Synthesis and evaluation of macrocyclic peptide aldehydes as potent and selective inhibitors of the 20S proteasome

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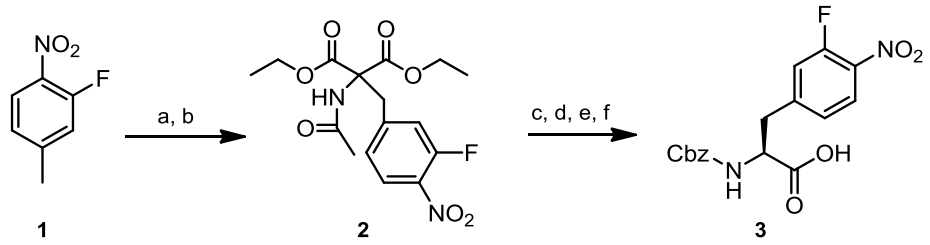
Contents

1. General methods

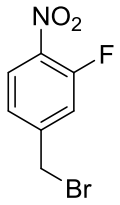
All reagents other than amino acids, Weinreb amide resin and 3-fluoro-4-nitrotoluene, were purchased from VWR International. The starting material 3-fluoro-4-nitrotoluene was purchased from Matrix Scientific. Materials for column chromatography were purchased from Sigma Aldrich and TLC silica plates from EMD Chemicals. All NMR spectra were collected on a Bruker Avance III 400 MHz Ultrashield Plus Spectrometer. Amino acids and coupling reagents were purchased from Aroz Technologies and Weinreb amide resin from NovaBiochem. 20S rabbit proteasome and bovine pancreatic α-chymotrypsin were purchased from Sigma Aldrich and Boston Biochem. Human liver cathepsin B and porcine erythrocytes calpain-1 were purchased from Millipore. All fluorogenic substrates were purchased from Calbiochem (now Millipore) and all fluorescence measurements were conducted on a BioTekFlx800 plate reader on 96 well plates. Bortezomic and carfilzomib were purchased from Advanced ChemBlocks Inc. Inhibitory constants were calculated using KaleidaGraph. Purification of final compounds was performed by reverse phase HPLC using an Agilent Technologies 1260 Infinity with a Discovery HS C18 preparatory column from SUPELCO; linear gradient 30% solvent A to 70% solvent B over 50 minutes (solvent A: 0.1% TFA in ACN; solvent B: 0.1% TFA in H_2O). Mass analysis was obtained using an Agilent Technologies 1220 and 6120 Quadropole LC/MS with the same solvent system and linear gradient over 30 minutes. High resolution mass spectral analysis was performed on a Bruker Apex-Qe FTMS (Arkansas Statewide Mass Spectrometry Facility, Fayetteville, AR).

2. Synthesis of 7, 8, and 9.

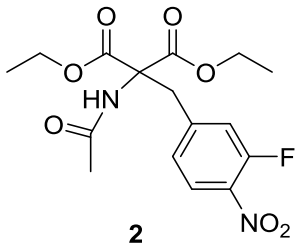
Scheme 1. Synthesis of enantiomerically pure phenylalanine analog[a]



[a]Reagents and conditions: (a) NBS, AIBN, CCl_4, reflux, 24 h. (b) diethyl acetamidomalonate, NaH, DMF, rt, 4 h. (c) HCl, reflux, 24 h. (d) Ac_2O, $NaHCO_3$, dioxane, H_2O, rt, 12 h. (e) acylase I, pH 7.5, 37 °C, 20 h. (f) Cbz-OSu, $NaHCO_3$, dioxane, H_2O, rt, 12 h.

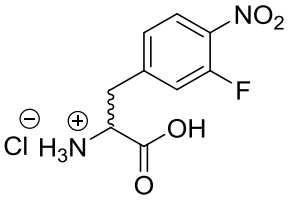


3-Fluoro-4-nitrobenzylbromide (not numbered in schemes)**:** Commercially available 3-fluoro-4-nitrotoluene (**1**) (4.24 g, 27.3 mmol) was dissolved in CCl_4 (100 mL) along with NBS (4.88 g, 27.4 mmol) and AIBN (0.506 mg, 3.08 mmol). The solution was refluxed at 95 °C for 24 hours. The resulting off-white precipitate was removed by vacuum filtration and the solvent was removed under reduced pressure yielding a yellow oil. The product of this reaction was purified by column chromatography (7:1 pet ether/EtOAc in silica gel) yielding 3-fluoro-4-nitrobenzylbromide as a yellow solid (3.68 g, 15.7 mmol, 57.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (t, J = 8.24 Hz, 1H, O_2N-C=CH), 7.35 (m, 2H, aromatic H), 4.48 (s, 2H, CH_2Br).



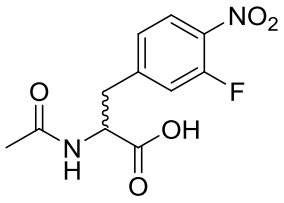
2

Diethyl 2-acetamido-2-(3-fluoro-4-nitrobenzyl)malonate (2): Diethyl acetamidomalonate (4.34 g, 20 mmol) was dissolved in dry DMF (30 mL) and stirred at 0 ˚C. Sodium hydride (0.80 g, 60% paraffin suspension, 20 mmol) was washed with pentane (10 mL), suspended in dry DMF (12 mL) and added dropwise to the reaction. A solution of 3-fluoro-4-nitrobenzylbromide (5.10 g, 21.7 mmol) in dry DMF (10 mL) was then added dropwise to the solution causing the reaction to turn dark purple. The reaction was stirred at room temperature for four hours during which the color faded to a light yellow. The solvent was removed under reduced pressure and the resulting solids were dissolved in EtOAc. The product was precipitated with increasing amounts of hexanes and collected by vacuum filtration yielding **2** as a white solid (6.00 g, 16.2 mmol, 75% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.28 (s, 1H, N*H*), 8.09 (t, *J* = 8.24 Hz, 1H, O_2N-C=C*H*), 7.17 (dd, *J* = 12.37, 1.54 Hz, F-C=C*H*), 7.08 (dd, *J* = 8.53, 1.40 Hz, 1H, O_2N-C=CH-C*H*), 4.17 (q, 4H, malonate CH_2), 3.54 (s, 2H, Phe CH_2), 1.95 (s, 3H, acyl CH_3), 1.17 (t, 6H, malonate CH_3).

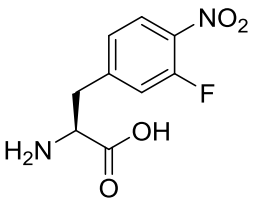


(*R*,*S*)-3-Fluoro-4-nitrophenylalanine Hydrochloride (not numbered in schemes)**:** The malonate ester **2** (14.96 g, 40.4 mmol) was suspended in concentrated HCl (75 mL) and refluxed at 120 °C for 24 hours. The solvent was removed under reduced pressure and the product was coevaporated three

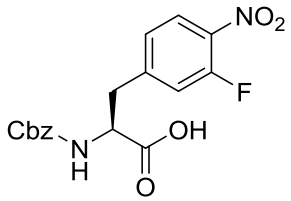
times with toluene and once with t-butyl methyl ether under reduced pressure yielding (*R*,*S*)-3-fluoro-4-nitrophenylalanine hydrochloride as a yellow solid (10.52 g, 39.7 mmol, 98.4% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.34 (s, broad, 3H, NH_3^+), 8.16 (t, *J* = 8.30 Hz, 1H, O_2N-C=C*H*), 7.56 (dd, *J* = 12.47, 1.60 Hz, 1H, F-C=C*H*), 7.37 (dd, *J* = 8.62 Hz, 1.19 Hz, 1H, O_2N-C=CH-C*H*), 4.33 (t, 1H, α C*H)*, 3.24 (dd, *J* = 14.24 Hz, 7.00 Hz, 2H, Phe CH_2).



(*R, S*)-*N*-Acetyl-3-fluoro-4-nitrophenylalanine (not numbered in schemes)**:** (*R*,*S*)-3-Fluoro-4-nitrophenylalanine hydrochloride (8.09 g, 30.6 mmol) was dissolved in a 1:1 mixture of dioxane and water (40 mL). The pH was adjusted to 8-9 by the addition of solid sodium bicarbonate. Acetic anhydride (9.37 g, 91.8 mmol) was added dropwise and the pH was confirmed. The reaction was stirred overnight and the solvent was removed under reduced pressure. The resulting solids were dissolved in EtOAc (50 mL) and washed with 5% $KHSO_4$ (3 x 75 mL), brine (3 x 75 mL) and then dried over $MgSO_4$. The solvent was removed under reduced pressure yielding (*R, S*)-*N*-acetyl-3-fluoro-4-nitrophenylalanine as a yellow solid (4.29 g, 15.9 mmol, 52% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.77 (s, broad, 1H, COO*H*), 8.25 (d, *J* = 8.19 Hz, 1H, N*H*), 8.07 (t, *J* = 8.31 Hz, 1H, O_2N-C=C*H*), 7.47 (d, *J* = 12.6, 1H, F-C=C*H*), 7.31 (d, *J* = 8.55, 1H, O_2N-C=CH-C*H*), 3.19 (dd, *J* = 13.68, 4.62 Hz, 2H, Phe CH_2), 2.97 (dd, *J* = 13.98, 9.87 Hz, 1H, α C*H*), 1.78 (s, 3H, acyl CH_3).



(*S*)-3-Fluoro-4-nitrophenylalanine (not numbered in schemes)**:** (*R, S*)-*N*-Acetyl-3-fluoro-4-nitrophenylalanine (956 mg, 3.57 mmol) was suspended in phosphate buffer (300 mL, 0.1 M KH_2PO_4/K_2HPO_4, pH 7.5) and dissolved by the addition of one equivalent of 0.1 M KOH (159 mL) and vigorous shaking. Acylase I isolated from *Asperigillus melleus* (0.55 U/mg, 4.08 g) was added to the solution and incubated at 37 °C overnight. The enzyme was removed by Amicon filtration under argon pressure (cut off > 10 kDa). The filtrate was acidified to a pH of 2-3 by the addition of 1 M HCl. The undesired *R*-isomer was extracted into EtOAc and discarded. The aqueous layer was neutralized to a pH of 7.0 by the addition of 1 M KOH and the water was removed under reduced pressure until the product (*S*)-3-fluoro-4-nitrophenylalanine formed as a yellow precipitate (184 mg, 0.806 mmol, 45.2%). ^{1}H NMR (400 MHz, D_2O) δ 8.06 (t, *J* = 8.14 Hz, 1H, O_2N-C=C*H*), 7.28 (dd, *J* = 12.23, 1.69 Hz, 1H, F-C=C*H*), 7.23 (dd, *J* = 8.51, 1.24 Hz, 1H, O_2N-C=CH-C*H*), 3.94 (t, 1H, α C*H*), 3.21 (m, 2H, Phe CH_2).

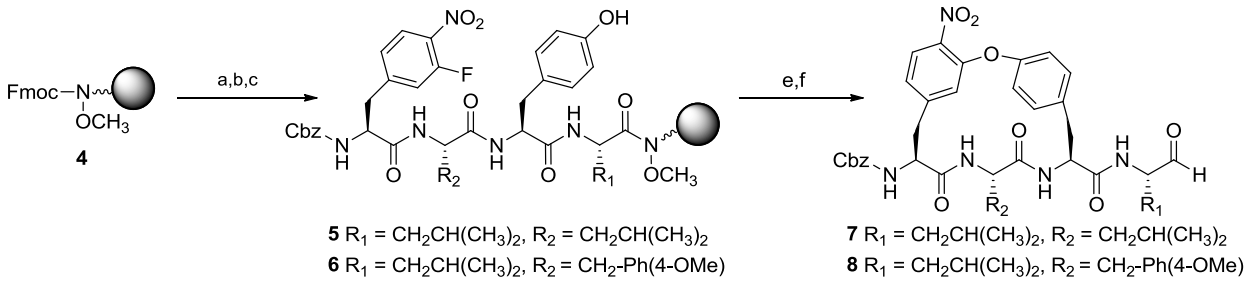


3

(*S*)-*N*-Benzyloxycarbonyl-3-fluoro-4-nitrophenylalanine (3): (*S*)-3-Fluoro-4-nitrophenylalanine (1.72 g, 10.48 mmol) was dissolved in a 1:1 mixture of dioxane and water (30 mL). The pH of the solution was adjusted to 8-9 by the addition of solid sodium bicarbonate and Cbz-OSu was added (2.61 g, 10.48 mmol). The solution was stirred overnight and the solvent removed under reduced pressure. The resulting solids were suspended in water and the pH adjusted to 2-3 by the addition of 1 M HCl. The product was extracted into EtOAc which was then removed under reduced pressure resulting in a yellow solid which was triturated in diethyl ether to remove excess unreacted Cbz-OSu

to give **3** as a yellow solid (1.60 g, 4.40 mmol, 42% yield). ^{1}H NMR (400 MHz, D_2O) δ 12.37 (s, broad, 1H, COO*H*), 8.08 (t, *J* = 8.06 Hz, 1H, O_2N-C=C*H*), 7.73 (d, *J* = 8.5 Hz, 1H, F-C=C*H*), 7.51 (d, *J* = 8.98 Hz, 1H, O_2N-C=CH-C*H*), 7.33 (m, 6H, Cbz aromatic and N*H*), 4.98 (s, 2H, Cbz CH_2), 3.23 (m, 2H, Phe CH_2), 2.96 (t, 1H, α C*H*).

Scheme 2. Solid phase synthesis of peptide aldehydes **7** and **8**[a]



[a]Reagents and conditions: (a) (i) 20% v/v piperidine, DMF, rt, 30 min; (ii) Fmoc-Leu-OH, HATU, DIPEA, DMF, rt, 6 h. (b) (i) 20% v/v piperidine, DMF, rt, 30 min; (ii) Fmoc-Tyr-OH, HATU, DIPEA, DMF, rt, 2 h. (c) (i) 20% v/v piperidine, DMF, rt, 30 min; (ii) Fmoc-Phe(4-OMe)-OH or Fmoc-Leu-OH, HATU, DIPEA, DMF, rt, 2 h. (d) (i) 20% v/v piperidine, DMF, rt, 30 min; (ii) **3** (1.5 equiv), HATU, DIPEA, rt, 2 h. (e) K_2CO_3, $CaCO_3$, 3 Å molecular sieves, DMF, 45 °C, 4 days. (f) (i) $LiAlH_4$, THF, 0 °C, 30 min; (ii) $KHSO_4$ (sat.), K, Na tartrate (sat.), THF, rt, 40 min.

Solid phase peptide synthesis (5 and **6):** Compounds **5** and **6** were synthesized via solid phase peptide synthesis using Weinreb amide functionalized resin beads (loading 0.76 mmol/g) according to the protocol outlined by Murphy *et al.*[1] and improved upon by Fehrentz *et al.*[2] The Fmoc protected resin was purchased from NovaBiochem and all reactions were carried out in 250 mL peptide synthesis vessels on an orbital shaker. The resin was first swelled in DMF (50 mL) for one hour using approximately 15 mL/g of resin. The Fmoc protecting group was then removed by shaking with a solution of 20% v/v piperidine in DMF (50 mL) for 30 minutes. Deprotection was qualitatively confirmed by either the chloranil test for secondary amines or the Kaiser test for primary amines described by Christensen *et al.*[3] and Kaiser *et al.*[4], respectively. The chloranil test was used after initial deprotection of the N-methoxy Weinreb amide and the Kaiser test was used for all subsequent cou-

pling reactions. Following successful deprotection, the resin was washed in DMF (3 x 20 mL) and the desired amino acid was coupled to the free amine by addition of 4 equivalents of the Fmoc-protected amino acid, HATU (4 eq) and DIPEA (10 eq.) in DMF (50 mL). The reaction was shaken for 6 hours if coupling directly to the N-methoxy amide or 2 hours for all other peptide couplings. Successful coupling was confirmed by the aforementioned chromophoric tests. The resin was then washed with DMF (3 x 20 mL) and DCM (4 x 20 mL) to shrink the beads and dried under constant vacuum flow for 15-20 minutes and stored under argon at -20 °C. This procedure was used to generate the peptide Cbz-Phe(3-F-4-NO_2)-Leu-Tyr-Leu **5** and Cbz-Phe(3-F-4-NO_2)-Phe(4-OMe)-Tyr-Leu **6** using Cbz-Phe(3-F, 4-NO_2) **3** as the final amino acid in the coupling sequence.

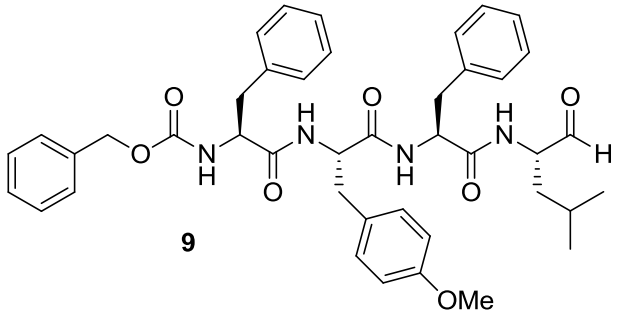
Macrocyclization. The resin-bound precursors to compounds **7** and **8** were generated by following the biaryl ether peptide cyclization procedure outlined by Boger *et al.* for the cyclization of vancomycin[5]. The resin-bound linear peptide precursor was suspended in dry DMF (50 mL) along with anhydrous potassium carbonate (10 eq), calcium carbonate (10 eq) and molecular sieves (3 Å, 250 mg). The solution was shaken in a 45 °C water bath for 4 days during which time the solution turned deep red. The resin was decanted off the salts and washed with DCM (4 x 20 mL) and dried under constant vacuum flow for 15-20 minutes and stored under argon at -20 degrees °C.

Resin Cleavage and purification of 7. Compound **7** was prepared using the procedure outlined by Fehrentz *et al.*[2] to cleave the peptide from the Weinreb amide resin under reductive conditions to produce a C-terminal peptide aldehyde. The resin was swelled in dry THF (50 mL) for an hour at 0 °C. Solid LAH (5 eq) was suspended in dry THF (10 mL) and added dropwise to the resin resulting in a dark red slurry. The mixture was stirred for 40 minutes and then quenched with saturated $KHSO_4$ (5 mL) and saturated potassium tartrate (5 mL). The reaction was brought to room temperature and the solids filtered off. The product was extracted from the resulting THF/water mix into

DCM and the solvent removed under reduced pressure. The resulting crude product was purified by HPLC. ^{1}H NMR (400 MHz, Acetone-d_6) δ 9.62 (s, 1H, C*H*O), 7.79 (d, *J* = 6.58 Hz, 1H, Leu N*H*), 7.72 (d, *J* = 8.23 Hz, 1H, O_2N-C=C*H*), 7.70 (m, 1H, Phe(3-F-4-NO_2 N*H*), 7.58 (m, 1H, Leu N*H*), 7.49 (m, broad, 7H, Cbz aromatic (5), Tyr aromatic (2)), 6.99 (m, 2H, Tyr aromatic), 6.68 (d, *J* = 8.12 Hz, 1H, O_2N-C=CH-C*H*), 6.18 (d, *J* =1 0.45 Hz, 1H, Tyr-O-C=C*H*), 5.76 (m, 1H, Tyr N*H*), 5.14 (dd, 2H, Cbz CH_2),4.88 (d, 1H, Phe(4-NO_2) α-C*H*), 4.69 (m, 2H, α-Tyr C*H*, Leu α-C*H*), 4.46 (m, 1H, Leu α-C*H*), 3.36 (m, 1H, Phe CH_2), 3.26 (m, 1H, Tyr CH_2), 2.86 (m, 1H, Tyr CH_2), 2.75 (m, 1H, Tyr CH_2), 1.48-1.92 (m, 6H, Leu C*H*, Leu CH_2), 0.82-1.00 (m, 12H, Leu CH_3). MS (ESI) *m/z* 716.3 (M+H). HRMS calcd for $C_{38}H_{46}N_5O_9$ 716.3290, found 716.3293 $[M+H]^+$.

Resin Cleavage and purification of 8. Compound **8** was cleaved using the same procedure as for compound **7.** The resulting crude product was purified by column chromatography (2% MeOH/DCM in silica gel). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.70 (s, 1H, C*H*O), 7.76 (d, *J* = 8.36 Hz, 2H, Leu N*H* and O_2N-C=C*H*), 7.54 (d, *J* = 6.25 Hz, 1H, Phe(4-OMe) N*H*), 7.40 (m, 5H, Cbz aromatic), 7.25 (d, *J* = 8.03 Hz, 1H, Tyr aromatic), 7.11 (m, 1H, Phe(4-NO_2) N*H*), 6.99 (d, *J* = 8.25 Hz, 1H, Tyr aromatic), 6.92 (d, *J* = 8.25 Hz, 3H, O_2N-C=CH-C*H*, Phe(4-OMe) aromatic (2)), 6.85 (d, *J* = 8.44 Hz, 1H, Tyr aromatic), 6.80 (d, *J* = 8.44, 1H, Tyr aromatic), 6.60 (d, *J* = 8.31 Hz, 3H, Tyr-O-C=C*H*, Phe(4-OMe) aromatic (2)), 5.71 (d, 1H, Tyr N*H*), 5.22 (dd, 2H, Cbz CH_2), 4.99 (s, broad, 2H, Tyr α-C*H*, Phe(4-NO_2) α-C*H*), 4.64 (m, 2H, Leu α-C*H*, Phe(4-OMe) α-C*H*) 3.52 (s, 3H, Phe-OCH_3), 3.32 (m, 1H, Tyr CH_2), 2.87 (m, 1H, Tyr CH_2), 2.60 (m, 4H, Phe(4-OMe) CH_2 Phe(4-NO_2) CH_2), 1.72-1.93 (m, 3H, Leu CH_2, Leu iso-C*H*) 0.88-1.09 (m, 6H, Leu CH_3). MS (ESI) *m/z* 780.3 (M+H). HRMS calcd for $C_{42}H_{45}N_5O_{10}$ 780.3239, found 780.3238 $[M+H]^+$.

Solid phase peptide synthesis of 9 (Cbz-Phe-Phe(4-OMe)-Phe-Leu-H)

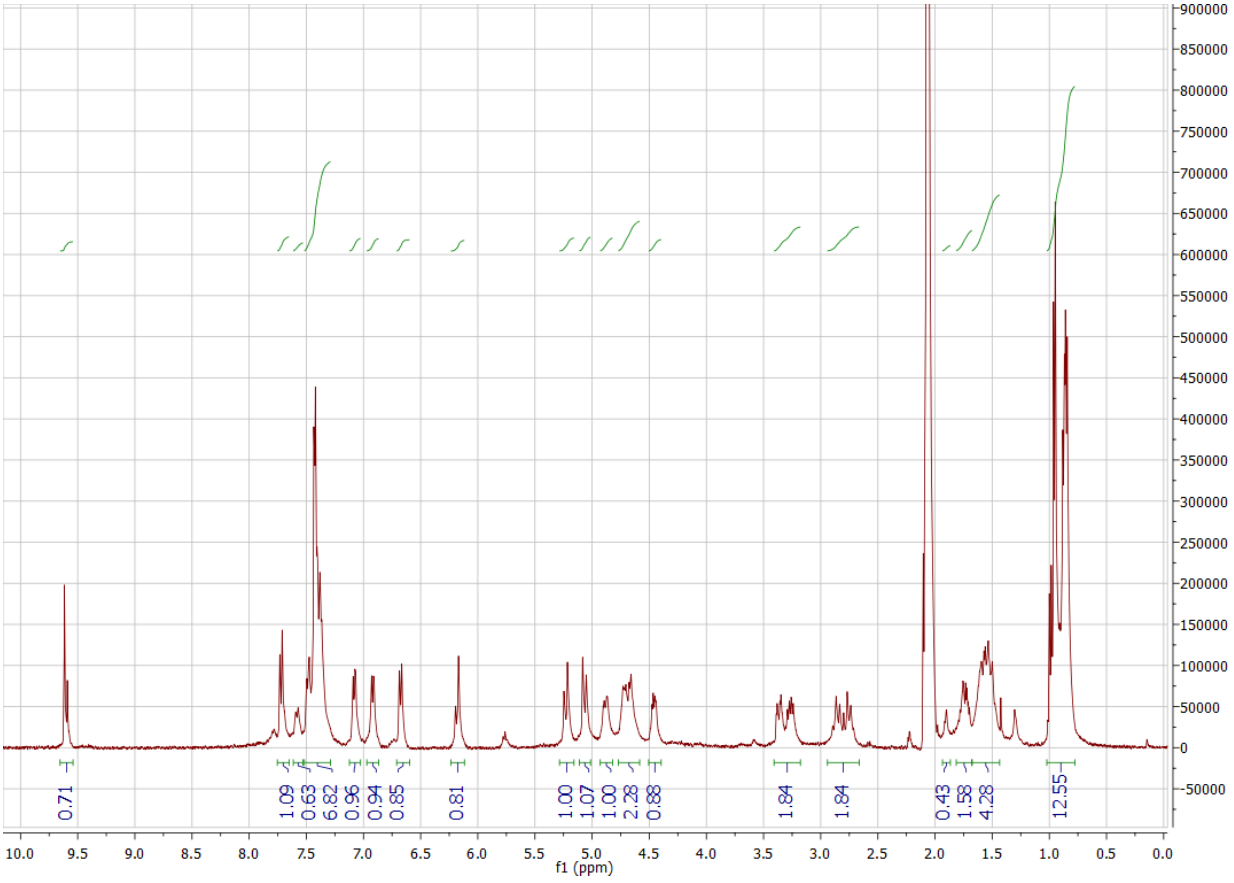


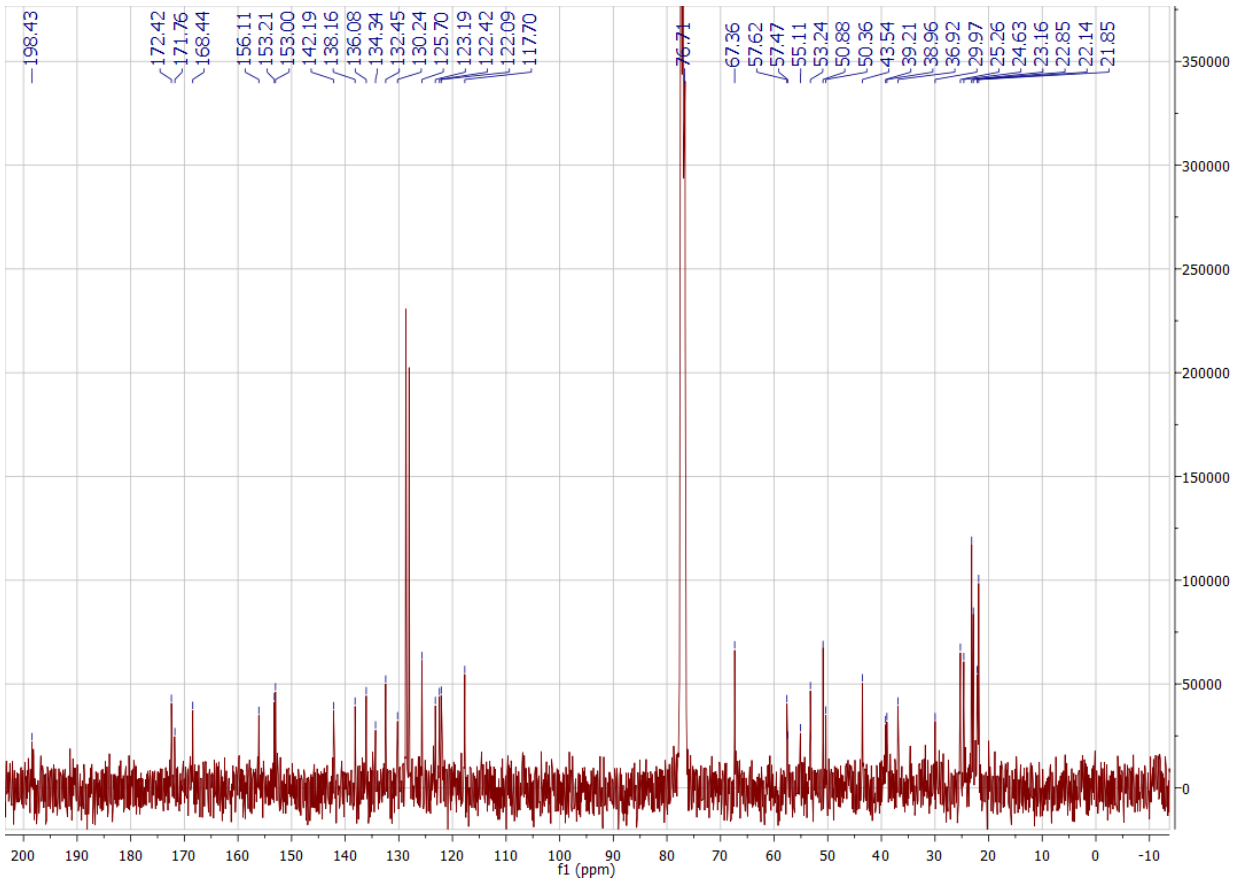
9

Tetrapeptide **9** was synthesized using the same procedure as for linear peptide precursors **5** and **6** using solid phase manual synthesis on a Weinreb amide resin (1.5 g, loading 5 mmols/g) with Fmoc/HATU chemistry. The procedure for reductive cleavage off the resin as described for compounds **7** and **8** provided 560 g of the tetrapeptide aldehyde as an off-white powder in a 62% overall yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.20 (s, 1H, C*H*O), 8.45 (d, 1H, NH), 8.33 (d, 1H, N*H*), 8.00 (d, 1H, NH), 7.48 (d, 1H, NH), 7.1 – 7.3 (m, 17H, aromatic Phe and Cbz), 6.77 (d, 2H, Phe(4-OMe) aromatic), 4.93 (s, 2H, Cbz CH_2), 4.60 (m, broad, 1H, α-C*H*), 4.51 (m, 1H, α-C*H*) 4.22 (m, broad, 1H, α-C*H*), 4.06 (m, 1H, α-C*H*), 3.69 (s, 3H, Phe(4-O-CH_3)), 3.02 (m, 2H, CH_2-Phe(4-OMe) , Phe-CH_2), 2.87 – 2.96 (m, 2H, Phe CH_2), 2.62 – 2.77 (m, 2H, CH_2-Phe(4-OMe) , Phe-CH_2), 1.33-1.54 (m, 3H, Leu CH_2, Leu iso-C*H*) 0.88-1.09 (m, 6H, Leu CH_3). MS (ESI) *m/z* 721.3 (M+H).

3. NMR Spectra of final compounds

Macrocycle **7** (^{1}H, ^{13}C and COSY spectra)

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22.14
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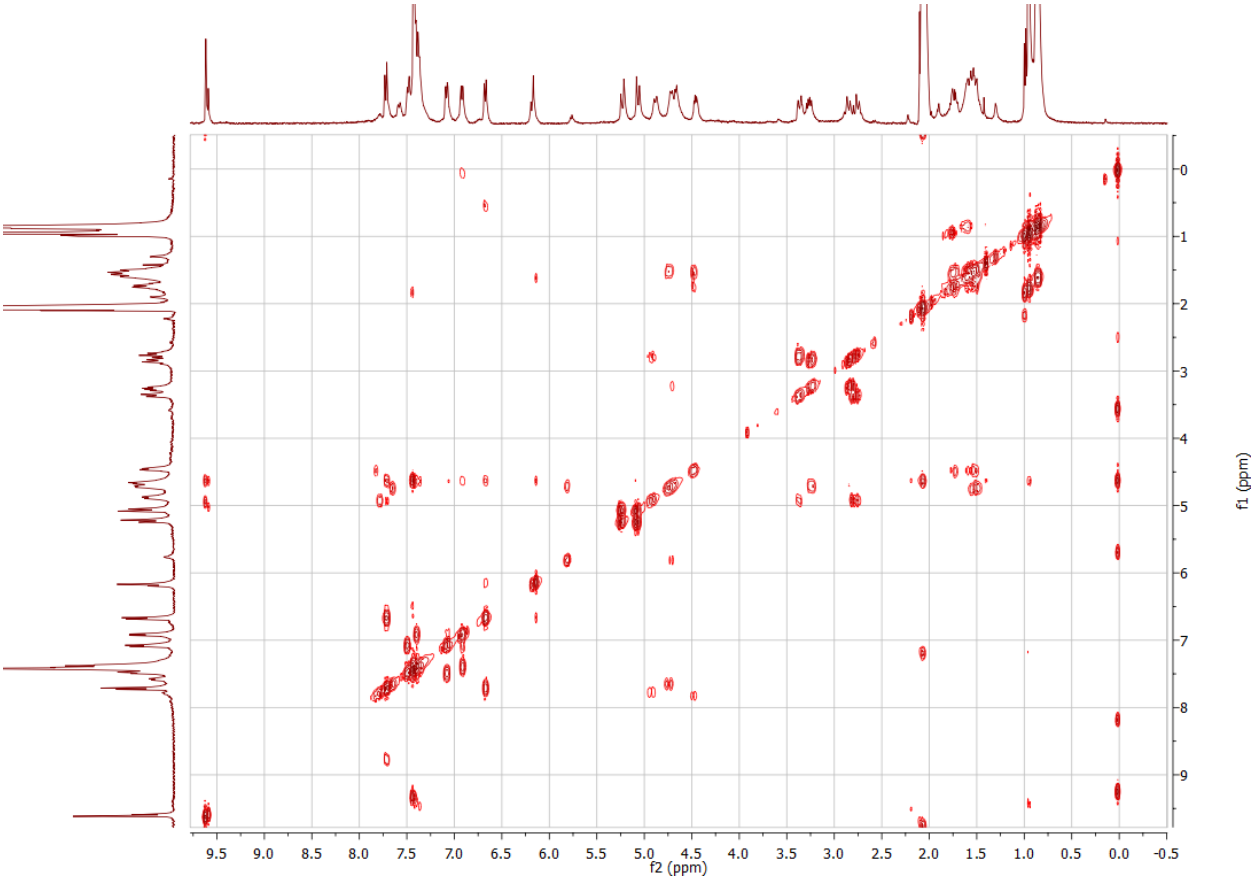
f1 (ppm)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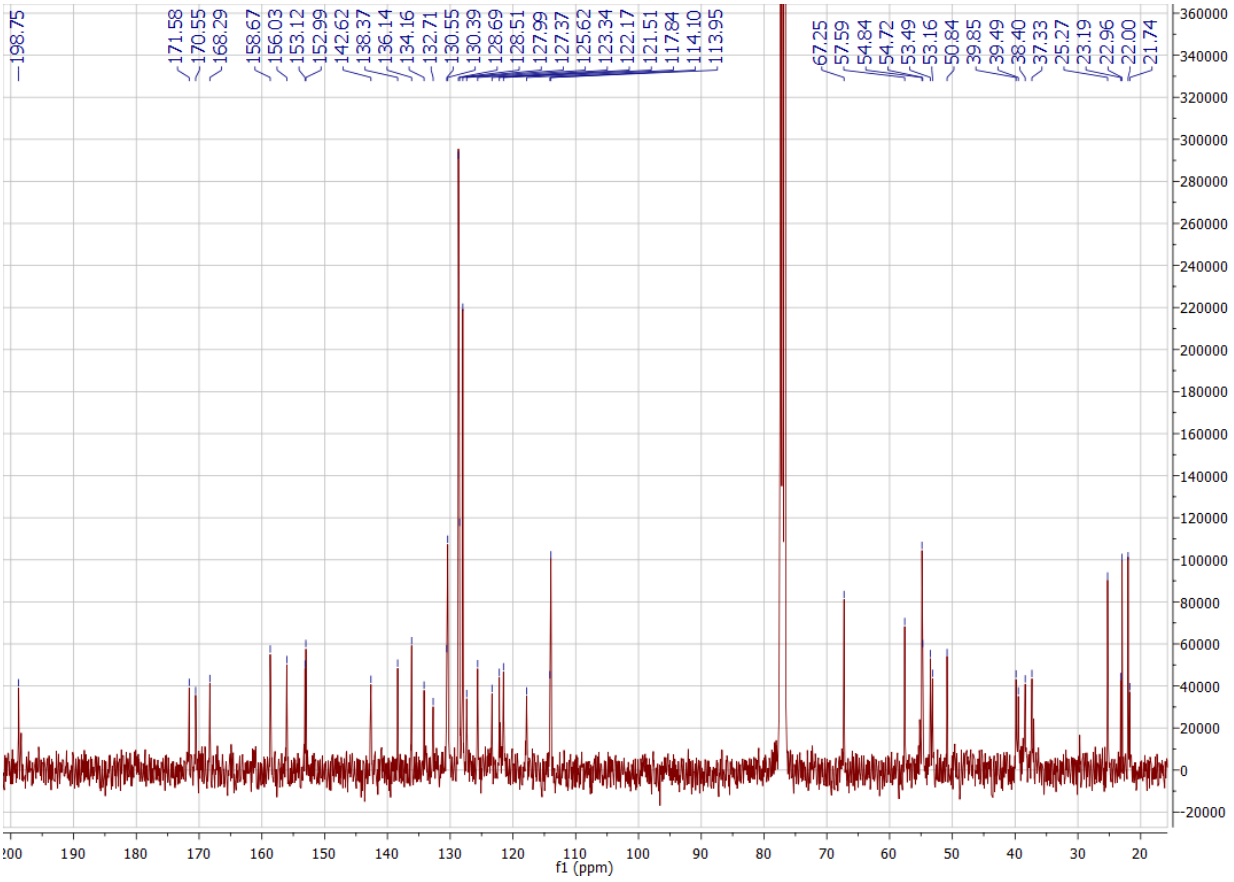
f1 (ppm)

Macrocycle **8** (^{1}H, ^{13}C and COSY spectra)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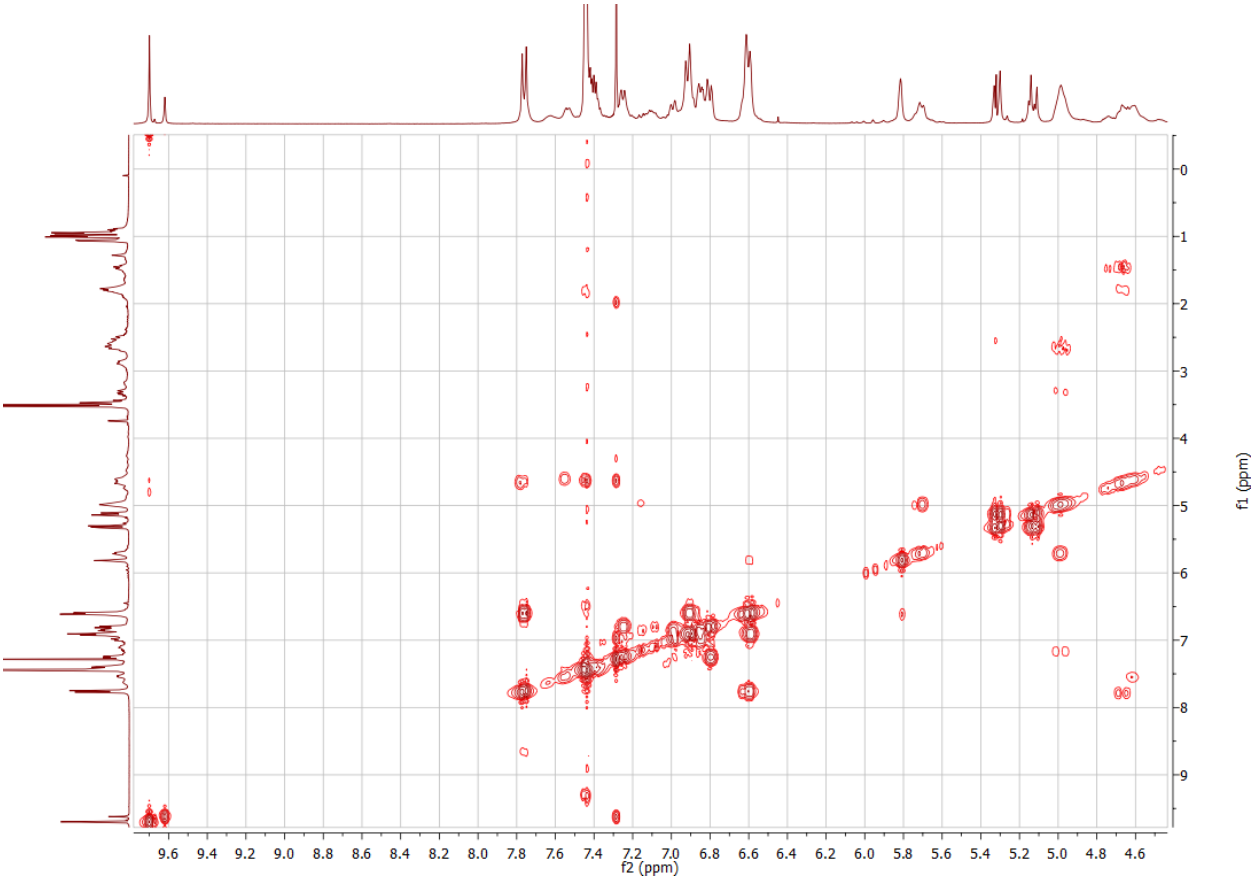
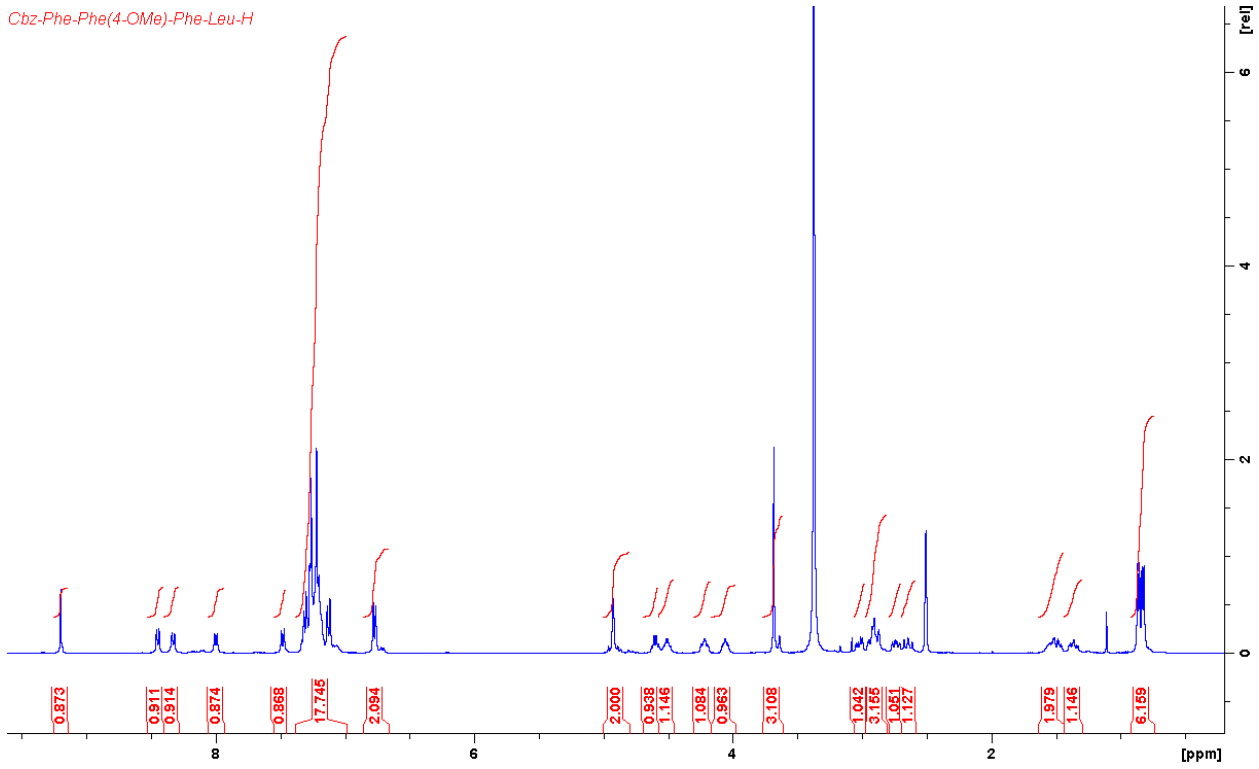
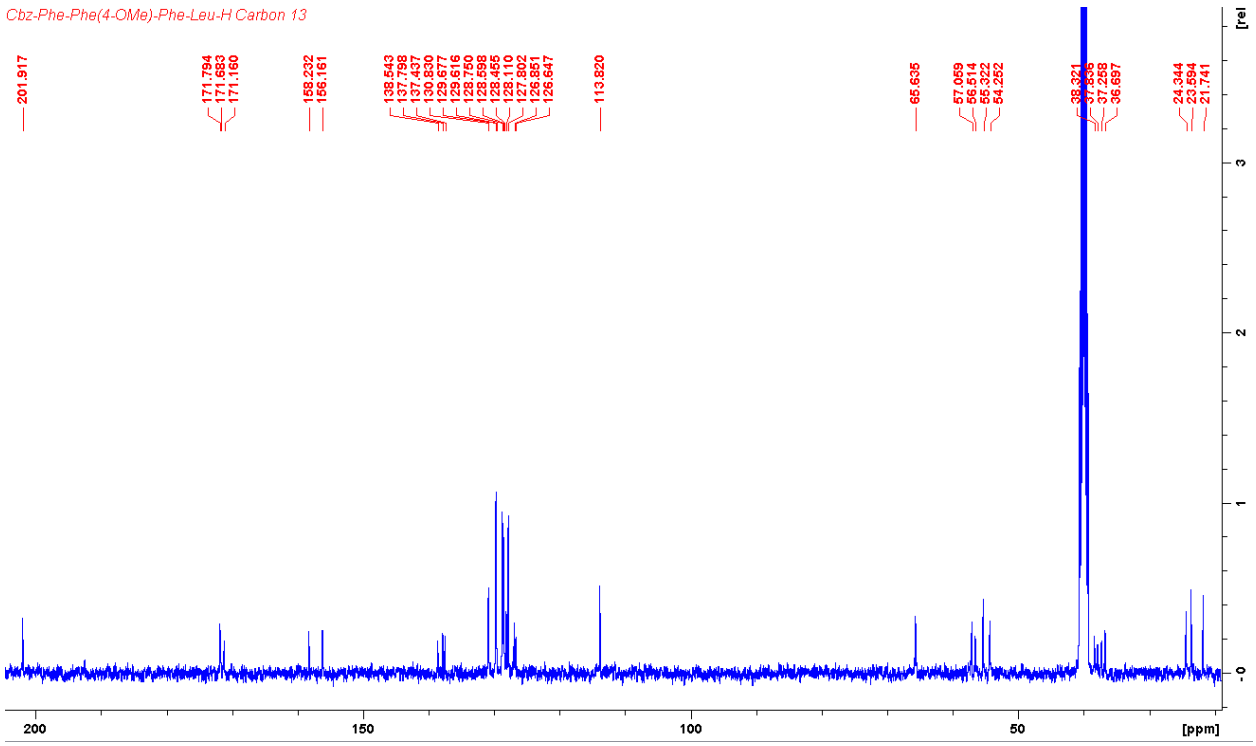
f1 (ppm)


f1 (ppm)
f2 (ppm)

Linear tetrapeptide aldehyde **9** (Cbz-Phe-Phe(4-OMe)-Phe-Leu-H) (^{1}H and ^{13}C spectra).





4. *In Vitro* Kinetic Enzyme Assays

Table 1. Inhibitory potency of **7** and **8** in comparison to **9**, MG-132, bortezomib and carfilzomib.

	K_i in nM					
	20S Proteasome					
Inhibitor	CL-Activity[a]	TL-Activity[b]	PGPH-Activity[c]	Chymotrypsin[d]	Cathepsin B[e]	m-Calpain[f]
7	241 ± 62.2	8,040	N.I.[g]	N.I.[g]	3047 ± 1	2028 ± 319
8	54.5 ± 4.9	N.I.[g]	N.I.[g]	N.I.[g]	30,345 ± 900	2983 ± 621
9	28.0 ± 9.9	N.T.[h]	N.T.[h]	N.I.[g]	2285 ± 1700	1708 ± 99
MG-132	63.5 ± 21.5	N.I.[g]	3,700	N.I.[g]	230 ± 206	653 ± 211
bortezomib	24.0 ± 2.8	N.T.[h]	N.T.[h]	N.I.[g]	N.I.[g]	N.I.[g]
carfilzomib	25.5 ± 12.0	N.T.[h]	N.T.[h]	N.I.[g]	N.I.[g]	N.I.[g]

Kinetic assays were performed on a fluorescent 96-well plate reader 20 S rabbit proteasome in a 50 mM HEPES, 150 mM NaCl, 1 mM DTT, pH 8.0 stock solution. AMC-substrate hydrolysis was measured at 380 nm excitation and 460 nm emission. The total DMSO concentration was below 1 %. K_i values were obtained through non-linear regression analysis from $v_i/v_o=1/(1+[I]/[K_i])$. [a]Chymotryptic activity was measured in 20 mM HEPES, 0.5 mM EDTA, 0.037 % SDS, pH 7.8 at 37 °C, using 20 μM Suc-LLVY-AMC as the fluorogenic substrate, and 0.5 nM enzyme. [b]Tryptic activity was measured in 50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5 at 37 °C, using 4.97 μM Cbz-ARR-AMC as the fluorogenic substrate, and 2.0 nM enzyme concentration. [c]PGPH activity was measured in 50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5 at 37 °C, using 39 μM Cbz-LLE-AMC as the fluorogenic substrate, and 2.0 nM enzyme concentration. [d]Inhibition of chymotrypsin was measured in 50 mM potassium phosphate buffer, pH 7.5 at 37 °C, using 20 μM Suc-LLVY-AMC as the fluorogenic substrate. [e]Cathepsin B inhibition was measured with cathepsin B from human liver in 0.1 M potassium phosphate, 1.25 mM EDTA, 0.01 % Brij, at pH 6.0 using Cbz-RR-

AMC as the fluorogenic substrate. m-Calpain inhibition was measured in 50 mM Hepes at pH 7.5 using Suc-LY-AMC as the fluorogenic substrate. [g]No inhibition at 100 μM inhibitor concentration. [h]Not tested.

5. Cell culture, Western Blot and electrophoretic mobility shift assay (EMSA)

HeLa cells were cultured in Dulbeco's modified eagles medium (DMEM) (10% FCS, 100 U/ml of penicillin-streptomycin). Compounds were dissolved in DMSO and administered to the cells for 2 h prior to stimulation with 10 ng/ml TNFα (Biomol) or 0,5 ng/ml IL-1β (R&D systems). Cells were lysed in whole cell lysis buffer [20 mM Hepes (pH 7.9), 350 mM NaCl, 20% glycerin, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 EGTA, 1% Nonidet P-40, 0.5 M NaF, 1 M DTT, 1 M β-glycerophosphate, 200 mM Na vanadate, and 25× Protease Inhibitor Mixture (Roche)]. For Western Blot whole cell extracts (WCE) were separated by SDS-PAGE and stained with antibodies anti-pS32/36 IκBα (5A5), anti-IκBα (L35A5) (both Cell Signaling) and anti-β-ACTIN (I-19; Santa Cruz). For EMSA, 2 μg of WCE were incubated with a ^{32}P-dATP–labeled, double-stranded NF-κB oligonucleotide probe (5′-CAGGGCTGGGGATTCCCCATCTCCACAGG-3′) and separated on native polyacrylamide gel electrophoresis before autoradiography.

6. QM/MM calculations

All QM/MM calculations were run using the 2013.08.02 release of the Molecular Operating Environment distributed by the Chemical Computing Group. Calculations were run using the Amber12:EHT force field and refined to an RMS gradient of 0.1 kcal/mol/Å^2. Each inhibitor was modeled as a hemiacetal adduct of the Thr1 residue of the β5 subunit. Energy minimizations were carried out to determine the minimum energy conformation of the inhibitor in the proteasome's binding pocket.

7. References

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