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Flexibility versus Rigidity for Orally Bioavailable Cyclic Hexapeptides

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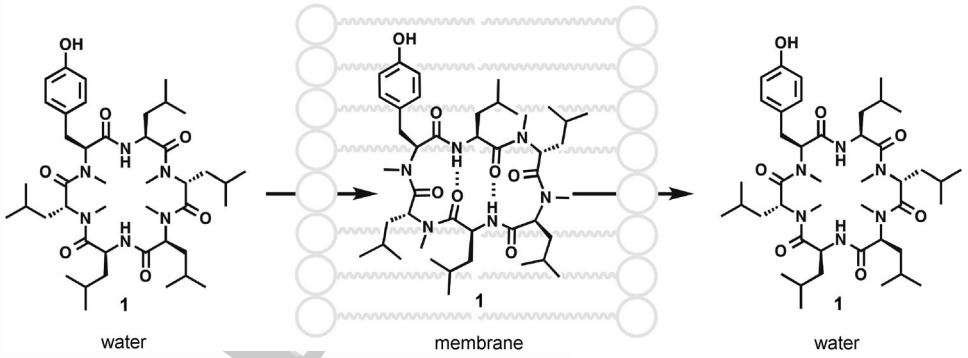
Cyclic peptides and macrocycles have the potential to be membrane permeable and orally bioavailable, despite often not complying with the "rule of five" used in medicinal chemistry to guide the discovery of oral drugs. Here we compare solvent-dependent three-dimensional structures of three cyclic hexapeptides containing D-amino acids, prolines, and intramolecular hydrogen bonds. Conformational rigidity rather than flexibility resulted in higher membrane permeability, metabolic stability and oral bioavailability, consistent with less polar surface exposure to solvent and a reduced entropy penalty for transition between polar and nonpolar environments.

Peptides are attracting renewed interest as pharmaceuticals[1] despite their key constraints (high polarity, metabolic instability, low membrane permeability, negligible oral bioavailability), which compromise delivery.[2] The immunosuppressant drug cyclosporine A (CsA)[3] is an unusual peptide in that it is 20–30% orally bioavailable, despite violating the "rule of five" and related parameters[4] associated with oral absorption of small-molecule drugs. This 11-residue cyclic peptide has seven N-Me amides and four NH protons intramolecularly hydrogen bonded, and is flexible in solution.[5] It has therefore been speculated that flexibility is important for passive membrane permeability and oral absorption of peptides,[6] by exposing the polar surface area maximally to water for solubility but minimally to lipids (by forming intramolecular hydrogen bonds) for passage through hydrophobic membranes (Scheme 1). Here we compare three analogous cyclic hexapeptides (**1**–**3**) that vary in the number of rigidifying prolines, for differences in three-dimensional structure, conformational flexibility, physicochemical properties, membrane permeability, metabolic stability and oral bioavailability.

Cyclic peptide **1** was synthesized by standard SPPS and cyclization in solution; **2** and **3** were synthesized on resin[7a] by selective N-methylation (see the Supporting Information). They were chosen because conformational flexibility has been proposed to be important for membrane permeability[6a,7] and oral bioavailability:[6a,7a] **2**, cyclo-[Leu-D-Leu(*N*-Me)-Leu(*N*-Me)-Leu-D-Pro-Tyr(*N*-Me)] was >20% orally bioavailable in rats.[7a] We aimed to increase flexibility by replacing D-Pro with D-Leu (**1**) and to decrease flexibility by replacing D-Leu with D-Pro (**3**). The circular dichroism (CD) spectra for **1**–**3** in aqueous media were compared against that for CsA (Figure 1), with increasing concentrations of sodium dodecyl sulfate (SDS, 0–10 mM) to simulate an aqueous–lipid environment. CsA and **1** clearly had different structures in water than in >2 mM SDS, as shown by a shift in the molar ellipticity minimum to lower wavelength (Figure 1). Neither **2** nor **3** showed a significant change over 0–10 mM SDS. Furthermore, **1**–**3** displayed similar spectra over 3–10 mM SDS, consistent with their having similar structures. Thus, although CsA and **1** changed structure between aqueous and water–lipid environments, **2** and **3** remained unchanged.



Scheme 1. Cyclic peptide **1** and possible conformational changes during transition from a water to membrane to water environment.

To investigate these structural differences for **1**–**3**, we compared their ^{1}H NMR spectra in nonpolar and polar solvents. Amide coupling constants ($^3J_{NHH\alpha}$) for Leu1–Leu4 of **1**–**3** varied by 0–0.5 Hz between solvents ($CDCl_3$, [D_6]DMSO, [D_6]DMSO/25%H_2O, CD_3CN/40%H_2O), with the order **1**>**2**>**3** (Table 1). The temperature coefficients ($\Delta\delta/T$) for Leu1–Leu4 NH chemical shifts in $CDCl_3$ and [D_6]DMSO were not especially revealing

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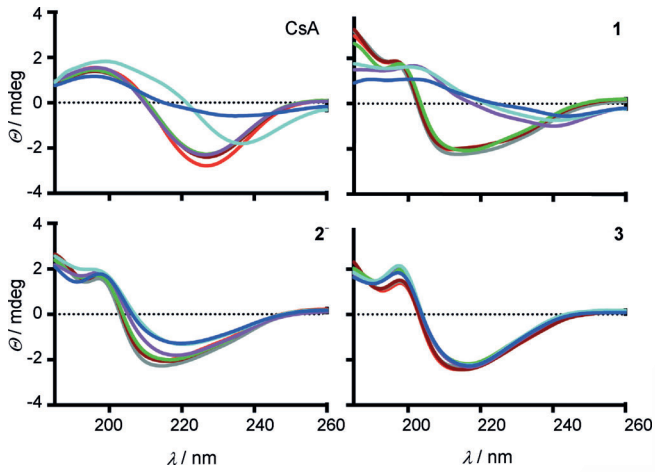

Figure 1. Circular dichroism spectra of CsA and **1**–**3** at 100 μM in 10 mM phosphate buffer (pH 7.4, 5% MeCN) with different concentrations of SDS (—: 0 mM, —: 1 mM, —: 2 mM, —: 3 mM, —: 4 mM, —: 5 mM, —: 10 mM).

Table 1. Comparison of NMR parameters for amides in **1**–**3**.

Leu1/ Leu4	Solvent	1	2	3
$^3J_{NHH\alpha}$ [Hz]	$CDCl_3$	9.4/9.5	9.7/9.1	9.2/9.3
$\Delta\,^3J_{NHH\alpha}$	[D_6]DMSO	9.1/9.0	9.5/8.9	9.2/9.2
	[D_6]DMSO/ 25% H_2O	9.3/9.1	9.5/8.9	9.2/9.4
	CD_3CN/ 40% H_2O	9.4/9.4	9.7/9.1	9.2/9.4
		0.3/0.5	0.2/0.2	0/0.2
$\Delta\delta/T$	$CDCl_3$	−1.8/−2.1	−1.4/−1.0	−1.6/−1.3
[ppb K^{-1}]	[D_6]DMSO	−3.2/−3.2	−1.3/−0.3	−3.2/−3.2
$t_{1/2}$ [min]	$CDCl_3$	$760/2.5\times10^3$	$4.1\times10^3/6.5\times10^3$	$6.4\times10^3/8.2\times10^3$
	[D_6]DMSO	55/24	$4.1\times10^3/5.4\times10^3$	$1.3\times10^5/1.3\times10^5$

(Table 1). However, amide NH exchange for Leu1–Leu4 in **1**–**3** was slow ($t_{1/2} > 700$ min) in $CDCl_3$, consistent with both NHs being hydrogen bonded in this nonpolar solvent. In [D_6]DMSO, slow H–D exchange for **2** and **3** supports Leu1–Leu4 being hydrogen bonded, whereas rapid H–D exchange (< 60 min) for **1** suggests no hydrogen bonds (Table 1). Together, these NMR data predict an order of flexibility **1** > **2** > **3**.

Three-dimensional structures were determined for **1**–**3** in $CDCl_3$ and [D_6]DMSO (Figure 2) from distance and H-bond restraints derived from NOEs and H–D exchange rates (Supporting Information). The structures were ■■calculated *"determined" (also please confirm you were using 23-year-old software [8])*■■ in XPLOR-NIH[8] by using a dynamic simulated annealing protocol in a geometric force field. In $CDCl_3$, the structures calculated for **1**–**3** (39, 52 and 19 distance restraints, respectively) formed two Leu1–Leu4 intramolecular hydrogen bonds, with no distance (≥ 0.2 Å) or angle (≥ 2°) violations (Figure 2A). Each structure consisted of anti-parallel β-sheets connected by type 1 β-turns at each end, thus facilitating intramolecular hydrogen bonding. In [D_6]DMSO, the structures of **2** and **3** (51 and 18 distance restraints, respectively; Figure 2B) superimposed well on the structures in $CDCl_3$, with the same two hydrogen bonds between Leu1 and Leu4 (no distance or angle violations). This correlates well with the slow H-D exchange rates in $CDCl_3$ and [D_6]DMSO for **2** and **3**. In contrast, the structure of **1** calculated in [D_6]DMSO from 46 distance restraints did not form any hydrogen bonds and did not superimpose well on the other structures (Figure 2). Although forming the β-sheet structure in $CDCl_3$, in [D_6]DMSO **1** formed a ring-shaped structure with N-methyl groups and carbonyls projecting perpendicular to the plane of the ring, and there was no transannular hydrogen bond.

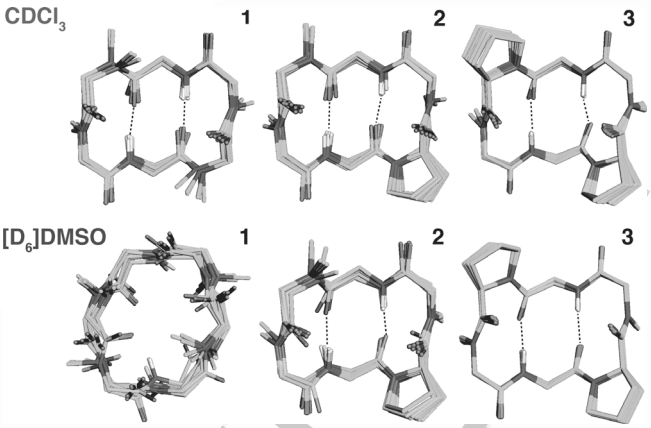

Figure 2. Ten NMR-derived lowest-energy backbone structures of **1**–**3** in nonpolar ($CDCl_3$) or polar ([D_6]DMSO) solvents, showing prolines, N-Me and Leu1···Leu4 intramolecular hydrogen bonds (dashed lines).

A comparison of the Ramachandran plots (Figure 3A; phi and psi angles for Leu1 (triangles) or Leu4 (circles)) for the 10 lowest-energy structures of **1**–**3** in a nonpolar ($CDCl_3$, red) or polar ([D_6]DMSO, blue) solvent is instructive: the structures for **1**–**3** were well defined in $CDCl_3$, more dispersed in [D_6]DMSO, and the order of flexibility was **1** > **2** > **3**. To analyse these structural differences in more detail, the variation between **1**–**3** in the angle ∢CO···H···N that defines the two hydrogen bonds was examined for each solvent (Figure 3B): 118.2–176.7° for **2** and 124.5–165° for **3** in both $CDCl_3$ and [D_6]DMSO; 144.9–171.0° for **1** in $CDCl_3$ but highly variable (80–175°) in [D_6]DMSO. Finally, the N··OC versus NH···O distances were also plotted for the two Leu1–Leu4 hydrogen bonds in the ten lowest-energy structures for **1**–**3** in $CDCl_3$ versus [D_6]DMSO (Figure 3C). The distances for NH···O and N···OC that define hydrogen bonds are typically 1.8–2.5 and 2.8–3.5 Å^2, respectively.[9] The plots shows such distances, but they are much less well-defined for **1** than for **2** and **3** (particularly in [D_6]DMSO), consistent with a greater flexibility of **1**. In summary, the results agree with the observations for H–D exchange, $^3J_{NHH\alpha}$ and low $\Delta\delta/T$ (Table 1) and structure calculations (Figure 2), thus strongly indicating the order of conformational flexibility as **1** > **2** > **3** in $CDCl_3$ and [D_6]DMSO.

Although **1**–**3** were not sufficiently soluble in water to permit NMR studies, the CD spectra (Figure 1) hint at the same order of flexibility in water, so we conducted molecular dynamics simulations (10 ns, 298 K) for **1**–**3** in a water box model (Figure 4A): the backbone RMSD variations also gave the order

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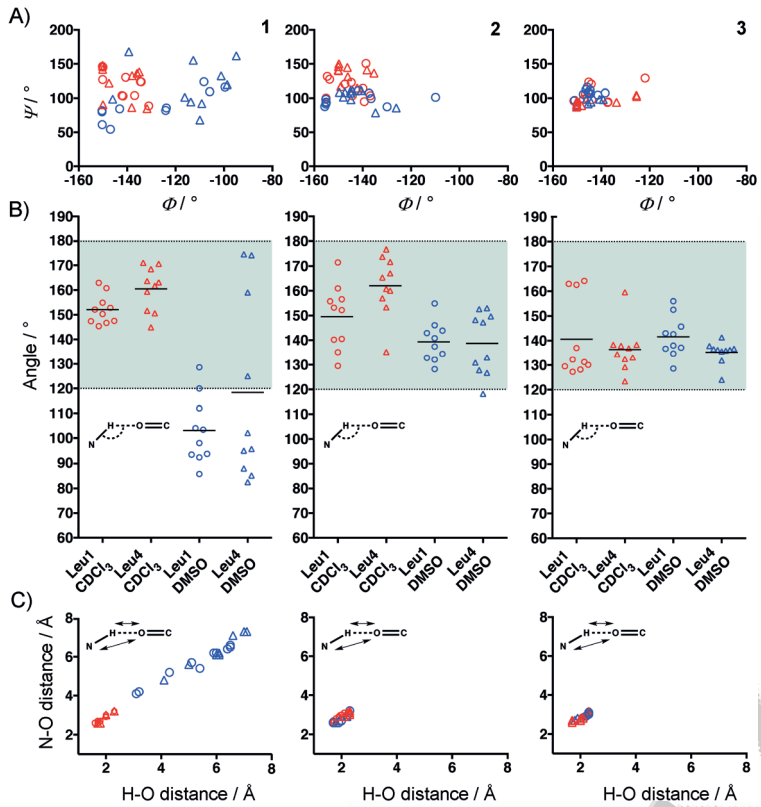

Figure 3. Angle and distance data from the ten lowest-energy NMR-derived solution structures for **1**, **2** and **3**. A) Phi/psi angle plot, B) Angle ∢N–H···O for hydrogen bonds (shaded areas are allowed angles for H-bonds), C) Plot of HN···OC distance against N···O= C distance, involving Leu1 ($CDCl_3$ Δ, $[D_6]$DMSO Δ) and Leu4 ($CDCl_3$ 0, $[D_6]$DMSO 0).

1 > **2** > **3** for flexibility in water. Finally, **2** was energy minimized in simulated-water and chloroform environments: there was no structural difference in the peptide backbone (Figure 4B). Thus, although **2** and **3** adopt the same rigid backbone conformations in polar and nonpolar solvents (Figures 2–5), **1** is much more flexible and adopts different, solvent-dependent conformations, as depicted in Scheme 1.

Having established the relative conformational flexibilities for **1**–**3**, we sought to investigate how these differences affect membrane permeability, liver microsomal stability and oral absorption. The passive membrane permeabilities (P_{app}) obtained from parallel artificial membrane permeability assay (PAMPA) data (Table 2) had the order **1** < **2** < **3** (the more rigid **3** had the lowest calculated polar component of surface area in 3D structures (FISA) and was the most permeable). These findings are in accordance with other studies that have reported that strong intramolecular hydrogen bonds facilitate passive permeability for cyclic hexapeptides of this class.[7,10] All three peptides were stable in plasma, and an in vitro metabolism assay with liver microsomes revealed an inverse relationship between clearance rate ■■(CL_{int}) *Is this usually an abbreviation for "intrinsic clearance? (and is this the same as "microsomal stability" at the beginning of the paragraph?)*■■ and rigidity: the most rigid peptide (**3**) displayed the lowest clearance rate, and the most flexible peptide (**1**) showed the highest

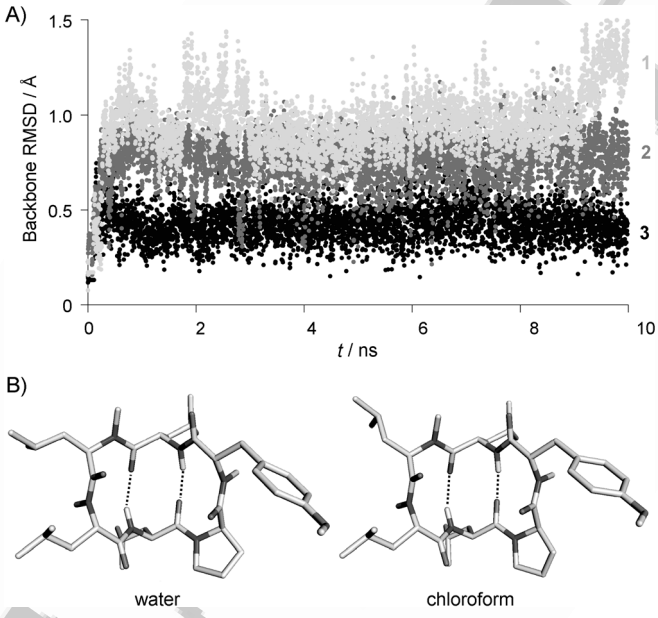

Figure 4. Molecular modelling of **1**–**3**. A) Molecular dynamics simulations of NMR solution structures (RMSD variations of backbones) of **1**–**3** over 10 ns in a water box model at 300 K: backbone flexibility **1** > **2** > **3** (for 100 ns simulations see the Supporting Information). B) Energy minimization of **2** in a dielectric medium simulating water and in chloroform (showing no structural difference).

Table 2. Hydrophilic surface area, PAMPA permeability and in vitro metabolic stability of **1**–**3**.

	1	**2**	**3**
FISA [Å²][a]	127.5	122.5	110.4
P_{app} [$\times 10^{-6}$ cm s^{-1}][b]	< 0.1	1.0 (0.5)	6.8 (2.1)
CL_{int} [μL min^{-1} mg^{-1}][c]	19.1 (0.4)	14.0 (1.8)	7.3 (1.6)
plasma $t_{1/2}$ [min][d]	> 60	> 60	> 60

[a] ■■Ave *"Average"? (please define)*■■ hydrophilic area of total solvent-accessible surface area of NMR solution structures in Figure 3 calculated in Maestro (Schrödinger). [b] PAMPA vs. propanol P_{aap} 2.1 × 10^{-5} cm s^{-1}. [c] Rat liver microsomal clearance. [d] Rat plasma half-life.

(Table 2). This metabolic stability in cells correlates with PAMPA permeability (most rigid and metabolically stable peptide has the highest permeability).

To investigate whether flexibility or rigidity is preferred for oral absorption in these hexapeptides, **1**–**3** were administered by oral gavage (10 mg kg^{-1} in olive oil) to male Wistar rats, and blood samples were removed periodically. CsA (10 mg kg^{-1} in olive oil) was used as a control; as these compounds are not soluble in water, intravenous doses were in DMSO at 1 mg kg^{-1}. There were clear ■■qualitative *"quantitative"?*■■ differences between the pharmacokinetic profiles of the compounds (Figure 5). The plasma oral concentration curves show more rapid absorption of **2** and **3** (maximum plasma concen-

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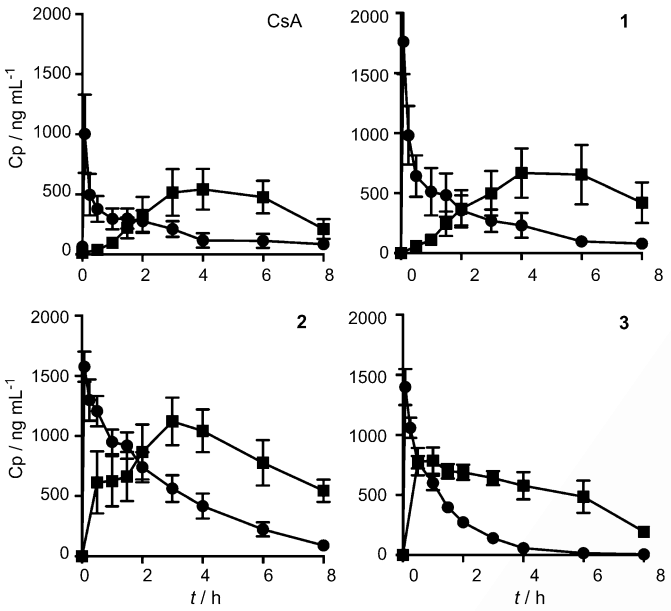

Figure 5. Plasma concentration–time curves for 1 mg kg^{-1} i.v. (administered in DMSO; ●) and 10 mg kg^{-1} oral (in olive oil; ●) for CsA, **1**, **2** and **3**. Cyclic peptide **2** has a reported oral bioavailability of 28% when administered in 10% self-emulsifying system/90% water.[7a] Pharmacokinetic parameters: CsA: $t_{1/2}=192\pm64$ min, $CL_{total}=24\pm15$ mL min^{-1} kg^{-1}, $C_{max}=576\pm192$ ng mL^{-1}, $AUC_{p.o.}=2918\pm882$ ng h mL^{-1}, $F=22\pm2$%, **1**: $t_{1/2}=121\pm24$ min, $CL_{total}=9.5\pm3.5$ mL min^{-1} kg^{-1}, $C_{max}=768\pm237$ ng mL^{-1}, $AUC_{p.o.}=3713\pm1270$ ng h mL^{-1}, $F=18\pm3$%, **2**: $t_{1/2}=122\pm12$ min, $CL_{total}=4.4\pm0.8$ mL min^{-1} kg^{-1}, $C_{max}=1144\pm178$ ng mL^{-1}, $AUC_{p.o.}=6394\pm1327$ ng h mL^{-1}, $F=16\pm3$%, **3**: $t_{1/2}=57.9\pm7.6$ min, $CL_{total}=10.7\pm1.1$ mL min^{-1} kg^{-1}, $C_{max}=878.5\pm90.6$ ng mL^{-1}, $AUC_{p.o.}=4320\pm676$) ng h mL^{-1}, $F=30\pm7$%.

trations at 1–3 h) than for **1** and CsA (maxima at 4–6 h). The fast absorption of **2** and **3** hints at the involvement of active transport, whereas the more flexible **1** and CsA can potentially vary their degree of polar surface exposure, thereby permitting greater partitioning to other compartments than for **2** and **3**. This appears to be supported by different volumes of distribution (CsA: 4.5 ± 3.0 L Kg^{-1}; **1**: 1.6 ± 0.5 L Kg^{-1}; **2**: $0.7(\pm0.6)$ L Kg^{-1}; **3**: $0.9(\pm0.05)$ L Kg^{-1}), with the more rigid **2** and **3** being more evenly distributed in the body water than the more flexible **1** and CsA, which might undergo more compartmental partitioning. The locked conformations of **2** and **3** might favour membrane permeability, or perhaps there is a transporter that shuttles peptides into epithelial cells of the intestinal lumen. Peptide transporters are highly expressed in the upper intestine, some with specificity for peptides with tyrosine side chains and thus improved oral bioavailability.[11] Rigid cyclic peptides like **2** and **3** retain the turn conformation that might be better recognized[10] by a transporter protein than the more flexible **1**. Oral bioavailabilities were 18 ± 3% (**1**), 16 ± 4% (**2**), 30 ± 7% (**3**), and 21 ± 3% (CsA), thus suggesting that rigidity leads to greater oral bioavailability.

In conclusion, we recently showed that protection of polar patches on cyclic peptide surfaces from water increased intestinal absorption after oral delivery to rats.[12] ■■Here =*the present publication or your 'recent' publication? Please clarify*■ ■ we compared compounds (**1**, **2** and **3**) with identical H-bond donors (HBD = 3), H-bond acceptors (HBA = 13) and ■■tPSA *please define*■■ (tPSA, 159.7), and found that reductions in molecular weight (785 > 755 > 725), rotatable bonds (12 > 10 > 8), and hydrophilic surface area (FISA 127.5 > 122.5 > 110.4 Å^2) all favour oral bioavailability. Successive incorporation into **1** of one proline (**2**), then two (**3**), was expected to rigidify the cyclic peptide. CD and NMR spectroscopy and molecular dynamics simulations were used to analyse their structures in polar and nonpolar solvents. The combination plots of Ramachandran phi/psi angles, N–H···OC angles, and NH···OC versus N···OC distances (Figure 4) proved to be a very sensitive measure of conformational flexibility/rigidity—more effective than simply inspecting the overall ensemble of NMR-derived structure calculations. It was found that replacing two N-Me-D-leucines (**1**) with two D-prolines (**3**) did indeed significantly rigidify the cyclic peptide backbone, substantially reduce polar surface area, significantly increase membrane permeability and liver microsome stability, and qualitatively increase oral bioavailability in rats. Conventional predictors of oral bioavailability for small organic molecules (e.g., calculated log *P*, tPSA, permeability assays, in vitro metabolic stability, etc.) did not seem to correctly predict oral bioavailability for these peptides, with CsA and **1**–**3** all violating rule-of-five and associated parameters.[4] In vitro passive permeability and liver microsomal stability (Table 2) did not predict good pharmacokinetic profiles, but in vivo measurements showed appreciable oral absorption into plasma and stability in the circulation. This study has suggested that both rigid and flexible compounds can have some oral bioavailability, perhaps for different pharmacokinetic reasons (■■Figure 6 A, B vs. C, D *Figure 5? (but this is not labelled "A"–"D")*■■). Overall the results are consistent with oral pharmacokinetics and bioavailability being enhanced by structural rigidity, as recently reported for cyclic heptapeptides,[12] rather than by a capacity for the conformational transition highlighted by Scheme 1.

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Keywords: cyclic peptides · NMR spectroscopy · oral bioavailability · peptides · permeability

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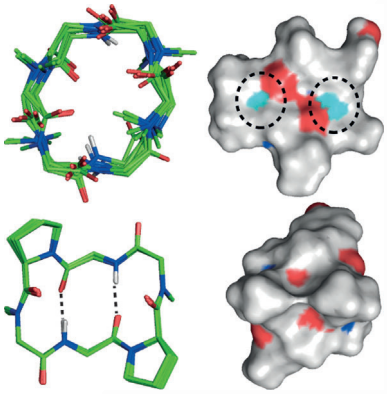

Three similar cyclic peptides with varying flexibility were compared for membrane permeability, oral bioavailability and metabolic stability. We show that more-rigid structures performed better, possibly as a result of the reduced exposure of polar surfaces and lower entropy penalty associated with crossing a membrane.

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