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# IDENTIFICATION OF KETOSES BY USE OF THEIR PERACETYLATED OXIME DERIVATIVES A G L C–M S APPROACH*

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ABSTRACT

A method based on peracetylated oxime (PAKO) derivatives has been developed for rapid g l c–m s survey of ketoses This derivatization procedure (and the chromatographic analysis of these derivatives) is identical to one previously employed to identify aldoses by means of peracetylated aldononitrile (PAAN) derivatives The production of chemically different derivatives from the aldoses and ketoses by the same derivatization procedure greatly simplifies the chromatographic separation of the derivatives of the ketoses from those of the aldoses, and also results in distinctively different, mass-spectral fragmentation-pathways for the two sets of derivatives Both the electron-impact (e i) and ammonia chemical-ionization (c i) mass spectra of PAKO derivatives have been examined Extensive differences between the fragmentation-pathways of the PAAN and the PAKO derivatives have been observed both by e i m s and ammonia c i m s The g l c–m s of these PAKO derivatives, in conjunction with various, isotopic variants of the derivatization process, can yield extensive structural information with regard to the starting saccharides associated with the known, or unknown, g l c peaks The g l c and mass-spectral properties of highly *O*-methylated PAKO derivatives of D-fructose are compared, and contrasted, to those of the PAKO derivatives of non-*O*-methylated saccharides The chromatographic properties of derivatives of oligosaccharides that result from the PAAN–PAKO derivatization procedure have also been studied

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## INTRODUCTION

The gas–liquid chromatography (g l c ) of ketoses and their derivatives has not been studied as extensively as that of the aldoses, and many studies of ketoses have dealt exclusively with D-fructose[1–4] Two fundamental approaches to saccharide separations by g l c , of the pertrimethylsilyl ($Me_3Si$) derivatives and the per-$Me_3Si$ oxime derivatives, were described in a single report by Sweeley *et al* [5], and the majority of the successive g l c studies of saccharides have dealt either with these derivatives or variants of them The aforementioned $Me_3Si$ derivatization procedure was adapted to combined aldose and ketose g l c studies for mixtures of D-glucose and D-fructose[6–8] It was noted[8] that the $Me_3Si$ derivative of D-fructose is formed less readily, or more slowly, than the corresponding derivative of D-glucose Further studies of the $Me_3Si$ derivatives of ketoses have been reported[9–11], and these will be discussed in the following section dealing with mass spectrometry In general, the introduction of $Me_3Si$ groups greatly increases the volatility of the saccharides, and provides readily separable derivatives However, the direct formation of $Me_3Si$ derivatives of saccharides leads to several problems, including (*a*) the production of multiple derivatives, and g l c peaks, from a given starting-sugar (due to $\alpha,\beta$ isomerism and to the formation of furanosyl and pyranosyl products), (*b*) the production of different ratios of these multiple peaks under different reaction-conditions, and (*c*) the relatively complex, mass-spectral patterns that result from the ring structure of these derivatives, and from the bulky $Me_3Si$ groups

In the report that described the g l c properties of the $Me_3Si$ derivatives, Sweeley *et al* [5] also described the production of the per-$Me_3Si$ oxime derivatives These analytical derivatives are produced by first treating the saccharide with hydroxylamine to yield the oxime, and then per(trimethylsilyl)ating the hydroxyl groups, including that of the oxime These per-$Me_3Si$ oxime derivatives are afforded both by aldoses and ketoses[5], and have the advantage, relative to the per-$Me_3Si$ saccharide derivatives, that fewer derivatives are produced from each starting saccharide. However, the introduction of an oxime group into a saccharide has the potential of resulting in two forms of the oxime, the *syn* and the *anti* Although such *syn* and *anti* isomers of the oxime from a single saccharide are often described as chromatographically unresolvable, this lack of resolution actually is usually an indication of the inefficiency of the g l c -separation conditions Sweeley *et al* [5] described the mutual separation of per-$Me_3Si$ oxime derivatives of 1,3-dihydroxy-2-propanone, D-*erythro*-2-pentulose, D-fructose, and 2,7-anhydro-D-*altro*-heptulose (sedoheptulosan), and the separation of these derivatives from corresponding derivatives of aldoses of similar molecular weight

Further developmental studies of the per-$Me_3Si$ oxime method, with applications to food technology, were made by Mason and Slover[12] In general, the retention times of the per-$Me_3Si$ oxime derivatives of ketoses were found to be somewhat shorter than those of the corresponding derivatives of aldoses of similar molecular weight The advantages of these acyclic, per-$Me_3Si$ oxime derivatives, compared to the cyclic

$Me_3Si$ derivatives, became apparent with applications to sugar phosphates[13] and aldonic acids[14] Petersson[14] also extensively studied the g l c and mass-spectral properties of per-$Me_3Si$ oxime derivatives of neutral saccharides, and Stroz[15] examined the quantitation of g l c data for the corresponding derivative of D-ribose In addition, Petersson[14] was able to correlate the g l c retention-times of the per-$Me_3Si$ oxime derivatives to structural features of these compounds Toba and Adachi[16] examined the chromatographic properties of per-$Me_3Si$ oxime derivatives of disaccharides, including ketodisaccharides

Laine and Sweeley[17,18] employed a fundamental variation on the per-$Me_3Si$ oxime derivatization procedure by employing methoxyamine instead of hydroxylamine, and produced the per-$Me_3Si$ *O*-methyloxime derivatives[18] It was shown that many of the chromatographic and mass-spectral properties of these derivatives were superior to those of the per-$Me_3Si$ oxime derivatives Although the reports of Laine and Sweeley[17 18] were principally concerned with derivatives of aldoses, the per-$Me_3Si$ *O*-methyloxime derivative of D-fructose, as a representative example of the ketoses, was also examined The g l c retention-times of these derivatives of D-fructose, and of the corresponding aldohexoses, were found to be quite similar[18] The chromatographic and mass-spectral studies of per-$Me_3Si$ *O*-methyloxime compounds were extended by application to phosphates of D-*erythro*-2-pentulose and D-fructose by Harvey and Horning[13], and to dialdoses and aldosuloses by Dizdaroglu *et al*[19]

Independent of the development of g l c conditions for the separation of the various trimethylsilyl derivatives of saccharides, improvements in g l c resolution allowed the utilization of peracetylated alditol derivatives of saccharides The original, effective, g l c separation of peracetylated alditols was reported by Sawardeker *et al*[20], and various modifications and applications of the technique have been summarized by Dutton[3] These alditol acetate derivatives are produced by an initial reduction of a saccharide to the corresponding alditol, followed by peracetylation Whereas the production of the per-$Me_3Si$, the per-$Me_3Si$ oxime, or the per-$Me_3Si$ *O*-methyloxime derivatives from a single, starting saccharide results in at least two subsequent products for any of the derivatization procedures, the production of an alditol acetate from a given starting-saccharide yields only a single derivative In general, most reductions have involved aldoses, but this technique is also applicable to ketoses[21] Therefore, it is possible to use the alditol acetate method to obtain more-meaningful chromatograms from much more complicated starting-mixtures of saccharides than can be analyzed by any of the three trimethylsilyl procedures However, one limitation to the alditol acetate derivatization procedure is that the reduction of the aldose (or the ketose) carbonyl group to a hydroxyl group lessens the asymmetry of the molecule, and, in many cases, two different saccharides yield the identical alditol acetate Ambiguity resulting from such reductions can be decreased by employing deuterated reducing agents in combination with mass-spectral analysis, although similar problems still remain when such information is dependent on separations by g l c

Saccharides containing *O*-methyloxime and acetylated hydroxyl groups, reported by Mawhinney *et al* [22], are intermediate between the alditol acetates and the per-$Me_3Si$ *O*-methyloxime derivatives, and have been described as useful in the g l c separation and analysis of hexosamines

The peracetylated aldononitrile (PAAN) derivatives are alternative, saccharide derivatives that have proved amenable to g l c separation and to mass-spectral analysis The facile, two-step, derivatization procedure employs hydroxylamine to convert an aldose into a hydroxyloxime derivative (as does the first step of the per-$Me_3Si$ oxime derivatization procedure), and then acetic anhydride, which both peracetylates all hydroxyl groups and reduces the oxime group to a nitrile group Therefore, the PAAN derivative from a given aldose is very similar to the corresponding alditol acetate derivative of that saccharide, but, in the case of the PAAN derivative, C-1 is converted into the chromatographically, and mass-spectroscopically, distinctive nitrile group A nitrile group involving C-1 neither adds nor subtracts a center of asymmetry (with regard to the $\alpha,\beta$ anomeric mixture from the original aldose), and therefore each aldose yields a single PAAN derivative

The production of these PAAN derivatives, for purposes of g l c separation and mass-spectral analysis, was described by Lance and Jones[23] and Dmitriev *et al* [24] Varma and co-workers extensively developed g l c conditions for PAAN derivatives of neutral saccharides and hexosamine derivatives[25–28], and also made extensive biomedical applications of this technique[29–32] Horning and co-workers employed these PAAN derivatives for g l c studies using glass-capillary columns[33] and for study of the electron-impact (e i ) mass spectra of these compounds[34], and applied the derivatization procedure for surveying the saccharides present in urine[35] We have employed extensive, isotopic substitution to study the g l c –m s of these PAAN derivatives[36,37], and Li *et al* [38] also examined the mass spectra of these compounds As the nitrile group of the PAAN derivative involves three of the bonds of the terminal, backbone carbon atom of the original aldose, there can be no corresponding nitrile analog for ketoses, because two of the bonds of the carbon atom of the ketone group are involved in carbon–carbon bonds However, at the end of the first step of the PAAN derivatization procedure, aldoses and ketoses are both converted into their respective oximes On addition of acetic anhydride in the second step of the derivatization procedure, the oximes of the ketoses afford peracetylated oximes, without the subsequent dehydration step that gives the nitrile group of the PAAN derivatives Although these saccharide derivatives may accurately be described as peracetylated oximes, we prefer to employ the term peracetylated ketooxime (PAKO) to emphasize the ketose origin of these derivatives, for direct comparison to the PAAN (peracetylated aldononitrile) nomenclature of the derivatives of the aldoses. Therefore, the uniform derivatization procedure can simultaneously convert aldoses into PAAN derivatives and ketoses into PAKO derivatives.

Szafranek *et al* [33] and Morrison[39] noted that the g l c properties of the acetylated alditols and the PAAN derivatives are similar, and both derivatives of the same saccharide could be readily chromatographed, Szafranek *et al* demonstrated this

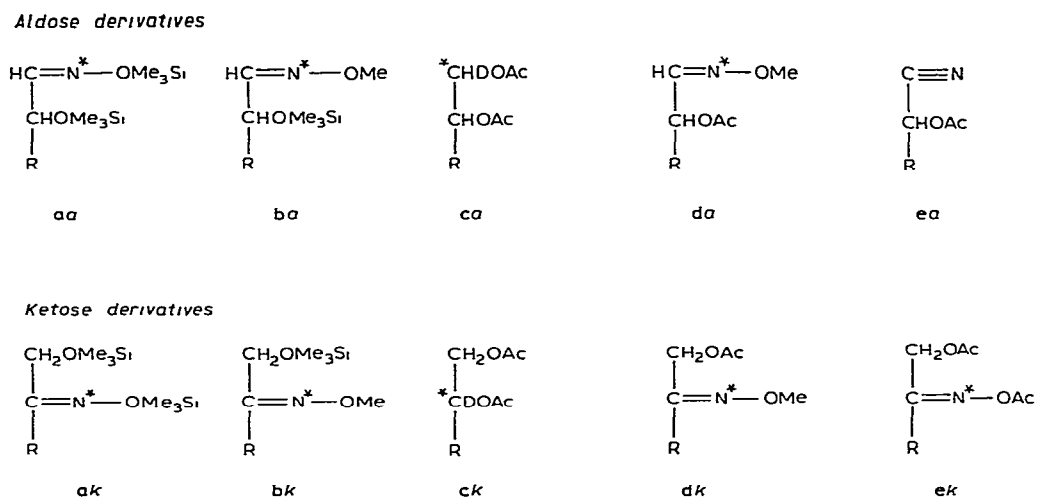



Fig 1 The generalized structures of the products from aldoses (*a*) and 2-ketoses (*k*), when these saccharides are derivatized by the following procedures per-$Me_3Si$ oxime (**a**), per-$Me_3Si$ *O*-methyloxime (**b**), alditol acetate (**c**), *O*-acetylated-*O*-methyloxime acetate (**d**), and PAAN–PAKO (**e**)

relationship with glass-capillary columns containing SE-30, whereas Morrison employed a variety of packed columns However, Morrison made the interesting observation that, when an oligosaccharide is subjected to the sequence of (*a*) reduction of the saccharide at the reducing end, (*b*) hydrolysis, and (*c*) formation of the PAAN derivatives of the saccharides in the hydrolyzate, followed by chromatographic analysis, the resulting chromatogram will contain one peak for an acetylated alditol and other peak(s) for the PAAN derivatives The degree of polymerization (d p ) of the oligosaccharide will then be represented by [(% of PAAN derivative/% of alditol acetate) + 1], and, for a hetero-oligosaccharide, the alditol group will help sequence the compound by identifying the reducing end Varma *et al* [28] employed this combined, alditol–aldononitrile method to evaluate the chain lengths of glycosaminoglycans In general, the PAAN derivative has a retention time distinctly shorter than that of the corresponding alditol acetate

The foregoing description of acyclic derivatives of saccharides commonly employed for g l c separations is summarized in Fig 1, which does not include the more-complex, cyclic structures arising from the simple per(trimethylsilyl)ation of saccharides. Fig 1 shows the structures of the saccharide derivatives that arise from the five acyclic-derivatization procedures, indicated by the bold-face letters **a** through **e** The per-$Me_3Si$ oxime derivative[5] is identified as **a**, the per-$Me_3Si$ *O*-methyloxime derivative[18] as **b**, the alditol acetate derivative[20] as **c**, the *O*-acetylated *O*-methyloxime derivative[22] as **d**, and the PAAN (or PAKO) derivative[23] as **e** Fig 1 is further subdivided to show the first two carbon-atom positions of an aldose derivative (*a*) and ketose derivative (*k*) of a given derivatization procedure This is not intended to be an exhaustive list of acyclic derivatives of saccharides, as other such derivatives

(*e g*, the peracetylated, diethyl dithioacetal derivatives[40]) can be chromatographed A comparison of vertical pairs of derivatives in Fig 1, which represent sets of derivatives from aldoses and ketoses that arise from a given derivatization procedure, indicates that, for most derivatization procedures, the aldoses and ketoses (for saccharides of a given molecular weight) yield derivatives that are chemically identical in terms of the number and type of functional groups present Therefore, chromatographic separations of the *a* and *k* sets (of a given chain-length) of the derivatives **a** through **d** are essentially dependent on properties resulting from the different stereochemistry of these derivatives

In contrast, the aldose and ketose products represented by e*a* and e*k* from the PAAN–PAKO derivatization procedure *are chemically different*, indicating that the derivatives of the aldoses and ketoses that result from this procedure will have distinctly different, chromatographic and mass-spectral properties As oxime derivatives can exist in the *syn* and *anti* dispositions, Fig 1 indicates that only derivatives c*a*, c*k*, and e*a* could yield a single product from a specific sugar When deuterated reagents are employed for the alditol acetate derivatization procedure (and such reagents are necessary in order to distinguish aldose from ketose products), a new center of asymmetry is created for both c*a* and c*k*, however, for c*a*, such a center of asymmetry, resulting from differences between deuterium and hydrogen, provides effects too subtle to affect currently available g l c resolution The center of asymmetry resulting from formation of c*k* yields derivatives of two different saccharides, and such differences are detectable by current g l c techniques

In addition, the reduction of saccharides to alditols may cause loss of molecular asymmetry, and two, or more, saccharides may yield identical, or essentially identical, products An examination of the structures represented by c*a* and c*k* shows that they are very similar, indicating problems in g l c separations and mass-spectral analyses of these derivatives The PAAN–PAKO derivatization procedure yields e*a* derivatives, the only structure depicted in Fig 1 that, for aldoses, yields a single derivative from a given saccharide, and, for ketoses, the chemically distinct e*k* derivatives Although each ketose can yield two e*k* products (the *syn* and *anti* isomers), the resulting chromatograms of the PAAN–PAKO derivatization procedure may be expected to be simple, as (*a*) due to differences of chemical structure, the retention times of the PAKO derivatives should be distinctly different from those of the PAAN derivatives, and (*b*) although the PAKO derivatives can show double g l c peaks for each starting ketose, the chromatograms of these ketose derivatives may be expected to be simpler than those of the corresponding aldose derivatives, as each 2-ketose has one center of asymmetry fewer than an aldose of corresponding molecular weight, and, therefore, correspondingly fewer possible derivatives for a given backbone chain-length

The foregoing discussion has dealt primarily with derivatives of saccharides that do not contain additional functional groups The g l c and g l c–m s analysis of *O*-methyl derivatives of saccharides has been of considerable interest[4], as these compounds result from methylation–fragmentation, structural analysis The principal,

developmental approaches for such derivatives have dealt with the derivatization procedures for *O*-methylated aldoses One of the most successful, and most widely applied, techniques for methylation–fragmentation analysis employs the alditol acetate derivatives[41 42] because of (*a*) the ease of formation, and the stability, of the derivatives, (*b*) the good chromatographic properties of the derivatives, and (*c*) the ease of interpretation of the mass spectra of these derivatives The adaptation of the alditol acetate method to methylation–fragmentation analysis of levans (polysaccharides composed of the ketose, D-fructose) has been reported by Lindberg *et al*[21], and further applied by Hancock *et al*[43] However, the alditol acetate derivatives of *O*-methylated saccharides also exhibit most of the limitations and inconveniences arising from reduction that have already been discussed for the alditol acetate derivatives of non-*O*-methylated saccharides In general, problems arise in the identification of products from methylation–fragmentation analyses, of levans, that employ the alditol acetate derivatization procedure, because of (*a*) the dependence on deuteration and mass-spectrometry (m s ) for the identification of the products, and (*b*) the great similarity between the products from the aldoses and ketoses (for example, similarities between the *O*-methyl derivatives of the hexoses and the 2-hexuloses)

We have adapted the PAAN derivatization procedure to the methylation–fragmentation analysis by g l c –m s of polysaccharides composed of aldose[44 45] (D-mannose and D-glucose) residues and groups, and have applied this technique to the structural analysis of D-mannans[46], D-glucans[47–51], and compounds containing *N*-acetylhexosamine residues[52] The increased asymmetry of these PAAN derivatives, compared to the alditol acetates, which results from the production of a nitrile at C-1, provides improved physical properties for the chromatographic separation and mass-spectral analysis of these derivatives

The e i m s of saccharide compounds in general, and the specific applications of m s for carbohydrate structural analyses, have been reviewed[53 54] Many of the difficulties encountered with early studies of g l c –m s resulted from rearrangements that occur in cyclic derivatives during fragmentation by e i , and also to complications arising from the rather bulky, trimethylsilyl protecting groups employed to promote volatilization of the saccharides However, despite these limitations, meaningful results have been obtained by g l c –e i m s for cyclic, trimethylsilyl derivatives of tetruloses, 2-pentuloses, and 3-pentuloses by Havlicek *et al*[9], and for 3-ketoses and 2-heptuloses by Okuda *et al*[11] The g l c –e i m s of the acyclic, per-$Me_3Si$ oxime derivatives of saccharide phosphates was examined by Harvey and Horning[13], and a more extensive, general analysis of these derivatives (including those of ketoses) was reported by Petersson[14] Laine and Sweeley[17,18] examined the g l c –e i m s of the $Me_3Si$ derivatives of *O*-methyloximes of saccharides, including the m s of that of D-fructose Both Laine and Sweeley[18] and Petersson[14] concluded that the mass-spectral patterns of these derivatives of the oximes of saccharides are dominated by an initial, random cleavage of the backbone chain of these acyclic compounds, and such mass-spectral fragmentation-pathways greatly facilitated the identification of these compounds Dizdaroglu *et al*[19] took advantage of this relatively simple,

fragmentation process of these derivatives to examine the g l c–e i m s of such products from dialdoses and aldosuloses, again demonstrating the usefulness of these oxime derivatives when dealing with saccharides that contain ketone groups The e i m s of PAAN derivatives of non-*O*-methylated, neutral saccharides has been examined by Szafranek *et al*[33] who concluded that the initial e i mass-spectral fragmentation-process of the acyclic derivatives also consists of random cleavage of the backbone chain We have employed various forms of isotopic substitution to examine the e i mass-spectral fragmentation-pathways of these PAAN derivatives further[37].

The partially *O*-methylated saccharides arising from methylation–fragmentation analysis have proved very amenable to identification and quantitation by g l c–m s analysis of the alditol acetate derivatives[41 42] These peracetylated derivatives of partially *O*-methylated saccharides have become useful for methylation–fragmention analysis by g l c–m s, as Bjorndal *et al*[41] have demonstrated that the different, partially *O*-methylated derivatives have markedly different e i mass-spectral patterns, and that these differences result from the favored backbone cleavage between adjacent carbon atoms to which are attached methoxyl groups However, as discussed in the foregoing section on g l c, the alditol acetate derivatization procedure involves an initial reduction of sugars to alditols, with some concomitant loss of molecular asymmetry Under some conditions, analysis of the mass spectra of these derivatives can be difficult, due to the presence of two terminal hydroxyl (or methoxyl) groups We have demonstrated that the e i m s of the PAAN derivatives of partially *O*-methylated aldoses follows the same general principles of favored backbone-cleavage under e i mass-spectral conditions that were observed for the alditol acetate derivatives of these saccharides[44] However, the e i m s of the PAAN derivatives of the partially *O*-methylated saccharides avoids ambiguities that were observed in the interpretation of the e i m s of the alditol acetate derivatives, partly as a result of even *m/e* values for most of the fragment ions originating from the nitrile end of the molecule, in contrast to the odd *m/e* values of fragment ions arising from the alditol end The fragment ions arising from the nitrile end of the PAAN derivatives are also relatively unstable, compared to those fragment ions arising from the non-nitrile end, therefore, the e i mass spectra of these PAAN derivatives of partially *O*-methylated aldoses contain spectral patterns that would be anticipated from "single-ended" alditol acetate derivatives

In addition to the well established e i m s, the more recently developed technique of chemical-ionization (c i) mass spectrometry has been employed for saccharide analysis C i m s involves the introduction of low concentrations (although higher than the concentrations of saccharides under analysis) of an ionizing gas into the ionization chamber of the mass spectrometer, ammonia, methane, and isobutane are currently the ionizing gases most commonly employed This additional molecular species in the ionizing chamber moderates the ion beam of the mass spectrometer, and affords, from the saccharide derivative, ions much larger than the fragment-ions produced from the corresponding compounds by e i m s

Early applications of the c i m s technique to sugars include those of Hogg and Nagabhushan[55], who employed ammonia c i m s on the peracetates of D-glucose and α,α-trehalose, and concluded that the ammonia c i mass spectra of these compounds consist of a single ion corresponding to the *m/e* of the molecular weight of the saccharide plus the molecular weight of the ammonium ion $[M + 18]^+$ They also observed that the methane c i mass spectra of these compounds contain a pattern principally composed of the molecular weight of the saccharide plus the hydronium ion less a multiple (*n*) of the molecular weight of acetic acid, $[MH - 60\ n]^+$ Dougherty *et al*[56] examined the c i spectra of the acetates of oligosaccharides, employing ammonia, methane, and isobutane, and concluded that ammonia c i m s yields ions at, or near, the molecular weight of oligosaccharides as large as the pentasaccharides, whereas methane and isobutane yielded c i spectra similar to the corresponding e i spectra of these compounds Horton *et al*[57] concluded that, when ammonia is employed as the ionizing reagent, saccharides containing no basic nitrogen atom yield ammonia c i spectra having a major capture-ion at $[M + NH_4]^+$, whereas saccharides containing a basic nitrogen atom yield ammonia c i spectra having a capture ion of major intensity at $[M + H]^+$ Cyclic $Me_3Si$ derivatives of saccharides have been examined by Murata *et al*[10] (employing ammonia) for neutral saccharides, and by Bowser *et al*[58] (employing methane) for *N*-acetylhexosamines, the studies respectively observed capture ions at $[M + NH_4]^+$ and $[M + H]^+$, but both investigations showed c i spectra having large percentages of fragment ions of smaller *m/e*

Hancock *et al*[43] reported the isobutane c i m s of alditol acetates, which are interesting because (*a*) the derivatives were products of methylation–fragmentation analysis, and (*b*) the original, partially methylated saccharides were ketoses The capture ions of $[M + 1]^+$ were reported for the c i m s of these derivatives of D-fructose, but the other *m/e*, and the percentage of these capture ions, relative to the total *m/e* of these c i spectra, were not discussed McNeil and Albersheim[59] extensively studied the methane and isobutane c i m s of alditol acetates of the products of methylation–fragmentation analyses of D-glucans, D-galactans, and D-mannans, and concluded that the isobutane c i m s of all of these peracetylated derivatives of partially *O*-methylated alditols contain prominent contributions of ions as $[M + 1]^+$, $[M + 1 - 32]^+$, and $[M + 1 - 60]^+$, attributable to the capture ion, $[M + 1]^+$, and ions resulting from the loss of either methanol or acetic acid from the capture ion An unusual feature of the report was the extensive study of mass-spectral differences that arise from different isomers of the same molecular weight and chemical structure, both for stereoisomers (*e g*, comparison of the c i m s of identically substituted alditol acetate derivatives of D-glucose, D-galactose, and D-mannose) and for positional isomers (*e g*, comparison of the c i m s of the peracetylated alditol acetate derivatives of 3,4- and 3,6-di-*O*-methyl-D-mannose)

McNeil and Albersheim[59] employed isobutane for the c i m s of alditol acetates, and reported that mass-spectral differences also exist when methane c i m s is employed on these derivatives, although the differences observed in the methane

c i m s were less pronounced than those for the isobutane c i m s Independently, Bowser *et al* [58] noted differences in the methane c i m s of the $Me_3Si$ derivatives of 2-acetamido-2-deoxy-D-glucose, -mannose, and -galactose Such differences in the mass spectra of various stereoisomers differ from many observations (based, in general, on e i m s ) that the stereochemistry of a molecule has no effect on its mass spectrum[53,54] However, it is important that, although both McNeil and Albersheim[59] and Bowser *et al* [58] observed differences in the c i m s of different stereoisomers, these differences were due to changes in the *relative intensity* of the ions present in these spectra, and not to the displacement of the *m/e* value of these ions, or, in general, to the disappearance of any given ions

We have extensively examined the e i and the ammonia c i mass spectra of the PAAN derivatives both of partially *O*-methylated aldoses[44,50] and non-*O*-methylated aldoses[36 37] The use of isotopically substituted reagents in the PAAN derivatization procedure provided an intimate knowledge of the fragmentation pathways for both e i m s and c i m s , in addition, such isotopic substitution, in combination with g l c–e i m s and g l c–c i m s , provides a swift and precise method of identifying the individual components in an unknown mixture of aldoses[36 37] In the course of our investigations with the e i m s and c i m s of the PAAN derivatives, we have observed little, if any, evidence for mass-spectral differences based on stereochemical differences of these compounds, in fact, we observed essentially no differences in the mass spectra for positional isomer differences in these derivatives (*e g* , the c i m s of the PAAN derivatives of 2-deoxy-D-*arabino*-hexose and 6-deoxy-D-glucose are identical) However, this does not necessarily mean that our observations are at variance with those of McNeil and Albersheim[59] and Bowser *et al* [58] McNeil and Albersheim[59] employed isobutane as the ionizing reagent, and extensively studied highly *O*-methylated derivatives We employed ammonia as the ionizing reagent, and our most extensive comparisons of saccharide analogs dealt with non-*O*-methylated derivatives

One of our objectives in the development of a general, saccharide-survey method[37 38] was to identify the molecular weight of the compound by c i m s while employing e i m s to establish the positional isomerism and the g l c retention-times to indicate the stereochemistry To further these objectives, we have employed ammonia as the ionizing reagent, insofar as we are aware, it is the ionizing reagent that yields c i mass spectra (of peracetylated saccharides) having the simplest mass-spectral pattern Once our mass spectrometer is tuned in the e i mode, we switch to the c i mode and, while monitoring successive g l c peaks of the PAAN derivative of D-glucose, so adjust the ammonia concentration that the two ions ($[M + 18]^+$ and $[M - 59]^+$) are of equal intensity Under these c i conditions, the spectra of most of the other PAAN derivatives also displayed only two ions, the $[M + 18]^+$ and the $[M - 59]^+$ (the ammonia c i spectrum of the PAAN derivative of 2-acetamido-2-deoxy-D-glucose being a notable exception) We also observed that the use of methane, instead of ammonia, greatly lessens the intensity of the ions in the region of the capture ion, $[M + 1]^+$, of the c i spectra, and promotes the contribution of ions of

lower *m/e* (primarily the result of stripping of acetic acid and ketene from the capture ion[37]).

Similarly, we also observed that the incorporation of *O*-methyl groups into D-glucose produces PAAN derivatives that yield an ammonia c i spectrum more complex than that of the PAAN derivative of D-glucose (recorded under conditions that yield only two fragments of equal intensity for the latter) We observed differences in the relative intensities of the methane c i and the ammonia c i spectra of the partially *O*-methylated derivatives However, we noted a "spectrum-to-spectrum" difference of ~20% in the relative intensities of these c i spectra, and currently have no basis for correlating stereochemical differences of derivatives to c i mass-spectral differences In view of the differences in the observations of the effects arising from c i m s , it is possible that (*a*) isobutane is much more effective than ammonia for revealing mass-spectral differences arising from stereochemical and positional differences of saccharides, and (*b*) the incorporation of relatively large proportions of different functional groups (*O*-methyl and *O*-acetyl) can promote the observation of such structural differences in the c i spectra In accord with this, Li *et al*[38] reported (see Table I in ref 38) differences in the relative intensity of the isobutane c i spectra of PAAN derivatives of different stereoisomers It is also possible that the differences in the methane c i m s of the $Me_3Si$ derivatives of different stereoisomers of *N*-acetylhexosamines[59] result from the conformational enhancement due to the cyclic structure of these compounds, in contrast to the acyclic structures of the alditol acetates and the PAAN derivatives

In addition to the g l c of derivatives of saccharides, the g l c properties of various derivatives of oligosaccharides have also been examined[2] Although $Me_3Si$ derivatives have been extensively employed for such analyses, the acetyl derivatives are also sufficiently volatile to be employed In general, it has proved relatively easy to chromatograph oligosaccharides up to a degree of polymerization (d p ) of 4, but more difficult to separate various oligosaccharides having the same d p

As described in Fig 1, the PAKO derivatives (e*k*) are the complementary derivatives, from ketoses, of the procedure that has been employed to convert aldoses into their PAAN derivatives (e*a*) Although, as already discussed, the PAAN derivatives have been the subject of extensive g l c and mass-spectral studies, and of applications to saccharide analysis, we are not aware of the use of the corresponding PAKO derivatives either for g l c or mass-spectral studies The retention properties of the g l c columns have possibly inhibited the development of chromatography employing the PAKO derivatives We have favored four column-conditions[37 44] for the chromatographic separation of these PAAN derivatives, namely, (*a*) a 5% 1,4-butanediol succinate (BDS) column, programmed at 185–210° at 1°/min for the highest resolution of partially *O*-methylated saccharides, (*b*) a 3% neopentylglycol succinate (NPGS) column, programmed at 120–150° at 1°/min for the high resolution of neutral saccharides through the hexoses, (*c*) a 2% OV-17 column, programmed from 130 to 300° at 5°/min for monosaccharide separations, including *N*-acetylhexosamines, and (*d*) a similar 2% OV-17 column programmed from 130 to 300°

at 20°/min for a rapid, comprehensive survey of the nonmethylated saccharides present in a mixture These column conditions have the advantage of giving "overlapping" regions of retention times so that compounds that emerge at ~30 min from the BDS column, emerge at ~5 min from the NPGS column, and compounds that emerge at ~1 h from the NPGS column emerge from the OV-17 column within a few minutes Our initial examination of the PAAN derivatives employed columns having BDS and NPGS packings, and when both columns were tested with D-fructose and with tetra-*O*-methyl-D-fructose which had been subjected to the PAAN derivatization procedure, no peaks were observed However, as indicated in our report on the PAAN survey method for aldoses[36], when the PAAN derivatization procedure was employed with ketoses, peaks did emerge from injections into OV-17 columns The g l c and mass-spectral examination of these PAKO products, under conditions identical to those previously described for the PAAN derivatives, is the subject of the following discussion

## RESULTS AND DISCUSSION

The principal results presented here deal with the g l c separations, and the mass-spectral analysis, of derivatives of non-*O*-methylated ketoses The g l c and mass-spectral conditions employed were identical to those previously reported for a survey method for the rapid identification and characterization of aldoses by use of their PAAN derivatives[37] Two additional sets of data, which directly pertain to the ketose survey method, will also be briefly discussed, these deal with (*a*) the chromatographic and mass-spectral properties of highly *O*-methylated PAKO derivatives, and (*b*) the chromatographic properties of compounds produced by PAAN derivatization of oligosaccharides The procedure that gives the saccharide derivatives discussed next has been described as the PAAN derivatization procedure when applied to aldoses However, as this same procedure can be applied to produce peracetylated oximes from ketoses, this technique will be designated in more-general terms as the "PAAN–PAKO derivatization procedure" For brevity in the following sections, the simple reference to PAKO and PAAN derivatives will, unless otherwise specifically stated, refer to derivatives of *non*-O-*methylated* saccharides

### *Peracetylated keto-oxime derivatives of non-*O*-methylated saccharides*

*Gas–liquid chromatography of peracetylated ketooxime derivatives* — The relative retention times (r r t ) for PAKO derivatives are listed in Table I, in conjunction with selected comparative r r t for PAAN derivatives, and additional r r t of derivatives of *O*-methylated saccharides and oligosaccharides to be discussed in subsequent sections The g l c conditions described refer exclusively to packed columns containing OV-17 as the active surface The BDS- and NPGS-packed columns, which provide such excellent chromatographic resolution for the PAAN derivatives, cannot be employed, as they retain the PAKO derivatives completely It is assumed that this retention of the PAKO derivatives is due to the polarity of the

TABLE I

RELATIVE G L C RETENTION-TIMES, ON A COLUMN OF OV-17, OF PERACETYLATED KETO-OXIMES FROM KETOSES, AND OF REFERENCE SACCHARIDES

| *Parent saccharide* | *Relative retention-time (condition 3[a])* |
|---|---|
| DL-Glyceraldehyde[b] | 0 34 |
| D-Erythrose[b] | **1 00**[c] |
| 1,3-Dihydroxy-2-propanone (type 14) | 1 34 |
| 2,3,4,6-Tetra-*O*-methyl-D-glucose[b] (1) | 1 38 |
| 1,3,4,6-Tetra-*O*-methyl-D-fructose (2) | 1 61 (*l*, 1 8[d]), 1 70 |
| 1,3,4-Tri-*O*-methyl-D-fructose (3) | 2 32, 2 35 (*l*, 1 5) |
| 3,4,6-Tri-*O*-methyl-D-fructose (4) | 2 38 |
| D-Glucose[b] | 2 42 |
| D-*erythro*-2-Pentulose (type 16) | 2 59, 2 67 (*l*, 1 7) |
| D-*glycero*-D-*gluco*-Heptose[b] | 2 95 |
| D-Fructose (type 17) | 3 18 |
| Sedoheptulosan[e] (type 18) | 3 18 |
| D-Tagatose (type 17) | 3 18, 3 26 (*l*, 2 3) |
| L-Sorbose (type 17) | 3 26 |
| D-*manno*-2-Heptulose (type 19) | 3 67, 3 77 (*l*, 1 8) |
| Maltose[b] | 4 79 |
| Cellobiose[b] | 4 89 |
| Melezitose[e] | 7 28 |
| Raffinose[e] | 7 49 |

[a]G l c in a glass column (1 23 m × 2 mm) packed with 2% of OV-17 on Chromosorb W HP (80–100 mesh), nitrogen flow 22 mL/min, programmed at 130 to 300° at 20°/min [b]These saccharides yielded peracetylated aldononitrile derivatives [c]1 00 equals 2 96 min [d]For saccharides yielding two peaks, the r r t of the largest peak is indicated by *l*, followed by the ratio of the area of the larger peak to the area of the corresponding smaller peak [e]These saccharides yielded the peracetylated derivatives

acetyloxime group In general, the order of the r r t of the PAAN derivatives remains the same for the OV-17, the BDS, and the NPGS columns Therefore, when the spectral "profiles" of dual chromatograms (OV-17 *vs* succinate column) are compared, peaks present in the chromatogram from OV-17 columns but absent from the chromatogram from the succinate column indicate the presence of derivatives of saccharides that contain moderately polar groups, or of PAKO derivatives

The following general observations can be made for the OV-17 compared to the succinate column (*a*) Although the OV-17 column does not give extremely high resolution, it yields sharp, symmetrical peaks for the PAKO derivatives (*b*) The rate of the temperature programming can be increased for the OV-17 column (*e g*, from 5°/min to 20°/min) to shorten the program time, with little loss of resolution (*c*) The relatively high, upper temperature-limit of the OV-17 column permits saccharides of large molecular weight to be chromatographed The chromatograms were produced by the direct injection of the chloroform extract from the PAAN–PAKO derivatization procedure Most of the chloroform solutions contained 1–10 mg of saccharide per mL of solvent, and injections were made in the 1-$\mu$L to 5-$\mu$L range, a range in which

the detector response (peak area) is essentially linear with the sample size Unless otherwise noted, all references to detector responses refer to the hydrogen-flame-detector technique employed for the preliminary analysis of each derivatization mixture The detector responses of the various PAKO derivatives (for intercomparison, and for comparison with the PAAN derivatives) are essentially proportional to the weight of the resulting derivatives However, for the comparison of similar aldoses and ketoses (*e g*, the hexoses and the 2-hexuloses), it should be noted that, owing to loss of acetic acid during production of the PAAN derivative, the PAKO derivative has a larger molecular weight (by 60 mass units) than the PAAN derivative When the mass spectrometer is employed in the repetitive-scan mode as the g l c detector, the detector responses of the PAAN and PAKO derivatives differ somewhat, those of the PAKO derivatives being the smaller For example, when employing e i m s, the detector response of the PAKO derivatives (on a weight basis) is ~70% of that of PAAN derivatives This relatively low, detector response of the PAKO derivatives is attributed to increased, total fragment-ion loss, due to the instability of the fragment ions produced from the acetoxime end of the molecule (see the following section on mass spectrometry)

In general, the r r t of these PAAN derivatives are proportional to their molecular weights A comparison of the r r t of PAAN and PAKO derivatives of similar size and structure indicated that the r r t of the PAKO derivative will be approximately one unit larger than that of the corresponding PAAN derivative For example, the following sets may be compared 1,3-dihydroxy-2-propanone PAKO (r r t 1 34) and DL-glyceraldehyde PAAN (0 34), D-fructose PAKO (r r.t 3 18) and D-glucose PAAN (2 42), and D-*manno*-2-heptulose PAKO (r r t 3 67) and D-*glycero*-D-*gluco*-heptose PAAN (2 95) We have not examined enough sets of stereoisomers of PAKO derivatives to reach any conclusions regarding the relationship of stereochemistry to r r t However, the PAKO derivatization procedure often produces two chromatographic peaks for each starting ketose, such peaks yield the same mass spectrum, and are presumed to represent the *syn* and *anti* orientations of the acetoxime group These double peaks are identified in Table I, which also describes the ratio of the areas of these peaks

The PAAN–PAKO derivatization procedure is applicable to hydrolyzates resulting from a wide variety of natural products, and is compatible with many common hydrolysts The general utility of this procedure for hydrolyzates of various origins has been discussed with regard to the PAAN derivatives[37], specifically, the procedure is useful with products originally containing large percentages of protein, as peaks from the resulting derivatives of the amino acids are not present in the chromatograms On comparing Table I presented herein with Table I of ref 37, it is observed that the g l c peaks of both the PAKO and PAAN derivatives have very little overlap when the OV-17 column conditions are employed, allowing simultaneous characterization of the PAAN and PAKO derivatives Possible ambiguities with regard to the identity of the g l c peaks can be readily resolved by mass spectrometry As previously described for the PAAN derivatives[37], the employment of the foregoing

TABLE II

AMMONIA CHEMICAL-IONIZATION, MASS SPECTROMETRIC FRAGMENT-IONS OF PERACETYLATED KETO-OXIME DERIVATIVES AND RELATED COMPOUNDS

| *Assignment of fragment-ion* | *Compound* | | | | |
|---|---|---|---|---|---|
| | *1,3-Dihydroxy-2-propanone PAKO (Type 14)* | D-erythro *2-Pentulose PAKO (Type 16)* | D-*Fructose PAKO (Type 17)* | D-manno *2-Heptulose PAKO (Type 18)* | *Peracetylated sedoheptulosan (Type 19)* |
| M + 18 | 249 (22[a], 1[b], 9[c], 3[d]) | 393 (2[a], 1[b], –[c,d], 3[e]) | 465 (2[a], 1[b], –[c], –[e]) | | 378 (22[a], 12[c]) |
| M + 1 | 232 (100, 1, 9, 3) | 376 (4, 1, 15, 3) | 448 (3, 1, 18, 3) | | 361 (68, 12) |
| M + 18 − 60 | | 333 (3, 1, –, 0) | | | |
| M + 1 − 58 (or X) | 174 (18, 1, 6, 0) | 318 (6, 1, 12, 0) | 390 (41, 1, 15, 0) | | |
| M + 1 − 60 | 172 (4, 1, 6, 0) | 316 (2, 1, 12, –) | 388 (6, 1, 15, –) | | 301 (38, 9) |
| M + 18 − 120 | 129 (50, 1, 3, 0) | | | | |
| X − 60 | 114 (55, 1, 3, 0) | 258 (22, 1, 9, 0) | 330 (100, 1, 12, 0) | | |
| M + 1 − 120 | 112 (32, 1, 3, 0) | 256 (8, –, 9, 0) | 328 (12, 1, 12, 0) | | 241 (100, 6) |
| X − 120 | | 198 (23, 1, 6, 0) | 270 (36, 1, 9, 0) | | |
| M + 1 − 180 | | | 268 (13, 1, 9, 0) | | 181 (31, 3) |
| X − 180 | | 138 (100, 1, 3, 0) | 210 (76, 1, 6, 0) | | |
| X − 240 | | | 150 (90, 0, 3, 0) | | |
| | 100 (19, 1, 4, 0)[f] | 240 (5, 1, 9, 0) | 333 (42, 1, 12, 0) | 447 (52[a], 0[b], 15[c]) | 199 (71, 3) |
| | 99 (20, 0, 3, 0)[g] | 217 (10, 0, 9, 0) | 289 (19, 0, 12, 0) | 430 (22, 0, 15) | 139 (21, 0) |
| | 60 (6, 0, 3, 0) | 156 (41, 1, 6, 0) | 256 (8, 1, 9, 0) | 405 (61, 1, 15) | 81 (64, 0)[g] |
| | | 140 (70, 1, 3, 0) | 187 (54, 0, 6, 0) | 328 (100, 1, 12) | |
| | | 120 (30, 0, 6, 0) | 168 (45, 1, 9, 0) | 322 (90, 0, 0) | |
| | | 114 (21, 1, 4, 0) | 115 (90, 0, 3, 0)[g] | 303 (100, 0, 12) | |
| | | 98 (15, 1, 1, 0) | | 259 (15, 0, 9) | |
| | | 80 (8, 0, 0, 0) | | 201 (18, 0, 6) | |
| | | 60 (8, 0, 0, 0) | | 138 (19, 0, 3) | |
| | | | | 81 (50, 0, 0) | |

[a]Intensity (percent) of the mass fragment relative to that of the most-intense mass-fragment [b]Hydroxyl[$^{15}$N]amine was employed in the derivatization procedure [c]Acetic anhydride-$d_0$ was employed in the derivatization procedure [d]The symbol "–" indicates that no corresponding *m/e* was observed [e]Acetic anhydride and acetic anhydride-$d_0$ were employed in a two step acetylation during the derivatization procedure, with a single acetyl-$d_3$ incorporated on the oxime [f]The *m/e* values below the line are unassigned [g]These fragment ions correspond to *m/e* in the e i spectrum

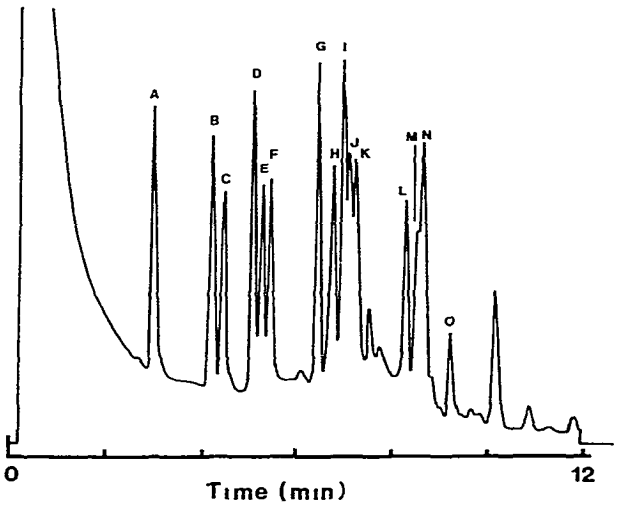



Fig 2 Gas–liquid chromatogram (hydrogen-flame detector) from a column of OV-17 on Chromosorb W (condition 3) [The PAAN derivatives of A, D-erythrose, B, digitoxose, C, 2-deoxy-D-*erythro*-pentose, D, D-ribose, E, D-arabinose, F, D-xylose, G, 2-deoxy-D-*arabino*-hexose, H, D-allose, I, D-mannose, J D-glucose, K, D-galactose, L, 2-acetamido-2-deoxy-D-glucose, M, 5-thio-D-glucose (peracetylated), N, D-*glycero*-D-*gluco*-heptose, and O, 2-acetamido-2-deoxy-D-galactose ]

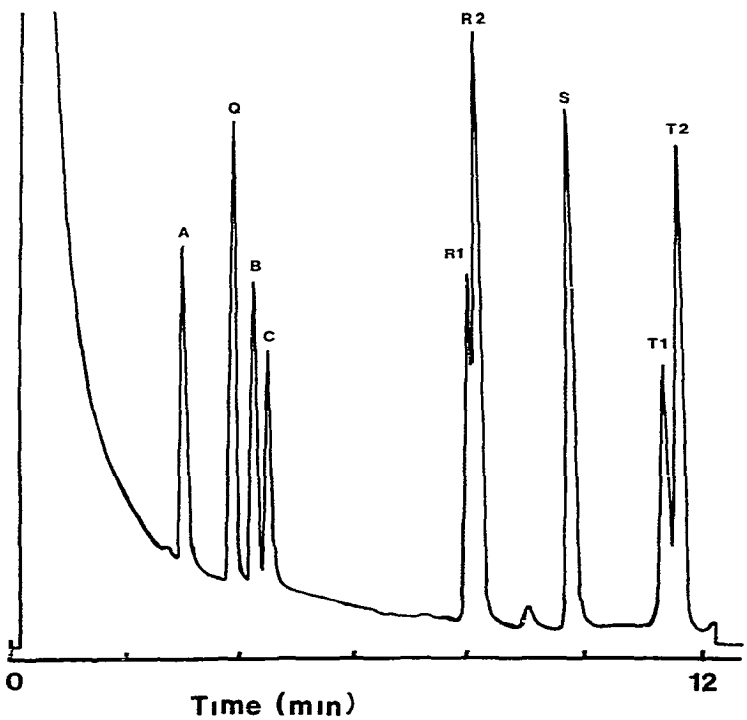



Fig 3 Gas–liquid chromatogram (hydrogen-flame detector) from a column of OV-17 on Chromosorb W (condition 3) (The PAKO derivatives of Q, 1,3-dihydroxy-2-propanone, R1 and R2, D-*erythro*-2-pentulose, S, D-fructose, and T1 and T2, D-*manno*-2-heptulose In addition, the PAAN derivatives of A, D-erythrose, B, digitoxose, and C, 2-deoxy-D-*erythro*-pentose )

chromatographic procedures, in conjunction with the various forms of mass spectrometry and isotopic variants of the derivatization procedure, allows the following information to be obtained with regard to the parent saccharide corresponding to a g l c peak of an unknown PAKO derivative (*a*) the molecular weight, (*b*) the number of hydroxyl groups, (*c*) the number of aldehyde or ketone functional groups, and (*d*) the presence, and position in the structure, of a variety of substituents

The chromatographic resolution of the PAKO derivatives may be evaluated by comparing the chromatogram in Fig 2, for PAAN derivatives, to the chromatogram in Fig 3, for PAKO derivatives The chromatograms in Figs 2 and 3 were obtained under identical chromatographic conditions (described as condition 3 in ref 37, and also herein) The mixture of PAAN derivatives employed in the foregoing Fig 2 (condition 3) is the same as the mixture employed in Fig 2 (condition 2) of ref 37, allowing direct comparison of these two chromatographic conditions, as both conditions employ an OV-17 column, but differ in the rate of temperature programming (condition 2, 5°/min, condition 3, 20°/min) For the PAAN derivatives, there is little difference in resolution between condition 2 and condition 3 Chromatography of the PAAN derivatives with the OV-17 column therefore differs from the succinate (NPGS and BDS) columns, when the latter phases are employed, slowing of the temperature-rate program can cause a pronounced increase in the resolution of the chromatographic peaks

When employing OV-17 columns, the dependence of the resolution of the peaks of the PAKO derivatives on the rate of the temperature program is similar to that of the PAAN derivatives Therefore, owing to the large r r t of the PAKO derivatives, it is more convenient to employ condition 3 for the separation of these derivatives, for example, a 12-min g l c program can survey the PAAN and PAKO derivatives from D-erythrose through the heptoses and 2-heptuloses The resolution of condition 3 is such that essentially base-line separation can be obtained between the peaks of the PAKO derivatives shown in Fig 3, with regard to any of the peaks of the PAAN derivatives shown in Fig 2 The use of condition 2 can be useful to (*a*) examine the PAKO derivatives of *O*-methylated saccharides (see the following section), and (*b*) increase the number of mass-spectral scans per chromatogram in order to allow a more accurate estimate of the homogeneity of each g l c peak For condition 2, when the retention time of the PAAN derivative equals 4 15 min, the retention times of the PAKO derivatives of the following ketoses are (in minutes) D-*erythro*-2-pentulose, 8 93 and 9 27, D-fructose, 12 4, and D-*manno*-2-heptulose, 14 6 and 15 1

*Ammonia chemical-ionization mass spectrometry* — The ammonia c i m s of the g l c peaks of each compound listed in Table I were recorded For those ketoses that yielded two, chromatographically separable, PAKO derivatives, the resulting g l c peaks gave identical ammonia c i spectra, in accord with the concept that these two peaks represent compounds differing only in respect to the *syn* and *anti* orientations of the acetoxime The effluent from the g l c column was repetitively scanned (~5 s for each scan), and no spectral differences were observed for mass spectra taken at different times as a given peak emerged from the column D-Fructose,

D-tagatose, and L-sorbose are members of the same set of stereoisomers, the 2-hexuloses, and the PAKO derivatives of these three compounds yielded identical ammonia c i spectra Therefore, the mass spectra of the PAKO derivatives are discussed in terms of "types", as was previously done with the PAAN derivatives[37], with each set of stereoisomers representing a given type, as described in Table II. Four different types of PAKO derivatives have been studied, and, as a continuation of the 13 designations of type of structure for the PAAN derivatives and related compounds[37], the PAKO derivatives have been designated types 14 through 18 A position, type 15, has been reserved for the PAKO derivatives of the 2-tetruloses, although no examples of this type are given in this report Table II also contains m s values for peracetylated sedoheptulosan as a representative of a set of stereoisomers designated type 19 Types 14 through 18 respectively represent the products from 1,3-dihydroxy-2-propanone, 2-tetruloses, 2-pentuloses, 2-hexuloses, and 2-heptuloses as their PAKO derivatives, formed by the procedure outlined in the Experimental section

The top section of Table II assigns the capture-ions observed in the ammonia c i spectrum of compounds of types 14, 16, and 17, on the basis of M, the molecular weight of the parent PAKO derivative, in combination with +18 (the ammonium ion) or +1 (the hydronium ion), and attributes many of the ions of smaller *m/e* value as resulting from the successive elimination of one, two, or three molecules of "neutral" acetic acid (mass = 60) from the $[M + 1]^+$ and $[M + 18]^+$ capture-ions The presence of the M + 1 and M + 18 peaks in the ammonia c i spectra of the PAKO derivatives is similar to that in the corresponding mass spectra of the PAAN derivatives (which display prominent M + 1 peaks in the spectra of PAAN derivatives that only contain nitrogen as the nitrile group, and M + 18 in those of PAAN derivatives that contain an additional nitrogen atom in an acetamido group) However, in contrast to the ammonia c i spectra of the PAAN derivatives, where the M + 18 peak (or M + 1 for the acetamido compounds), in conjunction with the M — 59 peak, dominates the spectrum, both the M + 1 and M + 18 peaks of the ammonia c i spectra of the PAKO derivatives are quite weak

For fine tuning of the mass spectrometer under ammonia c i conditions, the final step consisted of so adjusting the ammonia concentration in the ionization chamber that the c i spectrum of the PAAN derivative of D-glucose contained only two peaks, of equal intensity, M + 1 and M — 59, with these two peaks representing over 99% of all observable ions (for tuning, the spectrum was scanned from 100 to 450 mass units) The optimum conditions for obtaining maximum percentages of $[M + 1]^+$ and $[M - 59]^+$ ions in the ammonia c.i spectrum of the PAAN derivatives were also the optimum conditions that could be found for obtaining maximum percentages of ions of large *m/e* in the ammonia c i spectra of PAKO derivatives, the relatively weak intensities of these mass fragments (see Table II) indicate a lower stability of the PAKO derivatives, under c i m s conditions, than of the corresponding PAAN derivatives

It was fortunate that the $[M + 1]^+$ and $[M + 18]^+$ capture-ions of the PAKO derivatives could be observed, as these peaks provide direct evidence for the structure

of the derivative formed from ketoses under derivatization procedures that produce PAAN derivatives from aldoses Although ions of larger *m/e* obtained from the PAKO derivatives under ammonia c i m s conditions are less stable than those from the corresponding PAAN derivatives, the fundamental processes are the same, that is, $[M + 1]^+$ and $[M + 18]^+$ give prominent *m/e* values, and other prominent *m/e* values result from the loss of "neutral" acetic acid from the capture-ions If the concentration of ammonia in the ionization chamber is not correct, the c i spectra of the PAAN derivatives are quite similar to those observed for the PAKO derivatives, with weak $[M + 1]^+$ (or $[M + 18]^+$), and smaller ions (smaller than M + 1 by multiples of 60) dominating the spectrum as a result of the loss of acetic acid This enhanced tendency for $[M + 1]^+$ to lose acetic acid has also been noted for PAAN derivatives of partially *O*-methylated derivatives of D-glucose, where there is also a marked tendency to lose a neutral, methanol molecule[50].

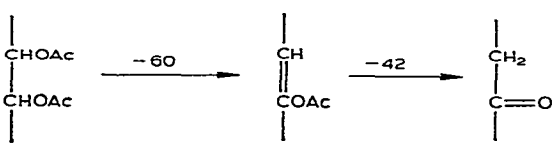
However, in addition to the ions observed in the ammonia c i spectra of PAKO derivatives, additional *m/e* are present in these spectra that apparently have no analog in the corresponding spectra of the PAAN derivatives Table II indicates that the ammonia c i spectra of PAKO derivatives contain major contributions from $[M + 1]^+$, $[M + 18]^+$, and ions smaller than these capture-ions by multiples of 60 The assignments of Table II were further confirmed by examining isotopically substituted variants of each of the types of PAKO derivative One simple, substitution technique is to employ hydroxyl[$^{15}$N]amine instead of hydroxylamine during the derivatization procedure, this produces a PAKO derivative yielding a mass spectrum in which all fragment-ions retaining the nitrogen atom of the acetoxime group are displaced (relative to those of the normal PAKO derivative) by +1 mass unit, such displacements are indicated in the columns, identified by *b*, following the *m/e* values (see Table II)

Similarly, use of acetic anhydride-$d_6$ instead of acetic anhydride during the derivatization procedure produces a PAKO derivative having a mass spectrum in which the *m/e* values of the ions are displaced (relative to those of the normal PAKO derivative) by +3 mass units for each acetoxyl group retained, such displacements are indicated in the columns, identified by *c*, following the *m/e* value (see Table II) The use of acetic anhydride-$d_6$ allows the easy determination of the number of acetyl groups remaining in each ion, and provides a good control on the assignment of a proposed structure to an ion of given *m/e* value For example, each backbone carbon atom of compounds of types 14, 16, and 17 is associated with an acetoxyl group, therefore, if $n$ represents the number of backbone carbon atoms for a given type, the *m/e* values of the ions of the corresponding ammonia c i spectra should increase, on acetylation with acetic anhydride-$d_6$, by $3n$ for M + 1 and M + 18, by $3(n - 1)$ for *m/e* values representing fragment-ions that have lost one molecule of acetic acid, and by corresponding decreases in units of 3 for successive losses of acetic acid A final, isotopic-substitution procedure consisted of first treating the ketose with hydroxylamine and then adding a slight excess of acetic anhydride, these steps then being followed by addition of a ten-fold molar excess of acetic anhydride-$d_6$ and a

normal, reaction period of 20 min at 70° The two-step acetylation produces PAKO derivatives that yield ammonia c i spectra which clearly indicate (for the PAKO derivatives of 1,3-dihydroxy-2-propanone, D-*erythro*-2-pentulose, and D-fructose) that the $[M + 1]^+$ and $[M + 18]^+$ capture-ions are completely displaced (relative to those of the normal PAKO derivative) by +3, and only +3, mass units; such displacements are listed in the columns, identified by *e*, following the *m/e* values (see Table II) As an excess of ~20 mole percent, relative to the ketose, of acetic anhydride was employed in the first step, which was then followed by a ten-fold increase in acetic anhydride-$d_6$, the effective molar ratio of deuterated to non-deuterated molecules of acetic anhydride was ~50 1 during the final acetylation step

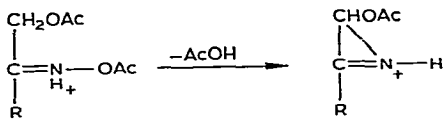
The ammonia c i spectrum of the PAKO derivative of 1,3-dihydroxy-2-propanone contains relatively intense M + 1 and M + 18 peaks, and for the ammonia c i spectrum of the two-step acetylation derivative, no peak intensity in excess of the expected ~50 1 ratio was observed for the undisplaced $[M + 1]^+$ ion (which would have been at *m/e* 232) or the undisplaced $[M + 18]^+$ ion (which would have been at *m/e* 249), a displacement effect paralleled, although less easily observed, by the PAKO derivatives of D-*erythro*-2-pentulose and D-fructose (see Table II) However, from Table II, it may also be seen that, although the nitrogen atom is retained in all of the assigned fragment-ions observed in the ammonia c i spectrum, the 3-mass-unit displacement resulting from the two-step acetylation occurs only in $[M + 1]^+$ and $[M + 18]^+$ ions, and not in any of the other assigned ions It should also be noted that most of the intense ion peaks, in both the c i. and e i spectra, are followed by a less intense peak one mass unit larger than the prominent peak Such additional ions of minor intensity result from the natural distribution of $^{13}C$, and, for simplicity, are not listed in the Tables

On the basis of these *m/e* shifts that result from isotopic substitution, it is concluded that (*a*) during the second acetylation step, only a single acetyl group is added to each molecule, (*b*) this single acetyl group is added to the position that, of the sites available for acetylation, is chemically the most different (*i e*, the oxime), (*c*) under ammonia c i m s conditions, the acetyl group of the oxime is lost very easily, and (*d*) the nitrogen atom of the acetoxime group resists loss It is assumed that there is an equal probability that any of the *O*-acetyl groups can then be lost from the parent ion, and that the mass-spectral peaks of M + 1 — 120 and M + 1 — 180 actually represent a group of fragment-ions corresponding to positional isomers, as a result of random loss of acetoxyl groups from different carbon atoms Therefore, no specific structures will be given to illustrate the ammonia c i m s fragmentation-pathways The loss of a "neutral" acetic acid molecule from a parent fragment-ion results in a product containing a double bond For e i m s, the following process is



normally observed firstly a loss of "neutral" acetic acid accompanied by double-bond formation in the capture-ion, and secondly, a rearrangement of the double bond and loss of ketene

However, for the ammonia c i spectra of the PAAN derivatives, little evidence was found for the second step (loss of ketene), and similarly, little evidence for loss of acetoxyl groups *via* ketene elimination has been found in the c i spectra of these PAKO derivatives under similar, mass-spectral conditions, deuterioacetylation makes such losses of ketene easy to observe, due to the resulting *m/e* displacements of 2 mass units for each acetoxyl group lost The loss of the *O*-acetyl group from the acetoxime presents a problem in interpretation, as C-2 of the ketose bears no hydrogen atom, and cannot easily accommodate further unsaturation The data provide little information on the type of structure involved, and it is possible that a three-member ring-system results, as shown, or that a more complex rearrangement is involved In summary, for these ammonia c i spectra, a set of peaks is observed that can be described as the M + 1 — 60*n* series for type 14 and 16 compounds, $n = 0$, 1, and 2, for type 17 compounds, $n = 0$, 1, 2, and 3




Although these spectra contain many peaks analogous to those observed in previous ammonia c i spectra of other derivatives of saccharides, the ammonia c i spectra of compounds of types 14, 16, and 17 contain significant contributions from ions that apparently have no analogs among the structures represented in the ammonia c i spectra of the PAAN derivatives For example, the ion of *m/e* 174 in the ammonia c i spectrum of the 1,3-dihydroxy-2-propanone PAAN derivative represents $[M - 57]^+$, and many ions that occur in the ammonia c i spectra of compounds of types 14, 16, and 17 can be described by $[M - 57]^+$, or by $[M - 57 - 60n]^+$, where *n* equals zero or a small integer. Despite, or, perhaps, because of, extensive isotopic substitution, we could not readily assign structures to this series of ions, however, the following relationships appear to hold true (*a*) the *O*-acetyl group attached to the oxime is always lost, (*b*) the nitrogen atom of the oxime group is never lost, (*c*) no other acetoxyl group need be lost, (*d*) the backbone chain always remains intact, and (*e*) these ions probably originate from $[M + 1]^+$, rather than from $[M + 18]^+$, on the basis that all *m/e* values of these ions are even numbers

In addition, as the ammonia c i spectrum of the PAKO derivative of 1,3-dihydroxy-2-propanone contains a member of this group, it is further indicated that only C-1 through C-3 need be involved in the production of the series containing the *m/e* = M — 57 — 60*n* fragment-ions Acetone has a molecular weight of 58, and it is tempting to describe the M — 57 — 60*n* series as resulting from the loss of this molecule However, the isotopic-substitution data preclude several possibilities for

acetone elimination, and, to fulfil such requirements, it would appear that at least three hydrogen atoms and, presumably, a corresponding carbon atom, would need to be abstracted from the backbone of the saccharide The ammonia c i spectrum of the deuterioacetyl isotopic variants of the PAKO derivative of 1,3-dihydroxy-2-propanone clearly shows the loss of one deuterioacetyl group, that of the acetoxime group, and the retention of both of the other acetyl groups For the simple molecule of the PAKO deuterioacetyl derivative of 1,3-dihydroxy-2-propanone, the only (nondeuterio) hydrogen atoms available are those on the backbone We therefore do not describe the precise structure of the $[M - 57 - 60n]^+$ ions, except to note that the final contribution of the C-1 through C-3 atoms of the original molecule are now represented by 173 mass units, and that the rest of the molecule appears to undergo sequential loss of molecules of acetic acid in a process similar to that described for the $M + 1 - 60n$ series For convenience of comparison, and for brevity of notation, the unit "M — 57" is also described as "X" in Table II In summary, this set of fragment-ions is observed for those ammonia c i spectra that can be described as the $M - 57 - 60n$ series for compounds of type 14, $n = 0$ and 1, for compounds of type 16, $n = 0$, 1. 2, and 3, and, for compounds of type 17, $n = 0$, 1, 2, 3, and 4 Despite the lack of an assignment of a precise structure, the $M - 57 - 60n$ series is a good diagnostic tool for differentiation of PAKO derivatives from PAAN derivatives

The ammonia c i spectrum of the D-*manno*-2-heptulose derivative, the example of type 18, has not been discussed with the other 2-glyculoses, as it is quite different from those recorded under corresponding conditions for compounds of types 14, 16, and 17 (see Table II) On the basis of the e i spectra discussed in the following section, it is believed that the product that results from subjecting D-*manno*-2-heptulose to the standard derivatization procedure, described in the Experimental section, produces a PAKO derivative that is a simple, linear homolog of the PAKO derivatives of 1,3-dihydroxy-2-propanone, D-*erythro*-2-pentulose, and D-fructose We are not certain why the ammonia c i spectrum of the PAKO derivative of D-*manno*-2-heptulose differs so greatly from those of the corresponding derivatives of the lower 2-ketoses However, it is probable that the differences in the ammonia c i spectra of these compounds, shown in Table II, result from a problem of instrumentation, rather than from any fundamental differences in the structure of the PAKO derivatives, or in the favored fragmentation-pathways for these PAKO derivatives The g l c instruments employed for the routine survey of the PAAN and PAKO derivatives are equipped with hydrogen-flame detectors, and these systems, when employing the OV-17 columns, can easily reach 350°, allowing production of sharp, symmetrical g l c peaks In contrast, the integrated, g l c –m s system has the g l c and the mass spectrometer components interfaced with a membrane separator, effectively limiting the upper g l c temperature to ~230°, a temperature close to the elution temperature of the PAKO derivative of D-*manno*-2-heptulose Therefore, although a peak for the PAKO derivative of D-*manno*-2-heptulose emerges from the g l c column of the integrated, g l c –m s system, and can be properly identified by e i m s , the resulting

TABLE III

ELECTRON-IMPACT, MASS-SPECTROMETRIC FRAGMENT-IONS OF PERACETYLATED KETO-OXIME DERIVATIVES AND RELATED COMPOUNDS

| *Compound* | | | | |
|---|---|---|---|---|
| *1,3-Dihydroxy-2-propanone PAKO (Type 14)* | D-erythro-*2-Pentulose PAKO (Type 16)* | D-*Fructose PAKO (Type 17)* | D-manno-*2-Heptulose PAKO (Type 18)* | *Peracetylated sedo-heptulosan (Type 19)* |
| 129[a] (81[b], 1[c], 3[d], 0[e]) | 273 (37[b], 1[c], 9[d], 0[e]) | 345 (16[b], 1[c], 12[d e f]) | 417 (16[b], 1[c], 15[d]) | 301 (3[b], 9[c]) |
| 99 (63, 0, 3, 0) | 231 (4, 1, –[g], 3) | 243 (10, 1, 7, [f]) | 315 (22, 1, 10) | 259 (3, 4) |
| 43 (100, 0, 3, [f]) | 213 (3, 1, 6, 0) | 213 (4, 1, 7, 0) | 255 (1, 1, [f]) | 258 (8, 7) |
| | 200 (2, 1, 6, 0) | 201 (6, 1, 4, 0) | 213 (5, 1, 5) | 199 (12, 4) |
| | 184 (6, 1, 6, 0) | 200 (5, 1, 6, 0) | 200 (8, 1, 6) | 157 (18, 6) |
| | 183 (5, 0, 6, 0) | 184 (10, 1, 3, 0) | 196 (9, 1, 3) | 141 (18, 3) |
| | 171 (31, 1, 4, 0) | 183 (33, 1, 4, 0) | 195 (23, 1, 3) | 115 (19, 0) |
| | 158 (22, 1, 4, 0) | 170 (8, 1, 6, 0) | 187 (8, 0, 6) | 112 (13, 3) |
| | 154 (9, 0, 3, 0) | 158 (17, 1, 4, 0) | 183 (3, 1, 5) | 101 (80, 3) |
| | 145 (12, 0, 6, 0) | 153 (18, 0, 3, 0) | 165 (17, 0, 3) | 98 (23, 1) |
| | 141 (23, 0, 4, 0) | 141 (32, 1, 2, 0) | 158 (5, 1, 4) | 81 (100, 0) |
| | 129 (19, 1, 2, 0) | 123 (23, 1, 1, 0) | 153 (18, 1, 2) | 43 (81, 3) |
| | 111 (38, 1, 1, 0) | 115 (21, 0, 3, 0) | 145 (19, 0, 6) | |
| | 99 (21, 0, 1, 0) | 111 (20, 0, 2, 0) | 139 (14, 0, 3) | |
| | 81 (6, 0, 1, 0) | 103 (19, 0, 1, 0) | 128 (16, 0, 4) | |
| | 43 (100, 0, 3, [f]) | 99 (12, 0, 1, 0) | 123 (16, 1, 1) | |
| | | 43 (100, 0, 0, [f]) | 115 (38, 0, 3) | |
| | | | 103 (20, 0, 1) | |
| | | | 43 (100, 0, 3) | |

[a]The assignments of the fragment ions of the e i mass spectra are made in Table IV, and in the following fragmentation-pathways for the e i mass spectra [b]Intensity (percent) of the mass fragment relative to that of the most-intense mass-fragment [c]Hydroxyl[$^{15}$N]amine was employed in the derivatization procedure [d]Acetic anhydride-$d_6$ was employed in the derivatization procedure [e]Acetic anhydride and acetic anhydride-$d_6$ were employed in a two-step acetylation during the derivatization procedure, with a single acetyl-$d_3$ incorporated on the oxime [f]Shifts of *m/e* of both 0 and 3 were observed [g]The symbol "–" indicates that no corresponding *m/e* was observed

peak is not symmetrical, and it is assumed that the effect of working near the upper temperature-limit of this system contributes to the unusual, ammonia c i spectrum of this PAKO derivative

Peracetylated sedoheptulosan (a representative of a group of stereoisomers designated type 19) is the remaining compound studied by ammonia c i m s (see Table II) The ammonia c i spectrum of this peracetylated product is readily distinguished from those of the PAKO derivatives Although both the $[M + 1]^+$ and $[M + 18]^+$ capture-ions are present in the ammonia c i spectra of compounds of type 19, the rest of the spectrum contains prominent contributions from the $M + 1 - 60n$ series of ions, when $n = 1$, 2, or 3

*Electron-impact mass spectrometry* — The e i spectra of the g l c peaks of

each PAKO derivative listed in Table I were recorded (see Table III) As with the ammonia c i spectra of these chromatograms, (*a*) no differences were observed in the e i spectra that were scanned at different time-intervals in a given g l c peak, (*b*) for ketoses that yielded two, chromatographically different, PAKO derivatives (*syn*- and *anti*-acetoxime), the e i spectra of these peaks were identical, and (*c*) stereoisomers within a given type (*e g*, the PAKO derivatives of D-fructose, D-tagatose, and L-sorbose) yielded identical e i spectra

Bishop *et al* [37] and others[34] have studied the e i m s of a variety of PAAN derivatives that can be produced from aldoses The two processes that dominate the e i m s fragmentation of the PAAN derivatives of non-*O*-methylated aldoses are (*a*) an initial, random cleavage of the backbone chain, followed by (*b*) the successive loss of acetic acid and ketene molecules from each of the fragment-ions produced by the backbone cleavage Such a fragmentation pattern can provide accurate information regarding the position of certain substituents, or structural features (*e g*, a deoxy group), in a given PAAN derivative and, presumably, information as to such features in the original saccharide For the e i m s of PAAN derivatives of aldoses containing methoxyl groups, the processes dominating the fragmentation pathways are (*a*) an initial, backbone cleavage, which occurs preferentially between two carbon atoms to which are attached methoxyl groups, or (*b*), alternatively, although less favored, an initial, backbone cleavage occurring between carbon atoms to which are respectively attached a methoxyl and an acetoxyl group, and (*c*) these initial steps, followed by the loss of methanol, acetic acid, and ketene molecules from each of the fragment-ions produced by the backbone cleavage (although, in practice, apparently as a result of the respective stabilities of the fragment-ions, the fragment-ions arising from the nitrile end of the chain make a small contribution to the total number of fragment-ions present in a given spectrum) For all e i spectra of the PAAN derivatives that have been studied, the great majority of the fragment-ions observed have *m/e* values of less than half of the molecular weight of the given PAAN derivative, and few, if any, *m/e* values are observed that are $>60\%$ of M

The e i spectra of the PAKO derivatives studied present a surprising contrast to the e i spectra of the PAAN derivatives In fact, owing to the radical divergence in the fragmentation pathways of the e i spectra, as between the PAAN and the PAKO derivatives, we were fortunate to have the ammonia, c i m s data available in order to confirm the actual molecular weights, and, by implication, the structures, of these PAKO derivatives Apparently, backbone cleavage is not the initial, or even a prominent, process in the fragmentation pathway of the e i m s of the PAKO derivatives For example, in the e i spectra of the PAKO derivatives there are (*a*) only a few, weak, fragment-ions that correspond to the C1, C2, etc, series (as described in ref 37), that would result from the non-acetoxime end of the PAKO derivative, and which should correspond exactly to the fragment-ions arising from the non-nitrile end of the PAAN derivatives, (*b*) relatively few fragment-ions of common *m/e* values in the spectra from D-*erythro*-2-pentulose, D-fructose, and D-*manno*-2-heptulose, further indicating no common fragment-ions from either end of the PAKO

derivatives, and (*c*) the nitrogen atom is present in most fragment ions, indicating either that no backbone cleavage occurs, or that most of the fragment-ions observed originate from the acetoxime end of the PAKO derivatives

Table III is constructed like Table II, with each observed *m/e* value being followed, in parentheses, by values representing intensity (relative to the most intense peak in that spectrum), designated column *b*, the mass increase of the fragment-ion when the e i spectrum of a non-isotopically-substituted PAKO derivative is compared to that of a PAKO derivative produced by use of hydroxyl[$^{15}$N]amine, designated column *c*, and two columns, designated *d* and *e*, which refer, respectively, to mass increases observed that result from PAKO derivative production that employs acetic anhydride-$d_6$, and to the two-step, acetic anhydride–acetic anhydride-$d_6$ derivatization procedure described in the ammonia c i m s section, and in the Experimental section As discussed in the ammonia c i m s section, it was concluded that only a single acetyl-$d_3$ group is incorporated into each PAKO derivative, and this incorporation occurs exclusively at the acetoxime position The isotopic-substitution data in Table III indicate that, for most fragment-ions of e i m s , (*a*) the nitrogen atom of the acetoxime group is retained, (*b*) the acetoxyl part of the acetoxime group is lost, (*c*) relatively large percentages of the total *m/e* are present as acetoxyl groups (from the relatively large numbers in column *d* of Table III), and (*d*) loss of acetyl groups occurs both *via* loss of acetic acid molecules and ketene molecules (from the many values in column *d* of Table III that are not multiples of 3)

It is convenient to examine the e i spectrum of the PAKO derivative of 1,3-dihydroxy-2-propanone (see Table III), as this spectrum is very simple, the various, isotopically substituted variants of this compound provide e i spectra having clearly displaced peaks, and the simplicity of the molecule precludes a number of alternatives for fragmentation pathways From the ammonia c i spectrum of the PAKO derivative of 1,3-dihydroxy-2-propanone, we established the molecular weight of this derivative to be 231, and therefore, the *m/e* 129 of the e i spectrum of this derivative corresponds to M — 60 — 42 For this fragment-ion of *m/e* 129, (*a*) the nitrogen atom of the acetoxime group is completely retained, (*b*) one of the (carbon) acetoxyl groups is completely lost, and the other is completely retained, and (*c*) there is no spectral evidence for an intermediate *m/e* value of M — 60, or M — 42 From these data for *m/e* 129, it is inferred that (*a*) the loss of both acetic acid and ketene involves a concerted mechanism (due to the absence of deuterium transfer to the saccharide backbone on loss of ketene), and (*b*) no backbone cleavage occurs, as the hydrogen atoms of the backbone of the original 1,3-dihydroxy-2-propanone molecule are retained The *m/e* 99 of the e i spectrum of the PAKO derivative of 1,3-dihydroxy-2-

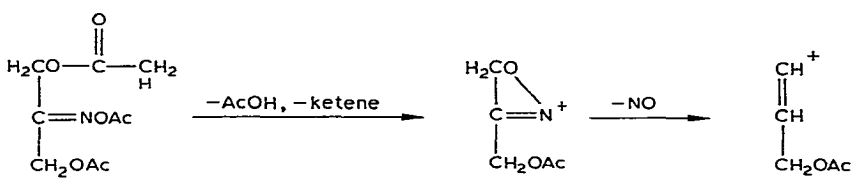


TABLE IV

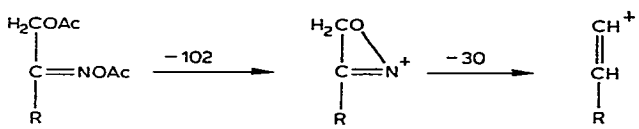
THE ASSIGNMENT OF GENERALIZED STRUCTURES TO ELECTRON-IMPACT, MASS-SPECTROMETRIC FRAGMENT-IONS OF PERACETYLATED KETO-OXIME DERIVATIVES[a]

| *Assignment of fragment-ion* | *Compound* | | | |
|---|---|---|---|---|
| | *1,3-Dihydroxy-2-propanone PAKO (Type 14)* | D-erythro-*2-Pentulose PAKO (Type 16)* | D-*Fructose PAKO (Type 17)* | D-manno-*2-Heptulose PAKO (Type 18)* |
| M − 102 | 129 | 273 | 345 | 417 |
| From C-4–C-5 cleavage | | 200 | 200 | 200 |
| M − 204 | | 171 | 243 | 315 |
| From C-4–C-5 cleavage | | 158 | 158 | 158 |
| M − 204 − 30 | | 141 | 213 | |
| M − 204 − 60 | | 111 | 183 | 255 |
| M − 306 | | | 141 | 213 |

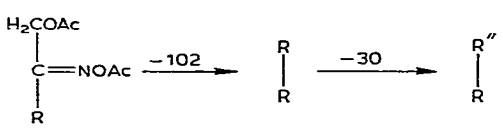
[a]The *m/e* values are taken from Table III

propanone corresponds to *m/e* 129 − 30, and, in terms of isotopic substitution, *m/e* 99 differs from *m/e* 129 in that only the nitrogen atom of the acetoxime group is completely lost It is, therefore, inferred that *m/e* 99 arises from *m/e* 129 by the loss of NO, and that this implies the pathway shown. Only C-1 and C-2 need be involved in such a process, and the foregoing observations therefore provide a general approach to interpreting the e i spectra of the PAKO derivatives

From the e i spectra of the PAKO derivative of 1,3-dihydroxy-2-propanone, and in conjunction with the *m/e* changes for various homologs of PAKO derivatives, shown in Table IV, it may be concluded that a general, fundamental process for the e i fragmentation-pathways of all PAKO derivatives may be described as follows



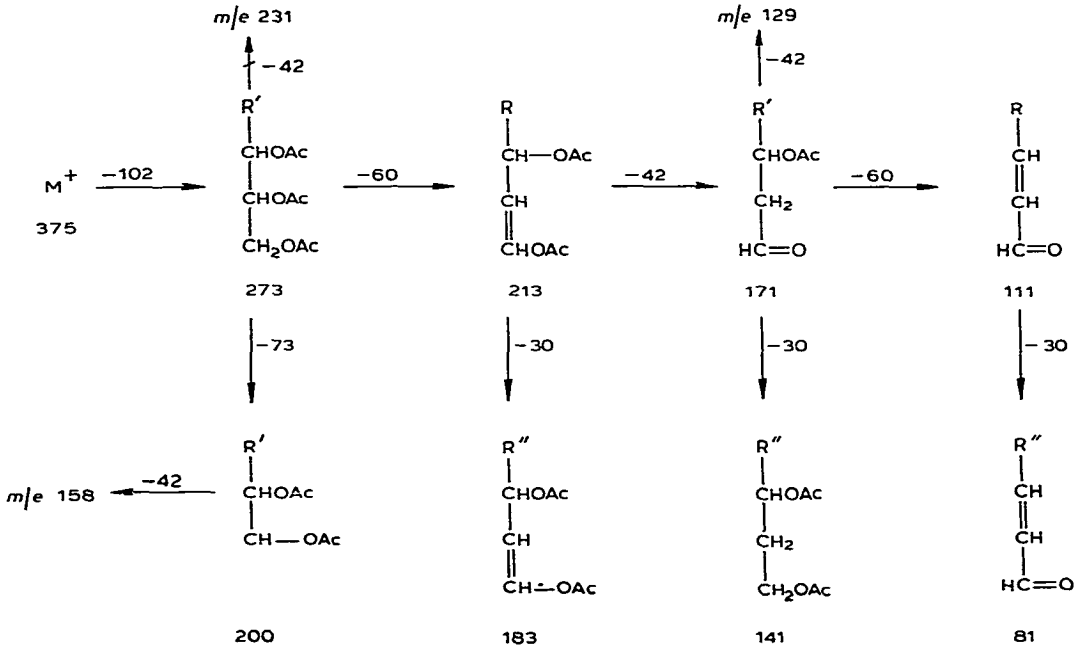

When, for notational simplicity, the $CH_2OCN^+$ group is defined as R′ and the $CHCH^+$ group as R″, this pathway may be written as follows




where R′ contains 56 mass units, and R″, 26 mass units The various homologs of the PAKO derivatives differ by $CH_2OAc$ (mass 72), and the resistance to backbone cleavage of these PAKO derivatives suggests that many of the *m/e* values in compari-

son e i spectra of these homologs will differ by 72 mass units Table IV, which both correlates *m/e* from various derivatives and indicates the structural nature of the fragment-ions, shows important parallels, in increments of 72 mass units, between the e i spectra of homologs of the PAKO derivatives Table IV also shows that fragment-ions of $M^+ - 102n$, the ion resulting from the successive loss of acetic acid and ketene, are very prominent in the e i spectra of the PAKO derivatives Most important of all, the similarities between the *m/e* arising from the derivatives produced by the standard derivatization procedure with hydroxylamine and acetic anhydride indicate that the derivatization product of D-*manno*-2-heptulose is the PAKO derivative

The proposed fragmentation-pathway for the e i m s for type 16 (data taken from the e i spectrum of the PAKO derivative of D-*erythro*-2-pentulose) is shown in

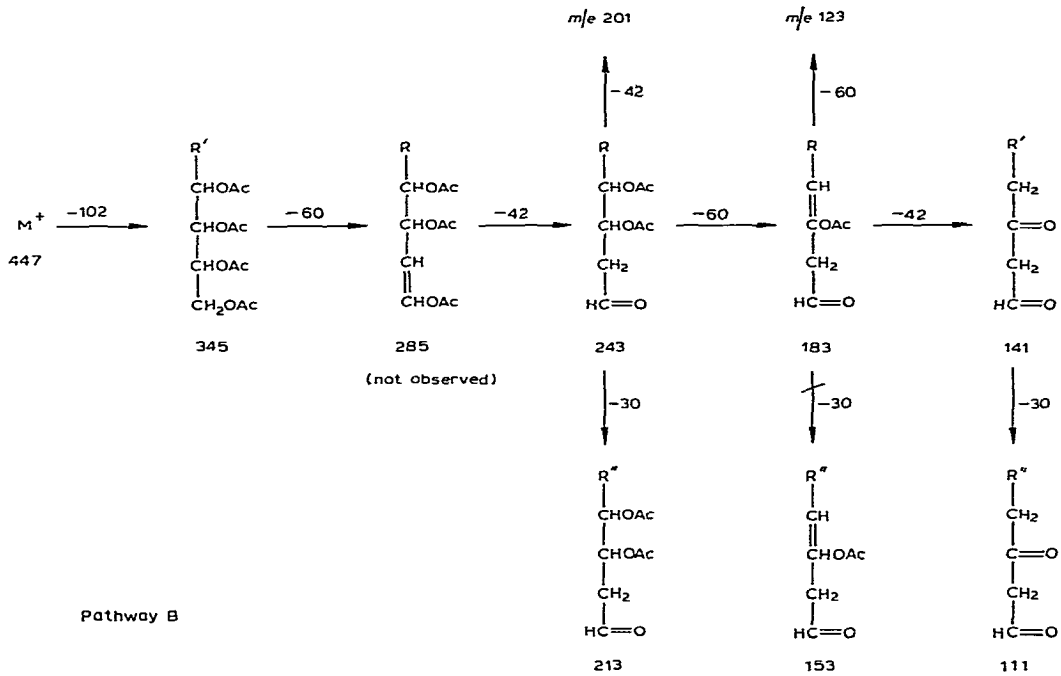



Pathway A

pathway A It should be noted that the structures shown for each *m/e* are *representative* structures, as a number of positional isomers can exist for most of the *m/e* shown Apparently, the loss of ketene occurs from *m/e* 171, to yield *m/e* 129, due to unsaturation in the R′ group A similar situation, between *m/e* 273 and *m/e* 231, apparently does not proceed in such a way, as isotopic substitution indicates that *m/e* 231 retains the complete acetoxime group Both *m/e* 158 and *m/e* 200 are rather unusual *m/e* values, (*a*) as, although these fragment-ions are of even *m/e*, they contain nitrogen, and (*b*) because they persist in the e i spectra of all of the PAKO derivatives of non-methylated saccharides that have been studied On the basis of isotopic-substitution data, it is concluded that these *m/e* values arise from the *m/e* fragment-ion (or frag-

ment-ions of corresponding structure of C-1–C-4 of the derivatives of the other ketose homologs), which then yields the *m/e* 200 by backbone cleavage at C-4–C-5 Apparently, *m/e* 200 then loses ketene (42 mass units) by a mechanism involving unsaturation in the R′ group, to yield *m/e* 158 Isotopic-substitution data also suggested that *m/e* 154 may come from backbone cleavage, as a similar *m/e* was observed from the non-nitrile end (C-series) in the e i. spectra of the PAAN derivatives[36] Unless specifically noted, all structures indicated for these fragmentation pathways are in agreement with all isotopic-substitution data currently available

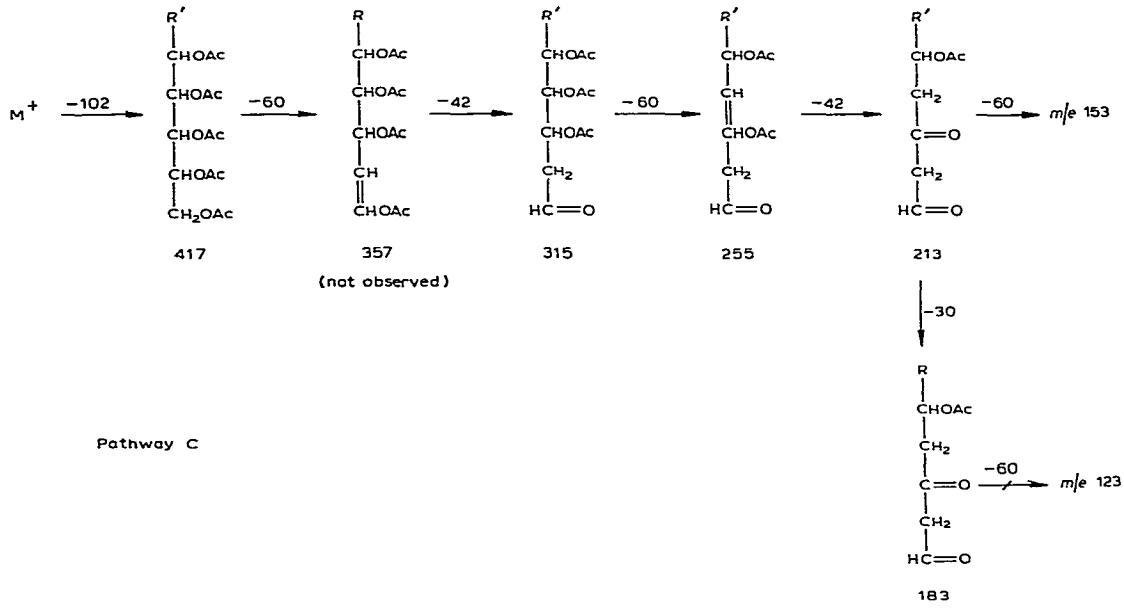
The proposed fragmentation-pathway for the e i m s. for type 17 (data taken from the e i spectrum of the PAKO derivative of D-fructose) is shown in pathway B




This pathway accounts for most of the more-intense *m/e* of the e i spectrum The expected *m/e* 285 was not observed, however, the large size of the fragment ion may be responsible for this instability, and, as already noted, *m/e* of even changes of 102 mass units are favored The analog of the *m/e* 99 of the e i spectrum of the PAKO derivative of 1,3-dihydroxy-2-propanone is not observed in the corresponding spectrum of the D-fructose derivative [expected *m/e* of (99 + 144) = *m/e* 243], nor are the corresponding *m/e* fragments observed in the e i. spectra of the PAKO derivatives of D-*erythro*-2-pentulose or D-*manno*-2-heptulose We are not certain why these expected *m/e* are not present The fragment ions *m/e* 103 and *m/e* 115 correspond to small *m/e* attributed to the C-series (products from the non-nitrile end of the PAAN derivatives after backbone cleavage), and probably arise from similar, backbone

cleavage of the PAKO derivative. However, although *m/e* 153 could arise from *m/e* 183, by loss of NO in a way similar to that observed for *m/e* 141 and *m/e* 243, isotopic substitution fails to confirm such an identity for this fragment-ion

The proposed fragmentation-pathway for the e i m s for type 18 (taken from the data for the PAKO derivative of D-*manno*-2-heptulose) is shown in pathway C.

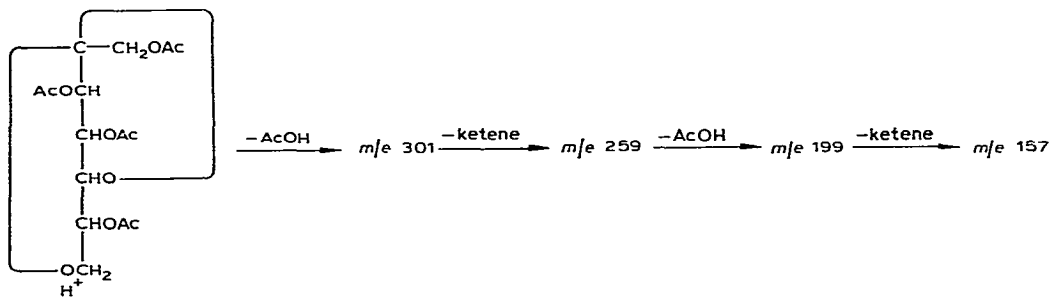



Most of the large *m/e* contain nitrogen, and are accounted for in this pathway Some of the expected *m/e* (*e.g* , *m/e* 255 and *m/e* 357) are not observed, and, as with the corresponding spectrum of the PAKO derivative of D-fructose, these larger *m/e* are assumed not to be observed due to (*a*) the instability of fragment-ions of large *m/e*, and (*b*) the preference for mass loss in terms of 102 mass units at a time However, the PAKO derivative of D-*manno*-2-heptulose is sufficiently large that some backbone cleavage now contributes fragment-ions to the e i spectra, for example, the *m/e* 103, 115, 139, 145, and 187 observed in this spectrum contain no nitrogen, and are typical e i fragment-ions that arise from the C-series (non-nitrile end) of the PAAN derivatives. The *m/e* 158 and *m/e* 200, previously described in the fragmentation pathway of the PAKO derivatives of D-*erythro*-2-pentulose as arising from the oxime end of C-4–C-5 cleavage, are also observed in the e i spectrum of the PAKO derivative of D-*manno*-2-heptulose The *m/e* 153 fragment-ion also contains nitrogen, and probably comes from *m/e* 213 by loss of acetic acid In terms of mass, it is conceivable that *m/e* 123 could arise from *m/e* 153 by loss of NO, or from *m/e* 183, by loss of acetic acid, however, such an origin and structure for *m/e* 123 is not supported by isotopic-substitution data. Very important for identification of this compound, the fragment-ions *m/e* 213, 315, and 417 correspond to the M — 102*n* series (when $n = 1, 2$, and 3),

thus confirming that this g l c peak is that of the PAKO derivative of D-*manno*-2-heptulose

These e i spectra also indicate that, for the e i m s of the (as-yet-unstudied) 2-tetrulose PAKO derivative (designated type 15), the prominent *m/e* will be *m/e* 69 and 111, which will not contain the nitrogen atom, and *m/e* 99, 141, 159, and 201, which will contain the nitrogen atom

The e i spectrum of peracetylated sedoheptulosan is shown in Table III, and




Generalized pathway D

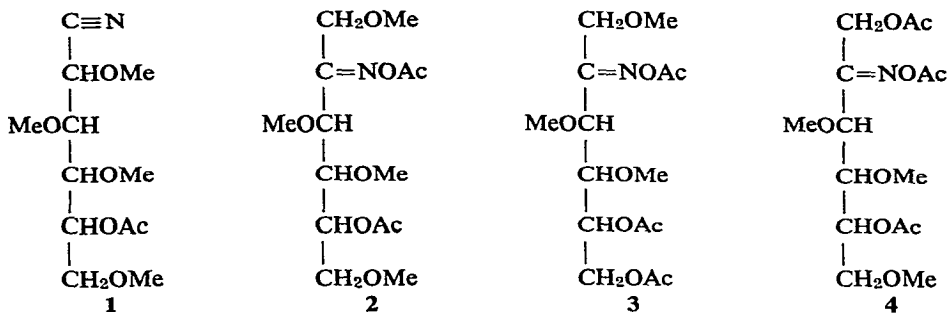
a generalized, e i fragmentation-pathway in pathway D This e i spectrum is similar to the corresponding mass spectrum of peracetylated L-idosan by having (*a*) the ions of larger *m/e* characterized by the successive loss of acetic acid and ketene, and (*b*) ions of small *m/e*, for which structure assignments are difficult, presumably resulting from complex fragmentation-processes that can occur because of the cyclic nature of these compounds However, the fragmentation pathway of the ions of the e i spectrum of this product, an example of compounds designated type 19, clearly serves to distinguish these derivatives from the e i spectra of the PAKO and PAAN derivatives

In general, the PAAN, the PAKO, and the peracetylated derivatives of saccharides can be readily identified and differentiated by the use of g l c in conjunction with e i m s and ammonia c i m s In contrast to the e i spectra of the PAAN derivatives, those of the PAKO derivatives provide much less information with regard to the possible positions of substituents, should such groups be incorporated into the parent saccharide However, the ease, speed, and precision of identifying saccharides allowed by the PAKO-derivative procedure makes this a very convenient, analytical approach, either for mixtures containing only ketoses, or for mixtures of aldoses and ketoses

*Peracetylated keto-oxime derivative of* O-*methylated saccharides*

In previous discussions of PAAN derivatives, some fundamental differences have been observed between the mass-spectral characteristics of the *O*-methylated[44,45 50,60] and the non-*O*-methylated derivatives[37] We shall now briefly indicate the general, chromatographic and mass-spectral properties of the PAKO derivatives of highly *O*-methylated saccharides in terms of comparing the PAAN

derivative (**1**) of 2,3,4,6-tetra-*O*-methyl-D-glucose to the PAKO derivatives (**2–4**) of 1,3,4,6-tetra-*O*-methyl-D-fructose, 1,3,4-tri-*O*-methyl-D-fructose, and 3,4,6-tri-*O*-methyl-D-fructose The production of **1–4** is quite simple, for example, an equimolar mixture (in terms of the starting saccharide groups) of **1** and **2** can be produced by the successive permethylation of sucrose, hydrolysis, and derivatization Similarly, **3** and **4** can, respectively, be obtained by the permethylation of levan and inulin, followed by hydrolysis, and derivatization




These permethylations and hydrolyses were performed as previously described for dextrans[45 48 50], except for the permethylation of sucrose, where a single water–chloroform extraction (with the product in the chloroform phase) replaced the dialysis step Compound **1** is a good mass-spectral reference-compound, as ~12 isotopically substituted variants of this compound (or of the analog of D-mannose) have been examined by e i m s [44 50 60]. We have not, at present, identified any differences in the mass spectra of these PAAN derivatives of *O*-methylated saccharides that can be attributed to differences in the stereochemistry of the compounds A comparison of C-1 and C-2 of **1** and **2** indicates that permethylated D-glucopyranose and D-fructofuranose yield distinctly different products by the PAAN–PAKO derivatization procedure For convenience, in the following discussion, reference to the tetramethyl ethers of D-glucose or D-fructose will specifically refer, respectively, to **1** and **2** The simple, unqualified reference to PAAN or PAKO derivatives will continue to refer to the derivatives of saccharides *not* containing *O*-methyl groups It may be noted that, for **1**, **2**, and **4**, C-3–C-6 are identical A comparison of the r r t in g l c of the PAAN derivatives of the variously *O*-methylated derivatives of D-mannose and D-glucose indicated that (*a*) the magnitudes of the retention times are principally dependent on the degree of *O*-methylation of the saccharides, and (*b*) these r r t are inversely proportional to the degree of *O*-methylation of a given saccharide

*G l c of O-methylated, peracetylated, keto-oxime derivatives* — The r r t for **1**, **2**, **3**, and **4** are listed in Table I Comparison of the two tetra-*O*-methyl derivatives, **1** and **2**, indicated that, for starting saccharides of equal molecular weight and degree of methylation (*e g*, tetra-*O*-methyl-D-glucose and tetra-*O*-methyl-D-fructose), the PAKO derivatives will have r r t larger than those of the PAAN derivatives Comparison of **3** and **4**, the tri-*O*-methyl PAKO derivatives, with **2**, the tetra-*O*-methyl

derivative, indicated that the r r t of the PAKO derivatives have the same inverse relationship to the degree of *O*-methylation that was observed for the PAAN derivatives Double peaks, presumably representing the *syn*- and *anti*-oxime orientations, were observed in the chromatograms of **2** and **3** (see Table I for the relative peak-areas from the hydrogen-flame detector) Although the OV-17 column fails to give the outstanding resolution that was obtained with the PAAN derivatives on 1,4-butanediol succinate columns, both the PAAN and PAKO derivatives can be chromatographed on this OV-17 column, and the chromatographic peaks emerging are quite sharp and symmetrical When sucrose is subjected to (*a*) permethylation, (*b*) hydrolysis, (*c*) PAAN–PAKO derivatization, and (*d*) g l c separation with condition 3 (hydrogen-flame detector), the resulting chromatogram displays sharp, well resolved peaks having distinctive ratios of peak heights of **1** **2**(peak 1) **2**(peak 2) = 1 00 0 73 0 51 Owing to the narrowness of these g l c peaks, it is, for mass-spectral analysis, more convenient to employ slower temperature–rate programs (either 3°/min or 5°/min) The hydrogen-flame-detector responses for these PAKO derivatives are similar to those of the PAAN derivatives Condition 3 allows base-line resolution between the two g l c peaks of **2**, however, the resolution between the two g l c peaks of **3** is similar to that of the PAAN derivatives of D-*erythro*-2-pentulose (see Fig 3) When employed in conjunction with m s , the identification of PAKO derivatives (either separately, or together with PAAN derivatives) by chromatography on OV-17 columns can, therefore, be effectively achieved

*Electron-impact, mass spectrometry of* O-*methylated keto-oxime derivatives* — The e i spectra of **1**, **2**, **3**, and **4** are summarized in Table V In general, for highly *O*-methylated saccharides, the e i spectra of the PAKO derivatives are not so informative as those of the PAAN derivatives, apparently for the following reasons (*a*) In contrast to the PAKO derivatives of non-*O*-methylated saccharides, and similarly to the PAAN derivatives, the major fragment-ions of the e i spectra of the PAKO derivatives of highly *O*-methylated saccharides result from initial, backbone cleavage (*b*) The acetoxime group, like the nitrile group, contributes instability to fragment-ions, and, therefore, there is little contribution to the mass spectrum from the acetoxime end of the molecule (*c*) Little cleavage of the backbone occurs between the carbon atom attached to the acetoxime group and the adjacent carbon atoms (an effect similar to that observed for the carbon atom adjacent to the nitrile group of the PAAN derivatives) (*d*) As the acetoxime group is in an interior position of the backbone chain, a PAKO derivative will have fewer favored cleavage-positions than will a corresponding PAAN derivative For example, a comparison of **1** and **2** indicates that **1** will yield fragment-ions resulting from intact-backbone cleavages between C-2 and C-3, whereas **2** will only yield fragment-ions resulting from initial cleavage between C-3 and C-4 with the concomitant loss of fragment-ions arising from the "upper" (nitrile or oxime) ends of these molecules Many of these PAKO derivatives of *O*-methylated saccharides will, therefore, give similar e i spectra For example, **2** and **4** will have favored cleavage-positions between C-3 and C-4 (or between higher-numbered carbon atoms) and, therefore, the principal contribution

TABLE V

ELECTRON-IMPACT, MASS-SPECTROMETRIC FRAGMENT-IONS OF PERACETYLATED ALDONONITRILE AND KETO-OXIME DERIVATIVES OF *O*-METHYLATED SACCHARIDES

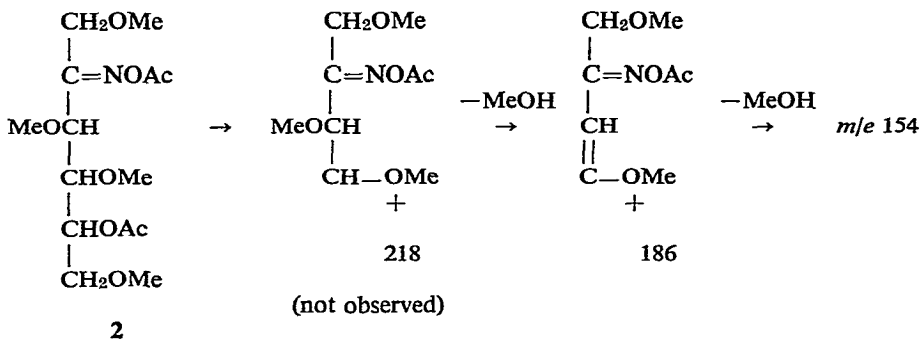
| *Parent saccharide* | | | |
|---|---|---|---|
| D-*Glucose* | D-*Fructose* | D-*Fructose* | D-*Fructose* |
| *Positions of* O-*methylation* | | | |
| *2,3,4,6* | *1,3,4,6* | *1,3,4* | *3,4,6* |
| *Compound designation* | | | |
| **1** | **2** | **3** | **4** |
| 205 (5[a], 0[b], 3[c], 9[d], B[e]) | 186 (33[a], 1[b], 6[c], –[e f]) | 233 (1[a], J[e]) | 161 (39[a], 0[b], A[e]) |
| 186 (1, 1, 1, –, –) | 161 (26, 0, 6, A) | 189 (12, K) | 145 (11, 0, B) |
| 161 (67, 0, 3 6, A) | 159 (9, 0, 3, –) | 173 (5, J) | 129 (90, 0, A) |
| 158 (6, 1, 0, 4, L) | 154 (42, 1, 3, –) | 159 (3, G) | 119 (3, 0, A) |
| 145 (33, 0, 0 9, B) | 129 (78, 0, 3, A) | 151 (10, –) | 117 (23, 0, –) |
| 129 (100, 0, 3, 3, A) | 112 (73, 1, 3, –) | 129 (95, K) | 102 (32, 0, –) |
| 126 (5, 1, 0, 6, L) | 101 (91, 0, 6, A) | 117 (6, –) | 101 (45, 0, A) |
| 119 (5, 0, 0, 6, A) | 99 (22, 0, 3, –) | 113 (5, J) | 87 (29, 0, A) |
| 117 (7, 0, 3, 3, –) | 96 (25, 1, 3, L) | 99 (28, J) | 71 (38, 0, A) |
| 114 (6, 1, 0, –, –) | 87 (27, 0, 3, A) | 87 (58, K) | 45 (38, 0, D) |
| 113 (10, 0, 0, 6, B) | 74 (16, 0, 0, –) | 71 (18, –) | 43 (100, 0, –) |
| 101 (38, 0, 0, 6, A) | 71 (14 0, 2, A) | 45 (21, D) | |
| 96 (6, 1, 0, 3, L) | 45 (38, 0, 3, A) | 43 (100, –) | |
| 88 (35, 0, 0, 6, C) | 43 (100, 0, 0, –) | | |
| 87 (35, 0, 1, 3, A) | | | |
| 71 (13, 0, 0, 3, A) | | | |
| 45 (83, 0, 0, 3, D) | | | |
| 43 (71, 0, 3, 0, –) | | | |

[a]Intensity (percent) of the mass fragment relative to the most-intense mass-fragment [b]Hydroxyl[$^{15}$N]-amine was employed in the derivatization procedure [c]Acetic anhydride-$d_6$ was employed in the derivatization procedure [d]Methyl-$d_3$ iodide was employed in the permethylation procedure [e]These letters correspond to the fragment-ion series that were identified and defined in ref 44 [f]The symbol "–" indicates either an unobserved *m/e* or an unassigned fragment ion

to the e i spectra of these compounds will come from the C-3–C-6 portion of the molecules (which are identical for **2** and **4**, as well as for **1**)

The foregoing discussion explains why the c i spectra of the PAKO derivatives (**2**, **3**, and **4** in Table V) are similar, and relatively simple, compared to the e i spectra of PAAN derivatives [*e g*, the e i spectrum of the PAKO derivative (**2**) of tetra-*O*-methyl-D-fructose compared to that of the corresponding PAAN derivative (**1**) of tetra-*O*-methyl-D-glucose] The fragment-ions arising from **2**, **3**, and **4** can be readily described in terms of the fragment-ion series, denoted A through S, which describes the fragmentation pathways of the PAAN derivatives of *O*-methylated saccharides[44] Such similarity of fragment-ions, and of nomenclature, is the result of the essential identity of C-3–C-6 of the PAAN or PAKO derivatives of D-mannose,

D-glucose, and D-fructose, and also of the predominant contribution of fragment-ions from the non-acetoxime (or non-nitrile) ends of the molecules Therefore, the separation and identification of a mixture of *O*-methylated PAAN and PAKO derivatives by g l c–e i m s is possible, although ambiguities can result For the e i spectrum of **2**, prominent fragment-ions at *m/e* 154 and 186 were observed, and isotopic substitution indicated that these fragment ions of even *m/e* arise from the acetoxime end of the molecule, as indicated by the accompanying fragmentation-pathway, the production of the *m/e* 154 ion could result from loss of the methoxyl group from C-1 or C-4 of the *m/e* 186 ion No corresponding fragment-ions of large, and even, *m/e* were observed in the e i spectra of **3** and **4**. In addition, no trace of fragment-ions attributable to the fragmentation-pathways dominating the e i spectra of the PAKO derivatives of non-*O*-methylated saccharides, which retain the backbone chain, have been observed in these e i. spectra, apparently, the introduction of *O*-methyl groups into the saccharide promotes e i backbone-cleavage, and completely suppresses the alternative fragmentation-pathways




*Ammonia chemical-ionization mass spectrometry of* O-*methylated PAKO derivatives* — The major ions resulting from the ammonia c i m s of **1** and **2**, and the identity of these ions as established by selective isotopic substitution, are shown in Table VI Table VI indicates that ammonia c i m s can readily distinguish between the PAAN and PAKO derivatives of similar, *O*-methylated starting-saccharides (*e g*, the tetramethyl ethers of D-glucose and D-fructose) Such data provide an effective complement to the e i m s data previously described, and eliminate most of the possible ambiguities that could be encountered in the g l c–m s separation and identification of a mixture of variously *O*-methylated PAAN and PAKO derivatives However, care must be taken with **2**, as the ammonia c i spectrum of this compound (and its various isotopic variants) is quite similar to those of PAAN derivatives of the tri-*O*-methylaldohexoses Such an ambiguity arises because the ammonia c i. spectrum of **2** fails to yield the $[M + 1]^+$ capture ion, in accord with the general observations that the relative intensity of the capture ion is lessened for (*a*) PAKO derivatives in general (relative to the ammonia c i m s of PAAN derivatives), and (*b*) saccharides of high degree of *O*-methylation In addition, the characteristic

TABLE VI

AMMONIA CHEMICAL-IONIZATION, MASS-SPECTROMETRIC IONS OF PERACETYLATED ALDONONITRILE AND KETO-OXIME DERIVATIVES OF TETRA-*O*-METHYLATED SACCHARIDES

| *Assignment of ion* | *Parent sugar* D-*Glucose* | D-*Fructose* |
|---|---|---|
| | *Positions of* O-*methylation* | |
| | *2,3,4,6* | *1,3,4,6* |
| | *Compound designation* | |
| | **1** | **2** |
| M + 18 | 293 (3[a], 1[b], 3[c], 12[d]) | |
| M + 1 | 276 (40, 1, 3, 12) | |
| M + 1 − 32 | 244 (35, 1, 3, 9) | 304 (22[a], 1[b], 6[c], 9[d]) |
| M + 1 − 32 − 32 | | 272 (38, 1, 6, 6) |
| M + 1 − 60 | 216 (100, 1, 0, 12) | |
| M + 1 − 60 − 32 | 184 (17, 1, 0, 9) | 244 (100, 1, 0, 9) |
| M + 1 − 60 − 32 − 32 | 152 (6, 1, 0, 6) | 212 (44, 1, 0, 6) |
| | | 186 (8, 1, –, 6) |
| | 161 (3, 0, –, 6)[e] | 161 (3, 0, 0, 6) |
| | 129 (10, 0, 3, 3) | 129 (10, 0, 0, 3) |
| | 101 (5, 0, 0, 6) | 101 (9, 0, 0, 6) |

[a]Intensity (percent) of the mass fragment relative to the most-intense mass-fragment [b]Hydroxyl[$^{15}$N]-amine was employed in the derivatization procedure [c]Acetic anhydride-$d_6$ was employed in the derivatization procedure [d]Methyl-$d_3$ iodide was employed in the permethylation procedure [e]The *m/e* values in the lower part of the Table indicate fragment ions previously identified in the e i mass spectra

$[M - 57 - 60n]^+$ ion-series, prominent in the ammonia c i spectra of the PAKO derivatives described in the preceding section, is not observed in the ammonia c i spectrum of **2** However, the e i spectrum of **2**, the tetramethyl ether, represents an extreme case for the c i m s of the PAKO derivative of D-fructose For less-highly *O*-methylated derivatives of D-fructose, both the capture-ion and the $[M - 57 - 60n]^+$ series of ions are observed, *e g*, the tri-*O*-methyl derivatives yield ammonia c i spectra with $[M + 1]^+$ at ~10% intensity (relative to the most-intense *m/e*, and with $[M - 57]^+$ at ~50% intensity In contrast to e i m s, similar fragmentation-pathways are observed in the ammonia c i spectra of PAKO derivatives of both *O*-methylated and non-*O*-methylated derivatives

Therefore, the application of the PAAN–PAKO derivatization procedure, in conjunction with e i m s, ammonia c i m s, and an appropriate selection of isotopic variations during derivatization, provides an effective technique for the methylation–fragmentation analysis of oligosaccharides or polysaccharides that contain ketoses, either as all of the residues and groups present, or combined with aldose residues and groups. Such applications yield specific information as to the nature of the branch-

point residues, and the frequency of such branching This specific, accurate information concerning polysaccharide branching is useful for correlation with structural data from other sources that often indicate differences between the structures of various polysaccharides, but that do not specifically identify the nature of these differences For example, such complementary data for levans are available from $^{13}$C-n m r techniques (see refs 61–64, and references cited therein) and by Fourier-transform, difference infrared-spectrometry[65]

*Gas–liquid chromatography of products of oligosaccharides after subjection to PAAN–PAKO derivatization conditions*

Several oligosaccharides have been studied by g l c on OV-17 columns after using the PAAN–PAKO derivatization procedure The r r t of four representative oligosaccharides are listed at the end of Table I These products are presumed to be the PAAN derivatives (or the peracetylated product, when no reducing group is present), although, owing to upper-temperature limitations on the membrane separator of the g l c –m s system, we have not examined the mass spectra of these g l c peaks Some oligosaccharides (*e g* cellobiose) dissolve slowly in the first step (oxime formation) of the derivatization procedure, and so Teflon-coated, screw-cap vials containing the reactants were heated for ~30 min at ~100° until a clear solution resulted, the normal derivatization procedure then being followed in the second step A typical chromatogram of the PAAN–PAKO derivatives of monosaccharides (D-erythrose and D-glucose), disaccharides (maltose and cellobiose), and trisaccharides (melezitose and raffinose) is shown in Fig 4 The g l c conditions for this chromatogram, which allow observation of the trisaccharides, differ from the normal condition

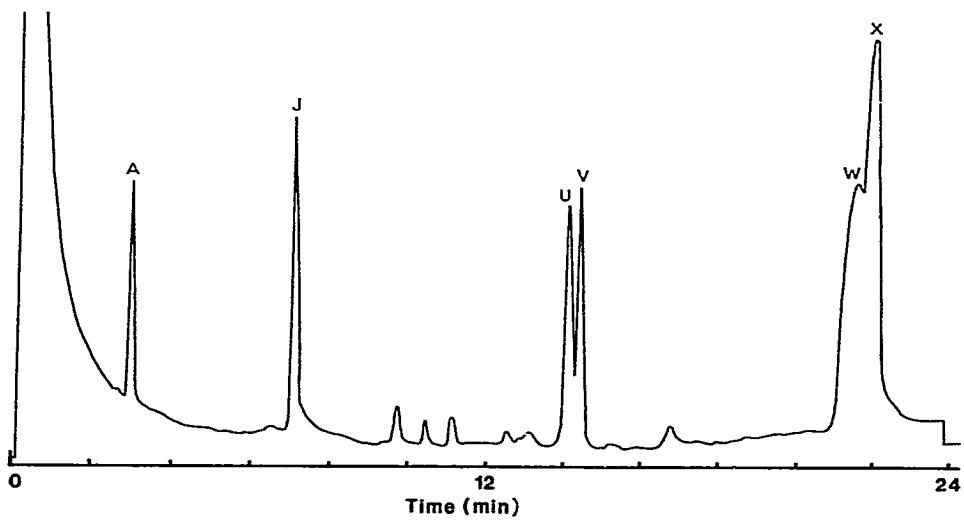



Fig 4 Gas–liquid chromatogram (hydrogen-flame detector) from a column of OV-17 on Chromosorb W (condition 3) (The PAAN derivatives of A, D-erythrose, J, D-glucose, U, maltose, and V, cellobiose In addition, the peracetylated derivatives of W, melezitose, and X, raffinose)

3, in that the injector and detector were at 390°, and the upper oven-temperature, program-limit was 370° The g l c properties of these compounds are similar to those previously observed for most of the various derivatives of oligosaccharides[3], *e g*, (*a*) the r r t of oligosaccharides are dependent on, and proportional to, the d p, and (*b*) for sets of saccharides having the same d p, the sets having the larger d p will be the most difficult to resolve

However, a variety of observations, with regard to the OV-17 column conditions, may be summarized as follows (*a*) These derivatives of oligosaccharides yield sharp, symmetrical peaks through d p = 3, and a g l c survey employing these derivatives may be performed in <25 min (*b*) Such a survey for oligosaccharides is compatible, and can be performed simultaneously, with the PAAN–PAKO derivatization survey for monosaccharides (*c*) The r r t of the various saccharides are well separated according to d p, such separations provide assurance that g l c peaks in the r r t range of 1–4 are monosaccharides (for analyses when permethylation is not employed), and the presence, or absence, of such peaks that represent oligosaccharides provides a simple and quick check for the completeness of hydrolysis for samples arising from hydrolyzates Although raffinose and melizitose respectively contain a D-fructofuranosyl group and residue, the r r t of these compounds is essentially the same as that of the trisaccharides composed exclusively of glycopyranosyl residues, or of the PAAN derivatives of reducing trisaccharides

The PAAN–PAKO derivatization procedure and chromatographic technique described herein therefore provides a rapid, accurate, discriminatory method for the identification and quantification of aldoses, ketoses, and small oligosaccharides This derivatization procedure is also compatible with the chromatographic separation, identification, and quantitation of products arising from methylation–fragmentation structural analyses

## EXPERIMENTAL

*Materials* — The origin of the aldoses employed for the reference PAAN derivatives has been described in ref 37 Other saccharides were used (after being dried *in vacuo* over $P_2O_5$) as obtained from Aldrich Chem Co, Milwaukee, WI (cellobiose, maltose, melezitose, and raffinose), Nutritional Biochemical Inc, Cleveland, OH (D-fructose), P-L Biochemicals Inc, Milwaukee, WI (D-*erythro*-2-pentulose and sedoheptulosan), and Sigma Chemical Co, St Louis, MO (1,3-dihydroxy-2-propanone, D-*manno*-2-heptulose, L-sorbose, and D-tagatose) Reagents were used as obtained from Fisher Scientific Co (hydroxylamine hydrochloride, pyridine, and acetic anhydride, all A C S grade) The pyridine was stored over potassium hydroxide For isotopic substitution, acetic anhydride-$d_6$ and hydroxyl[$^{15}$N]amine hydrochloride (Merck, Sharp and Dohme, Canada, Ltd, Montreal) were used OV-17 on Chromosorb W HP (80–100 mesh) was obtained from Supelco Inc, Bellefonte, PA Ammonia (Linde, ultra-high purity) was used as the chemical-ionization reagent-gas

*Equipment* — A Buchi rotary evaporator (Model R/A) and a Vortex Jr Mixer were respectively employed for the evaporations and vibromixing

The g l c surveys were performed in a Barber–Coleman Series 5000 dual-channel g l c instrument equipped with hydrogen-flame detectors and on-column injection (injector and detector temperatures, 330°), with nitrogen as the carrier gas

The mass-spectral analyses were performed with a Hewlett–Packard gas–liquid chromatograph–mass spectrometer system (Model 5980A) interfaced to a Hewlett–Packard data system (Model 5933A) Helium was employed as the carrier gas, with a membrane separator between the chromatograph and the mass spectrometer Ammonia, at ~1 torr, was injected into the mass spectrometer through a second port The maximum column-temperature for the gas–liquid chromatograph of the mass spectrometer was 230°, and the transfer lines were held at 250° The ion-source temperature of the mass spectrometer was 150°, the electron energy was 70 eV, and the filament was at 0 50 mA The mass spectrometer was tuned with perfluorotributylamine, and, for chemical-ionization experiments, the tuning was verified by examining the mass spectrum of the g l c peak of the D-glucose PAAN derivative, the concentration of ammonia in the ionization chamber being so adjusted that the *m/e* 328 and 405 peaks were of equal intensity and essentially no other *m/e* peaks observed For general conditions, the upper *m/e* value was set at 600, and the spectra were scanned at 5-s intervals, the data being stored in the computer-disc memory The total *m/e* value *vs* time was displayed in a manner analogous to that for the hydrogen-flame-detector chromatogram

The preparation of the columns and the conditions for g l c have been described for the PAAN derivatization procedures[36] In general, glass columns (1 23 m × 2 mm i d ) packed with 2% of OV-17 on Chromosorb W HP (80–100 mesh), a carrier-gas flow-rate of 22 mL/min, and on-column injection were employed Oven temperatures were programmed at 20°/min between 130 and 300° (condition 3 in Table I of ref 37), or at 5°/min for better discrimination of the g l c peaks when the mass spectrometer was employed as the detector

*Procedures* — Although most of the derivatives described herein were actually produced from pure, crystalline, commercial saccharides, the PAAN–PAKO derivatization procedure is compatible with most methods of hydrolysis (although the excess of sulfate must be removed[36]) For the final concentration of the hydrolyzate, it is convenient to employ thick-walled, cylindrical tubes (~12 × ~2 5 cm i d ) directly attached by a standard-taper fitting to the duct (inclined at ~45° from the vertical) of a rotary evaporator Vacuum from a water aspirator, and a bath temperature of 70°, adequately remove water and low-boiling solvents, without selectively altering the saccharide content of a hydrolyzate solution, even for hydrolyzates of permethylated polymers The addition of a few mL of absolute ethanol to wash the derivatives from the inside walls of the rotary flask, and evaporation of solvent followed by air drying for ~1 h, prepares the sample for PAAN–PAKO derivatization

The PAAN–PAKO derivatization procedure is readily performed on saccharide samples in the 5–10-mg range, and may conveniently be performed in parallel on

6–8 samples The derivatization can be performed in either (*a*) the thick-walled evaporation-tube, equipped with a Teflon-coated stirring-bar and a glass, standard-taper stopper, or (*b*) a hydrolysis vial (10 cm × 14 mm o d ) equipped with a similar stirring-bar and a Teflon-lined screw-cap Hydroxylamine hydrochloride (a weight either equal to, or in excess of, that of the saccharide) and pyridine (0 2 mL) are added to the vial, which is then immersed a few cm into an oil bath at 70°, and the contents magnetically stirred for 20 min In the case of the large, rotary-evaporator tubes, the reaction flasks are removed from the bath after 5 min, rotated so that the derivatization solution bathes the surface of the lower wall of the tube, and the heating and stirring are continued The vials are briefly cooled, acetic anhydride (0 1 mL) is added, and the heating and stirring are continued for a second 20-min period The resulting solution may then be directly injected for g l c analysis

However, the resulting PAAN–PAKO derivatives are most readily handled by an extraction and washing process, which can be conveniently performed in hydrolysis tubes (10 cm × 14 mm i d ), using bulb-equipped, Pasteur pipets for solvent transfers The reaction mixture is transferred to a vial containing water (2 mL) and chloroform (1 mL), and vibromixed for 10 s The chloroform extract is then transferred to a second vial containing water (2 mL) and again vibromixed for 10 s The final chloroform extract is dried over a few pellets of 4 A molecular sieve, and transferred to a small vial having a Teflon-lined screw-cap The derivatives are stable for months, most of the degradation of these products during prolonged periods of storage results from partial hydrolysis of the *O*-acetyl groups, and this may readily be remedied by evaporation of the solution and re-derivatization of the sample (without the hydroxylamine step) A 1-$\mu$mol aliquot of the chloroform extract is an adequate and convenient sample-size for chromatography, either when employing the hydrogen-flame detector, or a well tuned, mass spectrometer The chloroform extraction of the PAAN–PAKO derivatives is convenient because (*a*) the liquid phase of the g l c columns is protected from the derivatization reagents, (*b*) the PAAN–PAKO derivatives are stable for months, instead of a few days in the derivatization solution, and (*c*) the "tailing" of the chromatographic injection-front is greatly lessened The isotopically substituted derivatives were produced either by (*a*) employing methyl-$d_3$ iodide in permethylations preceding hydrolysis of the polymer, or (*b*) substituting either $[^{15}N]NH_2OH$ HCl for $NH_2OH$ HCl, or acetic anhydride-$d_6$ for acetic anhydride in the derivatization procedure

We are not aware of any parameters of this PAAN–PAKO derivatization procedure for which precise control is needed Neither the reaction temperature nor the reaction time is critical However, reaction temperatures below 60° may result in incomplete derivatization, and reaction temperatures appreciably above 80° may result in the selective loss of some of the more volatile, saccharide derivatives However, we have consistently performed, at 70° (bath temp ), derivatizations of relatively volatile (*e g* , *O*-methylated) saccharides by employing loosely stoppered vials, and have not observed any selective loss of these saccharides

The PAAN–PAKO derivatives are quite compatible with the chloroform solvent,

as they are degraded slowly, and the solvent can be evaporated without selective loss of the derivative We find it convenient to concentrate the solutions by positioning, directly over the surface of the solution, the tip of a Pasteur pipet attached by a vacuum line to a water aspirator By final concentration of the solution, it is possible to derivatize small (~10 μg) samples of saccharides An arbitrarily small weight (~1 mg) of hydroxylamine hydrochloride is employed, and the derivatization proceeds as for larger amounts (see ref 37 for a discussion of the effects of an excess of hydroxylamine hydrochloride on the PAAN derivatization of saccharides containing a deoxy group)

D-Erythrose is a convenient reference for the r r t values, because of the short retention time of its PAAN derivative However, as the sugar is normally available as a syrup, it is not convenient for use as an internal standard for quantitation We therefore prefer to perform a preliminary derivatization, without an internal standard, to identify the general "profile" of the chromatogram, and to establish the probable absence of the PAAN derivative of a sugar that can be added in pure, crystalline form (*e g*, D-arabinose or D-glucose) A second derivatization is then performed, with the addition of a measured weight of the reference sugar before the addition of hydroxylamine hydrochloride, a drop of chloroform containing the PAAN derivative of D-erythrose (from a stock reference-solution) being added to the final chloroform extract

The two-step acetylation is performed like the standard PAAN–PAKO derivatization, except for the following modification At the end of the 20-min heating-period after the addition of the pyridine, a relatively small volume of acetic anhydride (~20% mol excess) is added, and heating and stirring are continued for 1 h at 50° A larger amount of acetic anhydride-$d_6$ (at least 10 times that of the acetic anhydride) is then added, followed by heating and stirring for 20 min at 70°, and extraction

The OV-17 column does not require protection nor do the PAAN–PAKO derivatives require extensive drying before the injection Preliminary chromatography, and quantitation of the g l c peak areas, are performed with a hydrogen-flame detector Good chromatograms, employing the hydrogen-flame detector or mass spectrometer, are normally obtained on injection of ~1 μL of the chloroform extract from the derivatization

If anhydrous conditions are maintained, and the reaction mixture is directly injected into the column, it is possible to observe g l c peaks that represent derivatives of amino acids, compounds that could be present in a hydrolyzate However, contact with water for even a few seconds degrades these derivatives of the amino acids, and the water extraction of the chloroform phase apparently removes them Therefore, the PAAN–PAKO derivatization procedure is effective for the identification of saccharides (when present as a few mole-percent) in glycopeptides, although, for small weights of saccharides, a final concentration of the chloroform solution may be necessary

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