Supplementary material

Selective targeting of lysosomal cysteine proteases with radiolabeled electrophilic substrate analogs

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*Synthesis of diethyl (2*R,*3*S*)-2-bromo-3-hydroxy-butanedioate (**I**).* A 30% solution of HBr in AcOH (35 ml) was added to diethyl-D-tartrate (7.2 g, 6 ml, 35 mmol) and stirred for 1 h at 0°C. The solution was allowed to warm up to room temperature and stirred overnight. Then it was poured into ice-water, and extracted with ether (3×). The combined ether layers were washed with water and brine, dried (Na_2SO_4), and concentrated. The remaining oil was dissolved in EtOH (35 ml). A 30% solution of HBr in AcOH (21 ml) was added, and the resulting solution was refluxed for 4 h, and then concentrated *in vacuo*, removing most of the acetic acid by co-evaporation with toluene. The yellow oil was dissolved in EtOAc and washed with water, sat. $NaHCO_3$, brine and dried ($MgSO_4$). Silica column chromatography (16–25% EtOAc in hexanes) yielded I (5.67 g, 21.1 mmol, 60%). Diethyl (2*R*,3*S*)-2-bromo-3-hydroxy-butanedioate: 1H NMR (300 MHz, $CDCL_3$) δ 1.33 (m, 6H), 2.1 (d, 1H), 4.19–4.36 (m, 4H), 4.7 (dd, 1H).

*Synthesis of diethyl (2*S,*3*S*)-oxirane-2,3-dicarboxylate (**II**).* To I (2.67 g, 9.9 mmol) in ether (25 ml) DBU (1.63 ml, 10.9 mmol, 1.1 eq) was added dropwise. After 0.5 h TLC analysis revealed complete conversion of the starting material. The solid was filtered off, and the filtrate was washed with citric acid buffer (pH = 3), brine, and dried ($MgSO_4$). Silica column chromatography (9–13% EtOAc in hexanes) provided II (1.42 g, 7.53 mmol, 76%). Diethyl (2S,3S)-oxirane-2,3-dicarboxylate: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 6H), 3.68 (s, 2H), 4.19–4.36 (m, 4H).

*Synthesis of ethyl (2*S,*3*S*)-oxirane-2,3-dicarboxylate (**III**).* To a solution of II (1.35 g, 7.18 mmol) in EtOH (35 ml) at 4°C was slowly added a solution of KOH in ethanol (7.2 ml, 1.00 M). After 3 h the reaction was allowed to warm to room temperature and stirred for an additional hour. The solvent was removed under reduced pressure. The residue was dissolved in water, and extracted with EtOAc (2×). The resulting water layer was acidified to pH 1–2 with 3 M hydrochloric acid, and extracted with EtOAc (4×). The organic layers were washed with a 1:1 mixture of brine and 1 M hydrochloric acid, dried ($MgSO_4$), and concentrated *in vacuo*, yielding a colorless oil (872 mg, 5.45 mmol, 76%). The compound was used without further purification. Ethyl (2S,3S)-oxirane-2,3-dicarboxylate: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.22 (t, 3H), 3.60 (d, 1H), 3.69 (d, 1H), 4.17 (q, 2H).

*Synthesis of N-[[*L*-*trans*-3(ethoxycarbonyl)oxiran-2-yl]- 3-methylbutaneamide (**IV**).* Epoxy acid III (400 mg, 2.5 mmol) was dissolved in anhydrous THF under inert atmosphere and N-methyl morpholine (NMM; 303 µl, 2.75 mmol) added dropwise while stirring. The reaction was cooled to –10°C in an ice/sodium chloride slurry and isobutylchloroformate (IBCF; 358 µl, 2.75 mmol) was added slowly. To the stirring solution was added i-butylamine (273 µl, 2.75 mmol). The reaction was stirred for 1h at reduced temperature then allowed to warm to room temperature for 2h. The reaction mixture was diluted with ethyl acetate and the organic layer washed with 3× volumes of brine, water and 0.1 N HCl. The remaining organic layer was dried, concentrated by rotary evaporation resulting in a crude oil. Flash chromatography yielded the desired product as a pale yellow oil (430 mg, 2.0 mmol, 80%). N-[[L-*trans*-3(ethoxycarbonyl)oxiran-2-yl]-3-methylbutaneamide (IV): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83 (d, 6H), 1.32 (t, 3H), 1.92 (m, 1H), 3.24 (t, 2H), 3.60 (d, 1H), 3.72 (d, 1H), 4.32 (m, 2H), 6.19 (d, 1H).

*Synthesis of N-[[*L*-trans-3(ethoxycarbonyl)oxiran-2-yl]-tyramide (**V**).* Compound V was synthesized exactly as described for compound IV except tyramine was used instead of i-butylamine. N-[[L-*trans*-3(ethoxycarbonyl)oxiran-2-yl]-tyramine amide (V): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35 (t, 3H), 2.77 (m, 2H), 3.39 (d, 1H), 3.48 (m, 2H), 3.64 (d, 1H), 4.27 (m, (2H), 6. 33 (t, 1H), 6.81 (d, 2H), 6.99 (d, 2H).

*Synthesis of N-[(*L*-trans-3-carboxyoxirane-2-yl)carbonyl]-tyramide (**VI**) and N-[(*L*-trans-3-carboxyoxirane-2-yl)carbonyl]-3-methyl-butaneamide (**VII**).* Compounds VI and VII were synthesized by removal of the ethyl ester of compounds V and IV, respectively. Ester hydrolysis was achieved by dissolving each compound in a minimal volume of ethanol followed by addition of 1 equivalent of sodium hydroxide as a 1 N aqueous solution. The resulting reaction was stirred for 2 h at room temperature at which time 1 equivalent of acetic acid was added and water added to the mixture. The water solution was washed with 3 volumes of ethyl acetate. The organic layers were combined, dried and concentrated by rotary evaporation. The crude colorless oils VI and VII were used without further purification.

Synthesis of Boc-isoleucine-proline methyl ester (Boc-IP-OMe). Boc-isoleucine (3.17 g, 13.2 mmol) and hydroxybenzatriazole (HOBt; 1.95 g, 14.4 mmol) were dissolved in a minimal amount of a 1:1 (v:v) solution of DMF:CH_2Cl_2. Dicyclohexylcarbodiimide (DCC; 2.72 g, 13.2 mmol) was added as a solid in one portion to the stirring reaction mixture. The resulting reaction was stirred for 30 min at room temperature, the solid removed by filtration and the supernatant added to a flask containing proline methyl ester hydrochloride salt (2.0 g, 12 mmol). Diisopropylethylamine (DIEA, 4.25 ml, 24 mmol) was added and the reaction stirred at room temperature for 1 h. Solvent was removed by rotary evaporation resulting in a yellow oil, which was dissolved in ethyl acetate, washed with 3× volumes of 0.1 N hydrochloric acid, 3× volumes saturated sodium carbonate, dried and re-concentrated by rotary evaporation. The crude oil was purified by flash chromatography (1:3; v:v ethyl acetate:hexanes) yielding Boc-IP-OMe as a colorless oil (3.6 g, 10.3 mmol, 84%). Boc-IP-OMe: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.82 (t, 3H), 1.04 (d, 3H), 1.24 (m, 2H), 1.44 (m, 9H), 1.76 (m, 1H), 2.08 (m, 2H), 2.36 (q, 2H), 3.67 (m, 2H), 3.65 (s, 3H), 4.35 (t, 1H), 4.55 (m, 1H), 5.24 (d, 1H).

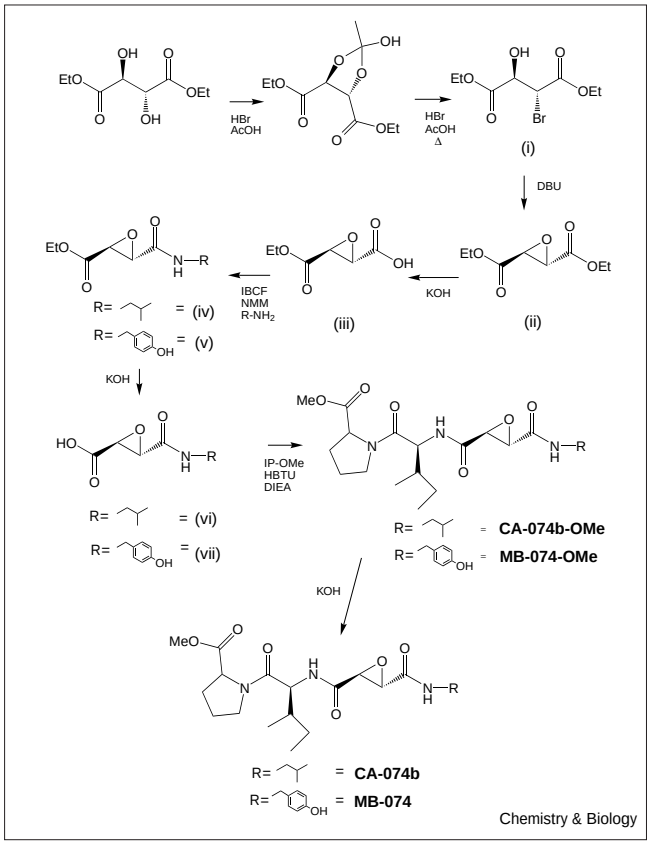
Synthesis of NH_2-isoleucine–proline-OMe (IP-OMe). The Boc group was removed by dissolving IP-OMe in a minimal amount of methylene chloride and adding an equal volume of trifluoroacetic acid (TFA). The reaction was stirred for 30 min at room temperature, a 2× volume of toluene added and the product concentrated by rotary evaporation. The resulting crude clear oil was used without further purification.

Synthesis of MB-074-OMe. Epoxy acid VI (71 mg, 308 µmol) was dissolved in a minimal volume of a 1:1 (v:v) mixture of methylene chloride:dimethylformamide. To the solution was added HBTU (140 mg, 369 µmol) followed by NH_2-IP-OMe (169 mg, 462 µmol) and then DIEA (136 µl, 770 µmol). The reaction was quenched by dilution into ethyl acetate followed by extraction with 3× volumes of brine and 0.1 N HCl. The resulting organic phase was dried and concentrated by rotary evaporation to yield a crude oil which upon purification by flash chromatography (4:1 ethyl acetate:hexanes) yielded MB-074-OMe as a white solid (60.6 mg, 125 µmol, 55%). MB-074-OMe ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.81 (t, 3H), 1.09 (d, 3H), 1.18 (m, 2H), 1.56 (m, 2H), 1.92 (m, 2H), 2.05 (m, 2H), 2.31 (m, 2H), 2.65-2.82 (dm, 2H), 2.83 (s, 2H), 3. 19 (s, 1H), 3.42 (m, 1H), 3.58 (m, 1H), 3. 77 (m, 2H), 3.78 (s, 3H), 4. 42 (m, 1H), 4.62 (t, 1H), 6. 36 (t, 1H), 6.79 (d, 2H), 6.99 (d, 2H).

Synthesis of CA-074b-OMe. CA-074b-OMe was synthesized exactly as described for MB-074-OMe except epoxy acid VII was used instead of epoxy acid VI. CA-074b-OMe 1H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 0.75 (m, 6H), 1.12 (m, 3H), 1.19 (m, 2H), 1.58-1.70 (dm, 1H), 1.85 (m, 1H), 2.16 (m, 2H), 2.34 (m, 2H), 2.82-2.95 (dd, 2H), 3.16 (m, 2H), 3.38 (s, 1H), 3.58 (s, 1H), 3.63–3.92 (dm, 2H), 3.76 (s, 3H), 4. 42 (m, 1H), 4.62 (t, 1H).

Synthesis of MB-074 and CA-074b. MB-074 and CA-074b were synthesized from MB-074-OMe and CA-074b by hydrolysis of the corresponding ester. Ester hydrolysis was achieved by dissolving each compound in a minimal volume of ethanol followed by addition of 1 equivalent of sodium hydroxide as a 1N aqueous solution. The resulting reaction was stirred for 2 h at room temperature or until the reaction was judged complete by TLC analysis. The reaction mixture was concentrated to dryness by rotary evaporation and the residue re-dissolved in a minimal amount of methanol. Ice cold diethyl ether was added and the product sodium salt was collected by centrifugation and washed with two portions of diethyl ether. The inhibitors were deemed pure by HPLC analysis and used directly for all further experiments. MB-074: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.81 (t, 3H), 1.09 (d, 3H), 1.18 (m, 2H), 1.56 (m, 2H), 1.92 (m, 2H), 2.05 (m, 2H), 2.31 (m, 2H), 2.75-2.92 (dm, 2H), 2.83 (s, 2H), 3. 19 (s, 1H), 3.42 (m, 1H), 3.58 (m, 1H), 3. 77 (m, 2H), 4.32 (t, 1H)4. 42 (m, 1H), , 6. 36 (t, 1H), 6.79 (d, 2H), 6.99 (d, 2H). CA-074b 1H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 0.75 (m, 6H), 1.12 (m, 3H), 1.19 (m, 2H), 1.58-1.70 (dm, 1H), 1.85 (m, 1H), 2.16 (m, 2H), 2.34 (m, 2H), 2.82–2.95 (dd, 2H), 3.16 (m, 2H), 3.38 (s, 1H), 3.58 (s, 1H), 3.63–3.92 (dm, 2H), 4. 42 (m, 1H), 4.62 (t, 1H).

Figure S1



Synthesis of the peptide epoxides CA-074b-OMe, MB-074-OMe, CA-074b and MB-074.