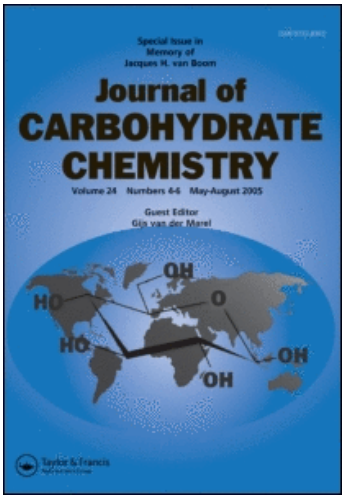


Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

New Techniques for Qligosaccharide Sequencing

Vernon N. Reinhold[a]; Eric Coles[a]; Steven A. Carr[a]
[a] Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

To cite this Article Reinhold, Vernon N. , Coles, Eric and Carr, Steven A.(1983) 'New Techniques for Qligosaccharide Sequencing', Journal of Carbohydrate Chemistry, 2: 1, 1 — 18
To link to this Article: DOI: 10.1080/07328308308058806
URL: http://dx.doi.org/10.1080/07328308308058806

J. CARBOHYDRATE CHEMISTRY, 2(1), 1-18 (1983)

NEW TECHNIQUES FOR OLIGOSACCHARIDE SEQUENCING

Vernon N. Reinhold*, Eric Coles and Steven A. Carr

Department of Biological Chemistry
Harvard Medical School
Boston, Massachusetts 02115

Received March 7, 1983

ABSTRACT

A new procedure is described for oligosaccharide sequencing that combines HPLC purification, the sensitive technique of fluorescence detection, and partial polymer degradation by ionic hydrogenation with component determination by direct chemical ionization mass spectrometry (DCI-MS) and GC-MS. The methodology is demonstrated for a series of model compounds and is shown to provide linkage information as well as sugar sequence.

INTRODUCTION

Structural characterization of polysaccharides by chemical methods are usually performed in two sequential steps; the first is the generation of a suitable mixture of overlapping oligomers to ascertain sequence,[1-3] and the second is the total degradation of the oligomer to monosaccharides from which linkage information is obtained. Oligomer permethylation[4] combined with acid catalyzed degradation to monosaccharides has been the most widely used

 0732-8303/83/0201-0001$3.50/0

method to determine sugar linkage. The partially methylated products are reduced, acetylated and separated for structural identification. Points of acetylation are determined by chromatographic comparison with standards, by MS, or a combination of both, GC-MS.[5]

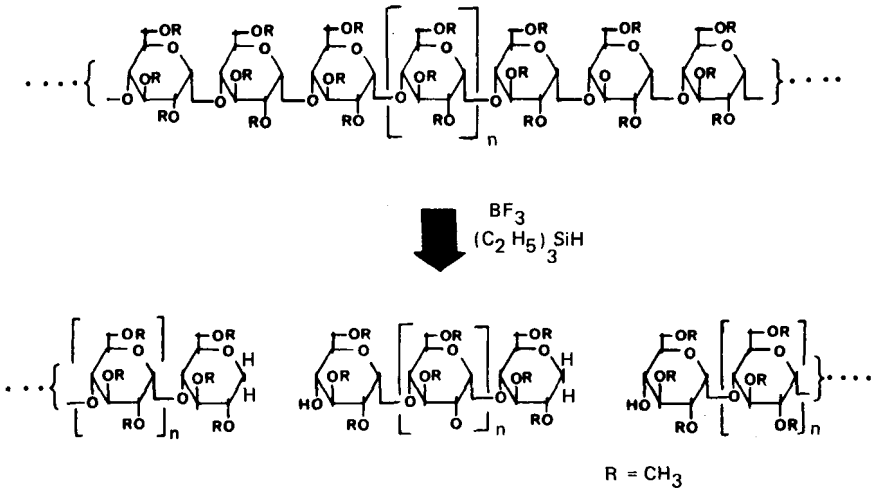
Unfortunately, acid-catalyzed polymer degradation initiates a series of problems related to the exposure of the reactive hemiacetal group and, in part, to the general instability of many sugar moieties under the conditions imposed. Furthermore, the original ring form (pyran or furan) may be irreversibly lost and it is difficult to generate a representative distribution of smaller oligosaccharides for overlap sequence analysis.

A recent report has appeared which describes the total depolymerization of a cyclic heptasaccharide to its constituent monomers using procedures that cause reductive cleavage at glycosidic linkages.[6] This simple, one-step, organosilane reduction offers several important advantages for oligosaccharide studies. Ionic hydrogenation, or reductive cleavage, as it is more descriptively called, not only avoids the complications inherent in acid-catalyzed degradations, but provides for the direct determination of the monosaccharide ring form and, in addition, produces a single structural entity free of anomeric complications that can be easily characterized by GC and GC-MS. Reductive cleavage, or hydrosilylation, of the glycosidic bond relies on the successful hydride transfer from silicon to an activated carbon center. Hydride transfer from silicon to electropositive carbon has been shown to be thermodynamically favored[7] and triethylsilane reductions of acetals, aldehydes and ketones in trifluoroacetic acid has been previously demonstrated.[8] Contrary to other boron and aluminum hydrides, silicon hydrides do require activation of the carbon center and both Brønsted and Lewis acids have been successful in promoting this hydride transfer. Boron trifluoride has been shown to be very effective in these hydrosilylation reactions.[9]

In efforts to incorporate these advantages into a sequencing strategy for permethylated (PM) polysaccharides, we have altered

the published reaction conditions[6] to generate mixtures of oligomers (i.e., products of partial reductive cleavage) that can provide an overlapping polymer series. Under the conditions chosen, glycosidic linkages which undergo cleavage yield an anhydro alditol on the terminal side of the oligomer chain and a free hydroxyl group on the reducing side (Scheme 1).

In combination with this unique degradation approach, we have tried to amplify the sensitivity for detection of carbohydrate samples by HPLC, via the incorporation of fluorescent groups prior to chromatographic purification and isolation. Two derivatives have been employed for this purpose; the pyridinylamine (PA) group[10] which is introduced at the reducing end of the polysaccharide chains before permethylation and reductive cleavage, and the naphthoyl (Np) group which is incorporated on the hydroxyl groups generated as a result of the partial reductive cleavage. In addition to enhancing detector sensitivity, the PA moiety provides a functional group responsive to solvent acidity. This



Scheme 1. Generalized accounting of partial reductive cleavage on linear glucopolymer. The oligomeric series will consist of three different series (i.e., terminal, reducing and intermediate).

feature allows selective HPLC isolation of reducing-end fragments from the oligomeric mixtures generated as a consequence of partial reductive cleavage. Analysis of these PA- and Np-derivatized, partially reductively cleaved polysaccharides by DCI-MS[12] yields molecular weight and sequence information for each oligomer, while linkage data is obtained by complete reductive cleavage to monosaccharides followed by GC-MS. These procedures are illustrated below with two model compounds, β-cyclodextrin and maltotriose.

RESULTS AND DISCUSSION

Reductive cleavage and acetylation of PM-β-cyclodextrin has been reported to yield a single product, the 4-O-acetyl-2,3,6-tri-O-methyl-1-anhydroglucitol.[6] We have found that this reaction may be modified to generate a mixture of intermediate lower molecular weight oligosaccharides, a process referred to here as partial reductive cleavage. Using the conditions described below (see Experimental), this reaction appears free of side products since all materials are observed to have either the hydroxyl or the 1-deoxy terminus or, in the case of the intermediate chain oligomers, both (see Scheme 1). For example, PM-β-cyclodextrin was subjected to partial reductive cleavage and the extent of depolymerization was monitored by HPLC with fluorescence detection after the nascent hydroxyl groups were derivatized with naphthoyl chloride. Seven cleanly resolved peaks were obtained which corresponded to the monomer through the open-chain heptamer (Fig. 1). The cyclized unreacted starting material would not be detected under these conditions. Each of these HPLC fractions has been characterized by ammonia DCI-MS which provides both molecular weight and sequence information. Ammonia DCI is uniquely capable of accentuating carbohydrate sequence by generating fragments at each glycosidic linkage with enhanced ion abundance.[11,12] Thus, for every glycosidic bond reductively cleaved in this cyclic heptamer, the combination of derivatization, HPLC, and fluorescence detection provided a qualitative and sensitive measure of sample depolymerization.

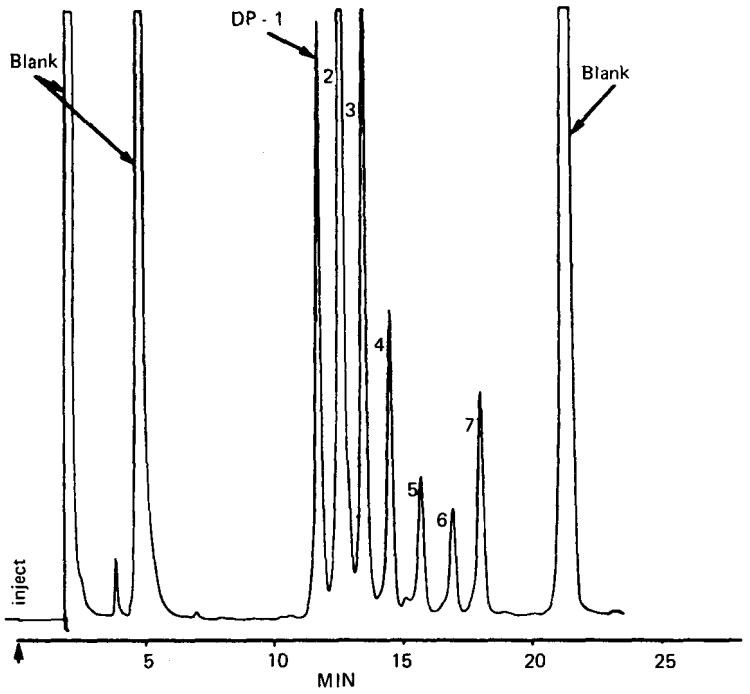


Figure 1. Mixture of naphthoylated products from partial reductive cleavage (4 minutes at 0°C) of permethylated β-cyclodextrin, approximately 0.01 µg total material. Column: C-6 Spherisorb, 25 X 4.6 mm (ID). Elution solvent: 50% to 60% acetonitrile in water (0.15M ammonium hydroxide), 1 ml/min program 15 min. Peaks not labeled are unidentified.

A faster and more direct method of following the extent of reductive cleavage is the removal of fractions from the reaction mixture at varying time intervals, acetylation and, without chromatographic separation, analysis of the products by ammonia DCI-MS. An example of this approach for the four-minute time interval is shown (Fig. 2). The ratio of molecular adduct ions (e.g., m/z 470, 674, 878, 1082, 1286 and 1446) reflects the distribution of oligomers (e.g., DP-2, 3, 4, 5, 6

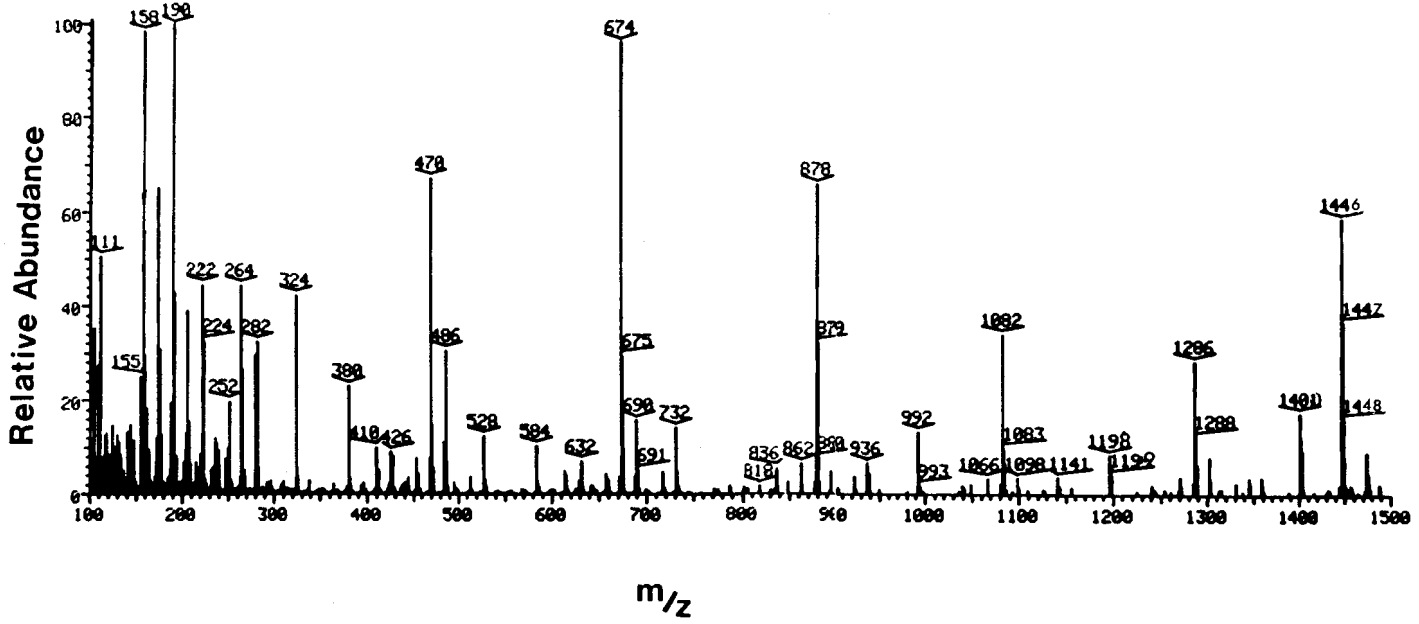


Figure 2. DCI-MS following reductive cleavage for 4 min at 0°C of PM-β-cyclodextrin. The products were acetylated prior to MS.

and 7, respectively) with the last ion, [m/z 1446, $(M + NH_4)^+$], being the unreacted cyclic heptamer starting material.

Oligosaccharides with the reducing end free can be first prepared as the PA-derivative, methylated, and reductively cleaved in a similar manner. The time course of the partial reductive cleavage may again be monitored with high sensitivity by periodic sampling and direct HPLC injection. For example, Figure 3 is the HPLC chromatogram of the products formed after four minutes of reductive cleavage of the PA-PM-trisaccharide, maltotriose. Analysis by ammonia DCI of peaks one and two showed them to be the 4-O-acetyl derivatives of PA-PM-Glc and PA-PM-Glc-Glc, respectively. Characteristic of this type of analysis is the data for peak 3 (Fig. 3) which proved to be the starting material, PA-PM-Glc-Glc-Glc (Fig. 4). Since the pyridinyl group imparts basicity to the molecule, fragments containing this residue are observed as protonated molecular ions and not ammonium adduct ions (e.g., m/z 751, $(M + H)^+$]. The introduction of the PA-group provides two important analytical advantages; first, a mass spectral label for reducing-end characterization and, second (as shown below), the compounds containing the basic pyridinyl residues can be separated chromatographically.

Reductive cleavage of PA-PM-oligosaccharides produces three groups of products which are related to the terminal, intermediate and reducing end (pyridinyl group) of the oligomer (see relationship to Scheme 1). In this case, however, the basic reducing-end fragments may be selectively separated from the other non-basic oligomers by taking advantage of the variation in elution time on C-18 columns with changes in solvent acidity. Residual polar sites present on the C-18 column prevent elution of the pyridinyl derivatized materials until the solvent is made basic with ammonium hydroxide. Thus, to generate a separation of the PA-PM-oligomers from other neutral components, a water-acetonitrile program is used with the solvents maintained at acid pH (Program I; see Legend, Fig. 5). Subsequently, the column is recycled to the

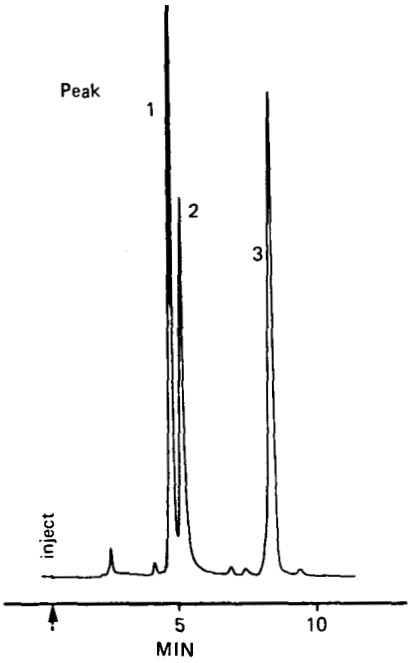


Figure 3. Products following reductive cleavage for 4 min at 0°C of PA-PM-maltotriose; reaction stopped with water; methylene chloride extract directly analyzed by HPLC.
Column: C-6 Spherisorb, 25 X 4.6 mm (ID).
Elution solvent: Acetonitrile:water 55:45 (0.15M ammonium hydroxide), 1 ml/min. Peaks not labeled are unidentified.

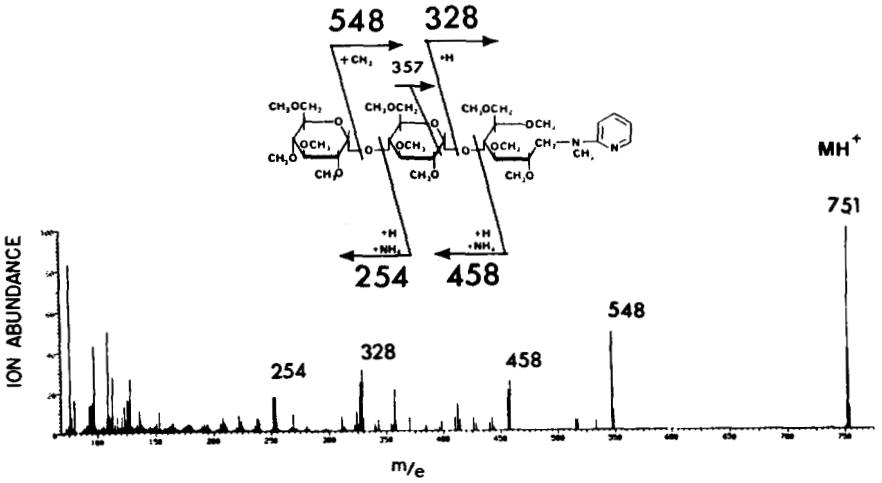


Figure 4. DCI-MS of peak 3 (Fig. 3).

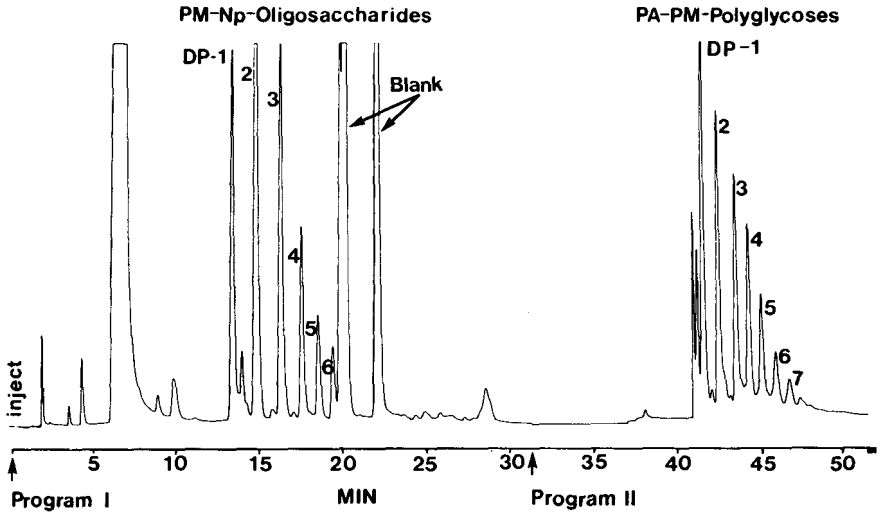


Figure 5. HPLC separation of mixture containing Np-derivatives(Fig. 1) and PA-PM-oligosaccharides obtained from corn syrup. Column: C-18 Spherisorb, 15 cm X 4.6 mm (ID). Program I, 40 to 100% acetonitrile:water (0.1 M Acetic acid), 1 ml/min, 30 min, recycled column to 40% acetonitrile:water (0.15 M ammonium hydroxide). Program II, 40 to 80% acetonitrile:water (0.15 M ammonium hydroxide), 1 ml/min, 20 min. Peaks not labeled are unidentified.

starting conditions and the program is repeated with the solvents maintained at basic pH (Program II). The separation is illustrated in Figure 5 for a complex mixture containing a series of linear PA-PM-polyglycoses (isolated and prepared from corn syrup), and the seven PM-Np-oligosaccharides generated from PM-β-cyclodextrin as discussed above. Under these conditions the family of naphthoyl oligomers is separated with Program I solvents, while the PA-PM-polyglycoses are eluted under the basic conditions of Program II. (Elution of the same mixture with acetonitrile/water gradients at basic pH alone results in an extremely complex chromatogram due to simultaneous elution of both families of materials.) Fluorescence derivatization combined with this chromatographic flexibility also provides an opportunity to

isolate larger oligomers, induce further chemical degradation, and recycle at great sensitivity.

To identify specific hydroxyl groups involved in each linkage, the samples are reductively cleaved completely and the products analyzed by GC-MS. To illustrate this point, a sample of PA-PM-maltotriose was prepared and reductively cleaved for four hours. The products were acetylated and analyzed by GC (Fig. 6) and GC-MS (Figs. 7-9). Electron ionization mass spectra provide many intense specific fragments which allow a direct assignment of linkage position. These assignments can also be corroborated by GC retention time.

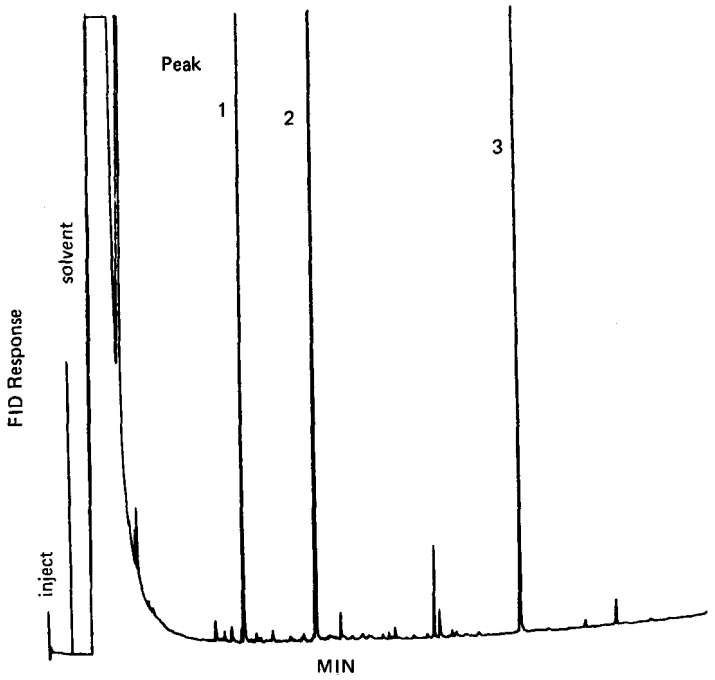


Figure 6. Gas-liquid chromatography of products obtained following two hours reductive cleavage and acetylation. Conditions as described in experimental section. Peaks not labeled are unidentified.

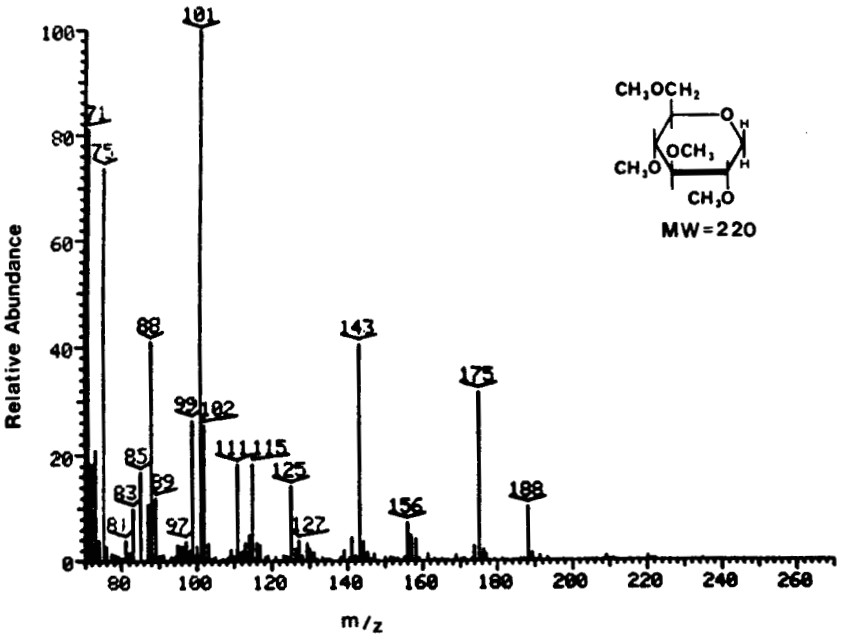


Figure 7. Electron ionization mass spectrum of peak 1 (Fig. 6); 1-deoxy-2,3,4,6-O-methyl-D-Glucopyranose.

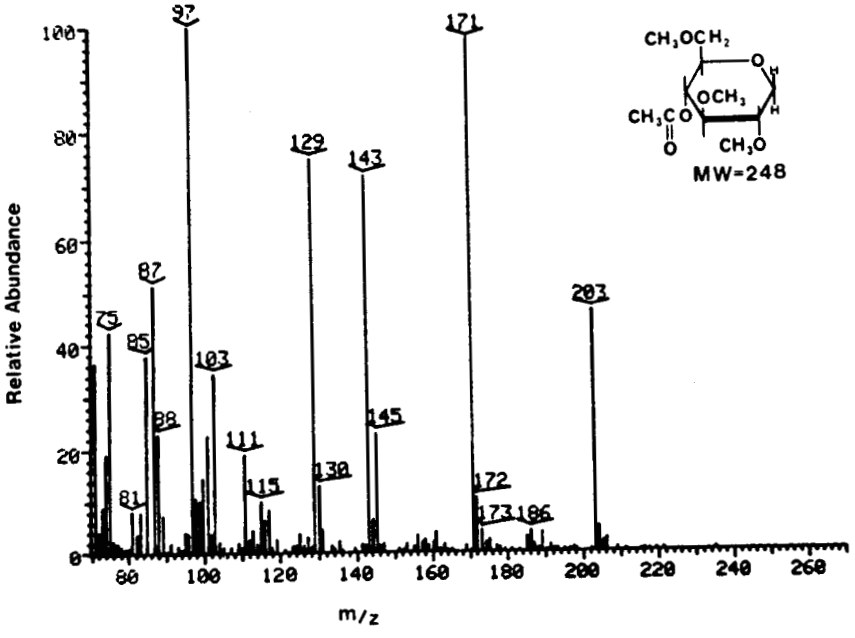


Figure 8. Electron ionization mass spectrum of peak 2 (Fig. 6); 1-deoxy-4-O-acetyl-2,3,6-O-methyl-D-Glucopyranose.

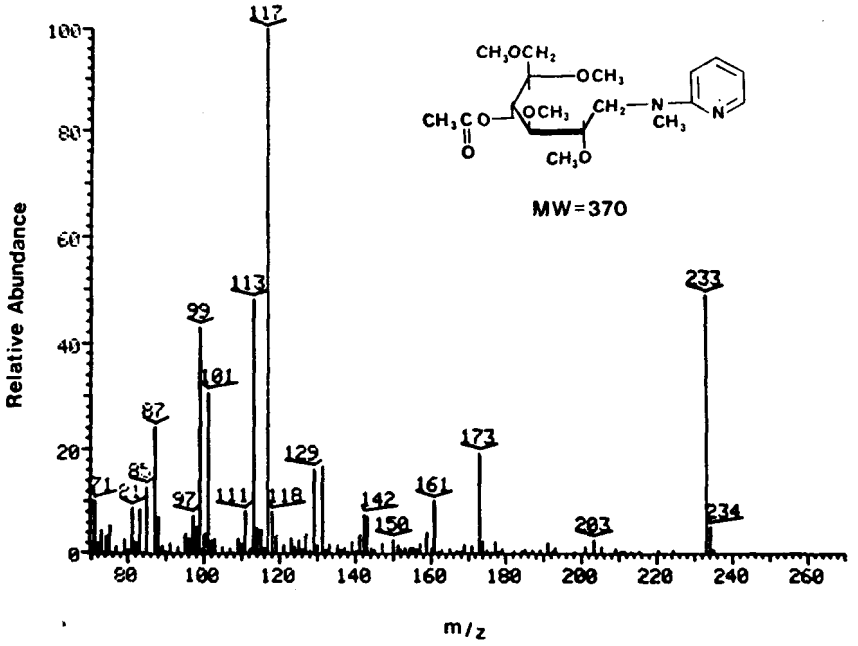


Figure 9. Electron ionization mass spectrum of peak 3 (Fig. 6); 1-(pyridinylmethylamine)-4-O-acetyl-2,3,6-O-methyl-D-glucositol.

It should be noted that following reductive cleavage the PA-PM-derivatized residues were resistant to naphthoylation under the conditions established for the other oligosaccharides.

In the work described above, all of the samples possessed the α(1-4) linkage. Exploration of such a reaction for polysaccharides, to allow differentiation of linkages, requires an indication of stability for each type. To assess this possibility, we prepared the PA-PM-derivatives of disaccharides containing different linkages and followed the kinetics of reductive cleavage using HPLC and fluorescence detection (Fig. 10). Considerable variation in glycosidic bond stability could be observed with each positional isomer, and even the anomers kojibiose and sophorose show surprising differences in reactivity. For example α(1-4) linkages are almost completely

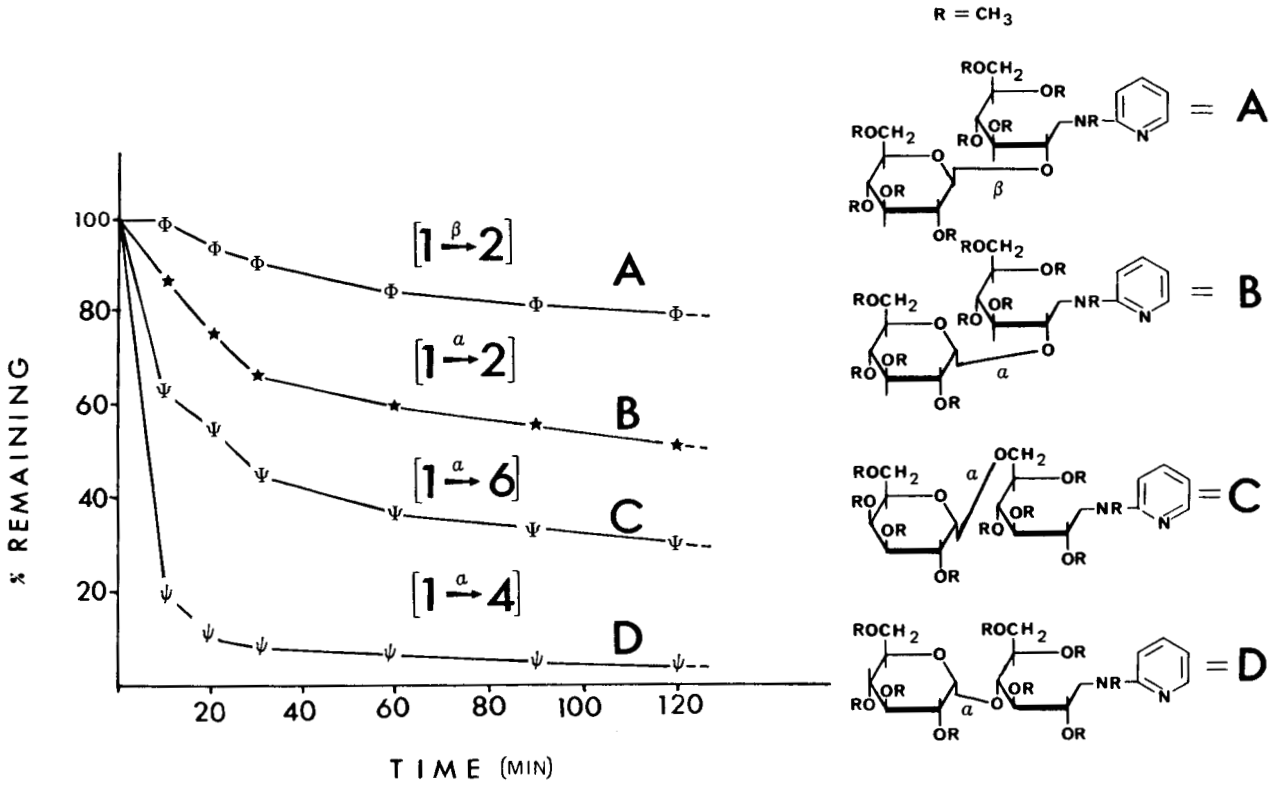


Figure 10. Linkage stability study of selected disaccharides under reductive cleavage conditions. Samples prepared as PA-PM-derivatives. The percent reductive cleavage was determined by the percent loss in peak area relative to an internal standard (PA-PM-glucose). Column: C-18 Spherisorb, 15 cm X 4.6 mm (ID).

cleaved while β(1-2) linkages remain essentially intact. Such reactivity in the role of reductive cleavage may possibly be used to aid structural analysis.

EXPERIMENTAL

A. High Performance Liquid Chromatography. Chromatography was performed with columns (15 cm or 25 cm X 4.6 mm ID) packed with either C-6 or C-18 Spherisorb (5 μm). Solvent gradients were controlled with dual pumps and a programmer (Waters Associates). For specific solvent conditions used, see appropriate figure legend. All solvents were HPLC grade from Burdick and Jackson. Fluorescence detection was performed using a Kratos Model FS970 Spectrofluoro Monitor, excitation at 232 nm, detection >320 nm.

B. Mass Spectrometry. The mass spectrometer utilized in this study was a Finnigan-MAT 312 with reverse geometry (magnetic sector preceding electric sector) and fitted with a combined CI/EI ionization source.

a). Direct chemical ionization: The instrument is equipped with a programmable DCI probe power supply unit for ramping the wire current. To maximize the desorption of intact neutral molecules this wire was coated with a polyimide surface.[11] Solutions containing the sample material are added to the coated wire surface, air dried and inserted directly into the CI chamber of the ionization source. Ammonia was used as the reagent gas.

b). Gas chromatography/mass spectrometry: Fused silica columns containing a chemically bonded liquid phase (Durabond-1701, J & W Scientific, Inc.) were directly coupled to the mass spectrometer and eluting peaks were ionized by both chemical and electron ionization methods. Temperature program: 75-300°C at 10°C/minute. The reagent gas used while in the CI mode was ammonia. Electron ionization voltage in the EI mode was 70 eV, ion source temperature 200°C.

C. Oligosaccharide Reductive Cleavage and Derivatization.

a). Reductive cleavage and acetylation: All samples were first permethylated as described below and reductive cleavage was carried out as previously described[6] with the following modifications. The reducing reagent (containing boron trifluoride etherate, 180 µl, triethylsilane, 240 µl, and trifluoroacetic acid, 40 µl, were combined at 0°C and brought into a homogenous solution using methylene chloride (200 µl). To a dried residue of PM-oligosaccharide(s) was added 100 µl of the above reducing reagent and the solution was brought to the desired reaction temperature (see figure legends for specific details). To terminate the reductive cleavage reaction and derivatize all liberated hydroxyl groups 50 µl of acetic anhydride was added and the solution warmed to 40°C for 10 minutes. This acetylation must precede extraction to overcome water solubility and loss of low molecular weight polar sample reaction products. Product purification was accomplished with methylene chloride extraction from an aqueous solution by the addition of 3 ml of methylene chloride and 3 ml of water. This total volume was vortexed for one minute or until the upper layer was clear. The methylene chloride layer was washed twice with 3 ml of water and concentrated under nitrogen. The last residues of liquid were removed by the addition of 0.5 ml of ethanol and this total solution was brought to dryness. The products were stored in acetonitrile.

b). Naphthoyl derivatization: To maximize oligomer detectability and characterize the products of partial reductive cleavage during the purification by HPLC, the liberated hydroxyl groups were derivatized with naphthoyl chloride. Since quantification of low molecular weight and water-soluble products were not of prime concern during the partial reductive cleavage, the reaction was stopped by the addition of 0.1 M sodium bicarbonate and the products extracted with an equal volume of methylene chloride. This extract was washed twice with distilled water and dried under a stream of nitrogen. To this residue was added

1 ml of pyridine containing 3 mg of 2-naphthoyl chloride and 5 mg of dimethylaminopyridine. The solution was heated for 16 hours at 60°C in a Teflon-lined screw cap reaction vial.[13] The pyridine was removed under a stream of nitrogen and the residue dissolved in methylene chloride and washed twice with an equal volume of dilute ammonium hydroxide (pH > 9.0).

c). Pyridinylamine derivatization: Introduction of the fluorescent pyridinylamine group was performed as previously described.[14-16] For these studies a methanolic solution (500 µl) containing 85 mg of 2-aminopyridine and 40 µl of acetic acid was added to the sample (1-5 mg) and heated at 100°C for 20 min. The reaction was cooled and 500 µl of methanol was added containing 20 mg of sodium cyanoborohydride. This sealed volume was heated for 3 hr. at 100°C. The PA-derivatized material was purified by passage over a Dowex 50 ion exchange resin (acid form) as described earlier[17] and eluted with 2 M pyridine.

d). Permethylation: Carbohydrate samples and their PA-derivatives were permethylated by the general procedure of Hakamori[4], as modified by Sanford and Conrad[18]. The potassium salt form of the anion was prepared[19] and methylations were carried out twice for 30 min. intervals. The reaction was cooled to 0°C before addition of the methyl iodide. Excess methyl iodide was removed under a stream of nitrogen preceding the second methylation step.

CONCLUSIONS

We have briefly outlined an alternative approach of great simplicity for oligosaccharide sequencing by HPLC, DCI-MS and the determination of linkage by GC-MS. The method is based on saccharide depolymerization by carefully controlling the conditions of ionic hydrogenation. A most important feature in this approach is the combination of fluorescence detection and the purification of oligomers by HPLC. Kinetic studies of different glycosidic linkages indicates considerable variation in stability under the conditions of reductive cleavage and these differences

may provide additional flexibility for structural determination. Further work is currently in progress with larger oligosaccharides of varying structure to assess the limitations and advantages of this approach.

ACKNOWLEDGEMENTS

We would like to thank Mr. C. Coretsopoulos for his excellent technical assistance and Dr. C. E. Costello for reviewing the manuscript. This work was supported by a grant (PO1 GM 26625) from the National Institutes of General Medical Sciences, National Institutes of Health, Bethesda, Maryland 20014.

REFERENCES

1. B. Lindberg, J. Lonngren and S. Svensson, Adv. Carbohydr. Chem. Biochem., 31, 185 (1975).
2. I. Danishefsky, R. L. Whistler and F. A. Bettelheim, Introduction to polysaccharide chemistry in "The Carbohydrates," Vol. IIA, W. Pigman and D. Horton, eds., Academic Press, New York, New York, 1970, p. 375.
3. L. D. Melton, L. Mindt, D. A. Rees and G. R. Sanderson, Carbohydr. Res., 46, 245 (1976).
4. S. Hakamori, J. Biochem. (Tokyo), 55, 205 (1964).
5. H. Bjorndal, C. G. Hellerqvist, B. Lindberg and S. Svensson, Angew. Chem. Int. Ed. Engl., 9, 610 (1970).
6. D. Rolf and G. R. Gray, J. Am. Chem. Soc., 105, 3539 (1982).
7. G. G. Hess, F. W. Lampe and L. H. Sommer, J. Amer. Chem. Soc. 87, 5327 (1965).
8. D. N. Kursanov, Z. N. Parnes and M. N. Loim, Synthesis, 9, 633 (1974).
9. M. P. Doyle, C. T. West, S. J. Donnelly and C. C. McOsker, J. Organometallic Chem., 117, 129 (1976).
10. S. Hase, S. Hara and Y. Matsushima, J. Biochem., 85, 217 (1979).
11. V. N. Reinhold and S. A. Carr, Anal. Chem., 54, 499 (1982).
12. V. N. Reinhold and S. A. Carr, Mass Spectr. Reviews, 2, in press (1983).
13. G. Golik and K. Nakanichi, Personal Communication (1983).
14. S. Hase, T. Ikenaka and Y. Matsushima, Biochem. Biophys. Res. Comm., 85, 257 (1978).

15. S. Hase, T. Ikenaka and J. Matsushima, Biochem. (Japan), 85, 989 (1979).

16. S. Hase, T. Ikenaka and Y. Matsushima, J. Biochem. (Japan), 90, 407 (1981).

17. M. Kasakai and Z. Yosisawa, J. Biochem. (Japan), 92, 295 (1982).

18. P. A. Sanford and H. E. Conrad, Biochemistry, 5, 1508 (1966).

19. B. S. Valent, A. G. Darville, M. McNeil, B. K. Robertson and P. Albersheim, Carbohydr. Res., 79, 165 (1980).