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Rational Design of a Highly Potent and Selective Peptide Inhibitor of PACE4 by Salt Bridge Interaction with D160 at Position P3

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PACE4, a member of the proprotein convertases (PCs) family of serine proteases, is a validated target for prostate cancer. Our group has developed a potent and selective PACE4 inhibitor: Ac-LLLLRVKR-NH_2. In seeking for modifications to increase the selectivity of this ligand toward PACE4, we replaced one of its P3 Val methyl groups with a basic group capable of forming a salt bridge with D160 of PACE4. The resulting inhibitor is eight times more potent than the P3 Val parent inhibitor and two times more selective over furin, because the equivalent salt bridge with furin E257 is not optimal. Moreover, the β-branched nature of the new P3 residue favors the extended β-sheet conformation usually associated with substrates of proteases. This work provides new insight for better understanding of β-sheet backbone–backbone interactions between serine proteases and their peptidic ligands.

Binding of peptide ligands to their complementary enzyme or receptor, is controlled by two important factors from internal and external origins; namely, their conformation and their binding interactions with the target. Thus, in the best scenario, their active conformation corresponds to their intrinsic most stable shape. Moreover, these ligands ideally develop as many noncovalent interactions as possible with the target biomolecule to ensure tighter binding. Here we show that proper substitution of a single key residue of a protease inhibitor can be leveraged to impact both factors in a concerted manner.

β-Strands are ubiquitous secondary structures. They have been identified as important features in antimicrobial peptides and natural ligands for biomolecular hosts like proteolytic enzymes, major histocompatibility complex (MHC) proteins and transferases.[1] It is well known that proteases universally bind to their substrates in extended β-strand conformation.[2] The β-sheet backbone hydrogen bonding pattern (mostly antiparallel) is crucial for peptide-based ligands to bind. In drug design targeting proteases, drug resistance is a serious issue. The active site geometry must be conserved to maintain the catalytic activity, so enhancing the backbone interactions, while stabilizing the active conformation, is a logic way to alleviate the effect of mutations.[3]

Statistic[4] and thermodynamic[5] studies clearly established that β-sheets prefer β-branched residues (Val, Ile, and Thr) in their structure rather than other residues. The greater steric clashes between the local backbone and the side chain of β-branched residues, favors β-sheets over all other conformations.[6] Moreover, β-sheet hydrogen bonds are shielded from interfering water molecules.[7] Consequently, Val and Ile are privileged residues in peptidic inhibitors of proteolytic enzymes.[8]

Side chain to side chain interactions, including those between charged residues,[9] cation–π interactions,[10] π–π interactions[11] and covalent bonds like disulfide bonds[12] have been employed to stabilize artificial β-sheet models. Recently, Gellman's group used a series of charged bearing β-branched amino acids making intramolecular salt bridges in their artificial β-sheets. These amino acids proved to be better β-sheet inducers and stabilizers than natural residues.[13] Here, we take advantage of this β-sheet propensity by incorporating such related residues in our peptide protease complexes.

Proprotein convertases (PCs) belong to the family of substilin–kexin serine proteases, which recognize their substrates through pairing with basic residues (R-P3-R/K-R-↓-P1′).[14] They proteolytically activate a range of proproteins, including hormones, receptors, growth factors, zymogens, etc. The seven members of proprotein convertases including PC1/3, PC2, PC4, PC5/6, PC7, furin and PACE4 have many resemblances in their active sites.[14] We have shown that PACE4 is a promising target in prostate[15] and ovarian cancer.[16] Optimization of binding selectivity is a major path toward drug candidates to alleviate potential side effects. Additionally, in vivo use necessitates the elaboration of stable compounds. Our research group has developed a PACE4 peptide inhibitor with the Ac-LLLLRVKR-NH_2 sequence[17] and named the Multi-Leu (ML) after its four leucine residues. The tetra-Leu tail makes the RVKR tetrapeptide warhead a cell penetrating peptide and increases the selectivity toward PACE4 (20-fold). We also reported the P1 Arg modification of ML with 4-amidinobenzylamine (Amba),

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which resulted in an inhibitor with improved activity and better stability in vivo.[18]

A P3 modification of a peptidomimetic furin inhibitor (Phac-RVK-Amba, where Phac stands for phenylacetyl) has been investigated by replacing Val with many other natural and modified amino acids.[19] That study suggested to us that β-branched residues[4,5] might be used to stabilize the β-sheet conformation of inhibitors, while concomitantly adding interactions with furin and its homologous PACE4 enzymes, with suitable β-substituents.

According to a homology model of PACE4[20] developed from the crystal structure of furin (1P8J),[21] from all amino acids within the S1-S4 sub-pockets, there is just a single difference between the two enzymes: Asp160 in PACE4 replaces Glu257 of furin in S3. We designed β-branched basic amino acids **1**–**4** (Figure 1) to replace Val at position P3 of ML, to bind Asp160 of PACE4, while stabilizing its β-sheet conformation (Figure 2).

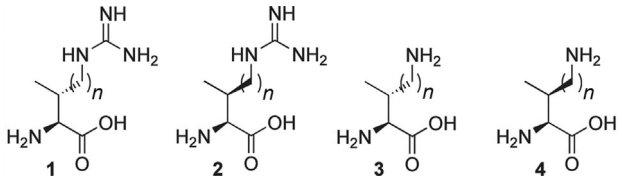


Figure 1. Structure of modified amino acids to be inserted at P3 of ML.

The binding affinities and selectivities (toward PACE4) of all the PACE4 inhibitors synthesized in the present work are summarized in Figure 2. Initial results from Val (**5**) substitution by Ala (**6**) suggest that β-branching is indeed important. We then endeavored to optimize the side chain length and the basic functional group. A series of ML P3 substituted analogues was

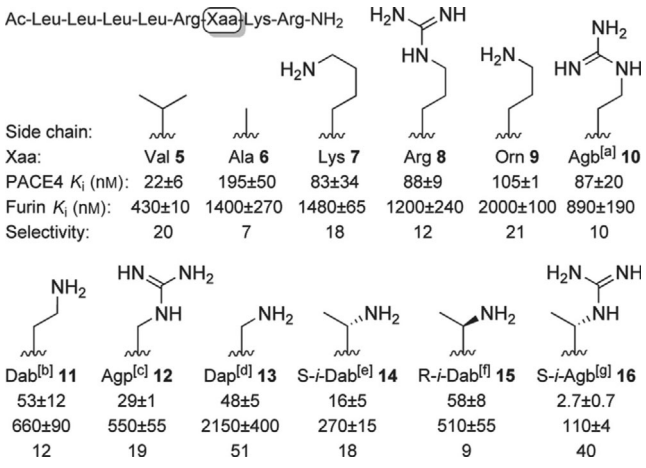
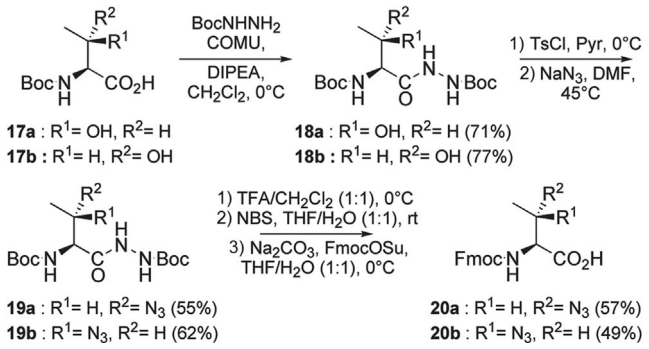


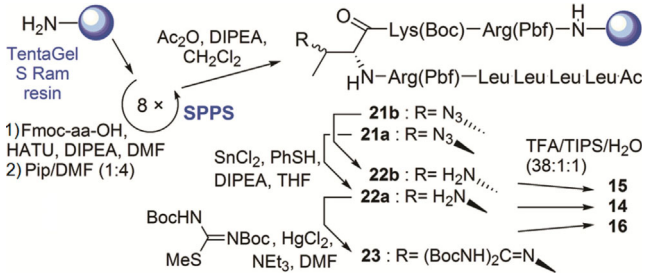
Figure 2. Structure and binding affinities of peptide inhibitors **5**–**16** for PACE4 and furin and their selectivity profile toward PACE4. All experiments were repeated at least twice, and data are shown as $K_i \pm$ SD. [a] 2-amino-4-guanidinobutyryl. [b] 2,3-diaminobutyryl. [c] 2-amino-3-guanidinopropionyl. [d] 2,3-diaminopropionyl. [e] 2*S*,3*S*-diaminobutyryl. [f] 2*S*,3*R*-diaminobutyryl. [g] 2*S*-amino-3*S*-guanidinobutyryl.

prepared from commercial linear basic residues (**7**–**13**) using solid-phase peptide synthesis (SPPS). Inhibitors **7**–**10** showed almost the same trend for PACE4 inhibition. Since **12** and **13**, gave the best results, it was then clear that the ideal chain length for the β-branched analogues was 0 ($n=0$ in Figure 1). We then logically prepared the corresponding inhibitors **14**–**16** from synthetic β-branched residues **20a** and **20b** (Scheme 1).



Scheme 1. Synthesis of Fmoc-protected residues **20a** and **20b**.

Thus, Boc-Thr-OH **17a** and Boc-*allo*-Thr-OH **17b** were protected as the Boc-hydrazides **18a** and **18b**. The β-hydroxy groups were converted into azides **19a** or **19b** through tosylation and subsequent azidation, with inversion of configuration at the β position. The Boc and hydrazide protecting groups were removed with TFA and NBS, respectively. Fmoc groups were installed on the resulting amino-acids to yield the protected amino acids **20a** or **20b**, ready for Fmoc-SPPS (Scheme 2). After eight cycles of deprotection and coupling



Scheme 2. Synthesis of peptide inhibitors **14**–**16** with modified residues.

with the adequate residues, the terminal amine was deprotected then acetylated. The resin bound peptide azides **21a** and **21b** were reduced to their corresponding amines **22a** and **22b**. The amine **22a** was converted into the guanidine derivative **23**, directly onto the resin. The cleavage and global deprotection of **22a**, **22b** and **23** gave the peptide inhibitors **14**, **15** and **16** respectively.

The data reported in Figure 2 show that, among the new inhibitors with straight chains (**7**–**13**), **12** is as potent as ML **5**. This suggests that the salt bridge between the guanidinium ion of Agp in **12** and the carboxylate of Asp160 in PACE4 is in

fact almost as effective as the β-sheet inducing effect of Val in **5**. The salt bridge is not as strong with a simple ammonium ion as in **13**. Methyl groups were then introduced at the β-position of Dap in **13** to transform it into a β-sheet inducer like Val in ML **5**. It appeared that only one isomer, S-*i*-Dab, produced the expected additive effect. The corresponding inhibitor **14** is now more potent than **5**, whereas its stereoisomer **15** is even less active than its parent **13**. The inhibitors **14** and **15** were docked into a model of PACE4 to compare their geometry with **5** (Figure 3). To satisfy the salt bridge structural requirements, the methyl group has to orient toward (for 3-S isomer) or away (for 3-R isomer) from the β-sheet hydrogen bond. The orientation of the β-methyl and β-ammonium groups of S-*i*-Dab (**14**) is the same as the Val methyl substituents in the crystal structure of furin.[21,22] We assume that only this orientation is prone to induce the β-sheet conformation of the inhibitor ligand. This is supported by the $\chi 1$ value of S-*i*-Dab (-37°) corresponding precisely to an antiparallel β-sheet.[23] Whereas, in inhibitor **15**, the salt bridge between the ammonium group of R-*i*-Dab and D160 forces the residue to adopt a $\chi 1$ value (-160°) normally found in parallel β-sheets. This phenomenon is obviously fighting the natural antiparallel mode of binding of inhibitors of PACE4. Consequently, the PACE4-**15** complex is probably less stable than the stereoisomeric complex between PACE4 and **14**.

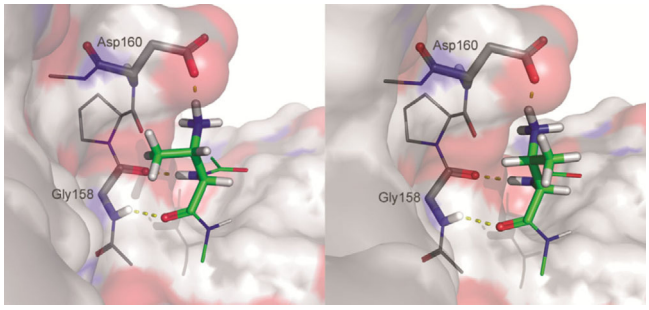


Figure 3. Docking of inhibitors **14** and **15** in PACE4. S-*i*-Dab from **14** (left) and R-*i*-Dab from **15** (right) in the S3 pocket of a PACE4 homology model.

On top of the beneficial β-sheet stabilization in peptide **14**, it is also likely that the isopropyl group orientation could affect the solvation profile of β-sheet hydrogen bonds, by screening them from external water molecules. Such shield effect is absent in inhibitor **15**.

Because inhibitor **14** has the correct stereochemistry for optimal binding, its equivalent guanylated analogue **16** was investigated. Some degree of synergy appears to be at work in its mode of inhibition, because it is eight times more potent for PACE4 than ML **5**, while two times more selective (20-fold vs. 40-fold, respectively).

To gather additional support for our hypothesis that β-branching of P3 residue might affect the conformation of peptide inhibitors; we recorded the CD spectra of inhibitors **5** and **6**. We were expecting to witness different conformational behaviors. Unfortunately, both octapeptides displayed identical random coil spectral patterns. We then turned our attention to molecular dynamics (MD) simulations on a PACE4 homology model in coordination with the two tetrapeptides Ac-R**V**KR-NH_2 and Ac-R**A**KR-NH_2 as ligands. Because the P3 side chains in these two model peptides are solvent exposed and have no interactions with the target enzyme, effect of β-branching on conformation could be investigated independently. The Ramachandran plot parameters, Φ and Ψ as descriptors of conformations, were analyzed for the P3 residue during the simulation (Figure 4a). The two tetrapeptides adopt different conformations described by the ∠*N*-H···O hydrogen bond angles (α_1 and α_2) between the P3 residue and Gly158 backbones (Figures 3 and 4b). During the MD simulations, the values of these angles for the Val residue remain consistently larger (average α_1 of 149° and α_2 of 159°) than for the Ala residue (average α_1 of 115° and α_2 of 130°). Because the ideal α_1 and α_2 angles for an antiparallel β-sheet, are $160^\circ \pm 10^\circ$,[24] the Val residue, in the model tetrapeptide, mostly adopts an antiparallel β-sheet conformation. Whereas the Ala equivalent in P3 is mainly in the poly-Pro II (PPII) helix region, which is often observed in β-turn residues.

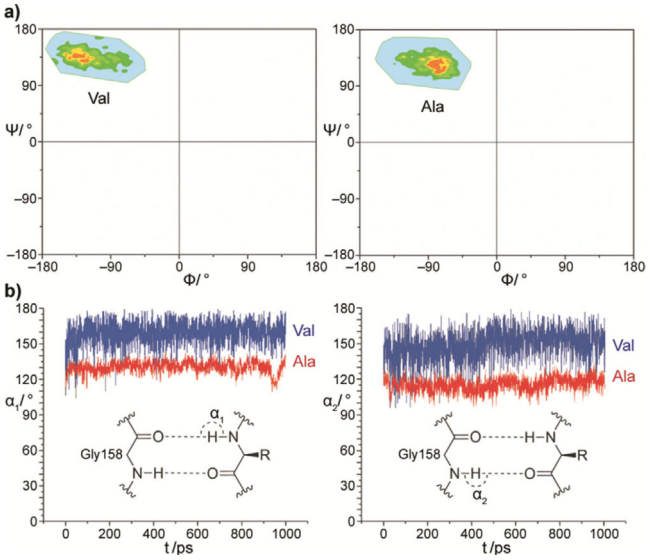


Figure 4. MD simulation (1 ns) of Ac-R**V**KR-NH_2 and Ac-R**A**KR-NH_2 docked in a PACE4 homology model. a) Ramachandran plots for the P3 residues. The Φ and Ψ angles for Val correspond to an antiparallel β-sheet, whereas those for Ala correspond to a PPII helix. b) Variations of the hydrogen bond angles α_1 and α_2 (∠N-H···O) between Gly158 of PACE4 and the P3 residues of the inhibitor backbones.

These computational data add credibility to the idea that β-branched residues like Val, S-*i*-Dab (as in **14**) and S-*i*-Agb (as in **16**) are β-sheet inducers when located at position P3 of PACE4 (as well as furin) inhibitors structurally related to **5**.

In summary, we introduced a new basic β-branched amino acid (S-*iso*-Agb in **16**) to the P3 position of the PACE4 inhibitor, ML **5**, leading to the discovery of a peptidic inhibitor with improved potency and selectivity over furin. We propose that this improvement is the result of three additive effects at the same P3 position: 1) additional stabilizing electrostatic interactions with Asp160; 2) stabilizing hydrophobic interaction be-

tween the β-methyl substituent (having the correct configuration as in Figure 3, left) and the Gly158-Pro159 region of PACE4, and 3) increase in β-sheet propensity resulting from β-branching. To the best of our knowledge, the correlation that might exist between β-sheet propensity of inhibitor residues and protease inhibition has not been studied. We suggest that β-sheet propensity improvement, coupled with other side chain stabilizing interactions, could be considered as a principle to target other proteases as well.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: hydrogen bonds · medicinal chemistry · proprotein convertases · proteases · β-sheet propensity

[1] W. A. Loughlin, J. D. Tyndall, M. P. Glenn, T. A. Hill, D. P. Fairlie, *Chem. Rev.* **2010**, *110*, PR32 – PR69.
[2] P. K. Madala, J. D. Tyndall, T. Nall, D. P. Fairlie, *Chem. Rev.* **2010**, *110*, PR1 – PR31.
[3] A. K. Ghosh, D. D. Anderson, I. T. Weber, H. Mitsuya, *Angew. Chem. Int. Ed.* **2012**, *51*, 1778 – 1802; *Angew. Chem.* **2012**, *124*, 1812 – 1838.
[4] P. Y. Chou, G. D. Fasman, *Trends Biochem. Sci.* **1977**, *2*, 128 – 131.
[5] a) M. B. Swindells, M. W. MacArthur, J. M. Thornton, *Nat. Struct. Biol.* **1995**, *2*, 596 – 603; b) D. L. Minor, Jr., P. S. Kim, *Nature* **1994**, *367*, 660 – 663.
[6] a) A. G. Street, S. L. Mayo, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9074 – 9076; b) D. Pal, P. Chakrabarti, *Acta Crystallogr. Sect. D* **2000**, *56*, 589 – 594.
[7] Y. Bai, S. W. Englander, *Proteins* **1994**, *18*, 262 – 266.
[8] a) D. J. Kempf, K. C. Marsh, J. F. Denissen, E. McDonald, S. Vasavanonda, C. A. Flentge, B. E. Green, L. Fino, C. H. Park, X. P. Kong, N. E. Wideburg, A. Saldivar, L. Ruiz, W. M. Kati, H. L. Sham, T. Robins, K. D. Stewart, A. Hsu, J. J. Plattner, J. M. Leonard, D. W. Norbeck, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2484 – 2488; b) J. M. Clements, R. P. Beckett, A. Brown, G. Catlin, M. Lobell, S. Palan, W. Thomas, M. Whittaker, S. Wood, S. Salama, P. J. Baker, H. F. Rodgers, V. Barynin, D. W. Rice, M. G. Hunter, *Antimicrob. Agents Chemother.* **2001**, *45*, 563 – 570; c) A. Yamamoto, K. Tomoo, T. Hara, M. Murata, K. Kitamura, T. Ishida, *J. Biochem.* **2000**, *127*, 635 – 643.
[9] a) L. H. Kuo, J.-H. Li, H.-T. Kuo, C.-Y. Hung, H.-Y. Tsai, W.-C. Chiu, C.-H. Wu, W.-R. Wang, P.-A. Yang, Y.-C. Yao, T. W. Wong, S.-J. Huang, S.-L. Huang, R. P. Cheng, *Biochemistry* **2013**, *52*, 7785 – 7797; b) F. A. Syud, H. E. Stanger, S. H. Gellman, *J. Am. Chem. Soc.* **2001**, *123*, 8667 – 8677.
[10] A. J. Riemen, M. L. Waters, *Biochemistry* **2009**, *48*, 1525 – 1531.
[11] L. Wu, D. McElheny, T. Takekiyo, T. A. Keiderling, *Biochemistry* **2010**, *49*, 4705 – 4714.
[12] A. M. Almeida, R. Li, S. H. Gellman, *J. Am. Chem. Soc.* **2012**, *134*, 75 – 78.
[13] S. J. Maynard, A. M. Almeida, Y. Yoshimi, S. H. Gellman, *J. Am. Chem. Soc.* **2014**, *136*, 16683 – 16688.
[14] a) F. Couture, F. D'Anjou, R. Day, *Biomol. Concepts* **2011**, *2*, 421 – 438; b) N. G. Seidah, A. Prat, *Nat. Rev. Drug Discovery* **2012**, *11*, 367 – 383.
[15] F. Couture, F. D'Anjou, R. Desjardins, F. Boudreau, R. Day, *Neoplasia* **2012**, *14*, 1032 – 1042.
[16] R. Longuespée, F. Couture, C. Levesque, A. Kwiatkowska, R. Desjardins, S. Gagnon, D. Vergara, M. Maffia, I. Fournier, M. Salzet, R. Day, *Transl. Oncol.* **2014**, *7*, 410 – 419.
[17] a) C. Levesque, M. Fugere, A. Kwiatkowska, F. Couture, R. Desjardins, S. Routhier, P. Moussette, A. Prahl, B. Lammek, J. R. Appel, R. A. Houghten, F. D'Anjou, Y. L. Dory, W. Neugebauer, R. Day, *J. Med. Chem.* **2012**, *55*, 10501 – 10511; b) C. Levesque, F. Couture, A. Kwiatkowska, R. Desjardins, B. Guerin, W. A. Neugebauer, R. Day, *Oncotarget* **2015**, *6*, 3680 – 3693.
[18] a) A. Kwiatkowska, F. Couture, C. Levesque, K. Ly, R. Desjardins, S. Beauchemin, A. Prahl, B. Lammek, W. Neugebauer, Y. L. Dory, R. Day, *J. Med. Chem.* **2014**, *57*, 98 – 109; b) A. Kwiatkowska, F. Couture, C. Levesque, K. Ly, S. Beauchemin, R. Desjardins, W. Neugebauer, Y. L. Dory, R. Day, *ChemMedChem* **2016**, *11*, 289 – 301.
[19] a) G. L. Becker, K. Hardes, T. Steinmetzer, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4695 – 4697; b) K. Hardes, G. L. Becker, Y. Lu, S. O. Dahms, S. Koehler, W. Beyer, K. Sandvig, H. Yamamoto, I. Lindberg, L. Walz, V. von Messling, M. E. Than, W. Garten, T. Steinmetzer, *ChemMedChem* **2015**, *10*, 1218 – 1231.
[20] S. Henrich, I. Lindberg, W. Bode, M. E. Than, *J. Mol. Biol.* **2005**, *345*, 211 – 227.
[21] S. Henrich, A. Cameron, G. P. Bourenkov, R. Kiefersauer, R. Huber, I. Lindberg, W. Bode, M. E. Than, *Nat. Struct. Mol. Biol.* **2003**, *10*, 520 – 526.
[22] S. O. Dahms, K. Hardes, G. L. Becker, T. Steinmetzer, H. Brandstetter, M. E. Than, *ACS Chem. Biol.* **2014**, *9*, 1113 – 1118.
[23] R. L. Dunbrack, Jr., M. Karplus, *J. Mol. Biol.* **1993**, *230*, 543 – 574.
[24] E. N. Baker, R. E. Hubbard, *Prog. Biophys. Mol. Biol.* **1984**, *44*, 97 – 179.

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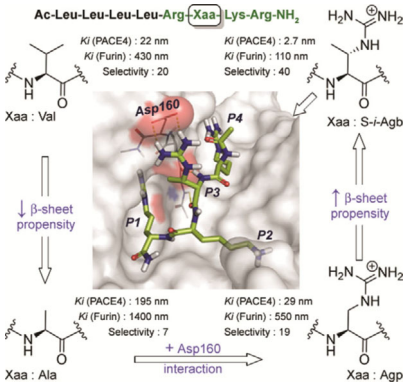
Setting the PACE with concurrent control of conformation and binding: Most substrates of proteases form a β-sheet with the active site upon binding. New β-branched residues, known to favor β-sheet conformation in linear peptides, were introduced at the P3 Val position of inhibitors of the serine protease PACE4. Through this P3 strategic position, it was possible to obtain potent and selective inhibitors, by additive β-sheet conformation control and salt bridge formation with PACE4 Asp160.



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