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Stretched Poly(methyl methacrylate) Gel Aligns Small Organic Molecules in Chloroform. Stereochemical Analysis and Diastereotopic Proton NMR Assignment in Ludartin Using Residual Dipolar Couplings and ^3J Coupling Constant Analysis

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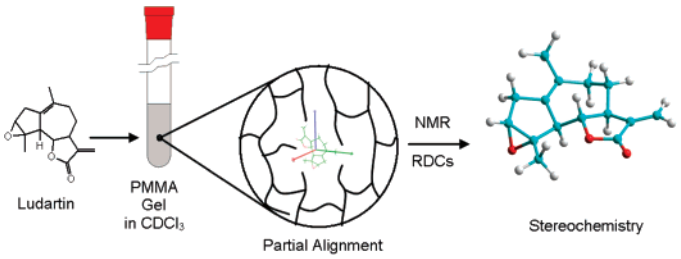

Poly(methyl methacrylate) (PMMA) gels prepared by copolymerizing methyl methacrylate (MMA) and various amounts of ethylene glycol dimethacrylate (EGDMA) in the presence of the radical initiator V-70 (2,2′-azobis(2,4-dimethyl-4-methoxyvaleronitrile)) can orient small organic molecules when swollen in NMR tubes with $CDCl_3$. The aligning properties of the stretched PMMA gels were evaluated by monitoring the quadrupolar splitting of the ^2H NMR signal of $CDCl_3$, and the aligning degree is proportional to the cross-linking density. Natural abundance one-bond $^1H-^{13}C$ residual dipolar couplings (RDCs) for menthol measured in the gels depended on the cross-link density. The stereochemistry and assignment of the diastereotopic protons of the gastroprotective and nonsteroidal aromatase inhibitor sesquiterpene lactone ludartin, isolated from *Stevia yaconensis var. subeglandulosa*, were unambiguously determined using a combination of natural abundance one-bond $^1H-^{13}C$ RDCs measured in a PMMA gel and a ^3J coupling constant analysis.

Introduction

Nuclear magnetic resonance is one of the most elegant spectroscopic techniques. In addition to supplying the information of the chemical environment of a given atom, it also provides connectivity information with its neighbors. In solution NMR, spin−spin connectivities are normally determined using experiments that involve homonuclear and heteronuclear scalar coupling interactions (COSY, TOCSY, HSQC, HMBC) and homonuclear dipolar coupling interactions (NOESY, ROESY), allowing the elucidation of the three-dimensional structure of a given molecule.[1]

Analysis of $^1H-^1H$ and $^1H-^{13}C$ three-bond J couplings (^3J) has proven to be a very powerful tool for the determination not only of relative stereochemistry but also of the preferred conformation of small rigid molecules. ^3J values strongly depend on the dihedral angle between the three bonds in consideration.[2] An empirical generalization of the Karplus equation, which includes the electronegativity of the substituents, developed by Altona and co-workers,[3] is nowadays widely used for the ^3J coupling constant analysis of small molecules. In addition, NOE[4] is one of the most powerful NMR tools, and it has been extensively used in the stereochemical and conformational analysis of organic molecules since the pioneering work by Anet and Bourn dating from 1965.[5] However, there are particular cases where the $1/r^6$ dependence of NOE sets a limitation in

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the distance information about a pair of remotely located protons. In addition, ambiguities can also arise from similarities in ^3J and NOE values among different stereoisomeric compounds. In cases like these, residual dipolar couplings (RDCs) become an extremely powerful and complementary NMR tool for the unambiguous determination of relative stereochemistry in small organic molecules and biological macromolecules.

Dipole−dipole interactions between spins in a given molecule give origin to an NMR parameter known as dipolar coupling. Different from spin−spin scalar couplings, dipolar couplings average to zero when molecules tumble in isotropic solutions. Dipolar couplings between the nuclei i and j (D_{ij}) contain important structural information since their values depend on the internuclear distance (for instance, bond length r) and on the angle Θ between the internuclear vector and the axis of the external magnetic field, according to the following equation:

$$D_{ij} = -\frac{\mu_0 \gamma_i \gamma_j h}{(2\pi r)^3}\left\langle \frac{3\cos^2\Theta - 1}{2} \right\rangle$$

where μ_0 is the permeability of vacuum, γ is the gyromagnetic constant for the corresponding nucleus, and h is the Planck constant. The brackets around the angular term represent averaging in solution. The dipolar couplings are of the order of several kilohertz and are responsible for the strong signal broadening in solid-state NMR.

However, if molecules in solution are forced to adopt a minor degree of alignment and no longer tumble isotropically, a measurable fraction of the original dipolar coupling (0.01−0.1%) known as residual dipolar coupling (RDC) is observed in the NMR spectrum. It is important to highlight that, with such a small degree of orientation, the molecules in solution still produce good quality NMR data since almost >99.9% of the dipolar couplings are removed. The RDCs still contain the same structural information as the original dipolar couplings and provide global orientation information between remote internuclear vectors. The great power of RDCs is that they provide information about the relative orientations between the internuclear vectors (H−H, H−C, H−N, C−C, etc.), regardless of the distance between them. The theoretical aspects of RDCs are beyond the scope of this work and can be found elsewhere.[6−11]

Since the introduction of the application of RDCs to the analysis of biological macromolecules by Tjandra and Bax in 1997,[12] they have been extensively used in structural biology. Initially, RDCs were applied to the analysis of proteins,[8,9,13−22] their application was later extended to nucleic acids[23−25] and studies of various nucleic acid−protein interactions,[26−28] and to dynamic studies of biological macromolecules in general.[29−32] However, RDCs were not as extensively used for the analysis of small organic molecules.[10,33,34] The main reason is that the traditional aligning media used for biomolecules were only applicable to water-soluble compounds. In order to be able to partially align small molecules, it is necessary to have access to orienting media compatible with organic solvents.

Courtieu et al.[35] introduced in the mid 1990s the use of chloroform solution of poly-γ-benzyl-L-glutamate (PBLG) to discriminate enantiomers using quadrupolar splittings, dipolar couplings, and chemical shift anisotropies.[35] More recently and almost simultaneously, Griesinger and co-workers[36] and Thiele and Berger[37] made significant contributions to the methodology of structure elucidation of small organic molecules by RDCs using PBLG solutions in organic solvents. Other liquid crystalline phases with lower degree of orientation in organic solvents were recently developed.[38,39]

Stretched polymer gels are another alignment media used for orienting molecules. Recently, Luy and co-workers[40] showed that molecules can be aligned in organic solvents using stretched polystyrene (PS) cross-linked with divinylbenzene. In a very original approach, a small cylindrical PS stick was introduced into an NMR tube together with $CDCl_3$, and the polymer swelled and stretched axially once it reached the tube wall. No additional tools were necessary. Luy and co-workers have reported the deuterium quadrupolar splitting of $CDCl_3$ and also the solution structure of strychnine using RDCs of C−H bonds measured from proton-coupled ^1H,^{13}C HSQC experiments. A more detailed study of the orientational properties of PS gels was later reported.[41] Several publications from the same group

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reported the alignment properties of other cross-linked polymer gels in organic solvents of different polarity: polydimethylsiloxane (PDMS),[42] poly(vinyl acetate) (PVAc),[43] polyacrylonitrile (PAN),[44] and even gelatin from commercial "gummy bears" with enantiomer discrimination properties.[45] In addition, Harbez et al.[46] have introduced a copolymeric polyacrylamide gel, the first alignment medium compatible with DMSO. All the solvent and alignment media combinations published thus far, except for PAN gels, are thoroughly summarized by Thiele in a recent review article.[10] For interested readers, a comprehensive brief history of RDCs is provided with the Supporting Information.

Here we introduce a poly(methyl methacrylate) gel (PMMA) with excellent scalable alignment properties in $CDCl_3$. We also report the unambiguous determination of the stereochemistry of the 3,4-epoxy group in the bioactive natural product ludartin (**1**) and the assignment of its diastereotopic protons at C-2 using RDCs, a case where the application of NOE and ^3J couplings was unsuccessful.

Results and Discussion

Rationale. One of the objectives of this work was to develop an orienting gel that would exhibit good alignment properties mainly in chloroform-*d* ($CDCl_3$) since it is the most common and inexpensive NMR solvent thanks to its good solubilizing properties and relatively unreactive nature. In the design of the gel, three major issues were considered: (i) the gel should not contain aromatic rings to avoid unwanted chemicals shift effects like those observed in stretched cross-linked polystyrene;[40,41] (ii) the polymer backbone should be rigid to induce shorter T_2 relaxation times in the polymer protons; and (iii) the gels should be easy to prepare and affordable. We thought that a cross-linked PMMA gel would meet all these characteristics.

Preparation and Alignment Properties of Cross-Linked PMMA. PMMA gel rods were prepared in disposable NMR tubes by polymerizing methyl methacrylate (MMA) in the presence of various amounts of the cross-linker ethylene glycol dimethacrylate (EGDMA), initiated by V-70 (2,2′-azobis(2,4-dimethyl-4-methoxyvaleronitrile)) at 50 °C. The reaction was carried out in acetone-d_6 to avoid the presence of significant residual acetone peak in the final gel (see Experimental Section). The cross-link densities in the PMMA gels are expressed as the molar fraction of the cross-linking agent EGDMA, in percent. Six PMMA samples with the following EGDMA percent molar fraction in the feed monomer mixture were prepared: 0.0034 (**A**), 0.01 (**B**), 0.068 (**C**), 0.17 (**D**), 0.542 (**E**), and 1.01 (**F**). The resulting polymers swelled very well in chloroform and markedly less in acetone. In all cases, the resulting sticks were ∼4 mm in diameter. They were cut into small cylinders, 15 mm long, then placed in NMR tubes and allowed to swell in $CDCl_3$. The quadrupolar splitting of the $CDCl_3$ ^2H NMR peak was monitored over time. The gels reached equilibrium (sharp doublet of the ^2H signal) over a period of 15−20 days.

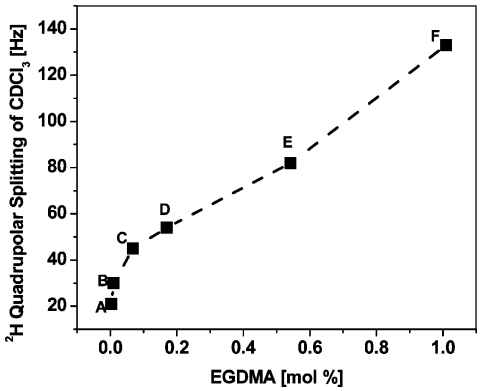

FIGURE 1. ^2H quadrupolar splitting of $CDCl_3$ as a function of cross-link density expressed as molar percent fraction of EGDMA.

Figure 1 shows the quadrupolar splitting of the $CDCl_3$ ^2H NMR doublet at equilibrium as a function of the cross-link density. The curve showed a profile similar to that observed previously for other gels.[40,41,43] In order to further evaluate the aligning properties of this new matrix, the gels were swollen in a solution containing 20 mg/mL of menthol in $CDCl_3$. Proton-coupled ^1H,^{13}C HSQC experiments were acquired on each sample when equilibrium was reached. In isotropic solution, $^1J_{CH}$ is the observed one-bond scalar CH coupling constant. In orienting media, the signal splitting changes by a factor of the RDC (D_{CH}), which may be positive or negative. One-bond C−H RDCs were measured for menthol taking the difference between the $^1J_{CH} + D_{CH}$ couplings of menthol in the swollen gels and the $^1J_{CH}$ couplings of menthol in the isotropic solution consisting of $CDCl_3$ only. Although menthol does not have at least five independent RDCs to perform an order matrix analysis,[6] its RDC values were used to choose the most appropriate cross-linking density to perform the structural analysis of a target small molecule. Gels **C** and **D** provided RDC values for menthol in a range of 0−22 Hz. In addition, no significant chemical shift differences were observed for menthol between the isotropic solution and the gel (see Figure 2, traces B and C). Only the ^1H NMR signals corresponding to the carbinolic methine proton and to the methine proton of the isopropyl group showed differences of less than 0.1 Hz. The lack of aromatic groups in the polymer gel might be advantageous for several reasons: (i) provides a clean area for the analysis of aromatic compounds, (ii) prevents the induction of chemical shift changes as those observed in polystyrene gels,[41] and (iii) eliminates flexible side groups as in the case of polystyrene gels which needed relaxation filter periods of 400 ms.[41] In case of the PMMA-based gels, the signals of the matrix could be eliminated in ∼30 ms, and it was not necessary to use relaxation filters in the HSQC experiments. Figure 2 shows the comparison of a regular proton 1D experiment with a 1D CPMG (Carr−Purcell−Meiboom−Gill)[1,47,48] experiment for a total relaxation filter of 30 ms (see Figure 2, traces A and B). It is clearly observed in Figure 2B how clean the resulting spectrum is. The PMMA signal decays quickly, resulting in a high-quality NMR spectrum. The presence of a methyl group in each monomer unit in the backbone of PMMA reduces the flexibility of the polymer network, leading to shorter relaxation times T_2.

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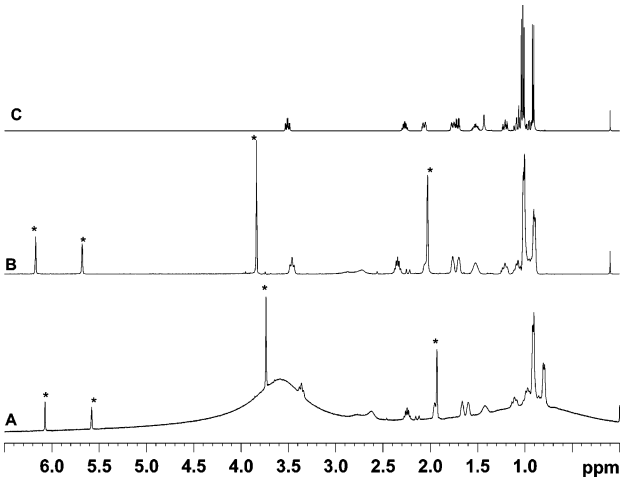

FIGURE 2. ^{1}H NMR spectrum of menthol in PMMA gel **D** in $CDCl_3$ (A); 30 ms 1D ^{1}H CPMG NMR spectrum of menthol in PMMA gel **D** in $CDCl_3$ (B); ^{1}H NMR spectrum of menthol in $CDCl_3$ (isotropic solution) (C). Signals marked with the asterisk correspond to the residual MMA monomer protons.

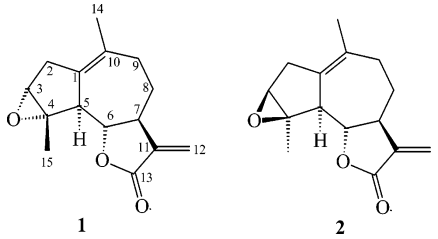

FIGURE 3. Absolute stereochemistry of ludartin (**1**) as previously determined using chemical transformation and NMR chemical shifts,[52] and its 3,4-β-epoxy isomer (**2**).

The NMR spectra of the gels show signals corresponding to the residual MMA monomer (see Figure 2, signals marked with an asterisk). This problem has also been observed in other orienting gels.[41] All of the NMR experiments were performed at 27 °C and at only one magnetic field strength (11.75 T, 500 MHz). It has been observed that the induced alignments by the stretched gels are significantly temperature-dependent but virtually field-independent.[42]

Unambiguous Determination of the Relative Stereochemistry of the Natural Product Ludartin Using RDCs from a PMMA Gel. Ludartin (**1**) is a bioactive natural product belonging to the sesquiterpene lactone family. It shows gastric cytoprotective effect[49] and also inhibits the aromatase enzyme, which is involved in hormone-dependent breast cancer.[50] Ludartin was first isolated in 1972 from *Artemisia carruthii* as a mixture with its 11,13-dihydro derivative by Geissman and Griffin, who proposed the stereochemistry displayed in **1** (Figure 3) based on the chemical shift and coupling constants exhibited by the proton H-6 NMR signal and on the chemical shift of H-15.[51] In 1989, it was shown that these criteria were not enough to unambiguously determine the stereochemistry of **1**, and its structure was confirmed using a combination of chemical transformations and NMR chemical shift analysis.[52] At the same time, a full proton and carbon NMR assignment was made. However, the diastereotopic protons of the methylenes C-2, C-8, and C-9 were not assigned.

Chemical transformations were the only option at that time since NOE experiments and ^3J coupling constant analysis for the protons of the five-membered ring could not be employed to determine the stereochemistry of the 3,4-epoxide group. Instead of performing tedious chemical transformations to analyze the structure of **1**, this is a good case to apply RDCs, which provide much easier access to the same type of structural information.

The five-membered ring of **1** is very rigid due to the presence of the 1,10-double bond and the 3,4-epoxide ring. The *trans* fusion of the lactone ring provides rigidity to the dihedral angle formed by C-5, C-6, C-7, and C-8. Some degree of flexibility in the seven-membered ring can only arise from variations in the C-7−C-8−C-9−C-10 dihedral angle that may lead to the presence of more than one conformer. Base structures for **1** and its 3,4-β-epoxide isomer (**2**) were initially generated in HyperChem,[53] and they were energy minimized using the molecular mechanics force field option MMX. A conformational search in HyperChem[53] using the MMX force field and randomly varying the value of the C-7−C-8−C-9−C-10 dihedral angle led to only two possible conformations for each ludartin isomers (**1** and **2**). The resulting structures, a total of four, were then energy minimized using the semiempirical method AM1 in order to obtain more accurate geometries. Calculations of the heat of formation of each of the four isomers showed that both ludartin isomers (**1** and **2**) have only one preferred conformer with a dihedral angle value for C-7−C-8−C-9−C-10 of +75° in each of them. The conformer with a dihedral angle value of −47° has an energy difference of +5.4 kcal/mol in the case of **1** and +6.3 kcal/mol in its 3,4-β-epoxide isomer (**2**). The PDB files of these four structures are provided with the Supporting Information.

Figure 4 shows a side to side view of the 3D structures of the preferred conformers of **1** and its hypothetical isomer 3,4-β-epoxy-ludartin (**2**). Figure 5 shows the root mean square (rms) fit and overlay of the heavy atoms of these two structures with an rms error of only 0.039 Å. Carbons C-14 and C-15 and the oxygen atom of the epoxy group were excluded in the rms fitting calculation. The geometry of the carbon skeleton in both isomers is almost identical.

As mentioned above, in the original publication[52] describing the isolation and structure elucidation of ludartin **1**, none of the diastereotopic protons were assigned (H-2, H-8, and H-9). The original NMR assignments were here verified by collecting a series of 1D and 2D NMR experiments at 500 MHz of ludartin **1** in $CDCl_3$, including ^{1}H NMR, COSY, NOESY, ^{1}H,^{13}C HSQC (coupled and decoupled), and HMBC. The combined analysis of these data confirmed the original assignments. The assignment of the protons H-8α, H-8β, H-9α, and H-9β is straightforward from a ^3J coupling constant analysis. The coupling constant values involving the diastereotopic protons H-8 and H-9 are not obvious from the multiplicity of the signals since protons H-9β and H-8α are strongly coupled, giving rise to a high-order spin system with very complex multiplicity patterns (see

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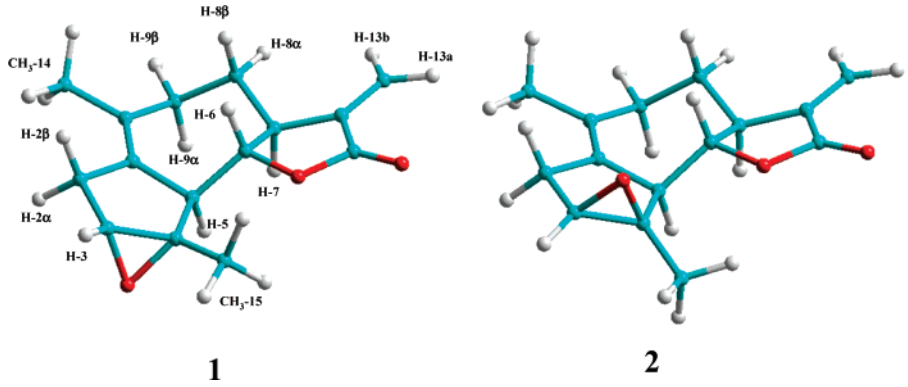

FIGURE 4. Side to side view of the 3D structures of ludartin (**1**) and 3,4-β-epoxy-ludartin (**2**).

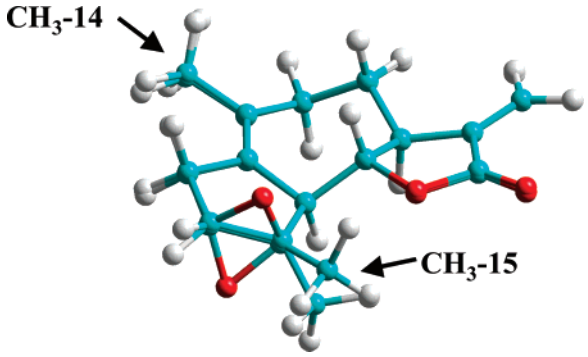

FIGURE 5. Root mean square fit and overlay of the 3D structures of ludartin (**1**) and 3,4-β-epoxy-ludartin (**2**) using only the heavy atoms belonging to the five-membered ring, the seven-membered ring, and the lactone ring. Rms error: 0.039 Å. The two methyl groups that do not completely overlap in the two isomers, **1** and **2**, namely, CH_3-14 and CH_3-15, are shown with arrows.

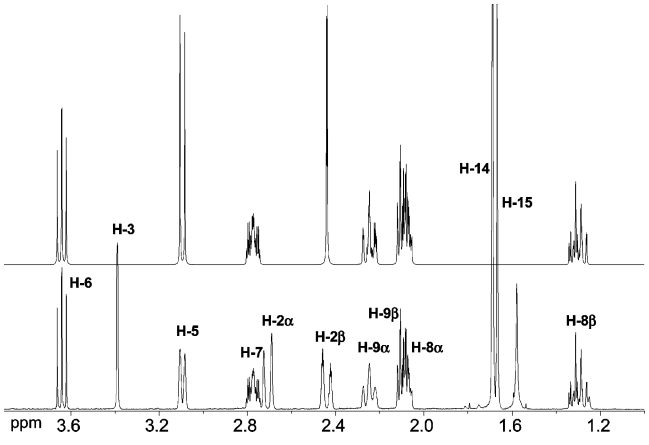

FIGURE 6. Expansion of the 500 MHz ^{1}H NMR spectrum of **1** in $CDCl_3$ (lower trace). The signals corresponding to H-13a and H-13b are outside the plot region. The upper trace shows the simulation of the spin system involving H-6, H-5, H-7, H-9α, H-9β, H-8α, and H-8β using the NUMMRIT[55] routine in SpinWorks.[56] The signal of H-2β was included because of the long-range coupling with H-9α observed in the COSY spectrum, which improved the quality of the simulation results.

Figures 6 and 7). The PDB file of the ludartin (**1**) structure generated in HyperChem[53] was opened in the MSpin[54] software package, and the ^3J coupling constants of the protons belonging to the seven-membered ring were estimated by the Altona equation.[3] The spin system involving H-5, H-6, H-7, H-8α,

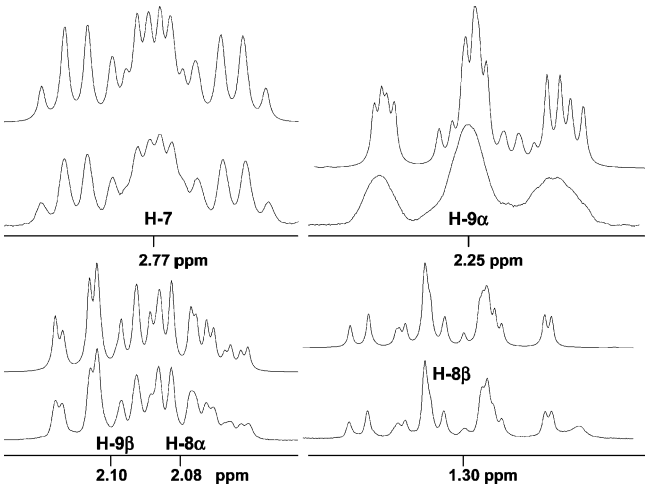

FIGURE 7. Expansion of the calculated (upper traces) versus the experimental (lower traces) of the ^{1}H NMR signals corresponding to H-7, H-8α,β, and H-9α,β.

H-8β, H-9α, H-9β, H-13a, and H-13b was simulated with the spectral analysis routine NUMMRIT[55] (including iteration) within the software SpinWorks.[56] The chemical shift of each signal from the ^{1}H NMR spectrum and the calculated coupling constants were used as initial input values for the spectral simulation. The coupling constants between H-7 and H-13a,b were measured from the spectrum. The geminal (^2J) coupling constants between H-8α and H-8β and between H-9α and H-9β were not obvious from the signals and were attributed to an initial guessed value of −13 Hz. The general profile of the initial calculated spectrum was similar to the experimental one. After a few rounds of automatic and manual iterations, the spectrum shown in Figure 6 (upper trace) was obtained. Table 1 shows the calculated versus experimental ^3J values. In addition, Table 1 also shows the values for the geminal coupling constants (^2J) for the diastereotopic protons H-8α,β and H-9α,β. The final simulated spectrum was almost identical to the experimental one. Figure 7 shows expansions of the signals corresponding to H-8α,β and H-9α,β. Note that the experimental signals of H-5 and H-9α are much broader than the calculated ones due to further long-range J couplings.

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(55) Quirt, A. R.; Martin, J. S. *J. Magn. Reson.* **1971**, *5*, 318−327.
(56) *SpinWorks 2.5.5*; Marat, K. University of Manitoba, Winnipeg, Manitoba, Canada, http://www.umanitoba.ca/chemistry/nmr/spinworks.

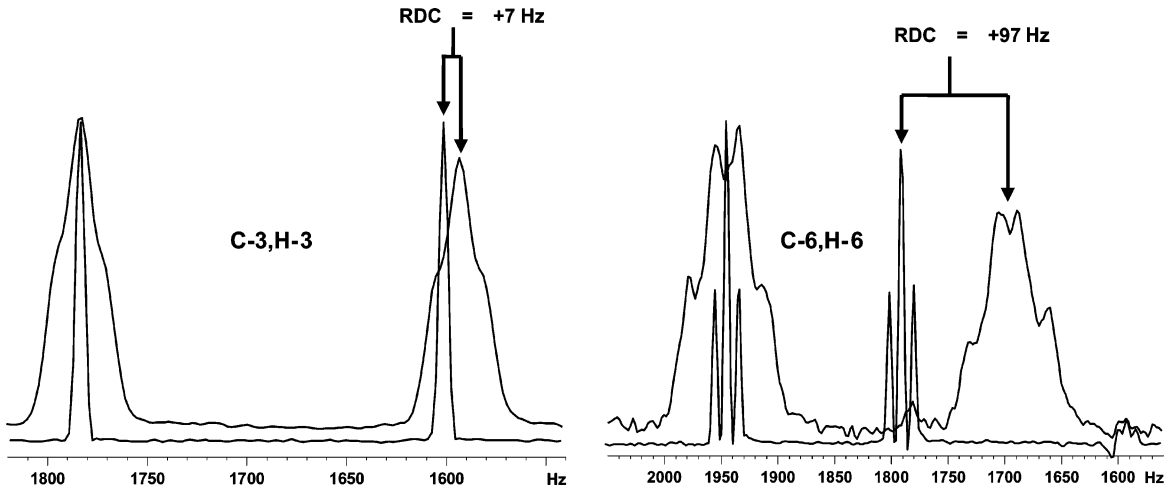

FIGURE 8. F2 slices extracted from the proton-coupled $^{1}H,^{13}C$ HSQC spectra of ludartin (**1**) in the gel (anisotropic medium − broad multiplets) and in $CDCl_3$ only (isotropic solution − sharp multiplets) for the signals corresponding to C-3 (left) and C-6 (right). The left submultiplet of each signal was aligned on top of each other so that the difference in hertz on the right submultiplets corresponds to the RDC. The coupling constant error for the isotropic spectrum was approximately 0.5 Hz and for the anisotropic spectrum about 1 Hz.

TABLE 1. Calculated ($^{3}J_{calc}$) versus Experimental ($^{3}J_{exp}$) ^{3}J Values for the Seven-Membered Ring Protons of 1

protons	$^{3}J_{calc}$ (Hz)	$^{3}J_{exp}$ (Hz)	$^{3}J_{exp} - {}^{3}J_{calc}$ (Hz)
5,6	10.9	10.51	−0.39
6,7	10.3	9.70	−0.60
7,8a	1.6	3.00	1.40
7,8b	11.8	11.20	−0.60
8a,8b		−13.50	
8a,9a	1.2	1.60	0.40
8a,9b	6.3	5.90	−0.40
8b,9a	13.1	12.50	−0.60
8b,9b	1.0	1.70	0.70
9a,9b		−14.60	

The excellent agreement between the calculated and the experimental ^{3}J coupling constants confirms the assignment of the diastereotopic protons H-8α,β and H-9α,β. In addition, these results reassure the quality of the structures generated by AM1 calculation which were later used as input for the RDC calculations. Figure 6 shows the corresponding assignments on top of each signal. A Q factor of 0.087 (Q = rms ($^{3}J_{obs} - {}^{3}J_{calc}$)/rms $^{3}J_{obs}$) was calculated for the comparison between experimental versus calculated ^{3}J values. Accordingly, the diastereotopic protons at C-8 and C-9 were unambiguously assigned, and coincidentally, the assignment reported previously[52] as H-8a, H-8b, H-9a, and H-9b corresponds to H-8α, H-8β, H-9α, and H-9β, respectively.

Epoxide rings have a very particular geometry. For example, when fused to five-membered rings (like in the present case), inversion of their stereochemistry does not produce significant changes in the orientation of the substituents. The angle of the C-3−H-3 bond and of the CH_3-15 methyl group with respect to the plane of the five-membered ring is only 27° in both isomers of ludartin (**1** and **2**) (see Figure 5). This geometry leads to a series of spectroscopical ambiguities as follows: (i) CH_3-14 can give NOE with H-2α and H-2β in both isomers; (ii) the distance between H-5 and C-15 is 3.12 Å in the α-epoxy isomer (**1**) and 2.70 Å in the β-epoxy isomer (**2**), leading to the observation of NOE between H-5 and CH_3-15 in both isomers; (iii) the distance between H-6 and C-15 is 3.46 Å in the α-epoxy isomer (**1**) and 3.94 Å in the β-epoxy isomer (**2**), borderline distance values to observe either weak or no NOE between H-6 and CH_3-15; and finally (iv) the Altona[3] equation predicts the same multiplicity and ^{3}J coupling constants for the ^{1}H NMR signal of H-3 (dd, J = 2 and 5 Hz). Note that the value of the vicinal coupling constants calculated by the Altona equation for five-membered rings are overestimated since this type of ring shows much wider H−C−C valence angles, hence reducing the observed ^{3}J value. The observed signal for H-3 in the ^{1}H NMR spectrum appears as a broad singlet.

Given all these NMR spectroscopical ambiguities, ludartin (**1**) is a good case for studying RDCs and for evaluating the aligning properties of the PMMA gels. Consequently, 5 mg of **1** was dissolved in $CDCl_3$, and a cross-linked PMMA stick (gel **D**, 0.17 mol % of EGDMA) was allowed to swell in this solution inside the NMR tube until equilibrium was reached. The evolution of the swelling process was monitored by the ^{2}H NMR signal of $CDCl_3$ until a sharp doublet was obtained (about 20 days). A regular proton-coupled $^{1}H,^{13}C$ HSQC experiment was collected on this sample, and RDCs were measured for the CH bonds by taking the differences between the $^{1}J_{CH} + D_{CH}$ of **1** in the PMMA gel and the $^{1}J_{CH}$ in the isotropic solution in $CDCl_3$ only. The best way to accurately measure the RDCs is to extract individual F2 slices from the HSQC experiment at each carbon chemical shift and manually measure the splitting of the signal ($^{1}J_{CH} + D_{CH}$) after properly phasing and correcting the baseline of each slice. A total of eight F2 slices at the chemical shift of carbons C-2, C-3, C-5, C-6, C-7, C-9, and C-13 were extracted. Figure 8 shows a couple of examples for the methine bonds C-3,H-3 and C-6,H-6 with corresponding RDC values of +7 and +98 Hz, respectively. For the methine CH bonds, the left submultiplets from the isotropic and the anisotropic spectrum were overlapped on top of each other so that the frequency difference (in hertz) between the right submultiplets corresponds to the RDC value (see Figure 8). The signals from the HSQC experiments collected in the PMMA gel are much broader and display a much more complex splitting pattern due to the presence of proton−proton and long-range proton−carbon RDCs. Also, the broadening arises from the field inhomogeneity caused by the gel itself. Figure 9 shows an example of the signals corresponding to the methylene carbon C-2. All the

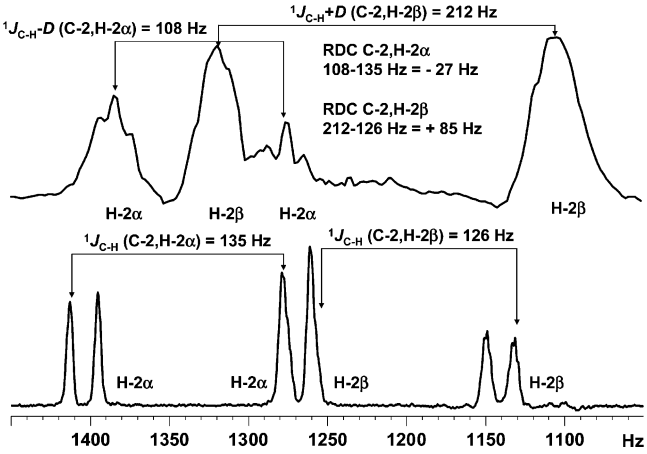

FIGURE 9. ^{1}H,^{13}C HSQC F2 slices at the chemical shift of C-2 of ludartin (**1**) in the gel (anisotropic medium − upper line) and in $CDCl_3$ only (isotropic solution − lower line). The lower trace shows the $^1J_{C-H}$, and the upper trace shows the splitting of the signal with the corresponding contribution from the RDCs ($^1J_{CH} \pm D$).

TABLE 2. Comparison of Observed (D_{obs}) versus Calculated RDCs (D_{calc}) for the Structure of 1, Its 3,4-*β*-Epoxy Isomer (2), and Ludartin with Permutated Assignments of H-2α and H-2*β* (P.A.)

CH bond	D_{obs} (Hz)	D_{calc} (Hz) **1**	**2**	P.A.
C-2,H-2α	−27	−24 (−3)[a]	−25 (−2)	−65 (38)
C-2,H-2*β*	85	93 (−8)	83 (2)	44 (41)
C-3,H-3	7	7 (0)	−17 (24)	2 (5)
C-5,H-5	98	98 (0)	81 (17)	86 (12)
C-6,H-6	97	93 (4)	85 (12)	42 (55)
C-7,H-7	81	78 (3)	78 (3)	104 (−23)
C-9,H-9α	89	89 (0)	86 (3)	67 (22)
C-9,H-9*β*	−34	−35 (1)	−42 (8)	−55 (21)
C-13,H-13a	−34	−36 (2)	−63 (29)	−18 (−16)
C-13,H-13b	−38	−37 (−1)	−53 (15)	−16 (−22)
Q[b]		0.048	0.221	0.488

[a] The numbers in parentheses are the difference between D_{obs} and D_{calc}. [b] *Q* or *Q* factor = rms ($D_{obs} - D_{calc}$)/rms D_{obs}, proposed by Cornilescu et al.[57] to validate structures using RDCs.

HSQC slices are shown in the Supporting Information. A total of 10 RDCs were measured for CH pairs of protons H-2α, H-2*β*, H-3, H-5, H-6, H-7, H-9α, H-9*β*, H-13a, and H-13b (see Table 2, D_{obs}). It was not possible to obtain clear RDCs for methylene C-8. The signals were significantly broad and complex, leading to ambiguous measurements. The experiment provided RDC values ranging from −40 to +100 Hz. This was advantageous in certain ways since higher values minimized the relative experimental errors of the RDCs.

As discussed above, Figure 5 shows that the geometry of the carbon skeleton in both isomers of ludartin (**1** and **2**) is almost superimposable, with an rms fit error of only 0.039 Å. The relative orientation of the internuclear vectors of all CH bonds in both isomers is almost identical except for the CH bond C-3,H-3. According to the theory,[10] parallel or antiparallel CH bonds will show the same RDC values since they will adopt the same relative angle with respect to the magnetic field B_0 regardless of the orientation of the molecule. The pseudoaxial CH bonds for protons H-5, H-6, H-7, and H-9α showed similar RDC values in a range of 81−98 Hz, in good agreement with the theory since they adopt a quasi-parallel structural relationship. On the other side, the CH bond of H-3, which adopts an orientation quasi-perpendicular to the pseudoaxial bonds, showed a significantly different RDC value (7 Hz).

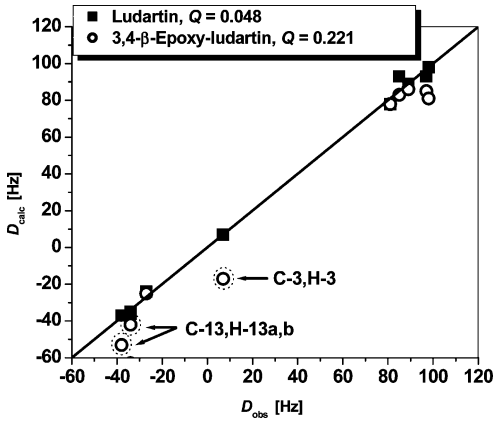

FIGURE 10. Comparison of observed (D_{obs}) versus calculated (D_{calc}) RDCs for the structures of ludartin (**1**) (3,4-α-epoxy configuration) (black squares) and for the structure of 3,4-*β*-epoxy-ludartin (**2**) (white circles).

For rigid structures, the use of RDCs is straightforward as long as more than five linearly independent RDCs are available in order to obtain the best fit with reliable trial structures. In our case, the matrix order analysis was performed using the single value decomposition method (SVD) incorporated in the software MestreSpin (MSpin).[54] The determination of the stereochemistry of the 3,4-epoxide group using RDCs was performed based on the best fit of observed RDCs with trial structures as described by Thiele[10] in her recent review article. The SVD calculations will lead to the best-fitting alignment tensor for each input structure irrespective of the trial structures quality. For each trial structure, RDCs will be estimated from the calculated alignment tensor and compared with the experimental RDCs. To calculate the RDCs, MSpin uses the C−H bond distances from the input structures. This is one of the reasons why it is important to generate structures using a good calculation theory level, such as AM1.

As long as judicious trial structures are used, the best agreement between calculated and experimental RDCs will correspond to the correct structure. In our case, on the basis of other spectroscopic data, we are certain that the correct structure of ludartin is either **1** or **2**.

The experimental RDC values shown in Table 2 were fit to the two structures **1** and **2**. In addition, the fitting was performed permutating the assignment of the diastereotopic protons at C-2. To validate the structure, MSpin generates the quality factor *Q* proposed by Cornilescu et al.,[57] defined as rms ($D_{obs} - D_{calc}$)/rms D_{obs}, where D_{obs} is the measured RDCs and D_{calc} is the calculated RDCs for the given trial structure. The best fitting was observed for the 3,4-α-epoxy isomer (**1**) as determined previously by chemical methods, with a *Q* of 0.048 (see Table 2). The 3,4-*β*-epoxy isomer (**2**) showed a larger *Q* value of 0.221 (Table 2). A comparison of the D_{obs} with the D_{calc} in Figure 10 clearly shows the deviation in the calculated value for the CH bonds of H-3, H-5, H-6, H-13a, and H-13b. In an attempt to fit the experimental RDC values to the wrong structure, in this case the structure of **2**, the SVD calculations will lead to an alignment tensor tilted with respect to the correct one, leading to wrong calculated RDCs not only for the C-3−H-3 bond but

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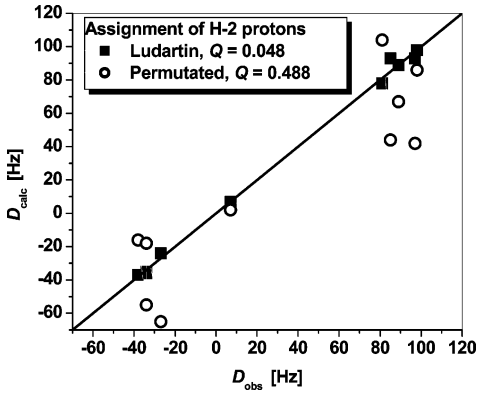

FIGURE 11. Comparison of observed (D_{obs}) versus calculated (D_{calc}) RDCs for the structure of **1** permutating the assignments of the signals corresponding to the diastereotopic protons H-2α and H-2β. A mis-assignment leads to a big dispersion and a poor Q factor (white circles).

also for other bonds in the molecule. It is very impressive how the RDC analysis can discriminate between two structures that are so similar and only slightly differ in the orientation of the C-3−H-3 bond (see Figure 10).

Another interesting application of RDCs in small molecules is the assignment of diastereotopic protons. As discussed above, in the original publication[52] describing the isolation and structure elucidation of ludartin, none of the diastereotopic protons were assigned (H-2, H-8, and H-9). The assignment of protons H-8α, H-8β, H-9α, and H-9β was straightforward from the ^3J coupling constant analysis. However, because of the ambiguity in the NOEs and ^3J coupling constant values shown by the diastereotopic protons at carbon C-2, their assignment was not possible by conventional NMR methods. The values of the RDCs for the CH bonds corresponding to H-2α and H-2β were permutated in the SVD calculations, and the results are shown in Figure 11. Coincidentally again, the assignments previously reported as H-2a and H-2b[52] agree with the assignments found here for H-2α and H-2β, respectively. Note the poor fitting with a $Q = 0.488$ when the assignments of the H-2 protons are permutated (Figure 11).

All the above calculations were performed using data collected from a sample of ludartin that was inside the gel during the whole swelling process (∼20 days). However, if the sample is not stable for such a long period of time, it is recommended to let the sample diffuse into an already swollen and "equilibrated" gel. To demonstrate this, about 5 mg of ludartin (**1**) was dissolved in 100 μL of $CDCl_3$, and this solution was put on top of the gel already swollen in the NMR tube. After approximately 3 days, the signals corresponding to ludartin were observed in the NMR spectrum of the gel. The proton-coupled HSQC experiment showed evidence of alignment, and its profile was almost identical to that obtained for ludartin that was inside the gel during the whole swelling process. Data are shown in the Supporting Information.

Conclusions

Cross-linked PMMA has very good aligning properties for small molecules in chloroform. The alignment is scalable and depends on the cross-link density, similar to other gels. Cross-linked PMMA showed some advantages over other aligning gels compatible with chloroform, such us polystyrene (PS) and poly(dimethylsiloxane) (PDMS). The presence of the aromatic ring as side pendant groups in PS not only creates an unwanted induced upfield chemical shift effect but also requires nearly 400 ms to be eliminated with a T_2 filter. The absence of aromatic rings in PMMA and the introduction of an extra methyl group in the backbone provide a gel with a clean region suitable for the analysis of aromatic molecules which is also a much less flexible gel, compared to cross-linked PS. The background signal of the PMMA-based gel can be eliminated in ∼30 ms with a T_2 filter, and this time is so short that no T_2 filters are necessary in the HSQC experiments. Similar to PS gels, monomer signals are still observed in the PMMA gels, and the swelling time is relatively long (15−20 days). The preparation of PDMS, another gel compatible with chloroform, is complicated: the cross-linking is achieved by irradiation with 10 MeV β-rays generated by an industrial electron accelerator.[42] The PMMA gels described in this work are both easy to prepare and affordable.

The application of RDCs for the structural analysis of small molecules in organic solvents is still in a very young stage of development. Thus far, only small molecules with very well-defined stereochemistry have been used to test the application of this new NMR technique to show its power to (i) determine the relative orientation of CH bonds (or other internuclear vectors) regardless of their position in the molecules, (ii) to assign diastereotopic protons, (iii) differentiate axial from equatorial protons in chair-like compounds, and (iv) discriminate enantiomers in chiral gels or chiral liquid crystal solutions. Much is yet to be done on the application of RDCs to flexible molecules where multiple conformations are present in solution. In the literature, particularly in the field of natural products, a large number of structures are described with unassigned stereochemistry at certain stereocenters due to the limitations imposed by NOE and ^3J coupling constant analysis. Nowadays, NMR instruments are equipped with standard software that makes it easy to perform multidimensional NMR experiments. The MSpin package includes modules for the calculations of ^3J coupling constants, RDCs, and NOEs from 3D structures. Its usage is straightforward, and it can run on multiple platforms. For non-NMR experts, solving the structure using RDCs would be no longer a difficult task.

Experimental Section

Preparation of Cross-Linked PMMA of Gels. The monomers methyl methacrylate (MMA, 99%) and ethylene glycol dimethacrylate (EGDMA, 98%) were purified prior to the experiments by passing the neat liquids through a short column filled with basic alumina in order to remove the polymerization inhibitor. A stock solution containing MMA (50 mL), V-70 (0.015 g), and acetone-d_6 (10 mL) was first prepared. Portions (10 mL) of the stock solution were added to four vials, and various amounts of cross-linker, EGDMA were added to each of them (10, 25, 80, and 150 μL in vials **C**, **D**, **E**, and **F**, respectively). The solutions were transferred to NMR tubes, which were then capped with rubber septa, and the septa were secured with tape. Each tube was evacuated for a short time and back-filled with nitrogen. The cycle was repeated three times, and the NMR tubes were inserted in an oil bath at 50 °C. The polymerizations were carried out for 4 h, and then the tubes were taken out of the heating bath, the septa were removed, and the gels were left to dry slowly at ambient conditions. The slow drying is essential for the preparation of uniform rod-shaped gels. When vacuum drying was attempted, the gels were deformed and some of them cracked. When the gels were dry, they shrank and could be easily removed from the tubes. Some tubes had to be broken to take the gels out. The gels prepared using the above

conditions were named **C**, **D**, **E**, and **F**. In another set of experiments, the same stock solution as described above was prepared first. To 50 mL of the stock solution was added EGDMA (5 μL). Part of this solution was retained for the synthesis of gel **A**. To 40 mL of the solution containing cross-linker was added an additional amount (4 μL) of the cross-linker. This solution was used to prepare gel **B**. The polymerization conditions and the handling of the gels were the same as that for the first batch. The molar percent concentrations of cross-linker in the six gels were 0.0034 (**A**), 0.01 (**B**), 0.068 (**C**), 0.17 (**D**), 0.542 (**E**), and 1.01 (**F**).

Ludartin. Authentic sample of ludartin (**1**) was reisolated from *Stevia yaconensis var. subeglandulosa*[52] by Dr. Viviana E. Nicotra from Córdoba National University, Córdoba, Argentina.

NMR Experiments. NMR experiments in isotropic $CDCl_3$ solutions and in PMMA gels swelled with $CDCl_3$ were collected at 500.13 MHz for ^{1}H, 125.77 MHz for ^{13}C, and 76.73 MHz for ^{2}H in a broad band inverse (BBI) probe with *Z*-only gradients and a 2H-TX board to perform ^{2}H and ^{1}H 3D gradshimming, and ^{2}H NMR experiments. ^{2}H 1D, ^{1}H 1D, ^{1}H 1D CPMG, COSY, NOESY, HSQC, and HMBC experiments were collected using standard NMR software.

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Supporting Information Available: A brief history of RDCs, the original data that include all the F2 slices from the HSQC experiments of ludartin in isotropic and anisotropic media, alignment tensor parameters, and the PDB files of the ludartin structures calculated using AM1. This material is available free of charge via the Internet at http://pubs.acs.org.

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