Bioorganic & Medicinal Chemistry Letters 19 (2009) 4427–4431

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Prolonged stability by cyclization: Macrocyclic phosphino dipeptide isostere inhibitors of β-secretase (BACE1)

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ARTICLE INFO

Article history:
Received 8 April 2009
Revised 13 May 2009
Accepted 14 May 2009
Available online 18 May 2009

Keywords:
BACE1
Alzheimer's disease
β-Secretase
Macrocyclic
Serum stability
Phosphino dipeptide isostere

ABSTRACT

Cyclization of recently reported linear phosphino dipeptide isostere inhibitors of BACE1 via side chain olefin metathesis yielded macrocyclic BACE1 inhibitors. The most potent compound **II-P1** (IC_{50} of 47 nM) and the corresponding linear analog **I** were tested for serum stability. The approach led to three times prolonged half life serum stability of 44 min for the macrocyclic inhibitor **II-P1** compared to the linear compound **I**.

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Almost 10% of today's population over the age of 65 and 40% over the age of 80 suffer from Alzheimer's disease (AD), a major neurodegenerative disorder.[1] A major factor in the pathogenesis of AD is the cerebral deposition of amyloid fibrils as senile plaques.[2] These plaques consist mainly of an insoluble form of Aβ amyloid, a 40–42 amino acid (AA) long peptide produced by proteolytic processing of the β-amyloid precursor protein (APP).[3,4] APP is cleaved by at least three proteases. β-secretase (BACE1) initiates the pathogenic processing of APP by cleaving at the N-terminus. The resulting C99 membrane bound C-terminal peptide can then be hydrolyzed by γ-secretase to form the 40 AA long peptide Aβ40. Thus, BACE1 is a molecular target for therapeutic intervention in AD.[5–10] The peptide Aβ42, also resulting from the cleavage of APP by BACE1, has an even higher propensity to aggregate and is the principal Aβ species found in amyloid plaques. Studies of BACE1 KO mice demonstrated the viability and the absence of gross phenotypic changes that are observed, for example, with presenilin knock-outs. Moreover, these mice showed the complete absence of Aβ in their brains.[11] BACE1 is a unique member of the pepsin family of aspartic proteases and has recently been identified as the principle β-secretase in neurons.[12]

In 2000 and 2002, Tang and co-workers reported nanomolar inhibitors of BACE1.[13,14] Subsequently, X-ray structures of these inhibitors in complex with BACE1 were available, showing the main features of the enzyme–inhibitor interactions. One of the best inhibitors of BACE1 is **OM00-3** (Fig. 1), which has an IC_{50} of 6 nM (Table 1) in our test system.

Our group has recently shown that the phosphino dipeptide (PDP) isostere is a suitable replacement of the hydroxy ethylene isostere in BACE1 inhibitors (Fig. 1). This exchange resulted in pseudo peptidic inhibitors (compound **I**) of same potency (Table 1) as **OM00-3**.[15,16] Consequently we were interested to develop a macrocyclic inhibitor containing a PDP isostere, as cyclization is known to enhance serum stability. In addition those molecules could be used as reporter molecules for ^{31}P NMR based screening.[17] In our search for conformationally restrained PDP isostere BACE1 inhibitors we speculated that the P1 and P3 cyclization would lock the active conformation (due to their close proximity in Tang's crystal structure) of the linear pseudo peptidic inhibitor **I** (Fig. 1) in the N-terminal region (Figs. 1 and 2A). Analysis of the crystal structure of **OM00-3** bound to BACE1,[14] using the programs Sybyl and AUTODOCK,[18] revealed that the ideal macrocycle should consist of a 13-membered cycle. The macrocycle consists of a peptidic backbone chain and of a hydrocarbon chain, arising from the side chains of unnatural amino acids (Fig. 1). During the course of our investigations this linkage has also been described for BACE1 inhibitors with structures derived from a hydroxyethylene[19] as well as an ethanolamine core.[20] Many other side chain cyclizations have also been reported.[20–23] A low-energy conformation of our macrocyclic inhibitor was able to perfectly emulate the bioactive conformation

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0960-894X/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2009.05.053

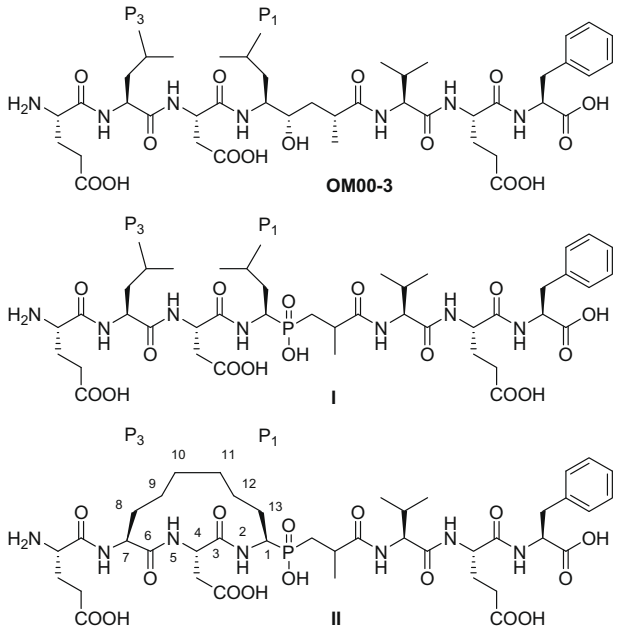


Figure 1. OM00-3, linear PDP inhibitor **I** and the corresponding macrocyclic inhibitor **II**.

Table 1
IC_{50} values against BACE1

Compound	Inhibition IC_{50}[a] (nM)	Retention time[b] (min)
OM-003	6 (±0.7)[c]	–
I	12 (±2)[c]	–
II-P1	47 (±10)	15.0
II-P2	320 (±30)	15.5
II–P3	522 (±50)	17.4
II–P4	808 (±70)	17.6

[a] Values are means of two experiments, standard deviation is given in parentheses.
[b] Detectable by HPLC–ESI-MS (gradient 15–40%, 30 min).
[c] Data taken from previous measurement with the full-domain of BACE1.[15]

of **OM00-3** (Fig. 2A). Superposition and docking of this low-energy conformation in BACE1 indicated that the macrocyclic PDP isostere ring system would fit and bind similar than **OM00-3** to the BACE1 binding cleft (Fig. 2B). Therefore, we decided to synthesize side chain cyclized PDP isostere inhibitors of BACE1.[24]

An essential tool for different macrocyclizations is the olefin metathesis. The functional group tolerance and the—during SPPS—unreactive alkene moieties, as well as the big arsenal of modern generation catalysts make the use of metathesis an extremely attractive option for peptide cyclization.[25–28] We used this concept for the synthesis of macrocycle **II** (Fig. 1), starting with the Fmoc protected tripeptide sequence Val-Glu-Phe on TCP-resin (Scheme 1). In this approach key fragments such as the PDP isostere **12**, Fmoc-Asp(O*tert*Bu)-OH, Fmoc-homoallylglycin-OH (**5**) and Fmoc-Glu(O*tert*Bu)-OH should be subsequently coupled to form the linear precursor for ring closing metathesis (RCM) (**13**, Scheme 1). Despite the commercially available Fmoc protected amino acids the two Fmoc building blocks carrying the alkene moieties for RCM had to be synthesized in solution. Synthesis of **5** was accomplished according to a route described by Rojo et al.[19] First, Boc-serine-OH (**1**) was transformed into the methyl ester **2** using methyl iodide. In order to substitute the hydroxy functionality for the corresponding iodide derivative triphenylphosphine, imidazol and iodide were used in line with a procedure published by Pavé et al.[29] However, the elimination side product **6** was predominantly formed instead of desired compound **3** (Scheme 2) contrary to an alternative procedure using the same reagents in inverse order reported by Trost et al.[30] Iodide **3** was transformed into a Knochel cuprate, which was subsequently reacted with allyl chloride to give allylic derivative **4** (Scheme 2).[31] The methyl ester was saponified by LiOH in a mixture of dioxane/water. After complete conversion acidification yielded H-homoallylglycine-OH. The aqueous solution was adjusted to pH 8–9 by addition of $NaHCO_3$ and Fmoc-OSu was added to give the final compound **5** ready for SPPS. Initial attempts to access the key fragment **12** using the chemistry reported by Baylis et al.[32] and Boyd et al.[33] failed. An alternative approach to synthesize **12** relies on a method worked out by Matziari et al. (Scheme 3).[34,35] The alkyl phosphinic acid **9** was synthesized according to literature procedures.[33] In this work the Matziari protocol was slightly modified as we used the carboxymethyl ester **9** to protect the phosphinic acid right away (Scheme 3). Compound **10** and 1-adamantyl bromide were refluxed in chloroform under successive addition of Ag_2O to introduce the adamantly protection group (Scheme 3).[36,37]

The obtained diastereomeric mixture was parted in three fractions by RP-HPLC in a ratio of 1:1:3 (**11a**/**11b**/**11c**). These fractions were handled separately in further reaction steps to facilitate the later purification of the four diastereomers of **II**. Next, the methyl ester was removed under standard conditions; saponification using one equivalent of LiOH under ice cooling did not result in a complete turnover. Hence, three equivalents were added in portions. Unfortunately, under these conditions also the Fmoc group was partially removed. Therefore, the free amine was in situ reprotected to yield **12**.

The resin loaded with the tripeptide sequence was split into three fractions with a ratio of 1:1:3 according to the three fractions of the PDP isostere **12** and the following synthetic steps were carried out in parallel for each fraction. The three fractions of PDP isostere **12** were attached using HATU/HOAt (1.25 equiv each) and

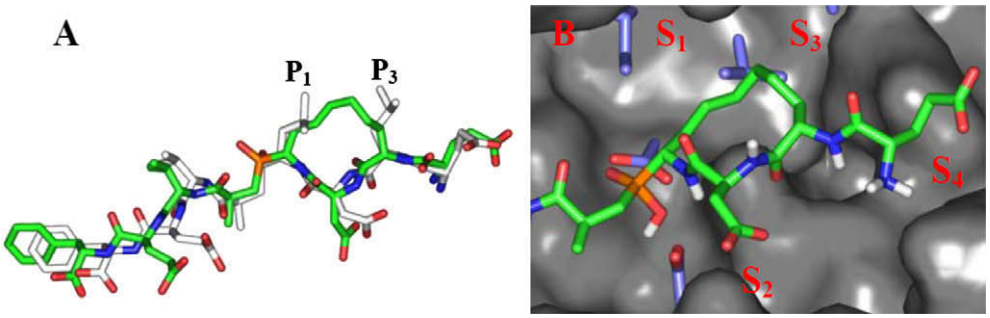
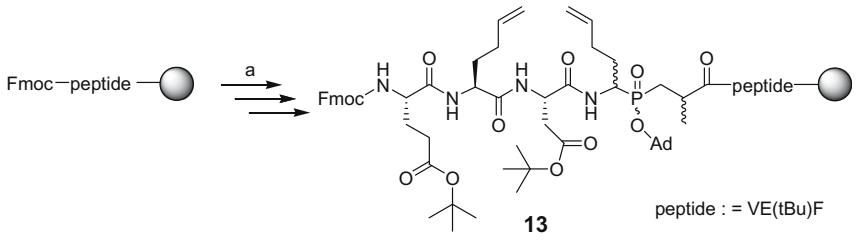
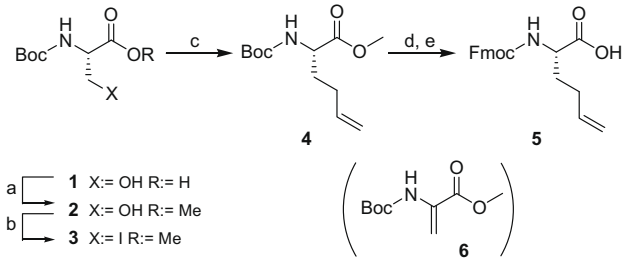


Figure 2. (A) Overlay of the X-ray conformation of **OM00-3** (grey) co-crystallized with BACE1 and the macrocyclic PDP isostere inhibitor **II** (green). (B) hypothetical binding mode of inhibitor **II** in the BACE1 binding site as predicted by AUTODOCK 3.0 (N-terminal region with macrocycle).



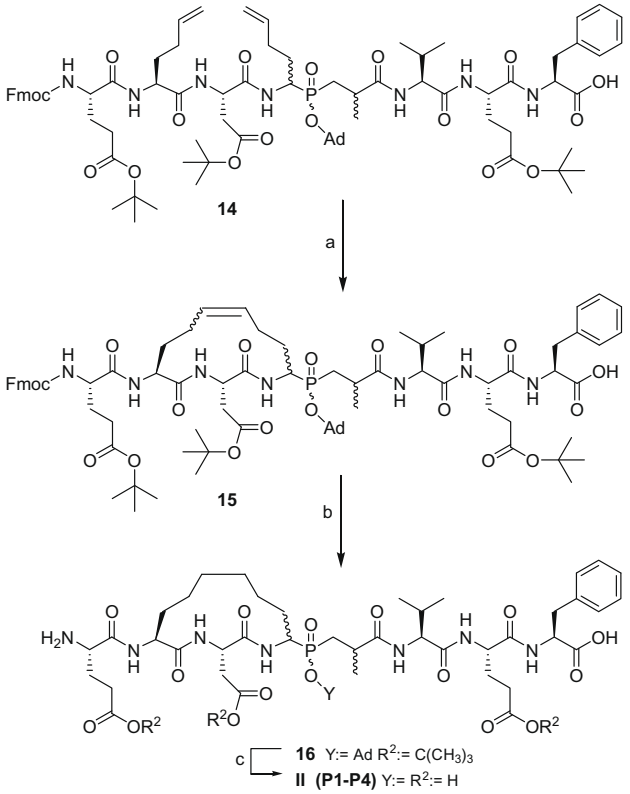
Scheme 1. SPPS of the linear precursor **13.** Reagents and conditions: (a) (i) 20% piperidine/NMP (2 × 10 min); (ii) **12** and HATU, HOAt (1.25 equiv each), DIPEA (5 equiv), NMP, 12 h; (iii) wash NMP (5 × 5 min); (iv) 20% piperidine/NMP (2 × 10 min) (v) amino acid, HOBt and TBTU (2.5 equiv each), DIPEA (5 equiv), NMP, 2.5 h; (vi) wash NMP (5 × 5 min)–repeat; (iv)–(vi) until sequence is complete, amino acids used in this order: Fmoc-Asp(O^{t}Bu)-OH, Fmoc-homoallylglycine-OH [HATU/HOAt (1.5 equiv) and 12 h instead] and Fmoc-Glu(O^{t}Bu)-OH.



Scheme 2. Synthesis of homoallylglycine **5** via Knochel cuprate. Reagents and conditions: (a) DMF, K_2CO_3, MeI (2 equiv), 12 h (76%); (b) (i) DCM, $P(Ph)_3$ (1.25 equiv), imidazole (1.3 equiv), I_2 (1.3 equiv), rt 10 min; (ii) then addition of **2**, 0 °C→rt 1 h (68%); (c) (i) Zn (6 equiv), THF, 1,2-dibromethane (0.3 equiv), 60 °C 3 min; (ii) TMS-Cl, 25 °C 30 min; (iii) **3** in THF, 45 °C 50 min; (iv) −10 °C, CuCN (1 equiv) and LiCl (2 equiv) in THF→0 °C 10 min; (v) −25 °C, allyl chloride (1.3 equiv)→rt 12 h (57%); (d) (i) dioxane/water (4:1), LiOH (2.2 equiv), 0 °C→rt 12 h; (ii) concd HCl (pH 1–1.5), 8 h; (e) satd $NaHCO_3$ (pH 8–9), Fmoc-OSu (1.3 equiv), 0 °C→rt 12 h (62% over three steps).

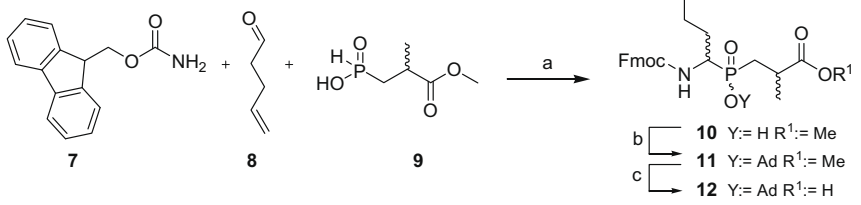
collidine (4 equiv) for 12 h in order to allow for complete coupling. The following couplings were again accomplished under standard SPPS conditions with just **5** to be coupled with HATU/HOAt and only 1.5 equiv each for a reaction time of 12 h (Scheme 1).

Encouraged by previously reported RCM reactions performed on solid support in our group by Schmiedeberg et al.[25] we first applied the solid phase methodology to this molecule. As indicated by HPLC–MS all of the starting material was consumed, but the product was cleaved from the resin. Therefore, hexafluoroisopropanole in DCM was used to cleave each of the peptidic sequences of the three fractions **13** from the resin whereby all protecting groups remained on the molecule to yield **14**. The RCM was accomplished in solution with each of the three fractions in parallel (Scheme 4) by use of Grubbs 2nd generation catalyst.[38,39] HPLC–MS analysis of conversion of **14** fraction c revealed clean conversion to the desired macrocycle **15**. Also the RCM conversion of the other fractions of **14** gave excellent results. The reduction of the olefin to give the saturated analog was accomplished by hydrogenation which



Scheme 4. Synthesis of compound **II** from the linear precursor **14**. Reagents and conditions: (a) (i) **14**, abs DCM (degassed), 2.5% Grubbs 2nd, reflux 4 h; (ii) 2.5% Grubbs 2nd, reflux 12 h (complete conversion as indicated by HPLC–MS); (b) DCM and *tert*-butanol (1:1), 20% Pd/C, H_2 atm, >36 h (quantitative conversion as indicated by HPLC–MS); (c) (i) TFA/TIPS/water (95:2.5:2.5), rt 12 h; (ii) semi preparative RP-HPLC.

results in simultaneous removal of the N-terminal Fmoc group. Several difficulties in first hydrogenation attempts led to the deci-



Scheme 3. Three component condensation to yield PDP isostere **12**. Reagents and conditions: (a) (i) AcCl/HOAc (3:1), **9**; (ii) **7**, 0 °C; (iii) **8**, 0 °C→rt 12 h (64%); (b) (i) $CHCl_3$, 1-Ad-Br, reflux; (ii) Ag_2O (45%; 40% **10** recovery); (c) (i) THF, water, LiOH (3 equiv), 0 °C→rt; (ii) 1 N HCl (pH 6–7); (iii) 2 N $NaHCO_3$ (pH 9), Fmoc-Cl (1 equiv) (60%, three fractions in ratio 1:1:3).

sion to use DCM together with *tert*-butanol as protic solvent (Scheme 4). The reaction time was quite long as it typically took more than 36 h for the conversion to compound **16**. Finally derivative **16** was stirred in a mixture of TFA/TIPS/water (95:2.5:2.5) for 12 h in parallel while monitoring deprotection by HPLC–MS. Finally, each mixture was purified and the different isomers separated by standard semi-preparative RP-HPLC. It was possible to separate all of the four diastereomers which were named after their chronological appearance from the HPLC-column as follows: **II-P1**, **II-P2**, **II-P3** and **II-P4**. Overall **II P1-4** was isolated in equimolar ratio with a yield of 60% over four steps and HPLC purification relative to the loading of the resin.

The inhibitory effects of these macrocyclic phosphino peptides against BACE1 were examined by an assay in which the ectodomain of BACE1 secreted from HEK393 cells was incubated with labeled substrate peptide and test compound as described in the Supplementary data. The results and analytical data are shown in Table 1. Two of the compounds have activities of 47 nM for **II-P1** and 320 nM for **II-P2.** The fraction containing **II-P3** has an activity of 522 nM whereas **II-P4** exhibited 808 nM. With this result it seems most likely that the most active macrocycle **II-P1** constitutes the chirality that belongs to the side chain orientation in natural amino acids. The most active compound **II-P1** was chosen together with the linear analog **I** to study enhancement of stability in serum achieved by cyclization.

It is well known that some major problems limit the use of peptides as effective drugs, among others are their often bad cellular activity, serum stability or brain penetration (across blood brain barrier). Particularly their fast degradation in vivo by proteases leads to their inherent instability. In order to protect biologically active peptides from in vivo decomposition there are different approaches, for example, alteration of the peptide bond, conjugation to carrier molecules, cyclization, N-methylation and the incorporation of non-proteinogenic amino acids.[40–42] For in vitro enzymatic stability studies mainly isolated enzymes such as carboxypeptidase A, aminopeptidase M, proteinase A, carboxypeptidase Y, α-chymotrypsin or complex biological fluids, such as human serum and urine, human plasma or rat liver lysosomes are in use. The present investigation used fresh human serum preparations. Therefore compounds **I** and **II-P1** were treated with fresh human serum of a single donor and the amount of intact ligand was determined by quantitative HPLC and HPLC–MS analysis over the time (Fig. 3). The linear inhibitor **I** is rapidly degraded with a half-life of about 14.8 min. As assumed, the cyclic inhibitor **II-P1** was more stable and degraded with a half-life of about 43.9 min. While inhibitor **I** is totally degraded and not detectable after approximately 120 min, cyclic inhibitor **II-P1** is still detectable after approximately 160 min. After this time about 20% of the inhibitor is still measurable by UV detection. In total the stability (half life) increased by a factor of approximately three.

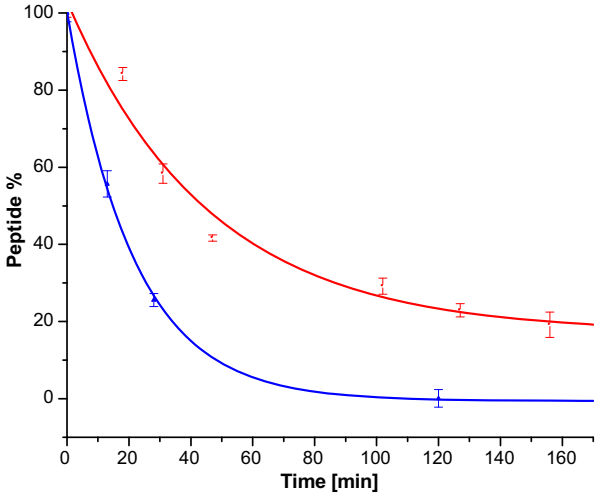


Figure 3. Stability in human serum of **I** (blue) and **II-P1** (red).

This study reports the synthesis of potent macrocyclic PDP isostere inhibitors with enhanced stability in human serum compared to their linear analogs. The synthesis was accomplished by SPPS of key fragments. A new useful protected PDP isostere building block for macrocyclization via metathesis bearing a terminal alkene functionality for SPPS was efficiently synthesized. After assembly of the precursor on solid support the RCM, hydrogenation and deprotection reactions were performed in high yield to obtain the desired macrocycle.

The data obtained from human serum incubation showed enhancement of stability against degradation in human serum by a factor of three compared to the linear analog. The remaining instability is possible due to the still very peptidic C-terminal part of the inhibitor and further development by replacing this part showed led to increased stabilities. Future investigations and optimization of pharmacokinetics together with known prodrug concepts, such as phosphinic acid esters (Monopril),[43] could led to macrocyclic PDP isostere inhibitors with improved pharmaceutical properties and thereby making them promising candidates for further drug development.

Acknowledgments

We thank B. Cordes for ESI-MS analysis and M. Wolff for technical support.

Supplementary data

Supplementary data (detailed protocols of the peptide digestion in human serum, molecular docking, SPPS, biological evaluation, experimental data of synthesized compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.053.

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