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Cyanobacterial Peptides as a Prototype for the Design of Potent β-Secretase Inhibitors and the Development of Selective Chemical Probes for Other Aspartic Proteases

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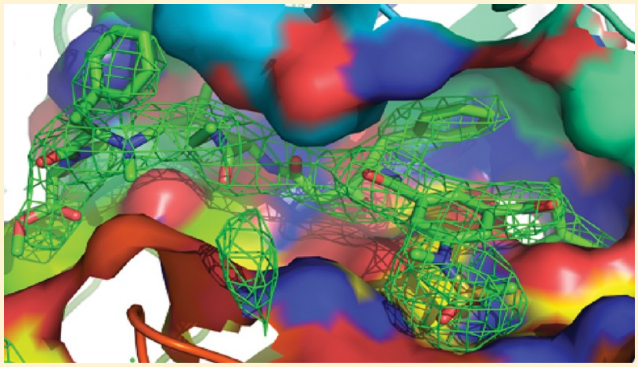
Supporting Information

ABSTRACT: Inspired by marine cyanobacterial natural products, we synthesized modified peptides with a central statine-core unit, characteristic for aspartic protease inhibition. A series of tasiamide B analogues inhibited BACE1, a therapeutic target in Alzheimer's disease. We probed the stereospecificity of target engagement and determined additional structure−activity relationships with respect to BACE1 and related aspartic proteases, cathepsins D and E. We cocrystallized selected inhibitors with BACE1 to reveal the structural basis for the activity. Hybrid molecules that combine features of tasiamide B and an isophthalic acid moiety-containing sulfonamide showed nanomolar cellular activity. Compounds were screened in a series of rigorous complementary cell-based assays. We measured secreted APP ectodomain (sAPPβ), membrane bound carboxyl terminal fragment (CTF), levels of β-amyloid (Aβ) peptides and selectivity for β-secretase (BACE1) over γ-secretase. Prioritized compounds showed reasonable stability in vitro and in vivo, and our most potent inhibitor showed efficacy in reducing Aβ levels in the rodent brain.



INTRODUCTION

Cyanobacteria are prolific producers of diverse secondary metabolites, many of which are linear or cyclic modified peptides and depsipeptides.[1] These compounds appear to have a propensity to inhibit various proteases with differential selectivity and may provide guidance for the development of therapeutic protease inhibitors or selective chemical probes. For example, cyclodepsipeptide-based serine protease inhibitors with specificity for elastase, chymotrypsin or trypsin are commonly produced by many terrestrial and marine cyanobacteria.[2] We also recently reported the discovery and characterization of grassystatins A and B, marine-derived linear depsipeptides that potently inhibit the aspartic proteases cathepsins D and E, with selectivity for cathepsin E.[3] Grassystatin C is a truncated, linear peptide of lower potency but similar protease selectivity.[3] While grassystatins were unable to inhibit the aspartic protease β-site APP Cleaving Enzyme type 1 (BACE1), which is the β-secretase implicated in the generation of the amyloid β-peptide (Aβ) of Alzheimer's disease (AD), we found here that the related cyanobacterial compound tasiamide B (**2**)[4,5] possesses broader specificity and also inhibits BACE1. Grassystatins and tasiamide B (**2**) contain the characteristic statine-core (γ-amino-β-hydroxy acid) unit that confers the affinity to the active site of certain aspartic proteases; however, the statine core in grassystatins is Leu-derived (Sta) and in tasiamide B (**2**) is phenylalanine-derived (phenylstatine). Certain synthetic aspartic protease inhibitors comprising a phenylstatine unit have previously been shown to inhibit BACE1.[6] We recently achieved the total synthesis of grassystatin A,[7] tasiamide B (**2**)[5] and the originally proposed (epimeric) structure of tasiamide B (**1**),[5] which allows us to

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characterize their biological activities more rigorously and deconvolute or selectively enhance certain overlapping activities. As part of this ongoing research, we engaged in efforts to design improved analogues with BACE1 inhibitory activity and with the ultimate goal of obtaining a molecule with potent cellular activity and in vivo efficacy.

BACE1 is a key factor in the proteolytic formation of the Aβ peptide, a major component of plaques in the brains of AD patients, and inhibition of this enzyme has emerged as a major strategy for pharmacologic intervention in AD.[8] Aβ is generated upon sequential action of BACE1 and γ-secretase to proteolytically cleave the amyloid precursor protein (APP).[9] The scission of APP by BACE1 liberates two cleavage products, viz. secreted APP ectodomain (sAPPβ) and membrane bound carboxyl terminal fragment (CTF), C99, which is further processed by γ-secretase (presenilins) to generate the C-terminus of the Aβ peptide (Aβ40 and Aβ42) and APP intracellular domain (Figure 1). BACE1 competes for APP with α-secretase

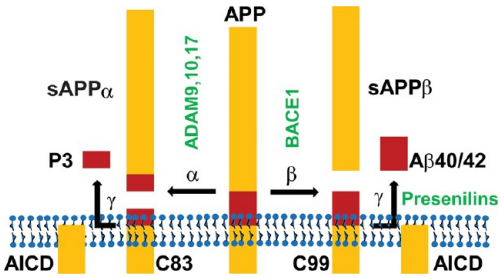

Figure 1. APP processing by secretases.

(metalloproteases ADAM9, 10 and/or 17), which diverts away from the potentially amyloidogenic Aβ genesis pathway by generating sAPPα and C83. The amyloid cascade hypothesis predicts that lowering Aβ in the brain should be disease modifying,[10] and the molecular pathways that affect Aβ levels have been major targets in efforts to develop therapeutic agents for AD.[11] BACE1 has been receiving considerable attention as a therapeutic target[12] since BACE1 knockout mice are viable[13] as compared to embryonic lethal presenilin (γ-secretase) knockouts.[14] Partial reduction of BACE1 in vivo leads to diminished AD-like pathology in transgenic mouse models.[15]

RESULTS AND DISCUSSION

Synthesis and Structure–Activity Relationship (SAR) Studies in Vitro. We have previously reported the lack of activity of grassystatins A–C against BACE1, while these compounds potently inhibit two other aspartic proteases, cathepsins D and E, and to different extents.[3] The central statine-type unit is crucial for the aspartic protease inhibitory activity. Tasiamide B (**2**), which we recently synthesized,[5] leading to the revision of the originally proposed structure (**1**, L-Phe),[4] contains a different statine unit, namely, phenylstatine. In light of the grassystatin data, we tested tasiamide B (**2**) against these three aspartic proteases in enzymatic in vitro assays and found that **2** inhibited BACE1 at sub-micromolar concentrations, but also lost the differential activity and potency against cathepsins D and E (Table 1). While the diastereomeric compounds **3** (L-Phe-D-Ala) and **4** (D-Phe) lost their aspartic protease inhibitory activity almost completely, tasiamide B analogue **5** lacking the prolyl methylester at the C-terminus relative to **2** not only restored but also improved on all activities, including BACE1 inhibition. This led us to design and synthesize other phenylstatine containing peptides **6**–**16** (Table 1) and directly compare them with compound **5** and with our previously published tasiamide B-like structures, compounds **1**–**4**.[5]

In order to prepare compounds **5**–**16**, several building blocks shown in Scheme 1 were synthesized first. Commercially available amino acids or other simple chemicals were coupled with other residues, as contained in the designed target molecules. The combination of EDC and HOAt as coupling reagents gave satisfactory results. With these building blocks and other commercially available amino acids in hand, the designed compounds were prepared smoothly, as described in Schemes 2–4. Removal of the benzyl ester protecting group of **17** gave the corresponding carboxylic acid, which was coupled with the amine released from **25**, yielding the protected tetrapeptide **37**. Treatment of this compound with TFA followed by coupling with the amine derivate from building block **29** gave the linear heptapeptide **39**. The desired target compound **5** was achieved smoothly after hydrogenation of **39** in methanol. A similar strategy yielded compound **8** by using other building blocks (**17**, **26**, and **36**). Amidation of carboxylic acid **5** with benzyl amine or isopropyl amine provided compound **6** or **7** in 76% yield (Scheme 2). Hydrogenation of compound **25** (Scheme 3) yielded the corresponding free amine, which was coupled with Cbz-Val-OH, giving tripeptide **43** in 82% yield. Treatment of **43** and **33** with TFA released the corresponding free carboxylic acid and amine, which were connected under the assistance of HATU/HOAt, resulting in the desired heptapeptide **9**. Removal of the Cbz group of **9** provided compound **10**. Blocking the free amine of **10** with the Boc group gave compound **11**. Replacement of the Val-MeGln residues in N-terminus of **9** with building blocks bearing aromatic sulfanilamide structures (**22**/**23**),[16] led to the desired peptides **12**/**13**. Acetylation of the free amine derivate from building block **24** (Scheme 4) gave compound **49**. Removal of the protecting group of **49** and coupled with the free amine deviate from **30** or **33**, yielded tetrapeptide **16** or **14**, respectively. Similarly, hexapeptide **15** was obtained smoothly over three steps using building blocks **27** and **33**.

Compound **8**, lacking *N*-methyl groups for the Phe and Gln residues compared with **2**, showed dramatically reduced activity against BACE1, indicating the importance of the tertiary amide functionalities. Since N-terminally truncated compounds **14** and **15** containing a secondary amide for Gln and an *N*-Me-Phe residue near the C-terminus have similar activity as **8**, it appears that the *N*-Me-Gln residue is particularly important for BACE1 inhibition of this series. Compound **9**, an N-terminally truncated Cbz analogue of **2** (Figure 2), appeared to be a potent broad-spectrum aspartic protease inhibitor in vitro, with potent activity against BACE1. BACE1 selectivity was slightly enhanced by replacing Cbz with Boc on the N-terminus (compound **11**), while the free amine (compound **10**) showed only 4-fold reduced activity against BACE1, and activities against cathepsin E and D were reduced by 30–137-fold. Compounds **12** and **13** (Figure 2) possessing isophthalic acid residues with sulfonamide functionalities at the Gln position (in **2**) retained significant activity against BACE1, and we expected these tasiamide B–isophthalic acid dipeptide hybrid molecules to possess better cellular activity (see below). The activity is consistent with previous reports that isophthalic acid derivatives at the S2 position possess potent BACE1 inhibitory activity.[6,16] Other modifications or further truncation on the N-terminus

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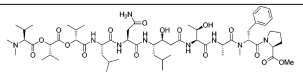
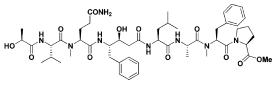
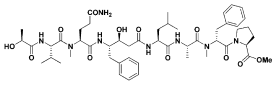
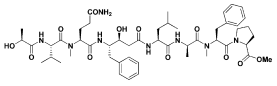
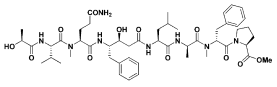
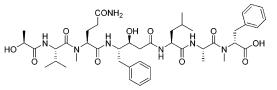
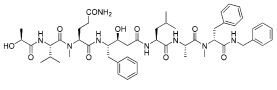
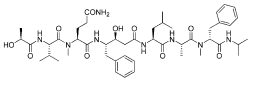
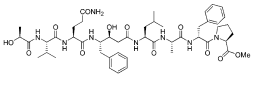
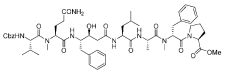
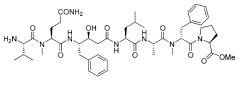
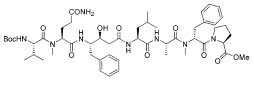
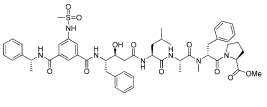
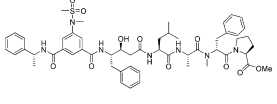
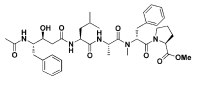
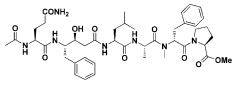
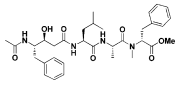
Table 1. Structures of Aspartic Protease Inhibitors and Inhibition of Enzymatic Activities

Compound ID	Structure	In vitro IC_{50} (nM)			Cathepsin selectivity
		BACE1[a] (BEI)[d]	Cathepsin D[b]	Cathepsin E[c]	IC_{50} D/IC_{50} E
Grassystatin A		>10,000	40	0.80	50
1		353 (0.0066)	251	37.5	6.7
2		189 (0.0069)	182	66	2.8
3		>10,000	3,480	1,710	2.0
4		>10,000	7,590	1,450	5.2
5		73 (0.0082)	54.1	20.8	2.6
6		124 (0.0072)	20.9	30.4	0.68
7		166 (0.0075)	254	51.9	4.9
8		5,430	89.3	4.94	18
9		54.2 (0.0070)	0.0783	0.724	0.11
10		211 (0.0074)	17.3	58.2	0.30
11		48.8 (0.0073)	0.126	1.92	0.066
12		128 (0.0068)	11.3	210	0.054
13		57.2 (0.0071)	5.84	36.4	0.16
14		>10,000	71.7	4,950	0.014
15		>10,000	7.81	77.5	0.10
16		>10,000	412	1,520	0.27

[a] IC_{50} positive control (β-secretase inhibitor IV) 250 nM. [b] IC_{50} positive control (pepstatin A) 0.693 nM. [c] IC_{50} positive control (pepstatin A) 0.446 nM. [d] Binding efficiency index, BEI = (pIC_{50})/(molecular weight).

led to reduced activities against all or a subset of the three proteases tested (Table 1).

BACE1–Inhibitor Crystal Structure Analyses. To interrogate the structural basis for activity and SAR, we determined

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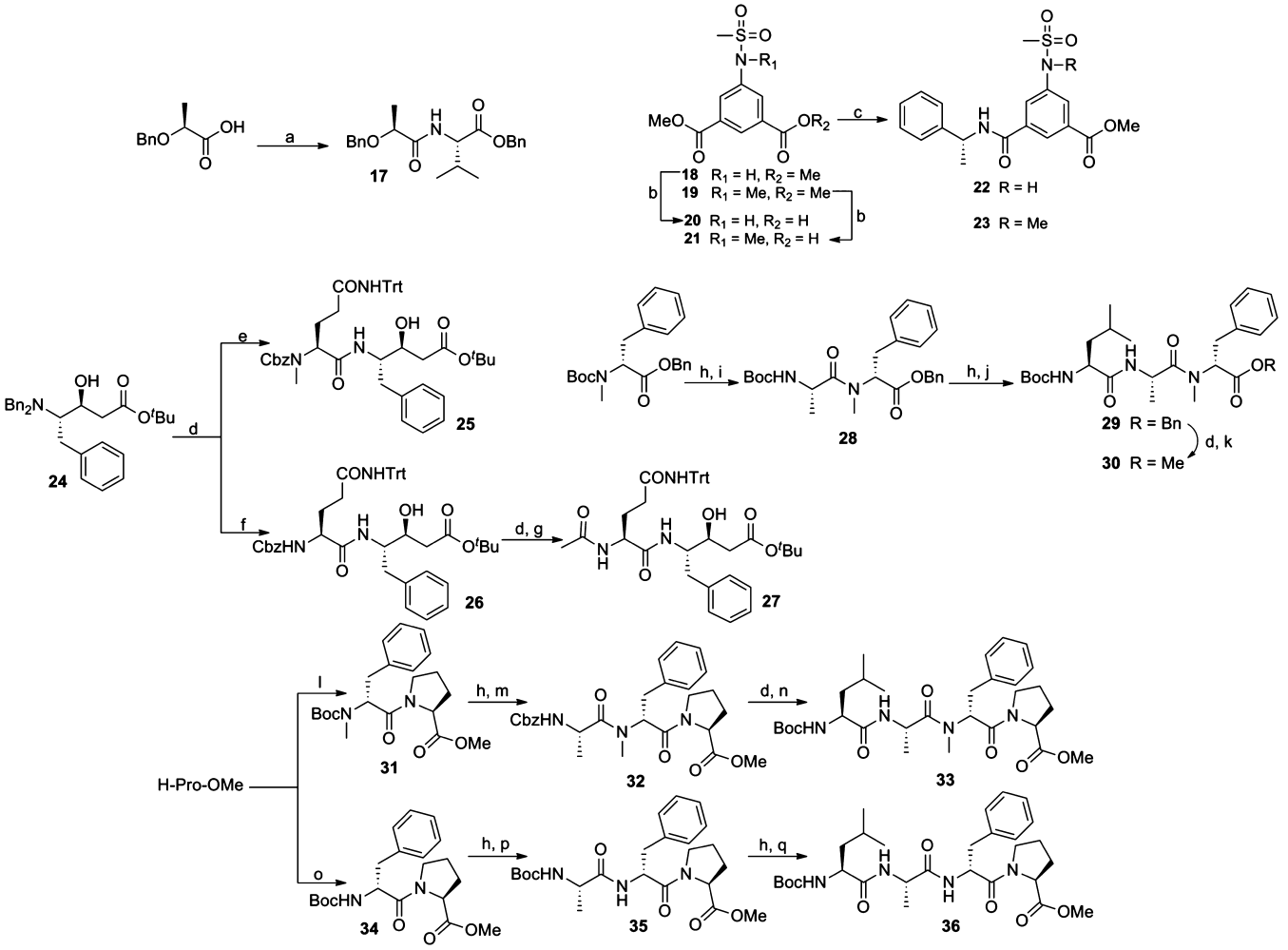
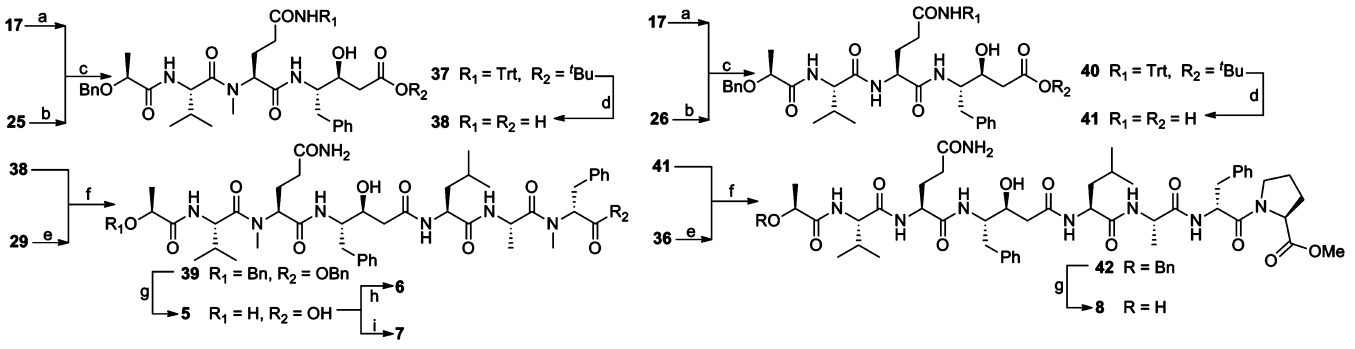
Scheme 1. Synthesis of Building Blocks[a]



[a]Reagents and conditions: (a) H-Val-OBn, EDC, HOAt, DIEA, DCM, 99%; (b) NaOH, THF/MeOH/H_2O, 64% for **20**, 97% for **21**; (c) (*R*)-1-phenylethanamine, EDC, HOAt, DIEA, DCM, 57% for **22**, 39% for **23**; (d) H_2, Pd−C, EtOAc; (e) Cbz-MeGln(Trt)−OH, EDC, HOAt, DIEA, THF, 64%; (f) Cbz-Gln(Trt)−OH, EDC, HOAt, DIEA, DCM, 65%; (g) Ac_2O, MeOH, 84%; (h) 4 M HCl-EtOAc; (i) Boc-Ala-OH, EDC, HOAt, DIEA, DCM, 74%; (j) Boc-Leu-OH, EDC, HOAt, DIEA, DCM, 69%; (k) K_2CO_3, MeI, DMF, 99%; (l) Boc-Me-D-Phe-OH, EDC, HOAt, DIEA, DCM, 96%; (m) Cbz-Ala-OH, EDC, HOAt, DIEA, DCM, 81%; (n) Boc-Leu-OH, EDC, HOAt, DIEA, DCM, 96%; (o) Boc-D-Phe-OH, EDC, HOAt, DIEA, DCM, 84%; (p) Boc-Ala-OH, EDC, HOAt, DIEA, DCM, 86%; (q) Boc-Leu-OH, EDC, HOAt, DIEA, DCM, 70%.

Scheme 2. Synthesis of Compounds 5−8[a]



[a]Reagents and conditions: (a) LiOH, THF/MeOH/H_2O; (b) Pd/C, EtOAc; (c) HATU, HOAt, DIEA, THF, 45% for **37**, 80% for **40**; (d) TFA, DCM; (e) 4 M HCl-EtOAc; (f) HATU, HOAt, DIEA, 56% for **39**, 20% for **42**; (g) H_2, Pd−C, MeOH; (h) $BnNH_2$, EDC, HOAt, $NaHCO_3$, 76%; (i) isopropyl amine, EDC, HOAt, $NaHCO_3$, 76%.

the crystal structures of compounds **2**, **5**, and **11**−**13** in complex with BACE1. The crystalline complexes of BACE1−**2**, BACE1−**5**, and BACE1−**11** were obtained by soaking the compounds into the orthorhombic apo BACE1 crystals (space group, $C222_1$), which diffracted to 2.08, 1.59, and 1.77 Å, respectively (Table S1). We were able to obtain the cocrystals

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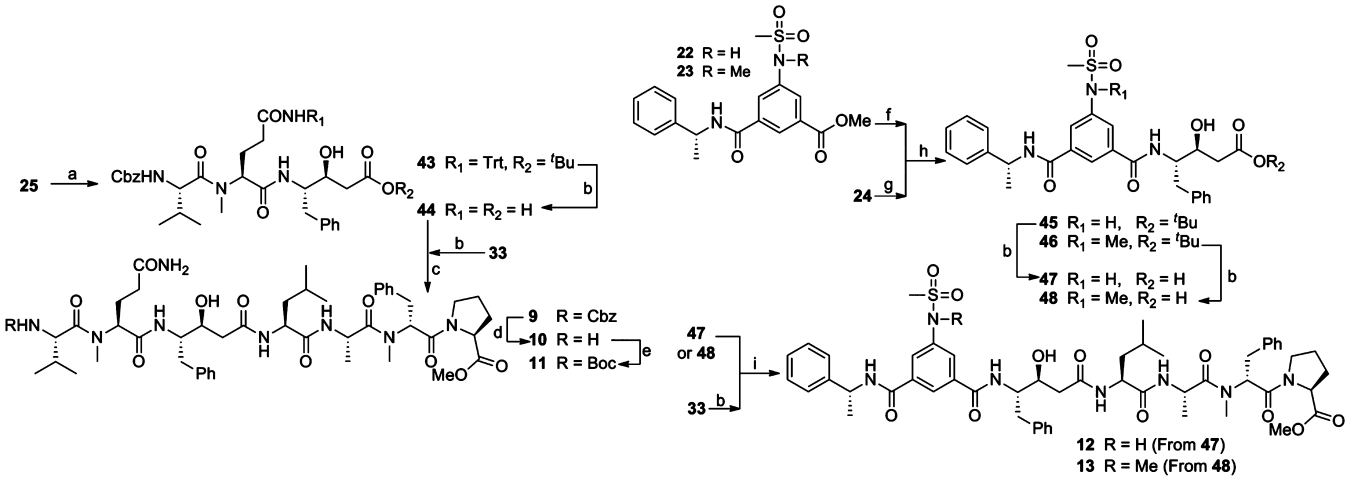
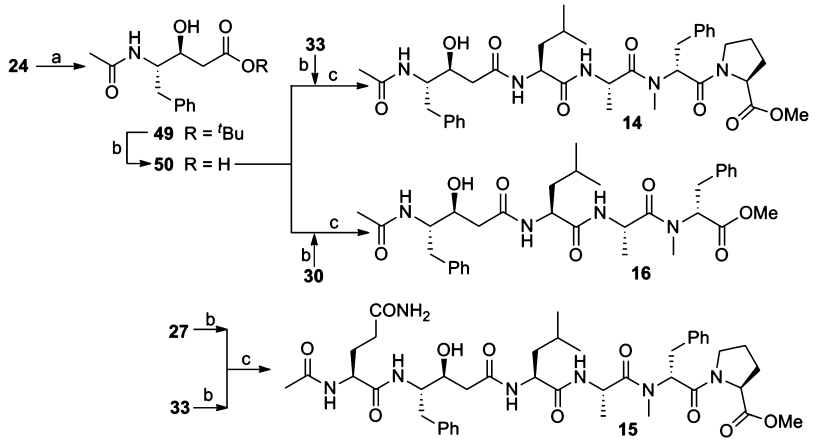
Scheme 3. Synthesis of Compounds 9−13[a]



[a]Reagents and conditions: (a) (i) Pd−C, H_2, EtOAc; (ii) Cbz-Val-OH, EDC, HOAt, DIEA, DCM, 82% over two steps; (b) TFA, DCM; (c) HATU, HOAt, DIEA, DCM, 64%; (d) Pd−C, H_2, MeOH, 83%; (e) Boc_2O, DCM, 60%; (f) NaOH, THF/MeOH/H_2O; (g) Pd−C, H_2, MeOH; (h) EDC, HOAt, DIEA, DCM, 81% for **45**, 70% for **46**; (i) HATU, HOAt, DIEA, DCM, 69% for **12**, 60% for **13**.

Scheme 4. Synthesis of Compounds 14−16[a]



[a]Reagents and conditions: (a) (i) Pd−C, H_2, EtOAc; (ii) Ac_2O, MeOH, 83% over two steps; (b) TFA; (c) HATU, HOAt, DIEA, THF, 86% over three steps for **14**; 75% over three steps for **15**; 98% over three steps for **16**.

of compounds **12** and **13** with the enzyme by mixing the solutions of the BACE1−**12** and BACE1−**13** complexes with 1.0 M ammonium sulfate/100 mM sodium citrate, pH 5.0. These crystals belonged to the $P2_12_12_1$ space group and diffracted to 1.8 and 3.06 Å for BACE1−**12** and BACE1−**13**, respectively (Table S1). In general, the compounds fit very well into the binding groove between the C-terminal and N-terminal lobes of BACE1 where the two catalytic residues D32 and D228 are located (Figure 3). The hairpin loop in the N-terminal lobe known as the 'flap' (residues 67−75) interacted directly with the bound compounds and thus adopted a close conformation, which is highly similar to the conformation seen in the first crystal structure of BACE1 in complex with peptoid OM99-2.[17] The binding position of the five compounds within the enzyme is very similar since the superimposition of the five compounds after fitting the Cα atoms of the protein shows that the compounds were overlapped (Figure 3). The program LIGPLOT[18] was used to examine the interactions between the compound and BACE1. Table 2 lists all the residues which formed the direct hydrogen bonds (H-bonds) with the compounds in the five complexes. The H-bond interactions of residues P70, T72, G230, and T232 with the compound were seen in all five complexes. In some structures, more than one H-bond formed between the residues and the compound. For example, in the complex of BACE1−**2**, three H-bonds formed between T232 and compound **2**. Residues such as N233 also interact with the compound via a water molecule, which can be found in the LIGPLOT plots (Figures 4 and S1). Due to the low diffraction resolution, water molecules were not visualized in the complex structure of BACE1−**13**. Residues Y71, F108, W115, and I126 interact with the compounds mainly through hydrophobic interactions, and Y71 in particular always contributes the most among all the residues' hydrophobic interaction with the compound in each complex (Table S2). The total number of paired atoms which formed hydrophobic interactions between the enzyme and the compound are 30, 24, 36, 51, and 57 in complex structures of BACE1/**2**, BACE1/**5**, BACE1/**11**, BACE1/**12**, and BACE1/**13**, respectively. Therefore, compounds **12** and **13** form more hydrophobic interactions with the enzyme than the other three compounds do because of the two additional benzene rings

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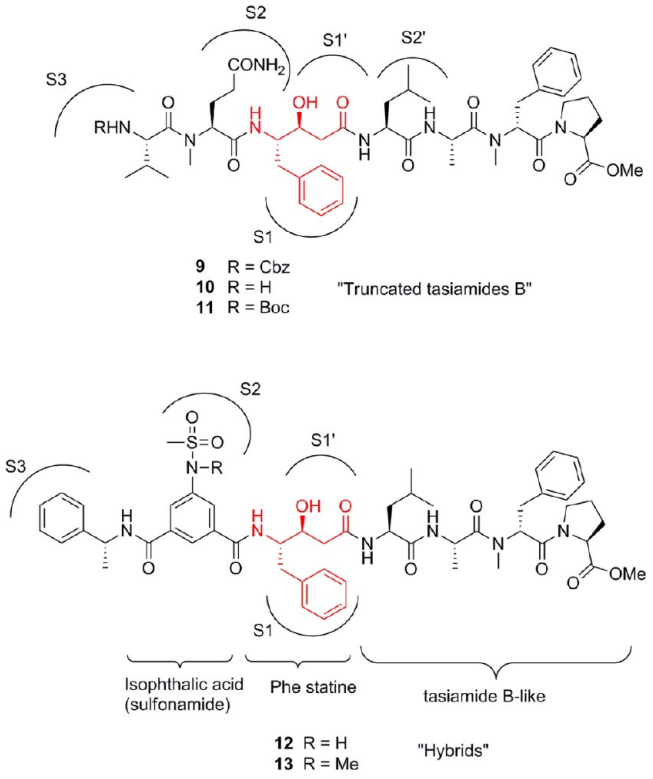

Figure 2. Fragments in compounds **9**–**13**.

(Table 2), which may explain why these compounds have high inhibition activity to BACE1 in the cell.

Activity against Cathepsins D and E. For all analogues we also assessed the selectivity index for cathepsins D/E, which may provide the basis for application of certain compounds as chemical tools to probe cathepsin D or cathepsin E function. For example, we had previously reported that the natural product grassystatin A shows 30-fold selectivity for cathepsin E,[3] and our synthetic compound showed the same selectivity trend (50-fold preference for cathepsin E).[7] Most notably in that regard, we found that **9** is extremely potent against cathepsin D (IC_{50} 78.3 pM) and 9-fold selectivity for cathepsin D over cathepsin E, a reverse selectivity trend. This selectivity is even more pronounced for **11** (15-fold) at the expense of some slight potency loss against cathepsins. This compound was also our most potent BACE1 inhibitor in our in vitro enzymatic assays (IC_{50} 48.8 nM). Both compounds **9** and **11** are characterized by their carbamate moiety on the N-terminus. When we substituted the two N-terminal residues with a modified dipeptide containing an isophthalic acid-based sulfonamide functionality at the Gln position (**12** and **13**), we retained all aspartic protease inhibitory activity. The *N*-methylated sulfonamide had slightly better activity against BACE1; however, activity against cathepsin D and particularly cathepsin E was also enhanced. Truncation of the N-terminus resulting in compounds **14** and **15** increased the selectivity for cathepsins D and E over BACE1; in fact **14** which is the simplest analogue showed 69-fold selectivity for cathepsin D over cathepsin E and >140-fold selectivity over BACE1. Removal of the Pro residue on the C-terminus (compound **16**) reduced the inhibitory effects on cathepsin D without dramatically changing the activity against cathepsin E. Thus, the

Table 2. The Number of H-Bonds between the Inhibitor and Residues in the Crystal Structures of BACE1 in Complex with Compounds 2, 5, 11, 12, and 13

	2	5	11	12	13
G11		1	1		
G34	1		1	1	
P70	1	1	1	1	1
T72	2	2	2	2	1
Q73	1		2	1	1
Y198	1		1	1	1
K224		2		1	1
G230	1	1	1	2	1
T231		1			
T232	3	3	2	1	1
N233				1	1
S325				1	1

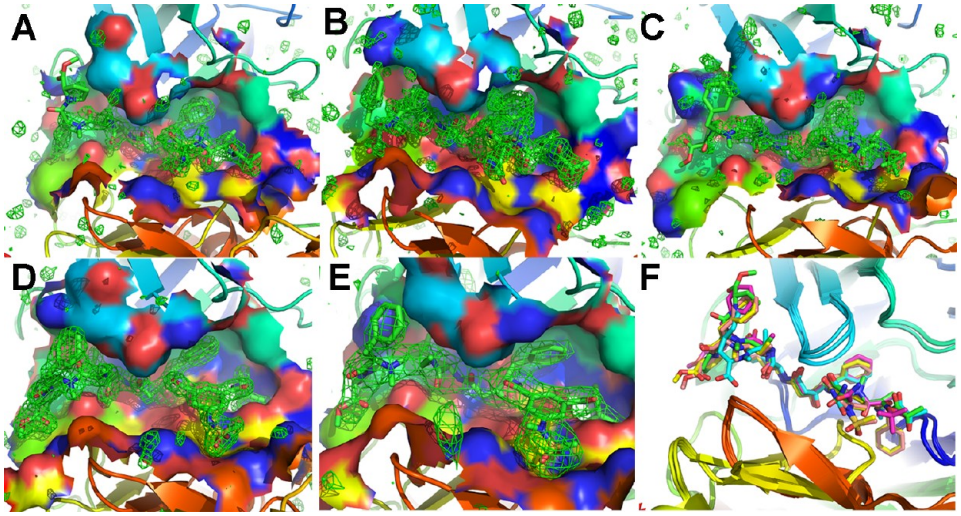

Figure 3. Molecular surface of the binding pocket for compounds **2** (A), **5** (B), **11** (C), **12** (D), and **13** (E) in their complex structures with BACE1. ($F_o - F_c$) difference electron-density maps contoured at 3.0 σ for all five crystal structures. (F) Superimposition of five crystal structures of BACE1–**2**, BACE1–**5**, BACE1–**11**, BACE1–**12**, and BACE1–**13** by fitting the Cα atoms of the protein. Five inhibitors **2** (green), **5** (cyan), **11** (magenta), **12** (yellow), and **13** (light brown) were displayed as sticks.

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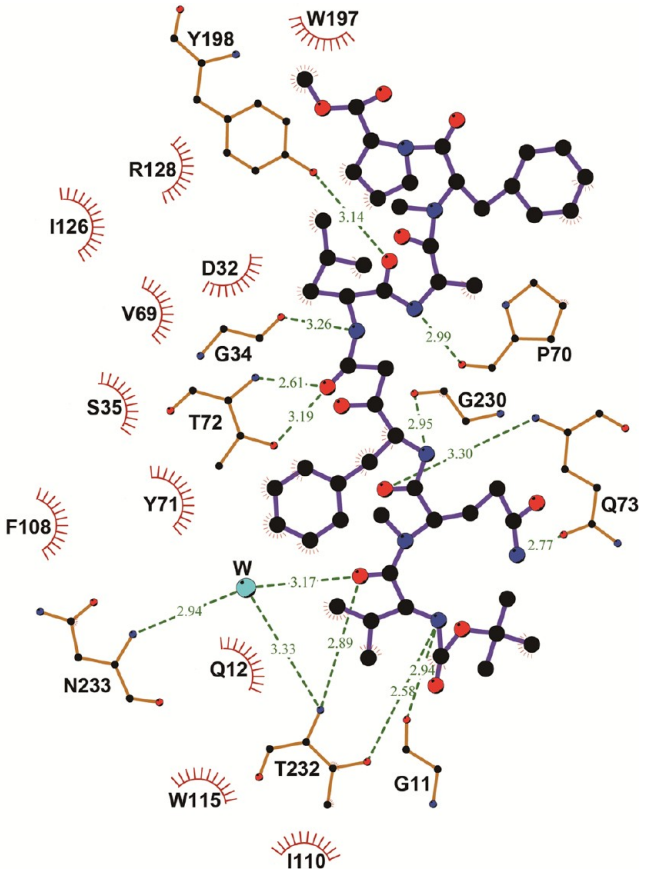

Figure 4. LIGPLOT representations of the interactions of inhibitor **11** with the enzyme in the crystal structure complex. Dashed lines represent hydrogen bonds, and spiked residues form hydrophobic interactions with **11**.

selectivity of tasiamide B-based protease inhibitors can be tuned through the editing or modification of both termini.

Measurement of Immediate Downstream Effects of Cellular BACE1 Inhibition. A human placental alkaline phosphatase-amyloid precursor protein (HPLAP-APP) fusion protein[19] was used to quantitatively measure secreted APP (sAPP). Control BACE1 inhibitors II (BI−II)[20] and IV (BI−IV)[21] (Figure S2) alone did not affect total sAPP (Figure 5B) due to the well-documented shift from β- to α-cleavage since α- and β-secretases both compete for the APP substrate.[22] In order to distinguish sAPPβ from total sAPP, transfected cells were treated with 25 μM of the metalloprotease inhibitor Batimastat (BB-94, Figure S2)[23] along with the various inhibitors in order to specifically quantitate effects on sAPPβ by inhibiting α-secretase activity (Figure 5). The production of sAPPβ, which corresponds to the total sAPP in the presence of Batimastat (BB-94, Figure 5B), was further reduced by BI−II and BI−IV (Figure 5B,C) and also by many test compounds (Figure 5C). Strongest reduction in sAPPβ at the 10 μM concentration was observed for compound **13** followed by **12**, **10** and then **2**.

We then tested the most active compounds from the reporter assay to determine effects on endogenous levels of the CTF, C99. Consistent with the reporter assay, compounds **10**, **12** and **13** prevented the generation of C99, without affecting C83 levels. These data clearly indicate that these compounds are BACE1 inhibitors with cellular activity and do not affect α-secretase (Figure 6). The γ-secretase inhibitor LY411575 (Figure S2)[24] increased the levels of C83 and C99 since both fragments are substrates of γ-secretase (Figure 1).

Measurement of Aβ Production Downstream of β- and γ-Secretase Activity. Next we measured secreted amounts of Aβ peptides formed upon sequential action of β- and γ-secretases in CHO 2B7 cells (clonal expression of APP695 wt). Specifically, we measured total Aβ and individually Aβ40 and Aβ42 upon treatment with the most promising compounds based on HPLAP cellular and BACE1 enzyme inhibitory activity. Cellular activity in CHO cells was validated for six compounds (Figure 6). Compounds **2**, **9**, **10** and **11** showed activity in the micromolar range (Figure 6) and therefore exhibited approximately 10−100-fold lower potency than in the enzymatic BACE1 assays. Compounds **12** and **13**, which together with **10** showed the best activity in the HPLAP cellular assay for sAPPβ reduction at 10 μM (Figure 7), were even active in the sub-micromolar range with IC_{50} values near 100 and 10 nM, respectively. These values for **12** and **13** closely correspond to the IC_{50} values for the in vitro BACE1 inhibition (Table 1), suggesting that both compounds have excellent cell penetrating ability.

Selectivity for β-Secretase over γ-Secretase. Our previous assays were unable to eliminate the possibility that the BACE1 inhibitors are additionally γ-secretase inhibitors. Specifically, sAPPβ levels are independent of γ-secretase action, while the observed inhibition of Aβ production is the net result of the sequential action of both β- and γ-secretase. Given that β- as well as γ-secretase have aspartic protease activity, and considering the observed effects on cathepsins D and E, it was quite possible that our aspartic protease inhibitors may not have been able to differentiate both secretases. To specifically test for γ-secretase inhibition, we used H4 cells overexpressing the BRI-C99 fusion protein and treated them with our compounds (10 μM). The BRI-C99 fusion protein utilizes a fusion between the BRI2 (ITM2B) portion and the terminal 99 amino acids of APP.[25] This construct does not require β-secretase for processing but is processed by furin and furin-like proteases to produce C99, which is equivalent to the β-secretase derived fragment from APP. Lack of accumulation with inhibitor treatment (Figure 8) indicates that these compounds are not γ-secretase inhibitors,[26] which would lead to an intensity increase of the C99 fragment, as seen for the positive control LY411575 (Figure 8).

Stability and in Vivo Activity. The in vitro plasma and cellular stabilities of **12** and **13**, together with identification of the major biotransformation products, were assessed using HPLC-MS. Both compounds showed reasonable stability and were primarily converted to their corresponding acids **51** and **52** (Figure 9A) in both mouse serum (Figure 9B) and when incubated with H4 cell lysates (Figure 9C). Notably, acids **51** and **52** retained nanomolar BACE1 inhibitory activity (IC_{50} 56.3 and 369 nM), suggesting that at least intracellular ester hydrolysis would not be detrimental to activity. Conversely, one might expect that the cellular activity would be reduced if hydrolysis occurs before cell penetration; e.g, the similar acid **5** (Table 1) displayed potent activity against the enzyme but not in a cellular context (see Figure 5C). However, the ester hydrolysis occurred only slowly in vitro; **12** and **13** possessed a presumably sufficient half-life of several hours (Figure 9B,C). To assess the potential to cross the blood−brain barrier (BBB), we tested **12** and **13** in the parallel artificial membrane permeability assay (PAMPA) using a filter-immobilized BBB lipid formulation. Compound **12** gave P_e ($\times 10^{-6}$ cm/s) of 10.7

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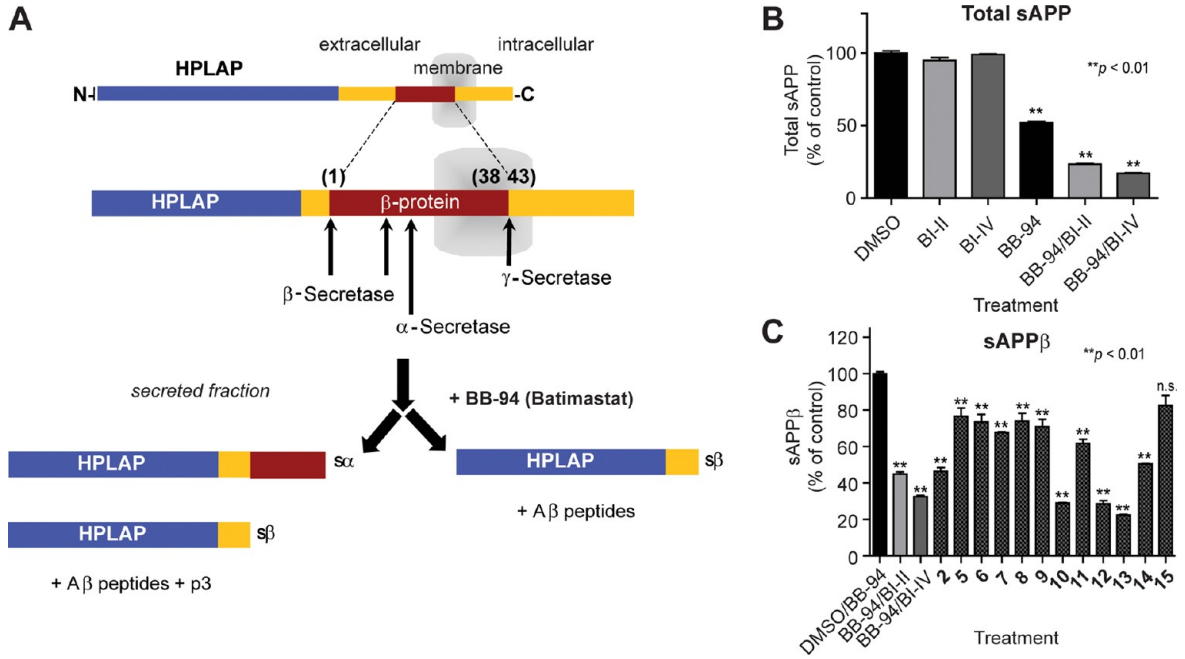

Figure 5. Reporter assay to detect cellular β-secretase activity. (A) Assay design. Our HPLAP-APP is processed by α- and β-secretase to generate HPLAP-sAPPα and HPLAP-sAPPβ, respectively. By inhibiting α-secretase with batimastat, only HPLAP-sAPPβ is generated and then quantitated. (B) Validation of detection of sAPPβ. Cells were treated for 16 h in serum-free media and sAPP was quantitated from the media by measuring HPLAP activity. Addition of β-secretase inhibitor II or IV was at a final concentration 10 μM. Only cells treated with BB-94 can differentiate the sAPPβ from total sAPP. Statistical analysis was by one-way Anova followed by Dunnett's multiple comparison test. (C) Transfected cells were simultaneously treated with BB-94 and the individual compounds at 10 μM for 16 h and assayed for HPLAP activity as in (B). The results of the individual compounds are shown. All compounds except **15** show statistically significant inhibition $p < 0.01$. Compounds **10**, **12**, and **13** were identified as the most potent in the set against the β-secretase activity.

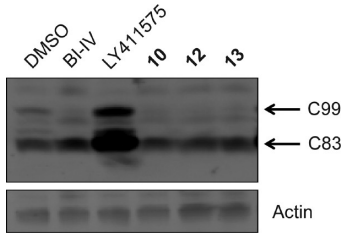

Figure 6. CTF immunoblot analysis. H4 cells were treated for 16 h with BACE1 inhibitor IV (BI–IV, 10 μM), γ-secretase inhibitor LY411575 (1 μM), or with compounds **10**, **12** and **13** (10 μM) or solvent control, and cell lysates analyzed. BI–IV and the prioritized test compounds prevented the generation of C99, indicating cellular BACE1 inhibition, while LY411575 led to accumulation of both fragments.

$\pm$ 1.41 and 8.58 $\pm$ 0.47 at pH 5.0 and 7.4, respectively, which corresponds to intermediate BBB permeability compared with controls (Table 3). We failed to accurately determine the permeability coefficient for **13**, which may be in part due to possible irreversible adsorption of the compound to the lipid-coated immobilized filter, giving variable concentration in the acceptor well. Small molecule transport across the BBB can occur through both passive and active modes, whereas PAMPA only accounts for the contribution of the former. It is possible that **12**, **13** and related congeners may be transported across the BBB in vivo via both mechanisms, thus only partially accounted for by this permeability assay, but additional processes cannot be ruled out either. Compared to other peptidomimetics, compound **12** has intermediate permeability in a BBB system based on the permeability coefficient; other peptides, modified peptides and peptidomimetics possess P_e values of 0.6–82.5 $\times$ 10^{-6} cm/s in a gastrointestinal tract model system.[27,28] Evaluation of the in vivo stability of **12** and **13** from mice treated with 30 mg/kg by intraperitoneal (i.p.) injection showed that after 3 h both compounds are present in the plasma together with lower levels of their corresponding acids **51** and **52** (Figure 9D). The same acute treatment led to a significant decrease in rodent Aβ40 for treatment with **13** but not with **12** (Figure 9E), which correlates with the 10-fold higher potency of **13** compared with **12** in the secreted Aβ assay using CHO 2B7 cells (Figure 7). In order to rationalize the observed in vivo Aβ40 levels for **12** and **13**, we determined the concentrations of these compounds in the cerebellum and cortex of test animals using HPLC-MS (Figure 9F). Relative to the plasma levels, 2.5% of **12** and 5.8% of **13** are delivered to the brain (Figure 9F). These values are about 2–4-fold lower compared to those reported for other BACE1 inhibitors such as GRL-8234, where approximately 10% brain penetration was reported.[29] The high molecular weight and number of hydrogen bonding groups are possible contributors to the lower levels of **12** and **13** in the brain. Nonetheless, because of the potent activity of **13**, it is able to significantly decrease Aβ40 levels in vivo. The detected concentration of **13** in the brain is around 20–40-fold in excess of its cellular IC_{50} for Aβ40 and Aβ42 secretion by ELISA (Figure 7). In contrast, brain concentration of **12** corresponds to only three times its cellular IC_{50}. Other BACE1 inhibitors previously demonstrated to reduce in vivo Aβ40 levels in cortical neurons typically showed greater than 10-fold excess of their cellular IC_{50} in brain tissues.[8,29–31]

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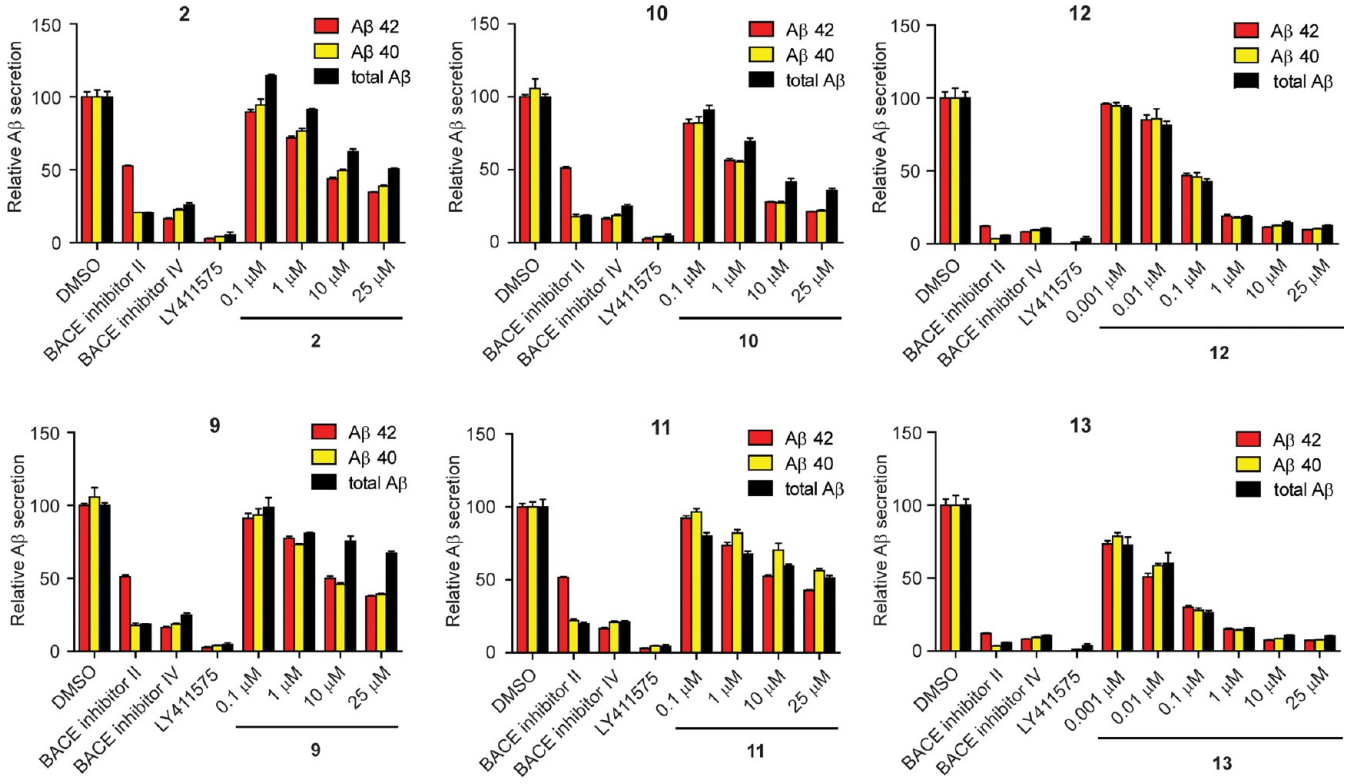

Figure 7. Secreted β-amyloid (Aβ) assay. CHO 2B7 cells (clonal expression of APP695 wt) were incubated for 16 h with compound and analyzed by direct sandwich Aβ ELISA. Control compounds: 10 μM BACE inhibitor II, 10 μM BACE inhibitor IV, and 1 μM LY411575 γ-secretase inhibitor. All treatments were performed in triplicate. Means and SEM are shown.

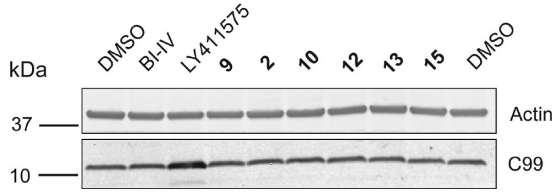

Figure 8. Immunoblot analysis for γ-secretase activity. H4 cells overexpressing the BRI-C99 fusion were treated for 16 h with the indicated compounds (10 μM). Lysates were separated by SDS-PAGE and probed for the amounts of C99 generated by furin-like proteases (instead of β-secretase). C99 is accumulated upon inhibition of γ-secretase with LY411575 (100 nM).

CONCLUSION

We identified a cyanobacterial metabolite as a BACE1 inhibitor, prepared a series of related aspartic protease inhibitors with differential selectivities against BACE1 and cathepsins D and E, and obtained inhibitor-bound BACE1 crystal structure complexes to determine the structural basis for BACE1 inhibition. We combined the natural product scaffold with structural features at the S2 position that are known to increase cellular activity against BACE1. This merger led us to hybrid molecules that showed inhibitory activity in a variety of assays, in which we probed the effects on APP processing. Among APP processing secretases, the resulting compounds specifically affected β-secretase. Hybrids **12** and **13** were fairly stable in vitro and in vivo, and compound **13** showed efficacy in reducing Aβ levels in a preliminary acute treatment study. This work exemplifies the power of a drug discovery platform combining natural products chemistry, chemical synthesis, structure-based design and protein−inhibitor complex crystallography, and a multiplexed disease-relevant molecular profiling platform to characterize potential lead compounds and chemical probes.

EXPERIMENTAL SECTION

Synthesis. *General Experimental Procedures.* Solvents were purified by standard methods. TLCs were carried out on Merck 60 F_{254} silica gel plates and visualized by UV irradiation or by staining with iodine absorbed on silica gel, ninhydrin solution or with aqueous acidic ammonium molybdate solution as appropriate. Flash column chromatography was performed on silica gel (200−300 mesh, Qingdao, China). Petroleum ether used as eluting solvent in this paper was the fraction of boiling range 60−90 °C. Optical rotations were measured using a JASCO P-1020 digital polarimeter. NMR spectra were recorded on a Varian 400 MHz spectrometer. Chemical shifts are reported in parts per million (ppm), relative to the signals due to the solvent. Data are described as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), integration, and assignment. Mass spectra were recorded on a Q-Tof Ultima Global mass spectrometer. Compound purity of ≥95% was confirmed by HPLC.

*Compound **37**.* A cold solution (ice bath) of **17** (86.0 mg, 0.31 mmol) in THF-MeOH (4:1, *V*/*V*, 5.0 mL) was treated with a solution of LiOH (1 M in H_2O, 2.0 mL). After being stirred at rt for 3 h, the mixture was recooled to 0 °C and adjusted to pH = 3 with 1 M HCl, then extracted with EtOAc (30 mL × 3). The combined organic layers were washed with brine (15 mL × 2), dried (Na_2SO_4), and concentrated in vacuo to give the free carboxylic acid as a white solid, which was used without further purification.

A solution of **25** (127.1 mg, 0.20 mmol) in EtOAc (10 mL) was hydrogenated for 2 h at 1 atm in the presence of a catalytic amount of Pd/C. The solution was filtrated and concentrated to give a colorless oil (amine), which was used without further purification.

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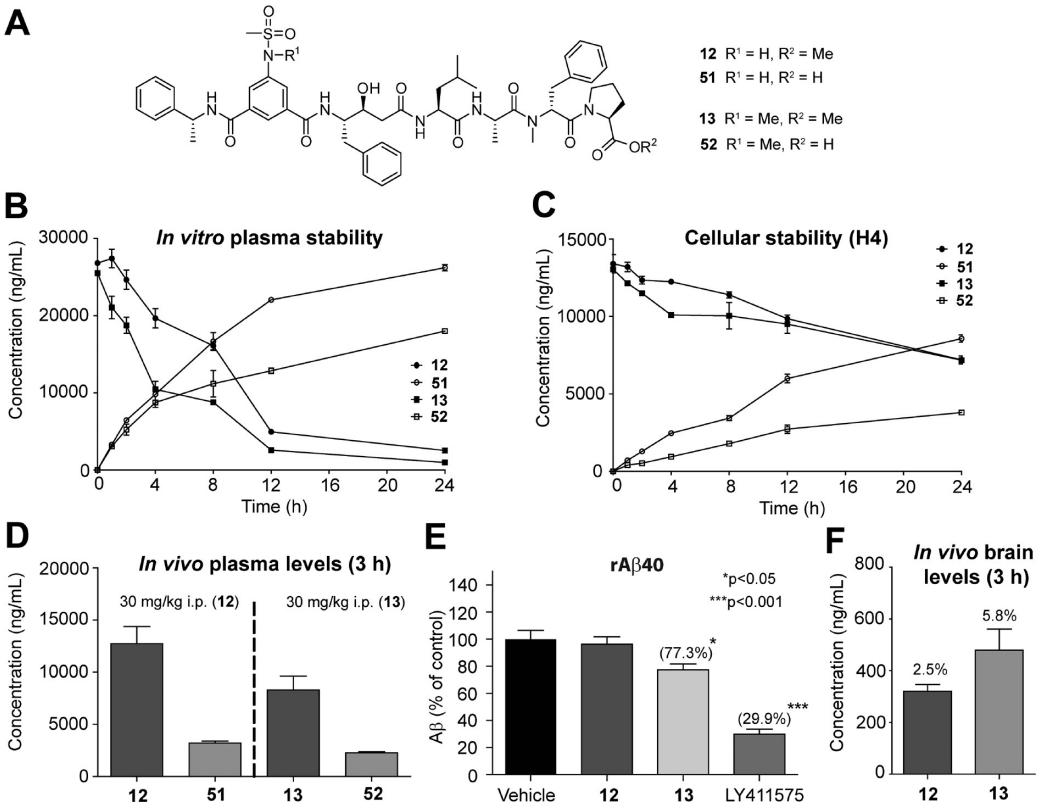

Figure 9. Stability and efficacy studies. (A) Structures of prioritized compounds **12** and **13** and their corresponding primary metabolites, ester hydrolysis products **51** and **52**, respectively. (B, C) Plasma and cellular stabilities of **12** and **13** were assessed by HPLC-MS following extraction of the compounds and their biotransformation products. (B) In vitro mouse serum stability of **12** and **13** showed a time-dependent decrease in concentration and accompanied by an increase in concentration of the corresponding ester hydrolysis product **51** or **52**. Data points depict mean ± SEM (n = 2). (C) Cellular stability of **12** and **13** assessed by incubation with whole-cell lysate of H4 cells (1.0 mg/mL). Data points depict mean ± SEM (n = 2). (D−F) Acute treatment of CF-1 mice. (D) Compound pairs **12**/**51** and **13**/**52** were detected in plasma of mice treated with 30 mg/kg of **12** or **13** (i.p. injection) after 3 h. Graphs illustrate mean + SEM (n = 6). (E) Brain concentrations of Aβ40 determined by sandwich ELISA, measured 3 h after i.p. injection with 10 mg/kg of LY411575 or 30 mg/kg of **12** and **13**, following diethylamine extraction. (F) In vivo brain levels of **12** and **13** (after 3 h) determined by HPLC-MS. Percentage of brain absorption is indicated (relative to plasma levels). Graphs for D−F illustrate mean + SEM (n = 6 per group). Differences from the vehicle-treated group: $^*p < 0.05$ or $^{***}p < 0.001$ by one-way Anova followed by Dunnett's post test.

Table 3. Permeability Coefficient (P_e, × 10^{-6} cm/s) Determined using the BBB Parallel Artificial Membrane Permeability Assay (mean ± SD)

compound	pH 7.4/7.4$_{sink}$	pH 5.0/7.4$_{sink}$
12	8.58 ± 0.47	10.7 ± 1.41
13	not determined	not determined
corticosterone[a]	17.30 ± 0.02	16.00 ± 0.94
theophylline[b]	0.09 ± 0.10	0.12 ± 0.20

[a]Highly BBB permeable control. [b]Impermeable control.

To the cold solution (ice bath) of the above carboxylic acid and amine in THF were added PyAOP (153.3 mg, 0.29 mmol), HOAt (40.1 mg, 0.29 mmol), and DIEA (97.2 μL, 0.59 mmol). The mixture was stirred at 0 °C for 2 h and then at room temperature for 26 h. After most of the solvent was removed at reduced pressure, the residue was dissolved in EtOAc (100 mL) and washed with 10% citric acid (10 mL × 2), 5% $NaHCO_3$ (10 mL × 2), and brine (10 mL × 2). After drying over Na_2SO_4, the organic layer was concentrated, giving crude **37**, which was purified by preparative TLC (petroleum ether/EtOAc = 1:1) (80.0 mg, 45%). $[\alpha]_D^{20}$ = −70.90 (c = 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.90 and 6.46 (d, 1 H, J = 9.4 Hz, Ahppa N*H*), 7.15−7.41 (m, 26 H, Ar-*H* × 25 + Val N*H*), 7.06 (s, 1 H, Gln δ-N*H*), 4.95 and 4.70 (m 1H, Gln α-*H*), 4.63 (m, 1H, PhC*H*aHbO), 4.51 (t, 1H, J = 6.9 Hz, Val α-*H*), 4.50 and 4.32 (m, 1H, PhCHa*H*bO), 4.23 (m, 1H, Ahppa NC*H*), 4.04 (m, 1H, La α-*H*), 3.94 (m, 1H, Ahppa C*H*OH), 3.77 and 3.65 (br, 1 H, Ahppa O*H*), 2.89 (m, 1H, Ahppa H-5a), 2.88 and 2.45 (s, 3H, *N*-CH_3), 2.87 (m, 1H, Ahppa δ-*Ha*Hb), 2.77 (m, 1H, Ahppa δ-Ha*Hb*), 2.51 (m, 1H, Gln γ-*Ha*Hb), 2.33−2.18 (m, 3H, Gln γ-Ha*Hb* + Ahppa α-H_2), 1.97 (m, 2H, Gln β-*Ha*Hb + Val β-*H*), 1.72 (m, 1 H, Gln β-Ha*Hb*), 1.51 and 1.27 (d, 3H, J = 6.8 Hz, La β-H_3), 1.42 and 1.37 (s, 9H, C(CH_3)$_3$), 1.27, 0.99, 0.94, and 0.80 (m, 6H, Val γ-*H* × 6); ESI-MS (m/z): 911.5 $[M + H]^+$, 933.5 $[M + Na]^+$ (Calcd 910.5).

Compound **5**. To a stirred solution of **37** (71.2 mg, 0.078 mmol) in dichloromethane (5 mL) was added TFA (2 mL). The mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure to provide crude **38**.

Building block **29** (47.63 mg, 0.086 mmol) in 4 M HCl/EtOAc (2 mL) was stirred at r.t. for 1 h, and then concentrated to dryness to give the corresponding amine, which was used without further purification.

To the cold solution (ice bath) of the amine obtained above and the crude **38** in THF were added HATU (60.8 mg, 0.16 mmol), HOAt (21.2 mg, 0.16 mmol), and DIEA (38.7 μL, 0.23 mmol). The mixture was stirred at 0 °C for 2 h, and then at r.t. for another 26 h. The solvent was removed under reduced pressure; the residue was dissolved in EtOAc (70 mL), washed with 10% citric acid (10 mL × 2), 5% $NaHCO_3$ (10 mL × 2) and brine (10 mL × 2), dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by chromatography on silica gel (DCM/MeOH = 30:1 → 20:1) to give the protected heptapeptide **39** (46.0 mg, 56%).

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A solution of **39** (41.9 mg, 0.04 mmol) in MeOH (5 mL) was hydrogenated overnight in the presence of a catalytic amount of Pd/C. The solution was filtrated, concentrated and purified by preparative TLC to give **5** (33.1 mg, 95.4%) as a white solid. $[\alpha]_D^{22} = -73.13$ (c = 0.34, $CHCl_3$); ^{1}H NMR (CD_3OD, 400 MHz) two rotamers δ: 7.13−7.25 (m, 10 H, Ar-*H* × 10), 5.36 and 5.24 (m, 1H, Phe α-*H*), 4.73 and 4.68 (m, 1H, Gln α-*H*), 4.60 and 4.55 (m, 1H, Ala α-*H*), 4.35 (m, 1H, Val α-*H*), 4.23 (m, 1H, Ahppa C*H*OH), 4.16 (q, 1H, *J* = 7.2 Hz, La α-*H*), 4.10 (m, 1H, Ahppa NC*H*), 3.46 (d-like, 1H, *J* = 10.7 Hz, Phe β-*Ha*), 3.00 (m, 2H, Phe β-*Hb* + Ahppa δ-*Ha*), 2.82 (m, 1H, Ahppa δ-*Hb*), 2.53 (m, 1H, Ahppa α-*Ha*), 2.45 (m, 1H, Ahppa α-*Hb*), 2.35 (m, 1H, Gln γ-*Ha*), 2.17 (m, 1H, Gln γ-*Hb*), 1.91−2.04 (m, 2H, Leu β-*Ha* + Leu γ-*H*), 1.75 (m, 1H, Leu β-*Hb*), 1.61−1.70 (m, 2H, Val β-*H* + Gln β-*Ha*), 1.55 (m, 1H, Gln β-*Hb*), 1.38 and 1.48 (d, 3H, *J* = 6.7 Hz, La CH_3), 0.90−1.35 (m, 12H, Val γ-*H* × 6 + Leu δ-*H* × 6), 0.87 (d, 3H, *J* = 7.6 Hz, Ala β-CH_3); ^{13}C NMR (CD_3OD, 100 MHz) δ: 178.5, 178.1, 177.8, 177.6, 175.4, 175.2, 175.0, 174.7, 174.6, 174.2, 174.1, 173.6, 172.0, 170.6, 139.9, 139.7, 139.5, 130.4, 130.3, 129.9, 129.7, 129.5, 129.4, 129.3, 127.5, 127.4, 71.3, 70.2, 69.1, 69.0, 61.0, 60.0, 57.9, 56.3, 55.8, 55.5, 55.3, 54.2, 53.2, 47.3, 46.8, 41.3, 41.2, 40.7, 38.7, 38.2, 36.2, 36.0, 32.5, 32.3, 32.0, 31.8, 30.8, 29.1, 26.6, 25.8, 25.3, 23.7, 23.5, 21.8, 21.7, 21.5, 20.2, 19.9, 18.9, 18.0, 17.2, 16.6; ESI-MS (*m*/*z*): 868.5 $[M + H]^+$, 890.5 $[M + Na]^+$ (Calcd 867.5).

Compound ***6***. To the cold solution (ice bath) of **5** (20.6 mg, 0.031 mmol) and benzylamine (4.3 μL, 0.037 mmol) in THF (5 mL) were added EDC (11.9 mg, 0.062 mmol), HOAt (8.5 mg, 0.062 mmol), and DIEA (14.8 μL, 0.093 mmol). The mixture was stirred at 0 °C for 2 h, and then at r.t. for another 16 h. The solvent was removed under reduced pressure; the residue was dissolved in EtOAc (70 mL) and washed with 10% citric acid (10 mL × 2), 5% $NaHCO_3$ (10 mL × 2) and brine (10 mL × 2), dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by TLC (DCM/MeOH = 12:1) to give compound **6** (17.2 mg, 76%). $[\alpha]_D^{20} = -52.99$ (c = 0.67, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.57 (d-like, 1H, Ala N*H*), 7.45 (d-like, 1H, Val N*H*), 7.40 (d, 1H, *J* = 5.9 Hz, BnN*H*), 7.07−7.28 (m, 17 H, Ar-*H* × 15 + Leu N*H* + Ahppa N*H*), 6.61 and 6.70 (s, 2H, Gln δ-CONH_2), 5.59 (dd, 1 H, *J* = 5.5, 11.0 Hz, Phe α-*H*), 4.92 and 5.02 (m, 1H, Gln α-*H*), 4.52 (q, 1H, *J* = 6.7, Ala α-*H*), 4.45 (dd, 1H, *J* = 6.7, 14.9 Hz, NHC*Ha*HbPh), 4.37 (m, 2H, Leu α-*H* + Val α-*H*), 4.29 (dd, 1H, *J* = 5.5, 14.9 Hz, NHCHa*Hb*Ph), 4.29 (m, 1H, Ahppa NC*H*) 4.13 (q, 1H, *J* = 5.9 Hz, La α-*H*), 4.02 (m, 1H, Ahppa C*H*OH), 3.40 (dd, 1H, *J* = 5.9, 13.5 Hz, Phe β-*Ha*), 2.94 (m, 5H, Phe β-*Hb* + Phe *N*-CH_3 + Ahppa δ-*Ha*), 2.80 (dd, 1H, *J* = 9.3, 13.7 Hz, Ahppa δ-*Hb*), 2.74 (s, 3H, Gln *N*-CH_3), 2.34 (m, 3H, Ahppa α-H_2 + Gln γ-*Ha*), 2.04 (m, 2H, Gln β-*Ha* + Gln γ-*Hb*), 1.87 (m, 2H, Gln β-*Hb* + Val β-*H*), 1.53 (m, 1H, Leu γ-*H*), 1.47 (m, 2H, Leu β-H_2), 1.36 (d, 1H, *J* = 6.7 Hz, La−CH_3), 0.92 (d, 3H, *J* = 7.0 Hz, Val γ-H_3), 0.89 (d, 3H, *J* = 6.7 Hz, Val γ-H_3), 0.84 (d, 3H, *J* = 6.3 Hz, Leu δ-H_3), 0.79 (d, 3H, *J* = 6.3 Hz, Leu δ-H_3), 0.76 (d, 3H, *J* = 6.7 Hz, Ala β-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 176.6, 175.9, 174.0, 172.6, 171.8, 170.1, 169.7, 138.4, 137.9, 136.9, 129.0, 128.7, 128.5, 128.4, 127.7, 127.1, 126.6, 126.4, 69.7, 68.6, 67.9, 56.9, 55.4, 54.3, 53.7, 52.0, 45.6, 43.4, 40.9, 40.2, 37.7, 34.2, 31.1, 30.9, 30.6, 30.0, 25.6, 24.6, 23.7, 23.0, 21.6, 20.7, 19.5, 17.7, 16.6; ESI-MS (*m*/*z*): 957.5 $[M + H]^+$, 979.5 $[M + Na]^+$ (Calcd 956.5).

Compound ***7***. Prepared using the same method described for **6**, but replacing the benzylamine with isopropyl amine. Yield 76%. $[\alpha]_D^{20} = -62.97$ (c = 0.64, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.63 (d, 1H, *J* = 5.9 Hz, Ala N*H*), 7.47 (d, 1H, *J* = 7.8 Hz, Val N*H*), 7.33 (d, 1H, *J* = 7.3 Hz, Leu N*H*), 7.24 (d, 1H, *J* = 4.4 Hz, Ahppa N*H*), 7.16 (m, 10 H, Ar-*H* × 10), 6.67 and 6.78 (s, 2H, Gln δ-CONH_2), 6.64 (d, 1H, *J* = 7.8 Hz, Isp N*H*), 5.50 (dd, 1 H, *J* = 5.7, 10.7 Hz, Phe α-*H*), 5.06 (m, 1H, Gln α-*H*), 4.50 (m, 1H, Ala α-*H*), 4.40 (m, 2H, Leu α-*H* + Val α-*H*), 4.26 (q, 1H, *J* = 6.6 Hz, Ahppa NC*H*), 4.14 (q, 1H, *J* = 6.8 Hz, La α-*H*), 0.4.03 (m, 2H, Ahppa C*H*OH + Isp NC*H*), 3.40 (dd, 1 H, *J* = 5.4, 15.1 Hz, Phe β-*Ha*), 3.00 (s, 3H, Phe *N*-CH_3), 2.82−2.96 (m, 3H, Phe β-*Hb* + Ahppa δ-H_2), 2.38 (m, 2H, Ahppa α-H_2), 2.16 (m, 2H, Gln β-*Ha* + Gln γ-*H*), 1.90 (m, 2H, Gln β-*Hb* + Val β-*H*), 1.59 (m, 2H, Leu β-H_2), 1.51 (m, 1H, Leu γ-*H*), 1.37 (d, 1H, *J* = 6.3 Hz, La CH_3), 1.12 (d, 3H, *J* = 6.3 Hz, NCHCH_3), 1.10 (d, 3H, *J* = 6.8 Hz, NCHCH_3), 0.89 (m, 12H, Val γ-H_3 × 2 + Leu δ-H_3 × 2), 0.79 (d, 3H, *J* = 6.3 Hz, Ala β-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 176.7, 175.9, 174.1, 173.5, 172.6, 171.9, 170.1, 168.6, 137.9, 137.1, 129.0, 128.7, 128.3, 126.5, 126.4, 69.6, 68.5, 57.0, 55.3, 54.3, 53.7, 52.0, 45.6, 41.5, 40.9, 37.7, 34.0, 31.6, 31.1, 30.8, 30.6, 30.0, 29.7, 24.6, 23.7, 23.0, 22.9, 22.4, 22.3, 21.8, 20.7, 19.5, 17.7, 16.3; ESI-MS (*m*/*z*): 909.5 $[M + H]^+$, 931.5 $[M + Na]^+$ (Calcd 908.5).

Compound ***8***. Similar as described for compound **5**. $[\alpha]_D^{20} = -81.45$ (c = 0.23, $CHCl_3$); ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.21 (d, 1 H, J = 7.8 Hz, N*H*), 8.17 (d, 1 H, J = 6.7, 9.0 Hz, Phe-α*H*), 7.90 (m, 2H, N*H* × 2), 7.67 (d, 1 H, J = 7.8 Hz, Phe-*NH*), 7.46 (d, 1 H, J = 9.4 Hz, N*H*), 7.24 (s, 1H, Gln-δCN*Ha*), 7.11−7.22 (m, 10H, Ar*H* × 10), 6.77 (s, 1H, Gln-δCN*Hb*), 5.99 (d, 1 H, J = 5.0 Hz, Ala-N*H*), 5.37 (d, 1 H, J = 3.7 Hz, Gln-N*H*), 5.04 (q, 1 H, J = 6.3 Hz, Ahppa-N*H*), 4.21 (m, 4H, Val-α*H* + La-α*H* + Leu-α*H*), 3.96 (m,1H, Ala-α*H*), 3.87 (m, 3H, Gln-α*H* + Ahppa-C*H*OH + Phe-α*H*), 2.77 (dd, 1H, J = 7.0, 13.4 Hz, Ahppa-δ*Ha*), 2.68 (m, 2H, Pro-βH_2), 2.48 (m, 1H, Ahppa-δ*Hb*), 1.87−2.09 (m, 4H, Ahppa-αH_2 + Pro-βH_2), 1.77 (m, 1H, Val-β*H*), 1.57−1.68 (m, 4H, Pro-δH_2 + Gln-γH_2), 1.41 (m, 1H, Leu-γ*H*), 1.23 (m, 2H, Leu-βH_2), 1.07 (d, 3H, J = 6.7 Hz, Ala-βCH_3), 0.81 (d, 3H, J = 7.0 Hz, La-CH_3), 0.59−0.70 (m, 12H, Val-γ*H* × 6 + Leu-δ*H* × 6); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 174.2, 173.8, 172.9, 172.2, 171.6, 170.9, 170.6, 169.3, 139.1, 137.2, 129.4, 129.2, 129.0, 128.1, 126.5, 67.5, 58.6, 56.4, 51.8, 47.9, 46.5, 31.4, 28.6, 28.0, 24.1, 23.2, 21.6, 21.3, 19.3, 18.4, 17.7; ESI-MS (*m*/*z*): 951.5 $[M + H]^+$, 973.5 $[M + Na]^+$ (Calcd 950.5).

Compound ***43***. A solution of **25** (205.9 mg, 0.26 mmol) in EtOAc (15 mL) was hydrogenated for 2 h at 1 atm in the presence of a catalytic amount of Pd/C. The solution was filtrated and concentrated to give the free amine, which was used without further purification.

To the cold solution (ice bath) of the amine obtained above and Cbz-Val-OH in DCM (20 mL) was added EDC (151.1 mg, 0.79 mmol), HOAt (107.2 mg, 0.79 mmol), and DIEA (208.7 μL, 1.31 mmol). The mixture was stirred at 0 °C for 2 h, and then at r.t. overnight. The reaction mixture was diluted with EtOAc (100 mL) and washed with 10% citric acid (10 mL × 2), 5% $NaHCO_3$ (10 mL × 2) and brine (10 mL × 2), dried (Na_2SO_4) and concentrated in vacuo, purified by TLC to give compound **43** (190.8 mg, 82%). $[\alpha]_D^{20} = -93.76$ (c = 0.14, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) two rotamers δ: 7.60 and 6.34 (d, 1 H, *J* = 8.6 Hz, Ahppa N*H*), 7.12−7.40 (m, 25 H, Ar-*H* × 25), 7.00 and 6.84 (s, 1 H, Gln δ-N*H*), 5.44 (m, 1H, Val N*H*), 5.22 and 5.01 (d, 1H, *J* = 12.5 Hz, PhC*Ha*Hb), 5.09 and 4.88 (d, 1H, *J* = 12.5 Hz, PhCHa*Hb*), 4.91 and 4.93 (m, 1H, Val α-*H*), 4.65 and 3.94 (d-like, 1H, *J* = 9.8 Hz, Ahppa C*H*OH), 4.25 and 4.11 (m, 1H, Ahppa NC*H*), 3.64 and 3.67 (s-like, 1H, Gln α-*H*), 2.71−2.94 (m, 2H, Phe β-H_2), 2.28−2.52 (m, 4H, Gln γ-H_2 + Ahppa α-H_2), 1.76−1.98 (m, 3H, Gln β-H_2 + Val β-*H*), 1.42 (s, 9H, ^{t}Bu), 0.99 and 0.83 (d, 3H, *J* = 6.3 Hz, Val γ-CH_3), 0.92 and 0.80 (d, 3H, *J* = 6.3 Hz, Val γ-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) two rotamers δ: 173.3, 172.6, 172.3, 171.0, 170.9, 170.7, 169.5, 168.5, 144.7, 144.6, 137.9, 137.7, 136.1, 135.6, 129.2, 128.7, 128.5, 128.1, 128.0, 127.9, 126.9, 126.5, 126.3, 81.7, 81.2, 70.4, 69.0, 67.6, 66.9, 66.7, 60.4, 58.9, 56.3, 56.0, 55.4, 54.1, 53.3, 40.9, 40.4, 39.5, 38.0, 37.6, 33.2, 31.2, 30.8, 30.7, 28.5, 28.0, 25.1, 23.3, 19.7, 19.2, 18.5, 17.3, 14.2; ESI-MS (*m*/*z*): 883.3 $[M + H]^+$, 905.4 $[M + Na]^+$ (Calcd 882.5).

Compound ***9***. To a stirred solution of **43** (88.8 mg, 0.101 mmol) in dichloromethane (2 mL) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure to give the corresponding free carboxylic acid (**44**), which was used without further purification.

To a stirred solution of **33** (69.35 mg, 0.121 mmol) in dichloromethane (2 mL) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure to give the corresponding amine, which was used in the next step without further purification.

To the cold solution (ice bath) of the carboxylic acid **44** and amine obtained above in DCM (10 mL) were added HATU (76.8 mg, 0.202 mmol), HOAt (27.5 mg, 0.20 mmol), and DIEA (64.2 μL, 0.404 mmol). The mixture was stirred at 0 °C for 2 h, and then at r.t. for another 24 h. The solvent was removed under reduced pressure; the residue was dissolved in EtOAc (100 mL) and washed with 10% citric

acid (10 mL × 2), 5% $NaHCO_3$ (10 mL × 2) and brine (10 mL × 2), dried (Na_2SO_4) and concentrated in vacuo, purified by chromatography on silica gel (DCM: MeOH = 60:1 → 20:1) to give compound **9** (67.1 mg, 64%). $[\alpha]_D^{20}$ = −49.15 (c = 0.47, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 7.00−7.34 (m, 21 H, Ar-*H* × 15 + N*H* × 6), 5.61 (dd, 1 H, J = 6.7, 9.0 Hz, Phe *α*-*H*), 5.06 (m, 2H, CO_2CH_2Ph), 4.93 (m, 1 H, Gln *α*-N*H*), 4.69 (m, 2H, Val *α*-*H* + Ala *α*-*H*), 4.38 (m, 3H, Pro *α*-*H* + Leu *α*-*H* + Ahppa NC*H*), 4.04 (m, 1H, Ahppa C*H*OH), 3.73 (s, 3 H, Pro OCH_3), 3.38 (m, 1H, Pro *δ*-*Ha*), 3.21 (m, 2H, Pro *δ*-*Hb* + Phe *β*-*Ha*), 3.00 (s, 3 H, Phe *N*-CH_3), 2.87−2.95 (m, 5H, Ahppa *δ*-*H* × 2 + Phe *β*-*Hb* + Gln *N*-*Me*), 2.44 (m, 1H, Ahppa *α*-*Ha*), 2.34 (m, 2H, Gln *β*-*H* × 2), 1.83−2.19 (m, 8H, Ahppa *α*-*Hb* + Pro *β*-*H* × 2 + Pro *γ*-*H* × 2 + Gln *γ*-*H* × 2 + Val *β*-*H*), 1.58 (m, 1H, Leu *γ*-*H*), 1.47 (m, 1H, Leu *β*-*H* × 2), 0.81−0.96 (m, 12H, Val *γ*-*H* × 6 + Leu *δ*-*H* × 6 + Ala *β*-C*H* × 3); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 173.4, 172.7, 172.4, 172.2, 171.7, 168.1, 156.5, 138.2, 136.6, 136.1, 129.5, 129.0, 128.8, 128.6, 128.5, 128.2, 127.9, 126.7, 126.4, 69.3, 67.0, 59.2, 56.1, 55.5, 52.3, 51.9, 46.9, 45.1, 40.9, 40.7, 36.9, 34.7, 31.6, 30.7, 30.4, 28.8, 25.3, 24.7, 23.9, 22.9, 21.8, 19.6, 17.3, 17.2; ESI-MS (*m*/*z*): 1041.6 $[M + H]^+$, 1063.5 $[M + Na]^+$ (Calcd 1040.6).

Compound ***10***. A solution of compound **9** (54.2 mg, 0.052 mmol) in MeOH (10 mL) was hydrogenated for 24 h at 1 atm in the presence of a catalytic amount of Pd/C. The mixture was filtrated, concentrated and purified by TLC to give compound **10** (39.4 mg, 83%) as a white solid. $[\alpha]_D^{20}$ = −29.85 (c = 0.068, $CHCl_3$); ^{1}H NMR (CD_3OD, 400 MHz) *δ*: 7.16−7.31 (m, 10 H, Ar-*H* × 10), 5.66 (dd, 1 H, *J* = 5.9, 9.4 Hz, Phe *α*-*H*), 5.04 (m, 1H, Gln *α*-*H*), 4.75 (m, 1H, Pro *α*-*H*), 4.67 (m, 1H, Ala *α*-*H*), 4.42 (t, 1H, *J* = 7.2 Hz, Val *α*-*H*), 4.34 (dd, 1 H, *J* = 6.3, 9.0 Hz, Leu *α*-*H*), 4.22 (m, 1H, Ahppa NC*H*), 4.09 (m, 1H, Ahppa C*H*OH), 3.72 (s, 3 H, Pro OCH_3), 3.66 (m, 1H, Pro *δ*-*Ha*), 3.44−3.51 (m, 3H, Pro *δ*-*Hb* + Gln *γ*-H_2), 3.13 (dd, 1 H, *J* = 6.3, 14.1 Hz, Phe *β*-*Ha*), 3.06 (s, 3 H, Phe *N*-CH_3), 2.93 (m, 2H, Ahppa *δ*-*Ha* + Phe *β*-*Hb*), 2.78 (m, 1H, Ahppa *δ*-*Hb*), 2.13−2.42 (m, 5H, Ahppa *α*-*H* × 2 + Pro *β*-*H* × 2 + Gln *β*-*Ha*), 1.95 (m, 1H, Gln *β*-*Hb*), 1.85 (m, 3H, Pro *γ*-*H* × 2 + Val *β*-*H*), 1.68 (m, 1H, Leu *γ*-*H*), 1.52 (m, 2H, Leu *β*-*H* × 2), 0.88−1.01 (m, 15H, Val *γ*-*H* × 6 + Leu *δ*-*H* × 6 + Ala *β*-CH_3); ^{13}C NMR (CD_3OD, 100 MHz) two rotamers *δ*: 188.5, 177.7, 177.6, 175.7, 175.5, 174.3, 174.2, 173.9, 173.8, 173.6, 171.8, 171.6, 170.7, 170.3, 139.9, 139.7, 138.3, 138.2, 130.8, 130.4, 130.2, 129.6, 129.4, 129.2, 126.7, 127.4, 127.2, 73.2, 72.1, 70.9, 69.9, 62.2, 60.9, 60.3, 59.5, 58.3, 57.6, 57.3, 57.0, 56.1, 55.3, 53.2, 53.0, 52.6, 46.5, 41.9, 41.6, 38.4, 35.5, 33.1, 32.9, 32.7, 32.1, 31.9, 31.3, 31.1, 30.8, 30.0, 28.5, 26.3, 25.8, 25.2, 23.6, 21.9, 20.6, 20.3, 20.2, 18.2, 16.9, 16.8; ESI-MS (*m*/*z*): 907.5 $[M + H]^+$, 929.5 $[M + Na]^+$ (Calcd 906.5).

Compound ***11***. To the cold solution (ice bath) of compound **10** (23.0 mg, 0.025 mmol) in DCM (5 mL) was added Boc_2O (6.7 mg, 0.031 mmol). The mixture was stirred at rt for 2 h, the solvent evaporated and product purified by chromatography (DCM/MeOH = 40:1 → 20:1) to give compound **11** (15.2 mg, 60%) as a white solid. $[\alpha]_D^{20}$ = −52.43 (c = 0.58, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 7.43 (d, 1H, *J* = 7.8 Hz, Leu N*H*), 7.34 (d, 1H, *J* = 7.8 Hz, Ahppa N*H*), 7.16−7.26 (m, 10 H, Ar-*H* × 10), 7.08 (d, 1H, *J* = 7.0 Hz, Ala N*H*), 6.13 and 6.24 (s, 2H, Gln *δ*-CON*H* × 2), 5.62 (dd, 1 H, *J* = 6.7, 9.4 Hz, Phe *α*-*H*), 5.13 (d, 1H, *J* = 8.2 Hz, Va N*H*), 4.95 (m, 1H, Gln *α*-*H*), 4.69 (m, 1H, Ala *α*-*H*), 4.43 (dd, 1 H, *J* = 6.5, 8.2 Hz, Pro *α*-*H*), 4.32 (m, 2H, Leu *α*-*H* + Val *α*-*H*), 4.04 (s-like, 2H, Ahppa C*H*OH + Ahppa NC*H*), 3.72 (s, 3 H, Pro OCH_3), 3.41 (m, 1H, Pro *δ*-*Ha*), 3.22 (m, 2H, Phe *β*-*Ha* + Pro *δ*-*Hb*), 3.01 (s, 3 H, Phe *N*-CH_3), 2.95 (m, 3H, Phe *β*-*Hb* + Gln *γ*-*H* × 2 + Pro *β*-*Ha*), 2.46 (m, 1H, Ahppa *α*-*Ha*), 2.18−2.34 (m, 4H, Ahppa *α*-*Hb* + Gln *γ*-*H* × 2, Pro *γ*-*Ha*), 2.08 (m, 3H, Gln *β*-*Ha* + Pro *β*-*Hb* + Leu *β*-*Ha*), 1.94 (m, 2H, Gln *β*-*Hb* + Pro *γ*-*Ha*), 1.84 (m, 3H, Val *β*-*H* + Pro *γ*-*Hb* + Ahppa *δ*-*H* × 2), 1.60 (m, 1H, Leu *γ*-*H*), 1.42 (s, 9H, ^{t}Bu), 0.85−0.95 (m, 12H, Val *γ*-*H* x 6 + Leu *δ*-*H* × 6), 0.82 (d, 3H, *J* = 6.7 Hz, Ala *β*-CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 175.0, 173.7, 172.6, 172.4, 172.2, 171.7, 170.8, 168.2, 156.0, 138.3, 136.6, 129.5, 129.1, 128.6, 128.3, 128.2, 126.6, 126.4, 79.9, 69.4, 59.2, 55.9, 55.5, 54.4, 52.3, 51.9, 46.9, 45.1, 40.8, 37.1, 34.7, 31.7, 30.7, 30.6, 30.4, 28.8, 28.4, 28.3, 25.3, 24.7, 24.2, 23.0, 21.9, 19.6, 18.5, 17.3, 17.2; ESI-MS (*m*/*z*): 1007.5 $[M + H]^+$, 1029.6 $[M + Na]^+$ (Calcd 1006.6).

Compound ***45***. To a cold solution (ice bath) of compound **22** (95.3 mg, 0.25 mmol) in THF/MeOH (4:1, 5.0 mL) was added a solution of NaOH (0.8 M in H_2O, 5.0 mL). The mixture was stirred at r.t. for 3 h before adjusting to pH = 2 and extracted with EtOAc (30 mL × 3). The combined organic layer was washed with brine (15 mL × 2), dried (Na_2SO_4), and concentrated in vacuo to give the free carboxylic acid as a white solid, which was used without further purification.

A solution of compound **24**[5] (104.0 mg, 0.23 mmol) in EtOAc (10 mL) was hydrogenated for 5 h at 1 atm in the presence of a catalytic amount of Pd/C. The solution was filtrated and concentrated to give the free amine, which was used without further purification.

To the cold solution (ice bath) of the acid and amine obtained above in DCM (15 mL) were added EDC (95.9 mg, 0.50 mmol), HOAt (68.1 mg, 0.50 mmol), DIEA (119.2 *μ*L, 0.75 mmol). The mixture was stirred at 0 °C for 2 h, and then at r.t. overnight. The reaction mixture was diluted with EtOAc (100 mL) and washed with 10% citric acid (10 mL × 2), 5% $NaHCO_3$ (10 mL × 2) and brine (10 mL × 2), dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether: EtOAc = 5:1) to give compound **45** (124.6 mg, 81%) as a white solid. $[\alpha]_D^{20}$ = −51.62 (c = 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 8.91 (s, 1H, SO_2N*H*), 8.01, 7.97, and 7.95 (s, 3H, Ar-*H* × 3), 7.12−7.40 (m, 10H, Ar-*H* × 10), 7.06 (d, 1H, *J* = 9.0 Hz, BnN*H*), 7.00 (d, 1H, *J* = 7.8 Hz, Ahppa N*H*), 5.35 (m, 1H, PhC*H*), 4.40 (q, *J* = 8.4 Hz, Ahppa NC*H*), 4.13 (d, 1H, *J* = 7.3 Hz, C*H*OH), 3.96 (s, 1H, CHO*H*), 3.01 (m, 2H, Ahppa *δ*-*H* × 2), 2.90 (s, 3H, SO_2*Me*), 2.44 (m, 2H, Ahppa *α*-*H* × 2), 1.60 (d, 3H, *J* = 6.7 Hz, PhCHC*H*$_3$), 1.40 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 172.7, 166.1, 165.2, 142.8, 138.8, 137.8, 135.9, 135.6, 129.4, 128.7, 128.5, 127.5, 126.5, 126.3, 122.4, 122.1, 121.4, 81.8, 67.7, 54.8, 49.7, 39.6, 39.5, 38.1, 34.4, 28.0, 21.9; ESI-MS (*m*/*z*): 610.3 $[M + H]^+$, 632.2 $[M + Na]^+$ (Calcd 609.3).

Compound ***12***. To a stirred solution of **45** (54.2 mg, 0.089 mmol) in dichloromethane (2 mL) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure to give the corresponding carboxylic acid (**47**), which was used in the next coupling without further purification.

To a stirred solution of **33** (61.4 mg, 0.11 mmol) in dichloromethane (2 mL) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure to give the corresponding amine, which was used without further purification.

To the cold solution (ice bath) of the carboxylic acid (**47**) and amine obtained above in DCM (10 mL) were added HATU (67.7 mg, 0.18 mmol), HOAt (48.5 mg, 0.18 mmol), and DIEA (56.6 *μ*L, 0.36 mmol). The mixture was stirred at 0 °C for 2 h, and then at r.t. overnight. The reaction mixture was diluted with EtOAc (100 mL) and washed with citric acid (10%, 10 mL × 2), 5% $NaHCO_3$ (10 mL × 2) and brine (10 mL × 2), dried (Na_2SO_4) and concentrated in vacuo. After purification by column chromatography (DCM/MeOH = 60:1→20:1), compound **12** (61.9 mg, 69%) was obtained as a white solid. $[\alpha]_D^{21}$ = −61.70 (c = 0.91, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 9.04 (s, 1H, SO_2N*H*), 8.02, 7.91, and 7.83 (s, 3H, Ar-*H* × 3), 7.56 (s-like, 1H, Ahppa N*H*), 7.05−7.33 (m, 18H, N*H* x 3 + Ar-*H* × 15), 5.63 (dd, 1H, *J* = 7.0, 8.6 Hz, Phe *α*-*H*), 5.30 (m, 1H, PhC*H*), 4.80 (s-like, 1H, Ala *α*-H), 4.41 (m, 2H, Leu *α*-*H* + Pro *α*-*H*), 4.24 (s-like, 1H, Ahppa NC*H*), 4.13 (s-like, 1H, C*H*OH), 3.65 (s, 3 H, Pro OCH_3), 3.26 (s-like, 1H, Pro *δ*-*Ha*), 3.26 (m, 1H, Pro *δ*-*Hb*), 3.15 (dd, 1H, *J* = 6.1, 14.3 Hz, Phe *β*-*Ha*), 3.00 (s, 3H, Phe *N*-CH_3), 2.95 (m, 2H, Ahppa *δ*-*H* × 2), 2.92 (dd, 1H, *J* = 9.7, 14.2 Hz, Phe *β*-*Hb*), 2.84 (s, 3H, SOO*Me*), 2.14−2.38 (m, 5H, Leu *β*-*Ha* + Pro *γ*-*H* × 2 + Ahppa *α*-*H* × 2), 1.86 (m, 1H, Leu *β*-*Hb*), 1.79 (m, 2H, Pro *β*-*H* × 2), 1.49 (d, 4H, PhCHCH_3 + Leu *γ*-*H*), 0.81 (d, 3H, *J* = 4.7 Hz, Ala *β*-*H* x 3), 0.77 (m, 6H, Leu *δ*-*H* × 6); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 172.6, 172.2, 171.8, 169.9, 168.8, 166.5, 165.4, 143.3, 138.5, 138.0, 136.6, 136.0, 135.3, 129.4, 129.3, 128.6, 128.5, 128.2, 127.2, 126.6, 126.5, 126.1, 122.9, 122.1, 121.4, 69.6, 60.9, 59.2, 55.6, 52.2, 51.8, 49.7, 46.9, 45.3, 41.3, 39.4, 37.7, 34.7, 33.8, 31.9, 31.6, 30.4, 29.7, 29.6, 29.5, 29.3, 28.8, 26.2, 25.3, 24.6, 22.8, 22.7, 21.9, 17.2, 14.1; ESI-MS (*m*/*z*): 1010.4 $[M + H]^+$, 1032.4 $[M + Na]^+$ (Calcd 1009.5).

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Compound ***13***. Obtained in the same way as described for **12**. $[\alpha]_D^{20}$ = −71.20 (c = 0.59, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ 8.17, 7.95, and 7.88 (s, 3H, Ar-*H* × 3), 7.57 (s-like, 1H, Ahppa-N*H*), 7.13−7.31 (m, 16H, PheCHMeN*H* + Ar*H* × 15), 6.87 (d, 1H, *J* = 6.7 Hz, Ala-N*H*), 6.71 (d, 1H, *J* = 7.0 Hz, Leu-N*H*), 5.59 (dd, 1H, *J* = 6.3, 9.4 Hz, Phe-*αH*), 5.26 (m, 1H, Ph−C*H*), 4.83 (m, 1H, Ala-*αH*), 4.44 (t, 1H, *J* = 7.4 Hz, Pro-*αH*), 4.00 (m, 1H, Leu-*αH*), 4.17 (q, 1H, *J* = 8.1 Hz, Ahppa-NC*H*), 4.06 (s-like, 1H, C*H*OH), 3.67 (s, 3 H, Pro-OCH_3), 3.41 (m, 1H, Pro-*δHa*), 3.30 (s, 3H, SO_2N*Me*), 3.24 (m, 1H, Pro-*δHb*), 3.13 (dd, 1H, *J* = 6.1, 14.5 Hz, Phe-*βHa*), 2.89−3.08 (m, 3H, Ahppa-*δ*H_2 + Phe *β*-*Hb*), 3.01 (s, 3H, Phe-NCH_3), 2.81 (s, 3H, S*Me*), 2.19 (m, 2H, Pro-*βHa* + Ahppa-*αHa*), 1.74−2.00 (m, 4H, Ahppa-*αHb* + Pro-*βHb* + Leu-*β*H_2), 1.48 (d, 3H, *J* = 6.7 Hz, Ph−CHCH_3), 1.48 (d, 3H, *J* = 6.7 Hz, Phe-NCH_3), 1.47 (m, 1H, Leu-*γH*), 0.89 (d, 3H, *J* = 7.1 Hz, Ala-*β*CH_3), 0.82 (d, 6H, *J* = 5.9 Hz, Leu-*δ*CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 191.0, 172.6, 172.5, 171.7, 168.2, 165.9, 164.8, 143.2, 141.9, 138.0, 136.5, 136.2, 135.0, 129.4, 129.2, 128.6, 128.2, 128.0, 127.2, 126.7, 126.6, 126.1, 59.2, 55.6, 52.2, 51.6, 49.7, 46.9, 45.3, 41.5, 37.9, 35.3, 34.7, 31.6, 30.4, 29.7, 29.6, 28.8, 25.3, 24.6, 22.9, 21.9, 21.7, 17.4; ESI-MS (*m*/*z*): 1024.5 $[M + H]^+$, 1046.4 $[M + Na]^+$ (Calcd 1023.5).

Compound ***49***. A solution of compound **24** (67.7 mg, 0.15 mmol) in EtOAc (10 mL) was hydrogenated for 5 h at 1 atm in the presence of a catalytic amount of Pd/C. The solution was filtrated and concentrated to give the free amine, which was dissolved in MeOH (10 mL), and then treated with Ac_2O (266 *μ*L, 15.2 mmol). The mixture was stirred at 0 °C for 1 h and then at rt for 2 h. After that, the solvent was removed and the residue was subjected to column chromatography (petroleum ether/EtOAc = 2:1) to give compound **49** (38.6 mg, 83%) as a colorless oil. $[\alpha]_D^{20}$ = −62.38 (c = 0.046, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ: 7.22−7.32 (m, 5H, Ar-H_5), 5.92 (d, 1H, *J* = 9.0 Hz, N*H*), 4.05 (q, 1H, *J* = 8.2 Hz, NC*H*), 3.97 (d-like, 1H, *J* = 9.4 Hz, Ahppa C*H*OH), 2.91 (d, 2H, *J* = 7.8 Hz, Ahppa *δ*-H_2), 2.43 (dd, 1H, *J* = 10.6, 17.2 Hz, Ahppa *α*-*Ha*), 2.28 (dd, 1H, *J* = 2.3, 17.2 Hz, Ahppa *α*-*Hb*), 2.00 (s, 3H, COCH_3), 1.42 (s, 9H, ^{t}Bu); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.0, 169.9, 138.0, 129.4, 128.5, 126.5, 81.6, 66.8, 53.8, 39.4, 38.2, 28.0, 23.4; ESI-MS (*m*/*z*): 308.2 $[M + H]^+$, 330.2 $[M + Na]^+$ (Calcd 307.2).

Compound ***14***. To a stirred solution of **49** (16.4 mg, 0.053 mmol) in dichloromethane (1 mL) was added TFA (1 mL). The reaction mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure to give the corresponding carboxylic acid **50**.

To a stirred solution of **33** (31.0 mg, 0.054 mmol) in dichloromethane (2 mL) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure to give the corresponding amine.

To the cold solution (ice bath) of the amine and carboxylic acid **50** obtained above in THF (8 mL) was added HATU (41.0 mg, 0.11 mmol), HOAt (14.7 mg, 0.11 mmol), and DIEA (33.7 *μ*L, 0.21 mmol). The mixture was stirred at 0 °C for 2 h, and then at r.t. overnight. After concentration under reduced pressure, the residue was dissolved in EtOAc (50 mL) and washed with citric acid (10%, 10 mL × 2), 5% $NaHCO_3$ (10 mL × 2) and brine (10 mL × 2), dried (Na_2SO_4) and concentrated in vacuo, purified by chromatography on silica gel (DCM/MeOH = 50:1 → 20:1) to give the compound **14** (32.7 mg, 86%) as a colorless oil. $[\alpha]_D^{20}$ = −15.1 (c = 0.19, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ: 7.14−7.28 (m, 10H, Ar-H_5 × 2), 6.81 (d, 1H, *J* = 7.8 Hz, Ala-N*H*), 6.53 (d, 1H, *J* = 7.8 Hz, Leu-N*H*), 6.29 (d, 1H, *J* = 9.0 Hz, Ahppa-N*H*), 5.55 (dd, 1 H, *J* = 6.3, 9.4 Hz, Phe-*αH*), 4.71 (m, 1H, Ala-*αH*), 4.45 (dd, 1H, *J* = 6.3, 7.8 Hz, Pro-*α*H), 4.32 (m, 2H, Leu-*αH*), 4.01 (m, 2H, AhppaCHO*H* + NC*H*), 4.01 (m, 2H, Leu-*αH* + Ahppa-NC*H*), 3.73 (s, 3H, Pro-COOCH_3), 3.36 (m, 1H, Phe-*βHa*), 3.19 (dd, 2H, *J* = 6.3, 14.9 Hz, Pro-*δ*H_2), 2.94 (m, 3H, Phe-*βHb* + Ahppa-*δ*H_2), 2.41 (dd, 1H, *J* = 9.4, 15.3 Hz, Ahppa-*αHa*Hb), 2.26 (dd, 1H, *J* = 3.5, 15.3 Hz, Ahppa-*α*Ha*Hb*), 2.22 (m, 1H, Pro-*δHa*), 1.81−1.94 (m, 3H, Pro-*γ*H_2 + Pro-*βHa*), 1.52−1.58 (m, 3H, Leu-*β*H_2 + Leu-*γH*), 0.87 (d, 6H, *J* = 6.3 Hz, Leu-*δ*CH_3), 0.80 (d, 3H, *J* = 7.0 Hz, Ala-*β*CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 172.4, 172.3, 171.2, 170.7, 168.1, 138.2, 136.6, 129.5, 129.2, 128.9, 128.4, 128.2, 126.6, 126.4, 68.1, 59.3, 55.6, 54.6, 52.2, 51.8, 46.9, 45.0, 41.2, 39.8, 37.8, 34.6, 31.6, 30.3, 29.7, 28.8, 25.3, 24.7, 23.3, 22.8, 21.9, 17.5; ESI-MS (*m*/*z*): 708.4 $[M + H]^+$, 730.3 $[M + Na]^+$ (Calcd 707.4).

Compound ***15***. Obtained similarly as described for the preparation of **14**. $[\alpha]_D^{20}$ = −19.66 (c = 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.00 (d, 1H, J = 6.7 Hz, Ala-N*H*), 7.76 (s-like, 1H, Leu-N*H*), 7.40 (d, 1H, *J* = 7.4 Hz, Ahppa-N*H*), 7.11−7.26 (m, 10H, Ar-H_5 × 2), 6.17 and 6.14 (s, 2H, Gln-CONH_2), 5.35 (d, 1 H, *J* = 7.8 Hz, Phe-*αH*), 5.10 (d, 1H, *J* = 5.9 Hz, Gln-N*H*), 4.70 (m, 1H, Gln-*αH*), 4.45 (m, 1H, Pro-*α*H), 4.29 (m, 2H, Leu-*αH* + Ahppa-NC*H*), 4.15 (m, 1H, Ahppa-C*H*OH), 3.73 (s, 3H, Pro-COOCH_3), 3.16 (m, 3H, Pro-*δHa* + Ahppa-*δHa*Hb + Phe-*βHa*), 3.00 (s, 3H, Phe-NCH_3), 2.94 (m, 2H, Phe-*βHb* + Ahppa-*δ*Ha*Hb*), 2.56 (dd, 1H, *J* = 7.2, 14.5 Hz, Ahppa-*αHa*Hb), 2.38 (m, 1H, Pro-*δHb*), 2.23 (m, 3H, Ahppa-*αHa*Hb + Gln-*γ*H_2), 1.98 (s, 3H, COCH_3), 1.85 (m, 3H, Gln-*β*H_2 + Leu-*βHb*), 1.61 (m, 1H, Leu-*γH*), 0.89 (d, 3H, *J* = 6.3 Hz, Leu-*δ*CH_3), 0.86 (d, 3H, *J* = 6.3 Hz, Leu-*δ*CH_3), 0.73 (d, 3H, *J* = 6.7 Hz, Ala-*β*CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 175.5, 173.0, 172.9, 172.6, 172.5, 171.3, 171,2, 168.1, 139.0, 136.7, 129.5, 129.2, 128.3, 128.1, 126.6, 126.4, 69.3, 59.4, 56.1, 53.5, 52.2, 46.9, 44.5, 41.1, 40.8, 36.8, 34.5, 31.2, 30.5, 29.6, 28.8, 26.7, 25.4, 24.7, 23.0, 22.8, 21.8, 16.9; ESI-MS (*m*/*z*): 836.5 $[M + H]^+$, 858.4 $[M + Na]^+$ (Calcd 835.5).

Compound ***16***. Obtained similarly as described for the preparation of **14**. $[\alpha]_D^{20}$ = −4.80 (c = 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ: 7.13−7.26 (m, 10H, Ar-H_5 × 2), 6.98 (d, 1H, *J* = 7.4 Hz, Ala-N*H*), 6.57 (d, 1H, *J* = 8.2 Hz, Leu-N*H*), 6.25 (d, 1H, *J* = 9.0 Hz, Ahppa-N*H*), 5.33 (dd, 1 H, *J* = 4.7, 11.7 Hz, Phe-*αH*), 4.69 (m, 1H, Ala-*αH*), 4.38 (m, 1H, Leu-*αH*), 4.02 (m, 2H, AhppaCHO*H* + NC*H*), 3.74 (s, 3H, Pro-COOCH_3), 3.40 (dd, 1H, *J* = 5.1, 14.9 Hz, Phe-*βHa*), 3.00 (dd, 1H, *J* = 12.1, 14.9 Hz, Phe-*βHb*), 2.92 (d, 2H, *J* = 7.4 Hz, Ahppa-*δ*H_2), 2.85 (s, 3H, Phe-N*Me*), 2.40 (dd, 1H, *J* = 9.4, 14.9 Hz, Ahppa-*αHa*Hb), 2.26 (dd, 1H, *J* = 3.5, 15.3 Hz, Ahppa-*α*Ha*Hb*), 1.95 (s, 3H, CO*Me*), 1.45−1.59 (m, 3H, Leu-*β*H_2 + Leu-*γH*), 0.89 (d, 6H, *J* = 6.3 Hz, Leu-*δ*CH_3), 0.80 (d, 3H, *J* = 6.7 Hz, Ala-*β*CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.0, 172.3, 170.9, 170.7, 170.6, 138.1, 136.3, 129.3, 128.6, 128.4, 126.9, 126.4, 68.1, 58.0, 54.4, 52.5, 51.8, 50.7, 45.5, 41.4, 39.9, 37.8, 34.7, 32.3, 31.6, 29.7, 24.7, 23.2, 22.9, 21.9, 19.9; ESI-MS (*m*/*z*): 611.3 $[M + H]^+$, 633.3 $[M + Na]^+$ (Calcd 610.3).

Compound ***51***. Compound **12** (500 *μ*g) was dissolved in 1 mL of the mixture of THF/MeOH/H_2O (4:1:2, *V*:*V*:*V*) and treated with LiOH monohydrate (200 *μ*g) at 0 °C. After TLC showed that all the starting material was consumed, the reaction mixture was diluted, adjusted to pH 5, and extracted with EtOAc. The combined EtOAc layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by RP-HPLC (Phenomenex Synergi 4*μ*, 250 × 10 mm, 1.0 mL/min, gradient elution with 80% to 100% MeOH−H_2O over 20 min), giving 253 *μ*g (estimated by UV) of the corresponding carboxylic acid **51** (t_R 7.75 min). HRESIMS calcd for $C_{52}H_{64}N_7O_{11}S$, $[M - H]^-$ 994.4390, found 994.4411.

Compound ***52***. Following the same procedure as described for compound **51** but using compound **13** (640 *μ*g) as starting material gave 107 *μ*g (estimated by UV) of carboxylic acid **52** (t_R 6.75 min). HRESIMS calcd for $C_{53}H_{66}N_7O_{11}S$ $[M - H]^-$ 1008.4541, found 1008.4547.

Protein Crystallography. *Protein Purification and Crystallization.* A detailed description of the production of recombinant human BACE1 has been provided in our previous publication.[32] Briefly, BACE1 was expressed in *E. coli* as inclusion bodies that were then denatured and refolded into the active monomer. A cDNA fragment encoding BACE1 residues 43−454 was cloned into pET28a with a TEV protease cleavage site following a six-residue His-Tag added at the N-terminus. After refolding, nickel beads (Ni Sepharose High Performance, Amersham Biosciences, Uppsala, Sweden) were used to concentrate the protein. The eluted BACE1 was then injected into a 124 mL Hiload Superdex75 column from which it was eluted with 1 mM DTT/0.5 M urea/150 mM NaCl/20 mM Tris-HCl, pH 7.5. In order to obtain crystals with different protein packing patterns, two mutations, K75A and E77A, were introduced into BACE1 together.

The active BACE1 monomer in the elution buffer was concentrated to 8−10 mg/mL. Cocrystallizations of compounds **12** and **13** with wild type BACE1 were carried out by mixing the solution of the

protein−ligand complex with an equal volume of a precipitant solution, 1.0 M ammonium sulfate/100 mM sodium citrate, pH 5.0. The protein−ligand complex was prepared by adding the compound to the protein solution to reach a final concentration of 1 mM ligand. Crystallization of apo mutated BACE1 was performed by mixing equal volumes of the protein stock solution and the precipitant (1.7 M Li_2SO_4/100 mM HEPES, pH 7.5). To obtain a crystalline complex with compounds **2**, **5**, and **11**, a 100 mM solution of the compound in DMSO was diluted 20-fold, to 5 mM, in the precipitant solution so as to generate a soaking drop. The crystal was transferred into the soaking drop and left for 24 h prior to data collection. All the crystallization utilized the vapor diffusion method in hanging drops. The perfluoropolyether, PFO-X175/08 (Hampton Research), was used as cryoprotectant for all the crystals.

Structure Determination and Refinement. Data were collected at 100 K on beamline BL17U (at wavelength 0.9793 Å) at the Shanghai Synchrotron Radiation Facility (SSRF) (Shanghai, China) for the soaked and the cocrystallized structures. The data were processed with the HKL2000[33] and XDS[34] software packages, and the structures were then solved by molecular replacement, using the CCP4 program MOLREP.[35] The search model used for the crystals was the apo wild type BACE1 structure (pdb code 3tpl). The structures were refined using the CCP4 program REFMAC5[35] combined with the simulated-annealing protocol implemented in the program PHENIX.[36] With the aid of the program Coot,[37] ligand, water molecules, and others were fitted into the initial F_o-F_c maps. The complete statistics, as well as the quality of the solved structures, are shown in Table S1.

Pharmacology and Biology. *Aspartic Protease Enzymatic Assays.* In vitro assays to determine inhibitory activity against BACE1, cathepsin D and cathepsin E were carried out as described.[3] Enzymes BACE1 (R&D Systems, Minneapolis, MN), cathepsin D (Enzo Life Sciences, Farmingdale, NY) and cathepsin E (R&D systems) and corresponding substrates were prepared freshly in their respective reaction buffers. The reaction buffer for BACE1 contained 100 mM NaOAc (pH 4.0) and the reaction buffer for cathepsins D and E contained 100 mM NaOAc/100 mM NaCl (pH 3.5). 1% DMSO was added into the buffer before use. Enzymes were added (final concentrations are 30 μg/mL for BACE1, 2 μg/mL for cathepsin D, 0.05 μg/mL for cathepsin E) followed by compounds dissolved in DMSO at 12 different concentrations (3-fold dilutions starting from 10 μM final assay concentration, up to ≤2% DMSO). Subsequently, substrates (final concentration of 10 μM) were added to initiate the reaction (substrate for BACE1: Mca-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Arg-Lys(Dnp)-Arg-Arg-NH_2 (R&D Systems); substrate for cathepsins D and E: Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-(Dnp)-D-Arg-NH_2 (Enzo Life Sciences); Mca = (7-methoxycoumarin-4-yl)acetyl; Dnp = 2,4-dinitrophenyl). The enzyme activities were detected as a time-course measurement of the increase in fluorescence signal (Ex 320 nm, Em 405 nm) from fluorescently labeled peptide substrate for 120 min at room temperature.

HPLAP-APP Reporter Assay.[19] In a 96-well tissue culture plate, stably transfected H4 (human neuroglioma) cells were seeded at a density of 2×10^4 cells/well in 100 μL of Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with fetal calf serum to final concentration of 10% and allowed to attach for approximately 6 h at 37 °C. Following the incubation period, the cells were washed with 100 μL of serum free media (Ultraculture, Lonza), and 100 μL of fresh serum free media was added to the cells which contained either test compounds, β-secretase inhibitor II or IV (Calbiochem) or the appropriate vehicle controls. Transfected cells were simultaneously treated with BB-94 (Calbiochem) and the individual compounds at 10 μM. Incubation was allowed to proceed for 16 h at 37 °C and samples were assayed for HPLAP activity to quantitate sAPPβ. Specifically, 50 μL of culture medium was transferred to a new 96-well assay plate and mixed with an equal volume of HPLAP assay solution containing 1 mM $MgCl_2$, 10 mM L-homoarginine, and 1 M diethanolamine, pH 9.8. It was empirically determined that heating the samples to 65 °C for 30 min to inhibit other phosphatase activities was not necessary. The reaction was started by the addition of 5 μL of 200 mM *para*-nitrophenyl phosphate (pNPP). Absorbance was read at 410 nm at 1 min intervals to determine pNPP hydrolysis rate to *para*-nitrophenol, which was expressed as an increase in absorbance units per minute. For ease of analysis, a single time point within the linear range of the reaction was selected for the data analysis. Statistical analysis was by one-way Anova followed by Dunnett's multiple comparison test.

CTF Analysis. H4 cells were plated at 3×10^5 per well in six well dishes and allowed to adhere for 4−6 h in DMEM supplemented with 10% fetal calf serum at 37 °C. The medium was replaced with Ultraculture (Lonza), to which test compounds were added to a final concentration of 10 μM and the incubation was allowed to proceed for an additional 16 h. The medium was removed, the cells were washed twice with phosphate buffered saline (PBS) and after the removal of the last wash, 50 μL of phospholysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate) was added to the center of the plate to initiate lysis. Using a cell scraper, the buffer was spread across the plate, and the resulting lysate was transferred to a 1.5-mL microcentrifuge tube. Nuclei were removed by pelleting in a microfuge for 30 s with the supernatant transferred to a fresh tube. Protein concentrations were determined by the bicinchoninic acid (BCA) method according to the manufacturer's instructions (Thermo Scientific). The samples were equated for protein concentration and diluted into 4× tricine sodium dodecyl sulfate sample buffer, denatured by heating at 95 °C for 5 min, and electrophoresed on 10−20% tricine polyacrylamide gels (BioRad). The proteins were transferred onto PVDF and blocked overnight. The blots were incubated with A8717, a rabbit polyclonal antibody, to the last 20 amino acids of APP at a 1:2000 dilution (Sigma). This was followed by incubation with a goat-anti rabbit IR antibody at a 1:20000 dilution. Blots were developed using the LiCor Odyssey Imager.

Secreted β-Amyloid (Aβ) Assay. CHO 2B7 cells (clonal expression of APP695 wt) were plated in 96-well plates in Opti-MEM with 1% serum. At 90% confluency cells were treated with positive controls (10 μM BACE1 inhibitor IV, 10 μM BACE1 inhibitor II, 1 μM LY411575 γ-secretase inhibitor) or test compounds (1% DMSO final) and incubated for 16 h. Supernatants were collected and analyzed by direct sandwich Aβ ELISA. Compound-treated media were captured onto ELISA plates with immobilized c-terminal specific Mabs to Aβ1−40 (13.1.1) or Aβ1−42 (2.1.3) and then detected with Aβ1−16 HRP-conjugated Mab (AB5-HRP). Total Aβ was measured with AB5 and detected with 4G8-HRP. ELISAs were developed with 3,3′,5,5′-tetramethylbenzidine (TMB), stopped with *o*-phosphoric acid solution, and absorbance was measured at 562 nm on a Molecular Devices Flexstation.

Measurement of Cellular γ-Secretase Inhibition. H4 cells overexpressing the Bri-C99 fusion protein were cultured in 12-well plates (400000/well) in Opti-MEM media with 1% serum and treated for 16 h with BACE1 inhibitor IV (1 μM), LY411575 γ-secretase inhibitor (100 nM) or test compounds (10 μM). Cells were lysed in TBS with 1% Triton X100 detergent and loaded onto a 12% bis-tris gel. Proteins were blotted onto sequencing grade 0.2 μm PVDF, probed with CT-20 antibody (Sigma 8717), scanned and analyzed on LiCor Odyssey.

BBB PAMPA. Permeability was assayed according to the manufacturer's instruction (pION Inc., Woburn, MA). In brief, 10 μM of the test samples were prepared in an aqueous buffer containing 2.5% Prisma HT buffer (P/N 110151), 5% acetonitrile and 0.5% DMSO, with pH of 5.0 and 7.4. Aliquots of the prepared test samples were added to donor wells of the PAMPA sandwich (P/N 110163). Immobilized filters of the acceptor plate were coated with 5 μL of pION artificial membrane forming BBB-1 lipid solution (P/N 110672) and the acceptor wells were filled with the Brain Sink Buffer (P/N 110674). The donor and acceptor plates were assembled to create the sandwich and left to incubate for 4 h under a humidified environment at room temperature. The absorbance of the donor, acceptor, and reference wells were scanned at 250−350 nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale CA). The permeability coefficient P_e was calculated using the PAMPA Explorer Command Software version 3.5 from pION. Assays were conducted in triplicate and results are expressed as mean ± SD. Theophylline and

corticosterone were used as controls for low and high BBB permeability, respectively.

Acute Treatment Study in CF-1 Mice. All compounds were prepared in a vehicle of 5% DMSO, 15% Solutol HS 15 (BASF, Ludwigshafen, Germany), 10% EtOH and 65% water. CF-1 mice (Charles River Laboratories, Wilmington, MA) were used at 8 weeks of age. Mice (25−35 g body weight) were administered either 10 mg/kg of LY411575 or 30 mg/kg of **12** and **13** by intraperitoneal injection, and euthanized by CO_2 asphyxiation 3 h postdosing. After extraction of the brain, olfactory bulbs and hindbrain were removed and the cerebral hemispheres were cut into two pieces for Aβ analysis. The cerebellum was removed for metabolite analysis. All tissue samples were weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis. Aβ analysis was carried out essentially as previously described with antibody 13.1.1 used for the analysis of rodent Aβ40.[38] All procedures were conducted according to NIH guidelines for animal care and approved by the University of Florida Institutional Animal Care and Use Committees.

Preparation of Plasma Samples. Whole blood was collected by cardiac puncture using a 22 gauge needle attached to a 1 mL syringe, as described.[39] Briefly, the sample was transferred to a 1.3 mL microtube containing potassium EDTA and placed on ice after gentle inversion 8−10 times to mix the anticoagulant additive with the blood. Tubes were maintained upright on ice until centrifugation within 30 min of collection. The samples were centrifuged (4−10 °C) for 20 min at 1300*g*. After centrifugation the plasma layer was removed and aliquoted into fresh microfuge tubes, flash frozen, and placed at −80 °C until needed.

Analytical Chemistry. *Materials.* HPLC-MS was done on a 3200 QTRAP (Applied Biosystems) equipped with a Shimadzu UFLC System. Mouse serum (S7273) was purchased from Sigma Aldrich.

Sample Preparation. Stock solutions (1 mg/mL) of **12**, **13**, **51**, and **52** were prepared in absolute EtOH. Aliquots were diluted in MeOH to give a 40 μg/mL solution and standard solutions with concentration of 25, 12.5, 2.5, 1.25, 0.25, 0.125 μg/mL were prepared from the serial dilution of the 40 μg/mL working solution. Stock solution (1 mg/mL) of the internal standard compound **8** was prepared in absolute ethanol, which was subsequently used to prepare a 100 μg/mL solution in MeOH. An aliquot of the 100 μg/mL solution of compound **8** was diluted with EtOAc to give the working internal standard solution (100 ng/mL).

*In Vitro Plasma Stability of **12** and **13**.* To evaluate the in vitro plasma stability of **12** and **13** and to monitor the formation of the corresponding acid metabolites **51** and **52**, 10 μL of the 25 μg/mL solution of **12** and **13** were separately incubated at 37 °C with 100 μL of mouse serum for the following durations: 0, 1, 2, 4, 8, 12, and 24 h. Reactions were quenched and metabolites were extracted at the end of the incubation period by adding 400 μL of EtOAc and followed by 200 μL of **8** in EtOAc (100 ng/mL). The reaction mixtures were left to shake on a plate mixer (750 rpm) for 5 min at room temperature and then further centrifuged at 16000*g* for 10 min. The EtOAc layer was collected, dried under nitrogen and reconstituted in 50 μL of MeOH.

*In Vivo Plasma Levels of **12** and **13**.* 100 μL-aliquots of plasma sample from test animals treated with **12** and **13** for 3 h (i.p. injection, see above) were extracted with EtOAc and spiked with the internal standard (**8**) using the same procedure as for the in vitro plasma stability.

*In Vitro Cellular Stability of **12** and **13**.* H4 cell lysates were diluted with 0.1 M phosphate buffer (pH 7.4) to give a 1 mg/mL protein concentration and volume of 100 μL. To diluted cell lysates were added 10 μL of **12** and **13** (12.5 μg/mL) and incubated at 37 °C for 0, 1, 2, 4, 8, 12, and 24 h. Extraction of **12** and **13** as well as the corresponding acid metabolites **51** and **52** from H4 cell lysates were performed according to the same procedure for the in vitro plasma stability.

*In Vivo Brain Levels of **12** and **13**.* Cerebellum and cortex from test animals were homogenized in acetonitrile (1:10 w:v) spiked with the internal standard and incubated on ice for 10 min before centrifugation at 16000*g* (15 min at 4 °C). The supernatant was collected and the pellet was re-extracted with acetonitrile. Supernatants from both extraction steps were pooled, the acetonitrile evaporated under nitrogen and further partitioned with ethyl acetate. The organic layer was collected, dried and reconstituted in 50 μL of MeOH. A 10 μL-aliquot was injected for HPLC-MS analysis.

*HPLC-MS Monitoring of **12** and **13** and Corresponding Acid Metabolites **51** and **52**.* Analysis of unmetabolized **12** and **13** and their corresponding acid products in plasma and cell lysates was done using HPLC-MS with multiple reaction monitoring (MRM) in negative ionization. A 10 μL-aliquot of the reconstituted methanolic solution was injected and chromatographic separation was done on an Onyx C18 monolithic column (Phenomenex, 3.0 × 100 mm) using a stepwise gradient of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in MeOH (solvent B). Elution was started using 70% solvent B and increased to 100% solvent B after 5 min and further maintained in 100% solvent B for another 10 min. The retention times and corresponding MRM pair (t_R, min; MRM) for the compounds are as follows: **12** (3.2; 1008.4/492.1), **51** (2.8; 994.4/492.1); **13** (3.4; 1022.4/506.2), **52** (3.1; 1008.4/506.2), **8** (2.2; 949.5/447.3). Compound- and solvent-dependent parameters were initially optimized using syringe infusion. Compound-dependent parameters were as follows. **12**: declustering potential (DP) −115, entrance potential (EP) −9.0, collision energy (CE) −59, collision exit potential (CXP) −15, collision entrance potential (CEP) −43; **51**: DP −95, EP −8.0, CE −70, CXP −15, CEP −8; 4−67: DP −140, EP −9.0, CE −38, CXP −12, CEP −46; **52**: DP −125, EP −10, CE −72, CXP −4.0, CEP −52; **8**: DP −80, EP −7.0, CE −72, CXP − 7.0, CEP −43. Solvent-dependent parameters were as follows: CUR 30, CAD Medium, IS −4500, TEM 450, GS1 60, GS2 60.

For brain analysis (cerebellum and cortex), monitoring for **12** was performed according to the procedure for plasma stability. Compound **13** was monitored in the positive ionization mode using the same chromatographic condition and using **8** as internal standard. The retention times and corresponding MRM pair (t_R, min; MRM) by positive ionization for **8** and **13** were as follows: **13** (3.4; 1024.4/895.1), **8** (2.2; 951.5/675.7). Compound-dependent parameters were as follows: **13**: DP 80, EP 9.0, CE 24, CXP 42, CEP 30; **8**: DP 95 EP 10, CE 37, CXP 34, CEP 30. Solvent dependent-parameters were as follows: CUR 30, CAD Medium, IS 5000, TEM 450, GS1 60, GS2 60.

*Quantitation of **12** and **13** and Corresponding Metabolites **51** and **52**.* Calibration curves for **12**, **51**, **13** and **52** were generated by least-squares linear regression analysis of standard solutions. Analyte peak area and internal standard peak area ratio was plotted against the analyte concentration and internal standard concentration ratio, and the concentrations of **12**, **13** and corresponding acids **51** and **52** at each time point in the in vitro plasma and cellular experiments and in vivo plasma experiment were determined through interpolation. All calculations were done using Analyst 1.4.2 Quantitation Mode.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Preparation of building blocks **22**, **23**, **26**, **27**, **29**, **30**, and **34**−**36**, copies of ^{1}H NMR and ^{13}C NMR of compounds **5**−**16**, Supplementary Figure S1 with LIGPLOT representations, Figure S2 with structures of control secretase inhibitors, Tables S1 and S2 with X-ray data collection/refinement statistics and hydrophobic interactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Ac_2O, acetic anhydride; Aβ, β-amyloid; BI−II, BACE1 inhibitor II; BI−IV, BACE1 inhibitor IV; BBB, blood−brain barrier; BCA, bicinchoninic acid; CAD, collision activated dissociation; CE, collision energy; CTF, carboxyl terminal fragment; CEP, collision entrance potential; CXP, collision exit potential; CUR, curtain gas; DIEA, *N*,*N*-diisopropylethylamine; DMEM, Dulbecco's Modified Eagle's medium; DP, declustering potential; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; EP, entrance potential; GS1, gas 1; GS2, gas 2; HATU, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HIs, hydrophobic interactions; HOAt, 1-hydroxy-7-azabenzotriazole; HPLAP, human placental alkaline phosphatase; HRP, horseradish peroxidase; i.p., intraperitoneal; IS, ionspray voltage; MRM, multiple reaction monitoring; PAMPA, parallel artificial membrane permeability assay; pNPP, *para*-nitrophenyl phosphate; PVDF, polyvinylidene fluoride; PyAOP, (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; sAPPβ, secreted APP ectodomain; sAPP, secreted APP; SSRF, Shanghai Synchrotron Radiation Facility; TEM, temperature; TMB, 3,3′,5,5′-tetramethylbenzidine

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