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Measurement of isotopic enrichments in ^{13}C-labelled molecules by 1D selective Zero-Quantum Filtered TOCSY NMR experiments

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Abstract

A NMR method based on the combination of selective pulses with Zero-Quantum Filtered TOCSY magnetization transfer was used to measure the specific ^{13}C-enrichment of labelled molecules. The analysis of a labelled lysine sample showed that DANTE-Z schemes give accurate measurements; meanwhile, DPFGSE schemes resulted in the underestimation of ^{13}C-enrichments. The former method can be applied to overcome signal overlap problems that are often encountered in the analysis of labelled molecules. *To cite this article: C. Nicolas et al., C. R. Chimie 11 (2008).*
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Résumé

Une méthode RMN basée sur l'association d'impulsions sélectives et de transfert d'aimantation de type TOCSY avec des filtres à zéro quantum a été utilisée pour mesurer l'enrichissement spécifique en ^{13}C de molécules marquées. L'analyse d'une solution de lysine marquée a permis de montrer que des modules de type DANTE-Z donnent des mesures fiables, contrairement à l'utilisation de modules DPFGSE qui conduisent à une sous-estimation de l'enrichissement. La première méthode permet de s'affranchir de problèmes de superposition de signaux fréquemment rencontrés dans l'analyse de molécules marquées. *Pour citer cet article : C. Nicolas et al., C. R. Chimie 11 (2008).*
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1. Introduction

NMR has been widely used over the past two decades to investigate the metabolism of living systems

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from ^{13}C-labelling experiments [1,2]. NMR provides direct information on the carbon position(s) of metabolites that receive the label, from which metabolic pathways can be identified. Moreover, the approach is quantitative and the amount of label incorporated in the various carbon positions of metabolites can be measured. In combination with appropriate mathematical models of metabolism, the NMR data can be exploited for the determination of metabolic fluxes, i.e. the actual rates of biochemical reactions in living systems [3,4].

The amount of label incorporated in a specific carbon position (also termed specific ^{13}C-enrichment) of a metabolite can be measured by ^{13}C NMR, but ^{1}H NMR is usually preferred because of the higher sensitivity of the latter nucleus. Due to the heteronuclear [13]C−^{1}H coupling, the NMR signal of protons attached to a ^{13}C nucleus consists of two satellite lines; meanwhile, the signal from ^{1}H attached to a ^{12}C remains central. The amount of ^{13}C incorporated in a protonated carbon can be measured from the intensity of the satellite lines relative to the total intensity of the corresponding ^{1}H resonance. This approach can be conveniently applied to pure compounds, but the narrow spectral width of the ^{1}H nucleus hampers seriously its application to complex mixtures of metabolites such as cell extracts or biomass hydrolysates, which are commonly considered in metabolic studies. To address this problem, different options have been proposed. A first option consists in separating the compounds of interest in order to record resolved spectra. But not all compounds can be always separated properly, and the process can be tedious and time-consuming. Furthermore, this is of no help where the overlapping signals are coming from the same molecule. A second option is the application of quantitative 2D NMR methods where the various signals are separated along the two spectral dimensions [5]. This allows ^{13}C-enrichments to be determined for a large number of metabolites from a single analysis, but the overlapping problems still remain for signals that are in close vicinity. A third option is the application of difference spin-echo methods to discriminate overlapping signals from labelled compounds [6]. The method is based on the selective inversion of the satellite lines, which can be further added or subtracted to the normal spectrum in order to extract the signals from the protons attached to a ^{12}C or a ^{13}C, respectively.

In this paper, we have evaluated the reliability of selective excitation experiments using either DPFGSE [7,8] or DANTE-Z [9,10] modules to provide an accurate measurement of ^{13}C-enrichments. This was achieved by applying the different experiments to a solution of lysine − a compound giving overlapping ^{1}H NMR signals − with known ^{13}C-enrichment. Further, a sequence containing a DANTE-Z module combined to a Zero-Quantum Filtered TOCSY was built-up to generate a method allowing the specific ^{13}C-enrichment to be determined from proton signals that are normally embedded into a crowded region of the ^{1}H NMR spectrum. This method was applied to the determination of the split ratio between the two lysine biosynthetic pathways occurring in the bacterium *Corynebacterium glutamicum*.

2. Experimental

2.1. Lysine sample preparation

A standard sample of lysine was prepared by mixing 10.9 mg of unlabelled − i.e. [13]C at natural abundance − L-lysine-monohydro-chloride (Sigma) to 15.5 mg of [U-[13]C$_6$]-L-lysine-dihydro-chloride (98% ^{13}C-label, Eurisotop, France). Labile protons were exchanged with deuterium by lyophilizing twice in 3 ml D$_2$O 99.9% (Eurisotop), and the final sample was suspended in 600 μl of 100 mM DCl (Eurisotop). The pH was adjusted to 7 with NaOH before the first lyophilization. Because the sample is prepared from a mixture of [U-[13]C$_6$]-lysine and unlabelled lysine, all carbon positions of lysine in the final sample have the same specific ^{13}C-enrichment. The ^{13}C-enrichment of the Cε carbon measured from the Hε signal in the 1D ^{1}H NMR spectrum recorded on the lysine sample was 53.8% (Fig. 1(1)).

2.2. Corynebacterium glutamicum *sample preparation*

C. glutamicum was grown on a medium containing [6-^{13}C]-glucose (99% ^{13}C-label) as sole carbon source. At the end of the culture, the supernatant was separated from the cells by centrifugation and dried under nitrogen flux. Labile protons were exchanged with deuterium as described above for the lysine standard sample.

2.3. NMR spectroscopy

All 1D and 2D NMR spectra were obtained on an Avance 500 MHz spectrometer (Bruker) equipped with a 5 mm *z*-gradient BBI probe. The data were acquired and processed using TOPSPIN® 1.3 software. The temperature was 298 K.

The 1D proton spectra were recorded by using quantitative conditions. To have full signal recovery, the relaxation delay between scans was 30 s and the pulse
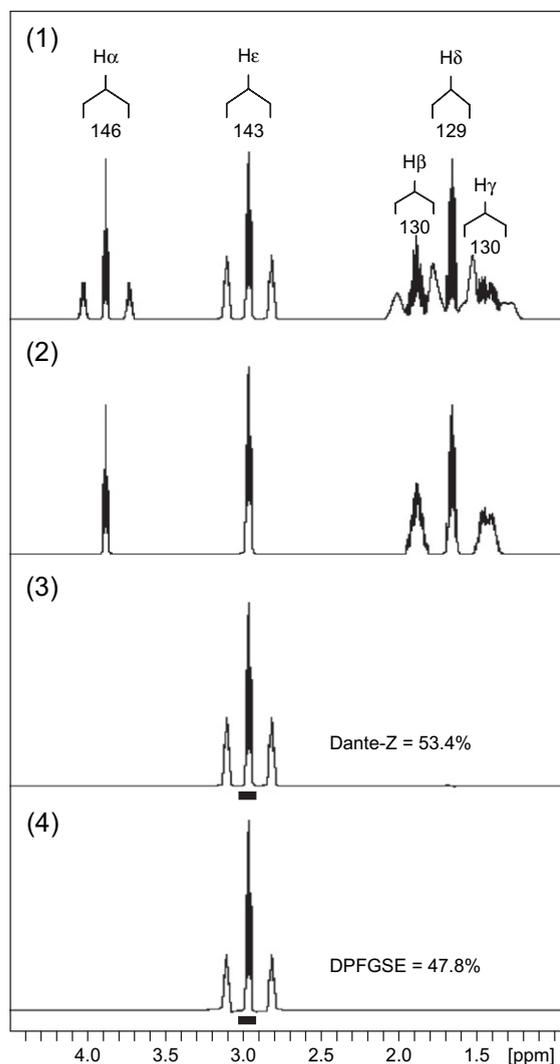
Fig. 1. Proton NMR spectra of ^{13}C-labelled lysine. (1) 1D ^{1}H quantitative spectrum. The assignments of the various resonances in the spectrum are provided as annotations above the peaks. Each resonance appears as a central line and two satellite lines separated by the value of the coupling constant — indicated for each resonance below the peak annotation. The spectrum shows that the resonances from the H$_\beta$, H$_\gamma$, H$_\delta$ protons overlap at the level of the satellite lines, and, therefore, the different contributions cannot be integrated properly. (2) 1D ^{13}C-decoupled ^{1}H spectrum. In this spectrum, all the signals appear as central lines only because the ^{13}C–^{1}H heteronuclear coupling has been suppressed. (3) 1D ^{13}C-decoupled DANTE-Z spectrum. The Hε protons were decoupled from ^{13}C nuclei and selectively excited by a DANTE-Z pulse scheme. Because the decoupling fuses all signals into the central lines, the selective excitation can be applied to a narrow 60-Hz bandwidth (black box below the spectrum). The spectra were recorded with no ^{13}C-decoupling during the acquisition period. (4) 1D ^{13}C-decoupled DPFGSE spectrum. Same experiment as in Fig. 1(3), except that DPFGSE pulse scheme was used instead of the DANTE-Z module for selective excitation (black box below the spectrum).

angle was 30°. The sweep width was 5000 Hz and the acquisition time was 1.6 s. A total of 16 scans were accumulated.

Selective excitations were performed with shaped pulses. The excitation range was 60 Hz except for the γ protons, for which it was 100 Hz because of the larger multiplet width. The 1D ^{13}C-decoupled DANTE-Z sequence consisted of eight consecutive scans of on-resonance Inversion BURP (I-BURP) pulses followed by eight consecutive scans of off-resonance I-BURP pulses [11]. This cycle was repeated four times. A 0.7 G/mm purge gradient was applied after the selective excitation. The 1D ^{13}C-decoupled DPFGSE sequence consisted of two REfocusing BURP (RE-BURP) pulses. The gradient powers were set at 0.7 and 0.3 G/mm, respectively. The decoupling of ^{13}C nuclei during the selective pulses was performed using a GARP4 sequence at a power of 3.8 kHz.

A ^{13}C-decoupled DANTE-Z, Zero-Quantum Filtered TOCSY (DANTE-Z ZQF-TOCSY) sequence (Fig. 2) was generated by inserting a ^{13}C-decoupled DANTE-Z pulse module into the previously described ZQF-TOCSY sequence [12]. The ^{13}C-decoupled DANTE-Z pulse was applied with the same parameters as above. The TOCSY transfer was carried out using a DIPSI-2 mixing sequence applied during 20 ms with 10 kHz field width. At the end of the mixing period, in-plane magnetization was suppressed by a square 2.6 G/mm purge gradient. A Zero-Quantum Filter [13] consisting of a 180° CHIRP adiabatic pulse with 10% smoothing, 20 kHz sweep width, and 30 ms duration at 330 Hz maximal RF power, was applied before acquisition. A square 0.2 G/mm gradient pulse was applied during the adiabatic pulse.

The pulse sequence (with phase cycles) and the AU program for Topspin® (Bruker spectrometers) of the DANTE-Z ZQF-TOCSY can be obtained freely upon request to Jean-Charles Portais (email: stephane.massou@insa-toulouse.fr).
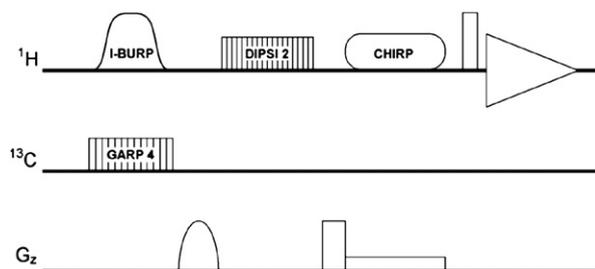

Fig. 2. DANTE-Z ZQF-TOCSY pulse sequence. See text for details.

3. Results and discussion

3.1. Selective excitation using DPFGSE and DANTE-Z

Fig. 1(1) shows the 1D ^{1}H NMR spectrum of a ^{13}C-labelled lysine sample, which contains the resonances from the five protonated carbons (α,β,γ,δ,ε) of this amino acid. Three resonances (β,γ,δ) are very close one to each other, with clear overlaps of the ^{1}H−^{13}C satellite lines that prevent the accurate integration of almost all signals in this region. This overlap still remains in 2D ^{1}H,^{1}H spectra (data not shown), which means that the specific ^{13}C-enrichments of Lys β,γ, and δ carbons cannot be measured from classical 1D ^{1}H or 2D ^{1}H,^{1}H NMR spectroscopy. The spectrum can be simplified by means of a selective excitation, but for the determination of ^{13}C-enrichments, the two satellite lines as well as the central one have to be recovered. If the selective excitation is applied over the spectral region containing the three lines, then the overlapping signals will be excited as well, which is not desired. This problem can be solved by decoupling the ^{13}C atoms during the selective excitation, so that the central and satellite lines fuse into only one signal that can be selectively excited (Fig. 1(2)). The signal acquisition itself is performed without ^{13}C-decoupling so that the actual spectrum contains the heteronuclear couplings.

To evaluate the effectiveness of DANTE-Z and DPFGSE pulses to achieve the selective excitation of ^{13}C-decoupled signals in a quantitative manner, they were applied first to a signal in a clear region of the spectrum (Hε). The two pulses were applied with the same excitation width (60 Hz), using I-BURP (D-DANTE-Z) and RE-BURP (DPFGSE) sequences. GARP4 ^{13}C-decoupling was applied during the selective excitation period. After the selective excitation, the ^{1}H NMR spectra were acquired without ^{13}C-decoupling. The results are displayed in Fig. 1(3) (DANTE-Z excitation) and 1.4 (DPFGSE excitation). In both cases, the satellites and the central lines of Hε multiplet have been well excited and the signal was roughly similar to that observed in the standard 1D ^{1}H spectrum (Fig. 1(1)). A close examination of the spectra revealed that the relative intensity of the satellite lines in the DANTE-Z spectrum was almost identical to that observed in the standard ^{1}H spectrum. As a result, the ^{13}C-enrichment measured from the DANTE-Z spectrum (53.4%) was in close agreement with the value determined from the standard ^{1}H spectrum (53.8%). In the DPFGSE spectrum, the relative intensity of the satellite lines was lower compared to the two other spectra, and the ^{13}C-enrichment measured from the former spectrum was only 47.8%. Such discrepancy between the measured value and the expected one is likely to be due to relaxation effects. Protons attached to a ^{13}C nucleus have shorter − transverse and longitudinal − relaxation times compared to the same protons attached to a ^{12}C nucleus. The duration of the DPFGSE module is too long and leads to the partial relaxation of all protons, so that a fraction of the signal is lost prior to the acquisition. Because the relaxation is faster with ^{13}C-coupled protons, the fraction of lost signal is wider for protons attached to ^{13}C nuclei compared to protons attached to ^{12}C nuclei. Such explanation is consistent with the underestimation of the ^{13}C-enrichment calculated from the DPFGSE spectrum. The duration of the DANTE-Z pulse scheme is shorter than that of DPFGSE, and the former sequence is expected to be less sensitive to relaxation effects [9,10], as emphasized by the ^{13}C-enrichment calculated from the spectrum displayed in Fig. 1(3), in close agreement with the expected one.

3.2. Specific ^{13}C-enrichment of lysine Cβ and Cδ

The ^{13}C-decoupled DANTE-Z sequence described above was applied for the determination of the ^{13}C-enrichments of carbons β, δ and γ of lysine from their ^{1}H signals, which tightly overlap in the ^{1}H spectrum (Fig. 1(1)). The corresponding spectra of the Hβ, Hδ and Hγ are shown in Fig. 3(1−3), respectively. To evaluate the quality of these experiments, the three signals were summed using appropriate scaling factors, and the reconstructed spectrum so-obtained (filled line in Fig. 3(4)) was compared to the standard ^{1}H-spectrum. The two spectra were closely similar, as shown from their difference (dotted line in Fig. 3(4)). This result indicated that the proposed method was reliable for providing selective excitations of the three lysine protons in a quantitative manner.

Because the lysine sample considered in these experiments consisted of a mixture of [U-^{13}C$_6$]- and unlabelled lysine, all carbons in the final sample have the same specific ^{13}C-enrichment. The ^{13}C specific enrichment measured from DANTE-Z spectra for the carbons β and δ of lysine were 52.6% and 53.9%, respectively. These values were in close agreement with the value obtained for the Cε carbon from the Hε signal (53.8%). The enrichment of the Cγ carbon could not be measured because the different satellite and central lines of this resonance are so large that they overlap in the actual

spectrum and therefore they cannot be integrated properly.

3.3. Selective ZQF-TOCSY

The ^{13}C-decoupled DANTE-Z experiment described above does not allow the measurement of ^{13}C-enrichments from protons that are still overlapped by other signals in the ^{13}C-decoupled ^{1}H spectrum, because it is not possible to selectively excite the resonance of interest without exciting the overlapping signal. In that case, a different strategy can be applied if the resonance
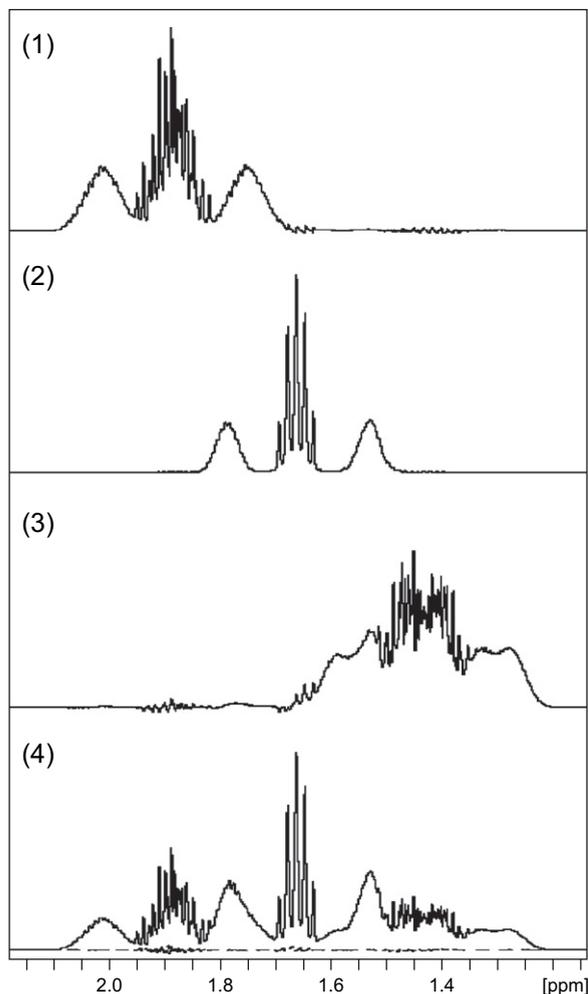

Fig. 3. Spectrum simplification using ^{13}C-decoupled DANTE-Z selective excitation. The spectrum obtained from the ^{13}C-decoupled DANTE-Z selective excitation of H$_\beta$ (1), H$_\delta$ (2) and H$_\gamma$ (3) protons of lysine. The spectra were recorded with no ^{13}C-decoupling during the acquisition period. The satellite lines appear in each spectrum but do not overlap with other signals due to the selective excitation. (4) Scaled sum (filled line) of spectra shown in Fig. 3(1—3). The dotted lines below the spectrum shows the difference between the reconstructed spectrum and the 1D ^{1}H spectrum of Fig. 1(1).

of interest can be excited via magnetization transfer. To test the efficiency of such approach, a pulse sequence combining ^{13}C-decoupled DANTE-Z excitation and magnetization transfer using Zero-Quantum Filtered (ZQF) TOCSY was generated. The advantage of ZQF-TOCSY with respect to the determination of ^{13}C-enrichments has been described elsewhere [5].

The DANTE-Z ZQF-TOCSY experiment was applied to transfer the magnetization from the Hε to the Hδ of lysine. The Hε protons were selectively excited using the ^{13}C-decoupled DANTE-Z module, and then the Hε magnetization was transferred to the Hδ protons by means of the TOCSY isotropic mixing. The TOCSY transfer was restricted to the nearest proton by applying an isotropic mixing time (20 ms) short enough to avoid further transfer to the Hγ and Hβ protons. The resulting spectrum is shown in Fig. 4, where only the two signals corresponding to the Hε and the Hδ were observed. The ^{13}C-enrichment of the Cδ carbon measured from the Hδ signal was 53.7%, a value which is in good agreement with the expected one.

The above results indicate that the combination of DANTE-Z and ZQF-TOCSY is a reliable tool for the determination of ^{13}C-enrichment from proton signals embedded into a crowded region of the ^{1}H NMR spectrum.

3.4. Ratio measurement between the two variants diaminopimelate pathway

In the bacterium *C. glutamicum*, lysine can be synthesized via two different processes: the dehydrogenase variant and the succinylase variant of the diaminopimelate pathway [14]. The two variants can be discriminated by means of ^{13}C-labelling experiments [15]. When bacteria are cultured with [6-^{13}C]-glucose as a carbon

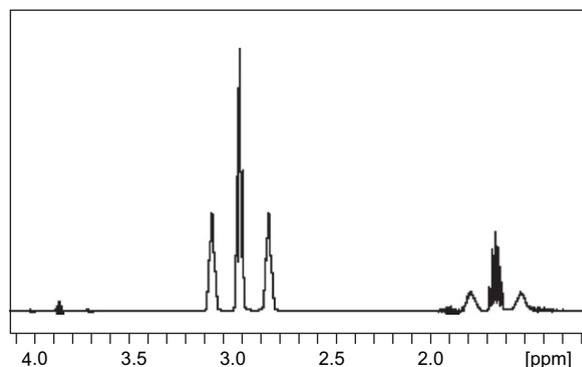Fig. 4. Selective ZQF-TOCSY spectrum. The lysine Hε protons were selectively excited by a ^{13}C-decoupled DANTE-Z pulse scheme. The Hε magnetization was subsequently transferred to the Hδ protons by a TOCSY mixing period of 20 ms. Zero-quantum coherences were suppressed by application of Zero-Quantum Filters.

source, the flux ratio between the two metabolic pathways can be evaluated from the following formula [15]:

$$\text{Flux ratio} = \frac{L_\delta - L_\beta}{2 \times A_\beta - L_\delta - L_\beta}$$

where L_δ, L_β and A_β are the enrichments of the carbons Cδ and Cβ of lysine, and Aβ of acetate, respectively, and the flux ratio is the ratio between the flux through the dehydrogenase pathway to the flux through the succinylase pathway.

We have applied the above-described selective pulse schemes to the determination of the ^{13}C-enrichments of the lysine and acetate produced by the bacteria, i.e. the lysine and acetate found in the culture supernatant. The ^{13}C-enrichments of lysine Cδ and Cβ were found to be 49.9 and 46.4%, respectively, and that of acetate Cβ was 52.4%. From these values, the split ratio was calculated to be 41.1%.

4. Conclusion

The above results show that ^{13}C-decoupled DANTE-Z pulse schemes can be profitably applied for the measurement of ^{13}C-enrichments from ^{1}H NMR spectra in situations where the signals of interest are embedded in a crowded region of the spectrum. Furthermore, such selective excitation can be combined to TOCSY experiment to transfer the magnetization to desired but not − directly or easily − accessible signals, which extends the range of NMR technique valuable for the measurement of ^{13}C-enrichment in complex mixtures of metabolites.

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