ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 330 (2001) 427–430

CARBOHYDRATE RESEARCH

Note

# Sulfated β-(1→4)-galacto-oligosaccharides and their effect on angiogenesis

Christian W. Käsbauer, Dietrich H. Paper,* Gerhard Franz

*Institute of Pharmaceutical Biology, University of Regensburg, Universitätstrasse 31, D-93040 Regensburg, Germany*

Received 11 July 2000; received in revised form 20 November 2000; accepted 20 November 2000

**Abstract**

Sulfated β-(1→4)-galacto-oligosaccharides were prepared from an arabino-galacto-rhamno-galacturonan from *Lupinus polyphyllus* Lindl. by successive partial hydrolysis and $SO_3$–pyridine sulfation in DMF. The resulting oligosaccharide polysulfates were analyzed by analytical GPC and the sulfate content was determined by ion chromatography. DP 5 and higher showed a pronounced antiangiogenic effect with scores of 0.9–1.2 for DP 7–9 using the CAM-assay. An interaction with the fibroblast growth factor FGF-2 was noticed for DP 4–12 depending on the degree of sulfation using the FGF-2-trypsin assay. 

*Keywords:* Angiogenesis inhibitor; CAM-assay; FGF-2, Galactan; Sulfated galactooligosaccharides

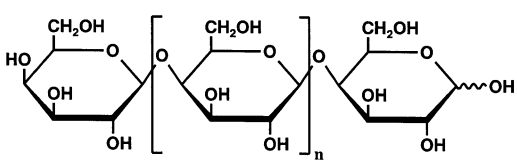
## 1. Introduction

Angiogenesis is a multi-step process leading to the formation of new capillaries, emerging from a pre-existing blood vessel system. Any imbalance in the control of this complex system may promote numerous angiogenesis dependent diseases such as cancer, rheumatoid arthritis or diabetic retinopathy. Not only heparins but also carrageenans and semi-synthetic sulfated polysaccharides like laminarin sulfate were shown to be potent angiogenesis inhibitors.[1–3] Because of the heterogeneity of these polymers, low molecular weight galactan sulfates were synthesized starting from defined oligosaccharides derived from a β-(1→4)-galactan obtained from *Lupinus polyphyllus* Lindl. and were tested in vivo and in vitro to study the dependence of the angiostatic effect on the degree of polymerization.

*Abbreviations:* Ara-Gal, arabino-galactan; CAM, chorioallantoic membrane; DS, degree of sulfation; FGF-2, fibroblast growth factor 2; Lu, oligosaccharide derived from a β-(1→4)-galactan; LuPS, β-(1→4)-galactan derived from a *Lupinus polyphyllus* Lindl. polysaccharide; PBS, phosphate buffered solution; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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

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




Scheme 1. Structure of the oligosaccharides derived from the galactan of *L. polyphyllus* Lindl. **Lu3**: $n=1$, **Lu4**: $n=2$, **Lu5**: $n=3$, **Lu6**: $n=4$, **Lu8**: $n=7$–9 (mixture).

0008-6215/01/$ - see front matter 
PII: S0008-6215(00)00305-0

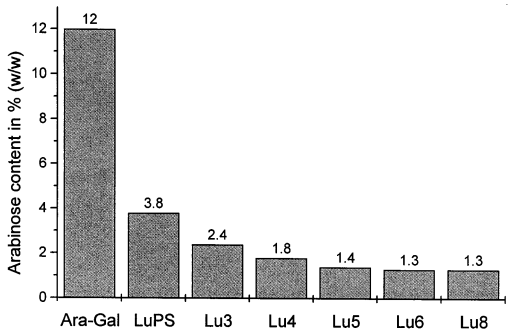



Fig. 1. Arabinose content of the polymer educts arabinogalactan (Ara-Gal), galactan (LuPS) and the oligomers from LuPS (**Lu3**…**Lu8**, DP = 3…7–9).

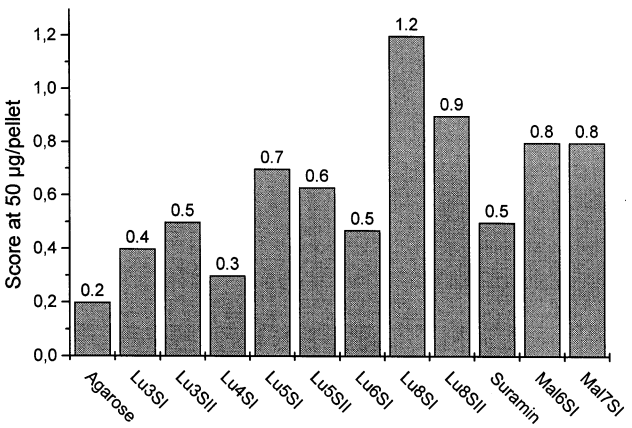



Fig. 2. Score values of the sulfated oligosaccharides (50 µg/pellet) in the CAM-assay with suramin as standard. Score $< 0.5$: no antiangiogenic effect; Score $\geq 0.5$: weak to strong antiangiogenic effect.

Table 1
DS (100% means sulfation of all hydroxyl groups) and hydrodynamic volumes of the sulfated galacto-oligosaccharides and maltohexaose and -heptaose

| | DS (%) | Hydrodynamic volume (kDa) |
|---|---|---|
| **Lu3SI** | 64 | 3.2 |
| **Lu3SII** | 64 | 3.4 |
| **Lu4SI** | 57 | 4 |
| **Lu5SI** | 59 | 4.8 |
| **Lu5SII** | 59 | 4.4 |
| **Lu6SI** | 57 | 5.4 |
| **Lu8SI** | 35 | 5.15 |
| **Lu8SII** | 58 | 6 |
| **Mal6SI** | 55 | 5.4 |
| **Mal7SI** | 50 | 6 |

## 2. Results and discussion

Oligosaccharides (DP 3–6 and a mixture of DP 7–9) (see. Scheme 1) were isolated after partial hydrolysis of the β-(1→4)-galactan (LuPS) of lupine seeds.[4] Although the glycosidic bond of the arabinose residues should be very sensitive against acid hydrolysis, small amounts of arabinose were still detectable in the oligosaccharide fractions (see Fig. 1). However, the adhering arabinose residues could not be substantiated in NMR-studies (data not shown). Due to the galactose content higher than 95%, the obtained oligosaccharides were considered as pure galactans.

Sulfation was carried out by a modified method according to Ochi et al.[1] with 2 (**-SI**) resp. 4 (**-SII**) equiv of $SO_3$. Under the chosen conditions, no complete sulfation of all free hydroxyl groups of the oligosaccharides was achieved. The hydrodynamic volumes (see Table 1) increase with the chain length and the degree of sulfation. The DS, calculated from the sulfate analysis, varied from 35 to 64% of full sulfation (see Table 1).

In previous investigations, it was shown that sulfated β-(1→4)-galactans derived from the arabinogalactan of lupine seeds have a good antiangiogenic effect in the CAM-assay (score 1), which was independent of the hydrodynamic volume (molecular weight) in a range between 10 and 80 kDa.[4] For other sulfated carbohydrates such as the heparins, it was stated that a minimum chain length of heparin fragments is required to demonstrate any antiangiogenic effect.[1,5–7]

Like in the case of heparin fragments, for the antiangiogenic effect of the sulfated galacto-oligosaccharides, a minimum chain length is required. **Lu3SI**, **Lu3SII** and **Lu4SI** with DP 3 resp. DP 4 showed no or only a weak antiangiogenic effect comparable to suramin (score 0.5), a weak angiogenesis inhibitor, which has been used in clinical trials[8,9] (see Fig. 2). The sulfated maltohexaose and -heptaose (**Mal6SI** and **Mal7SI**) which were reported to be oligosaccharides with antiangiogenic activity were used as positive controls.[10,11] At a chain length of five galactose units and higher, there is a pronounced antiangiogenic effect.

The long chain oligosaccharide sulfates **Lu8SI** and **Lu8SII** (DP 7–9) showed the best effects with score values at 0.9–1.2, although **Lu8SI** has a low DS. The sulfated maltohexaose **Mal6SI** and maltoheptaose **Mal7SI** were similarly active.

The FGF-2-trypsin-assay indicates whether a test compound binds to FGF-2 or not. The test is a modified version of the procedure according to Refs. 12,13 but needs lower amounts of FGF-2. A high band intensity means that less FGF-2 was degraded, because of being protected by a bound oligosaccharide sulfate.

The relative band intensity is the relation between the band intensity of the FGF-2 with, and that without test compound and trypsin. This experiment demonstrated that the galactan sulfates, especially **Lu6SI** and **Lu8SII** protect FGF-2 against proteolytic degradation (see Fig. 3). Therefore, these oligosaccharides might act as angiogenesis inhibitors via a possible interaction with the FGF-2-system.

In conclusion, this study reveals that defined sulfated β-(1→4)-galacto-oligosaccharides with a low degree of polymerization (even pentasaccharides) are potent angiogenesis inhibitors with an influence on FGF-2.

## 3. Experimental

*Materials.*—Basic fibroblast growth factor was from Strathmann Biotech GmbH and maltohexaose, maltoheptaose from Senn Chemicals. Trypsin Type IX was from Sigma.

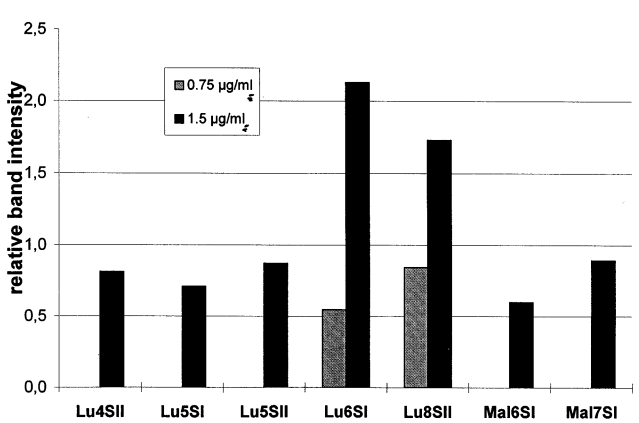



Fig. 3. Relative band intensities of the FGF-2 in the FGF-2-trypsin-assay.

*General methods*

*Sulfate content.* The sulfated oligosaccharide (2 mg) was hydrolyzed in 2 M TFA (1 mL, 1 h, 120 °C, 1 bar). After cooling, 2 mL water were added and the solution was freeze dried. The residue was dissolved in 2000 µL water and analysed by ion-chromatography (IC-Pak Anion, 4.6 × 50 mm, (Waters), eluent: borate–gluconate buffer)

*Molecular weight distribution.* The hydrodynamic volumes were determined by gel permeation chromatography on a Superdex™ S 75 column (30 cm) with 0.1 M NaCl as eluent and detection by refractive index. Linear pullulans were used as standards.

*Constituent sugars.* The relative proportions of the constituent sugars were determined by GC of the acetylated alditols according to Ref. 14.

*Production of the oligosaccharides.* The raw arabino-galacto-rhamnogalacturonan of *L. polyphyllus* Lindl. seeds was extracted and further modified to give a β-(1→4)-galactan according to Ref. 4. The galactan (3.00 g) was refluxed in 0.1 M HCl (60 mL) for 1 h. The mixture was neutralized (0.1 M NaOH) and water was added to 200 mL. This solution was dropped in EtOH (99%, 1130 mL). After centrifugation, the supernatant was concentrated under reduced pressure and then freeze dried.

Fractionation of the mixture was done by GPC on Biogel P-2 at 50 °C (140 × 2.4 cm, 40 mL/h) with water as eluent and detection by refractive index.

*Sulfation of the oligosaccharides.* Oligosaccharides (100–200 mg) were dissolved in dry DMF (5–10 mL) under an Ar atmosphere and dried with molecular sieve (0.4 g). $SO_3$–pyridine (2 or 4 equiv of OH) was added and the solution was stirred for 3 h at 60 °C. After pouring the solution off, the highly viscose residue was dissolved in water with ice-cooling. Immediately, the pH of the solution was adjusted to 8.0 and then the water and the formed pyridine were evaporated under diminished pressure. The residue was dissolved in water and the solution was lyophilized.

Purification of the sulfated oligosaccharides was carried out by GPC on a Sephadex G-10 column (100 × 1.5 cm, 18 mL/h) with water as eluent and detection by conductivity.

*Performance of the CAM-assay*[3]. Every test compound was dissolved in a 2.5% agarose-solution (final concentration: 5 mg/mL). All these preliminary steps were performed at approximately 60 °C. For the preparation of the pellets, 10 μL of the solution was applied on circular Teflon supports (∅ 3 mm) and then cooled to rt.

Fertilized hens' eggs were incubated for 65–70 h at 37 °C and a relative humidity of 80%. The eggs were positioned in a horizontal position and rotated several times. Then the eggs were opened on the snub side. Before opening, 10 mL of albumin were sucked off through a hole on the pointed side. At two thirds of the height (from the pointed side), every egg was traced with a scalpel and the shell top was removed with forceps. The aperture (cavity) was covered with keep-fresh film and the eggs were incubated at 37 °C at a relative humidity of 80% for 75 h. If 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 one further day and then evaluated under the stereomicroscope. For every test compound, 15–20 eggs were utilized. For the evaluation of the antiangiogenic effect, a score system was used. As positive control, suramin (50 μg/pellet) was tested. As blank, CAMs treated with only agarose-solution were included. Score $< 0.5$: no antiangiogenic effect; Score $\geq 0.5$: weak to strong antiangiogenic effect (details see Ref. 3).

*FGF-2-trypsin-assay*. Recombinant, human FGF-2 solution (4.5 μL, 50 μg in 1 mL PBS (pH 7.4) with 0.5% Tween 20) was incubated with the test compound solution (4.5 μL, 0.75 or 1.5 μg/mL) and freshly prepared trypsin solution (4,5 μL, 1 μg/mL in 25 mM Tris–HCl (pH 7.5)) for 8 h. Tests with water instead of the test compound and FGF-2 alone served as controls. To exclude inhibition of the trypsin by the test compound, controls with partially denaturated FGF-2 (65 °C, 1 min) were made. After incubation, 4.5 μL of a concentrated electrophoretic sample buffer (2.5 mL 1.5 M Tris–HCl pH 6.8, 6.0 mL glycerine, 1.20 g SDS, 0.5 mL bromophenol blue 0.3%, water ad 10 mL, soluble at 40 °C) was added. Electrophoresis was performed according to Refs. 15,16 with SDS-glycine gels at 16% acrylamide (100 × 100 × 1 mm), 12 μL sample, 125 V const. 100 min, 170 V const. 20 min, (NOVEX Xcell II mini cell).

## References


1. Ochi, K.; Watanabe, Y.; Okui, K.; Shindo, M. *Chem. Pharm. Bull.* **1980**, *28*, 638–641.
2. Paper, D. H.; Vogl, H.; Franz, G.; Hoffman, R. *Macromol. Symp.* **1995**, *99*, 219–225.
3. Paper, D. H.; Vogl, H.; Franz, G. In *Relevance of Tumor Models for Anticancer Drug Development*, *Contributions to Oncology*; Fiebig, H. H.; Burger, A. M., Eds. Low-molecular-weight galactan sulfates as angiogenesis inhibitors. Karger-Verlag: Basel, 1999; Vol. 54, pp. 191–199.
4. Vogl, H.; Paper, D. H.; Franz, G. *Carbohydr. Polym.* **2000**, *41*, 185–190.
5. Maccarana, M.; Casu, B.; Lindahl, U. *J. Biol. Chem.* **1993**, *268*, 23898–23905.
6. Faham, S.; Linhardt, R. J.; Rees, D. C. *Curr. Opin. Struct. Biol.* **1998**, *8*, 578–586.
7. Svahn, C. M.; Weber, M.; Mattson, C.; Neiger, K.; Palm, M. *Carbohydr. Polym.* **1992**, *18*, 9–16.
8. Braddock, P. S.; Hu, D. E.; An, T. P.; Stratford, I. J.; Harris, A. I.; Bicknell, R. *Br. J. Cancer* **1994**, *69*, 890–898.
9. Gradishar, W. J. *Investig. New Drugs* **1997**, *15*, 49–59.
10. Parish, C. R.; Freeman, C.; Brown, K. J.; Francis, D. J.; Cowden, W. B. *Cancer Res.* **1999**, *59*, 3433–3441.
11. Foxall, C.; Wei, Z.; Schaefer, M.; Casabonne, M.; Fugedi, P.; Peto, C.; Castellot, J. J.; Bradley, B. *J. Cell. Physiol.* **1996**, *168*, 657–667.
12. Sommer, A.; Rifkin, D. B. *J. Cell. Physiol.* **1989**, *138*, 215–220.
13. Coltrini, D.; Rusnati, M.; Zoppetti, G.; Oreste, P.; Isacchi, A.; Cacci, P.; Bergonzoni, L.; Presta, M. *Eur. J. Biochem.* **1993**, *214*, 51–58.
14. Stevenson, T. T.; Furneaux, R. H. *Carbohydr. Res.* **1991**, *210*, 277–298.
15. Lämmli, U. K. *Nature* **1970**, *227*, 680–685.
16. Westermeier, R. *Elektrophorese praktikum*; VCH: Weinheim, 1980; pp. 167–186.