Journal of Peptide Science
J. Peptide Sci. **7**: 45–55 (2002)
DOI: 10.1002/psc.367

Characterization of Polypeptide Antibiotics of the Polymyxin Series by Liquid Chromatography Electrospray Ionization Ion Trap Tandem Mass Spectrometry

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Received 18 July 2001
Accepted 1 October 2001

Abstract: A selective reversed phase liquid chromatography/mass spectrometry (LC/MS^n) method is described for the identification of related compounds in commercial polymyxin B samples. Mass spectral data for these polypeptide antibiotics were acquired on a LCQ ion trap mass spectrometer equipped with an electrospray ionization probe operated in the positive ion mode. The LCQ ion trap is ideally suited for the identification of the related substances because it provides on-line LC/MS^n capability. The main advantage of this hyphenated LC/MS^n technique is the characterization of novel related substances without time-consuming isolation and purification procedures. Using this method six novel related substances were partially identified in a polymyxin B bulk sample. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: polypeptide; antibiotic; polymyxin B; LC/MS; ion trap; electrospray ionization

INTRODUCTION

Polymyxin antibiotics, isolated from various strains of *Bacillus polymyxa* and related species are linear side-chain cyclic peptides, which were discovered in 1947 almost simultaneously by three independent groups [1–3]. 'Polymyxin' was accepted as the generic name for all the related antibiotics produced by *B. polymyxa*. They belong to the polypeptide antibiotics which all have relatively complex structures. The large variety of components other than amino acids, unusual peptide linkages and also for most of these antibiotics their cyclic nature makes their structural elucidation in many instances more complicated [4]. Many attempts have been made during the years to characterize the commercially available polymyxin B complex, which is marketed for the topical treatment of local cutaneous, otic, external ocular, and meningeal and mucosal infections caused by susceptible microorganisms, especially *Pseudomonas aeruginosa* [5]. In most cases structural elucidation was carried out after time-consuming isolation and purification procedures. Laborious classical amino acid and fatty acid analysis combined with partial hydrolysis resulted after many years of research in the structures described in Figure 1 [4,6–9]. Recently two new polymyxins, B_5 and B_6, also listed in Figure 1, were characterized using ^1H and ^{13}C NMR [10]. The polymyxins B have a general structure composed of a cyclic heptapeptide moiety and a side-chain consisting of a tripeptide with a fatty acyl residue on the *N*-terminus. They differ from each other in amino acid composition and on the basis of the fatty acid attached to the peptide.

The increase of serious infections with Gram-negative bacteria and the growing resistance to

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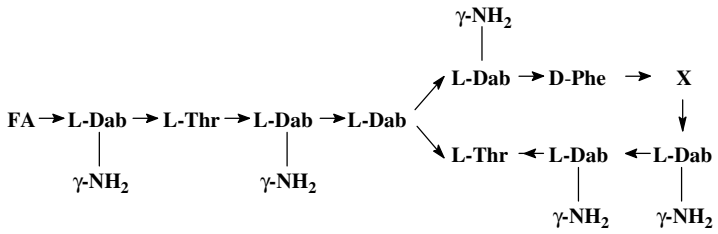
Contract/grant sponsor: Flemish Fund for Scientific Research; Contract/grant number: G.0355.98.



Polymyxin	X	Fatty acyl (FA) (Elemental formula)	Elemental formula Calculated monoisotopic mass	
B_1	L-Leu	6-methyloctanoyl ($C_9H_{17}O$)	$C_{56}H_{98}N_{16}O_{13}$	1202.7498
Ile-B_1	L-Ile	6-methyloctanoyl ($C_9H_{17}O$)	$C_{56}H_{98}N_{16}O_{13}$	1202.7498
B_2	L-Leu	6-methylheptanoyl ($C_8H_{15}O$)	$C_{55}H_{96}N_{16}O_{13}$	1188.7342
B_3	L-Leu	octanoyl ($C_8H_{15}O$)	$C_{55}H_{96}N_{16}O_{13}$	1188.7342
B_4	L-Leu	heptanoyl ($C_7H_{13}O$)	$C_{54}H_{94}N_{16}O_{13}$	1174.7185
B_5	L-Leu	nonanoyl ($C_9H_{17}O$)	$C_{56}H_{98}N_{16}O_{13}$	1202.7498
B_6	L-Leu	3-OH-6-methyloctanoyl ($C_9H_{17}O_2$)	$C_{56}H_{98}N_{16}O_{14}$	1218.7447

Figure 1 Chemical structures of known polymyxins B with fatty acyl moieties, elemental formulae and calculated monoisotopic masses. The elemental compositions of the fatty acyl moieties are shown between brackets. Dab, α, γ-diaminobutyric acid; FA, fatty acyl.

broad-spectrum antibiotics, have led to polymyxin again taking a major role in the therapy of multidrug-resistant Gram-negative bacterial infections [11]. With the development of a more performant LC method for polymyxin B, using a non-volatile mobile phase, more peaks were separated [12], but despite considerable efforts of isolation and purification [10], few structures were elucidated. Because polymyxins are again of growing importance and since the qualification and identification of impurities are critical issues in assessing the safety and quality of a pharmaceutical drug substance, identification of the still unidentified peaks is important. Therefore we considered developing a LC/MS^n method for the characterization of minor polymyxin components, thus preventing laborious preparative LC using large amounts of sample and solvents. The fact that linear side-chain cyclic peptides are claimed to be one of the most difficult peptide classes to sequence by MS/MS was an additional challenge [13].

MATERIALS AND METHODS

Chemicals

Acetonitrile (HPLC grade S) was purchased from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid was obtained from Riedel-de Haën (Seelze, Germany). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to further purify glass-distilled water.

Samples and Sample Preparation

Polymyxins B_1, B_2, B_3, B_4, B_5 and B_6 reference substances were available in the laboratory and were all characterized by NMR [10]. All reference substances were dissolved separately in water at a concentration of 0.05 µg/µl. Polymyxin B sulphate bulk powder, produced by growth of *Bacillus polymyxa* (*Prazmowski*) *Migula* (Fam. Bacillaceae) complying to the corresponding monograph in the United States Pharmacopeia [14], was

obtained from Ludeco (Brussels, Belgium). The sample was dissolved at a concentration of 0.25 μg/μl in water.

LC Instrumentation and Chromatographic Conditions

The LC apparatus consisted of a SpectraSYSTEM P1000XR quaternary pump, a SpectraSERIES AS100 autosampler equipped with a 20 μl loop, a variable wavelength Spectra 100 UV-VIS detector set at 215 nm, all from ThermoFinnigan (Fremont, CA, USA). The UV data were acquired with ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK). The YMC-Pack Pro C18 column (5 μm, 250 × 2.0 mm i.d.) (Waters, Milford, MA, USA) was immersed in a water bath at 30 °C. The following mobile phase was used for separation: 0.01 M trifluoroacetic acid (TFA)–acetonitrile (77 : 23, v/v %). The mobile phase was degassed by sparging helium. The LC pump was operated at a flow rate of 200 μl/min.

Electrospray Ionization Tandem Mass Spectrometry

The mass spectral data shown in this paper were acquired on a LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray interface operated in positive ion mode. The abundant doubly-charged ion with m/z 602.40, yielded by direct infusion of a 0.05 μg/μl solution of polymyxin B_1, was used to tune automatically the ionization source and MS parameters. A voltage of 5 kV applied to the electrospray ionization (ESI) needle resulted in a distinct signal. The temperature of the heated capillary was set at 210 °C . The number of ions stored in the ion trap was regulated by the automatic gain control. Nitrogen supplied by a Nitroprime TM membrane unit, type SNIFF (AGA, Lidingö, Sweden) was used as sheath and auxiliary gas. The flow rate of the sheath and the auxiliary gas was set at 90 and 20 (arbitrary units), respectively. Helium was used as the damping gas and as the collision gas at a pressure of 0.133 m^{-1} kg s^{-2}. Voltages across the capillary and the octapole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. The standard LCQ Navigator software package was used for instrument control, data acquisition and processing.

Mass Spectrometric Investigation of the Samples

Solutions of the reference substances (0.05 μg/μl) and the polymyxin B sulphate powder (0.25 μg/μl) were introduced on the column and analysed online with the ion trap. Full mass spectra were acquired over the mass range m/z 500–700. For MS/MS investigation doubly-charged polymyxin ions were isolated monoisotopically in the ion trap and collisionally activated with 30% collision energy (CE). The CE of the LCQ is set using a scale of 0–100%. The adjusted value corresponds with 30% of the available 5 V peak-to-peak of resonance excitation RF voltage. MS^3 spectra were acquired to confirm subsequent mass losses and if necessary, for the structure investigations of unknowns in the bulk sample.

RESULTS AND DISCUSSION

Development of the Liquid Chromatographic Method

Until now LC of the compounds in the polymyxin B complex has been performed with mobile phases containing non-volatile additives, incompatible with MS. Since polymyxins are polypeptides, LC conditions for separation of peptide mixtures were applied and optimized and a YMC-Pack Pro C18 column was used because it was described by Orwa *et al.* as a column with good selectivity for the polymyxins B [12]. An optimized combination of acetonitrile and aqueous TFA resulted in the UV profile of a 0.25 μg/μl sample, which is shown in Figure 2. All peaks discussed in this article are indicated with a number corresponding to the elution order under the specified conditions.

Optimizing Ionization Source and MS Parameters for LC/MS Investigation

The fragmentation behaviour of the main polymyxins B_1, B_2, E_1 and E_2 has been described elsewhere [15]. In this paper the polymyxins were investigated by direct infusion in Q/oaTOF and ion trap apparatus, resulting in a complete description of the fragmentation pattern, which is the guideline used in further structure investigations in this article. Other solvent conditions (water–TFA–acetonitrile instead of isopropanol–water) and higher LC flow (200 μl/min) instead of a low syringe flow [15] made adaptations of the ESI source and MS parameters necessary. Nitrogen supply was adapted and the parameters were tuned by an automated procedure to maximize the signal for the ion of interest. Changing these parameters did not affect the full

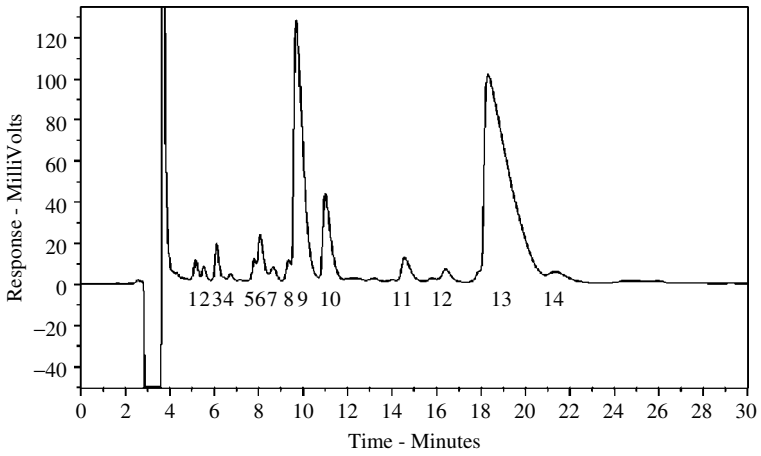


Figure 2 UV chromatogram of a commercial polymyxin sample (0.25 μg/μl). Conditions: YMC-Pack Pro C18, 5 μm, 250 × 2.0 mm i.d. maintained at 30 °C. Mobile phase of 0.01 M trifluoroacetic acid–acetonitrile (77 : 23, v/v) at a flow rate of 200 μl/min. Detection was by UV at 215 nm. 3, polymyxin B_4; 6, polymyxin B_6; 9, polymyxin B_2; 10, polymyxin B_3; 11, Ile-polymyxin B_1; 13, polymyxin B_1; 14, polymyxin B_5; other peaks are unknowns of which the identification is discussed further in this work.

MS spectra or fragmentation spectra. Infusions of solutions of polymyxin B_1 and B_2 into the mass spectrometer confirmed this.

Mass Spectrometric Results of the Reference Substances

In consistence with previous work [15], MS/MS experiments were performed with the doubly-charged ions as the precursor ions. On-line MS/MS spectra with 30% CE were recorded for polymyxin B_1 and B_2 and other available polymyxin B components and were used to compose a complete library. All compounds showed the fragmentation behaviour described for polymyxin B_1 and B_2. As an example Figure 3 shows the structure of polymyxin B_1 and the corresponding MS/MS spectrum with all diagnostic ions indicated with their m/z value. Amino acids are numbered (A*n*), starting from the fatty acyl side. Two important series of singly-charged product ions are present in the MS/MS spectrum. The first series of product ions (noted with an asterisk) displays a rather high relative abundance, whereas most product ions belonging to the second series are less intensely present. The first series of product ions is assumed to be formed by loss of the fatty acyl moiety plus the neighbouring α, γ-diaminobutyric acid (Dab) moiety and subsequent losses in the linear and circular part. The second series of product ions is yielded by a first loss of three ring amino acids (A5-A6-A7 or A6-A7-A8 in Figure 3) and subsequent losses of other amino acid moieties. Beneath the MS/MS spectrum a sketch is given with a short review of the proposed fragmentation routes. Characteristic ions offering information about the fatty acyl moiety and the ring amino acids A6 and A7 are shown above the MS/MS spectrum. The m/z values of the doubly-charged precursor ion and the singly-charged first (indicated with an asterisk) and second series of product ions are shown in Table 1 for polymyxins B_1, B_2, B_3, B_4, B_5 and B_6 for the purpose of comparison with product ion scans of unknown peaks in the bulk sample.

Mass Spectrometric Results of Polymyxin B Sulphate

Before starting the structure elucidation of the unknowns, it needs to be mentioned that the mass spectrometric technique used does not distinguish Leu from Ile, and that for the fatty acyl moieties, only the elemental composition could be defined and not the branching. No attempts were undertaken to isolate and purify the minor components, further discussed in this article, for characterization of the optical configuration of the amino acids.

Full mass spectral acquisition over the mass range m/z 500–700 was performed to gain information about the sample composition. The base peak chromatogram obtained with the ion trap is shown in Figure 4a. Full MS spectra of 14 peaks, which are indicated on the base peak (Figure 4a) and the UV (Figure 2) chromatogram, were investigated. Fragmentation spectra of all 14 compounds were

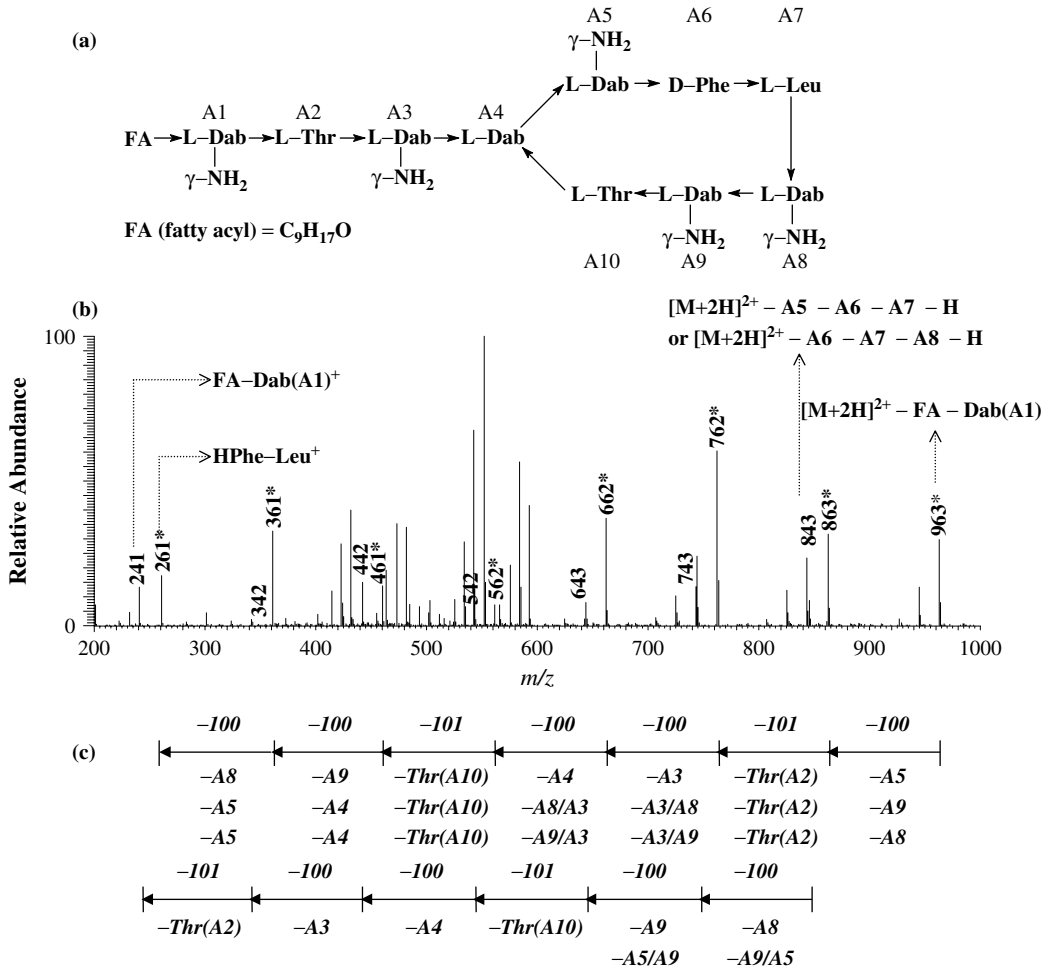


Figure 3 a) Structure of polymyxin B_1. The amino acids are indicated with a number A*n* starting from the fatty acyl side. FA, fatty acyl; Dab, α, γ-diaminobutyric acid. (b) Product ion spectrum acquired for polymyxin B_1, the result of isolation and collisional activation with 30% CE in the ion trap of the precursor ion with m/z 602.4. m/z values of the diagnostic product ions of the first series and the second series are noted with and without asterisk, respectively. The structures of four ions offering information about the fatty acyl moiety or the ring amino acids A6 and A7 are shown. (c) A review of the proposed fragmentation routes. Subsequent mass losses with the corresponding amino acid possibilities are noted in italic.

acquired with 30% collision energy using the doubly-charged precursor. Based on the retention time and the fragmentation pattern, peaks 3, 6, 9, 10, 13 and 14 were assigned as polymyxin B_4, polymyxin B_6, polymyxin B_2, polymyxin B_3, polymyxin B_1 and polymyxin B_5, respectively. It is striking that several peaks in the base peak chromatogram have the same m/z value. The extracted mass range chromatograms in Figure 4 clearly illustrate the plural presence of several m/z values. Figure 4b shows seven peaks with a doubly-charged ion with m/z 588.4 of which peak 3 corresponds with polymyxin B_4. Only peaks 2, 3 and 7 could be investigated since the other peaks coelute with compounds present in a huge amount in the sample. The small peak with m/z 588.4 eluted in front of peak 2 was too low in abundance for MS/MS investigation. Since the compound in peak 2 has the same m/z value as this peak, it was impossible to filter the product ions of the small peak from the product ions of the compound in peak 2. Six peaks are noticed in Figure 4c with a doubly-charged ion with m/z 595.4, of which peak 9 and peak 10 are polymyxin B_2 and B_3, respectively. Peak 7, which was already mentioned in the extracted mass range chromatogram of m/z 588.4 also seems to contain a compound with m/z 595.4. Peaks 5, 7, 8 and 12 were further investigated. Three peaks have a doubly-charged ion with m/z 602.4 of which peaks 13 and 14 correspond to

Table 1 Product Ions Acquired for the Polymyxin Reference Substances

Polymyxin peak	*m/z* doubly-charged ion	Product ions MS/MS scan							
B_1	602.4	963[a]	863[a]	762[a]	662[a]	562[a]	461[a]	361[a]	261[a]
Peak 13			843	743	643	542	442	342	241
B_2	595.4	963[a]	863[a]	762[a]	662[a]	562[a]	461[a]	361[a]	261[a]
Peak 9			829	729	629	528	428	328	227
B_3	595.4	963[a]	863[a]	762[a]	662[a]	562[a]	461[a]	361[a]	261[a]
Peak 10		929	829	729	629	528	428	328	227
B_4	588.4	963[a]	863[a]	762[a]	662[a]	562[a]	461[a]	361[a]	261[a]
Peak 3		915	815	715	615	514	414	314	213
B_5	602.4	963[a]	863[a]	762[a]	662[a]	562[a]	461[a]	361[a]	261[a]
Peak 14			843	743	643	542	442	342	241
B_6	610.4	963[a]	863[a]	762[a]	662[a]	562[a]	461[a]	361[a]	261[a]
Peak 6			859	759	659	558	458	358	257

[a] First series of product ions.

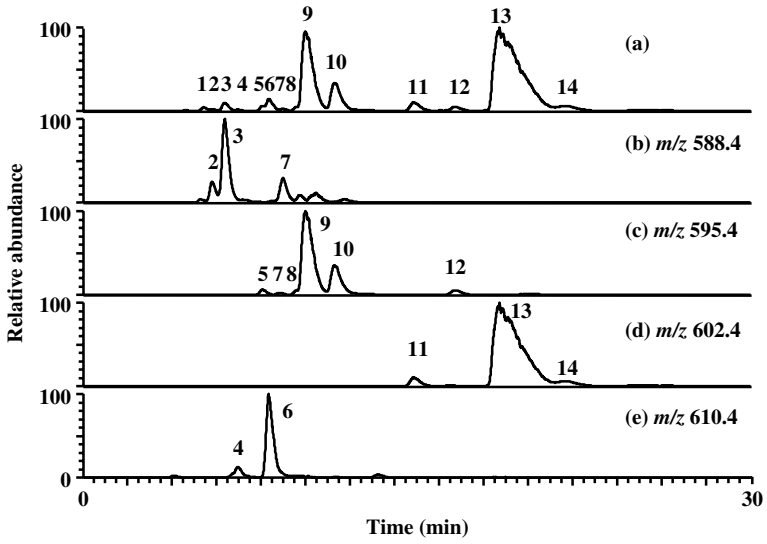


Figure 4 Typical chromatograms of a polymyxin bulk sample with concentration 0.25 μg/μl. Stationary phase, YMC-Pack Pro C18, 5 μm, 250 × 2.0 mm i.d. maintained at 30 °C; Mobile phase, 0.01 M trifluoroacetic acid–acetonitrile (77 : 23, v/v) at a flow rate of 200 μl/min. Top: (a) Base peak chromatogram; Bottom: Extracted mass range chromatograms for (b) *m/z* 588.4, (c) *m/z* 595.4, (d) *m/z* 602.4 and (e) *m/z* 610.4.

polymyxin B_1 and B_5, respectively. The extracted mass range chromatogram is shown in Figure 4d. Three peaks with *m/z* 610.4 are shown in the extracted mass range chromatogram in Figure 4e of which peak 6 corresponds with polymyxin B_6. The third peak was not investigated due to the low abundance. There remains peak 1 with *m/z* 603.4, of which no extracted mass range chromatogram is shown.

Investigation of Peaks with m/z 588.4

Three peaks (2,3 and 7) in the extracted mass range chromatogram in Figure 4b were investigated. The first and second series of product ions for peak 2 are shown in Figure 5. According to the fragmentation pattern described for the polymyxins, the first loss of mass 227 yielding the product ion with *m/z* 949 in the first series, corresponds with the fatty acyl

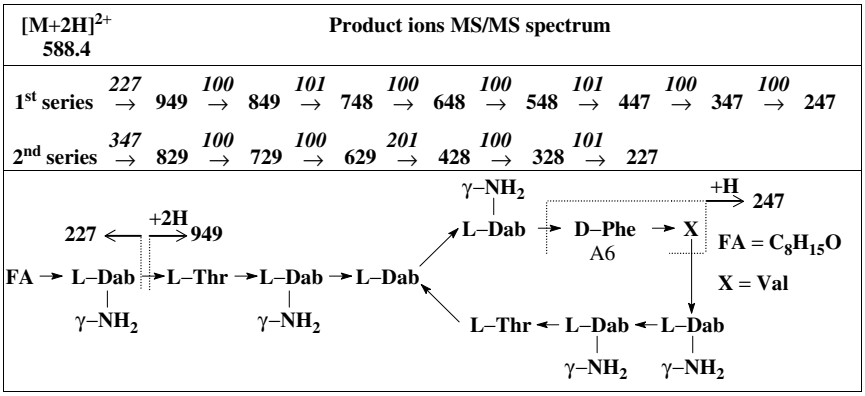


Figure 5 (a) First and second series of product ions acquired for peak 2, the result of isolation and collisional activation with 30% CE in the ion trap of $[M + 2H]^{2+}$ with m/z 588.4. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

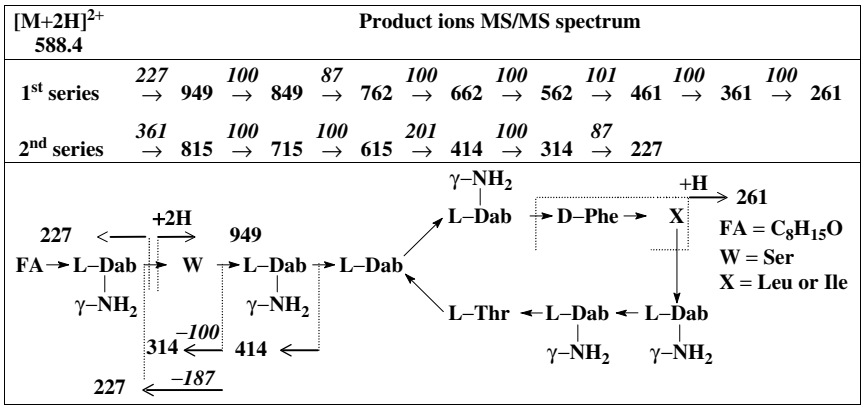


Figure 6 (a) First and second series of product ions acquired for peak 7, the result of isolation and collisional activation with 30% CE in the ion trap of $[M + 2H]^{2+}$ with m/z 588.4. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

moiety and the neighbouring Dab moiety. Based on experience with known polymyxins, we propose that the fatty acyl moiety has the same elemental composition as the fatty acyl moiety of polymyxin B_2 or B_3 (see Figure 1 for elemental compositions), but the branching of the fatty acyl, which cannot be defined, can differ. The ion with m/z 227 in the second series confirms this. The subsequent mass losses in both series are similar to the mass losses seen for polymyxin B_2 and B_3, except for the first loss in the second series (347 instead of 361 Da). For the polymyxins B_2 and B_3, this loss of 361 Da is due to the loss in the ring of Phe and Leu (see Figure 1) and a Dab moiety. The product ion 247 in the first series also confirms that there is a change in the ring amino acids. For all polymyxins in the library this product ion has mass 261, corresponding with HPhe–Leu$^+$. Based on the fragmentation pattern defined for the polymyxins and the feature that all polymyxins B in literature have a D–Phe at position A6 (Figure 5), it was deduced that the variable amino acid X in the circular part is Val instead of Leu. The presence of Val in the ring at the X position is substantiated by the description in the literature of a Val at the X position in the ring of related polymyxin E compounds [16].

The compound in peak 3 was assigned as polymyxin B_4 based on comparison with the retention time and fragmentation pattern of the polymyxin B_4 reference substance. For peak 3 the same series of product ions was recorded as shown in Table 1 for the polymyxin B_4 reference substance.

The first and second series of product ions identified in the MS/MS spectrum of peak 7 are shown in Figure 6. The first loss of 227 Da yielding the product ion with m/z 949 in the first series

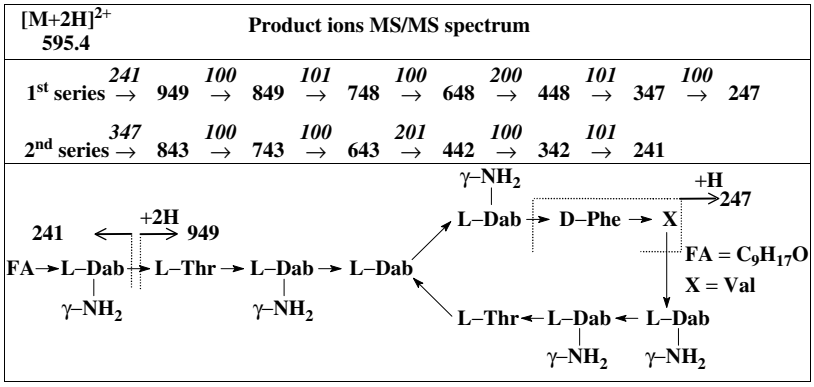


Figure 7 (a) First and second series of product ions acquired for peak 8, the result of isolation and collisional activation with 30% CE in the ion trap of $[M + 2H]^{2+}$ with m/z 595.4. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

and the ion with m/z 227 in the second series, indicate that the fatty acyl has the same elemental composition as the fatty acyl moiety ($C_8H_{15}O$) of polymyxin B_2 and B_3, but here also branching cannot be defined. The product ion with m/z 261 corresponding with the Phe–X combination in the ring indicates that the variable amino acid X has a mass of 113, which can correspond with Leu or Ile. Further MS^3 investigations of m/z 414 yielded the product ion with m/z 314 by loss of 100 Da, corresponding with a Dab moiety (Figure 6). The product ion with m/z 227 was formed out of m/z 414 by loss of 187 Da (Figure 6). Probably the loss of 187 Da corresponds with the loss of a Dab moiety (100 Da) and the loss of a serine moiety (87 Da). It was concluded that the sequence of this unknown polymyxin is similar to polymyxin B_2 or B_3, except for the Thr in the linear part, which is replaced by Ser. The amino acid at the X position can be Leu or Ile. The MS technique used did not allow differentiation.

Investigation of Peaks with m/z 595.4

The extracted mass range chromatogram in Figure 4c for m/z 595.4 shows six peaks. The three compounds in peaks 5, 7 and 10 have a similar MS/MS spectrum as polymyxin B_2 (peak 9). All those peaks are probably isomers of polymyxin B_2, however, replacement of leucines by isoleucines is possible and branching of the fatty acyl moiety can differ. Despite the high identity solving capacity of the ion trap, it is not possible to define the branching of a carbon chain or to distinguish between Leu and Ile. The compound eluted in peak 10 was assigned as polymyxin B_3 based on comparison with the retention time of polymyxin B_3, reference substance.

The first and second series of product ions of peak 8 are listed in Figure 7. The first loss of 241 yielding the product ion with m/z 949 and the product ion with m/z 241 shows that the fatty acyl part has the elemental composition of the fatty acyl part of polymyxin B_1 ($C_9H_{17}O$), though the carbon chain can be differently branched. All losses in the first series are the same as for polymyxin B_1. The product ion with m/z 247 points out that the amino acid combination Phe–X in the ring is different from polymyxin B_1. Based on the elucidated structure of the compound in peak 2, it is accepted that in this case too, Leu is replaced by Val.

The first and second series of product ions for peak 12 are shown in Figure 8. For peak 12 the doubly-charged ion fragments to the daughter ion m/z 949 by means of a loss of 241. This loss and the ion with m/z 241 in the second series indicate that the elemental composition of the fatty acyl is similar to that of polymyxin B_1 ($C_9H_{17}O$), however, branching of the fatty acyl moiety can not be defined. The product ion with m/z 261 indicates that the variable amino acid X (Figure 8) of the ring is Leu or Ile. MS^3 investigation of the low abundant m/z 328 showed that it fragments to 241 by loss of the Ser moiety (87 Da), which means that Ser replaces the Thr moiety in the linear side-chain.

Peaks with m/z 602.4

The extracted mass range chromatogram in Figure 4d shows three peaks with m/z 602.4. Peaks 13 and 14 are assigned as polymyxin B_1 and polymyxin B_5, respectively. Mass spectra are similar;

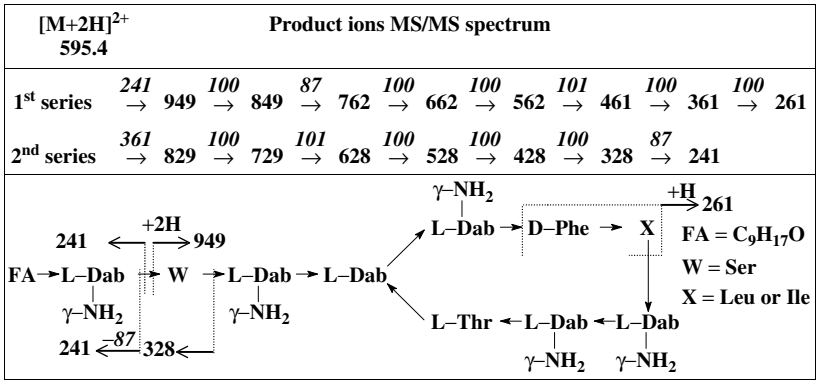


Figure 8 (a) First and second series of product ions acquired for peak 12, the result of isolation and collisional activation with 30% CE in the ion trap of $[M + 2H]^{2+}$ with m/z 595.4. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

no different product ions are formed. The list with product ions of the first and the second series is shown in Table 1. Based on the amount present in the sample, which was analysed with a non-volatile method by Orwa *et al.*, peak 11 is assigned as Ile–B_1 (see Figure 1).

Peak with m/z 603.4

The first and second series of product ions identified in the product ion scan of peak 1 are shown in Figure 9. The first loss of 243 Da yielding the ion with m/z 963 and the product ion with m/z 243 in the second series indicate that the fatty acyl moiety is probably a branched or linear octanoic acid, which is hydroxylated. Fragmentation of m/z 243 yields m/z 143 (fatty acyl moiety) and 101 (Dab moiety), but no product ions are formed offering information about the hydroxylation position. The compound has the same structure as polymyxin B_2, except for the fatty acyl moiety, which is probably replaced by a branched or linear monohydroxylated octanoic acid and has the elemental composition $C_8H_{15}O_2$. For the amino acid at the X position, it needs to be considered that both Leu or Ile are possible.

Peaks with m/z 610.4

The small peaks eluted besides peak 4 and 6 with m/z 610.4 in the extracted mass range chromatogram (Figure 4e) were not investigated due to the low abundancy. The diagnostic series of product ions of peak 4 are shown in Figure 10. The first loss (241 Da) in the first series yielding 979 and the product ion with m/z 241 yielded in the second series (see Figure 10 for corresponding structures), indicate that the fatty acyl moiety has the elemental composition of the fatty acyl

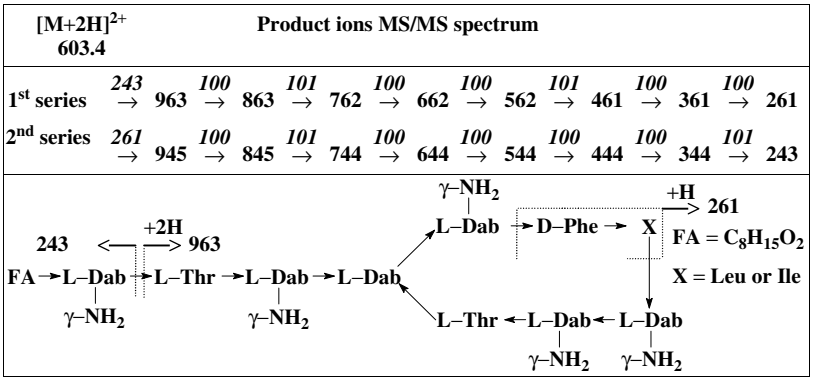


Figure 9 (a) First and second series of product ions acquired for peak 1, the result of isolation and collisional activation with 30% CE in the ion trap of $[M + 2H]^{2+}$ with m/z 603.4. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

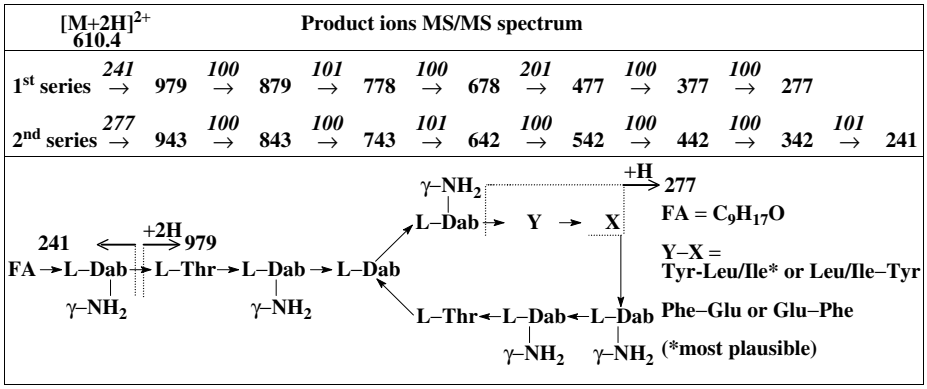


Figure 10 (a) First and second series of product ions acquired for peak 4, the result of isolation and collisional activation with 30% CE in the ion trap of $[M + 2H]^{2+}$ with m/z 610.4. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

moiety of polymyxin B_1, but branching cannot be defined. M/z 277 indicates that at the Phe–X position in the ring other amino acids are present than previously seen for the polymyxins B in the literature. Possible combinations of two amino acids with mass 276 Da are Tyr–Leu or Phe–Glu. We propose the combination Tyr–Leu as the correct one, since hydroxylation of the Phe seems plausible. But it needs to be considered that both Leu or Ile are possible.

The fragmentation spectrum of peak 6 matches the MS/MS spectrum of polymyxin B_6 in the library. Polymyxin B_6 has a similar sequence to polymyxin B_1, except that the fatty acyl moiety is 3-hydroxy-6-methyloctanoic acid. The product ions of polymyxin B_6 are listed in Table 1.

All the described compounds were present in an amount above 0.1%, as determined with UV normalization. Since most of the compounds were partly coeluted, the amount present is expressed by approximation. The amount of the compound with m/z 603.4 eluted in peak 1 was in the range of 0.8% to 0.9%. It should be noted that a small peak with m/z 588.4, which was not investigated, is coeluted with this compound. The compound in peak 2 with m/z 588.4 was present in an amount that ranged from 0.5% to 0.6%. For the other peaks, the following amounts defined with UV normalization were recorded: peak 4, 0.3%; peak 5, 0.9%; peak 7 (comprises 2 compounds), 0.9%; peak 8, 1%; peak 12, 1.2%. It has to be mentioned that peaks 5 and 7 were partly coeluted with peak 6 and peak 8 is partly coeluted with polymyxin B_2. Enlargement of the region 0–10 millivolts on the Y-axis of the UV chromatogram showed more peaks present in an amount above 0.1%. Those peaks are not visible on the UV chromatogram in Figure 2. However, in the base peak chromatogram some of those peaks were not abundant enough for MS/MS and MS^3 investigation. Four peaks eluted after polymyxin B_5 and present in an amount of 0.1% to 0.5%, were isomers of polymyxin B_1. Different branching of the fatty acyl moieties and replacement of Leu by Ile are possible.

CONCLUSIONS

The benefits and the drawbacks of the on-line MS determination of the sequence of complex cyclic polypeptides were exemplified by analysis of polymyxin B sulphate. The low content of the target components in the polymyxin sample mixture did not allow isolation and purification of the still unidentified minor components, which would have made unambiguous structure determination by amino acid analysis or NMR possible. Therefore a hyphenated LC/MS^n technique was developed providing on-line characterization of those minor components. Electrospray ionization in conjunction with an ion trap resulted in the production of distinct diagnostic ions of the ring peptide moieties, which made partial sequence determination of six novel related substances possible. Other peaks in the complex were assigned as isomers of the main polymyxins B_1 and B_2. The low resolution MS technique used, only provided suggestions about the sequence of the peptides. Though, the limited number of structural possibilities for the polymyxins made it possible to characterize six unidentified peaks, which were recently separated from the main

polymyxins. A drawback of the ESI-MS technique applied is that one cannot distinguish between the isomeric amino acids such as Leu and Ile, both of which occur in polymyxins. Additionally, further determination of the fatty acyl moiety still needs to be performed by NMR, since the technique only allowed the determination of the mass of the fatty acyl part, but not the branching of the carbon chain. Moreover, we cannot be sure that the optical configuration of all the amino acid residues in the new compounds is similar to the optical configurations reported for polymyxin B_1 or B_2. Chiral amino acid analysis could help to solve this problem, but then again, laborious preparative isolation and purification would be necessary.

Acknowledgement

This work was supported by the Flemish Fund for Scientific Research (Research Project G.0355.98).

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