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Proteasome inhibition by peptide-semicarbazones

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Abstract—Peptide-semicarbazones derived from Z-Trp-Trp-Phe-aldehyde inhibit the chymotryptic activity of the human proteasome at nanomolar concentrations, but are less active in a NFκB reporter gene assay. Cyclic semicarbazones, in contrast, combine a strong inhibitory effect on the enzyme with an inhibition of NFκB signaling in the nanomolar range. In addition, a practical synthesis for scale-up of such compounds was developed.

1. Introduction

The controlled degradation of damaged or misfolded proteins as well as the regulation of short lived proteins plays a crucial role for proper cellular functions. This is achieved by the ubiquitin/proteasome pathway, in which the target protein is marked by a polyubiquitin chain and, after recognition, is degraded in an ATP-dependent manner by the 26S proteasome.[1] The 26S proteasome by itself is a multicatalytic threonine protease consisting of two polyubiquitin recognizing 19S particles and the 20S proteasome core, which houses at least three different peptidase activities: post-glutamyl-, trypsin-, and chymotrypsin-like activities.[2] The latter is thought to be responsible for the rate limiting step in protein degradation.

Because of its central role in critical intracellular regulatory processes of cell division, growth activation, signaling, and transcription, proteasome inhibitors have been explored as agents to treat multiple myeloma. A peptide boronate inhibitor (PS-341) was approved recently under the name Velcade.[3] Most of the inhibitors address the chymotryptic activity via adduct formation with the N-terminal threonine hydroxyl group as part of the catalytically active center. These classes of covalent binding inhibitors belong to a diverse group of compounds such as epoxyketones, peptide aldehydes, peptide vinylsulfones, and peptide boronic acids. All these compounds act as electrophilic traps and create covalent intermediates, which can be in equilibrium with the unmodified enzyme.

Tripeptide aldehydes based on the hydrophobic Trp-Trp-Trp sequence were described in a patent as endothelin converting enzyme inhibitors.[4] In this patent, the Trp-Trp-Trp-aldehyde sequence was shown to be a superior inhibitor over the initial Ile-Ile-Trp-aldehyde sequence. Ketoepoxide peptides of this series were later published as potent proteasome inhibitors.[5] Further, all of the compounds of this patent[4] were later revised to be proteasome inhibitors.[6] We confirm here that these compounds are in fact inhibitors of the proteasome. In addition to the peptide semicarbazones, we synthesized cyclic semicarbazone analogs and evaluated their inhibitory activity on the proteasome, determined the inhibitory activity in a cell based NFκB reporter assay and confirmed the biological activity in a PHA stimulated T-cell proliferation assay.

2. Results and discussion

2.1. Synthesis

For the synthesis (Scheme 1), first the Boc-phenylalanine aldehyde was used.[7] The amino acid derived aldehyde was converted to the Schiff base with the cyclic semicarbazide (aminohydantoin) in the presence of TMOB and DIPEA. Deprotection with 3 N HCl in glacial acetic acid yielded the deprotected Schiff base. This compound

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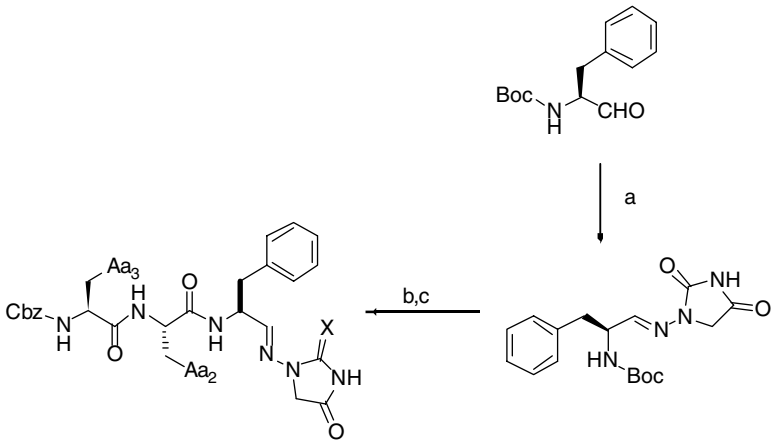


Scheme 1. Reagents and conditions: (a) Ahyd, TMOB, DIPEA, rt, 24 h; (b) 3 N HCl in AcOH, rt; (c) HBTU, Cbz-Trp-Trp-OH, DMF,DIPEA, rt, 24 h.

was coupled with commercial Cbz protected dipeptides in the presence of DIPEA with the coupling reagent, HBTU.

This method resulted in a LC–MS pure product with the expected mass. However, TLC indicated the presence of isomers in a ratio of 3:1, where the minor isomer termed Dia1 moved slightly further. The isomeric compounds could be separated by pTLC in pure form and were shown to have the same mass.

NMR experiments suggested that Dia1 (cpd **5**) and Dia2 (cpd **4**) differ only in the configuration of the α-carbon of the phenylalanine derivative in position P1 (see Table 1). For example, when pure isomer Dia2 was treated with 1 N HCl in dioxane for 1 h, again a mixture of isomers in the ratio 1:1 was obtained, as shown by NMR (Fig. 1). This experiment confirms that racemization occurs when the semicarbazone is treated with acid.

To avoid the acid catalyzed racemization of the imine we conceived an alternative synthesis route. This method proceeded through the coupling of deprotected Weinreb amide with the Cbz protected dipeptides and the reduction of the tripeptide Weinreb amide to the tripeptide aldehyde with LAH. The tripeptide aldehyde was converted to the semicarbazone by the reaction with aminohydantoin hydrochloride and DIPEA in the presence of TMOB for several days. By this method, single isomer Dia2 (cpd **4**) was obtained. Alternatively, exactly the same synthesis procedure starting with Boc-D-phenylalanine yielded a product, which was completely identical with the isomer Dia1 (cpd **5**). These results confirm the hypothesis about the nature of Dia1 and Dia2. Additionally, this synthesis was amenable to scale-up and with minor modifications a batch of 170 g of compound **4** was prepared (Scheme 2).

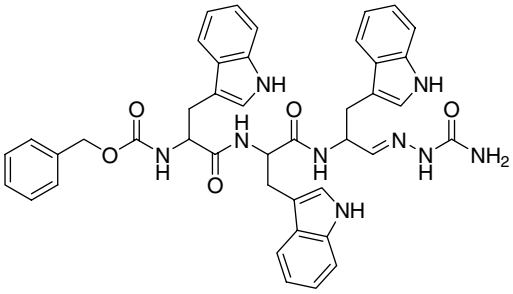
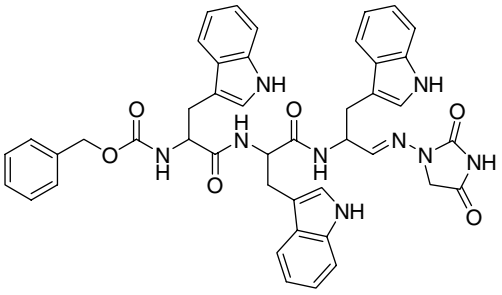
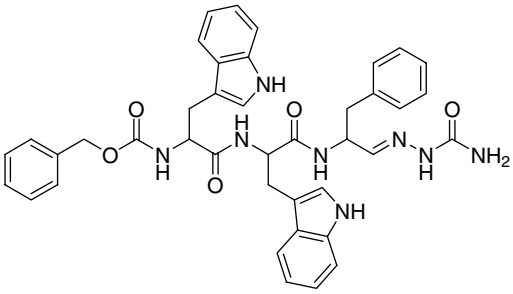
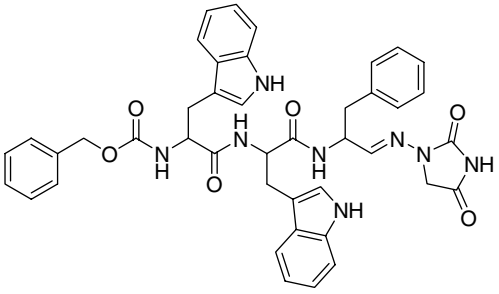
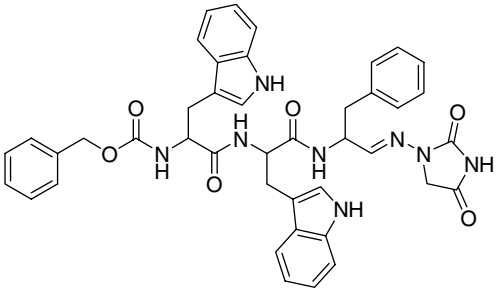
Additional analogs with varying capping groups at the N-terminus were prepared, by removing the Cbz group by catalytic hydrogenation. This procedure leaves the semicarbazone intact and acylation of the free amine with activated esters or by the help of a coupling agent resulted in additional analogs cpd **6**–**8** (Scheme 3).

The ability of the compounds to inhibit human 20S proteasome was determined by fluorescence spectroscopy using the substrate Suc-LLVV-AMC.[8] As a secondary assay, we used a NFκB gene reporter assay. These data are shown in Table 1. A few compounds were also further evaluated in their ability to inhibit PHA stimulated T-cell proliferation.

2.2. Structure–activity relationship

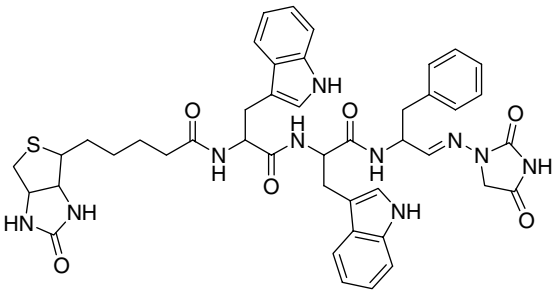
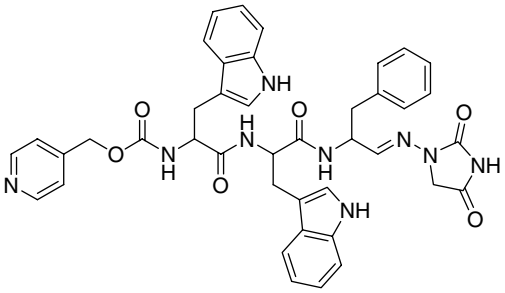
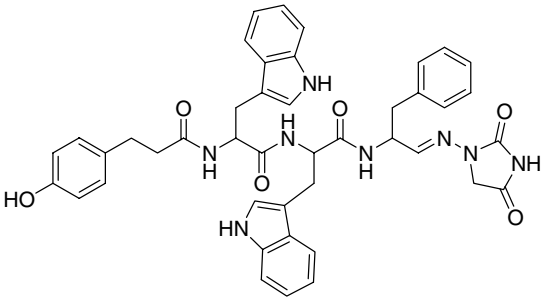
Compound **1** is a nanomolar proteasome inhibitor on the human enzyme and exhibits a micromolar inhibitory activity in the NFκB reporter assay. This activity is at least 20-fold higher as the activity of the corresponding aldehyde.[4] The aminohydantoin analog (cpd **2**) is at least 10-fold less active in inhibiting the enzyme compared to cpd **1**. When, for the sake of easier synthesis, the P1 amino acid of cpd **1** is replaced with phenylalanine, a minor reduction in enzyme inhibition is accompanied by a large reduction of inhibitory activity in the NFκB reporter gene assay (cpd **3**). The cyclic semicarbazone with the same amino acid sequence, however, retains the high activity in inhibiting the enzyme, but now in combination with a much better activity in inhibiting NFκB (cpd **4**). For the cyclic semicarbazone compounds, Trp to Phe substitution leads to an increase of both enzyme inhibition and cellular activity by a factor of ~10 (cpd **2** vs cpd **4**). The large difference in cellular activity between cpd **3** and cpd **4** is likely due to a better cell penetration of the cyclic semicarbazone compound. As expected, the D-Phe isomer (cpd **5**) is half as active on the enzyme as the L-Phe isomer (cpd **4**). A biotinyl group at the N-terminal does not affect potency significantly, but the activity in the NFκB reporter gene assay is lost (cpd **6**). A replacement of the Cbz group with a pyridylmethyl-oxycarbonyl, for the sake of better solubility, leads to lower activity (cpd **7**). The introduction of a *p*-hydroxy-phenyl-propionyl residue leads to slightly higher activity as compared to the Cbz group (cpd **8**); however, this advantage in enzyme inhibition is lost on the cellular NFκB assay. This result indicates that there exists a delicate balance between hydrophilicity and cellular activity.

Table 1. Inhibition of 20S proteasome (IC_{50}/μM, SD 0.2 μM) and NFκB signaling (EC_{50}/μM, SD 0.4 μM)

Compound	Structure	IC_{50}	EC_{50}
1		0.1	1.8
2		1.1	3.0
3		0.2	5.8
4		0.2	0.4
5		0.4	0.8

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	IC_{50}	EC_{50}
6		0.1	n.a.[a]
7		1.6	4.9
8		0.1	8.7

[a] Not active.

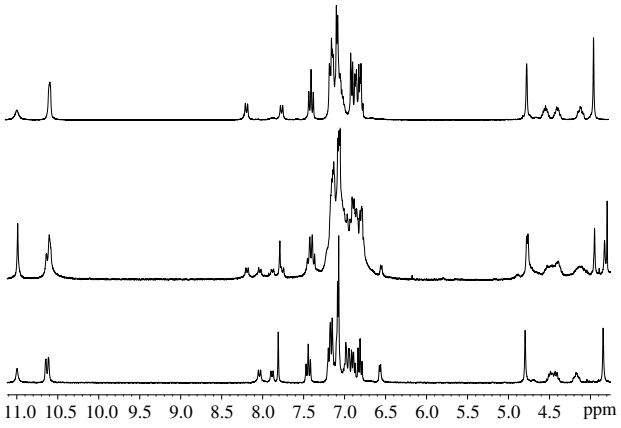
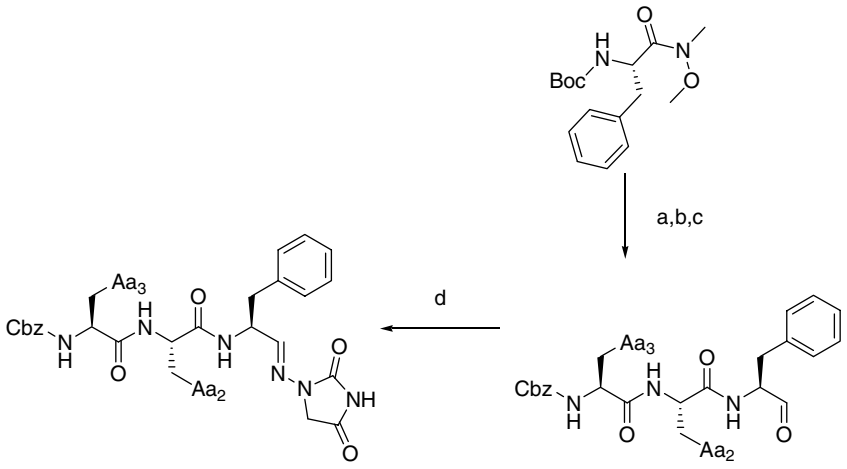


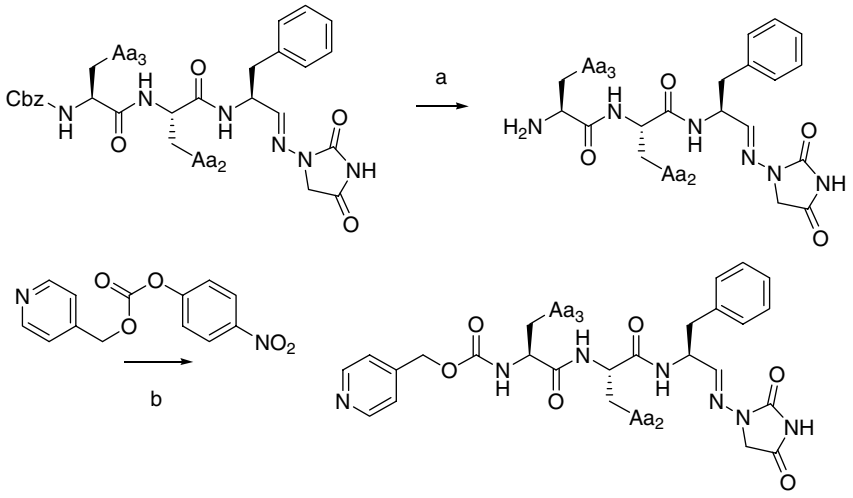
Figure 1. ^{1}H NMR spectra of Dia2 (bottom) after treatment with HCl in dioxane (middle), for comparison the spectra of pure Dia1 is shown above (top).

2.3. Mode of action

It is interesting to propose a mode of action for the inhibition of threonine proteases by semicarbazones. Adkison et al.[8] have reported that semicarbazones of small peptides are the inhibitors of Cathepsin K and that the mechanism was most likely the formation of the peptide aldehyde during the assay conditions.[8] We, however, have found that the compounds described here are extremely stable in acidic and basic conditions toward degradation and need boiling to be degraded. We therefore favor an alternative mode of action, where a covalent attack of the Thr1 hydroxyl of the enzyme to the imine carbon of the semicarbazone forms an aminal. Subsequent release of the aminohydantoin leads to an aldehyde adduct and blocks the Thr-1 nucleophile. Molecular modeling of the educt complex of cpd **4** and the 20S proteasome (PDB ID 1IRU) revealed that the 2-carbonyl of the hydantoin is likely to be coordinated by the N-terminal nitrogen of Thr1 with the semicarbazone coming close to the γ-oxygen of Thr1 (Fig. 2, distance 3.4 Å). The model therefore showed that the semicarbazone can—in principle—be cleaved by the chymotryptic site of the proteasome. Such a mechanism could be described as an enzyme activated prodrug approach, since the peptide aldehyde inhibitor is released from the semicarbazone (prodrug) only in the presence of the enzyme.



Scheme 2. Synthesis method B. Reagents and conditions: (a) 3 HCl dioxane, 1 h; (b) HBTU, DIPEA, Cbz-Trp-Trp^3-OH, DMF, rt, 24 h; (c) LAH, THF, −55 °C; (d) AhydHCl, DIPEA, TMOB, rt, 48 h.



Scheme 3. Synthesis method C. Reagents and conditions: (a) H_2, Pd/C 4 h, rt; (b) DIPEA, DMF, rt, 24 h.

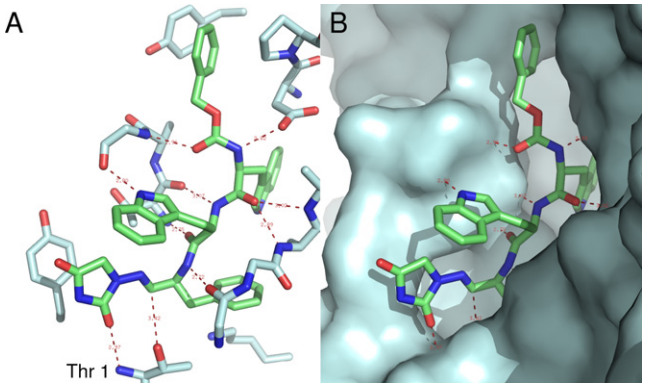


Figure 2. Model of the non-covalent complex of cpd **4** and the chymotryptic site of the 20S proteasome. (A) amino acids surrounding the binding site. (B) Surface representation of the binding site.

Peptide aldehydes as proteasome inhibitors are well known, but have not obtained a practical use for stability reasons. They tend to polymerize and racemize at the α-carbon adjacent to the aldehyde group. Further, they can form adducts with proteins. Compounds with protected aldehydes such as semicarbazones represent a stable alternative to peptide aldehydes as proteasome inhibitors.

2.4. Specificity of enzyme inhibition and immunosuppressive effect

In order to examine the subunit specificity of these compounds, proteasome inhibition of compounds **1** and **4** was measured at the peptidylglutamyl peptide hydrolyzing (PGPH) site. Inhibition values were compared to the values obtained at the chymotrypsin-like (CT) site. Compound **1** showed a 60-fold selectivity for the chymotryptic site (IC_{50}-CT = 0.06 μM, IC_{50}-PGPH = 3.7 μM) whereas compound **4** is only six times more selective toward the chymotryptic site (IC_{50}-CT = 0.2 μM, IC_{50}-PGPH = 1.2 μM). These results are in good agreement with the prevailing substrate specificity of the chymotrypsin-like (hydrophobic) and the peptidylglutamyl peptide hydrolyzing (acidic) site. Due to the incompati-

Table 2. Inhibition of human PBMC proliferation

Compound	IC_{50} (μM)
4	0.7
1	1.4
6	29

bility of the tryptic substrate with SDS used for proteasome activation, the tryptic site could not be characterized.

Proteasome inhibitors block the T-cell signaling pathway through their inhibition of NFκB and exert an immunosuppressive effect.[9] To solidify the assumption that these compounds have immunosuppressive activity, we tested a subset of compounds in a PBMC proliferation assay. Due to the phytohemagglutinin stimulus (PHA) used in the assay, the measured effect can be attributed mainly to T-cells. A significant suppressive effect was detected (Table 2).[9] As can be seen with the NFκB cell assay, the biotinylated analog has little activity despite its activity on the proteasome. The other compounds have potent activity in this assay, which is indicative of their immunosuppressive activity.

3. Conclusions

In conclusion, we have prepared a novel class of proteasome inhibitors derived from peptide aldehydes with good cellular activity in inhibiting NFκB signaling. The compounds have good stability in acidic and basic solutions and show low toxicity in vivo. Such compounds have great potential to be useful for the treatment of autoimmune diseases and cancer.

4. Experimental

4.1. Abbreviations

Ahyd, aminodydantoin; ATP, adenosine triphosphate; Boc, *tert*-butyloxycarbonyl; br, broad; BrdU, 5-brom-2-desoxyuridin; Cbz, benzyloxycarbonyl; cpd, compound; CT, chymotrypsin-like site; d, doublet; DCM, dichloromethane; Dia, diastereomer; DIPEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; EC_{50}, half maximal effective concentration, EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunoassay; g, gram, H_2, hydrogen; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate; HCl, hydrochloric acid; Hepes, 2-(4-2-hydroxyethyl)-1-piperazinyl sulfate; h(s) hour(s); HV, high vacuum; IC_{50}, half maximal inhibitory concentration; *J*, coupling constant; kV, kilovolt; LAH, lithium aluminum hydride; LC–MS, liquid chromatography mass spectroscopy; M, molar; m, multiplet; MD, molecular dynamics; MeCN, acetonitrile; MeOH, methanol; min(s), minute(s); ml, milliliter; mmol, millimol; NFκB, nuclear factor κB; nm, nanometer; NMR, nuclear magnetic resonance; PBMC, peripheral blood mononuclear cell; Pd/C, palladium on charcoal; PGPH, peptidylglutamyl peptide hydrolyzing site; PHA, phytohemagglutinin; Phe, phenylalanine; PDB, protein data bank; pHPLC, preparative HPLC; ps, picoseconds; pTLC, preparative thin-layer chromatography; quint., quintet; R_t, retention time; rt, room temperature; s, singlet; SD, standard deviation; SDS, sodium dodecyl sulfate; SEAP, secreted alkaline phosphatase; t, triplet; THF, tetrahydrofurane; TLC, thin-layer chromatography; TMOB, trimethyl-ortho-formate; TNF, tumor necrosis factor; Trp, tryphtophane; Ψ, pseudo; μl, microliter; μm, micrometer; μM, micromolar.

4.2. General methods

4.2.1. Analytical TLC. Merck aluminum sheets, silica gel 60 F_{254}.

4.2.2. Preparative TLC. Merck TLC plates, silica gel 60 F_{254}, 0.5, 1.0, or 2.0 mm.

4.2.3. Flash chromatography. Acros silica gel 60 A, 0.035–0.070 mm. Flash Master Personal or Flash Master II, Jones Chromatography, UK.

4.2.4. NMR spectra. Bruker Avance 300 MHz. The ^1H NMR spectra were recorded at 300 MHz; concentration, 1–5 mg/ml; temperature, 305 K. The ^{13}C NMR spectra at 75.5 MHz; concentration, 5–20 mg/ml; temperature, 305 K. The residual solvent peaks were used as the internal standards (DMSO-d_6: δ_H 2.49, δ_C 39.5; $CDCl_3$: δ_H 7.24, δ_C 77.0; CD_3OD: δ_H 3.30, δ_C 49.0). Alternatively, TMS was used as a standard (indicated with TMS).

4.2.5. Analytical LC–MS. Waters 2700 Autosampler. 2× Waters 600 Multisolvent Delivery System, Waters 600 Controller. Fifty micloliters of sample loop. Column, Chromolith Speed ROD RP18e (Merck, Darmstadt), 50 × 4.6 mm, with 2 μm prefilter (Merck). Eluent A, H_2O + 0.1% HCO_2H; eluent B, MeCN. Gradient, 2% B to 100% B within 4 min, then isocratic for 0.90 min, then back to 2% B within 0.15 min, then isocratic for 0.50 min; flow, 3 ml/min. Waters LCZ single quadrupole mass spectrometer with electrospray source. MS method, MS8minPM-80-800-20 V; positive/negative ion mode scanning, *m*/*z* 80–800 or 80–900 in 1 s; capillary, 3.5 kV; cone voltage, 20 V; multiplier voltage, 400 V; probe and desolvation gas temperature, 120 °C and 350 °C, respectively. Waters 2487 Dual λ Absorbance Detector, set to 254 nm. Software, Waters Masslynx V 4.0.

4.2.6. Preparative LC–MS. Waters 2700 Autosampler, Waters 600 Multisolvent Delivery System with preparative pump heads, Waters 600 Controller, 5000 μl Sample loop. At-column dilution: Waters 600 Multisolvent Delivery System with analytical pump heads; Waters 600 Controller; solvent, MeCN/MeOH 80: 20 (v/v); flow rate, 0.20 or 1 ml/min. Column, Waters X-Terra RP18, 7 μm, 19 × 150 mm with X-Terra RP18 guard cartridge 7 μm, 19 × 10 mm, used at flow rate 20 ml/min. Eluent

A, H_2O containing 0.1% (v/v) HCO_2H or H_2O containing 0.1% (v/v) NEt_3; eluent B, MeCN. Different linear gradients, individually adapted to sample. Injection volume, 0.5–5 ml, depending on sample. Make-up solvent, MeOH/MeCN/H_2O/HCO_2H 80:15:4.95:0.05 (v/v/v/v). Make-up pump, Waters Reagent Manager, flow rate 0.5 ml/min. Waters ZQ single quadrupole mass spectrometer with electrospray source. Positive or negative ion mode scanning *m*/*z* 105–950 in 1 s; capillary, 4 kV; cone voltage, 20 V; multiplier voltage, 600 V; probe and desolvation gas temperature, 120 °C and 250 °C, respectively. Waters Fraction Collector II with mass-triggered fraction collection. Waters 2487 Dual λ Absorbance Detector, set to 254 nm. Software, Waters Masslynx V 4.0.

4.3. Compound 1

Was prepared as described in.[4]

4.4. Compound 2

[1-[1-[1-Formyl-2-(1*H*-indol-3-yl)-ethylcarbamoyl]-2-(1*H*-indol-3-yl)-ethylcarbamoyl]-2-(1*H*-indol-3-yl)-ethyl]-carbamic acid benzyl ester was prepared as described in.[4] To a solution of 1.66 g of this product (2.39 mmol) in 100 ml TMOB, 0.6 g (2.39 mmol) aminohydantoin·HCl and 0.7 ml (2.629 mmol) DIPEA were added and the mixture was stirred for 24 h at rt. The solvent was removed in the vacuum and the residue became crystalline with water. After filtration and drying in the vacuum, 1.8 g crude product was obtained. The product was purified by flash chromatography on silica gel using DCM/MeOH 95:5 and 0.9 g (47%) pure product was obtained.

MS for $C_{44}H_{41}N_9O_6$ 792.00 (MH^+).

^{1}H NMR (300.13 MHz, 305 K, DMSO-d_6, ppm, TMS) δ 2.84–3.20 (6H, m); 3.94 (2H, m); 4.32 (1H, m); 4.60 (1H, m); 4.67 (1H, m); 4.94 (2H, s); 6.76 (1H, d, J = 5.5 Hz); 6.90–7.35 (19H, m); 7.60–7.52 (3H, m); 8.05 (1H, d, J = 8.1 Hz); 8.20 (1H, d, J = 7.8 Hz); 10.76 (3H, m); 11.11 (1H, s).

4.5. Compound 3

Boc-L-Phenylalanine-*n*-methyl-*o*-methylhydroxylamine 7.6 g was dissolved in 100 ml 2 M HCl in glacial acetic acid and stirred at rt for 2 h. The solvent was removed in the vacuum and the residue washed with ether several times. The residue was dried in the HV to yield 7.0 g of an oily residue. An aliquot of 3.0 g (12.25 mmol) was dissolved in 50 ml dry DMF. To this solution, 6.4 g (12.25 mmol) Cbz-Trp-Trp-OH, 4.6 g (12.25 mmol) HBTU, and 6.5 ml DIPEA (36.77 mmol) were added and the reaction mixture was stirred for 12 h at rt. The reaction mixture was diluted with 200 ml ethyl acetate and successively washed with 20% bicarbonate and citric acid. The ethyl acetate phase was dried with magnesium sulfate, the solvent removed in the vacuum and dried in the HV to yield 7.5 g white foam (86%).

MS for $C_{41}H_{42}N_6O_6$: 715.2 MH^+.

To a solution of 1.4 g (2 mmol) of Cbz-Trp-Trp-Phe-*N*-methyl *O*-methylhydroxylamine in 30 ml dry THF, 3 ml (3 mmol) of a 1 M solution of LAH was added dropwise in an ice bath. The reaction was stirred for another 5 h at rt and then quenched with 5% citric acid. The mixture was extracted with ethyl acetate and washed with 10% Na_2CO_3 and saturated NaCI. The organics were dried over $MgSO_4$, filtered, and concentrated in the vacuum. The completion of the reaction was shown by TLC and MS and the tripeptide aldehyde further reacted without purification. (1 g 100% crude yield).

MS for $C_{39}H_{37}N_5O_5$ 656.2 (MH^+).

To a solution of 1.0 g (1.5 mmol) of the above-made tripeptide aldehyde, 0.34 g (3 mmol) semicarbazide·HCl and 0.53 ml (3 mmol) DIPEA were added and the mixture was stirred for 48 h at rt. The solvent was removed in the vacuum. The product became crystalline after adding water and was collected by filtration. The product was purified by preparative HPLC. By this method, 0.57 g LC–MS pure product was obtained (53%).

MS for $C_{40}H_{40}N_8$ 713.16 (MH^+).

^{1}H NMR (300.13 MHz, 305 K, DMSO-d_6, ppm, TMS) δ 2.67–3.11 (6H, m); 4.30 (1H, m); 4.61 (2H, m); 4.93 (2H, m); 6.27 (2H, s); 6.93–7.33 (19H, m); 7.53–7.60 (2H, m); 8.00 (1H, d, J = 8.0 Hz); 8.17 (1H, d, J = 7.7 Hz); 9.88 (1H, s); 10.76 (2H, m).

4.6. Compound 4

4.6.1. Method A. To a mixture of 5.68 g (22.8 mmol) (1-benzyl-2-oxo-ethyl)-carbamicacid *tert*-butyl ester[9] and 3.45 g (22.8 mmol) 1-aminohydantoin hydrochloride (Aldrich) in 150 ml trimethylorthoformate, 3.4 ml DIEPA was added slowly under ice cooling. Ten microliters of dry DMF was added and the reaction mixture was stirred for 72 h at rt. The solvent was removed in the vacuum and the residue purified by flash chromatography on silica gel using DCM/MeOH 95:5. This procedure yielded 6.2 g (79.2% yield) pure [1-benzyl-2-(2,4-dioxo-imidazolin-1-ylimino)-ethyl]-carbaminic acid *tert*-butyl ester.

MS: MH^+-Boc 246.87 95% (346.39 Mass).

^{1}H NMR (300.13 MHz, 305 K, DMSO-d_6, ppm) δ 1.3 (9H, s); 2.6–3.0 (4H, m); 4.2 (2H, s), 4.3–4.4 (1H, m); 6.9–7.0 (H, m); 7.1–7.3 (5H, m); 11.0 (1H, s), 12.7 (1H, s).

1.0 g (3 mmol) of [1-benzyl-2-(2,4-dioxo-imidazolin-1-ylimino)-ethyl]-carbaminic acid *tert*-butyl ester was dissolved in 50 ml of 2 M HCl in glacial acetic acid and stirred for 1 h at rt. The solvent was removed on the rotavapor and the residue made crystalline with ether, collected by filtration and dried in the HV to get 0.8 g deprotected material.

This material was dissolved in 30 ml dry DMF and 1.48 g (2.8 mmol) Cbz-Trp-Trp-OH and 1.0 g (2.8 mmol)

HBTU were added. Finally, 1.5 ml (8.4 mmol) DIPEA was added and the solution was stirred at rt for 12 h. The solution was diluted with 200 ml ethyl acetate and washed with an 20% aqueous solution of sodium bicarbonate and citric acid. The ethyl acetate phase was dried with magnesium sulfate and the solvent removed in the vacuum. Purification by flash chromatography on silica gel using DCM/MeOH as eluent yielded 1.8 g (85%) of LC–MS pure material. The material showed two closely moving spots on TLC eluting with DCM/MeOH (95:5) and an estimated ratio of 3 to 1.

A batch of 100 mg of this material was submitted to preparative TLC and 20 mg of the major spot (lower moving) was obtained and this material was termed Dia2. Another 10 mg of the material moving faster was obtained and the material was termed Dia1.

Dia 1: MS ($C_{40}H_{40}N_8O_5$) 753.2 (MH^+).

Dia 1: ^{1}H NMR (300.13 MHz, 305 K, DMSO-d_6, ppm) δ 3.03–2.72 (6H, m, $H_{\beta,\beta'}$ Phe, Trp1, Trp2); 4.10 (2H, s, $CH_{2\ Hyd}$); 4.26 (1H, m, $H_{\alpha Trp2}$); 4.54 (1H, m, $H_{\alpha Trp1}$); 4.69 (1H, dddd, J = 9.1, 8.4, 5.9, 4.5 Hz, $H_{\alpha Phe}$); 4.92 (2H, m, $CH_{2\ Z}$); 6.95 (1H, d, J = 4.5 Hz, H_{Hyd}); 7.33–6.92 (19H, m, $H_{arom.(Trp1,\ Trp2,\ Z,Phe)}$; $H_{\delta(Trp1,\ Trp2)}$; NH_{Trp2}); 7.53 (1H, d, J = 7.6 Hz, $H\xi_{2\ Trp1}$); 7.56 (1H, d, J = 8.0 Hz, $H\xi_{2\ Trp2}$); 7.91 (1H, d, J = 8.3 Hz, NH_{Trp1}); 8.34 (1H, d, J = 8.4 Hz, NH_{Phe}); 10.73 (1H, d, J = 4.5 Hz, $H_{\varepsilon 1 Trp2}$); 10.74 (1H, d, J = 4.2 Hz, $H_{\varepsilon 1 Trp1}$); 11.73 (1H, s, NH_{Hyd})

Dia 2: MS ($C_{40}H_{40}N_8O_5$) 753.2 (MH^+).

Dia 2: ^{1}H NMR (300.13 MHz, 305 K, DMSO-d_6, ppm) δ 3.14–2.78 (6H, m, $H_{\beta,\beta'}$ Phe, Trp1, Trp2); 3.98 (2H, s, $CH_{2\ Hyd}$); 4.31 (1H, m, $H_{\alpha Trp2}$); 4.57 (1H, m, $H_{\alpha Trp1}$); 4.63 (1H, dddd, J = 8.2, 7.0, 6.7, 4.9 Hz, $H_{\alpha Phe}$); 4.93 (2H, m, $CH_{2\ Z}$); 6.70 (1H, d, J = 4.9 Hz, H_{Hyd}); 7.35–6.93 (19H, m, $H_{arom.(Trp1,\ Trp2,\ Z,\ Phe)}$; $H_{\delta(Trp1,\ Trp2)}$; NH_{Trp2}); 7.57 (1H, d, J = 7.9 Hz, $H\xi_{2\ Trp1}$); 7.60 (1H, d, J = 7.4 Hz, $H\xi_{2\ Trp2}$); 8.03 (1H, d, J = 8.4 Hz, NH_{Trp1}); 8.18 (1H, d, J = 8.2 Hz, NH_{Phe}); 10.75 (1H, d, J = 2.1 Hz, $H_{\varepsilon 1\ Trp2}$); 10.79 (1H, d, J = 1.9 Hz, $H_{\varepsilon 1\ Trp1}$); 11.14 (1H, s, NH_{Hyd}).

4.6.2. Method B. To a solution of 0.5 g (0.76 mmol) of the Cbz-tripeptide aldehyde as prepared in the synthesis of compound **2**, 0.115 g (0.76 mmol) AhydHCl and 0.133 ml (0.74 mmol) DIPEA were added and the mixture was stirred for 48 h at rt. The solvent was removed in the vacuum. The product became crystalline after adding water and was collected by filtration. By this method, 0.5 g product was obtained. Purification by flash chromatography yielded 0.3 g (50%) pure material. The product was identical (LC–MS, TLC, and NMR) with the product Dia2 obtained in compound **4**, method A.

4.7. Compound 5

Synthesis was completely identical to the synthesis of compound 4 with the exception of the use of Boc-D-Phe as a starting material. The product obtained was identical (NMR, LC–MS, and TLC) with Dia1 obtained in the synthesis method A of compound **4**.

4.8. Compound 6

One gram of compound **4** prepared by method B was dissolved in 100 ml MeOH and 3 ml acetic acid. Pd/C (10%) was added and the mixture was stirred under hydrogen for 3 h. The suspension was filtered over a short pad of Celite and washed with MeOH. The product was concentrated in vacuum. After diethyl ether was added, a precipitate was formed, filtered off and dried in vacuum. Yield: 0.8 g. This material was used for the syntheses of cpd **6**–**8**.

MS for $C_{34}H_{34}N_8O_4$ 619.2 (MH^+).

The product from above Trp-Trp-Phe-Ahyd 0.33 g (0.49 mmol) and D(+)-biotine 118 mg (0.49 mmol) was dissolved in 3 ml, DIPEA 169 μl (0.97 mmol) and HBTU 184 mg (0.49 mmol) were added and the reaction was stirred for 16 h at rt. After water was added, a precipitate was formed and filtered off, washed with water and dried. The product was further purified by preparative HPLC.

MS for $C_{44}H_{48}N_{10}O_6S$ 845.3 (MH^+).

^{1}H NMR (300.13 MHz, 305 K, DMSO-d_6, ppm, TMS) δ 1.16 (2H, m); 1.37 (4H, m); 2.00 (2H, m); 2.75–3.12 (6H, m); 3.99 (2H, s); 4.02 (1H, m); 4.24 (1H, m); 4.56 (3H, m); 6.32 (2H, m); 6.72 (1H, d, J = 5.0 Hz); 6.92–7.32 (14H, m); 7.55 (2H, m); 7.89 (2H, m); 8.11 (1H, d, J = 8.0 Hz); 10.73 (1H, d, J = 2.0 Hz); 10.80 (1H, d, J = 1.8 Hz); 11.15 (1H, s).

4.9. Compound 7

Carbonic acid 4-nitrophenyesterpyridin-4-methylester: to a vigorously stirred mixture of 0.6 g (5.5 mmol) of 3-pyridinylmethanol and 1 g of potassium carbonate in 20 ml of DCM under cooling (ice bath) was added dropwise 1.4 g (7 mmol) of *p*-nitrophenyl chloroformate in 10 ml of DCM, and a suspension was stirred for 24 h at rt. The precipitate was filtered off and DCM was evaporated at reduced pressure. The residue was washed with 100–150 ml of cold water and dried. Recrystallization of the residual solid from benzene afforded 1 g (73%) of desired compound. ^{1}H NMR (δ, ppm, $CDCl_3$): 5.33 (s, 2H, CH_2), 7.36–7.41 (m, 3H, Ar, HetAr), 7.79–7.82 (m, 1H, HetAr), 8.27–8.31 (m, 2H, Ar), 8.65–8.67, 8.72–8.73 (m, 2H, HetAr).

The intermediate product from cpd **6** Trp-Trp-Phe-Ahyd 300 mg (0.49 mmol) and carbonic acid 4-nitrophenyesterpyridin-4-methylester 133 mg (0.49 mmol) was dissolved in 3 ml dry DMF, DIPEA 169 μl (0.97 mmol) was added and the reaction stirred for 16 h at rt. After water was added, a precipitate was formed and filtered off, washed with water and ether and dried. The product was further purified by preparative HPLC.

MS for $C_{41}H_{39}N_9O_6$ 754.23 (MH^+).

^{1}H NMR (300.13 MHz, 305 K, DMSO-d_6, ppm, TMS) δ 2.80–3.18 (6H, m); 3.99 (2H, s); 4.31 (1H, m); 4.56 (1H, m); 4.63 (1H, m); 4.98 (2H, s); 6.71 (1H, d, J = 4.7 Hz); 6.92–7.40 (15H, m); 7.58 (2H, m); 8.05 (1H, d, J = 8.0 Hz); 8.18 (1H, d, J = 8.1 Hz); 8.50 (2H, m); 10.75 (1H, s); 10.80 (1H, s); 11.15 (1H, s).

4.10. Compound 8

The intermediate product from cpd **6** Trp-Trp-Phe-Ahyd 300 mg (0.49 mmol) and 3-(4-hydroxyphenyl)-propionic acid 81 mg (0.49 mmol) was dissolved in 3 ml dry DMF, DIPEA 169 μl (0.97 mmol) and HBTU 184 mg (0.49 mmol) were added and the reaction was stirred for 16 h at rt. After water was added, a precipitate was formed and filtered off, washed with water and dried. The product was further purified by pHPLC and 60 mg (30%) pure product was obtained.

MS for $C_{43}H_{42}N_8O_6$ 767.24 (MH^+).

^{1}H NMR (300.13 MHz, 305 K, DMSO-d_6, ppm, TMS) δ 2.24 (2H, t, J = 7.4 Hz); 2.54 (2H, t, J = 7.4 Hz); 2.78–3.10 (6H, m); 4.00 (2H, s), 4.47–4.66 (3H, m); 6.61 (2H, m); 6.72 (1H, d, J = 4.9 Hz); 6.95–7.33 (16H, m); 7.55 (2H, m); 7.92 (2H, m); 8.08 (1H, d, J = 8.1 Hz); 9.06 (1H, s); 10.74 (1H, d, J = 2.0 Hz); 10.79 (1H, d, J = 2.0 Hz); 11.09 (1H, s).

4.11. Proteasome assay

Compounds were characterized by monitoring the inhibitory effect on the chymotryptic activity of the human 20S proteasome (Biomol). The assay was performed using a Tecan Ultra plate reader and the fluorogenic substrates Suc-LLVY-AMC (Bachem) for the chemotryptic and Z-LLE-ßBachem) for the peptidyglutamyl peptide hydrolyzing and Bz-VGR-AMC (Bachem) for the tryptic activity, respectively. In a black 96-well polypropylene plate, 2 μl of compound, dissolved in 100% DMSO, was mixed with 50 μl substrate solution (25 mM Hepes, pH 7.5, 0.5 mM EDTA and 75 μM Suc-LLVY-AMC or Z-LLE-ßNA or Bz-VGR-AMC). Reaction was initiated by adding 150 μl proteasome solution (1.3 μg 20S proteasome in 25 mM Hepes, pH 7.5, 0.5 mM EDTA, 0.033% (w/v) SDS, pre-incubated for 10 min at room temperature). Substrate hydrolysis was followed by fluorescence spectroscopy (excitation wavelength: 360 nm; emission wavelength: 465 nm) for 20 min at 30 °C and signals were calculated as relative fluorescence units (RFU) per minute. However, no signal could be obtained when using Bz-VGR-AMC as a substrate. This substrate might be incompatible with SDS used for proteasome activation. For the determination of the IC_{50} values (concentration of inhibitor required for 50% inhibition), eight inhibitor concentrations were applied. Data points were recorded in triplicates on single measurement day. To obtain dose–response curves and IC_{50} values, data were fitted to a four parameter logistic function using SigmaPlot.

4.12. NFκB reporter gene assay

The NF-κB reporter gene assay was prepared with A549-NF-κB-SEAP cell line (CCS cell culture service, Hamburg, Germany) according to manufacturer's instructions. In short, A549 cells stable transfected with pNF-κB-SEAP reporter gene plasmid were plated at 2×10^4/well and allowed to attach overnight. The cells were subsequently incubated for 5 h with described compounds at 100, 30, 10, 3, 1, 0.3, 0.1, and 0 μM and then stimulated with 10 ng/ml TNF-α for 22 h. The supernatant of the cell was analyzed for SEAP activity using a chemiluminescent SEAP reporter gene assay (Roche, Mannheim, Germany), and a cell viability assay was prepared using a CellTiter-BluTM Cell Viability Assay (Promega, Mannheim, Germany). For each concentration of the compound, four replicates were measured.

4.13. T-cell proliferation assay

Cells (Mononuclear cells) were isolated from human peripheral blood by Accuspin™ System-Histopaque®-1077 (Sigma, Germany). After washing, the cells were diluted to approximately 100,000–200,000 cells/well in a sterile 96-well flat bottomed MP (Corning, Netherlands). T-lymphocytes were stimulated by the addition of 20 μg/ml phytohemagglutinin-L (Roche, Germany). The incubation at 37 °C, 5% CO_2, 90% humidity was made in the presence of different concentrations of the compounds. All cells were incubated for 48 h at 37 °C, 5% CO_2, 90% relative humidity over a concentration range of 0.4–50 μM compound solutions with a final volume per well of 100 μl. After the initial incubation period, 10 μM BrdU (Roche, Germany) was added for an additional 4 h incubation. The culture medium employed was RPMI 1640, which contained 10% heat inactivated fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptamycin sulfate. After 48 h incubation, the cells were labeled by adding 10 μl BrdU-Solution (Roche, Germany) and reinsulated for further 4 h. Following incubation, the media plus BrdU and drug was removed and the cells were fixed and the DNA was denatured in a single step using FixDenat (Roche, Germany) The anti-BrdU-POD (Roche, Germany) binds to the BrdU incorporated in newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction. The reaction product was quantified by measuring the absorbance at the respective wavelength using an ELISA reader. The EC_{50} values were determined using a fitting function.

4.14. Molecular modeling

The potential binding modes of the compounds were explored by refinement of docking poses generated by Pro-Pose.[10,11] The active site definition for docking and refinement contained subunits β5 and β6 of the 2.7 Å structure of the mammalian 20S proteasome (PDB ID 1IRU). Promising binding modes were selected manually and refined by molecular dynamics simulations (MD) utilizing Moloc.[12] The refinement protocol comprised an initial energy minimization, a short MD run (~10 ps), and a final energy minimization step. Fig-

ure 2 was prepared with the help of pymol 0.99 (open source).[13]

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