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Cyclic Penta- and Hexaleucine Peptides without *N*-Methylation Are Orally Absorbed

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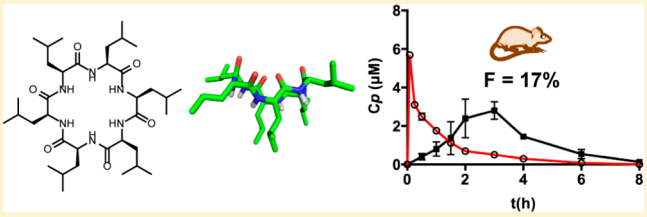
Supporting Information

ABSTRACT: Development of peptide-based drugs has been severely limited by lack of oral bioavailability with less than a handful of peptides being truly orally bioavailable, mainly cyclic peptides with *N*-methyl amino acids and few hydrogen bond donors. Here we report that cyclic penta- and hexaleucine peptides, with no *N*-methylation and five or six amide NH protons, exhibit some degree of oral bioavailability (4−17%) approaching that of the heavily *N*-methylated drug cyclosporine (22%) under the same conditions. These simple cyclic peptides demonstrate that oral bioavailability is achievable for peptides that fall outside of rule-of-five guidelines without the need for *N*-methylation or modified amino acids.



KEYWORDS: *Oral bioavailability, absorption, peptides, rule of five, permeability*

Lack of oral bioavailability and plasma stability have been limiting factors in the development and use by patients of peptide-based drugs.[1−3] Despite these deficiencies, peptides and proteins, especially antibodies, have become "blockbuster" injectable drugs in recent years.[3] Protein and peptide-based drugs also suffer from high costs of manufacture and delivery, low patient compliance with injection schedules and non-responsiveness in some patients. Oral delivery of drugs is still the preferred route for treating most chronic diseases where regular, cheaper, and longer term dosing is required.[3] Here we describe a step toward understanding factors that govern peptide oral bioavailability, reporting on comparative oral bioavailability of a simple cyclic penta- and hexapeptide in rats.

In medicinal chemistry, compounds are more likely to be orally absorbed if they obey "rule-of-five" (Ro5) parameters (MW < 500, cLogP < 5, hydrogen bond donors (HBD) ≤ 5, and hydrogen bond acceptors (HBA) ≤ 10).[4−6] A cyclic pentapeptide lies at the boundary of this range, with HBD = 5 and HBA = 10, while MW ≈ 500 and cLogP < 5 are dependent upon amino acid side chain composition. The chances of oral bioavailability are further increased if peptides are not rapidly cleaved before or after membrane penetration. Cyclic peptides are more resistant than linear peptides to degradation by proteases because there are no N- or C- termini available for recognition and cleavage by aminopeptidases and carboxypeptidases, respectively.[7−9] Nevertheless, most peptides, including cyclic peptides, have < 1% oral bioavailability. There are a handful of exceptions, mainly those containing *N*-methyl amino acids and thus fewer HBD atoms.[10−12] The best known exception is the widely used 11-residue immunosuppressive cyclic peptide drug cyclosporin A (CSA), with seven *N*-methylated amino acids, four hydrogen-bonded amide NH protons, and oral bioavailability (F = 15%−50%) that is dependent on the animal model, excipient/solvent formulation, and the concentration administered.[13−19]

Here we compare structures, membrane permeabilities, and oral bioavailabilities of simple cyclic peptides with properties that lie just outside rule-of-five parameters. Cyclic pentaleucine, cyclo-[Leu]$_5$ (**1**) (MW = 566, HBD = 5, HBA = 10, and cLogP = 7.6) is compared with cyclo-hexaleucine isomers cyclo-[(L-Leu)$_5$(D-Leu)] (**2**) (MW = 679, HBD = 6, HBA = 12, and cLogP = 9.1) and cyclo-[Leu]$_6$ (**3**). Leucines were chosen to ensure cLogP > 5. A D-leucine was inserted only in the hexpeptide, as it is known to be less constraining than in a

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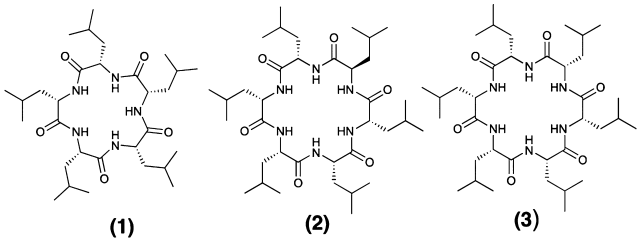
pentapeptide where it induces both a hydrogen bond and a beta turn.[20,21] The compounds violate rule-of-five guidelines[4−6] yet have some membrane permeability and oral bioavailability in rats. These findings provide a valuable platform for future studies aimed at understanding factors that determine peptide oral bioavailability.



Compounds **1**−**3** were synthesized using standard solid phase peptide synthesis protocols and Fmoc protected amino acids in DMF to produce linear precursors. These were cyclized under dilute conditions (10^{-3} M) and the cyclic peptides purified by rpHPLC (Supporting Information). Epimerization was observed duration formation of **3**, but diastereomers **2** and **3** were separable by rpHPLC and could be purified.

The membrane permeability of compounds **1**−**3** vs CSA was measured in RRCK cell monolayers (Figure 1), which lack

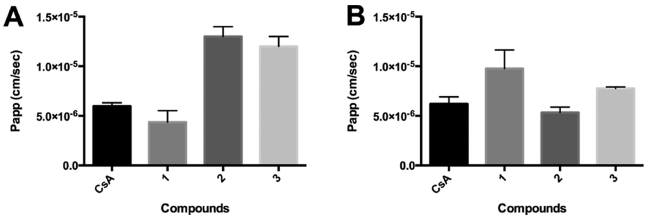


Figure 1. Apparent permeability ($cm \cdot s^{-1}$) of cyclic peptides **1**−**3** (A) at 8 μM vs CSA (8 μM) in RRCK cells or (B) at 10 μM vs CSA (30 μM) in CACO-2 cells. Cell medium = 0.05% DMSO in HEPES buffer, pH 7.4, 37 °C.

active transporters, and in CACO-2 cell monolayers, which have been more widely used to measure membrane permeability but also involve active transport.[10,11] Membrane permeability is often used to predict whether a compound will be orally absorbed since the first barrier after surviving the GI tract is membrane permeation and resistance to enterocyte metabolism.[22−25] In the RRCK assay, compound **1** had lower permeability (P_{app} = 1.7 × 10^{-6} $cm \cdot s^{-1}$) than CSA (P_{app} = 5 × 10^{-6} $cm \cdot s^{-1}$),[12] while cyclic hexapeptide isomers **2** and **3** had 6−7-fold greater permeability than **1** and 2−3-fold higher RRCK permeability than CSA (Figure 1A). By contrast, all three compounds were similarly permeable in CACO-2 cells (Figure 1B). When CACO-2 permeability was measured at 50 μM, compound **1** was not detected, while compounds **2** and **3** retained permeability (Figure S1, Supporting Information).

Given the differences in membrane permeability of **1** versus **2** and **3** and the reduced CACO-2 cell permeability of **1** at higher concentration, their structures were examined for potential influences on membrane permeability. The crystal structure of **1**, isolated from aqueous methanol, showed intermolecular (but no intramolecular) hydrogen bonds (Figure 2A) indicating some propensity for **1** to aggregate. Concentration-dependent changes in the line-shapes of circular dichroism spectra for **1** (30−500 μM) in TFE were consistent with a tendency to aggregate at concentrations > 60 μM in solution (Figure 2B),

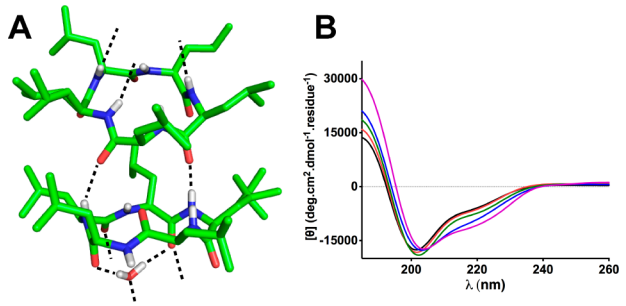


Figure 2. Aggregation of compound **1**. (A) Crystal structure of **1** isolated from MeOH/H_2O (80:20), showing intermolecular hydrogen bonds (dashes) (CCDC 1002286). (B) Concentration-dependent CD spectra of **1** (30 μM, purple; 60 μM, blue; 150 μM, green; 250 μM, red; 500 μM, black) in TFE at 298 K.

although this may not occur at physiologically relevant concentrations in aqueous conditions and so may not affect cell permeation *in vivo*.

By contrast the CD spectral line-shapes for compound **3** were concentration independent over the same range (Figure 3A), consistent with no aggregation for the cyclic hexapeptide.

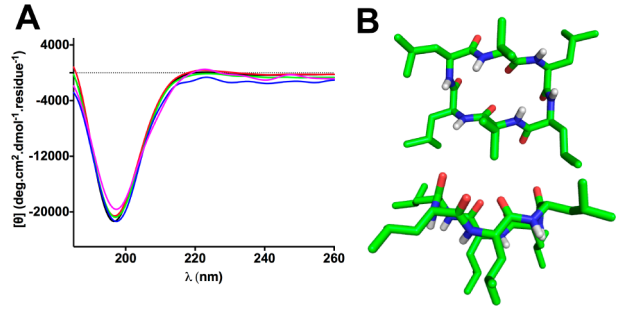


Figure 3. (A) Concentration-independent CD spectra for **3** in TFE (30 μM, purple; 60 μM, blue; 150 μM, green; 250 μM, red; 500 μM, black; 298 K). (B) NMR-derived structure of **3** in DMSO-d_6. Top and side views show two Leu side chains approaching closely to prevent intermolecular amide H-bonds.

To understand differences in aggregation properties between cyclic penta- and hexaleucine, NMR structures of the symmetrical **1** and **3** were also determined (in DMSO-d_6) using dihedral angle constraints (Supporting Information). Interestingly, whereas compound **1** had a pseudoplanar circular structure, compound **3** formed a more rectangular structure with two identical turns at each end. The leucine side chains in **1** were projected outward (Figure 3B), exposing the polar groups to solvent and allowing formation of intermolecular hydrogen bonds between exposed NH and CO groups, thereby encouraging aggregation. In contrast, for compound **3**, two of the leucine side chains were projected inward (Figure 3B) enabling shielding of the amide backbone from solvent. These leucine side chains were also on the same face as the amide NHs (Figure 3B), preventing formation of intermolecular hydrogen bonds, thereby hindering aggregation. These results are consistent with higher membrane permeability for **3** (no aggregation) than **1** (aggregation).

Since compounds **1**−**3** showed detectable permeability (P_{app} >1 × 10^{-6} $cm \cdot s^{-1}$) in RRCK or CACO-2 cells, oral bioavailability was measured in male Wistar rats (Figure 4) for **1**−**3** at 10 mg/kg/p.o. in olive oil vs 1 mg/kg/i.v. in DMSO

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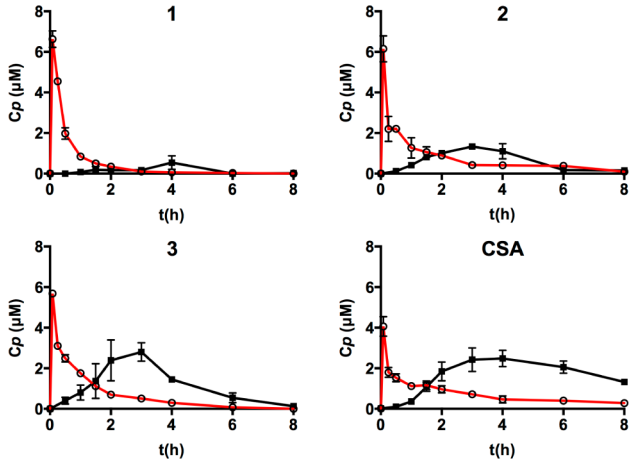

Figure 4. Relative concentrations in plasma (*Cp*) assessed with time (*t*) by LCMS up to 8 h after administration of peptides **1**–**3** or cyclosporin-A at 10 mg/kg/p.o. in olive oil (black) vs 1 mg/kg/i.v. in DMSO (red) to male Wistar rats. Oral bioavailability (*F*%) = 4.0 ± 1.7%, *n* = 3 (**1**); 8.5 ± 1.1%, *n* = 2 (**2**); 17.3 ± 5.7%, *n* = 3 (**3**); 22.5 ± 7.8%, *n* = 4 (CSA).

(Supporting Information). The systemic fraction of peptide (oral bioavailability, *F*%) was determined by comparing areas under the plasma concentration–time traces (AUC) over 8 h after i.v. and oral doses (D), namely, by $F\% = (AUC_{p.o.} \times D_{i.v.})/(AUC_{i.v.} \times D_{p.o.})$. The rank order of oral bioavailability (*F*%) was **1** (4%) < **2** (9%) < **3** (17%) < CsA (23%) and did not correlate with cell permeability (Figure 1). However, oral bioavailability is dependent on other factors in addition to membrane permeability, including solubility, metabolic stability (GI tract, enterocytes, plasma, and liver), clearance, tissue distribution, and protein binding.

The lower bioavailability of **2** vs **3** is consistent with the higher plasma clearance of **2** (24.1 mL/min/kg) vs **3** (4.7 mL/min/kg) after intravenous dosing. Different clearances of the two diastereomers might be due in part to differences in solvent exposure of the peptide backbone. For example, compound **3** had only one amide NH resonance in its ^{1}H NMR spectrum in DMSO-d_6, and it exchanged rather slowly ($t_{1/2}$ = 480 min, DMSO-d_6/D_2O 9:1, 298 K; Supporting Information). By contrast, **2** displayed well-dispersed resonances with different $^3J_{NHH\alpha}$ coupling constants for all six amide NHs, consistent with a well-defined asymmetric structure, with three more rapidly exchanging ($t_{1/2}$ = 60, 120, and 150 min) and three slowly exchanging ($t_{1/2}$ > 150 min) amide NH protons. This suggests that conformational influences of amino acid substitutions might reduce plasma clearance rates by controlling solvent exposure, and this should be investigated in detail in the future.

Conclusions. Very few peptides are orally bioavailable. They typically have backbone *N*-methylated amides and intramolecular hydrogen bonds. Here we find, surprisingly, that even simple cyclic peptides without *N*-methyl groups or intramolecular hydrogen bonds are able to permeate cell membranes and have a degree of oral bioavailability. Hydrophobic side chains of amino acids can sufficiently shield polar amides from solvation to permit membrane permeation and oral absorption. The peptide conformation may also impact oral bioavailability by influencing exposure to solvent and to proteins that dictate plasma clearance and metabolism. Peptide aggregation may hinder oral absorption despite reduced solvent exposure; this is likely due to reduced solubility and formation of higher molecular weight aggregates. This study adds to our understanding of oral bioavailability of compounds with different physicochemical properties than traditional drugs.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic methods, compound characterization, RRCK and CACO-2 experiments, rat pharmacokinetic experiments and data, CD methods, NMR calculations, NMR spectra, and crystal structure coordinates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

Oral bioavailabilities were disclosed at the 241st American Chemical Society National Meeting, "Towards orally bioavailable peptides and peptidomimetics" (Beyond the Rule of Five session), Anaheim, CA, March, 2011.

The authors declare no competing financial interest.

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