

CHEMBIOCHEM

Supporting Information

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for

Inhibition of Cathepsin L by Epoxysuccinyl Peptides Addressing Simultaneously Active-site and Remote-site regions

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Materials

Solvents and reagents were of the highest purity commercially available and were used without further purification. Amino acid derivatives, HBTU and HOBt were purchased from Iris Biotech GmbH (Marktredwitz, Germany) and TCP resins preloaded with the first Fmoc amino acid from PepChem (Reutlingen, Germany). Automated peptide synthesis was carried out on a batch synthesizer model 431A (Applied Biosystems, Foster City, USA). Manually performed synthetic steps on resin were carried out with a IKA KS 130 basic laboratory shaker (IKA[®] Werke GmbH & Co. KG, Staufen, Germany) using plastic syringes type Discardit II from Becton Dickinson (Franklin Lakes, USA) equipped with PE frits (35 µm pores size) and PE stoppers from Roland Vetter Laborbedarf (Ammerbuch, Germany). ¹H and ¹³C NMR spectra were recorded with a AMX 500 spectrometer from Bruker, ESI-MS spectra with a PE Sciex API 165 (Applied Biosystems, Framingham, USA), and HRMS (ESI) spectra with a micrOTOF (Bruker Daltonics, Bremen, Germany). TLC was performed on silica gel 60 plates and flash chromatography using silica gel 60 (230 – 400 mesh) from VWR International GmbH (Darmstadt, Germany), respectively. (2S,3S)-oxirane-2,3-dicarboxylic acid derivatives were visualized on TLC plates using 4-(4-nitrobenzyl)-

pyridine.^[1] Analytical RP-HPLC was performed with a system from Waters (Eschborn, Germany) using Nucleosil 100-5 C₈ RP columns (Macherey-Nagel, Düren, Germany) with a linear gradient from 100% eluent A (2% aq H₃PO₄/CH₃CN (95:5, v/v)) to 100 % eluent B (2% aq H₃PO₄/CH₃CN (10:90, v/v)) within 13 min.

Synthesis

(2*S*,3*S*)-oxirane-2,3-dicarboxylic acid *tert*-butyl ethyl ester (EtO-(2*S*,3*S*)-*tEps*-OtBu): (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid mono ethyl ester potassium salt (530 mg, 2.67 mmol)^[2,3] was dissolved in water (50 mL) and acidified by adding KHSO₄ (383 mg, 2.80 mmol). The resulting free acid was extracted with AcOEt (3×50 mL), the combined organic phases dried (Na₂SO₄) and concentrated under reduced pressure to a volume of app. 30 mL. According to a procedure reported by Pozdnev^[4] at RT under stirring *tert*-butanol (5 mL), pyridine (0.5 mL), DMAP (20 mg), and Boc₂O (583 mg, 2.67 mmol) were added. After 3 h, an additional portion of Boc₂O (583 mg, 2.67 mmol) was added and stirring continued over night. The solvent was evaporated and the resulting brownish oil dissolved in AcOEt (100 mL). The organic phase was washed with 5% aq KHSO₄ (3×50 mL), 5% aq. NaHCO₃ (3×50 mL), brine (1×50 mL), dried (Na₂SO₄), and the solvent evaporated. The resulting yellow oil was purified by flash chromatography (70 g silica gel, 3×25 cm; eluent: AcOEt/petroleum ether 1:9, v/v). The title compound was obtained as colorless oil; yield: 394 mg (68%); TLC (AcOEt/petroleum ether 1:6, v/v) R_f 0.55; ¹H NMR (DMSO-*d*₆): δ = 1.21 (t, 3H, *J* = 7.1 Hz, CH₂-CH₃), 1.43 (s, 9H, C(CH₃)₃), 3.58 (d, 1H, *J* = 1.8 Hz, CH), 3.67 (d, 1H, *J* = 1.8 Hz, CH), 4.17 (m, 2H, CH₂-CH₃); ESI-MS: *m/z* 217.3 [*M*+H]⁺, 161.2 [*M*+H-C₄H₉]⁺; calcd for C₁₀H₁₆O₅: 216.1.

(2*S*,3*S*)-oxirane-2,3-dicarboxylic acid mono *tert*-butyl ester sodium salt (NaO-(2*S*,3*S*)-*tEps*-OtBu): (2*S*,3*S*)-oxirane-2,3-dicarboxylic acid *tert*-butyl ethyl ester (394 mg, 1.82 mmol) was dissolved in dioxane/water (2:1, v/v; 15 mL) and at RT under stirring 1 N NaOH (1.8 mL) added. After 1 h, again 1 N NaOH (0.1 mL) was added, stirring continued for an additional hour and the solvent evaporated. Upon evaporation from toluene (5×) a colorless solid was obtained. The solid was suspended in a small amount of *i*PrOH, then *tert*-butyl methyl ether and petroleum ether was added. The powder was collected by centrifugation washed with petroleum ether (3×) and dried in vacuo. From the mother liquor an additional fraction was obtained; yield: 308 mg (81%); TLC (*n*-BuOH/AcOH/H₂O/AcOEt 3:1:1:5, v/v/v/v) R_f 0.6; ¹H NMR ([D₆]-

DMSO): $\delta = 1.41$ (s, 9H, C(CH₃)₃), 2.99 (d, 1H, $J = 1.8$ Hz, CH), 3.15 (d, 1H, $J = 1.8$ Hz, CH); ¹³C NMR (DMSO-*d*₆): $\delta = 27.51, 51.31, 54.90, 81.36, 167.7, 168.17$; ESI-MS: m/z 187.0 [$M-H$]⁻; calcd for C₈H₁₂O₅: 188.1.

General procedure for the peptide synthesis: The TCP resin preloaded with the first Fmoc-amino acid (0.25 mmol) was subjected to automated peptide synthesis according to the Fmoc/*t*Bu strategy using the FastMoc-protocol of Applied Biosystems for double coupling for each coupling step. Fmoc-protected amino acid with the following side-chain protecting groups were used: *Ot*Bu (Glu), Boc (Lys), Trt (Asn, Gln), and Pbf (Arg). The coupling was performed with Fmoc-Xaa-OH/HBTU/HOBt /DIEA (1:1:1:2, 4 equiv.) in NMP. After each coupling step the Fmoc group was cleaved with piperidine/NMP (1:4, v/v).

General procedure for manual coupling of NaO-(2S,3S)-tEps-OtBu. In a plastic syringe equipped with a PE frit and a PE stopper the N-terminally unprotected resin-bound peptide (0.05 mmol) was suspended in DMF (1 mL). In parallel, (2S,3S)-oxirane-2,3-dicarboxylic acid mono *tert*-butyl ester sodium salt (42 mg, 0.20 mmol) was suspended in DMF (2 mL), and HBTU (76 mg, 0.20 mmol) added. Upon preactivation of 5 min, the clear solution was added to the resin. After 30 min, the reaction was completed as monitored by the Kaiser test.^[5] The coupling solution was removed, the resin washed with DMF and *i*PrOH sequentially (3×3 mL each) and finally with DCM (3 mL).

General procedure for cleavage and work-up: In a plastic syringe equipped with a PE frit and a PE stopper the resin-bound inhibitor (0.05 mmol) was treated at RT for 2 h with TFA/H₂O/TIS (95:2.5:2.5, v/v/v; 3 mL). The peptide was isolated by adding the cleavage solution dropwise into ice-cold *tert*-butyl methyl ether/*n*-hexane (2:1, v/v; 50 mL). The formed colorless precipitate was collected by centrifugation, washed with petroleum ether (3×), and dried.

Inhibitor 4: yield: 70% (based on resin loading); HPLC t_R 3.9 min; HRMS (ESI) calcd for C₂₂H₂₇N₅O₁₀ + H⁺ ([$M+H$]⁺); m/z 522.18355, found: 522.18652.

Inhibitor 5: yield: 67% (based on resin loading); HPLC t_R 2.3 min; HRMS (ESI) calcd for C₅₁H₈₂N₁₈O₁₅ + H⁺ ([$M+H$]⁺); m/z 1187.62840, found: 1187.63590.

Inhibitor 6: yield: 72% (based on resin loading); HPLC t_R 4.6 min; HRMS (ESI) calcd for C₈₃H₁₃₀N₂₆O₂₄ + 2 H⁺ ([$M+2H$]²⁺); m/z 938.49528, found: 939.01498.

Inhibitor **7**: yield: 74% (based on resin loading); HPLC t_R 6.0 min; HRMS (ESI) calcd for $C_{117}H_{173}N_{31}O_{32} + 2 H^+$ ($[M+2H]^{2+}$); m/z 1263.15084, found: 1263.65880.

Kinetic Measurements

Enzyme assays with cathepsin L (~5 μM) and cathepsin B (~25 μM) were performed in 250 mM sodium acetate containing 2 mM EDTA, 0.015 % Brij-35 and 1 mM dithiothreitol (DTT, added immediately before use) at pH 5.5 with the fluorogenic substrate Z-Phe-Arg-AMC (4 μM for cathepsin L or 10 μM for cathepsin B) at 25 °C (cathepsin L) or 30 °C (cathepsin B). After a constant reaction rate was observed, 5-10 different inhibitor concentrations were added in maximal 1 % of the total test volume of 500 μL (dissolved and prediluted in DMSO). The final inhibitor concentrations ranged from 1 nM up to 100 μM , according to the inhibitory potencies. AMC release was continuously monitored with a SFM-25 fluorimeter (Biotek Kontron, Neufahrn/München, Germany) at 380 nm excitation and 460 nm emission running 4 cuvettes in parallel. The data were digitally recorded over 15-60 min using a self-programmed software as described in ref. [6].

The pseudo first order rate constants, k_{obs} , for the irreversible epoxide inhibitors were obtained by fitting the presteady-state progress data by nonlinear regression analysis with the integrated equation of Morrison.^[7] The apparent second order rate constants were calculated as $k_2/K_i = k_{obs}/I_t$. The rate constants for cathepsin L ($K_m = 2.9 \mu M$) were corrected for substrate competition by multiplication of the apparent values with a factor of $1+S/K_m = 2.38$, for cathepsin B ($K_m = 220 \mu M$) with a factor of 1.045. Each kinetic constant was calculated as a mean value of 5-10 replicates with a standard deviation of $(SD_{n-1}) \leq 10\%$.

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