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CAPILLARY GAS CHROMATOGRAPHY OF REDUCING DISACCHARIDES WITH NITROGEN-SELECTIVE DETECTION AND SELECTED-ION MONITORING OF PERMETHYLATED DEOXY(METHYLMETHOXYAMINO)ALDITOL GLYCOSIDES*

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SUMMARY

Reduction of disaccharide methoximes with methanolic sodium cyanoborohydride, followed by permethylation with methylsulphinyl–methyl iodide, yields the corresponding deoxy(methylmethoxyamino)alditol glycosides. Only one derivative is obtained for each glycosyl aldose, but glycosyl ketoses give two diastereomeric derivatives. The derivativatives of one ketose- and five aldose-terminated disaccharides could be separated on borosilicate glass capillaries coated with Superox 0.1, OV-101 or Chirasil-Val. Selective detection by a nitrogen–phosphorus-selective detector gave at least a five-fold increased response when compared to a non-selective flame ionization detector. The electron-impact mass spectra (70 eV) of the derivatives have characteristic fragmentation patterns, with specific diagnostic ions, which make possible the specific detection by selected-ion monitoring of aldose- or ketose-containing disaccharides, with different types of O-glycosidic linkages. The method is suitable for the assay of reducing disaccharides in complex mixtures of biological origins, and for end-group identification in methylation analysis.

INTRODUCTION

Because of their low volatility, disaccharidess must be subjected to chemical derivatization prior to gas chromatography (GC). The trimethylsilyl ethers first introduced by Bayer[1] and Sweeley *et al.*[2] are very popular, due to their ease of preparation and good volatility. Oligosaccharides as large as octaose have been successfully chromatographed[3]. Other volatile derivatives of oligosaccharides like the trifluoroacetates[4] have also been used. However, these derivatives are of limited usefulness because they do not affect the anomeric carbon atoms; and show a remarkable propensity for yielding multiple derivatives with reducing sugars, thus making identification and quantification very difficult, sometimes impossible. Destruction of the

* Dedicated to Professor Dr. Ernst Bayer on occasion of his 60th birthday.

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anomeric centres by oxime and methoxime formation[5] leads to two isomeric *syn*- and *anti*-forms that can be well separated by capillary GC, yielding two peaks for each sugar. With complex mixtures of sugars, overlapping of the peaks is unavoidable, even when long capillary columns are used[6]. Aldonitrile acetates[7–9], on the contrary, yield a single derivative for each aldose-terminated disaccharide, but they are not applicable to glycosyl ketoses. Reduction by sodium borohydride followed by acetylation[10], trimethylsilylation[11] or trifluoroacetylation[12–14] to obtain the corresponding alditol derivatives circumvents these problems, but creates new difficulties. Unequivocal identification of the parent sugar becomes impossible, because different reducing sugars yield identical alditols upon reduction. For example, D-arabinose and D-lyxose yield the same D-arabitol, and enantiomeric D- and L-glucitol are obtained after reduction of D-gulose and D-glucose respectively. The picture is further complicated by the fact that reduction of ketoses or ketose-containing saccharides introduces a new asymmetric centre in the molecule. Frutose and sorbose, for example, not only afford the same glucitol upon reduction, but each of them yields also a mixture of two diastereomeric alditols.

Chromatographic assays of carbohydrates in complex mixtures are limited by the lack of selectivity of the most detectors used in carbohydrate analysis. An highly selective detection can be achieved by gas chromatography–mass spectrometry (GC–MS) and an high degree of specificity is attainable by selected-ion monitoring (SIM). In spite of the drawbacks of GC, mass spectrometric and GC–MS techniques have been used with trimethylsilylated di- and trisaccharides[15,16], trimethylsilylated disaccharide oximes[17,18] and permethylated alditol derivatives of di-[19,20], tri[21] and tetrasaccharides[22]. Differences in the mass spectra could be related, in some instances, to the position of the O-glycosidic linkage. Recently, nitrogen–phosphorus-selective detection (NPD) has been used for the selective GC assay of amino sugars as their alditol peracetates[23].

We recently described a new derivatization procedure for obtaining a single derivative for each aldose, involving reduction of monosaccharide methoximes to the corresponding deoxy(methoxyamino)alditols, followed by trimethylsilylation or acetylation[24]. In this report, we describe the permethylated deoxy(methylmethoxyamino)alditol glycosides as suitable derivatives for the selective capillary GC assay of disaccharides with NPD. The characteristic mass spectra of the derivatives enable selective detection by SIM–GC–MS with differentiation between aldose- and ketose-containing disaccharides. Group detection according to the position of the O-glycosidic union is also possible.

EXPERIMENTAL

Materials

Standard disaccharides were purchased from Alltech Associates. Sodium cyanoborohydride (Aldrich Europe) was purified by recrystallization of its dioxane complex, and it was liberated from the complex by heating *in vacuo*[25]. Pyridine and dimethyl sulphoxide were reagent grade and used after appropriate drying and distillation. Reagent grade methoxyammonium hydrochloride (Tokyo Kasei), methyl iodide (BDH) and di-*tert.*-butyl peroxide (Ventron, Karlsruhe, F.R.G.) were used without further purification.

Instruments

Gas chromatography was performed with a Carlo Erba instrument (Mega Series 5300) equipped with a split–splitless injector, FID-40 and NPD-40 (Carlo Erba) detectors and two Carlo Erba EL-480 electrometers. A low dead-volume T-piece, supplied with 3 ml min^{-1} helium as make-up gas, was used to connect the column end to both detectors. Simultaneous recording of the detector signals was achieved with a Knauer dual-channel compensation recorder. Integration was performed with a Spectra Physics SP 4100 computing integrator. GC–MS was performed with a Shimadzu instrument, Model QP 1000.

Gas chromatography

The permethylated deoxy(methylmethoxyamino)alditol glycosides were separated in laboratory-made 25 m × 0.20 mm I.D. borosilicate glass capillaries coated statically with Superox 0.1 (Alltech). Hydrogen was used as a carrier gas, p_i = 70 kPa. The injector and detectors temperatures were maintained at 300°C. The NPD-40 detector was operated in the nitrogen–phosphorus mode, air flow-rate 350 ml min^{-1}, hydrogen flow-rate 35 ml min^{-1}, with helium as make-up gas, flow-rate 30 ml min^{-1}. The conditions for flame ionization detection (FID) were: air, 350 ml min^{-1}; hydrogen, 25 ml min^{-1}. The oven temperature was maintained at 240°C during the initial 5 min, then raised at 1° min^{-1} to 260°C, or maintained at 260°C during the analysis. Alternatively, glass capillaries coated with OV-101 (25 m × 0.20 mm I.D.) or Chirasil-Val (25 m × 0.25 mm I.D.) were used isothermally at 225 and 190°C respectively.

Gas chromatography–mass spectrometry

The injector and oven conditions were as described for GC. The column effluent was introduced directly into the ion source by means of a highly deactivated 1 m × 0.20 mm I.D. fused-silica column connected to the glass capillary through a Supeltex-M2 butt connector (Supelco). The temperatures of the transfer line and ion source were 300 and 200°C respectively. Continuous scanning from m/z 40 to 500 was effected every 1.2 s at 70 eV, with a multiplier voltage of 2450 V.

Derivatization

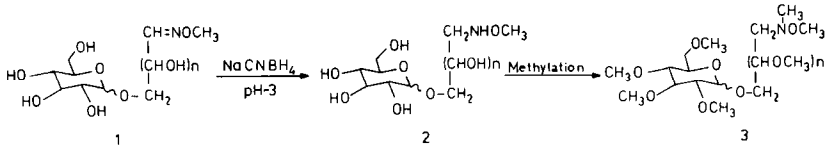
The disaccharides (0.8 mg of each) were derivatized together to the corresponding methoximes in a 1-ml Reacti-Vial (Pierce, Rockford, IL, U.S.A.) by heating with 100 µl of a solution of methoxyammonium hydrochloride in dry pyridine (25 mg ml^{-1}) at 80°C for 30 min. After evaporation of the solvent under a light stream of nitrogen, 50 µl of 1 *M* methanolic hydrochloric acid and 200 µl of a 1 *M* solution of sodium cyanoborohydride in absolute methanol were added to the residue. The mixture was sonicated for 45 min, with occasional addition of methanolic hydrochloric acid in order to maintain the pH at about 3. After concentration of the mixture under nitrogen, 100 µl of absolute methanol were added and the solution was heated at 80°C for 30 min. The solvent was evaporated under a stream of nitrogen and the residue dried overnight *in vacuo* over phosphorus pentoxide.

A solution of methylsulphinyl carbanion in dimethyl sulphoxide was prepared from dimethyl sulphoxide and di-*tert.*-butyl peroxide[26], and 100 µl were added to the dry residue. The mixture was sonicated for 15 min, then 40 µl of methyl iodide

were added and the sonication was continued for 20 min. The permethylated deoxy-(methylmethoxyamino)alditol glycosides were extracted three times with 100-μl portions of chloroform. The organic fractions were collected and dried over molecular sieves. The solvent was carefully evaporated under a stream of nitrogen. The residue was dissolved in 50 μl of dichloromethane. Aliquots of this solution were used directly for GC.

RESULTS AND DISCUSSION

The reduction of disaccharide methoximes with methanolic sodium cyanoborohydride proceeds at room temperature at pH 3, to yield the corresponding deoxy-(methoxyamino)alditol glycosides. After permethylation with the methylsulphinyl/methyl iodide reagent, the corresponding permethylated deoxy(methylmethoxyamino)alditol glycosides are obtained, as exemplified in Scheme 1. A single derivative is obtained for each glycosyl aldose, but ketose-terminated disaccharides yield two diastereomeric derivatives. The corresponding peaks appear well separated in the chromatograms. Unequivocal identification and quantitation of the derivatives of both types of reducing disaccharides can be achieved in the same chromatographic assay.



Scheme 1.

The derivatives of the following reducing disaccharides could be well separated in 15–20 min, on 25 m × 0.20 mm I.D. borosilicate glass capillaries coated with Superox 0.1: turanose, cellobiose, maltose, lactose, melibiose and gentiobiose (Fig. 1A). When OV-101 or Chirasil-Val was used as the liquid phase, the separation was achieved at lower temperatures in approximately the same time. Under isothermal conditions, all the derivatives were separated at 225°C with OV-101 or 190°C with Chirasil-Val as the liquid phase (Fig. 1B). Although the derivatives of maltose and lactose could not be completely resolved, enough separation was attained to allow electronic integration.

A standard sample was analysed with simultaneous detection by NPD and FID (Fig. 2). The column effluent was directed to both detectors simultaneously by means of a low dead-volume T-piece connected to the detectors by two 10-cm pieces of highly deactivated fused-silica tubing. In order to guarantee an efficient 1:1 splitting of the column effluent at the T-piece, a 3 ml min^{-1} stream of helium as make-up gas was supplied to the T-connection. Simultaneous recording of both detector signals was obtained by connecting each of the electrometers to a dual compensation recorder. With the NPD operating in the nitrogen–phosphorus mode, a five-fold increase in response was obtained as compared to FID. The selectivity of NPD was demonstrated by the fact that, under identical experimental conditions, no response was obtained for trimethylsilylated disaccharides.

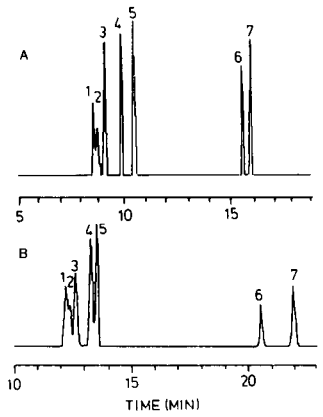


Fig. 1. Total ion current (TIC) chromatogram for the GC–MS assay of deoxy(methylmethoxyamino)alditol glycoside O-methyl ethers. Peaks (derivatives of): 1 = turanose-1; 2 = turanose-2; 3 = cellobiose; 4 = maltose; 5 = lactose; 6 = gentiobiose; 7 = melibiose. (A) 25 m × 0.20 mm I.D. glass capillary coated with Superox 0.1; oven temperature 240°C for 5 min, then raised at 1° min^{-1} to 260°C. (B) 25 m × 0.25 mm I.D. glass capillary coated with Chirasil-Val; oven temperature 190°C (other conditions as in Experimental).

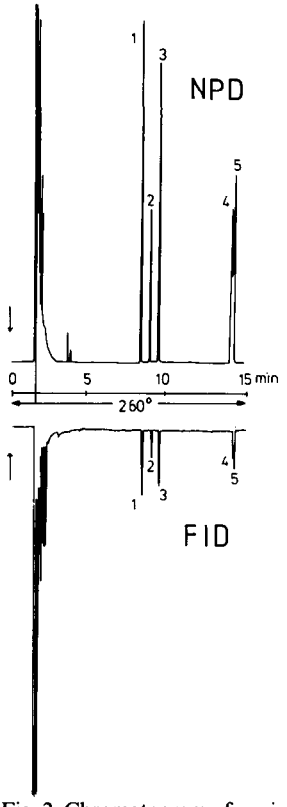


Fig. 2. Chromatogram of a mixture of disaccharides, consisting of 40 pmol of each deoxy(methylmethoxyamino)alditol glycoside O-methyl ether, with simultaneous FID and NPD. Column: glass capillary, 25 m × 0.20 mm I.D., coated with Superox 0.1; attenuation 1 × 64. Other conditions as in Experimental. Peaks (derivatives of): 1 = cellobiose; 2 = maltose; 3 = lactose; 4 = gentiobose; 5 = melibiose.

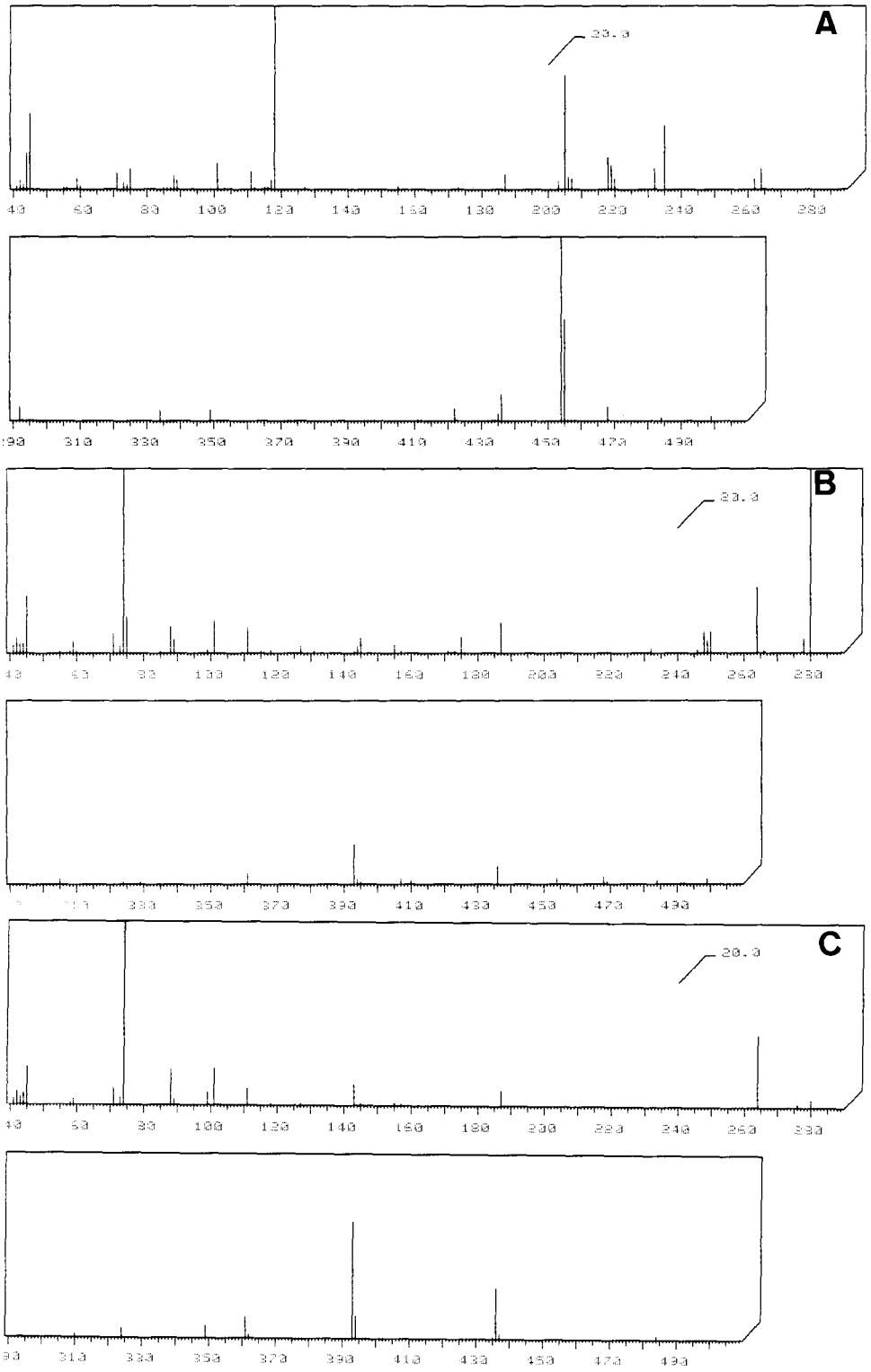
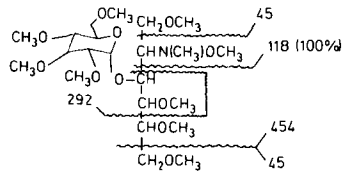


Fig. 3. Mass spectra (70 eV) of deoxy(methylmethoxyamino)alditol glycoside O-methyl ethers: Derivatives of turanose (A), cellobiose (B) and gentiobiose (C).

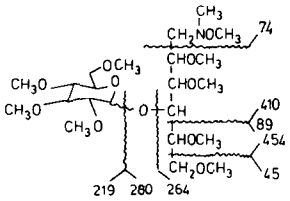
Based on a peak height four times the noise level, the minimum detectable amount of disaccharide analysed after derivatization by NPD in the nitrogen–phosphorus mode was 0.3 pmol under split injection conditions (1:20). The detector response was linear over a dynamic range of at least 10^2.

The electron impact (EI) mass spectra (70 eV) of the derivatives have characteristic fragmentation patterns according to whether they are aldose- or ketose-terminated, and to the position of the O-glycosidic union (Fig. 3). In the mass spectra of the derivatives of the glycosyl aldoses a base peak is observed at *m/z* 74, corresponding to the terminal group $-CH_2N(CH_3)OCH_3$. This ion is absent from the mass spectrum of the turanose derivative. In this case, the ketose termination is reflected in a base peak at *m/z* 118, and confirmed by an intense ion at *m/z* 454 arising from the loss of a CH_2OCH_3 fragment from the molecular ion (Scheme 2). The molecular and the $(M - 15)^+$ ions, at *m/z* 499 and 484 respectively, although present in the mass spectra of all the other derivatives, are very weak or even absent from the mass spectra of the derivatives of gentiobiose and melibiose. This is a consequence of the 6-O-substitution. The position of the O-glycosidic union is confirmed by the absence of a $(M - 45)^+$ ion. The terminal position of the O-glucopyranosyl group, allows extensive chain fragmentation from the molecular ion, yielding ions at *m/z* 484 ($M^+ - 15$), 436 ($M^+ - 31 - 32$) and 393 ($M^+ - 15 - 74$) as the most prominent in the higher mass region. These observations are of special value in the identification and specific detection of the derivatives of 6-O-glycosyl glycoses.



Scheme 2.

The presence or absence in the mass spectra of the permethylated deoxy(methylmethoxyamino)alditol glycosides, having ions at *m/z* 280 and 264, as well as their relative intensity ratios, may be related to the position of the O-glycosidic bond in the parent disaccharide. These ions result from cleavage of the O-glycosidic bond at either side of the oxygen atom, as exemplified in Scheme 3. Both ions are present in the mass spectra of the derivatives of cellobiose and lactose, in an intensity ratio of approximately 2:1. This ratio significantly increases to 4:1 in the mass spectrum of the maltose derivative. The relative intensities of the ions at *m/z* 280 and 264 are inverted in the mass spectra of the gentiobose and melibiose derivatives, where the ion at *m/z* 264 is approximately six times more intense than that at *m/z* 280. This



Scheme 3.

clearly indicates that preferential cleavage of the O-glycosidic linkage on the side of the open-chain moiety occurs in these compounds. The mass spectrum of the turanose derivative also shows an ion at *m/z* 264 but not at *m/z* 280. The presence of anion at *m/z* 292 confirms the 3-O-glycosyl substitution.

Fig. 4 illustrates the application of the characteristic fragmentation patterns of the derivatives to specific SIM–GC–MS detection of turanose, cellobiose, maltose, lactose, gentiobiose and melibiose. After derivatization, monitoring at *m/z* 74 specifically detects the aldose-terminated disaccharides, while the ions at *m/z* 118 and 454 are used in the specific SIM assay of the turanose derivative. A high degree of specificity of detection is attained by monitoring the ions at *m/z* 280, 484 and 499 for derivatives of 4-O-glucosyl glucoses, and at *m/z* 264, 393 and 436 for the 6-O-glucosyl glucoses.

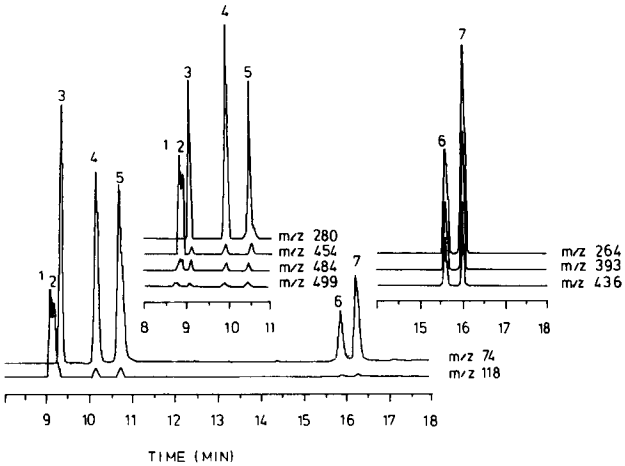


Fig. 4. SIM–GC–MS records of a standard mixture of 4 pmol of each deoxy(methylmethoxyamino)alditol glycoside O-methyl ether. Derivatives: 1 and 2 = turanose (*m/z* 118, 454, 484, 499); 3 = cellobiose (*m/z* 74, 280, 454, 484, 499); 4 = maltose (*m/z* 74, 280, 454, 484, 499); 5 = lactose (*m/z* 74, 280, 454, 484, 499); 6 = gentiobiose (*m/z* 74, 264, 393, 436); 7 = melibiose (*m/z* 74, 264, 393, 436). Glass capillary coated with Superox 0.1 (conditions as in Experimental).

CONCLUSIONS

The permethylated deoxy(methylmethoxyamino)alditol glycosides are suitable derivatives for the GC assay of reducing disaccharides. They are easily prepared from disaccharide methoximes, by reduction with methanolic sodium cyanoborohydride followed by permethylation, and are stable for long periods, even at room temperature. Except for the methoxime formation, all the derivatization steps take place at room temperature. Their EI mass spectra contain a series of intense diagnostic ions, which are suitable for highly sensitive specific SIM–GC–MS. This is an important feature, especially when only limited amounts of disaccharides are present in complex mixtures, as it is often the case in samples from biological origins. Mixtures of aldose- and ketose-terminated disaccharides can be analysed and the components unequivocally identified.

The introduction of a nitrogen atom in the molecule allows the use of NPD for selective detection with increased selectivity and sensitivity as compared to FID. This is an important aspect; owing to its high cost, a GC–MS instrument is not routinely available in most laboratories. Sensitive assays of reducing disaccharides in complex samples thus become possible.

Preliminary investigations have shown that the functional asymmetry introduced by the nitrogen group in the sugar molecule after derivatization makes these derivatives particularly interesting for end-group determinations in methylation analysis.

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