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CHARACTERIZATION OF STRUCTURALLY SIMILAR NEUTRAL AND ACIDIC TETRASACCHARIDES OBTAINED FROM THE ENZYMIC HYDROLYZATE OF A 4-*O*-METHYL-D-GLUCURONO-D-XYLAN

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ABSTRACT

Two similar tetrasaccharides, one neutral and one acidic, were isolated from the products released by the attack of a xylanase on the *in situ* reduced 4-*O*-methyl-D-glucurono-D-xylan from aspen (*Populus tremuloides*). Paper chromatography, gel filtration behavior, methylation followed by reduction, and mass spectrometry showed that the oligosaccharides were *O*-(4-*O*-methyl-α-D-glucopyranosyl)-(1→2)-D-xylotriose and *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-D-xylotriose. Independent of the acidic or neutral substituent on the present xylan chain, the enzymic cleavage led preferentially to oligosaccharides substituted at the nonreducing end. The existence, in wood, of a few uronic acid substituents of the D-xylan in the esterified form was confirmed, and their linkage to lignin postulated.

INTRODUCTION

Degradation of D-xylans with xylanases established unequivocally the linkage of L-arabinose residues to the xylan chain in the L-arabino-D-glucurono-D-xylan from wheat straw[1]. However, only in few instances, oligosaccharides formed by enzymic hydrolysis have been fully characterized[2–7]. The mode of action of the enzymes was difficult to interpret, since complex enzymic mixtures of fungal origin[3,4,8], or of cell-free enzyme systems and intact organisms from the protozoa and rumen bacteria[9–11] were used without previous purification.

Side-chains affect probably the enzymic degradation of the main chain. Thus, an enzyme from *Myrothecium verrucaria*, active on an arabinoxylan[1], and a commercial pectinase[4] active on a (4-*O*-methylglucurono)xylan catalyzed the hydrolysis near the branch points, both enzymes yielding oligosaccharides in which L-arabinose or 4-*O*-methyl-D-glucuronic acid residues are linked to the nonreducing end of a xylobiose or a xylotriose residue. However, Goldschmid and Perlin[7] reported the presence, in the hydrolyzate of an arabinoxylan of wheat by an enzymic preparation

from a *Streptomyces*, of a tetrasaccharide constituted of a L-arabinofuranosyl residue linked at C-3 of the central D-xylosyl residue of a xylotriose. Similarly, King and Fuller[12] found, after degradation of a glucuronoxylan from aspen with a xylanase from *Coniophora cerebella*, results differing from those of Timell[4] in that some of the acidic sugars had the uronic acid residues attached to a xylose residue other than the terminal nonreducing one. These various locations of cleavage observed by different authors could be explained by the varied origin of the xylanases. Indeed, various xylanases obtained from many sources and having a different function in the hydrolysis of xylan have been reported after fractionation and purification[12–15]. We obtained also two xylanases by purification of an enzymic mixture from a *Basidiomycete*[16]. In order to define their characteristics and mode of action on a specific D-xylan, the substrate was degraded exhaustively without removal of the products of attack by continuous dialysis.

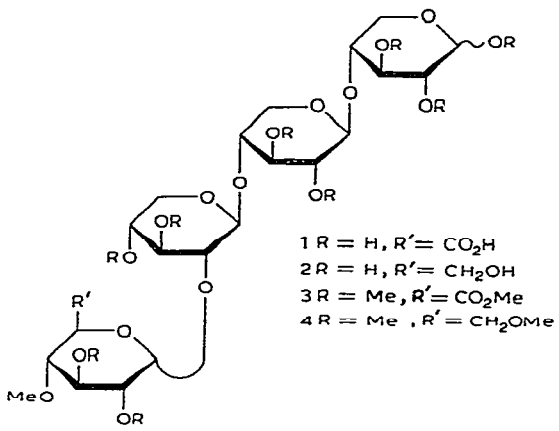
EXPERIMENTAL

General methods. — Paper chromatographic separations were performed on Whatman No. 1 and Schleicher–Schüll 2043 b Mgl papers, and preparative chromatography on Whatman No. 3 MM paper with the following solvent systems: 10:4:3 ethyl acetate–pyridine–water (Solvent A), 6:3:4 ethyl acetate–acetic acid–water (Solvent B), and 18:3:1:4 ethyl acetate–acetic acid–formic acid–water (Solvent C). Compounds were detected with the aniline oxalate reagent[17].

Gas–liquid chromatography (g.l.c.) was performed on a model 417 Becker gas chromatograph, equipped with glass columns (2 m × 2 mm i.d.) containing 3% ECNSS-M on Gas Chromosorb Q (100–120 mesh), and peak areas were determined by a digital integrator (Hewlett Packard 3370-B). The mass spectra were recorded on an A.E.I. model MS9 mass spectrometer by direct introduction at an ionising potential of 70 eV. The temperature in the ionising chamber was 180° for **2** and 200° for **1**.

Isolation of the D-*xylan*[18]. — Reduction and alkaline extraction of the 4-*O*-methyl-D-glucuronoxylan were performed as described by Zinbo and Timell[19].

Enzymic hydrolysis and fractionation. — The xylanase has been prepared in this laboratory[16] from a crude commercial cellulase mixture (Cellulase 1611, Sempa Chimie, Paris) by adsorption on a column of diethylaminoethyl(DEAE)Sephadex A-50 resin and elution with 0.06M sodium chloride in 0.01M sodium phosphate buffer (pH 7) at 4°. The reduced D-xylan (50 mg) was incubated for 48 h at 40° with the xylanase in acetate buffer solution (0.1M, 20 ml) at pH 5 containing sodium azide (5mM) as a preservative. The enzyme was inactivated at 100° for 10 min, and the mixture was dialyzed in a Visking tube (No. 18–32, pores of 24 Å) against distilled water for 3 days at 5°. The distilled water was frequently renewed until a negative Molisch test was obtained. Filtration of the dialyzate through a column (100 × 2,4 cm) of Bio-Gel P-2 (200–400 mesh) resin, maintained at a temperature of 60°, and elution with water afforded among other oligosaccharides the two, acidic (**1**) and neutral (**2**),



tetrasaccharides at vol. 193 ml and 282 ml, respectively (void volume, 139 ml). Purification of **1** and **2** was achieved on preparative paper chromatography (Whatman No. 3MM paper) in Solvent A, and their purity was checked in Solvent B (Whatman No. 1 paper).

Permethylation of **1** *and* **2**. — The two tetrasaccharides **1** and **2** were methylated by the method of Hakomori[20]. The oligosaccharide (to ~2 mg) was dissolved in dry dimethyl sulfoxide (2 ml) in a round-bottom flask fitted with a rubber cap and flushed with nitrogen. A solution of methylsulfinyl carbanion in dimethyl sulfoxide (2M, 0.5 ml) was then added and stirring maintained overnight. Methyl iodide (0.5 ml) was added in 0.1 ml portions over a period of 1.5 h, and the reaction solution was poured into cold water. The resulting mixture was extracted with chloroform (3 × 15 ml), and the combined extracts washed with water, dried with anhydrous sodium sulfate, and evaporated to dryness. The permethylated tetrasaccharides (**3** and **4**, respectively) were divided into two parts, one for mass spectroscopic examination, the other submitted to reduction and hydrolysis.

Reduction and hydrolysis of **3** *and* **4**. — The permethylated tetrasaccharide was dissolved in distilled tetrahydrofuran (2 ml), and an excess of lithium aluminum hydride was added. After stirring overnight at room temperature, the excess of lithium aluminum hydride was destroyed with ethyl acetate, and the mixture was diluted with water. The organic solvents were evaporated and the solution was concentrated. The aqueous solution was deionized on Amberlite IR-45 (AcO^-) and IR-120 (H^+) ion-exchange resins, and then evaporated to a syrup, which was subsequently hydrolyzed, in a sealed tube, with 0.5M sulfuric acid for 7 h at 100°. After neutralization with barium carbonate and passage through Amberlite IR-45 (AcO^-) and IR-120 (H^+) ion-exchange resins, the resulting partially methylated neutral sugars were reduced with sodium borohydride, acetylated according to Sawardeker *et al.*[21], and examined by g.l.c.

RESULTS AND DISCUSSION

Borohydride reduction *in situ*, prior to alkaline extraction of the D-xylan from aspen (*Populus tremuloides*), gave a polysaccharide having a chain terminating with a reduced end group[19]. This treatment provided the following advantages for enzymic degradation: (a) the xylan obtained was not modified by alkaline peeling during its extraction, and (b) the reducing power detected after enzymic degradation could be attributed only to the reducing ends brought about by cleavage in the polysaccharide chain, thus allowing an estimation of the enzymic activity. In a preceding study[18], we showed that the *in situ* reduced polysaccharide was a 4-*O*-methyl-D-glucurono-D-xylan analogous to that previously described by several authors[19,22–24] but differing in that a few 4-*O*-methyl-D-glucuronic acid residues had been transformed during the reduction into 4-*O*-methyl-D-glucose residues. The xylan has an average of four 4-*O*-methyl-D-glucose and five 4-*O*-methyl-D-glucuronic acid residues per molecule as side-chains linked to the (1→4)-β-D-xylosyl backbone.

In the present work, we have confirmed the presence of the neutral substituents by isolation and characterization of both a neutral (**2**) and an acidic (**1**) tetrasaccharide containing a 4-*O*-methyl-D-glucose and a 4-*O*-methyl-D-glucuronic acid residue, respectively, linked to C-2 of the nonreducing end residue of a D-xylotriose.

By incubation with xylanase, 60% of the D-xylan was solubilized as dialyzable sugars, as determined by the phenol–sulfuric acid method[25]. Fractionation of this mixture of saccharides on a column of Biogel P-2 resin (void volume, 139 ml) gave a broad peak (vol. 193 ml) containing three components, as shown by paper chromatography in Solvent A. One of these was isolated as compound **1** after mutiple-elution on Whatman 3 MM paper, and showed R_{Xyl} 0.59 (Solvent B, Whatman No. 1 paper) and 0.11 (Solvent C, Schleicher and Schüll paper). The two other components showed R_{Xyl} 0.42 and 0.24, respectively, (Solvent B, Whatman No. 1 paper). Compound **2** was eluted from the column of Biogel P-2 resin at an elution volume of 282 ml, and was isolated by chromatography on Whatman No. 3MM paper in Solvent A as just described with a single elution. It showed R_{Xyl} 0.57 (Solvent B, Whatman No. 1 paper), and 0.43 and 0.09 (Solvent A and C, respectively, on Schleicher and Schüll paper). Another product, present in a very small amount, was eluted at the same time from the column and had R_{Xyl} 0.39 (Solvent B, Whatman No. 1 paper). Two peaks having elution volumes of 325 ml and 340 ml showed, by comparison with authentic standards, the presence in various proportions of D-xylotriose (R_{Xyl} 0.31 in Solvent A and 0.48 in Solvent B) and D-xylobiose (R_{Xyl} 0.61 in Solvent A and 0.73 in Solvent B), and of D-xylotriose, D-xylobiose, and D-xylose, respectively. Free 4-*O*-methyl-D-glucuronic acid or 4-*O*-methyl-D-glucose were not produced by the action of the xylanase on the polysaccharide.

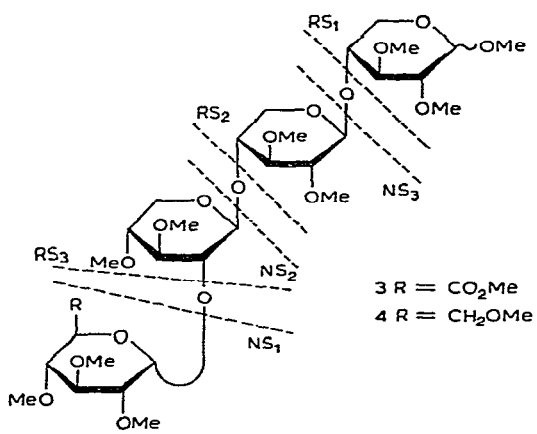
The structure of **1** and **2** was established by methylation, carboxymethyl ester reduction with lithium aluminum hydride in tetrahydrofuran, acid hydrolysis, and g.l.c. examination of the resulting, partially methylated sugars as their alditol acetates. After treatment, **1** showed only two chromatographic peaks corresponding to 1,4,5-

tri-*O*-acetyl-2,3-di-*O*-methyl-D-xylitol and to 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol in a relative molar ratio of 2.96 to 1.00.

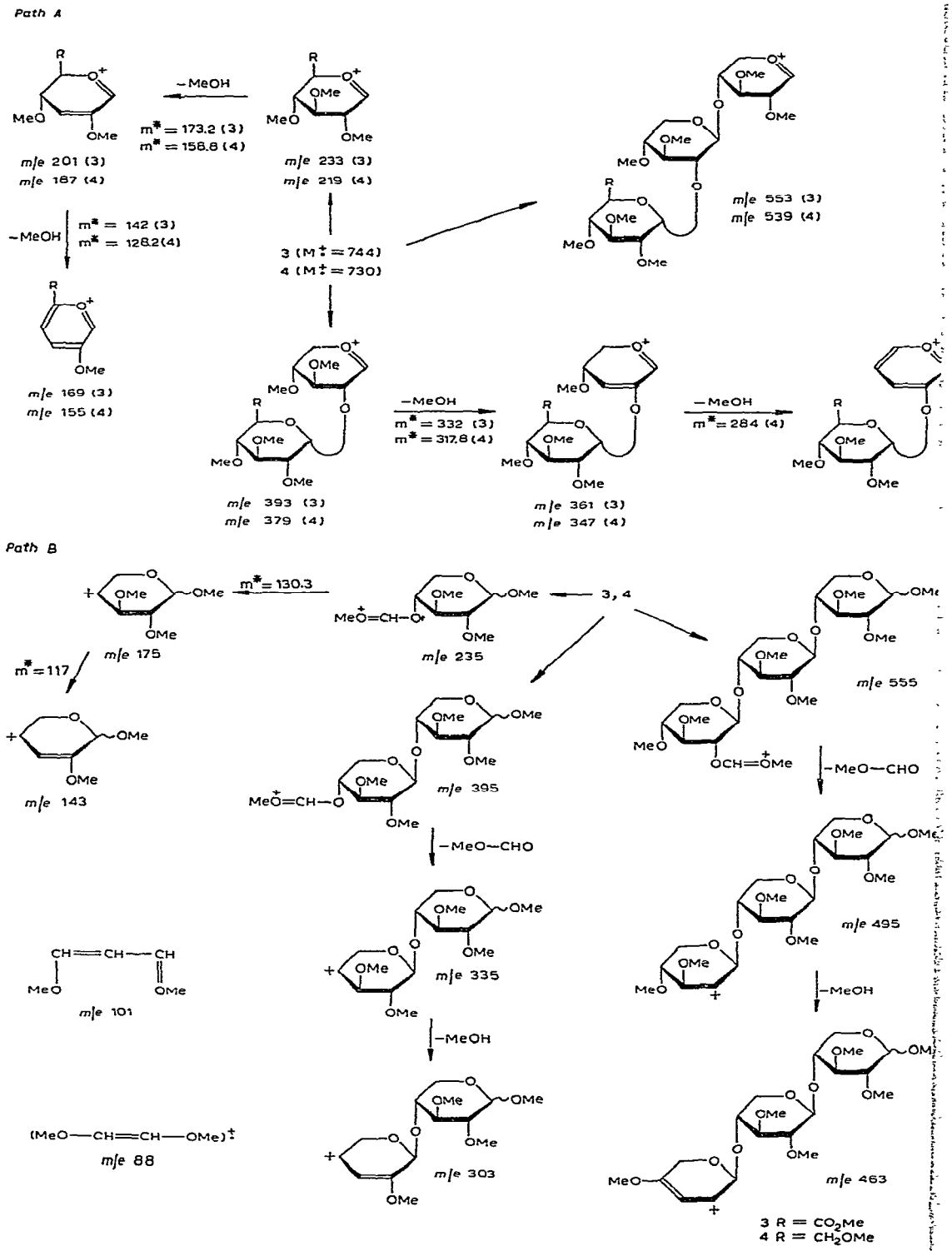
In agreement with the known β-(1→4) linkages of the D-xylan[19,26], this ratio indicates that **1** consists of three (1→4)-linked D-xylosyl residues, one of which being substituted at C-2 with a D-glucuronic acid residue. The absence of both 2,3,4-tri-*O*-methylxylitol and mono-*O*-methylxylitol in the chromatogram showed that the uronic acid residue is attached at C-2 of the nonreducing end-group of the D-xylotriose molecule, since 3,4-di-*O*-methyl- and 2,3-di-*O*-methylxylitol are D,L enantiomers chromatographically indistinguishable. 2,3,4-Tri-*O*-methyl-D-glucitol derived from the reduction of the D-glucuronic acid residue, esterified during methylation, and shown earlier[26] to be α-D-linked and substituted by a methoxyl group at C-4. Thus, the structure of **1** is *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose, identical with the uronic acid tetrasaccharide obtained by enzymic hydrolysis of a 4-*O*-methyl-D-glucurono-D-xylan from the wood of white birch[5].

After the same treatment, **2** showed only two peaks, in the g.l.c. analysis of the partially methylated alditol acetates, which corresponded to 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-D-xylitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, in the relative molar ratio of 2.92:1.00. This ratio shows that **2** is a tetrasaccharide consisting of three (1→4)-linked D-xylosyl residues. The nonreducing end of this short chain carries at C-2 a substituent identified by the isolation of 2,3,4,6-tetra-*O*-methyl-D-glucitol, which could arise only from a D-glucose derivative, since the tetrasaccharide had been reduced after methylation. As shown[19] by the structure of the substrate submitted to the enzymic degradation, this D-glucose derivative is 4-*O*-methyl-D-glucose, and thus, **2** has the structure *O*-(4-*O*-methyl-α-D-glucopyranosyl)-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose.

The structure of both **1** and **2** was confirmed by the analysis of the mass spectra



Scheme 1.



Scheme 2. Fragmentation pattern of **3** and **4**.

of the permethylated derivatives, which showed the characteristic fragmentations[27–29] of oligosaccharides derivatives to give fragments NS_1, NS_2, and NS_3, as well as fragments RS_1, RS_2, and RS_3, respectively (see Scheme 1). The most characteristic differences were found, as expected, for the path corresponding to the fragments NS_1, NS_2, and NS_3 (see *Path A*, Scheme 2); a difference of 14 mass units was observed between the ions derived from **1** and **2**. The ions corresponding to the fragments RS_1, RS_2, and RS_3 are shown in Scheme 2 (*Path B*). A few transitions have been confirmed by the presence of metastable ions (m*).

Xylanase treatment of the D-xylan, obtained from aspen and which had been previously reduced *in situ* prior to alkaline extraction, gave **2** in addition to **1**. This result suggests that some of the uronic acid residues of D-glucurono-D-xylans are linked to lignin through an ester bond, and is additional evidence for the suggestion advanced by Stewart[30] that "it is possible that small amounts of 4-*O*-methyl-D-glucose units and 3-*O*-methyl-L-gulitol (4-*O*-methyl-D-glucitol) units could be formed by borohydride reduction of ester bonds existing between stable uronic acids and lignin". According to the ratio of reduced to unreduced uronic acid residues (4:5), and taking into account the competition between borohydride reduction of the esters and their alkaline saponification, it seems that a high proportion of the 4-*O*-methyl-D-glucuronic residues of the D-xylan could exist in wood in their esterified form and play an important role in the bond to lignin.

It is of interest for our understanding of the mechanism of attack of the xylanase that we were not able to detect any oligosaccharide homologous to the two tetrasaccharides reported here but with a lower d.p.[31]. This suggests that not only a 4-*O*-methyl-D-glucuronic acid residue, as described by Timell[4], but also a neutral 4-*O*-methyl-D-glucose substituent on the D-xylan chain is a steric hindrance for the enzyme to reach the two (1→4)-β-D-xylosidic bonds immediately linked to the right of the substituted D-xylosyl residue. On the contrary, the D-xylosidic bond immediately to the left of the substituted D-xylose residues seems to be easily cleaved by the xylanase, independently of the acidic or neutral nature of the substituent. In order to obtain a better understanding of the mechanism of the xylanase, work is in progress in this laboratory to determine the influence of the nature of the substituent on the enzymic attack of D-xylans of different origins.

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