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Exploring the Analytical Potential of NMR Spectroscopy in Chiral Anisotropic Media for the Study of the Natural Abundance Deuterium Distribution in Organic Molecules

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The deuterium/hydrogen $(D/H)_i$ ratio measurement by quantitative ^2H NMR spectroscopy is a method of choice for the analysis of kinetic isotopic effects associated with enzyme-catalyzed reactions during a biosynthetic pathway. However, the efficiency of the current isotropic ^2H-{^1H} NMR can be limited by the rather small chemical shift dispersion of deuterium nuclei. In addition, this method does not allow the enantiotopic deuterons in prochiral molecules to be spectrally discriminated, hence precluding the quantification of isotopic fractionation on methylene prostereogenic sites. In this work, we explore another analytical strategy able to circumvent these disadvantages. This approach is based on the use of natural abundance ^2H 2D NMR experiments on solutes embedded in polypeptidic, chiral liquid crystalline solvent. Thus, we show that NMR in these oriented phases is a powerful way to separate deuterium signals on the basis of the quadrupolar interactions, providing a promising alternative to overcrowded ^2H NMR spectra obtained in liquid state. To illustrate our purpose, we have experimentally investigated the case of 1,1′-bis(phenylthio)hexane derived by cleavage from methyl linoleate of safflower. The ^2H NMR results in chiral liquid crystals are presented and discussed. We show, for the first time, that $(D/H)_{pro-R}$ and $(D/H)_{pro-S}$ can be measured at the same methylene position of a fatty acid chain.

The study of natural deuterium distribution in organic molecules by quantitative ^2H NMR spectroscopy is a method of choice for the analysis of kinetic isotopic effects (KIEs) associated with enzyme-catalyzed reactions during a biosynthetic pathway.[1−3] Applied to fatty acids, for instance, the variation in the $(D/H)_i$ ratio at the site i along the chain can provide useful information related to the biosynthesis.[3−9] The basic principle of the method consists of determining the deuterium/hydrogen $(D/H)_i$ ratio from the analysis of natural abundance deuterium (NAD) signals in the presence of an internal reference (TMU or pyridine) whose $(D/H)_i$ ratio is known.[4,5,10,11] Due to the very low probability of observing two interacting deuterons in the same isotopomer at natural abundance level, the $^2H-^2H$ scalar couplings are not detected and isotropic NAD spectra are only dominated by deuterium chemical shift interaction. If NAD spectra are a priori very simple to analyze, there being one resonance for each nonequivalent deuterium atom or group of atoms, such a situation is not always favorable when the number of deuterated sites in the investigated molecules is high, their chemical shifts are close, or both. Indeed for such molecules, the low chemical shift dispersion of deuterium nuclei (the magnetogyric ratio is 6.515 times smaller than that of proton) can produce strong signal overlaps, thus obscuring considerably their NAD 1D spectra. The use of modern NMR spectrometers operating at very high magnetic field could partly overcome such a major disadvantage, but this solution is not economical and does not ensure successful results when fortuitous isochronous resonances arise. Moreover, whatever the ^2H frequency used, the measurement of the $(D/H)_i$ ratio for each hydrogen (deuterium) nucleus bonded on prostereogenic carbon atoms of prochiral molecules is impossible using the isotropic NMR spectrum. Indeed, in achiral liquid solvents, enantiotopic deuterons are equivalent and cannot be spectroscopically discriminated. From an analytical point of view, the lack of

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data on $(D/H)_i$ ratios for several deuterated sites by the isotropic 1D NMR approach can lead to loss of valuable information. The use of chiral auxiliaries or chiral solvents could provide a solution to this problem, but to date, this approach has given rather poor results.[12] Consequently, the development of new analytical tools in the field of the quantification of isotopic fractionation merits some attention.

A potential solution to circumvent the analytical limitations mentioned above could be to record the NAD spectrum of the molecule under investigation when embedded in a weakly ordering system.[13−15] Indeed, NMR in achiral or chiral liquid crystals (noted hereafter ALC or CLC, respectively) is theoretically more informative than NMR in isotropic solvents, and the latest developments of this approach in the structural analysis of proteins, as well as natural or synthesized products, are particularly impressive.[16,17] The reason for this is that NMR spectroscopy in oriented systems gives access to order-sensitive NMR interactions such as dipolar couplings, quadrupolar splittings, and chemical shift anisotropies that are no longer averaged to zero as in an isotropic state.[14,18] Thus, for spin $I = 1$ nuclei such as deuterium, oriented spectra are dominated by quadrupolar interactions that can provide a simple way to separate the ^{2}H signals of two nonequivalent deuterons having fortuitous identical chemical shifts, for instance. Indeed, two isochronous deuterons do not necessarily exhibit identical quadrupolar splittings. Furthermore, the use of a chiral anisotropic medium is justified by the possibility of differentiating the NMR signals of two enantiomers or enantiotopic elements in a prochiral molecule.[19−21] In the first case, the difference in the enantioselective interactions between the polypeptide helices and the *S* and *R* isomers generates a sufficient molecular differential ordering effect to spectrally discriminate between them.[20] In the second case, the change in the symmetry of the intermolecular potential experienced by a prochiral compound when the environment is chiral (compared with an achiral medium) can yield separated NMR resonances in the spectra.[21] This methodology has proven to be an efficient technique in the field of enantiomeric and enantiotopic analysis; it has been applied successfully to a large variety of prochiral and chiral materials, including chiral hydrocarbons or molecules that are chiral by virtue of isotopic substitution.[15,22,23]

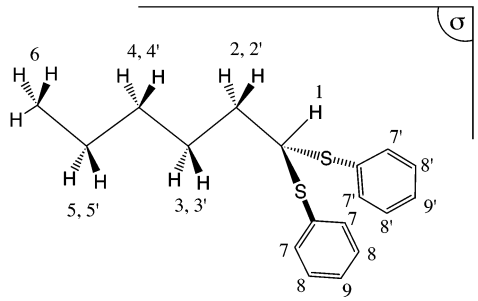

Figure 1. Chemical structure of 1,1′-bis(phenylthio)hexane (BPTH) showing the numbering system of the atoms used. No stereochemistry is defined here.

In this work, we explore the analytical potential of NAD NMR spectroscopy using weakly orienting CLC, made of organic solutions of polypeptide such as poly-γ-benzyl-L-glutamate (PBLG), dedicated to the study of $(D/H)_i$ ratios. We will focus our attention on the natural deuterium distribution of 1,1′-bis(phenylthio)hexane (BPTH) (see Figure 1). This molecule is obtained after chemical modification of methyl linoleate isolated from safflower seed oil.[5]

From an analytical point of view, this prochiral molecule is an interesting example for several reasons. First, the ^{2}H signals of the pairs of deuterons noted 4,4′ and 5,5′ are not spectrally separated in isotropic phase, even using a 500-MHz spectrometer.[24] As a consequence, the isotropic deuterium 1D NMR techniques provide only a mean value of the $(D/H)_i$ ratios for those methylene groups. Second, although this C_s symmetry prochiral molecule possesses a large conformational dynamic, previous results on similar entities suggest that deuterons belonging to the four prostereogenic methylene groups should give differentiated ^{2}H signals for each of them when the compound is dissolved in a chiral oriented environment.[15]

THEORETICAL ASPECTS

The spectral enantiodifferentiations observed on ^{2}H−{^{1}H} NMR spectra are based on a difference in quadrupolar splittings, $|\Delta\nu_Q^R - \Delta\nu_Q^S|$ (spin $I > {}^1/_2$):[15]

$$\Delta\nu_{Q_i}^{SorR} = \frac{3}{2} K_{C-D_i} S_{C-D_i}^{SorR} \quad \text{with} \quad K_{C-D_i} = \frac{e^2 Q_{D_i} q_{C-D_i}}{h} \quad (1)$$

where $S_{C-D_i}^{SorR}$ is the order parameter of the C−D$_i$ axis for the *R* or *S* isomers relative to the magnetic field axis and K_{C-D_i} is the quadrupolar coupling constant of the *i*th deuterium. In weakly ordering systems, it is assumed that the electric field gradient tensor is axially symmetric along the C−D bond. K_{C-D_i} is approximately equal to 170 ± 5, 185 ± 5, and 210 ± 5 kHz, for deuterium bonded to a carbon atom hybridized sp^3, sp^2, and sp, respectively.[18]

The narrowness of ^{2}H resonances due to the small quadrupole moment of deuterons actively contributes to the success of the method as well as the weak contribution to NMR line widths of

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order inhomogeneities in the sample.[14] Besides, the relatively large magnitude of K_{C-D} for deuterium nuclei compared to other order-sensitive NMR interactions, such as dipolar couplings or chemical shift anisotropies, is a great advantage[13] because the residual splittings observed may be sufficiently different to provide a spectral separation of quadrupolar doublets even if the difference in orientation between two internuclear C−D directions is small. This ability to discriminate between two C−D directions allows the signals of enantiomers or enantiotopic directions in prochiral molecules to be discriminated spectrally. More generally, it becomes possible to separate the signals of any deuterium nuclei having fortuitously the same chemical shift but not the same S_{C-D} Equation 1 indicates clearly that spectral enantiodiscrimination is detected when $|\Delta\nu^{A}_{Q_i} - \Delta\nu^{B}_{Q_i}|$ with A, B = *R*, *S* or pro-*R*, pro-*S*, including the case where one of two quadrupolar splittings is fortuitously equal to zero.

In practice, the $^2H-\{^1H\}$ spectrum of two monodeuterated ordinary enantiomers (i.e., chiral molecule of type (*R*,*R*′,*R*″)-C*D) dissolved in the PBLG phase generally consists of two independent quadrupolar doublets, one for each stereoisomer, centered approximately on the same chemical shift. The same spectral situation is observed on any NAD NMR spectra,[14,15] but in this case, we discriminate between isotopic enantiomers (chiral isotopomers by virtue of the isotopic substitutions, (*R*-C*HD-*R*′). Note here that the spectral enantiodiscrimination for isotopic enantiomers originates from the fact that two enantiotopic directions (in related prochiral molecules (*R*-CD_2-*R*′) are nonequivalent in CLC. This spectral nonequivalence enables us to distinguish between isotopic enantiomers through their NAD NMR spectrum.[19,21,25]

For large perdeuterated molecules or in NAD NMR, the identification of the two components for each quadrupolar doublet and, hence, their assignment on the basis of deuterium chemical shifts, is however, not always straightforward when numerous peak overlaps occur. This situation arises because the largest quadrupolar splittings in PBLG solutions have about the same amplitude as the deuterium chemical shift dispersion at 9.4 T. To correlate the two components of each deuterium doublet, and hence facilitate the interpretation of overcrowded 1D spectra, several proton-decoupled deuterium 2D NMR experiments have been developed such as the Q-COSY experiment. This was found to be the most efficient for NAD NMR applications in terms of signal sensitivity.[15,26] As on-diagonal peaks are absent in the Q-COSY 2D contour plot, we can tilt the spectral data as in a *J*-resolved 2D experiment.[15]

RESULTS AND DISCUSSION

Contribution of NAD NMR in an ALC. To illustrate the analytical potential of NAD NMR in oriented solvents and the advantages of analyzing organic molecules on the basis of the quadrupolar interaction, we have recorded the NAD spectrum of BPTH in an achiral liquid crystal. For this purpose, we have prepared a racemic mixture of PBLG and PBDG (the enantiomer of PBLG) dissolved in chloroform. In such a solvent, noted hereafter PBG, there is no spectral enantiodiscrimination.[27] When the measurement of $(D/H)_i$ ratios on prostereogenic methylenes in prochiral molecules is not studied, analysis of NAD NMR spectra recorded in an ALC is advantageous in terms of sensitivity. Indeed, the two deuterium atoms in such groups are equivalent, and hence, a single quadrupolar doublet is observed for both nuclei. Consequently, the signal-to-noise ratio is increased by a factor of 2 compared to S/N measured in a CLC for which two doublets are expected to be observed if the chiral discrimination occurs.

Figure 2 presents the natural abundance deuterium $^2H-\{^1H\}$ Q-COSY 2D map of BPTH recorded in the PBG/$CHCl_3$ phase at 300 K. Due to the absence of scalar and dipolar couplings between two rare atoms, the spectrum consists of the sum of independent subspectra corresponding to the various nonequivalent isotopomers of BPTH in the mixture. To simplify the discussion, we will disregard the chloroform signal (labeled with a star on the contour plots) in the analysis of the spectra. BPTH possesses 22 chiral or achiral isotopomers. Nevertheless, due to the equivalence of some of them, only 9 quadrupolar doublets (18 peaks) are expected to be observed on the NAD spectrum recorded in the achiral solvent if no fortuitous superpositions occur. Although this number is rather small, the F_1 and F_2 projections show a rather congested 1D spectrum, much more complex than the isotropic one, mainly owing to the crossing of quadrupolar doublets and peak overlapping. In particular, the analysis of aliphatic deuterium signals is not straightforward.

In this example, the various quadrupolar doublets of solute that are clearly detected on the 2D map show a S/N ratio varying between 8 (site 2,2′) and 63 (site 6). Note that the doublet of 9,9′ does not clearly emerge from noise (S/N < 3) and is not shown in Figure 2. Also, the signal from deuteron in position 1 was not clearly seen even using a strong exponential filtering. Note here that the fast dynamic of the aromatic core yields the two deuterium nuclei located in the ortho or in the meta position to be equivalent in the same aromatic ring. For the same reason, both rings are equivalent on average on the NMR time scale in an ALC. Consequently, two quadrupolar doublets are observed for all deuterons in the ortho and in the meta positions, one for each of them. On the basis of their chemical shift, we can assign the meta positions to the most shielded doublet to the deuterons 8,8′ as well as the aliphatic deuterons shown in Figure 2.

Except for the pairs 4,4′ and 5,5′, we were able to assign the quadrupolar doublets of aliphatic deuterons on the basis on the ^2H chemical shifts as reported in refs 4 and 5. The assignment of quadrupolar doublets for 4,4′ and 5,5′ was made on another basis and will be discussed below. As clear evidence, the pairs of equivalent deuterons noted 4,4′ and 5,5′ show two distinct quadrupolar splittings centered on the same chemical shift, one for each pair. This result implies that deuterons 4,4′ (or 5,5′) possess a single average orientational order parameter, S_{C-D}, but this parameter differs for the two pairs, thus leading to two quadrupolar splittings different in magnitude (absolute value). Contrary to the isotropic NAD spectrum, NAD NMR in the PBG phase allowed us to separate the signal of two pairs and hence perform a quantitative analysis of signals if necessary. The spectral data for each deuterium are reported in Table 1.

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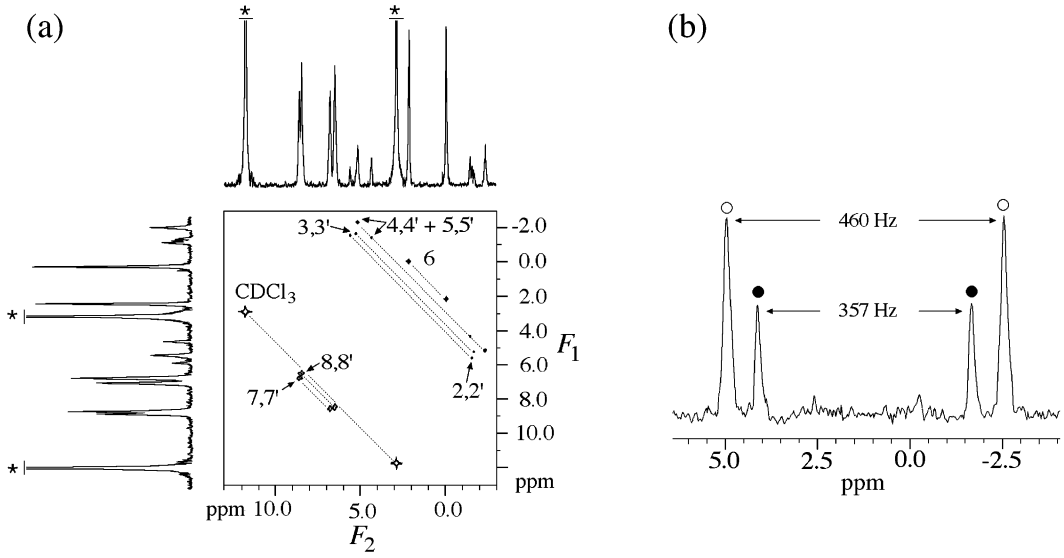

Figure 2. (a) 61.4-MHz NAD Q-COSY 2D spectrum of BPTH in the PBLG/$CHCl_3$ phase recorded at 300 K. The number of free induction decays added for each t_1 increment is 416. The data matrix was zero-filled to 256 (t_1) × 1024 (t_2) prior to 2D transformation. The recycling delay was of 0.6 s, and the spectral width in both dimensions (F_1 and F_2) is 1500 Hz. The 2D spectrum was symmetrized prior to tilting and displayed in magnitude mode. The contour plot is presented without any filtering. Dashed lines drawn on 2D maps facilitate the recognition of quadrupolar doublets. (b) Signals associated with the deuterons 4,4′ (open circles) and 5,5′ (solid circles) and extracted from the 2D map using a Lorentzian filtering (LB = 5 Hz) in both dimensions.

Table 1. Spectral Data of BPTH Recorded in the $CHCl_3$, PBG, and PBLG Phases at 300 K

deuterium numbering	chemical group	δ/ppm[a,b] ($CHCl_3$ sample)	$\lvert\Delta\nu_Q\rvert$/Hz[c] (PBG sample)	$\lvert\Delta\nu_Q\rvert$/Hz[c] (PBLG sample)
1	CD	4.5	not detected	513[e]
2,2′	CDH	1.96	439 (412)[d]	374/406 (390)[f]
3,3′	CDH	1.71	425 (399)[d]	352/397 (375)[f]
4,4′/5,5′	CDH	1.37	357 (335)[d]	301/333 (317)[f]
5,5′/4,4′	CDH	1.37	460 (434)[d]	393/434 (416)[f]
6	CDH_2	0.99	135 (127)[d]	122
7,7′	CD═	7.61	111 (104)[d]	88/96[g] (92)[f]
8,8′	CD═	7.48	119 (112)[d]	96/104[g](100)[f]
9	CD═	∼7.30	427 (400)[d]	360/437 (398)[f]
$CHCl_3$	CD	7.30	545	512

[a] For the chemical shift reference, the signal of $CDCl_3$ in natural abundance is set at 7.30 ppm. [b] The accuracy of the δ_i is around ±0.05 ppm. [c] The accuracy of the $\lvert\Delta\nu_{Q_i}\rvert$ values is ±2 Hz, respectively. [d] The value in parentheses corresponds to the quadrupolar splittings corrected by the factor 1.065, calculated from the ratio $\lvert\Delta\nu_Q$ ($CDCl_3$ in PBG)$\rvert/\lvert\Delta\nu_Q$ ($CDCl_3$ in PBLG)$\rvert$. [e] Signal of very weak intensity. [f] The value in parentheses corresponds to the average of $\lvert\Delta\nu_{Q_i}\rvert$. [g] Values measured after a Gaussian filtering.

The examination of spectral data indicates that the magnitude of the quadrupolar doublets varies between 90 and 434 Hz. The largest values correspond to the deuterons in the methylene groups. In contrast, the methyl group shows a rather small quadrupolar splitting, as generally found whatever the class of molecules concerned. This is due to the rotation of the methyl group around the C−CH_3 axis as theoretically described in ref 28. Similar arguments explain the small magnitude of quadrupolar splittings observed for the ortho and the meta deuterons of the aromatic rings.

Differences in peak intensity between the various doublets of BPTH are observed on the 2D contour plot. The origins of these variations are multiple. First, they reflect the number of equivalent atoms contributing to a given deuterium signal when quantitative measurement conditions are used and assuming identical line widths. This point will be discussed further in the next section. Second, in ordered media, the line widths increase with the magnitude of the quadrupolar splittings, leading to a reduction in peak intensity compared with signals of the same integral but smaller splitting. This spectral feature is a direct consequence of the "degree of disorder" of the solute due to the sample inhomogeneity. Third, but not least, the BPTH derived from methyl linoleate presents a nonstatistical distribution of deuterium along the aliphatic chain, and so the peak intensity for each doublet is expected to be different.[4,5]

Interestingly, it is that the intensity of the quadrupolar doublet associated with the pairs 4,4′ and 5,5′ is different while their line widths are roughly the same ($\Delta\nu_{1/2} \approx 12$ Hz). This observation has never been accessible in isotropic solvent thus far. On the basis of the established variation in deuterium intensity at the odd and even positions of aliphatic chains of fatty acids,[4,5] the inner quadrupolar doublet can be assigned to the deuteriums 5,5′. The measurement of the relative area of 4,4′ and doublets obtained on the filtered 2D spectrum gives a 69/31 ratio in favor of 4,4′ (see Table 2). Knowing the mean $(D/H)_i$ ratio for each deuterium in position 4, 4′, 5, or 5′ obtained in isotropic medium (denoted hereafter $(D/H)_{mean}$),[5] it becomes possible to estimate the $(D/H)_i$ ratios for the 4,4′ and 5,5′ sites in the achiral oriented phase. The mean $(D/H)_{mean}$ value is 109 ppm, thus leading to a $(D/H)_{4,4'}$ and $(D/H)_{5,5'}$ ratio equal to 150 and 68 ppm, respectively (see Table 3). It could be argued that the accuracy of these values is rather poor considering that the S/N ratios for the 4,4′ and 5,5′ sites are equal to 24 and 13, respectively. There is no doubt, however, that this result already provides a very useful estimation of the $(D/H)_i$ ratio, which suggests a significant depletion effect at the odd methylene 5,5′.

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Table 2. Relative Area of Doublets (%) Associated with the Methylene Sites 4,4′ and 5,5′ Measured in the PBG, PBLG, and PBDG Phases[a]

	deuterium sites i					
solvent	4,4′	4	4′	5,5′	5	5′
PBG	69			31		
PBLG	63[b]	38	62	37[b]	31	69
PBDG	68[b]	39	61	32[b]	31	69

[a] Measurements made by deconvoluting the two components of each quadrupolar doublet. The signal used corresponds to a sum of columns extracted from the tilted 2D Q-COSY maps. [b] Percentages calculated using the sum of separated signal area measured for each enantiomer.

Table 3. Evaluation of $(D/H)_i$ Ratios (ppm) Associated with the Methylene Sites 4,4′ and 5,5′ Measured in the PBG, PBLG, and PBDG Phases[a]

		deuterium sites i					
sample	solvent	4,4′	4	4′	5,5′	5	5′
1	PBG	150[b]			68[b]		
2	PBLG	137[b]	104[c]	171[c]	81[b]	50[e]	112[e]
3	PBDG	148[b]	116[d]	180[d]	70[b]	44[f]	96[f]
mean 1−3		145			73		
std dev		7			7		

[a] Values calculated from data reported in Table 2. [b] $(D/H)_{mean}$ = 109 ppm was used for the evaluation of $(D/H)_{4,4'}$ and $(D/H)_{5,5'}$. [c] $(D/H)_{mean}$ = 137 ppm was used for the evaluation of $(D/H)_4$ and $(D/H)_{4'}$ in PBLG. [d] $(D/H)_{mean}$ = 148 ppm was used for the evaluation of $(D/H)_4$ and $(D/H)_{4'}$ in PBDG. [e] $(D/H)_{mean}$ = 81 ppm was used for the evaluation of $(D/H)_5$ and $(D/H)_{5'}$ in PBLG. [f] $(D/H)_{mean}$ = 70 ppm was used for the evaluation of $(D/H)_5$ and $(D/H)_{5'}$ in PBDG.

Contribution of NAD NMR in a CLC. To discriminate between enantiomeric isotopomers of the mixture, we have recorded first the NAD spectrum of BPTH in the PBLG/$CHCl_3$ phase (see Table 4). Panels a and b of Figure 3 show expansions of the NAD Q-COSY 2D map of BPTH centered respectively on the aromatic and aliphatic deuterons of the molecules. The spectrum was recorded with the same NMR conditions as described above, but the number of t_1 increments and scans were increased by a factor of 1.4 and 2 to reach an acceptable S/N ratio on a 9.4-T spectrometer. Keep in mind that the S/N ratio is now decreased by a factor of 2 when the chiral discrimination occurs, compared to that measured on spectrum recorded in the achiral PBG phase.[13]

The comparison of the aromatic region spectrum (Figure 3a) shows the doubling of quadrupolar doublets for deuterium nuclei in the ortho and the meta position compared to the spectrum recorded in the achiral oriented phase. Here, the slight difference in peak intensity mainly arises because a strong Gaussian filtering was used to separate the aromatic doublets. The doubling of aromatic deuterium doublets in the CLC phase shows unambiguously that chiral discrimination is detected on the ortho and meta deuterons. Indeed, we can exclude any other origins for the doubling of signal (related to a very particular conformational dynamic, for instance) because no particular effect has been observed in the PBG phase. The corresponding quadrupolar splittings are given in Table 1. Examination of the magnitude of quadrupolar splittings allows us to conclude on their relative sign. This is possible because in the PBG phase we observe the algebraic average of the $\Delta\nu_Q$ values measured in the PBLG (assuming no solvent effect). In this example, the signs of $\Delta\nu_Q$ for the sites 8,8′ and 9,9′ are identical, namely, either positive or negative.

The comparison of signals in the aliphatic region (Figure 3b) with spectrum recorded in the achiral oriented phase is interesting. Except for the signal of the methyl group that *is not* discriminated (achiral isotopomer), we observe the doubling of quadrupolar doublets associated with deuterons on the four methylene groups, namely, eight distinct quadrupolar splittings. Here again, the result implies a spectral enantiodiscrimination on all sites 2,2′, 3,3′, 4,4′, and 5,5′. This result illustrates nicely the usefulness and the analytical potential of NAD 2D NMR in a CLC compared to NAD 1D NMR in an isotropic state that was not able to separate the resonances. As before, the examination of the quadrupolar splittings in PBLG compared to that measured in PBG indicates that the signs of quadrupolar doublets are the same.

Figure 4 presents three series of the sum of the slices corresponding to the pairs of deuterium 2,2′ (a), 3,3′ (b), and 4,4′/5,5′ (c), extracted from the Q-COSY spectrum when a tilt procedure is applied. As in the 2D map, the three 1D subspectra show the doubling of quadrupolar splittings. However, in this case, it is possible now to appreciate the variation in peak intensity for each enantiomer, the less intense peaks corresponding to the outer quadrupolar doublets. This result is interesting because it suggests significant differences in the $(D/H)_i$ ratio for the deuterium nuclei in the four methylene groups, assuming in a first step that the line widths for each doublet are strictly identical. However, the S/N ratio is rather weak and varies between 9 and 24; hence, it can be argued that this visible difference in peak intensity may reflect other effects: lack of sensitivity due to the low amount of solute per unit volume of the sample (compared with a neat compound), differences in line widths between the inner and the outer doublets, and the use of a relatively high recycling rate (T_R = 0.6 s) during the 2D experiment compared to the deuterium longitudinal relaxation time, T_1. In this specific case, T_1 measurements made on BTPH in the isotropic phase ($CHCl_3/CCl_4$ solvent) at 310 K have shown that T_1 values for deuterons in methylene groups vary between 0.09 and 0.22 s.[24] Using a T_R of 0.6−0.7 s, the ratio T_R/T_1 varies between around 7 and 3. Under these conditions, partial saturation due to the longest longitudinal relaxation time occurs during the repetition times but cannot induce intensity distortions over 5%. This distortion is probably less important because the working temperature is only 300 K and the deuterium T_1 and T_2 relaxation times are known to be generally smaller in oriented phases (<0.8 s) compared to the isotropic counterpart. Such a situation allows us to use shorter recycling rates in NMR experiments in liquid crystal solvents. It could also be argued that relaxation effects could be influential if the recycling rate used in the 2D experiment affects the accuracy of the measurement of enantiomeric excess. However, this argument is not valid because all our attempts to measure a difference in T_1 between two enantiomers failed within the experimental errors.

To check if the differences in peak intensity originate from experimental conditions or a real nonstatistical distribution of (D/H), we recorded the NAD NMR spectrum of BPTH embedded in the PBDG/$CHCl_3$ phase. Except that the degree of polymerization of PBDG was different, this new sample was prepared identically

Table 4. Compositions of Liquid-Crystalline NMR Samples

sample	solute	polymer	DP[a] PBLG/PBDG	cosolvent	polymer/ mg[b]	solute/ mg[b]	cosolvent/ mg[b]	wt % polymer
1	BPTH	PBG	562/914	$CHCl_3$	50/51	162	440	14.4
2	BPTH	PBLG	562	$CHCl_3$	100	200	399	14.3
3	BPTH	PBDG	914	$CHCl_3$	101	199	400	14.4

[a] DP, degree of polymerization. [b] The accuracy of the weighting is ±1 mg.

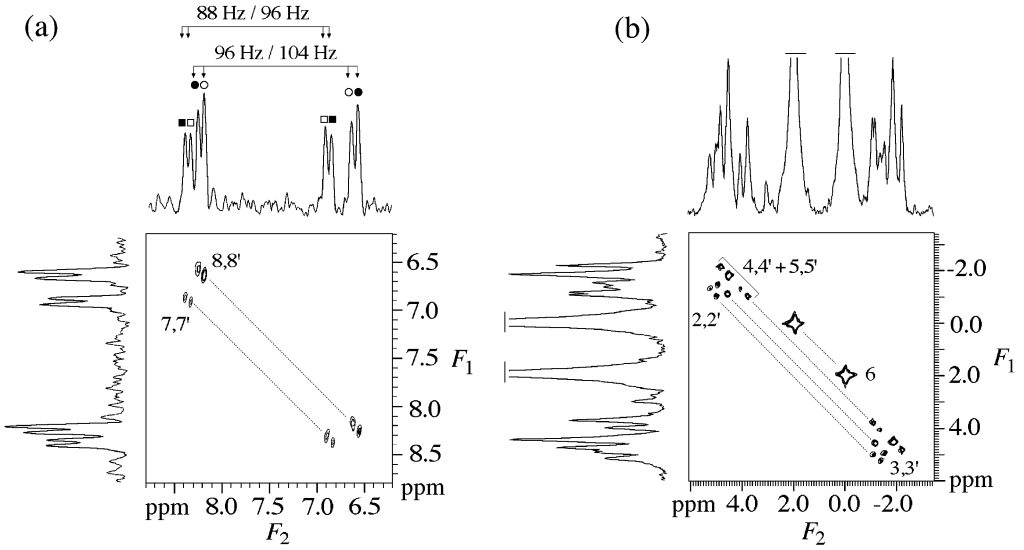

Figure 3. Expansion of the 61.4-MHz tilted NAD Q-COSY 2D spectrum showing separately the aromatic and aliphatic signals of BPTH in the PBLG/$CHCl_3$ phase. The data matrix was 300 (t_1) × 1300 (t_2) points, and the number of free induction decays added for each t_1 increment is 960. The recycling delay was of 0.6-s repetition time, and the spectral width in both dimensions is 1500 Hz. (a) A Gaussian filtering in both dimensions was used to enhance the separation of signals. (b) The 2D contour plot is shown with a Lorentzian filtering (LB = 5 Hz) in both dimensions. Dashed lines drawn on 2D maps facilitate the recognition of quadrupolar doublets.

to the PBLG sample (see Table 4). Using the enantiomer of PBLG, the chirality of the anisotropic system is changed. In the hypothesis where the polypeptide is modeled as a "molecular screw", the inversion of the absolute configuration for the stereogenic carbon atom can be seen as the inversion of the direction of the screw, thus modifying the "acting direction" of the interaction potential. As a direct consequence, the signals associated with each enantiomeric isotopomer should be inverted. In other words, the various quadrupolar doublets associated with the orientation of the *S*-enantiomeric isotopomers in PBLG correspond now to the doublets of the *R*-enantiomeric isotopomers in the PBDG phase and vice versa. Concretely, we should observe the inversion between the smallest and highest quadrupolar doublets. Figure 5 shows the signals of deuterons 4,4′ and 5,5′ (a sum of slices) extracted from the titled NAD Q-COSY spectrum recorded in the PBDG phase. Although the 2D spectrum was recorded using roughly half of the scans per t_1 transients compared to the 2D spectrum recorded in the PBLG phase, we can clearly observe the inversion of the peak intensities. This situation suggests a real depletion effect between the chiral isotopomers involving 4,4′ and 5,5′ sites and excludes the other phenomenon discussed above. Note here that the inversion was also observed on the two other methylene sites.

Measurements of the $(D/H)_i$ Ratio in the CLC Phase. The first goal of this work consisted of exploring the possible contributions of the NAD NMR spectroscopy in a CLC to the investigation of NAD distribution in prochiral, organic molecules. The possibility of observing the spectral enantiodifferentiation of deuterium signals in methylene groups shows that this aim has been largely reached. Although the S/N ratios obtained in this pioneer study are rather small, we have attempted to evaluate the NAD distribution using this analytical tool.

As in the isotropic phase, the surface area of the two quadrupolar components below the deuterium signal is theoretically directly proportional to the number of monodeuterated chiral or achiral isotopomers present in the mixture. The site-specific ratio $(D/H)_i$ expressed in parts per million (ppm) can be defined by the equation

$$(D/H) = D_i/H_i = N_{D_i}/(P_i N_H) \qquad (2)$$

where N_{D_i} is the number of monodeuterated chiral or achiral isotopomers of type *i*, N_H is the number of fully protonated molecules, and P_i is the number of equivalent hydrogens at site i.

As underlined before, the accuracy of the estimation of the $(D/H)_i$ ratio is dependent on several NMR-related parameters. In particular, standard deviation is a function of the S/N ratio, line shape, homogeneity in the NMR tube, and spectrometer stability during the acquisition. As a postacquisition control, the shape and the line width of chloroform signals can provide a valuable mean to check the stability of the magnet as well as possible time evolution of the sample homogeneity during the

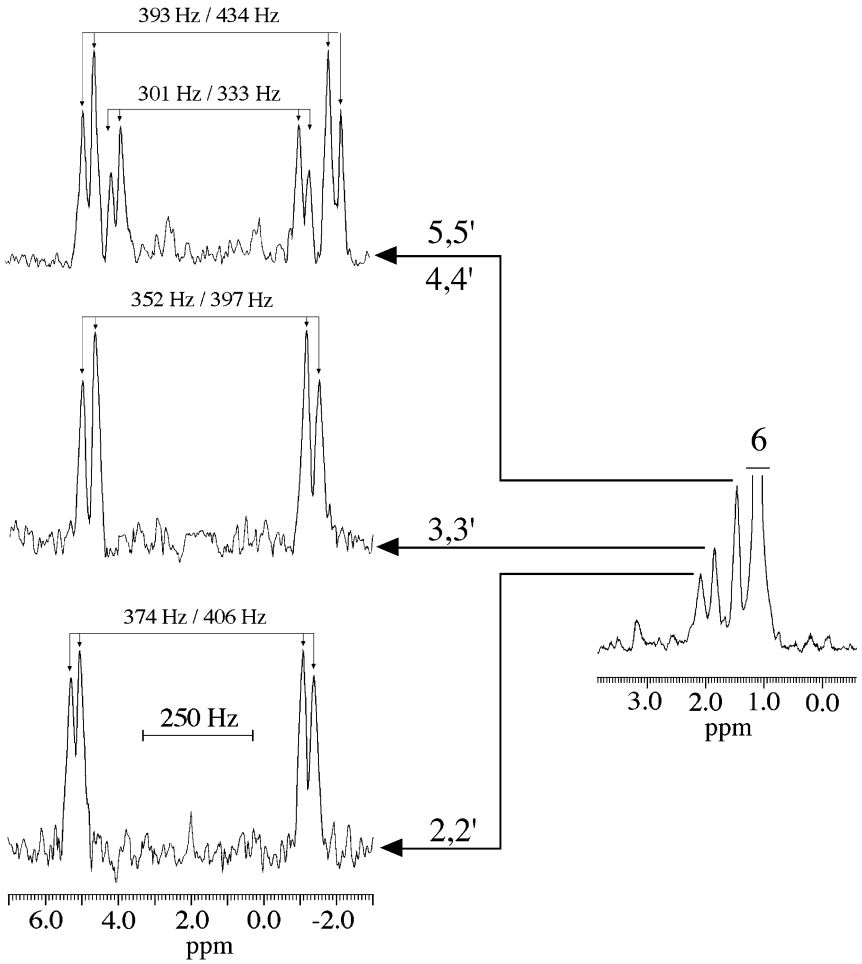

Figure 4. (Left) Signals of deuterons noted 2,2′, 3,3′ 4,4′, and 5,5′ (sum of columns) extracted from the previous NAD Q-COSY 2D map after the tilt procedure. (Right) Part of the F_2 projection observed on tilted spectrum. This ^{2}H spectrum is formally identical to that obtained in the isotropic phase.

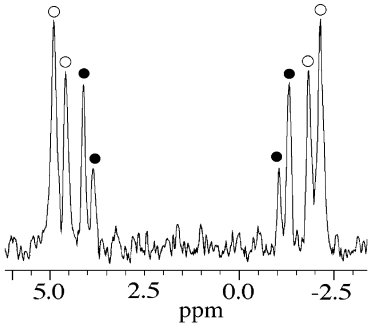

Figure 5. Signals (sum of columns) extracted from the tilted NAD Q-COSY 2D map recorded in the PBDG/$CHCl_3$ phase at 300 K and associated with the deuterons 4,4′ and 5,5′. The data matrix is 246 (t_1) × 1300 (t_2), and the number of free induction decays added for each t_1 increment is 416. The 2D spectrum was filtered using a Lorentzian filtering (LB = 3 Hz) in both dimensions and symmetrized. Note the inversion of peak intensities compared with slices shown in Figure 4.

NAD experiments. In the present study, the S/N ratios measured are rather weak. As a consequence, we have only focused our attention on the 4,4′ and 5,5′ sites that show an S/N ratio sufficient to perform reasonable evaluation of the $(D/H)_i$ ratios. The evaluation of the $(D/H)_i$ ratios was performed by deconvoluting the ^{2}H signals (a sum of slices) extracted from the tilted 2D Q-COSY spectrum. The best agreement was found using between 44 and 91% of Gaussian function. The use of a mixed Lorentzian/Gaussian function for deconvoluting line shapes, obtained by adding various slices extracted from the tilted spectrum, is required because the 2D contour plot has to be displayed in magnitude mode to avoid the "phase-twist" line shapes.[26] Tables 2 and 3 summarize the relative area of each quadrupolar doublet for the methylene sites 4,4′ and 5,5′ obtained in the achiral and chiral oriented phases and the first evaluations of $(D/H)_{4,4'}$ and $(D/H)_{5,5'}$ ratios for BPTH, respectively.

The calculation of $(D/H)_i$ ratios reported in Table 3 was performed as follows:

$$(D/H) = [2(\%\ \text{area})(D/H)_{\text{mean}}]/100 \qquad (3)$$

where $(D/H)_{\text{mean}}$ is the value determined together for the methylene sites 4 and 5 of BTPH. This value, equal to 109 ppm, was previously determined using quantitative deuterium 1D NMR spectroscopy.[5] The combination of the two results is possible because the BPTH in both studies used the same batch of methyl linoleate (Lot 65 H 0257 from Sigma) and identical chemical modifications.

The analysis of the data listed in Table 3 shows the excellent agreement between the measurements in the CLC phase (PBLG or PBDG) and the measurements made in the ALC phase (PBG) within the experimental error. In particular, the $(D/H)_{i,i'}$ ratio calculated using the sum of signal areas measured for each enantiomer gave 137 and 148 ppm in the PBLG and PBDG phases, respectively. These values are very consistent with the value of 150 ppm measured in the PBG sample regarding the standard deviation, equal to 7, calculated from data obtained on the spectra recorded in the PBG, PBLG, and PBDG phases. The coherence of these results provides good confidence in the $(D/H)_i$ values for each enantiomer in both phases (PBLG and PBDG); hence, these results could be analyzed as a real depletion between the enantiotopic directions 4 and 4′ as well as the directions 5 and 5′. This experimental finding is noteworthy because it shows, for the first time, a marked difference in $(D/H)_i$ between two enantiotopic directions of a prochiral molecule. This important result requires further investigation; it has important implications in terms of our present perception of the origin of the nonstatistical distribution of deuterium on the chain of fatty acids and of kinetic isotopic effects associated with enzyme-catalyzed reactions involving prochiral derivatives.

Regarding the S/N reached in this investigation, it is clear that the accuracy of $(D/H)_i$ ratios is much lower than that obtained using classical isotropic techniques.[4] This tool is, however, already very encouraging considering that the experiments reported here were performed using a 9.4-T spectrometer equipped with a deuterium 5-mm probe.

Evolution of This Approach. If the method provides valuable information that had not previously been obtained, it suffers from a lack of sensitivity. The main reason for this is that the S/N ratio for each deuterium resonance showing a spectral enantiodiscrimination in PBLG is basically reduced by a factor of 4 (racemic mixture) compared to that obtained on the isotropic spectra where single deuterium resonances are observed for any nonequivalent deuteron.

As the proton-to-deuterium polarization transfers give no good results for theoretical reasons,[29,30] the lack of sensitivity of this method can be overcome by different ways. First, improved sensitivity of NAD experiments can be easily achieved by increasing the molar concentration of the solute under investigation, the simplest procedure consisting of increasing the amount of solute dissolved in the oriented phase. However, this is very dependent on the nature of the solute and its compatibility with the polypeptide because some analytes can precipitate the polymer, and an excessive amount of solute compared to the other components of the mixture would eliminate the liquid-crystalline properties of the sample. A balanced solution could be found using a 10-mm NMR sample, for which the overall magnetization of the sample is much larger compared with a 5-mm NMR sample. Hovewer, this approach consumes large amounts of solute, not always available. Second, it is possible to take advantage of NMR spectrometers operating at very high magnetic fields, increasingly available in many laboratories. Indeed the S/N ratio is proportional to $(B_o)^{3/2}$ for a given experimental time (EXPT) while the EXPT is proportional to $1/(B_o)^3$ for a given S/N ratio. To be convinced, Figure 6 shows the evolution of the total EXPT and of the S/N ratio with respect to a 9.4-T spectrometer used in this study. Thus, when recording the NAD spectra of a chiral molecule at 122.8 MHz (18.8 T or 800 MHz for ^{1}H), the S/N ratios would be increased by a factor of 2.8 for the same EXPT. Conversely, the EXPT would be reduced by a factor of 6.6 to obtain the same S/N ratio that is obtained at 61.4 MHz with a 9.4-T field. The second advantage of using very high field magnets is a simpler analysis of the NAD NMR spectra due to a larger dispersion of deuterium signals. The third and probably the best solution consists of using selective cryogenic probes, which allow the thermal noise to be strongly reduced by cooling the receiver coil and the corresponding preamplifier.[31] This solution is much less expensive than the use of higher field strengths. From a sensitivity point of view, a very significant gain in signal sensitivity (a factor of ∼4) compared with selective standard probes is obtained. Obviously, the combination of a selective deuterium cryoprobe with a spectrometer operating at a very high field is the simplest way (not the cheapest!) to achieve an accurate determination of the $(D/H)_i$ ratios. Thus, calculations indicate that the sensitivity of a 600-MHz spectrometer equipped with a selective deuterium cryoprobe would be equivalent to a 1500-MHz spectrometer equipped with a standard probe. But currently ^{2}H-cryoprobes are not available yet.

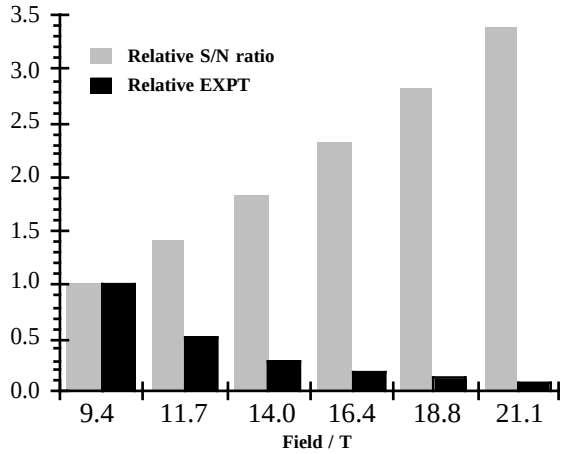

Figure 6. Evolution of the relative S/N ratio and the relative EXPT as a function of the field with respect to a 9.4-T (400 MHz for proton) spectrometer. The reference value at 9.4 T is 1.

CONCLUSION

In this paper, we propose a new analytical tool to study the natural abundance deuterium distribution at a maximum number of sites in prochiral molecules. This approach combines deuterium 2D NMR experiments at natural abundance level and the use of polypeptidic, chiral liquid crystals as enantioselective NMR solvents. As experimentally shown in this work, NMR in CLC offers complementary information that enhanced the classical

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method. First, it allows the deuterium sites exhibiting fortuitously isochronous resonances in liquid state to be separated on the basis of the quadrupolar splittings. Second, NMR in chiral oriented phase provides a unique possibility to investigate the $(D/H)_i$ ratio for deuterium nuclei bonded on prostereogenic carbon atoms of prochiral molecules, impossible to reveal using NMR method in the isotropic state.

In the present study, we show, for the first time, a probable marked difference in the (D/H)i between two enantiotopic directions of a prochiral molecule. This important result leads to new assumptions on the mechanisms of the kinetic isotopic effects associated with enzyme-catalyzed reactions involving prochiral derivatives.

These first results obtained with a routine 400-MHz spectrometer equipped with a selective deuterium 5-mm probe shows unambiguously that NAD NMR spectroscopy at natural abundance level in a CLC is a promising strategy in the investigation of the natural abundance deuterium distribution. As evidence, the next step of this study will consist mainly of determining the analytical possibilities for highly reliable quantitative applications.

EXPERIMENTAL SECTION

Materials. The synthesis of BPTH, from the commercial methyl linoleate, is reported in ref 4. The methyl linoleate (Lot 65 H 0257) and the PBLG and PBDG polymer were purchased from Sigma. The degree of polymerization (DP) of PBLG and PBDG is 562 and ($M_w \approx 120\,000$) and 914 ($M_w \approx 200\,000$), respectively. Chloroform (stabilized with ethanol) was dried using molecular sieves prior to use.

Sample Preparations. In NAD NMR applications, the preparation of oriented samples is crucial because a lack of macroscopic homogeneity may strongly affect the resolution of spectra, in particular, the line widths and, hence, the S/N ratio for solute signals. The usual procedure to prepare standard samples using 5-mm NMR tubes is described in detail in refs 13−15. In this study, it was possible to dissolve up to 200 mg of solute in about 200 mg of polypeptide and 400 mg of chloroform. The exact compositions of the samples are listed in Table 4. Note that it can be also useful to perform several freeze−pump−thaw cycles to remove paramagnetic oxygen dissolved in the sample that may contribute to NMR line widths of solutes, but this step is not usually required. To reach the best possible spectral resolution, various cycles of centrifugation and rehomogenization of the sample were carried out to remove concentration gradients in the sealed NMR tubes.

NMR Measurements. In this work, all NAD NMR spectra were recorded on a Bruker DRX-400 high-resolution NMR spectrometer (9.4 T) equipped with a 5-mm selective deuterium probe (61.4 MHz) with fluorine lock (376.5 MHz) developed by Bruker France. Note here that the fluorine field-frequency lock was not necessary because the field drift of the magnet is small enough to allow for a long time accumulation without significant line broadening. All NAD 1D and 2D NMR experiments were recorded by applying the WALTZ-16 composite pulse sequence to decouple protons. The NMR tube was not spun in the magnet, and its temperature was regulated carefully using the standard variable-temperature control unit (BVT 3000). Obviously, for each sample, the tuning and the matching of the deuterium coil were optimized to induce maximum NMR signals. Unless otherwise specified, the NAD Q-COSY 2D experiments were recorded using 320 FIDs for each t_1 increment, using a recycling delay of ~0.6 s. and the 2D spectra shown here were zero-filled to 1024 (t_1) × 2048 (t_2) data points prior to 2D FT and then symmetrized. Other details are given in the figure legends. Further methodological or experimental details can be found in refs 14 and 15. The area measurement was performed using a curve-fitting algorithm on complex least-squares treatment of the ^{2}H NMR signal.[32]

(32) Perch NMR Software, University of Kuopio, Finland.

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