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Towards subunit specific proteasome inhibitors

Linden, W.A. van der

Citation

Linden, W. A. van der. (2011, December 22). *Towards subunit specific proteasome inhibitors*. Retrieved from <https://hdl.handle.net/1887/18273>

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Towards subunit specific proteasome inhibitors

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 donderdag 22 december 2011
klokke 15:00 uur

door

Wouter Alexander van der Linden

Geboren te Rotterdam in 1983

Promotie commissie

Promotor: Prof. Dr. H. S. Overkleeft

Co-promotores: Prof. Dr. A. F. Kisselev
Dr. B. I. Florea

Overige leden: Prof. Dr. C. A. van Boeckel
Prof. Dr. M. Kaiser
Prof. Dr. J. Brouwer
Prof. Dr. G. A. van der Marel
Prof. Dr. M. Groll

The printing of this thesis was financially supported by the J. E. Jurriaanse stichting.

Typeset in L^AT_EX

Printed by Wöhrman Print Service

Van wie moet dat dan?

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List of Abbreviations

ABP	activity-based probe	EK	epoxyketone
ABPP	activity-based protein profiling	EL4	murine lymphoid cell line
Ac	acetyl	emPAI	exponentially modified protein abundance index
Ac ₂ O	acetic anhydride	equiv.	molar equivalent
AcCl	acetyl chloride	ER	endoplasmatic reticulum
AcOH	acetic acid	EtOH	ethanol
Ada	1-adamantyl acetic acid	FDA	U.S.A. food and drug administration
Ahx	6-aminohexanoic acid	Fmoc	(9 <i>H</i> -fluoren-9-yl)methoxycarbonyl
AMC	7-amino-4-methyl coumarin	GlbA	glidobactin A
APT	attached proton test	HBTU	2-(1 <i>H</i> -Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
aq.	aqueous	HCTU	2-(6-chloro-1 <i>H</i> -benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
ATP	adenosine triphosphate	HEK293T	human embryonic kidney cell line
BM	biotinylated molecular weight marker	HMB	3-hydroxy-2-methylbenzamide
Boc	<i>tert</i> -butoxycarbonyl	HOBt	<i>N</i> -hydroxybenzotriazole
Boc ₂ O	<i>tert</i> -butoxycarbonic anhydride	hr.	hour(s)
BODIPY	4,4-difluoro-4-bora-3 <i>a</i> ,4 <i>a</i> -diazas-indacene	HRMS	high resolution mass spectrometry
BSA	bovine serum albumin	HWE	Horner-Wadsworth-Emmons
Bu	butyl	Hz	Hertz
calcd.	calculated	<i>J</i>	coupling constant
cat.	catalytic	LC/MS	liquid chromatography/ mass spectrometry
Cbz	benzyloxycarbonyl	LMP2	low molecular weight protein 2, β1i
d	doublet	LMP7	low molecular weight protein 7, β5i
δ	chemical shift	M	molar
Da	Dalton	m	multiplet
DBU	diazabicyclo[5.4.0]undec-7-ene	<i>m/z</i>	mass to charge ratio
DC	dual colour molecular weight marker	Me	methyl
DCM	dichloromethane	MEC11	multicatalytic endopeptidase complex-like-1, β2i
dd	double doublet	MeCN	acetonitrile
ddd	double double doublet	MeOH	methanol
DiPEA	<i>N,N</i> -diisopropylethylamine	MHC I	major histocompatibility complex class I
DMF	<i>N,N</i> -dimethylformamide	min.	minute(s)
DMSO	dimethylsulfoxide	MS (ESI)	mass spectrometry (electronspray ionization)
dq	double quartet	NMR	nuclear magnetic resonance
dt	double triplet	o/n	overnight
DTT	dithiothreitol		
DUB	deubiquitinating enzyme		
EA	ethyl acetate		
EDTA	ethylenediaminetetraacetate		

OAc	acetate	sp.	species
PAI	protein abundance index	SPPS	solid phase peptide synthesis
PBS	phosphate buffered saline	subsp.	subspecies
Pd/C	palladium on charcoal	SylA	syringolin A
PE	petroleum ether	T	temperature
PEG	polyethylene glycol	t	triplet
Ph	phenyl	<i>t, tert</i>	tertiary
ppm	parts per million	<i>t</i> BuONO	tert-butyl nitrite
pv.	pathovar	TBDMS	<i>tert</i> -butyl dimethylsilyl
q	quartet	TBTA	tris-(benzyltriazolylmethyl)amine
quant.	quantitative	td	triple doublet
R_t	retention time	TEA	triethyl amine
RAJI	human lymphocyte cell line derived from Burkitt's lymphoma	TFA	trifluoroacetic acid
RAW264.7	mouse leukaemic monocyte macrophage cell line	THF	tetrahydrofuran
rcf	relative centrifugal force	TIS	triisopropyl silane
RT	room temperature	TLC	thin layer chromatography
s	singlet	TMR	tetramethylrhodamine
Sacc.	Pier Andrea Saccardo	Tol.	toluene
sat.	saturated	Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
SDS	sodium dodecyl sulfate	Ub	ubiquitin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis	UPS	ubiquitin proteasome system
		VS	vinyl sulfone
		Z	benzyloxycarbonyl

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

1

General Introduction

1.1 Introduction

Protein quality and half life are essential for the function of a cell and are controlled in a tightly regulated fashion. Most of this work is carried out by the ubiquitin-proteasome system (UPS), which degrades 60-90% of all proteins in the nucleus and cytoplasm of the eukaryotic cell.^{1,2} Central function in this process is proteolysis of substrates by the 26S proteasome, a large multicatalytic complex, crucial for cell viability. The proteasome degrades abnormal, damaged or misfolded proteins and is responsible for degrading short-lived regulatory proteins involved in cell differentiation, cell cycle regulation, transcriptional regulation, inflammation and apoptosis. Proteasomal degradation of substrates generates peptides, a fraction of which are used for MHC class I presentation. Hence, the proteasome is involved in almost all cellular processes and its deregulation is associated with many diseases. The proteasome contains catalytically active subunits with different substrate specificities and the role of these individual proteolytic activities of the proteasome in normal or diseased state is a question that is largely unanswered. This thesis aims at developing subunit specific inhibitors of the proteasome to study the individual function of proteasome active subunits *in vivo*.

1.2 Ubiquitin Proteasome System

The ubiquitin proteasome system is the major proteolytic machinery in the eukaryotic cell. Its central task is the degradation of proteins in an ATP and ubiquitin (Ub) dependent fashion. Ubiquitin, a 9 kDa highly conserved 76 residue protein, is attached to a target protein by an ATP-dependent cascade of E1, E2 and E3 enzymes.³ Ubiquitin is activated by one of the two E1 enzymes,⁴ which transfers ubiquitin to E2, a family of several dozen ubiquitin-conjugating enzymes. A member of the very large family of E3 enzymes then mediates the transfer of activated ubiquitin *via* its C-terminus to a lysine ϵ -amine of a pro-

tein substrate or an already linked ubiquitin.^{5,6} Ubiquitination is further regulated by the action of one of the many deubiquitinating enzymes (DUB).⁷ If a protein contains a chain of four or more Lys48-linked ubiquitin residues, it is recognised by the 26S proteasome as target for destruction (Figure 1.1A).⁸ Ubiquitin is recycled as the ubiquitinated substrate is transported into the catalytic chamber of the proteasome, where the peptide is cut into small fragments. The average length of peptide products is 3-22 residues, with an average length of 8-12 amino acids.⁹ Most peptides are degraded further into single amino acids downstream of the proteasome. About 1% escapes this fate and is trimmed N-terminally by cytosolic aminopeptidases, transported into the endoplasmatic reticulum (ER) to be loaded on a MHC class-I complex and presented at the cell surface. The proteasome is thus responsible for generating the C-terminus of most antigenic peptides.¹⁰

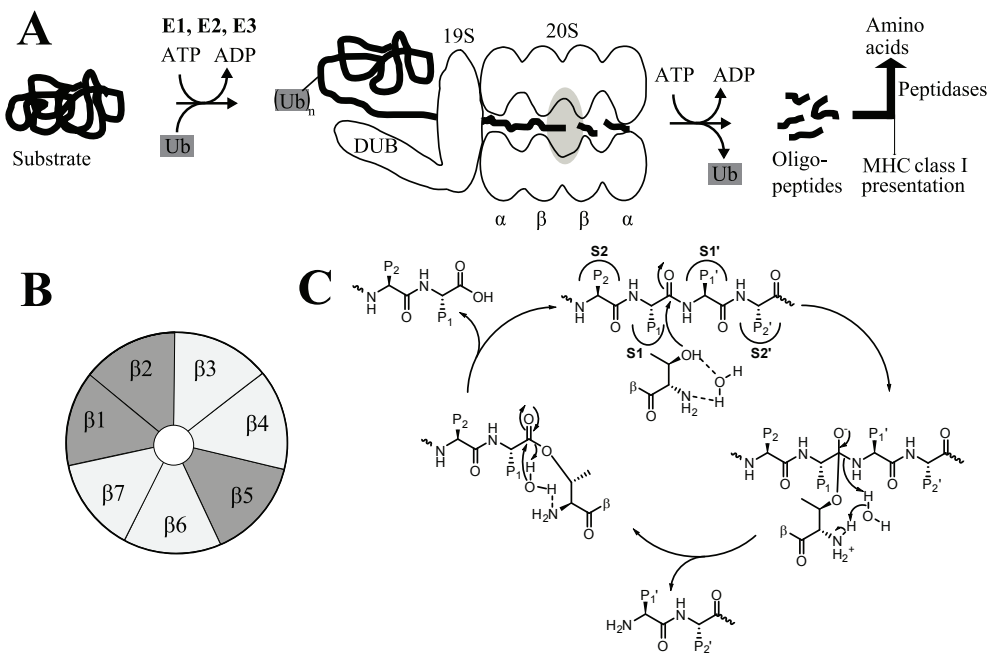


Figure 1.1: (A) Schematic presentation of protein catabolism by the proteasome. (B) Schematic top view of β ring of the constitutive proteasome. Proteolytic active subunits are depicted in dark gray (C) Proteolytic cycle of proteasome β subunit.

1.3 Structure and function of the proteasome

The term proteasome refers to a collection of different forms of proteasome, which all contain the catalytic 20S core particle. The eukaryotic 20S core particle is a 700 kDa complex and is built up from four heptameric rings, two rings of seven distinct α subunits and two rings of β_{1-7} subunits, stacked in the order $\alpha\beta\beta\alpha$ to form a hollow cylindrical structure 160Å by 120Å in dimension. The inside of this cylinder is partitioned into three cavities

of which the central cavity, lined by β subunits, contains the proteolytic active sites. Only $\beta 1$, $\beta 2$ and $\beta 5$ contain proteolytic activity performed by the N-terminal threonine of these subunits (Figure 1.1B). Via a water molecule, the N-terminal amine group activates the γ hydroxyl of Thr1, which performs a nucleophilic attack on the target amide bond.¹¹ A tetrahedral intermediate is formed, which then degrades into an ester-bound peptide, releasing the amine product of the substrate cleavage. The ester bond is hydrolysed by a water molecule, regenerating the proteasome Thr1 residue and releasing the carboxylic acid product of the substrate (Figure 1.1C).

The catalytically active β subunits differ in their substrate preference, which is induced by differences in their substrate binding pockets. The $\beta 1$ subunit (Y/ δ) prefers cleaving after acidic residues (caspase-like activity), $\beta 2$ (Z) prefers cleavage after basic residues (trypsin-like activity) and the $\beta 5$ (X) prefers to cleave after hydrophobic residues (chymotrypsin-like activity). Substrate preference of the active β has been determined with fluorogenic peptides and their activities are not as strictly defined as their names suggest and partly overlap.¹² For example, next to its preference for acidic residues, $\beta 1$ is able to cleave after branched amino acids, residues also processed by $\beta 5$.¹³

Entry of protein substrate into the channel of the 20S proteasome is hindered by α N-termini, preventing unregulated entry of substrates in the catalytic chamber.¹⁴ To open this gate to the proteasome, binding of a regulatory particle is required. Two 19S regulatory particle cap the 20S core particle to yield the 26S proteasome.¹⁵ The 19S regulatory particle recognises and deubiquitinates ubiquitinated substrates and promotes their unfolding, while the base of the particle that binds to the α -ring of 20S contains Rpt-subunits that regulate the opening of the channel into the 20S core particle in an ATP-dependent fashion.^{3,6,16,17}

In mammals, the cytokine interferon- γ induces the synthesis of three catalytic subunits with different substrate preference. These subunits, called $\beta 1i$ (LMP2), $\beta 2i$ (MECL1) and $\beta 5i$ (LMP7) replace their constitutive counterparts in newly formed immunoproteasomes.¹⁸ Furthermore, interferon- γ induces the expression of a different regulatory complex, the 11S or PA28 regulator, able to doubly cap the 20S core particle or form a hybrid proteasome (11S-20S-19S).^{19–21} The immunoproteasome displays substrate preferences slightly different from the constitutive proteasome.^{22,23} Peptides with an average length of 8–10 amino acids are generated, with more basic and hydrophobic residues at their C-terminus. These peptides are more optimal for binding to the MHC class I complex for presentation on the cell surface.¹⁰ For a long time, the function of the immunoproteasome was assumed to be only to increase the pool of antigenic peptides. However, the immunoproteasome displays increased efficiency in the degradation of oxidized proteins and its main function appears to preserve protein homeostasis after inflammation.^{24,25} In cortical thymic epithelial cells a seventh active β subunit is expressed, which is called $\beta 5t$. Compared to $\beta 5$ or $\beta 5i$, the $\beta 5t$ substrate pocket is more hydrophilic. Subunit $\beta 5t$ is found together with mainly $\beta 1i$ and $\beta 2i$ in so called thymoproteasomes. The chymotryptic activity of thymoproteasomes is decreased with respect to constitutive or immunoproteasomes and thymoproteasomes generate low-affinity MHC class I antigens. This altered pool of MHC class I ligands is important for the positive selection of developing thymocytes, although the exact role of $\beta 5t$ in this process is not yet clarified.^{26,27}

1.4 Proteasome inhibitors

The UPS has a central role in protein degradation, which ensures its involvement in many processes. Much knowledge about the proteolytic role of the proteasome in a cellular pathway has been gathered with the aid of proteasome inhibitors. Over the last decades, an increasingly large number of proteasome inhibitors has been identified, showing a broad range of structural features and cellular activities. Nature provides a large spectrum of complicated molecules able to block proteasomal function and the apparent medicinal benefits of many of these molecules inspired organic chemists to expand the versatility of proteasome inhibitors even more. Often, a proteasome inhibitor contains an electrophile that is able to covalently capture the Thr1 O γ of the proteasome and thereby blocking its catalytic activity. However, many inhibitors inhibit the proteasome in a non-covalent way. This Chapter gives an overview of classes of proteasome inhibitors.

1.5 Natural occurring proteasome inhibitors

In the search for new leads for medicine development, natural products are very often a source of inspiration for the development of new agents directed towards therapeutic targets. Natural products are commonly extracted from plant tissues, marine organisms or microorganism fermentation broths and screened for beneficial effects on various disease models. The cellular target of a biologically active molecule identified in such a screen is then sought for. In cases where natural products inhibit cell cycle progression, inflammation or microorganism growth, not rarely the proteasome is found to be inhibited. The variety of natural occurring proteasome inhibitors is very large and this diversity provides many lead structures for proteasome inhibitors.

1.5.1 Beta-lactones

The metabolite lactacystin (**1**, Figure 1.2A), produced by a *Streptomyces* strain, was identified as an inducer of differentiation of a mouse neuroblastoma cell line and inhibitor of cell cycle progression due to proteasome inhibition. Lactacystin itself is inactive against the proteasome, but upon lactonisation, the beta-lactone omuralide is formed (**2**, Figure 1.2A), a potent proteasome inhibitor.^{28–30} Three other families of beta-lactone natural products, structurally related to omuralide, have been identified to date. In extracts of the marine actinomycete *Salinospora tropica*, salinosporamides were identified as agents cytotoxic to cancer cell lines, with salinosporamide A (**3**) as most potent member.³¹ Highly related to salinosporamides are cinnabaramides, metabolites from terrestrial streptomycete *S. cinnabarinus* with cinnabaramide A (**4**) depicted in Figure 1.2A.³² Belactosin A (**5**), a metabolite from *Streptomyces* sp. UCK14, displays antitumour and antimicrobial activity through cell cycle inhibition, which was later ascribed to proteasome inhibition.^{33,34} Beta-lactones inactivate the proteasome by nucleophilic attack of the proteasome Thr1 O γ on the carbonyl of the lactone, thereby forming an ester adduct with the Thr1 O γ of the active subunits (Figure 1.2B). In the case of salinosporamide A, as a result of beta-lactone opening, a second reaction occurs, forming a tetrahydrofuran ring by displacement of the chloride atom. The ester bond mediated active site inactivation by beta-lactones is reversible, although this is a slow process because the orientation of the inhibitor-proteasome adduct is such that

the entry of water into the active site is prevented, thereby preventing deacylation of the proteasome's catalytically active moiety.³⁵

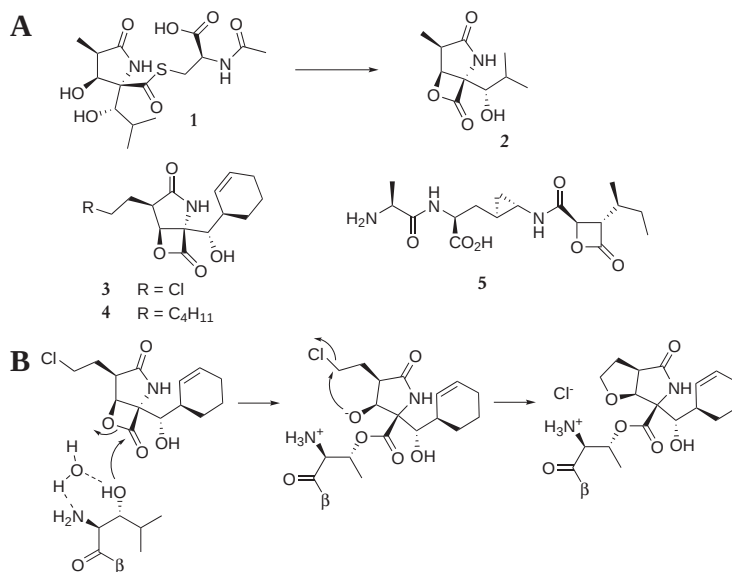


Figure 1.2: Structure (A) of beta-lactones found in nature and (B) proteasome inhibition mechanism of salinosporamide A.

1.5.2 Aldehydes

Leupeptin (6, Figure 1.3A) is a protease inhibitor with a broad range of enzyme families as target due to its reactive aldehyde electrophile. This compound, isolated from various strains of *Actinomycete*, inhibits several serine and cysteine proteases and was found to in-

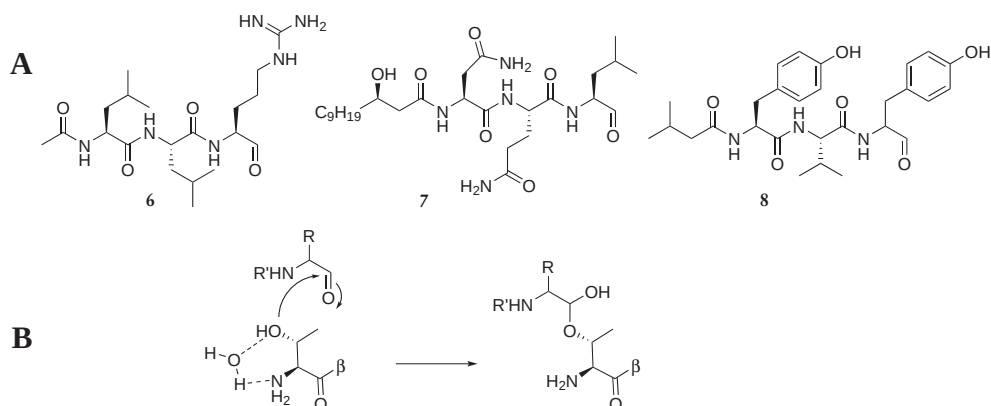


Figure 1.3: Structure (A) of aldehyde containing inhibitors found in nature and (B) their proteasome inhibition mechanism.

hibit the $\beta 2$ activity of the proteasome as well.^{36,37} Fellutamides are a family of cytotoxic peptides from marine fungus *Penicillium fellutanum*, and fellutamide A (**7**, Figure 1.3A) is a potent proteasome inhibitor with some preference for the $\beta 5$ subunit.^{38,39} Another natural occurring $\beta 5$ preferring proteasome inhibitor is tyropeptin A (**8**, Figure 1.3A) isolated from *Kitasatospora* bacteria in a search for new proteasome inhibitors.^{40,41} Proteasome inactivation by aldehydes occurs by attack of the proteasomal Thr1 O γ on the aldehyde, forming a hemiacetal bond (Figure 1.3B). The hemiacetal bond with the proteasome is reversible and aldehydes are rapidly inactivated by oxidation to the carboxylic acid *in vivo* and therefore the effect of aldehydes is not sustained.

1.5.3 α',β' -epoxyketones

In a screen for agents with antitumor activity, natural occurring α',β' -epoxyketone containing peptides epoxomicin (**9**) and eponemycin (**10**, Figure 1.4A) were discovered in an *Actinomycete* and *Streptomycete* strain, respectively.^{42,43} Closer analysis of fermentation broths of species of *Streptomyces* identified more linear α',β' -epoxyketone containing peptides analogous to epoxomicin or eponemycin, named TMC-86, TMC-89 and TMC-96.^{44,45} This marked the discovery of truly selective proteasome inhibitors.^{46,47} The selectivity of these compounds comes forth from the unique interaction of this electrophile with the proteasome Thr1 O γ (Figure 1.4B). First, the Thr1 O γ performs an attack on the carbonyl of the ketone, forming a reversible hemiacetal linkage. Next, the free amine from Thr1 opens the epoxide ring, irreversibly forming a morpholino ring.⁴⁸ Because many other common off-targets for proteasome inhibitors, such as cysteine or serine proteases, do not possess this N-terminal threonine functionality, these enzymes can not form the morpholino adduct as the proteasome does. Consequently, the proteasome is the only cellular target found for epoxomicin so far.^{46,47}

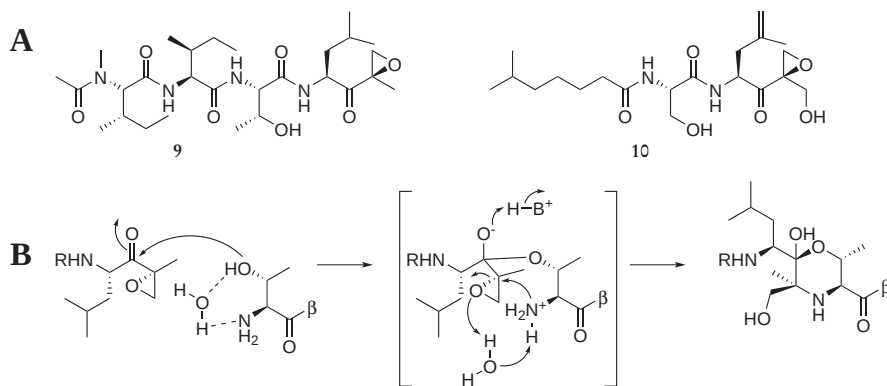


Figure 1.4: Structure (A) of α',β' -epoxyketone containing inhibitors found in nature and (B) their proteasome inhibition mechanism.

1.5.4 Syrbactins

Syrbactins are a class of structurally related proteasome inhibitors, containing syringolins, glidobactins and the closely related cepafungins⁴⁹ as subfamilies.⁵⁰ Glidobactins were isolated from a *Burkholderia* species, in a search for antitumour and antimicrobial agents, but

its cellular target was then not yet determined.⁵¹ Later, syringolin A (**11**, Figure 1.5A), secreted by plant pathogen *Pseudomonas syringae* pv. *syringae* as a virulence factor when invading a plant and the earlier found structurally related glidobactin A were both identified as potent inhibitors of the proteasome.^{52–54} Syrbactins share a twelve-membered unsaturated lactam core structure. The α,β -unsaturated amide in this lactam structure undergoes Michael addition of the Thr1 O γ of the proteasome catalytically active subunit to yield an irreversible ether band (Figure 1.5B).

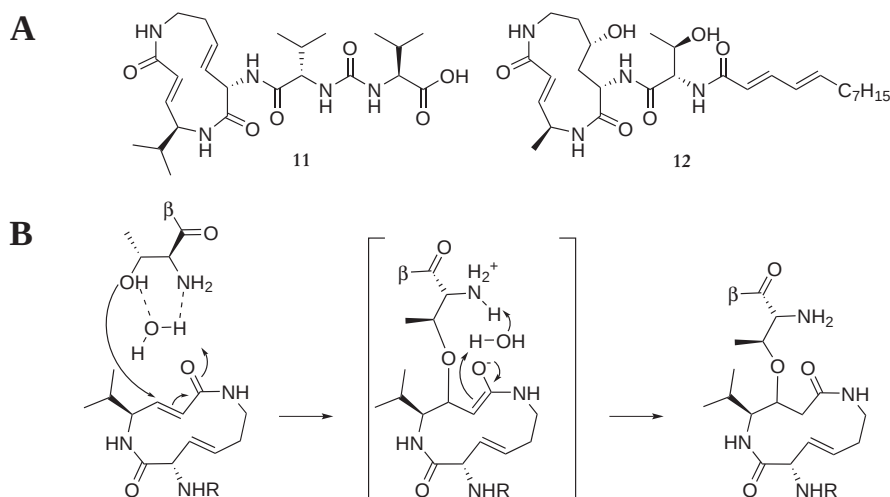


Figure 1.5: Structure (A) of syrbactins and (B) their proteasome inhibition mechanism.

1.5.5 Non-covalent natural peptoid proteasome inhibitors

The fungal plant pathogen *Apiospora montagnei* was found to produce complex cyclic peptoid molecules able to inhibit the proteasome with high selectivity.^{45,55} TMC95A (**13**, Figure 1.6), the most potent of this family, inhibits the proteasome in a non-covalent way, as was deduced from crystallographic studies. A network of hydrogen bridges accomplishes the formation of an anti-parallel β -sheet in the active β -sites of the proteasome, causing potent competitive binding of this peptide-like structure.⁵⁶ Another, are isolated from myxobacteria.⁵⁷ Argyrin A (**14**, Figure 1.6), member of a naturally occurring cyclic peptide family called argyryns, was found to stabilize a tumour suppressor protein through inhibition of the proteasome.⁵⁸ The exact binding mechanism remains to be determined, but an analogue of argyryns lacking the dehydroalanine moiety lacks proteasome inhibitory capacities.⁵⁹ Scytonemide A (**15**), a cyclic peptide-like molecule isolated from cyanobacterium *Scytonema hofmannii*, potently inhibits the proteasome. Its proteasome binding mechanism has not yet been elucidated, although covalent inactivation by the imine functionality is not excluded.⁶⁰ PR39 is an arginine and proline rich antibacterial peptide derived from porcine bone marrow that was found to inhibit the proteasome by perturbing the 20S core particle of the proteasome in an allosteric fashion.⁶¹

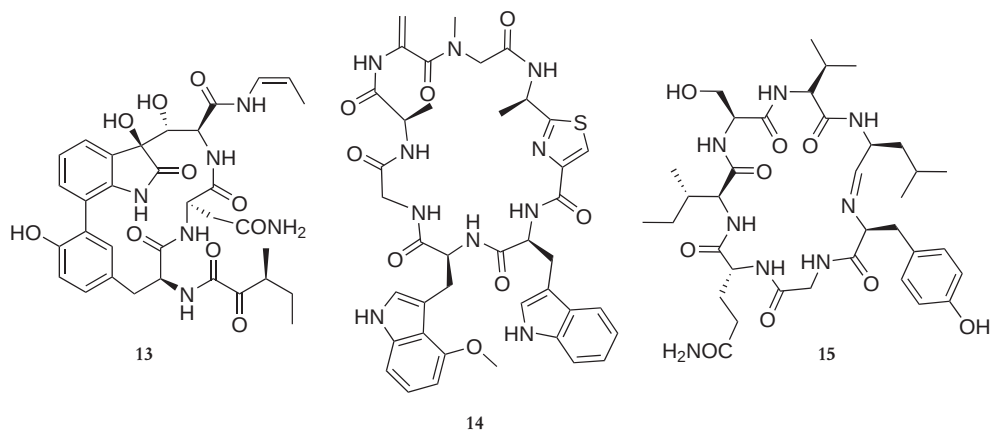


Figure 1.6: Structure of naturally occurring cyclic peptide proteasome inhibitors

1.5.6 Triterpenoids and flavonoid proteasome inhibitors

Triterpenoids form a diverse family of compounds, synthesised in plants from squalene, a precursor or steroids. A very large variety of triterpenoids have been isolated from plants used in traditional medicine. Subsequently, extracts of these plants were used in screens against various disease models.^{62–64} A large number of triterpenoids were identified as 20S proteasome inhibitors, however, these molecules often have multiple cellular targets. Agosterol C (16, Figure 1.7), extracted from marine sponge *Acanthodendrilla* reverses multidrug resistance in tumour cell lines and has been found to inhibit the chymotryptic activity of the proteasome, although the mechanism of inhibition is unclear.^{65,66} Withaferin A (17) and celastrol (18), terpenoids isolated from medicinal plants 'Indian Winter

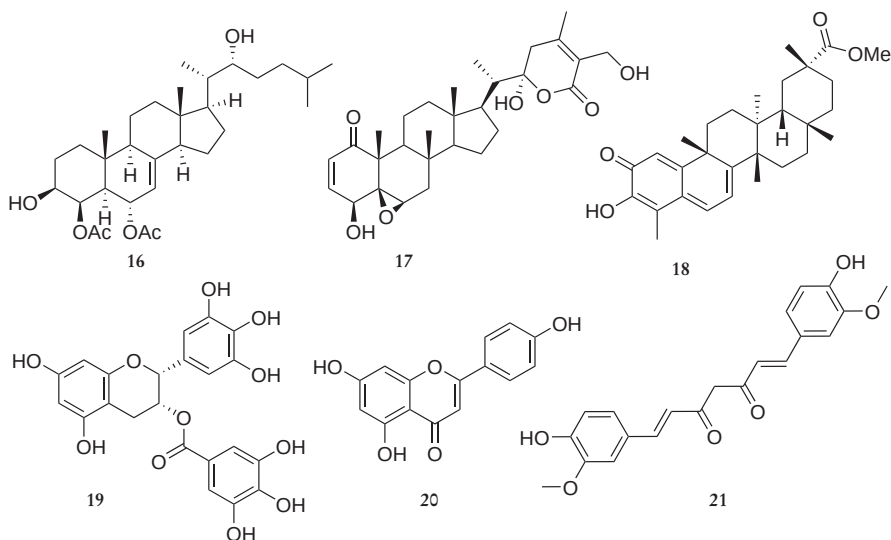


Figure 1.7: Structure of naturally occurring triterpenoid and flavonoid proteasome inhibitors.

Cherry' and 'Thunder of God vine', respectively, have been investigated for their anti-cancer properties. Withaferin A and celastrol inhibit $\beta 5$ of the proteasome, and hypothetically, these two compounds are able to form a covalent adduct with cellular targets *via* unsaturated carbonyl moieties present in these triterpenoids.^{67–69} Another large family of natural occurring compounds from which many show proteasome inhibition are the flavonoids. Green tea polyphenol (-)-epigallocatechin-3-gallate (**19**, Figure 1.7) potently inhibits the chymotryptic activity of the proteasome.⁷⁰ Docking studies suggest that the mechanism of proteasome inhibition could be acylation of the Thr1 hydroxyl.⁷¹ Many other flavonoids commonly found in vegetable and fruit inhibit the chymotryptic activity of the proteasome.^{72–74} Apigenin (**20**, Figure 1.7), found in celery seeds, is one of the most potent proteasome inhibitors and was found to have anticancer effects^{75,76} Curcumin (**21**), found in turmeric⁷⁷ is also a proteasome inhibitor. Although docking studies suggest that the unsaturated ketone in flavonoids could be susceptible to nucleophilic attack, covalent proteasome inhibition by flavonoids has not been proven. The binding mode of these compounds, however, is not clearly competitive.⁷³ It remains unclear whether the anticancer effects of triterpenoids and flavonoids are caused solely through proteasome inhibition.

1.6 Synthetic proteasome inhibitors

With a very large variety of natural product proteasome inhibitors as inspiration, chemists have been synthesising proteasome inhibitors for about two decades. Natural products inspired scaffolds were optimised for selectivity for the proteasome, increasing potency and pharmacological benefits, and several new pharmacophores able to bind the proteasome have been found. An overview of synthetic proteasome inhibitors follows.

1.6.1 Reversible covalent proteasome inhibitors

Simple peptide aldehydes, such as the natural occurring leupeptin and the synthetic calpain inhibitor I and II (**22**, **23**, Figure 1.8), were found to inhibit the proteasome, but suffered from many off-targets.^{1,37,78} This lack of selectivity sparked a large effort to develop more

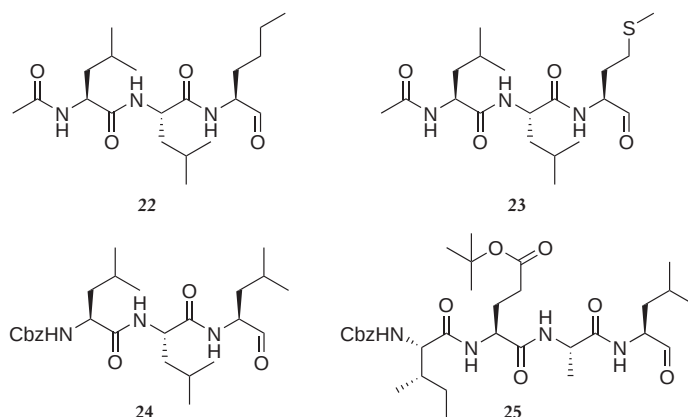


Figure 1.8: Structure of some synthetic aldehyde containing proteasome inhibitors.

proteasome specific peptide aldehydes. MG132 (**24**) displays more selectivity for the proteasome over other enzymes such as calpains or cathepsins and is still widely used as a model inhibitor of the proteasome.^{78–80} Compound **25** was developed later as an even more specific proteasome inhibitor.^{81,82} However, the reactivity of the aldehyde electrophile enables inhibition of targets other than the proteasome, limiting their use *in vivo*.

Since the discovery of peptide aldehydes, many other electrophiles have been synthesised and evaluated for their capacity to selectively inhibit the proteasome in a covalent reversible way. The boronic acid moiety yields inhibitors more potent than aldehyde counterparts and suffers less from cross-reactivity with cysteine proteases, although serine proteases can be modified by the boronic acid. Optimisation of the peptide sequence of a boronic acid inhibitor yielded bortezomib, which shows selectivity for the proteasome over most serine proteases (**26**, Figure 1.9A).⁸⁰ The empty orbital of boron acts as an electrophile, capturing the proteasomes active site hydroxyl in a reversible way (Figure 1.9B). Bortezomib has been approved by the FDA for the treatment of multiple myeloma. The success of this inhibitor in the treatment of cancer resulted in a surge of second generation boronic acid based inhibitors. For example, CEP18770 (**27**, Figure 1.9A) is an orally

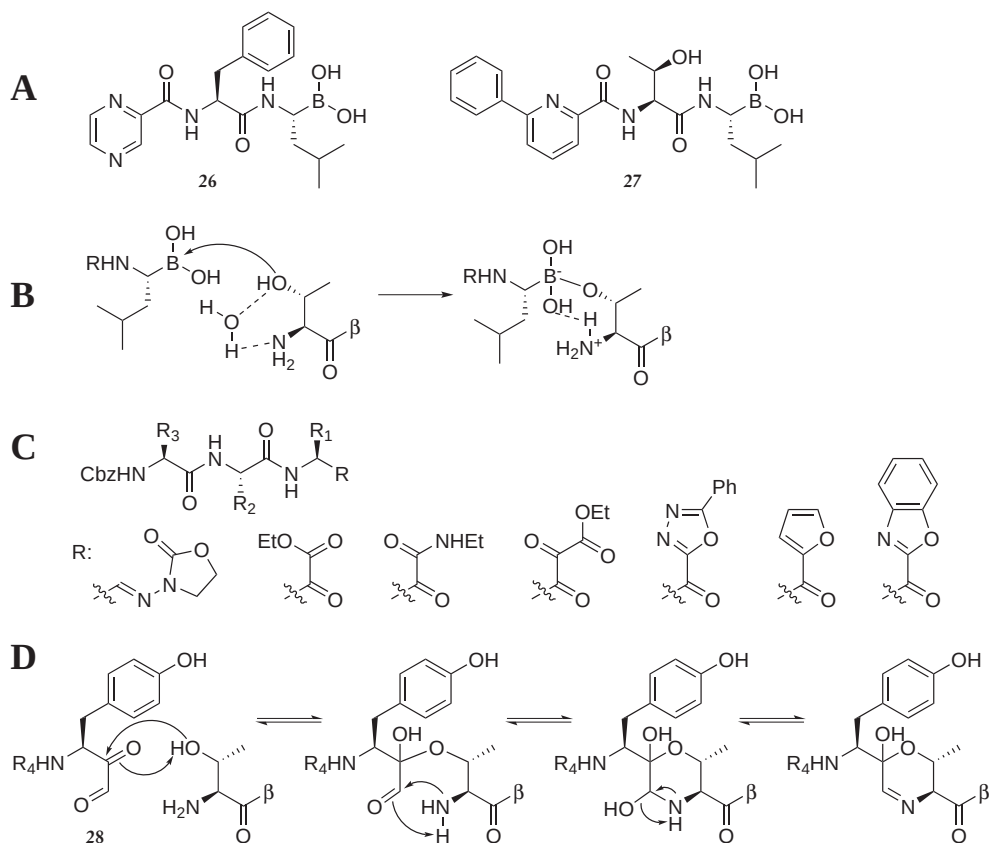


Figure 1.9: (A) Structure of boronic acid containing proteasome inhibitors with medicinal benefits. (B) Proteasome inhibition mechanism of boronic acid electrophile. (C) Structure of reversible covalent electrophiles (D) Proteasome inhibition mechanism of α -ketoaldehyde electrophile.

bioavailable selective proteasome inhibitor that is currently in clinical trials against multiple myeloma.⁸³

An overview of other electrophiles able to bind the proteasome in a covalent and reversible way is provided in Figure 1.9C.^{78,80,84–89} The mechanism of proteasome inhibition by the α -ketoaldehyde electrophile, present in the inhibitor Cbz-Leu-Leu-Tyr-COCHO (28), shows similarities with the epoxyketone electrophile. The catalytic Thr O γ first forms a reversible hemiacetal linkage with the ketone of the α -ketoaldehyde. Then, the N-terminal amine of the proteasome reacts with the aldehyde group and reversibly forms a 5,6-dihydro-2H-1,4-oxazine ring (Figure 1.9D).⁹⁰ This bivalent inhibition mechanism of N-terminal threonine proteases explains the high selectivity of Cbz-Leu-Leu-Tyr-COCHO for the proteasome over proteases with different catalytic mechanisms.

1.6.2 Non-covalent synthetic proteasome inhibitors

The reactivity of many pharmacophores to bind the proteasome in many cases leads to side reactions of these covalent inhibitors with targets other than the proteasome. Non-covalent proteasome inhibitors lacking a reactive electrophile were developed. A library of 5-methoxy-1-indanones was screened for 20S proteasome inhibition, resulting in several linear peptides able to inhibit the chymotryptic activity of the proteasome non-covalently, with compound 29 (Figure 1.10) able to inhibit proteasome activity in cells.^{91–93} The demanding synthesis of natural product TMC95A (13, Figure 1.6) led to the development of simplified cyclic analogues. These compounds, such as biaryl ether 30 (Figure 1.10) are less potent than TMC95A.^{94,95} Optimisation of simplified TMC95A analogues led to

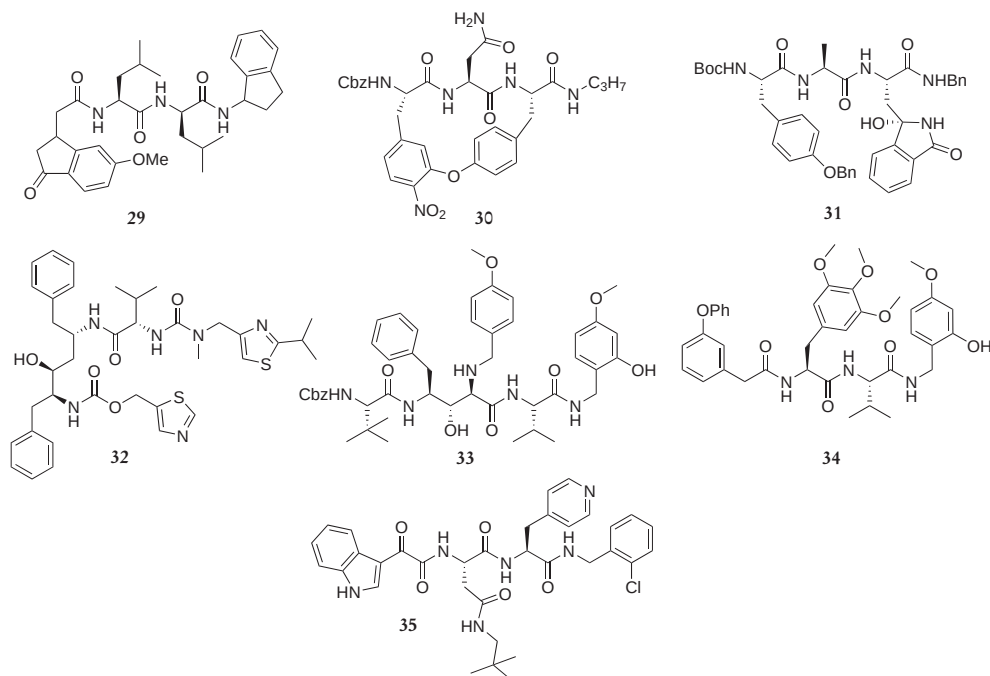


Figure 1.10: Structure of some noncovalent peptide based proteasome inhibitors.

linear analogue **31**, inhibiting the chymotryptic activity of the 20S proteasome.^{96,97} Another source of inspiration for peptoid non-covalent proteasome inhibitors was found in HIV protease inhibitor ritonavir (**32**, Figure 1.10), which was found to inhibit the proteasome.⁹⁸ The related benzylstatine peptide **33** was synthesised in the course of a HIV protease project and optimisation of this motif yields a wide range of $\beta 5$ selective proteasome inhibitors.^{99–101} Further optimisation of linear peptide proteasome inhibitors led to **34** (Figure 1.10), which displays better cell-permeability than the benzylstatine peptides.¹⁰² Independently, **35** was found to be a potent and cell permeable noncovalent inhibitor of proteasome chymotryptic activity.^{93,103,104}

1.6.3 Peptide vinyl sulfones

Peptide vinyl sulfones, originally designed as inhibitors of cysteine proteases,¹⁰⁵ were discovered to irreversibly inhibit the proteasome when the aldehyde group in MG132 was replaced by a vinyl sulfone to give **36** (Figure 1.11A).¹⁰⁶ The proteasome's active site Thr1 O γ attacks the vinyl sulfone in a Michael addition way, forming a stable ether linkage with the inhibitor (Figure 1.11B). The irreversibility of proteasome inhibition and ease of synthesis of these vinyl sulfone compounds enables the use of these scaffolds for activity-based probes (ABP). The first vinyl sulfone ABP for the proteasome is compound **37**, which is

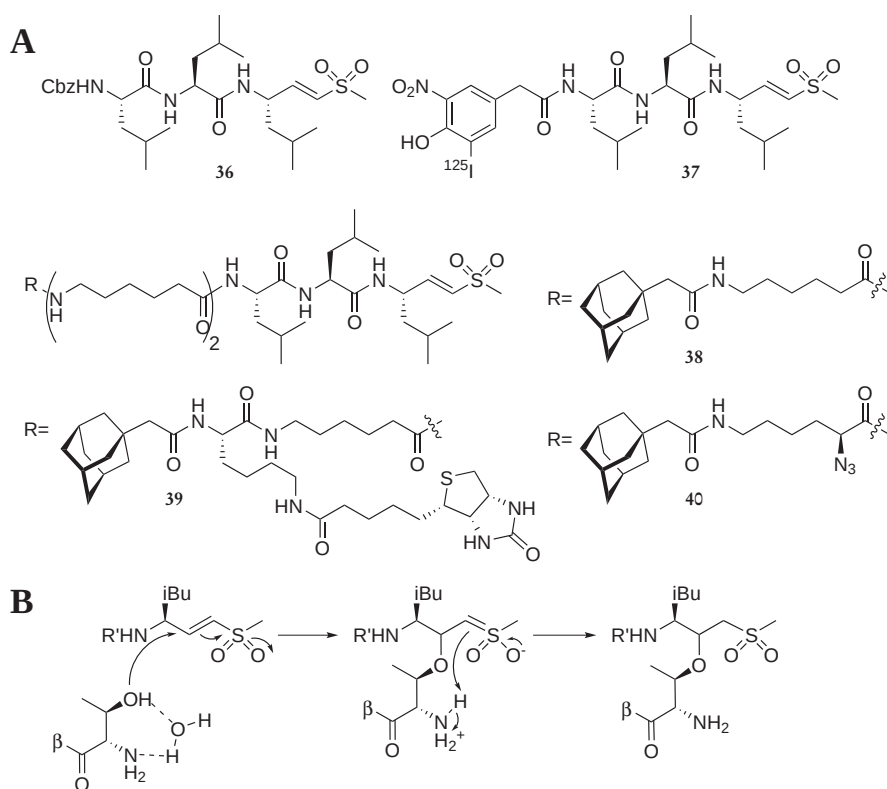


Figure 1.11: (A) Structure of vinyl sulfone inhibitors and ABPs. (B) Proteasome inhibition mechanism of vinyl sulfone electrophile.

labelled with ^{125}I and can be detected by autoradiography.¹⁰⁶ This probe has most affinity for the chymotryptic activity of the proteasome. N-terminal extension of the Leu₃-VS core, such as the adamantyl acetic acid-triaminohexanoic acid spacer in compound **38** yields an inhibitor with increased potency, good cell permeability and about equipotent activity for the three proteasomal subunits.¹⁰⁷ This finding was exploited by addition of a biotin as in compound **39** to yield an ABP that can be detected by immunoblotting. The presence of a biotin moiety, however, hampered cell permeability of this compound. To overcome this limitation, an azide group was introduced in the elongated inhibitor design to get a two-step labelling ABP, **40**. The azide moiety can be used to introduce a biotin after the probe bound its target, for example introduction of a biotin by the Staudinger-Bertozzi ligation.¹⁰⁸ This two-step labelling strategy is used often in cases where introduction of a large tag, such as biotin, negatively influences the biological characteristics of a probe. To be able to directly visualise labelled proteasomes in living cells, the Ahx₃-Leu₃-VS scaffold was decorated with a dansyl group to arrive at fluorescent ABP **41** (Figure 1.12).¹⁰⁹ This fluorescent ABP was used to study *in vivo* subunit specificity of anti-myeloma agent bortezomib. Substitution of the dansyl group by the BODIPY-TMR fluorophore yielded a probe (MV151, **42**) that allows detection of labelled proteasome subunits *in vivo* as well as directly in SDS-PAGE gels, greatly simplifying proteasome activity readout assays.¹¹⁰ Azido-BODIPY probe **43** combines direct readout by fluorescence with the presence of an azide moiety, opening up the possibility to directly study two-step labelling.¹¹¹

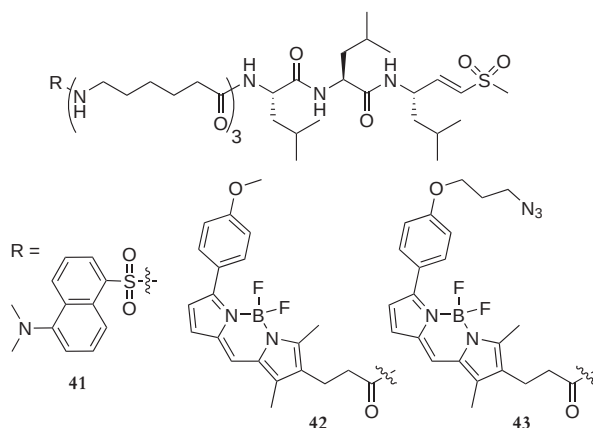


Figure 1.12: Structure of vinyl sulfone based fluorescent activity-based probes for the proteasome.

1.6.4 Synthetic peptide α',β' -epoxyketones

Synthetic epoxyketones were evaluated as proteasome inhibitors first by Spaltenstein et al. in 1996 (**44**, Figure 1.13).¹¹² Later, the cellular target of potent antitumour agents epoxomicin and eponemycin was determined to be the proteasome and the exclusive selectivity of epoxomicin for the proteasome was illustrated by biotinylated probe **45**.^{46,47,113} Attachment of BODIPY fluorophores to the epoxomicin scaffold yielded three epoxomicin analogues with different fluorescent properties. BODIPY-TMR-epoxomicin (MVB003, **49**) and azido-BODIPY-epoxomicin **50** show excellent subunit labelling in cells and have been used

to study the $\beta 5t$ subunit.^{114,115} The superior proteasome selectivity inherent to this electrophile lead to its widespread use in proteasome inhibitors and numerous epoxyketone peptides with remarkable potencies and selectivities have been synthesised. Several of these inhibitors will be discussed later in this Chapter.

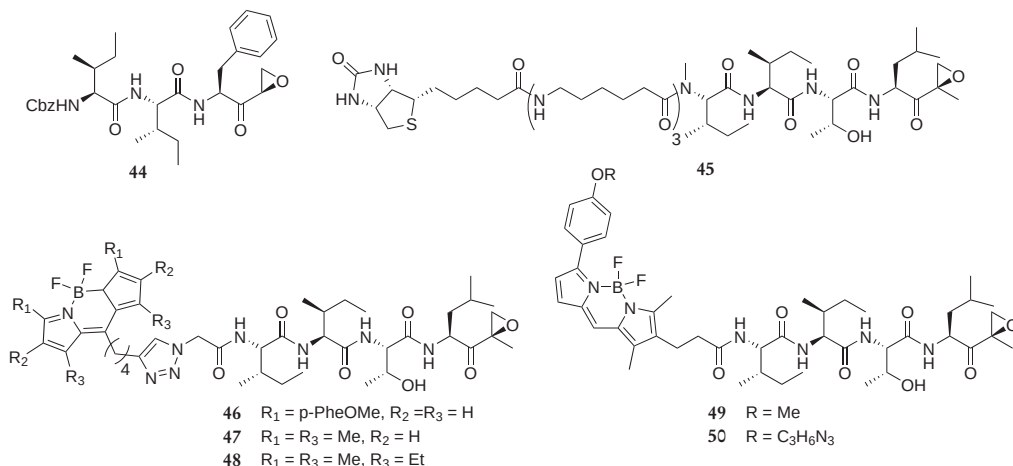


Figure 1.13: Structure of synthetic epoxyketone inhibitors and activity based proteasome probes.

1.7 Therapeutic implications of proteasome inhibition

Controlled protein degradation by the 26S proteasome is crucial for survival of eukaryotic cells. Among its targets are cell cycle regulators, tumour suppressors, oncogens and regulators of apoptosis. Tumour cells are more sensitive to proteasome inhibition than normal cells and this provides a therapeutic window for proteasome inhibitors to be used against cancers. Cytotoxicity of proteasome inhibitors in cancer cells is the result of several mechanisms.¹¹⁶ Interference with timed destruction of cell cycle regulators induces cell cycle arrest in malignant cells. $P27^{Kip1}$ is a tumour suppressor and various malignancies display enhanced ubiquitination of this protein, lowering its concentration due to degradation by the proteasome. Proteasome inhibition rescues this protein, causing cell cycle arrest and thus inhibits tumour cell proliferation.⁵⁸ The endoplasmic reticulum (ER) is responsible for the folding and maturation of proteins, and misfolded proteins are degraded by the proteasome. Exposure to proteasome inhibitors leads to prolonged accumulation of misfolded proteins which cause ER stress, a pro-apoptotic signal. Malignant cells are dividing quickly and have a high protein synthesis rate and are therefore more prone to pro-apoptotic ER stress. Apoptosis is tightly regulated by opposing activities of pro-apoptotic and anti-apoptotic proteins, whose levels are modulated by the proteasome. Inhibition of the proteasome leads to higher levels of pro-apoptotic proteins such as p53, Bax and Noxa, which are often inactivated in tumour cells.¹¹⁷ The proteasome is responsible for degradation of $I\kappa B$, an inhibitor of $NF-\kappa B$. $NF-\kappa B$ is a transcription factor that regulates various immune and inflammatory responses, stimulates proliferation and suppresses apoptosis and is upregulated in many cancer types. Bortezomib and other proteasome inhibitors prevent

the degradation of $\text{I}\kappa\text{B}$ and thereby $\text{NF-}\kappa\text{B}$ is inactivated, reducing proliferation and sensitizing the cells to apoptosis. Proteasome inhibitors show synergistic enhancement of cytotoxicity of other anticancer agents, such as melphalan and cyclophosphamide (inducers of DNA damage), dexamethasone (anti-inflammatory agent), doxorubicin (DNA intercalator that disrupts transcription and replication), and thalidomide.¹¹⁸

In 2003, bortezomib received FDA approval to be used against multiple myeloma and later against mantle cell lymphoma. Treatment of multiple myeloma with bortezomib is associated with many side effects, among which are peripheral neuropathy and thrombocytopenia, and this limits the dosage and therapeutic window of this drug.¹¹⁹ Upon prolonged exposure to bortezomib, cancers can become resistant to the action of bortezomib. This is thought to occur by a changed proteasome level and altered β subunit activity pattern.^{120,121} In cultured cells resistant to bortezomib, a mutation in the S1 of $\beta 5$ was found which reduces binding of bortezomib to this subunit. Whether this mutation plays a role in clinical resistance to bortezomib remains to be determined. Bortezomib displays no efficacy against solid tumours. These limitations of bortezomib sparked the generation of second generation proteasome inhibitors of which five are now in clinical trials (Figure 1.14).

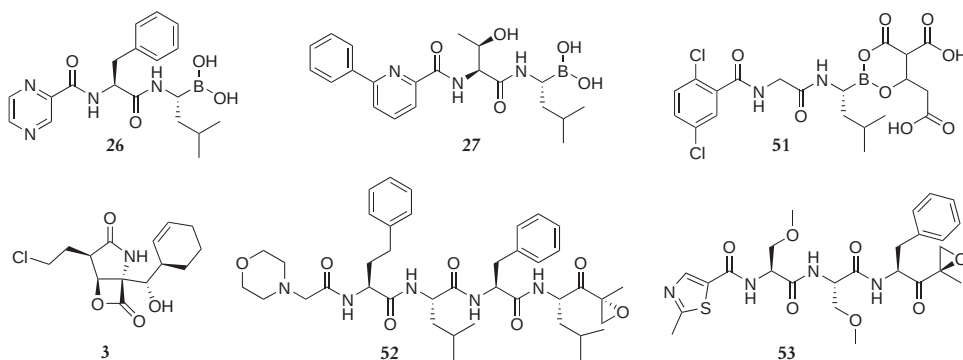


Figure 1.14: Structure proteasome inhibitor bortezomib (26) used clinically against multiple myeloma and structures of proteasome inhibitors in clinical trials against various types of cancer, CEP-18770 (27), MLN-9708 (51), salinosporamide A (marizomib, 3), carfilzomib (52), ONX-0912 (53).

Reversible covalent inhibitors 27 and 51 both can be administered orally and are in clinical trials against multiple myeloma, and 51 shows better activity against solid tumours than bortezomib. Irreversible binding epoxyketone based inhibitor carfilzomib (52) inhibits the chymotryptic-like proteasome activity and induces cancer cell death more potently than bortezomib, and was found to overcome resistance of tumour cells to bortezomib. Epoxyketone 53 displays similar subunit preference to carfilzomib and is optimised for oral bioavailability. In cultures of malignant cells, beta-lactone salinosporamide A (clinical name currently marizomib, 3), an inhibitor of chymotryptic and tryptic activity of the proteasome has been shown to overcome resistance to bortezomib, which inhibits chymotryptic and caspase-like proteasomal activity. Furthermore, salinosporamide A displays synergistic interaction with bortezomib, and is dependent on the stabilisation of pro-apoptotic proteasome substrates other than bortezomib for its anticancer efficacy.¹²² Compounds salinosporamide A (3), carfilzomib (52) and ONX-0912 (53) are in clinical trials against haematological tumours and in a number of solid tumours.¹¹⁷

Bortezomib displays a suppressive effect on activated cells of the immune system and its activity against proliferating cells is most likely at the basis of this effect. Bortezomib prolongs the function of transplants in mouse models of transplantation, most likely due to ER-stress triggered induction of apoptosis in cells producing antibodies against the transplant.¹²³ Proteasome inhibition also shows promising results in models of autoimmune diseases.¹²⁴

1.8 Subunit selective proteasome inhibitors

The proteasome is a very important part of cellular homeostasis in eukaryotic cells and modulators of proteasome activity have demonstrated a number of medicinal benefits. In mammals, seven distinct active β subunits are expressed and the individual role of each of these subunits in biological processes is not well understood. The function of proteolytic subunits of the proteasome cannot be studied individually as recombinantly expressed proteins since these proteolytic subunits only work in a fully assembled proteasome. Knockout of active β subunits is associated with defects in proteasome assembly and thereby obscures experimental outcome.¹²⁵ Subunit selective cell permeable inhibitors are useful tools to study individual subunit activities. Inhibition of individual proteasome subunits in vitro displayed reduction of degradation of model substrates depending on the subunit inactivated. Overall protein degradation in cells was halted only if two β sites were substantially inhibited, and cytotoxicity in most cancer cell lines could only be induced efficiently when, next to the important $\beta 5$ subunit, either $\beta 1$ or $\beta 2$ is inhibited as well.^{126–128} Whether the different mechanisms by which bortezomib and salinosporamide A induce apoptosis in malignant cells depends solely on their different proteasome subunit preference remains a question to be answered.

The proteasome and immunoproteasome are essential for the generation of MHC class I antigens but the role of each individual proteasome catalytic subunit on the generation of certain antigens is still subject of extensive study. However, mice lacking one or more immunoproteasome active β subunits show decreased efficiency of MHC class I presentation of pathogen epitopes and a lower T-cell response to these epitopes.^{10,129} These knockout studies suggest that antigen presentation may be changed by altering the activity of a single subunit. Very subunit selective and cell permeable proteasome inhibitors can be a great aid to verify results from knockout studies and determine therapeutic potential of proteasome inhibitor mediated shaping of the antigenic pool.

To date, an increasing number of subunit selective proteasome inhibitors has been reported. Subunit selectivity for a large part has been realized by optimisation of the P1 and P3 amino acids in oligopeptoid inhibitors, interacting with the S1 and S3 proteasome substrate binding pockets, which differ the most among subunits $\beta 1$, $\beta 2$ and $\beta 5$. This Chapter provides a summary of subunit selective inhibitors.

1.8.1 $\beta 1$ subunit

The proteasomal $\beta 1$ S1 selectivity pocket is characterised by a basic side chain of Arg45 of this subunit.¹¹ Consequently, peptides with an Asp or Glu at P1 show preference for $\beta 1$. However, the $\beta 1$ subunit also has the capacity to cleave after branched amino acids

and this promiscuity is demonstrated by the finding that Cbz-Gly-Pro-Phe-Leu aldehyde (**54**, Figure 1.15) shows preference for $\beta 1$.¹³ The proline at P3 is important in this motif since it seems to prevent binding to tryptic and chymotryptic sites. Optimisation of this motif led to YU102 (**55**) which is a less efficient $\beta 5$ inhibitor due to lack of aromaticity at its N-terminus.¹³⁰ A scanning positional library of P1 Asp-aldehyde yielded optimised P2-P4 sequence Ac-Ala-Pro-nLe.¹³¹ This P2-P4 motif was equipped with leucine p-phenol vinyl sulfone to give cell permeable inhibitor **56** with some selectivity for $\beta 1i$ over $\beta 1$, and introduction of an N-terminal azide gave probe **57** (Figure 1.15).¹³² Ultimately, the vinyl sulfone was changed for the more proteasome selective epoxyketone to arrive at NC001 (**58**) and azide analogue **59** as $\beta 1$ selective standard inhibitors to date.^{127,133} Attachment of a BODIPY fluorophore did not significantly decrease its $\beta 1$ selectivity and resulted in $\beta 1$ selective probe **60**.¹³³

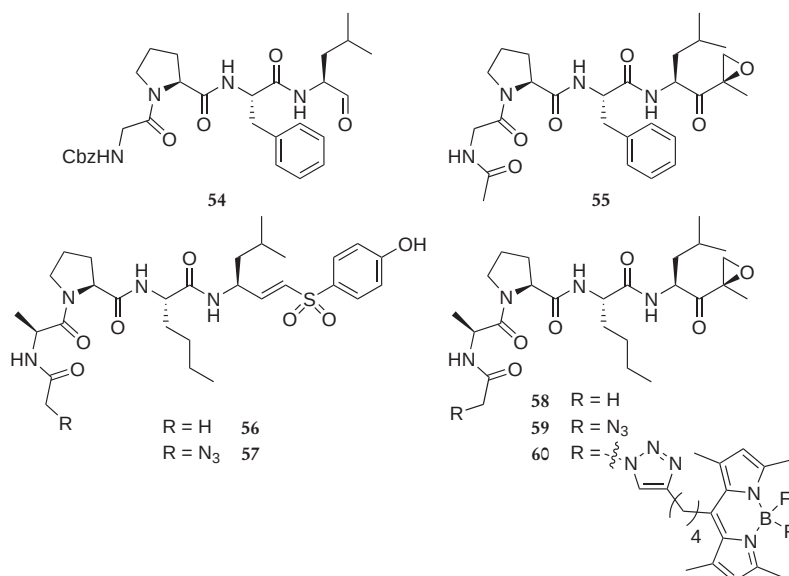


Figure 1.15: Structure of $\beta 1$ selective inhibitors and activity-based probes.

1.8.2 $\beta 1i$ subunit

The immunoproteasome more efficiently generates peptides with basic and hydrophobic amino acids at their C-termini while its caspase-like activity is reduced and this has been ascribed to an altered activity of $\beta 1i$ with respect to $\beta 1$. Subunit $\beta 1i$ of the immunoproteasome has changed significantly with respect to its constitutive counterpart.¹¹ Arg45 of $\beta 1$ is replaced for leucine which abrogates caspase-like activity and rather results in a more chymotryptic-like activity of this subunit.¹³⁴ The inhibitors presented in Figure 1.15 do not efficiently discriminate between $\beta 1$ and $\beta 1i$. Among analogues of eponemycin (**10**) in which bulk was introduced at the P1' position of the inhibitor, in the form of silyl ethers, *tert*-butyldimethylsilyl ether containing compound **61** (Figure 1.16) was found selective for $\beta 1i$ over $\beta 1$.¹³⁵ In this motif, P2 substitution for Lys and attachment of a BODIPY-650 or a fluorescein fluorophore resulted in $\beta 1i$ ABPs **62** and **63**.¹³⁶ Dipeptide aldehyde **64** has

selectivity for the immunoproteasome over constitutive proteasome and appears to target $\beta 1i$.¹³⁷

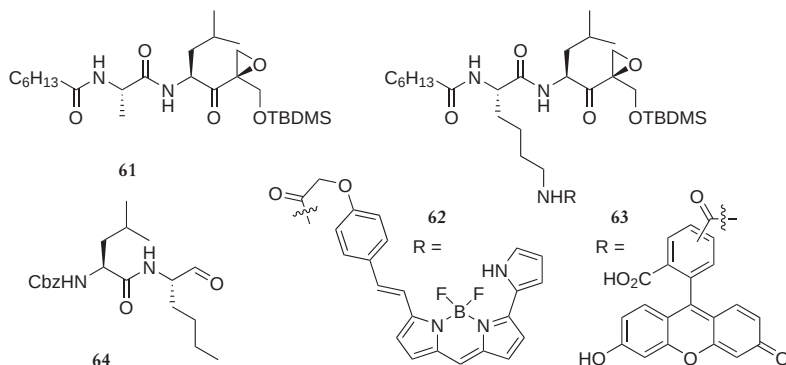


Figure 1.16: Structure of $\beta 1i$ selective inhibitors and activity-based probes.

1.8.3 $\beta 2$ subunit

The $\beta 2$ S1 pocket of the proteasome is spacious and characterised by Asp28 and Glu53 residues, explaining its preference for basic amino acid side chains.¹¹ Leupeptin is an inhibitor of $\beta 2$ due to its arginine aldehyde electrophile. The S3 pocket of $\beta 2$ contains a highly conserved cysteine side chain, Cys118, belonging to $\beta 3$. The reactivity of this moiety has been exploited for binding by the bifunctional aldehyde **65** (Figure 1.17). The aldehyde captures Thr1 O γ of $\beta 2$, while the N-terminal maleimide is positioned to capture Cys118 in the S3 binding pocket, resulting in potent and selective $\beta 2$ inhibition.¹³⁸

The barrel shaped structure of the proteasome, in which the β subunits are in a defined distance from each other, was exploited by bivalent aldehyde inhibitors in which the two electrophiles are separated by a long PEG spacer. The presence of a second aldehyde at a defined distance from the first greatly increased potency of the inhibitor with respect to its monovalent counterpart. When the sequence Arg-Val-Arg-aldehyde was used, such as in **66**, a profoundly $\beta 2$ selective inhibitor results.¹³⁹

The S3 pocket of $\beta 2$ has an acidic character due to the presence of two aspartic acids. A scanning positional library with asparagine vinyl sulfone at P1 stressed the importance of the P3 residue in a proteasome inhibitor for $\beta 2$ selectivity. Introduction of basicity at P3 yielded compound AcPRLN-VS (**67**) as selective $\beta 2$ inhibitor.¹⁴⁰ The requirement for basic amino acid residues in peptide based inhibitors decreases the ability of these compound to cross the cell membrane due to charged residues at physiologically pH. Ac-Arg-Leu-Arg-EK **68** is a recently discovered very specific inhibitor for $\beta 2$ in cell lysate, however, this compound is only active in cells at high concentration. Monobasic peptides such as HMB-Val-Ser-Arg-EK **69** and **70** display more efficient $\beta 2$ inhibition in cells.^{128,141} Compounds with selectivity for the $\beta 2i$ subunit over constitutive $\beta 2$, or vice versa, have not been reported to date.

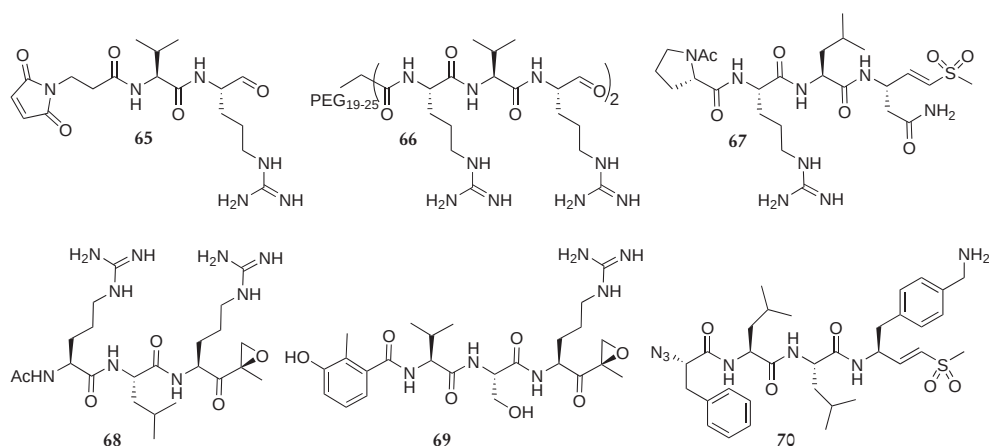


Figure 1.17: Structure of β_2 selective inhibitors.

1.8.4 β_5 subunit

The β_5 subunit prefers hydrophobic substrates, which is illustrated by a great number of hydrophobic noncovalent proteasome inhibitors that target the β_5 subunit, with examples **33**, **34**, **35** depicted in Figure 1.10. Epoxyketone YU101 (**71**, Figure 1.18), a very potent and selective inhibitor of chymotryptic activity of constitutive and immunoproteasome, was found by scanning the effect of introduction of large hydrophobic residues in the P2-P4 positions of a tetrapeptide epoxyketone.¹⁴² Modification of the N-terminus of this compound to improve its pharmacological profile led to carfilzomib (**52**), currently in clinical trials against various cancers.^{143,144}

In a medicinal chemistry effort that led to bioavailable epoxyketone ONX-0912 (**53**), multiple inhibitors have been discovered that inhibit chymotryptic activity of constitutive and immunoproteasome with about equal potency, for example **72** (Figure 1.18). However,

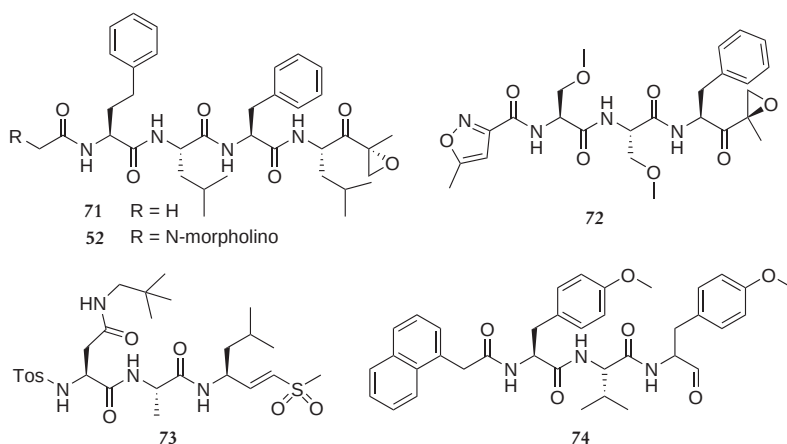


Figure 1.18: Structure of inhibitors selective for β_5/β_{5i} .

this study does not report on the effects of this compound on $\beta 1$ or $\beta 2$.¹⁴⁵ Hydrophobic bulky residues at the P3 position and aromaticity at the N-terminus of a proteasome inhibitor are factors beneficial for targeting of the chymotryptic activity of the proteasome. This is demonstrated by P3 neopentylasparagine containing potent and selective vinyl sulfone **73** (Figure 1.18), discovered when optimising a motif analogous to PSI (**25**) by screening different P3 residues and N-terminal caps.⁸⁷

Optimisation of inhibition of chymotryptic activity of tyropeptin A (**8**) afforded reversible inhibitor **74** (Figure 1.18).¹⁴⁶ Structural elements from compound **74** inspired the synthesis of azide containing ABP **75** (Figure 1.19), a very selective irreversible inhibitor of $\beta 5$.^{127,133} Change of the naphthyl group in **75** for a BODIPY dye preserved $\beta 5$ selectivity and afforded fluorescent and azide containing ABP **76**.¹³³ Incorporation of fluorinated amino acids at P2 and P3 of a tetrapeptide epoxyketone led to the discovery of compound **77**, which was found to be very selective for $\beta 5$.¹⁴⁷ This inhibitor could be transformed into $\beta 5$ selective fluorescent ABP by attachment of BODIPY fluorophore.¹⁴⁸

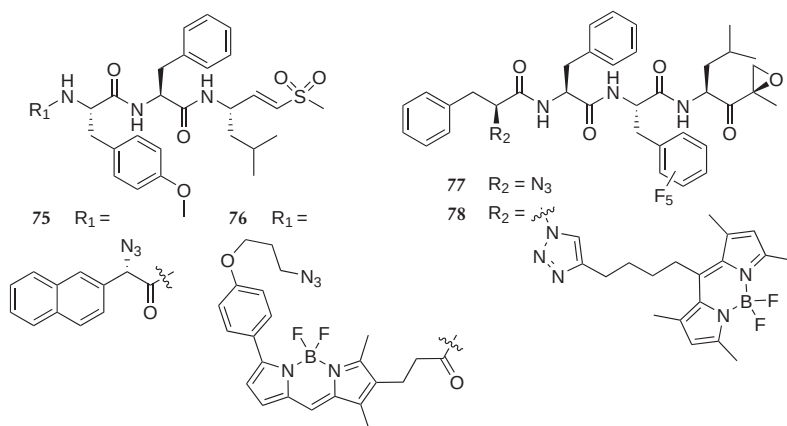


Figure 1.19: Structure of $\beta 5/\beta 5i$ selective activity-based probes.

1.8.5 Discrimination between $\beta 5$ and $\beta 5i$ subunit

The medicinal chemistry efforts that led to the discovery of ONX-0912 (**53**, Figure 1.14) identified compound **79** (Figure 1.20) as an inhibitor selective for $\beta 5$ over $\beta 5i$.¹⁴⁵ Interestingly, $\beta 5$ inhibitor **79** differs from the equipotent $\beta 5/\beta 5i$ inhibitor **72** (Figure 1.18) only in the amino acid at P1. Apparently, Phe at P1 is favourable for $\beta 5i$ preference while a $\beta 5$ inhibitor seems more selective carrying Leu at P1.¹⁴⁵ Compound **80** targets $\beta 5i$ before $\beta 5$ and this molecule indeed contains Phe at P1 and interestingly, it bears a D-alanine at P3.^{145,149,150} PR957 is another $\beta 5i$ selective inhibitor, carrying Phe at P1 and like **80** possesses an aromatic P2 group, which could be favourable for $\beta 5i$ inhibition. PR957 causes decreased MHC class I presentation of a $\beta 5i$ dependent epitope.¹⁵¹ This finding stresses the importance of the development of specific, cell permeable inhibitors of each of the active subunits of the proteasome to determine their ability to block presentation of certain epitopes.

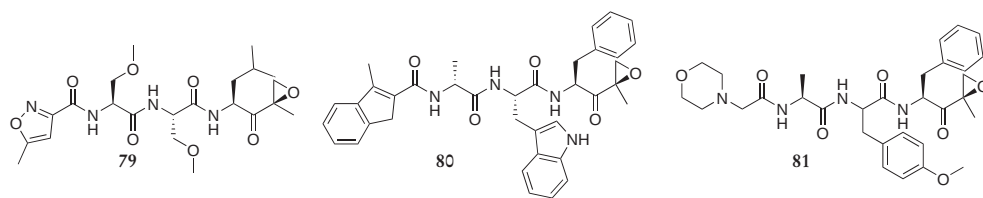


Figure 1.20: Structure of inhibitors able to discriminate between $\beta 5$ and $\beta 5i$.

1.9 Aim and outline of this Thesis

The research described in this Thesis aims at the development of novel subunit selective inhibitors of the proteasome. In Chapter 2 three vinyl sulfone analogues of three epoxyketone containing inhibitors described in literature are synthesised and characterised. Vinyl sulfones **82** and **83** (Figure 1.21) are more selective for $\beta 5$ activity than their epoxyketone analogues.

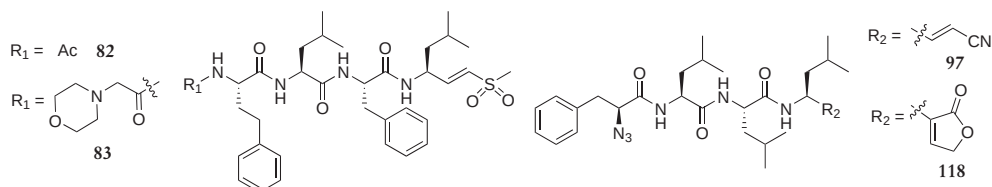


Figure 1.21: Structure of $\beta 5/\beta 5i$ selective inhibitors **82** and **83**. Structure of Michael acceptors **97** and **118**.

In Chapter 3, ten Michael acceptors are coupled to three peptoid tails yielding thirty peptide-like compounds. These compounds are assessed on their capacity to inhibit the proteasome in cell lysate but none of the peptoid Michael acceptors are efficient proteasome inhibitors. Using the Staudinger-Bertozzi ligation followed by affinity purification, tryptic digestion of the isolated proteins and LC/MS² identification, the targets of **97** and **118** (Figure 1.21) in HEK293T and RAW264.7 cells are determined.

Syringolins are potent proteasome inhibitors found in nature and contain an unusual valine-urea-valine motif. In Chapter 4 this motif was built in peptoid epoxyketones and vinyl sulfones yielding a 16-membered library of proteasome inhibitors. The distance between the urea and electrophile dictates subunit selectivity. Tripeptide **182** (Figure 1.22) is the most potent and selective inhibitor of $\beta 1$ in HEK cells reported to date. The longer tetrapeptides **190** and **192** preferentially inhibit $\beta 5$ and are more potent than the parent compound, syringolin A.

In Chapter 5, three cyclooctynes are compared to the Staudinger-Bertozzi two-step labelling strategy using azido-BODIPY labelled proteasome as test system. Although cyclooctynes quantitatively convert azide labelled proteasomes at a lower concentration than the Staudinger-Bertozzi phosphane, cyclooctyne two-step labelling is associated with tremendous background labelling.

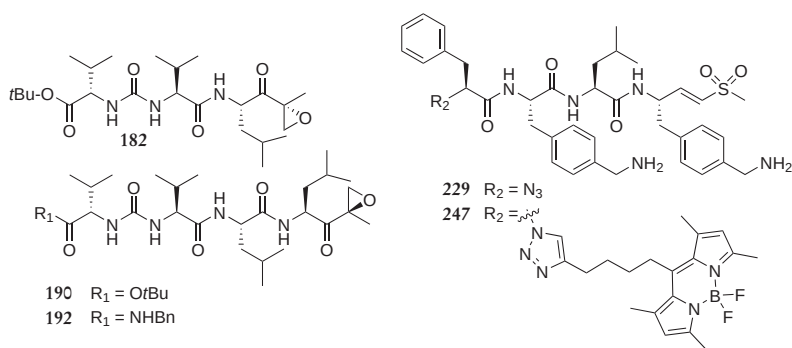


Figure 1.22: Structure of β 1 selective inhibitor **182**, β 5 selective inhibitors **190** and **192**, β 2 selective inhibitor **229** and fluorescent β 2 probe **247**.

Chapter 6 describes the synthesis and characterisation of a library of peptoid vinyl sulfones with basic amino acid side chains on P1 and P3. Compound **229**, with a benzylamine residue at both P1 and P3, is a potent and β 2 selective proteasome inhibitor. Attachment of a BODIPY fluorophore yielded β 2 selective probe **247** (Figure 1.22). The presence of two basic residues, however, reduced their entry into cells.

2

Subunit selectivity of a proteasome inhibitor is influenced by the electrophile*

2.1 Introduction

The 26S proteasome plays a crucial role in many cellular processes in eukaryotes. The eukaryotic 20S proteasome has three proteolytic activities, namely $\beta 1$ (caspase like), $\beta 2$ (trypsin like) and $\beta 5$ (chymotrypsin like). Upon stimulation of cells by interferon- γ , three catalytic subunits with altered substrate preference ($\beta 5i$, $\beta 2i$, $\beta 1i$) are expressed and built into the immunoproteasome.^{152,153} These activities were assigned according to fluorogenic substrate hydrolysis assays.¹² The prokaryotic 20S proteasome has only one proteolytic activity,¹⁵⁴ and the evolutionary role of the quite distinct functionalities in mammals is uncertain. Knockout studies in yeast showed that $\beta 5$ is the most important catalytic activity for viability since mutation of the active site threonine of this subunit caused the most phenotypic defects, including significant growth retardation, while inactivation of $\beta 1$ and $\beta 2$ caused less severe defects.^{155–157} The importance of $\beta 5$ for proteasome activity for cellular growth and viability, combined with the hydrophobic nature of most $\beta 5$ targeting inhibitors, favourable for cell permeability, focused the search for proteasome inhibitors on $\beta 5/\beta 5i$ inhibiting compounds.¹⁵⁸

Proliferating cells are more sensitive to proteasome inhibitors than non-proliferating cells and this makes proteasome inhibition a powerful strategy in the treatment of cancer.^{158–161} The approval of proteasome inhibitor bortezomib in 2003 to be used against multiple myeloma and mantle cell lymphoma validated proteasome inhibition as a strategy

*M. Screen, M. Britton, S. L. Downey, M. Verdoes, M. J. Voges, A. E. M. Blom, P. P. Geurink, M. D. P. Risseuw, B. I. Florea, W. A. van der Linden, A. A. Pletnev, H. S. Overkleeft, A. F. K. Kisselev, *J. Biol. Chem.* 2010, 285, 40125-40134.

against cancer.^{162,163} At the time of approval it was believed that bortezomib selectively targets $\beta 5/\beta 5i$, however, bortezomib was later found to also target $\beta 1/\beta 1i$ and $\beta 2i$ at clinically used doses.^{109,126,164} This raised the question what the clinical relevance of each of the proteasome subunit is. It was found that the effect of subunit selective proteasome inhibitors on proteasome substrate degradation was the largest for $\beta 5$ selective inhibition, but co-inhibition of $\beta 1$ or $\beta 2$ was necessary to completely stall degradation by the proteasome.¹²⁶ Next, a study on cell survival using selective proteasome inhibitors for $\beta 5$ and $\beta 1$ or $\beta 2$ showed that cytotoxicity of proteasome inhibition, in most cell lines, correlates badly with pure $\beta 5$ inhibition and that the partial co-inhibition of $\beta 1$ or $\beta 2$ greatly reduced the concentration of $\beta 5$ inhibitor necessary to induce cytotoxicity.^{127,128}

More recently, specific inhibitors of the immunoproteasome emerged.^{137,150,151} The specific role of the immunoproteasome in immunological processes remains to be elucidated and small molecule inhibitors selective for the immunoproteasome are valuable assets in these studies. Moreover, preliminary studies suggest these compounds may find applications both as a drug against certain cancers and against immunological diseases.^{137,151,165}

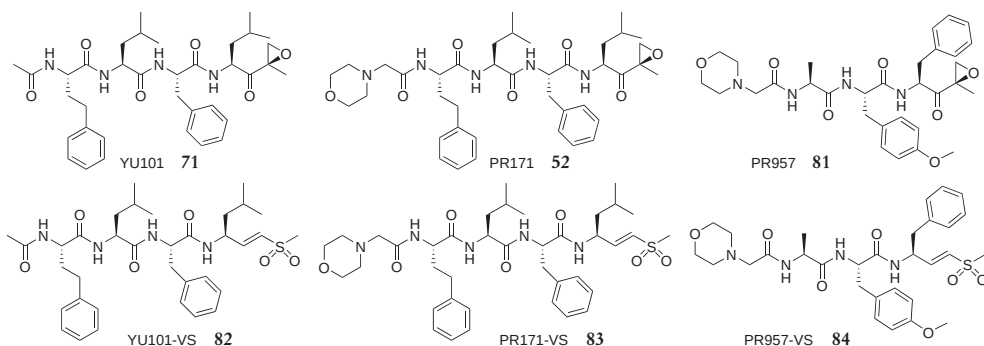


Figure 2.1: Two $\beta 5/\beta 5i$ selective inhibitors (71, 52) and $\beta 5i$ selective inhibitor 81, and their vinyl sulfone analogues 82, 83 and 84.

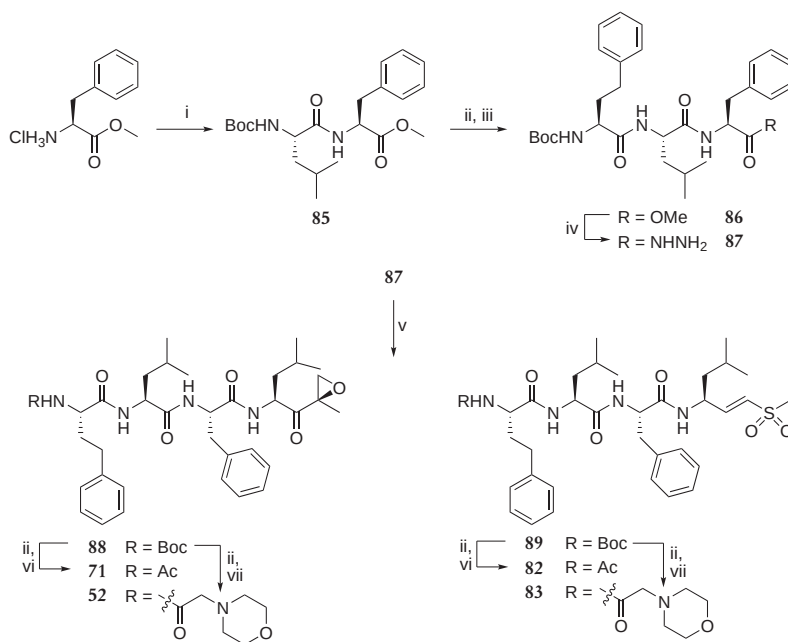
This chapter focuses on three proteasome inhibitors (52, 71, 81, Figure 2.1). YU101 was designed over a decade ago as a very potent and selective inhibitor of the chymotryptic activity of both constitutive and immunoproteasome.¹⁴² PR171, also known as carfilzomib, differs from YU101 only in the end cap, which optimises the pharmacological parameters of this compound. PR171 is now in clinical trials for the treatment of multiple myeloma.^{143,144} PR957 was identified as a specific inhibitor of the $\beta 5i$ subunit only.¹⁵¹ The three compounds in question have the epoxyketone as electrophilic trap to target the catalytically active amino acid of the preferred subunits of the proteasome. The epoxyketone is the most selective electrophile to target the proteasome since it reacts with N-terminal threonine proteases only, thereby abolishing inhibition of common side targets of other proteasome warheads, namely cysteine proteases (vinyl sulfones)¹⁰⁶ or serine proteases (boronic acids),¹⁶⁶ and it not surprising that epoxyketones are the electrophile of choice by medicinal chemists when designing proteasome inhibitors. However, an earlier study revealed that changing the epoxyketone electrophile in an inhibitor with $\beta 5$ preference for a vinyl sulfone electrophile yielded an inhibitor with even more selectivity for $\beta 5$, 75 (Figure 1.19, page 20).¹³³ In this chapter, therefore, the epoxyketone in YU101, PR171 and PR957 is re-

placed by the vinyl sulfone, to determine if this change in electrophile results in a different biological profile of the inhibitors.

2.2 Results and Discussion

The electrophiles necessary to synthesise the compounds shown in Figure 2.1, leucine and phenylalanine vinyl sulfone and epoxyketone, were synthesised using protocols for leucine vinyl sulfone (VS)¹⁰⁶ and epoxyketone (EK).¹⁶⁷ Compounds **71**, **52** and **82**, **83** contain an identical peptide portion, which was synthesised by standard Boc-protected solution peptide chemistry to arrive at **86** (Scheme 2.1). The peptide portion is block coupled to the appropriate electrophile. Since a peptide block coupling, in which a peptide-carboxylic acid

Scheme 2.1: Synthesis of PR171 and YU101 and their vinyl sulfone analogues.



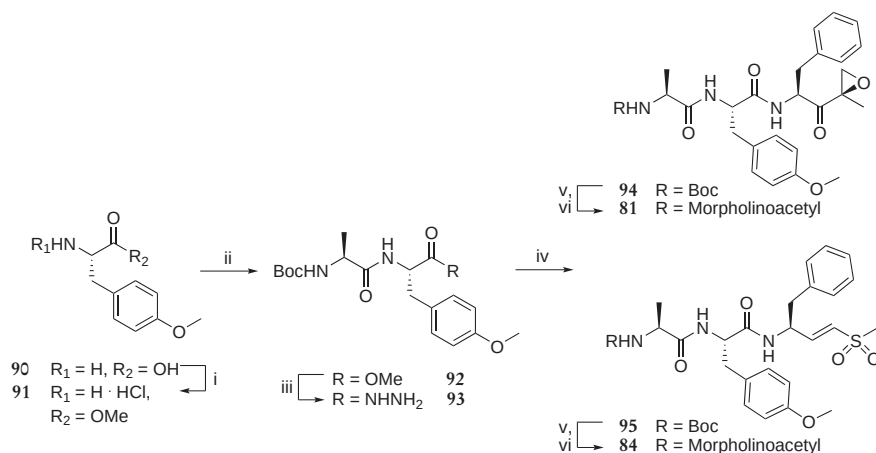
Reagents and conditions: i) Boc-Leu-OH (1.2 equiv.), HCTU, (1.2 equiv.), DiPEA (3.5 equiv.), DCM, o/n, quant. ii) DCM:TFA 1:1, 15-30 min, quant. iii) Boc-Phe-OH (0.9 equiv.), HCTU (1.1 equiv.), DIPEA (3.5 equiv.), DMF, 2 hr., 83%. iv) hydrazine hydrate, MeOH, reflux, 3 hr., 77%. v) *t*BuONO (1.1 equiv.), HCl (2.8 equiv.), DMF/DCM (1:1), -30°C, 3hr. then TFA·H-Leu-EK or TFA·H-Leu-VS (1.1 equiv.), DiPEA (5 equiv.), DMF, -30°C → RT, o/n, 92% (**88**), 78% (**89**). vi) Ac₂O (2.2 equiv.), DiPEA (2.1 equiv.), DCM, 36% (**71**), 90% (**82**). vii) 2-morpholinoacetic acid TFA salt (1.2 equiv.), HBTU (1.2 equiv.), DiPEA (4.5 equiv.), 2 hr., 24% (**52**), 69% (**83**).

is activated, can cause considerable racemisation at the carbon α of the carboxylic acid to be activated, a different method is preferred. To block-couple without considerable racemisation, the azide coupling was employed.^{115,168} The methyl ester in **86** was transformed to the hydrazide by treatment with hydrazine hydrate in methanol. In a one pot procedure, the hydrazide was converted to the acyl azide with *t*BuONO and HCl, which was then reacted with TFA·H-Leu-EK or TFA·H-Leu-VS to yield **88** and **89** as single diastereomers. Re-

removal of the Boc protecting groups in **88** and **89** followed by either acetylation with acetic anhydride or coupling to 2-morpholinoacetic acid under the influence of HBTU yielded inhibitors **71** and **52** and potential inhibitors **82** and **83**.

PR957 and vinyl sulfone analogue **84** were synthesised using a similar strategy. Boc protected dipeptide **92** was synthesised from Boc-Ala-OH and methyl ester **91** with HBTU as coupling reagent (Scheme 2.2). The methyl ester **92** was transformed to the hydrazide which was then coupled to TFA·H-Phe-EK or TFA·H-Phe-VS in the two step procedure mentioned above to yield tripeptides **94** and **95**. These were, after Boc-deprotection, coupled to 2-morpholinoacetic acid under the influence of HBTU to yield target compounds **81** and **84**.

Scheme 2.2: Synthesis of PR957 and PR957-VS.



Reagents and conditions: i) $SOCl_2$ (6 equiv.), MeOH, 24 hr., quant. ii) Boc-Ala-OH (1.2 equiv.), HBTU (1.2 equiv.), DiPEA (5.25 equiv.), DCM, 99%. iii) hydrazine hydrate, MeOH, reflux, 2.5 hr., 93%. iv) $tBuONO$ (1.1 equiv.), HCl (2.8 equiv.), DMF/DCM (1:1), $-30^\circ C$, 3 hr. then TFA·H-Phe-EK or TFA·H-Phe-VS (1.1 equiv.), DiPEA (5 equiv.), DMF, $-30^\circ C \rightarrow RT$, o/n, 72% (**94**), 64% (**95**). v) TFA:DCM (1:2), 1 hr., quant. vi) 2-morpholinoacetic acid TFA salt (1.2 equiv.), HBTU (1.2 equiv.), DiPEA (4.5 equiv.), 2 hr., 67% (**81**), 81% (**84**).

The effect of compounds **52**, **71** and **81-84** was assessed using both a fluorogenic peptide hydrolysis assay and competition versus fluorescent proteasome probe MVB003. The IC_{50} values were calculated from these data and the results are summarised in Table 2.1. Consistent with what is reported in literature, in the fluorogenic peptide hydrolysis assay (Table 2.1A) YU101 (**71**) and PR171 (**52**) are potent inhibitors of the constitutive proteasome, with a preference for $\beta 5$ and $\beta 5i$. PR957 (**81**), identified as a $\beta 5i$ specific inhibitor, indeed has a lower IC_{50} value against the immunoproteasome than the constitutive proteasome. The vinyl sulfones **82** and **84** are less potent than their epoxyketone analogues **71** and **81**. The most dramatic loss of potency is observed for PR957-VS (**84**), showing only slight inhibition of constitutive proteasome subunits, and selectivity among immunosubunits has decreased, in contrast to $\beta 5i$ selective epoxyketone **81**. Vinyl sulfone **83** on the other hand appears more potent versus $\beta 5$ than epoxyketone **52**. The IC_{50} values deduced from the competition assays (Table 2.1B) also reflect the trend in loss of potency when changing the

epoxyketone warhead to the vinyl sulfone. However, the subunit specificity improvement, as observed in the fluorogenic peptide hydrolysis assay, is less pronounced and the IC₅₀ values differ significantly. This discrepancy in outcome between the competition assay and fluorogenic substrate readout can be caused by the difference between purified proteasome and cell lysate. Next, one should be careful when comparing results from two different analytical methods and settings.

Table 2.1: Apparent IC₅₀ (μM) values calculated from semi log plots of residual proteasome activity against inhibitor concentration.

	Fluorogenic peptide hydrolysis assay						Quantified competition assay					
	β5	β1	β2	β5i	β1i	β2i	β5	β1	β2	β5/β5i	β1i	β2i
	26S proteasome			Immunoproteasome			HEK293T lysate			EL4 lysate		
Compound	IC ₅₀ (μM) A			IC ₅₀ (μM) A			IC ₅₀ (μM) B			IC ₅₀ (μM) B		
YU101 (71)	~0.001	2.6	2.2	0.26	4.1	2.0	0.023	1.2	0.20	0.037	0.40	0.21
YU101-VS (82)	~0.004	>100	>100	2.2	>100	>100	0.21	>50	21	2.2	2.2	12
PR171 (52)	0.069	2.7	1.5	~0.001	2.5	0.56	0.043	2.3	0.33	0.74	0.66	0.19
PR171-VS (83)	0.007	32	16	0.012	20	6.2	0.43	N.I.	8.8	6.9	4.7	8.9
PR957 (81)	0.27	6.1	1.2	0.012	5.7	1.0	0.61	>50	2.1	0.52	2.2	2.5
PR957-VS (84)	57	>100	82	1.4	54	47	39	N.I.	41	13	>50	N.I.

(A) Constitutive proteasomes purified from rabbit muscles or immunoproteasomes, purified from rabbit spleens, were incubated with different concentrations of inhibitors for 30 min at 37°C followed by measuring remaining activity with fluorogenic peptides (Suc-LLVY-AMC, β5/β5i, Ac-LPnLD-AMC, β1/β1i, Ac-RLR-AMC, β2/β2i). (B) Band intensities from each lane of the competition assay gels in Figure 2.2, Figure 2.3 or Figure 2.4 were quantified and used as input. N.I. no inhibition.

Ideally, a specific inhibitor for one of the proteasomes active sites totally silences one subunit while leaving the other subunits untouched. The IC₅₀ value of a compound is not a good measure for inhibitor selectivity since it gives no direct information about the co-inhibition of other sites when one subunit is fully inhibited. Therefore it is more informative to look at the effect of the inhibitor at all measured inhibitor concentrations.

YU101 (71) is a selective inhibitor of the β5 subunit of the proteasome. However, the maximal inhibition of β5, before other subunits are influenced, is about 90% at 0.1 μM in the fluorogenic substrate assay (Figure 2.2A) and thus this inhibitor can not fully inhibit β5 without influencing other subunits. Compound YU101 (71) is not very selective when it comes to immunoproteasome subunits; simultaneous reduction of activity of the three subunits is observed from 10 nM onwards, after which a sudden drop in β5i activity is seen for 0.1 μM onwards (Figure 2.2E). According to the fluorogenic substrate assays, YU101 (71) is able to inhibit 80% of β5 activity at 0.01 μM, a concentration at which it has no significant influence on the immunoproteasome. This finding, however, should be verified in a biologically more relevant setting. YU101-VS (82) is about 10-fold less potent for the β5 subunit with respect to YU101 (71). Only slight inhibition of β1 and β2 is observed at 100 μM for YU101-VS (82) according to fluorogenic substrate assay (Figure 2.2D). YU101-VS (82) is able to inhibit β5 almost completely (>95%) at 10 μM, at which point the β1 and β2 activities remain intact. YU101-VS (82) is also more selective within the immunoproteasome; about 80% reduction of β5i activity is reached before the other subunits are inhibited significantly (10 μM, Figure 2.2H). YU101-VS (82) retains apparent β5 selectivity over immuno-counterparts; 80% of β5 activity is inhibited at 0.01 μM, when other

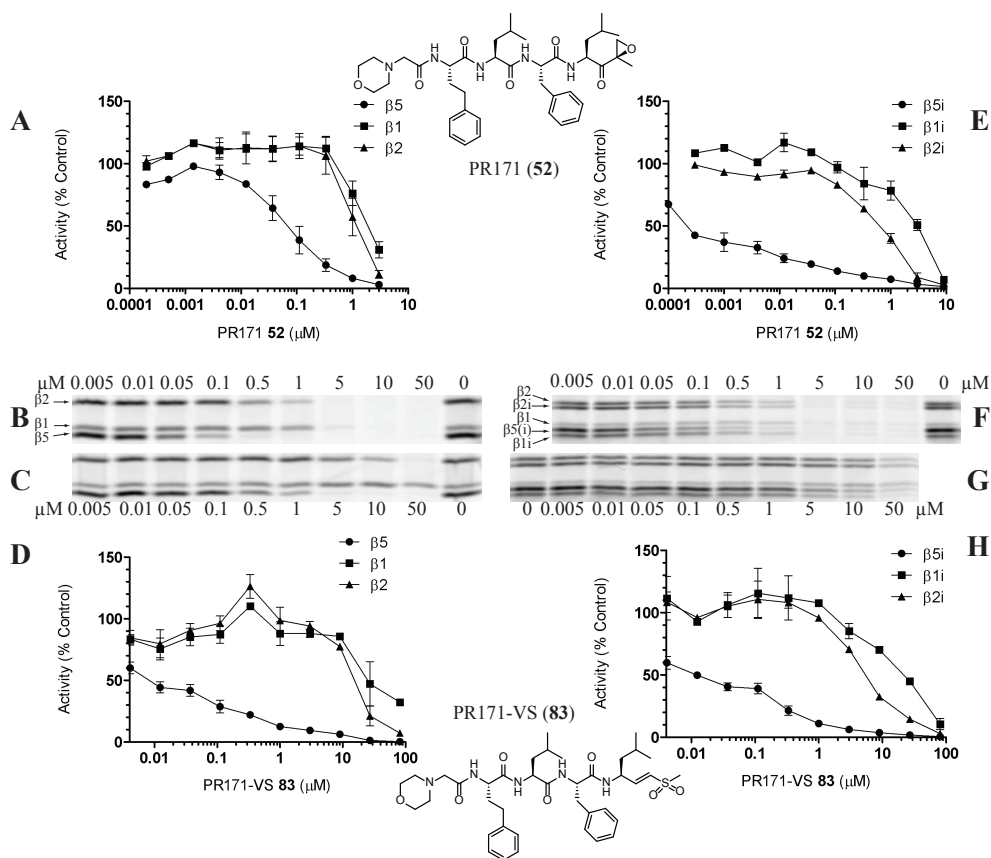


Figure 2.3: Fluorogenic peptide hydrolysis assay and competition assay of compounds PR171 (52) and PR171-VS (83). Fluorogenic peptide hydrolysis assay: 26S proteasomes isolated from rabbit muscles ((A) 52, (D) 83) or rabbit spleens ((E) 52, (H) 83) were incubated with inhibitors for 30 min. at 37°C followed by measurements of all three peptidase activities (circles, $\beta 5/\beta 5i$ activity (Suc-LLVY-AMC), squares, $\beta 1/\beta 1i$ activity (Ac-LPnLD-AMC), triangles, $\beta 2/\beta 2i$ activity (Ac-RLR-AMC). Competition assay in HEK293T lysates (15 μg) ((B) 52, (C) 83) or EL4 lysates (10 μg) ((F) 52, (G) 83) were incubated with indicated end concentrations of inhibitor for 1 hr. at 37°C. Residual proteasome activity was labelled by MVB003 (0.5 μM end concentration) for 1 hr. at 37°C.

$\beta 5$ at lower concentrations than PR171 (52), according to the fluorogenic peptide hydrolysis assay (Figure 2.3D versus A). The $\beta 1$ and $\beta 2$ subunits, in the presence of increasing concentrations of PR171-VS (83), first appear more active than the DMSO control in the fluorogenic peptide assay (Figure 2.3D). This upregulation effect has been observed earlier but is not understood.¹³¹ At 0.5 μM of vinyl sulfone 83, activities of $\beta 1$ and $\beta 2$ decrease again with a sudden drop from 10 μM and higher concentrations. PR171-VS (83) appears to achieve about 90% inhibition of $\beta 5$ at 5 μM , before the activity of remaining subunits becomes lower than the DMSO control. Thus, vinyl sulfone 83 is more selective than epoxyketone 52, since 52 is able to inhibit only 80% of $\beta 5$ activity at 0.5 μM before significantly inhibiting $\beta 1$ and $\beta 2$. Interestingly, the potencies of PR171-VS (83) for $\beta 5$ and $\beta 5i$, according to the fluorogenic substrate assay, are roughly equivalent. In the competition assay in HEK lysate, vinyl sulfone 83 is about tenfold less potent than epoxyketone 52, in contrast

to the fluorogenic peptide hydrolysis assay. PR171-VS (**83**) also shows relatively lower inhibitory activity versus the $\beta 1$ subunit (Figure 2.3B versus C) and thus also appears more selective for $\beta 5$ than epoxyketone **52**. This trend is not observed in EL4 lysate (Figure 2.3F versus G).

In the fluorogenic substrate assay graph of PR957 (**81**) on the constitutive proteasome it is apparent that onset of inhibition of the three subunits occurs simultaneously (0.01 μM), albeit $\beta 5$ inhibition proceeds more strongly at higher concentrations (Figure 2.4A). PR957 (**81**) appears to prefer to inhibit $\beta 5$ i in the fluorogenic substrate assay, although PR957 shows considerable inhibition of other subunits before $\beta 5$ i is silenced (Figure 2.4A and E). Again, selectivity for $\beta 5$ i can not be verified with the competition assay in EL4 lysate ((Figure 2.4F). Vinyl sulfone PR957-VS (**84**) is a poor inhibitor for the constitutive proteasome and is not selective according to the competition assay (Figure 2.4C and D) and

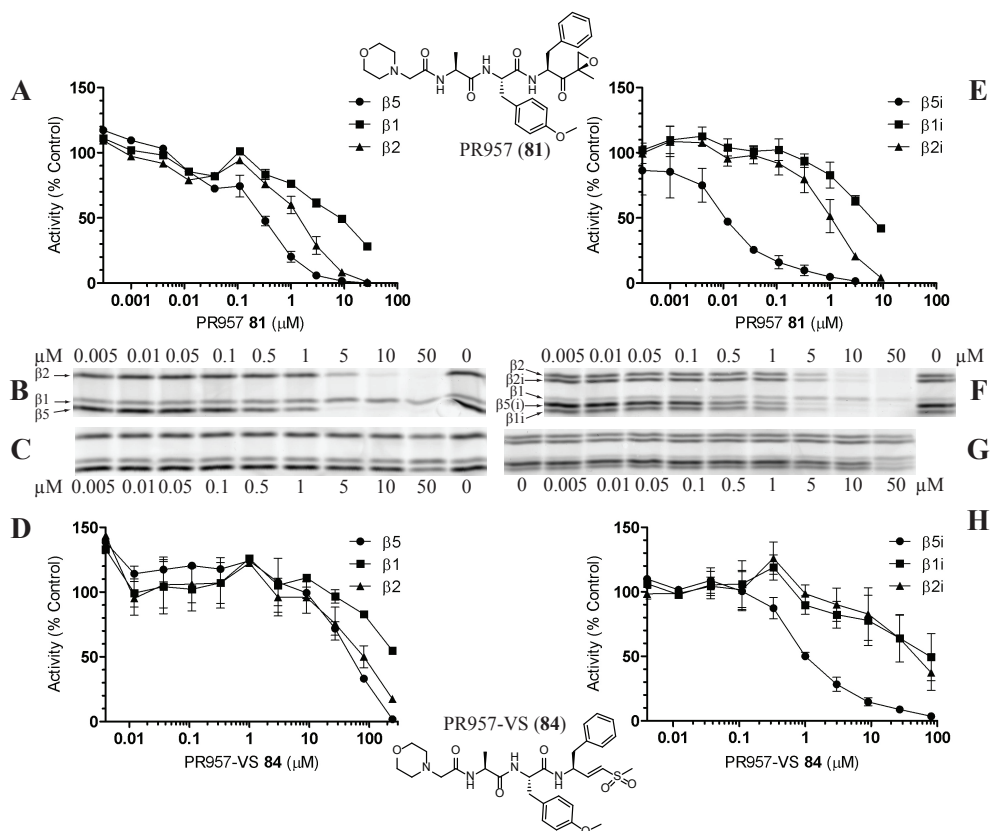


Figure 2.4: Fluorogenic peptide hydrolysis assay and competition assay of compounds PR957 (**81**) and PR957-VS (**84**). Fluorogenic peptide hydrolysis assay: 26S proteasomes isolated from rabbit muscles ((A) **81**, (D) **84**) or rabbit spleens ((E) **81**, (H) **84**) were incubated with inhibitors for 30 min. at 37°C followed by measurements of all three peptidase activities (circles, $\beta 5/\beta 5$ i activity (Suc-LLVY-AMC), squares, $\beta 1/\beta 1$ i activity (Ac-LPnLD-AMC), triangles, $\beta 2/\beta 2$ i activity (Ac-RLR-AMC). Competition assay in HEK293T lysates (15 μg) ((B) **81**, (C) **84**) or EL4 lysates (10 μg) ((F) **81**, (G) **84**) were incubated with indicated end concentrations of inhibitor for 1 hr. at 37°C. Residual proteasome activity was labelled by MVB003 (0.5 μM end concentration) for 1 hr. at 37°C.

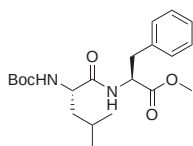
in EL4 lysate a considerable drop in potency with respect to PR957 (**81**) is also observed. The fluorogenic substrate assay shows that selectivity and potency of PR957-VS (**84**) for the $\beta 5i$ subunit are lower than that of PR957 (Figure 2.4E and H).

2.3 Conclusion

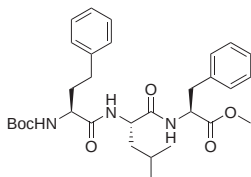
This Chapter describes the synthesis of three epoxyketone containing potent and selective proteasome inhibitors, their vinyl sulfone counterparts and their biological analysis by the competition assay in HEK293T and EL4 lysates as well as fluorogenic peptide hydrolysis assay with purified constitutive and immunoproteasome. Compound YU101 (**71**), designed as a selective $\beta 5$ inhibitor, shows co-inhibition of $\beta 1$ and $\beta 2$ before $\beta 5$ is completely silenced. This compound is more active against the constitutive than the immunoproteasome, as assessed with fluorogenic substrate hydrolysis assay. Epoxyketone YU101 (**71**) is about tenfold more potent than its vinyl sulfone analogue **82**, but vinyl sulfone **82** shows less co-inhibition of $\beta 1$ and $\beta 2$ and therefore is a more selective inhibitor of $\beta 5$ than the parent compound YU101 (**71**). YU101-VS (**82**) is selective for $\beta 5$ over $\beta 5i$ in the fluorogenic peptide hydrolysis and YU101-VS (**82**) could be a valuable tool for very selective $\beta 5$ inhibition. PR171 (**52**), in clinical trials against multiple myeloma, shows earlier onset of $\beta 5i$ inhibition than $\beta 5$ inhibition in the fluorogenic peptide hydrolysis assay. Again, vinyl sulfone **83** is able to reduce $\beta 5$ activity more before $\beta 1$ and $\beta 2$ are affected, and therefore is a more selective inhibitor than epoxyketone **52**. PR171-VS (**83**) displays about equipotent activity against immunoproteasome and constitutive proteasome chymotryptic activity. PR957 (**81**) shows preference for $\beta 5i$, as apparent from fluorogenic peptide hydrolysis assay data, although considerable reduction of other proteasomal subunits is observed before $\beta 5i$ is silenced. Vinyl sulfone PR957-VS (**84**) has lost selectivity between constitutive proteasome subunits, accompanied by a large drop in potency, with respect to epoxyketone **81**. This same observation is made for the potency and selectivity within the immunoproteasome. Different outcomes regarding inhibitor selectivity and potency have been observed between the competition assay and fluorogenic peptide hydrolysis assay. These can be caused by the presence of other proteasome forms in cell lysate (i.e. PA200 and PA28 activated proteasomes) or active site specificity affecting post-translational modifications that are lost when purifying proteasome for the fluorogenic substrate assay.

2.4 Experimental

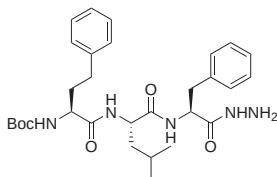
All reagents were commercial grade and were used as received unless indicated otherwise. Dichloromethane (DCM) and dimethyl formamide (DMF, Biosolve) were stored on 4 Å molecular sieves. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol or $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at $\sim 150^\circ C$ or by spraying with an aqueous solution of $KMnO_4$ (7%) and KOH (2%). Column chromatography was performed on silica gel from Screening Devices (0.040 - 0.063 nm). LC/MS analysis was performed on a LCQ Advantage Max (Thermo Finnigan) equipped with an Gemini C18 column (Phenomenex). HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^1H - and ^{13}C -APT-NMR spectra were recorded on a Bruker AV-400 (400/100 MHz) 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. All presented ^{13}C -APT spectra are proton decoupled. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, $\lambda = 589$ nm). Boc-Leu-EK, Boc-Phe-EK, Boc-Leu-VS and Boc-Phe-VS were synthesised according to literature procedures.^{106,167}



Boc-Leu-Phe-OMe (85). Boc-Leu-OH·H₂O (1.5 gr, 6 mmol, 1.2 equiv.) was co-evaporated with toluene (3×), before it was dissolved in DCM. After addition of HCTU (2.487 g, 6 mmol, 1.2 equiv.), H-Phe-OMe HCl (1.77 g, 5 mmol, 1 equiv.) and DiPEA (2.89 ml, 17.5 mmol, 3.5 equiv.) the reaction mixture was stirred overnight. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in EA, before being washed with 1 M HCl (3×), sat. aq. NaHCO₃ (2×) and Brine (2×). The organic layer was dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (EA: PE 10 → 25%) yielded the title compound (2.0 g, 5 mmol, 100%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.31-7.07 (m, 5H), 6.89 (d, *J* = 7.15 Hz, 1H), 5.21 (d, *J* = 7.89 Hz, 1H), 4.91-4.77 (m, 1H), 4.22-4.13 (m, 1H), 3.67 (s, 3H), 3.17-3.01 (m, 2H), 1.74-1.52 (m, 2H), 1.46-1.41 (m, 1H), 1.43 (s, 9H), 0.90 (t, *J* = 6.40 Hz, 6H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 172.21, 171.53, 155.34, 135.66, 129.07, 128.25, 126.78, 79.55, 53.03, 52.82, 51.98, 41.04, 37.65, 28.08, 24.41, 22.65, 21.77.



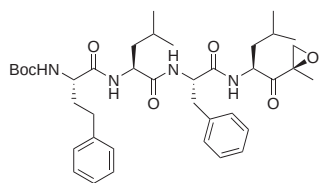
Boc-hPhe-Leu-Phe-OMe (86). Boc-Leu-Phe-OMe **85** (0.784 g, 2 mmol, 1.1 equiv.) was dissolved in TFA/DCM 1/1 (6 ml). The reaction mixture was stirred for 15 min., before being co-evaporated with toluene (3×). The crude TFA salt was dissolved in DMF and a solution of Boc-hPhe-OH (0.5 g, 1.79 mmol), HCTU (0.827 g, 2 mmol, 1.1 equiv.) and DiPEA (1.04 ml, 6.27 mmol, 3.5 equiv.) in DCM (20 ml) was added. The reaction mixture was stirred for 2 hr., before it was diluted with DCM. The reaction mixture was washed with 1M HCl (3x), sat. aq. NaHCO₃ (2×) and Brine (2×), before being dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (EA:PE 10 → 35%) yielded the title compound (0.818 g, 1.48 mmol, 83%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.28-7.03 (m, 10H), 7.01 (d, *J* = 7.72 Hz, 1H), 5.54 (d, *J* = 8.19 Hz, 1H), 4.81 (dd, *J*₁ = 14.07, *J*₂ = 6.38 Hz, 1H), 4.60-4.50 (m, 1H), 4.26-4.17 (m, 1H), 3.63 (s, 3H), 3.11-2.98 (m, 2H), 2.72-2.56 (m, 2H), 2.10-1.97 (m, 1H), 1.95-1.84 (m, 1H), 1.69-1.57 (m, 2H), 1.56-1.47 (m, 1H), 1.43 (s, 9H), 0.86 (dd, *J*₁ = 9.70, *J*₂ = 6.00 Hz, 6H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 172.01, 171.61, 171.55, 155.63, 140.88, 135.68, 129.03, 128.31, 128.26, 126.84, 125.89, 79.74, 53.88, 53.27, 52.05, 51.55, 41.02, 37.75, 34.07, 31.75, 28.18, 24.44, 22.61, 22.04.



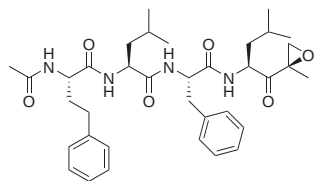
Boc-hPhe-Leu-Phe-NHNH₂ (87). After Boc-hPhe-Leu-Phe-OMe **86** (0.819 g, 1.48 mmol) was dissolved in MeOH (50 ml), hydrazine monohydrate (2.16 ml, 44.4 mmol, 30 equiv.) was added. The reaction mixture was refluxed for 3 hr., before being co-evaporated with toluene (3×). Purification by column chromatography (MeOH:EA:NEt₃ 0:99:1 → 20:79:1) yielded the title compound (0.632 g, 1.15 mmol, 77%). ¹H NMR (400 MHz, CD₃OD) ppm 7.32-7.10 (m, 10H), 4.53 (t, *J* = 7.42 Hz, 1H), 4.38 (dd, *J*₁ = 9.41, *J*₂ = 5.37 Hz, 1H), 4.04 (dd, *J*₁ = 8.29, *J*₂ = 4.95 Hz, 1H), 3.10 (dd, *J*₁ = 13.80, *J*₂ = 6.84 Hz, 1H), 2.95 (dd, *J*₁ = 13.71, *J*₂ = 8.12 Hz, 1H), 2.75-2.57 (m, 2H), 2.05-1.93 (m, 1H), 1.93-1.81 (m, 1H), 1.69-1.58 (m, 1H), 1.48 (s, 11H), 0.93 (d, *J* = 6.52 Hz, 3H), 0.89 (d, *J* = 6.42 Hz, 3H).

General protocol for azide couplings

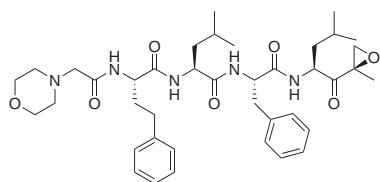
The Boc-protected warhead was dissolved in TFA:DCM (1:1, v/v) and stirred for 20 min. Coevaporation with toluene (3×) afforded the warhead TFA-salt, which was used without further purification. The appropriate hydrazide was dissolved in 1:1 DMF:DCM (v/v) and cooled to -30°C. *t*BuONO (1.1 equiv.) and HCl (4M sln. in 1,4-dioxane, 2.8 equiv.) were added, and the mixture was stirred for 3 hr. at -30°C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. The warhead-TFA salt was added to the reaction mixture as a solution in DMF with 1.1 equiv. of DiPEA. A further 3.9 equiv. of DiPEA were added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EA and extracted with H₂O (3×). The organic layer was dried over MgSO₄ and purified by flash column chromatography.



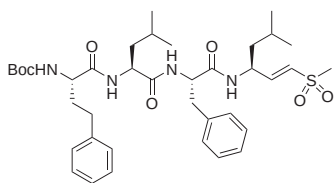
Hz, 1H), 2.70-2.53 (m, 2H), 2.08-1.86(m, 2H), 1.63-1.39 (m, 17H), 1.32-1.20 (m, 1H), 0.89-0.80 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 207.86, 172.16, 171.58, 170.70, 155.92, 140.87, 136.39, 129.11, 128.41, 128.31, 126.65, 126.03, 80.01, 58.81, 54.32, 53.83, 52.22, 51.84, 49.67, 41.26, 40.00, 38.06, 34.02, 31.91, 28.32, 25.01, 24.66, 23.19, 22.72, 22.07, 21.43, 16.56.



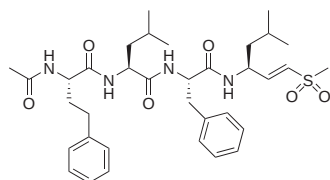
was then washed with H_2O (3 $\times$), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (MeOH:DCM 0 $\rightarrow$ 3%) yielded the title compound (11.3 mg, 17.8 μmol , 36%). $[\alpha]_{\text{D}}^{20}$ 4.5° (c = 1, MeOH). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/5 v/v)) ppm 7.33-7.07 (m, 10H), 4.62 (dd, $J_1 = 8.51$, $J_2 = 5.70$ Hz, 1H), 4.52 (dd, $J_1 = 10.41$, $J_2 = 3.29$ Hz, 1H), 4.33 (t, $J = 7.47$ Hz, 1H), 4.27 (dd, $J_1 = 8.60$, $J_2 = 5.52$ Hz, 1H), 3.20 (d, $J = 4.83$ Hz, 1H), 3.14 (dd, $J_1 = 14.01$, $J_2 = 5.64$ Hz, 1H), 2.91 (dd, $J_1 = 14.03$, $J_2 = 8.65$ Hz, 1H), 2.86 (d, $J = 5.01$ Hz, 1H), 2.72-2.55 (m, 2H), 2.01 (s, 3H), 1.97-1.84 (m, 2H), 1.48 (s, 3H), 1.70-1.52 (m, 2H), 1.44-1.12 (m, 4H), 0.95-0.83 (m, 12H). ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/5 v/v)): δ ppm 207.51, 172.19, 172.00, 171.49, 170.94, 140.30, 136.03, 128.43, 127.64, 127.51, 125.89, 125.29, 58.22, 53.30, 52.80, 51.43, 51.33, 49.53, 48.41, 39.78, 38.58, 36.86, 32.76, 31.24, 24.27, 23.92, 22.18, 21.78, 21.11, 20.43, 20.02, 15.49. LC/MS: R_t 9.45 min (linear gradient 10 $\rightarrow$ 90% TFA in MeOH, 15 min). HRMS: calcd. for $[\text{C}_{36}\text{H}_{50}\text{N}_4\text{O}_6\text{H}]^+$ 635.38031, found 635.37973.



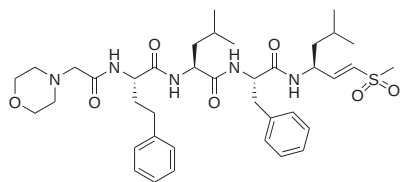
2-Morpholino-acetyl-hPhe-Leu-Phe-Leu-EK (PR171) (52). Boc-hPhe-Leu-Phe-Leu-epoxyketone **88** (0.0346 g, 0.05 mmol) was dissolved in TFA/DCM 1/1 (2 ml) and stirred for 25 min. The reaction mixture was co-evaporated with toluene (3 $\times$), before being dissolved in DCM (6.5 ml) and put under argon atmosphere. 2-morpholinoacetic acid TFA salt (8.8 mg, 0.06 mmol, 1.2 equiv.), HBTU (22.8 mg, 0.06 mmol, 1.2 equiv.) and DiPEA (38 μl , 0.225 mmol, 4.5 equiv.) were dissolved in DCM (5 ml) and this solution was added to the reaction mixture. The reaction mixture was stirred for 2 hr., before being washed with sat. aq. NaHCO_3 (4 $\times$), dried with Na_2SO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (MeOH:DCM 0 $\rightarrow$ 3%) yielded the title compound (8.6 mg, 11.9 μmol , 24%). $[\alpha]_{\text{D}}^{20}$ 0° (c = 0.17, MeOH). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/10 v/v)) ppm 7.32-7.08 (m, 10H), 4.61 (dd, $J_1 = 8.10$, $J_2 = 5.90$ Hz, 1H), 4.52 (dd, $J_1 = 10.46$, $J_2 = 3.27$ Hz, 1H), 4.42 (dd, $J_1 = 8.27$, $J_2 = 5.32$ Hz, 1H), 4.34 (t, $J = 7.44$ Hz, 1H), 3.80-3.71 (m, 4H), 3.19 (d, $J = 4.99$ Hz, 1H), 3.12 (dd, $J_1 = 13.99$, $J_2 = 5.89$ Hz, 1H), 3.02 (d, $J = 2.74$ Hz, 2H), 2.92 (dd, $J_1 = 14.05$, $J_2 = 8.17$ Hz, 1H), 2.86 (d, $J = 4.99$ Hz, 1H), 2.64-2.48 (m, 6H), 2.11-2.00 (m, 1H), 1.98-1.85 (m, 1H), 1.69-1.41 (m, 7H), 1.38-1.24 (m, 2H), 0.96-0.84 (m, 12H). ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/10 v/v)): δ ppm 172.42, 172.00, 171.35, 170.76, 140.84, 136.48, 129.12, 128.33, 128.25, 128.17, 126.58, 125.99, 66.75, 61.35, 53.87, 53.51, 52.43, 52.01, 51.95, 50.06, 40.55, 39.34, 37.57, 34.16, 31.81, 24.92, 24.56, 22.90, 22.47, 21.18, 20.76, 16.21. LC/MS: R_t 7.58 min (linear gradient 10-90% TFA in MeOH, 15 min). HRMS: calcd. for $[\text{C}_{40}\text{H}_{57}\text{N}_5\text{O}_7\text{H}]^+$ 720.43308, found 720.43265.



3.11 (m, 1H), 3.00-2.93 (m, 1H), 2.90 (s, 3H), 2.76-2.59 (m, 2H), 2.07-1.94 (m, 1H), 1.94-1.82 (m, 1H), 1.68-1.55 (m, 2H), 1.47 (s, 11H), 1.39-1.26 (m, 1H), 0.95-0.83 (m, 12H). ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/10 v/v)): δ ppm 178.43, 174.83, 173.89, 172.22, 148.19, 141.82, 137.62, 129.88, 129.78, 129.36, 129.23, 129.18, 127.76, 126.82, 80.77, 55.67, 55.50, 53.38, 48.72, 43.13, 42.85, 41.35, 38.20, 34.43, 32.69, 28.71, 25.41, 25.29, 23.34, 23.19, 22.11, 21.81.



μmol , 90%). $[\alpha]_{\text{D}}^{20}$ 30.5 °(c = 0.59, MeOH:DCM 1:1, v/v). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/10 v/v)): δ ppm 7.31-7.16 (m, 10H), 6.73 (dd, $J_1 = 15.13$, $J_2 = 4.49$ Hz, 1H), 6.17 (dd, $J_1 = 15.14$, $J_2 = 1.70$ Hz, 1H), 4.64-4.56 (m, 2H), 4.28-4.18 (m, 2H), 3.23-3.14 (m, 1H), 3.06-2.98 (m, 1H), 2.92 (s, 3H), 2.76-2.60 (m, 2H), 2.05 (s, 3H), 2.03-1.91 (m, 2H), 1.69-1.25 (m, 6H), 0.95-0.79 (m, 12H). ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/10 v/v)): δ ppm 172.82, 172.50, 171.91, 170.87, 146.89, 140.16, 136.29, 128.48, 128.23, 127.91, 127.84, 127.76, 126.32, 125.53, 54.45, 53.39, 52.16, 47.32, 41.72, 41.61, 39.51, 36.62, 32.62, 31.27, 24.07, 23.93, 22.01, 21.86, 21.42, 20.68, 20.58. LC/MS: R_t 8.73 min (linear gradient 10-90% TFA in MeOH, 15 min). HRMS: calcd. for $[\text{C}_{35}\text{H}_{50}\text{N}_4\text{O}_6\text{SH}]^+$ 655.35238, found 655.35184.



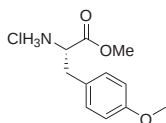
washed with sat. aq. NaHCO_3 (3 $\times$), dried with Na_2SO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (MeOH:DCM 0 $\rightarrow$ 3%) yielded the title compound (25.7 mg, 35 μmol , 69%). $[\alpha]_{\text{D}}^{20}$ 1.9 °(c = 0.51, MeOH). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/10 v/v)) ppm 7.34-7.16 (m, 10H), 6.67 (dd, $J_1 = 15.17$, $J_2 = 4.68$ Hz, 1H), 6.06 (dd, $J_1 = 15.18$, $J_2 = 1.69$ Hz, 1H), 4.66-4.58 (m, 2H), 4.48-4.37 (m, 2H), 3.80-3.70 (m, 4H), 3.09 (d, $J = 2.28$ Hz, 2H), 3.17-3.09 (m, 1H), 3.06-2.98 (m, 1H), 2.92 (s, 3H), 2.73-2.63 (m, 2H), 2.60-2.52 (m, 4H), 2.16-1.92 (m, 2H), 1.72-1.24 (m, 6H), 0.99-0.85 (m, 12H). ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/10 v/v)): δ ppm 174.19, 174.00, 172.76, 172.53, 148.38, 142.37, 138.13, 130.39, 129.71, 129.52, 128.11, 127.15, 67.88, 62.47, 56.33, 54.77, 54.14, 53.42, 49.09, 43.42, 42.88, 41.81, 38.65, 35.37, 33.07, 25.82, 25.62, 23.50, 23.32, 22.13, 21.83. LC-MS: R_t 6.91 min (linear gradient 10-90% TFA in MeOH, 15 min). HRMS: calcd. for $[\text{C}_{39}\text{H}_{57}\text{N}_5\text{O}_7\text{SH}]^+$ 740.40515, found 740.40474.

Boc-hPhe-Leu-Phe-Leu-VS (89). Following the general procedure for azide coupling the title compound was obtained from Boc-Leu-VS **356** (0.128 g, 0.44 mmol, 1.1 equiv.) and **87** (0.218 g, 0.4 mmol). Purification by column chromatography (MeOH:DCM 0 $\rightarrow$ 7%) yielded the title compound (0.221 g, 0.31 mmol, 78%). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ (1/10 v/v)): δ ppm 7.31-7.13 (m, 10H), 6.68 (dd, $J_1 = 15.06$, $J_2 = 4.22$ Hz, 1H), 6.10 (d, $J = 15.17$ Hz, 1H), 4.66-4.57 (m, 2H), 4.35-4.26 (m, 1H), 3.99 (dd, $J_1 = 8.69$, $J_2 = 5.35$ Hz, 1H), 3.21-

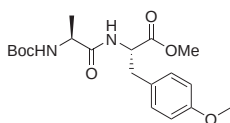
Ac-hPhe-Leu-Phe-Leu-VS (YU101-VS) (82). Boc-hPhe-Leu-Phe-Leu-VS **89** (35.6 mg, 50 μmol) was dissolved in TFA/DCM 1/1 (2 ml) and stirred for 30 min. The reaction mixture was co-evaporated with toluene (3 $\times$) yielding the crude hPhe-Leu-Phe-Leu-VS TFA salt, which was dissolved in DCM (5 ml) and cooled to 0°C. DiPEA (17 μl , 0.105 mmol, 2.1 equiv.) and Ac_2O (5 μl , 55 μmol , 1.1 equiv.) were added and the reaction mixture was stirred for 2 hr. The reaction mixture was then washed with H_2O (3 $\times$), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (MeOH:DCM 0 $\rightarrow$ 7%) yielded the title compound (29.5 mg, 45

2-Morpholino-acetyl-hPhe-Leu-Phe-Leu-VS (PR171-VS)

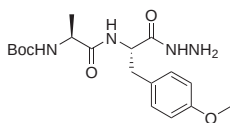
(83). Boc-hPhe-Leu-Phe-Leu-VS **89** (35.6 mg, 50 μmol) was dissolved in TFA/DCM 1/1 (2 ml) and stirred for 25 min. The reaction mixture was co-evaporated with toluene (3 $\times$), before being dissolved in DCM (6.5 ml) and put under argon atmosphere. 2-morpholinoacetic acid TFA salt (8.8 mg, 60 μmol , 1.2 equiv.), HBTU (22.8 mg, 60 μmol , 1.2 equiv.) and DiPEA (38 μl , 0.225 mmol, 4.5 equiv.) were dissolved in DCM (5 ml) and this solution was added to the reaction mixture. The reaction mixture was stirred for 2 hr., before being



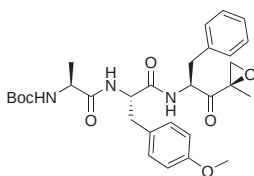
H-Tyr(Me)-OMe HCl salt (91). H-Tyr(Me)-OH (0.50 g, 2.56 mmol) was dissolved in MeOH (5.5 mL), brought under an argon atmosphere and cooled to 0°C. To this stirred solution SOCl_2 (1.12 mL, 15.37 mmol, 6 equiv.) was added. After 24 hr. the reaction mixture was co-evaporated with toluene (3 $\times$) to afford the title compound as a pale yellow solid (0.63 g, 2.55 mmol, >99%). ^1H NMR (400 MHz, CD_3OD): δ ppm 7.17 (d, J = 8.09 Hz, 2H), 6.92 (d, J = 8.04 Hz, 2H), 4.27 (t, J = 5.73 Hz, 1H), 3.81 (s, 3H), 3.79 (s, 3H), 3.17 (dq, J_1 = 14.55, J_2 = 5.95 Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 170.47, 160.88, 131.58, 126.93, 115.57, 55.85, 55.43, 53.71, 36.60.



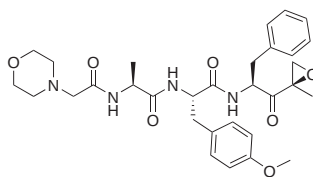
Boc-Ala-Tyr(Me)-OMe (92). Boc-Ala-OH (0.34 g, 1.8 mmol, 1.2 equiv.), HBTU (0.74 g, 1.8 mmol, 1.2 equiv.) and **91** (0.369 g, 1.5 mmol) were dissolved in DCM (10 mL). The reaction was initiated by addition of DiPEA (0.87 mL, 5.25 mmol, 3.5 equiv.) to the stirred solution. After 18 hr. the reaction mixture was concentrated, the concentrate was dissolved in EA and the solution was washed with 1M aq. HCl (3 $\times$), saturated aq. NaHCO_3 (5 $\times$), Brine, dried over MgSO_4 and concentrated. This yielded **92** (0.56 g, 1.48 mmol, 99%) as a viscous light brown oil. TLC analysis (eluent: 40% EA/ 60% PE) showed product at R_f 0.26, which was used without further purification. $[\alpha]_D^{20}$ 34.9° (c = 1, CHCl_3). ^1H NMR (400 MHz, CDCl_3): δ ppm 7.03 (d, J = 8.56 Hz, 2H), 6.89 (d, J = 6.39 Hz, 1H), 6.80 (d, J = 8.58 Hz, 2H), 5.38 (d, J = 6.34 Hz, 1H), 4.79 (dd, J_1 = 13.22, J_2 = 6.08 Hz, 1H), 4.21 (s, 1H), 3.75 (s, 3H), 3.69 (s, 3H), 3.04 (dq, J_1 = 13.96, J_2 = 6.10 Hz, 2H), 1.43 (s, 9H), 1.31 (d, J = 7.07 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.32, 171.64, 158.36, 155.14, 130.03, 127.55, 113.67, 79.59, 54.87, 53.20, 52.01, 49.78, 36.75, 28.06, 18.16. LC/MS: R_t 7.32 min (linear gradient 10 $\rightarrow$ 90% MeCN in H_2O , 0.1% TFA, 15 min). HRMS: Calculated for $[\text{C}_{19}\text{H}_{28}\text{N}_2\text{O}_6\text{Na}]^+$: 403.18396; found: 403.18352.



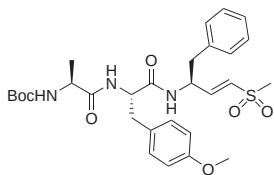
Boc-Ala-Tyr(Me)-NHNH₂ (93). To a solution of **92** (0.508 g, 1.34 mmol) in MeOH (22.5 mL) was added hydrazine hydrate (1.95 mL of 64% solution, 40.05 mmol, 30 equiv.). The reaction mixture was refluxed at 70°C. After 2.5 hr. the reaction mixture was co-evaporated with toluene (3 $\times$) and the residue purified with column chromatography (MeOH:EA:NEt₃ 0:99:1 $\rightarrow$ 20:79:1) to yield **93** (0.475 g, 1.25 mmol, 93%) as an off-white solid. ^1H NMR (400 MHz, CD_3OD): δ ppm 7.12 (d, J = 8.54 Hz, 2H), 6.83 (d, J = 8.69 Hz, 2H), 4.50 (t, J = 7.18 Hz, 1H), 3.96 (q, J = 7.18 Hz, 1H), 3.75 (s, 3H), 2.97 (ddd, J_1 = 21.78, J_2 = 13.65, J_3 = 7.12 Hz, 2H), 1.43 (s, 9H), 1.20 (d, J = 7.21 Hz, 3H). LC/MS: R_t 5.31 min (linear gradient 10 $\rightarrow$ 90% MeCN in H_2O , 0.1% TFA, 15 min). HRMS: Calculated for $[\text{C}_{18}\text{H}_{28}\text{N}_4\text{O}_5\text{H}]^+$: 381.21325; found: 381.21300.



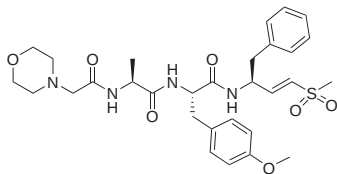
Boc-Ala-Tyr(Me)-Phe-EK (94). Following the general procedure for azide coupling the title compound was obtained from Boc-Phe-EK (84 mg, 0.28 mmol, 1.1 equiv.) and **93** (94.7 mg, 0.25 mmol, 1 equiv.). Purification by column chromatography (EA:PE 0 $\rightarrow$ 60%) yielded **94** (100 mg, 0.180 mmol, 72%). $[\alpha]_D^{20}$ +25.8° (c = 1, CHCl_3). ^1H NMR (400 MHz, CDCl_3): δ ppm 7.28-7.17 (m, 3H), 7.08 (d, J = 8.57 Hz, 2H), 7.04-6.99 (m, 2H), 6.78 (d, J = 8.63 Hz, 2H), 6.74 (d, J = 7.74 Hz, 1H), 6.44 (s, 1H), 5.09 (d, J = 7.22 Hz, 1H), 4.74 (dd, J_1 = 13.09, J_2 = 7.41 Hz, 1H), 4.54 (dd, J_1 = 13.94, J_2 = 7.31 Hz, 1H), 4.12 (dd, J_1 = 14.21, J_2 = 7.09 Hz, 1H), 3.76 (s, 3H), 3.26-2.63 (m, 6H), 1.44 (s, 3H), 1.42 (s, 9H), 1.26 (d, J = 7.07 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 206.95, 172.36, 170.30, 158.56, 155.35, 135.45, 130.32, 129.17, 128.43, 128.19, 126.99, 113.94, 80.08, 59.04, 55.11, 54.13, 52.30, 52.26, 50.15, 37.17, 28.23, 18.28, 16.31. LC/MS: R_t 8.92 min (linear gradient 10 $\rightarrow$ 90% MeCN in H_2O , 0.1% TFA, 15 min). HRMS: Calculated for $[\text{C}_{30}\text{H}_{39}\text{N}_3\text{O}_7\text{H}]^+$: 554.28608; found: 554.28539.



2-Morpholino-acetyl-Ala-Tyr(Me)-Phe-EK (PR957) (81). A solution of **94** (62.8 mg, 0.113 mmol) in 2 ml DCM/TFA (2/1) was stirred and coevaporated with toluene (3×) after 1 hr. The residue was dissolved in DCM and 2-morpholinoacetic acid TFA salt (33 mg, 0.125 mmol, 1.1 equiv.), HBTU (52 mg, 0.125 mmol, 1.1 equiv.) and DiPEA (84 μ l, 0.510 mmol, 4.5 equiv.) were added. After 18 hr. the reaction mixture was concentrated and the concentrate dissolved in EA. The organic solution was washed with sat. aq. NaHCO_3 (5×), brine, dried over MgSO_4 and concentrated. Purification by column chromatography (MeOH:DCM 0 $\rightarrow$ 3%) afforded the title compound **19** (44.3 mg, 76 μ mol, 67%). $[\alpha]_D^{20} +24.3^\circ$ (c = 1, CHCl_3). ^1H NMR (400 MHz, CDCl_3): δ ppm 7.44 (d, J = 7.58 Hz, 1H), 7.29-7.20 (m, 3H), 7.08 (d, J = 8.63 Hz, 2H), 7.02 (dd, J_1 = 7.62, J_2 = 1.56 Hz, 2H), 6.78 (d, J = 8.65 Hz, 2H), 6.69 (d, J = 7.55 Hz, 1H), 6.31 (d, J = 7.37 Hz, 1H), 4.74 (dt, J_1 = 7.87, J_2 = 5.01 Hz, 1H), 4.49 (q, J = 7.00 Hz, 1H), 4.37 (p, J = 7.10 Hz, 1H), 3.77 (s, 3H), 3.69 (t, J = 4.58 Hz, 4H), 3.26 (d, J = 4.92 Hz, 1H), 3.11-2.65 (m, 7H), 2.48-2.43 (m, 4H), 1.48 (s, 3H), 1.29 (d, J = 7.04 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 206.91, 171.79, 170.26, 170.09, 158.55, 135.53, 130.33, 129.19, 128.48, 128.22, 127.06, 113.96, 66.86, 61.58, 59.14, 55.17, 54.23, 53.70, 52.56, 52.41, 48.22, 37.03, 36.73, 17.65, 16.45. LC-MS: R_t 6.72 min (linear gradient 10 $\rightarrow$ 90% MeCN in H_2O , 0.1% NH_4OAc , 15 min). HRMS: Calculated for $[\text{C}_{31}\text{H}_{40}\text{N}_4\text{O}_7\text{H}]^+$: 581.29698; found: 581.29664.



Boc-Ala-Tyr(Me)-Phe-VS (95). Following the general procedure for azide coupling the title compound was obtained from Boc-Phe-VS (91 mg, 0.28 mmol, 1.1 equiv.) and **93** (94.7 mg, 0.25 mmol). Purification by column chromatography (EA:PE 50 $\rightarrow$ 80%) yielded **95** (0.186 g, 0.321 mmol, 64%) as a white solid. $[\alpha]_D^{20} +29.6^\circ$ (c = 1, CHCl_3). ^1H NMR (400 MHz, CDCl_3): δ ppm 7.31-7.13 (m, 5H), 7.06 (d, J = 4.2 Hz, 2H), 6.94 (d, J = 7.2 Hz, 1H), 6.80 (d, J = 8.4 Hz, 2H), 6.78-6.73 (m, 1H), 6.66 (d, J = 7.2 Hz, 1H), 6.19 (d, J = 14.8 Hz, 1H), 5.02 (m, 1H), 4.97 (m, 1H), 4.61 (q, J = 6.4 Hz, 1H), 4.03 (m, 1H), 3.76 (s, 3H), 3.14-3.10 (m, 2H), 3.00-2.89 (m, 2H), 2.80 (s, 3H), 1.37 (s, 9H), 1.31 (d, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.65, 170.40, 158.69, 156.07, 145.75, 136.03, 130.31, 130.22, 128.74, 128.47, 127.75, 127.11, 114.26, 80.87, 55.16, 54.28, 51.32, 50.40, 42.58, 40.15, 36.45, 28.25, 17.79. LC/MS: R_t 8.09 min (linear gradient 10 $\rightarrow$ 90% MeCN in H_2O , 0.1% TFA, 15 min). HRMS: Calculated for $[\text{C}_{29}\text{H}_{39}\text{N}_3\text{O}_7\text{SH}]^+$: 574.25815, found: 574.25755.



Morpholino-acetyl-Ala-Tyr(Me)-Phe-VS (PR957-VS) (84). A solution of **95** (0.106 g, 0.184 mmol, 1.0 equiv.) was dissolved in DCM/TFA (2/1), stirred for 1.5 hr. and co-evaporated with toluene (3×). The residue was dissolved in DCM (10 ml) and 2-morpholinoacetic acid TFA salt (54 mg, 0.203 mmol, 1.1 equiv.), HBTU (84 mg, 0.203 mmol, 1.1 equiv.) and DiPEA (0.137 mL, 0.829 mmol, 4.5 equiv.) were added. After 3.5 hr. the reaction mixture was concentrated and the concentrate was dissolved in EA, washed with sat. aq. NaHCO_3 (5×), brine, dried over MgSO_4 and concentrated. Purification by column chromatography (MeOH:DCM 0 $\rightarrow$ 4%) yielded **84** (90 mg, 0.150 mmol, 81%). $[\alpha]_D^{20} -19.6^\circ$ (c = 1, MeOH). ^1H NMR (400 MHz, DMSO-d_6): δ ppm 8.26 (d, J = 8.25 Hz, 1H), 8.10 (d, J = 8.21 Hz, 1H), 7.78 (d, J = 7.62 Hz, 1H), 7.32-7.15 (m, 5H), 7.10 (d, J = 8.57 Hz, 2H), 6.81 (d, J = 8.59 Hz, 2H), 6.67 (dd, J_1 = 15.25 Hz, J_2 = 4.86 Hz, 1H), 6.30 (dd, J_1 = 15.26, J_2 = 1.48 Hz, 1H), 4.74-4.65 (m, 1H), 4.44 (dd, J_1 = 14.90, J_2 = 8.16 Hz, 1H), 4.27 (p, J = 7.10 Hz, 1H), 3.70 (s, 3H), 3.59-3.54 (m, 4H), 3.04-2.65 (m, 9H), 2.40-2.37 (m, 4H), 1.12 (d, J = 6.96 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ ppm 171.56, 170.25, 168.37, 157.64, 145.33, 137.27, 130.08, 129.87, 129.11, 128.10, 127.99, 126.27, 113.38, 66.04, 61.16, 54.75, 54.19, 53.07, 50.26, 47.38, 42.08, 38.73, 36.82, 18.65. LC/MS: R_t 5.92 min (linear gradient 10 $\rightarrow$ 90% MeCN in H_2O , 0.1% NH_4OAc , 15 min). HRMS: Calculated for $[\text{C}_{30}\text{H}_{40}\text{N}_4\text{O}_7\text{SH}]^+$: 601.26905, found: 601.26838.

Competition experiments in cell lysate

HEK293T or RAJI cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin in a 7% CO_2 humidified incubator at 37°C . Cells were harvested, washed 2× with PBS 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 15 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 (Biorad). 15 µg (HEK293T) or 10 µg (RAJI) total protein in 9 µl lysis buffer per experiment was exposed to the inhibitors (10× solution in DMSO, 1 µl) for 1 hr. at 37°C prior to incubation with MVB003 (500 nM end concentration) for 1 hr. at 37°C. Reaction mixtures were boiled with Laemmli's buffer containing β -mercaptoethanol for 3 min. before being resolved on 12.5% SDS-PAGE. In-gel detection of fluorescently labelled 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 nm, λ_{em} 560 nm)

Fluorogenic substrate hydrolysis assay

26S proteasomes were purified from rabbit muscle as described.¹⁶⁹ To determine inhibition of purified proteasomes, proteasome (~ 10ng/ml) in assay buffer (50 mM Tris-HCl pH 7.5, 40 mM KCl, 2 mM EDTA, 1 mM DTT, 100 µM ATP, 50 µg/mL BSA) was incubated with inhibitors for 30 min. at 37°C followed by assay of activity with 100 µM fluorogenic substrates Suc-LLVY-AMC (chymotrypsin-like site), Ac-RLR-AMC (trypsin-like site), and Ac-nLPnLD-AMC (caspase-like site). Fluorescence of released AMC was measured (λ_{ex} 350 nm, λ_{em} 430 nm) continuously for 30 min at 37°C. Residual activity was determined from the slope of the reaction progress curves as a mean value from 3 experiments.¹⁴⁷

3

Proteasome selectivity towards Michael acceptor containing oligopeptide-based inhibitors*

3.1 Introduction

The potential of proteasome inhibitors to be used in the treatment of cancer and immunological disorders sparked many medicinal chemistry efforts to optimise peptide-based proteasome inhibitors in terms of selectivity, cell permeability and subunit specificity. In the past, much effort has been devoted to vary the peptide sequence of the recognition element.^{131,140,142} The electrophilic trap, in contrast, has not been an object of such extensive study. Well known electrophilic traps in peptide based proteasome inhibitors are the vinyl sulfone,¹⁰⁶ epoxyketone,⁴⁸ boronic acid⁸⁰ and aldehyde⁷⁸ functional groups. In this work, ten electrophilic traps, with the potential to target the proteasome, are investigated. As recognition elements used to assess proteasome inhibition capacity the Z-Leu₂ motif¹⁰⁶ and the AdaAhx₃Leu₂ motif are selected.¹⁰⁷ In addition, the N₃PheLeu₂ element yields potential inhibitors in which the azide moiety can be used to determine the reactivity of the inhibitors in cell extract using two-step labelling chemical proteomics experiments.^{108,170}

3.2 Results and Discussion

The C-terminally modified target peptides are depicted in Figure 3.1. The first five Michael acceptors in Figure 3.1 (compound 96-110) were derived from Boc-leucinal applying standard Horner-Wadsworth-Emmons (HWE) olefination procedures. Iodide substi-

*W. A. van der Linden, P. P. Geurink, C. Oskam, G. A. van der Marel, B. I. Florea, H. S. Overkleeft, *Org. Biomol. Chem.* **2010**, *8*, 1885-1893.

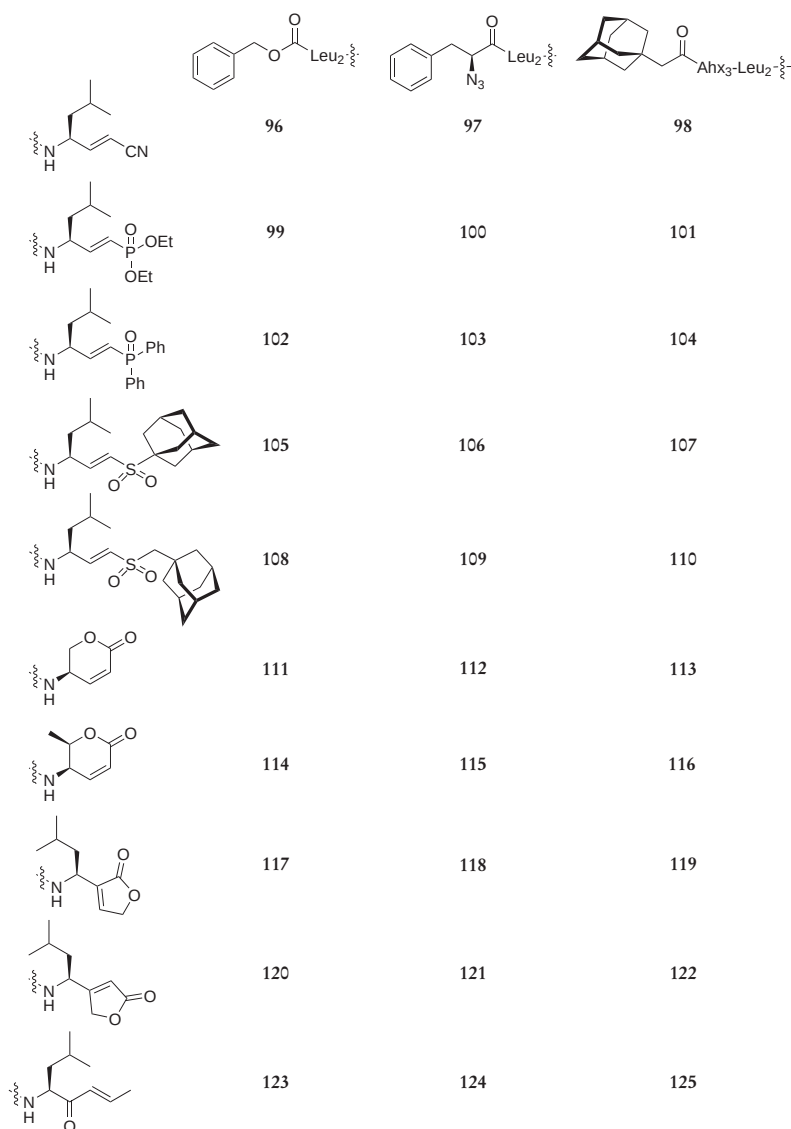
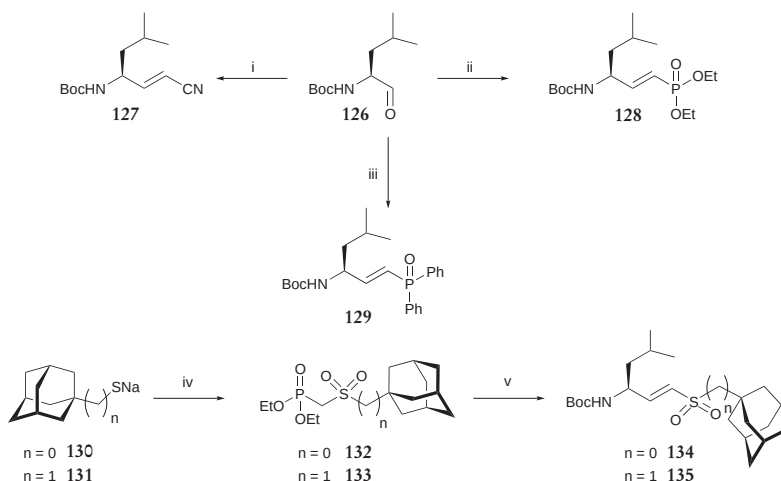


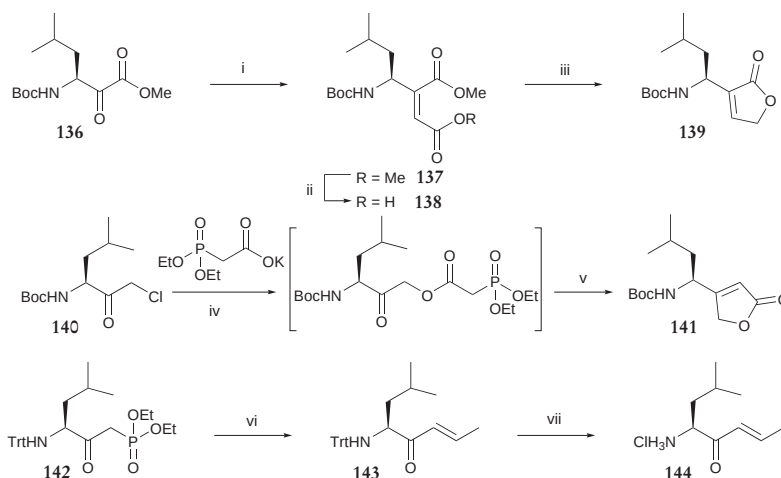
Figure 3.1: Overview of designed panel of potential proteasome inhibitors.

tution in diethyl iodomethylphosphonate by the sodium salt of 1-adamantylthiol or 1-adamantylmethylthiol¹⁷¹ followed by peracetic acid oxidation gave **132** and **133** in 79% and 65% yield over two steps, respectively (Scheme 3.1). Reaction of aldehyde **126** with five HWE reagents yielded **127-129**, **134** and **135**.

Horner-Wadsworth-Emmons olefination of **136**¹⁷² using diethyl methyl phosphonoacetate yielded diester **137** (Scheme 3.2), which could be selectively saponified to **138** in 73% yield. NaBH_4 reduction of the **138** derived mixed anhydride afforded unsaturated furanolactone **139**. Cyclic vinyl ester **141**, configurationally different from **139**, was obtained from chloromethylketone **140**¹⁷³ in two steps. Chloride substitution in **140** by potassium

Scheme 3.1: Synthesis electrophiles derived from Boc-leucinal.

Reagents and conditions: i) Diethyl cyanomethylphosphonate (1.5 equiv.), NaH (1.5 equiv.), THF, 0°C, 30 min., then **126** (1 equiv.), o/n, 42%. ii) Tetraethyl methylenediphosphonate (1.5 equiv.), NaH (1.5 equiv.), THF, 0°C, 30 min., then **126** (1 equiv.), 3 hr. 82%. iii) Diethyl methylphosphonate (1 equiv.), LiHMDS (2 equiv.), THF, -78°C, 15 min., then ClP(O)Ph₂ (1 equiv.), -40°C, 1 hr., then **126** (1 equiv.), -40°C, 3 hr., 5%. iv)(a) diethyl iodomethylphosphonate (1 equiv.), THF, 0°C, 1.5 hr. (b) 32% AcOOH in AcOH (4 equiv.), 1,4-dioxane, 0°C, 2 hr., 79% (**132**), 65% (**133**). v) **132** or **133** (1.1 equiv.), KOtBu (1.1 equiv.), THF, 0°C, 30 min., then **126** (1 equiv.), 62% (**134**), 89% (**135**).

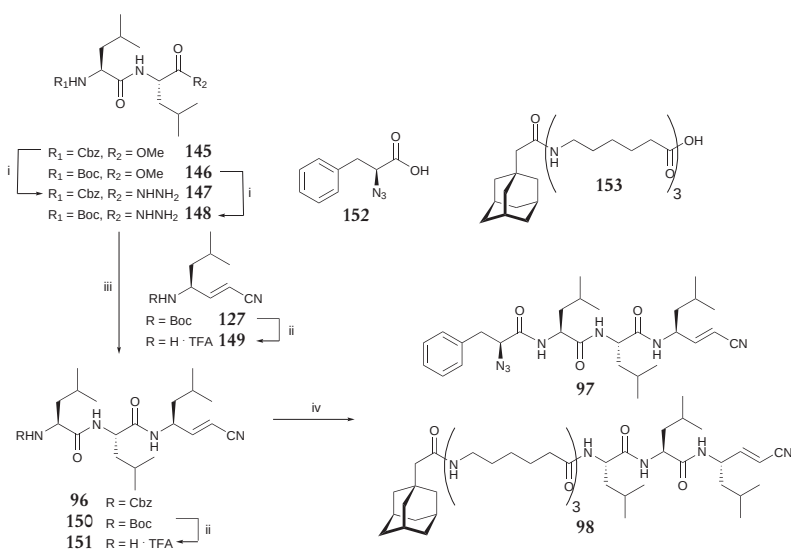
Scheme 3.2: Synthesis of electrophiles **139**, **141**, **144**.

Reagents and conditions: i) methyl dimethylphosphonoacetate (1.5 equiv.), NaH (1.5 equiv.), THF, 30 min., 0°C, then **136** (1 equiv.), 2.5 hr., 79%. ii) LiOH (1.15 equiv.), THF, H₂O, o/n, 73%. iii)(a) EtOCOCl (1.1 equiv.), TEA (1.1 equiv.), 15 min. (b) NaBH₄ (1.5 equiv.), H₂O, THF, 0°C, 1.5 hr., 71%. iv)(a) Potassium dimethylphosphonoacetate (2.2 equiv.), MeCN, 50°C, 3 hr. (b) LiCl (1.2 equiv.), NEt₃ (1 equiv.), 3 hr., 52%. vi) Acetaldehyde (8 equiv.), K₂CO₃ (1.06 equiv.), EtOH, o/n, 86%. vii) 4M HCl in 1,4-dioxane (2 equiv.), TIS (1.5 equiv.), Et₂O, 1 hr., 63%.

diethyl phosphonoacetate and subsequent intramolecular LiCl promoted HWE reaction yielded **141** in 52% yield over two steps. HWE olefination of **142**¹⁷⁴ with acetaldehyde afforded vinyl ketone **143** in 86% yield, which was detritylated using dry HCl/TIS to obtain free amine **144**.

The ten potential electrophilic traps were coupled to the peptide recognition parts using a general strategy, which is illustrated by the preparation of compounds **96-98** (Scheme 3.3). Vinyl nitrile **127** was deprotected using TFA to give **149** in quantitative yield. BocLeu₂OMe (**146**) was reacted with hydrazine to yield hydrazide **148**. In a one-pot procedure, hydrazide **148** was transformed into its acyl azide using *t*BuONO and HCl. This acyl azide was subsequently reacted with the vinyl nitrile-TFA salt **149** under the influence of DiPEA to yield the Boc-protected tripeptide **150** without observable epimerization during the coupling. In a similar fashion, Cbz-protected tripeptide **96** was synthesised from **147**. The tripeptide **150** was deprotected with TFA and subsequently block-coupled to either azido-phenylalanine **152**¹⁷⁵ or AdaAhx₃-OH **153** to yield potential inhibitors **97** and **98**, respectively (Scheme 3.3).

Scheme 3.3: Preparation of compounds **96**, **97** and **98** via block coupling strategy.



Reagents and conditions: i) $\text{H}_2\text{NNH}_2 \cdot \text{H}_2\text{O}$ (30 equiv.), MeOH, reflux, 98% (**147**), 75% (**148**). ii) 1:1 TFA:DCM, 30 min., quant. iii) (a) **147** or **148** (1 equiv.), *t*BuONO (1.1 equiv.), HCl (2.8 equiv.), 1:1 DMF/DCM, -30°C , 3 hr. (b) **149** (1.1 equiv.), DiPEA (5 equiv.), $-30^\circ \rightarrow \text{RT}$, o/n, 52% (**96**), 76% (**150**). iv) HBTU (1.1 equiv.), DiPEA (3.5 equiv.), **152** or **153** (1.1 equiv.), DMF/DCM 1/1, 5 min., then **151** (1 equiv.), 2 hr., 70% (**97**), 80% (**98**).

Next, the proteasomal inhibition capacity of the synthesised compounds was assessed. To this end a competition assay was employed in human embryonic kidney (HEK293T) cell lysates at a final inhibitor concentration of 0, 1, 10 and 100 μM versus fluorescent proteasome inhibitor MV151 (**42**, final concentration 500 nM).¹¹⁰ Only compounds **98**, **101**, **104**, **107**, **110**, **122** and **125** showed some loss of MV151 signal at 100 μM concentration, indicating proteasome inhibition (data not shown). However, this potency is very low compared to the known inhibitors AdaAhx₃Leu₃VS¹⁰⁷ and ZLeu₃VS.¹⁰⁶ For the two vinyl

sulfones in **105-110**, the steric bulk of the adamantane group could hamper active site entrance and thereby lowering potency. Given the broad range of known electrophilic traps capable of inhibiting the proteasome, such as vinyl sulfone, epoxyketones, aldehydes and boronic acids,¹⁶⁶ the lack of reactivity of the other inhibitors described in this chapter is quite surprising.

To test whether these compounds target other molecules in the cell, HEK293T and RAW264.7 cells were incubated with 50 μ M of compound **97** and **118**. After cell lysis, Staudinger ligation using biotin-phosphine **154**¹⁷⁶ (Figure 5.1, page 85) introduced a biotin to the azide modified proteins. Streptavidin pulldown enriched the pool of labelled enzymes, which were then digested on-bead and analysed by mass spectrometry. The observed peptides divided by the observable peptides yields the Protein Abundance Index (PAI). The exponentially modified PAI yields the emPAI ($(10^{\text{PAI}} - 1)$), which is a measure for absolute protein quantification of the labelled enzymes.^{177,178} Together with the total protein coverage by the peptides that were found back, hits were selected and depicted in Table 3.1 and Table 3.2.

Table 3.1: Determination of cellular targets of compound **97** in HEK293T and RAW264.7 cells by affinity purification and nano-LC/MS analysis.

HEK293T			RAW264.7		
Protein description	Coverage (%) ^a	emPAI ^b	Protein description	Coverage (%)	emPAI
Proteasome β 5	60.1	10.3	Proteasome β 1i	73.0	26.8
Proteasome β 2	38.3	1.1	Proteasome β 1	72.0	12.1
Proteasome β 1	39.3	0.5	Proteasome β 5i	54.0	6.1
Cathepsin L2	21.0	0.5	Proteasome β 5	72.0	11.6
Cathepsin L1	13.2	0.3	Proteasome β 2	46.1	1.6
Cathepsin F	3.1	0.1	Proteasome β 2i	11.0	0.2
tubulin		0.7	Cathepsin L1	44.0	2.5
			Cathepsin Z	49.8	4.1
			Cathepsin F	26.2	0.7
			Cathepsin D	23.4	0.4

^a Protein sequence coverage by peptides found back

^b $(10^{(\text{observed peptides}/\text{observable peptides})} - 1)$

Table 3.2: Determination of cellular targets of compound **118** in HEK293T and RAW264.7 cells by affinity purification and nano-LC/MS analysis.

HEK293T			RAW264.7		
Protein description	Coverage (%) ^a	emPAI ^b	Protein description	Coverage (%)	emPAI
Proteasome β 5	17.5	0.3	Proteasome β 1i	33.8	1.2
Proteasome β 2	10.1	0.1	Proteasome β 5	15.5	0.1
keratin		2.5	Proteasome β 1	8.0	0.3
			Cathepsin Z	12.1	0.4
			Cathepsin L1	32.3	0.5
			Cathepsin S	30.0	0.2

^a Protein sequence coverage by peptides found back

^b $(10^{(\text{observed peptides}/\text{observable peptides})} - 1)$

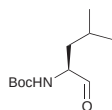
Surprisingly, the peptides found back with the highest emPAI and best protein coverage belong to the active subunits of the proteasome, which means that **97** and **118** targeted the proteasome in the HEK and RAW cells. This is remarkable since **97** and **118** do not show proteasome inhibition in lysates at concentrations up to 100 μ M in the competition assay in lysates versus MV151. The pull-down, tryptic digest/mass spectrometry analysis, in which even the smallest fraction of labelled proteasome is found back, appears to be more sensitive than the MV151 competition assay. A second class of peptides found with reasonable coverage belong to the cathepsin family. The emPAI of most of these cathepsin hits is low compared to the proteasome hits, which could be explained by the higher proteasome content in mammalian cells compared to cathepsins.

3.3 Conclusion

Despite the variety of potential proteasome inhibitors synthesised in this work, no new electrophilic trap was found which yields potent proteasome inhibitors. For future development of proteasome inhibitors, variation in the peptide recognition part is therefore a recommendable approach. Even though the potency of proteasome inhibition by compounds **97** and **118** in a gel based competition assay is low, these compounds are able to label the proteasome, as was confirmed with the biotin pulldown-MS² detection method. These results demonstrate that the biotin pulldown-MS² assay is an attractive alternative to the gel based competition assay, because the MS² is very sensitive and information about cross reactivity of an inhibitor is directly obtained. Therefore, incorporation of an azide in potential inhibitors is a viable strategy to determine its reactivity in cell extracts.

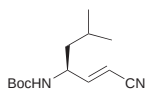
3.4 Experimental

All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.), ethyl acetate (EA), and light petroleum ether (PE) were obtained from Riedel-de Haën or Biosolve and were distilled prior to use. Dichloromethane (DCM), dimethyl formamide (DMF), and dioxane were stored on 4 Å molecular sieves. Tetrahydrofuran (THF) was distilled from LiAlH₄ prior to use. Reactions were conducted under an argon atmosphere. Reactions were monitored by TLC analysis by using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by UV absorption (254 nm), spraying with 20% H₂SO₄ in ethanol or (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid followed by charring at ~150°C or by spraying with an aqueous solution of KMnO₄ (7%) and KOH (2%). Column chromatography was performed on silica gel from Screening Devices (0.040-0.063 nm). LC/MS analysis was performed on a LCQ Advantage Max (Thermo Finnigan) equipped with an Gemini C18 column (Phenomenex). HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ¹H- and ¹³C-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. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Coupling constants are given in Hz. All presented ¹³C-APT spectra are proton decoupled. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, λ = 589 nm).

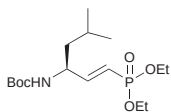


purification.

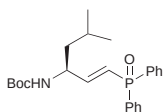
Boc-Leucinal (126). Boc-leucine Weinreb amide (1.7 g 6.2 mmol) was dissolved in Et₂O (30 mL) at 0°C. LiAlH₄ (0.24 g, 6.2 mmol, 1 equiv.) was added, and the mixture was stirred for 30 min. The reaction mixture was quenched by slow addition of 1M HCl. The water layer was extracted with Et₂O (3×). The combined organic layers were washed with brine and dried over Na₂SO₄, yielding the title compound, which was used without further



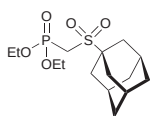
(S,E)-4-Boc-amino-6-methylhept-2-enenitrile (127). Diethyl cyanomethylphosphonate (1.39 g, 7.88 mmol, 1.5 equiv.) was dissolved in THF (30 mL) at 0°C. NaH (60% disp. in mineral oil, 0.315 g, 7.88 mmol, 1.5 equiv.) was added and the mixture was stirred for 30 minutes. Boc-leucinal **126** (1.11 g, 5.16 mmol, 1 equiv.) in THF was added, and the mixture was stirred o/n. EA was added, and the mixture was washed with 1M HCl (2×) and brine. The organic layer was dried with MgSO₄ and concentrated. Column chromatography (EA:PE (10 → 25%)) yielded the title compound (0.52 g, 2.18 mmol, 42%). ¹H NMR (200 MHz, CDCl₃): δ ppm 6.61 (dd, *J*₁ = 16.3 Hz, *J*₂ = 5.1 Hz, 1H), 5.49 (dd, *J*₁ = 16.1 Hz, *J*₂ = 1.5 Hz, 1H), 4.45-4.20 (m, 2H), 1.80-1.35 (m, 12H), 0.94 (d, *J* = 6.6 Hz, 6H). ¹³C NMR (50.1 MHz, (CDCl₃): δ ppm 155.44, 154.80, 116.71, 98.53, 79.18, 50.1, 42.37, 27.85, 24.22, 22.82, 21.41.



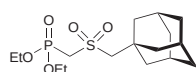
Boc-leucine vinyl diethylphosphonate (128). Tetraethyl methylenediphosphonate (0.9 g, 3.12 mmol, 1.5 equiv.) was dissolved in THF (10 mL) at 0°C. NaH (60% disp in mineral oil, 125 mg, 3.12 mmol, 1.5 equiv.) was added, and the mixture was stirred for 30 minutes at 0°C. Boc-leucinal **126** (448 mg, 2.08 mmol, 1 equiv.) in THF was added and the resulting mixture was stirred for 3 hr. 1M HCl and EA were added, and the layers were separated. The organic layer was washed with 1M HCl and brine and was dried with MgSO₄ and concentrated. Column chromatography yielded the title compound (596 mg, 1.71 mmol, 82%). [α]_D²⁰ -14.5° (c = 1, CHCl₃). ¹H NMR (200 MHz, CDCl₃): δ ppm 6.66 (ddd, *J*₁ = 21.9 Hz, *J*₂ = 17.2 Hz, *J*₃ = 4.8 Hz, 1H), 5.77 (ddd, *J*₁ = 17.2 Hz, *J*₂ = 3.7 Hz, *J*₃ = 1.5 Hz, 1H), 4.45-4.15 (m, 2H), 4.17-4.02 (m, 4H), 1.78-1.50 (m, 1H), 1.44 (s, 9H), 1.40-1.25 (m, 8H), 0.93 (d, *J* = 6.6 Hz, 6H). ¹³C NMR (50.1 MHz, CDCl₃): δ ppm 154.70, 153.08, 152.99, 116.85, 113.13, 78.13, 77.64, 77.00, 76.35, 60.88, 60.77, 50.63, 50.22, 42.53, 27.61, 23.97, 22.03, 21.30, 21.24, 15.49.



(S,E)-1-(diphenylphosphinoxy)-3-(Boc-amino)-5-methyl-1-hex-ene (129). Diethyl methylphosphonate (0.96 g, 6.32 mmol, 1 equiv.) was dissolved in THF and cooled to -78°C. LiHMDS (12.64 mL 1M sln. in THF/EtPh, 2 equiv.) was added and the solution was stirred for 15 min. Chlorodiphenylphosphinoxy (1.5 g, 6.32 mmol, 1 equiv.) was added and the solution was stirred for 1 h at -40°C. Boc-leucinal **126** (1.48 g, 6.3 mmol, 1 equiv.) in THF was added dropwise, and the mixture was stirred for 3 h at -40°C. The reaction was quenched with H₂O, and the resulting mixture was extracted with EA (3×). The combined organic layers were dried with Na₂SO₄ and concentrated. Flash column chromatography (EA:PE 40 → 80%, 2×) yielded the title compound (184 mg, 0.29 mmol, 5% isolated yield). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.78-7.36 (m, 10H), 6.79-6.51 (m, 1H), 6.45-6.32 (m, 1H), 4.98-4.71 (m, 1H), 4.42-4.20 (m, 1H), 1.88-1.13 (m, 12H), 0.98-0.83 (m, 6H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 155.08, 152.01, 133.35, 133.11, 132.30, 132.08, 131.60, 131.21, 131.12, 128.41, 128.29, 121.55, 120.54, 79.32, 51.58, 51.41, 43.19, 28.16, 24.56, 22.61, 21.92.

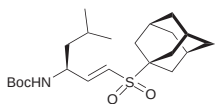


Diethyl 1-adamantylsulfonylmethylphosphonate (132). 1-adamantylthiol (1.77 g, 10 mmol, 1 equiv.) was dissolved in THF (50 mL) at 0°C. NaH (60% disp. in min. oil, 0.42 g, 10.5 mmol, 1.05 equiv.) was added, and the mixture was stirred for 1 hr. Diethyl iodo-methylphosphonate (2.78 g, 10 mmol, 1 equiv.) in THF was added, and the mixture was stirred for 1.5 hr. The reaction was quenched with 1M HCl, and the mixture was extracted with DCM (2×). The organic layer was dried with MgSO₄ and concentrated. The residue was dissolved in 1,4-dioxane and cooled to 0°C. AcOOH (8.4 mL 32% in AcOH (w/w), 40 mmol, 4 equiv.) was added and the mixture was stirred for 2 hr. Sat. aq. NaHCO₃ was added, and the mixture was extracted with EA (2×). The organic layer was dried with MgSO₄ and concentrated. The residue was recrystallised from EA:PE to yield the title compound (2.79 g, 7.95 mmol, 79%). ¹H NMR (400 MHz, CDCl₃): δ ppm 4.31-4.23 (m, 4H), 3.48 (d, *J* = 18.7 Hz, 2H), 2.21 (s, 3H), 2.04 (d, *J* = 2.3 Hz, 6H), 1.80-1.66 (m, 6H), 1.38 (t, *J* = 7.1 Hz, 6H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 63.61, 63.54, 42.81, 41.46, 35.60, 34.65, 28.12, 16.29, 16.22.



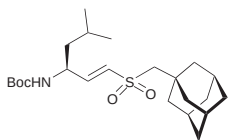
Diethyl 1-adamantylmethylsulfonylmethylphosphonate (133). 1-adamantyl-methylthiol¹⁷¹ (1.82 g, 10 mmol) was dissolved in THF (50 mL) at 0°C. NaH (60% disp. in mineral oil, 0.42 g, 10.5 mmol, 1.05 equiv.) was added and the solution was stirred for 1 hr. Diethyl iodo-methylphosphonate (2.78 g, 10 mmol, 1 equiv.) was added, and the mixture was stirred for 2 hr. The reaction was quenched by the addition of 1M HCl. The water layer was extracted with DCM (3×) and the combined organic layers were dried with MgSO₄ and evaporated.

The residue was dissolved in dioxane at 0°C and AcOOH (6.8 mL 39% sln in AcOH (w/w), 40 mmol, 4 equiv.) was added. The reaction was allowed to warm to RT and stirred for 1 hr. Sat. aq. NaHCO₃ was added, and this mixture was extracted with EA (3×). The combined organic layers were dried over MgSO₄ and evaporated. The residue was recrystallised from EA:PE, yielding two crops of the title compound (total 2.38 g, 6.54 mmol, 65%). ¹H NMR (400 MHz, CDCl₃): δ ppm 4.28-4.18 (m, 4H), 3.52 (d, *J* = 16.5 Hz, 1H), 3.21 (s, 2H), 2.04-1.97 (m, 3H), 1.90-1.87 (m, 6H), 1.78-1.60 (m, 6H), 1.37 (t, *J* = 7.1 Hz, 1H).



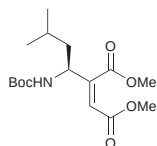
Boc-leucyl vinyl-1-adamantylsulfone (134). Diethyl 1-adamantylsulfonylmethylphosphonate **133** (1.9 g, 5.4 mmol, 1.1 equiv.) was dissolved in 50 mL THF at 0°C. KOtBu (607 mg, 5.4 mmol, 1.1 equiv.) was added and the mixture was stirred for 30 min. Boc-leucinal **126** (1.06 g, 4.92 mmol, 1 equiv.) in THF was added and the mixture was stirred at 0°C for 1 hr. Sat. aq. NH₄Cl was added and the mixture was extracted with EA (2×). The organic layer was dried with MgSO₄ and concentrated.

Column chromatography (EA:PE (5 → 30%)) and recrystallisation from DCM:PE yielded the title compound (1.22 g, 3.06 mmol, 62%). ¹H NMR (400 MHz, CDCl₃): δ ppm 6.69 (dd, *J* = 15.0, 5.33 Hz, 1H), 6.33 (d, *J* = 15.1 Hz, 1H), 5.17 (d, *J* = 7.6 Hz, 1H), 4.40-4.29 (m, 1H), 2.14 (s, 3H), 2.01-1.90 (m, 6H), 1.80-1.62 (m, 7H), 1.47-1.41 (m, 11H), 0.96-0.91 (m, 6H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 154.77, 150.76, 122.70, 79.16, 59.40, 49.75, 42.38, 35.45, 34.22, 27.97, 27.75, 24.35, 22.34, 21.73.



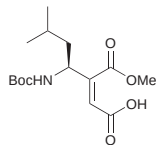
(S,E)-1-(1-adamantylmethylsulfonyl)-5-methylhex-1-en-3-Boc-amine (135). Diethyl 1-adamantylmethylsulfonylmethylphosphonate **133** (2.12 g, 5.83 mmol, 1.1 equiv.) was dissolved in THF (50 mL) and cooled to 0°C. KOtBu (0.65 g, 5.83 mmol, 1.1 equiv.) was added and the mixture was stirred for 30 min. Boc-leucinal **126** (1.14 g, 5.3 mmol, 1 equiv.) in THF (30 mL) was slowly added and the mixture was stirred for 90 min. The reaction was quenched with sat. aq. NH₄Cl. More sat. aq. NH₄Cl was added, and the resulting mixture was extracted with EA. The organic phase was washed with brine, dried over MgSO₄ and evaporated. Column chromatography (EA:PE (0 → 12%)) yielded the title compound (2.0 g, 4.7 mmol, 89%).

¹H NMR (400 MHz, CDCl₃): δ ppm 6.74 (dd, *J*₁ = 15.0, *J*₂ = 5.2 Hz, 1H), 6.46 (dd, *J*₁ = 15.1, *J*₂ = 1.2 Hz, 1H), 5.12 (d, *J* = 8.3 Hz, 1H), 4.44-4.33 (m, 1H), 2.81 (s, 1H), 2.02-1.97 (m, 3H), 1.87-1.79 (m, 6H), 1.75-1.63 (m, 7H), 1.47-1.41 (m, 11H), 0.94 (d, *J* = 6.7 Hz, 6H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 154.82, 147.16, 130.30, 79.19, 66.82, 49.10, 42.78, 41.73, 36.08, 33.91, 28.00, 24.37, 22.41, 21.65.



(S)-dimethyl-2-(1-Boc-amino-3-methylbutyl)maleate (137). 40 mL THF was cooled to 0°C. NaH (60% disp. in oil, 156 mg, 3.9 mmol, 1.5 equiv.) was added, followed by methyl dimethylphosphonoacetate (580 μL, 3.9 mmol, 1.5 equiv.) and the mixture was stirred for 30 min. at 0°C. (S)-3-(Boc-amino)-5-methyl-2-oxohexanoic acid methyl ester (**136**)¹⁷² (708 mg, 2.6 mmol, 1 equiv.) in THF was added and the mixture was stirred for 2.5 hr. Sat. aq. NH₄Cl was added, and the mixture was extracted with EA. The organic layer was washed with sat. aq. NaHCO₃, brine, dried with MgSO₄ and concentrated. Column chromatography (EA:PE (5 → 25%)) yielded the title compound (674 mg, 2.04 mmol, 79%).

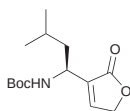
[α]_D²⁰ -31.1° (c = 1, CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ ppm 6.03 (s, 1H), 5.31 (d, *J* = 8.3 Hz, 1H), 4.49-4.40 (m, 1H), 3.81 (s, 3H), 3.72 (s, 3H), 1.84-1.62 (m, 1H), 1.60-1.33 (m, 11H), 0.96-0.89 (m, 6H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 167.15, 164.78, 154.55, 149.93, 119.54, 79.02, 51.78, 51.42, 51.32, 41.82, 27.81, 24.24, 22.29, 21.29.



(S,Z)-4-Boc-amino-3-(methylxycarbonyl)-6-methylhept-2-enoic acid (138). (S)-dimethyl-2-(1-Boc-amino-3-methylbutyl)-maleate **137** (674 mg, 2.04 mmol) was dissolved in THF (8 mL) at 0°C. LiOH (2.04 mL 1M aq sln, 2.04 mmol, 1 equiv.) was added and the solution was stirred for 2.5 hr. LiOH (306 μL 1M aq sln, 306 μmol, 0.15 equiv.) was added and the solution was allowed to warm to RT and stirred o/n. 1M aq. HCl was added, and the mixture was extracted with EA (2×). The combined organic layers were

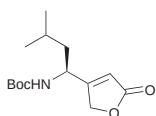
washed with brine, dried with MgSO₄ and concentrated. Column chromatography (EA:PE (30 → 70%)) yielded the title compound (467 mg, 1.48 mmol, 73%). [α]_D²⁰ -23.3° (c = 1, CHCl₃). ¹H NMR (400 MHz, CDCl₃, mixture of rotamers): δ ppm 10.55 (s, 1H), 6.57 (d, *J* = 7.1 Hz, 1H), 6.03 (s, 1H), 5.94 (s, 1H), 5.06 (d, *J* = 8.5 Hz, 1H), 4.50-4.41 (m, 1H), 4.28-4.19 (m, 1H), 3.80 (s, 3H), 3.78 (s, 3H), 1.81-1.63 (m, 2H), 1.53-1.40 (m, 22H), 0.97-0.87 (m, 12H). ¹³C NMR (100 MHz, CDCl₃, mixture of rotamers): δ ppm 168.30, 167.59, 167.19, 156.93, 154.88, 152.01,

150.71, 120.32, 119.37, 81.55, 79.79, 53.05, 52.21, 51.79, 42.16, 42.01, 28.09, 27.93, 24.50, 24.36, 22.67, 22.52, 21.63, 21.30.



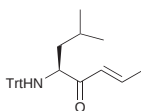
(S)-3-(1-Boc-amino-3-methylbutyl)furan-2(5H)-one (139). (S,Z)-4-Boc-amino-3-(methoxycarbonyl)-6-methylhept-2-enoic acid **138** (312 mg, 0.99 mmol) was dissolved in 20 mL THF at 0°C. NEt_3 (153 μL , 1.1 mmol, 1.1 equiv.) and EtOCOCl (110 μL , 1.1 mmol, 1.1 equiv.) were added and the mixture was stirred for 15 min. The mixture was filtered into a solution of NaBH_4 (60 mg, 1.5 mmol, 1.5 equiv.) in 20 mL H_2O at 0°C. The mixture was allowed to warm to RT and stirred for 1.5 hr. 1M HCl was added and the mixture was extracted with EA.

The organic layer was washed with 1M HCl, sat. aq. NaHCO_3 and brine. After drying with MgSO_4 and concentration, the residue was purified with column chromatography (EA:PE (10 $\rightarrow$ 50%)), yielding the title compound (190 mg, 0.70 mmol, 71%). ^1H NMR (400 MHz, CDCl_3): δ ppm 7.28 (s, 1H), 5.31 (d, J = 8.7 Hz, 1H), 4.82 (s, 2H), 4.66–4.55 (m, 1H), 1.66–1.53 (m, 3H), 1.43 (s, 9H), 0.99–0.89 (m, 6H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.60, 154.96, 145.43, 133.98, 79.29, 70.00, 45.88, 42.08, 28.10, 24.57, 22.30, 21.79. $[\alpha]_{\text{D}}^{20}$ -24.8° (c = 1, CHCl_3).



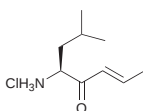
(S)-4-(1-Boc-amino-3-methylbutyl)-2(5H)-furanone (141). (S)-1-chloro-2-oxo-3-(Boc-amino)-5-methylhexane **140**¹⁷³ (0.79 g, 3 mmol) was coevaporated with toluene and dissolved in dry MeCN. Potassium dimethylphosphonoacetate (1.36 g, 6.6 mmol, 2.2 equiv.) was coevaporated with toluene and added to the reaction mixture, followed by stirring at 50°C for 3 hr. EA and 20% aq. NaH_2PO_4 were added, layers separated, and the water layer was extracted with EA (2 $\times$). The combined organic layers were dried over MgSO_4 and concentrated. The residue was dissolved in dry MeCN (50 mL) and LiCl (153 mg, 3.6 mmol, 1.2 equiv.) and NEt_3 (0.42 mL, 3 mmol, 1 equiv.). After 3 hr., the reaction mixture

was poured into 1M HCl, followed by extraction with EA. The organic layer was dried over MgSO_4 and concentrated. Flash column chromatography (EA:toluene (0 $\rightarrow$ 15%)) yielded the title compound (418 mg, 1.55 mmol, 52%). ^1H NMR (200 MHz, CDCl_3): δ = 5.91 (q, J = 1.5 Hz, 1H), 5.44 (d, J = 8.0 Hz, 1H), 4.95–4.75 (m, 2H), 4.74–4.60 (m, 1H), 1.85–1.57 (m, 3H), 1.44 (s, 9H), 0.99–0.89 (m, 6H). ^{13}C NMR (50.1 MHz, CDCl_3): δ ppm 173.4, 172.2, 155.2, 114.7, 79.6, 71.3, 47.2, 42.0, 28.0, 24.3, 22.7, 21.1. $[\alpha]_{\text{D}}^{20}$ -73.2° (c = 1, MeOH). HRMS: calcd for $[\text{C}_{14}\text{H}_{23}\text{NO}_4\text{Na}]^+$ 292.15193, found 292.15175.



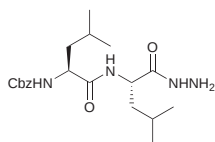
(S,E)-5-amino-7-methyloct-2-en-4-one (143). 1-(dimethylphosphonate)-2-oxo-3-(tritramino)-5-methylhexane **142**¹⁷⁴ (12 g, 23.64 mmol) was dissolved in EtOH (100 mL) and acetaldehyde (11 mL, 190 mmol, 8 equiv.) was added. K_2CO_3 (3.5 g, 25 mmol, 1.06 equiv.) was added in portions, and the reaction was stirred o/n. The reaction mixture was filtered and concentrated. The residue was dissolved in EA and extracted with sat. aq. NaHCO_3 and brine. The organic layer was dried with MgSO_4 and concentrated. Column chromatography (EA:PE (1 $\rightarrow$ 5%)) yielded the title compound (8.06 g, 20.27 mmol, 86%). ^1H NMR (400 MHz, CDCl_3): δ ppm

7.53–7.02 (m, 15H), 6.45–6.24 (m, 1H), 5.69 (dd, J_1 = 15.6, J_2 = 1.6 Hz, 1H), 3.59 (t, J = 6.6 Hz, 1H), 3.15–3.01 (m, 1H), 1.75–1.64 (m, 1H), 1.57 (dd, J_1 = 6.9, J_2 = 1.36 Hz, 3H), 1.44 (t, J = 6.8 Hz, 2H), 0.86 (d, J = 6.6 Hz, 3H), 0.84 (d, J = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 202.53, 146.29, 140.79, 128.76, 127.43, 125.98, 70.96, 58.07, 45.00, 24.33, 23.28, 22.51, 17.58.

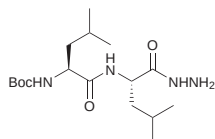


(S,E)-5-amino-7-methyloct-2-en-4-one HCl (144). (S,E)-5-amino-7-methyloct-2-en-4-one **143** (440 mg, 1.1 mmol) was dissolved in 6 mL Et_2O . HCl (550 μL 4M sln in dioxane, 2 equiv.) and TIS (341 μL , 1.65 mmol, 1.5 equiv.) were added and the mixture was stirred for 1 hr. The reaction mixture was filtered and the residue washed with Et_2O (4 $\times$). The residue was recrystallised from DCM/ Et_2O (3 $\times$), yielding the title compound (134 mg, 0.7 mmol, 63%).

^1H NMR (400 MHz, CD_3OD): δ ppm 8.60 (s, 3H), 7.07 (dq, J_1 = 13.9, J_2 = 6.9 Hz, 1H), 6.24 (dd, J_1 = 15.5, J_2 = 1.1 Hz, 1H), 4.53–4.43 (m, 1H), 2.14–2.00 (m, 1H), 1.97–1.92 (m, 3H), 1.92–1.86 (m, 1H), 1.71–1.60 (m, 1H), 1.04 (d, J = 6.4 Hz, 3H), 0.98 (d, J = 6.5 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 194.28, 146.46, 127.16, 56.14, 39.47, 24.46, 22.93.

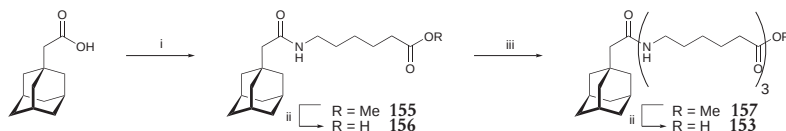


ZLeu₂NHNH₂ (147). ZLeu₂OMe **145** (6.67 g, 17 mmol) was dissolved in 50 mL MeOH. H₂NNH₂·H₂O (10 mL, 200 mmol, 30 equiv.) was added, and the mixture was refluxed o/n. The mixture was coevaporated with toluene (3×) and subjected to flash column chromatography (MeOH:EA:NEt₃ (0:99:1 → 20:79:1)) to yield the title compound (6.53 g, 16.6 mmol, 98%). ¹H NMR (400 MHz, CD₃OD): δ ppm 7.42-7.28 (m, 5H), 5.12 (s, 2H), 4.62 (s, 2H), 4.40 (dd, *J*₁ = 9.4, *J*₂ = 5.4 Hz, 1H), 4.17 (t, *J* = 7.6 Hz, 1H), 1.77-1.48 (m, 6H), 0.99-0.88 (m, 12H).

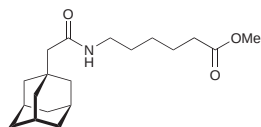


BocLeu₂NHNH₂ (148). BocLeu₂OMe **146** (1.62 g, 4.52 mmol) was dissolved in 50 mL MeOH. H₂NNH₂·H₂O (6.57 mL, 136 mmol, 30 equiv.) was added and the mixture was refluxed for 2 hr. Concentration and column chromatography yielded the title compound (1.21 g, 3.38 mmol, 75%). ¹H NMR (400 MHz, CD₃OD): δ ppm 4.46-4.39 (m, 1H), 4.11 (t, *J* = 7.5 Hz, 1H), 1.76-1.46 (m, 6H), 1.43 (s, 9H), 0.95-0.87 (m, 12H).

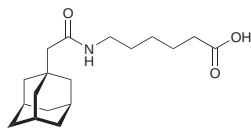
Scheme 3.4: Synthesis of AdaAhx₃OH tail **153**



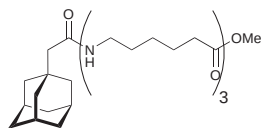
Reagents and conditions: i) HCl-H-Ahx-OMe (1 equiv.), Ada-AcOH (1.1 equiv.), HCTU (1.15 equiv.), DiPEA (3.25 equiv.), DCM, 3 hr., quant. ii) LiOH (1M aq. sln., 1.1 equiv.), THF, 0°C, 12 hr., 99% (**156**), 95% (**153**). iii) (a) Boc-Ahx₂-OMe, TFA/DCM 1/1, 30 min., quant. (b) **156** (1 equiv.), HCTU (1.15 equiv.), DiPEA (3.3 equiv.), DMF, 10 min., then TFA-H-Ahx₂-OMe (1.1 equiv.), 2 hr., 87%.



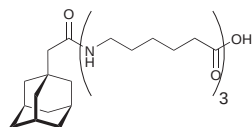
AdaAhxOMe (155). To a solution of methyl 6-aminoheptanoate HCl salt (0.78 g, 4.30 mmol) and 1-adamantanecarboxylic acid (0.94 g, 4.73 mmol, 1.1 equiv.) in DCM (25 mL) were added HCTU (2.0 g, 4.94 mmol, 1.15 equiv.) and DiPEA (2.31 mL, 14 mmol, 3.25 equiv.). The mixture was stirred for 3 hr., after which EA (100 mL) was added followed by washing of the mixture with 1M HCl (2×), sat. aq. NaHCO₃ (2×) and brine. Drying over MgSO₄ and concentration of the mixture resulted in crude product, which was further purified by column chromatography (EA:PE (10 → 100%)). The product (yield: 1.38 g, 4.30 mmol, quant.) was obtained as a colorless solid. LC/MS: linear gradient 10% → 90% MeCN/(0.1% TFA/H₂O): *R*_t (min): 8.71 (ESI-MS (*m/z*): 322.20 (M + H⁺)). ¹H NMR (400 MHz, CDCl₃): δ ppm 5.60-5.53 (m, 1H), 3.67 (s, 3H), 3.24 (dd, *J*₁ = 13.2, *J*₂ = 6.9 Hz, 2H), 2.32 (t, *J* = 7.4 Hz, 2H), 1.99-1.94 (m, 3H), 1.91 (s, 2H), 1.73-1.59 (m, 15H), 1.56-1.48 (m, 2H), 1.41-1.31 (m, 2H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 173.95, 170.81, 51.78, 51.42, 42.56, 39.01, 36.70, 33.79, 32.62, 29.33, 28.56, 26.36, 24.39.



AdaAhxOH (156). Methyl ester **155** (2.79 g, 8.68 mmol) was dissolved in THF (35 mL) and cooled to 0°C. An aqueous solution of LiOH (1M, 9.54 mmol, 9.54 mL, 1.1 eq.) was added and the reaction was stirred at 0°C for 12 hr. after which TLC analysis indicated complete consumption of starting material. Water (50 mL) was added and the mixture was extracted twice with EA. Next, the aqueous layer was acidified with concentrated HCl to pH 1 and extracted again with EA (2×). The latter two combined organic layers were dried (MgSO₄) and concentrated under reduced pressure, which yielded the title compound (yield: 2.63 g, 8.57 mmol, 99%) as a colorless solid. LC/MS: linear gradient 10% → 90% MeCN/(0.1% TFA/H₂O): *R*_t (min): 7.49 (ESI-MS (*m/z*): 308.13 (M + H⁺)). ¹H NMR (400 MHz, CDCl₃): δ ppm 10.87 (s, 1H), 5.95 (t, *J* = 5.6 Hz, 1H), 3.24 (dd, *J*₁ = 13.2, *J*₂ = 6.81 Hz, 2H), 2.34 (t, *J* = 7.3 Hz, 2H), 1.99-1.92 (m, 5H), 1.73-1.58 (m, 14H), 1.53 (dt, *J*₁ = 14.7, *J*₂ = 7.4 Hz, 2H), 1.43-1.33 (m, 2H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 177.96, 171.55, 51.49, 42.48, 39.15, 36.62, 33.86, 32.63, 29.11, 28.51, 26.24, 24.20.



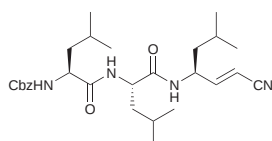
AdaAhx₃OMe (157). Methyl 6-(6-(tert-butoxycarbonylamino)hexanamido)hexanoate¹⁷⁹ (3.37 g, 9.39 mmol, 1.1 equiv.) was dissolved in DCM/TFA (1/1, v/v) and stirred for 30 minutes before being coevaporated with toluene (3×). AdaAhxOH **156** (2.63 g, 8.57 mmol, 1 equiv.) was dissolved in DMF. HCTU (4.08 g, 9.86 mmol, 1.15 eq) and DiPEA (5.12 mL, 31 mmol, 3.3 equiv.) were added and the mixture was stirred for 10 min. Methyl 6-hexanamido hexanoate TFA salt in DMF was added, and the mixture was stirred for 2 hr. The mixture was concentrated, dissolved in DCM, washed with 1M HCl (2×), sat. aq. NaHCO₃ (4×) and brine. The organic layer was dried with MgSO₄ and concentrated. The title compound was obtained as a colorless solid (yield: 4.10 g, 7.49 mmol, 87%) after column purification (EA 0 → 15% MeOH:EA). LC/MS: linear gradient 10% → 90% MeCN/(0.1% TFA/H₂O): *R_t* (min): 7.93 (ESI-MS (*m/z*): 548.33 (M + H⁺)). ¹H NMR (400 MHz, CDCl₃): δ ppm 6.42 (t, *J* = 5.7 Hz, 1H), 6.38 (t, *J* = 5.6 Hz, 1H), 6.13 (t, *J* = 5.6 Hz, 1H), 3.66 (s, 3H), 3.25-3.18 (m, 6H), 2.31 (t, *J* = 7.4 Hz, 2H), 2.18 (t, *J* = 7.4 Hz, 4H), 1.98-1.94 (m, 3H), 1.92 (s, 2H), 1.73-1.58 (m, 18H), 1.56-1.47 (m, 6H), 1.39-1.29 (m, 6H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 173.95, 172.97, 172.92, 171.02, 51.54, 51.37, 42.52, 39.07, 39.00, 36.65, 36.27, 33.75, 32.57, 29.28, 29.15, 28.51, 26.40, 26.38, 26.29, 25.18, 25.15, 24.39.



AdaAhx₃OH (153). Methyl ester **157** (4.10 g, 7.49 mmol) was dissolved in THF/MeOH 5:1 (35 mL) and cooled to 0°C. An aqueous solution of LiOH (1M, 8.24 mmol, 8.24 mL, 1.1 eq.) was added and the reaction was stirred at RT for 12 hr. after which TLC analysis indicated complete consumption of starting material. Water (50 mL) was added and the mixture was extracted twice with EA. Next, the aqueous layer was acidified with concentrated HCl to pH 1 and extracted with DCM (3×). The combined DCM layers were dried (MgSO₄) and concentrated under reduced pressure, which yielded the title compound (3.79 g, 7.12 mmol, 95%) as a colorless solid. LC/MS: linear gradient 10% → 90% MeCN/(0.1% TFA/H₂O): *R_t* (min): 7.21 (ESI-MS (*m/z*): 534.33 (M + H⁺)). ¹H NMR (400 MHz, CD₃OD): δ ppm 7.96-7.90 (m, 2H), 7.86-7.79 (m, 1H), 3.19-3.11 (m, 6H), 2.29 (t, *J* = 7.4 Hz, 2H), 2.17 (t, *J* = 7.4 Hz, 4H), 1.97-1.93 (m, 3H), 1.91 (s, 2H), 1.74 (d, *J* = 12.3 Hz, 3H), 1.69-1.57 (m, 15H), 1.55-1.46 (m, 6H), 1.40-1.28 (m, 6H). ¹³C NMR (100 MHz, CD₃OD): 177.40, 175.97, 173.74, 51.91, 43.79, 40.21, 37.95, 37.02, 34.83, 33.81, 30.24, 30.19, 30.14, 27.68, 27.58, 27.53, 26.74, 25.75.

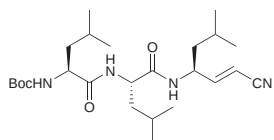
General protocol for azide couplings The Boc-protected warhead was dissolved in TFA:DCM (1:1, v/v) and stirred for 20 min. Coevaporation with toluene (3×) afforded the warhead TFA-salt, which was used without further purification. The appropriate hydrazide (**147** or **148**) was dissolved in 1:1 DMF:DCM (v/v) and cooled to -30°C. *t*BuONO (1.1 equiv.) and HCl (4M sln. in 1,4-dioxane, 2.8 equiv.) were added, and the mixture was stirred for 3 hr. at -30°C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. The warhead-TFA salt was added to the reaction mixture as a solution in DMF with 1.1 equivalent of DiPEA. A further 3.9 equivalents of DiPEA were added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EA and washed with H₂O (3×). The organic layer was dried over MgSO₄ and purified by flash column chromatography.

General protocol for block couplings The Boc-protected tripeptide was dissolved in TFA:DCM (1:1, v/v) and stirred for 20 min. Coevaporation with toluene (3×) afforded the tripeptide TFA-salt, which was used without further purification. The carboxylic acid (**152** or **153**, 1 equiv.) was dissolved in DCM:DMF (1/1, v/v). HBTU (1.1 equiv.), DiPEA (3.5 equiv.) were added and the mixture was stirred for 5 min. A solution of the tripeptide TFA salt in DMF was added and the mixture was stirred for 2 hr. before being concentrated. The residue was taken up in DCM, washed with 1M HCl (2×), sat. aq. NaHCO₃ (4×), brine, and dried with Na₂SO₄. The residue was purified by flash column chromatography.



(S,E)-4-Cbz-Leu₂-amino-6-methylhept-2-enenitrile (96). Following the general procedure for azide coupling the title compound was obtained from (S,E)-4-Boc-amino-6-methylhept-2-enenitrile **127** (141 mg, 0.59 mmol, 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (210 mg, 0.534 mmol, 1 equiv.). Purification by flash chromatography (EA:PE (0 → 40%)) gave the title compound (138 mg, 0.277 mmol, 52%). ¹H NMR (500 MHz, CD₃OD): δ ppm 7.35-7.29 (m, 6H), 6.74 (dd, *J*₁ = 16.3, *J*₂ = 5.0 Hz), 5.59-5.55 (m, 1H), 5.13-5.05 (m, 2H), 4.56-4.55 (m, 1H), 4.38-4.35 (m, 1H), 4.12-4.09 (m, 1H), 1.70-1.34 (m, 9H), 0.96-0.97 (m, 18H). ¹³C NMR (125 MHz, CD₃OD): δ ppm 175.11, 175.03,

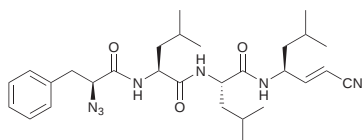
173.93, 173.85, 158.24, 156.50, 137.78, 129.34, 129.27, 128.87, 118.16, 99.83, 67.50, 54.90, 53.13, 50.20, 42.94, 41.95, 41.40, 25.76, 25.60, 25.54, 23.37, 22.26, 22.14, 22.00. HRMS: calcd. For $[C_{28}H_{43}N_4O_4]^+$ 499.32788, found 499.32764.



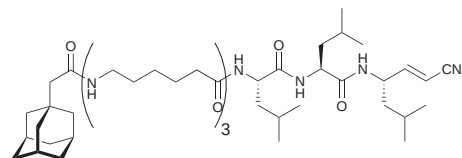
171.45, 156.17, 154.54, 117.31, 99.64, 80.81, 53.92, 52.23, 48.92, 42.69, 40.57, 40.09, 28.23, 24.88, 24.82, 24.69, 22.96, 22.89, 22.61, 21.86, 21.75, 21.67.

(S,E)-4-Boc-Leu₂-amino-6-methylhept-2-enenitrile (150). Following the general procedure for azide coupling the title compound was obtained from (S,E)-4-Boc-amino-6-methylhept-2-enenitrile **127** (49 mg, 0.21 mmol, 1.1 equiv.) and BocLeu₂NHNH₂ **148** (67 mg, 0.187 mmol, 1 equiv.). Purification by flash chromatography (EA:PE (0 → 30%)) gave the title compound (66 mg, 0.14 mmol, 76%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.00-6.90 (m, 1H), 6.62 (dd, *J*₁ = 16.4 Hz, *J*₂ = 4.9 Hz, 2H), 5.56 (d, *J* = 16.4 Hz, 1H), 5.12-5.00 (m, 1H), 4.67-4.58 (m, 1H), 4.41-4.35 (m, 1H), 4.08-4.00 (m, 1H), 1.82-1.25 (m, 18H), 1.10-0.85 (m, 18H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 172.89,

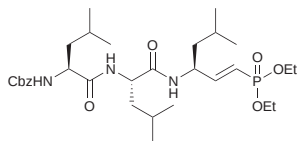
(S,E)-4-azido-Phe-Leu₂-amino-6-methylhept-2-enenitrile (97) Following the general procedure for block coupling, the title compound was obtained from (S,E)-4-Boc-Leu₂-amino-6-methylhept-2-enenitrile **150** (66 mg, 0.14 mmol, 1 equiv.) and azido-phenylalanine **152**¹⁷⁵ (29 mg, 0.154 mmol, 1.1 equiv.). Flash column chromatography (EA:tol (0 → 30%)) yielded the title compound (53 mg, 99 μmol, 70%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.35-7.20 (m, 5H), 7.06-6.98 (m, 1H), 6.96-6.86 (m, 1H), 6.59 (dd, *J*₁ = 16.3 Hz, *J*₂ = 5.5 Hz, 1H), 5.44 (d, *J* = 16.3 Hz, 1H), 4.57-4.42 (m, 3H), 4.24 (dd, *J*₁ = 7.8 Hz, *J*₂ = 4.1 Hz, 1H), 3.21 (dd, *J*₁ = 14.2 Hz, *J*₂ = 3.6 Hz, 1H), 3.05 (dd, *J*₁ = 14.2 Hz, *J*₂ = 7.9 Hz, 1H), 1.82-1.22 (m, 9H), 1.04-0.73 (m, 18H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 171.78, 171.43, 169.05, 154.39, 135.61, 129.37, 128.69, 127.35, 117.05, 99.73, 64.75, 51.95, 51.87, 49.17, 42.42, 41.10, 40.36, 38.00, 24.92, 24.67, 24.59, 22.80, 22.69, 22.61, 22.20, 21.77. HRMS: calcd. for $[C_{29}H_{44}N_7O_3]^+$ 538.35001, found 538.34979.



(S,E)-4-Ada-Ahx₃-Leu₂-amino-6-methylhept-2-enenitrile (98). Following the general procedure for block coupling, the title compound was obtained from (S,E)-4-Boc-Leu₂-amino-6-methylhept-2-enenitrile **150** (64 mg, 138 μmol) and AdaAhx₃OH **153** (81 mg, 152 μmol, 1.1 equiv.). Flash column chromatography (MeOH:DCM (2 → 8%)) yielded **98** (108 mg, 123 μmol, 80%). ¹H NMR (400 MHz, CD₃OD): δ ppm 8.12-8.04 (m, 2H), 8.00 (d, *J* = 8.2 Hz, 1H), 7.95 (t, *J* = 5.1 Hz, 2H), 7.87-7.82 (m,

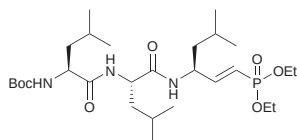


1H), 6.73 (dd, *J*₁ = 16.4, *J*₂ = 5.0 Hz, 1H), 5.55 (d, *J* = 16.4 Hz, 1H), 4.60-4.49 (m, 1H), 4.39-4.25 (m, 2H), 3.13 (dd, *J*₁ = 12.1, *J*₂ = 6.0 Hz, 6H), 2.23 (t, *J* = 7.3 Hz, 2H), 2.15 (t, *J* = 7.4 Hz, 4H), 1.95-1.87 (m, 5H), 1.76-1.24 (m, 38H), 0.96-0.84 (m, 18H). ¹³C NMR (100 MHz, CD₃OD): δ ppm 176.45, 175.95, 175.17, 174.37, 173.73, 156.76, 118.25, 99.86, 53.71, 53.45, 51.93, 50.40, 43.74, 43.12, 41.67, 41.51, 40.31, 40.26, 40.18, 37.90, 37.04, 36.66, 33.77, 30.22, 30.15, 30.12, 27.65, 27.59, 27.56, 26.73, 26.56, 25.94, 25.89, 25.78, 23.47, 23.42, 22.12, 21.93, 21.88. HRMS calcd. for $[C_{50}H_{86}N_7O_6]^+$ 880.66341, found 880.66397.

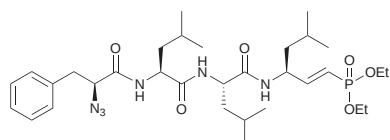


Cbz-Leu₂-leucinyl vinyl diethylphosphonate (99). Following the general procedure for azide coupling the title compound was obtained from Boc-leucinyl vinyl diethylphosphonate **128** (98 mg, 280 μmol, 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (100 mg, 254 μmol, 1 equiv.). Purification by flash chromatography (MeOH:DCM (0 → 3%)) gave the title compound **99** (71 mg, 117 μmol, 46%). $[\alpha]_D^{20}$ -50.6° (c = 1, CHCl₃). IR (film) 3269, 2955, 2870, 1699, 1645, 1539, 1468, 1387, 1367, 1232, 1024, 962 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ ppm 7.85-7.70 (m, 1H), 7.36-7.22 (m, 5H), 6.78-6.62 (m, 2H), 5.85-5.72 (m, 1H), 5.14-4.97 (m, 2H), 4.70-4.54 (m, 2H), 4.45-4.35 (m, 1H), 4.08-3.93 (m, 4H), 1.83-1.68 (m, 1H), 1.66-1.31 (m, 8H), 1.32-1.20 (m, 6H), 0.94-0.77 (m, 18H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 172.36, 171.29, 156.21, 153.02, 152.99, 136.31, 128.17, 127.69, 127.50, 116.21, 114.35, 66.43, 61.65, 61.58, 61.52, 53.24, 51.77, 49.28, 49.06, 42.64, 41.37, 40.74, 24.62, 24.53, 24.41, 24.36, 22.82,

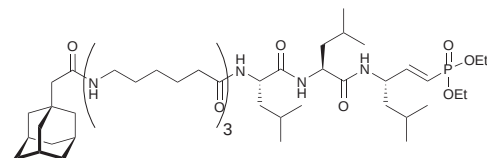
22.73, 22.62, 22.43, 22.17, 22.09, 21.57, 16.11, 16.08, 16.05, 16.02. HRMS: calcd. for $[C_{39}H_{53}N_3O_5P]^+$ 610.36156, found 610.36163.



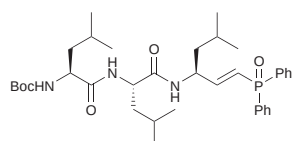
Boc-Leu₂-leucinyl vinyl diethylphosphonate (158). Following the general procedure for azide coupling the title compound was obtained from Boc-leucinyl vinyl diethylphosphonate **128** (86 mg, 0.246 mmol, 1.1 equiv.) and BocLeu₂NHNH₂ **148** (80 mg, 0.224 mmol, 1 equiv.). Purification by flash chromatography EA:tol (50 → 100%) gave the title compound (118 mg, 0.205 mmol, 92%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.48-7.40 (m, 1H), 6.72 (ddd, $J_1 = 22.2$ Hz, $J_2 = 17.2$ Hz, $J_3 = 5.2$ Hz, 1H), 5.81 (t, $J = 18.5$ Hz, 2H), 5.56-5.50 (m, 1H), 4.72-4.63 (m, 1H), 4.55-4.48 (m, 1H), 4.12-4.00 (m, 5H), 1.75-1.20 (m, 25H), 0.95-0.86 (m, 18H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 172.68, 171.22, 155.84, 152.60, 152.56, 116.90, 115.05, 80.00, 61.77, 61.72, 61.67, 53.26, 51.82, 49.48, 49.26, 43.14, 40.79, 40.61, 28.21, 24.64, 22.80, 22.70, 22.05, 21.95, 21.87, 16.26, 16.20.



Azido-Phe-Leu₂-leucinyl vinyl diethylphosphonate (100). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-leucinyl vinyl diethylphosphonate **158** (118 mg, 205 μmol, 1 equiv.) and azido-phenylalanine **152** (44 mg, 230 μmol, 1.1 equiv.). Flash column chromatography (EA:tol (50 → 100%)) yielded **100** (68 mg, 100 μmol, 50%). $[\alpha]_D^{20}$ -36.8° (c = 1, CHCl₃). IR (film) 3277, 2957, 2926, 2112, 1641, 1541, 1468, 1387, 1367, 1226, 1163, 1053, 1024, 964 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ ppm 7.33-7.19 (m, 5H), 7.08-6.99 (m, 1H), 6.68 (ddd, $J_1 = 22.2$, $J_2 = 17.2$, $J_3 = 5.3$ Hz, 1H), 5.77 (t, $J = 17.2$ Hz, 1H), 4.70-4.60 (m, 1H), 4.55-4.45 (m, 2H), 4.25 (dd, $J_1 = 8.3$, $J_2 = 4.2$ Hz, 1H), 4.12-3.96 (m, 4H), 3.22 (dd, $J_1 = 14.1$, $J_2 = 3.7$ Hz, 1H), 3.04 (dd, $J_1 = 14.1$, $J_2 = 8.3$ Hz, 1H), 1.77-1.16 (m, 15H), 1.00-0.78 (m, 18H). ¹³C NMR (100 MHz, CD₃OD): δ ppm 171.56, 171.05, 168.88, 152.42, 135.94, 129.37, 128.55, 127.14, 117.06, 115.20, 64.64, 61.89, 61.83, 61.76, 51.89, 51.62, 49.56, 49.34, 43.18, 41.03, 40.91, 38.00, 29.64, 24.81, 24.72, 24.48, 22.78, 22.74, 22.54, 22.50, 22.07, 21.99, 16.32, 16.29, 16.26, 16.23. HRMS: calcd. for $[C_{32}H_{54}N_6O_6P]^+$ 649.3837, found 649.38385.

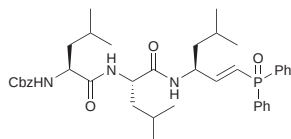


Ada-Ahx₃-Leu₂-leucinyl vinyl diethylphosphonate (101). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-leucinyl vinyl diethylphosphonate **158** (122 mg, 212 μmol, 1 equiv.) and AdaAhx₃OH **153** (124 mg, 233 μmol, 1.1 equiv.). Flash column chromatography (MeOH:DCM (2 → 10%)) yielded **101** (149 mg, 150 μmol, 71%). $[\alpha]_D^{20}$ -38.2° (c = 1, MeOH). IR (film) 3277, 3072, 2928, 2905, 2868, 1638, 1545, 1452, 1367, 1234, 1053, 1028, 968 cm⁻¹. ¹H NMR (400 MHz, CD₃OD): δ ppm 8.14-8.03 (m, 3H), 7.96 (t, $J = 5.1$ Hz, 2H), 7.89-7.84 (m, 1H), 6.67 (ddd, $J_1 = 22.1$, $J_2 = 17.2$, $J_3 = 4.8$ Hz, 1H), 5.86-5.74 (m, 1H), 4.64-4.54 (m, 1H), 4.45-4.30 (m, 2H), 4.05 (p, $J = 7.3$, 4H), 3.18-3.11 (m, 6H), 2.25 (t, $J = 7.3$, 2H), 2.17 (t, $J = 7.4$ Hz, 4H), 1.97-1.89 (m, 5H), 1.80-1.25 (m, 45H), 0.98-0.87 (m, 18H). ¹³C NMR (100 MHz, CD₃OD): δ ppm 176.23, 175.88, 174.85, 174.15, 173.66, 154.79, 154.74, 117.24, 115.37, 63.43, 63.37, 53.50, 53.29, 51.91, 51.05, 50.83, 43.73, 43.49, 41.82, 41.72, 40.29, 40.24, 37.90, 37.02, 36.66, 33.75, 30.21, 30.14, 30.10, 27.64, 27.58, 27.55, 26.71, 26.57, 25.93, 25.88, 25.85, 23.46, 23.38, 22.19, 22.12, 22.03, 16.72, 16.66. HRMS calcd for $[C_{53}H_{96}N_6O_9P]^+$ 991.69709, found 991.698.

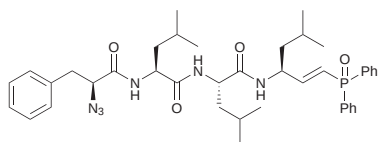


Boc-Leu₂-leucinyl vinyl diphenylphosphin oxide (159). Following the general procedure for azide coupling the title compound was obtained from Boc-leucinyl-vinyl diphenylphosphin oxide **129** (380 mg, 0.92 mmol, 1.1 equiv.) and BocLeu₂NHNH₂ **148** (300 mg, 0.84 mmol, 1 equiv.). Purification by flash chromatography EA:tol (30 → 70%, 3×) gave the title compound (221 mg, 0.345 mmol, 41%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.87-7.33 (m, 10H), 6.81 (d, $J = 7.1$ Hz, 1H), 6.73-6.42 (m, 2H), 5.47 (d, $J = 5.5$ Hz, 1H), 4.83-4.69 (m, 1H), 4.56-4.48 (m, 1H), 4.08-3.98 (m, 1H), 1.83-1.15 (m, 15H), 1.03-0.77 (m, 18H). ¹³C NMR (100 MHz, CDCl₃): δ

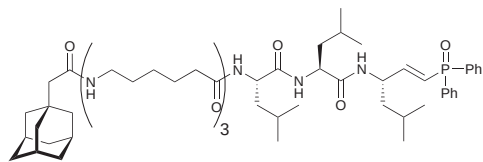
ppm 172.49, 171.33, 155.89, 151.63, 133.49, 133.01, 132.44, 131.96, 131.60, 131.24, 131.15, 128.49, 128.42, 128.30, 121.26, 120.26, 79.95, 53.46, 51.96, 49.73, 49.56, 43.09, 40.90, 40.75, 28.18, 24.74, 24.67, 24.59, 22.80, 22.70, 22.08, 21.80.



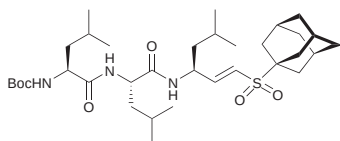
Cbz-Leu₂-leucinyl vinyl diphenylphosphinoxide (102). Following the general procedure for azide coupling the title compound was obtained from Boc-leucinyl-vinyl diphenylphosphinoxide **129** (179 mg, 280 μ mol, 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (100 mg, 254 μ mol, 1 equiv.). Purification by flash chromatography (MeOH:DCM (0 $\rightarrow$ 3%)) gave the title compound **102** (79 mg, 117 μ mol, 46%). $[\alpha]_D^{20}$ -56.8° (c = 1, CHCl₃). IR (film) 3271, 3059, 2955, 1701, 1645, 1539, 1437, 1387, 1367, 1263, 1236, 1171, 1121, 1105, 1043, 1028, 972 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ ppm 7.75 (d, *J* = 8.6 Hz, 1H), 7.71-7.60 (m, 4H), 7.54-7.24 (m, 11H), 6.90 (d, *J* = 7.9 Hz, 1H), 6.67-6.40 (m, 2H), 6.05 (d, *J* = 7.4 Hz, 1H), 5.12-4.95 (m, 2H), 4.78-4.68 (m, 1H), 4.59-4.51 (m, 1H), 4.22-4.13 (m, 1H), 1.80-1.19 (m, 9H), 0.92-0.72 (m, 18H). HRMS: calcd. for [C₃₉H₅₃N₃O₅P]⁺ 674.37173, found 674.37195.



Azido-Phe-Leu₂-leucinyl vinyl diphenylphosphinoxide (103). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-leucinyl vinyl diphenylphosphinoxide **159** (75 mg, 117 μ mol, 1.1 equiv.) and azidophenylalanine **152** (22 mg, 120 μ mol, 1.1 equiv.). Flash column chromatography (EA:tol (50 $\rightarrow$ 100%), 2 $\times$) yielded **103** (71 mg, 100 μ mol, 85%). $[\alpha]_D^{20}$ -43.6° (c = 1, CHCl₃). IR 3281, 3059, 2955, 2928, 2870, 2112, 1641, 1541, 1437, 1387, 1367, 1173, 1121, 1105, 1028, 997 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ ppm 7.86-7.34 (m, 10H), 7.28-7.13 (m, 5H), 7.05 (d, *J* = 8.0 Hz, 1H), 6.60-6.37 (m, 2H), 4.82-4.70 (m, 1H), 4.63-4.54 (m, 1H), 4.49-4.41 (m, 1H), 4.28-4.22 (m, 1H), 3.19-3.10 (m, 1H), 3.04-2.95 (m, 1H), 1.81-1.16 (m, 9H), 0.94-0.71 (m, 18H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 171.39, 171.09, 169.23, 151.88, 136.08, 133.01, 132.38, 131.92, 131.37, 131.27, 131.18, 131.08, 129.29, 128.66, 128.61, 128.54, 128.49, 127.00, 121.51, 120.50, 64.30, 51.95, 51.74, 49.79, 49.62, 43.16, 41.17, 40.59, 37.94, 24.85, 24.80, 24.46, 22.76, 22.70, 22.22, 22.00, 21.91. HRMS: calcd. for [C₄₀H₅₄N₆O₄P]⁺ 713.39387, found 713.39426.

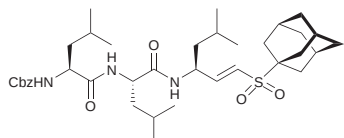


Ada-Ahx₃-Leu₂-leucinyl vinyl diphenylphosphinoxide (104). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-leucinyl vinyl diphenylphosphinoxide **159** (72 mg, 113 μ mol, 1.1 equiv.) and Ada-Ahx₃OH **153** (60 mg, 124 μ mol, 1.1 equiv.). Flash column chromatography (MeOH:DCM (5 $\rightarrow$ 11%)) yielded **104** (101 mg, 96 μ mol, 85%). $[\alpha]_D^{20}$ -41.0° (c = 1, MeOH). IR 3277, 2928, 2904, 2849, 1638, 1545, 1437, 1367, 1277, 1171, 1121, 1105 cm⁻¹. ¹H NMR (400 MHz, CD₃OD): δ ppm 8.14 (d, *J* = 8.3 Hz, 1H), 8.08 (d, *J* = 6.5 Hz, 1H), 8.04 (d, *J* = 7.4 Hz, 1H), 7.99-7.91 (m, 2H), 7.89-7.82 (m, 1H), 7.74-7.47 (m, 10H), 6.73-6.46 (m, 2H), 4.73-4.63 (m, 1H), 4.43-4.35 (m, 1H), 4.34-4.26 (m, 1H), 3.20-3.08 (m, 6H), 2.25 (t, *J* = 7.3 Hz, 1H), 2.16 (t, *J* = 7.2 Hz, 1H), 1.96-1.89 (m, 5H), 1.79-1.25 (m, 39H), 0.98-0.82 (m, 18H). ¹³C NMR (100 MHz, CD₃OD): δ ppm 176.41, 175.94, 174.98, 174.31, 173.73, 153.55, 133.90, 133.49, 132.84, 132.72, 132.35, 132.28, 132.25, 132.18, 130.02, 130.00, 129.90, 129.88, 121.76, 120.75, 53.75, 53.46, 51.93, 51.51, 51.34, 43.74, 43.43, 41.75, 41.62, 40.30, 40.25, 37.90, 37.03, 36.65, 33.76, 30.22, 30.12, 27.64, 27.60, 27.55, 26.71, 26.54, 25.98, 25.94, 25.89, 23.45, 23.40, 23.30, 22.14, 22.08, 21.98. HRMS: calcd. for [C₆₁H₉₆N₆O₇P]⁺ 1055.70726, found 1055.70846.

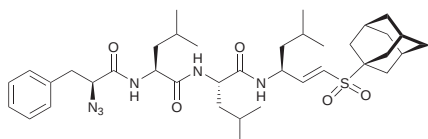


Boc-Leu₂-leucinyl vinyl 1-adamantylsulfone (160). Following the general procedure for azide coupling the title compound was obtained from Boc-leucinyl vinyl 1-adamantylsulfone **134** (410 mg, 1 mmol, 1.1 equiv.) and BocLeu₂NHNH₂ **148** (330 mg, 0.91 mmol, 1 equiv.). Purification by flash chromatography EA:tol (0 $\rightarrow$ 40%) gave the title compound (383 mg, 0.6 mmol, 66%). ¹H NMR (400 MHz, CDCl₃):

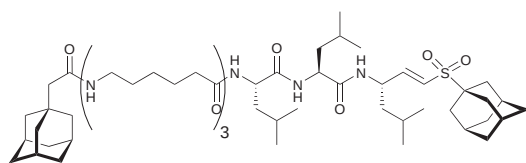
δ ppm 7.36-7.22 (m, 1H), 6.77 (dd, $J_1 = 15.2$, $J_2 = 4.9$ Hz, 1H), 6.73-6.71 (m, 1H), 6.42 (d, $J = 15.1$ Hz, 1H), 5.40 (d, $J = 4.7$ Hz, 1H), 4.81-4.71 (m, 1H), 4.47 (dd, $J_1 = 14.5$, $J_2 = 7.9$ Hz, 1H), 4.09-4.00 (m, 1H), 2.14 (s, 3H), 2.01-1.91 (m, 6H), 1.78-1.46 (m, 15H), 1.44 (s, 9H), 0.96-0.87 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.61, 171.23, 156.05, 150.22, 123.19, 80.19, 59.62, 53.61, 51.97, 48.14, 42.85, 40.75, 40.42, 35.71, 34.44, 28.23, 28.03, 24.87, 24.65, 22.80, 22.62, 22.19, 21.96, 21.82.



Cbz-Leu₂-leucinyln vinyl 1-adamantylsulfone (105). Following the general procedure for azide coupling the title compound was obtained from Boc-leucinyln vinyl 1-adamantylsulfone **134** (206 mg, 0.5 mmol, 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (177 mg, 0.45 mmol, 1 equiv.). Purification by flash chromatography (EA:tol (0 $\rightarrow$ 25%)) gave the title compound (271 mg, 400 μmol , 89%). $[\alpha]_{\text{D}}^{20}$ -44.4° (c = 1, CHCl_3). IR (film) 3287, 2957, 2914, 1699, 1645, 1539, 1454, 1263, 1134, 1047, 1028, 976 cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ ppm 7.44-7.28 (m, 5H), 6.88 (d, $J = 6.7$ Hz, 1H), 6.76 (dd, $J_1 = 15.1$, $J_2 = 5.1$ Hz, 1H), 6.37 (d, $J = 15.1$ Hz, 1H), 5.94 (d, $J = 6.6$ Hz, 1H), 5.14-5.01 (m, 2H), 4.79-4.69 (m, 1H), 4.51 (q, $J = 7.6$ Hz, 1H), 4.19 (dd, $J_1 = 13.6$, $J_2 = 6.9$ Hz, 1H), 2.11 (s, 3H), 1.99-1.87 (m, 6H), 1.84-1.33 (m, 15H), 0.93-0.82 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.41, 171.28, 156.69, 150.59, 136.25, 128.68, 128.35, 128.01, 123.19, 67.17, 59.86, 54.01, 52.08, 48.37, 42.94, 40.95, 35.89, 34.66, 28.20, 25.15, 24.89, 24.78, 22.97, 22.90, 22.68, 22.44, 22.19, 22.04. HRMS Calcd. for $[\text{C}_{37}\text{H}_{58}\text{N}_3\text{O}_6\text{S}]^+$ 672.40408, found 672.40442.

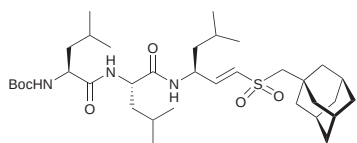


Azido-Phe-Leu₂-leucinyln vinyl 1-adamantylsulfone (106). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-leucinyln vinyl 1-adamantylsulfone **160** (140 mg, 220 μmol , 1 equiv.) and azido-phenylalanine **152** (46 mg, 242 μmol , 1.1 equiv.). Flash column chromatography (EA:tol (20 $\rightarrow$ 40%)) yielded the title compound (81 mg, 112 μmol , 52%). $[\alpha]_{\text{D}}^{20}$ -35.0° (c = 1, CHCl_3). IR (film) 3287, 2955, 2912, 2112, 1643, 1541, 1456, 1304, 1283, 1259, 1136 cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ ppm 7.49 (d, $J = 8.2$ Hz, 1H), 7.38-7.11 (m, 5H), 6.77 (dd, $J_1 = 15.1$, $J_2 = 5.3$ Hz, 1H), 6.38 (d, $J = 15.1$ Hz, 1H), 4.82-4.69 (m, 1H), 4.62-4.52 (m, 2H), 4.22 (dd, $J_1 = 8.3$, $J_2 = 4.0$ Hz, 1H), 3.19 (dd, $J_1 = 14.2$, $J_2 = 3.8$ Hz, 1H), 3.04 (dd, $J_1 = 14.1$, $J_2 = 8.5$ Hz, 1H), 2.13 (s, 3H), 1.99-1.88 (m, 6H), 1.84-1.32 (m, 15H), 0.94-0.81 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 171.65, 171.13, 169.03, 150.21, 135.95, 129.29, 128.58, 127.16, 123.33, 64.57, 59.72, 51.92, 51.68, 48.26, 42.87, 41.09, 41.06, 37.89, 35.71, 34.55, 28.04, 24.99, 24.73, 24.53, 22.72, 22.69, 22.24, 22.16, 22.04. HRMS calcd. for $[\text{C}_{38}\text{H}_{59}\text{N}_6\text{O}_5\text{S}]^+$ 711.42622, found 711.42633.

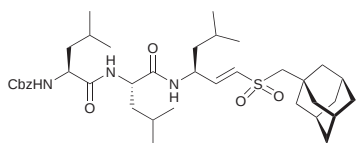


Ada-Ahx₃-Leu₂-leucinyln vinyl-1-adamantylsulfone (107). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-leucinyln vinyl 1-adamantylsulfone **160** (140 mg, 220 μmol , 1 equiv.) and AdaAhx₃OH **153** (252 mg, 242 μmol , 1.1 equiv.). Flash column chromatography (EA:MeOH (0 $\rightarrow$ 7%)) yielded **107** (158 mg, 152 μmol , 69%). $[\alpha]_{\text{D}}^{20}$ -34.0° (c = 1, MeOH). IR (film) 3285, 2910, 2851,

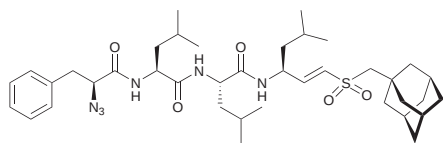
1639, 1541, 1454, 1279, 1134 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 8.08-7.98 (m, 1H), 7.96-7.87 (m, 2H), 7.84-7.78 (m, 1H), 6.69 (dd, $J_1 = 15.1$, $J_2 = 4.9$ Hz, 1H), 6.41 (d, $J = 15.1$ Hz, 1H), 4.67-4.60 (m, 1H), 4.34 (t, $J = 7.3$ Hz, 1H), 4.24 (dd, $J_1 = 8.5$, $J_2 = 6.4$ Hz, 1H), 3.19-3.04 (m, 6H), 2.27-2.18 (m, 2H), 2.18-2.04 (m, 7H), 2.00-1.82 (m, 12H), 1.84-1.38 (m, 29H), 1.37-1.22 (m, 6H), 0.97-0.82 (m, 18H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 176.40, 175.89, 175.81, 175.00, 174.18, 173.68, 152.03, 124.26, 60.82, 53.92, 53.32, 51.93, 51.88, 49.64, 43.74, 43.15, 41.56, 40.31, 40.26, 40.18, 40.13, 37.91, 37.04, 36.99, 36.83, 36.58, 35.64, 33.76, 30.22, 30.13, 29.59, 27.65, 27.62, 27.56, 26.72, 26.53, 26.03, 25.92, 25.89, 23.44, 23.40, 22.11, 22.01, 21.95. HRMS calcd. for $[\text{C}_{59}\text{H}_{101}\text{N}_6\text{O}_8\text{S}]^+$ 1053.73961, found 1073.74023.



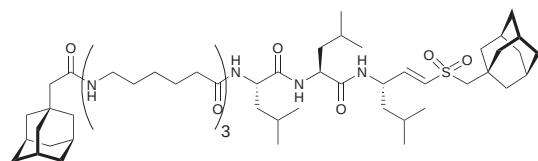
1H), 7.08 (d, $J = 5.8$ Hz, 1H), 6.78 (dd, $J_1 = 15.1$, $J_2 = 5.2$ Hz, 1H), 6.52 (d, $J = 15.0$ Hz, 1H), 5.55 (d, $J = 5.5$ Hz, 1H), 4.78-4.69 (m, 1H), 4.52-4.44 (m, 1H), 4.21-4.07 (m, 1H), 2.80 (s, 2H), 2.02-1.97 (m, 3H), 1.84-1.81 (m, 6H), 1.76-1.48 (m, 18H), 1.44 (s, 9H), 0.96-0.86 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.76, 171.37, 155.83, 146.35, 130.80, 79.89, 66.90, 53.26, 51.95, 47.60, 42.72, 41.93, 40.48, 36.27, 34.16, 28.19, 24.69, 24.56, 22.76, 22.70, 22.55, 22.12, 21.91.



Cbz-Leu₂-leucinyl vinyl 1-adamantylmethylsulfone (108). Following the general procedure for azide coupling the title compound was obtained from Boc-leucinyl vinyl 1-adamantylmethylsulfone **135** (309 mg, 0.73 mmol, 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (260 mg, 0.66 mmol, 1 equiv.). Purification by flash chromatography (EA:tol (0 $\rightarrow$ 25%)) gave the title compound **108** (365 mg, 530 μmol , 73%). $[\alpha]_{\text{D}}^{20}$ -50.6° (c = 1, CHCl_3). IR (film) 3290, 2959, 2907, 1699, 1645, 1539, 1456, 1263, 1240, 1132, 1139, 1028 cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ ppm 7.75-7.49 (m, 2H), 7.36-7.25 (m, 5H), 6.76 (dd, $J_1 = 15.0$, $J_2 = 5.2$ Hz, 1H), 6.47 (d, $J = 15.0$ Hz, 1H), 6.43-6.29 (m, 1H), 5.14-4.97 (m, 2H), 4.76-4.65 (m, 1H), 4.61-4.52 (m, 1H), 4.41-4.31 (m, 1H), 2.75 (s, 2H), 2.00-1.95 (m, 3H), 1.85-1.22 (m, 21H), 0.94-0.78 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.44, 171.37, 156.33, 146.66, 136.24, 130.61, 128.36, 127.94, 127.60, 66.92, 66.71, 53.47, 51.89, 47.62, 42.62, 41.96, 41.32, 40.64, 36.29, 34.20, 28.22, 24.82, 24.53, 22.78, 22.62, 22.56, 22.34, 21.76. HRMS calcd. for $[\text{C}_{38}\text{H}_{60}\text{N}_3\text{O}_6\text{S}]^+$ 686.41973, found 686.42012.

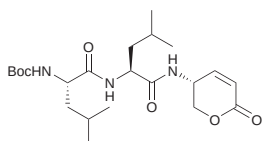


Azido-Phe-Leu₂-leucinyl vinyl 1-adamantylmethylsulfone (109). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-leucinyl vinyl 1-adamantylmethylsulfone **161** (232 mg, 357 μmol , 1.1 equiv.) and azido-phenylalanine **152** (62 mg, 320 μmol , 1 equiv.). Flash column chromatography (EA:tol (0 $\rightarrow$ 50%)) yielded **109** (144 mg, 200 μmol , 62%). $[\alpha]_{\text{D}}^{20}$ -35.0° (c = 1, CHCl_3). IR (film) 3277, 2957, 2907, 2851, 2110, 1638, 1541, 1454, 1387, 1313, 1265, 1132, 1119, 968 cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ ppm 7.69 (d, $J = 6.4$ Hz, 1H), 7.54 (d, $J = 8.2$ Hz, 1H), 7.48 (d, $J = 8.1$ Hz, 1H), 7.33-7.13 (m, 5H), 6.75 (dd, $J_1 = 15.1$, $J_2 = 5.7$ Hz, 1H), 6.44 (d, $J = 15.0$ Hz, 1H), 4.73-4.63 (m, 2H), 4.62-4.54 (m, 1H), 4.24 (dd, $J_1 = 8.6$, $J_2 = 4.2$ Hz, 1H), 3.15 (dd, $J_1 = 14.2$, $J_2 = 3.7$ Hz, 1H), 3.04 (dd, $J_1 = 14.2$, $J_2 = 8.7$ Hz, 1H), 2.77 (s, 2H), 2.02-1.95 (m, 3H), 1.85-1.32 (m, 21H), 0.97-0.80 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 171.72, 171.42, 168.96, 146.10, 135.97, 131.02, 129.23, 128.57, 127.14, 66.88, 64.41, 51.95, 51.54, 47.83, 42.75, 42.02, 41.42, 40.87, 37.84, 36.32, 34.29, 28.26, 24.90, 24.64, 24.55, 22.67, 22.65, 22.46, 22.37, 21.98. HRMS calcd. for $[\text{C}_{39}\text{H}_{61}\text{N}_6\text{O}_5\text{S}]^+$ 725.44187, found 725.44237.

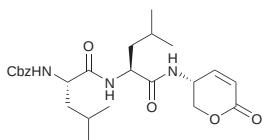


Ada-Ahx₃-Leu₂-leucinyl vinyl 1-adamantylmethylsulfone (110). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-leucinyl vinyl 1-adamantylmethylsulfone **161** (227 mg, 348 μmol , 1.1 equiv.) and AdaAhx₃OH **153** (170 mg, 320 μmol , 1 equiv.). Flash column chromatography (MeOH:DCM (0 $\rightarrow$ 7%)) yielded **110** (313 mg, 290 μmol , 92%). $[\alpha]_{\text{D}}^{20}$ -33.2° (c = 1, MeOH). IR (film) 3277, 3080, 2903, 2849, 1636, 1541, 1452, 1367, 1343, 1281, 1117, 1034 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 8.06 (t, $J = 7.3$ Hz, 2H), 7.91 (t, $J = 5.0$ Hz, 2H), 7.81 (t, $J = 4.9$ Hz, 1H), 6.72 (dd, $J_1 = 15.1$, $J_2 = 5.0$ Hz, 1H), 6.56 (d, $J = 15.1$

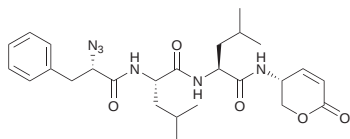
Hz, 1H), 4.70-4.61 (m, 1H), 4.43-4.29 (m, 2H), 3.21-3.09 (m, 6H), 2.90 (s, 2H), 2.32-2.21 (m, 2H), 2.17 (t, $J = 7.3$ Hz, 4H), 2.01-1.23 (m, 61H), 0.99-0.85 (m, 18H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 176.18, 176.10, 175.74, 175.65, 174.88, 174.80, 174.09, 174.01, 173.52, 147.66, 132.22, 67.65, 53.60, 53.21, 51.87, 49.15, 49.06, 43.66, 43.16, 43.01, 41.56, 41.47, 40.24, 40.19, 37.86, 37.52, 36.99, 36.57, 35.12, 33.69, 30.15, 30.10, 30.03, 29.75, 27.58, 27.54, 27.49, 26.65, 26.50, 25.87, 25.82, 25.76, 23.49, 23.44, 22.18, 22.06, 22.02. HRMS calcd. for $[\text{C}_{60}\text{H}_{103}\text{N}_6\text{O}_8\text{S}]^+$ 1067.75526, found 1067.75646.



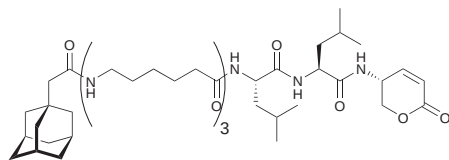
Boc-Leu₂-(R)-5-amino-5,6-dihydro-pyran-2-one (163). Following the general procedure for azide coupling the title compound was obtained from (R)-5-(Boc-amino)-5,6-dihydro-pyran-2-one **162**¹⁸⁰ (103 mg, 484 μmol , 1.1 equiv.) and BocLeu₂NHNH₂ **148** (158 mg, 440 μmol , 1 equiv.). Purification by flash chromatography (EA:tol (30 $\rightarrow$ 60%)) gave the title compound (129 mg, 290 μmol , 67%). ^1H NMR (400 MHz, CDCl_3): δ ppm 7.68-7.51 (m, 1H), 6.92-6.84 (m, 2H), 6.08 (d, $J = 9.7$ Hz, 1H), 5.32-5.22 (m, 1H), 4.83-4.71 (m, 1H), 4.51-4.43 (m, 2H), 4.33-4.24 (m, 1H), 4.13-4.03 (m, 1H), 1.78-1.48 (m, 6H), 1.43 (s, 9H), 0.99-0.86 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.87, 172.39, 162.76, 155.99, 144.85, 122.45, 80.39, 69.69, 53.58, 51.85, 41.77, 40.61, 28.19, 24.72, 22.85, 21.74.



Cbz-Leu₂-(R)-5-amino-5,6-dihydro-pyran-2-one (111). Following the general procedure for azide coupling the title compound was obtained from (R)-5-(Boc-amino)-5,6-dihydro-pyran-2-one **162**¹⁸⁰ (36 mg, 160 μmol , 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (59 mg, 150 μmol , 1 equiv.). Purification by flash chromatography (EA:tol (20 $\rightarrow$ 50%)) gave the title compound **111**. $[\alpha]_{\text{D}}^{20}$ -106.8° ($c = 1$, CHCl_3). IR (film) 3288, 2957, 2930, 1717, 1699, 1645, 1533, 1456, 1387, 1261, 1231, 1092, 1040 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 7.40-7.26 (m, 5H), 6.93 (dd, $J_1 = 9.7$, $J_2 = 4.5$ Hz, 1H), 6.10 (dd, $J_1 = 9.8$, $J_2 = 1.1$ Hz, 1H), 5.09 (s, 2H), 4.72-4.65 (m, 1H), 4.48 (dd, $J_1 = 11.6$, $J_2 = 4.4$ Hz, 1H), 4.38 (dd, $J_1 = 9.7$, $J_2 = 5.0$ Hz, 1H), 4.27 (dd, $J_1 = 11.5$, $J_2 = 5.0$ Hz, 1H), 4.11 (t, $J = 7.5$ Hz, 1H), 1.77-1.26 (m, 6H), 0.99-0.85 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.47, 172.20, 162.88, 156.69, 144.16, 135.91, 130.85, 128.55, 128.28, 127.91, 122.85, 69.91, 68.14, 67.31, 54.28, 51.83, 41.57, 40.88, 40.45, 24.88, 24.76, 22.87, 21.73, 21.68. HRMS calcd. for $[\text{C}_{25}\text{H}_{36}\text{N}_3\text{O}_6]^+$ 474.25986, found 496.24181.

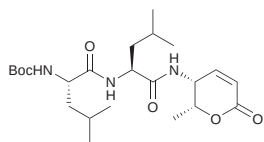


Azido-Phe-Leu₂-(R)-5-amino-5,6-dihydro-pyran-2-one (112). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-(R)-5-amino-5,6-dihydro-pyran-2-one **163** (44 mg, 100 μmol , 1 equiv.) and azido-phenylalanine **152** (21 mg, 110 μmol , 1.1 equiv.). Flash column chromatography (EA:PE (20 $\rightarrow$ 70%)) yielded **112** (19 mg, 37 μmol , 37%). IR (film) 3279, 2957, 2926, 2112, 1734, 1645, 1541, 1456, 1229, 1094 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 7.34-7.19 (m, 5H), 6.94 (dd, $J_1 = 9.8$, $J_2 = 4.6$ Hz, 1H), 6.11 (d, $J = 4.6$ Hz, 1H), 4.72-4.64 (m, 1H), 4.50 (dd, $J_1 = 11.5$, $J_2 = 4.4$ Hz, 1H), 4.41-4.34 (m, 2H), 4.30 (dd, $J_1 = 11.6$, $J_2 = 4.8$ Hz, 1H), 3.19 (dd, $J_1 = 14.0$, $J_2 = 4.9$ Hz, 1H), 2.98 (dd, $J_1 = 14.0$, $J_2 = 8.48$ Hz, 1H), 1.79-1.48 (m, 6H), 1.02-0.83 (m, 12H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 174.91, 174.33, 171.74, 146.37, 137.87, 130.47, 129.59, 128.04, 123.34, 71.12, 65.51, 53.36, 53.13, 43.16, 41.79, 41.76, 38.67, 25.92, 25.83, 23.45, 23.40, 22.03, 21.97. HRMS calcd for $[\text{C}_{26}\text{H}_{37}\text{N}_6\text{O}_5]^+$ 513.28199, found 513.28168.

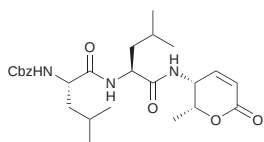


Ada-Ahx₃-Leu₂-(R)-5-amino-5,6-dihydro-pyran-2-one (113). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-(R)-5-amino-5,6-dihydro-pyran-2-one **163** (44 mg, 100 μmol , 1 equiv.) and AdaAhx₃OH **153** (57 mg, 110 μmol , 1.1 equiv.). Flash column chromatography (EA:MeOH (0 $\rightarrow$ 15%)) yielded **113** (76 mg, 89 μmol , 89%). $[\alpha]_{\text{D}}^{20}$ -54.0° ($c = 1$, MeOH). IR (film) 3285, 2905, 2851, 1732, 1641, 1545, 1439, 1202, 1175, 1134, 1094 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 8.49 (d, $J = 7.3$ Hz, 1H), 8.11 (d, $J = 6.7$

Hz, 1H), 8.07 (d, $J = 7.3$ Hz, 1H), 8.00-7.92 (m, 2H), 7.90-7.83 (m, 1H), 6.97-6.90 (m, 1H), 6.09 (d, $J = 9.7$ Hz, 1H), 4.74-4.63 (m, 1H), 4.54-4.45 (m, 1H), 4.43-4.24 (m, 3H), 3.22-3.06 (m, 6H), 2.25 (t, $J = 7.1$ Hz, 2H), 2.18 (t, $J = 7.0$ Hz, 4H), 1.01-0.86 (m, 12H), 1.99-1.89 (m, 5H), 1.81-1.20 (m, 36H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 176.46, 176.02, 175.93, 175.08, 174.84, 174.75, 173.80, 146.64, 123.18, 71.00, 55.82, 53.55, 53.15, 51.96, 43.77, 43.33, 43.24, 41.84, 41.63, 40.32, 40.27, 40.19, 40.14, 37.93, 37.06, 37.01, 36.68, 33.79, 30.23, 30.17, 30.12, 27.66, 27.57, 26.72, 26.59, 25.93, 25.90, 23.51, 23.43, 22.02, 21.93. HRMS calcd. for $[\text{C}_{47}\text{H}_{79}\text{N}_6\text{O}_8]^+$ 855.59539, found 855.59589.

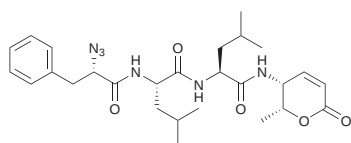


3H), 0.97-0.87 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.87, 172.71, 164.00, 155.84, 143.31, 122.73, 80.24, 75.98, 53.05, 51.94, 43.65, 40.83, 40.68, 28.14, 24.65, 22.86, 22.71, 21.88, 21.68, 15.98.

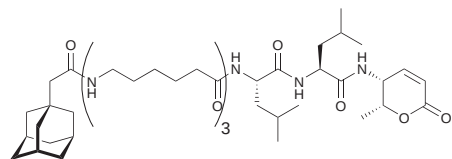


Boc-Leu₂-(5R,6R)-5-amino-6-methyl-5,6-dihydropyran-2-one (165). Following the general procedure for azide coupling the title compound was obtained from (5R,6R)-5-(Boc-amino)-6-methyl-5,6-dihydropyran-2-one **164**¹⁸¹ (112 mg, 495 μmol , 1.1 equiv.) and BocLeu₂NHNH₂ **148** (161 mg, 450 μmol , 1 equiv.). Purification by flash chromatography (EA:tol (30 $\rightarrow$ 60%)) gave the title compound **37** (168 mg, 370 μmol , 82%). ^1H NMR (400 MHz, CDCl_3): δ ppm 7.52 (d, $J = 5.6$ Hz, 1H), 7.12-7.00 (m, 1H), 6.85 (dd, $J_1 = 9.6$, $J_2 = 5.77$ Hz, 1H), 6.12 (d, $J = 9.6$ Hz, 1H), 5.28 (d, $J = 6.3$ Hz, 1H), 4.72-4.64 (m, 2H), 4.50-4.42 (m, 1H), 4.13-4.02 (m, 1H), 1.71-1.44 (m, 6H), 1.43 (s, 9H), 1.31 (d, $J = 6.3$ Hz, 3H), 0.97-0.87 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.87, 172.71, 164.00, 155.84, 143.31, 122.73, 80.24, 75.98, 53.05, 51.94, 43.65, 40.83, 40.68, 28.14, 24.65, 22.86, 22.71, 21.88, 21.68, 15.98.

Cbz-Leu₂-(5R,6R)-5-amino-6-methyl-5,6-dihydropyran-2-one (114). Following the general procedure for azide coupling the title compound was obtained from (5R,6R)-5-(Boc-amino)-6-methyl-5,6-dihydropyran-2-one **164**¹⁸¹ (36 mg, 160 μmol , 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (59 mg, 150 μmol , 1 equiv.). Purification by flash chromatography (EA:tol (25 $\rightarrow$ 60%)) gave the title compound **357** (13 mg, 27 μmol , 18%). $[\alpha]_D^{20}$ -278.8° (c = 1, CHCl_3). IR (film) 2957, 1734, 1717, 1699, 1684, 1668, 1639, 1558, 1541, 1506, 1456, 1387, 1250, 1067 cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ ppm 7.42-7.29 (m, 5H), 7.04 (d, $J = 8.5$ Hz, 1H), 6.83 (dd, $J_1 = 9.5$, $J_2 = 6.0$ Hz, 1H), 6.38 (d, $J = 6.8$ Hz, 1H), 6.15 (d, $J = 9.5$ Hz, 1H), 5.22 (d, $J = 4.0$ Hz, 1H), 5.19-5.03 (m, 2H), 4.71-4.60 (m, 2H), 4.48-4.38 (m, 1H), 4.11-4.03 (m, 1H), 1.83-1.39 (m, 6H), 1.28 (d, $J = 6.3$ Hz, 3H), 0.99-0.84 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 172.37, 143.02, 128.57, 128.34, 128.19, 123.17, 78.36, 75.97, 70.12, 67.59, 54.38, 52.23, 43.75, 40.73, 40.67, 31.42, 24.99, 24.83, 23.00, 22.88, 21.74, 21.51, 16.23. HRMS calcd. for $[\text{C}_{26}\text{H}_{38}\text{N}_3\text{O}_6]^+$ 488.27551, found 488.27512.

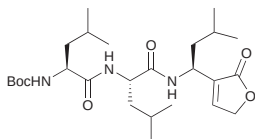


Azido-Phe-Leu₂-(5R,6R)-5-amino-6-methyl-5,6-dihydropyran-2-one (115). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-(5R,6R)-5-amino-6-methyl-5,6-dihydropyran-2-one **165** (32 mg, 70 μmol , 1 equiv.) and azido-phenylalanine **152** (15 mg, 77 μmol , 1.1 equiv.). Flash column chromatography (EA:PE (20 $\rightarrow$ 65%)) yielded **115** (22 mg, 42 μmol , 60%). IR (film) 3281, 2955, 2926, 2870, 2112, 1736, 1639, 1533, 1456, 1387, 1251, 1069 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 7.35-7.20 (m, 5H), 6.92 (dd, $J_1 = 9.6$, $J_2 = 6.1$ Hz, 1H), 6.15 (d, $J = 9.6$ Hz, 1H), 4.78-4.68 (m, 1H), 4.63 (dd, $J_1 = 5.9$, $J_2 = 3.3$ Hz, 1H), 4.44-4.35 (m, 2H), 4.16 (dd, $J_1 = 8.4$, $J_2 = 4.96$ Hz, 1H), 3.19 (dd, $J_1 = 14.02$, $J_2 = 4.93$ Hz, 1H), 2.98 (dd, $J_1 = 13.98$, $J_2 = 8.46$ Hz, 1H), 1.74-1.22 (m, 9H), 1.00-0.83 (m, 12H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 174.19, 144.85, 133.33, 130.49, 129.58, 128.03, 123.70, 77.58, 65.55, 53.25, 45.21, 41.91, 41.78, 38.70, 25.94, 25.82, 23.42, 23.35, 22.13, 22.04, 16.73. HRMS calcd. for $[\text{C}_{27}\text{H}_{39}\text{N}_6\text{O}_5]^+$ 527.29764, found 527.29736.

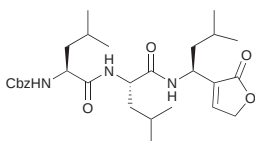


Ada-Ahx₃-Leu₂-(5R,6R)-5-amino-6-methyl-5,6-dihydropyran-2-one (116). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-(5R,6R)-5-amino-6-methyl-5,6-dihydropyran-2-one **165** (41 mg, 90 μmol , 1 equiv.) and AdaAhx₃OH **153** (51 mg, 100 μmol , 1.1 equiv.). Flash column chromatography (EA:MeOH (0 $\rightarrow$ 15%)) yielded **116** (31 mg, 35 μmol , 40%). $[\alpha]_D^{20}$ -100.0° (c = 1, MeOH). IR (film) 3285, 2905,

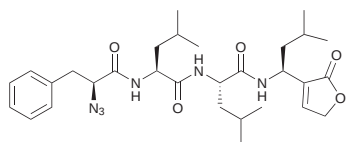
2851, 1734, 1639, 1541, 1456, 1369, 1257, 1204, 1069 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 8.42 (d, $J = 8.3$ Hz, 1H), 8.10 (d, $J = 6.6$ Hz, 1H), 8.06 (d, $J = 6.4$ Hz, 1H), 7.99-7.91 (m, 2H), 7.89-7.83 (m, 1H), 6.95-6.87 (m, 1H), 6.14 (d, $J = 9.4$ Hz, 1H), 4.77-4.67 (m, 1H), 4.67-4.59 (m, 1H), 4.45-4.31 (m, 2H), 4.15-4.05 (m, 1H), 3.21-3.10 (m, 6H), 2.29-2.12 (m, 6H), 1.99-1.89 (m, 5H), 1.80-1.18 (m, 41H), 0.98-0.87 (m, 12H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 176.28, 176.04, 175.08, 174.93, 174.85, 173.83, 144.87, 123.67, 77.55, 53.28, 53.19, 51.97, 45.20, 43.79, 41.87, 41.79, 40.34, 40.29, 37.94, 37.07, 36.72, 33.81, 30.25, 30.19, 30.13, 27.67, 27.59, 26.74, 26.63, 25.93, 23.46, 23.39, 22.11, 22.01, 16.73. HRMS calcd. for $[\text{C}_{48}\text{H}_{81}\text{N}_6\text{O}_8]^+$ 869.61104, found 869.61133.



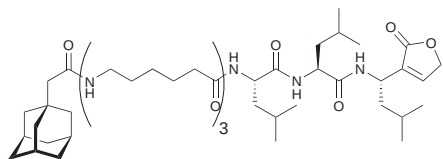
172.73, 171.32, 156.10, 145.85, 134.29, 80.52, 70.24, 53.63, 52.19, 44.78, 41.65, 40.56, 28.21, 24.78, 22.93, 22.79, 21.76, 21.70, 21.54.



Cbz-Leu₂-3-leucinylfuran-2(5H)-one (117). Following the general procedure for azide coupling the title compound was obtained from Boc-3-leucinylfuran-2(5H)-one **139** (56 mg, 208 μmol , 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (79 mg, 200 μmol , 1 equiv.). Purification by flash chromatography (EA:tol (20 $\rightarrow$ 50%)) gave the title compound (91 mg, 172 μmol , 86%). $[\alpha]_{\text{D}}^{20}$ -76.8° ($c = 1$, CHCl_3). IR (film) 3346, 3329, 2957, 2870, 1738, 1697, 1672, 1647, 1541, 1506, 1456, 1387, 1367, 1269, 1209, 1070, 1040 cm^{-1} . ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 1/1): δ ppm 7.72 (d, $J = 8.3$ Hz, 1H), 7.43-7.28 (m, 6H), 5.11 (q, $J = 12.3$ Hz, 2H), 4.45-4.41 (m, 3H), 4.40-4.35 (m, 1H), 4.15 (dd, $J_1 = 8.2$, $J_2 = 6.7$ Hz, 1H), 1.75-1.43 (m, 9H), 1.02-0.82 (m, 18H). ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 1/1): δ ppm 173.37, 172.96, 171.98, 156.70, 146.27, 135.73, 134.03, 128.08, 127.75, 127.32, 70.26, 66.56, 53.65, 51.68, 44.31, 41.23, 40.55, 39.76, 24.38, 24.26, 22.41, 22.32, 22.28, 21.05, 20.96, 20.82. HRMS calcd for $[\text{C}_{29}\text{H}_{44}\text{N}_3\text{O}_6]^+$ 530.32246, found 530.32230.

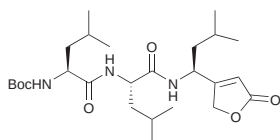


Azido-Phe-Leu₂-3-leucinylfuran-2(5H)-one (118). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-3-leucinylfuran-2(5H)-one **166** (24.6 mg, 50 μmol , 1 equiv.) and azido-phenylalanine **152** (12 mg, 64 μmol , 1.3 equiv.). Flash column chromatography (MeOH:DCM (0 $\rightarrow$ 2.5%)) yielded **118** (14 mg, 25 μmol , 50%). IR (film) 3277, 2957, 2932, 2110, 1749, 1639, 1541, 1457, 1387, 1340, 1287, 1207, 1067, 1049 cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ ppm 7.36-7.21 (m, 5H), 6.98 (d, $J = 8.6$ Hz, 1H), 6.80 (d, $J = 7.6$ Hz, 1H), 6.58 (d, $J = 7.9$ Hz, 1H), 4.90 (dd, $J_1 = 15.6$, $J_2 = 8.1$ Hz, 1H), 4.82-4.77 (m, 2H), 4.47-4.32 (m, 2H), 4.27 (dd, $J_1 = 7.7$, $J_2 = 4.1$ Hz, 1H), 3.29 (dd, $J_1 = 14.1$, $J_2 = 3.9$ Hz, 1H), 3.1 (dd, $J_1 = 14.1$, $J_2 = 7.8$ Hz, 1H), 1.80-1.20 (m, 9H), 0.95-0.81 (m, 18H). HRMS calcd for $[\text{C}_{30}\text{H}_{45}\text{N}_6\text{O}_5]^+$ 569.34459, found 569.34450.



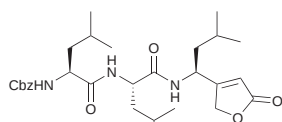
Ada-Ahx₃-Leu₂-3-leucinylfuran-2(5H)-one (119). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-3-leucinylfuran-2(5H)-one **166** (23 mg, 46 μmol , 1 equiv.) and AdaAhx₃OH **153** (27 mg, 51 μmol , 1.1 equiv.). Flash column chromatography (MeOH:DCM (3 $\rightarrow$ 8%)) yielded **119** (40 mg, 44 μmol , 95%). $[\alpha]_{\text{D}}^{20}$ -35.2° ($c = 1$, MeOH). IR (film) 3275, 2905, 1747, 1636, 1541, 1456, 1340, 1205, 1067, 1045 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 8.23-8.07 (m, 4H), 7.97 (t, $J = 4.8$ Hz, 1H), 7.50-7.47 (m, 1H), 4.87-4.82 (m, 2H), 4.80-4.70 (m, 1H), 4.41-4.26 (m,

2H), 3.22-3.09 (m, 6H), 2.28 (t, $J = 7.4$ Hz, 2H), 2.20 (t, $J = 7.3$ Hz, 4H), 1.98-1.88 (m, 5H), 1.79-1.23 (m, 39H), 0.99-0.84 (m, 18H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 176.67, 176.18, 176.15, 176.09, 175.25, 174.99, 174.27, 173.87, 149.49, 148.79, 135.42, 72.18, 53.92, 53.50, 51.94, 51.88, 49.86, 45.53, 43.71, 42.92, 41.60, 41.42, 40.32, 40.19, 40.13, 40.00, 37.86, 37.01, 36.70, 36.65, 33.79, 30.11, 30.01, 29.97, 27.51, 27.47, 27.32, 26.58, 26.51, 25.90, 25.88, 25.86, 23.59, 23.43, 23.41, 22.01, 21.90, 21.85. HRMS calcd for $[\text{C}_{51}\text{H}_{87}\text{N}_6\text{O}_8]^+$ 911.65799, found 911.65872.

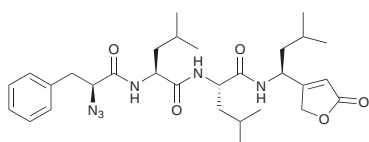


(s, 1H), 5.13-5.08 (m, 1H), 4.96-4.74 (m, 3H), 4.58-4.50 (m, 1H), 4.03-3.95 (m, 1H), 1.70-1.40 (m, 18H), 1.05-0.96 (m, 18H).

Cbz-Leu₂-4-leuciny-furan-2(5H)-one (120). Following the general procedure for azide coupling the title compound was obtained from Boc-4-leuciny-furan-2(5H)-one **141** (77 mg, 286 μmol , 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (102 mg, 260 μmol , 1 equiv.). Purification by flash chromatography (EA:tol (30 $\rightarrow$ 70%)) gave the title compound (112 mg, 211 μmol , 81%). $[\alpha]_{\text{D}}^{20}$ -80.2° ($c = 1$, CHCl_3). IR (film) 3269, 2957, 2934,

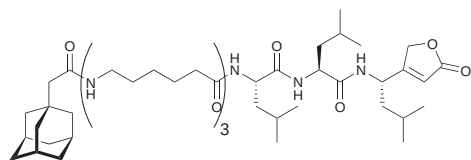


2870, 1782, 1751, 1699, 1668, 1641, 1539, 1471, 1456, 1387, 1369, 1261, 1238, 1153, 1120, 1030 cm^{-1} . ^1H NMR (400 MHz, CDCl_3) δ ppm 7.41 (d, $J = 8.2$ Hz, 1H), 7.36-7.24 (m, 5H), 6.13 (d, $J = 4.8$ Hz, 1H), 5.92-5.89 (m, 1H), 5.15-4.97 (m, 2H), 4.88-4.66 (m, 3H), 4.43 (dd, $J_1 = 14.2$, $J_2 = 7.3$ Hz, 1H), 4.25-4.17 (m, 1H), 1.86-1.31 (m, 9H), 0.95-0.81 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 173.74, 172.69, 172.05, 171.63, 156.78, 135.81, 128.53, 128.25, 127.59, 115.17, 71.61, 66.96, 54.24, 52.22, 45.81, 41.82, 41.07, 40.06, 24.92, 24.67, 24.44, 23.19, 22.69, 22.57, 21.96, 21.86, 21.16. HRMS calcd. for $[\text{C}_{29}\text{H}_{44}\text{N}_3\text{O}_6]^+$ 530.32246, found 530.32224.



Azido-Phe-Leu₂-4-leuciny-furan-2(5H)-one (121). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-4-leuciny-furan-2(5H)-one **167** (59 mg, 120 μmol , 1 equiv.) and azido-phenylalanine **152** (26 mg, 130 μmol , 1.1 equiv.). Flash column chromatography (EA:tol (30 $\rightarrow$ 60%)) yielded **121** (31 mg, 55 μmol , 45%). $[\alpha]_{\text{D}}^{20}$ -61.6° ($c = 1$, CHCl_3). IR (film) 3273, 2957, 2932, 2872, 2110, 1782, 1753, 1638, 1541, 1465, 1437, 1387, 1369, 1248, 1223, 1153, 1032 cm^{-1} . ^1H NMR (400 MHz, CDCl_3): δ ppm 7.36-7.20 (m, 5H), 7.06-7.02 (m, 1H), 6.82-6.78 (m,

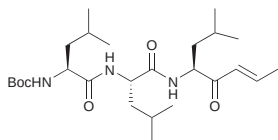
1H), 6.75-6.71 (m, 1H), 5.94-5.92 (m, 1H), 4.88-4.73 (m, 3H), 4.42-4.35 (m, 1H), 4.33-4.25 (m, 2H), 3.27 (dd, $J_1 = 14.1$ Hz, $J_2 = 4.2$ Hz, 1H), 3.08 (dd, $J_1 = 14.1$ Hz, $J_2 = 7.5$ Hz, 1H), 1.80-1.30 (m, 9H), 0.98-0.80 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 171.77, 171.66, 171.11, 169.34, 135.41, 129.39, 128.71, 127.40, 115.44, 71.59, 64.91, 52.34, 52.07, 45.94, 42.02, 40.60, 39.97, 38.05, 24.95, 24.64, 24.59, 23.16, 22.76, 22.72, 21.90, 21.87, 21.34. HRMS: calcd. for $[\text{C}_{30}\text{H}_{45}\text{N}_6\text{O}_5]^+$ 569.34459, found 569.34443.



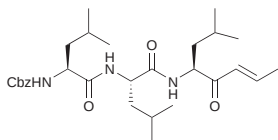
Ada-Ahx₃-Leu₂-4-leuciny-furan-2(5H)-one (122). Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-4-leuciny-furan-2(5H)-one **167** (59 mg, 120 μmol) and AdaAhx₃OH **153** (69 mg, 130 μmol , 1.1 equiv.). Flash column chromatography (MeOH:DCM (0 $\rightarrow$ 7%)) yielded **122** (87 mg, 96 μmol , 80%). $[\alpha]_{\text{D}}^{20}$ -54.0° ($c = 1$, MeOH). IR (film) 3285, 2928, 2903, 2870, 1780, 1747, 1638, 1541, 1439, 1367, 1277, 1242, 1155, 1032 cm^{-1} . ^1H

NMR (400 MHz, CD_3OD): δ ppm 7.85 (t, $J = 5.2$ Hz, 1H), 7.95 (t, $J = 5.4$ Hz, 2H), 5.95 (d, $J = 1.5$ Hz, 1H), 4.91-4.84 (m, 3H), 4.38-4.27 (m, 2H), 3.18-3.11 (m, 6H), 2.28-2.22 (m, 2H), 2.17 (t, $J = 7.5$ Hz, 4H), 1.96-1.92 (m, 3H), 1.92-1.90 (m, 2H), 1.83-1.44 (m, 33H), 1.39-1.29 (m, 6H), 0.98-0.88 (m, 18H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 176.41, 175.95, 175.86, 175.06, 174.52, 174.27, 173.73, 115.72, 73.25, 53.65, 53.35, 51.94, 47.29, 43.75, 42.60, 41.62, 41.40, 40.31, 40.26, 40.18, 37.91, 37.04, 36.99, 36.62, 33.77, 30.22, 30.14, 27.65, 27.60, 27.56,

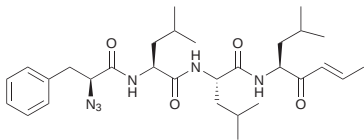
26.72, 26.54, 25.97, 25.90, 25.71, 23.70, 23.40, 22.11, 21.93, 21.49. HRMS: calcd. for $[C_{51}H_{87}N_6O_8]^+$ 911.65799, found 911.65874.



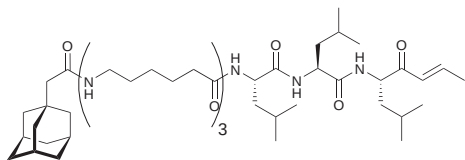
$J_1 = 6.9, J_2 = 1.6$ Hz, 3H), 1.73-1.34 (m, 18H), 0.99-0.85 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 197.79, 172.47, 171.49, 155.68, 144.85, 128.56, 54.37, 52.99, 51.65, 41.18, 40.97, 28.24, 24.80, 24.67, 24.58, 23.24, 22.80, 22.06, 21.85, 18.44.



15.7, $J_2 = 1.6$ Hz, 1H), 5.82 (d, $J = 1.6$ Hz, 1H), 5.16-5.01 (m, 2H), 4.88 (dt, $J_1 = 9.3, J_2 = 4.1$ Hz, 1H), 4.59 (dd, $J_1 = 15.2, J_2 = 7.5$ Hz, 1H), 4.35-4.26 (m, 1H), 1.90 (dd, $J_1 = 6.9, J_2 = 1.5$ Hz, 3H), 1.76-1.35 (m, 9H), 0.94-0.82 (m, 18H), 7.06-6.95 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 197.92, 172.09, 171.48, 156.19, 144.83, 136.34, 128.67, 128.40, 127.98, 127.84, 66.81, 54.21, 53.36, 51.66, 41.59, 41.19, 40.84, 24.78, 24.66, 24.57, 23.25, 22.72, 22.45, 22.19, 21.82, 18.41. HRMS calcd. for $[C_{29}H_{46}N_3O_5]^+$ 516.3432, found 516.34294.



$J_2 = 6.9$ Hz, 1H), 6.28 (dd, $J_1 = 15.6, J_2 = 1.7$ Hz, 1H), 4.70 (dd, $J_1 = 9.1, J_2 = 5.42$ Hz, 1H), 4.47-4.41 (m, 1H), 4.14 (dd, $J_1 = 8.5, J_2 = 5.0$ Hz, 1H), 3.18 (dd, $J_1 = 14.0, J_2 = 5.0$ Hz, 1H), 2.97 (dd, $J_1 = 14.0, J_2 = 8.5$ Hz, 1H), 1.91 (dd, $J_1 = 6.9, J_2 = 1.6$ Hz, 3H), 1.74-1.45 (m, 9H), 1.00-0.85 (m, 18H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 199.71, 174.31, 174.06, 145.97, 137.86, 130.44, 129.59, 129.37, 128.04, 65.49, 56.27, 53.10, 52.99, 42.06, 41.90, 40.91, 38.69, 25.97, 25.83, 23.68, 23.42, 23.33, 22.32, 22.14, 21.93, 18.54. HRMS calcd. for $[C_{30}H_{47}N_6O_4]^+$ 555.36533, found 555.36519.



1276, 1171, 1067, 972 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 8.22 (d, $J = 7.9$ Hz, 1H), 8.08 (t, $J = 6.7, 6.69$

Boc-Leu₂-(S,E)-5-amino-7-methyloct-2-en-4-one (168). Following the general procedure for azide coupling the title compound was obtained from (E,S)-1-isobutyl-2-oxo-pent-3-enyl-amine HCl salt **144** (100 mg, 520 μ mol, 1.1 equiv.) and BocLeu₂NHNH₂ **148** (170 mg, 470 μ mol, 1 equiv.). Purification by flash chromatography (EA:tol (0 $\rightarrow$ 25%)) gave the title compound (119 mg, 250 μ mol, 48%). ^1H NMR (400 MHz, $CDCl_3$): δ ppm 7.02 (dq, $J_1 = 15.5, J_2 = 6.9$ Hz, 1H), 6.85 (d, $J = 7.4$ Hz, 1H), 6.68 (d, $J = 7.9$ Hz, 1H), 6.21 (dd, $J_1 = 15.6, J_2 = 1.7$ Hz, 1H), 5.07 (d, $J = 6.6$ Hz, 1H), 4.89-4.82 (m, 1H), 4.54-4.45 (m, 1H), 4.17-4.07 (m, 1H), 1.93 (dd,

Cbz-Leu₂-(S,E)-5-amino-7-methyloct-2-en-4-one (123). Following the general procedure for azide coupling the title compound was obtained from (E,S)-1-isobutyl-2-oxo-pent-3-enyl-amine HCl salt **144** (38 mg, 200 μ mol, 1.1 equiv.) and CbzLeu₂NHNH₂ **147** (71 mg, 180 μ mol, 1 equiv.). Purification by flash chromatography (EA:tol (0 $\rightarrow$ 25%)) gave the title compound (62 mg, 120 μ mol, 67%). $[\alpha]_D^{20}$ -45.5° (c = 1, $CHCl_3$). IR (film) 3263, 2955, 2936, 2870, 1699, 1663, 1641, 1539, 1466, 1437, 1387, 1367, 1286, 1261, 1234, 1121, 1045, 1028, 970 cm^{-1} . ^1H NMR (400 MHz, $CDCl_3$): δ ppm 7.35-7.25 (m, 5H), 7.21 (d, $J = 8.3$ Hz, 1H), 6.20 (dd, $J_1 =$

Azido-Phe-Leu₃-(S,E)-5-amino-7-methyloct-2-en-4-one (124).

Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-(S,E)-5-amino-7-methyloct-2-en-4-one **168** (40 mg, 83 μ mol, 1 equiv.) and azido-phenylalanine **152** (23 mg, 120 μ mol, 1.45 equiv.). Flash column chromatography (MeOH:DCM (0 $\rightarrow$ 4%)) yielded **124** (36 mg, 66 μ mol, 79%). $[\alpha]_D^{20}$ -31.5° (c = 1, $CHCl_3$). IR (film) 3273, 2955, 2930, 2870, 2110, 1638, 1541, 1456, 1437, 1387, 1367, 1255, 1221, 1152, 970 cm^{-1} . ^1H NMR (400 MHz, CD_3OD): δ ppm 7.33-7.18 (m, 5H), 7.00 (dq, $J_1 = 20.7,$

Ada-Ahx₃-Leu₂-(S,E)-5-amino-7-methyloct-2-en-4-one (125).

Following the general procedure for block coupling, the title compound was obtained from Boc-Leu₂-(S,E)-5-amino-7-methyloct-2-en-4-one **168** (59 mg, 122 μ mol, 1 equiv.) and AdaAhx₃OH **153** (72 mg, 135 μ mol, 1.1 equiv.). Flash column chromatography (MeOH:DCM (2 $\rightarrow$ 8%)) yielded the title compound (88 mg, 98 μ mol, 80%). $[\alpha]_D^{20}$ -44.0° (c = 1, CD_3OD). IR (film) 3281, 2928, 2905, 2868, 1636, 1545, 1439, 1367,

Hz, 2H), 7.95 (t, $J = 5.0$ Hz, 2H), 7.88-7.82 (m, 1H), 7.04-6.94 (m, 1H), 6.32-6.26 (m, 1H), 4.68 (dd, $J_1 = 14.3$, $J_2 = 8.1$ Hz, 2H), 4.49-4.34 (m, 2H), 3.19-3.09 (m, 6H), 2.23 (t, $J = 7.4$ Hz, 2H), 2.17 (t, $J = 7.4$ Hz, 1H), 1.98-1.88 (m, 5H), 1.78-1.43 (m, 36H), 1.39-1.27 (m, 6H), 0.97-0.87 (m, 18H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 199.63, 176.09, 175.95, 174.78, 174.46, 173.74, 145.86, 129.25, 56.38, 53.17, 52.99, 51.93, 43.74, 41.92, 41.85, 40.85, 40.31, 40.25, 37.90, 37.03, 36.98, 36.68, 33.76, 30.21, 30.13, 27.64, 27.56, 26.71, 26.61, 25.91, 25.78, 23.69, 23.45, 23.40, 22.22, 22.06, 21.90, 18.56. HRMS calcd. for $[\text{C}_{51}\text{H}_{89}\text{N}_6\text{O}_7]^+$ 897.67873, found 897.67942.

Competition experiments in vitro

HEK293T cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin in a 7% CO_2 humidified incubator at 37°C. Cells were harvested, washed with PBS (2 $\times$) and permeated in digitonin lysis buffer (4 $\times$ pellet volume, 50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl_2 , 5 mM DTT, 0.025% digitonin) for 15 min. on ice followed by centrifugation 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 (Biorad). 10 μg total protein per experiment was exposed to the inhibitors (10 $\times$ solution in DMSO) for 1 hr. at 37°C prior to incubation with MV151 (500 nM) for 1 hr. at 37°C. Reaction mixtures were boiled with Laemmli's buffer containing β -mercaptoethanol for 3 min. before being resolved on 12.5% SDS-PAGE. In-gel detection of fluorescently labelled 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). Competition experiments were repeated twice.

Cell treatment

HEK293T or RAW264.7 cells (2 $\times$ 15 cm dishes) were incubated with 50 μM **97** or **118** or DMSO for 3hr at 37°C in culture medium (DMEM (PAA), 10% FCS (Hyclone), 10 U/ml penicillin and 10 mg/ml streptomycin (PAA)). Cells were washed and harvested in PBS, snap frozen in $\text{N}_2(\text{l})$ and kept at -80°C until analysis. Cell pellets were resuspended in 800 μl lysis buffer (50 mM Tris pH7.5, 250 mM sucrose, 1 mM MgCl_2 , 1 mM DTT, 0.025% digitonin) for 15 min. on ice, lysed by sonication with 2 pulses of 30 sec, 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. (BioRad).

Affinity purification

Some 2 mg of protein in 200 μl was precleared with 50 μl Streptavidin agarose beads (Pierce) under vigorous shaking for 1 hr. at RT to remove endogenously biotinylated proteins. Bead-free lysate (200 μl) was reacted with 300 μM biotin phosphin **154**¹⁷⁶ (1.5 μl from a DMF stock) for 2hr. at 37°C, denatured by boiling for 5 min. with 1% SDS and precipitated with chloroform/ methanol (C/M). The protein pellet was rehydrated in 180 μl 8M urea/100 mM NH_4HCO_3 , reduced with 10 μl 90mM DTT for 30 min. at 37°C, alkylated with 15 μl 200 mM iodoacetamide at room temp (RT) in the dark for 30 min., cleared by centrifugation at 13,000 g and desalted by C/M. The pellet was dispersed in 25 μl PD buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl) with 2% SDS in a heated (37°C) sonic bath. Stepwise (3 $\times$ 25 μl , 4 $\times$ 100 μl and 1 $\times$ 500 μl) addition of PD buffer afforded a clear solution that was incubated with 50 μl MyOne T1 Streptavidin grafted beads (Invitrogen) at RT with vigorous shaking for 2 hr. The beads were stringently washed with 2 $\times$ 300 μl PD buffer with 0.1% SDS, 2 $\times$ 300 μl PD buffer, 2 $\times$ 300 μl wash buffer I (4M urea/50 mM NH_4HCO_3), 2 $\times$ 300 μl wash buffer II (50 mM Tris HCl pH 7.5, 10 mM NaCl) and 2 $\times$ 300 μl water. Beads were taken up in 100 μl digest buffer (100 mM Tris HCl pH 7.8, 100 mM NaCl, 1mM CaCl_2 , 2% MeCN) and digested with 500 ng trypsin o.n. at 37°C. Supernatant containing peptides was acidified with 5 μl formic acid (FA) to pH<4, desalted on Stage tips and dissolved in 50 μl $\text{H}_2\text{O}/\text{MeCN}/\text{FA}$ (97/3/0.1%).

LC/MS analysis

Tryptic peptides were analysed on a Surveyor nanoLC system (Thermo) hyphenated with a LTQ-Orbitrap mass spectrometer (Thermo). Gold and carbon coated emitters (OD/ID=360/25 μm tip ID=5 μm), trap (OD/ID=360/100 μm packed with 25 mm robust Poros 10R²/ 15 mm BioSphere C18 5 μm 120Å) and analytical columns (OD/ID=360/75 μm packed with 20 cm BioSphere C18 5 μm 120Å) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% FA/ H_2O , B: 0.1% FA/MeCN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column via a 15 mm long TFE teflon tubing sleeve (OD/ID 0.3x1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a nano-source base (Upchurch scientific, Idex, USA). General mass spectrometric conditions were: an electrospray voltage of 1.8 kV was applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150°C, capillary voltage 41V, tube lens voltage 150V. Internal mass calibration was performed with

air-borne protonated polydimethylcyclsiloxane ($m/z = 445.12002$) and the plasticizer protonated dioctyl phthalate ions ($m/z = 391.28429$) as lock mass.¹⁸² For shotgun proteomics analysis, 10 μ l of the samples was pressure loaded on the trap column with a 10 μ l/min flow for 5 min followed by peptide separation with a gradient of 35 min 5-30% B, 15 min. 30-60% B, 5 min. A at a flow of 300 μ l/min split to 250 nl/min. by the LTQ divert valve. For each data dependent cycle, one full MS scan (300-2000 m/z) acquired at high mass resolution (60,000 at 400 m/z , AGC target 1×10^6 , maximum injection time 1,000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the LTQ linear ion trap (AGC target 5×10^3 , max inj. time 120 ms) from the three most abundant ions.¹⁸³ MS² settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 500 counts, activation $q = 0.25$ and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 60s and ions with $z < 2$ or unassigned were not analyzed.

4

Syringolin ureido-peptide moiety containing inhibitors show tunable subunit selectivity*

4.1 Introduction

Syringolins form a class of small molecule natural products that are secreted by strains of the plant pathogen *Pseudomonas syringae* pv. *syringae* (Pss) when these are invading a plant.⁵² Syringolin A and B (Figure 4.1) are of particular interest as these molecules act upon the plant proteasome to compromise plant defence mechanisms against invading pathogens.^{184,185} The eukaryotic 20S proteasome contains three catalytically active β subunits, $\beta 1$, $\beta 2$ and $\beta 5$, which display caspase-like, trypsin-like and chymotrypsin-like activity, respectively.¹² The plant 20S proteasome is homologous to the mammalian proteasome and syringolins are potent and selective inhibitors of the human 20S proteasome as well.⁵⁴ Small molecule proteasome inhibitors show promising antitumour activity and bortezomib is used in the clinic against multiple myeloma, whereas several other proteasome inhibitors are in clinical trials.^{144,145,186–188} Biological evaluation of syringolins on tumour cells has shown inhibition of cell proliferation and induction of apoptosis.^{189,190} This sparked the interest

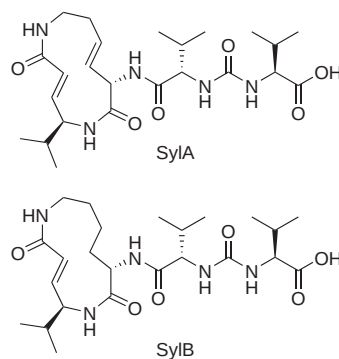


Figure 4.1: Structure of syringolin A and B

*W. A. van der Linden, L. I. Willems, T. B. Shabaneh, N. Li, M. Ruben, B. I. Florea, G. A. van der Marel, M. Kaiser, A. F. Kisselev, H. S. Overkleeft, *Org. Biomol. Chem.* 2011, in press.

of several organic chemists and lately, the total syntheses of several syringolins have been published.^{191–194}

With the total syntheses of syringolin A and B completed, the opportunity opened up to perform structure-activity relationship studies of syringolins and their analogues. Syringolins contain a 12-membered lactam core structure the nature of which differs among the members of this family of compounds. Syringolin A (SylA) has two (E)-configured double bonds resulting in a strained system, and syringolin B has only one ring unsaturation. The α,β -unsaturated amide in the ring system is responsible for covalently and irreversibly inactivating the catalytic Thr1 O γ of the 20S proteasome β subunits via Michael addition.⁵⁴ SylA and SylB inhibit β 2 and β 5 potently and β 1 at higher concentration.¹⁹⁵ The electrophilic lactam is quite rigid in structure and probably determines to a large extent the proteasome specificity. Attached to the lactam core structure is a peptoid fragment, which differs among the syringolins. SylA and SylB contain a valine-urea-valine moiety at their pseudo N-terminus. An ureido linkage induces chain reversal in a peptide, and for this reason has been installed in protease inhibitors.^{196–198} It is also found in several natural peptide-based compounds with antibiotic activity.^{199–201} However, the syringolins are the only examples of proteasome inhibitors that contain this moiety. Some research has already been conducted on the exocyclic part of syringolins, including a D-amino acid scan of the two valines (174, 175 and 176, Figure 4.2), revealing that the naturally occurring configuration yields the most potent compounds.¹⁹⁴ Addition of aliphatic (173)¹⁹³ or hydrophilic (170, 171)¹⁹⁴ tails yielded inhibitors with increased potency with respect to SylA. Addition of a fluorophore was tolerated, yielding proteasome probe 169.²⁰² Hybrids between SylA and a structurally related naturally occurring proteasome inhibitor glidobactin A were designed (177) that appear to possess improved potency compared to SylA.^{195,203}

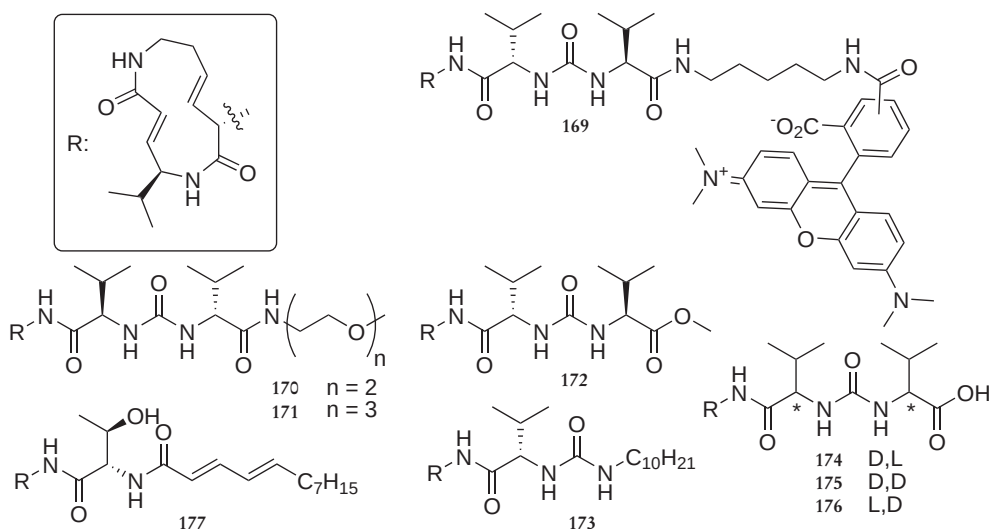


Figure 4.2: Literature SylA modifications

The proteasome subunit selectivity pattern of an inhibitor is important for its antitumour activity. Inhibition of $\beta 5$ is essential, but co-inhibition of $\beta 1$ or $\beta 2$ is usually needed for the compound to be cytotoxic in cell lines derived from various cancers.^{127,128,169} The structural elements in syringolins that are responsible for proteasome subunit selectivity are therefore useful in the design of new proteasome inhibitors. To determine the effect of the valine-urea-valine motif in the biological profile of syringolins this moiety is incorporated in a more conventional linear peptide based proteasome inhibitor design. In this design, the unsaturated lactam Michael acceptor is replaced with leucine vinyl sulfone (VS), another Michael acceptor commonly used to arrive at potent proteasome inhibitors. A leucine epoxyketone (EK) is used as well since this warhead often yields more potent and selective inhibitors than the vinyl sulfone.²⁰⁴

4.2 Results and Discussion

The first set of target compounds subject of the here presented study are displayed in Figure 4.3. Direct attachment of the leucine derived electrophilic trap to the valine-urea-valine moiety yields two tripeptide compounds (**179** and **183**). The carboxylic acid functionality is masked as a *tert*-butyl ester as its synthetic precursor (**178** and **182**). A benzylamide cap is also used (**180** and **184**), since aromaticity at the N-terminus of a short proteasome inhibitor could be beneficial for its potency.^{142,205} The decyl chain, improving syringolin A activity,¹⁹³ is also used in this library (**181** and **185**). When the tripeptide structure is compared to SylA, the distance between the electrophile of the inhibitor and the urea group is shorter in the tripeptide than in SylA. Therefore, one more amino acid is incorporated

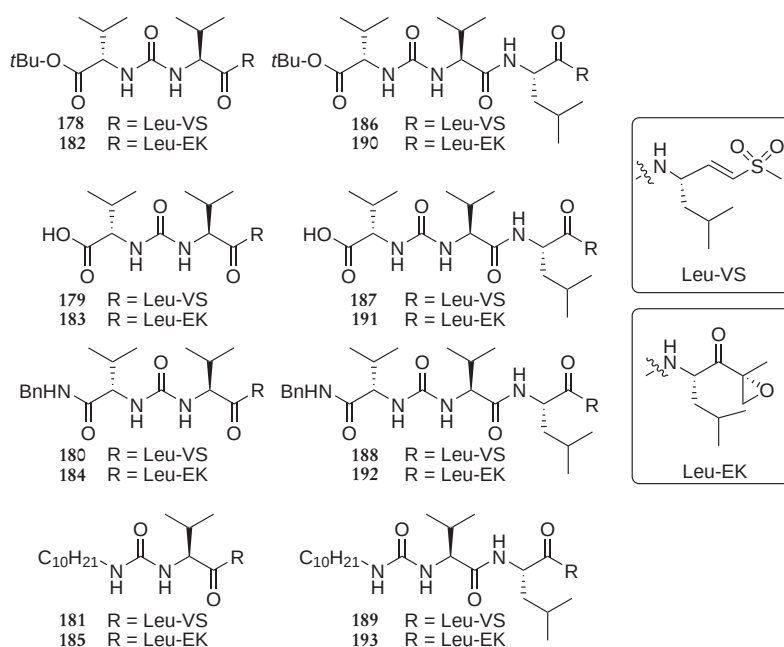


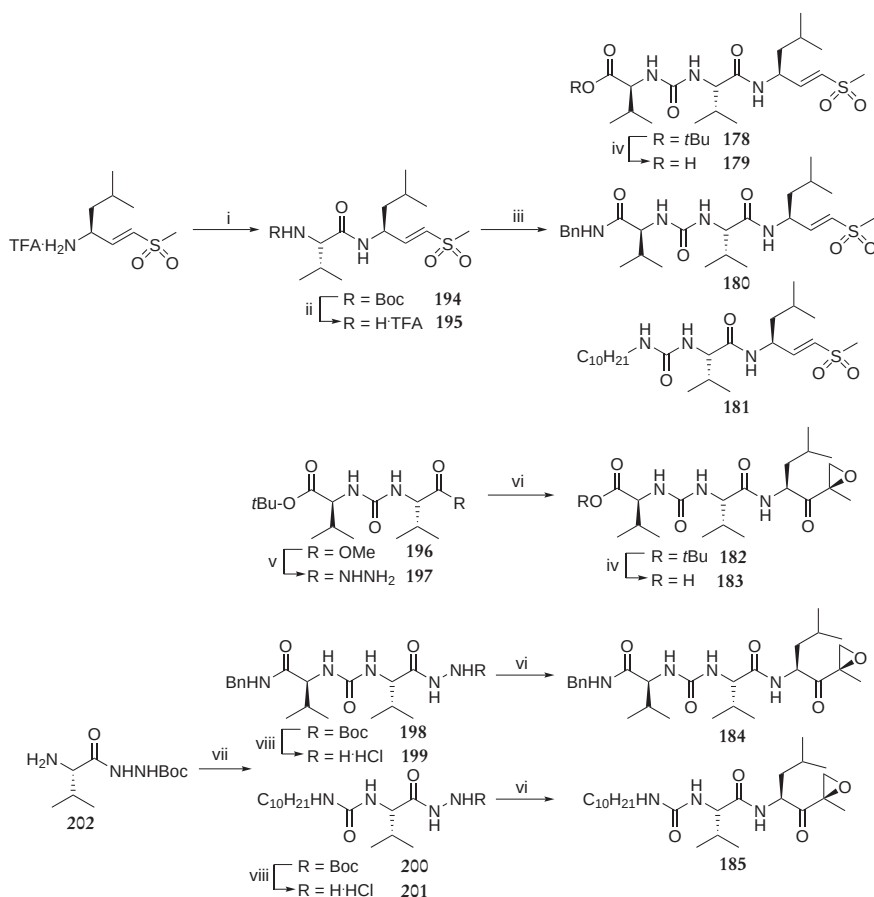
Figure 4.3: Panel of synthesised potential proteasome inhibitors.

between the urea and electrophile, yielding tetrapeptide inhibitors (**186-193**, Figure 4.3).

The synthesis of the tripeptide vinyl sulfones commenced with coupling of TFA·H-LeuVS¹⁰⁶ to Boc-Val-OH to yield Boc-protected dipeptide **194** from which the Boc protecting group was removed to yield dipeptide **195** (Scheme 4.1). This dipeptide was reacted with valine *tert*-butyl ester isocyanate, valine benzylamide isocyanate or decyl isocyanate to yield three inhibitors **178**, **180** and **181**. Peptide **178** was deprotected with TFA to yield **179**.

In an attempt to react dipeptide epoxyketone TFA·H-Val-Leu-EK with valine *tert*-butyl ester isocyanate, no formation of compound **182** was detected. Rather, dipeptide TFA·H-Val-Leu-EK cyclizes in this basic reaction medium. As an alternative approach to obtain

Scheme 4.1: Synthesis of tripeptide vinyl sulfones and epoxyketones.

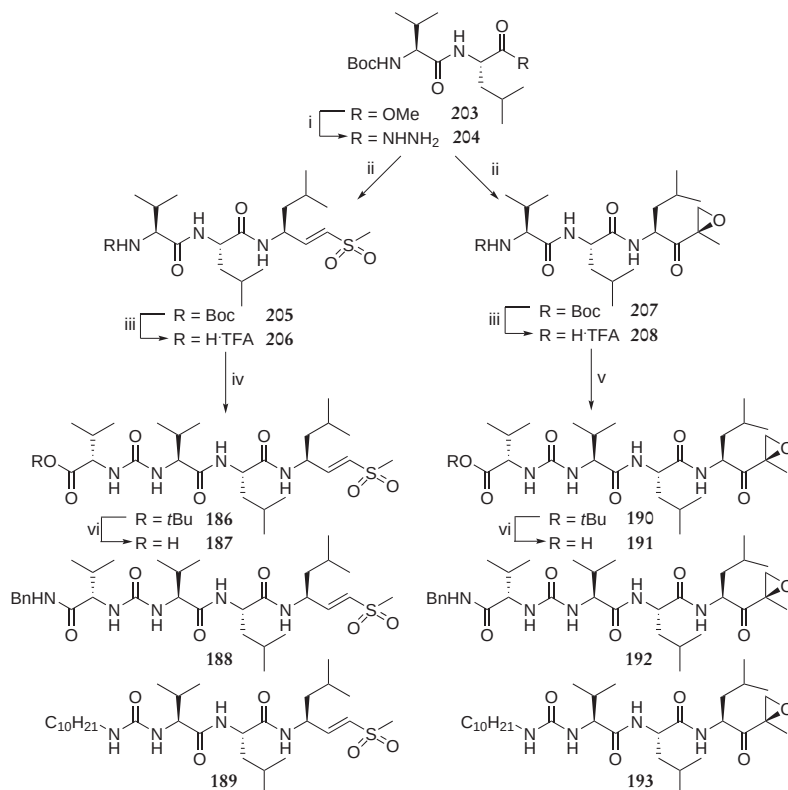


Reagents and conditions: i) Boc-Val-OH (1.1 equiv.), HBTU (1.2 equiv.), DiPEA (3.5 equiv.), EA, 4 hr., quant. ii) TFA:DCM 1:1, 30 min., quant. iii) 3 isocyanates (1 equiv.), DiPEA (2.2 equiv.), DCM, 1 hr.-o/n, 80% (**178**), 36% (**180**), 67% (**181**). iv) TFA, 45 min, quant. v) hydrazine hydrate (30 equiv.), MeOH, reflux, o/n. vi) *t*BuONO (1.1 equiv.), HCl (2.8 equiv.), DMF:EA 3:1, -30°C, 3 hr. then TFA·H-Leu-EK (1.1 equiv.), DiPEA (5 equiv.), DMF, -30°C → RT o/n, 47% (**182**), 20% (**184**), 35% (**185**). vii) valine benzylamide isocyanate (0.83 equiv.) or decyl isocyanate (1.05 equiv.), DiPEA (1.2 equiv.), DCM, 1-2 hr., 53% (**198**), 60% (**200**). viii) 4M HCl/dioxane, 1 hr.

compound **182**, the methyl ester in compound **196**¹⁹³ was transformed to the hydrazide (**197**) which was then in situ transformed to the acyl azide and coupled to TFA·H-Leu-EK to arrive at **182**, which was subsequently deprotected to gain **183** (Scheme 4.1). Compounds **184** and **185** were synthesised by a modified approach. Reacting **202**²⁰⁶ with valine benzylamide isocyanate or decyl isocyanate yielded **198** and **200**, which were then Boc-deprotected to yield hydrazides **199** and **201** which were transformed to the acyl azide in situ and coupled to TFA·H-Leu-EK to obtain **184** and **185**.

The synthesis of tetrapeptide vinyl sulfones and epoxyketones followed a general strategy (Scheme 4.2). Methyl ester **203**²⁰⁷ was transformed to the corresponding hydrazide **204** by hydrazine hydrate in methanol. This compound was in situ transformed to its acyl azide and then coupled to TFA·H-Leu-VS or TFA·H-Leu-EK to arrive at **205** and **207**. Carbamate **205** was deprotected with TFA in DCM to give **206**, which in the next step was reacted with the three isocyanates mentioned in the section above to yield tetrapeptides

Scheme 4.2: Synthesis of tetrapeptide vinyl sulfones and epoxyketones.



Reagents and conditions: i) hydrazine hydrate (20 equiv.), MeOH, reflux, 2 hr. ii) *t*BuONO (1.1 equiv.), HCl (2.8 equiv.), DMF:EA 3:1, -30°C, 3 hr. then TFA·H-Leu-VS (1.1 equiv.) TFA·H-Leu-EK (1.1 equiv.), DiPEA (5 equiv.), DMF, -30°C → RT o/n, 72% (**205**), 90% (**207**). iii) TFA:DCM 1:1, 30 min., quant. iv) valine *tert*-butylester isocyanate, valine benzylamide isocyanate or decyl isocyanate, DiPEA, DCM, 70% (**186**), 23% (**188**), 43% (**189**). v) valine *tert*-butylester isocyanate, valine benzylamide isocyanate or decyl isocyanate, DiPEA, DCM, 28% (**190**), 40% (**192**), 29% (**193**). vi) TFA, 1 hr., quant.

186, 188 and 189. Compound **186** was deprotected with TFA to yield **187**. The same strategy, employing **207**, led to **190-193**.

In a first assessment of inhibitory potency the sixteen compounds were subjected to a competition assay versus BODIPY-TMR-epoxomicin (MVB003, **49**).¹¹⁴ Extracts from HEK293T or EL4 cells were incubated with a broad concentration range of inhibitor for one hour, after which remaining proteasome activity was labelled with MVB003. After SDS-PAGE separation of the proteome, the wet gel slabs were scanned on a Typhoon fluorescence scanner. Results are displayed in Figure 4.4. Proteasome subunits were assigned based on earlier work.¹¹⁴

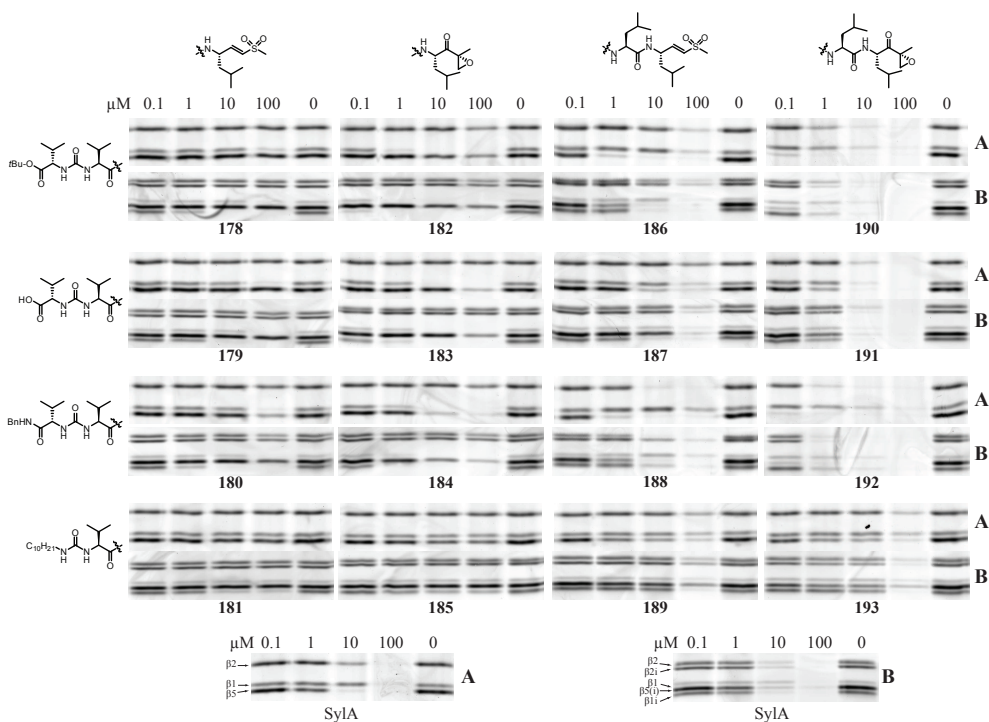


Figure 4.4: Competition assay in cell lysate versus MVB003. (A) HEK293T (15 μg) or (B) EL4 lysate (10 μg) was incubated with indicated end concentrations of inhibitor for 1 hr. at 37°C. Residual proteasome activity was labelled by MVB003 (0.5 μM end concentration) for 1 hr. at 37°C.

One first obvious conclusion from this broad concentration scan is that the syringolin A inspired ureido peptide moiety, coupled to leucine epoxyketone or vinyl sulfone, yields active proteasome inhibitors (Figure 4.4). Generally, the inhibitors containing a vinyl sulfone are less potent than their epoxyketone counterparts, which is in agreement with previous observations.^{169,204}

A second obvious trend is that tripeptide inhibitors show some selectivity for $\beta 1$. The most distinct selectivity for $\beta 1$ is displayed by **182** in HEK lysate (Figure 4.4). In EL4 lysate, tripeptide vinyl sulfones **178**, **179**, **180** appear to silence $\beta 1i$ before $\beta 1$ is targeted (Figure 4.4). Their epoxyketone counterparts, however, target $\beta 1$ and $\beta 1i$ more simultaneously. The tetrapeptide inhibitors appear to preferentially target $\beta 5$. Compounds contain-

ing the long aliphatic tail (**181**, **185**, **189**, **193**) are far less potent than their counterparts lacking this moiety. This is in sharp contrast to lipophilic SylA analog **173**, which is much more potent than parent compound SylA.¹⁹³

To gain better insight in the subunit selectivity of the most potent inhibitors, the competition assay was repeated with a more detailed concentration series (Figure 4.5). Tripeptide vinyl sulfones **178-181** as well as **185** and **189** were omitted because the inhibitory effect of these compounds falls mostly outside the more diluted concentration range. There is a difference in the outcome of the competition assay in HEK lysate and EL4 lysate (Figure 4.5A and B). The potency of tetrapeptide vinyl sulfones in HEK lysate does not seem to be matched in EL4 lysate. For example, **186** and **188** seem much less potent in EL4 lysate than in HEK lysate (Figure 4.5A and B). This could be explained by the overlap of $\beta 5$ subunits of the constitutive and immunoproteasome on gel. Inhibition of one of these signals is partially overshadowed by the untouched signal of the other subunit.

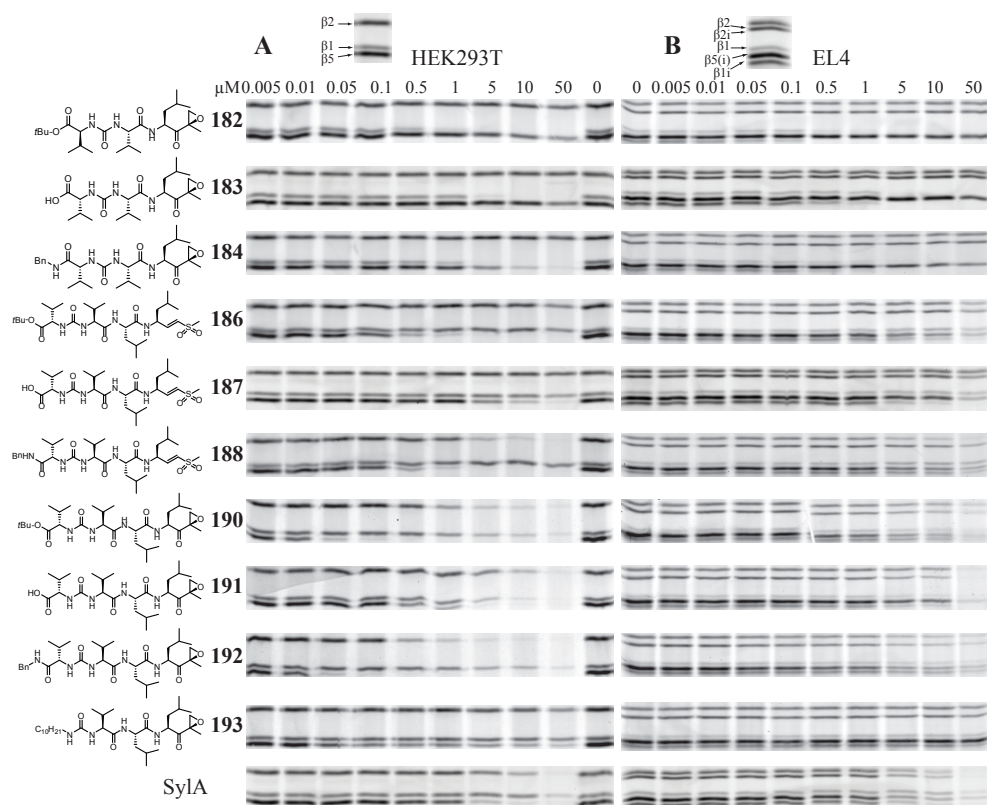


Figure 4.5: Competition assay in cell lysate versus MVB003. (A) HEK293T (15 μg) or (B) EL4 lysate (10 μg) was incubated with indicated end concentrations of inhibitor for 1 hr. at 37°C. Residual proteasome activity was labelled by MVB003 (0.5 μM end concentration) for 1 hr. at 37°C.

In HEK lysate (Figure 4.5A), several library members indeed are subunit selective; **186** inhibits $\beta 5$ by more than 90% at about 1 μM , at which concentration $\beta 1$ and $\beta 2$ appear untouched. Compound **190** fully inhibits $\beta 5$ at 100 nM, before inhibiting $\beta 1$ and $\beta 2$. Compound **182** is very selective for the $\beta 1$ subunit. Compared to NC001 (**58**, Table 4.1), a very selective tetrapeptide epoxyketone, compound **182** is only slightly less potent in HEK lysate.^{127,133} The benzyl amides in this library are generally less selective than their *tert*-butyl counterparts; **188** co-inhibits $\beta 2$ before complete $\beta 5$ inhibition is reached while **186** shows more selectivity. Benzyl amide **184** co-inhibits $\beta 5$ much earlier than the selective *tert*-butyl ester **182**. The difference in selectivity between **190** and **192**, however, is less pronounced. The parent compound, SylA, inhibits $\beta 5$ and $\beta 2$ in the low micromolar range and only inhibits $\beta 1$ at higher concentration (Figure 4.5). In this respect, the activities of the tetrapeptide inhibitors of this library in general more resemble that of SylA than the activities of the tripeptides do. Interestingly, there are several compounds in this library that display more potent proteasome inhibition than the parent compound, SylA. Compounds **190** and **192** are the most potent inhibitors in this study; compared to SylA in HEK lysate (Figure 4.5A), these two compounds inhibit $\beta 5$ at about 100-fold lower concentration.

The most potent and selective compounds were also analysed for their inhibitory capacities against purified proteasomes isolated from rabbit muscles (constitutive 26S proteasome) or rabbit spleens (immunoproteasome) with subunit specific fluorogenic peptides (Table 4.1A and B). To better compare these results with the competition assay gels from Figure 4.5, the gel bands were quantified and plotted against inhibitor concentrations. From these plots, the IC_{50} values were calculated and the results are summarised in Table 4.1C and D.

Table 4.1: Apparent IC_{50} (μM) values calculated from semi log plots of residual proteasome activity against inhibitor concentration.

	Fluorogenic peptide hydrolysis assay						Quantified competition assay					
	$\beta 5$	$\beta 1$	$\beta 2$	$\beta 5\text{i}$	$\beta 1\text{i}$	$\beta 2\text{i}$	$\beta 5$	$\beta 1$	$\beta 2$	$\beta 5/\beta 5\text{i}$	$\beta 1\text{i}$	$\beta 2\text{i}$
Compound	26S proteasome IC_{50} (μM) A			Immunoproteasome IC_{50} (μM) B			HEK293T lysate IC_{50} (μM) C			EL4 lysate IC_{50} (μM) D		
182	>30	1.7	>30	>100	24	N.I.	50	0.46	>100	N.I.	0.079	N.I.
183							50	1.4	>100	16	0.4	42
184	>10	1.2	>100	~20	1.0	N.I.	2.9	0.52	>100	24	0.67	N.I.
186	1.0	>30	>30	0.13	N.I.	N.I.	0.23	100	100	3.3	5.9	46
187							4.2	50	>100	13	2.3	32
188	0.64	18	10	0.077	>3	>3	0.17	100	2.1	4.4	32	22
190	~0.3	>0.3	>0.3	0.007	~1	~1	0.016	1.3	0.69	0.32	13	2.0
191							0.48	2.8	5.9	7.2	5.0	3.1
192	0.013	0.24	0.3	0.005	1.0	0.28	0.008	2.4	0.28	0.69	23	1.6
193							0.5	100	100	19	28	39
SylA							1.3	25	6.6	1.2	1.7	2.8
NC001 (58)							>50	0.31	N.I.			

Constitutive proteasomes purified from rabbit muscles (A) or immunoproteasomes, purified from rabbit spleens (B), were incubated with different concentrations of inhibitors for 30 min at 37°C followed by measurement of remaining activity with fluorogenic peptides (Suc-LLVY-AMC, $\beta 5/\beta 5\text{i}$, Ac-LPnLD-AMC, $\beta 1/\beta 1\text{i}$, Ac-RLR-AMC, $\beta 2/\beta 2\text{i}$). Band intensities from competition assay gels in HEK293T lysate (C) or EL4 lysate (D) in Figure 4.4 and Figure 4.5 were used as input. N.I. no inhibition.

The IC_{50} values found with the fluorogenic peptide hydrolysis assay are in most cases higher than those found in the competition assay, but one should be careful when comparing IC_{50} values obtained with two very different experimental setups. The subunit selectivity trends found with the fluorogenic peptide assay, however, in most cases agree with the results from the competition assay. In both settings, **182** is a selective $\beta 1$ proteasome inhibitor, while **192** is a selective $\beta 5$ inhibitor. In the fluorogenic substrate assay, compound **190** shows onset of inhibition of the constitutive $\beta 1$ and $\beta 2$ sites when $\beta 5$ is targeted, while in the competition assay in HEK293T lysate this compound appears more selective. Tetrapeptide vinyl sulfones **186** and **188** appear more potent towards $\beta 5$ than $\beta 5$ in the fluorogenic substrate assay (Table 4.1A and B). The differences in potency and selectivity between competition assay in cell lysate and fluorogenic substrate hydrolysis by purified proteasome can be caused by the presence of other proteasome forms in cell lysate (i.e. PA200 and PA28 activated proteasomes) or post-translational modifications that affect active site specificity and that are either species or tissue specific and may be lost during preparation of proteasomes from muscle or spleen.

Compounds **182**, **190** and **192** were evaluated on their proteasomal inhibition capacities in living HEK cells to assess cell permeability of these compounds. Subunit selective proteasome inhibitors NC001 (**58**) and NC005-VS were used for comparison.^{127,133} Living HEK cells were incubated with the inhibitors for 4 hr. after which remaining proteasomal activity was labelled by cell permeable probe MVB003 (Figure 4.6). The results are summarised in Table 4.2. Interestingly, **182** shows fivefold higher potency in living HEK cells than NC001, while NC001 is slightly more potent in cell extract.

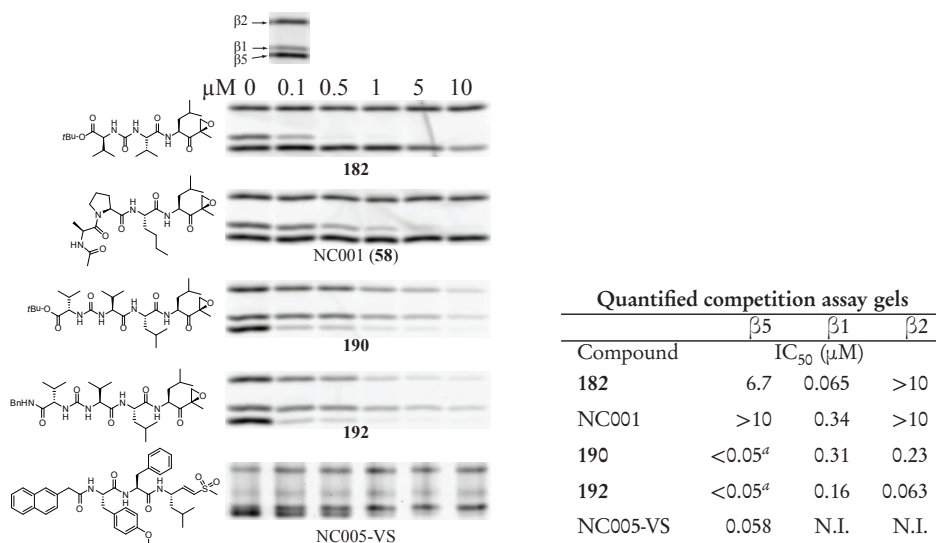


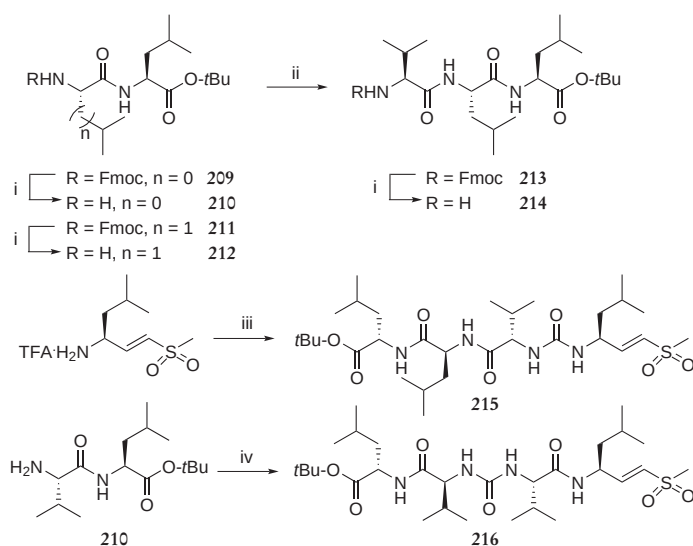
Figure 4.6: Competition assay in living HEK cells. Cells ($1 \cdot 10^6$) were incubated with indicated end concentrations of inhibitor (100 $\times$ stock in DMSO) for 4 hr. at 37°C. Residual proteasome activity was labelled with MVB003 (5 μM end concentration, 100 $\times$ DMSO stock) for 2 hr. at 37°C.

Table 4.2: Apparent IC_{50} (μM) values calculated from semi log plots of residual proteasome activity against inhibitor concentration. Band intensities from each lane of competition assay gels in living cells (Figure 4.6) were quantified and used as input. N.I. no inhibition. ^a about 80% inhibition of activity observed at 50 nM.

This difference can be ascribed to better cell permeability of **182**, possibly due to its smaller size than NC001. Compound **182** is the most potent and selective β 1 inhibitor to date. Compounds **190** and **192** do inhibit the proteasome in living HEK cells and therefore are cell permeable, but their β 5 selectivity is much lower than observed in HEK extracts and NC005-VS is much more β 5 selective in cells than **190** and **192**.

Having evaluated a small library of proteasome inhibitors containing tri- and tetrapeptides with the valine-urea-valine motif, a dramatic shift in selectivity from β 1 to β 5 is observed when the ureido linkage is moved one place in the molecule. A tetrapeptide vinyl sulfone scaffold was selected in which the ureido-linkage is 'shifted' through the molecule to arrive at two more potential proteasome inhibitors with the urea after the first or second amino acid in the peptide instead of the third (Scheme 4.3). The Fmoc group in **209**²⁰⁸ and **211** was removed by DBU.²⁰⁹ Dipeptide **212** was coupled to Fmoc-Val-OH and again deprotected with DBU to arrive at tripeptide **214**. TFA·H-Leu-VS was converted to its isocyanate by phosgene and DiPEA in DCM¹⁹³ and subsequently reacted with amine **214** which resulted in tetrapeptide **215**. Amine **210** was converted to the isocyanate by phosgene and sat. aq. NaHCO_3 ²¹⁰ and reacted with amine **195** which resulted in tetrapeptide **216**.

Scheme 4.3: Synthesis of two potential tetrapeptide proteasome inhibitors with ureido-linkage after P1 or P2



Reagents and conditions: i) EtSH (10 equiv.), DBU (0.1 equiv.), THF, 1.5-2hr., 97% (**210**), 93% (**212**), 91% (**214**). ii) Fmoc-Val-OH (1.05 equiv.), HBTU (1.15 equiv.), DiPEA (2.25 equiv.), DCM, 1.5 hr., 95%. iii) phosgene (1.1 equiv.), DiPEA (2.2 equiv.), DCM, 10 min, then **214** (1.1 equiv.), DiPEA (2.2 equiv.), 2 hr., 83%. iv) phosgene (1.2 equiv.), DCM/sat. aq. NaHCO_3 , 0°C, 10 min. then **195** (1 equiv.), (2.2 equiv.), 54%.

Compounds **215** and **216** were subjected to the competition assay in HEK and EL4 lysate versus MVB003 (Figure 4.7). In HEK lysate, only at high concentrations (100 μM), proteasome inhibition is observed. In EL4 lysate, on the other hand, compound **216** appears to inhibit β 1i selectively from 5 μM and higher concentrations, when β 1 remains

mostly untouched. However, the window of $\beta 1i$ specific inhibition is small. The activity of **216** resembles the activities of tripeptide vinyl sulfones **178**, **179** and **180**, where the ureido linkage is at the same distance from the warhead. Compound **215** only shows some inhibition of $\beta 1$ at 50 μM in EL4 lysate. Apparently, the place of the ureido-linkage in the peptide inhibitor determines its activity and selectivity for proteasome subunits. The relatively low activity of **215** could be the result of 'inversed' amino acid side chain configuration caused by chain reversal due to the ureido linkage. Substitution for D-amino acids at P2-4 for **215** might restore activity of this scaffold. Usage of D-amino acids at P3 and P4 for **216** might improve its activity as well.

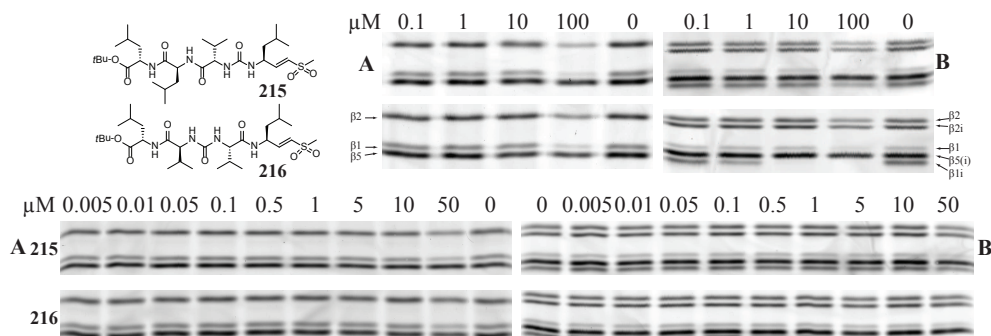


Figure 4.7: Competition assay in (A) HEK lysate (15 μg protein) or (B) EL4 lysate (10 μg protein). Lysates were incubated with indicated end concentrations of inhibitor for 1 hr. at 37°C. Residual proteasome activity was labelled by MVB003 (0.5 μM end concentration) for 1 hr. at 37°C.

4.3 Conclusion

Incorporation of the exocyclic valine-urea-valine motif, which is found in syringolin A, into a vinyl sulfone or epoxyketone oligopeptide results in a new set of potent proteasome inhibitors. In general, epoxyketone containing inhibitors are more potent than their vinyl sulfone counterparts, but this increase in potency is often accompanied by a decrease in subunit selectivity.^{133,169,204} The position of the ureido linkage with respect to the electrophilic trap has a profound effect on proteasome subunit selectivity. A short distance, as in compounds **182-184** and **178-180** results in preference for the $\beta 1$ subunit of the proteasome. In compounds **178-181** and **216**, a slight preference for $\beta 1i$ is observed in EL4 lysate. When an extra amino acid is incorporated in between the ureido linkage and the warhead, as in inhibitors **186-189** and **190-193**, a preference for the $\beta 5$ subunit is observed, with tetrapeptide epoxyketones **190** and **192** being quite selective inhibitors of the $\beta 5$ site. When the ureido linkage is incorporated after P1 in the peptide, as for compound **215** proteasomal inhibition is mostly lost.

Tripeptide epoxyketone **182** is a potent and very selective inhibitor of the $\beta 1$ subunit. In living cells, this compound is fivefold more potent than NC001 and is the most potent $\beta 1$ selective compound known to date. Further optimisation of this compound, for example by changing the P2 valine for a norleucine residue or changing the *tert*-butyl for a smaller methyl group, might make this molecule even more selective for $\beta 1$. Tetrapeptide epoxyketones **190** and **192** are the most potent compounds in this library, more potent

than the parent compound SylA. The subunit selectivity pattern of **192** shows similarities to that of SylA, but displays higher potency, indicating that the antitumour activity of this compound should be assessed.

Translating the findings of this study back to the case of syringolin A meets difficulties. One can envisage that the molecular basis of selectivity of **182**, **190** and **192** can be unravelled with the crystal structures of these compounds in complex with 20S proteasomes.^{35,54,211}

4.4 Experimental

All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.) (purum), ethyl acetate (EA) (puriss.), and light petroleum ether (PE) (puriss.) were obtained from Riedel-de Haën or Biosolve and were distilled prior to use. Dichloromethane (DCM), dimethyl formamide (DMF), and dioxane were stored on 4 Å molecular sieves. Tetrahydrofuran (THF) was distilled from LiAlH₄ prior to use. Reactions were conducted under an argon atmosphere. Reactions were monitored by TLC analysis by using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by UV absorption (254 nm), spraying with 20% H₂SO₄ in ethanol or (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid followed by charring at ~150°C or by spraying with an aqueous solution of KMnO₄ (7%) and KOH (2%). Column chromatography was performed on silica gel from Screening devices (0.040–0.063 nm). LC/MS analysis was performed on a LCQ Advantage Max (Thermo Finnigan) equipped with an Gemini C18 column (Phenomenex). HRMS was recorded on a LTQ Orbitrap (Thermo Finnigan). ¹H- and ¹³C-APT-NMR spectra were recorded on a Bruker DPX-300 (300/75 MHz) or Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Coupling constants are given in Hz. All presented ¹³C-APT spectra are proton decoupled. TFA·H-Leu-EK¹⁸⁴ and TFA·H-Leu-VS¹⁰⁶ were obtained by literature procedures. NC001 (**58**), NC005-mvs and SylA were obtained as described.^{127,133,193}

General procedure for the formation of isocyanates A¹⁹³

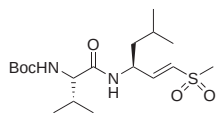
Amine or amine HCl salt (1 equiv.) was dissolved in DCM and DiPEA (2.2 equiv.) was added. This solution was slowly added to a solution of triphosgene (0.35 equiv.) or phosgene (1.1 equiv, 20 wt-% sln in Tol.) and the mixture was stirred for an additional 10 minutes after addition and used in the next step without any purification.

General procedure for the formation of isocyanates B²¹⁰

HCl·H-ValNHBN²¹² or H-Val-Leu-OtBu was dissolved in 1:1 DCM:sat.aq. NaHCO₃ at 0° C. The layers were allowed to separate and phosgene (1.2 equiv., 20 wt-% in Tol.) was added to the DCM layer and stirring was continued for 10 minutes after which the mixture was extracted with DCM (3×). The organic layers were combined and dried over Na₂SO₄ and concentrated. The resulting crude isocyanate was used without further purification.

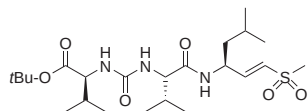
General procedure for azide coupling

Hydrazide or hydrazide HCl (1 equiv.) was dissolved in DMF:EA 3:1 and cooled to -30° C. tBuONO (1.1 equiv.) and HCl (4M in dioxane, 2.8 equiv.) were added and the resulting mixture stirred for 3 hr. TFA·H-Leu-EK or TFA·H-Leu-VS (1.1 equiv.) in DMF was added followed by DiPEA (5 equiv.) and the mixture was allowed to warm to RT overnight. The mixture was diluted with EA and washed with H₂O (3×). The organic layer was dried over Na₂SO₄ and concentrated before flash column chromatography.

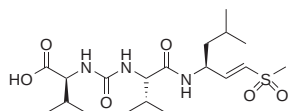


Boc-Val-Leu-VS (194). Boc-Val-OH (359 mg, 1.65 mmol, 1.1 equiv.) was dissolved in EA. HBTU (683 mg, 1.8 mmol, 1.2 equiv.), DiPEA 0.87 mL, 5.25 mmol, 3.5 equiv.) and TFA·LeuVS (1.5 mmol, 1 equiv.) were added and the mixture stirred for 4 hr. The mixture was washed with 1M HCl (3×), sat. aq. NaHCO₃ (3×) and brine before being dried over Na₂SO₄. The residue was purified by column chromatography (30% EA:PE → 60% EA:PE) to yield the title compound (587 mg, 1.5 mmol, quant.). ¹H NMR (400 MHz, CDCl₃): δ 6.88 (d, *J* = 6.6 Hz, 1H), 6.79 (dd, *J*₁ = 15.1, *J*₂ = 5.3 Hz, 1H), 6.52 (d, *J* = 15.1 Hz, 1H), 5.33 (d, *J* = 7.5 Hz, 1H), 4.87–4.62 (m, 1H), 3.86 (dd, *J*₁ = 8.3, *J*₂ = 7.1 Hz, 1H), 2.89 (s, 3H), 2.15–2.00 (m, 1H), 1.70–1.56 (m, 1H), 1.40 (s, 9H), 0.96–0.81 (m, 12H). ¹³C NMR (100 MHz, CDCl₃): δ 171.63, 155.89, 147.63, 129.16, 79.80, 60.25, 47.65, 42.62, 42.48, 30.17, 28.15, 24.50, 22.63, 21.65, 19.27, 17.85.

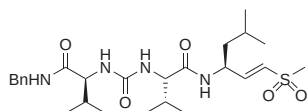
TFA·H-Val-Leu-VS (195). Boc-Val-Leu-VS (194) was stirred in 1:1 DCM:TFA for 30 minutes before coevaporation with toluene (3×) yielded the title compound, which was immediately used in the next reaction without further purification.



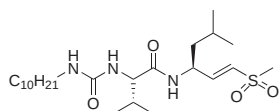
tBuO-Val-urea-Val-Leu-VS (178). A solution of TFA·H-Val-Leu-VS (195), 305 μmol , 1 equiv.) and DiPEA (111 μl , 671 μmol , 2.2 equiv.) in DCM was added to the isocyanate of valine *tert*-butyl ester (obtained from HCl·H-Val-OtBu (64 mg, 305 μmol , 1 equiv.) employing general procedure A). The remaining solution was stirred for 1 hr and concentrated. The residue was dissolved in EA and washed with 1M HCl (2×), sat. aq. NaHCO_3 and brine before being dried over Na_2SO_4 . Column chromatography (40% EA:PE $\rightarrow$ 70% EA:PE) yielded the title compound (119 mg, 243 μmol , 80%). LC/MS: R_t 10.28 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1%TFA, 15 min). ^1H NMR (400 MHz, CDCl_3): δ ppm 7.42 (d, $J = 7.98$ Hz, 1H), 6.80 (dd, $J_1 = 15.06$, $J_2 = 5.76$ Hz, 1H), 6.70 (d, $J = 15.16$ Hz, 1H), 6.34 (d, $J = 7.99$ Hz, 1H), 6.00 (d, $J = 8.97$ Hz, 1H), 4.76-4.67 (m, 1H), 4.24 (dd, $J_1 = 8.82$, $J_2 = 5.29$ Hz, 1H), 4.03 (t, $J = 8.40$, 1H), 2.93 (s, 3H), 2.13-1.94 (m, 2H), 1.73-1.58 (m, 1H), 1.47 (s, 9H), 1.46-1.38 (m, 2H), 0.97-0.84 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 219.35, 172.97, 172.34, 158.28, 147.04, 129.88, 81.59, 60.16, 58.69, 48.02, 42.66, 42.53, 31.37, 30.76, 28.04, 24.60, 22.56, 22.08, 19.47, 18.99, 18.56, 17.98. HRMS calcd. for $[\text{C}_{23}\text{H}_{44}\text{N}_3\text{O}_6\text{S}_1]^+$ 490.29453, found 490.29445.



HO-Val-urea-Val-Leu-VS (179). *t*-Bu ester 178 was stirred in 1 ml TFA for 45 min before being coevaporated with toluene (3×) to yield the title compound in quantitative yield. LC/MS: R_t 6.19 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1%TFA, 15 min). ^1H NMR (400 MHz, CD_3OD): δ ppm 6.78 (dd, $J_1 = 15.17$, $J_2 = 5.29$ Hz, 1H), 6.63 (dd, $J_1 = 15.18$, $J_2 = 1.34$ Hz, 1H), 4.73-4.64 (m, 1H), 4.19 (d, $J = 4.72$ Hz, 1H), 3.99 (d, $J = 6.60$ Hz, 1H), 2.95 (s, 3H), 2.21-2.09 (m, 1H), 2.08-1.99 (m, 1H), 1.78-1.62 (m, 1H), 1.60-1.50 (m, 1H), 1.49-1.38 (m, 1H), 1.03-0.84 (m, 18H). ^{13}C NMR (100 MHz, CD_3OD): δ ppm 176.02, 174.72, 160.55, 148.57, 130.92, 60.94, 59.47, 49.14, 43.35, 42.83, 32.18, 32.05, 25.86, 23.37, 21.94, 19.96, 19.75, 18.30, 17.94. HRMS calcd. for $[\text{C}_{19}\text{H}_{36}\text{N}_3\text{O}_6\text{S}_1]^+$ 434.23193, found 434.23186.



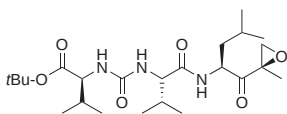
Benzylamide-Val-urea-Val-Leu-VS (180). Vinyl sulfone 195 (225 μM , 1 equiv.) and DiPEA (41 μl , 241 μmol , 1.1 equiv.) were dissolved in DCM and this mixture was added to the isocyanate of valine benzylamide (obtained from HCl·H-Val-NHBn²¹² (104 mg, 338 μmol , 1.5 equiv.) employing general procedure B). The remaining solution was stirred overnight and concentrated. The residue was dissolved in DCM and washed with H_2O (2×) and dried over Na_2SO_4 . Column chromatography (DCM $\rightarrow$ 10% MeOH:DCM) yielded the title compound (43 mg, 81 μmol , 36%). LC/MS: R_t 7.53 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1%TFA, 15 min). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1/1): δ ppm 7.36-7.21 (m, 5H), 6.81 (dd, $J_1 = 15.13$, $J_2 = 5.17$ Hz, 1H), 6.59 (dd, $J_1 = 15.12$, $J_2 = 1.49$ Hz, 1H), 4.75-4.65 (m, 1H), 4.41 (q, $J = 14.94$, 2H), 4.02 (d, $J = 6.69$ Hz, 1H), 3.96 (d, $J = 6.64$ Hz, 1H), 2.96 (s, 3H), 2.06 (m, 2H), 1.74-1.61 (m, 1H), 1.59-1.37 (m, 2H), 1.02-0.84 (m, 18H). ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$): δ ppm 172.64, 147.33, 128.73, 128.00, 127.02, 126.78, 59.24, 58.95, 47.35, 42.71, 41.92, 41.84, 30.66, 30.25, 24.22, 22.08, 20.90, 18.70, 18.60, 17.14, 17.09. HRMS calcd. for $[\text{C}_{26}\text{H}_{43}\text{N}_4\text{O}_5\text{S}_1]^+$ 523.29487, found 523.29478.



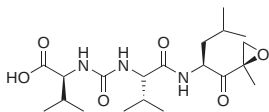
Decyl-urea-Val-Leu-VS (181). Vinyl sulfone 195 (266 μM , 1 equiv.) and DiPEA (100 μl , 585 μmol , 2.2 equiv.) were dissolved in DCM and this solution was added to 1-isocyanatodecane (obtained from decyl amine (58 μl , 293 μmol , 1.1 equiv.) employing general procedure A). The remaining solution was stirred for 1 hr and washed with 1M HCl (3×) and sat. aq. NaHCO_3 and the solution was dried over Na_2SO_4 . Column chromatography (40% EA:PE $\rightarrow$ 80% EA:PE) yielded the title compound (85 mg, 179 μmol , 67%). ^1H NMR (400 MHz, CDCl_3): δ ppm 8.28 (d, $J = 7.54$ Hz, 1H), 6.88 (dd, $J_1 = 15.10$, $J_2 = 6.04$ Hz, 1H), 6.68 (d, $J = 14.98$ Hz, 1H), 6.55-6.43 (m, 1H), 5.91-5.83 (m, 1H), 4.77-4.64 (m, 1H), 4.12-4.04 (m, 1H), 3.24-3.09 (m, 1H), 3.05-2.95 (m, 1H), 2.93 (s, 1H), 2.02-1.85 (m, 1H), 1.72-1.58 (m, 1H), 1.54-1.35 (m, 5H), 1.33-1.21 (m, 16H), 1.02-0.81 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 173.49, 158.70, 147.46, 129.73, 60.05, 47.94, 42.73, 42.69, 40.42, 31.87, 31.35, 30.47, 29.64,

29.60, 29.52, 29.32, 27.06, 24.66, 22.75, 22.64, 22.25, 19.52, 18.82, 14.06. HRMS calcd. for $[C_{24}H_{48}N_3O_4S_1]^+$ 474.33600, found 474.33595.

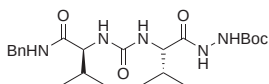
***t*BuO-Val-urea-Val-NHNH₂ (197).** Methyl ester **196**¹⁹³ (234 mg, 708 μ mol, 1 equiv.) was dissolved in MeOH. Hydrazine hydrate (1 ml, 21 mmol, 30 equiv.) was added and the mixture refluxed overnight. Coevaporation with toluene (4 $\times$) yielded the title compound which was used without further purification. ¹H NMR (400 MHz, CD₃OD): δ 6.41 (dd, $J_1 = 12.3$, $J_2 = 9.1$ Hz, 1H), 4.17-4.00 (m, 1H), 3.99-3.84 (m, 1H), 2.06 (dt, $J_1 = 12.1$, $J_2 = 6.8$ Hz, 1H), 2.02-1.89 (m, 1H), 1.46 (s, 9H), 1.01-0.85 (m, 12H). ¹³C NMR (100 MHz, CD₃OD): δ 173.87, 173.48, 160.37, 82.45, 60.25, 60.18, 59.27, 59.19, 32.58, 32.11, 28.32, 28.21, 19.67, 19.59, 18.53, 18.05.



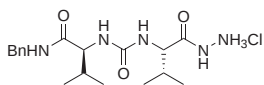
***t*BuO-Val-urea-Val-Leu-EK₂ (182).** Prepared by the general procedure for azide coupling employing **197** (33 mg, 100 μ mol, 1 equiv.) and TFA·H-Leu-EK (31 mg, 110 μ mol, 1.1 equiv.). Purification by column chromatography (DCM $\rightarrow$ 1% MeOH:DCM, 2 $\times$) yielded the title compound (22 mg, 47 μ mol, 47%). LC/MS: R_t 8.93 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1%TFA, 15 min). ¹H NMR (400 MHz, CDCl₃): δ ppm 6.49 (d, $J = 7.55$ Hz, 1H), 5.51 (d, $J = 8.52$ Hz, 1H), 5.39 (d, $J = 8.82$ Hz, 1H), 4.60 (ddd, $J_1 = 10.60$, $J_2 = 7.72$, $J_3 = 3.23$ Hz, 1H), 4.26 (dd, $J_1 = 8.78$, $J_2 = 4.62$ Hz, 1H), 4.08 (dd, $J_1 = 8.56$, $J_2 = 7.07$ Hz, 1H), 3.36 (d, $J = 4.98$ Hz, 1H), 2.88 (d, $J = 4.97$ Hz, 1H), 2.15-1.93 (m, 2H), 1.76-1.59 (m, 2H), 1.46 (s, 9H), 1.51 (s, 3H), 1.58-1.51 (m, 1H), 0.95-0.82 (m, 18H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 208.44, 172.72, 172.24, 157.72, 81.63, 59.27, 59.11, 58.47, 52.48, 50.41, 39.97, 31.49, 31.18, 29.71, 28.08, 25.13, 23.36, 21.33, 19.27, 18.98, 18.10, 17.65, 16.78. HRMS calcd. for $[C_{24}H_{44}N_3O_6]^+$ 470.32246, found 470.32233.



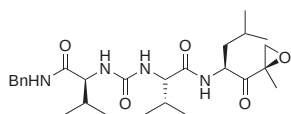
HO-Val-urea-Val-Leu-EK (183). *t*-Bu ester **182** (13 mg, 28 μ mol) was stirred with 2 mL TFA for 1 hr before being coevaporated with toluene (3 $\times$) to yield the title compound quantitatively. LC/MS: R_t 9.05 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1%TFA, 15 min). ¹H NMR (400 MHz, CD₃OD): δ 4.56 (dd, $J_1 = 10.6$, $J_2 = 3.0$ Hz, 1H), 4.20 (d, $J = 4.8$ Hz, 1H), 4.06 (d, $J = 6.6$ Hz, 1H), 3.29 (d, $J = 5.1$ Hz, 1H), 2.95 (d, $J = 5.1$ Hz, 1H), 2.16 (dq, $J_1 = 13.6$, $J_2 = 6.8$ Hz, 1H), 2.11-1.90 (m, 1H), 1.84-1.22 (m, 3H), 1.48 (s, 3H), 1.16-0.78 (m, 18H). ¹³C NMR (100 MHz, CD₃OD): δ 209.58, 176.05, 174.89, 160.41, 60.09, 59.98, 59.39, 53.06, 51.79, 40.25, 32.45, 32.03, 26.25, 23.75, 21.47, 19.79, 19.71, 18.24, 17.86, 17.04. HRMS calcd. for $[C_{20}H_{36}N_3O_6]^+$ 414.25986 found 414.25989.



Benzylamide-Val-urea-Val-NHNHBoc (198). A solution of H-Val-NHNHBoc²⁰⁶ (260 mg, 1.12 mmol, 1.2 equiv.) and DiPEA (203 μ l, 1.23 mmol, 1.2 equiv.) in DCM was added to a solution of valine benzylamide isocyanate (prepared from HCl·H-Val-NHBn (229 mg, 943 μ mol, 1 equiv.) using general procedure B) in DCM. The resulting mixture was stirred for 2 hr. The mixture was washed with 1M HCl (2 $\times$) and brine and dried over MgSO₄. Column chromatography (DCM $\rightarrow$ 5% MeOH:DCM) yielded the title compound (230 mg, 496 μ mol, 53%). LC/MS: R_t 7.08 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1%TFA, 15 min), (ESI-MS (m/z): 464.00 (M + H)⁺). ¹H NMR (400 MHz, CD₃OD, CDCl₃): δ 7.89 (t, $J = 5.7$ Hz, 1H), 7.37-7.18 (m, 5H), 4.43-4.29 (m, 2H), 4.02 (d, $J = 6.5$ Hz, 1H), 3.96 (d, $J = 6.7$ Hz, 1H), 2.19-1.91 (m, 2H), 1.44 (s, 9H), 1.00-0.83 (m, 12H). ¹³C NMR (100 MHz, CD₃OD, CDCl₃): δ 172.70, 158.11, 137.59, 127.78, 126.82, 126.52, 58.93, 57.01, 42.46, 30.72, 30.39, 27.16, 18.37, 18.27, 16.97.

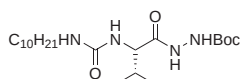


Benzylamide-Val-urea-Val-NHNH₃Cl (199). **198** (250 mg, 600 μ mol) was stirred with 4 ml 4M HCl in dioxane for 1 hr before coevaporation with toluene (3 $\times$), yielding the title compound which was used without purification. LC/MS: R_t 4.87 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1%TFA, 15 min), (ESI-MS (m/z): 364.00 (M - Cl)⁺). ¹H NMR (400 MHz, DMSO): δ 9.26 (t, $J = 5.9$ Hz, 1H), 8.31-7.77 (m, 5H), 7.31 (d, $J = 8.2$ Hz, 1H), 7.18 (d, $J = 9.1$ Hz, 1H), 5.11 (t, $J = 6.3$ Hz, 1H), 4.98-4.74 (m, 1H), 2.90-2.54 (m, 2H), 1.98-1.45 (m, 12H).



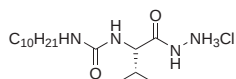
Benzylamide-Val-urea-Val-Leu-EK (184). Prepared by the general procedure for azide coupling employing **199** (39 mg, 100 μ mol, 1 equiv.) and TFA·H-Leu-EK (31 mg, 110 μ mol, 1.1 equiv.). Purification by column chromatography (DCM $\rightarrow$ 3% MeOH:DCM) yielded the title compound (10 mg, 20 μ mol, 20%). LC/MS: R_t 8.41 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, CDCl_3 : CD_3OD): δ 7.43-7.18 (m,

5H), 4.54 (dd, $J_1 = 10.6$, $J_2 = 3.1$ Hz, 1H), 4.37 (dd, $J_1 = 36.1$, $J_2 = 14.9$ Hz, 2H), 4.04 (dd, $J_1 = 6.7$, $J_2 = 4.9$ Hz, 2H), 3.29 (d, $J = 5.0$ Hz, 1H), 2.91 (d, $J = 5.1$ Hz, 1H), 2.09-1.93 (m, 2H), 1.80-1.64 (m, 1H), 1.48 (s, 3H), 1.57-1.21 (m, 1H), 0.99-0.85 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3 : CD_3OD): δ 208.39, 173.42, 173.29, 158.71, 138.24, 128.21, 127.28, 126.93, 59.28, 58.85, 58.71, 51.96, 50.41, 42.84, 39.06, 31.08, 31.05, 24.94, 22.69, 20.49, 18.74, 18.71, 17.30, 16.05. HRMS calcd. for $[\text{C}_{27}\text{H}_{43}\text{N}_4\text{O}_5]^+$ 503.32280, found 503.32271.



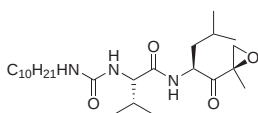
Decyl-urea-Val-NHNHBoc (200). A solution of H-Val-NHNHBoc²⁰⁶ (237 mg, 1.02 mmol, 1 equiv.) and DiPEA (185 μ l, 1.12 mmol, 1.1 equiv.) in DCM was added to a solution of decyl isocyanate (prepared from decylamine (213 μ l, 1.07 mmol, 1.05 equiv.) using general procedure A) in DCM. The resulting mixture was stirred for 1 hr. The mixture was washed with 1M HCl (2 $\times$) and brine and dried over MgSO_4 .

Column chromatography (PE $\rightarrow$ 40% acetone:PE) yielded the title compound (250 mg, 600 μ mol, 60%). LC/MS: R_t 10.23 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 415.00 ($\text{M} + \text{H}^+$)). ^1H NMR (400 MHz, CD_3OD : CDCl_3): δ 6.03 (d, $J = 8.8$ Hz, 1H), 5.98 (t, $J = 5.2$ Hz, 1H), 5.56 (t, $J = 5.1$ Hz, 1H), 4.07 (s, 1H), 3.22-2.99 (m, 2H), 2.09-2.00 (m, 1H), 1.47 (s, 9H), 1.34-1.20 (m, 16H), 0.98 (dd, $J_1 = 14.5$, $J_2 = 6.8$ Hz, 6H), 0.88 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD , CDCl_3): δ 172.75, 158.61, 57.04, 39.53, 31.38, 30.71, 29.71, 29.61, 29.10, 29.06, 28.89, 28.80, 27.43, 26.40, 22.12, 18.52, 17.27, 13.36.

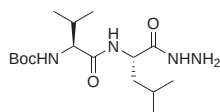


Decyl-urea-Val-NHNH₃Cl (201). **200** (250 mg, 600 μ mol) was stirred with 4 ml 4M HCl in dioxane for 1 hr before coevaporation with toluene (3 $\times$) yielded the title compound which was used without purification. LC/MS: R_t 7.39 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 315.00 ($\text{M} - \text{Cl}^-$)). ^1H NMR (400 MHz, CD_3OD : CDCl_3): δ 4.06 (d, $J = 6.2$ Hz, 1H), 3.28-3.01 (m, 2H), 2.17-2.05 (m, 1H), 1.56-1.44 (m, 2H), 1.41-1.14 (m, 14H), 0.99 (t, $J = 7.1$ Hz, 6H),

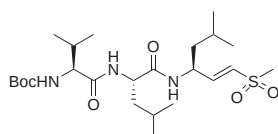
0.89 (t, $J = 6.8$ Hz, 3H).



Decyl-urea-Val-Leu-EK (185). Prepared by the general procedure for azide coupling employing **201** (53 mg, 151 μ mol, 1 equiv.) and TFA·H-Leu-EK (47 mg, 166 μ mol, 1.1 equiv.). Purification by column chromatography (DCM $\rightarrow$ 3% MeOH:DCM and 10% EA:Tol. $\rightarrow$ 30% EA:Tol.) yielded the title compound (24 mg, 53 μ mol, 35%). LC/MS: R_t 11.18 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, CDCl_3): δ 6.84 (d, $J = 7.4$ Hz, 1H), 5.56 (d, $J = 8.5$ Hz, 1H), 5.03 (t, $J = 5.6$ Hz, 1H), 4.71-4.43 (m, 1H), 4.10 (t, $J = 7.8$ Hz, 1H), 3.35 (d, $J = 5.0$ Hz, 1H), 3.25-3.00 (m, 2H), 2.89 (d, $J = 5.0$ Hz, 1H), 2.10-1.85 (m, 1H), 1.52 (s, 3H), 1.73-1.36 (m, 3H), 1.40-1.16 (m, 16H), 0.97-0.80 (m, 15H). ^{13}C NMR (100 MHz, CDCl_3): δ 208.37, 173.26, 158.22, 59.27, 59.15, 52.41, 50.56, 40.75, 40.57, 39.63, 31.88, 31.40, 30.28, 30.20, 29.68, 29.57, 29.40, 29.34, 29.31, 26.95, 25.16, 23.33, 22.66, 21.29, 19.19, 18.23, 16.78, 14.07. HRMS calcd. for $[\text{C}_{25}\text{H}_{48}\text{N}_3\text{O}_4]^+$ 454.36393, found 454.36392.

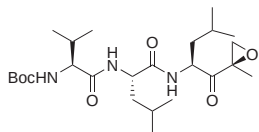


Boc-Val-Leu-NHNH₂ (204). Methyl ester **203**²⁰⁷ was dissolved in 50 ml MeOH and hydrazine hydrate (9.7 ml, 200 mmol, 20 equiv.) was added and the mixture refluxed for 2 hr. The reaction mixture was coevaporated with toluene. (5 $\times$) and the residue used without further purification. ^1H NMR (400 MHz, CD_3OD): δ 4.43 (dd, $J = 9.4$, 5.6 Hz, 1H), 3.86 (d, $J = 7.0$ Hz, 1H), 2.03 (dq, $J = 13.6$, 6.7 Hz, 1H), 1.91-1.49 (m, 3H), 1.48 (s, 9H), 1.10-0.79 (m, 12H).



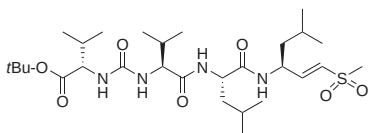
Boc-Val-Leu₂-VS (205). Prepared from **204** (344 mg, 1 mmol, 1 equiv.) and TFA·H-Leu-VS (336 mg, 1.1 mmol, 1.1 equiv.) using the general procedure for azide coupling. Column chromatography (Tol. $\rightarrow$ 80% EA:Tol.) yielded the title compound (362 mg, 719 μ mol, 72%). ^1H NMR (400 MHz, CDCl_3): δ 7.39-7.22 (m, 1H), 6.97 (d, $J = 6.8$ Hz, 1H), 6.85 (dd, $J_1 = 15.1$, $J_2 = 5.3$ Hz,

1H), 6.61 (d, $J = 15.1$ Hz, 1H), 5.45 (d, $J = 6.6$ Hz, 1H), 4.85–4.69 (m, 1H), 4.57–4.42 (m, 1H), 3.92 (t, $J = 6.7$ Hz, 1H), 2.94 (s, 3H), 2.14 (dq, $J_1 = 13.5$, $J_2 = 6.8$ Hz, 1H), 1.76–1.39 (m, 6H), 1.45 (s, 9H), 0.97–0.91 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ 172.02, 171.57, 156.30, 147.44, 129.36, 80.22, 60.69, 52.16, 47.72, 42.76, 42.71, 40.41, 30.09, 28.25, 24.87, 24.61, 22.83, 22.58, 21.91, 19.23, 17.94.



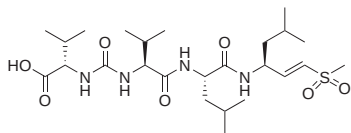
155.86, 79.18, 59.67, 59.13, 52.28, 51.33, 50.09, 41.00, 39.16, 30.99, 28.32, 24.83, 24.39, 23.21, 22.75, 22.32, 19.02, 18.38, 16.66.

Boc-Val-Leu-EK (207). Prepared from **204** (344 mg, 1 mmol, 1 equiv.) and TFA·H-Leu-EK (314 mg, 1.1 mmol, 1.1 equiv.) using the general procedure for azide coupling. Column chromatography (20% EA:Tol. $\rightarrow$ 60% EA:Tol.) yielded the title compound (437 mg, 903 μmol , 90%). ^1H NMR (400 MHz, CDCl_3): δ 7.59 (d, $J = 7.1$ Hz, 1H), 7.44 (d, $J = 6.9$ Hz, 1H), 5.95 (d, $J = 9.0$ Hz, 1H), 4.70–4.45 (m, 2H), 4.05 (t, $J = 8.5$ Hz, 1H), 3.45 (d, $J = 4.2$ Hz, 1H), 2.87 (d, $J = 5.0$ Hz, 1H), 2.09–1.94 (m, 1H), 1.75–1.30 (m, 6H), 1.51 (s, 3H), 1.45 (s, 9H), 0.98–0.82 (m, 18H). ^{13}C NMR (100 MHz, CDCl_3): δ 208.57, 172.02,



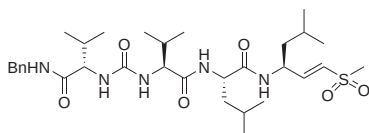
MgSO_4 . Column chromatography (DCM $\rightarrow$ 1% MeOH:DCM) afforded the title compound (42 mg, 70 μmol , 70%). LC/MS: R_t 8.80 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1/1): δ ppm 6.81 (dd, $J_1 = 15.13$, $J_2 = 4.91$ Hz, 1H), 6.62 (dd, $J_1 = 15.14$, $J_2 = 1.36$ Hz, 1H), 4.74–4.63 (m, 1H), 4.39 (t, $J = 7.37$, 1H), 4.04 (d, $J = 5.13$ Hz, 1H), 3.95 (d, $J = 5.78$ Hz, 1H), 2.97 (s, 3H), 2.16–2.03 (m, 2H), 1.73–1.57 (m, 6H), 1.47 (s, 1H), 1.04–0.88 (m, 24H). ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$): δ ppm 173.00, 172.27, 171.43, 158.72, 146.50, 128.55, 80.62, 59.40, 58.16, 51.38, 47.07, 41.18, 41.06, 39.34, 29.89, 29.78, 26.57, 23.94, 23.84, 21.59, 21.55, 20.09, 19.95, 17.97, 17.80, 16.43, 16.37. HRMS calcd. for $[\text{C}_{29}\text{H}_{55}\text{N}_4\text{O}_7\text{S}]^+$ 603.37860, found 603.37866.

tBuO-Val-urea-Val-Leu-VS (186). **205** (50 mg, 100 μmol , 1 equiv.) was treated with 1:1 TFA:DCM for 30 minutes, coevaporated with toluene (3 $\times$). The residue was dissolved in DCM and DiPEA (40 μmol , 244 μmol , 2.4 equiv.) was added and this mixture was added to a solution of valine *tert*-butyl ester isocyanate (prepared from HCl·H-Val-OtBu (23 mg, 110 μmol , 1.1 equiv.) using general procedure A). The resulting mixture was stirred for 30 minutes before being washed with 1M HCl and sat. aq. NaHCO_3 and dried over



175.91, 175.12, 174.46, 160.92, 148.35, 130.84, 61.26, 59.51, 53.42, 49.16, 43.26, 42.83, 41.44, 32.06, 31.83, 25.96, 25.84, 23.42, 21.91, 21.84, 19.85, 19.74, 18.34, 18.08. HRMS calcd. for $[\text{C}_{25}\text{H}_{46}\text{N}_4\text{O}_7\text{S}]^+$ 547.31600, found 547.31610.

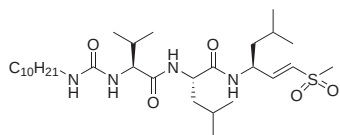
HO-Val-urea-Val-Leu-VS (187). *t*-Bu ester **186** (21 mg, 35 μmol) was stirred with 2 ml TFA for 1 hr before being coevaporated with toluene (3 $\times$) to yield the title compound quantitatively. ^1H NMR (400 MHz, $\text{CD}_3\text{OD}:\text{CDCl}_3$): δ ppm 7.94 (d, $J = 8.3$ Hz, 1H), 7.41 (d, $J = 7.5$ Hz, 1H), 6.82 (dd, $J_1 = 15.1$, $J_2 = 4.8$ Hz, 1H), 6.59 (dd, $J_1 = 15.1$, $J_2 = 1.5$ Hz, 1H), 4.78–4.64 (m, 1H), 4.45–4.31 (m, 1H), 4.22 (d, $J = 4.9$ Hz, 1H), 3.91 (d, $J = 5.6$ Hz, 1H), 2.98 (s, 3H), 2.29–2.02 (m, 2H), 1.76–1.36 (m, 6H), 1.10–0.80 (m, 24H). ^{13}C NMR (100 MHz, CD_3OD): δ



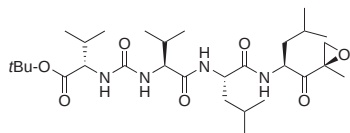
175.91, 175.12, 174.46, 160.92, 148.35, 130.84, 61.26, 59.51, 53.42, 49.16, 43.26, 42.83, 41.44, 32.06, 31.83, 25.96, 25.84, 23.42, 21.91, 21.84, 19.85, 19.74, 18.34, 18.08. HRMS calcd. for $[\text{C}_{25}\text{H}_{46}\text{N}_4\text{O}_7\text{S}]^+$ 547.31600, found 547.31610.

Benzylamide-Val-urea-Val-Leu-VS (188). **205** (51 mg, 101 μmol , 1 equiv.) was treated with 1:1 TFA:DCM for 30 minutes, coevaporated with toluene (3 $\times$). The residue was dissolved in DCM and DiPEA (37 μl , 222 μmol , 2.2 equiv.) was added and this mixture was added to a solution of valine benzylamide isocyanate (prepared from HCl·H-Val-NHBn (35 mg, 144 μmol , 1.4 equiv.) using general procedure B). The resulting mixture was stirred overnight. Upon quenching with H_2O the title compound precipitated as a white

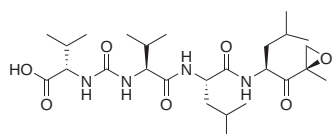
41.78, 41.68, 39.81, 30.97, 30.57, 24.29, 24.06, 22.07, 21.97, 21.01, 20.83, 18.53, 18.47, 17.40, 17.32. HRMS calcd. for $[C_{32}H_{54}N_5O_6S]^+$ 636.37893, found 636.37909.



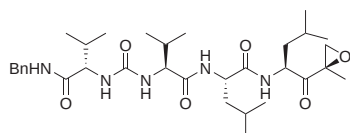
Decyl-urea-Val-Leu₂-VS (189). 205 (50 mg, 100 μ mol, 1 equiv.) was treated with 1:1 TFA:DCM for 30 minutes, coevaporated with toluene (3 $\times$). The residue was dissolved in DCM and DiPEA (36 μ mol, 220 μ mol, 2.2 equiv.) was added and this mixture was added to a solution of decyl isocyanate (prepared from decylamine (24 mg, 160 μ mol, 1.6 equiv.) using general procedure A). The resulting mixture was stirred for 3 hr before being washed with 1M HCl (3 $\times$) and H₂O (3 $\times$) and dried over Na₂SO₄. Column chromatography (DCM $\rightarrow$ 2% MeOH:DCM) afforded the title compound (25 mg, 43 μ mol, 43%). LC/MS: R_t 10.91 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CD₃OD): δ ppm 6.82 (dd, $J_1 = 15.2$, $J_2 = 4.9$ Hz, 1H), 6.66 (dd, $J_1 = 15.2$, $J_2 = 1.5$ Hz, 1H), 4.74-4.66 (m, 1H), 4.42 (dd, $J_1 = 9.6$, $J_2 = 5.3$ Hz, 1H), 3.97 (d, $J = 5.8$ Hz, 1H), 3.23-3.06 (m, 2H), 3.00 (s, 3H), 2.16-2.02 (m, 1H), 1.80-1.57 (m, 5H), 1.54-1.43 (m, 3H), 1.39-1.27 (m, 14H), 1.08-0.87 (m, 21H). ¹³C NMR (100 MHz, CD₃OD): δ 175.43, 174.52, 161.13, 148.39, 130.83, 61.47, 53.45, 43.22, 42.81, 41.27, 41.06, 33.07, 31.87, 31.30, 30.76, 30.70, 30.52, 30.46, 28.02, 26.01, 25.86, 23.74, 23.47, 23.42, 21.90, 21.69, 19.85, 18.30, 14.45. HRMS calcd. for $[C_{30}H_{59}N_4O_5S]^+$ 587.42007, found 587.42017.



tBuO-Val-urea-Val-Leu₂-EK (190). 207 (41 mg, 85 μ mol, 1 equiv.) was treated with 1:1 TFA:DCM for 30 minutes, coevaporated with toluene (3 $\times$). The residue was dissolved in DCM and DiPEA (31 μ l, 187 μ mol, 2.2 equiv.) was added and this mixture was added to a solution of valine *tert*-butyl ester isocyanate (prepared from HCl-H-Val-OtBu (27 mg, 128 μ mol, 1.6 equiv.) using general procedure A). The resulting mixture was stirred overnight. The reaction mixture was washed with 1M HCl (3 $\times$), H₂O (3 $\times$) and dried over Na₂SO₄. Column chromatography (DCM $\rightarrow$ 2% MeOH:DCM) yielded the title compound (14 mg, 24 μ mol, 28%). LC/MS: R_t 9.84 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CDCl₃): δ ppm 8.02 (s, 1H), 6.49 (d, $J = 7.5$ Hz, 1H), 6.19 (s, 1H), 4.74-4.60 (m, 1H), 4.53 (dd, $J_1 = 14.7$, $J_2 = 7.4$ Hz, 1H), 4.38 (dd, $J_1 = 9.2$, $J_2 = 4.9$ Hz, 1H), 4.25 (t, $J = 8.3$ Hz, 1H), 3.41 (d, $J = 4.8$ Hz, 1H), 2.87 (d, $J = 4.8$ Hz, 1H), 2.09-1.95 (m, 1H), 1.90-1.30 (m, 7H), 1.52 (s, 3H), 1.49 (s, 1H), 1.01-0.74 (m, 24H). ¹³C NMR (100 MHz, CDCl₃): δ ppm 173.37, 172.83, 171.92, 157.73, 81.41, 59.13, 58.85, 58.11, 52.56, 52.01, 50.32, 40.96, 39.12, 32.42, 31.96, 28.11, 24.92, 24.46, 23.37, 23.09, 22.17, 21.09, 19.25, 18.99, 18.74, 18.07, 16.89. HRMS calcd. for $[C_{30}H_{55}N_4O_7]^+$ 583.40653 found 583.40668.

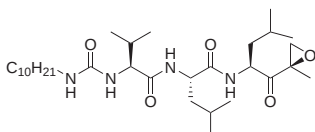


HO-Val-urea-Val-Leu₂-EK (191). *t*-Bu ester 190 (11 mg, 19 μ mol) was stirred with 1 ml TFA for 1 hr and coevaporated with toluene (3 $\times$) to yield the title compound quantitatively. ¹H NMR (400 MHz, CD₃OD): δ 4.54 (dd, $J_1 = 10.7$, $J_2 = 3.0$ Hz, 1H), 4.47 (dd, $J_1 = 8.7$, $J_2 = 6.3$ Hz, 1H), 4.20 (d, $J = 4.6$ Hz, 1H), 4.05 (d, $J = 6.4$ Hz, 1H), 3.27 (d, $J = 5.1$ Hz, 1H), 2.94 (d, $J = 5.1$ Hz, 1H), 2.24-2.12 (m, 1H), 2.10-1.97 (m, 1H), 1.82-1.26 (m, 6H), 1.48 (s, 3H), 1.18-0.58 (m, 24H). ¹³C NMR (100 MHz, CD₃OD): δ 171.60, 61.96, 57.29, 56.33, 50.10, 49.65, 48.90, 39.10, 37.12, 29.42, 29.06, 23.26, 22.75, 20.82, 20.41, 19.27, 18.45, 16.90, 16.77, 15.26, 14.91, 14.10. HRMS calcd. for $[C_{26}H_{47}N_4O_7]^+$ 527.34393 found 527.34387.

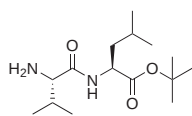


Benzylamide-Val-urea-Val-Leu₂-EK (192). 207 (50 mg, 101 μ mol, 1 equiv.) was treated with 1:1 TFA:DCM for 30 minutes, coevaporated with toluene (3 $\times$). The residue was dissolved in DCM and DiPEA (37 μ l, 222 μ mol, 2.2 equiv.) was added and this mixture was added to a solution of valine benzylamide isocyanate (prepared from HCl-H-Val-NHBn (34 mg, 144 μ mol, 1.4 equiv.) using general procedure B). The resulting mixture was stirred for 3 hr. The reaction mixture was washed with H₂O (3 $\times$) and dried over Na₂SO₄. Column chromatography (DCM $\rightarrow$ 4% MeOH:DCM) followed by crystallisation (DCM:PE) yielded the title compound (25 mg, 40 μ mol, 40%). LC/MS: R_t 8.51 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CD₃OD): δ 7.35-7.18 (m, 5H), 6.57 (dd, $J_1 = 8.9$, $J_2 = 6.1$ Hz, 1H), 4.58-4.49 (m, 2H), 4.44 (d, $J = 15.0$ Hz, 1H),

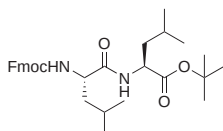
4.33 (d, $J = 14.9$ Hz, 1H), 4.18-4.04 (m, 2H), 3.25 (d, $J = 5.0$ Hz, 1H), 2.89 (d, $J = 5.0$ Hz, 1H), 2.05-1.88 (m, 2H), 1.77-1.24 (m, 6H), 1.48 (s, 3H), 0.99-0.83 (m, 24H). ^{13}C NMR (100 MHz, CD_3OD , CDCl_3): δ 207.71, 172.54, 172.42, 172.22, 157.96, 137.61, 127.50, 126.59, 126.20, 58.39, 58.15, 58.08, 51.24, 50.56, 49.71, 42.09, 39.95, 38.11, 30.74, 30.69, 24.17, 23.68, 22.01, 21.45, 20.82, 19.69, 18.06, 16.87, 16.79, 15.34. HRMS calcd. for $[\text{C}_{33}\text{H}_{54}\text{N}_5\text{O}_6]^+$ 616.40686 found 616.40698.



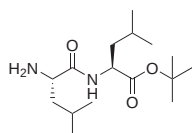
Decyl-urea-Val-Leu₂-EK (193). 207 (49 mg, 101 μmol , 1 equiv.) was treated with 1:1 TFA:DCM for 30 minutes, coevaporated with toluene (3 $\times$). The residue was dissolved in DCM and DiPEA (37 μL , 222 μmol , 2.2 equiv.) was added and this mixture was added to a solution of decyl isocyanate (prepared from decylamine (24 μL , 121 μmol , 1.2 equiv.) using general procedure A). The resulting mixture was stirred overnight. The reaction mixture was washed with 1M HCl (3 $\times$), H_2O (3 $\times$) and dried over Na_2SO_4 . Column chromatography (DCM $\rightarrow$ 2% MeOH:DCM) yielded the title compound (16 mg, 29 μmol , 29%). LC/MS: R_t 11.30 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, CD_3OD , CDCl_3): δ ppm 4.55-4.51 (m, 1H), 4.43 (dd, $J_1 = 8.9$, $J_2 = 5.5$ Hz, 1H), 4.03 (d, $J = 6.5$ Hz, 1H), 3.29 (d, $J = 5.0$ Hz, 1H), 3.20-2.99 (m, 2H), 2.90 (d, $J = 5.0$ Hz, 1H), 2.11-1.92 (m, 1H), 1.77-1.32 (m, 6H), 1.49 (s, 3H), 1.39-1.10 (m, 16H), 1.05-0.78 (m, 21H). ^{13}C NMR (100 MHz, CDCl_3 : CD_3OD): δ 208.04, 172.86, 172.33, 158.66, 58.57, 58.53, 51.76, 50.85, 49.99, 40.15, 39.50, 38.69, 31.34, 30.60, 29.65, 29.60, 29.04, 29.00, 28.84, 28.75, 26.34, 24.55, 24.04, 22.54, 22.07, 22.00, 21.11, 20.28, 18.50, 17.02, 15.97, 13.25. HRMS calcd. for $[\text{C}_{25}\text{H}_{47}\text{N}_4\text{O}_7\text{S}]^+$ 547.31600 found 547.31610.



H-Val-Leu-OtBu (210). Fmoc-Val-Leu-OtBu²⁰⁸ (956 mg, 1.88 mmol, 1 equiv.) was dissolved in THF and treated with EtSH (1.4 mL, 18.8 mmol, 10 equiv.) and DBU (28 μL , 188 μmol , 0.1 equiv.) for 2 hr and coevaporated with toluene. Column chromatography (EA:PE:MeOH 50:50:0 $\rightarrow$ 100:0:0 $\rightarrow$ 95:0:5) yielded the title compound (524 mg, 1.83 mmol, 97%). ^1H NMR (400 MHz, CDCl_3): δ 7.71 (d, $J = 8.5$ Hz, 1H), 4.57-4.42 (m, 1H), 3.27 (d, $J = 4.0$ Hz, 1H), 2.35-2.19 (m, 1H), 2.05 (s, 2H), 1.80-1.49 (m, 3H), 1.46 (s, 9H), 0.99 (d, $J = 7.0$ Hz, 3H), 0.97-0.89 (m, 6H), 0.85 (d, $J = 6.9$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ 173.83, 171.91, 81.09, 59.76, 50.58, 41.23, 30.57, 27.57, 24.56, 22.48, 21.51, 19.23, 15.87.

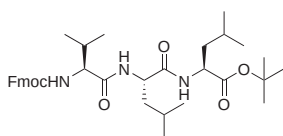


Fmoc-Leu₂-OtBu (211). Leucine *tert*-butyl ester (382 mg, 2.04 mmol, 1 equiv.) was dissolved in DCM and Fmoc-Leu-OH (757 mg, 2.14 mmol, 1.05 equiv.), HBTU (890 mg, 2.35 mmol, 1.15 equiv.) and DiPEA (759 μL , 4.59 mmol, 2.25 equiv.) were added and the mixture was stirred for 2 hr before being concentrated. The residue was taken up in EA and washed with 1M HCl (2 $\times$), sat. aq. NaHCO_3 (4 $\times$) and brine before drying over MgSO_4 . Column chromatography (Tol. $\rightarrow$ 16% EA:Tol.) afforded the title compound (1.09 gr, 2.04 mmol, quant.). ^1H NMR (400 MHz, CDCl_3): δ 7.70 (d, $J = 7.5$ Hz, 2H), 7.56 (t, $J = 7.0$ Hz, 2H), 7.40-7.29 (m, 2H), 7.23 (t, $J = 7.0$ Hz, 2H), 7.13 (d, $J = 7.6$ Hz, 1H), 7.02 (d, $J = 7.9$ Hz, 1H), 5.99 (d, $J = 8.7$ Hz, 1H), 4.49 (dd, $J_1 = 13.8$, $J_2 = 7.9$ Hz, 1H), 4.43-4.33 (m, 2H), 4.32-4.24 (m, 1H), 4.20-4.13 (m, 1H), 1.80-1.46 (m, 6H), 1.43 (s, 9H), 0.98-0.83 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ 172.11, 171.62, 156.05, 143.65, 143.50, 140.97, 127.38, 126.79, 124.89, 119.64, 81.33, 66.80, 53.11, 51.27, 46.84, 41.53, 41.18, 27.69, 24.60, 24.38, 22.77, 22.44, 21.88.

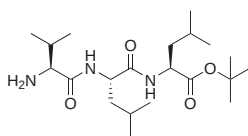


H-Leu₂-OtBu (212). Fmoc-Leu₂-OtBu 211 (1.09 gr, 2.04 mmol, 1 equiv.) was dissolved in THF and EtSH (1.5 mL, 20 mmol, 10 equiv.) and DBU (30 μL , 200 μmol , 0.1 equiv.) were added and the mixture was stirred for 1.5 hr. before being coevaporated with toluene. Column chromatography (EA:PE:MeOH 50:50:0 $\rightarrow$ 100:0:0 $\rightarrow$ 95:0:5) yielded the title compound (586 mg, 1.95 mmol, 93%). ^1H NMR (400 MHz, CDCl_3): δ 7.71 (d, $J = 8.6$ Hz, 1H), 4.46 (dt, $J_1 = 8.7$, $J_2 = 5.2$ Hz, 1H), 3.42 (dd, $J_1 = 9.6$, $J_2 = 4.2$ Hz, 1H), 1.87 (s, 2H), 1.81-1.29 (m, 6H), 1.46 (s, 9H), 1.07-0.81 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ 175.06, 171.77, 80.92, 53.03, 50.39, 43.69, 41.17, 27.46, 24.42, 24.28, 22.93, 22.39, 21.53,

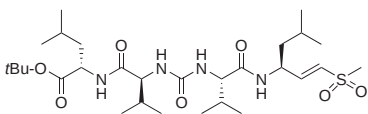
20.95.



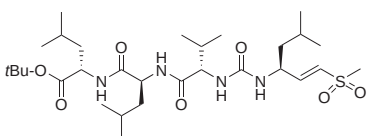
Fmoc-Val-Leu₂-OtBu (213). H-Leu-Leu-OtBu (212), 586 mg, 1.95 mmol, 93%), Fmoc-Val-OH (695 mg, 2.05 mmol, 1.05 equiv.), HBTU (850 mg, 2.24 mmol, 1.15 equiv.) and DiPEA (725 μ l, 4.39 mmol, 2.25 equiv.) were dissolved in DCM and stirred for 1.5 hr. before being washed with 1M HCl (2 $\times$), sat. aq. NaHCO₃ (4 $\times$) and dried over MgSO₄. Column chromatography (EA:Tol. 0 $\rightarrow$ 30%) yielded the title compound (1.15 gr, 1.85 mmol, 95%). ¹H NMR (400 MHz, CDCl₃): δ 7.73 (d, J = 7.5 Hz, 2H), 7.64-7.52 (m, 2H), 7.37 (dd, J_1 = 9.5, J_2 = 5.1 Hz, 2H), 7.30-7.22 (m, 2H), 7.15 (d, J = 8.0 Hz, 1H), 6.96 (d, J = 8.2 Hz, 1H), 6.11 (d, J = 9.1 Hz, 1H), 4.66 (dd, J_1 = 15.1, J_2 = 7.5 Hz, 1H), 4.53 (dd, J_1 = 13.5, J_2 = 8.5 Hz, 1H), 4.44 (dd, J_1 = 10.1, J_2 = 7.3 Hz, 1H), 4.30-4.22 (m, 1H), 4.19 (t, J = 7.2 Hz, 1H), 4.12 (t, J = 8.2 Hz, 1H), 2.15-1.99 (m, 1H), 1.77-1.46 (m, 6H), 1.43 (s, 9H), 0.94 (t, J = 6.4 Hz, 6H), 0.86 (dd, J_1 = 10.1, J_2 = 6.2 Hz, 12H). ¹³C NMR (100 MHz, CDCl₃): δ 171.88, 171.45, 171.35, 156.47, 143.82, 141.20, 127.60, 126.99, 125.21, 125.13, 119.86, 81.70, 67.07, 60.31, 51.58, 51.29, 47.09, 41.53, 41.31, 31.46, 27.91, 24.74, 24.60, 22.83, 22.52, 21.96, 19.05, 18.21.



H-Val-Leu₂-OtBu (214). Fmoc-Val-Leu-Leu-OtBu 213 (1.15 gr, 1.85 mmol, 1 equiv.) was dissolved in THF and treated with EtSH (1.37 ml, 18.5 mmol, 1 equiv.) and DBU (28 μ l, 185 μ mol, 0.1 equiv.) for 1.5 hr before being concentrated and purified on column chromatography (EA:PE:MeOH 50:50:0 $\rightarrow$ 100:0:0 $\rightarrow$ 95:0:5) to yield the title compound (672 mg, 1.68 mmol, 91%) ¹H NMR (400 MHz, CDCl₃): δ 7.85 (d, J = 8.8 Hz, 3H), 7.40 (d, J = 7.7 Hz, 3H), 4.74-4.58 (m, 3H), 4.40 (dd, J = 13.9, 7.9 Hz, 3H), 3.23 (d, J = 4.1 Hz, 3H), 2.36-2.18 (m, 3H), 1.74-1.49 (m, 6H), 1.46 (s, 9H), 0.98 (d, J = 6.9 Hz, 3H), 0.95-0.87 (m, 12H), 0.84 (d, J = 6.9 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 174.15, 171.75, 171.41, 80.93, 59.87, 51.25, 50.67, 41.00, 40.87, 30.63, 27.60, 24.46, 24.33, 22.64, 22.31, 21.86, 21.83, 19.31, 15.94.



tBuO-Leu-Val-urea-Val-Leu-VS (216). BocValLeuVS 194 (64 mg, 164 μ mol, 1 equiv.) was stirred in 1:1 TFA:DCM for 30 min. before being coevaporated with toluene (3 $\times$). The residue was dissolved in DCM and treated with the isocyanate of Val-Leu-OtBu (obtained from H-Val-Leu-OtBu 210 (54 mg, 188 μ mol, 1.15 equiv.) employing general procedure B) and DiPEA (60 μ l, 361 μ mol, 2.2 equiv.). The mixture was stirred for 2 hr before being washed with 1M HCl (2 $\times$) and dried over MgSO₄. Column chromatography (1% MeOH:DCM 1 $\rightarrow$ 3% MeOH:DCM) yielded the title compound (54 mg, 90 μ mol, 54%). LC/MS: R_t 9.02 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CD₃OD): δ 6.81 (dd, J_1 = 15.2, J_2 = 5.4 Hz, 1H), 6.68 (d, J = 15.2 Hz, 1H), 6.57-6.39 (m, 1H), 4.83-4.66 (m, 1H), 4.36 (t, J = 7.5 Hz, 1H), 4.15 (t, J = 6.5 Hz, 1H), 4.05 (t, J = 6.4 Hz, 1H), 2.98 (s, 3H), 2.04 (dq, J = 13.0, 6.5 Hz, 2H), 1.71 (td, J_1 = 13.3, J_2 = 6.6 Hz, 2H), 1.67-1.53 (m, 4H), 1.47 (s, 9H), 1.15-0.82 (m, 24H). ¹³C NMR (100 MHz, CD₃OD): δ 174.66, 174.59, 173.20, 148.49, 130.97, 82.56, 60.91, 60.84, 60.00, 52.90, 49.12, 43.34, 42.84, 41.66, 32.63, 32.30, 28.27, 25.91, 25.83, 23.37, 23.29, 22.10, 22.06, 20.00, 18.54, 18.49. HRMS: calcd. for [C₂₉H₅₅N₄O₇S]⁺ 603.37860 found 603.37881



tBuO-Leu₂-Val-urea-Leu-VS (215). TFA:H-Leu-VS (220 μ mol, 1 equiv.) was transformed to its isocyanate employing general procedure A. H-Val-Leu-Leu-OtBu 214 (97 mg, 242 μ mol, 1.1 equiv.) and DiPEA (80 μ l, 484 μ mol, 2.2 equiv.) were dissolved in DCM and added to the isocyanate solution and the resulting mixture was stirred for 2 hr, washed with 1M HCl (2 $\times$) and dried over MgSO₄. Column chromatography (DCM 0 $\rightarrow$ 3% MeOH:DCM) yielded the title compound (113 mg, 183 μ mol, 83%). LC/MS: R_t 9.40 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CDCl₃ CD₃OD): δ 7.93 (t, J = 7.9 Hz, 2H), 6.84 (dd, J_1 = 15.1, J_2 = 5.1 Hz, 1H), 6.53 (d, J = 15.1 Hz, 1H), 6.31 (d, J = 8.0 Hz, 1H), 6.02 (d, J = 8.8 Hz, 1H), 4.55-4.42 (m, 2H), 4.35 (dd, J_1 = 14.5, J_2 = 8.1 Hz, 1H), 4.13-4.03 (m, 1H), 2.98 (s, 3H), 2.09-1.95 (m, 1H), 1.85-1.50 (m, 9H), 1.47 (s, 9H), 1.10-0.77 (m, 24H). ¹³C NMR (100 MHz, CDCl₃ CD₃OD): δ 172.55, 172.15, 171.43, 157.68, 148.96, 127.92, 81.13, 51.14, 50.99, 48.16, 42.58, 41.69, 40.28, 40.11, 30.76, 26.96, 24.12, 23.96, 21.90, 20.96, 20.74, 18.39, 16.92. HRMS: calcd. for [C₃₀H₅₇N₄O₇S]⁺ 617.39425, found 617.39466.

Competition experiments in cell lysate

HEK293T and EL4 cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml

penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin in a 7% CO_2 humidified incubator at 37°C. Cells were harvested, washed 2 $\times$ with PBS and permeated in digitonin lysis buffer (4 $\times$ pellet volume, 50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl_2 , 5 mM DTT, 0.025% digitonin) for 15 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 (Biorad). 15 μg (HEK293T) total protein per experiment in 9 μl lysis buffer was exposed to the inhibitors (10 $\times$ solution in DMSO, 1 μl) for 1 hr. at 37°C prior to incubation with MVB003 (500 nM) for 1 hr. at 37°C. Reaction mixtures were boiled with Laemmli's buffer containing β -mercaptoethanol for 3 min. before being resolved on 12.5% SDS-PAGE. In-gel detection of fluorescently labelled 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 nm, λ_{em} 560 nm).

Competition experiments in living cells

1 $\times 10^6$ HEK cells were seeded and grown overnight. 100 $\times$ stock solution of inhibitor was added to 1 ml medium and the cells were incubated for 4 hr. at 37°C. MVB003 (100 $\times$ stock solution in DMSO, 5 μM end concentration) was added and incubated for 2 hr. at 37°C. Cells were harvested, washed 2 $\times$ with PBS and permeated in digitonin lysis buffer (4 $\times$ pellet volume, 50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl_2 , 5 mM DTT, 0.025% digitonin) for 15 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 (Biorad). 10 μg protein (dilution with lysis buffer) was boiled in Laemmli's sample buffer and resolved as described above. Coomassie Brilliant Blue staining of the gel verified protein loading.

Fluorogenic substrate assay

26S proteasomes were purified from rabbit muscle as described.¹⁶⁹ To determine inhibition of purified proteasomes, proteasome ($\sim 10\text{ng}/\text{ml}$) in assay buffer (50 mM Tris-HCl pH 7.5, 40 mM KCl, 2 mM EDTA, 1 mM DTT, 100 μM ATP, 50 $\mu\text{g}/\text{mL}$ BSA) was incubated with inhibitors for 30 min at 37°C followed by assay of activity with 100 μM fluorogenic substrates Suc-LLVY-AMC (chymotrypsin-like site), Ac-RLR-AMC (trypsin-like site), and Ac-nLPnLD-AMC (caspase-like site). Fluorescence of released AMC was measured (λ_{ex} 350 nm, λ_{em} 430 nm) continuously for 30 min at 37°C. Residual activity was determined from the slope of the reaction progress curves.¹⁴⁷

5

Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: a comparative study*

5.1 Introduction

Activity-based protein profiling (ABPP) has emerged in recent years as a powerful strategy to map enzyme activity in complex biological samples. Generally, an activity-based probe (ABP) consists of an electrophilic trap that reacts covalently and irreversibly with an enzyme active site residue, an enzyme (family) recognition element and a reporter group (biotin, epitope tag and/or fluorophore) to assist in visualisation and/or identification of the modified enzyme(s).^{213–215} ABPP has met with considerable success in the profiling of esterases^{216,217} and proteases^{218–220} and the numerous literature reports that have appeared over the years include examples in which photo-activatable groups are used to target enzymes (for instance, matrix metalloproteases)^{148,221} that do not employ an amino acid side chain functionality as the nucleophile in the hydrolysis of their substrates. In recent years other enzyme families (glycosidases,^{222–224} kinases)^{225–227} were successfully modified using ABPP protocols, pointing towards the general applicability of this strategy. Next to visualisation and identification of enzyme activities, ABPP has proven its merit in the assessment of potency and specificity of enzyme inhibitors in cell extracts and living cells.^{204,228} In these studies, cells or cell extract has first been treated with a prospective inhibitor, for instance designed to act against a specific member of an enzyme family, after which a broad-spectrum (ABP) aimed at the enzyme family at large has been added to the sample to visualise remaining enzyme activities.²²⁹ A specific class of ABPs and subject of this Chapter are those that employ bioorthogonal chemistry as a means to introduce the visu-

*W. A. van der Linden, N. Li, S. Hoogendoorn, M. Ruben, M. Verdoes, J. Guo, G.-J. Boons, G. A. van der Marel, B. I. Florea, H. S. Overkleef, *Bioorg. Med. Chem.* 2011, in press.

alisation/identification tag. Inspired by the cell-surface glycoprotein engineering work of Bertozzi and co-workers, who employed a Staudinger ligation handle to tag azide-modified sialic acid residues,¹⁷⁶ a protocol was developed in which the three proteasome catalytic activities have first been modified with a pan-reactive, cell permeable inhibitor. Next, the cells were lysed, the azides modified with a Staudinger-Bertozzi reagent and the proteins resolved by SDS-PAGE. In this fashion proteasome active sites could be modified with high efficiency and with relatively little background labelling.¹⁰⁸ In a later study it was revealed that Staudinger-Bertozzi ligation could be achieved with complete conversion of all azides, but that a large excess of the Staudinger-Bertozzi reagent is needed.¹¹¹ Cravatt and co-workers, in a study on esterase ABPP, demonstrated that essentially the same objective could be reached by making use of copper(I)-catalyzed azide-alkyne cycloaddition 'click' ligation.²³⁰ Two-step ABPP is now recognized as an attractive alternative for direct ABPP especially in cases where the tag interferes with enzyme recognition (such as where the enzyme/enzyme family at hand is rather specific with respect to the nature of its substrate/substrate analogue) or hampers bioavailability of the probe. In part for this reason several research groups worldwide are involved in the development and application of new, more efficient bioorthogonal ligation strategies. Two main developments in the field are the evaluation of the Diels-Alder ligation,^{231,232} with major advances especially in the use of inverse-electron-demand Diels-Alder reactions,^{233–235} and in the application of cyclooctynes in copper-free, strain-promoted click ligations.^{236–242} In this chapter the efficiency and selectivity of three recently reported cyclooctyne derivatives are compared in the two-step bioorthogonal ABPP of proteasome activities in cell extracts and in living cells, set against Staudinger-Bertozzi ligation as the benchmark.

5.2 Results and Discussion

The panel of reagents employed in this study is depicted in Figure 5.1. The strategy is based on previous work¹¹¹ in which an azide-modified BODIPY containing proteasome probe was employed, effectively a direct ABPP and two-step ABPP in one. After proteasome labelling, modification of the azide with a bioorthogonal tag can be monitored in two ways, either by fluorescence read-out and looking for a mobility-shift of the modified proteasome active sites or by biotin-streptavidin blotting if the bioorthogonal tag is equipped with a biotin. This experimental setup allowed the observation that Staudinger-Bertozzi phosphane **154** can be used to quantitatively tag all azide-modified proteasome active sites.¹¹¹ As a positive control in these studies biotin-BODIPY modified proteasome probe was employed. For the purpose of this study azido-BODIPY-epoxomicin **50**, biotin-BODIPY-epoxomicin **217**,¹¹⁴ biotin-cyclooctyne derivatives **218** (DIBO),²⁴² **219** (BCN)²⁴³ and **220** (MFCO)²⁴⁴ were used. Cyclooctyne derivatives **218–220** were selected for the two reasons, namely: a) they have proven their merit in extracellular bioorthogonal ligation and b) their synthesis has been well described.

As the first research objective (Figure 5.2) the labelling efficiency of the three strain-induced click reagents was determined to label azide modified proteasome active sites in cell extracts. To this end, lysates derived from human embryonic kidney (HEK293T) cells that express the constitutive proteasome as the single proteasome particle, were treated with azido-BODIPY-epoxomicin (**50**) at 5 μ M final concentration. As can be seen (for instance, Figure 5.2A lane 2) three bands are apparent after SDS-PAGE separation of the proteome

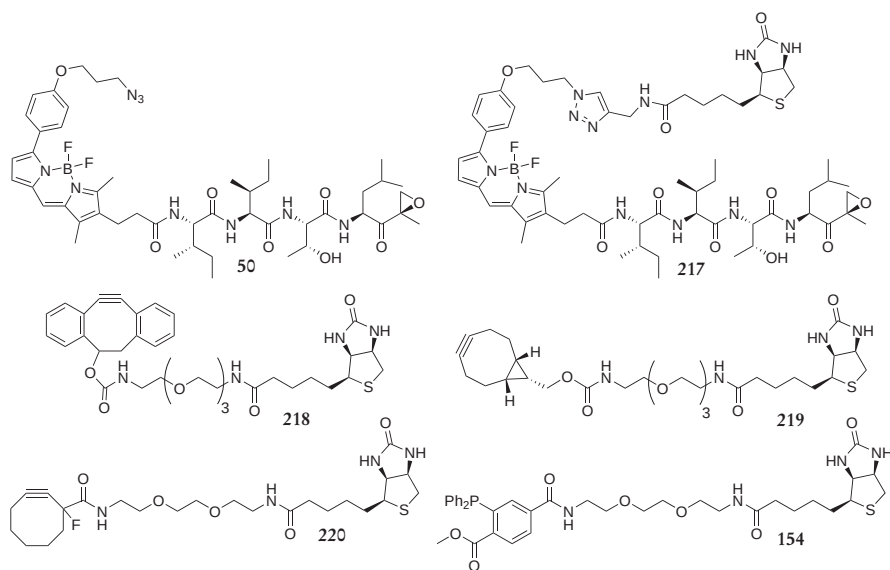


Figure 5.1: Structures of the compounds used in this study.

content followed by fluorescence read-out obtained by scanning the wet gel slabs using a Typhoon fluorescence scanner. Based on previous work the three bands were assigned to the constitutive proteasome active sites, $\beta 1$, $\beta 2$ and $\beta 5$, as indicated in Figure 5.2A.^{110,111} Biotin-cyclooctynes **218** (Figure 5.2A), **219** (Figure 5.2B) and **220** (Figure 5.2C) were added to the cell extracts to final concentrations ranging from 0.5 to 100 μM and incubated at 37°C for 1 hr. Proteasome active subunits labelled with **50** appear at a different position on the SDS-PAGE gel than subunits in which the azide of **50** has reacted with a two-step labelling reagent. This gel mobility shift indicates that all azides are (almost) quantitatively modified within the mentioned concentration range for all three cyclooctyne derivatives. The fully and bioorthogonally modified proteasome active sites now appear at a position approximating that of proteasome active sites labelled with the dual ABPP **217** (compare Figure 5.2A lane 1 versus lane 2).

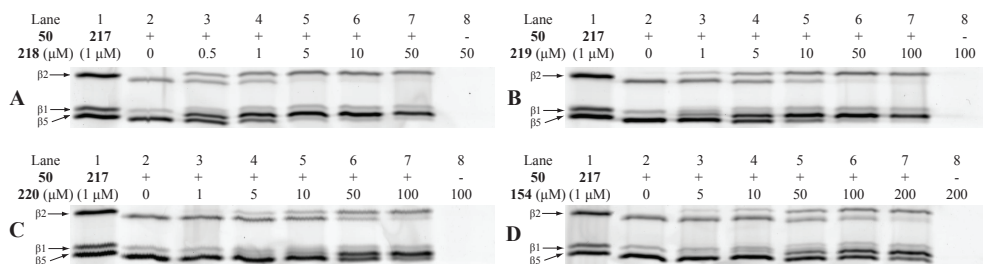


Figure 5.2: Determination of two-step label concentration for full conversion of labelled proteasome in cell lysate. HEK293T lysates labelled with fluorescent proteasome probe **50** were incubated with indicated concentrations of two-step labelling reagents ((A) DIBO (**218**), (B) BCN (**219**), (C) MFCO (**220**) and (D) Staudinger-Bertozzi (**154**)) for 1 hr. followed by SDS-PAGE separations of proteins and fluorescence readout of the wet gel slabs.

In contrast, labelling with Staudinger-Bertozzi reagent **154** (Figure 5.2D) is nearing completion only at 200 μM final concentration (a value corresponding to what is observed in previous work)¹¹¹ and from this experiment it is apparent that strain-induced click ligation is the more efficient approach at least where it concerns amounts of reagent used. Closer perusal of the experiments involving the three click reagents reveals a marked difference in efficiency, with ligation to dibenzylcyclooctyne derivative **218** complete at 5 μM final concentration and ligation to the two other reagents **219** and **220** complete between 50 and 100 μM final concentration. However, streptavidin readout of the Western blot indicated that the expected proteasome signals were overshadowed by tremendous background (Figure 5.5, page 89). This background increased proportionally with the amount of cyclooctyne added. Therefore, some preliminary optimization attempts were performed, none of which could significantly improve the signal to noise ratio of the cyclooctyne two-step labelling (Figure 5.6, page 90).

In the next experiment the time and temperature dependency and labelling efficiency of the panel of bioorthogonal ligation reagents was determined. In these experiments final concentrations for all compounds were applied that are below those identified in the first experiment as needed for full conversion (**218** (0.5 μM final concentration), **219** (5 μM), **220** (5 μM) and **154** (50 μM)). As expected, labelling at 0°C proceeded much slower than labelling at 37°C for all reagents (Figure 5.3). From the experiments at 37°C it is apparent that DIBO **218** is the most reactive of the series, with significant labelling after 30 minutes. Maximal labelling with compounds **219** was achieved after two hours, while compounds **220** and **154** show increase in labelling up to four hours of reaction time. These results roughly correspond to the reported reaction rates of the cyclooctynes in literature in which DIBO and BCN have a similar reaction rate,^{242,243,245} followed by MFCO²⁴⁴ with the lowest reaction rate in this series. Here, it should be noted that one has to be careful in comparing the literature reaction rates, since experimental conditions in which these were obtained might differ.

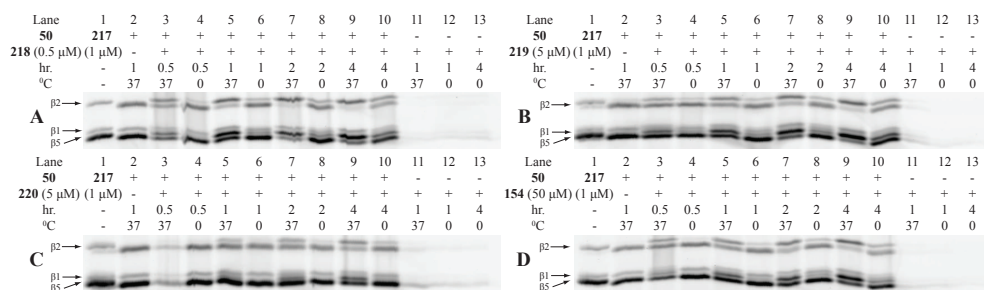


Figure 5.3: Time and temperature dependence of two-step labelling reaction in 50 labelled HEK293T cell lysates. Lysates (25 μg) were incubated with two-step reagents (**A**) DIBO (**218**), **B** BCN (**219**), **C** MFCO (**220**) and **D** Staudinger-Bertozzi (**154**) for indicated time and temperature followed by C/M precipitation. Proteins were resolved by SDS-PAGE and fluorescence detected in the wet gel slabs.

In a final experiment it was established whether both activity-based probe and bioorthogonal tag could be applied in living cells (Figure 5.4). Surprisingly, all three the click reagents, and indeed also the Staudinger-Bertozzi reagent, proved cell-permeable and in all four experiments significant bioorthogonal proteasome tagging was observed. This is apparent from the multiple bands that appear on gel as visualised by in-gel fluorescence

detection, and in most cases also after biotin-streptavidin blotting. With respect to this latter analysis some important differences were observed. As expected based on previous work, Staudinger ligation gives clean bands corresponding to the proteasome active sites after Western blotting.

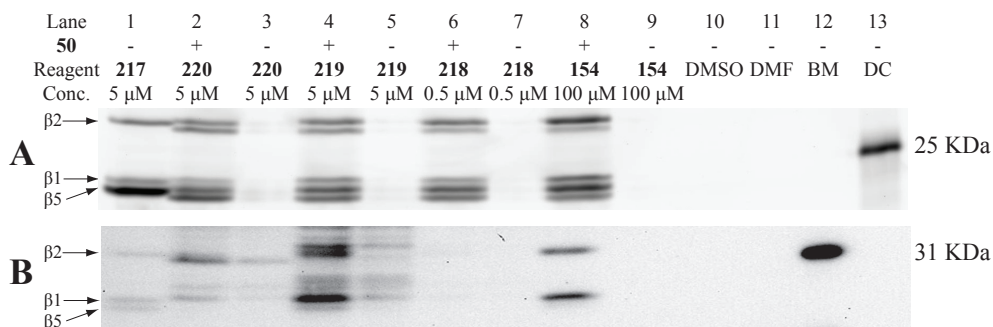


Figure 5.4: Cell permeability of two-step labelling reagents **218**, **219**, **220** and **154**. HeLa cells are incubated with **50** (5 μ M) for 2 hr. followed, after washing, by **218-154** for 4 hr. After washing, cells were lysed and proteins resolved by SDS-PAGE followed by western blotting. A. fluorescence readout and B. streptavidin blot of labelled cell lysates. BM = biotinylated molecular weight marker. DC = dual colour molecular weight marker.

Compound **219** and to a lesser extent also compound **220** delivered proteasome bands based on probing for a biotin moiety. The background labelling, albeit much lower than in experiments in lysates, is much more prominent for **219** and **220** compared to Staudinger-Bertozzi **154**. Rather surprisingly, and as yet not understood, is the almost complete absence (vague bands are visible) in the experiment employing biotin-cyclooctyne **218**. This result is at odds with the clear positive bioorthogonal labelling of proteasome subunits as visualised by fluorescence read-out.

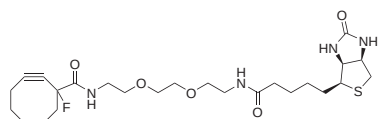
5.3 Conclusion

In conclusion, the results in this chapter underscore previous reports on the versatility of strain-promoted cyclooctynes in bioorthogonal chemistry, in particular with respect to two-step activity-based protein profiling. The three reagents tested, and in particular cyclooctyne **218**, outperform Staudinger-Bertozzi ligation reagent **154** where it comes to reactivity and stoichiometry. At the same time, it is equally clear that neither of the reagents is truly bioorthogonal in the sense that they modify the desired target, and nothing else. Background labelling as observed with the cyclooctynes in general is extensive, rather more so than observed by Staudinger-Bertozzi ligation, even though lower final concentrations of the cyclooctynes are needed. Apparently reactions between these highly strained systems and functional groups inherent to biological samples are more prominent, whereas the intrinsic sensitivity of the biotinylated phosphane towards oxidation,^{236,246} is the main causative for the need of large excesses of this reagent. Interestingly, all ligation reagents proved cell permeable and a general trend observed is that bioorthogonal ligation in living cells is much cleaner with respect to background labelling than performing this step in cell extracts. Future research involving pull-down followed by proteomics analysis is needed to unearth the nature of the proteins modified by the cyclooctyne reagents at functionalities other than an azide, and possibly also the nature of these functionalities. In addition, the

stability of the cyclooctyne reagents under physiological conditions should be studied. Recently, a report came out that describes the thiol-yne reaction, a free radical addition of two thiols to an alkyne under the influence of ultraviolet light and radical initiator.²⁴⁷ Whether this process plays a role in the background formation observed in this Chapter remains to be determined. In a more general sense, these results also underscore the need for the development of fast reacting, more selective bioorthogonal ligation reagents that can be used at low concentrations, especially for application in cell extracts.

5.4 Experimental

All reagents were of commercial grade and were used as received unless indicated otherwise. Dichloromethane (DCM) and dimethyl formamide (DMF, Biosolve) were stored on 4 Å molecular sieves. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kiesegel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H₂SO₄ in ethanol or (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid followed by charring at ~150°C or by spraying with an aqueous solution of KMnO₄ (7%) and KOH (2%). Column chromatography was performed on silica gel from Screening Devices (0.040 - 0.063 nm). HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ¹H- and ¹³C-APT-NMR spectra were recorded on a Bruker AV-400 (400/100 MHz) 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. All presented ¹³C-APT spectra are proton decoupled. DIBO 218,²⁴² BCN 219,²⁴³ Staudinger-Bertozzi reagent 154¹¹¹ and compound 217 and 50¹¹⁴ were synthesised as described in literature.



MFCO (220). 1-fluorocyclooct-2-ynecarboxylic acid (21.2 mg, 126 μmol) was coupled to biotinylated diamino triethyleneglycol¹¹¹ (60 mg, 160 μmol) by literature procedure²⁴⁴ to yield MFCO 220 (27 mg, 51 μmol, 41% over 2 steps). ¹H NMR (400 MHz, CD₃OD, mixture of diastereomers): δ ppm 4.52 (dd, *J*₁ = 7.7, *J*₂ = 4.9 Hz, 1H), 4.33 (dd, *J*₁ = 7.8, *J*₂ = 4.4 Hz, 1H), 3.73 - 3.54 (m, 8H), 3.47 - 3.35 (m, 4H), 3.29 - 3.15 (m, 1H), 2.95 (dd, *J*₁ = 12.7, *J*₂ = 4.9 Hz, 1H), 2.73 (d, *J* = 12.7 Hz, 1H), 2.40 - 2.21 (m, 6H), 2.18 - 1.56 (m, 9H), 1.54 - 1.41 (m, 3H). ¹³C NMR (100 MHz, CD₃OD, mixture of diastereomers): δ ppm 176.22, 176.13, 171.03 (*J* = 24.9 Hz), 166.07, 110.14 (d, *J* = 10.3 Hz), 95.2 (*J* = 185.4 Hz), 88.28 (*J* = 31.5 Hz), 71.27, 70.61, 70.20, 63.35, 61.60, 57.02, 47.69 (*J* = 24.8 Hz), 41.06, 40.42, 40.30, 36.73, 35.00, 30.12, 29.78, 29.50, 26.86, 26.76, 21.07, 21.05. HRMS Calcd. for [C₂₅H₄₀F₁N₄O₅S]⁺ 527.26980, found 527.26986.

Two-step labelling of fluorescently labelled proteasomes in lysate

HEK293T cells were cultured on DMEM supplemented with fetal calf serum (FCS, 10%), penicillin (10 units mL⁻¹), and streptomycin (10 mg mL⁻¹) in a CO₂ (5%) humidified incubator at 37°C. Some 15×10⁶ HEK293T cells were seeded on a 15 cm Petri dish and cultured overnight. 50 (5 μM final concentration, 100x DMSO stock) or DMSO was added in 10 mL fresh medium and incubated for 2 hr. at 37°C. The medium was removed and the cells were harvested in cold PBS and washed (3 ×) with cold PBS before the pellet was flash frozen (N₂ (l)). The pellet was resuspended in 400 μL digitonin lysis buffer [Tris pH 7.5 (50 mM), sucrose (250 mM), MgCl₂ (5 mM), dithiothreitol (DTT; 1 mM), digitonin (0.025 %)] and incubated on ice for 10 min. before spinning down at 16100 relative centrifugal force (rcf) at 0°C. The supernatant was collected and the protein concentration was determined by the Bradford assay. 25 μg protein in 9 μL lysis buffer was treated with 1 μL 10x stock of two-step labelling reagent (in DMSO for 218, 219, 220, in DMF for 154; no solubility issues were observed when dissolving the compounds in DMSO (20 μM) or adding the DMSO stocks to lysate) or 217 and incubated for 1 hr. at 37°C before the reaction was stopped by chloroform/methanol precipitation.²⁴⁸ The pellet was redissolved in 10 μL 2x Laemli's sample buffer. The proteins were resolved on 12.5% SDS-PAGE gel and the fluorescently labelled proteasome subunits visualised by scanning the wet gel slab on a Typhoon variable mode imager (Amersham Biosciences, using the Cy3/TAMRA setting (λ_{ex} 532 nm, λ_{em} 560 nm)). Proteins were electrotransferred to polyvinylidene (PVDF) membranes. The blots were blocked with BSA (1%) in TBS-Tween 20 (0.1% Tween 20) for 30 min. at RT, hybridized for 30 min. with streptavidin/HRP (1:10 000) in blocking buffer, washed, and visualised with the aid of an ECL+ kit (Amersham Biosciences).

Two-step labelling of fluorescently labelled proteasomes in living cells

HeLa cells ($75 \cdot 10^4$) were seeded and grown overnight on 2 mL medium as mentioned above. The medium was removed and replaced with 1 mL fresh medium containing $10 \mu\text{l}$ of a $100\times$ DMSO stock of **50** or **217** ($5 \mu\text{M}$ end concentration) followed by 2 hr. incubation. The medium was removed and the cells were washed with PBS. 1 mL fresh medium containing $10 \mu\text{l}$ of a $100\times$ stock solution of the click reagent (in DMSO for **218**, **219**, **220**, in DMF for **154**) was added and incubated for 4 hr., after which the cells were scrutinized under visual light microscope. The cells were washed with PBS, harvested and washed with $3\times$ with PBS before being lysed as described above. 25 μg protein was resolved on SDS-PAGE gel and western blotted as described above.

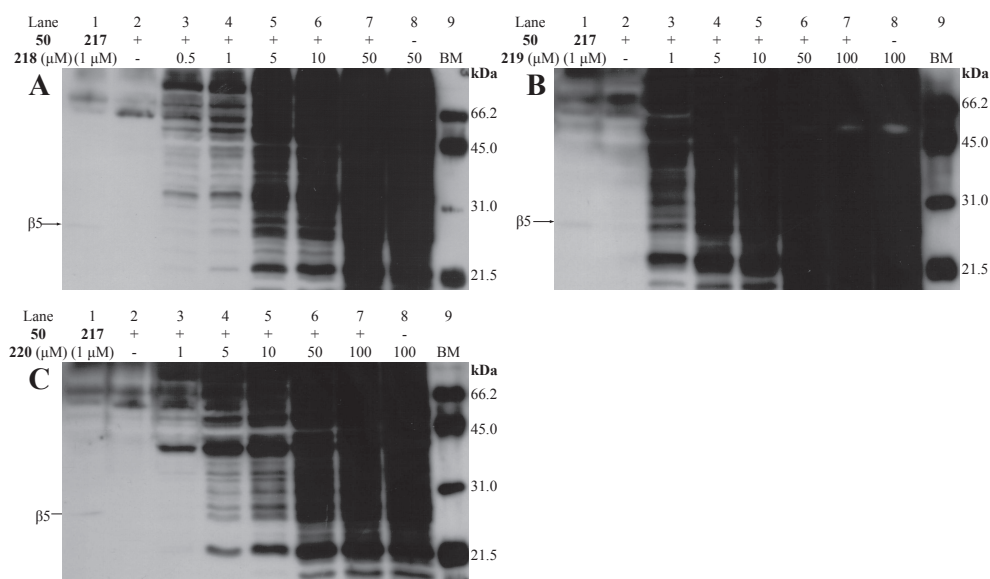


Figure 5.5: Western blots from the gels depicted in Figure 5.2, readout with Streptavidin-HRP and ECL+. (A) DIBO (**218**), (B) BCN (**219**), (C) MFCO (**220**). BM = biotinylated molecular weight marker.

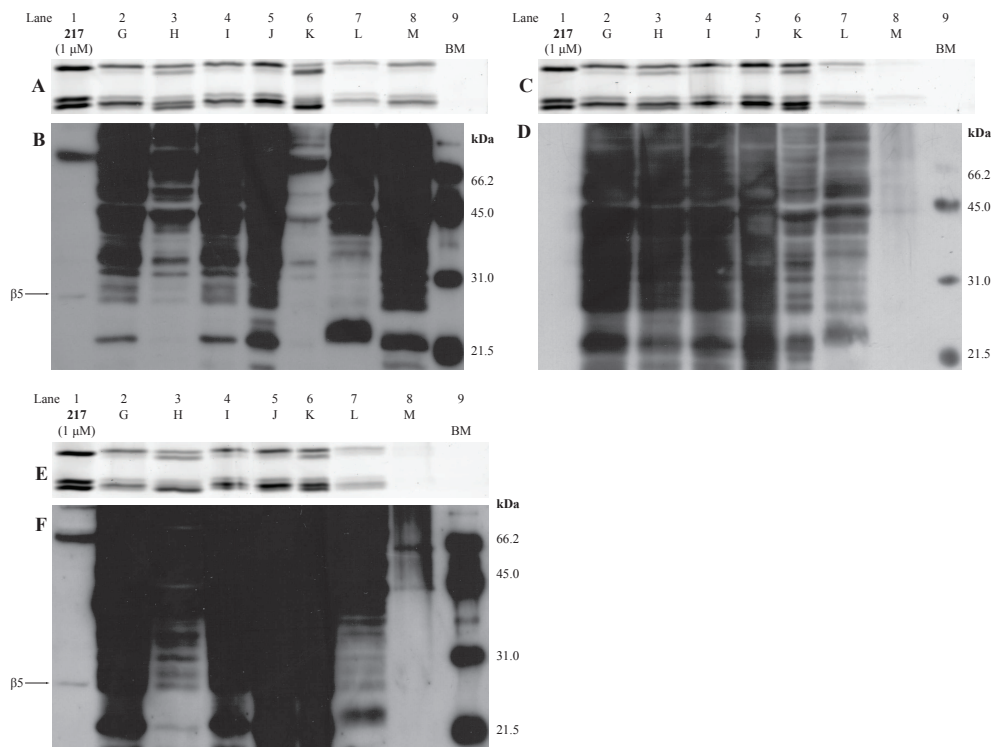


Figure 5.6: Attempt at optimization of the bioorthogonality of the cyclooctyne reagents **218**, **219**, **220** in lysates (25 μ g protein) of 50 incubated HEK cells. (A) Fluorescence and (B) Streptavidin readout of **218** (10 μ M), (C) Fluorescence and (D) Streptavidin readout of **219** (100 μ M), (E) Fluorescence and (F) Streptavidin readout of **220** (100 μ M). General procedure: Lysate (25 μ g in 9 μ l lysis buffer) was treated with 1 μ l 10 $\times$ octyne in DMSO followed by incubation for 1 hr. and chloroform/methanol precipitation (C/M). The proteins were redissolved in 10 μ l 2 $\times$ Laemli's sample buffer and boiled for 3 min. before loading on SDS-PAGE gel. (G) General procedure. (H) Incubation at 0°C. (I) Denature lysate + sample buffer at 55°C 15 min., load on gel. (J) No C/M (K) Lysate + 4 $\times$ sample buffer, 100°C 3 min. then cyclooctyne, 37°C 1 hr., load on gel (L) DTT/iodoacetamide capping. Lysate C/M, proteins taken up in 20 μ l 8M urea + 100 mM NH_4HCO_3 , 15 min. RT, DTT (5 mM) 30 min. 37°C, iodoacetamide (13 mM) 30 min. RT in the dark followed by C/M, take up in 90 μ l 0.1% SDS/ 50 mM Tris pH 7.4, add cyclooctyne, 37°C 1 hr., C/M, dissolve in 10 μ l 2 $\times$ SB, boil 3 min., load on gel. (M) DTT/DNTB capping. 9 μ l lysate was incubated with 80 μ l 9M urea for 15 min. at RT. Incubate with 0.5 μ l 1M DTT for 30 min. at 55°C. Add 4.7 μ l 1M 5,5'-dithiobis-(2-nitrobenzoic acid) in DMSO, 2 hr. RT, C/M, take up in 90 μ l 0.1% SDS/ 50 mM Tris pH 7.4, add cyclooctyne, 37°C 1 hr., C/M, dissolve 10 μ l 2 $\times$ SB, boil 3 min., load on gel. BM = biotinylated molecular weight marker.

6

Basicity at P1 and P3 induces $\beta 2$ selectivity in proteasome inhibitors

6.1 Introduction

The 26S proteasome is the central protein degrading machinery in the eukaryotic cell. In eukaryotes, seven distinct proteolytic active subunits are present in the 20S proteasome. The constitutive subunits are $\beta 1$ (cleaving after acidic amino acids), $\beta 2$ (cleaving after basic amino acids) and $\beta 5$ (cleaving after hydrophobic amino acids). Three immuno-subunits can replace the constitutive catalytic subunits and are named $\beta 1i$, $\beta 2i$, $\beta 5i$. In cortical thymic epithelial cells, $\beta 5t$ replaces $\beta 5i$ in so-called thymoproteasomes.²⁶ Amino acid preference at the cleavage site of these subunits has been determined using fluorogenic substrate cleavage assays.¹² Selective inhibitors of the proteasome, and subunit selective proteasome inhibitors in particular, are useful tools to determine the involvement of the proteasome in cellular processes. A number of selective inhibitors and probes of the $\beta 1/\beta 1i$ or $\beta 5/\beta 5i$ which display cell permeability has been reported in literature (See Chapter 1).^{127,130,132,133,147} For the $\beta 2$ subunit, several selective inhibitors have been described (Figure 6.1). Using a scanning positional library, Nazif et al. have demonstrated that the character of the P3 amino acid side chain in an inhibitor dramatically influences specificity for the $\beta 2$ subunit. Incorporation of arginine at P3 induced $\beta 2$ preference and gave structures **67** and **221** (Figure 6.1) as leads.¹⁴⁰ In this scanning positional library, the P1 position was fixed at asparagine, but substitution of this residue with leucine abrogated $\beta 2$ selectivity of the scaffolds. The preference of the $\beta 2$ subunit for basic substrates dictates the use of basic amino acid residues in the peptide fragment of a peptide based inhibitor, which, in most cases, renders the resulting inhibitor not able to cross cell membrane. Inhibitors **67** and **221** indeed suffer from poor cell permeability, limiting their use to cell lysates.

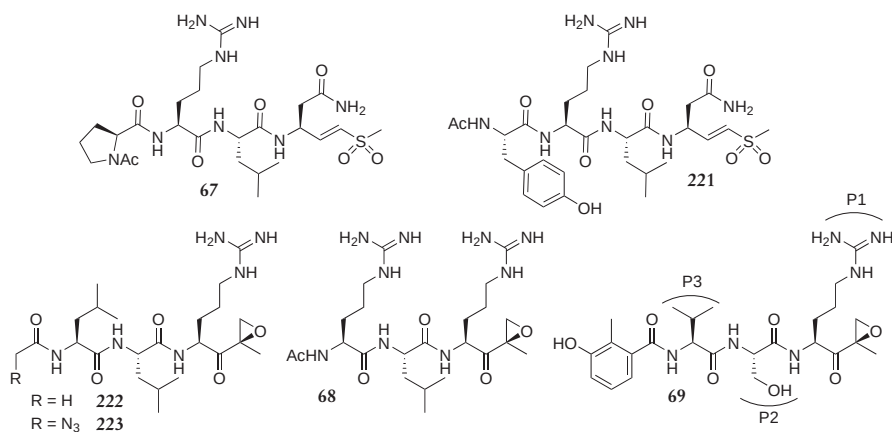


Figure 6.1: Literature β_2 selective inhibitors.

More recently, the synthesis of β_2 selective compound **222**, based on the leupeptin scaffold (Ac-Leu-Leu-Arg-aldehyde), has been described.¹²⁸ Overnight treatment of cells with this inhibitor indeed reduces β_2 activity in these cells. Incorporation of an azide gave activity based probe **223** which does not significantly bind cellular targets other than β_2 or β_2i . Substitution of the P3 leucine in **222** for arginine gave **68**, which was indeed a more potent and selective inhibitor for β_2 than **222** in cell lysate. Inhibitor **68**, however, displays very limited cell permeability. Compound **69** shows more preference for β_2 than **222** and at the same time shows cell permeability. This inhibitor was used in cellular assays to block the tryptic activity of the proteasome to sensitize multiple myeloma cells to antimyeloma agents such as bortezomib and carfilzomib.¹²⁸ However, compounds **222** and **69** contain the arginine epoxyketone, which is difficult to synthesise. These difficulties might be explained by the possibility of the P1 arginine side chain to cyclise onto the epoxyketone electrophile.

Potential cyclisation problems could be overcome by the use of phenylalanine derivatives at P1 since a para-substituted phenyl side chain prevents cyclisation of the basic moiety onto the electrophile. Basicity of the side chains could be varied, resulting in compounds **70**, **224** and **225** (Figure 6.2).¹⁴¹ The compound with the benzyl amine side chain at P1, **70**, displays selectivity for β_2 . However, the concentration range in which β_2 is silenced by **70** and the other subunits unaffected is rather small. Lowering the pK_a of the side chains at P1, namely by using an aniline or pyridine moiety (compound **224** and **225**, respectively) resulted in equipotent inhibition of β_2 and β_5 for **224** or even a slight preference for β_5 for **225**.¹⁴¹ This Chapter describes the synthesis and biological evaluation of a library of proteasome inhibitors with varying basicity at both the P1 and P3 position of the inhibitor to determine the influence on the proteasome subunit selectivity. The library consists of analogues of **70**, **224** and **225**, in which the P3 position is functionalised with a 4-aminomethylphenylalanine, 4-aminophenylalanine or 4-pyridylalanine side chain and at P1 either leucine or 4-aminomethylphenylalanine is incorporated (Figure 6.2).

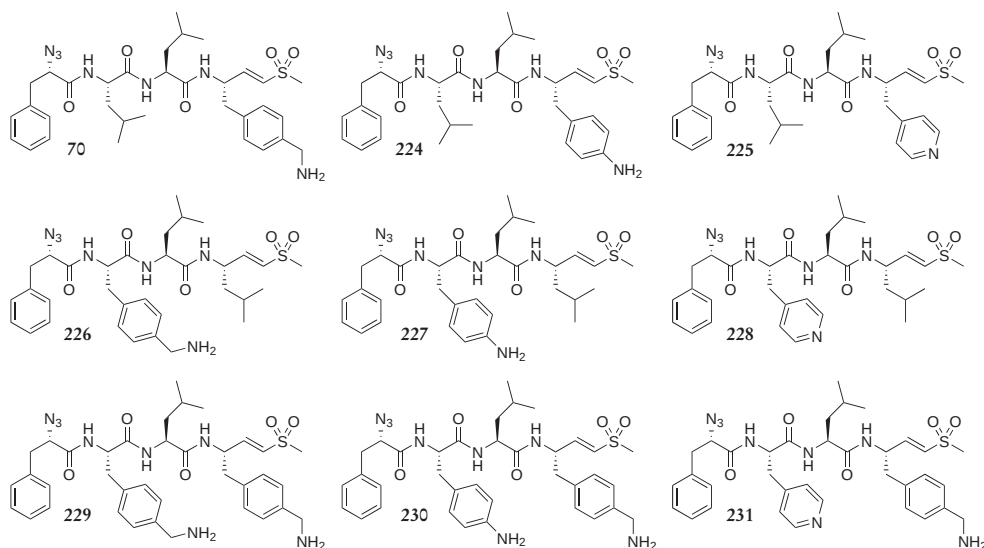


Figure 6.2: Structure of the compounds used in this study.

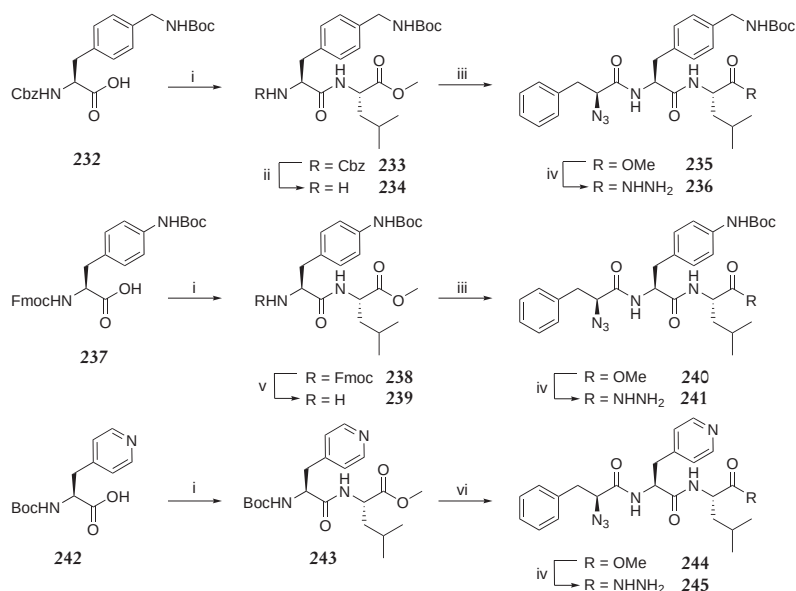
6.2 Results and Discussion

Retrosynthetically, compounds **226** and **229** could be made by coupling N_3 -Phe(4-CH₂-NHBoc)-Leu-NHNH₂ to H-Leu-VS or H-Phe(4-CH₂NHBoc)-VS using the azide coupling. In a similar fashion, **227** and **230** or **228** and **231** could be prepared from N_3 -Phe(4-NHBoc)-Leu-NHNH₂ or N_3 -Ala(4-pyridine)-Leu-NHNH₂, respectively. The synthesis of peptide hydrazides **236**, **241** and **245** is depicted in Scheme 6.1. Carboxylic acid **232**¹⁴¹ was coupled to leucine methyl ester and subsequently the Cbz protected N-terminus in **233** liberated by hydrogenation to yield **234**. Coupling to N_3 -Phe-OH and subsequent reaction with hydrazine hydrate yielded hydrazide **236**. Fmoc protected amino acid **237** was coupled to HCl·H-Leu-OMe after which the Fmoc group was removed in solution using EtSH and DBU.²⁰⁹ The resulting amine **239** was coupled to N_3 -Phe-OH to yield **240**, which was then treated with hydrazine hydrate to obtain **241**. Hydrazide **245** was prepared from hydrazine hydrate and methyl ester **244**, the latter of which was synthesised from commercially available **242** using standard Boc protected solution phase chemistry (Scheme 6.1).

Hydrazide **236** was coupled to H-Phe(4-CH₂NHBoc)-VS¹⁴¹ in the azide coupling reaction to yield Boc-protected inhibitor **246**, which was subsequently deprotected with TFA to yield compound **229** (Scheme 6.2). In a similar way, the five other inhibitors were prepared using the appropriate amines and hydrazides.

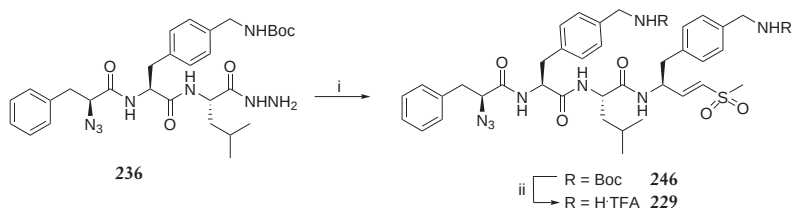
Compounds **70** and **224–231** were evaluated on their proteasome inhibition capacities in a competition assay versus MVB003 (**49**) in cell lysates from HEK293T and RAJI cells. HEK cells only express constitutive proteasome while RAJI, a lymphoblast cell line derived from Burkitt's lymphoma, displays both constitutive and immunoproteasome (Figure 6.3). The pK_a of the side chain of the amino acid at the P1 position of compounds **70** and **224–231** determines proteasome subunit selectivity. In HEK lysate, the benzylamine

Scheme 6.1: Synthesis of peptide hydrazides.



Reagents and conditions: i) HBTU (1.2 equiv.), HCl-H-Leu-OMe (1.1 equiv.), DiPEA (3.5 equiv.), DCM, 2 hr., quant. (233), 70% (238), 83% (243). ii) TFA (1.2 equiv.), MeOH, Pd/C (10 mol-%), H₂, 3 hr. then sat. aq. NaHCO₃, quant. iii) N₃-Phe-OH (1.2 equiv.), HBTU (1.3 equiv.), DiPEA (2.5 equiv.), DCM, 1.5-2 hr., 74% (235), 94% (240) iv) Hydrazine hydrate (60 equiv.), MeOH, o/n., quant. v) EtSH (10 equiv.), DBU (0.1 equiv.), THF, 30 min., quant. vi) (a) TFA:DCM 1:1, 30 min, quant. (b) N₃-Phe-OH (1.1 equiv.), HBTU (1.1 equiv.), DiPEA (4.5 equiv.), DCM, 1.5 hr., 76%.

Scheme 6.2: Example of azide coupling and subsequent deprotection to yield 229.



Reagents and conditions: i) *t*BuONO (1.1 equiv.), HCl (2.8 equiv.), DMF:DCM 3:1, -30°C, 3hr. then H-Phe(4-CH₂NHBoc)-VS, DiPEA (5 equiv.), DMF, -30°C → RT o/n, 30% ii) DCM:TFA 1:1, 30 min.

residue at P1 yields β 2 selective inhibitor 70 (Figure 6.3A). Inhibitors with aniline or pyridine as P1 substituent, compound 224 and 225, respectively, are roughly equipotent against β 2 and β 5. At physiological pH, the benzylamine side chain is presumably the only moiety of the three selected side chains that is charged. These results correspond to what was reported by Geurink.¹⁴¹ Basicity at the P3 position adds to β 2 preference, albeit not as absolute as P1 basicity, and P3 basicity in itself did not yield a β 2 selective inhibitor in this study. This result corresponds to what is observed by Nazif et al., who installed leucine at P1 in β 2 selective vinyl sulfone 67 (Figure 6.1) to yield Ac-Pro-Arg-Leu-Leu-VS, which

displayed restored activity against β_5 . In this study, compound **226**, with the benzylamine at P3, displays almost equipotent β_2/β_5 inhibition, while **227** and **228** show about 30-fold more potency against β_5 than β_2 (Figure 6.3A). Compounds **229-231** are selective β_2 inhibitors. Double benzylamine **229** is the most potent and β_2 selective compound found in this Chapter, more selective than **70**. Compounds with aniline or pyridine at P3 do show β_2 preference, about equivalent to that of compound **70**.

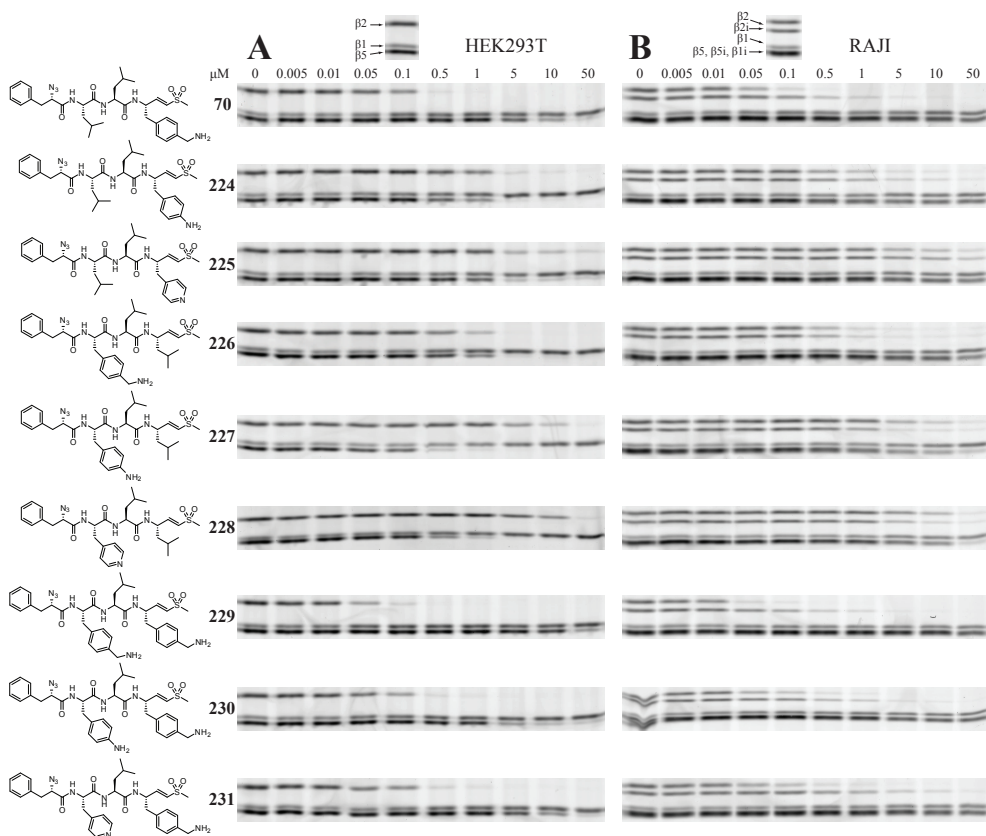


Figure 6.3: Competition assay of compounds **70** and **224-231** in cell lysate. (A) HEK293T lysate (15 μg) and (B) RAJI lysate (10 μg) was treated with 10 $\times$ DMSO stock of indicated inhibitor for 1 hr. at 37°C. Residual proteasome activity was labelled with MVB003 (0.5 μM end concentration) for 1 hr. at 37°C.

The selectivity of compounds **70** and **224-231** in RAJI lysate corresponds to what is observed in HEK lysate (Figure 6.3B versus Figure 6.3A). However, the effect of inhibitors on β_5/β_5i can not be determined directly since these subunits, in this experimental setup, appear to overlap with β_1i (Figure 6.3B). In all competition experiments in cell lysate for compounds **70** and **224-231**, the activity of β_1 , as was measured by MVB003 labelling, increased when activity of β_2 or β_5 decreased, sometimes to values up to 300% of the DMSO treated control. This effect has been observed earlier and is not well understood but could be a result of allosteric regulation within the proteasome.¹³¹ An upregulation effect on β_1i , similar to β_1 upregulation, is likely. This potential upregulation effect on

$\beta 1i$ overshadows a decrease of the $\beta 5/\beta 5i$ subunit signal. In RAJI lysate, the inhibitors with benzylamine at P1 appear to have limited preference for $\beta 2$ over $\beta 2i$. On the other hand, compounds **227** and **228**, with leucine at P1, show some more potency against $\beta 2i$ than for $\beta 2$ and this suggests that peptide sequence optimisation could yield inhibitors that can discriminate between tryptic activities of constitutive and immunoproteasome. An overview of quantified competition experiments is provided in Table 6.1.

Table 6.1: Apparent IC_{50} (μM) values calculated from semi log plots of residual proteasome activity, quantified from gel lanes from competition assays (Figure 6.3, Figure 6.4, Figure 6.5) against inhibitor concentration. $\beta 1$ is not inhibited in lysate and not significantly inhibited in cells and therefore it was omitted in this table.

Compound	HEK lysate		HEK cells		RAJI lysate			RAJI cells		
	$\beta 5$	$\beta 2$	$\beta 5$	$\beta 2$	$\beta 5, \beta 5i, \beta 1i$	$\beta 2i$	$\beta 2$	$\beta 5, \beta 5i, \beta 1i$	$\beta 2i$	$\beta 2$
	IC_{50} (μM)		IC_{50} (μM)		IC_{50} (μM)			IC_{50} (μM)		
70	5.8	0.084	>50	2.7	19	0.15	0.029	37	~0.5	<0.5
224	1.1	1.5	<0.5	~0.5	9.2	0.98	0.63	<0.5	<0.5	<0.5
225	1.5	2.5	0.98	1.8	7.3	5.5	2.0	2.0	1.9	1.2
226	0.48	0.19	5.8	17	17	0.49	0.32	10	0.56	0.46
227	0.093	2.7	<0.5	1.9	1.3	0.91	2.4	0.74	0.60	2.4
228	0.16	5.6	0.66	6.7	4.7	4.1	8.9	1.2	2.0	3.3
229	21	0.022	>50	50	~50	0.080	0.012	>50	18	4.4
230	2.8	0.047	>50	10	40	0.32	0.053	>50	2.2	0.69
231	5.5	0.051	>50	27	33	0.75	0.038	>50	6.4	0.80

Next, the ability of compounds **70** and **224-231** to cross cell membrane was assessed. Inhibitors were incubated with living HEK or RAJI cells for four hours, after which remaining proteasome activity was labelled with cell permeable MVB003 (Figure 6.4, Figure 6.5). Compound **70** inhibits $\beta 2$ with some selectivity, although it is not able to silence $\beta 2$ at a concentration where $\beta 5$ is unaffected (Figure 6.4). Inhibitor **229**, superior to **70** in selectivity and potency against $\beta 2$, in HEK cells inhibits only about 50% of tryptic activity at 50 μM , while **70** reaches this level already at 5 μM . Apparently, the presence of the benzylamine moiety in an inhibitor reduces its capability to inhibit proteasomes inside HEK cells (Figure 6.4). Compounds **230** and **231** also appear to have difficulties crossing the cell membrane. Compounds **224**, **225**, **227** and **228**, on the other hand, inhibit their target subunits at a lower concentration than the more basic benzylamine containing compounds (Figure 6.4). It is therefore remarkable that **70** is able to enter cells, given the large negative effect the benzylamine moiety has on cell permeability.

Selectivity trends from the competition assay in RAJI cells (Figure 6.5) in general agree with the selectivity trends found in the competition assay in HEK cells. One exception is that **226** targets $\beta 2$ more potently than $\beta 5$ in RAJI cells, while in HEK cells this preference is reversed. An explanation for this discrepancy can not be given. An important difference in potency trends between RAJI and HEK cell assays is that in RAJI cells, the benzylamine functionality hampers inhibitors less in the entrance to cells. At 0.5 μM , **70** inhibits more than 50% of both $\beta 2$ and $\beta 2i$, while in HEK cells, this level is only reached at 5 μM . In RAJI cells, **229-231** are also more active than in HEK cells. Compound **229**, while not able to fully inhibit $\beta 2$ in HEK cells at 50 μM , silenced $\beta 2$ in RAJI cells at 50 μM , and left about

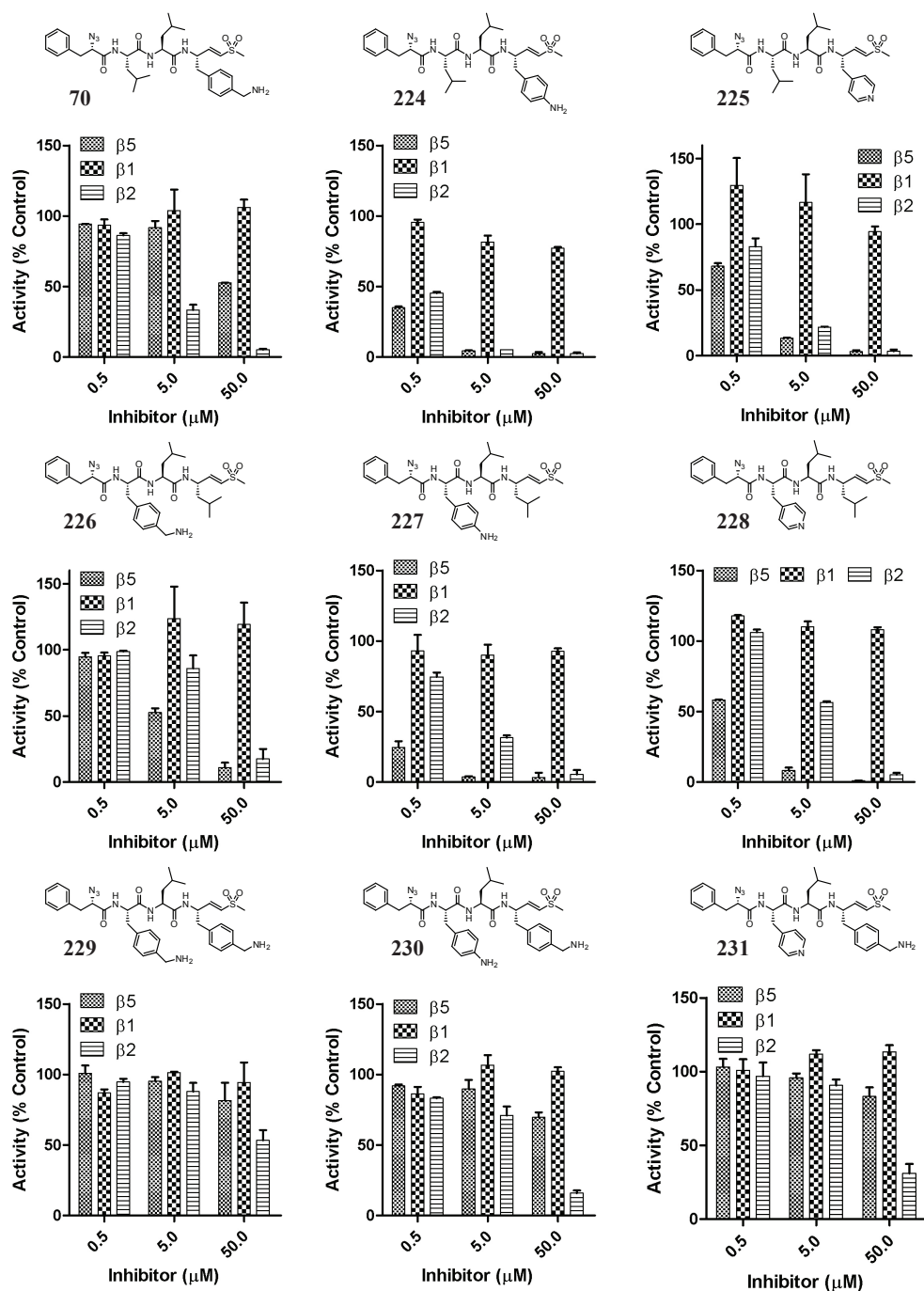


Figure 6.4: Competition assay in HEK293T cells. Cells ($1 \cdot 10^6$) were exposed to indicated concentration of inhibitor for 4 hr. at 37°C and remaining active proteasome was labelled with MVB003 ($5 \mu\text{M}$ end concentration) for 2 hr. at 37°C . Competition assay gels were quantified, corrected for gel loading and mean remaining activity values plotted against concentration.

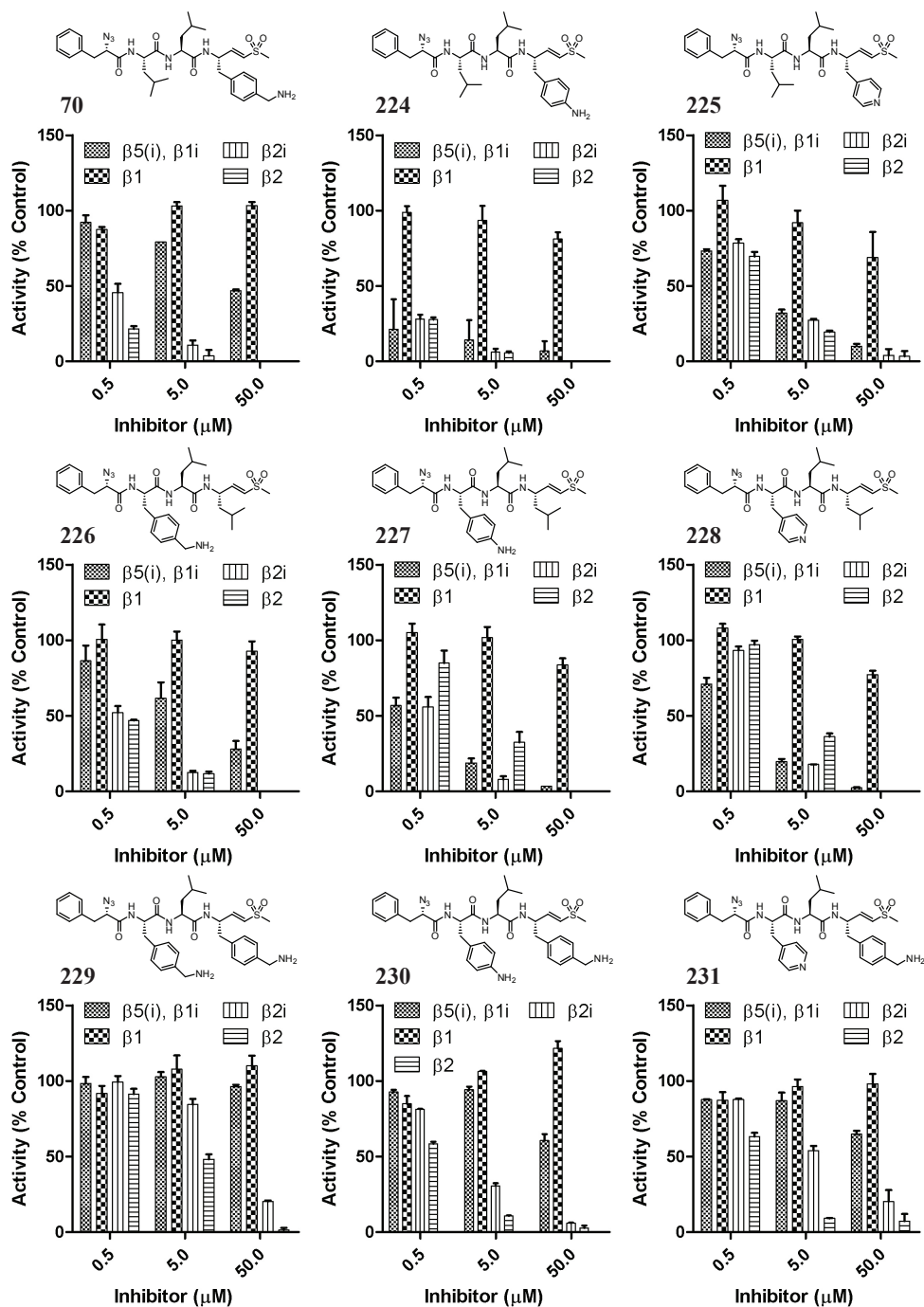
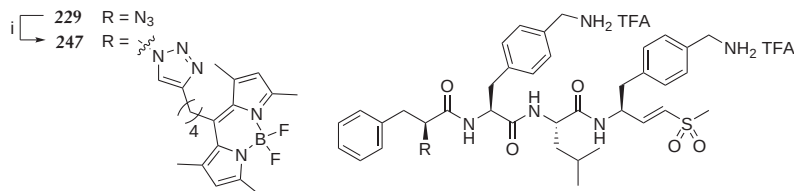


Figure 6.5: Competition assay in RAJI cells. Cells ($1 \cdot 10^6$) were exposed to indicated concentration of inhibitor for 4 hr. at 37°C and remaining active proteasome was labelled with MVB003 ($5 \mu\text{M}$ end concentration) for 2 hr. at 37°C . Competition assay gels were quantified, corrected for gel loading and mean remaining activity values plotted against concentration.

20% of $\beta 2i$ activity untouched. More importantly, **229** left $\beta 5$ activity almost intact at this concentration in RAJI cells. However, the combination of the signals for $\beta 5$, $\beta 5i$ and $\beta 1i$ in this experimental setup hampers the analysis of chymotryptic activity and selectivity of this compound should be analysed using different methods.

A fluorescent activity-based probe selective for the $\beta 2$ subunit has not been reported to date. Previously, attachment of a BODIPY dye to **70** afforded a molecule that targeted $\beta 2$ and $\beta 5$ simultaneously.¹⁴¹ Attachment of a BODIPY dye to the selective inhibitor **229** might yield a $\beta 2$ selective probe. Compound **229** was reacted with a green fluorescent BODIPY-alkyne¹¹⁵ in a copper(I) catalysed click reaction. For this reaction, the *in situ* generation of copper(I) from CuSO_4 and sodium ascorbate was avoided since this copper/ascorbic acid dyad is able to oxidise benzylic amines as present in **229**.^{141,249} Rather, direct addition of copper(I) by the use of $\text{Cu}(\text{MeCN})_4\text{PF}_6$ is preferred.²⁵⁰ This copper(I) catalysed 'click' reaction afforded target molecule **247** in 36% yield after HPLC purification (Scheme 6.3).

Scheme 6.3: Synthesis of fluorescent inhibitor **247**.



Reagents and conditions: i) BODIPY-alkyne (1.5 equiv.), TBTA (1 equiv.), $\text{Cu}(\text{MeCN})_4\text{PF}_6$ (1 equiv.), 1:1:1 $\text{H}_2\text{O}:\text{tBuOH}:\text{MeCN}$, o/n, then EDTA (5 equiv.), 36%.

First, **247** was incubated with HEK293T or RAJI lysate for 1 hr. after which the remaining proteasome activities were labelled with MVB003. Dual wavelength readout afforded simultaneous analysis of competition and labelling (Figure 6.6A-D). In the competition assay in HEK lysate, at 1 μM , activity of $\beta 2$ is almost completely competed away (Figure 6.6A). From 5 μM concentration and higher, inhibition of $\beta 5$ becomes visible by reduction of MVB003 signal. Labelling of $\beta 2$ becomes visible at 10 nM (Figure 6.6B) and labelling of $\beta 5$ starts to become visible already at 0.5 μM . Probe **247** thus does display some selectivity for $\beta 2$ over $\beta 5$ but compared to **229**, this probe clearly lost specificity due to the attachment of the BODIPY dye. Probe **247** is not able to fully label $\beta 2$ before $\beta 5$ is targeted. Note that in Figure 6.6B, in the lane where no **247** is added, faint bands are visible and this is presumably due to background fluorescence of MVB003 at this wavelength. The above trends are also seen in RAJI lysate (Figure 6.6C). In living HEK cells, **247** reduces MVB003 labelled $\beta 2$ slightly at 10 and 50 μM . This is in contrast with the effect of this compound in cell lysate, where at 0.5 μM the $\beta 2$ activity is silenced. Probe **247** thus suffers from poor cell permeability. In the direct readout of the probe (Figure 6.6F), again faint bands are observed in the lane where no **247** was added and this is again most likely background fluorescence from MVB003. At 10 μM , labelling of $\beta 2$ is observed with only faint binding to the $\beta 5$ subunit (Figure 6.6F). Interestingly, at 50 μM , $\beta 2$ labelling signal from **247** is decreased with respect to 10 μM . On the gel, a higher running fluorescent signal in the channel for compound **247** is observed (not shown). The nature of this background signal and whether this accu-

mulation of signal is the cause for the reduction in proteasome labelling both remain to be determined.

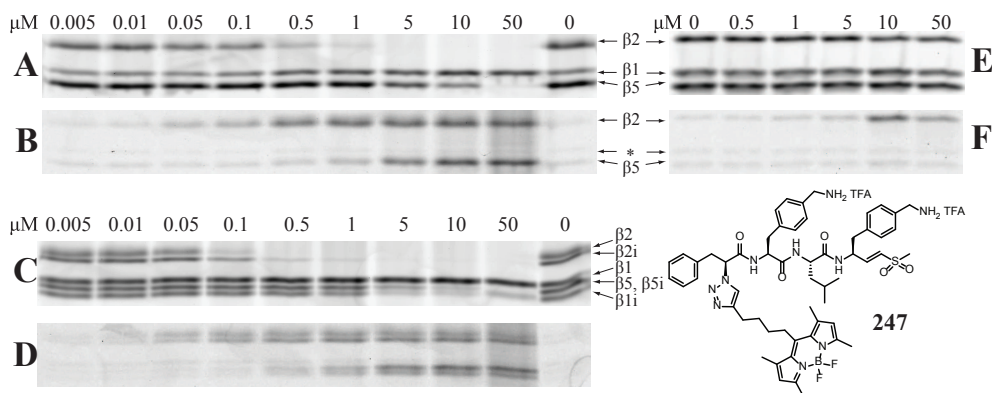


Figure 6.6: Analysis in cell lysate and living cells of **247**. Competition in HEK293T (A) and RAJI (C) lysate. Lysates (15 μg for HEK, 10 μg for RAJI) were incubated with indicated concentration of **247** for 1 hr. at 37°C followed by labelling of remaining proteasome activities by MVB003. (E) Competition assay in living HEK cells. Cells ($1 \cdot 10^6$) were incubated with **247** for 4 hr. at 37°C followed by MVB003 for 2 hr. at 37°C. (A, C, E) MVB003 readout, λ_{ex} 532 nm, λ_{em} 560 nm. (B, D, F) **247** readout, λ_{ex} 488 nm, λ_{em} 520 nm. * = background fluorescence of MVB003.

6.3 Conclusion

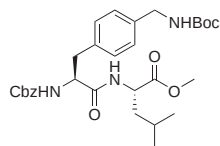
The biological effect of incorporation of basicity, in the form of benzylamine, aniline and pyridine moieties, at the P1 and P3 position of a proteasome inhibitor was studied. A residue that is charged at physiologically pH at the P1 position of the inhibitor is most important to get to β2 selectivity. This was illustrated by compounds **70**, **224**, **225**, from which only **70** displays selectivity for β2.¹⁴¹ Basicity at only P3 does not lead to a selective inhibitor for β2. A combination of basicity at both P1 and P3 gives inhibitors with good selectivity for β2, illustrated by the potent and selective inhibitor **229**. In RAJI lysate it appears that compounds with benzylamine on P1 prefer β2 more than β2i and this indicates that amino acid sequence optimisation of the benzylamine inhibitors may yield compounds able to discriminate between tryptic activity of constitutive and immunoproteasome. The presence of charged residues in a proteasome inhibitor hampers its entry into cells. In HEK293T cells, inhibitors **224**, **225** and **227**, **228** are more efficient at inhibiting the proteasome than benzylamine containing compounds. Compound **229**, superior in selectivity and potency in cell lysate, poorly enters HEK cells. RAJI cells, however, are entered more efficiently by benzylamine containing inhibitors than HEK cells. The better cell permeability in RAJI cells could be exploited for the treatment of Burkitt's lymphoma. Inhibition of proteasomes in untransformed cells by a proteasome inhibitor drug leads to side effects of this drug, and the better entry of inhibitors **229**, **230**, **231** in malignant cells can reduce severity of side effects. BODIPY containing compound **247** is the first example of a fluorescent probe that targets β2 with reasonable selectivity. However, its main usage would be in cell lysate since this probe displays poor cell permeability.

6.4 Experimental

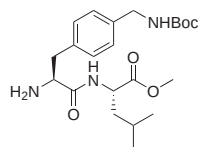
All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.), ethyl acetate (EA), and light petroleum ether (PE) were obtained from Riedel-de Haën or Biosolve and were distilled prior to use. Dichloromethane (DCM), dimethyl formamide (DMF), and dioxane were stored on 4 Å molecular sieves. Tetrahydrofuran (THF) was distilled from LiAlH₄ prior to use. Reactions were conducted under an argon atmosphere. Reactions were monitored by TLC analysis by using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by UV absorption (254 nm), spraying with 20% H₂SO₄ in ethanol or (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid followed by charring at ~150°C or by spraying with an aqueous solution of KMnO₄ (7%) and KOH (2%). Column chromatography was performed on silica gel from Screening Devices (0.040-0.063 nm). LC/MS analysis was performed on a LCQ Advantage Max (Thermo Finnigan) equipped with a Gemini C18 column (Phenomenex). HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ¹H- and ¹³C-APT-NMR spectra were recorded on a Bruker DPX-300 (300/75 MHz) or 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 an internal standard. Coupling constants are given in Hz. All presented ¹³C-APT spectra are proton decoupled.

General protocol for azide couplings

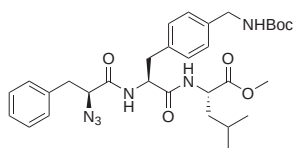
The appropriate hydrazide was dissolved in 1:1 DMF:DCM (v/v) and cooled to -30°C. *t*BuONO (1.1 equiv.) and HCl (4M sln. in 1,4-dioxane, 2.8 equiv.) were added, and the mixture was stirred for 3 hr. at -30°C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. The warhead as a free amine was added to the reaction mixture as a solution in DMF. DiPEA (5 equivalents) was added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EA and extracted with H₂O (3 $\times$). The organic layer was dried over MgSO₄ and purified by flash column chromatography.



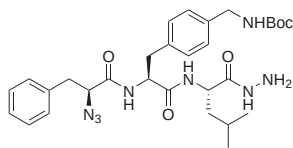
Cbz-Phe(4-CH₂NHBoc)-Leu-OMe (233). Cbz-Phe(4-CH₂NHBoc)-OH **232** (214 mg, 0.5 mmol, 1 equiv.) was dissolved in DCM. HBTU (228 mg, 0.6 mmol, 1.2 equiv.), HCl-H-Leu-OMe (100 mg, 0.55 mmol, 1.1 equiv.) and DiPEA (0.29 ml, 1.75 mmol, 3.5 equiv.) were added and the mixture was stirred for 2 hr before being concentrated. The residue was dissolved in EA and washed with 1M HCl (2 $\times$), sat aq NaHCO₃ (4 $\times$), brine and dried over MgSO₄. Column chromatography (tol $\rightarrow$ 30 EA:tol) yielded the title compound (282 mg, 508 μ mol, quant.). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.35-7.22 (m, 5H), 7.19 - 7.04 (m, 4H), 6.81 (d, *J* = 6.7 Hz, 1H), 5.76 (d, *J* = 8.4 Hz, 1H), 5.16 - 4.94 (m, 2H), 4.69 - 4.45 (m, 2H), 4.26 - 4.11 (m, 2H), 3.67 (s, 3H), 3.13 - 2.88 (m, 2H), 1.64 - 1.47 (m, 3H), 1.44 (s, 9H), 0.94 - 0.81 (m, 6H). ¹³C NMR (101 MHz, CDCl₃): δ ppm 172.83, 170.76, 155.81, 137.23, 136.04, 135.30, 129.37, 128.28, 127.92, 127.70, 127.57, 79.13, 66.70, 55.73, 52.06, 50.51, 44.19, 41.08, 37.94, 28.23, 24.51, 22.52, 21.69.



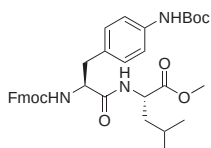
H-Phe(4-CH₂NHBoc)-Leu-OMe (234). Cbz-Phe(4-CH₂NHBoc)-Leu-OMe **233** (282 mg, 508 μ mol) was dissolved in MeOH. TFA (45 μ l, 607 μ mol, 1.2 equiv.) was added and the mixture was bubbled through with Ar for 20 min. 10% Pd/C was added (50 mg) and the flask was filled with an H₂ atmosphere for 3 hr. The mixture was filtered over celite and concentrated. The residue was dissolved in DCM and sat. aq. NaHCO₃ and the aqueous layer was extracted with DCM (3 $\times$). The organic layers were dried over Na₂SO₄ and concentrated to yield the title compound (221 mg, quant.), which was used without further purification. ¹H NMR (400 MHz, CDCl₃): δ ppm 7.67 (d, *J* = 8.6 Hz, 1H), 7.22 (d, *J* = 8.0 Hz, 2H), 7.17 (d, *J* = 8.1 Hz, 2H), 5.11 (s, 1H), 4.61 (td, *J* = 8.9, 5.0 Hz, 1H), 4.28 (d, *J* = 5.6 Hz, 2H), 3.72 (s, 3H), 3.61 (dd, *J* = 9.0, 4.0 Hz, 1H), 3.19 (dd, *J* = 13.7, 3.9 Hz, 1H), 2.72 (dd, *J* = 13.7, 9.0 Hz, 1H), 1.70 - 1.48 (m, 3H), 1.45 (s, 9H), 1.03 - 0.89 (m, 6H). ¹³C NMR (101 MHz, CDCl₃): δ ppm 173.91, 173.25, 155.77, 137.47, 136.49, 129.35, 127.56, 79.21, 56.06, 52.03, 50.13, 44.07, 41.22, 40.26, 28.23, 24.64, 22.69, 21.68.



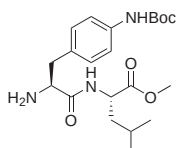
N₃-Phe-Phe(4-CH₂NHBoc)-Leu-OMe (235). H-Phe(4-CH₂NHBoc)-Leu-OMe **234** (68 mg, 161 μ mol, 1 equiv.) was dissolved in DCM (5 ml) and N₃-Phe-OH (37 mg, 193 μ mol, 1.2 equiv.), HBTU (79 mg, 209 μ mol, 1.3 equiv.) and DiPEA (67 μ l, 403 μ mol, 2.5 equiv.) were added and the mixture was stirred for 90 min. before being washed with 1M HCl (2 $\times$), sat. aq. NaHCO₃ (3 $\times$) and dried over Na₂SO₄. Column chromatography (10 EA:tol $\rightarrow$ 35 EA:tol) yielded the title compound (71 mg, 119 μ mol, 74%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.40 - 7.20 (m, 5H), 7.17 (d, J = 7.8 Hz, 2H), 7.04 (d, J = 7.9 Hz, 2H), 6.97 (d, J = 8.1 Hz, 1H), 6.34 (d, J = 7.5 Hz, 1H), 4.93 (s, 1H), 4.67 (dd, J = 14.3, 7.1 Hz, 1H), 4.51 (td, J = 8.2, 5.4 Hz, 1H), 4.26 (d, J = 5.2 Hz, 2H), 4.16 (dd, J = 8.2, 4.2 Hz, 1H), 3.70 (s, 3H), 3.24 (dd, J = 14.1, 4.1 Hz, 1H), 3.08 - 2.82 (m, 3H), 1.62 - 1.46 (m, 3H), 1.45 (s, 9H), 0.88 (d, J = 6.1 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃): δ ppm 172.71, 169.82, 168.38, 155.80, 137.63, 135.83, 135.00, 129.51, 129.42, 128.60, 127.81, 127.24, 79.36, 65.13, 54.03, 52.25, 50.80, 44.37, 41.36, 38.33, 37.89, 28.33, 24.70, 22.58, 21.91.



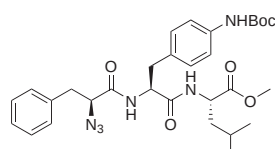
N₃-Phe-Phe(4-CH₂NHBoc)-Leu-NHNH₂ (236). Methyl ester **235** (71 mg, 119 μ mol) was dissolved in MeOH (5 ml). Hydrazine hydrate (350 μ l, 7.1 mmol, 60 equiv.) was added and the mixture was stirred o/n before being coevaporated with tol (3 $\times$). The residue was used without further purification. LC/MS: R_f 7.52 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 595.00 (M + H)⁺). ¹H NMR (400 MHz, CD₃OD): δ ppm 7.26 (ddd, J = 19.9, 13.4, 7.9 Hz, 7H), 7.15 (d, J = 8.0 Hz, 2H), 4.63 (t, J = 7.2 Hz, 1H), 4.36 (dd, J = 9.0, 6.0 Hz, 1H), 4.21 (s, 2H), 4.10 (dd, J = 8.7, 5.1 Hz, 1H), 3.13 (dd, J = 13.9, 5.1 Hz, 1H), 3.06 (dd, J = 13.7, 6.5 Hz, 1H), 2.96 - 2.80 (m, 2H), 1.70 - 1.51 (m, 3H), 1.46 (s, 9H), 0.96 (d, J = 6.4 Hz, 3H), 0.92 (d, J = 6.3 Hz, 3H).



Fmoc-Phe(4-NHBoc)-Leu-OMe (238). Fmoc-Phe(4-NHBoc)-OH (210 mg, 418 μ mol, 1 equiv.) was dissolved in DCM and HCl-H-Leu-OMe (77 mg, 460 μ mol, 1.1 equiv.), HBTU (190 mg, 502 μ mol, 1.2 equiv.) and DiPEA (242 μ l, 1.46 mmol, 3.5 equiv.) were added and the mixture stirred for 1 hr. after which it was washed with 1M HCl (2 $\times$), sat. aq. NaHCO₃ (4 $\times$) and brine and dried over MgSO₄. Column chromatography (10% acetone:PE $\rightarrow$ 20% acetone:PE) yielded the title compound (206 mg, 294 μ mol, 70%). ¹H NMR (400 MHz, CDCl₃): δ ppm 7.76 (d, J = 7.5 Hz, 2H), 7.54 (t, J = 6.9 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.35 - 7.20 (m, 4H), 7.11 (d, J = 7.1 Hz, 2H), 6.54 (s, 1H), 6.29 (d, J = 6.6 Hz, 1H), 5.44 (d, J = 7.2 Hz, 1H), 4.55 (td, J = 8.4, 5.4 Hz, 1H), 4.41 (dd, J = 10.3, 7.2 Hz, 1H), 4.32 (s, 1H), 4.18 (t, J = 6.9 Hz, 1H), 3.68 (s, 3H), 3.15 - 2.94 (m, 2H), 1.65 - 1.39 (m, 3H), 1.51 (s, 9H), 0.97 - 0.78 (m, 6H). ¹³C NMR (101 MHz, CDCl₃): δ ppm 172.74, 170.69, 155.89, 152.64, 143.67, 143.60, 141.16, 137.31, 130.58, 129.83, 127.64, 127.01, 124.98, 119.88, 118.55, 80.41, 67.11, 55.88, 52.17, 50.75, 46.95, 41.31, 37.78, 28.25, 24.63, 22.58, 21.81.

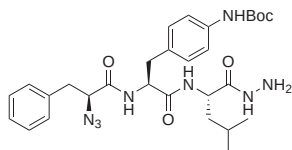


H-Phe(4-NHBoc)-Leu-OMe (239). Fmoc-Phe(4-NHBoc)-Leu-OMe (181 mg, 294 μ mol, 1 equiv.) was dissolved in THF and EtSH (220 μ l, 2.94 mmol, 10 equiv.) and DBU (one drop) were added and the mixture stirred for 30 min. After concentration, column chromatography (50% EA:PE $\rightarrow$ EA $\rightarrow$ 10% MeOH:EA) yielded the title compound (120 mg, 294 μ mol, quant). ¹H NMR (400 MHz, CDCl₃ CD₃OD): δ ppm 7.35 (d, J = 7.8 Hz, 2H), 7.13 (d, J = 8.2 Hz, 2H), 4.48 (t, J = 5.3 Hz, 1H), 3.71 (s, 3H), 3.68 - 3.61 (m, 1H), 3.04 (dd, J = 13.6, 4.8 Hz, 1H), 2.77 (dd, J = 13.6, 7.9 Hz, 1H), 1.66 - 1.56 (m, 3H), 1.52 (s, 9H), 1.08 - 0.81 (m, 6H). ¹³C NMR (101 MHz, CDCl₃ CD₃OD): δ ppm 173.84, 172.56, 153.26, 137.23, 130.17, 128.88, 118.11, 81.21, 55.02, 51.11, 49.95, 39.73, 39.23, 27.10, 23.88, 21.59, 20.30.



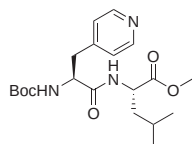
N₃-Phe-Phe(4-NHBoc)-Leu-OMe (240). H-Phe(4-NHBoc)-Leu-OMe (120 mg, 294 μ mol, 1 equiv.) was dissolved in DCM. N₃-Phe-OH (82 mg, 430 μ mol, 1.5 equiv.), HBTU (163 mg, 430 μ mol, 1.5 equiv.) and DiPEA (161 μ l, 975 μ mol, 3.3 equiv.) were added and the mixture stirred for 2 hr. before being washed with 1M HCl (2 $\times$), sat. aq. NaHCO₃ (4 $\times$) and brine and dried over MgSO₄. Column chromatography (10% EA:tol $\rightarrow$ 40% EA:tol) yielded the title compound (160 mg, 276 μ mol, 94%). ¹H

NMR (400 MHz, CDCl_3): δ ppm 7.55 - 7.20 (m, 7H), 7.01 (d, $J = 8.4$ Hz, 2H), 6.92 (d, $J = 8.0$ Hz, 1H), 6.60 (s, 1H), 6.26 (d, $J = 7.9$ Hz, 1H), 4.62 (dd, $J = 14.3, 7.2$ Hz, 1H), 4.50 (td, $J = 8.3, 5.5$ Hz, 1H), 4.18 (dd, $J = 8.2, 4.1$ Hz, 1H), 3.70 (s, 3H), 3.25 (dd, $J = 14.1, 4.1$ Hz, 1H), 3.02 - 2.75 (m, 3H), 1.68 - 1.37 (m, 3H), 1.51 (s, 9H), 0.88 (d, $J = 6.1$ Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3): δ ppm 172.67, 170.14, 168.53, 152.70, 137.36, 135.86, 130.22, 129.80, 129.36, 128.54, 127.15, 118.50, 80.40, 65.02, 53.99, 52.19, 50.86, 41.17, 38.30, 37.60, 28.22, 24.66, 22.53, 21.86.

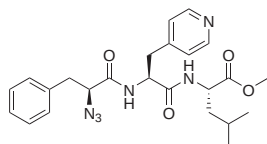


0.93 (d, $J = 6.0$ Hz, 3H), 0.89 (d, $J = 5.9$ Hz, 3H).

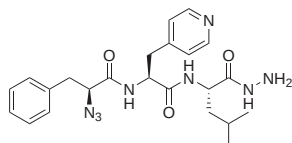
N₃-Phe-Phe(4-NHBoc)-Leu-NHNH₂ (241). N₃-Phe-Phe(4-NHBoc)-Leu-OMe (160 mg, 276 μmol) was dissolved in MeOH and hydrazine hydrate (800 μl , 16.6 mmol, 60 equiv.) was added and the mixture was stirred overnight. Coevaporation with toluene (3 $\times$) yielded the title compound which was used without further purification. LC/MS: R_t 7.54 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 581.13. ^1H NMR (400 MHz, CD_3OD): δ ppm 7.40 - 7.17 (m, 7H), 7.02 (d, $J = 8.5$ Hz, 2H), 4.61 (t, $J = 7.0$ Hz, 1H), 4.35 (t, $J = 7.3$ Hz, 1H), 4.13 (dd, $J = 8.6, 4.8$ Hz, 1H), 3.15 (dd, $J = 14.0, 4.7$ Hz, 1H), 3.00 - 2.81 (m, 3H), 1.60 - 1.45 (m, 3H), 1.51 (s, 9H),



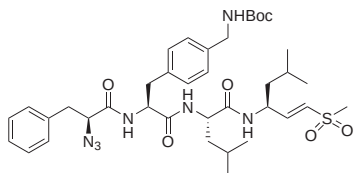
Boc-Ala(4-pyridine)-Leu-OMe (243). Boc-Ala(4-pyridine)-OH (133 mg, 500 μmol , 1 equiv.) was dissolved in DMF and HCl-H-Leu-OMe (92 mg, 550 μmol , 1.1 equiv.), HBTU (228 mg, 600 μmol , 1.2 equiv.) and DiPEA (290 μl , 1.75 mmol, 3.5 equiv.) were added and the mixture stirred for 2 hr. The mixture was diluted with EA and washed with sat. aq. NaHCO_3 . The aqueous layer was extracted with DCM and the combined organic fractions were dried over Na_2SO_4 and concentrated. Column chromatography (1:1 toleae $\rightarrow$ 1:2 $\rightarrow$ 4:1) yielded the title compound (164 mg, 417 μmol , 83%). ^1H NMR (400 MHz, CDCl_3 CD_3OD): δ ppm 8.43 (d, $J = 4.4$ Hz, 2H), 8.14 (d, $J = 7.7$ Hz, 1H), 7.29 (d, $J = 5.1$ Hz, 2H), 6.37 (d, $J = 8.7$ Hz, 1H), 4.57 - 4.49 (m, 1H), 4.49 - 4.41 (m, 1H), 3.72 (s, 3H), 3.18 (dd, $J = 13.8, 5.5$ Hz, 1H), 2.90 (dd, $J = 13.8, 8.7$ Hz, 1H), 1.77 - 1.57 (m, 3H), 1.38 (s, 9H), 0.94 (dd, $J = 9.7, 6.0$ Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3 CD_3OD): δ ppm 172.70, 171.29, 155.41, 148.08, 147.07, 124.75, 79.47, 53.90, 51.53, 50.40, 50.30, 48.39, 48.18, 47.97, 47.75, 47.54, 39.99, 39.94, 37.07, 27.39, 24.13, 22.02, 20.73.



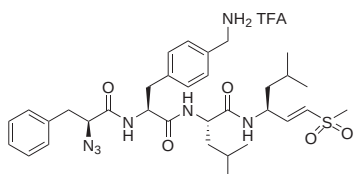
N₃-Phe-Ala(4-pyridine)-Leu-OMe (244). Boc-Ala(4-pyridine)-Leu-OMe (164 mg, 417 μmol) was dissolved in 1:1 DCM:TFA and stirred for 30 minutes before being coevaporated with toluene (3 $\times$). In a separate flask, N₃-Phe-OH (88 mg, 459 μmol , 1.1 equiv.) was dissolved in DCM and HBTU (174 mg, 459 μmol , 1.1 equiv.), DiPEA (310 μl , 1.88 mmol, 4.5 equiv.) were added. After 5 min. of stirring, this mixture was added to the flask containing TFA salt of H-Ala(4-pyridine)-Leu-OMe and this mixture was stirred for 1 hr. before being washed with H_2O (2 $\times$). Column chromatography (DCM $\rightarrow$ 2% MeOH:DCM) yielded the title compound (148 mg, 317 μmol , 76%). ^1H NMR (400 MHz, CDCl_3): δ ppm 8.44 (d, $J = 5.4$ Hz, 2H), 7.39 - 7.10 (m, 7H), 7.00 (d, $J = 5.7$ Hz, 2H), 4.82 (dd, $J = 14.7, 6.7$ Hz, 1H), 4.62 - 4.47 (m, 1H), 4.18 (dd, $J = 8.1, 4.3$ Hz, 1H), 3.71 (s, 3H), 3.23 (dd, $J = 14.0, 4.2$ Hz, 1H), 3.08 - 2.87 (m, 3H), 1.75 - 1.37 (m, 3H), 0.89 (d, $J = 6.1$ Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3): δ ppm 172.70, 169.52, 168.56, 149.45, 145.23, 135.63, 129.36, 128.56, 127.26, 124.71, 64.85, 52.85, 52.25, 50.87, 40.94, 38.12, 37.50, 24.71, 22.54, 21.73.



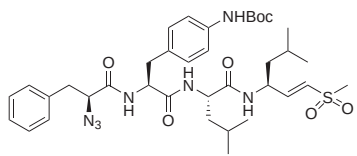
N₃-Phe-Ala(4-pyridine)-Leu-NHNH₂ (245). Methyl ester **244** (113 mg, 242 μmol) was dissolved in MeOH and hydrazine hydrate (700 μl , 14.5 mmol, 60 equiv.) was added and the mixture was stirred o/n. Coevaporation with toluene (3 $\times$) yielded the title compound which was used without further purification. LC/MS: R_t 4.86 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 467.20. ^1H NMR (400 MHz, CD_3OD): δ ppm 8.40 (d, $J = 5.7$ Hz, 2H), 7.35 - 7.17 (m, 5H), 7.14 (d, $J = 5.9$ Hz, 2H), 4.75 (dd, $J = 8.3, 5.6$ Hz, 1H), 4.40 - 4.29 (m, 1H), 4.10 (dd, $J = 8.3, 4.9$ Hz, 1H), 3.22 - 3.05 (m, 2H), 2.92 (ddd, $J = 14.0, 10.7, 8.5$ Hz, 2H), 1.67 - 1.51 (m, 3H), 0.95 (d, $J = 6.1$ Hz, 3H), 0.91 (d, $J = 6.0$ Hz, 3H).



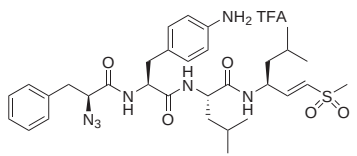
(s, 2H), 4.10 (dd, $J = 8.8, 4.5$ Hz, 1H), 3.22 - 3.11 (m, 1H), 3.05 (dd, $J = 14.0, 5.6$ Hz, 1H), 2.99 (s, 3H), 2.89 (ddd, $J = 27.2, 13.9, 8.4$ Hz, 2H), 1.79 - 1.46 (m, 6H), 1.45 (s, 9H), 1.12 - 0.72 (m, 12H). ^{13}C NMR (101 MHz, CD_3OD): δ ppm 172.02, 170.99, 169.39, 147.06, 137.40, 135.64, 134.44, 128.83, 128.75, 128.10, 127.03, 126.65, 64.29, 53.85, 51.71, 47.46, 43.44, 41.83, 41.73, 40.04, 37.57, 36.77, 27.61, 24.23, 24.20, 22.14, 22.06, 21.02, 20.83. HRMS calcd for $[\text{C}_{38}\text{H}_{56}\text{N}_7\text{O}_7\text{S}]^+$ 754.39564 found 754.39660



40.45, 37.43, 37.13, 24.55, 24.47, 22.00, 20.61. HRMS calcd for $[\text{C}_{33}\text{H}_{48}\text{N}_7\text{O}_5\text{S}]^+$ 654.34321 found 654.34325.



1H), 4.10 (dd, $J = 8.7, 4.5$ Hz, 1H), 3.14 (dd, $J = 13.4, 3.7$ Hz, 1H), 3.04 - 2.93 (m, 1H), 2.98 (s, 3H), 2.86 (dd, $J = 14.1, 9.0$ Hz, 2H), 1.71 - 1.37 (m, 6H), 1.50 (s, 9H), 1.13 - 0.74 (m, 12H). ^{13}C NMR (101 MHz, CDCl_3 , CD_3OD): δ ppm 172.02, 171.17, 169.50, 153.25, 147.01, 137.51, 135.61, 129.54, 129.01, 128.73, 128.67, 128.07, 126.64, 118.27, 64.23, 54.02, 51.82, 47.46, 41.80, 41.60, 39.94, 37.58, 36.36, 27.55, 24.24, 24.16, 22.12, 21.99, 20.94, 20.73. HRMS calcd for $[\text{C}_{37}\text{H}_{54}\text{N}_7\text{O}_7\text{S}]^+$ 740.37999 found 740.38116.



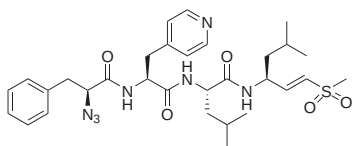
171.37, 170.11, 147.08, 137.83, 136.41, 130.86, 130.80, 129.79, 129.47, 128.96, 128.23, 126.70, 122.37, 64.09, 53.98, 52.23, 47.89, 41.82, 41.39, 40.46, 37.40, 36.90, 24.56, 24.47, 22.00, 20.59. HRMS calcd for $[\text{C}_{32}\text{H}_{46}\text{N}_7\text{O}_5\text{S}]^+$ 640.32756 found 640.32795.

N_3 -Phe-Phe(4- CH_2NHBoc)-Leu-Leu-VS (358). Prepared following the general procedure for azide coupling using N_3 -Phe-Phe(4- CH_2NHBoc)-Leu-NHNH $_2$ (27 mg, 45 μmol) and TFA·H-Leu-VS (50 μmol). Column chromatography (DCM $\rightarrow$ 4% MeOH:DCM) yielded the title compound (19 mg, 25 μmol , 56%). LC/MS: R_t 9.76 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, CD_3OD): δ ppm 7.38 - 7.16 (m, 7H), 7.09 (d, $J = 8.0$ Hz, 2H), 6.81 (dd, $J = 15.1, 5.2$ Hz, 1H), 6.54 (d, $J = 15.1$ Hz, 1H), 4.66 (dd, $J = 9.1, 4.8$ Hz, 1H), 4.62 - 4.59 (m, 1H), 4.35 (t, $J = 7.2$ Hz, 1H), 4.23

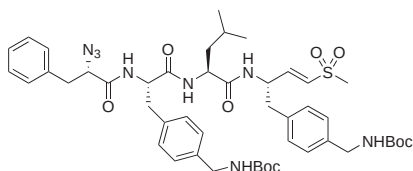
N_3 -Phe-Phe(4- $\text{CH}_2\text{NH}_2\cdot\text{TFA}$)-Leu-Leu-VS (226). N_3 -Phe-Phe(4- CH_2NHBoc)-Leu-Leu-VS was dissolved in 1:1 TFA:DCM and stirred for 30 min before coevaporation with toluene (3 $\times$). ^1H NMR (400 MHz, CD_3OD): δ ppm 7.49 - 7.09 (m, 9H), 6.82 (dd, $J = 15.2, 5.2$ Hz, 1H), 6.63 (dd, $J = 15.2, 1.5$ Hz, 1H), 4.99 - 4.61 (m, 2H), 4.38 (dd, $J = 9.5, 5.4$ Hz, 1H), 4.09 (s, 2H), 4.05 (dd, $J = 9.1, 4.9$ Hz, 1H), 3.23 - 3.08 (m, 2H), 3.00 (s, 3H), 2.98 - 2.81 (m, 2H), 1.82 - 1.43 (m, 6H), 1.13 - 0.77 (m, 12H). ^{13}C NMR (101 MHz, CD_3OD): δ ppm 172.92, 171.56, 170.18, 147.05, 138.08, 136.45, 131.50, 129.81, 129.49, 128.94, 128.68, 128.24, 126.70, 64.07, 54.24, 52.21, 47.90, 42.64, 41.83, 41.39,

N_3 -Phe-Phe(4-NHBoc)-Leu-Leu-VS (359). Prepared following the general procedure for azide coupling using N_3 -Phe-Phe(4-NHBoc)-Leu-NHNH $_2$ 241 (28 mg, 48 μmol) and TFA·H-Leu-VS (53 μmol). Column chromatography (DCM $\rightarrow$ 2% MeOH:DCM) yielded the title compound (18 mg, 24 μmol , 51%). LC/MS: R_t 9.86 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$): δ ppm 7.39 - 7.16 (m, 7H), 7.03 (d, $J = 8.4$ Hz, 2H), 6.77 (dd, $J = 15.1, 5.1$ Hz, 1H), 6.52 (dd, $J = 15.1, 1.3$ Hz, 1H), 4.66 - 4.60 (m, 1H), 4.57 - 4.52 (m, 1H), 4.31 (dd, $J = 9.3, 5.0$ Hz,

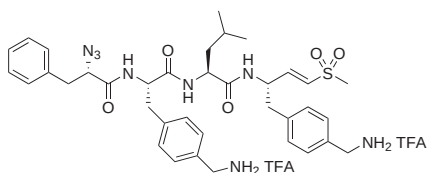
N_3 -Phe-Phe(4-NH $_2\cdot\text{TFA}$)-Leu-Leu-VS (227). N_3 -Phe-Phe(4-NHBoc)-Leu-Leu-VS 359 was stirred with 1:1 TFA:DCM for 30 min. and coevaporated with toluene (3 $\times$). ^1H NMR (400 MHz, CD_3OD): δ ppm 7.38 (d, $J = 8.4$ Hz, 2H), 7.35 - 7.18 (m, 7H), 6.82 (dd, $J = 15.2, 5.2$ Hz, 1H), 6.63 (dd, $J = 15.2, 1.5$ Hz, 1H), 4.78 - 4.61 (m, 2H), 4.38 (dd, $J = 9.6, 5.4$ Hz, 1H), 4.06 (dd, $J = 8.9, 5.0$ Hz, 1H), 3.22 (dd, $J = 14.0, 4.9$ Hz, 1H), 3.11 (dd, $J = 14.0, 5.0$ Hz, 1H), 3.00 (s, 3H), 2.99 - 2.91 (m, 1H), 2.85 (dd, $J = 13.9, 8.9$ Hz, 1H), 1.90 - 1.44 (m, 6H), 1.15 - 0.79 (m, 12H). ^{13}C NMR (101 MHz, CD_3OD): δ ppm 172.96,



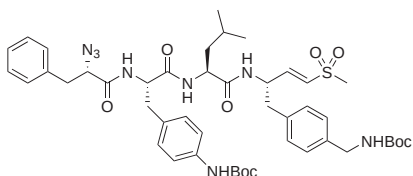
N₃-Phe-Phe(4-pyridine)-Leu-Leu-VS (228). Prepared following the general procedure for azide coupling using N₃-Phe-Phe(4-pyridine)-Leu-NHNH₂ **245** (41 mg, 88 μ mol) and TFA·H-Leu-VS (97 μ mol). Column chromatography (DCM $\rightarrow$ 4% MeOH:DCM + 0.1% AcOH) yielded the title compound (29 mg, 46 μ mol, 53%). LC/MS: R_t 6.48 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CD₃OD): δ ppm 8.42 (d, *J* = 6.0 Hz, 2H), 7.39 - 7.16 (m, 7H), 6.81 (dd, *J* = 15.2, 5.4 Hz, 1H), 6.63 (dd, *J* = 15.2, 1.3 Hz, 1H), 4.78 (dd, *J* = 9.3, 5.0 Hz, 1H), 4.72 - 4.65 (m, 1H), 4.38 (dd, *J* = 9.4, 5.5 Hz, 1H), 4.09 (dd, *J* = 8.5, 5.3 Hz, 1H), 3.25 - 3.18 (m, 1H), 3.12 (dd, *J* = 14.0, 5.3 Hz, 1H), 3.00 (s, 3H), 2.98 - 2.82 (m, 2H), 1.80 - 1.42 (m, 6H), 1.07 - 0.90 (m, 12H). ¹³C NMR (101 MHz, CD₃OD): δ ppm 174.21, 172.33, 171.52, 149.93, 148.87, 148.40, 137.73, 130.95, 130.36, 129.60, 128.09, 126.39, 65.41, 54.48, 53.60, 49.26, 43.29, 42.80, 41.93, 38.68, 38.02, 25.94, 25.85, 23.38, 23.35, 22.05. HRMS calcd for [C₃₁H₄₄N₇O₅S]⁺ 626.31191 found 626.31156



N₃-Phe-Phe(4-CH₂NHBoc)-Leu-Phe(4-CH₂NHBoc)-VS (246). Obtained by the general protocol for azide coupling from N₃-Phe-Phe(4-CH₂NHBoc)-Leu-NHNH₂ (45 mg, 76 μ mol) and H-Phe(4-CH₂NHBoc)-VS (30 mg, 84 μ mol, 1.1 equiv.). Column chromatography (10% acetone:tol $\rightarrow$ 25% acetone:tol) yielded the title compound (21 mg, 23 μ mol, 30%). LC/MS: R_t 10.04 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CDCl₃/CD₃OD): δ ppm 7.38 - 7.06 (m, 13H), 6.83 (dd, *J* = 15.4, 5.2 Hz, 1H), 6.60 - 6.45 (m, 2H), 4.90 - 4.77 (m, 1H), 4.62 - 4.53 (m, 1H), 4.35 - 4.26 (m, 1H), 4.26 - 4.14 (m, 4H), 4.09 (dd, *J* = 8.7, 4.6 Hz, 1H), 3.13 (dd, *J* = 14.1, 4.6 Hz, 1H), 3.05 - 2.80 (m, 7H), 1.59 - 1.49 (m, 3H), 1.45 (s, 9H), 0.93 (d, *J* = 6.0 Hz, 6H), 0.91 - 0.87 (m, 1H). ¹³C NMR (101 MHz, CD₃OD CDCl₃): δ ppm 171.81, 156.29, 145.77, 137.60, 134.82, 129.75, 129.08, 129.03, 128.35, 127.39, 126.95, 79.32, 64.57, 53.86, 51.86, 50.54, 43.78, 42.17, 40.13, 38.99, 37.86, 29.35, 27.96, 24.34, 22.40, 21.19. HRMS calcd for [C₄₇H₆₅N₈O₉S]⁺ 917.45897 found 917.45967.

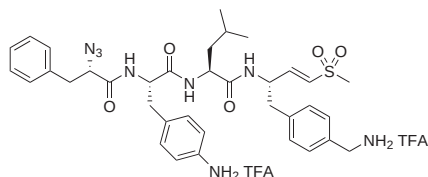


N₃-Phe-Phe(4-CH₂NH₂)-Leu-Phe(4-CH₂NH₂)-VS (229). Boc/protected compound **246** was stirred in 1:1 TFA:DCM for 30 minutes before being coevaporated with toluene (3 $\times$ to yield the title compound quantitatively. LC/MS: R_t 5.35 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CD₃OD): δ ppm 7.53 - 7.20 (m, 13H), 6.84 (dd, *J* = 15.2, 5.3 Hz, 1H), 6.60 (dd, *J* = 15.2, 1.4 Hz, 1H), 4.66 (dd, *J* = 9.6, 4.9 Hz, 1H), 4.33 (dd, *J* = 9.7, 5.0 Hz, 1H), 4.18 - 4.02 (m, 4H), 3.24 - 3.08 (m, 2H), 3.05 (d, *J* = 7.6 Hz, 1H), 2.96 (s, 3H), 2.98 - 2.80 (m, 3H), 1.67 - 1.55 (m, 2H), 1.54 - 1.44 (m, 1H), 1.04 - 0.85 (m, 6H). ¹³C NMR (101 MHz, CD₃OD): δ ppm 174.26, 173.08, 171.72, 146.65, 139.57, 139.45, 137.83, 133.01, 132.95, 131.90, 131.28, 131.14, 130.33, 130.24, 130.11, 129.63, 128.09, 65.50, 65.43, 55.94, 53.72, 52.50, 44.01, 42.80, 41.78, 40.13, 38.85, 38.34, 30.71, 25.87, 23.38, 21.92. HRMS calcd for [C₃₇H₄₉N₈O₅S]⁺ 717.35411 found 717.35415.

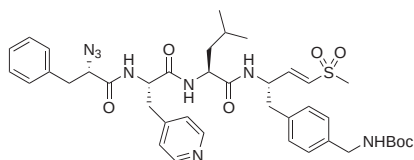


N₃-Phe-Phe(4-NHBoc)-Leu-Phe(4-CH₂NHBoc)-VS (360). Prepared following the general procedure for azide coupling using N₃-Phe-Phe(4-NHBoc)-Leu-NHNH₂ **241** (50 mg, 86 μ mol) and H-Phe(4-CH₂NHBoc)-VS (95 μ mol). Column chromatography (DCM $\rightarrow$ 1.5% MeOH:DCM) yielded the title compound (10 mg, 11 μ mol, 13%). LC/MS: R_t 10.15 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (400 MHz, CD₃OD): δ ppm 7.48 - 7.17 (m, 11H), 7.11 (d, *J* = 8.5 Hz, 2H), 6.83 (dd, *J* = 15.2, 5.2 Hz, 1H), 6.55 (d, *J* = 15.0 Hz, 1H), 4.85 - 4.78 (m, 1H), 4.60 (dd, *J* = 8.2, 6.1 Hz, 1H), 4.28 (dd, *J* = 8.7, 5.6 Hz, 1H), 4.18 (s, 2H), 4.13 (dd, *J* = 8.7, 5.0 Hz, 1H), 3.14 (dd, *J* = 14.0, 4.9 Hz, 1H), 3.02 (dd, *J* = 14.0, 6.0 Hz, 2H), 2.94 (s, 3H), 2.89 (dd, *J* = 14.0, 8.8 Hz, 3H), 1.51 (d, *J* = 4.4 Hz, 9H), 1.55 - 1.47 (m, 3H), 1.46 (s, 9H), 0.94 (d, *J* = 6.3 Hz, 3H), 0.89 (d, *J* = 6.3 Hz, 3H). ¹³C NMR (101 MHz, CD₃OD): δ

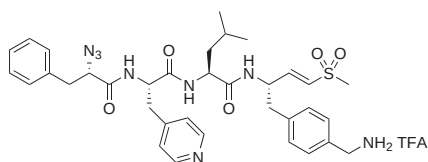
ppm 174.04, 173.26, 171.66, 155.20, 146.97, 139.57, 139.53, 137.80, 136.90, 131.79, 131.70, 130.71, 130.51, 130.38, 129.61, 128.47, 128.07, 119.84, 80.79, 80.16, 65.56, 55.95, 53.72, 52.54, 44.74, 42.83, 41.68, 40.01, 38.89, 37.98, 28.79, 28.71, 25.76, 23.38, 21.99. HRMS calcd for $[C_{46}H_{63}N_8O_9S]^+$ 903.44332 found 903.44439.



$J = 6.3$ Hz, 3H), 0.92 (d, $J = 6.3$ Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD): δ ppm 174.30, 172.89, 171.64, 146.60, 139.62, 139.53, 137.81, 133.03, 132.17, 131.92, 131.31, 130.91, 130.34, 130.24, 129.64, 128.11, 123.96, 65.52, 55.62, 53.71, 52.56, 44.02, 42.77, 41.82, 40.15, 38.82, 38.15, 25.88, 23.39, 21.90. HRMS calcd for $[C_{36}H_{47}N_8O_5S]^+$ 703.33846 found 703.33842.



1H), 6.56 (d, $J = 15.3$ Hz, 1H), 4.81 (s, 1H), 4.74 (dd, $J = 9.2, 5.2$ Hz, 1H), 4.42 - 4.28 (m, 1H), 4.19 (s, 2H), 4.09 (dd, $J = 8.4, 5.4$ Hz, 1H), 3.24 - 3.05 (m, 3H), 2.94 (s, 3H), 3.02 - 2.84 (m, 3H), 1.71 - 1.47 (m, 3H), 1.45 (s, 9H), 0.97 (d, $J = 6.3$ Hz, 3H), 0.92 (d, $J = 6.4$ Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD): δ ppm 172.69, 170.90, 170.13, 148.54, 147.45, 145.58, 136.32, 135.57, 130.35, 129.17, 128.97, 128.21, 127.08, 126.69, 125.02, 63.99, 53.12, 52.18, 51.30, 43.33, 41.41, 40.49, 38.65, 37.28, 36.63, 27.38, 24.45, 21.97, 20.63. HRMS calcd for $[C_{40}H_{53}N_8O_7S]^+$ 789.37524 found 789.37541.



1H), 1.73 - 1.32 (m, 3H), 0.98 (d, $J = 6.4$ Hz, 3H), 0.92 (d, $J = 6.4$ Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD): δ ppm 174.27, 171.64, 146.56, 143.83, 139.70, 137.62, 134.54, 133.05, 131.97, 131.34, 130.42, 130.25, 129.62, 128.82, 128.15, 65.27, 54.15, 53.76, 52.76, 44.01, 42.76, 41.87, 40.02, 38.88, 38.52, 25.88, 23.39, 21.86. HRMS calcd for $[C_{35}H_{45}N_8O_5S]^+$ 689.32281, found 689.32267.

N_3 -Phe-Phe(4- NH_2 -TFA)-Leu-Phe(4- CH_2NH_2 -TFA)-VS

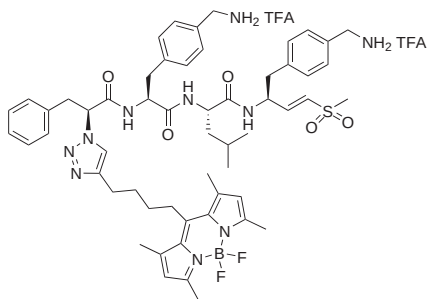
(230). N_3 -Phe-Phe(4-NHBoc)-Leu-Phe(4- CH_2NHBoc)-VS **361** was stirred with 1:1 TFA:DCM for 30 min. and coevaporated with toluene (3 $\times$). ^1H NMR (400 MHz, CD_3OD): δ ppm 7.51 - 7.08 (m, 13H), 6.83 (dd, $J = 15.2, 5.4$ Hz, 1H), 6.59 (dd, $J = 15.2, 1.3$ Hz, 1H), 4.88 - 4.79 (m, 1H), 4.69 (dd, $J = 9.9, 4.8$ Hz, 1H), 4.33 (dd, $J = 9.8, 5.0$ Hz, 1H), 4.09 (s, 2H), 4.08 - 3.96 (m, 1H), 3.23 (dd, $J = 14.1, 4.8$ Hz, 1H), 3.11 (dd, $J = 14.0, 5.0$ Hz, 1H), 3.05 (dd, $J = 10.3, 3.0$ Hz, 2H), 3.02 - 2.91 (m, 1H), 2.96 (s, 3H), 2.83 (dd, $J = 14.0, 9.0$ Hz, 1H), 1.72 - 1.40 (m, 3H), 0.98 (d,

N_3 -Phe-Phe(4-pyridine)-Leu-Phe(4- CH_2NHBoc)-VS (362).

Prepared following the general procedure for azide coupling using N_3 -Phe-Ala(4-pyridine)-Leu-NHNH $_2$ **245** (27 mg, 58 μ mol) and H-Phe(4- CH_2NHBoc)-VS (64 μ mol). Column chromatography (DCM $\rightarrow$ 6% MeOH:DCM) partially purified the title compound (7 mg, 9 μ mol, 15%, purity about 85%). LC/MS: R_t 7.15 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, CD_3OD): δ ppm 8.40 (d, $J = 6.1$ Hz, 2H), 7.47 - 7.19 (m, 11H), 6.85 (dd, $J = 15.3, 5.5$ Hz,

N_3 -Phe-Phe(4-pyridine)-Leu-Phe(4- CH_2NH_2 -TFA)-VS

(231). N_3 -Phe-Phe(4-pyridine)-Leu-Phe(4- CH_2NHBoc)-VS **362** was stirred with 1:1 TFA:DCM for 30 min. and coevaporated with toluene (3 $\times$). ^1H NMR (400 MHz, CD_3OD): δ ppm 8.67 (d, $J = 6.0$ Hz, 2H), 7.79 (d, $J = 6.3$ Hz, 2H), 7.48 - 7.34 (m, 4H), 7.32 - 7.09 (m, 5H), 6.82 (dd, $J = 15.2, 5.5$ Hz, 1H), 6.58 (dd, $J = 15.2, 1.4$ Hz, 1H), 4.85 - 4.65 (m, 2H), 4.30 (dd, $J = 9.9, 5.1$ Hz, 1H), 4.19 - 4.05 (m, 1H), 4.10 (s, 2H), 3.43 (dd, $J = 14.0, 5.4$ Hz, 1H), 3.23 - 3.00 (m, 4H), 2.95 (s, 3H), 3.00 - 2.88 (m,



BODIPY-triazole-Phe-Phe(4-CH₂NH₂·TFA)-Leu-Phe(4-CH₂NH₂·TFA)-VS (247). Azide **229** (3.94 mg, 4.17 μ mol) was dissolved in 1:1:1 *t*BuOH:H₂O:MeCN (0.5 ml) and the solution was degassed with Ar. BODIPY-alkyne¹¹⁵ (2 mg, 6.3 μ mol, 1.5 equiv.) and TBTA (2.2 mg, 4.17 μ mol, 1 equiv.) were added, followed by Cu(MeCN)₄PF₆ (1.56 mg, 4.17 μ mol, 1 equiv.). The mixture was stirred overnight under an argon atmosphere. EDTA (8.7 mg, 21 μ mol, 5 equiv.) in H₂O (300 μ l) was added and this mixture was directly subjected to HPLC purification (38–48% MeCN in H₂O + 0.1 % TFA) to afford the product (1.85 mg, 1.49 μ mol, 36%). LC/MS: *R*_t 6.48 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ¹H NMR (600 MHz, CD₃OD): δ ppm 7.44–7.30 (m, 4H), 7.28–7.14 (m, 4H), 7.01 (d, *J* = 13.3 Hz, 7H), 6.78 (dd, *J* = 15.1, 5.4 Hz, 1H), 6.55 (d, *J* = 15.1 Hz, 1H), 6.11 (s, 2H), 5.47 (d, *J* = 16.2 Hz, 1H), 4.60 (d, *J* = 5.3 Hz, 1H), 4.34–4.28 (m, 1H), 4.11–3.96 (m, 4H), 3.28–2.84 (m, 6H), 2.92 (s, 3H), 2.79–2.67 (m, 2H), 2.44 (s, 6H), 2.40–2.31 (m, 8H), 1.86 (s, 2H), 1.64–1.28 (m, 7H), 0.99–0.85 (m, 6H). HRMS calcd for [C₅₆H₇₂BF₂N₁₀O₅S]⁺ 1045.54635, found 1045.54639.

Competition experiments in cell lysate

HEK293T or RAJI cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 μ g/ml streptomycin in a 7% CO₂ humidified incubator at 37°C. Cells were harvested, washed 2 $\times$ with PBS and permeated in digitonin lysis buffer (4 $\times$ pellet volume, 50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl₂, 5 mM DTT, 0.025% digitonin) for 15 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 (Biorad). 15 μ g (HEK293T) or 10 μ g (RAJI) total protein per experiment was exposed to the inhibitors (10 $\times$ solution in DMSO) for 1 hr. at 37°C prior to incubation with MVB003 (500 nM) for 1 hr. at 37°C. Reaction mixtures were boiled with Laemmli's buffer containing β -mercaptoethanol for 3 min. before being resolved on 12.5% SDS-PAGE. In-gel detection of fluorescently labelled 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 nm, λ_{em} 560 nm) for MVB003 or Cy2 settings (λ_{ex} 488 nm, λ_{em} 520 nm) for **247**.

Competition experiments in living cells

1 $\times$ 10⁶ HEK293T or RAJI cells were seeded and grown overnight. 100 $\times$ stock solution of inhibitor was added to 1 ml medium and the cells were incubated for 4 hr. at 37°C. MVB003 (100 $\times$ stock solution in DMSO, 5 μ M end concentration) was added and incubated for 2 hr. at 37°C. Cells were harvested, washed 2 $\times$ with PBS and permeated in digitonin lysis buffer (4 $\times$ pellet volume, 50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl₂, 5 mM DTT, 0.025% digitonin) for 15 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 (Biorad). 10 μ g protein (dilution with lysis buffer) was boiled in Laemmli's sample buffer and resolved as described above. Coomassie Brilliant Blue staining of the gel verified protein loading.

7

Summary and Future Prospects

This thesis aims at finding proteasome inhibitors with unique characteristics and focuses on subunit selectivity. In **Chapter 1**, an overview of proteasome inhibitors has been provided, detailing both those found in nature and several originating from synthetic efforts. While proteasome-selective inhibition can be achieved with the natural product epoxomicin, among others, a full panel of subunit selective and cell-permeable inhibitors is not yet available. In particular, the discrimination of an inhibitor between a constitutive subunit and its immune-counterpart is a remaining challenge. Subunit selective inhibitors can greatly aid the determination of the role of a specific subunit in a biological process, for example the generation of peptides that are presented on the MHC class-I receptor. Subunit selective inhibitors can also help in determining the ideal proteasome inhibition profile for clinically used proteasome inhibitors targeted against various cancers. This is illustrated by the finding that while $\beta 5$ inhibition is essential for cytotoxicity against various cancer cell lines, pure $\beta 5$ inhibition is very often not enough to bring about the cytotoxic effect and partial co-inhibition of $\beta 1$ or $\beta 2$ is also necessary.^{127,128}

As has been described in **Chapter 2**, the nature of the electrophilic trap in three inhibitors selective for $\beta 5/\beta 5i$ influences the potency and proteasome subunit selectivity pattern. Epoxyketone YU101 (**71**) shows preference for $\beta 5/\beta 5i$ but this compound can also target $\beta 1$ and $\beta 2$ at concentrations where full $\beta 5$ inhibition was reached. Vinyl sulfone analogue YU101-VS (**82**) displays more selective $\beta 5$ inhibition than epoxyketone **71**. Carfilzomib (**52**) preferentially targets the $\beta 5/\beta 5i$ subunits and displays a slight bias against $\beta 5i$. The vinyl sulfone analogue of carfilzomib, PR171-VS (**83**), in contrast, is equipotent against $\beta 5$ and $\beta 5i$. Vinyl sulfone **83** is also more selective for $\beta 5/\beta 5i$ over the other active subunits than carfilzomib (**52**). Epoxyketone PR957 (**81**) is selective for $\beta 5i$ over the other subunits, but its vinyl sulfone analogue PR957-VS (**84**) displays low potency and subunit selectivity compared to PR957.

In an attempt to discover more electrophiles able to target the proteasome, ten Michael acceptors have been synthesised as described in **Chapter 3**. However, these electrophilic traps, when coupled to appropriate peptide fragments, do not potently bind the proteasome. This is remarkable, considering the breadth of range of electrophiles able to potently bind the proteasome. Two azide containing peptide Michael acceptors from this library were incubated in HEK293T and RAW264.7 cells. The cellular targets of these compounds were determined by introduction of a biotin with the Staudinger-Bertozzi ligation, pull-down of biotin-containing protein and analysis by LC/MS. Surprisingly, catalytically active proteasomal subunits were pulled out, indicating these two compounds do inhibit the proteasome, although with low potency. Along with the proteasomal subunits, several cathepsins were also identified and this encourages further screening of the compounds described in **Chapter 3** for their ability to inhibit cysteine protease family members.

Chapter 4 describes the effect of incorporation of the valine-urea-valine motif, present in syringolin A, into peptoid vinyl sulfone and epoxyketone inhibitors. A dramatic difference in subunit preference was found when the distance between ureido linkage and electrophile was changed. Tripeptide **182** (Figure 7.1) might well be the most potent β 1 inhibitor in living cells reported to date. Tetrapeptides **190** and **192**, on the other hand, prefer β 5 more. Compound **190** and **192** also potently inhibit the proteasome in cells, and are more potent than syringolin A.

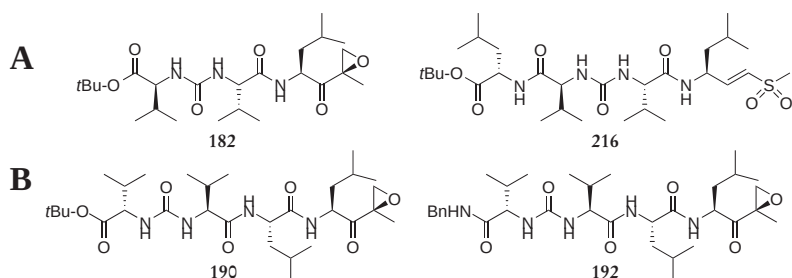
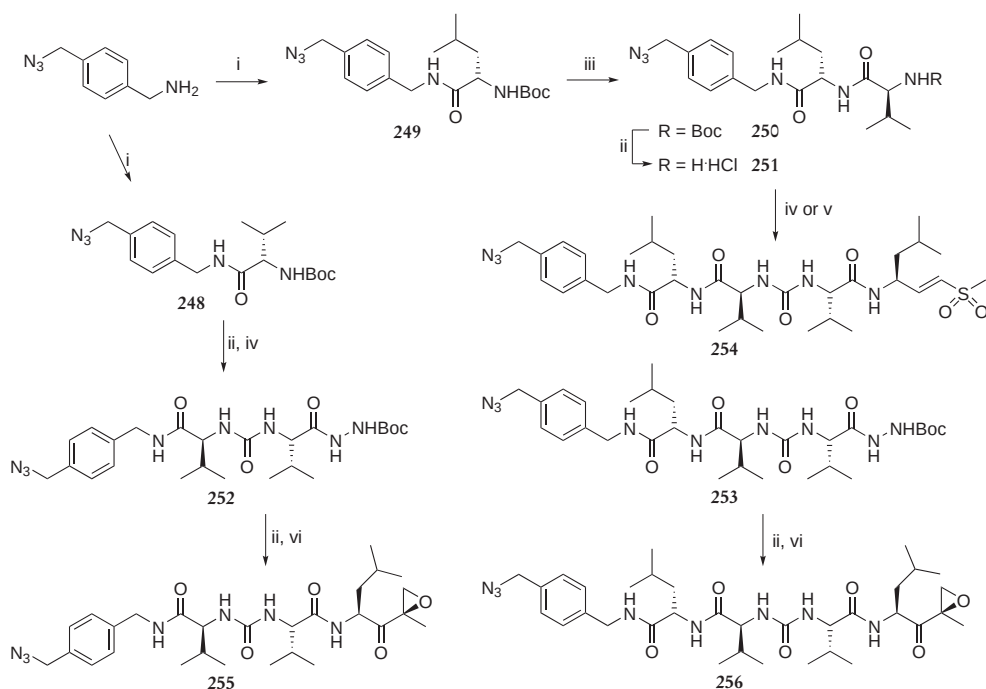


Figure 7.1: Ureido linkage containing proteasome inhibitors. Urea moiety after P2 (**A**) yields β 1 selective compounds **182** and **216**. Urea moiety after P3 (**B**) yields β 5 preferring inhibitors **190** and **192**.

An immunoproteasome specific inhibitor is a great asset to the biological community since it can be used to determine the role of the immunoproteasome in various processes. The immunoproteasome may be a tumour marker and a immunoproteasome selective fluorescent imaging probe can be very valuable as a tool for cancer screening.¹³⁶ Compounds **182** and **216** show some preference for β 1i over β 1. In an attempt to obtain β 1i selective activity based probes, the tBu ester in **216** and **182** was replaced by a para-azidomethylene benzylamide to lead to compounds **254** and **255**, respectively (Scheme 7.1). The epoxyketone version of **254**, compound **256**, was also synthesised.

Coupling 4-(azidomethyl)-benzylamine²⁵¹ to Boc-Val-OH and Boc-Leu-OH yielded amides **248** and **249** (Scheme 7.1). Boc protected compound **249** was deprotected with HCl and coupled to Boc-Val-OH to yield dipeptide **250**. The Boc group in **250** and dipeptide vinyl sulfone Boc-Val-Leu-VS (**194**) were removed with HCl. The amine group from HCl:H-Val-Leu-VS was transformed to its isocyanate and reacted with dipeptide **251** to

Scheme 7.1: Synthesis of potential β 1i selective probes.

Reagents and conditions: i) Boc-Leu-OH or Boc-Val-OH (1.1 equiv.), HBTU (1.2 equiv.), DiPEA (2.5 equiv.), DCM, 59% (**248**), 48% (**249**). ii) 4 M HCl/dioxane, 1 hr. quant. iii) (a) TFA:DCM 1:1, 30 min, quant. (b) Boc-Val-OH (1.1 equiv.), HBTU (1.15 equiv.), DiPEA (3.5 equiv.), DMF, 1 hr., 70%. iv) Phosgene (2 equiv.), DCM:sat. aq. NaHCO_3 , 10 min., 0°C , then H-Val-NHNHBoc (1.1 equiv.), DCM, 57% (**252**), 76% (**253**). v) First HCl-H-Val-Leu-VS (1.28 equiv.), phosgene (2 equiv.), DCM:sat. aq. NaHCO_3 , 10 min., 0°C , then **251** (1 equiv.), DiPEA (2 equiv.), DCM, 30% vi) *t*BuONO (1.1 equiv.), HCl (1.8 equiv.), DMF:EA 3:1, -30°C , 3hr. then H-Leu-EK (1.1 equiv.), DiPEA (5 equiv.), DMF, $-30^\circ\text{C} \rightarrow \text{RT}$ o/n, 41% (**255**), 53% (**256**).

yield **254**. Compound **248** was treated with HCl and transformed to its isocyanate counterpart before reaction with H-Val-NHNHBoc (**202**) to yield protected hydrazide **252**. Protected hydrazide **253** was synthesised from the isocyanate of **251** and H-Val-NHNHBoc (**202**). Boc-hydrazides **252** and **253** were deprotected and coupled to leucine epoxyketone to yield tripeptide **255** and tetrapeptide **256** in the azide coupling reaction.

To assess the subunit selectivity of probes **254**, **255** and **256**, these compounds were used in a fluorogenic substrate hydrolysis assay with purified proteasome. Next, 3T3 cells were incubated with or without interferon- γ for 48 hours to induce the immunoproteasome, after which the cells were incubated with the three probes for 2 hr. The cells were harvested, lysed and remaining proteasome activities labelled with MVB003 (**49**, $0.5 \mu\text{M}$). The results are summarised in Table 7.1A and B.

The fluorogenic peptide assay showed that **254** is not active against constitutive or immunoproteasome (Table 7.1A). This is in contrast to the competition assay, where **254** appears to inhibit β 1i and β 2i, although not potently (Table 7.1B). A big difference between MVB003 competition and fluorogenic substrate assay was also observed for **255**. This compound has almost the same potency for β 1 and for β 1i and its selectivity over

Table 7.1: Apparent IC₅₀ (μM) values calculated from semi log plots of residual proteasome activity against inhibitor concentration. (A) Fluorogenic substrate assay and (B) Competition assay in 3T3 cells versus MVB003.

	Fluorogenic peptide assay						Quantified competition assay					
	β1	β2	β5	β1i	β2i	β5i	β1	β2	β5	β1i	β2i	β5/β5i
	26S proteasome			Immunoproteasome			- INF-γ			+ INF-γ		
Cmp	IC ₅₀ (μM) A			IC ₅₀ (μM) A			IC ₅₀ (μM) B			IC ₅₀ (μM) B		
254	N.I.	N.I.	N.I.	>500	N.I.	>500	N.I.	N.I.	N.I.	11	22	>50
255	0.48	2.7	37	0.40	31	5.7	0.95	N.I.	9.5	0.10	N.I.	>10
256	135	N.I.	>500	104	>500	300	>10	N.I.	N.I.	0.27	≫10	N.I.

(A) Constitutive proteasomes purified from rabbit muscles or immunoproteasomes, purified from rabbit spleens, were incubated with different concentrations of inhibitors for 30 min. at 37°C followed by measuring remaining activity with fluorogenic peptides (Suc-LLVY-AMC, β5(i), Ac-LPnLD-AMC, β1(i), Ac-RLR-AMC β2(i)) or (B) 3T3 cells were grown for 48 hr. with or without 100 units interferon-γ and then incubated 2 hr. with inhibitor (**254** 0.05-50 μM, **255** 0.005-5 μM, **256** 0.01-10 μM). After cell harvest and lysis, residual proteasome activity readout with MVB003 (0.5 μM). band intensities from each lane of competition assay gels (not shown) were quantified and used as input. N.I. no inhibition.

β2/β2i and β5/β5i is only about five fold in the fluorogenic substrate assay (Table 7.1A). In the competition assay this compound has almost tenfold preference for β1i over β1. Tetrapeptide epoxyketone **256** shows low potency and subunit preference in the fluorogenic peptide assay, whereas the competition assay identified β1i as the only target inhibited with submicromolar potency. In a preliminary study to more directly analyze the specificity of compounds **254**, **255** and **256**, cell extracts from the inhibitor treated interferon-γ induced and uninduced 3T3 cells were labelled with biotin by means of the Staudinger-Bertozzi ligation (200 μM **154** for 1 hr.). Streptavidin-HRP readout of the Western blots revealed probe **254** labels β1 and β1i at a concentration of 5 μM and higher, **255** labels both β1 and β1i from 10 nM and higher and bands for β1 and β1i appeared at 0.5 μM and higher concentrations for **256**. Thus, none of the three inhibitors are selective for β1i over β1 (data not shown).

The selectivity and potency of epoxyketone probes **255** and **256** for β1i may be improved by application of previous optimisation efforts from literature.^{13,130,131,135} First, the bulky silyl ether at P1' as found in β1i preferring inhibitor **61** (Figure 1.16, page 18) can be introduced to have more discrimination between β1 and β1i (Figure 7.2A).¹³⁵ Next, activity towards β5 may be reduced by using smaller residues at the pseudo-N-terminus of the molecule, for example the use of glycine at P4 instead of leucine or an alkyl chain instead of the bulky aromatic group.¹³¹ Introduction of norleucine at P2 and proline at P3 may reduce β5 inhibition even more.^{13,131} The ureido linkage induces chain reversal in a peptide and thus the orientation of amino acid residues at P3 and P4 may be unfavourable. Although a D-amino acid scan for the two amino acids flanking the ureido linkage in syringolin A showed that the L-configuration yields the most active syringolin,¹⁹⁴ D-amino acids at P3 or P4 of peptoid epoxyketone analogues may give different results because of the structural differences between syringolin A and linear peptoid inhibitors.

Amino acid sequence alignment and modelling studies of the constitutive proteasome and immunoproteasome indicated that the β1i S1 pocket is more hydrophobic and constricted than β1 S1 pocket.^{5,11,252} Commonly, Leu or Asp has been used as P1 residue in

inhibitors selective for $\beta 1$. Use of a large acidic P1 residue could abrogate $\beta 1i$ inhibition while leaving $\beta 1$ inhibition unhampered. Candidate P1 residues for $\beta 1$ selectivity are glutamate, homoglutamate or alanine(p-benzoic acid) (Figure 7.2B).

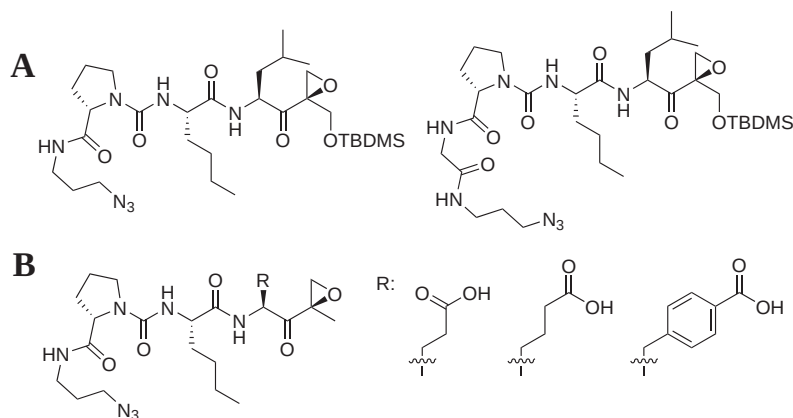


Figure 7.2: (A) Potential optimisation of $\beta 1i$ immunoproteasome probes. (B) Potential probes with selectivity for $\beta 1$ over $\beta 1i$.

The preference for existing $\beta 5$ selective inhibitors may be increased further by integrating the ureido linkage in their design. Hybridizing fluorinated peptide **77**¹⁴⁷ and tetrapeptide epoxyketones **190** and **192** (Figure 7.1B) yields potential $\beta 5$ selective compounds **257** or **258** (Figure 7.3). The incorporation of the ureido linkage in NC005 (**259**, Figure 7.3) can be accomplished by transforming commercially available 2-naphthylmethyleamine into its isocyanate and reaction with H-Phe-Tyr-Leu-EK, an intermediate in the synthesis of NC005,¹³³ to yield **260**. Then, the use of pentafluorophenylalanine at P2 yields the potentially even more selective inhibitor **261**.

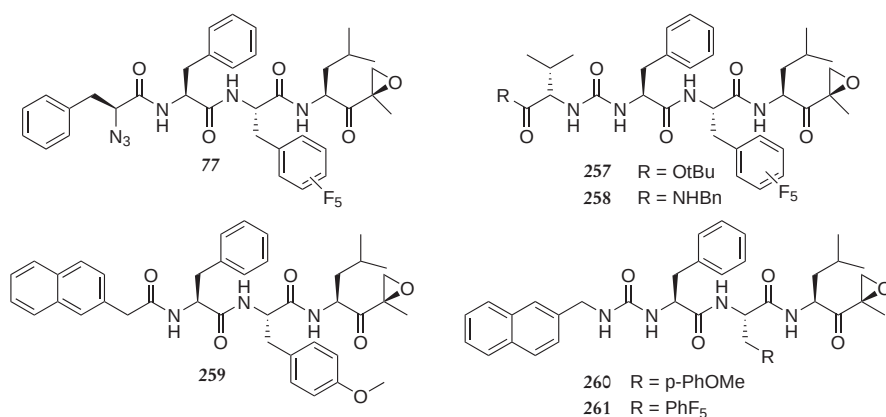


Figure 7.3: Combining structural elements favourable for $\beta 5$ selective inhibition.

Previously, N_3 -Phe-Leu₂-Phe(4-CH₂NH₂)-VS (**70**) was synthesised, which appears to be a cell permeable compound with reasonable selectivity for $\beta 2$.¹⁴¹ Chapter 6 has described the synthesis of a focused library of peptide vinyl sulfones with a basic residue at P1 and/or P3. Depending on the pK_a of the P1 residue and to a lesser extent the P3 residue, selectivity for $\beta 2$ can be tuned.

Compound **229**, with both at P1 and P3 the alanine-4-benzylamine residue (Figure 7.4), is a much more $\beta 2$ selective inhibitor than **70** in cell lysate. However, its entry into cells is much less efficient than **70**, probably due to the presence of a second benzyl amine. Interestingly, in RAJI cells, $\beta 2$ was much more inhibited by **229** than in HEK293T cells. Recently, other compounds with $\beta 2$ selectivity have been published which are based on arginine epoxyketone. Of these inhibitors, **69** (Figure 7.5) showed $\beta 2$ selectivity and cell permeability,¹²⁸ and this is in agreement with what is found for **262** (Figure 7.5).¹⁴¹ This shows that the peptide sequence of an inhibitor can be optimized for

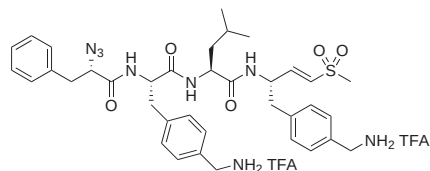


Figure 7.4: Structure of **229**.

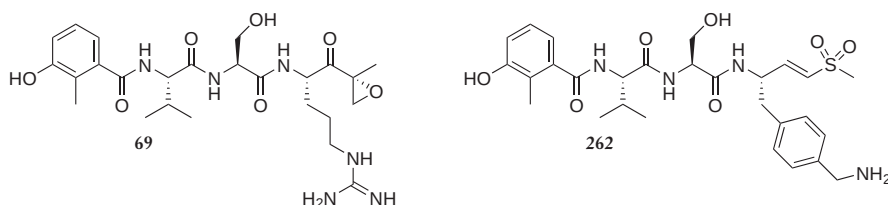
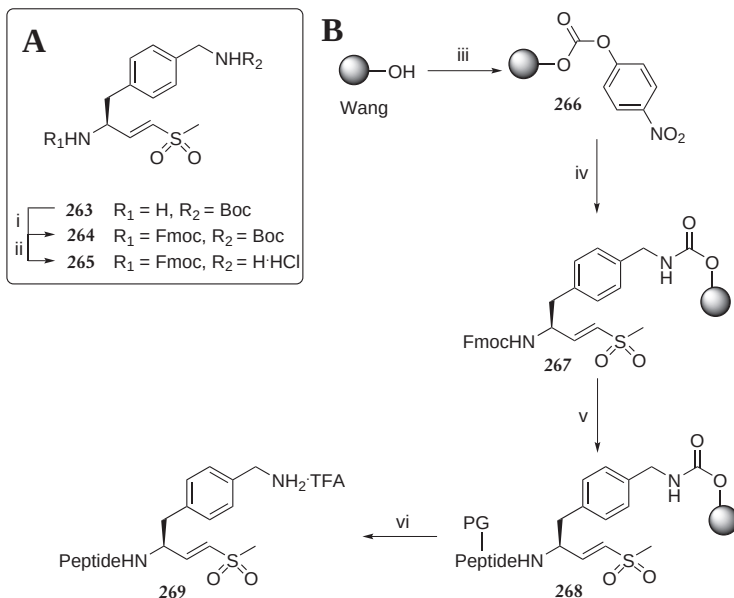


Figure 7.5: Improved $\beta 2$ selective proteasome inhibitors from literature.

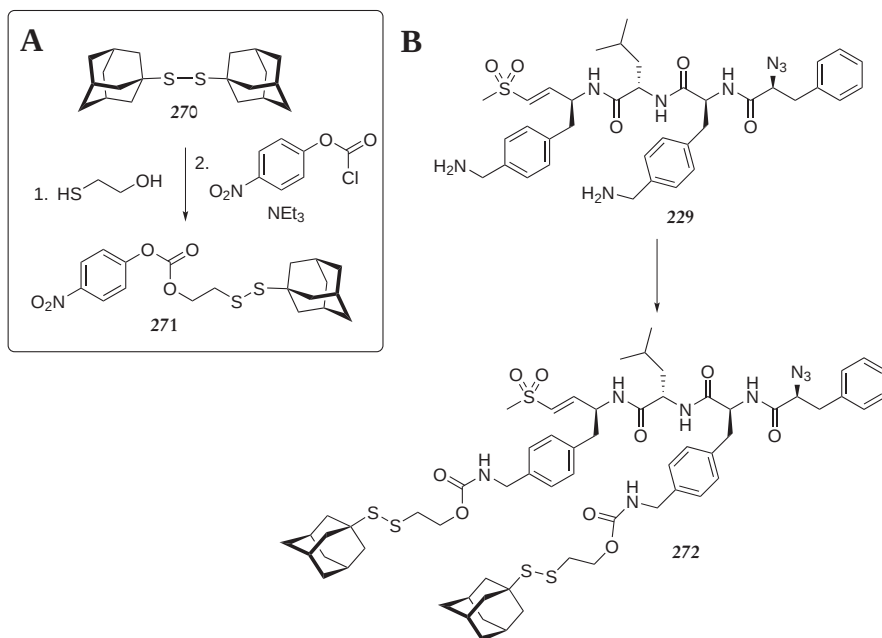
$\beta 2$ inhibition. A powerful strategy for amino acid sequence optimisation of an inhibitor is the synthesis of positional scanning libraries containing both natural and unnatural amino acids. A requirement of this strategy is solid phase peptide synthesis of the library, and this can be achieved by attaching the benzylamine side chain to a resin. To facilitate the attachment of the alanine-4-benzylamine vinyl sulfone on resin, amine **263** was protected with FmocCl to yield **264**, which was deprotected with HCl to yield **265** (Scheme 7.2). Wang resin was activated with p-nitrophenyl chloroformate to give resin **266**.²⁵³ This resin was allowed to react with **265** in the presence of base to yield carbamate resin **267**. Resin loading was about 50% of theoretical maximum loading. Next, standard Fmoc-based solid phase peptide chemistry led to the formation of resin-bound peptide **268**. Unfortunately, during the SPPS, accumulation of side products on the resin occurred, mainly due to esterification of activated amino acids with Wang-hydroxyls. Before the final product (**269**) was liberated from resin, saponification of the ester side products was attempted. However, many side products were released when the resin was subjected to strong acidic cleavage. Thus, before this strategy can be applied for the synthesis of libraries of $\beta 2$ selective candidates, resin loading should be optimised and subsequently, unreacted immobilized hydroxyl functions should be capped.

Scheme 7.2: (A) Synthesis of Fmoc protected vinyl sulfone to be coupled on resin. (B) Solid phase synthesis of β 2 selective inhibitors.



Reagents and conditions: i) Fmoc-Cl (1.1 equiv.), DiPEA (1.5 equiv.), EA:DMF 20:1, 0°C, 63%. ii) 4M HCl/dioxane, 1 hr., quant. iii) p-nitrophenylchloroformate (2 equiv.), N-methylmorpholine (2 equiv.), DCM, 0° $\rightarrow$ RT o/n. iv) 265 (2 equiv.), HOBt (3 equiv.), DiPEA (6 equiv.), DMF, RT, 24 hr., 50% theoretical loading. v) SPPS vi) (a) sat. LiOH in 1:1 THF:H₂O, 2 hr. (b) 95% TFA/H₂O, 1.5 hr.

Scheme 7.3: (A) Synthesis of disulfide containing protecting group. (B) Synthesis of potential prodrug 272.



Compound **229** displays very selective $\beta 2$ inhibition but a lack of cell permeability renders this compound unsuitable for in vivo studies. Transiently protecting the benzyl amines on **229** may improve cell permeability characteristics. This can be done by protecting the amines in **229** in the form of a self-immolative disulfide carbamate (**272**, Scheme 7.3).^{254–256} The cytosol of a cell contains high concentrations of glutathione and these free thiols are able to reduce the disulfide bridges in **272**, releasing the active inhibitor **229**. One very important requirement is that prodrug **272**, or the form in which one of the two protecting groups has been released, does not inhibit any proteasomal activities. Therefore, the large adamantyl group is chosen, since the bulky adamantyl group may increase cell-permeability and hamper proteasomal recognition due to steric hindrance. The disulfide linker can be synthesised by reacting 1-adamantyl disulfide (**270**)²⁵⁷ with β -mercaptoethanol, followed by conversion of the alcohol to activated carbonate **271**,²⁵⁵ which can be used to install the carbamate onto **229** to give bisprotected **272** (Scheme 7.3).

Loidl. et al. have demonstrated that a bivalent inhibitor, which contains two tripeptide aldehydes separated by a PEG-spacer, displays a 100-fold increase in potency with respect to its mono-aldehyde counterpart.¹³⁹ Depending on the amino acids in the tripeptide(s), these molecules potentially inhibit the $\beta 2$, $\beta 5$ or both $\beta 2$ and $\beta 5$ subunits of the proteasome. The synergy of proteasome inhibition displayed by two appropriately spaced electrophiles can be applied to yield proteasome inhibitors with unique subunit selectivity. The subunits of the 20S proteasome have characteristic intersubunit distances (Figure 7.6A) and a bivalent inhibitor of appropriate length can inhibit only a limited number of subunits with this synergy and thus potency.^{11,139} Furthermore, the profound increase in potency may only be induced by the unique shape of the proteasome. This may lower the concentration of inhibitor necessary to reach the desired inhibitory effect on the proteasome and this lower inhibitor concentration should attenuate the inhibition of off-targets.

Bivalent inhibitors of the proteasome can in theory be mimicked by two single proteasome inhibitors which show affinity for each other. A single agent should not be a very

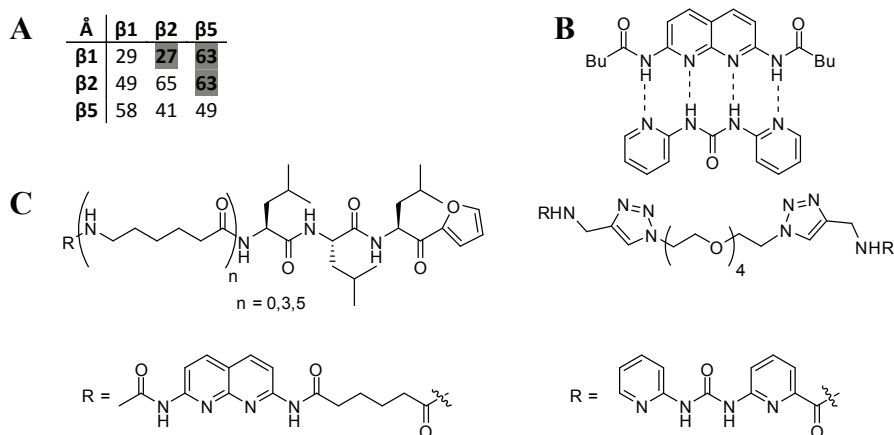
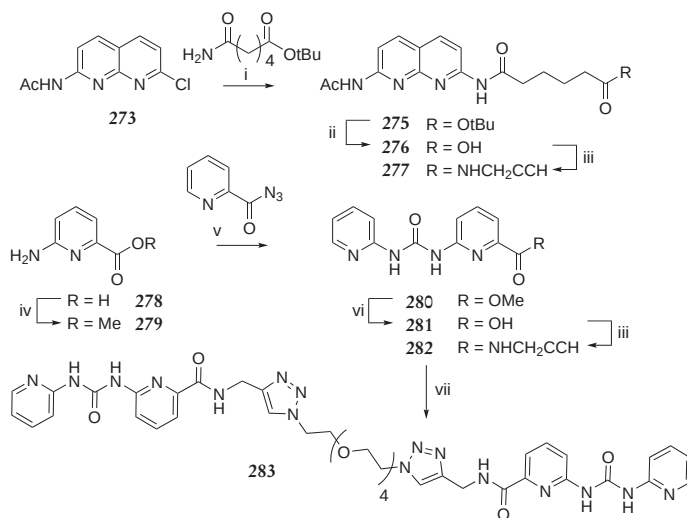


Figure 7.6: (A) Intersubunit distance of the proteasome. In grey are intersubunit distances within one β -ring (B) Supramolecular binding pair. (C) Potential supramolecular proteasome inhibitors and supramolecular spacer.

potent proteasome inhibitor, until a second, complementary proteasome inhibitor is introduced, which allows spatial arrangement in the proteasome to induce synergy in inhibition analogous to the work of Loidl et al.¹³⁹ In supramolecular polymer chemistry, quadruple hydrogen bonding templates are used that form dimers with each other with great affinity (Figure 7.6B).^{258,259} In this work, a chosen pair of hydrogen bonding templates was modified to allow attachment to proteasome inhibitors at their N-termini. A spacer of different lengths was incorporated between electrophile and N-terminal cap (Figure 7.6C). This should tune the length of the inhibitor pair and determine in between which proteasome subunits this inhibitor system may work synergistically (Figure 7.6A). A pair of inhibitors both containing a spacer of five aminohexanoic acid moieties should be able to span about the same distance as the PEG-bisaldehydes from Loidl et al.¹³⁹ A reversible covalent warhead was used to allow the molecule to arrange itself in the most optimal spatial configuration in the proteasome. The synthetically convenient ketofuran warhead was chosen, which yields inhibitors of low potency.⁸⁸ The desired synergistic enhancement of proteasome inhibition by hydrogen bonding may also be induced by a system in which the distance between the electrophiles of two proteasome inhibitors, containing a hydrogen bonding template, is managed by a separate entity that contains the complementary hydrogen bonding template separated by a spacer (Figure 7.6C). The decorated spacer, in itself potentially biologically inert, could enhance the potency of its complementary proteasome inhibitor. This concept should have significant pharmacological benefits since the concentration of inhibitor necessary to reach a certain degree of proteasome inhibition can be lowered by addition of an inert molecule.

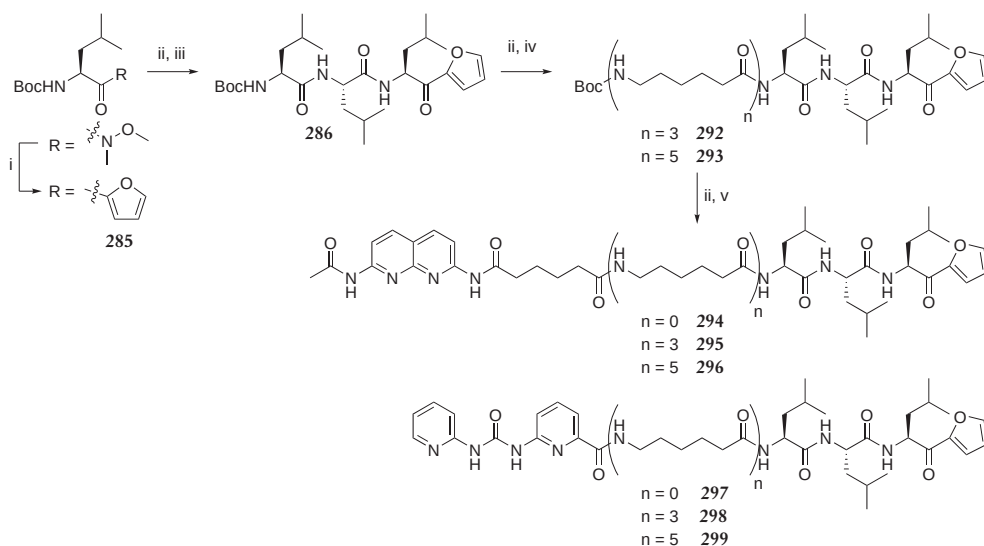
Scheme 7.4: Synthesis of quadruple hydrogen binding templates and PEG-dimer **283**.



Reagents and conditions: i) tert-butyl 6-amino-6-oxohexanoate (1.2 equiv.), Cs_2CO_3 (1.4 equiv.), Xantphos (8 mol-%) and Pd_2dba_3 (4 mol-%), dioxane, microwave 150°C , 20 min., 70%. ii) TFA, 1 hr., quant. iii) HBTU (1.1 equiv.), DIPEA (2.2 or 3.3 equiv.), propargylamine (2 equiv.), DMF or DCM, 1 hr., 99% (**277**), 54% (**282**). iv) HCl, MeOH, reflux, o/n, then sat. aq. NaHCO_3 , 84%. v) pyridine-2-carbonyl azide (1.3 equiv.), toluene, 100°C , 2 hr. then **279**, 100°C , o/n, 92%. vi) NaOH (1 equiv.), THF/ H_2O 1:1, 2 hr., 71%. vii) **282** (2.2 equiv.), tetraethyleneglycol diazide (1 equiv.), NaAsc (2.2 equiv.), CuSO_4 (0.44 equiv.), $t\text{BuOH}:\text{MeCN}:\text{H}_2\text{O}$, o/n, 46%

The synthesis of naphthyrindine template commenced with a Pd-catalyzed amidation²⁶⁰ using chloride **273**²⁵⁹ and *tert*-butyl 6-amino-6-oxohexanoate **274** to yield **275** in 70% yield (Scheme 7.4). The *tert*-butyl protecting group in **275** was removed by TFA to yield **276** quantitatively as a mono-TFA salt. The complementary template synthesis commenced with esterification of 6-aminopicolinic acid in acidic MeOH, followed by neutralization, to yield methyl ester **279** in 84% yield. The ureido-linkage was introduced by Curtius rearrangement of pyridine-2-carbonyl azide,²⁶¹ followed by addition of **279** and additional heating to yield urea **280** in 92% yield. Saponification yielded **281**, which was coupled to propargylamine and subsequently used in a Cu^I-catalyzed click reaction with tetraethyleneglycol diazide²⁶² to obtain **283**. In a similar way, **276** was coupled to propargylamine to yield alkyne **277**, which was attached to tetraethyleneglycol diazide to yield **284**. Ketofuran electrophile **285** was prepared by reaction of 2-lithiumfuran with Boc-leucine Weinreb amide in 82% yield (Scheme 7.5).⁸⁸ After deprotection with TFA this molecule was used in an azide coupling with BocLeu₂NHNH₂ to yield tripeptide **286**. Standard Boc-protected solution peptide chemistry (Scheme 7.5) using Boc-Ahx₃-OH or Boc-Ahx₅-OH and subsequently **276** or **281** afforded hydrogen bonding template containing inhibitors **294–299** shown in Scheme 7.5.

Scheme 7.5: Synthesis of inhibitors with N-terminal quadruple hydrogen bonding template.



Reagents and conditions: i) Furan (2.5 equiv.), *n*-BuLi (1.6 M in pentanes, 2.6 equiv.), 0°C, 90 min. then Boc-Leu-weinreb amide (1 equiv.), THF, -78°C, 3 hr., 82%. ii) TFA:DCM 1:1, 30 min, quant. iii) Boc-Leu₂NHNH₂ (1 equiv.), *t*BuONO (1.1 equiv.), HCl (1.8 equiv.), DMF:EA 3:1, -30°C, 3 hr. then TFA:H-Leu-2-furan (1.1 equiv.), DiPEA (5 equiv.), DMF, -30°C → RT o/n, 88% iv) Boc-Ahx₃-OH, HBTU, DiPEA, 79% (**292**, 92% (**293**). v) **276** or **281**, HBTU, DiPEA, DMF, 27-86%

Proteasome inhibition by the six inhibitors shown in Scheme 7.5 was assessed with a competition assay in HEK293T lysate versus MV151 (**42**). The results are summarised in Table 7.2. The potency of the inhibitors lies in the high micromolar range, as was anticipated by the choice for the ketofuran electrophile. The compounds show preference for β 5,

but this preference diminishes when the inhibitors were elongated. When naphthyridine **294**, **295** or **296** was coincubated with complementary urea **297**, **298** or **299**, no significant increase in proteasome inhibition was observed other than the addition of the effects of the two single inhibitors. Coincubation of spacer-templates **283** or **284** with their complementary inhibitor did not lead to increased proteasome inhibition (data not shown).

Table 7.2: Apparent IC_{50} (μM) values calculated from semi log plots of residual proteasome activity against inhibitor concentration. Band intensities from each lane of a competition assay (not shown) in HEK293T lysate (15 μg protein) versus MV151 (0.5 μM end concentration) were quantified and used as input. N.I. no inhibition.

Compound	Quantified competition assay gels		
	$\beta 5$	$\beta 1$	$\beta 2$
	IC_{50} (μM)		
294	17	~ 100	~ 100
297	>100	$\gg 100$	$\gg 100$
295	34	N.I.	>100
298	5.4	70	17
296	12	40	25
299	14	~ 100	41

reversible inhibitors could yield the desired supramolecular synergistic proteasome inhibitors.

Chapter 5 showed the efficiency of two-step labelling of three cyclooctynes compared to the Staudinger-Bertozzi ligation. A very high concentration of phosphane reagent was needed to completely convert the target azide with the Staudinger-Bertozzi ligation, which is in contrast with the much lower concentration of cyclooctynes necessary to quantitatively convert the azido-BODIPY-epoxomicin decorated proteasomes. However, background labelling of the cyclooctynes is high and their use in two-step activity based protein profiling is questionable since extensive background deteriorates the sensitivity and accuracy of two-step labelling. A possible explanation for this reactivity in cells or cell extracts can be a side reaction of the alkyne with free thiols, known as the thiol-yne reaction.²⁴⁷

Two-step activity-based protease profiling is a valuable tool for the chemical biology research field to determine the targets of their tag containing covalently reacting probes. An example in this work is in **Chapter 3**, where the the Staudinger-Bertozzi ligation was used to determine cellular targets of two azide containing compounds inactive towards the proteasome. An alternative for the Staudinger-Bertozzi reaction in activity-based protease profiling may be the inverse-electron-demand Diels-Alder ligation. In this ligation, a tetrazine reacts with either a functionalised trans-cyclooctene (Figure 7.7A)²³³ or a functionalised norbornene (Figure 7.7B),²³⁴ and these reactions occur with high efficiency and selectivity. A disadvantage of this strategy is that compared to the azide, the cyclooctene, norbornene or tetrazine are much larger and decoration of the target molecule with these moieties

Explanations for the lack of synergy can be found in the choice of either the spacer or the hydrogen bonding templates. The bivalent inhibitors of Loidl et al. contain a long PEG spacer, which is not able to form any secondary structure.¹³⁹ Incorporation of a shorter PEG spacer instead of the aminohexanoic acid spacer may improve characteristics of the inhibitors. Most of the hydrogen bonding templates from literature have been designed for and tested in hydrophobic media. In an aqueous environment, their recognition and binding strength may be lessened. A possible solution can be the use of other templates that are able to form more hydrogen bonds and thus show more affinity for each other, or templates specifically designed for use in aqueous media.²⁶³ The use of short non-self complementary peptide nucleic acid (PNA) sequences^{264,265} at the N-terminus of elongated

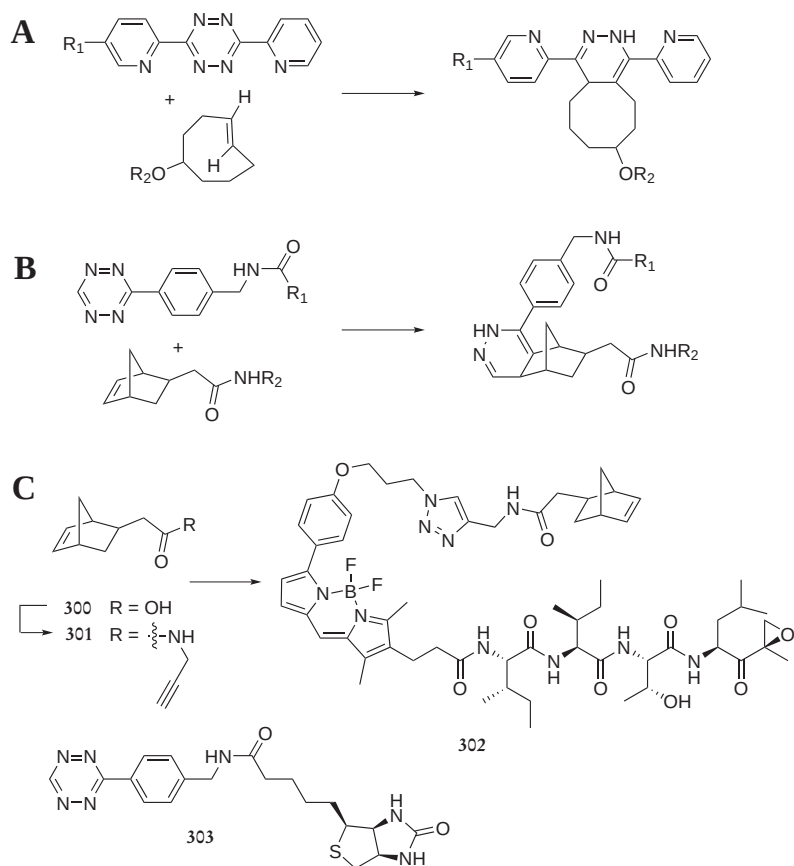


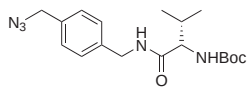
Figure 7.7: (A) Example of trans-cyclooctyne inverse demand Diels-Alder reaction with tetrazine derivative. (B) Example of norbornene inverse demand Diels-Alder reaction with tetrazine derivative. (C) Synthesis of a fluorescent norbornene epoxomicin potential proteasome inhibitor.

may influence its biological characteristics unfavourably. Nevertheless, the efficiency and bioorthogonality of the inverse-electron-demand Diels-Alder reaction should be studied. A possible test system may be the instalment of norbornene onto azido-BODIPY-epoxomicin **302** using copper catalysed click reaction (Figure 7.7C). This inhibitor can then be used to label proteasomes, after which the concentration of tetrazine-biotin **303** necessary to quantitatively convert norbornene labelled proteasome and background labelling can be determined simultaneously, analogous to what was done for three cyclooctynes in **Chapter 5**.

7.1 Experimental

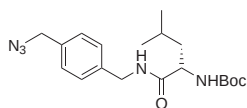
All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.), ethyl acetate (EA), and light petroleum ether (PE) were obtained from Riedel-de Haën or Biosolve and were distilled prior to use. Dichloromethane (DCM), dimethyl formamide (DMF), and dioxane were stored on 4 Å molecular sieves. Tetrahydrofuran (THF) was distilled from LiAlH_4 prior to use. Reactions were conducted under an argon atmosphere. Reactions were monitored by TLC analysis by using DC-fertigfolien (Schleicher & Schuell, F1500, LS254) with detection by UV absorption (254 nm), spraying with 20% H_2SO_4 in ethanol or $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (25

g/L) and $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4 \cdot 2\text{H}_2\text{O}$ (10 g/L) in 10% sulfuric acid followed by charring at $\sim 150^\circ\text{C}$ or by spraying with an aqueous solution of KMnO_4 (7%) and KOH (2%). Column chromatography was performed on silica gel from Screening Devices (0.040–0.063 nm). LC/MS analysis was performed on a LCQ Advantage Max (Thermo Finnigan) equipped with an Gemini C18 column (Phenomenex). HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^1H - and ^{13}C -APT-NMR spectra were recorded on a Bruker DPX-300 (300/75 MHz) or Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Coupling constants are given in Hz. All presented ^{13}C -APT spectra are proton decoupled. Microwave reactions were performed on an Emrys Optimizer (Biotage AB). Wattage was automatically adjusted to maintain the desired temperature.



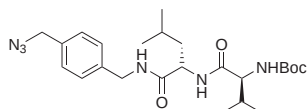
Boc-Val-(4-methyleneazide)-benzylamide (248). Boc-Val-OH (191 mg, 881 μmol , 1.1 equiv.) was dissolved in DCM. HBTU (304 mg, 961 μmol , 1.2 equiv.), 4-(azidomethyl)-benzylamine²⁵¹ (130 mg, 801 μmol , 1 equiv.), DiPEA (331 μl , 2 mmol, 2.5 equiv.) were added and the mixture stirred for 1 hr. before being concentrated. The residue was dissolved in EA and washed with 1M HCl (2 $\times$), sat. aq. NaHCO_3 (3 $\times$), brine and dried with MgSO_4 . Column chromatography (tol. $\rightarrow$

30% EA:tol.) afforded the title compound (170 mg, 470 μmol , 59%) ^1H NMR (400 MHz, CDCl_3) δ ppm 7.27 - 7.21 (m, 4H), 7.08 (s, 1H), 5.36 (d, J = 8.9 Hz, 1H), 4.39 (ddd, J = 47.0, 15.2, 5.9 Hz, 2H), 4.28 (s, 2H), 4.00 (t, J = 7.8 Hz, 1H), 2.09 (dt, J = 16.6, 8.3 Hz, 1H), 1.39 (s, 9H), 0.93 (dd, J = 9.6, 7.0 Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ ppm 171.89, 155.95, 138.35, 134.29, 128.35, 127.88, 79.68, 60.00, 54.29, 42.78, 30.75, 28.17, 19.24.



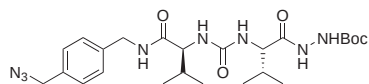
Boc-Leu-(4-methyleneazide)-benzylamide (249). Boc-leucine hydrate (213 mg, 855 μmol , 1.1 equiv.) was coevaporated with toluene (3 $\times$) and dissolved in DCM. HBTU (354 mg, 932 μmol , 1.2 equiv.), 4-(azidomethyl)-benzylamine²⁵¹ (126 mg, 777 μmol , 1 equiv.), DiPEA (321 μl , 1.94 mmol, 2.5 equiv.) were added and the mixture stirred for 1 hr. before being concentrated. The residue was dissolved in EA and washed with 1M HCl (2 $\times$), sat. aq. NaHCO_3 (3 $\times$), brine and dried with MgSO_4 . Column chromatography (tol. $\rightarrow$ 30% EA:tol.) afforded the title com-

compound (141 mg, 378 μmol , 48%). ^1H NMR (400 MHz, CDCl_3) δ ppm 7.34 - 7.14 (m, 4H), 5.35 (d, J = 7.8 Hz, 1H), 4.48 - 4.31 (m, 2H), 4.27 (s, 2H), 4.24 (d, J = 4.5 Hz, 1H), 1.77 - 1.46 (m, 3H), 1.38 (s, 9H), 0.91 (t, J = 6.5 Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ ppm 172.88, 155.79, 138.39, 134.16, 128.29, 127.71, 79.75, 54.26, 52.96, 42.68, 41.15, 28.13, 24.59, 22.78.



Boc-Val-Leu-(4-methyleneazide)-benzylamide (250). Carbamate 249 (141 mg, 376 μmol) was dissolved in TFA:DCM 1:1 for 30 min and coevaporated with toluene (3 $\times$). To this residue was added a solution of Boc-Val-OH (90 mg, 414 μmol , 1.1 equiv.), HBTU (164 mg, 432 μmol , 1.15 equiv.) and DiPEA (218 μl , 1.32 mmol, 3.5 equiv.) in DMF and the mixture stirred for 1 hr. The solution was diluted with EA, washed with 1M HCl (3 $\times$), sat. aq. NaHCO_3 (3 $\times$), brine and dried with MgSO_4 . Column

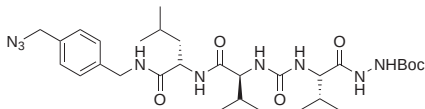
chromatography (tol. $\rightarrow$ 40% EA:tol.) afforded the title compound (125 mg, 263 μmol , 70%). ^1H NMR (400 MHz, CDCl_3) δ ppm 7.75 (s, 1H), 7.64 (d, J = 7.8 Hz, 1H), 7.26 - 7.12 (m, 4H), 5.66 (d, J = 8.8 Hz, 1H), 4.69 - 4.55 (m, 1H), 4.41 - 4.21 (m, 2H), 4.26 (s, 2H), 4.06 (t, J = 8.1 Hz, 1H), 1.99 (dt, J = 14.0, 7.0 Hz, 1H), 1.83 - 1.56 (m, 3H), 1.38 (s, 9H), 1.06 - 0.59 (m, 12H). ^{13}C NMR (101 MHz, CDCl_3) δ ppm 172.20, 172.06, 155.86, 138.55, 134.02, 128.21, 127.73, 79.38, 59.91, 54.31, 51.71, 42.66, 40.55, 30.72, 28.20, 24.69, 22.62, 22.23, 19.06, 18.19.



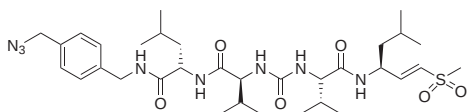
(4-methyleneazide)-benzylamide-Val-urea-Val-NHNHBoc (252). Carbamate 248 (170 mg, 470 μmol , 1 equiv.) was dissolved in 4M HCl/dioxane and stirred for 1 hr. before coevaporation with toluene (3 $\times$). The residue was dissolved in 1:1 DCM:sat.aq. NaHCO_3 at 0°C . Phosgene (495 μl 20 w-% in toluene, 940 μmol , 2 equiv.) was added to the DCM layer and the mixture stirred vigorously for 10 min.

The water layer was then extracted with DCM (3 $\times$), the organic layers dried with Na_2SO_4 and concentrated. The residue was dissolved in DCM and H-Val-NHNHBoc (202, 120 mg, 517 μmol , 1.1 equiv.) was added and the mixture stirred o/n. Addition of H_2O and evaporation of DCM caused precipitation of the title compound (138 mg, 266 μmol , 57%). LCMS: R_t 7.59 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), ESI-MS (m/z): 518.93 ($\text{M} + \text{H}^+$). ^1H NMR (400 MHz, CD_3OD) δ ppm 7.30 (q, J = 8.3 Hz, 4H), 4.41 (q, J = 15.1 Hz, 2H),

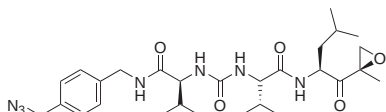
4.32 (s, 2H), 4.07 (d, $J = 5.9$ Hz, 1H), 4.00 (d, $J = 6.6$ Hz, 1H), 2.04 (dq, $J = 13.4, 6.7$ Hz, 2H), 1.46 (s, 9H), 1.11 - 0.79 (m, 12H). ^{13}C NMR (101 MHz, CD_3OD) δ ppm 172.77, 158.08, 137.88, 133.85, 127.74, 127.18, 80.17, 58.86, 56.91, 53.53, 41.96, 30.68, 30.30, 27.02, 18.25, 18.16, 16.81. HRMS calcd for $[\text{C}_{24}\text{H}_{39}\text{N}_8\text{O}_5]^+$ 519.30379 found 519.30385.



the water layer was then extracted with DCM (3 $\times$), the organic layers dried with Na_2SO_4 and concentrated. The residue was dissolved in DCM and H-Val-NHNHBoc (202, 43 mg, 187 μmol , 1.1 equiv.) was added and the mixture stirred o/n and concentrated. Column chromatography (DCM $\rightarrow$ 5% MeOH:DCM) afforded the title compound (79 mg, 124 μmol , 76%). ^1H NMR (400 MHz, CD_3OD) δ ppm 7.32 - 7.22 (m, 4H), 4.51 (t, $J = 7.2$ Hz, 1H), 4.36 (s, 2H), 4.31 (s, 2H), 4.15 - 4.09 (m, 1H), 4.05 (d, $J = 6.9$ Hz, 1H), 1.98 (ddd, $J = 20.8, 13.0, 7.4$ Hz, 2H), 1.67 - 1.60 (m, 3H), 1.45 (s, 9H), 1.03 - 0.82 (m, 18H). ^{13}C NMR (101 MHz, CD_3OD) δ ppm 172.81, 172.34, 158.19, 155.35, 137.91, 133.77, 127.72, 127.10, 80.20, 58.83, 56.68, 53.58, 51.18, 41.98, 39.92, 30.96, 30.40, 27.09, 24.03, 21.86, 20.76, 18.26, 18.14, 17.08, 16.95.

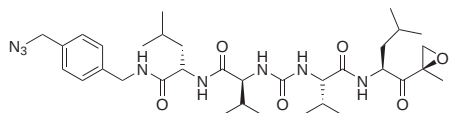


(4-methyleneazide)-benzylamide-Leu-Val-urea-Val-Leu-VS (254). Carbamate 250 (46 mg, 97 μmol , 1 equiv.) was dissolved in 4M HCl/dioxane and stirred for 1 hr. before coevaporation with toluene (3 $\times$) to yield HCl-H-Val-Leu-(4-methyleneazide)-benzylamide. Boc-Val-Leu-VS (194, 49 mg, 125 μM , 1.28 equiv.) was dissolved in 4M HCl/dioxane and stirred for 1 hr. before coevaporation with toluene (3 $\times$). The residue was dissolved in 1:1 DCM:sat.aq. NaHCO_3 at 0 $^\circ$ 3. Phosgene (132 μl 20 w-% in toluene, 250 μmol , 2 equiv.) was added to the DCM layer and the mixture stirred vigorously for 10 min. The water layer was then extracted with DCM (3 $\times$), the organic layers dried with Na_2SO_4 and concentrated. The residue was dissolved in DCM and a solution of HCl-H-Val-Leu-(4-methyleneazide)-benzylamide and DiPEA (33 μl , 200 μmol , 2 equiv.) in DCM was added and the resulting mixture stirred o/n before being concentrated. Column chromatography (DCM $\rightarrow$ 4% MeOH:DCM) afforded the title compound (20 mg, 29 μmol , 30%). LCMS: R_t 8.60 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, CD_3OD , CHCl_3) δ ppm 7.30 - 7.21 (m, 4H), 6.81 (dd, $J = 15.1, 5.5$ Hz, 1H), 6.66 - 6.58 (m, 1H), 4.72 - 4.63 (m, 1H), 4.61 - 4.51 (m, 1H), 4.35 (d, $J = 5.4$ Hz, 2H), 4.30 (s, 2H), 4.17 (d, $J = 7.1$ Hz, 1H), 4.02 (d, $J = 7.6$ Hz, 1H), 2.94 (s, 3H), 2.19 - 1.85 (m, 2H), 1.76 - 1.56 (m, 4H), 1.53 - 1.34 (m, 2H), 1.07 - 0.75 (m, 24H). ^{13}C NMR (101 MHz, CD_3OD , CHCl_3) δ ppm 172.73, 172.51, 172.26, 158.16, 147.00, 137.94, 133.76, 128.68, 127.74, 126.99, 58.93, 58.52, 53.60, 51.18, 47.14, 41.95, 41.66, 41.49, 39.94, 30.76, 30.49, 24.13, 24.04, 21.85, 21.81, 20.95, 20.75, 18.42, 18.31, 17.22, 17.17. HRMS calcd for $[\text{C}_{33}\text{H}_{55}\text{N}_8\text{O}_6\text{S}]^+$ 691.39598 found 691.39644.

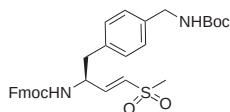


(4-methyleneazide)-benzylamide-Val-urea-Val-Leu-EK (255). Carbamate 252 (50 mg, 97 μmol) was dissolved in 4M HCl/dioxane and stirred for 1 hr. before coevaporation with toluene (3 $\times$). The residue was dissolved in DMF and cooled to -30 $^\circ\text{C}$. $t\text{BuONO}$ (14 μl , 107 μmol , 1.1 equiv.) and HCl (44 μl 4M in dioxane, 175 μmol , 1.8 equiv.) were added and the resulting mixture stirred for 3 hr. TFA-H-Leu-EK (107 μl , 1.1 equiv.) in DMF was added followed by DiPEA (80 μl , 485 μmol , 5 equiv.) and the mixture was allowed to warm to RT overnight. The mixture was diluted with EA and washed with H_2O (3 $\times$). The organic layer was dried over Na_2SO_4 and flash column chromatography (DCM $\rightarrow$ 5% MeOH:DCM) afforded the title compound (22 mg, 39 μmol , 41%). LCMS: R_t 8.72 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, CD_3OD , CHCl_3) δ ppm 7.35 - 7.24 (m, 4H), 4.54 (dd, $J = 10.6, 3.0$ Hz, 1H), 4.47 - 4.30 (m, 2H), 4.32 (s, 2H), 4.04 (dd, $J = 6.8, 4.8$ Hz, 2H), 3.31 (d, $J = 5.0$ Hz, 1H), 2.91 (d, $J = 5.0$ Hz, 1H), 2.14 - 1.92 (m, 2H), 1.78 - 1.63 (m, 1H), 1.50 (s, 3H), 1.56 - 1.25 (m, 2H), 0.97 - 0.87 (m, 18H). ^{13}C NMR (101 MHz, CD_3OD , CHCl_3) δ ppm 207.98, 172.83, 172.78, 158.11, 137.89, 133.87, 127.81, 127.25, 58.77, 58.42,

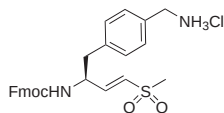
58.20, 53.62, 51.58, 49.87, 42.05, 38.61, 30.55, 30.50, 24.45, 22.33, 20.14, 18.37, 18.32, 17.00, 16.96, 15.74. HRMS calcd for $[C_{28}H_{44}N_7O_5]^+$ 558.33984, found 558.33990



was added followed by DiPEA (51 μ L, 310 μ mol, 5 equiv.) and the mixture was allowed to warm to RT overnight. The mixture was diluted with EA and washed with H_2O (3 $\times$). The organic layer was dried over Na_2SO_4 and flash column chromatography (DCM $\rightarrow$ 4% MeOH:DCM) afforded the title compound (22 mg, 33 μ mol, 53%). LCMS: R_t 9.33 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min). ^1H NMR (400 MHz, CD_3OD , $CHCl_3$) δ ppm 7.30 - 7.23 (m, 4H), 4.57 - 4.46 (m, 2H), 4.36 (s, 2H), 4.31 (s, 2H), 4.09 (d, J = 6.7 Hz, 1H), 4.05 (d, J = 7.1 Hz, 1H), 3.29 (d, J = 5.0 Hz, 1H), 2.90 (d, J = 5.0 Hz, 1H), 2.09 - 1.89 (m, 2H), 1.74 - 1.55 (m, 4H), 1.50 (s, 3H), 1.33 (ddd, J = 20.8, 12.6, 7.3 Hz, 2H), 0.90 (m, 24H). ^{13}C NMR (101 MHz, CD_3OD , $CHCl_3$) δ ppm 208.11, 172.86, 172.77, 172.33, 158.21, 137.93, 133.84, 127.84, 127.17, 58.66, 58.51, 58.09, 53.72, 51.66, 51.28, 49.94, 42.10, 40.02, 38.62, 30.84, 30.59, 24.55, 24.15, 22.41, 21.94, 21.02, 20.28, 18.39, 18.35, 17.15, 17.12, 15.86. HRMS calcd for $[C_{34}H_{55}N_8O_6]^+$ 671.42391, found 671.42439.

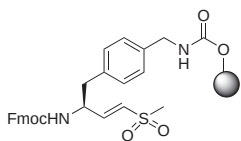


Fmoc-Phe(4- CH_2 NHBoc)-VS (264). Amine **263** (613 mg, 1.73 mmol, 1 eq.) was dissolved in a 20 mL mixture of EA and 5 drops of DMF and brought to 0°C under argon. DiPEA (0.45 mL, 2.60 mmol, 1.5 eq) was added followed by Fmoc-Cl (492 mg, 1.90 mmol, 1.1 eq). After stirring for 1 hr. the reaction mixture was washed with 0.1M HCl (2 $\times$), sat. aq. $NaHCO_3$ (1 $\times$) and brine (1 $\times$), dried with Na_2SO_4 and concentrated in vacuo. Crystallization with DCM/PE yielded the title compound (631 mg, 1.09 mmol, 63%) as a white solid. R_f = 0.3 (30% EA/Tol). ^1H NMR (400 MHz, $CDCl_3$) δ ppm 7.78 (d, J = 7.6 Hz, 2H), 7.58 - 7.48 (m, 2H), 7.42 (t, J = 7.4 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 7.22 (d, J = 7.5 Hz, 2H), 7.08 (d, J = 6.7 Hz, 2H), 6.87 (d, J = 14.4 Hz, 1H), 6.32 (d, J = 14.5 Hz, 1H), 4.85 - 4.65 (m, 2H), 4.49 (dd, J = 10.6, 6.4 Hz, 1H), 4.44 - 4.35 (m, 1H), 4.28 (d, J = 4.8 Hz, 2H), 4.17 (t, J = 6.3 Hz, 1H), 2.91 (d, J = 4.6 Hz, 2H), 2.87 (s, 3H), 1.78 (s, 1H), 1.46 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 156.00, 155.58, 146.78, 143.80, 143.62, 141.38, 138.09, 134.66, 130.07, 129.54, 127.87, 127.16, 124.95, 120.12, 120.09, 66.80, 52.52, 47.21, 44.28, 44.27, 44.26, 42.85, 39.87, 28.53, 28.51, 28.47. HRMS: calculated for $[C_{32}H_{36}N_2O_6S]^+$ 577.23668, found 577.23662.



Fmoc-Phe(4- CH_2 NH $_3$ Cl)-VS (265). Vinyl sulfone **264** (856 mg, 1.48 mmol) was dissolved in an 8 mL solution of 4M HCl in dioxane at rt under an argon atmosphere. After 1 hr. of stirring the deprotection was complete and the mixture was coevaporated with toluene (2 $\times$) yielding the title compound. ^1H NMR (400 MHz, CD_3OD) δ ppm 7.80 (d, J = 7.4 Hz, 2H), 7.67 - 7.50 (m, 2H), 7.44 - 7.26 (m, 8H), 6.85 (dd, J = 15.1, 4.9 Hz, 1H), 6.54 (d, J = 15.1 Hz, 1H), 4.59 (s, 1H), 4.31 (p, J = 10.7 Hz, 2H), 4.16 - 4.09 (m, 1H), 4.04 (s, 2H), 3.00 (dd, J = 13.7, 6.0 Hz, 1H), 2.94 (s, 3H), 2.85 (dd, J = 13.0, 9.4 Hz, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ ppm 157.95, 147.69, 145.27, 145.03, 142.63, 139.89, 132.89, 131.33, 131.24, 130.18, 128.86, 128.82, 128.36, 128.16, 126.13, 126.09, 120.97, 67.63, 54.30, 48.43, 43.99, 42.83, 40.49.

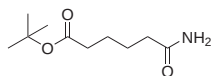
Wang resin p-nitrophenylcarbonate (266). Wang resin (1.1 mmol gr^{-1} , 1 gr, 1.1 mmol) was suspended in DCM at 0°C. N-methyl morpholine (242 μ L, 2.2 mmol, 2 equiv.) and p-nitrophenylchloroformate (443 mg, 2.2 mmol, 2 equiv.) were added and the resin agitated overnight, after which it was filtered and washed with DCM (4 $\times$).



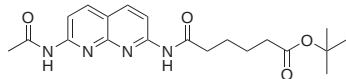
Fmoc-Phe(4-CH₂-NH-C(O)-Wang resin)-VS (267). Resin **266** (251.3 mg) was allowed to swell in DMF. HOBt (102 mg, 754 μ mol, 3 eq.), DiPEA (263 μ L, 1.51 mmol, 6 eq.) and vinyl sulfone **265** (257 mg, 503 μ mol, 2 eq.) were added and the mixture was agitated at rt for 24 h. The resin was washed with DMF (3 $\times$) and DCM (3 $\times$) and dried. Loading calculated by Fmoc-determination: 0.534 mmol/g. A small scale cleavage test was performed in 1 mL 95% TFA/H₂O for 1h, which afforded only the starting compound **265**. LCMS: R_t (min): 6.86, calculated for [C₂₇H₂₈N₂O₄S]⁺ 477.18, found 477.07.

General procedure solid phase peptide synthesis

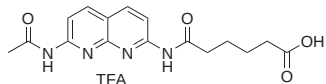
Fmoc containing resin was treated with of 20% piperidine/DMF and agitated for 0.5 h. The resin was washed with DMF (3 $\times$) and DCM (3 $\times$). Next, HCTU (3 equiv.), DiPEA (5 equiv.) and Fmoc-AA-OH (3 equiv) were dissolved in 10 mL DMF and added to the resin, which was agitated at RT for 4 hr. The resin was washed with DMF (3 $\times$) and DCM (3 $\times$) and dried. A small scale cleavage test was performed in 1 mL 95% TFA/H₂O for 1 hr, which afforded the corresponding Fmoc-AA_n-Phe-(4-CH₂NH₂-TFA)-VS peptide with Fmoc-AA_n-OH as a contamination. Finally, to remove unwanted esters the resin was treated with 10 mL of sat. aq. LiOH in THF/H₂O (1:1) and agitated for 2 hr. The resin was washed with H₂O (3 $\times$), MeOH (3 $\times$) and DCM (3 $\times$). Resin cleavage involved treatment with 5 mL of 95% TFA/H₂O for 1.5 hr., washing with MeOH (3 $\times$) and coevaporation with toluene (2 $\times$).



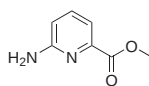
***t*-Butyl 6-amino-6-oxohexanoate (274).** Mono-*tert*-butyl adipate²⁶⁶ (7.23 gr, 35.8 mmol, 1 equiv.) was dissolved in THF and cooled to -10°C. NEt₃ (4.95 mL, 35.8 mmol, 1 equiv.) was added followed by dropwise addition of EtOCOC(=O)NH₂ (3.42 mL, 35.8 mmol, 1 equiv.) which caused the formation of white solids. The mixture was stirred for 15 min. at 0°C and NH₃ (g) was bubbled through the solution for 1 hr. The mixture was filtered, the residue washed with THF and the filtrate concentrated. Crystallisation (DCM/PE) yielded the title compound (4.21 gr, 20.92 mmol, 59%). ¹H NMR (400 MHz, CDCl₃) δ ppm 6.05 (s, 1H), 5.89 (s, 1H), 2.25 (t, *J* = 5.9 Hz, 4H), 1.64 (s, 4H), 1.44 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ ppm 175.50, 172.90, 80.21, 35.39, 35.01, 28.02, 24.75, 24.39.



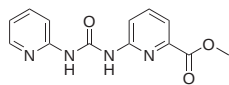
6-(7-acetamido-1,8-naphthyridin-2-ylamino)-6-oxohexanoic acid *t*-butyl ester (275). Synthesized by a procedure from Lighthart et al.²⁶⁰ N-(7-chloro-1,8-naphthyridin-2-yl)acetamide²⁵⁹ ((**273**, 222 mg, 1 mmol, 1 equiv.), *tert*-butyl 6-amino-6-oxohexanoate (**274**, 242 mg, 1.2 mmol, 1.2 equiv.) and Cs₂CO₃ (459 mg, 1.4 mmol, 1.4 equiv.) were suspended in dioxane and degassed by repeated reduction of pressure and Ar bubbling. Xantphos (46 mg, 8 mol-%) and Pd₂dba₃ (18 mg, 4 mol-%) were added and the mixture was heated in a microwave at 150°C for 20 minutes. The reaction mixture was filtered over celite and concentrated. Column chromatography (DCM $\rightarrow$ 3% MeOH/DCM followed by crystallization from DCM/PE) yielded the title compound (270 mg, 0.7 gr, 70%). LCMS: R_t 5.55 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 386.9 (M+H)⁺). ¹H NMR (400 MHz, CDCl₃) δ ppm 10.44 (s, 1H), 10.32 (s, 1H), 8.44 - 8.39 (m, 2H), 8.10 (d, *J* = 8.8 Hz, 2H), 2.43 (t, *J* = 7.3 Hz, 2H), 2.18 (s, 3H), 2.16 (d, *J* = 10.7 Hz, 2H), 1.70 - 1.48 (m, 4H), 1.42 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ ppm 172.94, 172.68, 170.30, 154.62, 153.21, 139.04, 117.86, 113.87, 113.73, 80.18, 36.69, 34.97, 27.98, 24.66, 24.40, 24.10.



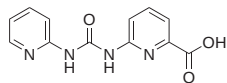
(6-(7-acetamido-1,8-naphthyridin-2-ylamino)-6-oxohexanoic acid mono TFA salt (276). *t*-Butyl 6-(7-acetamido-1,8-naphthyridin-2-ylamino)-6-oxohexanoate (**275**, 126 mg, 326 μ mol) was stirred with 2 mL TFA for 30 minutes before being coevaporated with toluene (3 $\times$) to yield the title compound (146 mg, quant). LCMS: R_t 2.96 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 331.2 (M+H)⁺). ¹H NMR (400 MHz, CDCl₃/CD₃OD) δ ppm 8.46 (d, *J* = 8.8 Hz, 2H), 8.39 (d, *J* = 9.0 Hz, 2H), 2.64 (t, *J* = 7.2 Hz, 2H), 2.39 (t, *J* = 7.2 Hz, 2H), 2.36 (s, 3H), 1.95 - 1.73 (m, 4H). ¹³C NMR (101 MHz, CDCl₃/CD₃OD) δ ppm 175.67, 173.95, 171.42, 153.57, 146.32, 141.92, 116.38, 113.69, 113.60, 36.46, 33.26, 23.84, 23.79.



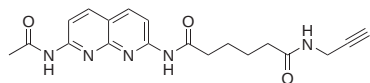
Methyl 6-aminopicolinate (279). AcCl (4.3 ml, 60 mmol, 12 equiv.) was added to MeOH (20 ml) at 0°C. 6-aminopicolinic acid (691 mg, 5 mmol, 1 equiv.) was added and the mixture was refluxed o/n after which it was concentrated. The residue was crystallised from MeOH/Et₂O, dissolved in sat. aq. NaHCO₃ and extracted with DCM. The organic layers were dried with Na₂SO₄ and concentrated, yielding the title compound (640 mg, 4.2 mmol, 84%). LCMS: *R*_t 1.82 min (linear gradient 0 → 20% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 152.87 (M + H)⁺). ¹H NMR (400 MHz, CDCl₃) δ ppm 7.54 - 7.48 (m, 1H), 7.44 (dd, *J* = 7.3, 0.8 Hz, 1H), 6.68 (dd, *J* = 8.2, 0.8 Hz, 1H), 5.21 (s, 2H), 3.93 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ ppm 165.82, 158.53, 145.76, 137.99, 115.17, 112.65, 52.36.



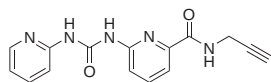
Methyl 6-(3-pyridin-2-ylureido)picolinate (280). pyridine-2-carbonyl azide²⁶¹ (322 mg, 2.17 mmol, 1 equiv.) was dissolved in toluene (3 ml) and heated to 100°C until gas evolution had stopped and TLC (1:1 EA:PE) indicated complete consumption of the starting material (2 hr.). Methyl 6-aminopicolinate (279, 248 mg, 1.63 mmol, 0.75 equiv.) in toluene was added and the mixture heated at 100°C o/n. The mixture was concentrated and crystallized from CHCl₃/PE to yield the title compound (409 mg, 1.5 mmol, 92%). LCMS: *R*_t 4.29 min (linear gradient 10 → 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 272.93 (M + H)⁺). ¹H NMR (400 MHz, DMSO) δ ppm 10.67 (s, 1H), 10.16 (s, 1H), 8.30 (d, *J* = 4.9 Hz, 1H), 8.03 (d, *J* = 8.1 Hz, 1H), 7.96 (t, *J* = 7.9 Hz, 1H), 7.77 (d, *J* = 3.8 Hz, 2H), 7.71 (d, *J* = 7.7 Hz, 1H), 7.08 - 7.02 (m, 1H), 3.89 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ ppm 164.67, 152.34, 152.20, 151.85, 147.57, 145.16, 139.68, 138.41, 119.17, 118.38, 116.19, 112.43, 52.42.



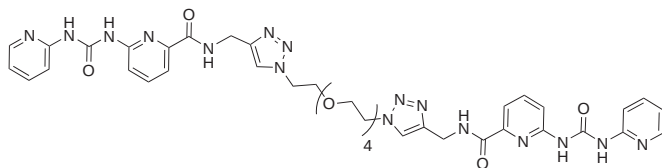
(6-(3-pyridin-2-ylureido)picolinic acid (281). Methyl 6-(3-pyridin-2-ylureido)picolinate (280, 279 mg, 1.02 mmol) was dissolved in 10 ml THF and 10 ml 0.1 M NaOH was added and the mixture stirred for 2 hr after which the THF was evaporated. 0.1 M HCl was added until crystallization commenced at pH 3. The solids were collected by centrifugation and the pellet was washed with 1 mM HCl (2x) and dried overnight at 40°C under vacuum to yield the title compound (188 mg, 0.73 mmol, 71%). LCMS: *R*_t 3.43 min (linear gradient 10 → 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 258.93 (M + H)⁺). ¹H NMR (400 MHz, DMSO) δ ppm 13.29 - 13.19 (m, 1H), 10.69 (s, 1H), 10.14 (s, 1H), 8.32 - 8.28 (m, 1H), 8.02 (d, *J* = 8.2 Hz, 1H), 7.94 (t, *J* = 7.9 Hz, 1H), 7.81 - 7.71 (m, 2H), 7.70 (d, *J* = 7.4 Hz, 1H), 7.08 - 7.03 (m, 1H).



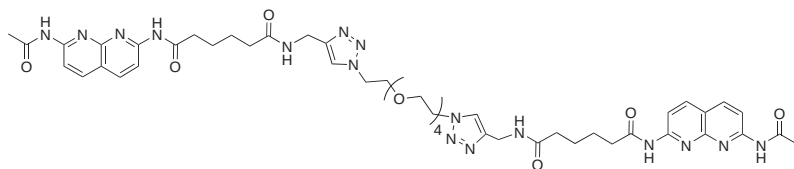
(Naphthyridine-alkyn (277). Carboxylic acid 276 (45 mg, 100 μmol), HBTU (42 mg, 110 μmol, 1.1 equiv.) and DiPEA (59 μl, 330 μmol, 3.3 equiv.) were dissolved in DMF and propargylamine (13 μl, 200 μmol, 2 equiv) was added and the mixture stirred for 1 hr. after which the mixture was concentrated. Column chromatography (5% MeOH:DCM → 10% MeOH:DCM) afforded the title compound (39 mg, 99 μmol, 99%). LCMS: *R*_t 3.81 min (linear gradient 10 → 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 368.07 (M + H)⁺). ¹H NMR (400 MHz, CD₃OD) δ ppm 8.29 (d, *J* = 8.8 Hz, 2H), 8.03 (d, *J* = 8.7 Hz, 1H), 7.98 (d, *J* = 8.7 Hz, 1H), 3.93 (s, 2H), 2.55 (s, 1H), 2.52 - 2.47 (m, 2H), 2.22 (s, 3H), 2.21 - 2.17 (m, 2H), 1.76 - 1.53 (m, 4H). ¹³C NMR (101 MHz, CD₃OD) δ ppm 175.30, 175.19, 172.69, 168.47, 155.09, 154.68, 140.61, 118.73, 114.70, 80.64, 72.11, 37.69, 36.33, 29.39, 26.11, 25.68, 24.49.



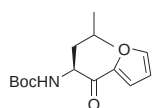
(Dipyridylurea-alkyn (282). Carboxylic acid 363 (26 mg, 100 μmol), HBTU (42 mg, 110 μmol, 1.1 equiv.) and DiPEA (36 μl, 220 μmol, 2.2 equiv.) were dissolved in DMF and propargylamine (13 μl, 200 μmol, 2 equiv) was added and the mixture stirred for 1 hr. after which the mixture was concentrated. The residue was dissolved in DCM and washed with sat. aq. NaHCO₃ (3x), H₂O (3x), brine and dried with Na₂SO₄. Column chromatography (DCM → 4% MeOH:DCM) afforded the title compound (16 mg, 54 μmol, 54%). ¹H NMR (400 MHz, CDCl₃:CD₃OD 1:1) δ ppm 8.36 (d, *J* = 4.5 Hz, 1H), 8.01 (br s, 1H), 7.91 - 7.81 (m, 2H), 7.73 (t, *J* = 7.5 Hz, 1H), 7.45 (s, 1H), 7.05 (dd, *J* = 6.7, 5.7 Hz, 1H), 4.29 (d, *J* = 2.4 Hz, 2H), 2.43 (t, *J* = 2.4 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃:CD₃OD 1:1) δ ppm 164.11, 161.79, 151.84, 150.99, 146.65, 146.51, 139.18, 138.34, 117.96, 116.82, 116.37, 112.34, 78.70, 71.04, 28.57.



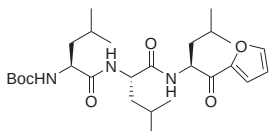
(Bis-(dipyridineurea)-PEG (283). Alkyne **282** (16 mg, 54 μ mol, 2.2 equiv.) and tetraethyleneglycol diazide²⁶² (7 mg, 24.5 μ mol, 1 equiv.) were dissolved in 2:1:1 *t*BuOH:MeCN:H₂O and degassed with Ar. Sodium ascorbate (54 μ l 1 M stock in degassed H₂O, 2.2 equiv.) and CuSO₄ (54 μ l 0.2 M stock in degassed H₂O, 0.44 equiv.) were added and the mixture stirred o/n. The mixture was concentrated and the residue dissolved in MeOH and subjected to LH20 Sephadex size exclusion chromatography to yield the title compound (10 mg, 11 μ mol, 46%). LCMS: *R*_t 4.58 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 879.20 (*M* + H)⁺). ¹H NMR (400 MHz, CD₃ODCDCl₃) δ ppm 8.95 (s, 2H), 8.27 (s, 2H), 8.02 (s, 2H), 7.97 (d, *J* = 11.3 Hz, 2H), 7.86 (t, *J* = 7.8 Hz, 2H), 7.78 (d, *J* = 7.4 Hz, 2H), 7.74 - 7.66 (m, 2H), 7.39 (s, 2H), 7.05 - 6.97 (m, 2H), 4.73 (s, 4H), 4.53 (t, *J* = 4.8 Hz, 4H), 3.84 (t, *J* = 4.8 Hz, 4H), 3.57 - 3.46 (m, 8H), 3.44 (s, 4H).



(Bis-(naphthyridine)-PEG (284). Alkyne **277** (28 mg, 76 μ mol, 2.2 equiv.) and tetraethyleneglycol diazide²⁶² (10 mg, 34 μ mol, 1 equiv.) were dissolved in DMF and degassed with Ar. Sodium ascorbate (80 μ l 1 M stock in degassed H₂O, 2.2 equiv.) and CuSO₄ (80 μ l 0.2 M stock in degassed H₂O, 0.44 equiv.) were added and the mixture stirred 3 hr. Column chromatography (5% MeOH:DCM $\rightarrow$ 20% MeOH:DCM) yielded the title compound (12 mg, 11 μ mol, 64%). LCMS: *R*_t 5.24 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 1023.47 (*M* + H)⁺). ¹H NMR (400 MHz, DMF-d₇) δ ppm 10.78 (s, 2H), 10.73 (s, 2H), 8.37 - 8.32 (m, 8H), 8.29 - 8.24 (m, 2H), 7.96 (s, 2H), 4.55 (t, *J* = 5.2 Hz, 4H), 4.41 (d, *J* = 5.6 Hz, 4H), 3.85 (t, *J* = 5.3 Hz, 4H), 3.57 - 3.48 (m, 12H), 2.64 - 2.55 (m, 4H), 2.29 - 2.23 (m, 12H), 1.75 - 1.61 (m, 8H).



Boc-Leu-2-furan (285). Furan (1.88 ml, 25.9 mmol, 2.5 equiv.) was dissolved in 100 ml THF and cooled to 0°C. *n*Bu-Li (16.8 ml 1.6M in pentane, 26.9 mmol, 2.6 equiv.) was added dropwise and the mixture was stirred at 0°C for 90 min. The mixture was cooled to -78°C and a solution of Boc-leucine weinreb amide (2.84 gr, 10.35 mmol, 1 equiv.) in THF (50 mL) was added dropwise and the mixture was stirred at -78°C for 3 hr after which it was allowed to warm to -30°C. The mixture was quenched with 1M HCl and EA was added and the mixture was washed with 1M HCl (3 $\times$), brine and dried with MgSO₄. Column chromatography (PE $\rightarrow$ 11% EA:PE) yielded the title compound (2.39 gr, 8.5 mmol, 82%). ¹H NMR (400 MHz, CDCl₃) δ ppm 7.66 (d, *J* = 1.0 Hz, 1H), 7.35 (d, *J* = 3.4 Hz, 1H), 6.58 (dd, *J* = 3.4, 1.5 Hz, 1H), 5.49 (d, *J* = 8.9 Hz, 1H), 5.11 (td, *J* = 9.4, 3.7 Hz, 1H), 1.86 - 1.74 (m, 1H), 1.69 - 1.45 (m, 2H), 1.43 (s, 9H), 1.05 (d, *J* = 6.5 Hz, 3H), 0.94 (d, *J* = 6.7 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ ppm 188.21, 155.18, 150.65, 146.68, 118.24, 112.10, 79.06, 53.67, 41.79, 27.91, 24.57, 22.91, 21.33.



Boc-Leu₃-2-furan (286). Carbamate **285** (1.6 gr, 5.69 mmol, 1.1 equiv.) was dissolved in 1:1 TFA:DCM and stirred for 30 min before coevaporation with toluene (3 $\times$ yielded TFA·H-Leu-2-furan. Boc-Leu₂-NHNH₂ (**148**, 1.85 gr, 5.2 mmol, 1. equiv.) was dissolved in DCM/DMF 9/1 and cooled to -40°C. *t*BuONO (0.76 ml, 5.69 mmol, 1.1 equiv.) and HCl (3.64 ml 4M sln. in 1,4-dioxane, 14.6 mmol, 2.8 equiv.) were added, and the mixture was stirred for 3hr. at -30°C after which TLC analysis (10% MeOH/DCM) showed complete consumption of the starting material. TFA·H-Leu-furan in DMF and DiPEA (4.3 ml, 26 mmol, 5 equiv.) was added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EA and extracted with H₂O (3 $\times$). The organic layer was dried over

MgSO₄ and purified by flash column chromatography (tol. → 30% EA:tol.) yielded the title compound (2.33 gr, 4.6 mmol, 88%). ¹H NMR (400 MHz, CDCl₃) δ ppm 7.63 (d, *J* = 1.1 Hz, 1H), 7.32 (d, *J* = 3.6 Hz, 1H), 6.62 (s, 1H), 6.57 (dd, *J* = 3.6, 1.7 Hz, 1H), 6.47 (d, *J* = 8.1 Hz, 1H), 5.36 (td, *J* = 9.6, 3.6 Hz, 1H), 4.86 (s, 1H), 4.47 (td, *J* = 8.5, 5.5 Hz, 1H), 4.17 - 4.02 (m, 1H), 1.74 - 1.46 (m, 4H), 1.44 (s, 3H), 1.01 (d, *J* = 6.0 Hz, 1H), 0.98 - 0.85 (m, 5H). ¹³C NMR (101 MHz, CDCl₃) δ ppm 187.51, 172.23, 171.69, 155.20, 150.89, 147.10, 119.15, 112.28, 79.06, 53.17, 52.14, 51.53, 41.80, 41.26, 40.92, 27.91, 24.78, 24.53, 23.17, 22.72, 22.59, 22.36, 22.14, 21.73.

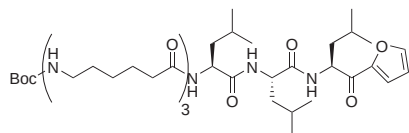
TFA·H-Leu₃-2-furan (287). Carbamate **286** (2.33 gr, 4.6 mmol) was dissolved in 1:1 TFA:DCM and stirred for 30 min before coevaporation with toluene (3× yielded TFA·H-Leu₃-2-furan, which was used without purification.

Boc-Ahx₃-OMe (288). Boc-Ahx₂-OH²⁶⁷ (2 gr, 5.8 mmol, 1 equiv.) was dissolved in DCM. HCl·H-Ahx-OMe (1.08 gr, 6.4 mmol, 1.1 equiv.), HBTU (2.7 gr, 7.0 mmol, 1.2 equiv.) and DiPEA (3.3 ml, 20 mmol, 3.5 equiv.) were added and the mixture stirred o/n. The mixture was washed with 1M HCl (2×), sat. aq. NaHCO₃ (4×), brine and dried with MgSO₄ to yield Boc-Ahx₃-OMe which was used without purification. ¹H NMR (400 MHz, CD₃OD) δ ppm 3.67 (s, 3H), 3.18 (dd, *J* = 12.8, 6.9 Hz, 4H), 3.04 (t, *J* = 7.0 Hz, 2H), 2.35 (t, *J* = 7.4 Hz, 2H), 2.23 - 2.16 (m, 4H), 1.69 - 1.59 (m, 6H), 1.58 - 1.47 (m, 6H), 1.45 (s, 9H), 1.42 - 1.31 (m, 6H).

Boc-Ahx₃-OH (289). The crude Boc-Ahx₃-OMe was dissolved in MeOH (10 ml) and KOH (10 ml 4 M in H₂O, 40 mmol, 7 equiv.) was added and the mixture stirred for 3 hr. Methanol was evaporated and the residue acidified with 3M HCl to pH 3 and extracted with CHCl₃ (3×). The organic layers were dried over MgSO₄ and concentrated to yield Boc-Ahx₃-OH (2.6 gr, 5.6 mmol, 97% over two steps). ¹H NMR (400 MHz, CD₃OD) δ ppm 3.19 - 3.11 (m, 4H), 3.01 (t, *J* = 7.0 Hz, 2H), 2.28 (t, *J* = 7.4 Hz, 2H), 2.19 - 2.13 (m, 4H), 1.66 - 1.55 (m, 6H), 1.55 - 1.39 (m, 6H), 1.42 (s, 9H), 1.38 - 1.27 (m, 6H). ¹³C NMR (101 MHz, CD₃OD) δ ppm 177.34, 175.96, 175.91, 158.46, 79.74, 41.17, 40.27, 40.14, 37.00, 36.95, 34.77, 30.63, 30.08, 28.80, 27.51, 27.46, 27.41, 26.73, 26.67, 25.69.

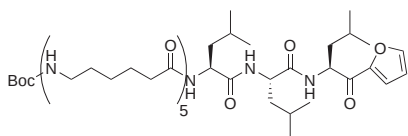
Boc-Ahx₅-OMe (290). Boc-Ahx₃-OMe (946 mg, 2 mmol, 1 equiv.) was dissolved in TFA:DCM 1:1 and stirred for 2 hr. The mixture was coevaporated with toluene (3×). Boc-Ahx₂-OH²⁶⁷ (689 mg, 2 mmol, 1 equiv.) was dissolved in DMF. HBTU (834 mg, 2.2 mmol, 1.1 equiv.) and DiPEA (1.16 ml, 7 mmol, 3.5 equiv.) were added and the mixture stirred for 10 min. before the mixture was added to TFA·H-Ahx₃-OMe and the resulting mixture stirred o/n. After evaporation, the residue was dissolved in CHCl₃/MeOH and addition of EA induced crystallisation of the title product (1.13 gr, 1.64 mmol, 71%). LCMS: R_t 6.78 min (linear gradient 10 → 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (*m/z*): 698.20 (M + H)⁺). ¹H NMR (400 MHz, DMSO) δ ppm 3.58 (s, 3H), 3.04 - 2.96 (m, 8H), 2.87 (dd, *J* = 12.9, 6.6 Hz, 2H), 2.28 (t, *J* = 7.4 Hz, 2H), 2.07 - 1.97 (m, 8H), 1.56 - 1.42 (m, 10H), 1.37 (s, 9H), 1.41 - 1.30 (m, 10H), 1.28 - 1.14 (m, 10H).

Boc-Ahx₅-OH (291). Boc-Ahx₅-OMe (376 mg, 539 μmol) was dissolved in MeOH (50 ml) and KOH (10 ml 4 M in H₂O) was added and the mixture stirred for 3 hr. Methanol was evaporated and the residue acidified with 3M HCl to pH 3, which caused precipitation of Boc-Ahx₅-OH (358 mg, 523 μmol, 97%). ¹H NMR (400 MHz, DMSO) δ ppm 3.05 - 2.95 (m, 8H), 2.87 (dd, *J* = 13.0, 6.5 Hz, 2H), 2.18 (t, *J* = 7.3 Hz, 2H), 2.02 (t, *J* = 7.3 Hz, 8H), 1.47 (dd, *J* = 14.7, 7.3 Hz, 10H), 1.36 (s, 9H), 1.36 (s, 10H), 1.22 (dt, *J* = 13.4, 6.8 Hz, 10H).

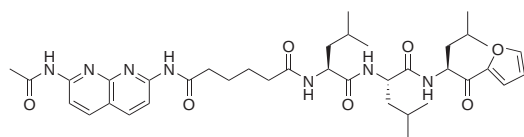


Boc-Ahx₃-Leu₃-2-furan (292). Boc-Ahx₃-OH (224 mg, 490 μmol, 1 equiv.) was dissolved in DMF HBTU (233 mg, 588 μmol, 1.2 equiv.) and DiPEA (283 μl, 1.72 mmol, 3.5 equiv.) were added, followed by compound **287** (281 mg, 539 μmol, 1.1 equiv.), and the mixture stirred o/n. The mixture was concentrated, the residue dissolved in DCM and washed with 1M HCl (2×), sat. aq. NaHCO₃ (4×), brine and dried with MgSO₄. Column chromatography (3% MeOH:EA → 10%

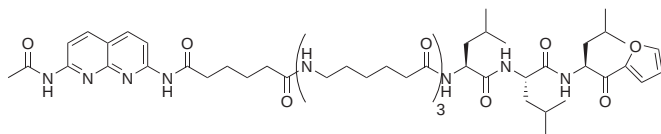
MeOH:EA) yielded the title compound (330 mg, 390 μmol, 79%). ¹H NMR (400 MHz, CD₃OD) δ ppm 7.83 (d, *J* = 1.0 Hz, 1H), 7.42 (d, *J* = 3.6 Hz, 1H), 6.66 (dd, *J* = 3.5, 1.6 Hz, 1H), 5.24 (dd, *J* = 9.7, 4.7 Hz, 1H), 4.52 (dd, *J* = 8.9, 6.0 Hz, 1H), 4.47 (t, *J* = 7.5 Hz, 1H), 3.14 (t, *J* = 6.9 Hz, 4H), 3.01 (t, *J* = 6.9 Hz, 2H), 2.28 - 2.21 (m, 2H), 2.16 (t, *J* = 7.4 Hz, 4H), 1.77 - 1.39 (m, 20H), 1.41 (s, 9H), 1.37 - 1.22 (m, 7H), 1.01 - 0.84 (m, 18H). ¹³C NMR (101 MHz, CD₃OD) δ ppm 188.94, 175.72, 175.65, 174.45, 174.22, 158.30, 152.21, 148.97, 120.01, 113.63, 79.59, 54.18, 52.80, 52.62, 41.95, 41.48, 41.12, 40.12, 40.10, 36.96, 36.91, 36.60, 30.58, 30.05, 28.83, 27.48, 27.36, 26.68, 26.62, 26.56, 26.02, 25.83, 25.65, 23.71, 23.48, 23.41, 22.36, 22.18, 21.98.



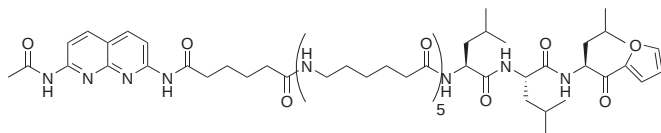
(192 mg, 184 μ mol, 92%). ^1H NMR (400 MHz, CD_3OD) δ ppm 7.85 (d, $J = 1.2$ Hz, 1H), 7.43 (d, $J = 3.6$ Hz, 1H), 6.70 (dd, $J = 3.6, 1.7$ Hz, 1H), 5.25 (dd, $J = 9.8, 4.6$ Hz, 1H), 4.60 - 4.45 (m, 1H), 4.43 (t, $J = 7.6$ Hz, 1H), 3.18 (t, $J = 7.0$ Hz, 8H), 3.04 (t, $J = 7.0$ Hz, 2H), 2.26 (t, $J = 7.4$ Hz, 2H), 2.19 (t, $J = 7.4$ Hz, 8H), 1.72 - 1.42 (m, 29H), 1.45 (s, 9H), 1.40 - 1.30 (m, 10H), 1.05 - 0.88 (m, 18H). ^{13}C NMR (101 MHz, CD_3OD) δ ppm 189.07, 175.98, 175.89, 174.66, 174.38, 158.48, 152.30, 149.01, 120.01, 113.67, 79.74, 54.32, 53.00, 52.76, 41.94, 41.81, 41.55, 41.18, 40.29, 40.16, 37.02, 36.98, 36.64, 30.64, 30.11, 28.81, 27.53, 27.43, 26.76, 26.70, 26.60, 26.10, 25.92, 25.74, 23.68, 23.45, 23.42, 22.21, 22.06, 21.90.



title compound (10 mg, 14 μ mol, 28%). LCMS: R_t 7.19 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 720.33 ($\text{M} + \text{H}^+$)). ^1H NMR (400 MHz, CDCl_3) δ ppm 8.45 (s, 2H), 8.07 (s, 2H), 7.78 (d, $J = 7.6$ Hz, 1H), 7.69 - 7.62 (m, 2H), 7.42 - 7.32 (m, 3H), 6.60 (s, 1H), 5.49 - 5.38 (m, 1H), 5.23 - 4.86 (m, 2H), 2.73 (s, 2H), 2.54 (s, 2H), 2.43 - 2.19 (m, 3H), 1.83 - 1.37 (m, 13H), 0.85 - 0.70 (m, 9H), 0.68 - 0.48 (m, 9H).

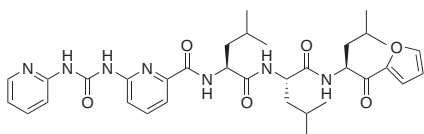


Naphthyridine-Ahx3-Leu3-2-furan (295). Compound 292 (52 mg, 66 μ mol) was dissolved TFA:DCM 1:1 and stirred for 30 min. before being coevaporated with toluene (3 $\times$). The residue was dissolved in DMF and 276 (44 mg, 132 μ mol, 2 equiv.), HBTU (50 mg, 132 μ mol, 2 equiv.) and DiPEA (55 μ l, 330 μ mol, 6 equiv.) were added and the mixture stirred for 2 hr. after which the mixture was concentrated. The residue was dissolved in MeOH and subjected to LH20 Sephadex size exclusion chromatography followed by column chromatography (5% MeOH:DCM $\rightarrow$ 10% MeOH:DCM) afforded the title compound (60 mg, 57 μ mol, 86%). LCMS: R_t 7.06 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 1059.53 ($\text{M} + \text{H}^+$)). ^1H NMR (400 MHz, CD_3OD) δ ppm 8.30 - 8.22 (m, 4H), 7.82 (d, $J = 1.2$ Hz, 1H), 7.40 (d, $J = 3.5$ Hz, 1H), 6.67 (dd, $J = 3.6, 1.7$ Hz, 1H), 5.24 (ddd, $J = 10.1, 7.8, 4.4$ Hz, 1H), 4.56 (p, $J = 7.5$ Hz, 2H), 3.14 (dd, $J = 13.3, 6.4$ Hz, 6H), 2.63 - 2.49 (m, 2H), 2.22 (s, 3H), 2.29 - 2.12 (m, 8H), 1.89 - 1.43 (m, 25H), 1.42 - 1.23 (m, 6H), 1.03 - 0.75 (m, 18H). ^{13}C NMR (101 MHz, CD_3OD) δ ppm 189.33, 176.09, 175.96, 175.91, 175.68, 175.04, 174.85, 172.58, 155.63, 155.00, 152.31, 149.04, 140.24, 140.20, 120.04, 119.32, 114.98, 114.86, 113.72, 54.60, 53.17, 52.99, 43.74, 41.87, 41.52, 40.33, 40.20, 40.12, 37.73, 37.03, 36.98, 36.89, 36.69, 30.10, 27.51, 26.70, 26.61, 26.11, 26.02, 25.90, 25.72, 24.37, 23.65, 23.50, 23.39, 22.08, 21.86.

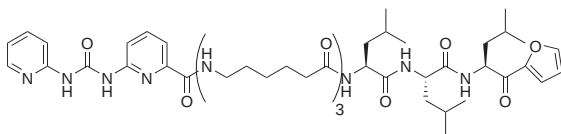


Naphthyridine-Ahx5-Leu3-2-furan (296). Compound 293 (16 mg, 15 μ mol) was dissolved TFA:DCM 1:1 and stirred for 30 min. before being coevaporated with toluene (3 $\times$). The residue was dissolved in DMF and 276

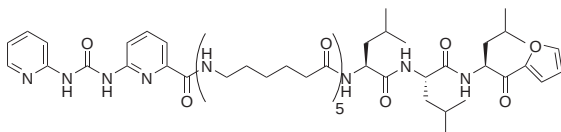
(10 mg, 30 μ mol, 2 equiv.), HBTU (12 mg, 30 μ mol, 2 equiv.) and DiPEA (12 μ l, 75 μ mol, 5 equiv.) were added and the mixture stirred for 2 hr. after which the mixture was concentrated. The residue was dissolved in MeOH and subjected to LH20 Sephadex size exclusion chromatography followed by column chromatography (5% MeOH:CHCl₃) afforded the title compound (5 mg, 4 μ mol, 27%). LCMS: R_t 7.01 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 1285.80 (M + H)⁺). ¹H NMR (400 MHz, CD₃OD) δ ppm 8.30 - 8.23 (m, 2H), 7.99 - 7.89 (m, 2H), 7.82 (s, 1H), 7.40 (d, J = 3.6 Hz, 1H), 6.66 (dd, J = 3.4, 1.5 Hz, 1H), 5.25 - 5.20 (m, 1H), 4.53 - 4.37 (m, 2H), 3.22 - 3.08 (m, 10H), 2.23 (s, 3H), 2.31 - 2.10 (m, 12H), 1.83 - 1.43 (m, 25H), 1.43 - 1.24 (m, 18H), 1.05 - 0.76 (m, 18H).



the title compound (17 mg, 26 μ mol, 52%). LCMS: R_t 7.89 min (linear gradient 10 $\rightarrow$ 90% MeCN + 0.1% TFA, 15 min), (ESI-MS (m/z): 648.20 (M + H)⁺). ¹H NMR (400 MHz, CDCl₃) δ ppm 8.31 (d, J = 4.4 Hz, 1H), 8.21 (d, J = 7.5 Hz, 1H), 7.97 (br s, 1H), 7.84 (d, J = 7.5 Hz, 1H), 7.75 (t, J = 7.8 Hz, 1H), 7.68 - 7.58 (m, 2H), 7.58 - 7.30 (m, 3H), 7.00 - 6.93 (m, 1H), 6.60 - 6.54 (m, 1H), 5.39 (td, J = 9.4, 3.9 Hz, 1H), 4.72 (q, J = 7.5 Hz, 1H), 4.67 - 4.60 (m, 1H), 2.09 - 1.46 (m, 9H), 1.08 - 0.75 (m, 18H). ¹³C NMR (101 MHz, CDCl₃) δ ppm 187.66, 172.21, 171.95, 164.37, 152.13, 151.27, 150.91, 147.10, 146.98, 139.37, 138.55, 118.82, 118.34, 117.40, 116.97, 112.85, 112.50, 52.80, 52.38, 52.02, 41.60, 40.69, 40.57, 25.03, 25.00, 24.69, 23.24, 22.66, 22.30, 22.07, 21.67.



μ mol, 4 equiv.) dissolved in DMF was added. This mixture was stirred for 1.5 hr. after which the mixture was concentrated. The residue was dissolved in MeOH and subjected to LH20 Sephadex size exclusion chromatography followed by column chromatography (5% MeOH:DCM $\rightarrow$ 7.5% MeOH:DCM) afforded the title compound (21 mg, 21 μ mol, 49%). ¹H NMR (400 MHz, CD₃OD) δ ppm 8.37 (dd, J = 5.0, 1.0 Hz, 1H), 8.02 (d, J = 8.1 Hz, 1H), 7.96 - 7.90 (m, 1H), 7.84 (d, J = 1.1 Hz, 1H), 7.81 - 7.75 (m, 2H), 7.50 (d, J = 7.6 Hz, 1H), 7.42 (d, J = 3.3 Hz, 1H), 7.11 - 7.06 (m, 1H), 6.68 (dd, J = 3.6, 1.7 Hz, 1H), 5.25 (dd, J = 9.8, 4.7 Hz, 1H), 4.53 - 4.47 (m, 1H), 4.43 (t, J = 7.6 Hz, 1H), 3.48 (t, J = 7.0 Hz, 2H), 3.19 - 3.13 (m, 4H), 2.21 (ddd, J = 19.7, 15.1, 7.5 Hz, 6H), 1.78 - 1.54 (m, 16H), 1.54 - 1.41 (m, 6H), 1.40 - 1.26 (m, 5H), 1.03 - 0.87 (m, 18H). ¹³C NMR (101 MHz, CD₃OD) δ ppm 189.13, 176.06, 175.93, 174.76, 174.46, 166.30, 154.07, 153.82, 152.93, 152.34, 149.27, 149.02, 148.21, 140.77, 139.91, 120.02, 119.52, 117.96, 117.40, 113.78, 113.67, 54.36, 53.04, 52.82, 41.94, 41.82, 41.56, 40.48, 40.18, 37.00, 36.65, 30.28, 30.09, 27.58, 27.54, 26.78, 26.67, 26.61, 26.13, 25.95, 25.77, 23.66, 23.43, 23.40, 22.18, 22.04, 21.88.



μ mol, 4 equiv.) in DMF was added. This mixture was stirred for 3 hr. after which the mixture was concentrated. The residue was dissolved in MeOH and subjected to LH20 Sephadex size exclusion chromatography followed by column chromatography (5% MeOH:DCM $\rightarrow$ 7.5% MeOH:DCM) afforded the title compound (26 mg, 21 μ mol, 75%). ¹H NMR (400 MHz, CD₃OD) δ ppm 8.37 (d, J = 4.4 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H), 7.93 (t, J = 7.9 Hz, 1H), 7.85 (s, 1H), 7.81 - 7.75 (m, 2H), 7.50 (d, J = 7.4 Hz, 1H), 7.42 (d, J = 3.5 Hz, 1H), 7.09 (dd, J = 6.9,

Dipyridylurea-Leu₃-2-furan (297). Compound **287** (26 mg, 50 μ mol) was dissolved in DMF and **363** (26 mg, 100 μ mol, 2 equiv.), HBTU (38 mg, 100 μ mol, 2 equiv.) and DiPEA (50 μ l, 300 μ mol, 6 equiv.) were added and the mixture stirred for 3 hr. after which the mixture was concentrated. The residue was dissolved in DCM:CHCl₃ 1:1 and washed with sat. aq. NaHCO₃ (3 $\times$), H₂O (3 $\times$) and dried with Na₂SO₄. Column chromatography (DCM $\rightarrow$ 3% MeOH:DCM) afforded the title

Dipyridylurea-Ahx₃-Leu₃-2-furan (298).

Compound **292** (37 mg, 44 μ mol) was dissolved TFA:DCM 1:1 and stirred for 30 min. before being coevaporated with toluene (3 $\times$). The residue was dissolved in DMF and a mixture of carboxylic acid **363** (17 mg, 66 μ mol, 1.5 equiv.), HBTU (27 mg, 70 μ mol, 1.6 equiv.) and DiPEA (29 μ l, 176

Dipyridylurea-Ahx₅-Leu₃-2-furan (299).

Compound **293** (30 mg, 28 μ mol) was dissolved TFA:DCM 1:1 and stirred for 30 min. before being coevaporated with toluene (3 $\times$). The residue was dissolved in DMF and a solution of carboxylic acid **363** (11 mg, 45 μ mol, 1.5 equiv.), HBTU (17 mg, 45 μ mol, 1.6 equiv.) and DiPEA (19 μ l, 112

5.4 Hz, 1H), 6.69 (dd, $J = 3.5, 1.7$ Hz, 1H), 5.25 (dd, $J = 9.8, 4.6$ Hz, 1H), 4.50 (t, $J = 7.5$ Hz, 1H), 4.43 (t, $J = 7.5$ Hz, 1H), 3.48 (t, $J = 7.0$ Hz, 2H), 3.16 (dd, $J = 11.5, 6.8$ Hz, 8H), 2.32 - 2.12 (m, 10H), 1.82 - 1.41 (m, 30H), 1.41 - 1.27 (m, 9H), 1.06 - 0.86 (m, 18H). ^{13}C NMR (101 MHz, CD_3OD) δ ppm 189.11, 176.04, 175.92, 174.73, 174.44, 166.27, 154.05, 153.81, 152.92, 152.32, 149.26, 149.03, 148.20, 140.76, 139.90, 120.03, 119.52, 117.94, 117.40, 113.77, 113.68, 54.36, 53.04, 52.81, 41.93, 41.81, 41.55, 40.48, 40.19, 36.99, 36.65, 30.28, 30.12, 27.55, 26.77, 26.71, 26.61, 26.12, 25.94, 25.76, 23.67, 23.44, 23.41, 22.18, 22.04, 21.88.

Competition experiments in cell lysate

HEK293T cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin in a 7% CO_2 humidified incubator at 37°C. Cells were harvested, washed 2 $\times$ with PBS and permeated in digitonin lysis buffer (4 $\times$ pellet volume, 50 mM Tris pH 7.5, 250 mM sucrose, 5 mM MgCl_2 , 5 mM DTT, 0.025% digitonin) for 15 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 (Biorad). 15 μg (HEK293T) total protein per experiment was exposed to the inhibitors (10 $\times$ solution in DMSO) for 1 hr. at 37°C prior to incubation with MVB003 (500 nM) for 1 hr. at 37°C. Reaction mixtures were boiled with Laemmli's buffer containing β -mercaptoethanol for 3 min. before being resolved on 12.5% SDS-PAGE. In-gel detection of fluorescently labelled 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 nm, λ_{em} 560 nm).

Fluorogenic substrate hydrolysis assay

26S proteasomes were purified from rabbit muscle as described.¹⁶⁹ To determine inhibition of purified proteasomes, proteasome ($\sim 10\text{ng}/\text{ml}$) in assay buffer (50 mM Tris-HCl pH 7.5, 40 mM KCl, 2 mM EDTA, 1 mM DTT, 100 μM ATP, 50 $\mu\text{g}/\text{mL}$ BSA) was incubated with inhibitors for 30 min. at 37°C followed by assay of activity with 100 μM fluorogenic substrates Suc-LLVY-AMC (chymotrypsin-like site), Ac-RLR-AMC (trypsin-like site), and Ac-nLpLD-AMC (caspase-like site). Fluorescence of released AMC was measured (λ_{ex} 350 nm, λ_{em} 430 nm) continuously for 30 min at 37°C. Residual activity was determined from the slope of the reaction progress curves as a mean value from 3 experiments.¹⁴⁷

Induction of immunoproteasome

3T3 cells (5×10^5) were seeded and grown for 6 hr in a 12-well plate. The medium was replaced for 1 ml fresh medium containing 100 units INF- γ per well. The cells were cultured for 48 hr. before inhibitor (1000 $\times$ stock in DMSO) was added and incubated for 2 hr. The cells were washed 2 $\times$ with PBS and harvested. Cell pellets were resuspended in hypotonic lysis buffer (50 mM Tris pH 7.4, 5 mM MgCl_2 , 5 mM DTT) for 15 min at 0°C and centrifuged at 16,100 rcf. for 20 min at 4°C. Lysate (20 μg protein) was incubated with MVB003 (0.5 μM end concentration) 1 hr. at 37°C before being treated as described above. For western blot analysis, cell lysate (100 μg protein) was treated with biotin phosphane (154, 200 μM end concentration) for 1 hr. at 37°C before the reaction was stopped by chloroform/methanol precipitation.²⁴⁸ The pellet was redissolved in 20 μl 2 $\times$ Laemmli's sample buffer. 10 μl of these samples was resolved on 12.5% SDS-PAGE gel. Proteins were electrotransferred to nitrocellulose membranes. The blots were blocked with BSA (1%) in TBS-Tween 20 (0.1% Tween 20) for 1 hr. at RT, hybridized for 1 hr. with streptavidin/HRP (1:3000) in blocking buffer, washed, and visualised with the aid of an ECL+ kit (Amersham Biosciences).

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Samenvatting

Op weg naar subunit-specifieke proteasoomremmers

De kwaliteit en hoeveelheid van elk eiwit is van cruciaal belang voor het overleven van een cel. Het proteasoom speelt een onmisbare rol in het afbreken van de meeste eiwitten in de menselijke cel. Het proteasoom, een groot eiwitcomplex dat strak gereguleerd wordt, is daarvoor uitgerust met subunits met verschillende proteolytische activiteiten die bij elkaar in het eiwitcomplex zitten. Het constitutieve proteasoom bevat subunits met caspase-achtige activiteit ($\beta 1$), trypsine-achtige activiteit ($\beta 2$) en chymotryptische activiteit ($\beta 5$). Elk van deze activiteiten prefereert het knippen van een eiwitketen op andere plekken. Dit wordt gestuurd door het aminozuur naast de te knippen plek in een eiwit. Afhankelijk van het celtype kunnen nog eens vier verschillende subunits met proteolytische activiteiten de plek van $\beta 1$, $\beta 2$ of $\beta 5$ van het proteasoom innemen. Op een gecontroleerde manier worden doeleiwitten door het proteasoom afgebroken tot korte peptiden. De meeste van deze peptiden worden verder afgebroken tot losse aminozuren. Een deel van de peptiden wordt echter geselecteerd voor presentatie op het celoppervlak en door het immuunsysteem bestudeerd. Op deze manier stelt het immuunsysteem vast of een cel lichaamsvreemde eiwitten bevat en wordt er zo nodig een immuunreactie opgewekt. Het proteasoom is dus een belangrijk studieobject in de immunologie. Daarnaast is het proteasoom een doelwit voor medicijnen tegen kanker.

Op basis van antiontstekings- of antitumoractiviteit zijn veel natuurstoffen gevonden die een remmende werking vertonen op proteolytische activiteiten van het proteasoom. Tegelijkertijd zijn er vele proteasoomremmers gesynthetiseerd en op het moment wordt de proteasoomremmer bortezomib in de kliniek gebruikt als medicijn tegen leukemie. Een aantal andere remmers wordt aan klinisch onderzoek onderworpen. De rol van elke afzonderlijke proteolytische activiteit in gezonde cellen of kankercellen of hun rol in het ontstaan van auto-immuunziekten is nog niet geheel duidelijk. Selectieve remmers die elke activiteit van het proteasoom afzonderlijk kunnen stilleggen zijn handig gereedschap om de rol van elk van deze activiteiten te onderzoeken. Om gebruikt te kunnen worden om proteasoomfuncties in cellen te bestuderen moeten deze selectieve remmers wel celpermeabel zijn. Veel proteasoomremmers bestaan uit een electrofiële val (om aan het katalytisch actieve residu van de proteolytische activiteit van het proteasoom te binden), een peptidensequentie (ver-

antwoordelijk voor herkenning), en een N-terminale extensie. Dit proefschrift beschrijft de ontwikkeling van enkele specifieke remmers van individuele proteasoomactiviteiten.

Hoofdstuk 1 geeft een overzicht van natuurlijk voorkomende en synthetische klassen proteasoomremmers. Daarnaast wordt een overzicht gegeven van proteasoomremmers die selectief zijn voor bepaalde proteolytische activiteiten van het proteasoom.

Hoofdstuk 2 beschrijft de synthese van drie proteasoomremmers die de chymotryptische activiteit van het proteasoom ($\beta 5$) remmen. Door het epoxyketon-electrofiel in deze drie stoffen, verantwoordelijk voor binding aan het nucleofiele katalytische residu van de proteasoomactiviteit, te vervangen door een vinylsulfon, werden drie analoga verkregen. Met behulp van een fluorescent epoxomicin-analoog werd het inhibitieprofiel van de zes stoffen in cellysaat bepaald. Door gebruik van een fluorogeen substraathydrolyse-assay met gezuiverd constitutief proteasoom en immunoproteasoom werd de activiteit op immunosubunits onderzocht. De activiteit van de drie vinylsulfon-analogen is lager dan die van de epoxyketon-gebaseerde remmers, maar twee van de vinylsulfon-analogen vertonen meer selectiviteit voor $\beta 5$. De electrofiel val beïnvloedt dus de selectiviteit van proteasoomremmers.

Hoofdstuk 3 beschrijft de synthese van tien electrofiel vallen die in staat zijn Michael-additie te ondergaan. Deze electrofielen werden gekoppeld aan drie verschillende peptidelen en dit leidde tot een dertigtal verbindingen. Geen van deze stoffen vertoonde potente inhibitie van proteasoomactiviteit. De cellulaire doelwitten van twee azide bevattende stoffen werd bepaald door middel van een bioorthogonale twee-staps labelingstrategie. De stoffen werden met cellen geïncubeerd, waarna de azide selectief gemodificeerd werd met biotine door middel van de Staudinger-ligatie. De gemodificeerde cellulaire doelwitten werden gescheiden op basis van de aanwezigheid van biotine en geanalyseerd met massaspectrometrie. Onder de teruggevonden eiwitten waren katalytisch actieve subunits van het proteasoom, ondanks het onvermogen van de gebruikte stoffen het proteasoom potent te remmen. Daarnaast werden cathepsines gevonden, een familie cysteine-proteases die in staat zijn Michael-additie te ondergaan.

Syringolin A is een potente proteasoomremmer die wordt uitgescheiden door een plantenpathogeen wanneer deze een plant binnendringt. Syringolin A is ook een potente remmer van het menselijke proteasoom. **Hoofdstuk 4** behandelt de inbouw van het bijzondere valine-ureum-valine motief, aanwezig in syringolin A, in een bibliotheek peptide-gebaseerde vinylsulfonen en epoxyketonen. Het inhibitieprofiel van de bibliotheek werd vastgesteld met een fluorescente analoog van epoxomicin. Stoffen waarbij de ureumband op een korte afstand van de electrofiel val is ingebouwd remmen de caspase-achtige activiteit ($\beta 1$) van het proteasoom. Een epoxyketon-gebaseerde remmer uit deze korte serie kwam naar voren als de meest potente selectieve remmer van $\beta 1$ in levende cellen tot nu toe beschreven. Remmers met een iets langere afstand tussen electrofiel en ureumband vertonen juist selectiviteit voor de chymotryptische proteasoomactiviteit ($\beta 5$). Verscheidene stoffen uit de in dit hoofdstuk beschreven bibliotheek zijn potenter dan syringolin A.

Hoofdstuk 5 beschrijft de vergelijking van twee-staps labeling door cyclooctynen en de Staudinger-Bertozzi ligatie. Proteasomen werden gelabeld met een proteasoomremmer die een BODIPY-fluorofor en een azide bevat. De reactie-efficiëntie van drie cyclooctynen,

bevestigd aan biotine, werd bestudeerd aan de hand van de verschuiving van proteasoomsignalen na gel-electroforese. De concentratie die nodig is om alle azide-BODIPY gemodificeerde proteasomen te decoreren met biotine is voor cyclooctynen veel lager dan voor de fosfine van de Staudinger-Bertozzi-ligatie. Bij het uitlezen van het signaal van biotine blijkt dat de cyclooctyn-ligatie gepaard gaat met veel ongewenste achtergrondlabeling, in tegenstelling tot de Staudinger-Bertozzi-ligatie.

In **Hoofdstuk 6** wordt de synthese van een serie proteasoomremmers met basische aminozuurzijketens beschreven. Het inhibitieprofiel van deze stoffen in cellysaat en levende cellen werd bepaald met behulp van een fluorescente epoxomicinanaloog. Selectiviteit voor de tryptische activiteit van het proteasoom ($\beta 2$) wordt verkregen wanneer een bij fysiologische pH positief geladen zijketen op P1 wordt gebruikt. Het inbouwen van een 4-aminomethyleen fenylalanine op de P1- en P3-positie van een tetrapeptide vinylsulfon levert een potente remmer met hoge selectiviteit voor $\beta 2$ op. Bevestiging van een BODIPY-fluorofoor aan dit molecuul resulteerde in een activiteits-gebaseerde probe voor $\beta 2$. Door de aanwezigheid van twee basische zijketens komen deze twee moleculen echter niet efficiënt de cel binnen.

List of Publications

‘A Fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes *in vitro* and *in vivo*.’ Verdoes, M.; Florea, B. I.; Menendez-Benito, V.; Maynard, C. J.; Witte, M. D.; van der Linden, W. A.; van den Nieuwendijk, A. M.; Hofmann, T.; Berkers, C. R.; van Leeuwen, F. W.; Groothuis, T. A.; Leeuwenburgh, M. A.; Ovaa, H.; Neefjes, J. J.; Filippov, D. V.; van der Marel, G. A.; Dantuma, N. P.; Overkleeft, H. S. *Chem. Biol.* **2006**, *13*, 1217 - 1226.

‘Mixing of peptides and electrophilic traps gives rise to potent, broad-spectrum proteasome inhibitors.’ Verdoes, M.; Florea, B. I.; van der Linden, W. A.; Renou, D.; van den Nieuwendijk, A. M. C. H.; van der Marel, G. A.; Overkleeft, H. S. *Org. Biomol. Chem.* **2007**, *5*, 1416-1426.

‘Azido-BODIPY acid reveals quantitative Staudinger-Bertozzi ligation in two-step activity-based proteasome profiling.’ Verdoes, M.; Florea, B. I.; Hillaert, U.; Willems, L. I.; van der Linden, W. A.; Sae-Heng, M.; Filippov, D. V.; Kisselev, A. F.; van der Marel, G. A.; Overkleeft, H. S. *ChemBioChem* **2008**, *9*, 1735-1738.

‘Proteasome selectivity towards Michael acceptor containing oligopeptide-based inhibitors.’ van der Linden, W. A.; Geurink, P. P.; Oskam, C.; van der Marel, G. A.; Florea, B. I.; Overkleeft, H. S. *Org. Biomol. Chem.* **2010**, *8*, 1885-1893.

‘Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit $\beta 5t$.’ Florea, B. I.; Verdoes, M.; Li, N.; van der Linden, W. A.; Geurink, P. P.; van den Elst, H.; Hofmann, T.; de Ru, A.; van Veen, P. A.; Tanaka, K.; Sasaki, K.; Murata, S.; den Dulk, H.; Brouwer, J.; Ossendorp, F. A.; Kisselev, A. F.; Overkleeft, H. S. *Chem. Biol.* **2010**, *17*, 795 - 801.

‘Nature of pharmacophore influences active site specificity of proteasome inhibitors.’ Screen, M.; Britton, M.; Downey, S. L.; Verdoes, M.; Voges, M.

J.; Blom, A. E. M.; Geurink, P. P.; Risseuw, M. D. P.; Florea, B. I.; van der Linden, W. A.; Pletnev, A. A.; Overkleeft, H. S.; Kisselev, A. F. *J. Biol. Chem.* **2010**, *285*, 40125-40134.

‘Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: A comparative study.’ van der Linden, W. A.; Li, N.; Hoogendoorn, S.; Ruben, M.; Verdoes, M.; Guo, J.; Boons, G.-J.; van der Marel, G. A.; Florea, B. I.; Overkleeft, H. S. *Bioorg. Med. Chem.* **2011**, *in press*.

‘Discovery of a potent and highly β 1 specific proteasome inhibitor from a focused library of urea-containing peptide vinyl sulfones and peptide epoxyketones.’ van der Linden, W. A.; Willems, L. I.; Shabaneh, T. B.; Li, N.; Ruben, M.; Florea, B. I.; van der Marel, G. A.; Kaiser, M.; Kisselev, A. F.; Overkleeft, H. S. *Org. Biomol. Chem.* **2011**, *in press*.

Curriculum Vitae

Wouter Alexander van der Linden werd op 18 februari 1983 geboren te Rotterdam. Na het behalen van het gymnasium-diploma aan het Rotterdams Montessori Lyceum in 2001 ving hij in dat jaar met de studie Scheikunde aan de Universiteit Leiden aan. Van september 2004 tot september 2005 en december 2005 tot februari 2006 werd als afstudeerproject onder leiding van M. Verdoes en dr. B. I. Florea in de vakgroep Bio-organische Synthese onder leiding van prof. dr. G. A. van der Marel en prof. dr. H. S. Overkleef onderzoek verricht. Dit onderzoek omvatte het synthetiseren en biologisch evalueren van een breed scala aan proteasoomremmers. Van juni 2006 tot augustus 2006 werd in het kader van een uitwisselingsproject aan het Occidental College in Los Angeles (USA) onder leiding van Prof. Dr. A. Nersissian het ontstaan van type 4-kopereiwitten in planten en gewervelden bestudeerd met behulp van sequentieanalyse. Het doctoraal examen (Master of Science) werd 'Cum Laude' behaald op 15 mei 2007. Van september 2007 tot december 2011 werd als assistent in opleiding het in dit proefschrift beschreven onderzoek uitgevoerd in de vakgroep Bio-organische Synthese onder leiding van dr. B. I. Florea en prof. dr. H. S. Overkleef. In november 2007 werd deelgenomen aan het 'International symposium on protein modification and degradation' in Peking, China en in september 2010 aan het symposium 'Chemical biology' van het European Molecular Biology Organisation (EMBO), Heidelberg, Duitsland. In juli 2009 werd deelgenomen aan de HRSMC summer school 'New horizons in synthetic methodology' in Maastricht.

Nawoord

Deze laatste pagina draag ik op aan iedereen die op wat voor manier dan ook de onmogelijkheid van de totstandkoming van dit proefschrift tenietgedaan heeft. Een stevige basis buiten het lab is erg belangrijk. Ik wil mijn ouders, broers, verdere familie en mijn vrienden bedanken voor de steun die zij mij hebben gegeven en voor alle welkome afleiding die zij hebben geleverd.

Onderzoek doen verloopt het beste in een omgeving waar wordt samengewerkt en gediscussieerd over plannen en resultaten. Bovendien is een goede sfeer op het lab onmisbaar als je plezier in je onderzoek wilt hebben. Voor alle zin en onzin, gezelligheid tijdens de lunch, borrels, labweekenden en andere activiteiten wil ik alle (ex)-BIOSYNers bedanken. Chris Oskam, Annet Blom, Thias Voges, Elliot Mock en Vincent van der Weijden, ik wil jullie bedanken voor jullie inzet en enthousiasme tijdens jullie stage en ik wens jullie veel succes met jullie carrière en daarbuiten.

Apparaten benodigd voor het uitvoeren van analyses zijn vaak zeer complex, en dit wordt altijd het beste duidelijk als er niet de beoogde resultaten behaald mee kunnen worden. Gelukkig kon ik dan altijd op de hulp rekenen van Fons, Kees, Karthick, Ine, Hans, Nico en Rian. De ama's Hennie, Arnold en Marco wil ik ook bedanken voor hun technische ondersteuning op het lab en Trudy en Arjan voor het stroomlijnen van de vele practica. Caroline, bedankt voor je hulp bij logistieke operaties.

Boudewijn, bedankt voor je hulp met $\text{\LaTeX}$.

Eric Reits, Marcel Raspe, Sabine Krom en Anita Stargardt, bedankt voor de vruchtbare samenwerking. My thanks go out to the people in the Bogyo-lab for all their help and good times during my time there. Thanks to Alexei Kisselev and members of his lab for the successful collaborations.

Sytske, ik vind het heel fijn met jou en ik ben blij dat je er altijd voor me bent. Ik hoop dat we binnenkort het regenachtige voor het zonnige gaan verruilen en een prachtige tijd gaan beleven!

