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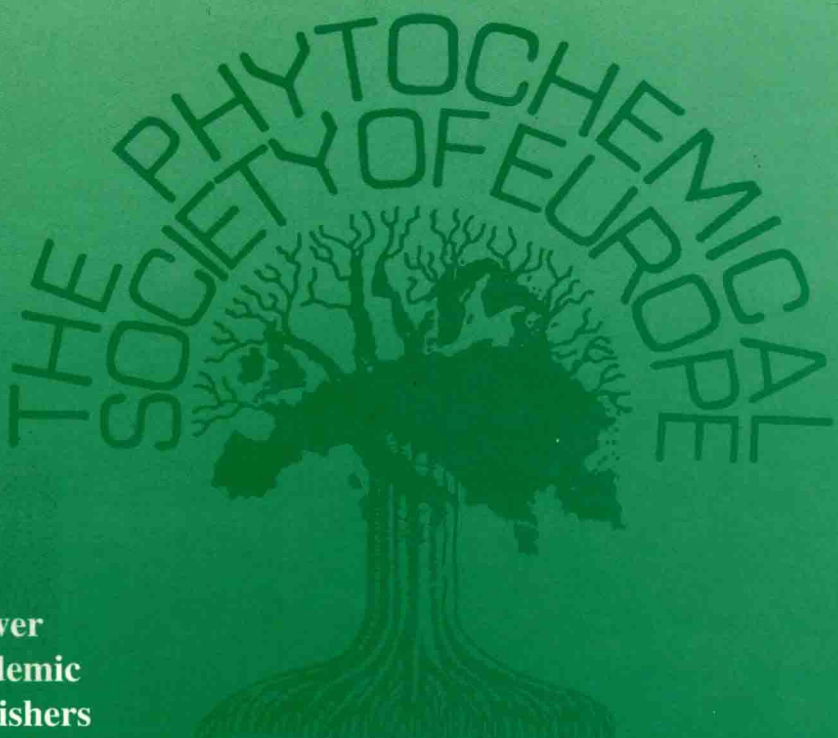
Bioactive Carbohydrate Polymers

Edited by Berit S. Paulsen



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Bioactive Carbohydrate Polymers

Edited by

Berit S. Paulsen

University of Oslo,
School of Pharmacy,
Oslo, Norway

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Volume 44

The titles published in this series are listed at the end of this volume.

Contributors

S. Alban; Institute of Pharmacy, University of Regensburg, Universitätsstrasse 31, 93040 Regensburg, Germany

C. Boisson-Vidal; Laboratoire de recherches sur les Macromolecules, CNRS, UMR 7540, Institute Galilee, Universite Paris-Nord, Av. J.B.Clement, 93430 Villateneuse, France

F. Chaubet; Laboratoire de recherches sur les Macromolecules, CNRS, UMR 7540, Institute Galilee, Universite Paris-Nord, Av. J.B.Clement, 93430 Villateneuse, France

L. Chevolot; Laboratoire de Biochimie et Molecules marines, IFREMER, B.P. 1105, Rue de lÍle d`Yeu, 44311 Nantes cedex, France

P.J.H. Daas; Department of Food Technology and Nutritional Sciences, Food Science Group, Bomenweg 2, 6703 HD Wageningen, The Netherlands

R.A. Dalmo; Institute of Marine Biochemistry, Norwegian College of Fishery Science, University of Tromsø, N-9037 Tromsø, Norway

P. Durand; Laboratoire de Biochimie et Molecules marines, IFREMER, B.P. 1105, Rue de lÍle d`Yeu, 44311 Nantes cedex, France

T. Espevik; Institute of Biotechnology and Insitutte of Cancer Research and Molecular Biology, Norwegian University of Science and Technology, N-7005 Trondheim, Norway

T. Flo; Institute of Biotechnology and Insitutte of Cancer Research and Molecular Biology, Norwegian University of Science and Technology, N-7005 Trondheim, Norway

G. Franz; Institute of Pharmacy, University of Regensburg, Universitätsstrasse 31, 93040 Regensburg, Germany

Ø. Halaas; Institute of Biotechnology and Insitutte of Cancer Research and Molecular Biology, Norwegian University of Science and Technology, N-7005 Trondheim, Norway

K. Ingolfsdottir, ; Department of Pharmacy, University of Iceland, Reykjavik, Iceland

J. Jozefonevicz; Laboratoire de recherches sur les Macromolecules, CNRS, UMR 7540, Institute Galilee, Universite Paris-Nord, Av. J.B.Clement, 93430 Villateneuse, France

S. kraus; Insitutte of Pharmacy, Pharmaceutical Biology, University of München, Karlstr. 29, D-80333 München, Germanty

J. Laencina; CEBAS-CSIC & Univeristy of Murcia, Department of Food Technology, 301'00 Murcia, Spain

D. Paper; Institute of Pharmacy, University of Regensburg, Universitätsstrasse 31, 93040 Regensburg, Germany

S.G. Ring; Department of Biochemistry, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

J.A. Robertson; Department of Biochemistry, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

J.M.Ros; CEBAS-CSIC & Univeristy of Murcia, Department of Food Technology, 301'00 Murcia, Spain

P. Ryden; Department of Biochemistry, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

A,B. Samuelsen; Department of Pharmacognosy, School of Pharmacy, University of Oslo, P.O.Box 1068 Blindern, N-0316 Oslo, Norway

H.A. Schols; Department of Food Technology and Nutritional Sciences, Food Science Group, Bomenweg 2, 6703 HD Wageningen, The Netherlands

G. Skjåk-Bræk; Institute of Biotechnology and Insitutte of Cancer Research and Molecular Biology, Norwegian University of Science and Technology, N-7005 Trondheim, Norway

A.G.J. Voragen; Department of Food Technology and Nutritional Sciences, Food Science Group, Bomenweg 2, 6703 HD Wageningen, The Netherlands

H. Wagner; Insitutte of Pharmacy, Pharmaceutical Biology, University of München, Karlstr. 29, D-80333 München, Germanty

H. Yamada; Oriental Medicine Research Center, The Kitasato Insitutte, okyo, 108-8642, Japan

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Preface

This volume is compiled of contributions from 11 scientists presenting either lectures or posters at the International Symposium of the Phytochemical Society of Europe entitled "Bioactive Carbohydrate Polymers" held at Sundøya, outside Oslo, Norway, from 13. to 16. September 1998. Present at the meeting were more than 50 participants from 19 different countries and the meeting provided a good opportunity for those interested in the fieldd of bioactive carbohydrates to meet and discuss future aspects of this growing field of science. The lectures dealt with bioactive polysaccharides from plants traditionally used in Japan and China, USA, Iceland and in other European countries. Substances from micro and macro algae were described, and the uses of the polymers ranged from woundhealing in human to immunestimulation in fish. The lectures covered most aspects of importance for studying biologically active polysaccharides, both chemical, enzymatic, chromatographic as well as various biological test-systems.

I am most grateful to all the contributors to this volume for their efforts both during the meeting and also afterwards in connection with finishing the proceedings from the meeting, and I will also like to thank PSE for wanting this meeting to be arranged in Norway.

Oslo May 1999-09-30
Berit Smestad Paulsen

Contents

Chapter 1

News on immunologically active plant polysaccharides

H. WAGNER and S. KRAUS
Institute of Pharmacy, Pharmaceutical Biology, University of Munich, Karlstr. 29, D-80333 Muenchen, Germany

Key words: polysaccharides, labeling, ELISA, mechanisms, antiinflammatory

Abstract: The medicinal use of plant polysaccharides as immunostimulatory, antitumoral or antiinflammatory agents has been hampered by the lack of bioavailability studies, by the scarce information available on the mechanism of action as well as the lack of studies on structure activity relationships in this class of compounds. Investigations of the possible mechanisms of action behind the noted inflammatory activity of polysaccharides have revealed that, beside influence on the complement cascade, endocrinal functions and cytokine induction, the effect on chemotaxis of leucocytes has to be considered as one important factor. In order to clarify the mechanism in pharmacological and clinical trials binding and resorption studies are needed. As a prerequisite for such studies we have developed methods for radio-, fluorescent- and gold labelling of polysaccharides. We also present the most recent results on production of polyclonal or monoclonal antibodies against polysaccharides, which can be used for the detection and quantification of polysaccharides in biological material and fluids.

1. Introduction

The rational use of polysaccharides isolated from plants as immunostimulatory, antitumoral or antiinflammatory agents was not possible previously, mainly due to:

- problems combined with the isolation and purification of sufficient amounts of pure bioactive polysaccharides in a reproducible manner;

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- unknown structure-activity relationships, as well as lack of information concerning the exact mechanism of action for the noted immunomodulatory/pharmacological activities;
- and lack of information concerning pharmacokinetics and bioavailability after *p.o.* and *parenteral* administration.

Due mainly to the first two problems listed above, major clinical studies with plant polysaccharides have hardly been possible. One exception has been Lentinan, a fungal β-1,3-glucan having antitumoral activity, which has been investigated in some clinical trials. In order to solve problems connected with the isolation and purification of plant polysaccharides, their production by cell cultures has been initiated. As has been proven by our laboratory for *Echinacea* polysaccharides, the use of the biotechnological route is possible [1] and also manageable on a larger scale [2], but has been associated with several handicaps. This is due mainly to the fact that the polysaccharides isolated using this technology differ from those obtained directly from the plant. Plant polysaccharides differed in molecular weight, sugar composition, mode of sugar interlinkages and immunological activities from those isolated using cell cultures. Additionally, large-scale isolation from cell cultures requires a large-scale fermentation equipment with a capacity of 2000 l or more. Hence, the routine biotechnological production of polysaccharides from plant cell cultures on an industrial scale has so far not yet been achieved.

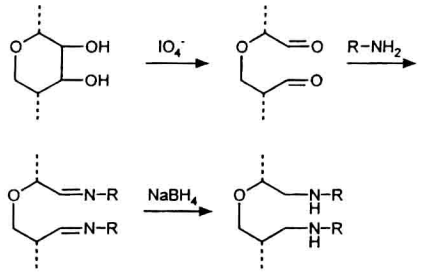
The difficulties concerning studies on mechanisms of action and the bioavailability of polysaccharides after parenteral or oral administration arise from the fact that various mechanisms are possible. Possible targets can be macrophages, T-lymphocytes, NK-cells, the complement cascades or a combination of two or more of these. One possibility to overcome these problems and enable the visualization of interactions between polysaccharides and the target molecules could be the use of labeled polysaccharides and/or specific ELISAs.

2. Labeling of polysaccharides by radio-, fluorescence and gold markers

The major problem concerning the synthesis of labeled bioactive polysaccharides for use in various immunological techniques is to find a suitable method, *i.e.*, a method that does not interfere with the biological activity and allows a highly sensitive detection of the polysaccharide after application to a living organism. With the exceptions of the non-radioactive iodine labeling and a fluorescence labeling procedure for glucans there are very few standard methods available which are applicable on all types of

polysaccharides. In order to tackle this problem we compared methods used for the labeling of other polymers.

Fig. 1a



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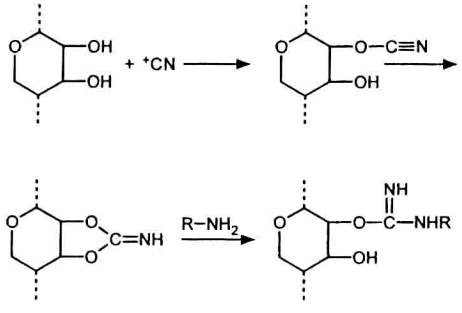

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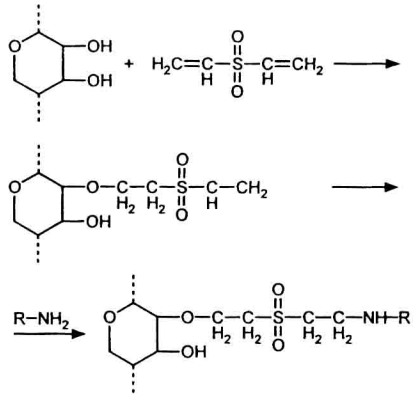

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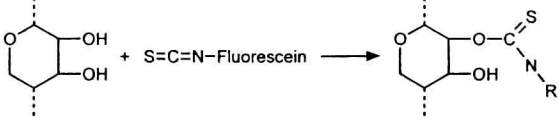

Fig. 1 Different reactions for labeling polysaccharides.

- In the first method hydroxyl groups are oxidized with periodate or dimethylsulfoxide, and the thus generated carbonyl groups transformed into Schiff's bases using an amine-marker, then into secondary amines through reductive amination [3]. This method allows to incorporate fluorescent dyes containing amines or Iodine^{131}-labeled tyramine (Fig. 1, a).
- In a second method, the hydroxyl groups are transferred into bromocyane or divinylsulfone derivatives, respectively, followed by the introduction of amine markers [4,5] (Fig. 1, b,c).
- In a third method, used for labeling proteins and antibodies, fluorescein isothiocyanate is introduced [6] (Fig. 1,d).

The usefulness of a method depends on its level of incorporated markers and to what extent the degradation of the polysaccharide occurs: High level of incorporation facilitates detection, whereas a low degree of alteration of the molecule or degradation ensures that enough free hydroxyls are left for binding reactions. Since Pfitzner and Moffat [7] showed that DMSO can also oxidize monosaccharides, we replaced periodate with DMSO. Using DMSO/acetic anhydride, 5 polysaccharides (2 dextrans, laminarin, larch-arabinogalactan and a neutral *Urtica*-glucan) were oxidized and the resulting carbonyl functions converted into the corresponding Schiff's bases with fluoresceinamin, Rhodamin B-amine and tyramine. Incubation with sodium borohydride produced the desired stable secondary amines. Control experiments with Concanavalin-A-sepharose (for the *Urtica*-glucan and dextran) and *Ricinus communis* agglutinine-agarose (for arabinogalactan) were used in order to investigate whether the obtained fluorescence labeled polysaccharides were still suitable for binding studies, *i.e.*, still contained intact glucan or arabinogalactan units. As confirmed by fluorescence microscopy all fluorescence-labeled polysaccharides bound to Con-A-sepharose were easily detectable. For measurement of tyramine incorporation, UV-absorption at 278 nm was used. After 120 min of incubation, a maximal degree of substitution (1 tyramine molecule per 21 sugar units) was obtained, indicating 5 - 6 tyramine units/arabinogalactan (mol.wt. ca. 20,000 D). Labeling using fluoresceinamine and Rhodamin B-amine was 5 - 6 times less effective, probably due to steric hindrance. When comparing the most optimal methods of oxidation (DMSO and periodate) using HPGPC-chromatography and RI or UV-detection respectively, we found that the elution profile of the product resulting from tyramine incorporation after oxidation with DMSO was not altered in comparison with native arabinogalactan. Labeled arabinogalactan resulting from oxidation with periodate, however, showed a drastic change in molecular

weight distribution, indicating a high level of degradation. In order to investigate in what position the oxidation with DMSO had taken place, and where the molecule had been labelled, the ^{13}C-NMR technique was used. These experiments showed that tyramine incorporation occurred primarily at the terminal C-6 and not at the reducing C-1 end. The third method, FITC-labeling was performed using dry DMSO according to the method of De Belder and Granath [8]. The highest degree of FITC-substitution (1/31) was achieved after 18 hrs of incubation. In the experiments performed by Winchester *et al.* [9] as well as those of Thornton *et al.* [6], the degree of substitution at aqueous condition reported was only 1:>1000. In order to investigate if the bioactivity of a polysaccharide was retained after FITC-labeling granulocyte chemotaxis, experiments were performed using native chemotactic *Urtica*-polysaccharide, UPS I (α,1,4-D-glucan), as well as labeled UPS I [10]. FITC-labeling reduced the chemotactic effect to ca. 55% as compared with the native polysaccharide. Using flow cytometry, we investigated if the FITC-labeled glucan binds specifically to an isolated granulocyte-fraction. Even after addition of a 100-fold excess of non-labeled glucan, it was not possible to displace the fluorescence signal of labeled glucan. Since the binding is not specific, the use of this labeling method will not enable UPS I binding studies. Another method for labeling biomolecules is the use of direct or indirect gold labeling [11,12]. Due to the high electron density of gold, gold conjugates are very suitable for detection with electron microscopy and, after silver enhancement, with light microscopy. For gold-labeling of the polysaccharide we chose an indirect two step method followed by silver enhancement. FITC-dextran was covalently bound to epoxy-active agarose and the FITC-dextran-agarose particle was incubated with anti-FITC-immunoglobulins (Fig. 2). After addition of silver reagent, the labeling was evaluated using an Epipolarisation microscope. The advantage of the use of the latter method, is that it enables the investigation of morphological changes. In judging the various methods for their usefulness for pharmacokinetic studies, we came to the conclusion that most of the methods investigated are only of limited value for the planned studies. In comparison to the fluorescence labeling the gold labeling technique seems to be much more sensitive. One important advantage of the use of fluorescence microscopy for detection is the possibility of evaluating morphological changes.