Identification of an endocyclic "structural pin" interaction and its significance for conformational control of macrocyclic scaffolds

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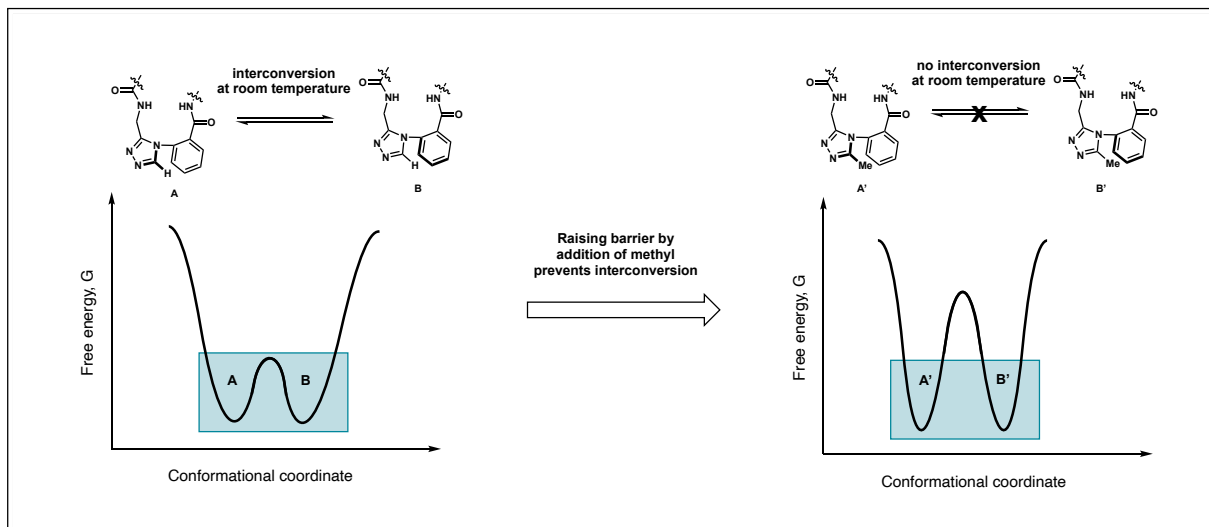   E-mail: andrei.yudin@utoronto.ca

Abstract: While peptide macrocycles with rigid conformations have proven to be useful in the design of chemical probes against protein targets, conformational flexibility and rapid conformational interconversion can be equally vital for biological activity. This study introduces the concept of a "structural pin", which represents an intramolecular hydrogen bond that controls overall ring conformation and can be used to explore macrocyclic conformational energy landscape. Characterization and structural analysis of macrocycles with an endocyclic Brønsted base using NMR and molecular modelling indicates that removal of the structural pin drastically influences the conformation of the entire ring, resulting in novel states with increased conformational heterogeneity. These results suggest that local interactions around structural pins can be effective in controlling overall macrocycle conformation, offering a useful conceptual framework for stabilizing bioactive molecules.

A variety of structural motifs have been developed for constraining peptides sequences.[1] These constraining motifs include, but are not limited to, D-amino acids,[2] hydrocarbon-stapled peptides,[3] and heterocyclic amide isosteres.[4] Recent studies in our lab have shown that incorporation of dominant rotors that limit rotational freedom can stabilize two major conformational ensembles, or "wells", in solution.[5] High rotational barriers embedded in these constructs significantly restrict the conformational space a peptide macrocycle can sample in solution and can prevent switching between the two conformational ensembles in solution (Figure 1A). Although rigidified conformations play an important role in the design of therapeutic agents and chemical probes, conformational heterogeneity can also be an important determinant of the functional behavior of a macrocycle.[6] For example, cyclosporine A relies on a switch between conformations to enter cell membranes.[7] Increasing backbone flexibility has led to the reduction of the k_{off} for stapled peptide macrocycles binding to receptor protein 14-3-3.[8] Conformational selection and multi-step, dynamic association processes are common in protein-protein interactions,[9] and strategies for controlling the conformational state of potential inhibitors are in demand. We have sought out to design a system with increased coverage of macrocycle conformational space. With a focus on the locations where key transannular interactions occur, we hypothesized that small modifications in those regions would engender the most significant effect on overall conformation. We term these interactions "structural pins," which represent hydrogen bonds that stabilize overall macrocycle backbone conformation by impeding local bond rotation. Identification of structural pins should enable a better selection of site modifications in conformation/activity studies. This is because minor structural modifications of a macrocycle can have a significant effect on overall conformation,[10] while the magnitude of modification can be unpredictable and highly dependent on local interactions.[10]
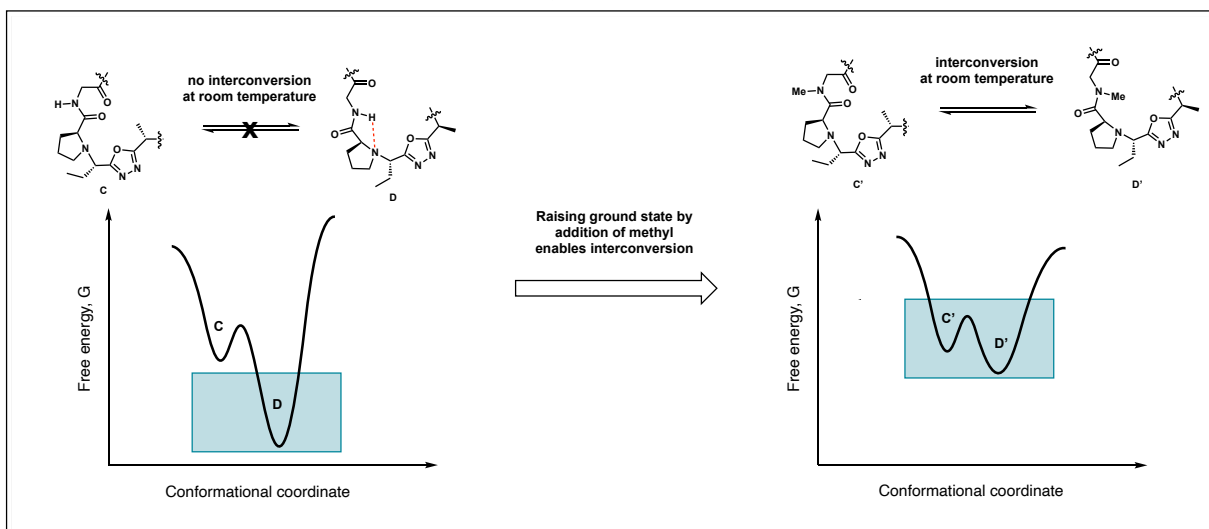
Using the dominant rotor systems described previously, the accessible conformational space of a macrocycle can be restricted by increasing the barrier to interconversion about a biaryl linkage[5] (Figure 1A). To provide a complementary method to the dominant rotor approach that can be used to increase conformational heterogeneity while also influencing the ground state conformation, we considered systems in which the ground state conformation is equipped with a hydrogen bond (a structural pin) that stabilizes overall macrocycle conformation by impeding local bond rotations. For a system in which a single

conformational state is stabilized by this construct, modification of the pin would raise the ground state conformational energy such that
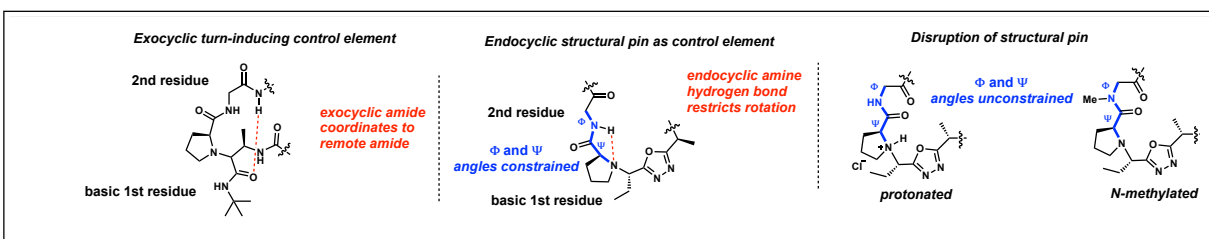

A. Dominant Rotor approch to controlling conformational heterogeneity (modifying barrier energy)

interconversion at room temperature

no interconversion at room temperature

Raising barrier by addition of methyl prevents interconversion

Free energy, G

Conformational coordinate

B. Structural Pin approach to controlling conformational heterogeneity (modifying ground state energy)

no interconversion at room temperature

interconversion at room temperature

Raising ground state by addition of methyl enables interconversion

Free energy, G

Conformational coordinate

C. Types of structural pins and methods for disruption

Exocyclic turn-inducing control element

2nd residue

exocyclic amide coordinates to remote amide

basic 1st residue

Endocyclic structural pin as control element

endocyclic amine hydrogen bond restricts rotation

2nd residue

Φ and Ψ angles constrained

basic 1st residue

Disruption of structural pin

Φ and Ψ angles unconstrained

protonated

N-methylated

Figure 1. A. & B. Depiction of hypothetical conformational spaces (blue box indicates conformational space accessible at room temperature) C. Comparison of exocyclic and endocyclic structural pins along with protonation and methylation as strategies for disruption of the pin

other conformational states become accessible (Figure 1B). The barrier to interconversion is expected to be reduced (Figure 1B). The appearance of a second conformation in solution would imply that the barrier to conformational transition is low

enough that it can be traversed at room temperature. Similar strategies aimed at ground state destabilization have been described in enzyme design, but have not received attention by the peptide community.[11]
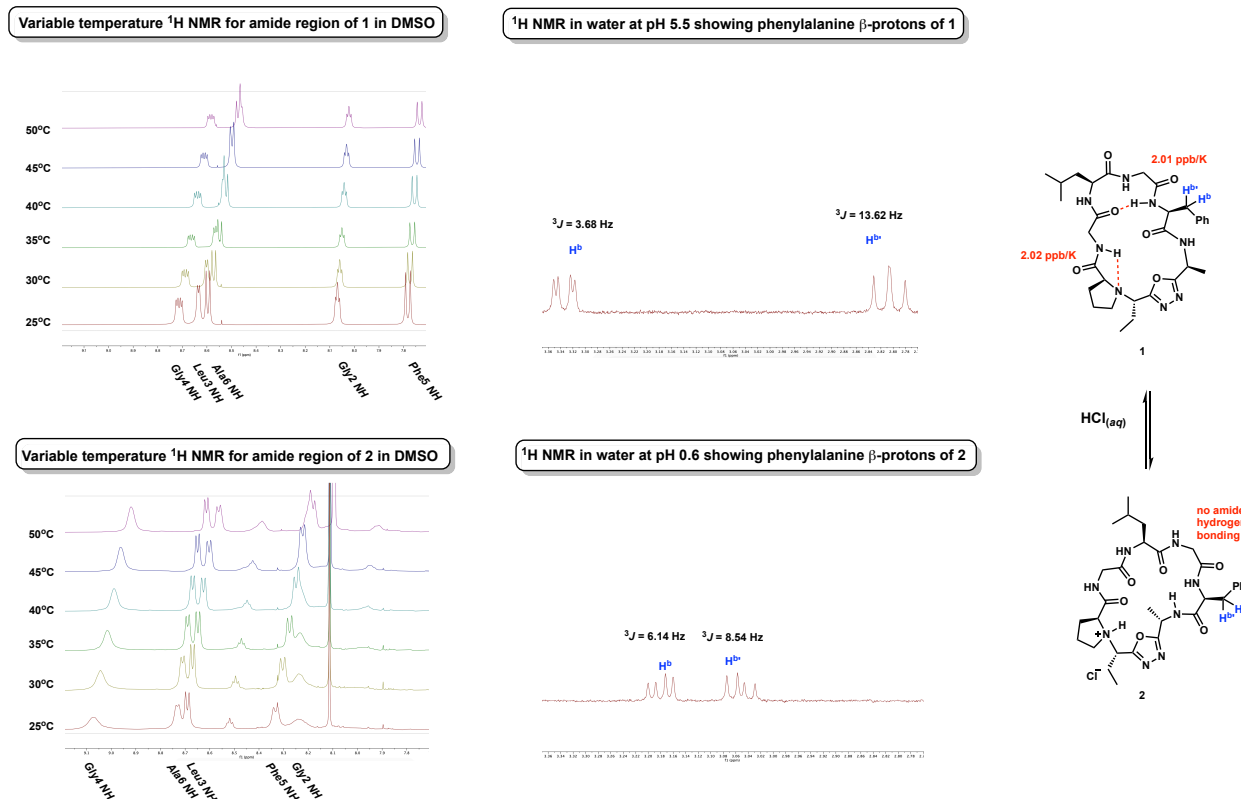

Figure 2. Disruption of intramolecular hydrogen bonding by amine protonation and remote side-chain flexibility via amine protonation

Earlier studies have identified that the combination of an endocyclic tertiary amine and an exocyclic amide in a macrocycle resulted in the exocyclic amide forming a hydrogen bond with the third residue N-H while the amine did not form interactions with the macrocycle[13] (Figure 1C). Investigations into amine/heterocycle combinations (reduced amide/heterocycle, or RAH motifs) revealed that tertiary backbone amines in oxadiazole-containing peptidic macrocycles routinely participate in hydrogen bonding to proximal amide protons and exhibit significantly reduced $pK_{B's}$[14] (Figure 1C). We hypothesized that this hydrogen bonding interaction is a structural pin for the entire macrocycle conformation. Reduced amide peptide linkages can induce turns and kinks in linear and cyclic peptides, however they typically interact with the rest of the peptide backbone via a protonated ammonium at physiological pH.[15] This is not the case for the RAH macrocycles. The RAH macrocycles have successfully been used as scaffolds to display the leucine-threonine-aspartate tripeptide motif in the same conformation as it adopts in mucosal addressin cell adhesion molecule-1 (MAdCAM-1). This enabled *in vitro* inhibition of integrin binding to MAdCAM-1, making these macrocycles biologically relevant scaffolds.[14] At neutral pH these RAH macrocycles adopt single conformations with spectral indications of reduced conformational heterogeneity relative to their homodetic analogs.

The hydrogen bond between the endocyclic amine and the adjacent amide is present in all macrocycles of this structural class and is presumed to play an important role in the conformational stability they display. The RAH macrocycles have a strong tendency to adopt β-turns, regardless of amino acid sequence.[14] Disrupting this conserved hydrogen bond on the overall organization of the macrocycle has enabled us to determine its importance for the overall conformational stabilization of the macrocycle (Figure 1C). Due to the coupled nature of bond rotations in cyclic compounds,[16] one can imagine that restricting the ψ torsional angle of the first residue and the ϕ torsional angle of the next residue influences the conformation of the entire macrocycle in a manner complementary to the "dominant rotor" effect described for biaryl-containing peptide macrocycles.[5] Specifically, the strong hydrogen bonding interaction seen in **1** behaves as a "structural pin" that significantly restricts the

pyrollidine ψ torsional angle and the φ torsional angle of the second residue (Figure 1C). By protonation of the backbone amine of **1**, the pyrollidine nitrogen is converted from a hydrogen bond acceptor to a hydrogen bond donor, allowing us to investigate the downstream effects of modification of this structural pin on the conformation. Treatment of **1** with aqueous HCl to furnish **2** resulted in significant backbone reorganization (Figure 2). To understand the differences in hydrogen bonding between the two macrocycles, variable temperature ^{1}H NMR experiments were conducted. A chemical shift dependence of less than 4 ppb/K typically indicates that a proton is involved in an intramolecular hydrogen bond.[17] Interestingly, VT ^{1}H NMR of the HCl salt in DMSO indicated that no amides were involved in intramolecular hydrogen bonding, which is in contrast to **1** that contains two H-bonds. There were also effects on the side chains in the proximal part of the macrocycle. Coupling constants of the phenylalanine β protons moved close to average values while the two resonances moved closer together (Figure 2), indicating free rotation and a greater occupancy of the phenylalanine χ-space.[18] This result highlights an increase in flexibility in an area of the macrocycle remote from the protonated amine. In our initial reports, pH titrations indicated a low pK_B of 1.9 for the amine. As such, we first set out to explain the low pK_B of **1**. In order to do so, we have investigated the thermodynamics of the equilibrium between freebase and protonated macrocycles (Figure 3). The presence of electron-poor heterocycle and adjacent amide do not completely account for the reduced basicity of the macrocyclic compound[14] We postulated that the reduction in measured pK_B of a functional group involved in a intramolecular interaction can be an indicator of its energetic role in entropic stabilization of conformation and can provide insight into its relevance as a structural pin. In order to address this, we measured the pK_B at different temperatures for both **1** as well as the amide containing linear analog (**3**). From this data one can compose a van't Hoff plot, which provides both the entropy and enthalpy change of the equilibrium.[19] The macrocyclic backbone reduces the entropic term of the free-energy of protonation, whilst at the same time reducing the enthalpic term (Figure 3).
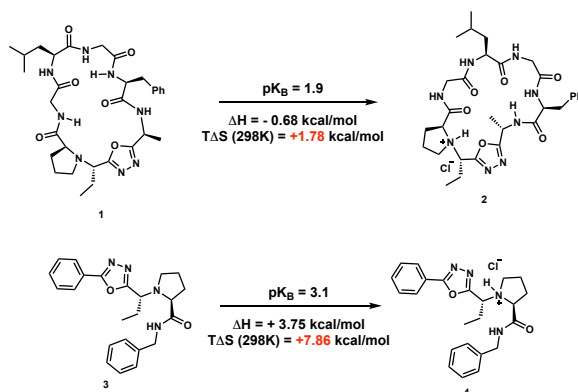
**Figure 3.** Thermodynamic parameters of amine protonation as determined by van't Hoff analysis

The change in the entropic factor is, however, greater than the change in the enthalpic one, causing an overall increase in relative ΔG of 1.64 kcal/mol. The reduction in entropy of protonation for macrocycle **1** relative to linear compound **3** suggests that there is a greater increase in available degrees of freedom when **3** is protonated to give **4**. This makes sense, as **4** is an unrestricted linear ammonium compound while the ammonium group of **2** is still constrained into a macrocycle scaffold. This result, in correlation with the spectral indicators of increased flexibility illustrated to us that protonation of the amine and the subsequent absence of conformational restriction by the structural pin was associated with a change in conformational entropy.[20] Molecular reorganization of proteins and peptidic molecules upon protonation of sidechains has been previously reported. For example, protonation of the lysine residues in the SNase protein results in significant conformational changes.[21] A link between protonation states and macrocycle conformation was also reported by Mügge *et al.*, when they found that cyclic pentapeptides containing a D-aspartate residue would adopt two distinct conformations at different pH values.[22] Based on the protonation state of the aspartate side chain, the macrocycle would either adopt a βII'-turn containing conformation (pH > 5) or a conformation that lacked this turn (pH < 4). However, previous investigations of backbone amine protonation in cyclic pentapeptides have not indicated significant conformational shifts occurring in DMSO.[23] While we were excited that protonation resulted in a new conformational state for **2**, the low basicity of the backbone amine means that the new conformational state will not be accessible at biologically relevant pH values.[24]
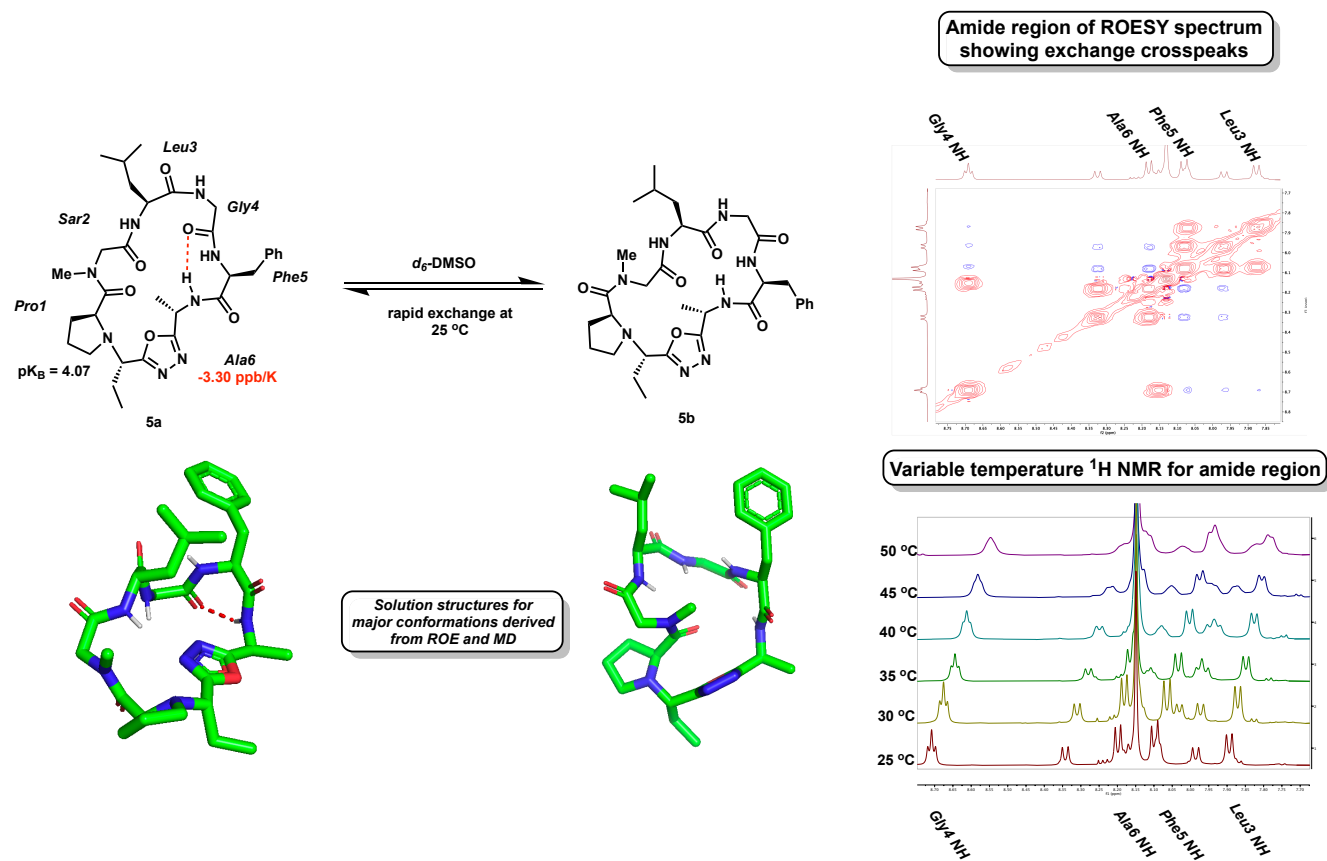
Figure 4. *N*-methylated macrocycle adopts novel hydrogen bonding pattern and backbone conformation while exchanging between *cis* and *trans*

Attempting to mimic the effects of amine protonation with a more stable modification, we moved on to eliminate the possibility of the hydrogen bond interaction via *N*-methylation of the adjacent amide to relax constraints on the local torsional angles. *N*-methylation of internal H-bond donors in peptidic macrocycles is an established method for removing hydrogen bonding interactions in peptides.[25] In small molecule design, methylation can be used to induce a specific conformation via steric interactions.[26] Backbone C-methylation of the 12-membered cyclic clinical candidate lorlatinib locks the conformation of a remote biaryl linkage.[27] The effects of N-methylating peptidic macrocycles are much less predictable. Methylation of heterocycle/peptide macrocycle natural product apratoxin A results in global conformational reorganization,[28] while mono-N methylation of homodetic macrocycles only significantly influences overall conformation if it is employed in combination with a D-residue.[29] N-methylated macrocycle **5** was prepared using previously reported methods[14] and characterized in d₆-DMSO. The appearance of two sets of peaks in the ¹H NMR spectrum was indicative of two conformations interconverting on the NMR timescale. This was confirmed by the appearance of EXSY cross-peaks in the ROESY spectrum (Figure 4).[30] Peak doubling was observed for the α protons of all residues except for Leu4. Additionally, the amide protons displayed peak doubling. The shifting of the backbone amide protons and α protons remote from the *N*-methylation is an indication that the conformational effects were being transmitted across the entire ring. We also evaluated the intramolecular hydrogen bonding pattern of the major conformation (**5a**) using VT ¹H NMR. Chemical shift/temperature coefficients indicated only the Ala6 amide proton was involved in intramolecular hydrogen bonding. NOE-guided MD simulations suggested that it was hydrogen bonding to the Gly4 carbonyl (Figure 4). This is significantly different than the secondary structure that was observed for **1**. Additionally, the $J_{\alpha\beta}{}^3$ coupling values for residue Leu3 was changed from 4.28 Hz for **1** to 7.67 Hz for **5a**, again indicating a disruption of the β-turn motif along with increased conformational flexibility. NOE-derived distances were used to generate solution structures. Conformational searches indicated the *trans* isomer of the Pro1-Sar2 linkage was the major conformation (**5a**). Molecular dynamics and VT ¹H NMR did not indicate any intramolecular hydrogen bonds for the *cis* isomer (**5b**). Overlaps made assessment of phenylalanine β proton coupling constants to analyze phenylalanine χ-space impossible. The basicity of **5,** as determined in water, was found to increase two orders of magnitude relative to **1**, which is another indication that the backbone amine was not involved in intramolecular hydrogen bonding and available to interact with solvent. We then investigated β-branched amino acids threonine (**6**, **7**) and isoluecine (**8**, **9**) at the residue 5 with the objective of observing a change in χ angle

preference, similarly to the effect observed in **2**. The corresponding amide (**6**, **8**) and *N*-methylated (**7**, **9**) derivatives were synthesized and characterized in d$_6$-DMSO as previously described for **5**. Both amide-containing macrocycles **6** and **8** adopted an analogous conformation to **1**, with the same hydrogen bond pattern and turn placement. However, *N*-Me macrocycles **7** and **9** each adopted distinct conformations. Both **7** and **9** displayed exchange between two conformations, analogous to the behaviour of **5**. Although both α to β coupling and MD simulations indicated the fifth residue sidechain did not change conformation upon incorporation of the *N*-Me, the hydrogen bonding patterns and overall backbone conformation changed significantly. The third and sixth residue amide protons were found to be involved in transannular hydrogen bonds for **7**, while MD simulations indicated the threonine side chain was hydrogen bonding with the threonine carbonyl. For **9**, the fourth and fifth residues amides were involved in intramolecular hydrogen bonding. Both **7** and **9** represent novel conformational states for RAH macrocycles. The difference between **7** and **9** is striking, considering the similarity between **6** and **8**. The only structural difference is the fifth residue sidechain. For threonine derivative **7** the side chain is hydrogen bonding to its amide C=O, while for isoleucine compound **9** there is no such interaction. This can account for the conformational difference between the two, however the question then becomes why this interaction does not influence the conformation of **6**. β-branched residues are known to exert significant conformational influence on homodetic cyclic peptides.[18] Presumably, when the amine/amide hydrogen bond is present in **6** and restricting the proline ψ angle this rigidifying effect is transmitted throughout the ring and dominates the conformational preference of the backbone so that the side chain does not affect it (Figure 6). The Thr OH---O=C interaction becomes the "structural pin" for **7**, but it is not a strong enough interaction to cause conformational rigidity, since two conformers are observed. This highlights the strength of the RAH as a unit to control conformation in peptides, but equally, that removal of this interaction is also useful if one wants to access flexible conformations. This enables the RAH unit to act as conformational "hotspot" that is predictably sensitive to N-methylation. This is in contrast to the unpredictable effects of backbone N-methylation in homodetic peptides.[28,29]
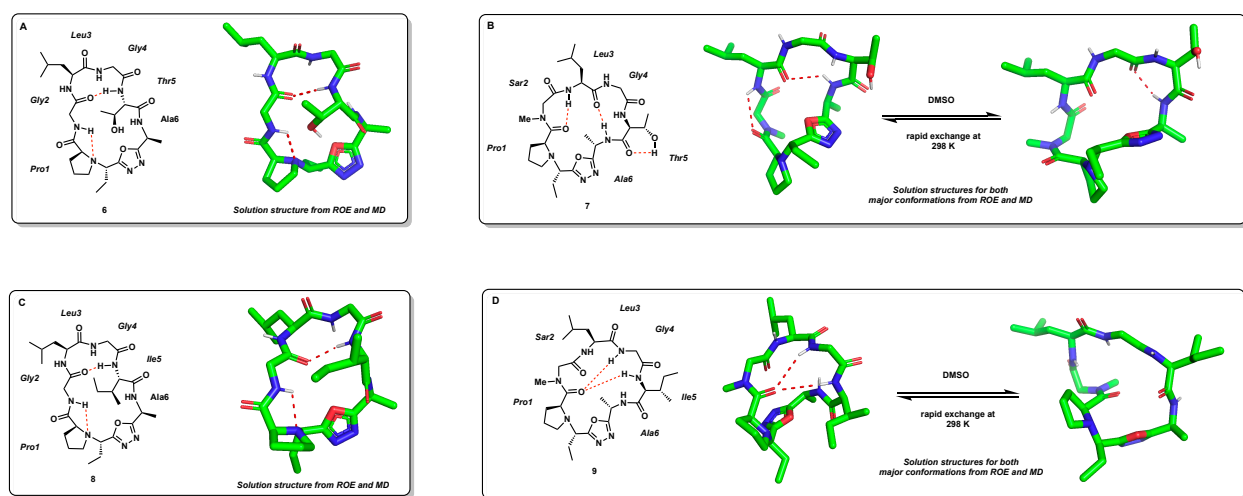
Figure 5. Solution structures illustrate the effect of removing amine/amide hydrogen bond on macrocycles with different side chains. Each inset depicts the chemical structure and the corresponding solution structure from ROE and MD. Insets A) and C) depict N-H macrocycles that adopt a single major conformation in solution. Insets B) and D) depict *N*-methylated macrocycles that adopt two major conformations exchanging

The physicochemical properties of a macrocycle are dependent on its conformational behaviour.[31] We were interested in relating the effects of increasing conformational heterogeneity on lipophilicity. Reverse phase HPLC times are often used as indicators of lipophilicity.[32] Accordingly, we compared the retention time of amide macrocycle **8** and *N*-methylated macrocycle **9**, which differ only by substitution of the amide proton for a methyl group. On a 15 minute gradient from 5% to 95% acetonitrile in water, **8** had a retention time of 5.69 minutes while **9** had a retention time of 5.63 minutes. These values are within the margin of error and suggest that compounds **7** and **8** have essentially the same lipophilicity. This is counterintuitive to conventional medicinal chemistry wisdom, as **9** is lacking a hydrogen bond donor and has an extra lipophilic alkyl group. *N*-methylation of macrocycles is expected to increase retention time versus all-NH congeners.[33] Fairlie *et al.* have predicted *in silico* that addition of methyl groups to the RAH macrocyclic scaffold can increase retention time if they connect hydrophobic surfaces.[34] While this is contrast with our results for **8** and **9**, it is clear that *N*-methylation that specifically prevents conformationally stabilizing transannular hydrogen bonds does not increase lipophilicity, likely due to an increase in backbone interactions with aqueous solvent. When a macrocyclic scaffold containing a non-canonical Brønsted base is modified in proximity to the base, predictive rules can change due to coupled intramolecular interactions. In our case, conventional strategies to influence permeability by extending hydrophobic surfaces no longer apply to the same extent. This is similar to Lokey *et al*'s finding that replacement of too many backbone amide protons with methyl groups can actually decrease

lipophilicity.[35] The ability of Cyclosporin A to permeate membranes while maintaining aqueous solubility and the capacity to engage polar binding is dependent on its conformational heterogeneity.[36] It could be that *N*-methylation of the oxadiazole/amine macrocycle scaffold provides a tool to for the rational design of such behaviour. The RAH macrocycle scaffold provides an approach to influencing permeability that is complementary to the strategy of extending hydrophobic surfaces that was reported by Fairlie.[34]

In summary, we have found that removal of key intramolecular hydrogen bonds that restrict bond rotation in a macrocycle backbone drastically influences the conformation of the entire ring. Introduction of an *N*-Me group on the second amide adjacent to a basic amine to prevent hydrogen bonding increases conformational heterogeneity and results in the appearance of novel conformations. The decreased rigidity of the backbone allows sidechain-backbone interactions to influence conformation and produces conformations distinct from those previously reported for this class of macrocycles. This offers further support for the strong influence backbone Brønsted base can have on overall macrocycle conformation when they can interact with other functionality, along with acting as a structural pin on the macrocycle that is predictably conformationally sensitive to modification. This is unlike the relatively insignificant effects of N-methylation of backbone amides in a homodetic peptide. The incorporation of the *N*-Me group did not increase the lipophilicity of the macrocycle although it is adjacent to other hydrophobic surfaces, suggesting that stabilizing effect of intermolecular interactions must be considered when optimizing the lipophilicity of a macrocycle. The finding that overall conformational heterogeneity changes when interactions of the basic amine are modified should be particularly useful for tuning the flexibility of macrocycles to optimizing entropy of protein binding or designing macrocycles capable switching conformations in various environments. It should also provide a tool for displaying peptide binding motifs in a range of complementary conformations to elaborate conformation-activity relationships while designing macrocycle-protein interactions. Additionally, these results provide a conceptual framework for the identification of structural pins in other macrocycles.

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Author Contributions

S. D. A. and A. K. Y. conceived the idea of the project and wrote the manuscript. S. D. A., N. H. and C. T. V. designed and conducted the experiments. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

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