# Structure Refinement of Cyclosporin A in Chloroform by Using RDCs Measured in a Stretched PDMS-Gel

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*New developments concerning alignment media for apolar solvents like chloroform make it possible to measure anisotropic parameters such as residual dipolar couplings (RDCs) at relatively low concentrations and natural isotopic abundance. As RDCs provide structural restraints with respect to an external coordinate system, long-range structural arrangements of the time-averaged structure can be determined with high precision. The method is demonstrated on the well-studied cyclo-undecapeptide Cyclosporin A (CsA), for which crystal and conventionally derived NMR structures are available. Neither crystal nor NMR structure are consistent with heteronuclear $D_{CH}$ RDCs measured in a stretched poly(dimethylsiloxane) gel, and refinement by using the anisotropic parameter results in a highly defined structure with a slightly changed backbone conformation. The applied methods and interpretation of the structural model are discussed.*

## Introduction

The measurement of anisotropic parameters, that is, parameters that depend on the orientation of a molecule to the external magnetic field, has revolutionized the NMR spectroscopy of biological macromolecules in recent years. In principal, any anisotropic parameter can be measured as the difference between measurements on an isotropic sample and a sample partially aligned by an appropriate alignment medium. The aim of such a partially aligned sample is to provide a measurable "residual" component of the anisotropic parameter, while retaining the favorable characteristics of isotropic samples in solution. The degree of alignment, and hence the choice of alignment medium, is therefore a critical factor. Although NMR on partially aligned systems was introduced as long ago as 1963 by Saupe[1]—who also provided the general theory describing the observable effects in a remarkable manuscript only one year later[2]—the measurement of anisotropic parameters has been limited to very small molecules until relatively recently. Only with the discovery of alignment media that provide alignment weak enough to allow practical measurements in larger molecules has the technique gained widespread use in biological systems.[3,4]

In general, two methods for the partial orientation of molecules without altering the molecule itself are of practical use: the autoalignment of liquid crystalline phases in a magnetic field and mechanically stretched polymer gels. In both cases, the alignment media work as oriented molecular lattices that partially align nonspherical molecules by steric and/or electromagnetic interactions. In aqueous solutions, a number of liquid crystalline phases[5–7] and polymer gels[8,9] are reported with weak alignment properties desirable for larger molecules. In organic solvents, liquid-crystalline phases are constantly being improved[10,11] but still do not allow the weak alignment necessary for studies of even medium-sized organic molecules, such as peptides. For these media, a workaround is provided by variable-angle sample spinning[12] if a high-resolution magic-angle spinning probe head is available. On the other hand, stretched polymer-gel-based alignment media, as introduced recently for apolar[13,14] and polar[15,16] organic solvents, are easily scalable with respect to their alignment properties, as they have no lower anisotropy limit. Among these polymer gels, poly(dimethylsiloxane) cross-linked by accelerated electrons and swollen in apolar organic solvents has the most interesting NMR properties, as it shows only a single $^1$H NMR signal at $\approx$0.1 ppm and therefore interferes only minimally with the signals of interest. In this article, we demonstrate the usefulness of stretched poly(dimethylsiloxane) gels in the partial alignment of a medium-sized organic molecules in apolar solvent, using the example of Cyclosporin A (CsA) in chloroform.

By far the most commonly measured anisotropic parameter is the residual dipolar coupling (RDC), which can be observed via its contribution ($D$) to scalar coupling constants ($J$). As dipolar coupling averages to zero in isotropic solutions, the RDC is simply the difference in couplings measured in isotropic and

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aligned media. For samples at natural isotopic abundance, $^1J_{HX}$ couplings are the most easily observed due to the good sensitivity and high resolution of heteronuclear correlation experiments. RDCs have proved applicable to the refinement of protein and nucleic acid structures due to their dependence on the orientation of the vector between the coupled nuclei with the external magnetic field. This provides information on the relative orientation of structural elements over practically unrestricted distances—even up to individual domains or proteins within a complex—that cannot be conferred by short-range NOE-derived distance restraints. We have used the partially aligned sample of CsA to measure $^1J_{CH}$ RDCs at natural abundance, and show that they confer significant information for structural refinement, even in this medium-sized system.

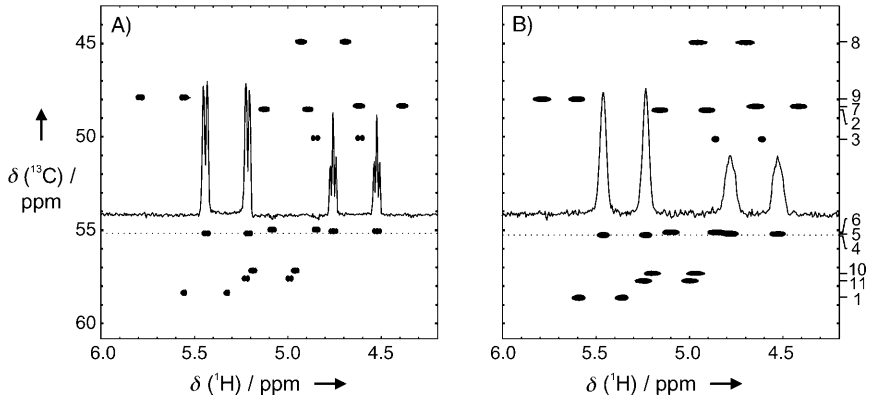


**Figure 1.** Hα–Cα regions of the $^{13}C,^1H$ HSQC spectra recorded on CsA in A) $CDCl_3$ and B) the PDMS/$CDCl_3$ gel without heteronuclear decoupling during acquisition. Slices along the dotted lines are shown for an impression of the spectral quality. Residue assignment is given on the right-hand side.

CsA, the cyclic undecapeptide cyclo(-MeBmt1-Abu2-Sar3-MeLeu4-Val5-MeLeu6-Ala7-D-Ala8-MeLeu9-MeLeu10-MeVal11-), is an immunosuppressant drug widely used clinically to prevent graft rejection in organ transplants. Its structure is well examined; crystal structures are available of free CsA[17] and several forms of CsA bound to Cyclophilin (CyP; e.g., refs. [18–20]), and a NOE-derived solution structure of CsA dissolved in chloroform has previously been determined.[17,21,22] In addition, structural changes of CsA in other solvents such as THF, THF with lithium chloride, and DMSO, as well as those of the closely related thiocyclosporins have been studied in great detail.[23–27] In chloroform and THF, CsA was found to have a conformation very similar to the crystal structure of free CsA,[24] with all sharing a common backbone conformation with a βII′ turn comprising residues 2–5 and an unconventional structured loop over residues 7–11. The RDC-refined structure of CsA reported here retains this common conformation, while showing considerable differences in overall structure from both the currently available crystal and the NOE-based solution structures.

## Results and Discussion

### RDC measurement

In this study we have used a PDMS-gel cross-linked by accelerated electrons[14] to partially align a sample of Cyclosporin A in chloroform in order to measure residual dipolar couplings. Conventional sensitivity-enhanced coherence order-selective $^{13}C,^1H$ HSQC spectra acquired without heteronuclear decoupling in the directly detected dimension are shown for both the partially aligned and isotropic samples in Figure 1. Most $^1J_{CH}$ coupling constants with the corresponding dipolar couplings ($D_{CH}$) could be measured from these spectra (see Table 1). For exact measurement we used the procedure described by Yan et al.[28] Altogether 35 $D_{CH}$ couplings in the range of −22.3 to 27.9 Hz could be obtained.

**Table 1.** $^1J_{CH}$ and $^1J_{CH}+D_{CH}$ couplings measured and $D_{CH}$ restraints used in structure calculations for CsA in $CDCl_3$ and a PDMS/$CDCl_3$-gel with quadrupolar deuterium splitting of $\nu_Q = 40.4$ Hz.

| Residue | Coupling | $^1J_{CH}$ [Hz] | $^1J_{CH}+D_{CH}$ [Hz] | $D_{CH}$ Restraints |
|---|---|---|---|---|
| MeBmt1 | $NCH_3$ | 139.6 | 139.0 | 1.7[a] |
| | CαHα | 138.9 | 139.5 | 0.6 |
| | CβHβ | 142.8 | 127.2 | −15.6 |
| | $C\delta H\delta^{pro\text{-}S}$ | 124.2 | 114.6 | −9.6 |
| | $C\delta H\delta^{pro\text{-}R}$ | 126.6 | 125.4 | −1.1 |
| Abu2 | CαHα | 139.6 | 148.3 | 8.8 |
| | $C\beta H\beta^{pro\text{-}S}$ | 128.7 | 135.6 | 6.9 |
| Sar3 | $NCH_3$ | 139.3 | 139.2 | 0.6[a] |
| | $C\alpha H\alpha^{pro\text{-}S}$ | 143.7 | 150.5 | 6.8 |
| | $C\alpha H\alpha^{pro\text{-}R}$ | 136.5 | 123.8 | −12.6 |
| MeLeu4 | $NCH_3$ | 138.6 | 137.8 | 2.2[a] |
| | CαHα | 136.1 | 136.4 | 0.3 |
| | $C\beta H\beta^{pro\text{-}S}$ | 129.4 | 157.3 | 27.9 |
| | $C\beta H\beta^{pro\text{-}R}$ | 126.1 | 119.8 | −6.3 |
| | CγHγ | 126.3 | 151.0 | 24.7 |
| Val5 | CαHα | 140.1 | 152.4 | 12.4 |
| | CβHβ | 130.9 | 140.2 | 9.3 |
| MeLeu6 | $NCH_3$ | 139.2 | 137.4 | 5.4[a] |
| | CαHα | 141.1 | 146.6 | 5.6 |
| | $C\beta H\beta^{pro\text{-}S}$ | 128.1 | 119.5 | −8.6 |
| | $C\beta H\beta^{pro\text{-}R}$ | 127.3 | 117.1 | −10.2 |
| | CγHγ | 126.6 | 117.0 | −9.6 |
| Ala7 | CαHα | 138.6 | 140.0 | 1.4 |
| Ala8 | CαHα | 142.1 | 158.9 | 16.8 |
| | CβHβ | 129.5 | 124.8 | 14.2[a] |
| MeLeu9 | $NCH_3$ | 139.3 | 142.6 | 10.0[a] |
| | CαHα | 139.1 | 118.0 | −21.2 |
| | $C\beta H\beta^{pro\text{-}S}$ | 129.6 | 109.5 | −20.2 |
| | $C\beta H\beta^{pro\text{-}R}$ | 125.1 | 139.3 | 14.2 |
| | CγHγ | 124.9 | 137.2 | 12.2 |
| MeLeu10 | CαHα | 136.0 | 139.5 | 3.5 |
| | $C\beta H\beta^{pro\text{-}S}$ | 128.8 | 106.5 | −22.3 |
| | $C\beta H\beta^{pro\text{-}R}$ | 125.8 | 113.7 | −12.1 |
| | CγHγ | 126.6 | 105.0 | −21.7 |
| MeVal11 | CαHα | 140.5 | 148.7 | 8.2 |
| | CβHβ | 129.6 | 134.1 | 4.5 |

[a] Residual dipolar couplings of methyl groups are multiplied by −3.

  

RDCs were then fitted to the existing crystal and the NOE-derived NMR structures by using the program PALES.[29] In both cases, a scattering of back-calculated versus measured RDCs was obtained with poor overall correlations of $R=0.586$ and 0.543 for the crystal and NOE-derived structure, respectively (Figure 2). We therefore conclude that neither one of the

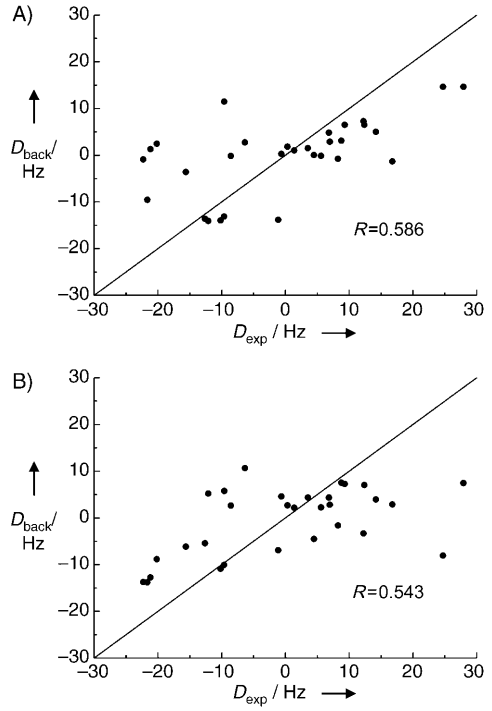


**Figure 2.** Experimental versus back-calculated $D_{CH}$ couplings for A) the crystal structure[17] and B) the NOE-derived NMR structure[22] of CsA. The correlation factors $R$ indicate a poor correlation for both structural models. RDCs were back-calculated by using the program PALES[29] with the bestFit option.

structures represents the time-averaged structure present inside the PDMS-gel. For side chains, the deviation of the $D_{CH}$ couplings can be explained by the inherent flexibility in the apolar solvent. For the backbone, however, it was previously found that a single, well-defined conformation is present in $CDCl_3$.[22] The correlations for the crystal and NOE-derived structures with only backbone RDCs considered improves to $R=$ 0.900 and 0.628, respectively, but the deviations between measured and fitted RDCs are still large. We therefore decided to use the measured RDCs for a structure refinement of CsA.

### RDC-refined NMR structure

As only $D_{CH}$ couplings were measured, we decided to use the susceptibility anisotropy (sani) implementation of XPLOR-NIH[30] to incorporate RDCs as angular restraints with respect to an introduced coordinate system representing the eigenvectors of the alignment tensor. Starting from the NOE-derived structure, a grid search for initial $D_A$ and $D_R$ values was performed and double checked by using the program PALES. Since the implementation of the sani function in XPLOR-NIH is known to converge slowly, considerable effort was put into testing various procedures for including RDCs into structure calculations. The best results were obtained by grouping RDCs in classes of equivalent C,H vectors on the basis of assumed flexibility and adding them successively to calculations, as described in the Experimental Section. By using this protocol, a highly defined set of structures could be obtained in which the best 10 out of 20 calculated structures showed a very small RMSD value of only 0.12 Å over all heavy atoms and in which all RDC restraints are very close to being fulfilled within experimental error, as shown in Figure 3. The correlation between measured and back-calculated RDCs is $R=0.997$ (Figure 3A and D). It is worth noting that, in addition to RDC restraints, the structure still fulfills all NOE-derived distance restraints used in the original solution structure.

For comparison, the backbone of the lowest-energy RDC-refined structure of CsA is shown in Figure 4, superimposed with the crystal and NOE-derived structures over the well-defined βII′ turn spanning residues 2–5 (Figure 4A) and over residues 7–9 (Figure 4B). The difference in backbone planarity is immediately obvious and explains the poor correlation of experimental versus back-calculated RDCs in the crystal and NOE-derived structures. While the crystal structure appears to be flattened, presumably due to crystal packing effects, the NOE-derived structure shows a slight bend in the backbone around residues 11–1 and 6–7. In the RDC-refined structure, however, this bend appears to be significantly stronger, caused by the sum of slight changes in backbone angles of residues 6–11. This clearly indicates that the short-distance NOE-derived restraints alone do not confer this long-range conformational arrangement.

### Side-chain conformations

As previously examined by $^3J_{HH}$ coupling constants as indicators for $\chi_1$ and $\chi_2$ angles,[31] the side chains of CsA in chloroform are most likely averaged over several conformations. In the RDC-refined structures, the side chains are all well defined, and practically no variation of dihedral angles is observed. However, the dynamic behavior of the CsA side chains is also visible in the structure, since the hydrophobic tails all point straight into the solvent—a behavior typical of an average structure for flexible parts of a molecule in an apolar solvent. In Figure 5, the differences of the side chains for the three different structures—crystal, NOE-derived, and RDC-refined structures—are displayed. While side chains are folded back due to crystal packing in the first case, they are oriented differently in the NOE-derived structure, with a low number of defining distance constraints. In the RDC-refined structure, a large number of RDCs fixes the side chains in space with all hydrophobic side chains pointing more or less into the solvent.

It is clear that the structure presented here is over-restrained, that is, that the variability within the ensemble is much lower than the expected conformational flexibility in solution, especially for the side chains. The result must therefore be viewed as a picture of the time-averaged structure in solution. This, in fact, is the case for all structures determined in

  

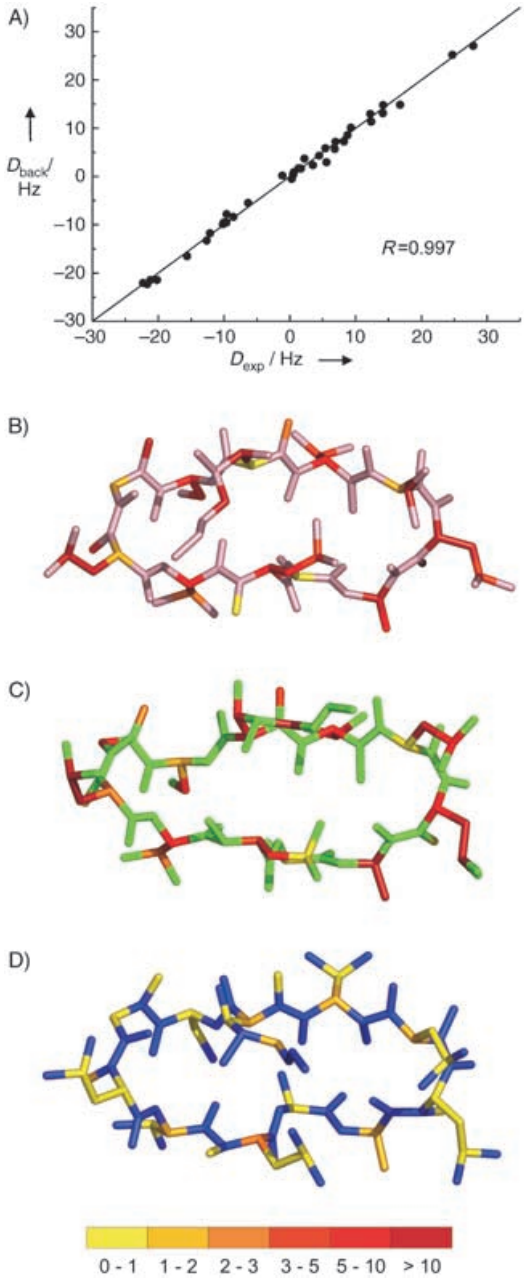


**Figure 3.** A) Experimental versus back-calculated $D_{CH}$ couplings for the RDC-refined structural model of CsA, and B)–D) visualization of the differences for various models. The correlation factor $R = 0.997$ of the RDC-refined structure represents the fact that all RDCs are very close to the experimental error (A, D, blue). The crystal structure (B, light red) and the NOE-derived structure (C, green) display strong deviations of experimental versus back-calculated RDCs (cf. Figure 1) all over the molecule. The color coding is given below with yellow indicating deviations of less than 1 Hz and "hotter" colors indicating stronger deviations.

solution, although rarely as precise and well-resolved as here. For a more complete structural model, additional data on local flexibility would be highly desirable.

A quantitative evaluation of the dynamical behavior of CsA side chains is not possible with only one set of RDCs measured in a single alignment medium. Each motionally averaged angle of interatomic vectors with the magnetic field is only defined on the distorted cone described by the alignment tensor. In the case that only a single alignment tensor is available, too few parameters are known to adequately define any structural information regarding an ensemble of conformations. However, the situation might change considerably if RDCs for a larger set of alignment media, such as stretched PS-gels, PVAc-gels, PBLG, PELG, or others, were used to measure further sets of RDCs.

### Structural influence of PDMS and general remarks

When measuring RDCs in an alignment medium, one always has to be aware that the gel or liquid crystal works as a cosolvent and might influence the structure of the solute to some extent (as was shown previously for oligosaccharides in liquid-crystalline alignment media[32]). In such a case, measured RDCs might be inaccurate because structural changes will also affect the size of the underlying scalar couplings. Apolar PDMS leaves no functional group exposed to the solvent that could specifically interact with the solute, so the probability of structural changes due to the gel is minimized. In the case of CsA in chloroform and the PDMS/chloroform gel, we compared the $^{1}H$ and $^{13}C$ chemical shifts of all relevant cross peaks and found only very minor deviations, which are generally less than 0.3 ppm for carbon atoms and less than 0.07 ppm for protons (cf. Table S1 in the Supporting Information). Taking into account that chemical-shift changes are expected due to residual chemical-shift anisotropy (RCSA), potential structural changes can be considered to be of minor importance. This is somewhat surprising for CsA, since this molecule is known to have a variety of conformations in different solvents.

Within the program PALES, it is possible to predict the alignment tensor from a given structure based on a rod model for the stretched polymer gel. For a rigid spiroindene molecule, the agreement between predicted and experimentally determined RDCs is very good and results in a correlation factor of $R = 0.983$.[14] In contrast, the prediction for CsA with the crystal, lowest energy NOE-derived, and RDC-refined structural models yield correlation factors calculated for all 35 RDCs of $R = 0.25$, $-0.24$, and 0.32, respectively. Predictions from $D_{C\alpha H\alpha}$ couplings only resulted in correlation factors of $R = 0.41$, $-0.3$, and 0.73, respectively. The poor correlations for CsA can easily be explained by the flexibility of the shape-determining side chains. A prediction that only makes use of a single structural model and not an ensemble of structures covering the whole conformational space of CsA must, of course, fail.

The RDC-supported structure calculations of CsA from a protocol as described above lead to a well-defined time-averaged structure. Several other protocols, however, failed to produce low-energy structures that obey the imposed restraints. Structure calculations based on measured RDCs alone resulted in strongly distorted structures of high energy, and neither did calculations starting from the NOE-derived structure with distance restraints and all RDCs added at once arrive at suitable structures. This can only be explained by the slow convergence of the sani implementation of RDCs in XPLOR-NIH, which seems to be responsible for structure calculations getting

  

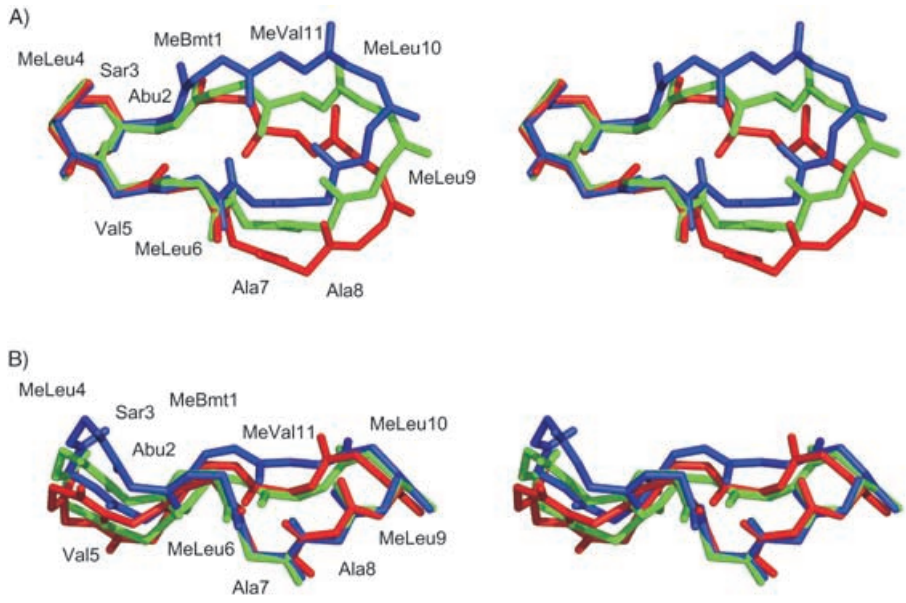


**Figure 4.** Stereoviews of the backbones of the crystal structure (red), the NOE-derived NMR structure (green), and the RDC-refined structure (blue) superimposed onto the highly defined βII′ turn comprising A) residues 2–5 and B) residues 7–9.

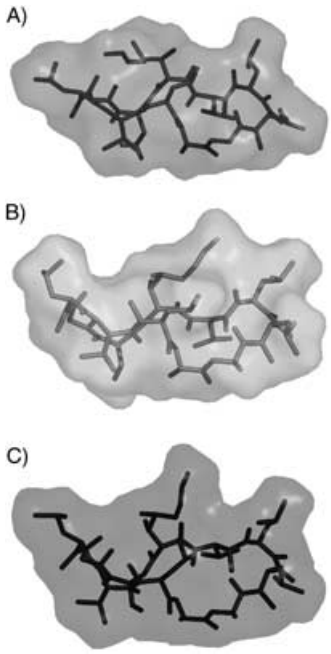


**Figure 5.** A) Crystal structure, B) NOE-derived structure, and C) RDC-refined structure of CsA displayed to highlight differences in side-chain conformations. While side chains in the crystal structure are compacted, most likely due to crystal packing artifacts, NOE-derived and RDC-refined structures show similar side-chain behavior, with side chains in the RDC-refined structure slightly more exposed to the solvent.

stuck in local minima. On the other hand, we tested the influence of initial $D_A$ and $D_R$ values on the structure refinement. We deliberately miss-set the alignment-tensor parameters by up to $\pm 30\%$ from the determined values for calculations. In all cases, the structures and alignment tensors finally obtained were practically identical to results obtained with the correct $D_A$ and $D_R$ values.

We also tried to determine a structure solely based on experimentally determined residual dipolar couplings. Unfortunately, the relatively few RDCs measured were not sufficient to fully define the structure of CsA. This situation might change if RDCs from different alignment media or further long-range $D_{CH}$ or $D_{HH}$ couplings were measured. However, at the concentration of CsA used in this study, barely any other known alignment medium can be used, and methods to measure long-range $D_{CH}$ and $D_{HH}$ couplings at natural abundance are scarcely feasible.

## Conclusion

In this article we have shown that a stretched PDMS gel cross-linked by accelerated electrons and swollen in $CDCl_3$ can be used to measure $D_{CH}$ RDCs at solute concentrations of $\approx 5$ mM at natural isotopic abundance. RDCs were measured for the well-studied cyclic undecapeptide Cyclosporin A and used for structure refinement. In contrast to the crystal structure and the previously reported NOE-derived structure, the resulting structure both explains the observed RDCs and fulfills the existing NOE restraints. The RDC-refined structure reveals a significant bend in the backbone, whilst side chains point into the solvent, as expected for averaged flexible parts of a molecule in apolar solvent. The potential impact of RDCs as angular structural restraints relative to an external coordinate system on the precision of structural models of the time-averaged conformation in solution has been demonstrated. The possibility of measuring RDCs at natural abundance in a variety of solvents opens up a wide range of future applications.

## Experimental Section

**Sample preparation**: CsA (cyclo(-MeBmt1-Abu2-Sar3-MeLeu4-Val5-MeLeu6-Ala7-D-Ala8-MeLeu9-MeLeu10-MeVal11)) was purchased from Fluka. For resonance assignment and measurement of *J* couplings in isotropic solution, the peptide was dissolved in $CDCl_3$ to a final concentration of 8.3 mM. The aligned sample was prepared from cross-linked poly(dimethylsiloxane) gel (PDMS, diameter = 3.6 mm, cross-linked with 100 kGy of accelerated electrons),[14] which was equilibrated in $CDCl_3$ (700 μL) in a NMR tube for several days. After 1 week, the sample showed a constant quadrupolar deuterium splitting of the solvent of $\Delta\nu_Q = 40.4$ Hz. Supernatant solvent was removed, and stock solution of CsA in $CDCl_3$ was added to a final concentration of approximately 5.8 mM in the gel. The sample could be analyzed after 2 days of incubation.

  

**NMR spectroscopy**: All NMR spectra were recorded at 285 K on a 600 MHz Bruker DMX spectrometer (Bruker, Karlsruhe, Germany) equipped with a quadruple-resonance probe head with actively shielded *x*-, *y*-, and *z*-gradients. All spectra were processed by using XWINNMR (Bruker) and analyzed with either XWINNMR or SPARKY.[33,34]

Resonance assignments were obtained from standard $^1$H TOCSY,[35,36] $^{13}$C,$^1$H HSQC,[37–39] $^{13}$C,$^1$H HMBC,[40] and $^1$H ROESY[41,42] spectra. Stereospecific assignments of the prochiral methylene and methyl groups were derived from the ROESY spectrum, which was acquired by using a pulsed spin-lock with a mixing time of 150 ms. $^1J_{CH}$ couplings in isotropic and anisotropic solution were determined from standard two-dimensional $^{13}$C,$^1$H HSQC spectra without decoupling during acquisition. The spectrum of the unaligned sample was recorded with 24 scans per increment. The spectral widths for $^1$H and $^{13}$C were 5513 Hz and 12078 Hz, sampled with 2048 and 512 complex points, respectively. The spectrum of the aligned sample was recorded with 96 scans per increment. The spectral widths for $^1$H and $^{13}$C were 6009 Hz and 15098 Hz, sampled with 4096 and 384 complex points, respectively. Linear prediction was applied to fill the indirect dimensions to 756 and 512 complex points for the isotropic and aligned samples, respectively. All dimensions were apodized with a $\pi/2$-shifted squared sine-bell function before zero filling to provide a processed spectrum of $4096\times2048$ complex points. RDCs were extracted from the spectra by using the procedure described in detail in ref. [28]. Altogether 35 reliable $D_{CH}$ residual dipolar couplings with an estimated error of 1 Hz could be measured for nonoverlapped signals.

**Structure calculations**: Structure calculations were performed by applying standard simulated annealing protocols implemented in the program XPLOR-NIH 2.9.9[30] optimized for proteins and peptides. For calculations, the artificial amino acids of CsA were built by using the program INSIGHT II (MSI) and appropriate topology and parameter files were obtained from the parameter learn function of XPLOR-NIH.

Initially, the NOE-based structure published by Kessler et al.[22] was reproduced, as the structure was not available electronically. Starting from a randomized structure, calculations were performed by using 114 NOE-derived distance restraints, as listed in ref. [22] four hydrogen bonds treated as pseudocovalent bonds,[44] and two side-chain dihedral restraints.[22] For distance restraints, lower and upper bounds of 5% and 10% of the extracted distance were applied, respectively; 1 Å was added to the upper boundary for methyl moieties. 20 structures were calculated; this resulted in eight structures of comparable energies occupying the same conformational space. The structure calculation was then repeated with the former lowest-energy structure as the starting structure. Again, 20 structures were generated, of which the best 17 structures showed comparable energy of $102.8\pm4.9$ kJ mol$^{-1}$ and highly similar conformations. Further repetition of the procedure did not decrease the overall energy. The best structures were then subjected to a final refinement in which the force constant specifying peptide-bond planarity was relaxed from 500 to 50 kJ rad$^{-1}$ to allow slight deviations from planarity. The 20 resulting structures all showed similar energies ($63.6\pm3.4$ kJ mol$^{-1}$) and occupied the same conformational space. As a test, calculations were also made by using the crystal structure as a starting point; these resulted in the same final conformation. The calculated structures had no restraint violations, and optical comparison with the published NOE-derived structure showed no obvious deviations.

For calculations, including RDCs, the lowest-energy structure without refinement was used as starting structure. The initial values for the axial and rhombic components[2] of the alignment tensor ($D_A=-19.2$ and $D_R=0.55$) were extracted by a grid search script implemented in XPLOR by using the formula for the alignment tensor $D_{CH}=D_A(3\cos^2\vartheta-1)+D_R\,{}^3/_2\sin^2\vartheta\cos2\phi$. The sani potential was used for RDCs, which treats dipolar couplings as angular restraints.[30] By using this protocol, RDCs determined from methyl groups can be included as pseudo CH groups pointing in the direction of the corresponding C–C or C–N bonds, if the value of the $D_{CH}$ coupling is multiplied by $-3$ (see e.g. ref. [45]). All 35 RDCs (see below) together with the existing distance and dihedral restraints were used in calculations. The RDCs were divided into five classes and added successively to the calculations. The first class included eleven out of twelve $C^\alpha H^\alpha$ couplings, the second class included one out of two $C^\beta$-methyl couplings and five out of seven N-methyl-couplings, the third class twelve out of 13 $C^\beta H^\beta$ couplings, the fourth all four $C^\gamma H^\gamma$ couplings, and the fifth the two $C^\delta H^\delta$ couplings from the MeBmt residue. During each calculation, 20 structures were generated and subsequently sorted by their total energy. The structure with the lowest energy was used both as the starting structure and to determine the alignment tensor (with PALES[29]) for the next run. After incorporation of all sets of RDCs, the resulting 20 structures were subjected to the same refinement protocol as was used for the calculation of the NOE-based structures.

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**Keywords:** Cyclosporin A · NMR spectroscopy · partial alignment · residual dipolar couplings · structure elucidation

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