicinal Chemistry' (Sint Petersburg, augustus 2007), de jaarlijkse NWO-CW meeting 'Design and Synthesis' (Lunteren, oktober 2007), 'HUPO 7th annual congress' (Amsterdam, augustus 2008), '1st RSC-SGC Symposium on Chemical Biology for Drug Discovery' (Oxford, december 2009) en 'Wageningen Symposium on Organic Chemistry' (Wageningen, april 2010).

Vanaf augustus 2010 is de auteur van dit proefschrift werkzaam als onderzoeker in de groep van Dr. H. Ovaa aan het Nederlands Kanker Instituut (NKI).

Nawoord

Deze laatste pagina wil ik besteden aan het bedanken van iedereen die, op wat voor manier dan ook, heeft bijgedragen aan de totstandkoming van dit proefschrift. Mijn dank gaat in de eerste plaats uit naar mijn familie en in het bijzonder naar mijn ouders. Jullie onvoorwaardelijke steun en rotsvaste vertrouwen in mij zijn van zeer grote waarde geweest in de vorming van mij als onderzoeker en als persoon in het algemeen.

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