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Supplementary information

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M24B aminopeptidase inhibitors selectively activate the CARD8 inflammasome

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Supplementary Table 1. IC_{50} values (nM) for the indicated enzymes and inhibitors.

	NPEPPS	ANPEP	RNPEP	LTA4H	PEPD	DPP9
Bestatin	<10	603	<10	89	>100,000	>100,000
Me-Bs	<10	913	61	49	>100,000	>100,000
CHR 2797	<10	<10	>75,000	1,791	>100,000	>100,000
Batimastat	<10	<10	>75,000	>75,000	>100,000	>100,000
CQ04	>100,000	>100,000	>100,000	>50,000	160	>100,000
CQ31	>100,000	>100,000	>100,000	>100,000	675	>100,000
VbP	>100,000	>100,000	>100,000	>100,000	>100,000	<10

Supplementary Table 2. IC_{50} values (nM) for the indicated inhibitors against M24A and M24B aminopeptidases.

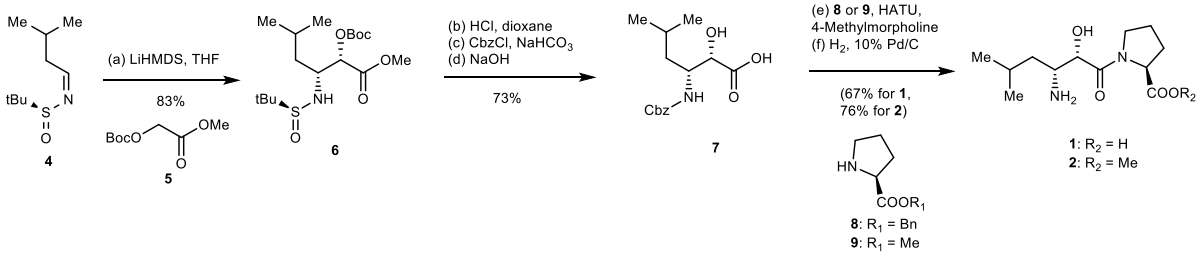
	METAP2	XPNPEP1	XPNPEP3	PEPD
Bestatin	>100,000	>75,000	>100,000	>100,000
Me-Bs	>100,000	>75,000	>100,000	>100,000
CHR 2797	>100,000	>75,000	31,410	>100,000
Batimastat	>100,000	>75,000	>100,000	>100,000
CQ04	>100,000	12,530	8,660	160
CQ31	>100,000	>100,000	54,410	675
VbP	>100,000	>100,000	>100,000	>100,000

Supplementary note.

Experimental Procedures and Spectroscopic Data of Compounds

General Procedures. All reactions were carried out under an argon atmosphere with dry solvents under anhydrous conditions, unless otherwise noted. Reagents were purchased from Aldrich, Acros, or Fisher at the highest commercial quality and used without further purification, unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC) carried out on MilliporeSigma glass TLC plates (silica gel 60 coated with F_{254}, 250 μm) using UV light for visualization and aqueous ammonium cerium nitrate/ammonium molybdate or basic aqueous potassium permanganate as developing agent. NMR spectra were recorded on a Bruker Avance III 600 MHz. The spectra were calibrated by using residual undeuterated solvents (for ^{1}H NMR) and deuterated solvents (for ^{13}C NMR) as internal references: undeuterated chloroform (δ_H = 7.26 ppm) and $CDCl_3$ (δ_C = 77.16 ppm); undeuterated methanol (δ_H = 3.31 ppm) and methanol-d_4 (δ_C = 49.00 ppm). The following abbreviations are used to designate multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. High-resolution mass spectra (HRMS) were recorded on a Waters Micromass LCT Premier XE TOF LC-MS.

Scheme S1. Syntheses of CQ04 (**1**) and CQ31 (**2**)



Methyl (2S,3R)-2-((tert-butoxycarbonyl)oxy)-3-(((S)-tert-butylsulfinyl)amino)-5-methylhexanoate (6): A solution of methyl 2-((tert-butoxycarbonyl)oxy)acetate **5** (5.02 g, 26.4 mmol) in dry THF (60 mL) maintained under an atmosphere of argon was cooled to –78 °C and then treated with LiHMDS (26.4 mL, 1.0 M solution in THF, 26.4 mmol). The reaction mixture was stirred for 1 h at the same temperature before imine **4** (1.00 g, 5.28 mmol) in THF (5 mL) was added slowly. The mixture was allowed to stir for 5 h before it was quenched with saturated aq. NH_4Cl (50 mL). The aqueous phase was extracted with

EtOAc (3 × 100 mL). The combined organic phases were washed with brine (100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The residue was passed through a short plug of silica gel with EtOAc/hexane (1:4) to give the desired methyl ester **6** (1.65 g, 82%) as a white solid. **6**: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 5.32 (s, 1 H), 4.26 (dd, *J* = 11.2, 5.3 Hz, 1 H), 3.91 (ddd, *J* = 11.2, 5.3, 3.7 Hz, 1 H), 3.71 (s, 3 H), 2.56 (t, *J* = 11.3 Hz, 1 H), 1.71 (ddd, *J* = 9.3, 6.5, 2.7 Hz, 1 H), 1.50 (s, 9 H), 1.31 – 1.23 (m, 1 H), 1.19 (s, 9 H), 0.94 (d, *J* = 6.6 Hz, 3 H), 0.91 (d, *J* = 6.6 Hz, 3 H) ppm; ^{13}C NMR (151 MHz, $CDCl_3$): δ = 173.4, 155.9, 84.2, 72.2, 60.4, 52.1, 49.2, 38.3, 28.3, 25.0, 24.1, 23.1, 21.1 ppm; HRMS (*m/z*): $[M+Na]^+$ calcd for $C_{17}H_{33}NO_6SNa^+$ 402.1926, found 402.1928.

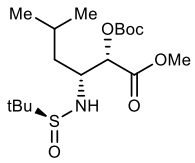
(2S,3R)-3-(((benzyloxy)carbonyl)amino)-2-hydroxy-5-methylhexanoic acid (7): To the solution of methyl ester **6** (1.20 g, 3.16 mmol) in dry CH_2Cl_2 (10 mL) was added HCl (10.0 mL, 4.0 M solution in 1,4-dioxane, 10.0 mmol) at 0 °C. The reaction mixture was warmed 22 °C and stirred for 12 h at the same temperature. The mixture was concentrated under vacuum to give a white solid which was used for the next step without further purifications. To a solution of crude solid from the last step in THF (30 mL) were sequentially added saturated aq. $NaHCO_3$ (10 mL) and CbzCl (0.90 mL, 6.30 mmol, 2.0 equiv) at 0 °C. The mixture was stirred for 2 h at the same temperature. The aqueous phase was extracted with EtOAc (3 × 30 mL). The combined organic phases were washed with brine (100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The residue was passed through a short plug of silica gel with EtOAc/hexane (1:1) to give the selectively protected amine as a white solid. This solid was dissolved in 1,4-dioxane/H_2O (1:1, 75 mL). To the stirred solution was added NaOH (152 mg, 3.80 mmol) and the reaction mixture was stirred at 22 °C for 1 h. The mixture was acidified to pH 3-4 with Dowex® 50W X8 resin. The resin was filtered and washed with CH_2Cl_2. The aqueous phase was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic phases were washed with brine (100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum. The residue was passed through a short plug of silica gel with CH_3OH/CH_2Cl_2 (1:10) to give the desired carboxylic acid **7** (680 mg, 73% for 3 steps) as a white solid. **7**: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.38 – 7.27 (m, 5 H), 5.20 (d, *J* = 10.0 Hz, 1 H), 5.16 (d, *J* = 12.3 Hz, 1 H), 5.07 (d, *J* = 12.3 Hz, 1 H), 4.25 (ddd, *J* = 9.9, 5.3, 1.8 Hz, 1 H), 4.19 – 4.10 (m, 1 H), 1.69 – 1.51 (m, 2 H), 1.42 (ddd, *J* = 13.9, 8.5, 5.3 Hz, 1 H), 0.95 (d, *J*

= 6.7 Hz, 2.45 H), 0.94 (d, *J* = 6.7 Hz, 2.45 H), 0.87 (d, *J* = 6.7 Hz, 0.55 H), 0.84 (d, *J* = 6.7 Hz, 0.55 H) ppm; ^{13}C NMR (151 MHz, $CDCl_3$, more than 15 ^{13}C signals for compound **7** were observed due to the presence of different rotameric species): δ = 175.9, 175.1, 157.9, 157.1, 135.9, 135.8, 128.7, 128.4, 128.2, 128.1, 72.1, 71.6, 67.8, 67.7, 52.4, 51.8, 41. 3, 40.9, 24.9, 24.7, 23.1, 22.8, 22.3, 22.1 ppm; HRMS (*m/z*): $[M+Na]^+$ calcd for $C_{15}H_{21}NO_5Na^+$ 318.1317, found 318.1321.

((2S,3R)-3-amino-2-hydroxy-5-methylhexanoyl)-L-proline (1): To a solution of carboxylic acid **7** (85.0 mg, 0.288 mmol) in CH_2Cl_2 (5 mL) were sequentially added L-Proline benzyl ester hydrochloride **8** (83.5 mg, 0.345 mmol, 1.2 equiv), HATU (131 mg, 0.344 mmol, 1.2 equiv), 4-Methylmorpholine (72.7 mg, 80 uL, 0.764 mmol, 2.5 equiv) at 0 °C. The reaction mixture was allowed to stir for another 6 h before it was quenched by addition of saturated aq. $NaHCO_3$ solution (5 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were combined, washed with brine (10 mL), dried over Na_2SO_4, and concentrated under vacuum. The resulting residue was purified by flash column chromatography (silica gel, EtOAc:hexane = 1:4, *v*/*v* → 1:1, *v*/*v*) to give the desired amide as a colorless oil. To a stirred solution of the obtained oil in MeOH (2 mL) was added Pd/C (30.6 mg, 0.0288 mmol, 10 wt%) at 22 °C. The resultant mixture was stirred under H_2 (1 atm) at that temperature for 2 h before it was diluted with EtOAc (10 mL) and passed through a plug of Celite. The volatile was removed under vacuum, and the residue was purified by recrystallization from MeOH/diethyl ether to give **1** (46.9 mg, 67% for 2 steps) as a white solid. **1**: ^{1}H NMR (600 MHz, methanol-d_4): δ = 4.59 (dd, *J* = 8.3, 2.9 Hz, 0.29 H), 4.53 (d, *J* = 2.5 Hz, 0.71 H), 4.38 (dd, *J* = 8.3, 5.0 Hz, 0.71 H), 4.27 (d, *J* = 3.5 Hz, 0.29 H), 3.80 – 3.73 (m, 0.71 H), 3.65 – 3.57 (m, 2 H), 3.55 – 3.49 (m, 0.29 H), 2.30 – 2.16 (m, 1.29 H), 2.10 – 2.00 (m, 0.71 H), 2.00 – 1.70 (m, 3.71 H), 1.60 – 1.46 (m, 1.29 H), 1.02 (d, *J* = 4.6 Hz, 2.13 H), 1.01 (d, *J* = 4.7 Hz, 2.13 H), 0.98 (d, *J* = 4.9 Hz, 0.87 H), 0.97 (d, *J* = 4.9 Hz, 0.87 H) ppm; ^{13}C NMR (151 MHz, methanol-d_4, more than 12 ^{13}C signals for compound **1** were observed due to the presence of different rotameric species): δ = 179.3, 178.7, 172.3, 171.5, 70.0, 68.2, 63.7, 63.0, 53.0, 52.6, 48.5, 48.2, 39.9, 39.4, 32.8, 30.5, 26.1, 25.3, 25.2, 23.2, 23.03, 23.02, 22.6, 22.5 ppm; HRMS (*m/z*): $[M+H]^+$ calcd for $C_{12}H_{23}N_2O_4^+$ 259.1658, found 259.1669.

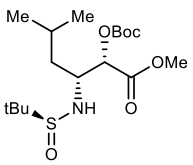
Methyl ((2S,3R)-3-amino-2-hydroxy-5-methylhexanoyl)-L-prolinate (2): To a solution of carboxylic acid **7** (180 mg, 0.601 mmol) in CH_2Cl_2 (10 mL) were sequentially added L-Proline methyl ester hydrochloride **9** (120 mg, 0.724 mmol, 1.2 equiv), HATU (274 mg, 0.721 mmol, 1.2 equiv), 4-Methylmorpholine (152 mg, 165 uL, 1.50 mmol, 2.5 equiv) at 0 °C. The reaction mixture was allowed to stir for another 4 h before it was quenched by addition of saturated aqueous $NaHCO_3$ solution (5 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were combined, washed with brine (10 mL), dried over Na_2SO_4, and concentrated under vacuum. The resulting residue was purified by flash column chromatography (silica gel, EtOAc:hexanes = 1:4, *v*/*v* → 1:1, *v*/*v*) to give the desired amide as a colorless oil. To a stirred solution of the obtained oil in MeOH (2 mL) was added sequentially AcOH (100 uL) and Pd/C (63.8 mg, 0.060 mmol, 10 wt%) at 22 °C. The resultant mixture was stirred under H_2 (1 atm) at that temperature for 2 h before it was diluted with EtOAc (10 mL) and passed through a plug of Celite. To the volatile was added HCl (400 uL, 2.0 M in Et_2O, 0.8 mmol) and the solvent was removed under vacuum. The residue was purified by recrystallization from MeOH/diethyl ether to give **2** (133 mg, 72% for 2 steps) as a white solid. **2**: ^{1}H NMR (600 MHz, methanol-d_4): δ = 4.80 (br.d, *J* = 7.4 Hz, 0.15 H), 4.49 (dd, *J* = 8.7, 4.6 Hz, 0.85 H), 4.46 – 4.43 (m, 1 H), 3.88 – 3.81 (m, 1 H), 3.74 / 3.73 (s, 3 H), 3.72 – 3.67 (m, 0.85 H), 3.67 – 3.55 (m, 0.30 H), 3.53 – 3.47 (m, 0.85 H), 2.34–2.25 (m, 1 H), 2.18 – 2.12 (m, 0.15 H), 2.11 – 1.95 (m, 2.85 H), 1.83 – 1.69 (m, 1 H), 1.63 – 1.54 (m, 2 H),, 1.03 (d, *J* = 6.4 Hz, 0.45 H), 1.02 (d, *J* = 6.2 Hz, 0.45 H), 1.00 (d, *J* = 6.5 Hz, 2.55 H), 0.98 (d, *J* = 6.3 Hz, 2.55 H) ppm; ^{13}C NMR (151 MHz, methanol-d_4, more than 13 ^{13}C signals for compound **2** were observed due to the presence of different rotameric species): δ = 175.10, 174.06, 171.6, 169.5, 70.6, 69.2, 61.5, 60.6, 53.10, 53.07, 53.0, 52.7, 48.6, 39.4, 39.2, 30.7, 30.0, 25.95, 25.90, 25.18, 25.15, 23.2, 22.8, 22.7, 22.2 ppm; HRMS (*m/z*): $[M+H]^+$ calcd for $C_{13}H_{25}N_2O_4^+$ 273.1814, found 273.1811.

^{1}H NMR Spectrum of 6 (600 MHz, $CDCl_3$)



6

^{13}C NMR Spectrum of 6 (151 MHz, $CDCl_3$)



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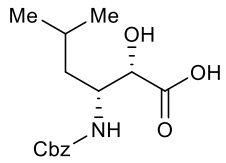
^{1}H NMR Spectrum of 7 (600 MHz, $CDCl_3$)



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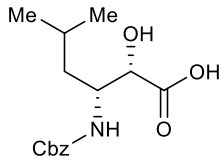
^{13}C NMR Spectrum of 7 (151 MHz, $CDCl_3$)



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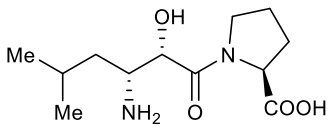
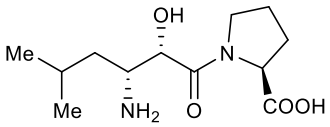
^{1}H NMR Spectrum of 1 (600 MHz, methanol-d_4)



1

^{13}C NMR Spectrum of 1 (151 MHz, methanol-d_4)



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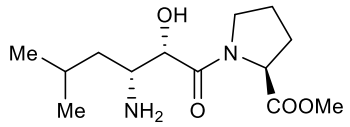
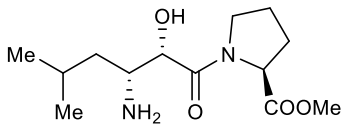
^{1}H NMR Spectrum of 2 (600 MHz, methanol-d_4)



2

^{13}C NMR Spectrum of 2 (600 MHz, methanol-d_4)



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