

# LaPSvS1, a (1 → 3)- $\beta$ -galactan sulfate and its effect on angiogenesis in vivo and in vitro

Jutta Bürgermeister,<sup>a</sup> Dietrich H. Paper,<sup>a,\*</sup> Horst Vogl,<sup>a</sup> Robert J. Linhardt,<sup>b</sup>  
Gerhard Franz<sup>a</sup>

<sup>a</sup>Institute of Pharmaceutical Biology, University of Regensburg, Universitätsstrasse 31, D-93040 Regensburg, Germany

<sup>b</sup>Department of Chemistry, Division of Medicinal and Natural Products Chemistry and Department of Chemical and Biochemical Engineering, University of Iowa, PHAR S328, Iowa City, IA 52242, USA

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## Abstract

LaPSvS1, a highly sulfated branched (1 → 3)- $\beta$ -galactan was prepared from the arabino-galactan from *Larix decidua* Miller by partial hydrolysis and subsequent sulfation with SO<sub>3</sub>-pyridine in DMF. The molecular weight was analyzed by GPC and the sulfate content was determined by ion chromatography. LaPSvS1 exhibited good antiangiogenic and antiinflammatory effects in two different modifications of the known CAM-assay. In vitro results obtained in the FGF-2-trypsin-assay and in fluorospectrometric experiments revealed that LaPSvS1 interacts with the fibroblast growth factor 2 system. This interaction is correlated with the in vivo effect of LaPSvS1 on FGF-2 induced angiogenesis. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Angiogenesis inhibitor; Antiinflammatory; CAM-assay; FGF-2; (1 → 3)- $\beta$ -Galactan-sulfate

## 1. Introduction

Angiogenesis, the formation of new capillaries from a preexisting vasculature is regulated by a complex interaction between angiogenic stimulators and inhibitors including, e.g., growth factors, cytokines, proteolytic enzymes, integrins and extracellular matrix components.<sup>1</sup> In the healthy adult body, it is switched on only during wound healing, embryonic development and the female reproductive cycle.<sup>2</sup> Disturbance of the angiogenic balance can lead to pathological angiogenesis and result in numerous diseases, e.g., cancer, diabetic

retinopathy and rheumatoid arthritis. In previous studies, it was demonstrated that galactan sulfates (carrageenans and low molecular weight galactan sulfates) are potent angiogenesis inhibitors in the chorioallantoic membrane model of fertilized hens' eggs (CAM-assay).<sup>3,4</sup> In this study, LaPSvS1, a highly sulfated branched (1 → 3)- $\beta$ -galactan sulfate, was synthesized starting from the AG of *Larix decidua* Miller and was investigated in vivo in various CAM-assays including embryonic as well as pathological angiogenesis induced by inflammation and FGF-2. The interactions of LaPSvS1 and the fibroblast growth factor 2 were studied also in two different in vitro assays.

## 2. Results and discussion

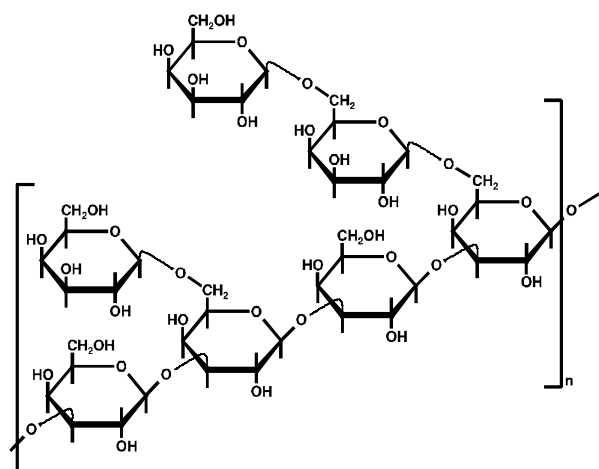
Previous investigations of naturally occurring and partially modified carrageenans and low-molecular weight galactan sulfates in the CAM-assay revealed their improved antiangiogenic activity compared to suramin, an angiogenesis inhibitor<sup>5</sup> used in clinical trials, e.g., for the treatment of metastatic melanoma and recurrent or progressive malignant glioma. Addi-

**Abbreviations:** AG, arabino-galactan; CAM, chorioallantoic membrane; DS, degree of sulfation; ECM, extracellular matrix; FGF-2, fibroblast growth factor 2; LaPSvS1, (1 → 3)- $\beta$ -galactan sulfate derived from *Larix decidua* Miller; LuPS S5, (1 → 4)- $\beta$ -galactan sulfate derived from a *Lupinus polyphyllus* Lindl. polysaccharide; MWCO, molecular weight cut off; PBS, phosphate buffered solution; PMAA, permethylated acetylated alditols.

\* Corresponding author. Tel.: +49-941-9434790; fax: +49-941-9434762

E-mail address: dieter.paper@chemie.uni-regensburg.de (D.H. Paper).

tionally, the results showed the good antiangiogenic effect of various (1→4)- $\beta$ -galactan sulfates derived from lupine seeds (e.g., LuPS S5).<sup>3,4,6</sup> In order to obtain further data about structure–activity relationships of the new class of antiangiogenic low-molecular weight galactan sulfates, a branched (1→3)- $\beta$ -galactan LaPSv (Scheme 1) was isolated after partial hydrolysis of the AG of larch wood and identified via GLC-analysis. Subsequent sulfation with  $\text{SO}_3$ -pyridine/DMF led to the (1→3)- $\beta$ -galactan sulfate LaPSvS1 with a molecular weight of 10 kDa. The DS, calculated from the sulfate analysis, was 2.0. The sulfate groups were not regularly distributed in the polysaccharide chain analogously to the (1→4)- $\beta$ -galactan sulfates (data not shown).<sup>7</sup> The used standard compound LuPS S5, a (1→4)- $\beta$ -galactan



Scheme 1. Structure of the (1→3)- $\beta$ -galactan LaPSv derived from the AG of *L. decidua* Miller. Sulfation led to the (1→3)- $\beta$ -galactan LaPSvS1 ( $M_w$  = 10 kDa, DS = 2.0).

Table 1

Score values for the evaluation of the antiangiogenic effect on the chorioallantoic membrane of the fertilized hens' eggs

Score value	Effects observed
0: no effect	—
0.5: very weak effect	no capillary free area  area with reduced density of capillaries around the pellet not larger than the area of the pellet
1: weak–medium effect	small capillary free area or area with significantly reduced density of capillaries. effects not larger than double the size of the pellet.
2: strong effect	capillary free area around the pellet at least double the size of the pellet

sulfate, was prepared after partial hydrolysis of the arabinogalactan–rhamnogalacturonan from *Lupinus polyphyllus* Lindl. and sulfation ( $M_w$ , 25 kDa, DS, 1.48) (for details see Refs. 3,7).

The CAM-assay has been shown to be a suitable in vivo model including many mechanisms relevant for physiological and pathological angiogenesis. It was further demonstrated that different modifications of the CAM-assay are versatile tools for the in vivo evaluation of small quantities (1–2 mg) of natural compounds enabling the detection of possible side effects of the test compound (e.g., embryotoxicity, membrane irritation, bleeding) in the same system as well.<sup>8–10</sup>

The (1→3)- $\beta$ -galactan sulfate LaPSvS1 was tested in different modifications of the CAM-assay with or without an external angiogenesis inducing agent (e.g., SDS, FGF-2). Because no angiogenesis inhibitor is used in the clinical practice and angiogenesis itself, is regulated by a complex redundant network of interacting molecules supporting the hypothesis that there is not only a single target to suppress uncontrolled angiogenesis, different standards with known activity were used in the respective in vivo and in vitro assays.

LaPSvS1 was tested in the CAM-assay for its antiangiogenic activity evaluated by a score system (see Table 1). Score 0 represents the normal growth of capillaries (see Fig. 1(a)), and score 2 indicates a strong antiangiogenic effect with a large capillary free area around the pellet (see Fig. 1(b)). LaPSvS1 showed a good inhibition of angiogenesis (score 1.2) in the CAM-assay compared to the control (agarose) ( $P < 0.05$ ) (Fig. 2). The treatment with suramin resulted in a weak effect, whereas the (1→4)- $\beta$ -galactan sulfate LuPS S5 was almost as effective as LaPSvS1. No side effects of the compounds were recognizable. The effect of suramin on the formation of capillaries in the CAM was in accordance to the observation by Gagliardi et al., who described a dose dependent antiangiogenic effect in the range of 25–200  $\mu\text{g}$  suramin/egg with an inhibition of 46% at a concentration of 50  $\mu\text{g}$ /egg.<sup>5</sup>

The HET-CAM-assay is an in vivo model for chronic inflammation, which is in this case induced by SDS. Inflammation and angiogenesis are codependent, with many mechanisms being involved in inflammation as well as in angiogenesis.<sup>11</sup> The biological effect was judged by a score system (see Table 2). SDS induced a characteristic strongly vascularized granuloma in the CAM with star-like capillaries surrounding the pellet (see Fig. 3(a)). If SDS is applied together with anti-inflammatory test compounds, complete normalization of the membrane irritation is observed (see Fig. 3(b)). LaPSvS1 exhibited a good antiinflammatory effect at a concentration of 50  $\mu\text{g}$ /pellet (see Fig. 4). At this concentration, LaPSvS1 was more potent than the standard antiinflammatory drug diclofenac sodium (50  $\mu\text{g}$ /pellet) ( $P < 0.05$ ) and less effective than phenylbuta-

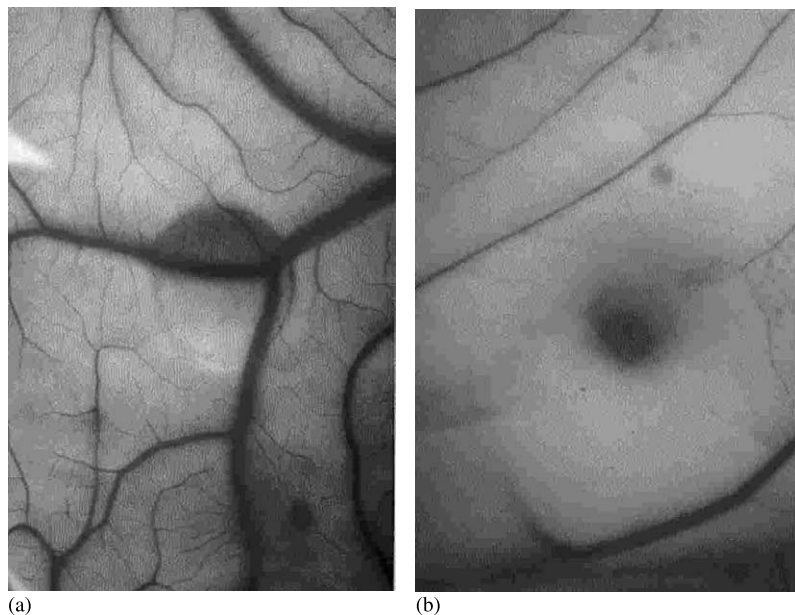


Fig. 1. CAM-assay: (a) shows an untreated CAM (2.5% agarose-solution) corresponding to score 0; (b) shows the best antiangiogenic effect (score 2) with a capillary free area around the pellet.

zone (50  $\mu\text{g}/\text{pellet}$ ) ( $P < 0.05$ ). There was no significant difference in potency compared to hydrocortisone (50  $\mu\text{g}/\text{pellet}$ ). None of the tested compounds showed any side effects.

FGF-2 is one of the 19 heparin-binding growth factors of the FGF family<sup>12</sup> which stimulates endothelial cell proliferation in vitro and angiogenesis in vivo.<sup>13</sup> FGF-2 exerts its biological effects after interaction with so-called low-affinity receptors (glycosaminoglycans, e.g., heparin, heparan sulfates) at the cell surface required for the binding to its high-affinity receptor tyrosine kinases.<sup>14</sup> The in vivo effects of FGF-2 are controlled by the interplay of free and cell-associated glycosaminoglycans. To examine the influence of LaPSvS1 on FGF-2 induced angiogenesis, the used CAM-assay had to be altered. Instead of agarose pellets—the preparation of which includes a temporary thermal load and therefore is not suitable for proteins—small gelatinized rings of agarose, either with a solution of FGF-2 in PBS (50 ng) with or without LaPSvS1 (50  $\mu\text{g}$ ) or PBS alone as blank, were applied to the CAMs of the 7 day-old chicken embryos. After 72 h of incubation (see Fig. 5), FGF-2 treatment led to a network-like growth of sprouting capillaries and increased vessel density within the ring (64% stimulation). No changes in vascular structure or density were observed with PBS. In contrast, LaPSvS1 (50  $\mu\text{g}$ ) clearly inhibited angiogenesis in the FGF-2-CAM-assay resulting in large capillary free areas within the ring (50% inhibition). One could speculate that LaPSvS1 prevents FGF-2 induced biological effects on endothelial cells by interfering with the growth factor/low affinity receptor interaction.

To study the interaction of LaPSvS1 with FGF-2, two different in vitro assays were performed. Sulfated carbohydrates like heparin are able to interact with growth factors, e.g., FGF-2. This keeps the growth factor in a biological active form in the ECM by protecting it from proteolytic cleavage and is the precondition for the interaction of the growth factor with its receptor tyrosine kinase.<sup>14</sup> The FGF-2-trypsin-assay indicates the binding of a test compound to FGF-2. LaPSvS1 exhibited a dose dependent protective effect against the tryptic cleavage of FGF-2. With increasing amounts of LaPSvS1 the band intensity of FGF-2 rose (see Fig. 6, lane 2–7), meaning that less FGF-2 was degraded because of being protected by the bound galactan sulfate. Without LaPSvS1, FGF-2 was completely degraded by trypsin (see Fig. 6, lane 1). To exclude the

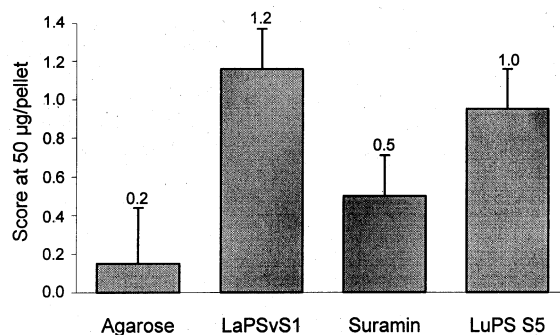


Fig. 2. Score values of the (1→3)- $\beta$ -galactan LaPSvS1 (50  $\mu\text{g}/\text{pellet}$ ) in the CAM-assay with suramin and the (1→4)- $\beta$ -galactan LuPS S5 as standard. Score  $< 0.5$ , no antiangiogenic effect; score 0.5–1, weak to medium antiangiogenic effect; score  $\geq 1$ , medium to strong antiangiogenic effect (\* $P < 0.05$  vs. agarose, \*\* $P < 0.05$  vs. suramin).

Table 2

Score values for the evaluation of the antiinflammatory effect on the chorioallantoic membrane of the fertilized hens' eggs

Category	Type	Effects observed
1	irritated	the granuloma is strongly vascularized
2	weakly irritated	a network of capillaries is formed starlike around the granuloma the granuloma is poorly vascularized
3	weakly normalized	a thin network of capillaries is formed starlike around the granuloma the granuloma is somewhat smaller than in category 1 and 2 and only poorly vascularized
4	normalized	the starlike network of vessels is hardly recognizable no granuloma or only a kind of “scar” can be observed (if the granuloma regresses a non-vascularized scar is left) the network of vessels is normal (as the control)

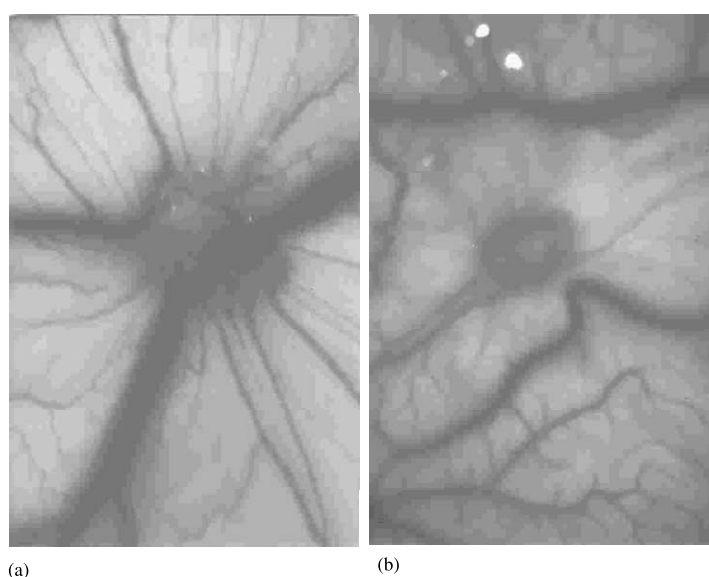


Fig. 3. HET-CAM-assay: (a) shows the CAM-irritation caused by SDS (50 µg/pellet); (b) shows the inhibition of the membrane irritation by LaPSvS1 (50 µg/pellet).

possibility that an inhibition of trypsin by LaPSvS1 was responsible for the observed effects, FGF-2 was denatured by heat thus destroying the integrity of the tertiary structure and preventing the binding of the (1 → 3)-β-galactan sulfate to FGF-2. Denatured FGF-2 was completely cleaved by trypsin in the presence of LaPSvS1 demonstrating that LaPSvS1 did not inhibit trypsin (data not shown).<sup>15</sup> Moreover, a time dependent inhibition of the proteolytic degradation of FGF-2 in the presence of LaPSvS1 similar to heparin was observed (see Fig. 7). The two–threefold higher  $IC_{50}$  value of LaPSvS1 (0.33 µg/mL) in contrast to that of heparin (0.14 µg/mL) leads to the conclusion that the affinity of LaPSvS1 to FGF-2 is lower than that of heparin.

More detailed information about site and strength of the interaction between sulfated carbohydrates and FGF-2 are obtained by fluorescence measurements. The

fluorescence emission of a single tryptophan residue in the FGF-2 molecule changes upon ligand binding to a cluster of basic residues located close to the tryptophan residue<sup>16,17</sup>. In the range of 40–80 nM LaPSvS1, a dose dependent increase in the fluorescence emission of the single tryptophan residue of the FGF-2 molecule was observed with subsequent decrease (see Fig. 8). An equilibrium dissociation constant  $K_D$  lower than 1 nM revealed a strong binding. Heparin tested under the same conditions had a  $K_D$  lower than 1 nM as well (data not shown). This is in agreement with fluorescence studies of Li & Seddon who determined a  $K_D \approx 1$  nM for the binding of heparin from porcine intestinal mucosa to FGF-2 without observing differences between FGF-2 expressed in *E. coli* or from a commercial source.<sup>17</sup> Additionally, published  $K_D$  values of heparin or heparin derivatives obtained by affinity

coelectrophoresis<sup>18</sup> and by analysis of FGF-2 bound to cell associated or isolated heparan sulfate proteoglycans<sup>19,20</sup> confirm our results.

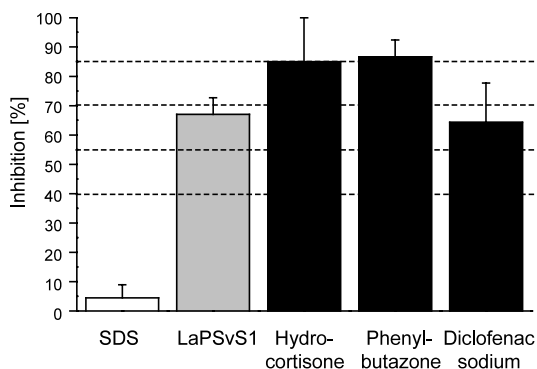


Fig. 4. Inhibition of the SDS induced inflammation by the (1 → 3)-β-galactan sulfate LaPSvS1 (50 μg/pellet) in the HET-CAM-assay with hydrocortisone, phenylbutazone and diclofenac sodium as standards. Inhibition < 40%, no antiinflammatory effect; inhibition 40–55%, uncertain antiinflammatory effect; inhibition 55–70%, weak antiinflammatory effect; inhibition 70–85%, good antiinflammatory effect; inhibition > 85%, strong antiinflammatory effect (\**P* < 0.05 vs. SDS).

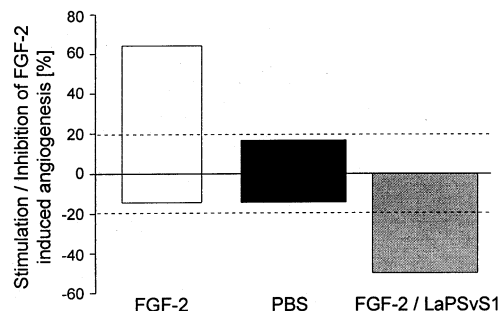


Fig. 5. Inhibition of the FGF-2 induced angiogenesis by the (1 → 3)-β-galactan sulfate LaPSvS1 (50 μg) in the CAM-assay. FGF-2 (50 ng) and PBS were used as positive control respectively blank. Positive or negative effects up to 20% corresponding to stimulation or inhibition of FGF-2 induced angiogenesis were considered as no activity.

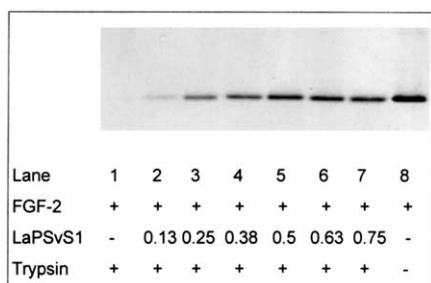


Fig. 6. Dose dependent protective effect of LaPSvS1 (final concentration: 0.13–0.75 μg/mL) in the FGF-2-trypsin-assay after incubation for 7 h. Without LaPSvS1, FGF-2 was degraded by trypsin (lane 1).

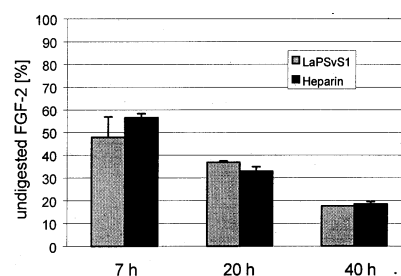


Fig. 7. Time dependent protective effect of LaPSvS1 in the FGF-2-trypsin-assay compared to heparin. The percentage of undigested FGF-2 was determined.

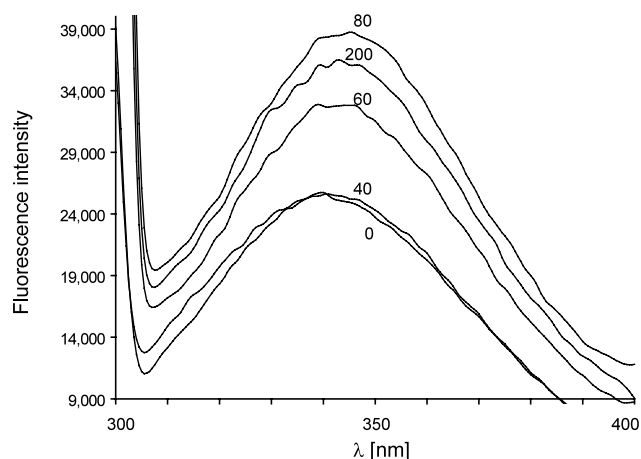


Fig. 8. Fluorespectrometric analysis: The different fluorescence spectra of FGF-2 show the change in the fluorescence emission of the protein solution by addition of LaPSvS1. The excitation wavelength was 290 nm and a maximum emission wavelength at 340 nm was observed. Concentration of LaPSvS1 in nM is given close to the respective spectra. (For a clear presentation a reduced number of data points is shown.)

The differences in the resulting FGF-2 binding affinities of LaPSvS1 and heparin in the two in vitro assays may be explained with possible additional contact points in the protein structure for heparin. Since changes of the intrinsic fluorescence of the tryptophan residue in the FGF-2 molecule are only detected by binding of a ligand to specific basic amino acids close to this residue, LaPSvS1 and heparin were shown to bind to this cluster with identical affinity. On the other hand, the proteolytic digestion is prevented only if the sulfated carbohydrates bind to FGF-2 in a way that the access for trypsin to the numerous theoretical possible cleavage points is at least partially prevented. Therefore, LaPSvS1 might exert its antiangiogenic and anti-inflammatory effects via the interaction with FGF-2.

In conclusion, this study demonstrates that the (1 → 3)-β-galactan sulfate LaPSvS1 is a potent inhibitor of natural and FGF-2 stimulated angiogenesis and inflammation in an in vivo model presumably by interacting with the FGF-2-system.

### 3. Experimental

**Materials.**—FGF-2 (fibroblast growth factor 2) was obtained from Strathmann Biotech GmbH. Arabino-galactan, trypsin type IX and heparin (sodium salt, from bovine intestinal mucosa) were from Sigma. All other chemicals were of analytical reagent grade (E. Merck Eurolab GmbH).

#### General methods

**Preparation of the galactan.** The AG of *L. decidua* Miller (25.12 g) was dissolved in 500 mL 0.005 N HCl and boiled. Degradation was controlled until GPC indicated that the molecular weight of the main peak decreased from 12 to 8 kDa (43 h). After cooling, the solution was neutralized, dialyzed (6 days; MWCO, 2 kDa) and lyophilized (yield, 8.5 g LaPSv).

**Sulfation of the galactan**<sup>7</sup>.—LaPSv (100 mg) was soaked in dry DMF (2 L). The sulfating agent SO<sub>3</sub>–pyridine was dissolved in 2.5 mL DMF and mixed with the polysaccharide. For every mol SO<sub>3</sub>–pyridine, 1 mol of pyridine was added to the mixture. The reaction was carried out with stirring under Ar atmosphere at 90 °C for 4 h. After cooling to rt, 25 mL of water was added with stirring and then the solution was adjusted to pH 10 with 1 N NaOH. The sulfated polysaccharide was precipitated with EtOH, redissolved in water and dialyzed (7 days; MWCO, 3500 Da) against alkaline water (pH 9, adjusted with NaOH) to remove pyridine. Finally the sulfated polysaccharide (LaPSvS1) was dialyzed against water and lyophilized.

**Molecular weight distribution.**—The hydrodynamic volume of LaPSv and the corresponding sulfate LaPSvS1 was determined by GPC using a Superose 12<sup>®</sup> column, 0.1 M NaCl as eluent and detection by refractive index. The system was calibrated with commercially available pullulan standards.<sup>21</sup>

**Sulfate content**<sup>7</sup>.—LaPSvS1 (1 mg) was hydrolyzed in 2 M TFA (1 mL/1 h/120 °C/100 kPa). After cooling to rt, 2 mL water were added and the solution was freeze-dried. The residue was dissolved in 2.0 mL of water, centrifuged and analyzed by ion-chromatography (IC-Pak Anion, 4.6 × 50 mm, (Waters), eluent: borate–gluconate buffer, conductivity detector). A calibration curve was measured for every analysis. All determinations were made in triplicate.

**Constituent sugars.**—The relative amounts of the constituent sugars were determined by GLC of the derived alditol acetates using the MMB-method of Stevenson & Furneaux<sup>22</sup> (HP 5890 equipped with a fused silica capillary column (FS-OV-225, 25 m, ID 0.25 mm), carrier gas: helium (1.0 mL/min, split 1:50), oven: 230 °C, injector: 280 °C, detector: FID, 290 °C).

The constituent sugar for LaPSv and LaPSvS1 was galactose represented by hexaacetylgalactitol in the GC-profile.

**Methylation analysis**<sup>22</sup>.—LaPSv was dissolved in Me<sub>2</sub>SO and methylated with CH<sub>3</sub>I and freshly prepared Dimethyl-K. Then 3 mL of 2:1 CH<sub>2</sub>Cl<sub>2</sub>–MeOH, 2 mL of water and some crystals of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> were added to the test tube. After mixing, the organic layer was washed four times with 2 mL of water, mixed with 2 mL of 2,2-dimethoxypropane and 20 µL of glacial AcOH and evaporated under a stream of nitrogen. The methylated polysaccharide was then converted into PMAA's according to the MMB-method of Stevenson & Furneaux.<sup>22</sup> The PMAA's were analyzed on a HP 5890 A gas-chromatograph with a mass-selective detector (HP 5970 B) (carrier gas: helium (1.0 mL/min), column: bonded phase fused silica capillary column (SP 2380, 30 m/0.25 mm), temperature-program: 140–180 °C (20 °C/min), 180–240 °C (5 °C/min), 240 °C (10 min isotherm)).

Molar ratio of PMAA (LaPSv): 2:1.6:1:2 = 2,3,4,6-tetramethyl-1,5-diacetylgalactitol (1-Gal):2,4,6-trimethyl-1,3,5-triacetylgalactitol (1,3-Gal):2,3,4-trimethyl-1,5,6-triacetyl-galactitol (1,6-Gal):2,4-dimethyl-1,3,5,6-tetramethylgalactitol.

**Performance of the CAM-assay**<sup>3,4,6</sup>.—Test compounds were dissolved in a 2.5% agarose-solution (final concentrations: 5 mg/mL). All these preliminary steps were performed at approximately 60 °C. For the preparation of the pellets, 10 µL of these solutions were applied dropwise on circular Teflon supports (Ø 3 mm) and then cooled to rt. As positive control, suramin at a concentration of 50 µg/pellet was applied. As blank, CAMs treated with agarose-solution were included only. The preparation of the fertilized hens' eggs was performed according to Refs. 3,4,6. After an incubation period of 65–70 h at 37 °C at a relative humidity of 80%, the eggs were opened and returned to the incubator for additional 75 h. When the formed chorioallantoic membrane (CAM) had approximately a diameter of 2 cm, one pellet (50 µg test compound) was placed on it. The eggs were incubated for 24 h and then evaluated under the stereo microscope. For each test compound, 12 eggs were utilized. Each experiment was performed in duplicate. For the evaluation of the antiangiogenic effect, a score system was used (see Table 1). Score < 0.5, no antiangiogenic effect; score 0.5–1, weak to medium antiangiogenic effect; score ≥ 1, medium to strong antiangiogenic effect (details see Ref. 3).

**Performance of the HET-CAM-assay**<sup>23,24</sup>.—The HET-CAM-assay was performed according to the CAM-assay, with the modification that each test compound was dissolved in a 2.5% agarose-solution containing 0.5% SDS as inflammatory agent (final concentration: 5 mg/mL). As negative control, CAMs treated with only agarose-solution containing 0.5% SDS were included. For each test compound, 14 eggs were utilized. Each experiment was performed in dupli-

cate. The evaluation was performed by using a score system (see Table 2), followed by the conversion of the score index in the proportional inhibition of inflammation: inhibition <40%, no antiinflammatory effect; inhibition, 40–55%: uncertain antiinflammatory effect; inhibition 55–70%: weak antiinflammatory effect; inhibition 70–85%: good antiinflammatory effect; inhibition >85%: strong antiinflammatory effect (details see Ref. 25).

**Statistical analysis.**—All results are expressed as mean values. For the statistical analysis, the SPSS for WINDOWS package was used. A 5% level of significance was selected. Evaluation was done using the non-parametric Mann–Whitney U (two independent samples).

**Performance of the FGF-2-CAM-assay.**—The preparation of the fertilized hens' eggs was performed according to Refs. 3,4,6. On day seven, rings of a gelatinized 5% agarose-solution with an inner diameter of 4 mm and a total diameter of 5 mm were placed on the CAM near, but not on top of a large blood vessel. Directly, 10  $\mu$ L of FGF-2 (50 ng) in PBS pH 7.4 with or without LaPSvS1 (50  $\mu$ g) was pipetted into the agarose ring. To exclude unspecific effects, some eggs were tested with PBS pH 7.4 alone. The eggs were incubated for 72 h at 37 °C and a relative humidity of 80%. Evaluation of the growing capillaries was done with a stereo microscope. Stimulation/inhibition of angiogenesis was given as percentage of eggs with enhanced/reduced capillary growth and density. For each experiment, a minimum of 12 eggs were used.

**FGF-2-trypsin-assay<sup>6</sup>.**—Recombinant, human FGF-2 solution was incubated with LaPSvS1 or heparin as standard compound and trypsin at 37 °C for 7, 20 and 40 h. Tests with water instead of test compound and FGF-2 alone served as controls. To exclude inhibition of the trypsin by the test compound, controls with denaturated FGF-2 (65 °C, 5 min) were made. At the end of the trypsin digestion, a concentrated buffer was added, then the sample was subjected to SDS/PAGE in a Tris–glycine-running buffer. The amount of undigested protein in a given lane was determined after silver staining by a densitometer (GS 710, Calibrated Imaging Densitometer, BIO RAD) (for details see Ref. 6). The IC<sub>50</sub> values were calculated from the dose response curve by sigmoidal fitting using software MICROCAL™ ORIGIN™ Version 4.1, Fa. Microcal Software, Inc., Northampton.

**Fluorespectrometric analysis<sup>17</sup>.**—Measurements were conducted at 25 °C using a Perkin–Elmer LS 50 B Luminescence spectrometer. The excitation and emission wavelengths were 290 and 340 nm, respectively. Small aliquots of the LaPSvS1 solution (1–20  $\mu$ L) were added to a 2 mL solution of FGF-2 (450 nM) in a Tris–HCl-buffer (pH 7.5). Fluorescence intensity was recorded after mixing and a subsequent 2 min incubation. To determine the equilibrium dissociation con-

stant ( $K_D$ ), the titration curve of a series of seven data points was fitted by non-linear regression according to Eq. (1)<sup>26</sup> using software MICROCAL™ ORIGIN™ Version 4.1, Fa. Microcal Software, Inc., Northampton.

$$F_0 = F_1 +$$

$$\frac{\Delta F([P]_t + K_D + [L]_t) - ([P]_t + K_D + [L]_t)^2 - 4[P]_t[L]_t^{0.5}}{2[P]_t} \quad (1)$$

$F_0$ , observed fluorescence intensity;  $F_1$ , initial fluorescence intensity;  $\Delta F$ , difference between initial and final fluorescence intensities;  $[P]_t$ , total concentration of the protein (FGF-2);  $[L]_t$ , total concentration of the carbohydrate ligand.

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