Fast Atom Bombardment Mass Spectrometry and Tandem Mass Spectrometry of Biologically Active Peptidoglycan Monomers from Neisseria gonorrhoeae

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Fast atom bombardment mass spectrometry (FAEMS) and tandem mass spectrometry (MS/MS) were employed to define the structures of Neisseria gonorrhoeae peptidoglycan monomers that were of interest because of their abilities to mediate diverse biological reactions ranging from arthritogenicity to somnogenicity. FAEMS-determined molecular weights of individual components present in several different enzymatically derived classes of gonococcal monomers revealed that each of these classes was a complex mixture of up to 13 distinct peptidoglycan fragments. These ranged from the predominant disaccharide tetrapeptides possessing reducing or nonreducing 1,6-anhydro-N-acetylmuramic acid ends to relatively minor constituents containing glycine or asparagine in addition to traditional peptidoglycan amino acids, i.e., alanine, glutamic acid, and diaminopimelic acid. FAEMS of high performance liquid chromatography-purified monomers yielded some sequence information; however, analysis even of unfractionated peptidoglycan mixtures using a JEOL HX110/HX110 tandem mass spectrometer operating at 10 kV provided unambiguous primary sequence data for the peptidoglycan monomers and defined the position of glycine in four compounds as well as the location of O-acetyl substituents (present on some compounds) on C-6 of the N-acetylmuramic acid residue.

Peptidoglycan is a uniquely bacterial heteropolymer that consists of a glycan backbone of alternating units of N-acetylglycosamine (GlcNAc) and N-acetylmuramic acid (MurNAc)1 with a short peptide side chain (typically 3–5 amino acids long) linked to the lecty moiety of muramic acid (1). Peptide cross-linking bonds between amino acid residues located on different glycan chains lead to the formation of a complex three-dimensional macromolecule that has been likened to an enormous, covalently closed basket surrounding the cytoplasmic membrane (2). Although nature has provided numerous subtle variations in the composition of peptidoglycan among the bacteria (3), this rather rigid arrangement of polymeric glycan (up to 100 disaccharide units long) cross-linked by peptides has been remarkably well conserved, a fact undoubtedly related to its role in maintaining the physical integrity of the bacterial cell. Yet, when taken from the host’s perspective, peptidoglycan is more than merely a biologically inert bacterial corset. Indeed, given access to host tissues and cells, soluble peptidoglycan derivatives are proving to be versatile biological effectors which, as a class, have a propensity to modulate immune and inflammatory reactions. Among the numerous peptidoglycan-mediated activities that have been well documented in recent years are adjuvanticity (4, 5), pyrogenticity (6, 7), activation of the metabolic and killing capacity of macrophages (8, 9), stimulation of leukocytes to release pharmacologically active mediators including interleukin-1 (10, 11), and arthritogenicity (12, 13). Very recently, certain peptidoglycan fragments have even been implicated as naturally occurring neuromodulators based on data showing that they accumulate in the brains and urine of sleep-deprived animals and induce excess slow-wave sleep (14, 15).

During the past several years, we have been testing the hypothesis that peptidoglycan fragments influence the host response during the natural course of bacterial infections. Toward this end, we have exploited Neisseria gonorrhoeae as a model organism in which peptidoglycan-host interactions might be particularly direct and extensive in vivo (16–20). To date, we have identified several sets of purified gonococcal peptidoglycan fragments that likely gain access to host tissues; these range from high molecular weight (>10^6 daltons) soluble fragments that are extensively substituted in the glycan with O-acetyl derivatives (19, 21, 22) to unusual anhydromuramic acid-containing disaccharide peptide monomers (~10^3 daltons), the major peptidoglycan compounds released by growing gonococci (16–18). Collectively, these gonococcal peptidoglycan fragments have been found to mediate diverse biological activities including arthritogenicity (23), toxicity for human fallopian tube mucosa (24), and complement activation (25). However, our aim to determine the structural requirements and molecular mechanisms of these activities is potentially compromised by the outdated procedures previously employed for purification and chemical analysis of peptidoglycan fragments. In fact, the whole field of peptidoglycan chemistry has, until recently, changed very little since the classical approaches offered by Ghysen (26) and others dur-
ing the late 1960s. There are several reasons for these difficulties in peptidoglycan chemistry that are related primarily to the unique structure of peptidoglycan, e.g. the relative inapplicability of peptide sequencing techniques to the fine structure analysis of peptidoglycan and the inability to hydrolyze selectively the various positions of the peptide side chain of peptidoglycan. Even the exemplary work of van Heijenoort and co-workers (27), applying conventional mass spectrometry to the analysis of derivatized peptidoglycan disaccharides containing 1,6-anhydro-N-acetylmuramic acid, has not been generally applicable to the study of the disaccharide peptide fragments of peptidoglycan.

Fortunately, vastly improved methodology for peptidoglycan chemistry is now being developed. First is the successful fractionation of low molecular weight peptidoglycan fragments by reverse phase high performance liquid chromatography (HPLC) as evinced in the original report of Glauner and Schwarz (28) and in the applications of Daugherty (29) and Martin et al. (30). These studies have demonstrated that muramidase digests of peptidoglycan from at least certain bacteria are considerably more complex chemically than previously appreciated. Yet, even with these excellent means of separation, the analysis by classical techniques of the numerous peptidoglycan products resolved requires a rather heroic effort and is indirect, thus typically falling well short of anything resembling unambiguous proof of structure.

The introduction of fast atom bombardment mass spectrometry (FABMS) to the early 1980s (31, 32), its application to peptidoglycans (30, 33, 34), and the more recent commercial availability of tandem magnetic four-sector mass spectrometers (35) seem to offer a novel approach to this problem by providing an efficient and unambiguous determination of the molecular weight and primary structure of peptidoglycan fragments. For conventional peptides FABMS produces primarily molecular weight information. Depending on several factors, including sample concentration and composition, some fragment ions indicative of the primary structure of the peptidoglycan may be observed and provide sequence information (36, 37), but their relative abundance is typically 5-10-fold less than that of the molecular ion. There are, however, several factors, including matrix interferences and the presence of other components of the extraction procedure, which may severely limit the value of sequence information derived from the sample (38). These observations also hold true for other compound classes, including peptidoglycan. The application of tandem mass spectrometry (MS/MS) to the analysis of peptidoglycan should overcome these limitations and increase the structural information available. In MS/MS the ions associated with the molecular weight of the compound of interest are selected in the first mass spectrometer (MS-1) at a resolution of one mass unit. These ions, which uniquely define the sample, collide with an inert gas such as helium, producing fragment ions which are mass analyzed in the second mass spectrometer, MS-2. The result of this two-stage process is a mass spectrum rich in structural information related only to the compound selected in MS-1 (39).

Accordingly, to study the structure-function relationship of activities mediated by gonococcal peptidoglycan fragments, we have used reversed phase HPLC, FABMS, and MS/MS to define the analogous disaccharide classes of disaccharide peptide monomers isolated from gonococcal peptidoglycan. Each of these classes, i.e. “Chalaropsis monomers” with reducing muramic acid ends and “anhydro monomers” with nonreducing disaccharide peptide ends, is biologically active in one or more experimental systems of interest (23, 24), and each likely interacts with host tissues in vivo (18, 19, 40).

**EXPERIMENTAL PROCEDURES**

**Preparation of Intact Peptidoglycan—Neisseria gonorrhoeae strains**

RD5 and FA19 (nonpiliated, transparent variants) (41) were grown at 37 °C in liquid medium (LBGB+, pH 7.3) containing 0.4% (w/v) pyruvate and d-[3-14C]- or d-[6-3H]glucosamine (ICN Pharmaceuticals, Inc., Irvine, CA). The radiolabeled glucosamine, which is incorporated into both amino acids comprising the 1,6-anhydro backbone of gonococcal peptidoglycan (40), was used to track the peptidoglycan during the purification procedure. Intact (insoluble) peptidoglycan was purified from exponential phase gonococci by a trichloroacetic acid-sodium dodecyl sulfate extraction procedure (42) as modified (22) to include (i) extraction with sodium dodecyl sulfate at pH 5.1 and (ii) treatment of the sodium dodecyl sulfate-insoluble residue with proteinase K. The final washed insoluble material (peptidoglycan) from either strain contained <0.9% (w/w) non-peptidoglycan amino acids. Intact peptidoglycan from strain FA19 has been shown previously to be extensively substituted in the glycan with O-acetyl derivatives; ~45% of the disaccharide subunits of FA19 peptidoglycan are O-acetylated (22, 43).

**Preparation of Peptidoglycan Monomers—** Purified intact peptidoglycan was used as starting material for two structurally related families of monomeric peptidoglycan fragments. Each of these sets, which are referred to as Chalaropsis monomers and anhydro monomers, respectively, was initially isolated as mixtures of peptidoglycan monomers. Chalaropsis monomers were isolated by gel filtration on connected columns of Sephadex G-50 and G-25 after complete digestion with Chalaropsis B muramidase (Miles Laboratories, Elkhart, IN) of intact extensively O-acetylated peptidoglycan from strain FA19 or of O-acetyl-deficient peptidoglycan from strain RD5, as we have described previously (24, 40). Pooled monomeric fractions were desalted by gel filtration on Sephadex G-15 eluted with pyrogen-free water. Chalaropsis monomers served as the source of peptidoglycan monomers with hydrated, reducing N-acetylmuramic acid ends.

Anhydro monomers were prepared from intact strain RD5 peptidoglycan with use of a partially purified enzyme preparation obtained from Escherichia coli ATCC 9637. This preparation contained both DD-endopeptidase and peptidoglycan-6-muramyl transerase (transglycosylase) activities. Several closely related procedures for purification of these peptidoglycan hydrolases have been reported (44–46). For our purposes, the optimal procedure was a variation of this basic method in which the key step involved chromatography on carboxymethyl-Sepharose CL-6B (Pharmacia P-L Biochemicals) of Triton X-100 extracts of sonicated E. coli. This chromatographic procedure was performed as described previously (22) except that Triton X-100 extracts of washed membranes (rather than extracts of combined cytoplasmic plus membrane fractions) served as the source of the enzymatic activity. Details of the protocol for the complete digestion of intact peptidoglycan with the E. coli transglycosylase-endopeptidase have been published (24). Anhydro monomers were isolated from the peptidoglycan digest by gel filtration and desalted as for Chalaropsis monomers. Using this procedure, the yield of anhydro monomers from intact peptidoglycan starting material was exceptionally high (~90%). The efficiency of this reaction was attributed to the virtually complete conversion of insoluble peptidoglycan to anhydro monomers by the novel use of an enzyme preparation which contained both glycan-splitting (transglycosylase) and peptide-splitting (endopeptidase) activities. Anhydro monomers served as the source of peptidoglycan monomers with nonreducing 1,6-anhydro-N-acetylmuramic acid ends.

Previous studies (18, 24, 40) employing traditional procedures for peptidoglycan chemistry have revealed that the major components of Chalaropsis monomers were N-acetylglicosaminyl-N-acetylmuramyl-alanyl-glutamyl-diaminopimelic acid and the corresponding disaccharide peptide with a COOH-terminal alanine. Anhydro monomers were composed predominantly of the respective disaccharide peptides containing the 1,6-anhydromuramic acid end.

**High Performance Liquid Chromatography—** Final purification of the individual components of Chalaropsis monomers and anhydro monomers was accomplished by reversed phase HPLC. Samples were separated using a Waters HPLC 510 binary pump system with solvent programmer and reversed phase columns. A Waters 491
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absorbance detector operated at 214 nm and a Hewlett-Packard 3396A integrator were used for detection. Initial separation employed 4 × 250-mm columns from Vydac (C₄ and C₆H₆. The Separations Group, Hesperia, CA) and Waters (C₁₈, Millipore Corp., Milford, MA). Several HPLC fractions were subjected to further chromatography using a Waters narrowbore (2 × 250-mm) reversed phase C₁₈ column to improve chromatographic resolution. The solvent gradient employed depended on the column, the complexity of the sample, and the resolution of the separation. Typical elution gradients were linear from 100% water (containing 0.050% CF₃COOH) to 25% CH₃CN (containing 0.035% CF₃COOH) over a period of 30 min. The flow rates were 1.0–1.5 ml/min for the 4 × 250-mm columns and 0.4 ml/min for the narrowbore column.

Sample Preparation—The samples for FAB must be dissolved in a liquid matrix (38, 47, 48) in order to observe abundant long-lasting secondary ion signals associated with the species of interest. Glycerol, which is the most widely used matrix for the analysis of biological molecules, was employed in the analysis of peptidoglycan monomers. The peptidoglycan monomer mixtures or individual fractions isolated by HPLC were placed in 1-ml conical vials to which glycerol and aqueous acetic acid were added in volume ratio of 5:1. Sample concentrations ranged from 0.1 to 10.0 nmol/μl of matrix with a total matrix volume of 3–5 μl.

Fast Atom Bombardment Mass Spectrometry—A double focusing (Finnigan MAT 731, Bremen, FRG) mass spectrometer of the MAT-tauq-Herzog geometry (38) and a tandem mass spectrometer (JEOL HX110/HX110, Tokyo) were employed in this work (35). The MAT 731 has a mass range of 2000 daltons at 8-kV accelerating potential and was employed for the initial characterization of peptidoglycan monomers, providing molecular weight and partial structural information. Approximately 0.5–0.7 μl of the glycerol matrix containing the sample (see above) was applied to a stainless steel sample stage mounted on the end of a high vacuum push rod. The sample was inserted via vacuum locks into the center of the ion source where a neutral xenon beam (10 FA, 7 kV, Ion Tech B12N neutral source, Teddington, UK) impinged upon the matrix containing the sample. Ions produced by the interaction of the neutral beam with the sample surface were accelerated, energy and mass selected, and detected with a secondary electron multiplier. Exact mass measurements were made in the peak matching mode (at a resolution of 1/10,000) employing [Sar⁻¹-Ala]³-glycylglycine II (Beckman Biochemical) protonated molecular ion (MH⁺) 926.5212 as a reference compound mixed with the sample on the probe tip at a ratio (w/w) of 1:3.

The JEOL HX110/HX110 tandem mass spectrometer consists of two consecutive double focusing mass spectrometers (MS-1 and MS-2) each employing an electric field (E) followed by a magnetic field (B) (49), i.e. an EBEB geometry and a mass range of 14,500 daltons at 10-kV accelerating potential. The methods of sample preparation, introduction, ionization, and detection were similar to those described for the MAT 731. The only difference is the use of a JEOL ion/neutral beam source (10 mA, 6 kV) to produce the xenon primary beam which strikes the sample probe. As in the case of the MAT 731 (double focusing mass spectrometer), the JEOL HX110/HX110 may be operated using only the first EB segment (MS-1) for recording the molecular weight and full mass spectra of the samples. The unique aspect of this instrument is that it may also be operated in the MS/MS mode. In this mode of operation, MS-1 is set to transmit only the ion of interest, generally the protonated molecular ion of the compound under investigation. These ions enter a region between MS-1 and MS-2 containing helium gas. Collisions of the precursor ion with the neutral gas convert a fraction of the translational energy of the precursor into vibrational energy resulting in bond cleavage. The fragment ions produced from these bond cleavages are referred to as product ions. Scanning MS-2 results in a mass spectrum (product ion spectrum) which contains only information relating to the compound whose mass was selected in MS-1 and can, therefore, be used to deduce its structure. In the experiments discussed below, the resolution of MS-1 and MS-2 was 1/1000 which resulted in unit resolution of both the precursor and product ion mass spectra (50).

Reported masses for both the structures and mass spectra are rounded down to the nearest integer mass for clarity. The data system-assigned mass values differed by ±0.3 daltons from that calculated to one decimal point for the proposed fragment components.

Results

Fast Atom Bombardment Mass Spectrometry—Characterization of Chalaropsis monomers and anhydro monomers of N. gonorrhoeae strain RD5 by FABMS prior to HPLC fractionation indicated the presence of several components in each preparation. The probability that all species in such a mixture could be detected by FABMS depends on several factors. First, as the number of components in the mixture increases, those compounds which are present at low molar concentrations compared to the major components are masked by the latter's abundant ion signal and the matrix background, which consists of ions from the sample and liquid matrix. Second, those compounds which are more surface active, i.e. have hydrophobic substituents, will be preferentially ionized (51). Third, in mixtures in which the various species differ by single amino acids or minor structural modifications such as cyclization with corresponding loss of O₂, it is frequently difficult to ascertain whether the observed ion is a protonated molecular ion or a fragment ion of a molecule of higher mass. The latter possibility is a prime consideration in the analysis of peptidoglycan monomers in which various structures differ by single amino acids or sugar residues and/or H₂O. Therefore, to determine the total number of com-

Table I

| MH* observed | CM | AM | Primary structure |
|--------------|----|----|------------------|
| 679          | -  | +  | GlcNAc-(1,6-anhydro)MurNAc-Ala-Glu |
| 697          | -  | +  | GlcNAc-MurNAc-Ala-Glu |
| 719*         | +  | -  | GlcNAc-(1,6-anhydro)MurNAc-Ala-Glu-Ala |
| 851*         | +  | +  | GlcNAc-(1,6-anhydro)MurNAc-Ala-Glu-Ala |
| 869*         | +  | -  | GlcNAc-MurNAc-Ala-Glu-Ala |
| 908*         | -  | +  | GlcNAc-(1,6-anhydro)MurNAc-Ala-Glu-Ala-Gly |
| 922*         | -  | +  | GlcNAc-(1,6-anhydro)MurNAc-Ala-Glu-Ala-Gly |
| 928*         | +  | -  | GlcNAc-MurNAc-Ala-Glu-Ala-Ala |
| 949*         | -  | +  | GlcNAc-(1,6-anhydro)MurNAc-Ala-Glu-Ala-Gly |
| 979*         | -  | +  | GlcNAc-MurNAc-Ala-Glu-Ala-Gly |
| 997*         | +  | -  | GlcNAc-MurNAc-Ala-Glu-Ala-Gly |
| 1011*        | +  | -  | GlcNAc-MurNAc-Ala-Glu-Ala-Ala |

* MH* determined by FABMS.

A + indicates this compound was detected in the corresponding preparation.

Separated by HPLC into single component fractions for structure function studies (15, 54, 55).

Exact mass measurement, Table II.

The structure of the disaccharide portion of this compound isolated from E. coli and Salmonella typhi was determined by electron ionization mass spectrometry previously by Taylor et al. (27) after derivatization.
Compositions of three peptidoglycan monomers were determined by exact mass measurements (Table II) as a further confirmation of their structure. It should be noted that several compounds were detected unambiguously only after HPLC purification. These include the compound of MH* 719 (Table I) which is masked in the mixture by an abundant fragment ion in the FAB mass spectrum of MH* 922 (Fig. 1A) and MH* 968 and 979 (Table I) which are present in relatively low molar concentrations and appear in the mass regions where the major components, MH* 922 and 993, exhibit abundant fragment ions. Another ion (not listed in Table I because its structure has not been proven unambiguously) which was always observed at m/z 1036 would correspond to MH* 922 asparagine. In addition, HPLC separation in conjunction with FABMS and MS/MS revealed an anhydro peptidoglycan monomer (MH* 851, Table I) as a minor component in the Chalaropsis monomer preparation. Pre-separation by HPLC

| Measured m/z | Theoretical m/z | Elemental compositiona | Elemental compositionb |
|--------------|----------------|------------------------|------------------------|
| 851.3568     | 851.3522       | C₆H₁₂N₄O₁₉             | C₂₀H₂₀N₂O₂             |
| 922.3904     | 922.3893       | C₂₀H₂₂N₂O₂             | C₂₀H₂₃N₂O₂             |
| 940.3968     | 940.3999       |                         |                        |

* MH* ion.

TABLE II
Exact mass measurements of three peptidoglycan monomers from N. gonorrhoeae strain RD5

Fig. 1. Comparison of the FAB mass spectra of GlcNAc-(1,6-anhydro)-MurNAc-Ala-Glu-Adp-Ala, MH* 922, (A) in a normal (MS-1) scan and (B) in the MS/MS mode. Several of the ions important for the structural verification of this compound are labeled in the mass spectra. In the normal mass spectrum (A), the glycerol cluster ions are labeled with asterisks. Typical concentrations are 10 µg/ml in A and 1 µg/ml in B. Furthermore, the compound underwent extensive HPLC purification prior to obtaining the spectrum in A, whereas spectrum B was measured by selectively fragmenting the ion of m/z 922 generated from the unseparated mixture of peptidoglycan monomers.
removed any ambiguity concerning the nature of these molecular ions. Recently, the presence of anhydromuramic acid-containing monomers was detected in muramidase digests of gonococcal peptidoglycan (57).

The HPLC separation of Chalaropsis monomers was complicated by the fact that two peaks are observed for each species due to the \( \alpha/\beta \) interconversion at C-1 of MurNAc (30, 52). The samples could not be reduced with sodium borohydride to the open form of MurNAc, which would have simplified the HPLC peak profile (28), because the separated, fully characterized compounds had to be tested for their ability to induce slow-wave sleep (53, 54). The anhydromonoglycerol gave a single peak by HPLC for each compound because the \( \alpha/\beta \) interconversion is blocked by the 1,6-anhydro linkage (30).

In addition to molecular weight information, FABMS may provide some sequence information dependent on several factors including sample concentration, composition, and purity. To increase the probability of observing fragment ions related to specific peptidoglycan monomers, HPLC fractions containing single components were analyzed by FABMS at concentrations of 10–15 nmol/\( \mu \)l of liquid matrix. The numerical values refer to the mass of the fragments produced by cleavage along the bonds indicated plus 1 or 2 if the notation \( "H \) or \( "2H \) indicates hydrogens transferred to the species which is observed in the mass spectra.

Although the mass spectrum shown in Fig. 1A exhibits several abundant ions indicative of the structure (Fig. 2), much of the ion current is due to the chemical background (the low intensity continuum along the \( m/z \) axis) and glycerol cluster ions (labeled with an asterisk). As already mentioned, unless a large sample is used (and available), the matrix ions will often mask the sample ions of interest, and frequently only matrix-related ions can be observed below \( m/z \) 300.

In addition to the Chalaropsis and anhydro monomers from strain RD5 characterized above, Chalaropsis monomer preparations from strain FA19 known to contain a large fraction of O-acetylated components (22, 43) were analyzed by HPLC and FABMS. A total of 150 \( \mu \)g of peptidoglycan from strain FA19 was separated by reversed phase HPLC. A typical HPLC chromatogram for the separation of this preparation is shown in Fig. 3. The observed protonated molecular ions of the peptidoglycan monomers present are listed in Table III along with their primary structures which were determined by FAB MS/MS (see below). Although the mixture of Chalaropsis peptidoglycan monomers derived from strain FA19 is even more complex than that obtained from strain RD5, only five components of the former mixture had not previously been detected in the latter (Table I). Three of these five, MH

![Fig. 2. Structure and associated sequence ions for GlcNAc-(1,6-anhydro)-MurNAc-Ala-Glu-Apm-Ala, MH* 922, observed in the normal and MS/MS mode of operation. The numerical values refer to the mass of the fragments produced by cleavage along the bonds indicated plus 1 or 2 if the notation "H or "2H indicates hydrogens transferred to the species which is observed in the mass spectra.](image)

![Fig. 3. Typical HPLC chromatogram of Chalaropsis monomer preparation from FA19. The observed MH* for several of the components are listed above the corresponding peak. Linear solvent programs from 4-10% CH3CN (containing 0.035% CF3COOH) in H2O (containing 0.05% CF3COOH) for 30 min at a flow rate of 1.0 ml/min.](image)
In order to remove the contribution of the matrix, peptidoglycan. The advantage of this approach is discussed in the previous section. Typical of normal FAB mass spectra, the abundance and number of ions in the mass spectrum, and increase the extent of structural information, MS/MS was employed in the analysis of FAB mass spectra of GlcNAc-(1,6-anhydro) monomers from strain RD5. As in strain RD5, the MS/MS spectrum was acquired without prior separation of the peptidoglycan monomers by HPLC.

An important aspect of a high performance tandem mass spectrometer is the ability to select a single mass for collision-induced dissociation. The result of this selectivity is that all the product ions observed in the MS/MS spectrum must be related to the precursor ion, MH* 922 in Fig. 1B. It should be reiterated that although the FABMS spectrum shown in Fig. 1A exhibits all the peaks that dominate Fig. 1B, one should keep in mind that peaks in the normal spectrum can also originate from either the matrix or other components of the matrix, possibilities which complicate the interpretation.

A summary of the sequence ions observed in the MS/MS spectrum and their related structural components are shown in Fig. 2 and have been discussed above in connection with Fig. 1A. In the MS/MS mass spectrum of GlcNAc-(1,6-anhydro)-MurNAc-Ala-Glu-Apm-Ala, MH* 851 (not shown), the ions characteristic of the loss of the carbohydrate moieties are shifted lower by 71 daltons (alanine), i.e. m/z 463, 461, and 446, respectively, indicating an anhydro disaccharide and a tripeptide that lacks the COOH-terminal alanine. Further evidence that MH* 922 contains (1,6-anhydro)-MurNAc is the absence of an abundant peak at MH* -18, i.e. m/z 904 in Fig. 1B. In the reducing forms of peptidoglycan, such as Chalaropsis monomers, a characteristic strong peak is observed at MH* -18 resulting from the loss of H₂O from the C-1 position of MurNAc (see below). In addition to the abundant ions which characterize the disaccharide portion of peptidoglycan monomer, i.e. m/z 747, 719, 534, and 204, a series of ions are observed which delineate the peptide sequence. However, unlike the disaccharide portion which exhibited the same set of fragment ions in both the MS and MS/MS modes, the majority of the fragment ions associated with the cleavage of a peptide portion in the MS/MS mode are those which retain charge on the COOH terminus, i.e. m/z 446, 391, and 262. In the normal FAB mass spectrum, cleavage of the peptide backbone with charge retention on both the carbohydrate (m/z 850, 678, 600, and 549) and the COOH-terminal peptide are observed. The mass difference between m/z 391 and 262 (129 daltons) defines the residue as glutamic acid and the difference between m/z 806 and 606 (200 daltons) further indicates that the glutamic acid moiety is present in the form of isoglutamic acid. The fragment ion would be m/z 634, a difference of 172 daltons, if cleavage occurred at a normal glutamic acid residue:

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| Structure | Sequence     |
|-----------|-------------|
| GlcNAc-(1,6-anhydro)-MurNAc-Ala-Glu-Apm-Ala | MH* 922 |
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The most important difference is the absence of the continuous background and the associated increase in the signal to noise ratio in the MS/MS spectrum (Fig. 1B). The concentration of GlcNAc-(1,6-anhydro)-MurNAc-Ala-Glu-Apm-Ala, MH* 922, was 1 nmol/μl in Fig. 1B, which is a factor of 10 less than that required to produce the spectrum shown in Fig. 1A. Furthermore, the MS/MS spectrum was acquired without prior separation of the peptidoglycan monomers by HPLC.

Although HPLC yielded fractions of Chalaropsis monomers from strain FA19 that contained only a single component, very little sequence information could be derived for any of the compounds containing an O-acetyl group. It was therefore impossible to assign the position of the O-acetyl group based on FABMS alone.

**Tandem Fast Atom Bombardment Mass Spectrometry**—In the normal mode of FABMS, the abundance and number of fragment ions is very dependent on several factors as discussed in the previous section. Typical of normal FAB mass spectra is the continuous background of ions associated with the matrix (Fig. 1A); this background makes it difficult to distinguish ions due to the sample from those associated with the matrix. In order to remove the contribution of the matrix, eliminate the ambiguity related to the origin of various sample ions in the mass spectrum, and increase the extent of structural information, MS/MS was employed in the analysis of peptidoglycan. The advantage of this approach is demonstrated by a comparison of the normal (Fig. 1A) and tandem (Fig. 1B) FAB mass spectra of GlcNAc-(1,6-anhydro)-

There are also a series of internal fragments including Glu-
Apm, m/z 302, and A_2pm, m/z 173, which provide further confirmation of the peptide sequence and indicate the location of amide groups if present. For example, in the fragment Glu-Apm there are two possible sites of amidation, and the mass shift from m/z 302 to 303 will not define its location. However, if in conjunction with this there is a shift from m/z 173 to 172 for Apm, then the replacement of -OH with -NH must have occurred at Apm. The location of the amide group is important since it affects the somnogenicity of these compounds (15). Furthermore, in work with peptides the low mass end provides information, in the form of immonium ions, which aids in determining the amino acids present in the compound (55). In addition to these ions there are several others which are observed below m/z 300 in the MS/MS spectrum (Fig. 1B) which are not observed in the normal FABMS mass spectra (Fig. 1A) regardless of sample concentration. For example, the absence of ions of m/z 128 and 173 in the MS/MS spectra derived from MH+ 679 and 697 (Table I) confirms the absence of Apm moieties in these two compounds.

\[ \begin{align*}
O & \quad \text{C-OH} \\
\text{CH-NH}_2 & \quad \text{HOC} \quad \text{H} \\
\text{H_2N} & \quad \text{CH-CO}^+ \\
m/z & \quad 173 \\
& \quad 128
\end{align*} \]

The interpretations discussed above were used to interpret the remaining FAB MS/MS spectra (Table I).

The above data demonstrate the power of MS/MS by providing detailed sequence information for individually selected components in a mixture of structurally related peptidoglycan analogs. As previously mentioned, peptidoglycan strain FA19 contains several components whose molecular weights correspond to the addition of an O-acetyl group, but FABMS of these HPLC-purified monomers gave only molecular weight information (thus simply confirming by homology the existence of O-acetylated monomers). MS/MS was therefore used to determine the position of the O-acetyl groups (Table III). The lack of structural information from the O-acetylated compounds in the normal FAB mass spectra was due to the fact that each of these pure compounds represents only a small fraction of the total amount of starting material, and this was not sufficient to observe fragment ions. The structural assignments were based on a comparison of the MS/MS spectra of two components present in Chalaropsis monomers derived from strain FA19. They were GlcNAc-MurNAc-Ala-Glu-Apm-Ala, MH+ 940, and the compound of MH+ 982 which differs from the former by 42 daltons corresponding to the replacement of -OH by -OCOCH₃. A comparison of the MS/MS spectra of these compounds (Fig. 4, A and B) indicates a number of similarities both in relative intensities and mass assignments of several ions. As discussed above, ions characteristic of loss of the disaccharide moiety are clearly observed in both compounds at m/z 534, 532, and 517. In addition to these ions, those at m/z 446, 391, 302, 262, and 173 are common to both spectra in Fig. 4 (and also Fig. 1B) and provide sequence ions related to the peptide portion of the molecule (Fig. 5, A and B).

Based on the observation that all the ions corresponding to the peptide portion were the same for MH+ 940 and 982 (and
also for MH+ 922, Fig. 1B), the structural difference must be in the disaccharide portion of the molecule. An ion characteristic of GlcNAc, m/z 204, was observed in both compounds (also in Fig. 1), thus eliminating it as the site of O-acetylation. Three characteristic ions associated with the disaccharide portion of the molecule were observed at m/z 765, 737, and 719 in Fig. 4A and are shifted by 42 daltons to m/z 807, 779, and 761, respectively, in Fig. 4B. These mass differences indicate that the O-acetyl group was located on the MurNAc moiety, either at C-1 or at C-6. The former can be eliminated as a possibility based on the very abundant ion at MH+ -18 in Fig. 4, A and B, i.e. m/z 922 and 964, respectively. This very facile loss is characteristic of a -OH group at the C-1 position of the reducing end of the disaccharide. If the molecule were acetylated at the C-1 position, a very abundant ion signal would be observed at MH+ -60 (i.e. m/z 922) corresponding to the loss of the components of acetic acid in Fig. 4B, but such an ion is not observed. Based on these results, O-acetylation has taken place at C-6 of muramic acid. This is confirmed by the peak at m/z 689 in Fig. 4, A and B corresponding to loss of the C-5 substituent, CH2-O-COCH3, followed by the elimination of the GlcNAc component including transfer of hydrogen from C-3 of MurNAc.

\[ \text{CH}_2-\text{C-} \text{peptide} \]

\[ \text{OH} \]

\[ \text{m/z 689} \]

These interpretations were then applied to the FAB MS/MS of other O-acetylated compounds as well as the other peptidoglycan monomers (Table III). The presence of the O-acetyl group on the MurNAc moiety is also supported by the observation that no O-acetyl derivative of the 1,6-anhydro component was found in this mixture (Table III).

**DISCUSSION**

These studies have employed FABMS and introduced MS/MS as powerful tools for structural analysis of low molecular weight peptidoglycan derivatives. The objective of this comprehensive analysis of diverse peptidoglycan compounds by FABMS and MS/MS was to define chemically two complex families of biologically active disaccharide peptide monomers isolated from gonococcal peptidoglycan. As such, the results seem significant in terms of both the chemistry of peptidoglycan generally and the pathobiology of *N. gonorrhoeae* specifically.

From the chemical perspective, we have shown that peptidoglycans are amenable to FABMS and MS/MS and that the amount of structural information revealed depends on the instrumentation and on the quantity, purity, and uniformity of peptidoglycan. Normal FABMS can provide molecular weight information from multicomponent mixtures, allowing rapid verification of the composition of the mixture. Separation by HPLC and analysis by FABMS may provide some sequence information if tens of nanomoles of purified sample is available. If more than one component is present or if impurities such as salts are part of the mixture, little if any structural information will be obtained. Furthermore, even if large quantities of material are available, the constant matrix background will mask some of the ions associated with the sample, especially below m/z 300.

Tandem mass spectrometry is a rapid, reliable method to resolve problems concerning the structure of low molecular weight peptidoglycan derivatives, e.g. unambiguous determination of the primary sequence of amino acids and amino sugars and of the location of substituents such as O-acetyl groups. Furthermore, in conjunction with HPLC it is possible to isolate single peptidoglycan components which retain their original, biologically active structure, to characterize these components by FABMS and MS/MS, and then to use these compounds in structure-function studies (15, 30, 53, 54).

In the long run, the real dividends from the applications of FABMS (and especially MS/MS) to peptidoglycan chemistry will likely result from our understanding the structural basis for biological activities mediated by naturally occurring peptidoglycan derivatives, a class of compounds currently under extensive examination for their role in health and disease (56). Indeed, the recent work of Krueger et al. (53, 54) defining the structural requirements for the potent somnogenic activity induced by naturally occurring peptidoglycan monomers relied on the diverse set of analogs present in preparations of gonococcal peptidoglycan monomers and the structural analysis of these compounds by FABMS. Thus, it was demonstrated (i) that the anhydromuramic acid residue (but not the glucosamine moiety) was essential for maximal somnogenic activity, (ii) that the activity was modulated by the length and composition of the peptide side chain, and (iii) that amidation of carboxyl groups on the peptide may regulate the sleep-inducing activity.

The results of the current study should also be of benefit to our studies dealing with the role of peptidoglycan in the
pathobiology of gonococcal disease, specifically. Thus, it should now be possible to define the structural basis by which gonococcal peptidoglycan fragments, e.g. anhydro monomers, produce arthritis (23) and damage human fallopian tube mucosa (24). Yet, beyond somnogenic peptidoglycan compounds and gonococcal infections specifically, the enhanced capacity to define peptidoglycan structurally will, we hope, contribute to other studies concerned with the physiological role of peptidoglycan in bacteria or with biologically relevant activities resulting from peptidoglycan-host interactions.

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