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Amidine Functionality As a Conformational Probe of Cyclic Peptides

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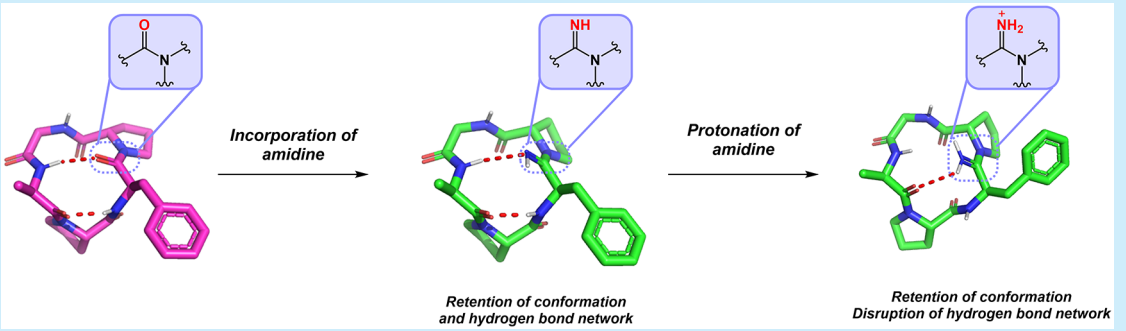


ABSTRACT: The amidine functionality switches between hydrogen bond donor and acceptor roles depending on pH. Herein, the amidine was incorporated to select amides in cyclo(D-Ala-Pro-D-Phe-Pro-Gly). The unprotonated amidine-containing macrocyclic conformation resembles its oxoamide counterpart. Upon protonation, minimal alterations in the macrocyclic conformation were observed despite changes to the hydrogen bond network. The amidine disrupts hydrogen bonding at minimal steric cost, making it a useful functionality to study the effect of hydrogen bonding on the macrocyclic conformation.

Cyclic peptides are a promising class of compounds in drug discovery owing to their resistance to proteolytic degradation relative to linear counterparts and capacity to adopt conformations that can mimic protein binding epitopes.[1,2] Stabilization of these conformations is often caused by the intramolecular hydrogen bond network set by the backbone amides.[3,4] However, the causality in this relationship is still poorly understood.[5]

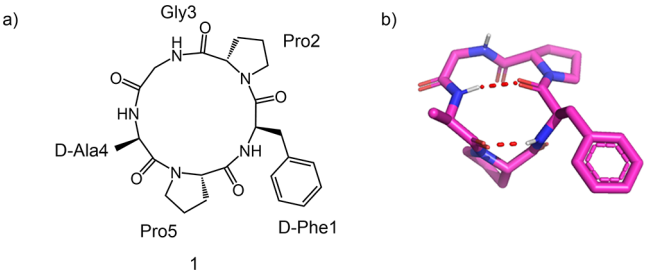
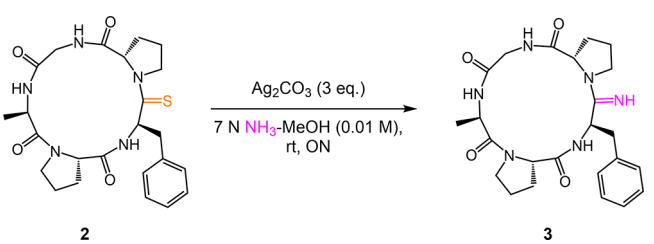


Figure 1. (a) Cyclo(D-Ala-Pro-D-Phe-Pro-Gly) and (b) its solution conformation (298 K, DMSO-d_6).

Scheme 1. Incorporation of Thioamide to Amidine via Thiophilic Lewis Acid



Replacement of the amide functionality with an isostere alters the torsional freedom of the backbone and the hydrogen bond donor/acceptor properties.[4,6] Amidines are relatively underexplored peptide bond isosteres with many physical properties similar to amides, particularly when it comes to barrier to rotation[7] due to resonance stabilization.[8] Amidines are found in nature, exemplified by birnbaurmin, pyrostatin, and noformycin.[9] They have also been incorporated into known macrocycles such as vancomycin[10,11] and FC131.[12,13] In Boger's study, the incorporation of amidine into vancomycin resulted in modulated binding to the precursor peptidoglycan termini, D-Ala-D-Ala and D-Ala-D-Lac, presumably because removal of the amide lone pair caused a repulsive interaction between D-Ala-D-Lac and vancomycin. In its unprotonated state, the amidine lone pair was shown to act as a hydrogen bond acceptor upon interaction with the NH amide of the D-Ala-D-Ala fragment. Upon protonation ($pK_a \sim 12.4$), the amidine functionality

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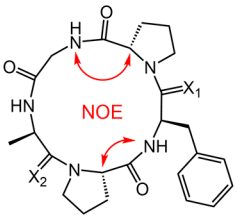
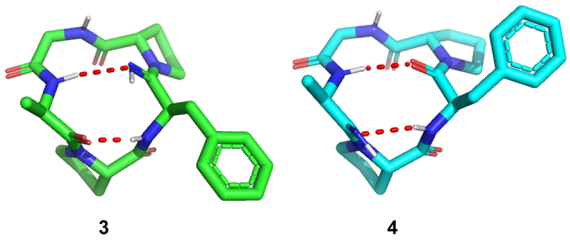


Compound 1: X_1 = O, X_2 = O

Compound 3: X_1 = NH, X_2 = O

Compound 4: X_1 = O, X_2 = NH

b)



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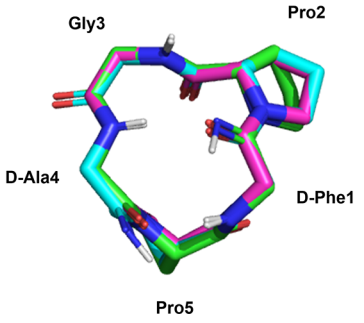

Temperature Coefficient (ppb/K)

Residues	Compound	1	3	4
	D-Phe1	-1.78	-3.93	-2.11
	Gly3	-5.62	-4.20	-6.00
	D-Ala4	-0.32	-1.96	-1.46
	Amidine-NH		-1.19	N/A

Figure 2. (a) ROESY and VT NMR of **1**, **3**, and **4**. (b) Conformation of **3** (green) and **4** (blue) with its hydrogen bonding (red dash). (c) Overlay with **1** (pink), **3** (green), and **4** (blue).

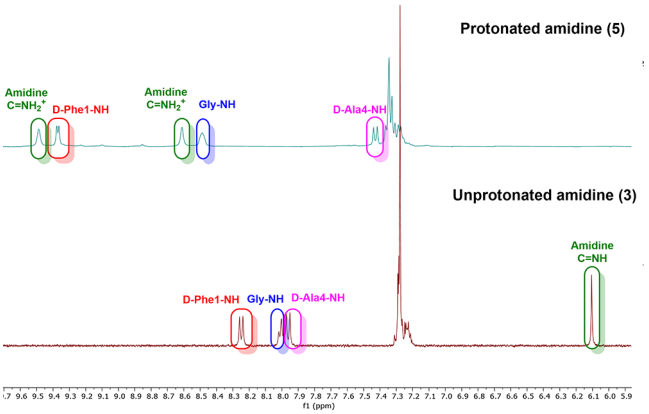

Figure 3. Amide region of ^{1}H NMR for **3** (below) and **5** (above).

switched to the hydrogen bond donor and bound to the modified D-Ala-D-Lac fragment. Despite these advances, little is known about the conformational preferences of cyclic peptides that bear amidines. The switch between the hydrogen bond acceptor to donor may impart significant changes to the backbone conformation of cyclic peptides. pH-dependent conformational switching has been shown to increase oral bioavailability in certain small molecules owing to their ability to adopt a more hydrophobic conformation,[14] which provides additional motivation to consider the "amidine effect" in peptides.

In this study, amidines were incorporated into carbonyl sites that act as hydrogen bond acceptors in cyclo(D-Ala-Pro-D-Phe-Pro-Gly) **1** via the thioamidated macrocycles upon treatment with a Lewis acid and ammonia. Using NMR analysis (^{1}H, ^{13}C, ROESY, variable temperature),[15,16] the effect of the protonated and unprotonated amidine functionality on the peptide conformation has been evaluated. We find that amino acid side chains appear to be the main conformational determinants, with intramolecular hydrogen bonding playing a relatively insignificant role. Overall, the amidine functionality offers a curious counterpart to better known reduced amide bond isosteres. The latter are also protonated under physiological conditions yet are wholly different in terms of flexibility and the resulting conformational properties.[4,12]

To compare and contrast amidine-containing macrocycles with their oxoamide counterparts, we turned to a known cyclic pentapeptide, cyclo(D-Ala-Pro-D-Phe-Pro-Gly) **1**.[17] This molecule adopts a type-II β-turn between Pro2-Gly3 and a γ-turn around Pro5 in both the solution conformation and the crystal structure (Figure 1). As a regioselective handle for amidine incorporation, thioamide was introduced into the linear peptide based on procedure by Rapoport et al.[18,19] Conversion of thioamides to amidines is typically accomplished utilizing thiophilic Lewis acids.[11,20] Using Ag_2CO_3 with ammonia in methanol solution afforded macrocycle **3** in 2 h with a modest yield of 59% (Scheme 1). ^{1}H NMR of **3** in DMSO-d_6 showed sharp, distinguishable peaks, consistent with a well-defined conformation on the NMR time scale.[21]

Using variable-temperature (VT) NMR, backbone NHs were determined to be either solvent exposed or undergoing H-bonding based on temperature coefficient values (<−4.6 ppb/K suggests solvent exposed, ≥−4.6 ppb/K suggests H-bonding).[16] The amidine macrocycle **3** featured two protons (D-Ala4-NH and amidine-NH) that were significantly above the −4.6 ppb/K threshold, indicating involvement in intramolecular hydrogen bonds. Additionally, temperature coefficients of Gly3-NH (−4.2 ppb/K) and D-Phe1-NH (−3.93 ppb/K) suggest that these protons are on the cutoff for intramolecular hydrogen bonds. The ROESY spectrum of compound **3** showed strong NOE coupling from Gly3-NH to CαH-Pro2 and D-Phe1-NH to CαH-Pro5 (Figure 2a).

To further elucidate the conformation of compound **3**, a low energy structure was generated using a Monte Carlo conformational search (OPLS3e) using distances derived from the ROESY experiment and dihedral angles from the ^{1}H NMR. A type-II β-turn was observed between Pro2-Gly3, where hydrogen bonding was evident between D-Ala4-NH and D-Phe1-C═O. A γ-turn was observed around Pro5 where

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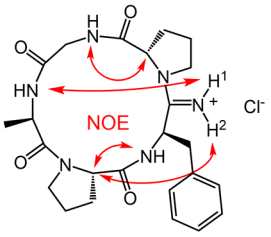

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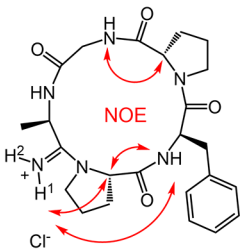

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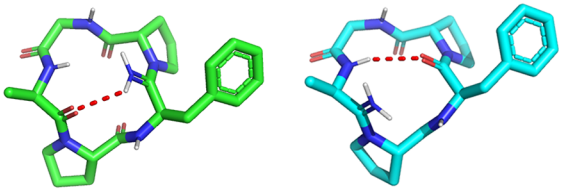

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c)

Temperature Coefficient (ppb/K)

Residues	5	6
D-Phe1	-6.20	-0.33
Gly3	10.6	-7.59
D-Ala4	-3.76	-0.95
Amidine-NH^1	4.02	-1.43
Amidine-NH^2	-2.39	-2.69

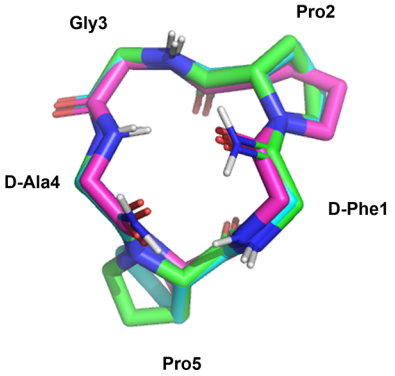

Figure 4. (a) ROESY and VT NMR of **5** and **6**. (b) Conformation of **5** (green) and **6** (blue) with its hydrogen bonding (red dash). (c) Overlay with **1** (pink), **5** (green), and **6** (blue).

hydrogen bonding occurred between D-Phe1-NH and Pro5-C═O (Figure 2b). These findings matched the solution conformation and the crystal structure of compound **1** and can be verified by the overlay of the solution structures of **1** and **3** (Figure 2 c). The macrocycle **4**, which featured amidine at the D-Ala residue, showed a similar conformation to **1** and **3** (Figure 2c). The incorporation of an unprotonated amidine did not perturb the conformation of **1** and maintained the hydrogen bonding pattern. This suggests that the amidine in its unprotonated state has similar hydrogen bond acceptor capacity as its carbonyl counterpart.

Upon protonating compound **3**, the ^{1}H NMR spectrum of **3** and **5** showed significant changes in chemical shifts, especially for the amidine-NH (Figure 3). The amidine proton of **3** (∼6.1 ppm) shifted downfield, and two peaks (∼8.6 ppm and ∼9.5 ppm) corresponding to the two protons of amidine of **5** were observed.

The VT NMR of compound **5** suggests a strong hydrogen bonding at amidine-NH^1 (−2.39 ppb/K). Weaker hydrogen bonds were observed for D-Ala4-NH (−3.76 ppb/K) and amidine-NH^2 (4.02 ppb/K). This hydrogen bonding pattern differed from compound **1** as no hydrogen bonding was observed for D-Phe1-NH in compound **5** (Figure 4a). In compound **6**, temperature coefficients of D-Phe1-NH (−0.33 ppb/K) and D-Ala4-NH (−0.95 ppb/K) indicated that these two residues were undergoing hydrogen bonding, while Gly3-NH (−7.59 ppb/K) did not. Additionally, hydrogen bonding appeared to occur for the two amidine protons (NH^1 = −1.43 ppb/K and NH^2 = −2.69 ppb/K). Similar NOE couplings were observed for **1**, **5**, and **6** where NOE signals of both Gly3-NH to CαH-Pro2 and D-Phe1-NH to CαH-Pro5 were observed. In compound **5**, additional NOE signals of D-Ala4-NH to amidine-NH^1 and CαH-Pro5 and amidine-NH^2 were observed. NOE signals of D-Phe1-NH to amidine-NH^1 and CαH-Pro5 and amidine-NH^1 in compound **6** were also observed.

The conformations of **5** and **6** given by the ROESY-restrained search (Figure 4b) showed similarity to macrocycle **1**, as seen in the overlay (Figure 4c). Slight deviation of the hydrogen bonding pattern from compound **1** was seen in compound **5** as D-Phe1-NH was solvent-exposed and not involved in hydrogen bonding. Additionally, no hydrogen bonding to D-Ala4-NH was observed, and a possible hydrogen bond between amidine-NH^2 and D-Ala4-C═O was seen in compound **5**. The conformation of **6** showed only D-Phe1-C═O and D-Ala4-NH participating in hydrogen bonding to form a type-II β-turn. This observation deviates from what the VT NMR showed for residue D-Phe1-NH (−0.33 ppb/K). The 20 minimized energy conformations for **5** and **6** showed retention of the β-turn portion of the molecule (see Figures S1 and S2). However, D-Phe-NH areas showed some flexibility, which explains the observed NH temperature coefficient. Alternatively, this discrepancy may also be due to the deshielding effects caused by the adjacent aromatic ring.[22]

Disruption of intramolecular hydrogen bonding in cyclic peptides has been explored in Snyder's work on roseotoxin B,[5] where hydrogen-bonded amides were substituted to esters, and in Robinson's work where amides undergoing hydrogen bonding were replaced with *N*-methyl amide.[23] The alteration of hydrogen bonds was done at the hydrogen bond donor site. In the case of the amidine, hydrogen bonding was disrupted by altering the hydrogen bond acceptor site upon protonation. This provides a complementary method to interrogate the conformational consequences of intramolecular hydrogen bonding. For macrocycles **5** and **6**, protonation of the amidine blocked off the lone pair that originally participated in an intramolecular hydrogen bond, switching the amidine role from the hydrogen

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bond acceptor to the hydrogen bond donor and changing the intramolecular hydrogen bonding pattern. However, the general backbone conformation of both macrocycles was retained. Conformational rigidity was previously recorded in Snyder's work on roseotoxin B, where hydrogen bonds displayed a considerably smaller effect on the conformation of small cyclic peptides.[5] Unlike proteins, where there are many cooperative hydrogen bonds that are stronger than a single hydrogen bond by up to 25%,[24] small cyclic peptides can have only a few hydrogen bonds. In addition, the partial double bond characteristics of the amide backbone, the rigidifying proline residue, and sterically bulky side chains may be determining the structure of small peptides. These features stabilize the conformation, which sets the intramolecular hydrogen bond network. With the parent cyclic pentapeptide **1**, stabilizing structural elements such as the proline side chains and the potentially sterically bulky D-Phe are present. Although the amidine functionality disturbs the original hydrogen bonding interaction of the cyclic peptide backbone, its steric contribution is insignificant and has a minimal role in setting the conformation of the cyclic peptide. These results highlight the dominance steric effects have over intramolecular hydrogen bonding when it comes to the conformation of cyclic peptides.

In summary, the conformational influence of the amidine functionality on cyclic pentapeptides has been investigated. When an amidine was substituted for a carbonyl group that acts as a hydrogen bond acceptor, the retention of both the backbone conformation and intramolecular hydrogen bonding was observed in unprotonated form of amidine. Upon protonation of the amidine, changes in the intramolecular hydrogen bonding interactions were recorded. However, minimal consequences to the overall conformation of the macrocycle have taken place. This sheds additional light on the balance between the conformational preferences induced by side chains and transannular hydrogen bonds in cyclic peptides. The replacement of hydrogen bond accepting carbonyls with amidines in flexible cyclic peptides may have a more significant effect on the overall backbone conformation. This is a topic of an ongoing investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c03369.

Experimental details, including synthesis, HRMS data, NMR spectra, VT-NMR, and solution structure determination (PDF)

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Notes

The authors declare no competing financial interest.

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