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Journal of Chromatography A, 726 (1996) 246–252

JOURNAL OF CHROMATOGRAPHY A

Short communication

New, stable halogenated derivative suitable for the gas chromatographic determination of muramic acid

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Received 28 August 1995; revised 30 October 1995; accepted 30 October 1995

Abstract

The formation of a new volatile derivative of muramic acid is described. The procedure entails a reaction with *O*-(pentafluorobenzyl)hydroxylamine in pyridine to give the corresponding oxime and a treatment with acetic anhydride in the presence of 4-*N*,*N*-dimethylaminopyridine. The structures of the obtained derivatives have been confirmed by MS. The influence of various catalyst amounts used during the acetylation step was studied and a mechanism was proposed in order to explain the different reaction courses.

Keywords: Derivatization, GC; Muramic acid; Peptidoglycan; Glycans

1. Introduction

Muramic acid (MA) is a constituent of the glycan backbone of peptidoglycan present in bacterial cell walls and it is not found elsewhere in nature; it is thus considered a marker for detecting both intact bacteria and bacterial debris. Many modern analytical techniques have been used for the determination of MA. HPLC has been performed either after pre-column derivatization using UV detection [1] or without derivatization using pulsed amperometric (PAD) [2] and plasma spray MS [3] detection. GC has been coupled to MS detection with either electron-impact (EI) [4] or negative-ion chemical ionization (NICI) [5]. In order to reach maximum sensitivity with the latter technique as well as with electron capture detection, stable halogenated derivatives have to be obtained. Trifluoroacetylated methyl glycosides used with NICI-MS detection [5] suffer low stability in the presence of water, thus not allowing a post-derivatization clean-up. Recently, two new approaches have been developed: direct injection electrospray tandem mass spectrometry (MS–MS) of underivatized MA [6] and EI-MS–MS coupled to GC of alditol acetate derivatives of MA [7]. The former allows maximum analysis speed and the latter improved selectivity. In order to achieve maximum improvement in sensitivity by exploiting electron capture and NICI-MS detection, our aim was to obtain a new halogenated MA derivative which was long-term stable, allowing post-derivatization clean-up. Among the derivatization proce-

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0021-9673/96/$15.00
SSDI 0021-9673(95)01234-6

dures suitable for GC determination alditol-acetate formation has been widely used [8,9], along with the treatment with *O*-alkylhydroxylamine of the carbonyl group, followed by acetylation of hydroxy groups [10,11]. The former procedure gives a single peak, but it is very time-consuming; the latter one affords a peak pair corresponding to *syn*- and *anti*-isomers of the resulting oxime. In order to obtain a single peak in the final GC profile after a procedure more rapid than the alditol-acetate one, the aldonitrile-acetate derivative was also reported [12,13], but no halogen atoms were yet allowed to be present in its structure. We introduced the use of *O*-(pentafluorobenzyl)hydroxylamine (PFBOA) for the formation of stable oximes of glycoprotein neutral [14] and amino sugars [15]; as in previous similar methods, the volatile derivatives were finally obtained by an acetylation step. In order to extend this procedure to the estimation of peptidoglycan components, in this work we tested the suitability of the treatments with PFBOA and acetic anhydride for the formation of a new stable halogenated derivative for the GC determination of MA (*O*-pentafluorobenzyloxime acetate), in order to achieve the improvements mentioned above.

2. Experimental

2.1. Materials

MA, PFBOA, meso-inositol, used as I.S., and 4-*N*,*N*-dimethylaminopyridine (DMAP) were purchased by Fluka (Buchs, Switzerland); other organic and inorganic reagents were of analytical grade; DMAP was recrystallized from ethyl acetate; pyridine and acetic anhydride were distilled over potassium hydroxide and anhydrous sodium acetate, respectively, before use. Aliquots of MA and I.S. were withdrawn from standard aqueous solutions (1 or 10 mmol/l) and brought to dryness under vacuum over potassium hydroxide pellets.

2.2. Apparatus and chromatographic conditions

A Packard Model 437A gas-chromatograph (Chrompack, Middelburg, Netherlands)) equipped with flame-ionization detector and connected to an HP 3396 Series II integrator (Hewlett-Packard, Palo Alto, CA, USA) was used. The separations were performed on a fused-silica capillary column (15 m × 0.25 μm I.D.) wall coated (0.25 μm film thickness) with Easy 52 chemically bonded stationary phase (Analytical Technology, Cernusco S/N, Italy). The column temperature was programmed from 160°C to 290°C at 10°C/min. Injector and detector temperatures were 250°C and 300°C, respectively. Helium was used as carrier gas at a flow-rate of 0.7 ml/min; air and hydrogen flow-rates were 22 ml/min and 250 ml/min, respectively. EI-MS was performed on a quadrupole Incos 50 instrument (Finnigan MAT, San Jose, CA, USA) at 70 eV ionizing voltage.

2.3. Derivatization procedure

MA (500 nmol) was treated with 0.2 ml of pyridine containing PFBOA (50 mg/ml) for 20 min at 80°C. After cooling 0.2 ml of pyridine containing DMAP (0, 20 or 40 mg/ml) and 0.5 ml of acetic anhydride were added and the samples were kept for different periods of time (10–60 min) at 80°C. After cooling the reaction mixtures were evaporated to dryness under a nitrogen stream, dissolved in dichloromethane (2 ml) and washed with 1 *M* HCl (2 ml) and water (2 × 2 ml). The organic phases were filtered over anhydrous sodium sulphate and brought to dryness under a nitrogen stream. The residues were dissolved in ethyl acetate (0.2 ml) and analysed by GC.

2.4. Quantitative analysis

Different aliquots (from 50 to 1000 nmol) of MA, together with the same amount (200 nmol) of I.S., were treated according to the above described procedure using 40 mg/ml DMAP solution and a 15-min heating time in the acetylation step. Calibration was performed by obtaining the ratios between the area of the MA peak and that of the I.S. and by reporting their values against the amounts of MA.

3. Results and discussion

In the structure of MA, along with carbonyl,

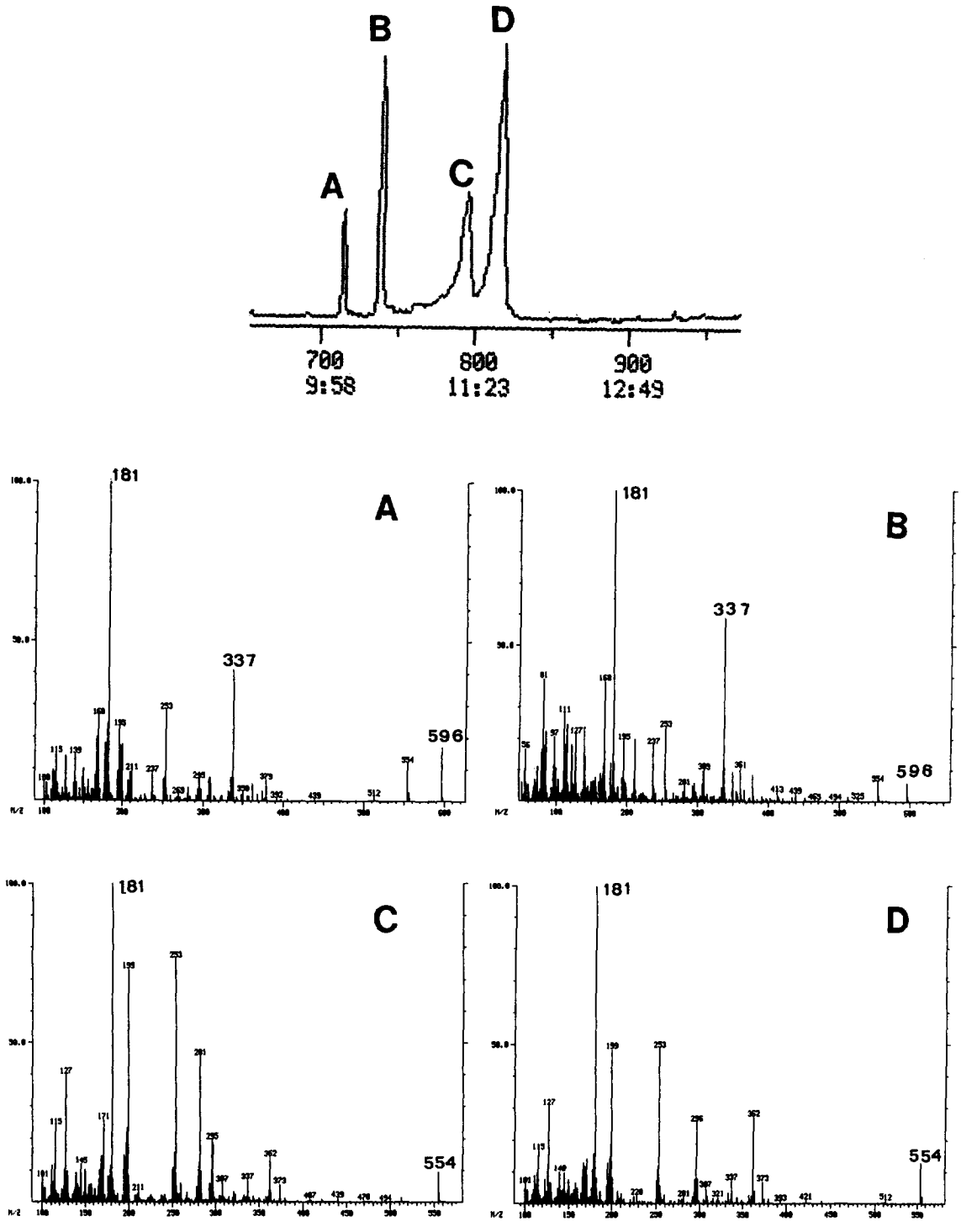

Fig. 1. Total ion current EI profile of MA derivatized without DMAP (top trace) and MS spectra corresponding to four obtained peaks (GC conditions are reported in Experimental section).

hydroxyl and amino groups, a carboxyl group is also present and another derivatization step appeared to be needed. However, since the alditol acetate procedure was studied, the dehydrating conditions of the acetylation step were shown to induce the formation of a lactamic linkage between the carboxyl and amino groups of MA, thus avoiding the need for the protection of the free carboxyl group [8]. In this connection, in preliminary work, we attempted to obtain a stable derivative of MA by simply applying the same procedure used for monosaccharides previously reported, which involves a final acetylation step [14]. The corresponding GC profile (Fig. 1, top trace) on the one hand confirms the formation of volatile derivatives, but, on the other hand, shows two peak pairs. Since the PFBOA treatment gives *syn*- and *anti*-isomers of the resulting oximes, two different derivatives were probably obtained. The MS analysis of the four peaks (Fig. 1) allows an explanation of the reaction course. Actually the early eluting peak pair corresponds to the peracetylated pentafluorobenzyl oximes (M_r=596), while the later eluting peak pair corresponds to derivatives (M_r= 554) lacking a fragment of *m/z* 42, corresponding to ketene produced by an acetyl group. Therefore, the difference in the later eluting compounds consists of the absence of an acetyl group, probably on the lactamic N atom (Fig. 2), as it occurred in the alditol acetate derivatization, when forcing acetylation conditions were not used [8]. Moreover the MS analysis confirms the lactamic structure of the derivatives; in fact the fragment resulting from the removal of *m/z* 217 after the ketene loss (*m/z* 337, clearly present in peracetylated oxime spectra 1 and 2), contains the stable six-membered lactam ring, as shown in Fig. 2.

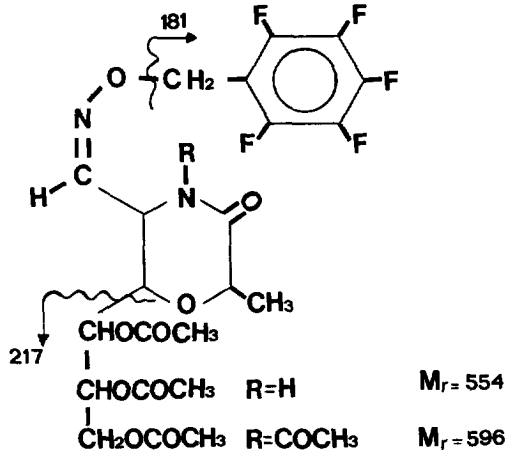

Fig. 2. Structure of MA derivative obtained according to the described procedure. Two of the main EI fragmentations are shown.

In order to simplify the GC profile and establish the conditions suitable to obtain a single volatile product we used forcing conditions in the acetylation step. The catalytic power of DMAP in the formation of *O*-methyloxime acetates of monosaccharides being known [16], we examined the effects of its use during the acetylation, in addition to that of reaction time. In the presence of 4 mg and 8 mg of DMAP the maximum GC response was reached after a treatment at 80°C for 45 min and 15 min, respectively, but qualitative results were clearly different. In Fig. 3 the GC profiles obtained by performing the acetylation step in the optimum conditions with 4 and 8 mg of DMAP are reported. The treatment with 4 mg of DMAP eliminated the *N*-deacetyl derivatives so giving an unambigous reaction course, only resulting in *syn*- and *anti*-isomers of the peracetylated derivative. When DMAP content was increased to 8 mg, a single significant peak, the latter

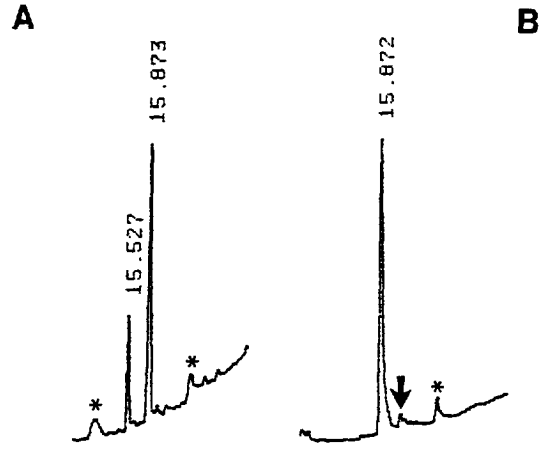

Fig. 3. Typical partial chromatograms corresponding to MA treated according to the described procedure in the presence of 4 mg for 45 min (A) and 8 mg for 15 min (B) of DMAP during acetylation step (GC conditions are reported in Experimental section). The arrow indicates the compound showing the same MS spectrum of MA derivative and probably corresponding to the D-manno analogue of MA (as mentioned in the text). The asterisks indicate unknown compounds.

between those obtained with 4 mg of DMAP, appeared in the final GC profile, so making the analysis more simple and sensitive. Probably the high concentration of DMAP catalysed the interconversion of *syn*- and *anti*-isomers of oximes and supported the thermodynamically more stable isomer. The proposed mechanism is shown in Fig. 4. DMAP induces the loss of an H^+ ion from C-2 of peracetylated oxime of MA and the shift of an electron pair toward the oxime N atom, which miss its isomerism center nature. When neutral oxime is again formed both isomers can be produced. The possible epimerization of C-2, giving the D-manno analogue of MA [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-mannose], is adversely affected by the resulting axial conformation of C-1 moiety. A trace of this epimer was probably obtained in the described conditions: in fact a small peak immediately after MA derivative, with an MS spectrum almost identical to those of MA peracetylated oximes (1 and 2), appeared in the final chromatogram. However the contribution of this MA epimer was always very low (area < 3% of the MA

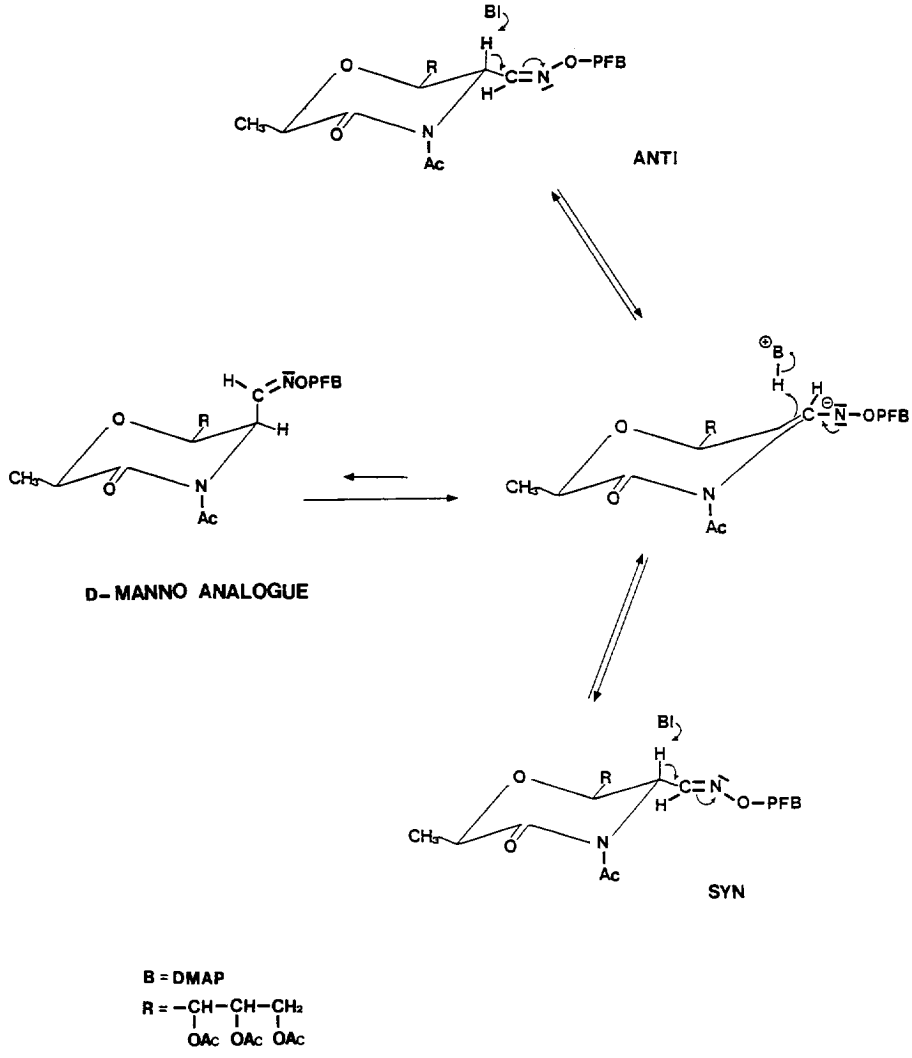

Fig. 4. Scheme of the proposed mechanism for the interconversion between MA peracetylated oxime isomers and for the epimerization at C-2 atom. B is the symbol for a general base; the dashes represent the electron pairs.

peak one) and did not interfere with the quantitative analysis of MA. The D-manno analogue of MA was actually detected in a Gram-positive bacterium in very low yields (1% of the total carbohydrate chain of peptidoglycan) [17]; if the D-manno analogue of MA is to be detected as PFBO-acetate, the procedure entailing only 4 mg of DMAP should be followed, so restricting the MA epimerization to a not significant extent.

The use of DMAP gave rise to brown products during the final heating step, but, after the post-derivatization clean-up, the samples became pale yellow and only some small background peaks, not affecting the MA estimation, appeared in the GC profile. The described method has been so far applied only to pure MA; possible modifications regarding the procedure (like additional clean-up steps) and/or the GC conditions (like different stationary phase and/or capillary column features) could be needed when the method will be used for the detection of MA in biological samples.

It is noteworthy that, for the first time, an *O*-alkyloxime acetate of MA gave a single significant peak, so affording two advantages: from a qualitative point of view the derivatization product appears unique as in the more time-consuming alditol-acetate method and in the aldonitrile-acetate one; from a quantitative point of view the response of a single product is obviously higher than that of two products resulting from a same amount of substrate. Actually the corrected area of the single peak obtained from 500 nmol of MA in the presence of 8 mg of DMAP was very similar to the sum of the peak pair areas obtained from the same amount of MA in the presence of 4 mg of DMAP.

As concerns quantitative analysis the relationship of peak-area (Ra) ratios between MA and meso-inositol (used as I.S. in 100-nmol amounts) against MA amounts was linear in the range 50–1000 nmol according to the equation:

$$\mathrm{Ra} = 0.0065 \times \mathrm{nmol\ MA} + 0.12 \quad (r = 0.998)$$

The limit of detection of MA at $S/N=3$ was about 10 pmol injected. In order to test the stability of the described derivative the response of a final solution resulting from 200 nmol of MA, kept at room temperature, was estimated: no significant decrease appeared for at least 2 days. The precision was evaluated by analyzing 200 nmol of MA five times: R.S.D. was 2.42%.

Because of the stability of the obtained volatile product, the easy and rapid handling of the samples and the selectivity of the final GC estimation, we can conclude that the method described offers a useful alternative to the reported derivatization procedures suitable for the GC determination of MA in view of the possibility of using electron capture and NICI-MS detection. Continuing work in our laboratory is concerned with the further development and application of the described procedure.

Acknowledgments

The authors wish to thank Prof. Severino Ronchi for helpful discussion and the Antidoping Center of the Italian Jockey Club in Milan for the assistance in MS analysis. This work was supported by M.U.R.S.T. grants (40% and 60% funds to P.A.B.) and by Regione Lombardia grant (project No. 1481). L.M.C. was recipient of a National Research Council (C.N.R.) fellowship (project R.A.I.S.A.).

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