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α-D-Glucuronosyl-(1→3)-L-galactose, an unusual disaccharide from polysaccharides of the hornwort *Anthoceros caucasicus*

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Dedicated to the memory of Professor Jeffrey B. Harborne

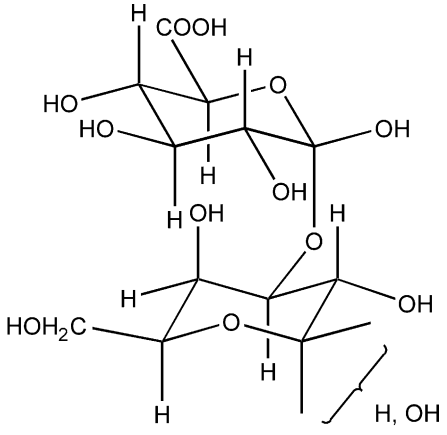
Abstract

Acid hydrolysis of cell wall-rich material from thalli of the hornwort *Anthoceros caucasicus* yielded substantial amounts of an unusual disaccharide (**1**). Hydrolysis of **1** yielded only GlcA, Gal and unhydrolysed **1**. Compound **1** was identified as α-D-Glc*p*A-(1→3)-L-Gal by ^{1}H and ^{13}C NMR spectroscopic analysis and by the susceptibility of its monosaccharide units to phosphorylation by enantiomer-specific kinases. Compound **1** was not detected in acid hydrolysates of other land plants including mosses, leafy and thalloid liverworts, lycopodiophytes and euphyllophytes; it was also absent from charophytes. The *Anthoceros* polysaccharide that yields **1** was partially extractable in cold aqueous buffer (pH 4.7) and Na_2CO_3, but not in EDTA or NaOH, suggesting that it was not a typical pectin or hemicellulose. The yield of **1** from various polysaccharide fractions correlated with the yields of Xyl, suggesting a previously unreported polymer containing D-GlcA, L-Gal and Xyl. The existence of a unique polysaccharide in an evolutionarily isolated plant (*Anthoceros*) supports the view that major steps in plant phylogeny were accompanied by significant changes in cell wall composition.

Keywords: Primary cell wall; L-Galactose; D-Glucuronic acid; Acidic disaccharide; Bryophyte; *Anthoceros*; Hornwort

1. Introduction

The major monosaccharide residues found in dicot and non-gramineous monocot primary cell walls (PCWs) are D-Glc, D-Gal, L-Ara, D-Xyl, L-Fuc, D-GalA, L-Rha, D-GlcA and D-Man (McNeil et al., 1984; Brett and Waldron, 1996; Fry, 2000); traces of L-Gal are also present (Roberts and Harrer, 1973; Baydoun and Fry, 1988). The PCW compositions of other euphyllophytes (=vascular plants other than lycopodiophytes) investigated differ only in the relative proportion of each monosaccharide. For example, the PCWs of gramineous monocots contain more Xyl and less Gal, Ara and Fuc (Burke et al., 1974; Carpita et al., 1996), whereas those of gymnosperms possess more Man (Thomas et al., 1987).



α-D-glucuronosyl-(1→3)-L-galactose **1**

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The PCWs of non-euphyllophyte land plants have been less well studied. The PCWs of all lycopodiophytes tested (*Selaginella*, *Lycopodium* and allied genera) have a high content of 3-*O*-methyl-D-galactose (MeGal), which is undetectable in the PCWs of other land plants (Popper et al., 2001). Among the bryophytes, liverwort PCWs resemble those of gymnosperms, the major neutral sugar residues being Glc > Gal > Man > Xyl≈Ara > Fuc≈Rha (Thomas, 1977; Popper and Fry, 2003). However, there is little information on the polysaccharides that contain these sugar residues. The acidic sugar residues of liverwort PCWs—previously thought to be mainly ManA (Das and Rao, 1966)—have now been shown to be principally GalA and GlcA (Popper and Fry, 2003). Bryophytes (hornworts, liverworts and mosses), like all other land plants, contain the hemicellulose xyloglucan, which is absent in their closest living algal relatives the charophytes (Popper and Fry, 2003).

These observations suggest a rapid evolution of PCW composition during charophyte→bryophyte→lycopediophyte evolution. Modern bryophytes are the surviving progeny of a great diversity of extinct taxa, among which were the first plants to colonise the land. Since the PCW plays several essential biological roles, including the regulation of cell expansion, tissue cohesion, defence (e.g. against microbes), ion-exchange and the production of oligosaccharins (Fry, 2000; Kaplan and Hageman, 1981; Brett and Waldron, 1996; Stübler and Buchenauer, 1996; Goldberg, 1995; Cassab, 1998), the demands placed on it—and therefore its optimal composition—may have changed rapidly during major upheavals in plant evolution. In particular, land colonisation by the first bryophytes is likely to have exposed these plants to new ecological problems whose solution may have required rapid evolution of the PCW. Presumably different problems would have had to be solved during vascularisation (the bryophyte→lycopodiophyte transition). It is therefore of interest to document any unique features of the PCWs of bryophytes. In the present paper we report on an unusual disaccharide (**1**) found in the PCW of the hornwort *Anthoceros caucasicus*.

2. Results

2.1. Chromatographic and electrophoretic characterisation of *1*

Acid hydrolysis of the alcohol-insoluble residue (AIR) from *Anthoceros* gametophytes yielded, in addition to the usual monosaccharides, a slow-migrating reducing sugar (**1**) (Fig. 1a). On paper chromatography (PC) in system 1, it migrated close to glucose 6-phosphate (Fig. 1b). On paper electrophoresis (PE) at pH 2.0, **1** was almost immobile, indicating the absence of strongly acidic groups such as phosphate or sulphate. On PE at pH 3.5, **1** migrated between GalA and ManA (Fig. 2a), supporting the presence of a –COOH group.

When **1** was subjected for a second time to acid hydrolysis, about half of it was hydrolysed. On PE at pH 3.5, the hydrolysis products were GlcA and a neutral sugar (Fig. 2a), which was identified by PC as Gal (Fig. 2b). No other products were detected, such as would have been obtained if **1** had been a trisaccharide or higher oligomer. Thus, **1** was a disaccharide of GlcA and Gal. The relative acid-stability of **1** and its staining (brown, not orange–yellow) with aniline hydrogenphthalate indicated that the GlcA was the non-reducing terminus (Harborne, 1998). Compound **1** resisted hydrolysis by Driselase, which contains β- but not α-D-glucuronidase activity (Gardner et al., 2002), indicating that **1** was α-GlcA-Gal.

2.2. Fractionation of Anthoceros polysaccharides

About 25% of the polysaccharide that yielded **1** was extracted by aqueous buffer, pH 4.7 (fraction W); none was detected in the EDTA extract (E) but additional amounts were extracted by Na_2CO_3 (C; 31%) and dilute NaOH (D; 15%). Little more was solubilised by concentrated alkali (A; traditionally expected to contain hemicelluloses) but 9% was present in fraction F_3, the first slightly acidic rinse *after* alkali treatment. About 18% remained in the α-cellulose residue (R).

On acid hydrolysis, all fractions yielded Glc (Fig. 3), probably reflecting its presence in diverse polymers, e.g. starch, callose and xyloglucan: the Glc yield is not considered further. Fractions W and C, rich in **1**, also yielded much GlcA, Xyl, and Gal. They yielded little Ara, GalA or Rha, indicating the paucity of pectins and arabinogalactans. Fraction A yielded little **1** and correspondingly little GlcA and Xyl, but it did yield Gal and Ara, suggesting arabinogalactans, and Man, suggesting mannan. Fraction D was intermediate in composition between C and A. Fraction F contained **1**, GlcA, Xyl and Gal (like fraction W), but also GalA, Rha and Ara, suggesting the additional presence of pectins. The final residue (R) yielded diverse sugars, including **1**.

Yields of **1** and of GlcA were highly correlated (Fig. 4). It appears that TFA released about half the GlcA residues as the monosaccharide and half as **1**. The Xyl yield also correlated with that of **1**, but with more scatter, suggesting that Xyl and **1** occur in the same polysaccharide but that Xyl also occurs in other polysaccharide(s). The only other monosaccharide to show some correlation with **1** was Gal, although the Gal curve (Fig. 4) did not pass through the origin, indicating that Gal occurs predominantly in other polysaccharide(s). The finding (see later) that the Gal in **1** was L- supports this conclusion, since most of the Gal in

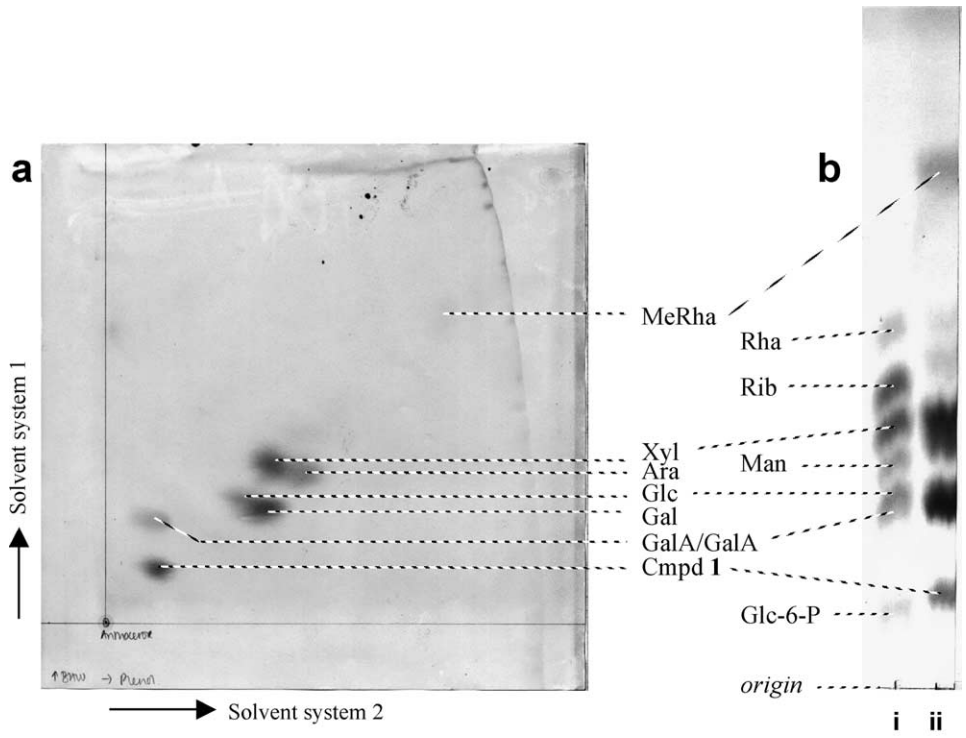

Fig. 1. PC of the acid hydrolysis products of *Anthoceros caucasicus* AIR. (a) A 2-D PC developed in solvent systems 1 and 2. (b) A 1-D PC developed in solvent system 1; track (i) = marker mixture containing the authentic sugars listed; track (ii) = fringe of the streak-loaded *Anthoceros hydrolysate* (the remainder of the streak of cmpd **1** was eluted and used for NMR spectroscopy). Both chromatograms were stained with aniline hydrogen-phthalate.

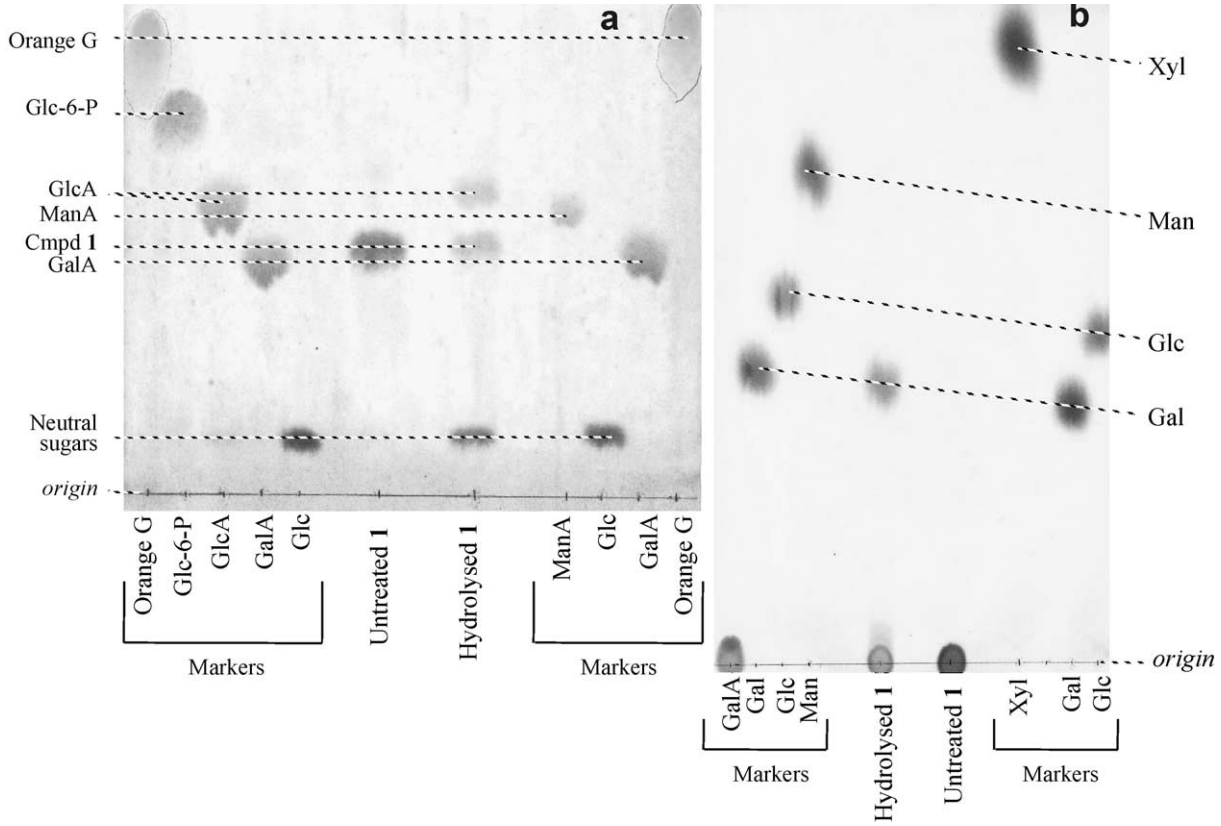

Fig. 2. Acid hydrolysis products of compound **1**. Hydrolysis products were separated by (**A**) PE at pH 3.5, 2.0 kV for 1 h and by (**B**) PC in solvent system 3. Reducing sugars were stained with aniline hydrogen-phthalate.

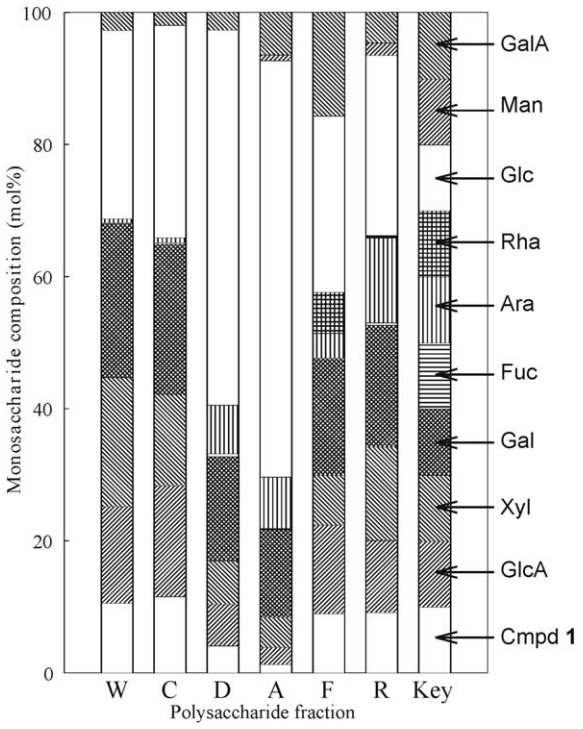

Fig. 3. Sugar composition of selected polysaccharide fractions extracted from *Anthoceros* AIR. The fractions were extracted serially with aqueous buffer at pH 4.7 (W), EDTA (data not shown), carbonate (C), dilute alkali (D) and 6 M alkali (A), followed by a final (F) wash in pH 4.7 buffer; the 'cellulosic' residue is labelled (R). Portions of each polysaccharide extract were dialysed, acid-hydrolysed, then analysed by HPLC. Total sugars (nmol/mg AIR) in the hydrolysates were W, 171; C, 193; D, 264; A, 123; F, 71; R, 136. Relative molar response factors in the pulsed amperometric detector were GalA, 1.0; GlcA 1.1; Fuc, 3.3; Rha, 3.0; Gal, 5.6; Glc, 5.7; Man, 4.7; Xyl, 3.6; Ara, 3.6. Quantification of compound **1** assumed the same molar response factor as GlcA.

the PCW is D-. The lack of correlation, or negative correlations, suggest that GalA, Rha, Ara, Man and Fuc are not major components of the polysaccharide that yields **1**.

2.3. Determination of the linkage of GlcA–Gal by NMR spectroscopy

The position of glucuronosylation of the disaccharide was deduced from its NMR spectra (Table 2). The 1D proton NMR spectrum (Fig. 5a) shows three anomeric proton doublets at δ 5.28 (H-1_A, $J=4.0$ Hz), δ 4.62 (H-1_B, $J=7.7$ Hz) and δ 5.22 (H-1_C, $J=3.8$ Hz) in area ratios 3:5:8 suggesting a reducing disaccharide where the first two signals arise from the two anomers A and B of the reducing terminus and the third from the non-reducing terminus (C). The remaining signals from residue C, H-2_C–H-5_C (identified from the 2D-COSY proton NMR spectrum—Fig. 5b), are clearly resolved and the inter-proton couplings show this residue to be an α-linked glucuronic acid.

The signals from H-2_A, H-3_B, H-4_A, H-2_B and H-4_B (also identified from the 2D-COSY spectrum) are also clearly resolved. The signal from H-5_A is partially obscured by H-5_C and the remaining signals from H-3_B, H-5_B and H-6 from residues A and B overlap in the region δ 3.63 to δ 3.68. Their positions were identified from the 2D 1-bond HSQC and long-range HMBC carbon–proton chemical shift correlation spectra (not shown), from which all the carbon chemical shifts were deduced. The inter-proton couplings for residues A and B showed these to be α and β anomers of galactose respectively. 1D-TOCSY experiments in which the signals from H-1_A and H-1_C were selectively inverted showed the connectivity of these resonances through to H-5_A and H-5_C respectively.

The position of the glycosidic linkage (see **1**) is shown by the observation of correlations between the H-1_C and C-3 of both anomers of the reducing residue in the 2D long-range HMBC carbon–proton shift correlation spectrum and also by the marked increase in the chemical shifts (8–10 ppm) of the ^{13}C resonances for C-3 of both galactose anomers compared with the non *O*-alkylated carbons.

2.4. Determination of D- versus L-isomers

Authentic D-Gal was completely phosphorylated by the native D-galactokinase present in the mung bean extract, whereas authentic L-Gal was unaffected. Boiled enzyme had no effect. At the same concentration as the authentic Gal samples, Gal isolated from **1** was largely unaffected by the kinase (Fig. 6), and is therefore concluded to be mainly the L-enantiomer.

This conclusion was confirmed by use of D-galactose oxidase. In this experiment, a trace of authentic D-[^{3}H]Gal, added as an internal standard, was completely oxidised whereas non-radioactive *Anthoceros* Gal in the same tube was largely unaffected (data not shown).

Authentic D-GlcA was largely phosphorylated by the native kinase preparation but not by the boiled control. No authentic L-GlcA was available for testing; however, L-glucuronokinase activity has not been reported from plants. The GlcA isolated from the *Anthoceros* disaccharide was phosphorylated by the kinase (Fig. 6), indicating that it was D-GlcA, the common enantiomer.

2.5. Occurrence of *1* in various green plants

Acid hydrolysates of AIR from various land plants and charophytes were analysed by two-dimensional PC to determine the presence of **1**. It was found only in the hornwort (Table 1), in which typical yields were about 15–20 mg per g AIR (estimated from staining intensity relative to authentic galactose)—similar to the concentration of GalA in the same AIR.

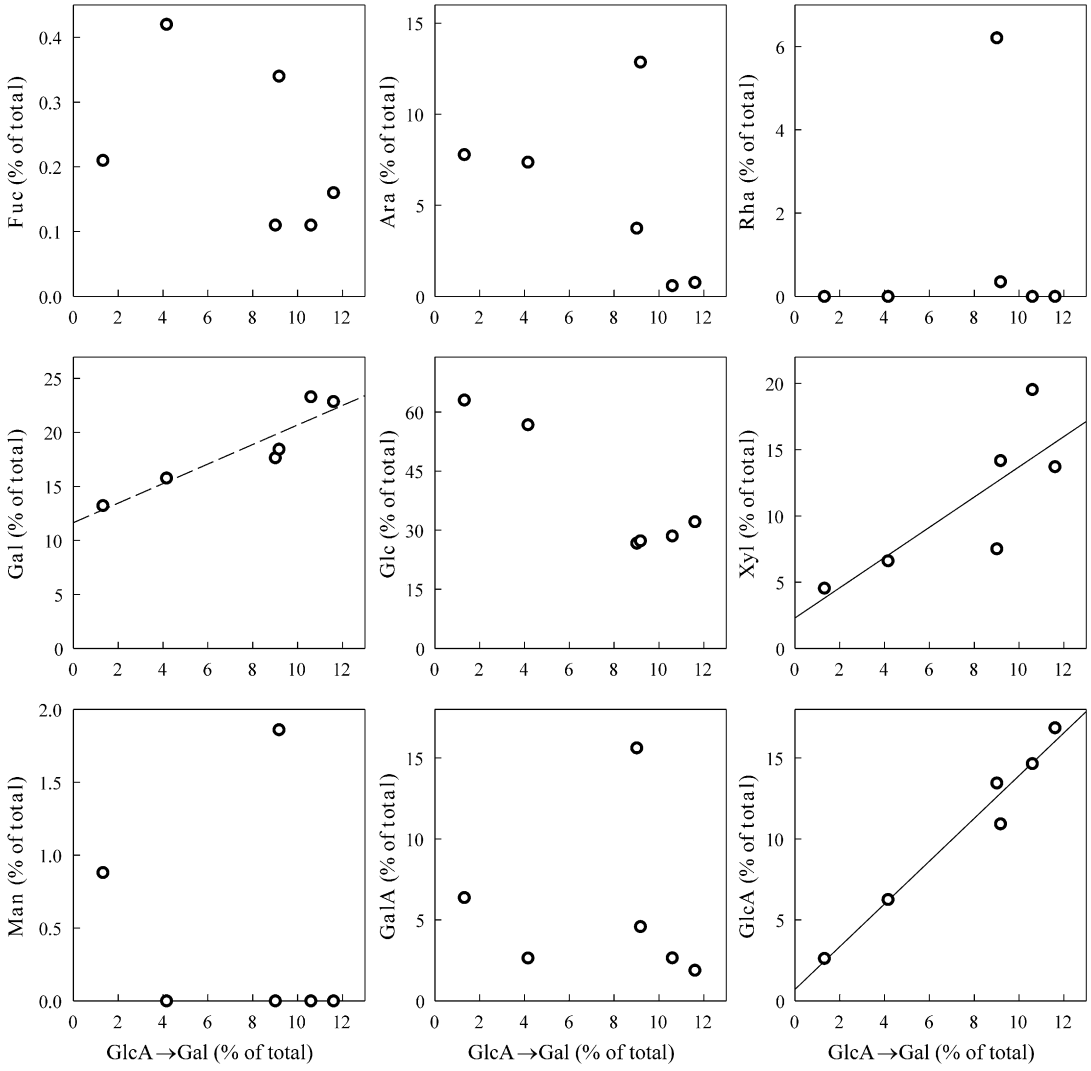

Fig. 4. Correlation between the occurrence of the disaccharide repeat-unit (**1**), and each of the other major products of acid hydrolysis of *Anthoceros* AIR, in six polysaccharide fractions. The six data-points on each graph represent (from left to right on the *x*-axis) fractions A, D, F, R, W and C. Linear regressions are drawn in where positive and significant.

3. Discussion

The observations indicate that *Anthoceros* contains a polysaccharide that is partially extractable with water and partially with Na_2CO_3, and that contains the repeat-unit α-D-Glc*p*A-(1→3)-L-Gal (**1**) and probably in addition Xyl. It is neither pectic (since it lacks GalA, Rha and Ara) nor hemicellulosic (since it is least well extracted by NaOH). The post-alkali extraction effected by pH 4.8 buffer has been observed with several other PCW polysaccharides (Shedletzky et al., 1992; Encina et al., 2002) but the explanation is unclear.

GalA is the major uronic acid residue in the PCWs of all land plants and charophytes tested. Charophyte and bryophyte PCWs are particularly rich in uronic acids compared with vascular plant PCWs, and contain high concentrations of both GalA and GlcA (Popper and Fry, 2003). However, among the diverse plants we studied, only the hornwort yielded **1**.

Most of the Gal in PCW polysaccharides is D-Gal, although L-Gal is also present in trace amounts (Roberts and Harrer, 1973; Baydoun and Fry, 1988) and may be incorporated into xyloglucan in place of L-Fuc in a mutant that is unable to produce this deoxyhexose (Zablackis et al., 1996).

Mucilage from a red alga contains α-D-GlcpA-(1→3)-D-Gal (Turvey and Griffiths, 1973), as do various proteoglycans such as those of human fibroblasts (Fransson et al., 1992, 2000). However, red algae and animals are not closely related to hornworts.

We considered the possibility that **1** might be non-hornwort-derived. *Anthoceros* and *Phaeoceros* have

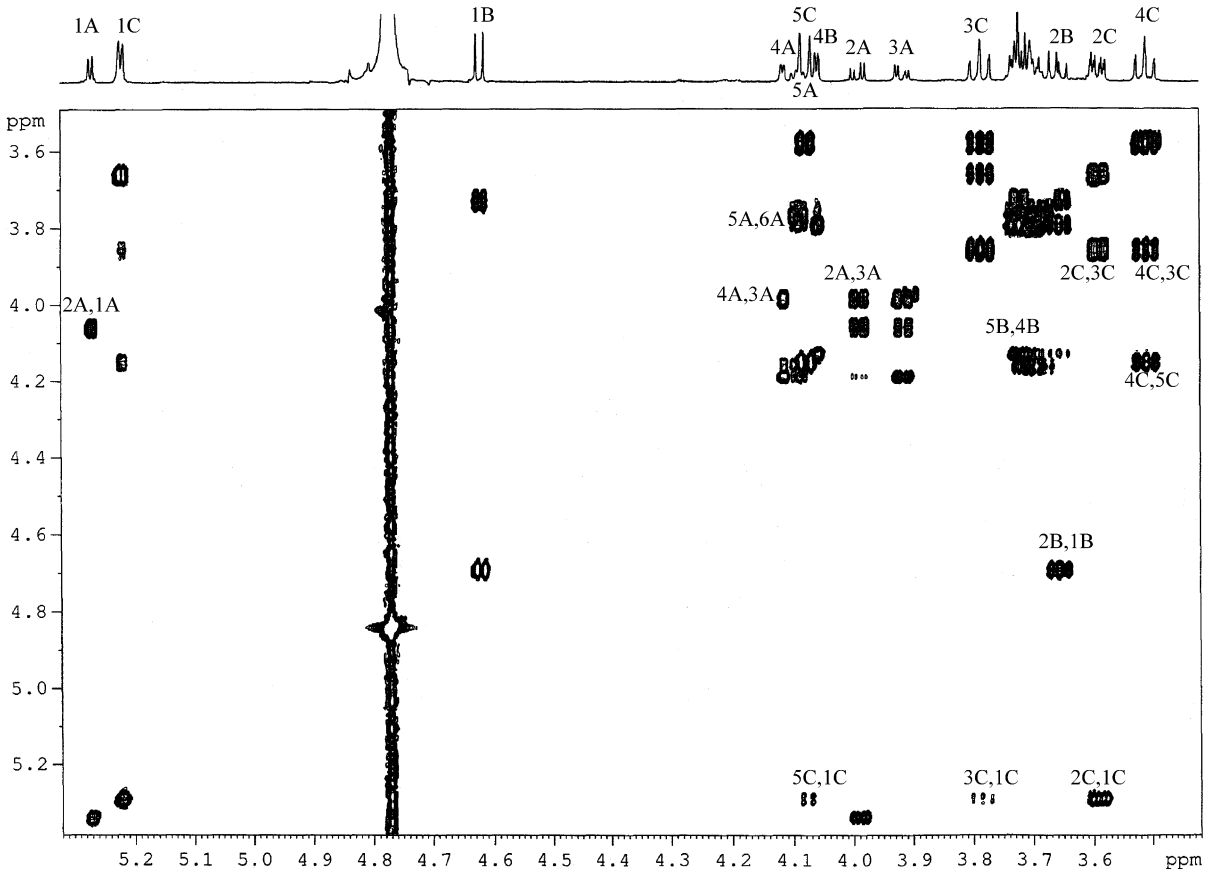

Fig. 5. NMR spectra of α-glucuronosyl-(1→3)-galactose. (a) 1-D single-pulse 600-MHz proton NMR spectrum over the region 3.3δ–5.3δ; the remaining resonances from residues A and B lie overlapping in the region 3.68–3.74δ (see text). (b) 2-D gradient-selected proton COSY NMR spectrum over the same region as in (a).

cavities in their thalli, some containing the cyanobacterium *Nostoc* (Watson, 1984). Partial acid hydrolysis of *Nostoc commune* yields the oligosaccharides

GlcA-(1 → 4/6)-Glc-(1 → 4)-Gal-(1 → 4)-Glc-(1 → 4)-Xyl

and

GlcA-(1 → 4)-MeGlc-(1 → 4)-Gal-(1 → 4)-Glc-(1 → 4)-Xyl,

where MeGlc = 6-*O*-methylglucose (Brull et al., 2000); their glucuronosyl linkages will be relatively resistant to hydrolysis under the conditions used by us. However, **1** has not been reported in *Nostoc*. *Nostoc commune* also secretes a polysaccharide that contains GlcNAc, GlcN and GalN (Hill et al., 1994). However, PE at pH 2.0, which readily detects these amino-sugars, did not show their presence in *Anthoceros*. We conclude that compound **1**, present in high concentrations as judged by staining, was derived from *Anthoceros* rather than from a symbiont.

Recent research has shown that major events in plant evolution, such as colonisation of the land (Popper and Fry, 2003), vascularisation (Popper and Fry, 2003), and the lycopodiophyte→euphyllophyte transition (Popper et al., 2001), were accompanied by major changes in PCW composition. It therefore seems likely that the diversification that occurred within the early Bryophyta during a period of rapid evolutionary experimentation—the mid-Ordovician (Kenrick and Crane, 1997)—was also accompanied by rapid changes in PCW composition. Such changes would have been essential for the colonisation of progressively more arid environments on the land. Although land plants are thought to be monophyletic (Bremer et al., 1987), relationships among the main basal extant groups are uncertain. Some phylogenies place liverworts as basal and either mosses or hornworts as the living sister group to vascular plants (Kranz and Huss, 1996; Bremer et al., 1987). Other phylogenies (Renzaglia et al., 2000) suggest that hornworts are the most primitive of the extant bryophytes: the present work suggests that hornworts experimented with a polysaccharide repeat-unit, compound **1**, which, however, was not retained by the more advanced bryophytes that evolved from (or alongside) them. Such a polysaccharide could possibly be a mucilaginous substance that helps to minimise desiccation. However, a precise definition of the ecological benefits, and costs, of synthesising the polysaccharide that

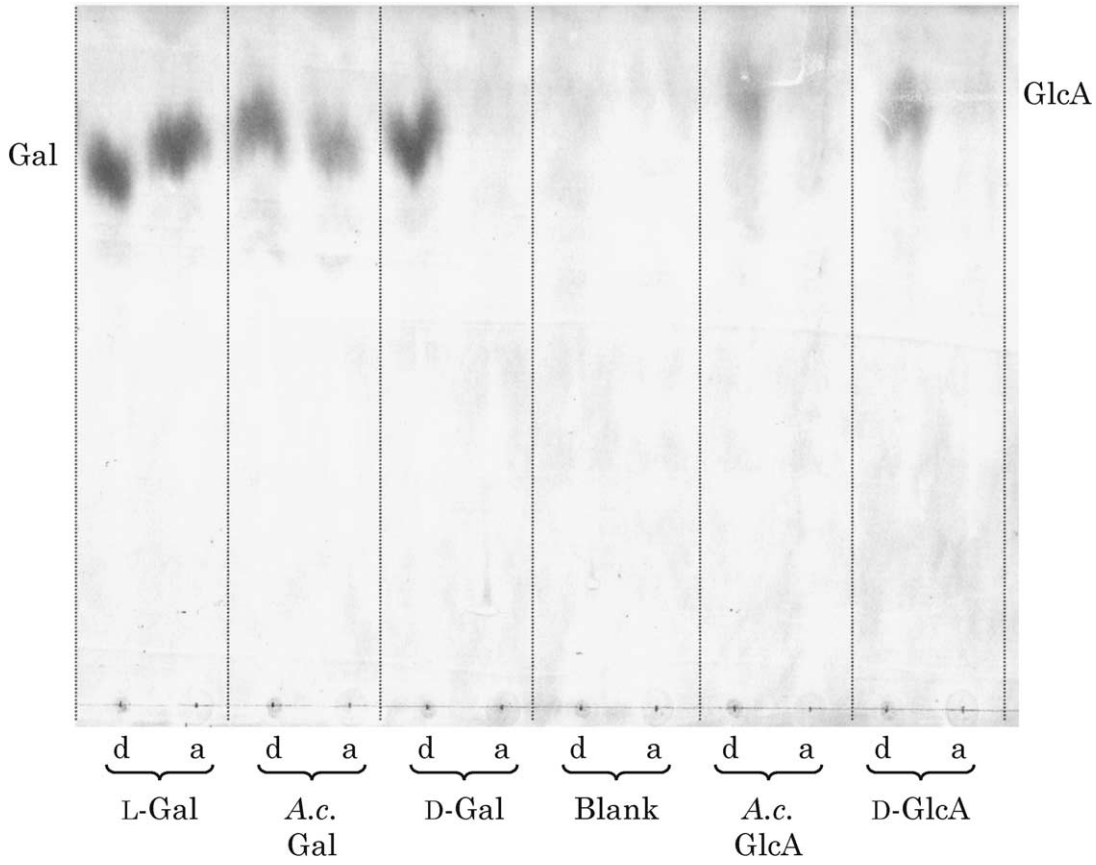

Fig. 6. Determination of the enantiomeric form of the sugar residues of the *Anthoceros* disaccharide, GlcA→Gal (**1**). Galactose [authentic L-Gal, the Gal isolated from the *Anthoceros caucasicus* (*A.c.*) disaccharide, and authentic D-Gal] at 4 mM was incubated with ATP and a preparation containing denatured (d) or active (a) D-galactokinase, and the products were analysed by PC in system (5). Glucuronic acid (the GlcA isolated from *A.c.* disaccharide and authentic D-GlcA) at 1.5 mM was incubated with ATP and a preparation containing D-glucuronokinase (denatured or active) and analysed similarly. The blank contained no added sugars.

contains **1** must await a more detailed characterisation of this polymer.

4. Experimental

4.1. NMR spectroscopy

NMR spectra were measured on D_2O solutions using a Bruker AVANCE 600-MHz spectrometer operating at 599.8 MHz for protons and 150.8 MHz for ^{13}C nuclei.

1-D TOCSY proton spectra were obtained (Dalvit et al., 1996) using a mixing time of 300 ms.

The 2-D gradient-selected COSY proton spectrum was obtained (von Kienlin et al., 1991) over the proton region 3.3–5.3 δ (2K data points), using 256 increments each with two transients per FID. The data were processed using sine-bell squared functions in both dimensions before transformation.

The 2-D proton-detected one-bond ^{1}H–^{13}C correlation (HSQC) spectrum was obtained (Schleucher et al., 1994) over the proton region 3.3–5.3 δ (2K data points) and carbon region 50–110 δ using 512 increments each with eight transients per FID. The experiment was preceded by 64 dummy scans to establish thermal equilibrium. An eight-step phase cycle (hypercomplex acquisition) was used with ^{13}C broad-band decoupling during acquisition of the proton signals. The data were processed using shifted sine-bell squared functions in both dimensions before transformation.

The 2-D proton-detected gradient selected long-range ^{1}H–^{13}C correlation (HMBC-g) spectrum was obtained (Ruiz-Cabello et al., 1992) over the proton region 3.3–5.3 δ (2K data points) and the carbon region 50–110 δ, optimised for proton–carbon couplings of ca. 8 Hz and using 128 increments each with 128 transients per FID. A 16-step phase cycle (hypercomplex acquisition) was used with no ^{13}C broad-band decoupling during the

Table 1
Sources of botanical material and occurrence of α-D-GlcpA-(1→3)-L-Gal (compound 1)

Classification		Species	Source[a]	Compd 1[b]
Algae	Charophytes	*Chara corallina* (Klein ex Willd. Em. R.D.W.)	Culture, D.S.	–
		Coleochaete scutata Brébisson	CCAP 414/4	*
		Klebsormidium flaccidium (Kützing) Silva, Mattox et Blackwell	Culture, H.S.	–
Bryophytes	Hornwort	*Anthoceros caucasicus* Spring.	Faial Island (R.S.)	+ + +
	Thalloid liverwort	*Pellia epiphylla* (L.) Corda	Roslin Glen	–
	Leafy liverworts	*Trichocolea tormentella* (Ehrh.) Dum.	Cairngorms	*
		Marsupellia emarginata (Lindb.) Dum.	Cairngorms	–
		Porella cordaena (Hüb.) Moore (*Madotheca cordaena* Hüb. Dum.)	Milngavie (D.S.R.)	–
	Mosses	*Sphagnum molle* Sull.	Cairngorms	–
		Andrea rupestris Hedw. (A. *petrophila* Ehrh.)	Cairngorms (W.M.M.E)[a]	–
		Hypnum cupressiforme Hedw.	Roslin Glen	–
Lycopodiophytes	Homosporous	*Huperzia selago* (L.) Bernh. Ex. Schrank and Mart.	Cairngorms	–
		Lycopodium pinifolium Blume	19835037(E)	–
		Diphasiastrum alpinum (L.) Holub.	Cairngorms	–
	Heterosporous	*Selaginella apoda* (L.) Spring.	19677705(E)	–
Euphyllophytes	Equisetophyte	*Equisetum debile* Roxb. Ex Vaucher	19731694(E)	–
	Psilotophyte	*Psilotum nudum* (L.) P. Beauv.	Edinburgh Univ.	–
	Eusporangiate fern	*Marattia fraxinea* Sm.	19697183(E)	–
	Leptosporangiate ferns	*Osmunda regalis* L.	19578631(E)	–
		Todea barbara (L.) Moore	19652792(E)	–
		Dryopteris crispifolia Rasbach et al.	19920813(E)	–
		Asplenium australassium (J.Sm.)Hook	19933661(E)	–
		Onoclea sensiblis L.	19662802(E)	–
		Phyllitis scolopendrum L.	19731529(E)	–
		Salvinia auriculatea Aubl.	19830813(E)	–
		Platycerium bifurcatum (Car.) C.Chr.	19734554(E)	–
		Blechnum spicant (L.) Roth.	Cairngorms	–
	Gymnosperm	*Gnetum gnemon* L.	19902511(E)	–
	Angiosperm, monocot	*Cyanotis longifolia* Wight	19672908(E)	–

[a] (E) = accession number for material provided by the Royal Botanic Garden, Edinburgh; CCAP = Culture Collection of Algae and Protozoa, Ambleside, Cumbria; some material was kindly provided by Dr D. Long and Dr H. Sluiman (Royal Botanic Garden, Edinburgh), Dr D.S. Rycroft (Glasgow University), Professor R. Schumacker (University of Liege), Dr W.M.M. Eddie (University of Texas, Austin) and Professor D. Sanders (University of York). Other material was collected by Z.A.P. Collection sites were: Cairngorm Hills (57°05–10′ N, 3°35–45′ W), Roslin Glen (55°51′ N, 3°10′ W), and Faial Island (38°40′ N, 28°40′ W).

[b] Key: – = Not detectable, + + + = present at high concentration, * = compound present which has a similar R_f value and stains the same colour with aniline hydrogen-phthalate. However, this spot was not confirmed to be compound **1**.

acquisition of the proton signals. The data were processed using shifted sine-bell squared functions in both dimensions before transformation.

4.2. Source of plant material

Sources of botanical material are listed in Table 1.

4.3. Preparation and hydrolysis of AIR

AIR was prepared as described by Popper et al. (2001).

4.4. Paper chromatography

Analytical PC was on either Whatman No. 1 paper or Schleicher and Schüll 2045b paper; preparative PC was on Whatman 3MM. One-dimensional PC was by the descending method; two-dimensional PC was by the ascending method. Solvent systems used were (1) BuOH–HOAc–H_2O, 12:3:5, v/v/v, (2) phenol–H_2O, 4:1, w/w, (3) EtOAc–pyridine–H_2O, 8:2:1, v/v/v, (4) BuOH–pyridine–H_2O, 4:3:4, v/v/v, and (5) EtOAc–HOAc–H_2O, 10:5:6, v/v/v. Chromatograms were stained with $AgNO_3$ or aniline hydrogen-phthalate; faint spots after the latter stain were more readily visible by their fluorescence when viewed under a 366-nm UV lamp (Harborne, 1998).

4.5. Paper electrophoresis

PE was on 57 × 40-cm sheets of Whatman No. 1 paper. Samples were loaded 12 cm from the cathode end. For PE at pH 3.5 the paper was wetted with 50%-strength running buffer (HOAc–pyridine–H_2O, 10:1:378

v/v/v, pH 3.5); the electrode troughs contained full-strength running buffer (HOAc–pyridine–water 10:1:189 v/v/v, pH 3.5). For PE at pH 2.0 the paper was wetted and run in (HOAc–HCOOH–water, 35:1:355 v/v/v, pH 2.0). Typical running conditions were 2.0 kV for 1 h. Electrophoretograms with radioactive markers were autoradiographed prior to staining with aniline hydrogen-phthalate.

4.6. Purification and chromatographic characterisation of compound 1

AIR (50 mg) from *Anthoceros caucasicus* thallus (mainly the thin, photosynthetic tissue, with little contamination from midribs or rhizoids) was hydrolysed in 2 ml of 2 M TFA at 120 °C for 1 h. The hydrolysate was fractionated by preparative PC on Whatman 3MM in system 1, and the fringes of the chromatogram were stained with aniline hydrogen-phthalate. The major, brown-staining, compound which migrated slower than marker GalA but slightly faster than glucose 6-phosphate (Fig. 1b) was eluted from the unstained portion of the chromatogram and freed of paper-derived polysaccharides by gel-permeation chromatography on Bio-Gel P-2 in a volatile buffer ['PyAW'; pyridine–HOAc–H_2O, 1:1:98, containing 0.5% chlorobutanol]. Eluted **1** was monitored by analytical PC in system 4 for 16 h followed by staining with aniline hydrogen-phthalate (R_{Gal} values of **1** and of GalA in system 4 were 0.50 and 0.58 respectively) and was analysed by analytical PC and PE in several solvent systems and by NMR spectroscopy.

4.7. Acid hydrolysis of compound 1

A portion of purified **1** was treated with 2 M TFA at 120 °C for 1 h and the products were analysed by PC in system 3 for 42 h (Fig. 2b) and by PE at pH 3.5 and 2 kV for 1 h (Fig. 2a). Prior to PE, the sample was dried, re-dissolved in 25 µl of 0.1 M NaOH, incubated at 20 °C for 2 min to hydrolyse lactones, then acidified with 6 µl of 0.5 M acetic acid and quickly (to minimise re-lactonisation) subjected to PE. Papers were stained with aniline hydrogen-phthalate.

4.8. Driselase hydrolysis of compound 1

A further portion of **1** was treated with 50 µl of PyAW containing 1% (w/v) Driselase [a fungal enzyme mixture from *Irpex lacteus*; Sigma Chemical Co.; partially purified as described by Fry (2000)] in PyAW. Controls lacked either Driselase or **1**. After incubation at 25 °C for 26 h, 16-µl samples of the digest were analysed by PE at pH 3.5 and 3 kV for 90 min or by PC in solvent system 3 for 40 h. The sheets were stained with aniline hydrogen-phthalate.

4.9. Fractionation of Anthoceros polysaccharides

A 50-mg portion of *Anthoceros* AIR was shaken at 25 °C with PyAW, pH 4.7 (3 × 10-ml aliquots; 1 h each), and the extracts (W_1, W_2, W_3) were pooled (fraction W). The extraction was then repeated (1 h each) with 2 × 10 ml 100 mM EDTA in the same buffer (fraction E); 2 × 10 ml 1.0 M Na_2CO_3 (fraction C); 2 × 10 ml 0.5 M NaOH containing 0.5% $NaBH_4$ (fraction D); 2 × 10 ml 6.0 M NaOH containing 0.5% $NaBH_4$ (fraction A); and finally 3 × 10 ml PyAW (fractions F_1, F_2, F_3). F_1 and F_2 still had pH > 7 owing to the persistence of the previously used NaOH; F_3 had pH 4.8. All the alkaline extracts (C, D, A, F_1 and F_2) were acidified with HOAc. All extracts were then dialysed against aqueous 0.1% chlorobutanol. All extracted fractions, and the final insoluble residue (fraction R), were freeze-dried.

Each dried extract was re-suspended in 5 ml H_2O and a portion of the suspension was hydrolysed in 2 M TFA as above; the products were analysed by PC in system 1 (Schleicher and Schüll paper for 56 h) and by HPLC on a Dionex CarboPac PA1 column. The elution programme for HPLC was 0–30 min, H_2O; 30–50 min, 0→800 mM NaOH, linear gradient; 50–55 min, 800 mM NaOH; 55–65 min, H_2O. The flow rate was 0.8 ml, with post-column addition of 0.5 M NaOH (0.5 ml/min) throughout.

4.10. Use of enzymes to distinguish D- and L-monosaccharide

The disaccharide was hydrolysed in 2 M TFA at 120 °C for 1 h and the resulting monosaccharides were isolated by PC in system 1 followed by system 3, eluted with water, dried and re-dissolved in 100 µl water.

A crude mixture of sugar kinases (Neufeld et al., 1961) was prepared from soaked mung bean seeds as described (Fry, 2000, Panel 5.4.1, steps 1–5). As a control, one portion of the kinase solution was heated at 100 °C for 10 min.

Aliquots (10 µl) of each monosaccharide sample (4 mM Gal isolated from the disaccharide, 4 mM authentic D-Gal, 4 mM authentic L-Gal, 1.5 mM GlcA from the disaccharide, 1.5 mM authentic D-GlcA) were mixed with 10 µl of substrate mixture [40 mM ATP, containing 80 mM NaF (a phosphatase inhibitor), final pH adjusted to 7.4 with NaOH]; 10 µl of the kinase or boiled kinase solution was added and the mixture was incubated at 25 °C for 7 h. The reaction products were then subjected to PC on Whatman No. 1 in system 5 for 18 h and stained with $AgNO_3$. Under these conditions, 10-µl aliquots of D-Gal up to 8 mM were completely phosphorylated whereas authentic L-Gal was unaffected even at concentrations as low as 1 mM; 10-µl aliquots of D-GlcA at 1 mM were completely phosphorylated, and at up to 4 mM largely phosphorylated.

Table 2
^{1}H and ^{13}C NMR spectral data of α-glucuronosyl-(1→3)-galactose

Site	δ_C[a]	δ_H	multiplicity[b]	J_{HH} (Hz)	Proton–carbon long-range correlations (from HMBC spectrum)
α-galactose (residue A)					
1′	95.5	5.27	(*d*)	4.0	C-3, C-5
2′	70.9	3.99	(*dd*)	10.3, 3.9	C-3′
3′	81.1	3.92	(*dd*)	10.3, 3.1.	
4′	72.4	4.12	(*d*)	2.9	C-3′
5′	73.8	4.09[c]	unresolved		C-6′
6′	63.4	3.71, 3.68[a]	unresolved		
β-*galactose (residue B)*					
1″	99.4	4.62	(*d*)	7.7	C-2″
2″	74.5	3.66	(*dd*)	9.9, 7.7	
3″	84.1	3.71[c]	unresolved		
4″	71.8	4.06	(*d*)	3.1	
5″	78.4	3.68[a]	unresolved	C-6″	
6″	63.3	3.71, 3.68[a]	unresolved		
α-glucuronic acid (residue C)					
1	103.8	5.22	(*d*)	3.9	C-3′, C-3″, C-5, C-3
2	74.8	3.59	(*dd*)	9.9, 3.9	
3	75.9	3.79	(*dd*)	9.9, 9.2	
4	75.1	3.51	(*dd*)	10.2, 9.2	C-6
5	75.8	4.08	(*d*)	10.3	C-6
6	179.5				

[a] from the HSQC spectrum
[b] (*d*) = doublet, (*dd*) = doublet of doublets.
[c] from the gCOSY spectrum

In a confirmatory experiment, a mixture of 47 nM D-[1-^{3}H]Gal (177 MBq/μmol; added as an internal standard, undetectable by staining) and 12 mM Gal obtained from the *Anthoceros* disaccharide was incubated with D-galactose oxidase as described by Popper et al. (2001). Both the stainable and the radioactive products were analysed by PC (system 1 for 16 h; system 3 for 48 h).

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