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Design of pentapeptidic BACE1 inhibitors with carboxylic acid bioisosteres at P'_1 and P_4 positions

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ABSTRACT

We previously reported potent BACE1 inhibitors KMI-420 and KMI-570 possessing a hydroxymethylcarbonyl isostere as a substrate transition-state mimic. Acidic moieties at the P'_1 and P_4 positions of KMI inhibitors are thought to be unfavorable in terms of membrane permeability across the blood–brain barrier. Herein, we replaced acidic moieties at the P_4 position with hydrogen bond accepting groups and acidic moieties at the P'_1 position with less acidic and similar molecular-size moieties (carboxylic acid or tetrazole bioisosteres). These inhibitors exhibited improved BACE1 inhibitory activities and a thorough quantitative structure–activity relationship study was performed.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder leading to the loss of memory and other intellectual abilities.[1,2] The diagnosis of AD can be confirmed by the presence of amyloid plaques,[3] neurofibrillary tangles (NFTs), synaptic loss and atrophy in specific areas of the brain.[4] Although the pathogenesis of AD is highly complex, several pathological traits characterize this disease such as amyloid plaques composed of clusters of β-amyloid peptide on the blood vessels and the outside surface of neurons in the brain. The generation of 40- and 42-residue amyloid β (Aβ) peptides in the human brain by proteolysis of the membrane anchored β-amyloid precursor protein (APP) is a key event in the progression of Alzheimer's disease. Two proteases, called β- and γ-secretases, cleave APP in the amyloidogenic pathway.[5–9] Aβ genesis is initiated by BACE1 (β-site APP Cleaving Enzyme type 1) cleavage of APP before the Asp residue of the Aβ sequence to form an N-terminus of the Aβ peptide.[10–13]

This scission librates two cleavage fragments: a secreted APP ectodomain (APPsβ) and membrane bound carboxyl terminal fragment (CTF), C-99, which is subsequently cleaved by γ-secretase to generate the C-terminus of the Aβ peptide and an APP intracellular domain (AICD). As BACE1 plays a critical role as a rate determining step in the progression of AD,[14,15] the development of BACE1 inhibitors is invaluable to elucidate the pathology of AD. Several transition-state analogues of BACE1 inhibitors modeled on the β-secretase cleaving sequence have been reported with relatively low IC_{50} values, although these inhibitors are often too large to be good drug candidates.[16,17]

Recently, we reported small-size BACE1 inhibitors with acidic moieties at the P'_1 position and tetrazolic acid at the P_4 position using hydroxymethylcarbonyl isosteres as a transition-state mimic (Fig. 1).[18–22] Although these inhibitors exhibited potent inhibitory activity, acidic moieties that are similar to carboxylic groups often

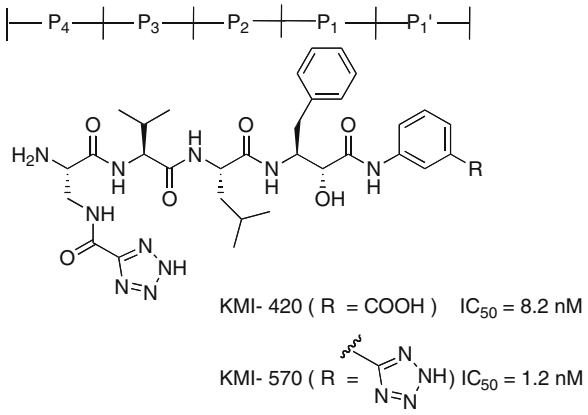

Figure 1. Pentapeptidic BACE1 inhibitors.

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possess low membrane permeability across the blood–brain barrier. Hence, there is a need to design and synthesize inhibitors with non- or less-acidic moieties or bioisosteres. Herein, we synthesize inhibitors with carboxylic acid bioisosteres. KMI inhibitors with tetrazole ring as a carboxylic acid bioisostere led to enhancement in BACE1 inhibitory activity. Our structure–activity relationship (SAR) study on KMI compounds showed that an acidic moiety at the P'_1 as well as P_4 position are essential for improving BACE1 inhibitory activity. Previous reports from our laboratory showed that replacement of the carboxylic acid group found in the P'_1 by less acidic and similar molecular-size carboxylic acid bioisosteres like tetrazole, oxadiazole, thiadiazole or triazole-type heterocycles showed high inhibitory activity.[23] Moreover, inhibitors with aromatic hydrogen bond acceptors such as tetrazole-5-carbonyl, 2,5-dihydroxybenzoyl or 5-fluoroorotyl groups at the P_4 position exhibited higher BACE1 inhibitory activity.[24] Herein, we designed and synthesized pentapeptidic KMI inhibitors with carboxylic acid bioisosteres such as triazole or thiazole derivatives at the P'_1 position, and 2,5-dihydroxybenzoyl or 5-fluoroorotyl groups at the P_4 position that exhibited IC_{50} values in the low nanomolar range (Fig. 2).

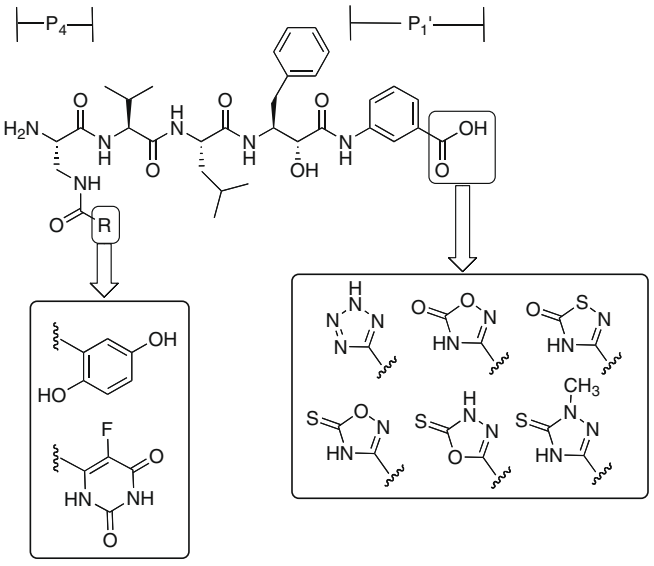
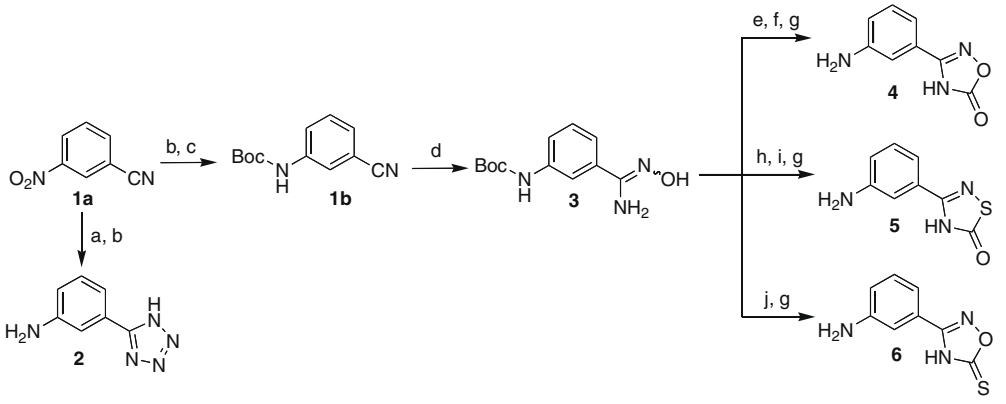


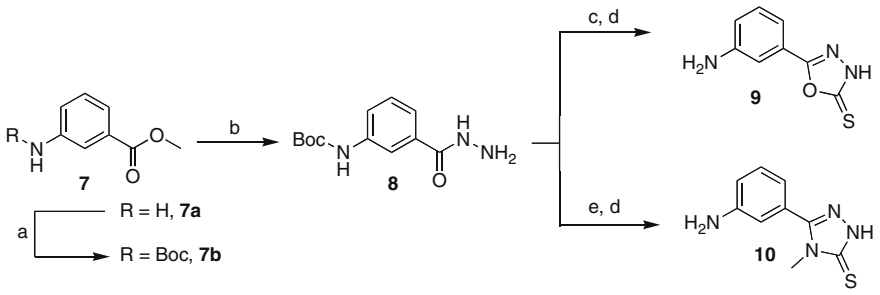
Figure 2. BACE1 inhibitors with carboxylic acid bioisostere at P'_1 and P_4 positions.

2. Chemistry

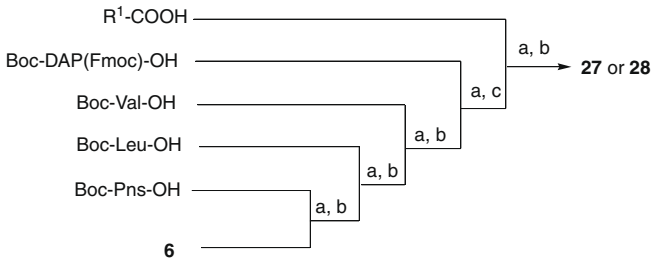
Acid bioisosteres corresponding to the P'_1 position of KMI inhibitors were synthesized. Heterocycles **2**, **4**–**6** were synthesized from 3-nitrobenzonitrile. Compound **2** was synthesized from 3-nitrobenzonitrile which was first converted into 3-nitrotetrazole by sodium azide mediated cyclization followed by reduction of the nitro group under catalytic hydrogenation condition using 10% Pd/C to give 3-(1*H*-tetrazol-5-yl)benzenamine **2**. On the other hand *tert*-butyl 3-amidinophenylcarbamate (aldoxime) **3** was synthesized from 3-aminobenzonitrile in a two-step protocol reported by Naka and co-workers.[25,26] Initially, the amino group was protected using di-*tert*-butyl dicarbonate [$(Boc)_2O$] and then converted into carbamate **3** using hydroxylamine in DMSO, and then was further converted into corresponding amino derivatives **4**–**6** as shown in Scheme 1. Carbamate **3** was reacted with 2-ethylhexylchloroformate and the obtained intermediate was cyclized by refluxing in xylene to afford **4**. 5-Oxo-1,2,4-thiadiazole **5** was synthesized by reacting 1,1′-thiocarbonyldiimidazole (TCDI) with aldoxime **3** followed by treatment with boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$). The synthesis of 5-thioxo-1,2,4-oxodiazole **6** was accomplished by treatment of aldoxime with TCDI and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The obtained Boc-protected derivatives were further converted to their respective free amine **5** and **6**. Analogues **9**[27,28] and **10**[29–31] were synthesized from the *N*-Boc protected methyl ester of 3-amino benzoic acid **7b**. Amino hydrazide derivative **8** was prepared by refluxing *tert*-butyl-3-(methoxycarbonyl)phenylcarbamate and hydrazine hydrate ($N_2H_4{\cdot}H_2O$) in tetrahydrofuran. The reaction of hydrazide **8** with carbon disulfide and KOH in ethanol followed by deprotection of the *tert*-butyloxycarbonyl afforded 5-(3-aminophenyl)-1,3,4-oxadiazole-2(3*H*)-thione **9**. Reacting the same benzoyl hydrazide **8** with methyl isothiocyanate (MITC) gave a thiosemicarbazide derivative which on heating in the presence 2–3 drops of Et_3N in EtOH or 1 N NaOH (aq) underwent smooth cyclization through dehydration to afford the *tert*-butyl-3-(4,5-dihydro-4-methyl-5-thioxo-1*H*-1,2,4-triazol-3-yl)phenylcarbamate. It was further exposed to 4 N HCl/dioxane to effectively remove the Boc group to give **10** (Scheme 2). The synthesis of the peptides was performed by traditional solution-phase peptide synthetic method, starting from *N*-Boc protected Pns [phenylnorstatine: (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] using appropriate amino acids. Peptide bond formation was carried out with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (EDC·HCl) and 1-hydroxybenzotriazole (HOBt). Boc (*tert*-butyloxycarbonyl) and Fmoc (9-fluorenylmethoxycarbonyl) protected amines were cleaved with a cocktail of anisole/4 N HCl/dioxane



Scheme 1. Reagents and conditions: (a) NaN_3, NH_4Cl, DMF, reflux; (b) H_2, 10% Pd/C, MeOH; (c) $(Boc)_2O$, Et_3N, THF, rt, 46 h; (d) $NH_2OH{\cdot}HCl$, Et_3N, DMSO, 75 °C, 15 h; (e) pyridine, 2-ethylhexyl chloroformate, THF, 0 °C, 30 min; (f) xylene, reflux, 2 h; (g) anisole, 4 N HCl/dioxane, rt, 1 h; (h) TCDI, THF, rt, 1 h; (i) $BF_3{\cdot}OEt_2$, THF, rt, 1 h; (j) TCDI, DBU, MeCN, rt,1 h.



Scheme 2. Reagents and conditions: (a) $(Boc)_2O$, Et_3N, THF, rt, 48 h; (b) $N_2H_4{\cdot}H_2O$, THF, reflux, 46 h; (c) CS_2, KOH, EtOH, reflux, 12 h; (d) anisole, 4 N HCl/dioxane, rt, 1 h; (e) methyl isothiocyanate, THF, rt, 6 h then aq 1 N NaOH, 45 °C, 3 h.



Scheme 3. Reagents: (a) EDC·HCl, HOBt·H_2O, DMF; (b) anisole, 4 N HCl/dioxane; (c) 20% piperidine/DMF.

and 20% piperidine in *N*,*N*-dimethylformamide (DMF), respectively, as shown in Scheme 3. The obtained crude peptides were further purified by column chromatography and finally purified by preparative RP-HPLC to afford pure peptides with >95% purity.

3. Results and discussion

3.1. BACE1 inhibition data

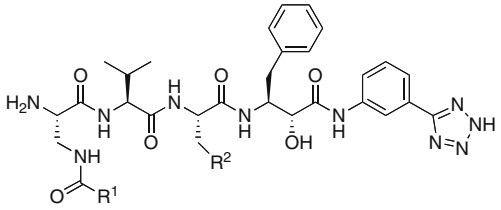
Previously, we reported compounds with 2,5-dihydroxybenzoic acid, 5-fluoroorotic acid or tetrazolic acid at the P_4 position that exhibited potent BACE1 inhibitory activity, and these moieties could be used as carboxylic acid bioisosteres in pentapeptidic inhibitors. Moreover Tables 1 and 2 shows pK_a data for P'_1 and P_4 moieties obtained from Advanced Chemistry Development (ACD/Labs) software for Solaris at 25 °C. Table 1 shows that the P_4 2,5-dihydroxybenzoyl (pK_a = 9.08) and 5-fluoroorotyl (pK_a = 6.02) amide moieties are less acidic than the P'_1 tetrazole (pK_a = 4.84) thus the latter one may contribute more to the overall acidity (pK_a = 4.25) of peptides **11**–**14**. Whereas the P_4 tetrazole derivatives having higher pK_a = 2.70 than the P'_1 position tetrazole residue lead to high acidity (pK_a = 2.86) in peptides **15** and **16**. From Table 2, we observed that P'_1 aniline derivatives **4**–**6**, **9**, **10** have much less acidity compared to that of tetrazole analogue **2** (pK_a = 4.84) and hence can be used as a bioisosteres. Thus from Tables 1 and 2, we decided to replace the 2,5-dihydroxybenzoyl and 5-fluoroorotyl moieties at the P_4 position, and used the aniline derivatives (**4**–**6**, **9**, **10**) at the P'_1 position to minimize the overall acidity of the pentapeptides. These inhibitors were synthesized from unnatural amino acid L-cyclohexyl-alanine (Cha) or natural amino acid Leu at the P_2 position, and their respective inhibitory activity is shown in Table 1.

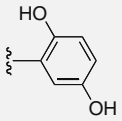
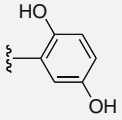
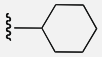
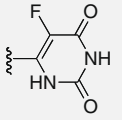
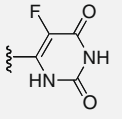
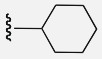
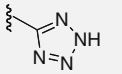
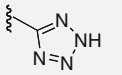
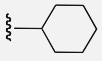
The 2,5-dihydroxybenzoic acid analogues, **11** and **12**, with Leu or Cha at the P_2 position, respectively, exhibited similar inhibitory activity. The high inhibitory activity of inhibitors **13** and **14** was due to the presence of a strong electron withdrawing fluorine atom in 5-fluoroorotic acid that led to a boost in the electrophilicity of the aromatic ring and enhanced the hydrogen bond accepting character of the P_4 side chain. Simultaneously, we observed the effect of Cha and Leu on BACE1 inhibitory activity at the P_2 position. The P_2 position's Cha seemed to display a slight enhancement in activity compare to that of Leu. We selected P_4 glutamic acid inhibitors with similar molecular-size, less acidic aniline derivatives at the P'_1 position as shown in Table 2.

Inhibitors **18**–**21** showed similar inhibitory activity as that of tetrazole analogue **17**. From these previous outcomes as shown in Tables 1 and 2, we fixed the P'_1 and P_4 position residues with less acidic and aromatic hydrogen bond acceptor carboxylic acid bioisosteres, respectively, and a P_2 Leu residue. The pentapeptides were synthesized starting from Pns with carboxylic acid isosteres at the P'_1 position and aromatic hydrogen bond acceptor heterocycles (2,5-dihydroxybenzoic acid and 5-fluoroorotic acid) at the P_4 position as shown in Fig. 2. The BACE1 inhibitory activity for inhibitors **23**–**32** with P'_1 position modifications is summarized in Table 3 alongside **11** and **13** tetrazole derivatives as references. With regards to the heterocyclic rings, 5-oxo-1,2,4-oxadiazole, 5-oxo-1,2,4-thiadiazole, 5-thioxo-1,2,4-oxadiazole and 2-thioxo-1,3,4-oxadiazole derivatives were found to be as potent as the tetrazole derivatives, suggesting that the spatial location of the P'_1 moiety interacting with the S'_1 hydrophobic regions of the BACE1 is important for improving BACE1 inhibitory activity. We synthesized compounds with 2,5-dihydroxybenzoic acid and 5-fluoroorotic acid at the P_4 position and varied the P'_1 position tetrazole residue with its bioisosteres like 5-oxo-1,2,4-oxadiazole, 5-oxo-1,2,4-thiadiazole, 5-thioxo-1,2,4-oxadiazole, 2-thioxo-1,3,4-oxadiazole and 2-methyl-3-thioxo-1,2,4-triazole derivatives. These five member ring heterocycles having similar size as that of tetrazole and possessing delocalized negative charges around the ring system[32] acted as hydrogen bond acceptors. An anionic group or hydrogen bond acceptor at the P'_1 carboxylic acids coordinated improved inhibitor site-interactions with the S'_1 pocket. In addition, these heterocycles possessed partial hydrophobic regions above and below the ring's plane that might interact favorably with the hydrophobic inner walls of the S'_1 pocket and led to enhancement in the inhibitory activity of compounds **11**, **13**, **17**–**30** (Tables 2 and 3). However inhibitors **22**, **31** and **32** which contained *N*-methyl heterocycles (2-methyl-3-thioxo-1,2,4-triazole) at the P'_1 position, resulted in a fall in BACE1 inhibitory activity at 0.2 μM, suggesting that the free amine functionality might be involved in hydrogen bond interactions and its *N*-methyl derivatives prevented hydrogen bond formation with the S'_1 pocket of BACE1.

When the inhibitory activity of compounds **17**–**21** was compared to that of **11**, **13**, **23**–**30**, the latter ones were more potent, suggesting a strong involvement of the P_4 position residue. In other words, both the P'_1 and P_4 are important for improving the BACE1 inhibitory activity. Compounds **11**, **13**, **23**–**30**, in which both P'_1 and P_4 positions were replaced by their respective carboxylic acid bioisosteres, showed potent BACE1 inhibitory activity. Considering that tetrazole displayed higher inhibitory activity when placed at the P_4 position,

Table 1
Pentapeptidic BACE1 inhibitors with modifications at P_2 and P_4 positions



Compound [a] (KMI No.)	R^1	R^2	BACE1 inhibition (%)		Predicted pK_a [b]		
			at 2 μM	at 0.2 μM	IC_{50} (nM)	Most acidic	P_4 moieties [c]
11 (KMI-573)			99	93	–	4.25	9.08
12 (KMI-575)			99	92	–	4.25	9.08
13 (KMI-572)			>99	98	6.5	4.25	6.02
14 (KMI-574)			>99	97	5.6	4.25	6.02
15 (KMI-570)			>99	98	4.8	2.86	2.70
16 (KMI-571)			>99	98	3.3	2.86	2.70

[a] See Ref. 24.
[b] Data from ACD/Labs software for Solaris 25 °C.
[c] Correspond to the pK_a of R^1CONH_2.

this position of the pentapeptide was modified with nitrogen containing heterocycles such as imidazole and triazole with Cha or Leu at the P_2 position and tetrazole at the P'_1 position as shown in Table 4. Synthesized inhibitors **33**–**36** having imidazole or triazole at the P_4 position showed a large loss of potency as compared to that of tetrazole analogues **15** and **16** which might be due to the difference in the number of binding regions between imidazole and triazole as compare to that of the tetrazole analogues. On the other hand, by increasing the number of nitrogen atoms in five member ring system, BACE1 inhibitory activity also increased, thus suggesting the involvement of hydrogen bond interactions.

3.2. Quantitative structure–activity relationship

To correlate chemical structure with BACE1 inhibition and statistically support our previous structure–activity relationship speculations, a formal quantitative structure–activity relationship (QSAR) study was performed. To increase the spread of data, that is, the BACE1 inhibition range, the inhibitory percentage at 0.2 μM was considered instead of 2 μM. However, inhibition at 0.2 μM was not determined for compound **22** because of its moderate inhibition at 2 μM. To include compound **22** in our quantitative structure–activity relationship study, we extrapolated its percent inhibition at 0.2 μM to 6% from a well-fitted linear correlation equation ($r^2 = 0.92$) between percent inhibitions at 2 μM and 0.2 μM, from compounds, **11**–**21**, **23**–**36**. We examined each position where moieties were varied, namely the P'_1, P_2 and P_4 residues. The spread of inhibitory activity values was small (63–74%) for compounds that only differed at the P'_1 residue (**17**–**21**).

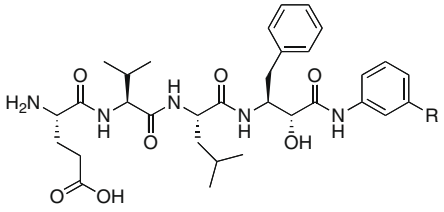
This narrow range suggested that, other than the *N*-methylthiooxotriazole outlier **22**, these moieties are effective bioisosteres of the tetrazole ring and that any parameterization for the P'_1 residue would contribute very little to the QSAR equation. Similarly, having a P_2 residue as either L-Leu or L-cyclohexyl-alanine led to inhibitors with only slight improvement in BACE1 inhibitory activity (cf. **11** and **12**, **13** and **14**, **15** and **16**, **33** and **34**, **35** and **36**). This suggested that the S_2 subsite could accommodate different hydrophobic moieties.

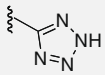
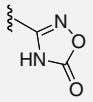
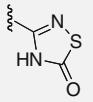
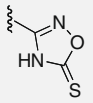
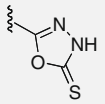
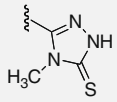
$$\begin{aligned} \mathrm{BACE} = &-(5.447\ \mathrm{Acc}_{P4} - 0.079\ \mathrm{Acc}_{P4}^2) - (5.064\ \mathrm{Don}_{P4} \\ &- 0.382\ \mathrm{Don}_{P4}^2) + 163.175 \\ n = 11, r^2 = 0.85, F = 9, p = 0.01 \end{aligned} \quad (1)$$

Acc_{P4} and Don_{P4} are the approximation to the sum of VDW surface areas (Å^2) of pure hydrogen bond acceptors and donors, respectively, on the P_4 side chain.

There is a hydrogen bonding region in the S_4 subsite that constitutes of Asn233, Arg235, Gly264, Arg307 and Lys321. Because the

Table 2
BACE1 inhibitory activity of P_4 glutamic compounds



Compound[a] (KMI No.)	R	BACE1 inhibition (%)		Predicted pK_a[b]	
		at 2 μM	at 0.2 μM	Most acidic	P'_1 moieties[c]
17 (KMI-569)		92	74	4.24	4.84
18 (KMI-596)		94	66	4.24	7.14
19 (KMI-683)		92	63	4.24	7.31
20 (KMI-879)		95	68	4.24	5.54
21 (KMI-666)		92	72	4.24	6.58
22 (KMI-686)		74	–	4.24	8.89

[a] See Ref. 23.
[b] Data from ACD/Labs software for Solaris at 25 °C.
[c] Correspond to the pK_a of $RPhNH_2$.

S_4 subsite has hydrogen accepting and donating potentials, we observed a good correlation (Eq. 1) between BACE1 inhibition and the approximation to the sum of van der Waals (VDW) surface areas of pure hydrogen bond acceptors and donors when compounds **11**–**17**, **33**–**36** were evaluated with the software Molecular Operating Environment (MOE, ver. 2008.10, Chemical Computing Group Inc., Canada). The well-fitted ($r^2 = 0.85$) and statistically significant ($p = 0.01$) equation was validated by leave-one-out cross-validation (r^2 range: 0.84–0.93).

Each descriptor was expressed in quadratic form such that the optimal or worst value for the descriptor could be calculated from the vertex of the parabola and a range of vertices could be obtained from cross-validation. The worst VDW surface areas of hydrogen bond acceptors and donors for the P_4 moiety were calculated to approximately 34–35 Å^2 and 6–7 Å^2, respectively, with both descriptors contributing almost equally to the equation.

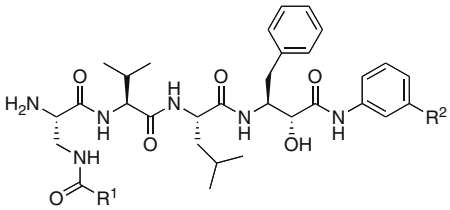
$$\begin{aligned} \mathrm{BACE} &= 39.520\ \mathrm{M_{P1'}} - (5.868\ \mathrm{Acc_{P4}} - 0.084\ \mathrm{Acc_{P4}^2}) \\ &\quad \times (2.616\ \mathrm{Don_{P4}} - 0.286\ \mathrm{Acc_{P4}^2}) + 130.284 \\ n &= 26, r^2 = 0.82, F = 18, p \leqslant 0.01 \end{aligned} \quad (2)$$

$M_{P1'}$ represents the P'_1 presence of the less effective *N*-methylthioxotriazole (0) or other more effective tetrazole bioisosteres (1).

We set out to derive a QSAR equation that correlates BACE1 inhibition at 0.2 μM with the physiochemical properties of the P'_1, P_2 and P_4 residues in our inhibitors. Because the change in the P_2 residue did not greatly affect BACE1 inhibition, the final QSAR equation would be composed of only the properties of the P'_1 and P_4 residues found in compounds **11**–**36**. Adopting a naïve assumption that the interactions at the S'_1 subsite would not interfere with the S_4 subsite, we derived Eq. 2 in which the physicochemical features of the P'_1 and P_4 residues were additive. In other words, Eq. 2 considered the less desired impact of the *N*-methylthioxotriazole in the P'_1 moiety, as well as the VDW surface areas of hydrogen bond acceptors and donors in the P_4 side chain. As with the previous equation, the derived well-fitted ($r^2 = 0.82$) and statistically significant equation ($p < 0.01$) was validated by leave-one-out cross-validation (r^2 range: 0.81–0.89).

Each descriptor contributed almost equally to the equation ($M_{P1'}$, 25%; Acc_{P4}, 38%; Don_{P4}, 36%) which meant that the P'_1 moiety contributed less to BACE1 inhibition than the P_4 moiety within this series of compounds. Eq. 2 revealed the worst VDW surface areas for Acc_{P4} and Don_{P4} as around 35 Å^2 and 3–6 Å^2, respectively, which were in agreement with the respective approximations obtained from Eq. 1. Hence, with these QSAR equations, we could obtain a more thorough understanding on the physicochemical features in the P'_1, P_2 and P_4 residues of a generic inhibitor that would be beneficial or detrimental to BACE1 inhibitory activity, while being able to statistically ascertain the reliability of our predictions (Fig.3).

Table 3
BACE1 inhibitory activity of pentapeptide with P'_1 modifications



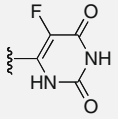
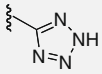
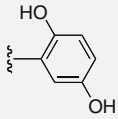
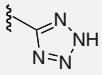
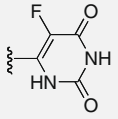
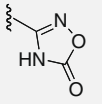
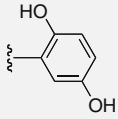
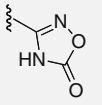
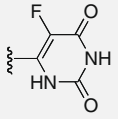
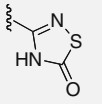
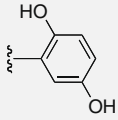
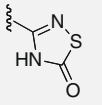
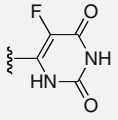
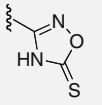
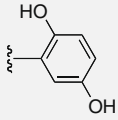
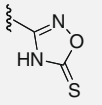
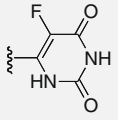
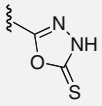
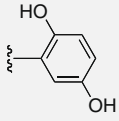
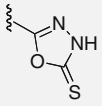
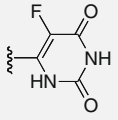
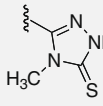
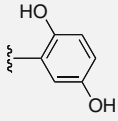
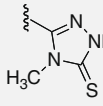
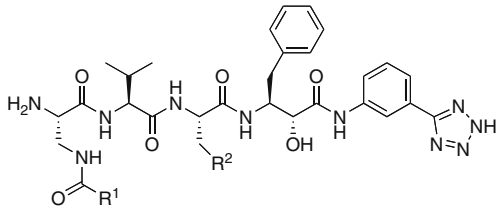
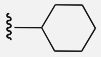
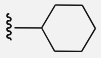
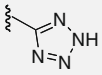
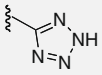
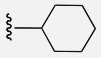
Compound (KMI No.)	R^1	R^2	BACE1 inhibition (%)		
			at 2 μM	at 0.2 μM	IC_{50} (nM)
13 (KMI-572)			>99	98	6.5
11 (KMI-573)			99	93	–
23 (KMI-707)			>99	96	7.9
24 (KMI-708)			>99	92	10.0
25 (KMI-1494)			99	93	–
26 (KMI-1436)			99	92	–
27 (KMI-809)			>99	95	10.2
28 (KMI-810)			>99	93	10.6
29 (KMI-1542)			>99	95	–
30 (KMI-1495)			99	89	–
31 (KMI-1543)			99	92	–
32 (KMI-1524)			94	64	–

Table 4
BACE1 inhibitory activity of pentapeptidic inhibitors with modifications at P_2 and P_4 positions



Compound (KMI No.)	R^1	R^2	BACE1 inhibition (%)		
			at 2 μM	at 0.2 μM	IC_{50} (nM)
33 (KMI-1544)			87	50	–
34 (KMI-1437)			83	45	–
35 (KMI-1545)			91	58	–
36 (KMI-1541)			94	69	–
15 (KMI-570)			>99	98	4.8
16 (KMI-571)			>99	98	3.3

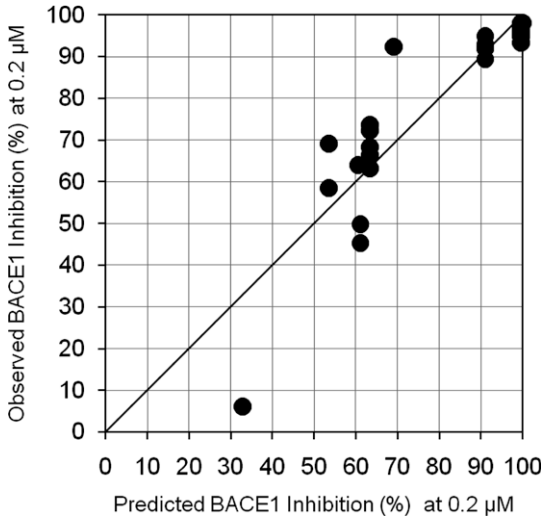


Figure 3. Comparative chart between calculated and observed BACE1 inhibition from Eq. 2.

3.3. Computer-assisted docking study

Replacing the Leu residue at the P_2 position slightly improved BACE1 inhibitory activity despite its big molecular size. Hence, we performed an in silico docking simulation using the software MOE as shown in Figure 4. Previously, we reported on the significant role of interactions between BACE1-Arg235 and a P_2 part of the inhibitor in the inhibition mechanism. The superimposed views of three crystal structures (PDB ID: 2B8L, 2IQG and 1W51) around the active site of BACE1 are shown in Figure 5A. The side chain of BACE1-Arg moves to accommodate the inhibitor's size along the wall of the β-sheet structure, which consists of four peptide strands behind BACE1-Arg235. The guanidine plane of BACE1-Arg235 pushes down on the P_2 part of the inhibitor causing affixing it in the active site of BACE1. Because the occupied area of inhibitor **16** in the S_2 pocket of BACE1 is in proximity of the inhibitor in the crystal structure of PDB 2B8L, the 3D-models in Figures 4 and 5B were depicted using the coordinates from PDB 2B8L. As shown in Figure 5B, the guanidine plane of BACE1-Arg235 is effectively packing down inhibitor **16** in the active site of BACE1.

4. Conclusions

In conclusion, pentapeptidic BACE1 inhibitors were designed and synthesized using various similar molecular-size lipophilic, hydrogen bond acceptor groups at the P'_1 position and polar groups at the P_4 position. From structure–activity relationship study, non-carboxylic acid residues at the P'_1 position and aromatic polar residues at the P_4 position were as effective as acidic moieties in promoting potent BACE1 inhibitory activity as tetrazole derivatives. Hence, these heterocycles act as bioisosteres of carboxylic acids as well as tetrazole ring at the P'_1 and at P_4 position.

5. Experimental section

5.1. Materials

Reagents and solvents were purchased from Wako Pure Chemical Ind., Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Aldrich Chemical Co. Inc. (Milwaukee, WI, USA), and Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan) and used without further purification.

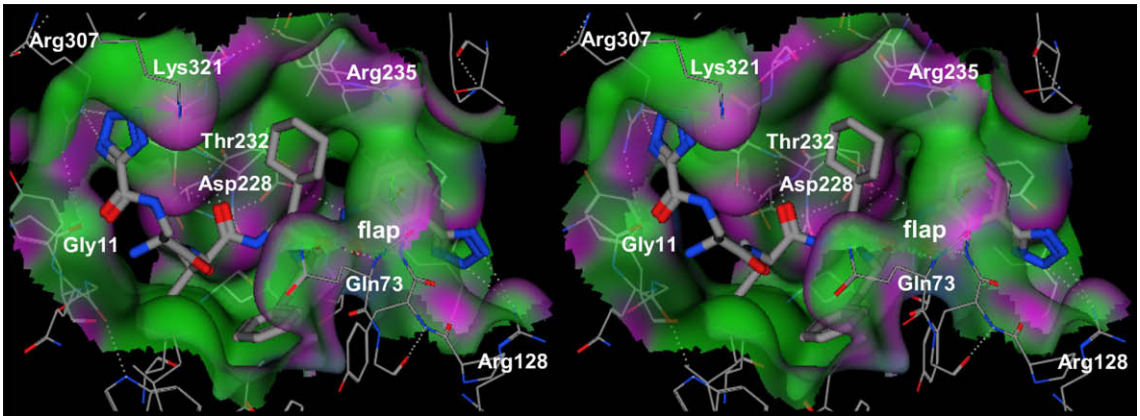


Figure 4. 3D View of docked inhibitor KMI-571 (**16**).

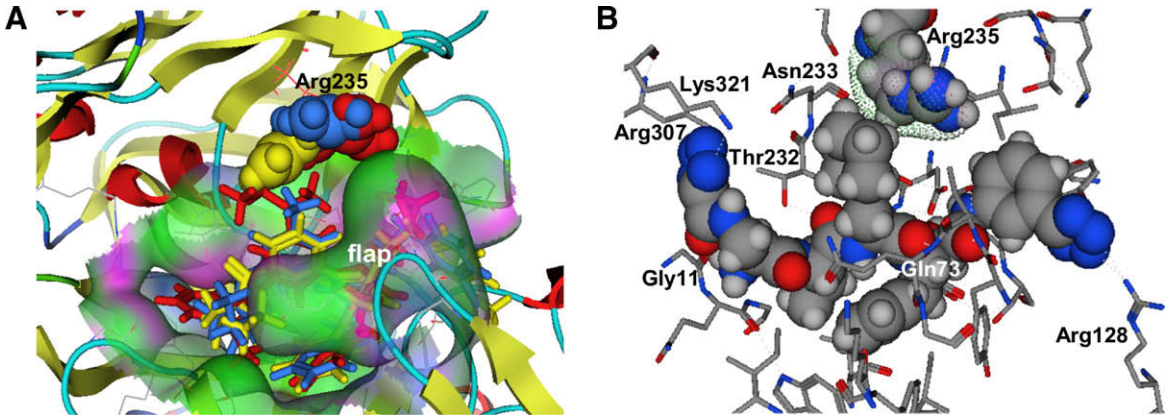


Figure 5. (A) Configurations of the Arg235-side chain in BACE1-inhibitor complexes. Red, blue and yellow models are derived from X-ray crystal structures, 2B8L, 2IQG and 1W51 in PDB ID, respectively. Color-coded space filling models indicate the side chain of BACE1-Arg235. Color-coded stick models indicate the inhibitors docked in BACE1. (B) Interactions between BACE1-Arg235 and the P_2 part of KMI-571 (**16**).

254-Column chromatography was performed on Merck 107734 Silica Gel 60 (70–230 mesh). Preparative HPLC was carried out on a C18 reverse phase column (250 × 20 mm; YMC-Pack-ODS-AM) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous trifluoroacetic acid (TFA) with a flow rate of 5.0 mL/min and detection at 230 nm. ^1H NMR spectra were obtained on a JEOL AL300 (300 MHz) and Varian Unity Inova 400NB (400 MHz) spectrometer with TMS as an internal standard. Mass spectra (electrospray ionization, MeOH as the mobile phase) were analyzed with SHIMADZU LCMS-2010 spectrometer. MALDI-TOF-MS was performed on a Voyager-DE RP spectrometer (Per Septive Biosystems, Inc.). Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC-Pack-ODS AM-302) with a binary solvent system: linear gradient of CH_3CN 0–100% in 0.1% aqueous TFA in 40 min at a flow rate of 0.9 mL/min, detected at 230 nm. The purity of the desired compounds were >95% pure.

5.1.1. General procedure for coupling reaction (A)

To a solution of amine (1.0 mmol) in DMF were added Boc-AA-OH or carboxylic acid (1.0 mmol), the reaction mixture was cooled to 0 °C, EDC·HCl (1.2 mmol) and HOBt·H_2O (1.2 mmol) and Et_3N (2 mmol) were added and the reaction was stirred for 12 h at room temperature. After removal of the solvent in vacuo, the residue was dissolved in ethyl acetate (20 mL), washed with 10% citric acid aq (3 × 5 mL), 10% $NaHCO_3$ aq (3 × 5 mL) and brine (1 × 5 mL), dried over anhydrous sodium sulfate (Na_2SO_4), evaporated to give the crude product which was further purified by column chromatography and subjected to next step, or purified by preparative HPLC in the case of the target compounds. The purified target compounds were immediately lyophilized to afford their respective amorphous powders.

5.1.2. General procedure for Boc-deprotection (B)

Anisole, 4 N HCl in 1,4-dioxane was added to the afforded Boc-protected peptide/amine (ca. 1.0 mmol) at 0 °C and stirred for 1 h at room temperature. The solution was evaporated in vacuo and used directly to the next step without purification. A small portion of the product was purified by preparative HPLC. The desired fractions were collected and immediately lyophilized to afford white amorphous powders, compounds **2**, **4**–**6**, **9**, **10**, **11**, **13**, **15**, **16** and **23**–**36**.

5.2. Synthesis of aniline derivatives for P_1' position

5.2.1. *tert*-Butyl 3-cyanophenylcarbamate (1b)

A mixture of 3-nitrobenzonitrile (5.00 g, 33.8 mmol) and 10% Pd/C (0.50 g) in dry MeOH was stirred at room temperature under H_2 at 1 atm pressure for 3 h. The reaction mixture was filtered and solvent was evaporated in vacuo to give 3-aminobenzonitrile (3.48 g). To a solution of 3-aminobenzonitrile (2.35 g, 20 mmol) in tetrahydrofuran (THF), Et_3N (2.76 mL, 20 mmol), $(Boc_2)O$ (4.80 g, 22 mmol) were added. The reaction mixture was stirred for 46 h at room temperature. The solvent was evaporated in vacuo and obtained residue was dissolved in ethyl acetate, extracted with 10% citric acid, the organic layer was washed with brine, dried over Na_2SO_4, evaporated to give desired compound which was further purified by column chromatography. Yield: 3.29 g (75%). Purity >98% by analytical HPLC

(t_R = 25.22 min). ESI-MS *m/z* 217.05 for [M−1] (calcd 218.25 for $C_{12}H_{14}N_2O_2$). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.52 (s, 9H), 7.26 (s, 1H), 7.29–7.32 (m, 1H), 7.37 (dd, *J* = 7.8, 1H), 7.49–7.53 (m, 1H).

5.2.2. 3-(1*H*-Tetrazol-5-yl)benzenamine (2)

A mixture of 3-nitrobenzonitrile (**1a**, 0.700 g, 2.10 mmol), sodium azide (0.411 g, 6.30 mmol) and ammonium chloride (0.337 g, 6.30 mmol) in DMF (10 ml) was stirred at 120 °C for 24 h, poured into water and extracted with ethyl acetate. The extract was washed with water, dried over Na_2SO_4, and concentrated in vacuo to give 5-(3-nitrophenyl)-1*H*-tetrazole which was further converted into title compound (**3**) by catalytic hydrogenation condition using 20 wt % of Pd/C under atmospheric pressure in dry MeOH at room temperature. Yield: 78% (over two steps). Purity >99% by analytical HPLC (t_R = 6.41 min). ESI-MS *m/z* 160 for [M−1] (calcd 161.16 for $C_7H_7N_5$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 6.88–6.91 (m, 1H), 7.26 (dd, *J* = 3.67, 1H), 7.28 (s, 1H), 7.32–7.34 (m, 1H).

5.2.3. *tert*-Butyl 3-amidinophenylcarbamate (3)

The Et_3N (6.18 g, 61.10 mmol) was added to a suspension of hydroxylamine hydrochloride (4.24 g, 61 mmol) in DMSO (20 mL). An insoluble material was filtered off and washed with THF. The filtrate was concentrated in vacuo to remove THF and *tert*-butyl-3-cynophenylcarbamate (**1b**) (5.00 g, 12.20 mmol) was added to the DMSO solution of hydroxylamine. After stirring at 75 °C for 15 h, the reaction mixture was diluted with water and extracted with ethyl acetate. The organic phase was extracted with 1 N HCl (25 mL). The aqueous solution was adjusted to pH 10 with 1 N NaOH and extracted with ethyl acetate. The organic layer was washed with water and dried over Na_2SO_4. The solvent was evaporated in vacuo and the obtained crude product was recrystallized from ethyl acetate–MeOH–hexane to give **3** Yield: 5.54 g (96%). Purity >97% by analytical HPLC (t_R = 16.26 min). ESI-MS *m/z* 250.10 for [M−1] (calcd 251.28 for $C_{12}H_{17}N_3O_3$). ^{1}H NMR (300 MHz, DMSO) δ (ppm) 1.47 (s, 9H), 5.9 (br s, 2H), 7.22 (m, 2H), 7.42 (m, 1H), 7.82 (s, 1H), 9.59 (br s, 1H).

5.2.4. 3-(3-Aminophenyl)-1,2,4-oxadiazol-5(4*H*)-one (4)

2-Ethylhexyl chloroformate (0.38 g, 0.20 mmol) was added dropwise to an ice-cooled mixture of **3** (0.50 g, 2.0 mmol) and pyridine (0.79 g, 2.1 mmol) in DMF (5 mL). The resulting mixture was stirred at 0 °C for 30 min, diluted with water, and extracted with ethyl acetate. The extract was washed with water and dried over Na_2SO_4. The solvent was evaporated in vacuo, and the residue was dissolved in xylene (5 mL). The solution was heated under reflux for 2 h. The solvent was evaporated in vacuo, the residue (0.255 g) obtained was directly subjected for *tert*-butylcarbamate (Boc) deprotection to give **4**. Yield: 0.103 g (62% overall two steps). Purity >97% by analytical HPLC (t_R = 11.92 min). ESI-MS *m/z* 175.95 for [M−1] (calcd 177.16 for $C_8H_7N_3O_2$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.31 (d, *J* = 7.34, 1H), 7.46–7.54 (m, 3H).

5.2.5. 3-(3-Aminophenyl)-1,2,4-thiadiazol-5(4*H*)-one (5)

A mixture of aldoxime **3** (3.00 g, 11.95 mmol) and TCDI (2.55 g, 14.34 mmol) in THF (25 mL) was stirred at room temperature for 1 h. It was diluted with water and extracted by ethyl acetate and the organic layer was washed with water, dried over Na_2SO_4. The solvent was evaporated in vacuo. The obtained residue was dissolved in THF (10 mL) and then $BF_3{\cdot}OEt_2$ (7.5 mL, 59.76 mmol) was added at 0 °C. The reaction mixture was stirred at room temperature for 1 h, diluted with water, extracted with ethyl acetate, washed with 1 N HCl, dried over Na_2SO_4. The solvent was evaporated in vacuo. The obtained crude product was washed with ethyl acetate to remove non-polar impurities. The obtained pure product was directly subjected for Boc-deprotection to give compound **5** in quantitative yield (over all two steps). Purity >97% by analytical HPLC (t_R = 12.06 min). ESI-MS *m/z* 191.95 for [M−1] (calcd 193.23 for $C_8H_7N_3OS$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.52–7.55 (m, 1H), 7.67 (dd, *J* = 7.89, 1H), 7.88 (m, 1H), 7.98 (dd, *J* = 3.67, 1H).

5.2.6. 3-(3-Aminophenyl)-1,2,4-oxadiazole-5(4*H*)-thione (6)

To a solution of aldoxime **3** (3.37 g, 13.43 mmol) in acetonitrile (12 ml), TCDI (3.53 g, 20.14 mmol), DBU (7.26 g, 53.72 mmol) was added and reaction was stirred at room temperature for 1 h. The reaction mixture was diluted with water; its pH was adjusted to 4 by using 1 N HCl and extracted with ethyl acetate. The extract was washed with water and dried over Na_2SO_4. The solvent was evaporated in vacuo; the residue obtained was directly subjected for Boc-deprotection (2.91 g). The obtained crude product was further purified by column chromatography to give compound **6**. Yield: 1.3 g (68%). Purity >96% by analytical HPLC (t_R = 13.47 min). ESI-MS *m/z* 192 for [M−1] (calcd 193.23 for $C_8H_7N_3OS$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.63–7.66 (m, 1H), 7.75 (dd, *J* = 7.89, 1H), 7.87 (dd, *J* = 1.38, 1H), 7.88–7.92 (m, 1H).

5.2.7. *tert*-Butyl 3-(methoxycarbonyl)phenylcarbamate (7b)

To a solution of 3-aminomethylbenzoate (0.100 g, 0.66 mmol) in THF (5 mL), Et_3N (0.100 g, 0.993 mmol) and di-*tert*-butyl dicarbonate (0.159 g, 0.728 mmol) was added at 0 °C. The reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated in vacuo. The residue was dissolved in ethyl acetate, extracted with $NaHCO_3$ followed by washed with brine. The organic layer was dried over Na_2SO_4, evaporated to give desired product. The obtained crude product was purified by column chromatography. Yield: 0.106 g (64%). Purity >99% by analytical HPLC (t_R = 25.62 min). ESI-MS *m/z* 274 for [M+Na] (calcd 251.28 for $C_{13}H_{17}NO_4$). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.52 (s, 1H), 3.9 (s, 3H), 7.36 (dd, *J* = 3.85, 1H), 7.69 (dd, *J* = 2.75, 1H), 7.72 (dd, *J* = 1.38, 1H), 7.97 (dd, *J* = 1.93, 1H).

5.2.8. *tert*-Butyl 3-aminobenzohydrazide (8)

The ester **7b** (0.450 g, 1.79 mmol) and 85% hydrazine hydrate (0.87 mL, 17.9 mmol) in 20 mL of THF were refluxed for 46 h. The excess solvent was removed in vacuo. The obtained solid was recrystallize by using hexane to furnish colorless crystalline benzoyl hydrazide intermediate (**8**). Yield: 0.414 g (92%). Purity >99% by analytical HPLC (t_R = 17.03 min). ESI-MS *m/z* 274 for [M+Na] (calcd 251.28 for $C_{12}H_{17}N_3O_3$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 1.47 (s, 9H), 7.28 (m, 1H), 7.35 (d, *J* = 8.99, 1H), 7.51 (d, *J* = 7.16, 1H), 7.93 (s, 1H).

5.2.9. 5-(3-Aminophenyl)-1,3,4-oxadiazole-2(3*H*)-thione (9)

To a solution of compound **8** (0.423 g, 1.68 mmol) in ethanol (10 mL) was added carbon disulfide (slightly more than one equivalent) and KOH (0.094 g, 1.68 mmol) at 0 °C. The resulting solution was refluxed for 12 h or until most of the hydrogen sulfide had evolved. The solvent was evaporated and the residue obtained dissolved in water and acidified with diluted solution of HCl. The solid obtained was filtered and recrystallized from MeOH and subjected for Boc-deprotection. Yield: 0.190 g (78%, overall two steps). Purity >95% by analytical HPLC (t_R = 12.59 min). ESI-MS *m/z* 192 for [M−1] (calcd 193.23 for $C_8H_7N_3OS$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 7.61–7.67 (m, 1H), 7.74 (dd, *J* = 8.82, 1H), 7.93–7.95 (m, 1H), 8.01–8.06 (m, 1H).

5.2.10. 5-(3-Aminophenyl)-4-methyl-2*H*-1,2,4-triazole-3(4*H*)-thione (10)

To a solution of benzoyl hydrazide **8** (0.500 g, 1.99 mmol) in THF (12 mL), methyl isothiocyanate (0.146 g, 1.99 mmol) and 2–3 drops of Et_3N were added and reaction mixture was stirred at room temperature for 6 h. Water was added and the precipitate obtained

was filter off, washed with water and dried well. The obtained product was dissolved in 4% aqueous NaOH (49.75 mL, 4.97 mmol) and stirred at 45 °C for 3 h. Resulting mixture was filter off and filtrate was acidified by 10% HCl until ~pH 3. Obtained precipitate was filtered, washed with water and dried well to give desire product, which was recrystallized from methanol and exposed for anisole, 4 N HCl/dioxane for Boc-deprotection. Yield: 62% over all three steps. Purity >99% by analytical HPLC (t_R = 12.48 min). ESI-MS *m/z* 205 for [M−1] (calcd 206.27 for $C_9H_{10}N_4S$). ^{1}H NMR (300 MHz, DMSO) δ (ppm) 2.51 (s, 3H), 7.45–7.48 (m, 1H), 7.58–7.65 (m, 3H), 14.01 (s, 1H).

5.3. General experimental procedure for synthesis of KMI compounds (C)

To an ice-cooled solution of amine (1.0 mmol) in DMF were added Boc-AA-OH or carboxylic acid (1.0 mmol), followed by EDC·HCl (1.2 mmol), HOBt·H_2O (1.2 mmol) and Et_3N (2 mmol) were added. The reaction mixture was stirred for 12 h at room temperature. After removal of the solvent in vacuo, the residue was dissolved in ethyl acetate (20 mL), extracted with 10% citric acid aq (3 × 5 mL), 10% $NaHCO_3$ aq (3 × 5 mL) and finally washed with brine (1 × 5 mL), then dried over Na_2SO_4, evaporated to give the crude product which was further purified by column chromatography and then subjected for *tert*-butyloxycarbamate deprotection by using General Procedure B. The obtained product was subjected to the next step or purified by preparative HPLC in the case of the target compound. The purified target compounds were immediately lyophilized to afford their respective amorphous powders.

5.3.1. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(2*H*-Tetrazol-5-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-2,5-dihydroxybenzamide (11)

Compound **11** was prepared from amine **2** and 2,5-dihydroxybenzoic acid by using General Method C. Yield: 47%. Purity >99% by analytical HPLC (t_R = 19.96 min). ESI/TOF-MS *m/z* 771.05 for [M−1] (calcd 772.37 for $C_{38}H_{48}N_{10}O_8$). ^{1}H NMR (300 MHz, DMSO) δ (ppm) 0.54 (d, *J* = 6.61, 3H), 0.59 (d, *J* = 6.06, 3H), 0.76–0.89 (m, 7H), 1.03 (d, *J* = 4.95, 2H), 1.23–1.30 (m, 3H), 1.94–1.99 (m, 1H), 2.25–2.28 (m, 1H), 2.74 (m, 1H), 2.83–2.93 (m, 2H), 4.26–4.37 (m, 2H), 6.76 (d, *J* = 8.62, 1H), 6.85 (dd, *J* = 5.78, 1H), 7.20–7.31 (m, 7H), 7.58–7.66 (m, 2H), 8.05 (dd, *J* = 4.14, 1H), 8.20 (s, 1H).

5.3.2. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(2*H*-Tetrazol-5-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-5-fluoro-1,2,3,6-tetrahydro-2,6-dioxopyrimidine-4-carboxamide (13)

Compound **13** was prepared from amine **2** and 5-fluoroorotic acid by using General Method C. Yield: 61%. Purity >99% by analytical HPLC (t_R = 18.23 min). ESI/TOF-MS *m/z* 791.35 for [M−1] (calcd 792.82 for $C_{36}H_{45}FN_{12}O_8$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.91–0.97 (m, 6H), 1.28–1.45 (m, 9H), 2.81–3.1 (m, 5H), 3.5 (m, 1H), 3.65 (m, 2H), 3.72 (m, 1H), 4.21 (d, *J* = 5.69, 1H), 7.48–7.64 (m, 5H), 7.76 (d, *J* = 7.34, 2H), 7.89 (d, *J* = 8.26, 2H), 8.17 (s, 1H).

5.3.3. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(2*H*-Tetrazol-5-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-2*H*-tetrazole-5-carboxamide (15)

Compound **15** was prepared from amine **2** and tetrazolic acid by using general method C. Yield: 45%. Purity >98% by analytical HPLC (t_R = 20.26 min). TOF-MS *m/z* 733.98 for [M+1] (calcd 732.36 for $C_{33}H_{44}N_{14}O_6$). ^{1}H NMR (400 MHz, DMSO) δ (ppm) 0.48 (d, *J* = 6.8, 3H), 0.55 (d, *J* = 6.4, 3H), 0.83 (m, 6H), 1.19 (m, 2H), 1.59 (m, 1H), 1.98 (m, 1H), 2.77 (m, 1H), 2.89 (m, 1H), 3.59 (m, 2H), 3.96 (br s, 1H), 3.98 (m, 1H), 4.19 (m, 1H), 4.33 (m, 2H), 7.23 (m, 1H), 7.28 (m, 4H), 7.50 (t, 1H), 7.68 (d, *J* = 7.6, 1H), 7.79 (d, *J* = 8, 1H), 7.86 (d, *J* = 8.8, 1H), 8.03 (d, *J* = 7.6, 1H), 8.49 (s, 1H), 8.54 (d, *J* = 8, 1H).

5.3.4. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(2*H*-Tetrazol-5-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-cyclohexylpropylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-2*H*-tetrazole-5-carboxamide (16)

Compound **16** was prepared from amine **2** and tetrazolic acid by using General Method C. Yield: 49%. Purity >98% by analytical HPLC (t_R = 21.95 min). TOF-MS *m/z* 773.82 for [M+1] (calcd 772.39 for $C_{36}H_{48}N_{14}O_6$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.82 (m, 11H+3H), 1.07 (m, 1H), 1.28 (m, 3H), 1.50 (d, *J* = 9.0, 1H), 1.94 (m, 1H), 2.74 (m, 1H), 2.88 (m, 1H), 3.55 (m, 2H), 3.95 (s, 1H), 4.09–4.28 (m, 3H), 4.37 (m, 1H), 7.27 (m, 5H), 7.49 (m, 1H), 7.67 (d, *J* = 7.5, 1H), 7.85 (d, *J* = 8.7, 1H), 8.01 (d, *J* = 8.4, 1H), 8.45 (s, 1H), 8.57 (d, *J* = 10.5, 1H).

5.3.5. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-5-oxo-1,2,4-oxadiazol-3-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-5-fluoro-1,2,3,6-tetrahydro-2,6-dioxopyrimidine-4-carboxamide (23)

Compound **23** was prepared from amine **4** and 5-fluoroorotic acid by using General Method C. Yield: 42%. Purity >99% by analytical HPLC (t_R = 19.78 min). ESI/TOF-MS *m/z* 807.05 for [M−1] (calcd 808.81 for $C_{37}H_{45}FN_{10}O_{10}$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.79–0.86 (m, 13H), 1.34 (m, 2H), 2.62–2.89 (m, 5H), 3.58 (m, 2H), 3.96 (d, *J* = 2.94, 1H), 4.28 (d, 1H), 4.21 (d, 1H), 7.17–7.27 (m, 6H), 7.38–7.56 (m, 1H), 7.70–7.81 (d, *J* = 2.94, 1H), 8.10 (d, *J* = 8.81 1H).

5.3.6. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-5-oxo-1,2,4-oxadiazol-3-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-2,5-dihydroxybenzamide (24)

Compound **24** was prepared from amine **4** and 2,5-dihydroxybenzoic acid by using General Method C. Yield: 51%. Purity >99% by analytical HPLC (t_R = 20.84 min). ESI/TOF-MS *m/z* 787.54 for [M−1] (calcd 788.35 for $C_{39}H_{48}N_8O_{10}$). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 0.59 (d, *J* = 6.4, 3H), 0.78–0.97 (m, 9H), 1.23–1.44 (m, 3H), 2.12 (m, 1H), 2.85–2.99 (m, 3H), 3.92–3.96 (m, 3H), 4.15 (d, *J* = 6, 1H), 4.11 (d, 1H), 4.17–4.22 (m, 1H), 6.76 (d, *J* = 8.8, 1H), 6.91–6.94 (dd, *J* = 8.8, 1H), 7.17 (m, 1H), 7.18–7.26 (m, 5H), 7.32 (d, *J* = 3.2, 1H), 7.38–7.42 (m, 1H), 7.62 (d, *J* = 8.8, 1H), 7.89 (d, *J* = 8, 1H), 8.06 (s, 1H).

5.3.7. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-5-oxo-1,2,4-thiadiazol-3-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-5-fluoro-1,2,3,6-tetrahydro-2,6-dioxopyrimidine-4-carboxamide (25)

Compound **25** was prepared from amine **5** and 5-fluoroorotic acid by using General Method C. Yield: 41%. Purity >99% by analytical HPLC (t_R = 19.96 min). ESI/TOF-MS *m/z* 823 for [M−1] (calcd 824.88 for $C_{37}H_{45}FN_{10}O_9S$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.54 (d, *J* = 6.6, 3H), 0.60 (d, *J* = 6.6, 3H), 0.78 (d, *J* = 6.6, 6H), 0.87 (m, 1H), 1.24–1.37 (m, 5H), 2.73 (m, 2H), 3.93 (m, 1H), 4.14 (m, 1H), 4.17 (t, *J* = 6.6, 1H), 4.30 (d, *J* = 7.8, 2H), 7.27–7.40 (m, 6H), 7.58 (d, *J* = 8.7, 1H), 7.67 (d, *J* = 7.2, 1H), 7.86 (d, *J* = 8.7, 1H), 8.26 (s, 1H).

5.3.8. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-5-oxo-1,2,4-thiadiazol-3-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-2,5-dihydroxybenzamide (26)

Compound **26** was prepared from amine **5** and 2,5-dihydroxybenzoic acid by using General Method C. Yield: 89%. Purity >99% by ana-

lytical HPLC (t_R = 21.50 min). ESI/TOF-MS *m/z* 803.03 for [M−1] (calcd 804.91 for $C_{39}H_{48}N_8O_9S$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.53 (d, *J* = 5.8, 3H), 0.85–0.97 (m, 9H), 1.1 (m, 2H), 1.25–1.40 (m, 3H), 2.92 (m, 2H), 3.56 (m, 1H), 4.09 (d, *J* = 4.5, 1H), 4.14 (d, *J* = 3.9, 2H), 4.21 (d, *J* = 6, 2H), 6.76 (d, *J* = 7.5, 1H), 6.97 (dd, *J* = 4.2, 1H), 7.18–7.26 (m, 7H), 7.32 (m, 1H), 7.76 (d, *J* = 8.9, 1H), 7.94 (m, 2H).

5.3.9. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-5-thioxo-1,2,4-oxadiazol-3-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-5-fluoro-1,2,3,6-tetrahydro-2,6-dioxopyrimidine-4-carboxamide (27)

Compound **27** was prepared from amine **6** and 5-fluoroorotic acid by using General Method C. Yield: 52%. Purity >99% by analytical HPLC (t_R = 21.70 min). ESI-MS (TOF-MS) *m/z* 822.95 (824.62) (calcd 824.31 for $C_{37}H_{45}FN_{10}O_9S$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.62 (d, *J* = 5.87, 3H), 0.66 (d, *J* = 5.87, 3H), 0.90 (d, *J* = 6.97, 6H), 1.17 (m, 2H), 2.21 (d, 2H), 2.73 (d, 2H), 3.07 (t, *J* = 6, 1H), 3.53 (t, *J* = 8.7, 1H), 4.08 (d, *J* = 2.1, 2H), 4.14 (d, *J* = 6.6, 1H), 4.57 (d, 2H), 7.24–7.30 (m, 6H), 7.37 (m, 1H), 7.52 (d, *J* = 8.26, 1H), 7.91 (d, 1H), 8.20 (s, 1H).

5.3.10. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-5-thioxo-1,2,4-oxadiazol-3-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-2,5-dihydroxybenzamide (28)

Compound **28** was prepared from amine **6** and 2,5-dihydroxybenzoic acid by using General Method C. Yield: 47%. Purity >99% by analytical HPLC (t_R = 19.78 min). ESI/TOF-MS *m/z* 803.87 for [M−1] (calcd 804.33 for $C_{39}H_{48}N_8O_9S$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.56 (d, *J* = 6.42, 3H), 0.60 (d, *J* = 6.79, 3H), 0.94–1.0 (m, 9H), 2.11–2.19 (m, 2H), 2.85–2.99 (m, 3H), 3.63–3.78 (m, 2H), 4.13–4.19 (m, 3H), 6.77 (d, *J* = 8.62, 1H), 6.95 (dd, *J* = 5.87, 1H), 7.15–7.30 (m, 7H), 7.37 (d, *J* = 2.75, 1H), 7.52 (s, 1H), 7.76 (m, 1H), 8.23 (s, 1H).

5.3.11. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-5-thioxo-1,3,4-oxadiazol-2-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-5-fluoro-1,2,3,6-tetrahydro-2,6-dioxopyrimidine-4-carboxamide(29)

Compound **29** was prepared from amine **9** and 5-fluoroorotic acid by using General Method C. Yield: 24%. Purity >99% by analytical HPLC (t_R = 20.48 min). ESI/TOF-MS *m/z* 823.90 for [M−1] (calcd 824.88 for $C_{37}H_{45}FN_{10}O_9S$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.69 (d, *J* = 6.3, 3H), 0.78 (d, *J* = 6.3, 3H), 0.86 (d, *J* = 6.6, 3H), 0.91 (d, *J* = 6.6, 3H), 1.26–1.29 (m, 2H), 2.74 (m, 2H), 3.01 (m, 2H), 3.82–3.89 (m, 2H), 3.98 (d, *J* = 6.8, 1H), 4.19 (d, *J* = 6.9, 2H), 4.31–4.33 (m, 2H), 7.27 (m, 5H), 7.46–7.7.51 (m, 2H), 7.75 (d, *J* = 9.9, 1H), 7.88 (d, *J* = 10.2, 1H), 8.18 (s, 1H).

5.3.12. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-5-oxo-1,3,4-oxadiazol-2-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-2,5-dihydroxybenzamide (30)

Compound **30** was prepared from amine **9** and 2,5-dihydroxybenzoic acid by using General Method C. Yield: 79%. Purity >99% by analytical HPLC (t_R = 22.06 min). ESI/TOF-MS *m/z* 803.50 for [M−1] (calcd 804.33 for $C_{39}H_{48}N_8O_9S$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.55–0.60 (m, 6H), 0.81–0.98 (m, 7H), 1.24 (m, 1H), 1.61 (m, 3H), 2.88 (m, 2H), 3.61 (m, 1H), 4.10–4.25 (m, 5H), 6.77 (d, *J* = 8.99, 1H), 6.94 (dd, *J* = 5.87, 1H), 7.25 (m, 7H), 7.41 (m, 1H), 7.62 (d, *J* = 10.09, 1H), 7.85 (d, *J* = 8.26, 1H), 8.19 (s, 1H).

5.3.13. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-4-methyl-5-thioxo-1*H*-1,2,4-triazol-3-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-5-fluoro-1,2,3,6-tetrahydro-2,6-dioxopyrimidine-4-carboxamide (31)

Compound **31** was prepared from amine **10** and 5-fluoroorotic acid by using General Method C. Yield: 75%. Purity >99% by analytical HPLC (t_R = 19.34 min). ESI/TOF-MS *m/z* 836.52 for [M−1] (calcd 837.34 for $C_{38}H_{48}FN_{11}O_8S$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.58–0.65 (m, 6H), 0.89–0.97 (m, 6H), 1.12 (m, 2H), 1.28–1.35 (m, 5H), 2.89–2.95 (m, 1H), 3.63 (d, *J* = 7.5, 2H), 3.89–3.96 (m, 2H), 4.07–4.10 (m, 1H), 4.19–4.21 (m, 2H), 4.60 (d, *J* = 5.7, 1H), 7.20 (d, *J* = 4.5, 1H), 7.27 (m, 5H), 7.40–7.47 (m, 1H), 7.79–7.82 (m, 2H), 8.08 (s, 1H).

5.3.14. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(4,5-Dihydro-4-methyl-5-thioxo-1*H*-1,2,4-triazol-3-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-2,5-dihydroxybenzamide (32)

Compound **32** was prepared from amine **10** and 2,5-dihydroxybenzoic acid by using General Method C. Yield: 67%. Purity >99% by analytical HPLC (t_R = 20.78 min). ESI/TOF-MS *m/z* 816.56 for [M−1] (calcd 817.36 for $C_{40}H_{51}N_9O_8S$). ^{1}H NMR (300 MHz, DMSO) δ (ppm) 0.53 (d, *J* = 6.61, 3H), 0.59 (d, *J* = 6.61, 3H), 0.77 (m, 6H), 0.85–0.87 (m, 2H), 1.18–1.28 (m, 3H), 1.9 (s, 3H), 2.78 (m, 2H), 2.87 (m, 1H), 3.95 (m, 1H), 4.14–4.18 (m, 2H), 4.29 (d, *J* = 7.71, 2H), 6.72 (d, *J* = 8.81, 1H), 6.83 (dd, *J* = 5.78, 1H), 7.22–7.28 (m, 6H), 7.39–7.48 (m, 2H), 7.71 (m, 1H), 7.83 (d, *J* = 8.62, 1H), 8.03 (s, 1H).

5.3.15. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(2*H*-Tetrazol-5-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-1*H*-imidazole-4-carboxamide (33)

Compound **33** was prepared from amine **2** and 4-imidazole carboxylic acid by using General Method C. Yield: 62%. Purity >98% by analytical HPLC (t_R = 18.32 min). ESI/TOF-MS *m/z* 729.59 for [M−1] (calcd 730.37 for $C_{35}H_{46}N_{12}O_6$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.58 (d, *J* = 6.24, 3H), 0.64 (d, *J* = 6.24, 3H), 0.99 (d, *J* = 6.61, 6H), 1.29 (m, 2H), 2.79 (m, 1H), 2.76–3.01 (m, 3H), 3.56–3.60 (m, 1H), 3.66–3.72 (m, 2H), 4.22 (d, *J* = 8.99, 3H), 4.33 (m, 1H), 7.24–7.29 (m, 6H), 7.50 (d, *J* = 5.1, 1H), 7.71 (dd, *J* = 10.92, 2H), 8.19 (s, 1H), 8.45 (s, 1H), 8.99 (s, 1H).

5.3.16. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(2*H*-Tetrazol-5-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-cyclohexylpropylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-1*H*-imidazole-4-carboxamide (34)

Compound **34** was prepared from amine **2** and 4-imidazole carboxylic acid by using General Method C. Yield: 73%. Purity >98% by analytical HPLC (t_R = 19.98 min). ESI/TOF-MS *m/z* 769.25 for [M−1] (calcd 770.40 for $C_{38}H_{50}N_{12}O_6$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.74 (d, *J* = 6.9, 3H), 0.88 (d, *J* = 6.6, 3H), 0.74 (d, *J* = 7.5, 2H), 1.12 (m, 1H), 1.23–1.55 (m, 10H), 1.89 (m, 2H), 2.75 (m, 2H), 2.95 (m, 1H), 3.92 (m, 1H), 4.19 (m, 2H), 4.31 (m, 2H), 7.24 (m, 6H), 7.64 (d, *J* = 8.4, 1H), 7.85 (d, *J* = 8.1, 1H), 8.03 (d, *J* = 7.8, 1H), 8.23 (s, 2H).

5.3.17. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(2*H*-Tetrazol-5-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-methylbutylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-1*H*-1,2,4-triazole-3-carboxamide (35)

Compound **35** was prepared from amine **2** and 4-triazole carboxylic acid by using General Method C. Yield: 61%. Purity >98% by analytical HPLC (t_R = 18.84 min). ESI/TOF-MS *m/z* 730.49 for [M−1] (calcd 731.8 for $C_{34}H_{45}N_{13}O_6$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.47–0.73 (m, 6H), 0.79–1.03 (m, 6H), 1.21–1.53 (m, 2H),

2.03–2.11 (m, 1H), 2.82–3.05 (m, 2H), 3.55–3.73 (m, 3H), 3.88 (q, J = 7.2, 1H), 4.07–4.27 (m, 2H), 4.30–4.38 (m, 1H), 4.61–4.70 (m, 1H), 7.13–7.34 (m, 6H), 7.45–7.55 (m, 1H), 7.71–7.79 (dd, J = 8.1, 2H), 8.39 (s, 1H), 8.54 (s, 1H).

5.3.18. *N*-((2*S*,5*S*)-5-((*S*)-1-((1*R*,2*S*)-1-(3-(2*H*-Tetrazol-5-yl)phenylcarbamoyl)-1-hydroxy-3-phenylpropan-2-ylcarbamoyl)-3-cyclohexylpropylcarbamoyl)-2-amino-6-methyl-3-oxoheptyl)-1*H*-1,2,4-triazole-3-carboxamide (36)

Compound **36** was prepared from amine **2** and 4-triazole carboxylic acid by using General Method C. Yield: 72%. Purity >97% by analytical HPLC (t_R = 20.66 min). ESI/TOF-MS m/z 770.60 for [M−1] (calcd 771.39 for $C_{37}H_{49}N_{13}O_6$). ^{1}H NMR (300 MHz, CD_3OD) δ (ppm) 0.73–0.79 (m, 6H), 0.85–0.90 (q, J = 5.1 2H), 1.03 (d, J = 5.4, 1H), 1.17–1.28 (m, 11H), 1.50 (m, 1H), 1.92–1.99 (m, 1H), 2.71–2.78 (q, J = 6.9, 2H), 2.84–2.92 (q, J = 7.8, 1H), 3.93 (m, 1H), 4.20 (m, 2H), 4.28–4.38 (m, 2H), 7.22–7.38 (m, 6H), 7.57 (d, J = 7.8, 1H), 7.64 (d, J = 8.1, 1H), 7.83 (d, J = 8.7, 1H), 8.23 (s, 1H).

5.4. BACE1 assay

BACE1 inhibitory activity of the inhibitors was determined by enzymatic assay using recombinant human BACE1 and FRET (fluorescence resonance energy transfer) substrate using previously reported methods.[16] After the enzymatic reaction with BACE1 and FRET substrate, (7-methoxycoumarin-4-yl)acetyl-Ser-Glu-Val-Leu*Asp-Ala-Glu-Phe-Arg-Lys(2,4-dinitrophenyl)-Arg-Arg-NH_2, in incubation buffer with 2 or 0.2 μM KMI-compounds, the N-terminal cleavage fragment of substrate was analyzed by fluorescence detection using RP-HPLC.

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