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GAS CHROMATOGRAPHY OF MONOSACCHARIDES: FORMATION OF A SINGLE DERIVATIVE FOR EACH ALDOSE

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SUMMARY

A novel method for the derivatization of monosaccharides is presented which generates only one derivative for each aldose. It involves formation of aldoximes, their reduction with borane to the corresponding aminopolyols and subsequent conversion in to the N-ethoxycarbonyl-O-trimethylsilyl derivatives. Although there are four steps, only small amounts of side-products are found in the gas chromatograms. The derivatives are stable, at least for several days, and are well suited for determination of carbohydrates. For ketoses the same derivatization is applicable but results, as expected, in two diastereomers.
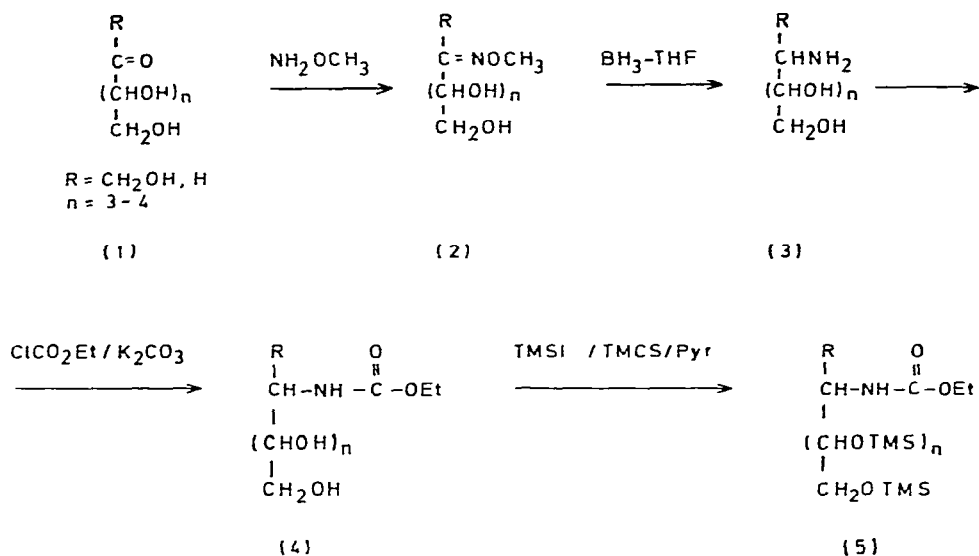
INTRODUCTION

The unequivocal identification of monosaccharides is of general importance for structural elucidation of natural products and in biochemistry. Capillary gas chromatography is the method of choice for analysis of complex mixtures due to its high separation efficiency. In the case of sugars, however, complications arise from derivatization.

Carbohydrates themselves are not directly amenable to gas–liquid chromatography and require the preparation of appropriate volatile derivatives. Since the pioneering work of Bayer[1] and Sweeley[2] on the gas chromatographic (GC) properties of trimethylsilyl ethers of monosaccharides, oligosaccharides and sugar alcohols, many other derivatives have been proposed. Alditol acetates[3], trifluoroacetates[4], n-butyl boronates[5,6], aldonitriles[7], O-methyl glycoside trifluoroacetates[8], methoxime- and oxime-trimethylsilyl ethers[9,10], and anhydrohexose dithioacetals[11] have been used in the GC analysis of carbohydrates. The major problems with many monosaccharides and reducing oligosaccharides is the generation of isomeric com-

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pounds during derivatization. This leads to multiple chromatographic peaks which interfere with the analysis of complex mixtures of carbohydrates. Peaks corresponding to different sugars may be superimposed, complicating their identification and quantitation[12]. Capillaries have been used in attempts to circumvent this difficulty[13-15]. The most successful approach to diminishing the number of peaks is the preparation of acyclic derivatives. Reduction to sugar alcohols, followed by acylation, has been used as a standard method for GC analysis of aldoses[3]. However, separation of some alditols is incomplete. Moreover, after reduction aldoses and ketoses afford identical alditols.

$$
\begin{array}{ccccc}
R & & R & & R \\
| & & | & & | \\
C=O & \xrightarrow{NH_2OCH_3} & C=NOCH_3 & \xrightarrow{BH_3-THF} & CHNH_2 \\
| & & | & & | \\
(CHOH)_n & & (CHOH)_n & & (CHOH)_n \longrightarrow \\
| & & | & & | \\
CH_2OH & & CH_2OH & & CH_2OH
\end{array}
$$

R = CH₂OH, H
n = 3 - 4

$R = CH_2OH, H$
$n = 3 - 4$

(1)   (2)   (3)

$$
\xrightarrow{ClCO_2Et / K_2CO_3}
\begin{array}{c}
R \quad\quad O \\
| \quad\quad\quad \| \\
CH-NH-C-OEt \\
| \\
(CHOH)_n \\
| \\
CH_2OH
\end{array}
\xrightarrow{TMSI / TMCS / Pyr}
\begin{array}{c}
R \quad\quad O \\
| \quad\quad\quad \| \\
CH-NH-C-OEt \\
| \\
(CHOTMS)_n \\
| \\
CH_2OTMS
\end{array}
$$

(4)   (5)

Scheme 1.

We now report a new method for the preparation of volatile acyclic derivatives of monosaccharides. Reduction of sugar methoximes yields aminodeoxyalditols (3), followed by ethoxycarbonylation (4) and trimethylsilylation to the N-ethoxycarbonyl-O-trimethylsilyl-aminopolyols (5) (Scheme 1). These derivatives are well separated in wall-coated open-tubular columns in a relatively short time. Aldoses give rise to only one peak, which allows their unequivocal identification and quantitation. For ketoses two well separated peaks of the corresponding diastereomers are obtained.

EXPERIMENTAL

Materials

Trimethylchlorosilane (TMCS) was obtained from Sigma (St. Louis, MO, U.S.A.), 1-trimethylsilylimidazole (TMSI) and ethyl chloroformate from E. Merck (Darmstadt. G.F.R.). Methoxyammonium chloride was purchased from Pierce (Rockford, IL, U.S.A.). Pyridine was dried over potassium hydroxide for 48 h,

refluxed over KOH and distilled. Samples of monosaccharides were purchased from Sigma.

Apparatus

Gas–liquid chromatography (GLC) was performed on a Dani instrument, Model 6800, equipped with splitter and flame ionization detector (FID). Two different capillaries were used: 25 m × 0.28 mm, coated with OV-101; and 25 m × 0.28 mm, coated with Chirasil-Val[16,17]. Injector temperature: 250°C. Detector temperature: 275°C. Oven temperature was programmed as shown in Figs. 2 and 3 at a rate of 1°/min. Mass spectrometry was performed on a Varian MAT 112 S instrument.

Preparation of derivatives

Aqueous standard solutions of the monosaccharides (each 1–2 mg/ml) with 2 mg/ml of D(−)-mannitol as internal standard were prepared. A 200-μl volume of each solution was transferred into a derivatization vial having a PTFE-lined rubber septum and screw-cap. The solvent was evaporated under a stream of nitrogen and the residue dried *in vacuo* over phosphorus pentoxide. A 100-μl volume of a solution of 250 mg of methoxyammonium chloride in 10 ml dry pyridine was added and the solution was heated to 80°C for 1 h. The solvent was evaporated under a stream of nitrogen or, if several samples were to be processed, in a vacuum centrifuge. A 100-μl volume of 1.0 M borane in tetrahydrofuran was added, the mixture agitated vigorously for 1 min and heated to 80°C for 2 h. After cooling to 0°C in an ice-bath, the excess of borane was destroyed by careful addition of methanol. The solvent was evaporated to dryness under nitrogen, 100 μl of 1 M HCl in methanol were added and the solution was heated to 80°C for 30 min. After cooling, the solvent was evaporated under a stream of nitrogen and the operation was repeated. The residue was dissolved in 50 μl of a saturated aqueous solution of K_2CO_3 and 25 μl of ethyl chloroformate were added. The mixture was vigorously agitated for 1 min and left at room temperature for 1 h. The liquid was then evaporated under nitrogen and the residue dried overnight *in vacuo* over phosphorus pentoxide. The dry residue was taken up in 40 μl of dry pyridine, 10 μl of trimethylchlorosilane (TMCS) and 10 μl of trimethylsilylimidazole (TMSI), agitated vigorously for 2 min and heated to 50°C for 30 min. After centrifugation, the supernatant was used for GC.

Calculation

Peak areas were calculated by means of a Spectra-Physics SP 4100 electronic integrator. The response factor of each sugar was calculated relative to D(−)-mannitol.

RESULTS AND DISCUSSION

Gas chromatography of sugars can greatly be simplified by the use of acyclic derivatives. However, for methoxime derivatives, two peaks are observed for each sugar[9], *i.e.*, the *syn-* and *anti*-forms. Oxime ethers can readily be reduced by borane in tetrahydrofuran to give the corresponding amines in high yields[18]. Borane offers a distinct advantage over lithium aluminium hydride: aluminium hydroxide is a strong adsorbent, and the yields of reduction are generally low[19]. The reaction conditions of

the borane reduction have not been optimized with respect to time and temperature. Considering the reactivity of borane, complete reduction may be achieved at lower temperatures and in shorter times.

The obtained aminopolyols can further be derivatized by different methods. Silylation was found to be difficult or gave two or more peaks with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) as silylating agent. This can be attributed to the fact that BSTFA can introduce either one or two silyl groups in primary amines[20]. On the other hand, the trifluoroacetyl derivatives were found to be unstable, and the more stable pentafluoropropionyl derivatives were not well separated.

Amino sugars can readily be converted with ethyl chloroformate into the corresponding N-ethoxycarbonyl derivatives[21]. These are stable and, after silylation, exhibit excellent GC properties. For silylation a mixture of pyridine–trimethylchloro-silane–trimethylsilylimidazole (4:1:1) gave the best results.

Identity of the products obtained was established by gas chromatography-mass spectrometry (GC–MS) of the derivatives obtained by reduction with trideuterio-borane. The fragmentation patterns observed are analogous to those of the trimethyl-silyl derivatives of the sugar oximes and methoximes. The mass spectrum of the glucose derivative (Fig. 1) shows no molecular ion, but a relatively intense $[M - 15]^+$ (m/e 600), corresponding to incorporation of two deuterium atoms, several chain cleavage fragments (m/e 103, 205, 307, 409, 410) and some cascades of ions from
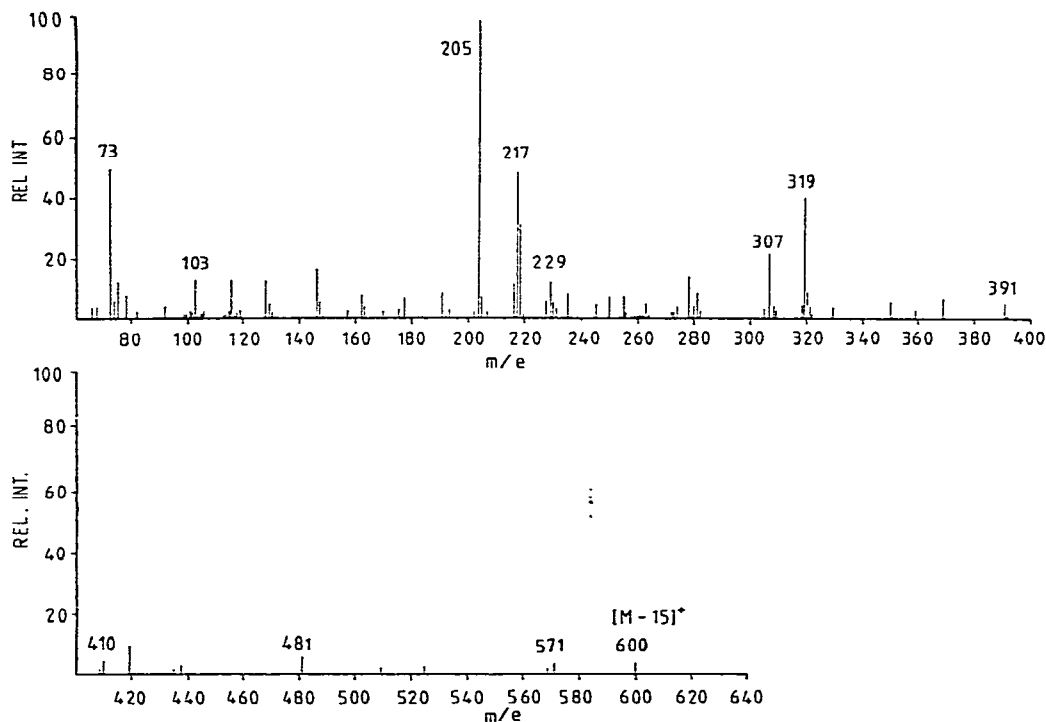

Fig. 1. Electron-impact (EI) mass spectrum of the N-ethoxycarbonyl-O-trimethylsilyl-aminodeoxy-alditol of glucose. Conditions: electron energy 70 EV; anode current 0.7 mA; GC-interface tempera-ture 250°C; ion source temperature 270°C.

sequential elimination of trimethylsilanol (571–481–391, 600–509–419–329, 410–319–229, 307–217). Other diagnostic ions are at m/e 571 ([M − 44]$^{+\cdot}$) and m/e 73 (trimethylsilyl).

Retention times for the derivatives of arabinose, ribose, rhamnose, fucose, fructose, galactose, mannose and glucose were determined in capillaries coated with Chirasil-Val (Fig. 2) and OV-101 (Fig. 3). As can be seen from the chromatograms, only one derivative is formed for each carbohydrate. The retention times on Chirasil-Val are relatively short with good separation of all sugars, except for galactose and mannose. A good separation of all sugars is obtained with OV-101, but with significantly higher retention times. However, if analysis is carried out isothermally at 180°C separation is achieved in 20 min. Interestingly, an inversion of the elution order of rhamnose and fucose is observed on changing from Chirasil-Val to OV-101 (peaks 4 and 5).
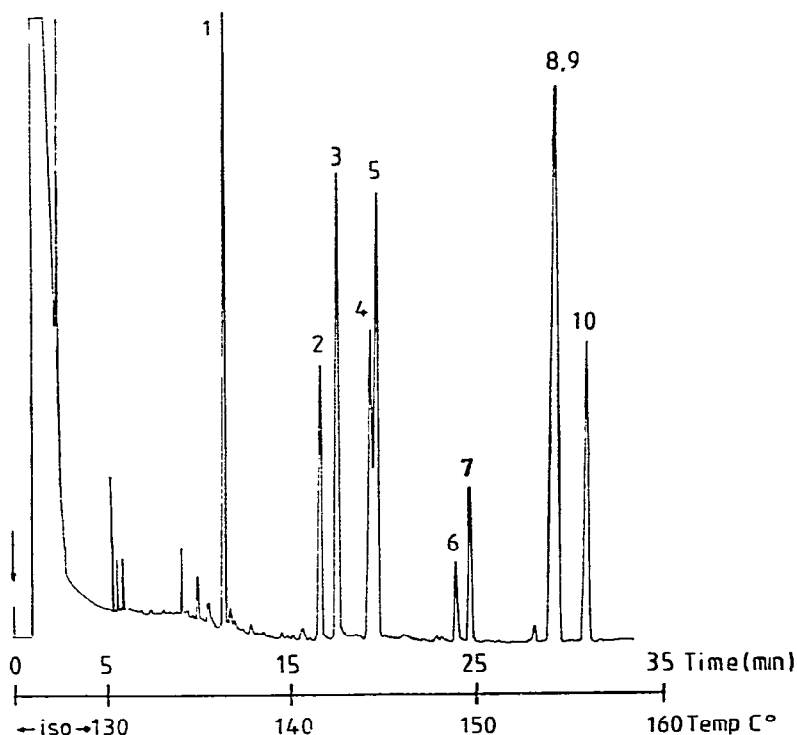

Fig. 2. Gas chromatogram of N-ethoxycarbonyl-O-trimethylsilyl-aminodeoxyalditols on Chirasil-Val (25 m × 0.28 mm). Carrier gas hydrogen: 0.4 kg/cm². Splitting ratio 1:25. Injector temperature: 250°C. FID temperature: 275°C. Peaks: 1 = TMS-mannitol as standard; 2 = arabinose; 3 = ribose; 4 = rhamnose; 5 = fucose; 6 = fructose 1; 7 = fructose 2; 8 = galactose; 9 = mannose; 10 = glucose.

In contrast to the aldoses, the ketosugar fructose, as expected, shows two well separated peaks, corresponding to the two possible diastereomers formed on reduction of the fructose methoxime. The reaction obviously proceeds with some stereoselectivity as the two peaks of fructose have an area ratio of ca. 1:2. Although
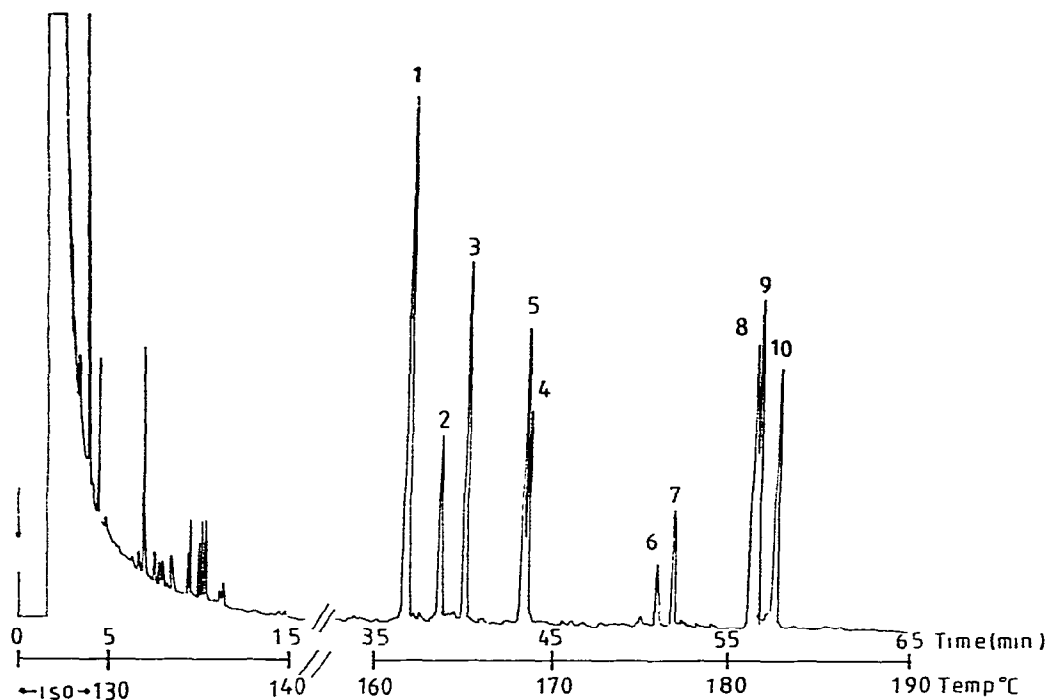
Fig. 3. Chromatogram of the N-ethoxycarbonyl-O-trimethylsilyl-aminodeoxyalditols on OV-101 (25 m × 0.28 mm). Conditions and numbering of peaks as in Fig. 2.
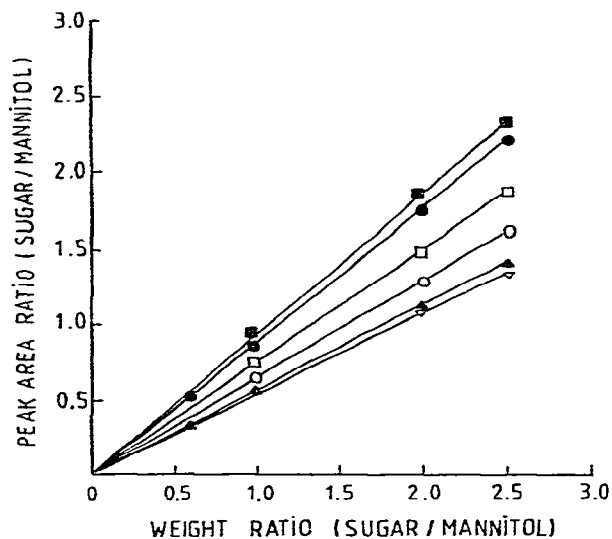
Fig. 4. Gas chromatographic response of N-ethoxycarbonyl-O-trimethylsilyl-aminodeoxyalditols relative to TMS-mannitol as internal standard: ▦, galactose; ●, ribose, rhamnose; □, fucose, mannose; ○, glucose; ▲, arabinose; ▽, fructose (sum of both peak areas).

formation of two derivatives constitutes a complication in the GC analysis of ketoses, the good separation of both peaks still allows quantitation. In any case, the proposed derivatization considerably simplifies the GC pattern[22].

Relative response factors were determined for each sugar, from mixtures containing various amounts of sugar and a fixed amount of mannitol as internal standard. Good linearity was observed (Fig. 4), but the detector response for the sugar derivatives is somewhat lower than for TMS-mannitol. A similar observation has been made previously[21].

CONCLUSIONS

The GC analysis of carbohydrates is greatly facilitated by a derivatization sequence which affords only one derivative for aldoses. In the case of ketoses two diastereomers are obtained. The derivatives are very stable and exhibit excellent GC properties. Most other derivatization procedures yield either more than one peak and/or easily decomposing derivatives. For instance, TMS-tagatose gives rise to seven or eight components[22]. The derivatization sequence involves four consecutive reactions, but offers the great advantage of unambiguous identification and simplified quantitation of sugars.

The identity of the compounds has been established by GC–MS. Formation of side- or decomposition products is negligible; consequently the sample amount required is low: derivatization was performed with about 1 μmol; GC was carried out with 0.1–1 nmol. We consider that this method is a significant contribution towards a more sensitive and accurate analysis of carbohydrates in natural products and biochemical samples.

ACKNOWLEDGEMENTS

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