

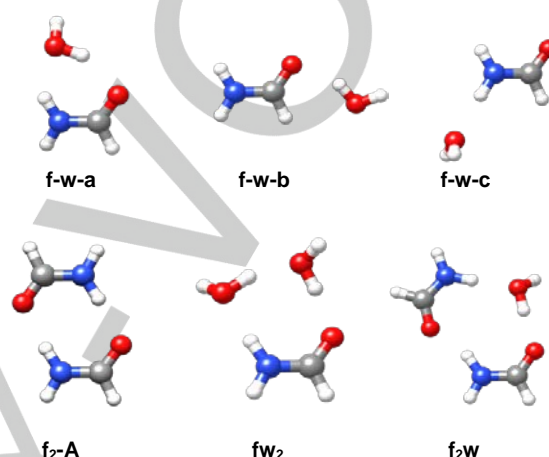
# Hydrogen Bond Cooperativity in Formamide<sub>2</sub>-water, a Model for Water Mediated Interactions.

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**Abstract:** The rotational spectrum of formamide<sub>2</sub>-H<sub>2</sub>O formed in a supersonic jet has been characterized by Fourier transform microwave spectroscopy. This adduct provides a simple model of water mediated interaction involving the amide linkages, as occur in protein folding or amide association processes, showing the interplay between self-association and solvation. Mono-substituted <sup>13</sup>C, <sup>15</sup>N, <sup>18</sup>O and <sup>2</sup>H isotopologues have been observed to investigate the structure. The adduct forms an almost planar three body sequential cycle. The two formamide molecules link on one side through an N-H...O hydrogen bond and on the other side through a water-mediated interaction with the formation of C=O...H-O and O...H-N hydrogen bonds. The analysis of the quadrupole coupling effects due to the presence of two <sup>14</sup>N-nuclei reveal the subtle inductive forces associated to cooperative hydrogen bonding. These forces are involved in the changes detected in the C=O and C-N bond distances with respect to bare formamide.

Hydrogen bonding (HB) is responsible for most of the inter- and intramolecular interactions that underpin protein folding, protein structure, biological activity and molecular recognition.<sup>[1-3]</sup> Protein folding is not random<sup>[4,5]</sup> and many theories identify hydrophobic effect and the entropy changes associated to the translational movement of water molecules as the main driving force for folding.<sup>[6]</sup> However, this point of view has been recently challenged<sup>[7-9]</sup> by analyzing the role of hydrophilic interactions in folding. In fact, both theory and experiment indicates that water may act as a mediator for the nucleation of folding through HB interactions. In the folded system the HB interactions N-H...O=C involving the amide linkages dominate in the hydrophobic core of the protein. The implication of these bonds for the formation of  $\beta$ -turns was first detected by crystallographic techniques.<sup>[10]</sup> However, it has been also found in proteins with turn structures, that the distances between aligned C=O and N-H groups are far to being involved in an HB.<sup>[11,12]</sup> The mediation of water to form these C=O...H-N in those cases has been proposed as far as the turns are located near the protein surface where they are exposed to interactions with water.<sup>[13]</sup> According to this, water mediated contacts may have an important role in the nucleation process of the protein folding by helping to form the C=O...H-N contacts when these groups are not aligned or beyond the standard HB distances. Another point of interest concerning folding is the role that cooperative forces<sup>[3,14]</sup> of amide hydrogen bonds plays in the stabilization of folded structures such as  $\alpha$ -helices or  $\beta$ -turns.<sup>[15,16]</sup> Cooperativity has been proposed to give contributions from entropy, due to the restrictions of internal motions, as well as from inductive polarization of the amide

groups not yet well established due to the short amount of experimental information on it.<sup>[17-20]</sup> The role of water to enhance, or not, HB cooperativity in those systems is still an open question.

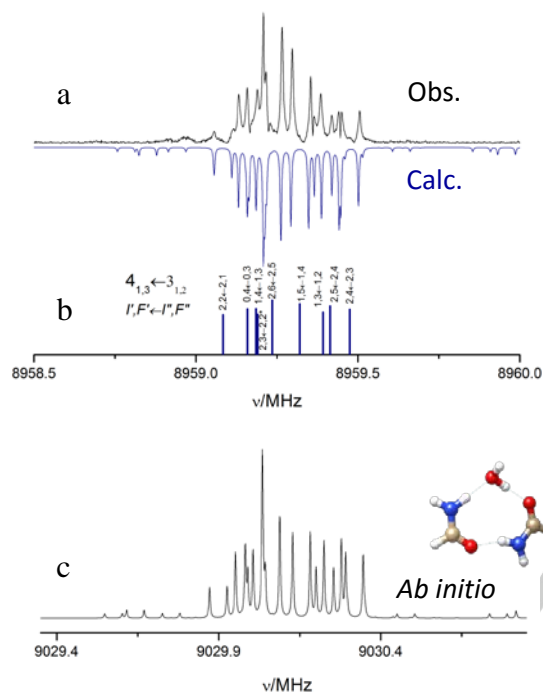


**Figure 1.** Adducts of formamide and water including the formamide<sub>2</sub> dimer. The structures of the heterodimers formamide-H<sub>2</sub>O (f-w-a, f-w-b, f-w-c), formamide-(H<sub>2</sub>O)<sub>2</sub> and the formamide<sub>2</sub>-H<sub>2</sub>O, studied in this work, are shown.

Direct structural investigations of isolated clusters of molecules modelling amide-water, amide-amide and amide-amide water mediated interactions are important to get a better understanding of the role of these interactions in larger systems. These models are also of help to better understand amide association processes showing the interplay between self-association and solvation. Under the isolated conditions of supersonic jets, clusters of molecules of different sizes can be formed in a controlled way. When jets are coupled to rotationally resolved techniques such as microwave spectroscopy accurate structural information can be obtained. However due to the experimental difficulty this research has been limited, with few exceptions,<sup>[21-23]</sup> to the study of complexes with a reduced number of ligands. Different examples of investigations on isolated hydrated amide clusters done in supersonic jets by microwave spectroscopy can be found in the literature.<sup>[20,24-30]</sup> These works, in particular the detailed investigation of the structures of formamide-(H<sub>2</sub>O)<sub>n</sub><sup>[25]</sup> (see Figure 1) (n=1,2), have led to interesting pieces of information. The observation of the conformers build from the different docking sites of formamide led to conclude that the dominant interaction results to be the HB of water with the amide carbonyl group as found in globular proteins.<sup>[2]</sup> In this paper some of the structural signatures of the cooperative HB effects were observed. However, inductive polarization effects have been scarcely reported.<sup>[20]</sup> The investigation of clusters modelling amide-amide and amide-amide water-mediated interactions is rarely found in the field of microwave spectroscopy. This implies the formation of adducts

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with at least two molecules possessing an amide group and a molecule of water. In the present work we have undertaken the study of one of the simplest system fulfilling those requirements, the complex formed by two molecules of formamide and one water molecule ( $f_2w$ , see Figure 1). This system can be thought as a microsolvated water cluster being formamide the solvent and water the solute. We have payed special attention to accurately determine the structure by using  $r_s$  and  $r_0$  methods which exploit the measurement of different monosubstituted isotopologues to search for the signatures of cooperative effects.



**Figure 2.** The transition observed around 8959.2 MHz shows the hyperfine structure characteristic of two  $^{14}\text{N}$  nuclei, complicated by the Doppler doubling (54 kHz). (a) Comparison of the observed (upwards) and calculated (downwards) spectrum for the  $4_{1,3} \leftarrow 3_{1,2}$ . (b) Assignment of the hyperfine components with the quantum numbers  $J, K_1, K_{-1}, l, F$  (coupled basis set  $(l_1, l_2, l, J, F)$ ,  $l_1 + l_2 = l$ ,  $l + J = F$ ) (c) *Ab initio* prediction for conformer  $f_2w$  centered at 9029.2 MHz with a hyperfine structure comparable to that observed experimentally.

The presence of two  $^{14}\text{N}$  atoms has the drawback of a complicated quadrupole hyperfine structure,<sup>[31]</sup> but it represents a fingerprint of the species of interest and contains direct information of the electronic environment around the N atoms. This hyperfine structure results from the interaction of the nuclear quadrupole moment of the  $^{14}\text{N}$ -nuclei ( $I=1$ ),  $eQ$ , with the electric field gradient,  $q$ , at the site of the N nuclei. The corresponding spectroscopic constants,  $\chi_{\alpha\beta}$  ( $\alpha, \beta = a, b, c$ ), are the elements of the nuclear quadrupole coupling tensor, set up in the principal inertial axis system representation. The quadrupole coupling constants are directly related to the electric field gradient tensor elements,  $q_{\alpha\beta}$ , by:  $\chi_{\alpha\beta} = eQq_{\alpha\beta}$ . As we will show later this is an invaluable probe to detect the subtle effects of cooperative HB forces, particularly those which cause the inductive polarization of the amide group.

We recorded the microwave spectrum of formamide-water complexes using the experimental setup described later. Once dropped all known frequencies,<sup>[25]</sup> several groups of unassigned lines appeared to show the quadrupole coupling hyperfine structure characteristic of species having two  $^{14}\text{N}$  nuclei (see Figure 2). In order to identify these new lines we considered the spectroscopic constants predicted (MP2/6-311++g(2d,p)) for the stable forms of formamide dimers<sup>[32]</sup> ( $f_2$ ) and formamide<sub>2</sub>-water ( $f_2w$ ) adducts (see Supplementary Data Tables S1, S2 and Figures S1 and S2). The most stable species of formamide dimer  $f_2$ -A, shown in Figure 1, expected to be dominant by far in the gas phase, has inversion center, and thus has no microwave spectrum. When the spectra predicted for the different species were compared with the observed spectrum we soon realize that the best match correspond to the predicted most stable  $f_2w$  conformer as can be seen in Figure 2. The assignment of the quadrupole coupling patterns for several R-branch  $\mu_a$ -type transitions lead to the complete assignment of the  $\mu_a$ - and  $\mu_b$ -type R-branch spectra.  $\mu_c$ -type spectrum was not observed. The rotational parameters determined from the analysis<sup>[31,33-35]</sup> of the spectrum are compared in Table 1 with the *ab initio* constants for the most stable form of  $f_2w$  complex.

**Table 1.** Rotational parameters obtained from the analysis of the spectrum of the observed formamide<sub>2</sub>-water adduct.

Fitted Parameters <sup>a</sup>	Exp. Value	<i>Ab initio</i> $f_2w$
A/MHz	2769.21599(63) <sup>b</sup>	2725
B/MHz	1232.34738(20)	1244
C/MHz	854.50622(10)	860
$P_{cc}/\text{u}\text{\AA}^2$	0.582715(89)	2.0317
$^{14}\text{N}_3 \chi_{aa}/\text{MHz}$	1.0685(46)	1.09
$^{14}\text{N}_3 \chi_{bb}/\text{MHz}$	1.9107(93)	2.05
$^{14}\text{N}_3 \chi_{cc}/\text{MHz}$	-2.9793(93)	-3.14
$^{14}\text{N}_9 \chi_{aa}/\text{MHz}$	1.2683(39)	1.20
$^{14}\text{N}_9 \chi_{bb}/\text{MHz}$	1.7235(91)	1.80
$^{14}\text{N}_9 \chi_{cc}/\text{MHz}$	-2.9917(91)	-3.00
N	205	
$\sigma/\text{kHz}$	1.60	

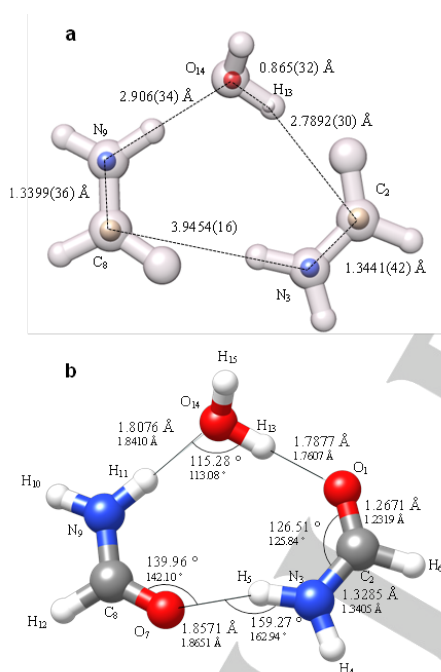
<sup>a</sup>A, B and C are the rotational constants.  $\chi_{aa}$ ,  $\chi_{bb}$  and  $\chi_{cc}$  are the quadrupole coupling constants for  $^{14}\text{N}_3$  and  $^{14}\text{N}_9$  nuclei (see Figure 3). N is the number of quadrupole hyperfine components fitted.  $\sigma$  is the rms deviations of the fit.  $P_{cc} = (I_a + I_b - I_c)/2 = (\sum_i m_i c_i^2)/I$ , is a planar moments of inertia (conversion factor 505679.1 MHz  $\text{u}\text{\AA}^2$ ); <sup>b</sup>Standard error is given in parentheses in units of the last digit.

Well established structural determination methods beyond the simple conformation identification based on the comparison of the observed and *ab initio* rotational parameters use data from the spectra of the parent and isotopically substituted species. In order to exploit those methods, the spectra of the monosubstituted species  $^{15}\text{N}_3$ ,  $^{15}\text{N}_9$ ,  $^{15}\text{N}_3$ - $^{15}\text{N}_9$ ,  $^{13}\text{C}_2$ ,  $^{13}\text{C}_8$ ,  $^{18}\text{O}_{14}$  and  $^2\text{H}_{13}$  (see Figure 3 for labeling) were measured. The observation of the  $^2\text{H}_{14}$  spectrum was not possible due to overlapping with other spectra. The complete sets of determined parameters, details of the analysis and observed frequencies are collected in the Supplementary Information, Tables S3-S12.

A first insight of the structure of the cluster comes from simple considerations. The nearly identical values of the B rotational constant (see Tables 1 and S4) for the parent and  $^{18}\text{O}_{14}$  species indicates that the water oxygen atom lies along the  $b$  inertial axis. The planar moment of inertia,  $P_{cc}$ , which gives a measure of the mass extension out of the  $ab$  inertial plane, takes a nearly constant value (around 0.58  $\text{u}\text{\AA}^2$ , see Tables 1 and S4) for all isotopologues. Therefore, a planar heavy atom skeleton practically coincident with the  $ab$  inertial plane is concluded. The water hydrogen atom involved in the HB is also in this plane.

The non-bonded water hydrogen atom, H<sub>15</sub>, is out of this plane. From  $P_{cc}$  the  $c$  coordinate of this atom is estimated to be 0.760 Å by taking  $r(\text{O-H}) = 0.965 \text{ \AA}$ <sup>[36]</sup> the dihedral angle  $\angle \text{H}_{15}\text{O}_{14}\text{O}_1\text{C}_2$  is estimated to be 128°.

The so-called substitution,  $r_s$ , method, which uses Kraitchman equations,<sup>[37,38]</sup> exploits changes in the moments of inertia upon mono-isotopic substitution to provide the atom coordinates of the substituted atoms in the principal inertial axis frame. This purely experimental approach allows to directly locating the different atoms of a molecule but has several drawbacks: i) It is of application with only mono-isotopic substitution data, ii) it only delivers the magnitudes of the atomic  $r_s$  coordinates and iii) it poses limitations for atoms located near the three principal axes. An alternative way to exploiting multi-isotopic information is to obtain the bond distances and angles from a least squares fit of all of the available rotational parameters.<sup>[39,40]</sup> Such a procedure involves a total or partial fitting of the effective ground state,  $r_0$ , structural parameters. The  $r_0$  principal inertial axis coordinates or those taken from *ab initio* computations can be used to assign the coordinate signs to the  $r_s$  coordinates allowing for the eventual unambiguous identification of a given cluster.

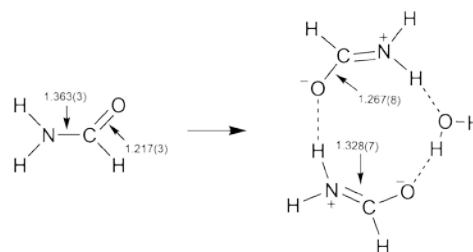


**Figure 3.** a) Comparison of the experimental  $r_s$  position of the isotopically substituted atoms of formamide<sub>2</sub>-H<sub>2</sub>O with the *ab initio* structure. The  $r_s$  interatomic distances are also given. b) The experimental  $r_0$  bond lengths and angles of formamide<sub>2</sub>-H<sub>2</sub>O (upper data) compared to the *ab initio* MP2/6-311++G(2d,p) predictions.

The results of the application of the  $r_s$  and  $r_0$  methods are summarized in Figure 3 where they show a good consistency with the *ab initio* data. The  $r_s$  coordinates are collected in Table S13 of the supplementary information. The  $r_0$  data and the details of the calculations are collected in Table S14 where they are compared to those of *ab initio* calculations. The experimental structure (see Figure 3) reflects that the two formamide subunits close a sequential cycle with water through three HBs,

C=O...H-N, N-H...OH and O-H...O=C. This is possible thanks to the HB donor/acceptor double character of both water and formamide molecules. The HB lengths indicate that the dominant interaction is O-H...O=C as reported for related systems<sup>[22,23]</sup> and agreement with the observed preference of water to interact with this group in globular proteins.<sup>[2]</sup>

The so called  $\sigma$ -bond cooperativity is associated to chains or cycles of hydrogen bonds between OH or any other group acting simultaneously as donor and acceptor.<sup>[3]</sup> Sequential cycles, as  $f_2w$  complex, are characterized by a shortening of the hydrogen bond distances and an increase of hydrogen bond energy as the size of the cycle is increased. The HB distances related to the O-H...O=C and N-H...O bonds of  $f_2w$  are shorter than those of related sequential cycles like formamide-(H<sub>2</sub>O)<sub>n</sub> (n=1,2),<sup>[25]</sup> the water dimers<sup>[41]</sup> and trimers<sup>[42]</sup> or those of formamide dimer in liquid phase<sup>[43]</sup> (see Table S16). The  $f_2w$  bond lengths are only comparable to those of the three body adduct formamide-(H<sub>2</sub>O)<sub>2</sub>. A similar conclusion can be reached by considering dissociation energies (see Table S16). Another aspect of hydrogen bond cooperativity described as resonance-assisted hydrogen bonding (RAHB)<sup>[19]</sup> or  $\pi$ -cooperative bonding<sup>[3]</sup> was first pointed out to occur in the case of the peptide linkage<sup>[44-46]</sup> from studies of the crystal structures of small amides. The main effect of this cooperativity on the structure of formamide is the C=O bond enlargement and the C-N peptide bond length decrease due to the formation of open and closed dimers. This effect not yet reported in gas phase form rotational spectroscopy has been observed in this work as illustrated in Scheme 1 where the  $r_0(\text{C-N})$  and  $r_0(\text{C=O})$  distances for formamide and  $f_2w$  (see Tables S14-S16) are shown. *Ab initio* calculations predict the same effect for related complexes (see Table S16).



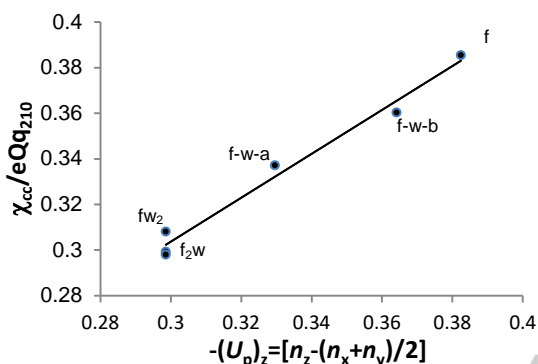
**Scheme 1.** Cooperative HB inductive effects in the formation of  $f_2w$  are evidenced by the C=O bond length enlargement and C-N bond length decrease.<sup>[3]</sup>

The <sup>14</sup>N quadruple coupling constants are expected to be very sensitive to the small changes in the electronic environment at these nuclei produced by inductive cooperative effects. Unfortunately, a complete picture of the electric field gradient can only be obtained from the complete determination of the quadrupole coupling tensor. In the present case, only the diagonal elements of this tensor have been determined, but we can still extract some interesting information from it. In bare formamide, the  $c$  inertial axis, perpendicular to the plane of the molecule, is parallel to one of the principal quadrupole coupling axis, say  $z$ , and thus  $\chi_{cc} = \chi_{zz}$ . Given the planarity of the heavy atom skeleton of  $f_2w$ , its  $c$  inertial axis is practically perpendicular to the formamide molecular planes and then  $\chi_{cc} \approx \chi_{zz}$ . This planar arrangement is common to all complexes of water and formamide studied so far.<sup>[25]</sup> It is thus interesting to compare if

there is some correlation in the experimental values of  $\chi_{cc}$  in formamide and its water complexes. These values are shown in Table S17 which also collect the complete *ab initio* quadrupole coupling tensors.

There is a progressive decrease in the value of  $\chi_{cc}$  going from formamide to its microsolvated complexes. The *ab initio* data based on a static picture of the complexes allow to discard intermolecular oscillations<sup>[47]</sup> as the main cause of this effect. This can be seen in Table S17 where there is a reasonable agreement between the experimental and calculated decrements in the values of  $\chi_{cc}$ . This correlation in the values of  $\chi_{cc}$  indicates that the electronic environment of the N atom of formamide is altered to some extent by the formation of one or successive HBs.

	f	f-w-a	fw <sub>2</sub>	f <sub>2</sub> wN <sub>3</sub>	f <sub>2</sub> wN <sub>9</sub>	f-w-b
$\chi_{cc}/eQq_{210}$	0.382	0.33	0.299	0.299	0.299	0.364
$-(U_p)_z$	0.385	0.337	0.308	0.299	0.298	0.360
$\eta_D$	0.042	0.224	0.348	0.346	0.384	0.038



**Figure 4.** Plot of the values (see upside values) of the quantity  $\chi_{cc}/eQq_{210}$  vs. the unbalanced  $2p_z$  electronic charge  $(U_p)_z$  obtained from a NBO analysis of formamide, formamide-H<sub>2</sub>O (f-w-a), formamide-(H<sub>2</sub>O)<sub>2</sub> (fw<sub>2</sub>) and formamide<sub>2</sub>-H<sub>2</sub>O (f<sub>2</sub>w). The slope of the line is 0.96.

The electric field gradient giving rise to nuclear quadrupole coupling of most molecules has been attributed primarily to the unequal filling of the p orbitals of the valence shell of the coupling atoms.<sup>[31,48]</sup> According to this the <sup>14</sup>N  $\chi_{zz}$  constants can be related to the unbalanced  $2p_z$  electronic charge  $(U_p)_z = [(n_x - n_y)/2 - n_z]$  by:

$$\chi_{zz}/eQq_{210} = -(U_p)_z \quad (1)$$

Where  $n_\alpha$  are the  $p_\alpha$  orbital occupation numbers and  $q_{210}$  the electric field gradient associated to a  $2p$  electron in an isolated atom ( $eQq_{210} \approx 10$  MHz for N). This definition of  $(U_p)_z$  is such that its positive or negative values correspond respectively to electron deficit or excess along the  $z$  reference axis.<sup>[31]</sup> The values of  $\chi_{zz}/eQq_{210}$  obtained from the experimental  $\chi_{cc}$  values are plotted in Figure 4 vs. those calculated using the natural atomic orbital populations from a natural bond orbital (NBO) analysis.<sup>[49]</sup> Despite the approximations, they show an excellent agreement. According to the definition of  $(U_p)_z$ , in formamide  $-(U_p)_z = 0.39$  there is an electron density excess along the  $z$  axis which can be identified with the nitrogen non-bonding orbital density. For the complexes there is a progressive decrease in the value of  $-(U_p)_z$  as the size of the cycle increases to values close to 0.30 in the three body adducts f<sub>2</sub>w and fw<sub>2</sub>. There is a small decrease even for f-w-b  $-(U_p)_z = 0.36$ , where the NH<sub>2</sub> is not involved in HB. This illustrates how the amide group is

progressively polarized due to inductive effects, which increases with the number of cooperating hydrogen bonds.

Another parameter constituting a source of information of the existence of cooperative effects is the asymmetry parameter,  $\eta_D = |(\chi_{yy} - \chi_{xx})/\chi_{zz}|$ . It measures the deviation of the charge distribution from axial symmetry and takes values between 0, for a symmetric distribution of charge around the  $z$  axis, and 1. The *ab initio* values of  $\eta_D$  (see Figure 4 and Table S15) show noticeable changes. Formamide and f-w-b are predicted to have an almost symmetric distribution of charge but this is not preserved in the other complexes so that  $\eta_D$  take values different than zero, 0.22 for formamide-H<sub>2</sub>O and 0.35-0.38 for three body clusters. The NBO analysis gives additional information about charge transfer in these cluster and allows to give an interpretation of the progressive enlargement of the C=O bond and decrease of C-N bond of formamide in the sequential cycle clusters based on the progressive increase of electron density on the carbonyl group  $\pi^*$  antibonding orbital and the progressive decrease of the small electron density in the C-N bond  $\sigma^*$  natural orbital respectively.

In conclusion the adduct formamide<sub>2</sub>-H<sub>2</sub>O studied in this work can be taken as a model to study the interactions of water with amides and the peptide group. It can be also considered as a microsolvated water adduct, being formamide the solvent in this case. In fact, both formamide and water are miscible. It may illustrate the way in which water acts as mediator to help in the formation of HB interactions involving the amide linkages during protein folding and in amide association processes showing the interplay between self-association and solvation. The structure of this adduct reflects clearly the effects of the so-called  $\sigma$  and  $\pi$ -cooperative hydrogen bonds.<sup>[3]</sup> It adopts a cyclic planar structure for the heavy atom skeleton and this directionality may be important in the process of protein folding. All structural parameters reflect the existence of strong cooperativity effects in the HB distances, shorter than in related sequential cycles, and in the C-N and C=O distances of formamide subunits which became shorter or larger, respectively, upon formation of the complex. But perhaps one of the most interesting aspects of this research is the confirmation that the quadrupole coupling interaction can be taken as a probe for the inductive processes accompanying cooperativity which alter the electronic structure of the amide group upon polarization. Another remarkable result concerning cooperative effects, is the similarity observed in the structural and quadrupole coupling data in both formamide<sub>2</sub>-H<sub>2</sub>O and formamide-(H<sub>2</sub>O)<sub>2</sub>. This suggests that the acid/base properties of formamide and water are not very different. Finally this work shows that water is able to enhance cooperative HB effects. This can be important for protein folding, in water mediated contacts between peptide groups.

## Experimental Section

Commercial samples of parent and isotopic species of formamide and water were used. The molecular-beam Fourier transform microwave spectrometer (MB-FTMW)<sup>[50]</sup> was operated in the frequency range 5-13 GHz. The supersonic jet was generated by expansion of a gas mixture He-Ne at backing pressures of about 12 bar through a small (0.9 mm diameter) pulsed heatable nozzle.<sup>[51]</sup> Formamide (b.p. 210°C) was placed in a nozzle at about 50°C and water in a reservoir inserted in the gas line just before the nozzle. Short (typ. 0.3  $\mu$ s, 10-300 mW) microwave pulses were used for polarization purposes. Typically, a ca. 400  $\mu$ s-length time domain spectrum was recorded in 40-100 ns intervals and converted to the frequency domain by a fast Fourier transformation. Due to the

collinear arrangement of the jet and resonator axis each rotational transition splits in two Doppler components so the resonant frequencies are taken as the arithmetic mean of both components. Frequency accuracy is better than 3 kHz. *Ab initio* calculations (MP2/6-311++G(2d,p)) were done using G09 package.<sup>[52]</sup> The counterpoise procedure<sup>[53]</sup> was used to calculate the dissociation energies. NBO analysis were also done using NBO 5.9.<sup>[54]</sup>

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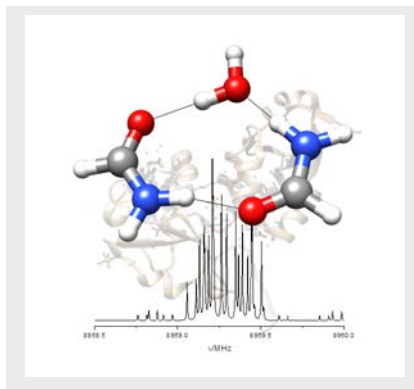
**Keywords:** Hydrogen bonds • Cooperative effects • Non covalent interactions • rotational spectroscopy • molecular modelling

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## Entry for the Table of Contents

## COMMUNICATION

Isolated formamide<sub>2</sub>-water can be taken as a simple model for water mediated interactions and amide self-association. The determined structure and the electric field gradient at the N nuclei measured from the rotational spectrum reveal the subtle inductive effects of cooperativity that polarize the amide group and alter the C=O and N-C distances of formamide as detected in this work.



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**Hydrogen Bond Cooperativity in Formamide<sub>2</sub>-water, a Model for Water Mediated Interactions.**

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