Synthetic tools to illuminate matrix metalloproteinase and proteasome activities

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Summary and Future Prospects

The research described in this Thesis aims at the development of chemical biology research tools to study proteolytic activities of metalloproteases (MMPs and ADAMs) and proteasomes. The common thread throughout the Thesis is the use of photocrosslinking in combination with activity-based protein profiling. **Chapter 1** describes the properties of the three most commonly applied photophores and examples of their use in chemical biology.

A potent class of MMP/ADAM inhibitors comprise the peptide succinyl hydroxamates. **Chapter 2** deals with the synthesis of *N,O*-diprotected succinyl hydroxamate building block **1** (Figure 1) and its use in the construction of enantiopure peptide hydroxamates in a highly efficient, linear solid-phase peptide synthesis (SPPS) protocol. With the aid of this building block, a biotinylated photoreactive probe was synthesized, which was applied in the covalent modification and visualization of ADAM-10. In addition, a library containing 96 inhibitors with general structure **2** was created and used to study the inhibitor preference of three metalloproteases (MMP-9, MMP-12 and ADAM-17) with respect to the substituents at the P2′ and P3′ positions. The presented highly efficient method for SPPS of peptide hydroxamates allows an easy extension of the inhibitor library by incorporation of other (both proteinogenic and non-proteinogenic) amino acids at all positions. Especially the modification of the P1′ substituent will be of great interest, since this position has a big influence on the enzyme's substrate preference.[1,2] It should however be noted that modification of this site will lead to a change in building block structure and hence a greater synthetic challenge. Extension of the inhibitor arsenal will hopefully lead to more potent, but especially more selective inhibitors for the MMP/ADAM family.

Another interesting modification is the change of the hydroxamic acid zinc binding group (ZBG) into a zinc coordinating electrophilic moiety, which would allow for the covalent capture of metalloproteases. This concept has proven to be useful in the covalent modification of histone deacetylases (HDACs), which represent another class of zinc dependent metalloenzymes. For example, it was shown that the epoxyketone moiety is able to coordinate the Zn^{2+} ion in HDACs and can subsequently trap a nearby nucleophile in the active site.[3,4] A possible mechanism of action for epoxyketone containing MMP inhibitors is shown in Figure 1 (insert).[5] As a first attempt diastereoisomers **3a** and **3b** were synthesized. These compounds are analogues of a known potent MMP inhibitor, which contains the hydroxamic acid ZBG.[6] Unfortunately, in a preliminary activity study it was shown that these compounds only display inhibitory activity in the millimolar range towards MMP-12. Therefore, variations in the epoxyketone moiety or the use of other electrophilic ZBGs will be necessary.

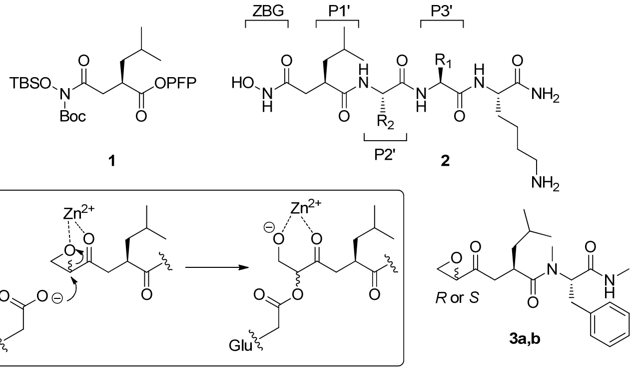


Figure 1. Structure of building block **1**, used for the construction of a library containing 96 metalloprotease inhibitors with general structure **2**. The epoxyketone ZBG containing MMP inhibitors **3a,b** are supposed to form a covalent adduct with the enzyme's active site via the mechanism shown in the insert.

The efficiency of photolabelling of ADAM-10 with the photoreactive probe described in Chapter 2 proved to be rather modest. Therefore, the effect of moving the photophore to the more tightly binding P1' pocket was studied and this is outlined in **Chapter 3**. The synthesis of building block **4** (Figure 2) and its application in the preparation of activity-based probe **5** is described herein. It was further demonstrated that **5** is indeed the more efficient MMP/ADAM probe in a head to head comparison towards a range of recombinant, purified metalloproteases. It has been shown that the use of other photophores in related MMP probes can also lead to efficient labelling.[7,8] However, the structural differences between these probes make mutual comparisons difficult. It is therefore of interest to synthesize the aryl azide (**6a**) and benzophenone (**6b**) analogues of **5** and study the differences with respect to inhibitory potency and labelling efficiency.

One of the most challenging tasks in photoaffinity labelling is the determination of the crosslinking site(s). Indeed, despite many efforts, locating the modified fragment after incubation and photocrosslinking MMPs with **5** has proven very difficult so far. A possible solution to this problem is the use of mixed isotope labelling (see Chapter 1). For this, the photoaffinity probe should be designed with incorporation of a stable isotope containing moiety. In this respect, a valuable set of stable isotope tags may be the ^{13}C-labelled PEG reagents **7a-g** (Figure 2),[9] which were initially designed as labelling reagents for the quantitative analysis of low molecular weight metabolites (intracellular amino acids and glutathione) by LC-MS. They can be easily incorporated into an ABP by means of a reaction between the activated PFP ester and an amine, both in solution-phase peptide chemistry and in SPPS. This would allow for the preparation of a set of structurally identical probes of different molecular weight, which can be used directly in mixed isotope labelling.

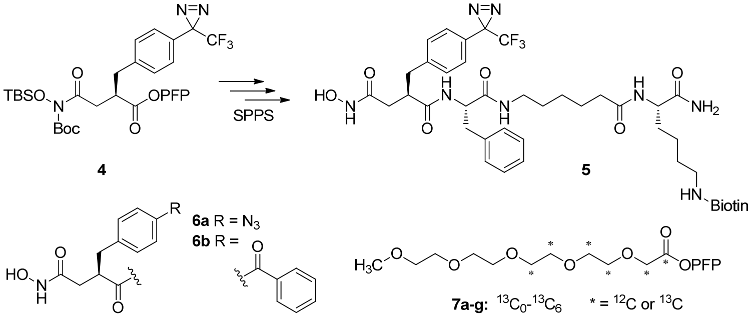


Figure 2. Structure of photoreactive building block **4**, used for the synthesis of MMP/ADAM probe **5**. Incorporation of the aryl azide or benzophenone photophores will lead to **6a** and **6b** respectively. The set of ^{13}C-labelled PEG derivatives **7a-g** was used for quantitative analysis of low molecular weight metabolites.[9]

The research described in **Chapter 4** entails the study towards the effect of fluorine incorporation into proteasome inhibitors on their specificity towards the different subunits. It was found that substitution of non-fluorinated Phe in peptide boronic ester **8** (Figure 3) with fluorinated versions (see insert) does not affect the selectivity. In addition, a set of peptide epoxyketones **9** was prepared, in which either one or both of the P2 and P3 positions is fluorinated. Interestingly, it was shown that fluorine incorporation, especially at the P2 site, led to a large decrease in potency towards the β1 and β2 subunits and hence resulted in selectivity for β5. Tripeptide epoxyketone **10** was identified as one of the most β5 selective inhibitors known to date and was subsequently converted into fluorescent probe **11**, which can be used for specific labelling and visualization of the β5 subunit.

It will be of interest to study the effect of fluorine incorporation at the P1 position, since this substituent is of great importance for substrate specificity. Also, the use of other fluorinated amino acids can give rise to improved (β5) selectivity. Especially fluorinated leucine (Hfl, Leu(F$_6$), Figure 3)[10,11] will be of interest, since it serves as a good,

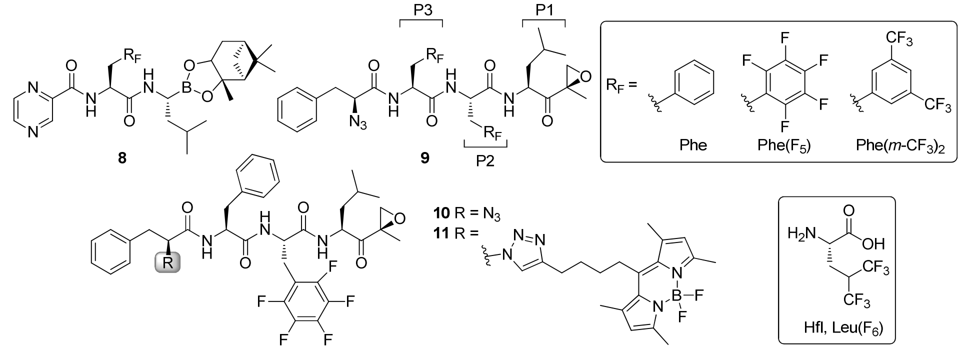


Figure 3. Structures of fluorinated proteasome inhibitors.

structurally similar, substitute for leucine, which is commonly used in proteasome inhibitors.

The development of selective inhibitors for the proteasome's trypsin-like sites (β2) with good cell penetrating properties is outlined in **Chapter 5**. Selectivity for β2 was achieved by instalment of basic side chain residues in the P1 position of tripeptide vinylsulfones and epoxyketones. Incorporation of a 4-aminomethylene phenylalanine analogue at this position resulted in compound **12** (Figure 4), which showed high inhibitory potency and selectivity for β2, both in cell extracts and in living cells. Attachment of this 4-aminomethylene-Phe-vinylsulfone warhead to the 3-hydroxy-2-methylbenzoyl-Val-Ser motif further increased β2 selectivity and even resulted in selectivity for β2 over its immuproteasome counterpart β2i.

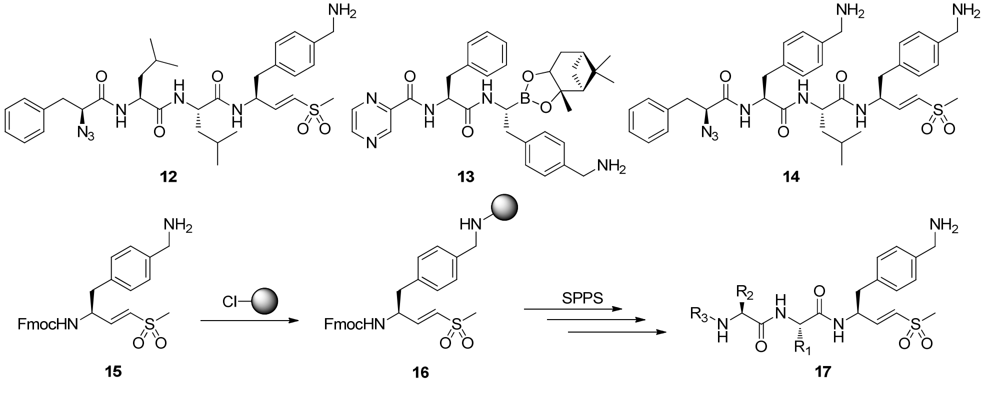
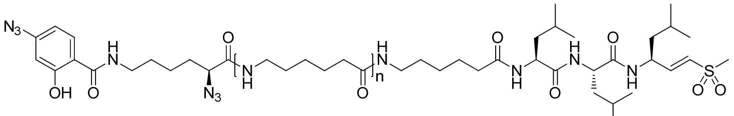


Figure 4. Structures of proteasome inhibitors functionalized with an amine at the P1 site and the use of this modification in SPPS.

It will be of interest to study the effect of incorporating the 4-aminobenzyl moiety in other peptide inhibitors. For example, incorporating the β2 directing substituent into β1,5 selective proteasome inhibitor Bortezomib (resulting in compound **13**) can lead to interesting insights into the selectivity determining part of the inhibitor. In addition, incorporation of 4-aminomethylene-Phe at the P3 position, resulting in **14**, can lead to an even higher selectivity for β2, since it was shown that the substituent at the P3 position is also of great importance in subunit selectivity.[12] It should be noted that this added basic residue may interfere with cell permeability. An interesting feature of the side-chain amine functionality is that it allows for an easy, linear SPPS of inhibitor libraries. For example, vinyl sulfone **15** (Figure 4) can be attached to a solid support via its side-chain amine, giving **16**, after which a peptide chain can be build up at the N-terminus. A subsequent cleavage step will then result in inhibitors with general structure **17**.

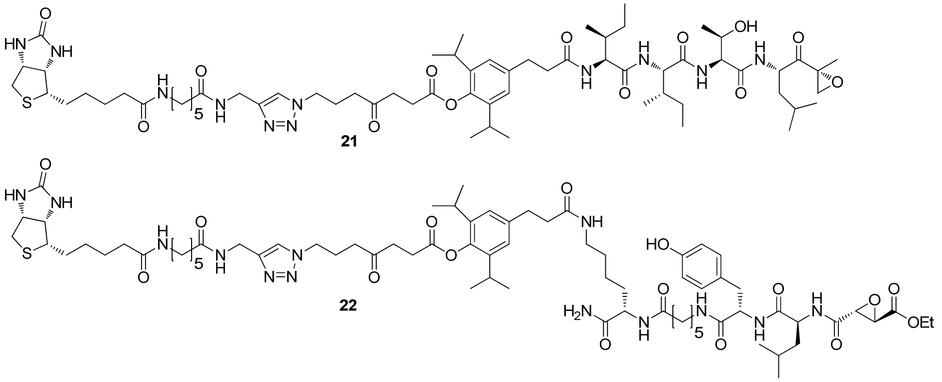
Chapter 6 deals with the study towards the orientation of extended peptide vinylsulfones inside the proteasome's catalytically active cavity. The study combined activity-based modification, by means of a warhead, with photocrosslinking. A panel of bifunctional two-step probes, comprising of an extended peptide vinylsulfone equipped with a N-terminal photophore, was prepared. Of these probes, compound **18** (Figure 5) was able to establish a crosslink between the active β5 subunit and a neighbouring β6 subunit, which could be identified by subsequent SDS-PAGE and LC-MS/MS analysis. Although these results showed the applicability of this approach, it still needs improvements in order to be used in studies towards an inhibitor's orientation within the proteasome's active 20S core particle. The use of longer photoreactive probes, for example compounds **19** and **20**, may be of interest in this respect and can lead to a more complete picture of the interactions of an inhibitor with the proteasome's internal structure in general. Besides the spacer length, also a change of its nature, for instance by introduction of a more hydrophilic (PEG) or a more rigid spacer, can lead to interesting structural insights. This may well be combined with stable isotope incorporation, for example ^{13}C labelled PEG spacers (modified versions of the PEG compounds in Figure 2), which allows an easier identification of the photocross-linked adducts.



18 n = 1
19 n = 2
20 n = 3

Figure 5. Structures of photoreactive proteasome probes.

The design and preparation of a novel cleavable linker system, based on the levulinoyl ester, is described in **Chapter 7**. The cleavable linker was incorporated into potent proteasome inhibitor epoxomicin and attachment of biotin resulted in ABP **21** (Figure 6). The optimal characteristics, in terms of stability and cleavability, of this linker system were reflected by the use of **21** for pull-down of proteasome active subunits from a cell lysate. Subsequent treatment with hydrazine allowed a chemoselective release of **21**-derived bound proteins. It was shown that the cleavable linker is well compatible with transformations often applied in chemical biology research, including click chemistry and Staudinger-Bertozzi ligation. In order to asses proteomics-wide applicability of the system, it needs to be used for the enrichment of other enzymes as well. In this respect, compound **22** was synthesized, in which the cleavable linker moiety was incorporated into DCG-04, for the ABPP of cysteine proteases.[13] However, this compound remains to be tested.



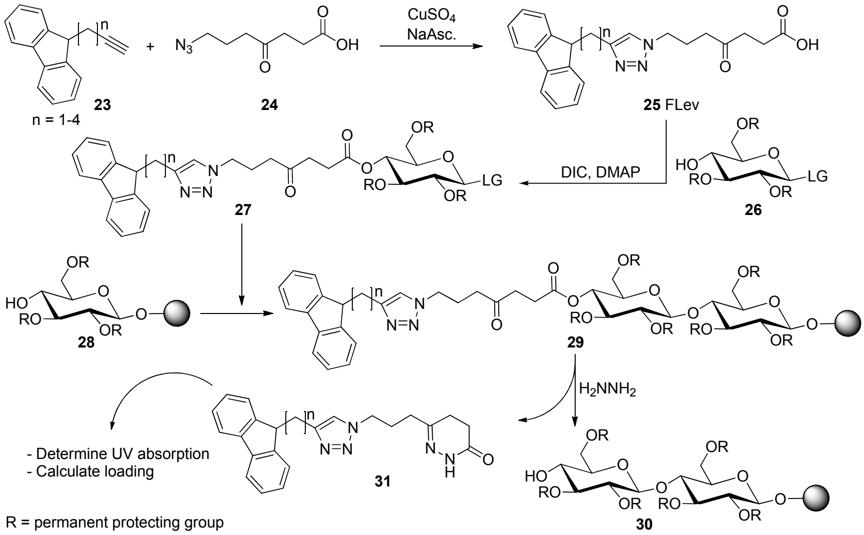
21

22

Figure 6. Structures of proteasome probe **21** and cysteine protease probe **22** containing the levulinoyl ester-based cleavable linker system.

The ease of cleavage of this linker system under relatively mild conditions and the fact that excess hydrazine can be easily removed by evaporation make that the use of this cleavable linker is not limited to activity-based enrichment of enzymes. For example, the linker system may find its use in affinity-based chromatography, or solid-phase extraction (SPE), of proteins. In addition, the synthetic strategy of the linker system allows for a straightforward modification of levulinic acid. A possible application of this can be in the field of (automated) solid-phase oligosaccharide synthesis.[14,15] This technique is used for the synthesis of oligosaccharides from their monomeric building blocks on a solid support, analogous to SPPS. The levulinoyl ester is often used as a temporal protecting group herein, because of the possibility to be cleaved orthogonally in the presence of other (permanent) protecting groups. A major difficulty in solid-phase oligosaccharide synthesis is the lack of straightforward methods to determine the loading of the resin, which indicates the coupling efficiency or yield. A possible solution for this is shown in Scheme 1. A click reaction of 9*H*-fluorene alkyne **23**, easily prepared from fluorene and the appropriate terminal alkyne halide,[16] with azido modified levulinic acid **24** (see Chapter 7) results in compound **25** (termed FLev). Subsequent condensation with **26** gives fully protected monomeric sugar building block **27**, which can be applied in solid-phase oligosaccharide synthesis. For example, a coupling between immobilized sugar **28** with donor **27** results in immobilized disaccharide **29**. Treatment of this construct with hydrazine results in a FLev deprotection and concomitant formation of fluorene containing compound **31**. Measuring the UV absorbance (λ = 301 nm) of the filtrate leads to the determination of the concentration of this species and hence, the loading on the solid support, which is analogous to Fmoc determination in SPPS.

Scheme 1. Suggested application of modified levulinic acid in solid-phase oligoaccharide synthesis.

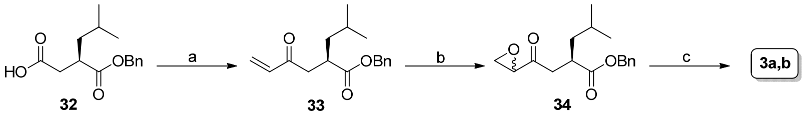


Experimental section

General

Tetrahydrofuran was distilled over $LiAlH_4$ 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). [1-$^{13}C_1$]acetic acid and [1,2-$^{13}C_2$]acetic acid were from Buchem b.v.. [1-$^{13}C_1$]bromoacetic acid and [1,2-$^{13}C_2$]bromoacetic acid were prepared from [1-$^{13}C_1$]acetic acid and [1,2-$^{13}C_2$]acetic acid via a reported procedure.[17] Amberlite IR120 H resin (Acros) was rinsed with MeOH (4×) and Et_2O (4×) before use. 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. 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. Automated column chromatography was performed on a CombiFlash Companion with a Silicycle FLH-R10030B-ISO12 Sillaflash 12 g cartridge. 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 F_{254}). Compounds were visualized by UV absorption (254 nm), 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, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (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. ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol JNM-FX-200 (200 MHz), a Bruker AV-400 (400 MHz) or a Bruker DMX-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, $CDCl_3$ or CD_3OD 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 LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at m/z = 400 (mass range m/z = 150-2,000) and dioctylpthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). 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 lm C18 50 × 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 × 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 × 4.6 mm). RP-HPLC purification was performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 × 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 × 4.6 mm).

Scheme 2. Synthesis of MMP inhibitors **3a,b** equipped with an epoxyketone ZBG.



Reagents and conditions: (a) i) $(ClCO)_2$, cat. DMF, DCM; ii) $Pd(PPh_3)_2Cl_2$, tributylvinyltin, toluene, 80°C, 81%; (b) H_2O_2, K_2CO_3, *t*BuOH, H_2O, 45%; (c) i) Pd/C, H_2, THF, 0°C; ii) TFA·MePheNHMe, HATU, DiPEA, DMF, 32%, 39%.

(R)-benzyl 2-isobutyl-4-oxohex-5-enoate (33)

Carboxylic acid **32** (2.65 g, 10 mmol) was dissolved in DCM (70 mL), put under an argon atmosphere and cooled to 0 °C. After addition of a few drops of DMF, oxalyl chloride (4 eq., 5.08 g, 40 mmol, 3.38 mL) was added to the solution. The mixture was stirred for 15 min. at 0 °C, then allowed to warm to room temperature and stirred until CO_2 formation seized after 1 h. Toluene was added and the solution was concentrated under reduced pressure followed by coevaporation three times with toluene. The resulting acyl chloride was dissolved in toluene (35 mL) before tributylvinyltin (1.05 eq, 3.07 mL, 3.33 g, 10.5 mmol) and $Pd(PPh_3)_2Cl_2$ (1 mol%, 70.2 mg, 0.1 mmol) were added. The mixture was then stirred for 20 h at 80 °C, a black colour was observed and TLC analysis indicated a complete conversion. The solution was allowed to cool to room temperature before being concentrated under reduced pressure. The product was obtained after purification by column chromatography (0% → 13% Et_2O/PE) as a clear yellow liquid (yield: 2.28 g, 8.32 mmol, 81%) ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.45-7.23 (m, 5H), 6.32 (dd, *J* = 17.7, 10.4 Hz, 1H), 6.20 (d, *J* = 17.6 Hz, 1H), 5.80 (d, *J* = 10.4 Hz, 1H), 5.11 (s, 2H), 3.07-2.98 (m, 2H), 2.71-2.62 (m, 1H), 1.69-1.49 (m, 2H), 1.39-1.23 (m, 1H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.86 (d, *J* = 6.3 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 198.3, 175.3, 136.1, 135.9, 128.3, 128.2, 127.9, 66.1, 41.5, 41.1, 38.2, 25.7, 22.4, 22.1 ppm. $[\alpha]_D^{23}$ = +14.6° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{17}H_{22}O_3$ $[M + H]^+$: 275.16417; found: 275.16432.

(R)-benzyl 4-methyl-2-(2-((S/R)oxiran-2-yl)-2-oxoethyl)pentanoate (34)

Compound **34** (1.1 g, 4.0 mmol) was dissolved in a mixture of *tert*-butanol (7.0 mL), hydrogen peroxide (2.5 eq, 1 mL, 10 mmol, 30% in H_2O) and H_2O (3.0 mL), before being cooled to 0 °C. An aqueous saturated solution of K_2CO_3 (344 μL) was added dropwise and the reaction was stirred for 1 h at 0 °C. The mixture was then allowed to warm to room temperature and stirred until TLC analysis indicated complete conversion after 2.5 h. Water was added to dilute the solution before being extracted three times with Et_2O. The organic layers were combined, dried over anhydrous

$MgSO_4$ and concentrated under reduced pressure. The crude mixture was then purified by column chromatography (3% → 8% EtOAc/PE) yielding 316 mg (1.09 mmol) of one diastereoisomer and 210 mg (0.72 mmol) of the other diastereoisomer. The total yield was 326 mg (1.81 mmol, 45.3%). Diastereoisomer A: ^1H NMR (400 MHz, $CDCl_3$): δ = 7.42-7.28 (m, 5H), 5.16-4.99 (m, 2H), 3.42-3.33 (m, 1H), 3.09-2.96 (m, 1H), 2.95-2.88 (m, 2H), 2.69 (dd, *J* = 18.2, 10.2 Hz, 1H), 2.37 (dd, *J* = 18.2, 4.1 Hz, 1H), 1.63-1.49 (m, 2H), 1.35-1.21 (m, 1H), 0.91 (d, *J* = 6.3 Hz, 3H), 0.86 (d, *J* = 6.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 206.2, 175.2, 135.8, 128.4, 128.1, 128.0, 66.3, 53.6, 45.8, 41.0, 37.7, 37.6, 25.6, 22.3, 22.2 ppm. $[\alpha]_D^{23}$ = +56.6° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{17}H_{22}O_4$ $[M + H]^+$: 291.15909; found: 291.15917. Diastereoisomer B: ^1H NMR (400 MHz, $CDCl_3$): δ = 7.39-7.25 (m, 5H), 5.11(s, 2H), 3.42-3.38 (m, 1H), 3.02-2.81 (m, 3H), 2.79 (d, *J* = 5.7 Hz, 1H), 2.40 (dd, *J* = 17.9, 4.4 Hz, 1H), 1.63-1.47 (m, 2H), 1.33-1.21 (m, 1H), 0.90 (d, *J* = 5.8 Hz, 3H), 0.85 (d, *J* = 5.7 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 205.4, 174.9, 135.7, 128.3, 128.0, 127.9, 66.2, 53.1, 46.0, 40.8, 38.9, 37.7, 25.6, 22.3, 22.1 ppm. $[\alpha]_D^{23}$ = –12.6° (*c* = 1 in $CHCl_3$). HRMS: calcd. for $C_{17}H_{22}O_4$ $[M + H]^+$: 291.15909; found: 291.15921.

(S)-2-((R)-N,4-dimethyl-2-(2-((R/S)-oxiran-2-yl)-2-oxoethyl)pentanamido)-3-phenylpropanoic acid (3a)

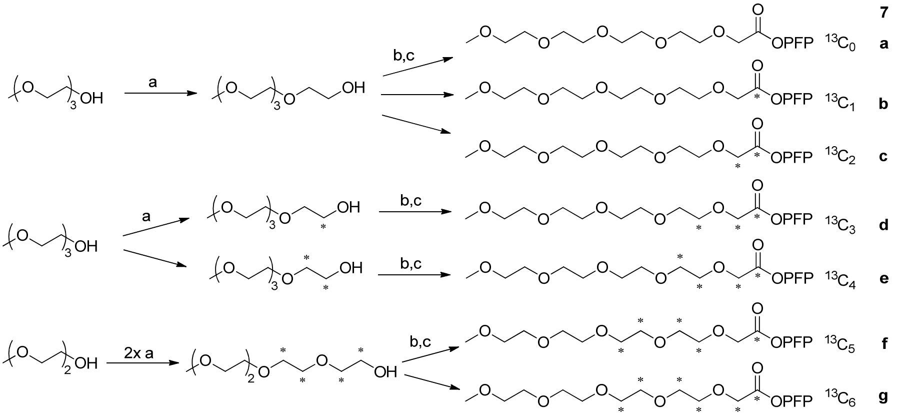
Compound **34** (156 mg, 0.54 mmol) was dissolved in THF (4 mL), put under an argon atmosphere and cooled to 0 °C. Next, 10% Pd/C was added and the mixture was subjected to hydrogen at 0 °C until TLC analysis indicated complete consumption of starting material after 4 h. The argon atmosphere was restored and the solution was diluted with 2 mL of DMF. HATU (1.2 eq., 246 mg, 0.65 mmol) and DiPEA (3.2 eq., 285 μL, 1.72 mmol) were added and the mixture was preactivated for 10 min. Next, a solution of Me-Phe-NHMe TFA salt (2 eq., 331 mg, 1.08 mmol) and DiPEA (2 eq., 178 μL, 1.08 mmol) in DMF (4 mL) was added and the mixture was stirred overnight while being allowed to slowly warm to room temperature. TLC analysis indicated the appearance of several spots, including one major spot at R_F 0.44 (50% EtOAc/PE). The mixture was filtrated over glassfiber patches and concentrated under reduced pressure. The residue was dissolved in DCM and washed with aqueous 1 M HCl, three times with a saturated aqueous $NaHCO_3$ solution and once with brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The product was isolated after purification by column chromatography (20% → 40% EtOAc/toluene) yielding 64.7 mg (0.17 mmol, 32%) of the title compound. ^1H NMR (400 MHz, $CDCl_3$, exists as a mixture of two rotamers): δ = 7.71 (d, J = 4.1 Hz, 0.5H), 7.35-7.08 (m, 5H), 6.08 (d, *J* = 4.1 Hz, 0.5H), 4.82 (t, *J* = 7.1 Hz, 0.5H), 4.46 (dd, *J* = 10.3, 3.6 Hz, 0.5H), 3.62 (dd, *J* = 14.5, 3.6 Hz, 0.5H), 3.41-3.28 (m, 1.5H), 3.15-3.04 (m, 1.5H), 3.01 (dd, *J* = 6.5, 3.9 Hz, 1H), 2.97 (s, 1.5H), 2.95 (d, *J* = 4.6 Hz, 2.5H), 2.86 (s, 1.5H), 2.86-2.83 (m, 0.5H), 2.73 (d, *J* = 4.9 Hz, 2H), 2.64 (dd, *J* = 18.4, 10.0 Hz, 0.5H), 2.34 (dd, *J* = 18.5, 3.4 Hz, 1H), 1.55-1.42 (m, 0.5H), 1.42-1.29 (m, 0.5H), 1.29-1.18 (m, 1H), 0.89 (d, *J* = 6.6 Hz, 1.5H), 0.86-0.76 (m, 2H), 0.61 (d, *J* = 6.6 Hz, 1.5H), 0.59 (d, *J* = 6.6 Hz, 1.5H), –0.19 (ddd, *J* = 14.2, 11.3, 1.5 Hz, 0.5H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, exists as a mixture of two rotamers): δ = 210.6, 207.6, 176.1, 175.8, 170.4, 169.9, 138.3, 137.7, 129.5, 129.1, 128.9, 128.4, 126.9, 126.4, 63.3, 60.6, 53.8, 53.6, 46.4, 46.0, 40.7, 38.7, 38.2, 37.8, 34.4, 33.9, 33.8, 33.4, 29.8, 26.7, 26.1, 25.5, 24.5, 23.3, 23.3, 21.6, 19.9 ppm. $[\alpha]_D^{23}$ –65.8° (*c* = 1 in $CHCl_3$). LC-MS: gradient 10% → 90% ACN(0.1% TFA/H_2O): R_t (min): 7.49. HRMS: calcd. for $C_{21}H_{30}N_2O_4$ $[M + H]^+$: 375.22783; found: 375.22784.

(S)-2-((R)-N,4-dimethyl-2-(2-((S/R)-oxiran-2-yl)-2-oxoethyl)pentanamido)-3-phenylpropanoic acid (3b)

Synthesized from its other diastereoisomer following the procedure described for compound **3a**. The compound was obtained in a yield of 114 mg (0.30 mmol, 39%). ^1H NMR (400 MHz, $CDCl_3$, exists as a mixture of rotamers): δ = 7.75 (d, J = 3.9 Hz, 0.5H, 7.33-7.12 (m, 5H), 6.16 (d, *J* = 4.1 Hz, 0.5H), 4.91 (t, *J* = 7.6 Hz, 0.5H), 4.46 (dd, *J* = 10.3, 3.5 Hz, 0.5H), 3.63 (dd, J = 14.5, 3.5 Hz, 0.5H), 3.43 (ddd, *J* = 25.1, 4.6, 2.4 Hz, 1H), 3.38-3.32 (m, 0.5H), 3.17-3.02 (m, 1.5H), 2.98 (d, *J* =

3.2 Hz, 1.5H), 3.01-2.94 (m, 1.5H), 2.93 (d, *J* = 4.7 Hz, 1.5H), 2.89 (d, *J* = 5.9 Hz, 1.5H), 2.85-2.79 (m, 0.5H), 2.79-2.75 (m, 1.5H), 2.74 (d, *J* = 4.9 Hz, 1.5H), 2.40 (ddd, *J* = 23.0, 18.8, 3.8 Hz, 1H), 1.53-1.40 (m, 0.5H), 1.40-1.29 (m, 0.5H), 1.29-1.19 (m, 1H), 0.94-0.86 (m, 2H), 0.83 (d, *J* = 6.5 Hz, 1.5H), 0.61 (d, *J* = 1.5 Hz, 1.5H), 0.59 (d, *J* = 1.5 Hz, 1.5H), –0.16 (ddd, *J* = 14.2, 11.3, 3.1 Hz, 0.5H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, exists as a mixture of rotamers) δ = 209.2, 176.1, 175.6, 170.4, 169.9, 138.3, 137.6, 129.5, 129.1, 128.9, 128.4, 126.9, 126.5, 63.4, 53.0, 47.0, 46.5, 40.7, 40.1, 39.9, 38.6, 34.5, 34.0, 33.9, 33.5, 29.9, 26.8, 26.1, 25.7, 24.6, 23.3, 23.2, 21.9, 19.9 ppm. $[\alpha]^{23}_{D}$ −100.9° (c = 1 in $CHCl_3$). LC-MS: gradient 10% → 90% ACN(0.1% TFA/H_2O): R_t (min): 7.28. HRMS: calcd. for $C_{21}H_{30}N_2O_4$ $[M + H]^+$: 375.22783; found: 375.22786.

Scheme 3. Synthesis of ^{13}C-labelled PEG-OPFP derivatives **7a-g**.



The asterisks (*) indicate the positions of ^{13}C atoms. Reagents and conditions: (a) i) $^{12}C_2$, 1-$^{13}C_1$, 1,2-$^{13}C_2$-BrAcOH, NaH, NaI, THF; ii) $LiAlH_4$, 15 minutes, then 2M NaOH (aq.), 75-95%; (b) i) $^{12}C_2$, 1-$^{13}C_1$, 1,2-$^{13}C_2$-BrAcOH, NaH, NaI, THF; ii) Amberlite H^+; (c) PFPOH, EDC, DCM, 55-60% over two steps.

General procedure A: extension of the PEG chain by one ethyleneglycol unit

To a solution of the alcohol (1 eq.), bromoacetic acid (either $^{12}C_2$, 1-$^{13}C_1$ or 1,2-$^{13}C_2$, 1.2 eq.) and NaI (0.01 eq.) in THF (5 mL/mmol) was carefully added NaH (60% in mineral oil, 4 eq.) in portions. After complete conversion of the alcohol (monitored by TLC analysis, usually after 2 h) $LiAlH_4$ (2.5 eq.) was carefully added in portions. The reaction was stirred for 30 min. after which NaOH (as a 2M aqueous solution, 2.5 eq.) was added dropwise. After 15 min. of stirring Et_2O (5 mL/mmol) and $MgSO_4$ were added. The reaction was stirred vigorously for another 5 min., after which the solid was filtrated over Celite. The filtrate was concentrated under reduced pressure and the resulting crude product was purified by column chromatography (100% EtOAc → 15% MeOH/EtOAc).

General procedure B: reaction of a PEG chain alcohol with bromoacetic acid followed by acidic work-up

To a solution of the alcohol (1 eq.), bromoacetic acid (either $^{12}C_2$, 1-$^{13}C_1$ or 1,2-$^{13}C_2$, 1.2 eq.) and NaI (0.01 eq.) in THF (5 mL/mmol) was carefully added NaH (60% in mineral oil, 4 eq.) in portions. After complete conversion of the alcohol (monitored by TLC analysis, usually after 2 h) Amberlyte IR120 H resin and Et_2O (2 mL/mmol) were added. After 30 min. of stirring all solids and the resin were filtered off over Celite and the filtrate was concentrated under reduced pressure. The crude product was subjected to the next step (general procedure C) without further purification.

General procedure C: conversion of the carboxylic acid to the pentafluorophenyl ester

The obtained (crude) carboxylic acid (obtained in general procedure B, 1 eq.) was dissolved in DCM (5 mL/mmol). To this solution were added pentafluorophenol (2 eq.) and EDC (2 eq.) and the reaction was stirred for 2 h. The mixture was then concentrated under reduced pressure and the product was purified by automated column chromatography (10% → 80% EtOAc/PE).

[13-^{13}C]2,5,8,11-tetraoxatridecan-13-ol

Prepared according to general procedure A, starting from 2-(2-(2-methoxyethoxy)ethoxy)ethanol (210 mg, 1.28 mmol). The product was obtained as a colourless liquid (yield: 199 mg, 0.95 mmol, 74%). ^{1}H-NMR (200 MHz, $CDCl_3$): $\delta = 3.70$ (dt, $J = 150$, 4.5 Hz, 2H), 3.70-3.53 (m, 14H), 3.39 (s, 3H), 2.61 (bs, 1H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): $\delta = 61.72$ ppm.

[12,13-$^{13}C_2$]2,5,8,11-tetraoxatridecan-13-ol

Prepared according to general procedure A, starting from 2-(2-(2-methoxyethoxy)ethoxy)ethanol (210 mg, 1.28 mmol). The product was obtained as a colourless liquid (yield: 194 mg, 0.92 mmol, 72%). ^{1}H-NMR (200 MHz, $CDCl_3$): $\delta = 3.70$ (dt, $J = 150$, 4.5 Hz, 2H), 6.67 (dt, $J = 150$, 4.5 Hz, 2H), 3.70-3.53 (m, 12H), 3.40 (s, 3H), 2.72 (bs, 1H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): $\delta = 72.53$ (d, $J = 41$ Hz), 61.70 (d, $J = 41$ Hz) ppm.

[1,2-$^{13}C_2$]2-(2-(2-methoxyethoxy)ethoxy)ethanol

Prepared according to general procedure A, starting from 2-(2-methoxyethoxy)ethanol (310 mg, 2.58 mmol). The product was obtained as a colourless liquid (yield: 398 mg, 2.39 mmol, 93%). ^{1}H-NMR (200 MHz, $CDCl_3$): $\delta = 3.73$ (dt, $J = 142$, 4.7 Hz, 2H), 3.62 (dm, $J = 139$ Hz, 2H), 3.69-3.53 (m, 8H), 3.38 (s, 3H), 2.68 (bs, 1H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): $\delta = 72.47$ (d, $J = 39$ Hz), 61.61 (d, $J = 41$ Hz) ppm.

[9,10,12,13-$^{13}C_4$]2,5,8,11-tetraoxatridecan-13-ol

Prepared according to general procedure A, starting from [1,2-$^{13}C_2$]2-(2-(2-methoxyethoxy)ethoxy)ethanol (398 mg, 2.39 mmol). The product was obtained as a colourless liquid (yield: 444 mg, 2.09 mmol, 87%). ^{1}H-NMR (200 MHz, $CDCl_3$): $\delta = 4.11$-3.96 (m, 4H), 3.67-3.53 (m, 8H), 3.40 (s, 3H), 3.38-3.24 (m, 4H), 2.61 (bs, 1H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): $\delta =$ 72.30 (d, $J = 41$ Hz), 70.18, 61.45 (d, $J = 39$ Hz) ppm.

[16-^{13}C]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from 2,5,8,11-tetraoxatridecan-13-ol (206 mg, 0.99 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): $\delta = 4.10$ (d, $J = 4.0$ Hz, 2H), 3.74-3.55 (m, 16H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): $\delta = 173.83$ ppm. LC-MS (system B): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 8.35 (ESI-MS (*m/z*): 268.1 ($M + H^+$)).

[15,16-$^{13}C_2$]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from 2,5,8,11-tetraoxatridecan-13-ol (201 mg, 0.97 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): $\delta = 4.19$ (dd, $J = 145$, 4.5 Hz, 2H), 3.80-3.57 (m, 16H), 3.39 (s, 3H). ^{13}C-NMR (50 MHz, $CDCl_3$): $\delta = 171.95$ (d, $J = 56$ Hz), 68.78 (d, $J = 56$ Hz) ppm. LC-MS (system B): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 7.97 (ESI-MS (*m/z*): 269.3 ($M + H^+$)).

[13,15,16-$^{13}C_3$]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from [13-^{13}C]2,5,8,11-tetraoxatridecan-13-ol (199 mg, 0.95 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): $\delta = 4.15$ (dt, $J = 145$, 4.4 Hz, 2H), 3.73 (dm, $J =$ 145 Hz, 2H), 3.72-3.54 (m, 14H), 3.39 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): $\delta = 172.13$ (d, $J =$

58 Hz), 70.78, 68.86 (d, *J* = 59 Hz) ppm. LC-MS (system B): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 8.37 (ESI-MS (*m/z*): 270.3 (M + H^+)).

[12,13,15,16-$^{13}C_4$]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from [12,13-$^{13}C_2$]2,5,8,11-tetraoxatridecan-13-ol (194 mg, 0.92 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.21 (dt, *J* = 145, 4.4 Hz, 2H), 3.73 (dm, *J* = 144 Hz, 2H), 3.80-3.57 (m, 14H), 3.40 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 172.25 (d, *J* = 58 Hz), 71.63, 69.90, 69.00 (d, *J* = 56 Hz) ppm. LC-MS (system B): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 8.37 (ESI-MS (*m/z*): 271.3 (M + H^+)).

[9,10,12,13,16-$^{13}C_5$]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from [9,10,12,13-$^{13}C_4$]2,5,8,11-tetraoxatridecan-13-ol (222 mg, 1.04 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.20-3.99 (m, 6H), 3.67-3.57 (m, 8H), 3.48-3.33 (m, 4H), 3.39 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 172.31, 71.88, 70.90, 70.33, 69.42 ppm. LC-MS (system A): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 7.59 (ESI-MS (*m/z*): 272.0 (M + H^+)).

[9,10,12,13,15,16-$^{13}C_6$]2,5,8,11,14-pentaoxahexadecan-16-oic acid

Prepared according to general procedure B, starting from [9,10,12,13-$^{13}C_4$]2,5,8,11-tetraoxatridecan-13-ol (222 mg, 1.04 mmol). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.18 (dt, *J* = 145 Hz, 4.4 Hz, 2H), 4.12-3.99 (m, 4H), 3.67-3.47 (m, 8H), 3.39 (s, 3H), 3.37-3.29 (m, 4H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 172.45 (d, *J* = 56 Hz), 71.88, 70.94, 70.36, 69.60 ppm. LC-MS (system A): gradient 0% → 20% ACN/(0.1% TFA/H_2O): R_t (min): 7.63 (ESI-MS (*m/z*): 273.1 (M + H^+)).

Perfluorophenyl 2,5,8,11,14-pentaoxahexadecan-16-oate (7a)

Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 230 mg, 0.53 mmol, 58% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (s, 2H), 3.85-3.55 (m, 16H), 3.39 (s, 3H) ppm. ^{13}C-NMR (150 MHz, $CDCl_3$): δ = 166.30, 140.75 (dd, *J* = 243, 12.0 Hz), 139.28 (dt, *J* = 252, 13.5 Hz), 137.41 (dt, *J* = 256, 15.0 Hz), 124.15 (t, *J* = 14.0 Hz), 71.46, 70.76, 70.12, 70.11, 70.01, 67.33, 51.18 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.05 (ESI-MS (*m/z*): 433.1 (M + H^+)). HRMS: calcd. for $C_{17}H_{21}F_5O_7$ $[M + H]^+$: 433.12802; found: 433.12818.

Perfluorophenyl [16-^{13}C]2,5,8,11,14-pentaoxahexadecan-16-oate (7b)

Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 226 mg, 0.52 mmol, 54% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (d, *J* = 4.4 Hz, 2H), 3.84-3.52 (m, 16H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.67 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.32 (ESI-MS (*m/z*): 434.0 (M + H^+)). HRMS: calcd. for $C_{16}{}^{13}CH_{21}F_5O_7$ $[M + H]^+$: 434.13138; found: 434.13145.

Perfluorophenyl [15,16-$^{13}C_2$]2,5,8,11,14-pentaoxahexadecan-16-oate (7c)

Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 223 mg, 0.51 mmol, 52% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (dd, *J* = 145, 4.7 Hz, 2H), 3.86-3.52 (m, 16H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.64 (d, *J* = 64 Hz), 67.87 (d, *J* = 64 Hz) ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.33 (ESI-MS (*m/z*): 435.0 (M + H^+)). HRMS: calcd for $C_{15}{}^{13}C_2H_{21}F_5O_7$ $[M + H]^+$: 435.13473; found: 435.13482.

Perfluorophenyl [13,15,16-$^{13}C_3$]2,5,8,11,14-pentaoxahexadecan-16-oate (7d)

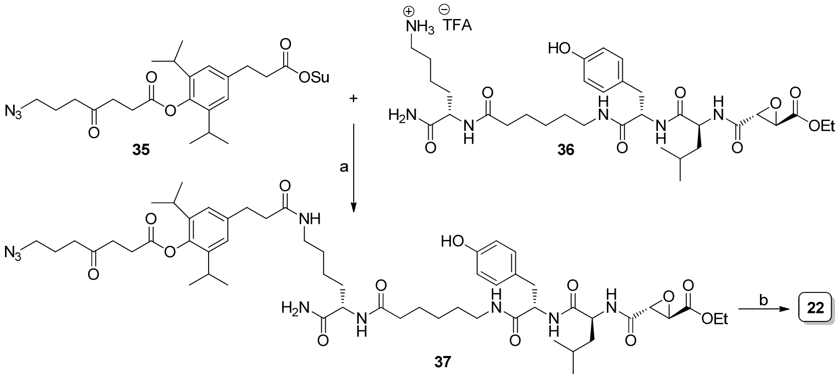
Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 67 mg, 0.15 mmol, 21% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (dt, *J* = 145, 4.8 Hz, 2H), 3.76-3.43 (m, 16H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.64 (d, *J* = 64 Hz), 71.27, 67.84 (d, *J* = 64 Hz) ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.05 (ESI-MS (*m/z*): 436.0 (M + H^+)). HRMS: calcd. for $C_{14}{}^{13}C_3H_{21}F_5O_7$ $[M + H]^+$: 436.13808; found: 436.13816.

Perfluorophenyl [12,13,15,16-$^{13}C_4$]2,5,8,11,14-pentaoxahexadecan-16-oate (7e)
Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 147 mg, 0.34 mmol, 37% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (dt, *J* = 146, 4.7 Hz, 2H), 3.79 (dm, *J* = 145 Hz, 2H), 3.67-3.50 (m, 14H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.67 (d, *J* = 63 Hz), 71.06, 70.84, 67.84 (d, *J* = 64 Hz) ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.03 (ESI-MS (*m/z*): 437.1 (M + H^+)). HRMS: calcd. for $C_{13}{}^{13}C_4H_{21}F_5O_7$ $[M + H]^+$: 437.14144; found: 437.14157.

Perfluorophenyl [9,10,12,13,16-$^{13}C_5$]2,5,8,11,14-pentaoxahexadecan-16-oate (7f)
Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 230 mg, 0.53 mmol, 53% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (t, *J* = 4.7 Hz, 2H), 4.19-3.93 (m, 4H), 3.66-3.32 (m, 12H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.64, 71.06, 70.87, 70.60 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.05 (ESI-MS (*m/z*): 438.1 (M + H^+)). HRMS: calcd. for $C_{12}{}^{13}C_5H_{21}F_5O_7$ $[M + H]^+$: 438.14479; found: 438.14491.

Perfluorophenyl [9,10,12,13,15,16-$^{13}C_6$]2,5,8,11,14-pentaoxahexadecan-16-oate (7g)
Prepared according to general procedure C. The product was obtained as a colourless oil (yield: 277 mg, 0.63 mmol, 63% over 2 steps). ^{1}H-NMR (200 MHz, $CDCl_3$): δ = 4.55 (dt, *J* = 146, 4.7 Hz, 2H), 4.16-4.01 (m, 4H), 3.66-3.32 (m, 12H), 3.38 (s, 3H) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 166.67 (d, *J* = 64 Hz), 71.06, 70.87, 70.60, 67.87 (d, *J* = 64 Hz) ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.05 (ESI-MS (*m/z*): 439.1 (M + H^+)). HRMS: calcd. for $C_{11}{}^{13}C_6H_{21}F_5O_7$ $[M + H]^+$: 439.14815; found: 439.14827.

Scheme 3. Synthesis of cleavable linker containing cathepsin probe **22**.



Reagents and conditions: (a) DiPEA, DMF, 73%; (b) Biotin-Ahx-propargylamide, $CuSO_4$, sodium ascorbate, DMF, 34%.

(2R,3R)-Ethyl 3-((9S,19S,22S)-1-(4-(7-azido-4-oxoheptanoyloxy)-3,5-diisopropylphenyl) -9-carbamoyl-19-(4-hydroxybenzyl)-24-methyl-3,11,18,21-tetraoxo-4,10,17,20-tetraaza-pentacosan-22-ylcarbamoyl)oxirane-2-carboxylate (37)

DCG-04 amine **36**[18] (0.12 g, 0.15 mmol) was dissolved in DMF (3 mL). To this were added NHS activated ester **35**[19] (1.1 eq., 89.0 mg, 0.165 mmol) and DiPEA (1.5 eq., 0.225 mmol, 37 μL) and the mixture was stirred for 15 h. LC-MS analysis showed complete consumption of starting material. The mixture was concentrated *in vacuo*. The remaining crude oil was crystallized in MeOH/Et_2O, filtered off and washed with Et_2O to give the title compound as a colourless solid (yield: 118 mg, 0.11 mmol, 73%). ^{1}H NMR (400 MHz, MeOD): δ = 7.02 (d, J = 8.4 Hz, 2H), 7.00 (s, 2H), 6.69 (d, J = 8.4 Hz, 2H), 4.5-4.4 (m, 2H), 4.3-4.2 (m, 3H), 3.66 (d, J = 1.6 Hz, 1H), 3.58 (d, J = 1.6 Hz, 1H), 3.14 (t, J = 6.8 Hz, 2H), 3.1-2.8 (m, 12H), 2.61 (t, J = 6.8 Hz, 2H), 2.44 (t, J = 7.6 Hz, 2H), 2.23 (t, J = 7.4 Hz, 2H), 1.9-1.8 (m, 2H), 1.6-1.1 (m, 15H), 1.36 (t, J = 3.8 Hz, 3H), 1.55 (d, J = 6.8 Hz, 12H), 0.91 (dd, J = 16.0, 6.0 Hz, 6H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 175.3, 173.7, 157.3, 141.7, 140.3, 131.4, 128.9, 124.9, 116.2, 63.2, 56.5, 54.4, 54.3, 53.4, 53.2, 51.8, 41.6, 40.2, 40.0, 39.2, 38.2, 37.8, 36.6, 32.9, 30.0, 29.8, 28.7, 28.5, 27.4, 26.5, 25.9, 24.3, 24.1, 23.3, 22.0, 14.4 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 8.66 (ESI-MS (*m/z*): 1076.33 ($M + H^+$)).

(2S,3R)-Ethyl 3-((9S,19S,22S)-9-carbamoyl-1-(3,5-diisopropyl-4-(4-oxo-7-(4-((6-(5-((3aS,4S,6aR)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)pentanamido)-hexanamido)methyl)-1H-1,2,3-triazol-1-yl)heptanoyloxy)phenyl)-19-(4-hydroxybenzyl)-24-methyl-3,11,18,21-tetraoxo-4,10,17,20-tetraazapentacosan-22-ylcarbamoyl)oxirane-2-carboxylate (22)

Compound **37** (118 mg, 0.11 mmol) was dissolved in DMF (2 mL). To this were added Biotin-Ahx-propargylamide[19] (1.4 eq., 0.154 mmol, 61.0 mg), 1 M $CuSO_4$ in H_2O (22 μL) and 1 M sodium ascorbate in H_2O (33 μL) and the mixture was stirred for 15 h, after which LC-MS analysis showed complete consumption of starting material. The mixture was concentrated *in vacuo* and purified by RP-HPCL (10% → 90% ACN/0.1% aqueous TFA), which afforded the title compound as a colourless solid (yield: 55.0 mg, 38.5 μmol, 35%). ^{1}H NMR (400 MHz, MeOD): δ = 6.87 (d, J = 8.4 Hz, 2H), 6.85 (s, 2H), 6.55 (d, J = 8.4 Hz, 2H), 4.4-4.2 (m, 6H), 4.2-4.0 (m, 2H), 3.52 (d, J = 1.6 Hz, 1H), 3.43 (d, J = 2.0 Hz, 1H), 3.2-2.9 (m, 7H), 2.8-2.6 (m, 15H), 2.55 (d, J = 12.8 Hz, 1H), 2.41 (t, J = 6.8 Hz, 2H), 2.30 (t, J = 7.6 Hz, 2H), 1.7-1.1 (m, 28H), 1.16 (t, J = 7.0 Hz, 3H), 1.00 (d, J = 6.8 Hz, 12H), 0.76 (dd, J = 16.0, 6.0 Hz, 6H) ppm. ^{13}C NMR (100 MHz, MeOD): δ = 209.4, 177.2, 176.2, 176.1, 176.0, 175.2, 173.8, 173.7, 173.1, 168.8, 168.4, 166.1, 160.1, 159.7, 157.3, 145.2, 141.7, 140.3, 131.4, 128.9, 124.9, 124.5, 116.3, 63.4, 63.2, 61.7, 57.0, 56.5, 54.4, 53.4, 53.2, 50.6, 41.7, 41.0, 40.2, 39.5, 39.1, 38.2, 37.8, 36.8, 36.7, 35.5, 32.9, 32.8, 30.1, 30.0, 29.8, 29.8, 29.5, 28.7, 28.5, 27.5, 27.4, 26.9 ppm. LC-MS (system B): gradient 10% → 90% ACN/(0.1% TFA/H_2O): R_t (min): 7.08 (ESI-MS (*m/z*): 1470.47 ($M + H^+$)).

References

(1) M. Whittaker, C. D. Floyd, P. Brown, A. J. H. Gearing, *Chem. Rev.* **1999**, *99*, 2735-2776.

(2) M. Uttamchandani, J. Wang, J. Q. Li, M. Y. Hu, H. Y. Sun, K. Y. T. Chen, K. Liu, S. Q. Yao, *J. Am. Chem. Soc.* **2007**, *129*, 7848-7858.

(3) P. Jones, S. Altamura, P. K. Chakravarty, O. Cecchetti, R. De Francesco, P. Gallinari, R. Ingenito, P. T. Meinke, A. Petrocchi, M. Rowley, R. Scarpelli, S. Serafini, C. Steinkuhler, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5948-5952.

(4) M. Kijima, M. Yoshida, K. Sugita, S. Horinouchi, T. Beppu, *J. Biol. Chem.* **1993**, *268*, 22429-22435.

(5) S. Brown, M. M. Bernardo, Z. H. Li, L. P. Kotra, Y. Tanaka, R. Fridman, S. Mobashery, *J. Am. Chem. Soc.* **2000**, *122*, 6799-6800.

(6) D. E. Levy, F. Lapierre, W. S. Liang, W. Q. Ye, C. W. Lange, X. Y. Li, D. Grobelny, M. Casabonne, D. Tyrrell, K. Holme, A. Nadzan, R. E. Galardy, *J. Med. Chem.* **1998**, *41*, 199-223.

(7) A. David, D. Steer, S. Bregant, L. Devel, A. Makaritis, F. Beau, A. Yiotakis, V. Dive, *Angew. Chem. Int. Ed.* **2007**, *46*, 3275-3277.

(8) A. Saghatelian, N. Jessani, A. Joseph, M. Humphrey, B. F. Cravatt, *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 10000-10005.

(9) N. Abello, P. P. Geurink, M. van der Toorn, A. J. M. van Oosterhout, J. Lugtenburg, G. A. van der Marel, H. A. M. Kerstjens, D. S. Postma, H. S. Overkleeft, R. Bischoff, *Anal. Chem.* **2008**, *80*, 9171-9180.

(10) X. Xing, A. Fichera, K. Kumar, *Org. Lett.* **2001**, *3*, 1285-1286.

(11) J. T. Anderson, P. L. Toogood, E. N. G. Marsh, *Org. Lett.* **2002**, *4*, 4281-4283.

(12) T. Nazif, M. Bogyo, *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 2967-2972.

(13) D. Greenbaum, K. F. Medzihradszky, A. Burlingame, M. Bogyo, *Chem. Biol.* **2000**, *7*, 569-581.

(14) P. H. Seeberger, W. C. Haase, *Chem. Rev.* **2000**, *100*, 4349-4394.

(15) O. J. Plante, E. R. Palmacci, P. H. Seeberger, *Science* **2001**, *291*, 1523-1527.

(16) J. A. Gautier, M. Miocque, Moskowit.H, *Bull. Soc. Chim. Fr.* **1965**, 1735-1740.

(17) N. Ouwerkerk, J. H. van Boom, J. Lugtenburg, J. Raap, *Eur. J. Org. Chem.* **2000**, 861-866.

(18) D. Greenbaum, A. Baruch, L. Hayrapetian, Z. Darula, A. Burlingame, K. F. Medzihradszky, M. Bogyo, *Mol. Cell. Proteomics* **2002**, *1*, 60-68.

(19) See Chapter 7.