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Electronic Supporting Information for:

Probing for improved selectivity with dipeptide-derived inhibitors of dipeptidyl peptidases 8 and 9: the impact of P1-variation.

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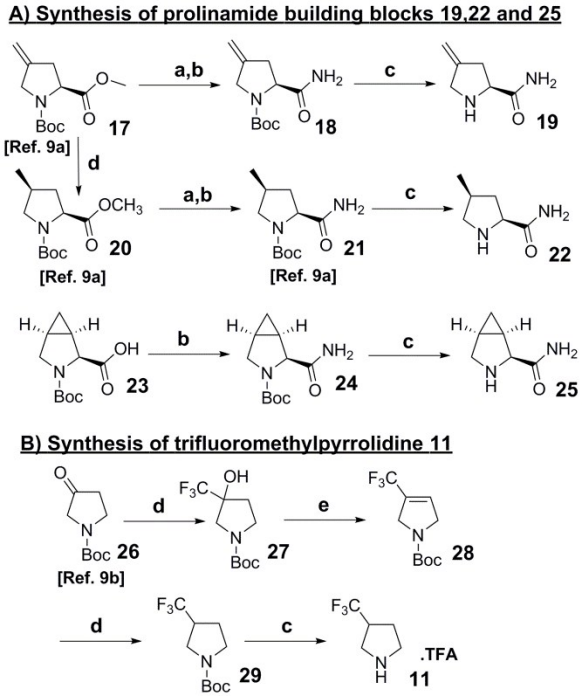
1 Synthesis of P1-building blocks not reported in literature

1.1 General data

Reagents were used from Sigma-Aldrich, Acros Organics, Apollo Scientific, TCI Europe, or Fluorochem and were used without further purification, unless otherwise mentioned.
Characterization of all compounds was done by ^{1}H and ^{13}C NMR spectroscopy and mass spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a 400 mHz Bruker Avance® III Ultrashield nanobay spectrometer. Chemical shifts are rendered in parts per million (ppm), coupling constants are in Hertz (Hz). Electrospray Ionisation (ESI) mass spectra were obtained from an Esquire™ 3000^{plus} iontrap mass spectrometer from Bruker Daltonics®.
LC-MS spectra were recorded on an Agilent 1100 Series HPLC system using an Alltech Prevail C18 column (2.1 × 50 mm, 3 µm) coupled with an Esquire 3000plus as MS detector and a 'method A' 5–100% B, 20 min gradient was used with a flow rate from 0.2 mL/min. Formic acid 0.1% was added to solvents A and B. UPLC-MS data were obtained from a Waters Acquity H-Class UPLC-MS system coupled with a Waters TQD ESI mass spectrometer and Waters TUV detector. A Waters Acquity UPLC-MS BEH C18 1.7 µm particle size, 2.1 x 50 mm column was used. Eluents were water with 0.1% formic acid (A), and MeCN with 0.1% formic acid (B). Method I: 0.15 min 95% A, 5% B isocratic, then a gradient from 95% A, 5% B to 95% B, 5% A in 1.85 min, followed by 0.25 min 95% B, 5% A isocratic (0.350 mL/min), and then a 0.75 min linear gradient to 95%A and 5% B. The wavelength for UV detection was 254 nm unless otherwise mentioned. Method II: flow 0.4 mL/min, 0.25 min 95% A, 5% B, then in 4.75 min to 95% B, 5% A, then 0.25 min 95% B, 5% A, followed by 0.75 min 95% A, 5% B. The wavelength for UV detection was 214 nm. Finally, reversed phase HPLC chromatograms were recorded on a Gilson instrument equipped with an Ultra sphere ODS column (4.6 x 250 mm x5µm) and a UV detector. A 10-100% acetonitrile, 35 min gradient was used with a flow rate of 1 ml/min. 0.1% trifluoroacetic acid was added to both solvents. An indicated purity of 100% indicates that no other peaks in the chromatogram occur.
Purity of all final products was determined to be > 95%

1.2 Synthetic procedures and analytical data for intermediates and building blocks not reported in literature.

The synthesis of intermediates **19**, **22, 25 and 11** had not been reported in the literature before. An overview of their preparation can be found in **Scheme S-1** (identical to **Scheme 1** in the manuscript)



Scheme S-1: Synthesis of unreported intermediates and building blocks.

1.2.a Synthesis of prolinamide building blocks 19, 22 and 25

1.2.a.1 Synthesis of building block 19

Synthetic data for starting material **17** are described in C. Gardelli et al. *J. Med.Chem.* 2007, **50**; 4953-4975.

(S)-*tert*-butyl 2-carbamoyl-4-methylenepyrrolidine-1-carboxamide (18)

(*S*)-1-(*tert*-butoxycarbonyl)-4-methylenepyrrolidine-2-carboxylic acid **17** (0.25 g, 1.100 mmol) was dissolved in dichloromethane (10 mL). 1-hydroxypyrrolidine-2,5-dione (0.139 g, 1.210 mmol) and DCC (0.250 g, 1.210 mmol) were added while cooling the reaction mixture in an ice bath. After stirring for 30 minutes, to the cloudy solution was added 7 N ammonia in methanol (10 mL), cooled to 0 °C. The reaction mixture was stirred for another 20 minutes and evaporated under reduced pressure. The residue was suspended in ethyl acetate (20 ml), mixed with celite and filtered. The filtrate was washed with saturated aqueous sodium bicarbonate (2 times 20 ml), dried over sodium sulfate and evaporated under reduced pressure. The residue was purified by column chromatography (hexanes to EtOAc).

Yield: 76%

MS (ESI) *m/z* 249.0 $[M+Na]^+$, 475.1 $[2M+Na]^+$

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.42-6.11 (br m, 2H); 5.21-5.08 (m, 2H), 3.59-3.39(m, 2H), 2.83-2.78 (m, 1H), 2.14-2.06 (m, 2H), 1.47(s, 9H).

(S)-4-methylenepyrrolidine-2-carboxamide-2,2,2-trifluoroacetate (19)

(S)-*tert*-butyl 2-carbamoyl-4-methylenepyrrolidine-1-carboxamide **18** (0.15 g, 0.67 mmol) was dissolved in 5 mL of TFA/DCM (1:1) and the reaction mixture was stirred for 1 hour at room temperature. Volatiles were then evaporated under reduced pressure and the residue

was washed two times with dry diethyl ether. The desired compound was obtained as an off-white hygroscopic solid.
Yield: 84%
MS (ESI) *m/z* 253.0 $[2M+H]^+$, 275.3 $[2M+Na]^+$
^1H NMR (400 MHz, $CDCl_3$) δ 6.4-6.06 (br m, 2H); 5.27-5.02 (br m, 2H), 3.49-3.23(m, 2H), 2.8-2.74 (m, 1H), 2.12-2.01 (m, 2H).

1.2.a.3 Synthesis of building block 25

The required methanoproline starting material for this compound is commercially available from Acros Organics.

(1*R*,2*S*,5*S*)-*tert*-Butyl 2-carbamoyl-3-azabicyclo[3.1.0]hexane-3-carboxylate (24)
A solution of commercially available (1*R*,2*S*,5*S*)-3-(*tert*-butoxycarbonyl)-3-azabicyclo[3.1.0]hexane-2-carboxylic acid (**23**) (2.62 g, 11.51 mmol) in DCM (120 mL) was cooled in an ice-bath. HONSu (1.46 g, 12.66 mmol) and a solution of DCC (2.61 g, 12.66 mmol) in DCM (110 mL) were added to the previous solution at vigorous stirring. The reaction reaction mixture was stirred for 30 min. A 7*N* NH_3 solution in MeOH (3.29 mL, 23.02 mmol) was then added and stirring was continued overnight. Before rotavaporation of volatile components, one spoon of Celite was added to the reaction mixture. The remaining crude was suspended in cold EtOAc (100 mL) and filtrated over a Celite path. The Celite path was washed with additional portions of cold DCM. The combined organic filtrates were washed with a saturated aqueous $NaHCO_3$ solution, dried over Na_2SO_4, filtrated and evaporated under reduced pressure. Further purification was performed by Isolera column chromatography, using a gradient of 2% MeOH in EtOAC to 20% MeOH in EtOAc to obtain the amide as a white to pale yellow solid (1.74 g, 67%).

UPLC-MS R_t 1.22, *m/z* 227.27 $[M+H]^+$; 249.27 $[M+Na]^+$ (purity: 99%).

^1H NMR (MeOD-d_4, 400 MHz) δ 0.63-0.73 (m, 2H), 1.42 (s, 9H), 1.63-1.69 (m, 1H), 1.88-1.94 (m, 1H), 3.49-3.57 (m, 2H), 4.25-4.26 (m, 1H).

^{13}C NMR (MeOD-d_4, 100 MHz) δ 9.02, 16.56, 22.41, 28.51, 51.37, 62.94, 81.79, 156.77, 176.57

(1*R*,2*S*,5*S*)-3-Azabicyclo[3.1.0]hexane-2-carboxamide 2,2,2-trifluoroacetate (25)
To a solution of (1*R*,2*S*,5*S*)-*tert*-butyl 2-carbamoyl-3-azabicyclo[3.1.0]hexane-3-carboxylate (1.74 g, 7.70 mmol) in DCM (75 mL) was added TFA (11.44 mL, 154.00 mmol). The reaction mixture was stirred for 1h at rt upon completion of the reaction. The volatile compounds were co-evaporated with additional portions of hexane, the obtained crude material was then treated multiple times with diethyl ether to yield the title compound as white powder (1.29 g, 70%).

UPLC-MS R_t 0.20, *m/z* 127.16 $[M+H]^+$ (purity: 99%).

^1H NMR (MeOD-d_4, 400 MHz) δ 0.62-0.66 (m, 1H), 0.77-0.82 (m, 1H), 1.85-1.91 (m, 1H), 2.11-2.17 (m, 1H), 3.44-3.52 (m, 2H), 4.46-4.47 (m, 1H).

^{13}C NMR (MeOD-d_4, 100 MHz) δ 4.83, 17.08, 20.09, 48.48, 61.96, 170.17.

1.2.b Synthesis of 4-trifluoromethylpyrrolidine (11)

tert-butyl 3-hydroxy-3-(trifluoromethyl)pyrrolidine-1-carboxylate (27)

A solution of *tert*-butyl 3-oxopyrrolidine-1-carboxylate **26** (0.65 g, 3.51 mmol) in THF (5 ml) was cooled to 0 °C, then trimethyl(trifluoromethyl)silane (1.097 ml, 7.02 mmol) and tetrabutyl-ammonium fluoride trihydrate (0.111 g, 0.351 mmol) were added. The mixture was warmed to room temperature and stirred overnight. After 10 hours, 2 eq of tetrabutylammonium fluoride trihydrate (2.214 g, 7.02 mmol) were added and the reaction was stirred for 3 hours. The solution was concentrated in vacuo to a brown oil, taken up in 20 ml of water and extracted with DCM (3 x 20 ml) The organic layers were dried over Na_2SO_4 and evaporated to give of a viscous brown liquor. The crude product was purified using FCC (hexanes to EtoAc) yielding *tert*-butyl 3-hydroxy-3-(trifluoromethyl)pyrrolidine-1-carboxylate (0.72 g, 2.82 mmol) as yellow crystals.

Yield: 80%

MS (ESI) *m/z* 278.2 $[M+Na]^+$, 533.3 $[2M+Na]^+$

^1H NMR (400 MHz, $CDCl_3$) δ 3.62-3.49(m, 4H), 2.25-2.17(m, 1H), 2.04-1.99(m, 1H), 1.45(s, 9H).

tert-butyl 3-(trifluoromethyl)-2,5-dihydro-1H-pyrrole-1-carboxylate (28)

A mixture of *tert*-butyl 3-hydroxy-3-(trifluoromethyl)pyrrolidine-1-carboxylate (0.72 g, 2.82 mmol), pyridine (4 ml) and thionyl chloride (2.68 ml, 36.7 mmol) was refluxed under nitrogen for 20 minutes. Then H_2O was added (10 ml) to quench the reaction. The aqueous layer was extracted with Et_2O (3 x 10 ml). The combined organic phases were washed with 1 N HCl (20 ml), saturated aqueous $NaHCO_3$ (10 ml), water (10 ml), and brine and dried over anhydrous Na_2SO_4. After removal of the solvent in vacuo, the residue was purified by flash chromatography (hexane to hexane:ethyl acetate (1:1) to give *tert*-butyl 3-(trifluoromethyl)-2,5-dihydro-1H-pyrrole-1-carboxylate (0.4 g, 1.686 mmol) as yellow cristals

Yield: 60%

MS (ESI) *m/z* 260.1 $[M+Na]^+$, 497.0 $[2M+Na]^+$

^1H NMR (400 MHz, $CDCl_3$) δ 6.32-6.28(m, 1H), 4.29-4.25(m, 4H), 1.47(s, 9H).

8.22 _tert_-butyl 3-(trifluoromethyl)pyrrolidine-1-carboxylate (29)

In a 25 ml round-bottomed flask was *tert*-butyl 3-(trifluoromethyl)-2,5-dihydro-1*H*-pyrrole-1-carboxylate (0.38 g, 1.602 mmol) dissolved in MeOH (10 ml) to give a yellow solution. After flushing with N_2, Pd/C (0.034 g, 0.160 mmol) was added carefully. The reaction was flushed with N_2 before H_2 gas was added via a balloon. The reaction was stirred at room temperature overnight and subsequently filtered over a small celite path. Next, the crude product was purified using a manual column (hexanes to hexanes:EtOAc (1:1)).

Yield: 44.4%

MS (ESI) *m/z* 262.1 $[M+Na]^+$, 501.2 $[2M+Na]^+$

^1H NMR (400 MHz, $CDCl_3$) δ 3.59-3.39(m, 4H), 2.92-2.86(m, 1H), 2.14-2.06(m, 2H) 1.47(s, 9H).

2) Analytical data for final products 4a-r

Typical procedures for coupling of P1 and P2 building blocks and the subsequent Boc-deprotection leading to final compounds can be found in, e.g., S. Van Goethem et al., *J. Med. Chem., 2011*, **54**, 5737-5746.

(*2S,4S*)-1-((*S*)-2-amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-4-oxobutanoyl)-4-azidopyrrolidine-2-carbonitrile bis(2,2,2-trifluoroacetate) (4a)
MS (ESI) *m/z* 522.34 $[M+H]^+$
HPLC rt: 14.51min, purity 98%, LCMS rt: 12.3min, purity >95%
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.5 (br m, 4H), 7.08-7.02 (m, 4H), 4.89-4.7(m, 2H), 4.46-4.31(m, 2H), 3.99-3.70(m, 6H), 2.89-2.83(m, 4H), 2.61-2.50(m, 3H), 1.31-1.24(m, 1H).

(2*S*)-1-((*S*)-2-amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-4-oxobutanoyl)-4-methylpyrrolidine-2-carbonitrile bis(2,2,2-trifluoroacetate) (4b)
MS (ESI) *m/z* 496.0 $[M+H]^+$
HPLC rt: 14.99min, purity 99%
^{1}H NMR (400 MHz, MeOD) δ 7.68-7.65(m, 4H), 7.20-7.15(m, 4H), 5.23-5.21(m, 1H), 4.89-4.68(m, 1H), 4.61-4.51(m, 1H), 3.69-3.76(m, 5H), 3.20-3.05(m, 5H), 2.97-2.91(m, 1H), 2.63-2.55(m, 1H), 2.41-2.32(m, 1H), 1.93-1.86(m, 1H), 1.19-1.14(m, 3H).

(2*S*,3*R*)-2-amino-3-methyl-1-(3-methylpyrrolidin-1-yl)pentan-1-one 2,2,2-trifluoroacetate (4d)
MS (ESI) *m/z* 213.2 $[M+H]^+$, 425.2 $[2M+Na]^+$
HPLC rt: 12.49min, purity 99%
^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19-4.14(dd, 4Hz-26.8H, 1H), 3.78-3.61(m, 1H), 3.56-3.48(m, 1H), 3.44-3.26(m, 1H), 3.13-2.91(m, 1H), 2.18-2.01(m, 2H), 1.89-1.88(m, 1H), 1.64-1.26(m, 5H), 1.02-0.84(m, 9H)

1-((*S*)-2-amino-5-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-5-oxopentanoyl)-4-methylenepyrrolidine-2*S*-carbonitrile bis(2,2,2-trifluoroacetate) (4e)
MS (ESI) *m/z* 508.1 $[M+H]^+$
HPLC rt: 15.12min, purity 100% LC-MS rt: 12.3min, purity 89%
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59-7.53(m, 4H), 7.08-7.00(m, 4H), 5.23(s, 2H), 5.04-4.86(m, 2H), 4.35-4.17(m, 3H), 4.12-3.77(m, 4H), 3.00-2.81(m, 1H), 2.62-2.52(m, 2H), 2.24-2.15(m, 2H), 1.26(s, 1H).

(*S*)-2-amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-1-(3-methylenepyrrolidin-1-yl)butane-1,4-dione bis(2,2,2-trifluoroacetate) (4f)
MS (ESI) *m/z* 470.4 $[M+H]^+$
HPLC rt: 14.29min, purity 97.04%
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64-7.58(m, 4H), 7.14-7.08(m, 4H), 5.08(s, 1H), 5.03-5.00(m, 2H), 4.60-4.49(m, 1H), 4.17-4.14(m, 1H), 4.04-4.00(m, 1H), 3.92-3.88(m, 2H), 3.78-3.66(m, 2H), 3.55-3.47(m,

1H), 3.21-3.17(m, 3H), 3.05-3.03(m, 2H), 2.84-2.83(m, 2H), 2.70-2.62(m, 1H), 2.57-2.51(m, 1H), 1.65-1.76(m, 1H)

(2*S*,3*R*)-2-amino-3-methyl-1-(3-methylenepyrrolidin-1-yl)pentan-1-one 2,2,2-trifluoroacetate (4g)

MS (ESI) *m/z* 197.0 $[M+H]^+$

HPLC rt: 10.18min, purity 99%

^{1}H NMR (400 MHz, MeOD) δ 5.11-5.08(m, 1H), 4.85-4.73(m, 2H), 4.24-4.02(m, 1H), 3.78-3.53(m,3H), 2.74-2.63(m, 1H), 2.01-1.97(m, 2H), 1.57-1.50(m, 1H), 1.41-1.29(m, 1H), 1.04-0.89(m, 6H).

(*S*)-2-amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-1-(3-methylenepyrrolidin-1-yl)butane-1,4-dione bis(2,2,2-trifluoroacetate) (4h)

MS (ESI) *m/z* 485.4 $[M+H]^+$

HPLC rt: 15.80min, purity 100%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64-7.58(m, 4H), 7.11-7.08(m, 4H), 4.93(s, 1H), 4.58-4.94(m, 1H), 3.84-3.49(m, 5H), 3.13-2.78(m, 8H), 2.14-2.02(m, 2H), 1.62-1.58(m, 1H), 1.42-1.26(m, 3H), 0.95-0.90(m, 3H).

(2*S*,3*R*)-2-amino-3-methyl-1-(3-methylpyrrolidin-1-yl)pentan-1-one 2,2,2-trifluoroacetate (4i)

Yield: quantitative

MS (ESI) *m/z* 213.2 $[M+H]^+$, 425.2 $[2M+Na]^+$

HPLC rt: 12.49min, purity 100%

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19-4.14(dd, 4Hz-26.8H, 1H), 3.78-3.61(m, 1H), 3.56-3.48(m, 1H), 3.44-3.26(m, 1H), 3.13-2.91(m, 1H), 2.18-2.01(m, 2H), 1.89-1.88(m, 1H), 1.64-1.26(m, 5H), 1.02-0.84(m, 9H).

(2*S*)-2-amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-1-(3-(trifluoromethyl)pyrrolidin-1-yl)butane-1,4-dione dihydrochloride (4j)

MS (ESI) *m/z* 525.2 $[M+H]^+$, 547.2 $[M+Na]^+$

HPLC rt: 15.53min, purity 95.05%

^{1}H NMR (400 MHz, MeOD) δ 7.89-7.84(m, 4H), 7.24-7.20(m, 4H), 5.63(br s, 1H), 5.29-5.11(m, 1H), 4.08-3.99(m, 2H), 3.96-3.60(m, 4H), 3.51-3.48(m, 1H), 3.48-3.31(m, 4H), 3.21-3.19(m, 2H), 2.92-2.87(m, 1H), 2.82(s, 1H), 2.34-2.01(3*m, 2H).

(*S*)-2-Amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-1-(pyrrolo[3,4-*c*]pyrazol-5(2*H*,4*H*,6*H*)-yl)butane-1,4-dione 2,2,2-trifluoroacetate (4k)

The title compound was synthesized by dissolving the corresponding Boc-protected precursor (0.042 g, 0.071 mmol) in DCM/TFA (50:50, 5 mL). After 1 hour, the volatiles were evaporated and the residue was washed several times with diethyl ether. The title compound was obtained as a pale brown powder (0.035 g, 81%).

UPLC-MS R_t 1.36, *m/z* 495.54 $[M+H]^+$, 517.54 $[M+Na]^+$ (purity: 99%).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 2.48-2.51 (m, 4H), 2.85 (ddd, 1H, *J* = 17.3, *J*′ = 9.4, *J*′′ = 2.6), 3.20 (ddd, 1H, *J* = 17.4, *J*′ = 8.9, *J*′′ = 4.2), 3.56-3.67 (m, 4H), 4.47-4.49 (m, 1H), 4.52-4.59 (m, 1H), 4.55-4.56 (m, 1H), 4.65-4.68 (m, 1H), 4.79-4.82 (m, 2H), 7.06 (t, 4H, *J* = 8.7), 7.46-7.49 (m, 4H), 7.52 (m, 1H).

^{13}C NMR (MeOD-d_4, 100 MHz) δ 32.73, 41.95, 45.37, 46.76, 47.01, 52.45, 52.71, 75.32, 116.71, 116.92, 118.11, 118.84, 124.15, 131.05, 136.83, 162.56, 168.68, 168.95.

(2*S*,3*R*)-2-Amino-3-methyl-1-(pyrrolo[3,4-*c*]pyrazol-5(2*H*,4*H*,6*H*)-yl)pentan-1-one 2,2,2-trifluoroacetate (4l)

The title compound was synthesized by TFA-mediated acidolysis of the corresponding Boc-protected precursor (0.26 g, 0.80 mmol), using a similar procedure as for **4k**. The title compound was obtained as a pale brown powder (0.18 g, 67%).

UPLC-MS R_t 0.26, *m/z* 223.29 $[M+H]^+$ (purity: 99%).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 0.97-1.07 (m, 6H), 1.37-1.48 (m, 1H), 1.55-1.65 (m, 1H), 2.02-2.13 (m, 1H), 4.23-4.25 (m, 1H), 4.51-4.55 (m, 1H), 4.67-4.74 (m, 2H), 4.82-4.87 (m, 1H), 7.51 (s, 1H).

^{13}C NMR (MeOD-d_4, 100 MHz) δ 10.87, 12.50, 25.86, 35.61, 45.50, 45.78, 55.08, 116.80, 122.96, 152.21, 168.18.

(*S*)-2-Amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-1-(4*H*-pyrrolo[3,4-*c*]isoxazol-5(6*H*)-yl)butane-1,4-dione 2,2,2-trifluoroacetate (4m)

The title compound was synthesized by TFA-mediated acidolysis of the corresponding Boc-protected precursor (0.27 g, 0.46 mmol), using a similar procedure as for **4k**. The title compound was obtained as a pale brown powder (0.21 g, 76%). UPLC-MS R_t 1.37, *m/z* 496.52 $[M+H]^+$ (purity: 99%).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 2.53-2.66 (m, 4H), 2.84-2.91 (m, 1H), 3.16-3.24 (m, 1H), 3.56-3.62 (m, 2H), 3.65-3.73 (m, 2H), 4.52-4.79 (m, 4H), 4.82-4.83 (m, 1H), 4.91-5.01 (m, 1H), 7.08 (t, 4H, *J* = 8.7), 7.51 (dd, 4H, *J* = 8.5, *J*′ = 5.3), 8.47-8.49 (m, 1H).

^{13}C NMR (MeOD-d_4, 100 MHz) δ 34.20, *34.23*, 42.34, 45.24, 45.35, 45.75, 49.76, *50.08*, 52.43, 52.71, 75.29, 116.57, 116.79, 119.06, 119.51, *120.03*, 130.86, 130.94, 137.59, 152.28, *152.46*, 162.45, 162.88, 164.90, 166.25, 167.28, 168.54, 169.07.

(2*S*,3*R*)-2-Amino-3-methyl-1-(4*H*-pyrrolo[3,4-*c*]isoxazol-5(6*H*)-yl)pentan-1-one 2,2,2-trifluoroacetate (4n)

The title compound was synthesized by TFA-mediated acidolysis of the corresponding Boc-protected precursor (0.035 g, 0.11 mmol), using a similar procedure as for **4k**. The title compound was obtained as a pale brown, fine powder (0.028 g, 77%).

UPLC-MS R_t 1.52, *m/z* 224.7 $[M+H]^+$, *m/z* 446.9 $[2M+H]^+$ (purity: 99%).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 1.03-1.08 (m, 6H), 1.36-1.48 (m, 1H), 1.54-1.64 (m, 1H), 2.02-2.13 (m, 1H), 4.22 (t, 1H, *J* = 5.4), 4.52-4.83 (m, 3H), 4.95-4.99 (m, 1H), 8.48 (s, 1H).

^{13}C NMR (MeOD-d_4, 100 MHz) δ 12.01, 13.64, 27.07, 36.74, 45.18, 45.30, 56.42, 119.08, 120.07, 152.43, 166.28, 167.33, 169.46.

(*S*)-2-Amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-1-(2-methyl-4*H*-pyrrolo[3,4-*d*]thiazol-5(6*H*)-yl)butane-1,4-dione 2,2,2-trifluoroacetate (4o)

The title compound was synthesized by TFA-mediated acidolysis of the corresponding Boc-protected precursor (0.12 g, 0.20 mmol), using a similar procedure as for **4k**. The title compound was obtained as a pale brown, fine powder (0.088 g, 69%).

UPLC-MS R_t 1.53, *m/z* 526.61 $[M+H]^+$; 548.61 $[M+Na]^+$ (purity: 99%).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 2.55 (br s, 2H), 2.59 (br s, 2H), 2.73 (s, 3H), 2.86 (dd, 1H, *J* = 17.3, *J*′ = 9.0), 3.20 (dt, 1H, *J* = 17.3, *J*′ = 4.0), 3.59 (br s, 2H), 3.69 (br s, 2H), 4.54-4.56 (m, 1H), 4.58 (t, 1H, *J* = 4.0), 4.67-4.72 (m, 1H), 4.81 (s, 1H), 4.84 (s, 1H), 4.98 (s, 1H), 7.05-7.09 (m, 4H), 7.49-7.52 (m, 4H).
^{13}C NMR (MeOD-d_4, 100 MHz) δ 19.37, 34.10, 42.53, 45.93, 49.28, 49.71, 50.04, 52.44, 52.74, 75.31, 116.50, 116.72, 128.46, 130.82, 130.90, 154.33, 162.39, 164.83, 168.56, 168.74, 174.32.

(2*S*,3*R*)-2-Amino-3-methyl-1-(2-methyl-4*H*-pyrrolo[3,4-*d*]thiazol-5(6*H*)-yl)pentan-1-one 2,2,2-trifluoroacetate (4p)

The title compound was synthesized by TFA-mediated acidolysis of the corresponding Boc-protected precursor (0.16 g, 0.45 mmol), using a similar procedure as for **4k**. The title compound was obtained as a pale brown, fine powder (0.12 g, 70%).

UPLC-MS R_t 1.08, *m/z* 254.36 $[M+H]^+$; 276.36 $[M+Na]^+$ (purity: 99%).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 1.03-1.07 (m, 6H), 1.36-1.45 (m, 1H), 1.54-1.64 (m, 1H), 2.02-2.13 (m, 1H), 2.73 (s, 3H), 4.20-4.22 (m, 1H), 4.54-4.79 (m, 2H), 4.88-5.02 (m, 2H).
^{13}C NMR (MeOD-d_4, 100 MHz) δ 12.01, 13.74, 19.37, 27.07, 36.78, 49.82, 49.94, 56.60, 128.59, 154.26, 169.18, 174.23.

(1*R*,2*S*,5*S*)-3-((*S*)-2-Amino-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-4-oxo-butanoyl)-3-azabicyclo[3.1.0]hexane-2-carbonitrile 2,2,2-trifluoroacetate (4q)

The title compound was synthesized by TFA-mediated acidolysis of the corresponding Boc-protected precursor (0.17 g, 0.29 mmol), using a similar procedure as for **4k,** but involving the use of acetonitrile instead of dichloromethane as a solvent. The title compound was obtained as a pale brown powder (0.094 g, 54%).

UPLC-MS R_t 1.45, *m/z* 494.55 $[M+H]^+$; R_t 1.50, *m/z* 494.55 $[M+H]^+$ (purity: 99%).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 0.60-0.64 (m, 1H), 1.03-1.12 (m, 1H), 1.94-2.01 (m, 1H), 2.10-2.15 (m, 1H), 2.81-2.92 (m, 5H), 3.11 (dt, 1H, *J* = 17.6, *J*′ = 4.8), 3.67-3.81 (m, 4H), 3.84-3.94 (m, 2H), 4.44 (ddd, 1H, *J* = 17.0, *J*′ = 8.7, *J*′′ = 4.8), 4.81 (dd, 1H, *J* = 23.3 , *J*′ = 5.1), 4.96-5.00 (m, 1H), 7.11-7.16 (m, 4H), 7.58-7.62 (m, 4H).
^{13}C NMR (MeOD-d_4, 100 MHz) δ 11.38, 19.44, 20.23, 34.08, 41.26, 44.71, 50.03, 50.76, 51.88, 52.49, 52.65, 75.34, 116.98, 117.20, 118.09, 131.18, 131.26, 135.04, 135.33, 162.81, 165.31, 168.33, 170.24.

(1*R*,2*S*,5*S*)-3-((2*S*,3*R*)-2-Amino-3-methylpentanoyl)-3-azabicyclo[3.1.0]hexane-2-carbonitrile 2,2,2-trifluoroacetate (4r)

The title compound was synthesized by TFA-mediated acidolysis of the corresponding Boc-protected precursor (0.17 g, 0.529 mmol), using a similar procedure as for **4k,** but involving the use of acetonitrile instead of dichloromethane as a solvent. The title compound was obtained as a (dark brown syrup (0.18 g, 100%).

UPLC-MS R_t 0.19, *m/z* 222.30 $[M+H]^+$; 244.30 $[M+Na]^+$ (purity: 99%).

^{1}H NMR (MeOD-d_4, 400 MHz) δ 0.52-0.60 (m, 1H), 0.93-1.00 (m, 6H), 1.04-1.12 (m, 1H), 1.15-1.25 (m, 1H), 1.35-1.45 (m, 1H), 1.90-2.03 (m, 2H), 2.07-2.16 (m, 1H), 3.83-3.87 (m, 1H), 3.99-4.11 (m, 1H), 4.43-4.48 (m, 1H), 4.79-4.85 (m, 1H).
^{13}C NMR (MeOD-d_4, 100 MHz) δ 11.41, *11.44*, 11.86, *11.92*, *14.89*, 15.09, *19.45*, 19.50, 19.97, *20.18*, 26.55, *27.06*, *37.24*, 38.07, *51.41*, 51.51, *51.77*, 51.85, 57.22, *57.33*, *118.10*, 118.22, *172.69*, 172.97.

3. Enzymatic potency assays (DPP8, DPP9, DPP IV, DPP II, FAP)

3.1 Enzyme purification

-Recombinant human DPP8 was expressed and purified as described. (Chen, Y. S.; Chien, C. H.; Goparaju, C. M.; Hsu, J. T.; Liang, P. H.; Chen, X. Purification and characterization of human prolyl dipeptidase DPP8 in SF9 insect cells. *Prot. Exp. Purif.* **2004**, *35*, 142-146)
-DPP9 was purified from bovine testes as described by Dubois et al. (Dubois, V.; Lambeir, A. M.; Van der Veken, P.; Augustyns, K.; Creemers, J.; Chen, X.; Scharpe, S.; De Meester, I. Purification and characterization of dipeptidyl peptidase IV-like enzymes from bovine testes. *Front. Biosci.* **2008**, *13*, 3558–3568)
-DPP IV and DPPII were purified from human seminal plasma as described previously. (De Meester, I.; Vanhoof, G.; Lambeir, A.; Scharpe, S. *J. Immun. Methods* **1996**, *189*, 99-105 and Maes, M. B.; Lambeir, A. M.; Gilany, K.; Senten, K; Van der Veken, P; Leiting, B.; Augustyns, K.; Scharpe, S; De Meester, I. Kinetic investigation of human dipeptidyl peptidase II (DPPII)-mediated hydrolysis of dipeptide derivatives and its identification as quiescent cell proline dipeptidase (QPP)/dipeptidyl peptidase 7 (DPP 7). *Biochem. J.* **2005**, *386*, 315-324)
-Recombinant murine FAP was purified from the cultured supernatant of HEK293 human embryonic kidney cell line as described elsewhere (Cheng, J. et al. *Cancer Res.* **2002**, *62*, 4767-4722).

3.2 IC_{50}-determination for DPP8, DPP9, DPPIV and DPPII

Initial rates were determined kinetically in a final volume of 200 µl for 10 minutes at 37°C by measuring the initial velocities of pNA release (405 nm) from the substrate using a Spectrafluor Plus reader (Tecan Benelux). The chromogenic substrate Gly-Pro-*p*-nitroanilide (100 µmol/l)was used at pH 8.3 for DPP IV, Lys-Ala-*p*-nitroanilide (1 mmol/l) at pH 5.5 for DPP II and Ala-Pro-*p*-nitroanilide (300 µmol/l) at pH 7.4 for DPP8 and DPP9-activity measurement. The substrate concentrations were chosen around the Km value obtained under the assay conditions used. Buffer compositions were reported before. (Dubois, V.; Lambeir, A. M.; Van der Veken, P.; Augustyns, K.; Creemers, J.; Chen, X.; Scharpe, S.; De Meester, I. Purification and characterization of dipeptidyl peptidase IV-like enzymes from bovine testes. *Front. Biosci.* **2008**, *13*, 3558–3568)

3.3 IC_{50}-determination for FAP

Enzyme activities were determined kinetically in a final volume of 200 µl for 10 minutes at 37°C by measuring the initial velocities of pNA release (405 nm) from the substrate using a Spectramax plus microtiterplate reader (Molecular devices). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 µmol pNA from the substrate per minute under assay conditions. All measurements were carried out in duplicate. The IC_{50} value was defined as the inhibitor concentration which caused a 50% decrease of the activity under assay conditions.
The chromogenic substrate Ala-Pro-*p*-nitroanilide (2 mmol/l) was used at pH 7.4 for FAP activity measurement. The substrate concentrations were chosen around the Km value obtained under the

assay conditions used. Buffer compositions for the DPP assays were previously reported in the purification articles– vide supra. The FAP assay buffer consisted of 50 mM Tris pH7.4 containing 100 mmol/l NaCl and 0.1 mg/ml bovine serum albumin.

3.4 General protocol

Test compounds were dissolved and diluted in DMSO (final concentration of DMSO during assay was 5% v/v for DPP9, PREP and DPP II and <1% for DPP IV and FAP). Inhibitors were pre-incubated with the enzyme for 15 min at 37 °C before starting by the addition of substrate. The concentrations of enzyme and inhibitor during the preincubation were the double of the final concentrations during the initial rate measurement. All measurements were carried out in duplicate. The initial evaluation of compounds was carried out at 100 µmol/l, or in case of solubility limits, the highest concentration possible. If vi/vo (initial velocity in presence of inhibitor/velocity in presence of DMSO) was < 0.5, an IC_{50} value was determined experimentally using at least 8 different concentrations of inhibitor. For those compounds with IC_{50} values below 5 µmol/l for one of the enzymes, the analysis was repeated using a new stock of compound. Generally, independent measurements of IC_{50} differed less than 20% from each other. The IC_{50}-value was defined as the inhibitor concentration, which caused a 50% decrease of the activity under assay conditions. The total inhibitor concentration is represented by I0. IC_{50}-values were calculated with the GraFit software (GraFit Version 5, Leatherbarrow, R.J., Erithacus Software Ltd., Horley, U.K.) using eq (1).

$$\frac{v_i}{v_o} = \frac{1}{1+\left(\frac{Io}{IC_{50}}\right)^s} + background \qquad \text{eq. (1)}$$

where s is the slope factor and background represents the estimated minimal vi/vo value. The errors given in the tables represent standard errors of the fit unless otherwise specified.

4. Molecular docking of compounds 4c and 4h

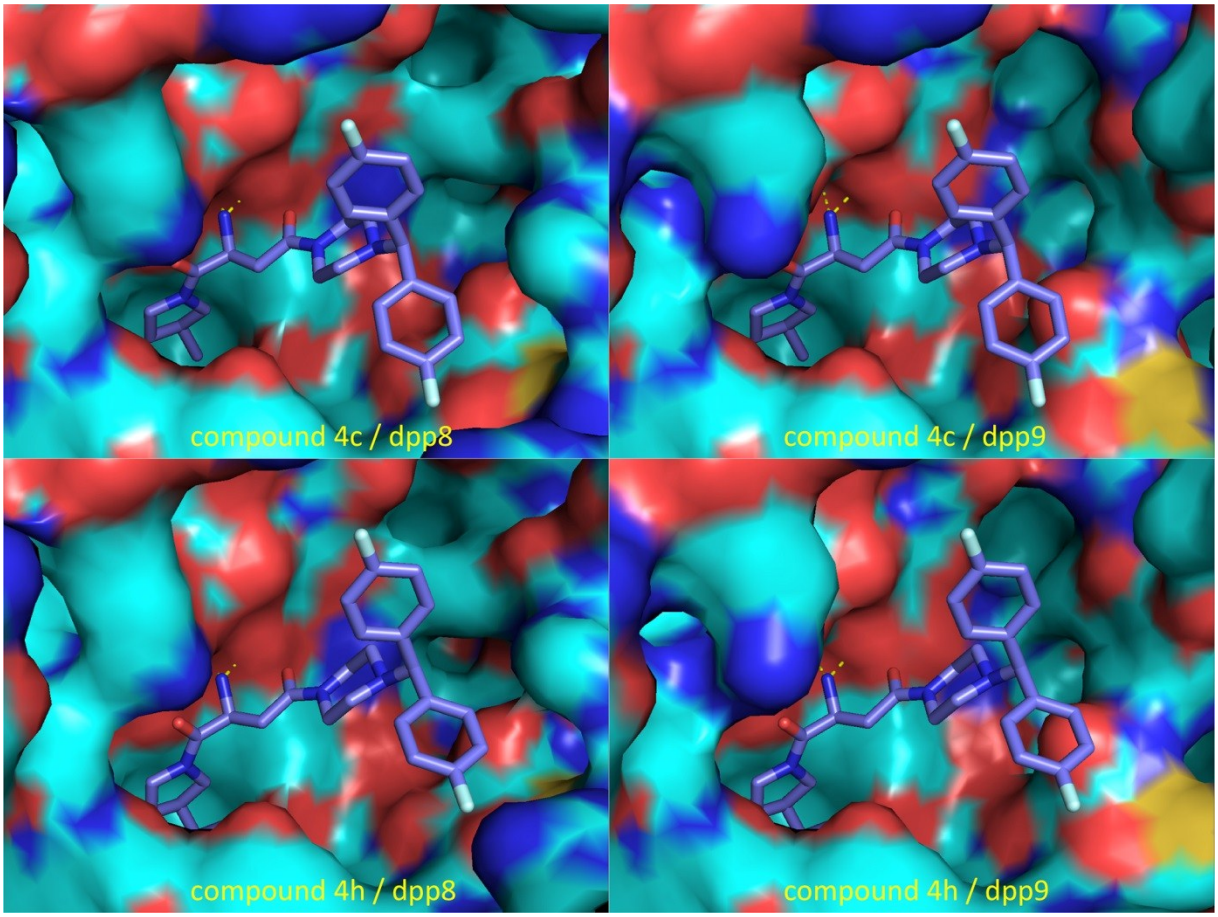


Figure S-1: Molecular docking of compounds 4c and 4h into the active centers of DPP8 and DPP9 (obtained via homology modeling by Rummey and Metz [reference S-1])

Docking of compounds **4c** and **4h** in the published homology models of both DPP8 and DPP9 [reference S-1] was performed by fitting the compounds onto the structurally related 2-amino-1-(pyrrolidin-1-yl)propan-1-one core of "compound 277" which has been co-crystallized in the structure of DPPIV (PDB-code 2OPH, [reference S-2]). For this purpose, the DPP8 and DPP9 structures were initially structure-aligned onto the corresponding structure of DPPIV. Side-chains of the compounds were manually rotated to fit within the available pockets, thereby taking care that the conformation of each compound stayed nearby its global energy minimum.

Rummey's work and this docking study also suggest that further improvement of DPP8 or DPP9 specificity could come from targeting the EE-helix at S209/A210 (the S2 loop). Although earlier work by us also roughly points in that direction, experimentally obtained, high-resolution structures of DPP 8 and DPP9 would be preferable to guide the design of specific inhibitors in a maximally reliable manner. [reference S-3]

References

[S-1] Rummey, C., Metz, G. (2007) 'Homology models of dipeptidyl peptidases 8 and 9 with a focus on loop predictions near the active site', *Proteins: Struct. Funct. Bioinf.* **66**, 160-171.

[S-2] Duffy, J.L., Kirk, B.A., Wang, L., Eiermann, G.J., He, H., Leiting, B., Lyons, K.A., Patel, R.A., Patel, S.B., Petrov, A., Scapin, G., Wu, J.K., Thornberry, N.A., Weber, A.E. (2007) '4-Aminophenylalanine and 4-aminocyclohexylalanine derivatives as potent, selective, and orally bioavailable inhibitors of dipeptidyl peptidase IV', *Bioorg. Med. Chem. Lett.* **17**: 2879-2885.

[S-3] Van Goethem, S.; Mateeussen V.; Joossens J.; Lambeir, A.M.; Chen, X.; De Meester, I.; Haemers, A.; Augustyns, K.; Van der Veken, P. Structure-Activity Relationship studies on isoindoline inhibitors of dipeptidyl peptidases 8 and 8 (DPP8, DPP9): is DPP8-selectivity attainable? *J. Med. Chem.* **2011**, *54*, 5737-5746.