Supporting Information

for

Synthesis of a TMC-95A Ketomethylene Analog by Cyclization via Intramolecular Suzuki Coupling

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General

^{1}H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively, on a Bruker DRX 500 spectrometer. For ^{1}H and ^{13}C, chemical shifts are reported in ppm (δ) downfield of tetramethylsilane (TMS) used as internal standard. Coupling constant values (J) are reported in Hz. 2D-TOCSY experiments were recorded by a MLEV-17 pulse sequence with a mixing time of 75 msec and 2D-ROESY spectra with a mixing time of 200 msec. LC-ESI mass spectrometry analysis was performed on a PE Sciex API165 instrument, equipped with a Microgradient System 140C (Applied Biosystems), a PE 785A UV-VIS-Detector, a PE Series 200 Autosampler and a PE Nelson 200 Interface using a Nucleosil C_8 100/5 column (125×2 mm) and a linear gradient from 5% CH_3CN in 0.05% aqueous TFA to 90% CH_3CN in 0.05% aqueous TFA in 15 min (flow: 250 µL/min). TLC was performed on SiO_2-F_{254} precoated glass plates (Merck AG, Darmstadt) and flash column chromatography on Kieselgel 60 (230-400 mesh). HPLC analysis was carried out with Waters equipments on Chromolith C_{18} 5 µm column (100×4.6 mm) from Merck by linear gradient elution from 5% CH_3CN in 2% aqueous H_3PO_4 to 90% CH_3CN in 2% aqueous H_3PO_4 in 7 min (flow rate: 3 mL/min). R_t values are expressed in min. Preparative reversed-phase HPLC was carried out on Abimed-Gilson chromatograph using a Nucleosil C_8

SP250/10 column (Macherey & Nagel, Düren) and gradients from 0.1% aqueous TFA to CH_3CN containing 0.1% TFA (flow rate: 3 mL/min). All reagents and solvents were purchased from Aldrich, Fluka, Merck and Lancaster and were used without further purification. Suzuki cross-coupling was carried out under an argon atmosphere in flame-dried glassware.

Experimental

Note: All MS analyses were routinely performed by LC-ESI-MS as described above. Under these conditions partial to complete hydrolysis of the boronic esters occurs during analysis. Thus, the masses of the corresponding free boronic acids are observed, although as shown by NMR analyses, in the single reaction steps only the boronic esters were obtained.

Boc-NleΨ[COCH$_2$]Gly-OH.[1] The title compound was prepared by converting Boc-Nle-OH into the Boc-Nle Weinreb amide, followed by reaction with butenylmagnesiumbromide and oxidative cleavage of the double bond by Corey oxidation with $RuCl_3 \times H_2O$ and $NaIO_4$.

Synthesis of Boc-Nle-N(OMe)Me. To a mixture of Boc-Nle-OH (2 g, 8.65 mmol), TBTU (1 equiv, 8.65 mmol, 2.78 g), HOBt (1 equiv, 8.65 mmol, 1.32 g) and DIEA (1 equiv, 8.65 mmol, 1.49 mL) in DCM (100 mL) was added at 0 °C a mixture of *N,O*-dimethylhydroxylamine hydrochloride (1.1 equiv, 9.52 mmol, 0.93 g) and DIEA (1.1 equiv, 9.52 mmol, 1.63 mL) in DCM (50 mL). After stirring at rt for 15 h the solvent was evaporated to dryness and the residue was taken up in EtOAc and 5% aqueous $KHSO_4$. The organic phase was washed with 5% aqueous $KHSO_4$, 5% aqueous $NaHCO_3$ and brine, dried (Na_2SO_4) and evaporated. The residue was taken up in a minimum amount of petroleum ether/EtOAc (3:1) and chromatographed on silica gel with petroleum ether/EtOAc (3:1) as eluent to yield 1.95 g (82%) of an oil. δ_H ($CDCl_3$): 0.80 (3 H, t, *J* 6.6, Me), 1.22-1.33 (4 H, m, MeCH_2CH_2), 1.38 (9 H, s, Me), 1.41-1.44 (1 H, m, $CHCH_2$), 1.52-1.58 (1 H, m, $CHCH_2$), 3.10 (3 H, s, NMe), 3.67 (3 H, s, OMe), 4.51-4.62 (1 H, m, α-CH) and 5.12 (1 H, d, *J* 9.8, NH); δ_C ($CDCl_3$): 13.6, 22.1, 27.3, 27.8, 31.9, 50.1, 61.3, 71.9, 79.2, 155.4 and 173.3; MS (ESI): *m/z* calcd. for $C_{13}H_{26}N_2O_4$: 274.4, found: 275.2 $[M+H]^+$.

Synthesis of Boc-Nle-$(CH_2)_2CH=CH_2$. The Boc-Nle Weinreb amide (2.48 g, 9.04 mmol) was dissolved in dry THF (200 mL), cooled to –78 °C and a solution of butenylmagnesiumbromide

[1] Loidl, G. *Ph.D. Dissertation*, Technische Universität München, München, Germany, 1999.

(5 equiv, 45.20 mmol, 90 mL) was added. The cooling bath was removed and the resulting mixture was stirred for further 3 h. Et_2O (600 mL) and 5% aqueous NH_4Cl (300 mL) were added, the organic phase separated, washed with water, dried (Na_2SO_4) and evaporated to dryness. The residue was taken up in a minimum amount of petroleum ether/EtOAc (4:1) and chromatographed on silica gel with petroleum ether/EtOAc (4:1) as eluent; yield: 2.29 g (94%). TLC (petroleum ether/EtOAc, 3:1): R_f = 0.72; δ_H ($CDCl_3$): 1.18 (3 H, t, *J* 6.3, Me), 1.58-1.69 (4 H, m, MeCH_2CH_2), 1.74 (9 H, s, Me), 1.77-1.89 (1 H, m, CHCH_2), 1.92-2.04 (1 H, m, CHCH_2), 2.11-2.18 (1 H, m, $COCH_2CH_2CHCH_2$), 2.57-2.64 (1 H, m, $COCH_2CH_2CHCH_2$), 3.91-3.98 (2 H, m, $COCH_2CH_2CHCH_2$), 4.87-5.02 (1 H, m, α-CH), 5.33 (1 H, dd, *J* 1.3 and *J* 9.3, $COCH_2CH_2CHCH_2$) 5.39 (1 H, dd, *J* 1.3 and *J* 17.2, $COCH_2CH_2CHCH_2$), 5.86 (1 H, d, *J* 9.7, NH), 6.12 (1 H, m, $COCH_2CH_2CHCH_2$); δ_C ($CDCl_3$): 14.6, 22.3, 22.7, 27.2, 28.0, 31.7, 36.4, 49.5, 60.8, 116.3, 135.2, 173.0 and 208.5; MS (ESI): *m/z* calcd. for $C_{15}H_{27}NO_3$: 269.4, found: 270.4 $[M+H]^+$.

Synthesis of title compound. To a mixture of Boc-Nle-$(CH_2)_2CH=CH_2$ (1.55 g, 5.76 mmol) in CH_3CN (100 mL) and $NaIO_4$ (7 equiv, 40.31 mmol, 8.62 g) in water (100 mL) was added at 0 °C $RuCl_3 \times H_2O$ (0.09 equiv, 0.51 mmol, 106 mg). After stirring for 75 min at rt, water and Et_2O were added and the phases separated. The water phase was re-extracted first with Et_2O and then with EtOAc. The combined organic phases were washed with water, dried (Na_2SO_4) and evaporated to dryness. The residue was taken up in a minimum amount of $CHCl_3$/MeOH (5:1) and chromatographed on silica gel using $CHCl_3$/MeOH as eluent to yield 1.37 g (83%) of a slightly brownish solid. TLC (EtOAc/petroleum ether, 3:1): R_f = 0.65; HPLC: R_t = 3.08; δ_H ($CDCl_3$): 0.89 (3 H, t, *J* 9.2, Me), 1.23-1.36 (4 H, m, MeCH_2CH_2), 1.39 (9 H, s, Me), 1.43-1.49 (1 H, m, CHCH_2), 1.52-1.58 (1 H, m, CHCH_2), 2.58-2.69 (1 H, m, CH_2CO_2 and 1 H, m, CH_2CO), 2.72-2.91 (1 H, m, CH_2CO_2 and 1 H, m, CH_2CO), 4.28-4.34 (1 H, m, α-CH), 5.16 (1 H, d, *J* 10.0, NH) and 10.21 (1 H, br s, CO_2H); δ_C ($CDCl_3$):14.5, 23.1, 27.9, 28.2, 29.0, 31.8, 34.9, 59.9, 156.3, 178.3 and 208.5; MS (ESI): *m/z* calcd. for $C_{14}H_{24}NO_5$: 287.4, found: 288.2 $[M+H]^+$.

Boc-Trp(7-Br)-OH. The brominated amino acid was obtained from 7-bromoindole and serine by enzymatic synthesis[2] with tryptophan synthetase and subsequent N^{α}-Boc-protection with $(Boc)_2O$.

[2] Lee, M.; Phillips, R.S. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1563-1564.

Synthesis of H-Trp(7-Br)-OH. An aqueous 100 μM solution of $\alpha_2\beta_2$ tryptophan synthetase (2 mL) was added to 1 M Tris×HCl buffer (pH 7.8, 5 mL), 5 M NaCl (2 mL), 0.5 m L-serine (2 mL) and 0.4 mM pyridoxal phosphate (5 mL). The mixture was diluted with water to a final volume of 45 mL and combined with a solution of 7-bromoindole (100 mg, 0.51 mmol) in toluene (20 mL). The reaction mixture was shaked for 48 h at 37 °C. The organic phase which still contained unreacted 7-bromoindole for subsequent batch reactions was separated and the aqueous phase was filtered through Amicon filter (cut-off > 3 kDa). The aqueous phase was adjusted to pH 10-11 by addition of 1 M NaOH, extracted twice with EtOAc, adjusted to pH 6-7 by addition of 1 M HCl, evaporated to a small volume and stored for 16 h at 4 °C. The resulting precipitate was collected and dried; yield: 13 mg (9%). HPLC: R_t = 2.40; δ_H (D_2O) 3.27 (1 H, dd, *J* 7.9 and *J* 15.4, CHC*H*$_2$), 3.43 (1 H, dd, *J* 4.9 and *J* 15.4, CHC*H*$_2$), 4.02 (1 H, dd, *J* 4.9 and *J* 7.9, α-CH), 7.06 (1 H, t, *J* 7.8, ArH), 7.33 (1 H, s, ArH), 7.42 (1 H, d, *J* 7.8, ArH) and 7.66 (1 H, d, *J* 7.8, ArH); δ_C (D_2O/MeOD) 27.8, 56.1, 105.4, 110.7, 118.8, 121.7, 125.5, 126.6, 129.1 and 175.2; MS (ESI): *m/z* calcd. for $C_{11}H_{11}BrN_2O_2$: 282.1, 284.1, found: 283.0, 285.0 $[M+H]^+$.

Synthesis of title compound. To a mixture of H-Trp(7-Br)-OH (42 mg, 0.418 mmol) and $NaHCO_3$ (3 equiv, 0.444 mmol, 37 mg) in dioxane/water (1:1, 30 mL) was added $(Boc)_2O$ (2 equiv, 0.296 mmol, 65 mg) and stirred over night at rt. The mixture was evaporated to dryness, the residue taken up in EtOAc and 5% aqueous $KHSO_4$, the organic phase was washed with 5% aqueous $KHSO_4$ and brine, dried (Na_2SO_4) and evaporated to dryness to yield 57 mg (>98%) of a white solid. TLC (DCM/MeOH, 9:1): R_f = 0.72; HPLC: R_t = 3.57; δ_H (DMSO-d_6): 1.35 (9 H, s, Me), 2.96 (1 H, dd, *J* 9.5 and *J* 14.4, CH_2), 3.12 (1 H, dd, *J* 4.4 and *J* 14.4, CH_2), 4.12 (1 H, m, α-CH), 6.94 (1 H, t, *J* 7.5, ArH), 6.99 (1 H, d, *J* 8.0, α-NH), 7.21 (1 H, d, *J* 2.0, ArH), 7.29 (1 H, d, *J* 7.5, ArH), 7.54 (1 H, d, *J* 7.5, ArH), 11.05 (1 H, s, NH) and 12.58 (1 H, br s, CO_2H); δ_C (DMSO-d_6): 28.1, 54.3, 78.0, 104.0, 111.7, 117.8, 119.8, 123.4, 125.0, 128.9, 134.3, 155.3 and 173.7; MS (ESI): *m/z* calcd. for $C_{16}H_{19}BrN_2O_4$: 382.2, 384.2, found: 383.1, 385.1 $[M+H]^+$.

Z-Tyr(3-boronic pinacol ester,4-Me)-OH. To a solution of Z-Tyr(3-I,4-Me)-OMe[3] (271 mg, 0.557 mmol) in dry DMSO (30 mL) under argon KOAc (164 mg, 1.67 mmol), bis(pinacolate)diboron (212 mg, 0.836 mmol) and [Pd(dppf)Cl_2]×DCM (14 mg, 0.017 mmol)

[3] This compound was prepared making use of standard procedures, i.e. esterification of commercial H-Tyr(3-I)-OH with $SOCl_2$/MeOH, followed by N^{α}-protection with Z-OSu in 1,4-dioxane /H_2O containing $NaHCO_3$ and methyl ether formation with MeI/K_2CO_3 in acetone.

were added, and the reaction mixture was stirred at 80 °C for 18 h. The resulting solution was cooled to rt and diluted with EtOAc (200 mL) and water (40 mL). The organic layer was separated and the aqueous phase extracted with EtOAc (3 × 50 mL). The organic extracts were combined, washed with 5% aqueous $KHSO_4$ (3 × 50 mL) and with brine (50 mL), dried (Na_2SO_4), and then evaporated to an oily residue which was purified by flash column chromatography (eluent: EtOAc/petroleum ether, 2:1) to yield 232.2 mg (89%) of a colorless oil. TLC (AcOEt/petroleum ether, 2:1): R_f = 0.85; HPLC: R_t = 4.17 (boronic pinacol ester) and 3.11 (free boronic acid); MS (ESI): *m/z* calcd. for $C_{25}H_{32}BNO_7$ (boronic pinacol ester): 469.4, found: 470.2 $[M+H]^+$; *m/z* calcd for $C_{19}H_{22}BNO_7$: 387.2, found: 388.0 $[M+H]^+$.

The Z-Tyr(3-boronic ester,4-Me)-OMe (232 mg, 0.495 mmol) was saponified with LiOH (13 mg, 0.545 mmol) in THF/H_2O (1:1, 20 mL). The progress of the reaction was monitored by TLC (EtOAc/*n*-hexane, 4:1) and after 16 h at rt the mixture was concentrated under reduced pressure and the aqueous solution was extracted with EtOAc (3 × 25 mL). The aqueous phase was acidified with 5% aqueous $KHSO_4$ and then extracted with EtOAc (3 × 25 mL). The combined organic extracts were washed with water (50 mL), dried (Na_2SO_4) and evaporated to dryness to yield 211.7 mg (94%) of a white solid. TLC (EtOAc/*n*-hexane, 4:1): R_f = 0.68; HPLC: R_t = 3.57 (boronic pinacol ester) and 2.63 (free boronic acid); δ_H (DMSO-d_6): 1.28 (12 H, s, Me), 2.76 (1 H, dd, *J* 8.5 and *J* 14.1, C*H*$_2$CH), 2.98 (1 H, dd, *J* 4.2 and *J* 14.1, C*H*$_2$CH), 3.78 (3 H, s, OMe), 4.10 (1 H, ddd, *J* 4.2, *J* 9.7 and *J* 14.1, α-CH), 4.98 (2 H, s, C*H*$_2$Ph), 6.88 (1 H, t, *J* 8.0, ArH), 7.23-7.36 (4 H, m, ArH), 7.39 (1 H, d, *J* 9.7, NH), 7.45 (1 H, dd, *J* 2.5 and *J* 12.7, ArH), 7.57 (1 H, dd, *J* 8.0 and *J* 12.7, ArH), 7.68 (1 H, s, ArH) and 12.80 (1 H, br s, CO_2H); δ_C (DMSO-d_6): 24.6. 35.3, 35.8, 55.3, 55.9, 65.2, 82.9, 110.1, 110.6, 127.4, 127.8, 128.1, 128.2, 129.1, 129.4, 132.2, 133.3, 136.1, 137.7, 155.9, 162.2, 162.5 and 173.3; MS (ESI): *m/z* calcd. for $C_{24}H_{30}BNO_7$ (boronic pinacol ester): 455.3, found: 456.2 $[M+H]^+$; *m/z* calcd. for $C_{18}H_{20}BNO_7$ (free boronic acid): 373.2, found: 374.2 $[M+H]^+$.

Synthesis of 5. Compound **4** (8 mg, 0.007 mmol) was dissolved in glacial acetic acid/conc. HCl (4:1, 15 mL) and stirred at rt for 5 min. DMSO (20 equiv, 0.14 mmol, 10 µL) was added and the resulting mixture was stirred for 4 h. Toluene (15 mL) was added and the mixture was evaporated to dryness. The addition of toluene (15 mL) followed by evaporation to dryness was repeated four times. The oxidation delivered a 1:1 diastereomeric mixture of **5** (76% HPLC yield) and as a side product, the 2-chlorotryptophan derivative (24% HPLC yield). As only the

sp^3-hybridized **5** can be cyclized, this mixture was used without further purification. HPLC: R_t = 3.55; MS (ESI): *m/z* calcd. for $C_{50}H_{66}BBrN_8O_{13}$ (free boronic acid): 1076.8; 1078.8, found: 1077.4; 1079.4 $[M+H]^+$.

Cyclization of 5 to 1. A solution of compound **5** (12 mg, 0.010 mmol) and K_2CO_3 (5 equiv, 0.052 mmol, 7 mg) in DME/H_2O (7:1, 30 mL) was degassed and flushed with argon. [Pd(dppf)Cl_2]×DCM (0.05 equiv, 0.5 µmol, 0.4 mg) was added, and the resulting solution heated for 5 h at 80 °C. The reaction mixture was evaporated, the residue suspended in DCM/MeOH (9:1) and chromatographed on a short silica gel column. The eluate was evaporated and purified by RP-HPLC on a C_8 column with a linear gradient of CH_3CN/H_2O (from 5:95 to 75:25 in 60 min) to yield **1** (5.4 mg, 57%) after lyophilization from *tert*-butanol/H_2O (4:1). HPLC: R_t = 3.48; δ_H (DMSO-d_6): 5.05-4.92 (2 H, m), 6.56 (1 H, s), 6.77-6.69 (1 H, m), 7.23-6.90 (6 H, m), 7.38-7.23 (5 H, m), 7.50-7.44 (1 H, m), 7.70-7.61 (2 H, m), 7.77 (1 H, d, *J* 7.5), 7.83 (1 H, d, *J* 7.2), 8.06 (1 H, d, *J* 6.8) and 9.71 (1 H, s), remaining signals overlap inseparable in alkyl area. The assignment of all signals was performed by 2D-TOCSY and 2D-ROESY experiments and these were consistent with the proposed structure. MS (ESI): *m/z* calcd. for $C_{50}H_{64}N_8O_{11}$: 953.1, found: 953.4 $[M+H]^+$. HRMS: *m/z* calcd. for $[C_{50}H_{64}N_8NaO_{11}]^+$: 975.45868, found: 975.45834 $[M+Na]^+$; *m/z* calcd. for $[C_{50}H_{65}N_8O_{11}]^+$: 953.47673, found: 953.47735 $[M+H]^+$.

Kinetic measurements

For measuring the chymotrypsin-like (CL), trypsin-like (TL) and peptidyl-glutamyl-peptide-hydrolase (PGPH) activities of yeast proteasome the conditions reported in Table 1 were used.

Table 1.

activity	*conditions*
CL	20 mM HEPES buffer (pH 7.5) containing 0.5 mM EDTA, 0.025% SDS and 5% DMSO; 7.5 µM Suc-LLVY-AMC (380 nm excitation, 460 nm emission); 0.34 nM yeast proteasome
TL	50 mM Tris/HCl buffer (pH 7.5) containing 0.5 mM EDTA, 100 mM NaCl, 0.01% Triton X-100 and 5% DMSO; 50 µM Z-ARR-AMC (380 nm excitation, 460 nm emission); 5.6 nM yeast proteasome
PGPH	50 mM Tris/HCl buffer (pH 7.5) containing 0.5 mM EDTA, 100 mM NaCl, 0.001% SDS, 5% DMSO and 1 mM DTT; 50 µM Z-LLE-β-Na (345 nm excitation, 425 nm

	emission); 3.4 nM yeast proteasome

Based on previous reports,[4] the detergents were optimized for each activity. The enzyme assays were carried out at 37 °C in a total volume of 500 μL on a spectrofluorimeter SM25 (Biotek-Kontron, Neufahrn, Germany). Inhibitors were dissolved in DMSO and used at 1-300 μM concentration in the assay. The K_i values were derived by non-linear regression analysis of the experimental v_i/v_0 data points and inhibitor concentrations, and fitting to the equation of classical inhibition [$v_i/v_0 = 1/(1+I_t/K_i)$]. When inhibition of the proteasome activity was not observed, K_i values ≥ 2000 μM were assumed to be at least 10-fold the highest used inhibitor concentration. Since the enzyme activities were measured at substrate concentrations < K_M, correction for substrate competition was not required.

[4] Arribas, J.; Castano, J.C. *J. Biol. Chem.* **1990**, *265*, 13969-13973.