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# The arginine mimicking β-amino acid $\beta^3$hPhe(3-$H_2N$-$CH_2$) as S1 ligand in cyclotheonamide-based β-tryptase inhibitors

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ABSTRACT

β-Tryptase, a mast-cell specific serine protease with trypsin-like activity, has emerged in the last years as a promising novel therapeutic target in the field of allergic inflammation. Recently, we have developed a potent and selective β-tryptase inhibitor based on the natural product cyclotheonamide E4 by implementing a basic P3 residue that addresses the determinants of the extended substrate specificity of β-tryptase. To further improve the affinity/selectivity profile of this lead structure, we have now investigated β-homo-3-aminomethylphenylalanine as S1 ligand. In contrast to the corresponding β-homo amino acids derived from lysine or arginine, we demonstrate that this particular basic β-homo amino acid is a privileged S1 ligand for the development of β-tryptase inhibitors. Besides affinity, selectivity and reduced basicity, these novel cyclotheonamide E4 analogs show excellent stability in human plasma and serum.



## 1. Introduction

Like other leukocytes, mast cells are derived from hematopoietic progenitor cells of the bone marrow. In contrast to basophiles that circulate as mature cells in the blood, mast cells reside in virtually all peripheral tissues of the human body, in particular the dermis, the lung, and the mucosa and submucosa of the intestine. It is well accepted that mast cells play a pivotal role in the pathogenesis of a variety of allergic and inflammatory diseases, most prominent among them allergic asthma, psoriasis, and rheumatoid arthritis.[1–3] Beside by other mechanisms, mast cells are activated upon crosslinking of FcεRI-bound IgE with multivalent allergens on the cells' surface. The signaling cascade elicited eventually results in downstream events that trigger the release of a set of preformed mediators, which are stored in cytoplasmic granules, into the surrounding tissue by degranulation.[4]

The mediators released by degranulation include, for example, histamine and heparin proteoglycan as well as β-tryptase, a trypsin-like serine protease that is abundantly and virtually exclusively expressed in mast cells.[1] The amount of catalytically active β-tryptase accounts for up to 20% of the entire protein of a mast cell and thus is higher than those of proteases like elastase and cathepsin G found in other granulocytes.[5] Therefore β-tryptase is used as a valuable marker for the activation and localization of mast cells.[6] In particular, increased levels of β-tryptase in the patient's circulation are utilized clinically to confirm anaphylactic reactions.[7] Typically, in insect sting-induced systemic anaphylaxis the level of β-tryptase reaches its maximum within 15–120 min after the sting and subsequently declines with a half live of 1.5–2.5 h.[8] The magnitude of β-tryptase levels observed in patients suffering from insect sting reactions correlate with the clinical severity as determined by the drop in mean arterial pressure.[9]

In addition to its well established role as clinical marker, β-tryptase has emerged as promising new target for therapeutic intervention in allergic asthma.[10] The secreted protease has been implicated in modulation of bronchial tone, airway inflammation and tissue remodeling, the key features of asthma. Indeed β-tryptase degrades and inactivates the neuropeptides vasoactive intestinal peptide (VIP)[11] and calcitonin gene-related peptide (CGRP)[12] and thus via its peptidolytic activity can modulate airway smooth muscle tone, leading to bronchoconstriction. In addition, β-tryptase was shown to induce the formation of edema,[13,14] to recruit neutrophils and eosinophils,[15–17] and to act as a mitogen for airway smooth muscle cells.

By implementing information derived from substrate specificity screening[18] onto the cyclotheonamide E4 scaffold we have recently developed a potent and selective β-tryptase inhibitor (**1**, Scheme 1).[19] For this, the natural product cyclotheonamide E4 was modified at two key positions: the α-amino function of (*S*)-2,3-diamino propionic acid was used as anchoring point for ε-amino hexanoic acid as optimized basic P3 residue. Based on modeling studies performed by Harris et al. to elucidate the structural features responsible for the extended substrate specificity of

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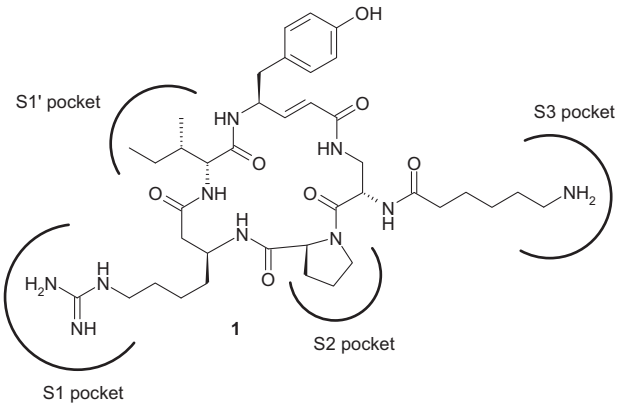
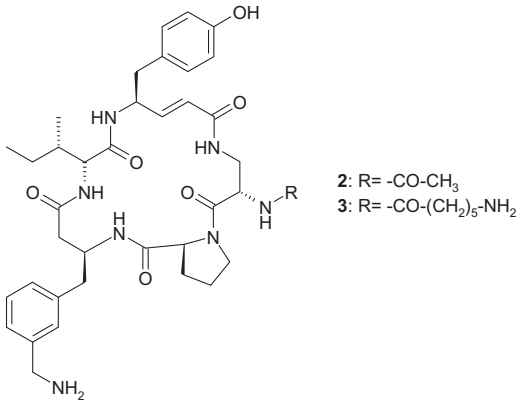



**Scheme 1.** Chemical structure of the cyclotheonamide E4-based β-tryptase inhibitor **1**. The proposed binding mode is indicated.

β-tryptase,[18] it is supposed that this S3 ligand interacts with the negatively charged residues Glu-217 of the cognate as well as Asp-60B' of the adjacent subunit of the β-tryptase tetramer. In addition, the S1 ligand present in the natural product, (*S*)-3-amino-6-guanidino-2-oxo-hexanoic acid, was substituted by the structurally related β-homoarginine to shift the binding from a covalent into a fully reversible mode.

In a previous study we have utilized 3-aminomethyl-phenylalanine as S1 ligand in bivalent β-tryptase inhibitors[20] and shown that Ac-DL-Phe(3-$H_2N$-$CH_2$)-OMe is a moderately potent and selective inhibitor of β-tryptase ($K_i$ (β-tryptase) 14 μM vs $K_i$ (trypsin) 400 μM). Taking advantage of these findings for the design and synthesis of cyclotheonamide E4-based β-tryptase inhibitors we have now investigated whether β-homo-3-aminomethylphenylalanine as S1 ligand in cyclotheonamide E4-based inhibitors increases selectivity for β-tryptase among trypsin-like serine proteases. Therefore, we have synthesized and assessed the inhibitory profile of inhibitor **2** (Scheme 2) which does not address the proposed determinants of β-tryptases' extended substrate specificity. To further improve the inhibitory profile of our lead compound **1**, the cyclotheonamide E4-based inhibitor **3** (Scheme 2) containing β-homo-3-aminomethyl-phenylalanine at P1 position was synthesized and its affinity and selectivity properties against a representative panel of trypsin-like serine proteases determined. In addition, the stability of both inhibitors against proteolytic degradation in human plasma and serum was determined.




**Scheme 2.** Chemical structures of the cyclotheonamide E4-based β-tryptase inhibitors **2** and **3** containing β-homo-3-aminomethylphenylalanine as S1 ligand.

## 2. Results and discussion

### 2.1. Chemistry

#### 2.1.1. Synthesis of Fmoc-$\beta^3$hPhe(3-BocHN-$CH_2$)-OH (4)

To incorporate β-homo-3-aminomethylphenylalanine as S1 ligand onto the cyclotheonamide E4 scaffold we have focused on a solid phase-assisted assembly based on the Fmoc/*t*Bu protecting group scheme. First, it was necessary to establish a synthetic approach towards the Fmoc amino acid building block Fmoc-$\beta^3$hPhe(3-BocHN-$CH_2$)-OH (**4**). For this, we took partially advantage from a synthetic approach towards Ac-DL-Phe(3-$H_2N$-$CH_2$)-OMe that we have established previously.[20] Thus, as starting material for the synthesis H-Phe(3-CN)-OH (**5**) was selected. Upon conversion into Pht-Phe(3-CN)-OMe (**7**) by standard procedures (Scheme 3), the nitrile function was reduced smoothly using hydrogen in the presence of 10% Pd-C, and the resulting amino function was Boc-protected yielding Pht-Phe(3-Boc-HN-$CH_2$)-OMe (**9**). After mild hydrazinolysis of the Pht-group by hydrazine acetate, saponification of the methyl ester, and Fmoc-protection of the free amino function Fmoc-Phe(3-Boc-HN-$CH_2$)-OH (**12**) was obtained over seven steps in 18% yield. Subsequently, applying the Seebach protocol[21] the protected amino acid **12** was converted into the corresponding β-homo amino acid **4**. Briefly, **12** was smoothly transferred into the diazoketone **13** which was then subjected to Arndt–Eistert homologation yielding the Fmoc amino acid building block **4**.

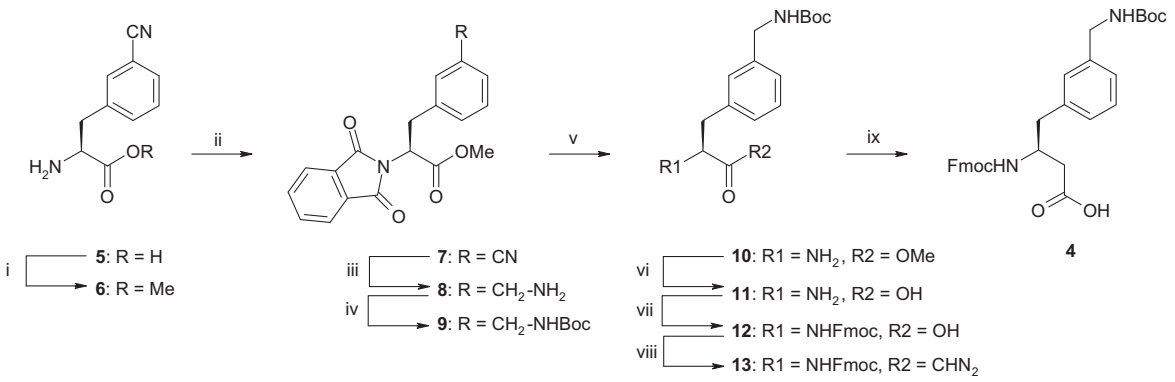
#### 2.1.2. Synthesis of the cyclotheonamide E4-based β-tryptase inhibitors 2 and 3 containing β-homo-3-aminomethyl-phenylalanine as S1 ligand

The synthesis of inhibitors **2** and **3** was performed by a combination of solid phase and solution phase chemistry following a strategy that we have utilized previously to establish an optimal basic S3 ligand (Scheme 4).[19] Briefly, after immobilization of H-Dap(Fmoc)-OAl through its α-amino function on a 2-chlorotrityl resin the linear pentapeptide was built stepwise according to the Fmoc/*t*Bu protocol for solid phase peptide synthesis using optimized excesses of each Fmoc-amino acid building block for the coupling step. After stepwise deprotection of the termini of the resin-bound and side chain protected linear pentapeptide **15**, cyclization was performed smoothly on-resin without appreciable oligomerization using PyBOP essentially according to a procedure described by the group of Albericio.[22] Then the fully protected cyclotheonamide E4 scaffold **16** was cleaved from the resin under mild acidic conditions, thereby liberating the α-amino function of (*S*)-2,3-diamino propionic acid for further functionalization in solution. Eventually **16** was reacted either with acetic anhydride or with Boc-protected ε-aminohexanoic acid in the presence of TBTU and subjected to the final deprotection step yielding the inhibitors **2** and **3** based on the initial resin loading with 29% and 32%, respectively, which were fully characterized by RP-HPLC, $^1$H NMR and HRMS.

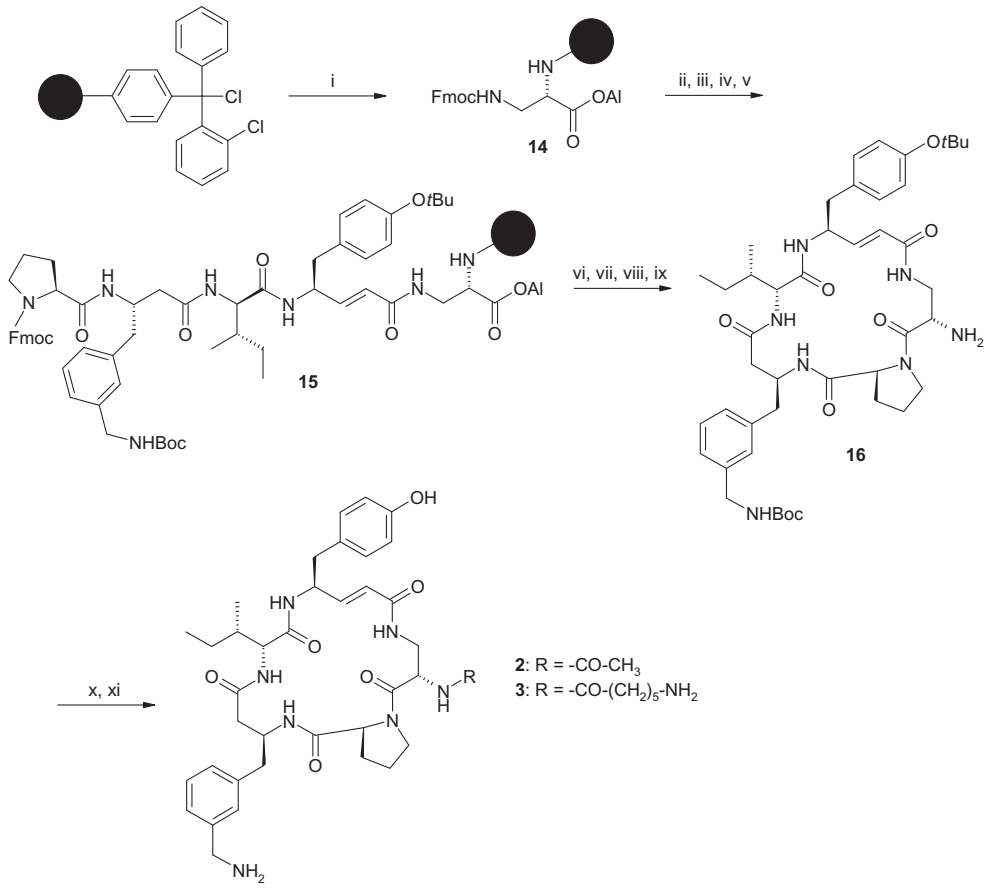
### 2.2. Biological activity

#### 2.2.1. Inhibitory profile of cyclotheonamide E4 analogs 2 and 3

The equilibrium dissociation constants $K_i$ for inhibition of β-tryptase by the inhibitors **2** and **3** reported in Table 1 were determined essentially as described previously.[19,23] In addition, to assess the selectivity of the inhibitors for β-tryptase among the trypsin-like serine proteases, $K_i$ values for trypsin were determined. The comparison of the different S1 ligands including β-homolysine (**17**), β-homoarginine (**18**) and β-homo-3-aminomethylphenylalanine (**2**) on the affinity/selectivity profile of



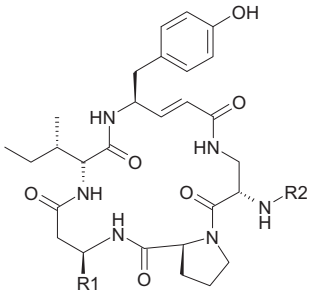

**Scheme 3.** Synthesis of Fmoc-$\beta^3$hPhe(3-BocHN-$CH_2$)-OH (**4**). Reagents and conditions: (i) MeOH, $SOCl_2$, 89%; (ii) *N*-ethoxycarbonylphthalimide, $Na_2CO_3$, dioxane/$H_2O$ (1:1, v/v), 87%; (iii) 10% Pd-C/$H_2$, AcOH, 52%; (iv) $(Boc)_2O$, $NaHCO_3$, dioxane/$H_2O$ (1:1, v/v), 96%; (v) $H_2N$-$NH_2$ × AcOH, MeOH, 76%; (vi) NaOH, dioxane/$H_2O$ (2:1, v/v), 82%; (vii) Fmoc-OSu, $NaHCO_3$, dioxane/$H_2O$ (2:1, v/v), 76%; (viii) (1) $Et_3N$, $ClCO_2Et$, THF, (2) $CH_2N_2$, $Et_2O$, 86%; (ix) $C_6H_5COOAg$, dioxane/$H_2O$ (5:3, v/v), 91%.

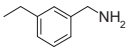
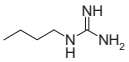
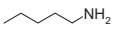
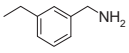
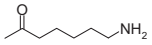
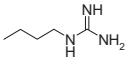
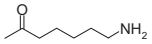



**Scheme 4.** Synthesis of the cyclotheonamide E4 analogs **2** and **3** containing β-homo-3-aminomethylphenylalanine as S1 ligand. Reagents and conditions: (i) (1) H-Dap(Fmoc)-OAl × HCl (2 equiv), DIPEA, $CH_2Cl_2$, (2) MeOH/$CH_2Cl_2$, DIPEA; (ii) (1) piperidine/DMF (1:4 v/v), (2) Fmoc-v-Tyr(*t*Bu)-OH/TBTU/HOBt/DIPEA (1:1:1:1, 2 × 1 equiv), DMF; (iii) (1) piperidine/DMF (1:4 v/v), (2) Fmoc-D-*allo*-Ile-OH/TBTU/HOBt/DIPEA (1:1:1:1, 4 equiv), DMF; (iv) (1) piperidine/DMF (1:4 v/v), (2) Fmoc-$\beta^3$hPhe(3-BocHN-$CH_2$)-OH (**4**)/TBTU/HOBt/DIPEA (1:1:1:1, 2 × 1 equiv), DMF; (v) (1) piperidine/DMF (1:4 v/v), (2) Fmoc-Pro-OH/TBTU/HOBt/DIPEA (1:1:1:1, 4 equiv), DMF; (vi) $Pd(PPh_3)_4$ (3 × 0.4 equiv), $PhSiH_3$ (3 × 24 equiv), $CH_2Cl_2$; (vii) piperidine/DMF (1:4 v/v); (viii) PyBOP/HOBt/DIPEA (1:1:2, 3 equiv), DMF; (ix) TFA/TIS/$CH_2Cl_2$ (1:1:98 v/v/v), over eight steps 96%; (x) BocHN($CH_2$)$_5$COOH/TBTU/HOBt/DIPEA (1:1:1:1, 2 equiv), DMF or $Ac_2O$/pyridine (1:1, 10 equiv), $CH_2Cl_2$; (xi) TFA/$H_2O$, (95:5 v/v), over two steps: **2**: 30%, **3**: 33%.

cyclotheonamide E4-based β-tryptase inhibitors clearly reveals that β-homo-3-aminomethylphenylalanine is by far the most potent one of the series (Table 1). Thus, the affinity of inhibitor **2** to inhibit the activity of β-tryptase is more than 600-fold higher than that of inhibitor **17** and about 70-fold higher than that of inhibitor **18**. Furthermore, inhibitors **17** and **18** do not discriminate between β-tryptase and trypsin, whereas compound **2** inhibits the activity of β-tryptase more than two orders of magnitude more effectively than that of trypsin. Substituting the acetyl group (inhibitor **2**) by a 6-aminohexanoyl group (inhibitor **3**) increases the affinity by a factor of ~25 (**2**: $K_i$ 0.6 μM vs **3**: $K_i$ 0.025 μM). These findings suggest that the basic S3 ligand exploits interactions with the acidic residues Glu-217 of the cognate as well as Asp-60B' of the adjacent subunit of the β-tryptase tetramer that have been proposed to be responsible for the extended substrate specificity of β-tryptase.[18] More importantly, the selectivity ratio $K_i$(trypsin)/$K_i$(β-tryptase)

**Table 1**
Affinity/selectivity profiles of the cyclotheonamide E4-based β-tryptase inhibitors **2** and **3**



| Inhibitor | R1 | R2 | $K_i$ (μM)[a] | | Selectivity[b] |
|---|---|---|---|---|---|
| | | | β-Tryptase | Trypsin | |
| **2** |  |  | 0.60 | 100 | 170 |
| **18** |  |  | 40 | 30 | 0.8 |
| **17** |  |  | 380 | 230 | 0.6 |
| **3** |  |  | 0.025 | 50 | 2000 |
| **1** |  |  | 0.007 | 10 | 1400 |

[a] The $K_i$ values of inhibitors **1**, **17** and **18** were taken from Ref. 19.
[b] $K_i$(trypsin)/$K_i$(β-tryptase).

**Table 2**
Expanded affinity/selectivity profiles of the cyclotheonamide E4-based β-tryptase inhibitors **2** and **3**

| Inhibitor | $K_i$ (μM) | | | | | |
|---|---|---|---|---|---|---|
| | β-Tryptase | Trypsin | Thrombin | Factor Xa | Tissue kallikrein | Plasma kallikrein |
| **2** | 0.60 | 100 | 680 | 310 | 1800 | 3300 |
| **3** | 0.025 | 50 | 330 | 360 | 1300 | 2000 |

is improved from 170 to 2000. A comparison of inhibitor **3** with our previous lead compound **1** shows that inhibitor **3** exhibits virtually the same affinity/selectivity profile as inhibitor **1** despite a considerably reduced basicity.

The affinity of inhibitor **3** and even inhibitor **2** towards β-tryptase prompted us to extend our selectivity studies on a broader panel of trypsin-like serine proteases. Therefore, the affinities of both inhibitors for thrombin, factor Xa, tissue kallikrein, and plasma kallikrein were determined.[23,24] The results summarized in Table 2 clearly show that both inhibitors exhibit an excellent selectivity profile for β-tryptase. Importantly, the introduction of ε-aminohexanoic acid as basic P3 residue (inhibitor **3**) increases the affinity towards β-tryptase but not those for other relevant trypsin-like proteases, resulting in a superior selectivity profile in comparison to inhibitor **2**.

### 2.2.2. Stability of the inhibitors 2 and 3 in human plasma and serum

In order to utilize cyclotheonamide E4-based inhibitors as tools for the analysis of β-tryptase activity in body fluids and tissues the

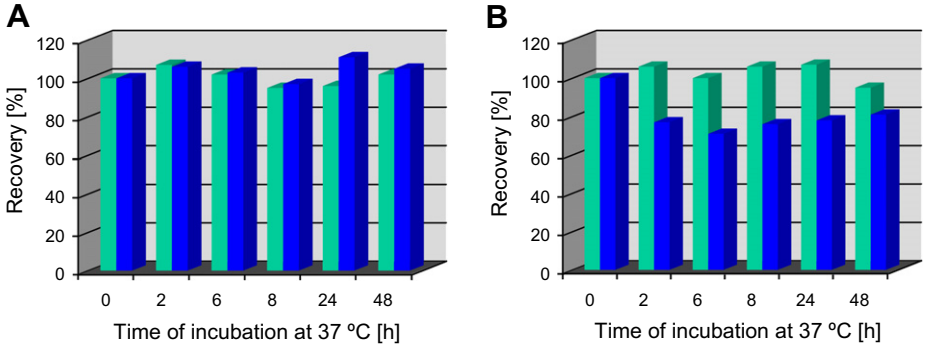



**Figure 1.** Stability of inhibitors **2** (●) and **3** (●) in (A) human plasma and (B) human serum.

stability of these peptidic compounds is an important issue that has to be addressed. To investigate this aspect, both inhibitors **2** and **3** were incubated in undiluted human plasma and human serum at 37 °C for up to 48 h, and the remaining amount of inhibitor was subsequently determined by analytical RP-HPLC. The data summarized in Figure 1 verify that both inhibitors are remarkable stable against proteolytic degradation or other modification in human plasma and even in serum containing the various activated trypsin-like serine proteases of the blood coagulation cascade. Even after 48 hours incubation at 37 °C the recovery of the inhibitors **2** and **3** is almost quantitatively in human plasma and only slightly reduced in human serum (inhibitor **2**: 95% and inhibitor **3**: 81%).

## 3. Conclusion

We have identified β-homo-3-aminomethylphenylalanine as a privileged S1 ligand for the development of β-tryptase inhibitors based on the cyclotheonamide E4 scaffold. In particular inhibitor **3** is a remarkable potent β-tryptase inhibitor that blocks the proteolytic activity of this enzyme within a panel of five physiological relevant trypsin-like serine proteases with exceptional high selectivity. Even inhibitor **2,** which lacks the basic group proposed to address the determinants of β-tryptases' extended substrate specificity, is still reasonably potent and selective. Furthermore, both inhibitors are remarkable stable even during prolonged incubation in human plasma and serum. Thus, inhibitors **2** and **3** are interesting new lead compounds for the development of β-tryptase inhibitors based on the cyclotheonamide E4 scaffold.

## 4. Experimental

### 4.1. Chemistry

Solvents and reagents were of the highest purity commercially available and were used without further purification. TBTU, HOBt, Fmoc-Pro-OH, and Fmoc-OSu were purchased from Iris Biotech (Marktredwitz, Germany), H-Phe(3-CN)-OH from Senn Chemicals (Dielsdorf, Switzerland), Fmoc-D-*allo*-Ile-OH from Bachem (Bubendorf, Switzerland), Diazald®, silver benzoate, and phenyl silane from Sigma–Aldrich, triisopropylsilane (TIS), $Boc_2O$, and hydrazine hydrate from Fluka, and 2-chlorotrityl chloride resin from Novabiochem/Merck Chemicals Ltd (Nottingham, UK). Fmoc-vTyr(*t*Bu)-OH and H-Dap(Fmoc)-OAl were synthesized as described previously[19] and diazomethane using Diazald® and the Diazald®-Distillation-Kit from Sigma–Aldrich according to a procedure of de Boer.[25] Manual peptide synthesis was carried out on a IKA KS 130 basic laboratory shaker (Staufen, Germany) using plastic syringes type Norm-Ject from Henke Sass Wolf (Tuttlingen, Germany) equipped with PE frits (35 μm pore size) and PE stoppers from Roland Vetter Laborbedarf (Ammerbuch, Germany). Precipitated peptides were collected by centrifugation using a Labofuge I from Heraeus Christ GmbH (Hanau, Germany). $^1H$ and $^{13}C$ NMR spectra were recorded with a DRX 500 spectrometer from Bruker, ESI-MS spectra with a Esquire 3000 from Bruker Daltonics, MALDI-MS spectra with a Voyager-DE Biospectroscopy from PerSeptive Biosystems using 2,5-dihydroxy benzoic acid as matrix, and HRMS (ESI) spectra with a APEX III FT-ICR mass spectrometer from Bruker Daltonics. TLC was performed on silica gel 60 plates with fluorescence indicator $F_{254}$ and flash chromatography using silica gel 60 (230–400 mesh) from VWR International GmbH (Darmstadt, Germany), respectively. Spots were visualized with UV light ($\lambda$ = 254 nm) using a lamp (type MinUVIS) from Desaga (Heidelberg, Germany). Analytical RP-HPLC was performed with a HPLC system from Thermo Separation Products using a column from Phenomenex (column I; type Jupiter 5 μm, C18, 300 Å, 250 × 4.60 mm) and eluting (flow rate: 1 mL/min) with the gradient I: 0–3 min, isocratic A/B (100:0); 3–35 min, linear gradient from A/B (100:0) to A/B (0:100) or using a column from Macherey-Nagel (column II; type ET 125/4 Nucleosil 100-5 C18 PPN) and eluting (flow rate: 1.5 mL/min) with the gradient II: 0–1 min, isocratic A/B (100:0); 1–14 min, linear gradient from A/B (100:0) to A/B (0:100) (eluent A: $H_2O$/MeCN/TFA, 95:5:0.1 v/v/v, eluent B: MeCN/$H_2O$/TFA, 95:5:0.1 v/v/v). Preparative RP-HPLC was performed with a HPLC system Type LaChrome from Merck Hitachi using a column from Phenomenex (Type Jupiter 10 μm, C18, 300 Å, 250 × 21.20 mm) and eluting (flow rate: 10 mL/min) with the following gradient: 0–5 min, isocratic A/B (100:0); 5–50 min, linear gradient from A/B (100:0) to A/B (50:50) (eluent A: $H_2O$/MeCN/TFA, 95:5:0.1 v/v/v, eluent B: MeCN/$H_2O$/TFA, 95:5:0.1 v/v/v).

#### 4.1.1. H-Phe(3-CN)-OMe × HCl (6)

To MeOH (30 mL, 739.70 mmol) was added under stirring dropwise at −10 °C within 10 min thionyl chloride (2.11 mL, 29.07 mmol) followed by H-Phe(3-CN)-OH (4.93 g, 25.92 mmol) as a solid. The resulting solution was stirred for 3.5 h at 45 °C and then over night at r.t. The solvent was evaporated under reduced pressure and the title compound was isolated as colorless solid by precipitation from MeOH/*tert*-butyl methyl ether; yield: 5.58 g (89%); TLC (*n*-butanol/AcOH/$H_2O$/AcOEt, 3:1:1:5, v/v/v/v) $R_f$ 0.4; $^1H$ NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 3.25 (m, 2H, β-C$H_2$), 3.69 (s, 3H, C$H_3$), 4.36 (t, $J$ = 6.6 Hz, 1H, α-C*H*), 7.55 (t, $J$ = 7.8 Hz, 1H, C5*H* Ar), 7.62 (d, $J$ = 8.2 Hz, 1H, C6*H* Ar), 7.77 (d, $J$ = 7.5 Hz, 1H, C4*H* Ar), 7.79 (s, 1H, C2*H* Ar), 8.78 (s, 3H, N$H_3$); $^{13}C$ NMR (125.7 MHz, DMSO-$d_6$, 25 °C): δ [ppm] = 35.4 (β*C*), 53.2 (α*C* and O$CH_3$), 111.9 (*C*3 Ar), 119.2 (*C*N), 130.2, 131.6, 133.7, 135.0 (*C*2, *C*4, *C*5, *C*6 Ar), 137.0 (*C*1 Ar), 169.6 (*C*=O); ESI-MS: $m/z$ 205.0 $[M+H]^+$; calcd for $C_{11}H_{12}N_2O_2$: 204.1.

#### 4.1.2. Pht-Phe(3-CN)-OMe (7)

H-Phe(3-CN)-OMe × HCl (**6**; 5.50 g, 22.85 mmol) was dissolved in 1,4-dioxane (60 mL) and a solution of $Na_2CO_3$ (2.42 g, 22.85 mmol) in $H_2O$ (40 mL) added. To the resulting solution at r.t., *N*-ethoxycarbonylphthalimide (6.51 g, 29.71 mmol) was added as solid under stirring and stirring was continued over night. The solvent was evaporated and the resulting pale yellow solid was distributed between AcOEt (100 mL) and $H_2O$ (50 mL). The organic phase was washed with 5% aq $KHSO_4$ (3 × 50 mL), brine (1 × 50 mL), dried ($Na_2SO_4$), and the solvent evaporated. The crude product was purified by flash chromatography (eluent: AcOEt/petroleum ether 1:6; v/v). The title compound was obtained as colorless solid; yield: 6.64 g (87%); RP-HPLC (column I, gradient I): $t_R$ 25.5 min; TLC (AcOEt/petroleum ether 1:6; v/v): $R_f$ 0.2; $^1H$ NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 3.31–3.36 (m, 1H, $\beta_2$-C$H_2$), 3.57 (dd, $J$ = 14.1, 4.9 Hz, 1H, $\beta_1$-C$H_2$), 3.70 (s, 3H, C$H_3$), 5.35 (dd, $J$ = 11.2, 4.9 Hz, 1H, α-C*H*), 7.39 (t, $J$ = 7.8 Hz, 1H, C5*H* Phe(3-CN)-Ar), 7.50 (d, $J$ = 7.5 Hz, 1H, C6*H* Phe(3-CN)-Ar), 7.61 (d, $J$ = 7.5 Hz, 1H, C4*H* Phe(3-CN)-Ar), 7.71 (s, 1H, C2*H* Phe(3-CN)-Ar), 7.86 (m, 4H, C*H* Pht-Ar); $^{13}C$ NMR (125.7 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 34.1 (β*C*), 52.6 (O$CH_3$), 53.3 (α*C*), 111.7 (*C*3 Phe(3-CN)-Ar), 119.1 (*C*N), 124.1, 130.0, 131.0, 133.1, 134.4, 135.6 (*C* Pht-Ar and *C* Phe(3-CN)-Ar), 139.1 (*C* Pht-Ar), 167.3 (*C*=O Pht), 169.3 (*C*=O); ESI-MS: $m/z$ 357.1 $[M+Na]^+$; calcd for $C_{19}H_{14}N_2O_4$: 334.1.

#### 4.1.3. Pht-Phe(3-$H_2$N-$CH_2$)-OMe × HCl (8)

To a solution of Pht-Phe(3-CN)-OMe (**7**; 3.66 g, 10.95 mmol) in AcOH (170 mL), aq HCl (2.2 mL, 10 M), and 10% Pd-C (3.60 g) were added. The reduction was performed at r.t. under static hydrogen pressure (4.8 bar) for 5 d. Subsequently, the catalyst was filtered off and the solvent evaporated under reduced pressure. The

resulting material was dissolved in a small amount of AcOEt, and the title compound was precipitated by addition of *tert*-butyl methyl ether and petroleum ether as colorless solid; yield: 2.12 g (52%); TLC (cyclohexane/$CHCl_3$/AcOH; 45:45:10; v/v/v): $R_f$ 0.1; RP-HPLC (column I, gradient I): $t_R$ 18.5 min; $^1$H NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 3.31–3.35 (m, 1H, $\beta_2$-C*H*$_2$), 3.51 (dd, *J* = 14.2, 4.8 Hz, 1H, $\beta_1$-C*H*$_2$), 3.70 (s, 3H, C*H*$_3$), 3.86 (s, 2H, C*H*$_2$-NH$_3$), 5.27 (dd, *J* = 11.0, 4.8 Hz, 1H, α-C*H*), 7.19–7.29 (m, 4H, Phe(3-H$_2$N-CH$_2$)-Ar), 7.87 (s, 4H, C*H* Pht-Ar), 8.35 (s, 3H, NH$_3$); $^{13}$C NMR (125.7 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 27.3, 34.3 (β*C*), 42.4, 49.2, 52.9 (O*C*H$_3$), 53.3 (α*C*), 124.1, 127.7, 129.1, 130.0, 131.1, 134.6, 135.6, 137.8, 167.4 (*C*=O Pht), 169.5 (*C*=O); ESI-MS: *m*/*z* 339.2 [M+H]$^+$; calcd for $C_{19}H_{18}N_2O_4$: 338.1.

#### 4.1.4. Pht-Phe(3-Boc-HN-CH$_2$)-OMe (9)

Pht-Phe(3-H$_2$N-CH$_2$)-OMe × HCl (**8**; 2.66 g, 7.09 mmol) was dissolved in 1,4-dioxane/$H_2O$ (250 mL; 3:2, v/v) and a solution of $NaHCO_3$ (715 mg, 8.51 mmol) in $H_2O$ (30 mL) added. To the resulting solution $Boc_2O$ (2.01 g, 9.22 mmol) was added as solid at r.t. under stirring and stirring was continued for 4.5 h. The solvent was evaporated under reduced pressure and the obtained material distributed between AcOEt (300 mL) and $H_2O$ (100 mL). The organic phase was washed with 5% aq $KHSO_4$ (3 × 50 mL), brine (1 × 50 mL), dried ($Na_2SO_4$), and the solvent evaporated. The title compound was obtained as colorless foam; yield: 2.97 g (96%); TLC (cyclohexane/$CHCl_3$/AcOH; 45:45:10; v/v/v): $R_f$ 0.5; RP-HPLC (column I, gradient I): $t_R$ 28.0 min; $^1$H NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 1.36 (s, 9H, Boc-C*H*$_3$), 3.28–3.35 (m, 1H, $\beta_2$-C*H*$_2$), 3.48 (dd, *J* = 14.1, 4.8 Hz, 1H, $\beta_1$-C*H*$_2$), 3.69 (s, 3H, C*H*$_3$), 3.96 (d, *J* = 6.3 Hz, 2H, C*H*$_2$-NH), 5.24 (dd, *J* = 11.2, 4.8 Hz, 1H, α-C*H*), 6.99–7.14 (m, 4H, Phe(Boc-HN-CH$_2$)-Ar), 7.24 (t, *J* = 6.0 Hz, 1H, Boc-*H*N-CH$_2$), 7.85 (s, 4H, C*H* Pht-Ar); $^{13}$CNMR (125.7 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 28.7 (Boc-*C*H$_3$), 34.4 (β*C*), 43.6 (*C*H$_2$-NH-Boc), 53.2 (α*C* and O*C*H$_3$), 78.2 (Boc-*C*CH$_3$), 124.0, 125.7, 127.4, 128.0, 128.7, 131.1, 135.5, 137.2, 140.7, 156.2 (*C*=O Boc), 167.4 (*C*=O Pht), 169.5 (*C*=O); ESI-MS: *m*/*z* 461.2 [M+Na]$^+$; calcd for $C_{24}H_{26}N_2O_6$: 438.2.

#### 4.1.5. H-Phe(3-Boc-HN-CH$_2$)-OMe × HCl (10)

To a stirred solution of Pht-Phe(3-Boc-HN-CH$_2$)-OMe (**9**; 2.30 g, 5.25 mmol) in MeOH (160 mL), hydrazine monohydrate (2.09 mL, 43.05 mmol), and AcOH (2.46 mL, 43.05 mmol) were added. The resulting reaction mixture was stirred at r.t. until the reaction was completed as monitored by analytical RP-HPLC. Subsequently, the solvent was removed under reduced pressure and $H_2O$ (170 mL) added. The formed pale yellow precipitate was filtered off and the pH of the filtrate was adjusted to 8-9 by $NaHCO_3$. The resulting aqueous phase was extracted with $CHCl_3$ (3 × 100 mL), and the combined organic phases were washed with brine (1 × 50 mL), dried ($Na_2SO_4$), and the solvent evaporated under reduced pressure. The obtained material was dissolved in MeOH (80 mL) and HCl in dioxane (1 M) was added to adjust the pH to ~4. Subsequently, the solvent was removed under reduced pressure and title compound was isolated as colorless solid by precipitation from *i*PrOH/*tert*-butyl methyl ether; yield: 1.38 g (76%); TLC ($CHCl_3$/MeOH/AcOH; 8:8:1; v/v/v): $R_f$ 0.9; RP-HPLC (column I, gradient I): $t_R$ 18.7 min; $^1$H NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 1.39 (s, 9H, Boc-C*H*$_3$), 3.07 (dd, *J* = 13.9, 7.7 Hz, 1H, $\beta_2$-C*H*$_2$), 3.21 (dd, *J* = 13.7, 5.0 Hz, 1H, $\beta_1$-C*H*$_2$), 3.65 (s, 3H, C*H*$_3$), 4.10 (d, *J* = 6.0 Hz, 2H, C*H*$_2$-NH), 4.18 (m, 1H, α-C*H*), 7.06–7.29 (m, 4H, Phe(Boc-HN-CH$_2$)-Ar), 7.38 (t, *J* = 6.0 Hz, 1H, Boc-*H*N-CH$_2$), 8.83 (s, 3H, NH$_3$); $^{13}$C NMR (125.7 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 28.7 (Boc-*C*H$_3$), 36.3 (β*C*), 43.7 (*C*H$_2$-NH-Boc), 52.9 (O*C*H$_3$), 53.7 (α*C*), 78.3 (Boc-*C*CH$_3$), 126.4, 128.2, 128.4, 129.0, 135.0, 140.9, 156.2 (*C*=O Boc), 169.8 (*C*=O); ESI-MS: *m*/*z* 309.2 [M+H]$^+$; calcd for $C_{16}H_{24}N_2O_4$: 308.2.

#### 4.1.6. H-Phe(3-Boc-HN-CH$_2$)-OH (11)

H-Phe(3-Boc-HN-CH$_2$)-OMe × HCl (**10**; 1.38 g, 4.00 mmol) was dissolved in 1,4-dioxane/$H_2O$ (120 mL; 2:1, v/v), and 1 N NaOH (7.60 mL, 7.60 mmol) was added at r.t. under stirring. After 3 h, the solution was neutralized with 1 N HCl and the solvent evaporated. The obtained solid was suspended in $H_2O$ (50 mL), and the aqueous phase extracted with $H_2O$-saturated 1-butanol (5 × 20 mL). The solvent was evaporated under reduced pressure from the combined organic phases. The resulting material was suspended in *tert*-butyl methyl ether (20 mL), petroleum ether (20 mL) added, collected by filtration, washed with petroleum ether and finally dried. The title compound was obtained as colorless solid; yield: 969 mg (82%); TLC ($CHCl_3$/MeOH/AcOH; 8:8:1; v/v/v): $R_f$ 0.5; RP-HPLC (column I, gradient I): $t_R$ 17.4 min; $^1$H NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 1.33 (s, 9H, Boc-C*H*$_3$), 2.84 (dd, *J* = 14.2, 8.4 Hz, 1H, $\beta_2$-C*H*$_2$), 3.11 (dd, *J* = 14.3, 4.1 Hz, 1H, $\beta_1$-C*H*$_2$), 3.54 (dd, *J* = 8.2, 4.4 Hz, 1H, α-C*H*), 4.07 (s, 2H, C*H*$_2$-NH), 7.08–7.11 (m, 3H, Phe(Boc-HN-CH$_2$)-Ar), 7.23 (m, 1H, Phe(Boc-HN-CH$_2$)-Ar); $^{13}$C NMR (125.7 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 28.5 (Boc-*C*H$_3$), 36.8 (β*C*), 43.7 (*C*H$_2$-NH-Boc), 55.9 (α*C*), 79.2 (Boc-*C*CH$_3$), 125.9, 128.3, 128.5, 129.1, 136.7, 140.2, 156.7 (*C*=O Boc), 171.7 (*C*OOH); ESI-MS: *m*/*z* 317.2 [M+Na]$^+$; calcd for $C_{15}H_{22}N_2O_4$: 294.2.

#### 4.1.7. Fmoc-Phe(3-Boc-HN-CH$_2$)-OH (12)

H-Phe(3-Boc-HN-CH$_2$)-OH (**11**; 904 mg, 3.07 mmol) was dissolved in a mixture of 1,4-dioxane (30 mL) and $H_2O$ (20 mL), and the pH was adjusted to 8-9 by 5% aq $NaHCO_3$. To the resulting solution Fmoc-OSu (1.00 g, 2.98 mmol) was added at r.t. as solid. After stirring over night the solvent was removed under reduced pressure. The obtained pale yellow solid was distributed between AcOEt/$H_2O$ (150 mL; 2:1, v/v), and upon addition of $KHSO_4$ (711 mg, 5.22 mmol), the free acid was extracted into the organic phase. The organic phase was washed with 5% aq $KHSO_4$ (3 × 50 mL), brine (1 × 50 mL), dried ($Na_2SO_4$), and the solvent evaporated. The title compound was isolated as colorless solid by precipitation from *tert*-butyl methyl ether/petroleum ether; yield: 1.20 g (76%); TLC ($CHCl_3$/MeOH/AcOH; 8:8:1; v/v/v): $R_f$ 0.8; RP-HPLC (column I, gradient I): $t_R$ 29.4 min; $^1$H NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 1.39 (s, 9H, Boc-C*H*$_3$), 2.87 (m, 1H, $\beta_2$-C*H*$_2$), 3.06 (m, 1H, $\beta_1$-C*H*$_2$), 4.10 (d, *J* = 5.4 Hz, 2H, C*H*$_2$-NH), 4.19 (m, 4H, α-C*H* and Fmoc-C*H*-C*H*$_2$), 7.08–7.41 (m, 9H, Phe(Boc-HN-CH$_2$)-Ar, Fmoc-Ar and Boc-N*H*), 7.66 (t, *J* = 8.8 Hz, 2H, Fmoc-Ar), 7.77 (d, *J* = 8.2 Hz, 1H, α-CH-N*H*), 7.88 (d, *J* = 7.2 Hz, 2H, Fmoc-Ar), 12.76 (s, 1H, COO*H*); $^{13}$C NMR (125.7 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 14.6, 21.2, 23.0, 27.3, 28.7 (Boc-*C*H$_3$), 36.9 (β*C*), 43.8 (*C*H$_2$-NH-Boc), 47.0 (Fmoc), 56.0 (α*C*), 60.2, 66.1 (Fmoc), 78.2 (Boc-*C*CH$_3$), 120.6 (Fmoc), 125.4, 125.7 (Fmoc), 127.5 (Fmoc), 127.9, 128.1 (Fmoc), 128.5, 138.4, 140.5, 141.1 (Fmoc), 144.2 (Fmoc), 156.3, 156.4, 173.9 (*C*OOH); ESI-MS: *m*/*z* 539.3 [M+Na]$^+$; calcd for $C_{30}H_{32}N_2O_6$: 516.2.

#### 4.1.8. Fmoc-Phe(3-Boc-HN-CH$_2$)-CHN$_2$ (13)

To a solution of Fmoc-Phe(3-Boc-HN-CH$_2$)-OH (**12**; 795 mg, 1.54 mmol) in dry THF (15 mL) at −15 °C under argon was added $Et_3N$ (214 μL, 1.54 mmol) and $ClCO_2Et$ (147 μL, 1.54 mmol). After 15 min, freshly distilled ethereal $CH_2N_2$ solution (5.70 mL, 1.54 mmol, *c* = 0.27 mol/L) was added to the resulting suspension at −5 °C. Stirring was continued over night at r.t. The solvent was evaporated under reduced pressure and the obtained material distributed between AcOEt (100 mL) and $H_2O$ (50 mL). The organic phase was washed with 5% aq $KHSO_4$ (3 × 30 mL), 5% aq $NaHCO_3$ (3 × 30 mL), brine (1 × 30 mL), dried ($Na_2SO_4$), and the solvent evaporated. The title compound was isolated as yellowish solid by precipitation from *tert*-butyl methyl ether/petroleum ether; yield: 714 mg (86%); TLC (AcOEt/petroleum ether 1:2; v/v): $R_f$

0.6; RP-HPLC (column II, gradient II): $t_R$ 12.2 min; $^1$H NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 1.39 (s, 9H, Boc-C$H_3$), 2.75 (m, 1H, $\beta_2$-C$H_2$), 3.00 (dd, $J$ = 13.8, 4.3 Hz, 1H, $\beta_1$-C$H_2$), 4.09 (d, $J$ = 5.9 Hz, 2H, C$H_2$-NH), 4.16–4.30 (m, 4H, $\alpha$-C$H$ and Fmoc-C$H$-C$H_2$), 6.04 (s, 1H, C$H$=N$_2$), 7.07–7.43 (m, 9H, Phe(Boc-HN-CH$_2$)-Ar, Fmoc-Ar and Boc-N$H$), 7.64 (m, 2H, Fmoc-Ar), 7.85–7.89 (m, 3H, $\alpha$-CH-N$H$ and Fmoc-Ar); $^{13}$C NMR (125.7 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 27.2, 29.1 (Boc-C$H_3$), 37.0 ($\beta$C), 44.2 (C$H_2$-NH-Boc), 47.5 (Fmoc), 66.4 (Fmoc), 78.6 (Boc-C$CH_3$), 120.9 (Fmoc), 125.8, 126.0 (Fmoc), 127.9 (Fmoc), 128.3, 128.4 (Fmoc), 128.6, 128.9, 138.6, 140.9, 141.5 (Fmoc), 144.6 (Fmoc), 156.6; ESI-MS: $m/z$ 563.2 [M+Na]$^+$; calcd for $C_{31}H_{32}N_4O_5$: 540.6.

#### 4.1.9. Fmoc-$\beta^3$hPhe(3-Boc-HN-CH$_2$)-OH (4)

To a solution of Fmoc-Phe(3-Boc-HN-CH$_2$)-CHN$_2$ (**13**; 710 mg, 1.31 mmol) in 1,4-dioxane (15 mL) and $H_2O$ (8 mL) was added silver benzoate (23 mg, 0.10 mmol). It was stirred at 90 °C for 5 h. The solvent was evaporated under reduced pressure and the obtained grey solid distributed between AcOEt (100 mL) and 5% aq $KHSO_4$ solution (50 mL). The organic phase was washed with 5% aq $KHSO_4$ (2 × 30 mL), brine (1 × 30 mL), dried ($Na_2SO_4$), and the solvent evaporated. The title compound was isolated as colorless solid by precipitation from *tert*-butyl methyl ether/petroleum ether; yield: 633 mg (91%); TLC ($CHCl_3$/MeOH/AcOH; 45:2:1; v/v/v): $R_f$ 0.4; RP-HPLC (column II, gradient II): $t_R$ 11.4 min; $^1$H NMR (500 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 1.35 (s, 9H, Boc-C$H_3$), 2.33 (m, 2H, $\alpha$-C$H_2$), 2.64 (dd, $J$ = 13.4, 6.4 Hz, 1H, $\gamma_2$-C$H_2$), 2.71 (dd, $J$ = 13.3, 7.6 Hz, 1H, $\gamma_1$-C$H_2$), 3.95 (m, 1H, $\beta$-C$H$), 4.05 (d, $J$ = 5.9 Hz, 2H, C$H_2$-NH), 4.13 (t, $J$ = 7.0 Hz, 1H, Fmoc-C$H$-CH$_2$), 4.19 (d, $J$ = 6.3 Hz, 2H, Fmoc-CH-C$H_2$), 7.00–7.39 (m, 10H, Phe(Boc-HN-CH$_2$)-Ar, Fmoc-Ar, Boc-N$H$ and $\beta$-CH-N$H$), 7.62 (d, $J$ = 7.5 Hz, 2H, Fmoc-Ar), 7.85 (d, $J$ = 7.5 Hz, 2H, Fmoc-Ar), 12.16 (s, 1H, COO$H$); $^{13}$C NMR (125.7 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 29.1 (Boc-C$H_3$), 39.5, 44.2 (C$H_2$-NH-Boc), 47.6 (Fmoc), 50.5, 66.1 (Fmoc), 78.6 (Boc-C$CH_3$), 120.9 (Fmoc), 125.7, 126.0 (Fmoc), 127.9 (Fmoc), 128.4 (Fmoc), 128.7, 128.9, 139.3, 140.9, 141.5 (Fmoc), 144.7 (Fmoc), 156.2, 156.6, 173.2 (*C*OOH); ESI-MS: $m/z$ 553.1 [M+Na]$^+$; calcd for $C_{31}H_{34}N_2O_6$: 530.6.

#### 4.1.10. FmocHN-CH$_2$-CH(NH®)-CO$_2$Al (14)

To pre-swollen 2-chlorotrityl chloride resin (2.00 g, 1.08 mmol Cl/g) was added a solution of HCl × H-Dap(Fmoc)-OAl (0.87 g, 2.16 mmol) and DIPEA (0.74 mL, 4.32 mmol) in dry dichloromethane (10 mL). The resin was gently agitated for 1.5 h at r.t. Subsequently, the solution was sucked off and to the resin was added a mixture of MeOH (2 mL), dichloromethane (8 mL), and DIPEA (0.37 mL) and agitation was continued for another 1 h at r.t. The resin was washed three times alternately with DMF (10 mL) and isopropanol (10 mL) then with *tert*-butyl methyl ether (1 × 10 mL), petroleum ether (1 × 10 mL), and dried. Loading was assessed by UV determination of the fulvene-piperidine adduct:[26] 0.36 mmol/g.

#### 4.1.11. Fmoc-Pro–$\beta^3$hPhe(3-BocHN-CH$_2$)–D-*allo*-Ile–vTyr(*t*Bu)–NH-CH$_2$-CH(NH®)-CO$_2$Al (15)

Using FmocHN-CH$_2$-CH(NH®)-CO$_2$Al (**14**; 0.56 g, 0.20 mmol), the linear pentapeptide was built up by coupling sequentially Fmoc-vTyr(*t*Bu)-OH (2 × 1 equiv), Fmoc-D-*allo*-Ile (4 equiv), Fmoc-$\beta^3$hPhe(3-BocHN-CH$_2$)-OH (**4**, 2 × 1 equiv) and Fmoc-Pro-OH (4 equiv). For each coupling step to a solution of equimolar amounts of Fmoc amino acid, TBTU, and HOBt in DMF (5 mL) was added at r.t. an equimolar amount of DIPEA. After 5 min of preactivation, the solution was added to the resin-bound peptide and the resin gently agitated for 1 h at r.t. The progress of coupling was monitored by using the chloranil test[27] and found to be completed by applying the indicated excesses, respectively. Subsequently, the resin was washed three times alternately with DMF (5 mL) and isopropanol (5 mL). Fmoc cleavage was performed prior to each coupling step by treating (2 × 15 min) the resin-bound peptide with piperidine/DMF (1:4, v/v, 5 mL). Subsequently, the resin was washed three times alternately with DMF (5 mL), and isopropanol (5 mL).

#### 4.1.12. *c*[–Pro–$\beta^3$hPhe(3-BocHN-CH$_2$)–D-*allo*-Ile–vTyr(*t*Bu)–NH-CH$_2$-CH(NH$_2$)-CO–] (16)

Prior on-resin cyclization the termini of the resin-bound linear pentapeptide **15** (0.20 mmol) were deprotected. For cleavage of the C-terminal allyl ester the resin-bound linear pentapeptide **15** was treated (3 × 15 min) under an argon atmosphere at r.t. with $Pd(PPh_3)_4$ (93 mg, 0.08 mmol, 0.4 equiv) and $PhSiH_3$ (593 μL, 4.8 mmol, 24 equiv) in dry dichloromethane (5 mL). Upon each treatment the resin was washed with dry dichloromethane (8 × 5 mL). Then, the N-terminal Fmoc-group was cleaved as described above. Subsequently, PyBOP (312 mg, 0.6 mmol, 3 equiv), HOBt (81 mg, 0.6 mmol, 3 equiv), and DIPEA (205 μL, 1.2 mmol, 6 equiv) in DMF (5 mL) were added to the resin-bound peptide. The resin was gently agitated at r.t. for 1 h. The progress of cyclization was monitored by using the chloranil test.[27] Then, the resin was washed three times alternately with DMF (5 mL) and isopropanol (5 mL). Eventually, at r.t., the cyclized resin-bound peptide was treated (10 × 5 min) with dichloromethane/TFA/TIS (98:1:1, v/v/v, 5 mL). The cleavage solutions were collected in MeOH/pyridine (45:5, v/v, 50 mL) and evaporated under reduced pressure. The resulting material was distributed between AcOEt (200 mL) and $H_2O$ (50 mL). The organic phase was washed with 5% aq $NaHCO_3$ (3 × 50 mL), brine (1 × 50 mL), dried ($Na_2SO_4$), and the solvent evaporated. The title compound was isolated as colorless solid by precipitation from *tert*-butyl methyl ether/petroleum ether and used for the next step without further purification; yield: 160 mg (96%, based on resin loading); TLC ($CHCl_3$/MeOH; 4:1; v/v): $R_f$ 0.74; RP-HPLC (column II, gradient II) $t_R$ 9.7 min; MALDI-MS: $m/z$ 854.8 [M+Na]$^+$; calcd for $C_{45}H_{65}N_7O_8$: 831.5.

#### 4.1.13. *c*[–Pro–$\beta^3$hPhe(3-H$_2$N-CH$_2$)–D-*allo*-Ile–vTyr–NH-CH$_2$-CH(NHAc)-CO–] (2)

To a stirred solution of the protected cyclic pentapeptide **16** (34 mg, 0.041 mmol) in dichloromethane (5 mL) at r.t. was added $Ac_2O$ (39 μL, 0.41 mmol) and pyridine (33 μL, 0.41 mmol) and stirring was continued over night. The solvent was removed under reduced pressure and the resulting oil was dissolved in AcOEt (100 mL). The organic phase was washed with 5% aq $KHSO_4$ (3 × 30 mL), 5% aq $NaHCO_3$ (3 × 30 mL), brine (1 × 30 mL), dried ($Na_2SO_4$), and the solvent evaporated. The acetylated compound was isolated by precipitation from AcOEt/*n*-hexane. The obtained material was treated at r.t. for 2 h with TFA/$H_2O$ (95:5, v/v; 3 mL). The peptide was isolated by adding the cleavage solution dropwise into ice-cold *tert*-butyl methyl ether/*n*-hexane (2:1, v/v; 50 mL). The formed colorless precipitate was collected by centrifugation, washed with petroleum ether (3×), and dried. The crude product was purified by preparative RP-HPLC and homogenous fractions were pooled and lyophilized; yield: 10 mg (30%); RP-HPLC (column II, gradient II) $t_R$ 6.1 min; $^1$H NMR (600 MHz, DMSO-$d_6$, 25 °C): $\delta$ [ppm] = 0.55 (d, $J$ = 6.7 Hz, 3H, $\gamma$-C$H_3$, D-*allo*-Ile), 0.65 (d, $J$ = 3.8 Hz, 3H, $\delta$-C$H_3$, D-*allo*-Ile), 0.66 (m, 1H, $\gamma_2$-C$H_2$, D-*allo*-Ile), 0.88 (m, 1H, $\gamma_1$-C$H_2$, D-*allo*-Ile), 1.29 (m, 1H, $\beta$-C$H$, D-*allo*-Ile), 1.84 (s, 3H, C$H_3$, Ac), 1.87 (m, 2H, $\gamma$-C$H_2$, Pro), 1.89 (m, 1H, $\alpha_2$-C$H_2$, $\beta^3$hPhe(3-H$_2$N-CH$_2$)), 2.14 (m, 2H, $\beta$-C$H_2$, Pro), 2.48 (m, 1H, $\delta_2$-C$H_2$, v-Tyr), 2.63 (m, 1H, $\beta_2$-C$H_2$, Dap), 2.67 (m, 1H, $\alpha_1$-C$H_2$, $\beta^3$hPhe(3-H$_2$N-CH$_2$)), 2.74 (m, 2H, $\gamma$-C$H_2$, $\beta^3$hPhe(3-H$_2$N-CH$_2$)), 2.88 (dd, $J$ = 13.8, 4.2 Hz, 1H, $\delta_1$-C$H_2$, v-Tyr), 3.41 (m, 1H, $\delta_2$-CH$_2$, Pro), 3.63 (m, 1H, $\delta_1$-C$H_2$, Pro), 4.01 (m, 2H, C$H_2$-NH$_3$, $\beta^3$hPhe(3-H$_2$N-CH$_2$)), 4.04 (m, 1H, $\beta_1$-C$H_2$, Dap), 4.17 (m, 1H,

α-C*H*, D-*allo*-Ile), 4.37 (m, 1H, α-C*H*, Pro), 4.39 (m, 1H, β-C*H*, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 4.46 (m, 1H, α-C*H*, Dap), 4.50 (m, 1H, γ-C*H*, v-Tyr), 5.93 (dd, *J* = 15.3, 1.7 Hz, 1H, α-C*H*, v-Tyr), 6.63 (d, *J* = 8.4 Hz, 2H, Ar, v-Tyr), 6.65 (m, 1H, β-C*H*, v-Tyr), 7.05 (d, *J* = 8.2 Hz, 2H, Ar, v-Tyr), 7.21–7.36 (2 m, 4H, Ar, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 7.65 (d, *J* = 9.3 Hz, 1H, N*H*, D-*allo*-Ile), 8.06 (m, 1H, α-N*H*, Dap), 8.07 (m, 1H, N*H*, v-Tyr), 8.10 (br s, 3H, $CH_2$-N$H_3$, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 8.27 (d, *J* = 9.6 Hz, 1H, N*H*, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 8.64 (d, *J* = 10.5 Hz, 1H, β-N*H*, Dap), 9.13 (s, 1H, OH, v-Tyr); HRMS (ESI) calcd for $C_{38}H_{51}N_7O_7$+$H^+$ ([M+H]$^+$); *m*/*z*: 718.39227, found: 718.39239.

#### 4.1.14. *c*[–Pro–$\beta^3$hPhe(3-$H_2$N-$CH_2$)–D-*allo*-Ile–vTyr–NH-$CH_2$-CH(NHCO-$(CH_2)_5$-$NH_2$)-CO–] (3)

To a stirred solution of the protected cyclic pentapeptide **16** (40 mg, 0.048 mmol) in DMF (4 mL) at r.t. was added upon 5 min preactivation a solution of BocHN-$(CH_2)_5$-COOH (22 mg, 0.096 mmol), TBTU (31 mg, 0.096 mmol), HOBt (13 mg, 0.096 mmol), and DIPEA (17 μL, 0.096 mmol) in DMF (1 mL) and stirring was continued over night. The solvent was removed under reduced pressure and the resulting oil was dissolved in AcOEt (100 mL). The organic phase was washed with 5% aq $KHSO_4$ (3 × 50 mL), 5% aq $NaHCO_3$ (7 × 50 mL), brine (1 × 50 mL), dried ($Na_2SO_4$), the solvent evaporated and further processed as described for inhibitor **2**; yield: 16 mg (33%); RP-HPLC (column II, gradient II) $t_R$ 5.8 min; $^1$H NMR (600 MHz, DMSO-$d_6$, 25 °C): δ [ppm] = 0.55 (d, *J* = 6.7 Hz, 3H, γ-C$H_3$, D-*allo*-Ile), 0.65 (d, *J* = 3.2 Hz, 3H, δ-C$H_3$, D-*allo*-Ile), 0.67 (m, 1H, $\gamma_2$-C$H_2$, D-*allo*-Ile), 0.87 (m, 1H, $\gamma_1$-C$H_2$, D-*allo*-Ile), 1.21–1.33 (m, 3H, β-C*H*, D-*allo*-Ile; γ-C$H_2$, ε-Ahx), 1.41–1.55 (m, 4H, β-C$H_2$, δ-C$H_2$, ε-Ahx), 1.86 (m, 2H, γ-C$H_2$, Pro), 1.89 (m, 1H, $\alpha_2$-C$H_2$, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 2.01–2.21 (m, 4H, β-C$H_2$, Pro; α-C$H_2$, ε-Ahx), 2.48 (m, 1H, $\delta_2$-C$H_2$, v-Tyr), 2.62–2.68 (m, 2H, $\beta_2$-C$H_2$, Dap; $\alpha_1$-C$H_2$, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 2.70–2.80 (m, 4H, γ-C$H_2$, $\beta^3$hPhe(3-$H_2$N-$CH_2$); ε-C$H_2$, ε-Ahx), 2.88 (dd, *J* = 13.7, 4.1 Hz, 1H, $\delta_1$-C$H_2$, v-Tyr), 3.41 (m, 1H, $\delta_2$-C$H_2$, Pro), 3.63 (m, 1H, $\delta_1$-C$H_2$, Pro), 4.00 (m, 2H, C$H_2$-$NH_3$, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 4.02 (m, 1H, $\beta_1$-C$H_2$, Dap), 4.17 (m, 1H, α-C*H*, D-*allo*-Ile), 4.37 (m, 1H, α-C*H*, Pro), 4.41 (m, 1H, β-C*H*, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 4.47 (m, 1H, α-C*H*, Dap), 4.51 (m, 1H, γ-C*H*, v-Tyr), 5.94 (d, *J* = 13.7 Hz, 1H, α-C*H*, v-Tyr), 6.63 (d, *J* = 8.2 Hz, 2H, Ar, v-Tyr), 6.66 (m, 1H, β-C*H*, v-Tyr), 7.05 (d, *J* = 8.2 Hz, 2H, Ar, v-Tyr), 7.20–7.35 (2 m, 4H, Ar, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 7.63 (d, *J* = 9.3 Hz, 1H, N*H*, D-*allo*-Ile), 7.70 (br s, 3H, ε-N$H_3$, ε-Ahx), 8.07 (m, 1H, α-N*H*, Dap), 8.08 (m, 1H, N*H*, v-Tyr), 8.19 (br s, 3H, $CH_2$-N$H_3$, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 8.26 (d, *J* = 9.5 Hz, 1H, N*H*, $\beta^3$hPhe(3-$H_2$N-$CH_2$)), 8.65 (d, *J* = 10.5 Hz, 1H, β-N*H*, Dap), 9.13 (s, 1H, O*H*, v-Tyr); HRMS (ESI) calcd for $C_{42}H_{60}N_8O_7$+$H^+$ ([M+H]$^+$); *m*/*z*: 789.46577, found: 789.46685.

### 4.2. Determination of the inhibitor stability in human serum and plasma

To determine the stability of the inhibitors **2** and **3** in biological fluids the compounds (200 μM) were incubated in human serum and human plasma (PAA Laboratories, Cölbe, Germany) at 37 °C. At various time points, samples were removed and any ongoing degradation stopped by acidification with an equal volume of 0.1% TFA. After centrifugation at 8000 × g for 10 min, the supernatants were injected onto a C18 column (LiChrospher 100 RP18, 125 × 4 mm, Merck, Darmstadt, Germany) and eluted (eluent A: $H_2O$/TFA, 99.9:0.1 v/v, eluent B: MeCN/TFA, 99.9:0.1 v/v) with a linear gradient from A/B (100:0) to A/B (0:100) over 200 min using a Sykam HPLC system (Fürstenfeldbruck, Germany). The residual inhibitors were quantified at 206 nm by comparison to standard curves obtained with the native compounds under identical conditions.

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