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New and simple procedure for the determination of muramic acid in chemically complex environments by gas chromatography–ion trap tandem mass spectrometry

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Abstract

A gas chromatographic–ion trap tandem mass spectrometric method was developed for the quantification of muramic acid, a marker of bacterial peptidoglycan, in environmental and clinical specimens. Samples (bacteria, house dust and urine) were heated in methanolic hydrochloric acid overnight and extracted with hexane for removal of hydrophobic compounds. The aqueous phase was evaporated and heated in acetic anhydride and pyridine after which the product, the acetate derivative, was washed with dilute hydrochloric acid and water. The described method is both rapid and simple to apply, and produces a stable derivative. It should become widely used for measuring peptidoglycan in chemically complex environments. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Muramic acid

1. Introduction

Muramic acid (MuAc), an amino sugar, has been suggested for use as a chemical marker in gas chromatographic–mass spectrometric determination of bacterial peptidoglycan in both clinical and environmental samples. Several derivatives of MuAc have been applied, including the alditol acetate [1,2], aldononitrile [3], trifluoroacetyl [4] and trimethylsilyl (TMS) [5,6] derivatives. The former two are chemically stable and produce a single chromatographic peak for the analyte; however, in particular the alditol acetate derivative is time-consuming to prepare. In addition, preparation of the aldononitrile derivative requires rigorously anhydrous conditions throughout the procedure [7], which we have found to be impractical for routine application (unpublished results). The trifluoroacetyl derivative is easily prepared, but it is unstable, can lead to rapid deterioration of the gas chromatography (GC) column, and requires access to negative ion-chemical ionization mass spectrometry (MS) for achieving good sensitivity [4]. We have previously used the TMS derivative following hydrolysis of samples [6]; the main disadvantage is its limited chemical stability which makes post-derivatization water or buffer

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washing impossible. Both the alditol acetate and TMS derivatives have proven suitable for use with GC–ion trap tandem MS (GC–MS–MS) [8,9].

The present report describes the development of an alternative method for the determination of MuAc in complex environments, aiming to be both convenient to use and to provide high detection sensitivity in ion trap GC–MS–MS.

2. Experimental

2.1. Chemicals and samples

Muramic acid was purchased from Sigma (St. Louis, MO, USA), *N*-methyl-D-glucamine from Aldrich (St. Louis, MO, USA), pyridine from Fluka (St. Louis, MO, USA), acetic anhydride from Acros (Geel, Belgium) and dichloromethane and chloroform from Lab-Scan (Dublin, Ireland). Lyophilized cells of ^{13}C-labelled blue–green algae were purchased from Isotec (Miamisburg, OH, USA).

Cells of *Staphylococcus lentus* were cultivated overnight on blood agar plates at 37°C. Five-ml aliquots of urine samples from patients both with and without urinary tract infection were centrifuged after which the pellets were dried using a stream of nitrogen. House dust samples were collected using a vacuum cleaner.

2.2. Sample preparation

In a typical experiment, samples (*S. lentus* cells, 1–2 mg; pellets of urine samples; settled house dust, 1–5 mg) were heated at 85°C overnight (16–20 h) in 1 ml of 4 *M* methanolic hydrochloric acid. After cooling to room temperature, two internal standards were added: *N*-methyl-D-glucamine (4 μg; 200 μl of a 0.02 mg/ml water solution) and ^{13}C-labelled MuAc (50–100 μl of a 1-ml aliquot of a methanolysate of 1 mg of algal cells). The mixture was extracted with 1 ml of hexane after which the lower phase was evaporated to dryness at 40°C under nitrogen and further dried under vacuum in a desiccator (1 h). Occasionally, when *N*-methyl-D-glucamine was not used, distilled water (200 μl) was added to improve phase separation upon the hexane extraction. Samples were acetylated by heating in a mixture of pyridine (100 μl) and acetic anhydride (100 μl) at 60°C for 1 h. The reaction mixture was evaporated with nitrogen (temperature below 40°C), dissolved in dichloromethane (2 ml), and washed, first with a 0.05 *M* HCl solution (1 ml) and then twice with distilled water (1 ml each). Samples were evaporated to dryness with nitrogen, dissolved in 130 μl of chloroform, and (when analysing dust samples only) centrifuged or filtered prior to GC–MS–MS.

2.3. Conditions for acetylation

An 8.6-mg sample of *S. lentus* cells was subjected to methanolysis and hexane extraction as described, and diluted with methanol to a volume of 20 ml. Of this volume, 16 1-ml portions were distributed to individual test tubes, evaporated to dryness, and subjected to acetylation. Four samples each were kept at room temperature and at 40, 60 and 80°C. After 3, 6, 18 and 26 h, respectively, one sample of each batch was analysed. The areas of the ions m/z 406 (MuAc derivative) and m/z 448 (*N*-methyl-D-glucamine, acetylated separately and added to the methanolysate immediately before injection onto the GC–MS system), were measured.

2.4. Reproducibility

A 1.0-mg portion of the ^{13}C-labelled algae was subjected to methanolysis, purified by hexane extraction, evaporated, and dissolved in 1.0 ml of methanol. Of this solution, 100-μl portions were used as internal standard.

Cells of *S. lentus* (1.0 mg) were methanolysed and *N*-methyl-D-glucamine was added after which the sample was washed with hexane and evaporated to dryness. The residue was dissolved in methanol (1 ml) and the methanolic phase was distributed into eight 100-μl portions. The ^{13}C-labelled MuAc standard was added to each sample which were then acetylated and analysed in the MS–MS mode. The preparations with the lowest and highest values of the ratio of m/z 145/150 [representing product ions of non-labelled (bacterial) and labelled (algal) MuAc, respectively] were injected repeatedly (five

times) and the mean values and standard deviations were calculated.

2.5. Stability, detection limit

Samples of derivatised MuAc prepared from *S. lentus* cells (0.4 mg) were stored at 4°C and analysed weekly during a 2-month period in order to assess the chemical stability of the derivative.

Cells of *S. lentus* (3.1 mg) were methanolysed, acetylated, and analysed for MuAc by GC–MS–MS after serial dilutions in order to determine the lower detection limit of the method.

2.6. Construction of a calibration curve

A 1-mg portion of the ^{13}C-labelled algae was heated in 1.0 ml of 4 *M* HCl_{aq} for 4 h at 100°C. The hydrolysate was washed with 1 ml of hexane, evaporated to dryness, and dissolved in 1 ml of distilled water. Of this solution, 100-μl portions were added to test tubes containing 0, 40, 80, 160, 320 and 640 ng of the reference MuAc standard. Since we were unsuccessful in methylating free MuAc (see Discussion), these samples were subjected to TMS derivatisation [6] before analysis.

2.7. GC–MS–MS

A fused-silica capillary GC column (CP-Sil 8-MS, 15 m×0.25 mm I.D., Chrompack, The Netherlands) was used. Volumes of 1 or 1.5 μl were injected in the splitless mode at an injector temperature of 290°C; the helium head column pressure was 69 kPa. The initial column temperature, 145°C, was increased to 245°C (6°C/min) and then to 290°C (30°C/min) where it was held for 5 min. The temperature of the interface (between GC and MS systems) was 290°C.

A Varian Saturn 2000 ion-trap MS instrument (Varian, Palo Alto, CA, USA) was used in the electron impact (EI) mode; the temperature of the trap was 200°C and of the manifold 50°C. The emission current was 20 μA in GC–MS and 50 μA in GC–MS–MS. The mass range was 70–650 *m/z* and the scan rate 1000 ms. Selected ejection chemical ionisation (SECI) was performed using methane as the reactant gas to achieve information of molecular mass and fragmentation patterns.

Conditions for MS–MS analysis were optimised by repeatedly injecting 500–1000 pg amounts of the MuAc derivative. Ions of *m/z* 187 (*m/z* 192 for the ^{13}C-labelled derivative) were fragmented in the non-resonance mode using a mass isolation window of 10 *m/z*. The collision-induced dissociation time was 50 ms and the amplitude 22.00 V, the low and high digital/analogue conversion values were 6 and 5, respectively, and the bandwidth was 1 kHz.

While samples were routinely analysed using the ion trap instrument described, a high-resolution instrument (Jeol Model JMS-SX102 MS system connected to a Shimadzu Model 14A GC system) was used to assist in the evaluation of the EI fragmentation.

3. Results

3.1. Mass spectra

The carboxyl and anomeric hydroxyl groups of MuAc were methylated and the three remaining hydroxyl groups (one primary and two secondary) were acetylated by the methanolysis and acetylation procedures. In accordance with previous findings [10] (protonated) molecular ions (M+H) for the non-labelled and labelled MuAc derivatives were found (at *m/z* 406 and *m/z* 415, respectively, Fig. 1); interestingly, none of these ions were found when using a conventional quadrupole type of instrument (data not shown). The suggested fragmentation scheme, elaborated with the help of previously published patterns [11], is shown (Fig. 2). There are three independent main fragmentations, two of which produce the three most abundant ions in the high-mass region, viz. *m/z* 145, *m/z* 187 and *m/z* 213 (*m/z* 150, *m/z* 192 and *m/z* 221 for the ^{13}C-labelled MuAc derivative). Fragmentation of *m/z* 187 (and *m/z* 192) resulted in the highest intensity of the product ions (*m/z* 145 and *m/z* 150, respectively) and was therefore used in GC–MS–MS (Fig. 3).

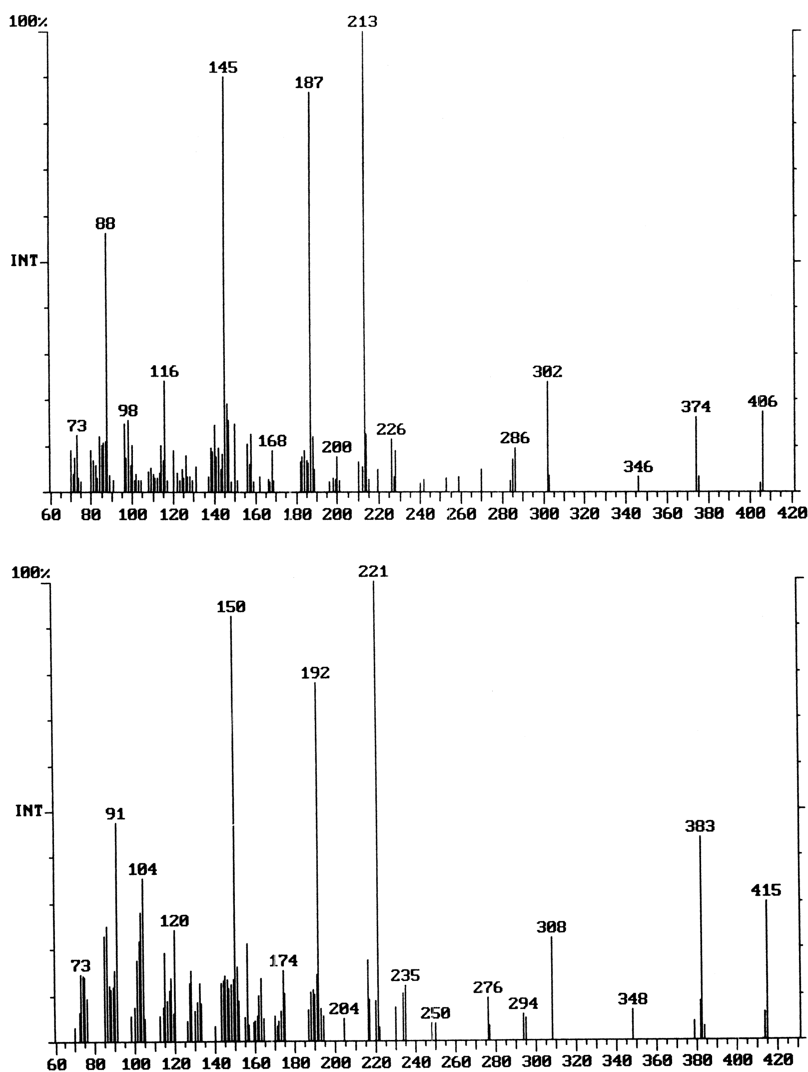

Fig. 1. EI spectra of derivatised bacterial (upper) and algal (lower) muramic acid.

Notably, none of the three dominating fragments were present in the CI spectra (Fig. 4).

3.2. Acetylation conditions, stability, reproducibility and detection limit

Acetylation for 1 h at 60°C was routinely used; at higher temperatures, a brownish colour was seen without any notably higher yield. No degradation of the derivative could be observed after storage of samples for several weeks at 4°C (data not shown).

The GC–MS–MS analyses of the 100-μl portions of the bacterial/algal methanolysates gave ratios of the product ions (m/z 145/150) of 3.23 (mean)±0.18 (standard deviation). The repeated injections of samples with the lowest and highest values of the same ratio gave mean values and standard deviations of 2.95±0.13 and 3.46±0.15, respectively.

The ^{13}C MuAc content in the algae was estimated to 2.6 ng/μg (dry mass) according to the linear calibration curve. Injection of a sample containing 500 pg of the MuAc derivative gave a signal-to-noise

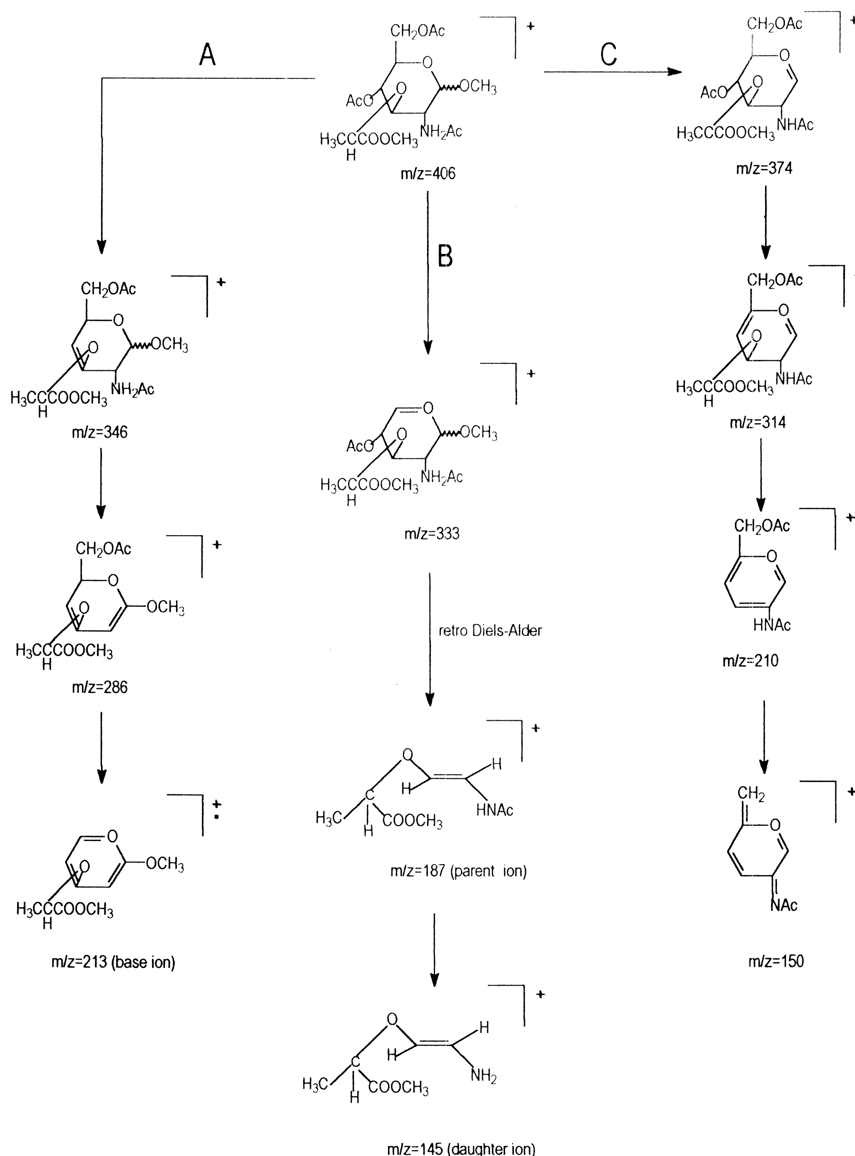

Fig. 2. Suggested fragmentation pattern of derivatised bacterial muramic acid.

ratio of ca. 400 upon GC–MS–MS (fragmentation of *m/z* 187 and monitoring *m/z* 145).

3.3. Urine and dust samples

MuAc was clearly detected in each of three analysed culture-positive urine samples and was absent, or found in trace amounts only, in all of three studied culture-negative samples. Results from analyses of a urine sample culture-positive for Group B streptococci and a culture-negative urine sample are shown (Fig. 5); the amount of MuAc in the former was ca. 170 ng/ml. The same figure also shows results from an analysis of a dust sample that contained 40 ng MuAc/mg. Due to the high detection specificity offered by GC–MS–MS, no extra-

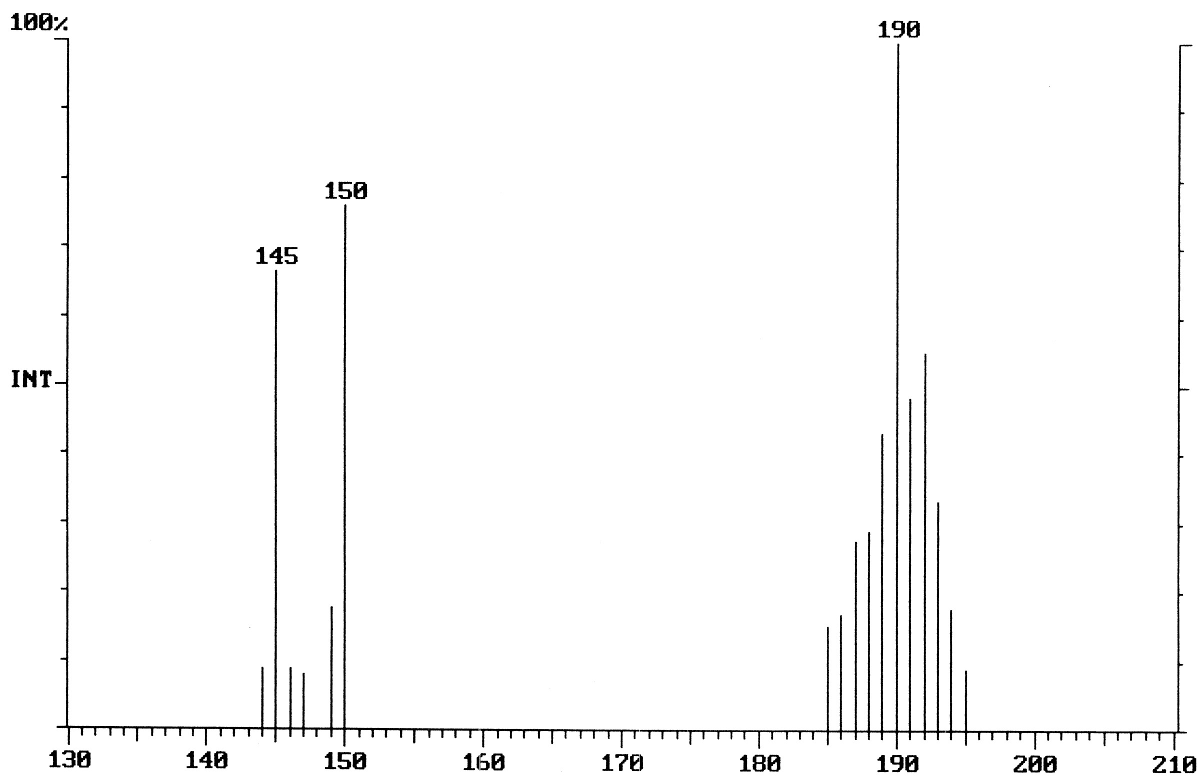

Fig. 3. MS–MS spectrum of derivatised muramic acid of a bacterial–algal mixture.

neous peaks of closely eluting compounds were observed in the chromatograms.

4. Discussion

The motivation for developing a specific and sensitive method for monitoring MuAc in complex environments can be found in different areas. MuAc has been used as a marker to demonstrate the presence of peptidoglycan in synovial fluids of patients with arthritis [12,13], and has also been identified in blood from healthy individuals [14]. It is being used as a bacterial marker in organic dust in research projects where the long-term goal is to correlate inhalation of microbe-contaminated air with the development of respiratory and other symptoms [15–17]. Clearly, a convenient and reliable method for the determination of MuAc, at trace levels, in complex environments will be required in order to render this chemical–analytical approach widespread acceptance.

A disadvantage of the described procedure is that free MuAc seems to be discriminated from being used as an external standard. Attempts to prepare the derivative from the MuAc reference compound by esterification (using diazomethane), methylation (with methanolic HCl), and, finally, acetylation, proved unsuccessful due to the formation of a lactam structure [18,19], since further treatment with *N*,*O*-bis-trimethylsilyltrifluoroacetamide resulted in the same product, the TMS derivative, as when hydrolysing samples with HCl followed by silylation (Fig. 6). The use of ^{13}C MuAc in algae as an internal standard, first described by Fox et al. [8] provides excellent quantification. With our method it should also be possible to prepare an internal standard from

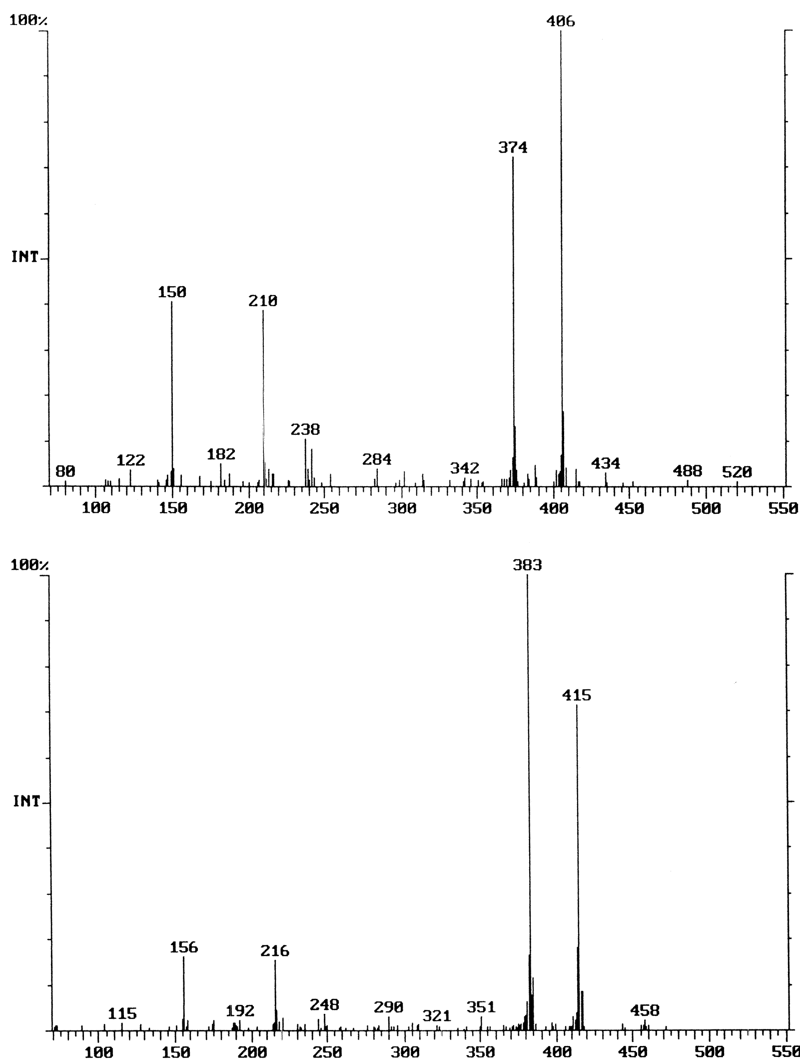

Fig. 4. CI (methane) spectrum of derivatised bacterial (upper) and algal (lower) muramic acid.

non-labelled bacteria or algae by using deuterated methanol in the methanolysis step. While a signal-to-noise ratio of ca. 400 was achieved upon injection of a 500-pg amount of derivatized MuAc of a pure bacterial culture, the actual detection limit must be determined for each type of environmental/clinical sample studied.

In short, we have found the described procedure to be suitable for routine application. It is simple and the derivative is chemically stable, producing abundant ions suitable for fragmentation in ion trap GC–MS–MS.

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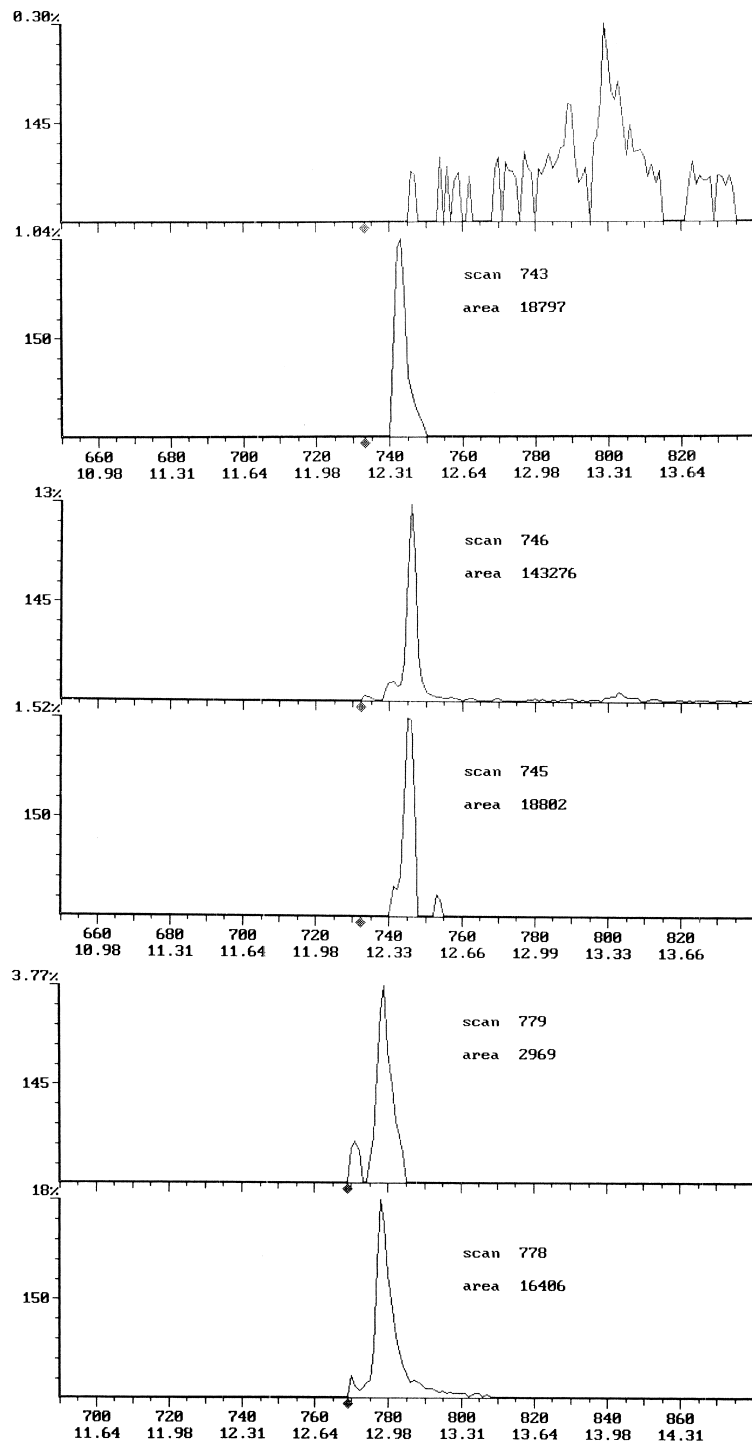

Fig. 5. Mass chromatograms obtained by GC–MS–MS analysis for muramic acid in a culture-negative urine sample (upper two tracings), a urine sample culture-positive for Group B streptococci (middle two tracings) and a house dust sample (lower two tracings), monitoring m/z 145 and m/z 150 (internal standard). The diamond symbols indicate the time segments used for monitoring the different ions.

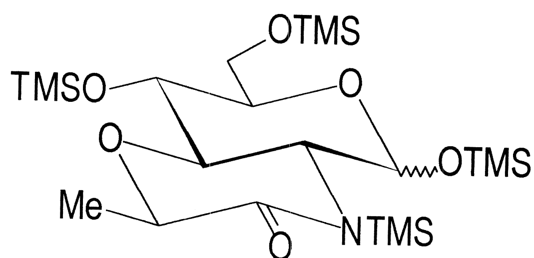

Fig. 6. Structure of a silylation product of muramic acid.

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