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Letter

Stabilization of Azapeptides by N_{amide}···H−N_{amide} Hydrogen Bonds

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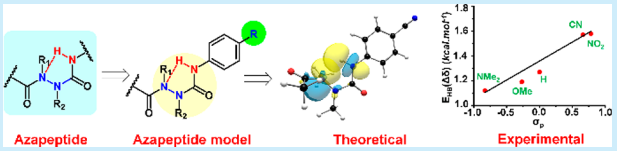
ABSTRACT: An unusual N_{amide}···H−N_{amide} hydrogen bond (HB) was previously proposed to stabilize the azapeptide β-turns. Herein we provide experimental evidence for the N_{amide}···H−N_{amide} HB and show that this HB endows a stabilization of 1−3 kcal·mol^{-1} and enforces the trans−cis−trans (t−c−t) and cis−cis−trans (c−c−t) amide bond conformations in azapeptides and *N*-methyl-azapeptides, respectively. Our results indicate that these N_{amide}···H−N_{amide} HBs can have stabilizing contributions even in short azapeptides that cannot fold to form β-turns.



Azapeptides are generated by isosterically substituting the α-carbon of one or more amino acid residues of a peptide with nitrogen atoms.[1] This substitution endows azapeptides with favorable drug-like properties including higher conformational rigidity and enhanced proteolytic stability compared with α-peptides.[2] Over the years, many azapeptide-based bioactive molecules have been discovered. Most notably, atazanavir (Reyatazt), a U.S. Food and Drug Administration (FDA)-approved antiretroviral drug, is a potent azapeptide inhibitor of the HIV protease.[3] In addition, azapeptide inhibitors of serine and cysteine proteases,[4] human neutrophil protease,[5] hepatitis A virus (HAV) 3C protease,[6] and hepatitis C virus NS3 serine protease[7] have also been developed.

Azapeptides predominantly adopt β-turn conformations (Figure 1A).[8] The conformational properties of azapeptides are explained by the lone-pair−lone-pair repulsion of the adjacent hydrazide nitrogen atoms (N_{lp}−N_{lp} repulsion). Some previous studies proposed two hydrogen bond (HB) interactions in azapeptide β-turns having aza-alanine,[8f] aza-asparagine[8f] (Figure 1A, X = O), and *N*-amidothiourea[8b,d] (Figure 1A, X = S) groups at the $(i + 2)$ position. One is the conventional $i \rightarrow (i + 3)$ C═O···H−N HB often found in peptide β-turns (Figure 1A, in blue), and the other is an unusual N_{amide}···H−N_{amide} HB between the hydrazide nitrogen at the $(i + 2)$ position and the NH hydrogen at the $(i + 3)$ position (Figure 1A, in red). These two HBs were also previously observed in the crystal structure of an azapeptide VI β-turn when aza-proline was incorporated at the $(i + 2)$ position, which induced two cis-amide bonds on both sides of the N−N bond.[8g] Lee et al. proposed a role of N_{amide}···H−N_{amide} HBs in the conformational properties of *N*-methyl-azapeptides using theoretical methods.[9] The N_{amide}···H−N_{amide} HB is unusual because the amide nitrogen lone pair is delocalized over the antibonding π orbital of the carbonyl (CO) group (π^*_{CO}) and should be less available to participate in the HB. Moreover, this HB orientation is not linear but perpendicular, which indicates the possibility of a weak HB. Nonetheless, the role of N_{amide}···H−N_{amide} HBs in the protein structure and stability was proposed.[10] More recently, Romesberg and coworkers performed experimental and theoretical studies to show the role N_{amide}···H−N_{amide} HBs in protein structure and stability.[11] Furthermore, N_{amide}···H−N_{amide} HBs were also studied in proteins using statistical and computational methods.[12,13] Lectka and coworkers also showed that in proteins, N_{amide}···H−N_{amide} HBs can work as intramolecular catalysis for the proline amide bond isomer-

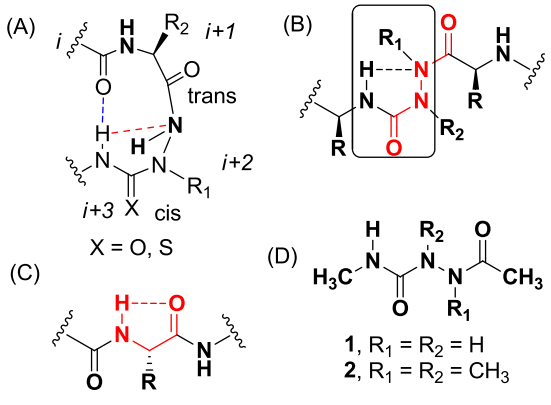


Figure 1. (A) Azapeptide β-turn having the proposed C═O···HN and N_{amide}···H−N_{amide} HBs. (B) Proposed N_{amide}···H−N_{amide} HB in azapeptides. (C) C5 C═O···H−N HB observed in peptide β-sheets.[15] (D) Chemical structures of azapeptide models **1** and **2**.

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ization, a key step in the folding of several proteins including dihydrofolate reductase.[14] In a recent study, Raines and coworkers also highlighted the importance of a perpendicular C5 C=O···H–N HB in proteins.[15] In this Letter, we show the stabilization of short azapeptides by N_{amide}···H–N_{amide} HBs by using spectroscopy, X-ray crystallography, and theoretical studies.

Ottersbach et al. showed that N-methylation of the peptide bond in model azadipeptides leads to the *cis*-amide bond (E) configuration (N–N–C=O ≈ 180°), but there was no mention of a N_{amide}···H–N_{amide} HB.[16] We envisaged that in the E conformation (cis) of azapeptide amide bonds, one of the hydrazide nitrogen lone pairs could interact with the vacant H–N σ^* orbital (σ^*_{H-N}) to form a N_{amide}···H–N_{amide} HB (Figure 1B). This prediction was supported by our recent observation of an n_N(amide) → σ^* interaction in *N,N′*-diacylhydrazines[17] and the observation of a N_{amide}···H–N_{amide} HB in the aza-Pro system by Lecoq et al.[8g] Because the azapeptide contains a biologically important semicarbazide[18] motif (the fragment within the box in Figure 1B) within its backbone, to probe the proposed N_{amide}···H–N_{amide} HB, we first carried out a detailed conformational analysis of 1-acetyl-4-methyl-semicarbazide (Ac-azaGly-NHMe (**1**)) and its 1,2-dimethyl analogue (Ac-(NMe)-azaAla-NHMe (**2**)) (Figure 1D) as azapeptide models. Compounds **1** and **2** were previously shown to be suitable models to study the conformational properties of azapeptides.[9] The theoretical calculations predicted the cis–cis–trans (c–c–t) and trans–cis–trans (t–c–t) conformations of **1** to be very close in energy (Figures S1 and S2A and Table S1); however, when the hydrazide nitrogen atoms were methylated (**2**), the c–c–t conformation with both of the hydrazide amide bonds in the cis (E) geometry was at least 3 kcal·mol^{-1} more stable than all other isomeric forms of **2** (Figure 2A and Table S2), which is

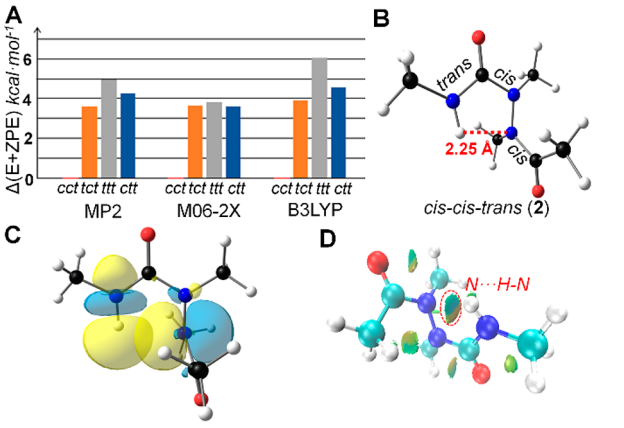

Figure 2. (A) Zero-point-energy-corrected relative electronic energies of **2** obtained using various computational methods. (B) Geometry of **2** optimized at the MP2/6-311+G(2d,p) level. (C,D) NBO orbital interaction and NCI plot showing the N_{amide}···H–N_{amide} HB in **2**.

in agreement with the previous reports.[9,16] Interestingly, in both t–c–t and c–c–t conformations, the amide NH hydrogen is ideally oriented to facilitate a N_{amide}···H–N_{amide} HB interaction with the hydrazide N atom far away from the NH group (Figure 2B and Figure S2B). Natural bond orbital (NBO) and noncovalent interaction (NCI) analyses also showed the presence of the N_{amide}···H–N_{amide} HB interactions in **1** and **2** (Figure 2C,D and Figure S2C,D). NBO analyses indicated a stabilization of ∼1 kcal·mol^{-1} due to this N_{amide}···H–N_{amide} HB, which is considerably higher than the C5 C=O···H–N HBs previously reported.[15]

Inspired by the theoretical results, we synthesized some 1-acyl-semicarbazides and their 1,2-dimethyl counterparts (compounds **2**–**14**) (Figure 3A) as azapeptide models. 2D-

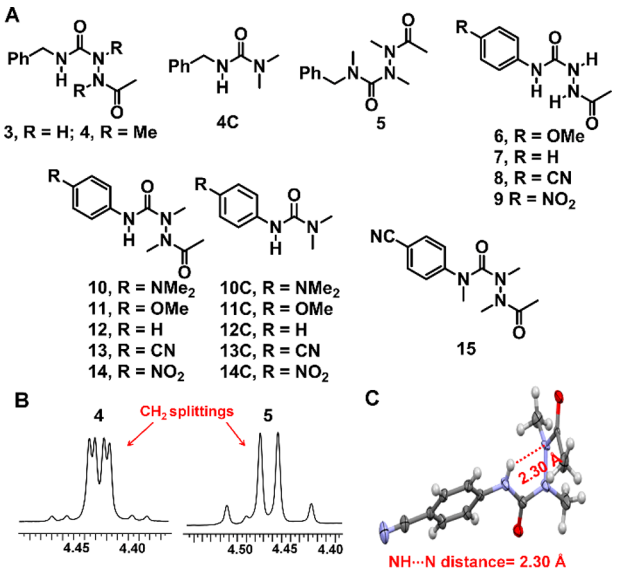

Figure 3. (A) Chemical structures of **3**–**15** and **4C** and **10C**–**14C**. (B) ^{1}H NMR splitting of the benzylic CH_2 hydrogen atoms of **4** and **5** in $CDCl_3$. (C) Crystal structure of **13** (50% probability density ellipsoids) in the c–c–t rotameric form.

NOESY data indicated that the azapeptide models **3** and **6**–**9** predominantly adopted the t–c–t conformation, but *N*-methyl-azapeptide models **2**, **4**, and **10**–**14** predominantly adopted the c–c–t conformation in solution ($CDCl_3$ and DMSO-d_6) (Table S6), which is consistent with the theoretical results. We observed the diastereotopic behavior of the benzylic CH_2 hydrogen atoms in **4** and **5** (Figure 3B). No such behavior of CH_2 hydrogen atoms was observed in **3** that was not N-methylated or in the control molecule **4C** that lacked the N–N bond. These observations indicate that N-methylation of azapeptide models induces chirality due to N–N bond-restricted rotation, which is consistent with a similar observation of Ottersbach et al.[16] We obtained single-crystal X-ray data for **4**, **11**, and **13**, which confirmed the preference of these azapeptide models to adopt the c–c–t geometry (Figure 3C and Figure S2). From the orientations and positions of the nitrogen atoms in these crystals, stabilization from a N_{amide}···H–N_{amide} HB can be anticipated. Furthermore, N-methylation of **4** led to the formation of 1:5.3 rotameric mixtures of c–c–t and t–c–t conformers in **5**, indicating the role of N_{amide}···H–N_{amide} HB in the conformational control of **4**. Similarly, compound **15** obtained from the N-methylation of **13** adopted c–c–c geometry wherein the NH–amide bond geometry changed from trans to cis after N-methylation, indicating the role of the N_{amide}···H–N_{amide} HB in stabilizing the c–c–t geometries of the azapeptide models **10**–**14**.

Furthermore, to probe the N_{amide}···H–N_{amide} HB, we carried out hydrogen–deuterium (H/D) exchange[19] and temperature-dependent NMR studies[20] of the azapeptide models in $CDCl_3$ at a low (5 mM) concentration wherein no intermolecular HB was observed (Figure S4). H/D exchange studies of azapeptide

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models **4**, **11**, and **13** and the corresponding controls **4C**, **11C**, and **13C** (Figures 4A and Figure S5) were carried out in 450

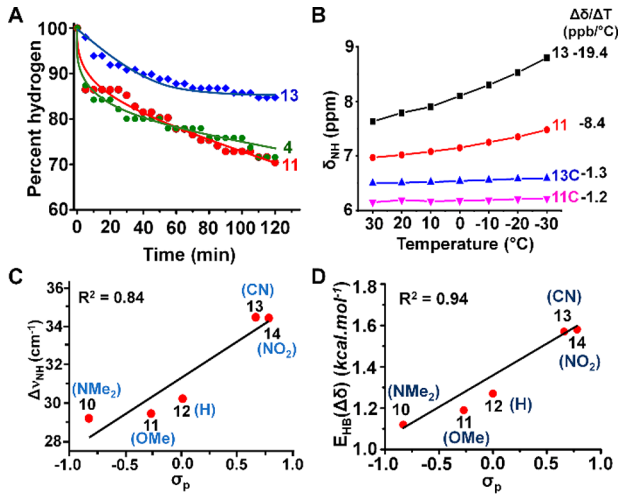

Figure 4. (A) H/D exchange studies of **4**, **11**, and **13** showing the exchange of the NH hydrogen. (B) VT NMR studies of **11**, **13**, **11C**, and **13C** showing the change in the chemical shift of the NH hydrogen. (C) Hammett correlation between $\Delta\nu_{N-H}$ and σ_ρ in **10**–**14**. (D) Hammett correlation between $E_{HB}(\Delta\delta)$ and σ_ρ in **10**–**14**.

μL of $CDCl_3$ by adding 50 μL of MeOH-d_4 to make a 5 mM solution. We observed that for the control urea molecules **4C**, **11C**, and **13C** that lacked a $N_{amide}\cdots H-N_{amide}$ HB, the NH hydrogen signals disappeared within 5 min of the addition of MeOH-d_4. On the contrary, the NH hydrogen atoms of **4**, **11**, and **13** showed only a ∼10−30% reaction in 2 h (Figure 4A), indicating the presence of an intramolecular $N_{amide}\cdots H-N_{amide}$ HB in the azapeptide models. Furthermore, the slower H/D exchange in **13** compared with **11** also supports a stronger $N_{amide}\cdots H-N_{amide}$ HB in **13**, which is expected due to the presence of an electron-withdrawing CN group in **13**. Furthermore, we observed a gradual downfield shift of the NH chemical shifts of **11** and **13** with a decrease in temperature, but no such effect was observed in **11C** and **13C**, indicating the strengthening of the intramolecular $N_{amide}\cdots H-N_{amide}$ HBs in **11** and **13** with the decrease in temperature (Figure 4B). Furthermore, the higher deshielding of the NH proton in **13** compared with **11** with lowering of the temperature was also consistent with the stronger intramolecular $N_{amide}\cdots H-N_{amide}$ HB in **13** compared with **11**. The infrared (IR) spectroscopic investigation of **10**–**14** and **10C**–**14C** indicated that the N−H stretching frequencies of the azapeptide models **10**–**14** were red-shifted compared with those of the control molecules **10C**–**14C** in $CDCl_3$ at 50 mM concentration, supporting the presence of a $N_{amide}\cdots H-N_{amide}$ HB in **10**–**14** (Table 1, Figure S7). The plot of $\Delta\nu_{N-H}$ (ν_{N-H}(control) − ν_{N-H}(azapeptide model)) with the $\sigma\rho$ values in **10**–**14** also indicated an increase in the HB strength from **10** to **14** with the increase in the electron deficiency of the phenyl ring (Figure 4C).

We further estimated the HB strength ($E_{HB}(\Delta\delta)$)[21a,b] of **10**–**14** in $CDCl_3$ by comparing their NH chemicals shifts with those of control molecules **10C**–**14C** (Table 1; see the SI for details). Unfortunately, azapeptide models **6**–**9** were insoluble in $CDCl_3$ and CD_2Cl_2 for such an HB strength estimation. The $E_{HB}(\Delta\delta)$ values ranged from 1.12 to 1.58 kcal·mol^{-1} in **10**–**14**, which can be considered weak HBs. In **10**–**14**, the increase in

Table 1. NH Chemical Shifts (δ(NH)) and N−H Stretching Frequencies (ν_{N-H}) of Azapeptide Models 10−14 and Control Urea Molecules and the HB Energies (E_{HB}) in kcal·mol^{-1} for 10−14 Calculated by Using Various Methods[a]

	$\Delta\delta$ (NH) (ppm)	$\Delta\nu_{N-H}$ (IR) (cm^{-1})	$E_{HB}(\Delta\delta)$ (NMR) (kcal·mol^{-1})	E_{HB} (NBO) (kcal·mol^{-1})	E_{HB} (ρ^{BCP}) (kcal·mol^{-1})
10	0.72	29.20	1.12	1.28	2.36
11	0.79	29.51	1.19	1.32	2.36
12	0.87	30.22	1.27	1.37	2.38
13	1.17	34.54	1.57	1.51	2.45
14	1.18	34.37	1.58	1.56	2.47

[a]$\Delta\delta$(NH) = (δ(NH) (azapeptide model) − δ(NH)(control)). $\Delta\nu_{N-H}$ = (ν_{N-H}(control) − ν_{N-H}(azapeptide model)).

the electron-withdrawing ability of the para-substituent (R) of the aromatic ring is expected to increase the acidity of the NH hydrogen atom, thereby leading to a stronger $N_{amide}\cdots H-N_{amide}$ HB. Accordingly, we observed a strong correlation between $E_{HB}(\Delta\delta)$ and the Hammett constant σ_ρ in **10**–**14** (Figure 4D), which indicated an increase in the $N_{amide}\cdots H-N_{amide}$ HB strength with the increase in the electron-withdrawing ability of the p-substituent on the phenyl ring. The NBO second-order perturbation energies (E^2) for the stabilization of the molecules due to the interactions (Figure 5A) between the HB acceptor nitrogen lone pair and the

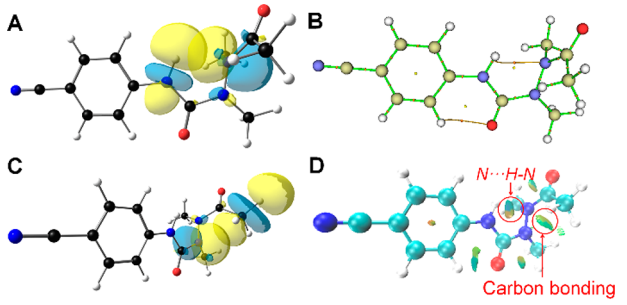

Figure 5. (A) NBO orbital interaction of the HB acceptor nitrogen lone pair and the σ^*_{N-H} orbital of the HB donor NH group in **13**. (B) AIM interaction showing the BCP between the HB acceptor nitrogen and the HB donor NH hydrogen atom in **13**. (C) NBO $n_N \rightarrow \sigma^*_{C-H}$ orbital interaction in **13**. (D) NCI plot showing the $n_N \rightarrow \sigma^*_{C-H}$ interaction in **13**.

σ^*_{N-H} orbital of the HB donor N−H bond for **10**–**14** were in the range of 1.28 to 1.56 kcal·mol^{-1} (Table 1), which correlates well with the HB strength obtained from NMR spectroscopy. We also estimated the HB strength using the electronic density (ρ^{BCP}) and the local potential energy (V) at the bond critical point (BCP) (Figure 5B, Table 1, and Table S11).[21] The HB strengths calculated by using atoms in molecules (AIM) analyses were relatively higher (2.36 to 2.53 kcal·mol^{-1}) than those obtained from NBO analysis (Table 1). The ρ^{BCP} values and positive values of the Laplacian ($\nabla^2\rho$) at the BCP indicate that these $N_{amide}\cdots H-N_{amide}$ HBs are primarily electrostatic in nature (Table S11), which is in agreement with the electrostatic nature of the $N_{amide}\cdots H-N_{amide}$ HBs in peptides.[11a] The strong electrostatic nature of the $N_{amide}\cdots H-N_{amide}$ HBs is consistent with the relatively higher strength of the HBs obtained by AIM compared with NBO, as NBO provides only the orbital interaction component of the $N_{amide}\cdots H-N_{amide}$ HB.

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We expected a decrease in the MeN → C=O conjugation and enhanced cis−trans isomerization of the amide bond involving the HB acceptor nitrogen atom. Although we observed the isomerization of **6**−**14** in DMSO-d_6 that increased with the increase in the N_{amide}···H−N_{amide} HB strength, no such cis−trans isomerization was observed in $CDCl_3$. Variable temperature (VT) NMR studies of **11** and **13** also indicated the presence of no additional rotamer in the −30 to +30 °C temperature range in $CDCl_3$. The lower tendency of isomerization could be due to repulsion between the lone pair of the central hydrazide nitrogen atom and the lone pair of the acetyl amide oxygen atom in the trans isomer. In addition, we also observed a noncovalent carbon bonding $n_N \rightarrow \sigma^*_{C-H}$ interaction in the c−c−t conformation of **10**−**14** (Figure 5C,D), which should favor the acetyl amide bonds in the cis conformation and impede their isomerization to the trans rotamer. We previously reported such noncovalent carbon bonding $n_N \rightarrow \sigma^*_{C-H}$ interactions in *N*-methyl-*N*,*N*′-diacylhydrazines.[17]

Because of the repulsion between the hydrazide nitrogen lone pairs, the hydrazide amide nitrogen atoms should become pyramidal,[22] which should decrease the amide resonance (N → CO) and increase the electron density at the nitrogen atoms of hydrazides.[23] Therefore, we expect hydrazide nitrogen atoms of azapeptides to be more electron-rich than amide nitrogen atoms and anticipate a stronger N_{amide}···H−N_{amide} HB in azapeptides compared with peptides. To test this hypothesis, we compared the strength of the N_{amide}···H−N_{amide} HBs in **1** and **2** with their amide versions **1-CH$_2$** and **2-CH$_2$** (Figure S13), generated by replacing the central NH and NMe groups of the c−c−t isomers of **1** and **2** with a CH_2 group. As expected, the N_{amide}···H−N_{amide} HBs in **2** were stronger compared with **2-CH$_2$** but similar for **1** and **1-CH$_2$** (Table S3 and Figure S13). Similarly, we also observed stronger N_{amide}···H−N_{amide} HB interactions (by both NBO and AIM analysis) in azapeptide models than in peptides that were previously[11] studied (Pro 185 and Pro 165 of nSH3 (PDB ID: 1CKA)) (Table 1 and Table S13). Previous studies have shown that N_{amide}···H−N_{amide} HBs are often found in the *β*-turn regions of proteins.[12,13] Interestingly, azapeptides predominantly adopt *β*-turns with the aza-amino acid residue at the (*i* + 2) position.[8] Therefore, we expect N_{amide}···H−N_{amide} HBs to play an important role in stabilizing the *β*-turns of azapeptides. To probe this, we analyzed two previously reported *β*-turn crystal structures of azapeptides (Figure S14).[8g,k] We carried out NBO, NCI, and AIM analyses on the crystal geometries of these two azapeptides (Figure S15). Although we did not observe BCPs for the N_{amide}···H−N_{amide} HBs, both NBO and NCI analyses indicated the presence of N_{amide}···H−N_{amide} HBs in the *β*-turns.

In conclusion, we have systematically studied the presence of an intramolecular N_{amide}···H−N_{amide} HB in small azapeptide models by using NMR and IR spectroscopies, X-ray crystallography, and various theoretical methods. We observed that the azapeptides and their N-methylated analogues are stabilized by a N_{amide}···H−N_{amide} HB of strength of ∼1−3 kcal·mol^{-1}. Our results reveal that a N_{amide}···H−N_{amide} HB stabilizes the t−c−t and c−c−t amide bond conformers of azapeptides and *N*-methyl-azapeptides, respectively. These N_{amide}···H−N_{amide} HBs should impose conformational rigidity in azapeptides, which is an essential property of drug-like molecules. Moreover, these intramolecular N_{amide}···H−N_{amide} HBs that engage the acidic NH hydrogen atoms of the azapeptides could also impede strong HB formation between the NH group and the surrounding water molecules, thereby improving the cell permeability of these molecules. Because azapeptides prefer *β*-turn structures, we are currently investigating if N_{amide}···H−N_{amide} HBs play a role in the stabilization of the azapeptide *β*-turns.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.1c01111.

Experimental details, various NMR studies, IR studies, computational studies, and Cartesian coordinates of optimized geometries (PDF)

Accession Codes

CCDC 2062577 and 2062582−2062583 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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