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Synthesis and biological evaluation of phosphino dipeptide isostere inhibitor of human β-secretase (BACE1)

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Abstract—Phosphino dipeptide (PDP) isosteres are known to be useful analogues of the transition state of metalloprotease substrates. Here we describe the use of this unit for the design of aspartic protease inhibitors. A PDP analogue of OM00-3, a potent BACE1 inhibitor, was synthesized and exhibited high biological activity ($IC_{50} \sim 12$ nM).

1. Introduction

A major factor in the pathogenesis of Alzheimer's disease (AD) is the cerebral deposition of amyloid fibrils as senile plaque.[1] The cerebral deposited amyloid fibrils consist of 40- and 42-residue long amyloid β peptides (Aβ) which are generated by proteolytic processing of β-amyloid precursor protein (APP).[2,3] Key players in deposition of Aβ are the proteolytic enzymes β- and γ-secretases. The mechanistic class of γ-secretase, which liberates the carboxy terminus of APP, is not fully understood but is thought to be an unusual aspartic protease.[4–8] Since β-secretase (BACE1), a unique member of the pepsin family of aspartic proteases, initiates the pathogenic processing of APP by cleaving at the N-terminus, it is a molecular target for therapeutic intervention in AD.[8–13] Several groups reported BACE1 inhibitors based on transition-state analogues for the scissile peptide bond, such as hydroxymethylcarbonyl,[13] hydroxyethylene,[10,14,15] statine,[11,14] hydroxyethylamine,[16] norstatine[17] and others, which are known to inhibit aspartic protease activity. We report here the use of the phosphino dipeptide (PDP) isostere for design of BACE1 inhibitors. During the course of our work we became aware of a new patent application which also uses PDP isosteres for BACE1 inhibition.[18] The PDP unit is biocompatible as it is used in approved drugs, such as ACE inhibitors.[19] Our compound was designed in such a way that the phosphinic acid replaces the corresponding dipeptide fragment (Fig. 1) of the counterpart peptide.

Thus, the phosphinic acid moiety is an excellent mimic of the tetrahedral transition state of amide bond hydrolysis. One could also assume to use a phosphonamide as tetrahedral transition state analogue, but several publications have alluded to the instability of the phosphonamide bond at acidic and even physiological pH.[20,21] Phosphino peptides have been used for rational drug design for inhibitors of proteases,[22] especially for inhibitors of the metalloprotease family.[23] One of the most potent BACE1 inhibitors OM00-3[15] ($K_i = 0.3$ nM) is a 2nd generation inhibitor of BACE1, based on the sequence of Swedish mutant APP. We present a novel potent octapeptide inhibitor (**5a**), based on OM00-3, with a PDP isostere as a transition-state mimic (Fig. 1).

2. Results

2.1. Synthesis of the phosphino dipeptide isostere

The synthesis of the PDP isostere started with the preparation of [1-[(Benzyloxycarbonyl)-amino]-3-methylbu-

Keywords: Alzheimer's disease; β-Secretase inhibitors; BACE1; Phosphino dipeptide (PDP) isostere.
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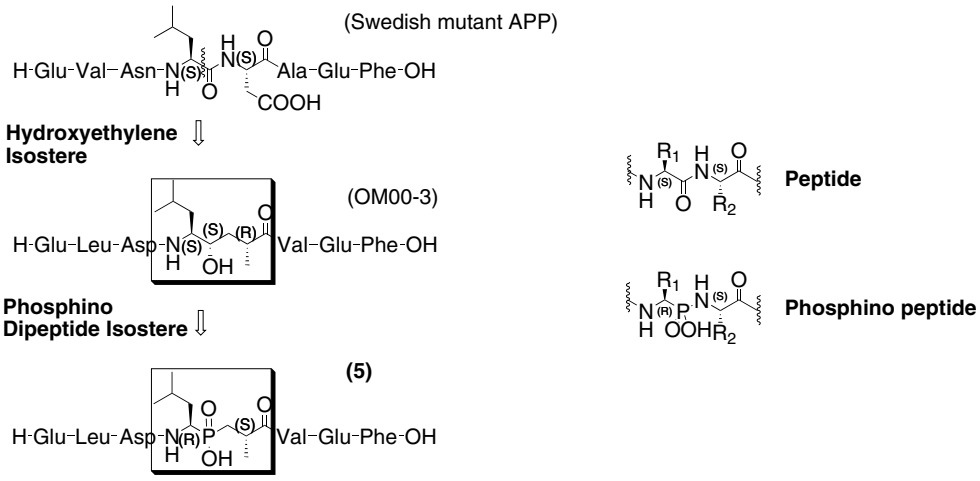
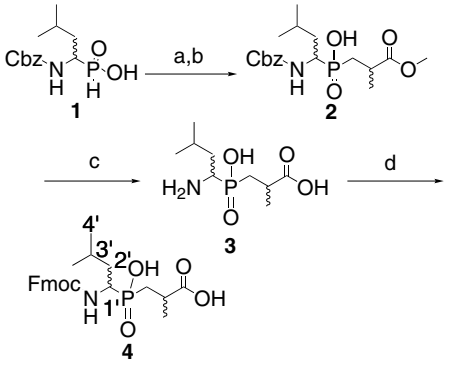


Figure 1. Design of phosphino dipeptide isostere inhibitor (5) of BACE1.

tyl] phosphinic acid (**1**) as described by Baylis et al.[24] Usually PDP isosteres are synthesized according to Boyd et al.[25] by addition of the amino phosphinate to an acrylate in the presence of strong base or an amino phosphinic acid preactivated as trivalent silylether.[26,27] We used the second procedure (Scheme 1). **1** was dissolved and activated by heating with freshly distilled hexamethyldisilazane under argon atmosphere to form the intermediate bis(trimethylsilyl)phosphonite (BTSP). The desired compound **2** is then formed in a Michael type reaction of BTSP to α methyl acrylate. Deprotecting and protecting steps to obtain the required Fmoc PDP isostere **4** for solid support synthesis of **5** proved to be difficult as the unprotected phosphinic acid is not optimal for the use of hydrogenation protocols.

However, clean and full deprotection was obtained by the use of HI, because benzyl carbamates are readily cleaved under strong acid conditions and the nucleophilic iodide can also cleave the methylester via an S_N2-type reaction. Therefore, compound **2** was refluxed for 2 h in 57% aqueous solution of HI. After evaporation of the solution, the crude product was dissolved in water. ESI-MS showed clean conversion to **3** which was directly transformed without further purification into the Fmoc-protected compound **4**. Recently an alternative way to compounds similar to **4** via a three-component condensation reaction of Fmoc-carbamate, aldehyde and alkylphosphinic acid as key step was reported without deprotection and protection steps.[28]



Scheme 1. Syntheses of Fmoc-LeuΨ[POOH-CH_2]-Ala-COOH **4**. Reagents and conditions: (a) 5 equiv HMDS, 100–110 °C, 2 h (Ar); (b) 60 °C, 1.25 equiv methacrylacidmethylester, 85–90 °C, 3 h (Ar) (91%); (c) 57% aq HI, 2 h; (d) 40% Na_2CO_3 (dioxane), 0 °C, 1.2 equiv Fmoc-Cl, 12 h (74% over two steps).

2.2. Synthesis of the PDP isostere containing inhibitor

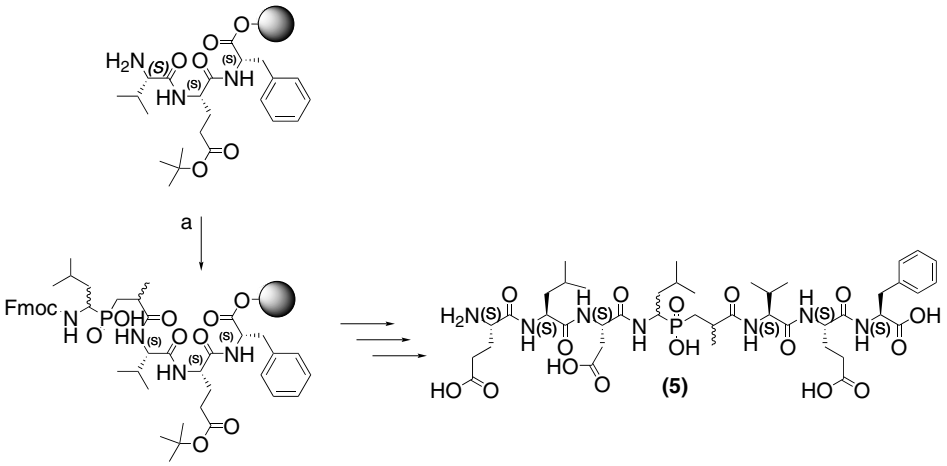
After synthesis of the key fragment **4** the amino acid sequence H-Val-Glu(O*t*-Bu)-Phe-OH was built up on trityl chloride polystyrene resin (TCP) using standard Fmoc solid-phase conditions.

Elongation of the peptide strand at the N-terminus with the analogue phosphinoalkylester of **4** has been successfully performed in solution.[29,30] However, the use of phosphino alkylesters complicates analysis, since ester protection introduces a chiral center on the phosphorus atom, thus increasing the number of diastereoisomers and finally deprotection of the phosphorus acid is required. High yields of the desired phosphino peptide can be obtained when using PyBOP® as coupling reagent.[31] Compound **4** was therefore coupled to the preloaded resin by activation with PyBOP® without conversion into the phosphino alkylester (Scheme 2). The amino acids aspartate, leucine and glutamate were coupled to the sequence using standard Fmoc conditions and *tert*-butyl ester as protection of the acid functionality.

After cleaving the phosphino peptide from the resin by adding TFA in DCM (1/1) for 2 h the fully unprotected phosphino peptide was purified by semi-preparative reversed phase HPLC. Three baseline fractions **5a**, **5b**, **5c** could be separated. The relative molar ratio of the fractions was approximately 1/2/1 for **5a/5b/5c**. Analytical HPLC-MS showed that one of the three fractions **5b** consisted of two diastereomers of equal amount while the others were optical pure compounds.

2.3. Biological evaluation of phosphino dipeptide isostere inhibitor

The inhibitory effects of these phosphino peptides against BACE1 were examined by a biochemical assay.



Scheme 2. Solid-phase synthesis of **5**. Reagents: (a) i—2.5 equiv **4**, 2.5 equiv. PyPOP, 5 equiv DIPEA,NMP; ii—wash with NMP.

The results and analytical data are shown in Table 1. The fraction containing two diastereomers **5b** in a ratio of 1:1 had an activity of 675 nM. The two pure diastereomers had activities of 12 nM for **5a** and 2.0 μM for **5c**. The activity of a single diastereomer of fraction **5b** could only reach an IC_{50} value of maximal ~340 nM (factor 2), if the other would have no activity at all. Therefore, separation of the two diastereomers in fraction **5b** was not necessary as **5a** is the most potent inhibitor of the four diastereomers.

While OM00-3 under these assay conditions has an activity of 6 nM, we loose only a factor of two in activity by using a PDP isostere.

2.4. Identification of the absolute chirality of position 1′ in the PDP isostere (Scheme 1) in 5a

We supposed the stereo centers of the PDP isostere in **5a** to have the same configuration as the native peptide, this means (*S*,*S*) for the peptide corresponds with (*R*,*S*) in the PDP isostere (The priority rule switches due to the phosphorus atom) (Fig. 1). Highly convenient routes for the synthesis of chiral α-aminophosphonic acids using the 'Pudovik reaction' are known.[32–34] The approaches are based on the addition of either hypo phosphorus acid salt of (*R*)- or (*S*)-α-methylbenzylamine to aldehydes in refluxing ethanol solution or on the acid-catalyzed addition of dialkyl phosphites to chiral aldimines, built from an aldehyde and (*R*)- or (*S*)-α-methylbenzylamine. As a rule, the amine in (*S*)-configuration introduces the new asymmetric center due to steric demands of the imine double bond similar to the 1,3 allylic strain in olefins in (*R*)-configuration, and the amine in (*R*)-configuration leads to amino phosphinic acid in (*S*)-configuration.[30–32] We decided to use (*S*)-1-(2-naphthyl)-ethylamine **6** as chiral auxiliary (Scheme 3). We synthesized the imine by condensation of isovaleraldehyde **7** and chiral amine **6**. The isolated imine was directly added to hypo phosphorus acid in dry THF at 0 °C, which formed overnight the phosphinic acids **8** and **9**. The diastereoselectivity of this reaction detected by analytical HPLC at 254 nM was found to be (2:1/SR:SS). Unfortunately the elimination of the chiral auxiliary to obtain the free amine was only achieved when using bromine. This was accompanied with the oxidation of the phosphor to the corresponding α-amino phosphonic acid.[35] Therefore, we separated the diastereomers **8** and **9** by semi-preparative HPLC and protected the secondary amine by acetylation. Attempts to react activated compound **11** with 2-Bromo, α-chiral propionic acid failed.

Table 1. IC_{50} values (nM) of the isolated diastereomeres against BACE1

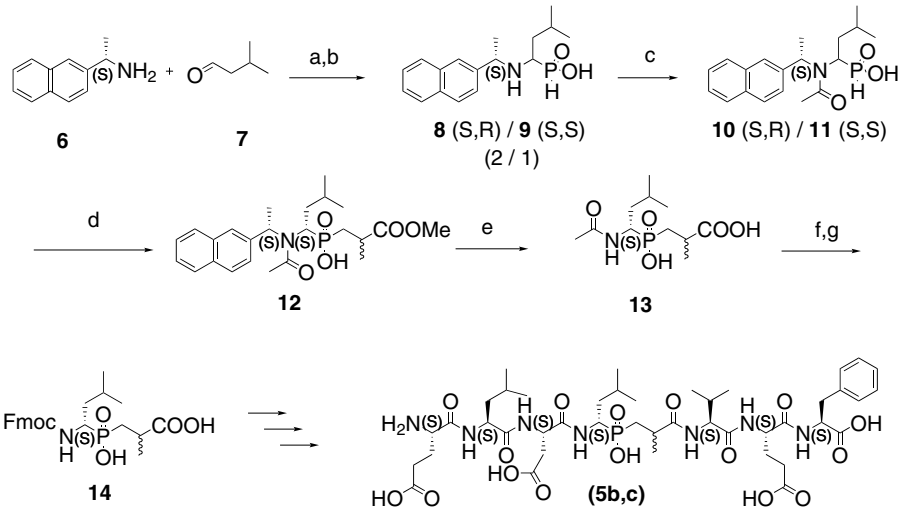
Compound	Inhibition IC_{50}[a] (nM)	Retention time (min)[b]	Amount of diastereomers in sample[b]
OM00-3	6 (±0.7)	—	1
5a	12 (±2)	11.4	1
5b	675 (±197)	12.6, 13.1	2
5c	2020 (±673)	13.7	1

[a] Values are means of two experiments, standard deviation is given in parentheses.
[b] Detected by HPLC ESI-MS (gradient 20–40%, 30 min).

The (*S*,*S*) diastereomer **11** was then activated by HMDS to obtain the second stereo center racemic **12**. Treatment of the protected PDP isostere **12** with HI gave the C-terminal free acid and the N-terminal acetamide **13** in good yields. The acetamide was cleaved under acid conditions, treated with sat. Na_2CO_3 to obtain a pH of 8 and addition of Fmoc-Cl gave **14**. Peptide synthesis yielded two diastereomers, which could be identified by HPLC retention times as **5b** and **5c**. Therefore, we verified the stereo center at position 1′ of the PDP isostere in **5a** to be *R*, as expected.

2.5. Selectivity against other aspartylproteases

Finally, we were interested in the selectivity of **5a** against other aspartic proteases like BACE2 (the closest relative of BACE1), cathepsin D and pepsin. The selectivity of BACE1 inhibitors against other human aspartic proteases is expected to be difficult to obtain since the catalytic domains of these proteases are highly similar.



Scheme 3. Synthesis of the PDP isostere with fixed chirality of the isopropyl group. Reagents and conditions: (a) Magnesium sulfate, 0 °C, 1 h (Ar), (benzene); (b) 5 equiv hypophosphorous acid, 0 °C, overnight (Ar), (THF), (40% over two steps); (c) 3 equiv NEt_3, 0 °C, 2 h (Ar), (THF), 1.5 equiv AcBr, rt, overnight (Ar), (THF), (73% for 8, 94% for 9); (d) 5 equiv HMDS, 100–110 °C, 2 h (Ar), 60 °C, 1.25 equiv methacrylacidmethylester, 85–90 °C, 3 h (Ar) (85%); (e) 57% aq HI, 100 °C, 2 h (62%); (f) 8 N HCl, 100 °C, 12h (g) sat. Na_2CO_3 (dioxane), 0 °C, 1.2 equiv Fmoc-Cl, 12 h (56% over two steps).

Table 2. IC_{50} values (nM) of the isolated diastereomers against BACE1, BACE2, Cathepsin D and Renin[a]

Compound	BACE1	BACE2	Cathepsin D	Pepsin
5a	12 (±2)	12 (±1)	28 (±8)	8 (±4)

[a] Values are means of two experiments, standard deviation is given in parentheses.

Cathepsin D plays an important role in cellular protein catabolism and is present in all cells.[36] Its inhibition would thus probable cause toxicity. Pepsin is responsible in the gut for protein break-down. The physiological function of BACE2 which is widely expressed in the body still needs to be identified.[37]

After testing we found **5a** IC_{50} values against the other aspartic proteases to be all in a low nanomolar range (Table 2). This displays no selectivity over the other aspartic proteases. Comparing with other reported inhibitors against BACE1 that also lack selectivity towards these proteases it becomes obvious that achieving the desired selectivity stays furthermore a major challenge in this field. Progress to understand the important features to develop selective inhibitors from potent unselective ones for BACE1 inhibition was made by Tangs et al.[38]

3. Conclusion

In conclusion, a novel class of BACE1 inhibitors was synthesized using a PDP isostere as transition state mimic. **5a** showed remarkable BACE1 inhibitory activity (IC_{50} = 12 nM) of about the same potency as OM00-3 (IC_{50} = 6 nM) under these assay conditions and therefore demonstrates the use of the PDP isostere in further development of potent BACE1 inhibitors.

4. Experimental

4.1. General

If no detailed information is given NMR spectra were recorded on a Bruker DMX 600 (600 MHz) spectrometer. Chemical shifts are reported in ppm with the solvent signal as the internal standard. Data are reported as follows: chemical shifts (ppm), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet), integration, coupling constants (Hz). Low resolution mass spectra were recorded on a Finnigan MAT LCQ with electro-spray ionization. Analytical HPLC was performed on a Pharmacia Biochem P-900 with detection at 220 and 254 nM with a linear gradient over 30 min (90:10 to 10:90 water/acetonitrile, containing 0.1% TFA). Semi preparative reverse phase chromatography was performed using a Beckman System GOLD and a S-5μm column (YMC-PACK ODS-A). Solvents for chromatography were of HPLC grade. TLC was performed on Merck Silica gel 60 F_{254} plates. Unless otherwise noted, all reactions were conducted in oven (60 °C)-dried glassware with magnetic stirring.

4.1.1. (1-Benzyloxycarbonylamino-3-methylbutyl) phosphinic acid (1). 1.56 g (10.32 mmol; 151.14 g/mol) of 1-amino-3-methylbutylphosphinic acid was dissolved in 20 mL of a 1N $NaHCO_3$-solution and 1.67 mL (11.87 mmol; 170.59 g/mol) benzylchloroformiate was added. After addition of 20 mL dioxane the solution was stirred for 24 h. The dioxane was removed and the solution diluted with 1N $NaHCO_3$. The aqueous phase was twice washed with ether. HCl (3N) was used to acidify the solution until no more precipitation was observed. After extraction with ethyl acetate and drying with Na_2SO_4, removal of the organic solvent provided 2.13 g (73%) of a white solid. ^1H NMR (500 MHz, DMSO-d_6) δ 7.54 (d, J = 9.2 Hz, 1H), 7.38–7.27 (m,

5H), 6.72 (d, J = 526.6 Hz, 1H), 5.03 (s(br), 2H), 3.67–3.55 (m, 1H), 1.69–1.58 (m, 1H), 1.54–1.43 (m, 1H), 1.40–1.30 (m, 1H), 0.88 (d, J = 6.5 Hz, 3H), 0.82 (d, J = 6.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 156.7, 137.5, 128.8, 128.2, 128.0, 66.0, 49.3 (d, $^{1}J_{PC}$ = 105.7 Hz), 35.4, 24.4 (d, $^{3}J_{PC}$ = 12 Hz), 23.6, 21.4. ^{31}P NMR (243 MHz, D_2O/NaOH) δ 33.1. MS (ESI) m/z 286.1 $(M+H)^+$, 593.4 $(2M+H)^+$, 609.4 $(2M+K)^+$, 878.3 $(3M+Na)^+$, 894.2 $(3M+K)^+$. RP–HPLC t_R = 16.8 (10–90%).

4.1.2. 2-Methyl-3-[(1-benzyloxycarbonylamino-3-methyl-butyl)-hydroxy-phosphinoyl]-propionic acid methyl ester (2). 1.2 g (4.2 mmol; 285.28 g/mol) of **1** was dissolved in freshly distilled hexamethyldisilazane and refluxed for 2 h under argon atmosphere. After cooling to 60 °C 0.57 mL (5.4 mmol; 100.12 g/mol; 0.943 g/mL) freshly distilled 2-methyl-acrylic acid methyl ester was added and the mixture was refluxed for 3 h. The suspension was cooled to 60 °C and 11 mL abs. ethanol was added. The solvent was evaporated, the white solid taken up in 50 mL ethyl acetate and the organic layer was washed twice with 5% HCl and with brine. After drying over Na_2SO_4 the solvent was removed to yield 1.47 g (91%) of a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.46 (d, J = 8.7 Hz, 1H), 7.40–7.26 (m, 5H), 5.06 (d, J = 36 Hz, 1H), 5.02 (d, J = 36 Hz, 1H), 3.72–3.62 (m, 1H), 3.57 (s, 3H), 2.77–2.69 (m, 1H), 2.03–1.93 (m,1H), 1.65–1.55 (m, 2H), 1.54–1.45 (m, 1H), 1.43–1.35 (m, 1H), 1.17–1.12 (m, 3H), 0.87 (d, J = 6.1 Hz, 3H), 0.80 (d, J = 6.1 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.5 (d, $^{3}J_{PC}$ = 11.7 Hz), 156.4, 137.7, 128.5, 127.9, 127.6, 127.5, 65.6, 51.7, 49.1 (d, $^{1}J_{PC}$ = 107.8 Hz, I), 48.7 (d, $^{1}J_{PC}$ = 107.8 Hz, II), 35.8 (d, $^{2}J_{PC}$ = 10.6 Hz), 33.4 (d, $^{2}J_{PC}$ = 10.6 Hz), 29.7 (d, $^{1}J_{PC}$ = 89 Hz, I), 29.5 (d, $^{1}J_{PC}$ = 89 Hz, II), 24.2 (d, $^{3}J_{PC}$ = 11.1 Hz), 23.4, 21.0, 19.0 (d, $^{3}J_{PC}$ = 8.3 Hz, I), 18.7 (d, $^{3}J_{PC}$ = 8.3 Hz, II). ^{31}P NMR (243 MHz, DMSO-d_6) δ 45.2. MS (ESI) m/z 386.3 $(M+H)^+$, 793.4 $(2M+H)^+$, 709.4 $(2M+K)^+$, 1194.5 $(3M+K)^+$. RP–HPLC t_R = 19.6 (10–90%).

4.1.3. 2-Methyl-3-[(1-9-fluorenylmethoxycarbonylamino-3-methyl-butyl)-hydroxy-phosphinoyl]-propionic acid (4). 500 mg of **2** (385.4 g/mol; 1.3 mmol) was taken up with 15 mL of 57% HI and refluxed for 2 h. After cooling the solvent was removed in vacuo with use of a nitrogen condenser. The residue was taken up with water and extracted with ethyl acetate (under addition of brine to get phase separation) until the water layer was clear. After the water was removed the residue was triturated with ethanol. The remaining NaCl was filtered off and evaporation of the solvent provided 330 mg (93%) chloride salt product as yellow oil. MS (ESI) m/z 238.1 $(M+H)^+$, 475.4 $(2M+H)^+$, 497.4 $(2M+Na)^+$, 513.5 $(2M+K)^+$, 712.4 $(3M+H)^+$, 734.4 $(3M+Na)^+$, 750.5 $(3M+K)^+$. TLC (DCM/MeOH/HOAc = 5/1/1) R_f = 0.23.

287 mg (1.05 mmol; 273.68 g/mol) of **3** hydrochloride was dissolved in 5 mL of 40% Na_2CO_3 and 5 mL of a water/dioxane mixture (2:3) was added. The mixture was cooled to 0 °C, 333 mg (1.25 mmol; 258.7 g/mol) Fmoc-Cl was added slowly and remained stirring overnight. The suspension was diluted with 20 mL water and acidified with 2N HCl to pH 2.5 to form a white precipitate. After extraction with ether the organic layer was washed with water, dried over Na_2SO_4 to yield 379 mg (79%) of a clear hygroscopic oil. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.87 (d, J = 7.7 Hz, 2H), 7.70 (d, J = 7.2 Hz, 2H), 7.56–7.48 (m, 1H), 7.44–7.36 (m, 2H), 7.35–7.26 (m, 2H), 4.34–4.25 (m, 2H), 4.23–4.17 (m, 1H), 2.70–2.59 (m, 1H), 2.04–1.94 (m, 1H), 1.65–1.48 (m, 3H), 1.46–1.36 (m, 1H), 1.26–1.19 (m, 1H), 1.14 (d, J = 6.7 Hz, 3H), 0.88 (d, J = 6.7 Hz, 3H), 0.79 (d, J = 6.7 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.8, 156.3, 144.1, 143.9, 140.9, 127.8, 127.3, 127.2, 125.4, 120.3, 65.7, 49.6 (d, $^{1}J_{PC}$ = 108.1 Hz, I), 48.8 (d, $^{1}J_{PC}$ = 108.1 Hz, II), 46.9, 36.0 (d, $^{2}J_{PC}$ = 8.6 Hz), 33.5 (d, $^{2}J_{PC}$ = 8.6 Hz), 30.0 (d, $^{1}J_{PC}$ = 87.1 Hz, I), 29.7 (d, $^{1}J_{PC}$ = 87.1 Hz, II), 24.2 (d, $^{3}J_{PC}$ = 11.3 Hz), 23.6, 21.1, 19.2 (d, $^{3}J_{PC}$ = 5.7 Hz, I), 18.9 (d, $^{3}J_{PC}$ = 5.7 Hz, II).

^{31}P NMR (243 MHz, DMSO-d_6) δ 45.5 (br). MS (ESI) m/z 460.3 $(M+H)^+$, 919.3 $(2M+H)^+$, 941.2 $(2M+Na)^+$, 1400.2 $(3M+Na)^+$. RP–HPLC t_R = 21.8 (10–90%).

4.1.4. 5H-(*S*)Glu-(*S*)Leu-(*S*)Asp-*rac*LeuΨ[POOH-CH$_2$]-*rac*Ala-(*S*)Val-(*S*)Glu-(*S*)Phe-OH. Peptide synthesis see Supplementary information.

4.1.5. H-(*S*)Glu-(*S*)Leu-Asp-(*R*)LeuΨ [POOH-CH$_2$]-Ala-(*S*)Val-(*S*)Glu-(*S*)Phe-OH (5a). ^{1}H NMR from (TOCSY) δ 8.78, 8.68, 8.51, 8.38, 8.03, 7.42, 7.23, 6.69, 4.48, 4.39, 4.20, 4.18, 4.07, 3.88, 3.85, 3.03, 2.98, 2.74, 2.65, 2.61, 2.34, 2.22, 2.15, 2.05, 1.95, 1.83, 1.79, 1.58, 1.57, 1.53, 1.50, 1.50, 1.47, 1.11, 0.88, 0.84, 0.82. ^{31}P NMR (364 MHz, 7.5 mM Na_2HPO_4-Buffer, pH 5.2, 20% D_2O) δ 38.7. MS (ESI) m/z 970.5 $(M+H)^+$. RP–HPLC t_R = 13.7 (10–90%).

4.1.6. H-(*S*)Glu-(*S*)Leu-Asp-(*R*)LeuΨ [POOH-CH$_2$]-Ala-(*S*)Val-(*S*)Glu-(*S*)Phe-OH and H-(*S*)Glu-(*S*)Leu-Asp-(*S*)LeuΨ [POOH-CH$_2$]-Ala-(*S*)Val-(*S*)Glu-(*S*)Phe-OH (5b). MS (ESI) m/z 970.5 $(M+H)^+$. RP–HPLC t_R = 14.2 (10–90%).

4.1.7. H-(*S*)Glu-(*S*)Leu-Asp-(*S*)LeuΨ [POOH-CH$_2$]-Ala-(*S*)Val-(*S*)Glu-(*S*)Phe-OH (5c). MS (ESI) m/z 970.6 $(M+H)^+$. RP–HPLC t_R = 14.5 (10–90%).

4.1.8. (1R)-1-((*S*)-1-(Naphthalen-6-yl)ethylamino)-3-methylbutylphosphinic acid (8). 2 g of (*S*)-(-)-1-(2-Naphthyl) ethylamine (11.68 mmol, 171.2 g/mol) and 3.5 g dry $MgSO_4$ (2.5 equiv 29.2 mmol) were dissolved in 25 mL of dry benzene under argon atmosphere. After cooling to 0 °C 1.4 mL isovaleraldehyde (1.1 equiv 12.85 mmol, 86.1 g/mol) was added and the reaction mixture was stirred for 2 h after which the solvent was removed to afford pure (*S*,*E*)-*N*-(3-methylbutylidene)-1-(naphthalen-2-yl)ethanamine in quantitative yield (2.8 g). 4 g anhydrous hypo phosphorus acid (5 equiv 60.6 mmol, 66 g/mol) was dissolved in 20 mL of dry THF under argon atmosphere and cooled to 0 °C. The freshly prepared (*S*,*E*)-*N*-(3-methylbutylidene)-1-(naphthalen-2-yl)ethanamine (2.8 g, 11.69 mmol) was

dissolved in 10 mL dry THF and added dropwise to the acid solution. The solution was stirred overnight and was allowed to slowly warm to room temperature. The solvent was removed and enantiomerically pure 1-*S*-(475 mg, 13%) and 1-*R*-((*S*)-1-(naphthalen-2-yl)ethylamino)-3-methylbutylphosphinic acid (954 mg, 27%) was obtained by HPLC purification.

^{1}H NMR (900 MHz, DMSO-d_6) δ 8.04–7.99 (m, 2H), 7.96–7.90 (m, 2H), 7.69 (d, J = 8.5 Hz, 1H), 7.58–7.55 (m, 2H), 6.99 (d, J = 526 Hz, 1H), 4.98 (q, J = 6.5 Hz, 1H), 2.57–2.52 (m, 1H), 1.70–1.66 (m, 1H), 1.65 (d, J = 6.5 Hz, 3H), 1.50–1.43 (m, 1H), 1.40–1.34 (m, 1H), 0.66 (d, J = 6.5 Hz, 3H), 0.39 (d, J = 6.5 Hz, 3H). ^{13}C NMR (225 MHz, DMSO-d_6) δ 134.5, 132.7, 132.5, 128.7, 127.8, 127.7, 127.5, 126.6, 124.3, 57.2, 52.5 (d,$^1J_{PC}$ = 88.7 Hz), 35.6, 23.7, 21.9, 21.1, 20.0. δ. ^{31}P NMR (243 MHz, DMSO-d_6) δ 19.6 MS (ESI) m/z 306.1 $(M+H)^+$, 611.3 $(2M+H)^+$, 938.4 $(3M+Na)^+$, 954.4 $(3M+K)^+$. RP–HPLC t_R = 14.3 (10–90%).

4.1.9. (1S)-1-((*S*)-1-(Naphthalen-6-yl)ethylamino)-3-methylbutylphosphinic acid (9). See Ref.8.

^{1}H NMR (600 MHz, MeOH-d_4) δ 8.05–8.01 (m, 1H), 7.99 (d, J = 9.2 Hz, 1H), 7.93–7.88 (m, 2H), 7.64 (d, J = 8.7 Hz, 1H), 7.58–7.52 (m, 2H), 7.00 (d, J = 530 Hz, 1H), 4.96–4.89 (m, 1H), 2.89–2.81 (m, 1H), 1.91–1.81 (m, 2H), 1.79 (d, J = 6.6 Hz, 3H), 1.65–1.51 (m, 1H), 0.94 (d, J = 5.7 Hz, 3H), 0.76 (d, J = 5.7 Hz, 3H). ^{13}C NMR (225 MHz, MeOH-d_4) δ. 135.1, 134.7, 130.6, 129.2, 129.0, 128.9, 128.2, 128.0, 125.2, 58.6, 55.2 (d, $^1J_{PC}$ = 88.8 Hz), 36.3, 26.1, 23.5, 21.9, 19.5. ^{31}P NMR (243 MHz, MeOH-d_4) δ 18.7. MS (ESI) m/z 306.1 $(M+H)^+$, 611.3 $(2M+H)^+$, 916.3 $(3M+H)^+$, 938.3 $(3M+Na)^+$, 954.3 $(3M+K)^+$. RP–HPLC t_R = 14.9 (10–90%).

4.1.10. (1R)-1-(N-((*S*)-1-(Naphthalen-7-yl)ethyl)acetamido)-3-methylbutylphosphinic acid (10). 110 mg of **8** (360.2 μmol, 305.4 g/mol) was dissolved in 10 mL absolute THF under argon. The solution was cooled to 0 °C and 3 equiv. of 151 μL NEt_3 was added. After 2 h 1.5 equiv. of 44 μL AcBr (540 μmol, 122.9 g/mol) was added dropwise and the reaction mixture was stirred at room temperature overnight. The next morning the solution was evaporated to dryness. The crude product was taken up in 1 N HCl and extracted three times with 20 mL DCM. The organic layers were dried over Na_2SO_4 and the DCM was removed under vacuum to yield 91 mg of **10** (73%) ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.05 (s, 1H), 7.94–7.91 (m, 1H), 7.91-7.58 (m, 1H), 7.87 (d, J = 7.9 Hz, 1H), 7.62–7.57 (m, 1H), 7.53–7.48 (m, 2H), 6.91 (d, J = 573 Hz, 1H), 5.34–5.30 (m, 1H), 3.05–2.90 (m, 1H), 2.15 (s, 3H), 1.78–1.65 (m, 5H), 1.33–1.24 (m, 1H), 0.89–0.82 (m, 6H). ^{13}C NMR (225 MHz, DMSO-d_6) δ 170.1, 137.7, 132.7, 132.2, 128.0, 127.7, 127.3, 126.4, 125.9, 125.5, 56.4, 53.1 (d, $^1J_{PC}$ = 99.6 Hz), 37.7, 24.9, 23.2, 21.8, 21.5, 18.4. ^{31}P NMR (146 MHz, DMSO-d_6) δ 34.0 MS (ESI) m/z 348.2 $(M+H)^+$, 717.4 $(2M+Na)^+$, 1064.5 $(3M+Na)^+$, 1080.4 $(3M+K)^+$. RP–HPLC t_R = 20.2 (10–90%).

4.1.11. (1S)-1-(N-((*S*)-1-(Naphthalen-7-yl)ethyl)acetamido)-3-methylbutylphosphinic acid (11). 50 mg of **9** was taken and treated like 10 to yield 53 mg of **11** (94 %). ^{1}H NMR (900 MHz, DMSO-d_6) δ 7.95 (d, J = 8.1 Hz, 1H), 7.93 (s(br), 1H), 7.92–7.89 (m, 2H), 7.55–7.50 (m, 3H), δ 6.95 (d, J = 571 Hz, 1H), 5.34 (q, J = 6.5 Hz, 1H), 2.89–2.80 (m, 1H), 2.28 (s, 3H), 1.88–1.81 (m, 1H), 1.65 (d, J = 6.5 Hz, 3H), 1.04–0.96 (m, 1H), 0.73–0.65 (m, 1H), 0.29 (d, J = 5.8 Hz, 3H), −0.09 (d, J = 5.8 Hz, 3H). ^{13}C NMR (225 MHz, DMSO-d_6) δ 169.9, 137.1, 132.8, 132.4, 127.9, 127.7, 127.3, 127.1, 126.3, 126.2, 126.0, 56.0, 52.5 (d, $^1J_{PC}$ = 99.6 Hz), 37.1, 24.1, 22.8, 21.9, 20.4, 17.2. ^{31}P NMR (146 MHz, DMSO-d_6) δ 34.4 MS (ESI) m/z 348.2 $(M+H)^+$, 717.4 $(2M+Na)^+$, 1080.4 $(3M+K)^+$. RP–HPLC t_R = 19.9 (10–90%).

4.1.12. 2-Methyl-3-[(1S)-1-(N-((*S*)-1-(naphthalen-7-yl)ethyl)acetamido)-3-methyl-butyl-hydroxy-phosphinoyl]-propionic acid methyl ester (12). A suspension of 10 mg of **11** (28.8 μmol, 347.4 g/mol) in 1 mL freshly distilled hexamethyldisilazane was heated at 110 °C for 1 h under argon atmosphere. 2-Methyl-acrylic acid methyl ester (1.1 equiv.) was added at this temperature and the reaction mixture was stirred for 3 h. Then, absolute ethanol (1 mL) was added dropwise. After cooling to room temperature, the mixture was evaporated in vacuo. The residue was dissolved in ACN/H_2O and purified by semi-preparative HPLC to yield 10.9 mg of **12** (85%). ^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 7.91–7.78 (m, 4H), 7.55–7.48 (m, 2H), 7.43–7.36 (m, 1H), 5.35–5.27 (m, 1H), 3.74/3,68 (two singlets due to 2 diastereomers, 3H), 3.54–3.42/3.30–3.19 (two multiplets due to 2 diastereomers, 1H), 3.15–3.05/3.03–2.93 (two multiplets due to 2 diastereomers, 1H), 2.41 (s(br), 3H), 2.01–1.85 (m, 2H), 1.79/1.75 (two doublets due to 2 diastereomers, J = 6.8 Hz, 3H), 1.49–1.44 (m, 1H), 1.34/1.30 (two doublets due to 2 diastereomers, J = 7 Hz, 3H), 1.00–0.78 (m, 2H), 0.33 (m, 3H), 0.05 (m, 3H). ^{31}P NMR (101 MHz, DMSO-d_6) δ 46.8, 46.6 (2 diastereomers) MS (ESI) m/z 448.3 $(M+H)^+$, 917.6 $(2M+Na)^+$, 933.6 $(2M+K)^+$, 1380.5 $(3M+K)^+$. RP–HPLC t_R = 23.3 and 23.4 (two diastereomers) (10–90%).

4.1.13. 2-Methyl-3-[(1S)-1-acetamido-3-methyl-butyl-hydroxy-phosphinoyl]- propionic acid (13). 10.9 mg of **12** (24.4 μmol, 447.5 g/mol) was dissolved in 2 mL of 57% HI and heated to 100 °C for 2 h. After cooling the mixture was evaporated in vacuo. The residue was dissolved in ACN/H_2O and purified by semi-preparative HPLC to yield 4.2 mg of **13** (62%). ^{1}H NMR (250 MHz, DMSO-d_6) δ 12.01 (s(br), 1H), 8.06–7.90 (m, 1H), 4.10–3.91 (m, 1H), 2.73–2.53 (m, 1H), 2.00–1.85 (m, 1H), 1.83 (s, 3H), 1.64–1.33 (m, 4H), 1.17–1.09 (m, 3H), 0.87 (d, J = 6.3 Hz, 3H), 0.78 (d, J = 6.3 Hz, 3H). ^{31}P NMR (101 MHz, DMSO-d_6) δ 46.5. MS (ESI) m/z 597.2 $(2M+K)^+$, 619.2 $(2M+K+Na-H)^+$, 914.2 $(3M+2K-H)^+$. RP–HPLC t_R = 8.6 (10–90%).

4.1.14. 2-Methyl-3-[(1S)-1-(N-(9-fluorenylmethoxycarbonyl))amino-3-methyl-butyl- hydroxy-phosphinoyl]-propionic acid (14). 4.2 mg of **13** (15 μmol, 279.3 g/mol) were dissolved in 1 mL of 8N HCl and heated to

100 °C for 12 h. After cooling Na_2CO_3 was added until the pH was 8. The solution was cooled to 0 °C and 5.8 mg Fmoc-Cl in dioxane was added dropwise. After 4 h at room temperature the solvent was evaporated in vacuo. The crude product was taken up in 1N HCl and extracted with DCM. The organic layer was washed with water and dried over Na_2SO_4. The organic layer was dried in vacuum and the residue was dissolved in ACN/H_2O and purified by semi-preparative HPLC to yield 3.9 mg of **13** (56%). ^{1}H NMR (250 MHz, DMSO-d_6) δ 7.88 (d, J = 7.3 Hz, 2H), 7.71 (d, J = 7.3 Hz, 2H), 7.55 (d, J = 9.8 Hz, 1H), 7.40 (t, J = 7.3 Hz, 2H), 7.35–7.25 (m, 2H), 4.34–4.26 (m, 2H), 4.25–4.15 (m, 1H), 2.72–2.59 (m, 1H), 2.11–1.93 (m, 1H), 1.68–1.32 (m, 4H), 1.27–1.20 (m, 1H), 1.15 (d, J = 7 Hz, 3H), 0.89 (d, J = 6.6 Hz, 3H), 0.79 (d, J = 6.6 Hz, 3H).

^{31}P NMR (101 MHz, DMSO-d_6) δ 46.0. MS (ESI) m/z 460.3 $(M+H)^+$, 919.3 $(2M+H)^+$. RP–HPLC t_R = 22.1 (10–90%).

4.2. Enzyme assay

BACE1 was expressed in HEK293 cells and BACE containing membranes were solubilized in 0.5% Triton X-100 in 20 mM MES containing protease inhibitors. An aliquot of these membranes was diluted in assay buffer (20 mM NaOAc, pH 4.4) and test compound diluted in 1% DMSO/assay buffer as well as substrate peptide to a final concentration of 1μM is added. The substrate used was SEVNLDAEFK labelled at the N-terminus with the Cy3 and at the C-terminus with the Cy5Q fluorophor (Amersham). The assay is run for 30 min at 30 °C and cleavage of the substrate is recorded by a fluorimeter (ex. 530 nm, em: 590 nm). The IC_{50} values for the test substances are calculated by standard software (GraphPad Prism®). The relative inhibition is calculated by the reduction of signal intensity in presence of the substance compared to the signal intensity without substance.

The BACE2 assay was performed under the same conditions except that the ectodomain of BACE2 fused to the Fc part of human IgG and secreted by HEK293 cells into the medium (OptiMEM, Invitrogen). Two microlitres of the concentrated BACE2 containing media was used within the assay.

The Cathepsin D assay was performed under the same assay conditions; Cathepsin D was obtained from Calbiochem.

For the Pepsin assay, 60 ng porcine pepsin (Calbiochem) is incubated with 6 ng/μL BODIPY-F1-casein (Molecular Probes) in 10 mM HCl, pH 2.2, over 60 min and fluorescence is recorded (ex 485 nm and em: 535 nm).

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Supplementary data

Synthesis of 1-amino-3-methylbutylphosphinic acid, solid phase synthesis protocols, HPLC and HPLC-(ESI)MS analysis of 5a,b,c, diastereoselectivity of 1-(2-naphthyl)-ethylamine as auxiliary, NMR, HPLC and ESI-MS of compound **4** and 1H, 1H – TOCSY of 5a with assignment are available free of charge via the Internet. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.03.072.

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