Chemical tools to probe the proteasome

Verdoes, M.

Stellingen

Behorende bij het proefschrift

Chemical Tools To Probe The Proteasome

1. In tegenstelling tot wat Marastoni en collega's stellen is 3-hydroxy-2-methylbenzoyl-valyl-seryl-leucine (*E*)-vinyl ethyl ester geen potente en selectieve remmer van de tryptische activiteit van het proteasoom.

Marastoni, M.; Baldisserotto, A.; Cellini, S.; Gavioli, R.; Tomatis, R. *J. Med. Chem.* **2005**, 48, 5038 – 5042.

Dit proefschrift

2. Röntgendiffractie studies van kristallen van 20S proteasomen uit gist welke zijn verzadigd met een gegeven remmer zullen niet bijdragen aan het ontwerpen van subunit-specifieke remmers.

Groll, M.; Berkers, C.R.; Ploegh, H.L.; Ovaa, H. *Structure* **2006**, *14*, 451-456.

Berkers, C.R.; Verdoes, M.; Lichtman, E.; Fiebiger, E.; Kessler, B. M.; Anderson, K.C.; Ploegh, H.L.; Ovaa, H.; Galardy, P.J. *Nat. Methods*. **2005**, *2*, 357-362.

Dit proefschrift

3. Het is opvallend dat de synthese en bijbehorende karakterisatie van bortezomib pas vijf jaar na de goedkeuring van deze verbinding als antikankermiddel in de literatuur is verschenen.

Beenen, M.A.; An, C.; Ellman, J.A. *J. Am. Chem. Soc.* **2008**, *130*, 6910-6911.

Dit proefschrift

4. Subunit specifieke proteasoom remmers zijn relevant voor de ontwikkeling van nieuwe methodes om kanker en auto-immuun ziektes te behandelen.

Dit proefschrift

5. Het nut van docking simulaties van remmers van het proteasoom wordt uitstekend geïllustreerd door Baldisserotto en collega's.

Baldisserotto, A.; Marastoni, M.; Fiorini, S.; Pretto, L.; Ferretti, V.; Gavioli, R.; Tomatis, R. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1849-1854.

6. Epoxomicin zou als een zeer intelligent ontworpen proteasoomremmer beschouwd kunnen worden, ware het niet dat het een natuurprodukt is.

7. Met het ontstaan van het proteasoom als proteolytische nanotube was de evolutie zijn tijd ver vooruit.

8. Het is jammer dat bij discussies over veiligheid na een ongeluk in een universitair chemisch laboratorium het menselijk tekort wordt uitvergroot, terwijl de infrastructurele tekortkomingen worden onderbelicht.

9. Wetenschappelijke congressen zijn niet meer van deze tijd.

10. De olympische gedachte is in strijd met de evolutietheorie.

11. De gedachte dat geluk een keuze is kan mensen ongelukkig maken.
Stelling Yves Gassler, 22 februari (rechtsgeleerdheid): Geluk is een keuze: het geluk van een persoon neemt toe naar mate hij meer energie steekt in door hem te beïnvloeden factoren.

12. Bij het bedrijven van wetenschap dient meer te vroeg gejuicht te worden!

Leiden, 19 december 2008 Martijn Verdoes

Chemical Tools To Probe The Proteasome

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 vrijdag 19 december 2008
klokke 13.30 uur

door

Martijn Verdoes

geboren te Leiden in 1980

Promotiecommissie

Promotores	:	Prof. dr. H. S. Overkleeft
	:	Prof. dr. G. A. van der Marel
Overige leden	:	Prof.dr. J. Brouwer
		Prof.dr. J.J. Neefjes
		Prof.dr. J. Lugtenburg
		Dr. B. M. Kessler

As long as we're having fun…

Table of contents

List of Abbreviations

ABP	Activity-based probe
ABPP	Activity-based protein profiling
Ac	acetyl
AcCl	acetyl chloride
Ac_2O	acetic anhydride
AcOH	acetic acid
Ada	1-adamantyl acetyl
Ahx	6-aminohexanoic acid
AMC	7-acetoxy-4-methyl-coumarin
AMP	adenosine monophosphate
APT	attached proton test
ATP	adenosine triphosphate
aq.	aqueous
βNA	β naphthylamide
Bax	Bcl-2-associated X protein
Bio	biotin
BM	biotinylated molecular marker
Boc	*tert*-butyloxycarbonyl
Boc_2O	*tert*-butyloxycarbonic anhydride
BODIPY	boron-dipyrromethene, boradiazaindacene
BOP	benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphonate
BSA	bovine serum albumine
Bu	butyl
calcd.	calculated
cat.	catalytic amount
CDI	carbodiimidazole
CLSM	confocal laser scanning microscopy
CtL	chymotrypsin-like
δ	chemical shift
d	doublet
Da	Dalton
DABCO	1,4-diazabicyclo[2.2.2]octane
DBU	diazabicyclo[5.4.0]undec-7-ene
DC	dual color marker
DCE	1,2-dichloroethane
DCM	dichloromethane
dd	double doublet
ddd	double double doublet
DIC	differential interference contrast

DIC	*N*,*N*′-diisopropyl carbodiimide
DIFO	difluorinated cyclooctyne
DiPEA	Diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMEM	dulbecco's modified eagle's medium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
dt	double triplet
DTNB	5,5′-dithio-bis(2-nitrobenzoic acid)
DTT	dithiothreitol
DUB	deubiquitinating enzyme
dq	double quartet
EDC·HCl	1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide HCl salt
EDTA	ethylenediaminetetraacetate
EL-4	murine lymphoid cell line
Et_3N	triethyl amine
Et_2O	diethyl ether
EtOH	ethanol
EtOAc	ethyl acetate
equiv.	molar equivalent
ES	ethyl methyl sulfone
FDA	United States food and drug administration
Fmoc	(9*H*-fluoren-9-yl)methoxycarbonyl
GFP	green fluorescent protein
HCTU	(2-(6-Chloro-1H-benzotriazole- 1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate)
HEK293T	human embryonic kidney cell line
HeLa	human cervix carcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMB	3-hydroxy-2-methylbenzamide
HMPB	4-hydroxymethylphenyl-butyric acid linker
HOBt	*N*-hydroxybenzotriazole
HOSu	*N*-hydroxysuccinimide
hr.	hour(s)
HRMS	high resolution mass spectrometry
Hz	Hertz
i.v.	intravenous
IFN-γ	interferon-gamma
IκB	inhibitor of NF-κB
J	coupling constant
K	kelvin
LC/MS	liquid chromatography/ mass spectrometry
LiHMDS	lithium hexamethyldisilazide
m	multiplet
M	molar
M	molecular marker
MBHA	*p*-methylbenzhydryl amine
Me	methyl
MeCN	acetonitrile
MelJuSo	metastasizing human melanoma cell line
MeOD	CD_3OD
MeOH	methanol
MES	2-(*N*-morpholino)ethane sulfonic acid
min.	minute(s)
*n*Bu	*n*-butyl
NEM	*N*-ethyl maleimide
MS (ESI)	mass spectrometry (electrospray ionization)
NF-κB	nuclear factor-κB
NIP	nitro-iodophenol
MHC I	major histocompatibility complex class I
NMM	*N*-methylmorpholine
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance

Ntn	*N*-terminal nucleophilic
m/z	mass-to-charge ratio
o/n	overnight
OAc	acetate
PBS	phosphate buffered saline
Pd/C	palladium on charcoal
PEG	polyethyleneglycol
PetEt	petroleum ether
PGPH	peptidylglutamyl peptide hydrolytic
Ph	phenyl
ppm	parts per million
pv.	pathovar
PyBOP	benzotriazol-1-yl-oxy-tripyrrolidino-phosphonium hexafluorophosphonate
q	quartet
quant.	quantitative
RAW	Mouse leukaemic monocyte macrophage cell line
RPMI	roswell park memorial institute medium
rt	room temperature
R_t	retention time
s	singlet
sacc.	Pier Andrea Saccardo
sat.	saturated
SDS-PAGE	sodium dodecyl sulfate-poly acrylamide gel electroforese
sp.	species
Su	succinimidyl
t	triplet
t, *tert*	tertiary
T	temperature
TAP	transporter associated with antigen presentation
TBAI	tetrabutylammonium iodide
TBS	*tert*-butyldimethylsilyl
*t*Bu	*tert*-butyl
*t*BuONO	*tert*-butyl nitrite
*t*BuOH	*tert*-butanol
td	triple doublet
TEA	triethyl amine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIS	triisopropyl silane
TL	trypsin-like
TLC	thin layer chromatography
TMR	tetramethylrhodamine
TMS	trimethylsilyl
Tol.	toluene
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
SDS	sodium dodecyl sulphate
SPPS	solid phase peptide synthesis
Su	succinimidyl
Ub	ubiquitin
UPS	Ubiquitin Proteasome System
VE	vinyl ethyl ester
$VO(acac)_2$	vanadium(IV)acetyl acetonate
VS	vinyl methyl sulfone
Z	benzyloxycarbonyl

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.

General Introduction

1.1 Introduction

Proteolysis, or the processing and degradation of proteins, has emerged as one of the most widely studied processes in biology today. Long viewed as a dead end process, of importance only for the removal of obsolete peptides and proteins, proteolytic events are now associated with numerous biological events. The main proteolytic pathway in the eukaryotic cytoplasm and nucleus, responsible for the degradation of 80-90% of all cellular proteins is known as the Ubiquitin Proteasome System (UPS). Proteasomes are the central proteases in this tightly controlled ATP- and ubiquitin-dependent proteolytic pathway. Proteasomes are multicatalytic, compartmentalized proteinase complexes. Their substrates include abnormal and damaged proteins, cell-cycle regulators, oncogens and tumor suppressors. Furthermore, proteasomal degradation is imperative for the generation of MHC class I antigenic peptides. The recent approval of a proteasome inhibitor as a cancer drug has boosted proteasome research. This Thesis is devoted to the development of chemical tools to study proteasome activity.

1.2 The Ubiquitin Proteasome System

The turnover of the majority of cellular proteins is controlled by the Ubiquitin Proteasome System (UPS, Figure 1). Being involved in the degradation of key regulatory proteins as well as clearance of misfolded and damaged proteins, the UPS plays a role in many cellular processes, such as cell cycle control, differentiation, apoptosis, transcription processes and immune response. Poly-ubiquitination is a substrates "molecular kiss of death", marking the protein for proteasomal degradation.[1] Ubiquitin, a 9 kDa, 76-residue

protein is attached via its glycine-glycine *C*-terminus to a substrate. A cascade of enzymes is responsible for this process. First, ubiquitin is activated in an ATP-dependent manner by the ubiquitin-activating enzyme E1 through adenylation of the *C*-terminus. Subsequent nucleophilic attack of the E1 active site cysteine on the activated ubiquitin results in a thioester linkage and liberates adenosine monophosphate (AMP). Before transthioesterification of ubiquitin to an ubiquitin-conjugating enzyme E2, a second ubiquitin is bound in the adenylation site of E1, thereby increasing the affinity for E2.[2] The E3 ubiquitin ligase enzymes facilitate the transfer of ubiquitin to a lysine residue in the substrate to form an isopeptide linkage. Two independent mechanisms are discerned. In the first, the E3 binds a substrate and a ubiquitin carrying E2 simultaneously, after which the E2 transfers the ubiquitin to the substrate. In the second mechanism, the E3 binds a charged E2 alone and ubiquitin is first transferred to the E3 via a transthioesterification. The ubiquitin carrying E3 then binds a substrate to transfer the ubiquitin. This highly controlled multistep mechanism involves only two E1 enzymes,[3] a large family of E2

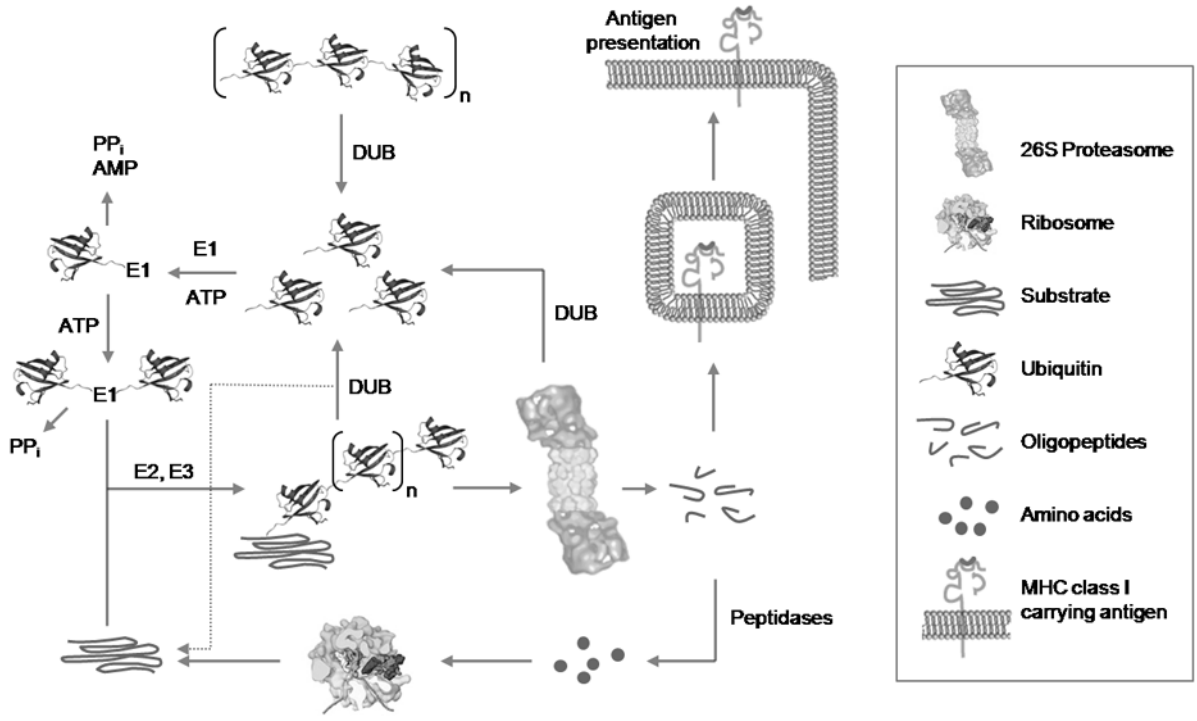

Figure 1. Schematic representation of the Ubiquitin Proteasome System.

enzymes and an even larger set of E3 enzymes. Of these, it appears that the E3 ligases confer selectivity in ubiquitin-mediated protein degradation processes.[1] A substrate can be rescued from degradation by cleavage of the ubiquitin by so called deubiquitinating enzymes (DUBs),[4] introducing an even higher degree of control. Poly-ubiquitin chains linked via the *C*-terminus to the side chain of lysine 48 are recognized by the 26S proteasome. During degradation of the substrate, ubiquitin is recycled by deubiquitinating

enzymes. A fraction of the generated peptide fragments (between 3 and 25 amino acids, with an average of 8 to 12 amino acids) is trimmed by specific aminopeptidases, translocated into the ER by the Transporter associated with Antigen Presentation (TAP) and loaded on Major Histocompatibility Complex class I (MHC I) molecules.[5,6] The trimeric MHC I epitope complex is then transported to the cell surface to be exposed to the immune surveillance system. The majority of the proteolysis products are further degraded to single amino acids by peptidases to maintain protein homeostasis.

1.3 The proteasome

Proteasomes are highly conserved compartmentalized protease complexes belonging to the family of *N*-terminal nucleophilic (Ntn) hydrolases.[5,7,8] The 20S proteasome, a proteolytic nanotube, is a 720 kDa cylindrical protein complex composed of four stacked rings. In prokaryotes, the two outer rings consist of seven identical α subunits,

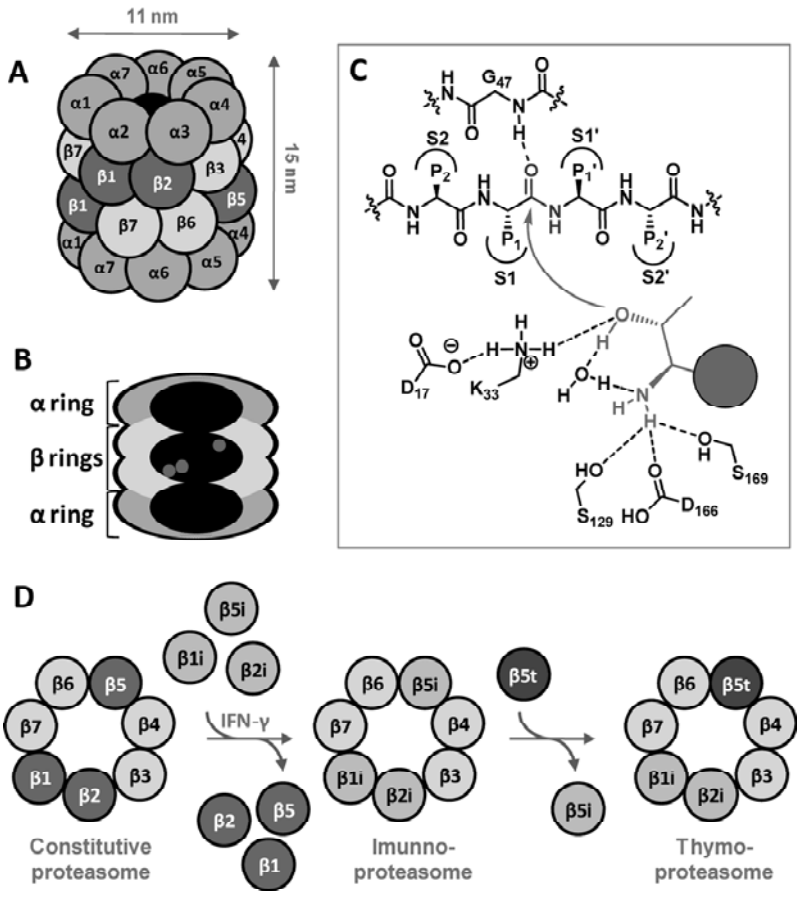

Figure 2. Schematic representation of the composition of the 20S proteasome.

(**A**) Subunit composition of the 20S proteasome core particle. (**B**) Cross-section of the 20S proteasome showing the position of the catalytic residues. (**C**) Representation of the catalytic active site. (**D**) Top-view of the β ring of the constitutive proteasome, the immunoproteasome and the thymoproteasome.

sandwiching the two inner rings which are build up of seven identical proteolytically active β subunits. During evolution, these subunits diverged into seven distinct α subunits and seven distinct β subunits, four of which lost their proteolytic character. Eukaryotic 20S core particles are build up of two rings consisting of seven different α subunits (α1-α7), capping the two inner rings which are build up of seven β subunits (β1-β7) (Figure 2A). The three remaining proteolytically active β subunits are the β1 (Y/δ), β2 (Z) and β5 (X) subunits (Figure 2B). The *N*-terminal threonine (Thr1) represents the proteolytically active residue and acts by nucleophilic attack of the γ-hydroxyl on the carbonyl of the peptide bond destined to be cleaved (Figure 2C). The α-amine of the threonine acts as the base in the catalytic cycle. Site-directed mutagenesis and kinetic studies using fluorogenic substrates revealed subtle differences in substrate specificities.[9] The active site located at the β5 subunit preferably cuts after hydrophobic residues and is termed 'chymotrypsin-like'. Having a preference for cleaving after basic amino acids the β2 site is named 'trypsin-like', whereas the β1 site cuts preferentially after acidic residues and is called 'caspase-like'. Despite the subunit preferences suggested by these designations, the proteasome subunits are rather more promiscuous. The proteolytic active sites reside inside the hollow proteasome microenvironment and can only be reached via the pores in the α rings, which are too narrow (10 to 15 Å) for folded proteins to enter.[10] The 2.5 MDa 26S proteasome is formed when the 20S core particle is capped at either side by the 19S regulatory complex (PA700). The 19S regulatory complex is composed of two functionally different units, the base and the lid. The lid is responsible for substrate recognition and cleavage of the poly-ubiquitin chains. The base is composed of eight subunits, six of which are ATPases, and interacts directly with the α rings, facilitating the energy dependent unfolding of the substrate, opening of the pores in the α rings and translocation of the substrate into the proteolytic chamber of the 20S core particle.

MHC class I antigen presentation is in constant competition with aminopeptidases that degrade the available pool of potential antigenic peptides. To enhance MHC I antigen presentation upon infection, proteasome activity is altered to increase the pool of antigenic peptides.[5,11] Besides being constitutively expressed in immune-relevant tissue, interferon-γ (IFN-γ) stimulates the expression of three additional catalytically active β subunits, β1i (Low Molecular weight Protein 2, LMP2), β2i (multicatalytic endopeptidase complex-like-1, MECL1) and β5i (LMP7). These subunits are incorporated in newly formed proteasome particles replacing their constitutive counterparts to form the so-called immunoproteasomes, which coexist next to the constitutive proteasomes. IFN-γ also upregulates the synthesis of the proteasome activator PA28 (11S cap).[12] PA28 binds to the α rings of the proteasome core particles to cause a conformational change in the *N*-terminal tails of the α subunits, resulting in the opening of the α-annulus, the gateway to the proteolytic chamber.

Recently, an additional, seventh proteolytically active proteasome β subunit was identified in cortical thymic epithelial cells, which are responsible for the positive selection of developing T cells.[13] This new catalytic β subunit proved to be most closely related to the β5 and β5i subunits and was therefore named thymus specific β5, abbreviated as β5t. In approximately 20% of the 20S proteasome population in the thymus, β5t replaces the β5i subunit in immunoproteasomes to form the thymoproteasome. The chymotrypsin-like activity of thymoproteasomes is reduced by 60 to 70% as compared to the constitutive- and immunoproteasome, without an effect on the trypsin- and caspase-like activities. Although the exact role of β5t remains to be unraveled, it is plausible that compared to the constitutive- and immunoproteasomes the thymoproteasomes produce low-affinity MHC I ligands rather than high-affinity ligands to support positive selection.

1.4 Inhibitors of the proteasome

The importance of the proteasome for viability was capitalized upon by several micro-organisms, demonstrated by the fact that these organisms produce natural products that are capable of blocking the proteasome. For example, the *Streptomyces* metabolite lactacystin (**1**)[14] is the precursor of the active proteasome inhibitor *clasto*-lactacystin β-lactone (**2**),[15] also known as ormulide, which is formed under neutral conditions by lactonization (Figure 3). A structurally related proteasome inhibitor salinosporamide A (**3**, NPI-0052) is produced by *Salinispora tropica*, a marine bacterium which is found in ocean sediment.[16] The mechanism by which these related compounds inhibit the proteasome is through nucleophilic attack of the *N*-terminal active site threonine of the proteasome on

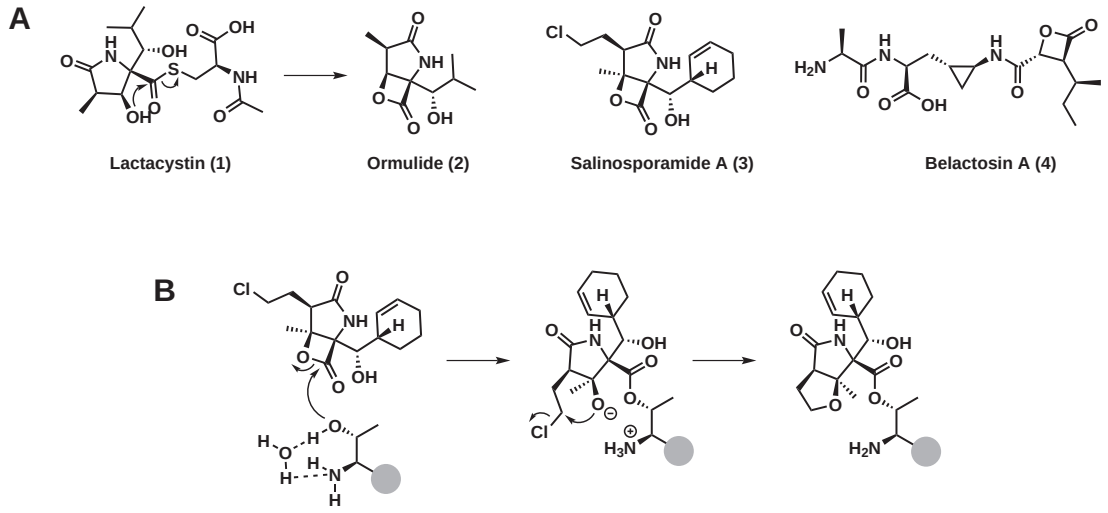

Figure 3. β-lactone containing natural products.

(**A**) Structures and (**B**) mechanism of inhibition of β-lactone containing natural products.

the β-lactone. This results in opening of the lactone and ester bond formation of the inhibitor to the active site threonine γ-hydroxyl.[17] In the case of salinosporamide A (**3**), the resulting oxanion ring-closes to an oxacycle by S_N2 displacement of the chlorine (Figure 3B).[17,18] The β-lactone containing belactosin A (**4**),[19] isolated from a fermentation broth of *Streptomyces* sp. UCK14 inhibits the proteasome via the same mechanism.[17] Acylation of the proteasome active site threonine by β-lactone inhibitors is reversible, with a half-life of approximately 20 hours.[20]

A search for substances capable of reversing multi drug resistance in tumor cells resulted in the isolation of the non-covalent proteasome inhibitor agosterol A (**5**, Figure 4) from a marine sponge of *Spongia* sp. collected in Mie Prefecture, Japan.[21] The highly selective, potent and non-covalent proteasome inhibitors TMC-95A-D[22] (TMC-95A (**6**) is shown in Figure 4) have been discovered in the fermentation broth of *Apiospora montagnei* sacc. TC 1093. The polyphenol component of green and black tea, (-)-epigallocatechin-3-gallate (**7**) was shown to inhibit the chymotrypsin- and caspase-like activities of the proteasome.[23] The latter reversibly deactivates the proteasome by acylation of the threonine γ-hydroxyl as a result of nucleophilic attack on the ester carbonyl and subsequent transesterification.

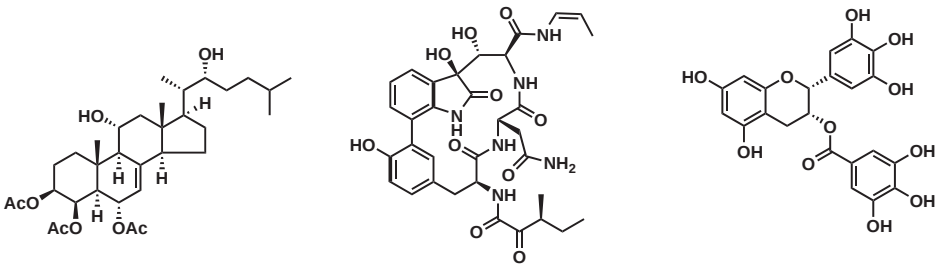

Figure 4. Structures of agosterol A (**5**), TMC-95A (**6**) and (-)-epigallocatechin-3-gallate (**7**).

Eponemycin (**8**, Figure 5A) was isolated from *Streptomyces hygroscopicus* based on its *in vivo* antitumor activity against murine B16 melanoma tumors.[24] The structurally homologues epoxomicin (**9**) was isolated from an *Actinomycetes* strain and was found to have antineoplastic activity.[25,26] The α′,β′-epoxyketone "warhead" (the electrophilic moiety that reacts with the active site nucleophile) containing natural products are highly specific inhibitors of the proteasome due to their unique inhibition mechanism (Figure 5B).[17,27] First, the ketone is attacked by the *N*-terminal threonine γ-hydroxyl, just like it would attack the carbonyl of the peptide bond destined for cleavage, to give a reversible hemiacetal linkage. Next, the α-amine attacks the epoxide resulting in the irreversible formation of a very stable morpholine ring.

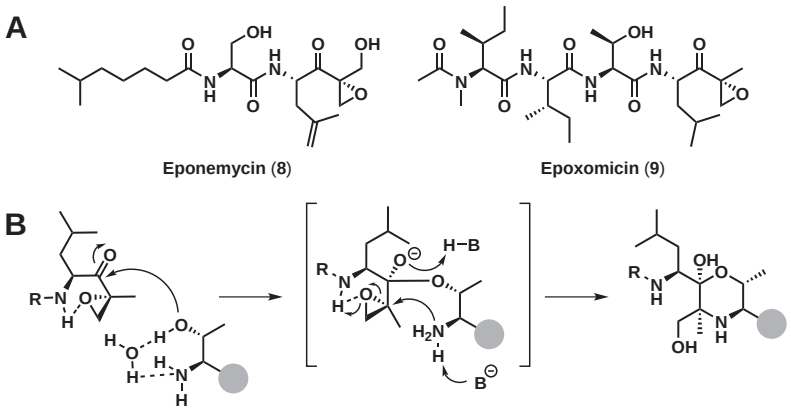

Figure 5. Epoxyketone containing natural products.

(**A**) Structures and (**B**) mechanism of inhibition of epoxyketone containing inhibitors.

Strains of the plant pathogen *Pseudomonas syringae* pv. *syringae* secrete syringolin A (**10,** Figure 6A), causing, for example, brown spot disease on bean.[28] The target of this virulence factor was found to be the proteasome, which is blocked after Michael addition of the threonine γ-hydroxyl on the macrocyclic α,β-unsaturated amide resulting in an irreversible ether bond formation.

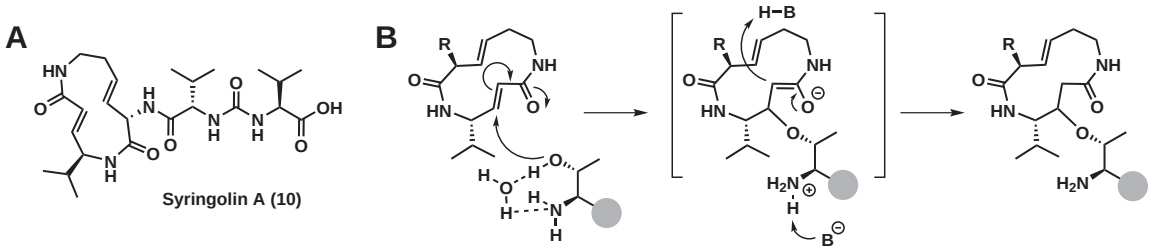

Figure 6. Syringolin A (**10**).

(**A**) Structure of syringolin A (**10**) and (**B**) its mechanism of proteasome inhibition.

Fellutamide B (**11,** Figure 7A) is a cytotoxic peptide isolated from a marine fish-possessing fungus *Penicillium fellutanum*.[29] Peptide aldehydes, such as fellutamide B inhibit the proteasome reversibly by the formation of a hemiacetal bond (Figure 7B).[17]

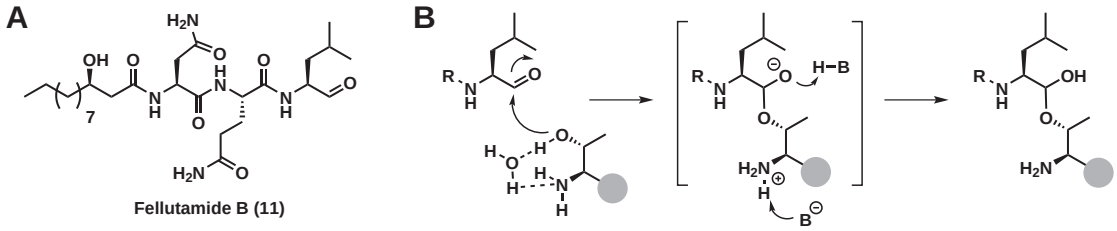

Figure 7. Fellutamide B (**11**).

(**A**) Structure of fellutamide B (**11**) and (**B**) mechanism of inhibition of aldehyde containing proteasome inhibitors.

The observation that leupeptin (**12**, Ac-LLR-al, Figure 8), an inhibitor of the calcium-dependent, non-lysosomal cysteine protease calpain, inhibited the trypsin-like activity of the proteasome meant the starting point of the development of synthetic peptide based proteasome inhibitors.[30] In a study performed by Vinitsky *et al.*, calpain inhibitor I (**13**, Ac-LLnL-al), calpain inhibitor II (**14**, Ac-LLM-al), Ac-LLF-al (**15**) and the α-ketoester Z-LLF-COOEt (**16**) were shown to inhibit the chymotrypsin-like activity of the proteasome.[31] Compared to its predecessors, Z-LLL-al (**17**, MG132) proved to be a more potent and more selective inhibitor of the proteasome as opposed to calpains and cathepsins,[32] and represents one of the most frequently used proteasome inhibitors to date. Since the development of these initial synthetic proteasome inhibitors, many aldehyde based peptide inhibitors have been described.[33]

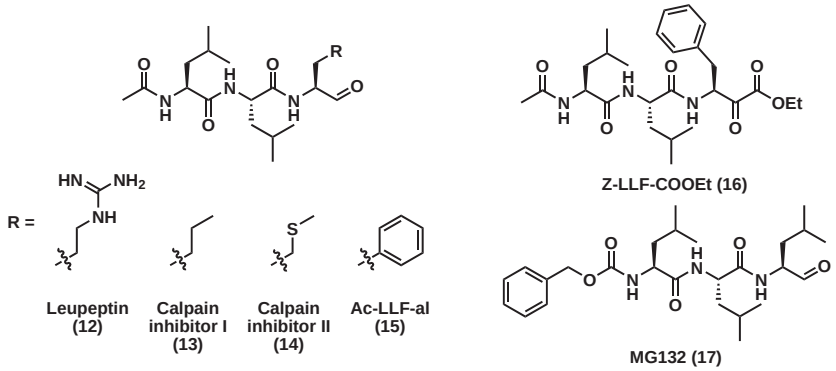

Figure 8. Synthetic peptide based proteasome inhibitors.

The challenging synthesis of lactacystin (**1**), combined with the fact that less synthetically demanding peptide aldehyde based inhibitors target calpains and cathepsins in addition to the proteasome, led to the development of the first synthetic peptide vinyl sulfone based proteasome inhibitors (Figure 9A).[34] Although still marginally targeting cathepsin S, Z-L_3VS (**18**) and NIP-L_3VS (**19**) covalently and irreversibly modified the γ-hydroxyl of the *N*-terminal threonine by ether bond formation via Michael addition (Figure 9B).[17] In a later study, Bogyo *et al*. showed that removal of the *N*-terminal benzyloxycarbonyl in Z-L_3VS (**18**) resulted in the loss of inhibitory potency.[35] By the installation of a fourth amino acid with an aromatic or aliphatic side chain however, like for example in YL_3VS (**20**), the inhibitory potency could be restored to even surpass that of Z-L_3VS (**18**), with the most dramatic increase of potency observed for the trypsin-like activity.[35] Substitution of the methyl for a phenol in the vinyl sulfone warhead, like in NIP-L_3VS-PhOH (**21**), dramatically increased the potency for the caspase-like activity and to a lesser extent for the trypsin-like activity.[35] A P2 to P4 side chain residue positional scanning library of asparagine derived vinyl sulfone based proteasome inhibitors resulted in the β2 selective inhibitor Ac-YRLN-VS (**22**).[36] The hypothesis that extended versions of the peptide based vinyl sulfone inhibitors would be better mimics of natural proteasome

substrates led to the synthesis of AdaAhx$_3$L$_3$VS (**23**).[37] Indeed, this inhibitor proved to be more potent and pan-reactive towards the proteolytically active β subunits of the proteasome in living cells in comparison with, for example, Z-L$_3$VS (**18**). Efforts to develop subunit specific proteasome inhibitors resulted in the β1 and β1i selective phenolic vinyl sulfone proteasome inhibitor **24**.[38]

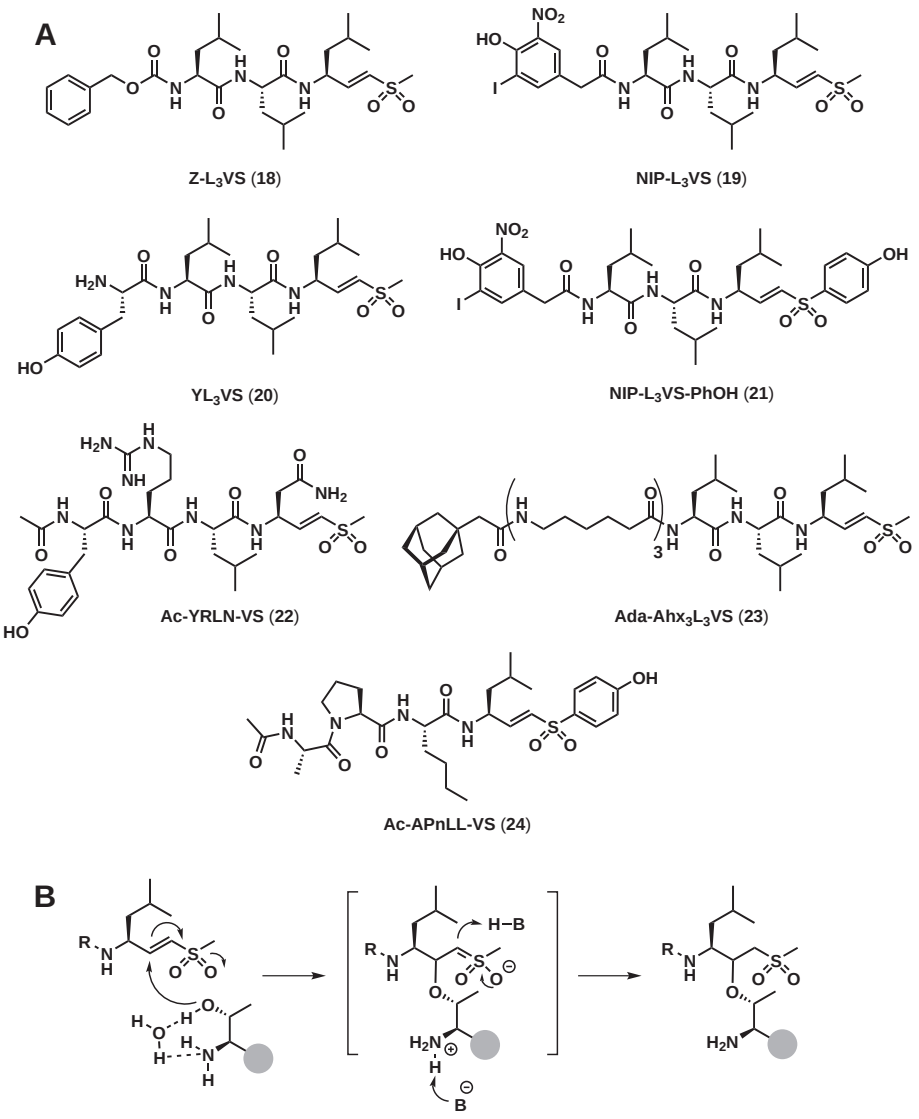

Figure 9. Vinyl sulfone based proteasome inhibitors.

(**A**) Structures and (**B**) mechanism of inhibition of vinyl sulfone based proteasome inhibitors.

Iqbal *et al.* described the derivatization of their previously developed peptide aldehyde proteasome inhibitor **25** into the corresponding α-ketoamide **26** and boronic ester **27** (Figure 10A).[39] The latter proved to be a very potent proteasome inhibitor and represents the first of a new generation of inhibitors armed with a boronate derived warhead. Soon thereafter, the boronic acid analogue of Z-LLL-al (**17**), MG262 (**28**) proved to be more than a 100-fold more potent then the parent compound MG132 (**17**).[40] In an

effort to reduce the molecular weight and to simplify the synthesis, the sub-nanomolar dipeptidyl boronic acid proteasome inhibitor **29** (PS-341) was developed.[40] Despite the fact that peptidyl boronic acids have been described as inhibitors of serine proteases,[41] PS-341 (**29**) exhibits a very high selectivity over common serine proteases for the proteasome. Interestingly, PS-341 (**29**) was shown to bind β1, β5, β1i and β5i exclusively at therapeutic concentrations, leaving β2 and β2i untouched.[42] The mechanism of reversible inhibition entails the formation of a tetrahedral adduct with the γ-hydroxyl of the active site threonine (Figure 10B).[17,43]

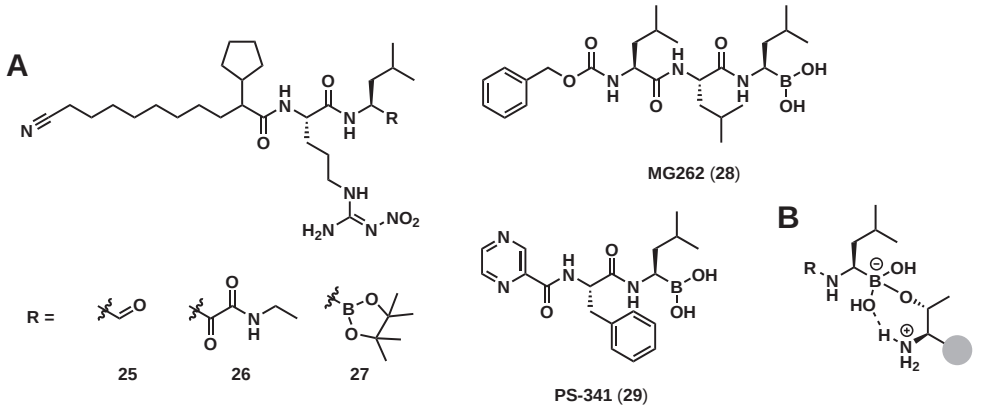

Figure 10. Boronate based proteasome inhibitors.

(**A**) Structures and (**B**) mechanism of inhibition of boronate based proteasome inhibitors.

Several synthetic analogues of the highly selective proteasome inhibitors eponemycin (**8**) and epoxomicin (**9**) have been synthesized (Figure 11). A structural hybrid library of the two inhibitors revealed that replacing the peptidic recognition element of epoxomicin (**9**) for the fatty acid *N*-terminal extension of eponemycin (**8**) resulted in a shift from a predilection for β2 and β5 towards β1 and β5.[44] The synthesis of a library of α′,β′-

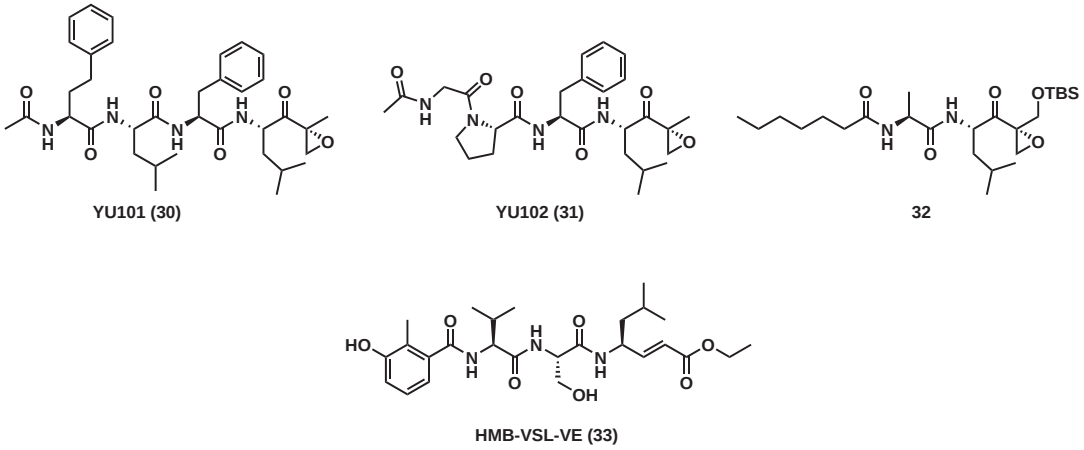

Figure 11. Synthetic epoxyketone and vinyl ethyl ester based proteasome inhibitors.

epoxyketone warhead armed peptide inhibitors, varying in length and amino acid sequence resulted in Ac-hFLFL-epoxyketone (**30**, YU101), a selective inhibitor of the chymotrypsin-like activity.[45] In a subsequent study, the same group developed Ac-GPFL-epoxyketone (**31**,YU102) as a selective inhibitor of the caspase-like activity of the proteasome.[46] The dihydroeponemycin analogue **32** was found to selectively inhibit the β1i subunit, over the other constitutive and immune-induced proteolytically active proteasome subunits.[47]

Marastoni *et al*. reported a new class of synthetic peptide based proteasome inhibitors possessing a leucine derived vinyl ethyl ester warhead. HMB-VSL-VE (**33**, Figure 11) was shown to be a very potent and highly selective inhibitor of the trypsin-like activity of the proteasome.[48]

1.5 Probing the proteasome

Activity-based probes are very useful tools in proteasome research that allow for direct visualization of the proteolytically active proteasome subunits, rather than looking at activity using fluorogenic substrates.[49] One of the first activity-based proteasome probes was the radio-labeled ^{125}I-NIP-L_3VS (**34**), developed by Bogyo *et al*. (Figure 12).[34] The biotinylated epoxomicin analogue **35** proved to be an important tool in the establishment of the proteasome being the target of epoxomicin.[26b] The biodistribution of PS-341 (**29**) after i.v. dosing of rat was determined by the use of the radiolabeled probe [^{14}C]PS-341.[50] Potent proteasome probes were derived from the extended vinyl sulfone inhibitor AdaAhx$_3$L$_3$VS (**23**) by the introduction of either a lysine ε-biotinamide to give AdaK(Bio)Ahx$_3$L$_3$VS (**36**), or a radioiodinated tyrosine to result in AdaY(^{125}I)Ahx$_3$L$_3$VS (**37**).[37]

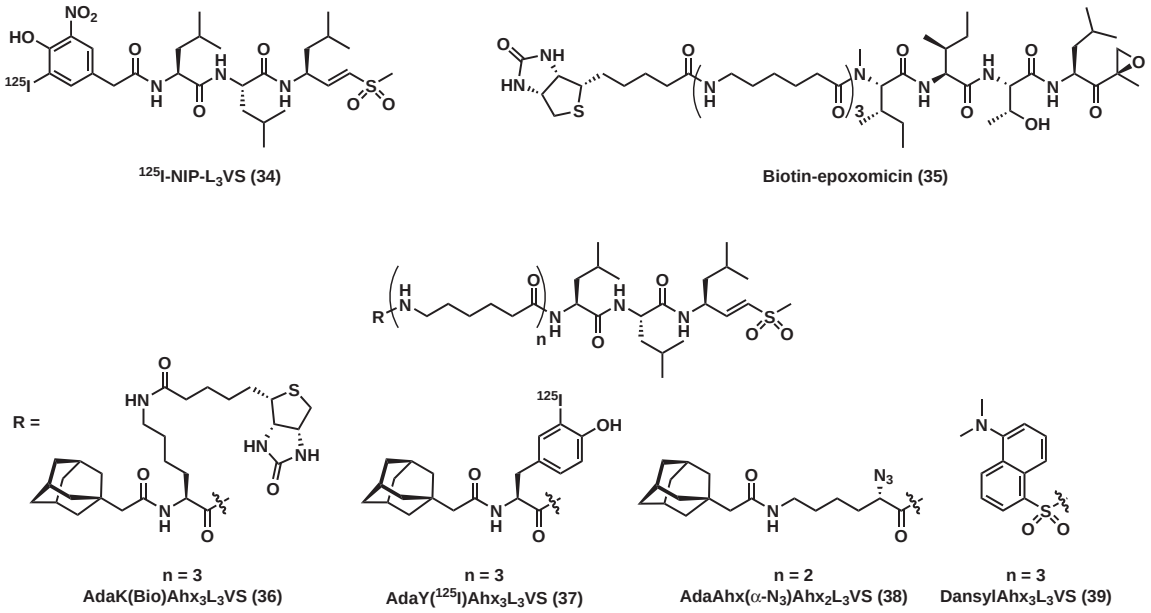

Figure 12. Epoxomicin-biotin and vinyl sulfone based proteasome probes.

The latter two probes are powerful tools for experiments in cell lysates or tissue homogenates, but the introduction of the reporter groups rendered the proteasome probes cell-impermeable. To alleviate this problem, the two-step labeling proteasome probe AdaAhx(α-N_3)Ahx$_2$L$_3$VS (**38**) was synthesized.[51] Indeed, the introduction of the small biocompatible azido functionality in AdaAhx$_3$L$_3$VS (**23**) did not affect the cell permeability. In a typical activity-based two-step proteasome labeling experiment living cells are exposed to AdaAhx(α-N_3)Ahx$_2$L$_3$VS (**38**) (Figure 13). After cell lysis, the azido functionalized proteasome subunits can be reacted with a ligation reagent equipped with a reporter group, allowing for the visualization of the labeled proteins. Ovaa *et al*. employed the biotinylated Staudinger-Bertozzi[52] reagent **40** (Figure 13) and proved that AdaAhx(α-N_3)Ahx$_2$L$_3$VS (**38**) is a cell permeable pan-reactive two-step proteasome probe, representing the first example of a two-step labeling approach in activity-based protein profiling. Simultaneously, Speers *et al.* reported a two-step protease labeling approach for the activity-based profiling of serine hydrolases.[53] In their study, an azide-derivatized phenyl sulfonate ester probe was used to tag serine hydrolases with an azide moiety *in vivo*. After tissue homogenation, the azide labeled proteins were reacted with a rhodamine functionalized alkyne reagent (**41**) employing the copper(I)-catalyzed Huisgen [2+3]-cycloaddition ("click reaction") (Figure 14A).[54] Since then, several bioorthogonal

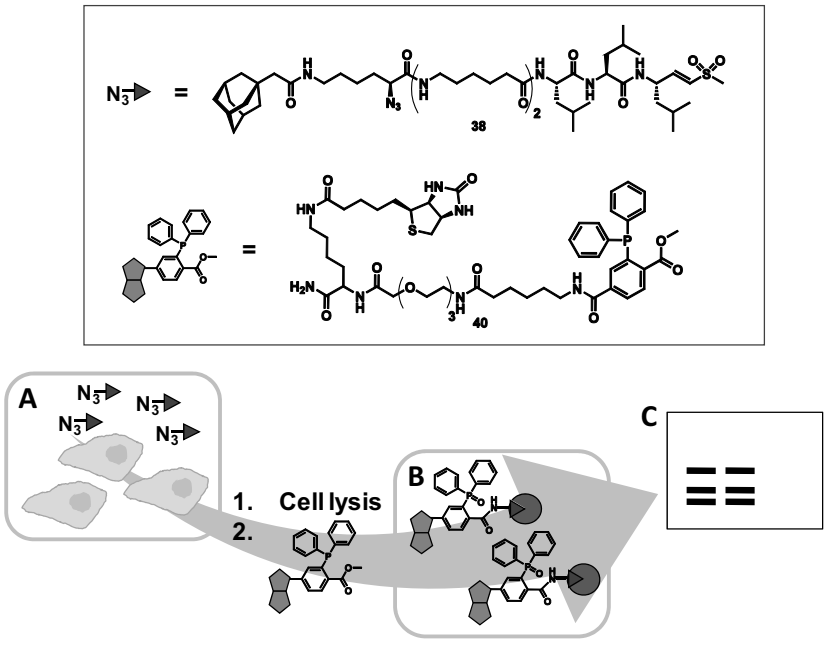

Figure 13. Schematic representation of a two-step proteasome labeling experiment.
(**A**) Living cells are being exposed to the cell-permeable azide containing proteasome probe (**38**), after which the cells are washed. (**B**) After cell lysis, the azide modified proteins are reacted with the Staudinger-Bertozzi reagent **40** to introduce a biotin moiety. (**C**) After denaturation of the proteins and separation on SDS-PAGE, the biotinylated proteins are visualized by streptavidin blotting.

chemoselective reagents have been developed for the ligation of a reporter group onto an azide functionalized biomolecule in cell-surface labeling experiments or activity-based protein profiling. To overcome the cytotoxicity of copper ions, the Bertozzi lab reported a series of strain promoted cyclooctyne copper-free click reagents.[55] The synthetically demanding DIFO (difluorinated cyclooctyne) **42** (Figure 14B) was applied for *in vivo* non-invasive imaging of metabolically azide labeled membrane-associated glycans in developing zebrafish.[56] Soon after, the easier accessible second-generation DIFO reagent **43** was reported.[57] In the same period, Boons and co-workers reported 4-dibenzocyclooctynol **44** for the visualization of metabolically labeled glycoconjugates on the surface of living cells.[58] In an effort to reduce aspecific labeling in *in vivo* imaging experiments, the quenched Staudinger-Bertozzi reagent **45** was synthesized.[59] Upon Staudinger reduction of the biomolecule bound azide, the quencher moiety is displaced and a fluorescent conjugate is formed (Figure 14C). Since the fluorophore on the unreacted ligation reagent is quenched, fluorescence will only appear after ligation with an azido modified biomolecule, making this tool ideal for imaging applications.

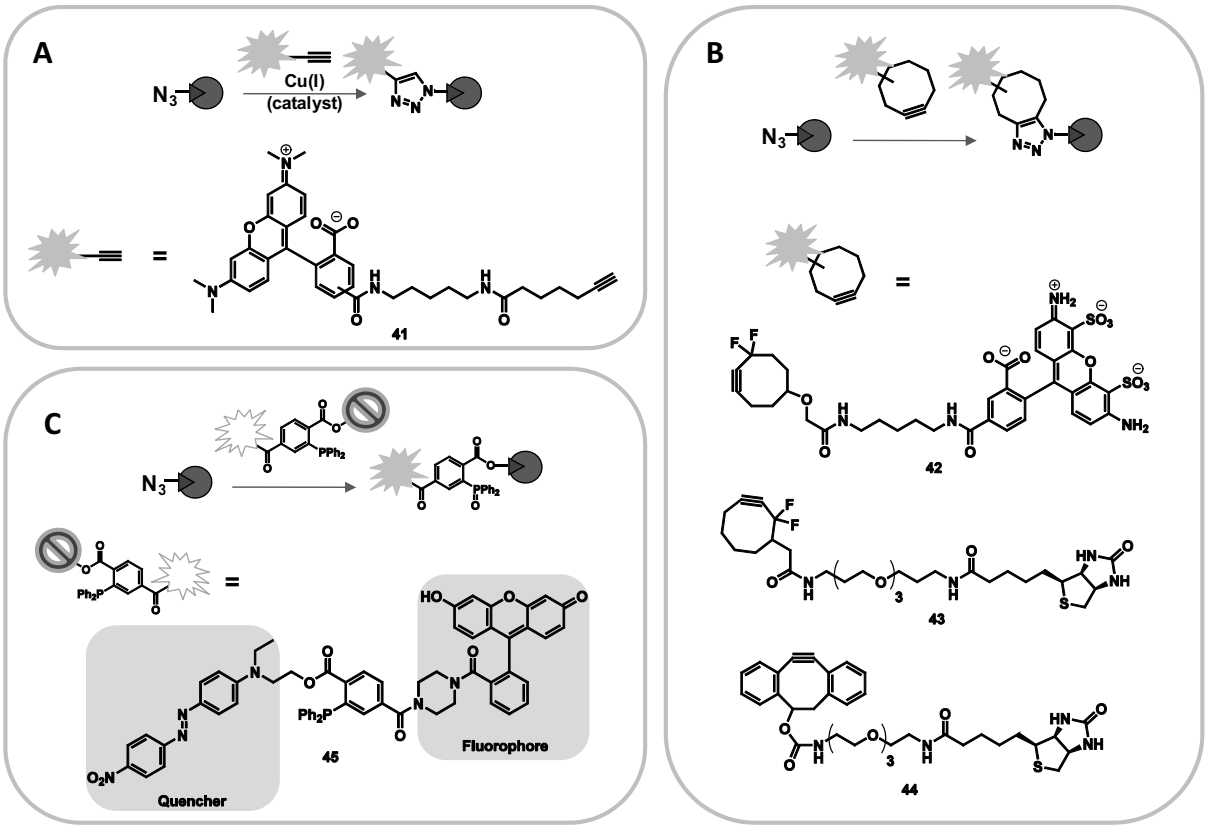

Figure 14. Schematic representation of two-step labeling approaches.

(**A**) The copper (I) catalyzed [2+3]-Huisgen cycloaddition. (**B**) Strain promoted [2+3]-cycloaddition. (**C**) Quenched Staudinger-Bertozzi reagent.

The subunit specificity of PS-341 (**29**) in living cells was determined with the use of the cell-permeable broad-spectrum proteasome probe dansylAhx$_3$L$_3$VS (**39**, Figure 12).[42a] The weak fluorescent dansyl hapten enables the fast detection of proteolytically active proteasome subunits with anti-dansyl antibodies by SDS-PAGE and Western-blot analysis.

1.6 The proteasome as a therapeutic target

The ubiquitin proteasome system plays an essential role in the regulation of proteins engaged in cell-cycle progression (e.g. cyclins, activators of the cyclin-dependent kinase family which effect cell cycle progression), oncogens (e.g. growth factors), tumor suppressors (e.g. the transcription factor p53 and IκB, the inhibitor of NF-κB, the transcription factor nuclear factor-κB), and proteins involved in apoptosis (e.g. Bax, the pro-apoptotic Bcl-2-associated X protein).[20] Dividing cells are particularly sensitive to proteasome inhibition due to the tight control of cell-cycle regulators. The stabilization of both promoters and repressors results in cell-cycle arrest and induction of apoptosis, making cancer cells in particular susceptible for proteasome inhibitors. In several malignancies, NF-κB is overexpressed and stimulates cell proliferation and protects the cancer cells from apoptosis. NF-κB is expressed in an inactive form and requires activation by proteasome-mediated processing. Blocking the proteasome prevents activation and furthermore results in the stabilization of IκB and consequently in the inhibition of the anti-apoptotic function of NF-κB. Therefore, besides the direct effect of proteasome inhibition, it also displays a chemosensitizing effect in making cancer cells more susceptible to conventional chemotherapeutics.[60] Multidrug resistance in cancer therapy is partly caused by the overexpression of the ATP-dependent efflux pump P-glycoprotein. Inhibition of the proteasome disrupts the maturation of P-glycoprotein and circumvents it reaching the cell membrane.[61] This is another means for proteasome inhibitors to sensitize cancer cells for chemotherapeutics.

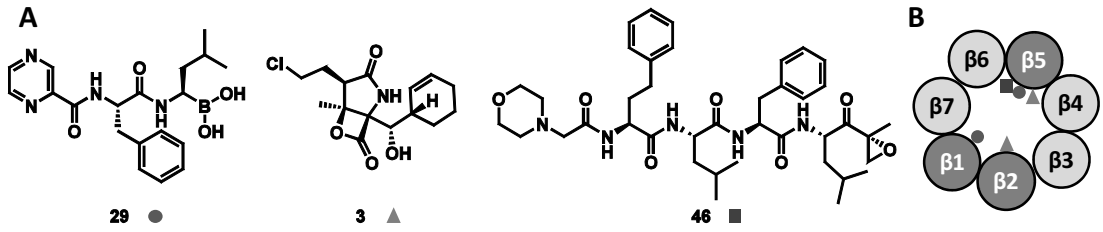

Figure 15. Proteasome inhibitor anti-cancer drugs.

(**A**) Structures of the anti-cancer drugs bortezomib (**29**), NPI-0052 (**3**) and carfilzomib (**46**). (**B**) Schematic representation of catalytic proteasome subunits targeted by bortezomib (circle), NPI-0052 (triangle) and carfilzomib (square).

The proteasome inhibitors PS-341 (**29**, bortezomib), salinosporamide A (**3**, NPI-0052) and the epoxyketone carfilzomib (**46**) have been studied extensively for their antineoplastic activity (Figure 15A).[62,63] On May 13, 2003 the FDA granted accelerated marketing approval to Millennium Pharmaceuticals for bortezomib (**29**) as a single agent against multiple myeloma (plasma cell cancer) in patients who have received at least two prior therapies and have demonstrated disease progression on the last therapy.[64] Later, on December 8, 2006 bortezomib (**29**) received marketing approval for the treatment of patients who have had at least one prior therapy for mantle cell lymphoma (a subtype of B-cell lymphoma and one of the rarer of the non-Hodgkin's lymphomas).[65] Promising results have been demonstrated for bortezomib (**29**) in combination with conventional drugs, like dexamethasone (member of the prednisone class of anti-inflammatory drugs), doxorubicin (a DNA intercalator, causing disruption of transcription and replication), melphalan and cyclophosphamide (nitrogen mustard alkylating agents with antiproliferative activity by the formation of non-replicating interstranded DNA) and as induction therapy prior to autologous stem cell transplantation.[62,63] Bortezomib (**29**) was shown to inhibit osteoclasts (bone tissue removing cells) and to enhance osteoblast (bone forming cells) activity, resulting in a positive effect on cancer-induced bone disease.[62] The most common adverse effects associated with bortezomib (**29**) are peripheral sensory neuropathy (effects the nerves that serve the hand and feet, causing pain, prickling, numbness and tingling of hands and feet), thrombocytopenia (decrease in the amount of platelets in blood), asthenia (symptoms of physical weakness and loss of strength), fatigue and gastrointestinal events. These side effects however are normally manageable. In contrast to the positive results with bortezomib (**29**) in hematologic malignancies, disappointing results were obtained in solid tumors. Resistance towards bortezomib (**29**) was shown to be correlated with the overexpression of proteasome subunits and a significant change in the proteasome activity profile.[66]

Two other proteasome inhibitors, NPI-0052 (**3**) and carfilzomib (**46**) have entered Phase I clinical trials.[62,63] NPI-0052 (**3**) has a preference for the chymotrypsin-like and the trypsin-like activity as opposed to the reversible inhibition of the chymotrypsin-like and caspase-like activity by bortezomib (Figure 15B). NPI-0052 (**3**) was shown to initiate cancer cell-death via a different mechanism as compared to bortezomib and primarily relies on pro-apoptotic caspase-8 signaling pathways.[67] Furthermore, NPI-0052 (**3**) was proven to be orally bioactive, omitting intravenous administration of the drug and was shown to be less cytotoxic against healthy lymphocytes.[67] Carfilzomib (**46**) is a highly specific irreversible proteasome inhibitor showing preference for the chymotrypsin-like activity. It was shown to have increased efficacy in inhibiting proliferation and activation of apoptosis in patient-derived multiple myeloma cells compared to bortezomib (**29**).[68] Both carfilzomib (**46**) and

NPI-0052 (**3**) have shown enhanced potency compared to bortezomib (**29**) and were proven to overcome resistance both to conventional drugs and bortezomib (**29**).[62,63,67,68]

1.7 Aim and outline of this Thesis

The research described in this Thesis aims at the development of novel chemical biology tools to study proteasome activity. **Chapter 2** describes the synthesis and characterization of the fluorescent, cell-permeable, and activity-based proteasome probe BODIPY TMR-Ahx$_3$L$_3$VS (**47**, MV151, Figure 16). This probe enables fast and sensitive direct in-gel fluorescence readout of proteasome activity *in vitro*, in cells, and in mice, is compatible with live-cell imaging techniques and facilitates screening and determination of the subunit specificity of novel proteasome inhibitors.

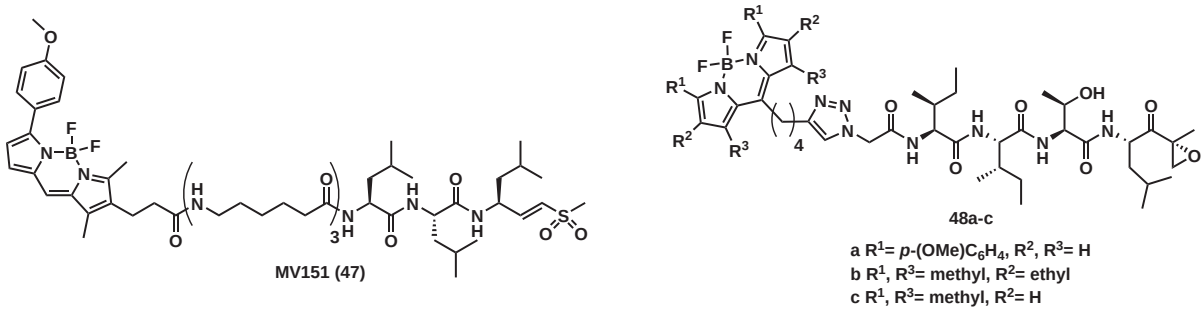

Figure 16. Structure of MV151 (**47**) and fluorescent epoxomicin derived proteasome probes **48a-c**.

The development of three easily accessible alkyne functionalized BODIPY dyes is discussed in **Chapter 3**. Using these fluorescent tags, three epoxomicin derived proteasome probes **48a-c** (Figure 16) were synthesized and shown to label the proteolytically active proteasome subunits *in vitro* and in living cells.

In **Chapter 4**, the synthesis of the bifunctional azido-BODIPY succinimidyl ester **49** (Figure 17) is described. Two sets of one- and two-step labeling proteasome probes were synthesized and were used to determine the efficiency of the Staudinger-Bertozzi ligation.

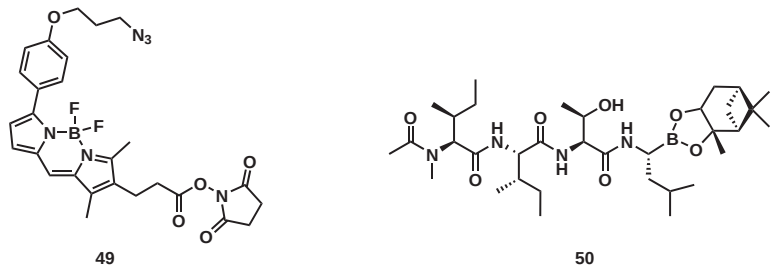

Figure 17. Azido-BODIPY-OSu (**49**) and epoxomicin derived boronic ester **50**.

Peptide based proteasome inhibitors can be divided in three structural entities, being 1) the warhead, the electrophilic trap that reacts with the active site nucleophilic residue, 2) the peptidic recognition element that serves as the homing sequence and 3) the *N*-terminal extension. In **Chapter 5** these three structural entities of five commonly used proteasome inhibitors are scrambled to afford a library of 15 hybrid proteasome inhibitors. Employing MV151, the inhibitory profile of the library was determined. One of the outcomes of this study is the discovery of the epoxomicin derived boronic ester **50** (Figure 17) as being one of the most potent peptide-based proteasome inhibitor reported to date.

A similar strategy was employed in **Chapter 6**, where warheads and peptidic recognition elements of previously reported subunit specific proteasome inhibitors were scrambled. A more potent β1 specific proteasome inhibitor **51** was developed, next to the β5 specific vinyl sulfone **52** (Figure 18). These hits were transformed into subunit specific two-step labeling probes **53** and **54** by the introduction of an azide moiety. Substitution of the naphthylacetamide in **52** for an azido-BODIPY moiety resulted in the fluorescent β5 specific two-step labeling proteasome probe **55**.

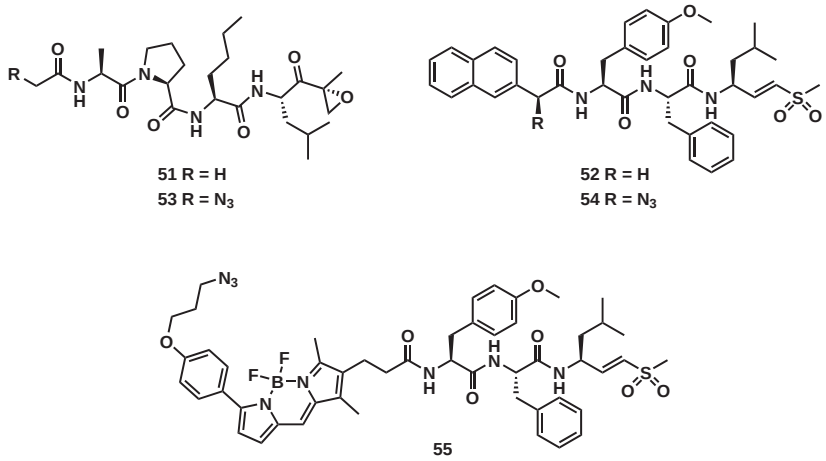

Figure 18. Subunit specific proteasome inhibitors and probes.

As discussed in the Chapter 1.5, two approaches for the two-step labeling of proteins have been developed, both relying on the azido moiety. In **Chapter 7** the applicability of the Diels-Alder reaction as a complementary two-step labeling approach of proteolytically active proteasome subunits is investigated. A panel of diene equipped epoxomicin analogues (like **56**, Figure 19) were synthesized and were shown to be able to inhibit the proteasome with potencies in the same order of magnitude compared to epoxomicin (**9**).

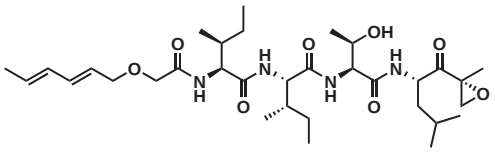

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Figure 19. Diene functionalized epoxomicin analogue **56**.

In the final Chapter, the work described in this Thesis is summarized and future applications of the developed tools are proposed, as well as some suggestions for further research in line with that described in this Thesis.

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2 A Fluorescent Broad-Spectrum Proteasome Inhibitor for Labeling Proteasomes *In Vitro* and *In Vivo*.

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2.1 Introduction

The 26S proteasome is the central protease in the ATP- and ubiquitin-dependent degradation of proteins in the eukaryotic cytoplasm and nucleus and is involved in the degradation of 80-90% of all cellular proteins. Proteasome substrates include abnormal and damaged proteins, cell-cycle regulators, oncogens and tumor suppressors, and the proteasome is imperative in the generation of MHC class I antigenic peptides.[1] Eukaryotic proteasomes contain two copies of seven distinct α and β subunits each. These subunits assemble into two types of hetero-oligomeric rings each composed of seven subunits [(α1-α7) and (β1-β7)]. The 20S proteasome is formed by two juxtaposed rings of β subunits flanked on top and bottom by a ring of α-subunits.[2] When capped by the 19S regulatory complex at both ends, the proteolytically active 26S proteasome is formed which is responsible for ATP-dependent proteolysis of poly-ubiquitinated target proteins.[3]

In the eukaryotic proteasome, three of the seven β subunits are responsible for the proteolytic activities of the proteasome. Characterization of the active β1, β2 and β5 subunits led to the classification of their substrate specificity as peptidylglutamyl peptide hydrolytic (PGPH, or caspase-like), trypsin-like and chymotrypsin-like, respectively. In immune competent cells three additional active β subunits (βi) are expressed upon interferon-γ stimulation, which replace their closely related, constitutively expressed β subunits in newly formed 20S proteasome particles.[2]

The proteolytic subunits β1, β2 and β5, and their immunoproteasomal counterparts, β1i, β2i and β5i act by nucleophilic attack of the γ-hydroxyl of the *N*-terminal threonine on the carbonyl of the peptide bond destined for cleavage. The α-amine of the threonine acts as a base in the catalytic cycle. The existence and evolutionary development of six different

active β subunits, their divergent substrate specificities, and their individual roles in cellular processes constitute a vast research field of interest in both academia and the pharmaceutical industry. This scientific demand can benefit from an activity-based proteasome probe that ideally (1) specifically targets the proteasome, (2) covalently and irreversibly binds to the active β and βi subunits indiscriminately, (3) facilitates direct, rapid, accurate, and sensitive detection, (4) is cell permeable, and (5) enables monitoring of the proteasome by microscopic techniques in living cells. To date, none of the available activity-based proteasome probes meet all of these requirements.[4,5] The compound that comes closest is the radiolabeled proteasome inhibitor AdaY(^{125}I)Ahx$_3$L$_3$VS (**1**, Figure 1).[6] This inhibitor is selective for the proteasome, labels the β subunits with equal intensity, and enables accurate and sensitive in-gel detection. However, usage of this activity-based probe is restricted to *in vitro* applications since this compound is not cell permeable. Recently, the weakly fluorescent and cell-permeable proteasome inhibitor dansylAhx$_3$L$_3$VS (**2**) was developed for profiling proteasome activity in living cells, enabling readout by anti-dansyl immunoblotting.[7] The low quantum yield and near-UV excitation of the dansyl makes this compound unsatisfactory for in-gel detection and standard fluorescence microscopic techniques.

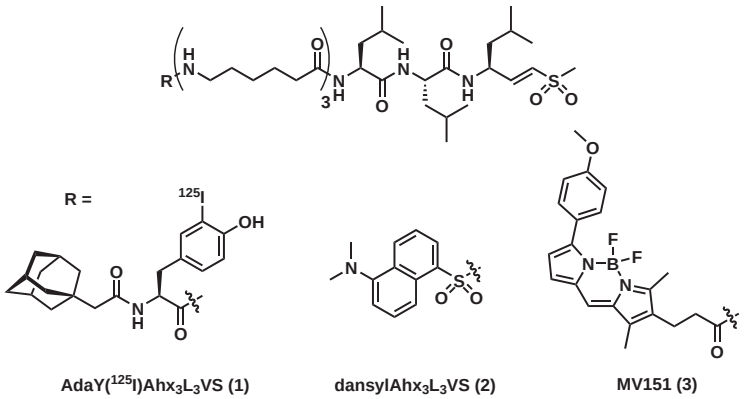


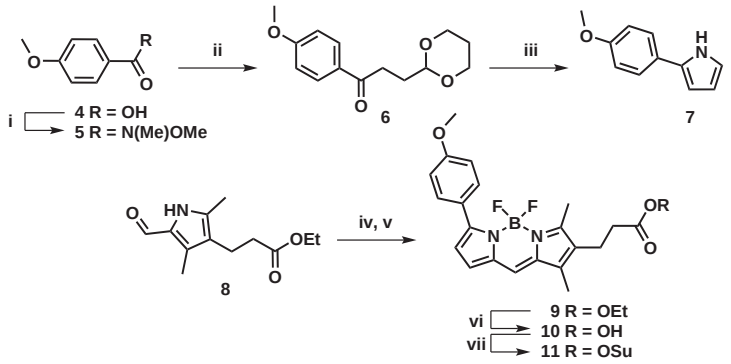
Figure 1. The proteasome probes AdaY(^{125}I)Ahx$_3$L$_3$VS (**1**), dansylAhx$_3$L$_3$VS (**2**) and MV151 (**3**).

In this chapter the synthesis and characterization of the fluorescent, cell-permeable, and activity-based proteasome probe BODIPY TMR-Ahx$_3$L$_3$VS (MV151, **3**, in which BODIPY stands for boron-dipyrromethene and TMR stands for tetramethylrhodamine) is described. After proteasome labeling and protein separation by SDS-PAGE, the labeled proteasome subunits are visualized by direct in-gel fluorescence readout. This compound enables fast and sensitive labeling of proteasome activity *in vitro*, in cells, and in mice, is compatible with live-cell imaging techniques and facilitates screening and determination of the subunit specificity of novel proteasome inhibitors.

2.2 Results and discussion

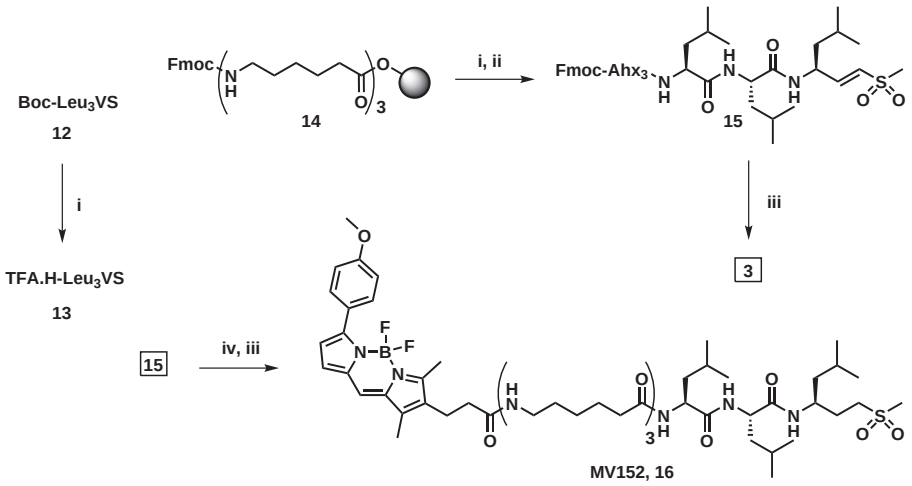
A proteasome probe typically consists of three parts, being a reporter group, a peptidic recognition element and a warhead. In BODIPY TMR-Ahx$_3$L$_3$VS (MV151, **3**), the warhead is the leucine derived vinyl methyl sulfone. This Michael acceptor mimics the carbonyl of a substrate peptide bond destined to be cleaved. Nucleophilic attack of the γ-hydroxyl of the *N*-terminal threonine on the vinyl sulfone covalently links the probe to the proteolytically active β subunit and renders the protease deactivated (see Chapter 1.4 for a more detailed description). BODIPY TMR-Ahx$_3$L$_3$VS (MV151, **3**) and the inactive, negative control compound BODIPY TMR-Ahx$_3$L$_3$ES (MV152, **16**), in which the vinyl sulfone moiety is reduced to an ethyl sulfone, were synthesized as depicted in Scheme 1 and 2. The synthesis commences with the preparation of the succinimidyl ester of the fluorophore BODIPY TMR (**11**) using a modified procedure of the synthesis described in the patents for related compounds (Scheme 1).[8-10] Anisic acid (**4**) was converted into Weinreb-amide **5**, which upon treatment with (1,3-dioxane-2-ylethyl)-magnesium bromide in THF yielded ketone **6**. *In situ* deprotection of the aldehyde and Paal-Knorr reaction afforded methoxy-phenyl pyrrole **7**, which was condensed with carboxyaldehyde pyrrole **8**.[11] Subsequent treatment of the resulting dipyrrole HBr salt with $BF_3{\cdot}Et_2O$ and triethylamine in refluxing dichloroethane afforded BODIPY TMR ethyl ester **9**. Saponification of the ethyl ester and subsequent condensation of **10** with *N*-hydroxysuccinimide furnished BODIPY TMR succinimidyl ester (**11**). Acidic cleavage of Fmoc-Ahx$_3$-Wang resin (**14**), synthesized employing standard Fmoc-based solid-phase peptide chemistry, gave the crude Fmoc-Ahx$_3$-OH, which was block coupled to TFA·H-Leu$_3$VS (**13**),[12] to yield Fmoc-protected hexapeptide **15** (Scheme

Scheme 1. Synthesis of BODIPY TMR-OSu **11**.



Reagents and conditions: i) HN(Me)OMe·HCl (1 equiv.), HCTU (1.1 equiv.), DiPEA (2 equiv.), DCM, 12 hr., 89%. ii) (1,3-dioxane-2-ylethyl)-magnesium bromide (1.5 equiv.), THF, 3 hr., 89%. iii) NH_4OAc (12 equiv.), Ac_2O (3.7 equiv.), AcOH, 3 hr., 70%. iv) **7**, HBr 48% in H_2O (1.5 equiv.), EtOH, 0 °C, 1 hr. v) $BF_3{\cdot}Et_2O$ (5 equiv.), TEA (3 equiv.), DCE, 90 °C, 30 min., 68% (2 steps). vi) 0.1M NaOH (1 equiv.), dioxane, 15 hr., 40%. vii) *N*-hydroxysuccinimide (4 equiv.), EDC·HCl (4 equiv.), DCM, 15 hr., 69%.

Scheme 2. Synthesis of BODIPY TMR-Ahx$_3$L$_3$VS (**3**), and the control compound BODIPY TMR-Ahx$_3$L$_3$ES (**16**).



Reagents and conditions: i) TFA/DCM 1/1 (v/v), 30 min. ii) **13** (2 equiv.), BOP (2.5 equiv.), DiPEA (6 equiv.), 12hr., 98%. iii) (a) DBU (1 equiv.), DMF, 5 min. (b) HOBt (4.5 equiv.), 1 min. (c) **11** (1 equiv.), DiPEA (6 equiv.), 30 min., **3** 99%, **16** 88%. iv) Pd/C, H_2, MeOH, 2 hr.

2). *In situ* deprotection of the Fmoc-protecting group with DBU and treatment with BODIPY TMR succinimidyl ester (**11**) afforded the potential proteasome probe MV151 (**3**). In order to obtain the inactive control compound **16**, hexapeptide **15** was first treated with hydrogen gas and palladium on charcoal in methanol to reduce the vinyl sulfone, followed by Fmoc cleavage and introduction of the BODIPY TMR moiety.

The potency of MV151 (**3**) to inhibit the proteasome was determined in a fluorogenic substrate assay.[13] EL-4 lysates were incubated with increasing concentrations of MV151, and the cleavage of the substrates Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity, β5), Z-Ala-Ala-Arg-AMC (trypsin-like activity, β2), and Z-Leu-Leu-Glu-βNA (PGPH activity, β1) was monitored. At concentrations of 1 μM and higher, MV151 completely inhibits all three activities. Below a 1 μM concentration, MV151 appears to inhibit the trypsin-like activity and the chymotrypsin-like activity more efficiently than it inhibits the PGPH activity (Figure 2A). This might be due to differences in activity between the subunits, to allosteric effects, to minor subunit specificities of the probe, or to nonsaturation kinetics. Having established the proteasome inhibitory capacity, direct in-gel visualization of MV151-labeled proteasome subunits was explored by using a fluorescence scanner. Treatment of purified human 20S proteasome with MV151 showed uniform labeling of the active subunits β1, β2, and β5 (Figure 2B). To determine the sensitivity of the in-gel detection, the fluorescence readout of the gel (Figure 2B) and the detection of proteasome subunits by silver staining (Figure 2C) was directly compared. The in-gel detection proved to be very sensitive since as little as 3 ng proteasome was sufficient to detect individual MV151-labeled proteasome

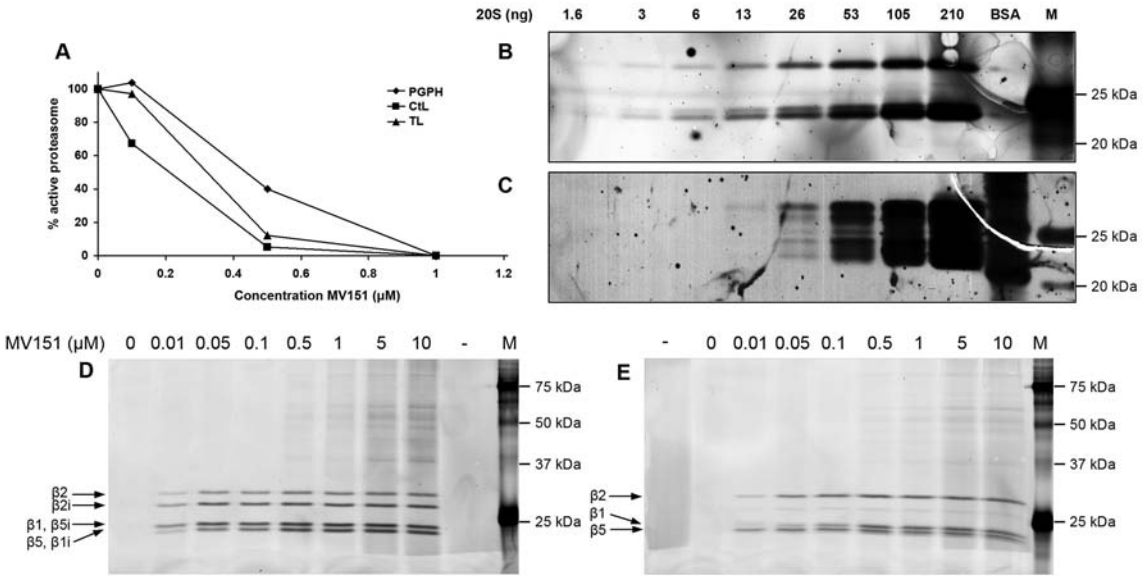


Figure 2. Proteasome labeling and in-gel detection from cell extracts.
(**A**) Measurement of proteasome activity with fluorogenic substrates after treatment of EL-4 lysates with the indicated concentrations of MV151 (**3**) (PGPH = peptidylglutamyl peptide hydrolytic activity, CtL = chymotrypsin-like activity, TL = trypsin-like activity). (**B**,**C**) Comparison between fluorescent in-gel detection and silver-staining. The indicated amounts of purified human 20S proteasome were incubated for 1 hr. at 37 °C with 300 nM MV151, resolved by SDS-PAGE and detected by (**B**) direct fluorescence in-gel read-out and by (**C**) silver staining. (**D**,**E**) Proteasome labeling profile in (**D**) EL-4 and (**E**) HeLa lysates (10 μg total protein) incubated for 1 hr. at 37 °C with the indicated concentrations of MV151. "M" represents the molecular marker (Dual Color, BioRad), "-" represents heat-inactivated lysates, incubated with 10 μM MV151 for 1 hr. at 37 °C.

subunits. In-gel detection of fluorescently labeled proteasome subunits is at least three times more sensitive than silver staining. To compare the labeling of the constitutive subunits β1, β2, and β5 with that of the immunoproteasome β1i, β2i, and β5i subunits, lysates of the human cervix carcinoma cell line HeLa (expressing constitutive proteasome) and the murine lymphoid cell line EL-4 (expressing both constitutive and immunoproteasome) were treated with increasing concentrations of MV151. All active constitutive and inducible β proteasome subunits were neatly and uniformly labeled by MV151 (Figures 2D and 2E). All subunits were already detectable at a concentration of 10 nM MV151 and reached saturation in fluorescence signal at 1 μM MV151. At higher concentrations of MV151, an increased nonspecific labeling was observed in the high molecular weight region.

Next, a set of competition experiments versus MV151 was performed to determine the potency and subunit specificity of a panel of known proteasome inhibitors (Figure 3). EL-4 and HeLa cell lysates (10 μg total protein) were exposed to the inhibitor of interest for 1 hr. After incubation with the proteasome inhibitor, the subunits that were still active were fluorescently labeled by treating the lysates with 100 nM MV151 for 1 hr. The proteins were

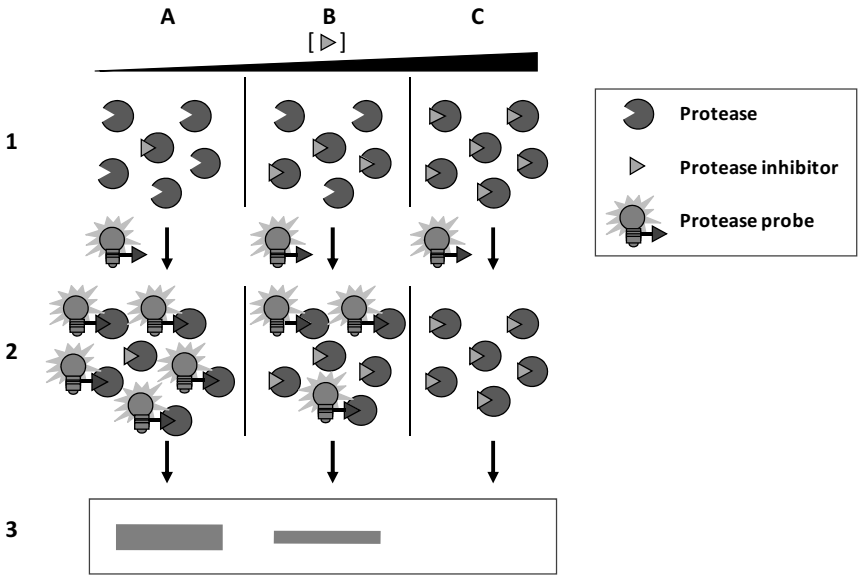


Figure 3. Schematic representation of a competition experiment.
Samples **A** to **C** are exposed to increasing concentrations of an inhibitor of interest in step **1**. In step **2** the residual protease activity is labeled with a fluorescent probe. Step **3** entails SDS-PAGE and fluorescence readout of the resulting gel. The more potent the inhibitor of interest, the sooner the fluorescent bands will disappear.

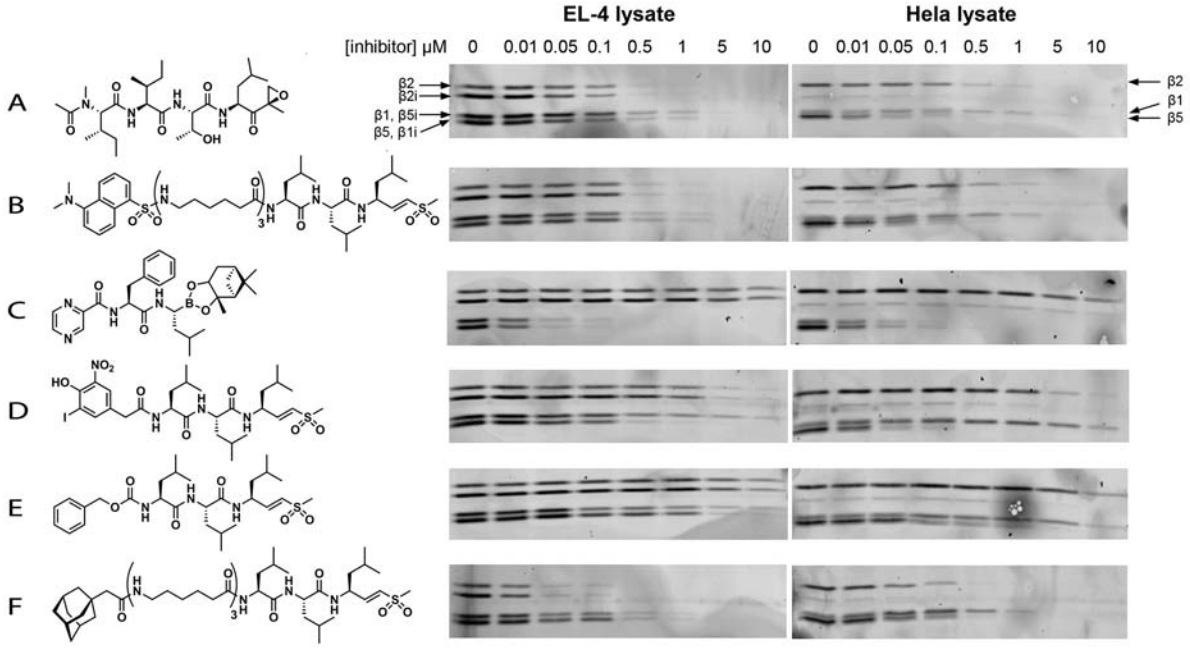


Figure 4. Proteasome profiling screen of known inhibitors using MV151.
(**A**-**F**) EL-4 and HeLa lysates (10 μg total protein) were incubated with the indicated concentrations of the proteasome inhibitor (**A**) epoxomicin, (**B**) dansylAhx$_3$L$_3$VS, (**C**) bortezomib pinanediol boronic ester, (**D**) NIP-LVS (**E**) Z-LVS and (**F**) AdaAhx$_3$L$_3$VS for 1 hr. at 37 °C. Residual proteasome activity was fluorescently labeled by incubation with 0.1 μM MV151 for 1 hr. at 37 °C.

separated on SDS-PAGE and the gels were scanned on a fluorescence scanner. In HeLa lysates, epoxomicin preferentially inhibits the β5 subunit, already visible at a 10 nM

concentration (Figure 4A, right panel). At epoxomicin concentrations over 100 nM, β1 and β2 are also targeted, with a slight preference for β2 (at 5 μM epoxomicin, β2 fluorescence is absent and a faint band of β1 is still visible). This is in accordance with the inhibition profile of epoxomicin determined with purified 20S proteasome.[14] Interestingly, in EL-4 lysates, epoxomicin preferentially inactivates β2 and β2i and is less active toward constitutive and immunoinduced β1 and β5 subunits (Figure 4A, left panel). DansylAhx$_3$L$_3$VS[7] inhibits all active constitutive and immunoinduced subunits in EL-4 from concentrations of 500 nM and greater (Figure 4B, left panel). In HeLa lysates, dansylAhx$_3$L$_3$VS has a preference for the β5 subunit, which is visible at 100 nM, and less of a preference for the β1 and β2 subunits, which are visible at slightly higher concentrations (Figure 4B, right panel). The dipeptidyl pinanediol boronic ester (pinanediol boronic ester of bortezomib[12]) shows a strong selectivity for the constitutive β1 and β5 subunits in HeLa lysates (Figure 4C, right panel) and β1, β1i, β5, and β5i in EL-4 lysates (Figure 4C, left panel). The inhibition profile of the dipeptidyl pinanediol boronic ester is comparable to the labeling profile of bortezomib, with potency in the same order of magnitude.[7] As previously reported, NIP-LVS[12] shows a predilection for β5 (Figure 4D), whereas Z-LVS[12] (Figure 4E) proves to be the least potent compound and shows some preference for the constitutive and immunoinduced β1 and β5 subunits. In EL-4 lysates, AdaAhx$_3$L$_3$VS[6] first targets the β2 and β2i subunits, and it shows a preference for β2 and β5 in HeLa lysates (Figure 4F). Altogether, this experiment shows that MV151 can be used for the determination of inhibition profiles of proteasome inhibitors. Exploiting the sensitivity of in-gel detection of MV151, it is possible to demonstrate that the inhibitors tested show subtle differences in the proteasome inhibition profile.

The capacity of MV151 to cross the cell membrane and label proteasome subunits in living cells was determined in the following experiment. Living EL-4 and HeLa cells were incubated with increasing concentrations of MV151 for 2 hr., before the cells were harvested, washed and lysed. Specific and sensitive labeling of all proteasome subunits was observed in EL-4 (Figure 5A) and HeLa cells (Figure 5B), although higher concentrations were required than for labeling of subunits in lysates. Labeling of the β1 subunit shows a lower intensity than in lysates, whereas β5 labeling looks more pronounced. This difference in the labeling profile between the proteasome in cell lysates and living cells has been previously reported,[7] however, the reason for this remains unclear. Importantly, incubation of EL-4 and HeLa cells with the inactive control compound MV152 (**16**), which is almost identical to MV151 but lacks the reactive vinyl sulfone warhead, showed no labeling of the proteasome or any other protein (Figures 5A and 5B).

To confirm the ability of MV151 to inhibit proteasome activity in living cells, HeLa cells stably expressing a green fluorescent protein (GFP) reporter proteasome substrate[16] were exposed to MV151. The ubiquitin^{G76V}-GFP fusion (Ub^{G76V}-GFP) expressed by these cells

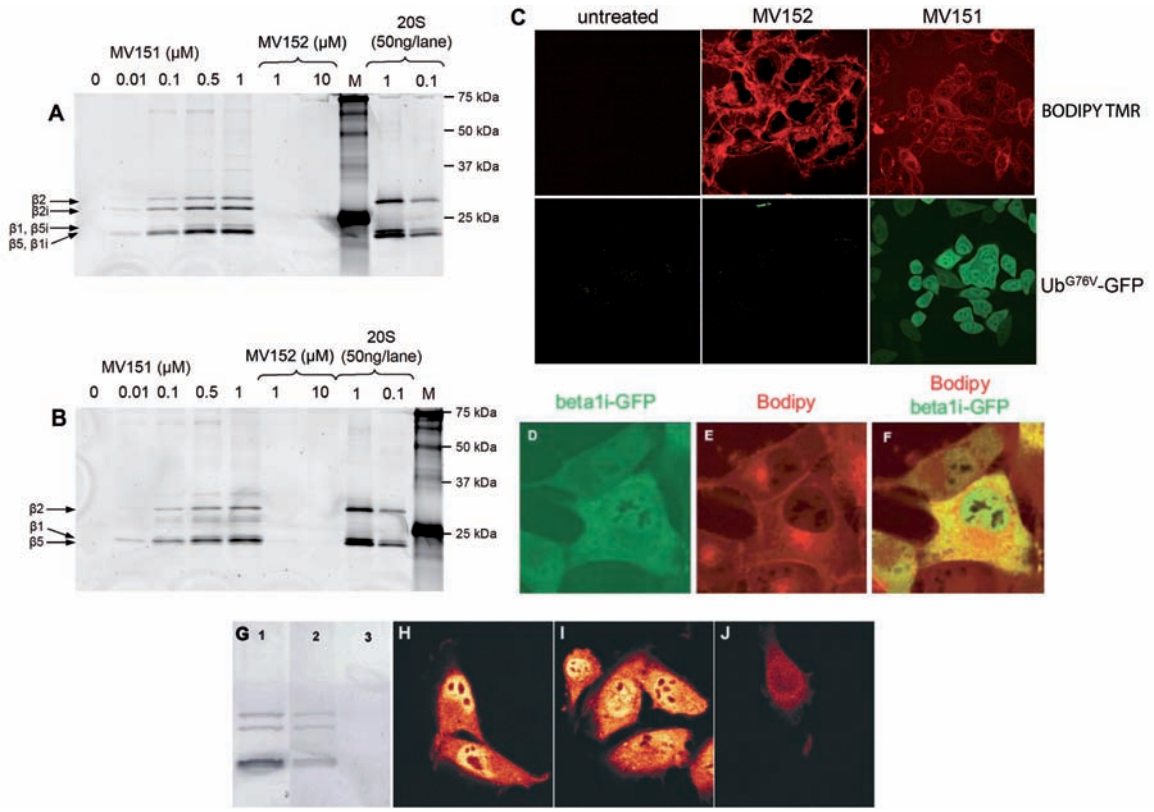


Figure 5. Functional proteasome inhibition in living cells.
(**A-B**) Proteasome profiling in living (**A**) EL-4 and (**B**) HeLa cells after 2 hr. incubation with the indicated concentrations of MV151. As a control, the cells were incubated with the inactive compound MV152. Purified 20S proteasome labeled with MV151 is also shown. (**C**) Representative micrographs of Ub^{G76V}-GFP HeLa cells that were untreated (left panel), incubated for 12 hr. with 10 μM inactive MV152 (middle panel), and 10 μM MV151 (right panel). BODIPY TMR and Ub^{G76V}-GFP fluorescence are shown. (**D-F**) Colocalization of GFP-labeled proteasome and MV151 in living GFP-β1i MelJuSo cells treated for 8 hr. with 10 μM MV151. (**D**) GFP-β1i, (**E**) BODIPY TMR fluorescence and (**F**) merged are shown. (**G**) In-gel visualization of proteasome labeling in living EL-4. Lane 1: 1 hr. incubation with MV151 (250 nM). Lane 2: 1 hr. incubation with MV151 (250 nM) followed by 1 hr. incubation with MG132 (5 μM). Lane 3: 1 hr. incubation with MG132 (5 μM) followed by 1 hr. incubation with MV151 (250 nM). (**H-J**) CLSM pictures of BODIPY TMR fluorescence in MelJuSo Ub-R-GFP cells after formaldehyde fixation, gain 700. (**H**) 1 hr. incubation with MV151 (500 nM). (**I**) 1 hr. incubation with MV151 (500 nM) followed by 1 hr. incubation with MG132 (5 μM). (**J**) 1 hr. incubation with MG132 (5 μM) followed by 1 hr. incubation with MV151 (500 nM).

is normally rapidly degraded by the proteasome. When the proteasome is blocked however, the reporter will accumulate and increased GFP fluorescence can be seen. Indeed, untreated HeLa cells emitted only low GFP fluorescence (Figure 5C, left panel). Cells that were exposed to 10 μM of the inactive MV152 for 12 hr. did accumulate the control compound, but they did not show increased levels of GFP fluorescence (Figure 5C, middle panel). During 12 hr. of exposure to 10 μM MV151, cells accumulated the inhibitor and showed significantly increased levels of GFP fluorescence (Figure 5C, right panel). Strong

BODIPY TMR fluorescence is apparent in the membranous compartments of cells treated with the inactive MV152. This fluorescence, which appears to be stronger than in MV151-treated cells, and which is not due to proteasome labeling (as judged from SDS-PAGE analysis), is due to accumulation of MV152 in the hydrophobic environment of the membranes. The active MV151 is likely to accumulate in the lipid bilayers to a lesser extent, because it is sequestered by the proteasome active sites. There was no visual evidence of cellular toxicity at the dose and exposure time used in this study. These results were confirmed by a study with the human melanoma cell line MelJuSo stably expressing the N-end-rule reporter proteasome substrate Ub-R-GFP[16] (data not shown). Next, it was determined whether the intracellular staining pattern of MV151 colocalized with the proteasome in living cells. To this end, a MelJuSo cell line that stably expresses a GFP-tagged β1i proteasome subunit, which is efficiently incorporated into the proteasome particles was used.[17] The GFP-β1i fusion construct shows ubiquitous distribution throughout the cytoplasm and nucleus, with exception of nucleoli and the nuclear envelope (Figure 5D). The GFP-β1i cells were incubated with 10 μM MV151 and the distribution of proteasomes and probe was compared. The intracellular permeation of MV151 was monitored in time and is characterized by a fast permeation phase (several minutes), followed by a slow distribution phase (several hours). During the permeation phase, the compound showed significant association with the plasma membrane, in discrete cytoplasmatic vesicular and membranous fractions and at the nuclear envelope. After 5 hours of distribution, MV151 is localized throughout the cell, with the exception of the nucleoli, similar to the GFP-β1i fusion protein (Figures 5D-5F). The fact that MV151 is excluded from the nucleoli is in line with the idea that the compound is associated with the proteasome. In some cells, granular accumulation of MV151 was observed in the cytoplasm in close proximity to the nucleus. To attest whether the in-gel readout could be correlated with the fluorescent microscopy data, MV151 was competed with the proteasome inhibitor MG132 (ZL_3-al)[18] in EL-4 cells (Figure 5G) and MelJuSo Ub-R-GFP cells (Figure 5H-J). EL-4 cells incubated with MV151 for 1 hour showed labeling of all the active proteasome subunits on gel (Figure 5G, lane 1). MelJuSo Ub-R-GFP cells treated in the same way showed, after fixation with formaldehyde, strong fluorescence in the cytoplasm and nucleus, with the exception of nucleoli (Figure 5H). Cells incubated with MV151 for 1 hour, followed by a 1 hour incubation with MG132, still showed labeling of the active proteasome subunits on gel (Figure 5G, lane 2) and a similar cellular localization to that shown in Figure 5H (Figure 5I). When the cells were first incubated with MG132 for 1 hour, followed by 1 hour incubation with MV151, in-gel readout proved negative (Figure 5G, lane 3) and the fluorescence in the cells had dramatically decreased (Figure 5J). This competition study proves that the vast majority of the fluorescence observed in cells, after fixation, is due to proteasome labeling.

The results obtained in cell lines raised the question whether MV151 could be used

to label proteasomes in mice. To test the bioavailability of MV151, C57Bl/6 mice were given a single intraperitoneal injection with MV151 (20 µmol/kg body weight) and were sacrificed 24 hours post-injection. Fluorescence microscopic analysis of mouse tissues revealed the capacity of MV151 to penetrate tissues *in vivo*. The highest BODIPY TMR fluorescence was detected in the liver (Figure 6A) and in the pancreas (Figure 6B). Interestingly, BODIPY TMR fluorescence was higher in the peripheries of the tissues, indicating that the probe might reach the liver most efficiently by diffusion from the peritoneal cavity rather than being distributed by entering the bloodstream. A recently developed transgenic mouse model for monitoring the ubiquitin-proteasome system, which is based on the ubiquitous expression of the Ub^{G76V}-GFP reporter[19] was employed to examine the effect of administration of the proteasome probe. It was previously shown that administration of the proteasome inhibitors epoxomicin and MG262 (ZL_3-boronic acid)[20] results in a substantial accumulation

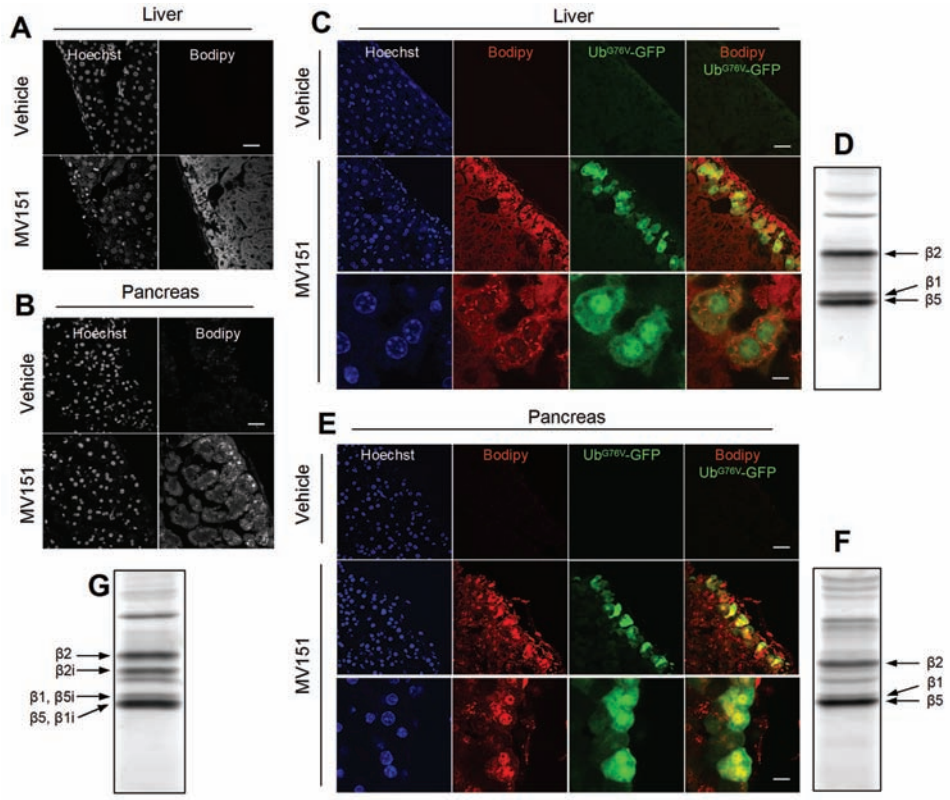


Figure 6. Functional proteasome inhibition in mice.
(**A**-**B**) Micrographs of (**A**) liver and (**B**) pancreas cryosections from C57Bl/6 mice that were treated with vehicle only or with MV151 (20 µmol/kg body weight). Hoechst staining and BODIPY TMR fluorescence are shown. Scale bar represents 40 µm. (**C**-**F**) Micrographs and in-gel fluorescence readout of (**C**, **D**) liver and (**E**, **F**) pancreas. (**C** and **E**) cryosections from Ub^{G76V}-GFP mice that were treated with vehicle only or with MV151 (20 µmol/Kg body weight). Hoechst staining, BODIPY TMR fluorescence, Ub^{G76V}-GFP fluorescence and BODIPY TMR and Ub^{G76V}-GFP merged images are shown. Scale bar represents 40 µm (upper and middle panels) and 5 µm (lower panels). (**D**-**G**) SDS-PAGE analysis and in-gel fluorescence readout of homogenates (10 µg total protein) from (**D**) liver and (**F**) pancreas tissues (shown in panels **C** and **E**) and (**G**) spleen.

of the Ub^{G76V}-GFP reporter in affected tissues.[19] The accumulation was primarily found in the liver and at higher inhibitor concentrations in other tissues. In the present experiment, the Ub^{G76V}-GFP reporter mice were given a single intraperitoneal injection with MV151 (20 µmol/kg body weight). A total of 24 hours post-injection, the mice were sacrificed and several tissues were analyzed by fluorescence microscopy. Cells accumulating Ub^{G76V}-GFP were detected in the liver (Figure 6C) and the pancreas (Figure 6E), which also contained the highest BODIPY TMR fluorescence of all of the examined tissues (spleen, intestine, kidney, liver, and pancreas). Importantly, all of the cells that accumulated the Ub^{G76V}-GFP reporter contained very high BODIPY TMR fluorescence. The proteasome probe was distributed both in the cytoplasm and nuclei of the cells that accumulated the reporter. Similar to the observations from experiments in cell culture, the affected cells in the mice contained granular accumulations of MV151 in the cytoplasm in close proximity to the nucleus. To verify that accumulation of Ub^{G76V}-GFP in the liver and pancreas coincided with proteasome labeling by MV151, tissue homogenates were analyzed by SDS-PAGE followed by in-gel fluorescence readout. Liver (Figure 6D) and pancreas (Figure 6F) homogenates of animals treated with MV151 revealed that the proteasome catalytic subunits were labeled as expected, although higher background labeling compared to *in vitro* studies was observed. (For Figures 6D and 6F, respectively, the tissues from the images in Figures 6C and 6E were used). SDS-PAGE followed by in-gel fluorescence analysis of spleen homogenates showed labeling of both constitutive and inducible proteasome catalytic subunits (Figure 6G).

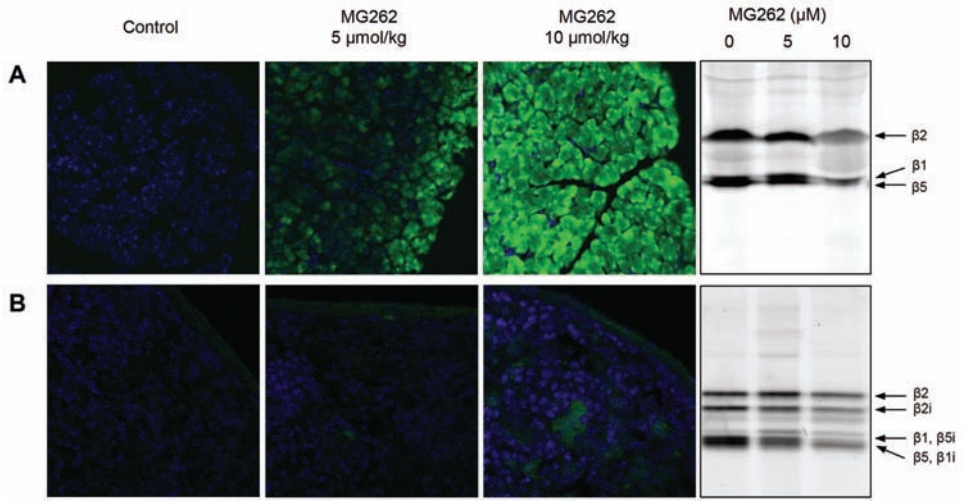


Figure 7. MG262 biodistribution study in Ub^{G76V}-GFP reporter mice.
(**A** and **B**) Ub^{G76V}-GFP reporter mice were treated with 0, 5 or 10 µmol/kg bodyweight MG262 by subcutaneous injection and sacrificed after 24 hr. (**A**) Pancreas and (**B**) spleen were analyzed. Cryosections of fixed (**A**) pancreas and (**B**) spleen were analyzed for accumulation of the reporter by confocal microscopy. GFP in green, Hoechst nuclear stain in blue. Remaining proteasome activity in the (**A**) pancreas and (**B**) spleen tissue homogenates was fluorescently labeled by incubation with 0.1 µM MV151 for 1 hr. at 37 °C.

As the final set of experiments, the biodistribution of MG262 in Ub^{G76V}-GFP transgenic mice was monitored. The boronic acid MG262 was selected for this purpose because it is both commercially available and most closely resembles the drug bortezomib. Animals were injected subcutaneously with either 5 µmol/kg, or 10 µmol/kg body weight of the boronic acid MG262 and were sacrificed 24 hours post-injection. Fluorescence microscopic analysis of spleen and pancreas tissues (Figure 7A and 7B, respectively) showed the concentration-dependent inhibition of the proteasome in MG262-treated Ub^{G76V}-GFP mice, as indicated by increased levels of Ub^{G76V}-GFP reporter. The same tissues were lysed and treated with MV151. SDS-PAGE analysis revealed significant reduction of labeled bands corresponding to the proteasome catalytic subunits when compared with tissue lysates from untreated animals (Figure 7A, pancreas and Figure 7B, spleen).

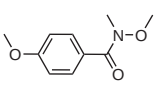
2.3 Conclusion

In summary, the synthesis of MV151 (**3**) as a new fluorescent, cell-permeable proteasome probe is described. MV151 enables broad-spectrum proteasome profiling, both in cell lysates and in living cells. The BODIPY TMR dye proved to be very useful for in-gel readout of labeled active subunits in that it provided a straightforward method for direct and sensitive proteasome profiling and omitted the need for western blotting, radioactivity, and gel drying. MV151 could be readily detected upon administration to mice and correlated with inhibition of the proteasome in the affected tissues. Finally, MV151-mediated proteasome labeling in combination with Ub^{G76V}-GFP transgenic mice is a useful strategy for monitoring the biodistribution of proteasome inhibitors.

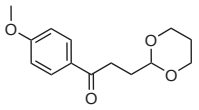
Experimental section

General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.)(purum), ethyl acetate (EtOAc) (puriss.) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, 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 followed by charring at ~150°C or spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on Merck silicagel (0.040 – 0.063 nm). Electrospray Ionization Mass spectra (MS (ESI)) were recorded on a PE/Sciex API 165 instrument interface and HRMS (SIM mode) were recorded on a TSQ Quantum (Thermo Finnigan) fitted with an accurate mass option, interpolating between PEG-calibration peaks. ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), a Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory or a Bruker AV-500 (500/125 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. Optical

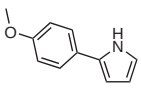
rotations were measured on a Propol automatic polarimeter (Sodium D line, λ = 589 nm) and ATR-IR spectra were recorded on a Shimadzu FTIR-8300 fitted with a single bounce DurasamplIR diamond crystal ATR-element. UV spectra were recorded on a Perkin Elmer, Lambda 800 UV/VIS spectrometer. Carboxyaldehyde pyrrole (**8**),[11] epoxomicin,[21] dansylAhx$_3$L$_3$VS,[7] NIP-LVS,[12] Z-LVS,[12] AdaAhx$_3$L$_3$VS[6] and Boc-L$_3$VS[12] were synthesized as described in literature.



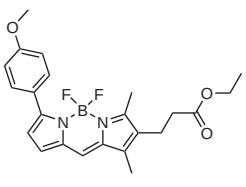
Anisoyl-*N,O*-di-methyl-hydroxamide (5). Anisic acid (**4**) (4.56 g, 30 mmol) was dissolved in DCM before being treated with HCTU (13.65 g, 33 mmol, 1.1 equiv.), *N,O*-dimethylhydroxylamine·HCl (2.93 g, 30 mmol, 1 equiv.) and DiPEA (9.92 ml, 60 mmol, 2 equiv.). The reaction mixture was stirred for 12 hr. before being washed with 1 M HCl (3×) and sat. aq. NaHCO$_3$ (2×). The DCM layer was separated and dried over MgSO$_4$ and concentrated. Purification by column chromatography (Tol. → 40% EtOAc in Tol.) gave 5.22 g (26.7 mmol, 89%) of the title compound. ^{1}H NMR (200 MHz, CDCl$_3$): δ ppm 7.72 (d, *J* = 9.1 Hz, 2H), 6.90 (d, *J* = 9.1 Hz, 2H), 3.83 (s, 3H), 3.55 (s, 3H), 3.35 (s, 3H). ^{13}C NMR (50 MHz, CDCl$_3$): δ ppm 168.41, 160.89, 129.81, 125.53, 112.52, 59.79, 54.33, 32.77. HRMS: calcd. for [C$_{10}$H$_{13}$NO$_3$H]$^+$ 196.09682, found 196.09678.



3-[1,3]Dioxan-2-yl-1-(4-methoxy-phenyl)-propan-1-one (6). Anisoyl-*N,O*-di-methyl-hydroxamide (**5**) (4.7 g, 24 mmol) was dissolved in freshly distilled THF, put under an argon atmosphere and cooled to 0 °C. (1,3-Dioxane-2-ylethyl)-magnesium bromide (72 ml 0.5M in THF, 36 mmol, 1.5 equiv.) was added dropwise over 1 hr. After 3 hr. the reaction mixture was quenched with sat. aq. NH$_4$Cl and extracted with EtOAc. The organic layer was separated, dried over Na$_2$SO$_4$ and concentrated. Column chromatography (Tol. → 20% EtOAc in Tol.) yielded **6** (5.35 g, 21.4 mmol, 89%). ^{1}H NMR (200 MHz, CDCl$_3$): δ ppm 7.96 (d, *J* = 9.1 Hz, 2H), 6.93 (d, *J* = 8.8 Hz, 2H), 4.66 (t, *J* = 5.1 Hz, 1H), 4.10 (dd, J_1 = 4.75, J_2 = 10.6 Hz, 2H), 3.87 (s, 3H), 3.78 (dt, J_1 = 2.56, J_2 = 12.23 Hz, 2H), 3.06 (t, *J* = 7.31 Hz, 2H), 2.06 (m, 3H), 1.33 (d, *J* = 13.16 Hz, 1H). ^{13}C NMR (50 MHz, CDCl$_3$): δ ppm 197.79, 163.06, 129.98, 129.74, 113.33, 100.84, 66.51, 55.11, 31.97, 29.24, 25.48. HRMS: calcd. for [C$_{14}$H$_{18}$O$_4$H]$^+$ 251.12779, found 251.12768.

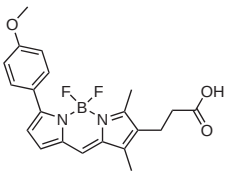


2-(4-Methoxy-phenyl)-1H-pyrrole (7). Ketone **6** (5.1 g, 20.4 mmol) was coevaporated with Tol. (2×), dissolved in glacial acetic acid (100 ml) and put under an argon atmosphere, before NH$_4$OAc (18.8 g, 244.8 mmol, 12 equiv.) and Ac$_2$O (7.14 ml, 75.5 mmol, 3.7 equiv.) were added. The reaction mixture was refluxed for 3 hr., poured into H$_2$O, neutralized with sat. aq. NaHCO$_3$ and extracted with DCM. The DCM layer was separated, dried over Na$_2$SO$_4$ and concentrated. Purification by column chromatography (PetEt → 10% EtOAc in PetEt) gained the title compound **7** (2.46 g, 14.2 mmol, 70%). ^{1}H NMR (200 MHz, CDCl$_3$): δ ppm 9.33 (s, 1H), 7.41 (2H), 6.87 (2H), 6.77 (1H), 6.40 (m, 1H), 6.24 (1H), 3.78 (s, 3H). ^{13}C NMR (50 MHz, CDCl$_3$): δ ppm 157.73, 131.89, 125.98, 124.94, 118.21, 114.03, 109.18, 104.26, 55.04.

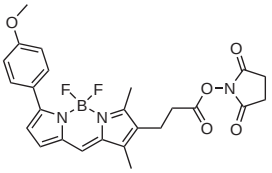


4,4-Difluoro-1,3-dimethyl-2-(2-(ethoxycarbonylethyl))-7-(4-methoxy-phenyl)-4-bora-3a,4a-diaza-s-indacene (9). Methoxyphenyl pyrrole **7** (5.79 g, 33 mmol, 1 equiv.) and carboxyaldehyde pyrrole **8**[11] (7.47 g, 33 mmol, 1 equiv.) were dissolved in EtOH (40 ml). The resulting mixture was cooled to 0 °C, and HBr (5.5 ml 48% solution in water, 50 mmol, 1.5 equiv.) was added. After 1hr. stirring, TLC analysis showed complete consumption of the starting materials. The purple precipitate was collected by filtration. The crude dipyrrole HBr salt was coevaporated with DCE and dissolved in DCE (750 ml) under argon atmosphere. TEA (7.74 ml, 55.5 mmol, 3 equiv.) and BF$_3$·Et$_2$O (11.7 ml, 92.5 mmol, 5 equiv.) were added, and the reaction was subsequently refluxed at 90 °C. After 30 min., the solution was cooled to room temperature, filtered over a plug of basic alumina and concentrated. Silica gel column chromatography (Tol. → 2.5% EtOAc in Tol.) gave

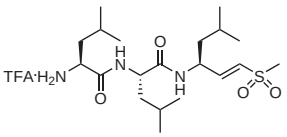
the title compound **9** (5.37 g, 12.6 mmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.87 (d, *J* = 8.8Hz, 2H), 7.06 (s, 1H), 6.96 (d, *J* = 8.8 Hz, 2H), 6.92 (d, *J* = 5.8 Hz, 1H), 6.52 (d, *J* = 4.4 Hz, 1H), 4.11 (q, *J* = 7.3 Hz, 2H), 3.84 (s, 3H), 2.71 (t, *J* = 7.1 Hz, 2H), 2.53 (s, 3H), 2.42 (t, *J* = 7.3 Hz, 2H), 2.18 (s, 3H), 1.23 (t, *J* = 7.3 Hz, 3H) . ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 172.13, 160.09, 158.64, 155.15, 139.53, 134.80, 133.95, 130.40, 129.67, 127.76, 125.16, 122.67, 117.91, 113.42, 60.23, 54.86, 33.67, 19.08, 13.86, 12.77, 9.10. HRMS: calcd.for $[C_{23}H_{25}BF_2N_2O_3H]^+$ 427.19991, found 427.20030.



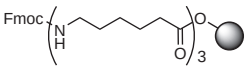
4,4-Difluoro-1,3-dimethyl-2-(2-carboxyethyl)-7-(4-methoxy-phenyl)-4-bora-3a,4a-diaza-s-indacene (10). Ethyl ester **9** (44 mg, 0.1 mmol) was dissolved in dioxane (2 ml). After addition of 0.1M aq. NaOH (1mL, 0.1 mmol, 1 equiv.) and stirring overnight, the purple suspension was diluted with EtOAc, extracted with 1M HCl, dried over $MgSO_4$ and concentrated. Column chromatography (Tol. → 1% EtOAc and 1% AcOH in Tol.) yielded BODIPY acid **10** (15 mg, 40 µmol, 40%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.87 (d, *J* = 8.8 Hz, 2H), 7.03 (s, 1H), 6.95 (d, *J* = 8.8 Hz, 2H), 6.89 (d, *J* = 4.4 Hz, 1H), 6.50 (d, *J* = 3.7 Hz, 1H), 6.24 (1H), 3.81 (s, 3H), 2.68 (t, *J* = 7.3 Hz, 2H), 2.51 (s, 3H), 2.45 (t, *J* = 7.3 Hz, 2H), 2.21 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 178.44, 160.33, 158.76, 155.75, 139.62, 135.04, 134.13, 130.65, 129.40, 128.13, 125.40, 122.91, 118.30, 113.66, 55.17, 33.76, 19.11, 12.98, 9.41. HRMS: calcd. for $[C_{21}H_{21}BF_2N_2O_3H]^+$ 399.16861, found 399.16904, calcd. for $[C_{21}H_{21}BF_2N_2O_3Na]^+$ 421.15055, found 421.15096.



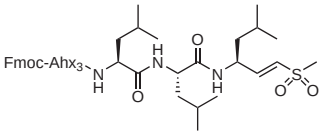
BODIPY TMR-OSu (11). BODIPY TMR-OH (**10**, 28 mg, 70 µmol) was coevaporated thrice with Tol., before being dissolved in DCM (1 ml). After the addition of HOSu (32 mg, 0.28 mmol, 4 equiv.) and EDC·HCl (54 mg, 0.28 mmol, 4 equiv.), the reaction mixture was stirred overnight. Next, the reaction was diluted with EtOAc, washed with 1M aq. HCl, dried over $MgSO_4$ and concentrated. Purification by column chromatography (Tol. → 5% EtOAc in Tol.) furnished title compound **11** (24 mg, 48 µmol, 69%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.88 (d, *J* = 8.8 Hz, 2H), 7.10 (s, 1H), 6.97 (m, 3H), 6.55 (d, *J* = 4.4 Hz, 1H), 3.85 (s, 3H, Me), 2.79 (m, 8H), 2.54 (s, 3H), 2.20 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 168.92, 167.58, 160.49, 158.21, 156.30, 139.62, 135.22, 133.98, 130.74, 128.46, 128.28, 125.31, 123.15, 118.61, 113.72, 55.23, 30.85, 25.51, 19.11, 13.01, 9.56. HRMS: calcd. for $[C_{25}H_{24}BF_2N_3O_5H]^+$ 496.18498, found 496.18453, calcd. for $[C_{25}H_{24}BF_2N_3O_5NH_4]^+$ 513.21153, found 513.21151, calcd. for $[C_{25}H_{24}BF_2N_3O_5Na]^+$ 518.16693, found 518.16706.



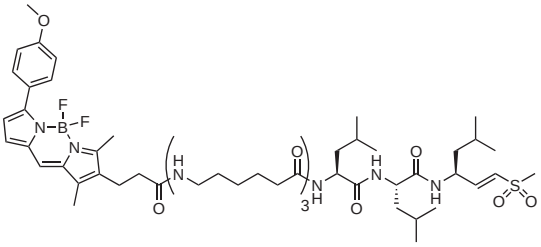
TFA·L$_3$VS (13). Boc-L$_3$VS[12] (**12**) (0.47 g, 0.9 mmol) was dissolved in a mixture of TFA/DCM (1/1) and was stirred for 30 min. The reaction mixture was concentrated *in vacuo* affording the TFA salt **13** as a white solid which was used without further purification.



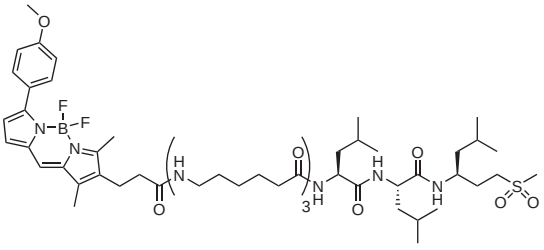
Fmoc-Ahx$_3$-Wang (14). Wang resin (1.8 g, 1.1 mmol/g, 2 mmol) was coevaporated with DCE (2×) and condensed with Fmoc-Ahx-OH (2.1 g, 6 mmol, 3 equiv.) under the influence of DIC (1.0 ml, 6.6 mmol, 3.3 equiv.) and DMAP (12 mg, 0.1 mmol, 0.05 equiv.) for 2hr. The resin was then filtered and washed with DCM (3×) and subjected to a second condensation sequence. The loading of the resin was determined to be 0.8 mmol/g (2.30 g, 1.84 mmol, 92%) by spectrophotometric analysis. The obtained resin was submitted to two cycles of Fmoc solid-phase synthesis with Fmoc-Ahx-OH, as follows: a) deprotection with piperidine/NMP (1/4, v/v, 15 min.); b) wash with NMP (3×); c) coupling of Fmoc-Ahx-OH (1.63 g, 4.6 mmol, 2.5 equiv.) in the presence of BOP (2.0 g, 4.6 mmol, 2.5 equiv.) and DiPEA (0.91 ml, 5.5 mmol, 3 equiv.) in NMP and shake for 2hr.; d) wash with NMP (3×) and DCM (3×). Couplings were monitored for completion by the Kaiser test.[22]



Fmoc-Ahx$_3$L$_3$VS (15). The tripeptide Fmoc-Ahx$_3$-OH was released from resin **14** (0.45 mmol) by treatment with TFA/DCM (1/1, v/v, 30 min., 3×). The fractions were collected and coevaporated with Tol. (3×). The crude Fmoc-Ahx$_3$-OH was dissolved in DCM/DMF (99/1, v/v) and condensed with the crude TFA·L$_3$VS (**13**, 0.9 mmol, 2 equiv.) under the influence of BOP (0.49 g, 1.13 mmol, 2.5 equiv.) and DiPEA (0.45 ml, 2.7 mmol, 6 equiv.). The reaction mixture was stirred overnight, before being concentrated *in vacuo*. The residue was dissolved in chloroform and washed with 1M HCl and sat. aq. NaHCO$_3$. The organic layer was dried over MgSO$_4$ and concentrated. Silica column chromatography (DCM → 4% MeOH in DCM) yielded the title compound **15** (0.41 g, 0.44 mmol, 98%). ^{1}H NMR (500 MHz, CDCl$_3$/MeOD): δ ppm 7.77 (d, *J* = 7.5 Hz, 2H), 7.61 (d, *J* = 7.5 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 6.82 (dd, J_1 = 15, J_2 = 5.0 Hz, 1H), 6.52 (d, *J* = 15 Hz, 1H), 4.68 (m, 1H), 4.36 (m, 4H), 4.21 (t, *J* = 6.8 Hz, 1H), 3.16 (m, 6H), 2.93 (s, 3H), 2.26-2.14 (m, 6H), 1.62-1.40 (m, 21H), 0.95-0.89 (m, 18H). ^{13}C NMR (125 MHz, CDCl$_3$): δ ppm 174.15, 173.95, 173.87, 173.04, 172.96, 171.98, 171.90, 156.82, 156.78, 147.50, 143.66, 141.00, 128.84, 127.42, 124.74, 124.58, 119.67, 66.25, 51.82, 51.75, 47.62, 47.52, 46.95, 42.35, 42.15, 40.41, 40.28, 39.95, 38.88, 38.75, 35.92, 35.81, 35.49, 29.09, 28.59, 26.02, 25.94, 25.88, 25.06, 24.96, 24.84, 24.54, 24.50, 25.44, 22.52, 22.50, 22.48, 21.46, 21.36. HRMS: calcd. for [C$_{53}$H$_{82}$N$_6$O$_9$SH]$^+$ 979.59368, found 979.59276.



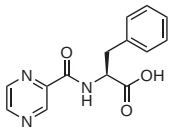
BODIPY TMR-Ahx$_3$L$_3$VS (3). DBU (30 µl, 0.2 mmol, 1 equiv.) was added to a solution of **15** (0.2 g, 0.2 mmol) in DMF. After 5 min. of stirring, HOBt (0.12 g, 0.9 mmol, 4.5 equiv.) was added. To this mixture, BODIPY TMR-OSu (**11**, 0.1 g, 0.2 mmol, 1 equiv.) and DiPEA (0.2 ml, 1.2 mmol, 6 equiv.) were added, and the mixture was stirred for 30 min., before being concentrated *in vacuo*. Purification by column chromatography (0.1% TEA in DCM → 3% MeOH and 0.1% TEA in DCM) afforded BODIPY TMR-Ahx$_3$L$_3$VS (**3**) (0.22 g, 197 µmol, 99%). ^{1}H NMR (500 MHz, CDCl$_3$): δ ppm 7.87 (d, *J* = 8.5 Hz, 2H), 7.52 (s, 1H), 7.51 (s, 1H), 7.35 (d, *J* = 8 Hz, 1H), 6.97 (m, 5H), 6.81 (dd, J_1 = 15, J_2 = 5 Hz, 1H), 6.79 (s, 1H), 6.55 (d, *J* = 4 Hz, 1H), 6.51 (d, *J* = 15 Hz, 1H), 4.67 (m, 1H), 4.33 (m, 2H), 3.86 (s, 3H), 3.17 – 3.10 (m, 6H), 2.96 (s, 3H), 2.74 (t, *J* = 7.3 Hz, 2H), 2.53 (s, 3H), 2.30 (t, *J* = 7.3 Hz, 2H), 2.21 (m, 5H), 2.14 (t, *J* = 7.3 Hz, 2H), 2.08 (t, *J* = 7.3 Hz, 2H), 1.66-1.17 (m, 27H), 0.95-0.89 (m, 18H). ^{13}C NMR (125 MHz, CDCl$_3$): δ ppm 174.40, 174.01, 173.94, 173.20, 172.53, 172.45, 172.06, 160.16, 159.44, 155.16, 147.61, 140.04, 134.77, 134.23, 130.46, 128.87, 128.30, 127.81, 125.29, 122.72, 118.10, 113.55, 55.12, 52.11, 52.04, 47.72, 45.98, 42.46, 42.26, 40.33, 40.00, 39.08, 38.94, 38.80, 35.93, 35.74, 35.62, 28.63, 28.49, 26.04, 25.99, 25.86, 25.03, 24.96, 24.88, 24.63, 24.60, 24.55, 22.66, 22.62, 22.58, 21.54, 21.48, 21.43, 20.09, 12.84, 9.27, 8.36. HRMS: calcd. for [C$_{59}$H$_{91}$BF$_2$N$_8$O$_9$SH]$^+$ 1137.67636, found 1137.67442, for [C$_{59}$H$_{91}$BF$_2$N$_8$O$_9$SNH$_4$]$^+$ 1154.70291, found 1154.70149, for [C$_{59}$H$_{91}$BF$_2$N$_8$O$_9$SNa]$^+$ 1159.65831, found 1159.65690. $[\alpha]_D^{23}$ = -44 (c 0.1, MeOH). λ_{max} (MeOH): 544.43 nm, ε: 60400 l mol^{-1}cm^{-1}.



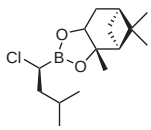
BODIPY TMR-Ahx$_3$L$_3$ES (16). A catalytic amount of 10% Pd/C was added to a solution of **15** (49 mg, 50 µmol) in MeOH. Hydrogen gas was bubbled through the solution for 2 hr., after which the catalyst was filtered of and the reaction mixture was concentrated *in vacuo*. The residue was dissolved in DMF and treated with DBU (7.5 µL, 50 µmol, 1 equiv.) for 5 min., before HOBt (30 mg, 0.23 mmol, 4.5 equiv.) was added. To this mixture, BODIPY TMR-OSu (**11**, 25 mg, 50 µmol, 1 equiv.) and DiPEA (50

µL, 0.3 mol, 6 equiv.) were added, and the mixture was stirred for 30 min., before being concentrated *in vacuo*. Purification by column chromatography (0.1% TEA in DCM → 3% MeOH and 0.1% TEA in DCM) yielded BODIPY TMR-Ahx$_3$L$_3$ES (**16**, 50.3 mg, 44 µmol, 88 %). ^{1}H NMR (500 MHz, $CDCl_3$/MeOD): δ ppm 7.78 (d, *J* = 8.5 Hz, 2H), 7.41 (m, 1H), 7.18 (m, 1H), 7.08 (m, 3H), 6.80 (m, 3H), 6.47 (d, *J* = 4.0 Hz, 1H), 4.18 (m, 2H), 3.92 (m, 1H), 3.78 (s, 3H), 3.07-2.99 (m, 6H), 2.87 (s, 3H), 2.65 (t, *J* = 7.5 Hz, 2H), 2.44 (s, 3H), 2.22 (t, *J* = 7.5 Hz, 2H), 2.14 (m, 5H), 2.06 (t, *J* = 7.3 Hz, 2H), 1.99 (t, *J* = 7.5 Hz, 2H), 1.95 (m, 1H), 1.77 (m, 1H), 1.56-1.11 (m, 27H), 0.88-0.78 (m, 18H). ^{13}C NMR (125 MHz, $CDCl_3$): δ ppm 174.57, 174.05, 173.14, 172.56, 172.39, 172.31, 160.10, 159.38, 155.08, 140.01, 134.72, 134.18, 130.39, 128.76, 127.96, 127.76, 125.23, 122.70, 117.34, 55.03, 52.26, 52.15, 51.28, 46.03, 45.95, 43.64, 40.17, 40.11, 39.90, 38.91, 38.78, 35.83, 35.77, 35.68, 35.45, 28.56, 28.41, 27.87, 26.02, 25.96, 25.84, 25.02, 24.96, 24.83, 24.63, 24.57, 24.53, 22.72, 22.52, 21.51, 21.37, 21.23. HRMS: calcd. for $[C_{59}H_{93}BF_2N_8O_9SH]^+$ 1139.69201, found 1139.69203.

Synthesis of dipeptidyl pinanediol boronic ester (Figure 4, entry C).

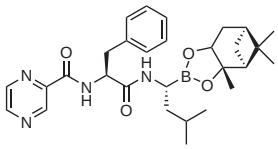


3-Phenyl-2-[(pyrazine-2-carbonyl)-amino]-propionic acid (Pyr-Phe-OH). Oxalylchloride (6.0 ml, 70 mmol, 1.9 equiv.) and 6 drops op DMF were added to a suspension of pyrazinecarboxylic acid HCl salt (5.26 g, 42.2 mmol, 1.7 equiv.) in Et_2O at 0 °C and stirred for 2 days, before being concentrated. The resulting purple residue was resuspended in Et_2O and cooled to 0 °C, before L-phenylalanine methyl ester (7.95 g, 36.8 mmol) was added. TEA (25 ml, 179 mmol, 4.9 equiv.) was added dropwise over 10 min. The reaction mixture was stirred for 12 hr. before being poured into H_2O and extracted with EtOAc (3 × 100 ml). The combined organic layers were dried over $MgSO_4$ and concentrated, giving a red oil. Purification by column chromatography (50% EtOAc in PetEt) gave 8.93 g (31.2 mmol, 85%) Pyr-Phe-OMe. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 9.37 (s, 1H), 8.75 (d, *J* = 2.2 Hz, 2H), 8.52 (d, *J* = 2.2 Hz, 1H), 8.22 (d, *J* = 8.0 Hz, 1H), 7.22 (m, 5H), 5.08 (m, 1H), 3.75 (s, 3H), 3.27 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 170.8, 162.0, 146.8, 143.5, 143.2, 142.1, 135.4, 128.5, 127.8, 126.3, 52.7, 51.6, 37.1. $[\alpha]_D^{23}$ = + 64.3 (c 1.25, DCM). To a solution of Pyr-Phe-OMe (8.40 g, 29.5 mmol) in dioxane/MeOH (5/2, v/v), NaOH (9.5 ml 4M in H_2O, 38 mmol, 1.3 equiv.) was added and the reaction mixture was stirred for 2 hr. 1M $NaHSO_4$ (36 ml) was added and the mixture was concentrated *in vacuo*. The residue was taken up in EtOAc, washed with brine, dried over $MgSO_4$ and concentrated to yield Pyr-Phe-OH as a white solid (7.88 g, 29.2 mmol, 99%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 9.40 (s, 1H), 9.37 (s, 1H), 8.76 (s, 1H), 8.54 (s, 1H), 8.21 (d, *J* = 8.0 Hz, 1H), 7.25 (m, 5H), 5.13 (m, 1H), 3.31 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 174.0, 164.7, 148.7, 145.6, 144.7, 144.6, 137.9, 130.3, 129.5, 127.9, 54.9, 38.2. MS (ESI): *m/z* 272.0 $[M+H]^+$. $[\alpha]_D^{23}$ = + 29.4 (c 1.0, EtOH).



(1*R*)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane. (2-methyl)-propylboronic acid (2.22 g, 21.8 mmol) was dissolved in THF and (+)-(1*S*,2*S*,3*R*,5*S*)-pinanediol was added. The reaction mixture was stirred overnight and concentrated *in vacuo.* Purification by column chromatography (3% EtOAc in PetEt) gave 4.54 g (20.3 mmol, 93%) of 4-isobutyl-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 4.25 (dd, J_1 = 8.8, J_2 = 2.2 Hz, 1H), 2.41-2.17 (m, 2H), 2.05 (m, 1H), 1.95-1.79 (m, 3H), 1.38 (s, 3H), 1.29 (s, 3H), 1.14 (d, *J* = 11 Hz, 1H), 0.94 (d, *J* = 7.3 Hz, 6H), 0.84 (s, 3H), 0.78 (d, *J* = 7.3 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 85.1, 77.4, 51.2, 39.5, 38.0, 35.5, 28.7, 27.0, 26.5, 25.2, 24.8, 24.0. $[\alpha]_D^{23}$ = 28.4 (c 1.0, DCM). *n*BuLi (7.0 ml 1.6M in hexane, 11.2 mmol, 1.12 equiv.) was added to a solution of DCM (0.90 ml, 14.0 mmol, 1.4 equiv.) in THF (40 ml) at -80°C and stirred for 1 hr. To the stirred reaction mixture a solution of 4-isobutyl-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane (2.36 g, 10.0 mmol) in THF (40 ml) was added and stirred for 1 hr. at -80°C before a solution of $ZnCl_2$ (20 ml 1M in diethyl ether, 20 mmol, 10 equiv.) was added dropwise over 10 min. The reaction mixture was allowed to warm up to -40°C in 2 hr., before it was allowed to warm up to room temperature and stirred for 2 hr. The resulting

reaction mixture was poured into 250 ml H_2O and extracted with EtOAc (3 × 100 ml). The combined organic layers were dried over $MgSO_4$ and concentrated. Purification by column chromatography (2% EtOAc in PetEt) gave the title compound as a colorless oil (2.68 g, 0.94 mmol, 94%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 4.36 (dd, J_1 = 9.1, J_2 = 1.8 Hz, 1H), 3.54 (dd, J = 9.9, J = 5.9 Hz, 1H), 2.44-2.18 (m, 2H), 2.09 (m, 1H), 1.99-1.73 (m, 4H), 1.68-1.55 (m, 1H), 1.42 (s, 1H), 1.30 (s, 1H), 1.19 (d, J = 10.9 Hz, 1H), 0.92 (m, 6H), 0.85 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 86.2, 78.1, 50.9, 42.5, 39.1, 37.9, 35.0, 28.2, 26.8, 26.0, 25.3, 23.7, 22.7, 21.0. $[\alpha]_D^{23}$ = + 44.2 (c 1.0, DCM).



Pyrazine-2-carboxylic acid (1-[3-methyl-1-(2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]dec-4-yl)-butylcarbamoyl]-2-phenyl-ethyl)-amide.

DIC (175 µl, 1.13 mmol, 1.3 equiv.) was added to a solution of Pyr-Phe-OH (239 mg, 0.88 mmol) and HOSu (129 mg, 1.12 mmol) in DCM (10 ml) and the reaction mixture was stirred for 12 hr., yielding the crude Pyr-Phe-OSu solution. Lithium bis(trimethylsilyl)amide (0.75 ml 1M in THF, 0.75 mmol, 1.3 equiv.) was added to a solution of (1*R*)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane (165 mg, 0.58 mmol) in THF (6 ml) at -80 °C. The reaction mixture was allowed to warm up to room temperature and was stirred for 12 hr. before it was cooled to -80 °C. HCl (2 ml 2M in diethyl ether) was added and the reaction mixture was allowed to warm to 5 °C, before being cooled to -80 °C. To the stirred solution DiPEA (1.2 ml, 7.3 mmol, 12.6 equiv.) and the crude Pyr-Phe-OSu solution (0.88 mmol, 1.5 equiv.) were added and the reaction mixture was allowed to warm up to room temperature. The reaction mixture was stirred for an additional 2 hr. before being filtered over hyflo and the filtrate was concentrated. Purification of the residue by column chromatography (10% EtOAc in PetEt → 50% EtOAc in PetEt) gave 185 mg (0.36 mmol, 62%) of the title compound. ^{1}H NMR (400 MHz, MeOD): δ ppm 9.1 (d, J = 1.4 Hz, 1H), 8.76 (d, J = 2.4 Hz, 1H), 8.64 (d, J = 2.4 Hz, 1H), 7.18-7.30 (m, 5H), 5.04 (m, 1H), 4.23 (m, 1H), 3.78 (m, 2H), 3.20 (m, 2H), 2.74 (m, 1H), 2.33 (m, 1H), 2.13 (m, 1H), 1.95 (m, 1H), 1.82 (m, 2H), 1.54 (m, 1H), 1.36 (s, 3H), 1.27 (s, 3H), 1.19 (d, J = 11 Hz, 1H), 1.08 (d, J = 6.6 Hz, 6H), 0.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD): δ ppm 171.9, 162.4, 146.9, 143.5, 143.3, 142.6, 135.4, 128.9, 127.9, 126.4, 84.1, 76.5, 52.2, 51.2, 40.9, 39.4, 39.2, 38.0, 37.5, 35.4, 28.1, 26.5, 25.8, 24.7, 23.4, 22.6, 21.1. MS (ESI): *m/z* 519.4 $[M+H]^+$, 367.1 [M-pinanediol-H_2O+H]$^+$.

Proteasomal activity measurement using fluorogenic substrates

Protein lysates from EL-4 (1 mg/ml) were incubated with various concentrations of MV151 (**3**) for 1 hr. at 37°C. For measurement of proteasomal activities, 10 µg of the labeled lysate were added to 100 µl of substrate buffer, containing 20 mM HEPES, pH 8.2, 0.5 mM EDTA, 1% DMSO, 1 mM ATP and 10 µM Z-Ala-Arg-Arg-AMC (trypsin-like), 60 µM Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like) or 60 µM Z-Leu-Leu-Glu-βNA (caspase-like). Fluorescence was measured every min. for 25 min. at 37°C using a Fluostar Optima 96 well plate reader (BMG Labtechnologies) ($\lambda_{ex}/\lambda_{em}$ = 355/450 nm for AMC and 320/405 nm for βNA) and the maximum increase in fluorescence per min. was used to calculate specific activities of each sample. Nonspecific hydrolysis was assessed by preincubation with 1 µM epoxomicin for 1 hr. at 37°C and was subtracted from each measurement.

In-gel detection of labeled proteasome subunits

Whole cell lysates were made in 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 colorimetric Bradford method. For the labeling reactions, 10 µg of total protein lysates were incubated for 1 hr. at 37°C with increasing concentrations of MV151 in a total reaction volume of 10 µl. Where indicated, 50 ng purified 20S proteasome (BioMol) was used. For competition studies, cell lysates (10µg) were exposed to the known inhibitors for 1hr

prior to incubation with MV151 (0.1 μM) for 1 hr. at 37°C. For assessment of background labeling, heat inactivated lysates (10μg, boiled for 3 min with 1% SDS) were treated with MV151. Reaction mixtures were boiled with Laemmli's buffer containing β-mercapto-ethanol for 3 min and resolved on 12.5% SDS-PAGE. In-gel visualization was performed in the wet gel slabs directly using the Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560) on the Typhoon Variable Mode Imager (Amersham Biosciences). Labeling profiles in living cells were determined by incubating approximately $1{\cdot}10^6$ cells with 1 to 10 μM MV151 in culture medium at 37°C for 8 hr. Cells were lysed followed by in gel detection as described above.

Cell culture

The human cervical epithelial carcinoma cell line HeLa, the human melanoma cell line MelJuSo and the murine lymphoid cell line EL-4 were cultured in RPMI medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich), 10 units/ml penicillin and 10 μg/ml streptomycin (Sigma-Aldrich).

Microscopy

Some $5{\cdot}10^5$ cells were seeded on 35 mm petridishes with 14 mm microwell nr 1.5 coverslips, glassbottom microwell dishes (MatTek Corp, Ashland, MA, USA) and allowed to attach overnight. Cells were visualized with a 60× oil immersion lens (Nikon) on a Nikon Eclipse TE 2000U microscope equipped with Radiance 2100 MP integrated laser and detection system (BioRad) and a Tsunami Multiphoton laser module (Spectra Physics). LaserSharp 2K (BioRad) software was used for microscope control and data acquisition and Image Pro 3DS 5.1 (Media Cybernetics Inc) software for image processing. GFP was excited at λ_{ex} 488 nm and detected at 500-530 nm. MV151 and MV152 were excited at λ_{ex} 543 nm and detected at 560-620 nm. CLSM images were adjusted for brightness and contrast using Photoshop software.

Mouse experiments

All animal experiments were approved by the Ethical Committee in Stockholm (Ethical permission number N-46/04 and N18/05). Mice were housed according to Swedish animal care protocols with a 12 hr. day/ light cycle, and fed standard laboratory chow and tap water ad libitum. Adult C57Bl/6 and $Ub^{G76V}GFP/1$ mice,[16] matched for sex and age were given a single intraperitoneal injection of vehicle (60% DMSO, 40% PBS), MV151 (20 μmol/Kg body weight), or MG262 (Affiniti) (5 or 10μmol/kg body weight) in a total volume of 200 μl. Based on prior experience in our lab, the boronic acid inhibitors proved to be more potent and show better tissue penetration *in vivo* compared to the vinyl sulfone inhibitors. Therefore, the 20 μmol/kg bodyweight dose was chosen for MV151, which showed no apparent toxicity in mice. Mice were euthanized 24h after injection by anesthetization with inhaled isoflurane (4.4% in oxygen) followed by transcardial perfusion with 50ml PBS for removal of contaminating blood. Tissues collected for immunocytochemical analysis were processed as described previously.[16] Briefly, 12μm cryosections were fixed for 15 min. in 4% paraformaldehyde/PBS, washed in PBS, and where mentioned, Hoechst nuclear stain (2μg/ml in H_2O), was applied for 15 min. in the dark followed by washing in PBS. Sections were mounted in a matrix containing 2.5% DABCO (Aldrich). Confocal microscopy was performed on a Zeiss LSM 510 META system. Tissues isolated for in gel analysis were lysed with a Heidolph tissue homogenizer in 300μl lysis buffer and further treated as described above.

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3

Acetylene functionalized BODIPY dyes and their application in the synthesis of activity based proteasome probes.

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3.1 Introduction

The ease of detection by direct in-gel readout and visualization in living cells make fluorophores powerful tools in activity-based proteomics. Tagging a probe with a fluorescent dye enables rapid assessment of the labeling profile, and in certain examples allows monitoring of intracellular localization of the target proteins. Tagging can be achieved by connecting the dye to the activity-based probe directly[1,2] or in a two-step labeling fashion.[3] Boradiazaindacenes (BODIPY dyes)[4] are fluorescent dyes with attractive properties, such as their photochemical stability, high molar absorptivity, high fluorescence quantum yield and the fact that their fluorescence properties can be altered by varying the substitution pattern on the core and the flanking pyrroles. Symmetrical BODIPY dyes are relatively easy to access by condensation of an acid chloride with two equivalents of any given 1*H*-pyrrole.[5] Condensing a variety of pyrroles with 6-heptynoic acid chloride would lead to a panel of optical diverse fluorescent dyes bearing a terminal alkyne. These dyes can be used to fluorescently label an azido functionalized inhibitor in a chemoselective manner employing the copper (I) catalyzed Huisgen 1,3-dipolar cycloaddition ('click' reaction),[6] gaining potential activity-based profiling probes.

In this Chapter the synthesis of three alkyne functionalized BODIPY dyes with different optical properties is described. Using these newly developed dyes, a set of fluorescently labeled epoxomicin analogues is prepared. The natural product epoxomicin (Figure 1) is a potent and selective proteasome inhibitor.[7] The selectivity of epoxomicin for the proteasome is governed by the α′,β′-epoxyketone warhead, which, upon reaction with the *N*-terminal threonine of the catalytically active β-

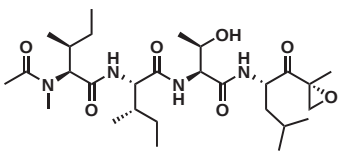


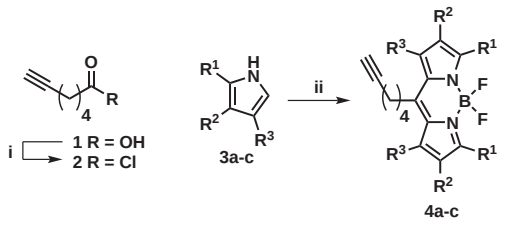
Figure 1. Epoxomicin

subunits of the proteasome, forms a very stable morpholine ring (see Chapter 1.4 for a more detailed description).[8] In Chapter 2 it is shown that the fluorescent proteasome probe MV151 is a powerful tool in proteasome research.[2] Because of the potency and selectivity of epoxomicin, fluorescently labeled analogues thereof could be very useful proteasome probes. Where the synthesis of the fluorophore BODIPY TMR, which is present in MV151, is rather laborious, the preparation of the acetylene functionalized BODIPY dyes described in this chapter is very straightforward.

3.2 Results and discussion

The synthesis of the alkyne functionalized BODIPY dyes commenced with the treatment of 6-heptynoic acid (**1**) with oxalyl chloride (Scheme 1). Crude acid chloride **2** was condensed with either 2-(4-methoxyphenyl)-1*H*-pyrrole (**3a**), 3-ethyl-2,4-dimethyl-1*H*-pyrrole (**3b**) or 2,4-dimethyl-1*H*-pyrrole (**3c**), followed by treatment with DiPEA and $BF_3{\cdot}OEt_2$, giving BODIPY dyes **4a** (pink), **4b** (orange) and **4c** (green). The extinction coefficients ε of the three new dyes were determined to be in the range of that reported for commercial BODIPY-TMR (around 60 000, see Scheme 1).

Scheme 1. Synthesis and spectroscopic data of acetylene functionalized BODIPY dyes.

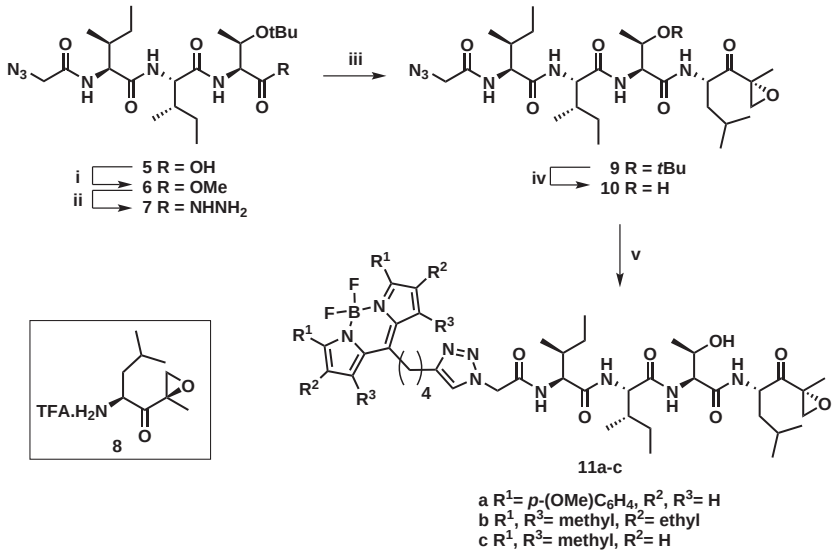


4	R^1	R^2	R^3	λ_{abs} (nm)	λ_{em} (nm)	stokes shift (nm)	ε (l/mol cm)
a	p-(OMe)C_6H_4	H	H	573	607	34	51 097
b	methyl	ethyl	methyl	519	529	10	66 807
c	methyl	H	methyl	496	503	7	84 161

Reagents and conditions: i) oxalyl chloride (1.5 equiv.), DMF (cat.), Tol., 3 hr. ii) (a) 1M **2** in DCE, **3a-c** (2.1 equiv.), 2 hr. 65 °C. (b) $BF_3{\cdot}OEt_2$ (5 equiv.), DiPEA (4 equiv.), **4a** 21%, **4b** 14%, **4c** 26%.

Next, the azido functionalized epoxomicin analogue "azidomicin" (**10**, Scheme 2) was synthesized. Tripeptide **5** capped with an *N*-terminal azidoacetyl moiety was prepared by standard Fmoc-based solid phase peptide synthesis. Treatment of **5** with TMS-diazomethane gave methyl ester **6**, which in turn was transformed into hydrazide **7** upon treatment with excess hydrazine in refluxing methanol. *In situ* generation of the acyl azide using *t*BuONO under acidic conditions and subsequent neutralization with DiPEA and treatment with leucine epoxyketone **8** gave protected epoxomicin analogue **9** as a single

Scheme 2. Synthesis of fluorescent epoxomicin analogues **11a-c**.



Reagents and conditions: i) TMS-diazomethane (2 equiv.), MeOH/Tol. (1/1), 15 min., 97%. ii) hydrazine monohydrate (60 equiv.), MeOH, reflux, 37%. iii) (a) *t*BuONO, HCl, dioxane/DMF. (b) DiPEA, **8**, 11%. iv) TFA, 30 min., quant. v) **4a-c**, $CuSO_4$ (10 mol%), sodium ascorbate (15 mol%), *t*BuOH/Tol./H_2O (1/1/1), 80 °C, 12hr., **11a** 91%, **11b** 82%, **11c** 65% (2 steps from **9**).

diastereomer.[9] Acidic deprotection of the threonine γ-hydroxyl gave azidomicin **10**. Conjugation of azidomicin was executed with the alkyne functionalized BODIPY dyes under the influence of $CuSO_4$ and sodium ascorbate resulting in the three fluorescent peptide epoxyketones **11a**, **11b** and **11c** in 91%, 82% an 65% yield, respectively.

Being a potential two-step labeling activity-based probe itself, the proteasome inhibitory potential of azidomicin (**10**) was assessed in a competition experiment versus MV151[2] (Figure 2A). Lysates of the murine EL-4 cell line (expressing both the constitutive proteasome and the immunoproteasome) were incubated with increasing concentrations of **10** for 1 hour, before the residual proteasome activities were labeled with MV151. The proteins were denatured, separated on SDS-PAGE and the resulting gel was scanned on a fluorescence scanner. Azidomicin proved to be a very effective proteasome inhibitor, with potency in the same order of magnitude as its parent compound epoxomicin.[2] Having synthesized three fluorescently labeled epoxomicin analogues, their labeling profile was determined in the following experiment. EL-4 cell lysates were incubated with increasing concentrations of **11a-c** at 37 °C. After 1 hour, the proteins were denatured and separated on SDS-PAGE, and the wet gel slabs were scanned on a fluorescence scanner. All three fluorescent epoxomicin analogues revealed bands of labeled proteins, the molecular weight of which correspond to the proteolytically active proteasomal β-subunits (Figure 2B).[2] Although there are no significant differences in potency, **11a** seems to be the least powerful

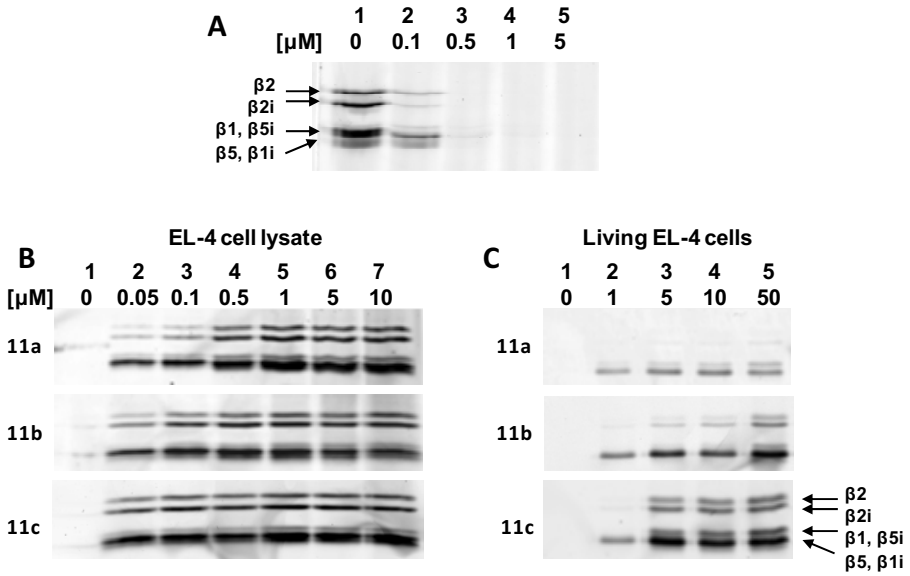


Figure 2. Competition study azidomicin and labeling profile of **11a-c.**
(**A**) EL-4 lysates (10 μg total protein) were incubated with the indicated concentrations of azidomicin (**10**) for 1 hr. at 37 °C. Residual proteasome activity was fluorescently labeled by incubation with 1 μM MV151 for 1 hr. at 37 °C. (**B** and **C**) In-gel readout of fluorescently labeled proteins by **11a-c** in (**B**) EL4 lysates and (**C**) living EL4 cells. (**B**) EL4 lysates (10μg total protein) were incubated with the indicated concentrations of **11a**, **11b** or **11c** for 1 hr. at 37 °C and resolved by SDS-PAGE. (**C**) Some $1{\cdot}10^6$ EL-4 cells were exposed to the indicated concentrations of **11a, 11b** or **11c** for 2 hr. at 37 °C. The cells were harvested, washed and lysed and the proteins were resolved by SDS-PAGE.

probe of the three. It should be noted that the efficiency of proteasome labeling is the combined result of binding affinity and the extinction coefficient of the probes, and proteasome probe **11a** contains the BODIPY dye that has the lowest ε value.

The cell permeability of the three probes was scrutinized by exposing living EL4 cells to increasing concentrations of **11a-c** for two hours. After washing, the cells were lysed, the proteins denatured and separated on SDS-PAGE. The scans revealed a marked difference in cell permeability between the three probes (Figure 2C). Probe **11b** seems to be better cell permeable than **11a**, which is carrying the biggest and most hydrophobic BODIPY dye of the three. This characteristic leads to a greater affinity of the probe for the cell membrane, resulting in a slower distribution throughout the cytosol and the nucleus. The smallest and least hydrophobic epoxomicin analogue **11c** appears to be the most efficient in crossing the cell membrane and labeling the active proteasome subunits in living cells.

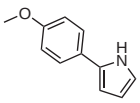
3.3 Conclusion

In conclusion, three acetylene functionalized BODIPY dyes **4a** (pink), **4b** (orange) and **4c** (green) have been synthesized and used in the synthesis of three fluorescently labeled epoxomicin analogues **11a-c**. The synthetic intermediate azidomicin (**10**) proved to be a very potent proteasome inhibitor, making this compound a potential activity-based

two-step labeling proteasome probe. This work results in the addition of three more fluorescently labeled proteasome probes to the proteasome profiling toolkit.[2,7,8,10,11] Apart from this, the alkyne functionalized BODIPY dyes can be conjugated any azide containing activity-based profiling probe and azido modified metabolite, potentially leading to valuable fluorescent biochemical tools.[12] Varying the substitution pattern on the core and the flanking pyrroles of the BODIPY not only changes the fluorescence properties of the dye, but also has a dramatic effect on the bioavailability of the fluorophore.

Experimental section

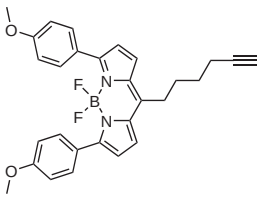
General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.)(purum), ethyl acetate (EtOAc) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, 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 followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on Screening Devices silica gel (0.040 – 0.063 nm). LC/MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.6 mm column (Phenomenex, Torrance, CA, USA) (detection at 200-600 nm), coupled to a Thermo Finnigan LCQ Advantage Max mass spectrometer (Breda, The Netherlands) with electrospray ionization (ESI; system 1). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. 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 = 60000 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). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), Bruker DPX-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory or a Bruker DMX-600 (600/150 MHz) with a cryoprobe. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. UV spectra were recorded on a Perkin Elmer, Lambda 800 UV/VIS spectrometer. Azidoacetic acid[13] and *tert*-butyl (S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylcarbamate[7b] were synthesised as described in literature.



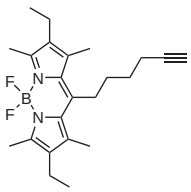
2-(4-methoxyphenyl)-1*H*-pyrrole (3a).[14] Pyrrole (3.50 ml, 50 mmol, 1.6 equiv.) was slowly added under an argon atmosphere to a cooled (0 °C) suspension of sodium hydride (2.0 g of a 60% dispersion in mineral oil, 50 mmol, 1.6 equiv.) in anhydrous THF (20 ml) and stirring was continued for 30 min. at room temperature. After cooling to 0 °C, a solution of $ZnCl_2$ (6.81 g, 50 mmol, 1.6 equiv.) in anhydrous THF (100 ml, 0.5M) was added *via canula*. After stirring for 10 min. at room temperature, di-*tert*-butyl-*o*-biphenylphosphine (75 mg, 0.25 mmol, 0.5 mol %) and $Pd(OAc)_2$ (56 mg, 0.25 mmol, 0.5 mol%) were added in one portion and the resulting mixture was degassed with Argon for 30 min. *p*-Bromoanisol

(3.50 ml, 31.25 mmol, 1 equiv.) was subsequently added dropwise to the reaction mixture and the resulting brown solution was stirred overnight at 65 °C. After cooling to room temperature, water (200 ml) and Et_2O (200 ml) were added and stirring was continued for 15 min., followed by filtration over celite. The filter cake was repeatedly washed with Et_2O (5 x 50 ml) and the filtrate was transferred to a separatory funnel. The organic layer was separated and the aqueous layer was extracted with Et_2O (2 x 50 ml). The combined organics was washed with brine (100 ml), dried over $MgSO_4$and concentrated under reduced pressure to afforded a yellow-brown solid which was purified by column chromatography (50 % PetEt in Tol → Tol) giving the title compound as a white solid (2.82 g, 16.3 mmol, 52%), which rapidly turned bluish upon exposure to air. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 8.57 (s, 1 H), 7.41 (d, *J* = 8.4 Hz, 2 H), 6.91 (d, *J* = 8.8 Hz, 2 H), 1.54 (s, 3 H); ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 156.76, 131.81, 125.96, 124.84, 118.16, 113.92, 108.95, 104.06, 54.94.

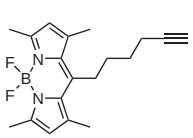
General procedure for the synthesis of BODIPY-alkyne (4a-c). Oxalylchloride (1.5 equiv.) was added dropwise to a solution of hept-6-ynoic acid (**1**, 1 equiv.) in anhydrous Tol. (10 ml). A catalytic amount of DMF (2 drops) was added and the solution was stirred at room temperature for 3 hr. After concentration *in vacuo*, the residue was co-evaporated with Tol. (2x) and the resulting crude hept-6-ynoyl chloride (**2**, yellow oil) was used without further purification. A 1M solution of hept-6-ynoyl chloride (**2**) in DCE was made and 1*H*-pyrrole (**3a-c**, 2.1 equiv.) was added. The resulting reaction mixture was stirred at 65 °C for 2 hr. After leaving to cool to room temperature, $BF_3.OEt_2$ (5 equiv.) was added over 5 min., followed by the dropwise addition of DiPEA (4 equiv.). Argon gas was then bubbled through the solution, and the reaction mixture was stirred for 12 hr. at ambient temperature, before being washed with H_2O and extracted with EtOAc. The organic layer was dried over Na_2SO_4 and the filtrate was immobilized on celite. Purification by flash chromatography afforded the crude product, which was then crystallized from Tol./heptane.



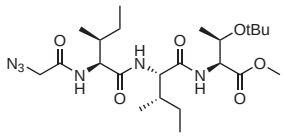
4,4-difluoro-8-(hept-6-yne)-3,5-di(2-(4-methoxyphenyl))-4-bora-3a,4a-diaza-s-indacene (4a). Following the general procedure, hept-6-ynoic acid (**1**, 0.69 ml, 5.48 mmol) and 2-(4-methoxyphenyl)-1*H*-pyrrole (**3a**, 2g, 4.8 mmol) were used. Purification by flash chromatography (PetEt → 10% EA in PetEt), followed by recrystallization afforded the title compound as golden cubes (0.48 g, 1.0 mmol, 21 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 7.83 (d, *J* = 9.0 Hz, 4H), 7.23 (d, *J* = 4.3 Hz, 2H), 6.92 (d, *J* = 9.0 Hz, 4H), 6.58 (d, *J* = 4.4 Hz, 2H), 3.80 (s, 6H), 2.93-2.84 (m, 2H), 2.25 (dt, J_1 = 7.0, J_2 = 2.6 Hz, 2H), 1.99 (t, *J* = 2.6 Hz, 1H), 1.96-1.88 (m, 2H), 1.70-1.63 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ ppm 160.48, 157.49, 144.53, 136.03, 130.91, 126.66, 125.12, 119.90, 113.64, 83.60, 69.01, 55.17, 32.39, 29.99, 28.27, 17.95. λ_{abs} 573 nm, λ_{em} 607 nm. HRMS: calcd. for $[C_{29}H_{27}BF_2N_2O_2H]^+$ 485.22064, found 485.22038, calcd. for $[C_{29}H_{27}BFN_2O_2]^+$ 465.21441, found 465.21403.



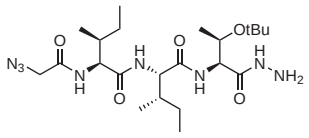
4,4-difluoro-8-(hept-6-yne)-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacene (4b). Employing the general procedure, hept-6-ynoic acid (**1**, 2 ml, 15.85 mmol) and 2,4-dimethyl-3-ethyl-1*H*-pyrrole (**3b**, 4.5 ml, 33.29 mmol) were used. Purification by flash chromatography (PetEt → 1% acetone in PetEt) yielded the title compound (1.2 g, 3.1 mmol, 20% yield). Crystallisation afforded **4b** as green metallic cubes (0.8 g, 2.1 mmol, 14%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 2.94-2.85 (m, 2H), 2.48 (s, 6H), 2.37 (q, *J* = 7.5 Hz, 4H), 2.28 (s, 6H), 2.25-2.20 (m, 2H), 1.94 (t, *J* = 2.5 Hz, 1H), 1.78-1.62 (m, 4H), 1.03 (t, *J* = 7.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 151.83, 144.16, 135.55, 132.33, 130.73, 83.61, 68.78, 30.28, 28.66, 27.65, 17.94, 16.97, 14.64, 13.01, 12.17. λ_{abs} 519 nm, λ_{em} 529 nm. HRMS: calcd. for $[C_{23}H_{31}BF_2N_2H]^+$ 385.26211, found 385.26212, calcd. for $[C_{23}H_{31}BFN_2]^+$ 365.25588, found 365.25581.



4,4-difluoro-8-(hept-6-yne)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (4c). Following the general procedures hept-6-ynoic acid (**1**, 1.24 ml, 9.83 mmol) and 2,4-dimethyl-1*H*-pyrrole (**3c**, 2.13 ml, 20.64 mmol) were used. Flash chromatography (PetEt → 10% acetone in PetEt) afforded the crude product, which was crystallised to yield the title compound **4c** as dark red-green crystals (811 mg, 2.5 mmol, 26%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.03 (s, 2H), 2.91-2.85 (m, 2H), 2.50 (s, 6H), 2.37 (s, 6H), 2.24 (dt, J_1 = 6.3, J_2 = 2.7 Hz, 2H), 1.95 (t, J = 2.6 Hz, 1H), 1.78-1.63 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 153.70, 145.86, 140.30, 131.30, 121.52, 83.60, 68.94, 30.45, 28.79, 27.71, 18.05, 16.15, 14.31. λ_{abs} 496 nm, λ_{em} 503 nm. HRMS: calcd. for $[C_{19}H_{23}BF_2N_2H]^+$ 329.19951, found 329.19961, calcd. for $[C_{19}H_{23}BFN_2]^+$ 309.19328, found 309.19333.

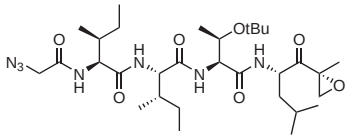


Azidoacetyl-Ile$_2$-Thr(*t*Bu)-OMe (6). MBHA functionalized polystyrene resin (3.57 g, 0.7 mmol/g, 2.5 mmol) was washed with NMP (3x) and DCM (3x) followed by addition of a preactivated mixture of HMPB linker (1.8 g, 7.5 mmol, 3 equiv.), BOP (3.3 g, 7.5 mmol, 3 equiv.) and DiPEA (2.48 ml, 15 mmol, 6 equiv.) in NMP. After 12 hr. of shaking, the resin was washed DCM (3x) and dried. The resin was transferred into a flask, coevaporated with DCE (2x), and condensed with Fmoc-Thr(*t*Bu)-OH (3.0 g, 7.5 mmol, 3 equiv.), under the influence of DIC (1.29 ml, 8.25 mmol, 3.3 equiv.) and DMAP (46 mg, 0.38 mmol, 5 mol%) in DCM for 2 hr. The resin was filtered and washed with DCM (2x), followed by a second condensation cycle. The loading of the resin was determined by spectrophotometric analysis (4.98 g, 0.44 mmol/g, 2.2 mmol, 88%). The obtained resin was submitted to two cycles of Fmoc solid-phase synthesis with Fmoc-Ile-OH as follows: a) deprotection with piperidine/NMP (1/4, v/v, 20 min.); b) washing with NMP (3×); c) coupling of Fmoc-amino acid (1.94 g, 5.5 mmol, 2.5 equiv.) in the presence of BOP (2.4 g, 5.5 mmol, 2.5 equiv.) and DiPEA (1.17 ml, 6.6 mmol, 3 equiv.) in NMP and shake for 2 hr.; d) wash with NMP (3×) and DCM (3×). Couplings were monitored for completion by the Kaiser test. After Fmoc-deprotection of the resin bound tripeptide (0.1 mmol), azidoacetic acid[13] (25 mg, 0.25 mmol, 2.5 equiv.), BOP (0.11 g, 0.25 mmol, 2.5 equiv.) and DiPEA (50 µl, 0.3 mmol, 3 equiv.) in NMP were added, and the resin was shaken for 12 hr. After washing with DCM (3×), the resin was subjected to mild acidic cleavage (TFA/DCM, 1/99, v/v, 10 min., 3x) and the collected fractions were coevaporated with Tol. (2x) to give the crude, partially protected tetrapeptide **5**, which was used without further purification. To a solution of peptide **5** in MeOH/Tol. (1/1, v/v), TMS-diazomethane (0.1 ml 2M in hexanes, 0.2 mmol, 2 equiv.) was added. The reaction mixture was stirred for 15 min., before being concentrated *in vacuo*. The crude title compound was crystallized from DCM/PetEt. Purification by flash chromatography (DCM → 2% MeOH in DCM) afforded Azidoacetyl-Ile$_2$-Thr(*t*Bu)-OMe as a white solid (28.5 mg, 97 µmol, 97%). ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 7.10 (d, J = 8.8 Hz, 1H), 6.92 (d, J = 8.7 Hz, 1H), 6.65 (d, J = 9.1 Hz, 1H), 4.53-4.44 (m, 3H), 4.23 (dq, J_1 = 6.1, J_2 = 1.6 Hz, 1H), 4.01 (q, J = 16.3, 2H), 3.71 (s, 3H), 1.88-1.80 (m, 2H), 1.61-1.46 (m, 2H), 1.28-1.17 (m, 2H), 1.16-1.07 (m, 13H), 0.96 (d, J = 6.8 Hz, 3H), 0.94-0.86 (m, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ ppm 171.10, 170.75, 170.46, 166.54, 77.00, 74.14, 67.10, 57.75, 57.47, 57.46, 52.37, 52.09, 37.84, 37.80, 28.25, 24.94, 20.71, 15.21, 14.98, 11.40, 11.26. HRMS: calcd. for $[C_{23}H_{42}N_6O_6H]^+$ 499.32386, found 499.32380, calcd. for $[C_{23}H_{42}N_6O_6Na]^+$ 521.30580, found 521.30539.

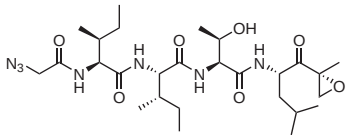


Azidoacetyl-Ile$_2$-Thr(*t*Bu)-hydrazide (7). Azidoacetyl-Ile$_2$-Thr(*t*Bu)-OMe (**6**, 46 mg, 92 µmol) was dissolved in MeOH and hydrazine monohydrate (0.27 ml, 5.5 mmol, 60 equiv.) was added. The reaction mixture was refluxed for 18 hr., before being concentrated in the presence of Tol. Purification by flash chromatography (DCM → 5% MeOH in DCM) afforded the title compound as a white solid (17.1 mg, 34 µmol, 37%). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD = 1/1, v/v): δ ppm 8.07 (d, J = 8.0 Hz, 2H), 7.81 (d, J = 8.7 Hz, 1H), 7.58 (d, J = 7.6 Hz, 1H), 4.40 (dd, J_1 = 7.9, J_2 = 3.3 Hz, 1H), 4.36-4.24 (m, 2H), 4.07 (dq, J_1 = 6.3, J_2 = 3.6 Hz, 1H), 3.96 (s,

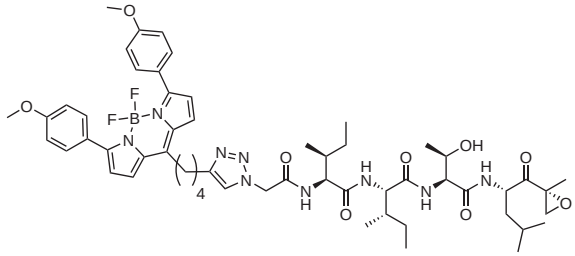
2H), 1.94-1.80 (m, 2H), 1.63-1.47 (m, 2H), 1.35-1.11 (m, 12H), 1.09 (d, *J* = 6.4 Hz, 3H), 0.96-0.88 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD = 1/1, v/v): δ ppm 174.89, 174.45, 172.85, 170.84, 77.70, 69.41, 61.09, 60.38, 60.34, 59.82, 54.56, 39.59, 39.09, 30.52, 27.55, 27.42, 21.10, 17.86, 17.76, 13.35, 13.26.



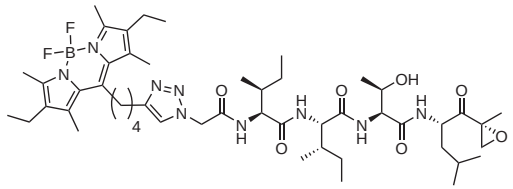
Azidoacetyl-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxiran (9). *Tert*-butyl (S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylcarbamate[7b] (9.5 mg, 35 µmol, 1.1 equiv.) was stirred in TFA, until TLC showed complete Boc deprotection. All volatiles were removed in the presence of Tol. (2×) yielding the crude leucine epoxyketone TFA salt (**8**), which was dissolved in DMF and neutralized with DiPEA (5.8 µl, 35 µmol, 1 equiv.). Azidoacetyl-Ile$_2$-Thr(*t*Bu)-hydrazide (**7**, 16 mg, 32 µmol) was dissolved in DMF/EtOAc (1/1, v/v), put under an argon atmosphere and cooled to -30 °C. HCl (22.5 µl 4M in dioxane, 90 µmol, 2.8 equiv.) and *tert*-butyl nitrite (4.2 µl, 35 µmol, 1.1 equiv.) were added and the reaction mixture was stirred for 3 hr. The leucine epoxyketone (**8**) solution and DiPEA (20.1 µl, 0.12 mmol, 3.8 equiv.) were added at -30 °C and the reaction mixture was stirred for 18 hr., before being concentrated *in vacuo*. Purification by flash chromatography (DCM → 1% MeOH in DCM) afforded a mixture of the title compound and a side product[15] as a white solid (14.7 mg). Column chromatography (*n*-hexane → 15% acetone in *n*-hexane) afforded azidoacetyl-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxiran (**9**) as a white solid (2.2 mg, 3.4 µmol, 11%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.66 (d, *J* = 7.5 Hz, 1H), 6.94-6.85 (m, 2H), 6.35 (d, *J* = 8.5 Hz, 1H), 4.46 (ddd, J_1 = 10.4, J_2 = 7.5, J_3 = 2.9 Hz, 1H), 4.37-4.26 (m, 3H), 4.13 (dq, J_1 = 6.4, J_2 = 4.3 Hz, 1H), 4.00 (q, *J* = 16.4 Hz, 2H), 3.34 (d, *J* = 5.1 Hz, 1H), 2.91 (d, *J* = 5.1 Hz, 1H), 1.94-1.76 (m, 2H), 1.75-1.54 (m, 3H), 1.52 (s, 3H), 1.29 (s, 9H), 1.27-1.24 (m, 2H), 1.19-1.09 (m, 2H), 1.07 (d, *J* = 6.5 Hz, 3H), 1.00-0.85 (m, 18H).



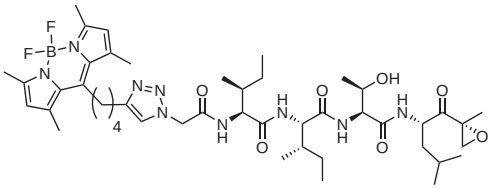
Azidoacetyl-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxiran (10). Azidoacetyl-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxiran (**9**, 10 mg, 15.6 µmol) was dissolved in TFA and stirred until TLC analysis revealed total consumption of **9**. The reaction mixture was coevaporated with Tol. (3×) resulting in azidomicin (**10**) as a white solid (9.1 mg, 15.6 µmol, quant.) and was used without purification.



Fluorescent epoxomicin analogue 11a. BODIPY-alkyne **4a** (7.6 mg, 15.6 µmol) and azidomicin (**10**, 9.1 mg, 15.6 µmol, 1 equiv.) were dissolved in a mixture of *t*BuOH and Tol. (1/1, 124.8 µl), before an aqueous solution of sodium ascorbate (31 µl 0.16 mM, 15 mol%) and an aqueous solution of $CuSO_4$ (31 µl 0.1 mM, 10 mol%) were added. The reaction mixture stirred at 60 °C for 12 hr., before being concentrated *in vacuo*. Purification by size-exclusion chromatography (Sephadex LH-20, eluens: MeOH) gave the title compound as a fine purple powder (19.4 mg, 14.2 µmol, 91%). ^{1}H NMR (400 MHz,$CDCl_3$ + drop MeOD): δ ppm 7.80 (d, *J* = 8.7 Hz, 4H), 7.52 (s, 1H), 7.23 (d, *J* = 3.7 Hz, 2H), 6.90 (d, *J* = 8.8 Hz, 4H), 6.54 (d, *J* = 4.0 Hz, 2H), 5.19-4.43 (m, 6H), 4.10-4.00 (m, 1H), 3.80 (s, 6H), 3.28 (d, *J* = 3.0 Hz, 1H), 2.93-2.83 (m, 3H), 2.68-2.49 (m, 2H), 1.90-1.15 (m, 15H), 1.14-0.99 (m, 4H), 0.94-0.69 (m, 18H). HRMS: calcd. for $[C_{56}H_{74}BF_2N_9O_9H]^+$ 1066.57434, found 1066.57512, calcd. for $[C_{56}H_{74}BF_2N_9O_9Na]^+$ 1088.55628, found 1088.55690.



Fluorescent epoxomicin analogue 11b. BODIPY-alkyne **4c** (6 mg, 15.6 µmol) and azidomicin (**10**, 9.1 mg, 15.6 µmol, 1 equiv.) were dissolved in a mixture of *t*BuOH and Tol. (1/1, 124.8 µl), before an aqueous solution of sodium ascorbate (31 µl 0.16 mM, 15 mol %) and an aqueous solution of $CuSO_4$ (31 µl 0.1 mM, 10 mol %) were added. The reaction mixture stirred at 60 °C for 12 hr., before being concentrated *in vacuo*. Column chromatography (DCM → 5% MeOH in DCM) yielded the title compound as an orange solid (12.4 mg, 12.8 µmol, 82%). ^{1}H NMR (400 MHz,$CDCl_3$ + drop MeOD): δ ppm 7.57 (s, 1H), 5.07 (dd, J_1 = 32.8, J_2 = 16.1 Hz, 2H), 4.54 (dd, J_1 = 10.5, J_2 = 2.8 Hz, 1H), 4.32 (d, J = 4.1 Hz, 1H), 4.25-4.19 (m, 2H), 4.14-4.06 (m, 1H), 3.30 (d, J = 4.9 Hz, 1H), 3.09-3.02 (m, 2H), 2.92 (d, J = 5.0 Hz, 1H), 2.83-2.77 (m, 2H), 7.57 (s, 1H), 2.48 (s, 6H), 2.41 (q, J = 7.7 Hz, 4H), 2.33 (s, 6H), 1.99-1.87 (m, 2H), 1.87-1.77 (m, 2H), 1.76-1.61 (m, 2H), 1.58-1.43 (m, 5H), 1.38-1.24 (m, 2H), 1.19-1.01 (m, 12H), 0.98-0.82 (m, 18H). HRMS: calcd. for $[C_{50}H_{78}BF_2N_9O_7H]^+$ 966.61581, found 966.61685, calcd. for $[C_{50}H_{78}BF_2N_9O_7Na]^+$ 988.59775, found 988.59809.



Fluorescent epoxomicin analogue 11c. BODIPY-alkyne **4b** (5.1 mg, 15.6 µmol) and azidomicin (**10**, 9.1 mg, 15.6 µmol, 1 equiv.) were dissolved in a mixture of *t*BuOH and Tol. (1/1, 124.8 µl), before an aqueous solution of sodium ascorbate (31 µl 0.16 mM, 15 mol %) and an aqueous solution of $CuSO_4$ (31 µl 0.1 mM, 10 mol %) were added. The reaction mixture stirred at 60 °C for 12 hr., before being concentrated *in vacuo*. Column chromatography (DCM → 5% MeOH in DCM) yielded the title compound as a fine orange powder (9.2 mg, 10.1 µmol, 65%). ^{1}H NMR (400 MHz,$CDCl_3$ + drop MeOD): δ ppm 7.57 (s, 1H), 6.08 (s, 2H), 5.08 (q, J = 16.1 Hz, 2H), 4.54 (dd, J_1 = 10.5, J_2 = 3.0 Hz, 1H), 4.31 (d, J = 4.1 Hz, 1H), 4.24-4.07 (m, 3H), 3.30 (d, J = 4.9 Hz, 1H), 3.06-2.98 (m, 2H), 2.91 (d, J = 5.0 Hz, 1H), 2.80 (t, J = 7.6 Hz, 2H), 2.50 (s, 6H), 2.41 (s, 6H), 1.97-1.88 (m, 2H), 1.87-1.78 (m, 2H), 1.77-1.64 (m, 2H), 1.51 (s, 3H), 1.50-1.28 (m, 6H), 1.13 (m, 4H), 0.98-0.83 (m, 18H). HRMS: calcd. for $[C_{46}H_{70}BF_2N_9O_7H]^+$ 910.55321, found 910.55425, calcd. for $[C_{46}H_{70}BF_2N_9O_7Na]^+$ 932.53515, found 932.53529.

***In vitro* proteasome labeling.** EL4 cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 µg/ml streptomycin in a 5% CO_2 humidified incubator at 37 °C. Cells were harvested and after flash freezing (N_2 (l)) the cells were lysed in digitonin containing lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl, 1 mM DTT, 0.025% digitonin) for 15 min. on ice and centrifuged at 20.000 g for 20 min. at 4 °C. The supernatant containing the cytosolic fraction was collected and the protein content was determined by Bradford assay. The resulting cell lysates (10µg total protein) were exposed to the probes **11a-c** with the indicated concentrations (10× solution in DMSO) for 1 hr. Reaction mixtures were boiled with Laemmli's buffer containing β-mercapto-ethanol for 3 min., before being resolved on 12.5% SDS-PAGE. Fluorescently labeled proteins were visualized by in-gel fluorescence scanning on a Typhoon variable mode imager (Amersham Biosciences, settings: **11a** λ_{ex} 532 nm, λ_{em} 610 nm; **11b** λ_{ex} 532 nm, λ_{em} 526 nm; **11c** λ_{ex} 488 nm, λ_{em} 520 nm).

Proteasome labeling in living cells. EL4 cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 µg/ml streptomycin in a 5% CO_2 humidified incubator at 37 °C. Some $1{\cdot}10^6$ cells were seeded in 6 cm Petri dishes and allowed to grow 12 hr. in 1 ml of medium. The cells were exposed to 0, 1, 5, 10 or 50 µM **11a-c** (100× solution in DMSO) for 2 hr., before being washed with PBS and harvested. After flash freezing (N_2 (l)) the cells were lysed in 60 µl digitonin containing lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl, 1 mM DTT, 0.025% digitonin) for 15 min. on ice and centrifuged at

20.000 g for 20 min. at 4 °C. The supernatant containing the cytosolic fraction was collected and the protein content was determined by Bradford assay. Some 20 µg protein was resolved by 12.5% SDS-PAGE. Fluorescently labelled proteins were visualised by in-gel fluorescence scanning on a Typhoon variable mode imager (Amersham Biosciences, settings: **11a** λ_{ex} 532 nm, λ_{em} 610 nm, **11b** λ_{ex} 532 nm, λ_{em} 526 nm, **11c** λ_{ex} 488 nm, λ_{em} 520 nm).

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14. The synthesis follows a procedure described by Rieth, R.D.; Mankad, N.P.; Calimano, E.; Sadighi, J.P. *Org. Lett.*, **2004**, *6*, 3981-3983.
15. LC/MS analysis of the reaction mixture before concentration *in vacuo* revealed a single product peak corresponding to **9** (R_f 10.17 min (linear gradient 10 → 90% B in 15 min), *m/z* 638.33 $[M+H]^+$). Purification by column chromatography (DCM → 1% MeOH in DCM) afforded a mixture of two compounds. LC/MS analysis (product 1: R_f 9.79 min (linear gradient 10 → 90% B in 15 min), *m/z* 674.40 $[M+H]^+$, showing the typical chlorine isotope pattern. **9**: R_f 10.17 min (linear gradient 10 → 90% B in 15 min), *m/z* 638.33 $[M+H]^+$) and ^1H NMR revealed partial opening of the epoxide by a chloride ion (probably originating from the DiPEA·HCl salt present in the reaction mixture), dramatically decreasing the yield.

4

Azido-BODIPY acid: a new tool in activity-based protein profiling

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4.1 Introduction

Activity-based protein profiling (ABPP) research aims at the development of tools and techniques that report on enzyme activity in complex biological samples.[1-4] With the aid of activity-based probes (ABP), small molecules designed to specifically, covalently and irreversibly react with the active site residues of an enzyme or enzyme family, enzymatic activity levels are detected, rather than the protein expression levels that are measured by means of conventional proteomics techniques. A typical ABP consists of three parts: 1) a warhead, the reactive group that binds covalently and irreversibly to the enzyme active site, 2) a recognition element targeting the ABP to a certain enzyme (family) and 3) an affinity tag or a fluorophore for visualization and/or enrichment purposes. In most ABPs described to date and that report on enzyme activity, the reporter group is directly attached to the probe, with obvious advantages with respect to experimental design. Incorporation of for instance a biotin or large fluorophore in an ABP may, however, have a detrimental effect on either bioavailability (cell permeability) or enzyme reactivity of the probe, or both. With the aim to alleviate these problems, the two-step labeling approach is an important alternative in ABPP. In two-step ABPP approaches a small biocompatible reactive group, normally an azide or an acetylene, is introduced in an ABP. After covalent modification of a target protein (family) a reporter group is introduced in a chemoselective manner, by means of either Staudinger-Bertozzi ligation[5-7] or Huisgen [2+3]-cycloaddition (the "click reaction", of which both copper(I)-catalyzed[8-13] and copper-free[14,15] versions exist). Speers *et al.* reported that this approach is versatile also in the profiling of serine hydrolases.[8] Simultaneously, Ovaa *et al.* reported the cell-permeable proteasome inhibitor **1** (Figure 1), which allows for profiling of proteasome activity in living cells by post-lysis

Staudinger-Bertozzi ligation of a biotin onto the azido moiety.[5] Key to the success of such two-step ABPP experiments is the selectivity (in terms of cross-reactivity towards endogenous functional groups in a biological sample) and efficiency (in terms of chemical yield with which the azide- or acetylene-modified proteins are converted) of the chemoselective ligation step in which the reporter group is attached to the modified proteins. There are several reports on the selectivity of both Staudinger-Bertozzi and click ligations,[11,14] but little information is available on the efficiency of these reactions.

In Chapter 2, the fluorescent broad-spectrum cell-permeable proteasome probe MV151 (**2**, Figure 1) is described. This probe allows for the rapid detection of the active proteasome pool in cells and lysates, and localization thereof in living cells and tissue.[16] In this Chapter, the better of two worlds is combined in the design of an azido functionalized BODIPY dye. Introducing such dye in a proteomics probe provides flexibility in designing the optimal ABP (one-step or two-step), depending on the nature of the ABPP experiment. The fluorophore enables the rapid and sensitive assessment of the labeling profile of a probe by in-gel fluorescence readout of the tagged proteins. On the other hand, the possibility to introduce an affinity tag, either before or after protein labeling, results in fluorescent affinity tagged proteins which can be purified and analyzed by mass-spectrometry. When performing a two-step labeling approach for the introduction of an affinity tag, the BODIPY allows for monitoring of the efficiency of the ligation reaction and will be of assistance in the optimization of the ligation conditions used.

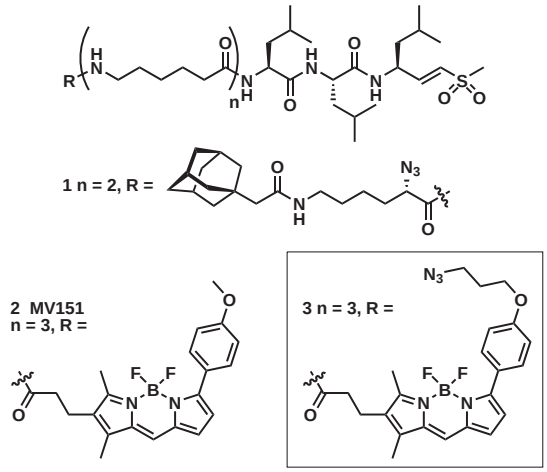


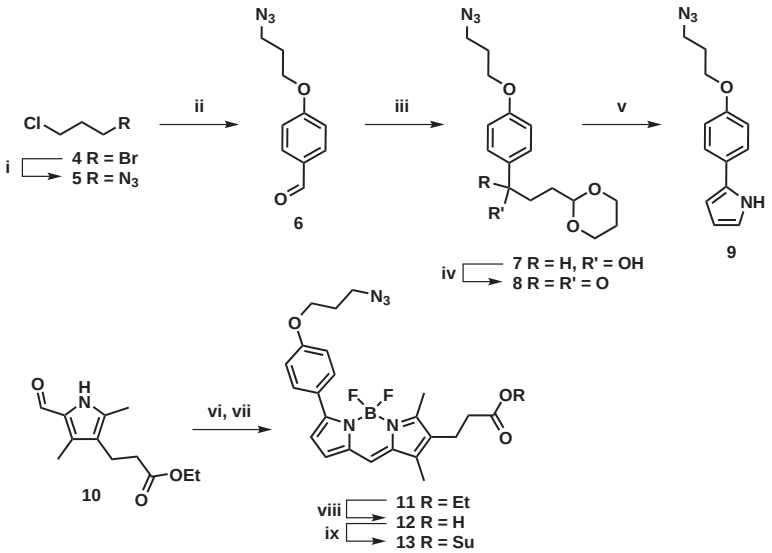
Figure 1. The proteasome probes AdaAhx(α-N_3)Ahx$_2$L$_3$VS (**1**), MV151 (**2**) and Azido-BODIPY-Ahx$_3$L$_3$VS (**3**).

This Chapter describes the development of the bifunctional fluorophore azido-BODIPY acid (**12**) and its application in the synthesis of two compatible sets of one-step and two-step proteasome ABPs. These probes were then used to demonstrate that the Staudinger-Bertozzi ligation in the two-step ABPP of the proteasome catalytic activities proceeds in a near quantitative fashion.

4.1 Results and discussion

It was reasoned that the substitution of the phenolic methyl group in BODIPY TMR for an azidopropyl spacer would gain an azido functionalized dye, without having a detrimental effect on the spectroscopic properties. Azido-BODIPY-OSu **13** was synthesized employing a strategy described in Chapter 2.[16] The synthesis commenced with the preparation of the azido functionalized pyrrole **9** (Scheme 1). Nucleophilic substitution of the better leaving group in 1-bromo-3-chloro-propane with an azide, followed by alkylation of 4-hydroxy-benzaldehyde gave aldehyde **6**. A Grignard reaction with (1,3-dioxane-2-ylethyl)-magnesium bromide and subsequent oxidation with manganese dioxide resulted in ketone **8**. Concomitant deprotection of the masked aldehyde and Paal-Knorr reaction afforded azidopropanoxy-phenyl pyrrole **9**. Condensation of carboxyaldehyde pyrrole **10**[17] with pyrrole **9** under the influence of hydrobromic acid, and subsequent treatment with $BF_3{\cdot}OEt_2$ and triethylamine in refluxing dichloroethane gave azido-BODIPY ethyl ester **11**.

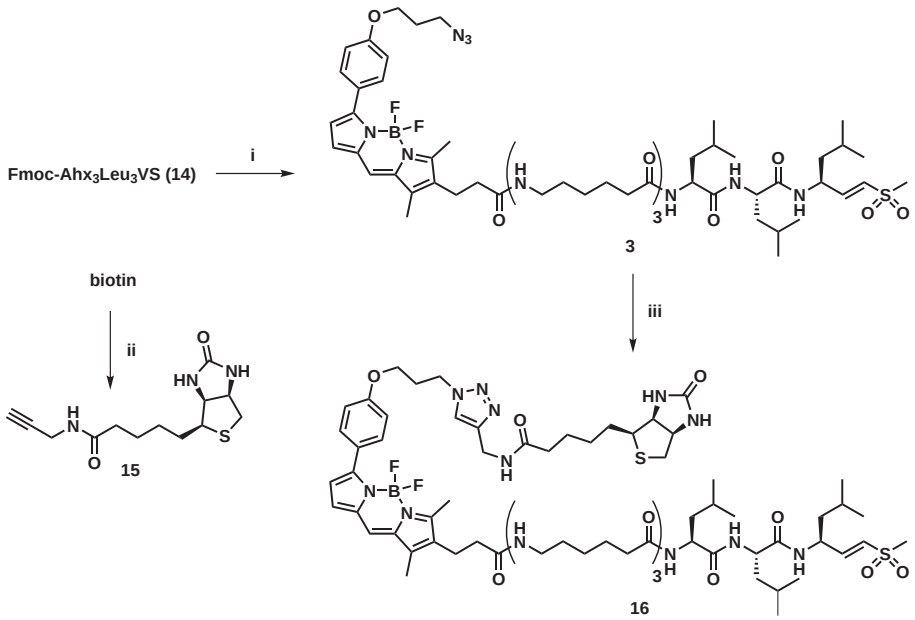
Scheme 1. Synthesis of Azido-BODIPY-OSu **13**.



Reagents and conditions: i) NaN_3 (1 equiv.), DMSO, 12 hr., quant. ii) 4-hydroxy-benzaldehyde (0.5 equiv.), K_2CO_3 (1 equiv.), DMF, 90 °C, 48 hr., 86%. iii) (1,3-dioxane-2-ylethyl)-magnesium bromide (1.5 equiv.), THF, -10 °C → RT, 12 hr., 45%. iv) MnO_2 (10 equiv.), DCM, 12 hr., 70%. v) NH_4OAc (12 equiv.), Ac_2O (3.7 equiv.), AcOH, reflux, 3 hr., 32%. vi) **9**, HBr (48% in H_2O, 1.5 equiv.), EtOH, 0 °C, 2 hr. vii) $BF_3{\cdot}Et_2O$ (5 equiv.), TEA (3 equiv.), DCE, 90 °C, 16 hr., 56% (2 steps). viii) 0.1 M NaOH (1.15 equiv.), dioxane/MeOH (1/1, v/v), 15 hr., 35%. ix) HOSu (4 equiv.), EDC (4 equiv.), DCM, 2 hr., 68%.

Saponification of the ethyl ester coincided with substitution of a fluorine for either water or methanol, dramatically reducing the yield of azido-BODIPY acid **12**. Condensation of **12** with *N*-hydroxysuccinimide completed the synthesis of azido-BODIPY-OSu **13**.

Scheme 2. Synthesis of the bifunctional proteasome probes **3** and **16**.



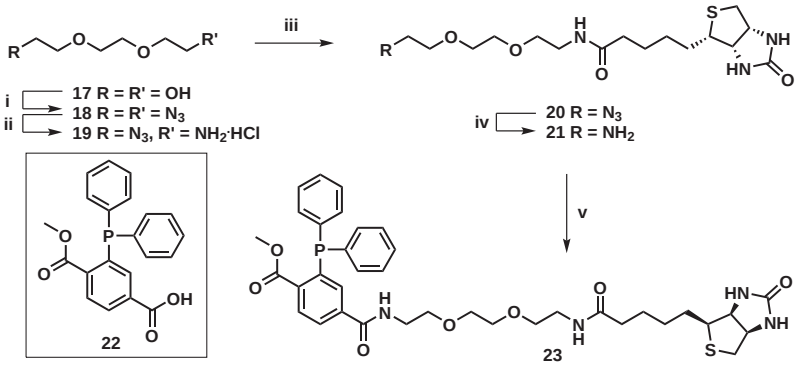
Reagents and conditions: i) (a) DBU (1 equiv.), DMF, 5 min. (b) HOBt (4.5 equiv.), 1 min. (c) **13** (1 equiv.), DiPEA (6 equiv.), 30 min., 86%. ii) Propargylamine (1 equiv.), HCTU (1 equiv.), DiPEA (2 equiv.), DMAP (cat.), DMF, 0 °C, 3 hr., 88%. iii) **15** (2 equiv.), 10 mol% $CuSO_4$, 20 mol% sodium ascorbate, *t*BuOH/H_2O 1/1, RT, 15 hr., quant.

Having the azido-BODIPY-OSu **13** in hand, the bifunctional proteasome probe **3** was synthesized (Scheme 2). Removal of the Fmoc protecting group in hexapeptide vinyl sulfone **14**,[16] followed by condensation with azido-BODIPY-OSu **13** afforded the proteasome probe **3**. Copper(I)-catalyzed Huisgen [2+3]-cycloaddition[9,10] with biotin-propargylamide **15**, which was obtained by condensation of biotin with propargylamine, gave rise to the fluorescent and affinity-tagged proteasome probe **16**.

For the two-step labeling approach the Staudinger-Bertozzi ligation was chosen. To this end, the biotin functionalized phosphane reagent **23** was synthesized as depicted in Scheme 3. Triglycol **17** was di-tosylated, followed by substitution with azide to give di-azide **18**. Mono Staudinger reduction of di-azide **18** afforded amine **19**. Condensation with biotin gave biotinylated azido-triethylene glycol **20**. Staudinger reduction of the azide to give amine **21** and subsequent condensation with phosphane **22**[6] resulted in the Staudinger-Bertozzi phosphane reagent **23**.

Having synthesized probes **3** and **16** their ability to label the proteolytically active proteasome subunits in both cell lysates and living cells was assessed. EL-4 cell lysates containing both the constitutive proteasome and the immunoproteasome[18] were treated with increasing concentrations of **3** or **16** for 1 hour at 37 °C. The lysates treated with **3** were then exposed to biotin-phosphane **23** for 1 hour at 37 °C. All samples were precipitated and the proteins were resolved on SDS-PAGE. Direct in-gel read-out of the wet gel slabs

Scheme 3. Synthesis of the Staudinger-Bertozzi phosphane reagent **23**.



Reagents and conditions: i)(a) Tosyl chloride (3 equiv.), TEA (3 equiv.), DMAP (cat.), DCM, 16 hr. (b) NaN_3 (2 equiv.), TBAI (cat.), DMF, 80 °C, 16 hr., 78% (2 steps). ii) PPh_3 (0.95 equiv.), 5% HCl (aq.), Tol., 0 °C, 16 hr., 79%. iii) D-(+)-biotin (1.01 equiv.), BOP (1.01 equiv.), DiPEA (3.03 equiv.), DMF, 16 hr., 60%. iv) (a) PPh_3 (1.5 equiv.), 1hr. (b) H_2O, DMF, 16 hr., 78%. v) **22** (1.1 equiv.), EDC·HCl (1.5 equiv.), DMF, 16 hr., 23%.

showed uniform labeling of the proteasome catalytic subunits (β1, β2, β5, β1i, β2i, β5i) by both ABPs in a concentration-dependent manner (Figure 2). The observed patterns are similar to those previously demonstrated (see for a representative example the labeling pattern of MV151 (**2**), Figure 2A lane 10).[16] Pre-incubation with epoxomicin[19,20] (Figure 2A lane 9, Figure 2C lane 8) abolished all labeling, which further proves the activity-based mechanism of ABPs **3** and **16**. ABP **3** appears slightly more reactive than its biotinylated counterpart **16** (compare Figure 2A lanes 3-5 and Figure 2C lanes 3-5). The near quantitative yield of the Staudinger-Bertozzi ligation on the proteasome subunits modified by ABP **3** is evidenced by the close to complete gel shift of those samples exposed to 100 µM biotin-phosphane **23** (Figure 2A, compare lanes 3-7 and 8). The efficiency of the ligation is also apparent when comparing the streptavidin blots prepared from the same gels (Figure2B/D). Again, both patterns are highly similar and the intensity of the signals is similar for those

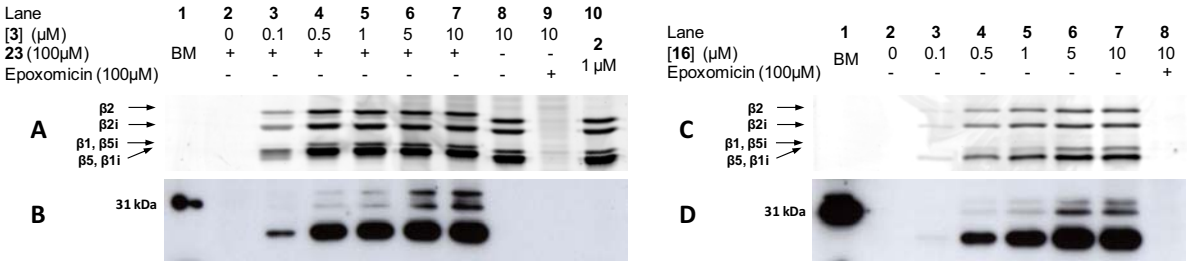


Figure 2. Fluorescence readout and streptavidin blot of **3** and **16** labeled proteasomes in cell lysate.
(**A**) Fluorescence readout and streptavidin blot (**B**) of EL-4 cell lysates (25µg total protein) treated with **3** for 1 hr. at 37 °C, followed by Staudinger-Bertozzi ligation (100µM biotin-phosphane **23,** 1 hr. at 37 °C) and SDS-PAGE. (**C**) Fluorescence readout and (**D**) streptavidin blot of EL-4 cell lysates (25µg total protein) treated with **16** for 1 hr. at 37 °C, followed by SDS-PAGE. BM = biotinylated marker.

experiments where 10 μM concentrations of either **3** or **16** (Figure 2B/D lanes 7) were applied.

The proteasome labeling potential of ABPs **3** and **16** in living cells was established by incubating living EL-4 cells with either of the two probes at various concentrations for 2 hours at 37 °C. The exposed cells were harvested, washed and lysed and the lysates were processed as before (Figure 3). The outcome of these experiments is highly reminiscent of the ABPP labeling of lysates depicted in Figure 2. However, the main, and important, difference is found in the diverged labeling efficiency now observed for the two probes. In contrast to the proteasome profiling experiments on lysates, where both probes appeared about equally efficient, two-step ABP **3** is estimated at least five-fold more efficient in targeting the proteasome catalytic activities in living cells. As both probes are equally efficient in labeling proteasomes in lysates this difference must be based on the relative cell permeability of the two probes.

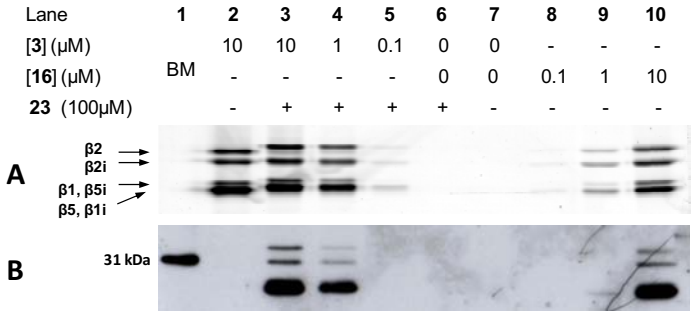
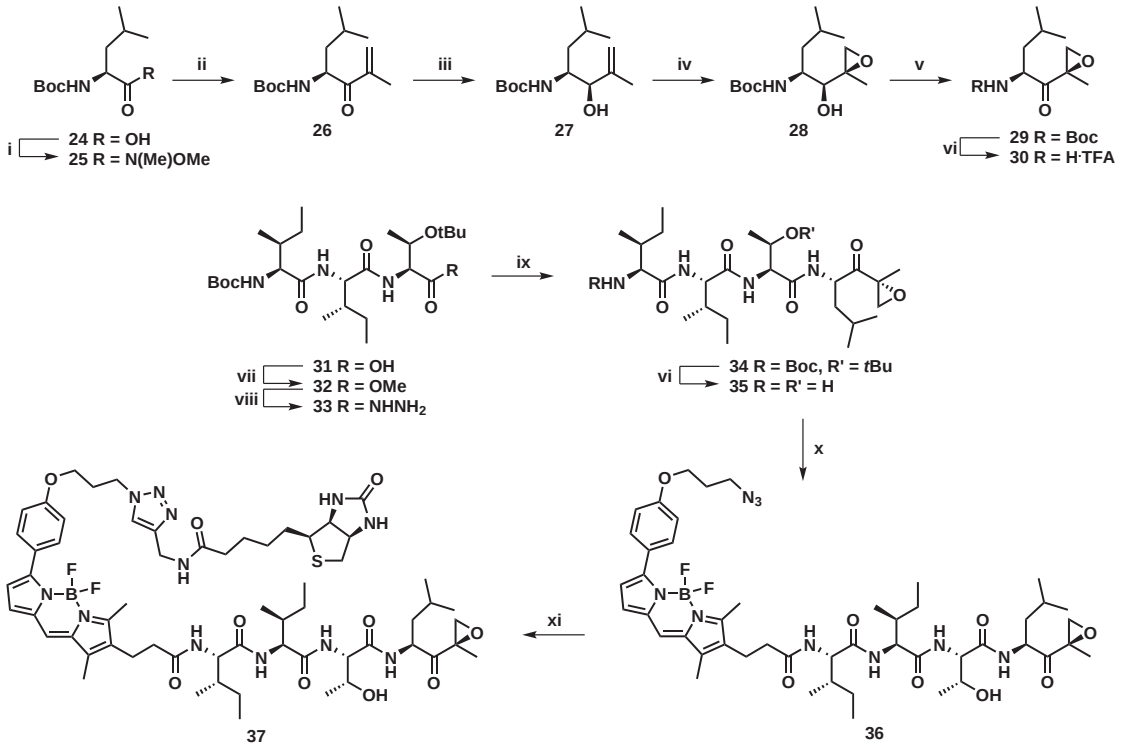


Figure 3. Fluorescence readout and streptavidin blot of **3** and **16** labeled proteasomes in living cells.
(**A**) Fluorescence readout and (**B**) streptavidin blot of living EL4 cells (some $2{\cdot}10^6$ cells) exposed to the indicated probes and concentrations for 2 hr. at 37 °C, before being harvested and lysed and separated on SDS-PAGE. Lane 3-6: 25 μg total protein was treated with biotin-phosphane **23** (100μM) for 1 hr. at 37 °C. Lane 7-10: 25 μg total protein was loaded on SDS-PAGE. BM = biotinylated marker.

Next to the bifunctional proteasome probes described in the above, a compatible set of epoxomicin derived one-step (**36**) and two-step (**37**) proteasome probes were synthesized (Scheme 4). The synthesis commenced with the preparation of the leucine derived α′,β′-epoxyketone warhead **29**.[21] Boc-leucine (**24**) was condensed with *N,O*-dimethyl-hydroxylamine and the resulting Weinreb amide **25** was reacted with 2-lithiumpropene to give the α′,β′-unsaturated ketone **26**. Stereoselective reduction to allylic alcohol **27** by $NaBH_4$ in the presence of $CeCl_3$ and subsequent asymmetric epoxidation using *t*butyl hydroperoxide and vanadyl acetylacetonate afforded epoxide **28**. Dess-Martin oxidation finalized the synthesis of the Boc-protected leucine α′,β′-epoxyketone warhead **29**. The *N*-terminally Boc-capped epoxomicin peptide sequence was synthesized employing standard Fmoc-based solid phase peptide chemistry. Mild acidic cleavage of

Scheme 4. Synthesis of the bifunctional epoxomicin derived probes **36** and **37**.



Reagents and conditions: i) *N,O*-dimethyl-hydroxylamine·HCl (1 equiv), BOP (1 equiv.), DiPEA (2 equiv.), DCM, 16 hr., 89%. ii) (a) *t*BuLi (4.5 equiv.), 2-bromopropene (3 equiv.), Et_2O, -78 °C, 15 min. (b) **25**, -78 °C to RT, 2 hr., 79%. iii) $NaBH_4$ (1.4 equiv.), $CeCl_3$ (2.2 equiv.), MeOH, 0 °C, 15 min., 91%. iv) *t*BuOOH (3 equiv.), $VO(acac)_2$ (4 mol%), DCM, 0 °C to RT, 2 hr., 51%. v) Dess-Martin periodinane (3 equiv.), DMSO, 0 °C to RT, 4 hr., 90%. vi) TFA, 30 min. vii) TMS-diazomethane (2 equiv.), MeOH/Tol. (1/1, v/v), 15 min., 64%. viii) Hydrazine monohydrate (60 equiv.), MeOH, reflux, 76%. ix) (a) *t*BuONO (1.1 equiv.), HCl (2.8 equiv.), EtOAc/DMF, 4 hr., -25 °C. (b) DiPEA (4 equiv.), **30** (1.1 equiv.), -25 °C to RT, 16 hr., 89%. x) **13** (1 equiv.), DiPEA (4 equiv.), 12 hr., 47%. xi) **15** (2 equiv.), 10 mol% $CuSO_4$, 20 mol% sodium ascorbate, *t*BuOH/H_2O/Tol. (1/1/1, v/v/v), RT, 12 hr., 85%.

resin-bound peptide gave **31** with the Boc and *tert*-butyl protecting groups still in place. To circumvent epimerization of the threonine α-position during condensation of the peptide to the leucine derived warhead an azide coupling was employed (Chapter 3). Therefore, the free carboxylic acid **31** was converted into methyl ester **32** by treatment with TMS-diazomethane, which was subsequently refluxed in methanol in the presence of a large excess of hydrazine to afford the protected peptide hydrazide **33**. After *in situ* generation of the peptide acyl azide, the deprotected leucine α',β'-epoxyketone warhead **30** was coupled to give the fully protected tetrapeptide epoxyketone **34**. After acidic deprotection of all protecting groups, the amine was acylated with azido-BODIPY-OSu (**13**) to give the bifunctional epoxomicin derived ABP **36**. The fluorescent affinity tagged analogue **37** was prepared by a Huisgen [2+3]-cycloaddition with biotin-propargylamide **15**.

Living EL-4 cells were exposed to increasing concentrations of two-step ABP **36** for 2 hours at 37 °C before being harvested, washed and lysed. The lysates were treated with 100 µM biotin-phosphane **23** for 1 hour at 37 °C prior to separation on SDS-PAGE, fluorescence readout (Figure 4A) and streptavidin blotting of the same gel (Figure 4B). Compared to **3**, azido-BODIPY-micin **36** appears to be a more potent proteasome probe in living cells, reaching saturation between 1 and 5 µM concentration. When comparing the ability of **36** and the fluorescent affinity tagged probe **37** to label active proteasome β-subunits in living EL-4 cells (Figure 4C), it becomes apparent that the difference between the one-step and two-step approach is even bigger for the epoxomicin derived probes as compared to probes **3** and **16** (Figure 3). Two-step ABP **36** was estimated to be at least one order of magnitude more efficient than the one-step ABP **37** in labeling the catalytically active proteasomes in living cells. Being smaller in size and less hydrophobic, the ligation of a biotin moiety onto epoxomicin derivative **36** has a more dramatic effect on the cell-permeability compared to introduction of a biotin into the much bigger and more hydrophobic azido-BODIPY-Ahx$_3$L$_3$VS **3**.

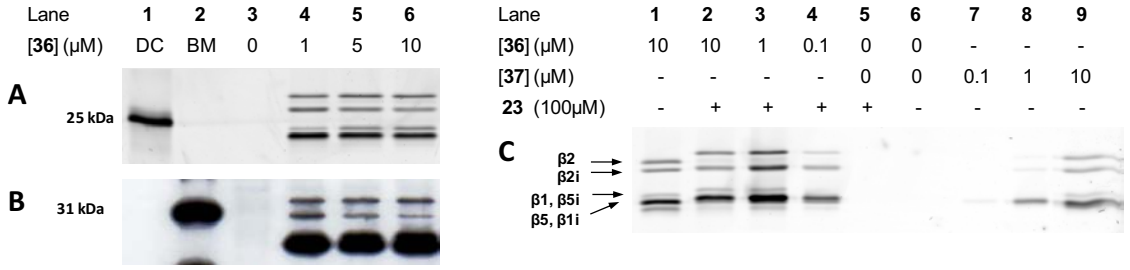


Figure 4. Fluorescence readout and streptavidin blot of **36** and **37** labeled proteasomes in living cells.
(**A**) Fluorescence readout and (**B**) streptavidin blot of living EL4 cells (some $2{\cdot}10^6$ cells) exposed to the indicated concentrations of **36** for 2 hr. at 37 °C, before being harvested and lysed. Some 80 µg total protein was treated with biotin-phosphane **23** (100µM) for 1 hr. at 37 °C, of which 25 µg total protein was separated on SDS-PAGE. (**C**) Fluorescence readout of living EL4 cells (some $2{\cdot}10^6$ cells) exposed to the indicated probes and concentrations for 2 hr. at 37 °C, before being harvested and lysed and separated on SDS-PAGE. Lane 2-6: 25 µg total protein was treated with biotin-phosphane **23** (100µM) for 1 hr. at 37 °C. Lane 6-9: 25 µg total protein was loaded on SDS-PAGE. DC = Dual Color molecular marker, BM = biotinylated marker.

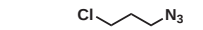
4.3 Conclusion

In conclusion, the versatility of the bifunctional fluorophore, azido-BODIPY-acid **12**, as a new tool in ABPP experiments is demonstrated. It was established that the Staudinger-Bertozzi ligation proceeds in a near quantitative yield under the conditions applied here. This result essentially means that two-step ABPP may proceed with equal efficiency with respect to protein tagging as contemporary one-step ABPP approaches. The efficiency thus depends on the reactivity of the ABP towards the target protein (family), and not on the

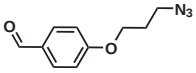
chemoselective ligation employed in the second step. The advantage of two-step ABPP is evident from the here presented results demonstrating that ABPs **3** and **36** are better capable of labeling proteasomes in living cells than their biotinylated analogues **16** and **37**. It is expected that BODIPY derivative **12** will be useful to the chemical biology community outside the proteasome field for several reasons. First, the system presented here should be of assistance in optimizing Staudinger-Bertozzi ligation conditions, for instance in reaction time and in the amount of phosphane reagent used with respect to the azido modified biomolecule. Further, azido-BODIPY acid **12** can be readily transposed to different ABPP experimental settings. These include those that aim for the profiling of different enzyme families (entailing the incorporation of **12** in other ABPs) but also those that aim to develop or employ other bio-orthogonal ligation strategies. An obvious extension of the here reported work is the evaluation of the efficiency of the Huisgen cycloaddition reaction, but modification of the azide in **12** to encompass a reaction partner of new bio-orthogonal ligations are envisaged as well. Another promising application can be found in the use of azido-BODIPY acid **12** as a linker moiety in the generation of fluorescent (bio)conjugates.

Experimental section

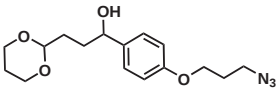
General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.)(purum), ethyl acetate (EtOAc) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, 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 followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on Screening Divices (0.040 – 0.063 nm). LC/MS analysis was performed on a LCQ Advantage Max (Thermo Finnigan) equipped with an Gemini C18 column (Phenomenex). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), Bruker DPX-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory or a Bruker DMX-600 (600/150 MHz) with cryoprobe. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. UV spectra were recorded on a Perkin Elmer, Lambda 800 UV/VIS spectrometer.



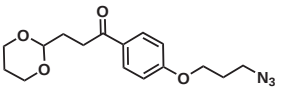
1-azido-3-chloro-propane (5). 1-Bromo-3-chloro-propane (**4**, 9.9 ml, 100 mmol) was dissolved in DMSO. NaN_3 (6.5 g, 100 mmol, 1 equiv.) was added and the solution was stirred for 12hr. before H_2O and pentane were added. The organic layer was separated, dried over $MgSO_4$ and concentrated to yield 1-azido-3-chloro-propane (11.96 g, quant.) as a colourless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 3.63 (t, *J* = 6.2 Hz, 2H), 3.50 (t, *J* = 6.6 Hz, 2H), 2.01 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 47.99, 41.35, 31.31.



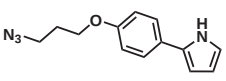
4-(3-Azido-propoxy)-benzaldehyde (6). 4-hydroxy-benzaldehyde (6.1 g, 50 mmol) was dissolved in DMF (200 ml) before 1-azido-3-chloro-propane (**5**, 11.96 g, 100 mmol, 2 equiv.) and potassium carbonate (13.82 g, 100 mmol, 2 equiv.) were added. The mixture was stirred 48hr. at 90 °C before being concentrated. The residue was taken up in DCM and washed with H_2O and brine, dried over $MgSO_4$ and concentrated. Silica column chromatography (0 → 20% EtOAc in PetEt) yielded the title compound (8.85g, 43 mmol, 86%) as a colourless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 9.88 (s, 1H), 7.83 (d, *J* = 9.1 Hz, 2H), 7.00 (d, *J* = 8.8 Hz, 2H), 4.13 (t, *J* = 5.8 Hz, 2H), 3.53 (t, *J* = 6.2 Hz, 2H), 2.08 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 190.22, 163.29, 131.45, 129.66, 114.34, 64.64, 47.60, 28.29.



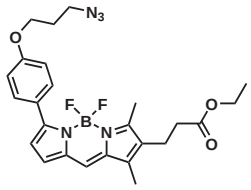
1-(4-(3-Azido-propoxy)-phenyl)-3-[1,3]dioxan-2-yl-propan-1-ol (7). 4-(3-Azido-propoxy)-benzaldehyde (**6**, 13.73 g, 67 mmol) was dissolved in freshly distilled THF (200 ml), put under an argon atmosphere and cooled to -10 °C. (1,3-Dioxane-2-ylethyl)-magnesium bromide (200 ml, 0.5M in THF, 100 mmol, 1.5 equiv.) was added dropwise over 1 hr. The reaction mixture was allowed to warm to room temperature and was stirred 12hr. before being quenched with sat. aq. NH_4Cl. and extracted with EtOAc. The organic layer was separated, dried over Na_2SO_4 and concentrated. Column chromatography (0% → 50% EtOAc in PetEt) yielded **7** (9.61g, 30 mmol, 45%) as a colourless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.24 (d, *J* = 8.8 Hz, 2H), 6.84 (d, *J* = 8.8 Hz, 2H), 4.63 – 4.50 (m, 2H), 4.11 – 3.99 (m, 4H), 3.73 (t, *J* = 11.5 Hz, 2H), 3.50 (t, *J* = 6.6 Hz, 2H), 2.92 (s, 1H), 2.13 – 1.25 (m, 8H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 157.24, 136.89, 126.55, 113.63, 101.47, 72.63, 66.14, 63.96, 47.62, 32.76, 30.88, 28.12, 25.08.



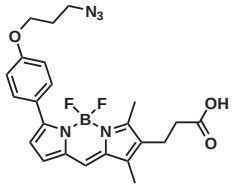
1-(4-(3-Azido-propoxy)-phenyl)-3-[1,3]dioxan-2-yl-propan-1-one (8). 1-(4-(3-Azido-propoxy)-phenyl)-3-[1,3]dioxan-2-yl-propan-1-ol (**7**, 0.8 g, 2.48 mmol) was dissolved in DCM and MnO_2 (2.16 g, 24.8 mmol, 10 equiv.) was added. The reaction mixture was stirred for 12 hr. before being filtered over HyFlo. The filtrate was concentrated *in vacuo* and purified by column chromatography (0% → 25% EtOAc in PetEt) yielding **8** (0.55 g, 1.72 mmol, 70%) as a slight yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.96 (d, *J* = 9.1 Hz, 2H), 6.84 (d, *J* = 8.8 Hz, 2H), 4.66 (t, *J* = 4.9 Hz, 1H), 4.14 – 4.06 (m, 4H), 3.83 – 3.69 (m, 2H), 3.53 (t, *J* = 6.6 Hz, 2H), 3.06 (t, *J* = 7.3 Hz, 2H), 2.13 – 1.99 (m, 5H), 1.37 – 1.29 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 197.37, 162.05, 129.81, 113.68, 100.67, 66.31, 64.40, 47.63, 31.86, 29.16, 28.16, 25.40.



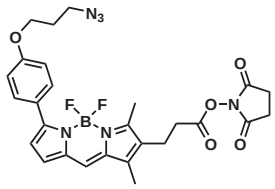
2-(4-(3-Azido-propoxy)-phenyl)-1H-pyrrole (9). To a solution of 1-(4-(3-Azido-propoxy)-phenyl)-3-[1,3]dioxan-2-yl-propan-1-one (**8**, 2.26 g, 7.1 mmol) in AcOH (50 ml) were added NH_4OAc (6.55 g, 85 mmol, 12 equiv.) and Ac_2O (2.5 ml, 26.3 mmol, 3.7 equiv.). The reaction mixture was refluxed for 3 hr., poured into ice water, neutralized with $NaHCO_3$ and extracted with DCM. The DCM layer was dried over Na_2SO_4 and concentrated. Purification by column chromatography (0% → 10% EtOAc in PetEt) gained an inseparable mixture of the title compound **9** and 1,3-diacetoxy-propane. The mixture was dissolved in MeOH and KO*t*Bu was added till pH 9. After stirring for 1 hr. the reaction mixture was neutralized with AcOH, H_2O and DCM were added and the DCM layer was separated, dried over Na_2SO_4 and concentrated *in vacuo* to yield 2-(4-(3-Azido-propoxy)-phenyl)-1H-pyrrole (0.55g, 2.26 mmol, 32%) as a purplish foam. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 8.39 (s, 1H), 7.39 (d, *J* = 8.4 Hz, 2H), 6.90 (d, *J* = 8.8 Hz, 2H), 6.82 (m, 1H), 6.41 (m, 1H), 6.28 (dd, J_1 = 2.6 Hz, J_2 = 5.5 Hz, 1H), 4.06 (t, *J* = 5.8 Hz, 2H), 3.52 (t, *J* = 6.6 Hz, 2H), 2.06 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 156.76, 131.49, 125.73, 124.67, 118.18, 114.54, 109.24, 104.32, 64.29, 47.83, 28.30.



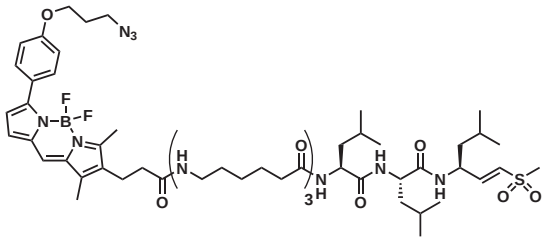
4,4-Difluoro-1,3-dimethyl-2-(2-(ethoxycarbonylethyl))-7-(4-(3-Azido-propoxy)-phenyl)-4-bora-3a,4a-diaza-s-indacene (11). 2-(4-(3-Azido-propoxy)-phenyl-1*H*-pyrrole **9** (0.99 g, 4.1 mmol, 1 equiv.) and carboxyaldehyde pyrrole **10**[17] (0.92 g, 4.1 mmol, 1 equiv.) were dissolved in EtOH (5 ml). The resulting mixture was cooled to 0 °C, and hydrobromic acid, 48% solution in water (0.7 ml, 6.15 mmol, 1.5 equiv.) was added. After 2 hr. stirring, TLC analysis showed complete consumption of the starting materials. The reaction mixture was concentrated *in vacuo*, coevaporated with DCE (3×). The resulting crude dipyrrole HBr salt was dissolved in DCE (50 ml) and put under an argon atmosphere. Triethylamine (1.7 ml, 12.3 mmol, 3 equiv.) and $BF_3{\cdot}Et_2O$ (5.4 ml, 20.5 mmol, 5 equiv.) were added, and the reaction mixture was stirred for 16 hr., before being concentrated *in vacuo* and purified by column chromatography (0% → 2.5% EtOAc in Tol.) yielding the title compound **11** (1.28 g, 2.58 mmol, 63%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.87 (d, *J* = 8.8 Hz, 2H), 7.09 (s, 1H), 6.97 (d, *J* = 8.8 Hz, 2H), 6.96 (d, *J* = 4.4 Hz, 1H), 6.54 (d, *J* = 4.4 Hz, 1H), 4.12 (m, 4H), 3.54 (t, *J* = 6.6 Hz, 2H), 2.73 (t, *J* = 7.7 Hz, 2H), 2.54 (s, 3H), 2.44 (d, *J* = 7.7 Hz, 2H), 2.22 (s, 3H), 2.08 (dt, *J* = 6.3 Hz, 2H), 1.24 (d, *J* = 7.1 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 172.07, 158.97, 158.51, 154.63, 139.50, 134.59, 133.74, 130.16, 129.52, 127.58, 125.07, 122.52, 117.61, 113.66, 64.02, 60.05, 47.62, 33.36, 28.09, 18.78, 13.53, 12.44, 8.71.



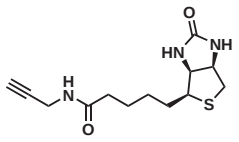
4,4-Difluoro-1,3-dimethyl-2-(2-carboxyethyl)-7-(4-(3-Azido-propoxy)-phenyl)-4-bora-3a,4a-diaza-s- indacene (12). Ethyl ester **11** (89 mg, 0.18 mmol) was dissolved in dioxane (2 ml) and MeOH (2 ml). After addition of 0.1M aqueous NaOH (0.2 mmol, 2 ml, 1.15 equiv.) and stirring overnight, the purple suspension was diluted with EtOAc, extracted with 0.1M HCl, dried over $MgSO_4$ and concentrated. Column chromatography (0% → 1% EtOAc and 1% AcOH in Tol.) yielded acid **12** (30 mg, 64 µmol, 35%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.86 (d, *J* = 9.1 Hz, 2H), 7.09 (s, 1H), 6.96 (d, *J* = 8.8 Hz, 2H), 6.96 (m, 1H), 6.54 (d, *J* = 4.0 Hz, 1H), 4.10 (t, *J* = 6.0 Hz, 2H), 3.53 (t, *J* = 6.6 Hz, 2H), 2.74 (t, *J* = 7.7 Hz, 2H), 2.53 (s, 3H), 2.49 (t, *J* = 6.9 Hz, 2H), 2.22 (s, 3H), 2.07 (dt, *J* = 6.3 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 175.32, 159.24, 155.18, 139.77, 134.80, 134.16, 130.49, 129.92, 127.80, 125.55, 122.79, 118.06, 114.03, 64.35, 48.01, 33.60, 28.48, 19.17, 12.80, 9.22.



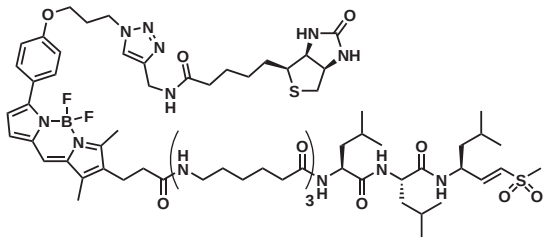
4,4-Difluoro-1,3-dimethyl-2-(2-(succimidyloxycarbonylethyl))-7-(4-(3-Azido-propoxy)-phenyl)-4-bora-3a,4a-diaza-s-indacene (13). Azido-BODIPY-acid **12** (30 mg, 64 µmol) was coevaporated thrice with toluene, before being dissolved in DCM (1 ml). After the addition of *N*-hydroxysuccinimide (29 mg, 0.25 mmol. 4 equiv.) and EDC (48 mg, 0.25 mmol, 4 equiv.), the reaction mixture was stirred for 2 hr. Next, the reaction was diluted with EtOAc, washed with 0.5M aq. HCl, dried over $MgSO_4$ and concentrated. Purification by column chromatography (0% → 4% EtOAc in Tol.) furnished title compound **13** (24 mg, 43 µmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.88 (d, *J* = 9.1 Hz, 2H), 7.12 (s, 1H), 6.97 (d, *J* = 8.8 Hz, 2H), 6.96 (m, 1H), 6.56 (d, *J* = 4.4 Hz, 1H), 4.11 (t, *J* = 5.8 Hz, 2H), 3.54 (t, *J* = 6.6 Hz, 2H), 2.79 (m, 8H), 2.56 (s, 3H), 2.14 (s, 3H), 2.08 (dt, *J* = 6.0 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 168.98, 167.55, 159.45, 158.30, 155.91, 139.71, 135.10, 133.95, 130.65, 128.37, 128.22, 125.43, 123.12, 118.45, 114.09, 64.35, 48.07, 30.69, 28.60, 25.39, 18.96, 12.95, 9.47. HRMS: calcd. for $[C_{27}H_{27}BF_2N_6O_5H]^+$ 565.21768, found 565.21783, for $[C_{27}H_{27}BFN_6O_5]^+$ 545.21145, found 545.21130.



N_3-BODIPY-Ahx$_3$L$_3$VS (3). DBU (3.3 µl, 22 µmol, 1 equiv.) was added to a solution of Fmoc-Ahx$_3$L$_3$VS (**14**)[16] (21.2 mg, 22 µmol) in DMF. After 5 min. of stirring, HOBt (13.4 mg, 0.1 mmol, 4.5 equiv.) was added. To this mixture, **13** (12.4 mg, 22 µmol, 1 equiv.) and DiPEA (22 µl, 0.13 mmol, 6 equiv.) were added, and the mixture was stirred for 30 min. before being concentrated *in vacuo*. Purification by column chromatography (0.1% TEA in DCM → 3% MeOH, 0.1% TEA in DCM) afforded N_3-BODIPY-Ahx$_3$L$_3$VS (**3**) (22.8 mg, 19 µmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD): δ ppm 7.86 (d, *J* = 8.85 Hz, 2H), 7.75-7.60 (m, 3H), 7.51-7.44 (m, 2H), 7.43-7.36 (m, 1H), 7.28-7.22 (m, 1H), 7.20 (s, 1H), 7.03-6.95 (m, 3H), 6.86-6.77 (m, 1H), 6.56 (m, 2H), 4.73-4.60 (m, 1H), 4.38-4.26 (m, 2H), 4.13 (t, *J* = 5.89 Hz, 2H), 3.55 (t, *J* = 6.62 Hz, 2H), 3.21-3.09 (m, 6H), 2.98 (s, 3H), 2.74 (t, *J* = 7.43 Hz, 2H), 2.54 (s, 3H), 2.31 (t, *J* = 7.34 Hz, 2H), 2.27-2.20 (m, 5H), 2.16 (t, *J* = 7.51 Hz, 2H), 2.13-2.05 (m, 4H), 1.73-1.18 (m, 27H), 1.03-0.84 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD): δ ppm 174.52, 174.45, 174.08, 173.20, 172.75, 172.72, 172.63, 159.31, 159.11, 154.85, 147.32, 139.97, 134.64, 134.09, 130.27, 128.72, 127.62, 125.36, 122.67, 117.85, 113.82, 64.23, 51.89, 51.85, 47.58, 46.09, 42.06, 41.95, 40.06, 39.87, 39.85, 38.80, 38.69, 35.66, 35.53, 35.38, 35.33, 28.42, 28.31, 25.96, 25.91, 25.80, 24.96, 24.90, 24.80, 24.41, 24.38, 24.33, 22.33, 22.29, 22.26, 21.12, 21.08, 21.01, 19.91, 8.85, 8.10. HRMS: calcd. for $[C_{61}H_{94}BF_2N_{11}O_9SH]^+$ 1206.70906, found 1206.71092, for $[C_{61}H_{94}BF_2N_{11}O_9SNa]^+$ 1228.69100, found 1228.69269, for $[C_{61}H_{94}BF_2N_{11}O_9SK]^+$ 1244.66494, found 1244.66770. λ_{abs} = 541.94 nm, λ_{em} = 570.00 nm, ε = 62488 liter mol^{-1} cm^{-1}.

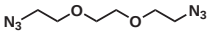


Biotin-propargylamide (15). Propargylamine (561 µl, 8.19 mmol, 1 equiv.) was added dropwise to a cooled solution (0 °C) of D-(+)-biotin (2.0 g, 8.19 mmol), HCTU (3.39 g, 8.19 mmol, 1 equiv.), DiPEA (2.85 ml, 16.38 mmol, 2 equiv.) and *N*,*N*-dimethyl-4-aminopyridin (cat.) in DMF (16 ml). The reaction mixture was allowed to reach room temperature over 3 hr., after which TLC indicated the disappearance of the starting material. After reduction of the volume under reduced pressure (~1/2), excess EtOAc was added and the resulting suspension was stored overnight at -20 °C. Filtration and rinsing with EtOAc and Et_2O ultimately afforded the title compound as an off-white powder (2.03 g, 7.21 mmol, 88%). ^{1}H NMR (400 MHz, DMSO-*d6*): δ ppm 8.21 (t, *J* = 5.4 Hz, 1H), 6.42 (s, 1H), 6.35 (s, 1H), 4.30 (dd, J_1 = 5.0 Hz, J_2 = 7.5 Hz, 1H), 4.08 – 4.16 (m, 1H), 3.83 (dd, J_1 = 2.5 Hz, J_2 = 5.6 Hz, 2H), 3.08 – 3.14 (m, 1H), 3.07 (t, *J* = 2.5 Hz, 1H), 2.82 (dd, J_1 = 5.0, J_2 = 12.5 Hz, 1H), 2.57 (d, *J* = 12.3 Hz, 1H), 2.08 (t, *J* = 7.5 Hz, 2H), 1.20 – 1.67 (m, 7H). ^{13}C NMR (100 MHz, DMSO-*d6*): δ ppm 171.8, 162.7, 81.4, 72.8, 61.0, 59.2, 55.4, 34.9, 28.2, 28.0, 27.7, 25.1. HRMS: calcd. for $[C_{13}H_{19}N_3O_2S]^+$ 282.12707, found 282.12714.

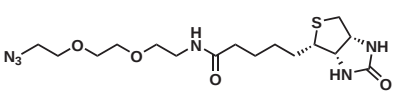


Biotin-BODIPY-Ahx$_3$L$_3$VS (16). N_3-BODIPY-Ahx$_3$L$_3$VS (**3**) (5.6 mg, 4.6 µmol) and Biotin-propargylamide (**15**) (2.6 mg, 9.2 µmol, 2 equiv.) were dissolved in *t*BuOH (0.25 ml) before aqueous solutions of $CuSO_4$ (125 µl 3.7 mM, 10 mol%) and sodium ascorbate (125 µl 7.4 mM, 20 mol%) were added. The reaction mixture was stirred for 12 hr., concentrated and purified by size-exclusion chromatography (Sephadex LH-20, eluens: MeOH) to give the title compound as a brown/red solid (6.9 mg, 4.6 µmol, quant.). ^{1}H NMR (600 MHz, $CDCl_3$/MeOD): δ ppm 7.88-7.85 (m, 1H), 7.82 (d, *J* = 8.74 Hz, 2H), 7.55 (s, 1H), 7.08 (d, *J* = 3.97 Hz, 1H), 6.95 (d, *J* = 8.77 Hz, 2H), 6.66 (dd, J_1 = 15.20 Hz, J_2 = 5.02 Hz, 1H), 6.62 (d, *J* = 3.97 Hz, 1H), 6.54 (d, *J* = 15.23 Hz, 1H), 4.57-4.48 (m, 3H), 4.34-4.27 (m, 3H), 4.27-4.19 (m, 2H), 4.13-4.08 (m, 1H), 4.01 (t, *J* = 5.89 Hz, 2H), 3.10-3.04 (m, 1H), 3.03-2.95 (m, 6H), 2.91 (s, 3H), 2.79 (dd, J_1 = 12.53 Hz, J_2 =5.00 Hz,

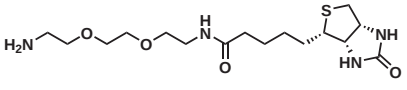
1H), 2.62 (t, *J* = 7.43 Hz, 2H), 2.58 (d, *J* = 12.54 Hz, 1H), 2.43 (s, 3H), 2.31-2.25 (m, 2H), 2.23-2.17 (m, 5H), 2.16-2.06 (m, 4H), 2.02 (t, *J* = 7.42 Hz, 2H), 1.99 (t, *J* = 7.47 Hz, 2H), 1.65-1.07 (m, 33H), 0.91-0.74 (m, 18H). ^{13}C NMR (150 MHz, $CDCl_3$/MeOD): δ ppm 173.79, 173.23, 173.03, 172.32, 172.19, 160.34, 159.72, 154.49, 147.35, 146.45, 145.65, 141.19, 135.36, 134.72, 131.72, 131.37, 131.02, 130.99, 130.96, 130.93, 130.07, 129.52, 128.61, 125.91, 124.40, 124.36, 123.39, 123.33, 118.35, 114.77, 114.71, 65.09, 61.78, 60.00, 56.07, 52.08, 51.97, 47.12, 42.50, 42.46, 40.87, 40.79, 40.30, 35.97, 35.93, 35.72, 35.57, 34.65, 30.14, 29.42, 29.33, 28.81, 28.59, 26.68, 26.64, 25.79, 25.69, 25.66, 25.62, 24.89, 24.86, 24.73, 23.17, 23.14, 21.83, 21.76, 21.52, 13.44, 13.12, 9.38. HRMS: calcd. for $[C_{74}H_{113}BF_2N_{14}O_{11}S_2]^+$ 1487.82885, found 1487.83093. λ_{abs} = 551.94 nm, λ_{em} = 574.05 nm, ε = 59325 liter mol^{-1} cm^{-1}.



1,2-bis(2-azidoethoxy)ethane (18). Triethyleneglycol (**17**, 0.3 g, 2 mmol) was dissolved in DCM and put under an argon atmosphere, before tosylchloride (1.14 g, 6 mmol, 3 equiv.), triethylamine (0.83 ml, 6 mmol, 3 equiv.) and *N*,*N*-dimethyl-4-aminopyridin (12 mg, 0.1 mmol, 5 mol%) were added. After 16 hr. the reaction mixture was washed with H_2O. The organic phase was separated, dried over $MgSO_4$ and concentrated *in vacuo*, resulting a yellowish oil which was dissolved in DMF. NaN_3 (0.26 g, 4 mmol, 2 equiv.) and tetrabutylammonium iodide (37 mg, 0.1 mmol, 5 mol%) were added and the reaction mixture was stirred at 80 °C for 16hr., before being washed with sat. aq. $NaHCO_3$. The organic phase was separated, dried over $MgSO_4$ and concentrated to a yellow oil. Purification by column chromatography (Tol. → 15% EtOAc in Tol.) afforded the bis-azide **18** as a colourless oil (0.31 g, 1.56 mmol, 78%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 3.68 (m, 8H), t, 3.39 (*t*, *J* = 5.1 Hz, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 70.3, 69.8, 50.3.

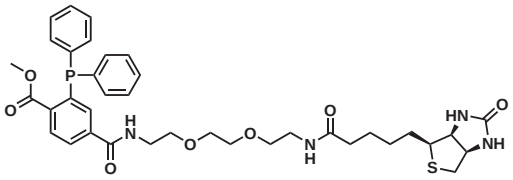


***N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)biotinylamide (20).** An aqueous 5% HCl solution (10 ml) was added to a cooled solution (0 °C) of 1,2-bis(2-azidoethoxy)ethane (**18**, 2.0 g, 10 mmol) in Tol. (10 ml), before triphenylphoshine (2.5 g, 9.5 mmol, 0.95 equiv.) was added. The reaction mixture was allowed to warm up to room temperature and was stirred 16 hr., after which the aqueous layer was separated and concentrated *in vacuo* to yield the crude 2-(2-(2-azidoethoxy)ethoxy)ethylamine HCl salt (1.67, 7.9 mmol, 79%). The HCl salt (0.83 g, 3.95 mmol) was coevaporated with Tol. (3×) and dissolved in DMF. D-(+)-biotin (0.98 g, 4 mmol, 1.01 equiv.), BOP (1.77 g, 4 mmol, 1.01 equiv.) and DiPEA (1.99 ml, 12 mmol, 3.03 equiv.) were added and the reaction mixture was stirred 16 hr. before the reaction volume was reduced under reduced pressure (~1/2). The crude title compound was crashed out with excess EtOAc. Recrystallization from MeOH/EtOAc gave *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)biotinylamide (**20**) as an off-white powder (0.95 g, 2.37 mmol, 60%). ^{1}H NMR (200 MHz, MeOD): δ ppm 4.50 (ddd, J_1 = 7.8 Hz, J_2 = 4.8 Hz, J_3 = 0.8 Hz, 1H), 4.31 (dd, J_1 = 7.9 Hz, J_2 = 4.4 Hz, 1H), 3.72-3.60 (m, 6H), 3.56 (t, *J* = 5.6 Hz, 2H), 3.42-3.34 (m, 4H), 3.27-3.15 (m, 1H), 2.93 (dd, J_1 = 12.7 Hz, J_2 = 4.9 Hz, 1H), 2.70 (d, *J* = 12.8 Hz, 1H), 2.22 (t, *J* = 7.2 Hz, 1H), 1.82-1.34 (m, 6H). ^{13}C NMR (50 MHz, MeOD/Acetone-*d6*): δ ppm 175.65, 71.36, 71.20, 70.96, 70.57, 63.11, 61.41, 56.88, 51.64, 41.09, 40.31, 36.70, 29.65, 29.40, 26.75.

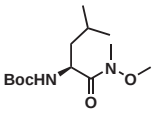


***N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)biotinylamide (21).** To a stirred solution of **20** (0.95 g, 2.37 mmol) in DMF, triphenylphosphine (0.93 g, 3.56 mmol, 1.5 equiv.) was added. After 1 hr. a drop of H_2O was added and the reaction mixture was stirred 16 hr., before an excess EtOAc was added. The title compound was filtered and washed with EtOAc, resulting an off-white powder (0.7 g, 1.86 mmol, 78%). ^{1}H NMR (200 MHz, MeOD): δ ppm 4.50 (dd, J_1 = 7.6 Hz, J_2 = 4.8 Hz, 1H), 4.31 (dd, J_1 = 7.7 Hz, J_2 = 4.3 Hz, 1H), 3.77-3.12 (m, 12H), 3.03-2.79 (m, 2H), 2.71 (d, *J* = 12.7 Hz, 1H), 2.23 (t, *J* = 7.0 Hz, 2H), 1.85-1.33 (m, 6H). ^{13}C NMR (50 MHz,

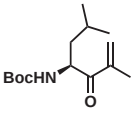
MeOD/Acetone-*d6*): δ ppm 176.27, 165.81, 72.38, 71.29, 70.93, 70.35, 63.13, 61.34, 56.73, 41.39, 40.99, 40.66, 40.05, 36.60, 29.50, 29.20, 26.59.



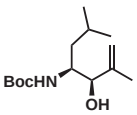
Biotin-phosphane (23). Amine **21** (0.11 g, 0.3 mmol) was coevaporated with Tol. (3×), dissolved in DMF and put under argon atmosphere, before 3-(diphenylphosphino)-4-(methoxycarbonyl)benzoic acid[6] (0.17 g, 0.33 mmol, 1.1 equiv.), EDC·HCl (85 mg, 0.45 mmol, 1.5 equiv.) and *N,N*-dimethyl-4-aminopyridin (cat.) were added. After 16 hr. the reaction mixture was concentrated *in vacuo*. Purification by column chromatography (first Acetone → 3% H_2O in acetone, followed by DCM → 6% MeOH in DCM) afforded Biotin-phosphane (**7**) (51 mg, 70 µmol, 23%). ^{1}H NMR (200 MHz, Acetone-*d6*, D_2O): δ ppm 7.99 (dd, J_1 = 8.1 Hz, J_2 = 3.5 Hz, 1H), 7.87 (dd, J_1 = 8.1 Hz, J_2 = 1.7 Hz, 1H), 7.47 (dd, J_1 = 3.9 Hz, J_2 = 1.7 Hz, 1H), 7.37-7.15 (m, 10H), 4.46 (dd, J_1 = 7.9 Hz, J_1 = 4.6 Hz, 1H), 4.26 (dd, J_1 = 7.9 Hz, J_2 = 4.4 Hz, 1H), 3.61 (s, 3H), 3.58-3.37 (m, 10H), 3.34-3.24 (m, 2H), 3.18-3.06 (m, 1H), 2.85 (dd, J_1 = 12.8 Hz, J_2 = 4.8 Hz, 1H), 2.64 (d, J = 12.7 Hz, 1H), 2.13 (t, J = 7.3 Hz, 2H), 1.79-1.14 (m, 6H). ^{13}C NMR (50 MHz, Acetone-*d6*, D_2O): δ ppm 176.16, 168.39, 142.29, 141.74, 139.01, 138.83, 138.61, 135.51, 135.09, 134.81, 134.75, 132.03, 130.70, 130.34, 130.21, 128.42, 128.38, 71.32, 70.81, 70.66, 63.24, 61.56, 57.05, 53.39, 41.45, 41.15, 40.48, 37.01, 29.83, 29.55, 26.95. ^{31}P NMR (81.1 MHz, Acetone-*d6*/D_2O): δ ppm -3.12. HRMS: calcd. for $[C_{37}H_{45}N_4O_7PSH]^+$ 721.28193, found 721.28186.



(Boc-leucinyl)-*N,O*-di-methyl-hydroxamide (25). To a solution of Boc-Leu-OH (**24**) (2.5 g, 10 mmol) in DCM were added BOP (5.3 g, 12 mmol, 1.2 equiv.), DiPEA (4.1 ml, 25 mmol, 2.5 equiv.) and *N,O*-dimethylhydroxylamine·HCl (2.9 g, 30 mmol, 3.0 equiv.) and the reaction mixture was stirred overnight, before being concentrated *in vacuo*. The residue was taken up in EtOAc, washed subsequently with 1M aqueous HCl solution (3x), saturated aqueous $NaHCO_3$ solution (3x) and Brine (1x). The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (10% EtOAc in Tol. → 20% EtOAc in Tol.), yielding the title compound **25** (2.2 g, 7.9 mmol, 79%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 5.06 (d, J = 9.0 Hz, 1H), 4.66 (td, J_1 = 8.0, J_2 = 7.2 Hz, 1H), 3.74 (s, 3H), 3.15 (s, 3H), 1.79-1.46 (m, 1H), 1.44-1.20 (m, 11H), 0.94-0.83 (m, 6H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 172.95, 154.85, 78.00, 60.56, 48.13, 40.82, 31.24, 27.51, 23.84, 22.51, 20.72.

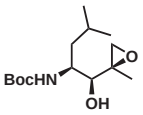


(Boc-leucinyl)-isopropene (26). 2-Bromopropene (1.2 ml, 14 mmol, 3.0 equiv.) was dissolved in freshly distilled Et_2O, put under argon atmosphere and cooled to -78 °C. After adding *t*BuLi (13 ml 1.6 M in pentane, 21 mmol, 4.5 equiv.), the reaction mixture was stirred for 15 min. Weinreb amide **25** (1.3 g, 4.6 mmol, 1.0 equiv.) was coevaporated with Tol., dissolved in freshly distilled Et_2O and put under argon atmosphere. The solution was cooled to -78 °C and added to the reaction mixture containing the lithium reagent using an argon-flushed *canula*. The resulting reaction mixture was stirred for 2 hr. and allowed to warm up to room temperature, before being quenched with sat. aq. NH_4Cl. The mixture was extracted with EtOAc (3x) and the combined organics were washed with Brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (Tol. → 1% EtOAc in Tol.) afforded compound **26** (0.93 g, 3.6 mmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.11 (s, 1H), 5.88 (s, 1H), 5.44 (d, J = 8.8 Hz, 1H), 5.12-5.06 (m, 1H), 1.90 (s, 3H), 1.75-1.68 (m, 1H), 1.49-1.34 (m, 11H), 1.01 (d, J = 6.4 Hz, 3H), 0.91 (d, J = 6.4 Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 201.04, 155.19, 141.96, 125.46, 78.86, 52.19, 42.52, 27.93, 24.55, 22.97, 21.34, 17.42.

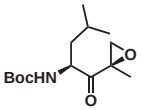


(1*S*,2*S*)-2-Boc-amino-4-methyl-1-(isopropenyl)pentan-1-ol (27). A solution of (Boc-leucinyl)-isopropene (**26**, 0.46 g, 1.8 mmol) in methanol was put under argon atmosphere and

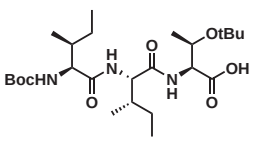
$CeCl_3 \cdot 7H_2O$ (1.0 g) was added. The solution was cooled to 0 ºC, before $NaBH_4$ (95 mg, 2.5 mmol, 1.4 equiv.) was added in 6 portions over 3 min. After stirring for 10 min., the reaction was quenched with glacial acetic acid and Tol. was added after an additional 20 min at 0 ºC. The solvents were removed *in vacuo* and the oily residue was dissolved in an EtOAc/H_2O mixture. The organic layer was washed with H_2O and Brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography (Tol. → 10% EtOAc in Tol.) yielded **27** (0.40 g, 1.6 mmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 5.02 (s, 1H), 4.97-4.92 (m, 1H), 4.72 (d, *J* = 8.6 Hz, 1H), 4.14 (s, 1H), 3.93-3.69 (m, 1H), 1.76 (s, 3H), 1.70-1.52 (m, 1H), 1.45 (s, 9H), 1.37-1.08 (m, 2H), 0.97-0.84 (m, 6H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 156.03, 144.69, 111.03, 79.03, 77.43, 50.65, 36.82, 28.18, 24.39, 23.60, 21.26, 19.17.



(1*S*,2*S*)-2-Boc-amino-4-methyl-1-((*R*)-2-methyloxiran-2-yl)pentan-1-ol (28). Compound **27** (0.53 g, 2.1 mmol) was dissolved in anhydrous DCM and cooled to 0 ºC. Next, $VO(acac)_2$ (22 mg, 0.080 mmol, 0.04 equiv.) and *t*BuOOH (1.1 ml 5.5 M in decane, 6.2 mmol, 3.0 equiv.) were added and the reaction mixture was stirred for 2 hr., allowing the temperature to rise slowly to RT. The resulting purple solution was concentrated *in vacuo* and the residue was dissolved in EtOAc and washed with a half saturated $NaHCO_3$ solution. The aqueous layer was extracted with EtOAc and the combined organic layers were washed with H_2O and Brine. The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (PetEt → 10% EtOAc in PetEt), yielding epoxide **28** (0.29 g, 1.1 mmol, 51%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 4.88 (d, *J* = 9.5 Hz, 1H), 3.91-3.85 (m, 2H), 2.99 (d, *J* = 5.1 Hz, 1H), 2.64 (d, *J* = 5.1 Hz, 1H), 1.73-1.63 (m, 1H), 1.45 (s, 9H), 1.38 (s, 3H), 1.23-1.00 (m, 2H), 0.96 (d, *J* = 4.0 Hz, 3H), 0.93 (d, *J* = 4.4 Hz, 3H).

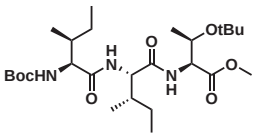


(Boc-leucinyl)-(*R*)-2-methyloxirane (29). Dess-Martin periodinane (1.2 g, 2.9 mmol, 3.0 equiv.) was dissolved in DMSO, put under argon atmosphere and cooled to 0 ºC. Epoxy alcohol **28** (0.26 g, 0.95 mmol) was coevaporated with Tol., dissolved in DMSO and added to the first solution. The reaction mixture was stirred for 4 hr. and allowed to warm up to room temperature, before sat. aq. $NaHCO_3$ was added. The layers were separated, the aqueous layer was extracted with EtOAc and the combined organic layers were washed with H_2O (3x), dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. Because DMSO was still present, the residue was dissolved in EtOAc and washed with H_2O (2x) and Brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo* and the crude product was purified by column chromatography (PetEt → 5% EtOAc in PetEt) to give the leucine-derived epoxyketone **29** (0.23 g, 0.85 mmol, 90%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 4.90 (d, *J* = 9.1 Hz, 1H), 4.32 (dt, J_1 = 10.4, J_2 = 3.08 Hz, 1H), 3.30 (d, *J* = 5.0 Hz, 1H), 2.90 (d, *J* = 5.0 Hz, 1H), 1.84-1.59 (m, 1H), 1.52 (s, 3H), 1.41 (s, 9H), 1.27-1.08 (m, 2H), 1.01-0.89 (m, 6H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 212.13, 155.69, 79.52, 58.74, 52.04, 51.28, 39.82, 27.96, 24.81, 23.02, 20.87, 16.41.

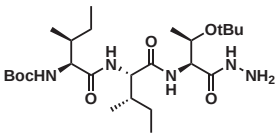


Boc-Ile-Ile-Thr(*t*Bu)-OH (31). 4-methylbenzhydrylamine (MBHA) functionalized polystyrene resin (5.0 g 1.2 mmol/g, 6.0 mmol) was coupled to 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker (4.3 g, 18 mmol, 3 equiv.) in the presence of BOP (8.0 g, 18 mmol, 3 equiv.) and DiPEA (6.3 ml, 36 mmol, 6 equiv.) in NMP. After shaking overnight, the resin was washed with NMP (3x) and DCM (3x). The coupling was monitored for completion by the Kaiser test. The resulting MBHA-HMPB resin (~6.0 mmol) was coevaporated with DCE (2x). The resin was then condensed with Fmoc-Thr(*t*Bu)-OH (7.2 g, 18 mmol, 3 equiv.) under the influence of DIC (3.1 ml, 20 mmol, 3.3 equiv.) and DMAP (0.11 g, 0.90 mmol, 15 mol%) in DCM for 2 hr. After the resin was filtered and washed with DCM, the condensation cycle was repeated. The resin was then washed with NMP (2x), DCM (2x) and ether (2x) and dried *in vacuo* overnight. The loading of the resin was

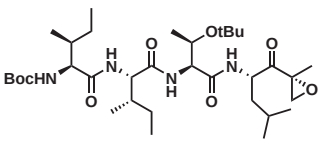
determined to be 0.43 mmol/g by spectrophotometric analysis. The obtained resin was washed with DCM and submitted to two cycles of Fmoc solid phase synthesis with Fmoc-Ile-OH and Boc-Ile-OH, respectively, as follows: after deprotection with 20% piperidine in NMP (20 min.), the resin was washed with NMP (2x) and DCM (2x) and coupled to Fmoc-Ile-OH (5.3 g, 15 mmol, 2.5 equiv.) or Boc-Ile-OH (3.5 g, 15 mmol, 2.5 equiv.) in the presence of BOP (6.6 g, 15 mmol, 2.5 equiv.) and DiPEA (3.1 ml, 18 mmol, 3 equiv.) in NMP. The reaction mixture was shaken overnight and for 5 hr. respectively, followed by washing with NMP (3x) and DCM (3x). Couplings were monitored for completion by the Kaiser test. Washing with ether and drying *in vacuo* overnight yielded the fully protected resin tripeptide. MBHA-HMPB-Thr(*t*Bu)-Ile-Ile-Boc resin (~6.0 mmol) was subjected to mild acidic cleavage with 1% TFA in DCM (10 min, 6x). The collected fractions were concentrated in the presence of toluene to yield the crude tripeptide **31**, which was used without further purification. LC/MS analysis: R_t 8.58 min (linear gradient 10 → 90% B in 13.5 min.), *m/z* 502.3 $[M + H]^+$, 524.5 $[M + Na]^+$, 1025.3 $[2M + Na]^+$.



Boc-Ile-Ile-Thr(*t*Bu)-methyl ester (32). The crude Boc-Ile-Ile-Thr(*t*Bu)-OH (**31**) (~3.0 g, ~6.0 mmol) was dissolved in MeOH/Tol (1/1) and treated with TMS-diazomethane (6 ml 2M in hexanes, 12 mmol, 2 equiv.) for 15 min. before being coevaporated with Tol. (3x). Purification by flash column chromatography (DCM → 0.5% MeOH in DCM) yielded the title compound as a white solid (2.0 g, 3.8 mmol, 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.51 (d, *J* = 8.1 Hz, 1H), 6.43 (d, *J* = 9.3 Hz, 2H), 4.48 (dd, J_1 = 9.1, J_2 = 1.6 Hz, 1H), 4.40 (dd, J_1 = 8.6, J_2 = 6.5 Hz, 1H), 4.24 (dq, J_1 = 6.2, J_2 = 1.6 Hz, 1H), 3.70 (s, 3H), 3.98-3.88 (m, 1H), 2.07-1.97 (m, 2H), 1.92-1.81 (m, 4H), 1.44 (s, 9H), 1.14 (d, *J* = 6.3 Hz, 3H), 1.11 (s, 9H), 0.99-0.86 (m, 12H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 171.052, 170.857, 67.206, 57.828, 57.502, 57.464, 52.138, 37.857, 37.233, 37.215, 37.195, 37.026, 28.320, 28.294, 24.875, 24.790, 24.759, 20.921, 15.532, 15.083, 11.414, 11.313.

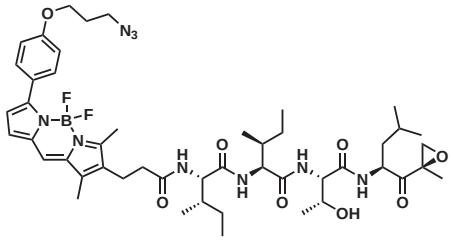


Boc-Ile-Ile-Thr(*t*Bu)-hydrazide (33). To the solution of methyl ester **32** (2.0 g, 3.8 mmol) in MeOH was added hydrazine monohydrate (11.1 ml, 228 mmol, 60 equiv.) and the reaction mixture was refluxed overnight, before being concentrated in the presence of toluene. The white precipitate was filtered and washed with MeOH to give the hydrazide **33** (0.88 g, 1.7 mmol, 45%). The filtrate was concentrated in the presence of toluene and recrystallized from toluene/MeOH to yield a second batch of product (**33**) (0.61 g, 1.2 mmol, 31%). (Total yield: 2.9 mmol, 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 8.50 (s, 1H), 7.79-7.71 (m, 1H), 7.54-7.45 (m, 2H), 6.03-5.95 (m, 2H), 4.47-4.35 (m, 1H), 4.28 (d, *J* = 7.3 Hz, 1H), 4.08 (d, *J* = 1.2 Hz, 1H), 3.94 (d, *J* = 6.4 Hz, 1H), 1.97-1.72 (m, 4H), 1.63-1.49 (m, 2H), 1.45 (s, 9H), 1.21 (s, 9H), 1.08 (d, *J* = 6.3 Hz, 3H), 0.92 (dd, J_1 = 14.7, J_2 = 7.6 Hz, 12H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 172.90, 171.27, 169.84, 79.50, 74.62, 66.18, 58.82, 58.74, 57.90, 57.84, 57.74, 56.79, 36.38, 36.14, 27.64, 27.51, 24.32, 24.24, 18.04, 14.96, 14.85, 14.75, 10.36.

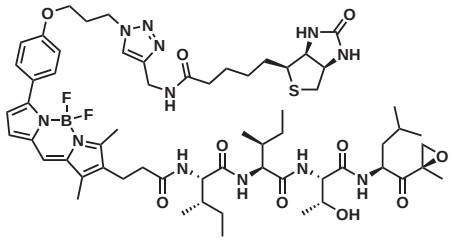


Boc-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (34). A solution of Boc-Ile-Ile-Thr(*t*Bu)-hydrazide (**33**) (0.52 g, 1.0 mmol) in DMF/EtOAc (1/1, v/v) was put under argon atmosphere and cooled to -30°C. After adding HCl (0.70 ml 4M in dioxane, 2.8 mmol, 2.8 equiv.) and *t*BuONO (0.13 ml, 1.1 mmol, 1.1 equiv.), the reaction mixture was stirred for 1 hr. to generate the acyl azide. (Boc-leucinyl)-(*R*)-2-methyloxirane (**29**) (0.30 g, 1.1 mmol, 1.1 equiv.) was stirred in TFA for 10 min. The reaction mixture was concentrated in the presence of toluene, the crude leucinyl-2-methyloxirane TFA salt (**30**) was dissolved in DMF and DiPEA (0.19 ml, 1.1 mmol, 1.1 equiv.) was added. The resulting solution was added to the acyl azide reaction mixture at -30°C. DiPEA was added (0.66 ml, 3.8 mmol, 3.8 equiv.) and the reaction mixture was allowed to warm up to room temperature overnight. EtOAc was added and the mixture was washed with H_2O (3x). The organic layer

was dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 25% EtOAc in PetEt) afforded the fully protected epoxyketone **34** as white crystals (058 g, 0.89 mmol, 89%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.64 (d, *J* = 7.1 Hz, 1H), 6.85 (d, *J* = 5.8 Hz, 1H), 6.46 (d, *J* = 8.6 Hz, 1H), 5.07 (d, *J* = 8.3 Hz, 1H), 4.54-4.38 (m, 1H), 4.37-4.25 (m, 2H), 4.20-4.06 (m, 1H), 3.99-3.87 (m, 1H), 3.13 (dd, J_1 = 93.1, J_2 = 5.0 Hz, 2H), 1.97-1.53 (m, 5H), 1.52 (s, 3H), 1.44 (s, 9H), 1.28 (s, 9H), 1.06 (d, *J* = 6.4 Hz, 3H), 1.37-1.02 (m, 4H), 0.99-0.82 (m, 18H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 208.01, 171.74, 171.13, 169.52, 155.91, 79.24, 75.27, 66.48, 59.11, 58.99, 57.29, 56.77, 52.35, 50.62, 39.64, 37.27, 37.06, 28.21, 27.96, 25.30, 24.90, 24.60, 23.26, 21.20, 16.62, 16.38, 15.35, 15.20, 11.23, 11.04.



Azido-BODIPY-epoxomicin (36). Boc-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (**34,** 7.9 mg, 12 µmol) was dissolved in TFA (1 ml) and stirred for 30 min., before being coevaporated with Tol. (3x). The crude TFA·H-Ile$_2$-Thr-Leu-epoxyketone (**35**) was dissolved in DMF and azido-BODIPY-OSu (**9**, 6.6 mg, 12 µmol, 1 equiv.) and DiPEA (8 µl, 48 µmol, 4 equiv.) were added and the reaction mixture was stirred for 12 hr. Concentration *in vacuo*, followed by purification by column chromatography (DCM → 2% MeOH in DCM) yielded the title compound as a brown/red solid (5.4 mg, 5.7 µmol, 47%). ^{1}H NMR (600 MHz, MeOD): δ ppm 7.88 (d, *J* = 8.7 Hz, 2H), 7.41 (s, 1H), 7.06 (d, *J* = 3.9 Hz, 1H), 6.99 (d, *J* = 8.7 Hz, 2H), 6.60 (d, *J* = 3.9 Hz, 1H), 4.55 (dd, J_1 = 10.7, J_2 = 2.8 Hz, 1H), 4.30 (d, *J* = 5.0 Hz, 1H), 4.22 (d, *J* = 7.8 Hz, 1H), 4.15-4.12 (m, 3H), 4.02 (p, *J* = 6.1 Hz, 1H), 3.54 (t, *J* = 6.7 Hz, 2H), 3.25 (d, *J* = 5.1 Hz, 1H), 2.92 (d, *J* = 5.1 Hz, 1H), 2.81 (m, 1H), 2.71 (m, 1H), 2.51 (s, 3H), 2.45-2.40 (m, 2H), 2.25 (s, 3H), 2.07 (p, *J* = 6.3 Hz, 2H), 1.89-1.79 (m, 1H), 1.75-1.66 (m, 2H), 1.65-1.52 (m, 2H), 1.53-1.41 (m, 5H), 1.41-1.21 (m, 15H), 1.20-1.06 (m, 5H), 1.05-0.97 (m, 1H), 0.97-0.85 (m, 16H), 0.82 (d, *J* = 6.7 Hz, 3H), 0.76 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (150 MHz, MeOD): δ ppm 209.51, 174.86, 174.06, 173.59, 172.23, 161.03, 160.67, 156.57, 141.79, 136.67, 135.83, 132.45, 131.92, 131.89, 131.86, 131.67, 131.65, 129.91, 129.28, 127.16, 124.70, 119.10, 115.27, 115.19, 69.14, 68.55, 65.98, 60.13, 59.82, 59.42, 59.41, 53.10, 51.84, 40.38, 38.02, 37.71, 36.45, 30.82, 29.90, 26.26, 26.03, 23.81, 21.52, 21.21, 20.02, 17.05, 15.92, 15.86, 11.47, 11.22, 9.67.



Biotin-BODIPY-epoxomicin (37). Azido-BODIPY-epoxomicin (**36,** 4.1 mg, 4.3 µmol) and Biotin-propargylamide (**15**) (2.4 mg, 8.6 µmol, 2 equiv.) were dissolved in *t*BuOH (0.25 ml) and toluene (0.25 ml) before $CuSO_4$ (125 µl 3.4 mM, 10 mol%) and sodium ascorbate (125 µl 6.9 mM, 20 mol%) were added. The reaction mixture was stirred at 80 °C for 12 hr., before being cooled to room temperature and concentrated *in vacuo*. Purification by column chromatography (PE → 50% acetone in PE) yielded the title compound as a brown/red solid (4.5 mg, 3.7 µmol, 85%). ^{1}H NMR (600 MHz, MeOD): δ ppm 7.95-7.78 (m, 3H), 7.42 (s, 1H), 7.07 (d, *J* = 4.1 Hz, 1H), 6.95 (d, *J* = 8.9 Hz, 2H), 6.61 (d, *J* = 4.1 Hz, 1H), 4.70-4.52 (m, 5H), 4.46-4.39 (m, 2H), 4.34-4.26 (m, 1H), 4.25-4.19 (m, 1H), 4.17-4.11 (m, 1H), 4.08-3.99 (m, 3H), 3.95 (t, *J* = 2.2 Hz, 1H), 3.25 (d, *J* = 5.0 Hz, 1H), 3.16-3.10 (m, 1H), 2.92 (d, *J* = 5.1 Hz, 1H), 2.71-2.64 (m, 2H), 2.60-2.56 (m, 1H), 2.51 (s, 3H), 2.46-2.37 (m, 4H), 2.26 (s, 3H), 2.24-2.17 (m, 2H), 1.95-1.21 (m, 32H), 1.21-1.10 (m, 5H), 1.06-0.85 (m, 17H), 0.82 (d, *J* = 6.8 Hz, 3H), 0.76 (t, *J* = 7.3 Hz, 3H).

Proteasome labeling in living cells.

EL4 cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 µg/ml streptomycin in a 5% CO_2 humidified incubator at 37 °C. Some $2{\cdot}10^6$ cells were seeded in 6 cm Petri dishes and allowed to grow O/N in 1 ml of medium. The cells were exposed to the indicated concentrations of

the probe (10 µl 100× solution in DMSO) for 2 hr., before being washed with PBS (2×) and harvested. After flash freezing (N_2 (l)) the cells were lysed in 50 µl digitonin lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl, 1 mM DTT, 0.025% digitonin) for 5 min. on ice and centrifuged at 16.100 rcf. for 20 min. at 4 °C. The supernatant containing the cytosolic fraction was collected and the protein content was determined by Bradford assay. In a one-step labeling experiment, at this point to some 25 µg protein 4x Laemli's sample buffer containing beta-mercapto ethanol was added and the samples were boiled for 5 min., before being separated on 12.5% SDS-PAGE. In a two-step labeling experiment some 25 µg protein was incubated with 100 µM biotin-phosphine **23** in 20 µl lysis buffer containing 5 mM DTT for 1 hr. at 37 °C. The reaction was terminated by a chloroform/methanol precipitation of the proteins.[22] The pellet was solubilized by boiling for 5 min. in 1x Laemli's sample buffer containing beta-mercapto ethanol. The proteins were resolved by 12.5% SDS-PAGE. Labeled proteasome subunits were visualized by in-gel fluorescence scanning on a Typhoon variable mode imager (Amersham Biosciences) followed 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 30 min. with Streptavidin-HRP (1:10000) in blocking buffer, washed and visualised using an ECL+ kit (Amersham Biosciences).

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5 Mixing of peptides and electrophilic traps gives rise to potent, broad-spectrum proteasome inhibitors.

M. Verdoes, B.I. Florea, W.A. van der Linden, D. Renou, A.M. van den Nieuwendijk, G.A. van der Marel, H.S. Overkleeft, Organic and Biomolecular Chemistry **2007**, *5*, 1416-1426.

5.1 Introduction

The development and use of proteasome inhibitors has found wide attention in recent years, both in fundamental and applied sciences.[1] The proteasome is a multicatalytic proteinase complex that is involved in many biological processes in man. Its primary function is the processing to oligopeptides of cytosolic and nuclear proteins, as well as *N*-linked glycoproteins that are rejected from the ER due to improper folding.[2] These substrates are marked for proteasomal degradation through the attachment of ubiquitin chains at specific sites. The ubiquitin modifications allow docking to one of the two 19S caps that, together with the inner catalytic 20S core, form the mammalian 26S proteasome. The approval of the peptide boronic acid PS341[3] (bortezomib, **1**, Figure 1) for the clinical treatment of multiple myeloma has led to a surge of activities in proteasome research. PS341 is a highly active proteasome inhibitor, but treatment with PS341 results in severe side effects. It is not clear yet whether this is the result of proteasome blockade or because of other factors that interact with the compound. The development of new and potentially more active or selective proteasome inhibitors may provide information on the mode of action of PS341 and open ways to develop more proteasome inhibitor based therapies in oncology.

Of interest also is the study of the role of the individual proteasomal proteolytic activities, which can be furthered greatly by the use of well-defined inhibitors. The mammalian proteasome differs from the corresponding prokaryotic particles in that the latter has only one type of catalytic activity. The overall shape of the prokaryotic 20S proteasome highly resembles the eukaryotic 20S proteasomes It has C2 symmetry and it

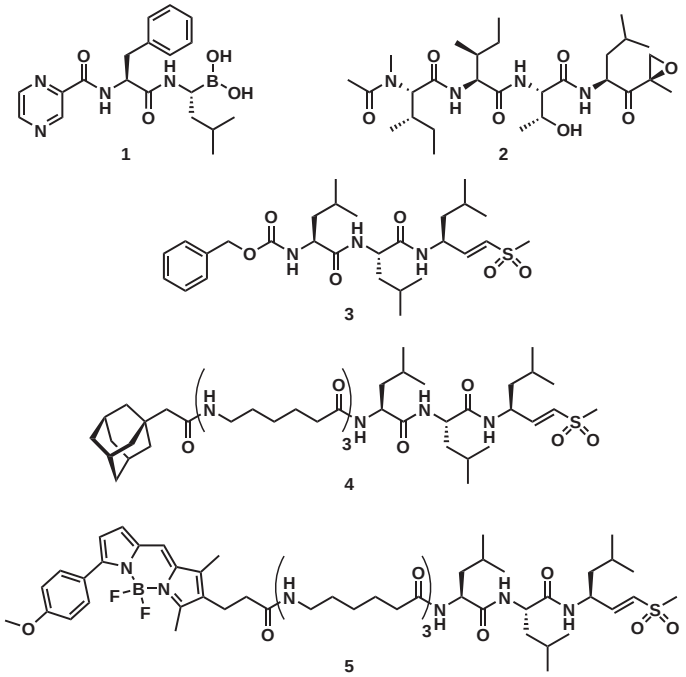


Figure 1. Proteasome inhibitors at the basis of this study.

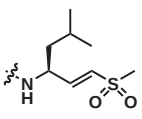
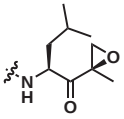
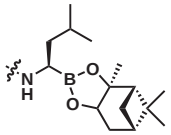
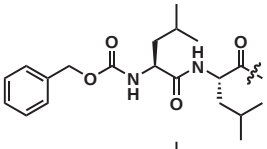
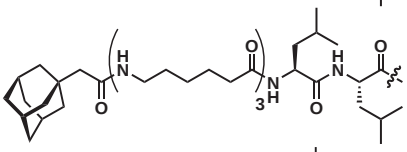
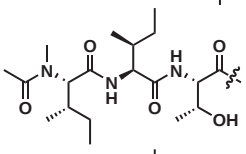
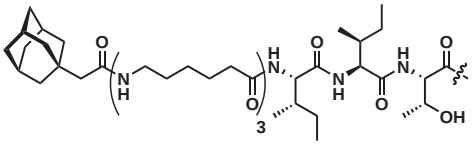
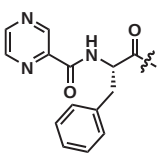
consists of two outer rings each containing seven identical α-subunits, and two inner seven-subunit rings containing identical catalytic β-subunits.[4] Eukaryotic proteasomes have evolved to such extent that each α-subunit in the outer ring has a unique sequence, as is the case with the inner β-rings. Remarkably, four of the β-subunits lost their proteolytic activities, whereas the three remaining subunits have a diverged substrate preference.[5] Based on fluorogenic substrate assays the substrate preference is loosely defined as caspase-like for the β1 subunit (cleaving after acidic residues), trypsin-like for β2 (cleaving after basic residues) and chymotrypsin-like for β5 (cleaving after neutral hydrophobic residues). However, many studies demonstrate that the subunits are much more promiscuous with respect to the amino acid residue at the cleavage site.

An important asset in proteasome research is the use of covalent and irreversible inhibitors such as the natural product epoxomicin (**2**),[6] the synthetic Michael acceptor ZL_3VS (**3**),[7] and their labelled (radio tag, affinity tag, fluorescent tag) counterparts.[7-10] Despite these studies the exact substrate preference of the individual catalytic activities, and the evolutionary benefit that results from the diversification, is not fully understood. The same holds true for the role of yet another proteasome particle, the immunoproteasome, which is formed upon challenge of the mammalian immune system and which contains three different catalytic subunits, namely β1i (LMP2), β2i (MECL1) and β5i (LMP7).[11] A much sought after research goal in immunology is to establish the impact of the immunoproteasome, in relation to the constitutively expressed proteasome, on the generation of specific oligopeptides that can be sequestered by the major histocompatibility complex class I pathway for presentation

to the immune system.[12] To aid these studies, several research groups are involved in the development of compounds that selectively target one catalytic subunit of either the constitutive proteasome or the immunoproteasome.

The pool of proteasome inhibitors reported to date encompasses numerous structurally diverse compounds. A large category within this pool has in common that they are peptide-based compounds equipped with an electrophilic trap at the *C*-terminus. *C*-terminal modifications include, next to boronic acid (as in **1**), epoxyketone (**2**), vinyl sulfone (**3**), aldehyde and other electrophiles.[1,13] The mechanism of inhibition is similar in all examples: the γ-hydroxyl of the *N*-terminal threonine within the active site of the catalytic subunits reacts with the electrophilic trap to form a covalent and (in most cases) irreversible bond. Some control over subunit specificity can be achieved by altering the nature of the amino acid residues. Several years ago, it was demonstrated that *N*-terminally extended, hydrophobic peptide vinyl sulfones, such as the adamantane containing compound **4**, are much more potent proteasome inhibitors than their truncated counterparts.[8] This gain in activity was accompanied by a loss in subunit selectivity. This finding was exploited by the development of the broad-spectrum cell permeable proteasome label MV151 (BODIPY TMR-Ahx$_3$L$_3$VS, Chapter 2, **5**).[10] One observation made is that the potency of peptide vinyl

Table 1. Panel of synthesized proteasome inhibitors.

			
	**3**	**9**	**13**
	**4**	**10**	**14**
	**6**	**2**	**15**
	**7**	**11**	**16**
	**8**	**12**	**17**

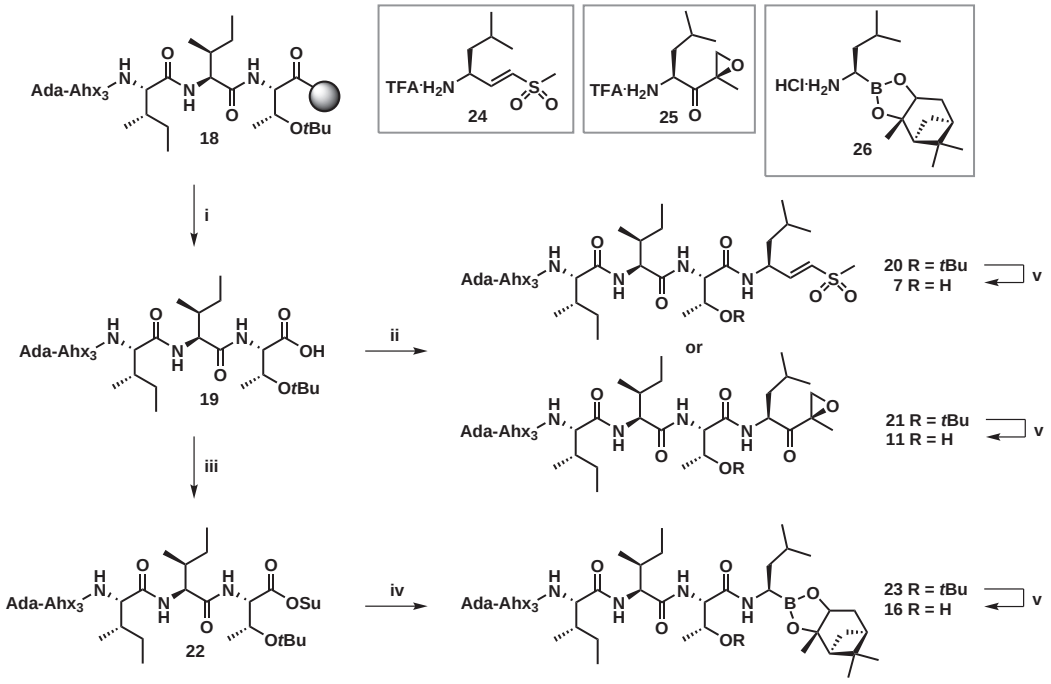
sulfone **4** to inhibit the proteasome approaches, or even surpasses that of bortezomib (**1**) and epoxomicin (**2**). This is remarkable, since the parent compound ZL_3VS (**3**) is a much weaker inhibitor. Indeed, in general oligopeptides containing epoxyketone or boronic acid warheads are found to be more potent proteasome inhibitors than their counterparts that have the same amino acid sequence but are equipped with a Michael acceptor. This observation raises the question whether recombining structural features of peptide-based proteasome inhibitors would lead to more potent compounds. To address this question, the structural characteristics of compounds **1**-**4** were scrambled to arrive at a number of *C*-terminally modified peptides, which were assessed on their proteasome inhibitory activity. Three distinct structural features were identified. These are a) the modified amino acid at the *C*-terminus, being boronic acid, vinyl sulfone and epoxyketone, b) the amino acid sequence, being the trileucine, the epoxomicin tetrapeptide and the bortezomib recognition element, and c) the presence (as in **4**) or absence (as in **3**) of the lipophilic *N*-terminal extension. These considerations led to the synthesis of the panel of 15 compounds listed in Table 1.

5.2 Results and discussion

In the design of the hybrid library a set of compounds that have the pyrazinoic acyl moiety in the bortezomib sequence replaced by the adamantane-spacer moiety was excluded. Such a set of compounds would likely resemble to a large extend the trileucine derivatives in their inhibition profile. Furthermore, it was decided to leave the pinanediol protection on the boronic ester, which stems from the enantioselective preparation of the leucine building block, in place. It has been reported that boronic esters of this nature have the same activity and specificity as their unprotected counterparts (Chapter 2).[10] Moreover, deprotection, for which there is no literature precedent (in fact there is no reliable literature synthesis of the drug bortezomib), proved to be detrimental in the course of the here described studies.

The preparation of all compounds follows the same general strategy: first the synthesis of the (*N*-terminaly extended) amino acid sequence and then coupling of these to the leucine derived warheads. As an example, the synthesis of compounds **7**, **11** and **16** is depicted in Scheme 1. Briefly, Fmoc-based solid phase peptide synthesis using HMPB functionalised MBHA resulted in **18**. Cleavage using 1% trifluoroacetic acid in dichloromethane gave partially protected oligopeptide **19**, which was condensed with either leucine vinyl sulfone **24**[7] or leucine epoxyketone **25**,[6] using BOP as the condensating agent. Acidic removal of the *t*Bu protecting group afforded the target compounds **7** and **11** after HPLC purification. Target compound **16** was obtained by coupling of succimidyl ester **22** with leucine boronic pinanediol ester **26** (synthesized as described in Chapter 2),[10] followed by acidic deprotection and HPLC purification.

Scheme 1. Synthesis of hybrid proteasome inhibitors **7**, **11** and **16**.



Reagents and conditions: i) 1% TFA in DCM, 3× 15 min., 73%. ii) **24** or **25** (1.1 equiv.), BOP (1.1 equiv.), DiPEA (2.2 equiv.), DMF, 12 hr., **21** 10%. iii) *N*-hydroxysuccinimide (1.7 equiv.), DIC (1.5 equiv.), DMF, 15 hr. iv) **26** (3.1 equiv.), DiPEA (24 equiv.), -80 °C to RT, 4 hr. v) TFA/DCM (1/1), 30 min., **7** 16% (2 steps), **11** 91%, **16** 4% (2 steps).

The inhibition potential of the panel of compounds was assessed in competition assays employing lysates of the murine EL4 cell line (expressing both the constitutive proteasome and the immunoproteasome, Figures 2 and 3) and the human HEK293T cell line (exclusively expressing the constitutive proteasome, Figure 4) in combination with fluorescent proteasome probe **5**. In a first set of experiments, EL4 cell lysates were incubated for one hour with each of the 15 compounds at 0, 0.1, 1, 10 and 100 μM final concentration, prior to treatment with 0.1 μM final concentration of MV151 (**5**). The samples were denatured and resolved by SDS-PAGE and the wet gel slabs were scanned on a fluorescence scanner (Figure 2). Lysates that have been exposed to the fluorescent label only display four bands corresponding to the six proteasome active sites (specified as depicted in the Figure 2).[10] The ability of the **15** compounds to inhibit the proteasome activities is reflected by disappearance of fluorescent labeling by MV151. Ten compounds from the panel of 15 (namely, **2, 4, 7, 9, 10, 11, 13** - **16**) proved to be potent proteasome inhibitors, with most or all labeling abolished at 1 μM. Vinyl sulfone derivatives **3, 6** and **8**, and epoxyketone **12** appear to be much weaker inhibitors. Boronic ester **17** is a weak inhibitor of the β2 and β2i subunits, while potently targeting the remaining subunits. As the next experiment the competition experiment was repeated, but with the difference that

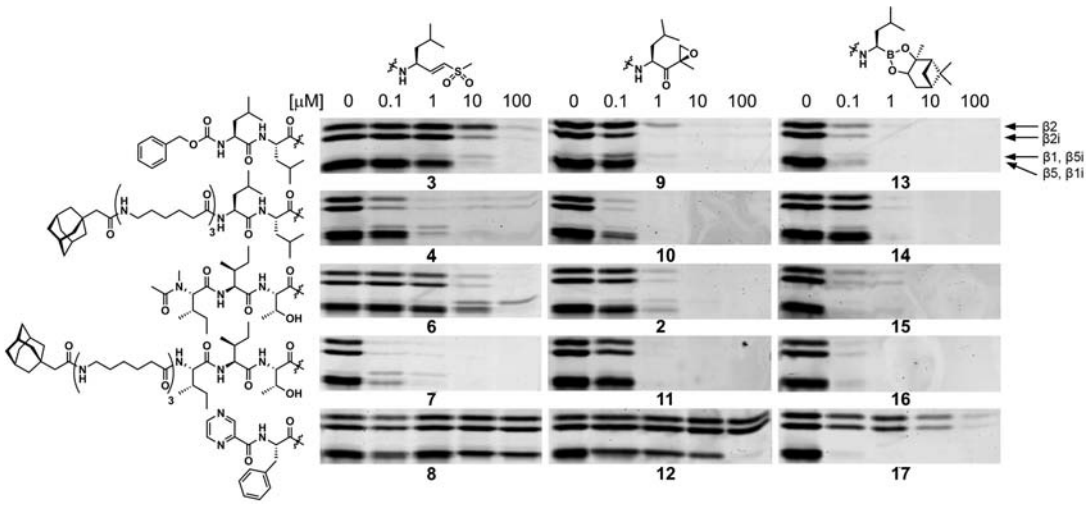


Figure 2. Competition assays of the hybrid library versus MV151 in EL4 cell lysates.
EL4 lysates (10 μg total protein) were incubated with the indicated concentrations of inhibitor for 1 hr. at 37 °C. Residual proteasome activity was labeled with 0.1 μM MV151 at 37 °C for 1 hr., prior to denaturation, SDS-PAGE and fluorescence scanning of the wet gel-slabs.

inhibitor concentrations were ranged from 0 to 500 nM (Figure 3, compounds **6**, **8** and **12** were excluded since these proved to be hardly effective between concentrations of 0 to 1000 nM in the first experiments). These results corroborate the earlier findings and allow the assessment of some more subtle differences between the different inhibitors. The most obvious result is that boronic ester **17** hardly targets β2 and β2i, a finding that corresponds to the reported specificity of the unprotected analogue, PS341 (**1**).[9] This selectivity is

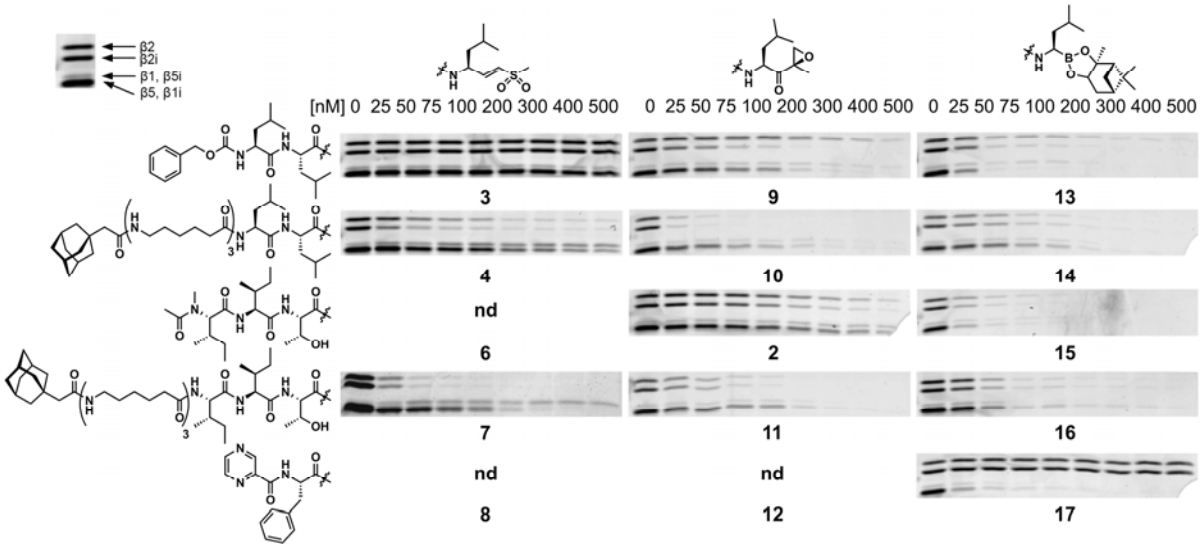


Figure 3. Competition assays of the hybrid library versus MV151 in EL4 cell lysates.
EL4 lysates (10 μg total protein) were incubated with the indicated concentrations of inhibitor for 1 hr. at 37 °C. Residual proteasome activity was labeled with 0.1 μM MV151 at 37 °C for 1 hr., prior to denaturation, SDS-PAGE and fluorescence scanning of the wet gel-slabs. nd = not determined.

abolished when keeping the boronic ester in place but substituting the peptide sequence, as in **13-16**. In general, the vinyl sulfone is the weakest electrophilic trap in each series, and the boronic ester the strongest, but there are some interesting differences. For instance, epoxyketone **10** is an equally potent inhibitor for each proteasome subunit as the boronic ester **14**, with a preference for the constitutive and immunoinduced β2 subunits. At the onset of the competition experiments it was assumed that elongation of a given proteasome inhibitor with the Ada(Ahx)$_3$ *N*-terminal cap leads to a more potent compound. This holds true to some extent, compare, for instance, vinyl sulfones **3** and **4**, vinyl sulfones **6** and **7**, epoxyketones **9** and **10**, and epoxyketones **2** and **11**. However the potency of ZL$_3$-boronic ester **13** (its unprotected counterpart has been described in the literature[3] and is known as MG262) belies the generality of this trend. Indeed, the potency of this compound is bested by boronic ester **15** only. Compound **15** is more potent than its *N*-terminally extended analogue **16** as well. In some cases, labeling of a specific active site increases as the other activities are inhibited. This finding, which could imply enhancement of a specific proteasome activity due to occupation of other active sites, has been noted before, however is not yet clearly understood.[14] When performing a similar competition experiment on human embryonic kidney HEK293T cell lysates (Figure 4), this effect is clearly visible in the case of the *N*-terminally extended epoxomicin sequence armed with the leucine vinyl sulfone warhead (**7**). This inhibitor potently inhibits β5 and β2, whereas β1 labeling increases dramatically. In general, the same trends are found as in the experiments in EL4 lysates.

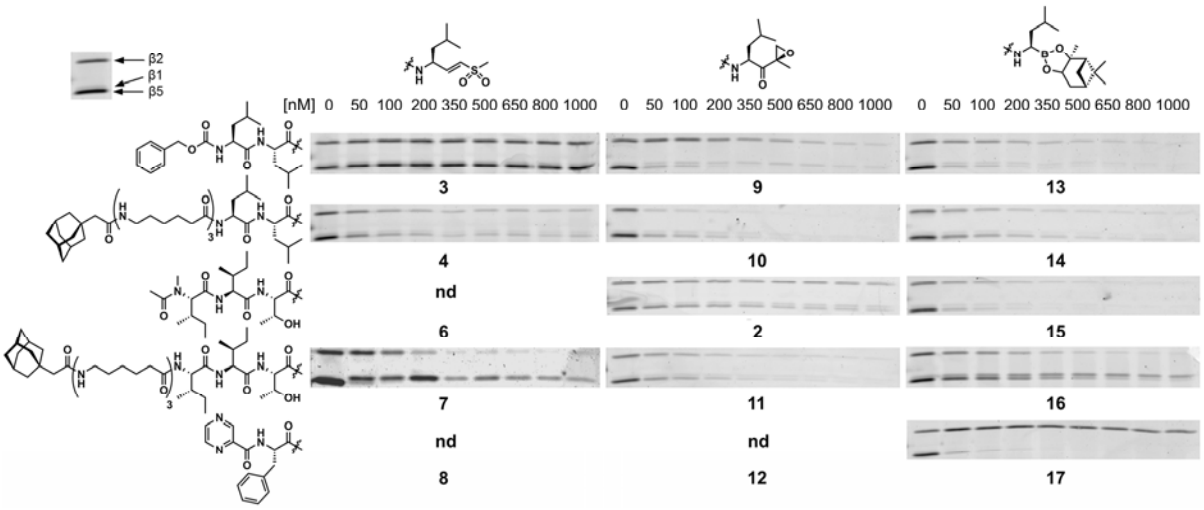


Figure 4. Competition assays of the hybrid library versus MV151 in HEK293T cell lysates.
HEK293T lysates (10 µg total protein) were incubated with the indicated concentrations of inhibitor for 1 hr. at 37 °C. Residual proteasome activity was labeled with 0.1 µM MV151 at 37 °C for 1 hr., prior to denaturation, SDS-PAGE and fluorescence scanning of the wet gel-slabs. nd = not determined.

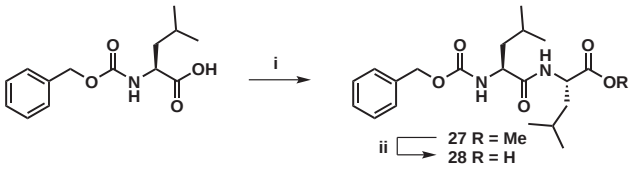
5.3 Conclusion

In conclusion, the work described in this Chapter demonstrates that scrambling of structural elements of known proteasome inhibitors is a viable strategy to arrive at potent new proteasome inhibitors. Prediction of the potency of a putative peptide-based inhibitor is not as straightforward as was considered at the onset of this study. For instance, while it is true that hydrophobic extension in most cases contributes to the potency, the most potent compound from the series tested in EL4 lysates presented here proved to be the boronic ester **15** bearing the epoxomicin tetrapeptide sequence without *N*-terminal extension. In the competition experiments performed in HEK293T lysates, inhibitor **10** proved to be at least equally potent as **15**. Although the potency of a certain inhibitor seems dependent on the cell type used or the type of proteasome (constitutive- or immunoproteasome) that is being expressed, compound **15** may well be the most potent peptide-based proteasome inhibitor reported to date. Possibly, such broad-spectrum proteasome inhibitors might find clinical application as an alternative for bortezomib. For instance, in cases where bortezomib resistance occurs, an event which is thought to be linked to upregulation of those proteasome activities that are left unmodified by bortezomib (bortezomib has a labeling profile similar to **17** and leaves the β2 and β2i subunits untouched at clinical doses).[9,10]

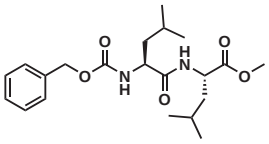
Experimental section

General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol..)(purum), ethyl acetate (EtOAc) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, 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 followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on Screening Devices (0.040 – 0.063 nm). HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), Bruker DPX-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory or a Bruker DMX-600 (600/150 MHz) with cryoprobe. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. UV spectra were recorded on a Perkin Elmer, Lambda 800 UV/VIS spectrometer. For RP-HPLC purifications a BioCAD "Vision" automated HPLC system (PerSeptive Biosystems, inc.) equipped with a semi-preparative Alltima C_{18} column was used. The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, λ = 589 nm). ZL_3VS (**3**),[7] Ada-Ahx_3L_3VS (**4**),[8] bortezomib pinanediol ester (**8**),[10] epoxomicin (**2**),[6] Boc-leucine-vinyl-(methyl)-sulfone (**35**)[7] and (Boc-leucinyl)-methyloxirane (**37**)[6] were synthesised as described in literature.

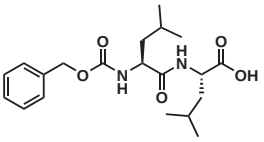
Scheme 2. Synthesis of Z-L_2-OH (**28**).



Reagents and conditions: i) HCl·Leu-OMe (1 equiv.), PyBOP (1 equiv.), DiPEA (2 equiv.), DCM, 3 hr., 68%. ii) NaOH (1.32 equiv.), dioxane/MeOH/H_2O, 3.5 hr., 92%.

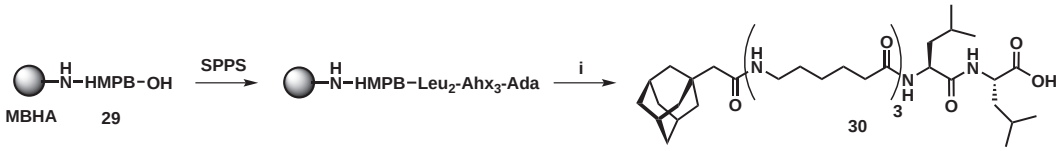


Z-leu$_2$-OMe (27). Z-Leu-OH (5.6 g, 21.5 mmol) and HCl·Leu-OMe (3.92 g, 21.5 mmol, 1 equiv.) were dissolved in 80 ml DCM and put under an Argon atmosphere. PyBOP (11.2 g, 21.5 mmol, 1 equiv.) and DiPEA (7.3 ml, 43 mmol, 2 equiv.) were added and the reaction mixture was stirred for 3 hr. The reaction mixture was washed with sat. aq. $NaHCO_3$, 1M HCl and brine, and the organic phase was dried over $MgSO_4$ and concentrated. Flash column chromatography (PetEt → 10% EtOAc/PetEt, v/v) gave the title compound (5.7 g, 14.5 mmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.52 (d, *J* = 7.7 Hz, 1H), 7.32-7.15 (m, 5H), 6.31 (d, *J* = 8.4 Hz, 1H), 5.08-4.90 (m, 2H), 4.60-4.18 (m, 2H), 3.59 (s, 3H), 1.70-1.35 (m, 6H), 0.95 – 0.68 (m, 6H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 172.7, 172.5, 155.9, 136.0, 127.9, 127.5, 127.3, 66.2, 52.9, 51.5, 50.3, 41.1, 40.4, 24.3, 24.1, 22.2, 21.7, 21.4.



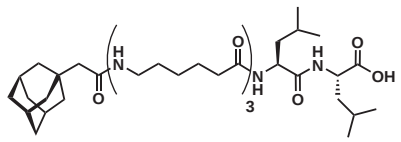
Z-Leu$_2$-OH (28). Z-Leu$_2$-OMe (**27**), (5.7 g, 14.5 mmol) was dissolved in a mixture of 61 ml dioxane, 21.7 ml MeOH and 4.78 ml 4M NaOH in H_2O (19.1 mmol, 1.32 equiv.) and stirred for 3.5 hr. The reaction mixture was acidified to pH 2 with 1M HCl and concentrated. The residue was dissolved in EtOAc, washed with H_2O. The water layer was extracted with EtOAc (2x), and the combined organic layers were dried over $MgSO_4$ and concentrated. Crystallisation from EtOAc/PetEt yielded the title compound (5.03 g, 13.3 mmol, 92%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.40-7.25 (m, 5H), 6.65 (d, *J* = 6.2 Hz, 1H), 5.48 (m, 1H), 5.1 (s, 2H), 4.65-4.50 (m, 1H), 4.38-4.19 (m, 1H), 1.81-1.43 (m, 6H), 1.05-0.80 (m, 6H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 175.8, 172.9, 156.5, 136.1, 128.4, 127.6, 127.8, 67.0, 53.3, 50.8, 41.0, 24.7, 24.4, 22.7, 21.7.

Scheme 3. Synthesis of Ada-Ahx$_3$-Leu$_2$-OH (**30**).



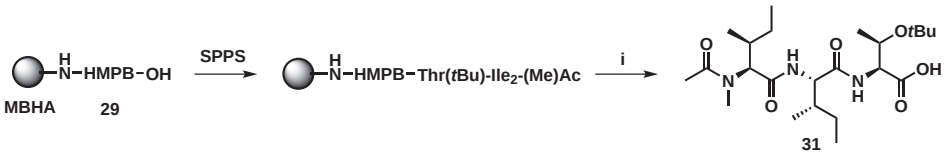
Reagents and conditions: i) 1% TFA in DCM, 3x 10 min.

HMPB-MBHA resin (29). 4-methylbenzhydrylamine (MBHA) functionalized polystyrene resin (0.56 g, 0.9 mmol/g, 0.5 mmol) was washed with NMP (3x) followed by addition of a preactivated mixture of 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker (0.36 g, 1.5 mmol, 3 equiv.), BOP (0.67 g, 1.5 mmol, 3 equiv.) and DiPEA (0.55 ml, 3 mmol, 6 equiv.) in NMP. After 2 hr. of shaking, the resin was washed with NMP (3x), MeOH (3x) and DCM (3x), dried and used as such.

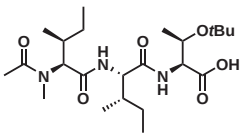


Ada-Ahx$_3$-Leu$_2$-OH (30). Resin **29** (0.5 mmol) was transferred to a flask, coevaporated with DCE (2x), and condensed with Fmoc-Leu-OH (0.53 g, 1.5 mmol, 3 equiv.) under the influence of DIC (0.26 ml, 1.67 mmol, 3.3 equiv.) and DMAP (10 mg, 0.075 mmol, 15 mol%) in DCM for 2 hr. The resin was filtered and washed with DCM (2x), followed by a second condensation cycle. The loading of the resin was determined to be 0.42 mmol/g (0.93 g, 0.39 mmol, 78%) by spectrophotometric analysis. The obtained resin was submitted to four cycles of Fmoc solid-phase synthesis with Fmoc-Leu-OH and Fmoc-Ahx-OH (3×), respectively, as follows: a) deprotection with piperidine/NMP (1/4, v/v, 20 min.), b) wash with NMP (3×), c) coupling of Fmoc amino acid (1.2 mmol, 3 equiv.) in the presence of BOP (0.53 g, 1.2 mmol, 3 equiv.) and DiPEA (0.4 ml, 2.3 mmol, 6 equiv.) in NMP and shaken for 2 hr., d) wash with NMP (3×) and DCM (3×). Couplings were monitored for completion by the Kaiser test. After deprotection of the resin bound pentapeptide, adamantylacetic acid (0.23 g, 1.2 mmol, 3 equiv.), PyBOP (0.63 g, 1.2 mmol, 3 equiv.), DiPEA (0.4 ml, 2.34 mmol, 6 equiv.) in NMP were added, and the resin was shaken for 2 hr. After washing with NMP (3×) and DCM (3×) the resin was subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 10 min, 3x) and the collected fractions were coevaporated with Tol. (2x) to give the crude title compound, which was used without any further purification. ^{1}H NMR (200 MHz, CD_3OD): δ ppm 4.51-4.34 (m, 2H), 3.25-3.05 (m, 6H), 2.22-2.10 (m, 6H), 1.93 (s, 2H), 1.82-1.20 (m, 39H), 1.01-0.83 (m, 12H). ^{13}C NMR (50.1 MHz, CD_3OD): δ ppm 176.0, 175.7, 174.8, 173.8, 53.0, 51.9, 51.7, 43.7, 41.7, 40.2, 37.8, 36.9, 36.6, 33.8, 30.1, 30.0, 27.5, 26.7, 25.9, 22.1, 21.9.

Scheme 4. Synthesis of Ac(Me)-Ile$_2$-Thr(*t*Bu)-OH (**31**).

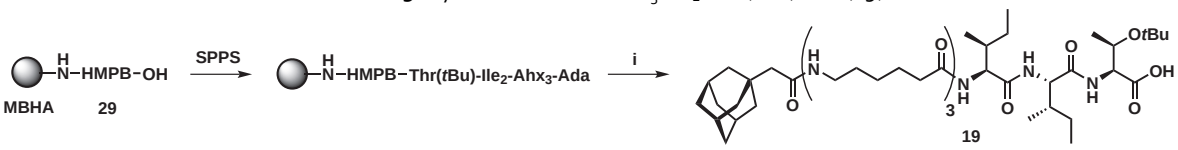


Reagents and conditions: i) 1% TFA in DCM, 3x 10 min.

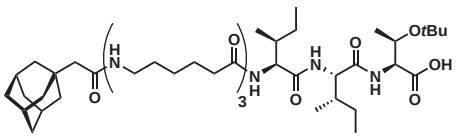


Ac(Me)-Ile$_2$-Thr(*t*Bu)-OH (31). Resin **29** (1 mmol) was transferred to a flask, coevaporated with DCE (2x), and condensed with Fmoc-Thr(*t*Bu)-OH (1.2 g, 3 mmol, 3 equiv.) under the influence of DIC (0.51 ml, 3.3 mmol, 3.3 equiv.) and DMAP (18 mg, 0.15 mmol, 15 mol%) in DCM for 2 hr. The resin was filtered and washed with DCM (2x), followed by a second condensation cycle. The loading of the resin was determined to be 0.55 mmol/g by spectrophotometric analysis. The obtained resin was submitted to two cycles of Fmoc solid-phase synthesis with Fmoc-Ile-OH and Fmoc(Me)-Ile-OH, respectively, as follows: a) deprotection with piperidine/NMP (1/4, v/v, 20 min.), b) wash with NMP (3×), c) coupling of Fmoc amino acid (2.5 mmol, 2.5 equiv.) in the presence of BOP (1.1 g, 2.5 mmol, 2.5 equiv.) and DiPEA (0.5 ml, 3 mmol, 3 equiv.) in NMP and shake for 2 hr., d) wash with NMP (3×) and DCM (3×). Couplings were monitored for completion by the Kaiser test. After Fmoc deprotection of the resin bound tripeptide, acetyl chloride (0.3 ml, 4 mmol, 4 equiv.) and DiPEA (0.66 ml, 4 mmol, 4 equiv.) in DCM were added, and the resin was shaken for 2 hr. After washing with DCM (3×) the resin was subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 10 min, 3x) and the collected fractions were coevaporated with Tol. (2x) to give the crude title compound, which was used without any further purification.

Scheme 5. Synthesis of Ada-Ahx$_3$-Ile$_2$-Thr(tBu)-OH (19).

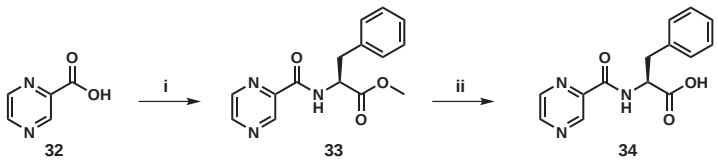


Reagents and conditions: i) 1% TFA in DCM, 3x 10 min.

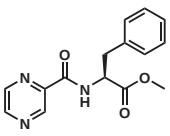


Ada-Ahx$_3$-Ile$_2$-Thr(tBu)-OH (19). HMPB-MBHA resin **29** (2 g, 0.75 mmol/g resin, 1.5 mmol) was coevaporated with DCE (2x) and condensed with Fmoc-Thr(tBu)-OH (1.8 g, 4.5 mmol, 3 equiv.) under the influence of DIC (0.7 ml, 4.5 mmol, 3 equiv.) and DMAP (10 mg, 75 µmol) for 2 hr. The resin was filtered, washed with DCM (3x) and subjected to a second condensation cycle. The loading of the resin was 0.67 mmol/g, as determined by spectrophotometric analysis. Next, the resin was subjected to five cycles of Fmoc solid-phase synthesis with Fmoc-Ile-OH (2x) and Fmoc-Ahx-OH (3x), respectively as follows: a) deprotection with piperidine/NMP (1/4, v/v, 15 min), b) washing with NMP (3x) and DCM (3x), c) coupling of the Fmoc amino acid (4.5 mmol, 3 equiv.) by shaking the resin for 2 hr. in the presence of HCTU (3 equiv., 4.5 mmol), DiPEA (6 equiv., 9 mmol) and NMP as solvent, d) washing with NMP (3x) and DCM (3x). Couplings were monitored by the Kaiser test for completion. This resin (1.7 g, 0.75 mmol) was deprotected using piperidine/NMP (1/4, v/v, 15 min), washed with NMP (3x) and DCM (3x) and condensed with Ada-OH (0.44 g, 2.25 mmol, 3 equiv.) using HCTU (0.93 g, 2.25 mmol, 3 equiv.) and DiPEA (0.75 ml, 4.5 mmol, 6 equiv.) in NMP for 2 hr. The resin was washed with NMP (3x) and DCM (3x), before being subjected to mild acidic cleavage (TFA/DCM, 1/99 v/v, 15 min, 3x). The fractions were collected and concentrated in the presence of Tol., yielding the title compound (505 mg, 0.55 mmol, 73 %). ^{1}H NMR (200 MHz, CD_3OD): δ ppm 4.48-4.23 (m, 4H), 3.25-3.08 (m, 6H), 2.35-2.12 (m, 7H), 2.04-1.10 (m, 52H), 1.00-0.83 (m, 12H). ^{13}C NMR (50.1 MHz, CD_3OD): δ ppm 175.8, 173.5, 173.6, 75.1, 68.8, 59.0, 51.8, 43.7, 40.1, 38.0, 37.9, 37.6, 36.9, 36.6, 33.7, 30.1, 30.0, 28.8, 27.5, 26.6, 26.0, 25.7, 21.1, 16.1, 15.9, 11.4, 11.2.

Scheme 6. Synthesis of pyrazine-2-carbonylphenylalanine (**34**).

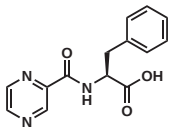


Reagents and conditions: i) H-Phe-OMe·HCl (1.1 equiv.), BOP (1.1 equiv.), DiPEA (3 equiv.), DMF, 1 hr., 70%. ii) NaOH (1.05 equiv.), dioxane/MeOH/H_2O, 1 hr., 69%.



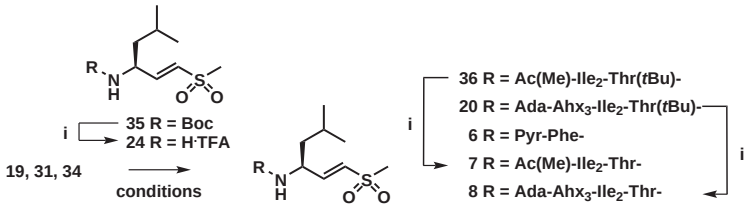
Pyrazine-2-carbonylphenylalanine methyl ester (33). Pyrazine-2-carboxylic acid (**32**) (0.37 g, 3 mmol) was dissolved in DMF (30 ml), put under an Argon atmosphere and preactivated with BOP (1.46 g, 3.3 mmol, 1.1 equiv.) and DiPEA (1.53 ml, 9 mmol, 3 equiv.) for 15 min. To this solution, phenylalanine methyl ester · HCl (0.712, 3.3 mmol, 1.1 equiv.) dissolved in 30 ml DMF was added, and the reaction mixture was stirred for 1 hr. Sat. aq. $NaHCO_3$ was added and the water layer was extracted with Et_2O (3x). The combined organic layers were dried over $MgSO_4$ and concentrated. The crude product was purified by column chromatography (Tol. → 40% EtOAc in Tol. v/v) to yield the title compound (0.6 g, 2.1 mmol, 70%). ^{1}H NMR (200 MHz, $CDCl_3$): δ

ppm 9.36 (d, *J* = 1.5 Hz, 1H), 8.71 (d, *J* = 2.6 Hz, 1H), 8.46 (m, 1H), 8.29 (d, *J* = 7.7 Hz, 1H), 7.25 (m, 5H), 5.14-5.04 (m, 1H), 3.74 (s, 3H), 3.35-3.15 (m, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 171.2, 162.4, 147.2, 144.0, 143.7,142.5, 135.5, 128.9, 128.4, 126.9, 53.1, 52.2, 37.8.

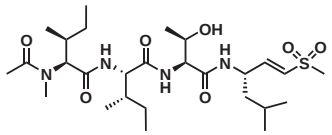


Pyrazine-2-carbonylphenylalanine (34). Methyl ester **33** (0.24 g, 0.85 mmol) was dissolved in 3 ml dioxane, 1.1 ml MeOH and 0.22 ml 4M NaOH. The reaction mixture was stirred for 1 hr., before 0.8 ml 1M $KHSO_4$ was added, and the solution was concentrated. The residue was taken up in H_2O/brine (1/1, v/v), and this solution was extracted with EtOAc (3x). The combined organic layers were dried over $MgSO_4$ and concentrated, yielding the title compound in 69% yield (0.16 gr, 0.59 mmol). LC/MS analysis: R_t 10.16 min (linear gradient 10 → 90% B in 20 min), *m/z* 272.0 $[M+H]^+$, 543.1 $[2M+H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 9.37 (s, 1H), 8.76 (s, 1H), 8.54 (s, 1H), 8.21 (d, *J* = 8.0 Hz, 1H), 7.3-7.19 (m, 5H), 5.18-5.07 (m, 1H), 3.42-3.20 (m, 2H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 174.0, 164.7, 148.7, 145.6, 144.7, 144.6, 137.9, 130.3, 129.5, 127.9, 54.9, 38.2.

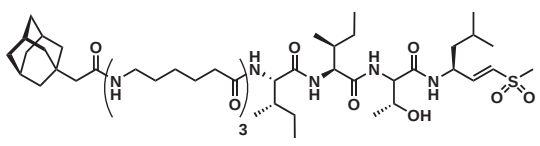
Scheme 7. Synthesis of the vinyl sulfone equipped hybrid inhibitors.



Reagents and conditions: i) TFA/DCM 1/1, 30 min.

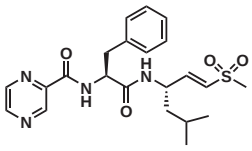


Ac(Me)-Ile$_2$-Thr-LeuVS (6). Boc-leucine-vinyl-(methyl)-sulfone (**35**)[7] (87 mg, 0.3 mmol) was stirred in TFA/DCM (1/1 v/v, 1 ml) until TLC analysis indicated complete deprotection. The reaction mixture was concentrated in presence of Tol. (2x) before being dissolved in DCM and put under an Argon atmosphere. Ac-(Me)-I$_2$-T(*t*Bu)-OH **31** (337 mg, 0.45 mmol, 1.5 equiv.), BOP (0.2 g, 0.45 mmol, 1 equiv.), DiPEA (0.12 ml, 0.76 mmol, 2.5 equiv.) were added, and the mixture was stirred for 12 hr., before being concentrated *in vacuo*. The crude vinyl sulfone was dissolved in TFA/DCM (1/1 v/v, 2 ml) and stirred at ambient temperature for 30 min., before being concentrated in presence of Tol. (2x), yielding Ac(Me)-Ile$_2$-Thr-LeuVS (**6**) as a mixture of two diastereomers. Semi-preparative RP-HPLC purification of the major product yielded the title compound as a white solid (1.2 mg, 2 µmol, 0.7% isolated yield). $[\alpha]_D^{20}$= -225 (c 0.024, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 6.81 (dd, J_1 = 14.6, J_2 = 4.0 Hz, 1H), 6.71 (d, *J* = 15.2 Hz, 1H), 4.78 (d, 11.2 Hz, 1H), 4.71 (m, 1H), 4.26 (m, 1H), 4.17 (m, 2H), 3.02 (s, 3H), 2.95 (s, 3H), 2.12 (s, 3H), 2.05 (m, 1H), 1.88 (m, 1H), 1.71 (m, 1H), 1.62-1.26 (m, 6H), 1.19 (m, 1H), 1.18 (d, *J* = 6.4 Hz, 3H), 1.02 (m, 1H), 0.92 (m, 18H). HRMS: calcd. for $[C_{27}H_{50}O_7N_4SH]^+$ 575.34730, found 575.34769.



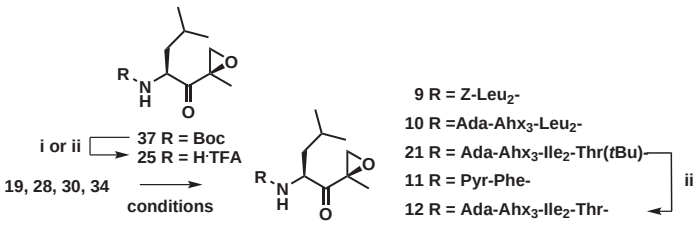
Ada-Ahx$_3$-Ile$_2$-Thr-LeuVS (7). Boc-leucine-vinyl-(methyl)-sulfone (**35**)[7] (76 mg, 0.26 mmol, 1.1 equiv.) was stirred in TFA/DCM (1/1 v/v, 1 ml) until TLC analysis indicated complete conversion of the starting material. The reaction mixture was concentrated in presence of Tol. (2x) before being dissolved in DMF and put under an Argon atmosphere. Ada-Ahx$_3$-I$_2$-T(*t*Bu)-OH **19** (217 mg, 0.24 mmol), BOP (117 mg, 0.26 mmol,

1.1 equiv.), DiPEA (90 µl, 0.53 mmol, 2.2 equiv.) were added, and the mixture was stirred for 12 hr., before being concentrated *in vacuo*. The crude vinyl sulfone was dissolved in TFA/DCM (1/1 v/v, 1 ml) and stirred at ambient temperature for 30 min., before being concentrated in presence of Tol. (2x), yielding Ada-Ahx$_3$-Ile$_2$-Thr-LeuVS (**7**) as a mixture of two diastereomers. Semi-preparative RP-HPLC purification of the major product yielded the title compound as a white solid (38.8 mg, 37.5 µmol, 16%). $[\alpha]_D^{20}$= -31 (c 0.2, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 7.52 (m, 2H), 6.86 (dd, J_1 = 4.3, J_2 = 15 Hz, 1H), 6.65 (d, J = 15 Hz, 1H), 4.72 (m, 1H), 4.32 – 4.10 (m, 4H), 3.18 (t, J = 6.9 Hz, 6H), 2.98 (s, 3H), 2.29 (t, J = 7.5 Hz, 2H), 2.18 (t, J = 7.4 Hz, 4H), 1.97 (m, 3H), 1.93 (s, 2H), 1.83 (m, 2H), 1.73 – 1.45 (m, 30H), 1.39 – 1.15 (m, 10H), 0.97 – 0.89 (m, 18H). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 175.2, 174.2, 172.7, 172.0, 171.7, 170.1, 147.4, 128.8, 66.5, 59.0, 58.7 (2x), 51.0, 47.8, 42.3, 42.2, 42.1, 38.8, 38.7, 36.4, 35.9, 35.8, 35.3, 32.4, 28.6 (2x), 28.5, 28.4, 26.0, 25.0, 24.9, 24.8, 24.6, 24.4, 22.5, 21.2, 19.2, 15.2, 15.1, 11.1, 10.6. HRMS: calcd. for $[C_{54}H_{95}N_7O_{10}SH]^+$ 1056.67533, found 1056.67658.

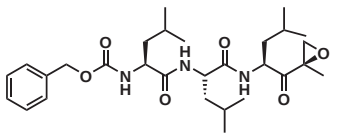


(Pyrazine-2-carbonylphenylalanyl)-leucine-vinyl-(methyl)-sulfone (8). Pyrazine-2-carbonylphenylalanine **34** (157 mg, 0.58 mmol) was preactivated with BOP (282 mg, 0.64 mmol, 1.1 equiv.) and DiPEA (0.3 ml, 1.7 mmol, 3 equiv.) in DCM. TFA·LeuVS **24** (212 mg, 0.7 mmol, 1.1 equiv.) was added, and the mixture was stirred for 4 hr. The reaction mixture was concentrated and subjected to flash column chromatography (Tol.. → EtOAc), yielding two epimers (both 82 mg, 0.18 mmol, 32%). Recrystallisation from PetEt/acetone yielded the title compound (50 mg, 0.113 mmol, 19%). $[\alpha]_D^{20}$= -12 (c 0.2, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 9.32 (s, 1H), 8.77 (d, J = 2.0 Hz, 1H), 8.59 (s, 1H), 8.50 (m, 1H), 7.43-7.22 (m, 5H), 7.16 (d, J = 8.0 Hz, 1H), 6.66 (dd, J_1 = 4.8, J_2 = 15.2 Hz, 1H), 5.99 (d, J = 15.2 Hz, 1H), 4.90-4.82 (m, 1H), 4.68-4.59 (m, 1H), 3.25-3.13 (m, 2H), 2.89 (s, 3H), 1.64-1.49 (m, 1H), 1.45-1.30 (m, 2H), 0.93-0.81 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 170.0, 163.0, 147.5, 147.2, 144.1, 143.8, 142.9, 136.0, 129.3, 128.9, 128.8, 127.3, 54.5, 47.9, 42.6, 42.5, 38.4, 24.5, 22.4, 21.7. HRMS: calcd. for $[C_{22}H_{28}O_4N_4SH]^+$ 445.19040 found 445.19031.

Scheme 8. Synthesis of the epoxyketone equipped hybrid inhibitors.

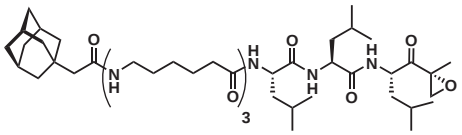


Reagents and conditions: i) TFA, 15 min. ii) TFA/DCM 1/1, 30 min.

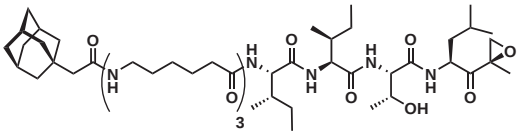


Z-Leu$_3$-2-methyloxirane (9). (Boc-leucinyl)-methyloxirane (**37**)[6] (67 mg, 0.25 mmol) was stirred in TFA (1 ml) until TLC analysis indicated complete consumption of the starting material. Tol.. was added, and the reaction mixture was concentrated. Z-Leu$_2$-OH **28** (104 mg, 0.275 mmol, 1.1 equiv.) was coevaporated with Tol.. (2x), dissolved in DCM and put under an Argon atmosphere. PyBOP (150 mg, 0.29 mmol, 1.16 equiv.) and DiPEA (0.13 ml, 0.75 mmol, 3 equiv.) were added, followed by the crude TFA·leucinyl-methyloxirane **25**. The reaction mixture was stirred for 1hr. The mixture was washed with H_2O and brine, dried over $MgSO_4$ and concentrated. Crystallisation from EtOAc/PetEt yielded crude title compound **9**, which was further purified by flash column chromatography (PetEt → 30%

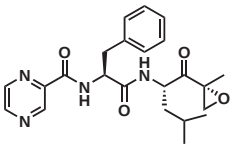
EtOAc/PetEt, v/v) yielding the title compound (18.7 mg, 44 µmol, 18%). $[\alpha]_D^{20}$ = -1 (c 0.2, MeOH). ^{1}H NMR (200 MHz, CD_3OD): δ ppm 7.35-7.25 (m, 5H), 5.08 (m, 2H), 4.62-4.38 (m, 2H), 4.22-4.05 (m, 1H), 3.24 (d, *J* = 5.1 Hz, 1H), 2.91 (d, 5.1 Hz, 1H), 1.77-1.25 (m, 9H), 0.95-0.83 (m, 18H). ^{13}C NMR (50.1 MHz, CD_3OD): δ ppm 209.5, 175.1, 174.5, 156.0, 129.5, 129.0, 128.8, 67.6, 60.1, 54.9, 53,1, 52.7, 51.9, 42.1, 41.9, 40.2, 26.2, 25.8, 25.7, 23.7, 23.4, 22.2, 22.0, 24.5, 17.0. HRMS: calcd. for $[C_{29}H_{45}O_6N_3H]^+$ 532.33811, found 532.33826.



Ada-Ahx$_3$-Leu$_2$-leucinyl-2-methyloxirane (10). (Boc-leucinyl)-methyloxirane (**37**)[6] (116 mg, 0.35 mmol) was stirred in TFA (1 ml) until TLC analysis indicated complete consumption of the starting material. The reaction mixture was concentrated in the presence of Tol.. (2x), dissolved in DCM/DMF (19/1, v/v) and put under an Argon atmosphere. Ada-Ahx$_3$-Leu$_2$-OH (**30**) (0.27 g, 0.35 mmol, 1 equiv.), PyBOP (0.2 g, 0.38 mmol, 1.1 equiv.) and DiPEA (0.17 ml, 1.1 mmol, 3 equiv.) were added and the mixture was stirred for 3 hr., before being concentrated and purified by flash column chromatography (DCM → 10% MeOH/DCM, v/v), yielding the title compound (130 mg, 0.14 mmol, 41%). $[\alpha]_D^{20}$= -1.5° (c 2, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 4.58-4.47 (m, 1H), 4.47-4.32 (m, 2H), 3.14 (t, *J* = 6.3 Hz, 6H), 3.24 (m, 1H), 2.92 (d, *J* = 5.1 Hz, 1H), 2.23 (t, *J* = 7.4 Hz, 2H), 2.17 (t, *J* = 7.4 Hz, 4H), 1.97-1.89 (m, 5H), 1.77-1.42 (m, 30H), 1.41-1.27 (m, 12H), 0.99-0.85 (m, 18H). HRMS: calcd. for $[C_{51}H_{88}N_6O_8H]^+$ 913.67390 found 913.67364



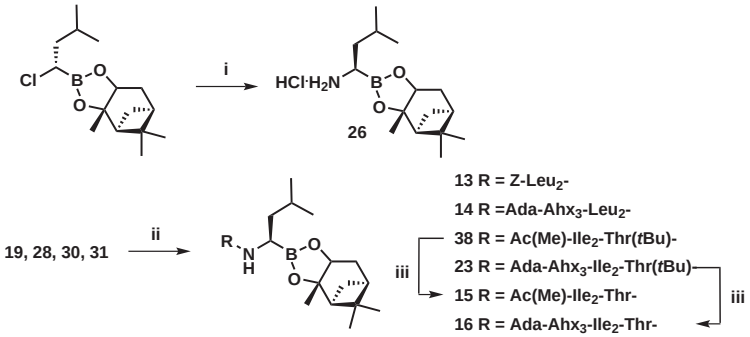
Ada-Ahx$_3$-Ile$_2$-Thr-leucinyl-2-methyloxirane (11). (Boc-leucinyl)-2-methyloxirane (**37**)[6] (78 mg, 0.29 mmol, 1.1 equiv.) was stirred in TFA/DCM (1/1 v/v, 1 ml) until TLC analysis indicated complete conversion of the starting material (20 min.). The reaction mixture was concentrated in presence of Tol. (2x) before being dissolved in DMF and put under an Argon atmosphere. Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OH (**19**) (236 mg, 0.26 mmol), BOP (0.13 g, 0.29 mmol, 1.1 equiv.), DiPEA (95 µl, 1.1 mmol, 4.4 equiv.) were added, and the mixture was stirred for 3hr. The crude Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-leucinyl-2-methyloxirane (**21**) was precipitated with EtOAc yielding a mixture of diastereomers. The major product was purified by semi-preparative RP-HPLC yielding **21** (27.3 mg, 25.5 µmmol, 10%). Tert-butyl ether **21** was dissolved in TFA/DCM (1/1 v/v) and stirred for 30 min., before being evaporated in the presence of Tol. (2x) yielding the title compound as a colourless oil (23.6 mg, 23.3 µmol, 91%). $[\alpha]_D^{20}$= -17.5 (c 0.24, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 4.55 (dd, J_1 = 2.6, J_2 = 10.6 Hz, 1H), 4.33 (d, *J* = 5.2 Hz, 1H), 4.27-4.21 (m, 2H), 4.04 (m, 1H), 3.25 (d, *J* = 5.2 Hz, 1H), 3.14 (t, *J* = 7.0 Hz, 6H), 2.91 (d, *J* = 5.2 Hz, 1H), 2.24 (t, *J* = 7.2 Hz, 2H), 2.16 (t, *J* = 7.4 Hz, 4H), 1.94 (m, 3H), 1.91 (s, 2H), 1.82 (m, 2H), 1.74-1.56 (m, 22H), 1.55-1.47 (m, 7H), 1.46 (s, 3H), 1.39-1.11 (m, 11H), 0.94 – 0.86 (m, 18H). ^{13}C NMR (100.2 MHz, $CDCl_3$): δ ppm 209.4, 176.0, 175.9, 174.0, 173.7, 173.4, 172.1, 68.4, 60.0, 59.6, 59.3, 59.1, 53.0, 51.9, 51.8, 43.7, 40.3, 40.2 (2x), 40.1, 37.9 (2x), 37.6, 37.0, 36.6, 33.7, 30.1 (3x), 27.6, 27.5, 26.6, 26.2, 26.0, 25.9, 23.8, 19.9, 17.0, 16.0, 15.9, 11.4, 11.2. HRMS: calcd. for $[C_{55}H_{95}N_7O_{10}H]^+$ 1014.72132 found 1014.72270, calcd. for $[C_{55}H_{95}N_7O_{10}Na]^+$ 1036.70326, found 1036.70421.



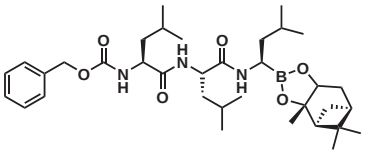
(Pyrazine-2-carbonylphenylalanyl)-leucinyl-2-methyloxirane (12). (Boc-leucinyl)-methyloxirane (**37**)[6] (103 mg, 0.38 mmol) was stirred in TFA (1 ml) until TLC analysis indicated complete consumption of the starting material. Tol. was added, and the reaction mixture was concentrated. Pyrazine-2-carbonylphenylalanine **34** (0.11 g, 0.4 mmol, 1.05 equiv.) was coevaporated with Tol. (2x) and dissolved in DCM (20 ml) and put under an Argon atmosphere. BOP (0.2 g, 0.44 mmol, 1.1 equiv.) and DiPEA (0.2 ml, 1.14 mmol, 3 equiv.) were added, followed by addition of the TFA·leucinyl-methyloxirane **25**. The

reaction mixture was stirred for 1hr., before washing with sat. aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated. Flash column chromatography (PetEt → 25% EtOAc/PetEt) yielded the title compound (8.6 mg, 20 µmol, 5.3% isolated yield). $[\alpha]_D^{20}$= +12 (c 0.1, MeOH). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 9.35 (d, *J* = 1.1 Hz, 1H). 8.75 (d, *J* = 2.2 Hz, 1H), 8.53 (d, *J* = 1.5 Hz, 1H), 8.37 (d, *J* = 8.0 Hz, 1H), 7.31-7.20 (m, 5H), 6.36 (d, *J* = 7.7 Hz, 1H), 4.91 (dd, J_1 = 6.9, J_2 = 8.0 Hz, 1H), 4.60-4.50 (m, 1H), 3.28 (d, *J* = 5.1 Hz, 1H), 3.20-3.15 (m, 2H), 2.90 (d, *J* = 4.7 Hz, 1H), 1.60 – 1.30 (m, 6H), 0.89-0.82 (m, 6H). ^{13}C NMR (50.1 MHz, $CDCl_3$): δ ppm 207.9, 170.3, 162.9, 147.5, 144.3, 143.9, 142.7, 136.2, 129.3, 128.6, 127.1, 58.9, 54.3, 52.3, 50.2, 40.2, 38.4, 25.0, 24.8, 23.1, 16.6. HRMS: calcd. for $[C_{23}H_{28}O_4N_4H]^+$ 425.21833, found 425.21835.

Scheme 9. Synthesis of the boronic ester equipped hybrid inhibitors.

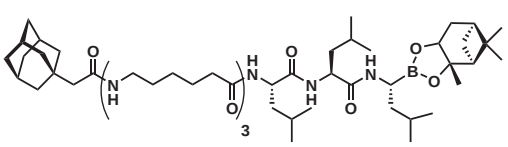


Reagents and conditions: i) (a) LiHMDS (1 equiv.), THF, -20 °C to RT, 12 hr. (b) HCl (5.7 equiv.), -90 °C to 0 °C, 2 hr. ii) (a) *N*-hydroxysuccinimide (1.4 equiv.), DIC (1.2 equiv.), DMF, 12 hr. (b) **26** (1 equiv.), DiPEA (1.02 equiv.), DMF/THF, -80 °C to RT, 4 hr. iii) TFA/DCM 1/1, 30 min.

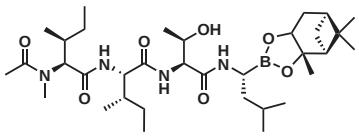


Z-Leu$_3$-boronic ester (13). Under argon atmosphere, Z-Leu$_2$-OH (**28**) (0.2 g, 0.53 mmol) and HOSu (85.1 mg, 0.73 mmol, 1.4 equiv.) were dissolved in DMF (1 ml) and DIC (100 µl, 0.64 mmol, 1.2 equiv.) was added. After a few min. a fine precipitate was formed. The mixture was stirred overnight at ambient temperature after which LC/MS analysis showed complete conversion of Z-Leu$_2$-OH (**28**) into Z-Leu$_2$-OSu. (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane[10] (152 mg, 0.53 mmol, 1 equiv.) was dissolved in THF (5 ml) in a flame dried two necked reaction flask which was put under an argon atmosphere. At -20 °C a LiHMDS solution (1M in THF, 600 µl, 0.60 mmol, 1.1 equiv.) was added dropwise. The mixture was allowed to warm to room temperature and stirred overnight. At -90 °C a HCl solution (2M in Et_2O, 1.50 ml, 3.0 mmol, 5.7 equiv.) was added drop wise. The mixture was slowly warmed to 0 °C (ca. 2 hr.) and re-cooled to -80 °C at which temperature the Z-Leu$_2$-OSu described above and DIPEA (0.90 ml, 5.4 mmol, 1.02 equiv.) were added. The mixture was slowly warmed to room temperature (ca. 2 hr.) and was stirred for another 2 hr. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silicagel column chromatography (10% EtOAc/PetEt → 25% EtOAc/PetEt) affording the title compound as a white solid (197 mg, 0.32 mmol, 59%). $[\alpha]_D^{20}$= -58 (c 1, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 7.37-7.28 (m, 5H), 6.79 (d, *J* = 8.1 Hz, 1H), 5.50 (d, *J* = 6.5 Hz, 1H), 5.12 (d, *J* = 12.0 Hz, 1H), 5.02 (d, *J* = 12.2 Hz, 1H), 4.58 (dd, J_1 = 13.4, J_2 = 7.5 Hz, 1H), 4.27 (dd, J_1 = 8.6, J_2 = 1.8 Hz, 1H), 4.22-4.13 (m, 1H), 3.10-2.97 (m, 1H), 2.36-2.27 (m, 1H), 2.15 (td, J_1 = 10.5, J_2 = 5.2 Hz, 1H), 2.01 (t, *J* = 5.4 Hz, 1H), 1.93-1.77 (m, 2H), 1.76-1.55 (m, 5H), 1.55-1.34 (m, 7H), 1.33-1.23 (m, 4H), 0.98-0.79 (m, 21H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 178.4, 175.3, 158.7, 138.0, 129.4(2x), 129.0, 128.8 (2x), 128.6, 84.0, 77.2, 67.7, 55.0, 53.5, 49.4, 42.0, 41.7,

41.3, 41.1, 39.1, 37.6, 29.9, 27.8, 27.4, 26.7, 25.8, 25.6, 24.6, 23.7, 23.5, 23.2, 22.5, 22.0, 21.8. HRMS: calcd. for $[C_{35}H_{56}BN_3O_6H]^+$ 626.43349, found 626.43408, calcd. for $[C_{35}H_{56}BN_3O_6Na]^+$ 648.41544, found 648.41578.

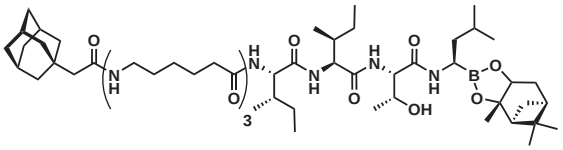


Ada-Ahx$_3$-Leu$_2$-leucinyl-boronic ester (14). Under argon Ada-Ahx$_3$-L$_2$-OH (**30**) (95 mg, 0.13 mmol) and HOSu (35 mg, 0.30 mmol, 2.3 equiv.) were dissolved in DMF (1 ml) and DIC (25 µl, 0.16 mmol, 1.2 equiv.) was added. After a few min. a fine precipitate was formed. The mixture was stirred overnight at ambient temperature after which LC/MS analysis showed complete conversion of Ada-Ahx$_3$-Leu$_2$-OH (**30**) into Ada-Ahx$_3$-Leu$_2$-OSu. (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane[10] (132 mg, 0.46 mmol, 3.5 equiv.) was dissolved in THF (4 ml) in a flame dried two necked reaction flask under an argon atmosphere. At -20 °C a LiHMDS solution (1M in THF, 460 µl, 0.46 mmol, 3.5 equiv.) was added drop wise. The mixture was allowed to warm to room temperature and stirred overnight. At -90 °C a HCl solution (2M in Et_2O, 1.00 ml, 2.0 mmol, 15.4 equiv.) was added drop wise. The mixture was slowly warmed to 0 °C (ca. 2 hr.) and re-cooled to -80 °C at which temperature the Ada-Ahx$_3$-Leu$_2$-OSu described above and DIPEA (0.60 ml, 3.6 mmol, 27.7 equiv.) were added. The mixture was slowly warmed to room temperature (ca. 2 hr.) at which it was stirred for another 2 hr. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silicagel column chromatography (PetEt/EtOAc/MeOH = 3/1/0 → 1/1/0 → 0/1/0 → 0/9/1 v/v/v) affording the title compound as a white solid (74 mg, 73 µmol, 59%). Further purification by RP-HPLC afforded **14** (20 mg, 20 µmol, 16%). $[\alpha]_D^{20}$= -21.7 (c 0.12, MeOH). ^{1}H NMR (600 MHz, CD_3OD): δ ppm 4.61 (dd, J_1 = 9.6, J_2 = 5.4 Hz, 1H), 4.37 (dd, J_1 = 9.6, J_2 = 5.4 Hz, 1H), 4.15 (dd, J_1 = 10.2, J_2 = 8.4 Hz, 1H), 3.17 (t, J = 7.2 Hz, 6H), 2.71 (t, J = 7.2 Hz, 1H), 2.36 (m, 1H), 2.27 (t, J = 7.2 Hz, 2H), 2.20 (t, J = 7.2 Hz, 4H), 2.15 (m, 1H), 1.97 (m, 4H), 1.94 (s, 2H), 1.88 (m, 1H), 1.81 (m, 1H), 1.79 – 1.61 (m, 23H), 1.58 (m, 2H), 1.56 – 1.50 (m, 6H), 1.48 (d, J = 10.2 Hz, 1H), 1.40 (s, 3H), 1.39 – 1.33 (m, 8H), 1.31 (s, 3H), 0.99 (s, 3H), 0.98 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H), 0.94 (s, 3H), 0.93 (s, 3H), 0.90 (s, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ ppm 178.5, 176.4, 175.8, 175.0, 173.6, 84.0, 77.2, 55.8, 53.5, 53.4, 51.9, 49.6, 43.8, 43.7, 41.8, 41.7, 41.4, 41.2, 40.2, 40.1, 39.2, 37.9, 37.7, 37.0, 36.6, 36.5, 33.8, 30.2, 30.1, 30.0, 29.9, 27.8, 27.6, 27.5, 27.4, 26.7, 26.6, 26.5, 26.3, 25.9, 25.7, 24.6, 23.6, 23.5, 23.4, 23.3, 23.2, 22.5, 22.1, 21.9, 21.8, 19.3, 18.8, 17.3, 13.2. HRMS: calcd. for $[C_{57}H_{99}BN_6O_8H]^+$ 1007.76722, found 1007.76902.



Ac(Me)-Ile$_2$-Thr-Leu-boronic ester (15). Under argon Ac(Me)-Ile$_2$-Thr(*t*Bu)-OH (**31**) (78 mg, 0.17 mmol) and HOSu (35 mg, 0.304 mmol, 1.8 equiv.) were dissolved in DMF (1 ml) and DIC (35 µl, 0.23 mmol, 1.4 equiv.) were added. The mixture was stirred overnight at ambient temperature after which LC/MS analysis showed complete conversion. (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane[10] (168 mg, 0.59 mmol, 3.5 equiv.) was dissolved in THF (5 ml) in a flame dried two necked reaction flask under an argon atmosphere. At -20 °C a LiHMDS solution (1M in THF, 550 µl, 0.55 mmol, 3.2 equiv.) was added drop wise. The mixture was allowed to warm to room temperature and stirred overnight. At -90 °C a HCl solution (2M in Et_2O, 1.50 ml, 3.0 mmol, 17.6 equiv.) was added drop wise. The mixture was slowly warmed to 0 °C (ca. 2 hr.) and re-cooled to -80 °C at which temperature the Ac(Me)-Ile$_2$-Thr(*t*Bu)-OSu described above and DIPEA (0.90 ml, 5.4 mmol, 31.8 equiv.) were added. The mixture was slowly warmed to room temperature (ca. 2 hr.) and was stirred for another 2 hr. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silicagel column chromatography (PetEt/EtOAc/MeOH = 3/1/0 → 1/1/0 →

0/1/0 → 0/9/1 v/v/v) affording Ac(Me)-Ile$_2$-Thr(*t*Bu)-Leu-boronic ester (**38**) as a white solid. This material was dissolved in TFA/DCM (1/1 v/v, 1 ml). After 30 min. Tol. (30 ml) was added and the solvents evaporated. Purification by RP-HPLC afforded the title compound (9 mg, 14 µmol, 8%). $[\alpha]_D^{20}$= -360 (c 0.04, MeOH). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.88 (s, 1H), 7.03 (d, *J* = 5.10 Hz, 1H), 6.77 (d, *J* = 8.0 Hz, 1H), 4.60-4.41 (m, 4H), 4.27 (d, *J* = 7.3 Hz, 1H), 4.09 (m, 1H), 3.82 (s, 1H), 2.94 (s, 3H), 2.87 (m, 1H), 2.32 (m, 1H), 2.14 (s, 3H), 2.02 (m, 2H), 1.96-1.71 (m, 4H), 1.41 (s, 3H), 1.34 (m, 3H), 1.28 (s, 2H), 1.18 (s, 1H), 1.14 (s, 1H), 0.96-0.81 (m, 27H). HRMS: calcd. for $[C_{34}H_{61}BN_4O_7H]^+$ 649.47061, found 649.47080, calcd. for $[C_{34}H_{61}BN_4O_7Na]^+$ 671.45255, found 671.45272.



Ada-Ahx$_3$-Ile$_2$-Thr-Leu-boronic ester (16). Under argon Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OH (**19**) (134 mg, 0.15 mmol) and HOSu (29 mg, 0.25 mmol, 1.7 equiv.) were dissolved in DMF (2 ml) and DIC (35 µl, 0.23 mmol, 1.5 equiv.) were added. After a few min. a fine precipitate was formed. The mixture was stirred overnight at ambient temperature after which LC/MS analysis showed complete conversion of Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OH (**19**) into Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OSu. (1R)-4-(1-chloro-3-methyl(butyl)-2,9,9-trimethyl-3,5-dioxa-4-bora-tricyclo[6.1.1.0^{2,6}]decane[10] (132 mg, 0.46 mmol, 3.1 equiv.) was dissolved in THF (4 ml) in a flame dried two necked reaction flask which was put under an argon atmosphere. At -20 °C a LiHMDS solution (1M in THF, 460 µl, 0.46 mmol, 3.1 equiv.) was added drop wise. The mixture was allowed to warm to room temperature and stirred overnight. At -90 °C a HCl solution (2M in Diethyl ether, 1.00 ml, 2.0 mmol, 13.3 equiv.) was added drop wise. The mixture was slowly warmed to 0 °C (ca. 2 hr.) and re-cooled to -80 °C at which temperature the Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-OSu described above and DIPEA (0.60 ml, 3.6 mmol, 24 equiv.) were added. The mixture was slowly warmed to room temperature (ca. 2 hr.) and was stirred for another 2 hr. After filtration through a path of hyflo and concentration *in vacuo* the crude product was purified by silicagel column chromatography (PetEt/EtOAc/MeOH = 3/1/0 → 1/1/0 → 0/1/0 → 0/9/1 v/v/v) affording Ada-Ahx$_3$-Ile$_2$-Thr(*t*Bu)-Leu-boronic ester (**23**) as a white solid. This material was dissolved in TFA/DCM (1/1 v/v, 1 ml). After 30 min. Tol. (30 ml) was added and the solvents evaporated. Purification by RP-HPLC afforded the title compound (7 mg, 6.3 µmol, 4%). $[\alpha]_D^{20}$= -72.5 (c 0.08, MeOH). ^{1}H NMR (300 MHz, CD_3OD): δ ppm 4.52 (dd, J_1 = 7.2, J_2 = 4.5 Hz, 1H), 4.21-4.13 (m, 4H), 3.14 (t, *J* = 6.9 Hz, 6H), 2.72 (dd, J_1 = 8.4, J_2 = 6.9 Hz, 1H), 2.36 (m, 1H), 2.25 (t, *J* = 7.5 Hz, 2H), 2.17 (t, *J* = 7.5 Hz, 4H), 1.94 (m, 4H), 1.91 (s, 2H), 1.79 (m, 2H), 1.77-1.43 (m, 30H), 1.41-1.26 (m, 15H), 1.23-1.11 (m, 5H), 0.94 (s, 3H), 0.93 (s, 3H), 0.92 (s, 3H), 0.91 (s, 3H), 0.89 (s, 3H), 0.88 (s, 3H), 0.87 (s, 3H). HRMS: calcd. for $[C_{61}H_{106}BN_7O_{10}H]^+$ 1108.81670, found 1108.81820, calcd. for $[C_{61}H_{106}BN_7O_{10}Na]^+$ 1130.79864, found 1130.79987.

Competition experiments.

Whole cell lysates of EL4 or HEK293T were made by sonication (30 sec., 11 Watt) in 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 (10µg total protein) were exposed to the inhibitors for 1 hr. prior to incubation with MV151 (**5**, 0.1 µM) for 1 hr. at 37 °C. Reaction mixtures were boiled with Laemmli's buffer containing β-mercapto-ethanol for 3 min. before being resolved on 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} 532, λ_{em} 560).

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6 Synthesis and evaluation of subunit specific proteasome probes.

6.1 Introduction

Prokaryotic proteasome core particles consist of two inner rings, composed of seven identical proteolytically active β subunits, stacked on top and bottom by two rings of seven identical α-subunits.[1] During evolution four of the seven β subunits lost their proteolytic activity and the three remaining proteolytically active β subunits diverged in their substrate specificity.[2] The activity of the eukaryotic β1 subunit is designated as caspase-like, the β2 subunit as tryptic-like and the β5 subunit as chymotryptic like, although the subunits are rather more promiscuous in their substrate preference than is suggested by this designation. Four additional proteolytically active proteasome subunits are expressed in specific cell types. The β1i, β2i and the β5i subunits replace the corresponding constitutive subunits in newly formed proteasome particles called immunoproteasomes.[3] Cortical thymic epithelial cells express an additional β5 subunit, β5t.[4] The individual role of the different proteolytically active proteasome subunits remains one of the big questions in proteasome research and pharmaceutical sciences. Bortezomib (Velcade, PS-341),[5] approved in the U.S. for treating relapsed multiple myeloma[6] and mantle cell lymphoma,[7] only targets β1 and β5 of the constitutive proteasome and β1, β5, β1i and β5i of the immuno-proteasome.[8] An interesting research question is what subunit or which combination of subunits should be targeted to get the optimal anti-cancer therapeutic. Being involved in the generation of antigenic peptides loaded in MHC class I complexes, the contribution of each separate active proteasome subunit to the epitope repertoire is yet another question to be answered. To unravel the evolutionary advantage and the individual roles of the different proteolytically active subunits in cellular processess, antigen

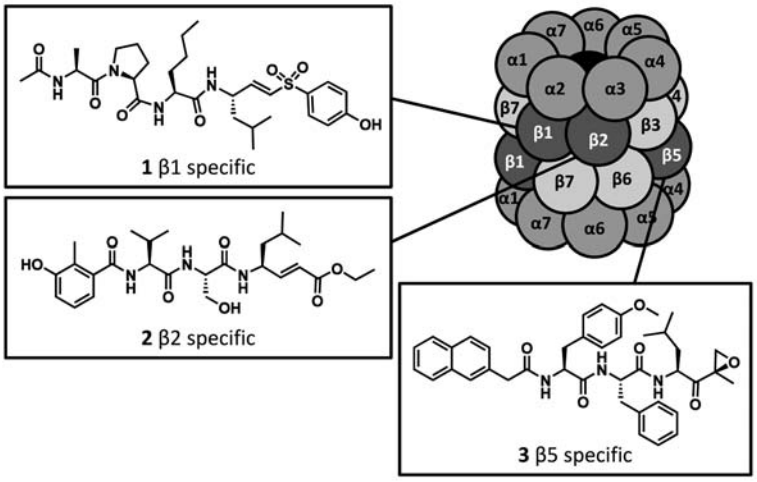


Figure 1. Eukaryotic 20S proteasome and previously described subunit specific proteasome inhibitors. The proteolytically active β subunits are marked in dark gray.

presentation and pharmacology, inhibitors that specifically target one proteolytic subunit would be highly valuable research tools.

Three modified peptides that have been described to have specificity for one of the three catalytic activities of the constitutive proteasome are depicted in Figure 1. Van Swieten *et al*. reported the β1 specific inhibitor Ac-Ala-Pro-Nle-LeuVS-PhOH (**1**).[9] HMBA-Val-Ser-LeuVE (**2**) was reported to be β2 specific by Marastoni *et al.*[10] In an unpublished study towards selective inhibitors for the chymotryptic activity of the proteasome by the Kisselev lab Napht-Tyr(OMe)-Phe-LeuEK (**3**) was discovered to have profound selectivity towards the β5-subunit.

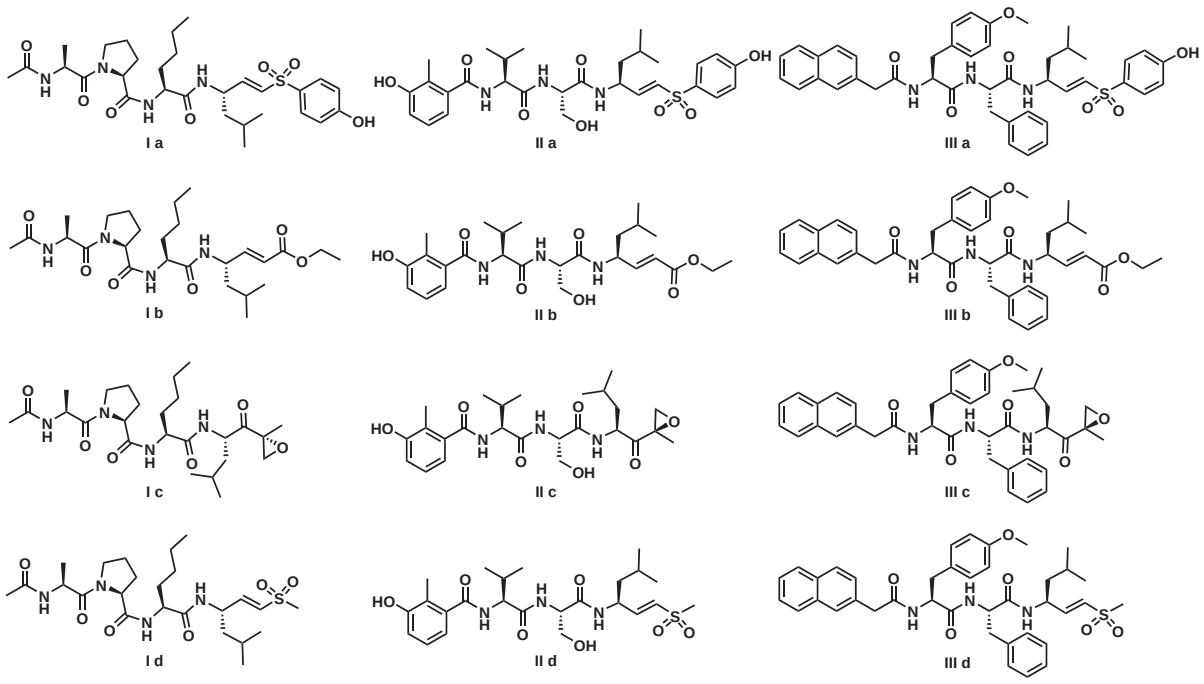


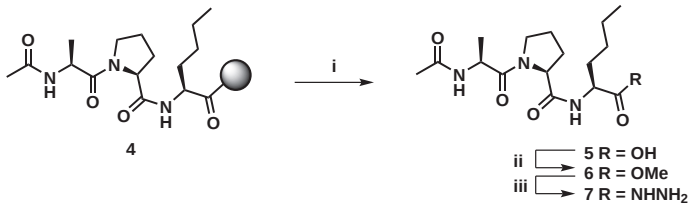
Figure 2. Hybrid library studied in this chapter.

In Chapter 5 it was demonstrated that scrambling of structural elements of known proteasome inhibitors is a viable strategy to arrive at potent new proteasome inhibitors.[11] Applying this strategy to the previously described inhibitors **1**-**3** could result in more potent and more selective subunit specific proteasome inhibitors. It was shown in Chapter 5 that prediction of the potency and subunit preference of a scrambled peptide-based inhibitor is not straightforward. Therefore, it was deemed appropriate to synthesize a library of hybrid structures of the subunit specific inhibitors **1**-**3**. This Chapter describes the coupling of the peptidic recognition elements of the inhibitors depicted in Figure 1 to four different warheads, being LeuVS-PhOH,[12] LeuVE,[10] LeuEK[13] and LeuVS[14] resulting in a library of 12 potential proteasome inhibitors (Figure 2), which were screened for their subunit specificity.

6.2 Results and discussion

For the generation of the hybrid library a strategy employing the azide coupling to condense the peptidic recognition element to the leucine derived warhead amines was chosen to prevent epimerization of the P2 position of the potential inhibitors.[15] Hence, the synthesis commenced with the preparation of the hydrazides of the peptidic recognition elements. Fmoc-based solid phase peptide synthesis using HMPB functionalized MBHA

Scheme 1. Synthesis of the β1 specific recognition peptide hydrazide **7**.

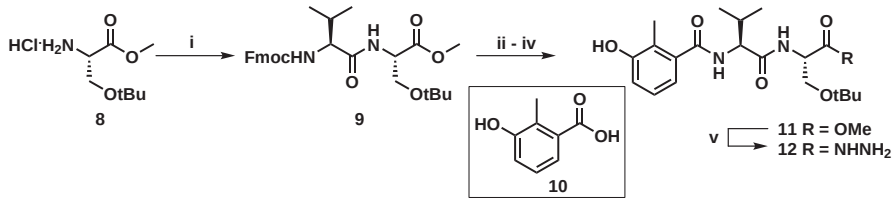


Reagents and conditions: i) 1% TFA in DCM, 30 min., 3×. ii) TMS-diazomethane (2 equiv.), 15 min., 85% from Fmoc-Nle-resin. iii) Hydrazine monohydrate (60 equiv.), MeOH, 1.5 hr., 92%.

resin gave immobilized acetyl capped tripeptide **4** (Scheme 1). After mild acidic cleavage from resin, the crude peptide was treated with TMS-diazomethane to give methyl ester **6**. Refluxing in methanol in the presence of an excess of hydrazine resulted in the β1 recognition element peptide hydrazide **7**.

For the synthesis of the β2 peptidic recognition element *tert*-butyl-protected serine methyl ester **8** was condensed with Fmoc-valine to give dipeptide **9** (Scheme 2). Deprotection of the *N*-terminus and subsequent capping with HMBA **10**, followed by refluxing in methanol in the presence of an excess of hydrazine gave the *tert*-butyl-protected peptide hydrazide **12**.

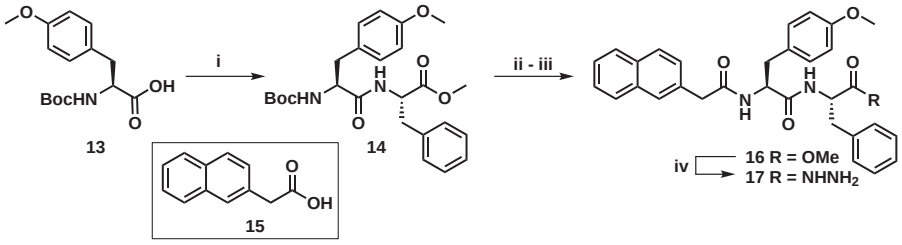
Scheme 2. Synthesis of the β2 specific recognition peptide hydrazide **12**.



Reagents and conditions: i) Fmoc-Val-OH (1 equiv.), BOP (1 equiv.), DiPEA (3.3 equiv.), DCM, 2 hr., quant. ii) DBU (1 equiv.), DMF, 5 min. iii) HOBt (4.5 equiv.), 1 min. iv) **10** (1 equiv.), BOP (1.1 equiv.), DiPEA (4 equiv.), 2 hr., 67%. v) Hydrazine monohydrate (60 equiv.), MeOH, 15 hr., 88%.

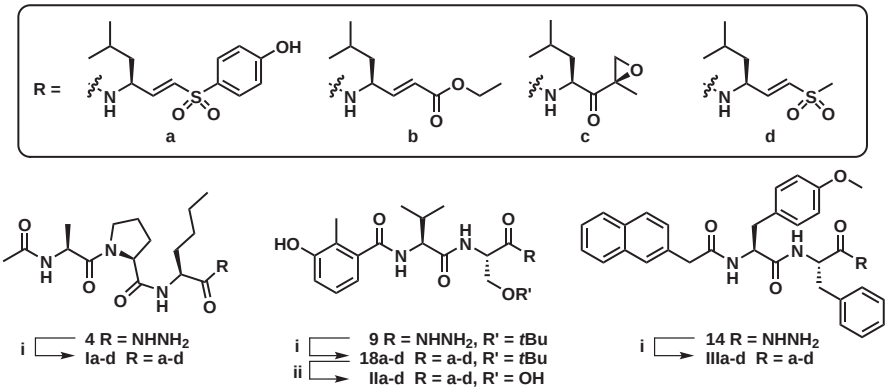
The final recognition peptide hydrazide was prepared by condensation of Boc-protected tyrosine methyl ether **13** with phenylalanine methyl ester resulting in dipeptide **14** (Scheme 3). Treatment with hydrazine in refluxing methanol gave the β5 recognition element peptide hydrazide **17**.

Scheme 3. Synthesis of the β5 specific recognition peptide hydrazide **17**.



Reagents and conditions: i) HCl·H-Phe-OMe (1 equiv.), BOP (1 equiv.), DiPEA (2.2 equiv.), DCM, 15 hr., quant. ii) TFA/DCM 1/1 (v/v), 15 min. iii) **15** (1 equiv.), BOP (1 equiv.), DiPEA (3.3 equiv.), DCM, 15 hr., 68%. iv) Hydrazine monohydrate (60 equiv.), MeOH, 2.5 hr., 95%.

Scheme 4. Synthesis of the hybrid library.



Reagents and conditions: i)(a) HCl (2.8 equiv.), *t*BuONO (1.1equiv.), DMF/EtOAc (1/1, v/v), -25 °C, 4 hr. (b) TFA·H-R^{a-d} (1.1 equiv.), DiPEA (3.8 equiv.), -25 °C to RT, 15 hr., **Ia** 77%, **Ib** 70%, **Ic** 63%, **Id** 34%, **18a** 63%, **18b** 82%, **18c** 89%, **18d** 75%, **IIIa** 77%, **IIIb** 89%, **IIIc** 66%, **IIId** 53%. ii) TFA/DCM 1/1 (v/v), 30 min., **IIa** 87%, **IIb** 89%, **IIc** 69%, **IId** 64%.

Having synthesized the three recognition peptide building blocks, they were coupled to the leucine derived warheads (Scheme 4), which were synthesized according to literature procedures.[10,12-14] The hydrazides were treated with *tert*-butyl nitrite under acidic conditions to generate an acyl azide *in situ*. After addition of base, the warhead amines were reacted with the activated peptides. Deprotection of the *tert*-butyl serine in compounds **18a-d** completed the synthesis of the library.

The proteasome inhibition profile of the panel of 12 modified oligopeptides was determined in competition experiments versus proteasome probe MV151[16] (see Chapter 2). Human Embryonic Kidney (HEK293T) cell lysates containing the constitutive proteasome were exposed to increasing concentrations of the inhibitors for one hour. Residual proteasome activity was fluorescently labeled with MV151 after which the proteins were denatured, separated on SDS-PAGE and visualized using a fluorescence scanner (Figure 3). Apparent IC_{50} values were determined by quantification of the fluorescent gel bands (Figure 4). As reported, inhibitor **Ia** (**1**) is selective for the β1 subunit. The putative β2 selective inhibitor **IIb** (**2**) turned out not to inhibit the β2-subunit at all in the competition assay and seems to have a preference for β5 at high concentrations. Although about 20 times more potent for β5, inhibitor **IIIc** (**3**) is capable to block the β2 subunit leaving β1 as the sole active proteasome subunit. The LeuVS equipped β1 recognition peptide **Id** is selective for β1 in the same order of magnitude as **Ia**, but when armed with the LeuEK warhead the β1 selective inhibitor (**Ic**) becomes one order of magnitude more potent. No selectivity for β2 was observed in the panel of inhibitors bearing the β2 peptidic recognition element. The

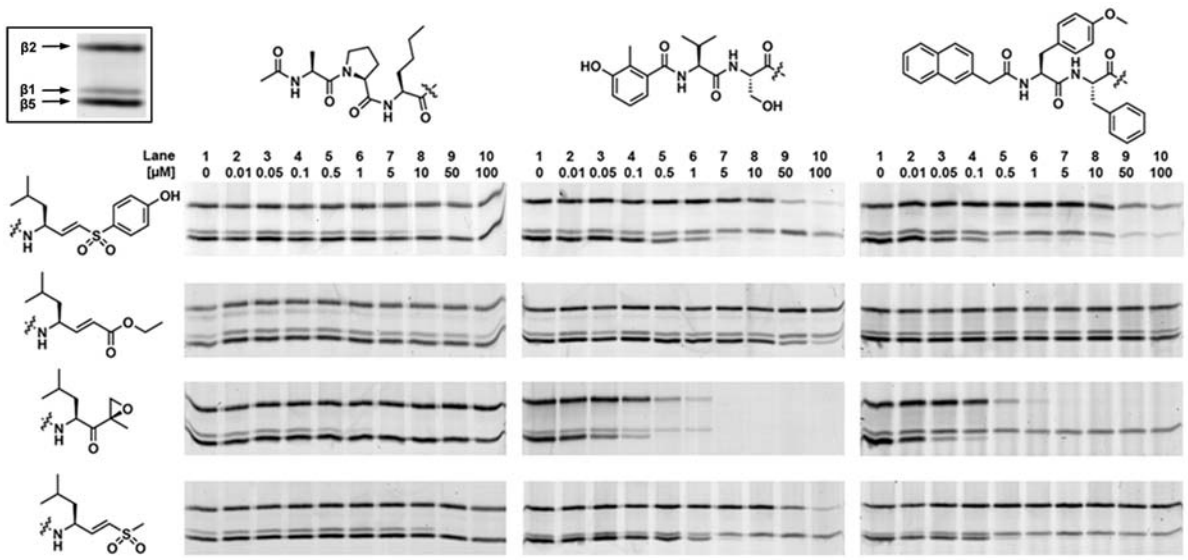


Figure 3. Proteasome profiling screen of hybrid library using MV151.
HEK293T lysates (10 μg total protein) were incubated with the indicated concentrations of hybrid compounds for 1 hr. at 37 °C. The residual proteasome activity was fluorescently labeled by incubation with 1 μM MV151 for 1 hr. at 37 °C.

preference for β5 becomes apparent in **IIa** and **IId** as seemed to be the case in the parent compound **IIb** (**2**). Once again, the LeuEK equipped inhibitor is the more potent member of the panel in this case blocking labeling of all subunits with MV151 potently. In the panel of inhibitors containing the β5 recognition element inhibitor **IIIa** is a more selective inhibitor for the β5-subunit than its parent compound **IIIc** (**3**), but still targets β1 and β2 at higher concentrations. However, inhibitor **IIId** turned out to be a very potent and very selective inhibitor for the β5-subunit.

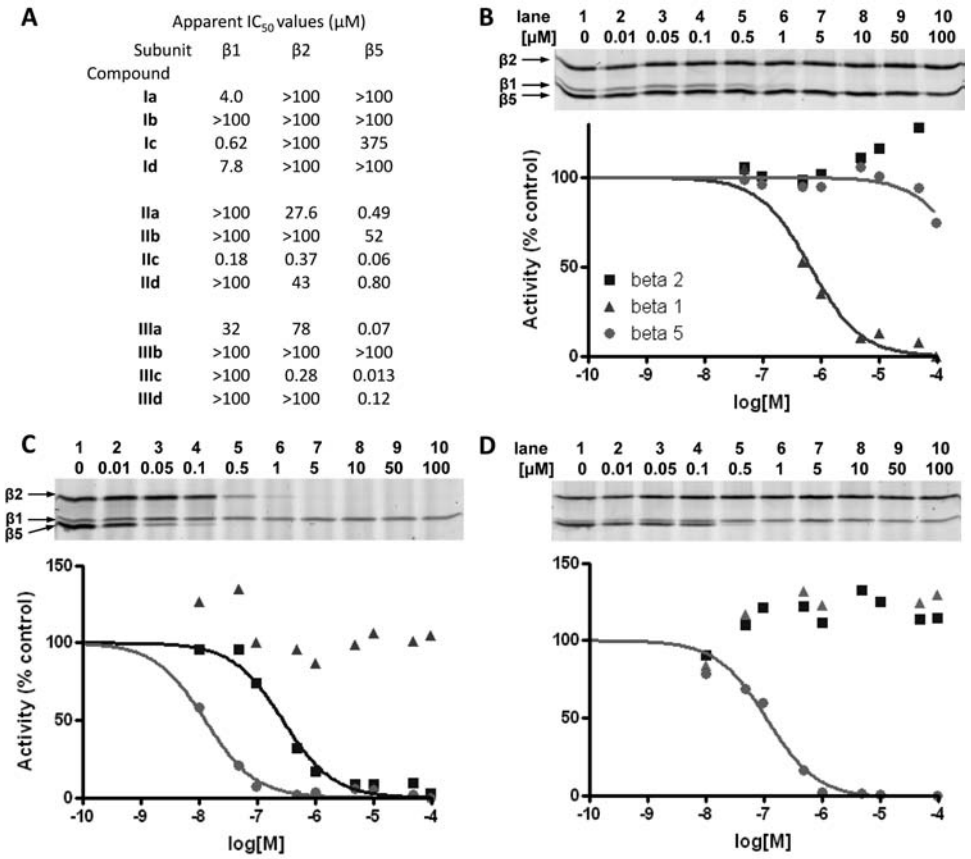
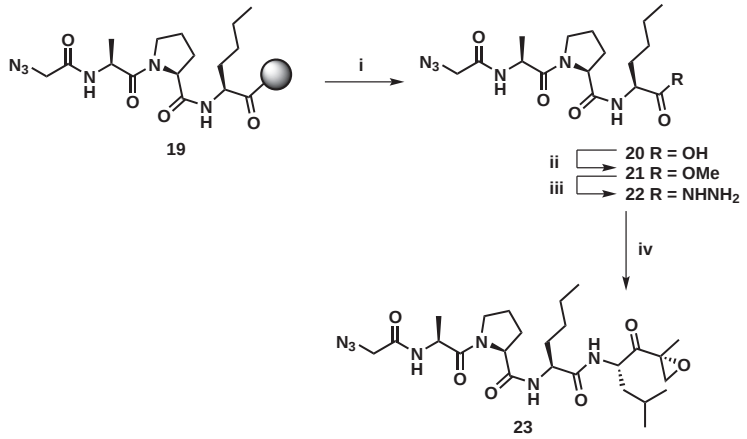


Figure 4. Apparent IC_{50} values of inhibitor library.
(**A**) Table with apparent IC_{50} values of the inhibitor library. (**B**-**D**) Gel scans and corresponding one-site competition plots of (**B**) **Ic**, (**C**) **IIIc** and (**D**) **IIId**.

Having identified three inhibitors with interesting subunit selectivities (figure 4), **Ic** being selective for β1, **IIIc** inhibiting β2 and β5 and **IIId** as a selective inhibitor for β5, activity-based probes were designed based on these. Introduction of an azido functionality in the selective inhibitors could lead to compounds able to selectively visualize the targeted subunits in a two-step labeling experiment (Chapter 1.5). A β1 selective probe is easily accessible by replacing the acetyl in **Ic** with an azido glycine. Standard solid phase peptide synthesis and subsequent capping with azido glycine afforded resin **19** (Scheme 5). Mild acidic cleavage, followed by reaction with TMS-diazomethane resulted in azido containing

Scheme 5. Synthesis of an azido containing β1 selective proteasome probe.

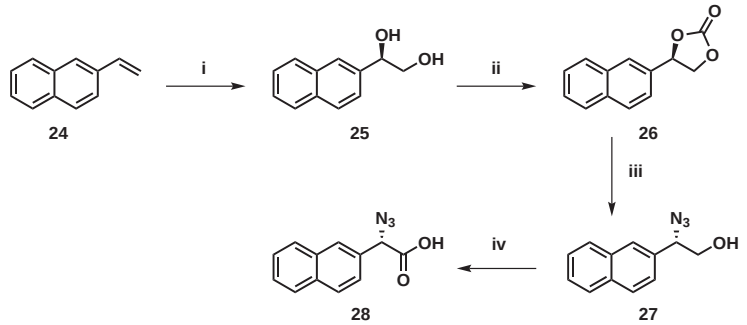


Reagents and conditions: i) 1% TFA in DCM, 30 min., 3×. ii) TMS-diazomethane (2 equiv.), 15 min., 90% from Fmoc-Nle-resin. iii) Hydrazine monohydrate (60 equiv.), MeOH, 1.5 hr., quant. iv) (a) HCl (2.8 equiv.), *t*BuONO (1.1 equiv.), DMF/EtOAc (1/1, v/v), -25 °C, 4 hr. (b) TFA·H-Leu-epoxyketone (1.1 equiv.), DiPEA (3.8 equiv.), -25 °C to RT, 15 hr., 23%.

peptide methyl ester **21,** which was converted to hydrazide **22**. The β1 selective probe was realized by performing an azide coupling with the LeuEK warhead giving **23**.

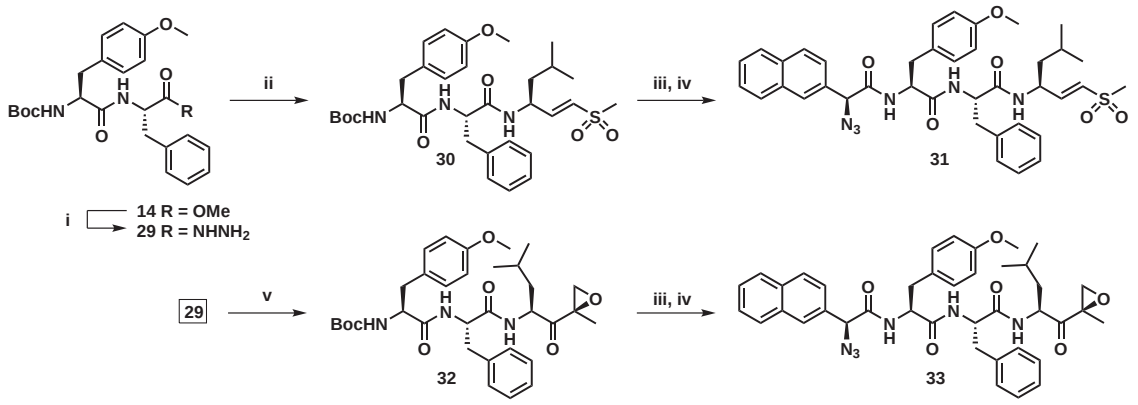
For the generation of the β5 selective probe and the probe labeling β2 and β5 azido naphthyl glycine **28** was synthesized (Scheme 6). Sharpless assymetric dihydroxylation of 2-vinylnaphthalene (**24**) gave optically pure *R*-1-(naphthhalen-2-yl)ethane-1,2-diol (**25**).[17] Opening of the cyclic carbonate in **26** at the benzylic position with azide,[18] followed by a TEMPO-BAIB oxidation[19] of the primary alcohol in **27** resulted in *S*-2-naphthyl azido glycine **28**. The synthesis towards the β5 selective probe **31** continued with the conversion of Boc-protected dipeptide methyl ester **14** to the corresponding hydrazide **29** (Scheme 7). Azide

Scheme 6. Synthesis of azido naphthyl glycine **28**.



Reagents and conditions: i) AD-mix-β, *t*BuOH/H_2O (1/1, v/v), 5 hr., 84%. ii) CDI (1.5 equiv.), MeCN, 45 °C, 1 hr., 93%. iii) NaN_3 (1.1 equiv.), H_2O (1 equiv.), DMF, 80 °C, 40 hr., 76%. iv) TEMPO (0.2 equiv.), BAIB (2.5 equiv.), DCM/H_2O (2/1, v/v), 1 hr., 80%.

Scheme 7. Synthesis of the β5 selective probe **31** and **33**.



Reagents and conditions: i) Hydrazine monohydrate (60 equiv.), MeOH, 2 hr., 88%. ii) (a) HCl (2.8 equiv.), *t*BuONO (1.1 equiv.), DMF/EtOAc (1/1, v/v), -25 °C, 4 hr. (b) TFA·H-LeuVS (1.1 equiv.), DiPEA (3.8 equiv.), -25 °C to RT, 15 hr., 75%. iii) TFA/DCM 1/1 (v/v), 30 min. iv) **28** (1.6 equiv.), EDC·HCl (1.6 equiv.), DiPEA (1 equiv.), DCM, 17 hr., **31** 70% (2 steps), **33** 26% (2 steps). v) (a) HCl (2.8 equiv.), *t*BuONO (1.1 equiv.), DMF/EtOAc (1/1, v/v), -25 °C, 4 hr. (b) TFA·H-LeuEK (1.1 equiv.), DiPEA (3.8 equiv.), -25 °C to RT, 15 hr., 71%.

coupling with LeuVS gave vinyl sulfone **30**. Acidic deprotection and subsequent capping of the free amine with azido acid **28** finished up the synthesis of probe **31**. Similarly, β2 and β5 targeting probe **33** was constructed from Boc-protected tripeptide epoxyketone **32**.

The potential of the probes to inhibit the proteasome in HEK293T cell lysate was determined in a competition experiment versus proteasome probe MV151 (Figure 5B, E and H). To assess the potential to cross the cell membrane, living HEK293T cells were exposed to the compounds with increasing concentrations for 2 hours at 37 °C. The cells were harvested, washed and the cytosolic content was screened for residual proteasome activity using MV151 (Figure 5C, F and I). The probe derived from the β1 selective inhibitor **Ic**, **23** still showed a predilection for β1 with an apparent IC_{50} value of 0.28 μM (Figure 5B and D). Compared to **Ic**, however, **23** targets β5 more potently with an apparent IC_{50} value of 75 μM (375 μM for **Ic**). The introduction of the relatively small azide moiety at the *N*-terminus of the inhibitor renders the compound slightly larger and more hydrophobic. In living cells, β1 selective probe **23** proved to be as potent as in cell lysates indicating that the probe can easily cross the cell membrane (Figure 5C and D). In this assay, β5 is targeted even more potently (but in the same order of magnitude as in lysates). It has been shown before that in living cells β5 activity is more pronounced relative to β2 and β1 compared to cell lysates, which could be the explanation for the increase potency for β5. Although slightly more potent, the selectivity of the β5 targeting probe **31** in lysates and in living cells is in the same order of magnitude as that of **IIId** (Figure 5E-G). This minimal increase in potency for β5 may again be explained by the introduction of some hydrophobicity at the *N*-terminus. Surprisingly, **31** seems more potent in labeling the β5 subunit in living cells compared to

lysates. This may be explained by the apparent difference in activity of β5 in intact cells and lysates as mentioned above. Azido probe **33** shows a more complex inhibition profile (Figure 5H-J). As for **IIIc,** probe **33** is a potent inhibitor of the β5 subunit with an apparent IC_{50} value of 21 nM in lysates and 24 nM in living cells. The parent inhibitor **IIIc** inhibits all β2 activity at concentrations as low as 10 μM. The azide decorated analogue **33** inhibits β2

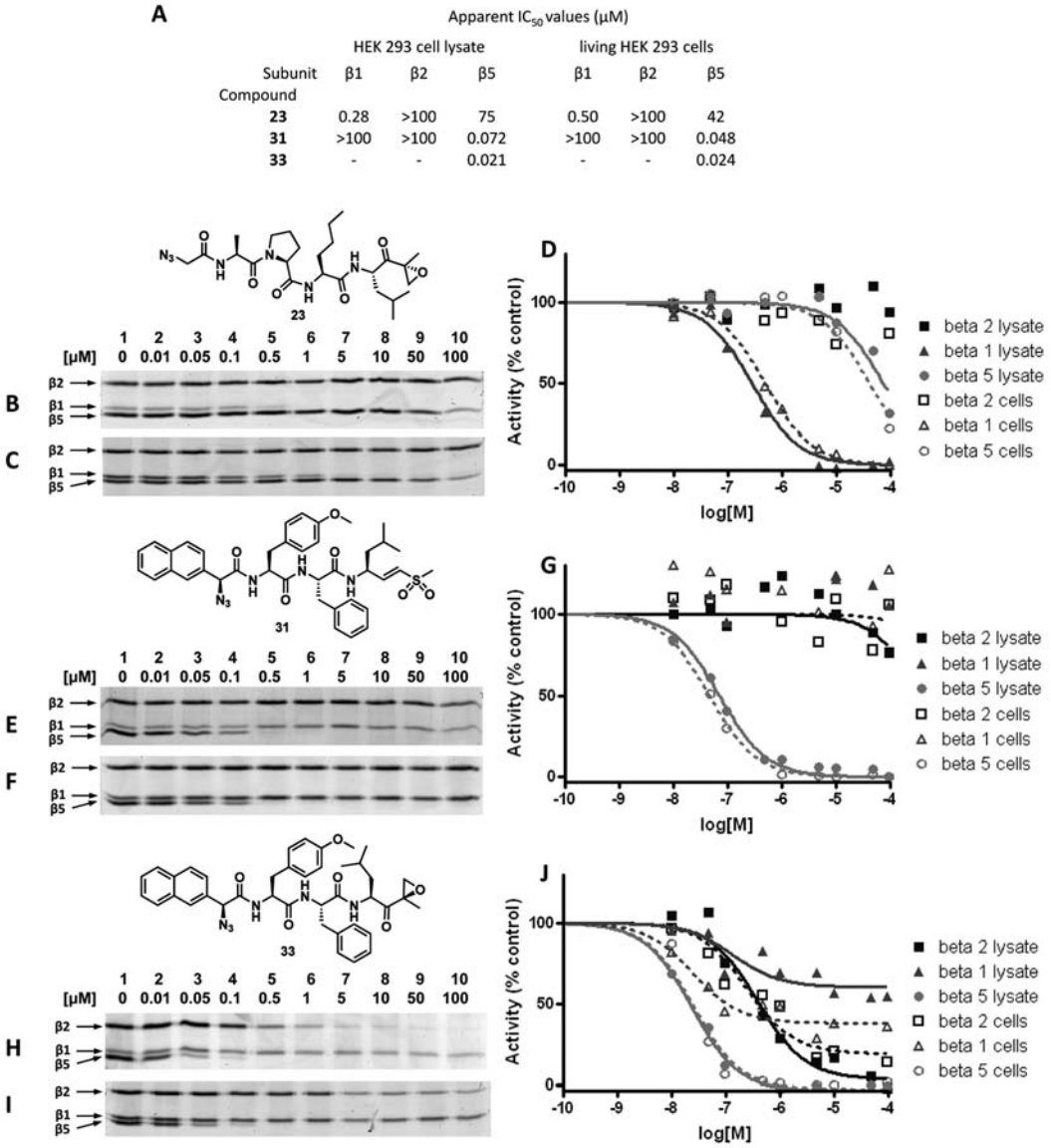
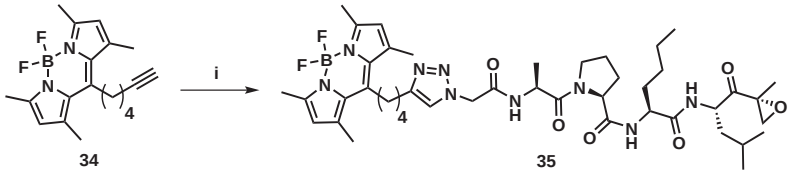


Figure 5. Proteasome profiling screen of specific proteasome probes using MV151.
(**A**) Table with apparent IC_{50} values of probes **23, 31,** and **33** in HEK293T cell lysate and living HEK293T cells. (**B, E, H**) HEK293T lysates (10 μg total protein) were incubated with the indicated concentrations of (**B**) β1 selective probe **23,** (**E**) β5 selective probe **31** and (**H**) β2 and β5 targeting probe **33** for 1 hr. at 37 °C. The residual proteasome activity was fluorescently labeled by incubation with 1 μM MV151 for 1 hr. at 37 °C. (**C, F, I**) HEK293T cells (some $5{\cdot}10^5$ cells) were exposed to the indicated concentrations of (**C**) **23,** (**F**) **31** and (**I**) **33** for 1 hr. at 37 °C. The residual proteasome activity was fluorescently labeled by incubation with 1 μM MV151 for 1 hr. at 37 °C. (**D, G, J**) One-site competition plots of (**D**) **23,** (**G**) **31** and (**J**) **33**. Solid lines: lysates, dashed lines: living cells.

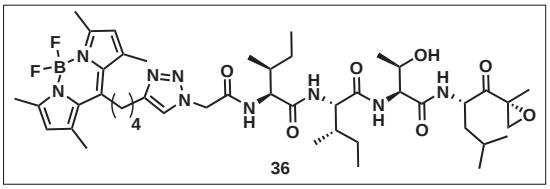
Scheme 8. Synthesis of fluorescent probe **35**.



Reagents and conditions: i) **23**, $CuSO_4$ (10 mol%), sodium ascorbate (15 mol%), Tol./H_2O/*t*BuOH (1/1/1, v/v/v), 80°C, 22 hrs, 65%.

comparable to the parent inhibitor, but the one-site competition curve starts to flatten before reaching 0% activity and at 100 μM a fluorescently labeled β2 band is still visible on gel (Figure 5H and I, lane 10). This effect is even more pronounced in the labeling profile of the β1 subunit.

Chapter 3 describes the synthesis and application of the green fluorescent acetylene functionalized BODIPY dye **34** in the synthesis of a fluorescently labeled epoxomicin analogue.[20] Having azido functionalized β1 selective probe **23** in hand, it was conjugated to BODIPY dye **34** by a copper catalyzed Huisgen [2+3] cycloaddition to give the fluorescently labeled probe **35** (Scheme 8). The labeling profile was determined by treating HEK293T lysates with increasing concentrations of the fluorescent probe (Figure 6A). As a reference, all proteolytically active proteasome subunits were labeled with the fluorescently tagged epoxomicin analogue **36** (see Chapter 3).[20] Probe **35** shows a preference for β1, but like the parent azido probe **23** starts labeling β5 at higher concentrations. To test the cell permeability, living HEK293T cells were exposed to increasing concentrations of the probe (Figure 6B). A similar labeling profile is observed.



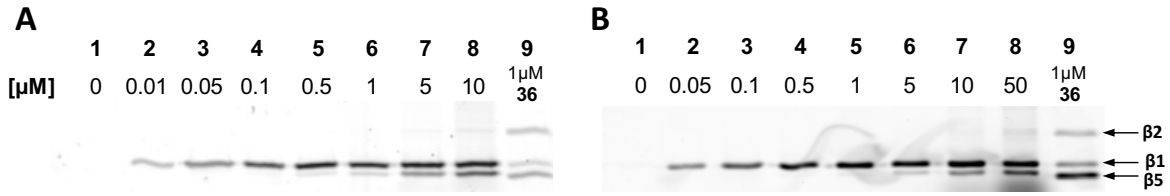
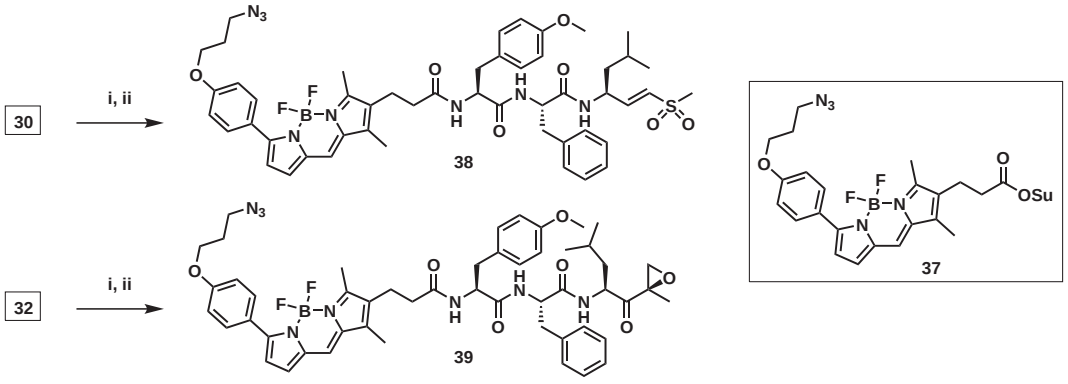


Figure 6. Labeling profile of probe **35**.

(**A**) HEK293T lysates (10 μg total protein) were incubated with the indicated concentrations of **35** for 1 hr. at 37 °C. (**B**) HEK293T cells (some $5{\cdot}10^5$ cells) were exposed to the indicated concentrations of **35** or 1 μM **36** (lane 10) for 1 hr. at 37 °C.

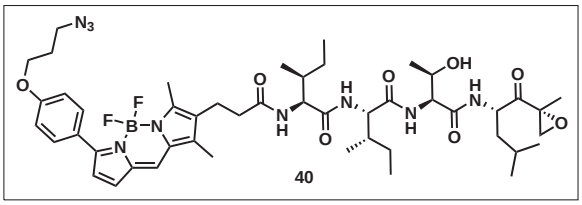
Scheme 9. Synthesis of azido-BODIPY probes **38** and **39**.



Reagents and conditions: i) TFA/DCM 1/1 (v/v), 30 min. ii) **37** (1 equiv.), DiPEA (1 equiv.), DCM, 15 hr., **38** 54%, **39** 27%.

It was reasoned that replacing the aromatic naphthyl azido acid in probes **31** and **33** by azido-BODIPY[21] (Chapter 4) would not have a dramatic effect on the specificity of the resulting fluorescent probes. The Boc-protected tripeptides **30** and **32** were deprotected and the resulting amines were reacted with azido-BODIPY-OSu **37** to give the fluorescent probes **38** and **39** (Scheme 9).

HEK293T cell lysates incubated with azido-BODIPY probe **38** show strong fluorescent labeling of the β5 subunit (Figure 7A, top panel). From about 0.1 μM on a faint band corresponding to the β2 subunit starts to appear. When using concentrations higher than 5 μM the labeling decreases and the background increases (data not shown). This is



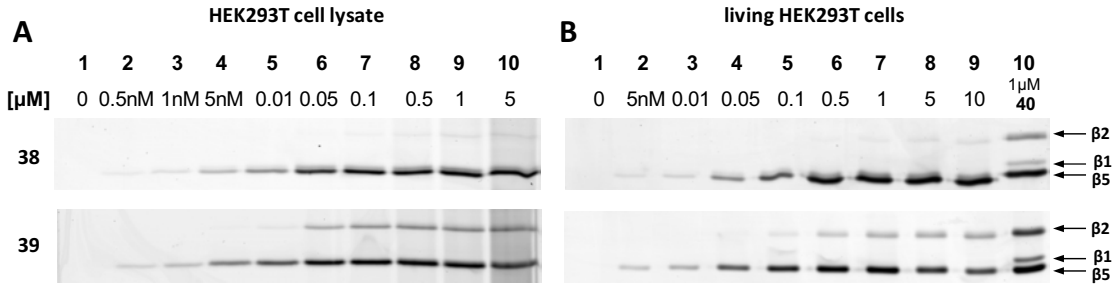


Figure 7. Labeling profile of Azido-BODIPY probes **38** and **39**.
(**A**) HEK293T lysates (10 μg total protein) were incubated with the indicated concentrations of **38** (top panel) or **39** (bottom panel) for 1 hr. at 37 °C. (**B**) HEK293T cells (some $5{\cdot}10^5$ cells) were exposed to the indicated concentrations of **38** (top gel), **39** (bottom gel) or 1 μM **40** (lane 10) for 1 hr. at 37 °C.

probably due to precipitation of the hydrophobic probe. An even cleaner labeling is observed when living HEK293T cells are exposed to the fluorescent probe (Figure 7B, toppanel). As a reference, HEK293T cells were exposed to **40** (see Chapter 4), labeling all proteolytically active proteasome subunits (Figure 7B, lane 10). Lysates treated with probe **39** show labeling of β5, followed at about a hundredfold increase in concentration by a band corresponding to β2 (Figure 7A, bottom panel). As observed for the azido modified probe **33** the β2 labeling seems to reach an optimum at a given probe concentration. In living cells β5 labeling appears to reach a maximum around 1 μM after which the labeling intensity starts to drop, whereas the labeling of the β2 subunit keeps increasing (Figure 7B, bottom panel).

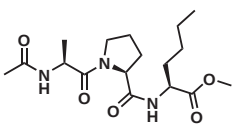
6.3 Conclusion

In conclusion, the synthesis and characterization of a library of hybrid compounds with structural characteristics of three known subunit specific proteasome inhibitors provided interesting results. Where the reported inhibitor Ac-Ala-Pro-Nle-LeuVS-PhOH (**1, Ia**)[9] indeed proved to be β1 selective, at least in the assays used here, the reported β2 specific HMBA-Val-Ser-LeuVE (**2, IIb**)[10] showed no inhibition at all. Naphth-Tyr(OMe)-Phe-LeuEK (**3, IIIc**) was shown to inhibit β2 and β5, leaving β1 as the sole active proteasome proteolytic subunit. A more potent β1 selective inhibitor (**Ic**) armed with a LeuEK warhead was identified. Inhibitor **IIId** proved to be selective for the β5 subunit. These hits were converted in two-step labeling and fluorescent activity-based probes. With the proteasome inhibitor bortezomib in mind, the here presented toolbox is useful in addressing the question which proteasome subunit or what combination of subunits should be targeted to give the optimal anti-cancer therapeutic. Of interest in the field of immune-therapy is the influence of the inhibition of a defined set of the proteasome subunits on the epitope repertoire. The inhibition profile of the synthesized library and the labeling profile of the in this Chapter designed probes remains to be determined for the immunoproteasome[3] and the thymus proteasome.[4]

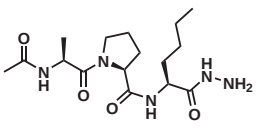
Experimental section

General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.)(purum), ethyl acetate (EtOAc) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, 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 followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on Screening Divices

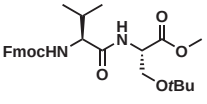
Silica gel (0.040 – 0.063 nm). LC/MS analysis was performed on a LCQ Adventage Max (Thermo Finnigan) equipped with a Gemini C18 column (Phenomenex). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), Bruker DPX-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory, a Bruker AV-500 (500/125 MHz) or a Bruker DMX-600 (600/150 MHz) with cryoprobe. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, λ = 589 nm). Boc-LeuVS-PhOH,[12] Boc-LeuVE,[10] Boc-LeuEK[13] and Boc-LeuVS[14] were synthesised as described in literature.



Ac-Ala-Pro-Nle-OMe (6). 4-methylbenzhydrylamine (MBHA) functionalized polystyrene resin (7.14 g, 0.7 mmol/g, 5 mmol) was washed with NMP (3x) followed by addition of a preactivated mixture of 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker (3.6 g, 15.0 mmol, 3 equiv.), BOP (6.6 g, 15 mmol, 3 equiv.) and DiPEA (5 mL, 30 mmol, 6 equiv.) in NMP. After 2 hr. of shaking, the resin was washed with NMP (3x) and DCM (3x), dried and used as such. Part of the resin (2 mmol) was transferred to a flask, coevaporated with DCE (2x), and condensed with Fmoc-Nle-OH (2.12 g, 6 mmol, 3 equiv.) under the influence of DIC (1.0 mL, 6.6 mmol, 3.3 equiv.) and DMAP (6.6 mg, 0.3 mmol, 5 mol%) in DCM for 2 hr. The resin was filtered and washed with DCM (2x), followed by a second condensation cycle. The loading of the resin was determined to be 0.46 mmol/g (4.28 g, 1.97 mmol, 98%) by spectrophotometric analysis. The obtained resin was submitted to two cycles of Fmoc solid-phase synthesis with Fmoc-Pro-OH and Fmoc-Ala-OH, respectively, as follows: a) deprotection with piperidine/NMP (1/4, v/v, 20 min.), b) wash with NMP (3×), c) coupling of Fmoc amino acid (5 mmol, 2.5 equiv.) in the presence of BOP (2.2 g, 5 mmol, 2.5 equiv.) and DiPEA (0.99 ml, 6 mmol, 3 equiv.) in NMP and shaken for at least 2 hr., d) wash with NMP (3×) and DCM (3×), yielding resin bound Fmoc-Ala-Pro-Nle. Couplings were monitored for completion by the Kaiser test. After Fmoc deprotection of 1.2 mmol, the resin bound tripeptide was capped with acetic anhydride (0.57 ml, 6 mmol, 5 quiv.) and DiPEA (1.98 ml, 12 mmol, 10 equiv.) for 15 min. Mild acidic cleavage with 1% TFA in DCM (3x 10 min.) resulted in Ac-Ala-Pro-Nle-OH **2** which was used without purification. The crude peptide **5** was dissolved in MeOH/Tol. (1/1) and treated with TMS-diazomethane (1.2 ml 2M in hexanes, 2.4 mmol, 2 equiv.) for 15 min. before being coevaporated with Tol. (3x). Purification by flash column chromatography (DCM → 3% MeOH in DCM) yielded the title compound as a white solid (0.36 g, 1.0 mmol, 85%). ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 7.42 (d, *J* = 7.8 Hz, 1H), 7.14 (d, *J* = 7.5 Hz, 1H), 4.78 (m, 1H), 4.64 (m, 1H), 4.50 (m, 1H), 3.78 (m, 1H), 3.74 (s, 3H), 3.59 (m, 1H), 2.29 (m, 1H), 2.12 (m, 1H), 2.06-1.93 (m, 6H), 1.80 (m, 1H), 1.66 (m, 1H), 1.36 (d, *J* = 6.9 Hz, 3H), 1.28 (m, 4H), 0.87 (t, *J* = 6.8 Hz, 3H).

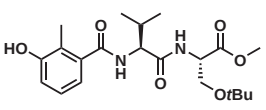


Ac-Ala-Pro-Nle-hydrazide (7). Ac-Ala-Pro-Nle-OMe (**6**, 0.36 g, 1.0 mmol) was dissolved in MeOH. Hydrazine monohydrate (2.9 ml, 60 mmol, 60 equiv.) was added and the reaction mixture was refluxed for 1.5 hr. Tol. was added and the resulting white solid was filtered to give the title compound (0.33 g, 0.92 mmol, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 4.60 (q, *J* = 7.0 Hz, 1H), 4.46 (dd, J_1 = 8.2, J_2 = 4.6 Hz, 1H), 4.25 (dd, J_1 = 8.4, J_2 = 6.0 Hz, 1H), 3.85-3.77 (m, 1H), 3.69-3.60 (m, 1H), 2.26-2.13 (m, 1H), 2.11-2.01 (m, 1H), 2.00-1.91 (m, 5H), 1.82-1.71 (m, 1H), 1.71-1.60 (m, 1H), 1.42-1.24 (m, 5H), 0.91 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 174.12, 173.77, 173.45, 172.80, 61.49, 53.48, 48.50, 48.46, 32.88, 30.31, 28.97, 26.00, 23.36, 22.26, 16.88, 14.28.

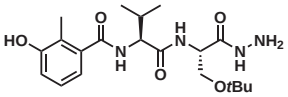


Fmoc-Val-Ser(*t*Bu)-OMe (9). HCl·H-Ser(*t*Bu)-OMe (**8**, 1.06 g, 5 mmol) and Fmoc-Val-OH (1.7 g, 5 mmol, 1 equiv.) were dissolved in DCM, before BOP (2.21 g, 5 mmol, 1 equiv.)

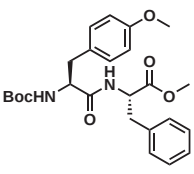
and DiPEA (2.73 ml, 16.5 mmol, 3.3 equiv.) were added. After 2 hr. the reaction mixture was washed with 0.5 M HCl (aq.) and sat. aq. $NaHCO_3$, separated and dried over $MgSO_4$. Purification by flash column chromatography (2% MeOH in DCM) yielded the title compound as a white solid (2.5 g, 5.0 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.75 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.3 Hz, 2H), 7.39 (t, J = 7.5 Hz, 2H), 7.30 (ddd, J_1 = 9.0, J_2 = 2.9, J_3 = 1.4 Hz, 2H), 6.57 (d, J = 8.2 Hz, 1H), 5.56 (d, J = 8.8 Hz, 1H), 4.73 (td, J_1 = 8.3, J_2 = 2.9 Hz, 1H), 4.42 (dd, J_1 = 10.3, J_2 = 7.6 Hz, 1H), 4.33 (dd, J_1 = 10.5, J_2 = 7.1 Hz, 1H), 4.22 (t, J = 7.1 Hz, 1H), 4.12 (dd, J_1 = 8.6, J_2 = 6.1 Hz, 1H), 3.83 (dd, J_1 = 9.0, J_2 = 2.5 Hz, 1H), 3.55 (dd, J_1 = 9.1, J_2 = 3.1 Hz, 1H), 2.15 (qd, J_1 = 13.5, J_2 = 7.0, J_3 = 6.7 Hz, 1H), 1.12 (s, 9H), 1.01 (dd, J_1 = 14.1, J_2 = 6.7 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 170.84, 170.55, 156.20, 143.85, 143.76, 141.22, 127.62, 127.00, 125.09, 125.03, 119.90, 119.88, 73.50, 67.00, 61.66, 60.00, 52.74, 52.29, 47.12, 31.66, 27.20, 18.93, 17.69.



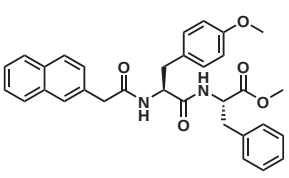
(Val-Ser(*t*Bu)-OMe)-3-hydroxy-2-methylbenzamide (11). DBU (0.15 ml, 1 equiv.) was added to a solution of Fmoc-Val-Ser(*t*Bu)-OMe (**9**, 0.5 g, 1 mmol) in DMF and stirred for 5 min., before HOBt (0.61 g, 4.5 mmol, 4.5 equiv.) was added. After 1 min. 3-hydroxy-2-methyl benzoic acid (**10**, 0.15 g, 1 mmol, 1 equiv.), BOP (0.49 g, 1.1 mmol, 1.1 equiv.) and DiPEA (0.66 ml, 4 mmol, 4 equiv.) were added and the reaction mixture was stirred for 2 hr. The reaction mixture was washed with 0.5 M HCl (aq.) and sat. aq. $NaHCO_3$, separated and dried over $MgSO_4$. Purification by flash column chromatography (PetEt → 50% EtOAc in PetEt) yielded the title compound as a white solid (0.27 g, 0.67 mmol, 67%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.06 (t, J = 7.8 Hz, 1H), 6.84 (d, J = 7.8 Hz, 2H), 4.64 (t, J = 4.0 Hz, 1H), 4.45 (d, J = 7.6 Hz, 1H), 3.83 (dd, J_1 = 9.3, J_2 = 4.2 Hz, 1H), 3.74 (s, 3H), 3.65 (dd, J_1 = 9.3, J_2 = 3.8 Hz, 1H), 2.30-2.06 (m, 4H), 1.17 (s, 9H), 1.04 (t, J = 7.0 Hz, 6H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 171.40, 171.13, 170.23, 155.00, 137.20, 125.79, 121.65, 117.30, 115.64, 73.13, 61.11, 58.36, 52.74, 51.49, 30.44, 26.30, 18.42, 17.28, 11.61.



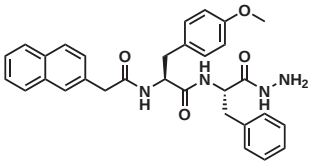
(Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (12). (Val-Ser(*t*Bu)-OMe)-3-hydroxy-2-methylbenzamide (**11**, 0.27 g, 0.67 mmol) was dissolved in MeOH. Hydrazine monohydrate (1.95 ml, 40.2 mmol, 60 equiv.) was added and the reaction mixture was refluxed for 15 hr., before being co-evaporated with Tol. (3×). Column chromatography (DCM → 7.5% MeOH in DCM) gave the pure title compound (0.24 g, 0.59 mmol, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.06 (t, J = 7.8 Hz, 1H), 6.87 (d, J = 7.8 Hz, 2H), 4.49 (dd, J_1 = 5.8, J_2 = 4.7 Hz, 1H), 4.41 (d, J = 6.9 Hz, 1H), 3.70 (dd, J_1 = 9.0, J_2 = 4.5 Hz, 1H), 3.56 (dd, J_1 = 9.0, J_2 = 6.2 Hz, 1H), 2.25 (s, 3H), 2.23-2.14 (m, 1H), 1.19 (s, 9H), 1.03 (dd, J_1 = 13.1, J_2 = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 171.53, 171.35, 169.92, 155.11, 137.01, 125.92, 121.82, 117.48, 115.87, 73.49, 60.91, 58.88, 52.06, 30.18, 26.51, 18.61, 17.44, 11.80.



Boc-Tyr(Me)-Phe-OMe (14). HCl·H-Phe-OMe (2.16 g, 10 mmol) and Boc-Tyr(Me)-OH (**13**, 2.95 g, 10 mmol, 1 equiv.) were dissolved in DCM. BOP (4.42 g, 10 mmol, 1 equiv.) and DiPEA (3.64 ml, 16.5 mmol, 2.2 equiv.) were added and the reaction mixture was stirred for 15 hr., before being washed with 0.5 M HCl (aq.) and sat. aq. $NaHCO_3$, separated and dried over $MgSO_4$. Purification by flash column chromatography (20% EtOAc in PetEt → 40% EtOAc in PetEt) yielded the title compound as a white solid (4.6 g, 10 mmol, quant.). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.32-7.17 (m, 4H), 7.15-7.05 (m, 3H), 7.04-6.94 (m, 2H), 6.81 (d, J = 8.7 Hz, 2H), 6.38 (d, J = 7.8 Hz, 1H), 4.78 (q, J = 5.9, 5.9, 5.9 Hz, 1H), 4.38-4.19 (m, 1H), 3.78 (s, 3H), 3.67 (s, 3H), 3.05 (dd, J = 5.9, 1.6 Hz, 2H), 2.96 (d, J = 6.7 Hz, 2H), 1.40 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 171.40, 158.15, 155.30, 135.62, 129.98, 128.86, 128.31, 128.10, 126.64, 113.54, 78.93, 55.41, 54.77, 53.13, 51.80, 37.46, 37.15, 27.78.

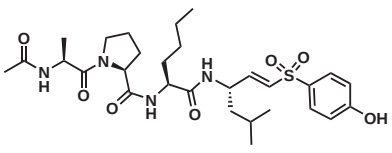


(Tyr(Me)-Phe-OMe)-2-(naphthalen-2-yl)-acetamide (16). Boc-Tyr(Me)-Phe-OMe (**14**, 4.6 g, 10 mmol) was dissolved in TFA/DCM 1/1 (v/v). The reaction mixture was stirred for 15 min. before being co-evaporated with Tol. (3×). The crude TFA salt was dissolved in DCM and 2-(naphthalen-2-yl)-acetic acid (**15**, 1.86 g, 10 mmol, 1 equiv.), BOP (4.42 g, 10 mmol, 1 equiv.) and DiPEA (5.46 ml, 33 mmol, 3.3 equiv.) were added. After being stirred for 15 hr. the reaction mixture was washed with 0.5 M HCl (aq.) and sat. aq. $NaHCO_3$, separated and dried over $MgSO_4$. Purification by flash column chromatography (PetEt → EtOAc, followed by a second column: DCM → 30% EtOAc in DCM), washing with H_2O (3×) and drying over $MgSO_4$ gave the pure title compound as a white solid (3.55 g, 6.8 mmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 8.53 (d, *J* = 7.5 Hz, 1H), 8.27 (d, *J* = 8.7 Hz, 1H), 7.91-7.70 (m, 3H), 7.59 (s, 1H), 7.54-7.40 (m, 2H), 7.32-7.16 (m, 6H), 7.11 (d, *J* = 8.7 Hz, 2H), 6.71 (d, *J* = 8.7 Hz, 2H), 4.62-4.41 (m, 2H), 3.65 (s, 3H), 3.62 (d, *J* = 13.7 Hz, 1H), 3.58 (s, 3H), 3.49 (d, *J* = 14.0 Hz, 1H), 3.13-2.83 (m, 3H), 2.67 (dd, J_1 = 13.7, J_2 = 9.9 Hz, 1H).



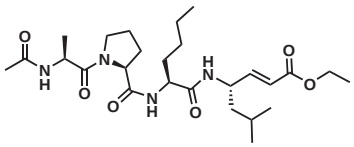
(Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide (17). To a solution of (Tyr(Me)-Phe-OMe)-2-(naphthalen-2-yl)-acetamide (**16**, 0.52 g, 1 mmol) in MeOH was added hydrazine monohydrate (2.91 ml, 60 mmol, 60 equiv.). The reaction mixture was refluxed for 2.5 hr. The title compound precipitated as a white solid and was filtered off and washed with MeOH (0.5 g, 0.95 mmol, 95%). ^{1}H NMR (600 MHz, $CDCl_3$): δ ppm 9.22 (s, 1H), 8.25 (t, *J* = 7.9, 7.9 Hz, 2H), 7.86 (d, *J* = 7.8 Hz, 1H), 7.79 (d, *J* = 7.9 Hz, 1H), 7.77 (d, *J* = 8.5 Hz, 1H), 7.62 (s, 1H), 7.50-7.44 (m, 2H), 7.29-7.21 (m, 5H), 7.20-7.15 (m, 1H), 7.10 (d, *J* = 8.6 Hz, 2H), 6.71 (d, *J* = 8.6 Hz, 2H), 4.59-4.51 (m, 2H), 4.29 (s, 2H), 3.65 (s, 3H), 3.64 (d, *J* = 12.5 Hz, 1H), 3.53 (d, *J* = 14.0 Hz, 1H), 2.99 (dd, J_1 = 13.7, J_2 = 5.6 Hz, 1H), 2.94 (dd, J_1 = 13.7, J_2 = 3.9 Hz, 1H), 2.86 (dd, J_1 = 13.7, J_2 = 8.7 Hz, 1H), 2.70 (dd, J_1 = 13.6, J_2 = 10.1 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 170.97, 170.00, 169.77, 157.66, 137.51, 133.91, 132.91, 131.71, 130.22, 129.50, 129.16, 128.07, 127.65, 127.41, 127.35, 127.21, 126.28, 125.93, 125.43, 113.30, 54.76, 53.98, 52.67, 42.34, 38.05, 36.70.

Synthesis of Ia-d, IIa-d and IIIa-d; general procedure azide coupling. The peptide hydrazide was dissolved in DMF/EtOAc (1/1, v/v) and cooled to -25 °C, before HCl (2.8 equiv., 4M in 1,4-dioxane) and *t*BuONO (1.1 equiv.) were added. The reaction mixture was stirred for 4 hr. at -25 °C to form the corresponding acyl azide. Boc-protected Leucine derived warhead Boc-LeuVS-PhOH,[12] Boc-LeuVE,[10] Boc-LeuEK[13] or Boc-LeuVS[14] was dissolved in DCM/TFA (1/1, v/v) and stirred for 30 min., before being coevaporated with Tol. (3×). The resulting warhead TFA-salt was dissolved in DMF and DiPEA (3.8 equiv.) was added, before the mixture was combined with the acyl azide mixture at -25 °C (**NOTE**: make sure the pH is 8-9. If not, add more DiPEA). The reaction mixture was allowed to warm up to room temperature overnight. EtOAc and water were added and the organic layer was separated, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography.

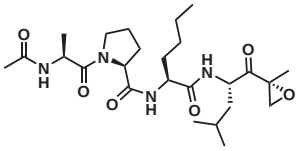


Ac-Ala-Pro-Nle-Leu-4-hydroxyphenyl-vinylsulfone (Ia). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS-PhOH (61 mg, 0.17 mmol, 1.1 equiv.) and Ac-Ala-Pro-Nle-hydrazide (**7**, 53.3 mg, 0.15 mmol). Purification by flash column chromatography (DCM → 6% MeOH in DCM) gave **Ia** as colorless oil (68.4 mg, 0.12 mmol, 77%). ^{1}H NMR (500 MHz, DMSO, T = 353K): δ ppm 7.68 (d, *J* = 8.8 Hz, 2H), 6.92 (d, *J* = 8.8 Hz, 2H), 6.79 (dd, J_1 = 15.0, J_2 = 5.1 Hz, 1H), 6.54 (dd, J_1 = 15.1, J_2 = 1.4 Hz, 1H), 4.69-4.59 (m, 1H), 4.56 (q, *J* = 7.0 Hz, 1H), 4.38 (dd, J_1 = 8.2, J_2 = 5.1 Hz, 1H), 4.17 (dd, J_1 = 8.2, J_2 = 6.0 Hz, 1H), 3.84-3.73 (m, 1H), 3.68-3.56 (m, 1H), 2.26-2.09 (m, 1H), 2.07-1.84 (m,

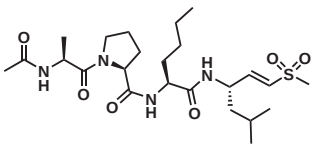
6H), 1.81-1.59 (m, 3H), 1.58-1.49 (m, 1H), 1.47-1.38 (m, 1H), 1.36-1.24 (m, 7H), 0.97-0.83 (m, 9H). HRMS: calcd. for $[C_{29}H_{44}N_4O_7SH]^+$ 593.30035, found 593.30046.



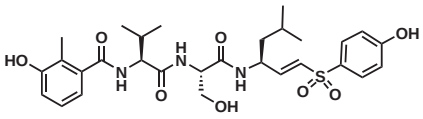
Ac-Ala-Pro-Nle-Leu-vinyl ethyl ester (Ib). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVE (49.7 mg, 0.17 mmol, 1.1 equiv.) and Ac-Ala-Pro-Nle-hydrazide (**7**, 53.3 mg, 0.15 mmol). Purification by flash column chromatography (DCM → 4% MeOH in DCM) gave **Ib** as white solid (53.1 mg, 0.1 mmol, 70%). ^{1}H NMR (500 MHz, DMSO, T = 353K): δ ppm 7.54 (d, J = 6.1 Hz, 1H), 6.79 (dd, J_1 = 15.7, J_2 = 5.5 Hz, 1H), 5.84 (d, J = 15.6 Hz, 1H), 4.60-4.53 (m, 1H), 4.53-4.45 (m, 1H), 4.41-4.32 (m, 1H), 4.21-4.15 (m, 1H), 4.13 (q, J = 7.1 Hz, 2H), 3.70-3.60 (m, 1H), 3.59-3.52 (m, 1H), 2.15-1.99 (m, 1H), 1.95-1.85 (m, 3H), 1.84 (s, 3H), 1.76-1.67 (m, 1H), 1.66-1.53 (m, 2H), 1.51-1.42 (m, 1H), 1.42-1.35 (m, 1H), 1.32-1.25 (m, 4H), 1.24-1.19 (m, 6H), 0.93-0.82 (m, 9H). HRMS: calcd. for $[C_{26}H_{44}N_4O_6H]^+$ 509.33336, found 509.33315.



Ac-Ala-Pro-NLe-Leu-epoxyketone (Ic). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuEK (47.4 mg, 0.17 mmol, 1.1 equiv.) and Ac-Ala-Pro-NLe-hydrazide (**7**, 53.3 mg, 0.15 mmol). Purification by flash column chromatography (DCM → 4% MeOH in DCM) gave **Ib** as colorless oil (47.1 mg, 95 µmol, 63%). ^{1}H NMR (500 MHz, DMSO, T = 353K): δ ppm 7.80 (s, 1H), 7.73 (d, J = 7.3 Hz, 1H), 7.50 (d, J = 5.6 Hz, 1H), 4.60-4.48 (m, 1H), 4.47-4.41 (m, 1H), 4.41-4.36 (m, 1H), 4.22 (dd, J_1 = 13.6, J_2 = 7.9 Hz, 1H), 3.72-3.59 (m, 1H), 3.57-3.47 (m, 1H), 3.18 (d, J = 5.2 Hz, 1H), 2.96 (d, J = 5.2 Hz, 1H), 2.08-1.95 (m, 1H), 1.95-1.86 (m, 2H), 1.83 (s, 3H), 1.73-1.61 (m, 2H), 1.56-1.46 (m, 1H), 1.42 (s, 3H), 1.41-1.31 (m, 2H), 1.30-1.23 (m, 4H), 1.20 (d, J = 6.7 Hz, 3H), 0.91 (d, J = 6.64 Hz, 1H), 0.88-0.83 (m, 6H). HRMS: calcd. for $[C_{25}H_{42}N_4O_6H]^+$ 495.31771, found 495.31755.

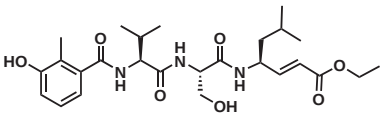


Ac-Ala-Pro-NLe-Leu-methyl vinylsulfone (Id). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS (50.7 mg, 0.17 mmol, 1.1 equiv.) and Ac-Ala-Pro-NLe-hydrazide (**7**, 53.3 mg, 0.15 mmol). Purification by flash column chromatography (DCM → 4% MeOH in DCM) gave **Ib** as white solid (26.1 mg, 51 µmol, 34%). ^{1}H NMR (500 MHz, DMSO, T = 353K): δ ppm 7.84 (s, 1H), 7.63-7.53 (m, 2H), 6.74-6.58 (m, 2H), 4.63-4.48 (m, 2H), 4.39-4.31 (m, 1H), 4.16 (dd, J_1 = 13.5, J_2 = 7.7 Hz, 1H), 3.69-3.61 (m, 1H), 3.60-3.52 (m, 1H), 2.95 (s, 3H), 2.10-2.00 (m, 1H), 1.98-1.86 (m, 3H), 1.84 (s, 3H), 1.77-1.67 (m, 1H), 1.67-1.55 (m, 2H), 1.54-1.46 (m, 1H), 1.45-1.35 (m, 1H), 1.33-1.24 (m, 4H), 1.22 (d, J = 6.8 Hz, 3H), 0.93-0.84 (m, 9H). HRMS: calcd. for $[C_{24}H_{42}N_4O_6SH]^+$ 515.28978, found 515.28961.

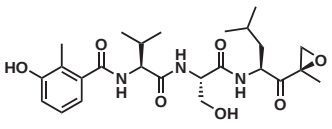


(Val-Ser-Leu-4-hydroxyphenyl-vinylsulfone)-3-hydroxy-2-methylbenzamide (IIa). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS-PhOH (61 mg, 0.17 mmol, 1.1 equiv.) and (Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (**12**, 61.3 mg, 0.15 mmol). Crystallization from EtOAc with Et_2O gave **18a** as a white solid (61.1 mg, 95 µmol, 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 8.00 (d, J = 8.5 Hz, 1H), 7.82 (d, J = 7.7 Hz, 1H), 7.68 (d, J = 8.8 Hz, 1H), 7.03 (t, J = 7.8, 7.8 Hz, 1H), 6.91 (d, J = 8.8 Hz, 2H), 6.86-6.79 (m, 3H), 6.60 (dd, J = 15.0, 1.7 Hz, 1H), 4.74-4.61 (m, 1H), 4.49-4.43 (m, 1H), 4.24 (d, J = 7.1 Hz, 1H), 3.69 (dd, J_1 = 8.8, J_2 = 3.7 Hz, 1H), 3.54 (dd, J_1 = 8.6, J_2 = 6.6 Hz, 1H), 2.19 (s, 3H), 2.18-2.10 (m, 1H), 1.73-1.60 (m, 1H), 1.58-1.47 (m, 1H), 1.43-1.32 (m, 1H), 1.13 (s, 9H), 1.01 (d, J = 6.8 Hz, 6H), 0.85 (dd, J = 12.0, 6.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 173.94, 173.44, 173.36, 172.07, 172.05,

163.78, 157.02, 146.69, 139.04, 131.85, 131.48, 131.19, 127.42, 123.30, 119.14, 117.13, 116.93, 74.81, 62.60, 61.45, 55.23, 49.45, 43.31, 31.32, 27.77, 25.72, 23.49, 21.72, 19.82, 19.06, 13.14. **18a** (61.1 mg, 95 µmol) was dissolved in TFA/DCM 1/1 (v/v) and stirred for 30 min., before being co-evaporated with Tol. (3×). Column chromatography (DCM → 5% MeOH in DCM) gave the title compound (48.8 mg, 83 µmol, 87%). ^{1}H NMR (400 MHz, MeOD): δ ppm 7.69 (d, *J* = 8.8 Hz, 2H), 7.04 (t, *J* = 7.8 Hz, 1H), 6.91 (d, *J* = 8.8 Hz, 2H), 6.86-6.82 (m, 2H), 6.80 (dd, J_1 = 15.2, J_2 = 4.0 Hz, 1H), 6.72 (dd, J_1 = 15.1, J_2 = 1.3 Hz, 1H), 4.71-4.65 (m, 1H), 4.42 (dd, J_1 = 6.2, J_2 = 5.1 Hz, 1H), 4.28 (d, *J* = 7.2 Hz, 1H), 3.81 (dd, J_1 = 10.6, J_2 = 5.0 Hz, 1H), 3.72 (dd, J_1 = 10.6, J_2 = 6.4 Hz, 1H), 2.19 (s, 3H), 2.18-2.09 (m, 1H), 1.74-1.61 (m, 1H), 1.52 (ddd, J_1 = 15.2, J_2 = 10.3, J_3 = 5.0 Hz, 1H), 1.42 (ddd, J_1 = 13.9, J_2 = 9.2, J_3 = 4.9 Hz, 1H), 1.00 (dd, J_1 = 6.8, J_2 = 2.9 Hz, 6H), 0.88 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, MeOD): δ ppm 173.81, 173.58, 172.03, 163.84, 157.03, 146.21, 139.20, 132.26, 131.56, 131.12, 127.47, 123.34, 119.13, 117.09, 116.98, 62.76, 61.24, 56.59, 49.43, 43.42, 31.59, 25.87, 23.39, 21.88, 19.86, 19.02, 12.99. HRMS: calcd. for $[C_{29}H_{39}N_3O_8SH]^+$ 590.25306, found 590.25312.

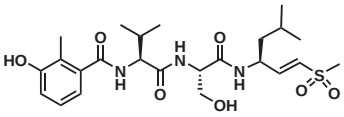


(Val-Ser-Leu-vinyl ethyl ester)-3-hydroxy-2-methylbenzamide (IIb). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVE (49.7 mg, 0.17 mmol, 1.1 equiv.) and (Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (**12**, 61.3 mg, 0.15 mmol). Column chromatography (n-hexane → 30% acetone in n-hexane) gave **18b** (69 mg, 0.12 mmol, 82%). ^{1}H NMR (400 MHz, MeOD): δ ppm 7.05 (t, *J* = 7.8 Hz, 1H), 6.90-6.82 (m, 3H), 6.01 (dd, J_1 = 15.7, J_2 = 1.7 Hz, 1H), 4.68-4.58 (m, 1H), 4.52 (dd, J_1 = 6.5, J_2 = 3.9 Hz, 1H), 4.34 (d, *J* = 7.2 Hz, 1H), 4.20-4.13 (m, 2H), 3.73 (dd, J_1 = 8.6, J_2 = 3.9 Hz, 1H), 3.60 (dd, J_1 = 8.6, J_2 = 6.7 Hz, 1H), 2.22 (s, 3H), 2.21-2.12 (m, 1H), 1.76-1.61 (m, 1H), 1.52 (ddd, J_1 = 15.2, J_2 = 10.7, J_3 = 4.7 Hz, 1H), 1.37 (ddd, J_1 = 13.9, J_2 = 9.6, J_3 = 4.6 Hz, 1H), 1.26 (t, *J* = 7.1 Hz, 3H), 1.21 (s, 9H), 1.03 (dd, J_1 = 6.7, J_2 = 3.6 Hz, 6H), 0.87 (dd, J_1 = 11.5, J_2 = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, MeOD): δ ppm 173.98, 173.34, 171.93, 168.07, 157.10, 149.94, 139.19, 127.46, 123.33, 121.36, 119.15, 117.12, 74.84, 62.80, 61.52, 61.41, 55.04, 49.87, 43.66, 31.44, 27.77, 25.79, 23.48, 21.88, 19.84, 19.08, 14.59, 13.10. **18b** (69 mg, 0.12 mmol) was dissolved in TFA/DCM 1/1 (v/v) and stirred for 30 min., before being co-evaporated with Tol. (3×). Column chromatography (DCM → 3.5% MeOH in DCM) gave the title compound (54.2 mg, 0.11 mmol, 89%). ^{1}H NMR (400 MHz, MeOD): δ ppm 7.05 (t, *J* = 7.8 Hz, 1H), 6.90-6.81 (m, 3H), 5.99 (dd, J_1 = 15.7, J_2 = 1.6 Hz, 1H), 4.66-4.59 (m, 1H), 4.50 (t, *J* = 5.7 Hz, 1H), 4.37 (d, *J* = 7.3 Hz, 1H), 4.17 (q, *J* = 7.1 Hz, 2H), 3.83 (dd, J_1 = 10.7, J_2 = 5.3 Hz, 1H), 3.78 (dd, J_1 = 10.8, J_2 = 6.1 Hz, 1H), 2.21 (s, 3H), 2.20-2.12 (m, 1H), 1.75-1.62 (m, 1H), 1.51 (ddd, J_1 = 15.1, J_2 = 10.1, J_3 = 5.2 Hz, 1H), 1.40 (ddd, J_1 = 13.9, J_2 = 9.0, J_3 = 5.2 Hz, 1H), 1.27 (t, *J* = 7.1 Hz, 3H), 1.03 (t, *J* = 6.9 Hz, 6H), 0.90 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, MeOD): δ ppm 173.75, 173.58, 171.83, 168.12, 157.04, 149.79, 139.24, 127.47, 123.34, 121.52, 119.13, 117.09, 62.92, 61.59, 61.16, 56.61, 49.86, 43.92, 31.70, 25.85, 23.39, 22.09, 19.90, 19.06, 14.58, 13.00. HRMS: calcd. for $[C_{26}H_{39}N_3O_7H]^+$ 506.28608, found 506.28592.

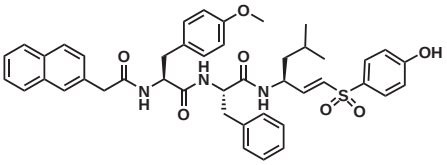


(Val-Ser-Leu-epoxyketone)-3-hydroxy-2-methylbenzamide (IIc). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuEK (47.4 mg, 0.17 mmol, 1.1 equiv.) and (Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (**12**, 61.3 mg, 0.15 mmol). Column chromatography (n-hexane → 25% acetone in n-hexane) gave **18c** (73 mg, 0.13 mmol, 89%). ^{1}H NMR (400 MHz, MeOD): δ ppm 7.04 (t, *J* = 7.8 Hz, 1H), 6.87-6.81 (m, 2H), 4.63 (dd, J_1 = 10.5, J_2 = 3.2 Hz, 1H), 4.51 (t, *J* = 5.1 Hz, 1H), 4.39 (d, *J* = 7.3 Hz, 1H), 3.69 (dd, J_1 = 8.9, J_2 = 4.7 Hz, 1H), 3.57 (dd, J_1 = 8.9, J_2 = 5.7 Hz, 1H), 3.25 (d, *J* = 5.0 Hz, 1H), 2.93 (d, *J* = 5.1 Hz, 1H), 2.21 (s, 3H), 2.20-2.14 (m, 1H), 1.76-1.63 (m, 1H), 1.52-1.42 (m, 4H), 1.42-1.32 (m, 1H), 1.18 (s, 9H), 1.04 (dd, J_1 = 10.5, J_2 = 6.8 Hz, 6H), 0.89 (dd, J_1 = 11.0, J_2 = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, MeOD): δ ppm 209.18, 173.70, 173.38, 172.03, 157.06, 139.30, 127.43, 123.28, 119.09, 117.06, 74.79, 62.84, 61.06, 59.99, 54.84, 52.94, 51.49, 40.67, 31.54, 27.73, 26.15, 23.74, 21.58, 19.93, 18.97, 17.02, 13.06. **18c** (73 mg,

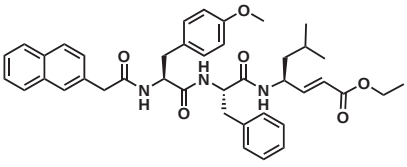
0.13 mmol) was dissolved in TFA/DCM 1/1 (v/v) and stirred for 30 min., before being co-evaporated with Tol. (3×). Column chromatography (DCM → 3.5% MeOH in DCM) gave the title compound (43.8 mg, 89 µmol, 69%). ^{1}H NMR (400 MHz, MeOD): δ ppm 7.05 (t, *J* = 7.8 Hz, 1H), 6.88-6.80 (m, 2H), 4.60 (dd, J_1 = 10.6, J_2 = 3.1 Hz, 1H), 4.51 (t, *J* = 5.6 Hz, 1H), 4.39 (d, *J* = 7.4 Hz, 1H), 3.77 (d, *J* = 5.6 Hz, 2H), 3.26 (d, *J* = 5.0 Hz, 1H), 2.93 (d, *J* = 5.1 Hz, 1H), 2.21 (s, 3H), 2.20-2.11 (m, 1H), 1.78-1.64 (m, 1H), 1.55-1.48 (m, 1H), 1.47 (s, 3H), 1.41-1.29 (m, 1H), 1.03 (dd, J_1 = 10.4, J_2 = 6.8 Hz, 6H), 0.92 (d, *J* = 6.5 Hz, 6H). ^{13}C NMR (100 MHz, MeOD): δ ppm 209.60, 173.65, 173.58, 172.07, 157.03, 139.32, 127.46, 123.33, 119.11, 117.05, 63.14, 60.89, 60.11, 56.39, 53.12, 51.93, 40.49, 31.83, 26.27, 23.77, 21.56, 19.94, 19.00, 17.07, 12.95. HRMS: calcd. for $[C_{25}H_{37}N_3O_7H]^+$ 492.27043, found 492.27033.



(Val-Ser-Leu-methyl vinylsulfone)-3-hydroxy-2-methylbenzamide (IId). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS (50.7 mg, 0.17 mmol, 1.1 equiv.) and (Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (**12**, 61.3 mg, 0.15 mmol). Column chromatography (n-hexane → 35% acetone in n-hexane) gave **18d** (63.5 mg, 0.11 mmol, 75%). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD): δ ppm 7.48-7.40 (m, 2H), 7.35-7.28 (m, 1H), 7.05 (t, *J* = 7.8, 7.8 Hz, 1H), 6.91-6.86 (m, 2H), 6.83 (dd, J_1 = 15.3, J_2 = 4.1 Hz, 1H), 6.62 (d, *J* = 15.1 Hz, 1H), 4.79-4.64 (m, 1H), 4.51-4.44 (m, 1H), 4.35-4.27 (m, 1H), 3.89-3.80 (m, 1H), 3.54 (dd, J_1 = 8.8, J_2 = 5.5 Hz, 1H), 2.96 (s, 3H), 2.25-2.21 (m, 1H), 2.19 (s, 3H), 1.70-1.60 (m, 1H), 1.59-1.49 (m, 1H), 1.44-1.31 (m, 1H), 1.19 (s, 9H), 1.05 (dd, J_1 = 15.7, J_2 = 6.8 Hz, 6H), 0.85 (dd, J_1 = 23.7, J_2 = 6.5 Hz, 6H). **18d** (18 mg, 31.7 µmol) was dissolved in TFA/DCM 1/1 (v/v) and stirred for 30 min., before being co-evaporated with Tol. (3×). Column chromatography (DCM → 4% MeOH in DCM) gave the title compound (10.4 mg, 20.3 µmol, 64%). ^{1}H NMR (400 MHz, MeOD): δ ppm 7.06 (t, *J* = 7.8 Hz, 1H), 6.89-6.73 (m, 4H), 4.77-4.66 (m, 1H), 4.47 (t, *J* = 5.5 Hz, 1H), 4.33 (d, *J* = 7.1 Hz, 1H), 3.87 (dd, J_1 = 10.6, J_2 = 4.9 Hz, 1H), 3.79 (dd, J_1 = 10.6, J_2 = 6.5 Hz, 1H), 2.96 (s, 3H), 2.21 (s, 3H), 2.20-2.12 (m, 1H), 1.78-1.66 (m, 1H), 1.61-1.51 (m, 1H), 1.49-1.40 (m, 1H), 1.04 (t, *J* = 6.0 Hz, 6H), 0.90 (d, *J* = 6.5 Hz, 6H). HRMS: calcd. for $[C_{24}H_{37}N_3O_7SH]^+$ 512.24250, found 512.24232.

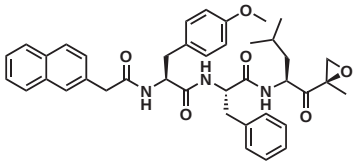


(Tyr(Me)-Phe-Leu-4-hydroxyphenyl-vinylsulfone)-2-(naphthalen-2-yl)-acetamide (IIIa). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS-PhOH (61 mg, 0.17 mmol, 1.1 equiv.) and (Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide (**17**, 78.7 mg, 0.15 mmol). Crystallization from EtOAc with PetEt gave **IIIa** as a white solid (88.1 mg, 0.12 mmol, 77%). ^{1}H NMR (400 MHz, DMSO): δ ppm 10.64 (s, 1H), 8.29 (d, *J* = 8.0 Hz, 1H), 8.25 (d, *J* = 8.4 Hz, 1H), 8.06 (d, *J* = 8.4 Hz, 1H), 7.85 (d, *J* = 7.0 Hz, 1H), 7.80-7.72 (m, 2H), 7.62 (d, *J* = 8.7 Hz, 2H), 7.58 (s, 1H), 7.50-7.42 (m, 2H), 7.25-7.20 (m, 1H), 7.18-7.13 (m, 5H), 7.07 (d, *J* = 8.6 Hz, 2H), 6.95 (d, *J* = 8.7 Hz, 2H), 6.71-6.61 (m, 3H), 6.22 (dd, J_1 = 15.1, J_2 = 1.3 Hz, 1H), 4.58-4.42 (m, 3H), 3.64 (s, 3H), 3.59 (d, *J* = 13.9 Hz, 1H), 3.48 (d, *J* = 13.9 Hz, 1H), 2.98-2.75 (m, 3H), 2.64 (dd, J_1 = 13.7, J_2 = 10.0 Hz, 1H), 1.62-1.50 (m, 1H), 1.39-1.25 (m, 2H), 0.81 (dd, J_1 = 12.6, J_2 = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, DMSO): δ ppm 170.99, 170.17, 169.71, 161.95, 157.58, 145.37, 137.14, 133.85, 132.81, 131.61, 130.10, 129.96, 129.85, 129.61, 129.39, 128.96, 127.99, 127.55, 127.32, 127.31, 127.24, 127.10, 126.35, 125.85, 125.35, 115.86, 113.21, 54.72, 54.02, 53.82, 47.03, 42.17, 41.96, 37.51, 36.59, 23.99, 22.88, 21.31. HRMS: calcd. for $[C_{44}H_{47}N_3O_7SH]^+$ 762.32075, found 762.32139.

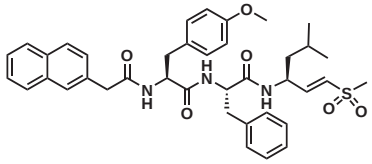


(Tyr(Me)-Phe-Leu-vinyl ethyl ester)-2-(naphthalen-2-yl)-acetamide (IIIb). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVE (49.7 mg, 0.17 mmol, 1.1 equiv.) and (Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide

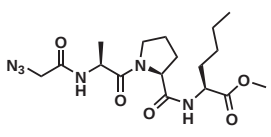
(**17**, 78.7 mg, 0.15 mmol). Upon washing the reaction mixture with EtOAc white precipitate forms. The crude product was filtered and redissolved in DCM. Crystallization with EtOAc gave the title compound as a white solid (70 mg, 0.13 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.92-7.86 (m, 1H), 7.85-7.79 (m, 2H), 7.60-7.53 (m, 2H), 7.52 (s, 1H), 7.30-7.24 (m, 3H), 7.11 (dd, J_1 = 8.4, J_2 = 1.6 Hz, 1H), 7.01 (dd, J_1 = 7.3, J_2 = 1.9 Hz, 2H), 6.70 (dd, J_1 = 15.7, J_2 = 5.7 Hz, 1H), 6.63 (d, J = 8.6 Hz, 2H), 6.45 (d, J = 8.6 Hz, 2H), 6.24 (d, J = 8.1 Hz, 1H), 6.19 (d, J = 8.3 Hz, 1H), 5.79 (dd, J_1 = 15.7, J_2 = 1.5 Hz, 1H), 5.76 (d, J = 7.1 Hz, 1H), 4.68-4.54 (m, 2H), 4.42 (q, J = 6.2 Hz, 1H), 4.19 (q, J = 7.1 Hz, 2H), 3.68 (s, 3H), 3.61 (d, J = 16.3 Hz, 1H), 3.44 (d, J = 16.2 Hz, 1H), 3.22 (dd, J_1 = 13.8, J_2 = 5.7 Hz, 1H), 2.89-2.78 (m, 3H), 1.50-1.33 (m, 2H), 1.30 (t, J = 7.1 Hz, 3H), 1.31-1.23 (m, 1H), 0.88 (t, J = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 171.71, 170.19, 169.65, 166.37, 158.60, 147.52, 136.19, 133.52, 132.58, 131.23, 129.85, 129.34, 129.14, 128.74, 128.37, 127.79, 127.64, 127.12, 127.07, 126.95, 126.70, 126.38, 120.84, 114.06, 60.35, 55.14, 54.86, 53.84, 48.56, 43.53, 42.83, 37.20, 35.86, 24.59, 22.78, 21.96, 14.27. HRMS: calcd. for $[C_{41}H_{47}N_3O_6H]^+$ 678.35376, found 678.35406.



(Tyr(Me)-Phe-Leu-epoxyketone)-2-(naphthalen-2-yl)-acetamide (IIIc). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuEK (76 mg, 0.28 mmol, 1.1 equiv.) and (Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide (**17**, 0.13 g, 0.25 mmol). Column chromatography (DCM → 2% MeOH in DCM) gave the title compound as a white solid (0.11 g, 0.17 mmol, 66%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.86-7.75 (m, 1H), 7.73-7.67 (m, 2H), 7.52 (s, 1H), 7.49-7.44 (m, 2H), 7.20-7.09 (m, 3H), 7.01-6.91 (m, 3H), 6.68 (d, J = 8.6 Hz, 2H), 6.57-6.47 (m, 1H), 6.40 (d, J = 8.3 Hz, 2H), 4.79-4.66 (m, 2H), 4.58 (dt, J_1 = 19.8, J_2 = 3.0 Hz, 1H), 3.65-3.45 (m, 5H), 3.24 (d, J = 4.9 Hz, 1H), 3.01-2.85 (m, 1H), 2.84-2.77 (m, 4H), 1.59-1.51 (m, 1H), 1.49 (s, 3H), 1.47-1.37 (m, 1H), 1.33-1.19 (m, 1H), 0.88 (dd, J_1 = 13.1, J_2 = 6.4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 207.99, 171.32, 170.57, 170.47, 158.50, 136.27, 133.56, 132.57, 131.70, 130.02, 129.36, 128.92, 128.87, 128.61, 128.56, 128.29, 128.25, 127.77, 127.72, 127.56, 127.12, 127.02, 126.98, 126.52, 126.19, 113.97, 79.36, 59.00, 55.11, 54.43, 54.01, 52.33, 50.11, 43.60, 39.94, 37.78, 36.31, 25.15, 23.30, 21.40, 16.71. HRMS: calcd. for $[C_{40}H_{45}N_3O_6H]^+$ 664.33811, found 664.33838.

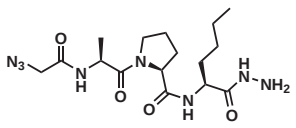


(Tyr(Me)-Phe-Leu-methyl vinylsulfone)-2-(naphthalen-2-yl)-acetamide (IIId). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS (32 mg, 0.11 mmol, 1.1 equiv.) and (Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide (**17**, 52 mg, 0.1 mmol). Column chromatography (DCM → 1.5% MeOH in DCM) gave the title compound as a white solid (36.2 mg, 53 µmol, 53%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 8.30 (d, J = 8.0 Hz, 1H), 8.24 (d, J = 8.3 Hz, 1H), 8.12 (d, J = 8.3 Hz, 1H), 7.85 (d, J = 7.3 Hz, 1H), 7.81-7.73 (m, 2H), 7.60 (s, 1H), 7.52-7.42 (m, 2H), 7.31-7.15 (m, 6H), 7.07 (d, J = 8.1 Hz, 2H), 6.68 (d, J = 8.3 Hz, 2H), 6.60 (dd, J_1 = 15.2, J_2 = 4.9 Hz, 1H), 6.32 (d, J = 15.3 Hz, 1H), 4.61-4.43 (m, 3H), 3.65 (s, 3H), 3.61 (d, J = 13.9 Hz, 1H), 3.49 (d, J = 14.0 Hz, 1H), 3.01 (dd, J_1 = 13.5, J_2 = 6.6 Hz, 1H), 2.95 (s, 3H), 2.91-2.82 (m, 2H), 2.66 (dd, J_1 = 14.6, J_2 = 11.1 Hz, 1H), 1.68-1.55 (m, 1H), 1.47-1.29 (m, 2H), 0.85 (dd, J_1 = 15.5, J_2 = 6.5 Hz, 6H). HRMS: calcd. for $[C_{39}H_{45}N_3O_6SH]^+$ 684.31018, found 684.31060.

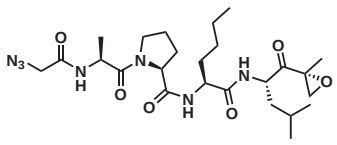


Az-Ala-Pro-Nle-OMe (21). Resin bound Fmoc-Ala-Pro-Nle (synthesis described above, 0.25 mmol) was deprotected with piperidine / NMP (1/4, v/v, 20 min.), washed with NMP (3×) and DCM (3×) and capped with azido acetic acid (63 mg, 0.63 mmol, 2.5 equiv) under the influence of BOP (0.28 g, 0.63 mmol, 2.5 equiv.) and DiPEA (0.12 ml, 0.75 mmol, 3 equiv.) for 15 hr. Mild acidic cleavage with 1% TFA in DCM (3x 10 min.) and co-evaporation with Tol. (3×) resulted in the crude Az-Ala-Pro-NLe-OH **20** which was used without

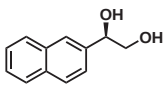
purification. The crude peptide was dissolved in MeOH/Tol. (1/1) and treated with TMS-diazomethane (0.25 ml 2M in hexanes, 0.5 mmol, 2 equiv.) for 15 min. before being coevaporated with Tol. (3x). Purification by flash column chromatography (DCM → 2.5% MeOH in DCM) yielded the title compound as a white solid (89.3 mg, 0.23 mmol, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.25 (d, *J* = 7.4 Hz, 1H), 7.11 (d, *J* = 7.7 Hz, 1H), 4.77 (p, *J* = 7.0 Hz, 1H), 4.62 (dd, J_1 = 8.1, J_2 = 2.6 Hz, 1H), 4.50 (dt, J_1 = 7.7, J_2 = 5.5 Hz, 1H), 3.98 (d, *J* = 3.6 Hz, 2H), 3.74 (s, 3H), 3.70-3.55 (m, 2H), 2.39-2.29 (m, 1H), 2.23-2.08 (m, 1H), 2.08-1.99 (m, 1H), 1.98-1.89 (m, 1H), 1.87-1.76 (m, 1H), 1.71-1.59 (m, 1H), 1.40 (d, *J* = 6.9 Hz, 3H), 1.35-1.21 (m, 2H), 0.88 (t, *J* = 7.0 Hz, 3H).



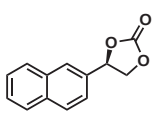
Az-Ala-Pro-Nle-hydrazide (22). Az-Ala-Pro-NLe-OMe (**21**, 89.3 mg, 0.23 mmol) was dissolved in MeOH. Hydrazine monohydrate (0.67 ml, 13.8 mmol, 60 equiv.) was added and the reaction mixture was refluxed for 2 hr. Tol. was added and the resulting white solid was filtered to give the title compound (90 mg, 0.23 mmol, quant.). ^{1}H NMR (400 MHz, MeOD): δ ppm 4.65 (q, *J* = 7.0 Hz, 1H), 4.46 (dd, J_1 = 8.2, J_2 = 4.6 Hz, 1H), 4.23 (dd, J_1 = 8.4, J_2 = 6.0 Hz, 1H), 3.89 (s, 2H), 3.86-3.77 (m, 1H), 3.70-3.60 (m, 1H), 2.30-2.12 (m, 1H), 2.12-1.91 (m, 2H), 1.83-1.72 (m, 1H), 1.71-1.61 (m, 1H), 1.44-1.25 (m, 7H), 0.92 (t, *J* = 6.9 Hz, 3H).



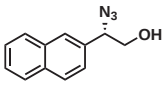
Az-Ala-Pro-NLe-Leu-epoxyketone (23). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuEK (38.8 mg, 0.14 mmol, 1.1 equiv.) and Az-Ala-Pro-Nle-hydrazide (**22**, 53.2 mg, 0.13 mmol). Purification by flash column chromatography (DCM → 2% MeOH in DCM) gave **23** (15.9 mg, 30 µmol, 23%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.16 (d, *J* = 7.4 Hz, 1H), 7.08 (d, *J* = 7.7 Hz, 1H), 6.40 (d, *J* = 8.0 Hz, 1H), 4.77 (p, *J* = 6.9 Hz, 1H), 4.62-4.54 (m, 2H), 4.30 (dt, J_1 = 7.8, J_2 = 5.7 Hz, 1H), 3.98 (d, *J* = 5.0 Hz, 2H), 3.71-3.62 (m, 1H), 3.61-3.55 (m, 1H), 3.31 (d, *J* = 5.0 Hz, 1H), 2.89 (d, *J* = 5.0 Hz, 1H), 2.35-2.27 (m, 1H), 2.20-2.09 (m, 1H), 2.07-1.99 (m, 1H), 1.98-1.90 (m, 1H), 1.85-1.75 (m, 1H), 1.69-1.53 (m, 2H), 1.51 (s, 3H), 1.39 (d, *J* = 6.8 Hz, 3H), 1.36-1.20 (m, 6H), 0.94 (dd, J_1 = 6.5, J_2 = 2.4 Hz, 6H), 0.88 (t, *J* = 7.1 Hz, 3H). HRMS: calcd. for $[C_{25}H_{41}N_7O_6H]^+$ 536.31911, found 536.31980.



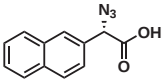
***R*-1-(naphthalen-2-yl)ethane-1,2-diol (25).** To a solution of 2-vinylnaphthalene (**24**, 1.54 g, 10 mmol) in *t*BuOH/H_2O (100 ml, 1/1, v/v) was added AD-mix-β (14.9 g). The reaction mixture was stirred for 5 hr., before sodium sulfite (6 g) was added. After the evaporation of *t*BuOH the aqueous mixture was extracted with EtOAc, separated, dried over $MgSO_4$ and concentrated *in vacuo*. The crude was dissolved in DCM/MeOH and the title compound was crystallized with PetEt (1.6 g, 8.4 mmol, 84%). $[\alpha]_D$= -66 (c 0.1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD): δ ppm 7.83-7.75 (m, 4H), 7.49-7.37 (m, 3H), 4.87 (dd, J_1 = 7.9, J_2 = 4.1 Hz, 1H), 3.71 (ddd, J_1 = 19.3, J_2 = 11.4, J_3 = 6.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD): δ ppm 138.31, 132.82, 132.57, 127.40, 127.30, 127.02, 125.46, 125.20, 124.47, 123.70, 74.23, 67.15.



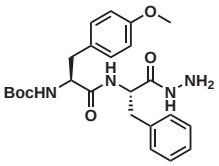
***R*-4-(naphthalen-2-yl)-1,3-dioxolan-2-one (26).** To a solution of *R*-1-(naphthalen-2-yl)ethane-1,2-diol (**25**, 1.47 g, 7.8 mmol) in MeCN was added CDI (1.9 g, 11.7 mmol, 1.5 equiv.). The reaction mixture was stirred 1 hr. at 45 °C, before it was allowed to cool to RT. DCM was added and the mixture was poured into sat.aq. NH_4Cl, extracted with DCM (3x) and dried over $MgSO_4$. Purification by flash column chromatography (Tol. → 5% EtOAc in Tol.) gave **26** as a white solid (1.56 g, 7.26 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.93 (d, *J* = 8.5 Hz, 1H), 7.90-7.82 (m, 3H), 7.59-7.52 (m, 2H), 7.42 (d, *J* = 8.5 Hz, 1H), 5.84 (t, *J* = 8.0 Hz, 1H), 4.86 (t, *J* = 8.4 Hz, 1H), 4.43 (t, *J* = 8.2 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 133.64, 132.92, 129.53, 128.10, 127.83, 127.12, 127.00, 125.76, 122.36, 78.13, 71.04.



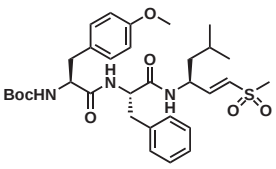
***S*-2-azido-2-(naphthalen-2-yl)ethanol (27).** *R*-4-(naphthalen-2-yl)-1,3-dioxolan-2-one (**26,** 1.54 g, 7.2 mmol) was dissolved in DMF, before H_2O (0.13 ml, 7.2 mmol, 1 equiv.) and NaN_3 (0.53 g, 7.92 mmol, 1.1 equiv.) were added. After being stirred at 80 °C for 40 hr., the reaction mixture was concentrated *in vacuo*. After resuspension of the crude in EtOAc the salts were filtered and the filtrate was evaporated and purified by flash column chromatography (Tol. → 6% EtOAc in Tol.) to give **27** as an off-white solid (1.16 g, 5.47 mmol, 76%). $[\alpha]_D$= +239 (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.84-7.77 (m, 3H), 7.74 (s, 1H), 7.50-7.43 (m, 2H), 7.35 (dd, J_1 = 8.5, J_2 = 1.6 Hz, 1H), 4.75 (t, *J* = 6.4 Hz, 1H), 3.78-3.74 (m, 2H), 2.64 (t, *J* = 5.2 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 133.54, 133.16, 133.03, 128.73, 127.88, 127.61, 126.48, 126.44, 126.38, 124.28, 67.79, 66.19.



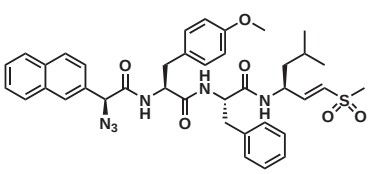
***S*-2-azido-2-(naphthalen-2-yl)acetic acid (28).** To a solution of *S*-2-azido-2-(naphthalen-2-yl)ethanol (**27,** 0.64 g, 3 mmol) in DCM/H_2O (2/1, v/v) were added TEMPO (94 mg, 0.6 mmol, 0.2 equiv.) and BAIB (2.42 g, 7.5 mmol, 2.5 equiv.). After 1 hr. of vigorous stirring the reaction was quenched with sat.aq. $Na_2S_2O_3$, extracted with DCM and dried over $MgSO_4$. Column chromatography (Tol. → 5% EtOAc in Tol., 1% AcOH in Tol.) gave the title compound as an off-white solid (0.55 g, 2.4 mmol, 80%). $[\alpha]_D$= +190 (c 0.1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 9.52 (s, 1H), 7.96-7.76 (m, 4H), 7.56-7.45 (m, 3H), 5.22 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 174.40, 133.55, 133.05, 130.54, 129.27, 128.22, 127.76, 127.42, 127.04, 126.80, 124.58, 65.38.



Boc-Tyr(Me)-Phe-hydrazide (29). Boc-Tyr(Me)-Phe-methyl ester (**14,** 1.17 g, 2.56 mmol) was dissolved in MeOH and hydrazine monohydrate (7.47 ml, 154 mmol, 60 equiv.) was added. The reaction mixture was refluxed for 2 hr., before being concentrated until white precipitate is formed. The resulting suspension was cooled and the product was filtered off (1.03 g, 2.25 mmol, 88%). ^{1}H NMR (400 MHz, MeOD/$CDCl_3$): δ ppm 7.26-7.08 (m, 5H), 7.03 (d, *J* = 8.6 Hz, 2H), 6.79 (d, *J* = 8.6 Hz, 2H), 4.55-4.50 (m, 1H), 4.18 (t, *J* = 6.6 Hz, 1H), 3.01 (dd, J_1 = 13.7, J_2 = 7.0 Hz, 1H), 2.96-2.85 (m, 2H), 2.73 (dd, J_1 = 13.5, J_2 = 8.1 Hz, 1H), 1.34 (s, 9H). NMR (100 MHz, MeOD/$CDCl_3$): δ ppm 171.77, 170.37, 158.08, 135.87, 129.70, 128.62, 128.08, 127.96, 126.37, 113.40, 79.72, 55.67, 54.53, 52.52, 37.25, 36.74, 27.46.

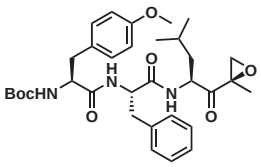


Boc-Tyr(Me)-Phe-Leu-methyl vinylsulfone (30). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS (0.24 g, 0.83 mmol, 1.1 equiv.) and Boc-Tyr(Me)-Phe-hydrazide (**29,** 0.39 g, 0.75 mmol). Purification by flash column chromatography (DCM → 1.5% MeOH in DCM) gave **30** (0.35 g, 0.56 mmol, 75%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.29-7.22 (m, 3H), 7.09 (d, *J* = 8.5 Hz, 2H), 7.03 (d, *J* = 6.1 Hz, 2H), 6.87 (d, *J* = 8.5 Hz, 2H), 6.82-6.65 (m, 2H), 6.47-6.37 (m, 2H), 4.84 (d, *J* = 3.0 Hz, 1H), 4.77-4.69 (m, 1H), 4.66 (dd, J_1 = 13.0, J_2 = 6.3 Hz, 1H), 4.16 (td, J_1 = 7.8, J_2 = 4.8 Hz, 1H), 3.80 (s, 3H), 3.32-3.23 (m, 1H), 3.03 (dd, J_1 = 14.3, J_2 = 5.0 Hz, 1H), 2.91 (s, 3H), 2.91-2.83 (m, 2H), 1.53-1.41 (m, 1H), 1.37-1.30 (m, 2H), 1.27 (s, 9H), 0.90 (d, *J* = 6.4 Hz, 3H), 0.86 (d, *J* = 6.5 Hz, 3H).

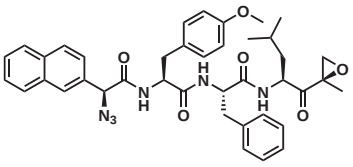


***S*-2-azido-2-(naphthalen-2-yl)acetamido-Tyr(Me)-Phe-Leu-methyl vinylsulfone (31).** Boc-Tyr(Me)-Phe-LeuVS (**30,** 0.12 g, 0.2 mmol) was dissolved in TFA/DCM 1/1 (v/v). The reaction mixture was stirred for 30 min., before being co-evaporated with Tol. (3×). The crude TFA salt was dissolved in DCM and *S*-2-azido-2-(naphthalen-2-yl)acetic acid (**28,** 73 mg, 0.32 mmol, 1.6 equiv.), EDC·HCl (62 mg, 0.32 mmol, 1.6 equiv.) and DiPEA (33 µl, 0.2 mmol, 1 equiv.) were added. After being stirred for 2 hr., the reaction mixture was washed with 1 M HCl (aq.), separated and dried

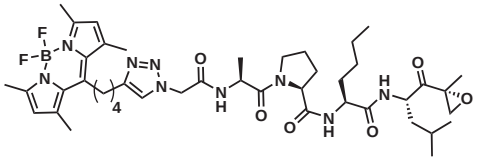
over $MgSO_4$. Purification by flash column chromatography (DCM → 2% MeOH in DCM) followed by washing with sat. aq. $NaHCO_3$ (3×) and drying over $MgSO_4$ gave the pure title compound as a white solid (0.1 g, 0.14 mmol, 70%). ^{1}H NMR (400 MHz, MeOD/$CDCl_3$): δ ppm 7.88-7.76 (m, 3H), 7.70 (s, 1H), 7.55-7.50 (m, 2H), 7.35-7.21 (m, 3H), 7.19 (d, *J* = 7.3 Hz, 2H), 7.11-7.03 (m, 3H), 6.74 (d, *J* = 8.5 Hz, 2H), 6.65 (dd, J_1 = 15.1, J_2 = 4.7 Hz, 1H), 5.95 (d, *J* = 15.1 Hz, 1H), 5.14 (s, 1H), 4.69 (dd, J_1 = 9.2, J_2 = 5.1 Hz, 1H), 4.64-4.58 (m, 2H), 3.74 (s, 3H), 3.12-3.02 (m, 2H), 2.97 (dd, J_1 = 13.5, J_2 = 6.6 Hz, 1H), 2.89 (s, 3H), 2.88-2.83 (m, 1H), 1.67-1.55 (m, 1H), 1.48-1.29 (m, 2H), 0.91 (dd, J_1 = 10.9, J_2 = 6.5 Hz, 6H). ^{13}C NMR (100 MHz, MeOD/$CDCl_3$): δ ppm 170.81, 170.44, 168.37, 158.14, 146.92, 135.81, 132.99, 132.61, 131.51, 129.70, 128.78, 128.47, 128.33, 128.17, 127.63, 127.17, 127.10, 126.63, 126.32, 126.11, 123.98, 113.50, 66.57, 54.47, 54.34, 53.90, 47.43, 41.85, 41.81, 37.30, 36.31, 24.11, 22.02, 20.99. HRMS: calcd. for $[C_{39}H_{44}N_6O_6SH]^+$ 725.31158, found 725.31214.



Boc-Tyr(Me)-Phe-Leu-epoxyketone (32). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuEK (0.22 g, 0.83 mmol, 1.1 equiv.) and Boc-Tyr(Me)-Phe-hydrazide (**29**, 0.39 g, 0.75 mmol). Purification by flash column chromatography (DCM → 1.5% MeOH in DCM) gave **32** (0.32 g, 0.54 mmol, 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.26-7.11 (m, 3H), 7.10-7.04 (m, 4H), 7.04-7.01 (m, 1H), 6.90 (s, 1H), 6.78 (d, *J* = 8.5 Hz, 2H), 5.31 (d, *J* = 7.7 Hz, 1H), 4.77 (q, *J* = 6.9 Hz, 1H), 4.56 (dt, J_1 = 9.8, J_2 = 3.2 Hz, 1H), 4.41 (s, 1H), 3.73 (s, 3H), 3.25 (d, *J* = 4.4 Hz, 1H), 3.02 (dd, J_1 = 14.0, J_2 = 6.8 Hz, 1H), 2.98-2.88 (m, 3H), 2.84 (d, *J* = 4.9 Hz, 1H), 1.58-1.51 (m, 1H), 1.49 (s, 3H), 1.48-1.38 (m, 1H), 1.36 (s, 9H), 1.27-1.18 (m, 1H), 0.88 (dd, J_1 = 12.3, J_2 = 6.4 Hz, 6H).

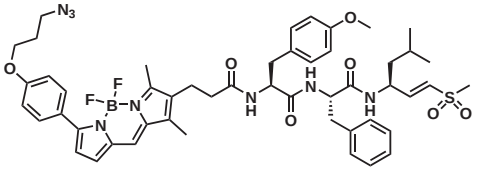


***S*-2-azido-2-(naphthalen-2-yl)acetamido-Tyr(Me)-Phe-Leu-epoxyketone (33).** Boc-Tyr(Me)-Phe-LeuEK (**32**, 0.12 g, 0.2 mmol) was dissolved in TFA/DCM 1/1 (v/v). The reaction mixture was stirred for 30 min., before being co-evaporated with Tol. (3×). The crude TFA salt was dissolved in DCM and *S*-2-azido-2-(naphthalen-2-yl)acetic acid (**28**, 50 mg, 0.22 mmol, 1.1 equiv.), EDC·HCl (84 mg, 0.44 mmol, 2.2 equiv.) and DiPEA (33 µl, 0.2 mmol, 1 equiv.) were added. After being stirred for 15 hr., the reaction mixture was washed with 1 M HCl (aq.), sat. aq. $NaHCO_3$, separated and dried over $MgSO_4$. Purification by flash column chromatography (DCM → 1% MeOH in DCM, followed by a second column: DCM → 10% EtOAc in DCM) gave the pure title compound as a white solid (36 mg, 51 µmol, 26%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.85-7.70 (m, 3H), 7.64 (s, 1H), 7.53-7.48 (m, 2H), 7.28-7.21 (m, 3H), 7.13-7.07 (m, 2H), 7.05-6.97 (m, 4H), 6.72 (d, *J* = 8.4 Hz, 2H), 6.66 (d, *J* = 7.6 Hz, 1H), 6.27 (d, *J* = 7.9 Hz, 1H), 5.04 (s, 1H), 4.70-4.58 (m, 2H), 4.58-4.50 (m, 1H), 3.73 (s, 3H), 3.24 (d, *J* = 4.9 Hz, 1H), 3.08-2.89 (m, 4H), 2.87 (d, *J* = 4.9 Hz, 1H), 1.50 (s, 3H), 1.49-1.41 (m, 2H), 1.23-1.13 (m, 1H), 0.89 (dd, J_1 = 15.9, J_1 = 6.1 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 207.85, 170.21, 167.99, 158.64, 136.09, 133.39, 132.99, 131.76, 130.15, 129.28, 129.12, 128.56, 128.14, 127.76, 127.68, 127.52, 126.98, 126.79, 126.59, 124.20, 114.12, 114.02, 67.33, 58.93, 55.10, 54.26, 54.11, 52.27, 50.10, 40.02, 37.89, 36.57, 25.07, 23.24, 21.32, 16.62. HRMS: calcd. for $[C_{40}H_{44}N_6O_6H]^+$ 705.33951, found 705.34004.

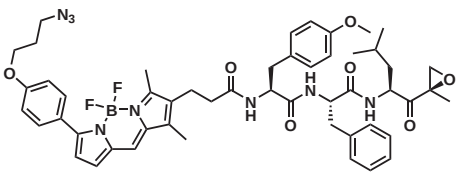


Green fluorescent β1 specific probe (35). β1 selective probe **23** (7.7 mg, 0.014 mmol) was reacted with acetylene-functionalized BODIPY **34**[20] (4.7 mg, 0.014 mmol, 1.0 equiv.) catalyzed by $CuSO_4$ (28mM in H_2O) (0.05 mL, 1.4 µmol, 10 mol%) and sodium ascorbate (44mM in H_2O) (0.05 mL, 2.2 µmol, 15 mol%) in a mixture of Tol./H_2O/*t*BuOH (final ratio 1/1/1, v/v/v, 0.6 mL) at 80°C for 22 hr. The mixture was then allowed to cool to room temperature and concentrated *in vacuo*. Purification by column chromatography (DCM → 2% MeOH in DCM) gave the fluorescent probe **35**

(8.6 mg, 9.3 µmol, 65%) as an orange solid. ^{1}H NMR (400 MHz, DMSO): δ ppm 8.66 (d, *J* = 6.8 Hz, 1H), 8.09 (d, *J* = 7.2 Hz, 1H), 7.80-7.77 (m, 2H), 6.23 (s, 2H), 5.07 (s, 2H), 4.56-4.53 (s, 1H), 4.38-4.33 (m, 2H), 4.24-4.17 (m, 1H), 3.58-3.55 (m, 2H), 3.19-3.17 (m, 1H), 3.02-2.96 (m, 3H), 2.72 (t, *J* = 7.2, 7.2 Hz), 2.41 (s, 6H), 2.40 (s, 6H), 1.98-1.94 (m, 2H), 1.89-1.81 (m, 4H), 1.64-1.62 (m, 4H), 1.47-1.44 (m, 1H), 1.41 (s, 3H), 1.36-1.31 (m, 2H), 1.24-1.20 (m, 7H), 0.91 (d, *J* = 6.8 Hz, 6H), 0.88-0.81 (m, 3H). ^{13}C NMR (100 MHz, DMSO): δ ppm 208.28, 171.87, 170.92, 170.39, 165.03, 153.06, 146.68, 140.90, 130.72, 123.50, 121.69, 59.18, 58.93, 51.99, 51.64, 51.22, 49.60, 46.70, 46.41, 38.33, 31.90, 30.81, 29.43, 28.78, 27.61, 27.20, 24.53, 24.40, 23.16, 21.92, 20.98, 17.07, 16.52, 15.83, 14.08, 13.92. HRMS: calcd. for $[C_{44}H_{64}BF_2N_9O_6H]^+$ 864.51134, found 864.51332.



Azido-BODIPY-Tyr(Me)-Phe-Leu-methyl vinylsulfone (38). Boc-Tyr(Me)-Phe-LeuVS (**30**, 22 mg, 35 µmol) was dissolved in TFA/DCM 1/1 (v/v). The reaction mixture was stirred for 30 min., before being co-evaporated with Tol. (3×). The crude TFA salt was dissolved in DCM and N_3-BODIPY-OSu[21] (**37**, 20 mg, 35 µmol, 1 equiv.) and DiPEA (6 µl, 35 µmol, 1 equiv.) were added. The reaction mixture was stirred for 5 hr., before being concentrated. Purification by flash column chromatography (DCM → 1.5% MeOH in DCM) afforded the title compound as a purple solid (18.2 mg, 18.9 µmol, 54%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.87 (d, *J* = 8.8 Hz, 2H), 7.29-7.20 (m, 3H), 7.07 (s, 1H), 7.04 (dd, J_1 = 7.4, J_2 = 1.5 Hz, 2H), 7.00-6.93 (m, 5H), 6.79 (d, *J* = 8.5 Hz, 2H), 6.71 (dd, J_1 = 15.1, J_2 = 4.6 Hz, 1H), 6.54 (d, *J* = 4.1 Hz, 1H), 6.52-6.44 (m, 2H), 6.21 (dd, J_1 = 15.1, J_2 = 1.1 Hz, 1H), 6.10-6.04 (m, 1H), 4.72-4.57 (m, 2H), 4.45 (q, *J* = 6.4 Hz, 1H), 4.10 (t, *J* = 5.9 Hz, 2H), 3.70 (s, 3H), 3.53 (t, *J* = 6.6 Hz, 3H), 3.19 (dd, J_1 = 13.8, J_2 = 5.4 Hz, 1H), 2.95-2.89 (m, 3H), 2.89 (s, 3H), 2.65-2.50 (m, 2H), 2.48 (s, 3H), 2.19-2.13 (m, 3H), 2.13 (s, 3H), 2.11-2.03 (m, 2H), 1.52-1.38 (m, 1H), 1.38-1.26 (m, 2H), 0.87 (t, *J* = 6.7 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 172.52, 170.81, 169.95, 159.55, 158.86, 158.32, 156.04, 147.21, 139.32, 135.87, 135.13, 134.04, 134.02, 130.77, 130.73, 130.69, 130.08, 129.60, 129.25, 129.21, 128.85, 128.80, 128.39, 127.41, 127.31, 125.53, 123.01, 118.64, 118.60, 118.58, 114.29, 114.20, 64.45, 55.19, 54.25, 48.20, 47.91, 42.74, 42.42, 37.17, 36.37, 35.82, 28.74, 24.61, 22.80, 21.74, 19.59, 13.09, 9.57. HRMS: calcd. for $[C_{50}H_{59}BF_2N_8O_7SH]^+$ 965.43613, found 965.43837.



Azido-BODIPY-Tyr(Me)-Phe-Leu-epoxyketone (39). Boc-Tyr(Me)-Phe-LeuEK (**32**, 21 mg, 35 µmol) was dissolved in TFA/DCM 1/1 (v/v). The reaction mixture was stirred for 30 min., before being co-evaporated with Tol. (3×). The crude TFA salt was dissolved in DCM and N_3-BODIPY-OSu[21] (**37**, 20 mg, 35 µmol, 1 equiv.) and DiPEA (6 µl, 35 µmol, 1 equiv.) were added. The reaction mixture was stirred for 15 hr., before being concentrated. Purification by flash column chromatography (DCM → 1% MeOH in DCM) afforded the title compound as a purple solid (8.8 mg, 9.3 µmol, 27%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.87 (d, *J* = 8.6 Hz, 2H), 7.27-7.19 (m, 3H), 7.11-7.05 (m, 3H), 7.00-6.92 (m, 5H), 6.76 (d, *J* = 8.2 Hz, 2H), 6.54 (d, *J* = 3.9 Hz, 1H), 6.32 (d, *J* = 7.6 Hz, 1H), 6.03 (d, *J* = 7.6 Hz, 1H), 5.88 (d, *J* = 7.0 Hz, 1H), 4.58-4.47 (m, 3H), 4.10 (t, *J* = 5.8 Hz, 2H), 3.73-3.70 (m, 3H), 3.53 (t, *J* = 6.6 Hz, 2H), 3.23 (d, *J* = 4.8 Hz, 1H), 3.08-2.89 (m, 4H), 2.88 (d, *J* = 4.8 Hz, 1H), 2.76-2.55 (m, 2H), 2.51 (s, 3H), 2.33-2.20 (m, 2H), 2.18 (s, 3H), 2.08 (td, J_1 = 12.1, J_2 = 6.1 Hz, 2H), 1.50 (s, 3H), 1.49-1.38 (m, 2H), 1.20-1.13 (m, 1H), 0.89 (dd, J_1 = 13.7, J_2 = 5.5 Hz, 6H). HRMS: calcd. for $[C_{51}H_{59}BF_2N_8O_7H]^+$ 945.46406, found 945.46639.

Competition and labeling experiments *in vitro*.

HEK293T cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 µg/ml streptomycin in a 7% CO_2 humidified incubator at 37 °C. Cells were harvested, washed with PBS (2×) and permeated in digitonin lysis buffer (4× pellet volume, 50 mM Tris pH 7.5, 250 mM sucrose, 5 mM

$MgCl_2$, 5 mM DTT, 0.025% digitonin) for 5 min. on ice and centrifuged at 16.100 rcf. for 20 min at 4 °C. The supernatant containing the cytosolic fraction was collected and the protein content was determined by Bradford assay. 10µg total protein per experiment was exposed to the inhibitors or fluorescent probes (10× solution in DMSO) for 1 hr. at 37 °C prior to incubation with MV151 (1 µM) for 1 hr. at 37 °C in case of a competition experiment. Reaction mixtures were boiled with Laemmli's buffer containing β-mercapto-ethanol for 3 min. before being resolved on 12.5% SDS-PAGE. In-gel detection of fluorescently labeled proteins was performed in the wet gel slabs directly on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560) for MV151 and the azido-BODIPY functionalized probes **38**, **39** and **40** or λ_{ex} 488 nm, λ_{em} 520 nm for probes **35** and **36**.

Competition and labeling experiments in living cells.

HEK293T cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 µg/ml streptomycin in a 7% CO_2 humidified incubator at 37 °C. Some $5{\cdot}10^5$ HEK293T cells were seeded in 6 cm Petri dishes and allowed to grow O/N in 2 ml of medium. The cells were exposed to the indicated concentrations of the inhibitors or fluorescent probes (100× solution in DMSO) for 2 hr., before being washed with PBS (2×) and harvested. The cells were permeated in digitonin lysis buffer (4× pellet volume, 50 mM Tris pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 5 mM DTT, 0.025% digitonin) for 5 min. on ice and centrifuged at 16.100 rcf. for 20 min at 4 °C. The supernatant containing the cytosolic fraction was collected and the protein content was determined by Bradford assay. In case of a competition experiment, the lysates were exposed to MV151 (1 µM) for 1 hr. at 37 °C. Some 10 µg protein/lane was boiled for 5 min. in Laemli's sample buffer containing beta-mercapto-ethanol and the proteins were resolved by 12.5% SDS-PAGE. Labelled proteasome subunits were visualised by in-gel fluorescence scanning on a Typhoon variable mode imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560) for MV151 and the azido-BODIPY functionalized probes **38**, **39** and **40** or λ_{ex} 488 nm, λ_{em} 520 nm for probes **35** and **36**.

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7 Diels-Alder two-step activity-based proteasome labeling

7.1 Introduction

As discussed in Chapter 1.5, two general strategies for two-step activity-based protein profiling have been described. These are the so called "click" ligation, based on the Huisgen [2+3]-cycloaddition[1] and the Staudinger-Bertozzi ligation.[2] Both these strategies rely on azido functionalized probes or reporter groups. To gain flexibility and the opportunity to examine multiple proteins in a single experiment, activity-based protein profiling research would greatly benefit from the availability of a larger panel of bioorthogonal two-step labeling techniques.[3] An optimal two-step ligation reaction should be chemoselective, bioorthogonal, efficient under mild reaction conditions and proceed in aqueous media. One of the most classical organic chemical reactions, the Diels-Alder [4+2] cycloaddition,[4] fits most of the aforementioned qualifications. This reaction of a conjugated diene and an electronically matched dienophile proceeds via a concerted mechanism and is highly chemoselective. The physiological conditions of a two-step labeling approach seem to fit the Diels-Alder reaction, since the reaction is accelerated in aqueous media by the hydrophobic effect.[5] The Diels-Alder reaction has successfully been applied in the bioconjugation of carbohydrates to proteins[6] and the tagging[7] and surface immobilization of oligonucleotides.[8] Waldmann and co-workers recently developed a Diels-Alder based site-specific chemoselective labeling, ligation and surface immobilization strategy for peptides and proteins.[9]

This Chapter describes the development of chemical tools to perform a Diels-Alder based two-step labeling strategy as depicted in Figure 1. The synthesis of four diene

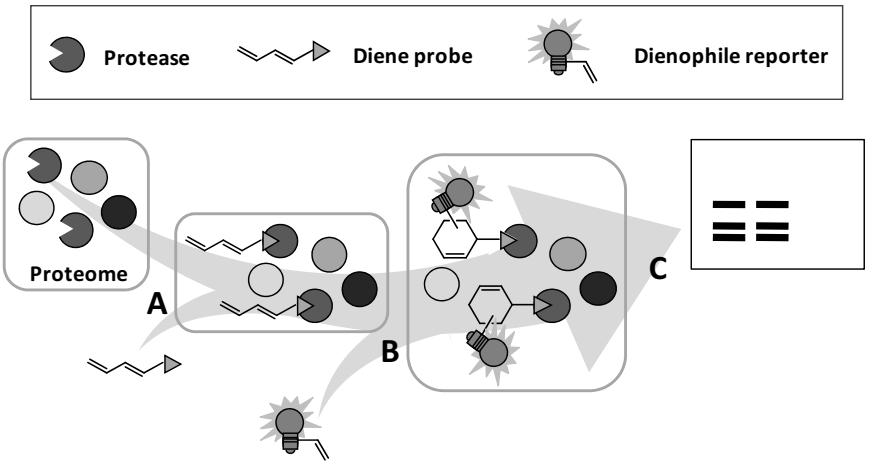


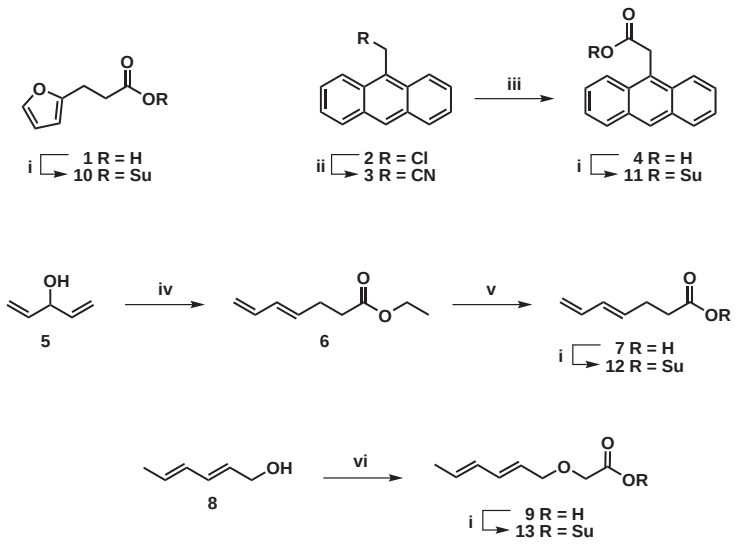
Figure 1. Schematic representation of a Diels-Alder based two-step protein profiling strategy.
(**A**) A protease is targeted by a diene functionalized probe. (**B**) Treatment of the diene tagged protease with a reporter group equipped dienophile results in the labeling of the protease. (**C**) SDS-PAGE, followed by detection results in the labeling profile of the diene functionalized probe.

functionalized epoxomicin analogues is described. The influence of the *N*-terminal diene modification on the capacity to inhibit the proteasome is determined in a competition assay versus MV151 (Chapter 2). A two-step labeling strategy was applied on purified 20S proteasomes spiked in RAW cell lysate, in which a BODIPY TMR tagged maleimide derivative was used as the fluorescent dienophile in the Diels-Alder ligation.

7.2 Results and discussion

The cyclic dienes furylpropionic acid **1** and 9-anthracenylacetic acid **4**, and the linear dienes hepta-4,6-dienoic acid **7** and (hexa-2,4-dienyloxy)acetic acid **9** were chosen for the synthesis of a panel of *N*-terminally diene modified epoxomicin analogues. Since some of these dienes are acid labile, it was reasoned that these functionalities are best introduced in the final step of the synthesis, after full deprotection of the epoxomicin synthon **14**. The syntheses commenced with the preparation of the diene functionalized carboxylic acids (Scheme 1). Apart from the commercially available 3-(2-furyl)propionic acid (**1**), the diene functionalized carboxylic acids were synthesized as follows. 2-(Anthracen-9-yl)acetic acid (**4**) was obtained from 9-chloromethyl anthracene (**2**) by reaction with KCN, followed by hydrolysis of the resulting nitrile **3** with NaOH in refluxing 2-methoxyethanol. (*E*)-hepta-4,6-dienoic acid (**7**) was synthesized in two steps from penta-1,4-dien-3-ol (**5**). Treatment with triethyl orthoacetate in refluxing toluene under mildly acidic conditions gave ethyl ester **6** via an orthoester Claisen rearrangement.[10] Hydrolysis of ethyl hepta-4,6-dienoate (**6**) with NaOH in methanol/dioxane mixture gave **7**. The final diene functionalized carboxylic acid, 2-((2*E*,4*E*)-hexa-2,4-dienyloxy)acetic acid (**9**), was obtained from

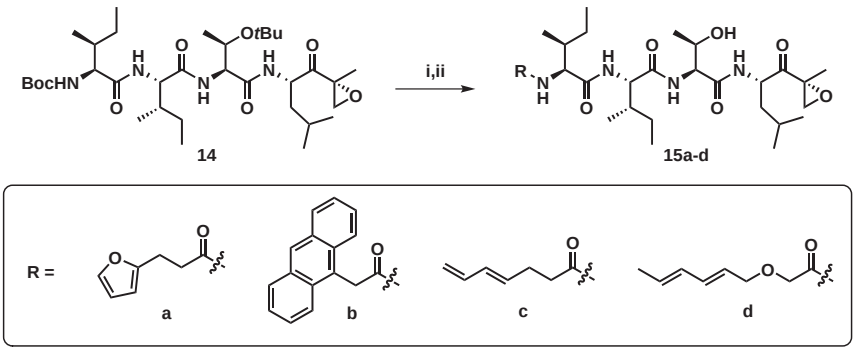
Scheme 1. Synthesis of diene succinimidyl esters.



Reagents and conditions: i) HOSu (4 equiv.), EDC·HCl (4 equiv.), DCE/DMF, rt, 16 hr., **10** 71%, **11** 81%, **12** 78%, **13** 80%. ii) KCN (1.5 equiv.), DMSO/H_2O, 60 °C, 1 hr. then rt, 16 hr., 94%. iii) (a) NaOH (2.5 equiv.), 2-methoxyethanol, reflux, 3 hr. (b) 1M HCl, rt, 16 hr., 56%. iv) triethyl orthoacetate (5 equiv.), propionic acid (cat.), Tol., reflux, 3 hr., 94%. v) 1M NaOH/MeOH/1,4-dioxane (1/1/1, v/v/v), rt, 1 hr., 68%. vi) (a) NaH (2 equiv.), THF, rt, 30 min. (b) 2-bromoacetic acid (1 equiv.), rt, 16 hr., 73%.

(2*E*,4*E*)-hexa-2,4-dien-1-ol (**8**) after deprotonation with NaH and reaction with bromoacetic acid. The four diene functionalized carboxylic acids **1, 4, 7** and **9** were converted into their corresponding succinimidyl ester by condensation with *N*-hydroxysuccinimide under the influence of EDC·HCl. The protected tetrapeptide epoxyketone Boc-Ile-Ile-Thr(*t*Bu)-LeuEK (**14**)[11] was fully deprotected, and condensed with the diene succinimidyl esters **10-13** to afford the four *N*-terminally diene modified epoxomicin analogues **15a-d** (Scheme 2).

Scheme 2. Synthesis of diene functionalized epoxomicin analogues.



Reagents and conditions: i) TFA/DCM (1/1, v/v), rt, 15 - 45 min, ii) R-OSu (**10-13**), DiPEA, DCE/DMF, rt, 1.5 - 18 hr., **15a** 82%, **15b** 39%, **15c** 64%, **15d** quant.

The proteasome inhibitory potential of the diene equipped epoxomicin analogues **15a-d** was assessed in a competition experiment versus the fluorescent broad-spectrum proteasome probe MV151 (Chapter 2).[12] Lysates of the murine EL-4 cell line (expressing both the constitutive proteasome and the immunoproteasome) were incubated with increasing concentrations of **15a-d** for 1 hr., before labeling of the residual proteasome activities with MV151. The proteins were denatured, separated on SDS-PAGE and the resulting gels were scanned on a fluorescence scanner (Figure 2). All four *N*-terminally

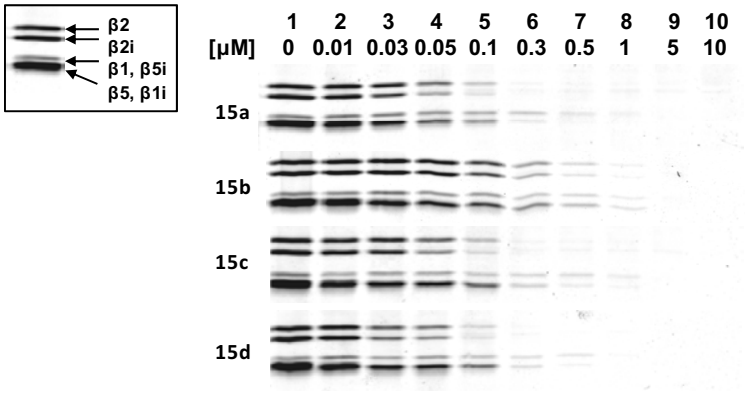
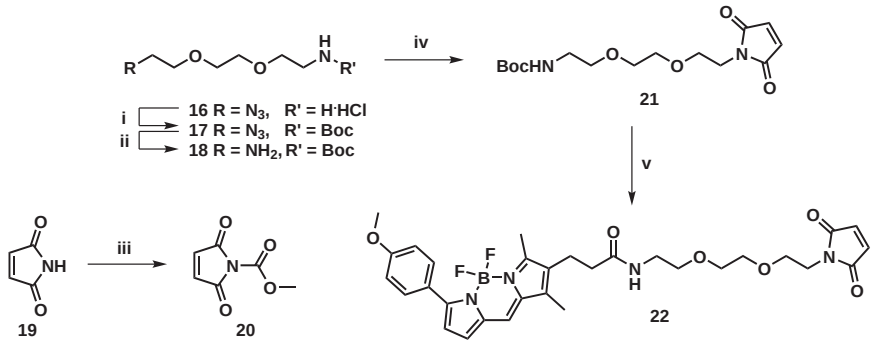


Figure 2. Competition assay of diene functionalized epoxomicin analogues **15a-d** versus MV151.
EL-4 lysates (10 μg total protein) were incubated with the indicated concentrations **15a-d** for 1 hr. at 37 °C. Residual proteasome activity was fluorescently labeled by incubation with 1 μM MV151 for 1 hr. at 37 °C.

diene modified epoxomicin analogues **15a-d** proved to be inhibitors of the proteasome with similar inhibitory profiles and potencies in the same order of magnitude as the parent inhibitor epoxomicin.[13] Although the differences in potency are small, the most bulky derivative, anthracenyl epoxomicin **15b** proved the least potent inhibitor of the four.

Scheme 3. Synthesis of fluorescent dienophile.



Reagents and conditions: i) Boc_2O (1.1 equiv.), Et_3N (2 equiv.), DCM, rt, 50 min., 90%. ii) (a) Ph_3P (1.2 equiv.), THF, rt, 5 hr., (b) H_2O (few drops), rt, 2 hr. iii) methyl chloroformate (1 equiv.), NMM (1 equiv.), EtOAc, 0 °C, 2 hr., 52%. iv) **20** (1.3 equiv.), sat. aq. $NaHCO_3$, 0 °C, 30 min., then rt, 45 min., 31% (2 steps). v) (a) TFA/DCM (1/1, v/v), rt, 10 min. (b) BODIPY TMR-OSu (1 equiv.), DiPEA (6 equiv.), DCE, rt, 16 hr., 34%.

Having synthesized a panel of diene functionalized proteasome inhibitors, their applicability in a Diels-Alder based two-step proteasome labeling approach was investigated. To this end, a reporter group equipped with a dienophile was designed and synthesized. Inspired by the results published by Waldmann and co-workers,[9] maleimide was chosen as the dienophile. A BODIPY TMR fluorophore tethered by a hydrophilic linker to the maleimide was synthesized as depicted in Scheme 3. After Boc-protection of the free amine in **16**,[11] the azide was reduced by a Staudinger reduction to give mono-protected di-amine **18**. Introduction of the maleimide moiety was carried out by treatment with Nefkens reagent analog **20**.[14] The synthesis was finalized by acidic deprotection of **21** and subsequent acylation with BODIPY TMR-OSu (Chapter 2) to give the fluorescently tagged dienophile **22**.

Initial Diels-Alder two-step proteasome labeling experiments were performed on purified 20S proteasomes, employing diene functionalized epoxomicin analogue **15d** as the potential probe as follows. Purified 20S proteasome was exposed to 5 µM **15d** or 1 µM MV151 as a positive control for 1 hr. at 37 ºC, before the proteins were denatured with urea and DTT. To prevent Michael addition of exposed cysteine residues on the fluorescently tagged maleimide, the samples were treated with Ellman's reagent[15] (5,5'-dithio-bis(2-nitrobenzoic acid), DTNB) to cap the free thiols. The same denaturation and capping procedure was performed on RAW cell lysate, which was added to prevent loss of diene functionalized proteasome subunits during workup.[16] The samples were subjected to chloroform/methanol precipitation to separate the proteins from the excess reagents. The precipitate was taken up in Diels-Alder buffer (5 mM NaH_2PO_4, 20 mM NaCl, 0.2 mM $MgCl_2$, pH 6.0)[9] containing 2 mM urea and 10 mM $Cu(NO_3)_2$ and the samples were incubated with 25 µM fluorescently tagged dienophile **22** for 16 hr. at 37 ºC. Copper nitrate was added to

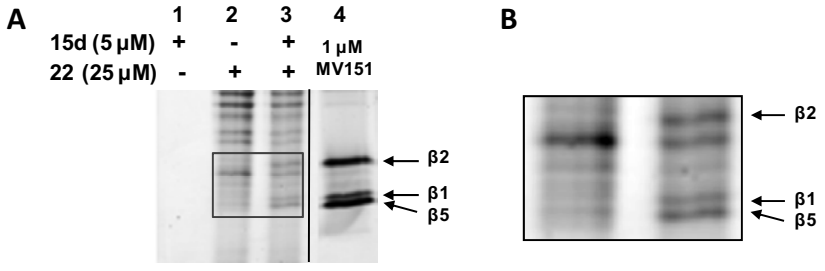


Figure 3. Diels-Alder two-step labeling of spiked purified 20S proteasome.
(**A**) Purified 20S proteasome (100 ng) was incubated with 5 µM **15d** for 1 hr. at 37 ºC, followed by denaturation with 8M urea and 5 mM DTT for 30 min. at 55 ºC and treatment with 30 mM DTNB for 3.5 hr. at rt. After addition of denatured and DTNB-treated RAW cell lysate, the samples were subjected to chloroform/methanol precipitation and redissolved in Diels-Alder buffer (pH 6.0) containing 2 mM urea and 10 mM $Cu(NO_3)_2$. Where indicated, the samples were exposed to 25 µM **22** for 16 hr. at 37 ºC. The reactions were terminated by chloroform/methanol precipitation. Proteins were separated by SDS-PAGE, followed by in-gel fluorescence readout. (**B**) Magnification of the box indicated in Figure 3A.

the ligation reactions, since copper(II) salts are known to catalyze Diels-Alder cycloadditions in aqueous media.[17,18] The ligation reactions were terminated by precipitation of the proteins. The precipitated proteins were resolved by SDS-PAGE and fluorescently labeled proteins were visualized by in-gel fluorescence readout (Figure 3). The gel revealed a considerable amount of labeling in the **15d** naïve sample (Figure 3A, lane 2), indicating aspecific fluorescent labeling by dienophile **22**. However, the sample treated with both the diene functionalized proteasome inhibitor **15d** and the fluorescently tagged dienophile **22** revealed three additional bands, not present in the **15d** naïve sample (Figure 3A, lane 3. Magnified in Figure 3B). This finding, and the fact that the labeling closely resembles that of the fluorescent proteasome probe MV151 indicates the Diels-Alder two-step labeling of proteolytically active proteasome β subunits.

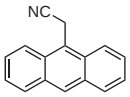
7.3 Conclusion

The synthesis of four *N*-terminally diene modified epoxomicin analogues **15a-d** is described. The introduction of the dienes did not influence the proteasome inhibitory potential as judged by the competition experiment versus MV151, which showed potencies in the same order of magnitude as for the parent inhibitor epoxomicin. Furthermore, the fluorescently tagged dienophile **22** was synthesized. Initial Diels-Alder two-step proteasome labeling experiments performed on purified 20S proteasome showed the potential of the Diels-Alder as an alternative ligation method in activity-based protein profiling, although considerable optimizations are needed to extrapolate this methodology to labeling of endogenously expressed proteasomes.

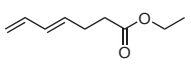
Experimental section

General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.) (purum), ethyl acetate (EtOAc) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and 1,4-dioxane (Biosolve) were stored on 4Å molecular sieves. Dimethyl sulfoxide (DSMO), methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), by spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, 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 followed by charring at ~150°C or by spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on silica gel (0.040-0.063 nm) obtained from Screening Devices. LC/MS analysis was performed on a LCQ Adventage Max (Thermo Finnigan) equipped with a Gemini C18 column (Phenomenex). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50) or Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory. Chemical shifts (δ) are given relative to tetramethylsilane as internal standard. All presented ^{13}C-APT spectra are proton decoupled.

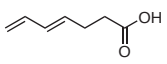
Coupling constants are given in Hz. Boc-Ile-Ile-Thr(*t*Bu)-Leu-ek **14** and 2-(2-(2-azidoethoxy)ethoxy)ethanamine·HCl **16** were synthesized as described in Chapter 4.



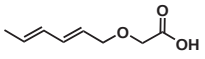
2-(anthracen-9-yl)acetonitrile (3). A solution of 9-(chloromethyl)anthracene (**2**) (2.27 g, 10 mmol) in DMSO (15 ml) was heated to 60 °C, before addition of a solution of KCN (0.98 g, 15 mmol, 1.5 equiv.) in H_2O (3 ml). The reaction mixture was stirred at 60 °C for 1 hr., then allowed to cool down to rt and stirred overnight. After addition of H_2O (40 ml), the precipitate was filtrated, washed with H_2O and dried *in vacuo* to yield **3** as yellow crystals (2.04 g, 9.4 mmol, 94%). ^{1}H NMR (200 MHz, $CDCL_3$): δ ppm 8.53 (s, 1H), 8.13 (ddd, J_1 = 20.7, J_2 = 8.4, J_3 = 0.4 Hz, 4H), 7.59 (dddd, J_1 = 14.8, J_2 =7.7, J_3 =6.6, J_4 =1.1 Hz, 4H), 4.61 (s, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 131.21, 129.33, 128.66, 127.07, 125.13, 122.77, 117.65, 77.62, 77.00, 76.35, 15.96.



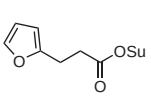
(*E*)-ethyl hepta-4,6-dienoate (6). To a solution of penta-1,4-dien-3-ol (**5**) (0.29 ml, 3.0 mmol) in toluene was added triethyl orthoacetate (2.8 ml, 15 mmol, 5 equiv.) and one drop of propionic acid. The reaction mixture was refluxed for 3 hr., before being concentrated *in vacuo*. Purification by column chromatography (PetEt → 5% EtOAc in PetEt) yielded compound **6** as a colorless oil (0.43 g, 2.8 mmol, 94%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 6.44-5.99 (m, 2H), 5.81-5.60 (m, 1H), 5.05 (ddd, J_1 = 13.3, J_2 = 11.5, J_3 = 1.6 Hz, 2H), 4.14 (q, J = 7.1 Hz, 2H), 2.37 (s, 4H), 1.25 (t, J = 7.1, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 172.12, 136.46, 132.18, 131.55, 115.05, 59.72, 33.31, 27.37, 13.75.



(*E*)-hepta-4,6-dienoic acid (7). (*E*)-ethyl hepta-4,6-dienoate (**6**) (0.43 g, 2.8 mmol) was dissolved in a mixture of 1,4-dioxane/MeOH/1M NaOH (1/1/1, v/v/v) and stirred for 1 hr. After adding DCM, the mixture was washed with sat. aq. $NaHCO_3$ (1x). The aqueous layer was acidified with 1M HCl, followed by extraction with DCM (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 25% EtOAc in PetEt) gave hepta-4,6-dienoic acid (**7**) (0.24 g, 1.9 mmol, 68%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 6.43-6.00 (m, 2H), 5.78-5.61 (m, 1H), 5.26-4.87 (m, 2H), 2.56-2.33 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 179.26, 136.67, 132.04, 115.74, 33.47, 27.26.

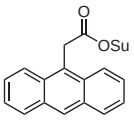


2-((2*E*,4*E*)-hexa-2,4-dienyloxy)acetic acid (9). (2*E*,4*E*)-hexa-2,4-dien-1-ol (**8**) (3.0 g, 30 mmol) was added to a suspension of NaH (60% in mineral oil, 2.5 g, 61 mmol, 2 equiv.) in freshly distilled THF under argon atmosphere. The reaction mixture was stirred for 30 min., after which bromoacetic acid (4.2 g, 30 mmol, 1 equiv.) was added. The reaction mixture was stirred overnight, before being quenched with a 3M KOH solution. The aqueous layer was then washed with Et_2O (2x), acidified with 6M HCl and extracted with $CHCl_3$ (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (DCM + 1% AcOH → 5% MeOH in DCM + 1% AcOH) yielded acid **9** (3.4 g, 22 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 11.58 (s, 1H), 6.20 (dd, J_1 = 15.2, J_2 = 10.5 Hz, 1H), 6.04 (ddd, J_1 = 14.7, J_2 = 10.5, J_3 = 1.3 Hz, 1H), 5.71 (dq, J_1 = 13.6, J_2 = 6.7 Hz, 1H), 5.62-5.53 (m, 1H), 4.08 (t, J = 3.2 Hz, 4H), 1.73 (d, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 175.58, 134.55, 130.60, 130.27, 124.61, 71.51, 65.80, 17.75.

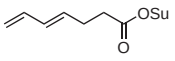


3-(2-furyl)propanoyl-OSu (10). A solution of 3-(2-furyl)propanoic acid (**1**) (0.98 g, 7.0 mmol) in DCE/DMF was put under argon atmosphere, before HOSu (3.2 g, 28 mmol, 4 equiv.) and EDC·HCl (5.3 g, 28 mmol, 4 equiv.) were added. The reaction mixture was stirred overnight, after which EtOAc was added and the mixture was washed with 1M HCl (2x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (PetEt

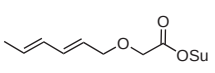
→ 40% EtOAc in PetEt), yielding OSu-ester **10** (1.2 g, 4.9 mmol, 71%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 7.38-7.28 (m, 1H), 6.29 (dd, J_1 = 3.2, J_2 = 1.9 Hz, 1H), 6.11 (dd, J_1 = 3.2, J_2 = 0.8 Hz, 1H), 3.14-2.90 (m, 4H), 2.84 (s, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 169.04, 167.46, 152.32, 141.50, 109.99, 105.60, 29.09, 25.14, 22.51.



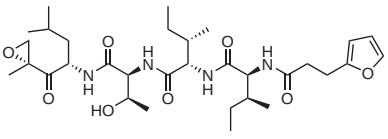
2-(anthracen-9-yl)acetyl-OSu (11). 2-(anthracen-9-yl)acetonitrile (**3**) (2.04 g, 9.4 mmol) was dissolved in 2-methoxyethanol (15 ml), before NaOH (0.94 g, 23.5 mmol, 2.5 equiv.) was added. The reaction mixture was refluxed for 3 hr., before addition of H_2O (60 ml). The mixture was extracted with Et_2O (2x) and the aqueous layer was acidified to pH 2 using 1M HCl, and left to stand overnight. The precipitate was filtered, washed with H_2O, dried *in vacuo* to give the acid **4** (1.24 g, 5.2 mmol, 56%), which was used without further purification. 2-(anthracen-9-yl)acetic acid (**4,** 0.86 g, 3.6 mmol) was coevaporated with Tol., dissolved in DCE/DMF and put under argon atmosphere, before HOSu (1.6 g, 14 mmol, 4 equiv.) and EDC·HCl (2.8 g, 14 mmol, 4 equiv.) were added. The reaction mixture was stirred overnight, after which EtOAc was added. The mixture was washed with 1M HCl (2x) and the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (PetEt → 40% EtOAc in PetEt), yielding OSu-ester **11** (0.98 g, 2.9 mmol, 81%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 8.48 (s, 1H), 8.22 (d, J = 9.1 Hz, 2H), 8.03 (d, J = 8.4 Hz, 2H), 7.64-7.45 (m, 4H), 4.92 (s, 2H), 2.76 (s, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 168.89, 166.58, 131.25, 130.43, 129.13, 128.16, 126.67, 125.01, 123.55, 122.85, 30.30, 25.27.



(*E*)-hepta-4,6-dienoyl-OSu (12). A solution of acid **7** (0.17 g, 1.3 mmol) in DCE/DMF was put under argon atmosphere, before addition of HOSu (0.60 g, 5.2 mmol, 4 equiv.) and EDC.HCl (1.0 g, 5.2 mmol, 4 equiv.). The reaction mixture was stirred overnight. EtOAc was added and the mixture was washed with 1M HCl (2x). The combined organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 40% EtOAc in PetEt) gave OSu-ester **12** (0.23 g, 1.0 mmol, 78%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.30 (td, J_1 = 16.9, J_2 = 10.2 Hz, 1H), 6.14 (dd, J_1 = 15.1, J_2 = 10.5 Hz, 1H), 5.76-5.65 (m, 1H), 5.09 (dd, J_1 = 51.1, J_2 = 13.5 Hz, 2H), 2.80 (s, 4H), 2.71 (t, J = 7.4 Hz, 2H), 2.54-2.47 (m, 2H).

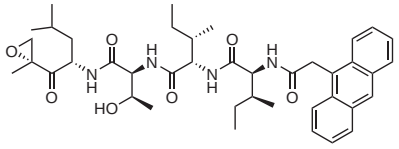


2-((2*E*,4*E*)-hexa-2,4-dienyloxy)acetyl-OSu (13). 2-((2*E*,4*E*)-hexa-2,4-dienyloxy)acetic acid (**9**, 3.42 g, 21.9 mmol) was dissolved in DCE/DMF and put under argon atmosphere, before HOSu (4.70 g, 40.5 mmol, 1.8 equiv.) and EDC·HCl (7.80 g, 40.5 mmol, 1.8 equiv.) were added. The reaction mixture was stirred overnight, before EtOAc was added and the mixture was washed with 1M HCl. The organics were separated and dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 50% EtOAc in PetEt) gave OSu-ester **13** (4.41 g, 17.4 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.26 (dd, J_1 = 15.2, J_2 = 10.4 Hz, 1H), 6.07 (dd, J_1 = 14.1, J_2 = 11.5 Hz, 1H), 5.81-5.71 (m, 1H), 5.66-5.53 (m, 1H), 4.41 (s, 2H), 4.16 (d, J = 6.6 Hz, 2H), 2.85 (s, 4H), 1.77 (d, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 168.72, 165.91, 135.31, 131.28, 130.35, 124.34, 72.06, 64.36, 25.52, 18.08.

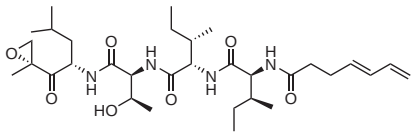


3-(2-furyl)propanoyl-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyloxirane (15a). Fully protected tetrapeptide epoxyketone **14** (0.16 g, 0.25 mmol) was treated with TFA/DCM (1/1, v/v) for 15 min., before being concentrated in the presence of toluene. The resulting TFA salt was dissolved in DCE/DMF and neutralized with DiPEA (0.17 ml, 1.0 mmol, 4 equiv.), followed by addition of a solution of OSu-ester **10** (0.18 g, 0.75 mmol, 3 equiv.) in DCE/DMF. The reaction mixture was stirred under argon atmosphere for 1.5 hr. Next, DCM was added and the mixture was washed with H_2O (1x). The aqueous layer was extracted with EtOAc (1x), the organic layers were combined and MeOH was added until the solution became clear. The organics were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*.

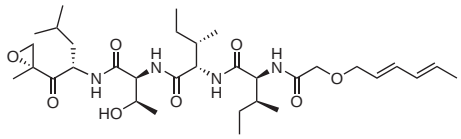
Purification by column chromatography (DCM → 4% MeOH in DCM) afforded title compound **15a** (0.13 g, 0.20 mmol, 82%) as a white solid. LC/MS analysis: R_t 8.50 min (linear gradient 10 → 90% B in 15 min), *m/z* 621.3 $[M+H]^+$, 1241.3 $[2M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$/MeOD): δ ppm 8.34 (d, *J* = 9.4 Hz, 1H), 7.87 (d, *J* = 7.4 Hz, 1H), 7.79 (d, *J* = 7.9 Hz, 1H), 7.26 (s, 1H), 7.15 (d, *J* = 8.8 Hz, 1H), 6.24 (dd, J_1 = 3.1, J_1 = 1.9 Hz, 1H), 6.00 (dd, J_1 = 3.2, J_1 = 0.7 Hz, 1H), 4.80-4.50 (m, 3H), 4.28-4.01 (m, 2H), 3.29 (d, *J* = 4.8 Hz, 1H), 3.03-2.90 (m, 2H), 2.88 (d, *J* = 4.9 Hz, 1H), 2.75-2.46 (m, 3H), 1.87-1.54 (m, 7H), 1.52 (s, 3H), 1.49-1.34 (m, 1H), 1.10 (d, *J* = 6.2 Hz, 3H), 0.93-0.71 (m, 18H).



2-(anthracen-9-yl)acetyl-Ile-Ile-Thr(*t*Bu)-leucinyl-(*R*)-2-methyl-oxirane (15b). Fully protected tetrapeptide epoxyketone **14** (0.16 g, 0.25 mmol) was treated with TFA/DCM (1/1, v/v) for 15 min, before being concentrated in the presence of toluene. The resulting TFA salt was dissolved in DCE/DMF, followed by the addition of DiPEA (0.09 ml, 0.50 mmol, 2 equiv.) and a solution of OSu-ester **11** (0.25 g, 0.75 mmol, 3 equiv.) in DCE/DMF. The reaction mixture was stirred under argon atmosphere for 18hr., before DCM was added and the mixture was washed with H_2O (1x). The aqueous layer was extracted with EtOAc (1x), the organic layers were combined and MeOH was added until a clear solution had formed. The organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (DCM → 2% MeOH in DCM) afforded title compound **15b** (70 mg, 98 µmol, 39%). LC/MS analysis: R_t 10.17 min (linear gradient 10 → 90% B in 15 min), *m/z* 717.3 $[M+H]^+$, 1455.4 $[2M+Na]^+$. ^1H NMR (200 MHz, $CDCl_3$/MeOD): δ ppm 8.455 (s, 1H), 8.18 (d, *J* = 8.8 Hz, 2H), 8.03 (d, *J* = 7.7 Hz, 2H), 7.61-7.41 (m., 5H), 6.74 (d, *J* = 8.0 Hz, 1H), 4.65 (s, 2H), 4.61-4.48 (m, 1H), 4.29-4.01 (m, 4H), 3.29 (d, *J* = 4.9 Hz, 1H), 2.90 (d, *J* = 4.9 Hz, 1H), 1.80-1.24 (m, 8H), 1.50 (s, 3H), 1.00-0.64 (m, 22 H).

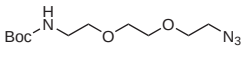


(*E*)-hepta-4,6-dienoyl-Ile-Ile-Thr(tBu)-leucinyl-(*R*)-2-methyloxirane (15c). Tetrapeptide epoxyketone **14** (0.13 g, 0.22 mmol) was treated with TFA/DCM (1/1, v/v) for 15 min, before being concentrated in the presence of toluene. The resulting TFA salt was dissolved in DCE/DMF and neutralized with DiPEA (0.14 ml, 0.80 mmol, 4 equiv.), followed by addition of a solution of OSu-ester **12** (0.13 g, 0.60 mmol, 3 equiv.) in DCE. The reaction mixture was stirred under argon atmosphere for 2 hr., before DCM was added and the mixture was washed with H_2O (1x). The aqueous layer was extracted with EtOAc (1x), the organic layers were combined and MeOH was added until a clear solution was obtained. The organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (DCM → 4% MeOH in DCM) afforded title compound **15c** (78 mg, 0.13 mmol, 64%). LC/MS analysis: R_t 8.94 min (linear gradient 10 → 90% B in 15 min), *m/z* 607.3 $[M+H]^+$, 1213.3 $[2M+H]^+$, 1819.1 $[3M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ ppm 8.27 (d, *J* = 8.1 Hz, 1H), 7.85 (d, *J* = 7.9 Hz, 1H), 7.63 (d, *J* = 6.9 Hz, 1H), 7.01 (d, *J* = 8.9 Hz, 1H), 6.37-6.00 (m, 2H), 5.66 (td, J_1 = 14.5, J_2 = 6.2 Hz, 1H), 5.03 (ddd, J_1 = 13.3, J_2 = 11.1, J_3 = 1.6 Hz, 2H), 4.79-4.49 (m, 3H), 4.28-4.08 (m, 2H), 3.30 (d, *J* = 4.9 Hz, 1H), 2.90 (d, *J* = 4.9 Hz, 1H), 2.86-2.82 (m, 1H), 2.52-2.28 (m, 4H), 1.85-1.56 (m, 6H), 1.53 (s, 3H), 1.48-1.33 (m, 2H), 1.11 (d, *J* = 6.5 Hz, 3H), 0.96-0.76 (m, 18H).

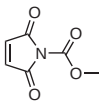


2-(hexa-2,4-dienyloxy)acetamido-Ile-Ile-Thr-leucinyl-2-methyloxirane (15d). The fully protected epoxketone **14** (0.16 g, 0.25 mmol) was deprotected in TFA/DCM (1/1 v/v) for 15 min, before being concentrated in the presence of toluene. The crude TFA salt was dissolved in DCE/DMF and neutralized with DiPEA (0.18 ml, 1.0 mmol, 4 equiv.), before a solution of OSu ester **13** (0.21 g, 0.81 mmol, 3.2 equiv.) in DCE/DMF was added. The reaction mixture was stirred under argon atmosphere for 2.5 hr., before DCM was added. The mixture was washed with H_2O and the aqueous

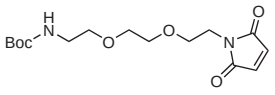
layer was extracted with EtOAc. MeOH was added to the combined organics until a clear solution was obtained. The solution was dried over $MgSO_4$, filtered and evaporated. Purification by column chromatography (10% acetone in hexane → 30% acetone in hexane) yielded the title compound **15d** (0.16 g, 0.25 mmol, quant.) as a white solid. ^{1}H NMR (400 MHz, $CDCL_3$): δ ppm 8.43 (d, *J* = 9.0 Hz, 1H), 7.94 (d, *J* = 7.4 Hz, 1H), 7.45 (d, *J* = 9.1 Hz, 1H), 7.30 (s, 1H), 6.19 (dd, J_1 = 15.2, J_2 = 10.4 Hz, 1H), 6.05 (dd, J_1 = 14.0, J_2 = 11.4 Hz, 1H), 5.79-5.66 (m, 1H), 5.63-5.54 (m, 1H), 4.86-4.69 (m, 1H), 4.55 (td, J_1 = 9.8, J_2 = 5.7 Hz, 3H), 4.18-3.99 (m, 1H), 3.97 (s, 2H), 3.89 (d, *J* = 15.4 Hz, 2H), 3.35 (d, *J* = 4.9 Hz, 1H), 2.89 (d, *J* = 4.9 Hz, 1H), 1.76 (d, *J* = 6.7 Hz, 2H), 1.71-1.58 (m, 2H), 1.52 (s, 3H), 1.43-1.17 (m, 2H), 1.11 (d, *J* = 6.3 Hz, 3H), 1.08-0.94 (m, 2H), 0.90 (d, *J* = 6.5 Hz, 6H), 0.87-0.75 (m, 12H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 208.20, 171.81, 171.21, 170.53, 169.80, 134.45, 130.80, 130.40, 125.02, 71.78, 68.65, 67.12, 59.17, 57.52, 56.86, 56.69, 52.35, 50.73, 39.13, 38.01, 37.92, 25.13, 25.05, 24.91, 23.21, 21.09, 17.98, 17.26, 16.74, 15.22, 15.20, 11.33, 11.25.



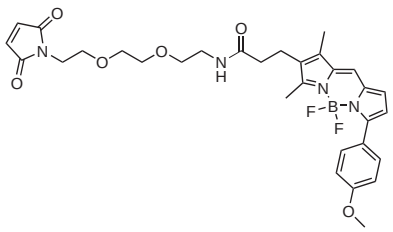
***t*Butyl 2-(2-(2-azidoethoxy)ethoxy)ethylcarbamate (17).** To a solution of 2-(2-(2-azidoethoxy)ethoxy)ethanamine·HCl (**16**) (1.5 g, 5.4 mol) in DCM was added triethylamine (1.5 ml, 11 mmol, 2 equiv.) and Boc_2O (1.3 g, 5.9 mmol, 1.1 equiv.). The reaction mixture was stirred for 50 min., before being concentrated *in vacuo*. Purification of the crude product by column chromatography (DCM → 3% MeOH in DCM) gave the title compound as a colorless oil (1.3 g, 4.9 mmol, 90%). LC/MS analysis: R_t 7.00 min (linear gradient 10 → 90% B in 15 min), *m/z* 274.8 $[M+H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 5.07 (s, 1H), 3.73-3.59 (m, 6H), 3.58-3.50 (m, 2H), 3.45-3.24 (m, 6H), 1.45 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 154.91, 77.46, 69.36, 69.12, 68.96, 49.50, 39.28, 27.27.



Nefkens reagent analog (20). A solution of maleimide (**19**) (0.99 g, 10 mmol) and *N*-methyl morpholine (1.1 ml, 10 mmol, 1 equiv.) in EtOAc was cooled to 0 °C. Methylchloroformate (0.80 ml, 10 mmol, 1 equiv.) was added and the reaction mixture was stirred 2 hr. The reaction mixture was washed sat. aq. $NaHCO_3$ (3x) and the aqueous layer was extracted with EtOAc (1x). The combined organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*, to yield the crude product **20** (0.80 g, 5.2 mmol, 52%) which was used without any further purification. LC/MS analysis: R_t 1.29 min (linear gradient 10 → 90% B in 15 min), *m/z* 156.0 $[M+H]^+$. ^{1}H NMR (200 MHz, acetone-d_6): δ ppm 7.05 (s, 2H), 3.89 (s, 3H). ^{13}C NMR (50 MHz, acetone-d_6): δ ppm 166.93, 148.80, 136.27, 54.12.



***t*Butyl 2-(2-(2-(2,5-dioxo-pyrrol-1-yl)ethoxy)ethoxy)ethylcarbamate (21).** Boc-protected azide **17** (1.0 g, 3.6 mmol) was dissolved in THF, before triphenylphosphine (1.2 g, 4.4 mmol, 1.2 equiv.) was added. The reaction mixture was stirred for 5 hr., after which a few drops of H_2O were added. After stirring for an additional 2 hr., toluene was added and the mixture was extracted with 1M HCl (4x). The combined aqueous layers were alkalized using sat. aq. $NaHCO_3$ and 3M KOH, and extracted with DCM (3x) and EtOAc (3x). The combined organics were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give amine **18** (0.65 g, 2.6 mmol, 72%). LC/MS analysis: R_t 3.86 min (linear gradient 10 → 90% B in 15 min), *m/z* 249.0 $[M+H]^+$, 496.9 $[2M+H]^+$. The crude compound **18** was dissolved in sat. aq. $NaHCO_3$ solution, cooled to 0 °C, before Nefkens reagent analog **20** (0.52 g, ~3.4 mmol, 1.3 equiv.) was added. The reaction mixture was stirred at 0 °C for 30 min., before being allowed to warm up to rt for 45 min. The mixture was extracted with chloroform (3x) and the combined organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 70% EtOAc in PetEt) yielded maleimide **21** (0.36 g, 1.1 mmol, 31% over two steps). LC/MS analysis: R_t 6.37 min (linear gradient 10 → 90% B in 15 min.), *m/z* 328.9 $[M+H]^+$, 351.1 $[M+Na]^+$. ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 6.72 (s, 2H), 5.04 (s, 1H), 3.85-3.41 (m, 10H), 3.39-3.20 (m, 2H), 1.45 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 170.37, 155.66, 133.86, 78.70, 69.81, 69.51, 67.42, 40.00, 36.67, 28.06.



(*N*)-BODIPY TMR-2-(2-(2-(2,5-dioxo-pyrrol-1-yl)ethoxy)ethoxy)ethyl-amine (22). Compound **21** (65 mg, 0.20 mmol) was treated with TFA/DCM (1/1, v/v) for 10 min, before being concentrated in the presence of toluene. The resulting TFA salt was dissolved in DCE and neutralized with DiPEA (0.20 ml, 1.2 mmol, 6 equiv.), followed by addition of BODIPY TMR-OSu (99 mg, 0.20 mmol, 1 equiv.). The reaction mixture was stirred under argon atmosphere overnight. DCE was added and the mixture was washed with H_2O (1x). The aqueous layer was extracted with EtOAc (2x), the organic layers were combined and MeOH was added until a clear solution was obtained. The organics were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (toluene → 30% acetone in toluene) afforded title compound **22** as a purple solid (41 mg, 68 µmol, 34%). LC/MS analysis: R_t 8.55 min (linear gradient 10 → 90% B in 15 min.), *m/z* 589.4 $[M-F]^+$, 1216.8 $[2M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$): δ ppm 7.89-7.83 (m, 2H), 7.07 (s, 1H), 6.99-6.95 (m, 2H), 6.94 (d, *J* = 4.1 Hz, 1H), 6.61 (s, 2H), 6.53 (d, *J* = 4.0 Hz, 1H), 6.35 (t, *J* = 4.9 Hz, 1H), 3.85 (s, 3H), 3.68 (t, *J* = 5.5 Hz, 2H), 3.58 (t, *J* = 5.4 Hz, 2H), 3.52-3.41 (m, 6H), 3.41-3.35 (m, 2H), 2.77 (t, *J* = 7.4 Hz, 2H), 2.53 (s, 3H), 2.36 (t, *J* = 7.5 Hz, 2H), 2.21 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 171.82, 170.72, 160.28, 159.69, 139.88, 134.80, 134.07, 130.77, 130.62, 127.67, 125.45, 122.63, 118.16, 113.66, 70.07, 69.76, 69.74, 67.74, 55.21, 39.17, 37.09, 36.16, 20.05, 13.12, 9.54.

***In vitro* competition assay in EL-4 cell lysates**

EL-4 cell lysates (10 µg total protein) were incubated for 1 hr. at 37 °C with the indicated concentrations of the inhibitor (10x solution in DMSO) in a total reaction volume of 10 µL (H_2O/DMSO 9/1, v/v), prior to incubation with MV151 (1 µM) for 1 hr. at 37 °C in a total reaction volume of 11 µL (H_2O/DMSO 9/2, v/v). The reaction mixtures were boiled for 3 minutes with 4 µL 4x Laemmli's sample buffer containing β-mercapto-ethanol and resolved on 12.5% SDS-PAGE. In-gel visualization of the fluorescent labeling was performed in the wet gel slabs directly on a Typhoon Variable Mode Imager (Amersham Biosciences) using Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560).

Diels-Alder ligation on denatured purified 20S proteasome

Purified 20S proteasome (100 ng per reaction) was exposed to 5 µM **15d** or 1 µM MV151 for 1 hr. at 37 °C in a total reaction volume of 10 µl (H_2O/DMSO 9/1, v/v). The samples were then denaturated with 8 M urea for 15 min. at rt followed by addition of DTT (5 mM final concentration) for 30 min. at 55 °C, and capping with 30 mM DTNB for 3.5 hr. at rt. After addition of RAW cell lysates (denatured and capped with 30 mM DTNB according to the same method as described above), the proteins were crashed out by chloroform/methanol precipitation. The proteins were taken up in Diels-Alder buffer (pH 6.0) containing 6M urea, followed by addition of 2 volumes of Diels-Alder buffer (5 mM NaH_2PO_4, 20 mM NaCl, 0.2 mM $MgCl_2$, pH 6.0) containing 10 mM $Cu(NO_3)_2$. The denatured proteins were incubated with 25 µM **22** for 16 hr. at 37 °C in a total reaction volume of 16.5 µL (H_2O/DMSO 10/1, v/v). The reaction was terminated by chloroform/methanol precipitation. The proteins were taken up in Laemmli's sample buffer containing β-mercapto-ethanol, heated at 55 °C for 15 min. and resolved on 12.5% SDS-PAGE, before in-gel fluorescence readout of the labeled proteins using the Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560) on the Typhoon Variable Mode Imager (Amersham Biosciences).

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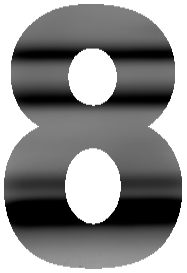


Summary and Future Prospects

Although subject of extensive studies in the last decades, a lot is still to be discovered in the field of proteasome research. The interdisciplinary efforts on the interface of chemistry and biology have boosted the understanding of the role of proteasomes in a variety of processes. A brief overview of the recent advances in the field of chemical biology-driven proteasome research is given in **Chapter 1**. The research described in this Thesis entailed the development of new chemical biology tools to study proteasome activity.

The synthesis and characterization of the fluorescent, cell-permeable, and activity-based proteasome probe BODIPY TMR-Ahx$_3$L$_3$VS (MV151) is described in **Chapter 2**. This probe enables fast and sensitive direct in-gel fluorescence readout of proteasome activity in a given sample. The probe has been used to label the active proteasome population in cell lysates and living cells. MV151 has some advantages over previously developed probes in that it omits the need for Western blotting, radioactivity, and gel drying. MV151 distribution was readily detected upon administration to Ub^{G76V}-GFP transgenic mice and correlated with inhibition of the proteasome in the affected tissues, as judged by the accumulation of the GFP-reporter. MV151-mediated proteasome labeling in combination with Ub^{G76V}-GFP transgenic mice is a useful strategy for monitoring the biodistribution of proteasome inhibitors. Finally, a competition experiment employing MV151 proved to be a fast and sensitive means of determining the inhibitory profile (in terms of potency and subunit preference) of a given proteasome inhibitor.

Recently, it was found that the level of circulating 20S proteasomes in the plasma of multiple myeloma patients is of clinical significance as a parameter reflecting disease

activity.[1] Moreover, the proteasome levels were shown to correlate with the response to chemotherapy. In patients (partially) responding to chemotherapy the circulating proteasome levels decreased significantly, whereas in non-responders no decrease was observed. Circulating proteasome levels were measured in serum samples by enzyme-linked immunoabsorbent assay (ELISA) techniques, after enrichment for 20S proteasomes. Since not only the abundance, but also proteasome activity is elevated in serum of various cancer patients,[2] the use of MV151 or analogues in a clinical setting would be a more straightforward and less expensive alternative for the quantification of circulating proteasome activity in plasma samples. Furthermore, differences in expression levels of proteolytically active proteasome subunits were shown to influence the sensitivity towards the proteasome inhibitor anti-cancer drug bortezomib.[3] In less sensitive cancer cells, the immunoproteasome levels as well as the constitutive β2 subunit levels are below the expression levels of that observed in sensitive cancer cells. Assessment of the proteasome labeling profile with MV151 could predict the chance of success of a proteasome inhibitor based antineoplastic therapy.

The development of three easily accessible alkyne functionalized BODIPY dyes **1a-c** is discussed in **Chapter 3** (Figure 1). These dyes can be conjugated to any azide containing activity-based profiling probe and azido modified metabolite chemoselectively, potentially leading to valuable fluorescent biochemical tools. The applicability of the alkyne equipped BODIPYs was demonstrated in the synthesis of three epoxomicin derived fluorescent proteasome probes **2a-c**.

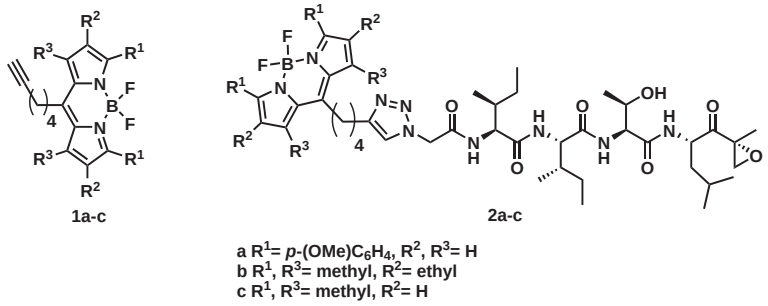


Figure 1. BODIPY dyes **1a-c** and epoxomicin derived fluorescent proteasome probes **2a-c**.

In **Chapter 4,** the synthesis of a bifunctional azido-BODIPY acid is described. In an activity-based protein profiling setting, this novel fluorophore provides flexibility compared to conventional monofunctionalized fluorescent dyes. Like the latter, azido-BODIPY tagged activity-based probes allow for rapid fluorescent readout of the protein labeling profile and is compatible with live cell imaging techniques. The azido moiety on the other hand, facilitates the ligation of an affinity tag, which enables the purification and identification of the fluorescently labeled proteins. With the aid of two sets of one- and two-step labeling proteasome probes based on azido-BODIPY (one set is depicted in Figure

2A), it was established that the Staudinger-Bertozzi ligation proceeds in a near quantitative yield under the conditions applied. This result implies that the efficiency of protein labeling thus depends on the reactivity of the activity-based probe towards the target protein (family), and not on the chemoselective ligation employed in the second step. This essentially means that two-step activity-based protein profiling may proceed with equal efficiency with respect to protein tagging as contemporary one-step approaches. The azido-BODIPY acid has proven to be valuable in the optimization of the Staudinger-Bertozzi ligation, since ligated proteins have increased masses and the corresponding fluorescent bands run higher on SDS-PAGE compared to unreacted labeled proteins. The efficiency of the Huisgen cycloaddition based ligation strategies can in theory

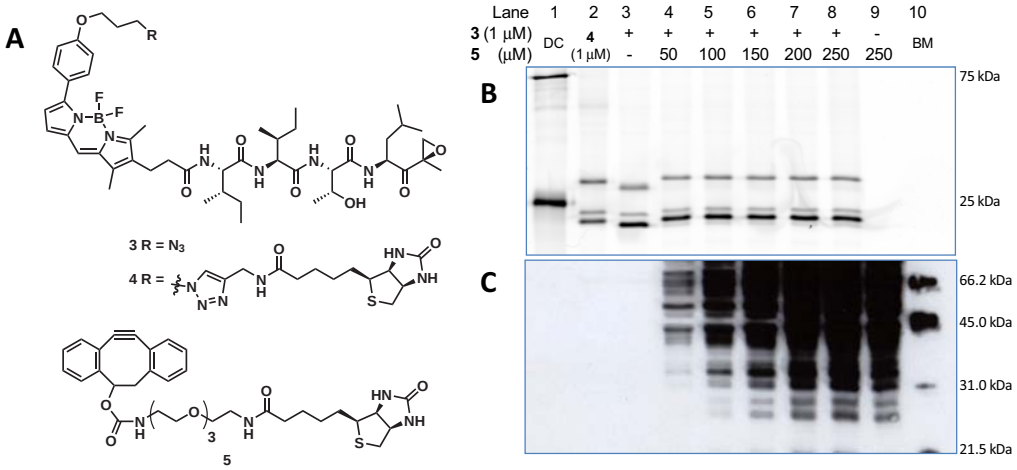


Figure 2. Azido-BODIPY based probes and their application in ligation optimization.
(**A**) The set of epoxomicin derived one-step and two-step labeling probes (**4** and **3**, respectively), and cyclooctyn **5**. (**B** and **C**) Living HEK 293 cells (some $15{\cdot}10^6$ cells) were exposed to 1 µM **3** or **4** for 2 hr. at 37 ºC, before being harvested, washed and lysed. The cytosolic fractions (25 µg total protein) of the **3** treated cells were incubated with the indicated concentrations of cyclooctyn **5** for 1 hr. at 37 ºC. (**B**) Fluorescent readout. (**C**) Streptavidin blot of the same gel. DC = dual color molecular marker, BM = biotinylated molecular marker.

be evaluated in the same way. In initial experiments, the biotin tagged, strain-promoted copper free click reagent **5** developed by Boons and co-workers[4] proved to react very efficiently with azido-BODIPY labeled proteasome subunits. Already at a concentration as low as 50 µM, all fluorescently labeled proteins had reacted with **5**, as judged from the complete shift of the corresponding fluorescent bands on SDS-PAGE (Figure 2B). For example, a typical Staudinger-Bertozzi ligation requires 100-250 µM reagent. The streptavidin blots however, showed a dramatic amount of aspecific labeling, overshadowing proteasome signals (Figure 2C). Further research is needed to determine whether this is due to cross-reactivity of **5** or that optimization will result in the application of cyclooctyn **5** in activity-based protein profiling.

Furthermore, the azido-BODIPY acid can find application in the screening of newly synthesized activity-based probes (Figure 3). Introduction of the bifunctional fluorophore enables rapid determination of the protein labeling potential of a novel probe. If specific protein bands do show up when performing in-gel fluorescence readout, a two-step labeling approach can be applied to purify and identify the labeled proteins. Such comprehensive screening assay will boost the discovery of probes targeting unstudied protein families.

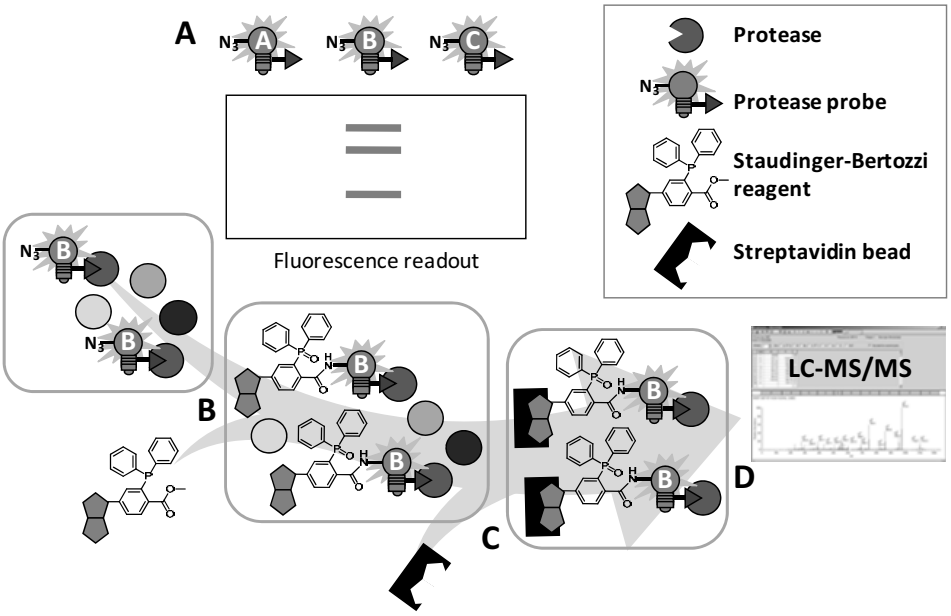
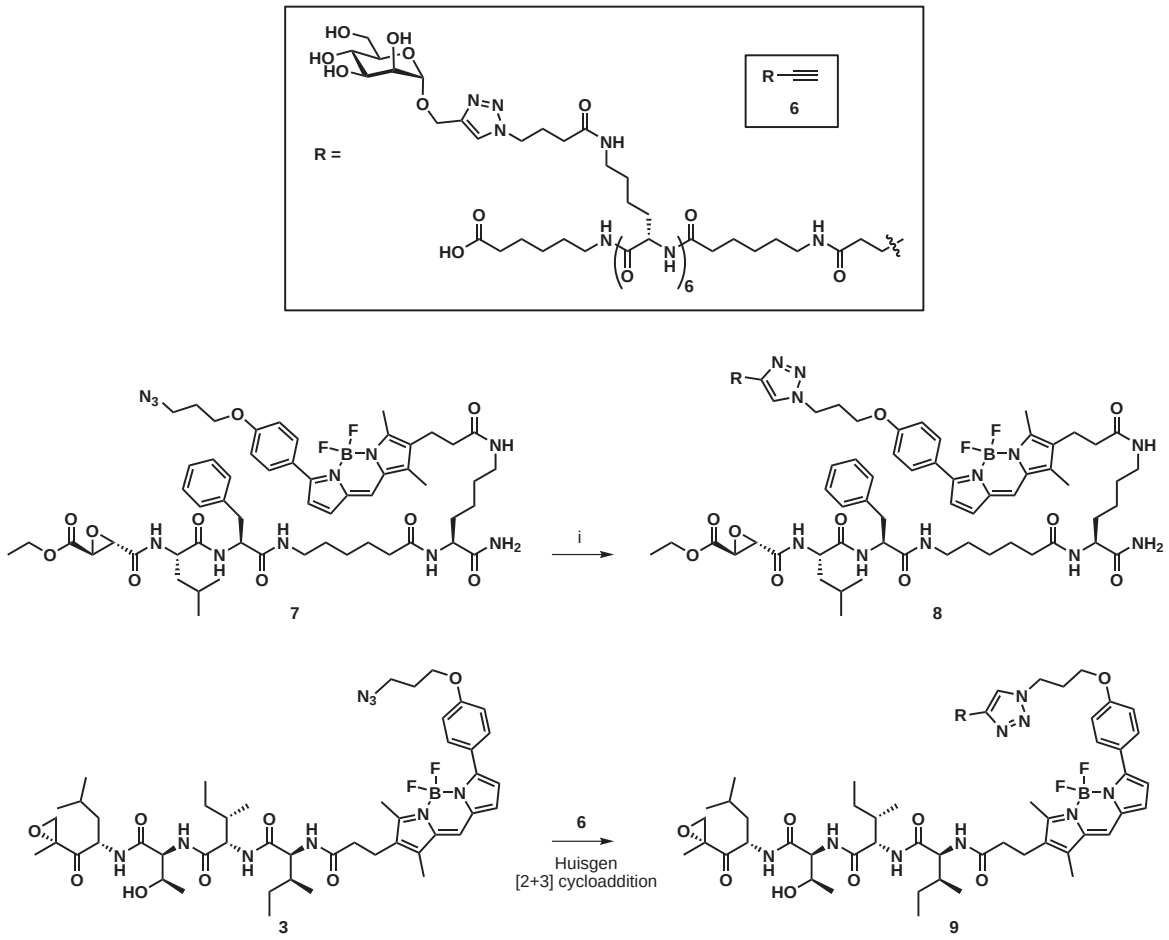


Figure 3. Screening of azido-BODIPY tagged activity-based probes.
(**A**) A proteome is exposed to probes A, B and C. SDS-PAGE, followed by direct in-gel fluorescence readout reveals specific labeling by probe B and no labeling by probes A and C. (**B**) The probe B labeled proteome is reacted with a biotinylated Staudinger-Bertozzi reagent and (**C**) the biotin tagged proteins are purified with streptavidin beads. (**D**) Identification of the proteins by trypsin digestion and LC-MS/MS analysis of the tryptic protein fragments.

Another promising application can be found in the use of azido-BODIPY acid as a fluorescent linker moiety. The bifunctional fluorophore allows for the elegant conjugation of for example peptide epitopes to TLR-ligands to generate fluorescently trackable adjuvant linked vaccines.[5] Furthermore, protease probes can be equipped with a homing element to direct the probes to specific organs or specific pathways. An example of the latter application is the fluorescent cathepsin probe conjugated to a synthetic mannose cluster (**8**, Scheme 1). The mannose cluster serves as a ligand for the mannose-receptor, targeting probe **8** for endosomal uptake. The fluorescent two-step activity-based probe azido-BODIPY-DCG04 (**7**) was conjugated to the acetylene functionalized, synthetic mannose cluster **6** to result in the fluorescent conjugate **8**. The intracellular uptake and

Scheme 1. Synthesis of mannose receptor ligand equipped fluorescent probes.



Reagents and conditions: i) **6** (0.98 equiv.), $CuSO_4$ (10 mol%), sodium ascorbate (15 mol%), *t*BuOH/H_2O (1/1.7), 48 hr., RT, then DMF (1.7), 80°C, 2hr. (14%).

distribution of probes **7** and **8** by immature murine bone-marrow derived dendritic cells (BM-DCs) was investigated by live-cell fluorescence microscopy (Figure 4A) and in-gel fluorescence readout of the labeled proteins (Figure 4B). Whereas probe **7** shows up in hydrophobic membranous compartments in the cells (e.g. endoplasmatic reticulum) almost instantly after administration, the mannose cluster decorated probe **8** enters the cells via discrete vesicles located near the cell membrane (Figure 4A). The fact that protein labeling in living BM-DCs by compound **8** is temperature dependent points towards active uptake (Figure 4B and C). Furthermore, the labeling of **8** can be inhibited by competition with the established mannose receptor ligand, mannan (poly-β-1-6-mannose, Figure 4B and C, lane 4) and the synthetic mannose cluster **6** (Figure 4B and C, lane 5), strongly pointing towards mannose receptor mediated endocytosis. Similar results were obtained by fluorescence-activated cell sorting (FACS) analysis (data not shown) of **7** and **8** exposed bone-marrow derived dendritic cells and macrophages.[6] In a similar fashion, a fluorescent epoxomicin analogue conjugated to a synthetic mannose cluster (**9**) could be synthesized (Scheme 1).

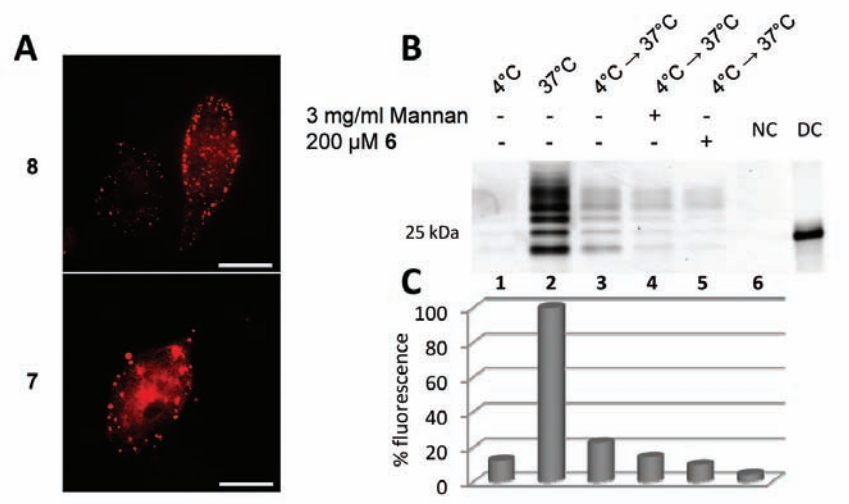


Figure 4. Cellular uptake of **7** and **8** by bone-marrow derived dendritic cells (BM-DCs).
(**A**) BODIPY fluorescence in BM-DCs treated with 10µM **8** (upper panel) or **7** (bottom panel) for 1 hr., washed and imaged by fluorescence microscopy. Scale bar represents 10µm. (**B**) SDS-PAGE, followed by in-gel fluorescence readout of BM-DC lysates (10µg protein/lane). Lane 1: Cells pulsed for 1 hr. with 10 µM **8** at 4 °C, washed and chased at 4 °C. Lane 2: Cells pulsed for 1 hr. with 10 µM **8** at 37 °C, washed and chased at 37 °C. Lane 3: Cells pulsed for 1 hr. with 10 µM **8** at 4 °C, washed and chased at 37 °C. Lane 4 and 5: Cells exposed to 3 mg/ml mannan or 200 µM **6** respectively for 30 min., before being pulsed for 1 hr. with 10 µM **8** at 4 °C, washed and chased at 37 °C. NC = no-probe control, DC = dual color molecular marker. (**C**) Quantification of fluorescence intensity of protein labeling in Figure 4B.

Often viewed as two independent proteolytic pathways, the lysosomal system, responsible for the degradation of endocytosed material, and the ubiquitin proteasome system do interplay. For example, it was found that in rat liver, lysosomes play a major role in the turnover of proteasomes.[7] Another example of interaction between the two pathways is the immunologically relevant process called cross-presentation. In cross-presentation, epitopes derived from antigens processed by the endocytic pathway end up being presented to the immune surveillance system by MHC class I molecules on the cell surface of certain antigen presenting cells. One of the mysteries in immunology is how these extracellular antigenic epitopes gain access to the MHC class I pathway,[8] which according to the dogma is restricted to the presentation of intracellular epitopes generated by the proteasome. Interestingly, the proteasome is essential for cross-presentation.[5,9] Probes specifically targeted for endocytosis, like the ones discussed in the above, might give insight in the involvement of protease activities in these processes.

The structure of peptide based proteasome inhibitors can be divided into three elements, being 1) the warhead, the electrophilic trap that reacts with the active site nucleophilic residue, 2) the peptidic recognition element that serves as the homing sequence and 3) the *N*-terminal extension. In **Chapter 5** these structural elements of five commonly used proteasome inhibitors were interchanged to result in a library of 15 hybrid proteasome inhibitors. Employing MV151 the inhibitory profile of the library was

determined. Scrambling of structural elements of known proteasome inhibitors proved to be a viable strategy to arrive at potent new proteasome inhibitors, since the epoxomicin derived boronic ester **10** (Figure 5) was characterized as being one of the most potent peptide-based proteasome inhibitor reported to date.

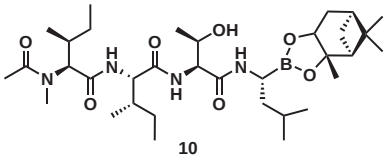


Figure 5. Epoxomicin derived boronic ester **10**.

A similar strategy was employed in **Chapter 6**, where warheads and peptidic recognition elements of previously reported subunit specific proteasome inhibitors were scrambled. Surprisingly, all vinyl ethyl ester based compounds, including the β2 specific inhibitor reported by Marastoni *et al.*[10] proved to be inactive in competition experiments versus MV151. A more potent epoxyketone based β1 specific proteasome inhibitor **11** was developed, next to the β5 specific vinyl sulfone **12** and a β2 and β5 targeting epoxyketone **13** (Figure 6A). These hits were transformed into subunit specific two-step labeling probes by the introduction of an azide moiety to result in compounds **14-16**. Substitution of the naphthylacetamide in **12** and **13** for an azido-BODIPY moiety resulted in the fluorescent two-step labeling proteasome probes **17** and **18**. Furthermore, conjugation of acetylene

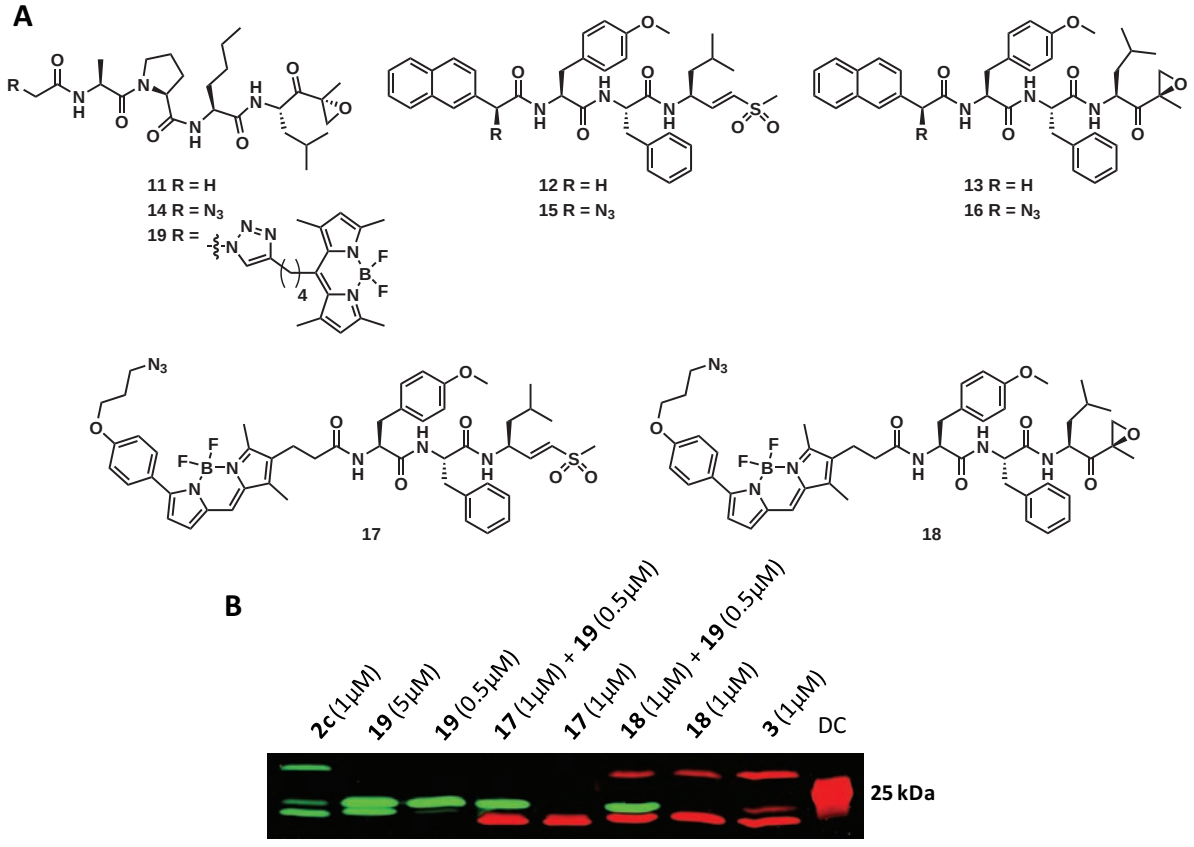


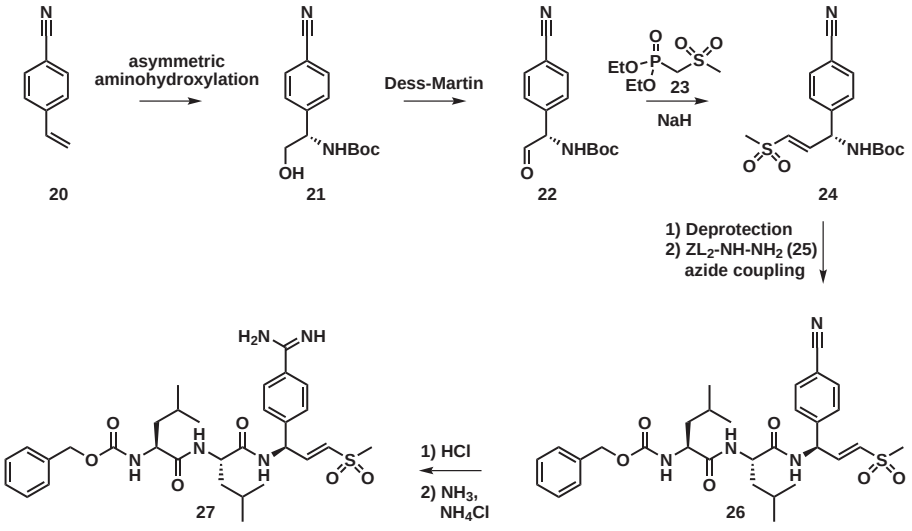
Figure 6. Subunit specific proteasome inhibitors and probes.
(**A**) Subunits specific inhibitors and probes described in Chapter 6. (**B**) Specific labeling of proteasome subunits with probes **2c, 3, 17-19** and combinations thereof. Merge of red and green fluorescence.

functionalized BODIPY dye **1c** with epoxyketone **11** resulted in the green fluorescent probe **19**, which has a predilection for β1, but at higher concentrations starts to label β5 as well.

The inhibition profile of the synthesized library and the labeling profile of the probes designed in this Chapter remains to be determined for the immunoproteasome. Applying a two-step labeling strategy, the subunits targeted by **14-18** could be identified by LC-MS/MS analysis. Preliminary results obtained from labeling experiments of murine thymus homogenates using the toolkit of fluorescently labeled proteasome inhibitors described in this Thesis did not give evidence for labeling of the thymus specific β5t.[11] The substitution of β5 for β5t was shown to result in a dramatic decrease of the chymotrypsin-like activity. This could imply two things. Either the β5t introduces an additional, fourth proteasomal activity or the β5t is proteolytically inactive. If the former is the case, inhibitors possessing hydrophilic P1 residues might target the β5t, since the S1 pocket of the β5t subunit differs from the S1 pockets of β5 and β5i in being more hydrophilic.[11]

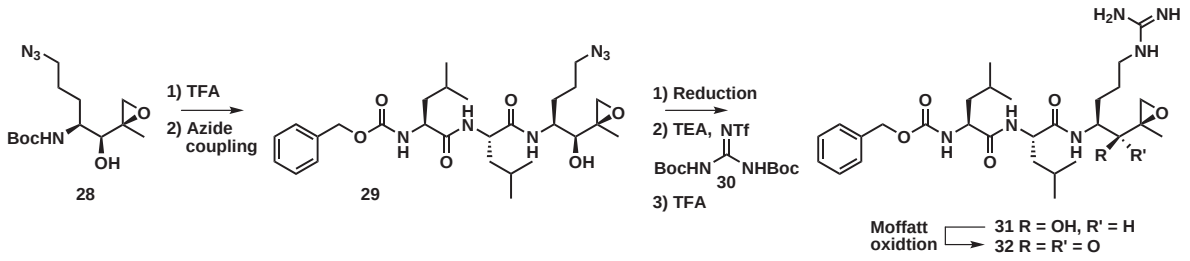
Figure 6B shows the fluorescent labeling of specific sets of proteolitically active proteasome subunits with a variety of probes developed in the work described in this Thesis. With the anti-cancer drug proteasome inhibitor bortezomib in mind, the here presented toolbox is useful in addressing the question which proteasome subunit or what combination of subunits should be targeted to give the optimal anti-cancer therapeutic. Of interest in the field of immune-therapy is the influence of the inhibition of a defined set of the proteasome subunits on the epitope repertoire.

Scheme 2. Synthesis of an arginine-like warhead containing inhibitor **27**.



An obvious addition to the panel of subunit specific proteasome inhibitors is a β2 specific inhibitor. Being responsible for the tryptic-like activity of the proteasome, the synthesis of an arginine-like warhead is likely to gain β2 specific inhibitors. The proposed

Scheme 3. Synthesis of an arginine-like warhead containing inhibitor **32**.



synthesis of two such inhibitors is depicted in Schemes 2 and 3. The phenylamidine based inhibitor **27** may be synthesized as follows. The Boc protected amino alcohol **21** can be obtained in one step from cyanostyrene **20** via Sharpless asymmetric aminohydroxylation.[12] Oxidation to aldehyde **22** and subsequent Horner-Wadsworth-Emmons reaction will result in the warhead **24**. Deprotection, followed by azide couplingwith dipeptidyl hydrazide **25** will give **26**. The synthesis is finalized by the introduction of the amidine to give the potential β2 specific proteasome inhibitor **27**. The arginine derived epoxyketone based inhibitor **32** may be synthesized as depicted in Scheme 3. Epoxy alcohol **28** can be synthesized from Boc protected δ-azido ornithine following the procedure described in Chapter 4. To minimize the chance of unwanted cyclization, the ketone functionality should be introduced in the final stage of the synthesis.

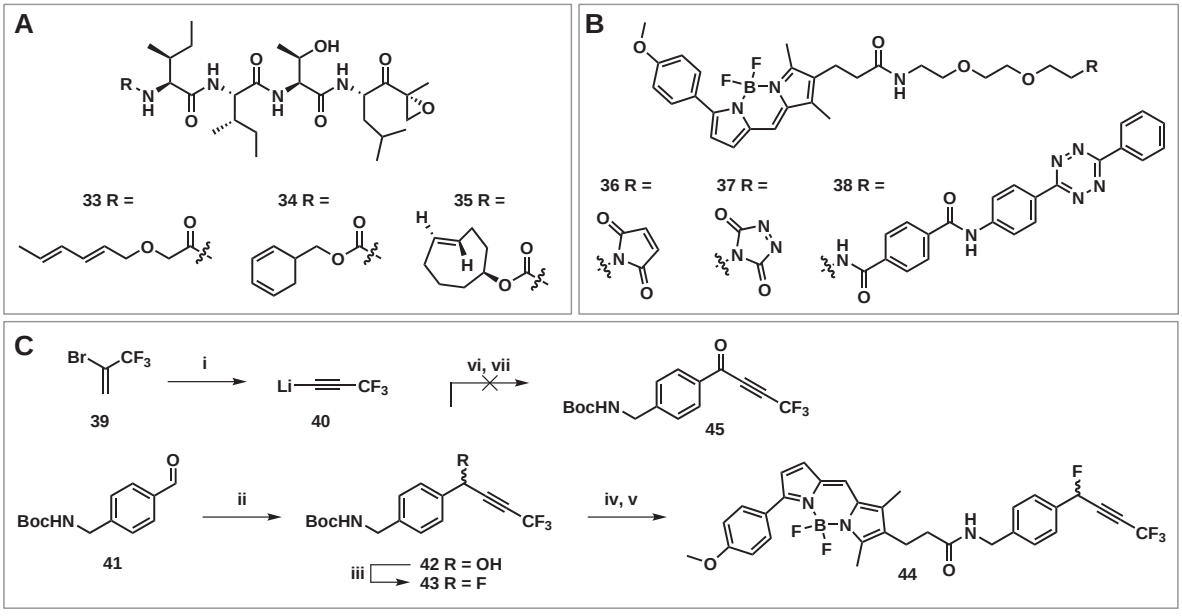


Figure 7. Diels-Alder two-step labeling toolbox.

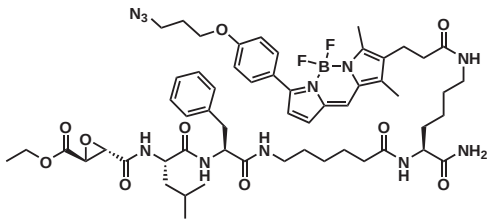
(**A**) Diels-Alder tagged epoxomicin analogues. (**B**) Fluorescent Diels-Alder ligation reagents. (**C**) Synthesis of tetrafluoroacetylene **44**. **Reagents and conditions**: i) LDA (2 equiv.), THF, -78 °C, 30 min. ii) **40** (2.2 equiv.), THF, -78 °C, 1 hr., 64% (2 steps). iii) DAST (1.25 equiv.), DCM, 16 hr., 67%. iv) TFA/DCM (1/1, v,v), 30 min. v) BODIPY TMR-OSu (1.1 equiv.), DiPEA (1.1 equiv.), DMF, 3 hr., 25% (2 steps). vi) MnO_2 (10 equiv.), DCM. vii) Dess-Martin periodinane (1.1 equiv.), DCM, 0 °C.

Chapter 7 deals with the synthesis of a panel of four diene equipped epoxomicin analogues. They were shown to inhibit the proteasome with potencies in the same order of magnitude as the parent compound epoxomicin. Initial Diels-Alder two-step proteasome labeling experiments performed on purified 20S proteasomes utilizing diene equipped proteasome inhibitor **33** and the Diels-Alder ligation reagent **36** showed the potential of the Diels-Alder as an alternative ligation method in activity-based protein profiling, although considerable optimizations are needed. The major problem in initial Diels-Alder two-step labeling experiments proved to be the large degree of aspecific "background" labeling. Several different conditions for denaturation and capping of nucleophiles need to beinvestigated, as well as the reaction temperature and different concentrations of $Cu(NO_3)_2$ and other Lewis acid Diels-Alder catalysts. Another way to decreased the amount of aspecific labeling is to shorten reaction times by increasing the reactivity of the reactants. A cyclohexadiene functionalized inhibitor, like for example epoxomicin analogue **34**, is expected to be more reactive than the inhibitors presented in Chapter 7. In a search for a non-Michael acceptor, electron poor dienophile, compound **44** was synthesized (Figure 7C). The crude trifluoroacetylide **40,** obtained by treatment of **39** with LDA,[13] was reacted with benzaldehyde **41** to give acetylene **42**. After introduction of the fluorine with DAST, the amine was deprotected and condensed with BODIPY TMR-OSu to give the fluorescently tagged, electron poor acetylene **44**. Attempts to oxidize **42** to the corresponding ketone (**45**), in order to get to the pentafluoroacetylene derivative, failed. Initial Diels-Alder ligations on 20S proteasomes labeled with **33** using **44** however proved unsuccessful and further research is needed to determine whether **44** is a useful tool in two-step Diels-Alder ligation. The dienophile triazolinedione **37** could be an attractive alternative for the Michael acceptor **36**. Application of **37** does not necessitate the prior capping of nucleophilic residues, before Diels-Alder ligation. Blackman *et al.* recently published a new method for bioconjugation based on the inverse-electron demand Diels-Alder reaction.[14] Translation of their findings to proteasome labeling will result in the *trans*-cyclooctene equipped epoxomicin analogue **35** and the inverse-electron demand Diels-Alder ligation reagent **38**.

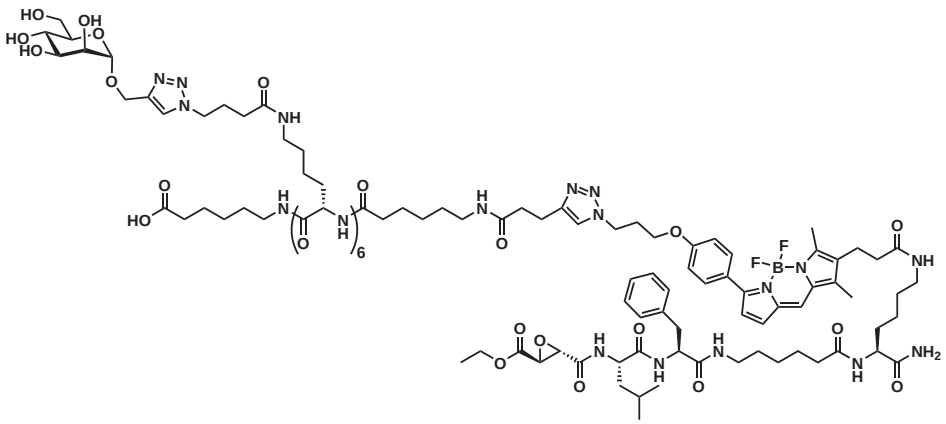
Experimental section

General: All reagents were commercial grade and were used as such. Toluene (Tol.) (purum), ethyl acetate (EtOAc) (puriss.) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën and distilled prior to use. Dichloroethane (DCE), dichloromethane (DCM) and dimethyl formamide (DMF) were stored on 4Å molecular sieves. Tetrahydrofuran (THF) (Biosolve) was distilled from $LiAlH_4$ prior to use. Reactions were monitored by TLC-analysis using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by UV-absorption (254 nm), C, 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 followed by charring at ~150°C. Column chromatography was performed on Merck silicagel (0.040-0.063 nm). LC/MS analysis was performed on a LCQ Adventage Max (Thermo Finnigan)

equipped with a Gemini C18 column (Phenomenex). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. HRMS (SIM mode) were recorded on a TSQ Quantum (Thermo Finnigan) fitted with an accurate mass option, interpolating between PEG-calibration peaks or a Waters LCT Premier XE TOF coupled to a Waters Alliance HPLC system or on a LTQ Orbitrap (Thermo Finnigan). 1H- and 13C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50), a Varian Mercury 300 (300/75) or a Bruker AV-400 (400/100) equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz.



Azido-BODIPY-DCG04 (7). To a solution of Azido-BODIPY-OSu[15] (59 mg; 0.105 mmol) and DiPEA (20 µl, 0.115 mmol, 1.1 equiv.) in DMF (1 ml), a solution of DCG-04 amine[16] (78 mg, 0.115 mmol, 1.1 equiv.) in DMF (1 ml) was added and the reaction mixture was stirred for 16 hr., before being concentrated *in vacuo*. Purification by flash column chromatography (5% MeOH in DCM → 10% MeOH in DCM) afforded the title compound **7** (110 mg, 98 µmol, 93%) as a deep-red solid. ^{1}H NMR (400 MHz, $CDCl_3$/MeOD): δ ppm 7.87 (d, *J* = 8.9 Hz, 2H), 7.63 (s, 1H), 7.25-7.20 (m, 1H), 7.17-7.10 (m, 1H), 7.05-6.95 (m, 5H), 6.73 (d, *J* = 8.5 Hz, 2H), 6.57 (d, *J* = 4.1 Hz, 1H), 4.47 (t, *J* = 7.5 Hz, 1H), 4.41 (t, *J* = 7.3 Hz, 1H), 4.33-4.23 (m, 3H), 4.13 (t, *J* = 6.0 Hz, 2H), 3.67 (d, *J* = 1.8 Hz, 1H), 3.57 (d, *J* = 1.8 Hz, 1H), 3.55 (t, *J* = 6.6 Hz, 2H), 3.21-3.11 (m, 3H), 3.04 (dd, J_1 = 13.5, J_2 = 6.8 Hz, 1H), 2.97 (dd, J_1 = 13.8, J_2 = 7.6 Hz, 1H), 2.86 (dd, J_1 = 13.7, J_2 = 7.5 Hz, 1H), 2.75 (t, *J* = 7.5 Hz, 2H), 2.53 (s, 3H), 2.31 (t, *J* = 7.5 Hz, 2H), 2.25 (s, 3H), 2.19 (t, *J* = 7.5 Hz, 2H), 2.08 (p, *J* = 6.3 Hz, 2H), 1.82-1.71 (m, 1H), 1.66-1.50 (m, 6H), 1.49-1.34 (m, 6H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.22-1.11 (m, 2H), 0.91 (dd, J_1 = 13.7, J_2 = 6.0 Hz, 6H).



Mannose cluster-BODIPY-DCG-04 (8). Azido-BODIPY-DCG04 (**7**, 30 mg, 26.7 µmol, 1.02 equiv.) and **6**[6] (80 mg, 26.07 µmol) were dissolved in a mixture of *t*BuOH/H_2O (1/1, v/v, 2 ml), before sodium ascorbate (0.77 mg, 15 mol%) and $CuSO_4$ (0.65 mg, 10 mol%) in H_2O (0.7 ml) were added. The reaction mixture was stirred for 48 hr., after which TLC analysis revealed incomplete conversion. DMF (1.7 ml) was added and the resulting mixture was stirred for 2 hr. at 80°C, before being concentrated *in vacuo*. HPLC purification afforded **8** as a dark red solid (20 mg, 18%). LC/MS analysis indicated that the ester moiety in **8** had partially been hydrolyzed (~10%) to the corresponding carboxylic acid. ESI-MS calcd. for $[C_{187}H_{291}BF_2N_{43}O_{63}]^{3+}$: 1399.5, found: 1399.6; HRMS calcd. for $[C_{187}H_{292}BF_2N_{43}O_{63}]^{4+}$: 1049.7774, found: 1049.7792.

Cell culture of primary cells

Immature dendritic cells and macrophages were obtained from the bone marrow of C57BL/6 mice. The use of animals was approved by the animal ethics committee of the Leiden University. After euthanasia, the bone marrow of tibiae and femurs was flushed out and washed with PBS. For DC selection, DC medium containing EMDM/R1 feeder cells conditioned medium 2:1 (v/v) supplied with 8% FCS, penicillin/streptomycin and

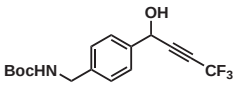
glutamax was used. After a 10 days selection, the DC populations were counted and 2x106 cells were seeded on 3cm non-tissue cultures petri dishes (Greiner) for pulse chase experiments. For imaging purposes, 2x104 cells were seeded on Labtek II, sterile, 4 chamber borosilicate coverglass systems (Nalge Nunc Int, Napperville, IL, SA). All experiments were conducted at least in duplicate.

Live cell imaging

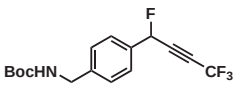
Microscopy studies were performed on an Olympus IX81 motorized inverted fluorescence microscope equipped with a DP72 digital camera and CellM operating software (Olympus, Zoeterwoude, The Netherlands). For live cell imaging we used a 37ºC thermostated chamber with 5% CO2 influx, 60x oil immersion objective, DIC for phase contrast and 565 RFP filter sets for fluorescence imaging of **7** and **8**.

Pulse chase experiments

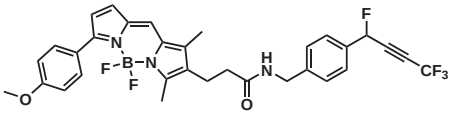
Compounds used (100x stocks) were dissolved in overnight conditioned DC medium as and thermostated at 4 ºC or 37 ºC. Cells were preincubated for 30 min. at 4 ºC or 37 ºC, then 30 min. incubation with cell culture medium or mannose receptor blockers followed by a 60 min. pulse with **8**. After 3 wash steps with PBS of 4 ºC or 37 ºC, the cells were chased for 2 hr. with conditioned DC of 4 ºC or 37 ºC, washed 3x with PBS, harvested in 1 ml PBS containing 4mM EDTA and kept on ice prior to analysis.The cells were washed with PBS and lysed in 50 mM TrisHCl (pH 7.5), 250 mM sucrose, 0.025% digitonin buffer with a 15 seconds sonication pulse on ice. Protein concentration was determined by the Bradford (BioRad) colorimetric method with a BSA calibration curve. Some 10µg (total protein/lane) was boiled with 4x Laemmli's sample buffer under reducing conditions and resolved 12.5% SDS-PAGE gel. In-gel detection of fluorescently labeled proteins was performed in the wet gel slabs directly on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560). The fluorescence was quantified using ImageQuant software.



***tert*-butyl 4-(4,4,4-trifluoro-1-hydroxybut-2-ynyl)benzylcarbamate (42).** 2-bromo-3,3,3-trifluoroprop-1-ene (**39**, 0.6 g, 3.48 mmol) was dissolved in THF (freshly distilled), put under argon atmosphere and cooled to -78 °C, before LDA (3.48 ml 2M in THF/heptane, 6.96 mmol, 2 equiv.) was added. The reaction mixture was stirred for 30 min., before *tert*-butyl 4-formylbenzylcarbamate (**41**, 1.57 mmol, 0.9 equiv.) in THF (freshly distilled) was added at -78 °C. After 1 hr., sat.aq. NH_4Cl was added and the mixture was extracted with DCM. The organic layer was dried over $MgSO_4$ and concentrated. Purification by column chromatography (Tol → 10% EtOAc in Tol) yielded the title compound (0.33 g, 1 mmol, 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.39 (d, *J* = 7.7 Hz, 2H), 7.20 (d, *J* = 7.8 Hz, 2H), 5.44 (s, 1H), 5.13-5.07 (m, 1H), 4.59 (s, 1H), 4.18 (d, *J* = 5.6 Hz, 2H), 1.43 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 156.21, 139.50, 137.46, 127.58, 126.72, 114.20 (q, $^1J_{CF}$ = 257.6 Hz), 87.32 (q, $^3J_{CF}$ = 6.4 Hz), 79.94, 72.80 (q, $^2J_{CF}$ = 52.7 Hz), 63.17, 44.03, 28.23.



***tert*-butyl 4-(1,4,4,4-tetrafluorobut-2-ynyl)benzylcarbamate (43).** DAST (32 µl, 0.25 mmol, 1.25 equiv.) was added to a mixture of *tert*-butyl 4-(4,4,4-trifluoro-1-hydroxybut-2-ynyl)benzylcarbamate (**42**, 66 mg, 0.2 mmol) in DCM under argon atmosphere and the reaction mixture was stirred overnight, before being poored in ice water. The mixture was extracted with DCM, washed with brine, dried over $MgSO_4$ and concentrated. Column chromatography (Tol → 5% EtOAc in Tol) gave the title compound (44.1 mg, 0.13 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.47 (d, *J* = 7.0 Hz, 2H), 7.37 (d, *J* = 7.9 Hz, 2H), 6.10 (dq, J_1 = 2.4, J_2 = 47.4 Hz, 1H), 5.01-4.92 (m, 1H), 4.35 (d, *J* = 5.0 Hz, 2H), 1.46 (s, 9H).



N-(4-(1,4,4,4-tetrafluorobut-2-ynyl)benzyl)BODIPY TMR-amide (44). *tert*-butyl 4-(1,4,4,4-tetrafluorobut-2-ynyl)benzylcarbamate (**43**, 22 mg, 66 µmol) was dissolved in TFA/DCM (1/1 v/v, 1 ml). After 30 min. the reaction mixture was coevaporated with Tol (3×). The crude was dissolved in DMF and neutralized with DiPEA (12 µl, 73 µmol, 1.1 equiv.), before BODIPY TMR-OSu (36 mg, 73 µmol, 1.1 equiv.). After 3 hr., the reaction mixture was concentrated *in vacuo*. Purification by column chromatography (Tol → 10% acetone in Tol, followed by (DCM → 10% EtOAc in DCM) yielded the title compound (10 mg, 16 µmol, 25%). LC/MS analysis: R_t 10.50 min (linear gradient 10 → 90% B in 13.5 min), *m/z* 592.27 $[M - F + H]^+$. ^1H NMR (400 MHz, $CDCl_3$): δ ppm 7.88 (d, *J* = 8.8 Hz, 2H), 7.39 (d, *J* = 7.0 Hz, 2H), 7.25 (d, *J* = 9.9 Hz, 2H), 7.05 (s, 1H), 6.99-6.96 (m, 3H), 6.56 (d, *J* = 4.0 Hz, 1H), 6.03 (dq, J_1 = 2.6, J_1 = 47.4 Hz, 1H), 5.73 (t, *J* = 5.2 Hz, 1H), 4.41 (d, *J* = 5.6 Hz, 2H), 3.86 (s, 3H), 2.78 (t, *J* = 7.3 Hz, 2H), 2.51 (s, 3H), 2.33 (t, *J* = 7.3 Hz, 2H), 2.14 (s, 3H).

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Samenvatting

Chemisch gereedschap voor het bestuderen van het proteasoom

Hoewel afgelopen decennia uitgebreide studies zijn verricht valt er nog veel te ontdekken in het veld van het proteasoom onderzoek. De interdisciplinaire inspanningen op het grensvlak van de chemie en de biologie hebben het begrip over de rol van het proteasoom in een breed scala van processen in een stroomverselling gebracht. Een beknopt overzicht van de recente ontwikkelingen in het veld van chemische biologie gedreven proteasoom onderzoek is uiteengezet in **Hoofdstuk 1**. Het onderzoek beschreven in dit proefschrift richt zich op de ontwikkeling van nieuw chemisch biologisch gereedschap waarmee proteasomen nauwgezetter bestudeerd kunnen worden.

De synthese en karakterisatie van de fluorescente, cell-permeabele en activiteits gebaseerde proteasoom probe BODIPY TMR-Ahx$_3$L$_3$VS (MV151) is beschreven in **Hoofdstuk 2**. Deze probe bewerkstelligt de snelle en gevoelige in-gel detectie van proteasoom activiteit in een gegeven monster gebruik makende van een fluorescentie scanner. De probe is gebruikt voor het labelen van de actieve proteasoom populatie in cel lysaten en in levende cellen. MV151 heeft een aantal voordelen vergeleken met eerder ontwikkelde probes in de zin dat Western blotten, radioactiviteit en gel drogen overbodig zijn. De distributie van MV151 was eenvoudig te detecteren in Ub^{G76V}-GFP transgene muizen en correleerde met de inhibitie van het proteasoom in het getroffen weefsel, gebaseerd op de accumulatie van de GFP-reporter. Het labellen van het proteasoom door middel van MV151 in combinatie met Ub^{G76V}-GFP transgene muizen is een bruikbare strategie voor het aantonen van de biodistributie van proteasoom inhibitoren. Ten slotte is een competitie experiment gebruik makende van MV151 een snelle en gevoelige methode

gebleken voor het vaststellen van het inhibitie profiel (in de zin van potentie en subunit preferentie) van een gegeven proteasoom inhibitor.

De ontwikkeling van drie makkelijk toegankelijke alkyn gefunctionaliseerde BODIPY fluoroforen wordt besproken in **Hoofdstuk 3**. Deze fluoroforen kunnen chemoselectief worden gekoppeld aan elke willekeurig azide bevattende activiteits gebaseerde karakterisatie probe en azide gemodificeerde metaboliet. De toepasbaarheid van de alkyn bevattende BODIPYs is gedemonstreerd aan de hand van de synthese van drie epoxomicin afgeleide fluorescente proteasoom probes.

In **Hoofdstuk 4** wordt de synthese van het bifunctionele azido-BODIPY zuur beschreven. In een activiteits gebaseerde eiwitprofilerings setting biedt deze nieuwe fluorofoor een keur aan flexibiliteit vergeleken met de conventionele monogefunctionaliseerde fluoroforen. Evenals de laatstgenoemde, maken azido-BODIPY uitgeruste activiteits gebaseerde probes snelle fluorescente detectie mogelijk en zijn deze probes compatibel met "live cell imaging" technieken. Gebruik makende van twee sets aan een- en twee-staps labeling proteasoom probes gebaseerd op azido-BODIPY werd vastgesteld dat de Staudinger-Bertozzi ligatie zo goed als quantitatief verloopt onder de toegepaste condities. Dit resultaat houdt in dat de efficiëntie van de eiwit labeling afhangt van de reactiviteit van de activiteits gebaseerde probe ten opzichte van het doel eiwit(familie) en niet van de chemoselectieve ligatie die gebruikt wordt in de tweede stap. Dit betekent in essentie dat twee-staps activiteits gebaseerde eiwit profilering met dezelfde efficiëntie kan verlopen als het labelen van eiwitten in een gebruikelijke een-staps strategie.

Op peptiden gebaseerde proteasoom inhibitoren bestaan uit drie structurele entiteiten, namelijk 1) het "warhead", de electrofiele val die reageert met het nucleofiele residu van het actieve centrum, 2) het peptidische herkenningselement dat fungeert als een sturingssequentie en 3) de *N*-terminale extentie. In **Hoofdstuk 5** wordt uitgelegd dat deze structurele elementen van vijf veel gebruikte proteasoom inhibitoren kunnen worden gecombineerd wat resulteerde in een bibliotheek van 15 hybride proteasoom inhibitoren. Met behulp van MV151 werd het inhibitie profiel van de bibliotheek bepaald. Het combineren van structurele elementen van bekende proteasoom remmers bleek een legitieme strategie voor de ontwikkeling van nieuwe potente proteasoom inhibitoren, daar een van epoxomicin afgeleide boor ester naar voren kwam als een van de meest potente peptide-gebaseerde proteasoom inhibitoren die zijn beschreven.

Een soortgelijke strategie wordt behandeld in **Hoofdstuk 6**. In dit geval werden warheads en peptide herkennings elementen van beschreven subunit specifieke proteasoom inhibitoren gecombineerd. Onverwachts bleken alle vinyl ethyl ester gebaseerde verbindingen, inclusief de door Marastoni *et al*. beschreven β2 specifieke inhibitor niet actief in competitie experimenten tegen MV151. Een potentere op epoxyketon gebaseerde β1 specifieke proteasoom inhibitor werd ontwikkeld, naast de β5 specifieke vinyl sulfon en een op β2 en β5 gerichte epoxyketon. Deze hits werden ongevormd tot subunit specifieke twee-staps labelings probes door de introductie van een azide. Door het vervangen van de naphthylacetamide voor een azido-BODIPY werden een β5 specifieke en een op β2 en β5 gerichte fluorescente twee-staps labelings probe verkregen. Daarnaast leverde de conjugatie van een alkyn gefunctionaliseerde BODIPY met de β1 specifieke twee-staps probe een groen fluorescente probe op met een voorkeur voor β1, welke bij hogere concentraties β5 begint te labelen.

In **Hoofdstuk 7** beschijft de synthese van vier epoxomicin analoga welke zijn uitgerust met een dieen. Deze probes bleken het proteasoom te inhiberen met een potentie in dezelfde orde van grootte als de moeder verbinding epoxomicin. Initiële Diels-Alder twee-staps proteasoom labeling experimenten, uitgevoerd op gezuiverd 20S proteasomen en gebruik makende van een dieen bevattende inhibitor en een fluorescent dienofiel, lieten zien dat de Diels-Alder een veel belovende alternatieve ligatie methode in activiteits gebaseerde eiwit profilering is. Om concurerend te worden met bestaande ligatie methoden is het nodig dat deze reactie wordt geoptimaliseerd.

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"Receptor-Mediated Targeting of Cathepsins in Professional Antigen Presenting Cells", Hillaert, U.; Verdoes, M.; Florea, B.I.; Saragliadis, A.; Habets, K.; Kuiper, J.; van Calenbergh, S.; Ossendorp, F.; van der Marel, G.A.; Driessen, C.; Overkleeft, H.S. *Submitted for publication.*

Curriculum Vitae

Martijn Verdoes werd op 6 maart 1980 geboren te Leiden. Na het behalen van het HAVO-diploma aan het Pieter-Groen College te Katwijk in 1997 werd in dat jaar begonnen met de opleiding Organische Chemie aan de Hogeschool Leiden. In het derde jaar van deze opleiding werd begonnen aan een zogenaamd sandwich programma met de studie Scheikunde aan de Universiteit Leiden. In het kader van het aftudeeronderzoek werd van september 2001 tot en met juni 2002 onderzoek verricht bij de vakgroep Bio-organische Synthese onder leiding van prof.dr. H. S. Overkleeft en prof.dr. J. H. van Boom. Dit onderzoek omvatte de vaste-drager synthese van een reeks pyranopyraanverbindingen die doormiddel van ringsluitingsmetathese van de hars werden afgesplitst. In augustus 2002 werd de opleiding Organische Chemie aan de Hogeschool Leiden met goed gevolg afgerond. Van oktober 2002 tot en met april 2003 werd een onderzoeksstage uitgevoerd aan de Harvard Medical School te Boston (USA) in de Department of Pathology onder leiding van prof.dr. H. L. Ploegh. Dit onderzoek was gericht op de synthese van verbindingen die gebruikt werden voor het bestuderen van het ubiquitine-proteasoom systeem. Het Master of Science examen werd in 2004 behaald.

Van juli 2004 tot oktober 2008 werd als assistent in opleiding het in dit proefschrift beschreven onderzoek uitgevoerd bij de vakgroep Bio-organische Synthese onder leiding van prof.dr. G. A. van der Marel en prof.dr. H. S. Overkleeft. In november 2007 werd deelgenomen aan het "international symposium on protein modification and degradation" te Peking, China.

Nawoord

Afsluitend wil ik de mensen bedanken die, zonder dat ze zich daar misschien van bewust zijn, een bijdrage hebben geleverd aan de totstandkoming van de "onderzoeker" Martijn Verdoes, met dit proefschrift als gevolg. Allereerst uiteraard mijn ouders, zussen en broer die mij respecteren, steunen en trots op me zijn.

Onderzoek is een teamsport. Wouter van der Linden, Lianne Willems en Boudewijn Duivenvoorden hebben met veel inzet en enthousiasme in het kader van hun bij- en/of hoofdvakstage een onmisbare bijdrage geleverd aan dit proefschrift en voor een onvergetelijke tijd gezorgd. I also really enjoyed working with Myra Sae-Heng. Peng-Ke, xie xie. Ik wens jullie allemaal heel veel succes en plezier toe in jullie wetenschappelijke loopbaan en daarbuiten.

Verder hebben alle leden van de vakgroep BIOSYN bijgedragen aan de fantastische tijd die ik heb gehad. De discussies en borrels zijn zeer waardevol geweest. Ik wil met name Bobby noemen voor de biologische kennis en vaardigheden die hij mij heeft bijgebracht. Verder heb ik veel geleerd van en gelachen met de ex-BIOSYNers Ulrik Hillaert, Michiel Leeuwenburgh, Mattie Timmer en Gijs Grotenbreg. I thank Alexei Kisselev for the fruitful collaboration. The same holds true for Victoria Menendez-Benito, Christa Maynard, Celia Berkers, Fijs van Leeuwen, Tom Groothuis, Huib Ovaa, Jaques Neefjes, Nico Dantuma as well as Renier van der Hoorn and his collegues.

Verder stonden de vaste waarden Caroline, Rian, Hans en Nico, als ook de ama's Hennie, Arnold en Marco altijd klaar voor logistieke en technische ondersteuning. Kees Erkelens en Fons Lefeber waren onmisbaar bij het opnemen van NMR-spectra en Trudie, Paul en Arjan bij de tijd op het practicum. De Katwijkse vogelaars en sympathisanten en Albert & Anke en Niels hebben voor de nodige afleiding naast het labwerk gezorgd.

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