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J. Med. Chem., **2005**, 48 (24), 7623-7627• DOI: 10.1021/jm050631+ • Publication Date (Web): 02 November 2005

Preorganization of the Hydroxyethylene Dipeptide Isostere: The Preferred Conformation in Solution Resembles the Conformation Bound to BACE

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Received July 1, 2005

Conformational analysis in solution of β-secretase inhibitors **1** and **2** by NMR spectroscopy reveals that the hydroxyethylene isostere, an apparently flexible fragment widely used as a scissile bond replacement in aspartic protease inhibitors, exists in one predominant conformation in solution. This preferred conformation is similar to that adopted by the hydroxyethylene core of **1** in complex with β-secretase and that adopted by hydroxyethylene cores of related compounds when bound to aspartic proteases, indicating that this structural unit is preorganized in solution.

Introduction

The aspartic proteases are a well-characterized class of enzymes that includes medicinally relevant proteases such as renin, plasmepsin, HIV protease, and BACE (β-secretase).[1] The active site of these enzymes contains a water molecule bound to two aspartate residues that undergoes a nucleophilic attack on the peptide amide carbonyl, resulting in the cleavage of the substrate.[2] Analogues of peptides in which the scissile dipeptide is replaced with a transition-state isostere have proven to be effective inhibitors for this type of protease.[1,3] Specifically, the hydroxyethylene isostere, initially designed for the inhibition of renin,[4] has also been applied to the development of HIV protease and β-secretase inhibitors[5,6] for the treatment of AIDS and Alzheimer's disease, respectively.

The three-dimensional structure of hydroxyethylene-based inhibitors complexed with aspartic proteases has received considerable attention.[7] Moreover, the analysis of more than 1500 protein−ligand crystal structures has shown that proteases commonly bind to the extended β-strand conformations of inhibitors of both peptidic and nonpeptidic origin.[8] In contrast, the conformational behavior of the hydroxyethylene core in solution, and comparison of this with the bound conformation, has not been investigated. This is a topic of major interest for determining to what extent the hydroxyethylene isostere mimics the β-strand conformation, because the preorganization of the ligand would increase the binding affinity through a considerable entropy saving.[9] Although the incorporation of a hydroxyethylene core within a peptide increases the number of rotatable bonds in the molecule, compounds that have flexible backbones, but nevertheless adopt a preferred conformation in solution, are found in nature.[10,11] A preferred conformation will be observed if every rotatable bond in the molecule has only a single low-energy local conformation. Analysis of saturated alkane chains with defined shapes has revealed that the conformational preference of such molecules results from the existence of destabilizing *syn*-pentane interactions in the other rotamers. These stereochemical considerations along with the allylic strain concept have already been used for the design of peptidomimetics with a desired conformation.[12]

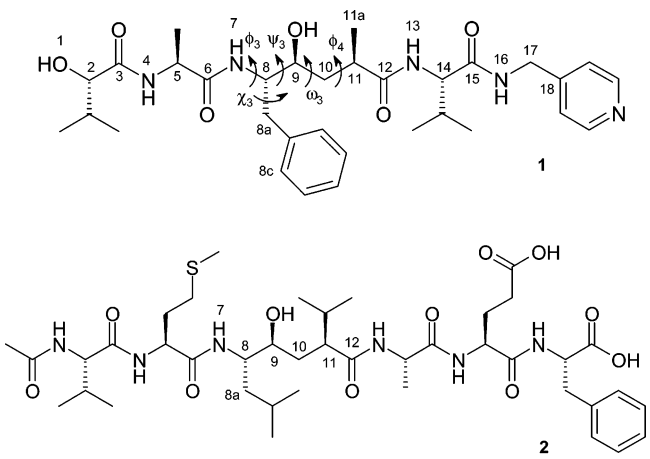

Figure 1. Molecular structures of **1** and **2** along with atom numbering and dihedral angles definition for the hydroxyethylene isostere.

Here we describe the conformational analysis of the hydroxyethylene dipeptide isosteres Phe*-Ala of **1** and Leu*-Val of **2** (Figure 1), in solution, by NMR spectroscopy. Compound **1** has demonstrated submicromolar enzyme inhibitory potency ($IC_{50} = 0.13\ \mu M$) and micromolar cell activity ($IC_{50} = 4.8\ \mu M$) against the human β-secretase,[13] which is hypothesized to be involved in Alzheimer's disease,[14] whereas compound **2** has shown significant inhibitory potency ($IC_{50} = 0.02\ \mu M$) against this enzyme[15]

Results and Discussion

All proton resonances of **1** were assigned through the combination of 1D and 2D NMR experiments (see

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10.1021/jm050631+ CCC: $30.25
Published on Web 11/02/2005

Table 1. $^3J_{HH}$ Values (in Hertz) for the Protons at the Hydroxyethylene Core of Inhibitors **1** and **2**

proton	**1**	**2**	proton	**1**	**2**
H9−H10	2.7	2.1	H11−H10′	3.3	2.5
H9−H10′	9.8	11.0	H8−NH7	9.1	9.2
H11−H10	9.7	11.0	H8−H9	1.8	2.4

Table 2. Relevant NOEs Involving Protons of the Hydroxyethylene Core for **1** and **2** along with Their Intensities[a]

NOE	**1**	**2**	NOE	**1**	**2**	NOE	**1**	**2**
HN7/H8	m	m	HN13/H10	w	w	H9/H10	s	s
HN7/H9	m	w	H8/H9	s	s	H9/H10′	m	w
HN7/H8a	w	w	H8/H8a	s	s	H9/H11a	w	w
HN7/H8a′	s	s	H8/H8a′	s	m	H11/H11a	s	s
HN7/H10	m	m	H8/H10	s	s	H11/H10	w	w
HN7/H10′	m	m	H8/H10′	w	m	H11/H10′	s	s
HN13/H9	m	m	H9/H8a	s	s	H11a/H10	m	m
HN13/H11	s	s	H9/H11	*	m	H11a/H10′	m	m
HN13/H11a	w	m	H9/H8a′	*	m			

[a] The NOE data were extracted from ROESY experiments acquired at 500 MHz with mixing times of 200, 300, and 500 ms. The relative cross-peak intensities were estimated from volume integration and classified as strong (s), medium (m), and weak (w). *, H8a′ shows partial overlapping with H11 in the ROESY spectra, and the intensities of these NOE were not determined.

Supporting Information). The analysis of ROESY spectra exclusively showed short-range NOEs. The absence of long-range NOEs indicates that the molecule adopts an extended conformation in solution. Experimental information on the dihedral angles can be obtained from vicinal ^{1}H−^{1}H coupling constants ($^3J_{HH}$) via Karplus-type equations.[16] Intermediate values usually reflect conformational averaging around the single bonds, whereas more extreme values (<4 or >9 Hz) are strong evidence for hindered rotation and decreased conformational flexibility.[17] These coupling constants were measured for the protons of the hydroxyethylene isostere from the ^{1}H spectrum and/or from homonuclear decoupling experiments. Remarkably, all of the values are extreme, reflecting the existence of a preferred conformation for the hydroxyethylene isostere rather than an ensemble of conformations.

The dihedral angles of the hydroxyethylene core of **1** were derived from the analysis of $^3J_{HH}$ in tandem with short-range NOEs observed in ROESY spectra (Tables 1 and 2), assuming staggered conformations around the single bonds, which are more stable than those eclipsed.[17] The large coupling constant between H7 and H8 (9.1 Hz) and the observation of NOEs between H7 and several protons of the pseudo-Phe side chain (H8a, H8a′, and H8c) are consistent with an anti relationship between H7 and H8, revealing the conformational preference around ϕ_3. The coupling constant between H8 and H9 is small (1.8 Hz), indicating that these protons are in a gauche relationship and, among the three staggered conformers available for ψ_3 (Figure 2a), conformer B can therefore be discarded. The observation of NOEs between H8 and the protons at C10, whereas no NOEs between the protons at C8a and the protons at C10 were detected, indicates that A is the most abundant conformer. The preferred conformation around ω_3 was determined in an analogous manner. The presence of two diastereotopic protons at C10 permitted the measurement of two couplings constants across this bond. H9 is coupled to H10 with a small coupling constant (2.7 Hz) and to H10′ with a large coupling

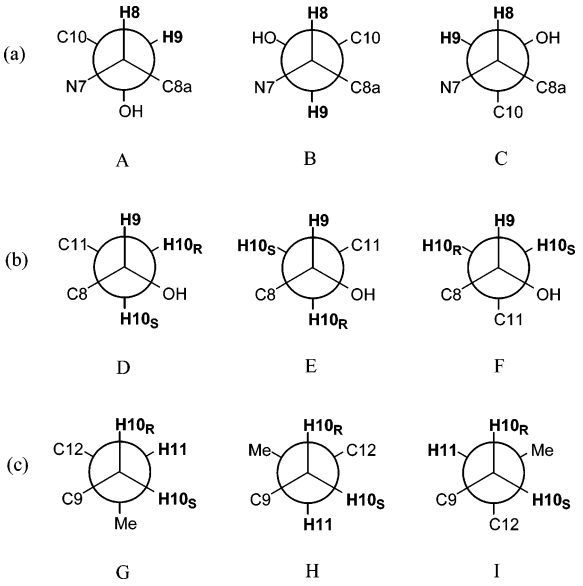

Figure 2. All possible staggered rotamers of **1** around the dihedral angles ψ_3 (a), ω_3 (b), and ϕ_4 (c).

constant (9.8 Hz), indicating that H9 is gauche to one of the protons and anti to the other, in agreement with conformer D or E (Figure 2b). H8 shows NOEs to both H10 and H10′, which is consistent with only conformer E, leading to the stereospecific assignment of H10 and H10′ resonances (proS and proR, respectively). Finally, H11 is coupled to H10 with a large coupling constant (9.7 Hz) and to H10′ with a small coupling constant (3.3 Hz). As the stereospecific assignment of the protons at C10 has been achieved, the preferred conformation around ϕ_4 can be directly deduced from the coupling constants to be conformer I (Figure 2c). On the other hand, the two $^3J_{HH}$ couplings across χ_3 are not as extreme as those involving backbone atoms (8.2 and 5.8 Hz), indicating a higher degree of rotational freedom for the pseudo-Phe side chain. The predominant conformation for **1** proved to be highly resistant to temperature changes, as the coupling constants at 75 °C are similar to those at 25 °C. In addition, the coupling constant analysis was also performed in a DMSO-d_6/D_2O mixture (4:1), close to the solubility limits of the compound. We observed no significant changes in either the chemical shifts or the coupling constants compared to those in DMSO, indicating that the conformational preference is maintained in the DMSO/water mixture.

The conformation derived from the NMR analysis was used as the starting point geometry for NOE-restrained molecular dynamics simulation using the MMFF94 force field[18] supplied with Sybyl software in order to get a more refined geometry. The calculations were performed on the hydroxyethylene core, flanked by Ala and Val residues (atoms 4−16). The observed NOEs were classified as strong, medium, and weak according to their intensities and introduced as restraints in the simulation. The protocol consisted of an initial equilibration period (200 ps) followed by a simulation time (2 ns) in which the structures were periodically saved (every 100 ps) and extensively minimized, resulting in structures having hydroxyethylene moieties presenting well-defined β-strand-like conformations that were consistent with the $^3J_{HH}$ values and free of NOE violations. The rmsd for the heavy atoms of the hydroxyethylene core

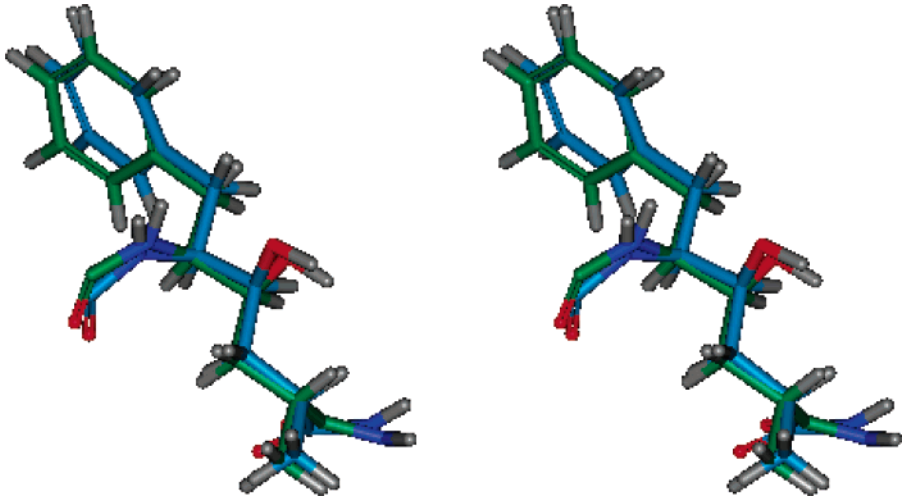

Figure 3. Stereoview of the NMR-derived conformation in solution for the hydroxyethylene core of **1** (in green) superimposed on the X-ray structure of **1** complexed with BACE (in blue).

(atoms N7−C12) among the 20 minimized structures was 0.29 Å.

As mentioned in the Introduction, compounds with flexible backbones can adopt a single distinct conformation as a result of the substituent pattern in the backbone that creates *syn*-pentane strain in the other rotamers.[10,11] Inspection of the NMR-derived structure of **1** indicates that this conformation is indeed free of such destabilizing interactions. On the basis of these precedents, which suggest that the conformational behavior of a saturated alkyl backbone is dictated by the relative configuration of the substitutents, it could be anticipated that the swapping of side chains on the hydroxyethylene dipeptide isostere would not produce significant changes in the overall conformation. Nevertheless, to investigate whether the type of side chain on the hydroxyethylene dipeptide isostere plays a role in defining its conformation in solution, a NMR study of BACE inhibitor **2** in DMSO solution was undertaken. The compound contains a Leu*-Val hydroxyethylene dipeptide isostere instead of the Phe*-Ala unit and also a different peptidic moiety. The vicinal coupling constants of the protons on the core are also extreme, and the relevant NOEs and their intensities are similar to those obtained for **1** (Tables 1 and 2), confirming that both hydroxyethylene moieties adopt the same predominant conformation in solution, irrespective of the nature of the side chain of the isostere. The $^3J_{HH}$ values for the protons at the hydroxyethylene core are slightly more extreme than those measured for **1**, indicating that the conformational preference is even more pronounced for **2**. It is noteworthy that the two $^3J_{HH}$ across χ_3 are also extreme (11.0 and 3.9 Hz), indicating the existence of a preferred conformation around this side-chain dihedral angle, as observed for the backbone.

Given the strong conformational preference of the hydroxyethylene core, we envisaged that this moiety would not undergo significant conformational changes when bound to BACE. Inspection of the X-ray crystal structure of **1** complexed with BACE confirmed our predictions. The superposition of the hydroxyethylene cores of the NMR-derived conformation closest to the average of the 20 saved structures and the conformation bound to BACE highlights their similarity (Figure 3). The rmsd of the heavy atoms (N7−C12) between the two hydroxyethylene backbones is 0.27 Å.

Furthermore, we have calculated the $^3J_{HH}$ for the protons of other hydroxyethylene-based inhibitors bound to aspartic proteases using Karplus-like equations[16] (a total of 16 crystal structures of the Protein Data Bank were examined) and compared them with the experimental values measured for **1** in solution and with the calculated values for bound **1** (Figure 4). The comparison reveals that the bound conformations of hydroxyethylene moieties are similar to the preferred conformation of **1** in solution, and the inhibitors can therefore accommodate the hydroxyethylene moiety in the protease active sites by small changes in the torsion angles displayed in solution. In contrast, the bound conformation of other protease inhibitors significantly differs from their conformations in solution as a result of intermolecular interactions with the enzyme.[19]

The entire structure of **1** complexed with BACE resembles an extended strand motif, as described for other aspartic proteases inhibitors.[7,8] Large backbone scalar coupling constants ($^3J_{HN-H\alpha}$ > 8 Hz) and Hα−HN(i,i+1) NOEs stronger than HN−HN(i,i+1) NOEs are hallmarks of highly populated β-strands.[20] Examination of the NMR data for the peptidic part of the inhibitor shows that the diagnostic HN−Hα couplings are 7.3 Hz ($^3J_{HN4-H\alpha5}$) and 8.5 ($^3J_{HN13-H\alpha14}$) and the αN(i,i+1)/NN(i,i+1) NOE intensity ratios are 7.0 (H5−HN7/HN4−HN7) and 10.7 (H14−HN16/HN13−HN16). The values deviate from the average coupling constants (6.6 Hz)[21] and NOE ratios (1.4)[22] predicted for a random coil conformation, indicating that the peptidic moiety, mainly the C-terminal, adopts an extended strand conformation in solution, similarly to the pseudopeptide moiety.

Conclusions

Because preorganization of a molecule to more closely resemble its bioactive conformation will increase the affinity for the receptor, a number of efforts to arrange peptide mimetics into extended strands have been investigated in order to favor the recognition by their protease targets.[9,23] The most common approach is the connection of two parts of the molecule through a

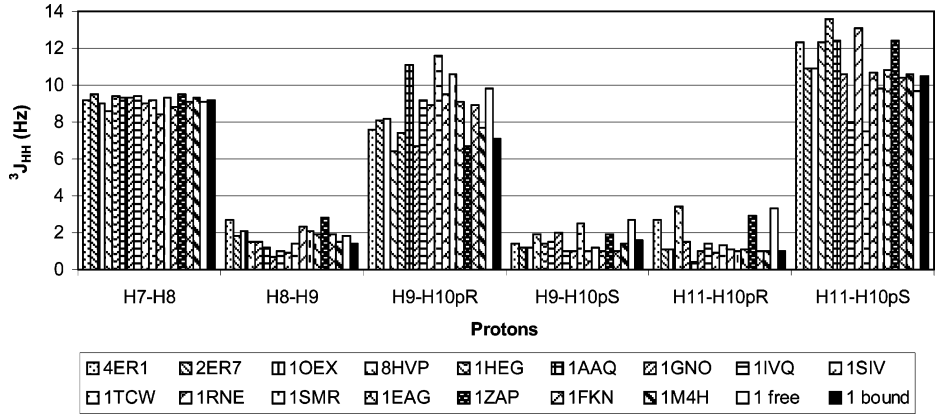

Figure 4. Comparison of the calculated vicinal proton−proton coupling constants for the bound conformations of 16 aspartic protease inhibitors containing the hydroxyethylene core (PDB codes: 4ER1, 2ER7, 1OEX, 8HVP, 1HEG, 1AAQ, 1GNO, 1IVQ, 1SIV, 1TCW, 1RNE, 1SMR, 1EAG, 1ZAP, 1FKN, 1M4H) with the experimental values measured for **1** in solution (**1** free) and the calculated values for the conformation of **1** complexed with BACE (**1** bound).

linkage to form a macrocycle, which is more conformationally restrained and more resistant to amide bond cleavage by peptidases.[24] Alternative strategies include the use of spacers such as aromatic rings that bridge the termini of the peptide[25] or the replacement of a segment of the backbone by a rigid scaffold.[26−29] Incorporation of a hydroxyethylene dipeptide isostere within a peptide is expected to confer higher flexibility to the backbone due to the increased number of rotatable bonds. However, the conformational study of **1** and **2** indicates that the hydroxyethylene dipeptide isostere adopts a predominant conformation in solution, which is similar to the conformation of **1** complexed with BACE and to the bioactive conformation of other hydroxyethylene-based compounds. The preorganization of the ligand reduces the entropy penalty associated with the binding process and explains the high affinity of this type of compound for aspartic proteases. Furthermore, our findings question current drug design strategies aimed at the synthesis of rigid variants of hydroxyethylene isosteres.[30]

Experimental Section

Materials. The synthesis of compound **1** has been previously described.[13] Compound **2** was purchased from Bachem. DMSO-d_6 and D_2O solvents were purchased from Merck.

NMR Experiments. NMR spectra were acquired on a Bruker DRX 500 Avance spectrometer equipped with a 5 mm inverse probe at 25 °C. A proton spectrum at 75 °C was also recorded. The experiments were performed in DMSO-d_6 and DMSO-d_6/D_2O 4:1 mixtures. Proton and carbon chemical shifts were referenced to the residual solvent signals at 2.50 and 39.5 ppm, respectively. One-dimensional spectra were acquired using 32K data points and zero filled to 64K. Complex multiplets were simplified by adding D_2O to remove NH and OH couplings or by using homonuclear decoupling techniques. Absolute value COSY, phase sensitive HSQC, and HMBC experiments were acquired using gradient selection techniques. Phase-sensitive ROESY experiments were recorded using three mixing times (200, 300 and 500 ms). Acquisition data matrices were defined by 1K × 256 points in F2 and F1, respectively. The 2D data matrices were multiplied by the appropriate window functions and zero-filled to 2K × 1K matrices. Linear prediction was applied prior to Fourier transformation, and polynomial baseline correction was used in both dimensions of the 2D spectra. Data were processed using the XWINNMR Bruker program on a Silicon Graphic computer.

Computational Methods. The molecular dynamic of **1** (atoms 4−16) was run on an SGI Octane workstation using version 6.5 of Sybyl (Tripos, St. Louis, MO). The initial structure of the compound was built on the basis of the proton−proton coupling constant values as described in the text. The Merck Molecular Force Field (MMFF94)[18] supplied with the software and its charges were used. A distance-dependent dielectric constant ($\epsilon = R_{ij}$) to simulate solvent effects and a 10 Å cutoff for nonbonded interactions were employed. Upper limits of 3.0, 4.0, and 5.0 Å were assigned for strong, medium, and weak NOE interactions, respectively, and incorporated as harmonic penalty functions to the empirical force field potential energy function (restraint set to 200 kcal/Å^2). The simulations were performed at 298 K with a 1 fs integration step. Thermal equilibrium was achieved by weak coupling ($\tau = 0.1$ ps) to an external bath at 298 K. After an equilibration period of 200 ps, the structures were saved every 100 ps for the 2 ns simulation time. The 20 saved structures were optimized by using conjugate gradient minimization until the rms derivative was <0.05 kcal/molÅ. The analysis of the structure set was performed using the molecular spreadsheet capabilities available in Sybyl. Back-calculation of proton−proton coupling constants on structures obtained from the molecular dynamic trajectory was done using the Karplus−Altona equation.[16]

Acknowledgment. We thank Rosario González for critical reading of the manuscript and Luis M. Granados for sample preparation.

Supporting Information Available: (a) Chemical shift assignments for compounds **1** and **2**, (b) superposition of the 20 NMR-derived structures of **1**, and (c) PDB codes, primary citations, aspartic proteases, and hydroxyethylene dipeptide isosteres of the X-ray structures used to generate Figure 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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