CHEMBIOCHEM

Supporting Information

CHEMBIOCHEM

Supporting Information

for

Novel Aza Peptide Inhibitors and Active-Site Probes of Papain Family Cysteine Proteases

Steven H. L. Verhelst, Martin D. Witte, Shirin Arastu-Kapur,
Marko Fonovic, and Matthew Bogyo*

Synthetic Procedures

Solid phase synthesis of (aza)glycine *O*-acylhydroxamates: Fmoc-hydroxylamine was loaded onto 2-chlorotritylresin as described.[1] Next, the protecting group was deprotected with 20 % piperidine in DMF. For azaglycine probes, the resin was reacted for 3 h with carbonyldiimidazole (6 equiv) in dichloromethane. The resin was washed and a 60-fold excess of hydrazine in DMF was added. After 1 h, resin was washed with DMF and elongated by solid phase peptide chemistry. For the glycine probes, Fmoc-glycine was coupled to the deprotected hydroxylamine resin, and further elongated by solid-phase peptide chemistry. Amino-termini were capped with Ac_2O (10 equiv) and DIEA (10 equiv) in DMF (15 min). Peptides were cleaved with 1% TFA in DCM (5 x 2 min), and dried by coevaporation with toluene. The crude peptide was dissolved in THF, cooled in ice, and subsequently pyridine (2 equiv) and *p*-anisoylchloride (1.9 equiv) were added. After LCMS analysis had proved complete conversion, the reaction was quenced with

methanol and evaporated to dryness. Crude inhibitors were deprotected with TFA/H_2O/TIS 95:2.5:2.5 for 1h, concentrated under reduced pressure and purified by HPLC.

Solid phase synthesis of *O*-acylhydroxyureas: Peptides were synthesized on Rink amide resin by standard protocols, using Fmoc-amino acids (3 equiv), DIC (3 equiv) and HOBt (3 equiv) for elongation. After final Fmoc-deprotection, the resin was reacted at 4 °C with carbonyldiimidazole (6 equiv) in DMF. After 5 min. the reactants were drained and the resin was washed with DMF. Subsequently, hydroxylamine (10 equiv) and DIEA (10 equiv) in DMF were added followed by shaking overnight. Acylation was performed on resin by treatment with *p*-anisoylchloride (1.9 equiv) and pyridine (2 equiv) in THF. Products were cleaved from the resin with with TFA/H_2O/TIS 95:2.5:2.5 (1 h), concentrated under reduced pressure and purified by HPLC.

***N*-Acetyl-(*O*-*tert*-butyl)tyrosylphenylalanine hydrazide (2)**: *N*-acetyl-(*O*-*tert*-butyl)-tyrosyl-phenylalanine methyl ester (**1**) was synthesized by coupling Fmoc-Tyr(*t*-Bu)-OH and phenylalanine methyl ester under influence of DIC/HOBt. Crude product was treated with 20 % piperidine, concentrated under reduced pressure and acetylated using an excess of acetic anhydride and DIEA. Crude **1** and hydrazine (60 equiv), were dissolved in methanol and the mixture was refluxed overnight, concentrated under reduced pressure and purified by silicagel column chromatography giving the title compound as a white solid in 53% yield over 4 steps. ESI-MS: *m/z* 441.4 $[M+H]^+$. ^{1}H NMR (500 MHz, [D_6]-DMSO): *d*=9.13 (s, 1H), 8.06 (d, 1H, *J* = 8.3 Hz), 7.98 (d, 1H, *J* = 8.4 Hz), 7.28-7.17 (m, 5H), 7.08 (d, 2H, *J* = 8.4 Hz), 6.83 (d, 2H, *J* = 8.4 Hz), 4.48-4.40 (m, 2H, 4.24 (br s, 2H), 2.94 (dd, 1H, *J* = 5.3 Hz, *J* = 13.8 Hz), 2.86 (dd, 1H, *J* = 4.3 Hz, *J* = 13.7 Hz), 2.80 (dd, 1H, *J* = 8.8 Hz, *J* = 13.7 Hz), 2.60 (dd, 1H, *J* = 10.0 Hz, *J* = 13.9 Hz), 1,71 (s, 3H), 1.25 (s, 9H).

{[*N*-Acetyl-(*O*-*tert*-butyl)tyrosyl]phenylalanyl}azaglycine hydroxyamide (4): Carbonyldiimidazole (6.0 equiv) was added to a soution of compound **2** (1.0 equiv) in DMF, and the reaction mixture was stirred for 4 h at room temperature. Next, hydroxylamine (10 equiv) and DIEA (10 equiv) were added and the resulting mixture was stirred for 1 h at 60 °C. Diluted $KHSO_4$ was added, and product was extracted with DCM (2x). Organic layers were concentrated under reduced pressure, and the crude product was purified by HPLC, yielding the title compound in 44%. ESI-MS: *m/z* 500.4 $[M+H]^+$. ^{1}H NMR (500

MHz, [D_6]DMSO): *d*=9.77 (s, 1H), 8.78 (s, 1H), 8.71 (s, 1H), 8.54 (s, 1H), 8.05 (d, 1H, *J* = 8.1 Hz), 7.98-7.94 (m, 1H), 7.29-7.13 (m, 5H), 7.08 (d, 2H, *J* = 8.0 Hz), 6.83 (d, 2H, *J* = 7.9 Hz), 4.62-4.56 (m, 1H), 4.47-4.41 (m, 1H), 3.10-3.06 (m, 1H), 2.87-2.79 (m, 2H), 2.59-2.54 (m, 1H), 1.70 (s, 3H), 1.25 (s, 9H).

([*N*-Acetyl-tyrosyl]phenylalanyl)azaglycine *p*-methoxybenzoyloxyamide (MW4): *P*-anisoyl chloride (1.9 equiv) and pyridine (2.0 equiv) were added to a stirred solution of compound **4** in THF, cooled to 4 °C. After 2 h, the reaction was quenched by addition of methanol. The mixture was concentrated under reduced pressure, treated with TFA/TIS/H_2O 95:2.5:2.5 for 1 h and again concentrated. HPLC purification gave the title compound in 21 % yield. ESI-MS: *m/z* 578.4 $[M+H]^+$. ^{1}H NMR (500 MHz, [D_6]DMSO): *d*= 10.26 (s, 1H), 9.93 (s, 1H), 9.14 (br s, 2H), 8.08 (d, 1H, *J* = 8.3 Hz), 8.00 (d, 2H, *J* = 8.8 Hz), 7.93 (d, 1H, *J* = 8.5 Hz), 7.28-7.18 (m, 5H), 7.10 (d, 2H, *J* = 8.8 Hz), 6.97 (d, 2H, *J* = 8.4 Hz), 6.61 (d, 2H, *J* = 8.3 Hz), 4.60-4.55 (m, 1H), 4.41-4.36 (m, 1H), 3.86 (s, 3H), 3.09 (dd, 1H, *J* = 3.4 Hz, *J* = 13.9 Hz), 2.85-2.78 (m, 2H), 2.53-2.48 (m, 1H), 1.70 (s, 3H).

([*N*-Acetyl-tyrosyl]phenylalanyl)glycine *p*-methoxybenzoyloxyamide (MW2): The title compound was isolated as a white solid in 12% yield. ESI-MS: *m/z* 577.3 $[M+H]^+$. ^{1}H NMR (500 MHz, [D_6]DMSO): *d*=9.16 (br s, 1H), 8.38-8.34 (m, 1H), 8.05 (d, 1H, *J* = 8.6 Hz), 8.01-7.96 (m, 3H), 7.27-7.18 (m, 6H), 7.11 (d, 2H, *J* = 8.5 Hz), 6.97 (d, 2H, *J* = 8.3 Hz), 6.61 (d, 2H, *J* = 8.1 Hz), 4,58-4.52 (m, 1H), 4.40-4.35 (m, 1H), 3.90-3.68 (m, 5H), 3.07 (dd, 1H, *J* = 4.1 Hz, *J* = 13.9 Hz), 2.84-2.78 (m, 2H), 2.56-2.50 (m, 1H), 1.71 (s, 3H).

{((*N*-[Methoxybenzoyloxyaminocarbonyl]leucyl)tyrosyl)-6-aminohexanoyl}lysyl-amide (MW6): The title compoud was isolated as a white solid in 52% yield. ESI-MS: *m/z* 728.7 $[M+H]^+$. ^{1}H NMR (500 MHz, [D_6]DMSO): *d*=9.23-9.10 (br s, 1H), 7.97 (d, 2H, *J* = 8.2 Hz), 7.90 (d, 1H, *J* = 8.5 Hz), 7.85-7.79 (m, 2H), 7.71 (br s, 2H), 7.34 (s, 1H), 7.12-7.07 (m, 3H), 7.03-6.94 (m, 3H), 6.63 (d, 2H, *J* = 8.4 Hz), 4.38-4.32 (m, 1H), 4.18-4.11 (m, 2H), 3.86 (s, 3H), 3.05-2.98 (m, 1H), 2.95-2.89 (m, 1H), 2.83 (dd, 1H, *J* = 5.8 Hz, *J* = 13.9 Hz), 2.76-2.67 (m, 3H), 2.10 (t, 2H, *J* = 7.4 Hz), 1.67-1.61 (m, 1H), 1.52-1.24 (m, 14H), 1.18-1.12 (m, 2H), 0.85-0.76 (m, 6H).

([*N*-Acetyl-tyrosyl]leucyl)azaglycine *p*-methoxybenzoyloxyamide (MW20): The title compound was isolated as a white solid in 10 % yield. ESI-MS: *m/z* 544.3 $[M+H]^+$. ^{1}H NMR (500 MHz, [D$_6$]DMSO): *d*=10.20 (s, 1H), 9.80 (s, 1H), 9.15 (br s, 1H), 9.08 (br s, 1H), 8.04 (d, 1H, *J* = 8.2 Hz), 7.98 (d, 2H, *J* = 9.0 Hz), 7.19 (s, 1H), 7.12-7.08 (m, 2H), 7.02 (d, 2H, *J* = 8.4 Hz), 6.63 (d, 2H, *J* = 8.4 Hz), 4.46-4.40 (m, 1H), 4.38-4.33 (m, 1H), 3.85 (s, 3H), 2.85 (dd, 1H, *J* = 3.3 Hz, *J* = 14.2 Hz), 2.64-2.51 (m, 3H), 1.74 (s, 3H), 1.62 (quint, 1H, *J* = 6.5 Hz), 0.89 (d, 3H, *J* = 6.5 Hz), 0.85 (d, 3H, *J* = 6.5 Hz).

([*N*-Acetyl-tyrosyl]-4-methylphenylalanyl)azaglycine *p*-methoxybenzoyloxyamide (MW21): The title compound was isolated as a white solid in 12% yield. ESI-MS: *m/z* 592.5 $[M+H]^+$. ^{1}H NMR (500 MHz, [D$_6$]DMSO): *d*=10.23 (s, 1H), 9.91 (s, 1H), 9.14 (br s, 1H), 9.12 (br s, 1H), 8.04 (d, 1H, *J* = 7.8 Hz), 7.99 (d, 2H, *J* = 8.9 Hz), 7.93 (d, 1H, *J* = 8.2 Hz), 7.15 (d, 2H, *J* = 8.1 Hz), 7.10 (d, 2H, *J* = 9.0 Hz), 7.05 (d, 2H, *J* = 7.9 Hz), 6.96 (d, 2H, *J* = 8.4 Hz), 6.61 (d, 2H, *J* = 8.6 Hz), 4.55-4.50 (m, 1H), 4.40-4.35 (m, 1H), 3.86 (s, 3H), 3.05-3.01 (m, 1H), 2.83-2.41 (m, 2H), 2.54-2.50 (m, 1H), 2.25 (s, 3H), 1.70 (s, 3H).

([*N*-Acetyl-tyrosyl]-4-methylphenylalanyl)glycine *p*-methoxybenzoyloxyamide (MW22): The title compound was isolated as a white solid in 43% yield. ESI-MS: *m/z* 591.5 $[M+H]^+$. ^{1}H NMR (500 MHz, [D$_6$]DMSO): *d*=11.9 (s, 1H), 9.15 (br s, 1H), 8.35 (s, 1H), 8.03-7.96 (m, 4H), 7,15-7.10 (d, 4H, *J* = 8.5 Hz), 7.05 (d, 2H, *J* = 7.6 Hz), 6.97 (d, 2H, *J* = 8.1 Hz), 6.61 (d, 2H, *J* = 8.3 Hz), 4.54-4.51 (m, 1H), 4.40-4.35 (m, 1H), 3.04-2.00 (m, 1H), 2.84-2,50 (m, 4H), 2.24 (s, 3H), 1.72 (s, 3H).

{(*N*-[Methoxybenzoyloxyaminocarbonyl]-4-methylphenylalanyl)alanyl}tyrosyl-amide (MW23): The title compound was isolated as a white solid in 42% yield. ESI-MS: *m/z* 606.5 $[M+H]^+$. ^{1}H NMR (500 MHz, [D$_6$]DMSO): *d*=9.83 (s, 1H), 9.16 (s, 1H), 8.20 (d, 1H, *J* = 7.1 Hz), 7.94 (d, 2H, *J* = 9.0 Hz), 7.78 (d, 1H, *J* = 8.0 Hz), 7.32 (s, 1H), 7.11-6.96 (m, 9H), 6.63 (d, 2H, *J* = 8.5 Hz), 4.38-4.30 (m, 2H), 4.26-4.22 (m, 1H), 3.86 (s, 3H), 2.94-2.86 (m, 2H), 2.78-2.70 (m, 2H), 2.23 (s, 3H), 1.19 (d, 3H, *J* = 7.0 Hz).

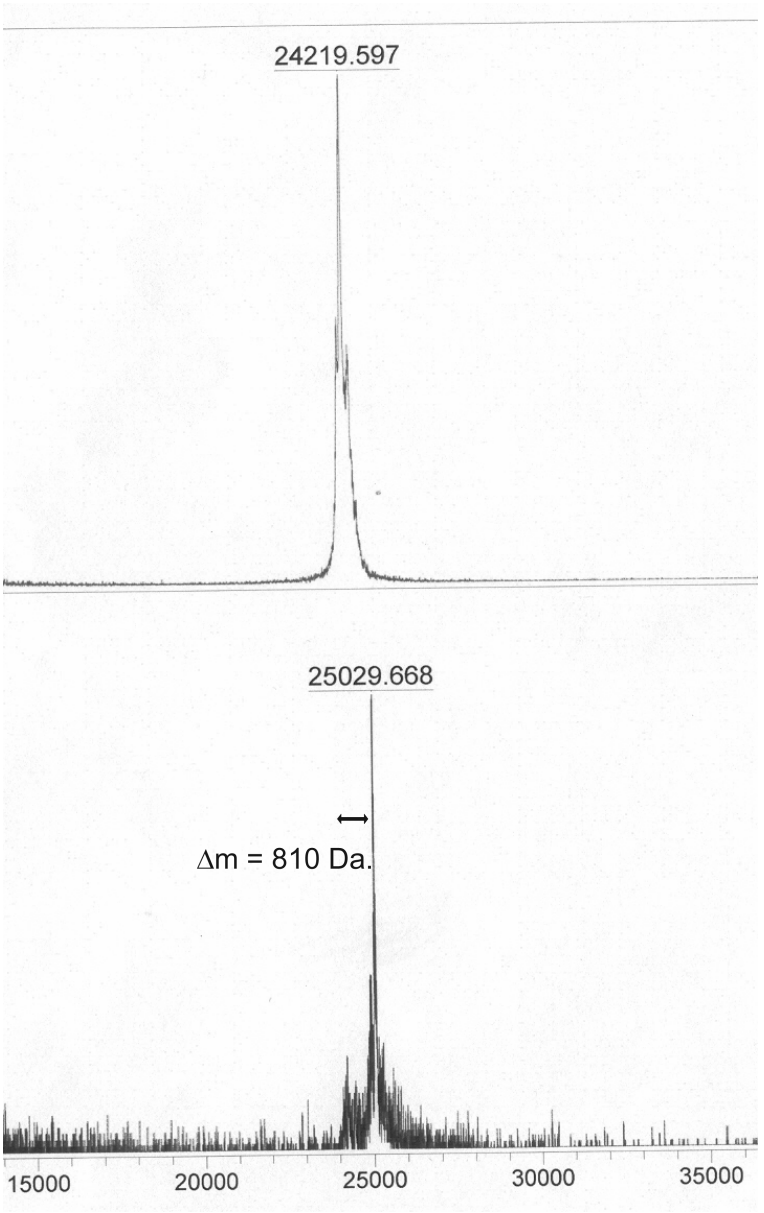

Figure S1. Parts of the MALDI-TOF spectrum of recombinant cathepsin L after incubation with control dmso (upper) or MW6bio (lower). Δm_{obs} = 810 Dalton. Δm for covalent modification of the enzyme with expulsion of the *p*-methoxybenzoate is 801.

---

[1] S. L. Mellor, C. McGuire, W. C. Chan, *Tetrahedron Lett.* **1997**, *38*, 3311- 3314.