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Note

Use of proton n.m.r. spectroscopy for detection of 2-acetamido-2-deoxy-D-mannose- and 2-acetamido-2-deoxy-D-mannuronate-containing carbohydrates*

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2-Amino-2-deoxy-D-mannose and 2-amino-2-deoxy-D-mannuronic acid occur, usually as their *N*-acetylated derivatives, in a variety of carbohydrates of prokaryotic[1–7] and eukaryotic origin[8–10]. Unfortunately, the similarity of these sugars to the widely occurring D-*gluco* and D-*galacto* stereoisomers has complicated the differentiation and resolution of these sugars by colorimetric[11] and chromatographic means[12–14]. Complicating the chemistry of analysis is the fact that some acetamido sugars, and especially some acetamidohexuronic acids, exhibit marked lability during acid hydrolysis[3,11,15,16]. Furthermore, the equilibrium between 2-acetamido-2-deoxy-D-mannose and 2-acetamido-2-deoxy-D-glucose, readily established under alkaline conditions, markedly favors the D-*gluco* epimer[17,18].

The cell wall of *Micrococcus luteus* contains a teichuronic acid consisting[3,19] of a repeating copolymer of 2-acetamido-2-deoxy-D-mannuronic acid and D-glucose, namely, $[\beta\text{-D-Man}p\text{NAcA-}(1\rightarrow6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow4)]_n$. During investigations on the structure and biosynthesis of this polysaccharide[20], it was observed that proton nuclear magnetic resonance spectroscopy (^{1}H-n.m.r.) showed three equimolar resonances in the downfield region of the spectrum (4.3–5.5 p.p.m.) normally associated with resonances of anomeric protons of polysaccharides. The unexpected third resonance has been attributed to H-2 of the 2-acetamido-2-deoxy-β-D-mannosyluronic acid residues. This resonance, as well as the H-2 resonances of the α and β anomers of 2-acetamido-2-deoxy-D-mannopyranose, occurs in a region of the ^{1}H-n.m.r. spectrum unencumbered by resonances from non-anomeric protons of other monosaccharides of frequent natural occurrence. This feature should per-

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mit the ready detection of, and assignment of anomeric configuration to, 2-acetamido-2-deoxy-D-mannose and 2-acetamido-2-deoxy-D-mannuronic acid residues in oligo- and poly-saccharides.

The ^{1}H-n.m.r. spectrum of teichuronic acid at 45° presented three major resonances in the downfield region (4.3–5.5 p.p.m.) which, for polysaccharides, normally includes only the resonances of anomeric (H-1) protons (Fig. 1A). As teichuronic acid consists of two monosaccharides in an alternating sequence, only two of these three resonance peaks could be attributed to anomeric protons. As the sharp resonance at 4.80 p.p.m. and the doublet at 4.48 p.p.m. in the spectrum of native teichuronic acid (Fig. 1A) are also present in the spectrum of periodate-oxidized, borohydride-reduced teichuronic acid as resonances at 4.77 and 4.46 p.p.m. (Fig. 1B), these resonances may be attributed to the 2-acetamido-2-deoxy-β-D-mannurosyluronic acid residues, which are resistant to oxidation by periodate. Coupling of these resonances was demonstrated in both preparations by double-resonance spin-decoupling. Additional evidence is given here to substantiate the assignment of these resonances to H-1 and H-2, respectively, of the 2-acetamido-2-deoxy-β-D-mannosyluronic acid residues. A major spectral change effected by the periodate oxidation is a change of the doublet at 5.38 p.p.m. ($J_{1,2}$ = 3.6 Hz) (Fig. 1A) to a triplet at 4.89 p.p.m. (Fig. 1B). These resonances are assigned respectively to H-1 of the α-D-glucosyl residues[21] and the corresponding proton after suc-

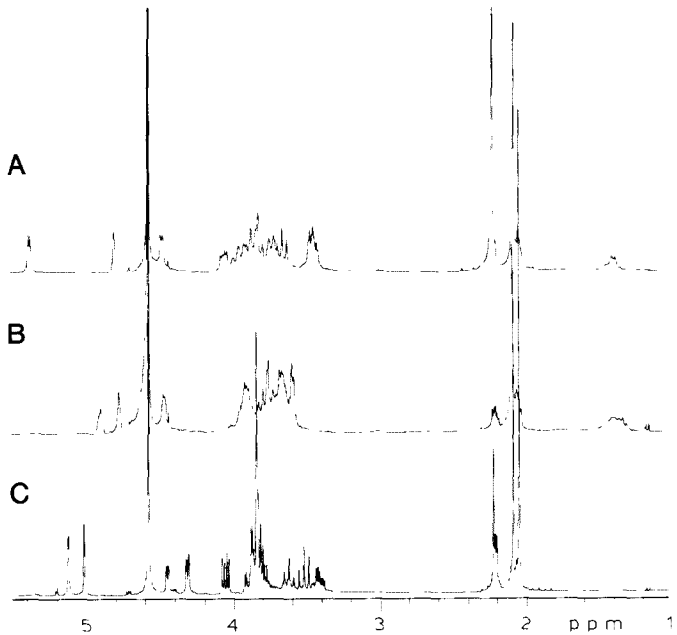

Fig. 1. ^{1}H-N.m.r. spectra at 300 MHz of (A) teichuronic acid, (B) periodate-oxidized, borohydride-reduced teichuronic acid, and (C) the equilibrium mixture of α and β anomers of 2-acetamido-2-deoxy-D-mannose Acetone was added to (A) and (C) for use as internal standard.

cessive oxidation and reduction of the glucosyl residues. Alteration of the temperature to 24 or 70° shifted the large HDO resonance peak at 4.57 p.p.m. (45°), but revealed no other signals.

The major resonance at 2.08 p.p.m. is assigned to the acetamido methyl protons. The 3.4–4.1-p.p.m. spectral region is crowded with overlapping resonances attributable, without specific assignments, to the remaining protons of teichuronic acid. As the teichuronic acid was prepared by digestion of cell walls with lysozyme, fragments of peptidoglycan covalently linked to the teichuronic acid were retained during purification and are responsible for numerous minor resonances in the spectra: acetamido methyl protons of 2-acetamido-2-deoxy-β-D-glucosyl and 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucosyl (*N*-acetyl-D-muramyl) residues at 2.05 and 2.03 p.p.m.; methyl protons of alanyl residues at 1.4 p.p.m.; γ-methylene protons of isoglutamyl residues at 2.3 p.p.m.; and δ-methylene protons[22] of lysyl residues at 3.0 p.p.m.

To verify the assignment of the H-2 resonance of 2-acetamido-2-deoxy-β-D-mannosyluronic acid residues of teichuronic acid at 4.48 p.p.m., commercial 2-acetamido-2-deoxy-D-mannose was examined under similar conditions. This monosaccharide readily forms an equilibrium mixture of α and β anomers for which the spectrum is shown in Fig. 1C. The two anomeric-proton resonances lie at 5.12 p.p.m. (H-1, α anomer) and 5.01 p.p.m. (H-1, β anomer) in the ratio of 11:9 in good agreement with the 57:43 ratio of α and β anomers reported by Horton *et al.*[23]. Double-resonance spin-decoupling experiments identified the H-2 (4.31 p.p.m.) and H-3 (4.04–4.08 p.p.m.) resonances of the α anomer and the H-2 (4.44 p.p.m.) and H-3 (3.81–3.86 p.p.m.) resonances of the β anomer. These H-2 assignments lie farther downfield than any previously reported H-2 resonances for other common monosaccharides.

D-Mannose is unique among the commonly occurring aldohexoses in that its H-2 ^{1}H-n.m.r. resonance lies sufficiently downfield (4.1–4.2 p.p.m.) from the envelope of the non-anomeric proton resonances that it does not overlap with other resonances and thus may be readily observed[24]. This characteristic has been exploited in the application of ^{1}H-n.m.r. to the structural identification of mannose-containing oligosaccharides of glycoproteins[25–27]. We have observed that the H-2 resonance of 2-acetamido-2-deoxy-D-mannose is located even farther downfield than the H-2 resonance of D-mannose. This is not unexpected, as the substitution of the acetamido group in 2-acetamido-2-deoxy-D-glucopyranose in place of the hydroxyl group in D-glucopyranose causes H-2 downfield shifts of 0.35 p.p.m. for the α anomer and 0.43 p.p.m. for the β anomer[28]. Similarly, the H-2 resonance of methyl 2-acetamido-2-deoxy-β-D-galactopyranoside[29] shows an approximately equivalent downfield-shift, relative to the H-2 resonance of β-D-galactopyranose[24]. The chemical shift of the H-2 resonance is little influenced by glycosidic substitution at C-1 as compared with the corresponding reducing monosaccharide[28]. Even so, the H-2 resonances of 2-acetamido-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-galactopyranose and their glycosides are often obscured by overlapping

resonances of other protons of the same residue. In contrast, the H-2 resonance of 2-acetamido-2-deoxy-D-mannopyranose is well separated from the envelope of overlapping resonances and, by comparison with D-mannose[24], is predicted to occur at 4.3 p.p.m. for the α anomer and at 4.4 p.p.m. for the β anomer, in good agreement with the experimentally observed chemical shifts of 4.31 and 4.44 p.p.m., respectively. These assignments are in agreement with those reported for 2-acetamido-2-deoxy-β-D-mannopyranose dissolved in hexafluoro-2-propanol[30] and for[31] 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate in D_2O.

Replacement of the hydroxymethyl group at C-5 by carboxyl leads to a downfield shift of the H-2 resonance of only 0.05 p.p.m. (methyl β-D-glucopyranoside[28] *vs.* methyl β-D-glucopyranosiduronic acid[29]). Thus one should expect the chemical shift of the H-2 resonance to be moderately insensitive to the state of oxidation at C-6. Indeed, there is relatively little difference between the shift of H-2 in 2-acetamido-2-deoxy-β-D-mannopyranose (4.44 p.p.m.) and that of H-2 in the 2-acetamido-2-deoxy-β-D-mannopyranosyluronic acid residues of teichuronic acid (4.48 p.p.m.).

The H-2 resonance of 2-acetamido-2-deoxy-D-mannose and its derivatives is located downfield from the corresponding resonance of the D-*gluco* stereoisomers, because of the equatorial orientation of H-2 of the former. It is to be expected that similar H-2 chemical shifts would be displayed by 2-acetamido-2-deoxy-D-talose and its uronic acid (recently reported as a constituent of *Methanobacterium* cell-walls[15]), and also by 2-acetamido-2-deoxy-D-altrose and 2-acetamido-2-deoxy-D-idose and their uronic acids (2-amino-2-deoxy-L-altruronic acid has been reported in the lipopolysaccharide of *Shigella sonnei*[32]). These monosaccharides have not been examined by ^{1}H-n.m.r. spectroscopy by us because of lack of availability. Nevertheless, the H-2 resonance described here may be considered diagnostic of the 2-acetamido-2-deoxy hexoses having H-2 equatorially oriented. Because of the apparent rarity of the D-*altro*, D-*talo*, and D-*ido* stereoisomers, the observation of a resonance in this region of the spectrum (4.3–4.5 p.p.m.) of a mono-, oligo-, or poly-saccharide is highly suggestive of the presence of 2-acetamido-2-deoxy-D-mannose or 2-acetamido-2-deoxy-D-mannuronic acid.

EXPERIMENTAL

Teichuronic acid was obtained from lysozyme digests of *Micrococcus luteus* ATCC 4988, and purified by anion-exchange chromatography[20]. Preparations of teichuronic acid were subjected to oxidation by sodium periodate and reduction by sodium borohydride as described previously[20]. 2-Acetamido-2-deoxy-β-D-mannose and D_2O (99.8 atom% deuterium) were purchased from Sigma Chemical Company.

The D_2O was freed of paramagnetic-ion contaminants by extraction with diphenothiocarbazone in carbon tetrachloride. The polysaccharide preparations were passed over Chelex-100 (Bio-Rad Laboratories). All carbohydrates were

lyophilized twice from D_2O and redissolved at a concentration of 25–50 mg/mL in D_2O.

Spectra were recorded at 45° with a Nicolet 300 MHz, n.m.r. spectrometer operated in the Fourier-transform mode. All chemical shifts are reported as p.p.m. downfield from 4,4-dimethyl-4-silapentane-1-sulfonate, sodium salt indirectly, by using acetone as the internal standard at 2.225 p.p.m.

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