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Simultaneous Assignment of All Diastereotopic Protons in Strychnine Using RDCs: PELG as Alignment Medium for Organic Molecules

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The concept of using residual dipolar couplings (RDCs) for the structure determination of organic molecules is applied to the simultaneous assignment of all diastereotopic protons in strychnine. To use this important NMR parameter the molecule has to be aligned in the magnetic field. Here we present a new alignment medium for organic substrates. The optimization of the alignment properties of mixtures of poly-γ-ethyl-L-glutamate (PELG) and $CDCl_3$ are described and the alignment properties of PELG at different concentrations are evaluated. A comparison of PELG with poly-γ-benzyl-L-glutamate (PBLG) shows considerable differences in the magnitude of alignment for strychnine in the two alignment media. PELG induces a lower degree of order and makes the measurement of residual dipolar couplings (RDCs) in strychnine possible. All one-bond C−H RDCs of strychnine in PELG were determined by using 2D heteronuclear single quantum coherence (HSQC) spectroscopy. The strategy for the extraction of RDCs for methylene groups is described in detail. The RDCs and order parameters are used to assign pairs of diastereotopic protons. This methodology can distinguish not only one pair of diastereotopic protons but it can be used to assign all pairs of diastereotopic protons simultaneously. Two different calculation approaches to achieve this task are described in detail.

Introduction

The determination of the relative stereochemistry of chiral compounds is one of the major tasks in structure elucidation by nuclear magnetic resonance (NMR) spectroscopy. For most organic molecules this is accomplished by a combined use of angular information provided by ^3J-coupling data and distance information from NOE spectra.[1] However, sometimes these interactions do not lead to unambiguous assignments due to either remoteness of the stereocenters, the absence of NOE, similar coupling constants, or almost identical distances within the molecule.

This problem can be circumvented by the use of another NMR parameter that contains both distance and angular information, namely the dipolar coupling. Although it has been known since the 1960s[2] that the dipolar coupling can yield a unique wealth of structural data, its use was limited to very few examples due to the problems associated with the necessary alignment of the molecule within the magnetic field. Only very strong alignment or alignment due to magnetic anisotropy[3] was possible before the advent of media that induce partial alignment.[4] With these new alignment media the observed dipolar couplings could be scaled down from many kilohertz (as was the case when nematic liquid crystals were used to induce strong alignment) to a few hertz, the result now being known as residual dipolar coupling (RDC). The information can be retrieved from the spectra much more easily; this explains the huge impact that this important NMR parameter has had on the determination of the spatial structure of proteins and oligosaccharides in recent years.[5]

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Applications to complicated organic molecules are few and far between. This is due to the fact that alignment media inducing a low degree of order were known only for water,[4,6,7] a solvent in which most organic molecules are insoluble. The excellent work on enantiomeric discrimination done by Courtieu's group[8,9] pointed us toward the use of poly-γ-benzyl-L-glutamate (PBLG) for weak alignment. We were able,[10] at the same time as two other groups,[11,12] to show that it is possible to align organic molecules in PBLG.

One of the interactions induced by weak alignment is the direct dipole−dipole interaction D_{IS}, which is defined for two isolated spins I and S as follows:[13]

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

The angle Θ_{IS} is the angle between the IS vector and the field, the symbols $\langle\ \rangle$ 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 given by

$$b_{IS} = -\frac{\mu_O}{4\pi}\frac{\gamma_I\gamma_S\hbar}{r_{IS}^{\ 3}} \qquad (2)$$

where μ_0 is the vacuum permeability, γ_I and γ_S are the magnetogyric ratios of the two spins I and S, and r_{IS} is the distance between the two spins I and S.

As can be seen from eqs 1 and 2, the residual dipolar couplings contain both distance and angular information. The strength of the dipolar interaction depends among other things on the angle Θ_{IS} between the internuclear vector of the two spins I and S and the magnetic field, but no information concerning the azimuthal angle is obtained. After measuring several RDCs the orientation of the molecular axis within the magnetic field can be calculated from the time average over internuclear angles. 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. Having determined the alignment tensor the orientation of other internuclear vectors can be predicted from a known structure. Thus it is possible to predict the size of the residual dipolar coupling expected for each vector. This can, for example, be applied to distinguish orientations of C−H vectors within a molecule.

The proposed method can be used for various tasks in structure elucidation. The assignment of diastereotopic protons[10,11] poses a challenge to this method equivalent to the determination of the relative configuration of stereocenters.[11,12] This approach will certainly revolutionize structure determination by NMR in the next years.

The crucial factor in its applicability, however, is the alignment medium. The search for suitable alignment media for organic substrates is in full swing at the moment.[14,15]

If the degree of alignment induced in the solute molecule is small enough, the contributions to the dipolar interaction from different spins can be distinguished and the line splitting J_{eff} (effective coupling constant) of a spin pair $^{13}C-^{1}H$ is given by the following equation:

$$J_{eff} = 2D_{C-H} + J_{C-H} \qquad (3)$$

where J_{C-H} is the scalar coupling constant in Hz. Thus the effective coupling constant J_{eff} is either slightly larger than J_{C-H} for a positive residual dipolar coupling D_{C-H} or smaller than J_{C-H} for a negative D_{C-H}.

This is of course only true if the observed residual dipolar couplings are smaller in size than the scalar coupling. When we used PBLG to orient strychnine, this was not the case.[10] We were forced to use ^{13}C-gated decoupled spectra to obtain the coupling information and also observed higher order effects in our spin systems. This was due to a too high degree of alignment for strychnine. A further dilution of PBLG to scale down the alignment was not possible, so we could use only 10 out of 22 C−H vectors in strychnine. To make this method generally applicable and the extraction of RDCs comfortable, an alignment medium has to be found that induces a lower degree of order in strychnine. This is accomplished by the use of PELG.

In this comprehensive report the alignment properties of PELG and PBLG[10] (and the recently developed polystyrene sticks,[14] see the Supporting Information) for strychnine as the solute molecule are compared. All three alignment media show considerable differences in the magnitude of alignment. PELG is therefore proposed as a new alignment medium for organic substrates which

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TABLE 1. Alignment Properties of PELG (85 mg)/$CDCl_3$ at Different Concentrations of Solvent for Strychnine (53 mg) as Solute

$m(CDCl_3)$/mg	$\Delta\nu_Q(CDCl_3)$/Hz[a]	D_a/Hz[b]	D_a^{NH}/Hz[b]	R[c]	α[d]	β[d]	γ[d]	n(RDC)[e]
817	693	1.19×10^{-3}	24.38	0.41	51.3	43.3	282.9	18
942	627	1.09×10^{-3}	22.50	0.37	53.3	40.6	278.7	18
1006	576	9.75×10^{-4}	21.10	0.40	54.3	40.1	278.9	18
1223	535	8.57×10^{-4}	19.69	0.32	54.9	40.1	279.0	20
1385	433	7.43×10^{-4}	16.20	0.38	60.2	41.0	278.5	18
1593	385	5.58×10^{-4}	13.53	0.45	69.9	40.2	273.9	22
1707	334	4.91×10^{-4}	11.59	0.42	74.3	41.1	274.4	18
1819	299	4.74×10^{-4}	10.33	0.53	77.5	40.1	275.1	22
1932	isotropic							

[a] $\Delta\nu_Q$: quadrupolar splitting of the solvent signal in the liquid crystal. [b] D_a: axial component of the alignment tensor. D_a^{NH}: axial component of the alignment tensor normalized to N−H vectors. [c] R: rhombicity. [d] α, β, and γ: the three Euler angles define the orientation of the solute molecule within the liquid crystal. Only one of the four possible orientations is reported. [e] n(RDC): number of RDCs (from gated decoupled ^{13}C NMR spectra, 400-MHz instrument, 300 K) used for the calculation of the parameters D_a, R, α, β, and γ with PALES.[19]

induces a low degree of order. A detailed description of alignment properties and their concentration dependence is given. A comparison of methods for obtaining RDCs from different kinds of HSQC spectra and ^{13}C gated decoupled spectra is presented. The concept of using RDCs in the structure determination of organic molecules is described in great detail for the assignment of diastereotopic protons. A short outline of the use of this methodology for the determination of relative configuration will be given.

Results and Discussion

Polyglutamates form liquid crystals within a certain concentration range. The structure in helicogenic cosolvents is as follows: the main chain of the synthetic polypeptide forms a rigid α-helical conformation, while the substituted glutamate side chains point away from the main helix. When brought into a magnetic field the supramolecular system behaves like a nematic liquid crystal. It can then be considered as parallel rods (formed by the α-helices of the main chain, pointing in the direction of the magnetic field) with glutamate side chains (as benzyl and ethyl ester, respectively) pointing out into the interstices between the rods.[16] The solute molecules are oriented in these interstices. A decrease in the degree of alignment for the solute molecule can either be achieved by dilution of the liquid crystalline phase increasing the temperature or by altering the side chain. Thus when using the ethyl ester (PELG) and not the benzyl ester (PBLG) the degree of alignment for strychnine is much reduced.

Optimization of PELG for the Alignment of Strychnine. The poly-γ-ethyl-L-glutamate/$CDCl_3$/strychnine mixture is prepared as described in the Experimental Section (see also ref 17). The degree of alignment is estimated by measuring the quadrupolar interaction $\Delta\nu_Q$ of the solvent signal in the ^2H NMR spectrum, and by measuring the RDCs of strychnine in the gated decoupled ^{13}C NMR spectrum. Solvent is added until the degree of alignment is suitable, as characterized by a quadrupolar interaction for $CDCl_3$ of 299 Hz and RDCs of less than 50 Hz for strychnine. Table 1 shows the results obtained by this procedure. The table also contains columns with some significant alignment properties: the degree of alignment can be judged from the axial component D_a of the alignment tensor. D_a is usually reported together with the rhombicity R of the alignment tensor[18] and the three Euler angles α, β, γ or the main axes of the tensor and the corresponding eigenvectors. To make comparisons with biomolecular systems possible D_a^{NH}(D_a normalized to NH vectors) is also reported in Table 1.

From the values of $\Delta\nu_Q$ and D_a with increasing amount of $CDCl_3$ it can clearly be seen that the degree of alignment decreases with dilution (see Table 1). When trying to further dilute the liquid crystal the solution becomes isotropic. We elected to use a concentration slightly higher than the lower concentration limit for the final measurements, as the phase is not stable over prolonged periods of time at the lower concentration boundary.

Another conclusion that can be drawn from the values in Table 1 is that not only the degree of alignment is concentration dependent but also its orientation (changes of the Euler angle α). This can be explained if the homopolypeptide is not considered to be a rod with a smooth surface, but if the fine structure of the homopolypeptide is also taken into account.

Comparison of PELG with PBLG.[10] For the comparison of PBLG to PELG two samples were chosen which showed a similar quadrupolar interaction on our 400-MHz instrument at 300 K.

A direct comparison of all alignment properties at the same magnetic field strength is not possible, as only five RDCs are known for the PBLG sample at 400 MHz.[20] It is nevertheless possible to draw the following conclusion: although the quadrupolar splitting of the solvent signal is similar at the same magnetic field strength and temperature, the degree of alignment induced in strychnine is much smaller in PELG.[21] The difference in the

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(18) $R = D_a/D_r$ (D_r: rhombic component of alignment tensor).

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(20) Although the alignment of strychnine in PBLG is dependent on magnetic field strength, the value of D_a is of the same order of magnitude ($D_a = -2.39 \times 10^{-3}$ at 400 MHz vs -2.44×10^{-3} at 700 MHz for PBLG, see ref 10).

(21) A different degree of polymerization was used for the two liquid crystals (PBLG: DP 562; PELG: DP 1707). It cannot be ruled out that the different orientational behavior is also due to different degrees of polymerization.

TABLE 2. Comparison of the Alignment Properties of Strychnine in Mixtures of PBLG and PELG with $CDCl_3$

	PBLG(700 MHz)[10]	PELG(400 MHz)
$\Delta\nu_Q$(61.4 MHz)/Hz[a]	423	433
$\Delta\nu_Q$(102.5 MHz)/ Hz[a]	464	n.d.[b]
D_a/Hz[c]	-2.44×10^{-3}	7.43×10^{-4}
D_a^{NH}/Hz[c]	−52.7	16.2
R[d]	0.43	0.61
n(RDC)[f]	10	18

[a] $\Delta\nu_Q$: quadrupolar splitting of the solvent signal in the liquid crystal (61.4 MHz: 400-MHz instrument; 102.5 MHz: 700 MHz instrument). [b] n.d. = not determined. [c] D_a: axial component of alignment tensor. D_a^{NH}: axial component of the alignment tensor normalized to N−H vectors. [d] R: rhombicity. [e] n(RDC): number of RDCs (from gated decoupled ^{13}C NMR spectra, 300 K) used for the calculation of the parameters D_a, D_a^{NH} and R with PALES.

orientation of alignment between PBLG and PELG is currently under investigation in our laboratory.

Thus it is not justified for small organic molecules to assess the degree of order induced in the solute from the size of the quadrupolar interaction of the solvent molecule. Specific interactions of the solute or solvent with the alignment medium cannot be ruled out.

A comparison of PELG with polystyrene sticks[14] shows considerable differences of the orientation for these two alignment media (see the Supporting Information), so that three different alignment media for organic substrates are available to date.

Measurement of Residual Dipolar Couplings: Comparison of Methods. RDCs are extracted from the difference of a coupling measurement (line splitting) in two samples, one isotropic and one anisotropic. So in principle any method that can be used to measure one-bond heteronuclear coupling constants can be used to extract one-bond C−H RDCs.

For the measurement of one-bond RDCs in biological macromolecules and oligosaccharides the splitting is usually observed in the indirect dimension (*F1* coupled HSQCs), but also HSQCs without decoupling during acquisition (*F2* coupled HSQCs) were used in protein NMR in cases of little or no spectral overlap (see ref 22, for example). In the few reports that have appeared considering the measurement of one-bond residual dipolar couplings in small organic molecules, different approaches were used. The measurement of one-bond C−H RDCS was achieved either from directly detected coupled ^{13}C NMR spectra[10,12] or by 1D or 2D heteronuclear multiple[7] (or single[6,11,14]) quantum coherence spectroscopy (HMQC/HSQC) without decoupling during acquisition (*F2* coupled HMQC/HSQC).

The success of indirectly detected spectra crucially depends on the knowledge of the coupling constant. In the case of the anisotropic sample, an optimization of the INEPT delay (τ in Figure 1) according to the effective coupling constant $J_{eff} = J + 2D$ must be carried out. If the spread of effective coupling constants is too large, as was the case for our PBLG sample,[10] the intensities of the cross-peaks are reduced to an extent depending on the deviation of the effective coupling constant from the coupling constant set in the experiment. In cases such

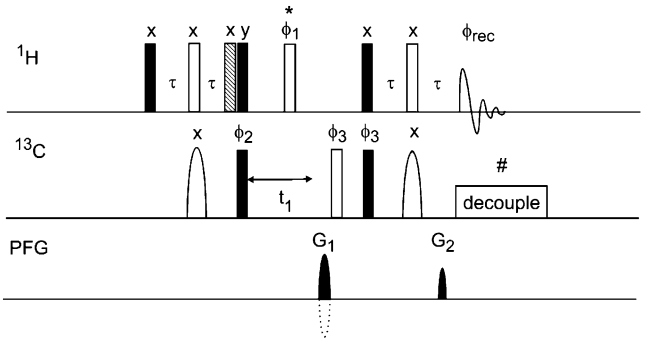
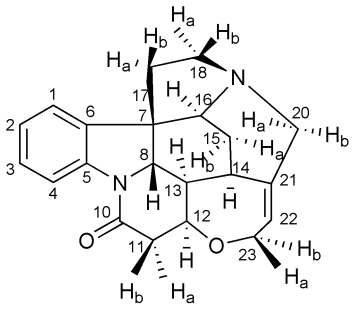


FIGURE 1. Basic HSQC sequence[24] with gradient selection. Black bars denote 90° pulses, open bars denote 180° pulses, the shaded bar is a trim pulse of 1 ms duration. The 180° pulses on carbon within the INEPT and reverse INEPT step are replaced by adiabatic pulses[25] (see Experimental Section). For *F2* coupled HSQCs the decoupler is off: the part of the pulse sequence marked with # is removed (pulse marked with an asterisk is conserved). For *F1* coupled HSQC the 180° proton pulse in the center of the evolution time (t_1) marked with an asterisk is removed; decoupling marked with # is conserved. τ: $^1/_4J$, $\phi_1 = (x)_2, (-x)_2$, $\phi_2 = x, -x$, $\phi_3 = (x)_4, (-x)_4$, $\phi_{rec} = x, -x, x, -x, -x, x, -x, x$. The experiment is recorded in an Echo/Antiecho manner: for each time t_1 increment, two FIDs are aquired, one with G_1 inverted. Field gradients are sine-bell shaped with durations of 0.5 ms and a ratio of amplitudes G_1:G_2 = 80:20.1.

CHART 1. Structure of Strychnine



as these, directly detected coupled ^{13}C spectra (or heteronuclear *J* resolved spectra) provide an alternative.

For the measurement of C−H RDCs of methine (CH) fragments the overall performance of the various methods tested (namely gated decoupled ^{13}C NMR spectra, *F1* and *F2* coupled HSQCs, see Table 3) is roughly comparable, although the amount of measurement time needed differs significantly. Strychnine (see Chart 1), however, contains several methylene groups (CH_2) with diastereotopic protons. The extraction of coupling constants for diastereotopic protons is not always straightforward.

The problems of ill-defined line shapes encountered in *F2* coupled HSQCs (vide infra) also have been described for coupled HMQCs.[7] The *F2* coupled HSQC used here is a standard pulse sequence,[23] in which the heteronuclear decoupling was off during acquisition to give the effective coupling in the direct dimension (see Figure 1, decoupler off).

The measurement of coupling constants is performed in *F2*; therefore, a high FID resolution in *F2* (16k data

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TABLE 3. Determination of Scalar (J) and Effective ($J_{Eff} = J + 2D$) Coupling Constants in Strychnine (All Measurements Were Performed at 300 K at a Field Strength Corresponding to a Proton Frequency of 700 MHz)

				isotropic sample[a] J/Hz				anisotropic sample[b] J_{eff}/Hz				
carbon	δ_C/ ppm[c]	proton	δ_H/ ppm[c]	^{13}C-GD[d]	*F2* coupled[e]	*F1* coupled[f]	J_{av}/ Hz[g]	^{13}C-GD[d]	*F2* coupled[e]	*F1* coupled[f]	$J_{eff,av}$/ Hz[g]	D/ Hz
C3	128.7	H3	7.26	159.6	159.85	159.6	159.6	134.0	131.20	134.1	134.0	−12.8
C22	127.9	H22	5.93	157.7	159.43	159.6	159.0	165.4	163.40	165.1	165.0	3.0
C2	124.3	H2	7.10	160.9	161.56	161.9	161.6	128.2	131.20	137.6	131.0	−15.3
C1	122.3	H1	7.17	159.0	159.00	159.5	159.0	68.0	71.38	72.2	72.0	−43.5
C4	116.3	H4	8.09	168.0	168.40	169.0	168.0	80.1	81.64	82.5	81.0	−43.5
C12	77.6	H12	4.29	149.4	149.17	150.1	149.2	n.d.	216.70	212.8	214.0	32.4
C23	64.6	H23a	4.16	144.3[h]	145.74	145.4	145.3	157.7[h]	n.d.[i]	158.4	158.0	6.3
		H23b	4.07	137.2[h]	137.20	137.1	137.2	218.6[h]	n.d.[i]	212.8	212.0	37.4
C16	60.3	H16	3.99	146.2	146.60	148.9	146.6	116.7	n.d.[i]	118.2	117.0	−14.8
C8	60.0	H8	3.87	145.5	144.89	147.8	144.9	193.6	n.d.[i]	196.2	194.0	24.6
C20	52.7	H20a	3.74	141.0[h]	138.91	138.5	139.3	176.3[h]	176.50	177.3	177.0	18.9
		H20b	2.77	141.0[h]	138.91	138.5	139.3	159.6[h]	159.43	159.6	159.6	10.2
C18	50.4	H18a	3.25	144.9[h]	146.17	146.6	146.2	125.7[h]	117.54	135.9	128.0	−9.1
		H18b	2.89	135.9[h]	131.20	131.2	131.2	89.8[h]	83.77	91.0	90.0	−20.6
C13	46.2	H13	1.28	124.4	124.38	126.5	125.7	151.3	144.89	150.1	150.0	12.2
C17	42.7	H17a	1.91	133.4[8]	132.93	133.6	132.9	82.7[h]	∼73	85.1	82.0	−25.5
		H17b	1.91	133.4[h]	132.93	133.6	132.9	160.3[h]	∼159	160.8	160.0	13.5
C11	42.5	H1a	3.13	135.9[h]	135.06	134.8	135.5	80.1[h]	n.d.[i]	85.1	84.0	−25.8
		H11b	2.67	126.3[h]	126.08	126.5	126.3	198.1[h]	195.76	192.7	194.0	33.9
C14	31.6	H14	3.16	130.1	130.36	132.4	132.0	194.9	196.18	197.4	195.0	31.5
C15	26.7	H15a	2.37	131.4[h]	130.36	133.6	131.2	116.7[h]	n.d.[i]	114.7	115.0	−8.1
		H15b	1.48	131.4[h]	129.93	126.5	130.3	125.7[h]	n.d.[i]	126.3	126.0	−2.2

[a] 3% solution of strychnine in $CDCl_3$, an INEPT delay corresponding to 145 Hz was used for HSQC. [b] 55 mg of PELG, 50 mg of strychnine, 850 mg of $CDCl_3$ with $\Delta\nu_Q = 356$ Hz, an INEPT delay corresponding to 170 Hz was used for HSQC. [c] Chemical shifts of the isotropic sample. The chemical shift anisotropies for carbon are of the size 0 to 0.5 Hz, for protons less than 0.1 Hz. [d] Gated decoupled ^{13}C NMR spectra. FID resolution: 0.64 Hz. After zero filling: digital resolution 0.32 Hz. [e] *F2* coupled HSQC with 16k data points in *F2* (SW = 10 ppm), giving a FID resolution of 0.42 Hz. After zero filling: digital resolution 0.21 Hz. An error of 1 Hz was assumed for the anisotropic sample. [f] *F1* coupled HSQC with 2k data points in *F1* (SW = 55 ppm), giving a FID resolution of 4.7 Hz. After zero filling and linear predicition (forward, 30 coefficients): digital reolution 1.2 Hz. An error of 2 Hz was assumed for the anisotropic sample. [g] The average of the coupling constant obtained by all methods was used to give one coupling constant in most cases. If one value was judged to be determined less reliably than others (due to broad lines, for example) it was removed from the calculation of the average; thus a weighting was performed. An error of 2 Hz (leading to 1 Hz on the RDC) was assumed. Calculations performed with data from *F1* coupled HSQCs only show essentially the same results (data not shown). [h] The coupling constants belonging to CH_2 groups cannot a priori be assigned to the corresponding protons. [i] Not determined (because of ill-defined line shapes, vide ante).

points) is chosen. The digital resolution of 0.43 Hz is certainly sufficient for the size of RDCs observed in this sample. An error of 1 Hz on J_{eff} (0.5 Hz on the RDC) is assumed as a conservative estimate.

A reliable extraction of coupling constants from *F2* coupled HSQCs is not possible for some of the methylene groups in this sample due to phase distortions. This effect is caused by large deviations of the effective coupling constant from the experimentally set coupling constant in anisotropic samples (see Figure 2b). Another very important complication is the observation of homonuclear scalar and dipolar couplings in the same dimension as the heteronuclear coupling. The additional complication by homonuclear dipolar couplings can become very strong in CH_2 groups. Some of the inconvienences caused by ill-defined line shapes can be seen in the insert in Figure 2b for the cross-peaks of H-11a and H-17a+b. The phase distortions observed lead to large errors in the measured coupling, thus an extraction of couplings is not possible for these protons.

The problem of ill-defined line shapes can be circumvented by using *F1* coupled HSQCs. To obtain heteronuclear couplings in the indirect dimension the 180° proton pulse in the center of the evolution period is removed (see Figure 1, pulse marked with an asterisk removed). Thus the homonuclear interactions remain in the direct dimension while the heteronuclear couplings can be extracted from the splitting in the indirect dimension.

The frequency difference in the indirect dimension is measured to give the scalar coupling (isotropic sample) or effective coupling (anisotropic sample) for methine fragments as can be seen from Figure 3. For the determination of coupling constants in CH_2 groups a number of pulse sequences have been developed, e.g. the SPITZE-HSQC.[26] It is, however, also possible to extract the one-bond C−H coupling constants from the HSQC recorded with the simplest possible pulse sequence. If the difference in effective coupling constants for the two protons exceeds 2× the natural line width, the cross-peak consists of the active and passive coupling of the two protons giving a doublet of doublet pattern (as in coupled ^{13}C spectra). Active and passive coupling can be distinguished from the phase behavior of the signal.[27] An extraction of

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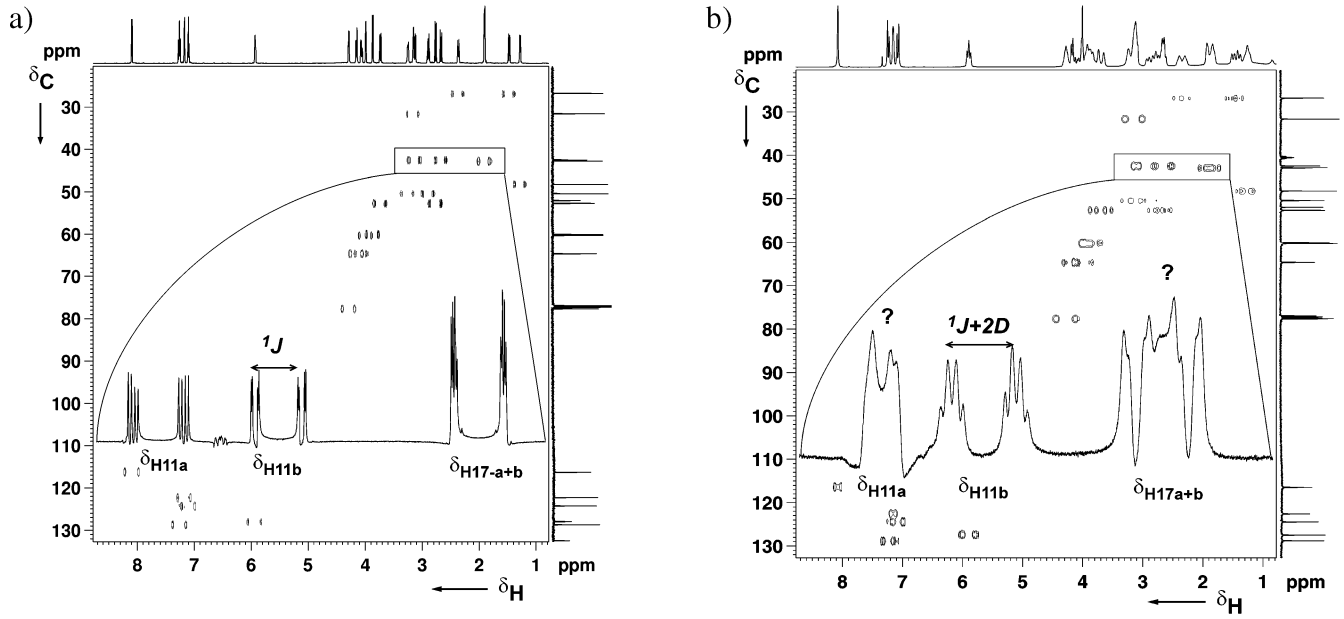

FIGURE 2. *F2* coupled HSQC of strychnine: (a) isotropic solution at 300 K and 700 MHz and (b) aligned in PELG/$CDCl_3$ at 300 K and 700 MHz. The experiment was performed with 16k × 256 data points and a spectral width of 10 × 170 ppm. An INEPT delay corresponding to 145 Hz for the isotropic and 170 Hz for the aligned sample was used, respectively. The inserts show a row taken from the 2D data set at a carbon chemical shift of 42.5 ppm.

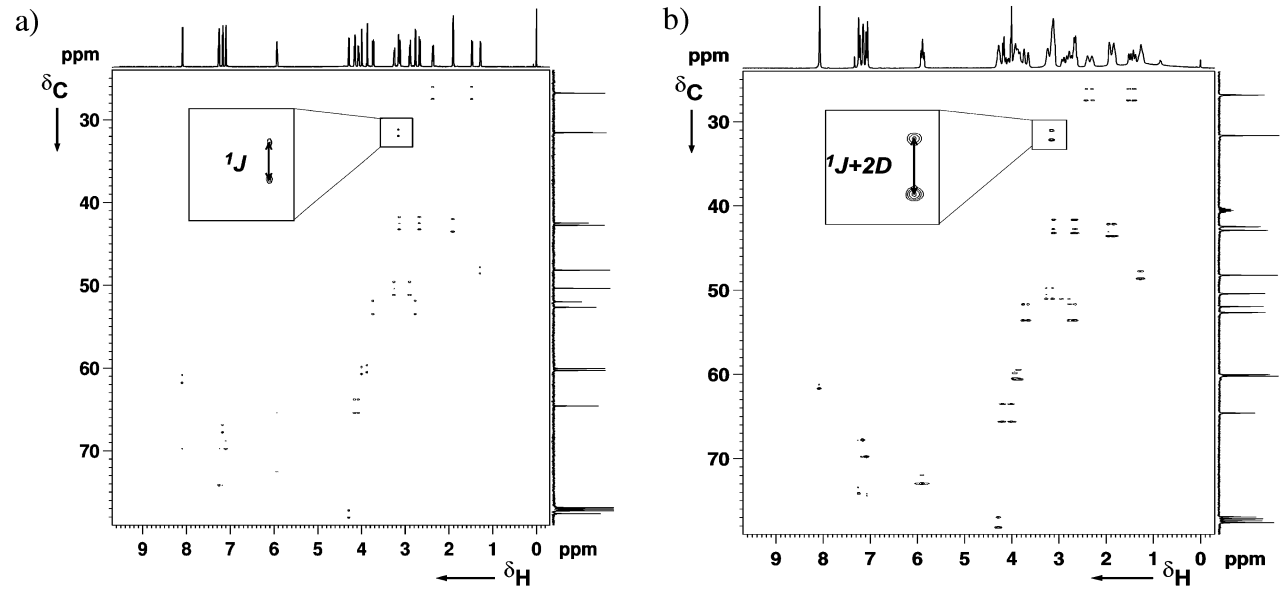

FIGURE 3. *F1* coupled HSQC of strychnine: (a) isotropic solution at 300 K and 700 MHz and (b) aligned in PELG/$CDCl_3$ at 300 K and 700 MHz. The experiment was performed with 1398 × 2k data points and spectral widths of 10 × 55 ppm. The signals of the aromatic region are folded in. INEPT delays corresponding to 145 Hz for the isotropic and 170 Hz for the aligned sample were used.

the coupling constants can be achieved as described in Figure 4 for the two cross-peaks of the C15−H_2 fragment. If the difference in effective coupling constants is smaller than 2× the natural line width the center peaks overlap or cancel. For overlapping center peaks the couplings cannot be determined as serious errors are to be expected due to the overlap of antiphase components. In the anisotropic sample all CH_2 groups show considerably different effective coupling constants, so that all CH RDCs can be determined reliably. For four out of six CH_2 groups of strychnine the scalar couplings (isotropic sample) are markedly different, so an extraction is done as described in Figure 4. For the other two CH_2 groups the scalar couplings are essentially of the same size (less than 0.4 Hz apart, see *F2* coupled HSQC, Table 3), so the difference of the two outer signals is divided by two to get the scalar coupling constant. The digital resolution

(27) This phase behavior can be verified by a simulation of the spectrum (only three CH_2 groups with corresponding chemical shifts and coupling constants entered) under otherwise identical conditions using the Bruker software NMR-Sim for MS-Windows NT, Version 3.1, Bruker Analytik GmbH, 2001.

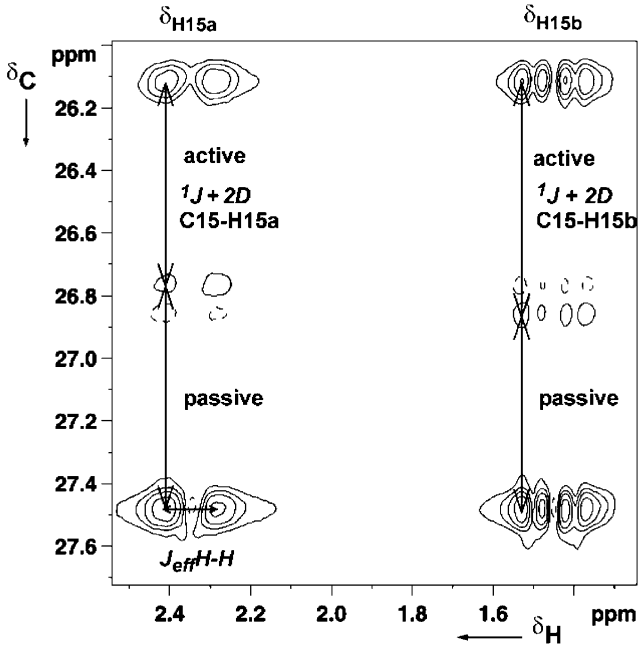

FIGURE 4. Region of *F1* coupled HSQC of strychnine aligned in PELG/$CDCl_3$ at 300 K and 700 MHz showing the cross-peaks for C15−H1 and C15−H2. The experiment was performed with 1398 × 2k data points and spectral widths of 10 × 55 ppm. An INEPT delay corresponding to 170 Hz was used. Full lines refer to positive signals, broken lines to negative signals. The active coupling constant for each proton−carbon pair can be extracted from the in-phase (anti-phase) doublet at lower (higher) ^{13}C frequency if the components are well-separated.

in the indirect dimension was increased to 1.2 Hz by zero filling and linear prediction. An error of 2 Hz for the effective coupling constant in *F1* coupled HSQCs of the anisotropic sample was assumed as a conservative estimate.

The only real complication is the much increased measurement time to get a reasonably high resolution in the indirect dimension (2048 increments for a spectral width of 55 ppm). Only very minor off-resonance effects for the signals of the aromatic region (folded back) were observed with the 400-MHz instrument. With the 700-MHz instrument, however, a separate recording of the aromatic region under otherwise identical conditions (FID resolution, etc.) was necessary due to off-resonance effects leading to incorrect coupling data. Other minor complications are the different relaxation of the two lines of the doublet (in the case of C−H vectors)[28] and the observation of C−H long-range couplings together with one-bond C−H RDCs. Pulse sequences to remove C−H long-range couplings from the indirect dimension of frequency-based HSQCs have been published, for example, see ref 29.

In Table 3 a comparison of measured scalar and effective coupling constants for the various methods (gated decoupled ^{13}C, *F2* coupled HSQC, *F1* coupled HSQC) at 300 K on a 700-MHz instrument can be found. The experiments were also performed on a 400-MHz instrument (see the Supporting Information) under otherwise identical conditions. For both kinds of HSQC experiments the INEPT delay was set to 145 Hz for isotropic samples and to 170 Hz for anistropic samples. A variety of different values for this delay were tested for the aligned sample in 1D-HSQCs. This value gave the best signal-to-noise ratio for all signals (data not shown).

It can be seen from the table that an extraction of all RDCs without ambiguous assignment is possible from *F1* coupled HSQCs only. The error of 2 Hz on the effective coupling constant in anisotropic samples is not a severe problem as RDCs are in the region of 50 Hz, but when RDCs of 10 Hz or less are to be measured a different approach has to be used.

Assignment of Diastereotopic Protons: Order Matrix Calculation. The approach of using RDCs for the determination of relative configuration or diastereotopicity of methylene protons in organic molecules has some basic prerequisites. A presumption of the structure is needed, which can be compared with experimental RDCs. In this case the crystal structure of strychnine was used (for details see the Experimental Section). To get information as to whether the assumed structure is correct, an order matrix calculation is necessary (exceptions: see refs 6 and 7). As soon as five linearly independent RDCs have been determined, the five elements of the symmetric and traceless 3 × 3 order matrix can in principle be determined. To get reliable results more than ten RDCs are usually used. The most popular methods for calculating an alignment tensor from a known structure and experimental RDCs are least-squares minimization or singular value decomposition (SVD).[30] In the program package PALES,[19] which is used in this report, both methods are available. When comparing the results from both methods (data not shown), no significant difference in output or calculation time was seen for this small organic molecule, so that all calculations were preformed with SVD. As soon as the order matrix is determined, the values of RDCs expected for other C−H vectors within the molecule can be predicted. Each C−H vector in the molecule stands at a certain angle to the magnetic field axis demanding a definite size of the RDC. PALES automatically calculates the size of RDCs expected for all C−H vectors within the molecule from the alignment tensor. So two sets of RDCs are compared, one of experimental RDCs and one calculated from the alignment tensor, which in turn is calculated from the experimental RDCs and the presumed structure. Only if a good agreement between RDCs and structure is obtained do we obtain a good fit between experimental and calculated RDCs.

It must be stated at this point that one size of RDC does not in turn belong to only one angle toward the magnetic field. Furthermore, no information concerning the azimuthal angle is obtained. So this method can fail in cases where the orientation of the vectors that need to be distinguished share angles toward the magnetic field that give rise to the same size of the RDC.

(28) Goldman, M. Interference effects in the relaxation of a pair of unlike spin-1/2 nuclei. *J. Magn. Reson.* **1984**, *60*, 437−452.

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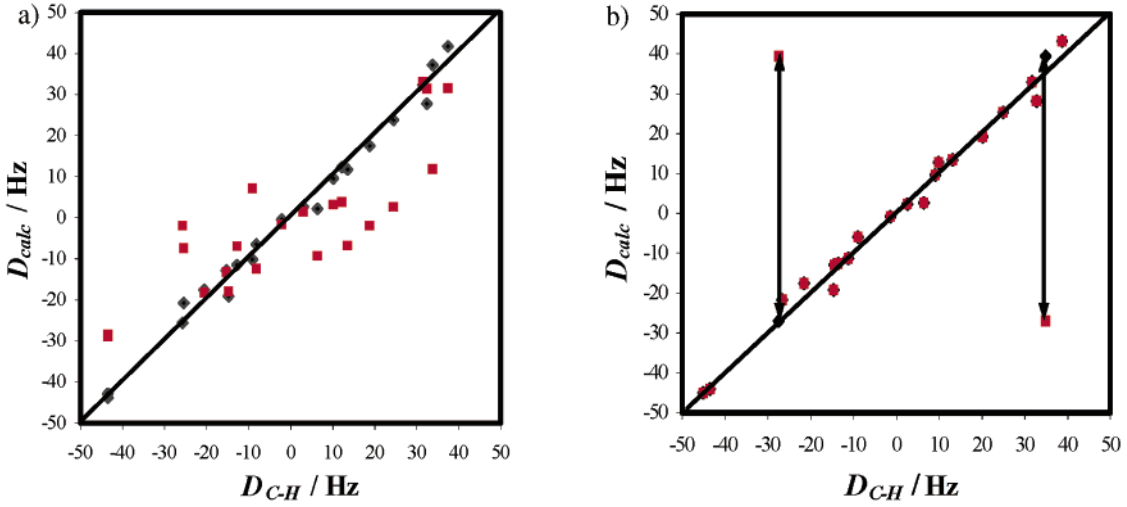

FIGURE 5. Comparison of the correlations of observed RDCs D_{C-H} and calculated RDCs D_{calc}. The diamond (♦) represents the values of D_{calc} vs D_{C-H} for correct assignment of the diastereotopic protons at C11; the square (■) depicts the values of D_{calc} vs D_{C-H} for interchanged RDCs at C11. In Figure 5a the values of the corresponding C−H bond vectors were included in the order matrix calculation. In Figure 5b the values of the protons in question were excluded from the calculation. The values were then predicted from the alignment tensor. An error of 1 Hz on the RDCs was assumed.

Another possible limitation to this method can occur with molecules that contain protons, which point into only a limited range of angles (cf. refs 6 and 7). Thus this method will work best for molecules with protons pointing in a wide range of angles with little internal dynamics.

With the values of RDCs obtained for strychnine we perform some tasks which can be encountered in structure elucidation. For the first task, the assignment of two diastereotopic protons with all other protons assigned correctly, there are two possibilities for performing the calculation:

The two protons are included in the group of RDCs used for the order matrix determination, then the calculation is performed twice: once with a presumably correct assignment and once with a presumably wrong assignment. The one that has a better fit of measured RDCs to calculated ones provides the correct solution. This is shown here for strychnine with the two diastereotopic protons of C11. In Figure 5a a comparison of experimental RDCs with calculated ones for the two possible assignments is shown. The good fit with correct assignment shows that the measured RDCs fit the assumed structure. As a parameter for the quality of the fit, the root-mean-square deviation (RMSD) of calculated and measured RDCs is chosen. An RMSD of 2.54 is obtained for the correct assignment. For the case of the wrong assignment a very poor fit is obtained (RMSD = 15.868).

This assignment procedure of diastereotopic protons is not limited to those of C11, but works for all CH_2 groups with markedly different observed RDCs for the two protons. In this context it is interesting that the coupling constants of the two protons of C17, which are isochronous, can be distinguished and assigned unambiguously. For the CH_2 groups C18, C15, and C20 the fit is only slightly better for the correct assignment (vide infra, calculation nos. 20−22); thus no safe assignment can be made.

The other possibility of performing the calculation for the assignment of one pair of diastereotopic protons is to exclude the two protons in question from the calculation of the order matrix. The size of the RDCs of these protons can then be predicted from the alignment tensor. This is again done for the two protons of C11. Which proton is which can be decided from the predicted RDC by comparison with the experimental value. The result is shown in Figure 5b, where the two protons with correct (♦) or false (■) assignment are indicated with arrows. A table containing the corresponding values of measured and calculated RDCs for both methods can be found in the Supporting Information. This approach allows even the assignment of the protons on C18, C15, and C20, which showed small differences in RMSDs for the case of right and wrong assignment (vide ante).

To be on the safe side, C−H long-range couplings should, nevertheless, be included in a real case study if these protons are to be distinguished. A measurement in a different alignment medium would also be very useful in such cases. The protons on C20, C15, and C18 show considerably different RDCs in PS[14] and can therefore be safely assigned by using PS as the alignment medium.

The approach using RDCs for the assignment of diastereotopic protons is completely different from the classical one. The assignment of all but two protons was assumed to be known, whereas only one pair of protons was not assigned. The assignment of the diastereotopic protons of C11 is conventionally achieved by NOE spectroscopy, where contacts of proton H11a to H12 and H13 are observed, whereas H11b shows a contact with H-8 (see Chart 1 for numbering). For other protons, the pairs on C15 and C20, for example, the assignment is usually conducted in pairs. Only H15a shows a contact to H20a, but no cross-peaks are observed for H15a/H20b or H15b/H20a, so that the assignment is unambiguous.

To show the usefulness of using RDCs for structure determination of organic compounds and to point out the difference to the classical approach a more demanding

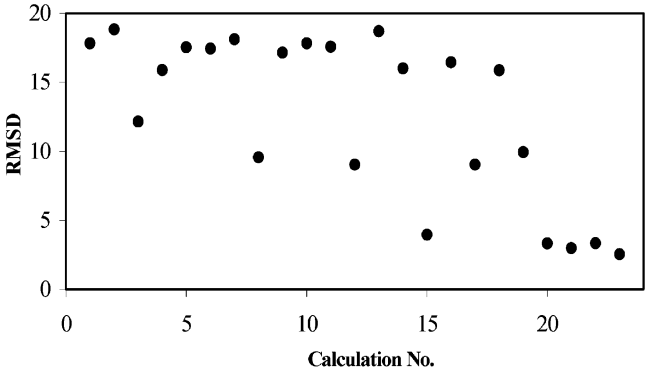

FIGURE 6. Assignment of all diastereotopic protons simultaneously. Plot of RMSD vs calculation number (details found in the Supporting Information).

task was performed. An attempt was made to find out whether it is possible to assign all protons on CH_2 groups simultaneously without any NOE data. The two approaches used to perform the calculation were similar to those used above:

In the first approach the alignment tensor was calculated with all RDCs included. A variety of deliberately incorrect assignments was chosen and the RMSD was used to monitor the outcome. If all protons were assigned incorrectly, this resulted in a RMSD of 17.834 indicating a very bad fit (calculation no. 1, Figure 6). A total of 23 calculations were carried out (for details see the Supporting Information): many cases (nos. 1−19, except 15, see Figure 6) show a considerably higher RMSD. These possibilities of assignments of diastereotopic protons can be ruled out. Only four calculations (nos. 15 and 20−22) lead to values for the RMSD close to that observed for correct assignment. No. 15 (RMSD = 3.96) is the calculation in which all protons on C15 and C20 were interchanged. Nos. 20−22 are the calculations where only one pair of diastereotopic protons is interchanged on C18 (RMSD = 3.318), C15 (RMSD = 2.986), and C20 (RMSD = 3.338). Thus in a real case study a definite assignment would not be possible for the protons on C15, C18, and C20 from comparing RMSDs. The possible solutions to this problem have already been pointed out when discussing the assignment of only one pair of diastereotopic protons.

In the other approach to performing the same task of simultaneous assignment of all protons in CH_2 groups, all protons in CH_2 groups were excluded from the calculation and the alignment tensor was calculated from the ten C−H vectors of C−H fragments only. From this alignment tensor (slightly different from the one obtained for 22 RDCs), the RDCs of all CH_2 fragments were predicted. As can be seen from Table 4, a reliable prediction is possible for all CH_2 groups. There are some deviations in predicted (D_{pred}) to experimental (D_{obs}) values (up to 7 Hz, due to the small number of RDCs used to obtain the alignment tensor), but the size and sign of predicted RDC are in all cases significant. Thus all diastereotopic protons can be assigned unambiguously. This is even possible for the protons on C15, C18, and C20.

It is possible to assign all pairs of diastereotopic protons showing considerably different RDCs simultaneously without recourse to NOE data. For CH_2 groups with a small difference in RDCs of the two protons a mere look at the RMSD is certainly not sufficient. It is, however, possible to assign these two protons if a calculation of the alignment tensor is performed without using their coupling data; from this alignment tensor the values of RDCs can be predicted for the protons in question. From a look at the size and sign an assignment can be assessed.

TABLE 4. Prediction of RDCs for Protons in Methylene Groups from the Alignment Tensor Obtained from the Ten Methine Fragments Only

proton	D_{pred}/Hz	D_{obs}/Hz
H23a	1.8	6.3
H23b	43.4	37.4
H20a	15.0	18.8
H20b	10.2	10.15
H18a	−6.4	−9.1
H18b	−16.6	−20.6
H17a	−18.6	−25.4
H17b	9.5	13.5
H11a	−21.6	−25.8
H11b	37.4	33.9
H15a	−9.6	−8.1
H15b	−0.4	−2.2

Future Prospects. Strychnine is a rather rigid molecule and therefore only one alignment tensor had to be determined: for more flexible molecules it might be necessary to determine alignment tensors for every flexible part of the molecule. This could perhaps be simplified if different alignment media are used. Another incentive for the development of alignment media would be the investigation of dynamic processes in molecules. For this at least five linearly independent alignment media would be needed. Whether it is possible to use this approach to investigate the dynamics of small organic molecules, as has been done for biomolecules,[31] remains to be seen.

The determination of diastereotopicity, however, is a comparable challenge to this method, as is the determination of relative stereochemistry on chiral centers. The future of this method clearly lies in the structure determination of natural products. We intend after this "proof-of-principle" to apply this methodology to molecules where conventional NMR methods are insufficient to determine the relative configuration. To achieve this goal, the measurement of different RDCs (e.g., long-range heteronuclear RDCs, homonuclear RDCs) will be necessary.

Conclusion

A new alignment medium for organic molecules is presented. It consists of poly-γ-ethyl-L-glutamate and deuteriochloroform and induces a low degree of order for strychnine. The degree of order it induces in strychnine

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is dependent on concentration, temperature (data not shown), and magnetic field strength. A comparison of the alignment properties for strychnine aligned in PELG, PBLG, and PS shows different alignment properties for the solute molecule in the three alignment media for organic substrates known to date.

For the measurement of residual dipolar couplings in this sample, *F1* coupled HSQCs were the method of choice. An error of 1 Hz on the RDCs was assumed because of the low digital resolution chosen. This is nevertheless accurate enough for the structure determination tasks performed with these data.

The diastereotopicity of single CH_2 fragments was determined by using two different calculation approaches. Either all data were included in the calculation and the RMSD was used to assess an assignment or the RDCs of the protons in question were excluded from the calculation and calculated from the alignment tensor that was determined by using the RDCs of the remaining C−H vectors. This was not only possible for one single CH_2 group, but could also be used to assign all pairs of diastereotopic protons simultaneously without the use of NOE data.

Experimental Section

Materials and Sample Preparation. Poly-γ-ethyl-L-glutamate (PELG), strychnine, DMSO-d_6, and $CDCl_3$ were commercially available. A degree of polymerization (DP) of 1707 for PELG was used as obtained from Sigma. The liquid crystal samples are highly viscous; the preparation was therefore performed directly within the NMR tube. For the alignment optimization procedure 85 mg of PELG were cut into small pieces and put into an NMR tube. Strychnine (53 mg) was added, followed by 817 mg of $CDCl_3$. After 2 days a clear solution was obtained. To provide a lock substance a capillary containing DMSO-d_6 was added. The alignment properties were checked by recording ^{2}H NMR spectra and gated decoupled ^{13}C NMR spectra. The sample was shaken until sharp lines were observed in the ^{2}H NMR spectrum. The amounts of solvent as described in Table 1 were added until the degree of alignment induced in strychnine was satisfactory. Further addition of solvent resulted in an isotropic solution. Approximately 350 mg of $CDCl_3$ evaporated during this process.

The sample for the HSQC measurements was prepared as above with 55 mg of PELG, 50 mg of strychnine, and 850 mg of $CDCl_3$. A capillary containing DMSO-d_6 was added and the sample tube was flame-sealed after 5 freeze and thaw cycles to prevent solvent evaporation.

NMR Experiments. All NMR experiments were performed either on a 700-MHz spectrometer with a triple-resonance inverse probe equipped with *z*-axis gradients or on a 400-MHz spectrometer with a triple-resonance inverse probe equipped with *z*-axis gradients (inverse experiments) or broadband observe probe with *z*-axis gradients (^{13}C gated decoupled spectra). All measurements were performed at 300 K. To ensure the same temperature on both spectrometers, the chemical shift difference of ethylene glycol[32] in DMSO-d_6 was adjusted on both spectrometers to the value corresponding to 300 K. The scalar and effective coupling were measured by various methods as line splittings in an isotropic solution of strychnine in $CDCl_3$ and the PELG/strychnine/$CDCl_3$ sample described above. The RDC is calculated from the difference of scalar and effective coupling by dividing by two. The experiments were performed in the same manner for both samples. For the aligned sample approximately double the number of transients was required, because of lower concentration of strychnine in the anisotropic sample and broader lines due to dipolar interactions.

Gated decoupled carbon spectra were recorded over the whole carbon chemical spectral width to give an FID resolution of 0.64 Hz on the 700-MHz instrument. INEPT delays corresponding to 145 Hz for the isotropic and 170 Hz for the anisotropic sample were used in all inverse experiments. The 180° pulses on carbon within the INEPT and reverse INEPT step are replaced by chirp pulses of 500 μs duration with a 60 kHz spectral width and 20% smoothing. *F2* coupled HSQCs were recorded by using the pulse sequence depicted in Figure 1 without decoupling during acquisition, using the Echo/Antiecho selection scheme. A total of 16k data points were sampled in the direct dimension over a spectral width of 10 ppm to give a FID resolution of 0.43 Hz on the 700-MHz instrument (8k data points corresponding to 0.68 Hz on the 400-MHz instrument). The resolution in the indirect dimension was reduced to a minimum: 256 data points were sufficient to prevent overlap of signals. The spectra were processed with use of an exponential window function with 0.2 Hz line broadening and zero-filling by a factor of 2 prior to Fourier transform in the direct dimension. In the indirect dimension a π/2 shifted sine squared function was applied. The overall experiment time for the *F2* coupled HSQC of the aligned sample was 5.5 hours. *F1* coupled HSQCs were recorded by using the pulse sequence depicted in Figure 1, using the Echo/Antiecho selection scheme. The 180° proton pulse in the center of the acquisition time (marked with an asterisk in Figure 1) was removed to observe couplings in *F1*. A total of 1398 data points (1118 data points) were sampled in the direct dimension over a spectral width of 10 ppm (14 ppm) to give a FID resolution of 5 Hz on the 700-MHz (400 MHz) instrument. A total of 2k data points were acquired in the indirect dimension over a spectral width of 55 ppm (70 ppm) to give an FID resolution of 4.7 Hz (3.4 Hz). A separate spectrum of the aromatic region was obtained on the 700-MHz intrument at the same FID resolution. This was not necessary on the 400-MHz instrument. The spectra were processed with use of an exponential window function with 5 Hz line broadening and zero-filling to 2k data points prior to Fourier transform in the direct dimension. In the indirect dimension zero-filling to 4k data points and forward linear prediction with 30 coefficents gave a digital resolution of 1.2 Hz/pt (1.7 Hz/pt). A π/2 shifted sine squared function was applied. An error of 2 Hz for the line splitting was assumed for the anisotropic sample. The overall experiment time for the *F1* coupled HSQC for the aligned sample was 24 h.

Calculations. For the order matrix calculation an input pdb-file is necessary. The crystal structure of strychnine with protons already attached was downloaded from the Indiana University Molecular Structure Center.[33] Due to the uncertainty of hydrogen coordinates taken from X-ray data and the large differences of bond lengths in the downloaded file a molecular mechanics simulation within SPARTAN[34] (force field: MMFF94[35]) was performed. For this the dihedral angles within the carbon framework were fixed, the bond lengths and angles to protons were allowed to be altered to get reasonable C−H bond lengths and dihedral angles for protons.

For the order matrix calculations, the calculations of RDCs from this order matrix, and the prediction of RDCs from an order matrix the program package PALES[19] was used. For the calculation of the alignment tensor from a known structure the SVD[30] module was used; using the Polar Fit module, which

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(33) www.iusmc.indiana.edu

(34) *SPARTAN* Version 5.1; Wavefunction, Inc.: 18401 Von Karman Avenue, Suite 370, Irvine, CA 92612.

(35) Halgren, T. A. Merck molecular force field. I. Basis, form, scope, parametrization, and performance of MMFF94. *J. Comput. Chem.* **1996**, *17*, 490−519.

performs a Levenberg−Marquardt nonlinear least squares minimization,[36] did not show different results. The RMSD obtained by 500× repetition of the SVD method or adding structural noise to the structure[37] was essentially the same. The structural noise addition method, however, could only be used when no (or only one) deliberately wrong assignment was used, due to increased calculation time. Therefore the SVD module with (usually) 500 to 1000 iterations was used. The RMSDs of two calculations (one with correct and one with wrong assignments) are compared to assess the correct assignments.

For the prediction of RDCs (as in Table 4) the corresponding experimental RDCs were excluded from the calculation, the order matrix calculation was performed as described above, and the order matrix obtained in this way was used in the Fix Saupe Module to obtain a prediction of RDCs for the excluded fragments from the calculation. The predicted RDCs obtained in this way are compared by eye with the experimentally determined ones to assess a correct assignment.

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Supporting Information Available: One table of coupling data used to obtain the alignment properties in Table 1, one table of coupling data obtained on the 400-MHz instrument, one table showing the magnetic field dependence of PELG, one table for the calculation when interchanging the protons on C11 (as in Figure 5), one table with the calculations used to obtain Figure 6, and one table in which the alignment properties of PELG,[10] and PS-sticks[14] are compared. This material is available free of charge via the Internet at http://pubs.acs.org.

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