# Structural Analysis of Partially Acetylated Glycosides by Refocused-Decoupled Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) Experiments: A Qualitative and Quantitative Method

Laurent Pouységu,[a] Bernard De Jéso,[a] Jean-Claude Lartigue,[b] Michel Pétraud,[b] and Max Ratier*,[c]

[a] *Institut du Pin (EA 494), Laboratoire de Chimie des Substances Végétales, Université Bordeaux I; 351 cours de la Libération, F-33405 Talence, France:* [b] *Centre d'Etude Structurale et d'Analyse des Molécules Organiques, Université Bordeaux I; 351 cours de la Libération, F-33405 Talence, France: and* [c] *Laboratoire de Chimie Organique et Organométallique, UMR 5802 CNRS, Université Bordeaux I; 351 cours de la Libération, F-33405 Talence, France.*
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**The application of the refocused-decoupled INEPT pulse sequence in $^{13}$C-NMR spectroscopy allows, in conjonction with computer simulations, a straightforward editing of the spectra of a raw mixture of glycosidic compounds.**

**Key words** carbohydrate chemistry; enzymatic hydrolysis; $^{13}$C-NMR; refocused-decoupled INEPT; computer simulations; acetylated glycosides

Selective acetylation-deacetylation methodologies are very commonly used in carbohydrate chemistry to generate a suitable protective-group strategy for hydroxyl functions.[1,2] Their effectiveness in the preparation of partially acetylated monosaccharides has received particular attention, owing to their great potential usefulness as precursors in the synthesis of oligosaccharides,[3] in the preparation of *O*-substituted derivatives,[4] as chiral building blocks in organic synthesis,[5] and as reference model compounds for the analysis of polysaccharides.[6]

Many enzymatic and chemical methods have been reported for the preparation of partially acetylated derivatives. As a rule, chemical transformations have the disadvantage that multiple synthetic steps are generally needed, with usually modest overall yields.[7—14] In contrast to chemical techniques, enzymic catalysis is conceivably a one-stage process in regioselective acylation[15—21] and deacylation[22—25] as well as in chemoselective deprotection.[26,27] Since the advent of enzymatic methods, numerous investigations have focused on the enzymatically catalyzed regioselective hydrolysis of peracetylated mono- and disaccharide model compounds to account for the nature and behaviour of both the enzyme and the substrate.[28—31] However, whatever the chosen strategy the question arises concerning the location of acetyl groups, which proved to be labile under basic conditions and prone to intramolecular migrations mainly during the purification steps.[32,33] Subsequent careful separation of the reaction products is needed to assign the deacetylated generated compounds unambiguously. Apart from some solvent change techniques[34] and selective deuteriation methods,[35,36] assignment of acetyl groups is inferred from diagnostic variations in $^{13}$C chemical shifts of the ring carbon atoms with substituent effects. These empirical and experimental methods have given rise to a flood of $^{13}$C-NMR data in carbohydrate chemistry.[37,38]

Obviously, the plurality of these factors makes the measurements time-consuming. From a practical point of view, it appears attractive to avoid the isolation and purification steps and also to preclude the prerequisite tedious steps of assigning $^{1}$H- or $^{13}$C-NMR resonances by the usual combination of $^{1}$H–$^{1}$H homonuclear and $^{1}$H–$^{13}$C heteronuclear 2D correlation experiments.

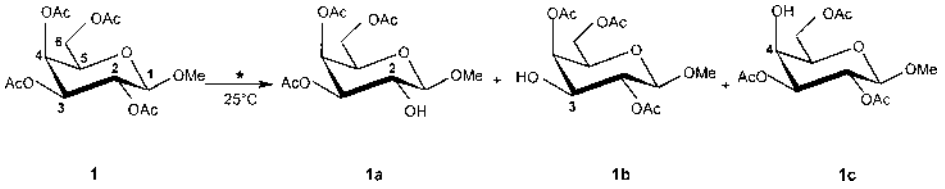
To solve such restrictive analytical problems, we recently applied the refocused-decoupled insensitive nuclei enhanced by polarization transfer (INEPT) pulse sequence to the structural analysis of acetylated carbohydrate model compounds.[39] By taking advantage of computed graphic representations of the refocused INEPT analytical relation,[40] we demonstrated that individual resonances can be assigned unambiguously to their specific positions in the pyranose ring, thanks to the exploitation of certain characteristic refocusing delay times $\Delta$, closely linked to the spatial proton environment around the different carbon atoms. Thus, the influence of homonuclear coupling contributions in $^{13}$C refocused-INEPT experiments was graphically visualized by monitoring the computed variations in the theoretical signal intensities as a function of the sequence refocusing delay time, enabling amplitude modulations of the signal response. The theoretical curves are thus plotted for typical axial/axial (aa), axial/equatorial (ae), and equatorial/equatorial (ee) H–H orientations associated with the vicinal protons close to each $sp^2$ carbon atom involved.[41]

## Results and Discussion

In this paper, we demonstrate the usefulness of the method for substrates of greater complexity and biological relevance in addressing the question of the regioselective enzymatic hydrolysis of peracetylated sugars. As an example, we investigated the regioselective deacetylation of methyl 2,3,4,6-tetra-*O*-acetyl-$\beta$-D-galactopyranoside **1** by *Aspergillus niger* lipase, under the conditions of Hsiao *et al.*[42] Enzymic hydrolysis resulted in the formation of a mixture of three monohydroxylated triacetylderivatives [2-OH, 3-OH, and 4-OH, respectively] (Chart 1).

The structural elucidation of the reaction products proceeded as follows: a series of simulations was carried out for various and characteristic $^{3}$J(HH) coupling constant values in pyranose rings (Table 1),[43,44] using two high J(HH) values (7.9, 10.4 Hz; aa/aa), two small J(HH) values (3.4, 0.9 Hz; ae/ae), and high and small J(HH) values (10.4, 3.4 Hz; aa/ae).

* To whom correspondence should be addressed. e-mail: m.ratier@lcoo.u-bordeaux.fr




* *A. niger* lipase conversion: 80%; 5.5 h.

Chart 1. Enzymatic Hydrolysis of the Methyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranoside **1** by *A. niger* Lipase (Yields According to Hsiao[42]); Methyl 3,4,6-Tri-*O*-acetyl-*β*-D-galactopyranoside **1a** (32%): Methyl 2,4,6-Tri-*O*-acetyl-*β*-D-galactopyranoside **1b** (9%): Methyl 2,3,6-Tri-*O*-acetyl-*β*-D-galactopyranoside **1c** (43%)

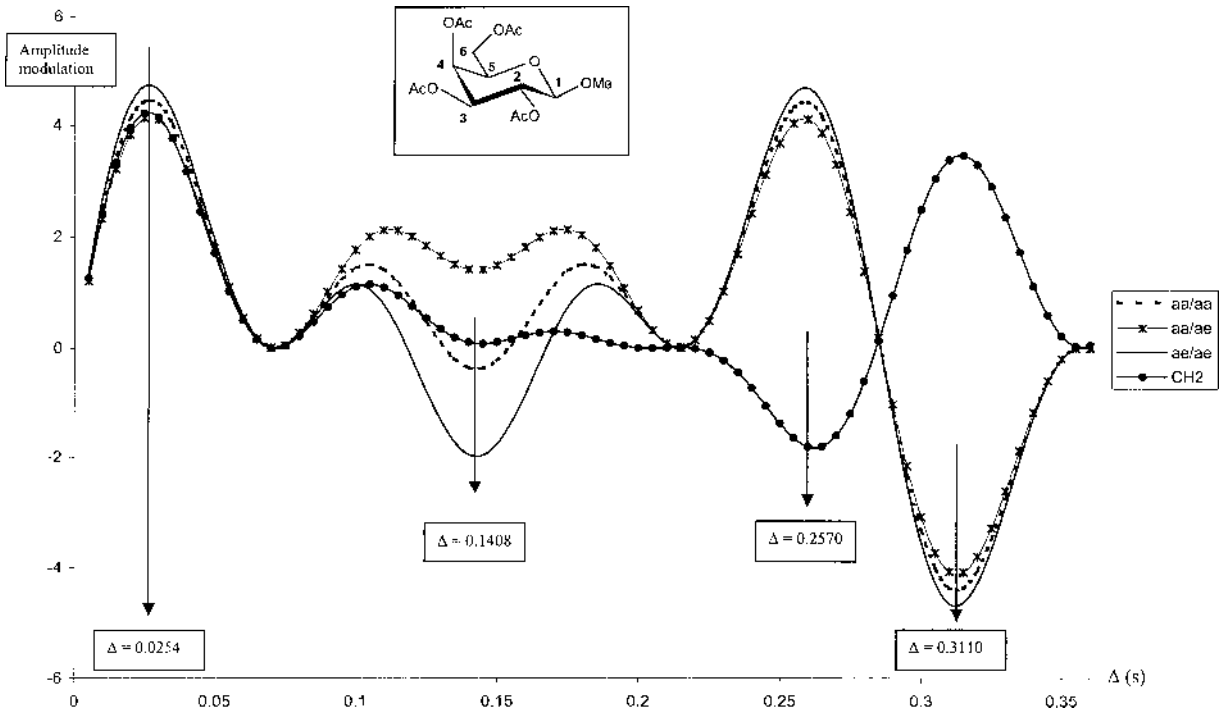



Fig. 1. Graphs of Decoupled Enhancements $E_{INEPTdec}=f(\Delta)$ in the 0 to 0.35 s Window, Showing the Dependence of the Signal Amplitude Modulation on the Spatial Environment around the $sp^2$ Carbonyl Carbon Atoms for the Methyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranoside **1** at $\tau=0.0357$ s.

Table 1. Coupling Constants of the Methyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranoside **1** in $CDCl_3$

| | $\delta$ $^{13}$C (ppm) | $^2$J(CH)[a] | $^3$(CH)[a] | $^3$J(HH) | Typical HH orientation |
|---|---|---|---|---|---|
| CO-2 | 169.4 | 7.0 | 3.5 | 7.9/10.4 | aa/aa |
| CO-3 | 170.0 | 7.0 | 3.6 | 10.4/ 3.4 | aa/ae |
| CO-4 | 170.2 | 7.0 | 3.4 | 3.4/ 0.9 | ae/ae |
| CO-6 | 170.3 | 7.0 | 2.5/3.5 | 6.3/ 6.7 | $CH_2$ |

*a*) Determined by non refocused 1D INEPT experiments.

Accurate knowledge of these coupling constants is not necessary to achieve computed simulations since these couplings are relatively constant throughout the hexopyranose series.[43,44] Profiles were recorded for the polarization delay $\tau=0.0357$ s value, which is maximum for $\tau=1/4\ ^2$J(CH). Superposition of all INEPT polarization curves displays characteristic $\Delta$ values, allowing spectral editing capability (Fig. 1).

The following observations could be made: at $\Delta=0.0254$ s, the amplitude modulation is positive for all $sp^2$ systems. At this $\Delta$ value, the intensity magnitude of the signal is optimum and allows the classical determination of the number of carbon atoms. Then, according to the chosen $\Delta$ value, the amplitude of the CO-6 signals could be positive ($\Delta=0.3110$ s) or negative ($\Delta=0.2570$ s). In the same way, at $\Delta=0.1408$ s one can discriminate the CO-2, CO-3, and CO-4 modulations thanks to their theoretical relative signal intensities, which are representative for the percentages of the corresponding monohydroxylated derivatives in the mixture. Moreover, it should be mentioned that opposite amplitude modulations were always observed between the CO-6 signals and the other types of carbon resonance.

Since the mono-deacetylation process is known not to modify the structure of the target compound significantly, the theoretical profiles obtained above for the methyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranoside **1** can account for the enzymatic hydrolysis selectivity.

Representative experimental refocused-decoupled INEPT spectra of the reaction mixture obtained from the hydrolysis of the methyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranoside **1** are depicted in Fig. 2, in which the refocusing time was varied according to the determined theoretical values ($\Delta=$

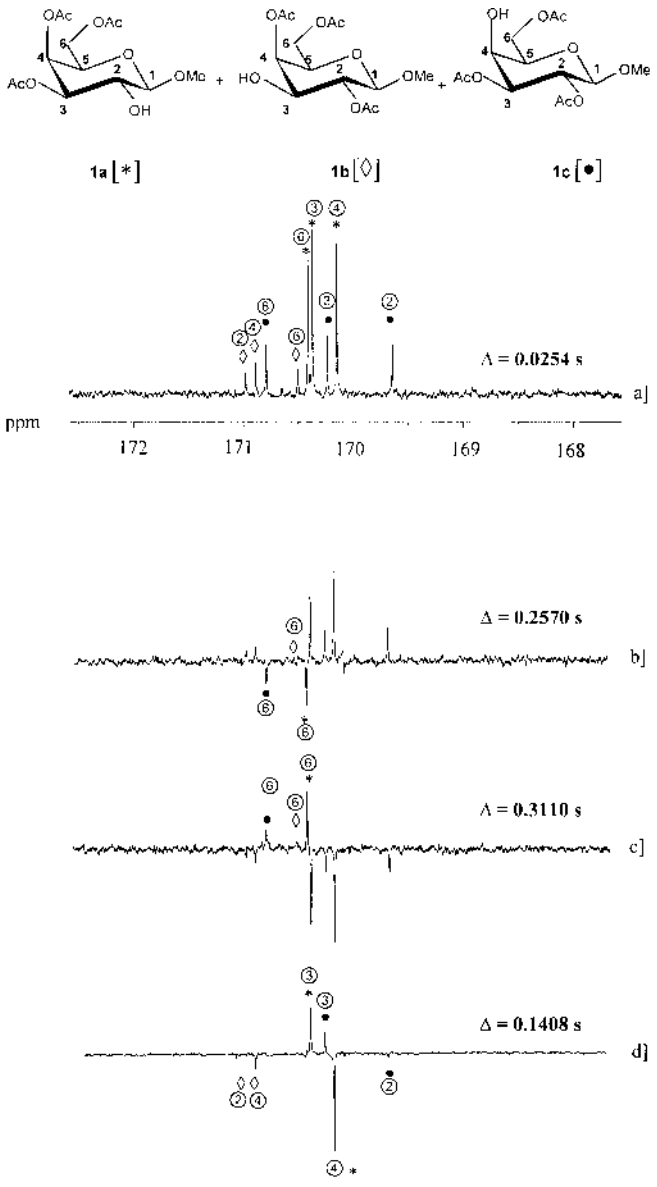



Fig. 2. Experimental Spectra (in $CDCl_3$) and Assignment of the Resonance Lines Obtained from the Enzymatic Hydrolysis of the Methyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranoside **1** by *A. niger* Lipase

(The resonances arising from the $sp^2$ carbon atoms are labeled and numbered with circles). a): broadband decoupling at $\Delta$=0.0254 s; b)—c): assignment of the CO-6 resonances at $\Delta$=0.2570 and 0.3110 s; d): assignment of the CO-2, CO-3, and CO-4 lines at $\Delta$=0.1408 s.

0.0254, 0.1408, 0.2570, 0.3110 s), to invert the $sp^2$ resonances (spectra a, b, c, d) selectively.

At $\Delta$=0.0254 s where the enhancement factor is maximum (spectrum a), nine signals are observed in the $sp^2$ carbon atom resonance window. Three sets of three signals can be constituted according to their relative intensities:

signals labeled with a star (*) [$\delta$ $^{13}C_{ppm}$: 170.1; 170.3; 170.4];

signals labeled with a circle (●) [$\delta$ $^{13}C_{ppm}$: 169.6; 170.2; 170.8]; and

signals labeled with a diamond (◇) [$\delta$ $^{13}C_{ppm}$: 170.5; 170.9: 171.0].

At $\Delta$=0.2570 s, the amplitude modulation of the CO-6 signals is negative and returns to a positive value at $\Delta$=0.3110 s (spectra b, c).

Assignment of labeled resonances to the corresponding compounds **1a**, **1b**, and **1c** is now inferred from the theoretical amplitude modulations observed at $\Delta$=0.1408 s. At this $\Delta$ value, the resonance at 170.3 ppm (*) is positive while that at 170.1 ppm (*) is highly negative. These relative intensities therefore correspond to CO-3 (aa/ae) and CO-4 (ae/ae) carbonyl functions. The absence of a CO-2 (aa/aa) resonance (*) demonstrates that the signals labeled with a star (*) are ascribable to compound **1a**.

Assignment of the signals labeled with a diamond (◇) to compound **1b** is based on the observation of a strong negative resonance at 170.9 ppm (CO-4: ae/ae), a resonance at 171.0 ppm with a signal intensity near zero (CO-2: aa/aa), and the absence of a CO-3 (aa/ae) resonance line.

Finally, assignment of the signals labeled with a circle (●) to compound **1c** is based on assignment of the positive resonance line at 170.2 ppm to a CO-3 (aa/ae) and that at 169.6 ppm (signal intensity near zero) to a carbonyl in position -2.

In conclusion, the $^{13}$C-NMR 1D refocused INEPT technique described here, associated with computer simulation experiments, proved to be useful to unravel accurately the $^{13}$C-NMR resonance lines of a raw mixture of glycosidic compounds. It should be noted that this qualitative spectral editing tool avoids the prerequisite tedious step of separation and purification of each compound, followed by unambiguous assignment of $^1$H-NMR spectra *via* 2D experiments. Moreover, only the $sp^2$ carbon atom window must be considered in the $^{13}$C-NMR spectra. To allow easy access to the method, the necessary computer SIMEPT program is available on request.

**Experimental**

The methyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranoside **1** was prepared according to the previously reported procedure[1,2] and its regioselective deacetylation by *A. niger* lipase was carried out under the conditions of Hsiao *et al.*[42]

$^1$H-NMR spectra were recorded on a Bruker AC-250 spectrometer operating at 250.133 MHz. $^{13}$C-NMR spectra were recorded on a Bruker AC-250 spectrometer operating at 62.896 MHz. $^{13}$C-INEPT decoupled experiments were recorded using the standard Bruker microprogram (INEPT. AU): [$D_1$-(90°$_x$$^1$H)-$\tau$-(180°$_x$$^1$H)(180°$_x$X)-$\tau$-(90°$_{\pm y}$$^1$H)(90°$_x$X)-$\Delta$/2-(180°$_x$$^1$H)(180°$_x$X)-$\Delta$/2-X acquisition with $^1$H broadband decoupling]. Optimization of the experimental polarization and the refocusing parameters ($\tau$, $\Delta$) was obtained using the previously mentioned computer program[41]: $\tau$=0.0357 s and $\Delta$=0.0254, 0.1408, 0.2570 and 0.3110 s. The following parameters were used: $D_1$=2 s; 90° $^1$H=9.0 $\mu$s; 180° $^1$H=18 $\mu$s; 90° $^{13}$C=4.1 $\mu$s; 180° $^{13}$C=8.2 $\mu$s; acquisition: SW=255.31 Hz; SI=4 K; LB=0.3 Hz.

Methyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranoside **1**: $^1$H-NMR ($CDCl_3$) $\delta$ ppm: 1.91, 1.98, 1.99, 2.08 ($CH_3CO$, 12H, 4s), 3.45 ($OCH_3$, 3H, s), 3.87 ($H_5$, 1H, broad t, $^3J$=6.7 eHz), 4.03 to 4.17 ($H_{6a}$+$H_{6b}$, 2H, m, $^2J$=−11.0 Hz), 4.35 ($H_1$, 1H, d, $^3J$=7.9 Hz), 4.95 ($H_3$, 1H, dd, $^3J$=10.4, 3.4 Hz), 5.12 ($H_2$, 1H, dd, $^3J$=7.9, 10.4 Hz), 5.32 ($H_4$, 1H, dd, $^3J$=3.4, 0.9 Hz). $^{13}$C-NMR ($CDCl_3$) $\delta$ ppm: 20.4, 20.5, 20.7, 20.7 ($CH_3CO$), 56.8 ($OCH_3$), 61.2 ($C_6$), 67.0 ($C_4$), 68.7 ($C_2$), 70.6 ($C_5$), 70.9 ($C_3$), 101.9 ($C_1$), 169.4 (CO-2), 170.0 (CO-3), 170.2 (CO-4), 170.3 (CO-6). IR (KBr) cm$^{-1}$: 1749, 1220, 1073.

Methyl 3,4,6-Tri-*O*-acetyl-*β*-D-galactopyranoside **1a**: $^{13}$C-NMR ($CDCl_3$) $\delta$ ppm: 170.1 (CO-4), 170.3 (CO-3), 170.4 (CO-6).

Methyl 2,4,6-Tri-*O*-acetyl-*β*-D-galactopyranoside **1b**: $^{13}$C-NMR ($CDCl_3$) $\delta$ ppm: 170.5 (CO-6), 170.9 (CO-4), 171.0 (CO-2).

Methyl 2,3,6-Tri-*O*-acetyl-*β*-D-galactopyranoside **1c**: $^{13}$C-NMR ($CDCl_3$) $\delta$ ppm: 169.6 (CO-2), 170.2 (CO-3), 170.8 (CO-6).

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