MASS SPECTROMETRY OF SYNTHETIC GLYCOPEPTIDE ANALOGS OF BACTERIAL PEPTIDOGLYCANS (MDP AND DERIVATIVES)

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1. Introduction

It is now well-established that the immunoadjuvant activity of mycobacteria and other bacteria is located in the water-soluble peptidoglycan fractions of their cell walls. Also, recent studies have demonstrated that a synthetic glycopeptide:

N-acetylmuramyl–L-Ala–D-isoGln (MDP) (I)

has the minimal structure required for this adjuvant activity [1,2]. The latter observation stimulated much interest in the possible therapeutic use of such small glycopeptides, and led to the synthesis of several analogs in order to evaluate their structure–activity relationship [3–9].

The correct structural assignment of these synthetic glycopeptides by conventional means is often a difficult task. Moreover, before employing such glycopeptides to biological tests it is necessary to establish their purity. Previous work on the structural elucidation of cell-wall peptides [10,11] obtained from peptidoglycans revealed that these problems may be solved by proper use of mass spectrometry, which also offers a possibility toward quick identification and characterization of such compounds.

We have therefore investigated the mass spectrometrical fragmentation of some of these synthetic glycopeptides after appropriate derivatization. The results obtained clearly indicate that mass spectrometry is a very suitable tool for the structural characterization of these glycopeptides. Here we wish to report the details of this investigation carried out with (I) and four of its analogs three of which contain a larger peptide chain:

2-(4-*O*-D-glucopyranose)–D-propionyl–L-Ala–D-isoGln (II)

N-acetylmuramyl–L-Ala–D-Glu–Gly (III)

N-acetylmuramyl–L-Ala–D-isoGln–L-Lys (IV)

N-acetylmuramyl–L-Ala–D-isoGln–L-Lys–D-Ala (V)

2. Materials and methods

The glycopeptides utilized in this work were synthesized by Lefrancier et al. [4,5].

2.1. *Glycopeptide derivatization*

Prior to mass spectrometric analysis the *N*-acetylated peptides were permethylated with methyl iodide in the presence of a base prepared by dissolving sodium hydride in dimethylsulfoxide. The *N*-acetyl-*N,O*-permethyl derivatives appeared to be the best in terms of ease of preparation, volatility and mass spectrometric fragmentation.

2.2. N-*Acetylation*

N-Acetylation of Lys-containing peptides (100 μg)

A preliminary account of the investigation has been given at the 15th European Peptide Symposium in Gdańsk, Poland, 1978

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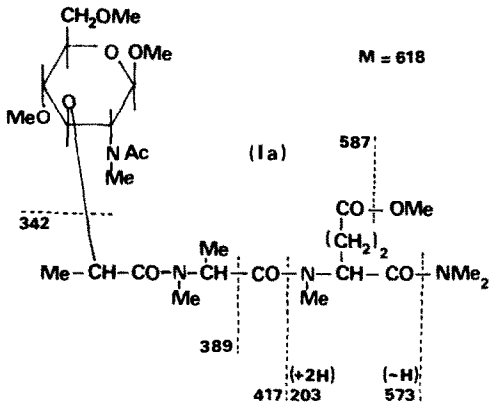
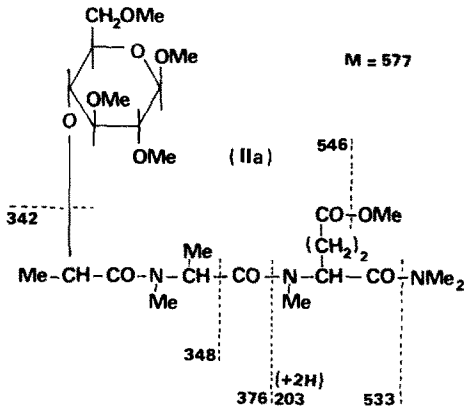
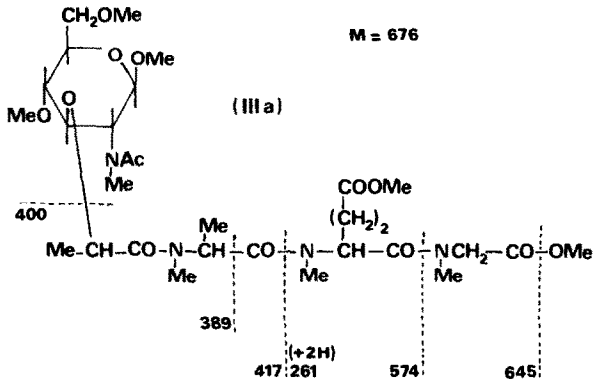
(IV) and (V) was carried out with an equimolecular mixture (100 μl) of acetic anhydride and deuterated (d_6) acetic anhydride. A trace of water was added to aid the dissolution of the sample.

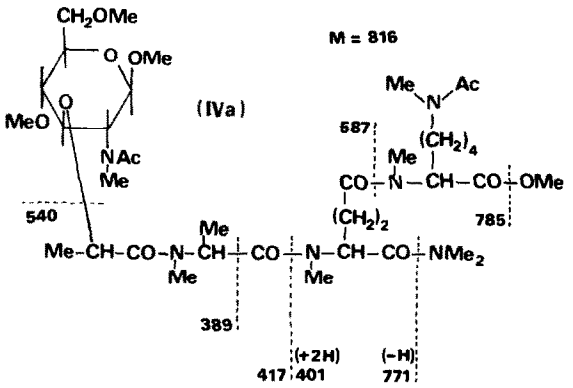
2.3. *Preparation of $Na^+CH_3SOCH_2^-$ base for permethylation [12]*

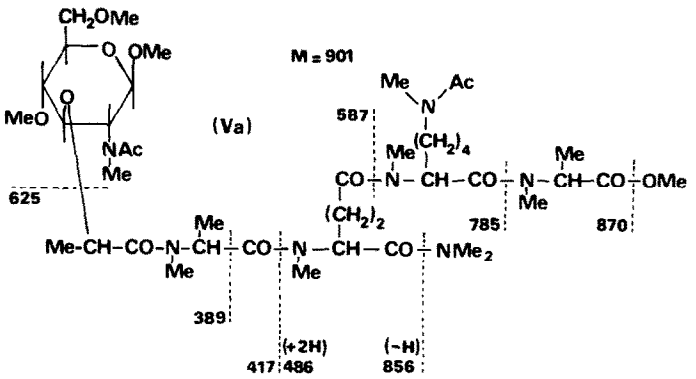
Sodium hydride (80 mg, 50% dispersion in oil, Fluka) was rinsed 3 times with anhydrous ether and











heated under nitrogen in dimethylsulfoxide (3 ml) at 80°C in an oil-bath until hydrogen evolution ceased. The solution which could be stored under nitrogen in a refrigerator for several weeks was used for permethylation of the glycopeptides.

2.4. *Permethylation of* N-*acetylated glycopeptides*

A solution of the $Na^+CH_3SOCH_2^-$ base (100 μl) was added to the *N*-acetylated peptide (100 μg) followed by methyl iodide (100–150 μl). The reaction was continued with stirring for ~30 min, after which water was added. The permethylated derivative was extracted with chloroform, the extract was washed 3 times with water, dried over anhydrous sodium sulfate, evaporated to dryness and then submitted to mass spectrometric analysis (electron impact and chemical ionization).

2.5. *Mass spectrometry*

The electron impact (EI) mass spectra were obtained with an AEI MS 50 mass spectrometer operating at 70 eV. The chemical ionization (CI) spectra were determined with a modified AEI MS 9 instrument [13] using methane as the reactant gas. The samples were introduced directly into the ion-source at 150–180°C.

3. Results and discussion

The derivatized glycopeptides (Ia), (IIa), (IIIa), (IVa) and (Va), in all cases yielded satisfactory EI mass spectra showing the molecular ion peaks as well as significant fragmentation patterns. The main fragment ions were obtained from degradation of the peptide chain and to a lesser extent from rupture of the carbohydrate moiety. Additional fragments originated by loss of methanol from the pyranose ring of the molecule.

In the high mass end of the spectra, the fragments below the molecular ions arose by loss of OCH_3 from the pyranose ring (presumably the glycosidic OCH_3) or from the carboxyl terminus and $N(CH_3)_2$ from the amide function of the isoglutamyl residue.

All the EI spectra exhibited the expected sequence peaks due to cleavage of the amide bonds of the peptide chain. An intense peak due to the N-terminal alanyl residue bearing the carbohydrate moiety is characteristic for all spectra. This is accompanied by peaks 28 and 60 mass units lower due to subsequent loss of CO and CH_3OH. Complementary C-terminal fragments with hydrogen rearrangements were also present in the spectra.

Although no peak belonging to the complete muramyl moiety was observed in the mass spectra, the presence of the carbohydrate residue was manifested by a peak at *m/e* 260 due to the loss of the entire substituent at the 3-position of the pyranose ring system. In the case of compound (IIa), a similar peak was observed at *m/e* 219 after loss of the substituent at the 4-position of the pyranose ring. Subsequent loss of a molecule of methanol then gave rise to a strong peak at *m/e* 228 (*m/e* 187 for compound (IIa)).

Fragment ions consisting of the entire peptide chain after cleavage of the ether linkage between the lactyl group and the pyranose ring of the carbohydrate residue were also characteristic of all spectra.

Another common fragmentation for these glycopeptide derivatives was the loss of 150 mass units from the molecular ions (M-149 in the case of compound (IIa)) by degradation of the carbohydrate part of the molecule. These fragment ions may be rationalized by way of elimination of carbons 1, 5 and 6 along with the pyranose ring oxygen and the loss of a molecule of methanol (loss of OCH_3 in the case of compound (IIa)).

It should be noted that the mass spectra of (IVa) and (Va) showed all fragments retaining the lysine residue as 2 peaks of equal intensity separated by 3 mass units, since its sidechain amino group was substituted with 1:1 mixed CH_3CO/CD_3CO (see section 2).

The fragmentation behaviour of the glycopeptide derivatives (Ia)-(Va) is indicated on their structural formulas. Figure 1 and 2 represent the entire mass spectra of compound (Ia) and (IVa). The above interpretation of the mass spectral fragmentation of the glycopeptides was confirmed by the mass spectra of the corresponding perdeuteriomethylated derivatives.

CI mass spectra with methane as ionizing gas were obtained from compounds (Ia–Va). The spectra of (Ia), (IIa) and (IIIa) showed strong MH^+ peaks, but fragment peaks were scarce and of low intensity. Also, the peaks observed were not different from those obtained under EI: the pyranose ring after elimination of CH_3OH at *m/e* 228 (for (Ia) and (IIIa)) and

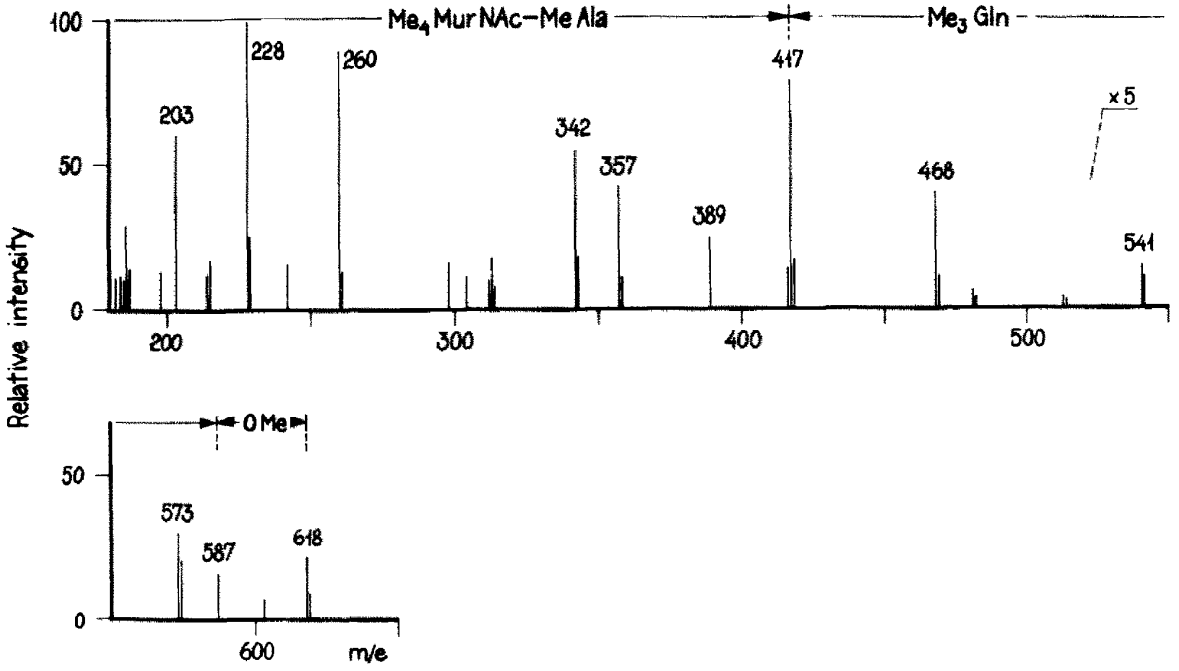


Fig.1. Mass spectrum of *N*-acetylmuramyl–L-Ala–D-isoGln, permethylated (Ia).

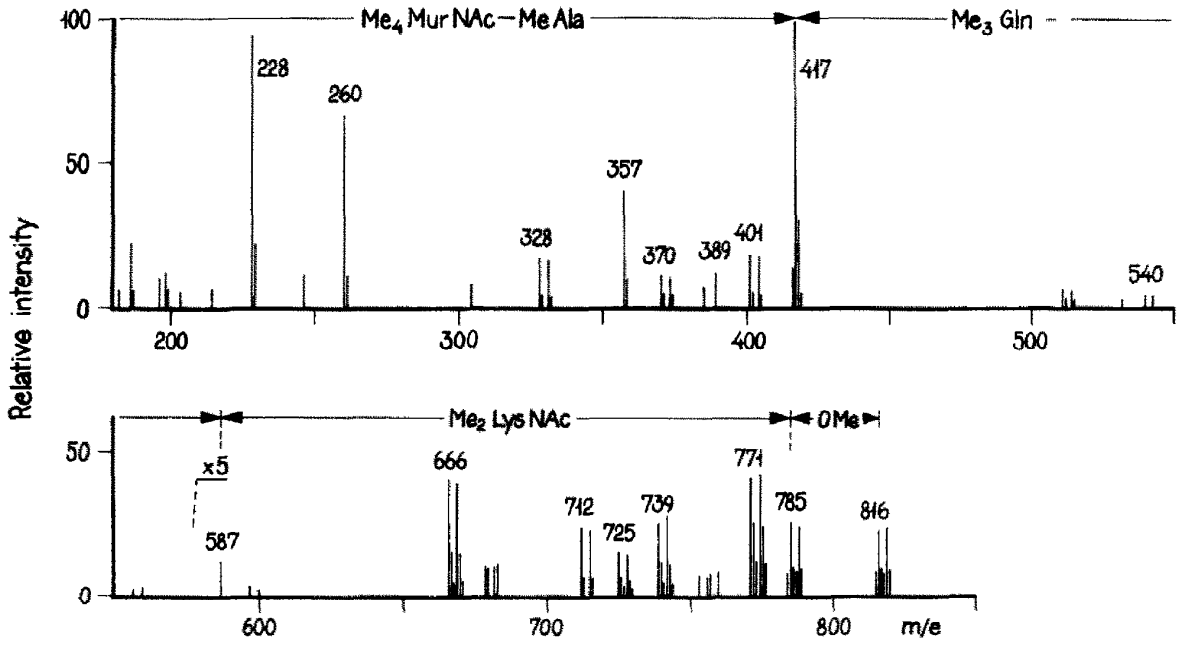


Fig.2. Mass spectrum of *N*-acetylmuramyl–L-Ala–D-isoGln–L-Lys, (CH_3CO/CD_3CO) acetylated and permethylated (IVa).

m/e 187 (for (IIa)), the carbohydrate part with alanine attached to it at *m/e* 417 (for Ia) and (IIIa)) and *m/e* 376 (for IIa)), and the C-terminal fragments at *m/e* 203 (for (Ia) and (IIa)) and 261 (for (IIIa)). The spectrum of (IVa) surprisingly did not show the MH^+ peak, whereas the spectrum of (Va) had a distinct MH^+ peak at *m/e* 902/905, although of low intensity. The fragmentation patterns of (IVa) and (Va) were not suitable to allow structural assignments.

As a result of this investigation it may be concluded that mass spectrometry can be successfully utilized for the characterization of complex glycopeptides containing a muramyl or similar residue. *N*-Acetyl permethyl derivatives are suitable for such investigation. Distinct molecular ion peaks are observed in the mass spectra from which the purity of the original glycopeptide sample may be evaluated. Moreover, the correctness of the structure can be established from the presence of the corresponding sequence peaks. It is expected that the results described here would facilitate the structural elucidation of similar glycopeptides.

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