

## Amides as models to study the hydration of proteins and peptides - spectroscopic and theoretical approach on hydration in various temperatures

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### Abstract

Interactions with water are one of the key factors which determine protein stability and activity in aqueous solutions. However, the protein hydration is still insufficiently understood. *N*-methylacetamide (NMA) is regarded as a minimal part of the peptide backbone and the relative simplicity of its structure makes it a good model for studies on protein–water interactions.

In this paper, the influence of NMA and *N,N*-dimethylacetamide (DMA) on surrounding water molecules in a range of temperature (25–75 °C) is studied by means of the FTIR spectroscopy. The results of the difference HDO spectra method are compared with the results of theoretical DFT calculations of NMA and DMA aqueous complexes.

Both NMA and DMA can be regarded as “structure-makers”, yet their hydration spheres are different. These molecules exhibit a mixed and mutually dependent types of hydration: hydrophilic and hydrophobic. In the case of a NMA molecule that has one methyl group less than DMA, the type of

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hydrophobic hydration is more important. The DMA hydration sphere is less stable: the interactions between water molecules around the methyl groups are strained. Moreover, the hydration of NMA is much more temperature dependant than in the case of DMA. The source of the differences may be hidden in the N–H···H<sub>2</sub>O interaction.

The delicate nature of water interactions with the peptide block models may be cautiously translated into the much more complicated interactions of proteins with their hydration shells.

*Keywords:* N-methylacetamide, hydration, FT-IR spectroscopy, DFT calculations

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## 1. Introduction

Proteins play key role in every biological process. Their functionality in living organism is determined by their secondary and tertiary structure, which is formed and preserved by relatively weak interactions called hydrogen bonds. Hydrogen bonds in protein-water systems consist mostly of intra- and intermolecular bonds between protein molecule and water in natural environment.

Interactions of water and proteins is one of the most important topics when it comes to studying behavior of proteins in their native state. Multiple studies have taken on this topic using various methods [1–12], however, it is still poorly understood. The study of proteins hydration experimentally is problematic due to the high level of complexity of these molecules. Additionally, overall hydration of proteins involves two general types of interactions, namely, hydrophilic hydration and hydrophobic hydration of the non-polar



15 fragments. Those contributions are hard to separate in such complex struc-  
16 tures.

17 *N*-methylacetamide (NMA) can be regarded as a single peptide bond unit  
18 that is used as a model for studying both intra- and intermolecular interac-  
19 tions that would occur within proteins backbone in their native states. It has  
20 been shown that the NMA's hydration sphere is very similar to the hydration  
21 sphere of lysozyme. The lysozyme-affected water spectrum mainly consists of  
22 the spectral share of water affected by NMA, which indicates the hydration  
23 of the protein backbone.[12] Recent studies have confirmed the resemblance  
24 of NMA to peptides as it seemed to form chain like structures similar to  
25 protein backbone [13]. Existence of NMA dimers and other oligomers have  
26 also been proposed [14–17]. Particularly, it was pointed out that NMA dimer  
27 might be even more convenient peptide bond model than NMA itself [15].

28 NMA and its water clusters have been extensively studied both experi-  
29 mentally [13–15, 18–28] (mainly by means of vibrational spectroscopy) and  
30 theoretically [13, 15, 18, 19, 25, 27–35]. It has been numerously investi-  
31 gated using C=O or N–D stretch as a probe of changes in its surrounding  
32 [13, 14, 18, 19, 26, 28–30]. Changes of water OD bands in isotopically diluted  
33 systems is also a popular probe for studying hydration of NMA [18, 34, 36].  
34 Using aforementioned approach it has been stated that at 25°C water in  
35 NMAs first hydration shell forms stronger hydrogen bonds than bulk water  
36 [36]. Recently, these observations have been supported by Salamatova *et. al*  
37 [18], with the results of MD simulations of NMA–water systems. Theoretical  
38 studies describing this molecule's hydration have shown that a water molecule  
39 forms stronger hydrogen bonds with the C=O group than with the N–H or



40 N–D [29, 37–39]. Also, it has been observed that  $C=O \cdots H_2O$  hydrogen  
41 bonds are stronger than between water molecules. However, computational  
42 studies of deuterated systems carried out by Yadav *et al.* [29] revealed that  
43 bonds formed by the C=O group and water are weaker than water–water  
44 hydrogen bonds outside NMA first hydration shell.

45 In other works, in which the hydration of this molecule was investigated,  
46 it has been pointed out that the dilution of the bulk NMA with water or the  
47 increase of temperature leads to the disruption of hydrogen bonded aggre-  
48 gates present in the bulk NMA at low temperatures [33]. Its hydration leads  
49 to the formation of NMA–water clusters in which at least some of NMA dy-  
50 namics seems to “freeze at a measurable time scale. This means that NMA  
51 experiences a kind of hydrophobic collapse while interacting with water [18].  
52 The *ab initio* analysis of  $1 \cdot NMA - 2 \cdot H_2O$  clusters and their deuterated deriva-  
53 tives has allowed to assign normal modes of the aqueous hydrogen bonded  
54 NMA [34]. The one water molecule has been hydrogen bonded to C=O group  
55 of NMA and another one to the N–H group of those molecule.

56 Yang and Qian [40] conducted MM studies on properties of the isolated  
57 *cis*- and *trans*-NMA and NMA–water complexes. Calculation of the interac-  
58 tion energies and cooperative effect have revealed that complex of the *trans*-  
59 NMA with two water molecules attached to the carbonyl oxygen is energet-  
60 ically unfavorable. Buck and Karplus on the basis of MD in a vacuum [39]  
61 have found that in the case of such a complex distance between the hydrogen  
62 atom of the first water molecule and the carbonyl oxygen atom is 1.71 Å and  
63 the second is 1.97 Å. Also, it has been shown that NMA with one hydrogen  
64 bonded water molecule on either C=O or N–H prefers a second one interact-



65 ing with the other group [40]. In addition, it has been found that complex  
66 with 3 water molecules is stable.

67 Several studies focus on a *cis-trans* equilibrium caused by rotation bar-  
68 rier around C–N bond in formamide and acetamide derivatives [23, 41–45].  
69 It has been shown that in the case of NMA similarly to proteins *trans* form of  
70 NMA is the dominant form in liquid phases. This form also dominates in the  
71 gas phase. Theoretical studies on properties of isolated NMA molecule and  
72 NMA in aqueous solution revealed also the existence of two other resonance  
73 structures in such systems [38, 46–48]. Neutral form of NMA is more pro-  
74 nounced in gas phase while zwitterionic structure in water. As a result, the  
75 elongation of C=O bond and the shortening of N–C bond passing from gas  
76 to water are observed. This effect is caused by the stabilization of zwitteri-  
77 onic structure of NMA by hydrogen bonding with water molecules. Also, the  
78 rotation of the amide methyl group as an effect of solvation NMA molecule  
79 has been observed.

80 The Born–Oppenheimer molecular dynamics and MD/MM have been  
81 used to study NMA in the gas phase and in a box of water molecules [48].  
82 The resultant frequencies of the amide bond vibrations confirmed other the-  
83 oretical and experimental studies [49, 50].

84 In this work we have investigated the water structure around NMA in  
85 wide temperature range by means of the FTIR spectroscopy. Vibrational  
86 spectroscopy is an excellent method for investigation of solute hydration  
87 [51, 52]. Isotopic dilution technique of HDO in water allows us to obtain  
88 bands which are more convenient to analyze and highly sensitive to changes  
89 in a solution. HDO spectra are narrower and less complex than the OH bands



90 of H<sub>2</sub>O. Moreover, the OD vibration is practically free from intramolecular  
91 and intermolecular couplings between oscillators. Using difference spectra  
92 method [53–55] it is possible to isolate the contribution of HDO affected by  
93 the solute from the OD band. Solute-affected HDO spectra can then be  
94 compared with HDO spectra of bulk water, thus determining the structural  
95 and energetic state of hydrogen bonds of water in the nearest surround-  
96 ings of solute. In isotopically diluted solutions of NMA there is significant  
97 amount of N-deuterated NMA. N–D bond vibrations happen to manifest in  
98 the IR spectrum in the same region as HDO bands. Spectra of water af-  
99 fected by *N,N*-dimethylacetamide (DMA) have also been obtained to isolate  
100 ND stretching vibration from NMA-affected HDO spectra and to compare  
101 those compounds in terms of hydration. To confirm the interpretation of  
102 spectral results theoretical calculations were performed.

## 103 2. Materials and Methods

### 104 2.1. Chemicals and Solutions

105 *N*-methylacetamide (99+%, Aldrich), *N,N*-dimethylacetamide (Acros Or-  
106 ganics, 99.5%) and deuterium oxide (Aldrich, 99.96%) were used to pre-  
107 pare solutions without purification. Water used was deionized. For each  
108 solute (NMA, DMA) stock solution in deionized water of maximum molal-  
109 ity 1 mol · kg<sup>-1</sup> was prepared. Less concentrated solutions were prepared by  
110 dilution of weighed amounts of stock solutions using deionized water. Each  
111 of the solutions was then divided into two parts in order to prepare sample  
112 and reference solutions. Sample solution was prepared by adding D<sub>2</sub>O to one  
113 of the parts in amount of 4% with respect to H<sub>2</sub>O (by weight). Reference

114 solution was prepared by adding the same molar amounts of H<sub>2</sub>O to the sec-  
115 ond part. The amount of used deuterium oxide was confirmed to be enough  
116 for reaction  $\text{H}_2\text{O} + \text{D}_2\text{O} = 2\text{HDO}$  ( $K \approx 4$ ) to give an almost quantitative  
117 amount of HDO. All solutions were prepared by weight. The prepared so-  
118 lutions were degassed before density and spectral measurements. Solution  
119 densities were measured using the Anton Paar DMA 5000 densitometer at  
120 all studied temperatures (25, 35, 45, 55, 65, and 75 °C, with the tolerance of  
121 0.001 °C).

## 122 2.2. FTIR Measurements

123 FTIR spectra of prepared solutions were recorded on Thermo Electron  
124 Co. Nicolet 8700 spectrometer with resolution of 4 cm<sup>-1</sup>. Each spectrum  
125 was an average of 128 independent scans. A liquid cell (model A145, Bruker  
126 Optics) with CaF<sub>2</sub> windows separated PTFE spacers was used. The path  
127 length was determined interferometrically and was 29.0 μm. The temperature  
128 of measurements was kept at 25, 35, 45, 55, 65, and 75 °C with a tolerance  
129 of ±0.1 °C and monitored using electronic thermometer with thermocouples  
130 placed in the sample. The temperature of 75 °C has been chosen as an upper  
131 temperature limit being the highest possible temperature due to technical  
132 capabilities of the experimental setup. In the case of DMA solutions, the  
133 maximum temperature was limited to 55 °C due to decomposition of solution  
134 components to gaseous products at higher temperatures. The spectrometer  
135 was purged with dry nitrogen to eliminate the influence of air components  
136 on recorded spectra, namely, CO<sub>2</sub> and water vapor.



137 *2.3. Spectral data analysis*

138 Procedures of recording and analyzing spectra were handled using the  
139 commercial PC software: OMNIC (Thermo Electron Co.), GRAMS/32 (Galac-  
140 tic Industries Corporation, Salem, NH) and RAZOR (Spectrum Square As-  
141 sociates, Inc., Ithaca, NY).

142 To obtain the desired information the difference spectra method [56] was  
143 applied. This method assumes that water in a solution can be divided into  
144 two additive contributions: the “bulk” water—identical to pure water—and  
145 “affected” water—under the influence of solute. Therefore information about  
146 solute-affected water can be isolated from solutions IR spectrum. The de-  
147 tailed procedure of spectral data analysis toward extraction of the solute-  
148 affected water spectra has been described in section S1 of Supplementary  
149 Data.

150 *2.4. Interpretation of the solute-affected water spectra*

151 To interpret given solute-affected spectra the Badger-Bauer rule is used  
152 [57]. This empirical rule states that energy of hydrogen bonds is proportional  
153 to the shift of the OD band position, thus it is a convenient method of com-  
154 paring energetic state of solute-affected water with bulk water and investigate  
155 the changes of this characteristics with temperature [54, 55]. The shape of an  
156 OD bands contain information about hydrogen bond energy distribution in  
157 water. With the existence of empirical curves that link the oxygen–oxygen  
158 intermolecular distance ( $R_{OO}$ ) with the OD stretching wavenumber ( $\nu_{OD}$ )  
159 it is possible to transform the spectral band shapes to the oxygen–oxygen  
160 distance distribution function  $P(R_{OO})$  [56, 58].





161 *2.5. Theoretical calculations*

162 All calculations involving optimization of NMA or DMA complexes with  
163 water molecules were performed with the Gaussian 09 v.D1 software [59]  
164 available at the Academic Computer Center in Gdansk (TASK). The analy-  
165 sis of resulting wavefunction files involving electron density difference calcu-  
166 lations and the reduced density gradient (RDG) method [60] was performed  
167 with the Multiwfn software v.3.3.9. [61]. Structures of NMA or DMA com-  
168 plexes with water molecules were optimized similarly to the procedure pre-  
169 sented in our previous papers [16, 62]. Final structures were optimized at  
170 the density functional theory (DFT) level with B3LYP hybrid exchange-  
171 correlation functional [63, 64] and 6-311++G(d,p) basis set [65] within the  
172 conductor-like polarizable continuum model (CPCM) of water as a solvent  
173 [66, 67]. All calculations were performed with the D3 version of Grimme’s  
174 empirical dispersion correction including Becke–Johnson damping [68]. Such  
175 a selection gives a good consistency of theoretical and experimental results.

176 Differences in electron densities caused by NMA or DMA interactions  
177 with water molecules were calculated and visualized according to section  
178 4.5.5 of the Multiwfn manual. Shortly, electron densities were calculated  
179 for the water complex with NMA or DMA and separately for their isolated  
180 fragments: NMA or DMA, and water molecules, and then appropriately  
181 subtracted from each other.

### 182 3. RESULTS AND DISCUSSION

#### 183 3.1. FTIR investigation of water structure

##### 184 3.1.1. Characteristic of solute-affected water spectra and “bulk” water spectra

185 Figure 1 presents the NMA-affected HDO spectra (Figure 1a), DMA-  
186 affected HDO spectra (Figure 1b), and the “bulk” water spectra (Figure 1c)  
187 as a function of temperature. Band shapes of these spectra were transformed  
188 into the oxygen–oxygen distance distribution function  $P(R_{OO})$  of the water  
189 molecules. The obtained distance probability distributions are shown in Figs.  
190 2a and 2b for solute-affected water,  $P^a(R_{OO})$ , and in Figure 2c for “bulk”  
191 water,  $P^b(R_{OO})$ . The band parameters for affected HDO bands, together  
192 with the bulk HDO bands, for measured temperatures are presented in Table  
193 1, along with intermolecular oxygen-oxygen distances,  $R_{OO}$ .

194 A comparison of the values of the mean oxygen–oxygen distances ( $R_{OO}^g$ )  
195 for solute-affected water and for “bulk” water (Table 1) points out that water-  
196 water hydrogen bonds are shorter in the presence of these solutes. The  
197 shift of the values of the gravity center of bands,  $\nu_{OD}^g$ , (related to the mean  
198 energy of water hydrogen bonds) towards lower values with respect to the  
199 ones corresponding to pure water at a given temperature (Table 1) suggests  
200 that water affected by NMA and DMA forms on average stronger H-bonds  
201 than pure water in the whole temperature range. In addition, the hydrogen  
202 bonds of water molecules around NMA are stronger and shorter than those in  
203 water affected by DMA. The above results indicate that both amides enhance  
204 the water structure in their nearest surrounding in the whole temperature  
205 range, and can be classified as “structure-making” solutes. This statement is  
206 supported by the analysis of radial distribution functions obtained by means

207 of MD simulations at 27°C [18]. Analyzing the effect of temperature variation  
208 on the affected HDO spectra, it can be seen that solute-affected water spectra  
209 at lower temperature are characterized by stronger hydrogen bonds than at  
210 higher temperatures. A similar relationship is shown by the “bulk” water  
211 spectrum. This is obvious because at higher temperatures the distances  
212 between water molecules increase and hydrogen bonds weaken. Furthermore,  
213 the number of moles of affected water molecules ( $N$  values from Table 1)  
214 around NMA decreases as the temperature increases, and as a result at the  
215 highest temperature, the two water molecules are affected by NMA. Water  
216 molecules in the hydration sphere of DMA are not susceptible to temperature  
217 variations, which is reflected the constant value of number of affected water  
218 molecules as a function of temperature.

219 *3.1.2. Differences in oxygen-oxygen distance distributions between “affected*  
220 *water” and “bulk” water*

221 To provide greater insight into the difference in intermolecular distances  
222 between affected water and “bulk” water the following procedure was used:  
223 the distance distribution function for “bulk” water,  $P^b(R_{OO})$ , (Figure 2c)  
224 was subtracted from the distribution function of water affected by solute,  
225  $P^a(R_{OO})$ , (Figures 2a and 2b) at a given temperature. The results of the  
226 subtraction,  $\Delta P(R_{OO})$ , each analyzed temperature are shown in Figures 3a  
227 and 3b for NMA and DMA, respectively. This operation allows to observe  
228 subtle changes in the population of hydrogen bonds of water affected by  
229 solute relative to “bulk” water at a given temperature.

230 The analysis of distance differences (Figure 3b) clearly shows that the  
231 hydration sphere of DMA contains two populations of hydrogen bonds of

232 water for all studied temperatures: the first one corresponds to the weak  
233 hydrogen bonds ( $R_{OO} \approx 2.9 \text{ \AA}$ ), while the second one corresponds to the  
234 strong water-water hydrogen bonds ( $R_{OO} \approx 2.73 \text{ \AA}$ ). The distances assigned  
235 to the second population is close to the distance typical to the water in the ice  
236 phase ( $R_{OO} = 2.76 \text{ \AA}$ ) [69]. The creation of the above-mentioned populations  
237 takes place mainly at the expense of very weak hydrogen bonds ( $R_{OO} \geq 3.0$   
238  $\text{ \AA}$ ) and to a lesser extent of hydrogen bonds with mean energy (the population  
239 of water-water hydrogen bonds only slightly longer than and equal to the  
240 most probable distance in bulk water, value  $R_{OO} = 2.826 \text{ \AA}$  at  $25^\circ\text{C}$ , see  
241 Table 1). A slightly larger population of strong water hydrogen bonds relative  
242 to the weak ones causes that in the surrounding of DMA the water structure  
243 is strengthened with respect to the “bulk” water. Furthermore, it can be  
244 seen that the differences between DMA-affected water and “bulk” water are  
245 practically the same at all temperatures. This means that DMA enhances  
246 to the same extent the water structure in the whole temperature range, and  
247 thus demonstrates the high stability of the DMA hydration shell.

248 A common feature of the differences in intermolecular distances distribu-  
249 tion for NMA,  $\Delta P(R_{OO})$ , (Fig. 3a) is the reduction of population of very  
250 weak hydrogen bonds of water ( $R_{OO} \geq 3.0 \text{ \AA}$ ) and simultaneous distinct  
251 increase the strong ones ( $R_{OO} \approx 2.73 \text{ \AA}$ ), in comparison to “bulk” water.  
252 The first population is practically unchanged with increasing temperature.  
253 The most significant changes relate to the population of water molecules with  
254 mean energy and weak hydrogen bonds of water ( $R_{OO} \approx 2.9 \text{ \AA}$ ). At the lowest  
255 temperatures ( $25^\circ\text{C}$  and  $35^\circ\text{C}$ ), it may be seen that population of such hy-  
256 drogen bonds of water around NMA decreases, relative to the “bulk” water.



257 However, starting from the temperature of 45 °C, the discussed population  
258 of hydrogen bonds increases with the temperature increase.

### 259 3.1.3. Differences between $\Delta P(R_{OO})$ vs. temperature 25 °C

260 Changes in populations of hydrogen bonds in the hydration sphere of  
261 NMA, with respect to the temperature of 25 °C as a reference point, are  
262 shown in Figure 4. As can be seen, the population of water molecules with  
263 mean and weak energy of hydrogen bonds is increased in comparison to  
264  $\Delta P(R_{OO})$  at 25 °C. This population seems to be responsible for the weakening  
265 of the hydrogen bond network around NMA when the temperature increases,  
266 relative to the course of  $\Delta P(R_{OO})$  at 25 °C. The NMA hydration sphere at  
267 35 °C is additionally characterized by a slightly larger population of strong  
268 hydrogen bonds (ca. 2.65 Å) relative to the temperature of 25 °C. Probably,  
269 such a population can be caused by the formation of NMA–NMA dimers  
270 [14–16] at this temperature, which may break up at higher temperatures.  
271 Such an interpretation is however speculative because the presence of dimers  
272 in aqueous solutions is difficult to observe.

### 273 3.2. DFT calculations: sources of differences in NMA and DMA hydration

274 A series of DFT calculations was performed to try to explain the differ-  
275 ences in strong and weak populations of hydrogen bonds recognized on the  
276 basis of NMA- and DMA-affected water spectra. Undoubtedly, the difference  
277 between NMA and DMA hydration lies in the presence of additional methyl  
278 group. Thus, we created a closed ring of water molecules encircling those  
279 compounds in the closest proximity of the amine proton or methyl group  
280 in the case of NMA and DMA, respectively, by a gradual addition of water

281 molecules to optimized NMA and DMA structures (see Figure 5). The water  
282 ring is a model of the full hydration shell, which could be too demanding  
283 to calculate for the selected set of method and basis set. In each case, the  
284 minimal number of water molecules needed to create a closed circle of water  
285 molecules was equal to eight. Animations showing optimized structures of  
286 these complexes (in two variants: with overlaid differences in electron density  
287 or with visualized places of weak interactions determined by RDG method)  
288 are included in Supplementary data.

### 289 3.2.1. Steric effect

290 For both NMA and DMA the ring of water molecules is skewed (Figure  
291 5, top view). The skewness seems to be connected with the steric effect  
292 that nitrogen-bounded methyl group exerts on water molecules approaching  
293 the carbonyl oxygen atom. The starting dihedral angle between C–H and  
294 C=O bonds in NMA or DMA isolated molecules is close to  $0^\circ$ . This result is  
295 in accordance with ref. [37]. The situation changes when interactions with  
296 water molecules occur. When water molecules approach the carbonyl bond of  
297 NMA the C–H bond easily adopts the dihedral angle to ca.  $30^\circ$  to fit the water  
298 ring encompassing the molecule (Figure 5, front view), hence the skewness  
299 of the water ring plane. The rotation of the amide methyl group of NMA,  
300 when going from gas phase to solution, has also been found by Mennuci *et.al*  
301 [37]. However, the examined clusters have been smaller (they contained up  
302 to 3 water molecules) and the value of the dihedral angle was different. The  
303 weak steric interaction between carbonyl group and methyl proton (marked  
304 with red arrow in Figure 6a) also shifts to one side and makes place for a  
305 water molecule to take the optimal location. The position of methyl proton

306 is not as fixed as in the case of DMA, where an additional steric interaction  
307 is placed between both amine methyl groups (large green/red patch under  
308 carbonyl group in Figure 6a). Moreover, carbonyl group (C=O) and water  
309 molecules interacting with it lies in one plane and the water ring is highly  
310 symmetrical (Figure 5, front view).

311 Interactions between water molecules or between water molecules and the  
312 carbonyl group of NMA or DMA are enhanced with respect to the “bulk”  
313 structure ( $R_{OO,bulk,av.} = 2.817 \text{ \AA}$ , see Fig. S5 in Supplementary Data), thus,  
314 an enhanced population is expected to be present in the  $R_{OO}$  distance dis-  
315 tribution. In fact, both compounds exhibit such an enhancement (Figure 3).  
316 The population origins not only in water–carbonyl interactions but can be  
317 the effect of the hydrophobic hydration, in which van der Waals interactions  
318 have a dominant role (green/olive patches around methyl groups in Fig. 6a).  
319 In the case of formamide, i.e. a molecule that does not contain hydrophobic  
320 groups in its structure, we observe only an increasing population of weak  
321 hydrogen bonds of water, in comparison to the “bulk” water (see Fig. 8a  
322 in ref. [36]) This indicates that in the presence of formamide, the hydrogen  
323 bonds between water molecules and the oxygen atom of carbonyl group and  
324 those between water molecules are weaker.

325 The interaction of carbonyl group of NMA with a single water molecule  
326 does not seem to be distinguished, and its energetics is similar to the one  
327 between water molecules [16, 29]. However, the closing of the water ring  
328 around NMA, involving weak hydrophobic interactions (green/olive patches  
329 in Fig. 6a), enhances significantly C=O $\cdots$ H<sub>2</sub>O binding. In such a situation,  
330 two water molecules interact strongly with carbonyl oxygen and almost to



331 the same degree ( $R_{OO} = 2.710 \text{ \AA}$  and  $2.728 \text{ \AA}$ ). Similar length ( $2.73 \text{ \AA}$ )  
332 between carbonyl oxygen and water oxygen has been obtained by means of  
333 MD simulations for a larger system in ref. [38]. The shape of the electron  
334 density differences caused by the interaction of NMA and the water ring  
335 confirms high similarity, or symmetry, in both water–carbonyl interactions.

336 Further analysis shows that under the influence of water molecules the  
337 electron density increases on the carbonyl oxygen. This is accompanied with  
338 the decrease of electron density along the C=O bond. Also, the decrease  
339 of electron on the amide hydrogen and its increase along the N–H bond  
340 towards the nitrogen atom can be observed. In addition, electrons of N–C  
341 bond are pushed towards the carbon atom. This results are in agreement  
342 with the analysis of Wannier Centers average positions for NMA molecule in  
343 gas phase and in water given in ref. [38]

344 A different picture emerges from the analysis of an analogous DMA data.  
345 Such a water ring closure in the DMA system is less favorable. The van der  
346 Waals interactions are less organized and differences in C=O $\cdots$ H<sub>2</sub>O inter-  
347 actions are larger than in the case of NMA. The introduction of additional  
348 methyl group simply inhibits the rotation of other two methyl groups in  
349 DMA molecule. The nitrogen-bonded methyl group of DMA cannot adopt  
350 an optimal angle to fit the water ring as in NMA system. This way, only one  
351 water molecule of water can easily interact with the carbonyl group while the  
352 second one has to find another—non-optimal with respect to NMA—place  
353 at the expense of the interaction energy with the carbonyl group. One water  
354 molecule is closer to the carbonyl oxygen atom ( $R_{OO} = 2.705 \text{ \AA}$ ), while the  
355 second one interacts poorly ( $R_{OO} = 2.886 \text{ \AA}$ , higher even than the average





356 “bulk” distance). Such an inequality may contribute to the presence of two  
357 distinct water hydrogen bond populations (stronger and weaker relative to  
358 the “bulk” water) in the vicinity of DMA molecule, as in experimental results  
359 presented in Figure 3. It should be stressed that the population of strong  
360 hydrogen bonds includes also interactions between water molecules around  
361 methyl groups. The symmetry of water–carbonyl bond–water double inter-  
362 action is broken. Such a division is also clearly visible in the electron density  
363 difference (Figure 5, top view), where the weakly interacting water molecule  
364 is pushed away from the plane of the water ring. The change in electron den-  
365 sity for the first water molecule is highly similar to the case of NMA, while  
366 the second one is non-symmetrical and exhibits a weaker electron density  
367 transfer.

368 According to the above discussion, the differences in the hydration prop-  
369 erties of both molecules are related to the formation of a water cage around  
370 them. Both NMA and DMA due to the presence of hydrophilic and hy-  
371 drophobic groups in their molecule are characterized by two types of hy-  
372 dration, which are mutually dependent. Moreover, hydrogen bonds between  
373 water molecules involved in the cage formation and the interaction of water  
374 molecules with the carbonyl oxygen atom are cooperative. The formation  
375 of a water cage around hydrophobic groups affects the equality/inequality  
376 of interactions of water molecules with the carbonyl group. In the NMA  
377 molecule, the hydrophobic type of hydration is more important, although  
378 it has one methyl group less than DMA. This is due to a worse fit of the  
379 “ice-like” water around methyl groups of DMA to the molecular shape of the  
380 molecule.



381 *3.2.2. Importance of N-H...H<sub>2</sub>O interaction*

382 The interaction of water molecule with hydrogen atom of amino group of  
383 NMA ensures the stability of the hydration sphere around NMA. It acts like  
384 a "clip" stabilizing the hydration cage around NMA. At lower temperatures,  
385 this interaction is incorporated in the hydration cage [36]. When the tem-  
386 perature increases, the hydrogen bonds around NMA weaken and eventually  
387 break, and as a result, the water molecule interacting with the hydrogen  
388 atom of amino group starts to lose its contact with other water molecules.  
389 The relatively weak N-H...water interaction (the distance between the ni-  
390 trogen atom of NMA and oxygen atom of water is 2.890 Å), freed from other  
391 interactions, contributes to the increase of the population of weak hydrogen  
392 bonds in the presence of NMA at higher temperatures (see Fig.3a).

393 The hydration shell around DMA, represented with the modeled ring of  
394 eight water molecules, is already less stable than in the case of NMA at  
395 ambient temperatures (see Figure 6a, the van der Waals interactions are  
396 scattered much more than in the case of NMA). The ring of water molecules  
397 around DMA is stretched from the very beginning due to the relatively large  
398 methyl group in the place of amine proton. In consequence, the already  
399 weakened hydration shell may be less prone to the temperature increase.

400 **4. Conclusions**

401 The effect of NMA and DMA on the water structure as a function of tem-  
402 perature has been studied by means of FTIR spectroscopy, supported with  
403 DFT calculations. The results of theoretical calculations helped to resolve  
404 spectral results. The agreement between experimental and computational re-

405 sults was satisfactory and gave a consistent picture of the amides hydration  
406 in aqueous solution.

407 The results revealed that hydrogen bonds of water molecules around these  
408 solutes are stronger and shorter than those in pure water in the whole tem-  
409 perature range. Although both solutes strengthened the water structure,  
410 significant differences in the characteristics of their hydration shells could be  
411 noticed. The essential difference in the hydration of NMA and DMA is the  
412 way they create the water cage around them, which is the result of both  
413 hydrophilic and hydrophobic hydration. These two types of hydration are  
414 mutually dependent. Thus, the increase in the population of strong hydrogen  
415 bonds of water in the surroundings of these solutes is the effect of the inter-  
416 actions of water molecules with the oxygen atom of carbonyl group and those  
417 between water molecules around hydrophobic groups. The hydration sphere  
418 of formamide, a molecule that does not contain any hydrophobic group, is  
419 characterized by an increased population of weak hydrogen bonds of water,  
420 with respect to the “bulk” water ref. [36], without the strengthening ob-  
421 served in the case of NMA or DMA. This suggest that the presence of a  
422 hydrophobic element in the molecule is a factor conditioning the enhanced  
423 network of hydrogen bonds around the solute.

424 The DMA molecule is additionally surrounded by a population of water  
425 molecules that forms weak hydrogen bonds with respect to the “bulk” water  
426 (at all given temperatures). The presence of this population results from the  
427 poor fit of the water cage to the solute’s geometry. Such a disadvantageous  
428 fit turn out to be less susceptible to the temperature change because the  
429 hydration cage is already distorted at lower temperatures, in comparison to



430 NMA.

431 On the other hand, the NMA water cage is better organized than in the  
432 case of DMA. The hydrophobic type of hydration is more important in the  
433 case of NMA molecule, although it has one methyl group less than DMA.  
434 The interaction of water molecule with the hydrogen atom of amino group is  
435 significant for the stability of the hydration sphere of NMA. Thus, at lower  
436 temperatures the hydration sphere of NMA contains only the population of  
437 stronger hydrogen bonds, in comparison to the “bulk” water. As the temper-  
438 ature increases, the additional population of water molecules, characterized  
439 with weak hydrogen bonds, is observed in the water population affected by  
440 NMA. It results from the weak N–H···water interaction. At lower tempera-  
441 tures, this interaction is incorporated into the hydration cage, while at higher  
442 temperatures it starts to be exposed when water molecule interacting with  
443 the hydrogen atom of amino group loses its contact with the cage around the  
444 molecule.

445 Although both NMA and DMA are simple molecules, their similarity  
446 to the basic building block of proteins makes them convenient models of  
447 protein–water interactions. The obtained results clearly show that the con-  
448 tributions to the hydration sphere of the solute are not additive due to the  
449 number of non-polar groups. A stable hydration sphere depends on the pos-  
450 sibility of building a cooperatively reinforced network of hydrogen bonding  
451 water molecules around the solutes. The changes in the hydration of solutes  
452 caused by the temperature increase and their explanation may be cautiously  
453 translated into phenomena accompanying the process of protein denatura-  
454 tion.

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459 **6. Declaration of interest**

460 The authors declare that they have no conflict of interest.

461 **7. References**

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Table 1: The parameters of HDO bands of water affected by NMA (Figure 1a), water affected by DMA (Figure 1b), “bulk” water (Figure 1c), and the respective intermolecular oxygen–oxygen distances.  $R_{OO}$  errors have been estimated on the basis of the HDO bands position errors.

$T^a$	$N^b$	$\nu^o_{OD}^c$	$\nu^g_{OD}^d$	$fwhh^e$	$I^f$	$R^o_{OO}^g$	$R^g_{OO}^h$
NMA-affected water spectrum							
25	3.0±0.5	2505±2	2486±2	177±4	13120	2.821±0.003	2.821±0.003
35	2.8±0.5	2517±2	2492±2	178±4	11836	2.821±0.003	2.828±0.003
45	2.6±0.5	2518±2	2495±2	167±4	10640	2.836±0.003	2.833±0.003
55	2.5±0.5	2526±2	2501±2	175±4	10059	2.836±0.003	2.838±0.003
65	2.4±0.5	2526±2	2505±2	175±4	9400	2.841±0.003	2.844±0.003
75	2.2±0.5	2534±2	2511±2	169±4	8447	2.851±0.003	2.849±0.003
DMA-affected water spectrum							
25	2.5±0.5	2517±2	2497±2	165±4	16169	2.836±0.003	2.836±0.003
35	2.5±0.5	2524±2	2503±2	167±4	14666	2.836±0.003	2.836±0.003
45	2.6±0.5	2528±2	2505±2	167±4	13467	2.841±0.003	2.841±0.003
55	2.5±0.5	2534±2	2511±2	166±4	12066	2.851±0.003	2.849±0.003
“bulk” water spectrum							
25	-	2509±2	2505±2	162±4	10434	2.826±0.003	2.844±0.003
35	-	2513±2	2509±2	164±4	10022	2.836±0.003	2.849±0.003
45	-	2519±2	2513±2	164±4	9670	2.836±0.003	2.854±0.003
55	-	2522±2	2517±2	167±4	9281	2.838±0.003	2.859±0.003
65	-	2528±2	2521±2	170±4	8965	2.844±0.003	2.861±0.003
75	-	2532±2	2524±2	169±4	8612	2.849±0.003	2.867±0.003

<sup>a</sup> Temperature (°C). <sup>b</sup> Affected number, equal to the number of moles of water affected by one mole of solute. <sup>c</sup> Band position at maximum (cm<sup>-1</sup>). <sup>d</sup> Band position at gravity center (cm<sup>-1</sup>). <sup>e</sup> Full width at half-height (cm<sup>-1</sup>). <sup>f</sup> Integrated intensity (dm<sup>3</sup> · mol<sup>-1</sup> · cm<sup>-1</sup>). <sup>g</sup> The most probable O···O distance (Å). <sup>h</sup> Mean O···O distance (Å).



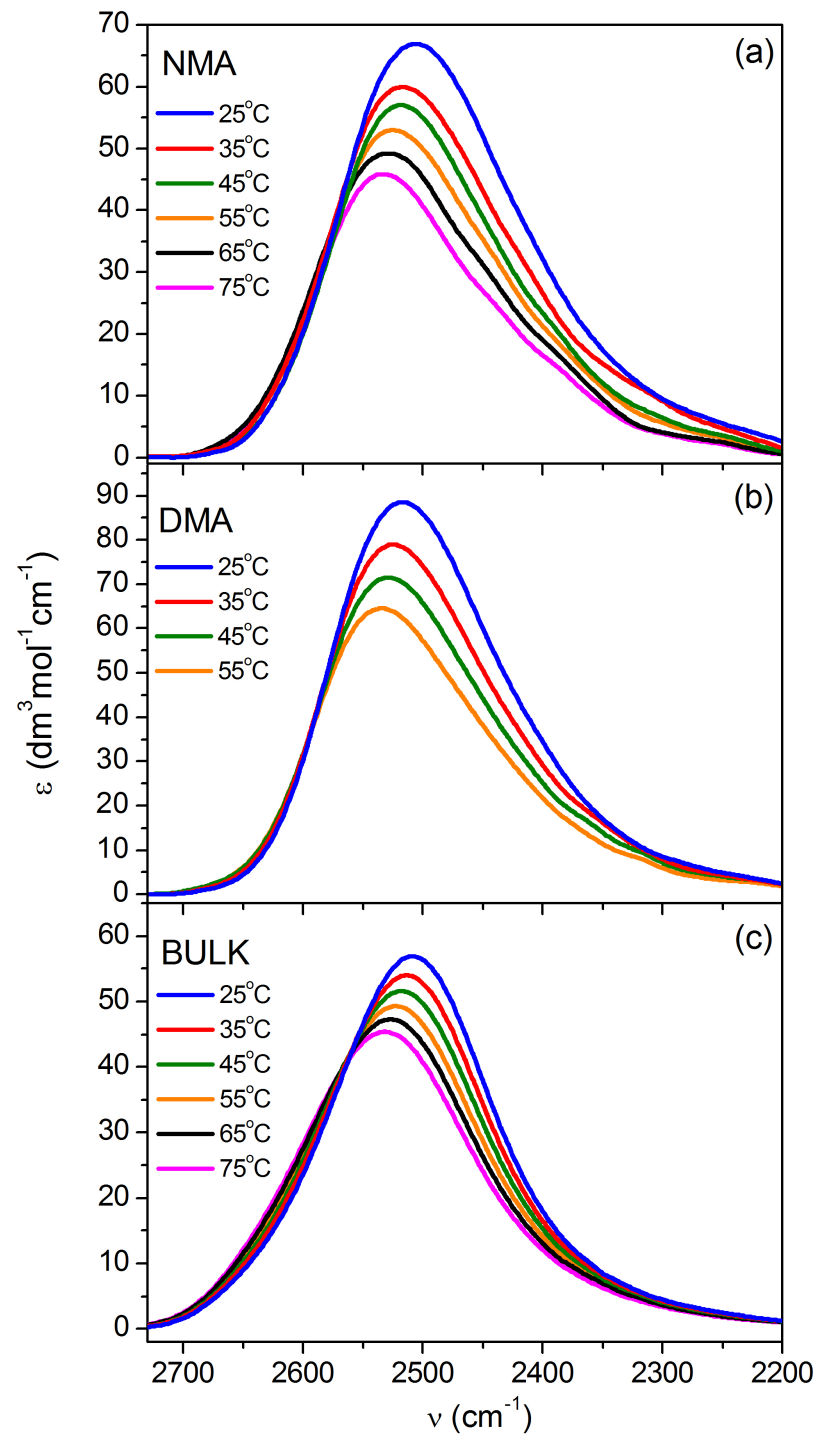


Figure 1: Solute-affected spectra in the OD stretching region for (a) NMA, (b) DMA, and (c) “bulk” HDO spectra as a function of temperature.

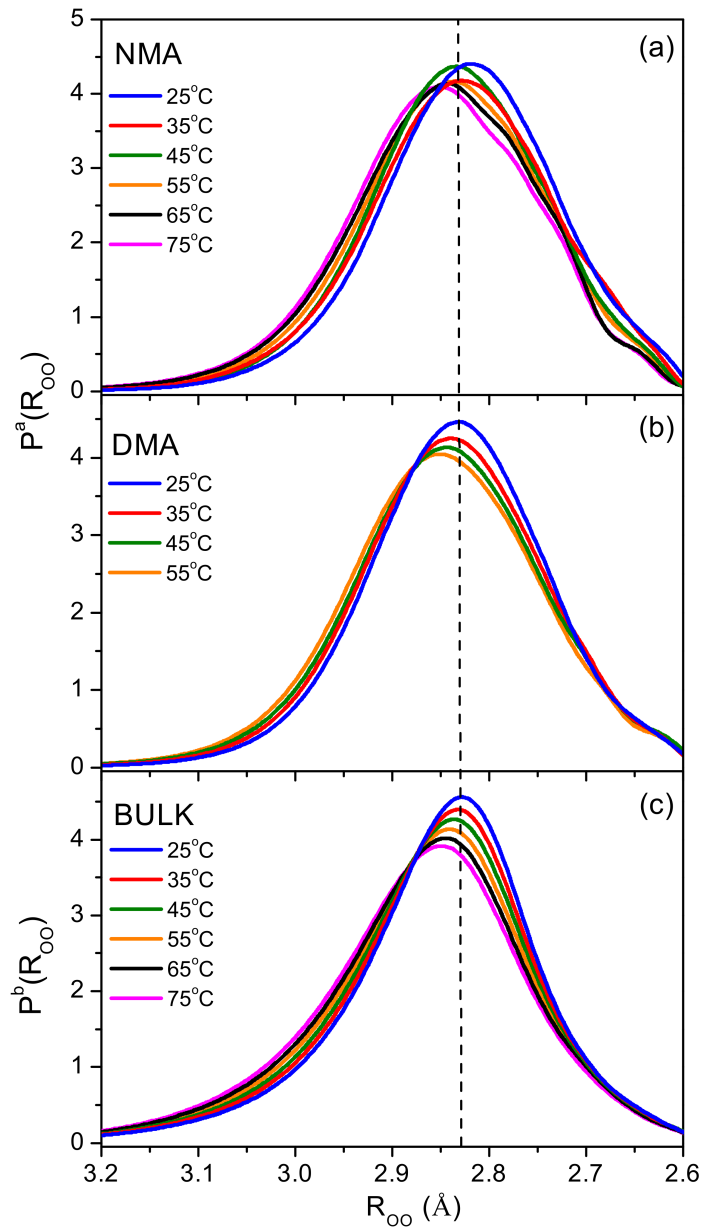


Figure 2: Interatomic oxygen-oxygen distance distributions function derived from the HDO spectra affected by (a) NMA, (b) DMA, and (c) “bulk” water spectra (Figure 1) for all studied temperatures. The vertical dashed line corresponds to the value of the most probable oxygen-oxygen distance in bulk water at 25 °C (2.826 Å, see Table 1).

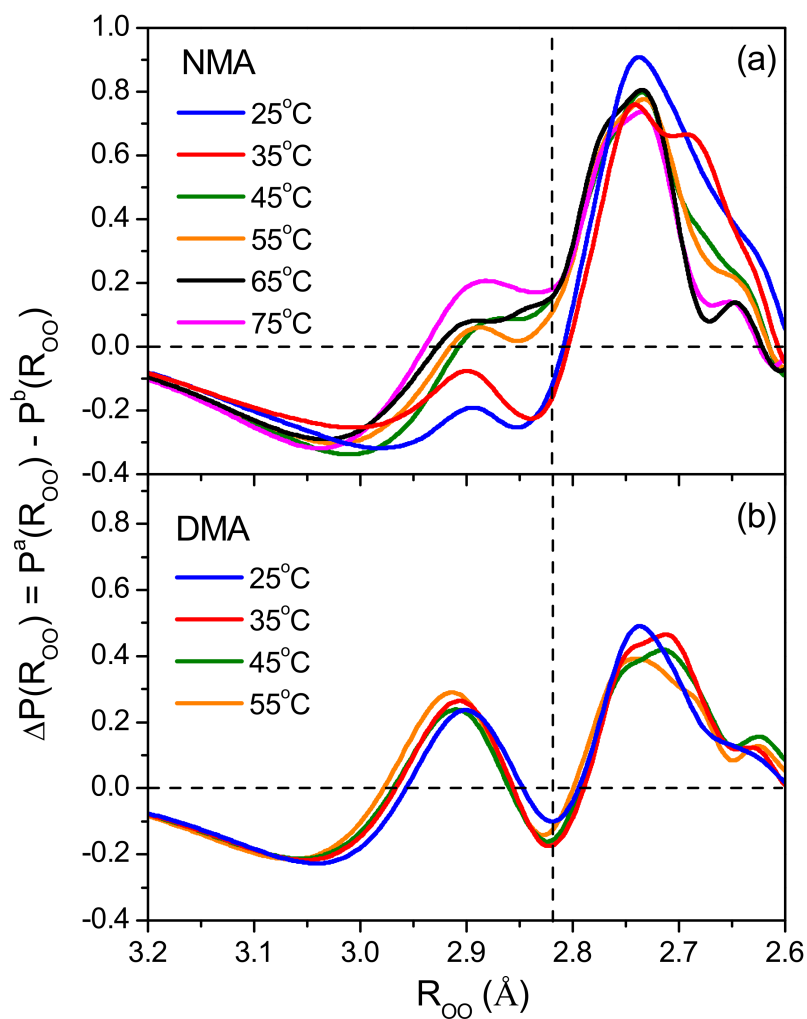


Figure 3: Differences between interatomic oxygen–oxygen distance distribution function of solute-affected water,  $P^a(R_{OO})$ , and the “bulk” water,  $P^b(R_{OO})$ , (Figure 2) at given temperature for (a) NMA and (b) DMA. The vertical dashed line corresponds to the value of the most probable oxygen-oxygen distance in bulk water at 25 °C (2.826 Å, see Table 1).

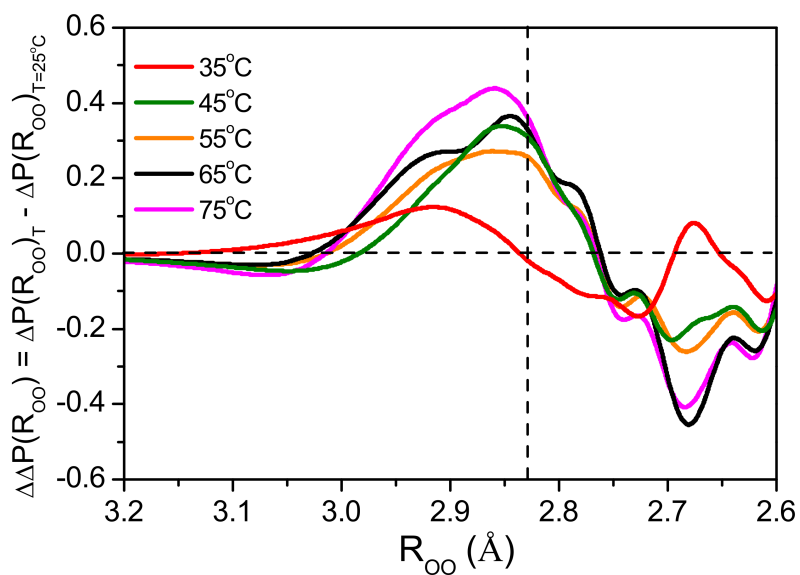


Figure 4: The result of subtraction of the oxygen-oxygen distance function at 25 °C,  $\Delta P(R_{OO})_{25\text{ }^\circ\text{C}}$ , (blue line in Figure 3a) from the distance function at different temperatures,  $\Delta P(R_{OO})_T$ , obtained for NMA. The vertical dashed line shown to the position of the most probable oxygen-oxygen distance in bulk water at 25 °C (2.826 Å, see Table 1).

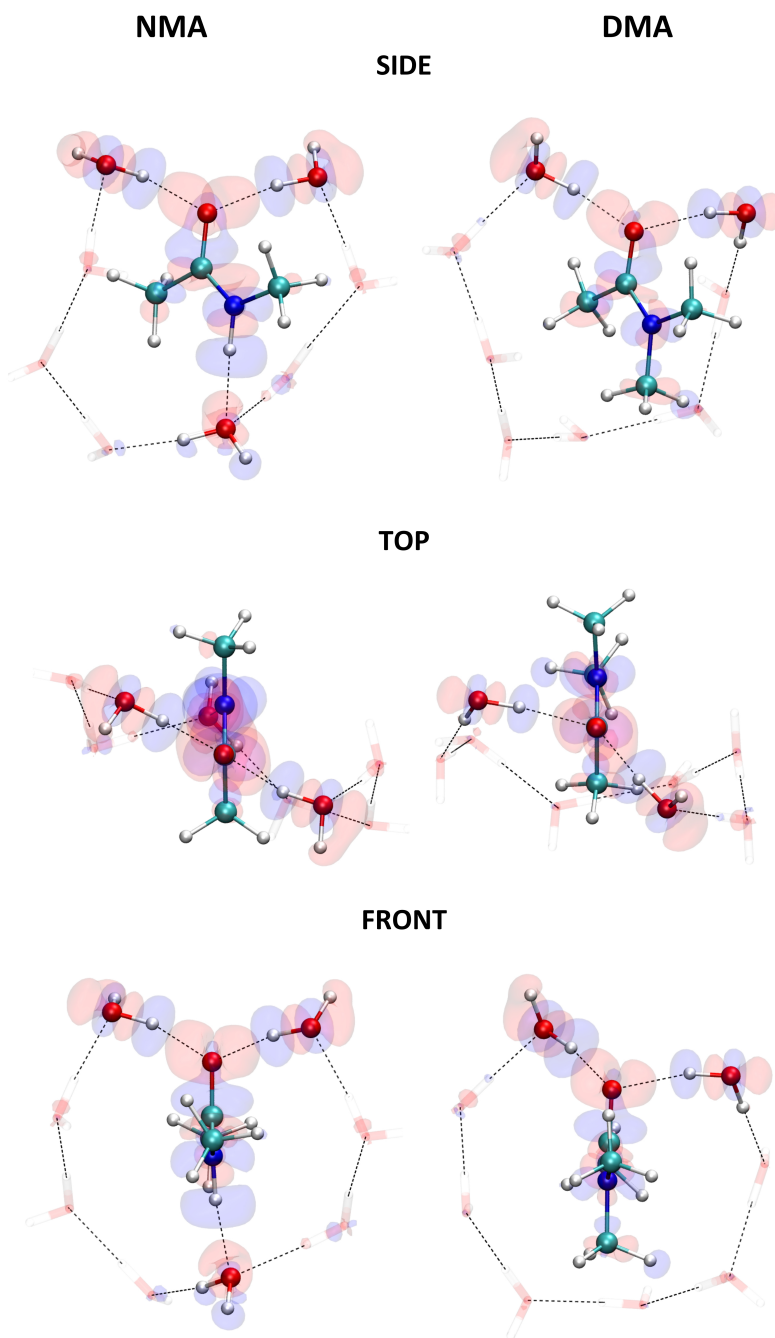
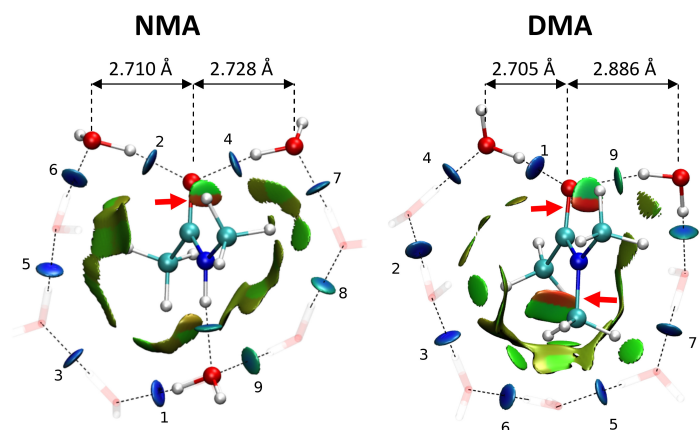


Figure 5: Differences in electron density of NMA and DMA complexes with water molecules caused by interactions of a central molecule with the water ring. Red regions correspond to increase of the electron density, blue regions mark its decrease. Additional movie files with the same structures are available in Supplementary Data.

(a)



(b)

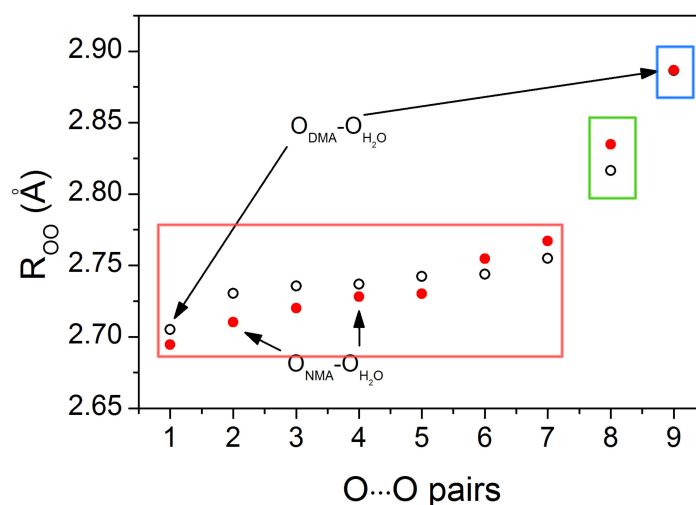


Figure 6: (a) Visualization of weak interactions in NMA and DMA complexes with water molecules. Blue disks correspond to hydrogen bonds, green/olive patches correspond to weak van der Waals interactions, red (marked with red arrows) correspond to steric repulsion. Selected water molecules are made transparent for clarity. Hydrogen bonds are marked with dashed black lines. Numbers indicate  $O \cdots O$  pairs sorted according to their length (as in Figure b). Additional movie files with the same structures are available in Supplementary Data. (b) Distribution of oxygen $\cdots$ oxygen distances in NMA (red) and DMA (white) complexes with water. Only distances shorter than 3 Å are shown, and pairs corresponding to interaction of water molecules with the carbonyl oxygen are marked with arrows. The  $O \cdots O$  pairs in red, green, or blue frames correspond to strengthened, “bulk-like”, and weakened hydrogen bonds, respectively.