Probing the Diastereotopicity of Methylene Protons in Strychnine Using Residual Dipolar Couplings
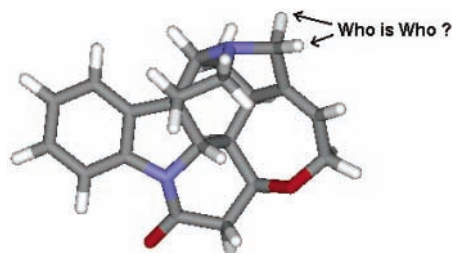
Christina M. Thiele* and Stefan Berger

University of Leipzig, Institute for Analytical Chemistry, Johannisallee 29, 04103 Leipzig, Germany

thiele@chemie.uni-leipzig.de

Received December 20, 2002

ABSTRACT

Residual dipolar couplings were successfully used to distinguish between the two diastereotopic protons on C-20 of strychnine dissolved in an organic liquid crystal (PBLG/CDCl$_3$). The results presented here strongly suggest that this method will be of help in organic structure determination, making the determination of relative stereochemistry in the absence of NOE data possible.

The determination of relative stereochemistry by nuclear magnetic resonance is often complicated by the absence of NOE data and/or ^3J coupling data. To demonstrate the utility of the use of residual dipolar couplings (rdcs) for organic structure determination, we have applied this method to a problem that can also be readily solved by conventional methods, namely, the differentiation between the two diastereotopic protons on C-20 of strychnine (cf. Figure 1). We acknowledge simultaneous but independent work by Verdier et al.[1]

NMR in orienting media is a very well-established field of research.[2] The application to complicated organic molecules was restricted so far only by the degree of alignment induced. Since new alignment media were introduced (e.g., aqueous solutions of phospholipid bicelles,[3] cellulose crystallites,[4] purple membrane fragments,[5] filamentous bacte-
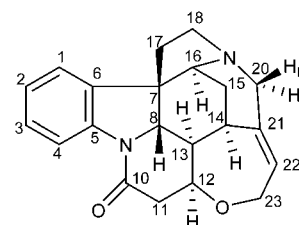

Figure 1. Structure of strychnine and numbering of atoms.

riophages,[6] or dilute lamellar phases), residual coupling information can be extracted more easily.

(1) Verdier, L.; Sakhaii, P.; Zweckstetter, M.; Griesinger, C. *J. Magn. Res.*, submitted.

(2) (a) Saupe, A. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 97−112. (b) Emsley, J. W.; Lindon, J. C. *NMR Spectroscopy Using Liquid Crystal Solvents*; Pergamon: Oxford, 1975.

(3) Tjandra, N.; Bax, A. *Science* **1997**, *278*, 1111−1114.

(4) Fleming, K.; Gray, D.; Prasannan, S.; Matthews, S. *J. Am. Chem. Soc.* **2000**, *122*, 5224−5225.

(5) (a) Koenig, B. W.; Hu, J. S.; Ottiger, M.; Bose, S.; Hendler, R. W.; Bax, A. *J. Am. Chem. Soc.* **1999**, *121*, 1385−1386. (b) Sass, J.; Cordier, F.; Hoffmann, A.; Rogowski, M.; Cousin, A.; Omichinski, J. G.; Lowen, H, Grzesiek, S. *J. Am. Chem. Soc.* **1999**, *121*, 2047−2055.

(6) (a) Hansen, M. R.; Mueller, L.; Pardi, A. *Nat. Struct. Biol.* **1998**, *5*, 1065−1074. (b) Clore, G. M.; Starich, M. R.; Gronenborn, A. M. *J. Am. Chem. Soc.* **1998**, *120*, 10571−10572.

Residual dipolar couplings have recently been used by various groups for the refinement of protein structures,[7] the de novo structure determination of proteins,[8] the conformational analysis of oligosaccharides,[9] and even the observation of protein backbone dynamics.[10]

The observation of residual dipolar couplings is only possible if the magnetic susceptibility anisotropy of the molecule is sufficiently large[11] or if the molecule is incorporated into one of the various alignment media.[12] Even mechanical orientation[13] has been used on biomolecules, but to our knowledge, no application of this weak alignment for the structure determination of a complicated organic molecule embedded in an organic liquid crystal has been reported so far.[14]

If any kind of molecule is introduced into a liquid crystalline phase, an anisotropic environment is created, orienting the molecule partially within the magnetic field. The observation of orientation-dependent nuclear spin interactions such as direct dipole−dipole interaction, chemical shift anisotropy, or quadrupole interaction (for nuclei with spin > 1/2 only) becomes possible. Mostly only the direct dipole−dipole interaction d_{IS} has been used for structural refinements so far, which is defined for two spins I and S in eq 1:[15]

$$d_{IS} = b_{IS}\left\langle \frac{3\cos^2 \Theta_{IS} - 1}{2} \right\rangle \qquad (1)$$

The angle Θ_{IS} is the angle between the IS vector and the field; the symbols < > indicate a time average over all orientations sampled by the molecules in the liquid crystal, and b_{IS} is the direct dipole−dipole coupling constant.[16]

If the degree of alignment is sufficiently small, the contributions from different spins can be distinguished and the linesplitting (in rad/s) in a ^{13}C spectrum of a spin pair $^{13}C-^{1}H$ is given by the eq 2:

$$2\omega_{C-H} = 2d_{C-H} + J_{C-H} \times 2\pi \qquad (2)$$

where J_{C-H} is the scalar coupling constant in hertz. Thus, the linesplitting is either larger than J_{C-H} for a positive residual dipolar coupling D_{C-H}[17] or smaller than J_{C-H} for negative D_{C-H}.

As can be seen from eq 1 and ref 16, the dipolar interaction is proportional to r^{-3}, making this method much more sensitive toward internuclear distances than NOE measurements, which are rather short-ranging due to their proportionality to r^{-6}.

Much more important for the current application is the dependence of the dipolar interaction on the angle Θ_{IS} between the vector of the two spins I and S and the magnetic field. From this time average over internuclear angles, the orientation of the molecular axis within the magnetic field can be calculated. This yields a second rank tensor called the alignment tensor, which can be determined as soon as five linearly independent I−S vectors are known (e.g., five D_{C-H} of different sizes are measured).

With the alignment tensor determined, the orientation of other internuclear vectors can be predicted from a known structure. Thus, it is possible to predict which size of the residual dipolar coupling is expected for each vector. This can be applied, for example, to C−H vectors. It is therefore possible to distinguish between two diastereotopic protons of a methylene group by comparison of the expected and observed size of the residual dipolar coupling.

To be able to apply this methodology to strychnine (depicted in Figure 1), which has become the standard test molecule for NMR developments,[18] a liquid crystal compatible with organic substrates and solvents was of necessity. The degree of alignment induced is of crucial importance so that the observed spin system is still first-order, rendering the interpretation of the NMR spectra possible. For this purpose we employed poly-γ-benzyl-L-glutamate (PBLG),[19] which has been used successfully by the group of Courtieu[20] for the NMR spectroscopic analysis of enantiomeric mixtures.

(7) Recent reviews: (a) de Alba, E.; Tjandra, N. *Prog. Nucl. Magn. Reson. Spectrosc.* **2002**, *40*, 175−197. (b) Prestegard, J. H.; Al-Hashimi, H. M.; Tolman, J. R. *Q. Rev. Biophys.* **2000**, *33*, 371−424.

(8) (a) Hus, J.-C.; Marion, D.; Blackledge, M. *J. Am. Chem. Soc.* **2001**, *123*, 1541−1541. (b) Tian, F.; Valafar, H.; Prestegard J. *J. Am. Chem. Soc.* **2001**, *123*, 11791−11796. (c) Andrec, M.; Peicheng, D.; Levy, R. M. *J. Biomol. NMR* **2001**, *21*, 335−347. (d) Rohl, C. A.; Baker, D. *J. Am. Chem. Soc.* **2002**, *124*, 2723−2729.

(9) (a) Neubauer, H.; Meiler, J.; Peti, W.; Griesinger C. *Helv. Chim. Acta* **2001**, *84*, 243−258. (b) Arzumendi, H. F.; Bush, C. A. *Carbohydr. Res.* **2002**, *337*, 905−915.

(10) (a) Meiler, J.; Pompers, J. J.; Peti, W.; Griesinger C.; Brüschweiler, R. *J. Am. Chem. Soc.* **2001**, *123*, 6098−6107. (b) Tolman J. R. *Curr. Opin. Struct. Biol.* **2001**, *11*, 532−539.

(11) (a) Alemany, L. B.; Gonzalez, A.; Billups, W. E. *J. Org. Chem.* **1997**, *62*, 5771−5779. (b) Tolman, J. R.; Flanagan, J. M.; Kennedy, M. A.; Prestegard, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9279−9283. (c) Tjandra, N.; Grzesiek, S.; Bax, A. *J. Am. Chem. Soc.* **1996**, *118*, 6264−6272.

(12) Prestegard, J. H.; Kishore, A. I. *Curr. Op. Chem. Biol.* **2001**, *5*, 584−590 and references therein.

(13) (a) Tycko, R.; Blanco, F. J.; Ishii, Y. *J. Am. Chem. Soc.* **2000**, *122*, 9340−9341. (b) Sass, H. J.; Musco, G.; Stahl, S. J.; Wingfield, P. T.; Grzesiek, S. *J. Biomol. NMR* **2000**, *18*, 303−309.

(14) Two posters have recently been communicated at the German Chemical Society NMR Group Meeting in Bremen, Germany, 2002: (a) Thiele, C. M.; Berger, S. Measurement of Residual Dipolar Couplings of Organic Substrates in an Organic Liquid Crystal. (b) Verdier, L.; Sakhaii, P.; Griesinger, C. Alignment of Organic Molecules in Nematic Liquid Crystal. A way to Determine Conformation by Detecting Dipole-Dipole Coupling in High Resolution NMR. (c) Note addd in proof: For a very recent communication, see: Mangoni, A.; Esposito, V.; Randazzo, A. *Chem. Commun.* **2003**, 154−155.

(15) Levitt, M. H. *Spin Dynamics: Basics of Nuclear Magnetic Resonance*; Wiley: Chichester, UK, 2001; Chapter 13.4, pp 425−431, and Errata at http://www.soton.ac.uk/~mhl/publications/books/SpinDynamics.

(16) $b_{IS} = -(\mu_0\gamma_I\gamma_S\hbar)/(4\pi \times r_{IS}^3)$ with μ_0 = vacuum permeability; γ_I and γ_S = magnetogyric ratios of the spins I and S, respectively; and r_{IS} = distance of spins I and S.

(17) D_{C-H} (in hertz) = d_{C-H} (in rad/s)/2π.

(18) Braun, S.; Kalinowski, H.-O.; Berger, S. *150 and More Basic NMR Experiments: A Practical Course*, 2nd Expanded ed.; Wiley-VCH: Weinheim, Germany, 1998.

(19) **Sample Preparation.** Poly-γ-benzyl-L-glutamate (145 mg; DP = 562, obtained from Sigma) was weighed in an NMR sample tube, and 50 mg strychnine was added together with 950 mg of CDCl₃. After a clear and uniform solution was obtained by shaking, five freeze and thaw cycles were performed and the sample tube was sealed to ensure constant conditions for the NMR experiments at different field strengths.

(20) For example, see: Aroulanda, C.; Sarfati, M.; Courtieu, J.; Lesot, P. *Enantiomer* **2001**, *6*, 281−287.

We recorded ^{2}H NMR spectra to check the degree of alignment from the size of the quadrupolar splitting of the deuterium signal of CDCl$_3$, which is assumed to follow the same alignment mechanism as strychnine and therefore should be a measure of the degree of alignment. For alignment media that are aligned solely due to their magnetic susceptibility anisotropy, there is a dependence on the square of the static magnetic field B_0;[7b] thus, the quadrupolar interaction (and with it also the dipolar interaction) should increase tremendously at higher field.

We acquired ^{2}H NMR spectra at 61.4, 92.1, and 107.5 MHz[21] and 300 K[22] but could not confirm a square dependence on B_0. We obtained a linear relationship with quadrupolar interactions $\Delta\nu_Q = 423$ Hz at 61.4 MHz, 454 Hz at 92.1 MHz, and 464 Hz at 102.5 MHz. Although this experimental finding is not yet fully understood, it is nevertheless of great importance, as it enables us to determine the sign of D_{C-H}.

As the sign of $^1J_{C-H}$ is known, the sign of D_{C-H} can be seen from the alteration of the linesplitting at different fields. If the linesplitting increases with increasing field, D_{C-H} is positive; if the linesplitting decreases with increasing field, D_{C-H} is negative.

As the observed dipolar and quadrupolar interactions for our sample are rather large,[23] we followed the most straightforward way to measure $^{13}C-^1H$ couplings. We recorded gated-decoupled ^{13}C NMR spectra,[22,24] where a full survey over the complexity of the spin system is provided and non-first-order cases can be seen immediately (cf. the triplet at $\delta = 27$ ppm, which changes into a doublet-type pattern in the aligned case in Figure 2).

From these spectra at different magnetic field strengths, the linesplitting ls can be extracted and the residual dipolar coupling can be calculated with the following formulas:

$$D_{C-H} = \frac{\text{ls} - J_{C-H}}{2} \text{ for } D_{C-H} > 0 \qquad (3)$$

$$D_{C-H} = -\frac{\text{ls} + J_{C-H}}{2} \text{ for } D_{C-H} < 0 \qquad (4)$$

As can be seen in Figure 2, not all spin systems are first order; it is therefore not safe in all cases to extract the dipolar interactions from the linesplittings. In ambiguous cases, a careful simulation of the spin system was performed[25] and the dipolar interaction was used only if good agreement between observed and simulated values was achieved. Ten D_{C-H} were considered to be safe (eight of those representing C−H vectors and one CH$_2$ group, the C−H vectors of which are to be assigned correctly).

Figure 2. Expansion of the aliphatic region of gated decoupled ^{13}C NMR spectra of strychnine at 300 K.[19,22] First (from top to bottom): strychnine in PBLG/CDCl$_3$ at 100.6 MHz; second at 150.9 MHz; and third at 176.1 MHz. For comparison, the spectrum of an isotropic solution of strychnine in CDCl$_3$ at 176.1 MHz is depicted at the bottom.

These rdcs were fed into the program package PALES,[26] which uses the mathematical concept of singular value decomposition (SVD)[27] to determine the alignment tensor from a given structure. As an input structure, the crystal structure of strychnine[28] was used after it had been minimized with the software package SPARTAN,[29] because of the uncertainty of hydrogen coordinates taken from X-ray data. After the alignment tensor was calculated from the experimental rdcs D_{C-H}, the expected rdcs D_{calc} are back calculated for this alignment tensor within PALES. The values of D_{calc} and D_{C-H} are compared in Table 1.

A good agreement between observed and calculated values ($\blacklozenge$, $R^2 = 0.99$) was obtained; hence, the structure matches the size of the observed residual dipolar couplings and the assignment of the two diastereotopic protons on C-20 was right.

(21) ^{2}H NMR spectra were acquired on Bruker instruments: DRX-400, DRX-600 (both equipped with a 5 mm BBO probe), and Avance 700 (equipped with a 5 mm TBI probe) without a frequency lock, which was not necessary due to the good stability of our instruments.

(22) To ensure the same temperatures on all spectrometers, the chemical shift difference of the two signals in the ^{1}H spectrum of ethylene glycol in DMSO-d_6 was monitored after at least 20 min and the temperature of the control unit adjusted so as to give the same chemical shift difference on every spectrometer; cf.: van Geet, A. L. *Anal. Chem.* **1968**, *40*, 2227−29.

(23) For biochemical applications, the quadrupolar splitting $\Delta\nu_Q$ is usually less than 20 Hz: Brunner, E. *Concept Magn. Reson.* **2001**, *13*, 238−259.
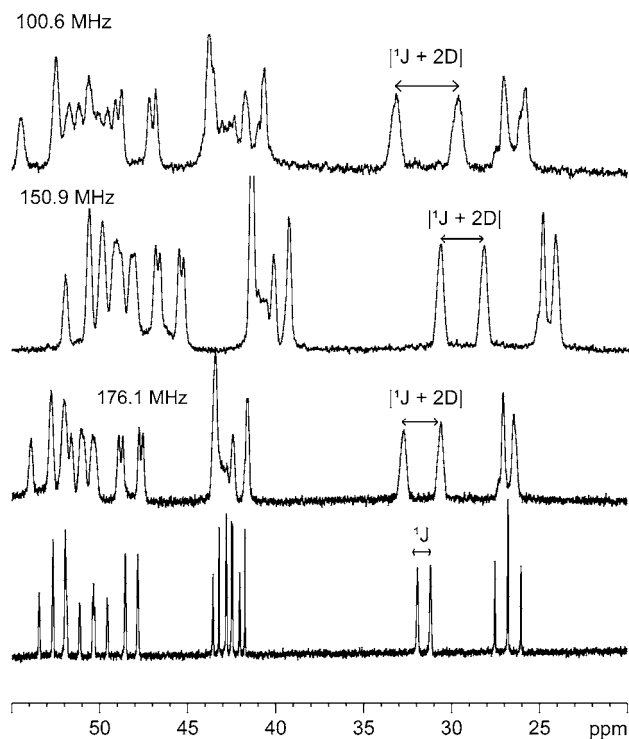
(24) Gated decoupled ^{13}C NMR spectra were acquired on Bruker instruments: DRX-400, DRX-600 (both equipped with a 5 mm BBO probe), and Avance 700 (equipped with a 5 mm TBI probe) at 300 K.

(25) For spin simulation, the program SPINWORKS 1.3 was used: Marat, K. *Spin Works version 1.3*; University of Manitoba, Winnipeg, MB, Canada, 2002. http://www.umanitoba.ca/chemistry/nmr/nmrsource2.html.

(26) Zweckstetter, M.; Bax, A. *J. Am. Chem. Soc.* **2000**, *122*, 3791−3792.

(27) Losonczi, J. A.; Andrec, M.; Fischer, M. W. F.; Prestegard, J. H. *J. Magn. Reson.* **1999**, *138*, 334−342.

(28) Provided as pdb file with protons already attached by the Indiana University Molecular Structure Center at www.iusmc.indiana.edu.

Table 1. Residual Dipolar Couplings D_{C-H} Extracted from a 176.1 MHz Gated Decoupled ^{13}C NMR Spectrum of Strychnine in PBLG/CDCl$_3$[19] Compared with the Values Back Calculated by PALES (D_{calc})

residue	D_{C-H} (Hz)	D_{calc} (Hz)
C-1	−6.8	−13.4
C-4	−15.6	−4.4
C-12	123.0	116.2
C-13	−42.3	−37.0
C-8	−75.5	−77.1
C-22	4.3	−12.1
C-14	123.8	133.0
C-16	−209.6	−210.0
C-20-H$_a$	98.7	95.7
C-20-H$_b$	−34.7	−30.2

To check for consistency, the rdcs of the two protons on C-20 were interchanged and the calculation was performed again. This time the agreement between calculated rdcs D_{calc} and observed rdcs D_{C-H} was poor, as can be seen from Figure 3 ($\square$, $R^2 = 0.88$). The two exchanged rdcs clearly deviate
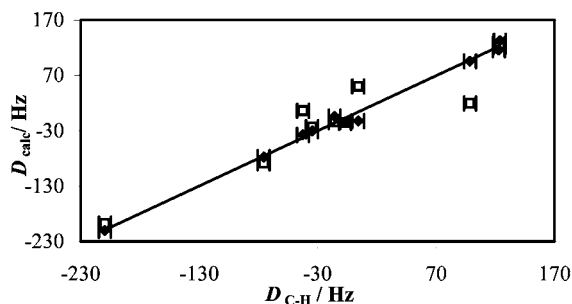

Figure 3. Comparison of the correlations of observed rdcs D_{C-H} and back-calculated ones D_{calc}. The diamond ($\blacklozenge$) represents the values of D_{calc} vs D_{C-H} for right assignment of the two diastereotopic protons on C-20; the square ($\square$) depicts the values of D_{calc} vs D_{C-H} for interchanged rdcs of C-20-H$_a$ and C-20-H$_b$. Due to the large linebroadening, an error of 5 Hz on the rdcs (10 Hz on the linesplitting) was assumed, which is shown as an error bar.

from the straight line (which is depicted only for the case of right assignment, $\blacklozenge$). Two other rdcs (belonging to C-13 and C-22) also deviate to a rather large extent from the line, indicating that the number of rdcs acquired is at the lower limit for solving this issue.

The good agreement in the case of right assignment and the poor agreement when the two diastereotopic protons are interchanged clearly shows that it is possible to use residual dipolar couplings to distinguish between diastereotopic protons.

The example of the use of rdcs presented here illustrates impressively the capacities of this approach for the determination of the diastereotopicity of methylene protons of a water-insoluble organic compound. An application to more flexible molecules[30] will be possible if five or more different rdcs (e.g., $^2D_{C-H}$, $^3D_{C-H}$) can be acquired for each flexible part of the molecule in order to be able to determine an alignment tensor for every flexible part.

The above results strongly suggest that it should also be possible to determine the relative stereochemistry of non-hydrogen-bearing stereocenters if ^{13}C−^{13}C residual dipolar couplings are obtained. This would revolutionize organic structure determination as conventional NMR techniques usually fail due to the lack of NOE data. For this purpose, the INADEQUATE pulse sequence[31] can certainly be used.[32] This method will be applicable to the structure determination of natural products as well, as it is possible to extract the molecule from the liquid crystal after performing the NMR experiments.[33]

We will now concentrate on improving the alignment properties of this liquid crystal toward a smaller degree of orientation so that the observed rdcs come into a range where it is not necessary to acquire spectra at different magnetic fields in order to determine the sign of the rdc.

Much work also has to be done on measurement techniques so that the spin system is fully understood and more and different kinds of rdcs (for example ^{13}C−^{13}C, ^{1}H−^{1}H, or long-range couplings) can be acquired, making this method generally applicable for organic structure determination and more flexible to different tasks.

Supporting Information Available: One table and one figure showing the linear dependence of the $\Delta\nu_Q$ and D_{C-H} from the magnetic field strength B_0, one table containing the observed rdc D_{C-H} and the back-calculated ones D_{calc} for the two cases of right and wrong assignment of the protons on C-20, two tables containing alignment tensor characteristics and Euler angles, and one figure depicting the alignment tensor. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0275163

(29) Minimizing was performed as follows: all dihedrals of the carbon framework were kept constant, and interatomic distances and bond angles to protons were allowed to be altered during a molecular mechanics calculation (force field: MM94).

(30) Strychnine is rather rigid, and therefore only one alignment tensor has to be determined for this molecule.

(31) Bax, A.; Freeman, R.; Frenkiel, T. A. *J. Am. Chem. Soc.* **1981**, *103*, 3, 2102−2104.

(32) It was demonstrated that the measurement of an INADEQUATE experiment is possible in PBLG: Sarfati, M.; Lesot, P.; Merlet, D.; Courtieu, J. *Chem. Commun.* **2000**, 2069−2081.

(33) Parenty, A.; Campagne, J.-M.; Aroulanda, C.; Lesot, P. *Org. Lett.* **2002**, *4*, 1663−1666.