ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 331 (2001) 203–208

CARBOHYDRATE RESEARCH

Synthesis and characterisation of dextran and pullulan sulphate

Christian Mähner, Manfred Dieter Lechner, Eckhard Nordmeier*

Department of Physical Chemistry, University of Osnabrück, Barbarastrasse 7, D-49069 Osnabrück, Germany

Received 19 July 2000; received in revised form 23 October 2000; accepted 1 December 2000

Abstract

Dextrans and pullulans of different molar masses in the range of 10^4–10^5 g/mol were sulphated via a SO_3–pyridine complex. The degree of substitution achieved was DS = 2.4 and DS = 1.4 for dextran sulphate and DS = 2.0 and DS = 1.4 for pullulan sulphate, respectively. Confirmation of sulphation was given by FTIR spectroscopy. Asymmetrical S=O and symmetrical C–O–S stretching vibrations were detected at 1260 and 820 cm^{-1}. Reactivity of the polysaccharide C-atoms was determined by ^{13}C NMR spectroscopy: For dextran this was C-3 > C-2 > C-4, while for pullulan it was C-6 > C-3 > C-2 > C-4.

Keywords: Dextran sulphate; Pullulan sulphate; NMR spectroscopy; FTIR spectroscopy

1. Introduction

Branched dextran sulphate and linear pullulan sulphate are native polyelectrolytes having several applications, e.g., as anticoagulants or agents for lowering cholesterol. Dextran sulphate has been already successfully applied in the treatment of sepsis, of AIDS, and due to its anti-metastatic effect, in the treatment of human prostatic carcinoma.[1–8] Scientific interest on sulphated polysaccharides was aroused in 1916 by the discovery of heparin,[9] followed by comprehensive work with sulphuric acid esters, particularly with dextran sulphate,[10] Husemann[11] sulphated linear celluloses and polyvinylalcohols. While these substances effectively restrained blood clotting, branched starch sulphates did not. Tsuji[12] showed that both dextran sulphates as well as pullulan sulphate were effective in the treatment of ulcers. However, since dextran was branched while pullulan was not, their activities were different.

Table 1
M_w, M_n and the polydispersity $U = (M_w/M_n) - 1$ for the samples used [a]

	M_w (g/mol)	M_n (g/mol)	U
Dextran 30	30.000	19.000	0.60
Dextran 65	57.000	29.000	0.97
Dextran 130	89.000	43.000	1.08
Dextran 200	105.000	32.000	2.36
Dextran 400	167.000	67.000	1.49
Pullulan 100	95.000	55.000	0.72
Pullulan 120	103.000	75.000	0.37
Pullulan 200	195.000	118.000	0.65
Pullulan 240	231.000	78.000	1.95
Pullulan 500	493.000	192.000	1.57

[a] The GPC measurements were performed in 0.2 M NaCl at 25 °C.

* Corresponding author. Fax: +49-541-9693324.
E-mail address: eckhard@uos.de (E. Nordmeier).

0008-6215/01/$ - see front matter
PII: S0008-6215(00)00315-3

Table 2
Degrees of substitution, determined gravimetrically, DS_g, and volumetrically, DS_v

	DS_g for $T = 65$ °C	DS_v for $T = 65$ °C	DS_g for $T = 30$ °C	DS_v for $T = 30$ °C
DexSul 30	2.42	1.98	1.38	1.34
DexSul 65	2.44	1.98	1.37	1.33
DexSul 130	2.39	2.06	1.42	1.37
DexSul 200	2.40	2.03	1.47	1.43
DexSul 400	2.39	2.02	1.36	1.43
PulSul 100	2.04	2.09	1.33	1.23
PulSul 120	1.99	1.92	1.42	1.25
PulSul 200	1.97	1.96	1.33	1.35
PulSul 240	2.09	1.99	1.44	1.26
PulSul 500	2.09	1.96	1.42	1.28

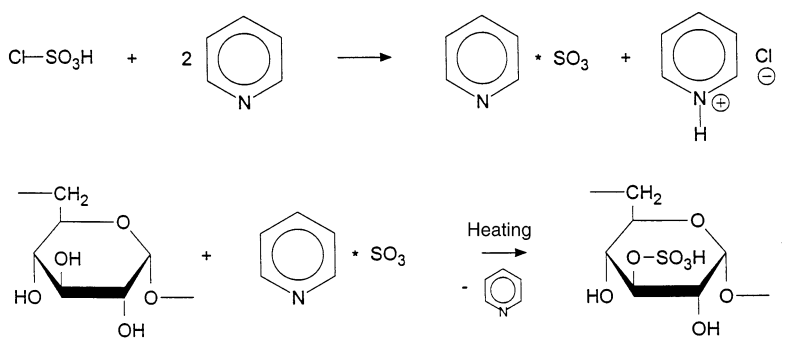

Fig. 1. Polysaccachride sulphation with chlorosulfonic acid and pyridine.

The aims of this paper were twofold: (1) a method is presented for homogeneous sulphation of dextran and pullulan to a sufficiently high degree of substitution; and (2) spectrocopic analysis is presented of the structures of dextran sulphate and pullulan sulphate. To our knowledge this is the first study by NMR and IR spectra of pullulan sulphate.

2. Experimental

Materials.—Dextrans were purchased from Sigma (Deishofen, Germany). Pullulan of molar mass 2×10^5 g/mol was from Bio-Chemicals (Eschwege, Germany) and the pullulan 1×10^5 g/mol from Sigma–Aldrich. The other pullulans were fractionated from a batch of Wacker Chemistry (Munich), following the conventional procedures.[13–16] The molar mass data were proved by GPC and static light scattering (Table 1).

Agents.—Reagents were of analytical grade. The dialysis tubes were from Reichelt (Heidelberg, Germany) and the acid ion exchanger was amberlites IR-120 obtained from Fluka (Neu-Ulm). The apparatus used for the titrations was a titroprozessor 633 combined with a pH glass electrode (6.0233.100) both purchased from Metrohm (Filderstadt, Germany). The results were listed in Table 2 where the subscripts g and v stood for gravimetrically and volumetrically. The average error of measurement was smaller than 1%.

Synthesis.—Dextran and pullulan sulphate were prepared by sulphating dextran and pullulan via a chlorosulphonic acid–pyridine complex as described in Refs. 17–24 (Fig. 1).

For the preparation of the SO_3–lewis base complexes, water-free pyridine was cooled at -15 °C using a 3:1 ice–salt mixture, adding drop by drop chlorine sulphate acid. 20 mL pyridine and 4 mL $ClSO_3H$ per gram polysaccharides (corresponding to a quadruple SO_3^- excess glucose unit). This large excess of pyridine was necessary because otherwise a depolymerisation occurred, not detected here by GPC. For a homogeneous reaction mixture the samples were dissolved in water-free formamide. Sulphation took over 4 h under con-

stant stirring. A temperature of 65 °C was necessary to obtain a high degree of sulphation (DS), i.e., DS = 2.4, and a temperature of 30 °C to achieve a small DS, i.e., DS = 1.4. The solutions were stirred with 100 mL ice water per gram polysaccharide, agitated for 15 min, and precipitated with MeOH. The precipitates were redissolved in water, transferred in 5 M NaOH to the salt form and precipitated again. They were first dialysed against water and then for 72 h against bidistilled water. Finally the samples were precipitated by acetone, pre-dried with Et_2O and dried at high vacuum at 45 °C.

Degree of substitution.—The DS was determined gravimetrically as well as volumetrically. For gravimetry 500–1000 mg water-free polysaccharide sulphate was hydrolysed in 250 mL of 10 vol% HCl and heated to simmering. Precipitation was achieved by addition of 35 mL of a 25 wt.% $BaCl_2$ solution. To make the precipitates chloride free they were washed several times followed by a transformation into an A1 frit which was heated at 800 °C (3 h). The sulphuric content, w(S), and the degree of substitution, DS, were calculated using the relationships:[21,24,25]

$$w(S)/\% = \frac{\text{mg } BaSO_4 \cdot 13.737}{\text{mg substance}} \quad \text{and}$$

$$DS = \frac{w(S) \cdot 162}{3200 - w(S) \cdot 102}$$

For the volumetric determination of DS, 200 mg polysaccharide sulphate was dissolved in 100 mL water containing 30 g of an activated IR-120 (amberlite) resin and shaken for 1 h. Then the ion exchanger was filtered off and the solutions were titrated with 0.1 M NaOH to the point of neutralisation. In the same way a blind regulation was accomplished.[21] The difference in the NaOH consumption of both experiments gave the polyion equivalent concentration and from this the sulphur content, w(S), was obtained:

$$w(S)/\% = \frac{(\text{mL } 0.1\text{ M NaOH - mL blind sample}) \cdot 320}{\text{mg substance}}$$

NMR.—The NMR spectra[26–28] were obtained using an AC 250 spectrometer (Bruker Analytics, Karlsruhe, Germany) applying the Pulse Fourier Transform procedure. The solvent used was D_2O (Merck, Darmstadt, Germany) where the sample concentration was 20% and the reference $(CH_3)_4Si$. The number of scans was 10,000 for pullulan and dextran and 100,000 for pullulan sulphate and dextran sulphate. The frequency was 63 MHz.

Infrared spectroscopy.—Fine pulverised, water-free samples were mixed with potassium bromide and then pressed at 1000 MPa to obtain a transparent pellet. The spectrometer used was a 22 FTIR spectrometer from Bruker (Karlsruhe) working in the wave number range between 4000 and 200 cm^{-1}. The reference measurement was performed with pure KBr. Final evaluation was done with a Fourier self deconvolution function.[29,30]

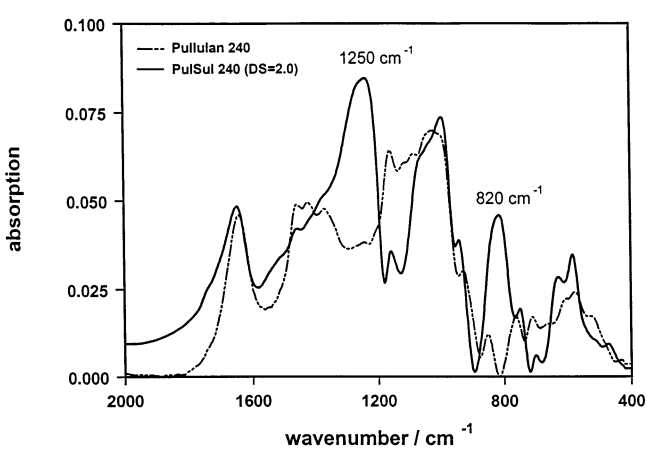

Fig. 2. FTIR spectra of pullulan 240 and pullulan sulphate 240.

3. Results and discussion

FTIR spectroscopy showed two characteristic absorption bands, one at 1260 cm^{-1} describing an asymmetrical S=O vibration and one at 820 cm^{-1} indicating a symmetrical C–O–S vibration associated to a C–O–SO_3 group[31–33] (Fig. 2). For pullulans the band at 900 cm^{-1} described α-(1→6) linkages. α-(1→4) linkages were observed at 925 cm^{-1}.[34] The absorptions at 850 and 765 cm^{-1} showed that pullulan had a $^4C_1$ chair conformation. Ring deformations and scaffold vibrations were observed at 710, 660, 600, 570, and 525 cm^{-1}. For pullulan sulphate the absorption band at

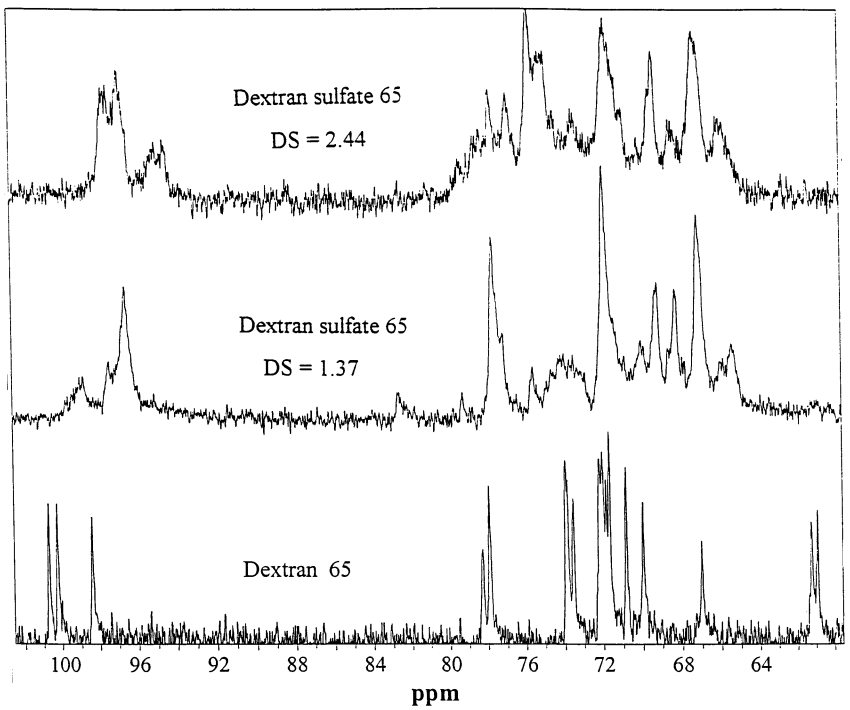

Fig. 3. ^{13}C NMR spectra of dextran 65, dextran sulphate 65/DS = 1.37, and dextran sulphate 65/ DS = 2.44 in comparison.

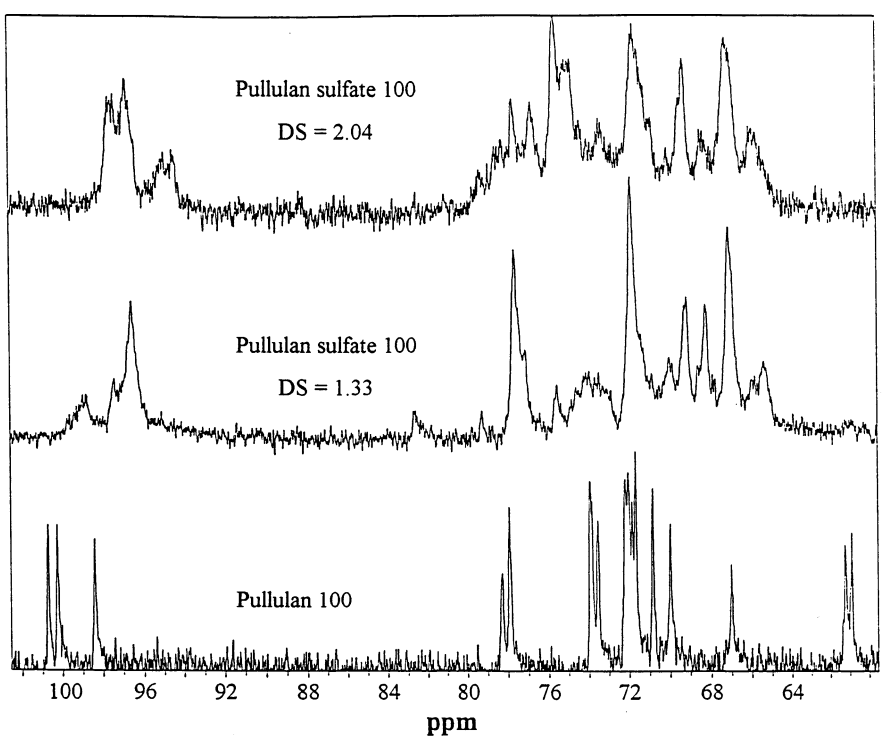

Fig. 4. ^{13}C NMR spectra of pullulan 100, pullulan sulphate 100/DS = 1.33, and pullulan sulphate 100/DS = 2.04.

815 cm^{-1} represented a symmetrical C–O–S vibration, with C–O–C bending vibration at 750 cm^{-1}. The ring deformation and scaffold vibrations appeared at 695, 640, 580 and 545 cm^{-1}. There was no influence of the polymer molar mass and only a marginal influence on the degree of substitution. With increasing DS the intensity of the OH absorption band decreased slightly. A similar result was found by Miyaji.[35]

Figs. 3 and 4 show the ^{13}C NMR spectra of dextran, dextran sulphate, pullulan, and pullulan sulphate. There was no influence of the molar mass on the positions of the peaks. Thus, only one typical plot for each species is presented.

Fig. 3 shows the ^{13}C NMR spectra of dextran and dextran sulphate. It could be seen that the C-1 glucose ring carbon absorbed at 98.15, C-2 at 71.90, C-3 at 73.90, C-4 at 70.03, C-5 at 70.67 and C-6 at 66.04 ppm.[36–41] Down field shifted resonances, which might be caused by branching at C-4 could not be detected. Thus introduction of sulphur groups on dextran has taken place preferentially at the C-3. The reactivity of the ring carbons was in the order C-3 > C-2 > C-4. The substitution of a H atom by a sulphur group led to a down field shift of the appropriate C resonance of approximately 4–6 ppm and to a high field shift at the neighbouring C atoms,[42,43] caused by magnetic anisotropy of the sulphate neighbouring groups. Since the local magnetic field was averaged over all segment movements and since these movements were slow, the line width was approximately 10 times larger than those of low-molecular materials. Due to the installation of the heavy sulphur groups this effect was stronger for the sulphates than for the pure saccharides.

For the dextrans (DS = 1.37) no visible high field effect was observed at the C-6 and C-5 positions. The C-3 signal appeared at 77.58 ppm. Two additional smaller C-3 signals appeared at 82.88 and 79.57 ppm, describing the sulphates at C-2 and C-4. Besides the high-field C-2 resonance at 71.87 ppm there was also a down field shifted signal of the sulphated C-2 at 75.51 ppm.

The signals at 70.85 and 70.27 ppm arose from the C-5. This double split could be the result from a high field shift due to the sulphation at the C-4. The down field shifted C-4 signal was negligible with respect to the resonance of the other signals. The 69.25 ppm peak was probably a high-field shifted resonance of the sulphated C-4 which occurred when a sulphate group was attached at the C-3.

In the spectrum of the highly sulphated dextran sulphates (DS = 2.44) the dextran C-2 signal was down field shifted at 75.20 ppm. Similarly, the C-3 resonance was broadened with a maximum at 76.93 ppm and a second absorption band at 79.52 ppm. The C-1 and C-6 signals were broad multiplets at around 97.7 and 66.5 ppm, respectively. Within the range of 71–68 ppm five resonances were observed at 70.63, 70.32, 69.87, 69.08, and 68.53 ppm. The two absorptions at 69.08 and 68.53 ppm were probably high field shifts caused by an unsulphated C-4 adjacent to sulphated C-3 and C-2. The absorption at 69.87 ppm described the high field shift of C-5 when both C-2, C-3 and C-4 atoms were sulphated. As a consequence, broad peaks occurred at 70.63 and 70.32 ppm. That is, when dextran was sulphated to a low degree, the C atoms had the reactivity C-3 > C-2 > C-4, while if the degree of sulphation was high, the order was C-3 ≥ C-2 > C-4.

Fig. 4 shows two typical NMR spectra of pullulan sulphate at two different DS-values. Again there was no influence of the molar mass on chemical shifts. For the pullulan sulphates of low DS (1.33) the primary hydroxyl groups at C-6 were more active than the secondary hydroxyl groups. The appropriate down field shifted C-6 resonance was a multiplet at 65.87 and 65.38 ppm. 67.02 ppm described unsulphated C-6 resonance and 69.14 and 68.21 ppm described the high field shifted C-4 resonance. At 69.93 ppm the C-2 and C-5 atoms adsorbed when C-3 or C-4 were sulphated. The C-5 signal, which originally was at 70.85 ppm, shifted down to 69.93 ppm. At 71.83 ppm C-2 and C-5 were active, but only if sulphation at neighbouring atoms has not taken place.

For pullulan sulphate of high DS strong broadening of all peaks occurred, for instance, the C-1 resonance was at 97.37, 96.61, 95.45, and 94.49 ppm. The unsulphated C-5 and C-2 absorbed at 71.60 ppm. The low field shifted resonances of the sulphated C-2 appeared between 76.0 and 74.5 ppm. The C-5 which was for pullulan at 70.83 ppm shifted for highly sulphated pullulan to 71.60 ppm. The resonances at 69.21 and 68.30 ppm were high field shifts of the C-4, where the C-3 had to be sulphated to observe them. At 65.86 ppm the sulphated C-6 absorbed, while between 79.5

and 76.5 ppm sulphated C-3 and unsulphated C-4 were active.

In conclusion, for pullulan sulphate C-6 was the most preferential position for sulphation, followed by C-3, whilst C-4 remained mostly unsulphated.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft for financial support.

References

1. Voigt, K. D.; Knabbe, C. US Patent 5055301, 1991.
2. Parish, C. R.; Snowden, J. M. US Patent 5541166, 1996.
3. Roufa, D.; Harel, A.; Frederickson, R.C.A.; George, T. US Patent 5605938, 1997.
4. Carroll, S.B.; Firca, R.J.; Pugh, C.; Padhye, N.V. US Patent 5545721, 1996.
5. Scranton, A. B.; Rangarajan, B.; Klier, J. *Adv. Polym. Sci.* **1995**, *1*, 122.
6. Schols, D. *Virology* **1991**, *175*, 556.
7. Lopalca, L. *AIDS Res. Hum. Retrovirus* **1994**, *10*, 787.
8. Yoshida, T. *Polym. J.* **1993**, *25*, 1069.
9. Ricketts, C. R. *Biochem. Z.* **1952**, *51*, 129.
10. Ricketts, C. R. *J.Chem. Soc.* **1956**, *2*, 3752.
11. Husemann, E. *Z. Naturforsch.* **1946**, *1*, 584.
12. Tsuji, K. Ger. Offen 2546699, 1974.
13. Tung, L. H. *Fractionation: Economic Polymer Science and Engineering*; Wiley: New York, 1989; Vol. 7.
14. Kawahara, K.; Ohta, K.; Miyamoto, H.; Nakamura, S. *Carbohyd. Polym.* **1984**, *4*, 335.
15. T. Igarashi, Germ. Offen. 3019895, 1980.
16. Kishino, K.; Kawai, T.; Nose, T.; Saitoh, M.; Kamide, K. *Br. Polm. J.* **1981**, *13*, 168.
17. Grönwall, A. *Upsala Lakaréfören. Forhandl.* **1945**, *50*, 397.
18. Tamba, R. *Biochem. Z.* **1923**, *141*, 274.
19. Engelskirchen, K. in: *Makromolekulare Stoffe*, Houben-Weyl, H. Bartl, Eds.; Georg Thieme Verlag:Stuttgart; 1987, Bd. E20, p. 2042.
20. Karrier, P. *Helv. Chem. Acta* **1943**, *26*, 1296.
21. Whistler, R. L.; King, A. H.; Ruffini, G.; Lucas, F. A. *Arch. Biochim. Biophys.* **1967**, *121*, 358.
22. Nagasawa, K.; Harada, H.; Hayashi, S.; Misawa, T. *Carbohydr. Res.* **1972**, *21*, 420.
23. Philip, B.; Wagenknecht, W. *Cellulose Chem. Technol.* **1983**, *17*, 443.
24. Guiseley, K. B. *Am. Chem. Soc. Symp. Ser.* **1978**, *77*, 148.
25. Whistler, R. L.; Spencer, W. W. *Arch. Biochim. Biophys.* **1961**, *95*, 36.
26. Fritsche, H. *Strukturuntersuchungen an Biopolymeren mit Spektroskopischen und Hydrodynamischen Methoden, Berlin:Akademie-Verlag*; 1976.
27. Bovey, F. A. In *NMR Spectroscopy: Comprehensive Polymer Science*; Allen, G., Ed.; Pergamon: Oxford, 1989.
28. Bovey, F. A. In *NMR Spectroskopy, Economic Polymer Science and Engineering*; Mark, H. F., Ed.; Wiley: New York, 1987; Vol. 10.
29. Coleman, M. M. In *Economic Polymer Science and Engineering*; Mark, H. F., Ed. Infrared-absorption spectroscopy. Wiley: New York, 1987; Vol. 8.
30. Hsu, S. L. In *Comprehensive Polymer Science*; Allen, G., Ed. IR Spectroscopy. Pergamon: Oxford, 1989.
31. Cabassi, F.; Casu, B.; Perlin, A. S. *Carbohydr. Res.* **1978**, *63*, 1.
32. P. T. Petrov, *Spectrosc. Biol. Mol.*, 6th European Conference, 1995.
33. Samal, R. K.; Sahoo, P. K.; Ray, S. S.; Nayak, S. N. *Angew. Makromol. Chem.* **1985**, *129*, 11.
34. Klaushofer, H.; Berghofer, E.; Diesner, L. *Starch* **1976**, *28*, 298.
35. Miyaji, H.; Misaki, A. *J. Biochem.* **1973**, *74*, 1131.
36. Colson, P. *J. Am. Chem. Soc.* **1975**, *96*, 8069.
37. Friebolin, H.; Frank, N.; Keilich, G.; Siefert, E. *Makromol. Chem.* **1976**, *177*, 845.
38. Arranz, F.; San Roman, J.; Sanchez-Chaves, M. *Macromolecules* **1987**, *20*, 801.
39. McIntyre, D. D.; Vogel, H. J. *Starch* **1991**, *43*, 69.
40. McIntyre, D. D. *Starch* **1995**, *45*, 406.
41. Ramirez, J. C.; Sanchez-Chaves, M.; Arranz, F. *Polymer* **1994**, *35*, 2651.
42. Franz, G. *Polysaccachride*; Springer: Berlin, 1991.
43. Nehls, I.; Wagenknecht, W.; Philipp, B.; Zschunke, A. *Acta Polym.* **1985**, *36*, 657.