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STRUCTURAL INVESTIGATIONS OF THE GALACTAN OF THE SNAIL *Lymnaea stagnalis*

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ABSTRACT

A galactan, isolated from the spawn of the snail *Lymnaea stagnalis*, contained D-galactose and 0.9% of nitrogen, but neither L-galactose nor phosphate groups. The $[\alpha]_D^{20}$ values of the galactan and its first Smith-degradation product were +19.5° and +20°, respectively. During each of two consecutive Smith-degradations of the galactan, 1 mol of periodate was consumed and 0.45 mol of formic acid was liberated per mol of "anhydrogalactose" unit. Methylation analyses of the galactan and its first Smith-degradation product yielded equal proportions of 2,3,4,6-tetra-*O*-methyl- and 2,4-di-*O*-methyl-galactose. Only small quantities of 2,4,6- (4.9 mol%) and 2,3,4-tri-*O*-methylgalactose (0.7 mol%) were formed from the galactan, whereas the first Smith-degraded product gave 15.6 and 20.4 mol%, respectively. The product of the second Smith-degradation disintegrated and the following oligosaccharides were identified: β-D-Gal-(1→1)-L-Gro, β-D-Gal-(1→3)-β-D-Gal-(1→1)-L-Gro, β-D-Gal-(1→6)-β-D-Gal-(1→1)-L-Gro, β-D-Gal-(1→6)-D-Gal-β-D-Gal-(1→3)-β-D-Gal-(1→1)-L-Gro, β-D-Gal-(1→3)-[β-D-Gal-(1→6)]-β-D-Gal-(1→1)-L-Gro, β-D-Gal-(1→3)-β-D-Gal-(1→6)-β-D-Gal-(1→1)-L-Gro, and β-D-Gal-(1→3)-β-D-Gal-(1→3)-β-D-Gal-(1→1)-L-Gro. Thus, the galactan is highly branched with the backbone containing sequences of either exclusively (1→6)-linked or of more or less regularly alternating (1→3)- and (1→6)-linked units. The side chains vary in length and in the degree of branching. In immunoprecipitin studies, a high degree of species-specificity was seen when various snail galactans were tested with the antiserum to the *Lymnaea stagnalis* galactan.

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INTRODUCTION

Snail galactans are highly branched polysaccharides having molecular weights of several million that are synthesised in the albumen glands of pulmonate and prosobranchiate snails and secreted as part of the perivitelline fluid in which the eggs are embedded. They constitute the only storage carbohydrate of the spawn and they are metabolised by the growing embryos or the freshly hatched snails[1]. Snail galactans[2,3] are composed mainly of D-galactose (Gal) residues (1→3)- and (1→6)-linked. Depending on the species, additional constituents have been found, such as L-galactose (L-Gal) in the galactans of *Helix pomatia* (*Hp*), *Arianta arbustorum* (*Aa*), and *Cepaea nemoralis* (*Cn*) (~14%)[2,3], L-fucose in that of *Ampullarius spec.* (~2%)[4], and phosphate groups in those of *Hp*, *Cn*[3,5], and *Biomphalaria glabrata* (*Bg*) (~2%)[3].

Methylation analyses of each of these polysaccharides gave 2,3,4,6-tetra- and 2,4-di-*O*-methyl-Gal in equal proportions, but various quantities of 2,3,4- and 2,4,6-tri-*O*-methyl-Gal were found[2–4,6,7]. The galactans of *Hp*, *Aa*, and *Cn* also gave 3,4,6-tri-*O*-methyl-Gal[3].

In contrast to this rather uniform pattern of structural elements is the highly species-specific immunochemical reaction with antisera raised in rabbits against certain snail galactans. Even the galactans of two species as closely related as *Hp* and *Aa*, which are almost identical, except that that from *Hp* contains phosphate groups, are easily distinguished by their reaction with various galactan-specific antisera. Similar species-specific difference were also detected among snail galactans by precipitin reactions with a lectin from the sponge *Geodia cydonium*[8] and with human IgM myeloma proteins[9] having specificity for pyruvylated D-Gal.

In seeking to determine the structural features responsible for this diversity, we have investigated the structure of a snail galactan which contains exclusively D-Gal and have compared its immunochemical properties with those of other snail galactans.

The galactan of the aquatic snail *Lymnaea stagnalis* (*Ls*) was reported[10] to be free of phosphate groups and devoid of L-Gal. We have now investigated its structure.

RESULTS AND DISCUSSION

*Ls*G* was purified by repeated ultracentrifugation; when examined by disc electrophoresis in 7.5% and 12% polyacrylamide gels, it did not enter the gel, because of its size, and contaminating proteins could not be detected. On Sepharose 4B, the single peak monitored by u.v. absorption at 280 nm corresponded to the galactan as detected by the orcinol method.

*Connotes the galactan of *Lymnaea stagnalis, etc.*

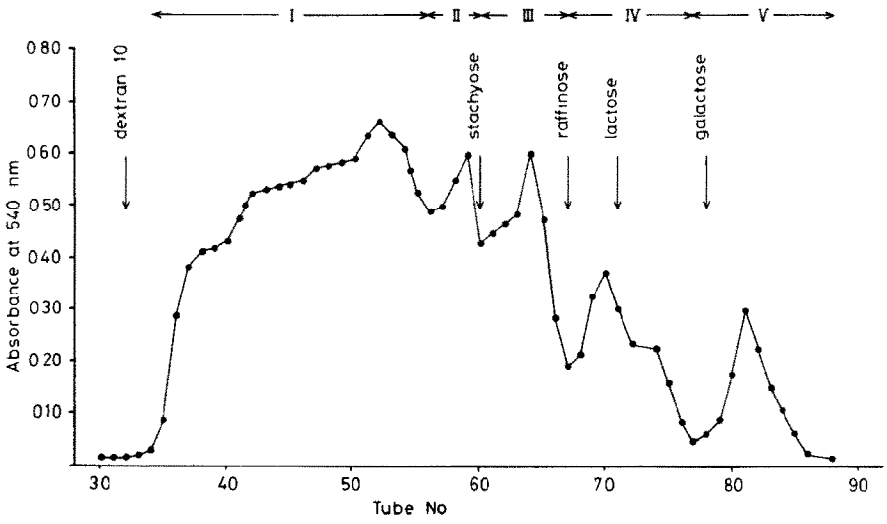


Fig. 1. Fractionation on Biogel P-2 of *Ls*G after two Smith-degradations.

Colorimetric methods showed that 6-deoxyhexoses and *N*-acetylhexosamines were absent and that the polysaccharide contained only hexose. Acid hydrolysis gave only galactose, identified as the alditol acetate by g.l.c. on either 3% ECNSS-M or 3% OV-225.

The $[\alpha]_D^{20}$ values of the galactan and its first Smith-degradation product were +19.5° and +20° (water), respectively (*cf.* +20.5° for *Ls*G reported earlier[11]). The hydrolysate of *Ls*G had $[\alpha]_D^{20}$ +79°, which was close to the value (+80.5°) expected for D-Gal at mutarotational equilibrium. The absence of L-Gal in the hydrolysate was confirmed by g.l.c. using a chiral stationary phase[11,12].

*Ls*G contained 0.03% of phosphorus (~20 times less phosphate than detected in *Hp*G) in good agreement with the findings of Fleitz and Horstmann[13]. As do most galactans isolated by repeated ultracentrifugation, *Ls*G contained 0.9% of nitrogen. Sulfate, pyruvate, and lactate were not detected, nor could short-chain carboxylic esters, lactones, and anhydrides as screened by the Hestrin[14] method. Unlike the cores of *Hp*G and *Aa*G[3] obtained after the first Smith-degradation, which contained ~5% of glycerol, the product of the first Smith-degradation of *Ls*G contained no glycerol.

The molecular weights of *Hp*G and *Ls*G were shown[10,11] to be 4.0 and 2.2 × 10^6, respectively, by sedimentation studies. In gel filtration on Sephacryl 500, *Ls*G was eluted in almost identical volumes as *Hp*G, *Aa*G, and *Bg*G. The product of the first Smith-degradation of *Ls*G was eluted slightly later than *Ls*G, and a molecular weight of 1.3 × 10^6 was calculated by using dextran 150 (mol. wt. 150,000) and arabinogalactan (mol. wt. 80,000) as standards in addition to *Hp*G and *Ls*G. However, after two Smith-degradations of *Ls*G, the product disintegrated into small fragments which were separated on Biogel P-2 into five fractions (Fig. 1).

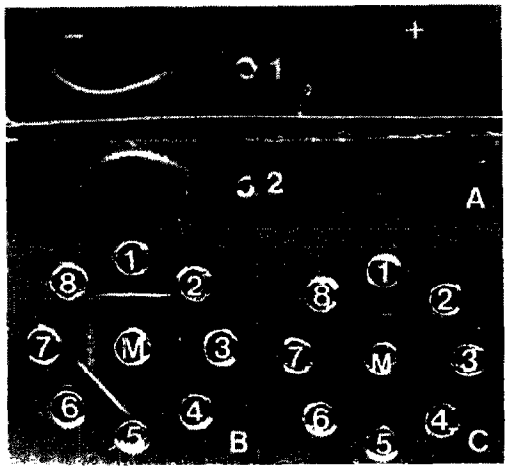


Fig 2. Immunoelectrophoretic (A) and immunodiffusion (B and C) patterns of the different snail galactan preparations with the anti-*Ls*G serum A. 1, *Ls*G (5 0 mg/mL), 2, *Ls*G 1st Smith-degradation (5 2 mg/mL), trough, anti-*Ls*G serum B: 1 and 6, *Ls*G (5 2 mg/mL); 2, *Hp*G (6 1 mg/mL), 3 and 7, *Bg*G (4.7 mg/mL); 4, *Cn*G (4.5 mg/mL); 5 and 8, *Aa*G (5 6 mg/mL) C 1 and 4, *Ls*G (5 0 mg/mL); 2, 5, and 7, *Ls*G 1st Smith-degradation (5.2 mg/mL); 3, 6, and 8, *Ls*G fraction I (5 1 mg/mL). M, in (B) and (C), anti-*Ls*G serum.

Fraction V was eluted slightly later than galactose, whereas fractions IV, III, and II were eluted somewhat earlier than lactose, raffinose, and stachyose, respectively. Fraction I had a broad elution profile indicative of heterogeneity, and the mol. wts. of its components, as calculated from the elution volume, ranged from 1000–3000.

Immunochemical studies. — In immunoelectrophoresis, *Ls*G formed one precipitin line with the antiserum raised against the purified *Ls*G (Fig. 2A); it migrated towards the cathode in agar, but did not move in agarose. Thus, *Ls*G is essentially uncharged. The product of the first Smith-degradation of *Ls*G gave one strong band and one faint diffuse band both of which moved towards the cathode in agar with a mobility the same as that of *Ls*G. After a second Smith-degradation, the product (fraction I on Biogel P-2) did not show any precipitin line either in immunoelectrophoresis or in the Ouchterlony test. The precipitin lines of *Ls*G and its first Smith-degradation product did not fuse completely in gel diffusion (Fig. 2C); a spur was observed, indicating only partial identity. Thus, *Ls*G lost specific determinants during the degradation process.

Some cross-reactivity of the anti-*Ls*G serum with other snail galactans was detected by the Ouchterlony technique. *Hp*G, *Aa*G, and *Cn*G reacted only faintly with this antiserum, whereas *Bg*G formed a significant precipitate. However, with *Bg*G, a spur was seen on the *Ls*G side. Thus, at least some determinants of these galactans differed from those of *Ls*G (Fig. 2B).

Quantitative precipitin reactions were also performed with the antiserum raised against *Ls*G. As seen in Fig. 3a, 40 μg of *Ls*G precipitated a maximum of

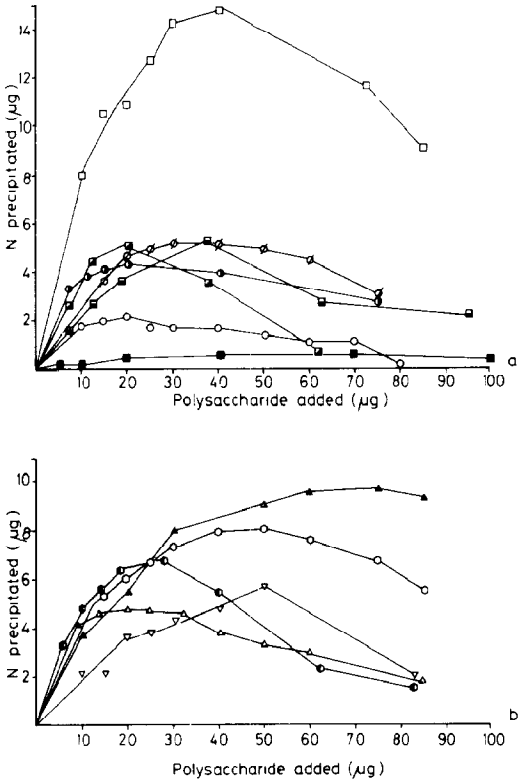


Fig. 3. Quantitative precipitin curves of anti-*Ls*G serum (40 μL) with the galactans (total volume, 250 μL): □, *Ls*G; □, *Ls*G, 1st Smith-degradation product; ■, *Ls*G, fraction I after 2nd Smith-degradation; □, *Ls* egg-shell polysaccharide; ○, *Hp*G; ⌀, *Hp*G, 1st Smith-degradation product; ◐, *Hp*G dephosphorylated; ▽, *Aa*G; ▲, *Aa*G, 1st Smith-degradation product; △, *Cn*G; ○, *Bg*G; ◐, *Bg*G, 1st Smith-degradation product.

14.8 μg of nitrogen (N) from 40 μL of anti-*Ls*G serum, and 7.5 μg of *Ls*G were required for 50% precipitation. The galactans of other snails were less active. *Bg*G (45 μg) gave about half of the precipitated N, *Aa*G (50 μg) and *Cn*G (25 μg) produced at the most 5.3 and 4.8 μg of N, respectively (Fig. 3b), and *Hp*G (20 μg) precipitated only 2 μg of N (Fig. 3a).

The addition of galactans in excess of the optimum quantities decreased the amount of precipitated N, probably due to the formation of soluble complexes. However, the addition of *Ls*G to the supernatant solution of the optimal precipitin reaction between *Aa*G (50 μg) and anti-*Ls*G serum (40 μL) gave a strong precipitate equivalent to the difference in N formed between the anti-*Ls*G serum and *Ls*G and *Aa*G, respectively. Corresponding results were obtained by prior absorption of the anti-*Ls*G serum with *Hp*G (Fig. 4). Thus, only a minor population of the antibodies cross-reacted with other snail galactans, whereas the major part of the antibodies was highly specific for *Ls*G.

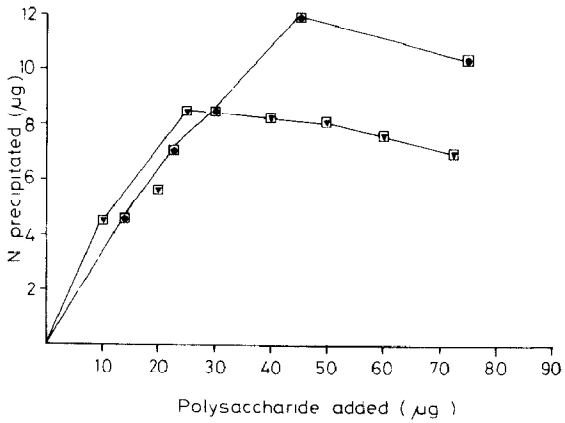


Fig. 4. Quantitative precipitin curves of *Ls*G and anti-*Ls*G serum (40 μL) maximally absorbed with *Hp*G (—□—) and *Aa*G (—▼—), respectively. The precipitate formed with these two galactans was removed prior to the addition of *Ls*G (total volume, 250 μL).

The first Smith-degradation product of *Ls*G was less active than the native material, giving only 5 μg of N with 20 μg of polysaccharide added (Fig. 3a). The corresponding products of *Hp*G and dephosphorylated *Hp*G were about as active as the first Smith-degradation product of *Ls*G and precipitated more than twice the amount of N obtained with *Hp*G (Fig. 3a). A similar increase in the precipitable N was observed when the first Smith-degradation product of *Aa*G was used instead of *Aa*G. Only the first Smith-degradation product of *Bg*G was slightly less active than the parent galactan (Fig. 3b). Fraction I derived from *Ls*G (see above) gave only negligible precipitation (0.6 μg of N).

A polysaccharide isolated[15] from the egg-shells of *Ls* and containing Gal (20%), fucose (14%), and uronic acids (16%) reacted with the anti-*Ls*G serum and gave 5 μg of N precipitate when 40 μg of polysaccharide were added, suggesting that the Gal residues formed determinants structurally related to those in the galactans of *Ls* and other snails.

*Ls*G was also tested for cross-reactivity with antisera raised against *Hp*G, *Aa*G, *Bg*G, and the first Smith-degradation product of *Hp*G. None of these antisera gave any precipitin reaction with *Ls*G, further emphasising the species-specific structure of *Ls*G.

Periodate oxidation. — *Ls*G consumed 1 mol of periodate per "anhydrogalactose" unit. In order to avoid incomplete oxidation due to the formation of intramolecular hemiacetals[16], the product was reduced with borohydride and again oxidised and reduced before proceeding with the Smith degradation. The resulting polymer also consumed 1 mol of periodate per "anhydrogalactose" unit during a second Smith-degradation. Elution of the final product from Biogel P-2 (Fig. 1) gave five fractions. Fraction I consumed 0.7 mol of periodate. *Ls*G, its first Smith-degradation product, and fraction I gave 0.45 mol of formic acid per "anhydrogalactose" unit.

TABLE I

RETENTION TIMES[a] (T) AND RELATIVE MOLAR RATIOS OF THE ACETYLATED AND METHYLATED GALACTITOL DERIVATIVES FROM *Ls*G, ITS FIRST SMITH-DEGRADATION PRODUCT, AND THE MAIN FRACTIONS OBTAINED AFTER THE SECOND SMITH-DEGRADATION

Substrate	*Relative molar ratio*				
	1,2-Me_2Gro (T 0.18)	*2,3,4,6-Me_4Gal[b] (T 1.0)*	*2,4,6-Me_3Gal (T 1.62)*	*2,3,4-Me_3Gal (T 2.29)*	*2,4-Me_2Gal (T 3.87)*
*Ls*G	—	47.8	4.9	0.7	46.6
1st Smith-degradation product	—	31.7	15.6	20.4	32.3
Fraction I	—	25.1	50.2	10.7	14.0
Fraction IV-2 and III-1	60.0	100.0	—	—	—
Fraction III-2 and II-1	28.7	51.2	48.8	—	—
Fraction III-3	22.1	52.1	—	47.9	—
Fraction II-3	11.0	31.5	34.1	34.4	—
Fraction II-4	12.1	64.1	—	—	35.9
Fraction II-5	9.7	32.4	33 0	34.6	—
Fraction II-9	9.6	32.3	67.7	—	—
Corresponding structural feature	→1)-Gro	Gal-(1→	→3)-Gal-(1→	→6)-Gal-(1→	→6)-Gal-(1→ ↑3

[a]Relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol on 3% OV-225 at 180°. Standards were those used and prepared by Bretting *et al.*[3]. [b]The relative mol. ratio of Me_2Gro and 2,3,4,6-Me_4Gal should be 1:1 in all oligosaccharides except fraction II-4, where it should be 0.5:1. Due to the high volatility of Me_2Gro, only 30–50% of the expected value was detected. However, the ^{1}H-n.m.r. spectra clearly show the corresponding mol. ratios.

The periodate-oxidation data are compatible with the (1→3) and (1→6) linkages assumed[17] to be present, and found in other snail galactans[2–4,6].

Methylation analysis. — Complete methylation of the *Ls*G, its first Smith-degradation product, and fraction I was achieved by two treatments using the Hakomori method. Conventional degradation and conversion of the products into alditol acetates gave the derivatives listed in Table I which were identified by g.l.c.–m.s. The derivatives obtained from *Ls*G were 2,3,4,6-tetra-*O*-methyl- (47.8 mol), 2,4-di-*O*-methyl- (46.6 mol), and 2,4,6-tri-*O*-methyl- (4.9 mol) and 2,3,4-tri-*O*-methyl-Galol (0.7 mol). Markedly different proportions of these derivatives were obtained from the first Smith-degradation product (31.7, 32.3, 15.6, and 20.4 mol) and fraction I (25.1, 14.0, 50.2, and 10.7 mol).

Thus, *Ls*G is a highly branched polysaccharide containing (1→3) and (1→6) linkages. (1→2) linkages, as found in *Hp*G, *Cn*G, *Aa*G[3], and the galactan of *Ampullarius* spec.[4], were absent. *Ls*G has the lowest proportion of linear sequences of all snail galactans, comparable only to *Bg*G[3] [8.3% of (1→3) linkages]. Both species are members of the order Basommatophora, whereas the galactans of the order Stylommatophora of the pulmonate snails show ~20% of linear sequences, so that the systematic classification is also reflected in the structure of the polysaccharides.

A substantial increase in the proportions of linear sequences of (1→3) and (1→6) linkages was found[3,6] in all the first Smith-degradation products of the snail galactans, indicating non-dichotomously branched polysaccharides. However, it was surprising to find that the second Smith-degradation product of *Ls*G comprised relatively small fragments, the largest of which contained 10–15 residues, and that >60% of the fragments were not larger than a pentasaccharide. In a dichotomously branched polysaccharide, the core should contain 2000–3000 residues.

This type of disintegration was not observed by Segura and Duarte[6] for the galactan of the snail *Strophocheilus oblongus*, although the molecular weight of their preparation was not given exactly.

Although a comparable increase in the proportion of the linear sequences of (1→3) linkages was observed in the second Smith-degradation products of the galactans of *Strophocheilus oblongus* and *Lymnaea stagnalis*, the structures seem to be different. Further studies are needed to determine whether the differences can be correlated with the systematic classification of the pulmonates into two different orders.

Isolation and identification of the oligosaccharides. — Fractions II–V were analysed by h.p.l.c. Fraction V was mainly glycerol contaminated with products which gave a positive orcinol reaction. Fraction IV gave glycerol and IV-2. Fraction III gave IV-2, III-2 (major component), and III-3 (25%). All of these fractions were pure by t.l.c. (solvents *A* and *B*). Fraction II gave a major peak and several minor peaks. T.l.c. showed that the major component was pure, whereas each of the peaks 5 and 6 contained 3–4 components. Peaks 1–2 contained little material and were not investigated further. T.l.c. (solvents *A* and *B*) of acetylated fraction

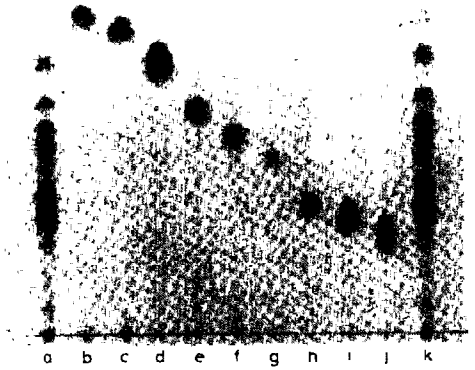


Fig. 5. T.l.c. pattern (solvent *A*) of the acetylated fraction II, and its purified components: a and k, fraction II: b, II-1; c, II-2; d, II-3; e, II-4; f, II-5; g, II-6; h, II-8; i, II-9; j, II-10.

II revealed twelve components (II-1/12 Fig. 5). Preparative t.l.c. (solvent *A*) gave 6 subfractions: (1) contained II-1, II-2, and traces of II-3; (2)–(4) contained II-3, II-4, and II-5, respectively; (5) contained II-6, II-7, some II-8, a small amount of II-9, and traces of II-5; and (6) contained mainly II-9, some II-8, and a small amount of II-10; II-11 and II-12 were not isolated due to insufficient material. The subfractions 1, 5, and 6 could be resolved into their constituents by t.l.c. (solvent *B*), to give II-1/2 and II-6/9 in pure form. All of the fractions except II-10 were homogeneous (Fig. 5). Fraction I contained a complex mixture of large oligosaccharides which was not studied further.

The quantities of each fraction obtained, the $[\alpha]_D^{20}$ values of the deacetylated oligosaccharides, and the relative mobilities of the acetylated derivatives in t.l.c. are given in Table II. Fractions IV-2, III-2, III-3, II-1, II-3, II-4, II-5, and II-9 were studied by ^{1}H-n.m.r. spectroscopy. Virtually all the signals of these di-, tri-, and tetra-saccharides could be assigned by using double-resonance experiments. The data for the multiplicities and coupling constants ($J_{1,2}$ 8.0 Hz) were almost uniform for all the galactose and glycerol units. The trisaccharides III-2 and III-3 were differentiated on the basis of the shift of the dd for H-3′ (δ 3.84) of III-2 and H-6′a,6′b (δ 3.38 and 3.73) in III-3. In a similar way, the tetrasaccharide derivatives II-3 [δ 3.63–3.78 (H-3′) and 3.84–3.93 (H-6″a,6″b)] and II-5 [δ 3.71–3.82 and 3.60 (H-6′a,6′b) and 3.73 (H-3″)] could be distinguished. The assignment was confirmed by enzymic studies. Finally, for II-4, the branching at the first galactose residue was

TABLE II

DATA ON THE FRACTIONS OBTAINED FROM *Ls*G AFTER TWO SMITH-DEGRADATION

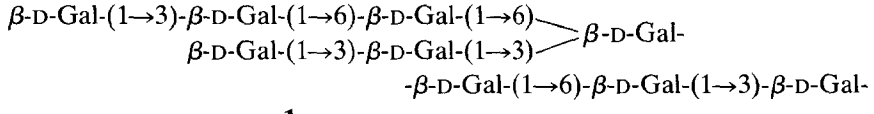
Fraction (LsG 2nd Smith-degradation)	*Quantity (mg)*	$[\alpha]_D^{20}$ *(degrees)*	*T.l.c. mobility*[b]		*Structure*
			Solvent A	*Solvent* B	
IV-2 and III-1	70.0	+3	n.d.[a]	n.d.	β-D-Gal-(1→1)-L-Gro
III-2 and II-1	57.0	+12	2.53	1.57	β-D-Gal-(1→3)-β-D-Gal-(1→1)-L-Gro
III-3	15.0	+8	3.14	n.d.	β-D-Gal-(1→6)-β-D-Gal-(1→1)-L-Gro
II-2	1.0	n.d.	2.42	1.57	n.d.
II-3	9.0	+11	2.13	1.66	β-D-Gal-(1→6)-β-D-Gal-(1→3)-β-D-Gal-(1→1)-L-Gro
II-4	7.5	+15	1.84	1.59	β-D-Gal-(1→3)-[β-D-Gal-(1→6)]-β-D-Gal-(1→1)-L-Gro
II-5	7.5	+13	1.64	1.55	β-D-Gal-(1→3)-β-D-Gal-(1→6)-β-D-Gal-(1→1)-L-Gro
II-6	1.0	n.d.	1.48	1.43	n.d.
II-7	1.0	n.d.	1.36	1.20	n.d.
II-8	1.0	n.d.	1.15	1.10	n.d.
II-9	35.7	+15.5	1.00	1.00	β-D-Gal-(1→3)-β-D-Gal-(1→3)-β-D-Gal-(1→1)-L-Gro
II-10	1.0	n.d.	0.82	0.80	n.d.

[a]Not determined. [b]Data for the acetylated compounds; 5 developments

indicated by the chemical shifts of the signals of the protons involved [δ 3.75 (H-3′), and 3.69–3.91 and 3.35 (H-6′a,6′b)]. The full n.m.r. data are listed in Tables III and IV.

The various oligosaccharides were deacetylated and subjected to methylation analysis. From the ^{1}H-n.m.r. and methylation analysis data, unambiguous structural assignments could be made for most of the components. However, due to the small differences in chemical shifts and coupling constants and the identical ratios for the derivatives of II-3 and II-5, their structures remained somewhat uncertain. Each substance was deacetylated and the products were treated with β-D-galactosidase from *E. coli*, then reacetylated, and subjected to t.l.c. The following components were detected. From II-5: D-Gal, β-D-Gal-(1→1)-L-Gro (traces), β-D-Gal-(1→6)-β-D-Gal-(1→1)-L-Gro, and II-5; from II-9: D-Gal, β-D-Gal-(1→1)-L-Gro (traces), β-D-Gal-(1→3)-β-D-Gal-(1→1)-L-Gro, and II-9. II-3 was not affected by the enzyme. Since II-5 contains (1→3) and (1→6) linkages and the enzymic removal of terminal non-reducing D-Gal generated β-D-Gal-(1→6)-β-D-Gal-(1→1)-L-Gro, II-5 must be β-D-Gal-(1→3)-β-D-Gal-(1→6)-β-D-Gal-(1→1)-L-Gro and the reverse sequence is ascribed to II-3.

The arrangement of the various isolated oligosaccharides in the native galactan cannot be deduced. Segura and Duarte[6] suggested for the galactan of *Strophocheilus oblongus* a linear core-structure of (1→3)-linked β-D-galactosyl residues to which multiple branched side-chains differing in length were attached. From the data obtained here, it is concluded that *Ls*G is neither dichotomously branched nor does it contain a linear (1→3)-linked backbone, otherwise the galactan should not disintegrate after the second Smith-degradation. However, it is possible that the main chain is built of (1→3)- and (1→6)-linked β-D-Gal residues in a more or less regular alternation and that the three main components IV-2, III-2, and II-9 are derived from the core structure. Thus, a tentative structure of the *Ls*G after one Smith-degradation would be **1**.



1

Alternatively, the main chain could consist only of (1→6)-linked D-Gal residues all substituted at C-3. The side chains, however, would contain one or more D-Gal residues. The first Smith-degradation uncovers a linear sequence of (1→6)-linked D-Gal residues in the main chain, thereby providing the points of fragmentation for the second Smith-degradation, releasing the mainly (1→3)-linked side-chains. An alternative structure of *Ls*G after one Smith-degradation, based on these assumptions, could be **2**.

TABLE III

^{1}H-N M R CHEMICAL SHIFT DATA (δ) FOR THE VARIOUS OLIGOSACCHARIDES ISOLATED FROM *Ls*G AFTER THE SECOND SMITH-DEGRADATION

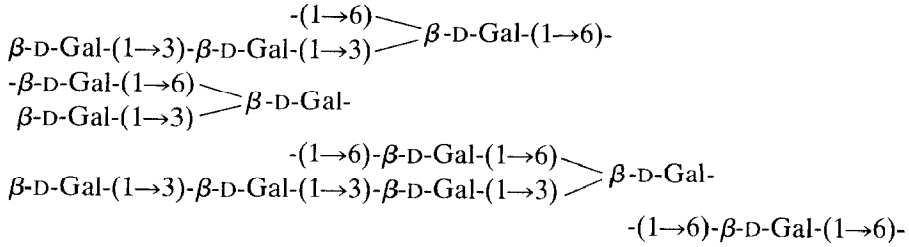
Proton	*Fraction*						
	IV-2	*III-2*	*III-3*	*II-3*	*II-4*	*II-5*	*II-9*
1a	3 97dd	3.94dd	3.98dd	3.84–3.93m	3.88dd	3.92dd	3.93dd
1b	3 69dd	3.65dd	3.70dd	3.58dd	3.56dd	3.59dd	3.64dd
2	5.17mc	5.15mc	5.18mc	5.09mc	5.11mc	5.11mc	5.15mc
3a	4.29dd	4 30dd	4.30dd	4 23dd	4.24dd	4.23dd	4.30dd
3b	4 10dd	4.08dd	4.10dd	4.03dd	4.01dd	3 98–4.07m	3 96–4.17m
1′	4.49d	4.39d	4.49d	4.34d	4.30d	4.32d	4.38d
2′	5.20dd	5 18dd	5.18dd	5.13dd	5 10dd	5.08dd	5.17dd
3′	5.00dd	3.84dd	4.98dd	3.63–3.78m	3 75dd	4.86dd	3.73dd
4′	5 39dd	5.39dd	5.37dd	5.33dd	5 25dd	5.28dd	5 36dd
5′	3.91ddd~dt	3.82ddd~mc	3 87mc	3 63–3.78m	3.69–3.91m	3 71–3.82m	3.75mc
6a′	4.18dd	4.14dd	3 83dd	3.98–4 09m			3 96–4.17m
6b′	4 12dd	4 07dd	3.73dd		3.35mc	3 60dd	
1″		4 55d	4.48d	4.43d	4 44d	4 41d	4.44d
2″		5 07dd	5 19dd	5.09dd	5.11dd	5 12dd	5.04dd
3″		4 90dd	5.00dd	4.82dd	4.86dd	3.73dd	3.80dd
4″		5.35dd	5.38dd	5 25dd	5.27dd	5.28dd	5.35dd
5″		3.85ddd~mc	3 91mc	3.63–3 78m	3.69–3.91m	3.71–3.82m	3.80mc
6a″		4 20dd	4 17dd	3.84–3.93m	3.98–4.13m	3.98–4.07m	3.96–4.17m
6b″		4.10dd	4.13dd				

1‴				4.49d	4.48d	4.49d	4.51d
2‴				4.98dd	5.00dd	5.00dd	5.05dd
3‴				4.94dd	4.93dd	4.91dd	4.91dd
4‴				5.34dd	5.31dd	5.31dd	5.34dd
5‴				3.63–3.78m	3.69–3.91m	3.71–3.82m	3.84mc
6a‴				4.15dd	3.98–4.13m	4.13dd	4.22dd
6b‴				3.98–4.09m		3.98–4.07m	3.96–4.17m
OAc	1.99, 2.06, 2.08(2) 2.10, 2.17	1.97, 2.01, 2.06, 2.09, 2.10, 2.11, 2.13, 2.17, 2.23	1.98(2) 2.06(3) 2.08, 2.09, 2.16(2)	1.89, 1.92, 1.94, 1.97, 1.98, 1.99, 1.99–2.00, 2.00, 2.12, 2.02, 2.04, 2.05, 2.07	1.89, 1.91, 1.94, 1.97, 1.99(2) 2.00, 2.01, 2.00, 2.02, 2.04, 2.05, 2.07, 2.13	1.90(2) 1.94, 1.97, 1.99(2) 2.00, 2.01, 2.00, 2.01, 2.02, 2.06, 2.07, 2.09	1.95, 2.00, 2.05(2) 2.06, 2.07, 2.08, 2.09, 2.12, 2.13, 2.14, 2.16

TABLE IV

COUPLING CONSTANTS (J, Hz) FOR VARIOUS OLIGOSACCHARIDES ISOLATED FROM *Ls*G AFTER THE SECOND SMITH-DEGRADATION

Protons	*Fraction*						
	IV-2	*III-2*	*III-3*	*II-3*	*II-4*	*II-5*	*II-9*
1a,1b	11.0	11.0	11.0	10.9	11.2	11 0	11.0
1a,2	4.6	5.0	4.8		5.2	4.8	5.2
1b,2	5.8	5.5	6.0	5 5	5.8	6.0	5.4
2,3a	3.8	3.8	3.8	3.8	3.8	4.0	3.8
2,3b	6.2	6 2	6.2	6.4	6.4		
3a,3b	12.0	12.0	12.0	12.0	12.0	12.0	12.0
1′,2′	8.0	8.0	8.0	8.0	8.0	8.0	8.0
2′,3′	10.4	10.2	10.4	10.2	10.0	10.4	10.2
3′,4′	3.6	3.6	3.4	3.4	3.4	3.4	3.4
4′,5′	1.0	1.0	1.0	1.0	1.0	1.0	1.0
5′,6a′	6.6	5.8	5.2				
5′,6b′	6.8	6.8	6.6			6.8	
6a′,6b′	11.0	11.6	10.6			10.6	
1″,2″		7.8	8.0	7.8	8.0	8.0	8.0
2″,3″		10.5	10.4	10.6	10.4	10.4	10.2
3″,4″		3.4	3.4	3.6	3.4	3.4	3.4
4″,5″		1.0	1.0	1.0	1.0	1 0	1.0
5″,6a″		6.0	6.4				
5″,6b″		7.0	7.6				
6a″,6b″		11.2	11.2				
1‴,2‴				8.0	8.0	7.8	7.8
2‴,3‴				10.4	10.6	10 4	10.5
3‴,4‴				3.6	3 4	3.4	3.4
4‴,5‴				1.0	1.0	1.0	1.0
5‴,5‴				4.6		5.8	5.8
5‴,6b‴							
6a‴,6b‴				11.2		11.2	11.4



2

EXPERIMENTAL

Galactans. — *Lymnaea stagnalis* snails were kept in culture in the Biological Institute of the University Amsterdam. The spawn was collected, lyophilised, and stored at −20°. Dried spawn (28 g) was homogenised in 0.9% saline (1.2 L) and extracted for 14 h at 4°. The mixture was centrifuged at 1000*g* for 30 min, and the supernatant solution at 150,000*g* for 2.5 h. The translucent pellet was rinsed twice with distilled water to remove a thin layer of insoluble material, and then dissolved in water. The ultracentrifugation was repeated five times. The sediment was finally dissolved in distilled water and the solution was lyophilised.

The sediment of the centrifugation at 1000*g* was resuspended in 0.9% saline (10 vol.), and the suspension was stirred for 1 h and sedimented at 1000*g*. The supernatant solution was discarded and this process was repeated five times until the supernatants were devoid of orcinol-positive material. The sediments were treated for 3 h with boiling, aqueous 25% potassium hydroxide, insoluble material was removed by centrifugation, and ice-cold aqueous 96% ethanol (3 vol.) was added to the clear supernatant. The precipitated white polysaccharide was collected by centrifugation, dissolved in water, and again precipitated with ethanol (3 vol.). After repeated precipitation, the egg-shell polysaccharide was dialysed for 5 days against distilled water and then lyophilised.

The galactans of the snails *Hp, Aa, Cn,* and *Bg* were prepared as described earlier[3]

Immunology. — The antiserum to *Ls*G was raised in a rabbit immunised once a week for 3 weeks with the galactan (250 μg) in Freund's complete adjuvant (0.5 mL). After 2 weeks, the immunising schedule was repeated.

Quantitative precipitin analysis in a total volume of 250–300 μL was performed by a microtechnique[18,19]. Total nitrogen in the washed precipitates was determined by the ninhydrin procedure[20]. Immunodiffusion was conducted by the Ouchterlony method[21] in 1.5% agar in 0.05M sodium barbital (pH 8.3). Immunoelectrophoresis was performed[22] at 220 V for 2 h.

Determination of sugars. — 6-Deoxyhexose, hexosamine, and hexose were determined by colorimetric methods[18]. Uronic acid was determined by the method of Dische[23], using 50–100-μg samples.

Identification and quantification of individual sugars were performed by g.l.c. of their alditol acetates[24]. After hydrolysis in 0.13M H_2SO_4 for 18 h, neutralisation ($BaCO_3$), and conversion into the alditol acetates, g.l.c. was performed using a Packard 419 gas chromatograph and a glass column filled with Chromosorb Q (80–100 mesh) coated with 3% of ECNSS-M. Inositol and erythritol were added before hydrolysis as internal standards.

Methylated galactans were treated with aqueous 90% formic acid for 1 h at 100° and then with 0.13M H_2SO_4 at 100° for 20 h. Each hydrolysate was neutralised ($BaCO_3$), and the sugars were reduced with $NaBH_4$ and then acetylated. The products were subjected to g.l.c. using a glass column (3.0 × 4 mm) of Chromosorb Q

coated with 3% of OV-225. Quantification of the corresponding galactose derivatives was effected with a Hewlett–Packard 3380-A integrator. The relative molar concentration was calculated from the peak areas. G.l.c.–m.s. was performed with a Finnigan MAT 311 A instrument operating in the c.i. mode with isobutane as the reagent gas.

Smith degradations. — Analytical periodate oxidation was performed with 9mM sodium periodate in the dark for 96 h at 0°. The galactan concentrations were ~10 mg/mL. Periodate consumption and liberation of formic acid were determined as described by Schiffmann *et al.*[25]. For Smith degradation, *Ls*G (6 g) was oxidised with 0.04M sodium metaperiodate for 96 h at 0° as described earlier[26,27]. The total volume was 1640 mL. The excess of oxidant was decomposed with ethylene glycol (80 mL), the solution was dialysed against distilled water for 10 days, sodium borohydride (20 g) was added, and, after 24 h at room temperature, the solution was dialysed against distilled water for 10 days.

In order to remove hemiacetals and complete the oxidation, the oxidised galactan was reduced with borohydride, oxidised with periodate, and reduced[16]. The periodate consumed during the second oxidation could not be determined exactly, due to insufficient removal of sodium borohydride after reduction. The resulting product was hydrolysed in 0.5M hydrochloric acid at room temperature for 24 h, and the hydrolysate was dialysed for 10 days against distilled water and lyophilised to give the first Smith-degradation product (2.6 g).

The first Smith-degradation product (1.5 g) was subjected to a second Smith-degradation as described above. However, the final hydrolysate was not dialysed, but neutralised with Ag_2CO_3. The supernatant solution and two washings of the residue were combined and passed over Amberlite MB-2 (20–50 mesh) mixed-bed resin. The eluate was concentrated under reduced pressure to 50 mL. Hexose determination (orcinol–sulphuric acid) indicated 553 mg of this product.

Methylation analyses. — Methylations of *Ls*G and its Smith-degradation products (10–15 mg) were readily accomplished by the Hakomori method[28,29]. The galactans were soluble in methyl sulfoxide at 1–2 mg/mL. After two methylations, the products showed no i.r. absorption for hydroxyl groups.

Separations. — Fractions II–IV were subjected to h.p.l.c., using a steel column (25 × 0.46 cm) filled with Spherosorb–propylamine, a refractive index detector, and elution with acetonitrile–water mixtures at 2 mL/min. The appropriate fractions were combined.

The acetylated oligosaccharides of fraction II were subjected to t.l.c. on Silica Gel 60 (Merck) with *A*, ethyl acetate–hexane (4:1); or *B*, chloroform–acetone (9:1). After 5 developments, marker strips were charred with sulfuric acid. The appropriate portions of the gel were extracted with ethyl acetate, and the eluted material was checked for purity by t.l.c. (solvents *A* and *B*).

Other determinations. — ^{1}H-N.m.r. spectra (400 MHz) were recorded with a Bruker WM 400 instrument for solutions in $CDCl_3$ (internal Me_4Si). Optical rotations were measured on aqueous solutions with a Perkin–Elmer MC 241

polarimeter. I.r. spectra were recorded for KBr discs with a Perkin–Elmer spectrophotometer 399. Phosphate determination was conducted according to Ames[30].

Analysis for short-chain carboxylic acid esters, lactones, and anhydrides was performed by the Hestrin method[14]. Glycerol (after Smith degradation), pyruvate, and lactate (after hydrolysis of the galactans) were determined enzymically by use of the appropriate kits (Boehringer).

Enzymic cleavage of oligosaccharides was performed[31] with purified β-D-galactosidase from *E. coli* (Sigma, 90 units/mg) in 0.01M phosphate buffer (pH 7.3) for 4 h. The enzyme was activated by $MgCl_2$ and 2-mercaptoethanol as proposed by the manufacturer. The degradation products were identified by t.l.c. (solvent *A*) of their acetylated derivatives after removal of salts and proteins with Amberlite MB-2. *Hp*G and *Bg*G were dephosphorylated[32] with 48% hydrofluoric acid at 0°.

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