New cationic amphiphilic compounds as potential antibacterial agents

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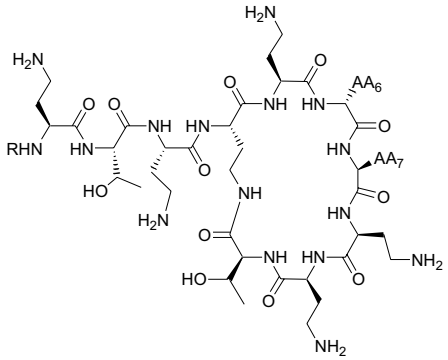
3.1 | Introduction

Polymyxins, potent antibiotics of the class of cationic antimicrobial peptides, are receiving increasing attention due to their unique combination of antibacterial and endotoxin-neutralizing properties.[1] Consequently, their chemical properties have been the subject of a number of studies, carried out mostly in the second half of the last century. These studies supply data on the stability of formulations of polymyxins B,[2,3] E,[4,5] and M[6] (see Table 1). Polymyxins B and M were found to be stable in neutral and mildly acidic media, but became inactivate after exposure to basic or strongly acidic conditions.[2a,7,8] This decrease in activity in both basic and acidic solutions were hypothesized to be the result of acyl or aminoacyl migration.[8] Studies on model systems established the possibility of both migrations in *Nα*-(amino)acylated Dab (α,γ-diaminobutyric acid) residues under basic and acidic conditions.[7,8,9]

TABLE 1 | Composition of polymyxins B, E and M. Different acyl chains (R) can be connected to the Dab1 residue – see Chapter 2. For polymyxin B1, R=*(S)*-6-methyloctanoyl; for polymyxin B3 R=octanoyl.

Polymyxin	AA_6	AA_7
B	DPhe	Leu
E	DLeu	Leu
M	DLeu	Thr



3.2 | Isomerization in Polymyxins

3.2.1 | Isolated polymyxin B1

In a recent analytical study by Orwa *et al.* devoted to the stability of single polymyxins isolated from commercial samples (*e.g.* a commercial sample of polymyxin B contains at least 7 compounds in varying amounts, see Chapter 2 Figure 1),[10] formation of degradation products was observed upon exposure to a range of conditions.[11] Interestingly, a number of these products showed different retention time on HPLC but possessed a molecular weight identical to that of

the substrate. For example, when polymyxin B1 (PMB1, Table 1) was subjected to an acid stability test, a side-product was formed with a longer Rt than PMB1 itself, but with identical molecular weight (Figure 1).

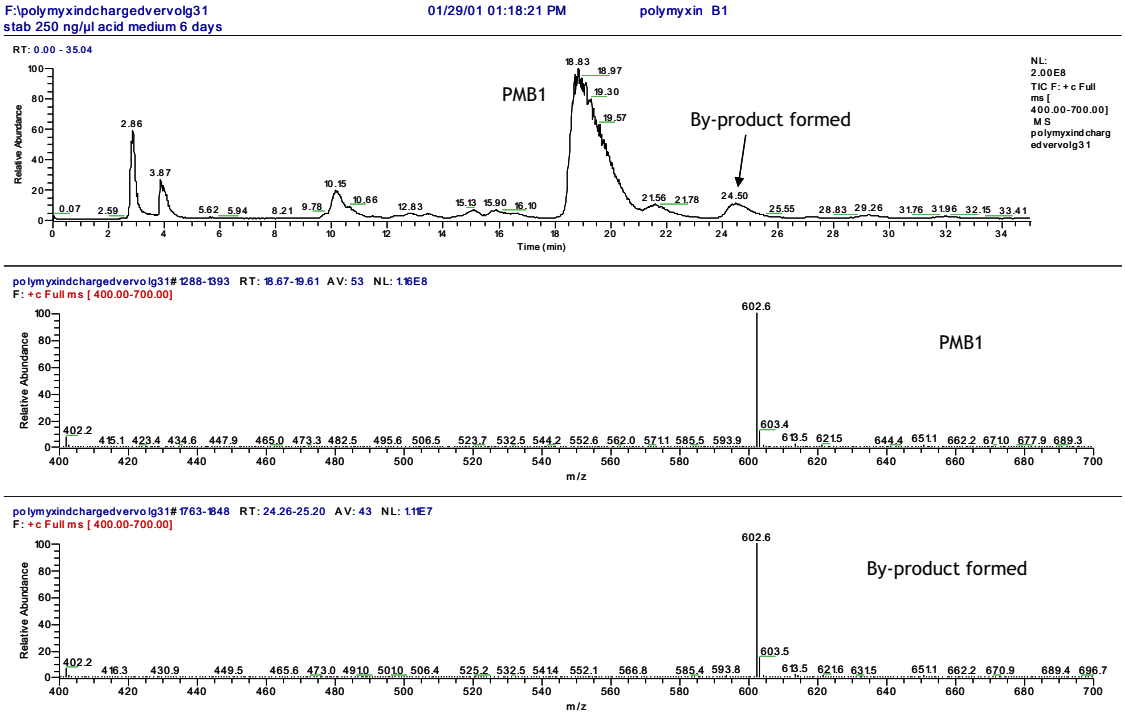


FIGURE 1 | TIC diagram of isolated PMB1 after acid stability test for 6d at pH 1.4, showing the presence of a side-product, and MS spectra of the two peaks. Peak at 602.6 represents $[M+2H]^{2+}$. Reprinted with permission from A. Van Schepdael, personal communication.

Interestingly, subtle differences were found (see Figure 2) in the MS/MS spectra of both peaks. For example, peaks present in both spectra are different in relative intensity, and a number of peaks are absent in the side-product's spectrum, including 241.0, 414.0, 432.3, 482.4, 863.3 and 963.4. The fatty acyl group (FA, in PMB1 this is *(S)*-6-methyloctanoyl) seems to be involved in all these fragments as in PMB1 these peaks represent FA+Dab1 (241.0), M-(FA+Dab1+Dab5) (863.3) and M-(FA+Dab1) (963.4).[10b-c,12] Peaks 482.4 (2^+ ion of 963.4), 432.3 (2^+ ion of 863.3) and 414.0 (432.3-H_2O) also relate to these fragments. In this study, it was concluded that this isomeric side-product arose from epimerization of PMB1.[11] However, besides the fact that in this study no data

was supplied to justify this theory (no attempts were made to localize the site of isomerization), amino acid epimerization is highly unlikely under acidic conditions as this process proceeds through an anionic species.[13]

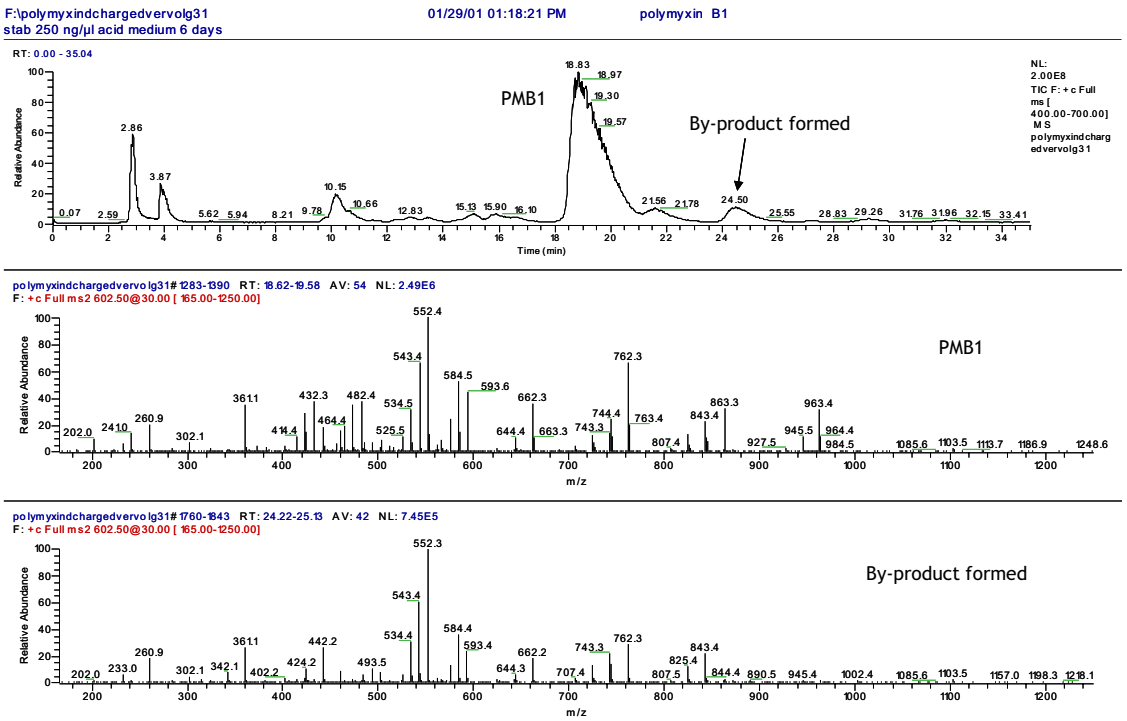


FIGURE 2 | TIC diagram of isolated PMB1 after acid stability test, showing the presence of a side-product, and MS/MS spectra (of the $[M+2H]^{2+}$ ion) of the two peaks. Reprinted with permission from A. Van Schepdael, personal communication.

3.2.2 | Synthetic polymyxins

As isolation of substantial amounts of every individual polymyxin from a commercial PMB sample requires an extremely time-consuming purification procedure,[10b] the chemical synthesis of PMB subfamily members was undertaken (Chapter 2). In the course of these studies, formation of a small amount (typically 5-10%) of a side-product with identical molecular weight but different LC retention time was encountered, reminiscent of the isolated PMB1 case (*vide supra*). Remarkably, after HPLC repurification, concentration *in vacuo* to remove solvents and redissolution of *e.g.* synthetic polymyxin B3 (PMB3), LCMS revealed the side-product had formed

again in a similar (5-10%) amount (Figure 3). Peaks in Figure 3 marked with arrows arose from two separate compounds both containing $[M+H]^+$ 1190.0 and $[M+2H]^{2+}$ 595.5 as major ions. Subjection of both peaks present in the mixture to MS/MS analysis displayed differences in abundances of a series of peaks between the spectra of PMB3 and the side-product (Figures 4 and 5).

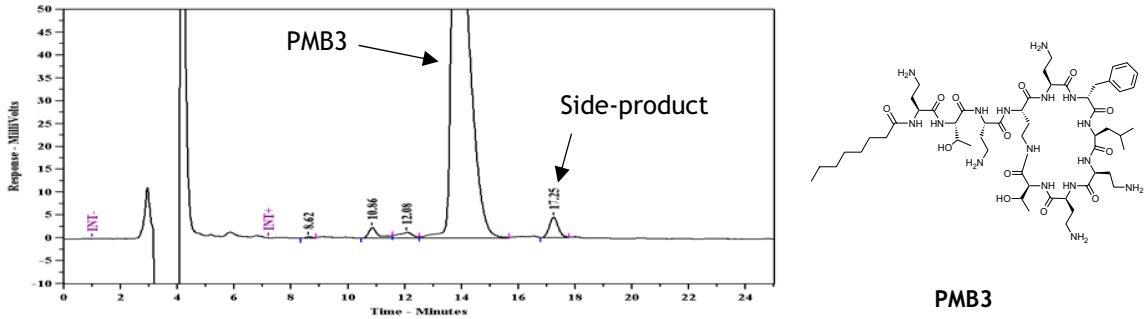


FIGURE 3 | LC diagram of purified PMB3, showing the presence of a side-product with identical molecular weight.

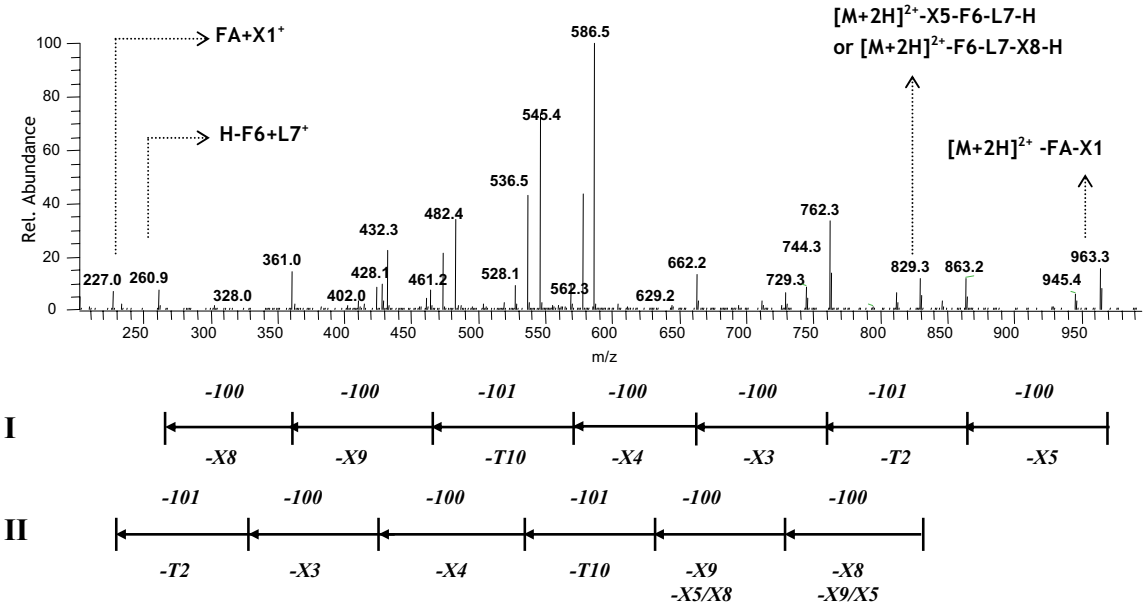


FIGURE 4 | $[M+2H]^{2+}$ MS/MS spectrum acquired for PMB3 and proposed fragmentation routes **I** and **II**.[10b-c,12] The structures of four ions offering information about the FA residue or the ring amino acids DF6 and L7 are shown (FA=fatty acyl (*i.e.* octanoyl), amino acids in one letter code (X=Dab)).

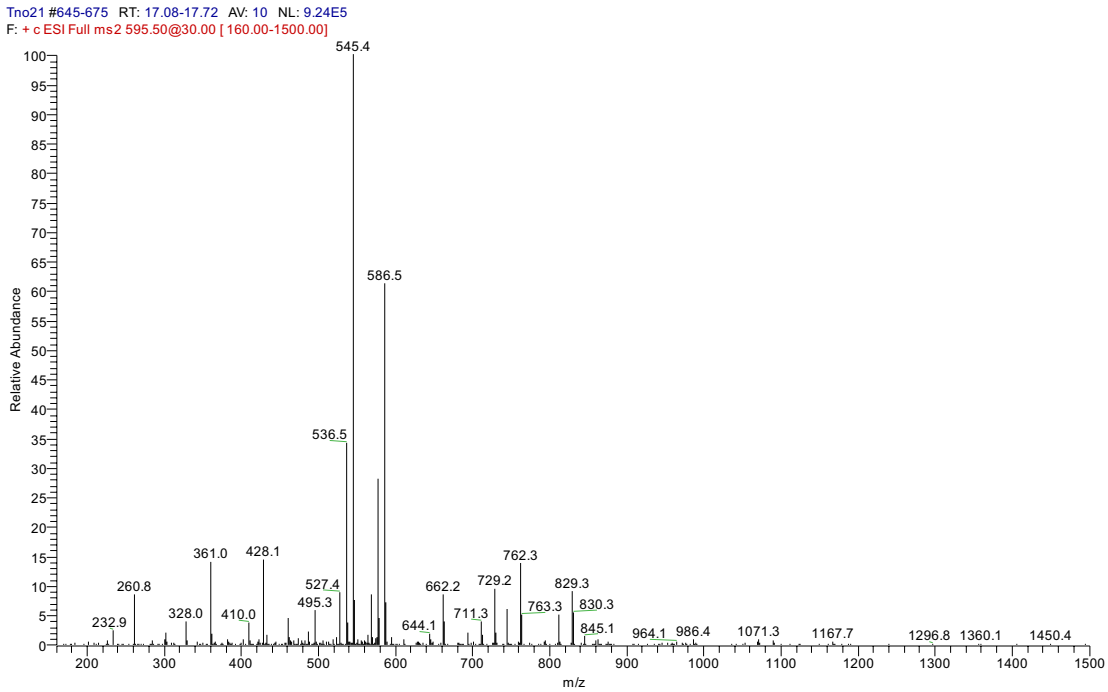


FIGURE 5 | $[M+2H]^{2+}$ MS/MS spectrum obtained from the side-product formed during PMB3 synthesis.

These differences are similar to those seen in the case of PMB1 and the corresponding side-product (*vide supra*); comparable peak disappearance in the side-product's MS/MS relating to fragments containing FA is found in the absence of fragments 227.0, 863.2, 963.3 and the doubly charged ions 432.3 and 482.4 (see Table 2 in the Experimental section). This indicates that a similar isomerization process has taken place in both isolated PMB1 and synthetic PMB3. As acyl migration between Nα and Nγ positions in Dab residues were found possible to occur in comparable systems (*vide supra*) through an energetically favorable 6-membered ring transition state, it was reasoned that acyl migration at Dab1 had taken place rather than epimerization.

3.3 | Synthesis & Analysis of the Acyl-Migrated Product

To obtain proof regarding the structure of the observed side-product resulting from Nα→Nγ acyl migration, the acyl-migrated product of PMB3 was synthesized. This regioisomer, the new polymyxin analogue Nγ-PMB3 (Figure 6B), was obtained following the safety-catch procedure

introduced in Chapter 2. Key step in this synthesis is the on-resin cyclization of the linear polymyxin with concomitant cleavage from the resin, which can only occur after activation of the resin by alkylation. This procedure generally yields a crude product of high purity that is not contaminated with its linear counterpart, obviating a tedious purification procedure. The reported procedure was slightly altered, applying Boc-Dab(Fmoc)-OH instead of Fmoc-Dab(Boc)-OH as the last amino acid residue to achieve acylation at the Nγ position of Dab1.

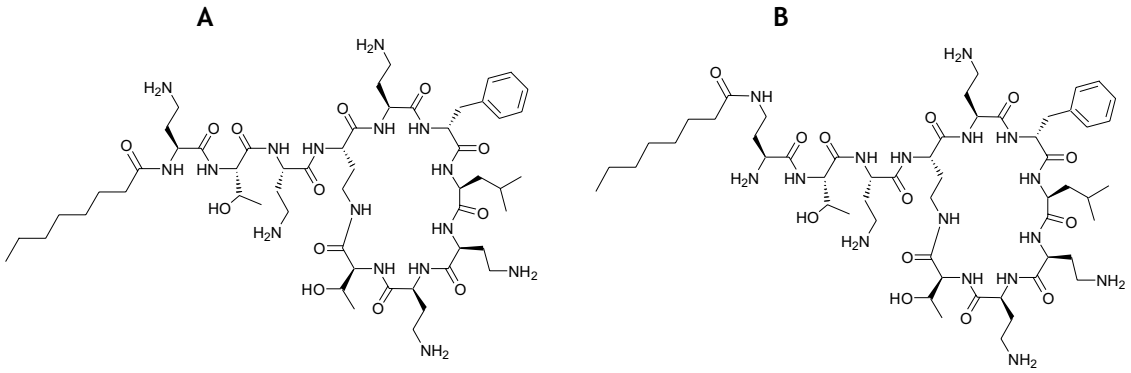


FIGURE 6 | Structures of PMB3 (A) and its regioisomer, Nγ-PMB3 (B).

Upon co-injection of the HPLC-purified, side-product-containing PMB3 mixture (Figure 3) with Nγ-PMB3, the Nγ-PMB3 retention time in LC was found to be identical with the one observed for the side-product (Figure 7). Moreover, comparison of the MS/MS spectra of the PMB3 isomeric side-product with the synthetic Nγ-PMB3 showed that they were identical (Figures 5 & 8).

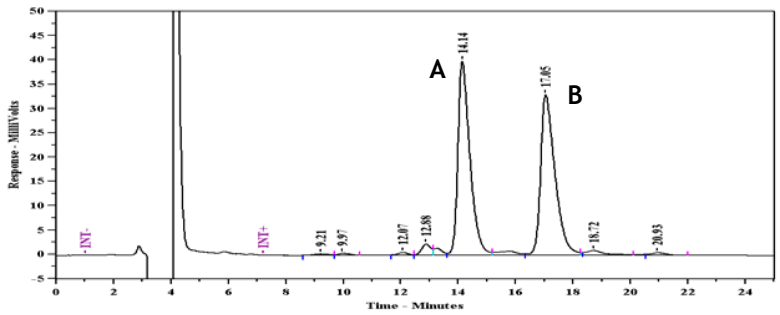


FIGURE 7 | LC chromatogram of crude PMB3 mixture co-injected with Nγ-PMB3. Peak **A**: PMB3, peak **B**: Nγ-PMB3 coincides with the obtained side-product as determined by MS/MS.

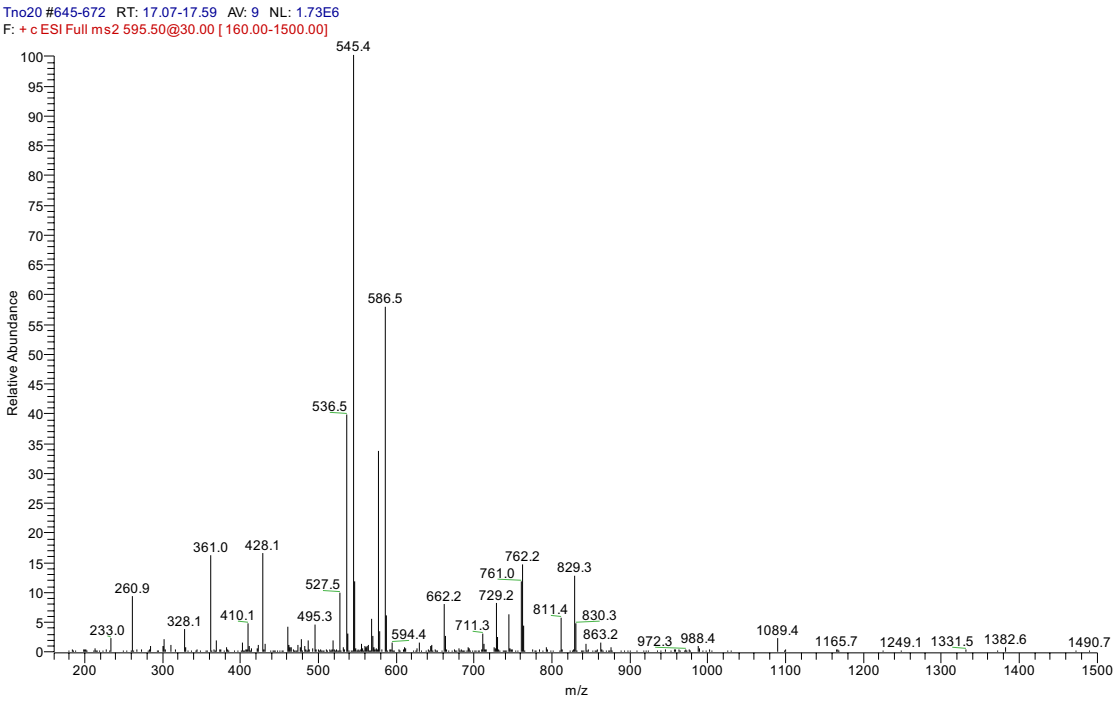


FIGURE 8 | MS/MS spectrum obtained from $[M+2H]^{2+}$ of synthetic Nγ-PMB3.

Based on these data it was concluded that the formed side-product corresponds to the structure in Figure 6B, being the Nγ-regioisomer of the desired polymyxin resulting from N→N′-acyl migration in the Dab1 residue.

3.4 | Determination of Favored Isomer

Acyl migration takes place from both *Nα*- and *Nγ*-acylated single Dab residues, in both cases favoring the Nα-product under slightly acidic conditions to yield approx. 60% Nα-product.[7a] To examine their chromatographic behavior, both *Nα*- and *Nγ*-octanoyl-Dab were synthesized by SPPS and treated thereafter in a similar way as was PMB3. In the final product mixture after synthesis of *Nγ*-octanoyl-Dab, only a small amount of this compound was obtained, accompanied by the major product *Nα*-octanoyl-Dab, whereas no *Nγ*-octanoyl-Dab was found in the synthesis

of *Nα*-octanoyl-Dab (by comparison of LCMS chromatograms: both $[M+H]^+$ 244.5, C_{18} RP column, 15→90% MeCN/0.1% aq. TFA in 26min; Nα Rt 8.79min, Nγ Rt 9.15min).

In order to examine the scope of the polymyxin N→N'-acyl migration reported here, a stability study of PMB3 and Nγ-PMB3 was performed as a function of temperature (RT and 60^0C) and pH (1.4, 4.4 and 7.4). The obtained LCMS/MS chromatograms of these samples showed that acyl migration does not take place at RT. The formation of Nγ-PMB3 from PMB3 at 60^0C at the different pH values is presented in Figures 9A-C.

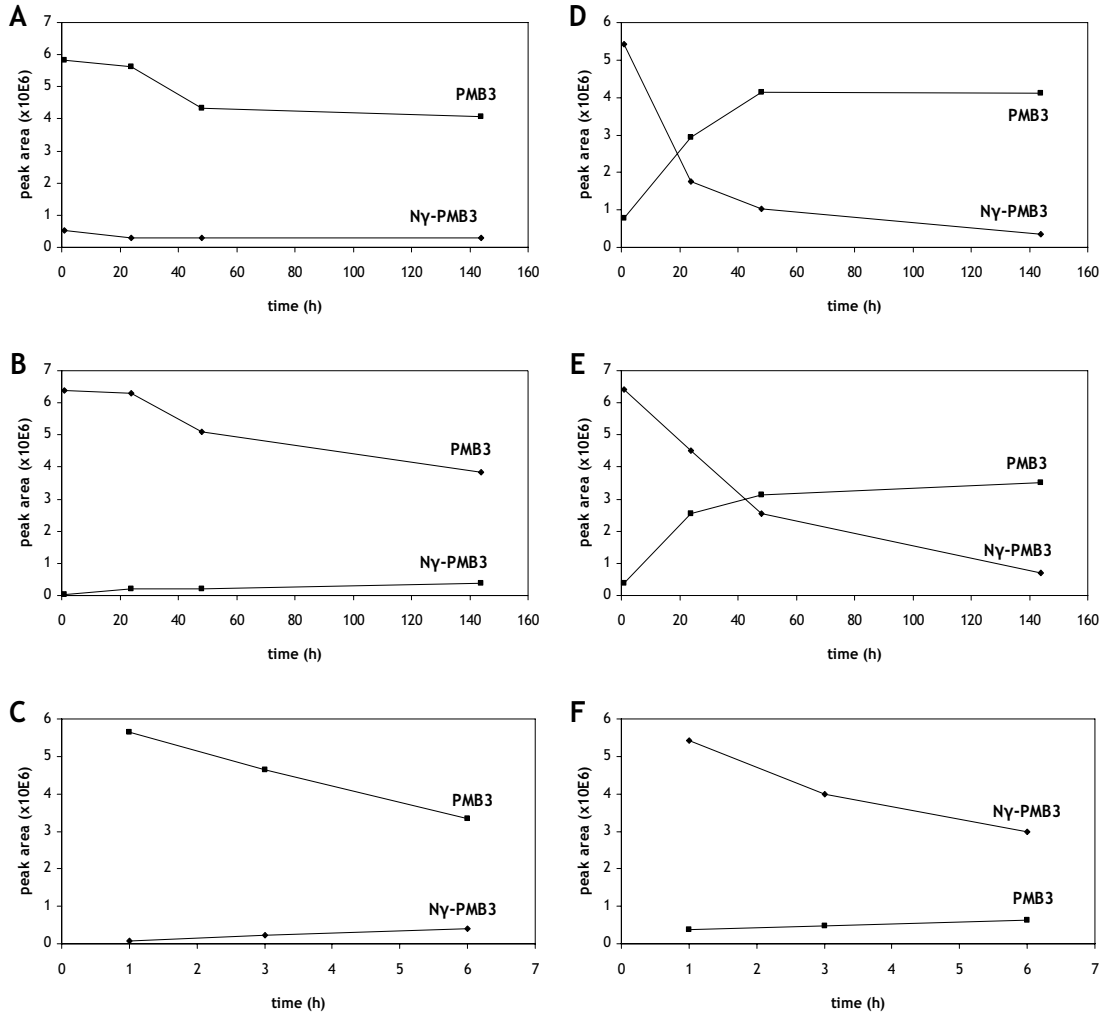


FIGURE 9 | Profiles showing the LC peak areas of PMB3 and Nγ-PMB3 in time at 60^0C as a function of pH. **A,B,C** starting from PMB3; **D,E,F** starting from Nγ-PMB3. pH 1.4 (**A,D**), 4.4 (**B,E**) and 7.4 (**C,F**).

The Nα→Nγ acyl migration reached a maximum in the medium with pH 1.4 after approx. 48h, by which time ~7% Nγ-PMB3 had formed (Figure 9A). Nγ-PMB3 is formed fastest at neutral pH, which can be explained by the fact that at this pH value the Nγ of Dab1 is more nucleophilic.

The formation of PMB3 out of Nγ-PMB3 (*i.e.* Nγ→Nα migration, Figures 9D-F) occurred most rapidly at low pH and equilibrated after approx. 48h. For pH 1.4, at this point ~20% of the Nγ-PMB3 was left. Conversion of either polymyxin went most rapid at pH 7.4 (Figures 9C and F). It should be noted that the decrease in PMB3 and Nγ-PMB3 levels might be partially ascribed to the formation of other degradation products that were not identified.

3.5 | Biological Evaluation

To investigate whether this N→N'-acyl migration has an effect on the potency of the polymyxin as antibiotic, minimal inhibitory concentrations (MIC) values were determined against *Escherichia coli* ATCC 11775 using a 2-fold serial dilution assay (see Chapter 2). PMB3 was found to have a MIC value of 0.6μM, whereas the antibacterial potency of Nγ-PMB3 had decreased to a MIC value of 2.5μM, showing that, at least in PMB3, Dab1 Nα→Nγ acyl migration has a negative effect on antibacterial activity.

3.6 | Summary

The structure of a side-product found in the synthesis of polymyxin B3 was elucidated. This side-product, with a mass identical to PMB3 but with different LC retention time, reappeared after purification of PMB3 and resulted from isomerization of the parent compound under acidic conditions. Acyl migration was hypothesized to have taken place at the alkanoylated Dab1 residue. Although the rare amino acid Dab occurs in some other natural products (*e.g.* in the peptide antibiotics friulimicins,[14] syringomycin,[15,16] syringopeptins,[15,17] gavaserin and saltavalin,[18] cepacidine A,[19] pseudomycins,[20] xylocandin,[21] fuscopeptins,[22] and tolaasins[23]), none of these compounds contain an N-terminally alkanoylated Dab residue,[24] providing no precedent for such a hypothesized N→N' acyl shift. LC and MS/MS analyses showed the side-product to be

identical with the synthetic PMB3 regioisomer having the Nα-Dab1 acyl chain migrated to its Nγ position. Analogously, this isomerization most likely also occurred during the acid stability test of isolated PMB1, rather than epimerization as proposed by Orwa *et al*.

3.7 | Experimental Section

3.7.1 | SPPS

PMB3 was prepared on 50µmol scale as reported previously (see Chapter 2). Nγ-PMB3 was prepared analogously on 50µmol scale, except for the coupling of the last residue to the peptide sequence. This moiety 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 equipped with an Alltima semi-preparative C_{18} column (250 x 10mm, 5 µm particle size) employing gradients of buffers A (0.1% TFA in 5% (v) MeCN/H_2O) and B (0.1% TFA in 80% (v) MeCN/H_2O) and simultaneous detection at 214 and 254nm. Collected product fractions were combined and evaporated or lyophilized using a LC1010 vacuum centrifuge (preventing isomerization) to yield PMB3 (1.9mg, 3.2%) and Nγ-PMB3 (2.1mg, 3.5%) respectively. ESI-MS (both compounds): $[M+H]^+$ 1190.0, $[M+2H]^{2+}$ 595.5. LC: PMB3 Rt 14.14min, Nγ-PMB3 Rt 17.05min.

3.7.2 | LCMS and MS/MS Analyses

The LC apparatus consisted of a Spectra HPLC system equipped with a YMC-Pack Pro C_{18} column (5µm, 250x2.0mm, Waters), immersed in a water bath at 30°C, employing 0.01M aq. TFA/MeCN (77/23 (v/v)) as buffer. Mass spectra were recorded on a LCQ ion trap mass spectrometer equipped with an ESI interface operated in the positive ion mode at 5kV and capillary temperature of 210°C. Helium was used as collision gas at a pressure of 0.1Pa. MS acquisition over the mass range 500-1500 was performed on-line with UV detection at 215nm. The doubly-charged ions of the products in the sample were isolated mono-isotopically in the ion trap.

3.7.3 | Stability Studies

Appropriate amounts of 0.1M aq. H_2KPO_4 and 0.1M aq. HK_2PO_4 were mixed to pH 7.4. Similarly, buffers of pH 1.4 and pH 4.4 were prepared from 0.1M aq. H_2KPO_4 and 0.1M aq. phosphoric acid. For the stability study at different pH values and temperatures, reactions were initiated by diluting stock aqueous solutions of 0.25mg/mL PMB3 and Nγ-PMB3 with equal amounts of buffers pH 1.4, 4.4 or 7.4 to give solutions of 0.125mg/mL. Aliquots in sealed vials were incubated at RT and at 60°C. Vials were removed at regular intervals and immediately frozen at -20°C until they were analyzed as a series. For pH 1.4 and pH 4.4 vials were removed after 1h, 24h, 48h and 144h. Those at pH 7.4 were removed after 1h, 3h and 6h.

3.7.4 | Antibacterial Assay

Antibacterial assays against *E. coli* ATCC 11775 were performed in 3-fold as previously described (Chapter 2).

TABLE 2 | Fragments found in MS/MS spectra of PMB3 and its isomer

Fragment[A]	Origin	PMB3	isomer
227.0	FA+Dab1	**+**	**-**
232.9	unknown	+	+
260.9	DPhe6+Leu7	+	+
328.0	FA+Dab1+Thr2	**-**	**+**
361.0	DPhe6+Leu7+Dab8	+	+
410.1	428-H_2O	+	+
423	2+ ion of 845	**+**	**-**
428.1	FA+Dab1+Thr2+Dab3	+	+
432.3	2+ ion of 863	**+**	**-**
461	DPhe6+Leu7+Dab8+Dab9	+	+
464	2+ ion of 963-2H_2O	**+**	**-**
473	2+ ion of 963-H_2O	**+**	**-**
482.4	2+ ion of 963	**+**	**-**
495.3	unknown[C]	-	+
527.4	2+ ion of 1090 (M-Dab)[B]-2H_2O	-	+
528.1	FA+Dab1+Thr2+Dab3+Dab4	+	-
536	2+ ion of 1090 (M-Dab)-H_2O	+	+
545.4	2+ ion of 1090 (M-Dab)	+	+
569	2+ ion of M-3H_2O	+	+
578	2+ ion of M-2H_2O	+	+
586.5	2+ ion of 1172 (M-H_2O)	+	+
662.2	DPhe6+Leu7+Dab8+Dab9+Thr10+Dab4	+	+
711.3	729-H_2O	+	+
729	FA+Dab1+Thr2+Dab3+Dab4+Thr10+Dab	+	+
744.3	762-H_2O	+	+
762.3	DPhe6+Leu7+Dab8+Dab9+Thr10+Dab4+Dab3	+	+
812	829-H_2O	+	+
829.3	M- DPhe6-Leu7-Dab	+	+
845.3	863-H_2O	+	+
863	M-FA-Dab1-Dab5	+	-
945.4	963-H_2O	+	-
963.3	M-FA-Dab1	+	-

These fragments are designated present (+) or absent (-) for PMB3 but are applicable for its isomer on the premise that identical parts in both molecules give identical fragment peaks. [A] Approximate values of peaks that are not denoted in spectra, or in which decimals differ by 0.1 are not given in decimal values; [B] M=1190; [C] might be 2+ ion of 990 (M-2Dab).

3.8 | Notes & References

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24. Gavaserin and saltavalin share analogy with known polymyxins regarding amino acid and fatty acid composition. As no chemical structures of the complete peptides were given, it cannot be excluded that an acylated Dab residue is present.

CHAPTER 4 | Design, Synthesis & Biological Evaluation of PMBN/CAP Conjugates