Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications


# Supplementary Data

## Novel Selective Inhibitors of Neutral Endopeptidase: Discovery by Screening and Hit-to-Lead Optimisation

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# Chemistry procedures

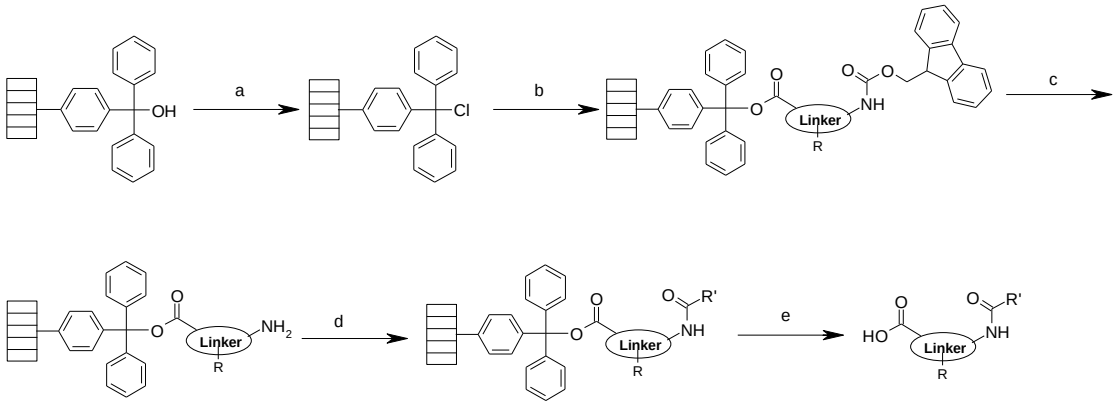
## General information

NMR spectra were recorded on a Bruker DRX-300 spectrometer. Chemical shifts are in parts per million (ppm). The assignments were made using one dimensional (1D) $^{1}H$ and $^{13}C$ spectra and two-dimensional (2D) HSQC, COSY and NOESY spectra. Mass spectra were recorded on a LCMS-MS triple-quadrupole system (Varian 1200ws) or a LCMS (Waters Alliance Micromass ZQ 2000). HPLC analysis were performed on a LCMS-Water Alliance system equipped with Waters 2695 pumps, Waters 2996 diode array, Waters 2747injector, using a $C_{18}$ TSK-GEL Super ODS 5 µm particle size column, dimensions 50 * 4.6 mm. A gradient starting from 100% $H_2O$/0.1% HCOOH and reaching 95% $CH_3CN$ /0.1% HCOOH within 7.5 min at a flow rate of 1 mL/min was used. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. All commercial reagents and solvents were used without further purification. Organic layers obtained after extraction of aqueous solutions were dried over $MgSO_4$ and filtered before evaporation *in vacuo*. Preparative HPLC were performed using a Varian PRoStar system using an OmniSphere 10 Column $C_{18}$ 250 x 41.4 mm Dynamax from Varian, Inc. Gradient starting from 20% $CH_3CN$/80% $H_2O$/0.1% formic acid and reaching 100% $CH_3CN$/0.1% formic acid at a flow rate of 80 mL/min or 20% MeOH/80% $H_2O$/ 0.1% formic acid reaching 100% MeOH/0.1% formic acid Purity (%) was determined by Reversed Phase HPLC, using UV detection (215 nM).

## Abbreviations

DIEA : diisopropylethylamine; DCM : dichloromethane ; TFA: trifluoroacetic acid ; DMF: dimethylformamide; DMSO : dimethylsulfoxide

## Supported Library design, synthesis and quality control




**Scheme 1. Reagents & Conditions:** (a) DCM, acetylchloride, 1 h. (b) HOOC-Linker-NH-Fmoc, DIEA, DCM/DMF (1/1), 24 h. (c) Piperidine, DMF, 30 min. (d) carboxylic acid, HBTU, HOBt, DCM/DMF (1/1) (e) TFA 5%/DCM, 1 h.

- **Selection of a diverse set of carboxylic acids R'COOH.**

Carboxylic acids were selected amongst a set of commercially available compounds (price < 80 € /g) from Maybridge™, Sigma-Aldrich-Fluka™, Acros™ and Matrix™ providers with a PipelinePilot™ protocol (**Figure S1**). Out of this set were manually selected 80 carboxylic acids to maximize diversity of : **i** MW, **ii** complexity, **iii** pharmacophoric pattern and **iv** groups in alpha of the carboxylic acid. Out of this set, a diverse set of 80 carboxylic acids was selected using clustering tools from PipelinePilot™.

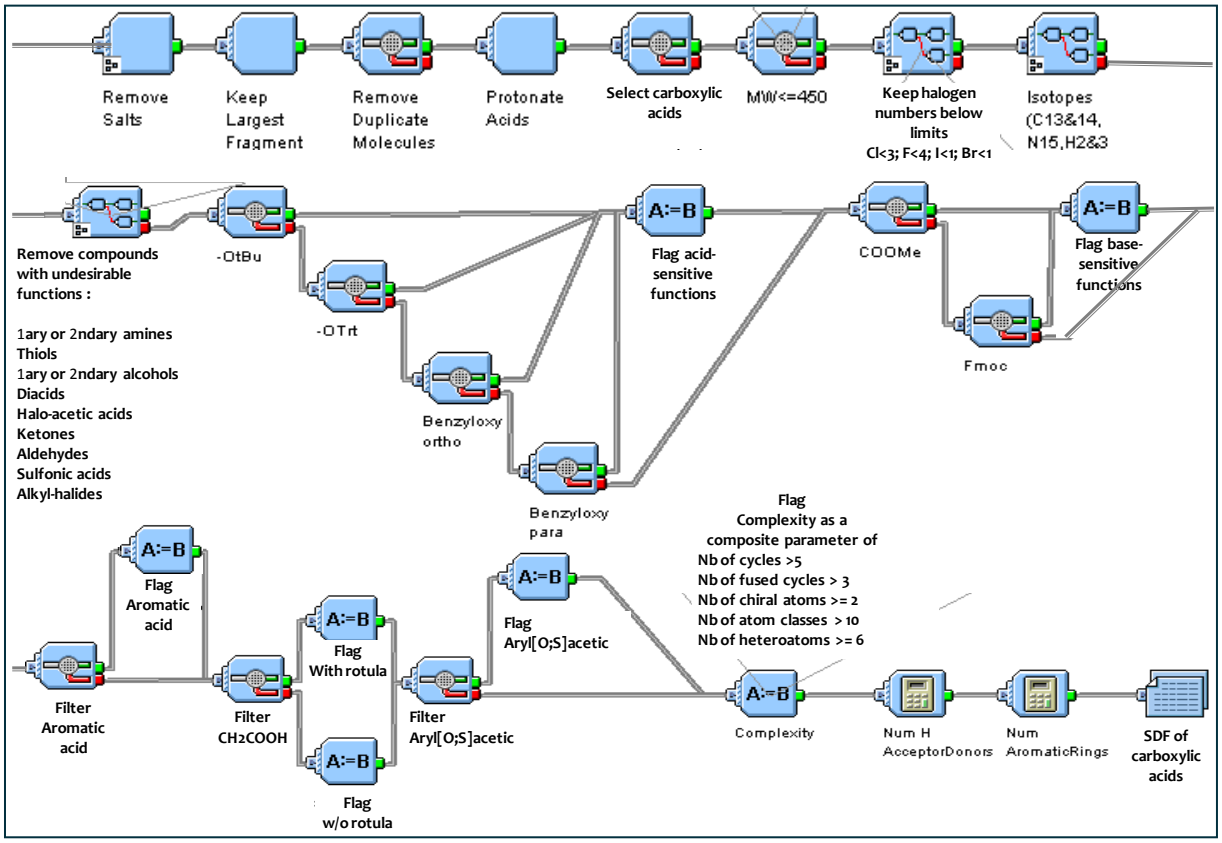



**Figure S1:** *In house* PipelinePilot™ protocol to sort and select a set of carboxylic acids.

- **Geometry of the library and plate format.**

The library format is 80 carboxylic acids * 5 precursors HOOC-Linker-NH2 = 400 final compounds stored as 2 aliquots of 15 µmol and 1 aliquot of 5 µmol in 2D-barcoded tubes in 96-well Matrix™ plates (column 1 and 12 free for controls).

- **Solid-support synthesis and quality controls .**

**Activation of lanterns**

Hydroxytrityl-functionnalized lanterns (PS-SynPhase Lanterns from Mimotopes™) are aliquoted in 5 batches of 85 lanterns in 250 mL round bottom flasks. Flasks are freezed then stored on a freeze-dryer for 24 h. Then, a solution of acetylchloride 10% in anh. DCM is added under argon and the flasks are stirred for 3 h.

**Loading of precursors HOOC-Linker-NH-Fmoc**

Each batch of lanterns is washed under argon with anh. DCM until complete removal of acetylchloride (at least 6 times). Then 3 eq of the corresponding HOOC-Linker-NH-Fmoc, 42 mL of anh. DMF, 42 mL of anh. DMF and 5.2 mL of DIEA are added. The 5 flasks are stirred overnight (Figure S2).
After 1 night, each batch of lanterns is washed 3 times with DMF and DCM. 1 lantern of each batch is used as a quality control of the loading step. The loading level is calculated from the quantity of fluorenyl-piperidine freed after the lantern is deprotected by 5 mL of a solution of 20% piperidine in DMF. After 30 min of stirring, 500 µL of this solution is diluted in 5 mL DMF and absorbance at 301 nm is measured to quantify fluorenyl-piperidine.

**Table S1:** Loading of each precursor on the resin

| Precursor | Loading |
|---|---|
| **A** (N-Fmoc-(Trt)-His) | 92 % |
| **B** (N-Fmoc-(Bz)-His) | 89 % |
| **C** (N-Fmoc-Serine) | 100 % |
| **D** (N-Fmoc-Gly) | 82 % |
| **E** (N-Fmoc-τ(isopentyl)His) | 85% |

In the 250-mL flask, the remaining 84 lanterns are reacted with a solution of piperidine 20% in DMF. The presence of the deprotected amine function is controlled by TNBS.

**Figure S2 :** Deprotection of Fmoc groups

**Acylation with the carboxylic acids**

Carboxylic acids are weighted in individual 8-mL tubes layout on a PP rack fitted for Tecan™ Genesis liquid-handler. Each carboxylic acid is given a specific position on the rack. Each lantern of each batch is then equipped with a transponder that is coded with the position in the rack and the linker.

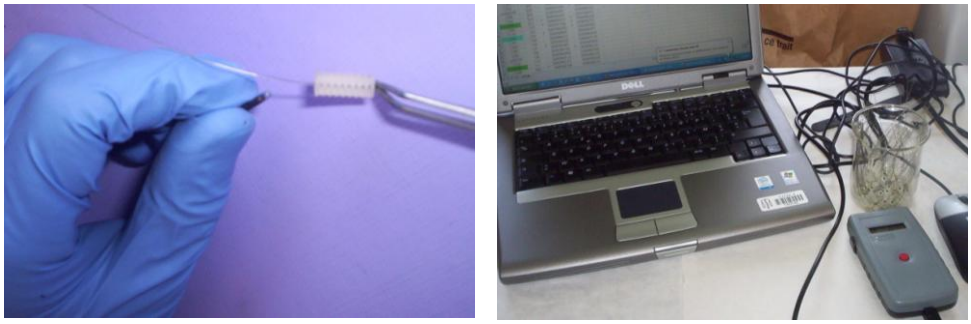


**Figure S3:** Insertion of the transpondors into lanterns and registering each lantern

In each reactor are introduced 5 lanterns (1 of each batch, loaded with a different –CO-Linker-NH2 amine). A solution of DMF containing 3.9 eq. of HOBt, 3.9 eq. of HBTU and 10 eq. of DIEA is added.

**Figure S4:** Distribution of the HOBt/TBTU solution in the 80 reactors.

The 80-position rack is stirred for one hour. After removal of solvents , the activation is reproduced. Lanterns are then washed with DMF, DMF/DCM (1/1) and DCM. Each lantern is then identified thanks to its transponder and placed in a 1.4 Matrix-2D-barcoded tube. Compounds are released from solid-support with a solution of TFA/TIS/DCM (50/5/45) during 1 h. Lanterns are removed and TFA solutions in plates are concentrated under reduced pressure on a Genevac™ evaporator.

**Quality control before reformatting in 15 µM batches.**

All matrix tubes were initially weighted to allow a yield calculation.

All compounds were checked by LCMS for both purity and identity.

### General procedure for amide synthesis (Procedure A).

To a solution of serine (1 equiv.), HOBt (1 equiv.), EDCI (1 equiv.) in DCM (5 mL/ mmol) were added succinic acid (1 equiv) and triethylamine (5 equiv). The resulting mixture was stirred from 2 to 14 h at room temperature. DCM was removed under reduced pressure and the residue solubilized in AcOEt. The organic layer was washed with 5% aqueous $NaHCO_3$ (3X), 0.1N aqueous $KHSO_4$ (3X), and saturated NaCl aqueous solution then dried over $MgSO_4$ and filtered before evaporation under reduced pressure.

### General procedure to remove t-butyl groups (Procedure B).

To a solution of **11a-d**, **14a-b**, **21a-b** or **26a-b** in DCM (1 mL/ mmol) was added TFA (1 mL/ mmol). The resulting mixture was stirred 2 h at room temperature. Solvent was removed under reduced pressure to give target compounds with good purity.

### General procedure for Urea synthesis (Procedure C).

A solution of *D*-leucine (1 equiv.) and carbodiimidazole (1.5 equiv.) in DMF (10 mL/ mmol) was stirred at rt. After 2 h a solution of (*S* or *R*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (1 equiv.) and DIEA (1 equiv.) in DMF (5 mL/ mmol) was added. The resulting mixture was stirred at room temperature for an additional 2 h. DMF was removed under reduced pressure and the resulting residue was solubilized in AcOEt and washed 3X with 1N HCl and satured NaCl solution. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure.

### General procedure for cleavage of methyl ester under neutral conditions (Procedure D).

**11a** or **11b** (200 mg, 0.52 mmol) and bis(tri-*n*-butyltin) oxide (450 µL, 1.03 mmol) were solubilized in 1.5 mL of toluene and irradiated for 2 h, at 110°C, under microwave. Toluene was removed under reduced pressure and the residue taken up in 5% aqueous KOH and washed with n-pentane (3X). The alkaline phase was acidified with 1N aqueous HCl (pH 3) and extracted with AcOEt (3X). The organic layer was then dried over $MgSO_4$, filtered and concentrated into a colorless oil which was purified by preparative LC-MS to give the desired product.

### General procedure for esterification with *t*-BuOH (Procedure E).

**23a** or **23b** (500 mg, 2.6 mmol) was solubilized in DCM/DMF (20 mL / 0.2 mL) and oxalyl chloride (250 µL, 2.6 mmol) was added dropwise. The resulting solution was stirred for 1 h at rt. *t*-BuOH (2 mL) was added and the mixture was stirred for an additional 12 h. solvents were distilled off and the residue was diluted with ethyl acetate and washed with saturated aqueous $NaHCO_3$ (3X) and saturated NaCl aqueous solution. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo* to give the target compound as an oil.

### General procedure for cleavage of methyl ester under alkaline conditions (Procedure F).

**24a** or **24b** was dissolved in MeOH and 2N NaOH (v/v). The mixture was stirred for 14 h at rt. MeOH was removed under reduced pressure and the residue was diluted in water, acidified with 1N HCl until

pH 3 and extracted with AcOEt (3X). This organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo to afford carboxylic acid **25a or 25b** , that was used directly in next step.

### General procedure for the synthesis of succinimid derivatives (Procedure G).

**11a** was solubilized in a mixture of 12 N aqueous HCl/HCOOH (40/60, v/v) and stirred for 48 h at room temperature. Solvents were evaporated to give pure succinimid derivative (**12a**) as a colorless oil.

**(*S*)-2-[((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid methyl ester (11a)** was prepared according to general procedure A, starting from (*S*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (254 mg, 1 mmol) and (*S*)-2-isobutylsuccimic acid 1-methyl ester (188 mg, 1 mmol) and was obtained as a colorless oil (256 mg, 66 %). $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.89-0.94 (2d, *J* = 6.3 Hz, 6H); 1.19 (s, 9H), 1.28-1.35 (m, 1H); 1.48 (s, 9H); 1.53-1.62 (m, 2H); 2.44 (dd, *J* = 15.0 Hz, *J* = 6.0 Hz; 1H); 2.61 (dd, *J* = 15.0 Hz, *J* = 8.4 Hz, 1H); 2.85-3.95 (m, 1H); 3.75 (dd, *J* = 8.7 Hz, *J* = 3.6 Hz, 1H); 3.7 (s, 3H); 3.75 (dd, *J* = 8.7 Hz, *J* = 3.6 Hz, 1H); 4.45 (t, *J* = 3.9 Hz, 1H). LC-MS (ESI+) m/z 388 (M+H)+.

**(*R*)-2-[((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid methyl ester (11b)** was prepared according to general procedure A, starting from (*S*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (254 mg, 1 mmol) and (*R*)-2-isobutylsuccimic acid 1-methyl ester (188 mg, 1 mmol) as a colorless oil (268 mg, 69%). $^1$H NMR ($CDCl_3$) $\delta$ (ppm) 0.89-0.94 (m, 6H); 1.15 (s, 9H); 1.24-1.33 (m, 1H); 1.46 (s, 9H); 1.53-1.62 (m, 2H); 2.37 (dd, *J* = 15.3 Hz, *J* = 5.1 Hz, 1H); 2.61 (dd, *J* = 15.3 Hz, *J* = 9.3 Hz, 1H); 2.93-3.00 (m, 1H); 3.50 (dd, *J* = 8.7 Hz, *J* = 2.7 Hz, 1H); 3.7 (s, 3H); 3.8 (dd, *J* = 8.7 Hz, *J* = 2.7 Hz, 1H); 4.59 (dd, *J* = 8.4 Hz, *J* = 3.0 Hz, 1H); 6.35 (d, *J* = 8.4 Hz, 1H). LC-MS (ESI+) m/z 388 $(M+H)^+$.

**(*S*)-2-[((*R*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid methyl ester (11c)** was prepared according to general procedure A, starting from (*R*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (254 mg, 1 mmol) and (*S*)-2-isobutylsuccimic acid 1-methyl ester (188 mg, 1 mmol) and was obtained as a colorless oil (250 mg, 64 %). $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.89-0.94 (2d, *J* = 6.3 Hz, 6H); 1.19 (s, 9H), 1.29-1.41 (m, 1H); 1.48 (s, 9H); 1.52-1.61 (m, 2H); 2.44 (dd, *J* = 15.3 Hz, *J* = 5.7 Hz, 1H); 2.60 (dd, *J* = 15.3 Hz, *J* = 8.7 Hz, 1H); 2.89-2.96 (m, 1H); 3.53 (dd, *J* = 9.0 Hz, *J* = 3.9 Hz, 1H); 3.7 (s, 3H); 3.75 (dd, *J* = 9.0 Hz, *J* = 3.9 Hz, 1H); 4.45 (t, *J* = 3.9 Hz, 1H). LC-MS (ESI+) m/z 388 (M+H)+.

**(*R*)-2-[((*R*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid methyl ester (11d)** was prepared according to general procedure A, starting from (*R*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (254 mg, 1 mmol) and (*R*)-2-isobutylsuccimic acid 1-methyl ester (188 mg, 1 mmol) as a colorless oil (252 mg, 65%). $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.89-0.94 (2d, *J* = 6.3 Hz, 6H); 1.19 (s, 9H); 1.25-1.35 (m, 1H); 1.48 (s, 9H); 1.52-1.60 (m, 2H); 2.44 (dd, *J* = 15.0 Hz, *J* = 6.1 Hz, 1H); 2.61 (dd, *J* =15.0 Hz, *J* = 8.4 Hz, 1H); 2.85-2.93 (m, 1H); 3.55 (dd, *J* = 8.7 Hz, *J* = 3.6 Hz, 1H); 3.68 (s, 3H); 3.75 (dd, *J* = 8.7 Hz, *J* = 3.6 Hz, 1H); 4.45 (t, *J* = 3.9 Hz, 1H). LC-MS (ESI+) m/z 388 $(M+H)^+$.

**(*S*)-2-[((*S*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid methyl ester (8a)** was prepared according to general procedure B, starting from **11a** (252 mg, 0.65 mmol) as a colorless oil (172 mg, 96%) ; Purity 97%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.89-0.94 (m, 6H); 1.29-1.37 (m, 1H); 1.52-1.62 (m, 2H); 2.45 (dd, *J* = 15.0 Hz, *J* = 5.1 Hz, 1H); 2.61 (dd, *J* = 15.0 Hz, *J* = 8.7 Hz, 1H); 2.86-3.00 (m, 1H); 3.67 (s, 3H); 3.79 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 3.89 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 4.47 (dd, *J* = 4.5 Hz, 1H). $^{13}$C RMN $\delta$ (ppm) 21.2, 25.9, 37.7, 40.0, 41.1, 52.2, 54.7, 61.5, 171.8, 172.3, 176.3. LC-MS (ESI+) m/z 274 (M+H)+.

**(*R*)-2-[((*S*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid methyl ester (8b)** was prepared according to general procedure B, starting from **11b** (141 mg, 0.36 mmol) as a colorless oil (45 mg, 45 %) ; Purity 97%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.89-0.94 (2d, *J* = 6.3 Hz, 6H);1.29-1.39 (m, 1H); 1.51-1.61 (m, 2H); 2.45 (dd, *J* = 15.0 Hz, *J* = 5.1 Hz, 1H); 2.61 (dd, *J* = 15.0 Hz, *J* = 8.7 Hz, 1H); 2.87-3.00 (m, 1H); 3.67 (s, 3H); 3.79 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 3.89 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 4.47 (t, *J* = 4.5 Hz, 1H). $^{13}$C RMN $\delta$ (ppm) 22.4, 27.1, 39.0, 41.2, 42.4, 52.2, 56.0, 62.9, 173.4, 173.8, 177.8. LC-MS (ESI+) m/z 274 (M+H)+.

**(*S*)-2-[((*R*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid methyl ester (8c)** was prepared according to general procedure B, starting from **11c** (252 mg, 0.65 mmol) as a colorless oil (152 mg, 85 %) ; Purity 97%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.89-0.94 (2d, *J* = 6.3 Hz, 6H); 1.29-1.39 (m, 1H); 1.51-1.63 (m, 2H); 2.45 (dd, *J* = 15.0 Hz, *J* = 5.1 Hz, 1H); 2.61 (dd, *J* = 15 Hz, *J* = 8.7 Hz, 1H); 2.86-3.00 (m, 1H); 3.67 (s, 3H); 3.79 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 3.89 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 4.47 (t, *J* = 4.5 Hz, 1H). $^{13}$C RMN $\delta$ (ppm) 21.0, 25.7, 37.6, 39.8, 40.9, 50.9, 54.6, 61.5, 172.0, 172.4, 176.4. LC-MS (ESI+) m/z 274 (M+H)+.

**(*R*)-2-[((*R*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid methyl ester (8d)** was prepared according to general procedure B, starting from **11d** (252 mg, 0.65 mmol) as a colorless oil ; Purity 97%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ ppm 0.89-0.94 (2d, *J* = 6.3 Hz, 6H); 1.30-1.37 (m, 1H); 1.52-1.62 (m, 2H); 2.45 (dd, *J* = 15.0 Hz, *J* = 5.1 Hz, 1H); 2.61 (dd, *J* = 15 Hz, *J* = 8.7 Hz); 2.87-2.93 (m, 1H); 3.67 (s, 3H); 3.79 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 3.89 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 4.47 (t, *J* = 4.5 Hz, 1H). $^{13}$C RMN $\delta$ (ppm) 21.0, 25.7, 37.6, 39.8, 40.9, 50.9, 54.6, 61.5, 172.0, 172.4, 176.4. LC-MS (ESI+) m/z 274 (M+H)+.

**(*S*)-3-Hydroxy-2-((*S*)-3-isobutyl-2,5-dioxo-pyrrolidin-1-yl)-propionic acid (12a)** was prepared according to general procedure G, from **11a** (25 mg, 0.06 mmol), as a colorless oil (14 mg, 62%). Purity 94%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ ppm 0.96 (d, *J* = 6.3 Hz, 3H); 0.99 (d, *J* =6.4 Hz, 3H); 1.44-1.50 (m, 1H); 1.73-1.79 (m, 2H); 2.39-2.43 (m, 1H); 2.84-2.94 (m, 2H); 4.02-4.17 (m, 2H); 4.64 (2d, *J* = 5.7 Hz, 1H). LC-MS (ESI+) m/z 244 (M+H)+.

**2-((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-4-methyl-pentanoic acid methyl ester (14a)** was prepared according to general procedure A, starting from (*S*)-(Ser(*t*-Bu)-O*t*-Bu), HCl

(348 mg, 1.37 mmol) and 2-isobutyl-malonic acid monomethyl ester (240 mg, 1.37 mmol) as a colorless oil (202 mg, 40 %). $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.92-0.96 (2d, $J$ = 3.3 Hz, 6H); 1.18 and 1.20 (2s, 9H), 1.48 and 1.49 (2s, 9H); 1.52-1.64 (m, 1H); 1.68-1.83 (m, 2H); 3.55-3.60 (m, 2H); 3.70 and 3.71 (2s, 3H); 3.74-3.82 (m, 1H); 4.47 (t, $J$ = 3.7 Hz, 1H). LC-MS (ESI+) m/z 374 (M+H)+.

**2-((*R*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-4-methyl-pentanoic acid methyl ester (14b)** was prepared according to general procedure A, starting from (*R*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (348 mg, 1.37 mmol) and 2-isobutyl-malonic acid monomethyl ester (240 mg, 1.37 mmol) as a colorless oil (230 mg, 45 %). $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.92-0.96 (2d, $J$ = 3.3 Hz, 6H); 1.20 and 1.21 (2s; 9H), 1.49 and 1.50 (2s, 9H); 1.56-1.67 (m, 1H); 1.72-1.80 (m, 2H); 3.55-3.61 (m, 2H); 3.72 and 3.73 (2s, 3H); 3.75-3.80 (m, 1H); 4.48 (t, $J$ = 3.9 Hz, 1H). LC-MS (ESI+) m/z 374 (M+H)+.

**2-((*S*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-4-methyl-pentanoic acid methyl ester (15a)** was prepared according to general procedure B, starting from **14a** (100 mg, 0.27 mmol) as a colorless oil; Purity 97%. $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.93-0.96 (2d, $J$ = 3.6 Hz, 6H); 1.52-1.69 (m, 1H); 1.74-1.83 (m, 2H); 3.54-3.60 (m, 1H); 3.71 and 3.72 (2s, 3H); 3.79-3.82 (m, 1H); 3.84-3.97 (m, 1H); 4.51 (t, $J$ = 4.4 Hz, 1H). LC-MS (ESI+) m/z 262 (M+H)+.

**2-((*R*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-4-methyl-pentanoic acid methyl ester (15b)** was prepared according to general procedure B, starting from **14b** (100 mg, 0.27 mmol) as a colorless oil (69 mg, 97 %) ; Purity 98%. $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.93-0.96 (2d, $J$ = 3.6 Hz, 6H); 1.52-1.69 (m, 1H); 1.74-1.83 (m, 2H); 3.54-3.60 (m, 1H); 3.71 and 3.72 (2s, 3H); 3.80-3.86 (m, 1H); 3.90-3.76 (m, 1H); 4.51 (t, $J$ = 4.4 Hz, 1H). LC-MS (ESI+) m/z 262 (M+H)+.

**(*S*)-2-[3-((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethyl)-ureido]-4-methyl-pentanoic acid methyl ester (17a)** was prepared according to general procedure C, from (*S*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (253 mg, 1 mmol) as a colorless oil (280 mg, 72%). $^1$H NMR ($CDCl_3$) $\delta$ (ppm) 0.90-0.94 (2d, $J$ = 3.0 Hz, 6H); 1.13 (s, 9H); 1.44 (s, 9H); 1.50-1.70 (m, 3H); 3.50 (dd, $J$ = 8.6 Hz, $J$ = 3.1 Hz, 1H); 3.71 (s, 3H); 3.74 (dd, $J$ = 8.6 Hz, $J$ = 3.1 Hz, 1H); 4.41-4.51(m, 2H); 5.20 (d, $J$ = 8.6 Hz, 1H); 5.43 (d, $J$ = 8.6 Hz, 1H). LC-MS (ESI+) m/z 389 (M+H)+.

**(*R*)-2-[3-((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethyl)-ureido]-4-methyl-pentanoic acid methyl ester (17b)** was prepared according to general procedure C, from (*R*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (253 mg, 1 mmol) as a colorless oil (264 mg, 68%). $^1$H NMR ($CDCl_3$) $\delta$ (ppm) 0.90-0.94 (2d, $J$ = 1.7 Hz, 6H); 1.11 (s, 9H);1.42 (s, 9H); 1.50-1.70 (m, 3H); 3.55 (dd, $J$ = 8.6 Hz, $J$ =3.0 Hz, 1H); 3.68 (s, 3H); 4.41-4.44(m, 2H); 5.43(d, $J$ = 8.2 Hz, 1H). 5.56 (d, $J$ = 8.2 Hz, 1H); LC-MS (ESI+) m/z 389 (M+H)+.

**(*S*)-2-[3-((*S*)-1-Carboxy-2-hydroxy-ethyl)-ureido]-4-methyl-pentanoic acid methyl ester (18a)** was prepared according to general procedure B, from **17a** (200 mg, 0.5 mmol), as a colorless oil (95 mg, 72%) ; Purity 95%. $^1$H NMR (MeOD-$d_4$) $\delta$ ppm 0.93-0.97 (2d, $J$ = 6.6 Hz, 6H); 1.52-1.58 (m, 2H); 1.72

(sept, *J* = 6.6 Hz, 1H); 3.71 (s, 3H); 3.78 (dd, *J* = 10.9 Hz, *J* = 3.7 Hz, 1H); 3.91 (dd, *J* = 11.1 Hz, *J* = 3.8 Hz, 1H); 4.29-4.35 (m, 2H). LC-MS (ESI+) m/z 277 (M+H)+.

**(*R*)-2-[3-((*S*)-1-Carboxy-2-hydroxy-ethyl)-ureido]-4-methyl-pentanoic acid methyl ester (18b)** was prepared according to general procedure B from **17b** (200 mg, 0.5 mmol) as a colorless oil (130 mg, 97%) ; Purity 98%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.93-0.97 (2d, *J* = 6.6 Hz, 6H); 1.52-1.58 (m, 2H); 1.75 (sept; *J* = 6.6 Hz, 1H); 3.71 (s, 3H); 3.78 (dd, *J* = 10.9 Hz, *J* = 3.7 Hz, 1H); 3.89 (dd, *J* = 11.1 Hz, *J* = 4.1 Hz, 1H); 4.27-4.34 (m, 2H). LC-MS (ESI+) m/z 277 (M+H)+.

**(*S*)-2-[((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid (21a)** was prepared according to general procedure D, from **11a,** as a colorless oil (92 mg, 47%). $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.91-0.95 (2d, *J* = 6.3 Hz, 6H); 1.19 (s, 9H); 1.25-1.34 (m, 1H); 1.48 (s, 9H); 1.60-1.73 (m, 2H); 2.44 (dd, *J* = 15.0 Hz, *J* = 6.6 Hz, 1H); 2.60 (dd, *J* = 15.0 Hz, *J* = 7.5 Hz, 1H); 2.81-2.91 (m, 1H); 3.56 (dd, *J* = 9.0 Hz, *J* = 3.9 Hz, 1H); 3.76 (dd, *J* = 9.0 Hz, *J* = 4.5 Hz, 1H); 4.47 (t, *J* = 3.9 Hz, 1H). LC-MS (ESI+) m/z 374 (M+H)+.

**(*R*)-2-[((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid (21b)** was prepared according to general procedure D, from **11b,** as a colorless oil (76 mg, 39%). $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.91-0.95 (2d, *J* = 6.3 Hz, 6H); 1.19 (s, 9H); 1.26-1.39 (m, 1H); 1.48 (s, 9H); 1.54-1.73 (m, 2H); 2.44 (dd, *J* = 15.0 Hz, *J* = 6.0 Hz, 1H); 2.60 (dd, *J* = 15.0 Hz, *J* = 8.1 Hz, 1H); 2.81-2.91 (m, 1H); 3.55 (dd, *J* = 9.0 Hz, *J* = 3.9 Hz, 1H); 3.76 (dd, *J* = 9.0 Hz, *J* = 3.9 Hz, 1H); 4.45 (t, *J* = 3.9 Hz, 1H). LC-MS (ESI+) m/z 374 (M+H)+.

**(*S*)-2-[((*S*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid (22a)** was prepared according to general procedure B, from **21a** (50 mg, 0.13 mmol), as a colorless oil after purification by preparative LC-MS (16 mg, 47%) ; Purity 99%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.91-0.95 (2d, *J* = 6.9 Hz, 6H); 1.22-1.31 (m, 1H); 1.54-1.71 (m, 2H); 2.38 (dd, *J* = 14.7 Hz, *J* = 6.3 Hz, 1H); 2.55 (dd, *J* = 14.7 Hz, *J* = 8.4 Hz, 1H); 2.76-2.81 (m, 1H); 3.80 (d, *J* = 4.5 Hz, 2H); 4.27 (t, *J* = 4.5 Hz, 1H). $^{13}$C RMN $\delta$ (ppm) 21.1, 21.9, 25.8, 38.2, 40.4, 41.0, 41.2, 56.1, 62.5, 172.3, 174.2, 178.4. LC $t_R$ = 1.77 min, MS (ESI+) m/z 262 (M+H)+.

**(*R*)-2-[((*S*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-methyl]-4-methyl-pentanoic acid (22b)** was prepared according to general procedure B, from **21b** (50 mg, 0.13 mmol), as a colorless oil after purification by preparative LC-MS (14 mg, 41%) ; Purity 99%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.91-0.95 (2d, 6H, *J* = 6.9 Hz); 1.23-1.32 (m, 1H); 1.54-1.71 (m, 2H); 2.35 (dd, *J* = 14.4 Hz, *J* = 5.7 Hz, 1H); 2.56 (dd, *J* = 14.4 Hz, *J* = 9.0 Hz, 1H); 2.77-2.83 (m, 1H); 3.80 (d, *J* = 4.5 Hz, 2H); 4.27 (t, *J* = 4.5 Hz, 1H). $^{13}$C RMN $\delta$ (ppm) 21.3, 21.8, 25.8, 38.7, 41.5, 41.5, 56.5, 62.7, 172.6, 175.0, 179.7. LC $t_R$ = 1.75 min, MS (ESI+) m/z 262 (M+H)+.

**(*S*)-4-*tert*-butyl 1-methyl 2-isobutylsuccinate (24a)** was prepared according to general procedure E, from **23a**, and used in the next step without further purification. $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.89-0.95 (2d, 6H, *J* = 6.0 Hz); 1.23-1.32 (m, 1H); 1.44 (s, 9H); 1.48-1.60 (m, 2H); 2.42 (dd, *J* = 16.2 Hz, *J* = 5.4 Hz, 1H); 2.52 (dd, *J* = 16.2 Hz, *J* = 9.3 Hz, 1H); 2.83-2.87 (m, 1H); 3.68 (s, 3H).

**(*R*)-4-*tert*-butyl 1-methyl 2-isobutylsuccinate (24b)** was prepared according to general procedure E, from **23b**, and used in the next step without further purification. $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.89-0.95 (2d, 6H, *J* = 6.0 Hz); 1.23-1.32 (m, 1H); 1.44 (s, 9H); 1.48-1.60 (m, 2H); 2.42 (dd, *J* = 16.2 Hz, *J* = 5.4 Hz, 1H); 2.52 (dd, *J* = 16.2 Hz, *J* = 9.3 Hz, 1H); 2.83-2.87 (m, 1H); 3.68 (s, 3H).

**(*S*)-2-(2-*tert*-butoxy-2-oxoethyl)-4-methylpentanoic acid (25a)** was prepared according to general procedure F, from **24a**, and used in the next step without further purification. $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.90-0.96 (2d, 6H, *J* = 6.5 Hz); 1.27-1.37 (m, 1H); 1.43 (s); 1.51-1.70 (m, 2H); 2.42 (dd, *J* = 16.7 Hz, *J* = 5.3 Hz, 1H); 2.60 (dd, *J* = 16.7 Hz, *J* = 9.2 Hz, 1H); 2.78-2.88 (m, 1H).

**(*R*)-2-(2-*tert*-butoxy-2-oxoethyl)-4-methylpentanoic acid (25b)** was prepared according to general procedure F, from **24b**, and used in the next step without further purification. $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.90-0.96 (2d, 6H, *J* = 6.5 Hz); 1.27-1.37 (m, 1H); 1.43 (s); 1.51-1.70 (m, 2H); 2.42 (dd, *J* = 16.7 Hz, *J* = 5.3 Hz, 1H); 2.60 (dd, *J* = 16.7 Hz, *J* = 9.2 Hz, 1H); 2.78-2.88 (m, 1H).

**(*S*)-3-((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-5-methyl-hexanoic acid *tert*-butyl ester (26a)** was prepared according to general procedure A, from **25a** (150 mg, 0.65 mmol) and (*S*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (165 mg, 0.65 mmol), as a colorless oil (125 mg, 45%). $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.90-0.98 (m, 6H); 1.19 (s, 9H); 1.25-1.35 (m, 1H); 1.45 (s, 9H); 1.48 (s, 9H); 1.52-1.62 (m, 2H); 2.33 (dd, *J* = 15.0 Hz, *J* = 6.0 Hz, 1H); 2.61 (dd, *J* =16.2 Hz, *J* = 8.4 Hz, 1H); 2.80-2.90 (m, 1H); 3.55 (dd, *J* = 8.4 Hz, *J* = 4.2 Hz, 1H); 3.75 (dd, *J* = 9.0 Hz, *J* = 4.5 Hz, 1H); 4.47 (t, *J* = 4.5 Hz, 1H). LC $t_R$ = 7.35 min, MS (ESI+) m/z 388 $(M+H)^+$.

**(*R*)-3-((*S*)-2-*tert*-Butoxy-1-*tert*-butoxycarbonyl-ethylcarbamoyl)-5-methyl-hexanoic acid *tert*-butyl ester (26b)** was prepared according to general procedure A, from **25b** (184 mg, 0.8 mmol) and (*R*)-(Ser(*t*-Bu)-O*t*-Bu), HCl (203 mg, 0.8 mmol), as a colorless oil (194 mg, 63%). $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.92 (d, *J* = 6.0 Hz, 3H); 0.98 (d, *J* = 6.0 Hz, 3H); 1.20 (s, 9H); 1.25-1.35 (m, 1H); 1.45 (s, 9H); 1.48 (s, 9H); 1.52-1.62 (m, 2H); 2.33 (dd, *J* = 15.0 Hz, *J* = 6.0 Hz, 1H); 2.61 (dd, *J* =16.2 Hz, *J* = 8.4 Hz, 1H); 2.80-2.90 (m, 1H); 3.55 (dd, *J* = 8.4 Hz, *J* = 4.2 Hz, 1H); 3.75 (dd, *J* = 9.0 Hz, *J* = 4.5 Hz, 1H); 4.47 (t, *J* = 4.5 Hz, 1H). LC-MS (ESI+) m/z 388 $(M+H)^+$.

**(*S*)-3-((*S*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-5-methyl-hexanoic acid** (**27a**) was prepared according to general procedure B, from **26a** (253 mg, 0.59 mmol), as a colorless oil after purification by preparative LC-MS (25 mg, 16%) Purity 96%. $^1$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.90-0.96 (m, 6H); 1.22-1.32 (m, 1H); 1.56-1.64 (m, 2H); 2.40 (dd, *J* = 15.0 Hz, *J* = 6.0 Hz, 1H); 2.61 (dd, *J* = 16.2 Hz, *J* = 8.4

Hz, 1H); 2.88-2.92 (m, 1H); 3.82 (dd, *J* = 11.1 Hz, *J* = 4.2 Hz, 1H); 3.89 (dd, *J* = 11.1 Hz, *J* = 4.5 Hz, 1H); 4.46 (dd, *J* = 4.5 Hz, 1H). LC-MS (ESI+) m/z 262 (M+H)+.

**(*R*)-3-((*S*)-1-Carboxy-2-hydroxy-ethylcarbamoyl)-5-methyl-hexanoic acid** (**27b**) was prepared according to general procedure B, from **26b** (150 mg, 0.35 mmol), as a colorless oil after purification by preparative LC-MS (25 mg, 27%) ; Purity 99%. $^{1}$H NMR (MeOD-$d_4$) $\delta$ (ppm) 0.92 (d, *J* = 6.0 Hz, 3H); 0.98 (d, *J* = 6.0 Hz, 3H); 1.23-1.30 (m, 1H); 1.55-1.69 (m, 2H); 2.37 (dd, *J* = 15.0 Hz, *J* = 6.0 Hz, 1H); 2.61 (dd, *J* =16.2 Hz, *J* = 8.4 Hz, 1H); 2.80-2.90 (m, 1H); 3.84 (dd, *J* = 4.7 Hz, *J* = 1.6 Hz, 2H); 4.40 (t, *J* = 4.5 Hz, 1H). $^{13}$C RMN $\delta$ (ppm) 21.3, 22.0, 25.4, 37.3, 40.8, 41.3, 55.6, 62.2, 173.6, 175.1, 176.2. LC-MS (ESI+) m/z 262 (M+H)+.

## Biology procedures

### *In vitro h*NEP activity assay.

*In vitro* NEP activity was measured with a quenched substrate N-Dansyl-D-Ala-Gly-p-nitro-Phe-Gly (Km = 350 µM) from Sigma.Inc (D2155, selective enkephalinase inhibitor, Florentin, D., et al. *Anal. Biochem.* **141**, 62, (1984)). Briefly, human NEP (R&D Systems) (200 ng/mL) was incubated 10 min at room temperature with compound in Hepes 50 mM, NaCl 100 mM, pH 7.4 and the enzymatic reaction is started by adding the substrate (final concentration 200 µM). After 2 h, samples (1% DMSO final) are excited at 340 nm and fluorescence emission at 535 nm is measured on a Victor3 V1420 Perkin Elmer spectrophotometer. All measurements were carried out as 8-point dose response curves and are reported as the average of at least three independent measurements. DL-thiorphan was used as a reference inhibitor ($IC_{50}$ = 1 (±0.3) nM).

### *In vitro h*ACE activity assay.

*In vitro* ACE activity was measured with a quenched substrate Abz-Gly-p-nitro-Phe-Pro-OH (Km = 180 µM). Briefly, human ACE (R&D Systems) was incubated 10 min at room temperature with compound in Tris 50 mM 1% NaCl pH7.4 and the enzymatic reaction is started by adding the substrate (final concentration 300 µM). After 17 h, samples (1% DMSO final) are excited at 340 nm and fluorescence emission at 420 nm is measured on a Victor3 V1420 Perkin Elmer spectrophotometer. All measurements were carried out as 8-point dose response curves and are reported as the average of at least three independent measurements. Captopril was used as a reference inhibitor ($IC_{50}$ = 11 nM).

### *In vitro h*ECE activity assay.

*In vitro* ECE activity was measured at CEREP.SA with a quenched substrate Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-DpaOH (Km = 14 µM). Briefly, human ACE (NCO cells) was incubated 45 minutes with compound and the enzymatic reaction is started by adding the substrate (final concentration 15 µM). After 45 min at room temp., samples (1% DMSO final) are excited at 320 nm and fluorescence emission at 405 nm is measured. All measurements were carried out as 8-point dose response curves and are reported as the average of at least three independent measurements. Phosphoramidon was

used as a reference inhibitor ($IC_{50}$ = 31 nM). The $IC_{50}$ values were determined by non-linear regression analysis of the inhibition curves generated with mean replicate values using Hill equation curve fitting (Y = D + [(A – D)/(1 + (C/C50)nH)], where Y = specific activity, D = minimum specific activity, A = maximum specific activity, C = compound concentration, C50 = IC50, and nH = slope factor). This analysis was performed using a software developed at Cerep (Hill software) and validated by comparison with data generated by the commercial software SigmaPlot® 4.0 for Windows® (© 1997 by SPSS Inc.).

### Solubility/LogD measurements.

The analysis was performed using a LC-MS/MS system (Varian 1200L) under SIM detection using the parameters optimized for each compounds. HPLC analysis was performed using a Luna C18 (50*2.1 mm, 5 µm); the gradient and the mobile phase (flow rate 600 µL.min$^{-1}$) used are determined in order to detect the compound of interest with satisfying retention time and peak shape. Acquisition and analysis of data were performed with MS WorkstationTM software (version 6.3.0 or higher).

10µL of a 10 mM solution in DMSO of the compound are diluted either in 490µL of PBS pH 7.4 or in organic solvent MeOH in a 700µL-microtube (in triplicate). The tubes are gently shaken 24 h at room temperature, then centrifuged for 5 minutes at 4000 rpm. The mixtures are filtered over 0.45 µm filters (Millex-LH Millipore). 20 µL of sample are diluted in 180 µL of MeOH. The solubility is determined by the ratio of mass signal area PBS/ organic solvent.

40µL of a 10 mM solution in DMSO of the compound were diluted in 1.960 mL of a 1/1 octanol /PBS at pH 7.4 mixture. The mixture was gently shaken 2 h at room temperature. 20 µL of each phase was diluted in 480 µL of MeOH and analyzed by LC-MS. Each compound is tested in triplicate. Log D was determined as the logarithm of the ratio of concentration of product in octanol and PBS respectively, determined by mass signals.

### GSH adducts detection

In a tube are introduced 30 µL of a 10mM/DMSO solution of compound. 435 µL of PBS (pH=7.4) and 435 µL of a GSH solution 5 mM/PBS are added and the tube is kept at 37°C for 24 h. During that period, aliquots of 150 µL are collected at 0, 1, 4, 6, 24 h and analyzed by LC-MS to look for the adduct (M+307+H)+.

### Stability in mouse plasma:

Incubations were performed in duplicate in Eppendorf tubes. The mouse plasma (Mouse Plasma Lithium Heparine from Sera Laboratories International Ltd) or the William's E medium supplemented with 10 % fetal calf serum was pre-incubated 5 min at 37°C before the addition of test compounds to a final concentration of 10µM (1% DMSO maximum). At the defined time points, 50 µL from each tube were removed to another tube containing 450µL of cold $CH_3CN$ + internal standard (1µM). After centrifugation (10 min at 10000 rpm), supernatants are analyzed. Analysis and quantification used a LC-MS/MS triple-quadrupole system (Varian 1200L) under MRM detection using the parameters optimized for each compounds. HPLC analysis was performed using a Luna $C_{18}$ (50*2.1 mm, 5µm);

the gradient and the mobile phase (flow rate 600µL/min-1) used are determined in order to detect the compound of interest with satisfying retention time and peak shape. Acquisition and analysis of data were performed with MS Workstation$^{TM}$ software (version 6.3.0 or higher). The degradation half-life (t1/2) values were calculated using the following equation: t1/2 = 0.693/k where k is the first-order degradation rate constant. The degradation rate constant (k) was estimated by one-phase exponential decay non-linear regression analysis of the degradation time course data.

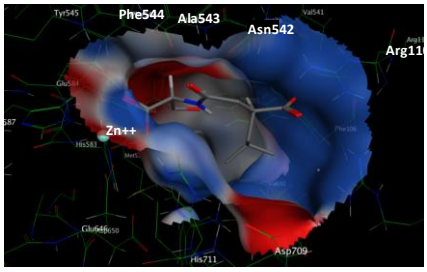
## Data and statistical analysis

Data analysis was performed using Xlfit v 5.0 from IDBS.Ltd and GraphPad Prism v 4.0 unless otherwise stated. Nonlinear curve fitting and statistical analysis was done using built-in functions. Data are plotted as mean ± s.e.m.

## Docking of 22a or 27a

Docking was performed using MOE software, version 2009.10 with default parameters, available from Chemical Computing Group Inc.,1010 Sherbrooke Street West, Suite 910, Montreal, Canada H3A 2R7.
Prior to docking, the target compounds **22a** or **27a** were built using MOE builder interface. Hydrogens were added and energy was minimized using implemented MMFF94 force field.
The X-ray crystallographic structure of *h*NEP complexed with a carboxylic acid inhibitor (PDB code 2QPJ) was obtained from the Protein Data Bank. The enzyme was prepared for docking studies where: (i) The ligand molecule was removed from the enzyme active site. (ii) Hydrogen atoms were added to the structure with their standard geometry. The coordinates of the residues with alternative charged states or flipped conformation were reassigned using Protonate3D implemented in MOE.
The docking mode with the highest score was employed. The number of docking runs was 100. The obtained ligand–enzyme complex model was then used in calculating the energy parameters using MMFF94x force field energy calculation and predicting the ligand–enzyme interactions at the active site. The top binding poses were refined using the LigX procedure (default parameters).

A.




B.

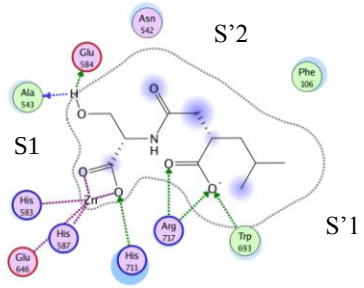



**Fig. S5**: Predicted binding mode of compound **22a** to the active site of *h*NEP obtained from docking simulation (using PDB code 2QPJ). A) Nitrogen, oxygen are blue and red and carbon atoms are grey or green respectively for inhibitor and protein. Zinc is represented as a blue sphere, water as a red sphere and key residues are labeled in white B) Schematic representation of the predicted binding mode of **22a** to *h*NEP pockets S1, S'1 and S'2 and Zinc. Nitrogen, oxygen and carbon are blue, red and black respectively. Polar residues are represented as a pink disk (circled red for acidic and blue for basic), neutral residues are represented as green disk. Hydrogen-bond interactions with side chains are represented with a green dotted arrow from donor to acceptor. Metal contacts are colored purple.