THE OUTER MEMBRANE PERMEABILITY-INCREASING ACTION OF DEACYLPOLYMYXINS

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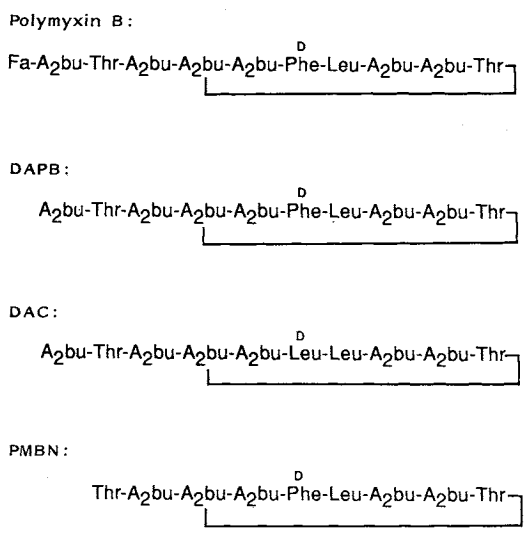
The outer membrane permeability-increasing action of deacylpolymyxins was compared to the well-known potent action of polymyxin B nonapeptide (PMBN). Deacylpolymyxin B (DAPB), prepared by treating polymyxin B with polymyxin acylase, was found to be a slightly more effective permeabilizer than PMBN. As low a DAPB concentration as 1 μg/ml sensitized *Escherichia coli* to the probe antibiotics (rifampin, fusidic acid, erythromycin, clindamycin, novobiocin) by factors 30~100 and *Salmonella typhimurium* by factors 10~100. A higher concentration (3 μg/ml) of DAPB elicited further sensitization. Also deacylcolistin (DAC) was found to be an effective permeabilizer.

CHIHARA and co-workers[1,2] have shown that the terminal *N*-fatty acyl amino acid moiety can be removed from polymyxin (PM) using ficin or papain to yield a PM-derived nonapeptide (Fig. 1). This PM nonapeptide lacks the bactericidal activity of the parent compound. It was then found by VAARA and VAARA[3~5] and confirmed by others[6~9], that PM nonapeptides still have one important property left, *viz.*, they drastically damage the outermost cell wall structure (the outer membrane: OM) of Gram-negative bacteria and increase its permeability to hydrophobic antibiotics.

However, it is not very clear, how selectively papain and ficin act in the hydrolysis of PM. In the light of the results of CHIHARA *et al.* (Table 7 in ref 1), it seems that up to 10% of PM cleaves to an octapeptide during the papain treatment. Even though many of such impurities can be removed by HPLC[10], the possibility that HPLC-purified PM nonapeptide preparations are still heteroge-

Fig. 1. Structure of PM B, DAPB, DAC, and PMBN.

Abbreviation: A_2bu, α,γ-diaminobutyric acid; Fa, fatty acid.



neous, exists.

PM acylase is a new enzyme, recently reported by KIMURA and co-workers, and deacylates PM and many other *N*-acyl compounds[11~13]. It removes only the fatty acyl group of PM and leaves the peptide moiety completely intact (Fig. 1).

In this study, we have compared the cell wall permeability-increasing potency of PM B nonapeptide (PMBN) and deacyl PMs. We will show that deacylpolymyxins are at least as effective permeabilizers as PMBN.

Materials and Methods

Bacterial Strains

Smooth *Escherichia coli* IH3080 (O18:K1:H8) and rough *Salmonella typhimurium* SH5014 (LPS chemotype Rb_2) are strains used in our earlier permeability studies[4,14~17].

PMBN Sulfate

PMBN was prepared essentially as described by VILJANEN and VAARA[14] and was a kind gift from Farmos Group Ltd. (Turku, Finland). Its residual content of PM B was approx 0.1%, as determined by reversed-phase HPLC[10].

Deacylcolistin (DAC) Sulfate

DAC sulfate was prepared from colistin sulfate (a kind gift from Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) by using PM acylase (acetone-dried cell powder of *Pseudomonas* M-6-3 strain) as described previously[11].

Preparation of Deacylpolymyxin B (DAPB) Sulfate

PM B sulfate, 2 g (Sigma Chemical Co., U.S.A.) was dissolved in 200 ml of deionized water and the solution was adjusted to pH 7.5 with 1 N NaOH. 400 mg of acetone-dried cell powder of *Pseudomonas* M-6-3 (the source of PM acylase, see ref 11) was added. The mixture was shaken at 37°C for 48 hours. Small samples were withdrawn at intervals to monitor the pH and the liberated fatty acids. When the pH dropped, it was raised to 7.5 with 0.1 N NaOH. After centrifugation (10,000 × *g*, 30 minutes, to remove the insoluble mass that formed), the pH of the supernatant was lowered to 2 with 1 N H_2SO_4, and the supernatant was washed with ether to remove free fatty acids. The water layer was adjusted to pH 4.0 with 1 N NaOH and was concd *in vacuo* to approx 4 ml, applied on a Sephadex G-15 column (90 × 1.6 cm, i.d., Pharmacia LKB, Uppsala, Sweden), and eluted with water. Fractions were analyzed by TLC and ninhydrin staining for the presence of DAPB, and the DAPB-containing fractions were concentrated.

DAPB was further purified by HPLC by using a YMC-Pack SH-343 column (25 × 2 cm i.d., particle size: 10 μm, Yamamura Chemical Institute, Kyoto, Japan), isocratic elution with 0.01 N H_2SO_4 in acetonitrile - water (15:85), the flow rate of 6 ml/minute, and a variable-wavelength UV detector (875-UV, Japan Spectroscopic, Tokyo, Japan; set at 220 nm).

The DAPB-containing fractions were collected and adjusted to pH 4 with saturated $Ba(OH)_2$ solution. After the precipitate formed was removed by centrifugation (10,000 × *g*, 30 minutes), the supernatant was lyophilized, yielding 800 mg of DAPB sulfate as white powder.

Purity Analysis of DAPB Sulfate

Purity analysis was performed using an analytical column YMC-Pack ODS AM-312 (150 × 6 mm, i.d.), elution with 0.1 M NaCl-HCl (pH 2.0) - MeOH (45:55), the flow rare of 1.0 ml/minute, and the detection at 210 nm.

Amino Acid Analysis of the DAPB Preparation

One mg of DAPB sulfate was hydrolyzed with 0.5 ml of constant-boiling hydrochloric acid at 110°C for 18 hours, the hydrolysate was then analyzed with a Hitachi L-8500 automatic amino acid analyzer.

FAB Mass Spectra

FAB mass spectra were obtained from a glycerol matrix with a JMS-DX 303 mass spectrometer (Nippon Densi, Tokyo, Japan).

GC of the Fatty Acids Liberated from PM B

The ether fraction (see above) was analyzed for the presence of PM B-derived fatty acids. Ether was removed by distillation, and the residue (approx 100 mg) was esterified with MeOH-BF_3 and analyzed in GC using the following conditions: a OV-1 column (2.1 m × 3.2 mm, i.d.), the flow rate of 50 ml/minute for the carrier gas, a FID-detector.

Antibiotics

Rifampin, fusidic acid (sodium salt) and novobiocin (sodium salt) were from Sigma, erythromycin ethylsuccinate from Orion (Helsinki, Finland), and clindamycin hydrochloride from Upjohn (Kalamazoo, U.S.A.).

The stock solution of rifampin was prepared by dissolving 10 mg of rifampin with 1 ml of MeOH, after which deionized water was added to a final volume of 10 ml. The stock solution of erythromycin was prepared by dissolving 10 mg with 4 ml of 96% EtOH, after which deionized water was added to a final volume of 10 ml.

Synergistic Growth Inhibition Assay

This was done essentially as in ref 17 by using the checkerboard technique, microdilution plates, L broth as the assay medium, and bacterial inoculum size of 10^4 cells per ml. After an incubation time of 18 hours at 37°C, the growth (A_{405}) of each well was measured with a Titertek Multiscan spectrophotometer (Labsystems, Helsinki, Finland). Before the reading, the spectrophotometer was blanked with corresponding uninoculated, drug-containing media. The MIC of an antibotic was defined as the lowest concentration of the antibiotic which reduced the growth of the target becteria by ≥95%.

Results

Preparation of DAPB

DAPB was prepared using PM acylase and the methodology previously described for the preparation of deacylcolistin[11]. Amino acid composition of the product (A_2bu, Thr, Leu, and Phe in a molar ratio of 6.5 : 1.8 : 1.0 : 0.9) corresponded to that expected for DAPB. Furthermore, in MS (Fig. 2), the protonated

Fig. 2. FAB mass spectrum of DAPB.

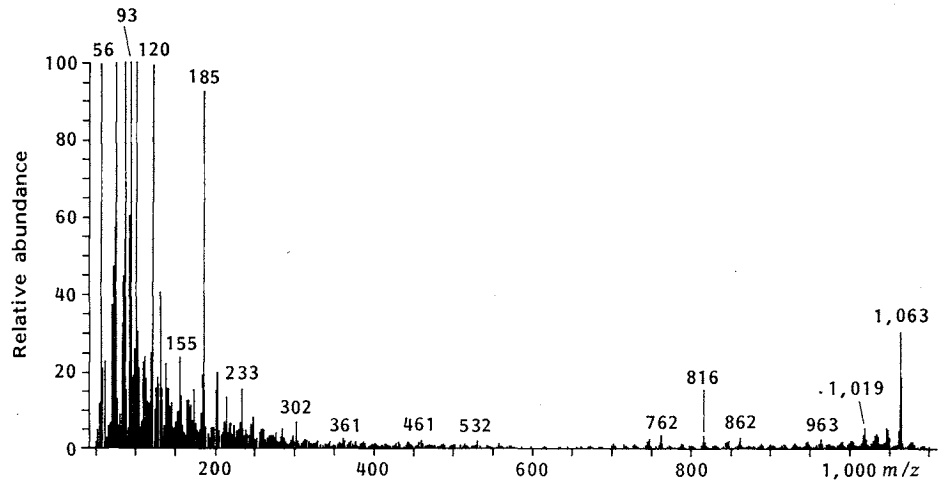


Fig. 3. HPLC profile of DAPB.

See Materials and Methods for analytical HPLC conditions. Sample size: 50 μg.

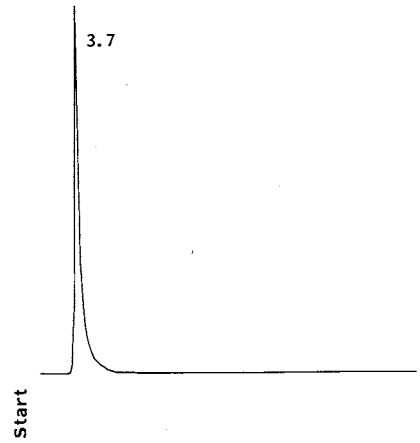


Table 1. Intrinsic antibacterial activity of PMBN and deacylpolymyxins[a].

Polymyxin derivative	MIC (μg/ml)	
	Escherichia coli IH3080	*Salmonella typhimurium* SH5014
PMBN	300	>300
DAPB	30	10
DAC	30	30

[a] As tested in L broth.

molecular ion $(M+H)^+$ had the *m/z* of 1,063, as expected. The mass difference of 100 between *m/z* 1,063 and 963 corresponded to the *N*-terminal amino acid A_2bu. The peaks at *m/z* 862 and 762 can be regarded as having been derived from the further successive elimination of Thr and A_2bu. GC-MS of the fatty acids released from PM indicated that both ante-isononanoic acid (*m/z* 172 (M^+) as the methyl ester) and isooctanoic acid (*m/z* 158 (M^+) as the methyl ester) were removed. Finally, analysis of the DAPB preparation (Fig. 3) indicated that its purity was approximately 99.7%. No peaks having the Rt of PM B1, B2, or B3 were observed in the DAPB preparation.

Intrinsic Antibacterial Activities of the Compounds

The intrinsic antibacterial activities of the compounds are shown in Table 1. As shown earlier, PMBN virtually lacks the direct antibacterial activity[4,5,14], and no inhibition of the growth of *E. coli* or *S. typhimurium* was observed when PMBN concentrations up to 300 μg/ml were tested. In contrast to PMBN, both DAPB and DAC had a measurable inhibitory action (MICs 10~30 μg/ml). Because the deacyl preparations contain no detectable amounts of residual PM, it is rather unlikely that the growth inhibition was due to contaminating PM.

Permeability-increasing Action

Rifampin, fusidic acid, erythromycin, clindamycin, and novobiocin are typical representatives of the large group of hydrophobic antibacterial agents against which the OM of Gram-negative cell wall is known to be an effective permeability barrier[15~18]. These antibiotics do not effectively permeate the intact OM of Gram-negative enteric bacteria but cross the OM of certain OM-defective mutants as well as the OM damaged by chelators or polycations. Accordingly, these five antibiotics were chosen to be probes to detect the OM permeability increase induced by the PM derivatives. Furthermore, instead of exceedingly artificial assay media of low ionic strength[19~21], see also[22], an isotonic NaCl-containing bacterial growth medium (L broth) was used. The results are shown in Tables 2 and 3. As low a DAPB concentration as 1 μg/ml sensitized *E. coli* to the probe antibiotics by a factor of approximately 30~100. *S. typhimurium* was sensitized by a factor of 10~100. A higher concentration of DAPB (3 μg/ml) elicited further sensitization. Also DAC was active. PMBN was clearly and reproducibly somewhat less active than DAPB.

The effective synergism between the hydrophobic antibiotics and all the PM derivatives is also evident in Fig. 4 where the growth of target bacteria is shown as absorbance readings. The results of Fig. 4 indicate

Table 2. MICs (μg/ml) of various hydrophobic antibiotics against *Escherichia coli* IH3080 in the absence and presence of PMBN or deacylpolymyxins.

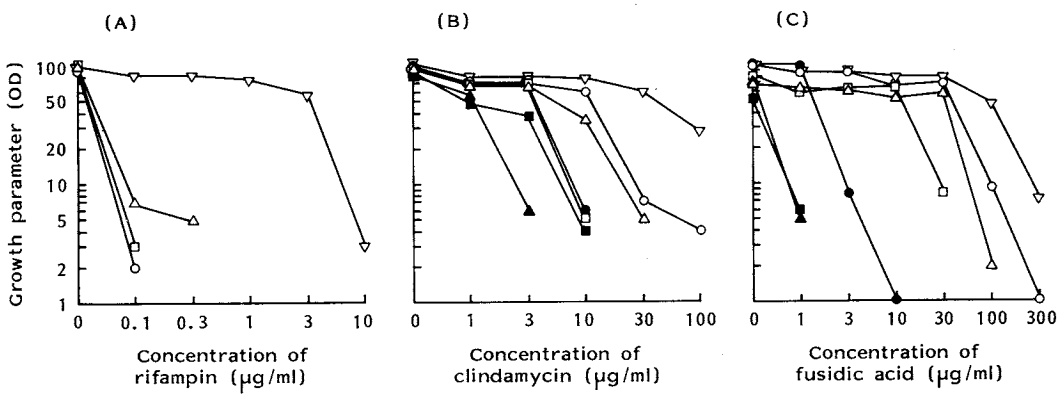
Polymyxin derivative	Rifampin	Fusidic acid	Erythromycin	Clindamycin	Novobiocin
None	10	300	100	300	30
PMBN (1 μg/ml)	0.1	30	30	30	3
DAPB (1 μg/ml)	0.1	10	3	10	1
DAC (1 μg/ml)	0.1	30	10	30	3
PMBN (3 μg/ml)	0.1	10	10	10	3
DAPB (3 μg/ml)	0.1	1	1	10	0.3
DAC (3 μg/ml)	0.1	3	3	3	0.3

Table 3. MICs (μg/ml) of various hydrophobic antibiotics against *Salmonella typhimurium* SH5014 in the absence and presence of PMBN or deacyl derivatives.

Polycation	Rifampin	Fusidic acid	Erythromycin	Clindamycin	Novobiocin
None	10	300	100	300	30
PMBN (1 μg/ml)	1	100	100	10	10
DAPB (1 μg/ml)	0.1	30	10	3	3
DAC (1 μg/ml)	0.1	100	100	100	3
PMBN (3 μg/ml)	0.1	3	10	10	3
DAPB (3 μg/ml)	0.1	1	0.3	1	0.1
DAC (3 μg/ml)	0.1	1	0.3	1	0.1

Fig. 4. PM derivatives as outer membrane permeability-increasing agents.

Bacteria (*Escherichia coli* IH3080 in panels A and B, *Salmonella typhimurium* SH5014 in panel C) were grown in L broth containing increasing concentrations of antibiotics (rifampin, clindamycin, fusidic acid) as well as 1 μg/ml (open symbols) or 3 μg/ml (solid symbols) of PMBN (○ and ●), DAPB (□ and ■), DAC (△ and ▲) or none (▽).



After the incubation of 18 hours at 37°C, the growth was measured (in a multichannel spectrophotometer) and was related to the value 100 (representing the growth in the absence of any antibiotics and PM derivatives).

for instance that 0.1 μg of rifampin per ml was sufficient to inhibit by 97% the growth of *E. coli* when 1 μg of DAPB per ml was present, while, in the absence of DAPB, a 100-fold higher concentration of rifampin was required for the same effect.

Discussion

Agents which increase the permeability of enterobacterial OM to antibiotics and other inhibitors could be expected to be potentially useful in antibacterial therapy. PMs have a high affinity for the lipopolysaccharide constituent of the OM[23,24]. As reviewed in introduction, the fatty acyl A_2bu-lacking PM derivatives (nonapeptides) are effective permeabilizers of the OM. Furthermore, the animal toxicity of PM nonapeptides is significantly lower than that of their parent compounds[2,25~27]. This communication indicates that deacylpolymyxins (PM decapeptides) are also potent OM permeability-increasing agents. DAPB appears to be slightly more active than PMBN as a permeabilizer. In contrast to PMBN, high concentrations of deacylpolymyxins are also directly antibacterial. Due to PM deacylase, which has recently been described by KIMURA *et al.*[11], deacylpolymyxins are easier to prepare and purify than PM nonapeptides and, accordingly, they might be more useful. It would be interesting to compare the toxicity profile of deacylpolymyxins with those of PMs and their nonapeptides.

Acknowledgments

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