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Tetrapeptides, as small-sized peptidic inhibitors; synthesis and their inhibitory activity against BACE1

Taeko Kakizawa, Koushi Hidaka, Daisuke Hamada, Ryoji Yamaguchi, Tsuyoshi Uemura, Hitomi Kitamura, Harichandra D. Tagad, Takashi Hamada, Zyta Ziora, Yoshio Hamada, Tooru Kimura and Yoshiaki Kiso*

β-Site amyloid precursor protein cleaving enzyme 1 (BACE1) is known to be involved in the production of amyloid β-peptide in Alzheimer's disease and is a major target for current drug design. We previously reported substrate-based peptidomimetics, KMI-compounds as potent BACE1 inhibitors. In this study, we designed and synthesized tetrapeptides as low molecular-sized inhibitors. These exhibited high potency against recombinant BACE1, with the highest IC_{50} value of 34.6 nM from KMI-927.

Supporting information may be found in the online version of this article

Keywords: Alzheimer's disease; BACE1 inhibitors; liquid-phase peptide synthesis; tetrapeptides

Introduction

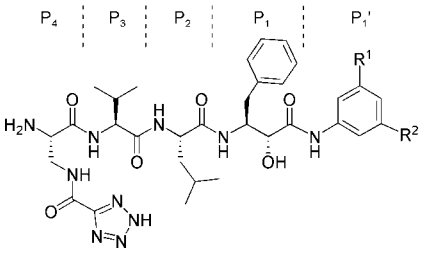
An aspartic protease, BACE1 [the β-site APP (amyloid precursor protein) cleaving enzyme] is a target protein for developing drugs against Alzheimer's disease, because it triggers amyloid β-peptide (Aβ) formation by cleaving APP at the *N*-terminus of the Aβ domain [1,2]. Numerous attempts to synthesize small-molecule inhibitors of BACE1 focusing on employing peptidomimetics have been examined [3–5]. Our initial investigation on BACE1 inhibitors focused on octapeptides that were derived from the sequence of the Swedish mutant APP [6]. A phenylnorstatine [Pns: (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] served as the central core at the P_1 position as a substrate transition-state analogue to bind with BACE1. These peptides were quite large in size encompassing the P_4–P_4' residues. We embarked on a truncation study that led to potent and shorter peptidic BACE1 inhibitors, while finding that removal of the non-prime side residues led to a considerable decrease in BACE1 affinity [7]. In contrast, removal of the prime side residues had a mild influence on the loss of inhibitory activity. After a structure-activity relationship study, we succeeded in removing the P_2'–P_4' positions to shorten the inhibitors down to pentapeptide size comprising the P_4–P_1' residues [8–12]. As shown in Figure 1, our previous pentapeptide inhibitors, known as KMI-compounds, possess a modified aromatic ring with acidic substituents at the P_1' position to exhibit a high affinity against BACE1 [8–13]. To obtain compounds with more potent activity but with low acidity, our current attempts are mainly focused on replacing the P_3–P_2 residues and modifying the P_4 and P_1' moieties. However, the overall MWs of these pentapeptides are >700 Da, which could be unfavorable as drugs. Thus, to develop more potent but low molecular weight BACE1 inhibitors, current studies have shifted to the design of less-peptidic molecules [3,14–20]. Considering the previous approaches for peptide-type BACE1 inhibitors, a further removal on the prime side amino acid still remains a challenge. Peptides are believed to possess good drug candidate properties, i.e. intrinsically potent, orally bioavailable, metabolically stable, brain penetrant, and safe. In this report, we describe the synthesis of tetrapeptidic KMI-compounds and their results on enzymatic assay. Indeed, our synthesized tetrapeptides exhibited high potency against BACE1, and thus are proven to be attractive candidates for drug development in Alzheimer's disease.

Results and Discussion

Tetrapeptides were prepared by classical approach for liquid-phase peptide synthesis on Boc chemistry (Boc: *tert*-butoxycarbonyl) [10–12]. The structure of exemplary inhibitor **1a** and its synthetic route from phenylnorstatine [Pns: (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] derivative Boc-Pns-OBn (Bn: benzyl) are summarized in Scheme 1. Boc deprotection was performed with 4 N HCl/dioxane solution in the presence of anisole. Coupling reactions with corresponding Boc-protected amino acids were carried out in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), *N*-hydroxybenzotriazole monohydrate (HOBt·H_2O), and

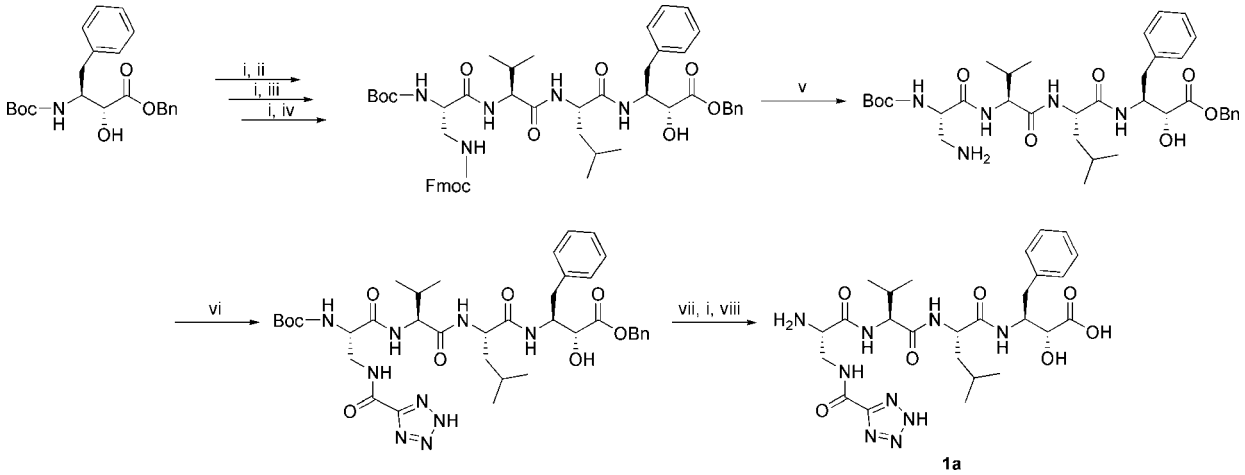
* *Correspondence to: Prof. Yoshiaki Kiso, Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan.*
E-mail: kiso@mb.kyoto-phu.ac.jp

Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan



Compound	R^1	R^2	MW	BACE1 inhibition at 2 μM (0.2 μM)	IC_{50} (nM)
KMI-429	COOH	COOH	752.77	>99.9% (98.1%)	3.9
KMI-570	H		732.79	>99.9% (98.1%)	4.8
KMI-684			800.83	>99.9% (>99.9%)	1.2

Figure 1. Pentapeptides containing a Pns at P_1 position and their BACE1 inhibitory activity.



Scheme 1. Reagents and conditions: (i) anisole, 4 N HCl/dioxane; (ii) Boc-Leu-OH, EDC·HCl, HOBt·H_2O, Et_3N in DMF; (iii) Boc-Val-OH, EDC·HCl, HOBt·H_2O, Et_3N in DMF; (iv) Boc-Dap(Fmoc)-OH, EDC·HCl, HOBt·H_2O, Et_3N in DMF; (v) 20% diethylamine/DMF; (vi) 1*H*-tetrazole-5-carboxylic acid, EDC·HCl, HOBt·H_2O, Et_3N in DMF; (vii) Pd/C, H_2 in MeOH; and (viii) preparative HPLC.

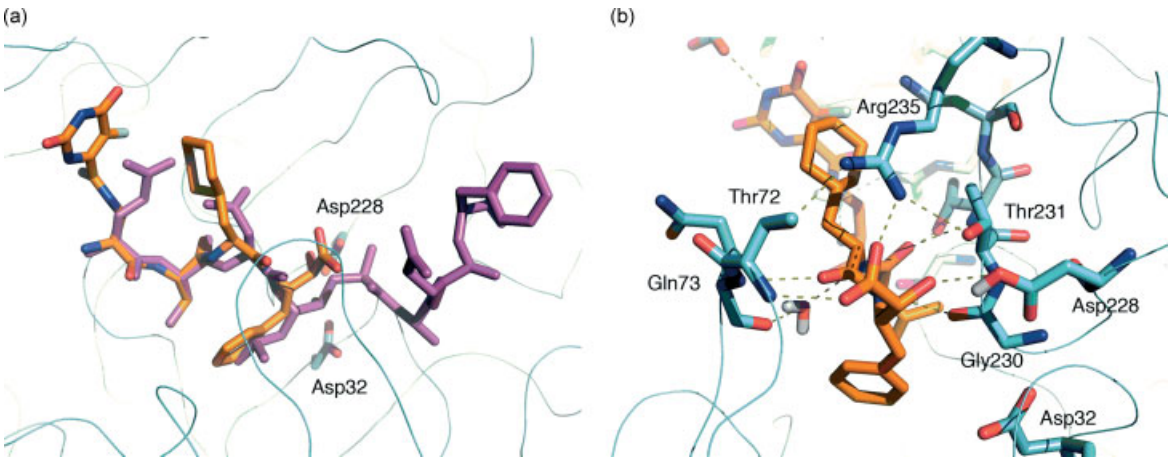


Figure 2. The molecular dynamics simulated poses of **2b** (KMI-927, orange stick) in the active site of BACE1 (PDB entry, 1FKN): (a) superimposed with an inhibitor OM99-2 (magenta stick). Catalytic two Asp residues (Asp32 and Asp228) are represented with cyan stick; (b) hydrogen bonding interactions (dotted line) around *C*-terminal Pns residue in the enzyme pockets.

Et_3N in DMF. After the introduction of Boc-Dap(Fmoc)-OH (Dap: 2,3-diaminopropionic acid, Fmoc: 9-fluorenylmethoxycarbonyl), Fmoc deprotection was carried out with 20% diethylamine/DMF solution. The tetrazole ring at the P_4 position was introduced with 1*H*-tetrazole-5-carboxylic acid, EDC·HCl, HOBt·H_2O, and Et_3N in DMF. Each coupling reaction or Boc deprotection proceeded in 64–100% yield. The deprotection of benzyl group was performed by catalytic reduction with Pd/C in MeOH under H_2 atmosphere. After removal of the Boc group, the crude peptides were purified on preparative HPLC and obtained as a white powder with a purity of >97% on HPLC (see Supporting Information). The mass-to-charge analysis showed an expected value (MS: $M + H_{calcd}$: 590.65 Da; $M + H_{found}$: 590.73 Da) to reveal a successful reduction in MW by ca. 143–211 Da from the parent *C*-terminal extended inhibitors depicted in Figure 1.

BACE1 inhibitory activity was determined using previously published methods [6]. After a reaction with 7 nM recombinant human BACE1 [21,22] and 25 μM fluorescence resonance energy transfer (FRET) substrate [23] in an incubation buffer with 2 μM or 0.2 μM inhibitors for 60 min at 37 °C, the *N*-terminal

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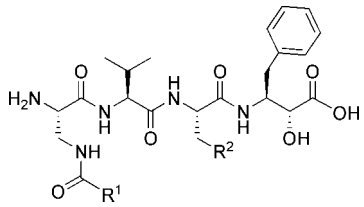
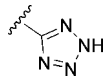
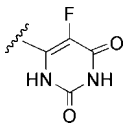
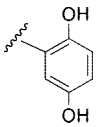
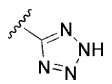
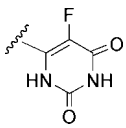
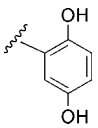
Table 1. Chemical data and BACE1 inhibitory activity of compounds **1a–c** and **2a–c**



Compound (KMI No)	R^1	R^2	Data	BACE1 inhibition (%) at 2 μM	BACE1 inhibition (%) at 0.2 μM	IC_{50} (nM)
1a (KMI-750)			Rt: 18.42 min[a] MALDI-TOF-MS (*m/z*): 590.73 for $[M + H]^+$ (calcd. 590.65 for $C_{26}H_{40}N_9O_7$)	92.3	64.3	75.0
1b (KMI-914)			Rt: 18.18 min[a] MALDI-TOF-MS (*m/z*): 650.68 for $[M + H]^+$ (calcd. 649.67 for $C_{29}H_{41}FN_7O_9$)	94.9	73.9	46.0
1c (KMI-915)			Rt: 19.92 min[a] MALDI-TOF-MS (*m/z*): 630.74 for $[M + H]^+$ (calcd. 630.71 for $C_{31}H_{44}N_5O_9$)	90.7	52.3	102
2a (KMI-961)			Rt: 19.95 min[a] MALDI-TOF-MS (*m/z*): 630.05 for $[M + H]^+$ (calcd. 630.72 for $C_{29}H_{44}N_9O_7$)	95.6	70.0	63.0
2b (KMI-927)			Rt: 19.96 min[a] MALDI-TOF-MS (*m/z*): 690.84 for $[M + H]^+$ (calcd. 690.74 for $C_{32}H_{45}FN_7O_9$)	95.9	82.8	34.6
2c (KMI-928)			Rt: 22.36 min[a] MALDI-TOF-MS (*m/z*): 670.79 for $[M + H]^+$ (calcd. 670.77 for $C_{34}H_{48}N_5O_9$)	93.0	63.3	87.6

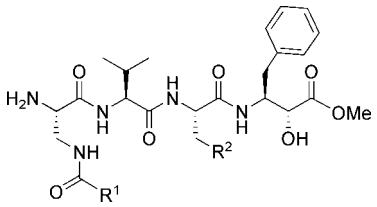
[a] Retention time on HPLC; linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm.

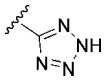
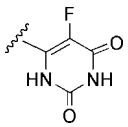
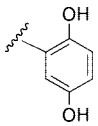
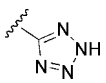
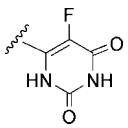
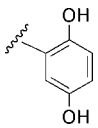
cleaved fragment of the substrate was analyzed by RP-HPLC (see Supporting Information). The activity of **1a** was 92.3% at 2 μM and 64.3% at 0.2 μM, respectively. Our previous pentapeptidic inhibitors with a modified aromatic ring at P_1' position possess moderate to excellent potency (51–100% inhibitory activity at 2 μM) [6–12]. Compared to these results, inhibitor **1a** shows a relatively high inhibitory activity.

Encouraged by this result, we decided to synthesize more tetrapeptidic derivatives. In our previous study, the introduction of 3-cyclohexylalanine (Cha) at P_2 and 5-fluoroorotyl or 2,5-dihydroxybenzoyl group at P_4 contributed to increase inhibitory activity [10]. Thus, we included these moieties into the tetrapeptides. In an analogous manner as in Scheme 1, P_2 Leu peptides **1b–c** and P_2 Cha derivatives **2a–c** were prepared. Their structures and obtained data (HPLC retention time [linear gradient of CH_3CN (0–100% for 40 min) in 0.1% aqueous TFA] and MALDI-TOF-MS) are shown in Table 1. All compounds were obtained with a purity of >97% on HPLC, and their MS data were consistent with expected values. The synthesized inhibitors showed 90.7–95.9% inhibitory activity at 2 μM, and 52.3–82.8% at 0.2 μM. In spite of the removal of the P_1' position, these tetrapeptides exhibited moderate BACE1 affinity. Particularly, inhibitors incorporating Cha at the P_2 position showed higher activities than Leu-containing peptides. IC_{50} values by enzymatic assay were evaluated for these compounds, and Cha P_2 peptide **2b** (KMI-927) showed the highest potency (IC_{50} = 34.6 nM).

Following removal of all prime side residues, we considered that modifications of the P_1 residue would be interesting because the *C*-terminal P_1 residue plays a key role in inhibitory activity by interacting with catalytic Asp residues [8,9]. Thus, as a first trial, we modified the Pns residue of inhibitors **1a–2c** to their corresponding methyl esters that may be favorable for improving permeability across the blood–brain barrier [24]. In addition, easy removals of their methyl group *in vivo* are expected [24], so that they are attractive prodrug candidates that release their corresponding parent compounds shown in Table 1. Starting with Boc-Pns-OMe, the peptide chain was elongated using similar procedure shown in Scheme 1 to give peptides **3a–4c**. The

Table 2. Chemical data and BACE1 inhibitory activity of compounds **3a–c** and **4a–c**



Compound (KMI No)	R^1	R^2	Data	BACE1 inhibition (%) at 2 μM	BACE1 inhibition (%) at 0.2 μM	IC_{50} (nM)
3a (KMI-1098)			Rt: 19.36 min[a] MALDI-TOF-MS (*m/z*): 604.82 for $[M + H]^+$ (calcd. 604.68 for $C_{27}H_{42}N_9O_7$)	71.1	–	–
3b (KMI-1100)			Rt: 18.88 min[a] MALDI-TOF-MS (*m/z*): 686.85 for $[M + Na]^+$ (calcd. 686.68 for $C_{30}H_{42}FN_7O_9Na$)	92.4	48.1	–
3c (KMI-1099)			Rt: 20.90 min[a] MALDI-TOF-MS (*m/z*): 645.02 for $[M + H]^+$ (calcd. 644.74 for $C_{32}H_{45}N_5O_9$)	63.1	–	–
4a (KMI-1078)			Rt: 22.72 min[a] MALDI-TOF-MS (*m/z*): 644.99 for $[M + H]^+$ (calcd. 644.74 for $C_{30}H_{46}N_9O_7$)	79.6	–	–
4b (KMI-1087)			Rt: 21.34 min[a] MALDI-TOF-MS (*m/z*): 704.41 for $[M + H]^+$ (calcd. 704.77 for $C_{33}H_{47}FN_7O_9$)	88.7	39.9	–
4c (KMI-1079)			Rt: 23.91 min[a] MALDI-TOF-MS (*m/z*): 684.79 for $[M + H]^+$ (calcd. 684.80 for $C_{35}H_{50}N_5O_9$)	74.4	–	–

[a] Retention time on HPLC; linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL^{-1} (temperature: 40 °C), detected at 230 nm.

obtained data are summarized in Table 2. In enzymatic assay, these were slightly less active than their parent compounds **1a–2c**. At 2 μM of the inhibitors, inhibitory activity was 63.1–92.4%. Compounds possessing >80% activity at 2 μM were subjected to low concentration assay at 0.2 μM. From the activity results of these methylated compounds, it is proven that the introduction of the 5-fluoroorotyl group at the P_4 position is favorable for inhibitory activity as shown in **3b** and **4b**.

We next turned our attention to the amidation of the *C*-terminus of the Pns residue. The amidated peptides **5a–6c** in Table 3 were prepared from Boc-Pns-NH_2 [25,26] by similar procedures described earlier. Through this modification, the inhibitors showed moderate inhibitory activity that was superior to the corresponding methylated compounds **3a–4c**. From the results of this series, it is again proven that the 5-fluoroorotyl group at P_4 position contributes significantly to affinity for BACE1. The P_2 Val and P_2 Cha derivatives containing the 5-fluoroorotyl group (**5b** and **6b**) exhibited 94.0 and 95.1% BACE1 inhibition at 2 μM, as well as 61.4 and 64.5% at 0.2 μM. Compounds having >50% inhibitory activity at 0.2 μM (**5b** and **6b**) were subjected for IC_{50} value evaluation. Compared to the compounds in Tables 1 and 2, the amidated peptides showed moderate potency as BACE1 inhibitors.

To better understand the coordination mode of the tetrapeptides toward BACE1, we performed a molecular modeling study between the structural data of BACE1 and the more potent compound **2b** (KMI-927, IC_{50} = 34.6 nM). The BACE1 binding model of **2b** was simulated using a modeling package (*MOE 2008.10*, Chemical Computing Group, Inc., Montreal, Canada). The X-ray crystal data of OM99-2 complex (PDB ID, 1FKN) [27] was used to build an initial conformation of the **2b** complex. A carboxyl group of Asp228 was intentionally protonated. Several energy minimization processes with an MMFF94x force field were performed with water molecules, followed by a molecular dynamics simulation (see Supporting Information). The obtained result is shown in Figure 2. Inhibitor **2b** was stably positioned along the main chain of *N*-terminal residues of OM99-2 during the simulation, especially at the P_3 and P_4 residues (Figure 2a). On the other hand, the P_1

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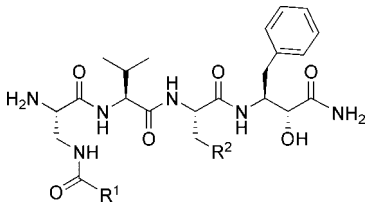
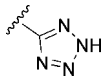
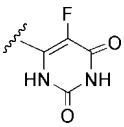
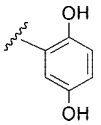
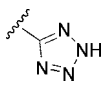
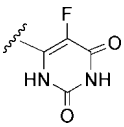
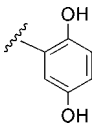
Table 3. Chemical data and BACE1 inhibitory activity of compounds **5a–c** and **6a–c**



Compound (KMI No)	R^1	R^2	Data	BACE1 inhibition (%) at 2 μM	BACE1 inhibition (%) at 0.2 μM	IC_{50} (nM)
5a (KMI-1338)			Rt: 17.43 min[a] MALDI-TOF-MS (*m/z*): 589.67 for $[M + H]^+$ (calcd. 589.67 for $C_{26}H_{41}N_{10}O_6$)	75.0	–	–
5b (KMI-1170)			Rt: 17.84 min[a] MALDI-TOF-MS (*m/z*): 649.81 for $[M + H]^+$ (calcd. 649.69 for $C_{29}H_{42}FN_8O_8$)	94.0	61.4	99.4
5c (KMI-1339)			Rt: 18.80 min[a] MALDI-TOF-MS (*m/z*): 629.73 for $[M + H]^+$ (calcd. 629.72 for $C_{31}H_{45}N_6O_8$)	75.2	–	–
6a (KMI-1167)			Rt: 21.15 min[a] MALDI-TOF-MS (*m/z*): 629.65 for $[M + H]^+$ (calcd. 629.73 for $C_{29}H_{45}N_{10}O_6$)	88.9	44.3	–
6b (KMI-1169)			Rt: 20.35 min[a] MALDI-TOF-MS (*m/z*): 689.91 for $[M + H]^+$ (calcd. 689.76 for $C_{32}H_{46}FN_8O_8$)	95.1	64.5	70.5
6c (KMI-1168)			Rt: 22.62 min[a] MALDI-TOF-MS (*m/z*): 669.69 for $[M + H]^+$ (calcd. 669.79 for $C_{34}H_{49}N_6O_8$)	87.8	41.8	–

[a] Retention time on HPLC; linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL^{-1} (temperature: 40 °C), detected at 230 nm.

and P_2 residues were slightly moved up from the pockets. In our previous *C*-terminal extended inhibitors, the modeling study suggested that the Pns residue at P_1 interacts with the catalytic two Asp residues (Asp32 and Asp228). However in **2b**, the hydroxyl group of Pns interacted with the carboxylic side chain of Asp228, but not with that of Asp32 (Figure 2b). The hydrogen bonding interactions of the Pns carboxylic group with the side chain of Arg235 and the amide NH of Thr72 in the flap were observed, and probably made the positioning of **2b** different from that of the *C*-terminal extended inhibitors. The modification of the carboxylic group by methyl ester and amide, compounds **4b** and **6b**, respectively, would prevent these interactions with Arg235 and Thr72, reducing the affinity to BACE1.

Conclusion

Tetrapeptidic KMI-compounds were prepared, and their potency against BACE1 *in vitro* was examined. In spite of the lack of all prime side residues, the synthesized compounds showed relatively high BACE1 affinity. We modified the P_1 residue of the synthesized KMI-compounds. In this study, the inhibitors with a free carboxylic group at the *C*-terminus exhibited better affinity than their corresponding methylated or amidated derivatives in enzymatic assay. The inhibitory effect of P_2 Cha derivative **2b** (KMI-927, IC_{50} = 34.6 nM) was superior to that of other tetrapeptides reported here.

We have just started investigating tetrapeptidic BACE1 inhibitors, and thus our attempts are yet at the early stage of development. To find more attractive drug candidates, further study is now in progress.

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Supporting Information

Supporting information is available free of charge via the Internet at www.interscience.wiley.com/journal/jpepsci.

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