Structure of a Hemicellulose A Fraction in Dietary Fiber from the Seed of Grape Variety Palomino (*Vitis vinifera* cv. Palomino)

José M. Igartuburu,*,† E. Pando,† F. Rodríguez-Luis,† and Antonio Gil-Serrano‡

Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, Apdo 40. 11510, Puerto Real, Cádiz, Spain, and Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, 41071 Sevilla, Spain

Received May 9, 1997

The structure of one of the hemicellulose A fractions (HA-1) extracted from the seeds of the grape variety Palomino (*Vitis vinifera* cv. Palomino) has been studied by means of methylation, ^{1}H NMR, ^{13}C NMR, and partial hydrolysis. This hemicellulose seems to be a homogeneous polysaccharide with an apparent molecular weight of 68 500. Its structure is that of an acidic xylan, a linear chain of *β*-D-xylopyranosyl units, bonded together by (1→4) glycosidic links, containing single α-D-xylopyranosyl and 4-*O*-methyl-α-D-glucopyranuronosyl residues joined by glycosidic links to position 2 of the xylose units of the main chain, in proportions of one residue to every 25 units of xylose.

Forecasts for the next century indicate the need for increased food production to meet ever-increasing demands, for both human and animal consumption. Defatted flours from oilseeds have been studied as potential sources of proteins, dietary fiber, and other nutrients to meet future demand.[1] The Spanish region of Andalucía is one of the major wine-producing regions of Europe, with viticulture and the production of wine and related products being important industries in this area. These industries generate various byproducts, such as grape pomace and fermentation lees, which are currently under exploited. The productive use of such byproducts could offer substantial economic benefits.

In the Jerez Denomination of Origin wine area, the main grape variety cultivated is Palomino (*Vitis vinifera* cv. Palomino); our research group has previously published findings on the composition of the Palomino grape seed in terms of its lipids, proteins, amino acids, and dietary fiber.[2−9] The importance of dietary fiber in the human and animal diet has been widely publicized in recent years, and the study of its components has been steadily increasing. Dietary fiber is the major component of grape seed, accounting for approximately 76 wt %.[6] Further study of this fiber is necessary to propose alternative methods of exploiting this byproduct.

The hemicellulose group comprises the most abundant fraction of the polysaccharides, which make up grape seed dietary fiber;[6] this paper describes the structural determination of a main polysaccharide isolated from the hemicellulose fraction. The description of the polysaccharide fraction made in this paper continues earlier studies we have made of the composition of the seed of the grape variety cv. Palomino.[2−9] The study of dietary fiber from *V. vinifera* cv. Palomino[6] by the Southgate method[10] has shown that hemicellulose is the most abundant fraction of the polysaccharides in this fiber (15%), followed by cellulose. Therefore, we have focused the present study on the fraction known as holocellulose (comprising the total of hemicelluloses and cellulose) from this material. This fraction represents about 24 wt % of the whole grape seed before treatment.

Results and Discussion

The milled seeds were extracted sequentially with hexane, ethanol, and chloroform−methanol (1:1), treated with 0.25 M sodium methoxide in methanol,[11] and then extracted with water. Previous work by our group has shown that there is no starch content in the Palomino grape seed;[6] therefore it was not necessary to include a prior extraction stage with hot water or dimethyl sulfoxide to solubilize this component. In addition, the fraction of mucilage, gums, and pectins was very small and was completely eliminated by extraction with water after the sodium methoxide treatment.

The residual solid from these extraction stages described was delignified with sodium chlorite−acetic acid to yield the holocellulose.[12] The hemicelluloses were extracted from this material by treatment with 10% NaOH[13] under nitrogen, and hemicellulose A was precipitated from the extract by acidification to pH 5 with 50% acetic acid. Hemicellulose B was isolated from the supernatant solution by precipitation with ethanol after dialysis against running water.

The hemicellulose A of grape seeds was treated with Fehling's solution to fractionate it into its components.[14] Purified hemicellulose HA-1 was isolated from the precipitate and gave a single narrow band on size-exclusion chromatography, having an apparent molecular weight of 68 500 and a specific rotation of $[\alpha]^{25}_{D}$ −47.4° (*c* 1.16, 1 M sodium hydroxide). This compound represents the most abundant fraction of the polysaccharides in grape seed dietary fiber. Acid hydrolysis[15,16] of the polysaccharide showed that it is composed of xylose and 4-*O*-methylglucuronic acid in a molar ratio of 36:1.

Methylation of HA-1[17] produced a pale-yellow solid product with a specific rotation of $[\alpha]^{25}_{D}$ −52.9° (*c* 1.34, chloroform), indicative of the existence of *β*-D-glycosidic linkages. This was confirmed by the NMR spectroscopic data (*δ* 4.25 for H-1 and *δ* 102.5 for C-1).[18,19] A portion

* To whom correspondence should be addressed. Tel.: int. + 34/56/830217. Fax: int + 34/56/834924. E-mail: josemanuel.igartuburu@uca.es.
† Universidad de Cádiz.
‡ Universidad de Sevilla.

S0163-3864(97)00240-1 CCC: $15.00
Published on Web 07/03/1998

Table 1. Methylation Analysis Data for Natural Polysaccharide, Reduced Polysaccharide, and Oligosaccharides Obtained by Partial Hydrolysis from the Hemicellulose Fraction HA-1

methylated sugar (as alditol acetate)	T^a	T^b	molar ratio[d]						
			A	B	NOS-1	NOS-2	NOS-3	AOS-2	AOS-3
Glc2,3,4Me$_3$[c]	2.49	1.13		1.0					
Xyl2,3,4Me$_3$	0.69	0.84	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Xyl2,3Me$_2$	1.54	0.96	51.5	49.7	1.2	2.1	3.0		1.1
Xyl3Me	2.78	1.04	2.1	2.4				1.1	1.2

[a] Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on a ECNSS-M column. [b] Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on a SPB-1 column. [c] Glc2,3,4Me$_3$ = 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol; Xyl2,3,4Me$_3$ = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol; Xyl2,3Me$_2$ = 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol; Xyl3Me = 1,2,4,5-tetra-*O*-acetyl-3-*O*-methylxylitol. [d] A, native polysaccharide; B, carboxyl-reduced polysaccharide; NOS-1/3, neutral oligosaccharides 1−3, obtained on partial acid hydrolysis; AOS-2/3, acidic oligosaccharides 2 and 3 obtained on partial acid hydrolysis.

of the methylated polysaccharide was hydrolyzed, and the resulting sugars were converted into their corresponding partially methylated alditol acetates and analyzed by GC[20] and GC−MS.[21] The results obtained are summarized in Table 1.

Another portion of methylated polysaccharide was reduced with $LiAlH_4$[22] and then hydrolyzed, and the resulting sugars were converted into their corresponding partially methylated alditol acetates; these were analyzed by GC and GC−MS and the results summarized in Table 1. The results of methylation analysis of the polysaccharide revealed that hemicellulose HA-1 is a linear acidic xylan having a backbone of β-D-xylopyranosyl residues bonded together by (1→4) glycosidic links. This linear chain has single-unit branches of 4-*O*-methyl-D-glucopyranuronosyl and D-xylopyranosyl residues attached at C-2. The relative proportions of Glc2,3,4Me$_3$ and Xyl2,3,4Me$_3$ shown in columns A and B of Table 1 indicate that there were approximately the same number of branch units due to each residue, and the molar ratio of Xyl3Me in both columns indicates that there is one branch point for every 25 units of xylose in the main chain.

Partial acid hydrolysis[23] of the natural polysaccharide enabled the isolation by preparative paper chromatography of six oligosaccharides (three neutral and three acidic oligosaccharides). Methylation analysis (see Table 1) and the spectroscopic data (δ 4.25 for H-1 and δ 102.5 for C-1) of the neutral oligosaccharides NOS-1, NOS-2, and NOS-3 showed that they belong to a homologous series of β-D-xylopyranose-containing oligosaccharides with (1→4) glycosidic links,[18,19,24] and it was possible for them to be identified as xylobiose, xylotriose, and xylotetraose, respectively.

The smallest of the acidic oligosaccharides (AOS-1) is an aldobiouronic acid having an $[\alpha]^{25}_D$ +102.0°, indicative of the existence of an α-D-glycosidic link; this was confimed by the NMR spectroscopic data (δ 5.18 for H-1 and δ 98.5 for C-1 of the 4-*O*-methyl-D-glucopyranuronosyl unit).[18,19] A portion of this oligosaccharide was converted into its corresponding methyl ester, methyl glycoside, and then analyzed by MS.[25] The mass spectrum of this product showed a fragmentation pattern consistent with the structure methyl 3,4-di-*O*-acetyl-2-α-*O*-(methyl 3,4-di-*O*-acetyl-4-*O*-methyl-D-glucopyranosyluronate)-D-xylopyranose. Therefore, AOS-1 was identified as 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylopyranose (α-D-Glc*p*A4Me-(1→2)-D-Xyl*p*).

Spectroscopic data of the other two acidic oligosaccharides (AOS-2 and AOS-3) were similar to those of the aldobiouronic acid (δ 5.18 for H-1 and δ 98.5 for C-1 of 4-Me-α-D-Glc*p*A). Methylation analysis of both oligosaccharides showed that their neutral residues were xylobiose and xylotriose. Therefore, we have identified these three acidic oligosaccharides as α-D-Glc*p*A4Me-(1→2)-D-Xyl*p*, β-D-Xyl*p*-(1→4)[α-D-Glc*p*A4Me-(1→2)]-D-Xyl*p*, and β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)[α-D-Glc*p*A4Me-(1→2)]-D-Xyl*p*. The presence of these oligosaccharides is consistent with the pattern of substitutions in the main chain that we have described above.

These findings indicate that hemicellulose HA-1 is an acidic xylan formed by a main chain of β-D-xylopyranosyl units joined by (1→4) glycosidic links that show branch units of 4-*O*-methyl-α-D-glucopyranosyluronic acid or D-xylopyranose joined at position 2, in a ratio of 1:25.

No precedents exist for the structural characterization of polysaccharides isolated from products related with grape bagasse. Various oligosaccharides that seem to show growth promotion activity in plants have been isolated from the xylem of the vine plant.[26,27] One possible application for xylans such as those described in this study is to improve the quality of paper.[28] Their widespread natural occurrence and the relative ease with which they can be extracted from biomass and hydrolyzed and converted into furfural has led to this group of materials being investigated as a source of "liquid fuel" for making chemically pure products.[29,30]

Experimental Section

General Experimental Procedures. Descending paper chromatography was performed using Whatman No. 3 MM paper, with ethyl acetate−acetic acid−formic acid−water (18:3:1:4) as eluant. Detection of the component sugars was performed with diphenylamine−aniline.[31] Optical rotations were recorded with a Perkin-Elmer 241 polarimeter, and the IR spectra were carried out with a Perkin-Elmer 257 spectrophotometer. ^{1}H NMR and ^{13}C NMR were performed with a Varian Unity-400 instrument. Spectra of the methylated polysaccharide were obtained in $CDCl_3$ and referenced by means of the residual peak of the solvent. Spectra of the oligosaccharides were obtained in D_2O; in this case, proton spectra were referenced by means of the residual peak of the solvent, whereas for the carbon spectra, MeOH was used as an internal reference standard.

GC of the alditol acetates was performed with a Hewlett-Packard Model 5890A gas chromatograph, fitted with a flame-ionization detector and a Supelcowax 10 M WCOT column (30 m × 0.53 mm i.d.), using a program that maintained an isocratic temperature of

220 °C for 15 min and then passed to 230 °C at a rate of 3 °C min^{-1}. For the partially methylated alditol acetates (PMAA), an SPB-1 WCOT column (30 m × 0.53 mm i.d.) was used, with a temperature program of 120−250 °C at a rate of 5 °C min^{-1}. GC−MS was performed with a Kratos MS-80 instrument fitted with a CP-SIL WCOT column (25 m × 0.32 mm i.d.), using a temperature program of 100−250 °C at a rate of 5 °C min^{-1}. The ionization potential was 70 eV.

Size-exclusion chromatography was performed with a FPLC Superose 10/30 column (Pharmacia, V_o 7.3 mL, V_i 21.9 mL) using 100 mM NaOH as eluent, at a rate of 0.5 mL min^{-1}. Solutions (0.2 mL) containing 1 mg mL^{-1} of the polysaccharide were injected, and the eluate was monitored by differential refractometry. The column was calibrated using dextrans of known molecular weights. HPAE-PAD HPLC of the monosaccharides resulting from total acid hydrolysis was performed with a Dionex DX-500 chromatograph equipped with a Dionex PA-1 column and precolumn. For analysis of the neutral sugars, the following were used as eluents: eluent A, NaOH (50 mM); eluent B, NaOH (300 mM); eluent C, deionized water. A program was used that maintained a 1:1 isocratic mixture of A and C for 15 min and then passed to a 100% solution of B for 15 min, using a No. 8 curve gradient.[32] Uronic acids were analyzed using the following as eluents: eluent A, a solution of 100 mM in NaOH and 600 mM in sodium acetate; eluent B, deionized water. The elution was performed under isocratic conditions using a mixture of 25% of A and 75% of B.[33]

Plant Material. Grape bagasse was obtained from the grapes (*V. vinifera* cv. Palomino) collected in the Jerez/Xerez/Sherry Zone in September 1992 and were provided by Domecq S.A. Grapes were pressed at Bodegas Domecq, and the bagasse was washed with water and dried at room temperature, in the dark.

Isolation and Purification of the Polysaccharide. Grape seeds (530 g) were triturated in a Braun AG-4050 mill, and the oil was extracted successively with hexane, ethanol and chloroform−methanol (1:1) for 18 h in a Soxhlet extractor. The residue (479 g) was stirred for 24 h at room temperature with sodium methoxide 0.25 M in methanol (1 L).[11] The insoluble residue was collected by centrifugation and washed with methanol until neutrality; it was then extracted with deionized water at room temperature for 24 h.

The remaining solid material (459 g) was delignified with sodium chlorite and acetic acid.[12] The resulting holocellulose (254 g) was stirred with aqueous 10% NaOH (5 L) containing 10 mM $NaBH_4$, for 24 h at room temperature under nitrogen. The resulting extract was vacuum filtered through filter cloth. Hemicellulose A was precipitated from this extract by acidification to pH 5 with 50% acetic acid. After storage for 3 h at 5 °C, the precipitate was collected by centrifugation, washed with water and ethanol, and then vacuum-dried to yield the crude polysaccharide. Hemicellulose B was later isolated from the supernatant solution by precipitation with ethanol, after its dialysis against running water.

A solution of the hemicellulose A (5.55 g) in 5% aqueous potassium hydroxide (550 mL) was treated with Fehling's solution[14] until precipitation was complete. The precipitated hemicellulose was collected by centrifugation, treated with 5% HCl in methanol, at 0 °C for 5 min, and then centrifuged. The residue was then washed four times with ethanol and twice with acetone and then vacuum-dried over phosphorus pentaoxide. This entire precipitation procedure was then repeated two more times; this preparation (2.64 g) was designated hemicellulose A-1 (HA-1), $[\alpha]^{25}_D$ −47.4° (*c* 1.16, 1 M sodium hydroxide).

Methylation Analysis of the Polysaccharide. A quantity (207.5 mg) of HA-1 was methylated by the Hakomori method.[17] The methylated product was purified by precipitation from benzene with light petroleum (bp 30−60 °C) (122.3 mg), $[\alpha]^{25}_D$ −53.0° (*c* 1.34, $CHCl_3$).

A portion of the methylated polysaccharide was hydrolyzed, and the resulting sugars were converted into the corresponding partially methylated alditol acetates (PMAA),[15] which were analyzed by GC[20] and GC−MS.[21]

To a solution of another portion of the methylated polysaccharide (22.7 mg) in dry tetrahydrofuran (10 mL) was added $LiAlH_4$ (lithium aluminum hydride) (380 mg).[22] The mixture was refluxed in an atmosphere of N_2 for 24 h, 2 mL of acetone was added to destroy the excess of hydride, and it was filtered through Whatman No. 1 paper. The product of the reaction was then extracted in $CHCl_3$ and vacuum-dried for 48 h over phosphorus pentaoxide. The resulting product showed IR absorption at 3600 cm^{-1} (OH), but not at 1735 cm^{-1} (ester C=O). The reduction product was hydrolyzed, and the resulting sugars were converted into PMAA and analyzed by GC[20] and GC−MS.[21]

The methylation analysis of the oligosaccharides was performed by the method of Harris et al.[34]

Monosaccharide Composition of the Polysaccharides and Oligosaccharides. (a) By Gas−Liquid Chromatography. Polysaccharide HA-1 (12.0 mg) was treated with 0.25 mL of 72% (w/w) H_2SO_4 at room temperature for 1 h,[15] after which time the sample was diluted to a concentration of 1 M (2.75 mL) and then heated for 3 h at 100 °C. When cool, the hydrolysate was neutralized with 0.6 mL of 15 M NH_3 solution, 50 μL of a solution of *myo*-inositol (20 mg mL^{-1}) was added as internal standard, and the hydrolyzate was clarified by centrifugation. A portion of 200 μL of the upper phase of centrifugate was reduced using 1 mL of $NaBH_4$ solution in DMSO (20 mg mL^{-1}) at 40 °C for 90 min. Then, 100 μL of 18 M acetic acid was added to decompose excess sodium borohydride. Quantities of 1-methylimidazole (200 μL) and acetic anhydride (2 mL) were added to the mixture of reduced sugars and mixed. After 10 min at room temperature, 10 mL of water was added to decompose the excess of acetic anhydride. When cool, 1 mL of dichloromethane was added, and the solution was mixed. After phase separation, the lower phase was removed with a Pasteur pipet, and the resulting alditol acetates were analyzed by GC.[16] The yield of recovery sugars was ∼95% from the polysaccharide samples.

(b) By High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD HPLC). A quantity of 2 mg of the polysaccharide was hydrolyzed with 0.3 mL of 2 M trifluoroacetic acid at 121 °C for 2.5 h. To the reaction mixture was added 50 μL of *myo*-inositol (20 mg L^{-1})

Table 2. ^{13}C NMR (δ, ppm) Data of Neutral Oligosaccharides Obtained by Partial Hydrolysis from Fraction HA-1

compd[a]	residue	C-1	C-2	C-3	C-4	C-5
NOS-1	β-Xyl*p*(1→4)-	102.8	73.7	76.6	70.1	66.1
	-β-Xyl*p*	97.5	74.8	74.9	77.4	63.9
	-α-Xyl*p*	93.0	72.3	71.9	77.5	59.8
NOS-2	β-Xyl*p*(1→4)-	102.8	73.7	76.5	70.1	66.2
	-β-Xyl*p*(1→4)-	102.6	73.6	74.6	77.3	63.9
	-β-Xyl*p*	97.5	74.8	74.9	77.3	64.4
	-α-Xyl*p*	92.9	72.3	71.8	77.5	59.8
NOS-3	β-Xyl*p*(1→4)-	102.9	73.8	76.6	70.2	66.2
	-β-Xyl*p*(1→4)-	102.7	73.7	75.0	77.4	64.1
	-β-Xyl*p*(1→4)-	102.7	73.7	75.0	77.4	64.1
	-β-Xyl*p*	97.5	74.7	74.7	77.4	63.9

[a] NOS 1/3 denote neutral oligosaccharides 1−3, obtained on partial acid hydrolysis.

solution as internal standard, and the mixture was completely dried in a current of N_2. The sample was then dissolved in 5 mL of deionized water and analyzed by HPAE-PAD HPLC.[32,33] Uronic acids were also determined by the carbazole method using D-glucuronic acid as the standard.[35]

Partial Acid Hydrolysis. Hemicellulose HA-1 (221 mg) was treated with 0.125 M sulfuric acid for 2 h at 100 °C.[23] The hydrolyzate was neutralized with $BaCO_3$, basified with 1 M KOH, and then passed through a column of Amberlite resin IR-120 (H^+) and concentrated. The syrupy residue was eluted from a column of Amberlite resin IRA-400 (AcO^-), first with water to yield the neutral sugars and then with aqueous 10% acetic acid to yield the acidic oligosaccharides. Components of the neutral fractions were separated by preparative paper chromatography, which enabled the isolation of three oligosaccharides (NOS-1, NOS-2, and NOS-3). These three oligosaccharides were crystallized from EtOH and were identified by methylation analysis and by their ^{1}H NMR and ^{13}C NMR data.

NOS-1. A white oligosaccharide (15 mg) was isolated (EtOH), $[\alpha]^{25}_D$ −30.0° (*c* 1.13, EtOH−H_2O 1:1), with the following spectroscopic data: ^{1}H NMR (D_2O, 400 MHz) δ 5.00 (1H, d, $J_{1,2}$ = 4.0 Hz, H-1 α-D-Xyl*p*), 4.40 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1 β-D-Xyl*p*), 4.27 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1 β-D-Xyl*p*-(1→4)-), 3.87 (1H, dd, $J_{5\alpha,5\beta}$ = 12.0 Hz, $J_{4,5\beta}$ = 5 Hz, H-5β β-D-Xyl*p*), 3.78 (1H, dd, $J_{5\alpha,5\beta}$ = 12.0 Hz, $J_{4,5\beta}$ = 5 Hz, H-5β β-D-Xyl*p*-(1→4)-), 3.65−3.53 (5H, group of signals corresponding to H-5α, H5β and H-4 of α-D-Xyl*p*, H-4 of β-D-Xyl*p*, and H-4 of β-D-Xyl*p*-(1→4)-), 3.47 (1H, t, $J_{2,3}$ = $J_{3,4}$ = 7.0 Hz, H-3 α-D-Xyl*p*), 3.43 (1H, dd, $J_{1,2}$ = 5.0 Hz, $J_{2,3}$ = 9.0 Hz, H-2 α-D-Xyl*p*), 3.36 (1H, t, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, H-3 β-D-Xyl*p*), 3.36 (1H, t, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, H-3 β-D-Xyl*p*-(1→4)-), 3.19 (1H, t, $J_{5\alpha,5\beta}$ = $J_{4,5\alpha}$ = 11.0 Hz, H-5α β-D-Xyl*p*), 3.12 (t, $J_{5\alpha,5\beta}$ = $J_{4,5\alpha}$ = 11.0 Hz, H-5α β-D-Xyl*p*-(1→4)-), 3.10−3.02 (3H, group of signals corresponding to the protons H-2 of β-D-Xyl*p*-(1→4)-, -β-D-Xyl*p*-(1→4)-, and β-D-Xyl*p*); ^{13}C NMR (D_2O, 100 MHz), see Table 2.

A quantity (8 mg) of this product was submitted to methylation analysis; the results are given in Table 1.

NOS-2. A white oligosaccharide (13.6) was isolated (EtOH), $[\alpha]^{25}_D$ −42.0° (*c* 1.05, EtOH−H_2O 1:1), with the following spectroscopic data: ^{1}H NMR (D_2O, 400 MHz) δ 5.01 (1H, d, $J_{1,2}$ = 4.0 Hz, H-1 α-D-Xyl*p*), 4.40 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1 β-D-Xyl*p*), 4.30 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1 -β-D-Xyl*p*-(1→4)-), 4.28 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1 β-D-Xyl*p*-(1→4)-), 3.92 (1H, dd, $J_{5\alpha,5\beta}$ = 12.0 Hz, $J_{4,5\beta}$ = 5 Hz, H-5β -β-D-Xyl*p*-(1→4)-), 3.87 (1H, dd, $J_{5\alpha,5\beta}$ = 12.0 Hz, $J_{4,5\beta}$ = 5 Hz, H-5β β-D-Xyl*p*), 3.79 (1H, dd, $J_{5\alpha,5\beta}$ = 12.0 Hz, $J_{4,5\beta}$ = 5 Hz, H-5β β-D-Xyl*p*-(1→4)-), 3.66−3.54 (6H, group of signals corresponding to protons H-5α, H5β and H-4 of α-D-Xyl*p*, H-4 of β-D-Xyl*p*, H-4 of -β-D-Xyl*p*-(1→4)-, and to H-4 of β-D-Xyl*p*-(1→4)-), 3.48 (1H, t, $J_{2,3}$= $J_{3,4}$ = 10.0 Hz, H-3 α-D-Xyl*p*), 3.45 (1H, dd, $J_{1,2}$ = 5.0 Hz, $J_{2,3}$ = 10.0 Hz, H-2 α-D-Xyl*p*), 3.37 (1H, t, $J_{2,3}$= $J_{3,4}$ = 10.0 Hz, H-3 -β-D-Xyl*p*-(1→4)-), 3.36 (1H, t, $J_{2,3}$= $J_{3,4}$ = 10.0 Hz, H-3 β-D-Xyl*p*), 3.25 (1H, t, $J_{5\alpha,5\beta}$ = $J_{4,5\alpha}$ = 10.0 Hz, H-5α -β-D-Xyl*p*-(1→4)-), 3.20 (1H, t, $J_{5\alpha,5\beta}$= $J_{4,5\alpha}$ = 10.0 Hz, H-5α β-D-Xyl*p*), 3.13 (1H, t, $J_{5\alpha,5\beta}$= $J_{4,5\alpha}$ = 10.0 Hz, H-5α β-D-Xyl*p*-(1→4)-), 3.10−3.03 (3H, group of signals corresponding to protons H-2 of β-D-Xyl*p*(1→4)-, -β-D-Xyl*p*-(1→4)-, and β-D-Xyl*p*); ^{13}C NMR (D_2O, 100 MHz) see Table 2.

A quantity (6.7 mg) of this product was submitted to methylation analysis; the results are given in Table 1.

NOS-3. A white oligosaccharide (6.3 mg) was isolated (EtOH), $[\alpha]^{25}_D$ −50.0° (c 1.03, EtOH−H_2O 1:1), with the following spectroscopic data: ^{1}H NMR (D_2O, 400 MHz) δ 4.91 (1H, d, $J_{1,2}$ = 4.0 Hz, H-1 α-D-Xyl*p*), 4.31 (1H, d, $J_{1,2}$ = 7.9 Hz, H-1 β-D-Xyl*p*), 4.21 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1 -β-D-Xyl*p*-(1→4)-), 4.09 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1 β-D-Xyl*p*-(1→4)-) 3.85 (1H, dd, $J_{5\alpha,5\beta}$ = 11.5 Hz, $J_{4,5\beta}$ = 5 Hz, H-5β -β-D-Xyl*p*-(1→4)-), 3.79 (1H, dd, $J_{5\alpha,5\beta}$ = 12.0 Hz, $J_{4,5\beta}$ = 6 Hz, H-5β β-D-Xyl*p*), 3.69 (1H, dd, $J_{5\alpha,5\beta}$ = 12.0 Hz, $J_{4,5\beta}$ = 5 Hz, H-5β β-D-Xyl*p*-(1→4)-), 3.58−2.95 (group of overlapped signals corresponding to the rest of protons); ^{13}C NMR (D_2O, 100 MHz) see Table 2.

A quantity (4.0 mg) of this product was submitted to methylation analysis; the results are given in Table 1.

The components of the acidic fractions were separated by preparative paper chromatography, which enabled the isolation of three oligosaccharides (AOS-1, AOS-2, and AOS-3) that were crystallized from EtOH and were identified by methylation analysis and by their ^{1}H NMR and ^{13}C NMR spectroscopic data.

AOS-1. A quantity (18.2 mg) of a yellowish-white oligosaccharide was isolated, $[\alpha]^{25}_D$ +102° (c 1.07, EtOH−H_2O 1:1), with the following spectroscopic data: ^{1}H NMR (D_2O, 400 MHz) δ 5.18 (1H, d, $J_{1,2}$ = 4.0 Hz, H-1 α-D-Glc*p*A(1→2)-α-D-Xyl*p*), 5.16 (1H, d, $J_{1,2}$ = 4.1 Hz, H-1 α-D-Glc*p*A4Me-(1→2)-β-D-Xyl*p*), 4.87 (1H, d, $J_{1,2}$ = 4 Hz H-1 α-D-Xyl*p*), 4.51 (1H, d, $J_{1,2}$ = 8 Hz, H-1 β-D-Xyl*p*), 4.09 (1H, d, $J_{4,5}$ = 10.2 Hz, H-5 α-D-Glc*p*A4Me-(1→2)-α-D-Xyl*p*), 4.99 (1H, d, $J_{4,5}$ = 10.1 Hz, H-5 α-D-Glc*p*A4Me-(1→2)-β-D-Xyl*p*), 3.73 (1H, dd, $J_{5\alpha,5\beta}$ = 11.2 Hz, $J_{4,5\beta}$ = 5.2 Hz, H-5β β-D-Xyl*p*), 3.28 (3H, s, CH_3O-C-4, α-D-Glc*p*A4Me-(1→2)-D-Xyl*p*), 3.64−2.96 3.58−2.95 (group of overlapped signals corresponding to the rest of protons); ^{13}C NMR (D_2O, 100 MHz) δ 110.0 (C-1 β-D-Xyl*p*), 98.5 (C-1 α-D-Glc*p*A4Me-(1→2)-α-D-Xyl*p*), 97.9 (C-1 α-D-Glc*p*A4Me-(1→2)-β-D-Xyl*p*), 97.2 (C-1 α-D-Xyl*p*), 90.6 (C-1 α-D-Xyl*p*), 83.5 (C-3 α-D-Glc*p*A4Me-(1→2)), 80.4 (C-2 α-D-Xyl*p*), 79.3 (C-2 β-D-Xyl*p*), 76.7 (C-4 α-D-Glc*p*A4Me), 75.3 (C-3 β-D-Xyl*p*), 73.1 (C-2 α-D-Glc*p*A4Me-(1→2) and C-3 α-D-Xyl*p*), 72.3 (C-5 α-D-Glc*p*A4Me-(1→2)), 72.2 (C-4 α-D-Xyl*p*), 70.4 (C-4 β-D-Xyl*p*), 65.9 (C-5 β-D-Xyl*p*), 61.8 (C-5 α-D-Xyl*p*), 60.8 (CH_3O-C-4 α-D-Glc*p*A4Me-(1→2)).

The oligosaccharide AOS-1 corresponds to an aldobiouronic acid; to identify it, it was converted into its corresponding methyl ester, methyl glycoside,[36] by

treatment with 3% HCl in methanol (5 mL) under reflux for 24 h. The product of this reaction was acetylated with acetic anhydride/pyridine (1:1) for 24 h at room temperature. Mass spectral analysis of the final product showed the following more relevant peaks: EIMS (70 eV) *m/z* 477 [abE_1] (0.3), 417 [abE_2] (0.6), 403 [baF_1] (0.4), 345 [abF_1] (3.4), 289 [aA_1] (30), 229 [aA_2] (35), 187 [aA_2-CH_2CO] (100), 155 [aA_3] (11), 127 [bC_3] (22), 85 [aK_2] (28), 43 [CH_3CO^+] (76).

Peaks are named according to the nomenclature used by Chizov and Kochetkov[37] for the different fragmentation series of permethylated glycosides, modified as suggested by Kováčik et al.[25] These data correspond to methyl 3,4-di-*O*-acetyl-2-*O*-(methyl 2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucopyranosyluronate)-D-xylopyranoside.

AOS-2. A quantity (10.3 mg) of a pale yellow oligosaccharide was isolated, $[\alpha]^{25}_D$ +98° (*c* 1.04, EtOH−H_2O 1:1), with the following spectroscopic data: ^{1}H NMR (D_2O, 400 MHz) δ 5.10 (1H, d, $J_{1,2} = 5.0$ Hz, H-1 α-D-Glc*p*A4Me-(1→2)-α-D-Xyl*p*), 5.09 (1H, d, $J_{1,2} = 4$ Hz, H-1 α-D-Glc*p*A4Me-(1→2)-*β*-D-Xyl*p*), 4.94 (1H, d, $J_{1,2} =$ 4 Hz, H-1 α-D-Xyl*p*), 4.37 (1H, d, $J_{1,2} = 8$ Hz, H-1 *β*-D-Xyl*p*), 4.33 (1H, d, $J_{1,2} = 8$ Hz, H-1 *β*-D-Xyl*p*-(1→4)), 4.08 (1H, d, $J_{4,5} = 10.0$ Hz, H-5 α-D-Glc*p*A4Me-(1→2)-D-Xyl*p*), 3.87 (1H, dd, $J_{5\alpha,5\beta} = 11.0$ Hz, $J_{4,5\beta} = 5.0$ Hz, H-5*β* *β*-D-Xyl*p*), 3.75 (1H, dd, $J_{5\alpha,5\beta} = 11.0$ Hz, $J_{4,5\beta} = 5.0$ Hz, H-5*β* *β*-D-Xyl*p*-(1→4)), 3.23 (3H, s, CH_3O-C-4, α-D-Glc*p*A4Me-(1→2)-D-Xyl*p*), 3.60−2.90 (group of overlapped signals corresponding to the rest of protons); ^{13}C NMR (D_2O, 100 MHz) δ 102.1 (C-1 *β*-D-Xyl*p*-(1→4)), 97.9 (C-1 *β*-D-Xyl*p*), 97.0 (C-1 α-D-Glc*p*A4Me-(1→2)), 92.5 (C-1 α-D-Xyl*p*), 83.0 (C-3 α-D-Glc*p*A4Me-(1→2)), 77.0 (C-2 *β*-D-Xyl*p*), 76.9 (C-4 *β*-D-Xyl*p*; C-4 α-D-Xyl*p*), 76.7 (C-3 *β*-D-Xyl*p*-(1→4); C-4 α-D-Glc*p*A4Me-(1→2)), 76.5 (C-2 α-D-Xyl*p*), 74.9 (C-3 *β*-D-Xyl*p*), 74.5 (C-2 *β*-D-Xyl*p*-(1→4)), 72.8 (C-2 α-D-Glc*p*A4Me-(1→2)), 71.9 (C-3 α-D-Xyl*p*), 71.8 (C-5 α-D-Glc*p*A4Me-(1→2)), 70.4 (C-4 *β*-D-Xyl*p*-(1→4)), 65.5 (C-5 *β*-D-Xyl*p*-(1→4)), 63.5 (C-5 *β*-D-Xyl*p*), 61.5 (CH_3O-C-4 α-D-Glc*p*A4Me-(1→2)), 59.2 (C-5 α-D-Xyl*p*).

A quantity (3.1 mg) of this product was submitted to methylation analysis; the results are given in Table 1.

AOS-3. A quantity (9.0 mg) of a pale yellow oligosaccharide was isolated, $[\alpha]^{25}_D$ +90° (*c* 1.20, EtOH−H_2O 1:1), with the following spectroscopic data: ^{1}H NMR (D_2O, 400 MHz) δ 5.11 (1H, d, $J_{1,2} = 4.0$ Hz, H-1 α-D-Glc*p*A4Me-(1→2)-α-D-Xyl*p*), 5.09 (1H, d, $J_{1,2} = 4$ Hz, H-1 α-D-Glc*p*A4Me-(1→2)-*β*-D-Xyl*p*), 4.98 (1H, d, $J_{1,2} = 4$ Hz, H-1 α-D-Xyl*p*), 4.42 (1H, d, $J_{1,2} = 8$ Hz, H-1 *β*-D-Xyl*p*), 4.41 (1H, d, $J_{1,2} = 8$ Hz, H-1 -*β*-D-Xyl*p*-(1→4)), 4.38 (1H, d, $J_{1,2} = 8$ Hz, H-1 *β*-D-Xyl*p*-(1→4)) 4.26 (1H, d, $J_{4,5} =$ 10.0 Hz, H-5 α-D-Glc*p*A4Me-(1→2)-D-Xyl*p*), 3.78 (1H, dd, $J_{5\alpha,5\beta} = 11.0$ Hz, $J_{4,5\beta} = 5.0$ Hz, H-5*β* *β*-D-Xyl*p*-(1→4)), 3.27 (3H, s, CH_3O-C-4, α-D-Glc*p*A4Me-(1→2)-D-Xyl*p*), 3.65−2.96 (group of overlapped signals corresponding to the rest of protons); ^{13}C NMR (D_2O, 100 MHz) δ 102.5 (C-1 *β*-D-Xyl*p*-(1→4)), 102.2 (C-1 -*β*-D-Xyl*p*-(1→4)), 98.0 (C-1 *β*-D-Xyl*p*), 97.0 (C-1 α-D-Glc*p*A4Me-(1→2)), 92.5 (C-1 α-D-Xyl*p*), 83.0 (C-3 α-D-Glc*p*A4Me-(1→2)), 77.3 (C-2 *β*-D-Xyl*p*), 76.9 (C-4 α-D-Xyl*p*), 76.6 (C-4 α-D-Glc*p*A4Me-(1→2), C-3 *β*-D-Xyl*p*-(1→4), C-4 -*β*-D-Xyl*p*-(1→4), C-4 *β*-D-Xyl*p*), 76.1 (C-2 α-D-Xyl*p*), 74.9 (C-3 *β*-D-Xyl*p*), 74.5 (C-3 -*β*-D-Xyl*p*-(1→4)), 73.3 (C-2 *β*-D-Xyl*p*-(1→4)), 73.2 (C-2 -*β*-D-Xyl*p*-(1→4)), 72.8 (C-2 α-D-Glc*p*A4Me-(1→2)), 71.8 (C-5 α-D-Glc*p*A4Me-(1→2)), 71.4 (C-3 α-D-Xyl*p*), 69.9 (C-4 *β*-D-Xyl*p*4Me-(1→4)), 65.7 (C-5 *β*-D-Xyl*p*-(1→4)), 65.3 (C-5 *β*-D-Xyl*p*), 63.3 (C-5 -*β*-D-Xyl*p*-(1→4)), 60.3 (CH_3O-C-4 α-D-Glc*p*A4Me-(1→2)), 59.3 (C-5 α-D-Xyl*p*).

A quantity (3.0 mg) of this product was submitted to methylation analysis; the results are given in Table 1.

Acknowledgment. We thank the Dirección General de Investigación Científica y Técnica (Grant No. AGR89-0615-C02-02) for financial support and the Servicio de Espectrometría de Masas de la Universidad de Sevilla for the GC-MS analysis.

References and Notes

(1) Fantozzi, P. *J. Am. Oil Chem. Soc.* **1981**, *58*, 1027−1031.
(2) Galán, M.; Massanet, G. M.; Montiel, J. A.; Pando, E.; Rodríguez Luis, F. *Grasas Aceites (Seville)* **1986**, *37*, 179−182.
(3) Massanet, G. M.; Montiel, J. A.; Pando, E.; Rodríguez Luis, F. *Grasas Aceites (Seville)* **1986**, *37*, 233−236.
(4) Massanet, G. M.; Montiel, J. A.; Pando, E.; Rodríguez Luis, F. *Grasas Aceites (Seville)* **1987**, *38*, 81−84.
(5) Massanet, G. M.; Montiel, J. A.; Pando, E.; Rodríguez Luis, F. *Grasas Aceites (Seville)* **1987**, *38*, 98−101.
(6) Igartuburu, J. M.; Massanet, G. M.; Montiel, J. A.; Pando, E.; Rodríguez Luis, F. *Grasas Aceites (Seville)* **1987**, *38*, 220−223.
(7) Igartuburu, J. M.; Massanet, G. M.; Montiel, J. A.; Pando, E.; Rodríguez Luis, F. *Grasas Aceites (Seville)* **1987**, *38*, 323−324.
(8) Igartuburu, J. M.; Martín del Río, R. Massanet, G. M.; Montiel, J. A.; Pando, E.; Rodríguez Luis, F. *J. Sci. Food Agric.* **1991**, *54*, 489−493.
(9) Igartuburu, J. M.; Martín del Río, R.; Montiel, J. A.; Pando, E.; Rodríguez Luis, F. *J. Sci. Food Agric.* **1991**, *57*, 437−440.
(10) Southgate, D. A. T. *J. Sci. Food Agric.* **1969**, *20*, 331−335.
(11) Morrison, I. M. *Carbohydr. Res.* **1997**, *57*, C4−C6.
(12) Whistler, R. L.; Brachrach, J.; Bowman, D. R. *Arch. Biochem.* **1948**, *19*, 25−33.
(13) Whistler, R. L.; Feather, M. S. *Methods Carbohydr. Chem.* **1965**, *5*, 144−145.
(14) Jones, J. N. K.; Stoodley, R. J. S. *Methods Carbohydr. Chem.* **1965**, *5*, 36−38.
(15) Harris, P. J.; Blakeney, A. B.; Henry, R. J.; Stone, B. A. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 272−275.
(16) Eldrige, A. C.; Black, L. T.; Wolf, W. J. *J. Agric. Food Chem.* **1979**, *27*, 799−802.
(17) Hakomori, S. *J. Biochem. (Tokyo)* **1964**, *55*, 205−208.
(18) Whyte J. N. C.; Englar, J. R. *Can. J. Chem.* **1971**, *49*, 1302−1305.
(19) Bock, K.; Pedersen, C.; Pedersen, H. *Adv. Carbohydr. Chem. Biochem.* **1984**, *42*, 193−226.
(20) Björndal, H.; Lindberg, B.; Svensson, S. *Acta Chem. Scand.* **1967**, *21*, 1081−1084.
(21) Lomax, J. A.; Gordon, A. H.; Chesson, A. *Carbohydr. Res.* **1985**, *138*, 177−188.
(22) Asensio, A. *Can. J. Chem.* **1988**, *66*, 449−453.
(23) Asensio, A. *Carbohydr. Res.* **1987**, *161*, 167−170.
(24) Hirsch, J.; Kovàc, P.; Petràkowà, E. *Carbohydr. Res.* **1982**, *106*, 203−206.
(25) Kováčik, V.; Bauer, Š.; Rosík, J.; Kovàc, P.*Carbohydr. Res.* **1968**, *8*, 282−290.
(26) Campbell, J. A.; Drake, A. M.; Lee, V. W. K.; Strother, S. *An. Bot.* **1995**, *75*, 359−363.
(27) Campbell, J. A.; Loveys, B. R.; Lee, V. W. R.; Strother, S. *Aust. J. Plant Physiol.* **1995**, *22*, 7−13.
(28) Thompson, N. S. In *Wood and Agricultural Residues*; Soltes, J., Ed.; Academic Press: New York, 1981; pp 101−119.
(29) Thompson, N. S. In *Organic Chemicals from Biomass*; Goldstein, I. S., Ed.; CRC Press: Boca Raton, FL, 1981; p 125.
(30) Gong, C. S.; Chen, L. F.; Flickinger, M. C.; Tsao, G. S. *Appl. Envir. Microbiol.* **1981**, *41*, 430−436.
(31) Bayley, R. W.; Bourne, E. J. *J. Chromatogr.* **1960**, *4*, 206−213.
(32) Kerhervé, P.; Charrière, B.; Gadel, F. *J. Chromatogr.* **1995**, *718*, 283−289.
(33) Quigley, M. E.; Englyst, H. N. *Analyst* **1994**, *119*, 1511−1518.
(34) Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, B. A. *Carbohydr. Res.* **1984**, *127*, 59−73.
(35) Bitter, T.; Muir, H. M. *Anal. Biochem.* **1962**, *4*, 330−334.
(36) Das Gupta, P. C.; Den, S. K.; Dey, A. *Carbohydr. Chem.* **1976**, *48*, 73−80.
(37) Chizhov, O. S.; Kochetkov, N. K. *Adv. Carbohydr. Chem.* **1966**, *21*, 29−93.

NP9702401