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Laboratory note

Synthesis and preliminary evaluation of peptidomimetic inhibitors of human β-secretase

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ABSTRACT

Based on the structure of OM99-2 and the X-ray crystal structure of its complex with β-secretase, a series of compounds containing the Leu*Ala hydroxyethylene isostere as a scissile bond substitution were designed. 31 compounds were synthesized and their β-secretase inhibition activities were measured. It was found that isobutyl group was a better R_3 substitution as C-terminus in our target compounds, and 4-nitrobenzyl group was the best R_2 side chain. With the aid of molecular modeling, the binding modes of compounds **9** and **22** with β-secretase were compared. The result revealed a stronger bonding mode of **22** than **9**. This explored that the optimal length of this series of peptidomimetic inhibitors was P3–P2′. The molecular weights of compounds with this length are around 600.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that afflicts more and more people worldwide. Intensive research efforts have focused on understanding the role of AD's neuropathological markers, plaques and tangles, in the disease process [1]. The amyloid cascade hypothesis centers on the amyloid β (Aβ) peptide, which is the principal component of the amyloid plaques detected in the brain of AD patients. Although the link between Aβ and AD neuronal dysfunction is still controversial, conspicuous biochemical, pathological and genetic evidence revealed that progressive accumulation of the Aβ peptide within the brain is critically responsible for the neurodegeneration that occurs in AD [2]. Therefore, a strategy aimed at lowering the concentration of neurotoxic Aβ represents an attractive way of clinical intervention [3]. The dominant anti-amyloid (Aβ lowering) approaches are sorted into two categories, increasing Aβ clearance by immunization [4] or decreasing Aβ production or aggregation. The latter approach is based primarily on two proteolytic enzymes, β- and γ-secretases, that participate in the processing of the amyloid precursor protein (APP) [5]. Our research project is targeted at β-secretase, because it plays a crucial role in the rate-limiting step of the amyloid cascade.

Former reports have revealed that natural APP substrate sequence of β-secretase is SEVKM/DAEFR while that of Swedish mutant type is SEVNL/DAEFR [6,7]. And the hydroxyethylene transition-state isostere has been proved to be a highly effective transition-state analogue for the inhibition of aspartic proteases. Moreover, Ala is a highly preferred residue in P1′ position of peptide β-secretase inhibitors [8]. So a hydroxyethylene isostere OM99-2 (Fig. 1) of EVNL/AAEF was found to be a potent β-secretase inhibitor [9]. And a series of peptidomimetics of OM99-2 were reported later [10].

On the basis of these conclusions and the X-ray crystal structure of the complex of β-secretase and its inhibitor OM99-2 [11], we designed a series of compounds containing the Leu*Ala hydroxyethylene isostere as a scissile bond substitution (Fig. 1). These compounds have low molecular weights and overlap the P3–P2′ of OM99-2. This was a part of efforts to explore the optimal length of peptidomimetic inhibitors. The compounds **1**–**31** were then synthesized with different amino acid substitutions, mainly at P3 position, in order to maximize the R_1 coverage (Table 1).

2. Chemistry

We have reported the synthetic method of compound **9** as a representative [12]. Scheme 1 showed the synthetic route of a key intermediate lactone **f**.

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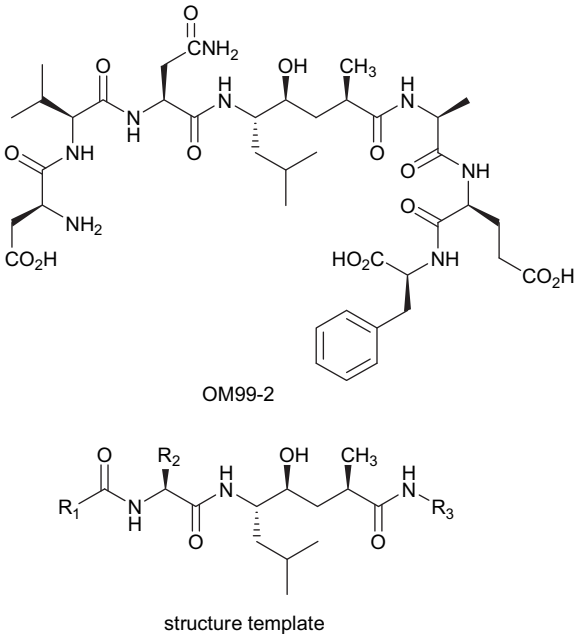


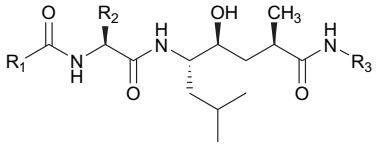
Fig. 1. Structure of OM99-2 and template design.

Scheme 2 outlined the synthesis of compound **9** from lactone **f**. The discussions on synthetic procedures of **f** had been presented therein [12]. The methyl group was smoothly introduced at α-C of **f** in 80% yield using methyl iodide and lithium hexamethyldisilazide (LiHMDs, Scheme 2). On a larger scale, the reaction duration should be prolonged to 35–40 min instead of 30 min as reported [13], for exhaustion of the starting materials. However, longer duration would also in the meantime lead to a dimethylated product (a white crystal, determined by ^{1}H NMR and MS) in a small amount. The α-C of compound **h** was identified as *R* configuration on the basis of NOE between α-CH_3 and C_4-α-H, as well as nonexistence of NOE between –OCH- and α-CH–. Subsequent hydrolysis of 2*R*,4*S*,5*S*-lactone **h** gave the hydroxyl acid derivative, which was protected by *tert*-butyldimethylsilyl chloride (TBDMSCl) to furnish **i**, followed by coupling with isobutylamine. The TBDMS group was then removed to afford hydroxyl amide **k** as an amorphous white solid, which proceeded from **l** to the model compound **9** through classic peptide condensation using *N*-Boc-L-phenylalanine and then 3-(trifluoromethyl)cinnamic acid in turn. The reagents for condensation involved *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) or *N*,*N*′-dicyclohexylcarbodiimide (DCC), accompanied by 1-hydroxybenzotriazole (HOBt) and *N*,*N*′-diisopropylethylamine (DIPEA) or *N*-methylmorpholine (NMM) in THF or DMF at room temperature. The reaction duration time varied depending on different acids used.

3. Bio-assay

The β-secretase inhibition activity was assayed as following. A β-secretase substrate was used, which contains a highly fluorescent group in one end and a quencher group in the other end, also a β-secretase cleavage site in the middle. The fluorescent group can be efficiently quenched by resonance energy transfer to the quencher group. When the substrate was cleaved by β-secretase, fluorescence will increase. Samples were added into 384-well black plate first. Then β-secretase (Recombinant Human BACE-1, R&D) was diluted with 0.1 M sodium acetate buffer (pH 4.0) to final concentration of 10 μg/mL, and added into 384-well black plate. After shaking for 5 min, substrate (Fluorogenic Peptide Substrate IV, R&D) was diluted with 0.1 M sodium acetate buffer (pH 4.0) to

Table 1
Structures of synthesized target compounds **1**–**31**.



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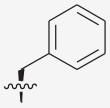
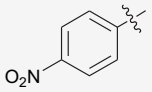
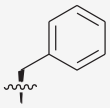
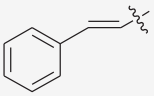
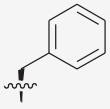
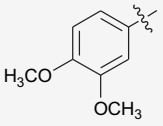
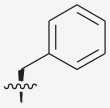
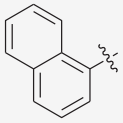
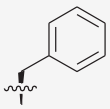
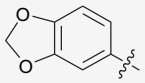
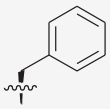
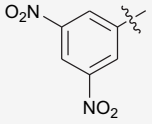
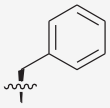
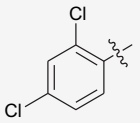
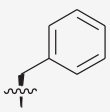
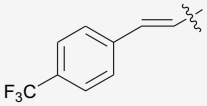
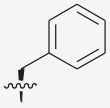
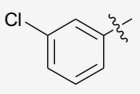
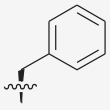
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

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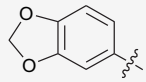
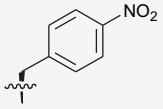
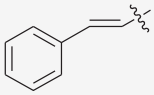
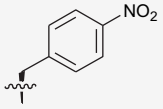
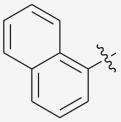
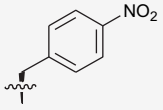
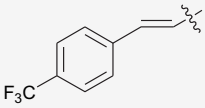
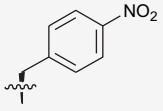
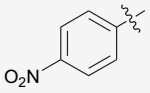
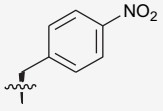
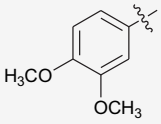
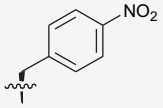
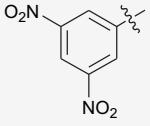
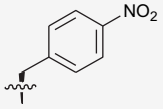
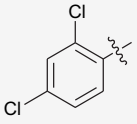
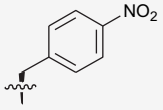
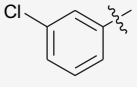
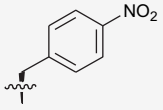
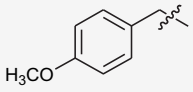
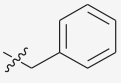
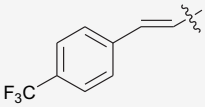
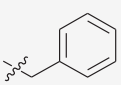
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			

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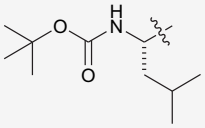
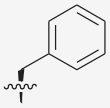
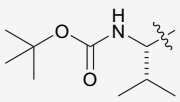
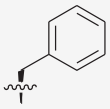
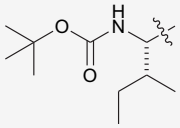
22			
23			
24			
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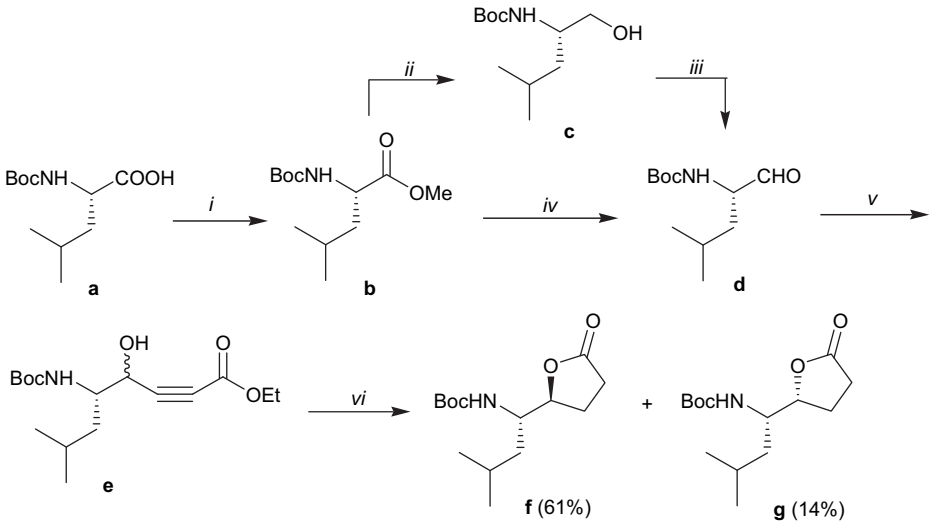
final concentration of 10 μM, and added into the 384-well black plate. The total volume of reactive solution was 50 μl. After shaking for 5 min, fluorescence was measured by excitation at 320 nm and emission at 405 nm. Δ*F*/min was expressed as the activity of β-secretase. If St represents the activity of standard and Sa

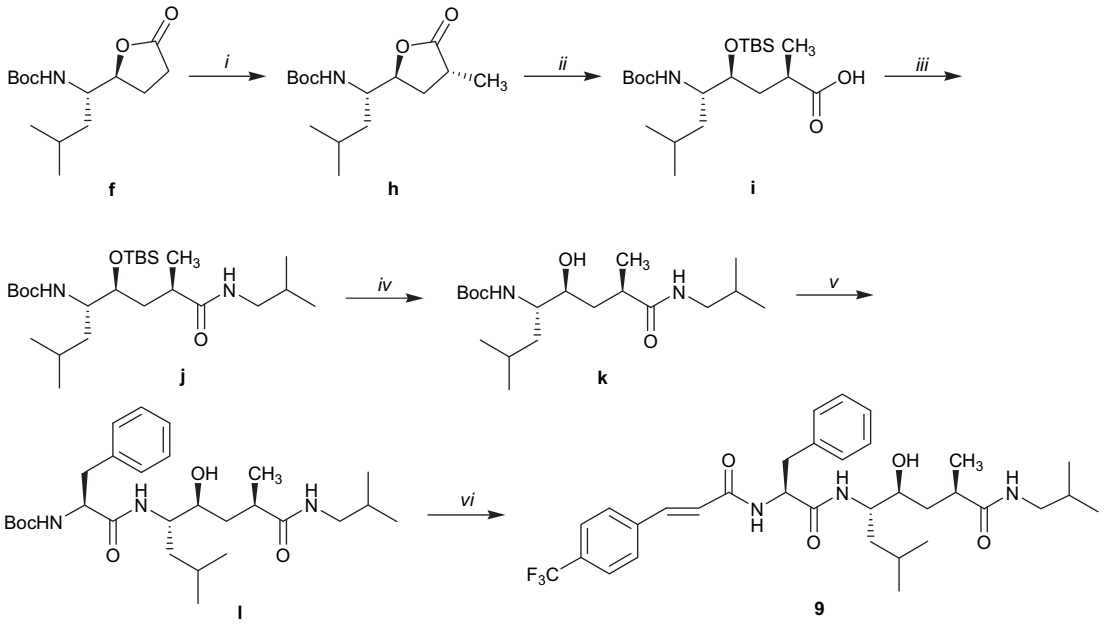


Scheme 1. Synthetic route to intermediate lactone **f**. Reagents and conditions: (i) $NaHCO_3$, CH_3I, DMF; (ii) $NaBH_4$, $CaCl_2$, THF/EtOH; (iii) $Py{\cdot}SO_3$, TEA, DCM/DMSO; (iv) DIBAL, toluene, 0 °C, 10 min; (v) ethyl propiolate, LDA (*n*-BuLi and *i*-Pr_2NH, −23 °C in THF, 1 h), −78 °C, N_2, 4.5 h; (vi) H_2/Pd, 60 psi, EtOAc, then reflux in toluene/acetic acid (97.5:2.5 v/v).

represents the activity of sample, the inhibition rate of sample = [(St − Sa)/St] × 100%. The β-secretase inhibition activities for compounds **1**–**31** are listed in Table 2.

4. Results and discussion

In our target compounds there were two C-terminal groups (R_3): isobutyl and benzyl. The compounds with isobutyl group showed obviously more potent β-secretase inhibition activity than that of benzyl group. This was in concordance with the result of Ref. [10]. With regard to the P2 substitution (R_2), three side chains were selected as representatives. It was observed that the two aromatic moieties (benzyl and 4-nitrobenzyl) were more active than hydroxymethyl group. It was also seen that the 4-nitrobenzyl group was more effective than the benzyl group, by comparing compounds **14**, **15** with **2**, **9**. The variation at P3 position (R_1) of N-terminus included phenyl vinyl, substituted phenyl, *tert*-butoxy, Boc-amino acids with side chains of Leu, Ile and Val. The result showed that compounds **1**, **5**, **6**, **14**, **15**, **16**, **18** and **22** were ten times more potent than **2** and **9**. How does the R_1 structure affect the inhibition activity? We have docked compound **9** into the active site of β-secretase structure (PDB code: 1FKN) using the docking program GOLD [14]. Fig. 2 shows the docked conformation of **9**. The scaffold of **9** overlayed very well with the original ligand OM99-2, which is in line with the computational work by Shen [10] except that the N-terminus extended to S′1 pocket due to its flexibility and lacking of additional polar interactions with S′3–S′4 subsites of 1FKN. Benzyl group (R_2) of **9** was accommodated in S2 pocket,



Scheme 2. Synthesis of compound **9** from lactone **f**. Reagents and conditions: (i) CH_3I, LiHMDS, −78 °C; (ii) a. LiOH (1 N), THF; b. TBDMSCl, imidazole, DMF; (iii) isobutylamine, EDC, HOBt, DIPEA, DMF/CH_2Cl_2; (iv) *n*-$Bu_4N^+F^-$, THF; (v) a. TFA/CH_2Cl_2; b. *N*-Boc-L-phenylalanine, EDC, HOBt, NMM, DMF; (vi) a. TFA/CH_2Cl_2; b. 4-(trifluoromethyl)cinnamic acid, EDC, HOBt, NMM, DMF.

Table 2
β-Secretase inhibition activities for compounds **1**–**31**.

Compd	Inhibition rate, % (10 μg/mL)	IC_{50}, μM
1	11.20	6.93
2	1.47	15.73
3	22.83	–
4	14.66	–
5	−0.10	4.67
6	16.20	8.07
7	0.91	–
8	20.26	–
9	59.66	19.54
10	16.92	–
11	40.29	–
12	37.22	–
13	−4.43	–
14	0.13	3.79
15	10.48	2.64
16	18.30	3.81
17	7.61	–
18	22.23	4.24
19	25.66	–
20	25.09	–
21	16.45	–
22	54.57	2.31
23	−2.37	–
24	−0.71	–
25	0.91	–
26	−1.56	–
27	−2.46	–
28	25.76	–
29	22.96	–
30	30.06	–
31	50.53	–

forming no hydrogen bond with guanidyl group of Arg235. It is supposed that if some polar substitutions were introduced onto R_3 or R_2 at proper position, additional hydrogen bonds might be formed within these relavant subsites and potent inhibitors might be achieved. The C-terminal 4-(trifluoromethyl)styryl group (R_1) of **9** oriented in the gap between S3 and S4 subsites, and that evoked us to change the rigid aromatic R_1 into some flexible groups so that it can enter either of the two subsites.

Compound **22** was also docked into the active site of 1FKN and the result is shown in Fig. 3. We are surprised to find that the binding mode of isobutyl group (R_3) of **22** is different from that of **9**. The positions of the adjacent N atom and carbonyl group were almost identical with OM99-2, which means an additional hydrogen bond was formed between the substituted amino group and Gly34 compared with **9**. This hydrogen bond also made the isobutyl group positioned into the hydrophobic S′2 pocket, so that more hydrophobic interaction was present than **9**. As for the R_1 moiety, the isobutyl group was found to extend to S4 subsite and the *tert*-butyloxy carbonylamino go upside to form strong hydrophobic interaction with Lys107. The rest of the parts of **22** overlayed quite well with OM99-2, and the binding mode is also the same with **9** in S1 and S2 subsites. So the docking results revealed a stronger bonding mode of **22** than **9**.

5. Conclusion

Based on the structure of OM99-2 and the X-ray crystal structure of its complex with β-secretase, a series of compounds containing the Leu*Ala hydroxyethylene isostere as a scissile bond substitution were designed and synthesized. It was found that isobutyl group was a better R_3 substitution as C-terminus in our target compounds, and 4-nitrobenzyl group was the best R_2 side chain. With the aid of molecular modeling, the binding modes of compounds **9** and **22** with β-secretase were compared. The result revealed a stronger bonding mode of **22** than **9**. This explored that the optimal length of this series of peptidomimetic inhibitors was P3–P2′. But the molecular weights of compounds with this length are around 600, which are too high to be an ideal drug. So a pharmacophore model has been produced from these compounds. That model is being modified and tested in our lab. It will be used to design non-peptide small molecular β-secretase inhibitors.

6. Experimental protocol

Unless otherwise mentioned, reagents were obtained commercially and used without further purification. Petroleum ether used here had a boiling point range from 60 to 90 °C. Melting points were taken with an X-4 apparatus and were uncorrected. IR (KBr), ^{1}H NMR, MS, and elemental analysis data were taken with PERKIN-ELMER983, INOVA-500, MDS SCIEX QSTAR, and FLASH EA 1112 instruments respectively.

The synthesis of compounds **b**–**l** followed the procedures in Ref. [12].

6.1. General procedure for the synthesis of target compounds (*1*–*31*)

Trifluoroacetic acid (TFA, 1 mL) was added to a stirred solution of amide **l** (65 mg, 0.13 mmol) in CH_2Cl_2. After 30 min, the solvent and most of TFA were removed by evaporation. In another flask, R_1COOH (0.15 mmol), *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI, 30 mg, 0.15 mmol) and 1-hydroxybenzotriazole (HOBt, 21 mg, 0.15 mmol) were dissolved in DMF (4 mL), to which were added the above deprotected amine (neutralized with *N*-methylmorpholine) and another *N*-methylmorpholine (0.1 mL, 0.3 mmol). The mixture was stirred at room temperature for 48 h. After this period, the mixture was poured into 10 mL of aqueous $NaHCO_3$ and extracted with EtOAc (30 mL × 3), and then washed successively with 10% aqueous citric acid (10 mL × 2), saturated aqueous $NaHCO_3$ (10 mL × 2) and brine, and dried over $MgSO_4$. After evaporation of the solvent, the residue was

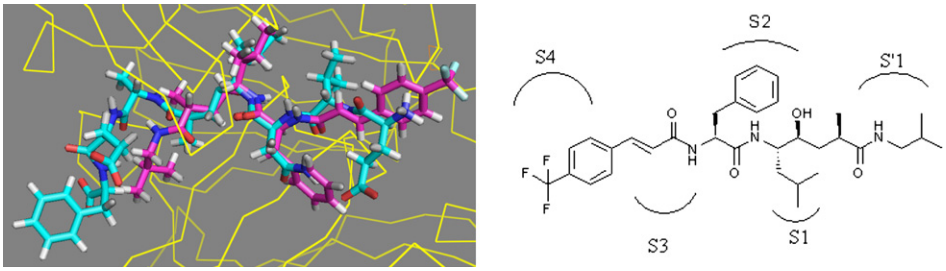


Fig. 2. The docked conformation of **9** (magenta) in comparison with OM99-2 (cyan) in the active site of 1FKN.

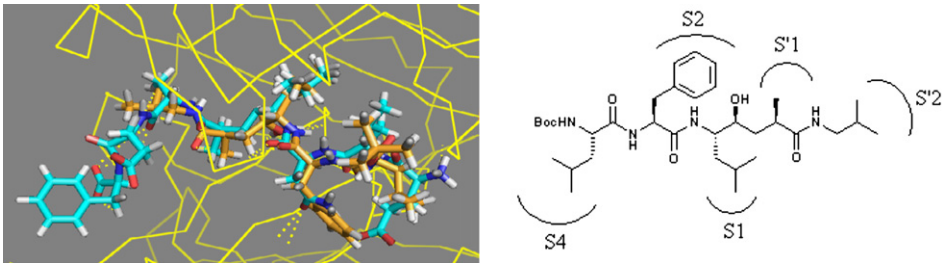


Fig. 3. The docked conformation of **22** (orange) in comparison with OM99-2 (cyan) in the active site of 1FKN.

dissolved in MeOH at 65 °C, to which ether and petroleum ether was added slowly. After 5 h at room temperature (lower temperature is desirable), the product crystal was obtained. Recrystallization can be repeated for several times. Synthesized target compounds **1**–**31** were characterized by melting point, MS, ^{1}H NMR and elemental analyses. Analyses indicated by the symbols of the elements (C, H, N) were within ±0.4% of the theoretical values.

*6.1.1. [(2R,4S,5S)-5-[N-(3,4-dimethoxy)benzoyl-L-phenylalanyl-amido]-4-hydroxy-2,7-dimethyloctal] N′-isobutyl amide (**4**)*

White solid, yield 53%, m.p. 212–214 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ = 8.43–8.41 (d, 1H, J = 8.5 Hz, –CONH–), 7.65–7.63 (m, 1H, –CONH–), 7.47–7.45 (d, 1H, J = 9.0 Hz, –CONH–), 7.41–7.39 (d, 1H, J = 8.5 Hz, Ph(3,4-dimethoxy)-C6H–), 7.36–7.12 (m, 5H, Ph–), 7.32 (s, 1H, Ph(3,4-dimethoxy)-C2H–), 6.98–6.97 (d, 1H, J = 8.5 Hz, Ph(3,4-dimethoxy)-C5H–), 4.69–4.65 (m, 1H, –NCHCO–), 4.61–4.60 (d, 1H, J = 5.5 Hz, –OCH–), 3.78–3.77 (s, 6H, 2CH_3O–), 3.73 (m, 1H, –NCH–), 3.14–3.10 (dd, 1H, J = 4.5 Hz and 14.0 Hz, PhCH_2–), 2.97–2.92 (dd, 1H, J = 10.5 Hz and 13.5 Hz, PhCH_2–), 2.85–2.82 (t, 2H, –CON–CH_2–), 2.48–2.44 (m, 1H, –CHCO–), 1.69–1.61 (m, 1H, –N–C–CH–), 1.60–1.55 (m, 1H, –CH–), 1.53–1.48 (m, 1H, –CH_2–C–CO–), 1.41–1.35 (m, 1H, –CH_2–C–CO–), 1.24–1.19 (m, 1H, –CH_2–), 1.17–1.11 (m, 1H, –CH_2–), 0.97–0.95 (d, 3H, J = 7.0 Hz, –CH_3), 0.83–0.80 (d, 12H, 4CH_3–). MS (ESI) calcd for $C_{32}H_{47}N_3O_6$ m/z: 570.35 [M + H$^+$], found 570.39. Elemental data: calculated: C, 67.46; H, 8.31; N, 7.38; measured: C, 67.60; H, 8.58; N, 7.10.

*6.1.2. [(2R,4S,5S)-5-[N-(4-trifluoromethyl)benzoyl-L-phenylalanyl-amido]-4-hydroxy-2,7-dimethyloctal] N′-isobutyl amide (**9**)*

White crystal, yield 26%, m.p. 246–247 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ = 8.41 (d, 1H, –CONH–), 7.76–7.72 (m, 4H, CF_3Ph–), 7.69–7.64 (m, 1H, –CONH–), 7.60 (d, 1H, J = 9.5 Hz, –CONH–), 7.40 (d, 1H, J = 16 Hz, CF_3PhCH═), 7.29–7.14 (m, 5H, Ph–), 6.82 (d, 1H, J = 16 Hz, ═CHCO–), 4.71–4.66 (m, 1H, –NCHCO–), 4.58 (d, 1H, J = 5.5 Hz, –OCH–), 3.73 (m, 1H, –NCH–), 3.11–3.08 (dd, 1H, J = 4.5 Hz and 14 Hz, PhCH_2–), 2.84–2.81 (m, 2H, –CON–CH_2–), 2.80–2.75 (dd, 1H, J = 10 Hz and 14 Hz, PhCH_2–), 2.46 (m, 1H, –CHCO–), 1.67–1.62 (m, 1H, –N–C–CH–), 1.60–1.55 (m, 1H, –CH–), 1.54–1.47 (m, 1H, –CH_2–C–CO–), 1.38–1.32 (m, 1H, –CH_2–C–CO–), 1.25–1.20 (m, 1H, –CH_2–), 1.12–1.07 (m, 1H, –CH_2–), 0.97 (d, 3H, J = 6.5 Hz, –CH_3), 0.84–0.79 (d, 12H, 4CH_3–). MS (ESI) calcd for $C_{33}H_{44}F_3N_3O_4$ m/z: 604.33 [M + H$^+$], found 604.41. Elemental data: calculated: C, 65.65; H, 7.35; N, 6.96; measured: C, 65.65; H, 7.57; N, 6.86.

*6.1.3. [(2R,4S,5S)-5-(N-Boc-L-leucyl-L-phenylalanylamido)-4-hydroxy-2,7-dimethyloctal] N′-isobutyl amide (**22**)*

White solid, yield 28.5%, m.p. 184–186 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.31–7.19 (m, 5H, Ph–), 6.52–6.51 (d, 1H, J = 5.0 Hz, –CONH–), 6.42–6.41 (d, 1H, J = 6.5 Hz, –CONH–), 6.15 (s, 1H, –CONH–), 4.80–4.79 (d, 1H, J = 4.5 Hz, BocNH–), 4.62–4.58 (m, 1H, Ph–C–CH–), 3.90–3.88 (m, 2H, –OH, –N–CHCO–), 3.60 (m, 1H, –OCH–), 3.49–3.48 (m, 1H, –NCH–), 3.27–3.25 (m, 1H, PhCH_2–), 3.16–3.00 (m, 3H, PhCH_2–, –CON–CH_2–), 2.53–2.52 (m, 1H, –C–CHCO–), 1.80–1.73 (m, 1H, –CH–), 1.64–1.56 (m, 2H, 2-CH–), 1.44–1.33 (m, 4H, 2-CH_2–C–CO–), 1.33 (s, 9H, Boc), 1.33–1.21 (m, 2H, –CH_2–), 1.15–1.13 (d, 3H, J = 7.0 Hz, CH_3–), 0.94–0.90 (d, 12H, J = 7.0 Hz, 4CH_3–), 0.86–0.84 (d, 6H, J = 6.0 Hz, 2CH_3–). MS (ESI) calcd for $C_{34}H_{58}N_4O_6$ m/z: 619.44 [M + H$^+$], found 619.49. Elemental data: calculated: C, 65.99; H, 9.45; N, 9.05; measured: C, 65.85; H, 9.77; N, 8.89.

The spectra data of other compounds are given in Supplementary information.

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Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2010.01.044.

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