SUPPORTING INFORMATION

Structure-guided design of substituted biphenyl butanoic acid derivatives as neprilysin inhibitors

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EXPERIMENTAL SECTION

I. Synthesis of key compounds

Experimental procedures and compound characterization for novel compounds

General Chemistry Information

Unless otherwise specified, all solvents and reagents were obtained from commercial suppliers and used without further drying or purification. All reactions were performed under nitrogen gas unless otherwise stated. ^{1}H NMR spectra were recorded using an internal deuterium lock at ambient temperature on a Varian 400 MHz spectrometer. Data are presented as follows: chemical shift (in ppm on the δ scale relative to δTMS = 0), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintuplet, m = multiplet, br = broad, dd = doublet of doublet, dt = doublet of triplet, dq = doublet of quartet), coupling constant (*J*/Hz) and integration. Low resolution mass spectra (LRMS) were recorded using an Agilent 1100 series LC-MS spectrometer. High resolution mass spectra (HRMS) were recorded using an Agilent 6220 mass spectrometer with electrospray ionization source and Agilent 1200 liquid chromatograph. The resolution of the HRMS system was approximately 11000 (FWHM definition). The purity of all exemplified compounds was ≥95%, as determined by both ^{1}H NMR and HPLC-UV at a wavelength of 214 nm. Unless otherwise stated, chiral starting materials were commercially available with e.e. ≥98%.

The relative stereochemistry was determined using two dimensional NMR. Under the reaction condition, it would be unexpected that the stereocenter bearing the bisphenyl-methyl group racemize. Therefore, the absolute stereochemistry was determined based on the relative stereochemistry and the absolute stereochemistry of the stereocenter bearing the bisphenyl-methyl group.

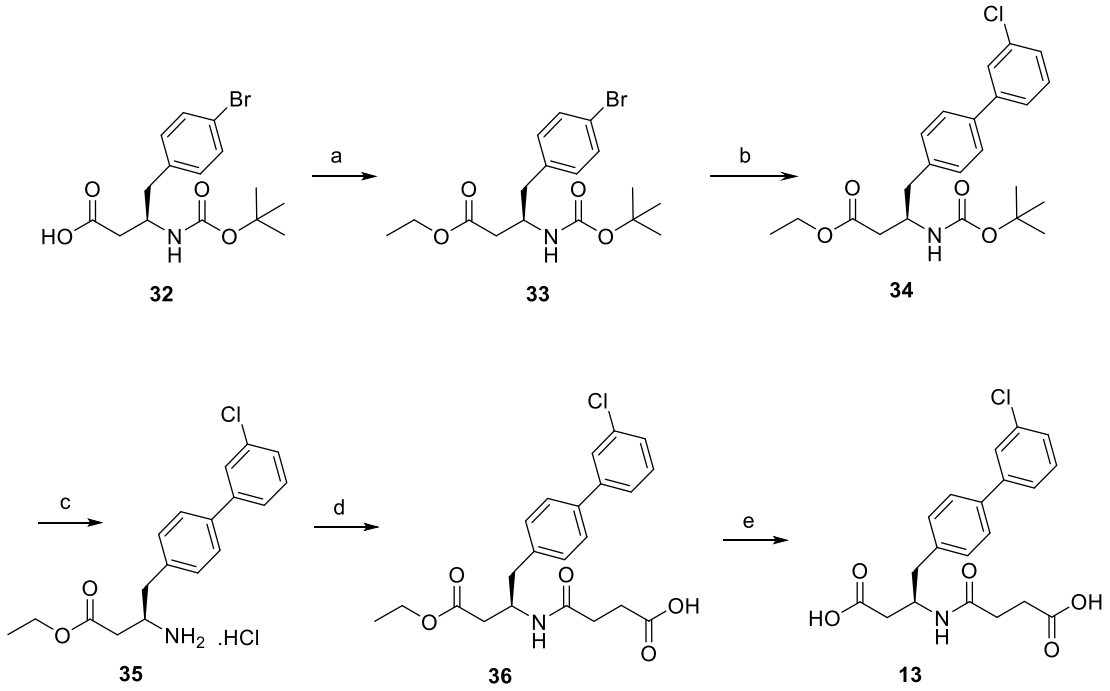
The conditions for determining the mass and the retention times were as follows:

Condition A (HRMS): 1.0 mL/min flow rate. 5% to 95% acetonitrile (with 0.1% formic acid) gradient in 9.50 min, Aqueous phase modified with 0.1% formic acid. Column: Inertsil ODS-4 C18, 3 μm, 3.0 x 100mm. LCUV/ESI-MS data was recorded on an Agilent 6220 with resolution of 11000 (FWHM).

Condition B (LRMS): Electrospray mass spectra (+) and (-), DAD-UV chromatogram 210-400 nm, Gradient: 40-95% acetonitrile with 5 mM ammonium formate in 2 min (2 mL/min), 2 μL injection. Column: Inertsil C8-3, 3.0 x 33mm x 3.0 μm, 50 °C.

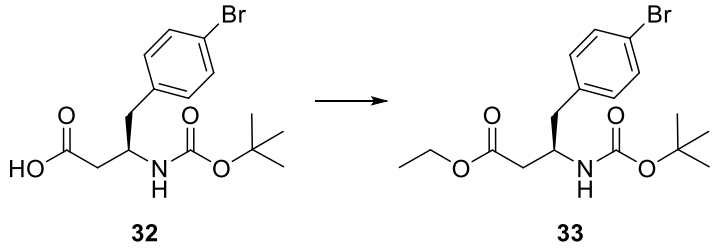
Condition C (LRMS): Electrospray mass spectra (+) and (-), DAD-UV chromatogram 210-400 nm, Gradient: 5-95% acetonitrile with 5 mM ammonium formate in 2 min (2 mL/min), 2 μL injection. Column: Inertsil C8-3, 3.0 x 33mm x 3.0 μm, 50 °C.

I-1: Synthesis of compound **36** and **13**.



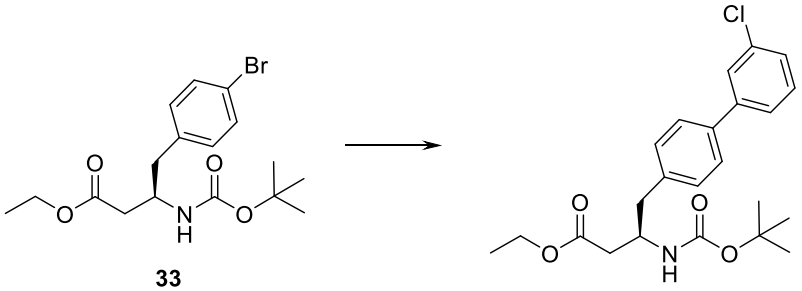
[a]Reagents and conditions: (a) EtI, $NaHCO_3$, DMF, rt (b) 3-Cl-C_6H_4-$B(OH)_2$, $Pd(PPh_3)_4$, aq. Na_2CO_3, DME, 95 ºC (c) 4 M HCl in 1,4-dioxane (d) succinic anhydride, DIPEA, DCM (e) 1 M aq. NaOH, THF, MeOH, rt; 1 M aq. HCl.

***(R)*-ethyl 4-(4-bromophenyl)-3-(tert-butoxycarbonylamino)butanoate (33)**



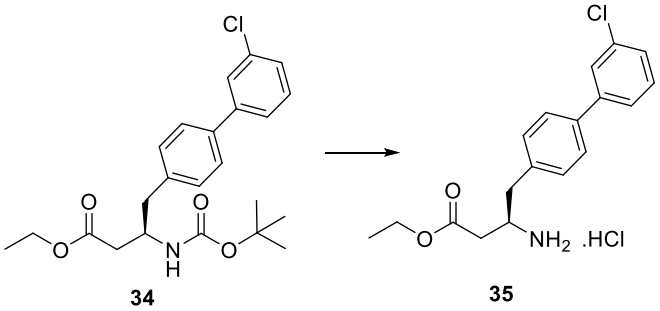
To a suspension of *(R)*-4-(4-bromophenyl)-3-(*tert*-butoxycarbonylamino)butanoic acid (**32**, 9.98 g, 27.9 mmol) and $NaHCO_3$ (4.68 g, 55.7 mmol) in DMF (45 mL) ethyl iodide was added (6.75 mL, 84 mmol) at room temperature under nitrogen. After stirring for 71 h, the reaction was quenched with H_2O (300 mL), and then precipitated solid was collected and washed with H_2O (500 mL) to give *(R)*-ethyl 4-(4-bromophenyl)-3-(*tert*-butoxycarbonylamino)butanoate (**33**, 10.25 g, yield 94%). LRMS (ESI^+) *m/z* calcd. for $C_{12}H_{17}BrNO_2$ $(M–Boc+2H)^+$, 286.0; found, 286.0; Tr = 1.48 min; >95% purity (Condition B). ^1H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.43–7.40 (m, 2 H), 7.07 (d, J = 8.3 Hz, 2 H), 5.04 (br d), 4.15 (q, J = 7.1 Hz, 2 H), 4.11 (br s), 2.90–2.74 (m, 2 H), 2.50 (B of ABX, J_{ab} = 15.8 Hz, J_{bx} = 5.4 Hz, 1 H), 2.43 (A of ABX, J_{ab} = 15.8 Hz, J_{ax} = 5.7 Hz, 1 H), 1.40 (s, 9 H), 1.27 (t, J = 7.2 Hz, 3 H).

(*R*)-ethyl 3-(tert-butoxycarbonylamino)-4-(3'-chlorobiphenyl-4-yl)butanoate (34)



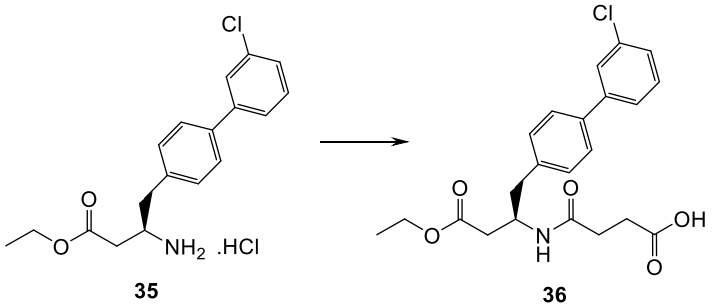
A mixture of (*R*)-ethyl 4-(4-bromophenyl)-3-(*tert*-butoxycarbonylamino)butanoate (**33**, 4.89 g, 12.66 mmol), 3-chlorophenylboronic acid (2.97 g, 18.99 mmol), $Pd(PPh_3)_4$ (1.463 g, 1.266 mmol) and 2 M aqueous Na_2CO_3 (12.66 ml, 25.3 mmol) in 1,2-dimethoxyethane (100 ml) is allowed to stir at 95 ºC under nitrogen for 3 h. The reaction mixture was cooled to room temperature and quenched with brine. The two phases were separated. The mixture was extracted twice with ethyl acetate from the aqueous layer. The combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The obtained residue was purified by silica gel flash column chromatography (heptane/EtOAc = 100:0 to 70:30) to give (*R*)-ethyl 3-(*tert*-butoxycarbonylamino)-4-(3'-chlorobiphenyl-4-yl)butanoate (**34**, 3.33 g, yield 62%). LRMS (ESI^+) *m/z* calcd for $C_{18}H_{21}ClNO_2$ $(M–Boc+2H)^+$, 318.1; found, 318.2; Tr = 1.44 min; >95% purity (Condition B). ^1H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.55 (br t, *J* = 1.8 Hz, 1H), 7.51–7.43 (m, 3H), 7.37–7.26 (m, 4H), 5.07 (br s, 1H), 4.18 (br s, 1 H) , 4.17 (q, *J* = 7.2 Hz, 2 H) , 3.00–2.95 (m, 1 H), 2.89–2.83 (m, 1 H), 2.52 (B of ABX, J_{ab} = 15.8 Hz, J_{bx} = 5.4 Hz, 1 H), 2.47 (A of ABX, J_{ab} = 15.8 Hz, J_{ax} = 5.9 Hz, 1 H), 1.41 (s, 9 H), 1.28 (t, *J* = 7.2 Hz, 3 H).

(*R*)-ethyl 3-amino-4-(3'-chlorobiphenyl-4-yl)butanoate hydrochloride (35)



To (*R*)-ethyl-3-(*tert*-butoxycarbonylamino)-4-(3'-chlorobiphenyl-4-yl)butanoate (**34**, 3.33 g, 7.97 mmol) a solution of 4 M HCl in 1,4-dioxane (19.9 mL, 18.0 mmol) was added at room temperature. After stirring for 0.5 h, the reaction mixture was concentrated under reduced pressure to give (*R*)-ethyl 3-amino-4-(3'-chlorobiphenyl-4-yl)butanoate hydrochloride (**35**, 2.90 g, yield quant.). LRMS (ESI^+) *m/z* calcd for $C_{18}H_{21}ClNO_2$ $(M–HCl+H)^+$, 318.1; found, 318.2 $(M+H)^+$; Tr = 0.70 min; >95% purity (Condition B). ^1H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 8.73 (br s, 3 H), 7.53–7.29 (m, 8 H), 4.17–4.14 (m, 2 H), 3.92 (br s, 1 H), 3.54–3.50 (m, 1 H), 3.11–3.05 (m, 1 H), 2.91–2.84 (m, 1 H), 2.78–2.73 (m, 1 H), 1.24–1.19 (m, 3 H).

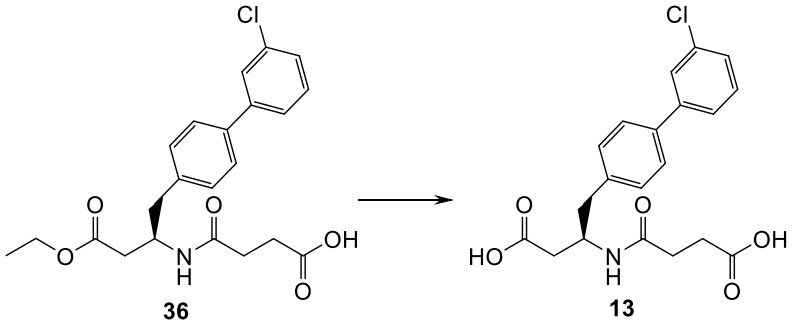
***(R)*-4-(1-(3'-chlorobiphenyl-4-yl)-4-ethoxy-4-oxobutan-2-ylamino)-4-oxobutanoic acid (36)**



35 **36**

A solution of (*R*)-ethyl 3-amino-4-(3'-chlorobiphenyl-4-yl)butanoate hydrochloride (**35**, 400 mg, 1.13 mmol), succinic anhydride (136 mg, 1.36 mmol) and DIPEA (0.237 mL, 1.36 mmol) in dichloromethane (5 mL) was allowed to stir for 2.5 h. The reaction was quenched with 1 M aqueous HCl and extracted with dichloromethane. The organic layer was separated and concentrated under reduced pressure. The resulting residue was purified by preparative HPLC using a gradient of 20% MeCN/water (0.1% TFA) to 100% MeCN to give (*R*)-4-(1-(3'-chlorobiphenyl-4-yl)-4-ethoxy-4-oxobutan-2-ylamino)-4-oxobutanoic acid (**36**, 255 mg, yield 53%). HRMS (ESI$^+$) *m/z* calcd for $C_{22}H_{25}ClNO_5$ $(M+H)^+$, 418.1416; found, 418.1406; Tr = 7.84 min; >95% purity (Condition A). ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.56–7.55 (m, 1 H), 7.52–7.48 (m, 2H), 7.46–7.43 (m, 1 H), 7.37–7.24 (m, 4 H), 6.50 (br d, J = 8.8 Hz, 1 H), 4.55–4.47 (m, 1 H), 4.24–4.12 (m, 2 H), 2.99 (B of ABX, J_{ab} = 13.6 Hz, J_{bx} = 6.6 Hz, 1 H), 2.87 (A of ABX, J_{ab} = 13.6 Hz, J_{ax} = 7.8 Hz, 1 H), 2.67–2.64 (m, 2 H), 2.58–2.46 (m, 4 H), 1.29 (t, J = 7.0 Hz, 3 H).

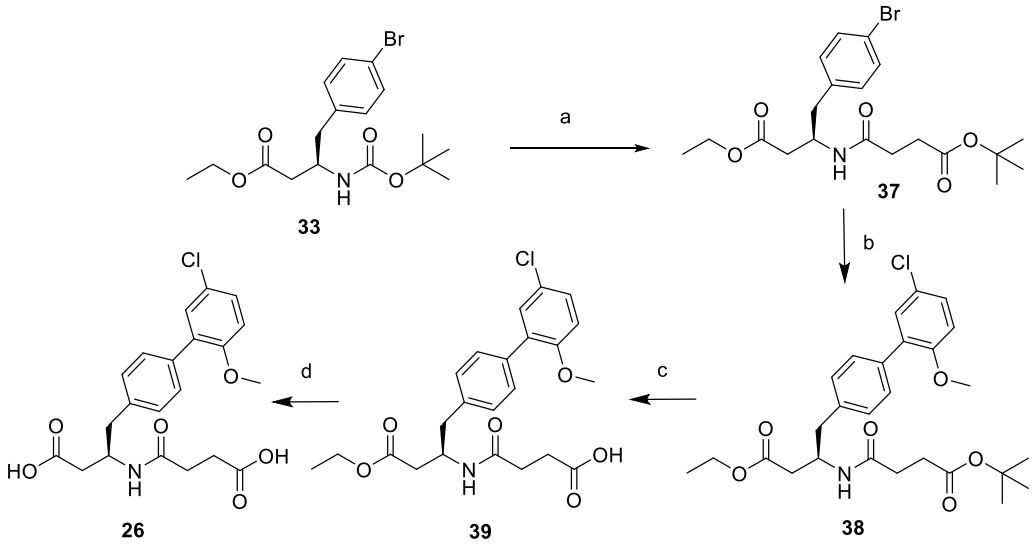
(*R*)-4-(1-carboxy-3-(3'-chlorobiphenyl-4-yl)propan-2-ylamino)-4-oxobutanoic acid (13)



36 **13**

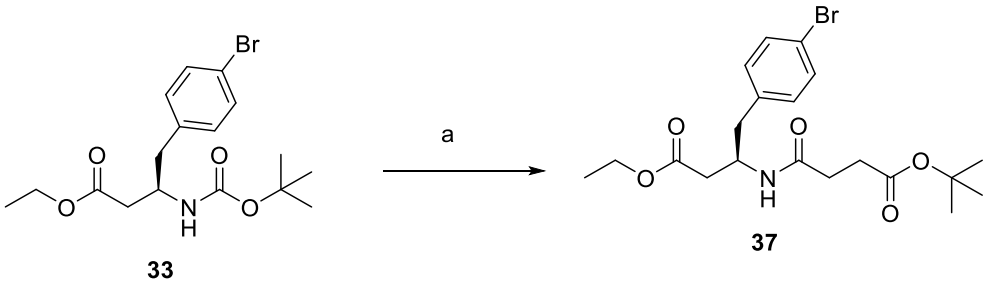
To a solution of (*R*)-4-(1-(biphenyl-4-yl)-4-ethoxy-4-oxobutan-2-ylamino)-4-oxobutanoic acid (**36**, 110 mg, 0.263 mmol) in THF (2 mL) and methanol (0.2 mL), aqueous 1M NaOH solution (1.053 mL, 1.053 mmol) was added at room temperature. After stirring for 1 h, the reaction was quenched with 0.1 M aqueous HCl, and the solution was diluted with DCM (15 ml) and allowed to stir for 1.5 h. The precipitated solid was collected on a funnel, washed with water, DCM, heptane and then DCM in that order, and dried under reduced pressure to (*R*)-4-(1-carboxy-3-(3'-chlorobiphenyl-4-yl)propan-2-ylamino)-4-oxobutanoic acid (**13**, 66 mg, yield 63%). HRMS (ESI$^+$) *m/z* calcd for $C_{20}H_{21}ClNO_5$ $(M+H)^+$, 390.1103; found, 390.1083; Tr = 7.15 min; >95% purity (Condition A). ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.60 (t, J = 1.8 Hz, 1 H), 7.56–7.51 (m, 3 H), 7.40 (t, J = 7.4 Hz, 1 H), 7.34–7.30 (m, 3 H), 4.49–4.42 (m, 1 H), 2.92 (B of ABX, J_{ab} = 13.6 Hz, J_{bx} = 6.2 Hz, 1 H), 2.86 (A of ABX, J_{ab} = 13.6 Hz, J_{ax} = 7.6 Hz, 1 H), 2.55–2.39 (m, 6 H).

I-2: Synthesis of compound **39** and **26**



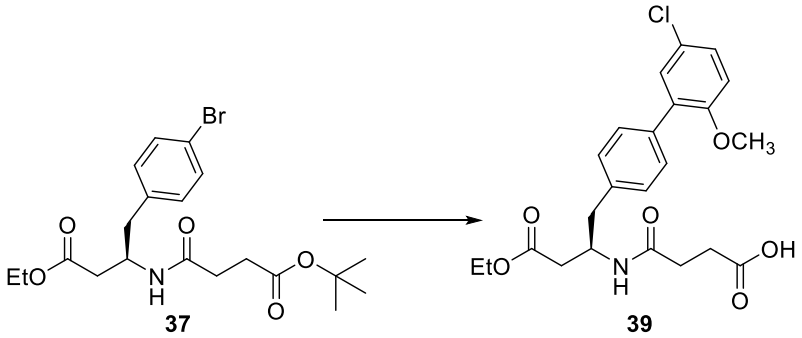
[a]Reagents and conditions: (a) HCl in dioxane; 4-*tert*-butoxy-4-oxobutanoic acid, DIPEA, EDC.HCl, HOAt, DMF, rt (b) 5-Cl-2-MeO-C_6H_4-B(OH)2, $Pd(PPh_3)_4$, aq. Na_2CO_3, toluene/EtOH, 90 ºC (c) 4 M HCl in DCM (d) 1 M aq. NaOH, MeOH, rt; 1N aq. HCl.

***(R)-tert*-butyl 4-(1-(4-bromophenyl)-4-ethoxy-4-oxobutan-2-ylamino)-4-oxobutanoate (37)**



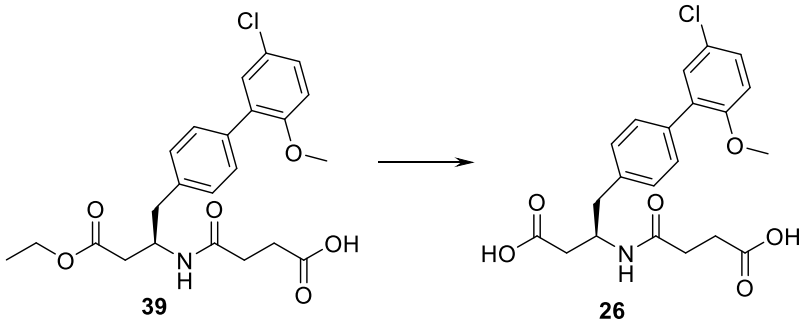
To (*R*)-ethyl 4-(4-bromophenyl)-3-(tert-butoxycarbonylamino)butanoate (**33**, 1.07 g, 2.78 mmol), a solution of 4 M HCl in 1,4-dioxane was added at rt. The mixture was allowed to stir for 1 h and concentrated to give the crude amine (0.96 g). To a suspension of the crude product, EDC.HCl (0.799 g, 4.17 mmol) and HOAt (0.568 g, 4.17 mmol) in DMF (14 ml), DIPEA (0.539 g, 4.17 mmol) was added at rt. After stirring for 2 h, the reaction mixture was quenched with 3% ammonia in H_2O, and then diluted with EtOAc, the organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give *(R)-tert*-butyl 4-(1-(4-bromophenyl)-4-ethoxy-4-oxobutan-2-ylamino)-4-oxobutanoate (**37**, 1.13 g, yield 92%). LRMS (ESI^+) *m/z* calcd for $C_{16}H_{21}BrNO_5$ $(M–tBu+H)^+$, 386.0; found, 386.1; Tr = 1.58 min; >95% purity (Condition C).

***(R)*-4-(4-ethoxy-1-(5'-chloro-2'-methoxybiphenyl-4-yl)-4-oxobutan-2-ylamino)-4-oxobutanoic acid (39)**



To a suspension of *(R)-tert*-butyl-4-(1-(4-bromophenyl)-4-ethoxy-4-oxobutan-2-ylamino)-4-oxobutanoate, (**37**, 200 mg, 0.45 mmol) and 5-chloro-2-methoxyphenylboronic acid (126 mg, 0.678 mmol) in toluene (2 mL) and EtOH (0.2 mL) was added $Pd(PPh_3)_4$ (52 mg, 0.045 mmol) and Na_2CO_3 (96 mg, 0.904 mmol). After stirring at 90 ^oC under nitrogen for 16 h, the solution was cooled to ambient temperature and then quenched with sat. aq. $NaHCO_3$. The crude was diluted with ethyl acetate, the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The obtained residue was purified by prep-TLC (heptane/EtOAc = 30:70) to give *(R)-tert*-butyl 4-(4-ethoxy-1-(5'-chloro-2'-methoxybiphenyl-4-yl)-4-oxobutan-2-ylamino)-4-oxobutanoate (**38**, 104 mg). A solution of *(R)-tert*-butyl 4-(4-ethoxy-1-(5'-chloro-2'-methoxybiphenyl-4-yl)-4-oxobutan-2-ylamino)-4-oxobutanoate, (**38**, 104 mg) in 4M HCl in 1,4-dioxane (1.03 ml, 4.14 mmol) was stirred at room temperature. After stirring for 2 h, the reaction mixture was concentrated under reduced pressure. The obtained residue was purified by RP-HPLC (SunFire C18, H_2O(0.1% TFA)/CH_3CN), and then lyophilized to give *(R)*-4-(4-ethoxy-1-(5'-chloro-2'-methoxybiphenyl-4-yl)-4-oxobutan-2-ylamino)-4-oxobutanoic acid (**39**, 73 mg, yield 79%). HRMS (ESI^+) *m/z* calcd for $C_{23}H_{27}ClNO_6$ $(M+H)^+$, 448.1521; found, 448.1506; Tr = 7.23 min; >95% purity (Condition A). ^1H NMR (400 MHz, CD_3OD) δ ppm 7.39 (d, *J* = 8.1 Hz, 2 H), 7.30–7.17 (m, 4 H), 7.01 (d, *J* = 8.6 Hz, 1 H), 4.57–4.40 (m, 1 H), 4.10 (q, *J* = 7.1 Hz, 2 H), 3.76 (s, 3 H), 2.85 (d, *J* = 7.1 Hz, 2 H), 2.58–2.36 (m, 6 H), 1.23 (t, *J* = 7.1 Hz, 3 H).

(R)-4-(1-carboxy-3-(5'-chloro-2'-methoxybiphenyl-4-yl)propan-2-ylamino)-4-oxobutanoic acid (26)



To a solution of *(R)*-4-(4-ethoxy-1-(5'-chloro-2'-methoxybiphenyl-4-yl)-4-oxobutan-2-ylamino)-4-oxobutanoic acid (**39**, 48 mg, 0.107 mmol) in MeOH (2 mL) was added 1N NaOH (4 mL, 4 mmol). After stirring at room temperature for 2 h, the crude was concentrated under reduced pressure to remove MeOH and then diluted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The obtained residue was purified by RP-HPLC (SunFire C18, H_2O(0.1% TFA)/CH_3CN), and then lyophilized to give *(R)*-4-(1-carboxy-3-(5'-chloro-2'-methoxybiphenyl-4-yl)propan-2-ylamino)-4-oxobutanoic acid (**26**, 33 mg, yield 69%). HRMS (ESI^+) *m/z* calcd

for $C_{21}H_{23}ClNO_6$ $(M+H)^+$, 420.1208; found, 420.1206; Tr = 7.12 min; >95% purity (Condition A). ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.39 (d, *J* = 8.1 Hz, 2 H), 7.31–7.18 (m, 4 H), 7.03 (d, *J* = 8.6 Hz, 1 H), 4.58–4.34 (m, 1 H), 3.77 (s, 3 H), 2.91 (dd, *J* = 13.4, 6.1 Hz, 1 H), 2.84 (dd, *J* = 13.4, 6.1 Hz, 1 H), 2.60–2.36 (m, 6 H).

II. Crystallographic structure determination of human NEP in complex with compound 13

For crystallization, the protein was used at a concentration of 17.6 mg/ml in 50 mM Tris pH 8.0 and 150 mM NaCl. Equal volumes (2 uL) of reservoir solution (0.2 M ammonium acetate, 0.1 M Bis-Tris pH 6.5, 25% w/v PEG 3350. 0.2 M ammonium acetate, 0.1 M Bis-Tris pH 6.5, 25% w/v PEG 3350) and protein solution were mixed. Compound **13** (100 mM in DMSO) was mixed with the protein to a final inhibitor concentration of 1mM. Crystallization experiments were performed at room temperature using the hanging drop vapor diffusion method. Crystals appeared overnight. Before data collection, the crystals were transferred to 25% PEG3350, 200 mM ammonium acetate, 100 mM BisTris at pH 6.5, 20 % (v/v) glycerol and flash frozen in liquid nitrogen. X-ray diffraction data were collected at the PXII beamline of the Swiss Light Source (Villigen, Switzerland) at a wavelength of 1.00 Å at 100°K and processed with XDS as implemented in the program package APRV.[1, 2] The crystals diffracted to 2.54 Å resolution and belong to the space group $P2_12_12_1$ with two monomers in the asymmetric unit. The structure was solved by molecular replacement using the coordinates of PDB entry 5JMY as a search model.[3] Iterative cycles of model building and refinement were carried out using programs COOT,[4] Refmac[5] and BUSTER.[6] The final R-factor is 20.5 % (Rfree 26.4 %). The data collection and refinement statistics are summarized in **Table S1**. The coordinates of the refined structure have been deposited in the Protein databank (6THP).

Table S1. Crystallographic data collection and refinement statistics

	Human NEP–**13**
PDB ID	6THP
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	59.79, 109.39, 248.018
Resolution (Å)	2.54 (2.54-2.62)
R_{sym} (%)	11.4 (53.6)
$I/\sigma I$	14.47 (3.91)
Completeness (%)	99.8 (99.9)
Redundancy	5.49 (5.59)
Refinement	
Resolution (Å)	65.9-2.54
No. reflections	51899
R_{work}/R_{free} (%)	20.5/26.4
No. protein /AU	2
No. atoms	11710
Protein	11178
Ligand/Zinc	58/2
Sugar	120
Water	352

B-factors (Å^2)	**40.6**
R.m.s deviations	
Bond lengths (Å)	0.011
Bond angles (°)	1.338

III. Inhibition of human NEP (biochemical assay)

Recombinant human NEP (Uniprot P08473 expressed in insect cells and purified using standard methods, final concentration 7 pM) was pre-incubated with test compounds at various concentrations for 1 hour at room temperature in 10 mM sodium phosphate buffer at pH 7.4, containing 150 mM NaCl and 0.05 % (w/v) CHAPS. The enzymatic reaction was started by the addition of a synthetic peptide substrate Cys(PT14)-Arg-Arg-Leu-Trp-OH to a final concentration of 0.7 µM. Substrate hydrolysis leads to an increased fluorescence lifetime (FLT) of PT14 measured by the means of a FLT reader as described by Doering et al.[7] The effect of the compound on the enzymatic activity was determined after 1 hour (t = 60 min) incubation at room temperature. The IC_{50} values, corresponding to the inhibitor concentration showing 50% reduction of the FLT values measured in absence of inhibitor, were calculated from the plot of percentage of inhibition *vs.* inhibitor concentration using non-linear regression analysis software.

IV. PK study of compounds 13 and 36 in rats

The PK of compound **13** was determined in Sprague Dawley rats. The compound was dosed intravenously (IV, via injection into a jugular vein catheter; 1 mg/kg, n=2 animals) and its prodrug **36** was dosed orally (PO, via oral gavage; 3 mg/kg, n=3 animals). The IV solution formulation was prepared in 2EQ 0.1N NaOH, 10% PG, 50(10%) Solutol, WFI solution. The PO formulation was a solution in 10% PG, 50(10%) Solutol, 40% WFI. Approximately 200 µL of whole blood was collected from the jugular vein catheter of each animal at 5 min (IV dose only), 15 min, 0.5, 1, 2, 4, 7, and 24 hours post-dose and was transferred to EDTA tubes. Blood was centrifuged at 3,000 rpm and the resultant plasma was transferred to a capped PCR 96-well plate, and frozen at –20 °C until subsequent analysis by HPLC-MS/MS. Both **13** and **36** drug levels in plasma were measured. The relevant PK parameters were estimated using non-compartmental methods using WinNonlin (Enterprise, Version 5.2) purchased from Pharsight Corporation (St. Louis, MO) or Watson LIMS (Thermo, Waltham, MA).

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