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Title:

Relative hydrophilicities of *cis* and *trans* formamides

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Abstract

Secondary formamides are widely encountered in biology and exist as mixtures of both *cis* and *trans* isomers. Here we assess hydrophilicity differences between isomeric formamides through direct competition experiments. Formamides bearing long aliphatic chains were sequestered in a water-soluble molecular container having a hydrophobic cavity with an end open to the aqueous medium. NMR spectroscopic experiments reveal a modest preference (<1 kcal/mol) for aqueous

solvation of the *trans* formamide terminals over the *cis* isomers. With diformamides, the supramolecular approach allows staging of intramolecular competition between short-lived species with subtle differences in hydrophobic properties.

Significance statement

Many studies have investigated secondary formamides as mixtures of *cis* and *trans* isomers. They are widespread in nature, but relatively low energetic barriers to interconversion prevents the isolation and characterization of the pure isomers. Here we determine hydrophilic differences between the isomers by binding in a water-soluble, synthetic molecular container. The container offers a range of environments from hydrophilic to hydrophobic and distinguishes subtle differences in the polarities of formamide isomers. Using NMR methods we find that *cis* formamides are more hydrophobic than the corresponding trans isomers. The conclusion holds for diformamides, where we staged an intramolecular competition between *cis* and *trans* formamides within the container. The advantage of this supramolecular method is that stable and isolable compounds are not required to make the determination.

Introduction

Decades of structural work with peptides have established that secondary amide bonds generally exist in *trans* conformations. Experimental[1] and computational[2] studies on model compounds such as *N*-methyl acetamide revealed that dipole/dipole and steric effects around the secondary amide bond destabilize the *cis* isomer (Fig. 1)[3]. The energetic differences are large enough that the *cis* isomer is usually present in only ~ 1%. Secondary formamides are different. They are widespread in nature as initiators of protein synthesis in bacteria and mitochondria and as triggers of immune responses in eukaryotic cells[4]. Typically, secondary formamides are 10-20% *cis* isomers, and the percentage increases with the bulk of the *N*-substituent (Fig. 1)[5]. The *trans/cis* equilibria can be determined by NMR methods[6-8], but the relatively low (15-20 kcal/mol) energetic barriers to interconversion – slow on the NMR chemical shift timescale but fast on the human timescale – prevent the isolation and characterization of the pure isomers. Dipole moments are calculated to be slightly higher for the *cis* formamides vs. *trans* (4.2 D vs. 4.0 D)[1,2], yet the amount of the *cis* isomer often increases slightly on transfer from water to organic solvents. Neither experimental[9,10] nor theoretical hydration studies offer clear answers[2]. As a result, the differences in polarity, hydrophilicity and their effects on chemical or biological behavior [11,12] remain elusive. Here we apply synthetic molecular containers (cavitands) to determine their relative hydrophilicities.

Results and discussion

The cavitand used is **1** (Fig. 2), a water-soluble version of a container introduced by de Mendoza[13] and Choi[14] for use in organic solvents. The cavitand acts as host with a hydrophobic interior and an open end exposed to water (D_2O)[15], and guest molecules of suitable size, shape and chemical surface are bound within. The cavity offers a range of chemical environments – from hydrophilic to hydrophobic – as well as magnetic environments: The 8 aromatic panels of the cavitand shield the guest nuclei within from the applied field of the NMR spectrometer. The effects on guest nuclei – calculated by the method of Schleyer[16] and mapped experimentally – are summarized in the cartoon of Fig. 2. Nuclei at the hydrophobic bottom experience the largest upfield shifts ($\Delta\delta$ = -4.0 to - 4.3 ppm)[17]. The magnetic effects gradually diminish as the positions rise to near the top of the rim ($\Delta\delta$ = 0 to -0.5 ppm) and nuclei outside the cavitand, exposed to the aqueous solvent are unaffected.

These effects emerge from comparison of the NMR spectra of free and bound *N*-octyl-formamides (Fig. 3). In the free spectra (Fig. 3A, $CDCl_3$ or D_2O solvent) the *cis* vs *trans* isomerism affects only signals near the polar end of the molecule, as indicated for the -CO-H and the *alpha*-CH_2-NH. Integration gives the relative concentrations of *cis* vs *trans* isomers of the free formamides as shown. The signals for CH_2 groups near the middle or the methyl end of the free molecule remain unaffected. Brief sonication of *N*-octyl-formamide (**2a**) with excess cavitand **1** in D_2O produces 1:1 complexes. Two species are present (*trans* and *cis* amides) whose spectra is shown in Fig. 3A (see also *SI Appendix* Fig. 1-1), and the chemical shifts are characteristic of extended conformations of the alkyl chains in the cavitand[15]. The assignments were obtained by 2D experiments (*SI Appendix* Fig. 1-2) and place the CH_3 (on average) closest to the bottom of the cavitand with $\Delta\delta$ = - 4.2 ppm for the *trans* isomer of **2a**. The other (polar) end of the guest is exposed to D_2O; the small shift ($\Delta\delta$ = - 0.7 ppm) of the -C*H*$_2$-CH_2-NH-CHO signal places it near the top of the cavitand with the NH-CHO group outside. The cartoon of Fig. 2b reflects this arrangement. The smaller set of signals for the *cis* isomer are shifted throughout the spectrum: Nuclei near the amide are shifted upfield indicating that the polar end – on average – is deeper in the hydrophobic cavitand, and nuclei near the methyl group are shifted downfield, indicating that the apolar end – on average – is shallower in the cavitand than the *trans* formamide. Parallel behavior is seen in the spectra of the longer *N*-decyl-formamide (**2b**) (*SI Appendix* Fig. 2-1 and 2-2).

The *trans* formamide guest **2a** is positioned with the methyl group fixed at the bottom (Fig. 3 and *SI Appendix* Fig. 11 and *SI Appendix* Table 7): its signal shows $\Delta\delta$ = - 4.1 ppm which is 98% of the maximum (- 4.2 ppm) seen in this cavitand (Fig. 3C). The signal for the methyl group of the *cis* amide shows $\Delta\delta$ = - 3.36 ppm or only 80% of the maximum value. Accordingly, this guest spends some 20% of the time in the "upside down" position with the formyl end deep in the cavitand (Fig. 3C). Rapid motion of the guest on the NMR chemical shift timescale between the two arrangements shown is consistent with the signals observed. The motion is facilitated by a coiled shape of the alkyl chain as seen in related cavitands[18], but the conformational details of the central atoms are unknown. In any case, the *cis* formyl group appears less hydrophilic than the *trans*.

We also staged intramolecular competitions for the hydrophobic cavitand with formamides of α,ω–diamines as guests. The NMR spectra of diformamides in solution are as expected (*SI Appendix*). But as guests in **1**, the diformamides of $(CH_2)_8$ to $(CH_2)_{11}$ (**3a-3d**) show starkly different signal patterns (Fig. 4A and *SI Appendix* Fig. 3). The major *trans,trans* isomers show simplified spectra indicating a time-averaged symmetrical arrangement of guest in the host. The signal for the beta methylene group of the *trans,trans* amide shows $\Delta\delta$ = - 2.1 ppm which is 50% of the maximum seen in this cavitand (- 4.2 ppm)[20] (Fig. 4B and *SI Appendix* Fig. 4). The minor *trans,cis* isomers show a superimposed set of signals with the more complex features expected of an unsymmetrical arrangement, in which one end spends more time inside the cavitand than the other. For example, the beta methylene of *cis* amide end signal shows $\Delta\delta$ = -3.38 ppm or only 80% of the maximum value. The biased arrangement of the unsymmetrical *trans,cis* guest arises from the differences in relative hydrophilicity and hydrophobicity of the two ends.

The detailed assignments of signals were established as follows for the $(CH_2)_9$ (**1:3b**) complex (Fig. 5; for this and other complexes see *SI Appendix* Figs. 3-10). The spectra were obtained under

conditions of excess diformamides in order to identify exchange processes between free and bound guests. Downfield regions of the 2D-EXSY spectrum are shown in Fig. 5 panel a and upfield regions in panel b. The NMR of the *trans,cis* isomer shows resolved upfield-shifted signals for each methylene group of the alkyl chain. The protons of the terminal formamide groups of the bound *trans,cis* isomer show chemical exchange cross-peaks with those of the corresponding free diamides (Fig. 5, panel a). The methylene *alpha* to the *cis*-amide (H^9) appears at -0.23 ppm and shows a selective cross peak to the formyl proton of the *cis* formamide in the complex at 5.81 ppm (*SI Appendix* Fig. 6). The *cis*-formyl proton in the bound *cis,trans* isomer of $(CH_2)_9$ (**3b**) is significantly upfield-shifted compared to its *trans* counterpart: it is deeper in the cavitand. Accordingly, the *trans* formyl prefers more exposure to the aqueous medium and is more hydrophilic than the *cis* formyl.

The guests are not static but move rapidly in the cavitand. The details are unknown but can involve a "yo-yo" like motion[21] of folded arrangements in the cavitand[22] or the simple tumbling of a compacted conformation. Additional evidence of hydrophilicity differences may be inferred from the integral values of the formyl protons of bound $(CH_2)_9$ in the two complexes. The ratio between the *trans,trans* and *trans,cis* isomers is 60:40, and indicates a binding preference of the cavitand for the *trans,cis* isomer. If the two complexes were isoenergetic, the expected ratio should be 82:18 (*SI Appendix*).

The hydrophilicity of stable structures can be directly compared by partitioning between water and a hydrophobic solvent such as octanol. The labile structures here are temporarily partitioned between an aqueous phase and the hydrophobic interior of the cavitand. The competition of the *cis,trans* isomers by this method is direct since both are present in the same solution and, in the case of the diformamides, in the same molecule. The shape and volume of the cavity can impose selectivity of its own[23], but the *cis* and *trans* formamides isomers are not expected to differ in size and shape enough to bias the competition. The possibility of hydrogen-bonded, dimeric structures exists in media of low polarity, but the competitive aqueous solvent (outside) and the limited size of the cavitand (inside) prevents self-association of these guests. The present results consistently indicate that the *cis* formamides are more hydrophobic than their *trans* isomers.

Conclusions

The advantage of the present application is that it does not require stable, isolable compounds. Their lifetimes need only to be long enough on the NMR chemical shift timescale – seconds or less – to make the determination. The choices between hydrophilic and hydrophobic offered by the cavitand resemble the workings of the Wilcox torsion balance[24] as they can operate on the sub-kilocalorie scale.

Materials and Methods

The experimental procedures for the synthesis of cavitand **1**, *N*-octyl-formamide **2a**, *N*-decyl-formamide **2b**, diformamide **3** and NMR spectra of the complexes are available in *SI Appendix*.

Acknowledgments

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Author Contributions

Y.S.L and Y.J.Z synthesized the formamides, carried out experiments and analyzed the experimental data. L.E performed 2D-NMR experiment of diformamides, DFT computations and data analysis. Y.S.L, Y.J.Z and L.E contributed equally to this work. Y.C reviewed the manuscript and provided advice. P.B analyzed the 2D-NMR data and revised the manuscript. J.R and Y.Y conceived the project, guided experiments and wrote the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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19. A typical *trans/cis* ratio of 4:1 for the formyl group results in a statistical distribution of diformamides: 64% *trans,trans*; 32% *trans,cis* and 4% *cis,cis*. The latter is rarely observed directly due to overlapped signals and low intensities.

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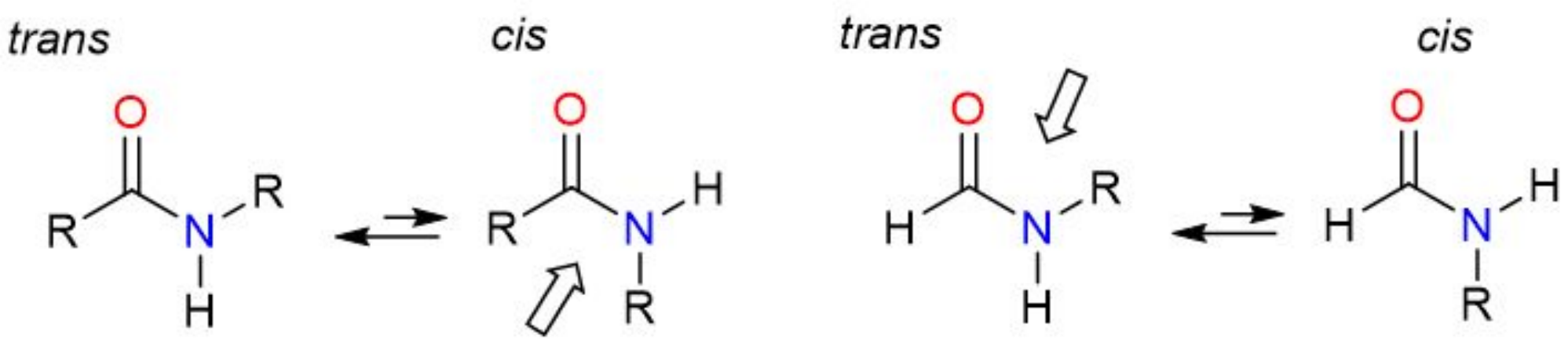
Fig. 1. Amide geometries and areas of steric clashes that effect *cis/trans* equilibria.

Fig. 2. Structures of cavitands **1** and cartoon for the complex of **1** with **2a**: (A) Chemical structure of the cavitand host **1**; (B) The cartoon abbreviation for its host/guest complex with *N*-octyl-formamide (**2a**). The observed Nucleus Independent Chemical Shifts (NICS)[16,17] for typical guest nuclei are given in -Δδ ppm. The magnetic effects increase gradually with depth in the cavitand as the chemical environment changes from hydrophilic at the top of the cavity to hydrophobic at the bottom.

Fig. 3. Partial ^{1}H NMR spectra (600 MHz, 298K) of free and bound **2a** and cartoons of its behavior in **1**: (A) The spectra of free *N*-octyl-formamide (**2a**) in $CDCl_3$ (top) and D_2O (bottom). The *cis/trans* ratio can be determined from the formyl C-H signals; (B) Upfield region of the spectrum of the formamide's complex of **2a** in **1** (see also Figures S1-1 and 1-2). The low intensity set of signals represents the *cis* isomer; (C) Cartoon of proposed conformations of *cis* and *trans* formamide in the cavitand.

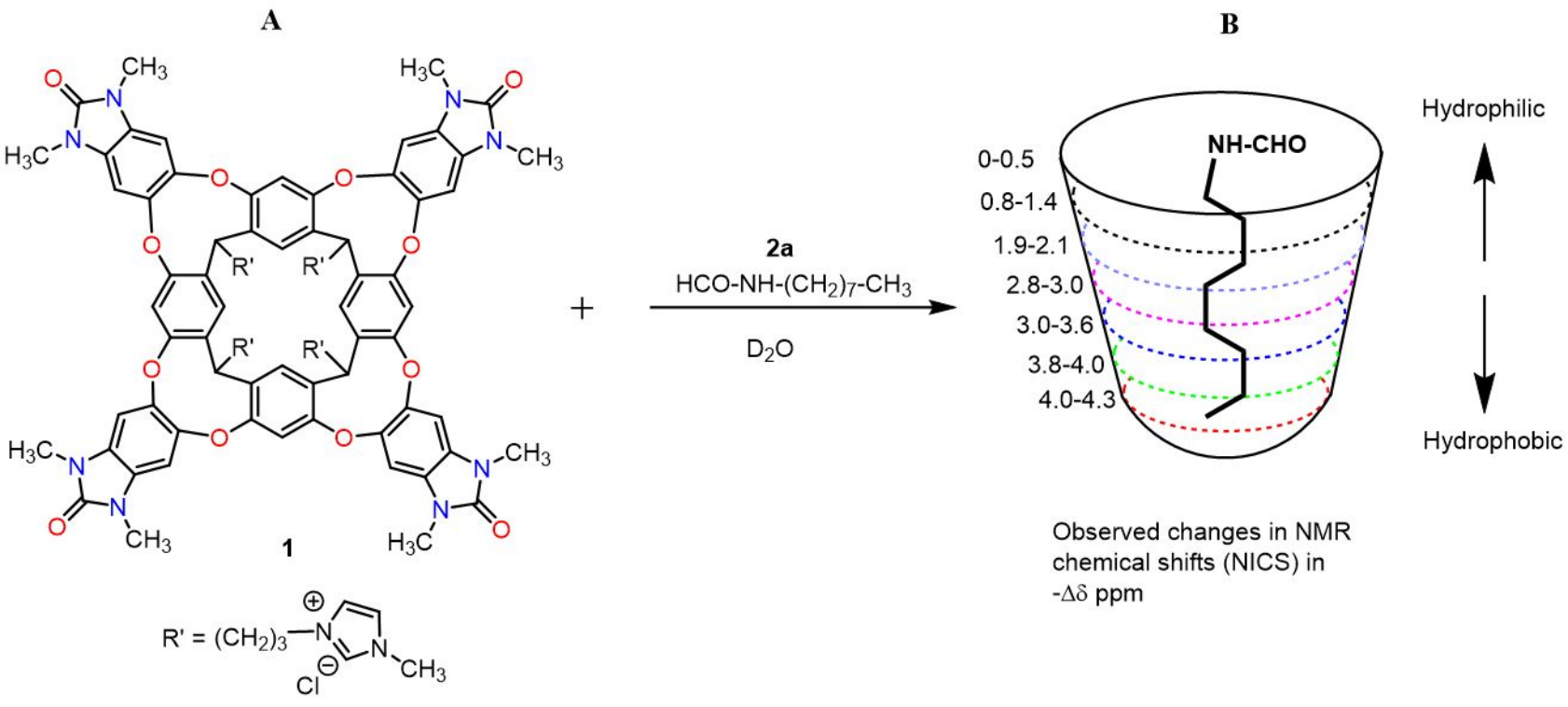
Fig. 4. Partial ^{1}H NMR spectra of the complexes of diformamide **3** and **1** and their cartoons in **1**: (A) Upfield regions of the ^{1}H NMR spectra (600 MHz, D_2O, 298 K) of the diformamide complexes of **1**. 1) n = 8 (**3a**); 2) n = 9 (**3b**); 3); n = 10 (**3c**); 4) n = 11 (**3d**); The larger signal clusters represent the symmetrical *trans*,*trans* isomers[19] while the smaller set represents the *trans*,*cis* isomers. (see also Figure S3); (B) Cartoons of the isomeric complexes and their proposed interconversion.

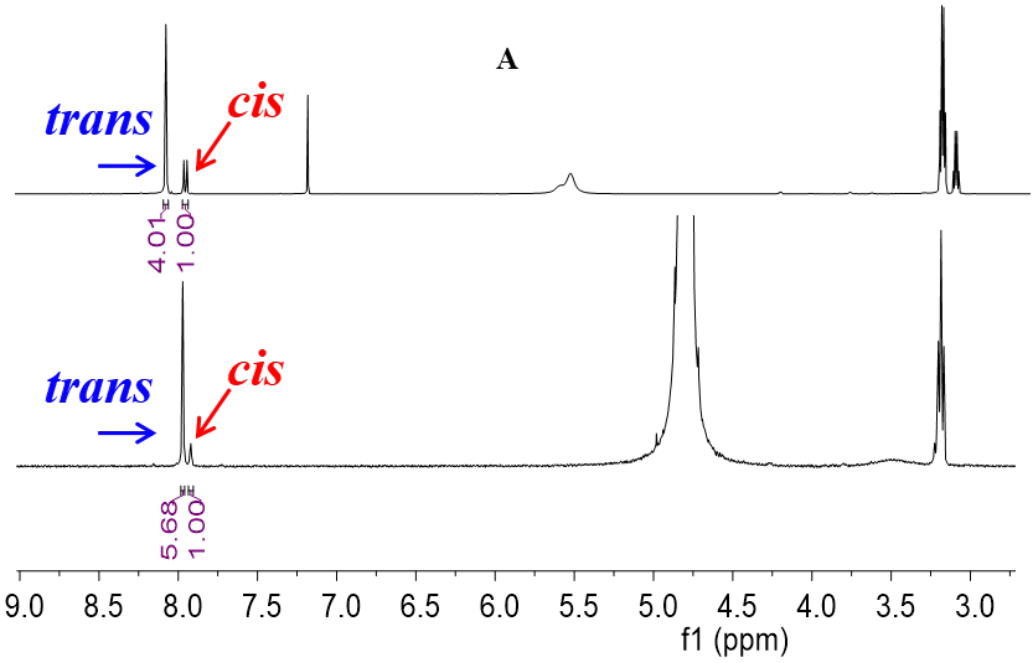
Fig. 5. Complexation of **3b**: (Top) The ^{1}H NMR spectrum of a D_2O solution of [**1**] = 1 mM and [**3b**][$(CH_2)_9$] = 2 mM. (Bottom) Selected downfield (panel a) and upfield (panel b) regions of the 2D-EXSY spectrum (mixing time 300 ms) showing the cross-peaks due to chemical exchange between the protons of free and bound $(CH_2)_9$ diformamide (see also Figures S5 and S6). Primed numbers are proton signals for the bound *trans*,*cis* isomer (black). Doubly primed labels correspond to the *trans,trans* counterpart (blue). Triply primed labels correspond to the *cis*,*cis* diformamide (red).[19]

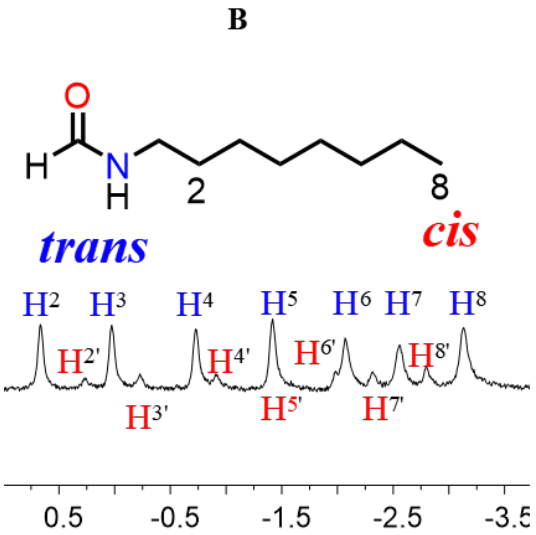


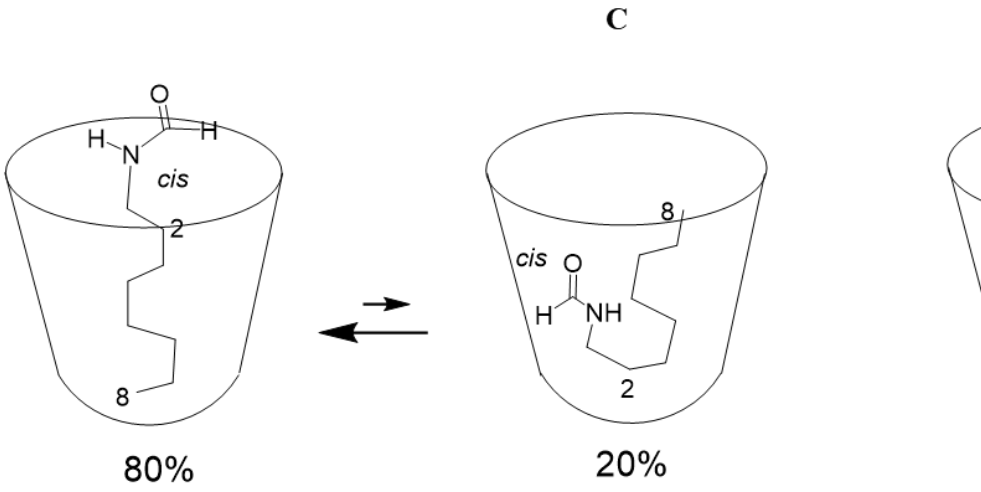
secondary amides

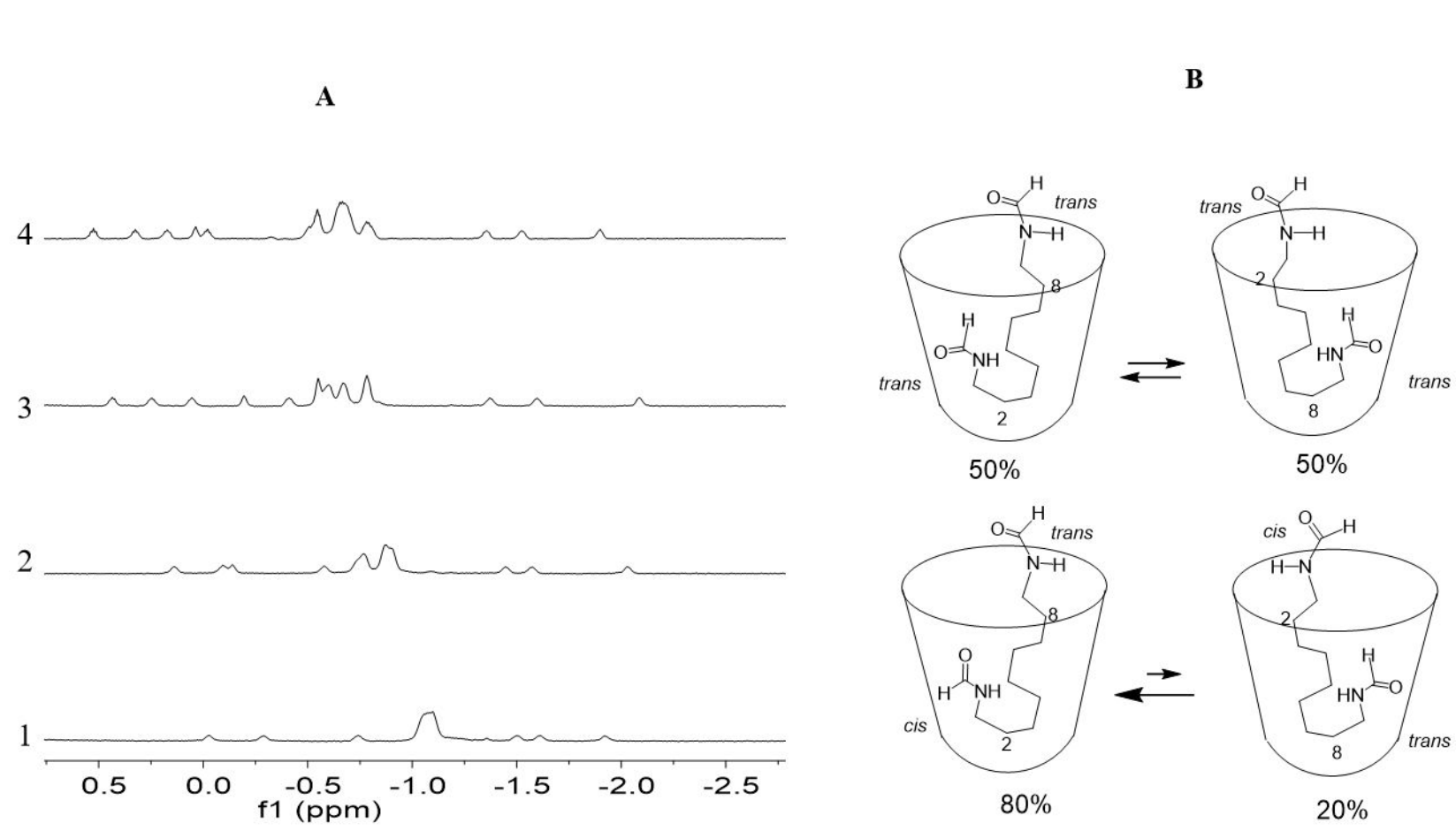
formamides


A
B
1
R' = (CH2)3
Cl
2a
HCO-NH-(CH2)7-CH3
D2O
NH-CHO
0-0.5
0.8-1.4
1.9-2.1
2.8-3.0
3.0-3.6
3.8-4.0
4.0-4.3
Hydrophilic
Hydrophobic
Observed changes in NMR chemical shifts (NICS) in -Δδ ppm


A
trans
cis
4.01
1.00
trans
cis
5.68
1.00
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0
f1 (ppm)


B
O
H
N
H
2
8
trans
cis
H2 H3 H4 H5 H6 H7 H8
H2' H4' H6' H8'
H3' H5' H7'
0.5 -0.5 -1.5 -2.5 -3.5


C
O
H
N
H
cis
2
8
80%
cis
O
H
NH
8
2
20%
H
O
trans
N
H
2
8
~100%


A
4
3
2
1
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
f1 (ppm)
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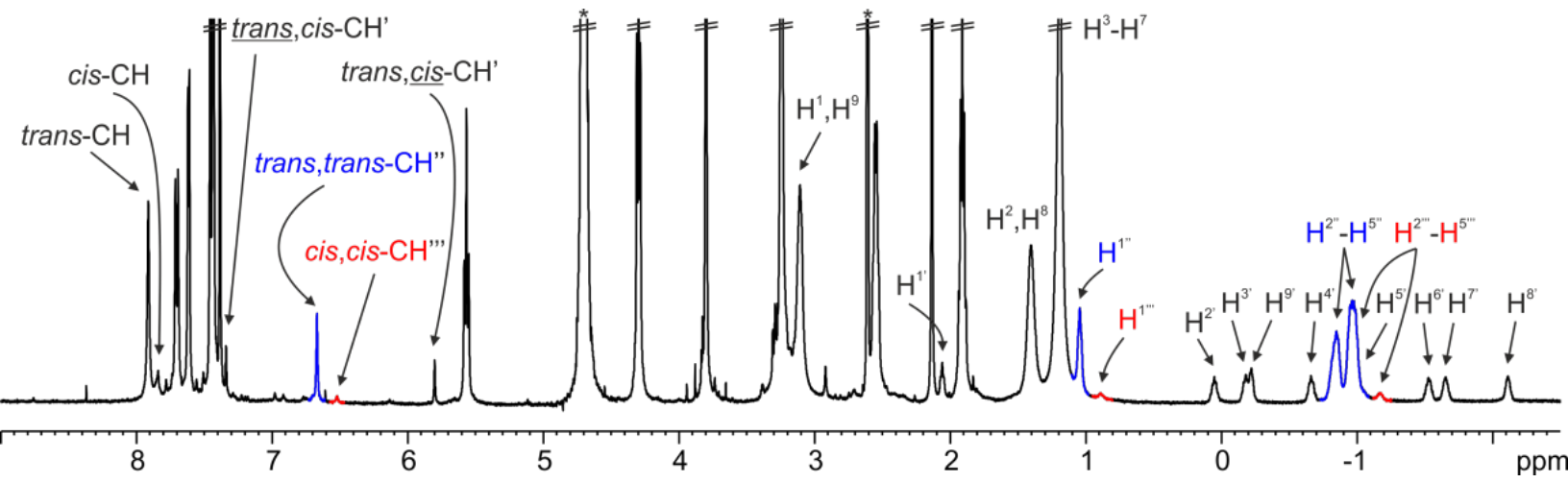
50%
50%
cis
trans
cis
trans
80%
20%


cis-CH
trans-CH
trans,cis-CH'
trans,cis-CH'
trans,trans-CH''
cis,cis-CH'''
H1,H9
H3-H7
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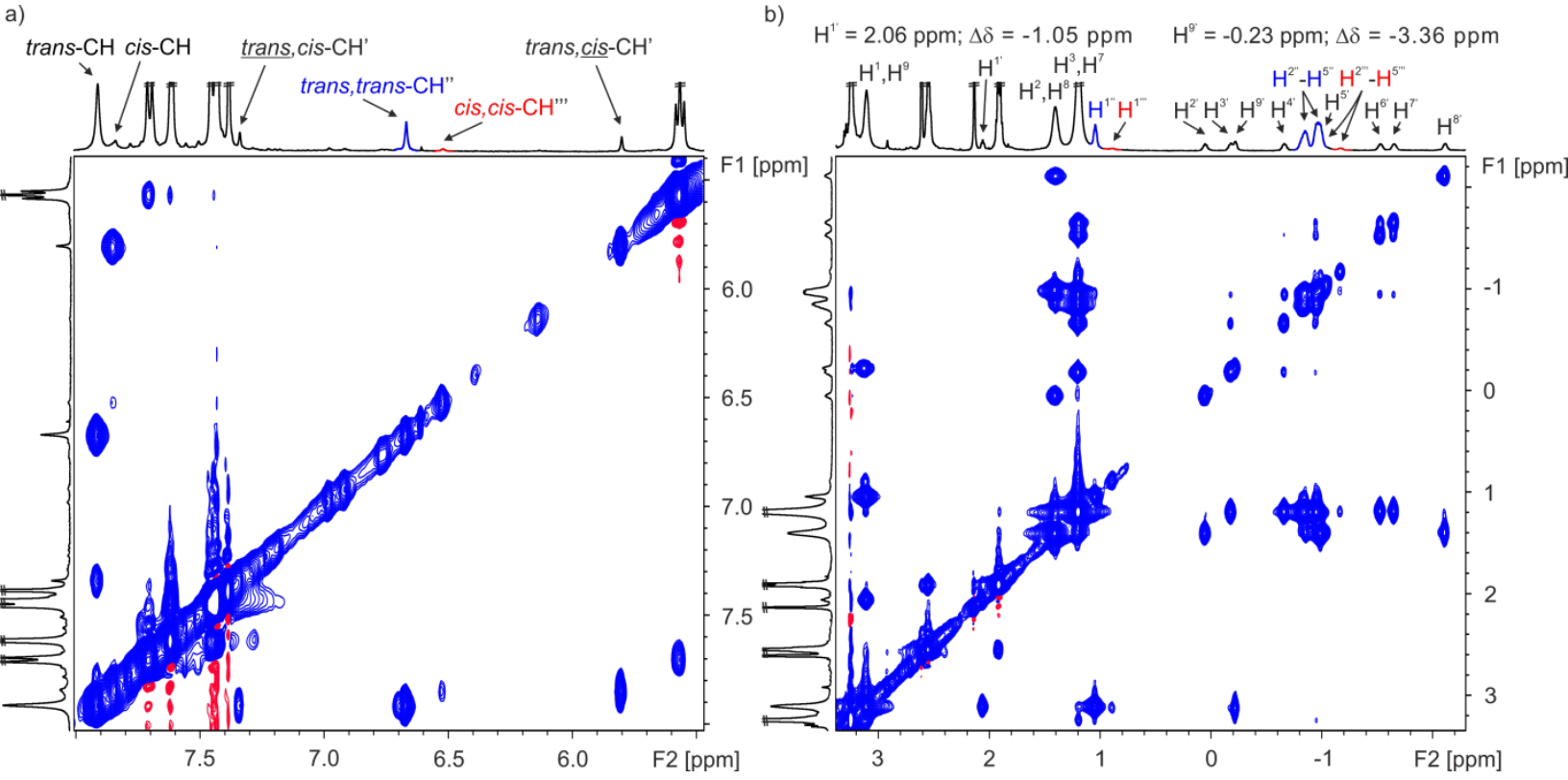
H1''
H1'
H1'''
H2'
H3'
H9'
H4'
H2''-H5''
H2'''-H5'''
H5'
H6'
H7'
H8'
8
7
6
5
4
3
2
1
0
-1
ppm


a)
trans-CH
cis-CH
trans,cis-CH'
trans,trans-CH''
cis,cis-CH'''
trans,cis-CH'
F1 [ppm]
6.0
6.5
7.0
7.5
7.5
7.0
6.5
6.0
F2 [ppm]
b)
H1'' = 2.06 ppm; Δδ = -1.05 ppm
H9' = -0.23 ppm; Δδ = -3.36 ppm
H1,H9
H1'
H3,H7
H2,H8
H1''
H1'''
H2'
H3'
H9'
H4'
H2''-H5''
H2'''-H5'''
H5'
H6'
H7'
H8'
F1 [ppm]
-1
0
1
2
3
3
2
1
0
-1
F2 [ppm]