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Synthetic tools to illuminate matrix metalloproteinase and proteasome activities

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

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4.1 Introduction

The majority of all cytosolic and nuclear proteins in eukaryotic cells are degraded by the ubiquitin-proteasome pathway. In this system, proteins destined for degradation are modified with a poly-ubiquitin chain, which serves as a recognition tag for the 26S proteasome where proteolysis occurs. The 26S proteasome contains one or two 19S regulatory caps together with the proteolytically active, cylindrical 20S core (see Figure 1A). Within the mammalian constitutive 20S core three pairs of proteolytically active sites are present displaying different substrate specificity. Of these, the β 1 subunits (caspase-like) cleave after acidic residues, the β 2 subunits (trypsin-like) cleave after basic residues and the β 5 subunits (chymotrypsin-like) cleave after bulky, hydrophobic residues.^{1,2} Next to the constitutive proteasome some specific mammalian cells also express the so-called immunoproteasome, in which the active subunits are replaced by their immuno counterparts β 1i, β 2i and β 5i. The peptidyl boronic acid proteasome inhibitor Bortezomib (also known as PS-341 or Velcade, see Figure 1B)³ is used for the treatment of multiple myeloma and targets the β 5(i) and β 1(i) subunits. In order to study the role of the individual active subunits, inhibitors that specifically target one active subunit are imperative. Inhibitors with moderate to good selectivity for either one of the subunits have been developed.⁴ There remains, however, room for improvements, for instance in the direction of inhibitors that can distinguish between a constitutive proteasome active site and its immunoproteasome counterpart.

The search for subunit selective inhibitors is predominantly conducted by either screening of natural products,⁵ rational design⁶ or compound library building.^{4b,7} Interestingly, in these studies the effect of fluorine functionality in proteasome inhibitors is relatively uncharted.⁸ In contrast, fluorine has found wide interest in bioorganic and structural chemistry over the past decade and has become an important feature in drug design.⁹ This is predominantly due to the typical characteristics of fluorine (when bound to carbon), such as its comparable size to hydrogen, its electron withdrawing ability, superhydrophobicity of fluorocarbons and self-association between fluorinated moieties. In protein structure design introduction of fluorine can mimic functional groups, alter structural properties and thereby (de)stabilize protein structures or function as recognition motifs.¹⁰ In addition, the beneficial ¹⁹F nuclear magnetic characteristics have found their use in structure analysis by (solid state) ¹⁹F NMR spectroscopy or ¹⁹F MRI to study, for example, protein aggregation.¹¹

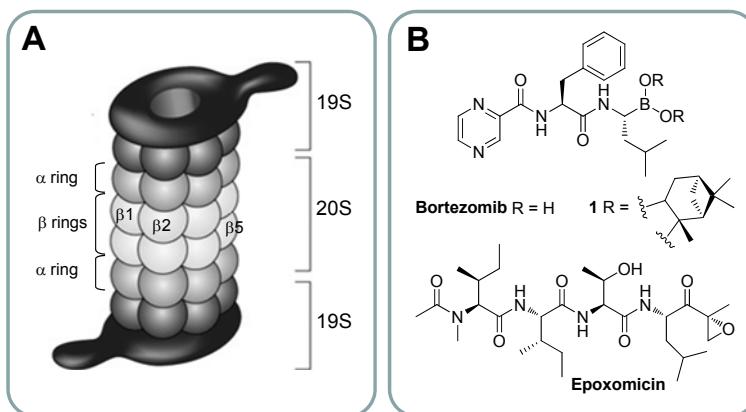


Figure 1. (A) Schematic representation of the 26S proteasome. (B) Structures of potent proteasome inhibitors.

The set of fluorinated proteasome inhibitors prepared in the context of the here presented studies are depicted in Figure 2. Compounds **2a** and **2b** containing *penta*-fluorophenylalanine (Phe(F₅)) and 3,5-bis(trifluoromethyl)phenylalanine (Phe(*m*-CF₃)₂) respectively, are based on Bortezomib derivative **1** (having a comparable potency towards the $\beta1$ and $\beta5$ proteasome subunits with respect to Bortezomib)¹² and differ in that the phenylalanine in **1** is replaced by the corresponding fluorinated analogue. In addition, incorporation of fluorinated phenylalanines at different positions in tripeptide epoxyketones² led to compounds **3-6** in which systematically either one or both of the P2 and P3 positions were altered. Fluorinated amino acids Phe(*m*-CF₃)₂ and Phe(F₅) were used for the dual reason that these are readily available and that hydrophobic amino acids (that is the non-fluorinated analogues) are in principle accepted by all proteasome active sites. The epoxyketone electrophilic trap was selected based on the natural product epoxomicin (see Figure 1B). The epoxyketone warhead featured by epoxomicin displays a specific and selective reactivity towards the N-terminal threonine residue that makes up the

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

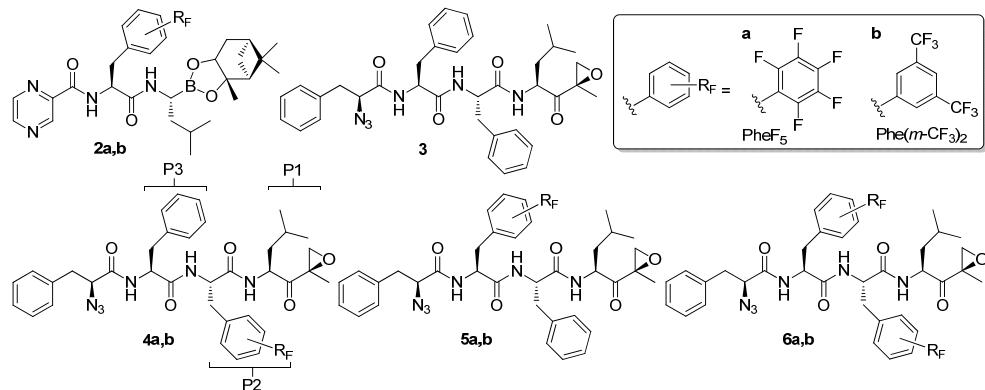
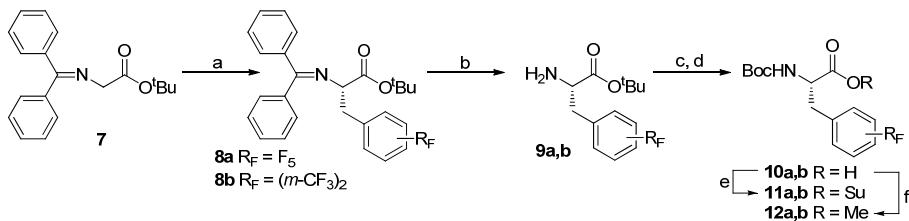


Figure 2. Synthesized fluorinated proteasome inhibitors. Indicated are the enzyme pockets (P1, P2, P3).

4.2 Results and Discussion

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

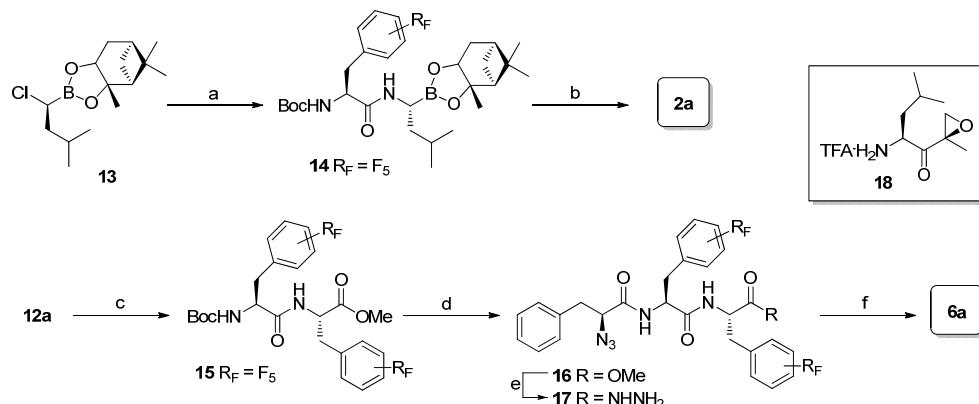
Scheme 1. Synthesis of fluorinated phenylalanine derivatives.



Reagents and conditions: (a) R_FBnBr , KOH, 2,7-bis(O9-allylhydrocinchonidinium-N-methyl) naphthalene dibromide, H_2O , $CHCl_3$, toluene, $-20\text{ }^\circ C$, quant., e.e. >98%; (b) citric acid, H_2O , THF, 95%; (c) TFA, DCM; (d) Boc_2O , Na_2CO_3 , H_2O , 1,4-dioxane, 96%; (e) $HOSu$, DIC, DCM; (f) $TMSCHN_2$, $MeOH$, toluene, quant.

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

Scheme 2. Synthesis of the fluorinated proteasome inhibitors.



Reagents and conditions: (a) i) LiHMDS, THF, $-78\text{ }^{\circ}\text{C}$; ii) HCl; iii) **11a,b**, DiPEA; (b) i) TFA, DCM; ii) PyrOH, HCTU, DiPEA, DCM, 16% from **13** after RP-HPLC; (c) i) TFA, DCM; ii) **10a,b**, HCTU, DiPEA, DCM, 79-95%; (d) i) TFA, DCM; ii) N_3PheOH , HCTU, DiPEA, DCM, 48-93%; (e) $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$, MeOH, reflux, quant.; (f) i) $t\text{BuONO}$, HCl, DCM, DMF, $-30\text{ }^{\circ}\text{C}$; ii) **18**, DiPEA, $-30\text{ }^{\circ}\text{C} \rightarrow \text{RT}$, 7-99%.

The inhibition potential of compounds **2a** and **2b**, in comparison with their non-fluorinated analogue boronic ester **1** (the pinanediol analogue of the clinical drug Bortezomib), was assessed in a competition assay employing cell lysates from human embryonic kidney cells (HEK-293T) and mouse lymphoma cells (EL4) in combination with the fluorescent broad spectrum proteasome probe MV151.¹² Cell lysates were incubated with each of the three compounds at 0.05, 0.1 and 1 μM final concentrations, prior to treatment with 0.5 μM final concentration of MV151. The samples were denatured, resolved by SDS-PAGE and the wet gel slabs were scanned on a fluorescence scanner. The gel images are shown in Figure 3. HEK-293T lysates treated with the fluorescent probe display three bands that correspond to the three active subunits ($\beta 1$, $\beta 2$ and $\beta 5$) as depicted in Figure 3A lane 1. The ability of a compound to inhibit the

proteasome active sites is reflected by disappearance of the bands. As apparent from this image the fluorinated compounds **2a** and **2b** are at least as potent as their non-fluorinated counterpart **1**, since they show complete inhibition of the $\beta 1$ and $\beta 5$ subunits between 0.1 and 1 μ M. In addition, incorporation of fluorinated Phe leaves the selectivity of $\beta 1$ and $\beta 5$ over $\beta 2$ subunits for this type of inhibitor unchanged. The experiments in EL4 cell lysate (Figure 3B) show a similar selectivity for these three compounds towards the $\beta 1(i)$ and $\beta 5(i)$ subunits. This result is also apparent from the activity (IC_{50}) measurements of the inhibitors towards the different subunits in purified rabbit 26S proteasome as shown in Table 1.

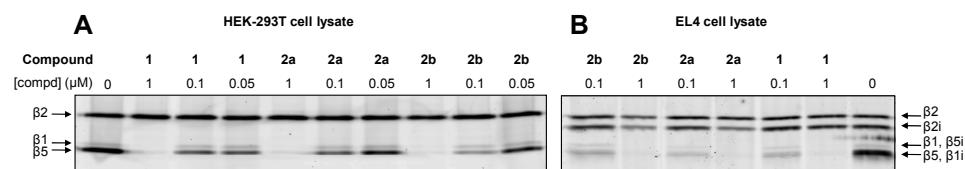


Figure 3. Characterization of the specificity of the fluorinated dipeptide boronates. Competition assay in (A) HEK-293T lysate and (B) EL4 lysate. Lysates were incubated for one hour with compounds **1**, **2a** and **2b** at the indicated final concentrations. Residual proteasome activity was labeled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

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

Compound	$\beta 1$ (nLPnLD)	$\beta 2$ (RLR)	$\beta 5$ (LLVY)
2a	0.44	>15	0.031
2b	0.16	11	0.0030
3	>15	1.8	0.0010
4a	>15	>15	0.0020
4b	>15	>15	0.10
5a	>15	>15	0.20
5b	>15	>15	0.13

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

Next, the inhibition properties of the seven epoxyketone containing compounds were determined in a similar competition assay against MV151 at 1 and 100 μ M final concentrations employing HEK-293T cell lysates (Figure 4) and by measuring inhibition of purified proteasomes for the most active compounds (Table 1). When comparing non-fluorinated compound **3** to the fluorinated ones (**4-6**), it becomes apparent that none of these compounds inhibit the $\beta 1$ subunit at concentrations up to 100 μ M and that introducing fluorines (in either position) leads to a decrease in the inhibition of the $\beta 2$ subunit and hence to an increase in $\beta 5$ specificity. When comparing Table 1 with Figure 4 it appears that the results from the two independent assays deviate in places. For example, the $\beta 2$ - IC_{50} value for **4a** is >15 μ M (Table 1), whereas the majority of the corresponding band in Figure 4 is gone at 1 μ M. Intrinsic differences in both assays,

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

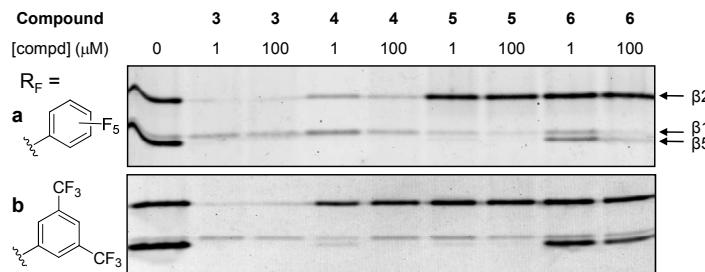


Figure 4. Characterization of the specificity of fluorinated peptide epoxyketones. Competition assay in HEK-293T lysate. Lysates were incubated for one hour with compounds **3**, **4a,b**, **5a,b** or **6a,b** at the indicated final concentrations. Residual proteasome activity was labeled with 0.5 μ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

Interestingly, the presence of fluorine substituents at both P2 and P3 positions (**6a,b**, Figure 4) has a dramatic effect on the inhibition. This effect is most pronounced for the Phe(*m*-CF₃)₂ analogues (**b** series). In general the Phe(F₅) compounds (**a** series) are more active against the $\beta 5$ subunit than their hexafluoro-Phe analogues (**b** series). Thus, it appears that introduction of Phe(F₅) in the P2 position generates a highly specific inhibitor of the $\beta 5$ site. This most potent and $\beta 5$ selective inhibitor (compound **4a**) was further investigated. The inhibitory potential at much lower concentrations (1 nM to 1 μ M) is shown in Figure 5 (competition assay against MV151). Inhibition of $\beta 5$ is already apparent at 5 nm and between 100 and 250 nM all $\beta 5$ subunits are saturated, while $\beta 1$ and $\beta 2$ are unaffected or even upregulated (a phenomenon which is not fully understood, but has been observed by others as well).¹⁷ Having an IC_{50} value of 2 nM for the $\beta 5$ subunit against >15 μ M for $\beta 1$ and $\beta 2$, this compound is one of the most $\beta 5$ selective inhibitors known to date. For instance, this compound compares well with NC005, a $\beta 5$ selective inhibitor recently discovered.⁶ Comparison of non-fluorinated compound **3** with **4a** reveals that both compounds are equally active towards the $\beta 5$ subunit. Enhanced selectivity for $\beta 5$ arises by the dramatic drop in activity for the $\beta 2$ subunit when fluorine is introduced, as in **4a**.

For the direct labeling of $\beta 5$ a new fluorescent probe was made by reacting compound **4a** with a Bodipy-alkyne¹⁸ in a Cu(I) mediated Huisgen 1,3-dipolar cycloaddition giving green fluorescent probe **19** (Figure 6A). The potential of this probe to label the $\beta 5$ subunit was explored in a competition assay against MV151 as explained before. The gel was scanned on a fluorescence scanner at two different wavelengths allowing visualization of one of the two fluorescent dyes at a time. Figure 6B shows the read-out at 520 nm visualizing the appearance of one band: labeling of the $\beta 5$ subunit

by compound **19**. The labeling is already visible at a concentration of 1 nM and the subunit appears to be saturated (no more increase in the bands intensity) between 50 and 100 nM. At this point only a faint β 2 band is visible. The fluorescence read-out at 560 nm in Figure 6C (displaying labeling with MV151 of remaining activities) reveals that the β 5 band disappears while leaving the remaining subunit-bands intact. The IC_{50} values of compound **19** towards each active subunit were determined in the same manner as for the other compounds: β 1 > 10 μ M, β 2 > 10 μ M, β 5 = 0.30 μ M.

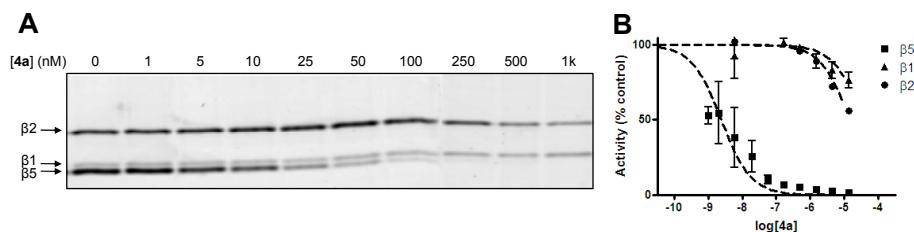


Figure 5. Characterization of the specificity of compound **4a**. (A) Competition assay in HEK-293T lysate. Lysates were incubated for one hour with compound **4a** at the indicated final concentrations. Residual proteasome activity was labeled with 0.5 μ M MV151 for one hour. (B) Remaining subunit activity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

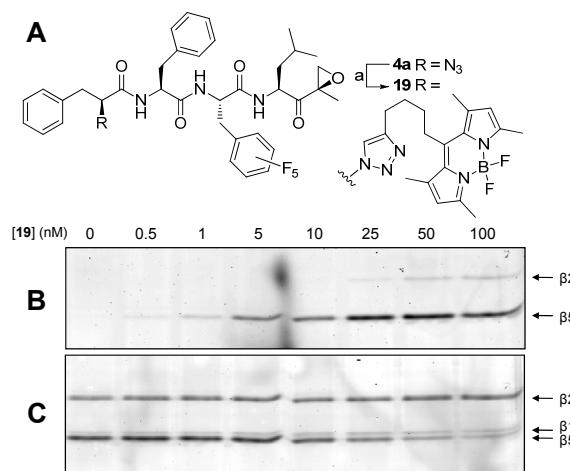


Figure 6. (A) Construction of a novel probe for the β 5 site. Reagents and conditions: a) Bodipy-alkyne,¹⁸ 10 mol% CuSO₄, 15 mol% sodium ascorbate, toluene/H₂O/tBuOH 1:1:1, 80 °C, 87%. (B,C) Competition assay in HEK-293T cell lysate. Lysates were incubated for one hour with compound **19** at the indicated final concentrations. Residual proteasome activity was labeled with 0.5 μ M MV151 for one hour. Fluorescence read-out at (B) λ_{ex} 488 nm, λ_{em} 520 nm (compound **19**) and (C) λ_{ex} 532 nm, λ_{em} 560 nm (MV151).

4.3 Conclusion

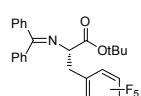
In summary, the effect of incorporation of fluorinated Phe in proteasome inhibitors was studied. It was found that substitution of non-fluorinated Phe in Bortezomib analogue **1** with fluorinated versions does not affect its selectivity for the different active subunits whereas the potency is slightly increased for the Phe(*m*-CF₃)₂ version. In addition, the effect of incorporation of fluorinated Phe in peptide epoxyketone proteasome inhibitors appeared to depend on the site of substitution. Fluorination of both the P2 and P3 sites decreases potency dramatically, however fluorinated Phe at the P2 position hardly affects the potency, but instead yields much more β 5 selective inhibitors. Comparison of the results obtained with the boronic esters with those from the epoxyketone studies invites the tentative conclusion that β 2 is most sensitive towards fluorine substituents. Compound **1** is ineffective towards β 2 and introduction of fluorines into this sequence has no apparent effect. When comparing epoxyketone **3** with **4-6**, however, the major difference is that the latter, fluorinated analogues leave β 2 largely intact. Further studies, for instance making use of different fluorine amino acids, are needed to substantiate this finding. Finally, compound **4a** was identified as one of the most β 5 selective inhibitors known to date and was converted to a β 5 selective fluorescent probe **19**, which can be used to label and visualize the β 5 subunit selectively.

Experimental section

General

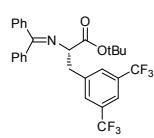
Tetrahydrofuran was distilled over LiAlH₄ before use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Fischer, Merck, Sigma-Aldrich) were used as received. *O*-(1*H*-6-Chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased at Iris Biotech (Marktrewitz, Germany). Traces of water were removed from reagents used in reactions that require anhydrous conditions by coevaporation with toluene. Solvents that were used in reactions were stored over 4 Å molecular sieves, except methanol and acetonitrile, which were stored over 3 Å molecular sieves. Molecular sieves were flame dried before use. Unless noted otherwise all reactions were performed under an argon atmosphere. Column chromatography was performed on silicagel (Screening Devices b.v.) with a particle size of 40-63 µm and a pore size of 60 Å. The eluents toluene, ethyl acetate and petroleum ether (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F₂₅₄). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₂·2H₂O (10 g/L) in 10% sulfuric acid, a solution of KMnO₄ (20 g/L) and K₂CO₃ (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 ml/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ¹H- and ¹³C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) or a Bruker DMX-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CDCl₃ or CD₃OD as internal standard. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 1/1; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTO Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at *m/z* = 400 (mass range *m/z* = 150-2,000) and dioctylphthalate (*m/z* = 391.28428) as a "lock mass". The high

resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations $[\alpha]_D^{23}$ were recorded on a Propol automatic polarimeter at room temperature. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 μ m C18 50 \times 4.60 mm column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI (system A) or on a Finnigan Surveyor HPLC system with a Gemini C18 50 \times 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI (system B). Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 \times 4.6 mm). RP-HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μ m 250 \times 10 mm column and a GX281 fraction collector. Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 \times 4.6 mm).



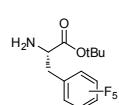
(S)-tert-butyl 2-(diphenylmethyleneamino)-3-(perfluorophenyl)propanoate (8a)

To a solution of *tert*-butyl 2-(diphenylmethyleneamino)acetate (**7**, 5.10 g, 17.3 mmol) in toluene/CHCl₃ (7/3 v/v, 80 mL) 2,3,4,5,6-pentafluorobenzylbromide (3.6 mL, 25.6 mmol, 1.5 eq.) and 2,7-bis[O(9)-allylhydrocinchonidinium-*N*-methyl]naphthalene dibromide (0.045 g, 0.045 mmol, 0.003 eq.) were added. The mixture was stirred at -20 °C for 30 min.. Hereafter, a precooled 50% aqueous solution of KOH (30 mL) was added and the resulting mixture was stirred at -20 °C for 3.5 days. Diethyl ether (150 mL) was added and the mixture was washed with H₂O (2 \times) and brine before being dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography (100% PE \rightarrow 9% EtOAc/PE) to give the title compound (yield: 8.22 g, 17.3 mmol, quant.) as a yellowish oil. Using chiral HPLC the e.e. was determined >98%. ¹H NMR (400 MHz, CDCl₃): δ = 7.57 (d, J = 1.2 Hz, 2H), 7.42-7.28 (m, 6H), 6.88 (d, J = 6.4 Hz, 2H), 4.26 (dd, J = 8.8, 5.2 Hz, 1H), 3.37-3.31 (m, 1H), 3.27-3.22 (m, 1H), 1.46 (s, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 171.41, 169.63, 146.53, 144.08 (t, J = 9.00 Hz), 140.96, 139.01, 138.51, 135.88, 135.65, 129.95, 128.18, 127.97, 127.76, 127.45, 111.76 (t, J = 24.0 Hz), 81.78, 64.33, 27.84, 26.10 ppm. $[\alpha]_D^{23} = -155.6$ (c = 1 in CHCl₃). HRMS: calcd. for C₂₆H₂₂F₅NO₂ [M + H]⁺ 475.15707; found 475.15705.



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

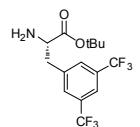
This compound was prepared by the same method described for compound **8a**, using 2,4-trifluoromethylbenzylbromide as alkylating agent. The product was obtained as a colourless oil (yield: 2.21 g, 4.23 mmol, quant.). Using chiral HPLC the e.e. was determined >98%. ¹H NMR (400 MHz, CDCl₃): δ = 7.70 (s, 1H), 7.58 (s, 2H), 7.54 (d, J = 7.22 Hz, 2H), 7.40-7.27 (m, 6H), 6.68 (d, J = 6.95 Hz, 2H), 4.16 (dd, J = 8.32, 4.87 Hz, 1H), 3.38-3.27 (m, 2H), 1.45 (s, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 169.87, 140.94, 130.50, 130.20, 131.2 (q, J = 33.14 Hz), 128.67, 128.34, 127.99, 127.32, 123.31 (q, J = 272.51 Hz), 120.20, 81.82, 66.66, 39.11, 27.99 ppm. $[\alpha]_D^{23} = -165.7^\circ$ (c = 1 in CHCl₃). HRMS: calcd. for C₂₈H₂₅F₆NO₂ [M + H]⁺ 522.18622; found 522.18587.



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

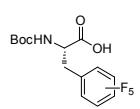
Aqueous citric acid (15% w/w, 110 mL) was added to a solution of compound **8a** (8.22 g, 17.3 mmol) in THF (90 mL) and the mixture was stirred overnight at RT. Aqueous K₂CO₃ (sat.) and EtOAc were added and the layers were separated before the aqueous layer was extracted twice with EtOAc. The combined organic layers were dried over MgSO₄ and concentrated. The product (yield: 4.92 g, 15.8 mmol, 91%) was obtained as a yellowish oil after column chromatography (100% PE \rightarrow 50% EtOAc/PE). ¹H NMR (400 MHz, CDCl₃): δ =

3.58 (dd, $J = 8.4, 6.4$ Hz, 1H), 3.10 (dd, $J = 13.6, 6.0$ Hz, 1H), 2.90 (dd, $J = 14.0, 8.8$ Hz, 1H), 1.63 (bs, 1H), 1.45 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 173.39, 146.57\text{--}146.38$ (m), 144.13–143.94 (m), 141.21–141.02 (m), 138.70–138.37 (m), 135.99 (t, $J = 17.0$ Hz), 111.70 (t, $J = 20.0$ Hz), 81.57, 54.38, 27.75, 27.60 ppm. $[\alpha]_D^{23} = +12.1$ ($c = 1$ in CHCl_3). HRMS: calcd. for $\text{C}_{13}\text{H}_{14}\text{F}_5\text{NO}_4$ [$\text{M} + \text{H}]^+$ 312.10175; found 312.10182.



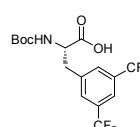
(S)-tert-butyl 2-amino-3-(3,5-bis(trifluoromethyl)phenyl)propanoate (9b)

This compound was prepared by the same method described for compound **9a**. The product was obtained as a colourless oil (yield: 1.37 g, 3.83 mmol, quant.). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.76$ (s, 1H), 7.73 (s, 2H), 3.66 (t, $J = 6.61, 6.61$ Hz, 1H), 3.07 (ddd, $J = 20.92, 13.78, 6.62$ Hz, 2H), 1.57 (s, 1H), 1.42 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 173.62, 140.34, 131.43$ (q, $J = 33.16$ Hz), 129.58, 123.28 (q, $J = 272.53$ Hz), 120.55, 81.66, 55.75, 40.51, 27.72 ppm. $[\alpha]_D^{23} = +12.6^\circ$ ($c = 1$ in CHCl_3). HRMS: calcd. for $\text{C}_{15}\text{H}_{17}\text{F}_6\text{NO}_2$ [$\text{M} + \text{H}]^+$ 358.12362; found 358.12364.



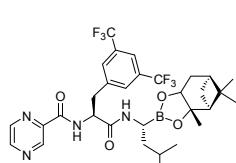
(S)-2-(tert-butoxycarbonylamino)-3-(perfluorophenyl)propanoic acid (10a)

Compound **9a** (1.25 g, 4.0 mmol) was treated with TFA (15 mL) for 30 min., after which the mixture was coevaporated three times with toluene. The resulting product was dissolved in water (40 mL) containing Na_2CO_3 (1.61 g, 15.2 mmol) and cooled to 0 °C. A solution of di-*tert*-butyldicarbonate (0.96 g, 4.43 mmol) in 1,4-dioxane (25 mL) was slowly added and the resulting mixture was stirred for 12 h slowly warming up to RT. Next, water was added followed by extracting twice with EtOAc. The aqueous layer was acidified with 4M aq. HCl to pH 2 and extracted with EtOAc (3×). The latter organic layers were combined, dried over MgSO_4 and concentrated under reduced pressure. The title compound was purified by column chromatography (30% EtOAc/PE → 100% EtOAc) and obtained as a white solid (yield: 1.35 g, 3.80 mmol, 95%). ^1H NMR (200 MHz, CDCl_3): $\delta = 10.57$ (bs, 1H), 5.14 (d, $J = 8.4$ Hz, 1H), 4.63–4.43 (m, 1H), 3.40–3.11 (m, 2H), 1.37 (s, 9H) ppm. ^{13}C NMR (50 MHz, CDCl_3): $\delta = 173.88, 156.68, 148.35, 143.16, 139.83, 134.88, 110.11, 82.43, 53.38, 27.94, 26.61$ ppm. $[\alpha]_D^{23} = +22.3^\circ$ ($c = 1$ in CHCl_3). HRMS: calcd. for $\text{C}_{14}\text{H}_{14}\text{F}_5\text{NO}_4$ [$\text{M} + \text{H}]^+$ 356.09158; found 356.09165.



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

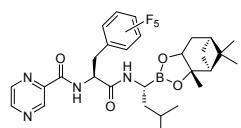
This compound was prepared by the same method described for compound **10a**. The product was obtained as a colourless solid (yield: 1.47 g, 3.67 mmol, 96%). Spectroscopic data are given for a mixture of rotamers. ^1H NMR (400 MHz, CDCl_3): $\delta = 10.27$ (s, 1H), 7.79 (s, 1H), 7.77 (s, 1H), 7.68 (s, 2H), 7.65 (s, 2H), 6.99 (d, $J = 6.83$ Hz, 1H), 5.13 (d, $J = 7.38$ Hz, 1H), 4.66 (d, $J = 6.05$ Hz, 1H), 4.43 (d, $J = 4.43$ Hz, 1H), 3.45–3.30 (m, 2H), 3.21–3.02 (m, 2H), 1.41 (s, 9H), 1.29 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 175.16, 174.64, 156.69, 155.18, 139.21, 138.70, 131.84\text{--}131.51$ (m), 129.69, 123.23 (q, $J = 272.57$ Hz), 121.05, 121.02, 82.54, 80.85, 55.53, 53.91, 38.97, 37.55, 28.08, 27.86 ppm. HRMS: calcd. for $\text{C}_{16}\text{H}_{17}\text{F}_6\text{NO}_4$ [$\text{M} + \text{H}]^+$ 402.11345; found 402.1134.



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

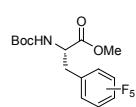
N,N-Diisopropylcarbodiimide (1.3 eq., 0.41 mmol, 64 μL) was added to a solution of BocPhe(*m*-(CF_3)₂-OH (**10b**, 1 eq., 127 mg, 0.32 mmol) and *N*-hydroxysuccinimide (1.12 eq., 0.35 mmol, 41 mg) in DCM (5 mL) and the reaction mixture was stirred for 12 h yielding the crude BocPhe(*m*-(CF_3)₂-OSu (**11b**)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

200 mg, 0.70 mmol) in THF (10 mL) at -78°C . The reaction mixture was allowed to warm to RT and was stirred for 12 h before it was cooled to -78°C . HCl (4.6 mmol, 1.15 mL 4M in 1,4-dioxane) was added and the reaction mixture was allowed to warm to RT, before being cooled to -78°C . To the stirred solution DiPEA (8.0 mmol, 1.32 mL) and the crude BocPhe(*m*-(CF₃)₂-OSu solution (0.32 mmol) were added and the reaction mixture was allowed to warm to RT. The reaction mixture was stirred for an additional 2 h before being filtered over Celite and the filtrate was concentrated under reduced pressure. Purification of the residue by column chromatography (10% \rightarrow 20% EtOAc/PE) resulted in the dipeptide (yield: 40 mg, 61 μmol , 19%). This compound was dissolved in a 1/1 (v/v) mixture of DCM/TFA (2 mL) and stirred for 30 min. before being coevaporated three times with toluene. The crude TFA salt was dissolved again in DCM (2 mL) and HCTU (1.5 eq., 92 μmol , 38 mg), 2-pyrazine carboxylic acid (1.5 eq., 92 μmol , 115 mg) and DiPEA (4 eq., 0.25 mmol, 41 μL) were added and the mixture was stirred for 3 h before being concentrated under reduced pressure and purified by HPLC (linear gradient 80% \rightarrow 100% ACN in H₂O, 0.1% TFA, 15 min). The product was obtained as a colourless solid (yield: 7.4 mg, 11 μmol , 18%). ¹H NMR (400 MHz, CDCl₃): δ = 9.34 (s, 1H), 8.78 (s, 1H), 8.55 (s, 1H), 8.38 (d, J = 8.47 Hz, 1H), 7.75 (s, 3H), 6.04 (d, J = 4.24 Hz, 1H), 4.85 (dd, J = 14.30, 6.92 Hz, 1H), 4.28 (d, J = 8.48 Hz, 1H), 3.41-3.20 (m, 3H), 2.34-2.28 (m, 1H), 2.23-2.14 (m, 1H), 2.01 (t, J = 5.19, 5.19 Hz, 1H), 1.94-1.88 (m, 1H), 1.81 (d, J = 14.60 Hz, 1H), 1.57-1.46 (m, 1H), 1.45-1.41 (m, 2H), 1.40 (s, 3H), 1.28 (s, 3H), 1.15 (d, J = 10.88 Hz, 1H), 0.89-0.81 (m, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 169.62, 163.01, 147.66, 144.19, 142.85, 138.99, 131.77 (q, J = 33.23 Hz), 129.83, 129.80, 123.19 (q, J = 272.80 Hz), 121.07, 86.23, 77.91, 53.60, 51.23, 39.87, 39.50, 37.97, 35.37, 28.38, 27.04, 26.26, 25.47, 24.00, 22.80, 22.05 ppm. $[\alpha]_D^{23} = -16.2^{\circ}$ (c = 1 in CHCl₃). LC-MS: system B, gradient 50% \rightarrow 90% ACN/0.1% TFA/H₂O): R_t (min): 9.33. HRMS: calcd. for C₃₁H₃₇BF₆N₄O₄ [M + H]⁺ 655.28848; found 655.28839.



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

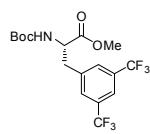
Prepared according to the procedure as described for compound **2b** using BocPhe(F₅)-OH. The product was obtained as a colourless solid (yield: 12 mg, 19.7 μmol , 14%). ¹H NMR (600 MHz, CDCl₃): δ = 9.31 (s, 1H), 8.77 (d, J = 2.20 Hz, 1H), 8.56 (d, J = 1.29 Hz, 1H), 8.40 (d, J = 8.63 Hz, 1H), 6.07 (d, J = 4.53 Hz, 1H), 4.87 (dd, J = 14.43, 8.57 Hz, 1H), 4.30 (d, J = 8.64 Hz, 1H), 3.38-3.31 (m, 2H), 3.23 (dd, J = 13.98, 8.84 Hz, 1H), 2.36-2.31 (m, 1H), 2.23-2.17 (m, 1H), 2.02 (t, J = 5.41, 5.41 Hz, 1H), 1.94-1.90 (m, 1H), 1.82 (d, J = 14.61 Hz, 1H), 1.65-1.54 (m, 3H), 1.40 (s, 3H), 1.29 (s, 3H), 1.16 (d, J = 10.87 Hz, 1H), 0.92-0.82 (m, 9H) ppm. ¹³C NMR (150 MHz, CDCl₃): δ = 169.13, 163.14, 147.68, 144.24, 143.57, 142.85, 86.32, 78.08, 51.98, 51.17, 40.03, 39.43, 38.18, 35.35, 29.69, 28.43, 27.03, 26.24, 25.44, 24.01, 22.89, 22.09 ppm. $[\alpha]_D^{23} = -18.9^{\circ}$ (c = 1 in CHCl₃). LC-MS: system B, gradient 10% \rightarrow 90% ACN/0.1% TFA/H₂O): R_t (min): 12.15. HRMS: calcd. for C₂₉H₃₄BF₅N₄O₄ [M + H]⁺ 609.26660; found 609.26664.



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

TMS-diazomethane (3.0 mL as a 2M solution in hexanes, 6.0 mmol, 4 eq.) was added dropwise to a solution of BocPhe(F₅)-OH (**10a**, 0.52 g, 1.5 mmol) in MeOH/toluene (1/1 (v/v), 8 mL) and the mixture was stirred until TLC analysis revealed a completed reaction after 2 h. The mixture was concentrated to obtain the product (yield: 0.54 g, 1.5 mmol, quant.) as a white solid without further purification. ¹H NMR (400 MHz, CDCl₃): δ = 5.27 (d, J = 7.6 Hz, 1H), 4.60 (d, J = 4.4 Hz, 1H), 3.79 (s, 3H), 3.34 (dd, J = 13.2, 3.6 Hz, 1H), 3.08 (dd, J = 13.6, 6.8 Hz, 1H), 1.40 (s, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ 171.19, 154.79, 146.75, 144.30, 141.70-141.3 (m), 139.10-138.21 (m), 136.10-135.91 (m), 110.05 (t, J = 18.0 Hz), 80.07,

52.54, 27.91, 25.94 ppm. $[\alpha]_D^{23} = +37.8^\circ$ ($c = 1$ in CHCl_3). HRMS: calcd. for $\text{C}_{15}\text{H}_{16}\text{F}_5\text{NO}_4$ $[\text{M} + \text{H}]^+$ 370.10723; found 370.10718.

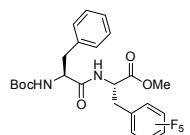


(S)-methyl 3-(3,5-bis(trifluoromethyl)phenyl)-2-(*tert*-butoxycarbonylamino)propanoate (12b)

This compound was prepared by the same method described for compound **12a**. The product was obtained as a colourless solid (yield: 623 mg, 1.5 mmol, quant.). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.77$ (s, 1H), 7.59 (s, 2H), 5.14 (d, $J = 7.09$ Hz, 1H), 4.63 (d, $J = 6.25$ Hz, 1H), 3.75 (s, 3H), 3.26 (ddd, $J = 72.60, 13.71, 5.68$ Hz, 2H), 1.42 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.42, 154.82, 138.85, 131.63$ (q, $J = 32.67$ Hz), 129.59, 123.22 (q, $J = 272.66$ Hz), 120.98, 80.33, 54.08, 52.46, 37.92, 28.11 ppm. $[\alpha]_D^{23} = +45.2^\circ$ ($c = 1$ in CHCl_3). HRMS: calcd. for $\text{C}_{17}\text{H}_{19}\text{F}_6\text{NO}_4$ $[\text{M} + \text{H}]^+$ 416.12910; found 416.12899.

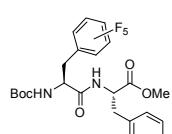
General procedure A: peptide coupling

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



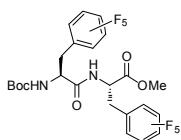
(S)-methyl 2-((S)-2-(*tert*-butoxycarbonylamino)-3-phenylpropanamido)-3-(perfluorophenyl)propanoate, BocPhePhe(F₅)OMe

Prepared via general procedure A using Boc-protected amine **12a** and BocPheOH. The product was obtained as a colourless solid (yield: 759 mg, 1.47 mmol, 95%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.30\text{--}7.19$ (m, 3H), 7.17 (d, $J = 6.68$ Hz, 2H), 6.62 (d, $J = 7.58$ Hz, 1H), 4.95 (d, $J = 7.80$ Hz, 1H), 4.79 (dd, $J = 13.52, 7.00$ Hz, 1H), 4.36–4.29 (m, 1H), 3.73 (s, 3H), 3.27 (dd, $J = 14.06, 5.85$ Hz, 1H), 3.06 (dd, $J = 15.68, 6.89$ Hz, 2H), 2.98–2.89 (m, 1H), 1.39 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.33, 170.45, 155.28, 145.44$ (dd, $J = 237, 11.0$ Hz), 140.17 (dt, $J = 251, 11.0$ Hz), 137.30 (dt, $J = 251, 11.0$ Hz), 136.37, 129.08, 128.56, 126.87, 109.60 (t, $J = 19.0$ Hz), 80.29, 55.77, 52.73, 51.21, 38.06, 28.05, 25.55 ppm. $[\alpha]_D^{23} = +2.45^\circ$ ($c = 1$ in CHCl_3). LC-MS: system A, gradient 10% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 9.68. HRMS: calcd. for $\text{C}_{24}\text{H}_{25}\text{F}_5\text{N}_2\text{O}_5$ $[\text{M} + \text{H}]^+$ 517.17564; found 517.17563.



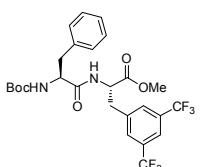
(S)-methyl 2-((S)-2-(*tert*-butoxycarbonylamino)-3-(perfluorophenyl)propanamido)-3-phenylpropanoate, BocPhe(F₅)PheOMe

Prepared via general procedure A using Boc-protected amine BocPheOMe and carboxylic acid **10a**. The product was obtained as a colourless solid (yield: 497 mg, 0.96 mmol, 91%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.30\text{--}7.20$ (m, 3H), 7.10 (d, $J = 6.62$ Hz, 2H), 6.72 (d, $J = 7.23$ Hz, 1H), 5.24 (d, $J = 8.58$ Hz, 1H), 4.86 (td, $J = 7.88, 6.08$, 6.08 Hz, 1H), 4.47–4.39 (m, 1H), 3.71 (s, 3H), 3.19 (dd, $J = 14.17, 5.27$ Hz, 1H), 3.10 (dd, $J = 7.87, 6.29$ Hz, 2H), 2.95 (dd, $J = 14.02, 8.91$ Hz, 1H), 1.37 (s, 9H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 171.54, 169.72, 155.09, 145.44$ (dd, $J = 237, 11.0$ Hz), 140.17 (dt, $J = 251, 11.0$ Hz), 137.30 (dt, $J = 251, 11.0$ Hz), 135.50, 129.15, 128.52, 127.13, 110.49 (t, $J = 19.0$ Hz), 80.27, 53.16, 52.30, 37.87, 27.97, 25.87 ppm. $[\alpha]_D^{23} = +28.5^\circ$ ($c = 1$ in CHCl_3). LC-MS: system A, gradient 10% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 9.67. HRMS: calcd. for $\text{C}_{24}\text{H}_{25}\text{F}_5\text{N}_2\text{O}_5$ $[\text{M} + \text{H}]^+$ 517.17564; found 517.17542.



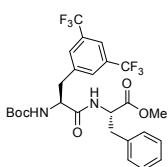
(S)-methyl 2-((S)-2-(*tert*-butoxycarbonylamino)-3-(perfluorophenyl)propanamido)-3-(perfluorophenyl)propanoate, BocPhe(F₃)Phe(F₃)OMe (15)

Prepared via general procedure A using Boc-protected amine **12a** and carboxylic acid **10a**. The product (yield: 1.18 g, 1.9 mmol, 79%) was obtained as a white solid after purification by column chromatography (1% - 13% EtOAc/toluene). ¹H NMR (400 MHz, MeOD): δ = 4.77 (dd, J = 9.6, 5.2 Hz, 1H), 4.37-4.25 (m, 1H), 3.79 (s, 3H), 3.43 (dd, J = 14.4, 5.2 Hz, 1H), 3.23 (dd, J = 14.0, 9.6 Hz, 1H), 3.15-3.08 (m, 1H), 2.98-2.91 (m, 1H), 1.29 (s, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 170.63, 170.09, 155.15, 146.70 (m), 144.25 (m), 141.61 (m), 139.25-138.66 (m), 136.15 (m), 110.32, 80.68, 53.40, 52.98, 51.58, 27.90, 25.54, 25.07 ppm. $[\alpha]_D^{23} = +6.5^\circ$ (c = 1 in CHCl₃). LC-MS analysis: R_t 10.43 min (linear gradient 10-90% ACN in H₂O, 0.1% TFA, 15 min). HRMS: calcd. for C₂₄H₂₀F₁₀N₂O₅ [M + H]⁺ 607.12853; found 607.12834.



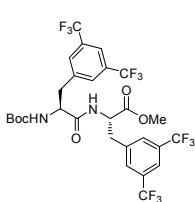
(S)-methyl 3-(3,5-bis(trifluoromethyl)phenyl)-2-((S)-2-(*tert*-butoxycarbonylamino)-3-phenylpropanamido)propanoate, BocPhePhe(F₆)OMe

Prepared via general procedure A using Boc-protected amine **12b** and BocPheOH. The product was obtained as a colourless solid (yield: 823 mg, 1.46 mmol, 96%). ¹H NMR (400 MHz, CDCl₃): δ = 7.76 (s, 1H), 7.53 (s, 2H), 7.31-7.21 (m, 3H), 7.18 (d, J = 6.87 Hz, 2H), 6.64 (d, J = 7.24 Hz, 1H), 4.97 (d, J = 7.52 Hz, 1H), 4.82 (dd, J = 12.66, 5.98 Hz, 1H), 4.37-4.30 (m, 1H), 3.67 (s, 3H), 3.21 (dq, J = 13.89, 13.88, 13.88, 5.88 Hz, 2H), 3.10-2.97 (m, 2H), 1.38 (s, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 171.30, 170.61, 155.37, 138.59, 136.25, 131.64 (q, J = 33.0 Hz), 129.54, 129.11, 128.66, 127.02, 123.14 (q, J = 271 Hz), 121.03, 80.35, 55.97, 52.95, 52.40, 37.63, 28.03 ppm. $[\alpha]_D^{23} = +26.1^\circ$ (c = 1 in CHCl₃). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H₂O): R_t (min): 10.41. HRMS: calcd. for C₂₆H₂₈F₆N₂O₅ [M + H]⁺ 563.19752; found 563.19753.



(S)-methyl 2-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-2-(*tert*-butoxycarbonylamino)propanamido)-3-phenylpropanoate, BocPhe(F₆)PheOMe

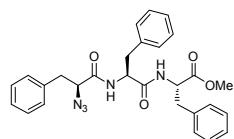
Prepared via general procedure A using Boc-protected amine BocPheOMe and carboxylic acid **10b**. The product was obtained as a colourless solid (yield: 551 mg, 0.98 mmol, 93%). ¹H NMR (400 MHz, CDCl₃): δ = 7.73 (s, 1H), 7.64 (s, 2H), 7.28-7.17 (m, 3H), 7.05 (d, J = 6.55 Hz, 2H), 6.70 (d, J = 7.15 Hz, 1H), 5.33 (d, J = 7.66 Hz, 1H), 4.83 (dd, J = 13.80, 6.14 Hz, 1H), 4.51-4.43 (m, 1H), 3.69 (s, 3H), 3.21 (dd, J = 13.92, 5.89 Hz, 1H), 3.13-2.99 (m, 3H), 1.38 (s, 9H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 171.53, 170.17, 155.18, 139.25, 135.47, 131.48 (q, J = 33 Hz), 129.69, 129.13, 128.56, 127.17, 123.24 (q, J = 271 Hz), 120.76, 80.34, 54.82, 53.19, 52.55, 37.77, 28.05 ppm. $[\alpha]_D^{23} = +28.2^\circ$ (c = 1 in CHCl₃). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H₂O): R_t (min): 9.88. HRMS: calcd. for C₂₆H₂₈F₆N₂O₅ [M + H]⁺ 563.19752; found 563.19745.



(S)-methyl 3-(3,5-bis(trifluoromethyl)phenyl)-2-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-2-(*tert*-butoxycarbonylamino)propanamido)propanoate, BocPhe(F₆)Phe(F₆)OMe

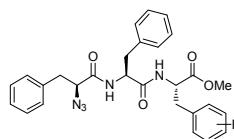
Prepared via general procedure A using Boc-protected amine **12b** and carboxylic acid **10b**. The product was obtained as a colourless solid (yield: 971 mg, 1.39 mmol, 93%). ¹H NMR (400 MHz, CDCl₃): δ = 7.77 (s, 1H), 7.76 (s, 1H), 7.65 (s, 2H), 7.54 (s, 2H), 6.67 (d, J = 7.28 Hz, 1H), 4.98 (d, J = 7.51 Hz, 1H), 4.87-4.78 (m, 1H), 4.37 (dd, J = 14.62, 7.09 Hz, 1H), 3.71 (s, 3H), 3.32-3.16 (m, 4H), 1.38 (s, 9H) ppm. ¹³C NMR

(100 MHz, CDCl_3): δ = 170.610, 170.322, 138.987, 138.367, 131.79 (q, J = 33.31 Hz), 129.513, 123.15 (q, J = 267.69 Hz), 121.220, 80.940, 53.013, 52.634, 37.592, 27.985 ppm. LC-MS: system A, gradient 10% \rightarrow 90% ACN/0.1% TFA/ H_2O : R_t (min): 10.94. HRMS: calcd. for $\text{C}_{28}\text{H}_{26}\text{F}_{12}\text{N}_2\text{O}_5$ $[\text{M} + \text{H}]^+$ 699.17229; found 699.17228.



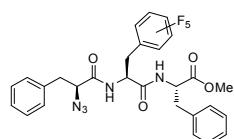
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-phenylpropanamido)-3-phenylpropanoate, N₃PhePhePheOMe

Prepared via general procedure A using Boc-protected amine BocPhePheOMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 217 mg, 0.43 mmol, 87%). ^1H NMR (400 MHz, CDCl_3): δ = 7.32-7.12 (m, 9H), 7.07-6.94 (m, 6H), 4.95 (dd, J = 14.23, 6.97 Hz, 1H), 4.81 (dd, J = 12.13, 5.88 Hz, 1H), 3.87 (dd, J = 4.76, 3.55 Hz, 1H), 3.64 (s, 3H), 3.17 (dd, J = 13.84, 2.01 Hz, 1H), 3.08-2.96 (m, 2H), 2.92 (d, J = 6.47 Hz, 2H), 2.81 (dd, J = 13.87, 8.70 Hz, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 171.04, 169.96, 168.22, 135.96, 135.89, 135.52, 129.32, 129.28, 129.06, 128.40, 128.26, 127.03, 126.90, 126.76, 64.80, 53.45, 53.16, 52.02, 38.43, 38.20, 37.77 ppm. $[\alpha]_D^{23} = +55.2^\circ$ (c = 1 in CHCl_3). LC-MS: system A, gradient 10% \rightarrow 90% ACN/0.1% TFA/ H_2O : R_t (min): 8.96. HRMS: calcd. for $\text{C}_{28}\text{H}_{29}\text{N}_5\text{O}_4$ $[\text{M} + \text{H}]^+$ 500.22923; found 500.22909.



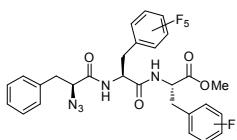
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-phenylpropanamido)-3-(perfluorophenyl)propanoate, N₃PhePhe(F₅)OMe

Prepared via general procedure A using Boc-protected amine BocPhePhe(F₅)OMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 205 mg, 0.35 mmol, 71%). ^1H NMR (400 MHz, CDCl_3): δ = 7.34-7.18 (m, 8H), 7.05 (dd, J = 7.64, 1.44 Hz, 2H), 6.92 (d, J = 8.00 Hz, 1H), 6.87 (d, J = 8.34 Hz, 1H), 4.77 (ddd, J = 19.50, 14.54, 7.08 Hz, 2H), 4.19 (dd, J = 8.47, 3.99 Hz, 1H), 3.72 (s, 3H), 3.28-3.19 (m, 2H), 3.03 (dd, J = 14.05, 7.07 Hz, 1H), 2.92-2.89 (m, 2H), 2.85 (dd, J = 14.12, 8.49 Hz, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 170.23, 170.17, 168.52, 145.44 (dd, J = 237, 11.0 Hz), 140.17 (dt, J = 251, 11.0 Hz), 137.30 (dt, J = 251, 11.0 Hz), 135.84, 135.75, 129.34, 129.12, 128.52, 128.41, 127.15, 126.89, 109.51 (dt, J = 18.60, 3.47 Hz), 65.07, 53.81, 52.66, 51.15, 38.33, 37.75, 25.37 ppm. $[\alpha]_D^{23} = +13.7^\circ$ (c = 1 in CHCl_3). LC-MS: system A, gradient 10% \rightarrow 90% ACN/0.1% TFA/ H_2O : R_t (min): 9.58. HRMS: calcd. for $\text{C}_{28}\text{H}_{24}\text{F}_5\text{N}_5\text{O}_4$ $[\text{M} + \text{H}]^+$ 590.18212; found 590.18204.



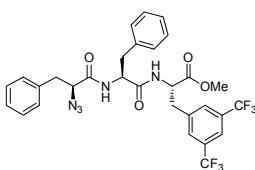
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-(perfluorophenyl)propanamido)-3-phenylpropanoate, N₃PhePhe(F₅)PheOMe

Prepared via general procedure A using Boc-protected amine BocPhe(F₅)PheOMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 117 mg, 0.20 mmol, 62%). ^1H NMR (400 MHz, CDCl_3): δ = 7.45 (d, J = 8.00 Hz, 1H), 7.30-7.20 (m, 8H), 7.11 (d, J = 8.80 Hz, 1H), 7.03 (d, J = 6.85 Hz, 2H), 5.09 (dd, J = 15.60, 7.20 Hz, 1H), 4.93 (dd, J = 12.66, 5.98 Hz, 1H), 3.72 (s, 3H), 3.55 (dd, J = 8.47, 3.99 Hz, 1H), 3.18 (dd, J = 14.00, 3.60 Hz, 1H), 3.13-3.08 (m, 3H), 2.93 (dd, J = 13.20, 6.00 Hz, 1H), 2.78 (dd, J = 13.80, 9.00 Hz, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 171.14, 169.18, 168.72, 145.44 (dd, J = 237, 11.0 Hz), 140.17 (dt, J = 251, 11.0 Hz), 137.30 (dt, J = 251, 11.0 Hz), 135.96, 135.62, 129.41, 129.26, 128.43, 128.39, 127.21, 127.10, 109.65 (dt, J = 18.57, 3.61 Hz), 64.74, 53.36, 52.27, 51.24, 38.31, 37.95, 25.86 ppm. $[\alpha]_D^{23} = +18.0^\circ$ (c = 1 in CHCl_3). LC-MS: system A, gradient 10% \rightarrow 90% ACN/0.1% TFA/ H_2O : R_t (min): 9.84. HRMS: calcd. for $\text{C}_{28}\text{H}_{24}\text{F}_5\text{N}_5\text{O}_4$ $[\text{M} + \text{H}]^+$ 590.18212; found 590.18212.



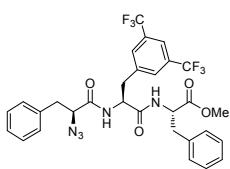
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-(perfluorophenyl)propanamido)-3-(perfluorophenyl)propanoate, N₃PhePhe(F₅)Phe(F₅)OMe (16)

Prepared via general procedure A using Boc-protected amine **15** and azidophenylalanine. The product was obtained as a colourless solid (yield: 0.22 g, 0.32 mmol, 61%). ¹H NMR (400 MHz, CDCl₃): δ = 7.31-7.21 (m, 5H), 4.90-4.85 (m, 1H), 4.83-4.77 (m, 1H), 4.21 (dd, J = 8.0, 3.2 Hz, 1H), 3.80 (s, 3H), 3.35-3.23 (m, 2H), 3.12-3.08 (m, 2H), 2.99-2.90 (m, 2H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 170.47, 169.22, 169.02, 146.69 (m), 144.17 (m), 141.69 (m), 138.60 (m), 136.08, 135.64, 129.39, 128.69, 126.01, 109.57 (m), 65.05, 52.93, 51.70, 51.38, 38.21, 25.58, 24.81 ppm. $[\alpha]_D^{23}$ = +12.6° (c = 1 in CHCl₃). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H₂O): R_t (min): 10.59. HRMS: calcd. for C₂₄H₁₉F₁₀N₅O₄ [M + H]⁺ 680.13501; found 680.13494.



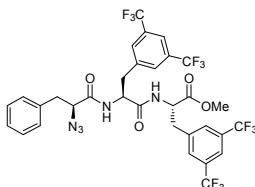
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-phenylpropanamido)-3-(3,5-bis(trifluoromethyl)phenyl)propanoate, N₃PhePhePhe(F₆)OMe

Prepared via general procedure A using Boc-protected amine BocPhePhe(F₆)OMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 149 mg, 0.23 mmol, 48%). ¹H NMR (400 MHz, CDCl₃): δ = 7.74 (s, 1H), 7.52 (s, 2H), 7.34-7.19 (m, 8H), 7.05 (d, J = 6.25 Hz, 2H), 6.84 (d, J = 7.90 Hz, 1H), 6.67 (d, J = 7.37 Hz, 1H), 4.76 (dd, J = 13.42, 6.17 Hz, 1H), 4.67 (dd, J = 14.62, 7.20 Hz, 1H), 4.15 (dd, J = 8.15, 4.04 Hz, 1H), 3.66 (s, 3H), 3.22 (dd, J = 14.19, 3.92 Hz, 1H), 3.16 (dd, J = 14.08, 6.13 Hz, 2H), 3.00-2.84 (m, 3H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 170.49, 170.15, 168.57, 138.52, 135.77, 135.67, 131.61 (q, J = 33.29, Hz), 129.44, 129.14, 128.60, 127.26, 127.13, 123.12 (q, J = 272.76 Hz), 121.04, 65.04, 54.24, 53.10, 52.42, 38.33, 38.04, 37.63 ppm. $[\alpha]_D^{23}$ = +37.5° (c = 1 in CHCl₃). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H₂O): R_t (min): 10.14. HRMS: calcd. for C₃₀H₂₇F₆N₅O₄ [M + H]⁺ 636.20400; found 636.20406.



(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-(3,5-bis(trifluoromethyl)phenyl)propanamido)-3-phenylpropanoate, N₃PhePhe(F₆)PheOMe

Prepared via general procedure A using Boc-protected amine BocPhe(F₆)PheOMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 193 mg, 0.30 mmol, 93%). ¹H NMR (400 MHz, CDCl₃): δ = 7.73 (s, 1H), 7.55 (s, 2H), 7.33-7.16 (m, 9H), 7.13 (d, J = 8.65 Hz, 1H), 6.99 (dd, J = 6.92, 2.33 Hz, 2H), 5.10 (dd, J = 15.02, 6.46 Hz, 1H), 4.85 (td, J = 7.62, 5.62, 5.62 Hz, 1H), 3.77 (dd, J = 8.95, 3.78 Hz, 1H), 3.72 (s, 3H), 3.24 (dd, J = 14.00, 3.68 Hz, 1H), 3.06 (dd, J = 14.03, 5.99 Hz, 4H), 2.80 (dd, J = 14.00, 8.99 Hz, 1H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 171.14, 169.20, 168.77, 138.47, 135.89, 135.48, 131.47 (q, J = 33.21 Hz), 129.77 (d, J = 2.42 Hz), 129.37, 129.13, 128.58, 128.45, 127.25, 127.21, 123.18 (q, J = 272.74 Hz), 121.01, 64.89, 53.15, 52.84, 52.23, 38.53, 38.31, 37.78 ppm. $[\alpha]_D^{23}$ = +41.8° (c = 1 in CHCl₃). LC-MS: system A, gradient 10% → 90% ACN/(0.1% TFA/H₂O): R_t (min): 10.20. HRMS: calcd. for C₃₀H₂₇F₆N₅O₄ [M + H]⁺ 636.20400; found 636.20406.



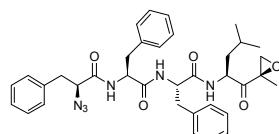
(S)-methyl 2-((S)-2-((S)-2-azido-3-phenylpropanamido)-3-(3,5-bis(trifluoromethyl)phenyl)propanamido)-3-(3,5-bis(trifluoromethyl)phenyl)propanoate, N₃PhePhe(F₆)Phe(F₆)OMe

Prepared via general procedure A using Boc-protected amine BocPhe(F₆)Phe(F₆)OMe and azidophenylalanine. The product was obtained as a colourless solid (yield: 0.34 g, 0.44 mmol, 88%). ¹H NMR

(400 MHz, CDCl_3): δ = 7.76 (s, 2H), 7.51 (s, 2H), 7.49 (s, 2H), 7.34-7.23 (m, 5H), 4.81-4.75 (m, 1H), 4.70 (dd, J = 14.0, 7.2 Hz, 1H), 4.19 (dd, J = 8.0, 4.0 Hz, 1H), 3.70 (s, 3H), 3.30-3.24 (m, 2H), 3.20 (dd, J = 16.8, 5.9 Hz, 2H), 3.00-2.93 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 170.48, 169.18, 168.72, 138.26, 135.50, 131.81 (q, J = 11.0 Hz), 129.62, 129.51, 129.25, 128.73, 123.12 (q, J = 127 Hz), 121.27, 64.97, 53.56, 53.03, 52.66, 38.41, 37.59, 37.48 ppm. $[\alpha]_D^{23} = +2.2^\circ$ (c = 1 in MeOH). LC-MS: system A, gradient 10% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 11.66. HRMS: calcd. for $\text{C}_{32}\text{H}_{25}\text{F}_{12}\text{N}_5\text{O}_4$ [$\text{M} + \text{H}$]⁺ 772.17877; found 772.17899.

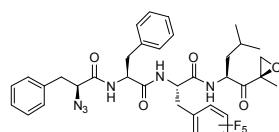
General procedure B: azide coupling of the peptidehydrazides to the epoxyketone warhead (18)

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



(S)-2-azido-N-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)-3-phenylpropanamide, N₃PhePheLeu-EK (3)

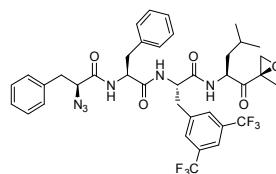
Prepared via general procedure B. The product was obtained as a colourless solid (yield: 157 mg, 0.25 mmol, 77%). ^1H NMR (400 MHz, CDCl_3): δ = 7.33-7.12 (m, 12H), 7.04 (d, J = 6.38 Hz, 1H), 7.00 (d, J = 7.69 Hz, 3H), 6.94 (d, J = 7.93 Hz, 1H), 6.63 (d, J = 8.15 Hz, 1H), 4.80 (dd, J = 14.46, 6.89 Hz, 1H), 4.74 (dd, J = 14.29, 6.80 Hz, 1H), 4.59-4.53 (m, 1H), 4.03 (dd, J = 8.63, 4.02 Hz, 1H), 3.21-3.15 (m, 2H), 3.03-2.87 (m, 4H), 2.87-2.79 (m, 2H), 1.56-1.50 (m, 2H), 1.49 (s, 3H), 1.22 (dd, J = 16.68, 7.16 Hz, 1H), 0.90 (dd, J = 9.10, 6.22 Hz, 6H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 207.96, 170.12, 168.61, 135.96, 135.87, 129.35, 129.27, 129.19, 128.62, 128.56, 128.52, 128.39, 128.33, 127.18, 127.01, 126.82, 64.96, 58.80, 53.91, 53.86, 52.10, 49.74, 40.13, 38.25, 37.88, 25.01, 23.20, 21.38, 16.50 ppm. $[\alpha]_D^{23} = +47.5^\circ$ (c = 1 in CHCl_3). LC-MS: system A, gradient 50% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 5.82. HRMS: calcd. for $\text{C}_{36}\text{H}_{42}\text{N}_6\text{O}_5$ [$\text{M} + \text{H}$]⁺ 639.32894; found 639.32906.



(S)-2-azido-N-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-(perfluorophenyl)propan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)-3-phenylpropanamide, N₃PhePhe(F₅)Leu-EK (4a)

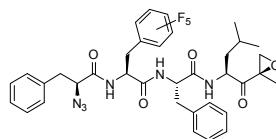
Prepared via general procedure B. The product was obtained as a colourless solid (yield: 234 mg, 0.32 mmol, quant.). ^1H NMR (400 MHz, CDCl_3): δ = 7.38 (d, J = 8.10 Hz, 1H), 7.33-7.13 (m, 10H), 7.03 (d, J = 6.67 Hz, 2H), 4.91 (dd, J = 12.63, 6.05 Hz, 2H), 4.67 (t, J = 7.71, 7.71 Hz, 1H), 4.21 (dd, J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

3.28 Hz), 65.09, 58.88, 53.85, 52.13, 51.90, 50.02, 40.67, 38.33, 38.18, 26.03, 25.20, 23.31, 21.51, 16.50 ppm. $[\alpha]_D^{23} = +8.7^\circ$ ($c = 1$ in CHCl_3). LC-MS: system B, gradient 50% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 7.73. HRMS: calcd. for $\text{C}_{36}\text{H}_{37}\text{F}_5\text{N}_6\text{O}_5$ $[\text{M} + \text{H}]^+$ 729.28184; found 729.28213.



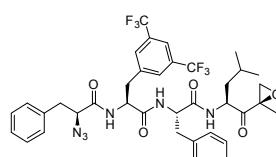
(S)-2-azido-N-((S)-1-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxopropan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)-3-phenylpropanamide, $\text{N}_3\text{PhePhe}(\text{F}_6)\text{Leu-EK}$ (4b)

Prepared via general procedure B. The product was obtained as a colourless solid (yield: 156 mg, 0.20 mmol, 95%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.71$ (s, 1H), 7.53 (s, 2H), 7.34-7.16 (m, 8H), 7.05 (d, $J = 7.63$ Hz, 1H), 7.00 (d, $J = 6.14$ Hz, 3H), 6.93 (d, $J = 7.75$ Hz, 1H), 4.76 (dd, $J = 13.13, 6.42$ Hz, 1H), 4.68 (dd, $J = 14.27, 7.10$ Hz, 1H), 4.54 (dd, $J = 13.00, 5.25$ Hz, 1H), 4.15 (dd, $J = 8.35, 3.84$ Hz, 1H), 3.24-3.14 (m, 2H), 3.07 (dd, $J = 13.92, 5.77$ Hz, 2H), 2.92 (d, $J = 6.64$ Hz, 2H), 2.84 (d, $J = 4.68$ Hz, 2H), 1.67-1.57 (m, 1H), 1.53 (dd, $J = 13.48, 2.57$ Hz, 1H), 1.49 (s, 3H), 1.30-1.22 (m, 1H), 0.95-0.91 (m, 6H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 208.28, 170.34, 169.28, 168.61, 162.53, 138.62, 135.86, 135.66, 131.27$ (q, $J = 33.14$ Hz), 129.80, 129.39, 129.03, 128.57, 128.54, 127.20, 127.06, 123.19 (q, $J = 272.84$ Hz), 120.84, 64.98, 59.02, 54.20, 53.43, 52.14, 50.21, 39.79, 38.23, 38.00, 37.84, 36.44, 31.36, 25.07, 23.24, 21.14, 16.39 ppm. $[\alpha]_D^{23} = +41.9^\circ$ ($c = 1$ in CHCl_3). LC-MS: system B, gradient 50% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 8.73. HRMS: calcd. for $\text{C}_{38}\text{H}_{40}\text{F}_6\text{N}_6\text{O}_5$ $[\text{M} + \text{H}]^+$ 775.30371; found 775.30428.



(S)-2-azido-N-((S)-1-((S)-1-((R)-4-methyl-1-((2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-ylamino)-1-oxo-3-(perfluorophenyl)propan-2-yl)-3-phenylpropanamide, $\text{N}_3\text{PhePhe}(\text{F}_5)\text{PheLeu-EK}$ (5a)

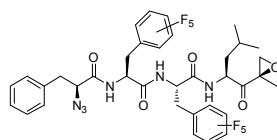
Prepared via general procedure B. The product was obtained as a colourless solid (yield: 135 mg, 0.18 mmol, 92%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.69$ (d, $J = 6.88$ Hz, 1H), 7.31-7.15 (m, 9H), 7.09 (d, $J = 6.46$ Hz, 2H), 7.04 (d, $J = 6.34$ Hz, 1H), 6.73 (d, $J = 6.27$ Hz, 1H), 5.03 (dd, $J = 14.61, 7.19$ Hz, 1H), 4.83 (dd, $J = 13.94, 6.69$ Hz, 1H), 4.59 (dd, $J = 12.37, 5.10$ Hz, 1H), 3.91 (dd, $J = 8.90, 3.47$ Hz, 1H), 3.22 (dd, $J = 13.83, 3.23$ Hz, 1H), 3.13-3.06 (m, 2H), 3.02-2.97 (m, 3H), 2.83 (dd, $J = 13.94, 9.17$ Hz, 1H), 2.76 (d, $J = 4.71$ Hz, 1H), 1.64-1.52 (m, 2H), 1.50 (s, 3H), 1.29-1.22 (m, 1H), 1.00-0.82 (m, 6H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 208.27, 169.95, 169.31, 168.94, 145.44$ (dd, $J = 237, 11.0$ Hz), 140.17 (dt, $J = 251, 11.0$ Hz), 137.30 (dt, $J = 251, 11.0$ Hz), 135.98, 129.33, 128.46, 128.27, 127.09, 127.04, 126.91, 109.66 (dt, $J = 19.00, 2.82$ Hz), 64.79, 58.83, 54.07, 52.04, 51.48, 49.82, 40.22, 38.73, 38.21, 25.62, 25.06, 23.10, 21.30, 16.45 ppm. $[\alpha]_D^{23} = +27.0^\circ$ ($c = 1$ in CHCl_3). LC-MS: system B, gradient 50% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 7.86. HRMS: calcd. for $\text{C}_{36}\text{H}_{37}\text{F}_5\text{N}_6\text{O}_5$ $[\text{M} + \text{H}]^+$ 729.28184; found 729.28211.



(S)-2-azido-N-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-1-((S)-1-((R)-4-methyl-1-((2-methyloxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-ylamino)-1-oxopropan-2-yl)-3-phenylpropanamide, $\text{N}_3\text{PhePhe}(\text{F}_6)\text{PheLeu-EK}$ (5b)

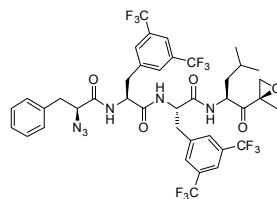
Prepared via general procedure B. The product was obtained as a colourless solid (yield: 220 mg, 0.28 mmol, 95%). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.85$ (d, $J = 7.85$ Hz, 1H), 7.71 (s, 1H), 7.60 (s, 2H), 7.40 (d, $J = 8.60$ Hz, 1H), 7.32-7.08 (m, 9H), 7.05 (d, $J = 6.92$ Hz, 2H), 5.13 (dd, $J =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

128.51, 128.16, 127.07, 126.72, 123.11 (q, $J = 272.74$ Hz), 120.75, 65.00, 58.77, 53.95, 52.89, 51.99, 49.81, 40.08, 38.53, 38.39, 37.86, 25.04, 23.01, 21.32, 16.37 ppm. $[\alpha]_D^{23} = +43.4^\circ$ ($c = 1$ in CHCl_3). LC-MS: system B, gradient 50% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 8.73. HRMS: calcd. for $\text{C}_{38}\text{H}_{40}\text{F}_6\text{N}_6\text{O}_5$ $[\text{M} + \text{H}]^+$ 775.30371; found 775.30404.



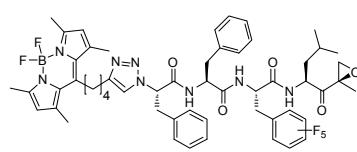
(S)-2-azido-N-((S)-1-((S)-4-methyl-1-((R)-2-methoxyxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxo-3-(perfluorophenyl)propan-2-ylamino)-1-oxo-3-(perfluorophenyl)propan-2-yl-3-phenylpropanamide, $\text{N}_3\text{PhePhe}(\text{F}_5)\text{Phe}(\text{F}_5)\text{Leu-EK}$ (6a)

Prepared via general procedure B from **16**. Purification by HPLC (linear gradient 72 \rightarrow 82% ACN in H_2O , 0.1% TFA, 15 min) furnished the title compound (yield: 16.4 mg, 20.1 μmol , 10%) as a white solid. ^1H NMR (400 MHz, MeOD): $\delta = 7.31\text{--}7.23$ (m, 5H), 4.63 (dd, $J = 14.4, 6.8$ Hz, 2H), 4.54 (dd, $J = 10.0, 3.2$ Hz, 1H), 4.04 (dd, $J = 8.8, 5.2$ Hz, 1H), 3.28 (d, $J = 5.2$ Hz, 1H), 3.17-3.09 (m, 3H), 3.01-2.98 (m, 1H), 2.96-2.83 (m, 3H), 1.74-1.60 (m, 1H), 1.44 (s, 3H), 1.38-1.34 (m, 1H), 1.32-1.31 (m, 1H), 0.93 (t, $J = 6.8$ Hz, 6H) ppm. ^{13}C NMR (100 MHz, MeOD): $\delta = 209.55, 171.41, 171.12, 148.20, 145.78, 143.0\text{--}142.50$ (m), 140.15-139.8 (m), 137.75, 137.68, 130.36, 129.60, 128.06, 111.54 (q, $J = 18$ Hz), 65.49, 59.95, 53.09, 52.77, 51.27, 40.46, 38.69, 26.33, 26.02, 23.70, 21.58, 16.73 ppm. $[\alpha]_D^{23} = +5.5^\circ$ ($c = 1$ in MeOH). LC-MS: system A, gradient 50% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 8.63. HRMS: calcd. for $\text{C}_{36}\text{H}_{32}\text{F}_{10}\text{N}_6\text{O}_5$ $[\text{M} + \text{H}]^+$ 819.23473; found 819.23498.



(S)-2-azido-N-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-1-((S)-3-(3,5-bis(trifluoromethyl)phenyl)-1-((S)-4-methyl-1-((R)-2-methoxyxiran-2-yl)-1-oxopentan-2-ylamino)-1-oxopropan-2-ylamino)-1-oxopropan-2-yl-3-phenylpropanamide, $\text{N}_3\text{PhePhe}(\text{F}_6)\text{Phe}(\text{F}_6)\text{Leu-EK}$ (6b)

Prepared via general procedure B. The product was obtained by HPLC purification (linear gradient 62 \rightarrow 69% ACN in H_2O , 0.1% TFA, 15 min) as a yellowish oil (yield: 29.3 mg, 32.1 μmol , 7%). ^1H NMR (400 MHz, MeOD): $\delta = 7.84$ (s, 2H), 7.79 (s, 4H), 7.25-7.14 (m, 5H), 4.76-4.59 (m, 2H), 4.49 (dd, $J = 10.8, 2.8$ Hz, 1H), 3.97 (dd, $J = 8.8, 2.8$ Hz, 1H), 3.28 (d, $J = 5.7$ Hz, 1H), 3.25 (d, $J = 4.8$ Hz, 1H), 3.21 (d, $J = 5.2$ Hz, 1H), 3.04-2.98 (m, 3H), 2.91 (d, $J = 4.8$ Hz, 1H), 2.74 (dd, $J = 13.9, 9.1$ Hz, 1H), 1.74-1.70 (m, 1H), 1.51-1.47 (m, 1H), 1.45 (s, 3H), 1.39-1.31 (m, 1H), 0.95 (d, $J = 6.4$ Hz, 3H), 0.91 (d, $J = 6.4$ Hz, 3H) ppm. ^{13}C NMR (100 MHz, MeOD): $\delta = 209.65, 172.28, 171.92, 171.25, 141.46, 137.81, 132.60$ (q, $J = 33.0$ Hz), 131.20, 130.25, 129.57, 128.07, 124.90 (q, $J = 332$ Hz), 121.77, 65.51, 60.18, 55.05, 54.78, 52.98, 51.94, 40.08, 38.88, 38.33, 26.32, 23.73, 21.47, 16.95 ppm. $[\alpha]_D^{23} = +29.4^\circ$ ($c = 1$ in MeOH). LC-MS: system A, gradient 50% \rightarrow 90% ACN/(0.1% TFA/ H_2O): R_t (min): 10.09. HRMS: calcd. for $\text{C}_{40}\text{H}_{38}\text{F}_{12}\text{N}_6\text{O}_5$ $[\text{M} + \text{H}]^+$ 911.27848; found 911.27902.



Bodipy-triazole-Phe-Phe(F₅)-Leu-EK (19)

Compound **4a** (70.7 mg, 97 μmol) was dissolved in a mixture of toluene/*t*BuOH/ H_2O (1/1/1 v/v/v, 2 mL) and to this were added Bodipy-alkyne¹⁸ (1 eq., 97 μmol , 32 mg), CuSO_4 (10 mol%, 9.7 μmol , 9.7 μL ; 1M in H_2O) and sodium ascorbate (15 mol%, 14.5 μmol , 14.5 μL ; 1M in H_2O). The

mixture was stirred at 80 °C for 2.5 h after which TLC analysis indicated complete consumption of the starting material. The mixture was concentrated under reduced pressure and the compound was purified by column chromatography (DCM \rightarrow 2.5% MeOH/DCM) and obtained as a brownish solid (yield: 89.2 mg, 84.4 μmol , 87%). ^1H NMR (400 MHz, CDCl_3) $\delta = 8.07$ (d, $J = 5.49$ Hz, 1H), 7.62 (s, 1H), 7.52 (d, $J = 8.17$ Hz, 1H), 7.19 (d, $J = 5.94$ Hz, 1H), 7.08 (d, $J = 4.92$ Hz, 4H), 7.03-7.00 (m, 2H), 6.95 (d, $J = 4.02$ Hz, 4H), 6.84 (d, $J = 4.15$ Hz, 2H), 6.02 (s, 2H), 5.77 (t, $J = 7.40$,

7.40 Hz, 1H), 5.30-5.24 (m, 1H), 4.82 (dd, J = 13.07, 6.73 Hz, 1H), 4.75 (dd, J = 12.64, 5.94 Hz, 1H), 3.44-3.32 (m, 2H), 3.24 (d, J = 4.58 Hz, 1H), 3.05 (dd, J = 13.96, 6.43 Hz, 1H), 3.00-2.63 (m, 8H), 2.50 (s, 6H), 2.37 (s, 6H), 1.95-1.86 (m, 2H), 1.73-1.58 (m, 4H), 1.54 (s, 3H), 1.31 (dd, J = 6.89, 3.54 Hz, 1H), 0.92 (dd, J = 21.75, 6.16 Hz, 6H) ppm. ^{13}C NMR (150 MHz, CDCl_3): δ = 208.40, 170.33, 169.33, 167.56, 153.79, 147.36, 145.92, 140.22, 135.57, 135.24, 131.33, 129.02, 128.72, 128.42, 128.23, 127.09, 126.60, 121.56, 120.54, 64.65, 58.79, 54.28, 52.03, 51.56, 49.92, 40.50, 38.83, 38.64, 31.29, 29.58, 28.06, 25.43, 25.13, 23.19, 21.44, 16.46, 16.27, 14.35 ppm. $[\alpha]_D^{23}$ = +27.5° (c = 1 in CHCl_3). LC-MS: system A, gradient 50% → 90% ACN/(0.1% TFA/ H_2O): R_t (min): 8.95. HRMS: calcd. for $\text{C}_{55}\text{H}_{60}\text{BF}_7\text{N}_8\text{O}_5$ $[\text{M} + \text{H}]^+$ 1057.47407; found 1057.47562.

Competition assays

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

IC₅₀ determinations

Purified 26S proteasome (~10 ng/mL) was incubated with various concentrations of inhibitors at 37 °C for 30 min. in the assay buffer (50 mM Tris-HCl, pH 7.5, 40 mM KCl, 2 mM EDTA, 1 mM DTT, 100 μM ATP, 50 $\mu\text{g}/\text{mL}$ BSA). In the meantime, 100 μM solution of the fluorogenic peptide substrates (Suc-LLVY-7-amido-4-methyl-coumarin (amc) for the β 5 site, Ac-nLPnLD-amc for the β 1 site and Ac-RLR-amc or Ac-RQR-amc for the β 2 site) in the assay buffer were pre-incubated at 37 °C. Immediately after the end of this incubation, an aliquot of the inhibitor-treated proteasome was mixed with the substrate, and fluorescence of released amc was measured continuously for 30 min. at 37 °C. The rate of reaction was determined from the slope of the reaction progress curves. Mock-treated proteasomes served as control. Residual activity in inhibitor treated samples were plotted against concentration of inhibitors and IC₅₀ values were determined from these plots.

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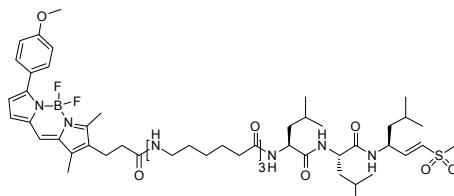
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