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Original article

Design and synthesis of 1,4-dihydropyridine derivatives as BACE-1 inhibitors

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ABSTRACT

BACE-1 has been shown to be an attractive therapeutic target in Alzheimer's disease (AD). Using a 1,4-dihydropyridine (DHP) scaffold, we synthesized new inhibitors of BACE-1 by modifying the known BACE inhibitor **2** containing a hydroxyethylamine (HEA) motif. Using structure-based drug design based on computer-aided molecular docking, the isophthalamide ring of **2** was replaced with a 1,4-dihydropyridine ring as a brain-targeting strategy. Several of the new dihydropyridine derivatives were synthesized and their BACE-1-inhibitory activities were evaluated using a cell-based, reporter gene assay system that measures the cleavage of alkaline phosphatase (AP)-APP fusion protein by BACE-1. Most of the 1,4-DHP analogs showed BACE-1-inhibitory activities with IC_{50} values in the range 8–30 μM, suggesting that the 1,4-DHP skeleton may be utilized to develop brain-targeting BACE-1 inhibitors.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and no disease modification therapy is currently available [1]. Much research on AD is based on the amyloid cascade hypothesis, which suggests that accumulation of amyloid-β-42 ($Aβ_{42}$) peptides and the formation of plaques in the brain early in the course of the disease are significant in most patients with AD. These $Aβ_{40}$ and $Aβ_{42}$ peptides are secreted as a result of proteolytic processing of a large transmembrane protein, amyloid precursor protein (APP), by two enzymes, the β- and γ-secretases [2,3]. Therefore, the development of specific inhibitors of these key proteases, to block Aβ production, has been a major therapeutic target in AD treatment. Particularly, β-secretase (β-site APP cleaving enzyme or BACE-1), a type I membrane-associated aspartyl protease [5], has been considered to be attractive therapeutic target in AD, because the enzyme catalyzes the first step in Aβ production [4].

A strategy frequently employed in designing BACE-1 inhibitors is replacement of the scissile amide bond of APP with noncleavable transition state isosteres such as statine, hydroxyethylene, norstatine, or hydroxyethylamine (HEA), which can interact with aspartates in the active site of BACE-1 [6–9]. Among known motif of the aspartyl protease inhibitors, hydroxyethylamine (HEA) dipeptide isoster has been extensively applied to HIV protease and renin inhibitors [10] as well as several BACE-1 inhibitors (Fig. 1) [9,11–13]. For example, Elan Corporation reported an isophthalamide-HEA based derivative, **1**, as a potent and cell permeable BACE-1 inhibitor [11]. Introduction of a pyrrolidine scaffold led to development of a conformationally constrained HEA inhibitor, compound **3** (by Schering) [12], and the 5-substituted isophthalamide analog, **2** (by Merck), inhibited BACE-1 enzymatic activity (IC_{50} = 15 nM) and in a cell-based assay (IC_{50} = 29 nM), with impressive selectivity against other biologically relevant aspartyl proteases [9]. Because of impermeability through the blood–brain barrier and P-glycoprotein (Pgp)-mediated efflux, however, compound **2** was poorly distributed within the CNS. Recently GSK reported a modified isophthalamide inhibitor, **4**, which was the first orally bioavailable compound but had low brain penetration because of Pgp-efflux [13].

Abbreviations: 1,4-DHP, 1,4-Dihydropyridine; $Aβ_{40}$, 40-Residue β-amyloid peptide; $Aβ_{42}$, 42-Residue β-amyloid peptide; AD, Alzheimer's diseases; AP, Alkaline phosphatase; APP, β-Amyloid precursor protein; APPsβ, Soluble APP product from β-secretase cleavage; BACE-1, β-Site APP cleaving enzyme; Bn, Benzyl; BSA, Bovine serum albumin; CNS, Central nervous system; DCM, Dichloromethane; DIPA, Diisopropylamine; DIPEA, Diisopropylethylamine; DMAP, Dimethylaminopyridine; DMEM, Dulbecco's modified eagle medium; DMF, Dimethylformamide; EDAC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; FBS, Fetal bovine serum; HATU, 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium; HEA, Hydroxyethylamine; HEK, Human embryonic kidney; pNPP, *p*-Nitrophenylphosphate; PyBOP, (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; SAR, Structure-activity relationship; TEA, Triethylamine; THF, Tetrahydrofuran.

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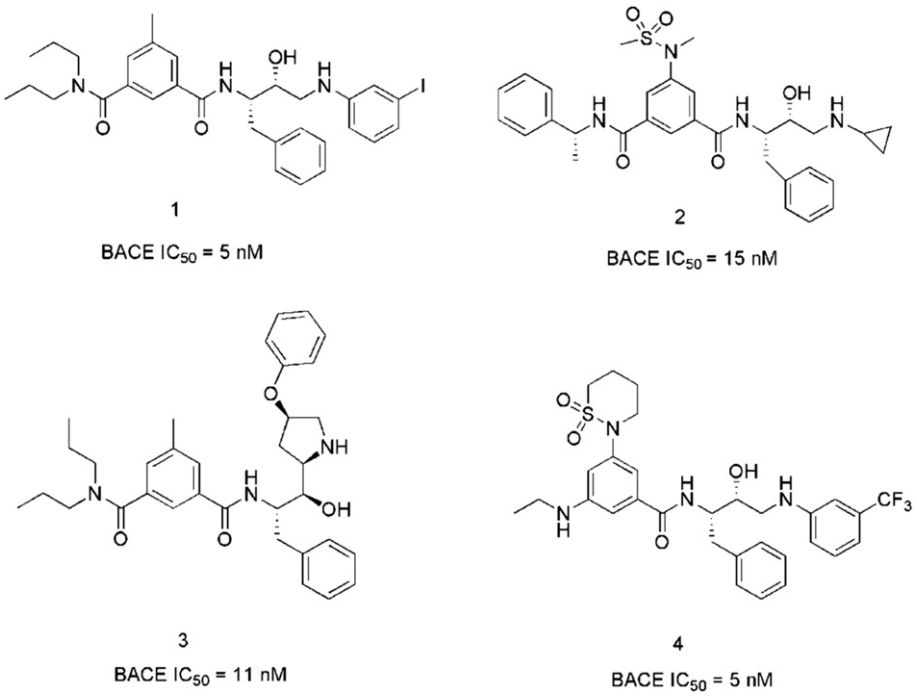


Fig. 1. Hydroxyethylamine (HEA) BACE-1 inhibitors.

As a BACE-1 inhibitor must act in the brain, the material must be able to cross the blood–brain barrier and achieve sustainable drug levels in the brain [14]. To introduce brain-targeting properties for BACE-1 inhibitors, we attempted to replace the isophthalamide ring of **2** with a 1,4-dihydropyridine (DHP) scaffold, which has been utilized to target the brain, because of an ability to be metabolized into charged quaternary amine molecules, resulting in the material becoming locked into the brain [15]. Moreover, 1,4-DHP scaffolds can be used to design diverse analogs through derivatization at various positions (Fig. 2). In this perspective, 1,4-DHP scaffold was investigated as a new skeleton of BACE-1 inhibitor by incorporation of structural information from known BACE-1 inhibitors such as compound **2**, estimation of binding through molecular docking, and evaluation of the inhibitory activity in a cell-based assay system.

2. Results and discussion

2.1. Inhibitor design and molecular docking study

To estimate the binding of 1,4-DHP-based BACE-1 inhibitors, we used a molecular modeling program, Discovery Studio (Accelrys), to dock **9a** into the active site of BACE-1. Fig. 3a and b show the interaction models of docked compounds **2** and **9a**, respectively. Overlay of docking structures showed that the two compounds occupied the same binding pocket in the active site of BACE-1 (Fig. 3a). Hydrophobic interactions of the α-methylbenzyl group, hydrogen bonding of the sulfonamide group, and interactions of the cyclopropyl amine moieties in the S3, S2, and S1′ pockets, respectively, were the same for the two compounds. A detailed analysis of the hydrogen bonding network of **9a** is shown in Fig. 3b. The sulfonamide group of **9a** was hydrogen-bonded to the carbonyl oxygen of Gln73 and the amine nitrogen to Gly230, and the HEA isostere of **9a** was hydrogen-bonded to both catalytic aspartates, Asp32 and Asp228, findings consistent with those observed for compound **2**.

Based on molecular docking results, we designed 1,4-DHP derivatives as BACE-1 inhibitors using five strategies. These were (1) replacement of the bulky α-methylbenzamide group in **2** with a benzyl ester or smaller acetyl group for binding in the S3 pocket; (2) modification of the sulfonamide group in the aromatic scaffold of **2** with alkyl ester or amide groups, maintaining the important hydrogen bonding with Asn233 in the S2 binding pocket; (3) incorporation of additional hydrophobic interactions into the S1 binding pocket by introduction of alkyl or aryl groups, including methyl, ethyl, propyl, isopropyl, and phenyl groups; (4) alteration of the cyclopropyl group at the R_4 position to other aromatic groups, thus changing hydrophobic interactions in the S2′ binding pocket by extension toward the prime-side of the enzyme; and, (5) alterations at the 2 and 6 positions of the 1,4-DHP scaffold by synthesis of 2-monomethyl and 2,6-unsubstituted analogs.

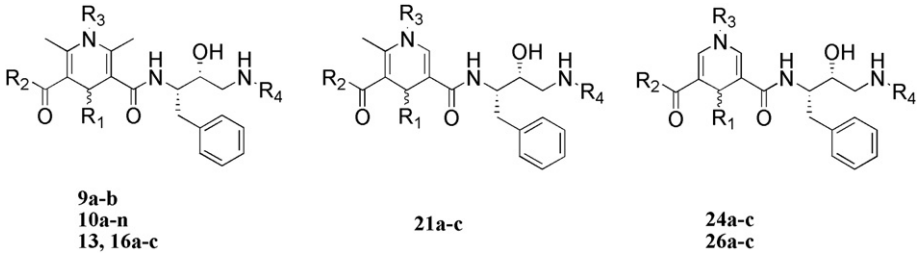


Fig. 2. 1,4-dihydropyridine (DHP) BACE-1 inhibitors.

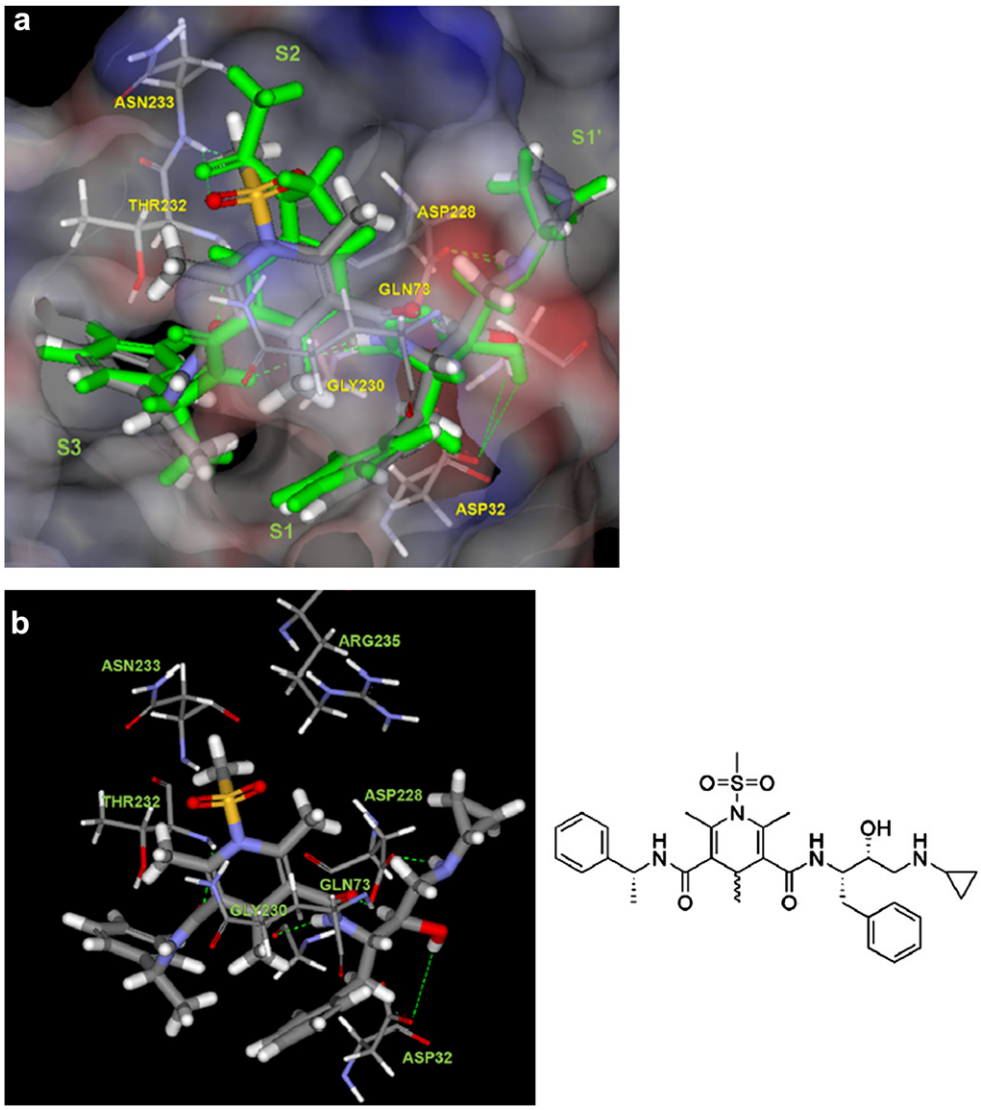


Fig. 3. a. Overlay of **2** (green) and **9a** in the BACE-1 active site, b. Interactions of **9a** in the active site of BACE-1. Hydrogen bonds are shown with dotted lines (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.2. Chemistry

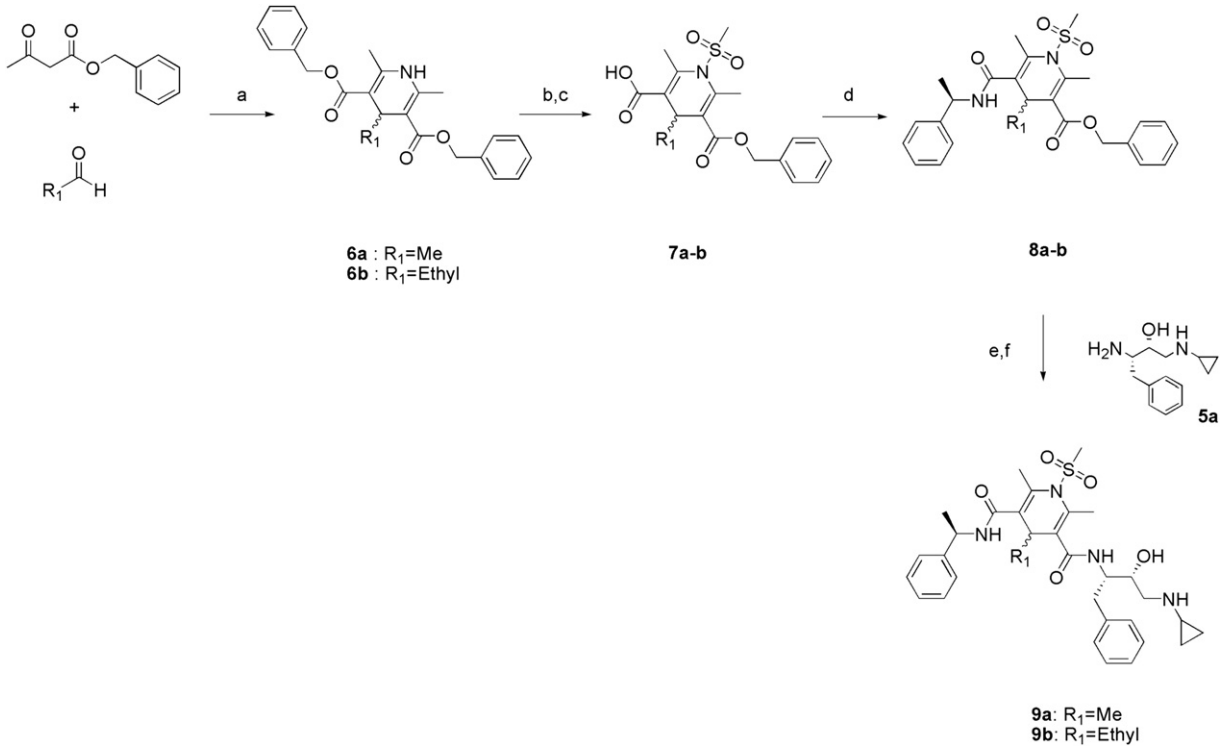
2.2.1. 2,6-Dimethyl-1,4-dihydropyridine derivatives

As molecular docking suggested that the 1,4-DHP analog, **9a**, and the 5-amino isophthalate analog, **2**, had similar binding properties, we synthesized various 1,4-DHP derivatives by Hantzsch condensation using three components; a β-ketoester, an aldehyde, and a β-enaminoester, as shown in Schemes 1–6 [16]. The initial 2,6-dimethyl-1,4-DHP analogs, **9a**–**b**, corresponding to **2**, were synthesized as shown in Scheme 1. Briefly, the N-1 positions of the 1,4-DHP dibenzyl esters, **6a**–**b**, were mesylated, followed by monohydrolysis at 0 °C with aluminum chloride as a Lewis-acid, resulting in compounds **7a**–**b** [17]. PyBOP-mediated amide coupling of **7a**–**b** with (*R*)-methylbenzylamine, hydrolysis of the resulting esters, **8a**–**b**, and coupling with compound **5a**, an HEA isoster moiety [9], yielded **9a**–**b**. The HEA isoster moieties, **5a**–**d**, were prepared in two steps as described [9].

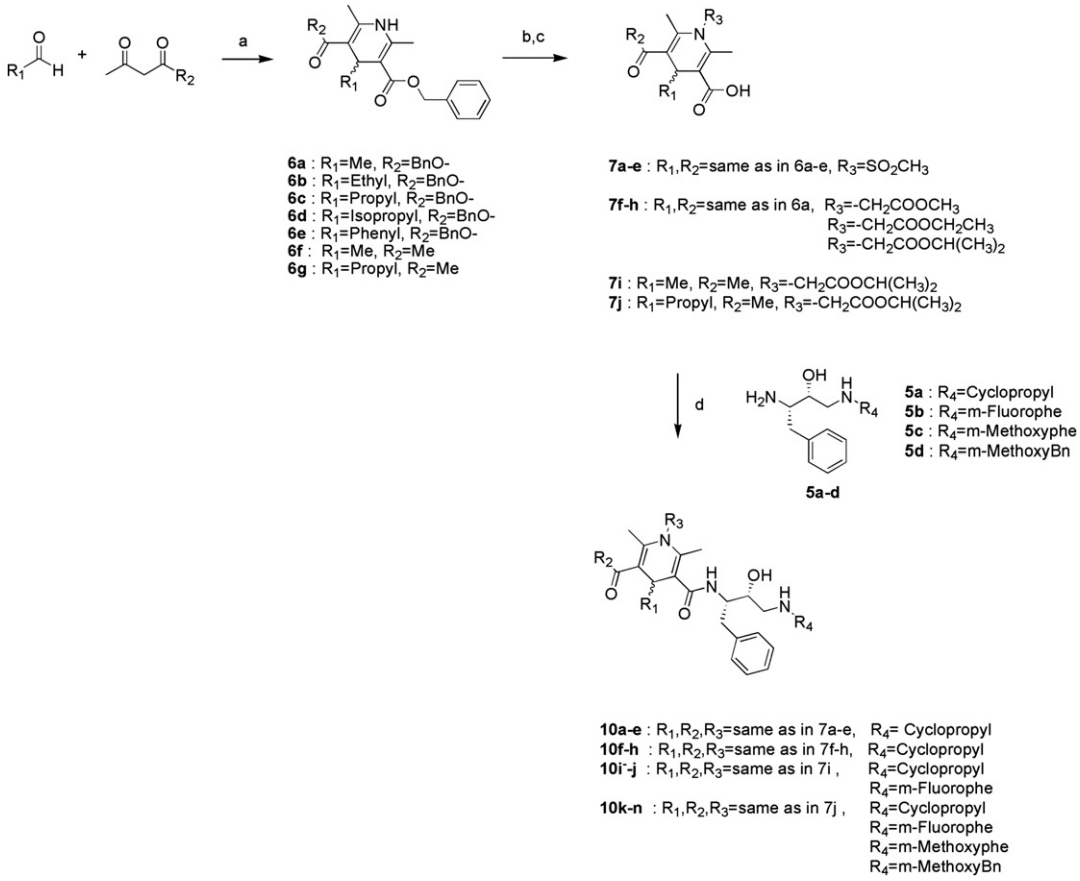
A second series of 2,6-substituted 1,4-DHP-based inhibitors, **10a**–**n**, was prepared as depicted in Scheme 2. Compounds **10a**–**n** contained various alkyl chains (methyl, ethyl, propyl, or isopropyl), or a phenyl group, at the R_1 position, and a benzyl ester or acetyl group instead of the (*R*)-methylbenzylamine at the R_2 position. We also replaced the sulfonamide moiety at the R_3 position with several alkyl ester groups (methyl, ethyl, or isopropyl) for extension of carbon chains. The aromatic building blocks (*m*-fluorophenyl, *m*-methoxyphenyl, or *m*-methoxybenzyl) at the R_4 position were introduced in place of the cyclopropyl group (**10j**, **10l**–**n**).

Compound **13** was synthesized to assess the effects of an alkyl chain linker on the R_3 group (Scheme 3). Compound **11** was hydroborated using borane-methylsulfide; subsequent oxidation with H_2O_2 resulted in an alcohol compound [18,19], which was converted to **12** by acetylation. Monohydrolysis of one of the benzyl ester groups followed by a coupling reaction with **5a** yielded the carbon chain-extended analog, **13**.

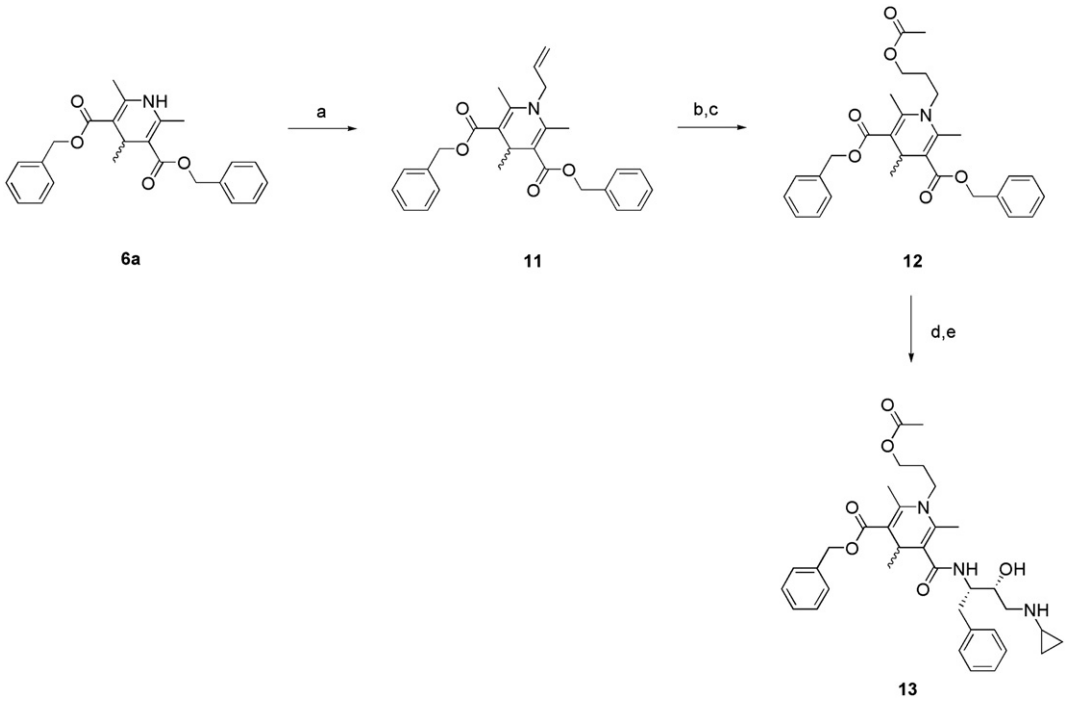
Compounds **16a**–**c**, containing amides at the N-1 position instead of esters, were prepared as outlined in Scheme 4. To introduce an amide moiety into the secondary amine group of 1,4-DHP, the tert-butyl acetate group of **14a** was selectively hydrolyzed and coupled with several secondary amine building blocks to yield **15a**–**c**. Monohydrolysis of the benzyl ester group



Scheme 1. Synthesis of 1-methylsulfonamide-2,6-dimethyl-1,4-dihydropyridine derivatives. Reagent: (a) NH_4OAc, Ethanol, 90 °C, 24 h, 98%; (b) Methane sulfonylchloride, NaH, DMF, 0–60 °C, 4 h, 35%; (c) $AlCl_3$, Anisole, DCM, −50 °C to RT, 2 h, 21%; (d) *R*-methylbenzylamine, PyBOP, DIPA, DCM, 1 h, 70%; (e) $AlCl_3$, Anisole, DCM, −50 °C to RT, 2 h, 30%; (f) Compound **5a**, PyBOP, DIPEA, DCM, 15 min, 65%.



Scheme 2. Synthesis of 1-alkyl acetate-2,6-dimethyl-1,4-dihydropyridine derivatives. Reagent: (a) NH_4OAc, Ethanol, 90 °C, 24 h, 98%; (b) R_3 building blocks, NaH, DMF, 0–60 °C, 5 h, 30%; (c) $AlCl_3$, Anisole, DCM, −50 °C to RT, 1 h, 20%; (d) Compounds **5a**–**d**, PyBOP, DIPEA, DCM, 15 min, 49%.

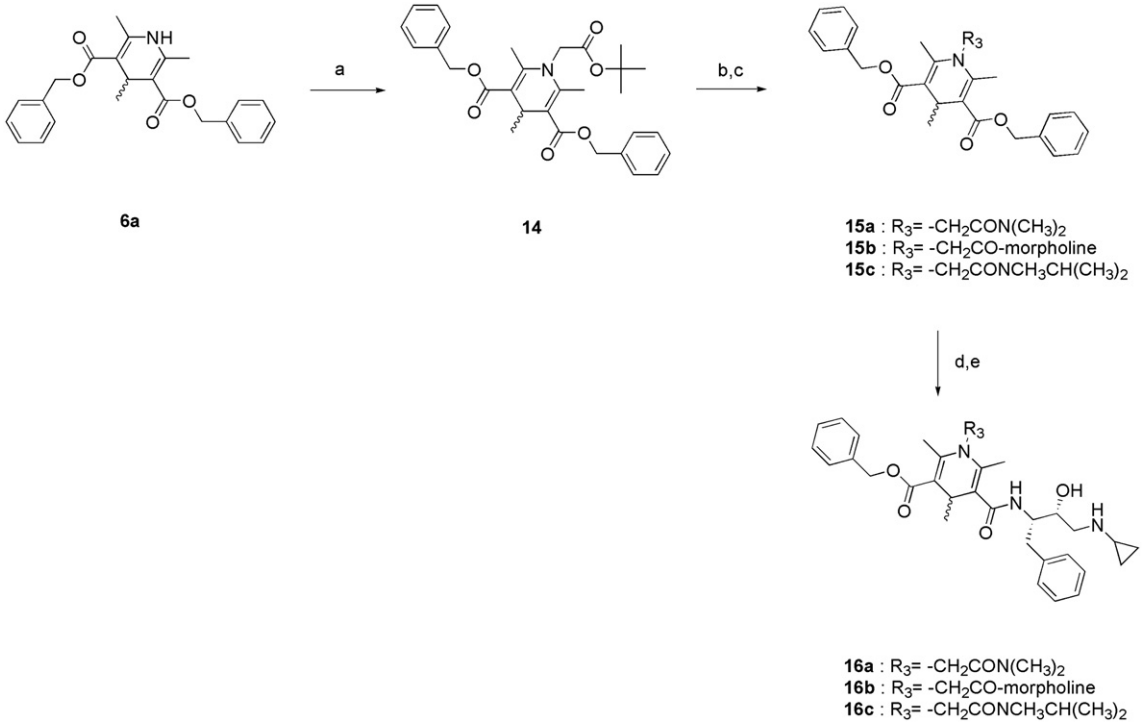


Scheme 3. Synthesis of 1-(3-acetoxypropyl)-2,6-dimethyl-1,4-dihydropyridine derivatives. Reagent: (a) Ally bromide, NaH, DMF, 1 h, 45%; (b) i. Borane-methylsulfide, THF, 2 h ii. 3N NaOH, 0 °C, iii. 30% H_2O_2 in water, 0 °C, 35%; (c) AcCl, TEA, DCM, 2 h, 50%; (d) $AlCl_3$, Anisole, DCM, −50 °C to RT, 2 h, 21%; (e) Compound **5a**, PyBop, DIPEA, DCM, 15 min, 45%.

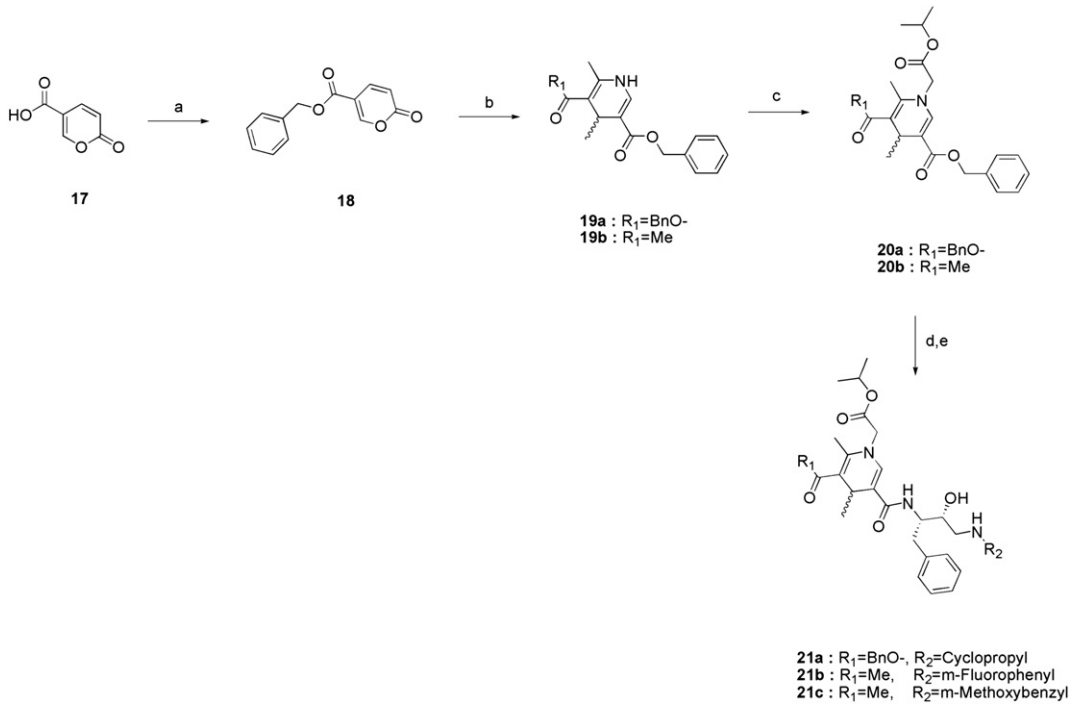
and PyBOP-mediated amide coupling with **5a** afforded compounds **16a**–**c**.

2.2.2. 2-Monomethyl 1,4-dihydropyridine derivatives

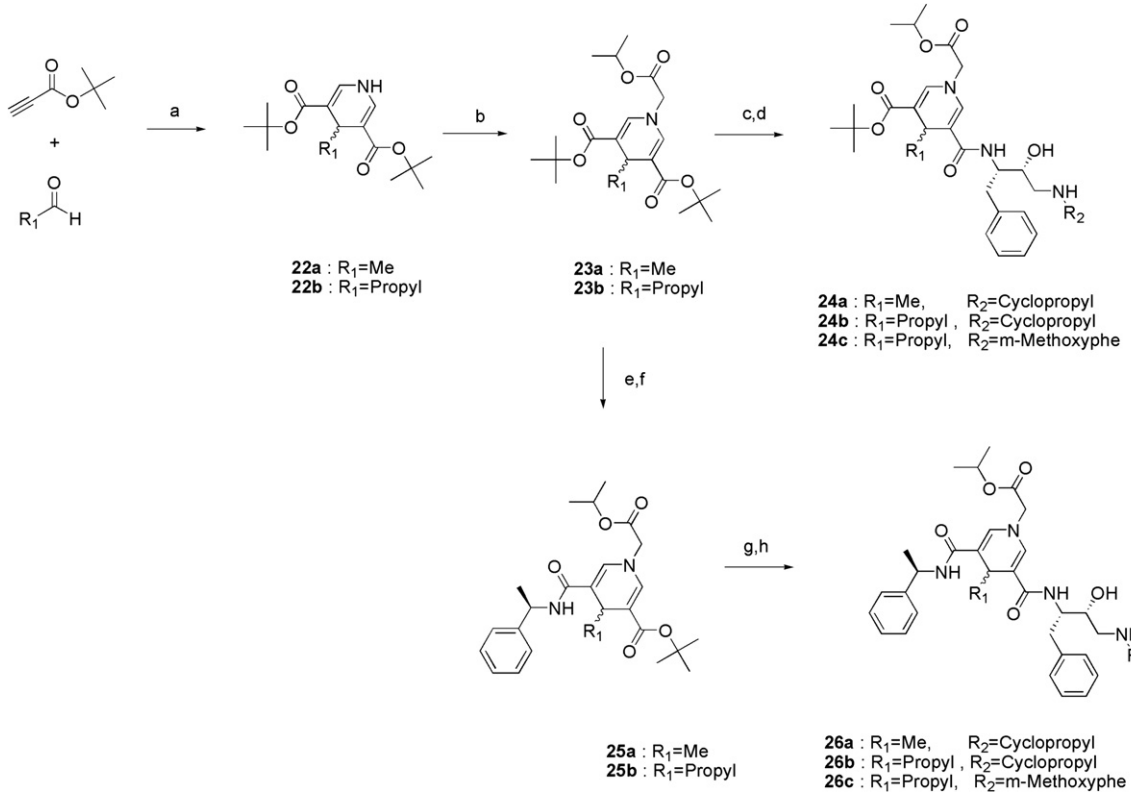
The 2-monomethyl-1,4-dihydropyridines, **21a**–**c**, were synthesized to assess the possible steric effects of methyl groups of dimethyldihydropyridines in the binding site of BACE-1 (Scheme 5). Benzyl coumalate, **18**, was subjected to a condensation reaction with benzylacetoacetate or acetylacetone in the presence of ammonium acetate, yielding **19a**–**b** [20]. Subsequent reactions, including *N*-alkylation, hydrolysis, and coupling with **5a** and **5b**–**d** were the same as described in the reaction schemes above. Compounds **21a**–**c** were



Scheme 4. Synthesis of 1-acetamide-2,6-dimethyl-1,4-dihydropyridine derivatives. Reagent: (a) t-butyl bromoacetate, NaH, DMF, 0–60 °C, 4 h, 33%; (b) TFA 50% in DCM, 2 h, 47%; (c) PyBop, R_3 building blocks, DCM, 2 h, 49%; (d) $AlCl_3$, Anisole, DCM, −40 °C to RT, 2 h, 29%; (e) Compound **5a**, PyBop, DIPEA, DCM, 15 min, 40%.



Scheme 5. Synthesis of 2-monomethyl-1,4-dihydropyridine derivatives. Reagent: (a) Benzyl alcohol, DMAP, EDC, DCM, 5 h, 70%; (b) Benzylacetoacetate or acetylacetone, acetic acid, NH_4OAc, 24 h, 33%; (c) Isopropylbromoacetate, NaH, 0–60 °C, 4 h, DMF, 69%; (d) $AlCl_3$, Anisole, DCM, −50 °C to RT, 2 h, 20%; (e) Compounds **5a** or **5b** or **5d**, HATU, DIPEA, DCM, 1 h, 42%.



Scheme 6. Synthesis of 2,6-unsubstituted-1,4-dihydropyridine derivatives. Reagent: (a) NH_4OAc, Ethanol, 90 °C, 24 h, 15%; (b) Isopropylbromoacetate, NaH, DMF, 0–60 °C, 4 h, 58%; (c) 96% formic acid, 0 °C, 20 min, 45%; (d) Compounds **5a**,**c**, HATU, DIPEA, DCM, 1 h, 55%; (e) 96% formic acid, 0 °C, 20 min, 49%; (f) *R*-methylbenzylamine, PyBop, DIPA, DCM, 1 h, 59%; (g) 96% formic acid, 0 °C, 30 min, 60%; (h) Compounds **5a**,**c**, HATU, DIPEA, DCM, 1 h, 48%.

also prepared as a mixture of diastereomers and confirmed by NMR spectrum of R_1-group.

2.2.3. 2,6-Unsubstituted 1,4-dihydropyridine derivatives

We synthesized the 2,6-unsubstituted-1,4-dihydropyridine analogs **24a**–**c** and **26a**–**c** using **22a**–**b**, which had been prepared by reaction with tert-butylpropiolate and acetaldehyde or butylaldehyde (Scheme 6) [21,22]. *N*-alkylation with isopropylbromoacetate followed by monohydrolysis of a t-butyl ester with 96% formic acid [23], and the HATU-mediated coupling of a carboxylic acid group with derivatives of **5a**, **c** yielded **24a**–**c**. Compounds **26a**–**c** were synthesized by monohydrolysis of **23a**–**b**, followed by amide coupling with (*R*)-methylbenzylamine, hydrolysis of the remaining ester, and final amide coupling, as shown for **24**.

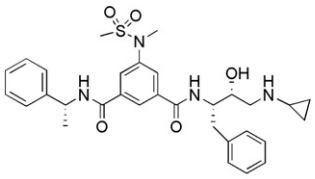
In the case of compound **9a** and **9b**, each single diastereomer could be separated by silica gel column chromatography. However, all other final compounds were obtained and tested as diastereomeric mixtures.

2.3. BACE-1-inhibitory activity

We utilized an alkaline phosphatase (AP)-APP-cell-based assay system to evaluate the BACE-1-inhibitory activity of the synthesized 1,4-DHP derivatives. A plasmid encoding a fusion protein of APP with AP, and containing the BACE-1 cleavage site, was stably transfected into HEK293 cells, allowing BACE-1-inhibitory activity to be measured indirectly by quantification of secreted AP after cleavage of the β-site of APP by BACE-1. The BACE-1-inhibitory activities of the 2,6-dimethyl-1,4-DHP derivatives are summarized in Table 1. The known BACE-1 inhibitor, **2** (reported IC_{50} = 29 nM), was utilized as a positive control. We found that the IC_{50} value of **2** was 0.25 μM in our indirect cell-based assay system, which exhibited less than 8-fold loss of BACE-1-inhibitory activity compared to the direct measurement of BACE-1 activity in cells [9].

Initially, to test the effect on BACE-1 enzyme activity of the replacement of the isophthalate ring of **2** by a 1,4-DHP scaffold, we determined the activities of **9a** (R_1 = methyl) and **9b**

Table 1
BACE-1-inhibitory activities of 2,6-dimethyl-1,4-DHP analogs.



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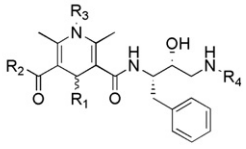
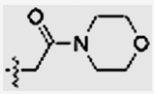
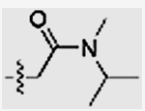


9a-b, 10a-n, 13, 16a-c

Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)[a]
2[b]					0.25 ± 0.18
9a[c]	Methyl	–NH-(α)methylBn	$-SO_2CH_3$	Cyclopropyl	16.1 ± 2.1
9b[c]	Ethyl	–NH-(α)methylBn	$-SO_2CH_3$	Cyclopropyl	16.2 ± 0.6
10a[d]	Methyl	–OBn	$-SO_2CH_3$	Cyclopropyl	14.9 ± 0.6
10b[d]	Ethyl	–OBn	$-SO_2CH_3$	Cyclopropyl	9.5 ± 0.4
10c[d]	Propyl	–OBn	$-SO_2CH_3$	Cyclopropyl	8.1 ± 0.8
10d[d]	Isopropyl	–OBn	$-SO_2CH_3$	Cyclopropyl	11.0 ± 3.0
10e[d]	Phenyl	–OBn	$-SO_2CH_3$	Cyclopropyl	21.4 ± 3.5
10f[d]	Methyl	–OBn	$-CH_2COOCH_3$	Cyclopropyl	15.2 ± 3.1
10g[d]	Methyl	–OBn	$-CH_2COOCH_2CH_3$	Cyclopropyl	15.5 ± 0.3
10h[d]	Methyl	–OBn	$CH_2COOCH(CH_3)_2$	Cyclopropyl	9.0 ± 0.6
10i[d]	Methyl	$-CH_3$	$CH_2COOCH(CH_3)_2$	Cyclopropyl	29.3 ± 2.3
10j[d]	Methyl	$-CH_3$	$CH_2COOCH(CH_3)_2$	*m*-Fluorophe	26.4 ± 0.4
10k[d]	Propyl	$-CH_3$	$CH_2COOCH(CH_3)_2$	Cyclopropyl	33.1 ± 3.1
10l[d]	Propyl	$-CH_3$	$CH_2COOCH(CH_3)_2$	*m*-Fluorophe	27.1 ± 1.3
10m[d]	Propyl	$-CH_3$	$CH_2COOCH(CH_3)_2$	*m*-Methoxyphe	26.6 ± 2.1
10n[d]	Propyl	$-CH_3$	$CH_2COOCH(CH_3)_2$	*m*-MethoxyBn	23.2 ± 0.4
13[d]	Methyl	–OBn	$-(CH_2)_3OCOCH_3$	Cyclopropyl	26.9 ± 2.8
16a[d]	Methyl	–OBn		Cyclopropyl	39.1 ± 2.5
16b[d]	Methyl	–OBn		Cyclopropyl	28.4 ± 3.0
16c[d]	Methyl	–OBn		Cyclopropyl	28.2 ± 3.1

[a] Calculated as the average from four experiments.
[b] The known BACE-1 inhibitor **2** was synthesized as described [9] and used as a positive control in the BACE-1 cell-based assay.
[c] Although each single diastereomer of **9a**–**b** was purified and tested, there was no difference of the inhibitory activity between each single diastereomer of **9a** and **9b**.
[d] Compounds **10a**–**n**, **13**, and **16a**–**c** were obtained and tested as diastereomeric mixtures.

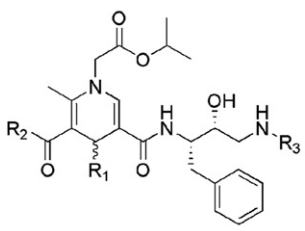
(R_1 = ethyl), which had the same side chains as **2**. We found that these compounds showed a 60-fold decrease in inhibitory activity compared with **2**. There was no difference of the inhibitory activity between each single diastereomer of **9a** and **9b**. In the course of an effort to reduce the peptidic nature, benzyl ester for R_2 group instead of (*R*)-methylbenzylamine of **9a**,**b** was found to be tolerated as shown in the activity of **10a**,**b**. Replacement of side chains at the R_1 position of 2,6-dimethyl-1,4-DHP (**10a**–**e**), such as methyl, ethyl, propyl, isopropyl, and phenyl, showed that longer or bulkier alkyl groups slightly increased BACE-1-inhibitory activity (**10a**–**c**), whereas a phenyl group (**10e**) at the R_1 position diminished activity more than two-fold. Therefore, following the first round of optimization of 1,4-DHP-based BACE-1 inhibitors, we found that **10c**, with a propyl group at the R_1 position and a benzyl ester group at the R_2 position, showed a two-fold enhancement of inhibitory activity compared with the initial compound **9**.

The sulfonamide group in the isophthalate scaffold of **2** has been shown to contribute to important hydrogen bonding interactions with the backbone residues of Asn233 and Arg235 in the active site of BACE-1 [9]. We therefore performed SAR at the R_3 position of 1,4-DHP derivatives by assessing the effects of ester groups (**10f**–**h**) or amide groups (**16a**–**c**) instead of the sulfonamide group of **10a**–**e**. Compound **10f**, with a methylacetate group at the R_3 position, showed an inhibitory activity similar to that of **10a**, suggesting that the oxygen molecules of the ester could provide a degree of hydrogen bonding similar to that of the sulfonamide group of **10a**. Among the ester groups of **10f**–**h**, the isopropyl ester group of **10h** resulted in increased inhibitory activity, with an IC_{50} value of 9 μM. To determine the effect of the carbon chain spacer of the R_3 group, we synthesized **13**, with three carbon chains. However, **13** showed an approximately two-fold loss of BACE-1-inhibitory activity compared with **10f**, suggesting that space in the S2 binding pocket was limited. Amide substitutions (dimethyl, morpholine, and *N*-isopropyl-*N*-methyl amide) at the R_3 position (**16a**–**c**) showed an average three-to-four-fold loss of BACE-1-inhibitory activity compared with compounds containing ester group substitutions (**10f**–**h**), suggesting that two hydrogen bond donors could enhance binding.

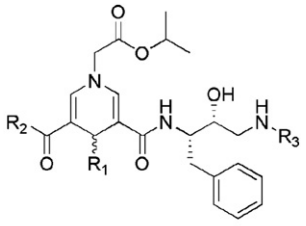
We also attempted to incorporate different hydrophobic interactions at the R_2 and R_4 positions [11,24] by reducing the size of the R_2 group (*e.g.*, to an acetyl group) and by introducing aromatic building blocks at the R_4 position for extensions toward the prime-side of BACE-1 (*i.e.*, an *m*-fluorophenyl, *m*-methoxyphenyl, or *m*-methoxybenzyl group instead of a cyclopropyl group [**10i**–**n**]). However, replacement of the benzyl group at the R_2 position (**10h**) by an acetyl group (**10i**) resulted in a three-fold decrease in BACE-1-inhibitory activity. The remaining compounds designed according to this concept also showed significant decreases in potency.

As 2,6-dimethyl groups may increase steric hindrance in the maintenance of hydrogen bonding interactions between the two oxygens at the R_3 position and the S2 binding site, we tested the effects of 2,6-dimethyl groups in DHP derivatives on BACE-1-inhibitory activity by evaluating 2-monomethyl and 2,6-unsubstituted derivatives (Table 2). However, we found that **21a**, with a monomethyl group, showed a two-fold reduction in inhibitory potency compared with **10h**. In addition, an acetyl group at the R_2 position (*e.g.*, **21b**–**c**) did not enhance inhibitory activity, compared with the corresponding benzyl group of **21a**. 2,6-Unsubstituted 1,4-DHPs (**24a**–**c** and **26a**–**c**), containing combinations of various active groups around the DHP skeleton, also showed decreased BACE-1-inhibitory activities, without a clear trend in SAR. These results indicated that methyl groups at the 2 and 6 positions could contribute to inhibitory activity without interfering with the hydrogen bonding of the ester group at the N-1 position of DHP.

Table 2
BACE inhibitory activities of 2-methyl-1,4-DHP analogs and 2,6-unsubstituted-1,4-DHP analogs.



21a-c



24a-c, 26a-c

Compound[a]	R_1	R_2	R_3	IC_{50} (μM)
21a	Methyl	–OBn	Cyclopropyl	16.1 ± 1.3
21b	Methyl	Methyl	*m*-Fluorophenyl	29.3 ± 2.1
21c	Methyl	Methyl	*m*-MethoxyBn	26.1 ± 0.9
24a	Methyl	–O^{t}Bu	Cyclopropyl	31.9 ± 1.7
24b	Propyl	–O^{t}Bu	Cyclopropyl	24.6 ± 0.9
24c	Propyl	–O^{t}Bu	*m*-Methoxyphe	30.4 ± 2.3
26a	Methyl	–NH-(α)methylBn	Cyclopropyl	32.8 ± 0.8
26b	Propyl	–NH-(α)methylBn	Cyclopropyl	29.6 ± 0.3
26c	Propyl	–NH-(α)methylBn	*m*-Methoxyphe	31.2 ± 2.8
2				0.3 ± 0.11

[a] All compounds were obtained and tested as diastereomeric mixtures.

Whereas molecular modeling predicted that 1,4-DHP derivatives could have similar docking fits, the synthesized compounds were experimentally less potent than **2**.

3. Conclusion

We have described a series of BACE-1 inhibitors, synthesized using a 1,4-dihydropyridine scaffold to replace the isophthalate ring of the known BACE-1 inhibitor **2**. The designed analogs showed BACE-1-inhibitory activities with IC_{50} values of 8–30 μM, suggesting that a chemical probe may block APP processing induced by BACE-1. Further study of chemical delivery systems for BACE-1 inhibitors with the 1,4-DHP scaffold are currently under investigation.

4. Experimental protocols

4.1. Chemistry

Proton nuclear magnetic resonance spectroscopy was performed on a JEOL JNM-LA 300WB spectrometer, and spectra were taken in $CDCl_3$ or DMSO-d_6. Unless otherwise noted, chemical shifts are expressed as ppm downfield from internal tetramethylsilane, or relative ppm from DMSO (2.5 ppm). Data reported include chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; b, broad), coupling constants, and integration. Mass spectroscopy was performed on ESI. Chromatographic purifications were achieved using kieselgel 60 (Merck) 0.040–0.063 mm column chromatography. TLC was performed on fluorescent silica gel plates (60 F_{254} from Merck).

4.1.1. General procedure for synthesis of derivatives of 2,6-substituted 1,4-DHP (*6a–g*)

For compounds **6a**–**e**: 2 equimolar amounts of benzylacetoacetate, R_1-aldehyde, and ammonium acetate (1.5 eq) were dissolved in anhydrous ethanol [16]. The mixture was refluxed for 24 h. After cooling to room temperature, the solvent was evaporated and the residue was purified by silica gel column chromatography.

For compounds **6f**–**g**: Benzylacetoacetate (1 eq), acetylacetone (1 eq), and ammonium acetate (1.5 eq) were dissolved in anhydrous ethanol. The reaction condition and purification was same as **6a**–**e**.

*4.1.1.1. Dibenzyl 2,4,6-trimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**6a**).* Yield: 65%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36 (m, 10H), 5.59 (s, 1H, N–H), 5.19 (s, 1H), 5.17 (s, 1H), 3.97 (q, J = 6.3 Hz, 1H), 2.27 (s, 6H), 1.01 (d, J = 6.6 Hz, 3H)

*4.1.1.2. Benzyl 5-acetyl-2,6-dimethyl-4-propyl-1,4-dihydropyridine-3-carboxylate (**6g**).* Yield: 31%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.35 (m, 5H), 5.6 (s, 1H, N–H), 5.21 (d, J = 2.7 Hz, 2H), 3.94 (t, J = 5.7 Hz, 1H), 2.30 (s, 3H), 2.26 (s, 3H), 2.25 (s, 3H), 1.25 (m, 4H), 0.80 (t, J = 6.9 Hz, 3H)

*4.1.2. General procedure for the Lewis-acid mediated hydrolysis reaction (**7a**–**j**)*

To a solution of 2,6-substituted 1,4-DHP (100 mg, 0.25 mmol) and NaH (15 mg, 0.3 mmol) dissolved in DMF (5 mL) was added methane sulfonylchloride or bromoacetate (0.025 mL, 0.3 mmol) dropwise at 0 °C. The reaction mixture was heated to 60 °C and stirred for 4 h. After cooling, the reaction mixture was partitioned between a saturated ammonium chloride solution and ethylacetate. The organic layer was dried over sodium sulfate, filtered and evaporated under vacuum. The resulting product was purified by silica gel column chromatography. To a solution of 0.13 mmol of each product in DCM was added anisole (0.13 mmol) dropwise. The reaction mixture was stirred at −50 °C for 30 min and $AlCl_3$ (51 mg, 0.39 mmol) was added at the same temperature. The reaction mixture was stirred for 1 h with warming to room temperature, quenched with water, and partitioned between DCM and water. The resulting inorganic precipitates were removed by filtration and the filtrate was dried over sodium sulfate, filtered, and evaporated. The product was purified by silica gel column chromatography ($CHCl_3$: CH_3OH = 20:1).

*4.1.2.1. 5-(benzyloxycarbonyl)-2,4,6-trimethyl-1-(methylsulfonyl)-1,4-dihydropyridine-3-carboxylic acid (**7a**).* Yield: 21%, ^{1}H NMR (300 M Hz, $CDCl_3$) δ (ppm) 7.37 (m, 5H), 5.25 (s, 2H), 3.84 (q, J = 5.1 Hz, 1H), 3.38 (s, 3H), 2.50 (s, 3H), 2.48 (s, 3H), 1.21 (d, J = 6.6 Hz, 3H)

*4.1.2.2. 5-(Benzyloxycarbonyl)-1-(2-methoxy-2-oxoethyl)-2,4,6-trimethyl-1,4-dihydropy ridine-3-carboxylic acid (**7f**).* Yield: 16%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.38 (m, 5H), 5.20 (q, J = 12.6 Hz, 2H), 4.35 (s, 2H), 3.92 (q, J = 6.3 Hz, 1H), 3.78 (s, 3H), 2.37(s, 3H), 2.34 (s, 3H), 1.01 (d, J = 6.6 Hz, 3H)

*4.1.2.3. 5-(Benzyloxycarbonyl)-1-(2-isopropoxy-2-oxoethyl)-2,4,6-trimethyl-1,4-dihydro pyridine-3-carboxylic acid (**7h**).* Yield: 25%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.34 (m, 5H), 5.18 (s, 2H), 5.01 (m, 1H), 4.28 (s, 2H), 3.99 (q, J = 6.3 Hz, 1H), 2.37(s, 3H), 2.34 (s, 3H), 1.25 (d, J = 3.9 Hz, 6H), 1.01 (d, J = 6.6 Hz, 3H)

*4.1.3. (R)-Benzyl 2,4,6-trimethyl-1-(methylsulfonyl)-5-(1-phenylethylcarbamoyl)-1,4-dihydropyridine-3-carboxylate (**8a**)*

A solution containing **7a** (135 mg, 0.36 mmol) in 5 mL DCM, PyBOP (222 mg, 0.42 mmol), (*R*)-methylbenzylamine (0.055 mL, 0.42 mmol), and DIPA (0.151 mL, 1.07 mmol) was stirred at ambient temperature for 1 h. The solvent was evaporated and the product was purified by silica gel column chromatography. Yield: 70%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.35 (m, 10H), 5.87 (s, 1H), 5.20 (m, 3H), 3.58 (q, J = 6.3 Hz, 1H), 3.29(s, 3H), 2.46 (s, 3H), 2.30 (s, 1H), 1.54 (d, J = 6.9 Hz, 3H), 1.67 (d, J = 6.9 Hz, 3H)

*4.1.4. General procedure for the coupling reaction with HEA moieties (**9a**–**b**, **10a**–**n**, **13**, **16a**–**c**, **21a**–**c**, **24a**–**c**, **26a**–**c**)*

A solution containing a monoacid compound such as **7** in DCM was treated sequentially with an HEA moiety (**5a**–**d**) (1.19 eq), DIPEA (2.2 eq), and PyBop (1 eq) or HATU (1 eq). The reaction mixture was stirred at ambient temperature for 15 min. After evaporation, the product was purified by silica gel column chromatography.

In the case of compound **9a**–**b**, two diastereomers could be separated by silica gel column chromatography. However, compound **10a**–**n**, **13**, **16a**–**c**, **21a**–**c** were only obtained as a mixture of diastereomers.

*4.1.4.1. N3-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-yl)-2,4,6-trimethyl-1-(methylsulfonyl)-N5-((R)-1-phenylethyl)-1,4-dihydropyridine-3,5-dicarboxamide (**9a**).* (**9a**–**1**: single diastereomer) Yield: 65%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.4 (m, 4H), 7.25–7.15 (m, 6H), 7.08 (s, 1H, N–H), 6.97 (s, 1H, N–H), 5.17 (q, J = 7 Hz, 1H), 4.28 (m, 1H), 3.69 (m, 1H), 3.18 (s, 3H), 3.10 (m, 1H), 3.08 (q, J = 6.9 Hz, 1H), 2.85 (m, 3H), 2.09 (s, 3H), 1.85 (s, 3H), 1.52 (d, J = 7.2 Hz, 3H), 0.99 (d, J = 6.9 Hz, 3H), 0.49–0.39 (m, 4H). ESI [M + H] = 595.5

(**9a**–**2**: single diastereomer) Yield: 60%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.39–7.30 (m, 5H), 7.26–7.13 (m, 5H), 6.16 (s, 1H, N–H), 6.01 (s, 1H, N–H), 5.18 (q, J = 6.9 Hz, 1H), 4.28 (m, 1H), 4.13 (q, J = 6.9 Hz, 1H), 3.60 (m, 1H), 3.21 (s, 3H), 3.12 (m, 2H), 2.86–2.73 (m, 2H), 2.13 (s, 3H), 2.10 (m, 1H), 1.84 (s, 3H), 1.53 (d, J = 6.9 Hz, 3H), 0.99 (d, J = 6.9 Hz, 3H), 0.47–0.35 (m, 4H).

*4.1.4.2. N3-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-yl)-4-ethyl-2,6-dimethyl-1-(methylsulfonyl)-N5-((R)-1-phenylethyl)-1,4-dihydropyridine-3,5-dicarboxamide (**9b**).* (**9b**–**1**: single diastereomer) Yield: 68%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36–7.29 (m, 5H), 7.22–7.14 (m, 5H), 6.33 (s, 1H, N–H), 6.04 (s, 1H, N–H), 5.18 (q, J = 7.2 Hz, 1H), 4.29 (m, 1H), 3.70 (m, 1H), 3.18 (s, 3H), 3.10 (t, J = 4.8 Hz, 1H), 3.02–2.78 (m, 4H), 2.21 (m, 1H), 2.10 (s, 3H), 1.97 (s, 3H), 1.51 (d, J = 7.2 Hz, 3H), 1.31 (m, 2H), 0.76 (t, J = 7.5 Hz, 3H), 0.55–0.48 (m, 4H). ESI [M + H] = 609.5

(**9b**–**2**: single diastereomer) Yield: 51%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.34–7.28 (m, 5H), 7.26–7.13 (m, 5H), 6.76 (s, 1H, N–H), 6.38 (s, 1H, N–H), 5.18 (q, J = 6.2 Hz, 1H), 4.25 (m, 1H), 3.83 (m, 1H), 3.23 (s, 3H), 3.11 (t, J = 6.5 Hz, 1H), 3.10–2.94 (m, 3H), 2.80 (m, 1H), 2.24 (m, 1H), 2.14 (s, 3H), 1.93 (s, 3H), 1.56 (d, J = 6 Hz, 3H), 1.27 (m, 2H), 0.72–0.64 (m, 7H). ESI [M + H] = 609.5

*4.1.4.3. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,4,6-trimethyl-1-(methylsulfonyl)-1,4-dihydropyridine-3-carboxylate (**10a**).* Yield: 59.1%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.35 (m, 5H), 7.28–7.14 (m, 5H), 6.11 (s, 1H, N–H), 4.34 (m, 1H), 3.71 (m, 1H), 3.45 (q, J = 6.6 Hz, 1H), 3.26 (s, 3H), 3.18–3.12 (m, 1H), 2.99–2.95 (m, 1H), 2.89–2.83 (m, 2H), 2.43 (m, 3H), 2.19 (m, 1H), 1.78 (s, 3H), 1.08 and 0.96 (2d, J = 6.6 Hz, 3H, mixture of two diastereomers), 0.53 (m, 4H). ESI [M + H] = 582.6

*4.1.4.4. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-4-ethyl-2,6-dimethyl-1-(methylsulfonyl)-1,4-dihydropyridine-3-carboxylate (**10b**).* Yield: 51%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.39–7.31 (m, 5H), 7.25–7.13 (m, 5H), 6.2 (s, 1H, N–H), 5.22 (m, 2H), 4.33 (m, 1H), 3.81 (m, 1H), 3.48 (t, J = 7.2 Hz, 1H), 3.26 (s, 3H), 3.17–2.76 (m, 4H), 2.45 (s, 3H), 2.09 (s, 1H), 1.76 (s, 2H), 1.48–1.42 (m, 2H), 0.78 (t, J = 7.5 Hz, 3H), 0.71–0.64 (m, 4H). ESI [M + H] = 596.5

*4.1.4.5. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,6-dimethyl-1-(methylsulfonyl)-4-propyl-1,4-dihydropyridine-3-carboxylate (**10c**).* Yield: 65%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36–7.30 (m, 5H), 7.21–7.16 (m, 5H), 6.11 (s, 1H,

N–H), 5.27–5.10 (m, 2H), 4.41–4.23 (m, 1H), 3.68 (m, 1H), 3.56 (t, J = 7.2 Hz, 1H), 3.28 (s, 3H), 3.11–2.81 (m, 4H), 2.42 (s, 3H), 2.16 (s, 1H), 2.08 (s, 1H), 2.04 (m, 1H), 1.77 (s, 2H), 1.39–1.05 (m, 4H), 0.80–0.69 (m, 3H), 0.48–0.40 (m, 4H). ESI [M + H] = 610.9

*4.1.4.6. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenyl butan-2-ylcarbamoyl)-4-isopropyl-2,6-dimethyl-1-(methylsulfonyl)-1,4-dihydropyridine-3-carboxylate (**10d**).* Yield: 67%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.39–7.35 (m, 5H), 7.26–7.16 (m, 5H), 6.25 (s, 1H, N–H), 5.18 (s, 2H), 3.68 (m, 1H), 3.41 (d, J = 3 Hz, 1H), 3.39 (m, 1H), 3.27 (s, 3H), 2.96–2.7 (m, 4H), 2.41 (s, 3H), 2.17 (m, 1H), 1.95 (m, 1H), 1.82 (s, 3H), 1.58 (m, 1H), 0.75 (d, J = 6.6 Hz, 3H), 0.66 (d, J = 7.2 Hz, 3H), 0.51–0.39 (m, 4H). ESI [M + H] = 610.8

*4.1.4.7. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenyl butan-2-ylcarbamoyl)-2,6-dimethyl-1-(methylsulfonyl)-4-phenyl-1,4-dihydropyridine-3-carboxylate (**10e**).* Yield: 62.6%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.30–7.28 (m, 5H), 7.24–7.10 (m, 10H), 5.84 (s, 1H, N–H), 5.11 (s, 2H), 4.80 (s, 1H), 4.39–4.21 (m.1H), 3.42 (m, 1H), 3.19 (s, 3H), 3.0 (m, 1H), 2.79 (m, 1H), 2.73–2.62 (m, 2H), 2.51 (s, 3H), 2.04 (m, 1H), 1.79 (s, 3H), 0.44–0.26 (m, 4H). ESI [M + H] = 644.8

*4.1.4.8. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenyl butan-2-ylcarbamoyl)-1-(2-methoxy-2-oxoethyl)-2,4,6-trimethyl-1,4-dihydropyridine-3-carboxylate (**10f**).* Yield: 21%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36 (m, 5H), 7.26–7.15 (m, 5H), 7.35 (s, 1H, N–H), 5.25–5.15 (m, 2H), 4.24 (s, 2H), 3.76 (s, 3H), 3.58 (m, 1H), 3.38 (q, J = 4.2 Hz, 1H), 3.17 (m, 1H), 3.16–3.08 (m, 2H), 2.90–2.78 (m, 2H), 2.35 (s, 3H), 1.98 (s, 2H), 1.80 (s, 1H), 0.94 and 0.78 (2d, J = 6.5 Hz, 3H, mixture of two diastereomers), 0.48–0.41 (m, 4H). ESI [M + H] = 576.4

*4.1.4.9. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenyl-butan-2-ylcarbamoyl)-1-(2-ethoxy-2-oxoethyl)-2,4,6-trimethyl-1,4-dihydropyridine-3-carboxylate (**10g**).* Yield: 20%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36–7.27 (m, 5H), 7.22–7.16 (m, 5H), 5.81 (s, 1H, N–H), 5.24–5.15 (m, 2H), 4.25–4.18 (m, 4H), 3.79 (m, 1H), 3.63 (t, J = 8.1 Hz, 1H), 3.35–2.89 (m, 5H), 2.32 (s, 3H), 2.27 (s, 3H), 1.65 (m, 1H), 1.24 (t, J = 7.8 Hz, 3H), 0.87 and 0.79 (2d, J = 8.1 Hz, 3H, mixture of two diastereomers), 0.59–0.41 (m, 4H). ESI [M + H] = 590.9

*4.1.4.10. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenyl-butan-2-ylcarbamoyl)-1-(2-isopropoxy-2-oxoethyl)-2,4,6-trimethyl-1,4-dihydropyridine-3-carboxylate (**10h**).* Yield: 37%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.37–7.34 (m, 5H), 7.21–7.15 (m, 5H), 5.77 (s, 1H, N–H), 5.19 (m, 2H), 5.09 (m, 1H), 4.23 (m, 1H), 4.18 (s, 2H), 3.56 (m, 1H), 3.37 (q, J = 6.6 Hz, 1H), 3.07 (m, 1H), 2.87–2.78 (m, 3H), 2.31 (s, 3H), 2.16 (m, 1H), 1.94 (s, 2H), 1.80 (s, 1H), 1.25 (d, J = 15.9 Hz, 3H), 0.94 and 0.78 (2d, J = 6.6 Hz, 3H, mixture of two diastereomers), 0.46 (m, 4H). ESI [M + H] = 604.9

*4.1.4.11. Isopropyl 2-(3-acetyl-5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,4,6-trimethylpyridin-1(4H)-yl)acetate (**10i**).* Yield: 10%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.41–7.19 (m, 5H), 5.75 (s, 1H, N–H), 5.07 (q, J = 6.6 Hz, 1H), 4.32 (m, 1H), 4.17 (s, 2H), 3.63 (m, 1H), 3.2–3.0 (m, 2H), 2.83 (m, 3H), 2.23 (s, 3H), 2.19 (s, 3H), 2.14 (m, 1H), 1.83 (s, 3H), 1.26 (d, J = 6 Hz, 6H), 0.91 and 0.85 (2d, J = 6.6 Hz, 3H, mixture of two diastereomers), 0.47 (m, 4H). ESI [M + H] = 512.2

*4.1.4.12. Isopropyl 2-(3-acetyl-5-((2S,3R)-4-(3-fluorophenylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,4,6-trimethylpyridin-1(4H)-yl)acetate (**10j**).* Yield: 11%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.39–7.21 (m, 5H), 7.12 (q, J = 6.6 Hz, 1H), 6.42 (m, 3H), 5.64 (m, 1H), 4.47 (m, 1H), 4.36 (m, 1H), 4.19 (s, 2H), 3.94 (q, J = 6.6 Hz, 1H), 3.34 (m, 1H), 3.22–3.02 (m, 3H), 2.29 (s, 3H), 2.12 (s, 3H), 1.93 (s, 2H), 1.87 (s, 1H), 1.25 (dd, J = 4.5, 1.5 Hz, 6H), 0.92 and 0.86 (2d, J = 6.6 Hz, 3H, mixture of two diastereomers). ESI [M + H] = 566.7

*4.1.4.13. Isopropyl 2-(3-acetyl-5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,6-dimethyl-4-propylpyridin-1(4H)-yl)acetate (**10k**).* Yield: 35%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.33–7.19 (m, 5H), 5.73 (s, 1H, N–H), 5.09 (m, 1H), 4.29 (m, 1H), 4.17 (s, 2H), 3.62 (m, 1H), 3.26–2.79 (m, 5H), 2.22 (s, 3H), 2.19 (s, 3H), 2.12 (m, 1H), 1.86 (s, 3H), 1.26 (dd, J = 6.4, 1.5 Hz, 6H), 1.19–1.06 (m, 4H), 0.76 (t, J = 6.3 Hz, 3H), 0.49–0.35 (m, 4H). ESI [M − H] = 540.2

*4.1.4.14. Isopropyl 2-(3-acetyl-5-((2S,3R)-4-(3-fluorophenylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,6-dimethyl-4-propyl-pyridin-1(4H)-yl)acetate (**10l**).* Yield: 28.1%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.40–7.28 (m, 5H), 7.12 (m, 1H), 6.47 (m, 1H), 6.41 (m, 1H), 6.36 (m, 1H), 5.65 (s, 1H, N–H), 5.07 (m, 1H), 4.38 (m, 1H), 4.17 (s, 2H), 3.85 (m, 1H), 3.32 (dd, J = 12.3, 3.6 Hz, 1H), 3.17–3.02 (m, 3H), 2.80 (m, 1H), 2.19 (d, J = 6.3 Hz, 6H), 1.89 (s, 3H), 1.25 (d, J = 6.3 Hz, 6H), 1.05 (m, 2H), 0.81 (m, 2H), 0.70 (t, J = 6.6 Hz, 3H). ESI [M − H] = 592.2

*4.1.4.15. Isopropyl 2-(3-acetyl-5-((2S,3R)-3-hydroxy-4-(3-methoxy phenylamino)-1-phenylbutan-2-ylcarbamoyl)-2,6-dimethyl-4-propylpyridin-1(4H)-yl)acetate (**10m**).* Yield: 21%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.32–7.28 (m, 3H), 7.22 (m, 2H), 7.09 (t, J = 8.4 Hz, 1H), 6.33–6.24 (m, 3H), 5.71 (m, 1H), 4.39 (m, 1H), 4.16 (s, 2H), 3.9 (m, 1H), 3.77 (s, 3H), 3.33 (m, 1H), 3.18 (m, 1H), 3.14–2.85 (m, 3H), 2.20 (s, 3H), 2.19 (s, 1H), 2.13 (s, 2H), 1.89 (s, 3H), 1.25 (dd, J = 4.5, 2.7 Hz, 6H), 1.19–1.11 (m, 4H), 0.72 (t, J = 6.9 Hz, 3H). ESI [M − H] = 604.2

*4.1.4.16. Isopropyl 2-(3-acetyl-5-((2S,3R)-3-hydroxy-4-(3-methoxy benzylamino)-1-phenyl butan-2-ylcarbamoyl)-2,6-dimethyl-4-propyl pyridin-1(4H)-yl)acetate (**10n**).* Yield: 12%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.29–7.23 (m, 5H), 7.16 (dd, J = 6.9, 1.5 Hz, 1H), 6.92 (m, 1H), 6.85(m, 2H), 5.09 (m, 1H), 4.25 (m, 1H), 4.16 (s, 2H), 3.82 (s, 3H), 3.74 (s, 2H), 3.65 (t, J = 3.9 Hz, 1H), 3.16 (m, 1H), 2.97–2.73 (m, 4H), 2.19 (s, 6H), 1.86 (s, 3H), 1.27 (d, J = 6.3 Hz, 6H), 1.18–0.85 (m, 4H), 0.76 (t, J = 6.6 Hz, 3H). ESI [M + H] = 620.2

*4.1.5. Dibenzyl 1-allyl-2,4,6-trimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**11**)*

To a solution of compound **6a** (100 mg, 0.25 mmol) in DMF (5 mL) and NaH (15 mg, 0.3 mmol) was added dropwise allyl bromide (0.05 mL, 0.4 mmol) at 0 °C. The solution was stirred at ambient temperature for 1 h and partitioned between a saturated ammonium chloride solution and ethylacetate. The organic layer was dried over sodium sulfate, filtered and evaporated under vacuum. The product was purified by silica gel column chromatography, using hexane:ethylacetate 7:1 (v/v).

Yield: 45%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36–7.28 (m, 10H), 5.82–5.78 (m, 1H), 5.26–5.21 (m, 2H), 5.19 (s, 2H), 4.22 (m, 2H), 3.97 (q, J = 6.6 Hz, 1H), 2.40 (s, 6H), 0.95 (d, J = 6.6 Hz, 3H).

*4.1.6. Dibenzyl 1-(3-acetoxypropyl)-2,4,6-trimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**12**)*

0.12 mL of 2 M borane-methylsulfide complex solution was slowly added dropwise to compound **11** (100 mg, 0.232 mmol) in THF and under nitrogen gas. The reaction mixture was stirred for 2 h, cooled in an ice-bath, and quenched with one drop of NaOH solution. The flow of nitrogen was stopped when effervescence

ceased. After the addition of 3 M aqueous NaOH (1.5 mL) and 30% aqueous H_2O_2 (0.69 mL), the reaction mixture was stirred at room temperature for 2–3 h. The solution was saturated with solid NaCl and products were extracted with diethyl ether. The ether extract was washed with brine, dried over sodium sulfate, filtered and evaporated under vacuum. The residue was purified by silica gel column chromatography using chloroform: methanol 20:1. To a solution of the purified product in 6 mL of DCM was added dropwise acetic anhydride (0.02 mL) and TEA (0.019 mL). The reaction mixture was stirred for 2 h and partitioned between saturated sodium bicarbonate solution and DCM. The organic layer was dried over sodium sulfate, filtered and evaporated under vacuum. The product was purified by silica gel column chromatography using hexane:ethylacetate 4.5:1.

Yield: 50%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.31 (m, 10H), 5.19 (s, 4H), 4.09 (t, J = 6.9 Hz, 2H), 3.97 (q, J = 6.9 Hz, 1H), 3.75 (t, J = 7.8 Hz, 2H), 2.41 (s, 6H), 2.06 (s, 3H), 1.83 (m, 2H), 0.93 (d, J = 6.9 Hz, 3H).

4.1.7. Benzyl 1-(3-acetoxypropyl)-5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,4,6-trimethyl-1,4-dihydropyridine-3-carboxylate (**13**)

The methods for Lewis-acid mediated hydrolysis and the coupling reaction with the HEA moiety are described above.

Yield: 45%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36–7.29 (m, 5H), 7.22–7.15 (m, 5H), 5.16 (m, 2H), 4.24 (m, 1H), 4.07 (m, 2H), 3.67 (m, 1H), 3.57 (m, 2H), 3.34 (q, J = 6.6 Hz, 3H), 3.11 (m, 1H), 2.88–2.82 (m, 3H), 2.36 (s, 3H), 2.17 (m, 1H), 2.07 (s, 3H), 1.94 (s, 2H), 1.89 (s, 1H), 1.81 (m, 2H), 0.85 and 0.72 (2d, J = 6.6 Hz, 3H, mixture of two diastereomers). ESI [M + H] = 604.8

4.1.8. Dibenzyl 1-(2-(dimethylamino)-2-oxoethyl)-2,4,6-trimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**15a**)

Synthesis of **15a** was identical to that of **11** except for using t-butyl bromoacetate. Briefly, *N*-alkylated **14a** (50 mg, 0.099 mmol) was hydrolyzed with TFA (50%, v/v) in DCM for 2 h at room temperature. After removing the solvent by evaporation, the acid product was extracted with DCM and washed with sodium bicarbonate. The organic layer was dried over sodium sulfate, filtered and evaporated under vacuum. After purification by silica gel column chromatography using chloroform: methanol 10:1 (v/v), the acid compound was reacted PyBop (60 mg) and R_3 amine building blocks in DCM (6 mL). The reaction mixture was stirred for 2 h and washed with saturated ammonium chloride solution. The organic layer was extracted and dried over sodium sulfate. The product was purified by silica gel column chromatography using hexane:ethylacetate 1: (v/v).

Yield 49%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.35–7.29 (m, 10H), 5.18 (s, 4H), 4.36 (s, 2H), 4.02 (q, J = 6.6 Hz, 1H), 3.06 (s, 3H), 3.02 (s, 3H), 2.33 (s, 6H), 1.04 (d, J = 6.6 Hz, 3H). ESI [M + H] = 477.9

4.1.9. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-1-(2-(dimethylamino)-2-oxoethyl)-2,4,6-trimethyl-1,4-dihydropyridine-3-carboxylate (**16a**)

The methods of Lewis-acid mediated hydrolysis and the coupling reaction with the HEA moiety have been described above.

Yield: 40%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36–7.30 (m, 5H), 7.21–7.14 (m, 5H), 5.81 (s, 1H, N–H), 5.24–5.09 (m, 2H), 4.23 (s, 2H), 4.20 (m, 1H), 3.55 (m, 1H), 3.40 (q, J = 6.3 Hz, 1H), 3.07 (m, 1H), 3.03 (s, 3H), 3.00 (s, 3H), 2.81–2.70 (m, 3H), 2.30 (s, 3H), 2.09–2.08 (m, 1H), 1.91 (s, 2H), 1.78 (s, 1H), 1.0 and 0.81 (2d, J = 6.3 Hz, 3H, mixture of two diastereomers), 0.41–0.30 (m, 4H). ESI [M + H] = 589.7

4.1.10. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,4,6-trimethyl-1-(2-morpholino-2-oxoethyl)-1,4-dihydropyridine-3-carboxylate (**16b**)

Yield: 61%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.37–7.29 (m, 5H), 7.21–7.13 (m, 5H), 5.86 (s, 1H, N–H), 5.14 (m, 2H), 4.27 (s, 2H), 4.20 (m, 1H), 3.71–3.68 (m, 4H), 3.62–3.54 (m, 4H), 3.45 (m, 1H), 3.38 (q, J = 6.6 Hz, 1H), 3.06 (m, 1H), 2.83–2.75 (m, 3H), 2.29 (s, 3H), 1.89 (s, 2H), 1.79 (s, 1H), 0.97 and 0.81 (2d, J = 6.6 Hz, 3H, mixture of two diastereomers), 0.46–0.35 (m, 4H). ESI [M + H] = 631.9

4.1.11. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-1-(2-(isopropyl(methyl)amino)-2-oxoethyl)-2,4,6-trimethyl-1,4-dihydropyridine-3-carboxylate (**16c**)

Yield: 54%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.39–7.28 (m, 5H), 7.21–7.13 (m, 5H), 5.79 (s, 1H, N–H), 5.24–5.10 (m, 2H), 4.83 (q, J = 6.6 Hz, 1H), 4.30 (s, 2H), 3.53 (m, 1H), 3.37 (q, J = 6 Hz, 1H), 3.19–3.05 (m, 2H), 2.85 (s, 3H), 2.84–2.75 (m, 2H), 2.29 (s, 3H), 2.09 (s, 1H), 1.83 (s, 3H), 1.25 and 0.82 (2d, J = 6 Hz, 3H, mixture of two diastereomers), 1.11 (dd, J = 6.6, 2.4 Hz, 6H), 0.45–0.41 (m, 4H). ESI [M + H] = 617.9

4.1.12. General procedure for synthesis of derivatives of 2,6-monosubstituted 1,4-DHP (**19a**–**b**)

4-DMAP (348 mg, 0.2 mmol) was added to a stirred suspension of coumalic acid (2 g, 14.27 mmol) in DCM/THF solution maintained at room temperature. After stirring for 15 min, benzyl alcohol (1.5 mL) and EDC (2.73 g, 14.27 mmol) were added. The reaction mixture was stirred for 5 h and partitioned between water and DCM. The organic layer was dried over sodium sulfate, filtered and evaporated under vacuum. The product (**18**) was purified by silica gel column chromatography using hexane:ethylacetate 4.5:1 (yield: 70%)

To a solution of compound **18** (100 mg, 0.434 mmol), and acetylacetone (0.045 mL, 0.434 mmol) or benzylacetoacetate (0.075 mL, 0.434 mmol) in ethanol (1 mL)/acetic acid (0.1 mL) [20], was added ammonium acetate (50.2 mg, 0.651 mmol) and the reaction mixture was stirred for 24 h. Ethanol was removed by evaporation and the products (**19a**–**b**) were extracted with DCM, washed with saturated sodium bicarbonate solution, and purified by silica gel column chromatography using hexane:ethylacetate 3:1 (v/v) (yield: 33%)

4.1.12.1. Benzyl 5-acetyl-4,6-dimethyl-1,4-dihydropyridine-3-carboxylate (**19b**).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.38–7.32 (m, 5H), 7.19 (s, 1H), 6.07 (s, 1H, N–H), 5.21 (s, 2H), 3.83 (q, J = 6.6 Hz, 1H), 2.28 (s, 3H), 2.21 (s, 3H), 1.03 (d, J = 6.6 Hz, 3H). ESI [M − H] = 284.0

4.1.13. Benzyl 5-acetyl-1-(2-isopropoxy-2-oxoethyl)-4,6-dimethyl-1,4-dihydropyridine-3-carboxylate (**20b**)

The synthetic method was the same as that for compound **11**, except for using isopropylbromoacetate.

Yield: 69%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.37–7.31 (m, 5H), 7.04 (s, 1H), 5.22 (s, 2H), 5.08 (m, 1H), 4.14 (s, 2H), 3.80 (q, J = 6.6 Hz, 1H), 2.31 (s, 3H), 2.17 (s, 3H), 1.26 (d, J = 5.7 Hz, 6H), 1.06 (d, J = 6.6 Hz, 3H).). ESI [M − H] = 384.2

4.1.14. Benzyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-1-(2-isopropoxy-2-oxoethyl)-2,4-dimethyl-1,4-dihydropyridine-3-carboxylate (**21a**)

The methods of Lewis-acid mediated hydrolysis and the coupling reaction with the HEA moiety have been described above.

Yield: 37%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.39–7.27 (m, 5H), 7.20–7.15 (m, 5H), 6.98 (s, 1H), 5.81 (s, 1H, N–H), 5.19 (m, 2H), 5.03 (m, 1H), 4.28 (m, 1H), 4.03 (s, 2H), 3.58 (q, J = 6 Hz, 1H), 3.38 (m, 1H), 3.17–2.79 (m, 4H), 2.18 (m, 1H), 1.81 (s, 3H), 1.25 (d, J = 6.3 Hz, 6H),

1.12 and 0.82 (2d, J = 6 Hz, 3H, mixture of two diastereomers), 0.52–0.35 (m, 4H). ESI [M + H] = 590.2

*4.1.15. Isopropyl 2-(3-acetyl-5-((2S,3R)-4-(3-fluorophenylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-2,4-dimethylpyridin-1(4H)-yl)acetate (**21b**)*

Yield: 45%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36–7.34 (m, 2H), 7.24–7.20 (m, 3H), 7.11 (q, J = 7.8 Hz, 1H), 6.78 (s, 1H), 6.46–6.33 (m, 3H), 5.07 (m, 1H), 4.27 (m, 1H), 4.27–4.0 (m, 2H), 3.64 (q, J = 6.6 Hz, 1H), 3.29 (m, 1H), 3.17–2.99 (m, 3H), 2.31 (s, 3H), 2.28 (s, 3H), 1.26 (d, J = 5.7 Hz, 6H), 1.06 and 0.71 (2d, J = 6.6 Hz, 3H, mixture of two diastereomers). ESI [M + H] = 552.2

*4.1.16. Isopropyl 2-(3-acetyl-5-((2S,3R)-3-hydroxy-4-(3-methoxybenzylamino)-1-phenylbutan-2-ylcarbamoyl)-2,4-dimethylpyridin-1(4H)-yl)acetate (**21c**)*

Yield: 59%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.30–7.24 (m, 5H), 7.21 (m, 1H), 6.99–6.82 (m, 2H), 6.73 (s, 1H), 6.20 (s, 1H, N–H), 6.14 (s, 1H, N–H), 5.05 (m, 1H), 4.24 (m, 1H), 4.1 (s, 2H), 3.96 (m, 1H), 3.82 (s, 3H), 3.80 (s, 2H), 3.39 (q, J = 6 Hz, 1H), 3.24–3.09 (m, 2H), 2.96–2.79 (m, 2H), 2.28 (s, 3H), 2.13 (s, 3H), 1.26 (m, 6H), 0.81 and 0.78 (2d, J = 6 Hz, 3H, mixture of two diastereomers). ESI [M + H] = 578.2

*4.1.17. General procedure for synthesis of derivatives of 2,6-unsubstituted 1,4-DHP (**22a**,**b**)*

A solution of ammonium acetate (1.5 eq), tert-butylpropiolate (2 eq) and butylaldehyde (1 eq) (acetaldehyde or butylaldehyde) was refluxed at 90 °C, and stirred for 24 h [21,22]. After cooling to room temperature, the solvent was removed by evaporation and the residue purified by silica gel column chromatography.

*4.1.17.1. Di-tert-butyl 4-methyl-1,4-dihydropyridine-3,5-dicarboxylate (**22a**).* Yield: 12.8%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.10 (s, 1H), 7.08 (s, 1H), 5.82 (s, 1H, N–H), 3.75 (q, J = 6.6 Hz, 1H), 1.46 (s, 18H), 1.08 (d, J = 6.6 Hz, 3H)

*4.1.17.2. Di-tert-butyl 4-propyl-1,4-dihydropyridine-3,5-dicarboxylate (**22b**).* Yield: 17%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.17 (s, 1H), 7.16 (s, 1H), 5.92 (s, 1H, N–H), 3.79 (t, J = 5.1 Hz, 1H), 1.43 (s, 18H), 1.37–1.32 (m, 4H), 0.82(t, J = 7.2 Hz, 3H)

*4.1.18. Di-tert-butyl 1-(2-isopropoxy-2-oxoethyl)-4-methyl-1,4-dihydropyridine-3,5-dicarboxylate (**23a**)*

Yield: 37.8%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.1 (s, 1H), 6.87 (s, 1H), 5.13 (m, 1H), 4.62 (m, 1H), 4.19 (s, 2H), 1.43 (s, 18H), 1.28 (d, J = 2.1 Hz, 9H)

*4.1.19. General procedure for the hydrolysis reaction and the coupling reaction with the HEA moiety (**24a**–**c**)*

N-alkylated compounds (**23a**,**b**) were hydrolyzed with 96% formic acid for 20 min at 0 °C to room temperature. The solvent was removed by evaporation and the residue was purified by silica gel column chromatography using hexane:ethylacetate 3:1. The coupling reaction with the HEA moiety was as described above.

*4.1.19.1. Tert-butyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-1-(2-isopropoxy-2-oxoethyl)-4-methyl-1,4-dihydropyridine-3-carboxylate(**24a**).* Yield = 55%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.31–7.24 (m, 5H), 6.84 (s, 1H), 6.81 (s, 1H), 6.13 (s, 1H, N–H), 5.06 (m, 1H), 4.21 (m, 1H), 3.98 (d, J = 3.9 Hz, 2H), 3.65 (m, 1H), 3.42 (q, J = 1.2 Hz, 1H), 2.98–2.82 (m, 4H), 2.03 (m, 1H), 1.47 (s, 9H), 1.26 (dd, J = 5.4 Hz, 1.8 Hz, 6H), 0.95 and 0.92 (2d, J = 6.3 Hz, 3H, mixture of two diastereomers), 0.61 (m, 4H). ESI [M + H] = 542.2

*4.1.19.2. Tert-butyl 5-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-1-(2-isopropoxy-2-oxoethyl)-4-propyl-1,4-dihydropyridine-3-carboxylate (**24b**).* Yield: 34.2%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.35–7.22 (m, 5H), 6.94 (s, 1H), 6.91 (s, 1H), 5.97 (s, 1H, N–H), 5.08 (m, 1H), 4.25 (m, 1H), 3.91 (d, J = 11.4 Hz, 2H), 3.78 (q, J = 6.6 Hz, 1H), 3.65 (m, 1H), 3.17 (m, 2H), 2.86 (m, 2H), 2.2 (m, 1H), 1.49 (s, 9H), 1.25 (d, J = 2.1 Hz, 6H), 1.15 (m, 4H), 0.80 (t, J = 6.6 Hz, 3H), 0.49 (m, 4H). ESI [M − H] = 568.3

*4.1.19.3. Tert-butyl 5-((2S,3R)-3-hydroxy-4-(3-methoxyphenylamino)-1-phenylbutan-2-ylcarbamoyl)-1-(2-isopropoxy-2-oxoethyl)-4-propyl-1,4-dihydropyridine-3-carboxylate (**24c**).* Yield: 42.1%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.38–7.24 (m, 5H), 7.05 (t, J = 7.5 Hz, 1H), 6.94 (s, 1H), 6.93 (s, 1H), 6.31–6.25 (m, 3H), 5.65 (s, 1H, N–H), 5.06 (m, 1H), 3.93 (d, J = 6.9 Hz, 2H), 3.86 (m, 1H), 3.81 (d, J = 2.4 Hz, 3H), 3.48 (m, 1H), 3.29 (m, 2H), 3.15–3.08 (m, 2H), 1.44 (s, 9H), 1.27 (d, J = 4.8 Hz, 6H), 1.25–1.01 (m, 2H), 0.68 (t, J = 2.4 Hz, 3H). ESI [M − H] = 634.2

*4.1.20. Tert-butyl 1-(2-isopropoxy-2-oxoethyl)-4-methyl-5-((R)-1-phenylethylcarbamoyl)-1,4-dihydropyridine-3-carboxylate (**25a**)*

Yield = 45.9%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.35 (s, 1H), 7.21 (m, 5H), 7.04 (s, 1H), 6.00 (s, 1H, N–H), 5.22 (m, 1H), 5.13 (m, 1H), 4.49 (d, J = 6.3 Hz, 2H), 3.63 (t, J = 3.9 Hz, 1H), 1.50 (s, 3H), 1.47 (s, 9H), 1.25 (d, J = 3.6 Hz, 6H), 1.08 (d, J = 6.3 Hz, 3H).

*4.1.21. Isopropyl 2-(3-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-4-methyl-5-((R)-1-phenylethylcarbamoyl)pyridin-1(4H)-yl) acetate(**26a**)*

Yield = 28.4%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.34–7.22 (m, 10H), 6.79 (s, 1H), 6.72 (s, 1H), 5.23 (m, 1H), 5.06 (m, 1H), 4.23 (m, 1H), 3.94 (d, J = 6.0 Hz, 2H), 3.79–3.68 (m, 2H), 2.98–2.86 (m, 4H), 2.08 (m, 1H), 1.56 (t, J = 4.2 Hz, 3H), 1.24 (d, J = 2.1 Hz, 6H), 0.98 and 0.91 (2d, J = 6.3 Hz, 3H, mixture of two diastereomers), 0.55–0.53 (m, 4H). ESI [M + H] = 589.3

*4.1.22. Isopropyl 2-(3-((2S,3R)-4-(cyclopropylamino)-3-hydroxy-1-phenylbutan-2-ylcarbamoyl)-5-((R)-1-phenylethylcarbamoyl)-4-propylpyridin-1(4H)-yl)acetate (**26b**)*

Yield: 48.2%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.32 (m, 5H), 7.21 (m, 5H), 6.94 (s, 1H), 6.85 (s, 1H), 5.97 (s, 1H, N–H), 5.32 (m, 1H), 5.04 (m, 1H), 4.25 (m, 1H), 3.79 (d, J = 7.8 Hz, 2H), 3.68 (m, 2H), 2.93–2.82 (m, 4H), 2.14 (m, 1H), 1.51 (m, 3H), 1.24 (q, J = 3.9 Hz, 9H), 1.18–1.05 (m, 4H), 0.73 (t, J = 3.2 Hz, 3H), 0.48 (m, 4H). ESI [M + H] = 617.2

*4.1.23. Isopropyl 2-(3-((2S,3R)-3-hydroxy-4-(3-methoxyphenylamino)-1-phenylbutan-2-ylcarbamoyl)-5-((R)-1-phenylethylcarbamoyl)-4-propylpyridin-1(4H)-yl)acetate (**26c**)*

Yield: 12.7%, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.31–7.19 (m, 10H), 7.08 (t, J = 7.8 Hz, 1H), 6.89 (s, 1H), 6.79 (s, 1H), 6.28 (m, 1H), 5.23 (m, 1H), 5.05 (m, 1H), 4.19 (m, 1H), 3.89 (d, J = 5.4 Hz, 2H), 3.76 (s, 3H), 3.42 (t, J = 3.9 Hz, 1H), 3.28 (m, 1H), 3.05 (m, 4H), 1.52 (s, 3H), 1.21 (q, J = 6.6 Hz, 6H), 1.12 (m, 4H), 0.74 (t, J = 6.9 Hz, 3H). ESI [M + H] = 683.2

4.2. BACE-1-inhibitory activities

4.2.1. Cell-based assay

To evaluate the BACE-1-inhibitory activity, an alkaline phosphatase (AP)-APP-cell-based assay was carried out in HEK293 cells with pCDNA-neo-SEAP-APP plasmid. The plasmid encoding a fusion protein of APP and AP containing the BACE-1 cleavage site, was stably transfected into HEK293 cells. BACE-1-inhibitory activity of test compounds was measured indirectly by quantification of secreted AP after cleavage of the β-site of APP by BACE-1. Cells in

DMEM containing 10% FBS and antibiotics were plated at a density of 2×10^4 cells per well of a 96-well plate and allowed to adhere for 24 h. The culture medium was aspirated and DMEM containing 1 mg/mL BSA was added to each well. A BACE-1 inhibitor, diluted in DMEM containing 1 mg/mL BSA and 5 μM forskolin (Sigma; catalog no F6886), was added, and the cells were incubated for 6 h. A 50 μL aliquot of conditioned medium was transferred to each well of a 96-well fluorescence assay plate and 50 μL of a yellow liquid alkaline phosphatate substrate was added. Absorbance was measured at 405 nm with a microplate reader.

4.3. Molecular docking studies

Molecular modeling was performed using CDOCKER, a CHARMm-based molecular dynamics docking algorithm on Discovery Studio 2.0 (Accelrys). The BACE-1 structure co-crystallized with **2** was obtained from the PDB data bank (PDB code: 2B8L) [25]. A protein clean process and a CHARMm-force field were sequentially applied. The area around **2** was chosen as the active site, with the radius set as at 8 Å. After removing **2** from the structure of the complex, a binding sphere in the three axis directions was constructed around the active site. All default parameters were used in the docking process. CHARMm-based molecular dynamics (1000 steps) were used to generate random ligand (**9a**) conformations and the position of any ligand (**9a**) was optimized in the binding site using rigid body rotation followed by simulated annealing at 700 K. Final energy minimization was set as the full potential mode. The final binding conformation of **9a** was determined on the basis of energy.

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