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Purification and analysis of growth-regulating xyloglucan-derived oligosaccharides by high-pressure liquid chromatography

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ABSTRACT

The retention times of 10 oligosaccharides, generated from the xyloglucans of *Rosa* and *Tropaeolum* by the action of *Trichoderma* cellulase, and of 17 related carbohydrates, in h.p.l.c. on an amino-substituted silica (Amino-Spheri-5) depended largely on the number of hydroxyl groups per molecule, whereas h.p.l.c. on a pellicular anion-exchange resin (CarboPac PA1) was strongly influenced by the nature of the sugar residues present, especially L-fucose, and by their linkages. The major nonasaccharide (XG9, D-Glc$_4$–D-Xyl$_3$–D-Gal–L-Fuc) obtained from *Rosa* xyloglucan, after purification on Amino-Spheri-5, retained biological activity as an inhibitor of auxin-induced growth in a *Pisum* stem-segment bioassay. H.p.l.c. on Amino-Spheri-5 was used to monitor the action of "Driselase" in stripping the non-reducing terminal α-D-Xyl*p*-(1→6)-β-D-Glc*p* units from XG9 to yield a pentasaccharide (XG5, D-Glc$_2$–D-Xyl–D-Gal–L-Fuc).

INTRODUCTION

Xyloglucan, a hemicellulose found in all higher plants examined[1,2], occurs in tight association with the cellulosic microfibrils of the primary cell wall[3] and may contribute significantly to the extensibility of the cell wall[4].

In addition to this structural rôle, a signalling rôle has been proposed. Thus, a specific nonasaccharide (XG9, Scheme 1), derived from xyloglucan, antagonised the auxin-promoted[5–7] and H^+-promoted[8] elongation of pea-stem segments and the optimal concentration was ~nM. XG9 is produced *in vitro* by exhaustive digestion of xyloglucan with cellulase, and *in vivo* in small amounts[9], apparently by the partial hydrolysis of xyloglucan rather than *de novo* as the nonasaccharide[10]. The closely related oligosaccharides XG7 and XG8 were inactive in this bioassay[5,6,11] as was methyl α-L-fucopyranoside[12], which indicated a crucial rôle for the terminal α-L-Fuc*p*-(1→2)-β-D-Gal*p* group. On the other hand, XG5 and 2′-fucosyl-lactose were active auxin-antagonists[12], which indicated that the α-D-xylose residues of XG9 are not necessary for activity.

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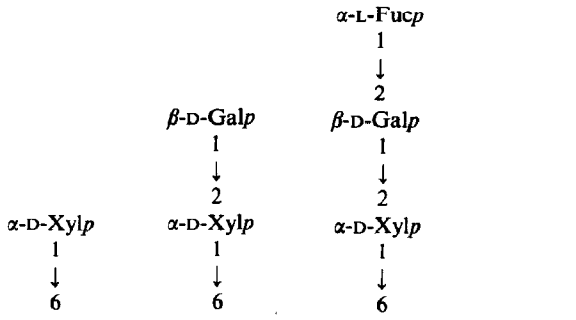
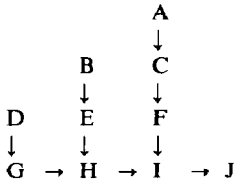


XG10

If the structure of XG10 is represented as



then the other xyloglucan-derived oligosaccharides referred to in the text are believed to be:

XG9	=	A CDEFGHIJ
XG9n	=	BCDEFGHIJ
XG8	}	CDEFGHIJ
XG8′	}	B DEFGHIJ
XG7	=	DEFGHIJ
XG5	=	A C F IJ
XG3	=	F IJ
XG2	=	F I

Scheme 1. Proposed structure of XG10 (from refs. 15 and 23).

A second growth-regulating activity has also been reported[13]; several xyloglucan-derived oligosaccharides promoted the growth of pea-stem segments in the absence of exogenous auxin[13]. This effect is not dependent on the presence of an α-L-fucose residue, and the optimal concentration was ~μM. In addition, ~nM (but not μM) XG9 reduces the beneficial effect of 2,4-dichlorophenoxyacetic acid on the regeneration of callus cultures from isolated protoplasts of carrot[7,14]. Therefore, there is considerable current interest in xyloglucan-derived oligosaccharides.

XG9 and XG7 are the major end products of the action of cellulase on xyloglucan from the primary cell walls of Dicots; other relatively minor products are XG5, XG8, and XG10[2,15–18]. In assessing structure–activity relationships, it is important to work with pure oligosaccharides and to have analytical methods for assessing homogeneity and for monitoring changes in structure during enzymic dissection[19]. These oligosaccharides have been purified by gel-permeation chromatography[6,15–19], paper chromatog-

raphy[9,18,19], and preparative t.l.c.[20]. We now describe semi-preparative and analytical h.p.l.c. methods for the isolation and characterisation of xyloglucan-derived oligosaccharides and for monitoring the enzymic cleavage of the non-reducing terminal α-D-Xyl*p*-(1→6)-D-Glc*p* units which occur in many xyloglucan-derived oligosaccharides.

RESULTS AND DISCUSSION

Analytical h.p.l.c. of xyloglucan-derived and related oligosaccharides. — The retention times of 27 carbohydrates, in two h.p.l.c. systems, namely, an amino-substituted silica (Amino-Spheri-5) and an anion-exchange resin (CarboPac PA1), are listed in Table I. In each system, an increase in M_r was correlated in general with an increase in retention time. However, the two systems differed in certain respects, so that they could be employed usefully in combination.

H.p.l.c. on Amino-Spheri-5 in aqueous 60% acetonitrile fractionated the oligosaccharides in an order that was related closely to the number of hydroxyl groups per molecule (Fig. 1a), regardless of the nature of the monosaccharide residues and their anomeric configurations. In particular, the seven L-fucose-containing carbohydrates

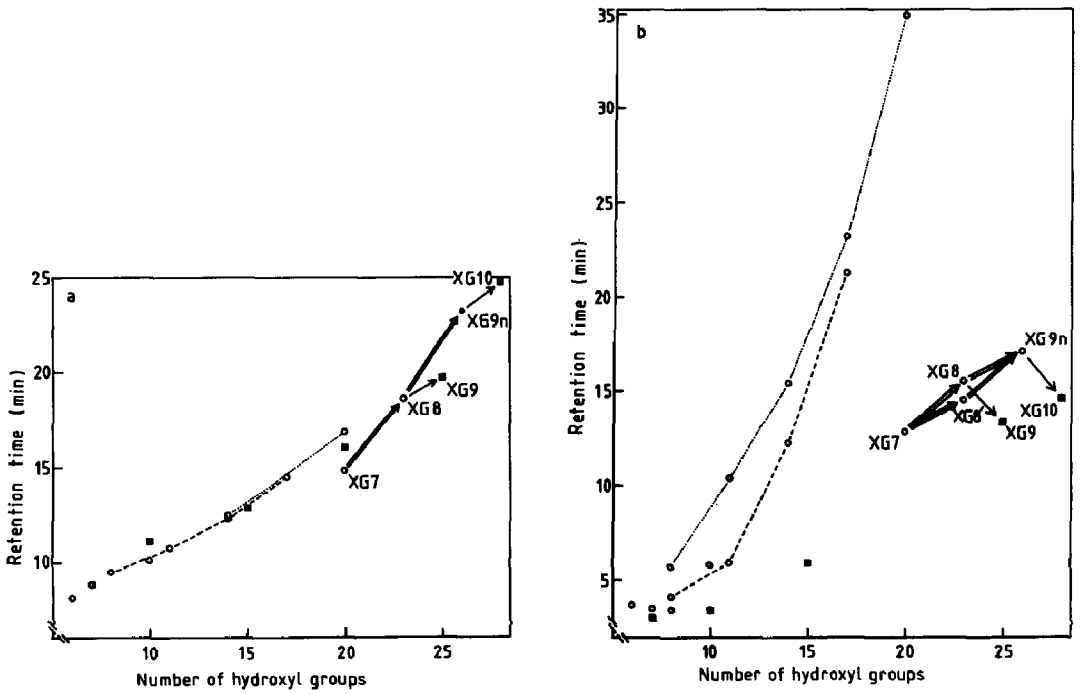


Fig. 1. Effect of structure on the behaviour of oligosaccharides in h.p.l.c. on (*a*) Amino-Spheri-5, (*b*) CarboPac PA1: ○, non-fucosylated compounds; ■, fucosylated compounds; ○·····○, malto-oligosaccharides; ○-------○, cello-oligosaccharides; →, shift in behaviour due to fucosylation; ⇒, shift due to galactosylation.

TABLE I

Retention times of xyloglucan-related carbohydrates

Compound	M_r	*No. of OH groups*[a]	*Retention time (min)*	
			CarboPac	*Amino-Spheri-5*
XG10 (Glc_4–Xyl_3–Gal_2–Fuc)	1532	28	14.6	24.7
XG9 (Glc_4–Xyl_3–Gal–Fuc)	1370	25	13.3	19.7
XG9n (Glc_4–Xyl_3–Gal_2)	1386	26	17.1	23.2
XG8, XG8′ (isomeric	1224	23	14.5	18.6
Glc_4–Xyl_3–Gal)	1224	23	15.5	18.6
XG7 (Glc_4–Xyl_3)	1062	20	12.8	14.8
XG7f (Glc_3–Xyl_2–Gal–Fuc)	1072	20	nd	16.0
XG5 (Glc_2–Xyl–Gal–Fuc)	782	15	5.9	12.8
XG3 (Glc_2–Xyl)	474	10	5.8	10.1
XG2 [α-Xyl-(1→6)-Glc]	312	7	3.5	8.79
α-Fuc-(1→2)-Gal	326	7	3.0	8.80
α-Fuc-(1→2)-β-Gal-(1→4)-Glc	458	10	3.4	11.1
β-Gal-(1→4)-Glc	342	8	3.4	nd
β-Xyl-(1→4)-Xyl	282	6	3.7	8.11
[β-Glc-(1→4)]$_4$-Glc	828	17	21.2	14.5
[β-Glc-(1→4)]$_3$-Glc	666	14	12.2	12.3
[β-Glc-(1→4)]$_2$-Glc	504	11	5.9	10.7
β-Glc-(1→4)-Glc	342	8	4.1	9.44
[α-Glc-(1→4)]$_5$-Glc	990	20	35.0	16.8
[α-Glc-(1→4)]$_4$-Glc	828	17	23.2	nd
[α-Glc-(1→4)]$_3$-Glc	666	14	15.4	12.4
[α-Glc-(1→4)]$_2$-Glc	504	11	10.4	nd
α-Glc-(1→4)-Glc	342	8	5.7	nd
Gal	180	5	3.1	8.74
Glc	180	5	3.1	8.38
Fuc	164	4	2.5	7.81
Xyl	150	4	3.1	7.63
Solvent peak	–	–	1.8	5.11

[a] Including the anomeric hydroxy group.

fitted the same curve as those that lacked fucose. Reduction of the concentration of the acetonitrile from 60 to 50% reduced the retention times (*e.g.*, that of maltohexaose was approximately halved), but the order of elution was unchanged.

H.p.l.c. on CarboPac PA1, on the other hand, resolved pairs of isomers (*e.g.*, XG8 and XG8′, cellotetraose and maltotetraose) which were not resolved on Amino-Spheri-5 (Table I; Fig. 1b). In addition, CarboPac was more responsive to the nature of the monosaccharide residues present. For example, 2-α-L-fucosylation of a β-D-galactose residue caused a *decrease* in retention time (*e.g.*, galactose → 2-*O*-fucosyl-galactose, lactose → 2′-*O*-fucosyl-lactose, XG8/XG8′ → XG9, and XG9n → XG10), similar to, though less pronounced than, the effect of 3- or 4-L-fucosylation of lactosamine and lacto-*N*-biose oligosaccharides[21]. Addition of other residues *increased* the affinity of oligosaccharides for CarboPac. Thus, the retention times were increased by 6-α-D-

xylosylation of β-D-glucose (*e.g.*, cellobiose → XG3, and cellotetraose → XG7) and by 2-β-D-galactosylation of α-D-xylose (*e.g.*, XG7 → XG8/XG8′, XG8/XG8′ → XG9n, and XG9 → XG10). It is known that the retention of oligosaccharides on CarboPac is affected by the monosaccharide composition, the glycosidic linkages, and the conformation in solution[21,22].

H.p.l.c. of the products of digestion of xyloglucan with cellulase. — The products obtained by the digestion of *Rosa* xyloglucan with *Trichoderma* cellulase were fractionated on Bio-Gel P-2 (Fig. 2a), then on Amino-Spheri-5 (*e.g.*, Fig. 3a–c). This analysis yielded k_{av} values for individual oligosaccharides (Fig. 2b), and these data, coupled with sugar composition, supported some of the tentative identifications. Two of the oligosaccharides, XG7 and XG9, were generated in large proportions (21 and 38%, respectively, of the total material detected). Several other oligosaccharides were generated in lesser proportions, two of which had sugar compositions consistent with their being XG10 and XG5 (2 and 6%, respectively). The xyloglucan-derived oligosaccharides obtained in this way were found to be nearly homogeneous by analytical h.p.l.c. on CarboPac. The small number of major oligosaccharides detected supports the view that most of the structure of xyloglucan consists of a few, highly conserved units.

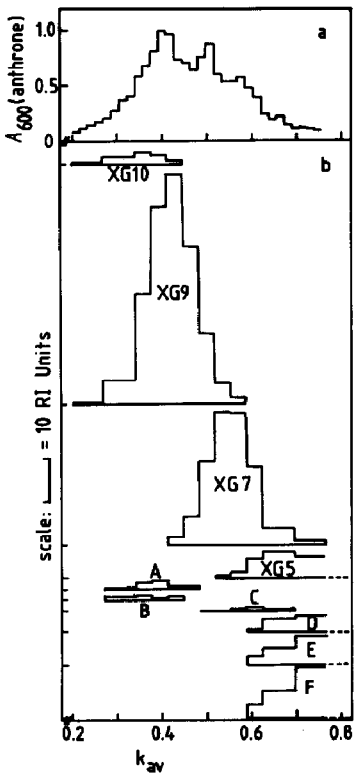


Fig. 2. Gel-permeation chromatography on Bio-Gel P-2 of cellulase-digested *Rosa* hemicellulose. Elution of (*a*) total hexose-reactive material, (*b*) oligosaccharides subsequently resolved by h.p.l.c. on Amino-Spheri-5 (for the identification of A–F, see Fig. 3).

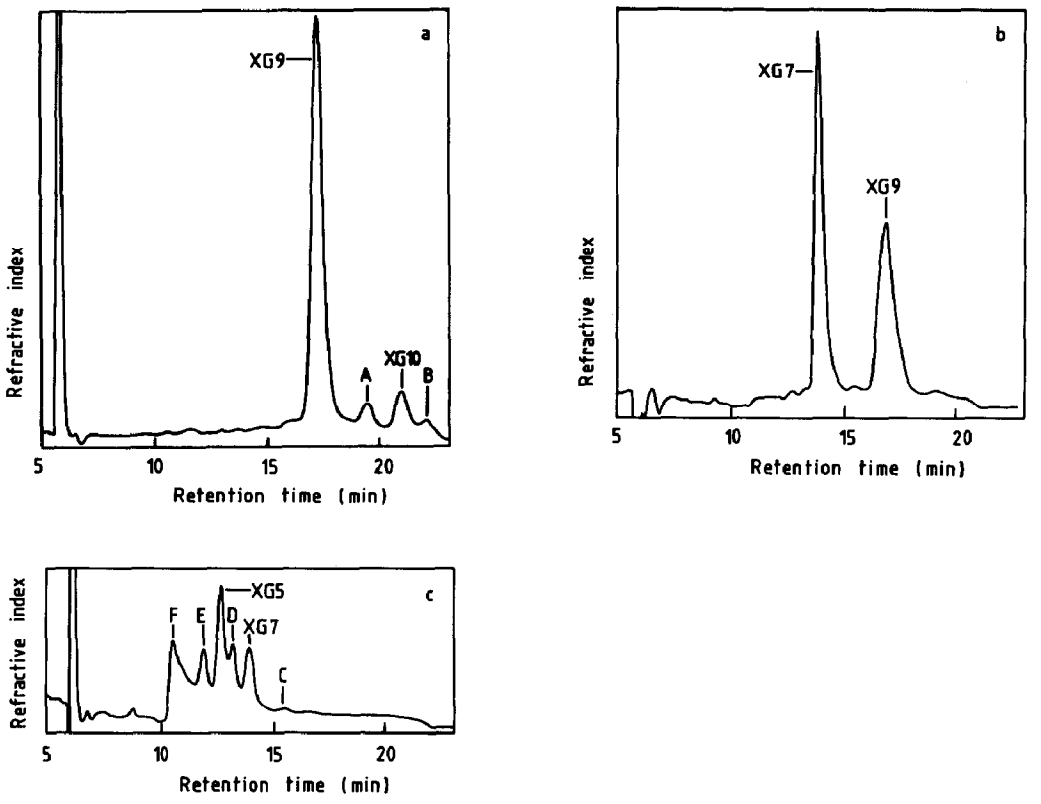


Fig. 3. Representative h.p.l.c. traces on Amino-Spheri-5 used to obtain the data in Fig. 2. H.p.l.c. of (*a*) k_{av} 0.34–0.38 material, (*b*) k_{av} 0.48–0.52 material, (*c*) k_{av} 0.62–0.70 material.

Biological activity of h.p.l.c.-purified XG9. — Samples of XG9 exhibited growth-inhibiting activity after partial purification by gel-permeation chromatography alone[5–7] and with subsequent paper chromatography[11]. In the present work, XG9 was purified further by semi-preparative h.p.l.c. on Amino-Spheri-5. This additional step removed some XG7 and XG10 (Fig. 4), and the purified XG9 still retained growth-inhibiting activity similar to that reported[11] for less pure samples (Fig. 5).

Biological activity and structural analysis of h.p.l.c.-purified XG5. — XG5, obtained from *Rosa* xyloglucan and purified by h.p.l.c. on Amino-Spheri-5, has already been reported to inhibit auxin-induced growth[12]. XG5 was characterised as follows. (*a*) The monosaccharide composition agreed closely with Glc:Xyl:Gal:Fuc in the ratios 2:1:1:1. (*b*) XG5 co-chromatographed in both h.p.l.c. systems and on paper chromatography with authentic XG5. (*c*) the k_{av} value (~0.65) on Bio-Gel P-2 was consistent with a d.p. of 5. (*d*) An [^{3}H]oligosaccharide that was identical chromatographically to XG5 was generated from [fucosyl-^{3}H]XG9 by the action of an unusual β-D-glucosidase ("xyloglucosidase") present in Driselase (Fig. 6). This enzyme catalyses the hydrolysis of β-D-Glc*p* residues that are 6-α-D-xylosylated and unsubstituted at O-4. Thus, it

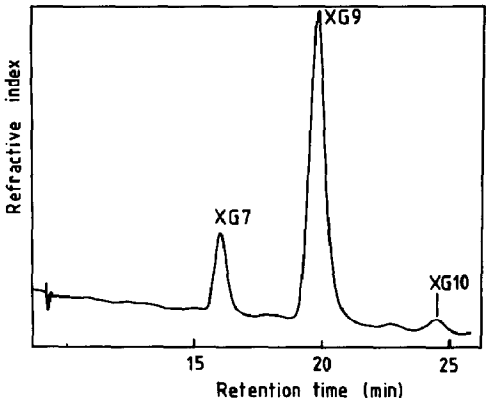


Fig. 4. Semi-preparative h.p.l.c. on Amino-Spheri-5 (in 50% aqueous MeCN at 0.4 mL/min) of XG9 partially pre-purified by gel-permeation and paper chromatography.

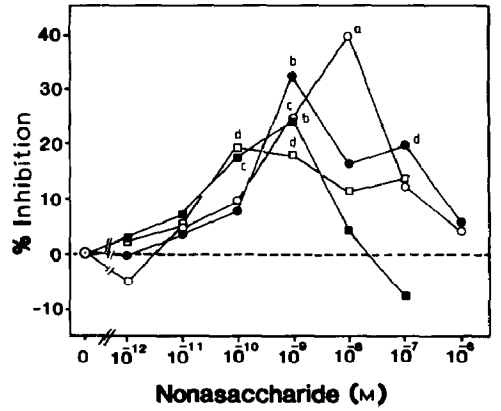


Fig. 5. Inhibition of 2,4-dichlorophenoxyacetic acid-induced elongation in etiolated pea-stem segments by h.p.l.c-purified XG9: ○—○, ●—●, □—□, ■—■ are the results of experiments conducted on 4 separate days. The significance of the apparent deviation of the XG9-treated segments from the corresponding XG9-free controls (-----) is indicated by a ($P \leqslant 0.001$), b ($0.001 < P < 0.01$), c ($0.01 < P < 0.02$), or d ($0.02 < P < 0.05$); elsewhere, $P \geqslant 0.05$.

releases α-D-Xyl*p*-(1→6)-D-Glc from XG9 and appears to be similar to an enzyme reported from *Aspergillus*[23]. The action of this enzyme should prove useful in the elucidation of structures of other xyloglucan-derived oligosaccharides. Driselase contains α-L-fucosidase and β-D-galactosidase that also attack xyloglucan-derived oligosaccharides, and steps were taken to minimise the action of these enzymes (inclusion of free monosaccharides[24] and Hg^{2+}). These precautions, coupled with the use of a short incubation time, rendered it unnecessary to purify the "xyloglucosidase" from Driselase.

The foregoing results indicate that h.p.l.c. on Amino-Spheri-5 and CarboPac PA1 will be valuable in investigations of the structure and function of xyloglucan-derived, biologically-active oligosaccharides.

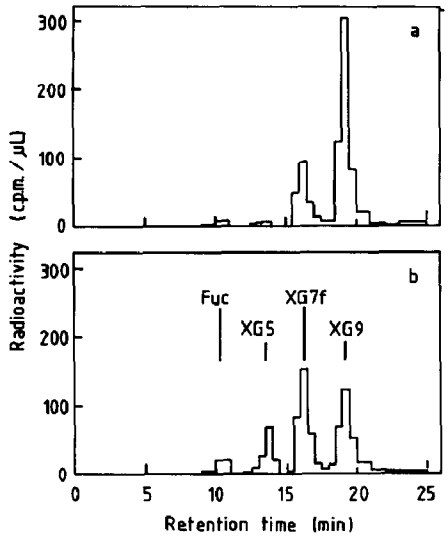


Fig. 6. Effect of Driselase on [fucosyl-^{3}H]XG9, monitored by h.p.l.c. on Amino-Spheri-5 after digestion for (*a*) 10 min, (*b*) 15 min.

EXPERIMENTAL

Extraction of polysaccharides. — Suspension-cultured cells of "Paul's Scarlet" rose (*Rosa* sp.) were maintained as described[6]. Cell walls were isolated and de-proteinised by stirring for 24 h at 25° in phenol–acetic acid–water (2:1:1, w/v/v)[19]. Hemicellulose was extracted from the phenol-insoluble material by stirring for 24 h at 25° in 6M NaOH that contained 10mM $NaBH_4$, and the mixture was neutralised with acetic acid, dialysed against water, and freeze-dried[6]. Xyloglucan, devoid of L-fucose residues, was isolated from *Tropaeolum* seeds as described[11].

Source of the oligosaccharides. — For the preparation of XG5, XG7, XG9, and XG10, hemicellulose (100 mg), isolated from cultured *Rosa* cells, was stirred for 20 min at 25° in 1% *Trichoderma viride* cellulase (Sigma, not purified further) in 20mM sodium acetate buffer (pH 4.7, 10 mL), then centrifuged at 2500*g* for 5 min. The supernatant solution was eluted from a column (180 × 1.4 cm) of Bio-Gel P-2 with water and fractions were assayed for hexose residues by the anthrone method[25]. XG9-enriched fractions were combined and subjected to preparative p.c. on Whatman 3MM paper in ethyl acetate–acetic acid–water (10:5:6). XG8, XG8′, and XG9n were prepared by the above procedure from *Tropaeolum*-seed xyloglucan. XG7f was obtained as an intermediate product during the treatment of XG9 with Driselase in the presence of L-fucose, D-galactose, and Hg^{2+} (see below). Authentic[18,23] XG3, XG5, XG7, XG9, and XG10 were generously donated by Dr. Y. Kato (Hirosaki University, Japan). α-L-Fuc*p*-(1→2)-D-Gal[26] (H-disaccharide) was kindly donated by Professor R. R. Schmidt (Konstanz University, F.R.G.). α-D-Xyl*p*-(1→6)-D-Glc (isoprimeverose) and β-D-Xyl*p*-(1→4)-D-Xyl (xylobiose) were produced by digestion of *Tropaeolum* xyloglucan and

Avena xylan, respectively, with Driselase and purified by preparative p.c. in ethyl acetate–pyridine–water (8:2:1)[19]. Cello-oligosaccharides were prepared by partial, H_2SO_4-catalysed hydrolysis of cellulose powder[8]. Malto-oligosaccharides, lactose, and 2′-*O*-fucosyl-lactose were obtained from Sigma.

Sugar composition of the oligosaccharides. — Each oligosaccharide was hydrolysed in 2M trifluoroacetic acid at 120° for 1 h and the hydrolysate was analysed by t.l.c. on cellulose first in 1-butanol–acetic acid–water (3:1:1) then, after drying, in ethyl acetate–pyridine–water (10:4:3) in the same dimension[19]. Sugars were detected with aniline hydrogenphthalate and scanned in a densitometer[6].

H.p.l.c. — Each fraction from Bio-Gel P-2 was dried *in vacuo*, and a solution of the residue in h.p.l.c.-grade water (100 μL) was centrifuged at 2000*g* for 5 min. Samples (20 μL) of the supernatant solution were injected via a Rheodyne sample loop on to a column (220 × 4.6 mm) of Amino-Spheri-5 (Brownlee) and eluted with aqueous 60% MeCN at 0.4 mL/min and ambient temperature. Detection was by refractive index.

For analysis of purified oligosaccharides on a column (250 × 4 mm) of CarboPac PA1 (Dionex), standard Dionex hardware was used. Eluents were sparged and pressurised with helium. The eluents were *A*, 100mM NaOH in 50mM NaOAc; and *B*, 100mM NaOH in 100mM NaOAc. The sample size was 20 μL, and the eluent flow rate was 1.0 mL/min, with a linear gradient of 100% *A* → 100% *B* in 20 min at ambient temperature. Detection was by means of a Dionex pulsed amperometric detector fitted with a gold electrode.

Limited digestion of [fucosyl-^{3}H]XG9 with "Driselase". — A solution of ~1 μCi of [fucosyl-^{3}H]XG9 (prepared as described[27]) in 1% "Driselase" (Sigma, partially purified[19]) in 20mM sodium acetate buffer (pH 4.7, 0.5 mL) that contained 40mM L-fucose, 40mM D-galactose[24], and 0.1mM $HgCl_2$ was kept at 25°. At intervals, the digestion was halted by the addition of ice-cold h.p.l.c.-grade MeCN to 60%. The samples were centrifuged (5 min at 2000*g*) and each supernatant solution was analysed by h.p.l.c. on Amino-Spheri-5. Fractions were collected, then dried *in vacuo*, and solutions of the residues in water were assayed for ^{3}H by scintillation counting in 10 vol. of 0.33% PPO–0.033% POPOP in toluene–Triton X-100 (2:1).

Bioassay. — H.p.l.c.-purified XG9 and XG5 were assayed for their ability to inhibit 2,4-dichlorophenoxyacetic acid-stimulated straight-growth in etiolated pea-stem segments as described[6].

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