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PAPER

Synthesis and biological activity of optimized belactosin C congeners†

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Successful biochemical studies of the natural products belactosin A and C as well as their more stable acylated derivatives have proved them to be powerful proteasome inhibitors and thereby potential candidates as pharmacologically relevant active compounds. In order to understand their structure–biological activity relations in detail and to find ways of improving their biological activity, four new modified belactosin congeners have been synthesized and tested. One of them (compound **6**) turned out to be a more potent inhibitor against HeLa cells than the known proteasome inhibitor MG132.

Introduction

Because of their potent proteasome inhibitory effects[1] and other important biological activities of the natural products belactosin A and C and especially several of their acylated as well as otherwise modified congeners, these β-lactone derivatives have attracted considerable interest.[1–3] Despite this interest, however, only a small number of detailed investigations concerning their biological activities have been carried out so far.[4,5]

In order to better understand their proteasome inhibitory power and the important structure–activity relationships, the prevailing interactions between such molecules and the proteasome should be known. Towards this end, a protected homobelactosin C was initially cocrystallized with the 20S proteasome from *Saccharomyces Cerevisiae*, and the structure of this crystal was elucidated by X-ray diffraction.[1] On the basis of this analysis it was decided which groups in the inhibitor should be modified in order to improve potentially favorable interactions with the proteasome. In this paper we report the synthesis and detailed structure–activity investigations of some new belactosin C congeners.

Chemistry

Six different total syntheses of members of the belactosin family have been reported up to date.[6] Our own approach to the belactosins A **1** and C **2** as well as homo-C **3** relied on the fact that 2-isobutylmalic acid monophenylthio ester **4** (Fig. 1) as a key building block could be easily prepared in enantiomerically pure form.[6b] Subsequent peptide coupling of **4** with the corresponding dipeptide and ensuing *in situ* β-lactone ring closure provided the diprotected derivatives of belactosins A and C and homobelactosin C, **1**, **2** and **3**, respectively, which were eventually completely deprotected.

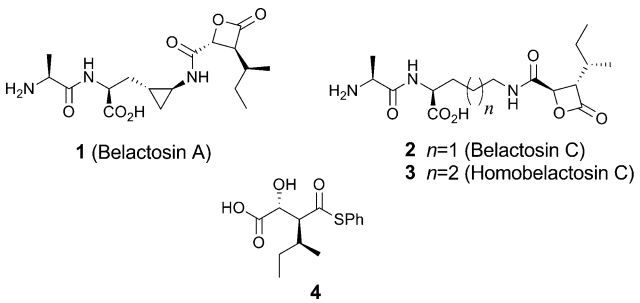

Fig. 1 The naturally occurring proteasome inhibitors belactosin A and C, its non-natural analogue homobelactosin C and a key building block **4** in our total syntheses of **1**–**3**.[6b]

Computer modelling of possible modifications of the belactosin structure showed that attachment of a 2-naphthylethoxycarbonyl (CNAP) group onto the amino terminus in the alanine residue in belactosin C could be favorable for hydrophobic interactions and thus improve its biological activity. On the other hand, introduction of a 4-carboxybenzyl residue capable of both hydrophilic and hydrophobic interactions, onto the ornithine part of belactosin C could also lead to an improved biological activity.

To test these hypotheses, we prepared the analogues **6**, **11** and **15** of belactosin C (**2**) as well as the known derivative **16** according to our previously published strategy.[6b] Compound **6** with a 2-naphthylmethoxycarbonyl protective group on the amino terminus was obtained starting from the 2-naphthylmethoxycarbonyl-protected alanine (CNAP-Ala-OH) **5** by coupling it with the appropriately decorated ornithine and subsequently installing

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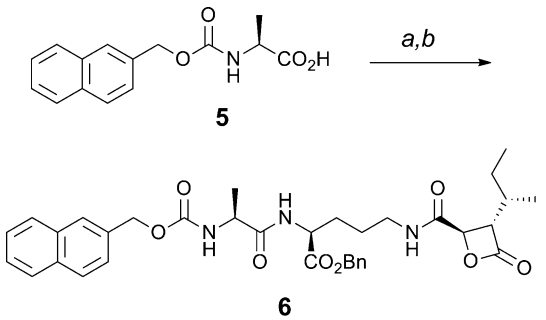
the β-lactone moiety by a one-pot acylation/β-lactonization[6b] reaction employing the building block **4** (Scheme 1).

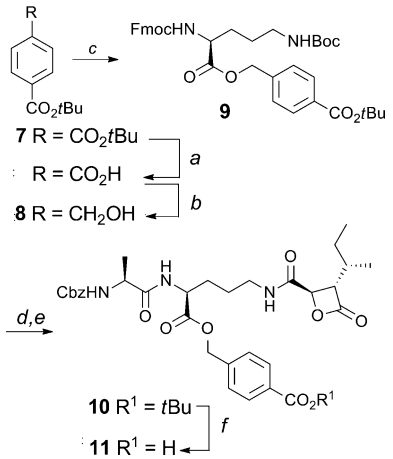


Scheme 1 Synthesis of the new belactosin C derivative **6** with a (2-naphthylmethoxy)carbonyl substituent at the amino terminus. Reagents and conditions: (*a*) H_2N-Orn(Boc)-OBn, EDC, HOAt, TMP, 0 to 20 °C, 24 h, 77%. (*b*) (1) HCl, EtOAc, r.t., 12 h (2) **4**, TMP, HOAt, EDC, –20 to 20 °C, 48 h, 33%.

The synthesis of the belactosin C analogues **10** and **11** containing a *tert*-butoxycarbonyl (**10**) and a free carboxylic acid group, respectively, in the benzyl ester moiety, commenced with partial hydrolysis of di-*tert*-butyl terephthalate **7** followed by a chemoselective reduction of the free carboxylic acid group with tetrabutylammonium borohydride to furnish the benzyl alcohol **8** (Scheme 2).

The DCC-mediated condensation of **8** with the correspondingly protected ornithine gave rise to the N-terminally Boc-protected intermediate **9**. This was converted into the tripeptide **10** by way of Fmoc removal, peptide coupling with Cbz-Ala-OH, selective removal of the *N*-Boc group, and the one-pot acylation/β-lactonization reaction as a key step. Cleavage of the *tert*-butyl ester under acidic conditions (trifluoroacetic acid in dichloromethane) yielded the desired belactosin C congener **11** with a free carboxylic acid functionality in the benzyl ester moiety.

Finally, the stable and storable crystalline hydrotrifluoroacetate salt of belactosin C **15** was prepared along the route outlined in Scheme 3. Thus, H-Orn(Cbz)-O*t*Bu **12** was transformed into



Scheme 2 Synthesis of the belactosin C analogues **10** and **11** with functionally substituted benzyl ester moieties. Reagents and conditions: (*a*) KOH, *t*BuOH, 50 °C, 3 h, 63%. (*b*) Bu_4NBH_4, r.t., 18 h, 75%. (*c*) Fmoc-Orn(Boc)-OH, DCC, CH_2Cl_2, 0 °C, 6 h, 74%. (*d*) (1) Et_2NH, THF, 3 h; (2) Cbz-Ala-OH, EDC, TMP, HOAt, 0 to 20 °C, 24 h, 65%. (*e*) (1) HCl, EtOAc, r.t., 3 h; (2) **4**, TMP, HOAt, EDC, –20 to 20 °C, 48 h, 37%. (*f*) CF_3CO_2H, CH_2Cl_2, –18 °C, 18 h, 99%.

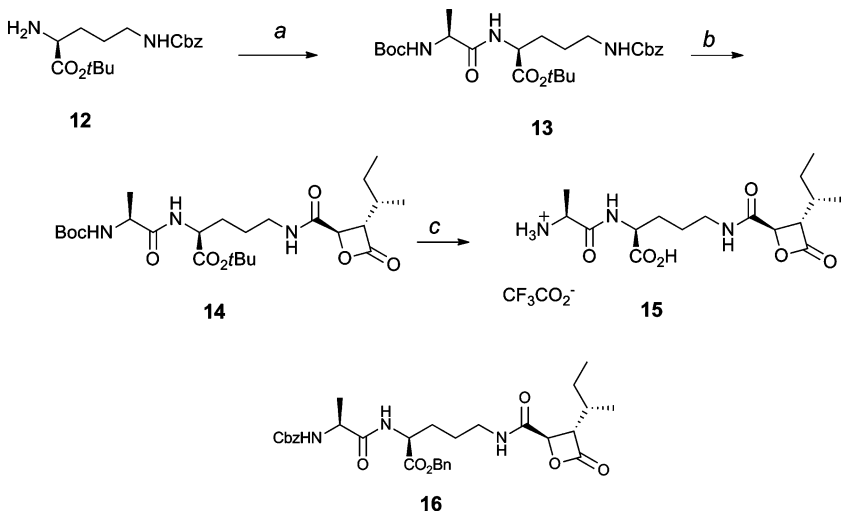
the dipeptide **13**, which after hydrogenolytic deprotection was condensed with the hydroxyacid monothioester **4** to give the diprotected belactosin C derivative **14**. Complete deprotection was effected upon treatment with trifluoroacetic acid to yield the hydrotrifluoroacetate salt **15** of belactosin C.

The belactosin C derivative **16** was synthesized according to the previously published protocol.[6b]

Thus, the novel derivatives of belactosin C **6**, **11** and **15** were prepared in sizable quantities employing our original synthetic protocol.[6b]

Results and discussion

To investigate the effect of the obtained belactosin C congeners on proteasomal activity in mammalian cells, HeLa cells were treated with varying concentrations of compounds **6**, **10**, **14**, and



Scheme 3 Preparation of the belactosin C hydrotrifluoroacetate salt. Reagents and conditions: (*a*) Boc-Ala-OH, EDC, HOAt, TMP, 0 to 20 °C, 24 h, 87%. (*b*) (1) H_2/Pd/C, r.t., 16 h; (2) **4**, EDC, HOAt, TMP, –20 to 20 °C, 48 h, 57%. (*c*) CF_3CO_2H, CH_2Cl_2, –18 °C, 18 h, 99%.

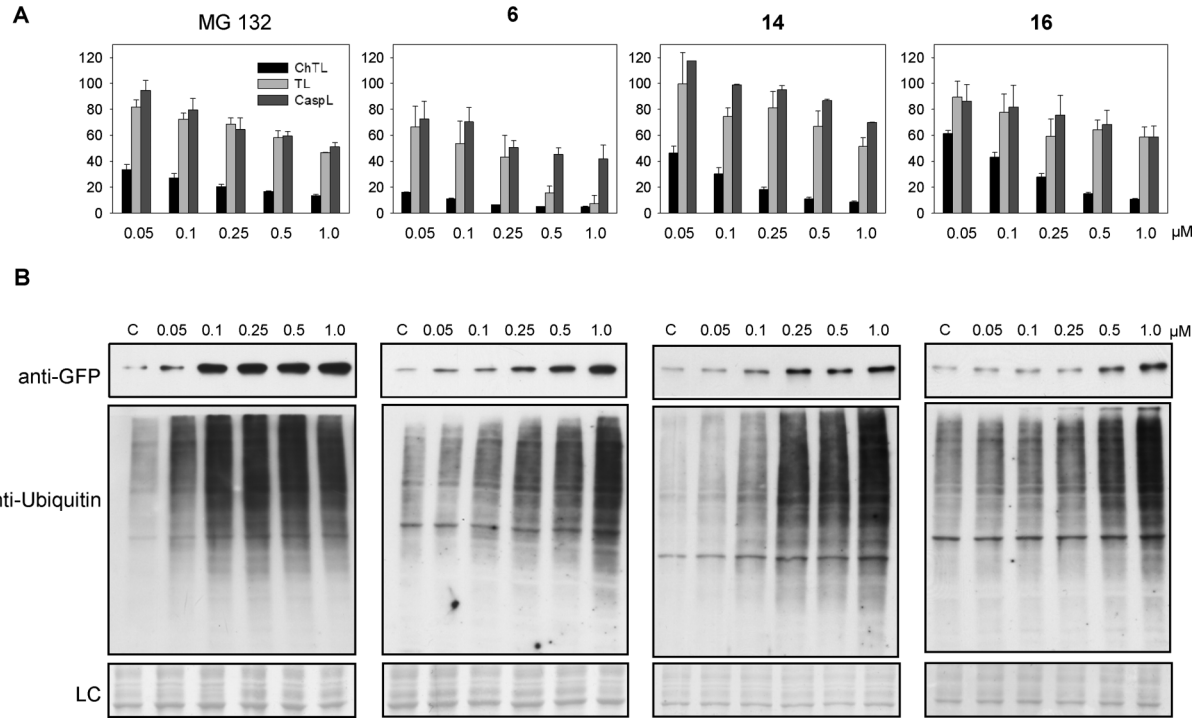


Fig. 2 Biological activities of belactosin C congeners **6**, **14** and **16** in comparison to MG132. A) HeLa cells were treated with the indicated concentrations of MG132 and belactosin C congeners **6**, **14** and **16** or solvent for 4 h. Cells were lysed, followed by measurement of chymotrypsin-like (ChTL), trypsin-like (TL) and caspase-like (CaspL) activities in the *in vitro* activity assay employing fluorogenic substrates. Activities are expressed as percentage of a solvent-treated control. Values are given as the means of three independent experiments ± SEM. B) Ear fibroblasts of Ub^{G76V}-GFP1 transgenic mice were treated with increasing concentrations of MG132 and belactosin C congeners **6**, **14**, and **16** or solvent (C) for 4 h. Accumulation of degradation-prone proteins was analyzed in cell lysates by western blot, using anti-GFP and anti-ubiquitin antibodies. Amido black staining of western blot membranes served as a control for equal protein loading (LC).

16 for 4 h as well as with the widely used proteasome inhibitor MG132. Subsequently, an *in vitro* activity assay was performed in cell lysates employing the fluorogenic substrates SSLVY-AMC, BzVGR-AMC and ZLLE-AMC to measure the chymotrypsin-like (ChTL), the trypsin-like (TL) and the caspase-like (CaspL) activities respectively. All compounds dose dependently inhibited proteasomal degradation. The ChTL activity was predominantly affected by all substances. As shown in Fig. 2A, the inhibitory effects of compounds **14** and **16** were similar to those of MG132, albeit MG132 was more effective in concentrations below 0.25 μM. Compound **6** turned out to be the most effective inhibitor. Notably, besides its potent inhibitory effect on the ChTL activity, compound **6** showed the most pronounced effect of all tested substances on TL activity, in particular in concentrations above 0.25 μM. Compound **10** displayed a considerably lower inhibitory potency compared to MG132.

The accumulation of polyubiquitinated proteins or proteins that contain a constitutively active degradation signal is one characteristic effect of proteasome inhibition in cells. To detect the levels of degradation-prone proteins that accumulated upon incubation with belactosin C congeners, fibroblasts obtained from ears of Ub^{G76V}-GFP1 mice were treated with varying concentrations of compounds **6**, **14**, **16**, and MG132 for 4 h. Ub^{G76V}-GFP1 mice contain the green fluorescent protein (GFP) fused to a constitutively active degradation signal (Ub^{G76V}), thereby allowing us to monitor the accumulation of this specific reporter protein in addition to overall polyubiquitinated protein. Following treatment, cell lysates were analyzed by western blot, using anti-GFP and anti-ubiquitin antibodies. As shown in Fig. 2 B, a concentration-dependent accumulation of endogenous polyubiquitinated proteins, as well as elevated levels of Ub^{G76V}-GFP were caused by substances **6**, **14**, and **16**, thereby confirming their inhibitory effect on proteasomal activity in living cells. In concentrations below 1μM, compounds **14** and **16** lead to a considerably lower accumulation of polyubiquitinated proteins and Ub^{G76V}-GFP compared to MG132. It is noteworthy that a similar effect was observed for compound **6**, which appeared to be a more potent inhibitor compared to MG132 as estimated in the *in vitro* activity assay.

In order to get insight into the mechanism of biological activities of these compounds, compound **6** was co-crystallized with the yeast 20S proteasome (see Fig. 3).

Yeast 20S proteasome crystals were soaked with compound **6** for 24 h at a final concentration of 5 mM. Data collected from the soaked crystals were evaluated by molecular replacement using the coordinates of the yeast 20S proteasome.[7] Subsequent bulk solvent correction and positional refinement performed with CNS yielded an R_{free} of 23.7% (Table S1†). The $2F_o$-F_c-electron density map calculated after density averaging visualized compound **6** in the primed site of the chymotrypsin-like substrate binding channel, for which it exerts a high selectivity (Fig. 3A). In close analogy to what has been observed for bis-protected homobelactosin C (hBelC),[1] the carbonyl carbon atom of the ligand derived from the beta-lactone ring is covalently bound to the free hydroxyl

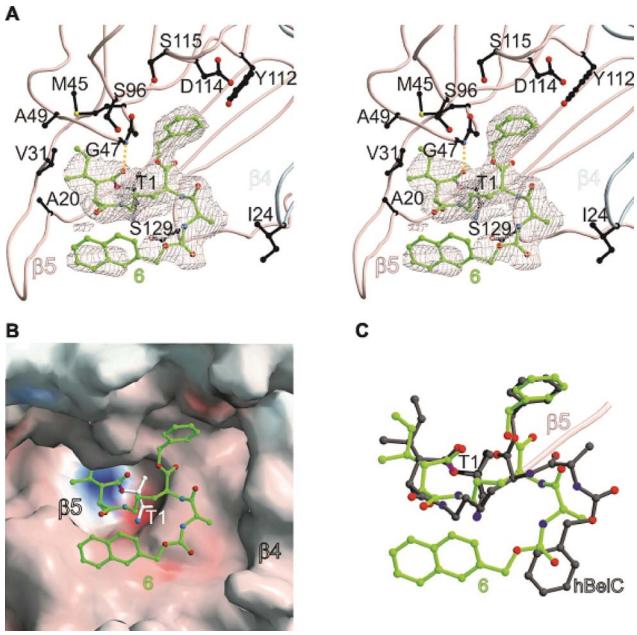


Fig. 3 The belactosin C derivative **6** specifically binds to the chymotryptic-like active site by formation of an ester. (*a*) Stereorepresentation of the chymotryptic-like active site of the yeast 20S proteasome (colored in bisque) in its complex with **6** (colored in green). The covalent linkage of the inhibitor with β5-Thr1O$^{\gamma}$ is drawn in magenta. The electron density map (colored in grey) is contoured from 1σ around Thr-1 (colored in black) with $2F_o - F_c$ coefficients after twofold averaging. Temperature factor refinement indicates full occupancies of the inhibitor-binding site except for the 2-naphthylmethoxycarbonyl side chain. **6** has been omitted for phasing. Amino acid residues stabilizing **6** are colored in black. (*b*) Surface representation of the chymotryptic-like active site in its complex with **6** covalently bound to Thr-1 (depicted in white). Surface colors indicate positive and negative electrostatic potentials contoured from 15 kT/e (intense blue) to −15 kT/e (intense red). (*c*) Structural superposition of **6** and bisbenzyl-protected homobelactosin C (hBelC), including Thr-1 with respect to subunit β5. **6** is shown in green, hBelC as well as the active site Thr1 are drawn in black.

group of the catalytic N-terminal Thr1O$^{\gamma}$ of subunit β5 (Fig. 3B). Except for the interaction of the isoleucine side chain with the S1 specificity pocket, the molecule targets only the primed site of the substrate binding channel. Structural superposition of **6** and hBelC bound to the subunit β5 reveals that shortening of the linker by a methylene group in **6** compared to hBelC only affects the conformation of the inhibitor backbone but not that of the side chains. The perfect match of the benzyl side chains of both compounds indicates an important proteasomal primed site for substrate binding at this position (Fig. 3C). The restricted size of the primed specificity pockets in the yeast β1- and β2-subunits[8] explains the selectivity of **6** and hBelC to only bind by their extended benzyl side chain to the chymotrypsin-like active site. Furthermore, comparison of the primary sequences of mammalian subunits β5 and β5i reveals a conspicuous alteration in this primed specificity channel: Ser115 and Glu116 of the human constitutive subunit β5 are replaced by Glu and His in subunit β5i, respectively. The putative primed substrate binding channels in the β1- and β2-subunits modelled on the basis of subunit β5 show similar architectures and exhibit similar significant differences between constitutive and immune subunits.[9] Thus, the novel chemical, biological and structural insights into the binding mechanism of belactosin C derivatives might contribute to the development of new anti-inflammatory or anti-cancer drugs targeting the proteasome.

Conclusion

Four novel belactosin congeners have been synthesized according to an established protocol[6b] in order to check the hypothesis concerning their structure–activity relations based on the computer modelling of their complexes with the proteasome. The best substances turned out to have an improved biological activity against HeLa cells at least comparable or even superior to the activity of the known proteasome inhibitor MG132. The most active compound **6** is more potent than MG132, thus becoming a promising candidate for further investigations and possible application in cancer therapy. A detailed investigation of the complex of **6** with the proteasome showed an identical binding mode (and thus mechanism of its activity) as the known β-lactone inhibitor homobelactosin C.

Experimental section

Synthesis of belactosin C congeners

General remarks. ^{1}H and ^{13}C NMR spectra were recorded at 250, 300, 600 (^{1}H), and 75.5, 151 MHz (^{13}C), additional APT (Attached Proton Test)] with Bruker AM 250, Varian AMX 300 and Inova 600 instruments on compounds in $CDCl_3$ solutions if not otherwise specified, chemical shifts are reported as δ values in ppm, coupling constants J in Hz. IR: Bruker IFS 66 (FT-IR) spectrometer, measured as KBr pellets or as films between KBr plates. MS (ESI): Finnigan MAT 95 spectrometer. Optical rotations: Perkin-Elmer 241 digital polarimeter, 1 dm cell. Starting materials: CH_2Cl_2 was distilled from P_4O_{10}. 4-(*tert*-Butyloxycarbonyl)phenylmethanol (**8**) was synthesized from di-*tert*-butyl terephthalate (**7**) according to a published procedure.[10] (2*S*)-[(2*S*)-Benzyloxycarbonylaminopropionylamino]-5-{[(3*S*)-((1*S*)-methylpropyl)-4-oxo-oxetane-(2*R*)-carbonyl]amino}pentanoic acid benzyl ester (**16**) was synthesized according to a known procedure.[6b] All other chemicals were used as commercially available. All reactions were performed under an atmosphere of dry nitrogen. Organic extracts were dried over Na_2SO_4.

((2*S*)-Naphthylmethoxycarbonylamino))propionic acid (CNAP-Ala) (5). To a stirred ice-cold solution of L-alanine (1.8 g, 20 mmol) and NaOH (1.6 g, 40 mmol) in H_2O (10 mL) was added 2-naphthylmethyl chloroformate[11] (CNAP–Cl) (6.3 g, 29 mmol). Then water (10 mL) and THF (10 mL) were added, upon which the solution became clear. The reaction mixture was stirred at r. t. for 2 h, and the reaction was then quenched by adding a saturated solution of $NaHCO_3$ (20 mL). The mixture was extracted with Et_2O (1 × 30 mL), the ethereal phases were discarded, and the pH value of the aqueous phase was adjusted to 2–3 with 12 M aqueous HCl. Then it was extracted EtOAc (3 × 30 mL), and the combined organic phases were dried. Evaporation of the solvent gave 4.4 g (80%) of the acid **5** as a colorless solid, m.p. 132–133 °C. – ^{1}H NMR (500 MHz, CD_3OD): 1.48 (3 H, d, J = 7 Hz), 4.31 (1 H, q, J = 7 Hz), 5.32 (2 H, s), 7.50–7.56 (3 H, m),

7.86–7.92 (4 H, m) ppm. – ^{13}C NMR (125.7 MHz): 17.8 (CH_3), 50.8 (CH), 67.6 (CH_2), 126.5 (CH), 127.1 (CH), 127.2 (CH), 127.6 (CH), 128.6 (CH), 128.9 (CH), 129.1 (CH), 134.4 (C), 134.6 (C), 135.6 (C), 158.4 (C), 176.5 (C) ppm. – IR (KBr): ν = 3329 cm^{-1}, 3048, 1692, 1535, 1461, 1253, 1076, 820, 737, 623. – $[\alpha]_D^{20}$ −8.1 (*c* 1.0, MeOH). – MS (DCI) *m*/*z* = 564 ([2 M + NH_4^+], 4), 291 ([M + NH_4^+], 100). – Calcd. for $C_{15}H_{15}NO_4$: C 65.92%, H 5.53%, N 5.13%, found: C 65.65%, H 5.31%, N 4.89%.

General procedure (GP1) for Fmoc-deprotection of the benzyl esters and subsequent peptide condensation. To a solution of the respective benzyl ester (1.70 mmol) in THF (3.3 mL) was added at r.t. Et_2NH (3.3 mL). The mixture was stirred at r. t. for 1 h, then another 2 mL of Et_2NH was added, and the mixture was stirred for an additional 2 h. The volatiles were removed under reduced pressure at 35 °C. The oily residue was azeotropically distilled off with toluene (2 × 8 mL) under reduced pressure at 45–50 °C, and the crude amine was taken up in CH_2Cl_2 (5 mL).

A separate flask was charged with the corresponding protected alanine (2.06 mmol), CH_2Cl_2 (7.5 mL) and HOAt (0.26 g, 1.92 mmol). EDC (0.32 g, 2.04 mmol) was added dropwise at 0 °C within 10 min, and the resulting mixture was stirred at 0 °C for 20 min. To the solution of the prepared crude amine in CH_2Cl_2 was added TMP (0.66 g, 0.72 mL, 5.43 mmol), and the resulting suspension was added through a cannula to the stirred reaction mixture. This was left to attain r.t. within 16 h and then concentrated under reduced pressure. The residue was taken up in EtOAc (30 mL). The cloudy solution was washed with 1 N aqueous $KHSO_4$ solution (2 × 30 mL), and saturated aqueous $NaHCO_3$ solution (2 × 30 mL), then dried and concentrated under reduced pressure. The oily residue was taken up in EtOAc (5 mL) and purified by column chromatography [SiO_2 (50 g), hexane–EtOAc 1 : 1] to give the desired dipeptide.

2-Naphthylmethoxycarbonyl-(*S*)-alanyl-(*S*)-(N^{δ}-*tert*-butyloxycarbonyl)ornithine benzyl ester [CNAP-Ala-Orn(Boc)-OBn]. 2-Naphthylmethoxycarbonyl-(*S*)-alanyl-(*S*)-(N^{δ}-*tert*-butyloxycarbonyl)ornithine benzyl ester (756 mg, 77%) was prepared from **5** (560 mg, 2.06 mmol) and N^{δ}-(*tert*-butyloxycarbonyl)-N^{α}-(9*H*-9-fluorenyloxycarbonyl)-L-ornithine benzyl ester (900 mg, 1.70 mmol) according to GP1 as a colorless solid. R_f 0.40 [hexane–EtOAc (1 : 1)], m. p. 112–113 °C. – ^{1}H NMR (300 MHz): 1.30–1.50 (2 H, m), 1.38 (3 H, d, *J* = 7 Hz), 1.41 (9 H, s), 1.60–1.92 (2 H, m), 2.90–3.11 (2 H, m), 4.26–4.40 (1 H, m), 4.53–4.64 (1 H, m), 4.65–4.80 (1 H, m), 5.07–5.20 (2 H, m), 5.21–5.32 (2 H, m), 5.64–5.77 (1 H, br m), 7.05–7.12 (1 H, m), 7.28–7.40 (4 H, m), 7.40–7.52 (3 H, m), 7.75–7.85 (4 H, m) ppm. – ^{13}C NMR (75.5 MHz): 18.6 (CH_3), 26.0 (CH_2), 28.3 (CH_3), 28.9 (CH_2), 39.7 (CH_2), 50.4 (CH), 52.1 (CH), 67.0 (CH_2), 67.1 (CH_2), 79.2 (C), 125.7 (CH), 126.1 (CH), 126.2 (CH), 127.0 (CH), 127.6 (CH), 127.9 (CH), 128.2 (CH), 128.3 (CH), 128.4 (CH), 128.5 (CH), 133.0 (C), 133.1 (C), 133.6 (C), 135.1 (C), 155.9 (C), 156.1 (C), 171.7 (C), 172.3 (C) ppm. – IR (film): ν = 3336 cm^{-1}, 2926, 1718, 1700, 1685, 1669, 1653, 1521, 1507, 1457, 1368, 1251, 1159, 1027, 912, 738. – $[\alpha]_D^{20}$ −6.0 (*c* 1.0, $CHCl_3$). – MS (ESI) positive ion mode: *m*/*z* = 1177 ([2 M + Na^+], 100), 600 ([M + Na], 40). Negative ion mode: *m*/*z* = 622 ([M + CH_3COO^-], 100). – HRMS (ESI) [M + H^+]. – Calcd. for $C_{33}H_{40}N_3O_7$ 578.2866, found 494.2861. – Calcd. for $C_{32}H_{39}N_3O_7$: C 66.53%, H 6.80%, N 7.27%, found: C 60.44%, H 6.65%, N 7.08%.

General procedure (GP2) for Boc-removal and subsequent sequential acylation/β-lactonization. To a solution of the respective dipeptide (1.56 mmol) in EtOAc (3 mL) was added 3 N HCl in EtOAc (12 mL). The mixture was stirred at r. t. for 12 h, then concentrated under reduced pressure at 40 °C. The resulting crude solid product was dried *in vacuo* (0.05 mbar) at r. t. for 3 h, then dissolved in DMF (6 mL) and the solution cooled to −30 °C. A solution of the thioester **4** (440 mg, 1.57 mmol) in CH_2Cl_2 (20.6 mL) was added first, then HOAt (255 mg, 1.54 mmol), TMP (377 mg, 3.11 mmol), as well as EDC (561 mg, 3.11 mmol), and the resulting mixture was stirred at −30 °C for 6 h, then at r. t. for 30 h. It was then washed with 1 N aqueous $KHSO_4$ (80 mL), dried and concentrated under reduced pressure. The oily residue was purified by column chromatography [SiO_2 (60 g), hexane–EtOAc (1 : 2)] to give the desired product.

(2*S*)-[(2*S*)-(2-Naphthyl)methoxycarbonylaminopropionylamino]-5-{[(3*S*)-((1*S*)-methylpropyl)-4-oxo-oxetane-(2*R*)-carbonyl]amino}pentanoic acid benzyl ester (6). Compound **6** was prepared according to GP2 from 2-naphthylmethoxycarbonyl-(*S*)-alanyl-(*S*)-(N^{δ}-*tert*-butyloxycarbonyl)ornithine benzyl ester (900 mg, 1.56 mmol), the thioester **4** (440 mg, 1.57 mmol), TMP (377 mg, 3.11 mmol), HOAt (255 mg, 1.54 mmol) and EDC (561 mg, 3.11 mmol) in dichloromethane (20.6 mL). Yield 320 mg (33%), R_f 0.46 [hexane–EtOAc (1 : 2)]. – ^{1}H NMR (300 MHz): 0.88 (3 H, t, *J* = 7 Hz), 0.97 (3 H, d, *J* = 7 Hz), 1.20–1.27 (1 H, m), 1.37 (3 H, d, *J* = 7 Hz), 1.35–1.48 (2 H, m), 1.54–1.64 (2 H, m), 1.78–1.92 (2 H, m), 3.05–3.12 (1 H, m), 3.20–3.28 (1 H, m), 3.53 (1 H, dd, *J* = 8, 5 Hz), 4.33–4.40 (1 H, m), 4.48 (1 H, d, *J* = 5 Hz), 4.57–4.62 (1 H, m), 5.09–5.18 (2 H, m), 5.20–5.27 (2 H, m), 5.66–5.70 (1 H, d, *J* = 8 Hz), 6.82 (1 H, m), 7.03 (1 H, d, *J* = 8 Hz), 7.29–7.37 (4 H, m), 7.40–7.44 (1 H, m), 7.46–7.49 (2 H, m), 7.78–7.84 (4 H, m) ppm. – ^{13}C NMR (75.5 MHz): 18.2 (CH_3), 25.5 (CH_2), 27.9 (CH_3), 28.2 (CH_3), 29.6 (CH_2), 40.4 (CH_2), 50.1 (CH), 52.3 (CH), 66.5 (C), 80.0 (C), 82.2 (CH_2), 128.0 (CH), 128.4 (CH), 136.5 (CH), 155.4 (C), 156.5 (C), 171.0 (C), 172.5 (C) ppm. – IR (film): ν = 3306 cm^{-1}, 3059, 2964, 2877, 1830, 1734, 1717, 1700, 1696, 1684, 1669, 1653, 1539, 1250, 1098, 1072, 908, 734. – $[\alpha]_D^{20}$ = +5.4 (*c* 0.96, $CHCl_3$). – MS (ESI) positive ion mode: *m*/*z* = 1285 ([2 M + Na^+], 100), 654 ([M + Na^+], 90). Negative ion mode: *m*/*z* = 676 ([M + $HCOO^-$], 100), 630 ([M – H^+], 30). – HRMS (ESI) [M + H^+]. – Calcd. for $C_{35}H_{42}N_3O_8$ 632.2972, found 632.2966.

5-*tert*-Butyloxycarbonylamino-(2*S*)-(9*H*-fluoren-9-ylmethoxycarbonylamino)pentanoic acid (4-*tert*-butyloxycarbonylphenyl)-methyl ester (9). Dicyclohexylcarbodiimide(1360 mg, 6.60 mmol) was added at 0 °C to a mixture of 5-*tert*-butoxycarbonylamino-(2*S*)-(9*H*)-fluoren-9-ylmethoxycarbonylamino)pentanoic acid (1530 mg, 3.37 mmol) and 4-(*tert*-butyloxycarbonyl)phenylmethanol **8** (700 mg, 3.37 mmol) in CH_2Cl_2 (6.5 mL) and DMF (3.5 mL). The reaction mixture was stirred at r. t. for 3 h, then the solvent was distilled off under reduced pressure, and the residue was purified by column chromatography, yielding 800 mg (37%) of the product (R_f 0.28 [hexane–Et_2O (1 : 1)]) along with 350 mg (50%) of starting alcohol (R_f 0.45 [hexane–Et_2O (1 : 1)]). – ^{1}H NMR (300 MHz): 1.43 (9 H, s), 1.58 (9 H, s), 1.88–2.00 (4 H, m), 3.04–3.20 (2 H, m), 3.40–3.54 (1 H, m), 4.08–4.24 (1 H, m), 4.30–4.44 (2 H, m), 5.07 (2 H, s), 5.19–5.22 (2 H, m), 7.26–7.44 (6 H, m), 7.56–7.64 (2 H, m), 7.76 (2 H, d, *J* = 8 Hz), 7.96 (2 H, d, *J* = 8 Hz) ppm. – ^{13}C NMR

(75.5 MHz): 24.9 (CH_2), 25.6 (CH_2), 28.1 (CH_3), 28.4 (CH_3), 29.6 (CH_2), 33.9 (CH_2), 47.0 (CH), 47.1 (CH), 66.5 (CH_2), 66.9 (CH_2), 81.2 (C), 120.0 (CH), 127.0 (CH), 127.7 (CH), 129.7 (CH), 141.2 (C), 141.3 (C), 141.1 (C), 143.6 (C), 156.7 (CO), 165.2 (CO), 167.6 (CO), 172.1 (CO) ppm. – IR (film): ν = 3334 cm^{-1}, 2923, 1710, 1700, 1685, 1669, 1653, 1521, 1507, 1457, 1368, 1251, 1159, 1027, 912, 738. – $[\alpha]_D^{20}$ −6.0 (*c* 1.0, $CHCl_3$). – MS (ESI) *m*/*z* = 683 ([M + K^+], 100), 622 ([M + Na^+], 80). – Calcd. for $C_{32}H_{39}N_3O_7$: C 66.53%, H 6.80%, N 7.27%, found: C 60.44%, H 6.65%, N 7.08%.

(*S*)-Benzyloxycarbonylalanyl-(*S*)-(N^{δ}-*tert*-butyloxycarbonyl)-ornithine 4-(*tert*-butoxycarbonyl)phenylmethyl ester. (*S*)-Benzyloxycarbonylalanyl-(*S*)-(N^{δ}-*tert*-butyloxycarbonyl)ornithine 4-(*tert*-butoxycarbonyl)phenylmethyl ester (343 mg, 44%) was prepared from **9** (800 mg, 1.24 mmol) and Cbz-Ala (330 mg, 1.42 mmol) according to GP1 as a colorless solid. R_f 0.11 [hexane–EtOAc (2 : 1)], m. p. 118–119 °C. – ^{1}H NMR (300 MHz): 1.38 (3 H, d, *J* = 7 Hz), 1.41 (7.7 H, s), 1.43 (1.3 H, s), 1.37 (9 H, s), 1.38–1.76 (2 H, m), 1.87–1.96 (2 H, m), 3.00–3.17 (2 H, m), 3.80–3.96 (1 H, m), 4.10–4.32 (1 H, m), 4.55–4.69 (1 H, m), 5.05–5.11 (2 H, m), 5.15–5.19 (2 H, m), 5.47–5.57 (1 H, br m), 6.93–7.02 (1 H, br m), 7.30–7.40 (7 H, m), 7.97 (2 H, d, *J* = 8 Hz) ppm. – ^{13}C NMR (75.5 MHz): 18.4 (CH_3), 25.8 (CH_2), 28.1 (CH_3), 28.3 (CH_3), 29.3 (CH_2), 39.7 (CH_2), 50.5 (CH), 52.2 (CH), 66.5 (CH_2), 67.0 (CH_2), 79.4 (C), 81.2 (C), 127.7 (CH), 128.0 (CH), 128.2 (CH), 128.9 (CH), 129.7 (CH), 132.0 (C), 136.2 (C), 139.6 (C), 156.2 (C), 156.7 (C), 157.3 (C), 165.3 (C), 172.3 (C) ppm. – IR (film): ν = 3336 cm^{-1}, 2926, 1718, 1700, 1685, 1669, 1653, 1521, 1507, 1457, 1368, 1251, 1159, 1027, 912, 738. – $[\alpha]_D^{20}$ −6.0 (*c* 1.0, $CHCl_3$). – MS (ESI) positive ion mode: *m*/*z* = 1177 ([2 M + Na^+], 100), 600 ([M + Na], 40). Negative ion mode: *m*/*z* = 622 ([M + CH_3COO^-], 100). – HRMS (ESI) [M + H^+]. – Calcd. for $C_{33}H_{46}N_3O_9$ 628.3234, found 628.3229.

(2*S*)-[(2*S*)-Benzyloxycarbonylaminopropionylamino]-5-{[(3*S*)-((1*S*)-methylpropyl)-4-oxooxetane-(2*R*)-carbonyl]amino}pentanoic acid 4-(*tert*-butyloxycarbonyl)phenylmethyl ester (10). A solution of (*S*)-benzyloxycarbonylalanyl-(*S*)-(N^{δ}-*tert*-butyloxycarbonyl)ornithine 4-(*tert*-butoxycarbonyl)phenylmethyl ester (340 mg, 0.54 mmol) and triethylsilane (316 mg, 2.70 mmol) in EtOAc (6 mL) was added to a saturated solution of HCl in EtOAc (5 mL), and the mixture was stirred for 3 h. The volatiles were distilled off, and the residue was dried under reduced pressure (0.05 mbar). A solution of the dry solid in DMF (3 mL) was treated with a solution of the thioester **4** (157 mg, 0.56 mmol) in CH_2Cl_2 (6 mL), TMP (146 mg, 1.21 mmol), HOAt (100 mg, 0.60 mmol) and EDC (200 mg, 1.11 mmol) in CH_2Cl_2 (2 mL) according to GP2 to yield 130 mg (35%) of the product **10** as a glassy colorless solid, R_f 0.30 [hexane–EtOAc (1 : 2)]. – ^{1}H NMR (300 MHz): 0.90 (3 H, t, *J* = 7 Hz), 1.01 (3 H, d, *J* = 7 Hz) 1.20–1.30 (2 H, m), 1.35 (3 H, d, *J* = 7 Hz), 1.44–1.53 (1 H, m), 1.59 (9 H, s), 1.58–1.68 (2 H, m), 1.82–1.95 (2 H, m), 3.14–3.21 (1 H, m), 3.25–3.33 (1 H, m), 3.40–3.46 (1 H, m), 3.54 (1 H, dd, *J* = 8, 5), 4.28–4.34 (1 H, m), 4.52 (1 H, d, *J* = 5 Hz), 4.57–4.63 (1 H, m), 5.00–5.10 (2 H, m), 5.12–5.22 (1 H, m), 5.50–5.54 (1 H, m), 6.71–6.77 (1 H, m), 6.90–6.95 (1 H, m), 7.27–7.40 (7 H, m), 7.95 (2 H, d, *J* = 8 Hz) ppm. – ^{13}C NMR (75.5 MHz): 10.9 (CH_3), 16.2 (CH_3), 18.3 (CH_3), 25.3 (CH_2), 26.5 (CH_2), 28.1 (CH_3), 29.6 (CH_2), 38.3 (CH_2), 50.4 (CH), 51.7 (CH), 62.8 (C), 66.6 (CH), 66.9 (CH), 70.6 (CH), 81.18 (C), 127.7 (CH), 128.0 (CH), 128.1 (CH), 128.5 (CH), 129.7 (CH), 132.0 (C), 136.0 (C), 139.3 (C), 168.2 (C), 169.2 (C), 171.5 (C), 172.5 (C) ppm. – IR (film): ν = 3297 cm^{-1}, 2933, 2877, 1836, 1734, 1717, 1700, 1684, 1669, 1653, 1559, 1540, 1293, 1256, 1167, 1112, 756. – $[\alpha]_D^{20}$ = +1.6 (*c* 1.8, $CHCl_3$). – MS (ESI): *m*/*z* = 1385 ([2 M + Na^+], 48), 704 ([M + Na^+], 100), 682 ([M + H^+], 7). – HRMS (ESI) [M + NH_4^+]. – Calcd. for $C_{36}H_{51}N_4O_{10}$ 699.3605, found 699.3600.

(2*S*)-[(2*S*)-Benzyloxycarbonylaminopropionylamino]-5-{[(3*S*)-((1*S*)-methylpropyl)-4-oxooxetane-(2*R*)-carbonyl]amino}pentanoic acid 4-carboxylphenylmethyl ester (11). To a solution of the product **10** (15 mg, 0.22 mmol) in CH_2Cl_2 (1 mL) was added trifluoroacetic acid (1 mL). The mixture was left overnight at –15 °C in a freezer. All the volatiles were distilled off under reduced pressure, and the residue was dried *in vacuo*. Yield 13 mg (99%). – ^{1}H NMR (300 MHz): 0.91 (3 H, t, *J* = 7 Hz), 1.02 (3 H, d, *J* = 7 Hz) 1.20–1.30 (2 H, m), 1.37 (3 H, d, *J* = 7 Hz), 1.44–1.53 (1 H, m), 1.58–1.68 (2 H, m), 1.82–1.95 (2 H, m), 3.15–3.35 (2 H, m), 3.54 (1 H, dd, *J* = 8, 5 Hz), 4.28–4.38 (1 H, m), 4.57–4.63 (1 H, m), 4.59 (1 H, d, *J* = 5 Hz), 5.02–5.12 (2 H, m), 5.15–5.25 (1 H, m), 5.84–5.90 (1 H, m), 6.71–6.77 (1 H, m), 6.90–7.00 (1 H, m), 7.27–7.43 (7 H, m), 8.05 (2 H, d, *J* = 8 Hz), 9.00–9.40 (1 H, br s) ppm. – ^{13}C NMR (75.5 MHz): 11.0 (CH_3), 16.2 (CH_3), 25.2 (CH_2), 26.5 (CH_2), 29.7 (CH_2), 33.7 (CH), 38.6 (CH_2), 50.6 (CH), 52.1 (CH), 63.0 (CH_2), 66.5 (CH_2), 67.3 (CH), 70.6 (CH), 127.8 (CH), 127.9 (CH), 128.5 (CH), 129.4 (C), 130.5 (CH), 132.0 (C), 135.9 (C), 140.8 (C), 169.0 (C), 169.2 (C), 170.1 (C), 171.3 (C) ppm. – IR (film): ν = 3022 cm^{-1}, 1844, 1792, 1772, 1734, 1717, 1700, 1696, 1684, 1669, 1653, 1559, 1539, 1457, 1250, 1098, 734. – $[\alpha]_D^{20}$ −5.5 (*c* 0.65, $CHCl_3$). – MS (ESI): *m*/*z* = 1273 ([2 M + Na^+], 100), 648 ([M + Na^+], 90). HRMS (ESI) [M + H^+]. – Calcd. for $C_{32}H_{40}N_3O_{10}$ 626.2714, found 626.2708.

(*S*)-*tert*-Butyloxycarbonyl-alanyl-(*S*)-(N^{δ}-benzyloxycarbonyl)-ornithine *tert*-butyl ester [Boc-Ala-Orn(Cbz)-OtBu] (13). The dipeptide **13** (2.60 g, 84%) was prepared from *N*-*tert*-butyloxycarbonyl-(*S*)-alanine (1.44 g, 7.62 mmol) and (N^{δ}-benzyloxycarbonyl)-(*S*)-ornithine *tert*-butyl ester (2.00 g, 6.30 mmol) according to GP1 as a yellow viscous oil. R_f 0.25 [hexane–EtOAc (1 : 1)] – ^{1}H NMR (300 MHz): 1.26 (3 H, d, *J* = 7 Hz), 1.34 (9 H, s), 1.37 (9 H, s), 1.38–1.76 (4 H, m), 3.14–3.22 (2 H, m), 4.10–4.20 (2 H, br m), 4.38–4.47 (1 H, m), 5.07 (2 H, s), 5.09–5.19 (1 H, br m), 6.80 (1 H, d, *J* = 6 Hz), 7.30–7.34 (5 H, m) ppm. – ^{13}C NMR (75.5 MHz): 18.2 (CH_3), 25.5 (CH_2), 27.9 (CH_3), 28.2 (CH_3), 29.6 (CH_2), 40.4 (CH_2), 50.1 (CH), 52.3 (CH), 66.5 (C), 80.0 (C), 82.2 (CH_2), 128.0 (CH), 128.4 (CH), 136.5 (CH), 155.4 (C), 156.5 (C), 171.0 (C), 172.5 (C) ppm. – IR (film): ν = 3336 cm^{-1}, 2926, 1718, 1700, 1685, 1669, 1653, 1521, 1507, 1457, 1368, 1251, 1159, 1027, 912, 738. – $[\alpha]_D^{20}$ −3.0 (*c* 1.0, $CHCl_3$). – MS (ESI) positive ion mode: *m*/*z* = 1009 ([2 M + Na^+], 100), 516 ([M + Na^+], 34). Negative ion mode: *m*/*z* = 552 ([M + CH_3COO^-], 70), 538 ([M + $HCOO^-$], 100), 492 ([M – H], 48). – HRMS (ESI) [M + H^+] – calcd. for $C_{25}H_{40}N_3O_7$ 494.2866, found 494.2861. – Calcd. for $C_{25}H_{40}N_3O_7$: C 60.83%, H 7.96%, N 8.51%, found: C 60.60%, H 7.86%, N 8.29%.

(2*S*)-[(2*S*)-*tert*-Butyloxycarbonylaminopropionylamino]-5-{[(3*S*)-((1*S*)-methylpropyl)-4-oxooxetane-(2*R*)-carbonyl]amino}pentanoic acid *tert*-butyl ester (14). A mixture of the dipeptide **13** (1.0 g, 2.1 mmol) and Pd/C (50 mg, 10%) in methanol (50 mL) was

stirred at r. t. overnight under an atmosphere of hydrogen, then it was filtered through a pad of Celite, and the volatiles were removed in vacuo. The crude amine obtained was treated with the thioester **4** (510 mg, 1.83 mmol), TMP (438 mg, 3.6 mmol), HOAt (255 mg, 1.8 mmol) and EDC (561 mg, 3.6 mmol) according to GP2 to give 400 mg (38%) of **14**. R_f 0.28 [hexane–EtOAc (1 : 1)]. – ^{1}H NMR (300 MHz): 0.91 (3 H, t, J = 7 Hz), 1.03 (3 H, d, J = 7 Hz), 1.32 (3 H, d, J = 7 Hz), 1.40 (9 H, s), 1.42 (9 H, s), 1.48–1.64 (4 H, m), 1.78–1.86 (1 H, m), 1.88–2.00 (1 H, m), 3.20–3.40 (2 H, m), 3.58 (1 H, dd, J = 9, 5 Hz), 4.10–4.20 (1 H, m), 4.36–4.44 (1 H, m), 4.57 (1 H, d, J = 5 Hz), 5.16–5.22 (1 H, m), 6.80–6.88 (1 H, m) ppm. – ^{13}C NMR (75.5 MHz): 10.9 (CH_3), 16.3 (CH_3), 18.2 (CH), 25.0 (CH_3), 28.2 (CH_3), 29.6 (CH_2), 33.7 (CH_3), 38.5 (CH_2), 50.0 (CH), 52.1 (CH), 62.8 (CH), 70.7 (CH), 80.0 (C), 82.3 (C), 155.5 (C), 168.1 (C), 169.2 (C), 170.8 (C), 172.6 (C) ppm. – IR (film): ν = 3318 cm^{-1}, 2976, 2934, 2878, 1837, 1669, 1540, 1507, 1457, 1368, 1251, 1161, 1098, 738. – $[\alpha]_D^{20}$ = +9.7 (c 1.5, $CHCl_3$). – MS (ESI) positive ion mode: m/z = 1049 ([2 M + Na^+], 100), 536 ([M + Na^+], 64). Negative ion mode: m/z = 558 ([M + $HCOO^-$], 100), 512 ([M − H], 26). – HRMS (ESI) [M + H^+]. – Calcd. for $C_{25}H_{44}N_3O_8$ 514.3128, found 514.3123.

(2*S*)-[(2*S*)-Aminopropionylamino]-5-{[(3*S*)-((1*S*)-methylprop-yl)4-oxooxetane-(2*R*)-carbonyl]amino}pentanoic acid (15). The tripeptide **14** (75 mg, 0.15 mmol) was mixed with CH_2Cl_2 (2 mL) and trifluoroacetic acid (2 mL) at 0 °C, and this mixture was kept at −15 °C overnight. The volatiles were distilled off, and the obtained hydrotrifluoroacetate salt was dried *in vacuo*. Yield 71 mg (99%). – ^{1}H NMR (CD_3OD, 300 MHz): 0.94 (3 H, t, J = 7 Hz), 1.03 (3 H, d, J = 7 Hz), 1.25–1.32 (1 H, m), 1.52 (3 H, d, J = 7 Hz), 1.60–1.71 (2 H, m), 1.90–2.00 (2 H, m), 3.25–3.32 (2 H, m), 3.65 (1 H, dd, J = 8, 4 Hz), 3.93–4.00 (1 H, m), 4.40–4.46 (1 H, m), 4.70 (1 H, d, J = 4 Hz) ppm. ^{13}C NMR (75.5 MHz): 11.3 (CH_3), 16.6 (CH_3) 17.5 (CH_3), 26.7 (CH_2), 27.7 (CH_2), 29.6 (CH_2), 36.9 (CH), 39.7 (CH_2), 50.2 (CH), 53.4 (CH), 63.8 (CH), 72.9 (CH), 170.7 (C), 171.0 (C), 171.1 (C), 174.5 (C) ppm. – IR (film): ν = 3420 cm^{-1}, 2967, 2934, 1830, 1684, 1540, 1457, 1362, 1203, 1140. – $[\alpha]_D^{20}$ −3.6 (*c* 1.1, MeOH). – MS (ESI) positive ion mode: m/z = 715 ([2 M + H^+], 14), 358 ([M + H^+], 100). Negative ion mode: m/z = 713 ([2 M − H], 96), 356 ([M − H], 100). – HRMS (ESI) [M + H^+]. – Calcd. for $C_{16}H_{28}N_3O_6$ 358.1978, found 358.1973.

Investigation of biological activities of the new belactosin C congeners

Methods

1. Cell culture. HeLa cells (epithelial cells derived from human cervix carcinoma) were obtained from the American Type Culture Collection. The cells were maintained in RPMI (Gibco, Karlsruhe, Germany) supplemented with 10% FCS, 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin in a humidified 5% CO_2 atmosphere.

Primary mouse ear fibroblasts were isolated from Ub^{G76V} GFP1 mice.[12] Mouse ears were washed in 70% EtOH for 2 min and cut into small pieces in DMEM using a scalpel. Pieces were transferred into a 15 mL tube containing 2 mL of 0.25% trypsin. After incubation for 1 h at 37 °C, trypsin was inactivated by adding 5 mL of DMEM/10%FCS. The suspension was centrifuged at 1000 rpm for 5 min. The pellet was resuspended in 1 mL of DMEM) supplemented with 10% FCS, 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin and transferred into a well of a 6 well-plate. Fibroblasts had grown after 1–2 days and covered the well bottom after 3–5 days.

2. Assay of proteasomal activity. Proteasome chymotrypsin-like, trypsin-like and caspase-like activities from HeLa cell lysates were determined fluorometrically in a spectramax GEMINI-EM (MolecularDevices) by using synthetic peptides linked to the fluorophor methylcoumarine. ChTL activity was measured by SLLVY-AMC hydrolysis, TL by BzVGR-AMC and CaspL activity by ZLLE-AMC hydrolysis with 360-nm excitation and 460-nm emission wavelengths.

Cells were treated for indicated times with indicated substances or solvent (DMSO) as a control. Cells were subsequently washed with PBS, and then scraped and lysed under hypotonic conditions with repeated cycles of thawing and freezing in liquid nitrogen. Lysates were centrifuged, and the protein content of the supernatant was estimated by BCA protein assay (Pierce). Lysates were incubated for 30 min at 37 °C in incubation buffer containing an ATP-regenerating system (225 mM Tris–HCl, pH 8.2, 45 mM KCl, 7.5 mM $Mg(CH_3COO)_2$, 7.5 mM $MgCl_2$, 1.1 mM dithiothreitol, 6 mM ATP, 5 mM phosphocreatine, 0.2 unit of phosphocreatinekinase) and 0.2 mM of the appropriate fluorogenic substrate. Enzymatic activity was normalized to protein concentration and expressed as a percentage of activity of the solvent-treated control. The values are given as the means of three independent experiments ± S.E.M.

3. Western blot. Cells were lysed in extraction buffer containing 50 mM Tris/HCl 5 (pH 7.4), 150 mM KCl, 5 mM glucose, 50 mM EDTA, 1 mM PMSF, 2 mM DTT 2, and 1% Triton X-100. Total protein (20 µg per lane) was subjected to SDS-PAGE and membranes were probed with the respective antibodies: rabbit anti-GFP (Epitomics); rabbit anti-Ubi (DAKO). Bands were visualized by employing the ECL detection system (Amersham). Amido black staining of membranes served as control for equal protein loading.

4. Co-crystallisation. Crystals of the 20S proteasome from *S. cerevisiae* were grown in hanging drops at 24 °C as has previously been described[13] and incubated for 48 h with the chemical compound **6** at 10 mM. The protein concentration used for the crystallization was 45 mg mL^{-1} in Tris-HCl (10 mM, pH 7.5) and EDTA (1 mM). The drops contained 1 µL of protein and 1 xµL of the reservoir solution, containing 20 mM of magnesium acetate, 100 mM of morpholino-ethanesulfonic acid (pH 6.9) and 10% of MPD.

The space group belongs to $P2_1$ with cell dimensions of a = 134.9 Å, b = 301.6 Å, c = 144.2 Å and β = 112.9° (see Table S1†). Data to 2.7 Å for the proteasome : inhibitor-complex were collected using synchrotron radiation with λ = 1.0 Å at the X06SA-beamline in SLS/Villingen/Switzerland. Crystals were soaked in a cryoprotecting buffer (30% MPD, 20 mM of magnesium acetate, 100 mM of morpholino-ethanesulfonic acid pH 6.9) and frozen in a stream of liquid nitrogen gas at 100 K (Oxford Cryo Systems). X-ray intensities were evaluated employing the XDS program package.[14] The anisotropy of diffraction was corrected by an overall anisotropic temperature factor by comparing

observed and calculated structure amplitudes using the program CNS.[15,16] Electron density was improved by averaging and back transforming the reflections 10 times over the twofold noncrystallographic symmetry axis using the program package MAIN.[17] Conventional crystallographic rigid body, positional and temperature factor refinements were carried out with CNS using the yeast 20S proteasome structure as a starting model.[7] Modelling experiments were performed with the program MAIN with current crystallographic values of $R_{cryst} = 0.212$, $R_{free} = 0.237$ [18] (Table S1†).

Accession number. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (entry code 3TDD).

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Notes and references

1 M. Groll, O. V. Larionov, M. Huber and A. de Meijere, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 4576.
2 A. Asai, A. Hasegawa, K. Ochiai, Y. Yamashita and T. Mizukami, *J. Antibiot.*, 2000, **53**, 81.
3 A. Asai, T. Tsujita, S. V. Sharma, Y. Yamashita, S. Akinaga, M. Funakoshi, H. Kobayashi and T. Mizukami, *Biochem. Pharmacol.*, 2004, **67**, 227.
4 K. Yoshida, K. Yamaguchi, A. Mizuno, Y. Unno, A. Asai, T. Sone, H. Yokosawa, A. Matsuda, M. Arisawa and S. Shuto, *Org. Biomol. Chem.*, 2009, **7**, 1868.
5 T. Muthny, M. Koverik, L. Sispera, A. de Meijere, O. V. Larionov, I. Tilser and M. Holecek, *J. Physiol. Biochem.*, 2009, **65**, 137.
6 (*a*) A. Armstrong and J. N. Scutt, *Chem. Commun.*, 2004, (5), 510; (*b*) O. V. Larionov and A. de Meijere, *Org. Lett.*, 2004, **6**, 337; (*c*) G. Kumaraswamy, M. Padmaja, B. Markondaiah, N. Jena, B. Sridhar and M. U. Kiran, *J. Org. Chem.*, 2006, **71**, 337; (*d*) S. W. Cho and D. Romo, *Org. Lett.*, 2007, **9**, 1537; (*e*) G. Kumaraswamy and B. Markondaiah, *Tetrahedron Lett.*, 2007, **48**, 1707; (*f*) K. Yoshida, K. Yamaguchi, T. Sone, Y. Unno, A. Asai, H. Yokosawa, A. Matsuda, M. Arisawa and S. Shuto, *Org. Lett.*, 2008, **10**, 3571.
7 M. Groll, L. Ditzel, J. Löwe, D. Stock, M. Bochtler, H. D. Bartunik and R. Huber, *Nature*, 1997, **386**, 463.
8 L. Borissenko and M. Groll, *Chem. Rev.*, 2007, **107**, 687.
9 L. Borissenko and M. Groll, *Biol. Chem.*, 2007, **388**, 947.
10 (*a*) M. Graffner-Nordberg, J. Marelius, S. Ohlsson, Å. Persson, G. Swedberg, P. Andersson, S. E. Andersson, J. Åqvist and A. Hallberg, *J. Med. Chem.*, 2000, **43**, 3852; (*b*) S. Narasimhan, S. Swarnalakshmi and R. Balakumar, *Synth. Commun.*, 2000, **30**, 941.
11 2-Naphthylmethyl chloroformate (CNAP-Cl) was prepared according to: E. A. Papageorgiou, M. J. Gaunt, J.-q. Yu and J. B. Spencer, *Org. Lett.*, 2000, **2**, 1049.
12 K. Lindsten, V. Menéndez-Benito, M. G. Masucci and N. P. Dantuma, *Nat. Biotechnol.*, 2003, **21**, 897.
13 M. Groll and R. Huber, *Methods Enzymol.*, 2005, **398**, 329.
14 W. Kabsch, *J. Appl. Cryst.*, 1993, **26**, 795.
15 A. T. Brünger, P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J.-S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Rice, T. Simonson and G. L. Warren, *Acta Crystallogr D Biol Crystallogr.*, 1998, **1**, 905–921.
16 A. Brünger, *X-PLOR version 3.1. A system for X-ray crystallography and NMR*, Yale University Press, New Haven, (1992).
17 D. Turk, *Improvement of a program for molecular graphics and manipulation of electron densities and its application for protein structure determination*, Dissertation, Technische Universitaet Muenchen, (1992).
18 A. T. Brünger, *Nature*, 1992, **355**, 472.

