Electronic Supplementary Information (ESI)

Design of Potent and Highly Selective Inhibitors for Human β-Secretase 2 (Memapsin 1), a Target for Type 2 Diabetes

Arun K. Ghosh, Bhavanam Sekhara Reddy, Yu-Chen Yen, Kalapala Venketeswara Rao, Emilio Cardenas, Deborah Downs, Xiangping Huang, Jordan Tang and Andrew D. Mesecar

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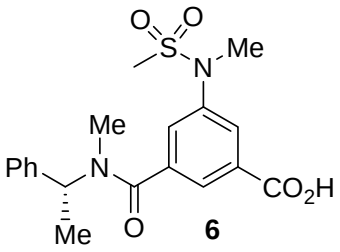
1. General Information: Those reactions which required anhydrous conditions were carried out under an argon atmosphere using oven-dried glassware (120 ^{o}C). All chemicals and reagents were purchased from commercial suppliers and used without further purification. Anhydrous solvents were obtained as follows: anhydrous diethyl ether and toluene were distilled from sodium metal under argon, and anhydrous dichloromethane and tetrachloromethane were dried *via* distillation from CaH_2 immediately prior to use under argon. All other solvents were reagent grade. TLC analysis was conducted using glass-backed Thin-Layer Silica Gel Chromatography Plates (60 Å, 250 μm thickness, F-254 indicator). Flash chromatography was performed using 230-400 mesh, 60 Å pore diameter silica gel. ^{1}H NMR spectra were recorded at 400 or 500 MHz. ^{13}C NMR spectra were recorded at 100 or 150 MHz. Chemical shifts are reported in parts per million and are referenced to the deuterated residual solvent peak. NMR data is reported as: δ value (chemical shift, *J*-value (Hz), integration, where s = singlet, d = doublet, t = triplet, q = quartet, brs = broad singlet). IR spectra were recorded on a Varian 2000 Infrared spectrophotometer and are reported as cm^{-1}. LRMS spectra were recorded at the Purdue University Department of Chemistry Mass Spectrometry Center. Melting point was measured on a melting point apparatus and was uncorrected.

2. Experimental details for the synthesis of inhibitors:

Synthesis of isophthalic acid derivative 6:



6

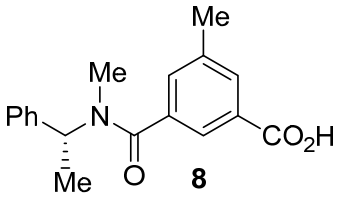
To a solution of Boc-(*R*)-*N*-methyl-1-phenylethylamine[1] (132 mg, 0.56 mmol) in CH_2Cl_2 (18 mL), trifluoroacetic acid (1 mL) was added at 0 °C and the reaction mixture was allowed to stir at room temperature for 1.5 h. Trifluoroacetic acid and CH_2Cl_2 were removed under reduced pressure, the resulting residue was dissolved in ethyl acetate and washed with saturated aqueous $NaHCO_3$ solution. The organic layer was dried over anhydrous Na_2SO_4 and solvent was removed under reduced pressure. The residue was used directly for the next reaction.

To a solution above (*R*)-*N*-methyl-1-phenylethylamine and known[2] 5-(*N*-methylmethylsulfonamido)isophthalic acid methyl ester (135.2, 0.47 mmol) in CH_2Cl_2 (6 mL) at 23 °C was added EDC·HCl (180 mg, 0.94 mmol), HOBt (127 mg, 0.94 mmol) and *i*-Pr_2NEt (0.25 mL, 1.4 mmol). The resulting mixture was stirred at 23 °C for 12 h. After this period, the reaction was quenched with saturated aqueous $NaHCO_3$ solution. The mixture was extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4 and solvent was removed under reduced pressure. The residue was purified by silica gel chromatography to provide the corresponding amide derivative (125.5 mg, 66% yield).

To above ester (125 mg, 0.31 mmol) in a mixture of water (1 mL) and THF (2 mL), $LiOH·H_2O$ (49 mg, 0.93 mmol) was added and the resulting mixture was stirred for 12 h at at 23 °C. The solvent was removed under reduced pressure, and the resulting mixture was diluted with H_2O and washed with diethyl ether. The aqueous layer was acidified with aqueous 1 N HCl and extracted with ethyl acetate. The combined organic fractions were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to

furnish carboxylic acid **6** (103 mg, 91% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10-7.85 (brs, 2H), 7.71 (s, 1H), 7.46-7.05 (m, 6H), 4.95-5.05 (br, 2H), 3.45 (brs, 3H), 2.85 (brs, 3H), 2.78 (s, 2H), 2.61 (s, 1H), 1.62 (d, *J* = 6.4 Hz, 3H).

Synthesis of 5-methyl isophthalic acid derivative 8:

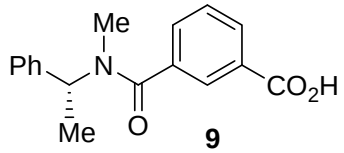


8

Methyl (*R*)-3-methyl-5-((1-phenylethyl)carbamoyl)benzoate was prepared as described in the literature.[3] To the stirred solution of above methyl ester (128 mg, 0.43 mmol) in dry THF (4 mL), NaH (34 mg, 60% NaH in mineral oil, 0.86 mmol) was added at 23 °C, and the resulting reaction mixture was stirred for 15 min. Iodomethane (305 mg, 2.15 mmol) was added and stirring was continued for 8 h at 23 °C. The reaction mixture was cooled to 0 °C and quenched by adding methanol drop wise and then diluted with EtOAc (10 mL). The resulting reaction mixture was washed with water, brine and the organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (15-20% EtOAc/hexanes) to furnish the corresponding N-methyl derivative (69 mg, 51% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (brs, 2H), 7.56-7.10 (m, 6H), 6.17 and 5.02 (br, 1H), 3.97 (s, 3H), 2.84 (brs, 1H), 2.6 (brs, 2H), 2.44 (s, 3H), 1.62 (d, *J* = 6.8 Hz, 3H).

To the solution of the above ester (69 mg, 0.22 mmol) in a mixture of water (1 mL) and THF (2 mL), $LiOH{\cdot}H_2O$ (28 mg, 0.66 mmol) was added and stirred for 12 h at 23 °C. The solvent was removed under reduced pressure, and the resulting mixture was diluted with H_2O and washed with diethyl ether. The aqueous layer was acidified with aqueous 1 N HCl and extracted with ethyl acetate. The combined organic fractions were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to furnish carboxylic acid **8** (60 mg, 92% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06-7.90 (m, 1H), 7.95 (s, 1H), 7.51 (s, 1H),7.46-7.30 (m, 3H), 7.30-7.27 (m, 1H), 7.25-7.15 (m, 1H), 6.18 (s, 1H), 5.00 (s, 1H), 2.82 and 2.61 (brs, 3H), 2.44 (s, 3H), 1.62 (d, *J* = 6.8 Hz, 3H).

Synthesis of isophthalic acid derivative 9:



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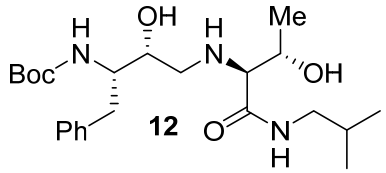
To the stirred solution of commercially available methyl (*R*)-3-((1-phenylethyl)carbamoyl)benzoate (141 mg, 0.5 mmol) in dry THF (4 mL), NaH (24 mg, 60% NaH in mineral oil, 0.60 mmol) was added at 23 °C, and the resulting reaction mixture was stirred for 15 min. Iodomethane (141 mg, 1 mmol) was added and stirring was continued for 18 h at 23 °C. The reaction mixture was cooled to 0 °C and quenched by adding methanol drop wise and dilute with EtOAc (10 mL). The resulting reaction mixture was washed with water, brine and the organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (5-15% EtOAc/hexanes) to furnish methyl (*R*)-3-methyl-(1-phenylethyl)carbamoyl)benzoate (60 mg, 40% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20-8.05 (m, 2H), 7.64 (d, *J* = 7.2 Hz, 1H), 7.49 (t, *J* = 8.0 Hz, 1H), 7.42-7.33 (m, 3H); 7.30 (d, *J* = 7.2 Hz, 1H), 7.26-7.15 (m, 1H), 6.17 & 5.00 (2s, 1H) 3.92 (s, 3H), 2.82 & 2.60 (2s, 3H), 1.61 (d, *J* = 6.8 Hz, 3H).

To the solution of the above ester (59 mg, 0.2 mmol) in a mixture of water (1 mL) and THF (2 mL), $LiOH{\cdot}H_2O$ (14 mg, 0.6 mmol) was added and the resulting mixture was stirred at 23 °C for 12 h. The solvent was removed under reduced pressure, and the resulting mixture was diluted with H_2O and washed with diethyl ether. The aqueous layer was acidified with aqueous 1 N HCl and extracted with ethyl acetate. The combined organic fractions were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to furnish (*R*)-3-[methyl(1-phenylethyl)carbamoyl]benzoic acid **9** (47 mg, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.62 (b s, 1H), 8.30-8.17 (m, 1H), 8.14 (d, *J* = 7.6 Hz, 1H), 7.70 (d, *J* = 7.2 Hz, 1H), 7.52 (t, *J* = 8.0 Hz, 1H), 7.48-7.34 (m, 3H), 7.30 (d, *J* = 5.2 Hz, 1H), 7.25-7.14 (m, 1H), 6.18 & 5.00 (2s, 1H), 2.84 & 2.61 (2s, 3H), 1.62 (d, *J* = 6.4 Hz, 3H).

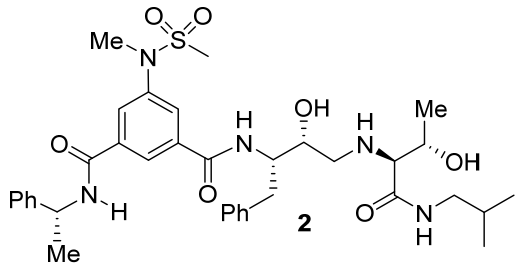
Synthesis of Boc-aminoalcohol 12:



To a solution of *tert*-butyl [(2*S*,3*S*)-3-hydroxy-1-(isobutylamino)-1-oxobutan-2-yl]carbamate **10**[4] (573 mg, 2.08 mmol) in CH_2Cl_2 (18 mL), trifluoroacetic acid (6 mL) was added at 0 °C and the reaction mixture was allowed to stir at 23 °C for 2 h. Trifluoroacetic acid and CH_2Cl_2 were removed under reduced pressure and the resulting residue was purified by silica gel column chromatography [1-5% (5%NH_3/MeOH)/CH_2Cl_2] to furnish (2*S*, 3*S*)-2-amino-3-hydroxy-*N*-isobutylbutanamide in 94% yield (341 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55 (s, 1H), 4.94 (quin, *J* = 6.4 Hz, 1H), 3.22 (d, *J* = 76.8 Hz, 1H), 3.08 (t, *J* = 6.4 Hz, 2H), 2.46 (b s, 2H), 1.85-1.72 (m, 1H), 1.23 (d, *J* = 6.4 Hz, 3H), 0.91 (d, *J* = 6.4 Hz, 6H).

To a solution of above (2*S*, 3*S*)-2-amino-3-hydroxy-*N*-isobutylbutanamide (209 mg, 0.62 mmol) in CH_2Cl_2 (3 mL), commercially available optically active oxirane **11** (263 mg, 1 mmol) and silica gel (3 g) were added as described in the literature.[5] After thoroughly stirring in CH_2Cl_2, the solvent was removed under reduced pressure and the reaction mixture was kept at 23 °C for 2 days. The resulting reaction mixture was purified by silica gel column chromatography [1-5% MeOH/CH_2Cl_2] to obtain *tert*-butyl [(2*S*,3*R*)-3-hydroxy-4-([(2*S*,3*S*)-3-hydroxy-1-(isobutylamino)-1-oxobutan-2-yl]amino)-1-phenylbutan-2-yl]carbamate **12** (180 mg, 41% yield) along with 48% unreacted (2*S*,3*S*)-2-amino-3-hydroxy-*N*-isobutylbutanamide. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.27 (m, 2H), 7.26-7.16 (m, 4H), 4.55 (d, *J* = 8.0 Hz, 1H), 4.08-3.96 (m, 1H), 3.88-3.74 (m, 1H), 3.59-3.50 (m, 1H), 3.18-3.02 (m, 3H), 3.03-2.92 (m, 1H), 2.84-2.68 (m, 3H), 1.84-1.72 (m, 1H), 1.35 (s, 9H), 1.21 (d, *J* = 6.4 Hz, 3H), 0.91 (d, *J* = 6.4 Hz, 6H).

Synthesis of Inhibitor 2:

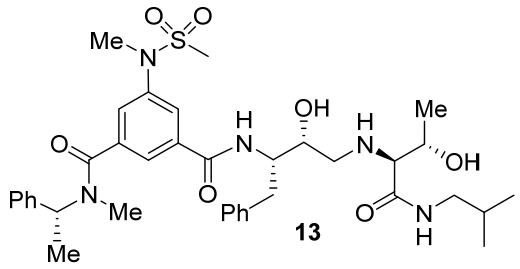


(2*S*, 3*S*)-2-([(2*R*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyl]amino)-3-hydroxy-*N*-isobutylbutanamide was synthesized from Boc-aminoalcohol **12** by treating with trifluoroacetic acid following the procedure described for the synthesis of (2*S*,3*S*)-2-amino-3-hydroxy-*N*-isobutylbutanamide as described above. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34-7.27 (m, 3H), 7.25-7.13 (m, 3H), 4.06 (quin, *J* = 5.6 Hz, 1H), 3.75-3.68 (m, 1H), 3.44-3.25 (br s, 2H), 3.25-3.16 (m, 3H), 3.09 (td, *J* = 6.8 Hz & 2.0 Hz, 2H), 2.93-2.70 (m, 3H), 2.57 (dd, *J* = 13.6 Hz & 10.0 Hz, 1H), 1.84-1.72 (m, 1H), 1.18 (d, *J* = 6.4 Hz, 3H), 0.91 (d, *J* = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.73, 137.95, 129.21, 128.76, 126.76, 71.99, 68.05, 67.99, 56.29, 50.84, 46.65, 37.98, 28.47, 20.20, 18.07.

To a solution of the above amine (15 mg, 0.044 mmol) in dry CH_2Cl_2 (4 mL), $^{i}Pr_2NEt$ (18 μL, 0.11 mmol), HOBt·H_2O (7.2 mg, 0.053 mmol), (*R*)-3-(*N*-methylmethylsulfonamido)-5-(1-phenylethyl)carbamoyl)benzoic acid **5**[2] (20 mg, 0.05 mmol), and EDC·HCl (10.1 mg, 0.05 mmol) were added simultaneously at 23 °C and the resulting reaction mixture was stirred for 15 h at the same temperature. The reaction mixture was quenched by adding saturated aqueous $NaHCO_3$ solution and extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (1-5% MeOH/CH_2Cl_2) to furnish the inhibitor **2** (19.4 mg, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (s, 1H), 7.98 (s, 1H), 7.86 (s, 1H), 7.78 (s, 1H), 7.41-7.34 (m, 2H), 7.27-7.34 (m, 3H), 7.24-7.13 (m, 6H), 5.33-5.22 (m, 1H), 4.48-4.32 (m, 1H), 4.15-4.03 (m, 1H), 3.86-3.70 (m, 1H), 3.25-3.16 (m, 1H), 3.24 (s, 3H), 3.09-3.02 (m, 2H), 3.00-2.85 (m, 4H), 2.85-2.81 (m, 1H), 2.80 (s, 3H), 1.80-1.68 (m, 1H), 1.60 (d, *J* = 6.8 Hz, 3H), 1.13 (d, *J* = 5.6 Hz, 3H), 0.88 (d, *J* = 6.4 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, at 37 °C): δ 172.45, 166.36, 165.18, 143.00, 142.02, 137.76, 135.95, 135.76,

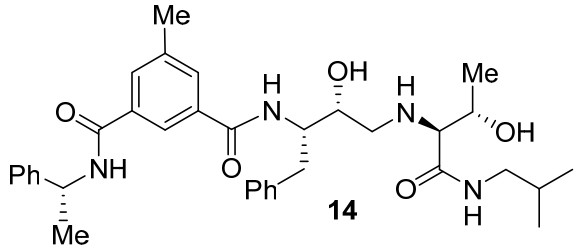
129.24, 128.79, 128.73, 127.88, 127.78, 127.59, 126.77, 126.39, 124.31, 71.17, 68.18, 67.81, 55.50, 50.60, 49.94, 46.67, 37.94, 36.53, 35.70, 28.53, 21.80, 20.26, 18.86. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{36}H_{49}N_5O_7S$, 696.3425; found, 696.3426.

Synthesis of Inhibitor 13:



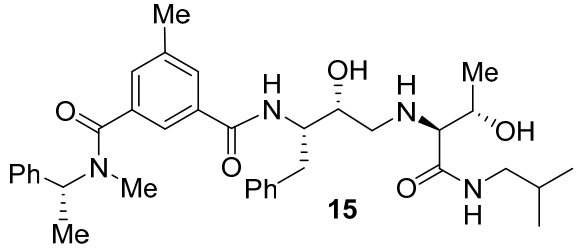
Inhibitor **13** was synthesized by coupling (2*S*, 3*S*)-2-([(2*R*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyl]amino)-3-hydroxy-*N*-isobutylbutanamide and carboxylic acid **6** using the general procedure described above for inhibitor **2**. The desired inhibitor **13** was obtained as an oil (35% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69 (d, *J* = 39.9 Hz, 1H), 7.57 (d, *J* = 2.0 Hz, 1H), 7.27 (m, 14H), 6.12 (s, 1H), 4.46 (s, 1H), 4.10 (s, 1H), 3.75 (d, *J* = 10.4 Hz, 1H), 3.29 (d, *J* = 27.3 Hz, 3H), 3.17 (s, 1H), 3.13 – 3.07 (m, 2H), 3.03 (d, *J* = 8.2 Hz, 1H), 2.97 (d, *J* = 6.1 Hz, 1H), 2.79 (d, *J* = 35.3 Hz, 6H), 2.64 (s, 2H), 1.78 (dt, *J* = 13.3, 6.7 Hz, 2H), 1.62 (d, *J* = 7.0 Hz, 3H), 1.25 (s, 1H), 1.21 (d, *J* = 6.4 Hz, 3H), 0.91 (d, *J* = 6.7 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, 37 ºC): δ 170.66, 169.79, 166.47, 141.87, 139.54, 137.66, 137.57, 136.03, 129.21, 128.81, 128.62, 127.79, 126.67, 125.14, 70.76, 67.69, 67.41, 54.95, 50.52, 46.88, 37.71, 36.52, 35.77, 29.69, 28.42, 20.16, 18.95, 17.53, 15.53. MS-ESI (m/z): $[M+H]^+$ calcd for $C_{37}H_{51}N_5O_7S$, 710.4; found, 710.7.

Synthesis of Inhibitor 14:



Inhibitor **14** was synthesized by coupling (2*S*, 3*S*)-2-([(2*R*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyl]amino)-3-hydroxy-*N*-isobutylbutanamide and carboxylic acid **7** using the general procedure described above for inhibitor **2**. The desired inhibitor **14** was obtained as an oil (41% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.80 (s, 1H), 7.61 – 7.48 (m, 3H), 7.38 – 7.30 (m, 4H), 7.30 – 7.22 (m, 6H), 7.18 (td, *J* = 5.9, 2.5 Hz, 1H), 6.81 (d, *J* = 7.7 Hz, 1H), 5.29 (dd, *J* = 8.5, 5.7 Hz, 1H), 4.49 – 4.38 (m, 1H), 4.10 – 4.00 (m, 1H), 3.75 (q, *J* = 4.8 Hz, 1H), 3.14 (d, *J* = 4.3 Hz, 3H), 3.09 – 2.90 (m, 4H), 2.82 (p, *J* = 7.7, 6.8 Hz, 2H), 2.29 (s, 3H), 1.73 (dt, *J* = 13.5, 6.8 Hz, 1H), 1.59 (d, *J* = 6.9 Hz, 3H), 1.25 (m, 1H), 1.14 (d, *J* = 6.4 Hz, 3H), 0.95 – 0.77 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, 37 ºC): 171.95, 167.51, 166.12, 143.11, 139.10, 137.50, 134.77, 130.79, 129.22, 129.08, 128.93, 128.70, 128.62, 127.44, 126.70, 126.27, 71.05, 67.91, 67.59 53.53, 49.52, 41.88, 29.69, 28.35, 21.73, 21.12, 20.05, 11.73. MS-ESI (m/z): $[M+H]^+$ calcd for $C_{35}H_{46}N_4O_5$, 603.4; found, 603.6.

Synthesis of Inhibitor 15:

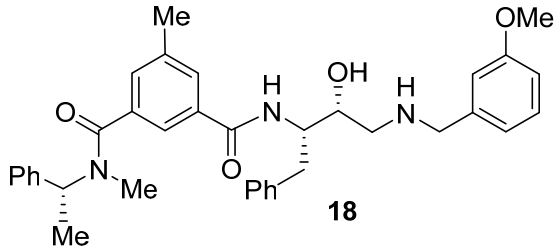


15

Inhibitor **15** was synthesized by coupling (2*S*, 3*S*)-2-([(2*R*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyl]amino)-3-hydroxy-*N*-isobutylbutanamide and carboxylic acid **8** using the general procedure described above for inhibitor **2**. The desired inhibitor **15** was obtained as an oil (40% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 – 7.01 (m, 13H), 6.1 (brs, 1H), 4.95 (brs, 1H), 4.29 (brs, 1H), 3.95 (brs, 1H), 3.72 (brs, 1H), 3.15 (s, 1H), 3.07-2.95 (m, 4H), 2.85 (brs, 3H), 2.6 (brs, 2H), 2.22 (s, 3H), 1.85 (m, 1 H), 1.71 – 1.54 (m, 3H), 1.5 (brs, 1H), 1.18 (d, *J* = 6.28 Hz, 3H), 0.95 – 0.77 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.52, 168.76, 167.58, 144.69, 137.66, 136.59, 134.73, 129.12, 128.49, 128.62, 127.57, 127.24, 126.52, 122.45, 77.14, 71.52, 68.21, 67.62, 54.48, 50.53, 46.46,

36.23, 36.23, 28.38, 21.17, 20.08, 18.95, 14.02. MS-ESI (m/z): $[M+H]^+$ calcd for $C_{36}H_{48}N_4O_5$, 617.37; found, 617.7. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{36}H_{48}N_4O_5$, 617.3703; found, 617.3700.

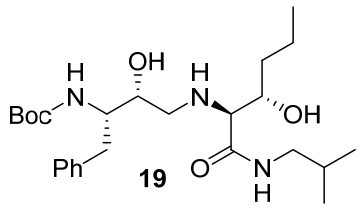
Synthesis of Inhibitor 18:



18

Inhibitor **18** was synthesized by coupling known[2] aminoalcohol **16** and carboxylic acid **8** using the general procedure described above for inhibitor **2**. The desired inhibitor **18** was obtained as an oil (65% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52-7.40 (m, 2H), 7.40-7.32 (m, 3H), 7.32-7.28 (m, 3H), 7.25-7.18 (m, 4H), 7.18-7.07 (m, 1H), 6.96-6.88 (m, 2H), 6.86-6.76 (m, 2H), 4.43-4.28 (m, 1H), 3.98-3.58 (m, 6H), 3.72 (s, 3H), 3.14-2.92 (m, 2H), 2.90-2.74 (m, 2H), 2.74-2.50 (m, 2H), 2.33 (s, 3H), 1.58 (d, J = 6.4 Hz, 3H) ; ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.72, 167.31, 159.72, 139.79, 138.90, 137.73, 136.85, 129.55, 129.21, 128.59, 128.41, 127.48, 126.41, 120.77, 113.98, 113.21, 70.45, 55.12, 53.93, 53.24, 50.74, 50.42, 36.22, 29.61, 21.14, 17.43. MS-ESI (m/z): $[M+H]^+$ calcd for $C_{35}H_{46}N_4O_5$, 580.7; found, 580.69.

Synthesis of Boc-aminoalcohol **19**:



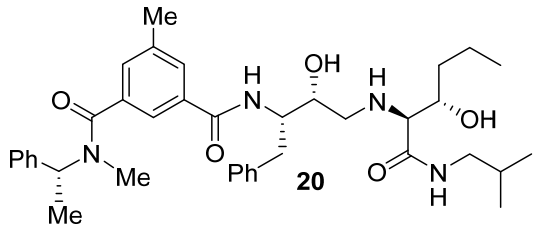
19

(2*S*, 3*S*)-2-amino-3-hydroxy-*N*-isobutylhexanamide was synthesized from the known[4] *tert*-butyl [(2*S*, 3*S*)-3-hydroxy-1-(isobutylamino)-1-oxohexan-2-yl]carbamate **17** by treating with trifluoroacetic acid following the procedure described for the synthesis of (2*S*, 3*S*)-2-amino-3-hydroxy-*N*-isobutylbutanamide. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48

(s, 1H), 3.74 (dt, *J* = 7.2, 2.8 Hz, 1H), 3.24 (d, *J* = 6.8 Hz, 1H), 3.08 (t, *J* = 6.4 Hz, 2H), 1.84-1.72 (m, 1H), 1.62-1.30 (m, 5H) 0.93 (t, *J* = 6.8 Hz, 3H), 0.91 (d, *J* = 6.4 Hz, 6H).

Compound **19** was prepared from (2*S*, 3*S*)-2-amino-3-hydroxy-*N*-isobutylhexanamide by treating with commercially available oxirane **11** with silica gel following the procedure described for the synthesis of compound **12** (yield 57%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37-7.27 (m, 2H), 7.25-7.15 (m, 3H), 7.12 (s, 1H), 4.54 (d, *J* = 7.6 Hz, 1H), 3.90-3.74 (m, 2H), 3.57-3.49 (m, 1H), 3.18-3.10 (m, 2H), 3.10-3.03 (m, 1H), 3.03-2.93 (m, 1H), 2.89 (d, *J* = 7.2 Hz, 1H), 2.86-2.76 (m, 1H), 2.74 (d, *J* = 4.0 Hz, 2H), 1.85-1.72 (m, 1H), 1.60-1.50 (m, 1H), 1.50-1.38 (m, 3H), 1.35 (s, 9H), 0.93 (t, *J* = 5.2 Hz, 3H), 0.92 (d, *J* = 6.4 Hz, 6H).

Synthesis of Inhibitor 20:



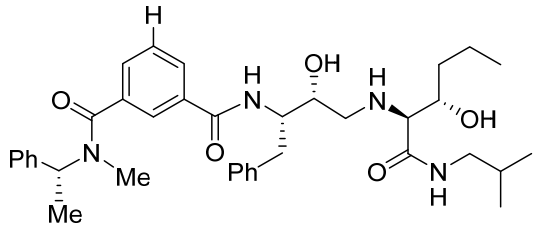
20

(2*S*, 3*S*)-2-{[(2*R*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyl]amino}-3-hydroxy-*N*-isobutylhexanamide was synthesized from Boc-aminoalcohol **19** by treating with trifluoroacetic acid following the procedure described for the synthesis of (2*S*,3*S*)-2-amino-3-hydroxy-*N*-isobutylbutanamide. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.34-7.29 (m, 2H), 7.25-7.21 (m, 1H), 7.20-7.16 (m, 2H), 7.13-7.08 (m, 1H), 3.83 (dd, *J* = 6.5, 5.5 Hz , 1H), 3.64-3.59 (m, 1H), 3.17-3.08 (m, 4H), 2.87 (dd, *J* = 13.5 Hz & 4.0 Hz, 1H), 2.82 (dd, *J* = 12.0 Hz & 3.0 Hz, 1H), 2.73 (dd, *J* = 12.0 Hz & 9.0 Hz, 1H), 2.48 (dd, *J* = 13.5 Hz & 10.0 Hz, 1H), 1.83-1.75 (m, 1H), 1.59-1.51 (m, 1H), 1.50-1.44 (m, 2H), 1.44-1.34 (m, 1H), 0.93 (t, *J* = 6.5 Hz, 3H), 0.92 (d, *J* = 6.5 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.06, 138.72, 129.28, 128.77, 126.65, 73.17, 71.97, 67.67, 55.95, 51.02, 46.65, 39.08, 34.84, 28.58, 20.28, 19.34, 14.11.

The resulting aminoalcohol was coupled with carboxylic acid **8** using the general procedure described above for inhibitor **2**. The desired inhibitor **20** was obtained as an oil (49% yield). ^{1}H NMR (500 MHz, $CDCl_3$, at 37 °C): δ 7.43 (s, 1H), 7.38-7.26 (m, 4H),

7.26-7.19 (m, 2H), 7.19-7.14 (m, 5H), 7.14-7.08 (m, 2H), 7.06 (t, *J* = 6.0 Hz, 1H), 6.06 & 4.87 (2s, 1H), 4.33 (p, *J* = 7.0 Hz, 1H), 3.6 (p, *J* = 4.0 Hz, 1H), 3.72-3.53 (m, 2H), 3.09 (d, *J* = 4.0 Hz, 2H), 3.05-2.96 (m, 2H), 2.95 & 2.93 (2s, 3H), 2.81-2.67 (m, 3H), 2.49 (b s, 2H), 2.27 (s, 3H), 1.68 (sept, *J* =6.5 Hz, 1H), 1.51 (b s, 3H), 1.47-1.41 (m, 1H), 1.41-1.34 (m, 2H) 1.34-1.26 (m, 1H), 0.82. (t, *J* = 8.0 Hz, 3H), 0.81(d, *J* = 5.0 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, at 37 °C): δ 172.94, 171.17, 167.75, 140.02, 139.14, 137.78, 137.00, 135.05, 130.26, 129.42, 128.89, 128.80, 127.81, 127.53, 126.85, 122.48, 72.40, 71.41, 67.12, 64.40, 54.84, 50.35, 46.77, 36.84, 35.87, 29.84, 28.63, 22.79, 21.40, 20.32, 19.24, 14.14; MS-ESI (m/z): $[M+H]^+$ calcd for $C_{38}H_{52}N_4O_5$, 645.4; found, 645.8

Synthesis of Inhibitor 3:



Inhibitor **3** was synthesized by coupling amine derived from **19** with (*R*)-3-[methyl(1-phenylethyl)carbamoyl]benzoic acid **9** following a similar procedure described for the synthesis of inhibitor **2**. The desired inhibitor **3** was obtained as an oil (44% yield). ^{1}H NMR (500 MHz, $CDCl_3$, at 47 °C): δ 7.62 (d, *J* = 7.5 Hz, 1H), 7.60 (s, 1H), 7.40 (d, *J* = 7.5 Hz, 1H), 7.35-7.26 (m, 3H), 7.25-7.20 (m, 2H), 7.19-7.14 (m, 5H), 7.13-7.06 (m, 2H), 7.02-6.96 (m, 1H), 6.04 & 4.90 (2s, 1H), 4.34 (p, *J* = 7.0 Hz, 1H), 3.75 (dt, *J* = 7.5 Hz & 5.0 Hz, 1H), 3.67-3.60 (m, 1H), 3.07 (d, *J* = 4.5 Hz, 1H), 3.05-2.97 (m, 2H), 2.95 & 2.94 (2s, 3H), 2.80-2.69 (m, 3H), 2.58 (b s, 3H), 1.68 (sept, *J* =6.5 Hz, 1H), 1.51 (d, *J* = 6.5 Hz, 3H), 1.48-1.41 (m, 1H), 1.41-1.35 (m, 2H) 1.35-1.25 (m, 1H), 0.82 (t, *J* = 7.5 Hz, 3H), 0.81 (d, *J* = 9.0 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, at 47 °C): δ 172.92, 171.20, 167.46, 140.05, 137.83, 137.17, 135.26, 129.44, 128.92, 128.83, 128.66, 127.84, 126.88, 125.44, 72.48, 71.52, 67.23, 54.92, 50.53, 46.84, 36.85, 35.94, 28.66, 20.32, 19.24, 14.11. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{37}H_{50}N_4O_5$, 631.3854; found, 631.3860.

3. Analytical Purity of Inhibitors:

Table 2. Analytical Purity of Inhibitors determined by HPLC.

Inhibitor	Retention Time (min)	Purity (%)	Column	Flow rate (mL/min)
2	10.10	93	YMC-Pack-ODS-A 5μm 250X4.6mm	1
13	20.08	96	YMC-Pack-ODS-A 5 μM 250X4.6 mm	1
14	26.29	98	YMC-Pack-ODS-A 5 μM 250X4.6 mm	1
15	23.15	98	YMC-Pack-ODS-A 5 μM 250X4.6 mm	1
18	25.04	99	YMC-Pack-ODS-A 5 μM 250X4.6 mm	1
20	25.97	97	YMC-Pack-ODS-A 5 μM 250X4.6 mm	1
3	12.44	98	YMC-Pack-ODS-A 5μm 250X4.6mm	1

HPLC Conditions: t = 0-1 min [CH_3CN:H_2O (with 0.05% TFA) 20:80], t = 1-15 min [*gradient to* CH_3CN:H_2O (with 0.05% TFA) 90:10], 15-20 min [CH_3CN:H_2O (with 0.05% TFA) 90:10]; single detection wavelength λ = 215 nm, T = 25 °C.

4. Co-crystallization and X-ray structure determination of Memapsin 2 in complex with inhibitor 2

Crystals of memapsin 2 in complex with inhibitor **2** (GRL-0211)[6] were obtained using the hanging-drop, vapor-diffusion method. A solution of memapsin 2 protein (3-6 mg/ml) was mixed with inhibitor **2** (200 μM) and allowed to incubate for at least 1 h in order to form the Memapsin 2 and inhibitor **2** complex. 2 μL of the Memapsin-2 and inhibitor **2** complex was then mixed with 1 μL of the reservoir solution that contained 0.2 M $MgSO_4$, 0.1 M Na citrate (pH varied from 5.0 to 6.0) and 14 % to 20 % PEG4000.

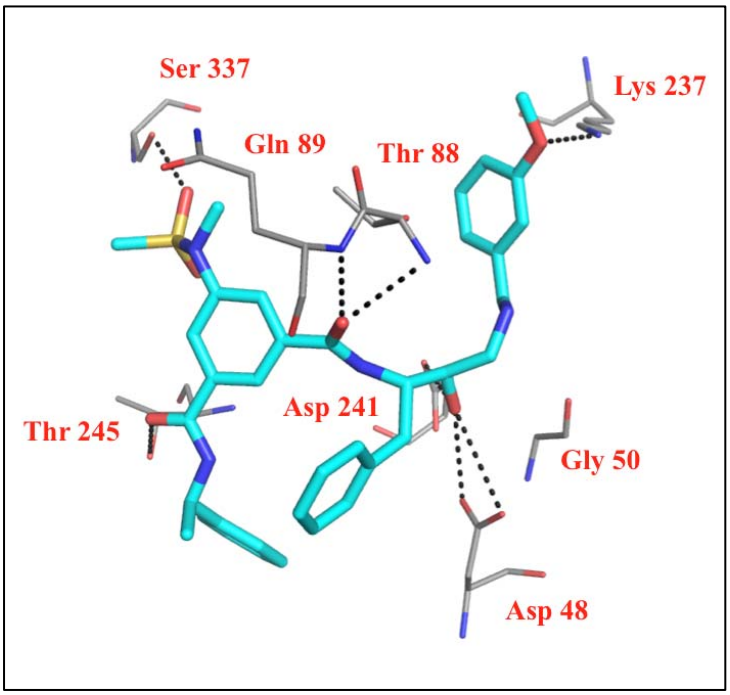
Crystals suitable for X-ray data collection grew within two weeks. Crystals were retrieved with a nylon loop, which was then used to swipe the crystals through the well solution supplemented with 30 % glycerol. The crystals were immediately flash-cooled by plunging into liquid nitrogen. Crystals were stored in shipping dewars containing liquid nitrogen until X-ray data collection. All diffraction data were collected at beamline 21-ID-G at the Life Science Collaborative Access Team (LS-CAT), at the Advanced Photon Source (APS), at Argonne National Laboratories. X-ray data were processed and scaled using the program HKL2000.[7] Scaled and merged intensity data were converted to structure factor amplitudes using CCP4.[8] The structure of the complex belonged to the primitive monoclinic space group $P2_1$ with three molecules in the asymmetric unit. Molecular library files and coordinates for the inhibitors were built using the program Phenix.[9] The inhibitors were manually modeled into electron density using the program Coot.[10] Fourier maps were calculated and visualized using the program Coot, and the structure was refined using the program Phenix. Iterative rounds of manual building and refinement were continued until R-work and R-free values reached their lowest values. Electron density maps presented in the figures were calculated using Phenix and the figures were generated using the program PyMoL molecular graphics system, Version 1.7.2.1 Schrodinger, LLC.

5. Docking studies of inhibitors in BACE2 active site

The protein and the ligand were prepared for docking studies using AutoDockTools (ADT).[11] The 3D structure of BACE2 (PDB ID: 2EWY) was downloaded from the protein databank (PDB). Only one of the monomers in the asymmetric unit, Chain A, was extracted from the file using the program PyMoL molecular graphics system, Version 1.7.2.1 Schrodinger, LLC. The polar hydrogens were added to the protein by using ADT. The edited protein structure was saved in PDBQT format for later molecular modeling. The ligand coordinates were extracted from PDB files (2VKM for inhibitor **1** (GRL-8234) and 5DQC for inhibitor **2** (GRL-0211)), and the information of rotatable bonds was added to the PDB file and saved in PDBQT format. The binding site for docking was selected by using ADT. The center of the grid box was set at 104.817, 33.522, -0.254. The grid box dimensions were chosen to cover the whole

binding site by using the Grid option widget in ADT. The docking was performed with AutoDock Vina.[12]

[A]



[B]

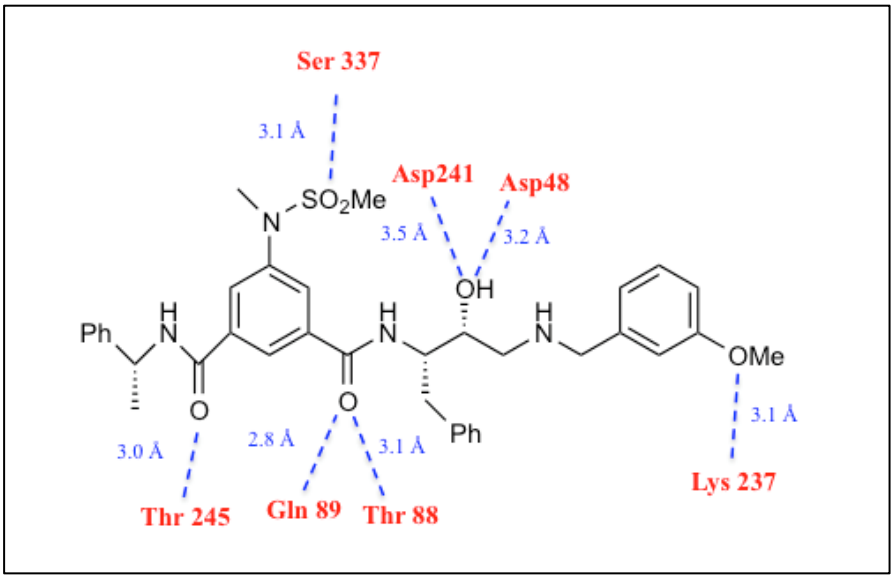


Figure 5. An energy-minimized model of inhibitor **1** (turquoise) in BACE2 active site (**Figure A**). All strong hydrogen bonding interactions are shown as dotted lines (**Figure B**).

6. Table 3. Data collection and refinement statistics

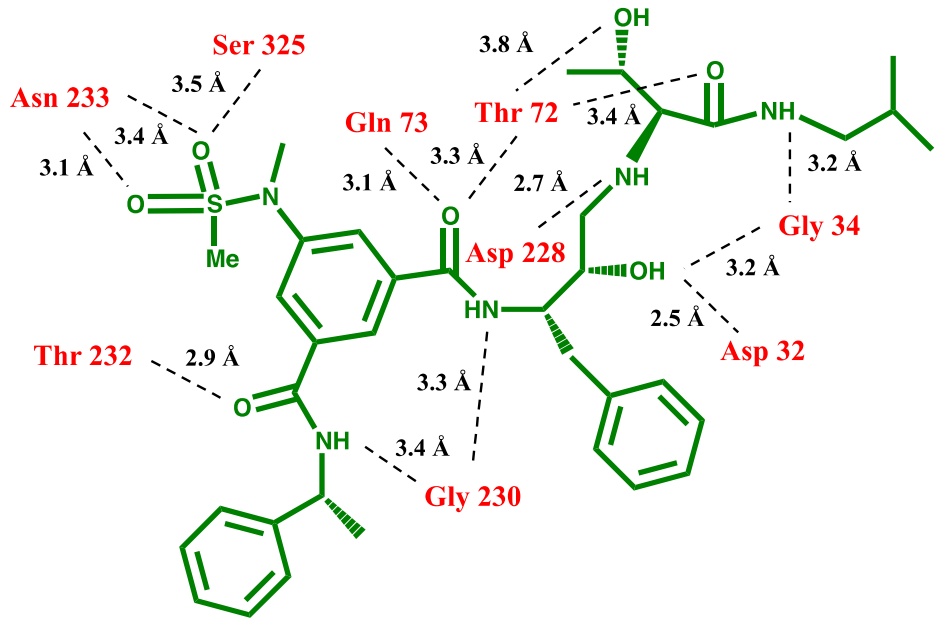
Memapsin 2 in complex with inhibitor **2** (GRL0211)	
Data Collection	
X-ray source and detector	LS-CAT Sector 21 ID-G
Wavelength (Å)	0.9786
Space Group	$P2_1$
Unit Cell dimensions:	
a, b, c (Å)	81.95, 103.40, 100.83
α, β, γ (°)	102.6
Data Processing Statistics	**Overall [Last Shell]**
Resolution range (Å)	100-2.5 [2.54-2.50]
No. reflection recorded	1,173,727
No. averaged reflections	58,492
Average Redundancy	3.4 [3.2]
R_{merge} (%)[a]	6.5 [69.1]
Rpim[b]	4.0 [45.3]
I/σI	26.5 [1.7]
% Completeness	90.6 [86.1]
Refinement	
Resolution Range (Å)	45.76-2.46 [2.52-2.46]
No. Reflections in Working Set	52,944
No. Reflections in Test Set	1999
R_{work} (%) [c]	18.47
R_{free} (%) [d]	23.52
Average B-factor (Å²)	60
RMSD from ideal geometry:	
Bond Lengths (Å)	0.005
Bond Angles (degrees)	0.963
Ramachandran Plot	
Most Favored (%)	96.81

Allowed (%)	3.10
Disallowed (%)	0.09

[a] $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of a given reflection, and $\langle I(hkl)\rangle$ is the mean intensity of symmetry-related reflections.
[b] $R_{pim} = \sum_{hkl}\sqrt{1/n-1}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where n is the multiplicity. R_{pim} is multiplicity-weighted R_{merge}.
[c] $R_{work} = \sum_{hkl}||F_{obs}| - |F_{calc}|| / \sum_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.
[d] R_{free} was calculated using 5% of the data set chosen at random that were excluded from the refinement.

7. Distances in Angstroms of all key H-bonds of inhibitor **2** in BACE 1 and BACE 2 active site.

(A)



(B)

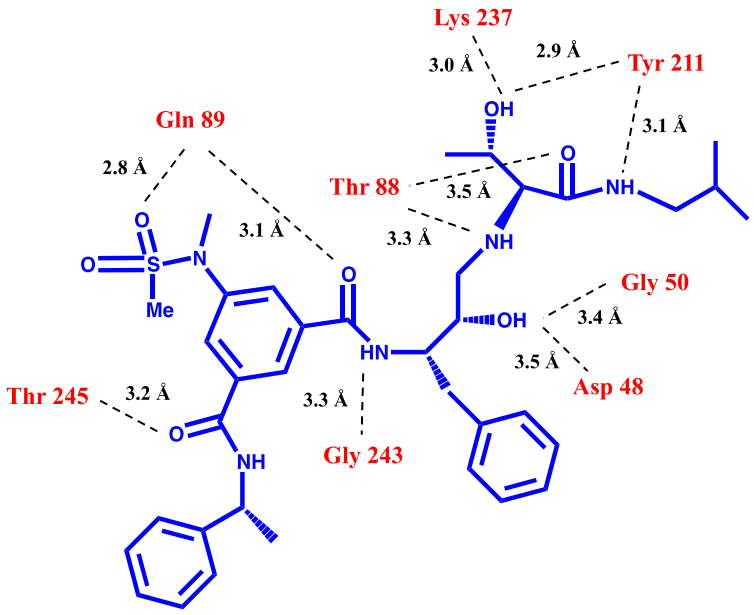


Figure 6. **A.** representation of polar contact interactions of the X-ray structure of inhibitor 2 (green color) in complex with BACE 1 (memapsin 2) (PDB code: 5DQC); **B.** the energy-minimized model of inhibitor **2** (turquoise) bound within the BACE2 (memapsin 1) active site. All strong hydrogen bonding interactions are shown as dash lines and the distances are shown in between.

8. Determination of Ki values against BACE1 and BACE2

A FRET-based (Fluorescence resonance energy transfer) assay was used to monitor the enzymatic activity of BACE1[13] and BACE2.[14] The assay was performed with a fluorogenic, 8-mer peptide substrate (Mca-S-E-V-N-L-D-A-E-F-K-Dnp). Assays (100 µL final volume) were conducted in black, half-area 96-well plates (Corning Glass). Each assay contained a final concentration of 100 nM for BACE1 or BACE2, and the inhibitor concentrations were varied in the reaction buffer (0.1 M acetic acid, pH 4). The inhibitors were first incubated in the assay buffer for 5 minutes at 37°C. The BACE enzyme was then added to the above solution and further incubated for an additional 10 minutes at 37°C. The enzymatic reactions were then initiated by the addition of peptide substrates to a final concentration 1 µM. The increase in the fluorescence of the reaction at 393 nm (excitation wavelength = 328 nm) was measured using a BioTek Synergy plate reader.

The initial slopes (RFU/min) of each reaction were determined and then analysed using the Morrison equation. All reactions were carried out in triplicate.

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