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Sugar amino acid based peptide epoxyketones as potential proteasome inhibitors

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ABSTRACT

This paper describes the synthesis and biological evaluation of nine epoxomicin-derived sugar amino acid containing peptide epoxyketones. The title compounds are assembled from six sugar amino acid dipeptide isosteres and are synthesized using solution-phase peptide synthesis protocols. Although neither of the compounds displays inhibitory activity towards any of the proteasome active sites, our approach holds promise towards the development of structurally new proteasome inhibitors. It is likely that the central sugar amino acid dipeptide isoster needs to be designed such that it closely resemble dipeptides at position P2 and P3 in proteasome substrates inhibitors, such as the Thr-Ile dipeptide present in the lead compound, epoxomicin.

1. Introduction

The 26S proteasome is involved in the majority of cytosolic and nuclear proteolysis events in mammalian cells [1]. Proteasomal proteolysis takes place in an ATP- and ubiquitin-dependent fashion [2]. The proteasome products themselves, small oligopeptides ranging from three to about twenty amino acid residues, are further processed by a range of aminopeptidases to ultimately deliver amino acids that can be reused in ribosomal protein synthesis. A small portion of the oligopeptides produced by the proteasome is recruited by the immune system and ends up on major histocompatibility complex class I molecules on the cell surface for immune surveillance [3]. Next to the constitutively expressed 26 proteasome two immune tissue specific proteasome particles have been identified to date, namely the immunoproteasome [4] and the thymoproteasome [5]. The biochemistry, biology and immunology of the proteasome, as part of the ubiquitin–proteasome pathway [6] has been extensively reviewed [1–7] in recent years. Of relevance for the here described research is the nature and differences in activity of the catalytic entities that reside in the core proteasome particles [8]. The 26S proteasome is assembled from two distinct multiprotein subunit species. Of these, the 19S cap contains amongst others the ubiquitin-recognising domains and ATPase activities that together control proteasomal processing. Either one or two 19S caps are associated with the core 20S proteasome particle. The 20S proteasome has a C2-symmetrical barrel-like overall shape and consists of 28 protein subunits stacked in four rings of seven subunits each. The two outer rings consist of seven homologous but sequentially distinct α-subunits that provide structural integrity to the 20S complex and that are the interaction sites for specific proteins in the 19S caps. The 20S inner rings contain seven distinct β-subunits and in eukaryotes three of these possess proteolytic activity. With the aid of fluorogenic substrates, oligopeptides of a specific sequential nature and equipped with a fluorogenic leaving group situated at the C-terminus, the specificity of these is determined as chymotryptic for the β5-subunit (cleaving preferentially C-terminal of hydrophobic amino acid residues), tryptic for the β2-subunit (cleaving preferentially C-terminal of basic amino acid residues) and caspase-like for the β1-subunit (cleaving preferentially C-terminal of acidic amino acid residues) [9]. The substrate specificity however is not as narrow as the designations indicate, and in fact determination of the actual substrate specificity of the proteasome catalytic activities and their role in human health and disease is subject of extensive research [8–10]. The same holds true for the catalytic subunits unique to the immunoproteasome (β5i, β5i and β5i) [11] and the thymoproteasome (β5t) [12]. The clinical relevance of these research activities is underscored by the recent development of the peptide boronic acid Bortezomib as a drug for the treatment of multiple myeloma [13] Bortezomib inhibits both β1 and β5 while leaving β2 largely intact and it is thought that this specificity is at the basis of the mode of action of this clinically approved drug [14].

The development of proteasome inhibitors is a widely pursued research objective both for fundamental and practical (clinical) reasons [15]. With a few notable exceptions [16] most effective proteasome inhibitors are composed of a short oligopeptidic sequence equipped C-terminally with an electrophilic trap [8–10]. A prominent example is the natural product and proteasome inhibitor, epoxomicin [17] (Fig. 1, left structure) in which the C-terminal epoxyketone features as the electrophilic trap. The N-terminal threonine that resides in the proteasome catalytic sites reacts with the epoxyketone moiety to form a morpholine ring and in this

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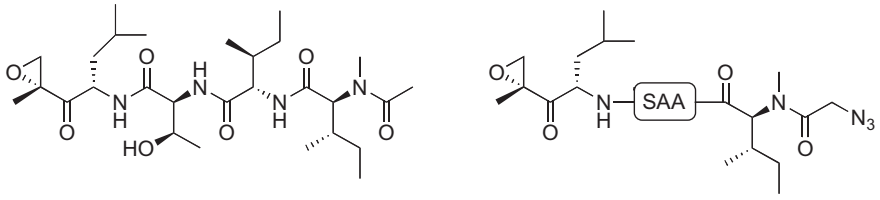


Fig. 1. Epoxomicin (left) and general design of the tagged sugar amino acid containing analogues (right).

fashion the active sites are covalently and irreversibly modified [18]. Epoxomicin is a broad-spectrum proteasome inhibitor and targets all catalytic subunits, with some preference for β2 and β5. Next to potency, subunit specificity is considered a major research theme in the research community elaborating proteasome inhibitors for both fundamental reasons (which subunit is responsible for the generation of a given peptide, for instance in the context of antigen presentation) and applied reasons (consider the subunit specificity of the therapeutic agent Bortezomib, is this the optimal pharmacological profile?). For these reasons considerable efforts are aimed at the generation and evaluation of synthetic peptide based proteasome inhibitors. These efforts can be roughly divided in two distinct research themes, with the first characterised as research aimed at variation of the C-terminal electrophilic trap and the second as research aimed at variation of the oligopeptidic proteasome recognition segment. With respect to the first strategy, next to peptide epoxyketones [17,19] and peptide boronic acids [13,20] peptide aldehydes [21], peptide vinyl sulphones [22] and other peptide-based Michael acceptors [22] have been studied. With respect to the latter, both peptide fragments composed of proteinogenic [23] and non-proteinogenic [13] α-amino acids have been generated, also in a combinatorial approach [24] Pharmacophores composed of molecular entities different from the α-amino acid configuration are remarkably scarce in the proteasome inhibitor literature [25]. Given the fact that sugar amino acids, subject of this work, have met with some success in research on the design of conformationally constrained and physiologically stable inhibitors of other enzymes (RNAse [26], protein:farnesyl transferase [27]) we set out to investigate whether the assembly of sugar amino acid based peptide epoxyketones would lead to effective and possibly subunit-selective proteasome inhibitors.

The general structure of the sugar amino acid containing peptide epoxyketones envisaged is depicted in Fig. 1 (right structure). The C-terminal leucine epoxyketone moiety present in epoxomicin is recognised by the three proteolytic activities of the 26S proteasome and it is known [28] that some subunit specificity can be achieved by variation of the threonine–isoleucine–isoleucine sequence at the positions P2–P4. We decided to keep the N-terminal, N-methylated isoleucine residue intact and replace the threonine–isoleucine dipeptide sequence with the panel of sugar amino acids **a**–**f** depicted in Fig. 2. Of the panel of sugar amino acids used derivatives **a**–**e** were subject of studies described elsewhere by us [29] and others [30]. The N-terminal acetyl cap as present in epoxomicin finally is replaced by azidoacetyl, enabling activity-based profiling [31] as an alternative strategy compared to competition assays to establish potential proteasome reactivity. We here report on the synthesis and evaluation as potential proteasome inhibitors of a panel of eight peptide epoxyketones encompassing sugar amino acids **a**–**f**.

2. Results

The synthesis of the panel of peptide epoxyketones commences with N-methylation of Fmoc-Ile–OH **1** according to the literature procedure [32] to give Boc-(*N*-Me)-isoleucine **2** (Scheme 1). TFA-mediated removal of the Boc protective group was followed by N-acylation with bromoacetyl bromide and subsequent bromide displacement by azide to give the protected, azide tagged N-terminal residue **3**. Treatment with lithium hydroxide in a water/dioxane mixture provided the free carboxylic acid **4** in 72% overall yield.

With the suitably protected sugar amino acids and N-terminal N-methylated isoleucine in hand the synthesis of the peptide epoxyketones was undertaken. The strategy followed is depicted in Scheme 2. Leucine epoxyketone **5**, prepared according to the literature procedure [19a], was condensed with sugar amino acid **a**–**f** under the agency of HCTU and DIPEA. Staudinger reduction of the azide in the resulting dipeptides **6** was followed by condensation with isoleucine derivative **4**, and for this transformation the reagent combination consisting of EDC, HOAt and lutidine in a mixture of DMF and dichloromethane proved most efficient. These conditions have been succesfully applied previously in peptide couplings of *N*-methyl-*N*-acyl amino acids with minimal racemization. Epimerization of the α-carbon of the *N*-terminal isoleucine residue could however not be avoided. This provided stereomeric mixtures of peptide epoxyketones **7a**–**f**. Sugar amino acids **b**, **c** and **d** gave rise to separable pairs of diastereomers (**7bI**/**7bII**, **7cI**/**7cII** and **7dI**/**7dII**), although we were not able to identify the nature of the chiral carbon (marked ∗ in the scheme) of the individual diastereomers. Sugar amino acids **a**, **e** and **f** produced intractable mixtures of diastereoisomers, at least after HPLC purification.

Epimerisation such as described above is a known hindrance in peptide chemistry, especially during block couplings of (oligo)peptidic fragments such as executed here. An attractive strategy to avoid or suppress epimerisation of the carboxylate partner in peptide block couplings is to make use of acyl azides as the activated species [32]. This strategy was investigated here as well, and to this end, the required Boc-protected isoleucine hydrazone **10** was readily prepared starting from N-methyl-Fmoc-isoleucine **8** (Scheme 3). Ensuing *in situ* formation of the acyl azide by treatment with HCl and *tert*-butyl nitrite followed by addition of amines **7a**–**f**, obtained after Staudinger reduction of **6a**–**f** (see Scheme 2) and DIPEA proved however abortive, and no tripeptides were isolated from the resulting reaction mixtures.

In the next experiment, peptide epoxyketones **7a**–**f** were screened on their proteasome inhibitor potential in a competition experiment using the BODIPY–peptide–vinyl sulphone, MV151 (Fig. 3), as a read-out [22a–c]. In this experiment, lysates of the human hybridoma kidney cell line, HEK 293T, were treated with final concentrations of 0, 1, 10 and 100 μM and incubated for one hour prior to treatment with MV151. The proteins were denatured, resolved by SDS PAGE and monitored by a Typhoon fluorescence scanner to identify fluorescent protein bands. As can be seen (Fig. 4 lane 0) tissue cell lysate treated with MV151 without preincubation with one of the putative inhibitors reveal three fluorescent bands corresponding to the proteasome catalytic actives (from top down) β2, β1, and β5. Essentially the same picture emerges irrespective of the nature and end concentrations of the peptide epoxyketones added to the tissue lysate, indicating that neither of the produced compounds holds any inhibitory potential towards the proteasome. In some cases, there seems to be a slight reduction of fluorescence at 100 μM final concentrations.

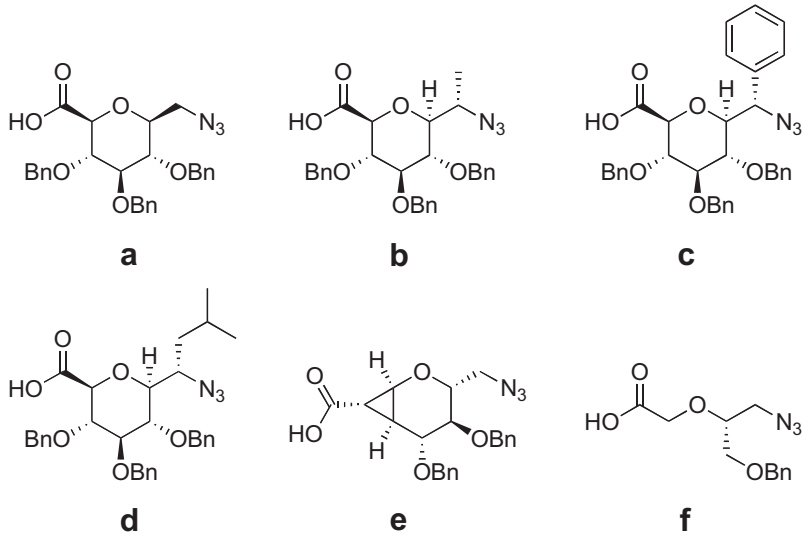
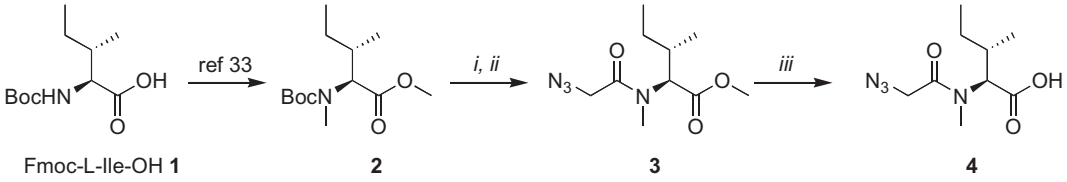
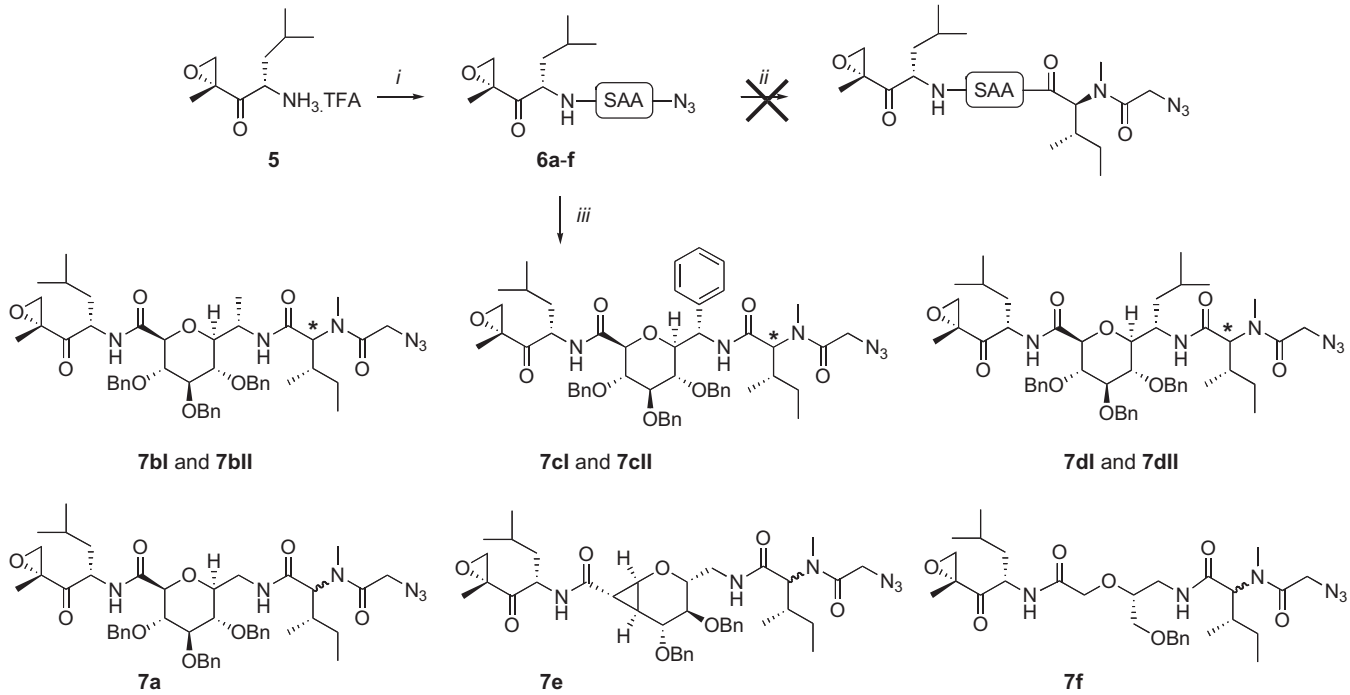


Fig. 2. Structure of the sugar amino acid scaffolds used in the here described study.

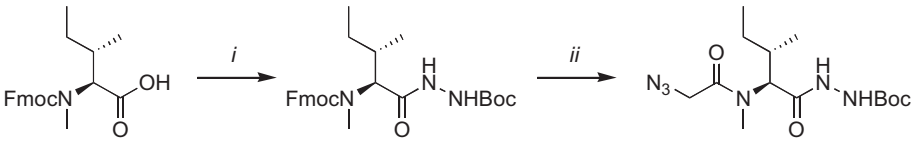


Scheme 1. Reagents and conditions: (i) TFA, CH_2Cl_2. (ii) First bromoacetyl bromide, DBU, DMF, then NaN_3 (76% over the two steps). (iii) LiOH, H_2O/dioxane 1:1 (95%).



Scheme 2. Reagents and conditions: (i) sugar amino acid **a**–**f**, HCTU, DIPEA, CH_2Cl_2 (79–93%). (ii) a. Me_3P, H_2O/THF, b. **10**, TFA, rt, c. tBuONO, HCl, −30 °C, then DiPEA, DMF, EtOAC, rt. (iii) First Me_3P, H_2O/THF, then **4**, EDC, HOAt, lutidine, DMF, CH_2Cl_2 followed by HPLC purification (11–55% two steps).

However, incubation of tissue cell lysate with a 1 mM final concentration of these specific examples led to no significant increase in labelling inhibition (data not shown). In contrast with the SAA-based compound library, epoxomicin shows a very potent proteasome inhibition, competing out MV151 at concentrations as low as 1 μM.



8 9 10

Scheme 3. Reagents and conditions: (i) $BocNHNH_2$, HCTU, DIPEA, CH_2Cl_2 (85%). (ii) First DBU, DMF, then bromoacetyl bromide, then sodium azide (72%).

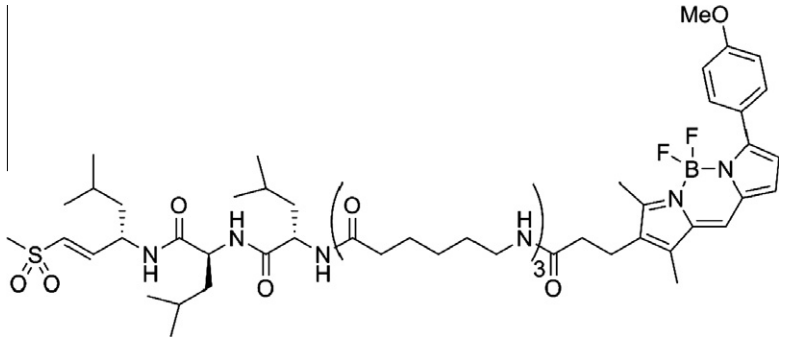


Fig. 3. Structure of the fluorescent proteasome inhibitor MV151.

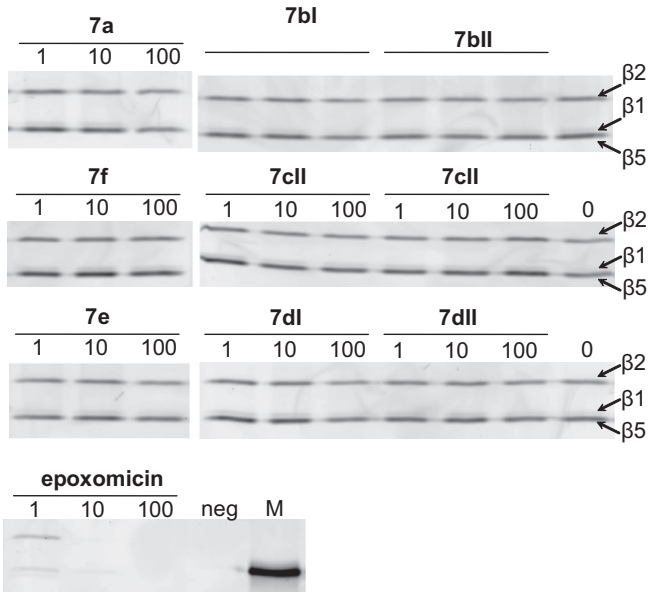


Fig. 4. Compounds **7a**–**f** do not inhibit the active proteasome subunits.

3. Conclusion

This paper details the design and synthesis of nine sugar amino acid containing peptide epoxyketones. Evaluation of these in a proteasome inhibitor competition assay revealed that none of these compounds possess affinity of one of the proteasome catalytic actives. Thus, replacement of the central isoleucine–threonine dipeptide as present in the parent compound, epoxomicin, with either of the six sugar amino acids applied appears detrimental to the inhibitory activity. Lack of activity may be caused by the secondary structure inherent to the sugar amino acids used and that imposes secondary structural features that are at odds with proteasomal recognition. Alternatively, either the substitution pattern or the presence of benzyl ethers, or a combination thereof, prohibits proteasomal inhibition. Future research on compounds free of protective groups, on compounds featuring sugar amino acids of a different structural and stereochemical nature, and on compounds featuring sugar amino acids structurally closely related to the Thr-Ile dipeptide are needed to form a definite conclusion whether sugar amino acids are useful building blocks in the construction of effective, selective and possibly physiologically inert proteasome inhibitors.

4. Experimental

4.1. Synthesis

All reactions described were performed under an argon atmosphere and at ambient temperature unless stated otherwise. All solvents, except water, were purchased from Biosolve and used as received. Amino acids were purchased from Novabiochem. All other reagents were purchased from Sigma–Aldrich or Acros and were used as received. Reactions were monitored by TLC analysis using TLC aluminium sheets (Merck, Silica gel 60, F_{254}) with detection by spraying with a solution of $(NH4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in H_2SO_4 (10%) followed by charring. Column chromatography was performed on 60 Å silica gel (40–63 μm). High resolution spectra were recorded with a Finnigan LTQ Orbitrap Mass spectrometer. ^{1}H- and ^{13}C-APT-NMR spectra were recorded with a Bruker AV-400 (400/100 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard (^{1}H NMR) or $CDCl_3$ (^{13}C NMR). Coupling constants are given in Hz. Optical rotations were measured with a Propol automatic polarimeter (λ = 589 nm) and IR (ATR-IR) spectra were recorded with a Perkin Elmer Paragon 1000 FT-IR Spectrometer. Preparative HPLC purifications were carried out using a Gilson preparative HPLC system equipped with a Phenomenex Gemini C18 column (150 × 21.20 mm, 5 μm) using a water/acetonitrile/TFA gradient system.

4.1.1. Compound 3

Boc-N,O-dimethyl-isoleucine [32] (2.6 g, 10 mmol) was taken up in TFA (50 mL) allowed to stand for 10 min and concentrated *in vacuo*. The residue was coevaporated twice with toluene (50 mL) and taken up in DMF (100 mL). Bromoacetylbromide

(1.8 mL, 20 mmol) and DBU (3.0 mL, 20 mmol) were added and stirring is continued for 4 h during which the reaction mixture became deep red in colour. Sodium azide (6.5 g, 100 mmol) was added and stirring is continued over night. The reaction mixture is diluted with ethyl acetate (500 mL) and washed with HCl (1 M, 200 mL, twice) and NaCl (satd. aq., 200 mL, twice). The organic fraction was dried (Na_2SO_4) and taken to dryness. Silica gel chromatography (0 → 25% EtOAc in toluene) yielded the product (1.90 g, 7.29 mmol, 73%) as a pale yellow oil. (major rotamer) ^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.00 (d, J = 10.8 Hz, 1H), 4.09–3.91 (m, 2H), 3.72 (s, 3H), 2.96 (s, 3H), 2.07–1.97 (m, 1H), 1.40–1.34 (m, 1H), 1.13–1.05 (m, 1H), 1.00–0.88 (m, 6H),13C NMR (100 MHz, $CDCl_3$) δ = 171.25, 168.05, 60.20, 51.77, 50.55, 33.36, 30.42, 24.99, 15.47, 10.52; IR neat (cm^{-1}): 2968.9, 2361.6, 2105.4, 1735.9, 1660.1, 1452.0, 1265.6, 1138.6, 996.8, 740.6; $[\alpha]_D^{20}$ −99.1 (c 2.0, $CHCl_3$); ESI-MS: [M+H]: 243.3.

4.1.2. General procedure I

Condensing the epoxyketone warhead to the azido acids.

Boc–Leucine–epoxyketone (68 mg, 0.25 mmol) is taken up in CH_2Cl_2 (2 mL) and trifluoroacetic acid (2 mL) is added. The mixture is allowed to stand for 30 min after which it is concentrated *in vacuo*. The residue is coevaporated twice with toluene (3 mL) and is taken up in DMF (4 mL). To the solution the appropriate amino acid is added (0.20 mmol) along with HCTU (0.25 mmol, 104 mg) and DIPEA (1 mmol, 174 μl). The reaction mixture was stirred overnight and diluted with 50 mL ethyl acetate. This solution was extracted with citric acid (10% aq., 30 mL, twice) and $NaHCO_3$ (satd. aq., 30 mL, twice). The organic fraction was dried on Na_2SO_4, and concentrated. The residue was purified on column chromatography (0 → 50% EtOAc in toluene) yielding the product as a viscous oil (79–93%).

4.1.3. Compound 6a

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.33–7.22 (m, 15 H), 6.65 (d, J = 8.4 Hz, 1H), 4.90–5.55 (m, 7H), 3.90 (d, J = 8.4 Hz, 1H), 3.73–3.67 (m, 2H), 3.65–3.58 (m, 3H), 3.34 (d, J = 4.8 Hz, 1H), 3.30 (dd, J = 13.6 Hz, J = 3.2 Hz, 1H), 2.87 (d, J = 4.8 Hz. 1H), 1.64 (m, 1H), 1.55–1.47 (m, 4H), 1.26 (m, 1H), 0.95–0.85 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.22, 168.52, 138.03, 137.63, 137.51, 128.47, 128.42, 128.32, 128.18, 137.99, 127.92, 127.80, 127.73, 127.69, 85.14, 80.32, 77.93, 77.56, 77.49, 75.27, 74.83, 74.79, 59.07, 52.37, 50.80, 49.66, 39.81, 25.12, 23.23, 21.11, 16.66; IR neat (cm^{-1}): 2871.6, 2363.9, 2100.8, 17.18.2, 1667.9, 1454.6, 1095.4, 734.9, 697.6, 460.5; $[\alpha]_D^{20}$ −12.8 (c 2.0, $CHCl_3$); HRMS: calcd for $C_{37}H_{45}O_7N_4$ ([M+H]) 657.32828 found 657.32864.

4.1.4. Compound 6b

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.31–7.21 (m, 15H), 6.62 (d, J = 8.4 Hz, 1H), 4.89–4.55 (m, 7H), 3.92 (m, 1H), 3.77–3.71 (m, 2H), 3.69–3.59 (m, 2H), 3.52–3.47 (m, 1H), 3.28 (d, J = 4.8 Hz, 1H), 2.86 (d, J = 4.8 Hz, 1H), 1.66–1.60 (m, 1H), 1.55–1.48 (m, 4H), 1.27–1.20 (m, 1H), 1.17 (d, J = 6.8 Hz, 3H), 1.00–0.85 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 207.74, 168.214, 137.67, 137.44, 137.13, 128.14, 128.10, 127.98, 127.73, 127.68, 127.65, 127.41, 127.33, 84.61, 79.83, 79.48, 77.71, 76.67, 74.69, 74.16, 73.66, 58.63, 56.58, 51.98, 49.39, 39.88, 24.80, 22.92, 20.95, 16.33, 12.68; IR neat (cm^{-1}): 3397.1, 2960.6, 2360.2, 2103.1, 1718.2, 1689.2, 1519.8, 1077.2, 733.3, 695.4; $[\alpha]_D^{20}$ +9.6 (c 1.0, $CHCl_3$); HRMS: calcd for $C_{38}H_{46}O_7N_4$ ([M+H]) 671.34393 found 671.34434.

4.1.5. Compound 6c

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.36–7.16 (m, 20 H), 6.58 (d, J = 8.8 Hz, 1H), 4.87–4.40 (m, 8H), 4.03 (d, J = 7.6 Hz, 1H), 3.93 (dd, J = 9.6 Hz, J = 7.6 Hz, 1H), 3.74 (t, J = 7.2 Hz, 1H), 3.60 (t, J = 7.6 Hz, 1H)3.32 (d, J = 5.0 Hz, 1H) 3.23, (dd, J = 9.6 Hz, J = 7.2 Hz, 1H), 2.89 (d, J = 5.0 Hz, 1H), 1.55 (s, 3H), 1.52–1.45 (m, 2H), 1.18–1.13 (m, 1H), 0.86 (d, J = 6.4 Hz, 3H), 0.83 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.25, 168.81, 137.73, 137.57, 134.45, 128.92, 128.60, 128.50, 128.41, 128.33, 128.12, 127.88, 127.78, 127.68, 127.62, 84.09, 79.65, 78.91, 77.72, 76.97, 74.50, 74.20, 73.20, 64.66, 58.94, 52.29, 49.26, 40.31, 24.96, 23.24, 21.14, 16.63; IR neat (cm^{-1}): 3065.6, 2100.7, 1718.9, 1638.8, 1451.2, 1092.5, 734.5, 698.0, 535.6; $[\alpha]_D^{20}$ +18 (c 1.0, MeOH); HRMS: calcd for $C_{43}H_{49}O_7N_4$ ([M+H]) 733.35958 found 733.36023.

4.1.6. Compound 6d

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.30–7.16 (m, 15 H), 6.63 (d, J = 8.4 Hz, 1H), 4.87–4.53 (m, 7H), 3.96 (d, J = 7.6 Hz, 1H), 3.80–3.75 (m, 2H), 3.73–3.64 (m, 3H), 3.32 (d, J = 5.0 Hz, 1H), 2.87 (d, J = 5.0 Hz, 1H), 1.80–1.62 (m, 3H), 1.55–1.48 (m, 3H), 1.29–1.20 (m, 2H), 0.96–0.85 (m, 12 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.07, 168.69, 138.018, 137.78, 137.52, 128.42, 128.30, 128.08, 127.85, 127.78, 127.73, 127.60, 84.21, 80.48, 79.60, 78.19, 77.34, 74.78, 74.31, 74.01, 61.31, 59.05, 52.35, 49.76, 39.99, 37.44, 25.14, 25.09, 23.29, 23.22, 21.28, 21.24, 16.69;; IR neat (cm^{-1}): 2960.2, 2103.4, 1718.1, 1638.5, 1512.0, 1092.6, 734.6, 695.4, 457.2; $[\alpha]_D^{20}$ +48 (c 1.0, $CHCl_3$); HRMS: calcd for $C_{41}H_{53}O_7N_4$ ([M+H]) 713.39088 found 713.39151.

4.1.7. Compound 6e

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.36–7.24 (m, 10H), 6.08 (d, J = 8.4 Hz, 1H), 4.75–4.50 (m, 5H), 3.80 (d, J = 5.2 Hz, 2H), 3.69–3.65 (m, 1H), 3.61–3.55 (m, 1H), 3.44 (t, J = 5.2 Hz, 1H), 3.29 (d, J = 5.0 Hz, 1H), 3.28–3.23 (m, 1H), 2.86 (d, J = 5.0 Hz, 1H), 1.92–1.88 (m, 1H), 1.83–1.80 (m, 1H), 1.81 (m, 1H), 1.57–1.50 (m, 4H), 1.30–1.24 (m, 1H), 0.99–0.95 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.65, 170.15, 137.59, 137.47, 128.42, 127.91, 127.87, 127.81, 127.65, 75.26, 74.26, 73.34, 72.92, 71.15, 58.94, 56.01, 52.30, 51.12, 50.13, 40.41, 25.49, 25.10, 23.30, 22.50, 21.37, 16.59; IR neat (cm^{-1}): 3293.9, 2959.4, 2096.0, 1718.0, 1635.8, 1533.7, 1072.0, 735.1, 696.6; $[\alpha]_D^{20}$ +79 (c 2.0, $CHCl_3$); HRMS: calcd for $C_{31}H_{39}O_6N_4$ ([M+H]) 563.28641 found 563.28614.

4.1.8. Compound 6f

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.45 (m, 1H), 7.37–7.29 (m, 5H), 4.68–4.62 (m, 1H), 4.61–4.52 (m, 2H), 4.14–4.02 (m, 2H), 3.72–3.69 (m, 1H), 3.54–3.47 (m, 3H), 3.43–3.37 (m, 1H), 3.32 (d, J = 5.0 Hz, 1H), 2.87 (d, J = 5.0 Hz, 1H), 1.68 (m, 1H), 1.54–1.46 (m, 4H), 1.26–1.20 (m, 1H), 0.96–0.88 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.01, 169.54, 137.07, 128.33, 127.78, 127.65, 78.46, 73.39, 69.26, 68.75, 58.87, 52.11, 51.81, 49.43, 39.47, 24.94, 23.15, 21.01, 16.50; IR neat (cm^{-1}): 2959.7, 2102.7, 1719.0, 1675.8, 1522.1, 1454.0, 1096.2, 532.2; $[\alpha]_D^{20}$ +60.4 (c 2.0, $CHCl_3$); HRMS: calcd for $C_{21}H_{31}O_7N_4$ ([M+H]) 419.22890 found 419.22877.

4.1.9. General procedure II

Condensing the N-methyl-leucine azidoacetamide to the AA epoxyketones.

Compound **3** (61 mg, 0.25 mmol) is taken up in 2 mL THF. To this solution are added methanol (0.5 mL) and LiOH (250 μL, 4.0 M) and the reaction is stirred at 0 °C for 2 h. The reaction mixture is acidified with HCl (1 M) to pH 2 and extracted with CH_2Cl_2 (2 × 2 mL). The organic layers are pooled dried (Na_2SO_4) and concentrated. The residue was used without further purification. The appropriate epoxyketone amide (150 μmol) was taken up in THF (2 mL) and water (0.1 mL). To this solution Me_3P in THF (3 mL, 1.0 M) was added and the solution was stirred for 1 h. The reaction mixture is concentrated *in vacuo* and coevaporated twice with

toluene (3 mL). The crude amine was taken up in CH_2Cl_2 (2.5 mL) and DMF (0.5 mL) and the freshly prepared *N*-methyl-leucine azidoacetamide (~250 μmol) was added. The solution was cooled to −30 °C and under stirring EDC (144 mg, 750 μmol), HOAt (35 mg, 250 μmol) and 2,6 lutidine (30 μl, 250 μmol) are added. Stirring is continued for 16 h after which the reaction mixture is diluted with ethyl acetate (20 mL). The ethyl acetate fraction was extracted with citric acid (10% aq., 10 mL, twice) and $NaHCO_3$ (satd. aq., 10 mL, twice). The organic fraction was dried on Na_2SO_4, and concentrated. The products were purified and where possible separated on reversed phase HPLC using a water/acetonitrile/TFA gradient system. The yields were 35–55% for epimeric mixtures and 11–27% for diastereomerically pure compounds.

4.1.10. Compound 7a

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.28–7.25 (m, 15 H), 7.07 (d, *J* = 8.0 Hz, 1H), 6.91 (m, 1H), 4.91–4.55 (m, 8H), 4.03–3.93 (m, 2H), 3.84 (d, *J* = 9.2 Hz, 1H), 3.73–3.60 (m, 3H), 3.55–3.49 (m, 1H), 3.45–3.40 (m, 1H), 3.35 (d, *J* = 5.0 Hz, 1H), 3.19 (t, *J* = 8.8 Hz, 1H), 2.97 (s, 3H), 2.89 (d, *J* = 5.0 Hz, 1H), 2.17–2.10 (m, 1H), 1.69–1.63 (m, 1H), 1.54–1.46 (m, 4H), 1.38–1.22 (m, 2H), 1.08–0.98 (m, 1H), 0.94–0.85 (m, 12 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.60, 169.79, 169.75, 168.76, 138.15, 137.63, 137.48, 128.58, 128.46, 128.36, 128.17, 127.97, 127.84, 127.75, 127.61, 85.69, 80.40, 77.98, 77.84, 76.95, 75.41, 75.04, 74.81, 61.94, 59.21, 52.53, 51.06, 50.33, 39.51, 39.36, 31.64, 30.53, 25.21, 24.68, 23.24, 21.10, 16.75, 15.32, 10.29; IR neat (cm^{-1}): 2963.7, 2361.7, 2107.9, 1718.1, 1651.9, 1270.5, 1096.2, 732.2; ESI-MS: [M+H]: 841.7.

4.1.11. Compound 7bI

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.35–7.25 (m, 15 H), 4.92–4.59 (m, 7 H), 4.50 (d, *J* = 11.2 Hz, 1H), 4.40 (m, 1H), 4.00–3.91 (m, 2H), 3.80 (d, *J* = 9.0 Hz, 1 H), 3.76–3.70 (m, 1H), 3.65–3.58 (m, 1H), 3.47–3.37 (m, 2H), 3.35 (d, *J* = 4.9 Hz, 1H), 3.00 (s, 3H), 2.90 (d, *J* = 4.9 Hz, 1H), 2.11 (m, 1H), 1.62 (m, 1H) 1.52 (m, 4H), 1.36–1.17 (m, 2H), 1.01–0.80 (16 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.86, 168.93, 168.70, 168.37, 138.05, 137.74, 137.53, 128.49, 128.45, 128.34, 128.28, 128.18, 128.05, 127.94, 127.78, 127.75, 127.71, 85.96, 80.38, 79.93, 77.79, 77.11,75.36, 74.81, 74.16, 59.17, 52.57, 50.90, 50.07, 44.37, 44.05, 39.86, 32.27, 30.83, 25.28, 24.73, 23.26, 21.33, 16.74, 15.24, 13.26, 10.40; IR neat (cm^{-1}): 2963.8, 2360.3, 2108.7, 1718.1, 1653.3, 1090.3, 699.9, 526.1; ESI-MS: [M+H]: 855.2.

4.1.12. Compound 7bII

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.33–7.25 (m, 15 H), 4.92–4.71 (m, 5H), 4.69–4.61 (m, 2H), 4.04–3.92 (m, 2H), 3.82–3.68 (m, 3H), 3.41–3.33 (m, 2H), 3.27 (dd, *J* = 9.9 Hz, *J* = 2.2 Hz, 1H), 2.94 (s, 3H), 2.87 (d, *J* = 5.0 Hz, 1H), 2.11 (m, 1H) 1.70 (m, 1H), 1.56–1.46 (m, 5H), 1.30 (m, 1H), 1.06 (m, 1H), 1.00–0.78 (m, 15 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.25, 168.81, 168.27, 168.20, 138.17, 138.09, 137.53, 128.53, 128.46, 128.29, 128.00, 127.95, 127.73, 127.70, 127.65, 86.36, 80.52, 79.67, 78.35, 77.07, 75.40, 74.76, 74.45, 62.21, 59.11, 51.08, 50.20, 44.11, 52.45, 51.08, 50.20, 44.11, 39.74, 31.91, 30.16, 25.87, 25.34, 23.32, 21.38, 16.77, 14.76, 13.46, 11.07; IR neat (cm^{-1}): 2970.2, 2361.9, 2108.8, 1664.0, 1454.5, 1049.9, 528.4; ESI-MS: [M+H]: 855.2.

4.1.13. Compound 7cI

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.38–7.23 (m, 20H), 5.38 (dd, *J* = 8.7 Hz, *J* = 2.6 Hz, 1H), 4.82–4.74 (m, 2H), 4.71–4.56 (m, 5H), 4.51–4.46 (m, 1H), 4.01 (d, 8.0 Hz, 1H), 3.96–3.82 (m, 3H), 3.74 (t, *J* = 7.5 Hz, 1H), 3.55 (t, *J* = 7.9 Hz, 1H), 3.37 (d, *J* = 4.9 Hz, 1H), 3.12 (dd, *J* = 9.7 Hz, *J* = 7.5 Hz, 1H), 2.93 (d, *J* = 4.9 Hz, 1H), 2.71 (s, 3H), 2.08 (m, 1H), 1.57 (s, 3H), 1.54–1.46 (m, 2H), 1.33–1.16 (m, 2H), 1.06–0.97 (m, 1H), 0.93–0.82 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.84, 174.96, 168.98, 168.45, 137.85, 137.65, 137.49, 137.29, 128.68, 128.47, 128.35, 128.32, 128.28, 128.05, 127.92, 127.75, 127.71, 127.62, 127.44, 84.52, 79.82, 79.01, 77.62, 77.10, 61.56, 59.06, 53.02, 52.49, 50.59, 49.66, 39.91, 31.60, 30.26, 24.99, 24.49, 23.19, 21.08, 16.76, 15.29, 10.27; IR neat (cm^{-1}): 2963.7, 2360.1, 2107.8, 1718.0, 1669.9, 1270.3, 1028.0, 530.6; ESI-MS: [M+H]: 917.20.

4.1.14. Compound 7cII

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.39–7.22 (m, 20 H), 5.34 (dd, *J* = 8.4, *J* = 2.8, 1H), 4.82–4.54 (m, 7H), 4.43–4.38 (m, 1H), 4.00–3.92 (m, 3H), 3.76–3.70 (m, 2H), 3.66 (t, *J* = 8.3 Hz, 1H), 3.39 (d, *J* = 5.0 Hz, 1H), 3.10 (dd, *J* = 9.8 Hz, *J* = 7.6 Hz, 1H), 2.96 (s, 3H), 2.91 (d, *J* = 5.0 Hz, 1H), 2.02 (m, 1H), 1.62 (m, 1H), 1.58–1.47 (m, 4H), 1.34–1.20 (m, H2), 0.94–0.75 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.49, 175.18, 168.84, 168.55, 137.94, 137.84, 137.82, 137.09, 128.86, 128.52, 128.41, 128.39, 128.31, 128.22, 128.02, 127.95, 128.87, 127.78, 127.73, 85.28, 79.67, 79.48, 78.06, 77.14, 74.76, 74.40, 73.52, 61.77, 59.09, 52.93, 52.44, 50.95, 49.84, 39.87, 32.24, 38.87, 32.24, 30.13, 25.50, 25.19, 23.33, 21.15, 16.73, 14.70, 10.81; IR neat (cm^{-1}): 2963.7, 2361.8, 2108.3, 1718.0, 1675.8, 1272.2, 1090.8, 531.6; ESI-MS: [M+H]: 917.20; ESI-MS: [M+H]: 917.13.

4.1.15. Compound 7dI

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.50–7.20 (m, 15 H), 4.90–4.46 (m, 9 H), 4.05–3.36 (m, 10H) 2.96–2.89 (m, 4H), 2.25 (m, 1H), 1.70–1.20 (m, 10 H), 1.01–0.80 (m, 19H), ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.88, 170.20, 169.12, 168.80, 168.70, 168.31, 138.15, 137.82, 137.64, 137.55, 137.51, 128.65, 128.62, 128.56, 128.49, 128.47, 128.45, 128.42, 128.36, 128.24, 128.14, 128.11, 128.08, 128.02, 127.93, 127.80, 127.75, 127.72, 127.69, 127.57, 85.92, 85.83, 82.62, 81.12, 80.63, 80.35, 80.21, 79.16, 78.49, 77.81, 77.46, 77.26, 76.72, 75.39, 75.32, 75.16, 75.02, 74.89, 74.77, 74.35, 69.23, 68.89, 59.28, 54.44, 52.63, 51.11, 51.02, 50.17, 47.14, 46.91, 46.62, 39.75, 36.65, 31.93, 31.79, 31.70, 25.47, 25.33, 24.98, 24.66, 23.70, 23.31, 21.98, 21.61, 21.44, 16.79, 15.38, 10.51; IR neat (cm^{-1}): 2959.0, 2360.1, 2108.4, 1652.0, 1094.3, 735.1, 699.0, 529.9; ESI-MS: [M+H]: 898.0.

4.1.16. Compound 7dII

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.45–7.19 (m, 15 H), 4.90–4.60 (m, 7 H), 4.55–4.30 (m, 2H), 4.00 (d, *J* = 3.7 Hz, 2H), 3.88–3.65 (m, 3H) 3.51–3.42 (m, 2H) 3.36 (d, 5.0 Hz, 1H), 3.24 (dd, *J* = 9.9 Hz, J = 1.8 Hz, 1H) 2.94 (s, 3H), 2.84 (d, *J* = 5.0 Hz, 1H), 2.11 (m 1H), 1.59–1.34 (m, 8H), 1.10 (m, 2H), 1.01–0.78 (m, 19 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.03, 169.00, 168.54, 168.16, 138.32, 138.28, 137.62, 128.61, 128.49, 128.42, 128.37, 128.29, 128.15, 128.12, 127.98, 127.93, 127.74, 127.70, 127.64, 127.60, 127.51, 86.54, 81.84, 79.39, 78.70, 77.51, 75.36, 74.86, 74.69, 62.83, 59.14, 52.44, 51.31, 50.30, 46.51, 39.61, 36.50, 31.38, 30.22, 25.75, 25.41, 24.85, 23.93, 23.32, 21.47, 21.30, 16.82, 14.81, 10.86; IR neat (cm^{-1}): 2958.7, 2361.4, 2108.1, 1718.1, 1669.2, 1455.0, 1090.7, 910.7, 732.2, 698.2; ESI-MS: [M+H]: 898.0.

4.1.17. Compound 7e

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.36–7.24 (m, 10 H), 4.75–4.47 (m, 6H), 4.02–3.88 (m, 2H), 3.82–3.78 (m, 1H), 3.76–3.70 (m, 1H), 3.63–3.57 (m, 1H), 3.51–3.44 (m, 2H), 3.36–3.31 (m, 2H), 2.98–2.90 (m, 3H), 2.88 (d, J = 4.9 Hz, 1H), 2.09 (m, 1H), 1.94–1.90 (m, 1H), 1.87–1.80 (m, 1H), 1.74 (m, 1H), 1.53–1.46 (m, 4H), 1.37–1.23 (m, 2H), 1.02–0.77 n(m, 13H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 209.08, 208.92, 170.56, 170.53, 169.86, 169.63, 168.75, 168.68, 138.63, 137.50, 137.45, 128.28, 127.90, 127.82, 127.73, 127.69, 127.62, 127.60, 127.47, 76.14, 76.10, 74.76, 74.38, 72.88,

72.74, 72.63, 72.06, 70.99, 70.95, 61.61, 61.16, 58.93, 56.13, 55.85, 52.30, 50.51, 50.42, 50.29, 42.40, 39.87, 39.70, 31.77, 31.55, 30.04, 29.86, 25.68, 24.97, 24.89, 24.70, 24.49, 23.19, 21.95, 21.14, 16.54, 15.23, 14.38, 10.96, 10.19; IR neat (cm^{-1}): 2963.9, 2366.2, 1718.0, 1654.0, 1268.7, 1025.8, 526.7; ESI-MS: [M+H]: 748.0.

4.1.18. Compound 7f

^1H NMR (400 MHz, $CDCl_3$) δ = 7.40–7.25 (m, 5H), 4.72–4.64 (m, 1H), 4.62–4.50 (m, 3H), 4.18–3.82 (m, 4H), 3.70–3.40 (m, 4H), 3.37–3.21 (m, 3H), 3.00–2.85 (m, 4H), 2.08 (m, 1H), 1.68 (m, 1H), 1.54–1.23 (m, 6H), 1.07–0.76 (m, 13H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 208.73, 208.59, 170.34, 170.19, 170.19, 170.02, 169.83, 168.86, 168.54, 137.43, 137.31, 129.54, 128.42, 127.87, 127.79, 127.77, 127.71, 127.61, 78.85, 78.55, 73.39, 73.34, 70.28, 70.09, 69.31, 69.15, 61.46, 61.29, 58.99, 58.95, 52.37, 52.31, 50.59, 50.53, 49.75, 49.71, 39.81, 39.63, 39.47, 39.26, 31.75, 31.62, 30.06, 29.81, 25.73, 25.05, 25.02, 24.52, 23.19, 21.07, 21.05, 16.65, 16.63, 15.13, 14.35, 10.92, 10.18; IR neat (cm^{-1}): 2963.8, 2360.4, 2107.6, 1718.2, 1654.0, 1027.8, 523.6; ESI-MS: [M+H]: 747.6.

4.1.19. Compound 9

Fmoc-*N*-methyl-isoleucine (3.80 g, 10.3 mmol) was taken up in DMF (50 mL). To this solution were added *tert*-butyl carbazate (2.0 g, 15.1 mmol), HCTU (6.20 g, 15 mmol) and DIPEA (5.2 mL, 30 mmol). The reaction mixture was stirred for 4 h after which the reaction mixture was diluted with ethyl acetate (250 mL). The solution was washed with HCl (1 M, 150 mL, thrice) and $NaHCO_3$ (satd. aq., 150 mL, thrice). The organic fraction was dried (Na_2SO_4) and taken to dryness. Silica gel chromatography (0 → 25% EtOAc in toluene) yielded the product as a hard white foam (4.27 g, 8.86 mmol, 86%). ^1H NMR (400 MHz, DMSO, 352 K) δ = 7.87 (d, J = 7.2 Hz, 2H), 7.65 (d, J = 7.2 Hz, 2H), 7.41 (t, J = 7.2 Hz, 2H), 7.33 (t, J = 7.2 Hz, 2H), 4.50–4.45 (m, 1H), 4.38–4.31 (m, 2H), 4.18 (m, 1H), 2.78 (s, 3H), 1.91 (m, 1H), 1.40 (s, 9H), 1.26–1.17 (m, 1H), 0.88–0.80 (m, 7H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 168.00, 155.10, 154.60, 143.57, 140.39, 127.09, 126.59, 126.54, 124.51, 124.43, 119.49, 78.69, 66.40, 60.80, 46.53, 32.00, 29.12, 27.64, 23.93, 14.77, 9.82; IR neat (cm^{-1}): 2969.8, 2360.1, 1651.7, 1668.1, 1451.9, 1311.7, 1147.3, 758.1, 739.5; $[\alpha]_D^{20}$ −103.2 (c 0.5, $CHCl_3$); HRMS: calcd for $C_{27}H_{36}O_5N_3$ ([M+H]) 482.26495 found 482.26477.

4.1.20. Compound 10

Compound **9** (2.83 g, 5.88 mmol) is taken up in DMF (60 mL). To this solution is added DBU (1.8 mL, 12.0 mmol) and the reaction mixture is stirred. After 10 min, bromoacetylbromide (1.0 mL, 11.5 mmol) is added and stirring is continued for 4 h during which the reaction mixture became deep red in colour. Sodium azide (4.0 g, 61.5 mmol) is added and stirring is continued over night. The reaction mixture is diluted with ethyl acetate (500 mL) and washed with HCl (1 M, 200 mL) and NaCl (satd. aq., 200 mL). The organic fraction was dried (Na_2SO_4) and taken to dryness. Silica gel chromatography (0 → 25% EtOAc in toluene) yielded the product (1.31 g, 3.82 mmol, 65%) as a pale yellow wax. ^1H NMR (400 MHz, $CHCl_3$, major rotamer)) δ = 4.70 (d, J = 11.2 Hz, 1H), 4.04–3.91 (m, 2H0, 2.92 (s, 3H), 2.17–2.13 (m, 1H), 1.45 (s, 9H), 1.38–1.34 (m, 1H), 1.07–1.03 (m, 1H), 0.97 (d, J = 6.4 Hz, 3H), 0.89 (t, J = 7.6 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.61, 169.27, 155.03, 81.48, 59.42, 50.95, 31.38, 30.12, 28.10, 24.56, 15.32, 10.18; IR neat (cm^{-1}): 3289.4, 2964.7, 2103.7, 1725.1, 1651.8, 1367.6, 1158.3, 1016.3; $[\alpha]_D^{20}$ −142 (c 1.0, $CHCl_3$); ESI-MS: [M+H]: 343.1.

4.2. Proteasome inhibition assay

Five 15 cm petri dishes of exponentially growing Hek293T were harvested in PBS, flash frozen in N_2(l) and lysed with 2 mL lysis buffer (50 mM Tris HCl pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin) for 15 min on ice. After 2 × 30 s sonication pulses, cell debris were removed by cold centrifugation, yielding a protein concentration of 5 μg/μl determined by Bradford assay with a BSA calibration curve. For the competition study, every compound was diluted at the indicated concentration in 10 μl reaction volume containing 10 μg protein incubated at 37 °C for 1 h and the residual proteasome activity was captured with 0.5 μM MV151 for the next hour at 37 °C. Proteins were resolved by 12.5% PAA SDS–PAGE gels and the wet gel slabs were imaged with a Typhoon fluorescence scanner (GE Healthcare). As negative control, 10 μg protein was denatured by boiling with 1% SDS prior to 1 h exposure to 0.5% MV151.

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