Natural chirality of methylene sites applied to the recognition of origin and to the study of biochemical mechanisms

Maryvonne L. Martin, Benli Zhang and Gérard J. Martin

Chimie Organique Physique ERA 315, CNRS, Université de Nantes, 44072 Nantes-Cedex, France

Received 5 April 1983

Naturel abundance deuterium NMR has been applied to the quantitative determination of natural methylene chirality in amino acids of different configurations and in ethanols of different origins. An enantiomeric enrichment is detected for aspartic acid of the L series obtained from fermentation and the technique therefore provides an absolute method for identifying the natural origin of a given substance. In ethanols of different origins the pro-*R* and pro-*S* positions appear as naturally equally populated at the present level of precision of the experiments. These results provide a new source of information about reaction mechanisms in the natural conditions.

Isotopic ratios *^2H NMR* *Natural methylene chirality*

1. INTRODUCTION AND METHOD

We have shown that the natural distribution of deuterium in the different sites of a molecule may exhibit very large deviations with respect to a statistical distribution and, on this basis, we have proposed a new procedure [1,2] for identifying the origin of natural and synthetic products [3,4]. Thus the quantitative ^2H NMR technique at the natural abundance level enables the specific isotropic fractionation to be determined and we have therefore at our disposal a method of labelling without the need for enrichment, to investigate the mechanisms of chemical [5] and biochemical [6] reactions. In this method, the distinction between natural and synthetic species, for example, is achieved by comparing the specific isotropic fractionation in the investigated compounds of unknown origin, with the values measured in test samples of authenticated origin. Here we show that, in appropriate cases, it is possible to make a distinction between synthetic or natural origin without the need for reference samples. This absolute method is based on the determination of the enantiomeric purity of the naturally present monodeuterated species which involve methylene sites pertaining to chiral or achiral compounds. In addition, the method offers a source of interesting information about the mechanism and stereospecificity of the reactions which possibly involve the methylene groups of achiral compounds originating from biosynthetic pathways.

2. RESULTS AND DISCUSSION

In the natural abundance ^2H spectra of aspartic acid recorded at 38.4 MHz or 61.2 MHz the α-site and the β- and β'-sites of the methylene group can be distinguished. As a result of the very low natural abundance of deuterium ($\simeq$0.015%) the monodeuterated species exist as a mixture of threo- and erythro-diastereoisomers involving the enantiomeric $-\underset{\beta}{C}HD-\underset{\alpha}{C}H\begin{matrix}\diagup\\ \diagdown\end{matrix}$ fragments. In aqueous media at pH $\sim$ 13.1 and at 298 K the diastereotopic β- and β'-sites are significantly differentiated and the deuterium chemical shifts measured with respect to HMDS are:

$\delta(\alpha)=3.49$ ppm $\delta(\beta)=2.56$ ppm $\delta(\beta')=2.27$ ppm

Published by Elsevier Science Publishers B.V.
00145793/83/$ 3.00

Table 1

Relative deuterium contents R_β and $R_{\beta'}$ at the diastereotopic methylene sites β and β' of aspartic acid, measured with respect to the deuterium content at site α

Origin of the sample	Configuration	R_β	$R_{\beta'}$	$R_{\beta'}/R_\beta$
AEC-R-P	DL	0.94	0.95	1.0
AEC-R-P	D	0.75	0.73	1.0
AEC-R-P	L	0.78	0.95	1.2
Fluka	L	0.91	1.04	1.15

The ratio $R_{\beta'}/R_\beta$ characterizes the optical purity at the methylene site. The accuracy in the R parameters is about ±0.05. The samples have been obtained from the firms Fluka and AEC-Rhône–Poulenc

According to the techniques already described [4], we have determined the specific isotopic distribution in the different non exchangeable sites. This distortion may be characterized by the ratios:

$$R_\beta = S_\beta/S_\alpha \quad \text{and} \quad R_{\beta'} = S_{\beta'}/S_\alpha$$

which represent the enrichment at site β with respect to the deuterium content in position α taken as a reference (S_i denotes the integral of signal i). For an optically pure D or L sample of aspartic acid the isotopic ratios R_β and $R_{\beta'}$ reflect the optical purity at the methylene site. A racemic distribution of deuterium at this site should correspond to a value $R_\beta/R_{\beta'} = 1$.

Table 1 gives the values of the isotropic ratios R corresponding to samples of aspartic acid of different configurations: DL, D and L. The D sample was obtained by chemical synthesis whereas the L sample results from a biochemical reaction. It is observed that in most cases the β site exhibits a slight deuterium depletion with respect to the α position. Moreover, it appears that within experimental accuracy, no difference is detected between the enantiomeric populations at site β in the sample with stereochemistry D which results from chemical synthesis. By contrast, the β' site is significantly enriched with respect to the β position in the samples with stereochemistry L which had been obtained by fermentation. The method therefore provides a new tool for recognizing, in an absolute way, the natural origin of a compound.

This method can be of particular interest for the investigation of compounds devoid of an asymmetric centre and we have applied it to the detection of the possible chirality of ethanol samples obtained by fermentation of sugars. In this case, it is necessary to introduce a diastereotopy of the methylene sites and we have reacted each ethanol sample with (−)camphanoyl chloride [7] to obtain the corresponding ester. In this camphanate ester, the prochiral methylene protons are differentiated and the diastereotopy of the corresponding deuterium nuclei may be enhanced by addition of the shift reagent Eu $(dpm)_3$ at suitable concentrations [7]. Thus for a solution ≈0.6 M in CCl_4 containing about 32 mol% of the europium complex the difference between the chemical shifts of the ^2H signals of the S and R methylene sites reaches 0.34 ppm.

Therefore we could determine the parameters:

$$R_R = S_R/3S \text{ (methyl)} \quad \text{and} \quad R_S = S_S/3S \text{ (methyl)}$$

Table 2

Relative deuterium contents in the pro-R, R_R and pro-S, R_S, methylene sites of ethanol measured with respect to the deuterium content in the methyl group which is given the statistical weight 3

No.	Origin of the ethanol	^2H content of the fermentation water (ppm)	R_R (integral)	R_S (integral)	R_R/R_S (heights)
1	Chemical synthesis	–	1.2	1.2	1.0
2	Beet saccharose	150	1.3	1.3	1.0
3	Corn starch	150	1.1	1.1	1.0
4	Beet saccharose	395	2.1	2.1	1.0

The ratio R_R/R_S characterizes the optical purity at the methylene site. The accuracy in the R_R and R_S parameters is about ±0.1. Owing to the equality of the transverse relaxation times of the deuterium nuclei in the R and S positions the ratio R_R/R_S can also be obtained from line height measurements with an accuracy of about 0.05

which characterize the deuterium content in the pro-*R* and pro-*S* methylene sites with respect to the methyl site taken as a reference and given the statistical weight of 3 [4].

We have checked that in the camphanate ester derived from synthetic ethanol, 1, the enantiomeric monodeuterated species involving the methylene site exist as a racemic mixture (table 2). In the case of a natural ethanol sample, 2, obtained from sugar-beet no significant enantiomeric enrichment is detected within the limits of experimental accuracy (±5% in the present experimental conditions). A similar result is obtained in the case of an ethanol sample, 3, derived from hydrolysis, followed by fermentation, of corn starch and also for a sample of ethanol, 4, prepared from beet saccharose fermented in an aqueous medium slightly enriched in deuterium (D/H = 395 ppm) (table 2). Therefore, we provide *direct* proof that, under *natural conditions,* the biosynthesis of ethanol occurs with complete or nearly complete racemization at the methylene site. This equality or near equality in the populations of the enantiomers involving the methylene site is in fact the result of intermolecular exchange mechanisms occurring at two distinct steps. In the course of the fermentation process, the reduction of acetaldehyde by the couple NADH-alcohol dehydrogenase is stereospecific and only the pro-*R* position of ethanol undergoes a hydrogen exchange with the aqueous medium [8,9]. The pro-*S* hydrogen which corresponds to the formyl hydrogen of acetaldehyde also comes from fermentation water but at a previous step, when the pyruvate leads to acetaldehyde. Therefore, we may conclude that eventual differences in the isotropic effects occurring in the course of the normal reaction mechanisms are too small to result in an enantiomeric imbalance detectable in the present state of our technical conditions. In fact, the observation of an enantiomeric enrichment at a methylene site of chiral or achiral species characterizes the natural origin of the compound, since in usual conditions chemical synthesis does not involve asymmetric steps. However, the absence of enantiomeric enrichment is obviously not a sufficient condition for discarding a natural origin. More generally, the method offers a source of valuable information on the stereospecificity of the biochemical pathways involving methylene sites under the natural conditions.

ACKNOWLEDGEMENTS

The authors wish to thank M. Jouffret and M.G. Farges from AEC–Rhône–Poulenc (Commentry) for providing them with samples of DL, a D,L-aspartic acid.

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