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Liquid chromatography–mass spectrometry study towards the pH and temperature-induced *N*-acyl migration in polymyxin B

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Abstract

In the course of the synthesis and purification of new polymyxins and analogues, formation of a by-product with identical mass was observed and it was believed that this might be the result of acyl migration from the *N*α- to the *N*γ-position of residue α,γ-diaminobutyric acid 1 (Dab1) under acidic conditions. Therefore, a LC–MS/MS study was initiated to establish the stability of polymyxin B_3 in aqueous solution at room temperature and 60 °C, as well as different pH values (i.e. 1.4, 4.4 and 7.4). It was shown that the by-product, which is actually formed in the course of the purification of polymyxin B_3 after evaporation in acidic medium, has a retention time similar to *N*γ-polymyxin B_3. Acyl-migration occurred most rapidly at 60 °C and pH 7.4. Furthermore, it was established that migration of the acyl from the *N*α- to the *N*γ-position of residue Dab1 is reversible and that the equilibrium seems to be in favor of the *N*α-acylated compound.

Keywords: pH; Temperature; Acyl migration; Stability study; Polymyxin

1. Introduction

Polymyxins, a family of cyclic lipopeptides, belong to the class of cationic antimicrobial peptides, isolated from *Bacillus polymyxa* [1]. Of the polymyxins, polymyxin B (PMB, Fig. 1), a complex mixture containing mainly six compounds differing only in the composition of the acyl chain [2,3] is the most potent, displaying sub-micromolar MIC values against a variety of Gram-negative bacteria [4,5]. Polymyxin B_1 (PMB_1) is the major component. In an on-going project towards the synthesis and evaluation of antibiotics, a convenient synthetic route towards polymyxins and polymyxin analogues was recently published [6]. The synthetic method involves a so-called "cleavage-by-cyclisation" step and the only possible by-products that can be obtained upon cleavage are the molecules missing any of the four N-terminal residues (i.e. acyl chain, α,γ-diaminobutyric acid 1 (Dab1), Thr2, or Dab3), and these truncated sequences are capped through acetylation.

Unexpectedly, LC–MS analysis of the crude polymyxin derivatives revealed the presence of a small amount (5–10%) of a by-product with identical mass as compared to the desired compound. After semi-preparative LC purification of the crude mixture it was noticed that the by-product had formed again in a similar 5–10% amount (see Fig. 2).

Interestingly, the formation of the same by-product was also observed during a stability study on natural PMB_1 ($nPMB_1$) upon treatment with acid at elevated temperature [7]. Mass spectrometric analysis of $nPMB_1$ and of the by-product showed that they have the same mass, however small differences in the relative abundances of the fragment ions were observed. It has been proposed that these products were the result of acid-catalyzed epimerization. However, such a process proceeds via a sp^2-hybridized planar carbanion as a result of initial hydrogen abstraction (see Fig. 3). In other words, epimerization is more likely to occur under basic reaction conditions.

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Polymyxin	R
B_1	(*S*)-6-methyloctanoyl
B_2	6-methylheptanoyl
B_3	octanoyl
B_4	heptanoyl
B_5	nonanoyl
B_6	3-hydroxy-6-methyloctanoyl†

† Configurations of C3 and C6 have not been established

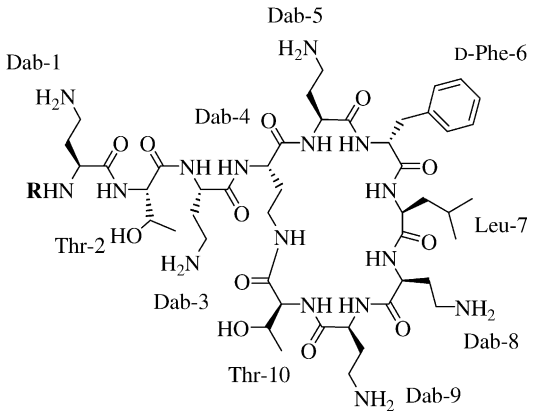

Fig. 1. Structures of polymyxins B_1–B_6.

The previous observations led us to believe that the by-product might be the result of an acyl migration of the acyl chain from the *N*α- to the *N*γ-position of residue Dab1, strengthened by the knowledge that this migration would proceed via an energetically favorable six-membered ring transition state. Unlike $S \rightarrow N$ [8] and $O \rightarrow N$-acyl [9] migrations, $N \rightarrow N'$-acyl migration are rarely reported, and mostly deal with a 1,2-migration [10] instead of 1,3-migration [11]. To verify our hypothesis, a LC–MS/MS study was initiated towards the stability of polymyxin in aqueous solution at room temperature and 60 °C, as well as different pH values (i.e. 1.4, 4.4 and 7.4), the outcome of which is reported below.

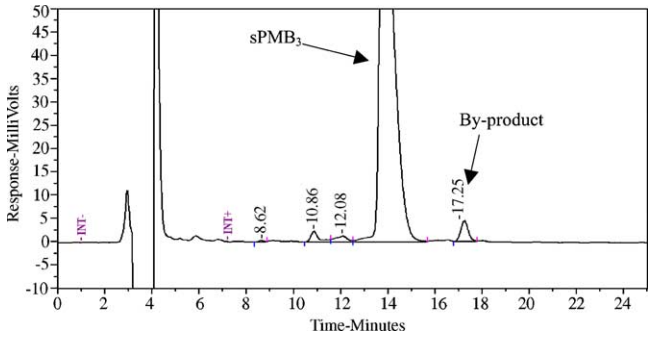

Fig. 2. Appearance of a by-product after semi-preparative LC purification of $sPMB_3$. Conditions: YMC-Pack Pro C_{18}, 5 μm, 250 mm × 2.0 mm i.d. maintained at 30 °C. Mobile phase of 0.01 M TFA–acetonitrile (77:23, v/v) at a flow rate of 200 μL/min. Sample concentration: 0.05 mg mL^{-1} $sPMB_3$ in H_2O. Injection volume: 20 μL. Detection was by UV at 215 nm.

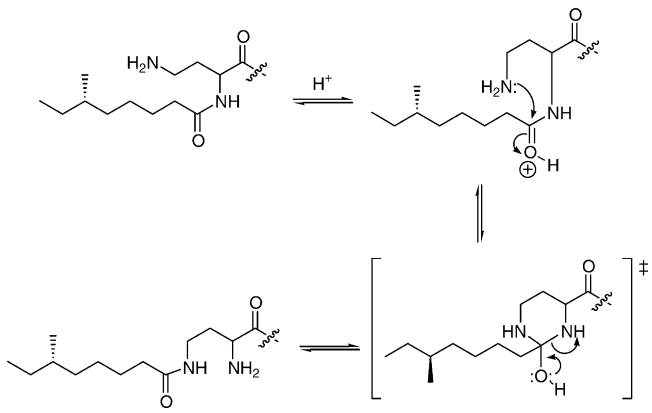

Fig. 3. Acyl migration of the acyl chain from *N*α-Dab1 to *N*γ-Dab1, illustrated for polymyxin B_1.

2. Experimental

2.1. Solvents and reagents

2.1.1. Solvents and reagents used for synthesis and semi-preparative purification

All solvents used in the automated peptide synthesis, acetonitrile, diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade and purchased from Biosolve (Valkenswaard, The Netherlands). All amino acids were purchased from Senn Chemicals, except for Boc-Dab(Fmoc)-OH and Fmoc-Dab(Boc)-OH (Neosystem Laboratoire, Strasbourg, France) and Fmoc-Dab(Mtt)-OH (NovaBiochem, Läufelfingen, Switzerland). All other reagents were obtained from Acros Organics (Geel, Belgium). Water was purified using a Milli-Q Plus purification system (Millipore, Bedford, MA, USA). All reagents were used as supplied by the manufacturer unless stated otherwise.

2.1.2. Solvents and reagents used for LC–MS

Acetonitrile (HPLC grade S) was purchased from Biosolve. TFA was obtained from Riedel-de Haën (Seelze, Germany). A Milli-Q water purification system was used to further purify glass-distilled water. Chemicals used for the preparation of buffers were of analytical grade: potassium dihydrogen phosphate (Merck, Darmstadt, Germany), dipotassium hydrogen phosphate, phosphoric acid (85%, m/m) and potassium chloride (Acros Organics).

2.2. Isolation of $nPMB_3$ and synthesis and purification of synthetic PMB_3 and $N\gamma$-PMB_3

$nPMB_3$ was isolated from commercially available polymyxin B sulphate as described previously [2]. $sPMB_3$ was prepared on 50 μmol scale using a safety-catch approach as reported previously [6]. $N\gamma$-PMB_3 was prepared analogously on 50 μmol scale, except for the coupling of the last residue to the peptide sequence. This moiety, i.e. Dab1, was incorporated using Boc-Dab(Fmoc)-OH instead of Fmoc-Dab(Boc)-OH. After liberating the peptides from the solid support, the compounds were purified using an ÄKTA Explorer™ LC system (Amersham Pharmacia Biotech) equipped with an Alltech Alltima (Deerfield, IL, USA) semi-preparative C_{18} column (250 mm × 10 mm, 5 μm particle size) and an Alltech Alltima analytical C_{18} column (250 mm × 4.6 mm, 5 μm particle size), employing gradients of buffers A (0.1% TFA in acetonitrile–water (5:95, v/v)) and B (0.1% TFA in acetonitrile–water (80:20, v/v)). Collected fractions were combined and evaporated using a LC1010 (Jouan, St. Herblain, France) vacuum centrifuge and yielded $sPMB_3$ (1.9 mg, 3.2%) and $N\gamma$-PMB_3 (2.1 mg, 3.5%), respectively. The purified polymyxins were analyzed by MS using a Q-TOF mass spectrometer (Micromass, Manchester, UK) at a cone voltage of 20 V.

2.3. Buffer and sample preparation for the stability study of $nPMB_3$, $sPMB_3$ and $N\gamma$-PMB_3

Appropriate amounts of 0.1 M potassium dihydrogen phosphate and 0.1 M dipotassium hydrogen phosphate were mixed to pH 7.4. Similarly, 0.1 M buffers of pH 1.4 and 4.4

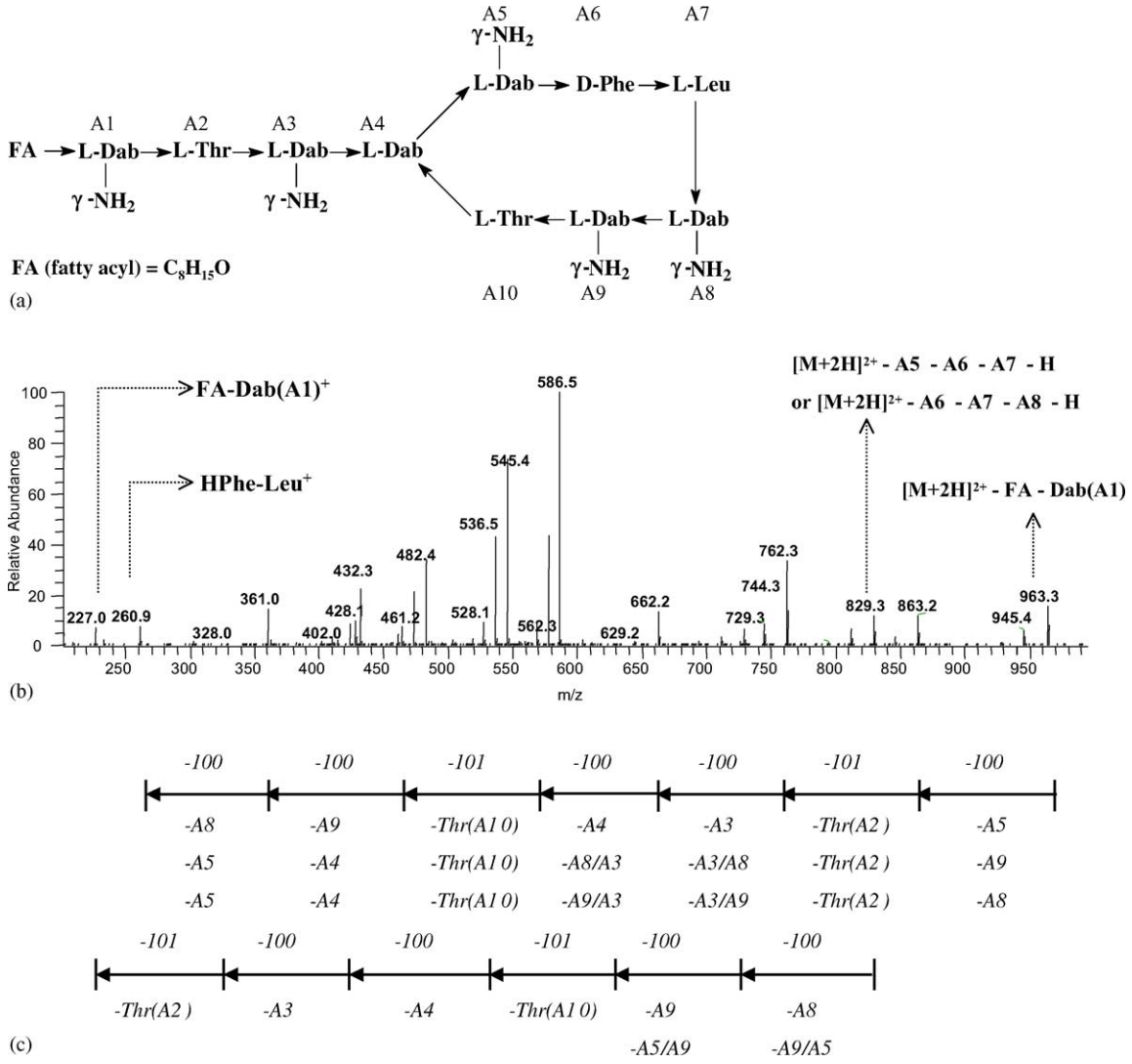

Fig. 4. (a) Structure of $nPMB_3$ or $sPMB_3$. The amino acids are indicated with a number An starting from the FA side. FA, fatty acyl; Dab, α,γ-diaminobutyric acid. (b) $[M + 2H]^{2+}$ CID spectrum acquired for $nPMB_3$, the result of isolation and collisional activation at 30% collision energy level in the ion trap of the precursor ion at *m/z* 595.5. The structures of four ions offering information about the FA residue or the ring amino acids A6 and A7 are shown. (c) A review of the proposed fragmentation routes. Subsequent losses with the corresponding amino acid possibilities are noted in italics.

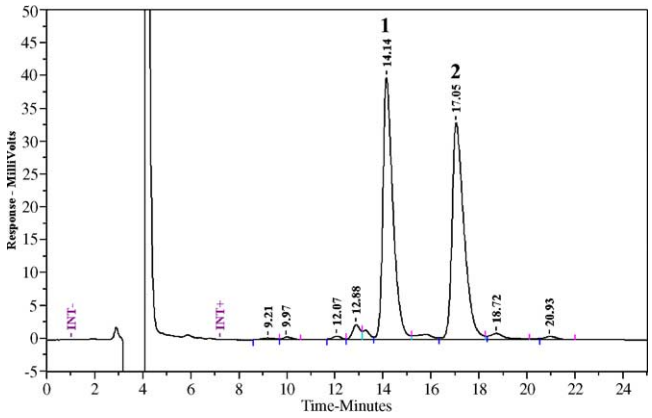

Fig. 5. $sPMB_3$ (1) coinjected with $N\gamma$-PMB_3 (2). Conditions: YMC-Pack Pro C_{18}, 5 μm, 250 mm × 2.0 mm i.d. maintained at 30 °C. Mobile phase of 0.01 M TFA–acetonitrile (77:23, v/v) at a flow rate of 200 μL/min. Sample concentration: 0.025 mg mL^{-1} $sPMB_3$ and $N\gamma$-PMB_3 in H_2O. Injection volume: 20 μL. Detection was by UV at 215 nm.

were prepared from 0.1 M potassium dihydrogen phosphate solution and 0.1 M phosphoric acid. For the stability study at different pH values and temperatures, reactions were initiated by diluting stock aqueous solutions of 0.25 mg mL^{-1} $nPMB_3$ and $N\gamma$-PMB_3 with equal amounts of buffers pH 1.4, 4.4 or 7.4 to give solutions of 0.125 mg mL^{-1}. Aliquots in sealed vials were incubated at room temperature and at 60 °C. The vials were kept in sand inside the oven to ensure a constant temperature of 60 °C. Vials were removed at regular intervals (i.e. 1 h, 24 h, 48 h and 144 h for experiments at pH 1.4 and 4.4 and 1 h, 3 h and 6 h for experiments at pH 7.4, respectively) and immediately frozen at −20 °C until they were analyzed as a series.

2.4. LC and LC–MS/MS 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 C_{18} column (5 μm, 250 mm × 2.0 mm i.d.) (Waters, Milford, MA, USA) was immersed in a waterbath at 30 °C. The following mobile phase was used: 0.01 M TFA–acetonitrile (77:23, v/v). It was degassed by sparging helium. The LC pump was operated at a flow rate of 200 μL/min.

The mass spectral data were acquired on a LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with the electrospray ionization (ESI) interface operated in the positive ion mode. A voltage of 5 kV applied to the 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™ membrane unit, type SNIFF (Hoek Loos, Niel, Belgium) was used as sheath and auxiliary gas. The flow rates of the sheath and the auxiliary gas were set at 90 and 20 arb, respectively. Helium was used as the damping gas and as collision gas at a pressure of 0.1 Pa.

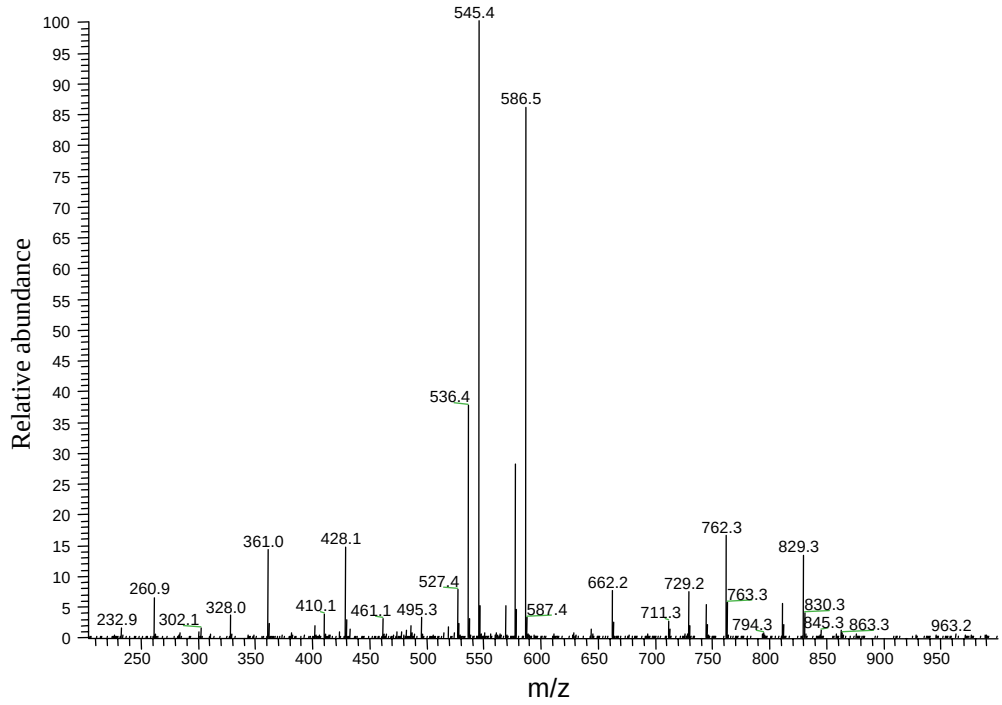

Fig. 6. $[M + 2H]^{2+}$ CID spectrum acquired for $N\gamma$-PMB_3, the result of isolation and collisional activation at 30% collision energy level in the ion trap of the precursor ion at *m/z* 595.5.

Voltages across the capillary and the octapole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. The capillary voltage was set at 46 V and the tube lens offset voltage was set at 5 V. The octapole 1 offset voltage, the interoctapole lens voltage and the octapole 2 offset voltage were set at −3, −24 and −5.5 V, respectively. The Xcalibur software package was used for instrument control, data acquisition and processing. Full MS acquisition over the mass range 500–1500 was performed on-line with UV detection at 215 nm. The doubly-charged ions of the products in the sample were isolated monoisotopically in the ion trap and collisionally activated with 30% CE (collision energy).

3. Results and discussion

PMB_3 was selected as the molecule of investigation as this member of the polymyxin B family does not require laborious synthesis of a chiral acyl chain. The compounds under investigation were either isolated from commercially available polymyxin B sulphate (i.e. $nPMB_3$), or synthesized and purified semi-preparatively ($sPMB_3$ and *N*γ-PMB_3) according to literature procedures [2,6]. It was established that $nPMB_3$ and $sPMB_3$ co-elute under the conditions described in the experimental part. When separately analyzed with the ion trap mass spectrometer identical *m/z* values of 595.5 ($[M + 2H]^{2+}$) and 1189.5 ($[M + H]^{+}$) were observed for $nPMB_3$ and $sPMB_3$. Collision-induced dissociation (CID) spectra of *m/z* 595.5 are identical and show a characteristic first series of product ions of rather high relative abundance and a second series of product ions of a lower abundance, as presented in Fig. 4. The first series is assumed to be formed by loss of the fatty acyl residue plus the neighboring Dab residue and subsequent losses in the linear and cyclic part. The second series of product ions is the result of loss of three ring amino acid residues (A5–A6–A7 or A6–A7–A8) and subsequent losses of other amino acid residues. Beneath the $[M + 2H]^{2+}$ CID spectrum a sketch is given with a short review of the proposed fragmentation routes, which are based on a complete ion trap fragmentation study of PMB_1 and reference substances [12,13]. Characteristic ions offering information about the fatty acyl part and the ring amino acids A6 and A7 are shown above the CID spectrum.

Samples of $sPMB_3$ and *N*γ-PMB_3 were confirmed to have different retention times by co-injection in HPLC (Fig. 5), *N*γ-PMB_3 showing a different retention time with respect to $sPMB_3$. It was also observed that the retention time of *N*γ-PMB_3 is identical to the retention time of the by-product that appears upon concentration of PMB_3 in the presence of acid. This indicates that the unknown isomer found (Fig. 2) might correspond with *N*γ-PMB_3. Comparison of mass spectra of $nPMB_3$ and sPMB3 with *N*γ-PMB_3 showed that they have the same mass and follow a similar fragmentation route, revealing similar product ions in the CID spectrum, for which however the relative abundances are different (compare Figs. 4 and 6). This feature was also observed during a stability study on $nPMB_1$ [7].

To verify the hypothesis that acyl migration takes place in acidic medium and that the product formed corresponds with *N*γ-PMB_3 or the observed by-product, a stability study of polymyxin B_3 in aqueous solution at room temperature and 60 °C, as well as different pH values (i.e. 1.4, 4.4 and 7.4) was undertaken, using $nPMB_3$ and *N*γ-PMB_3 as starting materials. Reactions were initiated by diluting stock aqueous solutions of 0.25 mg mL^{-1} $nPMB_3$ and *N*γ-PMB_3 with equal amounts of buffers pH 1.4, 4.4 and 7.4 to give solutions of 0.125 mg mL^{-1}. Vials were removed at regular intervals (i.e. 1 h, 24 h, 48 h and 144 h for experiments at pH 1.4 and 4.4 and 1 h, 3 h and 6 h for experiments at pH 7.4, respectively).

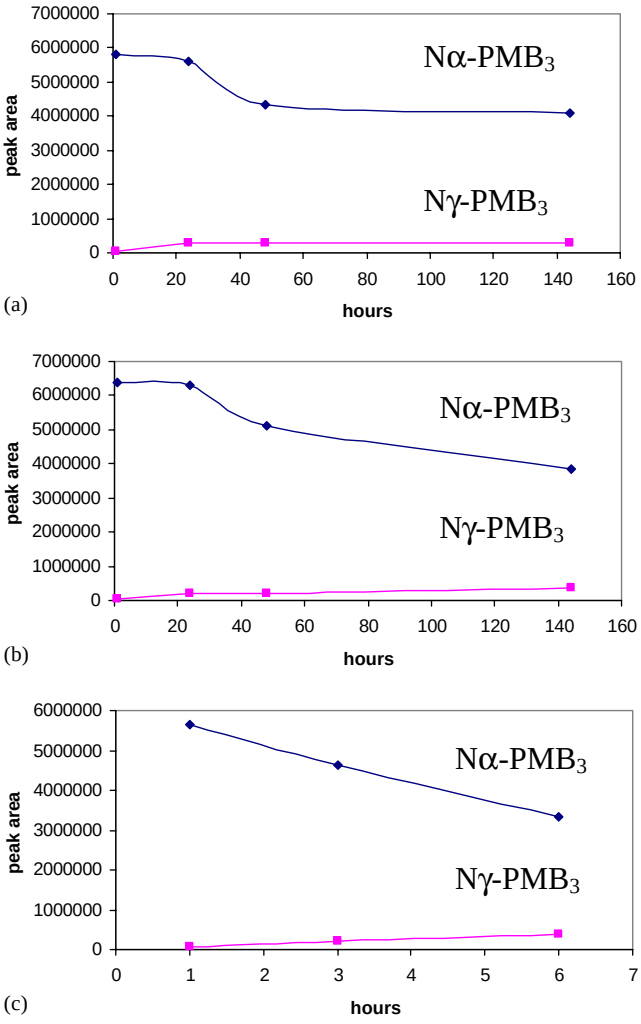

Fig. 7. Profiles showing the peak areas of *N*α-PMB_3 and *N*γ-PMB_3 observed in the chromatograms in function of time at a temperature of 60 °C at pH 1.4 (a), pH 4.4 (b), pH 7.4 (c). Conditions: YMC-Pack Pro C_{18}, 5 μm, 250 mm × 2.0 mm i.d. maintained at 30 °C. Mobile phase of 0.01 M TFA–acetonitrile (77:23, v/v) at a flow rate of 200 μL/min. Injection volume: 20 μL. Detection by UV at 215 nm. Samples were containing 0.125 mg mL^{-1} $nPMB_3$ in H_2O–buffer solution (1:1).

A migration of the acyl chain from *N*α-Dab1 to *N*γ-Dab1 is not observed at room temperature (figures not shown). The formation of *N*γ-PMB_3 from *N*α-PMB_3 at 60 °C at the different pH values is presented in Figs. 7a–c, in which the peak areas of *N*α-PMB_3 and *N*γ-PMB_3 are plotted in function of time. Similarly, the results obtained for the reversible migration starting from *N*γ-PMB_3 are summarized in Figs. 8a–c. Figs. 7a (pH 1.4) and 7c (pH 7.4) clearly indicate that the formation of *N*γ-PMB_3 from *N*α-PMB_3 is faster at neutral pH, which can be explained by the fact that *N*γ of Dab1 will be more nucleophilic at this pH and thus more capable to attack the carbonyl function of the acyl-chain. It should be noted that the decrease in *N*α-PMB_3 may also partly be ascribed to the formation of other degradation products, which were not identified. The acyl migration reached a maximum in the medium with pH 1.4 after approximately 48 h, by which time 7.3% *N*γ-PMB_3 had formed. Starting from *N*γ-PMB_3, it was observed that the formation of *N*α-PMB_3 occurred most rapidly at low pH (see Figs. 8a–c), and equilibrated in this buffer after approximately 48 hours. At this sampling point, 8.6% of the reaction mixture could be attributed to *N*γ-PMB_3. In each chromatogram, the identities of the peaks were confirmed with CID ion trap mass spectrometry based on the fragmentation scheme in Fig. 4.

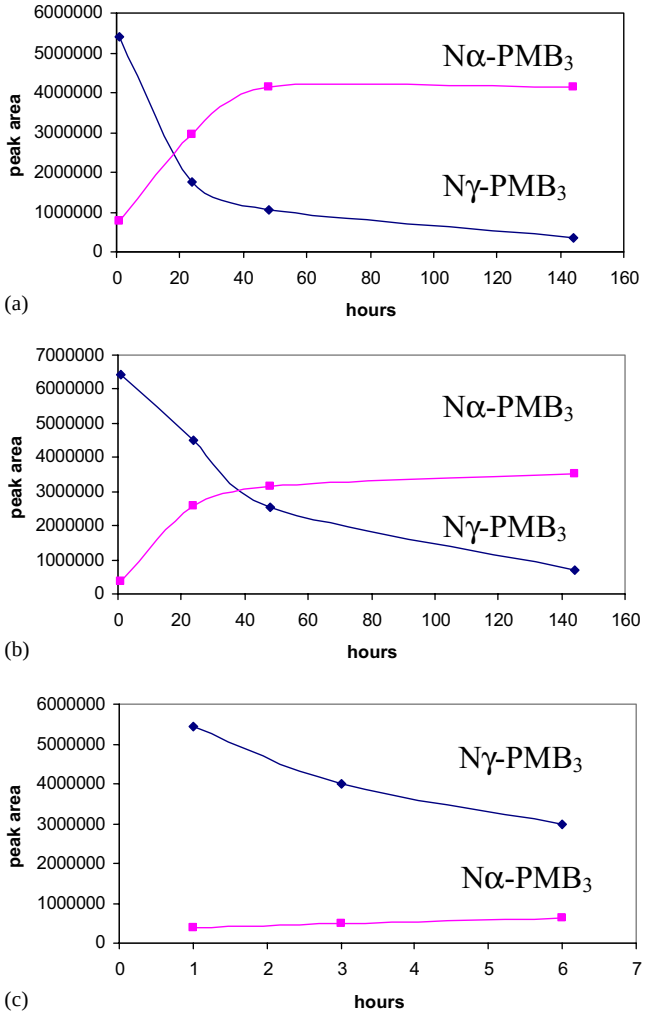

Fig. 8. Profiles showing the peak areas of Nα-PMB_3 and *N*γ-PMB_3 observed in the chromatograms in function of time at a temperature of 60 °C at pH 1.4 (a), pH 4.4 (b), pH 7.4 (c). Chromatograms were obtained under the conditions specified in Fig. 7. Samples were containing 0.125 mg mL^{-1} *N*γ-PMB_3 in H_2O–buffer solution (1:1).

4. Conclusion

A study was undertaken to identify the nature of a by-product formed in the course of the synthesis and purification of polymyxins during evaporation in acidic environment. By studying polymyxin B_3 as a model compound, stability studies carried out at different pH values (i.e. 1.4, 4.4 and 7.4) and temperatures (room temperature and 60 °C) indicated that the by-product is the result of migration of the acyl chain from *N*α-Dab1 to *N*γ-Dab1. The migration was only observed at elevated temperature. It was established that the rate of *N*α → *N*γ migration was highest at pH 7.4. Furthermore, it was established that migration of the acyl from the *N*α- to the *N*γ-position of residue Dab1 is reversible and that the equilibrium seems to be in favor of the *N*α-acylated compound. It is highly likely that the *N* → *N*′-acyl migration observed is not limited to PMB_3 only.

Acknowledgements

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