Proteasome selectivity towards Michael acceptor containing oligopeptide-based inhibitors†‡

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The synthesis and biological evaluation of ten Michael acceptors containing potential proteasome inhibitors are described. Cellular targets of azide containing inhibitors **Ib** and **VIIIb** were assessed in HEK293T and RAW264.7 cells by a two step labeling strategy, followed by biotin-pulldown, affinity purification, on-bead tryptic digestion and LC-MS^2 identification. This strategy appears to be an attractive alternative to gel-based competition assays.

Introduction

The 26S proteasome is the central protein degrading enzyme machinery in the eukaryotic cell. Present in both the cytoplasm and nucleus, the proteasome degrades 80-90% of all cellular proteins in an ATP and ubiquitin-dependent pathway.[1] The proteasome is responsible for the degradation of abnormal or damaged proteins, oncogenes, tumor suppressors and cell cycle regulators, and partakes in the generation of MHC class I restricted peptides.[2] The eukaryotic 26S proteasome contains a 20S core particle that is capped with either one or two 19S regulatory domains. The 20S particle is build up from 28 subunits and contains the proteolytic activities. In eukaryotes, the proteasome consists of two copies of seven distinct α subunits and seven distinct β subunits. These subunits are ordered in four juxtaposed heptameric rings [(α_1-α_7) (β_1-β_7) (β_1-β_7) (α_1-α_7)] forming a C_2-symmetrical barrel shaped structure.[3] The β1, β2 and β5 subunits display caspase-like, trypsin-like and chymotrypsin-like activity, respectively.[4] In immunocompetent cells, stimulation by interferon-γ causes expression of three additional proteolytic subunits, namely β1i, β2i and β5i, with substrate preferences different from their constitutive counterparts.[5] These subunits are incorporated in newly formed immunoproteasomes, which coexist with the constitutive proteasome.[6] Fluorescent substrate assays and inhibitors of the proteasome have proven useful tools to elucidate the proteolytic function of the proteasome. A fluorescent substrate assay, however, cannot provide direct information about which subunit cleaves the substrate. Covalent and irreversible inhibitors act in a more defined way upon the proteasome. When an inhibitor is equipped with a tag, such as a fluorescent label,[7] radio tag[8] or affinity tag,[9] covalent and irreversible modification allows detection and identification of the catalytic β-subunits targeted by the inhibitor. Furthermore, the proteasome is a validated clinical target in oncology and several covalent inhibitors of the proteasome are considered as leads for the development of therapeutics.[10] Most proteasome inhibitors known to date are built up from a peptide recognition element, equipped with an electrophilic trap to capture the γ-hydroxyl of the catalytically active N-terminal threonine.[11] Well known electrophilic traps are the vinyl sulfone,[8] epoxyketone,[12] boronic acid[13] and aldehyde functional groups.[14] In the past, much effort has been devoted to vary the peptide sequence of the recognition element.[15] In this work, ten electrophilic traps, with the potential to target the proteasome, are investigated. As recognition elements used to assess proteasome inhibition capacity we selected the Z-Leu_2 motif[8] and the $AdaAhx_3Leu_2$ motif.[9] In addition, the $N_3PheLeu_2$ 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 labeling chemical proteomics experiments.[16]

Results and discussion

The C-terminally modified target peptides are depicted in Table 1. We first set out to synthesize ten amino acid derived Michael acceptors. The first five Michael acceptors in Table 1 (compounds **I–V**) were derived from Boc-leucinal applying standard Horner–Wadsworth–Emmons (HWE) olefination procedures. For example, iodide substitution in diethyl iodomethylphosphonate by the sodium salt of 1-adamantylmethylthiol[17] followed by peracetic acid oxidation yielded diethyl 1-adamantylmethylsulfonylmethylphosphonate **1** in 65% over two steps (Scheme 1). Reaction of aldehyde **2** with HWE reagent **1** yielded **3** in 89% yield. The synthesis of four other Boc-leucinal derived electrophilic traps of compounds **I-IV**, as well as the traps in compounds **VI-VII**, are described in the supplementary information.

Horner–Wadsworth–Emmons olefination of **4**[18] using diethyl methyl phosphonoacetate yielded diester **5**, which could be selectively saponified to **6** in 73% yield. $NaBH_4$ reduction of the **6** derived mixed anhydride afforded unsaturated furanolactone **7**. Cyclic vinyl ester **9**, configurationally different from **7**, was obtained from chloromethylketone **8**[19] in two steps. Chloride substitution in **8** by potassium diethyl phosphonoacetate and

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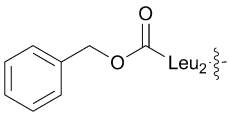
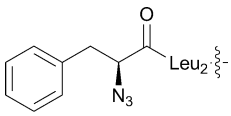
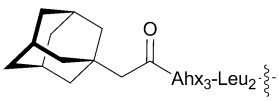
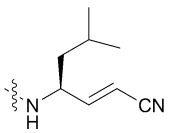
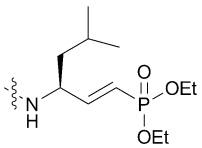
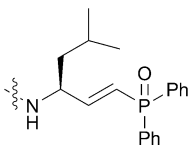
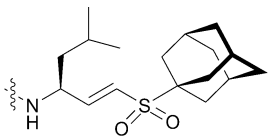
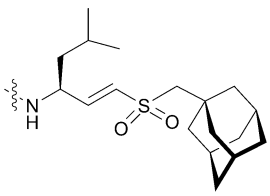
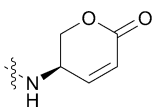
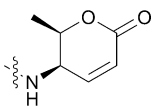
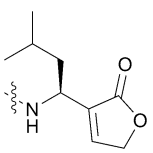
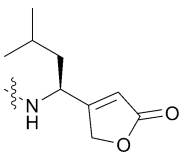
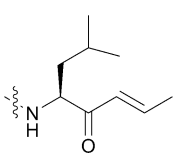
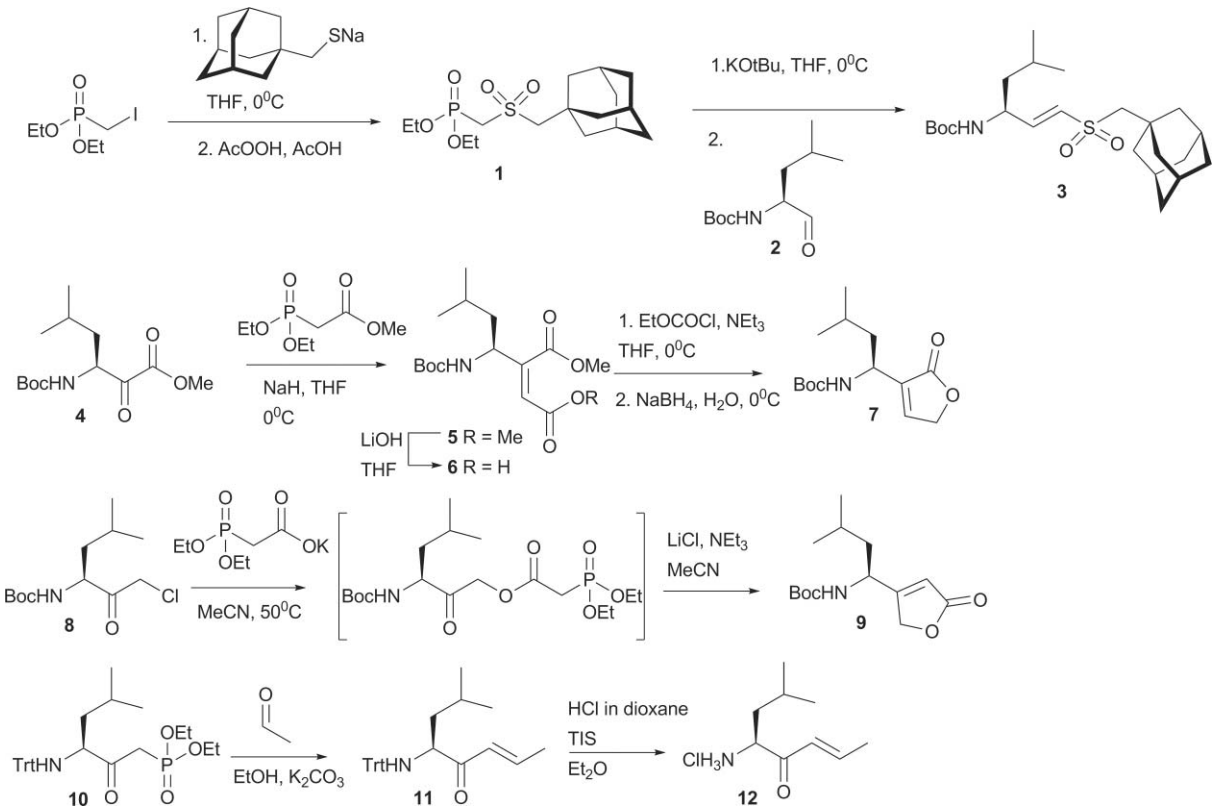
‡ Electronic supplementary information (ESI) available: ^1H and ^{13}C spectroscopic and analytical data of new compounds, competition assay results and raw LC/MS peptide data. See DOI: 10.1039/b924134e

Table 1 Overview of designed panel of potential proteasome inhibitors

			
	**Ia**	**Ib**	**Ic**
	**IIa**	**IIb**	**IIc**
	**IIIa**	**IIIb**	**IIIc**
	**IVa**	**IVb**	**IVc**
	**Va**	**Vb**	**Vc**
	**VIa**	**VIb**	**VIc**
	**VIIa**	**VIIb**	**VIIc**
	**VIIIa**	**VIIIb**	**VIIIc**
	**IXa**	**IXb**	**IXc**
	**Xa**	**Xb**	**Xc**



Scheme 1 Synthesis of selected Michael acceptors.

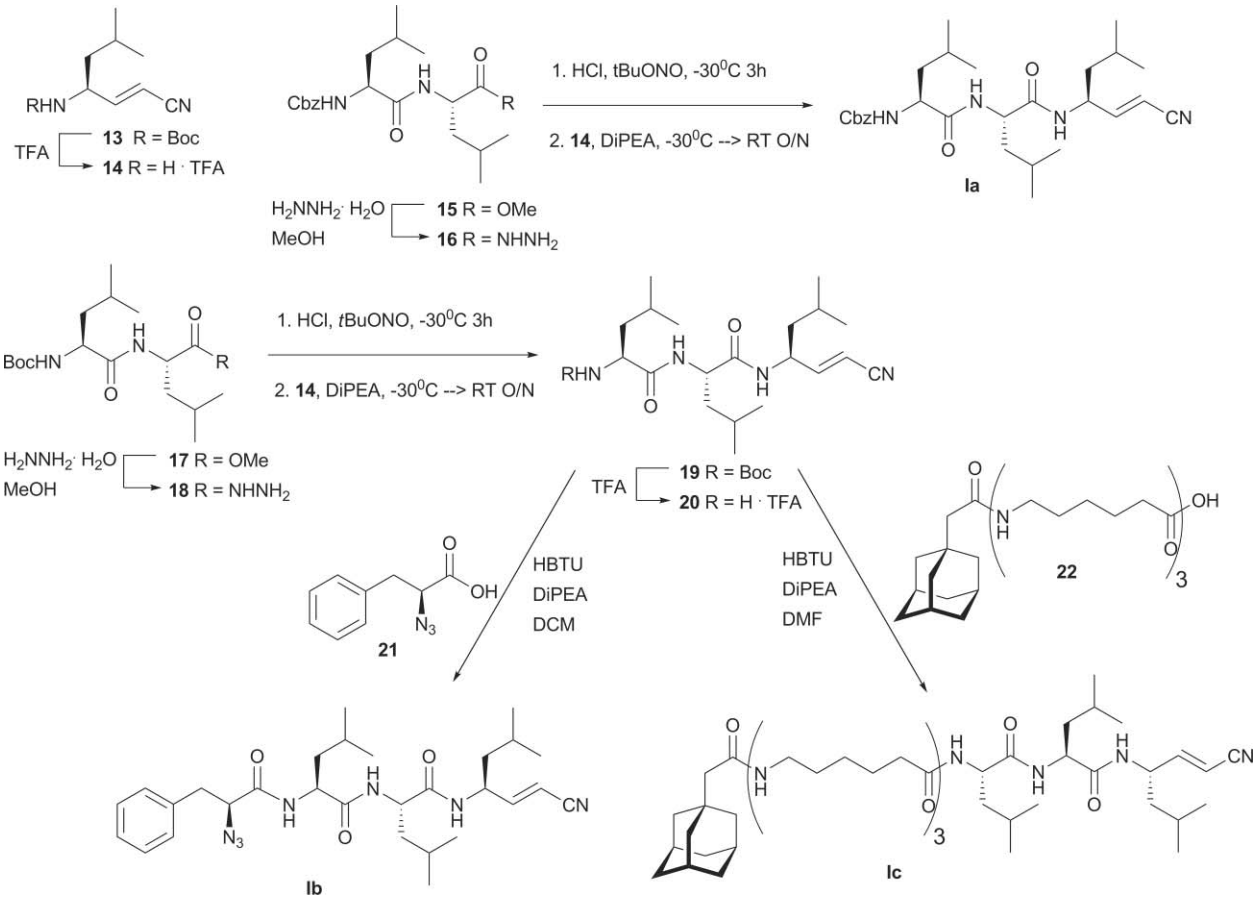
subsequent intramolecular LiCl promoted HWE reaction yielded **9** in 52% yield over two steps. HWE olefination of **10**[20] with acetaldehyde afforded vinyl ketone **11** in 86% yield, which was detritylated using dry HCl/triisopropylsilane (TIS) to obtain free amine **12** (Scheme 1).

The ten potential electrophilic traps were coupled to the peptide recognition parts using a general strategy, which is illustrated by the preparation of the **I**-series (Scheme 2). Vinyl nitrile **13** was deprotected using TFA to give **14** in quantitative yield. BocLeu$_2$OMe **17** was reacted with hydrazine to yield hydrazide **18**. In a one pot procedure, hydrazide **18** was transformed into its acyl azide using *t*BuONO and HCl. This acyl azide was subsequently reacted with the vinyl nitrile-TFA salt **14** under the influence of DiPEA to yield the Boc-protected tripeptide **19** without observable epimerization during the coupling. In a similar fashion, Cbz-protected tripeptide **Ia** was synthesized from **16**. The tripeptide **19** was deprotected with TFA and subsequently block-coupled to either azido-phenylalanine[21] **21** or AdaAhx$_3$-OH **22** to yield potential inhibitors **Ib** and **Ic**, respectively (Scheme 2).

Next, the proteasomal inhibition capacity of the synthesized compounds was assessed. To this end we employed a competition assay in human embryonic kidney HEK293T cell lysates at a final concentration of 0, 1, 10 and 100 μM *versus* fluorescent proteasome inhibitor MV151[7] (Supplementary information figure 2, 500 nM final conc.). Only compounds **Ic**, **IIc**, **IIIc**, **IVc**, **Vc**, **IXc** and **Xc** showed some loss of MV151 signal at 100 μM concentration, indicating proteasome inhibition (Supp. Info S1). However, this potency is very low compared to the known inhibitors AdaAhx$_3$Leu$_3$VS and ZLeu$_3$VS.[22]

For the two vinyl sulfones in **IV** and **V**, 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, vinyl ethyl esters,[23] epoxyketones, aldehydes and boronic acids, the lack of reactivity of the other inhibitors described in this article is quite surprising. To test whether these compounds target other molecules in the cell, HEK293T and RAW264.7 cells were incubated with 50 μM of compound **Ib** and **VIIIb**. After cell lysis, Staudinger ligation using biotin-phosphine[24] introduced a biotin to the azide modified proteins. Streptavidin pulldown enriched the pool of labeled enzymes, which were then digested on-bead and analysed by mass spectrometry. The observed peptides divided by the observable peptides (Protein Abundance Index, PAI), exponentially modified, yields the emPAI ($10^{PAI} - 1$), which is a measure for absolute protein quantification of the labeled enzymes.[25] Together with the total protein coverage by the peptides that were found back, hits were selected and depicted in Table 2-3.

Surprisingly, the peptides found back with the highest emPAI and best protein coverage belong to the active subunits of the proteasome, which means that **Ib** and **VIIIb** targeted the proteasome in the HEK and RAW cells. This is remarkable since **Ib** and **VIIIb** do not show proteasome inhibition in lysates at concentrations up to 100 μM in the competition assay in lysates *versus* MV151. The pull-down, tryptic digest/mass spectrometry analysis, in which even the smallest fraction of labeled 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



Scheme 2 General coupling strategy of electrophilic traps to peptide elements.

Table 2 Determination of cellular targets of compound **Ib** in HEK293T and RAW264.7 cells by affinity purification and nano-LCMS analysis

HEK293T			RAW264.7		
Protein description	Coverage (%)[a]	emPAI[b]	Protein description	Coverage (%)	emPAI
Proteasome subunit β5	60.1	10.25	Proteasome subunit β1i	73	26.78
Proteasome subunit β2	38.3	1.07	Proteasome subunit β1	72	12.08
Proteasome subunit β1	39.3	0.45	Proteasome subunit β5i	54	6.13
Cathepsin L2	21	0.52	Proteasome subunit β5	72	11.6
Cathepsin L1	13.2	0.29	Proteasome subunit β2	46.1	1.56
Cathepsin F	3.1	0.06	Proteasome subunit β2i	11	0.24
Tubulin		0.66	Cathepsin L1	44	2.49
			Cathepsin Z	49.8	4.13
			Cathepsin F	26.2	0.73
			Cathepsin D	23.4	0.41

[a] Protein sequence coverage by peptides found back. [b] (10^(observed peptides/observable peptides)) – 1.

Table 3 Determination of cellular targets of compound **VIIIb** in HEK293T and RAW264.7 cells by affinity purification and nano-LCMS analysis

HEK293T			RAW264.7		
Protein description	Coverage (%)[a]	emPAI[b]	Protein description	Coverage (%)	emPAI
Proteasome subunit β5	17.5	0.25	Proteasome subunit β1i	33.8	1.22
Proteasome subunit β2	10.1	0.11	Proteasome subunit β5	15.5	0.12
Keratin		2.54	Proteasome subunit β1	8	0.28
			Cathepsin Z	12.1	0.44
			Cathepsin L1	32.3	0.52
			Cathepsin S	30	0.18

[a] Protein sequence coverage by peptides found back. [b] (10^(observed peptides/observable peptides)) – 1.

is low compared to the proteasome hits, which could be explained by the higher proteasome content in mammalian cells compared to cathepsins.

Conclusion

Despite the variety of potential proteasome inhibitors synthesized 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 **Ib** and **VIIIb** in a gel based competition assay is low, these compounds are able to label the proteasome, as was confirmed with the biotin pulldown-MS^2 detection method. These results demonstrate that the biotin pulldown-MS^2 assay is an attractive alternative to the gel based competition assay, because the MS^2 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.

Experimental

All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.) (purum), ethyl acetate (EtOAc) (puriss.), and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën or Biosolve and were distilled prior to use. Dichloromethane (DCM), dimethylformamide (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, followed by charring at ~150 °C; by spraying with a solution of $(NH_4)_6Mo_7O_{24}.4H_2O$ (25 g L^{-1}) and $(NH_4)_4Ce(SO_4)_4.2H_2O$ (10 g L^{-1}) in 10% sulfuric acid, followed by charring at ~150 °C; or by spraying with an aqueous solution of $KMnO_4$ (7%) and KOH (2%). Column chromatography was performed on 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). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Jeol JNM-FX-200 (200/50 MHz), Bruker DPX-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory or a Bruker AV-500 (500/125 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. IR spectra were recorded on a Shimadzu FTIR-8300 spectrometer. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, $\lambda = 589$ nm).

Diethyl 1-adamantylmethylsulfonylmethylphosphonate (1)

1-Adamantylmethylthiol[17] (1.82 g, 10 mmol) was dissolved in THF (50 mL) at 0 °C. NaH (0.42 g 60% disp. in mineral oil, 10.5 mmol, 1.05 equiv.) was added and the solution was stirred for 1 h. Diethyl iodomethylphosphonate (2.78 g, 10 mmol, 1 equiv.) was added, and the mixture was stirred for 2 h. The reaction was quenched by the addition of 1 M HCl. The water layer was extracted with DCM (3×) and the combined organic layers were dried with $MgSO_4$ 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 h. Sat. aq. $NaHCO_3$ was added, and this mixture was extracted with EA (3×). The combined organic layers were dried over $MgSO_4$ and evaporated. The residue was recrystallised from EA : PE, yielding two crops of the title compound (total 2.38 g, 6.54 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 1.37 (t, $J = 7.06$, 7.06 Hz, 1H), 1.78-1.60 (m, 6H), 1.90-1.87 (m, 6H), 2.04-1.97 (m, 3H), 3.21 (s, 2H), 3.52 (d, $J = 16.47$ Hz, 1H), 4.28-4.18 (m, 4H).

Boc-leucinal (2)

Boc-leucine Weinreb amide (1.7 g 6.2 mmol) was dissolved in Et_2O (30 mL) at 0 °C. $LiAlH_4$ (0.24 g, 6.2 mmol, 1 equiv.). was added, and the mixture was stirred for 30 min. The reaction mixture was quenched with 1 M HCl. More 1 M HCl was added, and the water layer was extracted with Et_2O (3×). The combined organic layers were washed with brine and dried over Na_2SO_4, yielding the title compound, which was used without further purification.

(*S*,*E*)-1-(1-Adamantylmethylsulfonyl)-5-methylhex-1-en-3-Boc-amine (3)

Diethyl 1-adamantylmethylsulfonylmethylphosphonate **1** (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 **2** (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. NH_4Cl. More sat. aq. NH_4Cl was added, and the resulting mixture was extracted with EA. The organic phase was washed with brine, dried over $MgSO_4$ and evaporated. Column chromatography (EA : PE (0-12%)) yielded the title compound (2.0 g, 4.7 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 0.94 (d, $J = 6.65$ Hz, 6H), 1.47-1.41 (m, 11H), 1.75-1.63 (m, 7H), 1.87-1.79 (m, 6H), 2.02-1.97 (m, 3H), 2.81 (s, 1H), 4.44-4.33 (m, 1H), 5.12 (d, $J = 8.28$ Hz, 1H), 6.74 (dd, $J = 15.04$, 5.22 Hz, 1H), 6.46 (dd, $J = 15.05$, 1.21 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 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 (5)

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 (**4**)[18] (708 mg, 2.6 mmol, 1 equiv.) in THF was added and the mixture was stirred for 2.5 h. Sat. aq. NH_4Cl was added, and the mixture was extracted with EA. The organic layer was washed with sat. aq. $NaHCO_3$, brine, dried with $MgSO_4$ and concentrated. Column chromatography (EA : PE (5-25%)) yielded the title compound (674 mg, 2.04 mmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 6.03 (s, 1H), 5.31 (d, $J = 8.32$ 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). ^{13}C NMR (100 MHz, $CDCl_3$): 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. $[\alpha]_D^{20}$ –31.1 (*c* 1.00, $CHCl_3$).

(*S*,*Z*)-4-Boc-amino-3-(methoxycarbonyl)-6-methylhept-2-enoic acid (6)

(*S*)-Dimethyl-2-(1-Boc-amino-3-methylbutyl)-maleate **5** (674 mg, 2.04 mmol) was dissolved in 8 mL THF at 0 °C. LiOH (2.04 mL 1 M aq sln, 2.04 mmol, 1 equiv.) was added and the solution was stirred for 2.5 h. LiOH (306 µL 1 M aq sln, 306 µmol, 0.15 equiv.) was added and the solution was allowed to warm to RT and stirred overnight. 1 M aq. HCl was added, and the mixture was extracted with EA (2×). The combined organic layers were washed with brine, dried with $MgSO_4$ and concentrated. Column chromatography (EA : PE (30-70%)) yielded the title compound (467 mg, 1.48 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers): δ ppm 10.55 (s, 1H), 6.57 (d, J = 7.09 Hz, 1H), 6.03 (s, 1H), 5.94 (s, 1H), 5.06 (d, J = 8.48 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). ^{13}C NMR (100 MHz, $CDCl_3$, 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. $[\alpha]_D^{20}$ –23.3 (*c* 1.00, $CHCl_3$).

(*S*)-3-(1-Boc-amino-3-methylbutyl)furan-2(5*H*)-one (7)

(*S*,*Z*)-4-Boc-amino-3-(methoxycarbonyl)-6-methylhept-2-enoic acid **6** (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 h. 1 M HCl was added and the mixture was extracted with EA. The organic layer was washed with 1 M HCl, sat. aq. $NaHCO_3$ and brine. After drying with $MgSO_4$ and concentration, the residue was purified with column chromatography (EA : PE (10 : 50%)), yielding the title compound (190 mg, 0.70 mmol, 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.282, 1H), 5.31 (d, J = 8.66 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. $[\alpha]_D^{20}$ –24.8 (*c* 1.00, $CHCl_3$).

(*S*)-4-(1-Boc-amino-3-methylbutyl)-2(5*H*)-furanone (9)

(*S*)-1-Chloro-2-oxo-3-(Boc-amino)-5-methylhexane **8**[19] (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 h. EA and 20% aq. NaH_2PO_4 were added, layers separated, and the water layer was extracted with EA (2×). 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 TEA (0.42 mL, 3 mmol, 1 equiv.). After 3 h, the reaction mixture was poured into 1 M HCl, followed by extraction with EA. The organic layer was dried over $MgSO_4$ and concentrated. Flash column chromatography (EA : toluene (0-15%)) yielded the title compound (418 mg, 1.55 mmol, 52%). ^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 5.91 (q, J = 1.5 Hz, 1H), 5.44 (d, J = 8.0 Hz, 1H), 4.95-4.75 (m, 2H), 4.61 (m, 1H), 1.85-1.57 (m, 3H), 1.44 (s, 9H), 0.95 (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. $[\alpha]_D^{20}$ –73.2 (*c* 1.00, MeOH). HRMS: calcd for $[C_{14}H_{23}NO_4Na]^+$ 292.15193, found 292.15175.

(*S*,*E*)-5-Amino-7-methyloct-2-en-4-one (11)

1-(Dimethylphosphonate)-2-oxo-3-(tritylamino)-5-methylhexane **10**[20] (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 overnight. 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 (1-5%)) yielded the title compound (8.06 g, 20.27 mmol, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.53-7.02 (m, 15H), 6.32 (qd, J = 13.76, 6.86 Hz, 1H), 5.69 (dd, J = 15.55, 1.58 Hz, 1H), 3.59 (t, J = 6.60 Hz, 1H), 3.15-3.01 (m, 1H), 1.75-1.64 (m, 1H), 1.57 (dd, J = 6.87, 1.36 Hz, 3H), 1.44 (t, J = 6.75 Hz, 2H), 0.86 (d, J = 6.60 Hz, 3H), 0.84 (d, J = 6.59 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 (12)

(*S*,*E*)-5-Amino-7-methyloct-2-en-4-one **11** (440 mg, 1.1 mmol) was dissolved in 6 mL Et_2O. HCl (550 µL 4 M sln in dioxane, 2 equiv.) and triisopropylsilane (341 µL, 1.65 mmol, 1.5 equiv.) were added and the mixture was stirred for 1 h. The reaction mixture was filtered and the residue washed with Et_2O (4×). The residue was recrystallised from DCM–Et_2O (3×), yielding the title compound (134 mg, 0.7 mmol, 63%). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 8.60 (s, 3H), 7.07 (dq, J = 13.87, 6.88 Hz, 1H), 6.24 (dd, J = 15.54, 1.05 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 = 6.43 Hz, 3H), 0.98 (d, J = 6.46 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.

Z-Leu$_2$-NHNH$_2$ (16)

Z-Leu$_2$-OMe (6.67 g, 17 mmol) was dissolved in 50 mL MeOH. $H_2NNH_2{\cdot}H_2O$ (10 mL, 200 mmol, 30 equiv.) was added, and the mixture was refluxed overnight. The mixture was coevaporated with toluene (3×) and subjected to flash column chromatography (MeOH : EA : NEt_3 (0 : 99 : 1-20 : 79 : 1)) to yield the title compound (6.53 g, 16.6 mmol, 98%). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 7.42-7.28 (m, 5H), 5.12 (s, 2H), 4.62 (s, 2H), 4.40 (dd, J = 9.37, 5.39 Hz, 1H), 4.17 (t, J = 7.57 Hz, 1H), 1.77-1.48 (m, 6H), 0.99-0.88 (m, 12H).

Boc-Leu$_2$-NHNH$_2$ (18)

Boc-Leu$_2$-OMe (1.62 g, 4.52 mmol) was dissolved in 50 mL MeOH. $H_2NNH_2{\cdot}H_2O$ (6.57 mL, 136 mmol, 30 equiv.) was added and the mixture was refluxed for 2 h. Concentration and column

chromatography yielded the title compound (1.21 g, 3.38 mmol, 75%). ^{1}H NMR (400 MHz, CD_3OD): δ ppm 4.46-4.39 (m, 1H), 4.11 (t, J = 7.45, 7.45 Hz, 1H), 1.76-1.46 (m, 6H), 1.43 (s, 9H), 0.95-0.87 (m, 12H).

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 (**16** or **18**) was dissolved in 1 : 1 DMF–DCM (v/v) and cooled to −30 °C. *t*BuONO (1.1 equiv.) and HCl (4 M sln. in 1,4-dioxane, 2.8 equiv.) were added, and the mixture was stirred for 3 h. 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 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 H_2O (3×). The organic layer was dried over $MgSO_4$ 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 (**21** or **22**, 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 h. before being concentrated. The residue was taken up in DCM, washed with 1 M HCl (2×), sat. aq. $NaHCO_3$ (4×), brine, and dried with Na_2SO_4. The residue was purified by flash column chromatography.

(*S*,*E*)-4-Boc-Leu$_2$-amino-6-methylhept-2-enenitrile (19)

Following the general procedure for azide coupling the title compound was obtained from (*S*,*E*)-4-Boc-amino-6-methylhept-2-enenitrile **13** (49 mg, 0.21 mmol, 1.1 equiv.) and Boc-Leu$_2$-$NHNH_2$ (**18**, 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%). ^{1}H NMR (400 MHz, $CDCl_3$): 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). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 172.89, 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-Cbz-Leu$_2$-amino-6-methylhept-2-enenitrile (Ia)

Following the general procedure for azide coupling the title compound was obtained from (*S*,*E*)-4-Boc-amino-6-methylhept-2-enenitrile **13** (141 mg, 0.59 mmol, 1.1 equiv.) and Cbz-Leu$_2$-$NHNH_2$ (**16**, 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%). $[\alpha]_D^{20}$ −58.2 (*c* 1.00, $CHCl_3$). IR (film) 3263, 2959, 1699, 1666, 1641, 1537, 1263, 1238, 1121, 1045, 1028, 961 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD): 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), 1.70-34 (m, 9H), 0.96-0.97 (m, 18H). ^{13}C NMR (125 MHz, CD_3OD): 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.

(*S*,*E*)-4-Azido-Phe-Leu$_2$-amino-6-methylhept-2-enenitrile (Ib)

Following the general procedure for block coupling, the title compound was obtained from (*S*,*E*)-4-Boc-Leu$_2$-amino-6-methylhept-2-enenitrile **19** (66 mg, 0.14 mmol, 1 equiv.) and azido-phenylalanine[21] **21** (29 mg, 0.154 mmol, 1.1 equiv.). Flash column chromatography (EA : tol (0-30%)) yielded the title compound (53 mg, 99 µmol, 70%). $[\alpha]_D^{20}$ −55.6 (*c* 0.72, $CHCl_3$). IR (film) 3271, 2957, 2110, 1639, 1541, 1456, 1387, 1224, 961 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): 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). ^{13}C NMR (100 MHz, $CDCl_3$): 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-Ahx$_3$-Leu$_2$-amino-6-methylhept-2-enenitrile (Ic)

Following the general procedure for block coupling, the title compound was obtained from (*S*,*E*)-4-Boc-Leu$_2$-amino-6-methylhept-2-enenitrile **19** (64 mg, 138 µmol) and Ada-Ahx$_3$-OH **22** (81 mg, 152 µmol, 1.1 equiv.). Flash column chromatography (MeOH–DCM (2-8%)) yielded **Ic** (108 mg, 123 µmol, 80%). $[\alpha]_D^{20}$ −38.2 (*c* 1.00, MeOH). IR (film) 3277, 2928, 2905, 2849, 1636, 1541, 1456, 1368, 1244, 1171, 962 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ ppm 8.12-8.04 (m, 2H), 8.00 (d, J = 8.24 Hz, 1H), 7.95 (t, J = 5.06, 5.06 Hz, 2H), 7.87-7.82 (m, 1H), 6.73 (dd, J = 16.36, 5.04 Hz, 1H), 5.55 (d, J = 16.35 Hz, 1H), 4.60-4.49 (m, 1H), 4.39-4.25 (m, 2H), 3.13 (dd, J = 12.14, 6.00 Hz, 6H), 2.23 (t, J = 7.27, 7.27 Hz, 2H), 2.15 (t, J = 7.39, 7.39 Hz, 4H), 1.95-1.87 (m, 5H), 1.76-1.24 (m, 38H), 0.96-0.84 (m, 18H). ^{13}C NMR (100 MHz, CD_3OD): 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.

Competition experiments *in vitro*

HEK293T cells were cultured on DMEM supplemented with 10% Fetal Calf Serum (FCS), 10 units/ml penicillin and 10 µg ml^{-1} streptomycin in a 7% CO_2 humidified incubator at 37 °C. Cells were harvested, washed with PBS (2×) and permeated in digitonin lysis buffer (4× pellet volume, 50 mM Tris pH 7.5, 250 mM

sucrose, 5 mM $MgCl_2$, 5 mM DTT, 0.025% digitonin)[26] 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 total protein per experiment was exposed to the inhibitors (10× solution in DMSO) for 1 h. at 37 °C prior to incubation with MV151 (500 nM) for 1 h at 37 °C. Reaction mixtures were boiled with Laemmli's buffer containing 2-mercaptoethanol for 3 min before being resolved on 12.5% SDS-PAGE. In-gel detection of fluorescently labeled proteins was performed in the wet gel slabs directly on the Typhoon Variable Mode Imager (Amersham Biosciences) using the Cy3/Tamra settings (ex 532, em 560). Competition experiments were repeated twice.

Cell treatment

HEK293T or RAW264.7 cells (2 × 15 cm dishes) were incubated with 50 µM **Ib** of **VIIIb** or DMSO for 3 h at 37 °C in culture medium (DMEM (PAA), 10% FCS (Hyclone), 10 U/ml penicillin and 10 mg ml^{-1} streptomycin (PAA)). Cells were washed and harvested in PBS, snap frozen in N_2(l) and kept at –80 °C until analysis. Cell pellets were resuspended in 800 µl lysis buffer (50 mM Tris pH 7.5, 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 s, 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 h at RT to remove endogenously biotinylated proteins. Bead-free lysate (200 µl) was reacted with 300 µM biotin phosphin[24] (1.5 µl from a DMF stock) for 2 h. at 37 °C, denatured by boiling for 5 min with 1% SDS and precipitated with chloroform/methanol (C/M).[27] The protein pellet was rehydrated in 180 µl 8 M urea/100 mM NH_4HCO_3, reduced with 10 µl 90 mM DTT for 30 min. at 37 °C, alkylated with 15 µl 200 mM iodoacetamide at 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 × 25 µl, 4 × 100 µl and 1 × 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 h. The beads were stringently washed with 2 × 300 µl PD buffer with 0.1% SDS, 2 × 300 µl PD buffer, 2 × 300 µl wash buffer I (4 M urea/50 mM NH_4HCO_3), 2 × 300 µl wash buffer II (50 mM Tris HCl pH 7.5, 10 mM NaCl) and 2 × 300 µl water. Beads were taken up in 100 µl digest buffer (100 mM Tris HCl pH 7.8, 100 mM NaCl, 1 mM $CaCl_2$, 2% ACN) and digested with 500 ng trypsin overnight at 37 °C. Supernatant containing peptides was acidified with 5 µl formic acid (FA) to pH < 4, desalted on Stage tips[28] and dissolved in 50 µl H_2O/ACN/FA (97/3/0.1%).

LCMS 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 = 360/25µm tip ID = 5 µm), trap (OD/ID = 360/100µm packed with 25 mm robust Poros $10R^2$/15 mm BioSphere C18 5 µm 120 Å) and analytical columns (OD/ID = 360/75µm packed with 20 cm BioSphere C18 5 µm 120 Å) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% FA/H_2O, B: 0.1% FA/ACN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column *via* a 15 mm long TFE teflon tubing sleeve (OD/ID 0.3 × 1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a 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 41 V, tube lens voltage 150 V. Internal mass calibration was performed with air-borne protonated polydimethylcyclosiloxane (m/z = 445.12002) and the plasticizer protonated dioctyl phthalate ions (m/z = 391.28429) as lock mass.[29]

For shotgun proteomics analysis, 10 µl of the samples was pressure loaded on the trap column with a 10 µl min^{-1} flow for 5 min followed by peptide separation with a gradient of 35 min 5-30% B, 15 min. 30-60% B, 5 min. A at a flow of 300 µl min^{-1} split to 250 nl min^{-1}. 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 1000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the LTQ linear ion trap (AGC target 5 × 103, max inj time 120 ms) from the three most abundant ions.[30]

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 60 s and ions with z < 2 or unassigned were not analyzed.

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