Chemical tools to probe the proteasome

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6 Synthesis and evaluation of subunit specific proteasome probes.

6.1 Introduction

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

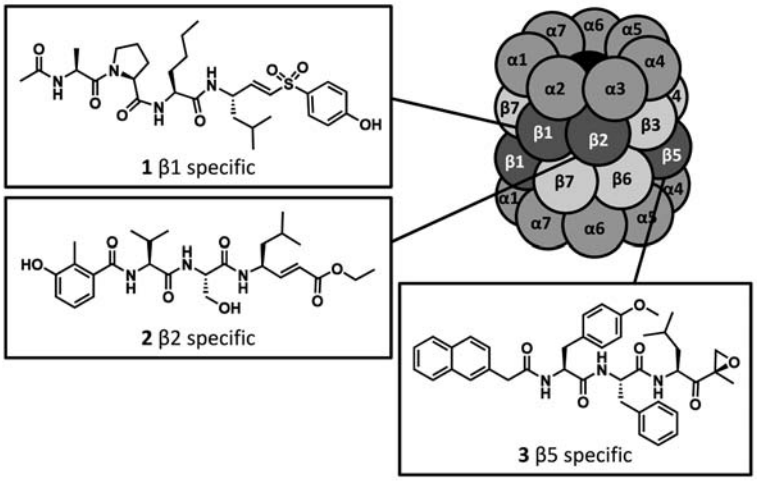


Figure 1. Eukaryotic 20S proteasome and previously described subunit specific proteasome inhibitors. The proteolytically active β subunits are marked in dark gray.

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

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

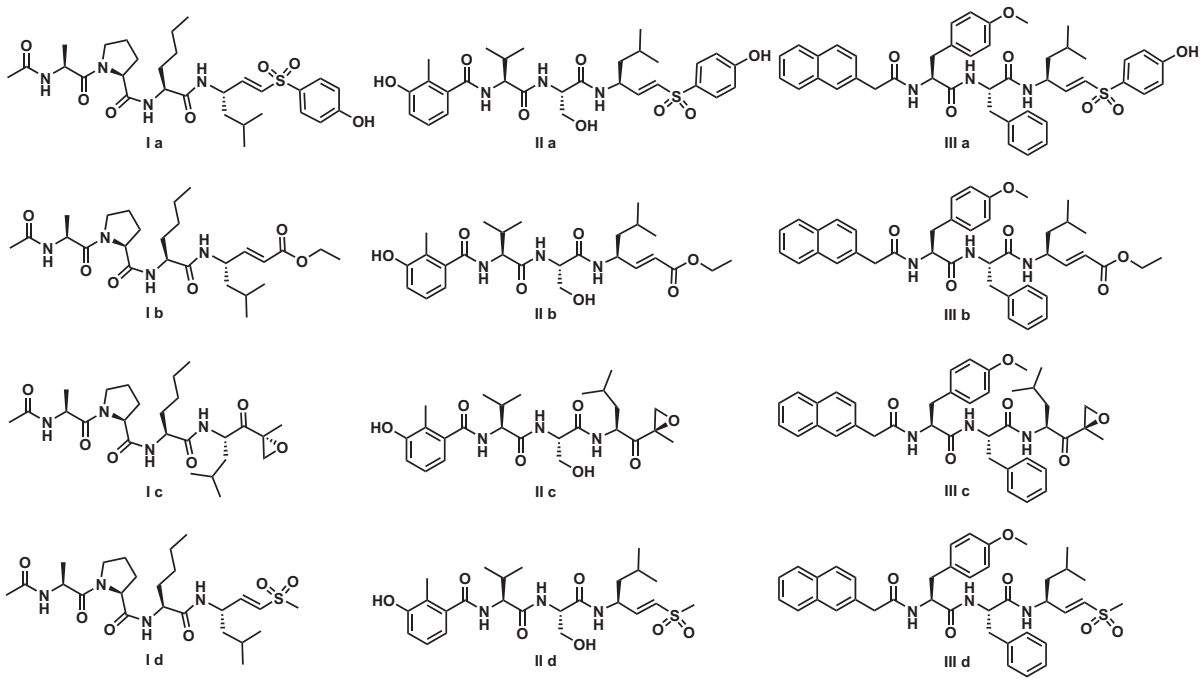


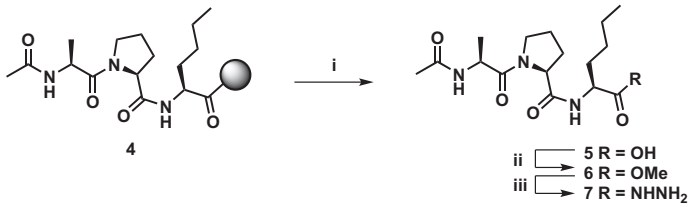
Figure 2. Hybrid library studied in this chapter.

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

6.2 Results and discussion

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

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

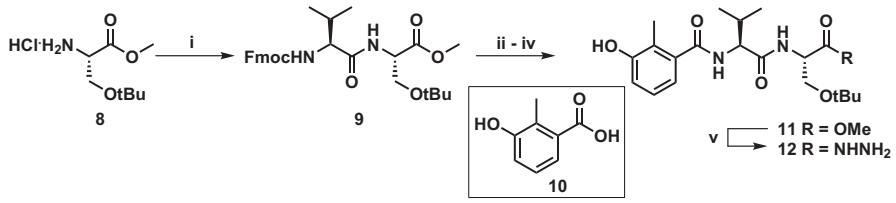


Reagents and conditions: i) 1% TFA in DCM, 30 min., 3×. ii) TMS-diazomethane (2 equiv.), 15 min., 85% from Fmoc-Nle-resin. iii) Hydrazine monohydrate (60 equiv.), MeOH, 1.5 hr., 92%.

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

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

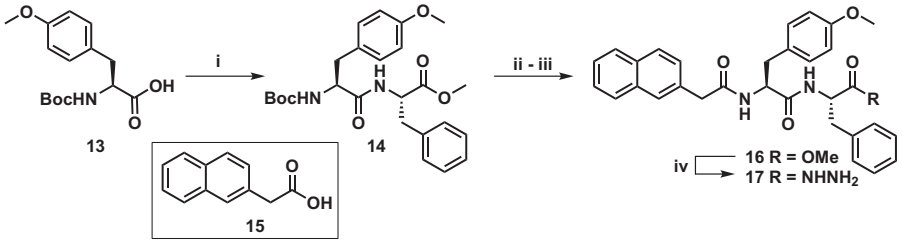
Scheme 2. Synthesis of the β2 specific recognition peptide hydrazide **12**.



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

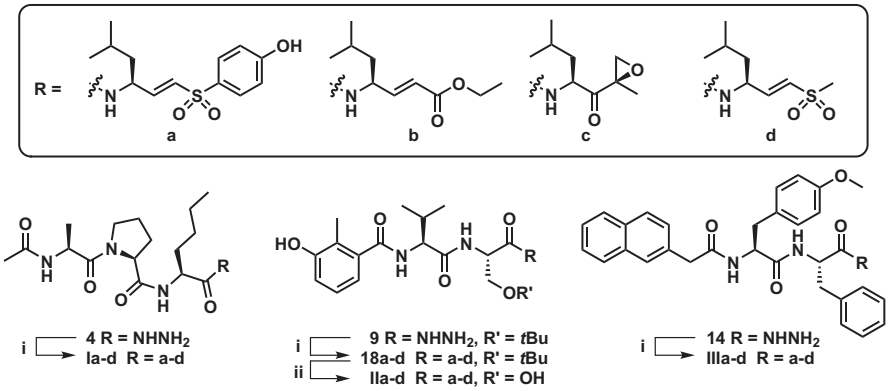
The final recognition peptide hydrazide was prepared by condensation of Boc-protected tyrosine methyl ether **13** with phenylalanine methyl ester resulting in dipeptide **14** (Scheme 3). Treatment with hydrazine in refluxing methanol gave the β5 recognition element peptide hydrazide **17**.

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



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

Scheme 4. Synthesis of the hybrid library.



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

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

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

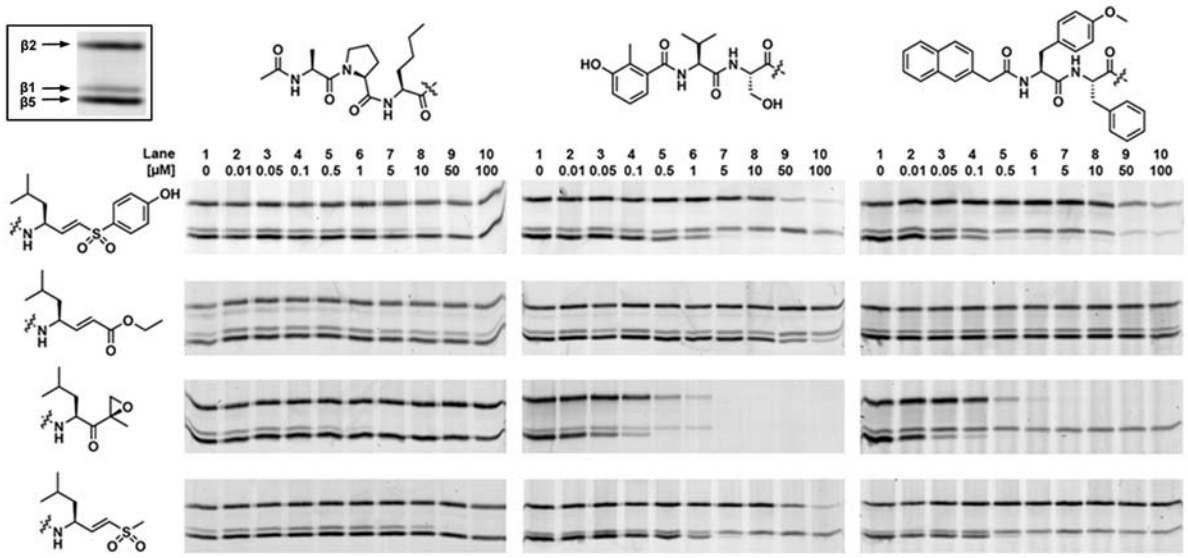


Figure 3. Proteasome profiling screen of hybrid library using MV151.
HEK293T lysates (10 μg total protein) were incubated with the indicated concentrations of hybrid compounds for 1 hr. at 37 °C. The residual proteasome activity was fluorescently labeled by incubation with 1 μM MV151 for 1 hr. at 37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

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

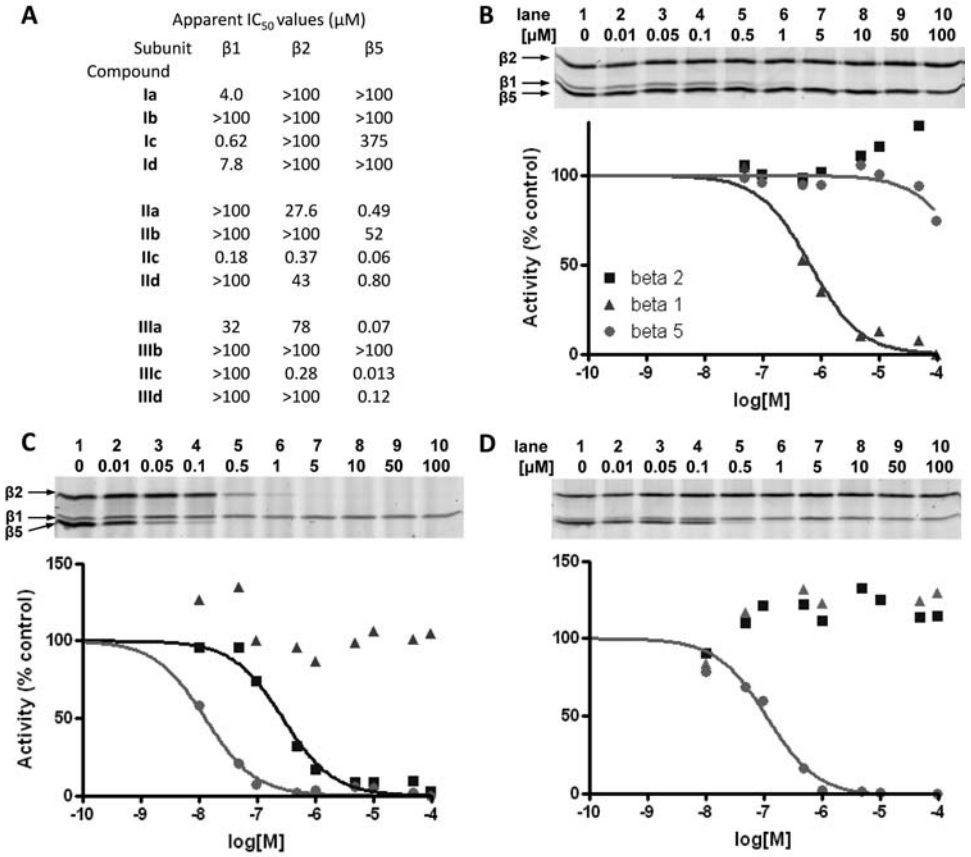
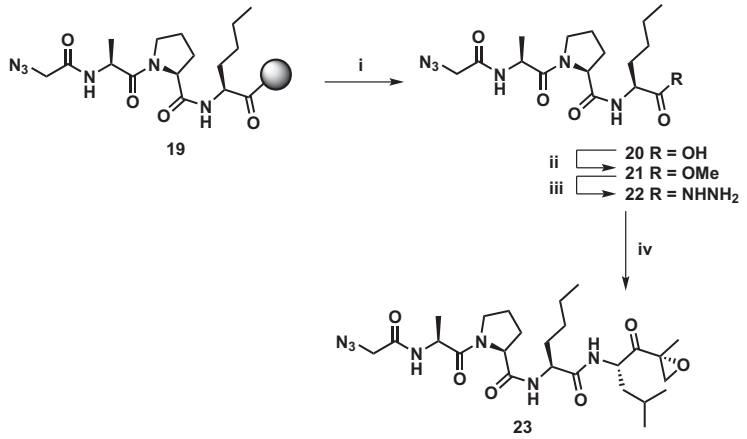


Figure 4. Apparent IC_{50} values of inhibitor library.
(**A**) Table with apparent IC_{50} values of the inhibitor library. (**B**-**D**) Gel scans and corresponding one-site competition plots of (**B**) **Ic**, (**C**) **IIIc** and (**D**) **IIId**.

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

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

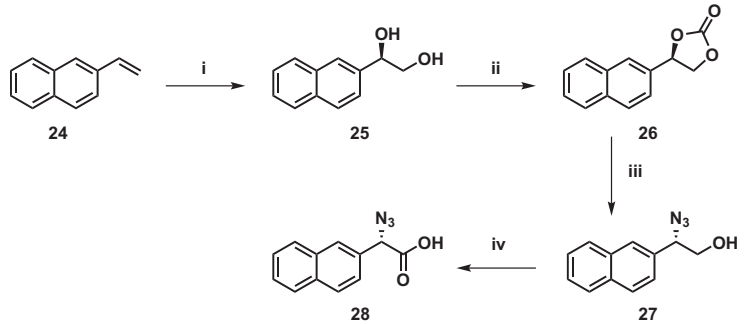


Reagents and conditions: i) 1% TFA in DCM, 30 min., 3×. ii) TMS-diazomethane (2 equiv.), 15 min., 90% from Fmoc-Nle-resin. iii) Hydrazine monohydrate (60 equiv.), MeOH, 1.5 hr., quant. iv) (a) HCl (2.8 equiv.), *t*BuONO (1.1 equiv.), DMF/EtOAc (1/1, v/v), -25 °C, 4 hr. (b) TFA·H-Leu-epoxyketone (1.1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

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

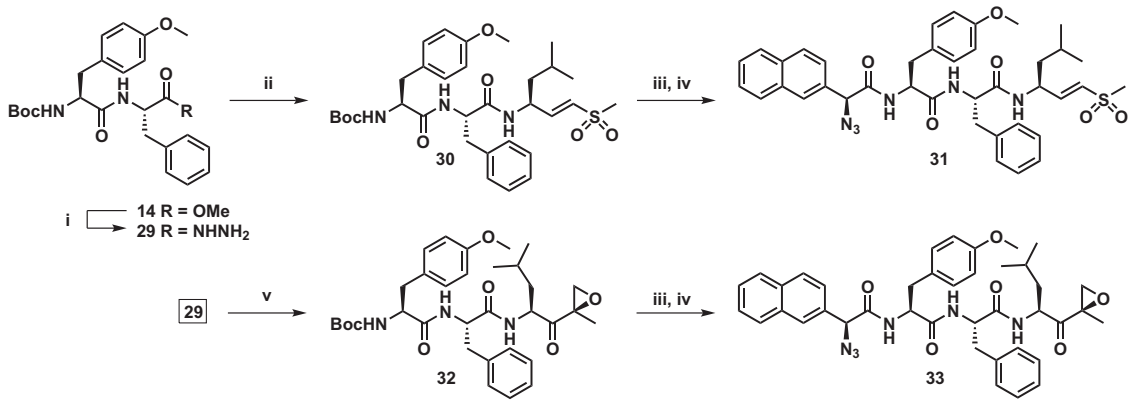
For the generation of the β5 selective probe and the probe labeling β2 and β5 azido naphthyl glycine **28** was synthesized (Scheme 6). Sharpless assymetric dihydroxylation of 2-vinylnaphthalene (**24**) gave optically pure *R*-1-(naphthhalen-2-yl)ethane-1,2-diol (**25**).[17] Opening of the cyclic carbonate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

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



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

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



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

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

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

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

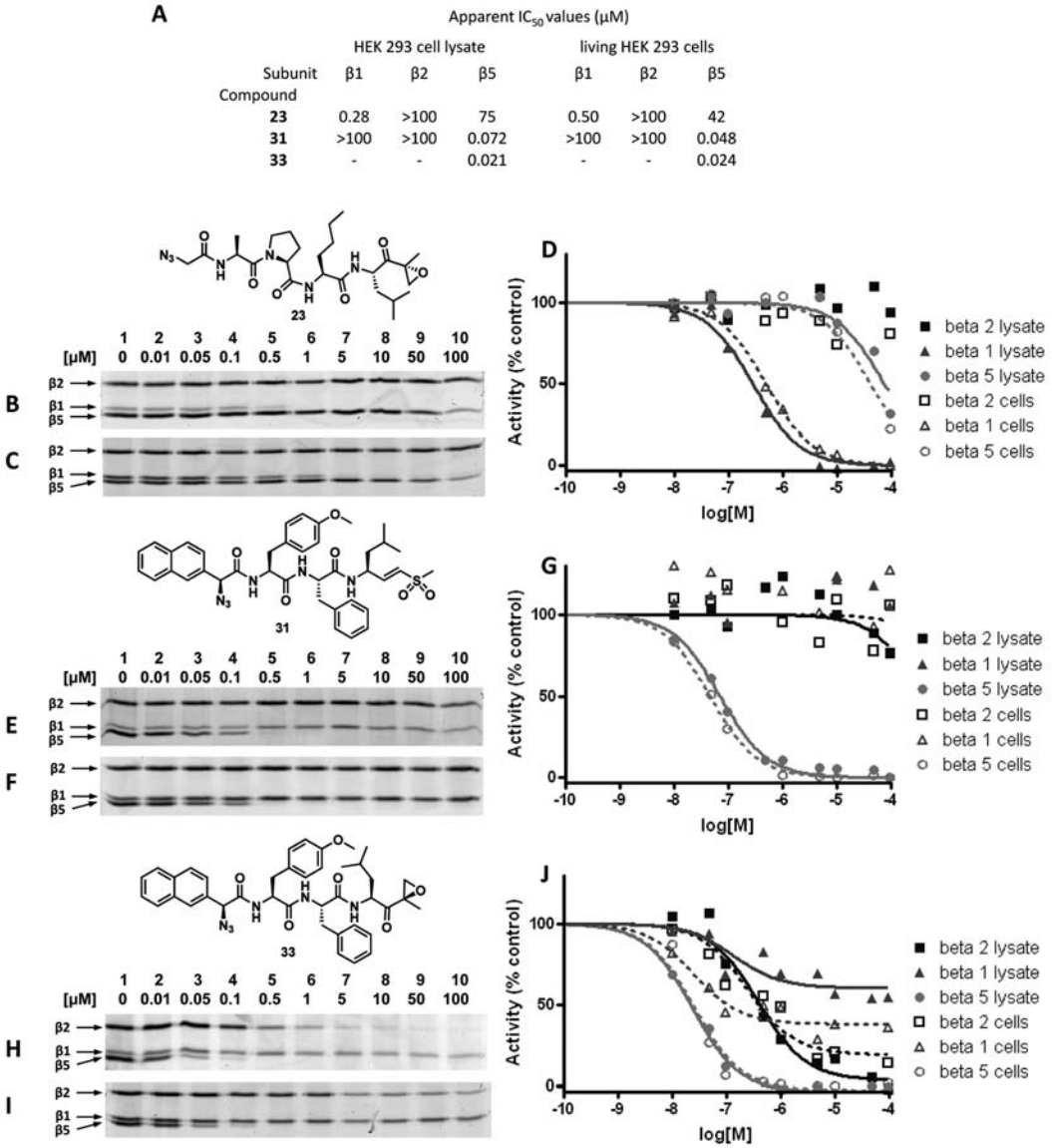
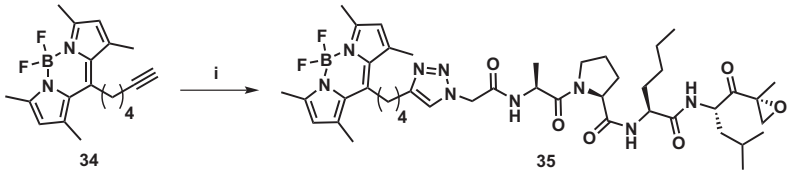


Figure 5. Proteasome profiling screen of specific proteasome probes using MV151.
(**A**) Table with apparent IC_{50} values of probes **23, 31,** and **33** in HEK293T cell lysate and living HEK293T cells. (**B, E, H**) HEK293T lysates (10 μg total protein) were incubated with the indicated concentrations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

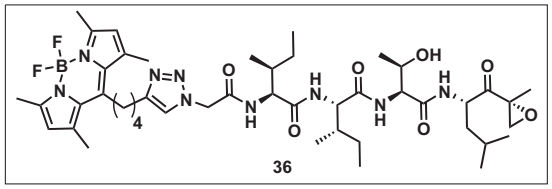
Scheme 8. Synthesis of fluorescent probe **35**.



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

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

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



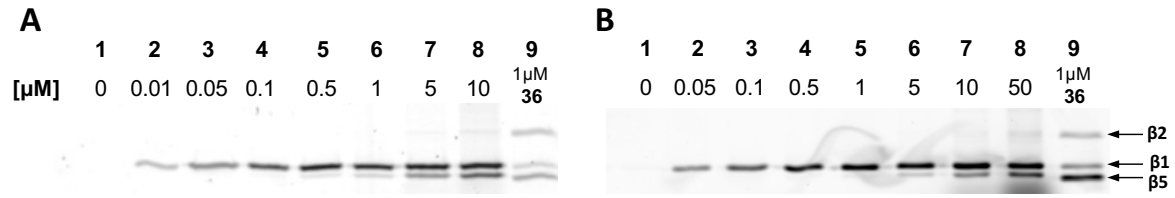
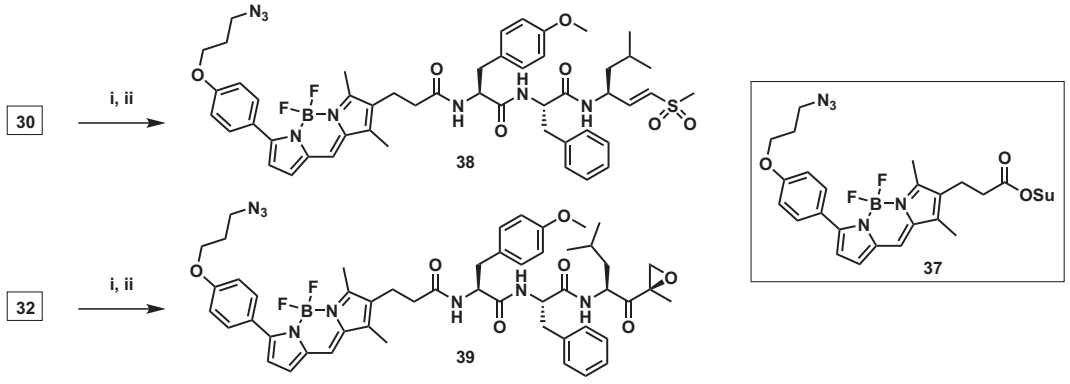


Figure 6. Labeling profile of probe **35**.

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

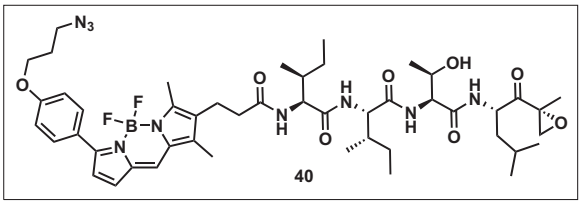
Scheme 9. Synthesis of azido-BODIPY probes **38** and **39**.



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

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

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



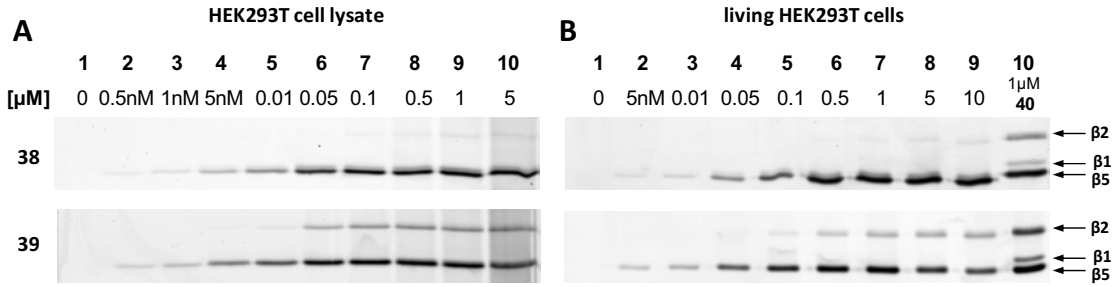


Figure 7. Labeling profile of Azido-BODIPY probes **38** and **39**.
(**A**) HEK293T lysates (10 μg total protein) were incubated with the indicated concentrations of **38** (top panel) or **39** (bottom panel) for 1 hr. at 37 °C. (**B**) HEK293T cells (some $5{\cdot}10^5$ cells) were exposed to the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

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

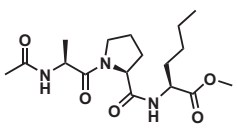
6.3 Conclusion

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

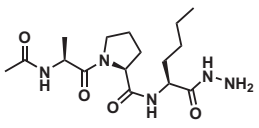
Experimental section

General: All reagents were commercial grade and were used as received unless indicated otherwise. Toluene (Tol.)(purum), ethyl acetate (EtOAc) (puriss.), diethyl ether (Et_2O) and light petroleum ether (PetEt) (puriss.) were obtained from Riedel-de Haën. Dichloroethane (DCE), dichloromethane (DCM), dimethyl formamide (DMF) and dioxane (Biosolve) were stored on 4Å molecular sieves. Methanol (MeOH) and *N*-methylpyrrolidone (NMP) were obtained from Biosolve. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on Screening Divices

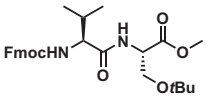
Silica gel (0.040 – 0.063 nm). LC/MS analysis was performed on a LCQ Adventage Max (Thermo Finnigan) equipped with a Gemini C18 column (Phenomenex). The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra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



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

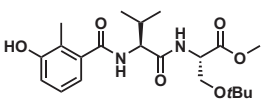


Ac-Ala-Pro-Nle-hydrazide (7). Ac-Ala-Pro-Nle-OMe (**6**, 0.36 g, 1.0 mmol) was dissolved in MeOH. Hydrazine monohydrate (2.9 ml, 60 mmol, 60 equiv.) was added and the reaction mixture was refluxed for 1.5 hr. Tol. was added and the resulting white solid was filtered to give the title compound (0.33 g,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

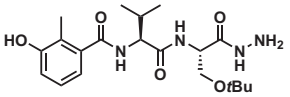


Fmoc-Val-Ser(*t*Bu)-OMe (9). HCl·H-Ser(*t*Bu)-OMe (**8**, 1.06 g, 5 mmol) and Fmoc-Val-OH (1.7 g, 5 mmol, 1 equiv.) were dissolved in DCM, before BOP (2.21 g, 5 mmol, 1 equiv.)

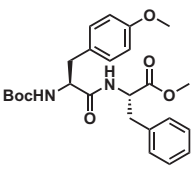
and DiPEA (2.73 ml, 16.5 mmol, 3.3 equiv.) were added. After 2 hr. the reaction mixture was washed with 0.5 M HCl (aq.) and sat. aq. $NaHCO_3$, separated and dried over $MgSO_4$. Purification by flash column chromatography (2% MeOH in DCM) yielded the title compound as a white solid (2.5 g, 5.0 mmol,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



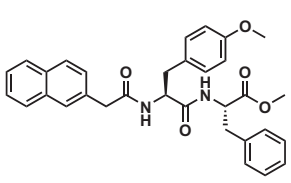
(Val-Ser(*t*Bu)-OMe)-3-hydroxy-2-methylbenzamide (11). DBU (0.15 ml, 1 equiv.) was added to a solution of Fmoc-Val-Ser(*t*Bu)-OMe (**9**, 0.5 g, 1 mmol) in DMF and stirred for 5 min., before HOBt (0.61 g, 4.5 mmol, 4.5 equiv.) was added. After 1 min. 3-hydroxy-2-methyl benzoic acid (**10**, 0.15 g, 1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



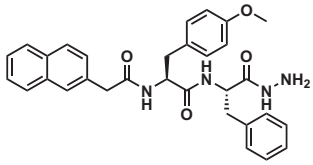
(Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (12). (Val-Ser(*t*Bu)-OMe)-3-hydroxy-2-methylbenzamide (**11**, 0.27 g, 0.67 mmol) was dissolved in MeOH. Hydrazine monohydrate (1.95 ml, 40.2 mmol, 60 equiv.) was added and the reaction mixture was refluxed for 15 hr., before being co-evaporated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



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

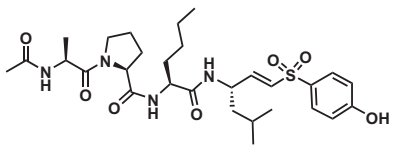


(Tyr(Me)-Phe-OMe)-2-(naphthalen-2-yl)-acetamide (16). Boc-Tyr(Me)-Phe-OMe (**14**, 4.6 g, 10 mmol) was dissolved in TFA/DCM 1/1 (v/v). The reaction mixture was stirred for 15 min. before being co-evaporated with Tol. (3×). The crude TFA salt was dissolved in DCM and 2-(naphthalen-2-yl)-acetic acid (**15**,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



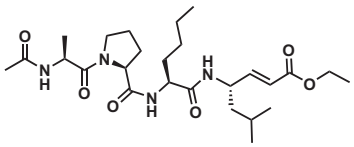
(Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide (17). To a solution of (Tyr(Me)-Phe-OMe)-2-(naphthalen-2-yl)-acetamide (**16**, 0.52 g, 1 mmol) in MeOH was added hydrazine monohydrate (2.91 ml, 60 mmol, 60 equiv.). The reaction mixture was refluxed for 2.5 hr. The title compound precipitated as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

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

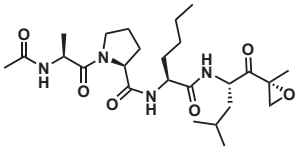


Ac-Ala-Pro-Nle-Leu-4-hydroxyphenyl-vinylsulfone (Ia). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS-PhOH (61 mg, 0.17 mmol, 1.1 equiv.) and Ac-Ala-Pro-Nle-hydrazide (**7**, 53.3 mg, 0.15 mmol). Purification by flash column chromatography (DCM → 6% MeOH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

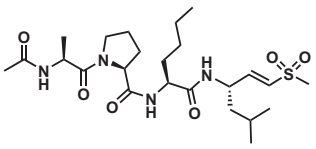
6H), 1.81-1.59 (m, 3H), 1.58-1.49 (m, 1H), 1.47-1.38 (m, 1H), 1.36-1.24 (m, 7H), 0.97-0.83 (m, 9H). HRMS: calcd. for $[C_{29}H_{44}N_4O_7SH]^+$ 593.30035, found 593.30046.



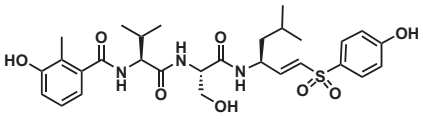
Ac-Ala-Pro-Nle-Leu-vinyl ethyl ester (Ib). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVE (49.7 mg, 0.17 mmol, 1.1 equiv.) and Ac-Ala-Pro-Nle-hydrazide (**7**, 53.3 mg, 0.15 mmol). Purification by flash column chromatography (DCM → 4% MeOH in DCM) gave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



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

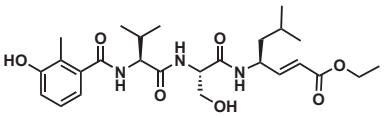


Ac-Ala-Pro-NLe-Leu-methyl vinylsulfone (Id). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS (50.7 mg, 0.17 mmol, 1.1 equiv.) and Ac-Ala-Pro-NLe-hydrazide (**7**, 53.3 mg, 0.15 mmol). Purification by flash column chromatography (DCM → 4% MeOH in DCM)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

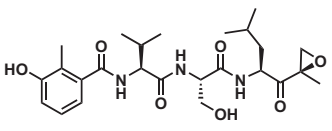


(Val-Ser-Leu-4-hydroxyphenyl-vinylsulfone)-3-hydroxy-2-methylbenzamide (IIa). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS-PhOH (61 mg, 0.17 mmol, 1.1 equiv.) and (Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (**12**, 61.3 mg, 0.15 mmol).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

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

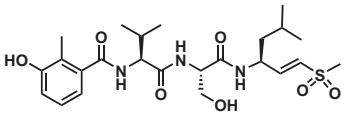


(Val-Ser-Leu-vinyl ethyl ester)-3-hydroxy-2-methylbenzamide (IIb). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVE (49.7 mg, 0.17 mmol, 1.1 equiv.) and (Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (**12**, 61.3 mg, 0.15 mmol). Column chromatography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

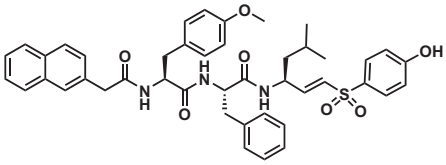


(Val-Ser-Leu-epoxyketone)-3-hydroxy-2-methylbenzamide (IIc). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuEK (47.4 mg, 0.17 mmol, 1.1 equiv.) and (Val-Ser(*t*Bu)-hydrazinyl)-3-hydroxy-2-methylbenzamide (**12**, 61.3 mg, 0.15 mmol). Column chromatography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

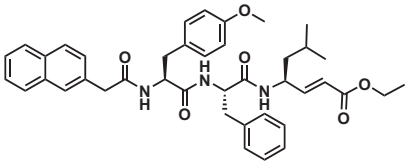
0.13 mmol) was dissolved in TFA/DCM 1/1 (v/v) and stirred for 30 min., before being co-evaporated with Tol. (3×). Column chromatography (DCM → 3.5% MeOH in DCM) gave the title compound (43.8 mg, 89 µmol, 69%). ^{1}H NMR (400 MHz, MeOD): δ ppm 7.05 (t, *J* = 7.8 Hz, 1H), 6.88-6.80 (m, 2H), 4.60 (dd, J_1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



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

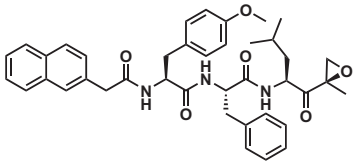


(Tyr(Me)-Phe-Leu-4-hydroxyphenyl-vinylsulfone)-2-(naphthalen-2-yl)-acetamide (IIIa). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS-PhOH (61 mg, 0.17 mmol, 1.1 equiv.) and (Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide (**17**, 78.7 mg, 0.15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

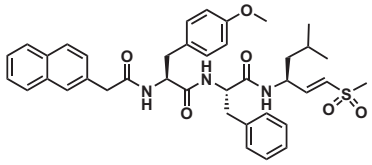


(Tyr(Me)-Phe-Leu-vinyl ethyl ester)-2-(naphthalen-2-yl)-acetamide (IIIb). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVE (49.7 mg, 0.17 mmol, 1.1 equiv.) and (Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide

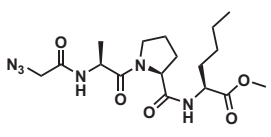
(**17**, 78.7 mg, 0.15 mmol). Upon washing the reaction mixture with EtOAc white precipitate forms. The crude product was filtered and redissolved in DCM. Crystallization with EtOAc gave the title compound as a white solid (70 mg, 0.13 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 7.92-7.86 (m, 1H),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



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

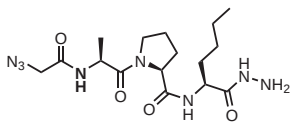


(Tyr(Me)-Phe-Leu-methyl vinylsulfone)-2-(naphthalen-2-yl)-acetamide (IIId). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS (32 mg, 0.11 mmol, 1.1 equiv.) and (Tyr(Me)-Phe-hydrazinyl)-2-(naphthalen-2-yl)-acetamide (**17**, 52 mg, 0.1 mmol). Column chromatography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

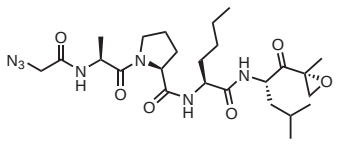


Az-Ala-Pro-Nle-OMe (21). Resin bound Fmoc-Ala-Pro-Nle (synthesis described above, 0.25 mmol) was deprotected with piperidine / NMP (1/4, v/v, 20 min.), washed with NMP (3×) and DCM (3×) and capped with azido acetic acid (63 mg, 0.63 mmol, 2.5 equiv) under the influence of BOP (0.28 g, 0.63 mmol, 2.5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

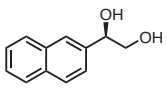
purification. The crude peptide was dissolved in MeOH/Tol. (1/1) and treated with TMS-diazomethane (0.25 ml 2M in hexanes, 0.5 mmol, 2 equiv.) for 15 min. before being coevaporated with Tol. (3x). Purification by flash column chromatography (DCM → 2.5% MeOH in DCM) yielded the title compound as a white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



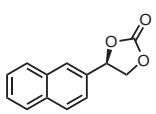
Az-Ala-Pro-Nle-hydrazide (22). Az-Ala-Pro-NLe-OMe (**21**, 89.3 mg, 0.23 mmol) was dissolved in MeOH. Hydrazine monohydrate (0.67 ml, 13.8 mmol, 60 equiv.) was added and the reaction mixture was refluxed for 2 hr. Tol. was added and the resulting white solid was filtered to give the title compound (90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



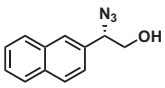
Az-Ala-Pro-NLe-Leu-epoxyketone (23). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuEK (38.8 mg, 0.14 mmol, 1.1 equiv.) and Az-Ala-Pro-Nle-hydrazide (**22**, 53.2 mg, 0.13 mmol). Purification by flash column chromatography (DCM → 2% MeOH in DCM) gave **23**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



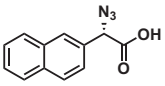
***R*-1-(naphthalen-2-yl)ethane-1,2-diol (25).** To a solution of 2-vinylnaphthalene (**24**, 1.54 g, 10 mmol) in *t*BuOH/H_2O (100 ml, 1/1, v/v) was added AD-mix-β (14.9 g). The reaction mixture was stirred for 5 hr., before sodium sulfite (6 g) was added. After the evaporation of *t*BuOH the aqueous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



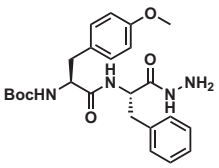
***R*-4-(naphthalen-2-yl)-1,3-dioxolan-2-one (26).** To a solution of *R*-1-(naphthalen-2-yl)ethane-1,2-diol (**25**, 1.47 g, 7.8 mmol) in MeCN was added CDI (1.9 g, 11.7 mmol, 1.5 equiv.). The reaction mixture was stirred 1 hr. at 45 °C, before it was allowed to cool to RT. DCM was added and the mixture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



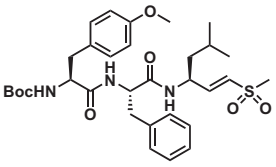
***S*-2-azido-2-(naphthalen-2-yl)ethanol (27).** *R*-4-(naphthalen-2-yl)-1,3-dioxolan-2-one (**26,** 1.54 g, 7.2 mmol) was dissolved in DMF, before H_2O (0.13 ml, 7.2 mmol, 1 equiv.) and NaN_3 (0.53 g, 7.92 mmol, 1.1 equiv.) were added. After being stirred at 80 °C for 40 hr., the reaction mixture was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



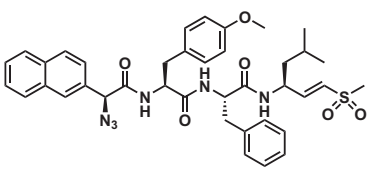
***S*-2-azido-2-(naphthalen-2-yl)acetic acid (28).** To a solution of *S*-2-azido-2-(naphthalen-2-yl)ethanol (**27,** 0.64 g, 3 mmol) in DCM/H_2O (2/1, v/v) were added TEMPO (94 mg, 0.6 mmol, 0.2 equiv.) and BAIB (2.42 g, 7.5 mmol, 2.5 equiv.). After 1 hr. of vigorous stirring the reaction was quenched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



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

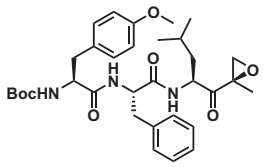


Boc-Tyr(Me)-Phe-Leu-methyl vinylsulfone (30). Following the general procedure for azide coupling the title compound was obtained from Boc-LeuVS (0.24 g, 0.83 mmol, 1.1 equiv.) and Boc-Tyr(Me)-Phe-hydrazide (**29,** 0.39 g, 0.75 mmol). Purification by flash column chromatography (DCM → 1.5% MeOH in DCM)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

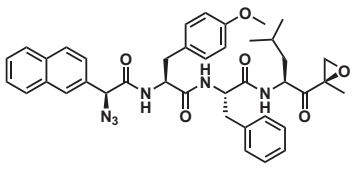


***S*-2-azido-2-(naphthalen-2-yl)acetamido-Tyr(Me)-Phe-Leu-methyl vinylsulfone (31).** Boc-Tyr(Me)-Phe-LeuVS (**30,** 0.12 g, 0.2 mmol) was dissolved in TFA/DCM 1/1 (v/v). The reaction mixture was stirred for 30 min., before being co-evaporated with Tol. (3×). The crude TFA salt was dissolved in DCM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

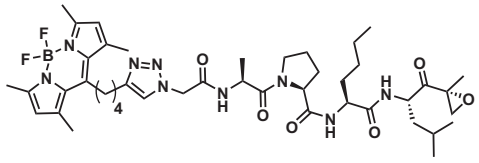
over $MgSO_4$. Purification by flash column chromatography (DCM → 2% MeOH in DCM) followed by washing with sat. aq. $NaHCO_3$ (3×) and drying over $MgSO_4$ gave the pure title compound as a white solid (0.1 g, 0.14 mmol, 70%). ^{1}H NMR (400 MHz, MeOD/$CDCl_3$): δ ppm 7.88-7.76 (m, 3H), 7.70 (s, 1H),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



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

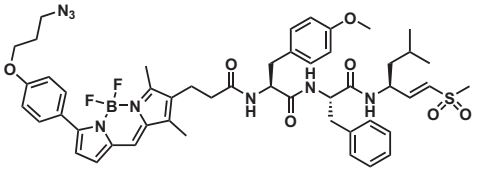


***S*-2-azido-2-(naphthalen-2-yl)acetamido-Tyr(Me)-Phe-Leu-epoxyketone (33).** Boc-Tyr(Me)-Phe-LeuEK (**32**, 0.12 g, 0.2 mmol) was dissolved in TFA/DCM 1/1 (v/v). The reaction mixture was stirred for 30 min., before being co-evaporated with Tol. (3×). The crude TFA salt was dissolved in DCM and *S*-2-azido-2-(naphthalen-2-yl)acetic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

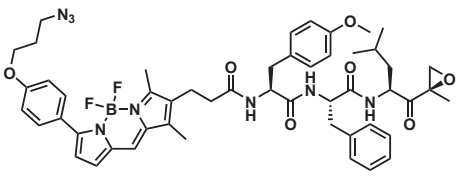


Green fluorescent β1 specific probe (35). β1 selective probe **23** (7.7 mg, 0.014 mmol) was reacted with acetylene-functionalized BODIPY **34**[20] (4.7 mg, 0.014 mmol, 1.0 equiv.) catalyzed by $CuSO_4$ (28mM in H_2O) (0.05 mL, 1.4 µmol, 10 mol%) and sodium ascorbate (44mM in H_2O) (0.05 mL, 2.2 µmol,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

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



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



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

Competition and labeling experiments *in vitro*.

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

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

Competition and labeling experiments in living cells.

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

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