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CHARACTERISATION OF FOUR LACTOSE MONOPHOSPHATES BY APPLICATION OF ^{31}P-, ^{13}C-, AND ^{1}H-N.M.R. SPECTROSCOPY*

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ABSTRACT

By a combination of ion-exchange chromatography and h.p.l.c., two fractions (*A* and *B*) have been obtained from pharmaceutical-grade lactose, each containing a mixture of lactose monophosphates. ^{31}P-N.m.r. and ^{13}C-n.m.r. spectroscopic analysis indicated *A* to contain the 6- and 6′-phosphates and *B* to contain the 3′- and 4′-phosphates. Application of 2D-^{13}C–^{1}H COSY and 2D-^{1}H–^{1}H COSY afforded ^{1}H-n.m.r. assignments for all protons in all four compounds. The observed ^{31}P–^{1}H and ^{31}C–^{1}H couplings are interpreted in terms of preferred orientations of the phosphate group in each compound.

INTRODUCTION

Upon crystallisation of pharmaceutical-grade lactose, an unknown acidic substance, which has a retarding effect on the growth rate of the crystal, is incorporated into the growing α-lactose hydrate crystal[1]. The acidic material has been separated from lactose by using ion-exchange chromatography, and tentatively characterised by g.l.c. and n.m.r. spectroscopy as a mixture of isomeric lactose monophosphates[2] with the phosphate group linked to the galactose moiety of the molecule. By h.p.l.c., the mixture of lactose monophosphates could be further separated into two fractions, each being a mixture of at least two compounds. However, these mixtures could not be characterised by conventional ^{1}H-n.m.r. spectroscopy[2].

We now report the identification of the various components based on the

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application of ^{13}C- and ^{31}P-n.m.r. spectroscopy. Application of 2D-^{1}H–^{13}C and -^{1}H–^{1}H shift-correlation spectroscopy allowed complete assignment of the ^{1}H-n.m.r. spectra of each component.

EXPERIMENTAL

The inhibitor of crystal-growth of lactose was concentrated into the crystal by repeated crystallisation[1], followed by separation of the ionic compounds from lactose by ion-exchange chromatography to yield a mixture (*T*) of disaccharide phosphates. Application of h.p.l.c. afforded[2] two sub-fractions (*A* and *B*).

Prior to ^{31}P- and ^{13}C-n.m.r. spectroscopy, the material was dissolved in D_2O (99.75% atom D), and the pD was adjusted to ~3.6 using NaOD or DCl (see Discussion). The pD values are non-corrected readings from a standard pH meter. 81-MHz ^{31}P-n.m.r. spectra were obtained at 27°, using a Bruker WP-200 spectrometer operating in the pulsed F.t. mode and controlled by an Aspect 2000 computer; ^{31}P chemical shifts (δ) are expressed in p.p.m. relative to that of external orthophosphoric acid[3]. 50-MHz ^{13}C-n.m.r. spectra were obtained at 27°, using a Bruker WM-200 spectrometer equipped with a 5-mm broad-band probe-head, operating in the pulsed F.t. mode and controlled by an Aspect 2000 computer (SON hf-NMR-facility, Department of Biophysical Chemistry, Nijmegen University). ^{13}C Chemical shifts (δ) are expressed in p.p.m. relative to that of internal acetone at 31.55 p.p.m., with an accuracy of 0.02 p.p.m. 500-MHz ^{1}H-n.m.r. spectra were recorded at 27° with a Bruker AM-500 spectrometer operating in the pulsed F.t. mode and controlled by an Aspect 3000 computer (SON hf-NMR-facility). Prior to ^{1}H-n.m.r. spectroscopy, the pD-adjusted samples were repeatedly exchanged in D_2O after which the pD was readjusted to 3.6. Chemical shifts (δ) are expressed in p.p.m. downfield from that for sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS), but were actually measured by reference to internal acetone (δ 2.225 p.p.m. in D_2O at 27°) with an accuracy of 0.002 p.p.m. ^{31}P-Decoupling for ^{1}H- as well as for ^{13}C-n.m.r. spectra was effected using an external source with in-built phase-cycling necessary for Waltz-decoupling[4]. The ^{31}P-decoupled ^{1}H-n.m.r. spectrum was obtained by using a broad-band probe tuned for both ^{1}H and ^{31}P; the ^{31}P-decoupled ^{13}C-n.m.r. spectrum was obtained with a dual-probe, tuned for ^{13}C, ^{31}P, and ^{1}H, simultaneously.

The 2D-^{13}C–^{1}H shift-correlation experiments were performed with simultaneous suppression of ^{1}H homonuclear couplings[5,6], using the standard Bruker pulse program XHCORRD. In this experiment, the phase cycling of the refocusing pulse, as described by Wilde[7], was used in addition. Refocusing delays as required in the experiment were adjusted to an average $^{1}J_{C,H}$ coupling constant of 150 Hz[8]. In order to determine the 90° and 180° ^{1}H pulse-width, the spectrometer configuration was set for the 2D-experiment and the pulse-sequence 90°(^{13}C) – 1/(2*J*) – ϕ(^{1}H) – acq(^{13}C) was employed using a D-glucose sample in which C-1 was enriched 90% with ^{13}C. In this pulse-sequence, *J* is the one-bond C–H coupling for

glucose C-1α and the ^{1}H pulse-widths are determined by a zero C-1α signal for ϕ = 90° and a maximum reversed signal, with respect to the signal at ϕ = 0, for ϕ = 180°. The ^{13}C and ^{1}H 90° pulse-widths were 8 and 12 μs, respectively. A 128 × 4K data matrix was acquired which was zero-filled prior to Fourier-transformation to obtain a 2K × 8K spectral data matrix. A 1/6 π shifted sine-squared function for ^{13}C-sub-spectra and a non-shifted sine-bell function for ^{1}H-sub-spectra were applied to enhance resolution.

2D-^{1}H–^{1}H correlation spectra were obtained by a three-pulse sequence, 90° $- t_1 -$ 90° $-$ 90° $-$ acq, which, in the proper phase-cycling, allowed for coherence transfer through a double quantum filter[9]. Phase-sensitive handling of the data in ω_1 dimension became possible by TPPI[10]. A 480 × 4K (fraction *A*) or 800 × 4K (fraction *B*) data matrix was obtained, which was zero-filled to 2K × 4K prior to Fourier-transformation. Resolution enhancement in ω_2 and suppression of truncation effects in ω_1 were obtained by a Gaussian window function in t_2 and a cosine-squared-bell function in t_1.

RESULTS AND DISCUSSION

All n.m.r. spectroscopic studies were performed at a pD of ~3.6, which is in between the pK_{a1} and pK_{a2} values of the phosphate group, at ~1 and ~6, respectively. At this pD value, ^{13}C-n.m.r. chemical shifts of sugar phosphate resonances are not influenced[11] by slight variations in the actual pD. The same pD-dependence will probably also hold for ^{1}H- and ^{31}P-n.m.r. chemical shifts of sugar phosphates.

For the mixture *T* of lactose monophosphates, the 81-MHz ^{31}P-n.m.r. spectra without and with ^{1}H-decoupling are shown in Fig. 1. The ^{1}H-coupled ^{31}P-n.m.r. spectrum (Fig. 1a) exhibits two doublets at δ 0.53 and 1.45 with $^{3}J_{POCH}$ of 8.2 and 9.5 Hz, respectively, and occurring in an intensity ratio of 9:11. Furthermore, two triplets are present at δ 0.92 and 1.11, with $^{3}J_{POCH}$ of 4.5 and 6.9 Hz, respectively, in an intensity ratio of 2:3. The total intensities of the doublets amount to three times that of the triplets.

For fraction *B*, obtained after h.p.l.c. of *T*, the ^{31}P-n.m.r. spectrum only shows the presence of two doublets at δ 0.53 and 1.45. This feature points to the presence of at least two secondary monophosphates[12]. Consequently, the two triplets in the ^{31}P-n.m.r. spectrum of the mixture *T*, which correspond to two or more primary monophosphates, belong to the compounds in fraction *A*. However, the amount of material in fraction *A* was too low for experimental verification by ^{31}P-n.m.r. spectroscopy.

The 50-MHz ^{13}C-n.m.r. spectra of fractions *B* and *A* are shown in Figs. 2 and 3, respectively. Phosphorylation of a sugar unit will result in a down-field shift of 1.7–4.8 p.p.m. of the resonance of the carbon atom to which the phosphate group is attached, whereas those of the neighbouring carbons are shifted up-field, but usually to a lesser extent[13–16]. Furthermore, this spectrum will show $^{2}J_{POC}$ and, for the neighbouring atoms, $^{3}J_{POCC}$ couplings.

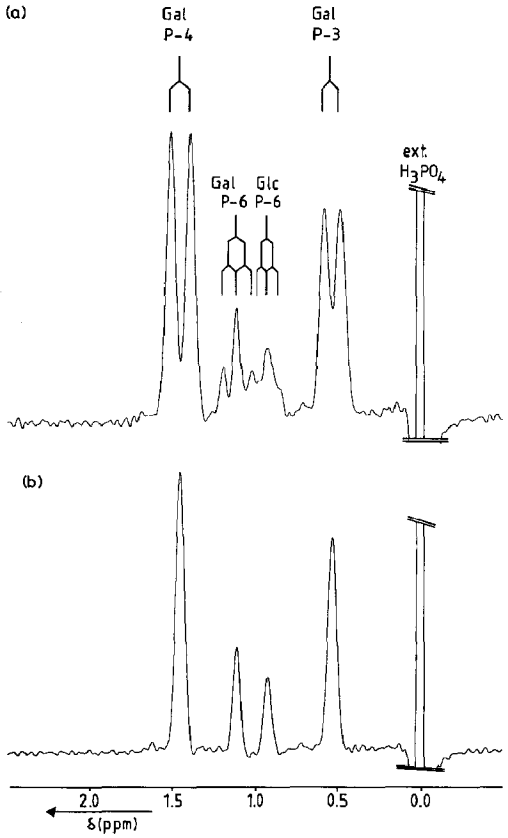


Fig. 1. 81-MHz ^{31}P-n.m.r. spectrum of fraction *T*, (*a*) without and (*b*) with ^{1}H-decoupling. The assignments are based upon further ^{13}C- and ^{1}H-n.m.r. spectroscopic analysis.

The ^{13}C-n.m.r. spectrum of fraction *B* (Fig. 2) contains C-1α and C-1β signals for the glucose moiety at δ 93.1 and 97.1, respectively. The positions and intensity ratio (3:5 for α:β) are equal to those of unsubstituted lactose (see Table I). This holds also for the other Glc signals, except those of C-4α and C-4β, which have slightly altered positions and are each present with two signals of differing intensities, *i.e.*, 3:4. These findings indicate the presence of at least two types of lactose, in which Glc is not phosphorylated. For the galactose moiety, three signals occur for C-1 at $\delta \sim 104$. Those at δ 104.28 and 104.32 are present in an intensity ratio equal to the $\alpha\beta$-ratio. Two signals are present for Gal C-6 at δ 62.31 and 62.04 in an intensity ratio of 3:4. The positions of these signals are analogous to those for unsubstituted lactose (see Table I). Therefore, Gal C-2,3,4 have to be considered as possible sites of phosphorylation. When ^{1}H- and ^{31}P-decoupling are applied simultaneously, 6 signals are recognised in the ^{13}C-n.m.r. spectrum of fraction *B* containing a ^{13}C–^{31}P coupling. In Fig. 2, these signals are indicated with splitting

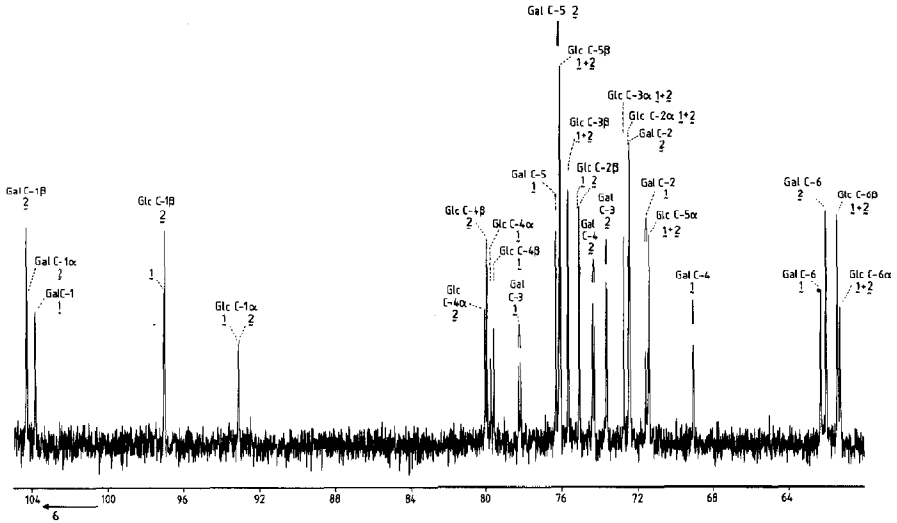


Fig. 2. 50-MHz ^{13}C-n.m.r. ^{1}H-decoupled spectrum of fraction *B*, containing lactose 3′- (**1**) and 4′-phosphate (**2**). ^{13}C–^{31}P couplings are indicated with splitting patterns in the spectrum.

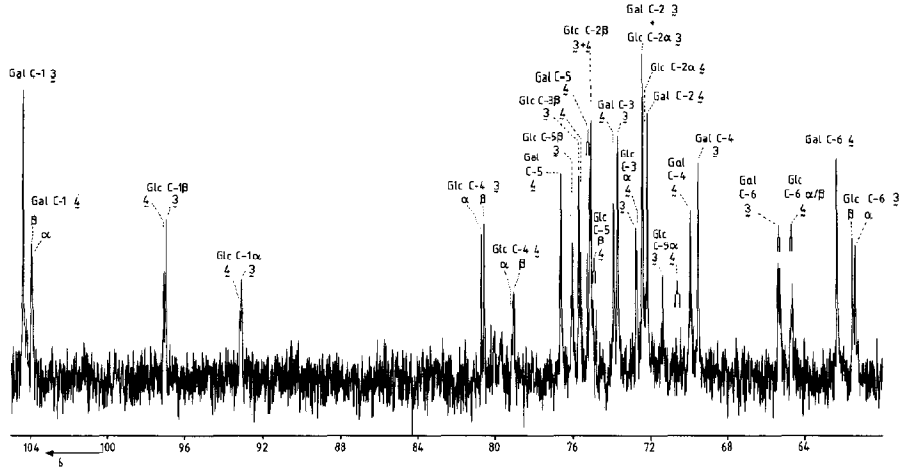


Fig. 3. 50-MHz ^{13}C-n.m.r. ^{1}H-decoupled spectrum of fraction *A*, containing lactose 6′- (**3**) and 6-phosphate (**4**). ^{13}C–^{31}P couplings are indicated by splitting patterns in the spectrum.

patterns. Taking into account the above-mentioned effects of phosphate substitution, the ^{13}C-n.m.r. spectrum is now interpreted in terms of a 3:4 mixture of lactose 3′- (**1**) and 4′-phosphate (**2**). The ^{13}C-n.m.r. assignments for these compounds together with the chemical shift differences relative to lactose are listed in Table I. The identification of the compounds accords with the conclusions reached from the ^{31}P-n.m.r. spectra of fraction *B*.

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS[a] (δ) OF LACTOSE AND ITS 3'-, 4'-, 6'-, AND 6-PHOSPHATES

Atom	*Compound*				
	Lactose	*3'-Phosphate*	*4'-Phosphate*	*6'-Phosphate*	*6-Phosphate*
Glc 1α	93.12	93.12 (0)[b]	93.09 (−3)	93.09 (−3)	93.17(+5)
2α	72.47	72.46 (−1)	72.43 (−4)	72.41 (−6)	72.28 (−19)
3α	72.72	72.71 (−1)	72.71 (−1)	72.75 (+3)	72.69 (−3)
4α	79.78	79.75 (−3)	80.05 (+27)	80.73 (+95)	79.10 (−68)
5α	71.39	71.39 (0)	71.39 (0)	71.33 (−36)	70.51 (−88)
6α	61.27	61.25 (−2)	61.25 (−2)	61.42 (+15)	64.66 (+339)
Glc 1β	97.07	97.07 (0)	97.03 (−4)	97.00 (−7)	97.13 (+6)
2β	75.12	75.12 (0)	75.08 (−4)	75.05 (−7)	75.05 (−7)
3β	75.68	75.67 (−1)	75.67 (−1)	75.70 (+2)	75.61 (−7)
4β	79.65	79.60 (−5)	79.95 (+30)	80.57 (+92)	79.10 (−55)
5β	76.09	76.08 (−1)	76.08 (−1)	76.02 (−7)	74.87 (−122)
6β	61.39	61.39 (0)	61.39 (0)	61.56 (+17)	64.70 (+331)
Gal 1α	104.23	103.87 (−36)	104.28 (+5)	104.40 (+17)	103.91 (−32)
1β			104.32 (+9)		103.96 (−27)
2	72.26	71.51 (−75)	72.39 (+13)	72.41 (+15)	72.17 (−9)
3	73.84	78.24 (+440)	73.63 (−21)	73.69 (−15)	73.90 (+6)
4	69.86	69.05 (−81)	74.32 (+446)	69.50 (+36)	69.94 (+8)
5	76.65	76.30 (−35)	76.11 (−54)	75.20 (−145)	76.60 (−5)
6	62.33	62.31 (−2)	62.04 (−29)	65.34 (+301)	62.33 (0)

[a]Expressed relative to the signal for acetone at 31.55 p.p.m. [b]In brackets are the chemical shift differences relative to lactose (× 100).

The 50-MHz ^{1}H-decoupled ^{13}C-n.m.r. spectrum of fraction *A* is shown in Fig. 3. The spectrum contains signals for Glc C-1α and C-1β and for Gal C-1 at positions that are shifted only slightly with respect to those for unsubstituted lactose. As in fraction *B*, the anomeric signals are split into two signals, the intensity ratio being 3:2. The Gal C-1 signal at δ 103.9 shows a doubling due to anomerisation of the Glc residue. These findings point to the presence of two or more lactose monophosphates, in which the phosphate is not attached to Glc C-1. From a simultaneously ^{31}P- and ^{1}H-decoupled ^{13}C-n.m.r. spectrum of fraction *A*, a number of doublets due to ^{13}C–^{31}P coupling are recognised, as indicated in Fig. 3 together with the splitting patterns. Two of these signals, at δ 64.7 and 65.3 (the former being resolved into an α and a β signal), are ~3 p.p.m. down-field from those of Glc and Gal C-6, respectively, in free lactose. These signals are ascribed to C-6 of a Glc and a Gal residue with a phosphate at C-6. The bulk-region of the spectrum shows 3 additional signals with a ^{13}C–^{31}P coupling. These are at positions expected for C-5 of Gal and Glc residues of lactose with phosphate at C-6 or C-6'. The total spectrum is then interpreted to be derived from a 3:2 mixture of lactose 6'- (**3**) and 6-phosphate (**4**). The ^{13}C-n.m.r. assignments together with the chemical shift differences relative to lactose are listed in Table I.

For all four lactose phosphates, the ^{13}C-n.m.r. shift-effects induced by phos-

phate substitution are restricted to the α, β, and γ carbons, *i.e.*, the α-carbon resonance is shifted down-field 4.5 p.p.m. (Gal C-3 and C-4 in **1** and **2**, respectively) and 3.0 p.p.m. (Gal C-6 and Glc C-6 in **3** and **4**, respectively). The β- and γ-carbon resonances are shifted up-field <1 p.p.m., the effect on the β-carbon resonance being the largest. For lactose 4′- and 6′-phosphate, an additional down-field shift is observed for the resonances of Glc C-4α and C-4β. Furthermore, for lactose 6-phosphate, there is an up-field shift for the resonance of Gal C-6. Since these extra shift effects are restricted to atoms involved in the glycosidic linkage, they probably originate from small alterations of the conformation about this linkage.

The 500-MHz ^{1}H-n.m.r. spectra of fractions *A* and *B* are presented in Fig. 4, which includes the difference spectra obtained after subtracting the corresponding ^{31}P-decoupled 500-MHz spectra. As expected from the ^{31}P-n.m.r. spectrum (see Fig. 1), several ^{31}P–^{1}H couplings are present. The ^{1}H-n.m.r. spectra were unravelled by application of heteronuclear ^{13}C–^{1}H spectroscopy. The 2D-^{13}C–^{1}H COSY spectrum of fraction *T* at a ^{1}H-n.m.r. frequency of 200 MHz is depicted in Fig. 5. In this experiment, ^{1}H–^{1}H couplings were eliminated in order to obtain maximum resolution and sensitivity. The resolution in the ^{1}H domain is sufficient to resolve the ^{31}P–^{1}H couplings, when present. This is so for Gal C-3,4,6 in **1**–**3**, respectively (see the enlarged cross-peaks in Fig. 5). The cross-peak for Glc C-6α/β is below the lowest level of the contour plot. The 2D-^{13}C–^{1}H COSY spectrum affords ^{1}H-n.m.r. chemical shifts for all atoms in all four lactose monophosphates and allows proper assignment of all structural reporter-groups in the 500-MHz ^{1}H-n.m.r. spectrum of fractions *A* and *B*. Refinement of these assignments is obtained by 2D-^{1}H–^{1}H correlation spectroscopy at 500 MHz (see Fig. 6, ^{1}H–^{1}H COSY spectrum of fraction *B*). For several atoms, only approximate ^{1}H-n.m.r. chemical shifts are obtained due to interference of cross-peaks with diagonal-peaks (see Table II). The precise values of the Glc H-6 resonances in lactose 6-phosphate are difficult to establish since the H-6a and H-6b signals are nearly isochronous and, furthermore, the resonances of their respective α and β protons have slightly different chemical shifts, giving rise to a complex multiplet for these signals. The signals for H-6a and H-6b of Gal in lactose 6′-phosphate are superimposed at δ 4.024. For all compounds, phosphate attachment induces a down-field shift for nearly all the protons of the monosaccharide unit to which phosphate is attached. The effect on the α-proton is the largest, *i.e.*, 0.45 and 0.55 p.p.m. for the 3- and 4-phosphate, respectively, and 0.25–0.30 p.p.m. for the 6-phosphate. The effect on the resonance of the β-proton can also be substantial, *e.g.*, up to 0.22 p.p.m. for H-4 of lactose 3′-phosphate.

The various ^{31}P–^{1}H and ^{31}P–^{13}C coupling constants determined from the ^{13}C-, ^{31}P-, and ^{1}H-n.m.r. spectra (see Table III) are interpreted in terms of a distribution among a number of preferred orientations of the phosphate group relative to the sugar residue to which it is attached. For lactose 3′-phosphate, 3 possible rotamers are assumed. When the HCOP torsion-angle (θ) is taken as reference, these orientations are *trans* (t), *gauche*$^{+}$ (g^{+}), and *gauche*$^{-}$ (g^{-}), with the fractional

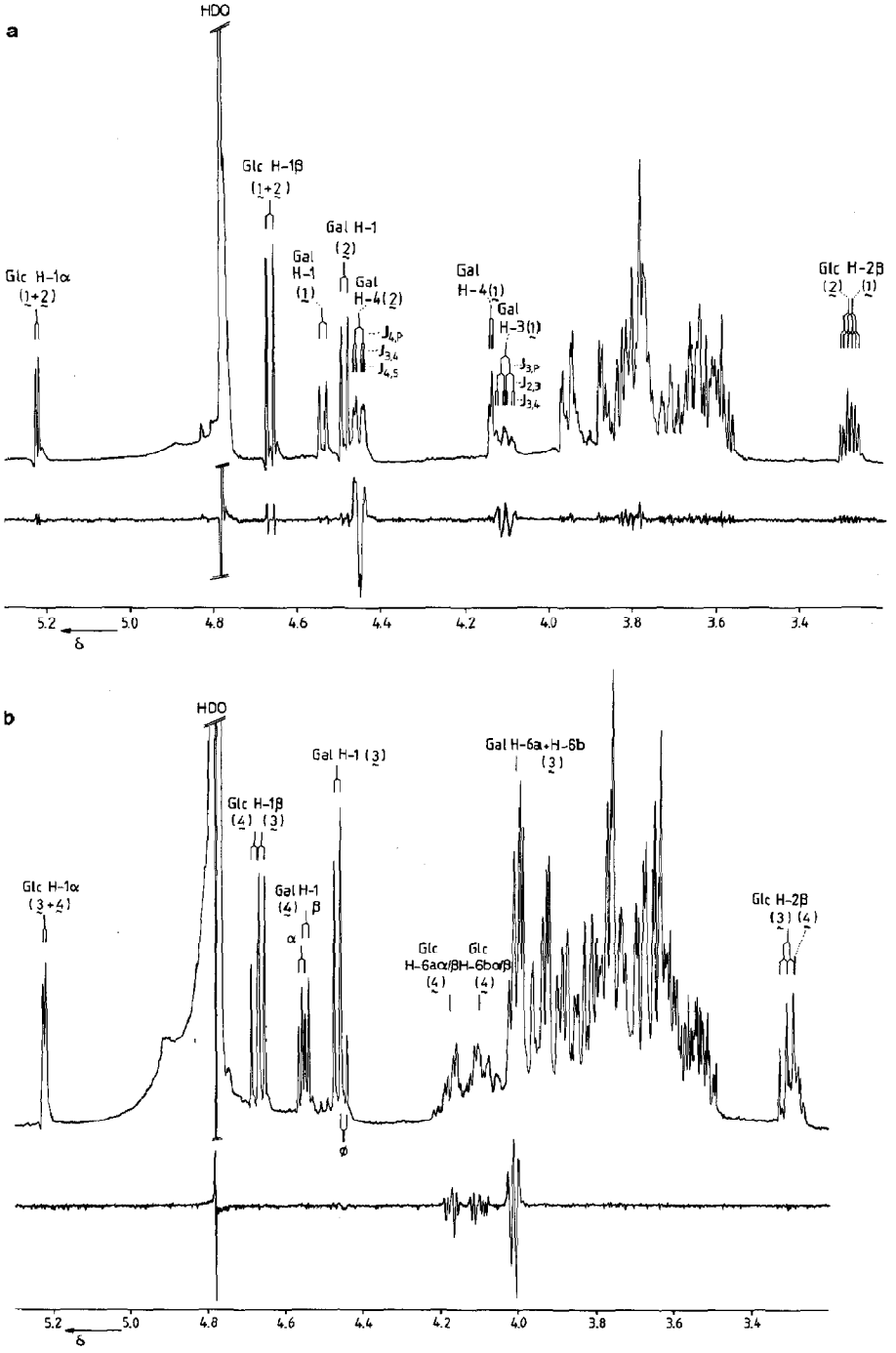


Fig. 4. 500-MHz ^{1}H-n.m.r. spectra of (*a*) fraction *A*, containing lactose 3′- (**1**) and 4′-phosphate (**2**), and (*b*) fraction *B*, containing lactose 6′- (**3**) and 6-phosphate (**4**). Included are the difference spectra with their corresponding ^{31}P-decoupled ^{1}H-n.m.r. spectra.

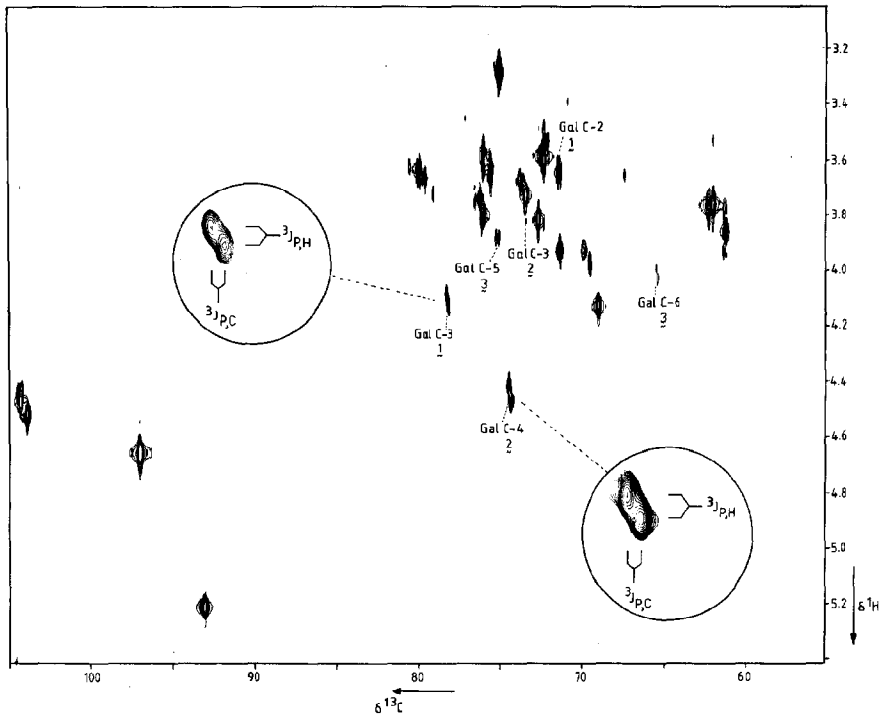


Fig. 5. 2D-^{13}C–^{1}H heteronuclear shift-correlation spectrum of fraction *T*, containing all four lactose phosphates, at a ^{1}H-frequency of 200 MHz. Indicated in the Figure are only those signals that exhibit a ^{31}P–^{13}C coupling. In the two insets, the cross-peaks of Gal C-3 and Gal C-4 of **1** and **2**, respectively, have been enlarged, *i.e.*, with different enlargement factors in the ^{1}H- and ^{13}C-dimensions.

populations of these orientations being P_t, P_{g^+}, and P_{g^-}. The observed (time-averaged) coupling constants are then determined by the following equations.

$$^3J_{POCH} = P_{g^-}J(g^-) + P_{g^+}J(g^+) + P_tJ(t) \quad (1)$$

$$^3J_{POCC\text{-}2'} = P_{g^-}J'(g^-) + P_{g^+}J'(g^+) + P_tJ'(t) \quad (2)$$

$$^3J_{POCC\text{-}4'} = P_{g^-}J''(g\ ) + P_{g^+}J''(g') + P_tJ''(t) \quad (3)$$

$$P_{g^-} + P_{g^+} + P_t = 1 \quad (4)$$

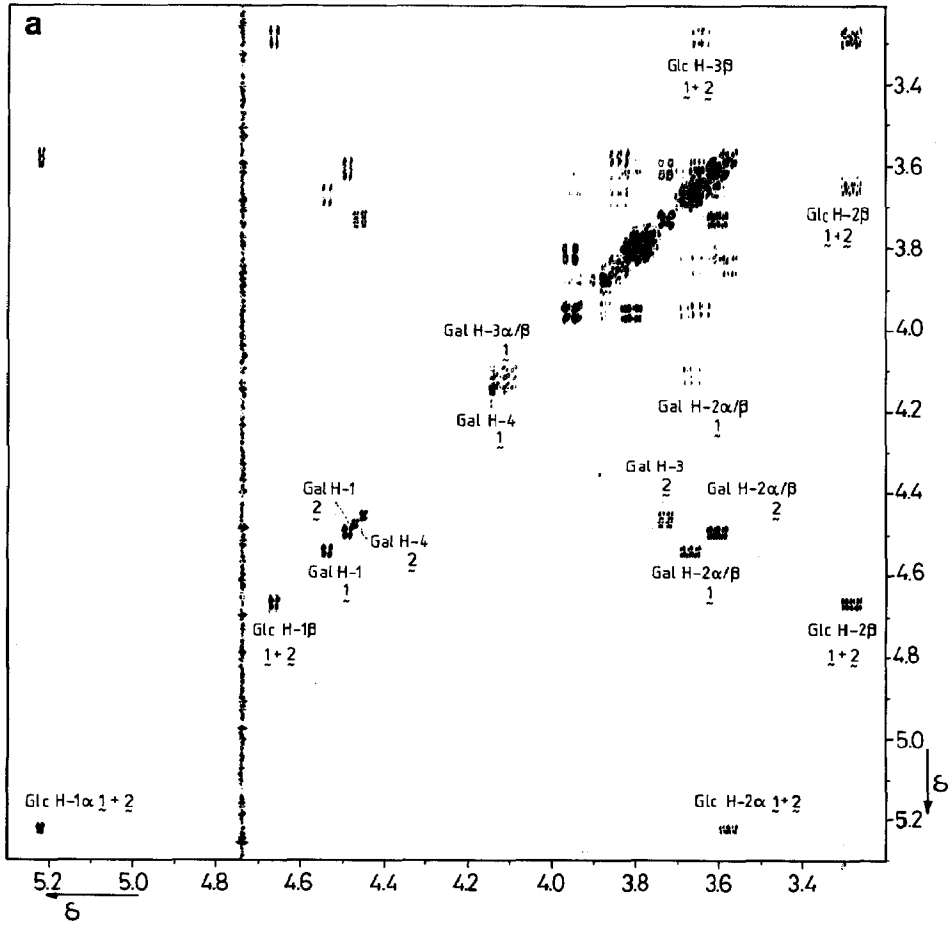
Since no information is available about the exact value of θ for the different orientations, the classical values -60, $+60$, and $+180$ degrees are used for g^-, g^+, and t, respectively. For the calculation of the coupling constants for the three orientations, a set of Karplus equations (*5* and *6*) is used for vicinal coupling-constants in CCOP and HCOP fragments[17–19].

$$^3J_{POCH} = 15.3 \cos^2\theta - 6.1 \cos\theta + 1.6 \quad (5)$$

$$^3J_{POCC} = 6.9 \cos^2\theta - 3.4 \cos\theta + 0.7 \quad (6)$$

The parameters in equations *5* and *6* were derived simultaneously, using a large data set of vicinal ^{31}P–^1H and ^{31}P–^1H coupling-constants[17]. These equations indicate, for the two *gauche* orientations, $^3J_{POCH}$ and $^3J_{POCC}$ to be ~2 and ~0.5 Hz, respectively, while, for the *trans* orientation, $^3J_{POCH}$ and $^3J_{POCC}$ are ~23 and ~11 Hz, respectively. On the basis of equations *1–4,* the populations are found for the HCOP fragment in lactose 3′-phosphate, *i.e.,* $P_{g^-}:P_{g^+}:P_t \approx 0.6:0.1:0.3$. These findings indicate the g^+ orientation to be unfavourable, probably due to steric interactions with O-2.

For lactose 4′-phosphate, a completely analogous handling of the vicinal coupling constants involving the phosphate group indicates the three orientations



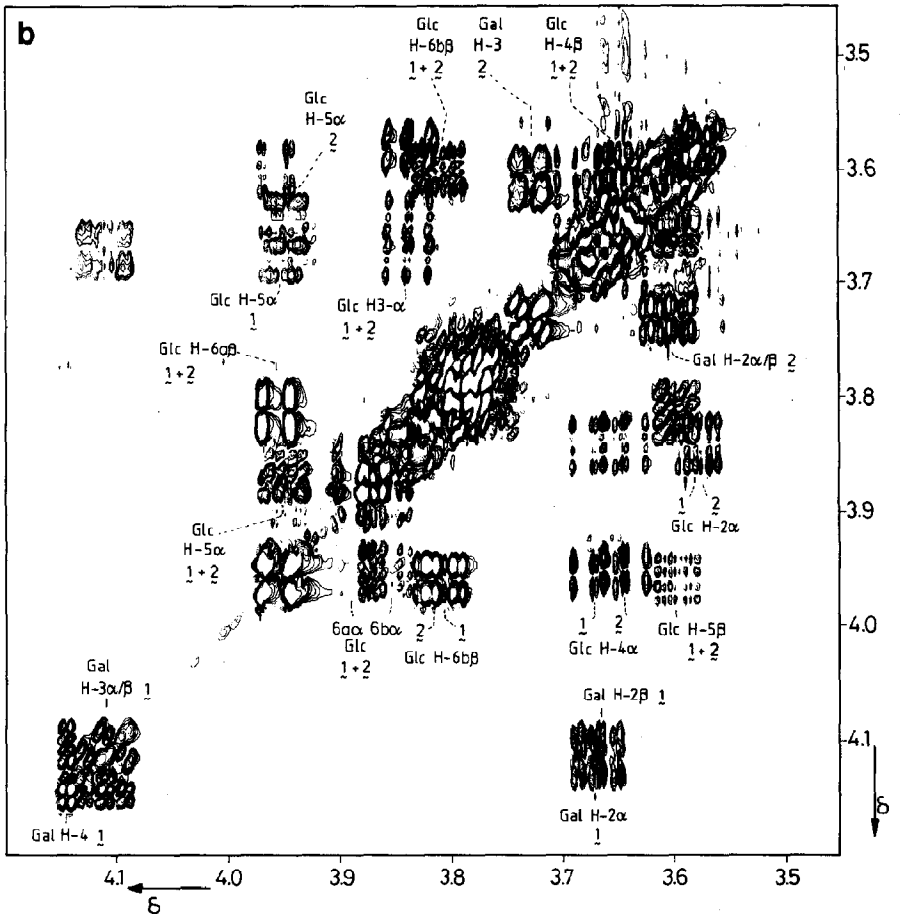


Fig. 6. 500-MHz 2D-^{1}H–^{1}H double-quantum-filtered shift-correlation spectrum of fraction *B*, containing lactose 3′- (**1**) and 4′-phosphate (**2**). Shown are (*a*) the total spectrum and (*b*) the region of skeleton protons.

for the HCOP fragment to be populated as $P_{g^-}:P_{g^+}:P_t \approx 0.2:0.4:0.4$. This indicates a slightly decreased tendency for the phosphate group to adopt the g^- orientation, which is probably due to steric interaction with the Gal hydroxymethyl group.

For lactose 6′-phosphate and lactose 6-phosphate, no separate vicinal coupling constants are observed with the two H-6 in each phosphorylated hydroxymethyl group. In that case, only the population can be determined of the orientation for which the phosphate group is *trans* with respect to C-5, using the equation[17]:

$$P_t = (25.5 - \Sigma')/20.5, \qquad (7)$$

where $\Sigma' = {}^{3}J_{POCH\text{-}6a} + {}^{3}J_{POCH\text{-}6b}$.

TABLE II

^{1}H-N.M.R. CHEMICAL SHIFTS[a] (δ) FOR LACTOSE AND ITS 3′-, 4′-, 6′-, AND 6-PHOSPHATES

Proton	*Compound*				
	Lactose[b]	*3′-Phosphate*	*4′-Phosphate*	*6′-Phosphate*	*6-Phosphate*
Glc 1α	5.221	5.222 (+1)[c]	5.222 (+1)	5.221 (0)	5.224 (+3)
2α	3.577	3.575 (−2)	3.571 (−6)	3.586 (+9)	3.607 (+30)
3α	3.832	3.844 (+12)	3.840 (+8)	3.830 (−2)	3.830 (−2)
4α	3.646	3.674 (+28)	3.647 (+1)	3.624 (−2)	3.734 (+88)
5α	4.95	3.95 (0)	3.95 (0)	3.947 (0)	4.062 (+110)
6aα	3.878	3.88 (0)	3.88 (0)	3.88 (0)	4.18 (+300)
6bα	3.852	3.85 (0)	3.85 (0)	3.85 (0)	4.11 (+260)
Glc 1β	4.660	4.665 (+5)	4.665 (+5)	4.662 (+2)	4.682 (+22)
2β	3.285	3.280 (−5)	3.289 (+4)	3.314 (+29)	3.295 (+10)
3β	3.630	3.643 (+13)	3.646 (+16)	3.639 (+9)	3.639 (+9)
4β	3.65	3.67 (+2)	3.66 (+1)	3.65 (0)	3.65 (0)
5β	3.595	3.594 (−1)	3.594 (−1)	3.60 (0)	3.75 (+15)
6aβ	3.950	3.960 (+10)	3.960 (+10)	3.947 (−3)	4.18 (+23)
6bβ	3.797	3.817 (+20)	3.809 (+12)	3.787 (−10)	4.11 (+31)
Gal 1α	4.448	4.542 (+94)	4.493 (+45)	4.469 (+21)	4.556 (+108)
2α	3.549	3.676 (+127)	3.612 (+63)	3.554 (+5)	3.521 (−28)
3α	3.663	4.114 (+451)	3.733 (+70)	3.686 (+23)	3.678 (15)
4α	3.926	4.147 (+221)	4.463 (+537)	3.985 (+59)	3.922 (−4)
5α	3.72	3.76 (+40)	3.81 (+90)	3.885 (+170)	3.72 (0)
6aα	3.76	3.76 (0)	3.76 (0)	4.024 (+260)	3.76 (0)
6bα	3.79	3.79 (0)	3.79 (0)	4.024 (+230)	3.79 (0)
Gal 1β	4.448	4.540 (+92)	4.492 (+44)	4.469 (+12)	4.546 (+89)
2β	3.540	3.668 (+128)	3.604 (+64)	3.554 (+14)	3.518 (−22)
3β	3.660	4.111 (+451)	3.733 (+73)	3.686 (+26)	3.678 (+18)
4β	3.926	4.147 (+221)	4.463 (+537)	3.985 (+59)	3.922 (−4)
5β	3.72	3.76 (+40)	3.81 (+90)	3.885 (+170)	3.72 (0)
6aβ	3.76	3.76 (0)	3.76 (0)	4.015 (+260)	3.76 (0)
6bβ	3.79	3.79 (0)	3.79 (0)	4.015 (+230)	3.79 (0)

[a]Chemical shifts are relative to the signal of DSS (using internal acetone at δ 2.225 p.p.m.) in D_2O. [b]Chemical shifts for lactose have been obtained by ^{13}C–^1H and ^1H–^1H 2D spectroscopy. [c]In brackets are the chemical shift differences relative to lactose (× 1000).

From the experimental values in Table III, it then follows that, for lactose 6′-phosphate, the t orientation is adopted for ~60% of the time; for lactose 6-phosphate, this is ~80%. The predominance of the t orientation for both lactose 6′-phosphate and lactose 6-phosphate is in agreement with the large $^3J_{POCC\text{-}5}$ for both disaccharides, *i.e.*, application of equation *2* for this linkage yields 7 and 9 Hz, respectively. The orientation around the C-5–C-6 bond cannot be determined solely from the observed ^{31}P coupling-constants, but the estimated coupling-constants of H-5 with H-6a and H-6b for the phosphorylated hydroxymethyl groups do not suggest a dramatic deviation from the situation for free lactose.

The results presented indicate that the combined use of different n.m.r. spectroscopic techniques is needed when identifying a carbohydrate compound for which not enough reference data are available. The complexity of the ^{1}H-n.m.r.

TABLE III

^{31}P–^{1}H AND ^{31}P–^{13}C COUPLING CONSTANTS (Hz) FOR LACTOSE PHOSPHATES AS DETERMINED FROM ^{13}C-, ^{31}P-, AND ^{1}H-N.M.R. SPECTRA

Linkage	*Compound*			
	3'-Phosphate	*4'-Phosphate*	*6'-Phosphate*	*6-Phosphate*
POCC-2'	6.1[a]			
POC-3'	5.2[a]			
POCC-3'		1.9[a]		
POC-4'		5.5[a]		
POCC-4'	1.5[a]			
POCC-5'		4[a]	8[a]	
POC-6'			5[a]	
POCC-5				8.9[a]
POC-6				6[a]
POCH-3'	8.2[b]			
POCH-4'		9.5[b]		
POCH-6'			6.9[c]	
POCH-6				4.5[c]

[a]Determined from ^{1}H-decoupled ^{13}C-n.m.r. spectra. [b]Determined from ^{1}H-n.m.r. and ^{31}P-n.m.r. spectra. [c]Determined from ^{31}P-n.m.r. spectra.

spectra of the fractions *A* and *B* precluded application of an approach using structural reporters only[20,21]. However, the combined use of ^{31}P- and ^{13}C-n.m.r. spectroscopy afforded enough data to identify the constituents of the two mixtures. Application of shift effects in ^{13}C-n.m.r. spectroscopy may prove more fruitful than in ^{1}H-n.m.r. spectroscopy when completely new compounds are concerned. The pH was of major importance when comparing the n.m.r. spectra of the different lactose phosphate-containing fractions.

Phosphate-containing oligosaccharides from biological sources are usually obtained only in low amounts, which might preclude application of ^{13}C- and ^{31}P-n.m.r. spectroscopy, and the identification of the primary structures of these oligosaccharides might still rely on analysis of structural reporter-groups, combined with analysis of sub-spectra using spinlock techniques[22]. A proper understanding of ^{31}P-induced shift effects in the ^{1}H-n.m.r. spectra is therefore of value when identifying primary structures of oligosaccharides containing phosphate groups. Also, the use of 2D-^{13}C–^{1}H-n.m.r. spectroscopy may be suitable for the study of sulphated oligosaccharides for which larger quantities are available and might provide data for structural analysis using only the structural reporter-groups in a high-resolution ^{1}H-n.m.r. spectrum.

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