^{1}H NMR CONFORMATIONAL STUDIES IN WATER OF ANGIOTENSIN II ANALOGUES MODIFIED AT THE N- AND C-TERMINI: INTERACTIONS OF THE AROMATIC SIDE CHAINS AND FOLDING OF THE N-TERMINAL DOMAIN

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The conformation of [Sar^1]angiotensin II in water at neutral pH has been examined by proton magnetic resonance spectroscopy at 400 MHz and in particular by comparing its ^{1}H NMR spectral data with those of analogues modified at positions 1,4 and 6, namely [Sar^1,Cha^8]ANGII, [Des Asp^1,Cha^8]ANGII, [Aib^1,Tyr(Me)^4]ANGII, [Aib^1,Tyr(Me)^4,Ile^8]ANGII, [N-MeAib^1,Tyr(Me)^4]ANGII, [N-MeAib^1,Tyr(Me)^4,Ile^8]ANGII, ANGIII and [Sar^1,Ile^8]ANGII. Assignment of all proton resonances in these analogues was made possible by 2D COSY NMR experiments. The H-2 and H-4 protons for the histidine ring in [Sar^1]ANGII, ANGII and ANGIII were shielded compared with the same protons in [Sar^1,Ile^8]ANGII, [Sar^1,Cha^8]ANGII and [Des Asp^1,Cha^8]ANGII; this shielding effect was not disturbed upon methylation of the tyrosine hydroxyl and/or replacement of residue 1 (sarcosine or aspartic acid) with aminoisobutyric acid (Aib) or *N*-methyl aminoisobutyric acid (N-MeAib). These data are consistent with our previous suggestion based on NMR studies in neutral DMSO that a characteristic folded conformation for ANGII previously observed in non-polar solvents can also be detected in water at neutral pH, but to a lesser degree.

The linear octapeptide angiotensin II (ANGII, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is a potent myotropic and pressor agent and knowledge of its conformation is critical for the design and synthesis of improved antagonists. One important feature in assessing the

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conformation which is assumed by ANGII in solution is the conformation of the His^6-Pro^7 peptide bond and the positioning of the aromatic rings in relation to each other. Our previous NMR studies have been done exclusively in dimethyl sulfoxide and have suggested that there is an interaction of the histidine and phenylalanine rings[1]; it was of interest, therefore, to determine if this interaction was also a feature of the conformation assumed by ANGII and analogues in water.

With the intent of understanding the aromatic side-chain intramolecular interactions in ANGII in water, the ^{1}H NMR spectra of [Sar^1]ANGII*, ANGII and ANGIII at 400 MHz were compared with those of analogues in which phenylalanine has been replaced by an aliphatic amino acid such as cyclohexylalanine[2] (Cha) or isoleucine. In particular we have compared the aromatic proton resonances between [Sar^1]ANGII and [Sar^1,Cha^8]ANGII, ANGIII and [$DesAsp^1$,Cha^8]ANGII, [N-$MeAib^1$,$Tyr(Me)^4$]ANGII and [N-$MeAib^1$, $Tyr(Me)^4$,Ile^8]ANGII, and also between [Aib^1,$Tyr(Me)^4$]ANGII and [Aib^1,$Tyr(Me)^4$,Ile^8]ANGII. All analogues under investigation showed similar shielding/deshielding differences upon replacement of the phenylalanine residue with an aliphatic one. We have also compared the Sar N-CH_3 resonance of [Sar^1]ANGII with those of [Sar^1,Ile^8]ANGII and [Sar^1,Cha^8]ANGII (see Scheme 1 and Table I).

[Sar^1]ANGII
Sar-Arg-Val-Tyr-Ile-His-Pro-Phe

[Sar^1,Ile^8]ANGII
Sar-Arg-Val-Tyr-Ile-His-Pro-Ile

ANGIII
Arg-Val-Tyr-Ile-His-Pro-Phe

SCHEME 1

From our previous NMR studies on these peptides in dimethyl sulfoxide, folding of the N-terminal domain and proximity of the histidine and phenylalanine side chains in [Sar^1]ANGII was suggested[3,4]. In the present investigation these characteristics were also found to be consistently present for ANGII and agonist analogues in water, suggesting that certain conformational feature of ANGII can be maintained to some degree in diverse dielectric environments.

EXPERIMENTAL

Peptides were prepared by the solid-phase method on a Schwarz Bioresearch Model 105 peptide synthesizer. L-Cyclohexylalanine has been prepared as previously described[5] and was converted to

* Abbreviations are used in accordance with the rules of IUPAC-IUB Commission on Biochemical Nomenclature, Eur. J. Biochem. *138*, 8 (1984). Cha, cyclohexylalanine.

Boc-L-Cha by the method of Nagasawa et al.[6]. Aib, N-MeAib and Tyr(Me) were purchased from Bachem and converted to Boc-derivatives by the same method. The completed peptide was removed from the resin and simultaneously deprotected by reaction with anhydrous HF at 0 °C for 1 h in the presence of *p*-cresol, indole and ethyl methyl sulfide. HF was removed in vacuo, the peptide was dissolved in trifluoroacetic acid and the resin was removed by filtration. The solvent was removed on a rotary evaporator and the peptide was obtained as a white amorphous solid by trituration with ether. Purification of the product was achieved by RP HPLC using a preparative automated repetitive injection technique that has been described in detail previously[7]. The rat isolated uterus assay was carried out on uteri from diethyl stilbestrol-primed female Sprague–Dawley rats as described previously[8].

Samples for NMR measurement were made by dissolving 5 mg peptide in 0.35 ml of 25 mM aqueous phosphate (10% D_2O–90% H_2O) buffer pH 7.0 solution containing 0.1 M KCl. Adjustment of pH was accomplished by adding small amounts of diluted NaOH or HCl solution to the sample. At pH 7.2 small amounts of cotton-like precipitate formed and spectra were recorded without spinning the sample-tube. All NMR measurements were carried out on a Bruker AM 400 wide bore spectrometer equipped with an Aspect-3000 computer. A jump-return sequence was used to null the solvent peak. Sample temperature was controlled to 298 ± 0.1 K. Two dimensional (2D) COSY experiments were acquired in absolute value mode. The spectral width was 4 100 Hz and the carrier was set at the H_2O peak. The solvent H_2O was suppressed by irradiation during relaxation delay and with lower power during evolution time. The 2D COSY data consist of 256 individual 1 K size experiments with incremental t_1 values; these were apodised with sine-bell function and zero-filled to produce a 1 K × 1 K matrix. Typically 64 scans were collected for each experiment. In some cases 128 or 32 scans were collected.

RESULTS

Assignment of all proton resonances was possible by recording magnitude mode COSY spectra in water for all analogues synthesized in this study[9]. The resulting contour plot for [DesAsp^1,Cha^8]ANGII is shown in Figs 1 and 2. The 1D NMR spectra (aromatic region) at 400 MHz for [Sar^1]ANGII. [Sar^1,Cha^8]ANGII, [N-MeAib^1, Tyr(Me)^4]ANGII and [N-MeAib^1,Tyr(Me)^4,Ile^8]ANGII are shown in Fig. 3. The chemical shifts for the aromatic protons of the side chains for Tyr, His and Phe residues of all analogues under investigation are given in Table I. By comparison of the H-2 and H-4 proton chemical shifts of His in [Sar^1]ANGII and ANGIII, the chemical shifts for the His residue in [Sar^1,Cha^8]ANGII and [DesAsp^1,Cha^8]ANGII are characterized by downfield shifts. Thus, the His H-2 and H-4 protons for [Sar^1]ANGII appear at δ_1 7.77 ppm and δ_2 7.02 ppm, while the same protons in [Sar^1,Cha^8]ANGII occurred at lower field (δ_1 7.90 ppm and δ_2 7.10 ppm). Similarly the His H-2 and H-4 protons for ANGIII appeared at δ_1 7.95 ppm and δ_2 7.05 ppm while the same protons in [DesAsp^1,Cha^8]ANGII occurred at lower field (δ_1 8.05 ppm and δ_2 7.15 ppm). The same downfield shift for the His H-2 and H-4 protons is also observed on going from [Sar^1]ANGII to the [Sar^1,Ile^8]ANGII. In [Sar^1,Ile^8]ANGII these protons appear accordingly at lower field (δ_1 7.82 ppm, δ_2 7.08 ppm), when compared with those of [Sar^1]ANGII (Table I).

The Phe/His shielding effect appears to be persistently present regardless of changes made at position 1 and 4. Thus, replacement of sarcosine with the bulky Aib or N-MeAib, and methylation of the Tyr hydroxyl group results in analogues in which the Phe/His interaction remains present. Comparison of the His H-2 and H-4 proton resonances of [N-MeAib^1,Tyr(Me)^4]ANGII with those of [N-MeAib^1,Tyr(Me)^4),Ile^8]ANGII, show again a downfield shift on going from the former (δ_1 7.78 ppm, δ_2 7.02 ppm) to the latter (δ_1 7.85 ppm, δ_2 7.08 ppm). Similar downfield shifts for the His H-2 and H-4 protons are observed on going from [Aib^1,Tyr(Me)^4]ANGII (δ_1 7.81 ppm, δ_2 7.02 ppm) to [Aib^1,Tyr(Me)^4,Ile^8]ANGIII (δ_1 7.85 ppm, δ_2 7.10 ppm). These studies show that when the Phe ring is absent in ANGII analogues a shielding influence on the His ring

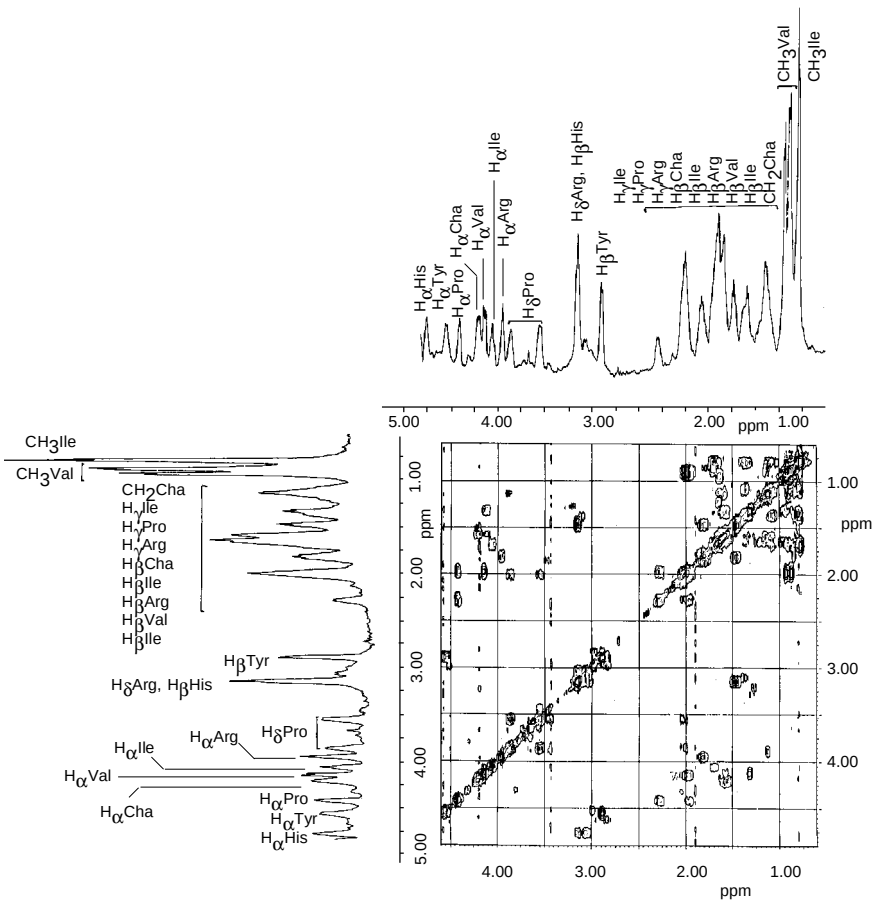

FIG. 1
Completely assigned COSY spectrum for [DesAsp^1,Cha^8]ANGII in H_2O; aliphatic region (0 – 5.00 ppm)

is removed. This interaction has been also indicated in DMSO (refs[3,4]) by NOE spectroscopy in the Rotating Frame. The N-Me proton resonances for [Sar[1],Ile[8]]ANGII and [Sar[1],Cha[8]]ANGII appeared at lower field (δ_1 2.70 ppm and δ_2 2.72 ppm, respectively) compared to the same resonance in [Sar[1]]ANGII (δ 2.62 ppm) indicating a shielding effect on NCH_3 in the agonist (Table I).

Cis–trans isomers due to restricted rotation of the His-Pro bond were observed at pH 7.2. One isomer predominated over the other at a 1 : 5 – 1 : 7 ratio as judged by the relative intensities of the His H-2, H-4 and Tyr *ortho*-, *meta*-protons. Only one isomer was observed at pH 4.6.

At neutral pH values the signal line width for all resonances is broader than for spectra obtained at acid pH. This appear to be due to conformational averaging which on the NMR scale becomes slow enough at neutral pH to cause peak broadening. This effect is not the result of dimerisation, because it is known that aggregation of the molecule does not occur in water even at higher concentration than that used here[10].

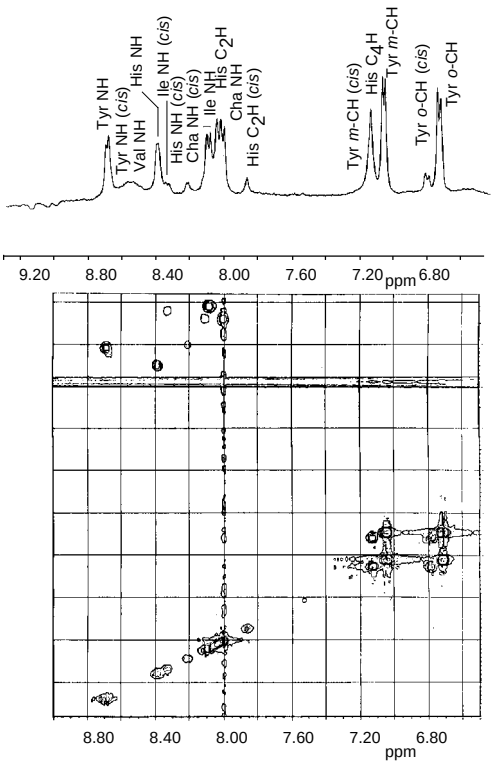

FIG. 2
Completely assigned COSY spectrum for [DesAsp[1],Cha[8]]ANGII in H_2O; aromatic region (6.00 – 9.00 ppm)

DISCUSSION

The biologically active conformation of ANGII has been the subject of many investigations utilizing a variety of different spectral techniques[10–15]. These studies have been largely driven by the established role of ANGII in blood pressure regulation and in the development and maintenance of hypertension. Several conformational models have been suggested ranging from random coil[16] to α-helix[17], β-pleated sheet, β- and γ-turn[18,19], ion–dipole[20] and charge relay[21,22]. The solvent of choice for studying the conformation of biologically active peptides has been the subject of some debate in the past. On the one hand it can be argued that the aqueous environments at neutral pH provide the

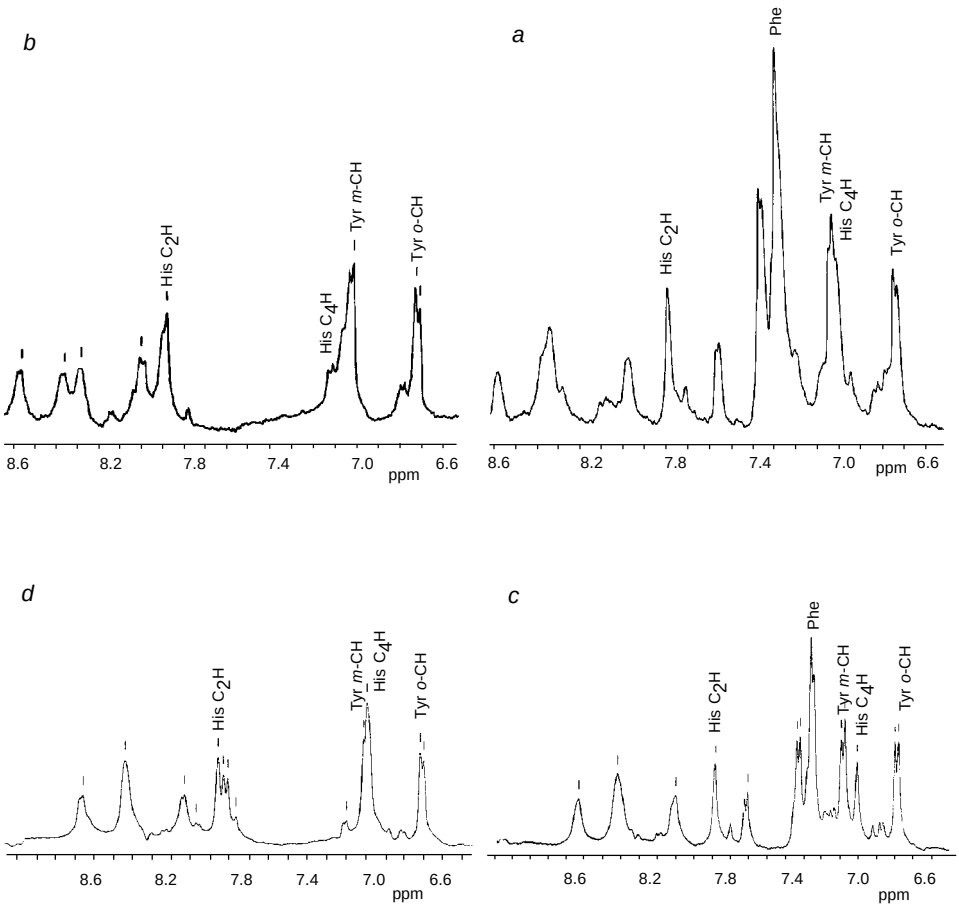

FIG. 3

Proton magnetic resonance spectra (aromatic protons) at 400 MHz in water at pH 7.2. *a* [Sar[1]]ANGII; *b* [Sar[1],Cha[8]]ANGII; *c* [N-MeAib[1],Tyr(Me)[4]]ANGII; *d* [N-MeAib[1],Tyr(Me)[4],Ile[8]]ANGII

conformation of the peptide present in body fluids as it approaches the receptor. On the other hand solvents of intermediate polarity such as DMSO are more representative of the lipid environment which the peptide will encounter at the membrane receptor. In DMSO, ANGII in its neutral form takes up a compact conformation characterized by marked shielding influences on the aromatic side chains in particular[23], whereas the protonated form exists as an extended structure in DMSO (refs[22,23]). Furthermore, 2D-ROESY studies in DMSO have demonstrated a folded conformation characterized by clustering of the three aromatic rings[3]. Unfortunately ANGII analogues in water at neutral pH gave very poor 2D-ROESY (and NOESY) spectra (ref.[24] and unpublished results), making this a questionable area of investigation. Furthermore, NOE studies in water often fail for molecules of the size of ANGII (ref.[25]). However, in the present study, 1D NMR experiments in neutral aqueous environments have demonstrated in a clear way structural features similar to that observed in DMSO. Thus, the present 1D NMR comparative studies in water have shown deshielding of the His H-2 and H-4 protons in analogues containing a non-aromatic amino acid (cyclohexylalanine or isoleucine) at position 8, suggesting interaction of the Phe and His rings in ANGII and other Phe[8] containing ANGII analogues. The positioning of the His ring close to the Phe ring appears to be important for the active conformation assumed by ANGII at the receptor[3,4] and appears to be mediated by the α-carboxylate. Thus, formation of an ion

TABLE I

Chemical shifts for the aromatic protons and methyl protons of N-terminal amino acid analogues in H_2O at 400 MHz at pH 7.2

Peptide	His		Tyr/Tyr(Me)			Phe	Sar	Aib
	H-2	H-4	*meta*[a]	*ortho*[a]	OCH_3	C_6H_5	NCH_3	CH_3
[Sar[1]]ANGII	7.77	7.02	7.02	6.73	–	7.33 m	2.62	–
[Sar[1],Ile[8]]ANGII	7.82	7.08	7.02	6.72	–	–	2.70	–
[Sar[1],Cha[8]]ANGII	7.90	7.10	7.05	6.72	–	–	2.72	–
ANGIII	7.95	7.05	7.05	6.78	–	7.38 m	–	–
[DesAsp[1],Cha[8]]ANGII	8.05	7.15	7.05	6.72	–	–	–	–
[Aib[1],Tyr(Me)[4]]ANGII	7.81	7.02	7.08	6.78	3.75	7.28 m	–	1.55
[Aib[1],Tyr(Me)[4],Ile[8]]ANGII	7.85	7.10	7.00	6.70	3.70	–	–	1.55
[N-MeAib[1],Tyr(Me)[4]]ANGII	7.78	7.02	7.08	6.78	3.75	7.28 m	2.55	1.55
[N-MeAib[1],Tyr(Me)[4],Ile[8]]ANGII	7.85	7.08	7.08	6.78	3.75	–	2.55	1.55

[a] *ortho* and *meta* refer to the TyrOH group.

quadrupole bond between $-CO_2^-$ and the Phe ring[26] positions the $-CO_2^-$ group close to His[21] resulting in shielding of the imidazole ring by the carboxylate anion.

The [Sar[1],Cha[8]]ANGII and [DesAsp[1],Cha[8]]ANGII analogues, while retaining some agonist activity (5 – 10%), antagonize the effects of angiotensin II at uterine smooth muscle receptors (pA_2 = 8). The conformational constraints produced by the replacement of the C-terminal aromatic side chain in ANGII with the non-aromatic cyclohexyl moeity are not well understood in terms of the hormone–receptor interaction. The agonist activities of the analogues investigated herein are higher than that of [Sar[1],Ile[8]]ANGII (< 0.1%) and may be due to the fact that the cyclohexylalanine side chain can mimic to some degree the phenylalanine ring at the receptor. However, the Phe ring of ANGII is believed to constrain the motion of the C-terminal carboxylate by the formation of an ion–quadrupole bond[26], an interaction which is not possible for Cha. Possibly the steric influence of the Cha residue, which is significantly larger than either the Ile or the Phe side chains, may sufficiently limit the motion of the α-carboxylate in constrained environment at the receptor. Therefore, the carboxylate may spend significant time close to the imidazole group resulting in charge relay formation (5% ?) and consequent agonist activity[22]. Recent fluorescence studies have confirmed the presence of the charge relay system in [Sar[1],Cha[8]]ANGII and [DesAsp[1],Cha[8]]ANGII but not in [Sar[1],Ile[8]]ANGII[27].

Although changes at the N-terminus do not appear to markedly affect the anisotropic environment of the Phe ring, changes at the C-terminal do influence the chemical shift for the Sar[1] N-CH_3 protons. Thus replacement of the Phe[8] in [Sar[1]]ANGII with Ile or Cha causes a downfield shift of the Sar[1] N-CH_3 protons. The shielding effect on the N-CH_3 of [Sar[1]]ANGII is believed to result from a folding of the N-terminal tetrapeptide which places the sarcosine N-CH_3 very close to the aromatic ring cluster[23]. Recent 2D-ROESY (refs[3,4]) in DMSO have indicated that the Tyr residue may be the source of the shielding effect on Sar N-CH_3 suggesting that the influence of the Phe residue for shielding of the N-CH_3 is a secondary or indirect effect (dependent on the influence of Phe in generating and positioning the tyrosinate species[22,24]). We suggest that the Phe ring positions the C-terminal carboxylate close to His, which in turn promotes interaction of His with Tyr; consequently the resulting Tyr O^- species thereby generated[24] is close to the N-CH_3 group of Sar[1] (ref.[3]), resulting in shielding of the latter. The proposed hydrogen bond between the Tyr OH and one of the His imidazole nitrogen atoms is thought to be important for the expression of agonist activity since its absence in Sarmesin produces a competitive antagonist[7]. The chemical shifts for the N-CH_3 protons of N-CH_3Aib remain the same in [N-MeAib[1],Tyr(Me)[4]]ANGII (δ 2.55 ppm) and [N-MeAib[1],Tyr(Me)[4],Ile[8]]ANGII (δ 2.55 ppm) indicating a more relaxed conformation for these two analogues at the N-terminus. Both of these analogues accordingly have low antagonist activity (pA_2 = 6.0 and 5.1, respectively).

The configuration of the His-Pro peptide bond is very important for the conformation of ANGII and other active analogues and it has been the subject of many investigations using mostly ^{1}H and ^{13}C NMR spectroscopy[10–12,28]. *Cis–trans* isomers of the His-Pro bond were found in water for a variety of analogues, with one isomer predominating over the other at a 1 : 5 – 1 : 7 ratio, at pH 7.2 but not at pH 4.6 (ref.[24]). ^{13}C NMR studies in water[29] have suggested that the major isomer is the *trans*. Our results, based on ^{13}C NMR studies in water also suggest that the major isomer is the *trans*. NOE studies in water often fail for molecules of the size of ANGII, irrespective of the internuclear distances involved, because the tumbling rate for these solutes is close to that at which the maximum possible NOE passes through zero[25]. However in dimethyl sulfoxide, which allows a stronger build up of the NOE effect, we were able to observe strong interactions between the His C_α proton and the two Pro C_δ protons in all analogues under investigation, suggesting also a major *trans* configuration for the His-Pro bond in DMSO. The present NMR findings using water as the solvent are reminiscent of the behavior of ANGII analogues in DMSO (refs[1,3,4,30,31]). On the whole, many of the conformational features of ANG seen in neutral DMSO are also present in neutral water, though to a lesser extent. It is our impression that more conformations may be sampled in neutral H_2O than in DMSO, and that the folded bioactive conformer of interest is considerably less prevalent in aqueous environments.

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