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Comparison of distance information in [TOAC^{1}, Glu(OMe)^{7,18,19}] alamethicin F50/5 from paramagnetic relaxation enhancement measurements with data obtained from an X-ray diffraction-based model†

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Peptaibol antibiotics are membrane-active linear peptides of fungal origin that are characterized by a high population of the C^{α}-tetrasubstituted, strongly helicogenic, α-amino acid, α-aminoisobutyric acid, an *N*-terminal acetyl group, and a *C*-terminal 1,2-amino alcohol. Alamethicins (Alms), among the longest peptaibiotics, are a group of closely sequence-related peptides composed of 19 amino acid residues. [TOAC^{1}, Glu(OMe)^{7,18,19}] Alm and [TOAC^{16}, Glu(OMe)^{7,18,19}] Alm are synthetic, nitroxide free-radical labeled analogs of [Glu(OMe)^{7,18,19}] Alm F50/5. In this work, nitroxide to peptide NH proton distance information obtained from paramagnetic relaxation enhancement (PRE) studies on [TOAC^{1}, Glu(OMe)^{7,18,19}] Alm is compared with distances derived from an X-ray diffraction-based model. The methodology for PRE determination, as well as the generation of the X-ray diffraction-based model three-dimensional structures, is discussed. The distances obtained from PRE measurements are in close agreement with the information derived from the X-ray diffraction-based model. This finding suggests that this type of information could be implemented as long-range distance restraints in NMR-based structure determination. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: NMR; paramagnetic probe; peptide conformation; X-ray diffraction; C^{α}-tetrasubstituted α-amino acid

Introduction

Besides X-ray diffraction crystallography, NMR is one of the most valuable tools in biomolecular three-dimensional structure determination. With this technique, the global structure of well-folded systems is determined based on a number of relatively short distance restraints obtained from NOE effects between protons with internuclear distances less than about 5 Å. In short peptides, however, a more rigorous analysis of the system has to be performed, as in this case it is usually more complicated to obtain enough restraints to fully define the structure.

In a number of specific cases, the data set from NOE measurements can be supplemented with long-range paramagnetic relaxation enhancement (PRE) restraints [1]. PRE provides information about the distance between a paramagnetic center (usually a metal with unpaired electrons or a stable radical) and an NMR-active nucleus. The magnitude of this PRE effect is proportional to r^{-6} in the relaxation equations (r being the electron–nucleus distance in the system under study). Unlike the NOE effect, PRE can provide significantly longer distance information [2].

The goal of this article is to compare intramolecular nitroxide to peptide NH proton distance information obtained from PRE measurements on a spin-labeled analog of the long peptaibiotic [3] alamethicin (Alm) [4] with data extracted from an X-ray diffraction-based model [5]. The spin label used in this study is the C^{α}-tetrasubstituted α-amino acid 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-4-carboxylic acid (TOAC, Figure 1) [6–8]. As this nitroxide probe is rigidly fixed into the backbone, the distance results will be directly correlated to the conformation of the peptide, without being hampered by the potential flexibility of linkers typically used to attach paramagnetic probes (*vide infra* 'Choice of the spin label' section).

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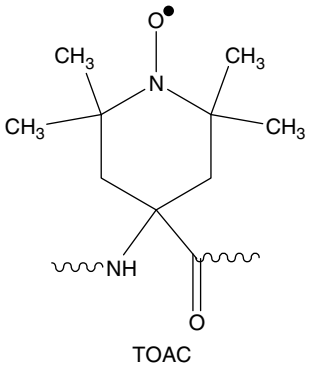

Figure 1. The nitroxide-based, paramagnetic TOAC probe.

Materials and Methods

Materials

The total chemical syntheses in solution of [TOAC^{1}, Glu(OMe)^{7,18,19}] Alm and [Glu(OMe)^{7,18,19}] Alm were performed according to Peggion *et al*. [4,9]. The analytical HPLC profile and the ESI–TOF mass spectrum of purified [Glu(OMe)^{7,18,19}] Alm are shown in Ref. 9. The analytical HPLC profile of purified [TOAC^{1}, Glu(OMe)^{7,18,19}] Alm is reported in Ref. 4. The ESI–TOF mass spectrometry data (Mariner Perseptive Biosystem) for [TOAC^{1}, Glu(OMe)^{7,18,19}] Alm are as follows: (*m/z*) calcd. for $C_{101}H_{165}N_{21}O_{28}$, 2119.14; found 1061.05 $[M+2H]^{2+}$, 1072.54 $[M+H+Na]^{2+}$, and 1083.03 $[M+2Na]^{2+}$. For a detailed discussion on the problems which might arise during the synthesis of TOAC-containing peptides, the reader should refer to a recent review article [10].

The amino acid sequences of the three Alm analogs discussed in this article are as follows:

[Glu(OMe)^{7,18,19}] Alm : Ac-U-P-U-A-U-A-Q*-U-V-U-G-L-U-P-V-U-U-Q*-Q*-Fol [9]
[TOAC^{1},Glu(OMe)^{7,18,19}] Alm : Ac-TOAC^{1}-P-U-A-U-A-Q*-U-V-U-G-L-U-P-V-U-U-Q*-Q*-Fol [4]
[TOAC^{16},Glu(OMe)^{7,18,19}] Alm : Ac-U-P-U-A-U-A-Q*-U-V-U-G-L-U-P-V-TOAC^{16}-U-Q*-Q*-Fol [4,5]

[Ac, acetyl; Q*, Glu(OMe); Fol, the 1,2-aminoalcohol phenylalaninol; U, Aib (α-aminoisobutyric acid)].

PRE Measurements

Measurements of PRE were carried out on a Bruker Avance 600 MHz NMR spectrometer (at Leuven) equipped with a 5-mm TXI-probe at 298 K. The TOAC^{1}-labeled samples were prepared in six concentrations (starting from 1 mM) by dilution with CD_3OH. Dilutions were performed by adding each time 50 µl of CD_3OH to the sample. The solvent was degassed using freeze-thawing cycles.

For the T_1 measurements, a standard inversion recovery sequence, modified with the water suppression using excitation sculpting with gradients, was exploited. Per sample, 20 data sets were collected with delay values ranging from 0.05 to 20 s. Per data set, 96 scans of 64 K points each were recorded over a 6000 Hz sweep width. Data were processed with Topspin 2.2. T_1 values were obtained from nonlinear least-square fit of the intensities according to the equation $I[t] = I[0] + P \times \exp(-t/T_1)$.

Results and Discussion

Theory

To quantify the relaxation enhancing effect exerted by a paramagnetic center on a proton, a comparison has to be made between the relaxation of the nuclei in the molecule in the presence and absence of the paramagnetic center. Hence, measurements need to be performed on a labeled and on an unlabeled sample in the case of a nitroxide probe [it is also possible to carry out measurements on the oxidized (paramagnetic) and the reduced (diamagnetic) form of the probe].

The paramagnetic contribution to the longitudinal relaxation rate of protons in NMR (R_{1para} or ${T_{1para}}^{-1}$) can be calculated from the relaxation rate of the unlabeled peptide (R_{1alm}) and the labeled peptide (R_{1toac}) according to:

$$R_{1para} = (R_{1toac} - R_{1alm}) \text{ with } R_x = T_x^{-1} \quad (1)$$

The distance r (in Å) between the nitroxide and the observed proton can be estimated from $R_{1para[0]}$ (which is the value of R_{1para} extrapolated for zero concentration of the spin label to neglect intermolecular contributions to R_{1para}), using a simplified form of the Solomon–Bloembergen equation:

$$r = C\left\{\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right)R_{1para[0]}^{-1}\right\}^{1/6} \quad (2)$$

where C is a constant having a value of 540 Å and ω_I and ω_S the nuclear and electron Larmor frequencies [11]. In the application of these equations, it is assumed that the rigid dipole–dipole vector distance r is tumbling isotropically in solution. The correlation time τ_c is described by the sum of contributions from the relaxation of the electron plus motions of the electron–proton vector:

$$\frac{1}{\tau_c} = \frac{1}{\tau_S} + \frac{1}{\tau_R} \quad (3)$$

where τ_S is the longitudinal relaxation time of the free electron and τ_R the effective rotational correlation time of the vector. For nitroxide free radicals, τ_c is essentially the same as τ_R. For the Alm analogs, the rotational correlation time is 0.85 ± 0.13 ns in methanol [12]. This value was also used for the measurements in CD_3OH.

Choice of the Spin Label

To measure a PRE effect, the peptide needs to be labeled with a paramagnetic probe. This probe should not influence the original conformation of the peptide it is built into. Traditional spin label probes are attached to proteins after biosynthesis *via* a flexible linker that has several rotational degrees of freedom [13–16]. This procedure, that implies the conformational space occupied by the linker probe system is relatively large, might introduce an error in the measurement (indeed, distances will be underestimated as, using the r^{-6} relationship in the equation, the short distances have a more pronounced effect on the PRE). Hence, the rigid vector approximation of the Solomon–Bloembergen equation will not be valid in this case. Moreover, in this approach it is extremely important that labeling would be uniform (>95% of the molecules need to carry the label), otherwise the magnitude of the relaxation effect will be underestimated, resulting in turn in longer apparent distances.

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Table 1. Distances of the paramagnetic center to the peptide NH protons in the two conformers of the [TOAC^{1},Glu(OMe)^{7,18,19}] Alm model

[TOAC^{1},Glu(OMe)^{7,18,19}] Alm model	*U*3	*A*4	*U*5	*A*6	*Q**7	*U*8	*V*9	*U*10	*G*11	*L*12	*U*13	*V*15	*U*16	*U*17	*Q**18	*Q**19	Fol20
Nitroxide–NH proton distance for conformer 1 (Å)	8.4	7.4	6.8	9.4	11.0	10.8	11.7	14.2	15.4	15.9	17.9	21.2	21.4	23.5	25.5	25.9	27.0
Nitroxide–NH proton distance for conformer 2 (Å)	8.0	6.8	6.9	9.5	10.8	11.0	13.0	14.9	15.7	17.3	19.6	21.9	22.9	25.2	26.6	27.3	29.0

An alternative approach to label peptides is to incorporate the paramagnetic residue TOAC directly as a part of the backbone [6–8]. TOAC is a non-natural, C^{α}-tetrasubstituted, α-amino acid containing a stable nitroxide radical. It can be used to replace the Aib residue in peptides without changing their overall conformation [5,17]. Moreover, it is a rigid label, the mobility of which is determined almost exclusively by that of the peptide backbone. An obvious result from incorporating this label into the backbone during synthesis is that labeling is 100% complete.

The aim of this investigation was to use PRE measurements to determine long-range, nitroxide to peptide NH proton distances in [TOAC^{1},Glu(OMe)^{7,18,19}] Alm and to compare them to those in a model compound derived from the X-ray diffraction structure obtained for [TOAC^{16},Glu(OMe)^{7,18,19}] Alm [5].

[TOAC^{1}, Glu(OMe)^{7,18,19}] Alm Model Construction and Nitroxide to Peptide NH Distance Determination

We have reasonably assumed that the isomeric [TOAC^{1}, Glu(OMe)^{7,18,19}] Alm and [TOAC^{16},Glu(OMe)^{7,18,19}] Alm, which differ only in the position of TOAC in the sequence (both positions are originally occupied by an Aib residue) share a similar overall conformation [5,7,8,18]. The [TOAC^{1},Glu(OMe)^{7,18,19}] Alm model 3-D structure was generated starting from the X-ray diffraction structure of [TOAC^{16},Glu(OMe)^{7,18,19}] Alm [5] using the molecular operating environment (MOE, Chemcomp modeling tools) software. For this study, the two different conformers present in the asymmetric unit of the X-ray diffraction structure of [TOAC^{16},Glu(OMe)^{7,18,19}] Alm were exploited. These two conformations, both largely α-helical and only differing slightly in their C═O···H-N intramolecular hydrogen-bonding scheme, were converted into [TOAC^{1},Glu(OMe)^{7,18,19}] Alm models by replacing Aib^{1} with TOAC^{1} and TOAC^{16} with Aib^{16}. Subsequently, the local geometry of the side chains of residues TOAC^{1} and Aib^{16} were optimized by keeping all the other atoms fixed. In these two conformers, the distances from the paramagnetic center to the peptide NH protons were measured (Table 1).

NMR Results

Experimental data for spin–lattice relaxation times were acquired on two samples, unlabeled [Glu(OMe)^{7,18,19}] Alm and labeled [TOAC^{1},Glu(OMe)^{7,18,19}] Alm (both dissolved in CD_3OH solution) using an inversion recovery sequence modified with solvent suppression using excitation sculpting with gradients. Assignments of the NH protons were performed in a previous study of our group [18].

PRE measurements were carried out on [TOAC^{1},Glu(OMe)^{7,18,19}] Alm and not on [TOAC^{16},Glu(OMe)^{7,18,19}] Alm as too many peaks are blanked in the NMR spectrum on the latter molecule. In any case, the solution conformations of unlabeled and (TOAC^{1}- and TOAC^{16}-) labeled Alms can be easily verified by spectral comparisons of the two-dimensional heteronucear multiple quantum coherence (HMQC) experiments which show that the unblanked signals of the labeled Alms overlap with the corresponding signals of the unlabeled [Glu(OMe)^{7,18,19}] Alm (these results are illustrated in Figure 2 for [TOAC^{1},Glu(OMe)^{7,18,19}] Alm. From these data it is reasonable to assume that the solution conformation of the unlabeled and labeled Alm analogs would be the same.

When comparing the NMR spectra of the labeled and the unlabeled Alm systems in more detail, it can be seen that the peptide NH proton signals up to five residues away from the TOAC paramagnetic moiety are completely blanked due to extreme line broadening. This result indicates that at a field strength of 600 MHz TOAC is able to suppress the peptide NH proton signals within a radius of about 10 Å (value measured in the X-ray diffraction-based models of [TOAC^{1},Glu(OMe)^{7,18,19}] Alm).

The paramagnetic component of the relaxation was deduced by comparing the T_1 values for the unlabeled Alm with the corresponding values for the TOAC^{1}-labeled systems (Figure 3) according to Eqn (1). To account for the effects of intermolecular PRE, the T_1 values for the [TOAC^{1},Glu(OMe)^{7,18,19}] Alm were measured at different concentrations and the effect was extrapolated to zero concentration ($R_{1\text{para}[0]}$) as described in literature [19]. An average intermolecular contribution to $R_{1\text{para}}$ of about 0.16 s^{-1}/mol was observed. Owing to the line broadening effect exerted by the free radical, the distance analysis for the peptide NH residues was limited (only nine nitroxide to peptide NH proton distances could be determined). The results, summarized in Table 2, should be compared with the corresponding distances found in the X-ray diffraction-based models in Table 1.

Figure 4 clearly shows the distances determined via PRE measurements are in close agreement with those extracted from the X-ray diffraction-based structures, especially for conformer 1. This finding suggests that the overall conformation in solution as well as in the crystal state is very similar for these molecules. Also, it validates the backbone geometry of [TOAC^{1},Glu(OMe)^{7,18,19}] Alm. The apparent underestimation of the distances might be due to systematic errors in the distance calculation or to motion of the helix backbone (e.g. bending). Indeed, in an ensemble of (slightly) bent structures, shorter proton–nitroxyl distances will contribute more to the observed $R_{1\text{para}}$ *via* the r^{-6} dependence (Eqn (2)).

It is worth noting that, as the distance between nitroxide radical and peptide NH protons increases, the difference between the PRE results and those from the X-ray diffraction-derived models is enhanced [20]. It has to be noted that small changes in a variety of factors like sample concentration errors, instrumental errors, errors in processing the data (baseline correction, integration), and the possibility of helix bending (*vide supra*), will influence the observed results. It is hard to estimate how much each of these parameters exactly contributes to the final result. In literature, sometimes standard errors on the measurements

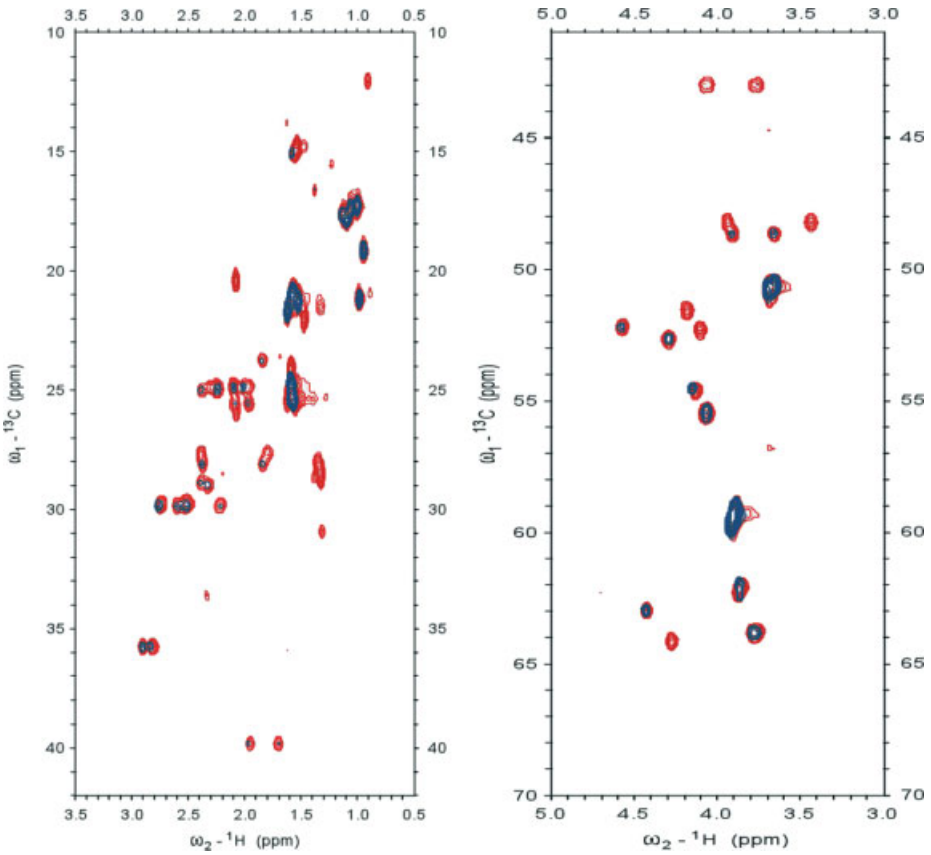

Figure 2. Expansions of the HMQC spectra (red: [Glu(OMe)^{7,18,19}] Alm, blue: [TOAC^{1},Glu(OMe)^{7,18,19}] Alm.

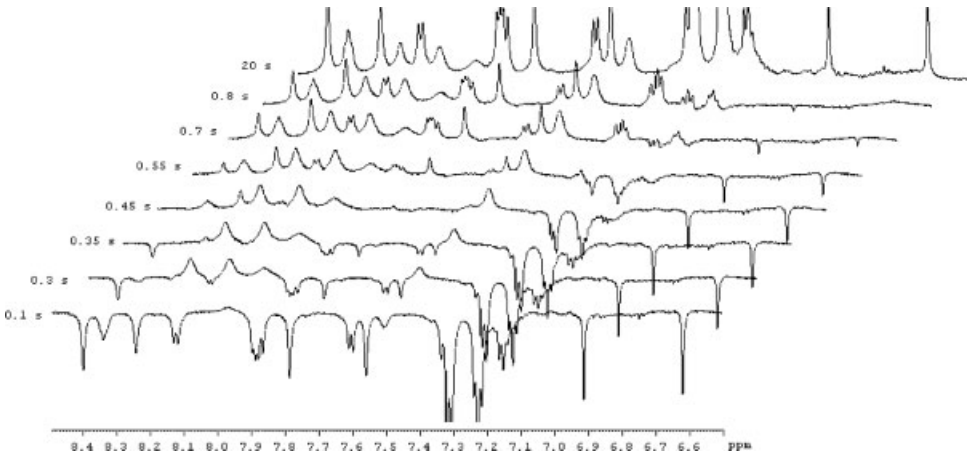

Figure 3. T_1 inversion recovery experiment for [TOAC^{1},Glu(OMe)^{7,18,19}] Alm.

Table 2. Calculated nitroxide to peptide NH proton distances obtained from the T_1 experiments on [TOAC^{1},Glu(OMe)^{7,18,19}] Alm

Residue (NH)	*U*8	*V*9	*U*10	*G*11	*L*12	*U*13	*U*17	*Q**18	*Q**19
$R_{1para[0]}$ (s^{-1})	3.0	1.95	0.84	0.73	0.43	0.25	0.07	0.03	0.04
Distance (Å) (calculated)	11.1	11.9	13.7	14.0	15.3	16.8	20.9	23.9	22.8
Distance (Å) (upper bound)	10.1	11.1	12.8	13.1	14.3	15.7	19.5	22.3	21.3
Distance (Å) (lower bound)	12.4	13.3	15.4	15.8	17.2	18.9	23.3	26.8	25.6

Upper and lower bounds were calculated by assuming a 50% error on the value $R_{1para[0]}$.

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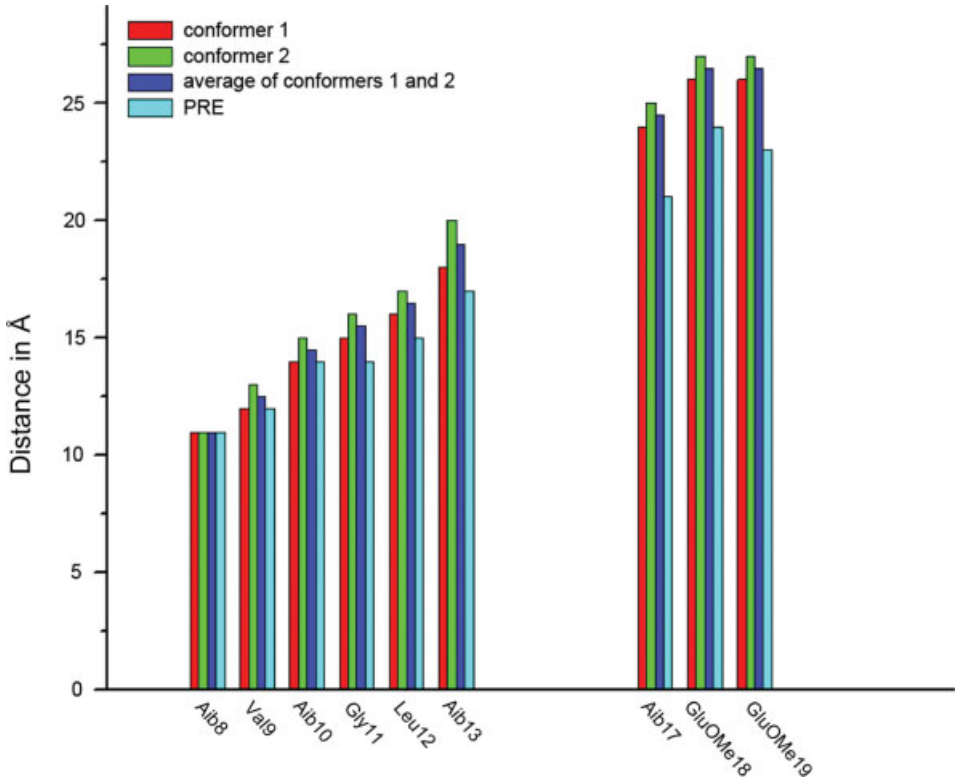

Figure 4. Nitroxide to peptide NH proton distance comparison between PRE *versus* X-ray diffraction-based models for [TOAC[1],Glu(OMe)[7,18,19]] Alm.

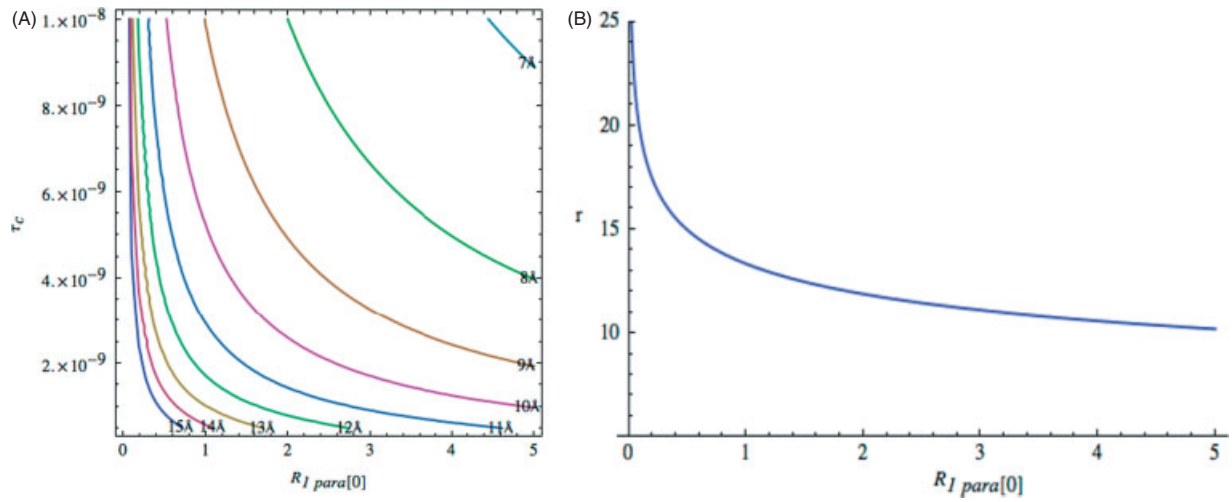

Figure 5. Relationship between the distance *r* with respect to τ_c and $R_{1\text{para}[0]}$ for [TOAC[1],Glu(OMe)[7,18,19]] Alm (calculated according to Eqn (2) for a 600 MHz NMR spectrometer). (A) Isocontours for the distances 7-15 Å; (B) Distance *r* as a function of $R_{1\text{para}[0]}$ for a fixed τ_c of 0.85 ns.

are reported. However, these data merely reflect the error on the fitting procedure and do not fully account for other sources of experimental uncertainties. Nevertheless, even when assuming a 50% error on the value of $R_{1\text{para}[0]}$, useful upper and lower distance bounds can be obtained to supplement NOESY-based restraints for NMR calculations (Table 2). Table 2 also indicates that a constant error on R_1 (in our assumption 50%) has a much more pronounced effect when longer distances are examined. This phenomenon is further exemplified in Figure 5, which shows the relationship between the distance *r* with respect to the parameters τ_c and $R_{1\text{para}[0]}$, calculated according to Eqn (2) for a 600 MHz spectrometer, and the dependence of *r* on the value of $R_{1\text{para}[0]}$ for a fixed τ_c of 0.85 ns.

On the basis of the present experimental and theoretical considerations, we believe that the nitroxide to peptide NH proton distance estimations are reliable up to about 20 Å if a sufficiently large error margin is considered. This conclusion should also be taken into account when implementing these values as long-range NMR restraints.

To evaluate the agreement between the nitroxide to peptide NH proton distances derived from the X-ray diffraction and PRE

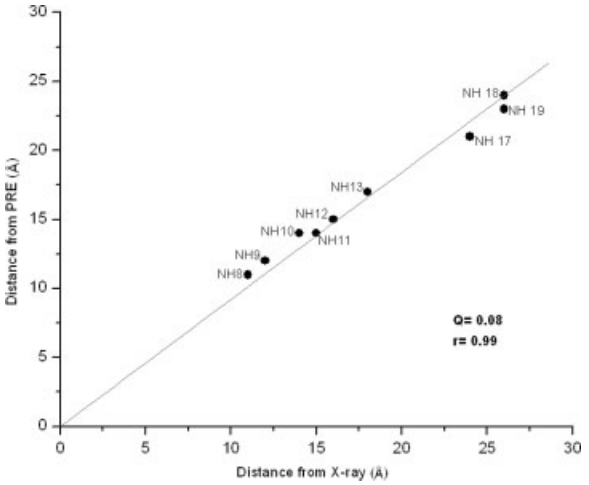

Figure 6. Correlation between the average nitroxide to peptide NH proton distances in X-ray diffraction-derived models and the corresponding PRE distances for [TOAC^{1},Glu(OMe)^{7,18,19}] Alm.

measurements, we extended the use of the quality factor (*Q* factor) [21–23] according to the equation

$$Q = \sqrt{\frac{\sum_i (D_{obs} - D_{calc})^2}{\sum_i (D_{obs})^2}} \qquad (4)$$

where D_{obs} is the distance of residue *i* derived from the averaged distances in the X-ray diffraction-based models and D_{calc} the distance obtained from the PRE measurements. For [TOAC^{1},Glu(OMe)^{7,18,19}] Alm, the *Q* factor is 0.08 with a correlation coefficient of 0.99 (Figure 6).

Conclusions

In this study, quantification of nitroxide to peptide NH proton distance information *via* PRE measurements is in good agreement with results from X-ray diffraction-derived models for distances from 10 to about 20 Å. Shorter distances cannot be assessed because the peaks are too broadened to be observed and at longer distances the error on the determination propagates too much in the distance calculation. In this specific peptide system examined, the conformation in solution is in close agreement with that in the crystal state (with a preference for conformer 1). Finally, it seems that these long-range restraints can complement NMR-based structure determination at least if large enough upper restraints are used in the calculations. Implementation of these restraints in structure calculation on these peptaibotics is a topic for further research.

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