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Testing the Conformational Hypothesis of Passive Membrane Permeability Using Synthetic Cyclic Peptide Diastereomers

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Cyclic peptides provide ideal scaffolds for exploring structure−activity relationships in ligand−receptor interactions. Cyclization serves to increase membrane permeability by eliminating charged termini and facilitating internally hydrogen bonded conformations.[1] Cyclic peptides are also more resistant to proteolytic degradation than their linear counterparts,[2] and their restricted conformations can provide increased affinity for protein targets by diminishing the entropy loss upon binding.[3] While most cyclic peptides have been designed to target extracellular receptors, relatively few are known that act on intracellular targets, presumably due to their low intrinsic membrane permeability. One prominent exception is the cyclic undecapeptide cyclosporine A (CSA), an orally active immunosuppressive drug that binds to the cytoplasmic protein cyclophilin.[4] CSA penetrates the cell membrane by passive diffusion despite its high molecular weight (∼1200 Da) and the fact that it violates most classic criteria for assessing "drug-likeness".[5] It has a highly N-methylated backbone and adopts a conformation in chloroform[6] in which all of its backbone N−H groups are involved in intramolecular hydrogen bonds. In aqueous solution, CSA adopts multiple conformations in which most of its backbone N−H groups point toward solvent.[7,8] This conformational switching, along with observations in smaller cyclic peptides,[9−11] suggests that the ability of cyclic peptides to form intramolecular hydrogen bonds in hydrophobic media facilitates membrane diffusion by reducing the energetic cost of amide N−H desolvation (Figure 1). We set out to systematically examine the relationship between conformation and membrane permeability by testing the diffusion of simple cyclic peptide diastereomers across model lipid membranes in vitro.

We synthesized a collection of nine cyclic hexapeptide diastereomers based on the sequence cyclo[Leu-Leu-Leu-Leu-Pro-Tyr]. These sequences were chosen based on molecular modeling studies showing that their low-energy structures exhibit a variety of intramolecular hydrogen bonding patterns in low dielectric medium. A single proline residue was included to increase conformational rigidity and facilitate turn structures.

Diastereomers **1**−**9** (Table 1) were synthesized by solid-phase methods, and their permeabilities were examined using a parallel artificial membrane permeability (PAMPA) assay.[12] The PAMPA assay measures the diffusion of a compound from a donor compartment to an acceptor compartment through a phospholipid-impregnated membrane, the results of which correlate well with passive diffusion across living cells.[13] Compounds were quantitated using LC/MS by comparison with an internal standard, and apparent permeability coefficients (P_E) were calculated using established methods.[12]

Table 1 shows that the experimental log P_E values for compounds **1**−**9** span nearly two log units, from the most permeable diastereomer **1** (log $P_E = -6.2$) to the least permeable diastereomers **8** and **9** (log $P_E \leq -8.1$). Consistent with the conformational hypothesis, a terminally protected linear version (**1-lin**) of the most permeable compound, **1**, showed greatly diminished permeability relative to its cyclic counterpart. We selected two compounds for further study, **1** and **9**, whose log P_E values differed by the widest margin.

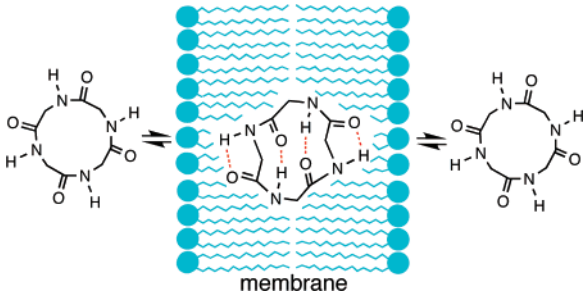

Figure 1. Schematic representation of conformational basis of membrane permeability in cyclic peptides.

Table 1. The log P_E Values of Cyclic Peptide Diastereomers **1**−**9**

compound	sequence	log P_E[a]
1	cyclo[D-Leu-D-Leu-Leu-D-Leu-Pro-Tyr]	−6.2
2	cyclo[D-Leu-D-Leu-D-Leu-D-Leu-Pro-Tyr]	−7.0
3	cyclo[Leu-Leu-Leu-D-Leu-Pro-Tyr]	−7.1
4	cyclo[Leu-D-Leu-D-Leu-D-Leu-Pro-Tyr]	−7.2
5	cyclo[Leu-Leu-Leu-Leu-D-Pro-Tyr]	−7.3
6	cyclo[D-Leu-D-Leu-D-Leu-D-Leu-D-Pro-Tyr]	−7.3
7	cyclo[Leu-Leu-D-Leu-D-Leu-Pro-Tyr]	−7.3
8	cyclo[Leu-D-Leu-Leu-D-Leu-D-Pro-Tyr]	<−8.1[b]
9	cyclo[Leu-D-Leu-Leu-Leu-D-Pro-Tyr]	<−8.1[b]
1-lin	Ac-D-Leu-D-Leu-Leu-D-Leu-Pro-Tyr-OAllyl	<−8.1[b]
cyclosporine A		−6.6

[a] Determined using PAMPA assay according to ref 10. The compounds were quantitated using LC/MS with 9-aminofluorene as an internal standard. [b] These compounds could not be detected by LC/MS in the acceptor well of the PAMPA assay after 72 h. An upper limit log P_E value of −8.1 was set according to the detection limit of the LC/MS.

NMR was used to determine the solution conformations of **1** and **9** in $CDCl_3$, a non-hydrogen bonding solvent with a dielectric constant similar to that calculated for the center of a lipid bilayer.[14] Both compounds exhibited well-defined conformations in $CDCl_3$ with single proline rotamers in the *trans* amide configuration (Figure 2).

Compound **1** adopts a pseudo-symmetric saddle conformation with two transannular hydrogen bonds between Leu[3] and Tyr[6] that define a 2:2 β-hairpin structure[15] and two inverse γ-turns[16] centered on Leu[3] and Tyr[6]. In contrast to the symmetry of **1**, compound **9** exhibits an irregular, twisted conformation defined by two consecutive β-turns flanked by a classic γ-turn. The D-Pro[5] residue occupies

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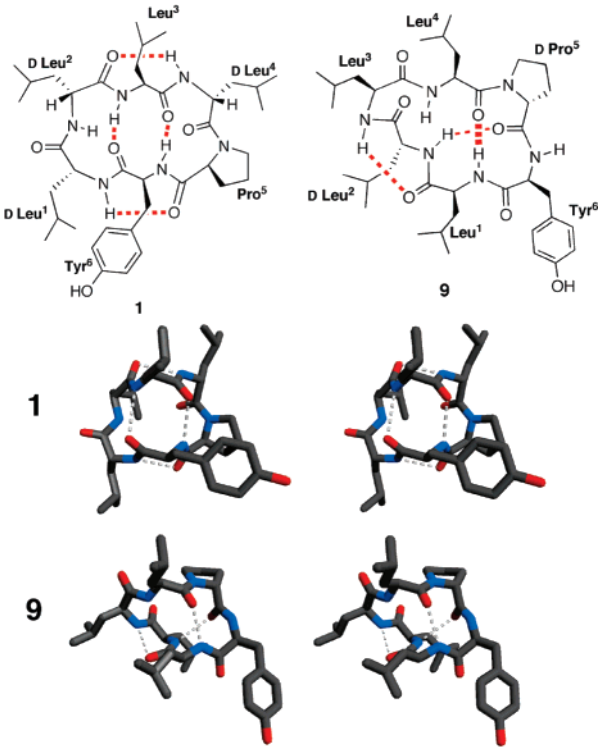

Figure 2. NMR solution structures of **1** and **9** in $CDCl_3$. Above: Schematic structures of **1** and **9** illustrating their hydrogen bonding networks. Below: Stereoviews of **1** and **9**.

the $i + 1$ position of a type II′ β-turn, which is followed by a type I β-turn centered on Tyr^6 and Leu^1 at the $i + 1$ and $i + 2$ positions, respectively. The amide N−H of Tyr^6 points away from the ring into solvent, while the amide N−H of Leu^4 points inward, toward the γ-turn centered on D-Leu^2.

To further examine the relationship between amide N−H solvent accessibility and membrane permeability, we carried out acid-catalyzed hydrogen/deuterium (H/D) exchange experiments with **1** and **9** by shaking their $CDCl_3$ solutions with 0.2 equiv of TFA in D_2O. Only one amide N−H in **1**, that of D-Leu^2, exchanged with D_2O over 19 h, whereas all five of the amide N−H groups in **9** exchanged completely within 19 h. The amides of Leu^1 and Leu^4 of **9** were relatively slow to exchange (over hours), whereas D-Leu^2 of **9** exchanged more rapidly (over minutes). Thus, the number of exposed N−H groups determined in the H/D exchange experiments correlated with the observed permeability differences between **1** and **9**. However, the observation that all of the amide N−H groups of **9** exchanged with solvent was not consistent with its NMR structure showing that the amide N−H groups of Leu^1, D-Leu^2, and Leu^3 participate in intramolecular hydrogen bonds.

To reconcile the solution structure of compound **9** with the results of the H/D exchange experiments, we investigated its low-energy conformations in water and $CHCl_3$ using a computational search/minimization algorithm (see Supporting Information). This algorithm successfully predicted the NMR structures in chloroform of both **1** and **9** within 0.1 Å RMSD. The low-energy conformation predicted for **9** in water was identical to its solution structure in $CDCl_3$. However, an alternate conformation for **9** in water was calculated to lie just 0.3 kcal/mol above its low-energy structure, in which the exchangeable amide N−H groups of D-Leu^2, Leu^3, and Tyr^6 point toward solvent, with the more slowly exchanging amides of Leu^1 and Leu^4 involved in transannular hydrogen bonds (see Supporting Information). Significant population of this conformer in water, in which three out of five amide N−H groups point toward solvent, may contribute to the low membrane permeability of compound **9**.

We have identified a novel cyclic peptide scaffold whose backbone contains none of the modifications (e.g., N-methylation) commonly found in cyclic peptide natural products, yet whose passive membrane diffusion rate parallels that of cyclosporine A. Detailed structural analysis suggests that membrane permeability in cyclic peptides may be governed by a combination of factors: intramolecular hydrogen bonding, steric protection of amide NH groups from solvation, and the relative stability of impermeable "open" conformers in water. Future studies aimed at elucidating the relative importance and control of these factors may advance our understanding and design of membrane-permeable cyclic peptides. Regardless of the detailed mechanism, synthetic scaffolds, such as **1**, promise to extend the application of cyclic peptides to include a greater share of intracellular targets.

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Supporting Information Available: NMR data for **1** and **9**, detailed experimental procedures on compound synthesis, PAMPA assay, H/D exchange, and details of computational methods and calculated structure of **9** in water. This material is available free of charge via the Internet at http://pubs.acs.org.

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