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Preparation of a sulfated linear (1 → 4)-β-D-galactan with variable degrees of sulfation

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Abstract

A linear 1,4-β-D-galactan has been prepared by partial acid hydrolysis of the major lupin seed polysaccharide on a preparative scale. The galactan has been characterized by acetylation and methylation analysis, by GPC, as well as by its ^{1}H- and ^{13}C-NMR-spectra. Sulfation with increasing amounts of SO_3-pyridine yielded a series of galactan sulfates with increasing DS. In all derivatives, the sulfate groups had a similar pattern of distribution, as shown by methylation analysis. Beside unsulfated parts of the molecule, there were also parts with a multiple substitution pattern.

1. Introduction

It was found that the carrageenans, linear galactans with alternating (1 → 3)-α-D- and (1 → 4)-β-D-glycosidic linkages, were active angiogenesis inhibitors (Hoffman & Paper, 1993; Paper, Vogl, Franz, & Hoffman, 1995). The individual carrageenans differ mainly in their content of 3,6-anhydrogalactose and their sulfation pattern. However, the high molecular weight and the structural heterogeneity are major drawbacks for a possible therapeutical application. The aim of this study was the preparation of smaller and regular built, linear galactan sulfates for the investigation of their antiangiogenic activity in the future.

2. Experimental

2.1. General

Seeds of *Lupinus polyphyllus* Lindl. were purchased in the local market. All chemicals were of analytical reagent grade (E. Merck, Darmstadt, Germany).

2.2. Uronic acid content

Hexuronic acids were measured according to Blumenkrantz and Asboe-Hansen (1973). At low amounts of uronic acid, 20 μl of 4 M amidosulfonic acid were added to the test tubes, to reduce the brownish colour of the neutral sugars (Filisetti-Cozzi & Carpita, 1991).

2.3. Constituent sugars

The proportions of the constituent sugars were determined by GLC of the derived alditol acetates using the MMB-method of Stevenson and Furneaux (1991). The analysis was conducted on a HP5890 equipped with a fused silica capillary column (FS-OV-225, 25 m, ID 0.25 mm) and helium (1,0 ml/min, Split 1:50) as the carrier gas. Oven: 230°C, injector: 280°C, detector: FID, 290°C.

2.4. Methylation analysis

Unsulfated polysaccharides were directly dissolved in DMSO, and methylated with CH_3I and freshly prepared Dimsyl-K. Then 3 ml CH_2Cl_2/CH_3OH (2:1), 2 ml water and some crystals of $Na_2S_2O_3$ were added to the test tube. After mixing, the organic layer was washed four times with 2 ml water, mixed with 2 ml 2,2-dimethoxypropane, 20 μl glacial acetic acid and evaporated with a stream of nitrogen (Harris, Henry, Blakeny, & Stone, 1984).

The sulfated polysaccharides had to be converted into DMSO-soluble triethylammonium salts prior to methylation with CH_3I and Dimsyl-K. After methylation, water was added and a stream of N_2 was passed through the cloudy

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CTABr, cetyltrimethylammoniumbromide; DMF, dimethylformamide; DS, degree of sulfation; L.p., *Lupinus polyphyllus*; LuPS, *Lupinus polyphyllus* polysaccharide; MMB, 4-methylmorpholine borane; MWCO, molecular weight cut-off; PMAA, partially methylated alditol acetates; PS, polysaccharide.

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solution to remove the residual CH_3I. The methylated, sulfated polysaccharides were further purified by dialysis (2 d, MWCO: 1000 Da) and then freeze-dried.

The methylated polysaccharides (sulfated and unsulfated) were then converted into PMAAs according to the MMB-method of Stevenson and Furneaux (1991). The PMAAs were analysed on a HP5890 A gaschromatograph with a mass-selective detector (HP5970 B). Carrier gas: Helium (1.0 ml/min), column: bonded phase fused silica capillary column (SP2380, 30 m × 0.25 mm), temperature-program: 140–180°C (20°C/min), 180–240°C (5°C/min), 240°C (10 min isotherm).

2.5. The carbohydrate content

The carbohydrate content was determined according to Morris (1948).

2.6. Sulfate content

Sulfated polysaccharide (1 mg) was hydrolysed with 1 ml of 2 M TFA (1 h/120°C). After cooling to room temperature, 2 ml water was added and the solution was freeze-dried. The residue was dissolved in 2.0 ml water, centrifuged and analysed by ion-chromatography on a Waters HPLC system equipped with an autosampler and conductivity detector. Column: IC-Pak Anion, 4.6 × 50 mm, *Waters*; eluent: borate–gluconate buffer; injection volume: 50 μl. A calibration curve was measured for every analysis. All determinations were made in triplicate.

The degree of sulfation (DS) was obtained as: sulfate content (mol/g)/carbohydrate content (mol/g).

2.7. Molecular weight determination

The molecular weight of the galactans and their sulfates was performed by GPC using a Superose 12® column and 0.1% sodium chloride as solvent. The system was calibrated with commercially available pullulan standards.

2.8. NMR

NMR-spectra were performed on a Bruker ARX-400 (400 MHz), solvent D_20, internal standard: TMS.

2.9. Isolation of the Lupinus polyphyllus polysaccharide

Lupinus polyphyllus seeds were swollen overnight in water, manually stripped of their peel, dried and ground. The ground seeds (277 g) were first stirred with 10% NaCl-solution (4 × 3 h) and then with 0.2% NaOH-solution (5 × 12 h) at room temperature to remove the protein. The pre-extracted residue was boiled with 0.2% NaOH (1.5 l) and filtered. Twofold ethanol precipitation of the resulting viscous, orange solution gave 50.33 g of arabino-galacto-rhamnogalacturonan (AGR).

2.10. Preparation of LuPS

AGR (15 g) dissolved in 735 ml of water were mixed with 750 ml 0.02 N-oxalic acid, adjusted to pH 3.5 and refluxed. During hydrolysis, samples were taken, neutralized and analysed by GPC. After 51 h the heating was stopped, as the molecular weight distribution was equivalent to the profile of the best pilot test. The reaction mixture was neutralized with $Ca(OH)_2$ solution and centrifuged after standing for 2 h at 4°C. The clear yellow solution was stirred three times with 20 g strong anion exchanger resin (Dowex 1 × 2, analyt. grade) at 4°C, 1.5 d in each case. The resulting clear and colourless solution was dialysed at 14 d against distilled water (MWCO: 3500 Da), filtered and freeze-dried. → 5.5 g LuPS crude (5.5% Ara).

2.11. Second partial hydrolysis

About 3.5 g of crude LuPS was dissolved in 350 ml of 0.01 N oxalic acid, refluxed 2 h, neutralized, centrifuged and dialysed (7 d, MWCO: 1000 Da). Adjusting to pH 7.35, filtering and freeze-drying gave 2.09 g LuPS.

2.12. Sulfation

Prior to sulfation the polysaccharide was lyophilisized. If a high DS was required, the freeze-dried polysaccharide was additionally soaked in dry DMF and most of the DMF was distilled off in vacuo. In most cases however, the polysaccharide (100 mg) was simply soaked in dry DMF (2 ml). The sulfating agent SO_3-pyridine was dissolved in 2.5 ml DMF and mixed with the polysaccharide. For every mole of SO_3-pyridine, 1 mol of pyridine was added to the mixture. The reaction was carried out with stirring under argon atmosphere at 90°C. The standard reaction time was 4 h, but was reduced if a brownish colour appeared. After cooling to room temperature, 25 ml water was added with stirring and then sufficiently with 1 N-NaOH to produce a pH of 10. The sulfated polysaccharide was precipitated with ethanol, re-dissolved in water and dialysed (7 d, MWCO: 3500 Da) against slightly alkaline water (pH 9) to remove pyridine. Finally the sulfated polysaccharide was dialysed against distilled water, until the contents of the dialysis tube were slightly acidic, neutralized and freeze-dried.

Table 1
Analytical data of the polysaccharides of *Lupinus albus* L. and *Lupinus polyphyllus* Lindl. (AnGalA: anhydrogalacturonic acid = polymeric form)

Polysaccharide derived from	$[\alpha]_D^{20°}$	AnGalA (%)
Lupinus albus L.	52	8
Lupinus polyphyllus Lindl.	52.6	8.7

Table 2
Fractionation experiments with the polysaccharide(s) of *Lupinus polyphyllus* Lindl.

Reagent	Precipitation	Precipitation described with:	Reference
Neutral $CuSO_4$-sln.	−	*Lupinus albus* galactan	(Deuel & Neukom, 1949)
$CaCl_2$-sln.	−	Polygalacturonan	(Hirst et al., 1947)
CTABr-sln.	−	Acidic polysaccharides	(Scott, 1965).
$Ba(OH)_2$-sln	+	many PS, e.g. 1,4-β-D-galactan	(Meier, 1965; Bouveng & Meier, 1959)

Table 3
Monosaccharide composition of the polysaccharides of *Lupinus angustifolius* L. and *Lupinus polyphyllus* Lindl.

Polysaccharide	Monosaccharides (%)[a]							
	Rha	Fuc	Ara	Xyl	Man	Gal	GalA	MW (D)[b]
Lupinus polyphyllus	2.5	0.7	11.8	1.7	–	74.6	8.7	> 853 000
Lupinus angustifolius	3.67	–	9.89	0.7	0.24	78.17	5.13	> 758 000

[a] Percentages apply to the anhydroform of the sugars.
[b] Estimation of the average molecular weight by GPC.

Table 4
Hydrolysis of the *Lupinus polyphyllus* polysaccharide with 0.01 N oxalic acid. Analytical data of the isolated galactan sidechains at different times of hydrolysis

	Time of hydrolysis (h)			
	8	28	53	72
Yield(%)	56	46	35	32
AnGalA[a] (%)	n.d.[b]	1.76	0.7	0.48
MG[c] (D)	200 000	10 000	6000	5500

[a] AnGalA: anhydrogalacturonic acid = polymeric form.
[b] n.d. = not determined.
[c] Estimation of the molecular weight by GPC.

3. Results and discussion

3.1. Preparation of the 1,4-β-D-galactan

Linear 1,4-β-D-galactans have been isolated from *Strychnos nux-vomica* L. seeds (Andrews, Hough, & Jones, 1954), from potato tubers (Wood & Siddiqui, 1972), from the pectin fraction of lemon peels (Labavitch, Freeman, & Albersheim, 1976) and from white lupin seeds (Hirst, Jones & Walder, 1947). Only lupin seeds seem to be a suitable source for isolation on a preparative scale, as the galactan content of the other materials is relatively low. Instead of white lupin seeds, which are available in small amounts, seeds of *Lupinus polyphyllus* Lindl. were used in this study. The isolation followed the procedure of Hirst et al. (1947). The specific rotation and the galacturonic acid content of the product were in good agreement with the data of the *Lupinus albus* polysaccharide (Table 1). Hirst et al. (1947) removed most of the uronic acid by precipitation with Ca^{2+} from the white lupin extract, which they described as a mixture of galactan, arabinan and polygalacturonan. The extract of *Lupinus polyphyllus* Lindl.; however, gave no precipitate with calcium chloride. Likewise the selective precipitation with $CuSO_4$, CTABr, $Ba(OH)_2$, or further GPC or ion-exchange-chromatography were unable to split the extract into two distinct fractions (Table 2). This result suggested that the extract was a homogenous polysaccharide. Its structure probably resembles the structure of the *Lupinus angustifolius* polysaccharide, which has recently been established by Cheetham, Cheung and

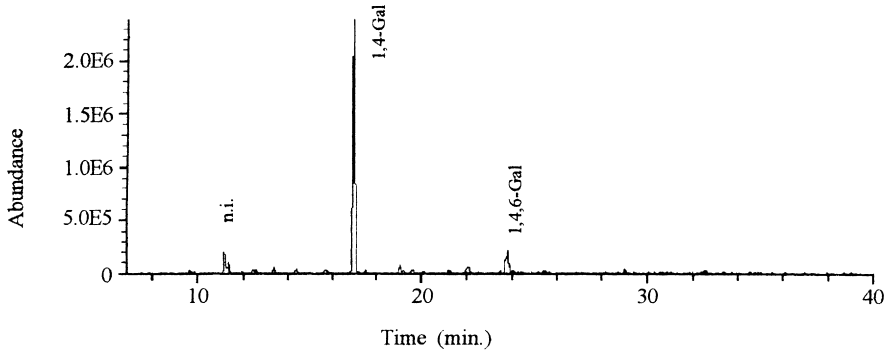

Fig. 1. Methylation analysis: GLC of the PMAA of LuPS. The PMAA were identified with a mass-selective detector (EI-MS).

Table 5
Monosaccharide composition of the *Lupinus polyphyllus* polysaccharide, LuPS crude and LuPS in percent of the dry weight

	Monosaccharides in (%)[a]						
	Rha	Fuc	Ara	Xyl	Gal	GalA	MW (D)[b]
L.p.-polysacharide	2.5	0.7	11.8	1.7	74.6	8.7	> 853 000
LuPS crude	0.5	0.5	5.5	–	92.6	0.9	9500[c]
LuPS	0.6	0.5	1.7	–	96.3	0.9	8500[c]

[a] Percentages apply to the anhydroform of the sugars.
[b] Estimation of the average molecular weight by GPC.
[c] On a Superose 12® column.

Table 6
^{13}C NMR-data of LuPS, methyl-α- and methyl-β-galactoside. All δ-values in ppm

Carbon atom	LuPS	Methyl-β-D-galactoside	Methyl-α-D-galactoside
C1	104.4	104.5	100.1
C2	71.9	71.7	69.2
C3	73.4	73.8	70.5
C4	77.7[a]	69.7	70.2
C5	74.6	76.0	71.6
C6	60.8	62.0	62.2

[a] Downfield shift due to glycosidic linkage.

Evans (1993), as both polysaccharides are very similar in their monosaccharide composition (Table 3). In this case, the galactan and the arabinan represent sidechains of a rhamno-galacturonan backbone, rather than individual polysaccharides. Prior to isolation of the galactan it seemed necessary to degrade the *Lupinus polyphyllus* polysaccharide in order to split off the galactan sidechains. This was achieved by refluxing a 1% solution of the polysaccharide in 0.01 N oxalic acid. Under these conditions, mainly furanosidic linkages such as occurring in the arabinan sidechains are cleaved, but also the linkages of the galactan sidechains to the backbone. In a series of experiments, the hydrolysis time was optimized to obtain a maximum yield of galactans with a molecular weight as high as possible and a low content of non-galactose sugar residues (Table 4). After 51 h most of the arabinose and the uronic acid containing polysaccharide fragments could be removed by treatment with a strong anion exchange resin and subsequent dialysis. On a preparative scale, however, a product was obtained, which still had an arabinose content of 5.5% (LuPS crude). A part of this crude galactan was refluxed for an additional 2 h with 0.01 N oxalic acid. After dialysis, an almost pure galactan (LuPS) with an arabinose content of 1.7% was obtained (Table 5). The molecular weight estimated by GPC on a calibrated column was 8500 Da. Methylation analysis established LuPS as essentially linear polysaccharide (Fig. 1). About 5% of the galactose monomers are branched at position C-6. To determine the anomeric configuration of LuPS, ^{1}H and ^{13}C NMR-spectra were recorded. The position of the anomeric signal at 104.2 ppm indicated the β-configuration, which was confirmed by a doublett ($\delta = 4.53$ ppm, $J = 7.78$ Hz) in the ^{1}H-NMR-spectrum (Agrawal, 1992). The other five signals of the ^{13}C NMR-spectrum were in good agreement with the signals of methyl-β-D-galactoside (Guiseley, 1978), except the C4-signal, which was shifted downfield indicating the glycosidic linkage at this position (Fig. 2, Table 6).

3.2. Sulfation of the 1,4-β-D-galactan (LuPS)

LuPS was sulfated with a solution of SO_3-pyridine in dry DMF under anhydrous conditions. In order to maximize the reacting surface of the substrate, the galactan had been freeze-dried prior to sulfation. When lyophilized galactan was subjected to a distillative drying step, analogous to the hydration-drying method of Guiseley, LuPS S1 was

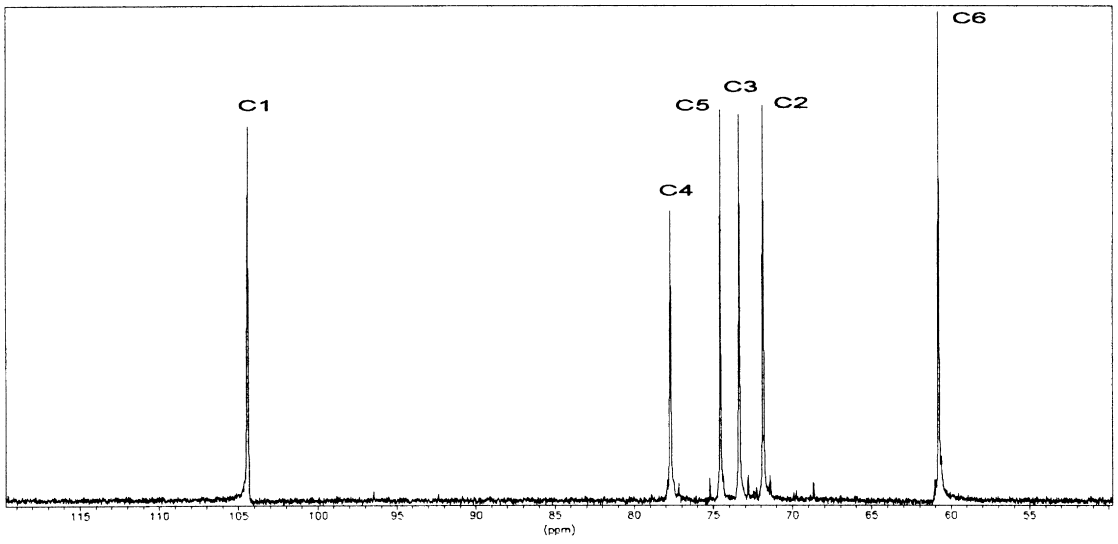

Fig. 2. ^{13}C NMR-spectrum of LuPS with assignment of the signals. Solvent = D_2O.

Table 7
DS and MW of the galactan sulfates in dependence on the reaction conditions

	DS	MW (Da)[a]	$n(SO_3\text{-Pyr})/n(AGU)$[b]	c (SO_3) mol/l	T (°C)	t (h)
LuPS S1	1.70	27 000	2.6	0.37	90	4
LuPS S2	0.56	5000	1.5	0.21	90	4
LuPS S3	1.65	10 000	4.0	0.54	90	4
LuPS S5	1.48	25 000	4.0	0.49	50	7
LuPS S6	0.14	10 000	0.5	0.07	90	4
LuPS S7	0.27	12 000	1.0	0.14	90	4
LuPS S8	0.53	19 000	1.5	0.21	90	4
LuPS S9	0.72	20 000	2.0	0.28	90	4

[a] The actual molecular weights of the LuPS sulfates are smaller than the given values, as the calibration curve had been determined with (unsulfated) pullulans.
[b] Mol sulfating agent/mol monomeric sugar (AGU).

Table 8
Methylation data of the LuPS sulfates

Partially methylated alditol acetates (%)	LuPS S1	LuPS S2	LuPS S3	LuPS S5	LuPS S7	LuPS S8	LuPS S9
2,3,6-Me_3-Gal [1,4-Gal]	8	64	18	27	86	73	56
2,6-Me_2-Gal [1,3,4-Gal]	8	7	9	5	4	8	9
3,6-Me_2-Gal [1,2,4-Gal]	–	5	2	–	2	3	4
2,3-Me_2-Gal [1,4,6-Gal]	7	11	8	12	8	11	11
2,4-Me_2-Gal [1,3,6-Gal]	3	–	2	–	–	–	–
6-Me-Gal [1,2,3,4-Gal]	–	3	–	–	–	–	–
2-Me-Gal [1,3,4,6-Gal]	48	4	42	41	–	3	14
3-Me-Gal [1,2,4,6-Gal]	11	–	8	4	–	2	–
Hexa-Ac-Gal [1,2,3,4,6-Gal]	15	6	11	11	–	5	5

obtained (Guiseley, 1978; Guiseley & Whitehouse, 1973). Different DS were produced by varying the amount and the concentration of the sulfating agent. In Table 7 the reaction conditions and the corresponding DS values of the galactan sulfates are given. The DS showed a linear increase with the reagent amount and concentration, provided that, the other reaction conditions remained constant. As shown with LuPS S5, the variation of temperature and duration led to a lower DS. LuPS S1, however, which had been subjected to a distillative drying with DMF prior to sulfation, had the highest DS. The removal of residual water resulted in a higher effective SO_3-pyridine concentration. The molecular weight of LuPS S1 was not affected by the additional drying. Therefore, it seems unlikely that the low molecular weight of LuPS S2 and LuPS S3 is due to a hydrolytic process. Both galactan sulfates had been prepared with a somewhat decomposed sulfating agent, which degraded the polysaccharide during sulfation in an unknown, most likely radical mechanism. The distribution of the sulfate groups was determined by methylation analysis (Table 8). As the consequence of a heterogenous reaction, the sulfate groups were distributed unevenly. Beside unsulfated galactose monomers, also monomers with multiple sulfation were present. Under the chosen reaction conditions preferred sites of substitution were the positions C-6 and C-3 (Table 4).

4. Conclusions

Seeds of *Lupinus polyphyllus* Lindl. contains a galacto-arabino-rhamno-galacturonan, which has a structure similar to the polysaccharide of *Lupinus angustifolius* L. seeds. The polysaccharide is a suitable source for the large-scale preparation of a linear 1,4-β-D-galactan. The galactan has been sulfated to derivatives with varying DS. In this study, controlling the DS with the reagent amount gave better results than the control via reaction temperature, as proposed by Guiseley (1978). The sulfate groups were not regularly distributed in the polysaccharide chain. For an overall characterization of the galactan sulfates in addition to molecular weight and DS, information about the distribution of the sulfate groups is important.

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