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Liquid chromatography–ion trap tandem mass spectrometry for the characterization of polypeptide antibiotics of the colistin series in commercial samples

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Abstract

A selective reversed-phase liquid chromatography–tandem mass spectrometry method is described for the characterization of related substances in the colistin complex. Mass spectral data were acquired on an LCQ ion trap mass spectrometer equipped with an electrospray ionization probe operated in the positive ion mode. The main advantage of this technique is the characterization of novel related substances without time-consuming isolation and purification procedures. Using this method seven new related substances were partially identified in colistin bulk sample and tablets. Four components were assigned as isomers of the main components of colistin.

Keywords: Pharmaceutical analysis; Polypeptides; Antibiotics; Colistins; Polymyxins

1. Introduction

Colistin or polymyxin E is a complex of chemically related acylated linear side-chain cyclized peptides, belonging to the polypeptide antibiotics, which all have relatively complex structures. The large variety of components other than amino acids, unusual peptide linkages and for most of these antibiotics also their cyclic nature, makes their structural elucidation in many instances more complicated [1]. Following the establishment of the structure of polymyxin E in 1965 [2–4], many attempts have been made over the years to identify all components in the commercial colistin sulphate, which is marketed for oral administration in the treatment of intestinal infections and used topically in therapy of external ear infections [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 Fig. 1 [3,4,6–8]. Recently polymyxin E_7 and isoleucine–polymyxin E_8 were isolated and characterized by ^{1}H and ^{13}C NMR [9]. As shown in Fig. 1 colistins 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 and are characterized by the presence of 6 α,γ-diaminobutyric acid (Dab) residues and a hydrophobic D-Leu at position A6 (Fig. 1).

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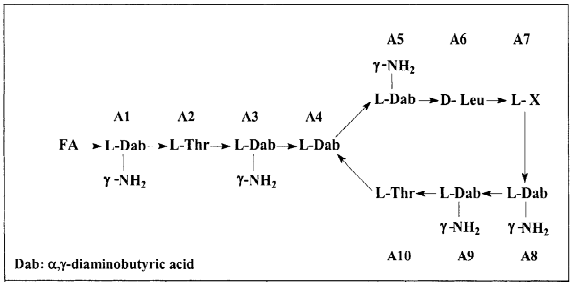


Polymyxin (Elemental formula)	Calculated monoisotopic mass	Fatty acyl (FA)	Elemental formula FA	X
E_1 ($C_{53}H_{100}N_{16}O_{13}$)	1168.7655	6-methyloctanoyl	$C_9H_{17}O$	Leu
E_2 ($C_{52}H_{98}N_{16}O_{13}$)	1154.7498	6-methylheptanoyl	$C_8H_{15}O$	Leu
F_3 ($C_{52}H_{98}N_{16}O_{13}$)	1154.7498	octanoyl	$C_8H_{15}O$	Leu
E_4 ($C_{51}H_{96}N_{16}O_{13}$)	1140.7342	heptanoyl	$C_7H_{13}O$	Leu
E_7 ($C_{53}H_{100}N_{16}O_{13}$)	1168.7655	7-methyloctanoyl	$C_9H_{17}O$	Leu
Ile-E_1 ($C_{53}H_{100}N_{16}O_{13}$)	1168.7655	6-methyloctanoyl	$C_9H_{17}O$	Ile
Ile-E_2 ($C_{52}H_{98}N_{16}O_{13}$)	1154.7498	6-methylheptanoyl	$C_8H_{15}O$	Ile
Ile-E_8 ($C_{54}H_{102}N_{16}O_{13}$)	1182.7811	7-methylnonanoyl	$C_{10}H_{19}O$	Ile
Nva-E_1 ($C_{51}H_{96}N_{16}O_{13}$)	1140.7342	6-methyloctanoyl	$C_9H_{17}O$	Norval
Val-E_1 ($C_{52}H_{98}N_{16}O_{13}$)	1154.7498	6-methyloctanoyl	$C_9H_{17}O$	Val
Val-E_2 ($C_{51}H_{96}N_{16}O_{13}$)	1140.7342	6-methylheptanoyl	$C_8H_{15}O$	Val

Fig. 1. Chemical structures of known polymyxins E with elemental formulae, calculated monoisotopic masses, fatty acyl moieties and elemental formulae of fatty acyl moieties. Amino acids are indicated with a number A*n* starting from the fatty acyl side.

The increase of serious infections with Gram-negative bacteria and the growing resistance to broad-spectrum antibiotics, have led to colistin taking again a major role in the therapy of multidrug-resistant Gram-negative bacterial infections [10]. With the development of a more performant LC method for colistin, using a non-volatile mobile phase, more peaks were separated [11], but despite considerable efforts of isolation and purification [9], few peaks were elucidated. Because colistin is again of growing importance and since 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 an LC–multiple mass spectrometry (MS^n) method for the characterization of unknown colistin components, preventing laborious preparative LC using large amounts of sample and solvents. Recently fast atom bombardment tandem mass spectrometry (FAB-MS–MS) was used in an attempt to determine the structures of some minor colistin components. FAB-MS–MS produced only a few prominent fragment ions and did not yield distinct diagnostic ions of the ring peptide moieties important for full identification of the structures [12,13]. Our group recently investigated the major polymyxins of the B (polymyxins B_1 and B_2) and E (polymyxin E_1 or colistin A and polymyxin E_2 or colistin B) series by direct infusion in Q/oaTOF and ion trap apparatus, resulting in a complete description of the fragmentation behaviour. Based on this work it was decided to use the LCQ ion trap mass spectrometer, providing on-line LC–MS^n capability [14]. The final objective of the study was to confirm, in the commercial samples, the presence of the known colistins depicted in Fig. 1 and to identify all unknown compounds, present in an amount above 0.1%. The fact that linear side-chain cyclized peptides are claimed to be one of the most difficult peptide classes to sequence by MS–MS was an additional challenge [15].

2. Experimental

2.1. Chemicals

Acetonitrile (HPLC grade S) was purchased from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid (TFA) 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.

2.2. Samples and sample preparation

The sample obtained from Asahi Kasei Shiraoi (Hokkaido, Japan) was dissolved at a concentration of 0.25 μg/μl in water. Colimycin tablets were obtained from Rhône-Poulenc Rorer (Neuilly-Sur Seine, France). The tablets were pulverized and extracted with water by sonication. After centrifugation the upper layer was filtered through a 0.2 μm filter. A solution of ~0.15 μg/μl in water was used for investigation. Polymyxin E_1, E_2, Ile-E_1 and E_7 reference substances were available in the laboratory and were all characterized by NMR [9].

2.3. 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 processed with ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK). The YMC-Pack Pro C_{18} column (5 μm, 250× 2.0 mm I.D.) (Waters, Milford, MA, USA) was immersed in a waterbath at 30 °C. The following mobile phase was used for separation: 0.01 *M* aqueous TFA–acetonitrile (79:21, v/v%). The mobile phase was degassed by sparging helium. The LC pump was operated at a flow-rate of 200 μl/min.

2.4. Electrospray ionization tandem mass spectrometry

The MS data shown in this paper were acquired on an LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray interface operated in the positive ion mode. The abundant doubly-charged ion with *m/z* 585.3, yielded by direct infusion of a 0.05 μg/μl solution of polymyxin E_1, was used to tune automatically the ionization source and MS parameters. Infusion into the mass spectrometer was performed as follows: the flow of sample solution coming from the built-in syringe pump at a flow-rate of 10 μl/min was mixed with mobile phase (200 μl/min) through a T-piece. A voltage of 5 kV applied to the ESI (electrospray ionization) 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 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 and collision gas at a pressure of 0.133 Pa. 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 software package was used for instrument control, data acquisition and processing.

2.5. Mass spectrometric investigation of the reference substances

Solutions of the reference substances (0.05 μg/μl) were directly infused similar to the tuning procedure. Full mass spectra were acquired over the mass range *m/z* 500–700. For MS–MS investigation the doubly-charged ions were isolated monoisotopically in the ion trap and collisionally activated with different collision energies (CE) to find the optimal CE for a distinct fragmentation. A CE of 30% generated a distinct product ion scan and was chosen as the final value. The CE of the LCQ is set using a scale of 0–100%. This adjusted value corresponds with 30% of the available 5 V peak-to-peak of resonance excitation radio frequency voltage. MS^3 spectra were acquired to confirm subsequent mass losses and if necessary for the structure investigation of unknowns.

2.6. Mass spectrometric investigation of the bulk and tablet samples

Solutions of the commercial samples (bulk sample 0.25 μg/μl, tablet sample 0.15 μg/μl) were injected onto the column and analyzed on-line. In a first run, full MS acquisition over the mass range 500–700 was performed to gain some idea of the commercial sample composition. The doubly-charged polymyxin ions were isolated monoisotopically in the ion trap and collisionally activated with 30% CE. LC–MS^3 spectral data were obtained in the next run, if necessary, for identification of the unknowns in the sample. CE was optimized as described above for MS–MS.

3. Results and discussion

3.1. Development of the liquid chromatographic method

Important features in combining LC and MS are the incompatibility with MS of non-volatile mobile phase additives and the incompatibility of high flow-rate with the electrospray interface. Since the polymyxin E complex is a polypeptide mixture, LC conditions for separation of peptide mixtures were applied and optimized [16]. Using a 250×2.0 mm I.D. YMC-Pack Pro C_{18} column, which allows a flow-rate of 200 μl/min, the chromatographic procedure with acetonitrile and aqueous TFA, described

in Section 2, was developed. The YMC-Pack Pro C_{18} column was used, because it was described by Orwa et al. as a column with good selectivity for colistin [11]. UV detection was performed at 215 nm on-line with the MS detection.

3.2. Mass spectrometric results of the reference substances

MS–MS spectra were recorded for the polymyxin E reference substances. In previous work [14] the main polymyxins of the B and E series were investigated by direct infusion (3 μl/min) in Q/oaTOF and ion trap apparatus, resulting in a complete description of the fragmentation behaviour. Other solvent conditions (water–TFA–acetonitrile instead of isopropanol–water) and higher LC flow (200 μl/min) compared to that study [14] resulted in different ESI source and MS parameters. Nitrogen supply had to be adapted in this case and the parameters were tuned by an automated procedure to maximize the signal for the ion of interest. Those adaptations did not affect the fragmentation behaviour, which is going to be the guideline in further structure investigations in this article. As an example Fig. 2 shows the structure of polymyxin E_1 and the corresponding MS–MS spectrum recorded under the solvent and high flow conditions described in Section 2 for the reference substances. The MS–MS spectrum shows singly- and doubly-charged product ions formed out of the doubly-charged precursor 585.3. Structural information is derived from the singly-charged product ions coming from the doubly-charged precursors, which may, therefore, be observed at higher m/z values than the precursor. Two important series of product ions are present in the spectra. The first series of product ions (noted with an asterisk in the spectrum) 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 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 Fig. 2) 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. Polymyxin E_2 (m/z 578.3) has an identical first series of product ions as polymyxin E_1. The m/z values of the product ions in the second series are 14 mass units lower than the m/z values of the corresponding second series ions observed for polymyxin E_1. Product ions for polymyxins E_7 (m/z 585.3) and Ile-E_1 (m/z 585.3) are similar to those for polymyxin E_1 and are therefore not shown. It was observed that the MS–MS spectra of Ile-E_1 and E_1 do not differ, because with the ion trap it is not possible to distinguish Leu from Ile, both having a residue mass of 113. The fragmentation spectrum of polymyxin E_7 is also similar to E_1 since the difference in branching of the fatty acyl moiety cannot be revealed by the ion trap. Indeed, it is only offering information about the elemental composition of the fatty acyl part. No charge-remote fragmentations [17] offering more information about the fatty acyl moiety were observed. Also 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.

3.3. Mass spectrometric results of the bulk and tablet samples

Full mass spectral acquisition over the mass range m/z 500–700 was performed. The base peak chromatogram obtained for the bulk and tablet sample is shown in Fig. 3 (a, bulk; b, tablet). Full MS spectra of 21 peaks, which are indicated on the base peak chromatogram in Fig. 3a were investigated. For the tablet sample (Fig. 3b), some peaks seen in the bulk were lacking and two additional peaks, not noticed in the bulk, are indicated with a character. Fragmentation spectra of all compounds present in an amount above 0.1% in the UV spectrum (not shown) were acquired with 30% CE using the doubly-charged precursor. The peaks are mentioned in the legend of Fig. 3 with their corresponding doubly-charged precursor ion. All peaks were characterized, except peaks 13 and 15 due to low abundance of the product ions. Increasing the CE did not increase the abun-

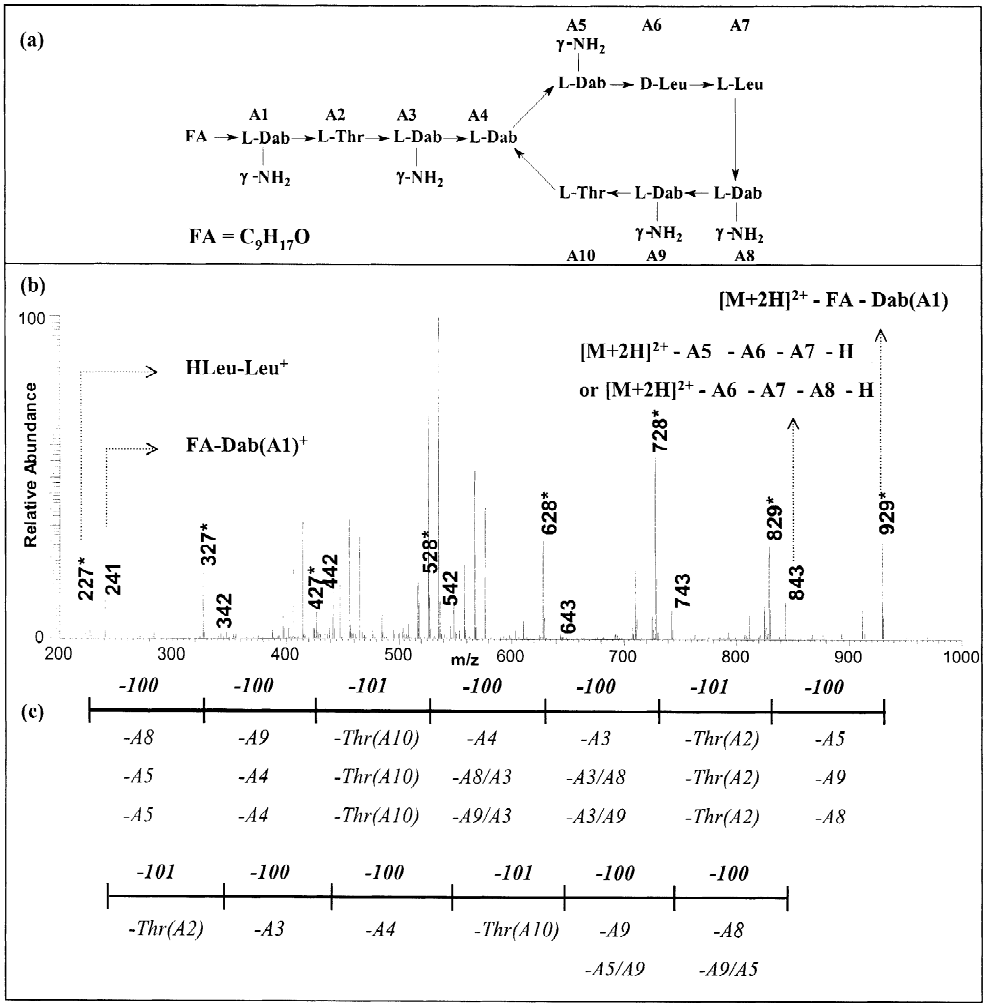


Fig. 2. (a) Structure of polymyxin E_1.The amino acids are indicated with a number A*n* starting from the fatty acyl side. FA, fatty acyl; Dab, α,γ-diaminobutyric acid. (b) Product ion spectrum acquired for polymyxin E_1, the result of isolation and collisional activation with 30% CE in the ion trap of the precursor ion with *m*/*z* 585.3. *M*/*z* values of the diagnostic product ions of the first series and the second series are noted with and without asterisks, 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 italics.

dance of the product ions for further investigation. It was also remarkable to notice that when a new compound was identified, a second compound with a similar sequence was also found in the same sample. Each time the fatty acyl moiety of this compound differed 14 mass units, corresponding with the mass of a methylene unit. For this reason these compounds will be discussed together.

3.3.1. Investigation of peaks 1 (m/z 586.3) and 7 (m/z 593.3)

The MS–MS spectrum of peak 1 is shown in Fig. 4. The first and second series of product ions are listed below the spectrum. Out of the product ions and the losses in the series it was concluded that the sequence of polymyxin E_1 or E_2 is retained, but the fatty acyl moiety differs. According to the frag-

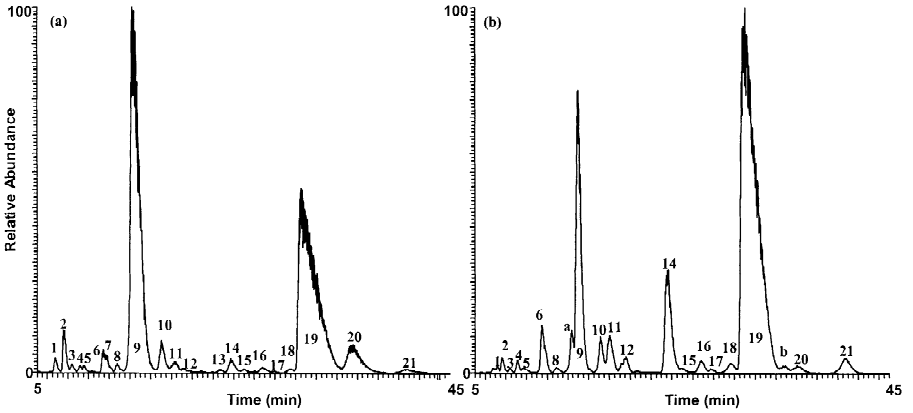


Fig. 3. Base peak chromatograms of polymyxin E samples: (a) bulk sample 0.25 μg/μl; (b) tablet sample 0.15 μg/μl. Conditions: YMC-Pack Pro C_{18}, 5 μm, 250×2.0 mm I.D. maintained at 30 °C. Mobile phase of 0.01 *M* TFA–CH_3CN (79:21, v/v) at a flow-rate of 200 μl/min. 1, *m*/*z* 586.3; 2, *m*/*z* 571.3; 3, *m*/*z* 571.3; 4, *m*/*z* 571.3; 5, *m*/*z* 587.3; 6, *m*/*z* 578.3; 7, *m*/*z* 593.3; 8, *m*/*z* 571.3; 9, *m*/*z* 578.3, polymyxin E_2; 10, *m*/*z* 578.3; 11, *m*/*z* 578.3; 12, *m*/*z* 594.3; 13, *m*/*z* 569.3; 14, *m*/*z* 585.3, isoleucine–polymyxin E_1; 15, *m*/*z* 576.3; 16, *m*/*z* 578.3; 17, *m*/*z* 585.3; 18, *m*/*z* 584.3; 19, *m*/*z* 585.3, polymyxin E_1; 20, *m*/*z* 585.3, polymyxin E_7; 21, *m*/*z* 585.3; a, *m*/*z* 578.3; b, *m*/*z* 578.3. The peaks without a compound name are discussed in this work.

mentation pattern described for polymyxin E_1 in Fig. 2, the first loss of mass 243 yielding the product ion with *m*/*z* 929 in the first series, corresponds with the fatty acyl moiety (143 u) and the neighbouring Dab moiety (100 u). Based on experience with known polymyxins, we propose that the fatty acyl moiety is a branched or linear octanoic acid, which is hydroxylated ($C_8H_{15}O_2$). The product ion with *m*/*z* 243, corresponding with FA-Dab$^+$ (FA, fatty acyl), in the second series confirms this. For the component eluted in peak 7 the first series of product ions is identical to the first series observed for peak 1. The *m*/*z* values of the product ions of the second series are 14 mass units higher than the corresponding *m*/*z* values of the second series of peak 1. According to the fragmentation pattern this difference is due to the fatty acyl moiety, which is probably a branched or linear hydroxylated nonanoyl with elemental composition $C_9H_{17}O_2$ (157 u).

For both unknowns the branching and the hydroxylation place of the fatty acyl moiety could not be defined with MS^3 fragmentations. Probably the fatty acyl moieties of peak 1 and peak 7 correspond with the fatty acyl moieties of polymyxin E_2 ($C_8H_{15}O$) and E_1 ($C_9H_{17}O$), respectively, which are additionally hydroxylated, but it needs to be considered that the fatty acyl moieties of these new compounds can be differently branched, since components of the polymyxin E complex are described with the sequence of E_1 or E_2 and with fatty acyl moieties with similar elemental composition, but differently branched (see Fig. 1) [9]. The presence of a hydroxylated fatty acyl moiety is substantiated by the description in the literature of polymyxin B_6 in the polymyxin B complex. NMR experiments proposed a 3-hydroxy-6-methyloctanoic acid structure for the fatty acyl of polymyxin B_6 [18]. Assuming that all polymyxins E have D-Leu at position A6, it is concluded that the two compounds under investigation here have the Leu–Leu or Leu–Ile sequence (A6–A7 positions). Both possibilities need to be considered, since related compounds having an Ile instead of Leu at the A7 or X position (Fig. 4) were observed by others (Fig. 1) [12], but as was already mentioned, it is not possible to distinguish Leu from Ile with the ion trap. The proposed structures are shown in Fig. 9.

3.3.2. Investigation of peaks 2 (m/z 571.3), 4 (m/z 571.3), a (m/z 578.3), 11 (m/z 578.3)

The MS–MS spectrum for peak 2 and the product ions important for the characterization are shown in Fig. 5. For peak 4 two identical series of product ions were observed. According to the fragmentation pattern described for the polymyxins, the first loss of mass 227 yielding the product ion with *m*/*z* 915 in

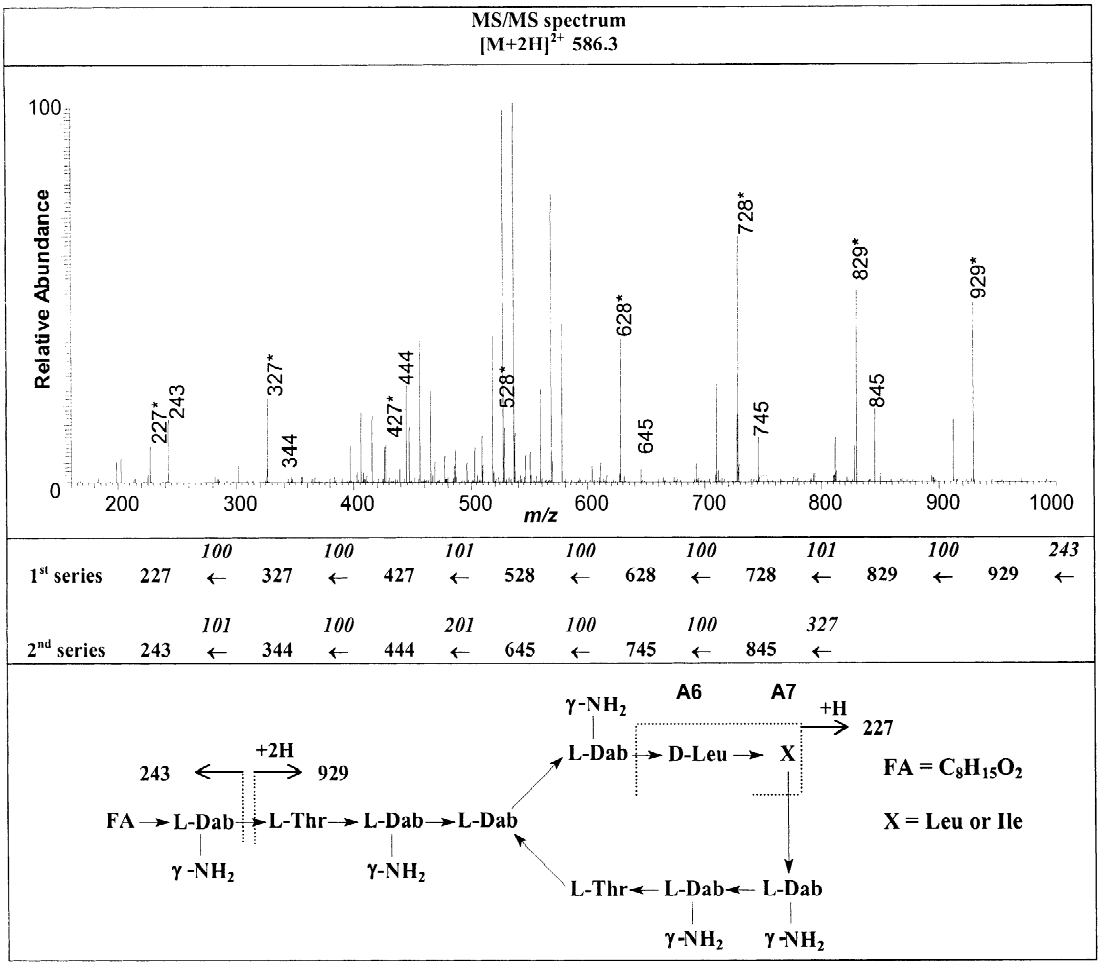


Fig. 4. (a) MS–MS spectrum with first and second series of product ions acquired for peak 1, the result of isolation and collisional activation with 30% CE in the ion trap of $[M+2H]^{2+}$ with m/z 586.3. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

the first series corresponds with the fatty acyl moiety and the neighbouring Dab moiety. Based on the experiments with known polymyxins collected in the library, we propose that the fatty acyl moiety has the elemental composition of the fatty acyl moiety of polymyxin E_2 (see Fig. 1 for elemental compositions of described fatty acyl moieties). The ion with m/z 227 in the second series confirms this. Though the branching of the fatty acyl can differ. The subsequent mass losses in both series are similar to the mass losses in the MS–MS spectrum of polymyxin E_2 except for the first loss in the second series (213 instead of 227 u, observed for polymyxin E_2). The product ion 213 ($HA6–A7^+$ in Fig. 5) in the first series also confirms that there is a change in those amino acids. For polymyxin E_1 and E_2 this product ion has mass 227 and corresponds with $HLeu–Leu^+$ (residue mass Leu: 113). Probably, one of the Leu is replaced by a Val with residue mass 99. One of the two peaks (2 or 4, which are probably isomers) corresponds with the valine–polymyxin E_2 (Val-E_2), first isolated and characterized by Elverdam et al., but which of both cannot be defined [8]. The structure of Val-E_2 was already shown in Fig. 1. For the component eluted in peak 11 the first series of product ions is similar to the first series of product

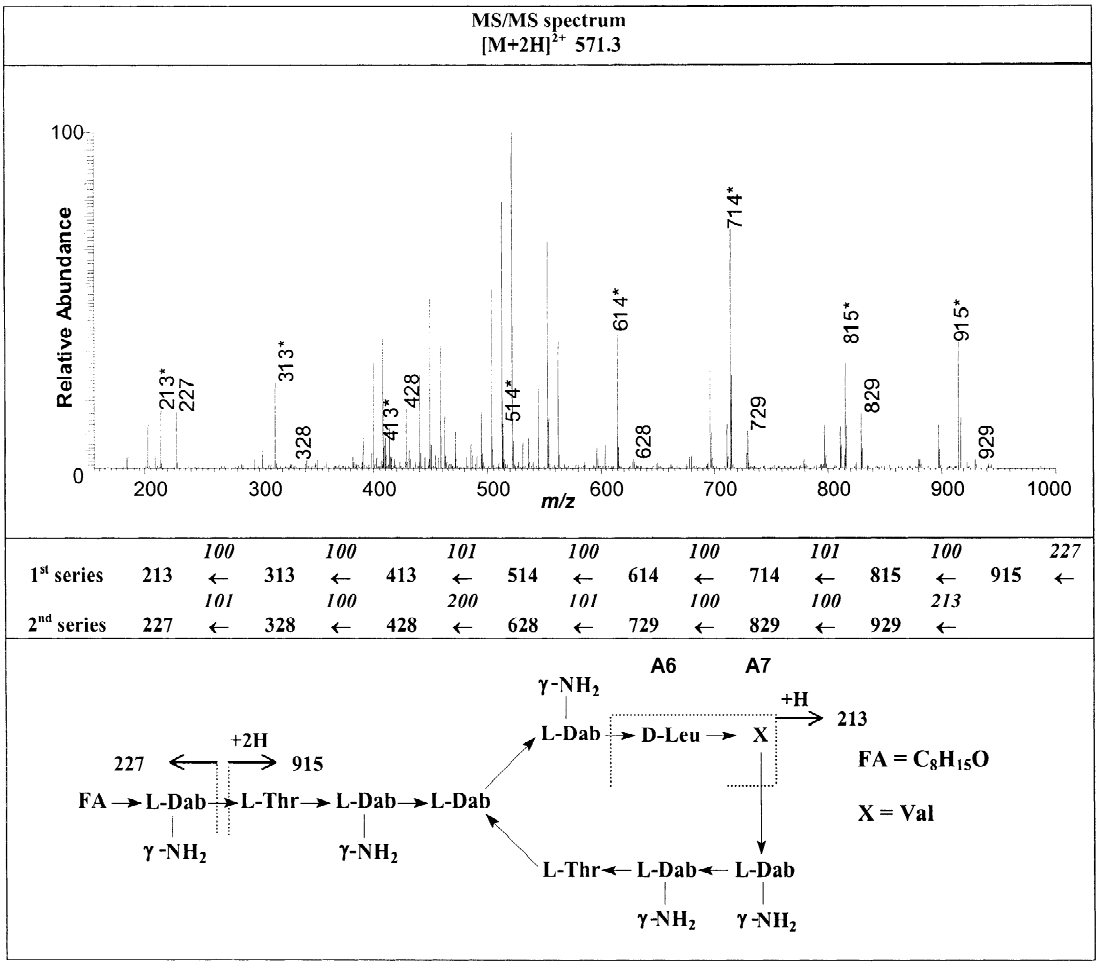


Fig. 5. (a) MS–MS spectrum with first and second series of product ions acquired for peak 2, the result of isolation and collisional activation with 30% CE in the ion trap of $[M+2H]^{2+}$ with m/z 571.3. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

ions for peaks 2 and 4. The m/z values of the product ions of the second series are 14 u higher in mass than the corresponding m/z values of the second series of peaks 2 and 4. The fatty acyl moiety is probably a branched or linear nonanoic acid ($C_9H_{17}O$) and it can even be assumed that the fatty acyl moiety of this new compound corresponds with the fatty acyl moiety of polymyxin E_1 (Fig. 1). Here also, branching of the fatty acyl moiety cannot be defined. The base peak chromatogram of the tablet sample shows peak ''a'' eluted just in front of polymyxin E_2, with the same m/z and product ions as peak 11. As was seen for Val-E_2, probably two isomers are also present for valine–polymyxin E_1 (Val-E_1) and one of those two isomers can correspond with the Val-E_1 described by Ikai et al. (Fig. 1) [12], but which of both cannot be defined. The structure of Val-E_1 was already shown in Fig. 1. All compounds are mentioned in Fig. 9.

3.3.3. Investigation of peak 3 (m/z 571.3)

In the MS–MS spectrum of peak 3 (not shown), we recognize starting from the product ion with m/z 929, the first series of product ions with m/z 829, 728, 628, 528, 427, 327 and 227. Recognizing the second series of product ions was difficult, because

peak 2 with the same m/z and partly coeluted with peak 3, has some abundant product ions in the first series in common with the second series of peak 3. The following product ions belong to the second series: m/z 915, 815, 715, 615, 414, 314 and 213. Out of the product ions and the calculated losses in the series it was concluded that the sequence of this unknown polymyxin is similar to polymyxin E_1, but the fatty acyl moiety is different. The first loss of 213 u yielding the product ion with m/z 929 indicates that the fatty acyl has the elemental composition of a heptanoyl moiety ($C_7H_{13}O$) as seen for polymyxin E_4 [7], but a different branching is possible. Since polymyxin E_4 reference substance has never been isolated, confirmation by retention time was not possible.

3.3.4. Investigation of peaks 5 (m/z 587.3) and 12 (m/z 594.3)

The MS–MS spectrum and the first and second series of product ions for peak 5 are shown in Fig. 6. For peak 5 the doubly-charged ion with m/z 587.3 fragments to the product ion m/z 947 by means of a loss of 227. This loss shows that the fatty acyl part has the elemental composition of the fatty acyl of polymyxin E_2 ($C_8H_{15}O$), though an isomeric fatty acyl is possible. The second series is identical to the second series of polymyxin E_2. The product ion with

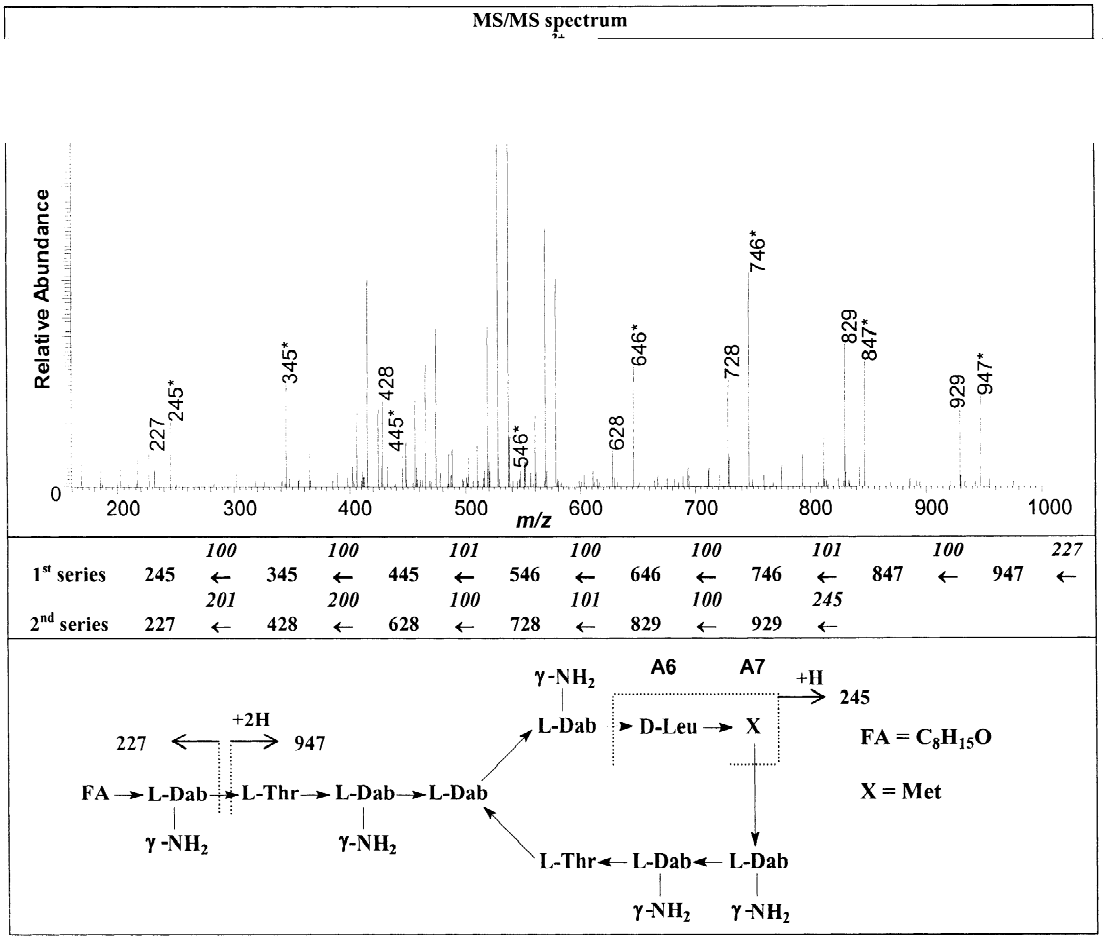


Fig. 6. (a) MS–MS spectrum with first and second series of product ions acquired for peak 5, the result of isolation and collisional activation with 30% CE in the ion trap of $[M+2H]^{2+}$ with m/z 587.3. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

m/*z* 245 in the first series points out that the amino acid combination A6–A7 is different from polymyxin E_2. Several combinations of amino acids are possible. Since all colistins identified before contain a D-Leu in the ring at position A6, which means that D-Leu at position A6 can be considered as a characteristic of the colistin series, it is assumed for this compound that probably a Met moiety (131 u) replaces the Leu in the ring at position A7 or X (Fig. 6). For peak 12 the first series of product ions was identical to the first series for peak 5, resulting in the tentative conclusion that a Met also replaces the Leu in the ring at position A7 or X. All product ions of the second series are each time 14 mass units higher in mass than the corresponding product ions of the second series of peak 5. This indicates that the fatty acyl moiety of peak 5 is 14 mass units less than the fatty acyl moiety of peak 12. Probably the fatty acyl moiety of peak 12 corresponds with the fatty acyl moiety of polymyxin E_1, while the fatty acyl moiety of peak 5 corresponds with the fatty acyl moiety of E_2. However, similar to the other identified compounds, the branching, which cannot be defined with MS, of the fatty acyl moiety can differ from the original fatty acyl moieties of polymyxin E_1 or E_2. The final proposed structures are shown in Fig. 9.

3.3.5. Investigation of peaks 6, 9 and 10 (m/z 578.3)

The MS–MS spectrum of peak 9 matched the MS–MS spectrum of the isolated polymyxin E_2. Based on two observations it is assumed that the components eluted in peaks 6 and 10 are isomers of E_2. They have the same doubly-charged *m*/*z* value and identical product ion series, but different retention times. It is possible that they differ in branching of the fatty acyl moiety or that Leu at the X position (Fig. 1) in the structure is Ile, which could not be defined with the described technique. The isomers are mentioned in Fig. 9.

3.3.6. Investigation of peaks 8 (m/z 571.3), 16 (m/z 578.3), b (m/z 578.3)

The MS–MS spectrum and the first and second series of product ions identified in this MS–MS spectrum of peak 8 are shown in Fig. 7. The first loss of 227 u yielding the product ion with *m*/*z* 915 in the first series and the ion with *m*/*z* 227 in the second series indicate that the fatty acyl moiety has the elemental composition $C_8H_{15}O$. Here too, branching cannot be defined. Both series have some product ions in common, which makes the structure elucidation anyhow more complex. The product ion with *m*/*z* 227 in the second series corresponds with FA-Dab^+, but is also indicative in the first series for the two amino acids in the ring at the A6–A7 position. MS^3 investigations (Fig. 8) of *m*/*z* 414 yielded the product ion with *m*/*z* 314 by loss of 100 u, corresponding with a Dab moiety. The product ion with *m*/*z* 227 was also formed out of *m*/*z* 414 by loss of 187 u. The loss of 187 u corresponds with the loss of a Dab moiety (100 u) and the loss of a serine moiety (87 u). It was concluded that the sequence of this unknown polymyxin is similar to polymyxin E_2, except for the Thr in the linear part, which is replaced by Ser. The presence in the first series of the product ions 815 and 728 confirms this. A characteristic of all colistins previously identified is the amino acid D-Leu at the A6 position, while the amino acid at the A7 or X position (Fig. 7) in this case can be Leu or Ile. For the compound eluted in peak 16 (*m*/*z* 578.3) the first series of product ions was identical to the first series for peak 8, resulting in the conclusion that a Ser also replaces the Thr in the ring. All product ions of the second series are each time 14 mass units higher in mass than the corresponding product ions of the second series of peak 8. Isolation and collisional activation of *m*/*z* 428 results in two fragment ions 328 and 241. *M*/*z* 241 corresponds with FA-Dab^+. This indicates that the fatty acyl moiety of peak 8 is 14 mass units less than the fatty acyl moiety of peak 16. Probably the fatty acyl moiety of peak 16 corresponds with the fatty acyl moiety of polymyxin E_1, while the fatty acyl moiety of peak 8 corresponds with the fatty acyl moiety of E_2. However, similar to the other identified compounds, the branching of the fatty acyl moiety, which cannot be defined with MS, can differ from the original fatty acyl parts of polymyxin E_1 and E_2. This variant (peak 16) was already synthesized by Kline et al. [19], but there it was mentioned that the compound was not readily observed in native unenriched fractions. It is the first time that this compound was analyzed on-line in a higher amount in an unenriched bulk sample.

The compound eluted in peak b in the tablet

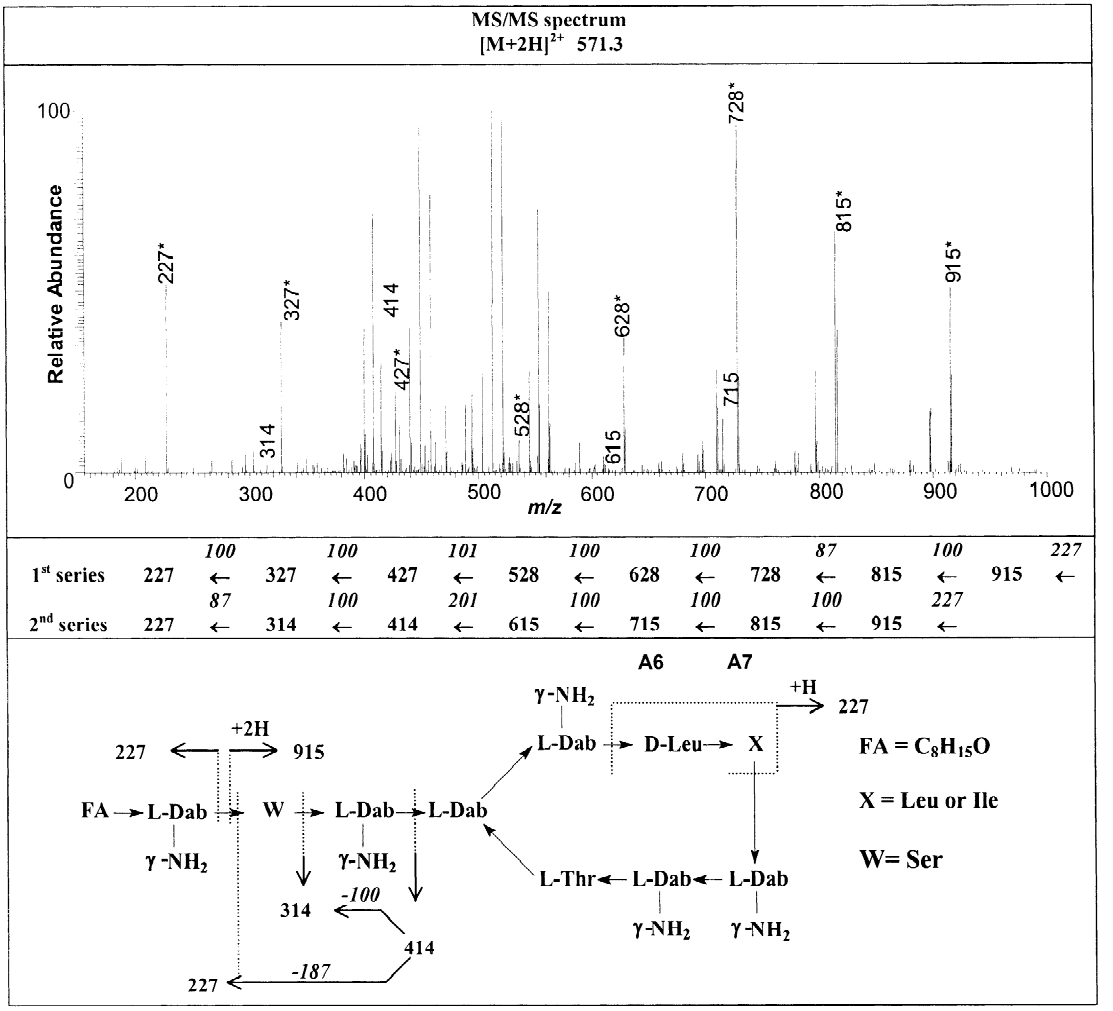


Fig. 7. (a) MS–MS spectrum with first and second series of product ions acquired for peak 8, the result of isolation and collisional activation with 30% CE in the ion trap of $[M+2H]^{2+}$ with m/z 571.3. The mass differences are indicated above the arrows. (b) The proposed structure with important ions leading to the characterization of the structure.

sample has the following first series of product ions: 915, 815, 714, 614, 514, 427, 327 and 227. The second series comprises the product ions 829, 729, 629, 442, 342 and 241 (not shown in a figure). Probably this compound is similar to the compound eluted in peak 16, except that the Thr in the ring is replaced by Ser instead of the Thr in the side-chain. The loss of the Ser instead of the Thr in the ring could not be shown by MS^3 experiments since the product ion 514 is very low in abundance. However the presence of the ion 442 corresponding with FA-Dab–Thr–Dab^+ and the fragmentation of 815 yielding the ion 714 by loss of 101 u show that the Thr in the side-chain is not replaced. The product ion with m/z 629 was too low in abundance for MS^3 investigation. The final proposed structures are shown in Fig. 9.

3.3.7. Investigation of peaks 14, 17, 19, 20 and 21 (m/z 585.3)

Mass spectra for the compounds eluted in peaks 14, 17, 20 and 21 are identical to polymyxin E_1 eluted in peak 19. No different product ions are formed. The list with product ions for E_1 is shown in

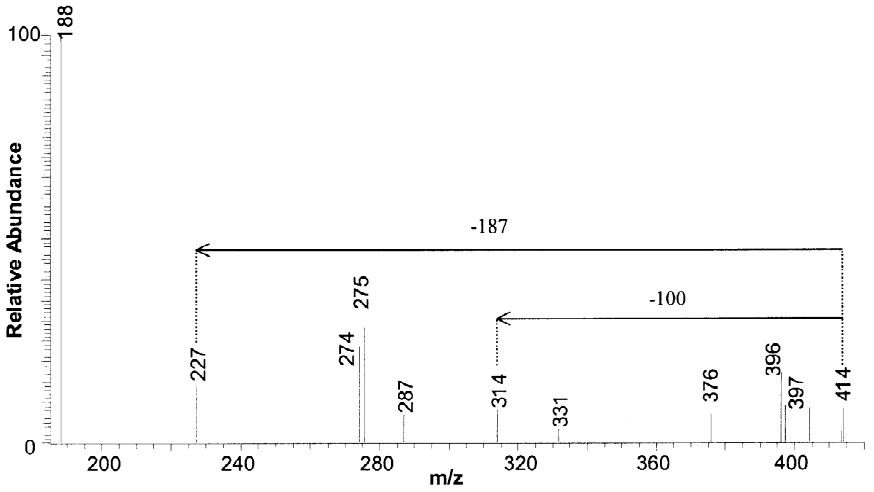


Fig. 8. Isolation and collisional activation with 15% CE in the ion trap of the product ion with m/z 414, which was the result of isolation and collisional activation with 30% CE of $[M+2H]^{2+}$ with m/z 571.3 (peak 8).

Fig. 2. Based on the UV retention time, peak 14 is assigned as Ile-E_1 and peak 20 is assigned as polymyxin E_7. Both compounds were characterized by Orwa et al. [9]. The other peaks are isomers of polymyxin E_1 and are all mentioned in Fig. 9.

3.3.8. Investigation of peak 18 (m/z 584.3)

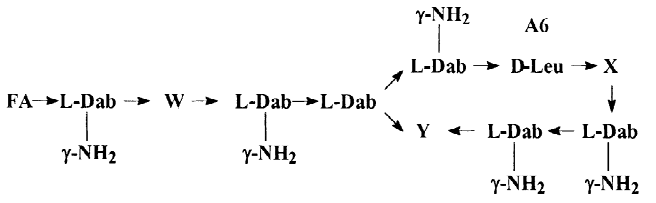
The compound eluted as peak 18 has m/z 584.3. The first series of product ions matches the first series acquired for polymyxin E_1. Starting from the product ion 841 the second series of product ions with m/z 741, 641, 440, 340 and 239 is identified. According to the fragmentation pattern described for the polymyxins, the first loss of mass 227 in the second series corresponds with the Leu–Leu part in the ring. The last ion with m/z 227 in the first series confirms this. The first loss of 239 u in the first series and the product ion with m/z 239 in the second series indicate that the fatty acyl moiety differs from the fatty acyl moieties previously seen for the known polymyxins E. It is plausible that the fatty acyl moiety has the elemental composition $C_9H_{15}O$, by which we assume that this compound has the fatty acyl moiety of polymyxin E_1, however with a double bond somewhere in the carbon chain. The proposed structure is shown in Fig. 9.

Peaks 13 (m/z 569.3) and 15 (m/z 576.3) could not be identified. MS–MS spectra of those peaks are similar to MS–MS spectra observed for degradation products of polymyxins E_1 and E_2 [20]. Probably these compounds are formed by loss of H_2O from either polymyxin E_1 (m/z 585.3) or polymyxin E_2 (m/z 578.3). The part of the molecule which loses H_2O could not be defined, due to the low abundancy of the observed product ions in the MS–MS spectra which hampers MS^3 fragmentations.

All described compounds were present in an amount above 0.1%, as defined with UV normalization. The amounts recorded for the novel related substances are noted in Fig. 9. It has to be mentioned that peaks 6 and 7 were partly coeluted. Peak ‘‘a’’ is partly coeluted with polymyxin E_2. Enlargement of the region 0–10 mV on the y-axis of the UV chromatogram showed more peaks present in an amount around 0.1%. However, in the base peak chromatogram some of those peaks were not abundant enough for MS–MS and MS^3 investigation. Other related substances, which are not mentioned in the table, are present in the following amounts: peak 13, bulk 0.4%; peak 14 (Ile-E_1), tablet 7.2%, bulk 1.1%; peak 15, tablet 0.5%, bulk 0.5%; peak 20 (polymyxin E_7), tablet 0.7%, bulk 4.5%.

4. Conclusions

Liquid chromatography–ion trap tandem mass spectrometry was used for study of the polymyxin E



Peak	M/z doubly-charged ion	Elemental formula FA	W	X	Y	% in bulk	% in tablet
Peak 1	586.3	$C_8H_{15}O_2$	Thr	Leu/Ile	Thr	0.7	0.4
Peak 2	571.3	$C_8H_{15}O$	Thr	Val	Thr	2.1	0.5
Peak 3*	571.3	$C_7H_{13}O$	Thr	Leu	Thr	0.4	0.1
Peak 4	571.3	$C_8H_{15}O$	Thr	Val	Thr	0.2	0.5
Peak 5	587.3	$C_8H_{15}O$	Thr	Met	Thr	0.6	0.6
Peak 6	578.3	$C_8H_{15}O$	Thr	Leu/Ile	Thr	0.9	3.0
Peak 7	593.3	$C_9H_{17}O_2$	Thr	Leu/Ile	Thr	1.6	/
Peak 8	571.3	$C_8H_{15}O$	Ser	Leu/Ile	Thr	0.7	0.4
Peak 10	578.3	$C_8H_{15}O$	Thr	Leu/Ile	Thr	1.9	1.3
Peak 11	578.3	$C_9H_{17}O$	Thr	Val	Thr	0.9	1.7
Peak 12	594.3	$C_9H_{17}O$	Thr	Met	Thr	0.6	1.1
Peak 16**	578.3	$C_9H_{17}O$	Ser	Leu/Ile	Thr	0.5	1.0
Peak 17	585.3	$C_9H_{17}O$	Thr	Leu/Ile	Thr	0.1	0.4
Peak 18	584.3	$C_9H_{15}O$	Thr	Leu/Ile	Thr	0.9	2.1
Peak 21	585.3	$C_9H_{17}O$	Thr	Leu/Ile	Thr	0.6	1.4
Peak a	578.3	$C_9H_{17}O$	Thr	Val	Thr	/	1.9
Peak b	578.3	$C_9H_{17}O$	Thr	Leu/Ile	Ser	/	0.6

Dab: α,γ-diaminobutyric acid
FA: fatty acyl
* probably polymyxin E_4
**synthesized by Kline *et al.*

Fig. 9. Proposed structures of the novel related substances with the m/z of the doubly-charged ion and the elemental formulae of the fatty acyl moieties. The amino acid at position A6 is assumed to be D-Leu, which is a characteristic for all polymyxins E. The amounts defined with UV normalization for the novel related substances are mentioned in the table. Peaks 9, 13, 14, 15, 19 and 20 are not mentioned. Peaks 13 and 15 were not identified and the other peaks correspond with known polymyxins shown in Fig. 1.

complex. The benefits and the drawbacks of this on-line MS method for sequence determination of these complex linear side-chain cyclized peptides were exemplified. The low content of the target components in the colistin sample mixture did not allow isolation and purification of the still unidentified minor components, which would have allowed unambiguous structure determination by amino acid analysis or NMR. Therefore an LC–MS^n technique was used, 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 in contrast to a former FAB-MS–MS study. The low-resolution MS technique used provided suggestions about the sequence of the peptides. The limited number of structural possibilities for the polymyxins made partial characterization of seven novel related substances possible without time-consuming isolation and purification procedures. Four additional peaks in the complex were assigned as isomers of the main polymyxins E_1 and E_2 and two peaks are assumed to be isomers of valine–polymyxin E_1 and E_2. This confirms that many impurities in colistin are isomeric in nature. Often the isomeric fatty acids are the source of structural variation.

A drawback of the ESI-MS technique applied is that it does not allow to 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 MS 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 of the new compounds is similar to the optical configurations reported for polymyxins E_1 or E_2. Chiral amino acid analysis can help to solve this problem, but then again, laborious preparative isolation and purification is necessary.

Acknowledgements

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