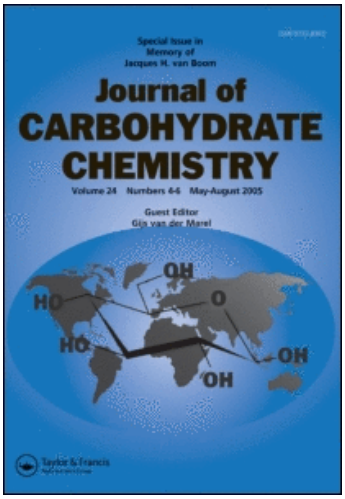

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SYNTHESIS OF OLIGOSACCHARIDES CORRESPONDING TO STRUCTURES FOUND IN CAPSULAR POLYSACCHARIDES OF *CRYPTOCOCCUS NEOFORMANS*. PART 3. TWO REGIOSELECTIVELY ACETYLATED TETRASACCHARIDES.

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ABSTRACT

Regioselective acetylation of a tetrasaccharide has been accomplished using acetyl chloride and collidine in dichloromethane at low temperature. The degree of acetylation depended on the time and equivalents of reagents used. However, only acetylation of primary positions was observed. The acetylated oligosaccharides obtained after hydrogenolysis correspond to structures found in the capsular polysaccharide of *Cryptococcus neoformans*. The acetylation pattern in the target compounds was determined both by NMR and methylation analysis.

INTRODUCTION

Cryptococcus neoformans, a fungus, is an opportunistic pathogen, that has emerged as the fourth leading cause of death among AIDS-patients. It is surrounded by a polysaccharide capsule, especially prominent during starvation periods and in the infectious mode. The capsule is an important virulence factor and governs the serotype specificity. At least four different serotypes, A- D, are known.[1] The major part of the capsule is a polysaccharide consisting of D-mannose, D-xylose, D-glucuronic acid and *O*-acetyl groups. A common feature to all serotype structures is an α-D-Man*p*-(1→3)-α-D-Man*p* backbone with D-xylopyranosyl and D-glucuronopyranosyl groups at the 2-

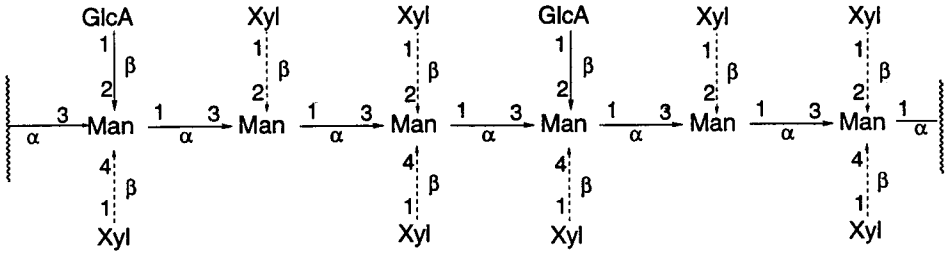


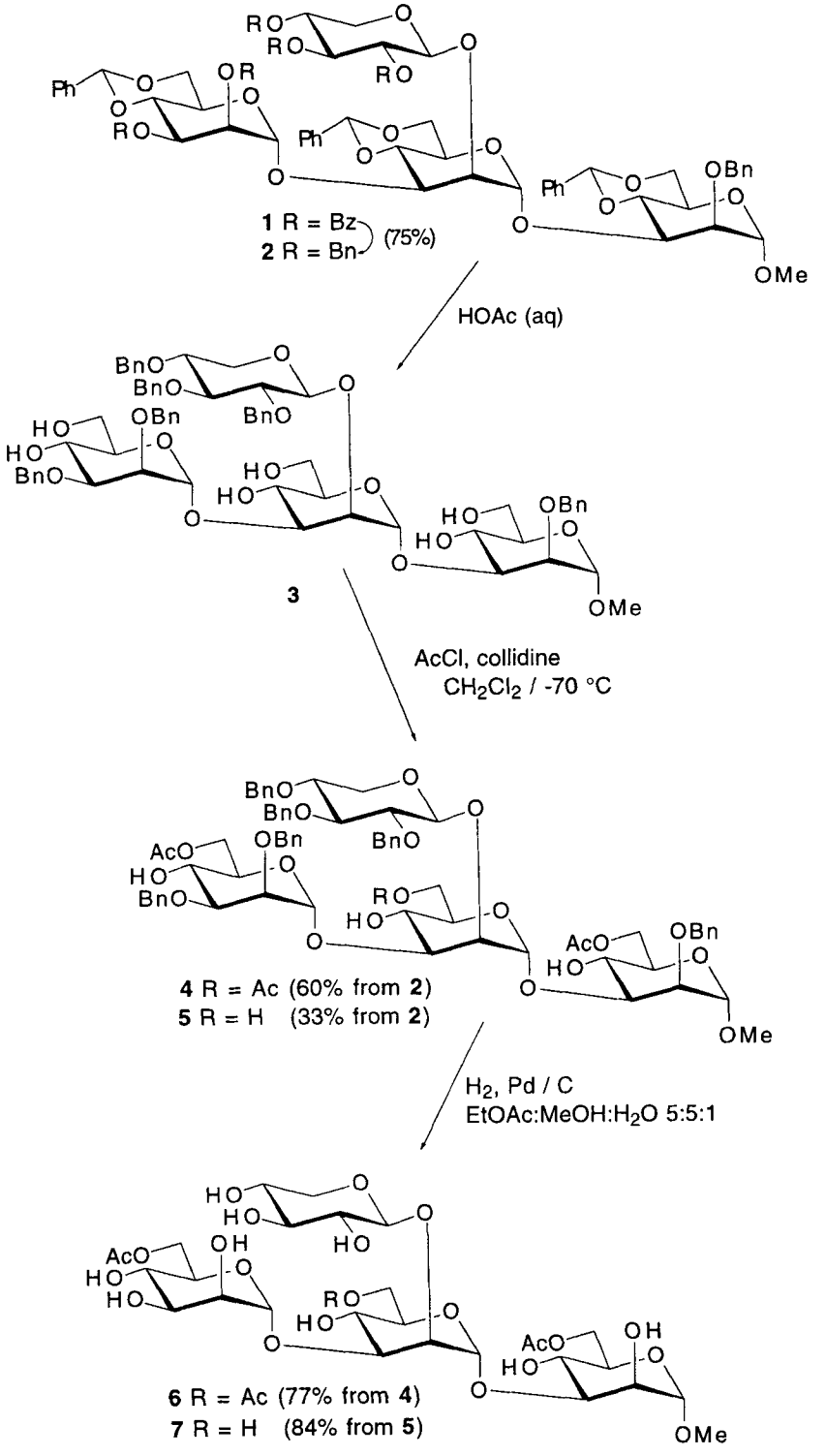
Figure 1. Generalised structure of *Cryptococcus neoformans* capsular polysaccharide.

positions of the mannosyl residues (see Fig.1). Amount and site of xylose substitution distinguish between the serotypes.

The 6-position of the mannosyl residues are often acetylated.[2] The acetates are known to be important for the conformation of the polysaccharide, and thus often determine many biological activities of the polysaccharide.[3] Recent results indicate that the acetylation patterns within each serogroup is conservative[4] and therefore probably also a cause of the serological differences together with the glycosyl side-chain pattern. Thus, to synthesise biologically active oligosaccharide structures of the polysaccharide, acetyl substituents should be included in the target structures. As a first attempt we here describe the syntheses of the tetrasaccharide **6**, in which all three mannose residues are regioselectively acetylated in the 6-positions, and the di-*O*-acetylated analogue **7**.

RESULTS AND DISCUSSION

Since our earlier syntheses of *C. neoformans* structures[5,6] contain many promising intermediates for a late introduction of the acetates, the following strategy was chosen. In the earlier synthesized tetrasaccharide **1**,[6] the benzoyl protecting groups of the xylose residue were changed into benzyl protecting groups, through Zemplén deacylation followed by benzylation with benzyl bromide and sodium hydride, to give compound **2** (75%), devoid of acyl protecting groups. In tetrasaccharide **2**, the only primary hydroxyl groups to be acetylated are on the mannose residues. Furthermore, selective removal of the benzylidene acetals would give a partially protected derivative, soluble in organic solvents, and with only three other (secondary) hydroxyl groups not to be acetylated, which makes a good candidate for a successful regioselective introduction of the acetyl groups.



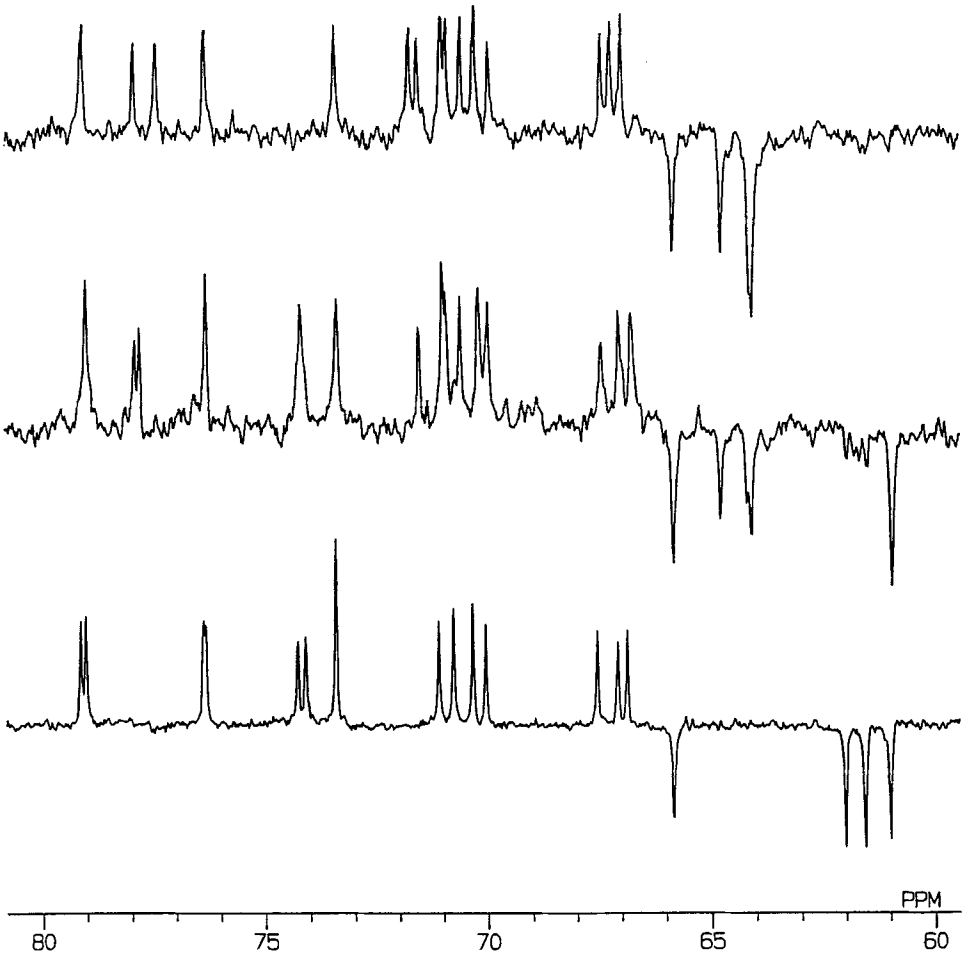


Figure 2. ^{13}C NMR spectra run in the DEPT mode of compound **6** (top), **7** (middle) and its non-acetylated analogue (bottom).

Removal of the benzylidene acetals in **2** by acidic hydrolysis gave the hexahydroxyl derivative **3**. Since the acetylation pattern of the native polysaccharide is heterogeneous and not definitely elucidated, various acetylation patterns could be of interest. The important thing was to avoid acetylation at secondary positions. Of various methods[7-10] used for selective acetylation of primary hydroxyl groups, the procedure using acetyl chloride and collidine in CH_2Cl_2 at low temperatures[7] looked promising and convenient. When this method was applied to compound **3**, with 5.25 equivalents of acetyl chloride at -70 °C for 3.3 hours, a clean reaction was observed. Separation of the

few products gave 60% of the tri-*O*-acetylated derivate **4**, together with 15% of the di-*O*-acetylated derivative **5**. If less acetyl chloride (3.6 equiv) and shorter time (50 min) were used, the di-*O*-acetyl derivate **5** became the main product (33%) together with compound **4** (17%) and various monoacetylated derivatives. In none of the reactions was any acetylation of secondary hydroxyl groups detected.

Deprotection of derivatives **4** and **5** using catalytic hydrogenolysis gave the target products **6** (77%) and **7** (84%) respectively, without any indication of acetyl migration, as shown by NMR. The acetylation patterns of the target molecules **6** and **7** were elucidated using NMR (^{13}C experiment ran in DEPT mode to identify methylene carbons, see Fig. 2) and methylation analysis. It was found that the methylating procedure developed by Prehm[11] involving methyl triflate in trimethylphosphate was useful for mild methylation of the tetrasaccharides leaving the *O*-acetyl substituents untouched.

In compound **6** all three primary carbons are shifted downfield 2.5-3.1 ppm compared to the previously synthesized non-acetylated analogue (see Fig. 2) indicating acetylation in these positions. Methylation analysis further confirmed this acetylation pattern and the carbohydrate structure (see experimental section). NMR experiments on compound **7** showed the presence of two primary acetates (see Fig. 2). The positions of these were determined by methylation analysis and found to be at the terminal and the reducing-end mannose residues.

EXPERIMENTAL

General methods. Organic phases were dried with $MgSO_4$ before concentrations, which were performed under reduced pressure at <50 °C (bath). NMR spectra were recorded in $CDCl_3$ at 25 °C (internal Me_4Si, $\delta = 0.00$) unless otherwise stated, using a JEOL GX-270 instrument at 67.5 MHz (^{13}C) or 270 MHz (^{1}H). Optical rotations were recorded at room temperature with a Perkin-Elmer 241 polarimeter. TLC was performed on Silica Gel 60 F_{254} (Merck) with detection by UV light and/or by charring with 8% sulfuric acid. Silica gel (0.040-0.063 mm, Amicon) was used for column chromatography. Semipreparative HPLC was carried out on a Dynamax 60-A silica gel column with a flow rate of 2 mL/min, detection was achieved by measuring the absorbance at 260 nm.

Methyl *O*-(2,3-Di-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→3)-[*O*-(2,3,4-tri-*O*-benzyl-β-D-xylopyranosyl)-(1→2)]-*O*-(4,6-*O*-benzylidene-α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside (2).

Methanolic sodium methoxide (0.2 mL, 1M) was added to a solution of compound **1**[6] (214 mg, 0.142 mmol) in MeOH:CH_2Cl_2 (20 mL, 2:1). After stirring overnight, debenzoylation was complete according to TLC and the mixture was neutralised with Dowex 50 (H^+) resin, filtered, concentrated and dried in vacuum. The dried residue was dissolved in *N*,*N*-dimethylformamide (10 mL) and to the solution was added sodium hydride (57 mg, 1.42 mmol, 60% suspension) and 5 min later benzyl bromide (110 μL, 0.92 mmol). After 2 h, the reaction was quenched with MeOH (2 mL) and the mixture partitioned between toluene-H_2O. The organic phase was washed with water, dried and concentrated. Silica gel flash chromatography, (toluene-EtOAc 12:1) gave **2** (156 mg, 0.11 mmol, 75%). HPLC was used to purify an aliquot for optical rotation and elemental analysis. The elution system was an isocratic flow of *n*-hexane-EtOAc 7:3. $[\alpha]_D$ -22° (*c* 1.1, $CHCl_3$). NMR data: ^{13}C, δ 54.8 (CH_3-O), 63.0, 63.8, 64.2, 64.3, 68.7 (two signals), 68.9, 71.4, 72.2, 72.6, 73.0, 73.4, 73.6, 74.4, 75.1, 75.6, 76.5, 77.0 (internal $CDCl_3$), 77.3, 77.4, 77.6, 78.7, 79.1 (two signals), 80.9, 82.9 (C-2-6, O-$\underline{C}H_2$-Ar), 99.9, 99.9, 100.0, 101.0, 101.5, 102.0, 103.4 (C-1 and O_2-$\underline{C}$H-Ar), 125.8-129.2, 137.2, 137.5, 137.8, 137.9, 138.1, 138.7 (aromatic C).

Anal. Calcd for $C_{87}H_{90}O_{20}$: C, 71.79; H, 6.23. Found: C, 71.62; H, 6.22.

Methyl *O*-(6-*O*-Acetyl-2,3-di-*O*-benzyl-α-D-mannopyranosyl)-(1→3)-[*O*-(2,3,4-tri-*O*-benzyl-β-D-xylopyranosyl)-(1→2)]-*O*-(6-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-6-*O*-acetyl-α-D-mannopyranoside (4). The tetrasaccharide **2** (75 mg, 0.052 mmol), dissolved in MeCN (1 mL), was added to prewarmed (65 °C) aqueous HOAc (15 mL, 70%) and the mixture was stirred for 4 h at 65 °C before the solvent was evaporated. Coevaporation twice with toluene and drying in vacuum gave debenzylidenated material **3** [NMR data: ^{13}C, δ 54.9 (CH_3-O), 61.9, 62.8, 63.2, 63.8, 66.2, 67.9, 71.7, 72.5, 72.7, 72.8, 73.1, 73.2, 73.4, 74.6, 75.3, 75.6, 77.7, 77.8, 78.8, 79.8, 79.9, 81.3, 83.8 (C-2-6, O-$\underline{C}H_2$-Ar, overlap), 98.9, 99.9, 100.3, 103.7 (C-1), 127.5-128.6, 137.8, 138.0, 138.2, 138.3, 138.6, 138.7 (aromatic C)]. The dried residue and sym-collidine (0.29 mmol, 38 μL) were dissolved in CH_2Cl_2 (6 mL) and the solution was cooled to -70 °C. After 15 min of stirring, acetyl chloride (0.18 mmol, 500 μL of stock solution) was added from an acetyl chloride stock solution (128 μL, 1.79 mmol of acetyl chloride in 5.0 mL CH_2Cl_2). Additional adding of acetyl chloride (100 μL of stock solution each time) were done after 100 and 150 min. The reaction was quenched with MeOH after 200 min and the mixture was allowed to attain room temperature. Evaporation of the solvent and purification of the remaining material by silica gel flash chromatography (toluene-EtOAc 2:1) gave **4** (41 mg, 0.031 mmol, 60%) and **5** (10 mg, 7.8 μmol, 15%). HPLC was used to purify an analytical sample of **4** for optical rotation

and elemental analysis. The elution system was an isocratic flow of *n*-hexane-EtOAc 4:6. $[\alpha]_D$ +11° (*c* 0.7, $CHCl_3$). NMR data: ^{13}C, δ 20.6, 20.9 (two signals) (CH_3 acetyl), 54.9 (CH_3-O), 63.4, 63.5, 63.7, 64.6, 66.3, 66.8, 67.6, 70.5, 71.2, 71.3, 71.7, 72.5 (two signals), 73.3, 73.6, 74.9, 75.6, 77.2, 77.7, 78.3, 78.4, 79.4, 81.5, 83.7 (C-2-6, O-$\underline{C}H_2$-Ar, one overlap), 98.7, 99.4, 100.0, 103.7 (C-1), 127.6-128.6, 137.9, 138.0, 138.1, 138.2, 138.3, 138.7 (aromatic C), 171.5, 171.6, 171.9 (C=O acetyl).

Anal. Calcd for $C_{72}H_{84}O_{23}$: C, 65.64; H, 6.43. Found: C, 65.38; H, 6.43.

Methyl *O*-(6-*O*-Acetyl-2,3-di-*O*-benzyl-α-D-mannopyranosyl)-(1→3)-[*O*-(2,3,4-tri-*O*-benzyl-β-D-xylopyranosyl)-(1→2)]-*O*-(α-D-mannopyranosyl)-(1→3)-2-*O*-benzyl-6-*O*-acetyl-α-D-mannopyranoside (5). Debenzylidenation of the tetrasaccharide **2** (58 mg, 0.040 mmol) followed by treatment with 3.6 equiv of acetyl chloride (0.14 mmol) and 6 equiv of sym-collidine (0.23 mmol, 30 μL) for 50 min using the procedures described for compound **4** gave the di-*O*-acetylated compound **5** in 33% yield (17 mg, 0.013 mmol) together with 17% of product **4** (9 mg, 0.076 mmol). **5**: $[\alpha]_D$ +16° (*c* 0.7, $CHCl_3$). NMR data: ^{13}C, δ 20.9 (two signals) (CH_3 acetyl), 55.0 (CH_3-O), 62.9, 63.5, 63.8, 64.6, 66.8, 67.1, 67.5, 70.6, 71.2, 71.8, 72.6 (two signals), 72.7, 73.2, 73.8, 74.7, 75.5, 76.5, 77.6, 77.7, 77.9, 78.5, 79.3, 83.7 (C-2-6, O-$\underline{C}H_2$-Ar, one overlap), 98.7, 99.2, 99.9, 103.3 (C-1), 127.6-128.5, 138.0, 138.1 (two signals), 138.1, 138.5, 138.7 (aromatic C), 171.5, 171.9 (C=O acetyl).

Methyl *O*-(6-*O*-Acetyl-α-D-mannopyranosyl)-(1→3)-[*O*-β-D-xylopyranosyl-(1→2)]-*O*-(6-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-6-*O*-acetyl-α-D-mannopyranoside (6). Compound **4** (40 mg, 0.30 mmol) in EtOAc:MeOH:H_2O (15 mL, 5:5:1) was hydrogenolyzed over Pd/C (10%, 25 mg) in a Parr apparatus (120 psi) for 5 h. The mixture was filtered through Celite, concentrated, dissolved in water (5 mL), washed with diethyl ether and freeze-dried. After gel filtration on a Bio-gel P2-column eluted with H_2O:1-butanol 99:1 and freeze-drying, compound **6** was collected in 77% yield (18 mg, 0.024 mmol). $[\alpha]_D$ + 52° (*c* 1.0, water). NMR data (D_2O) : ^{13}C, δ 20.9, 21.0, 21.1 (CH_3 acetyl), 31.0 (internal acetone), 55.6 (CH_3-O), 64.1, 64.1, 64.8, 65.8, 67.0, 67.2, 67.4, 70.0, 70.3, 70.6, 70.9, 71.0, 71.6, 71.7, 73.4, 76.3, 77.4, 77.9, 79.1 (C-2-6), 100.8 ($J_{C\text{-}1,H\text{-}1}$ 174 Hz), 101.6 ($J_{C\text{-}1,H\text{-}1}$ 170 Hz), 103.6 ($J_{C\text{-}1,H\text{-}1}$ 174 Hz), 104.3 ($J_{C\text{-}1,H\text{-}1}$ 160 Hz) (C-1), 174.8 (two signals), 174.4 (C=O acetyl), ^1H (70 °C), selected signals, δ 2.13 (6 H, s, 2xCH_3 acetyl), 2.16 (3 H, s, CH_3 acetyl), 2.21 (internal acetone), 4.73 (d, $J_{1,2}$ 1.6 Hz), 5.10 (d, $J_{1,2}$ 1.4 Hz), 5,19 (d, $J_{1,2}$ 1.5 Hz) (H-1). DEPT-experiment showed methylene carbons at δ 64.1, 64.1, 64.8 and 65.8. Identical DEPT-experiment on previously synthesised analogous compound without *O*-acetyl substituents showed methylene carbons at δ 61.0, 61.6, 62.0 and 65.8 (see Fig. 2). These data support the postulated

structure of **6** with three 6-*O*-acetyl substituents, the signal at 65.8 comes from C-5 of xylose. HRMS calculated for $C_{30}H_{48}O_{23}$ $[M-H]^-$: 775.2508. Found: 775.2455.

Methylation analysis of compound **6**, using the methylating procedure described by Prehm,[11] showed the presence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylpentitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol and 1,2,3,5,6-penta-*O*-acetyl-4-*O*-methylhexitol. These data are in agreement with the postulated structure of **6** with acetyl substitution at the 6-position of all the three mannose residues.

Methyl *O*-(6-*O*-Acetyl-α-D-mannopyranosyl)-(1→3)-[*O*-β-D-xylopyranosyl-(1→2)]-*O*-(α-D-mannopyranosyl)-(1→3)-6-*O*-acetyl-α-D-mannopyranoside (7). Compound **5** (29 mg, 0.23 mmol) was deprotected as described for compound **4** and the product **7** was collected in 84% yield (14 mg, 0.019 mmol). $[\alpha]_D$ + 52° (*c* 1.2, water). NMR data (D_2O): ^{13}C, δ 21.0, 21.1 (CH_3 acetyl), 31.0 (internal acetone), 55.6 (CH_3-O), 60.1, 64.1, 64.8, 65.9, 66.8, 67.1, 67.5, 70.0, 70.2, 70.6, 70.9, 71.0, 71.5, 73.4, 74.2, 76.3, 77.8, 77.9, 79.0 (C-2-6), 100.6 ($J_{C\text{-}1,H\text{-}1}$ 174 Hz), 101.6 ($J_{C\text{-}1,H\text{-}1}$ 170 Hz), 103.7 ($J_{C\text{-}1,H\text{-}1}$ 172 Hz), 104.0 ($J_{C\text{-}1,H\text{-}1}$ 160 Hz) (C-1), 174.8, 174.9 (C=O acetyl), ^1H (70 °C), selected signals, δ 2.13 (3 H, s, CH_3 acetyl), 2.16 (3 H, s, CH_3 acetyl), 2.21 (internal acetone), 4.72 (d, $J_{1,2}$ 1.5 Hz), 5.10 (d, $J_{1,2}$ 1.5 Hz), 5,27 (d, $J_{1,2}$ 1.8 Hz) (H-1). DEPT-experiment showed methylene carbons at δ 60.1, 64.1, 64.8 and 65.9. Identical DEPT-experiment on previously synthesised analogous compound without *O*-acetyl substituents showed methylene carbons at δ 61.0, 61.6, 62.0 and 65.8 (see Fig. 2). These data support the structure of **7** with two 6-*O*-acetyl substituents, the signal at 65.8 comes from C-5 of xylose. HRMS calculated for $C_{28}H_{46}O_{22}$ $[M-H]^-$: 733.2403. Found: 733.2382.

Methylation analysis of **7**, using the methylating procedure described by Prehm,[11] showed the presence of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylpentitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol, 1,2,3,5,-tetra-*O*-acetyl-4,6-di-*O*-methylhexitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol. These data are in agreement with the postulated structure of **7** with acetyl substitution at the 6-position of the terminal and the reducing end mannose residues.

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