

Phe*-Ala-based pentapeptide mimetics are BACE inhibitors: P2 and P3 SAR

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Abstract—We describe herein the syntheses and evaluation of a series of C-termini pyridyl containing Phe*-Ala-based BACE inhibitors (**5–19**). In conjunction with four fixed residues at the P1 (Phe), P1' (Ala), P2' (Val), and P2' cap (Pyr.), rather detailed SAR modifications at P2 and P3 positions were pursued. The promising inhibitors emerging from this SAR investigation, **12** and **17** demonstrated very good enzyme potency ($IC_{50} = 45$ nM) and cellular activity ($IC_{50} = 0.4$ μ M).

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With increasing life expectancy of the population and a prevalence of 5.5% in the age group above 60 years of age, Alzheimer's disease (AD) will become a major social and financial burden for the coming decades.¹ The Alzheimer's Association has estimated that as many as 14 million Americans could be affected by AD in year 2005. The currently available therapy for AD is limited only to cholinesterase inhibitors, a new class of drugs that are quite effective in reducing the symptoms, but are not capable of curing or stopping AD progression.² In light of this situation, scientists around the world are confronting AD from several angles including identifying small molecules that can slow down the production and accumulation of the β -amyloid peptide ($A\beta$) in the brain.^{3,4} The rationale for this approach is based on the fact that a gradual and chronic imbalance between production and clearance of $A\beta(40/42)$ peptides results in its build-up in the brain. This $A\beta$ accumulation process leads to subsequent aggregation ($A\beta_{42}$)⁵ and plaque formation,⁶ thereafter the phenotype of Alzheimer's disease. Since $A\beta(40/42)$ were produced by two sequential protease cleavages (β -secretase and γ -secretase)^{7,8} of the transmembrane protein β -amyloid precursor protein (APP), thus inhibition of β -secretase (BACE) activity⁹ could in principle slow down $A\beta$ formation and thereby progression of Alzheimer's disease.

Guided by this amyloid hypothesis, several structural types of BACE inhibitors have been reported recently including the polar termini bearing octapeptides such as **1** ($K_i = 1.6$ nM)¹⁰ and **2** ($K_i = 49$ nM)¹¹ as shown in Figure 1. Unfortunately, however, owing to their poor membrane permeability, these highly polar inhibitors failed to show any cellular activity. To address the polarity issue mentioned above, Tang's group reported a neutral series of pentapeptidyl Leu*-Ala-based inhibitors including compound **3** with excellent enzyme potency ($K_i = 2.5$ nM).¹² In light of Tang's finding, we launched systematic SAR investigations surrounding Phe*-Ala based pentapeptides such as **4**. Biological evaluation of **4**, a P1-Phe containing derivative of **3** (Leu as P1), revealed that there is a poor correlation between its enzyme inhibitory effect ($IC_{50} = 28$ nM) and its cellular activity ($IC_{50} = 22$ μ M). We hypothesized that the poor whole cell activity displayed by **4** might be attributed to its poor aqueous solubility. To prove this hypothesis, we carried out rather extensive C-termini modifications,¹³ and found that replacement of the C-termini phenyl moiety with a pyridine ring led to inhibitor **5** possessing greatly improved aqueous solubility and whole cell activity ($>10\times$) relative to **4**.¹³ To take advantage of these exciting results, we decided to keep P1, P1' and P2' residues constant and perform further P2 and P3 modifications on **5** with a goal of identifying more potent BACE inhibitors. In this communication, we describe the synthesis and evaluation of these novel BACE inhibitors (**6–19**).

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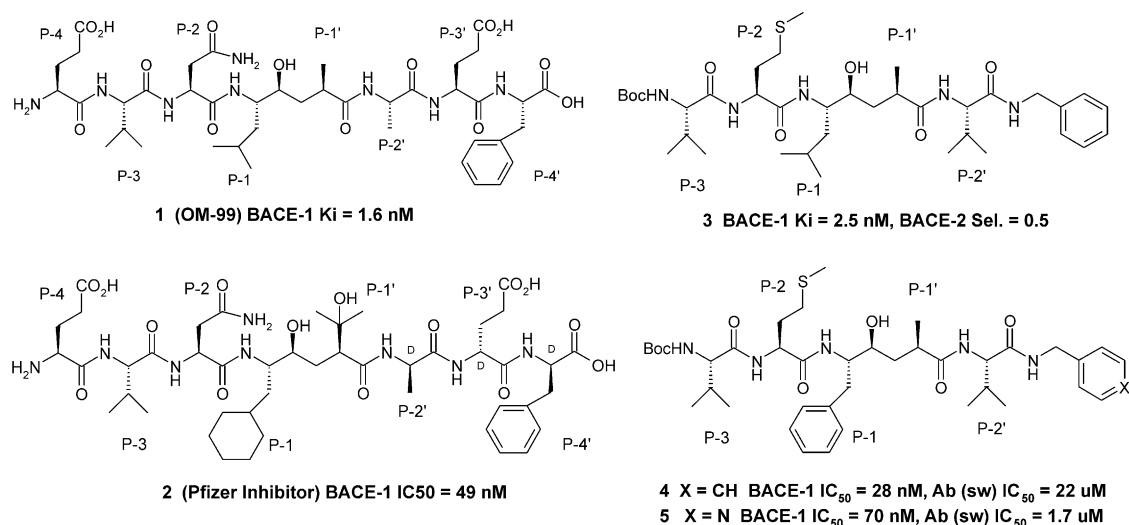
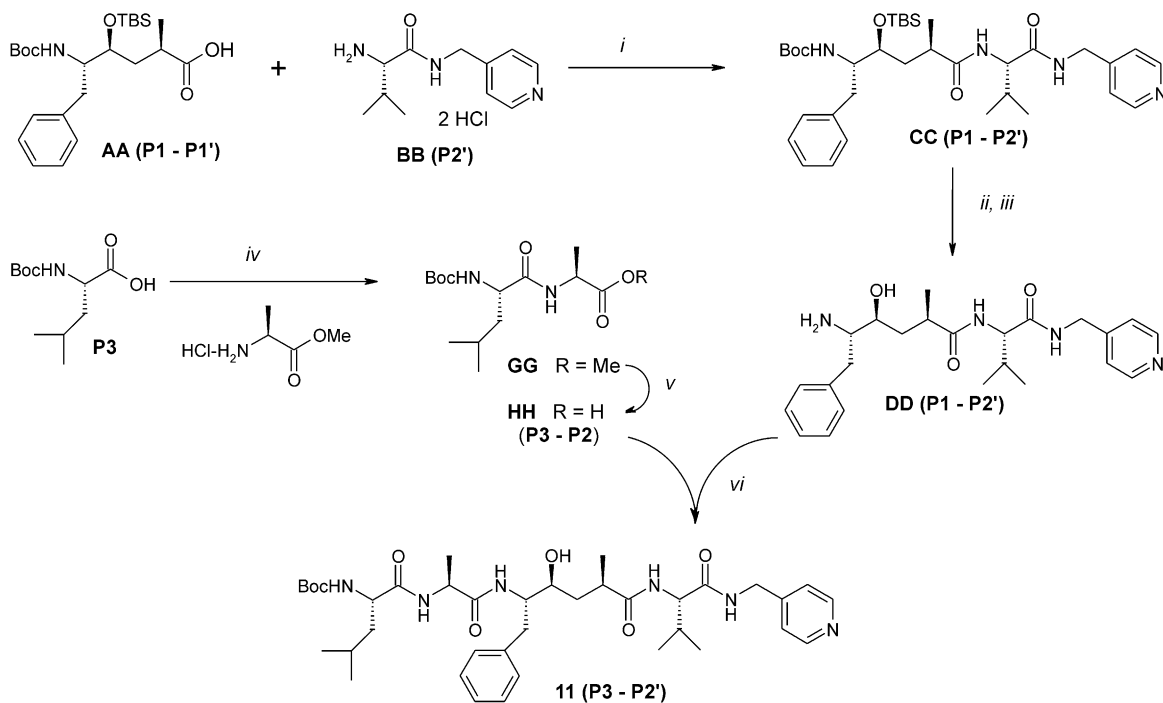


Figure 1. Representative BACE inhibitors.



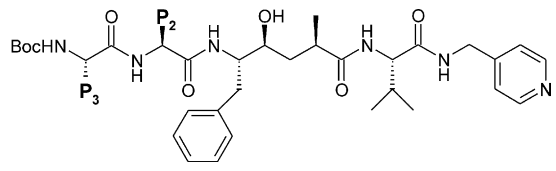
Scheme 1. Synthesis of Phe*-Ala bearing BACE inhibitor **11**. Reagents and conditions: (i) HOBt/EDCI/THF, 87%; (ii) TBAF/THF, 90%; (iii) 4N HCl/dioxane, 95%; (iv) PyBOP/CH₂Cl₂/*i*-Pr₂EtN, 66%; (v) 2N NaOH/MeOH, 77%; (vi) HOAt/EDCI/THF/*i*-Pr₂EtN, 42%.

The general synthetic route employed for the preparation of pentapeptide inhibitors discussed in this manuscript is exemplified for inhibitor **11** as shown in Scheme 1. HOBt/EDCI mediated coupling¹⁴ of the known P1–P1' acid **AA**^{12,15} with the P2'-Val derivative **BB** [itself prepared in 65% via two steps from Boc-valine and 4-(aminomethyl)pyridine] provided the adduct **CC**, which was further converted to its corresponding free amine **DD** via standard *N*-deprotection. Subsequent coupling of **DD** with the P3–P2 acid **HH** (obtained in 51% via a two-step sequence from Boc-Leu and Ala-OMe-HCl) afforded the desired product **11** in modest yield.

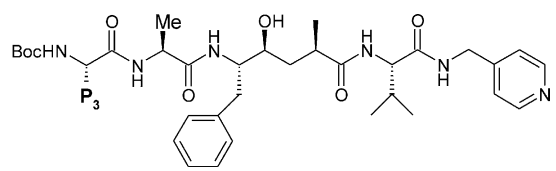
Following essentially identical procedures as described for compound **11**, all other BACE inhibitors listed in

Tables 1–3 were obtained via coupling of **DD** (P1–P2') with various requisite P3–P2 dipeptidyl acids **HH**. It should be pointed out that the synthetic procedures used for the preparation of P2 residues of **13**,¹⁶ **14**,¹⁷ and **17**¹⁸ are included in the Reference section. The structures of all BACE inhibitors were confirmed on the basis of their respective proton NMR and mass spectra analyses.

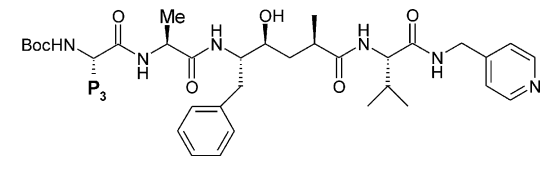
All pentapeptidyl BACE inhibitors synthesized were evaluated in the following assays: (1) *in vitro* homogenous BACE FRET (Fluorescent Resonance Energy Transfer) enzyme assay,¹⁹ and (2) whole cell Aβ lowering assay in HEK293/APP751swe cells.²⁰ A few representative inhibitors were also tested in the S³⁵

Table 1. P2 SAR trends


Compd	P3	P2	BACE-1 IC ₅₀ (nM)	Abeta (sw) IC ₅₀ (μM)
5'	CHMe ₂	CH ₂ CH ₂ OMe	127	2.9
5	CHMe ₂	CH ₂ CH ₂ SMe	70	1.7
6	CHMe ₂	Me	82	2.1
7	CHMe ₂	Me (D-isomer)	1058	48
8	<i>n</i> -Pr	CH ₂ CH ₂ SMe	60	0.99
9	<i>n</i> -Pr	Me	41	1.4

Table 2. P3 SAR trends


Compd	P3	BACE-1 IC ₅₀ (nM)	Abeta (sw) IC ₅₀ (μM)
6	CHMe ₂	82	2.1
9	CH ₂ CH ₂ CH ₃	41	1.4
10	CH ₂ CH ₃	75	2.8
11	CH ₂ CHMe ₂	63	0.86
12	(s)-MeCHEt (Ile)	42	0.41
13	CH ₂ NMe ₂	2.53 μM	–
14	CH ₂ CH ₂ NMe ₂	89 μM	–
15	CH ₂ OMe	118	4.99
16	CH ₂ CH ₂ OMe	705	–

Table 3. Fluorination effect at P3


Compd	P3	BACE-1 IC ₅₀ (nM)	Abeta (sw) IC ₅₀ (μM)
10	CH ₂ CH ₃	75	2.8
17	CH ₂ CHF ₂ (diFABu)	45	0.40
18	(rac)-CH ₂ CF ₂ CHF ₂	281	1.62
19	(rac)-(CH ₂) ₃ CF ₃	419	13.4

Methionine incorporation assay to check the effect of inhibitor treatment on the protein synthesis.²¹

1. P-2 SAR

Four new P2 modified analogues were prepared and evaluated in the enzyme and in vitro whole cell assays. Careful inspection of the data shown in Table 1 reveals the following SAR trends: (1) when compared with their P2-Met bearing analogues 5 and 8, the corresponding

P2-Ala containing counterparts 6 and 9 showed similar enzyme inhibitory activity as well as cellular activity; (2) comparison of the enzyme potencies displayed by inhibitors 6 and 7 revealed that L-Ala is preferred (> 12×) over D-Ala at the P2 pocket; (3) the most potent inhibitor within P2 SAR, inhibitor 9 demonstrated impressive whole cell activity with IC₅₀ value less than 1 μM; and (4) replacing the 'S' atom at P2 Met in 5 with an 'O' atom as seen in 5' resulted in slight reduction of biological activity in both the enzyme and whole cell assays.

2. P-3 SAR

Replacement of the P3 Val in 6 with other non-polar aliphatic residues (Nva, Abu, Leu and Ile) led to four P3 modified analogues 9–12. In addition, two P3 basic amine bearing and P3-methyl Ser as well as homomethyl Ser containing inhibitors 13–16 were also prepared in order to improve biopharmaceutical properties (e.g., aqueous solubility). After careful analysis of the data shown in Table 2, we discovered the following trends: (1) consistent with the published BACE subsite specificity,^{7f} five P3 non-polar residue bearing inhibitors (6 and 9–12) exhibited superior enzyme and whole cell activities to that obtained with the P3 polar moiety bearing counterparts such as 15 and 16, which in turn showed better activity than the P3 basic residue containing ones such as 13 and 14; (2) inhibitors with heteroatom (O or N) linked through α-carbon at P3 (e.g., 13 and 15) showed improved enzyme inhibitory activities than those containing heteroatom linked through β-carbon at P3 (e.g., 14 and 16); (3) P3 Leu and Ile containing compounds 11 and 12 were found to be the most promising inhibitors with whole cell IC₅₀ values of 0.86 and 0.41 μM, respectively.

Incorporation of fluorine in P3 resulted in three analogues 17–19. Based on the data obtained for the P3-Abu bearing analogue 10 and its corresponding P3-diFABu counterpart 17, it is clear that P3 fluorination (for 17) resulted in 2- or 6-fold improvement in enzyme and whole cell activity, respectively. Further P3 side-chain elongation (based on 17) led to inhibitors 18 (by one carbon) and 19 (by two carbons). When compared with 17, the P3 tetrafluoro-Nva bearing inhibitor 18 showed ~2-fold reduction (after adjustment for P3 chirality) in enzyme and whole cell activity. Likewise, inhibitor 19 was found to be ~5-fold less potent in the enzyme assay relative to the P3-diFABu bearing inhibitor 17.

3. Cytotoxicity assessment

A few selected BACE inhibitors were evaluated in the S³⁵-methionine incorporation assay in HEK293/APP751swe cells to measure the effect of compound treatment on protein synthesis. In this event, inhibitors 8, 11, and 12 (see Tables 1 and 2 for structures) were tested and found to be non-toxic at 100 μM. Thus, the TC₅₀ values determined for all three compounds were at least >100-fold high than their respective whole cell

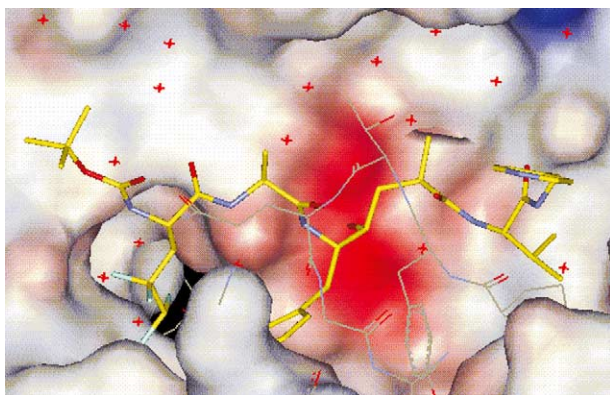


Figure 2. Crystal structure of the (S)-18 binding with BACE-1.

IC_{50} values, indicating that the whole cell activities obtained with these inhibitors are reliable.

4. Enzyme–inhibitor interaction

The co-crystal structure of the P3 (S)-isomer of inhibitor **18** with hu-BACE enzyme was obtained using standard protocol.^{22,23} As can be seen from **Figure 2**, the inhibitor side chains were positioned at the expected binding pockets, with the P3-tetrafluoroNva occupying the S3 pocket. The types of interactions made by the N-termini Boc as well as the C-termini pyridyl moieties suggest that these functional groups may be modified without compromising enzyme inhibitory activity. It is also interesting to point out that the more potent (S)-isomer [relative to its (R)-isomer at P3] was preferentially co-crystallized with the enzyme target.

Beginning with the C-termini Val-Pyridine bearing inhibitor **5** ($A\beta$ IC_{50} = 1.7 μ M),¹³ we have designed and synthesized, on the basis of BACE substrate specificity, a novel series of C-termini pyridyl bearing Phe*-Ala based inhibitors endowed with good water solubility. As a result of systematic P2 and P3 modifications, we found that replacement of the Val-Met (P3–P2) sequence as seen in **5** with Ile-Ala (P3–P2) led to inhibitor **12** ($A\beta$ IC_{50} = 0.41 μ M) displaying improved enzyme (2 \times) and whole cell activity (4 \times) as well as reduced molecular weight in comparison to inhibitor **5**. Furthermore, our data also shows that fluorination at the P3 residue such as diFABu seen in **17** ($A\beta$ IC_{50} = 0.40 μ M) resulted in 6 \times enhancement of the whole cell activity relative to its P3 non-fluorinated counterpart **10**. In summary, in contrast to the poor cellular activity found with the pentapeptidyl BACE inhibitors reported by Tang's group,¹² we have discovered a novel Val-Pyridine C-termini bearing inhibitors endowed with impressive enzyme as well as whole cell activity (<0.5 μ M).

Acknowledgements

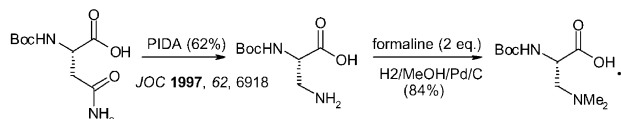
We are indebted to L. Patterson, E. E. Bunel for providing valuable fluorinated amino acid and Dr. J.

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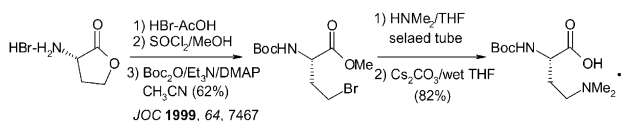
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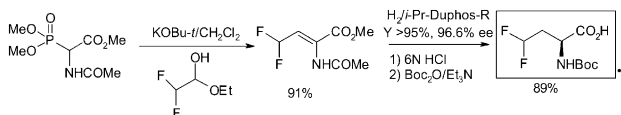
14. cf. Carpino, L. A.; El-Faham, A. *Tetrahedron* **1999**, 55, 6813.
15. AA was prepared, according to the route outlined in ref 12, from the known gamma-lactone reported by Fray, A. H.; Kaye, R. L.; Kleinman, E. F. (c.f. *J. Org. Chem.* **1986**, 51, 4828).
16. This amino acid was prepared according to the following route:



17. This amino acid was synthesized via five-step sequence shown below:



18. *N*-Boc protected diFluoroAbu was prepared according to the following sequence:



19. BACE FRET assay uses 20 nM purified recombinant human BACE/Fc. The standard assay format contains

100 μM ELGY-9 (an aminobenzoate based FRET peptide containing Swedish mutation) in 50 mM ammonium acetate, pH 4.6, 1 mg/mL BSA and 1 mM Triton X-100 at room temperature for 4 h. The enzyme activity is determined by an increase in the relative fluorescent of reaction mixture, with umbilliferone excitation/emission filter set.

20. Whole cell Aβ lowering assay measures intracellular inhibition of endogenous BACE expression in HEK293 (Human Embryonic Kidney) cells by BACE inhibitors. This cell line stably overexpresses APP751swe substrate. Testing subjects are incubated with cells for 4 h at 37 °C and 5% CO₂. The conditioned media are removed from the culture wells. The Aβ ELISA is used to measure amount of Aβ(total) peptide produced during the incubation time.
21. S³⁵-Methionine assay is conducted with 35,000 cells per well in 96-well plate in DMEM containing 10% FBS. Compounds are incubated for 5 or 24 h at 37 °C and 5% CO₂. The cell pallets are lysed and radioactivity of S³⁵ is counted.
22. Crystals of the BACE catalytic domain were grown in the presence of a 5-fold molar excess of inhibitor (rac)-**18** using minor modifications of previously described methods cited in ref 23. Diffraction data to 2.05 Å resolution were collected at 100 K using synchrotron beamline 17ID at Argonne National Laboratory. The (s)-**18** model was fit into well-defined active site electron density using QUANTA (Accelrys, Inc) and O (Jones et al. *Acta Crystallogr.* **1991**, A47 110), and the structure was refined to a crystallographic *R*-factor of 0.194 (*R*_{free} = 0.222) using CNX (Accelrys, Inc).
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