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Synthesis of Bivalent Inhibitors of Eucaryotic Proteasomes

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Abstract: Based on the peculiar spatial array of the active sites in the internal chamber of the multicatalytic proteasome, as derived from the X-ray structure of yeast proteasome, homo- and heterobivalent inhibitors were designed and synthesized to exploit the principle of multivalency for enhancing inhibition potency. Peptidic bis-aldehyde compounds of the octapeptide size were synthesized to address adjacent active sites, whilst a PEG spacer with a statistical length distribution of 19–25 monomers was used to link two identical or different tripeptide aldehydes as binding heads. These bis-aldehyde compounds were synthesized applying both methods in solution and solid phase peptide synthesis. Bivalent binding was observed only for the PEG-spaced inhibitors suggesting that binding from the primed side prevents hemiacetal formation with the active site threonine residue. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: synthetic inhibitors; peptide bis-aldehydes; pegylation; bivalent inhibition; proteasome

INTRODUCTION

The proteasome is a multicatalytic protease complex that is found in all three kingdoms of life. It is the central enzyme of intracellular protein turnover both in the cytosol and the nucleus where it degrades abnormal, misfolded, or improperly assembled proteins and generates antigenic peptides for presentation via MHC class I molecules. These cellular functions are linked to a ubiquitin- and ATP-requiring pathway involving the 26S proteasome, whose proteolytic chamber is formed by the 20S proteasome [1]. The determination of the crystal structure of the 20S proteasome from *Saccharomyces cerevisiae* revealed a cylindrical particle with an overall assembly of the 28 subunits as a $(\alpha 1\text{-}\alpha 7,\ \beta 1\text{-}\beta 7)_2$ complex consisting of four stacked heptameric rings [2]. Only three of the seven different β-subunits are fully processed during assembly and maturation of the enzyme with release of the *N*-terminal nucleophile, Thr^1, in $\beta 1$, $\beta 2$, and $\beta 5$. These three β-subunits are responsible for the three major proteolytic activities of the complex against small chromogenic substrates and a large protein, i.e. $\beta 1$ for the PGPH, $\beta 2$ for the trypsin-like, and $\beta 5$ for the chymotrypsin-like, as could be shown by mutational studies in yeast [3–5].

Despite the differentiated specificities of the $\beta 1$, $\beta 2$, and $\beta 5$ active sites, the crystal structure of the yeast proteasome in complex with the tripeptide aldehyde Ac-Leu-Leu-Nle-H revealed binding of this inhibitor to all six active sites via hemiacetal formation with the Thr^1 hydroxyl function [2]. This observation indicates the relatively low substrate specificity of the proteasome, which can cleave

Abbreviations: AcOEt, ethyl acetate; AMC, 7-amido-4-methyl-coumarin; NA, naphthylamide; DEA, diethyl acetal; DIEA, diisopropylethylamine; DMF, dimethylformamide; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide; ESI-MS, electron spray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; HATU, so-called *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; Lev, laevulinic acid; NMM, *N*-methylmorpholine; NMP, *N*-methyl-pyrrolidone; PEG, polyethyleneglycol; PGPH, post-glutamyl-peptide hydrolytic; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; Saa, succinic acid aldehyde; Sc, semicarbazone; SPPS, solid phase peptide synthesis; TBTU, so-called 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

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protein substrates at almost every position [6–10]. In consequence, the development of selective inhibitors of this protease is very difficult. The *Streptomyces* metabolite lactacystin [11] acts via its intermediate *clasto*-lactacystin-β-lactone and inhibits irreversibly mainly the chymotrypsin-like activity of the proteasome [12,13], but surprisingly shows activity also against cathepsin A [14]. More selective irreversible inhibitors were obtained with peptidyl-vinylsulfones [15,16] and α',β'-epoxyketones [17,18]. With regard to clinical applications the reversible boronic-acid based inhibitors seem more promising [19].

Besides tryptase [20] the proteasome is the only oligomeric protease in eucaryotes with a known and well-defined display of the proteolytic centres, which makes this protease an ideal target for multivalent inhibition. Multivalency has first been used as a tool to increase affinity in chelate chemistry [21,22] and was later discovered in nature as a principle that is ubiquitously exploited to enhance selectivity in molecular recognition processes [23,24]. We have applied this principle for the inhibition of 20S proteasome by bivalent inhibitors [25,26]. In the present communication will be described the synthesis of this new class of proteasome inhibitors, which were developed on the basis of the unique arrangement of a 2-fold set of three different active sites (Figure 1).

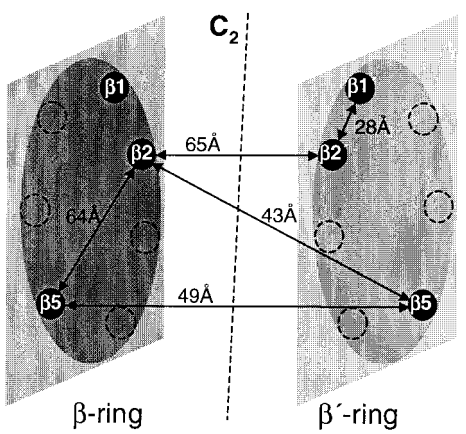


Figure 1 Schematic representation of the two β-rings of the yeast proteasome with selected inter-active site distances as derived from the crystal structure [2].

MATERIALS AND METHODS

All solvents and reagents used in the synthesis were of the highest quality commercially available, and when required were further purified and dried by standard methods. TLC silica gel 60 plates were from Merck AG (Darmstadt, Germany) and compounds were visualized with the chlorine/tolidine or permanganate reagent. The polymeric spacer $(PEG)_{19-25}$-[NH-CO-$(CH_2)_2$-COOH]$_2$ was from Rapp Polymere (Tübingen, Germany). Analytical HPLC was performed with a flow rate of 1 ml/min on Nucleosil 100/C18 columns (Macherey-Nagel, Düren, Germany) using linear gradients of MeCN/2% H_3PO_4 from 20:80 to 85:15 in 25 min (A) or MeCN/2% H_3PO_4 from 5:95 to 80:20 in 30 min (B) or MeCN/2% H_3PO_4 from 5:95 to 80:20 in 13 min with 2 min additional isocratic elution (C). Preparative HPLC was carried out on Nucleosil RP 18 (Macherey-Nagel) and size exclusion chromatography was performed on Fractogel TSK HW 40 S (Merck AG, Darmstadt, Germany) using MeOH as eluent. FAB-MS spectra were recorded on a Finnigan MAT 900 and ESI-MS spectra were recorded on a PE API 165 mass spectrometer (Perkin Elmer, Langen, Germany).

The substrates Z-Leu-Leu-Glu-βNA, Bz-Phe-Val-Arg-AMC and Suc-Leu-Leu-Val-Tyr-AMC as well as H-Gly-Pro-Gly-Gly-OH were purchased from Bachem (Heidelberg, Germany). Proteasome from *S. cerevisiae* was prepared as described previously [2].

Synthesis of Intra-Ring Bivalent Inhibitors

H-Leu-Leu-Nle-Sc trifluoroacetate and Ac-Leu-Leu-Nle-H (1). The title compounds were prepared as described previously [27].

Boc-Gly-Pro-Gly-Gly-OH (2). To a solution of H-Gly-Pro-Gly-Gly-OH (0.57 g; 2.0 mmol) in 4 ml of 0.5 N NaOH and 4 ml of dioxane was added Boc_2O (0.55 g; 2.5 mmol). After 2 h, the solution was concentrated *in vacuo* and distributed between *n*-butanol/AcOEt (1:2) and 5% $KHSO_4$. The aqueous phase was reextracted once more with AcOEt and the combined organic phases washed with brine. The organic phase was evaporated and the resulting oil crystallized from AcOEt/methyl tert-butyl ether/diisopropyl ether; yield: 0.50 g (65%); TLC ($CHCl_3$/MeOH/AcOH/H_2O; 60:40:5:10) R_f 0.7; ES-MS: $m/z = 387.4$ ($M + H^+$); calc. $M_r = 386.2$.

Boc-Gly-Pro-Gly-Gly-Leu-Leu-Nle-Sc (3). To a solution of Boc-Gly-Pro-Gly-Gly-OH (0.14 g; 0.36 mmol) in 2 ml of DMF at 0°C was added H-Leu-Leu-Nle-Sc × TFA (0.15 g; 0.36 mmol), PyBOP (0.19 g; 0.36 mmol) and NMM (0.11 ml). After stirring for 15 h at room temperature, the solution was concentrated and the resulting oil treated with H_2O. The solid residue was crystallized from MeOH/methyl tert-butyl ether; yield: 0.23 g (100%); TLC ($CHCl_3$/MeOH/pyridine/H_2O; 60:15:2:1) R_f 0.6; FAB-MS: $m/z =$ 767.4 ($M + H^+$); calc. $M_r = 766.5$.

H-Gly-Pro-Gly-Gly-Leu-Leu-Nle-Sc × TFA (4). To a suspension of Boc-Gly-Pro-Gly-Gly-Leu-Leu-Nle-Sc (0.21 g; 0.27 mmol) in 12 ml of CH_2Cl_2 was added at 0°C 4 ml of TFA. After 1 h at room temperature, the solution was evaporated, redestilled with toluene and the residue treated with methyl tert-butyl ether; yield: 0.17 g (81%); TLC ($CHCl_3$/MeOH/pyridine/H_2O; 60:15:2:1) R_f 0.1; FAB-MS: $m/z = 667.4$ ($M + H^+$); calc. $M_r = 666.4$.

Di-ethylthio-acetic acid potassium salt (5). Glyoxylic acid ethyl ester diethylmercaptal (3.90 ml; 20 mmol) in 5 ml of EtOH was treated with KOH (1.12 g; 20 mmol) in 20 ml of EtOH. After 3 h, the resulting suspension was diluted with 150 ml of methyl tert-butyl ether and the precipitate collected; yield: 3.70 g (85%); TLC (cyclohexane/$CHCl_3$/AcOH; 45:45:10) R_f 0.2.

Di-ethylthio-acetyl-Leu-OH (6). Di-ethylthio-acetic acid potassium salt (0.87 g; 4.0 mmol), H-Leu-OMe × HCl (0.73 g; 4.0 mmol), HOBt (0.54 g; 4.0 mmol) and EDC × HCl (0.77 g; 4.0 mmol) were stirred in 20 ml of DMF over night at room temperature. After concentration *in vacuo*, the residue was distributed between AcOEt and 5% $NaHCO_3$. The organic phase was washed with 5% $KHSO_4$ and H_2O, dried (Na_2SO_4) and evaporated; yield of di-ethylthio-acetyl-Leu-OMe: 1.15 g (93%); TLC (cyclohexane/$CHCl_3$/AcOH, 45:45:10) R_f 0.8.

The methyl ester (0.26 g; 0.85 mmol) was dissolved in 7 ml of dioxane and treated with 2 ml of 0.5 N NaOH at room temperature. After 2 h, the solvents were evaporated and the residue distributed between AcOEt and 1 N H_2SO_4. The organic layer was washed with H_2O, dried (Na_2SO_4) and evaporated *in vacuo*. The resulting oil solidified in the cold to a wax-like solid; yield: 0.24 g (96%); TLC (cyclohexane/$CHCl_3$/AcOH; 45:45:10) R_f 0.7.

Di-ethylthio-acetyl-Leu-Gly-Pro-Gly-Gly-Leu-Leu-Nle-Sc (7). To a solution of di-ethylthio-acetyl-Leu-OH (67 mg; 0.23 mmol) and H-Gly-Pro-Gly-Gly-Leu-Leu-Nle-Sc × TFA (0.17 g; 0.21 mmol) in 3 ml of DMF was added at 0°C HATU (87 mg; 0.23 mmol) and DIEA (114 µl; 0.23 mmol). After stirring over night at room temperature, the solvent was removed *in vacuo*. The oily residue was distributed between *n*-butanol and H_2O and the organic phase was concentrated to an oil. After dilution with MeCN, the product was precipitated with methyl tert-butyl ether. The crude material was chromatographed on 80 g silica gel with $CHCl_3$/MeOH/pyridine/H_2O (60:15:2:1) as eluent; yield: 0.11 g (57%); TLC ($CHCl_3$/MeOH/pyridine/H_2O; 60:15:2:1) R_f 0.8; FAB-MS: $m/z = 942.4$ ($M + H^+$); calc. $M_r = 941.5$.

Glyoxylyl-Leu-Gly-Pro-Gly-Gly-Leu-Leu-Nle-H (8). Di-ethylthio-acetyl-Leu-Gly-Pro-Gly-Gly-Leu-Leu-Nle-Sc (55 mg; 0.06 mmol) in 3.8 ml of MeCN and 0.2 ml of H_2O was treated at room temperature with thallium nitrate trihydrate (53 mg; 0.12 mmol). After 20 min, the precipitated thallium (I) salt was filtered off, the solution diluted with 0.25 ml of H_2O and adjusted to pH 5–6 with $NaHCO_3$. The salts were once again removed by filtration, the filtrate was diluted with H_2O and lyophilized. The crude product was chromatographed in batches on Lichroprep RP-18 using a linear gradient of MeCN/H_2O from 20:80 to 80:20 in 5 h. Fractions containing pure product as monitored by HPLC were pooled and lyophilized; yield: 15 mg (33%); HPLC (gradient A): t_R 13.60 min; FAB-MS: $m/z = 779.6$ ($M + H^+$); calc. $M_r = 778.5$.

Fmoc-Glu(OtBu)N(OMe)Me (9). To a solution of Fmoc-Glu(OtBu)-OH × H_2O (10.0 g; 22.6 mmol), TBTU (7.24 g; 22.6 mmol) and DIEA (3.85 ml; 22.6 mmol) in 220 ml of CH_2CL_2 was added at 0°C HOBt (3.05 g; 22.6 mmol), *N,O*-dimethylhydroxylamin × HCl (2.42 g; 24.8 mmol) and DIEA (4.24 ml; 24.8 mmol) dissolved in 30 ml of CH_2Cl_2 and 10 ml of DMF. The mixture was allowed to warm to room temperature and upon completion of the reaction, the solvent was evaporated. The oily residue was distributed between EtOAc and 5% $KHSO_4$, the organic layer washed twice with 5% $KHSO_4$, 5% $NaHCO_3$ and H_2O, dried ($MgSO_4$) and evaporated; yield: 10.6 g (100%); TLC (EtOAc/PE; 2:1) R_f 0.7.

Fmoc-Glu(OtBu)-H (10). Fmoc-Glu(OtBu)N(OMe)Me (10.48 g; 22.4 mmol) was dissolved in 350 ml of dry diethyl ether and $LiAlH_4$ (0.87 g; 22.8 mmol) was added at 0°C in small portions under argon atmosphere. After stirring for 30 min at 0°C and 30 min at room temperature, the reaction was quenched by careful addition of 0.1 M HCl (240 ml; 24.0 mmol).

The resulting mixture was distributed between 5% $KHSO_4$ and EtOAc, the aqueous layer was extracted with EtOAc, the combined organic layers were washed with 5% $KHSO_4$, dried ($MgSO_4$) and evaporated; yield: 8.67 (95%); TLC (EtOAc/petrol ether; 2:1) R_f 0.6.

Fmoc-Glu(OtBu)-Sc (11). Semicarbazid × HCl (2.59 g; 23.2 mmol) and sodium acetate (1.91 g; 23.2 mmol) in 60 ml of H_2O was added to Fmoc-Glu-(OtBu)-H (8.65 g; 21.1 mmol) in 120 ml of EtOH. After stirring overnight, the solvent was evaporated, the residue distributed between EtOAc and H_2O, the organic layer washed with 5% $NaHCO_3$ and H_2O, dried ($MgSO_4$) and evaporated. The crude product was purified by silica gel chromatography using EtOAc/EtOH (12:1) as eluent; yield: 6.17 g (63%); TLC (EtOAc/EtOH; 20:1) R_f 0.6.

Fmoc-Glu(OH)-Sc (12). To a solution of Fmoc-Glu-(OtBu)-Sc (3.57 g; 7.65 mmol) in 375 ml of CH_2Cl_2 was added at 0°C 125 ml of TFA and H_2O (0.28 ml; 15.3 mmol). After stirring for 1 h at room temperature, the solvent was removed and the residue redistilled three times with toluene. The crude product was precipitated with diethyl ether; yield: 2.96 g (94%); TLC (EtOAc/EtOH; 10:1) R_f 0.6; FAB-MS: $m/z = 411.2$ ($M + H^+$); calc. $M_r = 410.2$.

4-oxo-Butyric acid semicarbazone (13). To a solution of 4-oxo-butyric acid (15% in H_2O; 5.0 ml; 7.35 mmol) was added dropwise semicarbazid × HCl (0.90 g; 8.08 mmol) and sodium acetate (0.66 g; 8.08 mmol) in 2 ml of H_2O. After 4 h, the white precipitate was collected, washed with cold water and dried *in vacuo* over KOH; yield: 1.14 g (98%); TLC (MeCN/H_2O/AcOH; 3:1:0.1) R_f 0.5; ES-MS: $m/z = 159.8$ ($M + H^+$); calc. $M_r = 159.1$.

Solid Phase Synthesis of CH_3-CO-CH_2-CH_2-CO-KKGEVSLE-H (14), OHC-CH_2-CH_2-CO-KKGEVSLE-H (15) and OHC-CH_2-CH_2-CO-AAEVSLE-H (16). The synthesis on solid support was carried out manually at a 0.03–0.14 mmol scale using 2-chlorotritylchloride resin, which was loaded by addition of Fmoc-Glu-Sc (0.99 g; 2.40 mmol) and DIEA (1.23 ml; 7.20 mmol) in 7 ml of CH_2Cl_2 and 1.5 ml of DMF to the preswollen resin (2.40 g; 3.14 mmol) in 10 ml of CH_2Cl_2. After 45 min, the reaction was quenched with 3 ml of MeOH, the resin was filtered off and washed with CH_2Cl_2, 80% NMP/CH_2Cl_2, isopropanol, MeOH and diethyl ether, and dried *in vacuo* over KOH. The degree of loading was 0.46 mmol/g as determined by quantitative Fmoc cleavage and monitoring of the piperidinium adduct at 301 nm using an ε of 7800 l/mol/cm. Coupling of the Fmoc-amino acids and levulinic acid was performed in double with a 4-fold excess of Fmoc-AA-OH or levulinic acid/HBTU/HOBt/DIEA (1:1:1:2) in NMP/CH_2Cl_2 (4:1). 4-oxo-Butyric acid semicarbazone was coupled with a 10-fold excess of 4-oxo-butyric acid semicarbazone/PyBOP/HOBt/DIEA (1:1:1:2) in NMP/CH_2Cl_2 (4:1). The Fmoc-group was cleaved with 20% piperidine in NMP/CH_2Cl_2 (4:1) in two cycles of 3 and 12 min, respectively. Coupling efficiency was monitored by the Kaiser test. The final product was deprotected and cleaved from the resin with 95% TFA (3 h) and was precipitated after filtration by addition of cold diethyl ether. The crude product was purified by preparative HPLC using linear gradients of MeCN/H_2O from 5:95 to 35:65 in 90 min; fractions containing homogeneous material as monitored by HPLC were combined and lyophilized.

CH_3-CO-CH_2-CH_2-CO-KKGEVSLE-H (14). Yield: 11 mg (11%); HPLC (gradient B): t_R 9.38 min; ES-MS: $m/z = 971.5$ ($M + H^+$); calc. $M_r = 970.5$.

OHC-CH_2-CH_2-CO-KKGEVSLE-H (15). Yield: 1.6 mg (5%); HPLC (gradient B): t_R 9.15 min; ES-MS: $m/z = 957.5$ ($M + H^+$); calc. $M_r = 956.5$.

OHC-CH_2-CH_2-CO-AAEVSLE-H (16). Yield: 4.4 mg (4%); HPLC (gradient B): t_R 11.93 min; ES-MS: $m/z = 786.4$ ($M + H^+$); calc. $M_r = 785.4$.

Synthesis of Inter-Ring Bivalent Inhibitors

H-Val-Arg(Adoc)$_2$-DEA. The title compound was prepared as described previously [28].

Z-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (17). Z-Arg-(Adoc)$_2$-OH (0.30 g; 0.45 mmol), TBTU (0.15 g; 0.45 mmol), HOBt (62 mg; 0.45 mmol) and DIEA (0.16 ml; 0.90 mmol) were dissolved in 2 ml of DMF, and at 0°C a solution of H-Val-Arg(Adoc)$_2$-DEA (0.31 g; 0.45 mmol) in 2 ml of DMF was added. After stirring overnight at room temperature, the solvent was removed and the residue distributed between AcOEt and 5% $KHSO_4$. The organic layer was washed with 5% $KHSO_4$, 5% $NaHCO_3$ and water, and dried over $MgSO_4$. The solvent was removed and the residue redestilled with toluene; yield: 0.57 g (93%); TLC ($CHCl_3$/MeCN/AcOH; 20:1:0.1) R_f 0.6.

H-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (18). Z-Arg-(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (0.53 g; 0.40 mmol) was hydrogenated in 100 ml of 95% EtOH over Pd/C (10%). After 1 h, the catalyst was filtered off and the solvent evaporated; yield: 0.47 g (98%); TLC ($CHCl_3$/MeOH/AcOH; 20:1:0.1) R_f 0.6; HPLC (gradient C): t_R 13.36 min.

Ac-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (19). To a solution of H-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (0.18 g; 0.15 mmol) in 10 ml of DMF was added acetic anhydride (0.14 ml; 1.50 mmol) and DIEA (0.26 ml; 1.50 mmol). After 1 h, the solvent was removed under reduced pressure and the residue distributed between AcOEt and 5% $NaHCO_3$. The organic layer was washed with 5% $KHSO_4$ and H_2O, dried over $MgSO_4$ and evaporated; yield: 0.18 mg (97%); TLC ($CHCl_3$/MeOH; 20:1) R_f 0.6; HPLC (gradient C): t_R 14.63 min.

Ac-Arg-Val-Arg-H × 2 TFA (20). Deprotection of Ac-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (41 mg; 33 μmol) was carried out by reaction with 1 ml of 95% TFA at 0°C. After 90 min, the product was precipitated with cold diethyl ether; yield: 18 mg (80%); TLC (AcOEt/BuOH/AcOH/H_2O; 2:1:1:1) R_f 0.3; ES-MS: $m/z = 456.4$ ($M + H^+$); calc. $M_r = 455.3$.

HOOC-(CH$_2$)$_2$-CO-NH-(PEG)$_{19-25}$-NH-(CH$_2$)$_2$-CO-Leu-Leu-Nle-Sc (21). The succinyl-amino-$(PEG)_{19-25}$ (0.10 g; 88 μmol), H-Leu-Leu-Nle-Sc trifluoroacetate (45 mg; 88 μmol), TBTU (28 mg; 88 μmol), HOBt (12 mg; 88 μmol) and DIEA (45 μl; 0.26 mmol) were dissolved in 6 ml of DMF. After 3 h, the solvent was evaporated and the oily residue was distributed between CH_2Cl_2 and 5% $KHSO_4$. The aqueous phase was extracted twice with CH_2Cl_2 and the combined organic layers were washed with 5% $NaHCO_3$, dried over Na_2SO_4 and evaporated; yield: 90 mg (68%); HPLC (gradient C): t_R 7.70 min.

(PEG)$_{19-25}$-(NH-CO-(CH$_2$)$_2$-CO-Leu-Leu-Nle-Sc)$_2$ (22). The succinyl-amino-$(PEG)_{19-25}$ (0.10 g; 88 μmol), H-Leu-Leu-Nle-Sc trifluoroacetate (90 mg; 0.18 mmol), TBTU (57 mg; 0.18 mmol), HOBt (24 mg; 0.18 mmol) and DIEA (90 μl; 0.53 mmol) were dissolved in 1 ml of DMF. After 4 h, the solvent was evaporated and the oily residue was purified by preparative HPLC with a linear gradient of MeCN/H_2O from 15:85 to 60:40 in 70 min. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized; yield: 80 mg (49%); HPLC (gradient C): t_R 8.73 min.

HOOC-(CH$_2$)$_2$-CO-NH-(PEG)$_{19-25}$-NH-(CH$_2$)$_2$-CO-Leu-Leu-Nle-H (23). HOOC-$(CH_2)_2$-CO-NH-$(PEG)_{19-25}$-NH-$(CH_2)_2$-CO-Leu-Leu-Nle-Sc (80 mg; 53 μmol) was dissolved in a mixture of 5 ml of MeOH, 2 ml of acetic acid and 2 ml of formaldehyde (37% in water). After 2 h, the solvent was removed and the oily residue was immediately purified by preparative HPLC with a linear gradient of MeCN/H_2O from 15:85 to 60:40 in 70 min. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized; yield: 8 mg (10%); HPLC (gradient C): t_R 7.99 min; ES-MS: $m/z = 1508.6 \pm (44.0)_n$ with $n = 0–3$ ($M + H^+$); calc. $M_r = 1507.9 \pm (44.0)_n$ with $n = 0–3$.

(PEG)$_{19-25}$-(NH-CO-(CH$_2$)$_2$-CO-Leu-Leu-Nle-H)$_2$ (24). $(PEG)_{19-25}$-[NH-CO-$(CH_2)_2$-CO-Leu-Leu-Nle-Sc]$_2$ (80 mg; 53 μmol) was dissolved in a mixture of 5 ml of MeOH, 2 ml of acetic acid and 2 ml of formaldehyde (37% in H_2O). After 2 h, the solvent was removed and the oily residue was immediately purified by preparative HPLC with a linear gradient of MeCN/H_2O from 15:85 to 60:40 in 70 min. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized; yield: 35 mg (20%); HPLC (gradient C): t_R 9.56 min; ES-MS: $m/z = 1832.6 \pm (44.0)_n$ with $n = 0–3$ ($M + H^+$); calc. $M_r = 1831.2 \pm (44.0)_n$ with $n = 0–3$.

HOOC-(CH$_2$)$_2$-CO-NH-(PEG)$_{19-25}$-NH-CO-(CH$_2$)$_2$-CO-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (25). A mixture of the succinyl-amino-$(PEG)_{19-25}$ (0.20 g; 0.18 mmol), H-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (0.23 g; 0.19 mmol), TBTU (62 mg; 0.19 mmol), HOBt (26 mg; 0.19 mmol) and DIEA (96 μl; 0.56 mmol) was stirred at room temperature for 3 h. After removal of the solvent, the residue was distributed between CH_2Cl_2 and 5% $KHSO_4$. The aqueous phase was extracted twice with CH_2Cl_2 and the combined organic layers were washed with 5% $NaHCO_3$, dried over Na_2SO_4 and evaporated to a colourless oil. The crude material was purified by size exclusion chromatography. Fractions containing homogeneous material as monitored by HPLC were pooled and evaporated; yield: 0.14 g (35%); HPLC (gradient C): t_R 12.3 min; ES-MS: $m/z = 2368.6 \pm (44.0)_n$ with $n = 0–3$ ($M + H^+$); calc. $M_r = 2368.4 \pm (44.0)_n$ with $n = 0–3$.

(PEG)$_{19-25}$-(NH-CO-(CH$_2$)$_2$-CO-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA)$_2$ (26). To a solution of HOOC-$(CH_2)_2$-CO-NH-$(PEG)_{19-25}$-NH-CO-$(CH_2)_2$-CO-Arg-(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (43 mg; 18 μmol), TBTU (12 mg; 37 μmol), HOBt (5 mg; 37 μmol) and DIEA (6.3 μl; 37 μmol) in 1 ml of DMF was added H-Arg-(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (45 mg; 37 μmol) in 1 ml of DMF. After stirring at room temperature for 2 h, the solvent was removed and the oily residue distributed between CH_2Cl_2 and 5% $KHSO_4$. The organic layers were washed with 5% $NaHCO_3$, dried over Na_2SO_4 and evaporated. The crude material was purified by size exclusion chromatography; fractions containing homogeneous material as monitored by HPLC were pooled and evaporated; yield: 44 mg (68%); HPLC (gradient C): t_R 14.60 min; ES-MS:

$m/z = 1775.6 \pm (22.0)_n$ with $n = 0–3$ $(M + 2H^{2+})$; calc. $M_r = 3552.2 \pm (44.0)_n$ with $n = 0–3$.

(PEG)$_{19–25}$-(NH-CO-(CH$_2$)$_2$-CO-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA)(NH-CO-(CH$_2$)$_2$-CO-Leu-Leu-Nle-Sc) (27). HOOC-(CH$_2$)$_2$-CO-NH-(PEG)$_{19–25}$-NH-CO-(CH$_2$)$_2$-CO-Arg-(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (54 mg; 23 μmol), H-Leu-Leu-Nle-Sc trifluoroacetate (12 mg; 23 μmol), TBTU (7.4 mg; 23 μmol), HOBt (3.1 mg; 23 μmol) and DIEA (3.9 μl; 23 μmol) were reacted in 3 ml of DMF for 3 h. After removal of the solvent, the oily residue was distributed between CH_2Cl_2 and 5% $KHSO_4$. The aqueous phase was extracted twice with CH_2Cl_2 and the combined organic layers were washed with 5% $NaHCO_3$, dried over Na_2SO_4 and evaporated; yield: 58 mg (92%); HPLC (gradient C): t_R 13.3 min; ES-MS: $m/z = 2748.3 \pm (44.0)_n$ with $n = 0–3$ $(M + H^+)$; calc. $M_r = 2748.7 \pm (44.0)_n$ with $n = 0–3$.

HOOC-(CH$_2$)$_2$-CO-NH-(PEG)$_{19–25}$-NH-CO-(CH$_2$)$_2$-CO-Arg-Val-Arg-H × 2TFA (28). HOOC-(CH$_2$)$_2$-CO-NH-(PEG)$_{19–25}$-NH-CO-(CH$_2$)$_2$-CO-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (31 mg; 13 μmol) was reacted with 95% TFA for 2 h. Then the solvent was evaporated and the oily residue purified by preparative HPLC using a linear gradient of MeCN/H_2O from 25:75 to 70:30 in 90 min. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized; yield: 19 mg (81%); HPLC (gradient C): t_R 7.95 min; ES-MS: $m/z = 1582.0 \pm (44.0)_n$ with $n = 0–3$ $(M + H^+)$; calc. $M_r = 1579.9 \pm (44.0)_n$ with $n = 0–3$.

(PEG)$_{19–25}$-(NH-CO-(CH$_2$)$_2$-CO-Arg-Val-Arg-H)$_2$ × 4TFA (29). (PEG)$_{19–25}$-[NH-CO-(CH$_2$)$_2$-CO-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA]$_2$ was deprotected by reaction with 95% TFA. After 2 h, the solvent was removed and the oily residue distributed between H_2O and diethyl ether. The aqueous phase was evaporated and the residue lyophilized; yield: 15 mg (88%); HPLC (gradient C): t_R 7.36 min; ES-MS: $m/z = 989.2 \pm (22.0)_n$ with $n = 0–3$ $(M + 2H^{2+})$; calc. $M_r = 1975.2 \pm (44.0)_n$ with $n = 0–3$.

(PEG)$_{19–25}$-(NH-CO-(CH$_2$)$_2$-CO-Arg-Val-Arg-H)-(NH-CO-(CH$_2$)$_2$-CO-Leu-Leu-Nle-H) (30). (PEG)$_{19–25}$-[NH-CO-(CH$_2$)$_2$-CO-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA][NH-CO-(CH$_2$)$_2$-CO-Leu-Leu-Nle-Sc] (52 mg; 19 μmol) was dissolved in a mixture of 0.5 ml of MeOH, 0.2 ml of acetic acid and 0.2 ml of formaldehyde (37% in H_2O). After 2 h, the mixture was distributed between CH_2Cl_2 and 5% $KHSO_4$. The aqueous phase was extracted twice with CH_2Cl_2 and the combined organic layers were washed with 5% $NaHCO_3$, dried over Na_2SO_4 and evaporated. The residue was reacted with 95% TFA for 2 h and after evaporation of the solvent, the crude product was immediately purified by preparative HPLC using a linear gradient of MeCN/H_2O from 5:95 to 65:35 in 100 min. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized; yield: 19 mg (47%); HPLC (gradient C): t_R 9.03 min; ES-MS: $m/z = 952.4 \pm (22.0)_n$ with $n = 0–3$ $(M + 2H^{2+})$; calc. $M_r = 1903.2 \pm (44.0)_n$ with $n = 0–3$.

Proteasome Assay

A solution of proteasome from *Saccharomyces cerevisiae* in Tris-buffer (pH 7.5; 450 μl; 6.67 nM for PGPH, 5.56 nM for trypsin-like and 1.11 nM for chymotrypsin-like activity) was incubated with the inhibitors at varying concentrations (1 nM to 100 μM) for 1 h at 37°C. The fluorogenic substrates for PGPH (Z-Leu-Leu-Glu-βNA, 40 μM), trypsin-like (Bz-Phe-Val-Arg-AMC, 8 μM) and chymotrypsin-like assays (Suc-Leu-Leu-Val-Tyr-AMC, 8 μM) were dissolved in the same Tris-buffer with a minimum amount of DMSO and added to the enzyme solution at 37°C to reach a final volume of 500 μl. Fluorescence excitation/emission wavelengths were 360/460 nm for AMC and 335/410 nm for βNA. The rates of hydrolysis were monitored by the fluorescence increase and the initial linear portions of curves (100–300 s) were used to calculate the IC_{50} values.

RESULTS AND DISCUSSION

Synthesis of Intra-Ring Bivalent Inhibitors

The crystal structure of the yeast proteasome in complex with the calpain inhibitor I Ac-Leu-Leu-Nle-H revealed binding of the inhibitor to all six active sites [2] indicating the relatively low substrate specificity of the different proteolytic activities [6–10]. This observation prompted us to elongate the tripeptide aldehyde to an octapeptide bis-aldehyde (Figure 2A), a length that correlates well with the average length of antigenic peptides (8–10 residues) generated by the proteasome. The introduction of an *N*-terminal glyoxylic acid residue yielded a bis-aldehyde (**8**), which could bridge the distance of 28 Å between the active sites in β1 and β2. Upon bivalent binding from the non-primed side in β1 and from the primed side in β2, respectively, should inhibit both the PGPH and the trypsin-like activity of the proteasome.

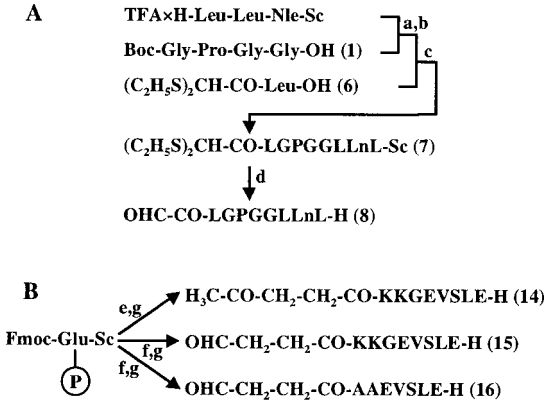


Figure 2 Synthesis of intra-ring bivalent inhibitors. (a) PyBOP/NMM; (b) 25% TFA in CH_2Cl_2; (c) HATU/DIEA; (d) $Tl(NO_3)_3 \times 3H_2O$; (e) (i) Fmoc SPPS, (ii) H_3C-CO-$(CH_2)_2$-CO_2H, HBTU/HOBt/DIEA; (f) (i) Fmoc SPPS, (ii) Sc-$(CH_2)_2$-CO_2H, PyBOP/HOBt/DIEA; (g) 95% aq. TFA.

The semicarbazone-protected tripeptide aldehyde H-Leu-Leu-Nle-Sc [27] was coupled with Boc-Gly-Pro-Gly-Gly-OH (**2**) by the PyBOP method and subsequent N^{α}-Boc-cleavage was performed with 25% TFA in CH_2Cl_2. The *N*-terminal glyoxylic acid moiety was introduced as the thioacetal-protected fragment $(C_2H_5S)_2$-CH-CO-Leu-OH (**6**), which was obtained by saponification of glyoxylic acid ethyl ester diethylmercaptal and reaction with H-Leu-OMe/EDC followed by saponification of the methyl ester moiety. The two fragments were coupled by using HATU/DIEA and final deprotection of both aldehyde moieties was carried out simultaneously using $Tl(NO_3)_3 \times 3H_2O$ in $MeCN/H_2O$.

A second approach to develop bivalent inhibitors which bind concomittantly to $\beta 1$ from the non-primed side and to $\beta 2$ from the primed side was derived from the X-ray structure of a $\beta 1$ Thr^1Ala mutant [29]. This mutation abolishes the ability of the subunit to autoprocess the propeptide between Thr^1 and Gly^{-1} with release of the *N*-terminal nucleophile Thr^1. Correspondingly, the propetide is only partially processed between Arg^{-10} and Leu^{-9} by the adjacent $\beta 2$ active site. This propeptide was then used as a possible lead structure to design bivalent inhibitors carrying *C*-terminally a glutamic acid aldehyde for binding to $\beta 1$ and *N*-terminally the levulinic or (4-oxo-butyric) acid as anchor for the $\beta 2$ active site (Figure 2B).

For the synthesis of compounds **14**–**16** on solid support, Fmoc-Glu(OtBu)-OH was converted to the corresponding Weinreb amide **9** using HN(OMe)Me/TBTU/HOBt. Upon reduction with $LiAlH_4$ the resulting aldehyde **10** was protected as semicarbazone and after cleavage of the OtBu protecting group with 25% TFA in CH_2Cl_2, the resulting Fmoc-Glu(OH)-Sc (**12**) was attached to the resin via the side chain carboxylic acid moiety. Cleavage of the Fmoc-group was carried out in two cycles by treatment with 20% piperidine. Elongation of the peptide chain was performed using Fmoc/tBu chemistry with double couplings of a 4-fold excess of Fmoc-AA-OH or levulinic acid/HBTU/HOBt/DIEA (1:1:1:2). The *N*-terminal succinic acid aldehyde was introduced as the corresponding semicarbazone derivative (**13**), which was obtained from commercially available 4-oxo-butyric acid by reaction with semicarbazid. As in this case the HBTU/HOBt/DIEA procedure did not yield the desired product, coupling was performed using a 10-fold excess of 4-oxobutyric-acid semicarbazone/PyBOP/HOBt/DIEA (1:1:1:2). Final deprotection and cleavage from the resin was performed by reaction with 95% aqueous TFA for 3 h, and the crude product was purified using preparative HPLC.

Synthesis of Monovalent PEG-Conjugates

To develop bivalent inhibitors that approach two active sites from the non-primed side, the crystal structure of Ac-Leu-Leu-Nle-H bound to $\beta 5$ and $\beta 5'$ [2] was used to design a spacer that has to span distances of 50 Å or more. As peptides of this size, like gastrin (17 mer) or secretin (27 mer), were in full agreement with previous studies [7,30] found to be rapidly degraded by the yeast proteasome (unpublished results), we chose the highly flexible polyethylene glycol [31] to mimic hydrophilic and unstructured, random coiled peptide chains. A PEG with a statistical length distribution of 19–25 monomers and with the terminal amino groups capped with succinic acid was selected as spacer to allow for attachment of tripeptide aldeydes as binding heads via their *N*-termini. To analyse the effects of the PEG moiety on the inhibition potency of monovalent tripeptide aldehydes, we linked the spacer to the *N*-terminus of H-Leu-Leu-Nle-H (Figure 3) by coupling of equimolar amounts of the PEG and the semicarbazone-protected peptide aldehyde using the TBTU/HOBt/DIEA procedure. After deprotection of the semicarbazone **21** using acetic acid and formaldehyde as scavenger, the mono-derivatized PEG–peptide aldehyde conjugate **22** was separated from the bis-derivatized conjugate, which was formed as a by product, using preparative HPLC.

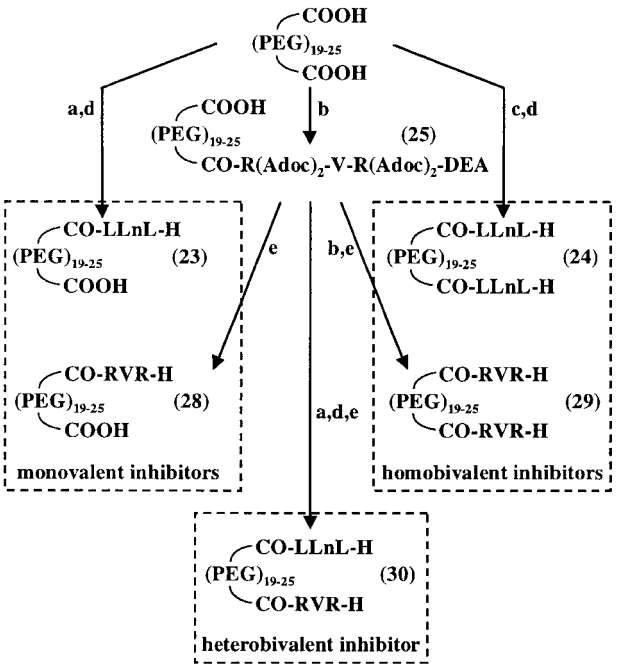


Figure 3 Synthesis of the PEG/peptide aldehyde conjugates. (a) 1 equivalent H-Leu-Leu-Nle-Sc, TBTU/HOBt/DIEA; (b) 1 equivalent H-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA, TBTU/HOBt/DIEA; (c) 2 equivalents H-Leu-Leu-Nle-Sc, TBTU/HOBt/DIEA; (d) AcOH, HCHO; (e) 95% aq. TFA.

To develop a monovalent inhibitor of the trypsin-like activity of the proteasome, the tripeptide aldehyde Ac-Arg-Val-Arg-H (**20**) was synthesized (Figure 4). H-Val-Arg(Adoc)$_2$-DEA [28] was elongated *N*-terminally by reaction with Z-Arg(Adoc)$_2$-OH/TBTU/HOBt/DIEA, and after hydrogenolytic cleavage of the Z-group and acetylation with acetic anhydride, final deprotection of the arginine side chains and the *C*-terminal aldehyde moiety was performed using 95% aqueous TFA. The intermediate H-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA (**18**) was also pegylated according to the procedure described above. Due to the hydrophobicity of the conjugates the mono-amidated compound **25** was isolated by size exclusion chromatography. The monovalent PEG–

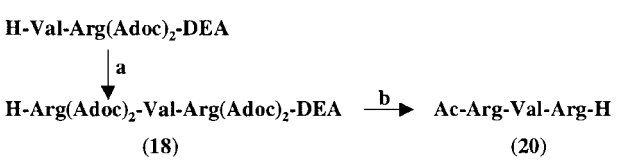


Figure 4 Synthesis of Ac-Arg-Val-Arg-H. (a) (i) Z-Arg-(Adoc)$_2$-OH, TBTU/HOBt/DIEA, (ii) H_2, Pd/C (10%); (b) (i) Ac_2O, DIEA, (ii) 95% aq. TFA.

peptide aldehyde conjugate **28** was obtained upon deprotection with 95% aqueous TFA.

Synthesis of Inter-Ring Homobivalent Inhibitors

The homobivalent inhibitor **24** (Figure 3) was obtained by complete amidation of both carboxylic acid moieties of the PEG spacer with 2 equivalents of H-Leu-Leu-Nle-Sc/TBTU/HOBt/DIEA (1:1:1:2) followed by deprotection of the aldehyde groups as described above. To address simultaneously the two trypsin-like active sites we synthesized the homobivalent inhibitor **29**. The second peptide aldehyde moiety was introduced by amidation of the monovalent key intermediate **25** with H-Arg(Adoc)$_2$-Val-Arg(Adoc)$_2$-DEA/TBTU/HOBT/DIEA and final deprotection was carried out with 95% aqueous TFA.

Synthesis of an Inter-Ring Heterobivalent Inhibitor

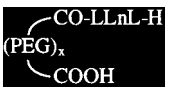
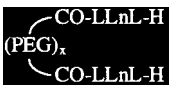
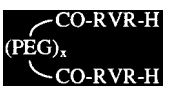
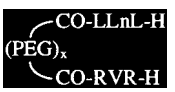
For the synthesis of a PEG conjugate carrying two different peptide aldehydes as binding heads to address concomittantly the trypsin-like and the chymotrypsin-like activities of the proteasome, H-Leu-Leu-Nle-Sc was acylated with the intermediate **25** using TBTU/HOBt/DIEA and the heterobivalent inhibitor **30** was obtained after cleavage of the semicarbazone and final deprotection with 95% TFA.

Inhibition of 20S Proteasome

The inhibitory potency of the bis-aldehyde **8** for the chymotrypsin-like activity was significantly increased if compared to the monovalent Ac-Leu-Leu-Nle-H, but no inhibition was found for the trypsin-like and the PGPH activity (Table 1), although the *N*-terminal glyoxylic acid residue was designed to bind to the $\beta 2$ active site. These data suggest an enhanced monovalent binding of the *C*-terminal peptide aldehyde moiety to the $\beta 5$ active site, whilst the *N*-terminal glyoxylic acid does not interact with the active site of the $\beta 2$ subunit. The crystal structure of the proteasome/**8** complex [25] fully confirmed this assumption, indicating improved binding of the *C*-terminal moiety from the S1 to the S6 subsite in $\beta 5$, while access of the glyoxylic acid function to the adjacent $\beta 2$ active site is impeded.

The propeptide-derived, double-headed inhibitors **14**–**16** inhibit only weakly the PGPH activity and the residual activities are not affected (Table 1). Consequently, these inhibitors bind only in a monovalent manner with the *C*-terminal peptide

Table 1 Inhibition of 20S Proteasome by Mono- and Bivalent Inhibitors (IC_{50}, μM)

Inhibitor	PGPH	Trypsin-like	Chymotrypsin-like
Ac-LLnL-H (**1**)	>100	>100	2.1
HOC-CO-LGPGGLLnL-H (**8**)	>100	>100	0.08
Lev-KKGEVSLE-H (**14**)	102	>100	>100
Saa-KKGEVSLE-H (**15**)	82	>100	>100
Saa-AAGEVSLE-H (**16**)	146	>100	>100
Ac-RVR-H (**20**)	>100	6.4	>100
 (**23**)	>100	>100	1.8
 (**24**)	>100	>100	0.017
 (**28**)	>100	8.2	>100
 (**29**)	>100	0.071	>100
 (**30**)	>100	0.097	0.031

aldehyde, while the *N*-terminal second reactive moiety does not interact with the $\beta 2$ active site from the primed side as described for compound **8**.

By linking the *N*-termini of two tripeptide aldehydes with a polymeric spacer that allows for simultaneous binding of the two monovalent binding heads to two different active sites from the non-primed side in $\beta 5$ and $\beta 5'$ (compound **24**) or $\beta 2$ and $\beta 2'$ (compound **29**), we obtained homobivalent inhibitors with binding affinities enhanced by two orders of magnitude (Table 1). A comparison of the IC_{50} values of the acetylated (compounds **1** and **20**) and the pegylated tripeptide aldehydes (compounds **23** and **28**) shows, that the spacer itself does not affect the affinity of the binding heads to the enzyme.

In the case of heterobivalent inhibitors, two molecules are required for inhibition of one proteolytic activity due to the 2-fold set of three different active sites in one enzyme particle. If this stoichometry is taken into account, the increase in inhibition potency of the hetrobivalent inhibitor **30** is similar to the effect obtained in the case of homobivalent inhibition.

Conclusions

An approach to develop highly potent and selective inhibitors of eucaryotic proteasomes is to exploit the unique display of a 2-fold set of three different active sites in the central cavity of this multicatalytic protease. By linking the *N*-termini of tripeptide aldehydes with a polymeric spacer we obtained homo- and heterobivalent inhibitors of two orders of magnitude enhanced binding affinities [25]. This principle of bivalent inhibition can be also be applied to other multicatalytic proteases. Recently, Burgess *et al.* [32] obtained bivalent subnanomolar inhibitors of the bisbenzamidine type for tryptase, another protease with a known and well defined display of multiple active sites [20].

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