*Carbohydrate Research*, 143 (1985) 175–183
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

# OCCURRENCE OF β-D-GALACTOPYRANOSYL UNITS ESTERIFIED AT O-6 WITH 2-AMINOETHYLPHOSPHONIC ACID IN THE D-GALACTAN OF ALBUMEN GLANDS OF THE SNAIL *Megalobulimus paranaguensis*†

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(Received June 26th, 1984; accepted for publication in revised form, March 18th, 1985)

ABSTRACT

The albumen glands of the snail *Megalobulimus paranaguensis* were previously found to contain a branched β-D-galactopyrananan consisting mainly of nonreducing end-groups and 3,6-di-*O*-substituted residues with smaller proportions of 3-*O*- and 6-*O*-substituted units. $^{13}$C-n.m.r. spectroscopy has now shown that 2-aminoethylphosphonic acid (**1**) is an additional component and its presence was confirmed by cellulose-plate chromatography. Furthermore, the 2-aminoethylphosphonic acid-substituted oligosaccharides, formed on partial hydrolysis of the polysaccharide, were isolated, examined by fast-atom-bombardment mass spectrometry, and found to have molecular weights consistent with monophosphonic esters of **1**. The $^{13}$C-n.m.r. spectrum of this mixture indicated substitution of D-galactopyranosyl units at O-6 with esters of **1**, and this was confirmed by spectral examination of a monosubstituted D-galactose isolated after strong acid hydrolysis of the polymer. The snail D-galactan contained one ester of **1** for every 34 residues, it was free of other components such as protein, lipid, and nucleic acid, and is the first polysaccharide of this type that has been identified.

## INTRODUCTION

D-Galactans and D-galactose-containing polysaccharides are components of albumen glands and egg masses (or eggs in some cases) of snails. Considerable structural variation occurs between polysaccharides of different species of snail, but polysaccharides obtained from the albumen glands and egg masses of the same snail are generally similar. All polysaccharides are highly branched with β-D-

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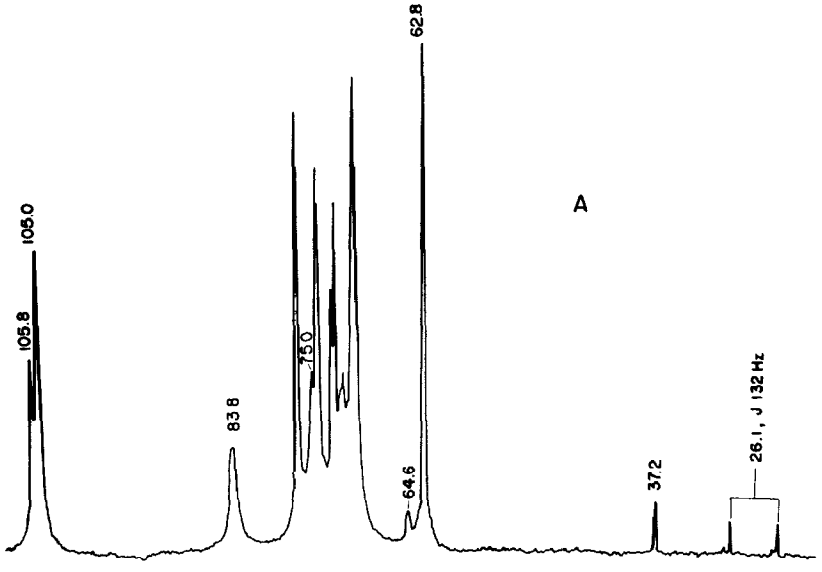
0008-6215/85/$ 03.30 

galactopyranosyl residues, which can comprise the entire structure or can constitute a core. In the latter case, the other residues found in the side chains are α-L-fucopyranose, α-L-galactopyranose, α-D-glucopyranose, or 3,4-*O*-(1-carboxyethyl idene)-β-D-galactopyranose[1]. The present study concerns the detection of 2-aminoethylphosphonic acid (**1**) linked to the branched β-D-galactopyranan isolated from albumen glands of the snail *Megalobulimus paranaguensis*. Methylation analysis previously showed it to contain nonreducing end-groups (43%), and 3-*O*-(11%), 6-*O*- (3%), and 3,6-di-*O*-substituted (43%) residues[2].

## RESULTS AND DISCUSSION

During the course of examination of a number of snail polysaccharides by $^{13}$C-n.m.r. spectroscopy, the β-D-galactopyranan of *M. paranaguensis* was found to give a spectrum having some unusual high-field signals in addition to the ones at δ 62.8–105.8 which pertain to the carbohydrate residue (Fig. 1A). These signals at δ 23.4–37.2 resembled those of free **1** at δ 36.8 (-$CH_2NH_2$), 28.2, and 26.8 (-$CH_2$-P(O)(OH)-O-; $^1J_{C,^{31}P}$ 132 Hz). This doublet centered at δ 27.5 is similar to that of the polysaccharide at δ 26.1 with a coupling constant of 134 Hz. Acid hydrolysis of the polysaccharide gave a compound that showed, according to chromatography on Merck cellulose plates, a spot which was nitrogen- and phosphorus-positive and carbohydrate-negative, and whose mobility corresponded to that of **1** ($R_{Gal}$ 0.51). The phosphorus content of the D-galactan indicated that one of every 34 units of D-galactopyranose was substituted by an ester of **1**.

Since the $^{13}$C-n.m.r. spectrum of the D-galactan contained only signals corresponding to carbohydrate and **1**, the two components were linked probably *via* the



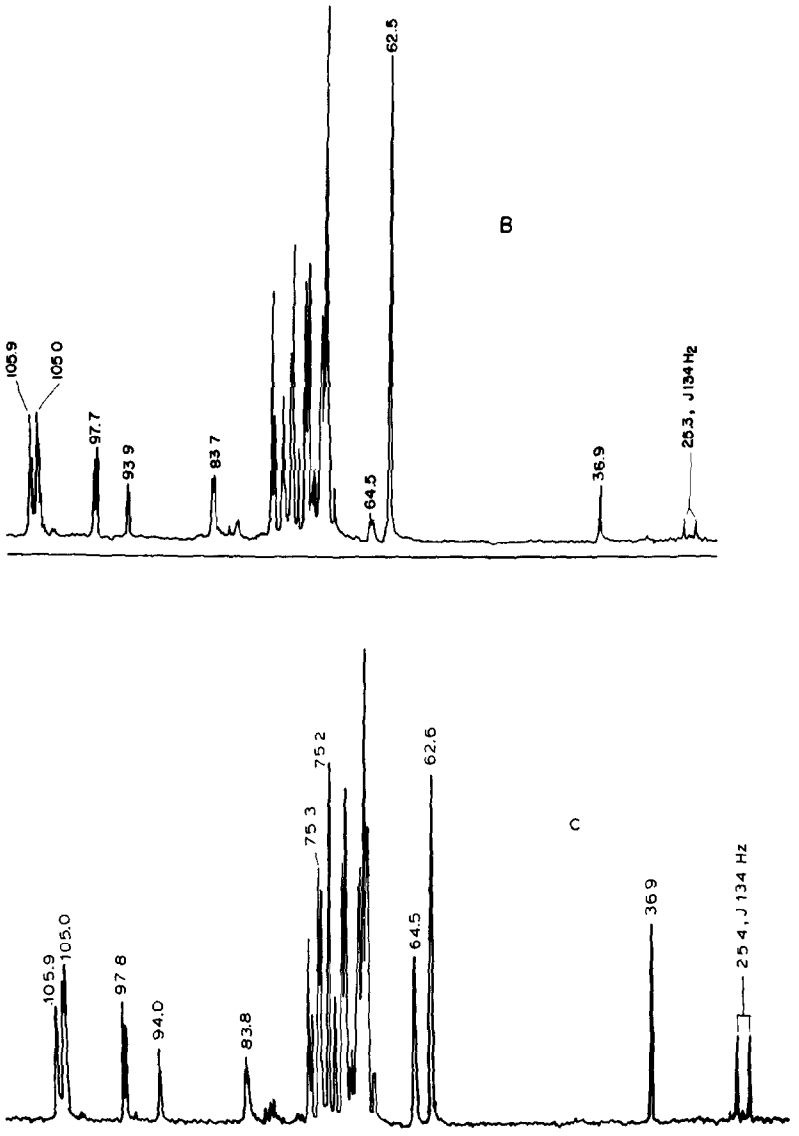



Fig. 1. (A). $^{13}$C-N.m.r. spectrum of D-galactan alkylphosphonate of *M. paranaguensis* for a solution in $D_2O$ at 70° recorded with a spectrometer operating at 100 MHz. Values ($\delta$) relative to that of an external $Me_4Si$ signal. (B). $^{13}$C-N.m.r. spectrum of Fraction A. (C). $^{13}$C-N.m.r. spectrum of Fraction C. The chemical shifts of each component of the upfield doublet arising from $^{13}$C,$^{31}$P coupling differ in the polysaccharide spectrum (Fig. 1A) from those of the oligosaccharide mixtures (Fractions A and C; Figs. 1B and C) and of **1** as the experiment illustrated in Fig. 1A was performed at 100 MHz, whereas a 360-MHz instrument was used for the other three experiments.

phosphonic group which would form an ester with hydroxyl groups. Thus, in order to avoid phosphonate migration prior to determination of location of the phosphonic ester, D-galactan was extracted from albumen glands by avoiding alkaline conditions. Extraction was carried out with aqueous mercuric chloride adjusted to pH 5.5, conditions that render protein and nucleic acid components insoluble[3]. Addition of the extract to ethanolic excess precipitated a polysaccharide which was isolated in 11% yield and whose $^{13}$C-n.m.r. spectrum was identical to that (Fig. 1A) obtained from a polysaccharide isolated[2] *via* a step where the pH was 8.5.

Partial acid hydrolysis of the polysaccharide with M trifluoroacetic acid at 100° for 3 h gave oligosaccharide fragments linked to **1**. These were isolated and characterized as follows. Fractionation of the partial hydrolysis product was carried out by cellulose-column chromatography; elution with 7:1 (v/v) acetone–water gave galactose, with 3:1 acetone–water Fraction A (1.49% yield), and further elution with 5:2 acetone–water Fraction B (5.6% yield). This was not examined in detail since it was found to be a mixture of components of Fraction A and those of Fraction C, eluted later from the column by 2:1 acetone–water (3.5% yield).

Fraction A had nitrogen and phosphorus contents corresponding to one residue of **1** for every 21 D-galactosyl units. The low content in **1** was reflected by the $^{13}$C-n.m.r. spectrum (Fig. 1B) which contained weak high-field signals (their chemical shifts corresponded to combined rather than free **1**). The fast-atom-bombardment (f.a.b.) mass spectrum[4], obtained from a water–glycerol solution, indicated a mixture of hexose-containing di-, tri-, and tetra-saccharide having molecular ion peaks ($Na^+$) of mol. wts. 365 (30%), 527 (35%), and 689 (30%), respectively (Fig. 2A). Peaks corresponding to **1**-linked oligosaccharides were not observed.

Fraction C gave a $^{13}$C-n.m.r. spectrum (Fig. 1C) whose high-field signals indicated a proportion of **1** higher than that of the original polysaccharide (compare with Fig. 1A). Each signal was shown to arise from a $CH_2$ group by examination of a $^{13}$C-n.m.r. spectrum obtained without $^{13}$C,$^1$H decoupling. As would be expected, three triplets were present. Also observed in Fig. 1C, when compared with Fig. 1A, was a proportional increase in the height of the signal at $\delta$ 64.5. This suggests that it is closely associated with the unit **1** and probably arises from C-6 of D-galactosyl units esterified by phosphonate groups. The resonance is 1.9 p.p.m. downfield from that of a free $CH_2OH$-6 group, smaller than the shift of +4.0 p.p.m. occurring on 6-*O*-phosphorylation of $\alpha,\beta$-galactopyranose. The mol. wts. of components of Fraction C were deduced from the f.a.b.–mass spectrum (Fig. 2B) which contained molecular-ion peaks ($H^+$) corresponding to mol. wts. of 450 (48%), 612 (21%), and 774 (2%). These corresponded to one molecule of **1** linked as a phosphonic ester to di-, tri-, and tetra-saccharides composed of hexose units, respectively. A parallel series of peaks, 472 (16%), 634 (11%), and 796 (2%) arose from the $Na^+$ forms of the oligosaccharides. The tendency to form the $Na^+$ form of the molecular ion is less than for phosphoric esters. For example, dihydrogen $\alpha,\beta$-

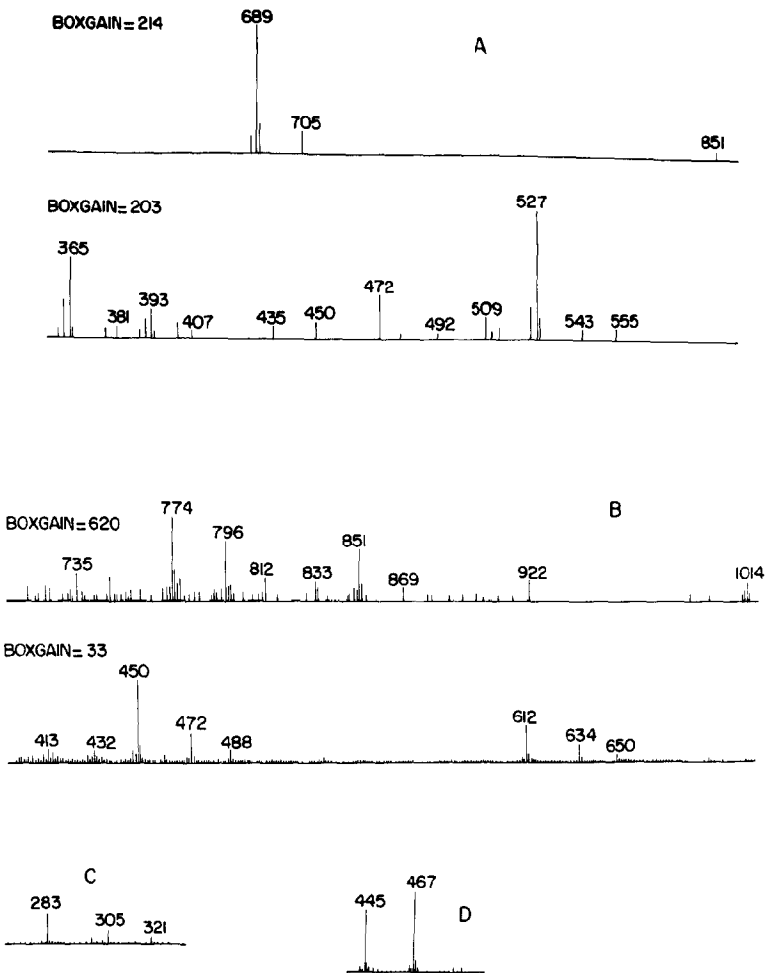
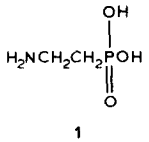



Fig. 2. (A) F.a.b.–m.s. of Fraction A obtained for a water–glycerol solution. (B) F.a.b.–m.s. of Fraction C. (C) F.a.b.–m.s. of dihydrogen $\alpha,\beta$-D-glucose 6-phosphate. (D) F.a.b.–m.s. of 2-*O*-$\alpha$-D-mannopyranosyl-D-mannose (dihydrogen 6-phosphate)[5].

D-glucose 6-phosphate and mannobiose 6-phosphate either ionize in the monosodium or disodium form (see Fig. 2C and D). The nitrogen and phosphorus contents of Fraction C indicated that one residue of **1** was present for every 2.6 units.

The data obtained thus indicate that the snail polysaccharide contains $\beta$-D-galactopyranosyl units esterified at O-6 with **1** (**2**). In order to determine whether esterification occurred elsewhere, the following experiments were carried out. Partial hydrolysis of the polysaccharide under stronger conditions (M trifluoroacetic acid, 18 h, 100°) gave products that corresponded, on cellulose plates, to galactose, a disaccharide ($R_{Gal}$ 0.68), and **1** ($R_{Gal}$ 0.51). In addition, a new compound having

$R_{Gal}$ 0.17, which was phosphate-, nitrogen-[6] and carbohydrate-positive, was detected. It was isolated by cellulose column chromatography, elution with 6:1 (v/v) acetone–water giving D-galactose and, with a 5:1 solvent ratio, **1** and a carbohydrate- and phosphate-negative, and nitrogen-positive material. Elution with 2:1 (v/v) acetone–water provided, in 4.8% yield, a material ($R_{Gal}$ 0.17) having $[\alpha]_D^{25}$ +22°. Strong acid hydrolysis with 6M trifluoroacetic acid for 18 h at 100° gave D-galactose and **1**, which were detected on a cellulose plate. $^{13}$C-N.m.r. data indicated that α,β-D-galactose was esterified at O-6 by the phosphonate group of **1**, corresponding to structure **2** of the polymer. Signals at δ 97.6 (β-D anomer) and 93.4

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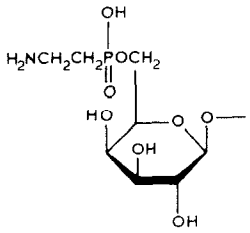


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(α-D anomer) were the only ones detected in the C-1 region, indicating a monosaccharide derivative. In the C-6 region, signals were observed at δ 64.2 and 64.4 (*O*-substituted C-6), and a small one at δ 62.2 (< 10%). Resonances of **1** were observed at δ 36.4 (-$CH_2NH_2$) and 26.0 [centre of doublet, $^1J_{C,^{31}P}$ 134 Hz, -$CH_2$-P(O)(OH)-O-]. Analytical figures for nitrogen and phosphorus confirmed the structure.

A report has been published[7] on the presence of a lipid-free glycan in eggs of the planorbid snail *Helisoma* sp. Preliminary studies indicated structurally similar macromolecules in egg masses of *Biomphalaria glabrata*[7] and in the garden snail *Helix aspersa*[8]. The galactan of albumen glands of *Pomacea lineata* gave a high-field $^{13}$C-n.m.r. signal at δ 25.3 arising from 3,4-*O*-(1-carboxyethylidene) groups and another at δ 35.3, which is weak and could be due to the $CH_2NH_2$ group of **1**. It disappeared following Smith degradation[9].

Alkylphosphonates have been investigated mainly in organisms other than snails and have been found in the free and combined form. Detailed structural studies have been carried out on the phosphonate-containing complexes of invertebrates, namely, protozoans, amoeba, sea anemones, and molluscs other than snails. The compound most closely resembling that of the alkylphosphonate derivatives of the D-galactan is 1-*O*-[6-*O*-(*N*-methylaminoethylphosphonyl)-D-galactopyranosyl] ceramide, which was isolated by Matsuura[10] from the viscera of the marine mollusc *Turbo cornutus*.

The most common, naturally-occurring phosphonic acid is **1**, found mainly in glycerolipids, sphingolipids, and more polar macromolecules[11]. The last-named compounds have various structures. For example, the plasma membrane of the

amoeba, *Acanthamoeba castellanii,* contains[12] a lipoglycoprotein having four neutral sugars, including glucose, mannose, and xylose (30%); hexosamines (6%); **1** and its 1-hydroxy derivative (13%), protein (3%), and unidentified lipids (~50%). Structurally different glycoproteins of high-molecular weight occur in the sea anemones, *Metridium senile* and *M. dianthus,* and have carbohydrate (20–30%) and **1** (10–15%) (refs. 13–16), likely combined as a phosphonic ester[16,17]. The amino group of **1** is also substituted[18]. Kittredge *et al.*[19] found an ester of **1** of glycerol (substitution position unknown) in the free state in the sea anemone, *Anthopleura elegantissima.* Phosphonoglycoproteins are present in the ciliate protozoan, *Tetrahymena thermophila,* and contain carbohydrate components (30%) that are linked *via N*- and *O*-glycosyl linkages to the polypeptide chain; **1** (8%) is attached to an *O*-linked chain and also contains a substituted amino group[20]. Compound **1** is also a component of *Trypanosoma cruzi*[21] and a fraction known as lipopeptidophosphoglycan was degraded with hot, aqueous sodium borohydride–sodium hydroxide to give a low-molecular-weight polysaccharide containing mannose, galactose (in the furanosyl form), and 2-amino-2-deoxyglucose. Its $^{13}$C-n.m.r. spectrum showed[22] high-field signals typical of **1**.

## EXPERIMENTAL

*$^{13}$C-N.m.r. spectroscopy.* — The $^{13}$C-n.m.r. spectra of *M. paranaguensis* D-galactan and D-galactose monoalkylphosphonate were recorded with a Varian XL-100-15 spectrometer in the F.t. mode for solutions in $D_2O$ (0.85 mL) of the compounds (100 mg and 20 mg, respectively) contained in a coaxial glass cylinder fitting snugly within a tube (1.2 diam. × 20.3 cm) maintained at 70°. The spectral width was 5000 Hz, the acquisition time 0.8 s, the pulse width 9.5 μs, and the number of transients 100 000.

Oligosaccharide fractions (14–56 mg), obtained from the polysaccharide following partial hydrolysis, and **1** were examined at 70° as solutions in $D_2O$ (2 mL) in a tube (0.10 cm diam.), with a Bruker AM-360-WB n.m.r. spectrometer in the F.t. mode. The spectral width was 18 519 Hz, the acquisition time 0.44 s, the pulse width 21.0 μs, and the number of transients 25 000.

In each series of experiments, chemical shifts are in δ relative to that of $Me_4Si$, obtained in a different experiment.

*Cellulose-plate chromatography.* — This was carried out on Merck cellulose plates backed with aluminium foil, which were developed with 10:7:3 (v/v) pyridine–acetic acid–water. To detect the various components encountered in the investigation, the following reagents were used: (a) ammoniacal $AgNO_3$ and *p*-anisidine hydrochloride for carbohydrate, (b) ninhydrin for N-containing compounds, and (c) $H_2MoO_4$–$HClO_4$ for P (ref. 23).

*Preparation of polysaccharide.* — Albumen gland (50 g) was suspended in Folch reagent (500 mL; 2:1 chloroform–methanol)[24], the mixture centrifuged, and the pellet washed with the same solvent. The defatted material (16 g) was treated

with 3% aqueous $HgCl_2$, adjusted to pH 5.5 (50 mL), and the process repeated three times. Soluble material was obtained following centrifugation and filtration through glass wool, and added to ethanol (3 vol.) to precipitate the polysaccharide. This was filtered off, washed with ethanol, and dried (yield 5.4 g). It contained N, 0.29%; P, 0.56%.

*Partial hydrolysis of polysaccharides and isolation of fractions containing oligosaccharide alkylphosphonates.* — The polysaccharide (1.0 g) was partially hydrolyzed in M trifluoroacetic acid (30 mL) for 3 h at 100°, and the solution evaporated to a sirup which was then fractionated on a column of cellulose. Elution was carried out successively with mixtures of acetone–water (2 L each) containing different ratios of each component (v/v). The eluent having a 7:1 ratio provided D-galactose and that having a 4:1 ratio eluted a disaccharide. The eluent having a 3:1 ratio gave a mixture of oligosaccharides (Fraction A; 14 mg), $R_{Gal}$ 0.4–0.6, $[\alpha]_D^{25}$ +25° (*c* 0.2, water).

*Anal.* N, 0.9; P, 1.6.

The eluent having a 5:2 ratio gave Fraction B (56 mg), and the eluent having a 2:1 ratio gave Fraction C (35 mg), $R_{Gal}$ 0.04–0.06, $[\alpha]_D^{25}$ +23° (*c* 0.2, water).

*Anal.* N, 3.2; P, 6.0.

*Partial hydrolysis of polysaccharide to give D-galactose monoalkylphosphonate and its isolation.* — The polysaccharide (1.5 g) was partially hydrolyzed in M trifluoroacetic acid (50 mL) for 18 h at 100°, and the solution evaporated to a sirup. Examination on a cellulose plate showed several spots, namely, galactose (carb. posit., N and P negat.); a disaccharide, $R_{Gal}$ 0.68 (carb. posit., N and P negat.); **1**, $R_{Gal}$ 0.51 (N and P posit., carb. negat.); several spots (N posit., carb. and P negat.); and a spot having $R_{Gal}$ 0.17 (carb., N, and P posit.). The mixture was fractionated on a column of cellulose with acetone–water mixtures as eluent. 6:1 Eluent gave D-galactose; 5:1 eluent gave 6-*O*-β-D-galactopyranosyl-D-galactose, $R_{Gal}$ 0.68, followed by ($R_{Gal}$ 0.51), and then compounds ($R_{Gal}$ 0.42 and 0.34) that were N-positive, and carbohydrate and P-negative. Elution with 2:1 acetone–water gave D-galactose monoalkylphosphonate, $R_{Gal}$ 0.17 (68 mg), $[\alpha]_D^{25}$ +22° (*c* 0.2, water).

*Anal.* N, 4.7; P, 10.2.

Hydrolysis with 6 M trifluoroacetic acid for 18 h at 100° gave a mixture containing D-galactose and **1** (cellulose plate).

*F.a.b.–m.s.* — This was performed by Prof. Alan Hogg, Department of Chemistry, University of Alberta, Edmonton, Canada. Samples were dissolved in a small quantity of water, and glycerol was added prior to examination. Other solvents such as sulfolane, thioglycerol, and glycerol–HCl were unsuitable.

ACKNOWLEDGMENTS

The authors thank Mr. M. Mazurek of The Plant Biotechnology Institute, National Research Council, Saskatoon, Saskatchewan, Canada for recording the $^{13}C$-n.m.r. spectra.

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