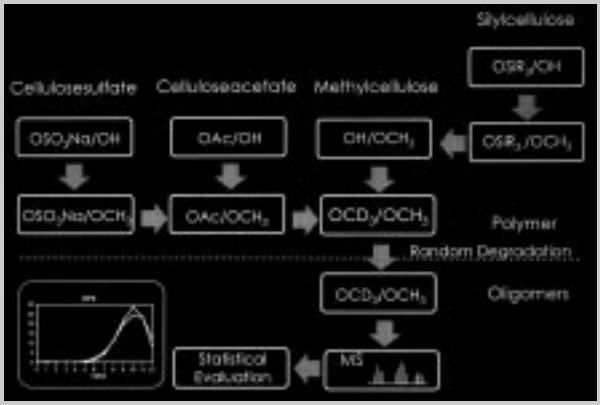
Feature Article: Cellulose, starch and cyclodextrin ethers and esters are produced for many fields of application as for example oil recovery, building and ceramic materials, textile and paper industry, food, cosmetics, and pharmaceuticals. For further development of new types of derivatives and also for a better understanding of their properties a detailed analysis of the usually complex mixtures is required. By complete degradation, separation and quantification of appropriate monomer derivatives the regioselectivity in the glucose unit can be determined. For the determination of the substitution pattern in the polymer chain partial random degradation, oligomer analysis by mass spectrometry, and subsequent statistical evaluation can be applied. The composition of products obtained by enzymatic degradation also reflects the pattern in the polymer chain. In addition a possible heterogeneity of the material must be taken into consideration, since any degradation averages the structural information.



Oligomer analysis of cellulose derivatives for the determination of the substitution pattern in the polymer chain.

Structure analysis of 1,4-glucan derivatives

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Introduction

Before petrol oil based polymer chemistry explosively developed, biomacromolecules were the only polymeric organic materials. Due to its strong mechanic properties and its abundant occurrence in nature of about 10^{11} t/a cellulose was and still is the most important representative of the polysaccharides. However, the native properties as non-solubility in common solvents had to be overcome to extend the area of applications. So, about 160 years ago, the first derivatives of cellulose were produced,[1] long before the structure and the polymeric character was discussed and proved by Haworth,[2] Meyer and Mark,[3] Staudinger[4] and Freudenberg.[5]

Cellulose nitrate was the first derivative which was produced in an industrial scale. But also cellulose sulfate[6] is mentioned as early as 1844. Since then a wide spectrum of cellulose ethers and esters, of organic and inorganic derivatives, of neutral, anionic, cationic or amphoteric compounds, has been synthesized. (Fig. 1). Only a few of them have gained commercial importance – mainly carboxymethyl, methyl, hydroxyethyl, hydroxypropyl ethers and cellulose acetates, while others are only of scientific interest. While the ethers are used "for what they do", celluloses esters are used "for what they are".[1] Cellulose derivatization is mainly the chemistry of its alcoholic hydroxy groups. Strong hydrogen bonds and supramolecular structures must be overcome to get the monomer units of this 1,4-β-glucan accessible. This is usually achieved by alkaline swelling, and under these basic conditions alcoholate ions are formed as nucleophiles that can attack e. g. alkyl halides, oxiranes, or sul-

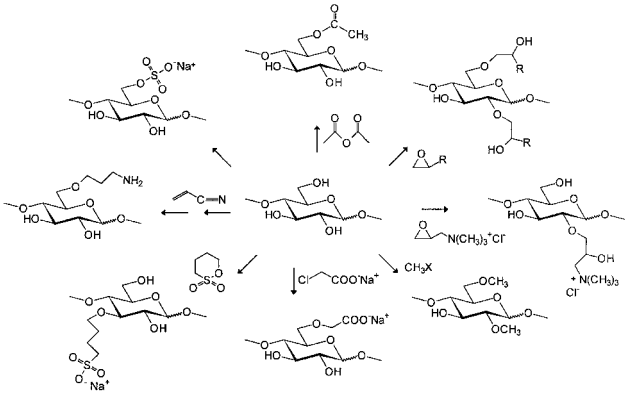

Fig. 1. Derivatization reactions of 1,4-glucans.

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tones to form ethers. Esters can also be prepared under acid catalysis. An overview is given by Klemm et al.[7]

Starch is a further representative of the renewable resources. It is also a 1,4-glucan, but with α-linkages and comprises additional α-1,6-branching points. It is more heterogeneous than cellulose and has a higher molecular weight, (10^5-10^6 for amylose and 10^7-10^8 for amylopectin). So the properties of the native polysaccharide completely differ from those of its linear β-linked counterpart. However, the types of derivatives that are produced are the same, although low degrees of substitution (*DS*) are common for starch in contrast to high *DS* values for cellulose ethers and esters.

Cyclodextrins (CDs) are the third type of 1,4-glucans, that will be considered in this feature. Since enzymatically produced from starch, the helix of amylose is conserved in fixed ring structures, mainly comprising 6 (α-CD), 7 (β-CD) or 8 (γ-CD) glucose units. In principle, the same chemistry is applicable to these cyclic oligoglucans, however, the less flexible conformation and the ability to include suitable compounds into their cavity favors reactions with higher and unusual regioselectivity.

While the latter types of compounds are used as chiral selectors in chromatography[8] and electrophoresis,[9] as excipients for pharmaceuticals[10] (methyl, hydroxypropyl, sulfobutyl) or for controlled release of drugs, aroma compounds, pesticides etc., starch and cellulose derivatives are used in oil recovery, in textile, building and paper industry, for detergents, cosmetics, food and pharmaceuticals, in medicine and as chromatographic materials. New fields of application lie in the biomedical area, in membrane or sensor technology.[7] Required properties are viscosity control, adhesion, water retainment, film and fiber forming properties, improvement of freeze-thaw-stability, prevention of crystallization, biocompatibility, stabilization against enzyme digestion, to mention only a few.

Beside other structural features the number and distribution of the introduced groups determine the properties of these complex compound mixtures. Therefore, our efforts are directed to the development of analytical tools for the elucidation of these patterns as detailed as possible.

Substitution pattern in the monomer unit

In 1,4-linked glucans three OH groups are available for further functionalization The terminal 4-OH can normally be neglected. If only one type of substituent is introduced, up to 2^3 patterns in the anhydro glucose unit (AGU) can result, their molar ratios have been assigned as s_0, s_2, s_3, s_6, s_{23}, s_{26}, s_{36}, s_{236} by Spurlin.[11] Two different substituents and still free hydroxy groups form up to 3^3 different monomer units.

NMR Spectroscopy

NMR spectroscopy can give a first insight in the partial degrees of substitution in the positions 2, 3 and $6-x_2$, x_3 and x_6. ^{13}C-NMR signals of the glucose C atoms are shifted downfield, if this position is substituted, e.g. C-6 from about 60 to about 70 ppm. C-1 suffers an upfield-shift, when O-2 is derivatized.[12] Tezuka has investigated even mixed acetates/butyrates by ^{13}C-NMR, where all C═O resonances were resolved.[13] A limitation of this approach is given by the solubility and viscosity of the samples, since viscous solutions of low concentration only give broad signals with poor resolution and sensitivity. However, this problem has been overcome by enzymatic degradation or complete hydrolysis.[14] Ultrasound degradation has successfully been applied by Baar et al.[15] for sample preparation. Signals also become narrower and more distinct when the derivative is more uniform. Therefore, Miyamoto et al. perdeuteroacetylated cellulose acetates and deduced the partial *DS* values from the now well resolved C*H*$_3$—CO signals in the ^{1}H-NMR spectrum.[16] Recently, Kern et al. gave an impressive comparison of NMR spectra from more or less regioselectively substituted 2,3-di-*O*-methyl celluloses. Resolution of the signals increases with increasing uniformity of these ethers and minor signals could be assigned and correlated with special structural features.[17] Starch derivatives with their amylose/amylopectin heterogeneity and usually very low *DS* values have been far less the objective of NMR studies. In contrast, cyclodextrins with their defined core structures and often regioselective substitution patterns are usually analyzed by NMR spectroscopy as it is common in structure elucidation and synthesis control of organic compounds. However, non-uniform substituted CD derivatives give complex spectra of higher order that are difficult to interpret. Typical random type CD ethers again give broad unresolved signals. A downfield shift of H-1 for about 0.14 ppm effected by 2-*O*-substitution allows to estimate the partial *DS* in this position (x_2), while the total *DS* can be calculated from the ratio of a substituent signal, e.g. of CH_3 in hydroxypropyl (HP) or in quaternary ammonium groups, to the summarized H-1 signals.[18, 19]

Degradation to monomers, chromatography and mass spectrometry

Liquid Chromatography: While NMR experiments predominantly supply the partial *DS* values x_i, hydrolysis and HPLC analysis can complete this information, provided the substituents are stable under the acidic conditions applied (most of the ethers). Separation of the un-, mono-, di- and trisubstituted glucose fractions c_0, c_1, c_2, and c_3, can easily be achieved due to differences in mass and polarity. Methyl ethers can be separated by RP-HPLC or on amine modified silica columns with RI or polarimetric detec-

tion,[20] carboxymethyl ethers were resolved on an Aminex HPX 87 H column (Biorad).[21, 22] Anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was also successfully applied to glucan ether hydrolysates. Naturally, anionic ethers as carboxymethyl and sulfoethyl glucoses are the preferred substrates and even regioisomer separation was achieved on a CarboPac column.[23, 24] However, for the strongly acidic sulfonic acid derivatives this special anion exchange columns for carbohydrate analysis cannot be recommended since at higher *DS* values, when multiple charged components are present, the separation efficiency of the CarboPac-PA1 completely broke down.[25] Also water soluble *O*-methyl glucoses can be separated due to the acidity of free sugar OH groups.[26] A drawback is the strong decrease of the response in the electrochemical detector with increasing *DS* of the glucose derivatives and the significant differences for the mono- and disubstituted regioisomers that requires complete resolution for any quantitative evaluation. In addition response factors vary with the stage of the electrode.[27]

Gas chromatography-mass spectrometry: To determine the complete monomer composition (s_0, s_2, s_3, s_6, s_{23}, s_{26}, s_{36}, s_{236}) capillary gas chromatography (GLC) has proved a success in many cases. Besides its high separation efficiency coupling with mass spectrometry (GLC-MS) is well established and provides additional structural information. Therefore, peaks can unambiguously be assigned without synthesis of standard compounds, which is essential when very complex mixtures are analyzed as obtained from hydroxyalkyl ethers. The molecular mass and therefore the number of substituents of a glucose derivative can be deduced from the CI mass spectrum, preferably with ammonia as reactant gas. The fragmentation pattern under EIMS conditions allows to localize the position of substitution, based on the well investigated fragmentation behavior of the permethylated ground structures.[28–31] GLC-MS also favors the detection and identification of small side-products and artefacts. A further profit of GLC analysis is the connection with the flame ionization detector (FID). The response of the FID depends on the number of carbon atoms in a molecule and their neighborhood. The "effective carbon response" (ecr) can be calculated from experimental data for certain structural features.[22, 23] So, the relative molar composition of the constituents can be determined after correction of the peak areas by the ecr-concept.

A prerequisite for GLC analysis is sufficient thermal stability and volatility of the analytes. While this is easily achieved for neutral ethers, ionic derivatives must be transformed to neutral ones. A general requirement is that the monomer composition remains representative over all steps of sample preparation. That means that transformations performed on the polymer level, e. g. permethylation of free hydroxy groups, should be quantitative, while during and after degradation to monomers discrimination of any constituents must be excluded. Of course, this is the ideal case, which should be the goal of all optimization efforts.

In the following, sample preparation for certain types of glucan derivatives is reported.

Alkyl ethers: Alkyl ethers are chemically stable and can directly be hydrolyzed. The eight constituents of methylcelluloses or starches can be separated by HPAEC-PAD[26] (no differentiation of anomers) or reduced and acetylated to yield partially methylated glucitol acetates which can be analyzed by GLC-FID and GLC-MS. After reductive amination with *p*-aminobenzonitrile or another appropriate amine these compounds can be separated by capillary electrophoresis in a borate buffer.[34] With increasing number of methylene groups aqueous acid hydrolysis becomes less appropriate.

Since side reactions as dehydration and intramolecular reactions during acid hydrolysis are favored by free OH groups it is advantageous to permethylate – or in the case of methyl ethers perethylate – the polysaccharide prior to degradation. If hydrophobicity of the derivatives renders hydrolysis more difficult, methanolysis can be used for prehydrolysis. However, in this case, reductive cleavage is the method of choice.[35] This alternative type of methylation analysis is performed in dichloromethane under promotion of a Lewis acid and with triethylsilane as reducing agent.[36] The resulting 1,5-anhydro-glucitol derivatives are acetylated and can be analyzed as well as the open-chain glucitols by GLC-FID and GLC-MS. 5-*O*-Acetyl-1,4-anhydro-D-glucitols may occur as a result of ring-isomerisation.

Hydroxyalkyl ethers: As a consequence of tandem reaction of the hydroxyalkyl substituent the number of constituents is theoretically unlimited. Permethylation with NaOH/MeI in Me_2SO according to Ciucanu and Kerek[37] or by a modified Hakomori procedure[38] with Li dimsyl as the base, is recommended prior to hydrolysis since 2-*O*-hydroxyalkyl-glucose is in equilibrium with its intramolecular bicyclic acetal. After permethylation, acid hydrolysis followed by reduction and acetylation can be performed with both *O*-hydroxyethyl (HE) and *O*-hydroxypropyl (HP) glucans, while reductive cleavage is less appropriate for the latter.[39] The ratio of oxirane addition on the glucose core and oligoether chain formation is strongly influenced by base concentration. Low amounts of base favor the etherification of the sugar-OH with the 2-OH being the predominated one due to the order of acidity. At higher concentration of NaOH as used for cellulose activation, the tandem reaction is preferred. A statistical evaluation of the monomer pattern has been reported by Reuben.[40] A critical investigation of methylation analysis of HECs with higher molar degrees of substitution (MS) has been performed by Arisz et al.[41] Mixed ethers (EHEC, HPMC) were analyzed by Lindberg et al.[42] and by Mischnick.[43]

Cationic ethers: The main representatives of cationic ethers are *O*-diethylaminoethyl derivatives (DEAE) and *O*-(2-hydroxy-3-trimethylammonium) propyl ethers. From the latter appropriate monomer compounds for GLC analysis could be obtained by the sequence methanolysis, permethylation, and Hofmann-elimination.[19, 44] A strongly preferred reaction with the cationic oxirane was observed in position 2 of starch derivatives (*DS* 0.02–0.9), followed by nearly equal ratios of 3- and 6-*O*-substitution. Comparison of the monomer composition with the models of Spurlin[11] and Reuben[13] indicates a negative intramonomeric effect, i.e. a reduced reactivity of 3-OH, when position 2 is substituted. This small effect might be caused by intramolecular masking of the deprotonated 3-OH and/or electrostatic repulsion of the cationic reagent.[44]

Carboxymethyl ethers: The most important cellulose ether is carboxymethylcellulose (CMC). Beside the commercial products new types of CMC have been synthesized by Heinze et al.[21, 22, 45] and Mann et al.[46] The substitution pattern has been analyzed by NMR,[12, 13, 15] by HPLC,[21] HPAEC-PAD[23, 27] and by reductive cleavage of the permethylated sample.[47] CE after reductive amination of the hydrolysate was also successful.[34] Direct acid hydrolysis favors intramolecular lactone formation. Under pH-control and activation with a water soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) the carboxymethyl residues can be reduced to hydroxyethyl groups with sodium borohydride[48] and then be analyzed as described above.

Sulfoalkyl ethers: The analysis of sulfoethyl cellulose by HPAEC has already been mentioned[24] While these derivatives can be produced *via* Michael addition of the vinyl sulfonic acid, the higher homologues are prepared by ring-opening-addition of the 1,3-propane sultone or 1,4-butane sultone, respectively. β-Cyclodextrin sulfobutylethers (SBE-β-CD) are of great interest as carrier for water-insoluble drugs. Attempts to use the sulfonic acid esters as neutral derivatives for GLC failed due to thermal instability. Therefore, sulfofluorides were prepared and successfully analyzed by GLC and GLC-MS.[25] Surprisingly, an unexpected high *DS* was observed for the 3-position, which usually shows very low reactivity in CD etherification. From NMR and microcalorimetry experiments it is evident that the sultone is inlcuded in the β-CD cavity when water is used as a solvent.[49] In contrast, no host-guest interaction was observed in methylsulfoxide, and now the more acidic 2-OH is favored as usual. Sulfobutyl amylose prepared in water also showed strongly preferred 2-*O*-substitution.

Discrimination of higher substituted constituents could not be prevented, because the multi-step sample preparation is not quantitative. Investigations are in progress to calculate the molar fractions c_i from CE analysis after reductive amination of the hydrolysate.

Trialkylsilyl ethers: Trialkylsilyl ethers are mainly used as protective groups for regioselective modification of cellulose and cyclodextrins or as soluble intermediates.[50] Bulky *tert*-butyldimethylsilyl (TBDMS) of *tert*-hexyldimethylsilyl (THxDMS) residues can selectively be introduced in the primary 6-position, but 2,6-di-*O*-silylated derivatives have also been produced. Since the O-Si-linkages are not stable under both Bronsted and Lewis acid conditions, permethylation is essential to save the information of the substituent distribution as the complementary methyl pattern. Such indirect methods require stability of the primary substituents under the methylation conditions. For the β-linked trialkylsilyl celluloses this requirement could be fulfilled, while in the corresponding α-glucans nearly complete migration of the SiR_3 group from O-2 to O-3 occurred under the alkaline methylation conditions, and the 2-position was irreversibly methylated.[51,52] This rearrangement interferes with the analytical target, but is a valuable tool for regioselective synthesis of 2-*O*-methyl-α-glucans. Methylation with methyl triflate and 2,6-*tert*-butylpyridine occurs without silyl migration, since no active nucleophile for the intramolecular attack is formed. However, this method was effective for CD silyl ethers, while amylose could not completely be methylated. Since the silyl ethers are cleaved in the subsequent hydrolysis step, complete degradation to monomers was difficult to achieve for high *DS* silyl celluloses. Stepwise hydrolysis with trifluoroacetic acid[53] or sulfuric acid[54] or a combination of methanolysis and hydrolysis could improve the recovery of the constituents. If a 6-OH group is formed during hydrolysis, intramolecular attack of the carboxonium ion always yields small amounts of 1,6-anhydroglucose derivatives, which can easily be identified by GLC-MS. Reductive cleavage was also applicable but a high excess of reagents (40 equivalents), Lewis acid and triethylsilane was necessary to recover the highly silylated constituents. Glucose-1-acetates were formed as by-products since intramolecular stabilization by the 6-*O*-SiR_3 group prevents complete reduction.

Acetates: Acetates suffer from both, acid and base lability. Carbamates[55] and ethyl vinyl ethers[56] have been prepared to generate the inverse substitution pattern. Methylation with methyl triflate/2.6-*tert*-butylpyridine in trimethyl phosphate according to Prehm[57] or with trimethyloxonium tetrafluoroborate in dichloromethane[58] can be applied without significant side reactions to cellulose acetates with a $DS > 2$. At lower *DS* migration and chain degradation could not be excluded.[59] In principle acyl residues are stable under reductive cleavage conditions.[60] However, complete cleavage of the polysaccharide is best accomplished with a high excess of Lewis acid/triethylsilane under complete reduction of the acyl residues to the corresponding alkyl ethers as has been demonstrated by Lee and Gray.[61] By this approach even

mixed esters can be analyzed.[62] As an alternative, direct hydrolysis under acyl cleavage and formation of partially methylated glucitol acetates[59] or acetyl-alkyl exchange under alkaline conditions with subsequent reductive cleavage[58] can be applied.

For commercial cellulose acetates (*DS* 2.5) that were prepared by ester hydrolysis of the "triacetate" preferred 2,3-acetylation was found, while direct acetylation to a *DS* of 2.66 under alkaline conditions[59] resulted in predominant esterification of positions 2 and 6. There are also significant differences with respect to the pattern in the polymer backbone, as will be reported later on.

Sulfates: Regioselective sulfation has been extensively investigated with respect to medical applications.[7] Due to the lability of these inorganic esters again an indirect determination via the complementary methyl pattern is performed. Polysaccharide sulfates can be permethylated under alkaline conditions, however, in *trans*-1,2-diol structures as present in 1,4-linked glucans an intramolecular nucleophilic displacement of sulfate under formation of an oxirane structure as intermediate is possible. Optimization of the methylation conditions could avoid this side-reaction.[63] Due to the strongly enhanced acid lability of glucosyl linkages in 2-sulfates reductive hydrolysis that was introduced by Lindberg[64] was applied as described by Stevenson and Furneaux[65] to stabilize early liberated glucose residues by direct reduction to glucitols. After complete hydrolysis, reduction and acetylation again the partially methylated glucitol acetates are obtained.

Distribution in the polymer chain

It is well known that gel strength of alginates with a certain ratio of L-guluronic acid is higher for those with a blockwise structure than for alginates with a random distribution of guluronic acids.[66] This is attributed to the cooperative effect of weak interactions as is well known for hydrogen bonds.[67] Methylcellulose (MC) showed cold-water-solubility at a *DS* of 0.6 and 1.2, respectively.[68] Hirrien et al.[69] reached water solubility at *DS* 0.9, while commercial samples require at least a *DS* of 1.3. MC and HPMC show thermoreversible gelation, a property which is explained by hydrophobic interactions of permethylated sequences in the polymer chain, when the solvate shells are melting away.[70] Heinze et al. reported water solubility of CMC at *DS* 0.4 when prepared in isopropyl alcohol/water slurry, while those prepared with solid NaOH in an aprotic solvent required an average of 1.3 carboxymethyl groups per glucose unit due to much higher local density of the substituents, which is less effective in mediating solubility.[45] Therefore, the distribution of the monomer units and substituents in the polymer chain considerably influence the properties.

Fundamental considerations

Polymer analogous reactions on cellulose or starch produce copolymers of eight or even more different monomer units. The regioselectivity and the possible influence of primary substitution on relative reactivities within the glucose moiety determine the monomer composition. If all glucose units are accessible at the same probability, a random distribution of these monomers along the polymer chain should result. This pattern can be calculated for small sequences of the polymer by binomial functions according to Bernoulli statistic. If the glucose units are not accessible in the same way, e.g. due to residual crystalline regions that have not been activated, then a more heterogeneous pattern compared to the random distribution is expected. In addition, the reactivity of monomers might change during the reaction, e.g. because the local reactivity is enhanced when first changes in polarity have modified the "local accessibility", or it may be decreased due to sterical effects or electrostatic repulsion. In the latter case a more regular or narrower distribution of substituents in the polymer chain would be expected, compared to the random pattern, which is always the reference model.[43] The random pattern is expected for homogeneous reactions of molecularly dissolved polysaccharides, provided that the relative reactivities of all AGUs remain equal over the course of the reaction, in analogy to the Spurlin model for the random pattern in the AGU.[11] Especially for heterogeneously prepared derivatives it must be considered that a heterogeneity of first order may superpose that within the polymer chains (second order).

Statistical approach

How can experimental data be obtained to compare the model and the reality? The complex mixture of polymer molecules with a distribution in molecular weight must partially be degraded first. Provided the glucosidic linkages are randomly cleaved, the composition of the oligomeric fractions (*DP* 2,3,4...) reflects the situation in the polymer chain.

Methyl ethers: Arisz et al.[71] checked the partial acid hydrolysis of commercial methylcelluloses (*DS* 1.75) under this aspect and analyzed the resulting mixture after perdeuteromethylation by FAB-MS. The relative composition for the trisaccharide fraction (DP3) was compared with the data calculated for a random pattern. A slight deviation indicating some heterogeneity in the material was observed. We applied a similar approach to methyl amyloses prepared in different homogeneous and heterogeneous systems. The samples were permethylated first with MeI-d_3 to get a chemically uniform product for random degradation[72] (Fig. 2). Partial degradation was performed by methanolysis, hydrolysis or mild reductive cleavage. Reliability of quantitative mass spectrometry

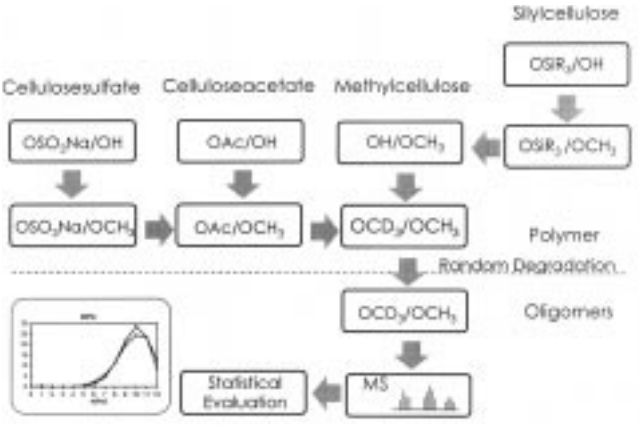

Fig. 2. Oligomer analysis of cellulose derivatives for the determination of the substitution pattern in the polymer chain.

was proved for mixed OCH_3/OCD_3-ethers with synthesized standards for FAB-MS as well as for MALDI-TOF-MS. If no discrimination had occurred, the average *DS* of each oligomeric fraction should be in agreement with the total *DS* of the sample. Indeed, methyl amylose prepared in homogeneous reaction with water as the solvent showed an ideal random pattern in the polymer chain. When amylose was dissolved in methylsulfoxide and methylated with pulverized sodium hydroxide and MeI, a bimodal distribution (average *DS* 1.72) with a superposition of a nearly random pattern (average *DS* 1.09) and nearly permethylated sequences was observed. These sample could be fractionated by extraction with solvents of different polarity, indicating that heterogeneity of first and second order are overlapping. Light scattering experiments showed that even when the sodium hydroxide particles could no longer be recognized their diameter was still about 200 nm.[73] After filtration of a solution of amylose in methylsulfoxide with and without addition of pulverized NaOH the amylose was recovered in the filtrate in the first case but was retained by the sodium hydroxide in the latter. When the solid base is added to the amylose solution in an aprotic solvent, it is adsorbed on the solid particles according to the model of Fleer and Scheutjens[74] and methylated with a high rate on this liquid solid interphase (trains), while non-adsorbed molecules or parts of it (tails, loops) react with a much lower rate in a more random way. So, the bimodal pattern is the result of two competing mechanisms. It has to be considered that adsorption and desorption is a dynamic process. This phenomenon has also been recognized and used by Heinze et al. as synthetic concept for CMC with a unusual distribution of substituents.[22, 45, 75] These new type compounds have been thoroughly investigated by enzymatic degradation and further structure elucidation.[76]

Fractionation of a heterogeneously prepared methyl cellulose yielded four portions with average *DS* values from 0.61 (main portion) to 2.82. From oligomer analysis

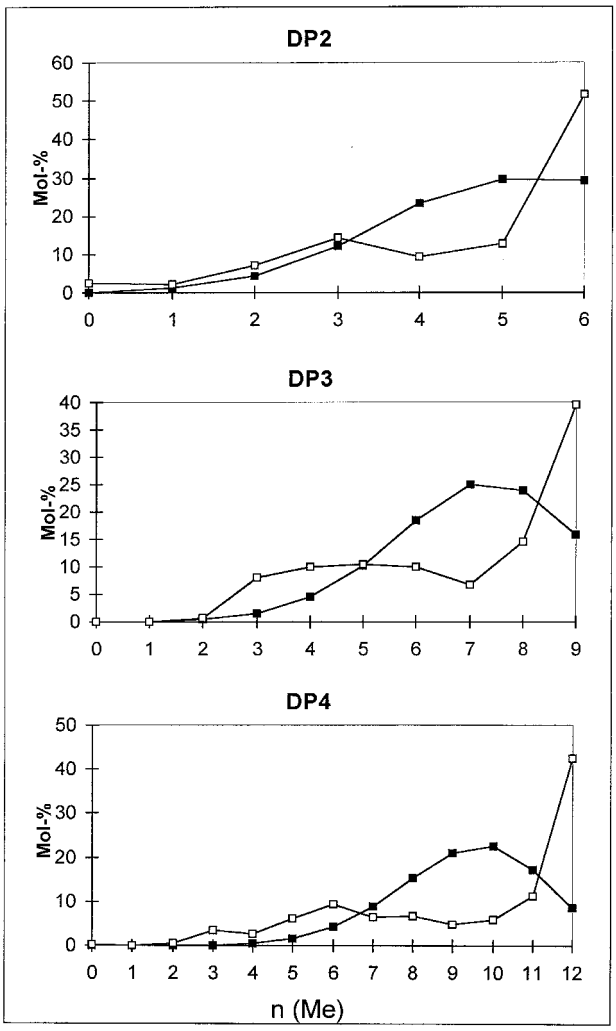

Fig. 3. Substituent distribution in the $CHCl_3$/MeOH (2:1)-soluble fraction of heterogeneously prepared methyl cellulose, *DS* 2.32. Relative composition of the di-, tri-, and tetrasaccharides obtained after deuteropermethylation, partial random cleavage, and remethylation. ■ = calculated, □ = experimental.

of the fraction with a *DS* of 2.32 (extracted with $CHCl_3$/MeOH, 2:1) a bimodal pattern is obvious (Fig. 3).[77] Since the sample has already been fractionated both patterns should be located on single macromolecules.

To quantify the heterogeneity, Arisz et al.[71] introduced a heterogeneity parameter H_i, where i is the *DP* of the oligomeric fraction observed. The squares of the difference of the experimental and the calculated data for a random pattern for each component with n substituents are summarized, the root of which is defined as H_i.

Regioselectively substituted methyl celluloses: To study the phenomenon of thermoreversible gelation Kern et al.[17] synthesized regioselectively substituted 2,3-di-*O*-methyl-celluloses via 6-*O*-(*p*-methoxy)trityl cellulose. How close these products came to the ideal uniform structure was investigated by NMR, monomer and oligomer analysis. The method of oligomer analysis after par-

tial degradation allowed to determine the distribution of under- and over-methylated glucose units in the products with high reproducibility. An over-methylated sample showed a clustered arrangement of the 2,3,6-tri-*O*-methylated monomers that reflected the positions in the cellulose that could not be tritylated in the first step, presumably due to insufficient activation. In contrast, under-methylated moieties of a completely 6-*O*-tritylated cellulose were more randomly distributed in the cellulose chain. The products of highest uniformity showed no phase separation in water solution at elevated temperatures, while phase transitions were observed for over- and also for under-methylated pruducts by DSC.[17]

Cellulose sulfates: Quantitative mass spectrometry is still a limitation for the application of the just described oligomer analysis. Therefore, the method was first extended to such derivatives that can be transformed to the required OCH_3/OCD_3-ethers. Permethylated sulfates must first be desulfated, which was best achieved under acetylation conditions. Acetyl groups were subsequently changed against CD_3 (see Fig. 2). Then random degradation, remethylation and FAB-MS analysis could be carried out.[78] Independently on the method of preparation (homogeneous or heterogeneous start conditions) a random pattern was observed in the cellulose chain. This result was explained by thermodynamic control of the sulfation reaction. While in kinetically controlled etherification no "correction" of heterogeneity is possible during the course of the reaction, the reversibility of sulfation allows an equilibration of the substituent pattern. Sulfation of partially protected intermediates reflect the distribution of the primary introduced groups. So, only a sulfate that was produced from a cellulose acetate showed a broad and heterogeneous distribution.

Acetates: As already outlined in the above section acetates must be permethylated to save the information about their structure as the complementary methyl pattern. After acetyl-CD_3-exchange again the required isotopically labeled methyl ethers are obtained (Fig. 2). Application to cellulose acetates[59] with a *DS* around 2.5 showed slight heterogeneity for commercial samples produced with acetic anhydride/sulfuric acid and subsequent partial ester hydrolysis of the "triacetate". In contrast, a sample that was directly acetylated to a *DS* of 2.66 after alkaline swelling was randomly substituted along the cellulose backbone. Good activation of the cellulose and thermodynamic control seem to be responsible for the random outcome, while the heterogeneity of the commercial sample might reflect uncomplete activation under acidic conditions and a "triacetate" intermediate with a $DS < 3$.

Trialkylsilyl cellulose: Trialkylsilyl celluloses can also be prepared by direct silylation to a certain *DS* or by partial desilylation of a fully *O*-silylated polysaccharide. Since silyl cellulose can be permethylated without silyl migration as outlined above and silyl groups can easily be cleaved by fluoride the statistical approach is also applicable to these derivatives (Fig. 2).

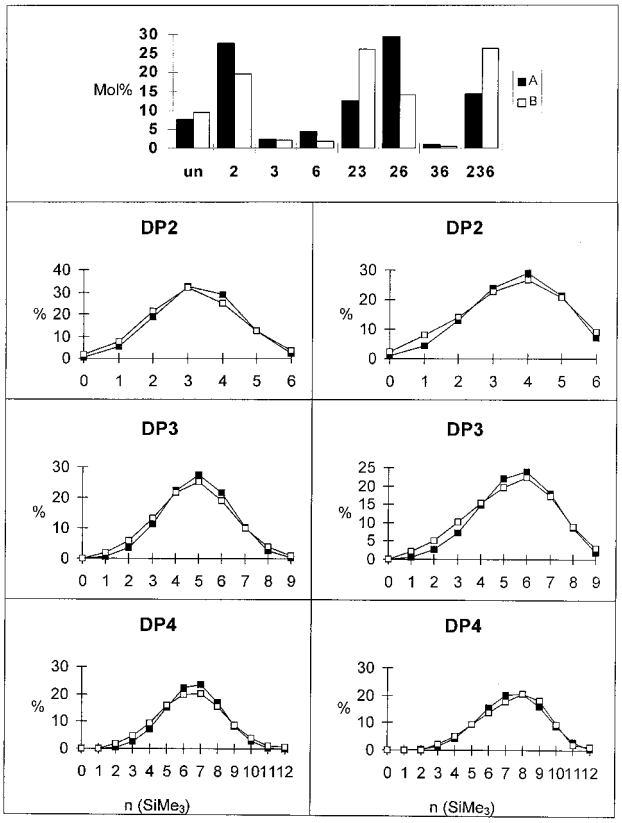

Fig. 4. Monomer and oligomer composition of *O*-trimethylsilylcelluloses A (direct heterogeneous silylation, *DS* 1.64) and B (prepared via the persilylated cellulose by homogeneous partial hydrolysis, *DS* 1.84) ■ = calculated, □ = experimental.

Monomer and oligomer analysis was performed with two trimethylsilylcelluloses. One of them, TMSC1, was prepared by direct silylation to a *DS* of 1.65 in a heterogeneous reaction with hexamethyldisilazane in liquid ammonia,[79] while TMSC2 was produced via homogeneous desilylation of a persilylated cellulose (*DS* 1.86).[80] The monomer composition showed significant differences with preferred 2- and 2,6-di-*O*-trimethylsilylation for the direct modified sample, while the indirect method mainly yielded 2,3,6-, 2,3- and 2-*O*-substitution, similar to directly and indirectly prepared acetates. The oligomer analysis showed a slight heterogeneity for both samples with no significant differences (Fig. 4).

Labeling for mass spectrometry: Mass spectrometry of chemically non-uniform derivatives as for example $OCH_3/O[CH_2CHRO]_nH$ ethers do not give correct quantitative results. Bartsch et al.[81] showed that strong discrimination of smaller constituents occurs when oligomeric mixtures of ethoxylated surfactants are analyzed by MALDI-TOF-MS. However, the relative peak intensities could be corrected after calibration with standard compounds. To extend the statistical oligomer analysis to other types of

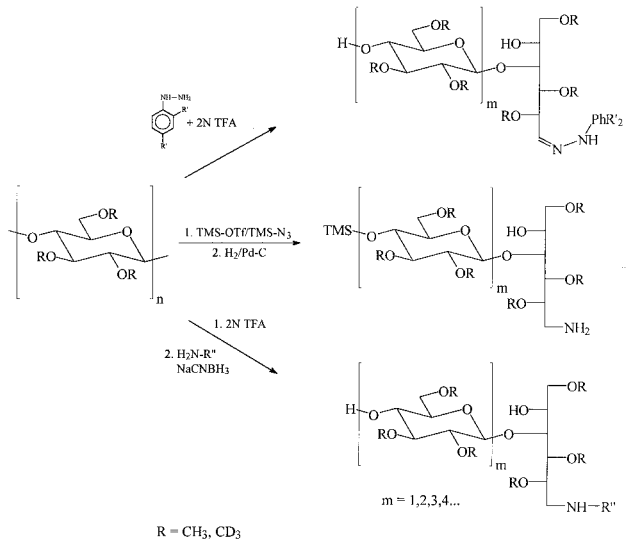

Fig. 5. Labeling of oligosaccharides with nitrogen containing tags.

derivatives it was attempted to level the differences in ion yield by labeling each oligosaccharide with an appropriate group.[77] Quaternary ammonium compounds are known to give mass spectra of high abundance. In addition, no multiple ion detection due to sodium and potassium adduct formation will occur. Therefore, different strategies were checked (see Fig. 5). Hydrazinolysis and reductive cleavage with TMS azide turned out to be inappropriate, but reductive amination with propylamine and subsequent peralkylation yielded the desired labeled oligosaccharides. For control OCH_3/OCD_3 substituted oligosaccharides were labeled first. FAB-MS, MALDI-TOF-MS and ESI-MS all gave the same results as for the unlabeled compounds with an improved signal/noise. Then a well characterized methylcellulose was perethylated und submitted to statistical oligomer analysis. Under FAB ionisation conditions oligomers were discriminated with increasing number of methyl groups, and the quantitative results were not improved by the labeling procedure.[77] Under MALDI conditions the reverse discrimination was observed, but to a lesser extent. Labeling caused insufficient improvement. Electrospray ionization (ESI) of a methanolic solution of the analytes showed only a slight discrimination of higher ethylated compounds for DP2, but the *DS* was not constant over the *DP* range, indicating that relative ion intensities were influenced by the composition and concentration of the components. Similar observations were made by Bartsch et al.[81] Nevertheless, ESI is promising for further investigations which must systematically check all parameters. MALDI-MS is also not yet kicked out, while FAB-MS is not suitable for quantitative analysis of mixed derivatives.[82] Reductive amination allows to introduce further residues with other functional groups that might also effect absolute and relative ion yields.

Enzymatic approach

Another approach to partial degradation is the use of enzymes. The specificity of cellulose and starch-degrading enzymes is modified by the presence of substituted glucose residues. Glucosidic bonds adjacent to modified monomers are resistant or less accessible to the enzymes, which need a minimum of amenable glucose moieties for enzyme-substrate complex formation. Steeneken and Woortman[83] compared methyl starches that were prepared in granular suspension and in solution. After digestion with the *endo*-enzyme α-amylase and further degradation by the *exo*-enzyme amyloglucosidase more large fragments with an enhanced DS_{Me}, and also more glucose was obtained from the granular starch, indicating that the methylation in suspension occurred more heterogeneously than in solution. We compared a series of methyl amyloses prepared under various conditions. After exhaustive degradation with α-amylase and amyloglucosidase the amount of glucose liberated was determined and referred to the unsubstituted glucose fraction. The amount of glucose that could be liberated decreased with increasing *DS*. At the same *DS* the ratio was lower for methyl amyloses prepared under homogeneous conditions (in water solution) than for heterogeneously prepared ones. By further structure analysis of the degradation products according to the reaction scheme in Fig. 6 un-, 2-*O*-, 6-*O*-, and even 2,6-di-*O*-methylated glucose moieties could be identified as terminal units (Fig. 7), indicating that the amyloglucosidase accepts these methylation patterns while a methyl group at O-3 of the aglycon prevents the cleavage of the glucosidic bond.[84] Similar results were obtained for cationic starch ethers when the ratio of enzymatically liberated glucose was compared for samples of a certain *DS* prepared in solution and in a slurry reaction, respectively.[44] Recently, Van der Burgt et al.[85] compared normal methyl starch with highly branched amylopectin methyl starch both prepared in suspension. After partial degradation with α-amylase linear and branched fragments were separated and independently analyzed. It turned out that in amylopectin branched regions were preferably methylated, while amylose was higher substituted than linear regions of amylopectin. Richardson et al.[86] investigated hydroxypropyl starches (HPS) prepared in granular suspension and from gelatinised starch. Treatment with α-amylase and amyloglucosidase again liberated more glucose from the heterogeneously prepared HPS. Debranching of the starch with isoamylase and pullulanase with subsequent SEC analysis yielded larger fragments with a higher dispersity for the granular HPS compared to the gelatinized sample.

For cellulose similar investigations are known. Wirick[87] and Gelman[88] reported on the digestibility of water soluble HEC and CMC with cellulase, which was a mixture of endoglucanases and cellobiohydrolases. The

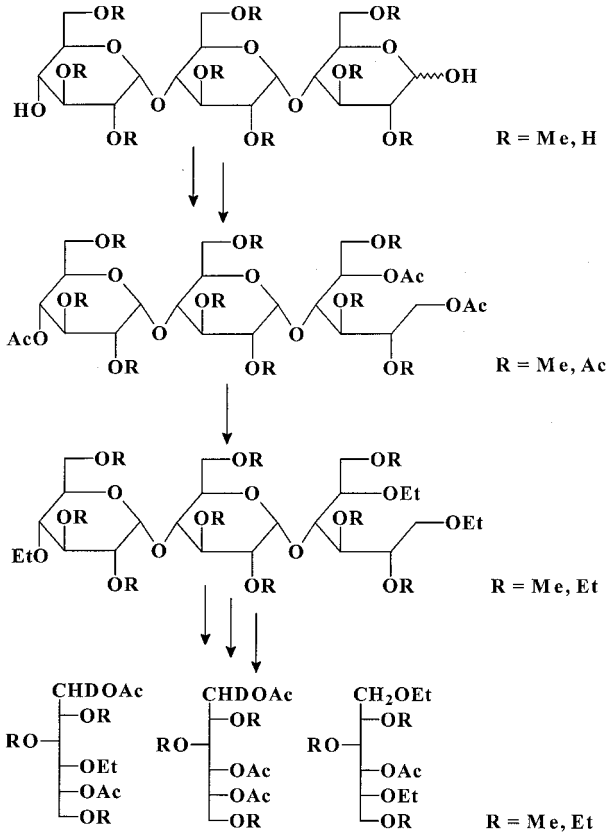

Fig. 6. Labeling and degradation of enzyme-resistant maltooligosaccharides for the independent determination of the substitution pattern of terminal and 1,4-linked-glucosyl residues and reducing glucose units.

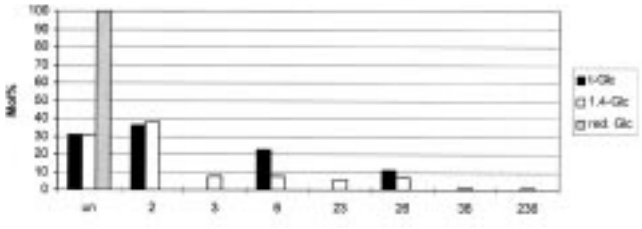

Fig. 7. Relative monomer composition in the fraction of terminal glucosyl residues (t-Glc), 1,4-linked glucosyl residues (1,4-Glc) and reducing glucose in the enzyme-resistant fraction of a methyl amylose (DS 0.69) after exhaustive degradation with α-amylase and amyloglucosidase. Each fraction is normalized to 100%. Average *DP* = 9.8. Analysis was performed according to Fig. 6.

amount of unsubstituted glucose at a certain *DS* as well as the amount liberated by the enzymes was related to average sequence length of unsubstituted glucose moieties. Exhaustive enzymatic digestion of cellulose sulfates showed the same correlation of glucose yield and distribution of the substituents in the polymer chain, as was independently determined by the above mentioned statistical approach.[78]

Horner et al.[76] thoroughly investigated various CMCs after exhaustive degradation by endoglucanases by means of SEC and further structure analysis of the obtained fractions by HPAEC-PAD.[27] Their results are reported in this issue in detail.[89]

One limitation of the enzymic approach lies in the fact that the question whether a glucosidic linkage with a certain position in the sequence of a glucan derivative is attacked by an appropriate enzyme cannot be simply answered by "yes" or "no", but the rates may differ for some orders of magnitude[90] and do not only depend on the substitution pattern of the directly neighbored glucose units but also on the pattern of further ones as on the distance to the chain terminus,[91] since an oligomeric sequence is usually involved in the formation of the active complex.

Outlook

Modern MS techniques with soft ionization as MALDI-TOF- and ESI- or APCI-MS with or without coupling to LC or CE and appropriate labeling are promising to extend the random approach in oligomer analysis to other types of derivatives.[82] Mathematical models which include mutual influences on the relative reactivities of glucose units must be developed to better fit the experimental data with a calculated distribution curve. Since any analytical approach gives an average picture of the complex material combination of fractionation steps, enzymatic digestion, and chemical degradation with statistical evaluation promises to give the most detailed information.

Experimental part

Permethylation of glucan ethers: Permethylation is performed according to a modified Hakomori procedure[38] with lithium methyl sulfinyl methanide (Li dimsyl), prepared from methyl lithium and dimethyl sulfoxide (DMSO), and methyl iodide (MeI) under nitrogen, or according to Ciucanu and Kerek[37] with NaOH and CH_3I in DMSO. Methylated products are isolated by extraction with dichloromethane, by dialysis, or – for small quantities – by solid phase extraction from a RP-cartridge. Optionally the crude product is purified on Sephadex LH20. Cationic ether derivatives are permethylated after methanolysis to methyl glucosides[19, 44] with NaOH/ CH_3I in DMSO and purified by direct chromatography on Sephadex LH20 with methanol.

Permethylation of 1,4-glucan sulfates: The sodium sulfates of the glucan derivatives are transformed to the triethylammonium salts by ion exchange to achieve solubility in DMSO. Permethylation must be carried out under optimized conditions with Li dimsyl and CH_3I in DMSO/tetramethylurea to prevent sulfate loss. The methylated product is directly isolated via Sephadex LH 20 chromatography with methanol as the eluent.[63]

Permethylation of glucan acetates and trialkylsilyl ethers of 1,4-α-glucans: Permethylation is performed according to Prehm[57] with methyl triflate/2,6-di-*tert*-butylpyridine in trimethyl phosphate[59] or with trimethyl oxonium tetrafluorobo-

rate/2,6-di-*tert*-butylpyridine in dichloromethane.[58] The product is isolated and purified as described for ether derivatives.

Permethylation of sulfoalkyl ethers: O-Sulfobutyl-β-cyclodextrins are first hydrolyzed with perchloric acid (see under *Acid hydrolysis*). Methylation is carried out after cation exchange with the triethylammonium salt with NaH and CH_3I in DMF, and the permethylated methyl glucosides are directly isolated by means of chromatography on Sephadex LH20 with methanol.

Acid hydrolysis: About 1-2mg of the permethylated sample in a 1ml-V-vial is treated with 2 m trifluoroacetic acid (TFA) at 120 °C for 2–3 h. After cooling the solvent is evaporated in a stream of nitrogen and the residue co-distilled with toluene to remove residual acid. Especially for cellulose derivatives with low *DS* or acid labile substituents as methylated silyl ethers or acetates, the procedure of Fengel et al. starting with concentrated TFA can be used.[92] An often applied procedure is the hydrolysis with perchloric acid, starting with 0.1 mL 70% perchloric acid at room temperature for swelling. After dilution to 1.2 M $HClO_4$ the sample is heated for 16 h to 100 °C. After cooling, the solution is neutralized with 2 M KOH, centrifuged and the supernatant evaporated to dryness or freeze dried.[23] This procedure can be modified according to Horner et al.[27, 76] For sulfates the reductive hydrolysis method of Garegg et al.[64] as described by Stevenson and Furneaux[65] was applied to prevent losses of early liberated glucose-2-sulfates.[63]

Methanolysis: The permethylated sample (1–2 mg) in a 1 mL V-vial is treated with 1.5 M methanolic HCl at 100 °C for 2 h. The solvent is removed in a stream of nitrogen. Methanolysis can be applied prior to acid hydrolysis to hydrophobic trialkyl silyl ethers in a two-step procedure.[51] Methyl glucosides obtained from cationic starches via methanolysis are isolated on an anion exchange column (Bio-Rad, AG 1-X4, [OH^-]) with water as eluent.[44]

Reduction and acetylation: Free sugars obtained by hydrolysis are reduced with 0.5 M $NaBD_4$ in 2 M ammonia for 1 h at 60 °C. Excess of $NaBD_4$ is destroyed with glacial acetic acid and the borate removed as its methyl ester by evaporation with methanol/acetic acid for five times at 30–40 °C. The residue is acetylated with pyridine and acetic anhydride at 90 °C for 3 h. Partially methylated and substituted glucitol acetates are extracted with dichloromethane, and thoroughly washed with aq. $NaHCO_3$ and water. The glucitol derivatives obtained are analyzed by GLC and GLC-MS.

Reductive cleavage: Reductive cleavage is carried out according to Rolf and Gray[36]. About 2 mg of the permethylated alkyl or hydroxyethyl ether[39] is dissolved in dichloromethane to a 0.05 M solution and 5 equiv. of each triethylsilane and the Lewis acid (TMS triflate or TMS mesylate/BF_3 etherate, 5:1) are added. After 2–24 h at room temperature acetic anhydride is added (20 μL) and after 2 h the reaction is quenched with aq. $NaHCO_3$. The products are extracted, washed and dried as described above. For BF_3-containing samples, methylation is performed under TFA-catalysis at 50 °C.[93] Application of reductive cleavage to methylated acyl derivatives is described by Sherman[60] and Yu and Gray.[62] Anhydroglucitol derivatives obtained are analyzed by GLC and GLC-MS.

Oligomer analysis: Sample preparation, FAB-MS and MALDI-TOF-MS analysis and statistical calculation have been described for methyl ethers,[71, 72] cellulose sulfates[78] and acetates.[59] First results with labeled oligosaccharides have been reported.[77, 82]

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