Journal of Medicinal Chemistry

BRIEF ARTICLE

pubs.acs.org/jmc

On a Possible Neutral Charge State for the Catalytic Dyad in β-Secretase When Bound to Hydroxyethylene Transition State Analogue Inhibitors†

Fredy Sussman,*[,‡] José M. Otero,[§] M. Carmen Villaverde,[‡] Marian Castro,[∥] José L. Domínguez,[‡] Lucía González-Louro,[‡,§] Ramón J. Estévez,[§] and J. Carlos Estévez*[,§]

[‡]Molecular Modeling Research Group, Departamento de Química Orgánica, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[§]Carbohydrate Research Group, Departamento de Química Orgánica, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[∥]Departamento de Farmacología, Instituto de Farmacia Industrial, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

Supporting Information

ABSTRACT: β-Secretase is one of the aspartic proteases involved in the formation of amyloid plaques in Alzheimer's disease patients. Our previous results using a combination of surface plasmon resonance experiments with molecular modeling calculations suggested that the Asp dyad in β-secretase bound to hydroxylethylene containing inhibitors adopts a neutral charged state. In this work, we show that the Asp dyad diprotonated state reproduced the binding ranking of a set of these inhibitors better than alternative protonation states.

INTRODUCTION

Alzheimer's disease (AD) is a widespread, neurodegenerative, dementia-inducing disorder, characterized by the formation of amyloid plaques in the brain.[1] The genesis of this construct is catalyzed by a tandem of two proteases identified as β- and γ-secretases.[1,2] It is known that the former enzyme (also referred to as BACE-1 and Memapsin 2) participates in the rate-limiting step of the hydrolytic process that leads to the APP fragments,[1−3] a fact that has converted β-secretase into a major target for drugs against Alzheimer's disease.[2,4,5]

There are several issues related to the computer-aided design of novel ligands that could enhance the success of in silico high throughput screening protocols but that have not been fully addressed yet. One of the most vital ones is the protonation state of the many buried acidic residues found in this protein, including the active site Asp dyad, which lends to this enzyme an optimal catalytic activity at low pH.[3]

There have been many attempts to predict the charged state of the active site Asp dyad by computational methods.[6−9] The results of those calculations support the hypothesis that the most favored Asp dyad charge state is the one that has only one of the Asp residues protonated. Nevertheless, quantum mechanical (QM) based calculations have left open the possibility that the Asp dyad might be neutral at the acidic optimal pH of the protein.[8]

Recently, we have determined the effect of the pH on the affinities of a set of inhibitors with a variety of chemical motifs to the ectodomain BACE-1 region by a surface plasmon resonance (SPR) biosensor based assay.[10] To understand the molecular interactions that underlie the diverse optimum pH for the binding of the various inhibitors as observed experimentally, we calculated the titration curves for a set of BACE-1 ligand complexes.[10] One of our most striking results of that work relates to the protonation state of peptidic inhibitors with hydroxylethylene (HE) based isosteres. For these inhibitors, our calculations predicted that the Asp dyad will be diprotonated at low pH, as opposed to all previous studies that pointed to a monoprotonated Asp dyad state.[10]

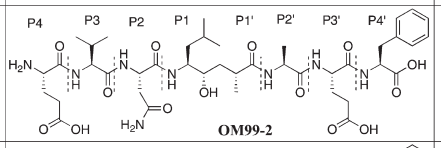
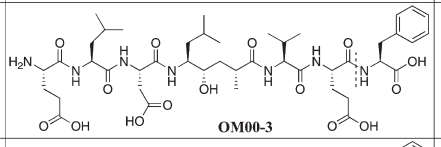
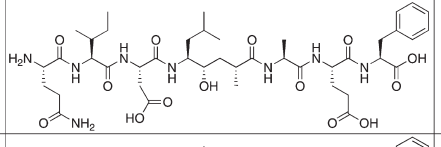
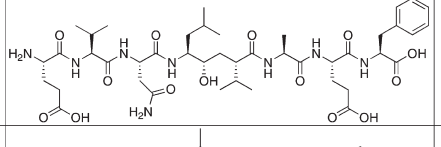
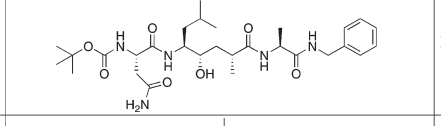
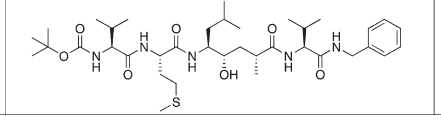
To further investigate this issue, in this work we have assembled a set of transition state analogue peptidic inhibitors with HE isosteres (see Table 1), some of which have been already synthesized and tested by other authors, while others have been produced and assayed by our group. Using molecular mechanics based protocols we show that the diprotonated state reproduced the binding ranking of our set of peptidic inhibitors better than the ones based on either of the monoprotoned Asp dyad bearing enzymes proposed by previous studies,[6−9] a result that provides further support to our enzyme's charge assignment when bound to this type of transition state analogue inhibitors. Finally, we discuss the proposed Asp dyad protonation state in relation to the possible existence of low barrier hydrogen bonds (LBHBs) in these systems.

RESULTS AND DISCUSSION

Chemistry. The δ-amino acid isosters of inhibitors **3** and **4** were prepared as shown in Scheme 1, through a methodology that allows the introduction of a variety of side chain groups at P1/P1′ using as a key intermediate the oxiranelactone **9**, easily

Received: October 18, 2010
Published: March 31, 2011

Table 1. Inhibitors Studied in This Work and Their Experimental BACE-1 Inhibitory Activity

Name	Structure	Inhibitory activity
1	 OM99-2	IC_{50}= 19.11 nM[a] Ki = 1.6 nM[b]
2	 OM00-3	Ki = 0.3 nM[c]
3		IC_{50}= 45.5 nM[a]
4		IC_{50}= 164.5 nM[a]
5		Ki = 22,423.0 nM[b]
6		Ki = 2.5 nM[b]

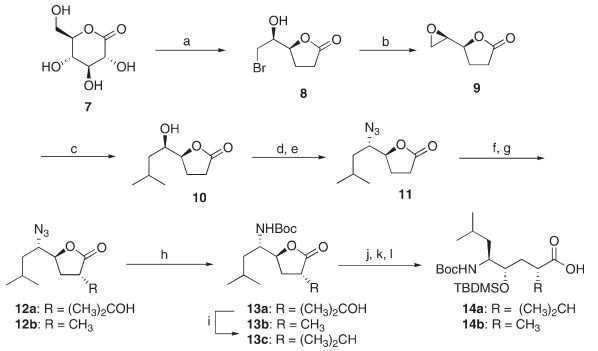
[a] See SI for details on IC_{50} evaluation. [b] Data from ref 17. [c] Data from ref 16.

prepared from D-glucono-1,5-lactone (**7**). Thus D-glucono-1,5-lactone (**7**) was transformed into lactone **8** by following a procedure described in the literature,[11] and this lactone, **8**, when treated in basic media with potassium carbonate in acetone, gave quantitatively the expected oxiranelactone **9**.

Key intermediate lactone **9** was first alkylated at C-6 when treated with isopropylmagnesium chloride to give compound **10** in 50% yield. The hydroxyl group in **10** was then transformed into the corresponding mesylate by treatment with methanesulfonyl chloride, followed by reaction with sodium azide to give azidolactone **11** in 89% yield. Alkylation of lactone **11** at C-2 position with LDA and subsequent addition of acetone afforded, in a 67% yield, compound **12a**, with all the functionality needed for the final isoster. To transform compound **12a** into the δ-amino acid isoster **14a**, compound **12a** was reduced at the azido position with hydrogen, using a mixture of palladium/carbon as catalyst, and the resulting amine treated in the reaction mixture with *tert*-butoxycarbonyl anhydride to give compound **13a** in 95% yield. Elimination of the alcohol group in **13a** when submitted to reaction with methyl chlorooxoacetate and triphenyltin hydride, using AIBN as starting radical reaction reactive, gave the expected compound **13c** in 70% yield. Finally compound **13c** was transformed into the isoster **14a** in a 76% yield by following a previously described procedure.[12]

The isoster **14b** was prepared by an easier version of the protocol that afforded compound **14a** because alkylation at position C-2 of the lactone can be done directly. Thus, the

Scheme 1. Total Synthesis of δ-Amino Acid Isosters 14a and 14b[a]



[a] Reagents and conditions: (a) as in ref 11; (b) K_2CO_3, acetone, rt, 20 h; (c) *i*PrMgCl, Li_2CuCl_4, THF, −78 °C, 30 min; (d) MsCl, pyridine, 0 °C, 5 h; (e) NaN_3, DMF, 80 °C, 12 h; (f) LDA, THF, −78 °C, 1 h; (g) acetone for **12a** and MeI for **12b**; (h) H_2 (1 atm), Pd−C (10%), $(Boc)_2O$, AcOEt, rt, 5 h; (i) $ClCOCO_2Me$, DMAP, Et_3N, THF, 0 °C, 1 h and then HPh_3Sn, AIBN, toluene, 110 °C, 3 h; (j) LiOH (1 M), DME, rt, 12 h; (k) ClTBDMS, imidazole, DMF, rt, 15 h; (l) MeOH, rt, 2 h.

intermediate lactone **11** was alkylated at C-2 position by treating it with LDA followed by methyl iodide to give **12b** in 70% yield. Azide **12b** was then reduced to amine with hydrogen, using palladium/carbon as catalyst, and the amine protected in the reaction medium with *tert*-butoxycarbonyl anhydride to give **13b** in 95% yield. Finally compound **13b** was transformed into the isoster **14b** in 82% yield following a described procedure.[12]

The above synthetic procedure for preparing the δ-amino acid isosters presents two advantages over previous approaches described in the literature. In the first place, the protocols presented here improve previous synthetic routes for the preparation of the oxirane intermediate **9** starting from a sugar.[13] Second, our synthetic strategy affords the insertion of any alkyl group at position C-5 in the lactone, a feature that will enable the synthesis of isosteres with a variety of side chain fragments beyond those that could be obtained from commercially available α-aminoacids.[14]

Binding Affinity Prediction. Table 1 lists the peptidic inhibitors studied here and their BACE-1 inhibitory activity expressed as IC_{50} and/or K_i values whenever available. This table also displays the partition into fragments for inhibitor **1** (OM99-2) following the rules of Schechter and Berger.[15] Having as a reference inhibitor **1**, compound **4** introduces an Ala → Val replacement in P1′. Studies on the substrate subsite specificity of BACE-1 by the group of J. Tang[16] indicate that the S1′ site is ill suited for bulky chains like those in Trp, Phe, or Val, the one that is found in the inhibitor **4**. Perusal of the IC_{50} values indicates that its presence increases the IC_{50} value (as compared to **1**) by 9-fold, in agreement with the predictions of the substrate fragment specificity.[16]

On the other hand, the ligand **3** introduces three aminoacid substitutions in the N-terminal segment of **1**: the Asn → Asp at P2, the Val → Ile at P3, and the Glu → Gln at P4. The substrate specificity studies predict that while Val → Ile replacement at P3 should increase the enzyme affinity, the Asn → Asp at P2 and the Glu → Gln at P4 modifications will decrease it.[16] In accordance with this line of thought, inhibitor **3** has a lower BACE-1 inhibitory activity (higher IC_{50} value) than **1**, indicating that the substitution at P3 does not compensate for the detrimental effect of the modifications at P2 and P4.

Finally, inhibitors **5** and **6** were originally the result of a second iteration drug design cycle aimed at reducing the size and molecular weight of BACE-1 inhibitors like **1** and **2** (OM00-3), based on the realization that in spite of the large size of this protein, the active site of BACE-1 is smaller than that of other aspartic protease enzymes.[17] While the parent compounds extended from fragment P4 to P4′, the analogues **5** and **6** cover a shorter span from either P4 to P3′ in **6**, or from P3 to P3′ in **5**. It was found that in agreement with the design premises, some of the shorter compounds were very potent BACE-1 inhibitors.[17]

As seen in Table 1, the experimental data obtained in this work using an in vitro peptide cleavage FRET assay, together with the data extracted from the literature,[16,17] point toward the following BACE-1 inhibitory activity:

$$\mathbf{2} > \mathbf{1} \sim \mathbf{6} > \mathbf{3} > \mathbf{4} > \mathbf{5}$$

One of the main aims of this work is to compare the ligand binding affinities generated by a diprotonated BACE-1 Asp dyad proposed in our recent work[10] with that of monoprotonated states proposed by other groups.[6−9] For this sake, we carried out a very careful and involved docking by homology, having as a template the X-ray structure of the complex between BACE-1 and inhibitor **1** (pdb code 1FKN).[18] Once the starting docked structures were obtained, we proceeded to calculate the pK_a values for all titratable residues by the protocol of Spassov and Yan[19] at pH 4.5, a value close to the optimal pH of the enzyme (see Supporting Information (SI) for details). These calculations produce titration curves that depict the degree of protonation for each ionogenic residue at every pH value. Our pK_a calculations indicate that the catalytic Asp dyad of all BACE-1/inhibitor complexes studied here will be neutral. To illustrate this issue, we display the titration curves for the Asp dyad residues (see Figure S1, SI) elicited by the presence of **1** and the shortest inhibitors **5** and **6**. As seen from this figure, both Asp residues have an almost constant protonation fraction close to 1, even at pH values around 7, indicating that the Asp dyad will remain neutral in the acidic medium optimum for the enzymatic activity. Moreover, the protonation fraction pH dependence graphs indicate that the Asp 32 has a higher pK_a value than Asp 228 and hence will remain protonated at higher pH.

To include the alternative monoprotonated states, we selected additional starting structures with single charged Asp dyads, predicted by previous studies.[7,8] To assess the effect of the monoprotonated Asp dyad on the charge state of the remainder titratable residues, we fixed the Asp dyad in a monoprotonated state (with the Asp 228 charged) and proceeded to recalculate the pK_a values of the remainder titratable residues, using the protocol described above, and in detail in the SI. The specific Asp dyad charge assignment for this calculation was based on the ionization proclivity (see Figure S1, SI). Our results indicate that the pK_a values of the other residues do not vary, implying that in this protocol the Asp dyad charge has a marginal effect on the titration of the other ionogenic residues (results not shown). The resulting structures with the protonation states that included monoprotonated and diprotonated catalytic dyads were used in our molecular mechanics (MM)/molecular dynamics (MD) protocol, aimed at generating binding poses for each protonation state. The final production stage of the MD protocol was used to calculate the binding affinity scoring function for each of the inhibitors listed in Table 1 (see SI for details).

Table 2 lists the binding scoring function values for the three Asp dyad protonation states studied here. The first column contains the scoring function values for the charge distributions calculated in this work, which include a neutral Asp dyad. The other columns list the scoring function for the enzyme with the Asp dyad in the monoprotonated states supported by previous calculations.[6−9] To assess the effect of the rest of the ionogenic residues, we carried out two simulations for the case where the Asp 32 is protonated. In the first one, we used the predicted pK_a values of the titratable residues other than the Asp dyad, and in the second one, we left those residues with their individual charge assignment in solution at pH 7.

Table 2. Binding Affinities Scoring Function Values Calculated for the Docked Poses with the Catalytic BACE-1 Asp Dyad in Three Different Protonation States[a]

inhibitor	AspH32/ AspH228[b]	AspH32/ Asp(−)228[b]	Asp(−)32/ AspH228[c]	AspH32/ Asp(−)228[c]
1 (OM99-2)	−95.5 ± 2.1	−89.2 ± 1.7	−91.8 ± 2.8	−80.9 ± 1.0
2 (OM00-3)	−99.1 ± 1.5	−91.6 ± 2.0	−84.8 ± 1.7	−77.7 ± 1.5
3	−95.7 ± 1.4	−98.0 ± 2.0	−90.1 ± 2.3	−98.4 ± 2.6
4	−95.2 ± 1.2	−97.0 ± 2.6	−101.9 ± 2.4	−93.9 ± 2.8
5	−75.0 ± 1.4	−76.6 ± 2.0	−76.8 ± 2.0	−77.0 ± 2.1
6	−96.2 ± 2.2	−94.6 ± 1.7	−95.7 ± 1.1	−91.4 ± 2.0

[a] Energies are in kcal/mol. [b] The ionizable residues charge states were calculated according to the protocol described in the SI. [c] The ionizable residues, other than those in the Asp dyad, were assigned following their pK_a values in solution at pH 7.

Perusal of Table 2 indicates that the predictions based on the Asp dyad for the two possible monoprotonated states are unsuccessful in correctly ranking these ligands. For instance, the ranking prediction based on the protonated Asp 228 points to **4** as the top binder, while **2** is estimated to be one of the worst, contrary to the experimental observations. The binding affinities calculated with protonated Asp 32, predict that **1** is one of the worst binders, while **4** one of the best, at variance with the experimental results, regardless of the charge state of the rest of the titrable residues. As seen from this table, only the calculations carried out with an Asp dyad in the doubly protonated state came closer to rank properly the inhibitors studied here. For instance, calculations carried with a neutral Asp dyad are the only ones to identify **2** as the inhibitor with the highest affinity and **4** as next to worst binder, in agreement with the experimental results (see Table 1).

All previous calculations started from the premise that the Asp dyad is monoprotonated, but they disagreed on which Asp residue the proton resides.[2] The earliest attempt to assign a charge to the catalytic dyad was based on MD simulations of the X-ray structure of the β-secretase complexed with inhibitor **1** in solution.[7] Their results indicate that a neutral Asp32 and ionized Asp228 combination is the only option that maintains the intricate network of hydrogen bonds around the Asp dyad observed in the X-ray structure.[7] This outcome is in agreement with our recent results[10] for the **1**/BACE-1 complex around a neutral pH, the one used for the determining its structure by X-ray crystallography.

Nevertheless, as pointed out above, our calculations suggest that the dyad is totally neutralized at the pH at which the FRET based enzyme inhibition assays have been carried out and at which the enzyme presents its highest activity (pH 4.5). Other calculations carried out on the **1**/BACE-1 complex, like the linear scaling quantum approach,[8] supported a monoprotonated state Asp dyad with a neutralized Asp 228 and a charged Asp 32, also in

disagreement with the conclusions based on our binding affinity predictions. Nevertheless, the authors of this QM study found out that a diprotonated state could be accessible at optimal enzyme pH given the rather small energy difference found with the monoprotonated state.[8] The only other study that evaluated the protonation state for the Asp dyad from the calculated titration curves is the one carried out by Polgar and Keserü.[6] From these curves, they surmised that the Asp dyad should be monoprotonated, with the proton located at Asp 32. Nevertheless, perusal of their titration curves indicate that the protonation fraction at pH 4.5 (close to the enzyme's optimal pH) is well above 0.5 for both Asp residues, indicating that the Asp dyad should be diprotonated at that relevant pH value.

There have been a large number of studies on the Asp dyad protonation state of other aspartic proteases. The results are not unambiguous, but they support the idea that the dyad charge will depend on the nature of the inhibitor and of the aspartic protease studied. For instance, NMR studies combined with Poisson–Boltzmann calculations carried out on the HIV-1 PR, an aspartic protease that is the target for many AIDS drugs, support the premise that there are some HIV-1 PR inhibitors (cyclic ureas) that elicit a diprotonated Asp dyad, while others, like KNI-272, produce a monoprotonated Asp dyad.[20] Our recent results performed on a chemically diverse set of BACE-1 inhibitors also support the ligand dependence of the Asp dyad protonation state.[10]

One of the most stringent experimental tests on the Asp dyad protonation state proposals will be a neutron diffraction structural determination for some of the BACE-1 inhibitor complexes studied here. Previously, neutron diffraction combined with X-ray studies in endothiapepsin (a fungal aspartic protease) bound to a gem-diol inhibitor implied the possible existence of low barrier hydrogen bonds (LBHB), characterized by short distances between one of the inhibitors hydroxyl groups and the oxygens of the Asp dyad (~2.50 Å).[21] There has been some debate about which kind of experimental observables or computed variables should be used to unambiguously identify a LBHB, and some authors have even disputed its very existence.[22] Nevertheless, the compact hydrogen bonds (HBs), which support the existence of LBHBs, have been observed in several X-ray structures of ligand bound aspartic proteases, besides endothiapepsin, like in HIV-1 PR[23] and BACE-1 bound to peptidic inhibitors,[18] suggesting that LBHBs may be a common feature for some aspartic protease–inhibitor complexes. It has been posited that in LBHBs, the proton that mediates the HB could undergo tunnelling, from the energy well located next to hydroxyl oxygen to the one next to the carboxylate oxygen.[21] In such a system, the proton could be found at both positions (see Figure 1B), resulting in the dispersion of the negative charge of the Asp residue. It may be argued that the tunneling in the LBHB populates the Asp dyad diprotonated state, and hence it may be claimed that among the three active site charge distributions studied here (two monopronated and one diprotonated states), the latter could be a compromise molecular mechanics representation of the entities implied by LBHB (Figure 1). The improved ranking provided by including a neutral Asp dyad over the alternative monoprotonated states (shown in this work) could be a result of a better representation of the LBHB in molecular mechanics simulations. There has been an increased body of work aimed at identifying and characterizing LBHBs in enzymes since they were proposed among others by the group of Frey.[24] In the case of aspartic proteases, quantum mechanical

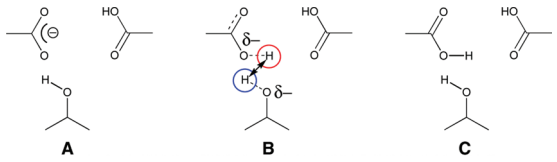


Figure 1. (A) and (C) represent two of the three possible protonation states (mono- and diprotonated) for the Asp dyad, while (B) schematically represents a LBHB, showing the possible proton positions enclosed by red and blue circles.

(QM) or quantum mechanical/molecular mechanics (QM/MM) studies have been applied to the unbound enzymes of this family[25] and enzymes bound to phosphinate and phosphanate containing inhibitors.[26] Nevertheless, no calculations of this kind have been applied to aspartic proteases bound to HE peptidomimetic transition state analogue inhibitors. At present, we are involved in the development of quantum mechanical protocols that would overcome some of the shortcomings of this kind of calculations, like the representation of the quantum region. The results of our QM calculations will be presented elsewhere.

CONCLUSIONS

To further validate the Asp dyad protonation state in BACE-1 bound to HE transition state analogues, found in our laboratory,[10] we have compared the binding ranking predictions for a set of HE peptidomimetic inhibitors based on alternative Asp dyad protonation states by a molecular mechanics method developed in our group. Some of the inhibitors studied were synthesized in our laboratory by a protocol that can afford many isostere variants at P1/P1′. Our calculations indicate that Asp dyad neutral state reproduces better the binding ranking that the Asp dyad charged alternatives. The results are discussed in the light of possible LBHB between the Asp dyad components and the HE isostere hydroxyl group.

EXPERIMENTAL SECTION

Synthesis of Inhibitors. Inhibitors **4** and **3** were prepared from the corresponding δ-amino acids isosters **14a** and **14b** at the Serveis Cientificotècnics Universitat de Barcelona (Spain) by a Boc/Bzl strategy following standard protocols. The purity was greater than 95% as judged by HPLC. See SI for details.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures for the synthesis of isosters **14a** and **14b** and inhibitors **3** and **4** as well as IC_{50} evaluation. Detailed docking and affinity prediction protocols. This material is available free of charge via the Internet at http://pubs.acs.org..

AUTHOR INFORMATION

Corresponding Author

*For F.S.: phone, 34-881-814402; fax, 34-981-595012; E-mail, fredy.sussman@usc.es. For J.C.E.: phone, 34-881-815730; fax, 34-981-591014; E-mail, juancarlos.estevez@usc.es.

■ ACKNOWLEDGMENT

This work was supported by financial aid from the Xunta de Galicia (to F.S. and grant PGIDIT 08CSA002209PR to M.C.V.) and Ministerio de Educación y Ciencia (grants CTQ2005-00555/BQU and CTQ2008-03105 to R.J.E. and fellowships to J.M.O. and J.L.D.). The Supercomputing Center of Galicia (CESGA) provided computer time.

DEDICATION

[†]Dedicated to the memory of Professor Rafael Suau, recently deceased.

■ ABBREVIATIONS USED

AD, Alzheimer's disease; APP, amyloid precursor protein; BACE, β-site APP cleaving enzyme; QM, quantum mechanics; SPR, surface plasmon resonance; HE, hydroxyethylene; LBHB, low barrier hydrogen bond; FRET, fluorescence resonance energy transfer; MM, molecular mechanics; MD, molecular dynamics; HIV-1 PR, human immunodeficiency virus type 1 protease; HB, hydrogen bond

■ REFERENCES

(1) Selkoe, D. J. Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* **1999**, *399*, A23–A31.

(2) Villaverde, M. C.; González-Louro, L.; Sussman, F. The Search for Drug Leads Targeted to the β-Secretase: An Example of the Roles of Computer Assisted Approaches in Drug Discovery. *Curr. Top. Med. Chem.* **2007**, *7*, 980–990.

(3) Lin, X.; Koelsch, G.; Wu, S.; Downs, D.; Dashti, A.; Tang, J. Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1456–1460.

(4) Silvestri, R. Boom in the Development of Non-peptidic β-Secretase (BACE1) Inhibitors for the Treatment of Alzheimer's Disease. *Med. Res. Rev.* **2009**, *29*, 295–338.

(5) (a) Ghosh, A. K.; Gemma, S.; Tang, J. β-Secretase as a Therapeutic Target for Alzheimer's Disease. *Neurotherapeutics* **2008**, *5*, 399–408. (b) Ghosh, A. K. Harnessing Nature's Insight: Design of Aspartyl Protease Inhibitors from Treatment of Drug-Resistant HIV to Alzheimer's Disease. *J. Med. Chem.* **2009**, *52*, 2163–2176.

(6) Polgár, T.; Keserü, G. M. Virtual Screening for β-Secretase (BACE1) Inhibitors Reveals the Importance of Protonation States at Asp32 and Asp228. *J. Med. Chem.* **2005**, *48*, 3749–3755.

(7) Park, H.; Lee, S. Determination of the Active Site Protonation State of β-Secretase from Molecular Dynamics Simulation and Docking Experiment: Implications for Structure-Based Inhibitor Design. *J. Am. Chem. Soc.* **2003**, *125*, 16416–16422.

(8) Rajamani, R.; Reynolds, C. H. Modeling the Protonation States of the Catalytic Aspartates in β-Secretase. *J. Med. Chem.* **2004**, *47*, 5159–5166.

(9) Yu, N.; Hayik, S. A.; Wang, B.; Liao, N.; Reynolds, C. H.; Merz, K. M., Jr. Assigning the Protonation States of Key Aspartates in β-Secretase Using QM/MM X-ray Structure Refinement. *J. Chem. Theory Comput.* **2006**, *2*, 1057–1069.

(10) Domínguez, J. L.; Christopeit, T.; Villaverde, M. C.; Gossas, T.; Otero, J. M.; Nyström, S.; Baraznenok, V.; Lindström, E.; Danielson, U. H.; Sussman, F. Effect of the Protonation State of the Titratable Residues on the Inhibitor Affinity to BACE-1. *Biochemistry* **2010**, *49*, 7255–7263.

(11) (a) Bock, K.; Lundt, I.; Pedersen, C. Preparation of some bromodeoxyaldonic acids. *Carbohydr. Res.* **1979**, *68*, 313–319. (b) Lundt, I.; Pedersen, C. Preparation of some 2,3-Dideoxylactones by an Unusual Catalytic Hydrogenolysis. *Synthesis* **1986**, 1052–1054.

(12) Ghosh, A. K.; Shin, D.; Downs, D.; Koelsch, G.; Lin, X.; Ermolieff, J.; Tang, J. Design of Potent Inhibitors for Human Brain Memapsin 2 (β-Secretase). *J. Am. Chem. Soc.* **2000**, *122*, 3522–3523.

(13) Ghosh, A. K.; McKee, S. P.; Thomson, W. J. An Efficient Synthesis of Hydroxyethylene Dipeptide Isosteres: The Core Unit of Potent HIV-1 Protease Inhibitors. *J. Org. Chem.* **1991**, *56*, 6500–6503.

(14) (a) D'Aniello, F.; Taddei, M. A Stereoselective Method for the Preparation of HIV-1 Protease Inhibitors Based on the Lewis Acid Mediated Reaction of Allylsilanes and *N*-Boc-α-amino Aldehydes. *J. Org. Chem.* **1992**, *57*, 5247–5250. (b) Ghosh, A. K.; Cappiello, J.; Shin, D. Ring-closing metathesis strategy to unsaturated γ- and δ-lactones: synthesis of hydroxyethylene isostere for protease inhibitors. *Tetrahedron Lett.* **1998**, *39*, 4651–4654.

(15) Schechter, I.; Berger, A. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157–162.

(16) Turner, R. T., III; Koelsch, G.; Hong, L.; Castenheira, P.; Ghosh, A.; Tang, J. Subsite Specificity of Memapsin 2 (β-Secretase): Implications for Inhibitor Design. *Biochemistry* **2001**, *40*, 10001–10006.

(17) Ghosh, A. K.; Bilcer, G.; Harwood, C.; Kawahama, R.; Shin, D.; Hussain, K. A.; Hong, L.; Loy, J. A.; Nguyen, C.; Koelsch, G.; Ermolieff, J.; Tang, J. Structure-Based Design: Potent Inhibitors of Human Brain Memapsin 2 (β-Secretase). *J. Med. Chem.* **2001**, *44*, 2865–2868.

(18) Hong, L.; Koelsch, G.; Lin, X.; Wu, S.; Terzyan, S.; Ghosh, A. K.; Zhang, X. C.; Tang, J. Structure of the Protease Domain of Memapsin 2 (β-Secretase) Complexed with Inhibitor. *Science* **2000**, *290*, 150–153.

(19) Spassov, V. Z.; Yan, L. A fast and accurate computational approach to protein ionization. *Protein Sci.* **2008**, *17*, 1955–1970.

(20) Trylska, J.; Antosiewicz, J.; Geller, M.; Hodge, C. N.; Klabe, R. M.; Head, M. S.; Gilson, M. K. Thermodynamic linkage between the binding of protons and inhibitors to HIV-1 protease. *Protein Sci.* **1999**, *8*, 180–195.

(21) Coates, L.; Tuan, H.-F.; Tomanicek, S.; Kovalevsky, A.; Mustyakimov, M.; Erskine, P.; Cooper, J. The Catalytic Mechanism of an Aspartic Proteinase Explored with Neutron and X-ray Diffraction. *J. Am. Chem. Soc.* **2008**, *130*, 7235–7237.

(22) Schutz, C. N.; Warshel, A. The Low Barrier Hydrogen Bond (LBHB) Proposal Revisited: The Case of the Asp···His Pair in Serine Proteases. *Proteins* **2004**, *44*, 711–723.

(23) Jaskólski, M.; Tomaselli, A. G.; Sawyer, T. K.; Staples, D. G.; Heinrikson, R. L.; Schneider, J.; Kent, S. B. H.; Wlodawer, A. Structure at 2.5 Å Resolution of Chemically Synthesized Human Immunodeficiency Virus Type 1 Protease Complexed with a Hydroxyethylene-Based Inhibitor. *Biochemistry* **1991**, *30*, 1600–1609.

(24) Frey, P. A.; Whitt, S. A.; Tobin, J. B. A low-barrier hydrogen bond in the catalytic triad of serine proteases. *Science* **1994**, *264*, 1927–1930.

(25) Porter, M. A.; Molina, P. A. The Low-Barrier Double-Well Potential of the $O^{\delta 1}-H-O^{\delta 1}$ Hydrogen Bond in Unbound HIV Protease: A QM/MM Characterization. *J. Chem. Theory Comput.* **2006**, *2*, 1675–1684.

(26) Vidossich., P.; Carloni, P. Binding of Phosphinate and Phosphonate Inhibitors to Aspartic Proteases: A First-Principles Study *J. Phys. Chem. B* **2006**, *110*, 1437–1442.