POLYMYXIN B OCTAPEPTIDE AND POLYMYXIN B HEPTAPEPTIDE ARE POTENT OUTER MEMBRANE PERMEABILITY-INCREASING AGENTS

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Polymyxin B octapeptide (PBOP) and polymyxin B heptapeptide (PBHP) were found to be effective permeabilizers of the outer membrane of *Escherichia coli* and *Salmonella typhimurium*. PBOP was as effective as polymyxin B nonapeptide (PMBN), the known very potent permeabilizer. As low a PBOP concentration as 1 μg/ml sensitized *E. coli* to rifampicin by a factor of 100. Three μg of PBOP per ml was sufficient to sensitize this target to all the other tested hydrophobic antibiotics (erythromycin, fusidic acid, clindamycin, and novobiocin) by a factor of 30. Only a slightly higher (3-fold) concentration of PBHP was required for a similar sensitizing effect.

The outer membrane (OM) of Gram-negative enteric bacteria and *Pseudomonas aeruginosa* is an effective permeability barrier towards noxious drugs including antibiotics[1]. Agents such as divalent chelators and certain polycations are known to damage and permeabilize the OM and to sensitize the cell to many of those drugs. Chelators remove the stabilizing divalent cations inherent in the OM whereas polycations bind to the acidic lipopolysaccharide constituent of the OM[1]. While the OM permeability-increasing action of chelators is severely blocked by physiologically relevant concentrations of cations[2], that of the most potent polycations (such as polymyxin B nonapeptide, PMBN) is not[2].

Polymyxin nonapeptides (five positive charges, 5+) are significantly less toxic in animal studies than polymyxins[3~6], but able to sensitize, usually at as low concentrations as 1~3 μg, the target bacteria to lipophilic antibiotics (such as erythromycin, clindamycin, rifampicin and fusidic acid) by a factor of 30~300[7~11]. Also deacylpolymyxins[12] (6+), the lysine polymer with approx 20 residues[8,9] (20+) and the arginine-rich peptide protamine[8,9] (17+) are effective sensitizers, while the permeabilizing activity of aminoglycosides (streptomycin; 3+, gentamicin; 5+) is weak or absent in biologically relevant conditions[8,13] (numbers in parentheses indicate the net charge). Weak or very weak permeabilizers also include pentalysine[13] (5+) and the human granulocyte defensins[14] (5+). Polyamines, including spermine (4+), are not permeabilizers[8]. Perhaps surprisingly, linear polymyxin-resembling synthetic peptides (linear arginylpolymyxin B decapeptide; 6+, linear lysylpolymyxin B nonapeptide; 5+) also lack the activity[15].

In order to develop an effective and possibly therapeutically useful polycationic permeabilizer, it should be important to evaluate which factors (total number of positive charges, charge density, favorable conformation of the polycation) are critical for the OM-permeabilizing activity. We will show in this communication that the OM permeability-increasing potency of the octapeptide derivative (5+) of

polymyxin B equals that of PMBN. Furthermore, even the heptapeptide derivative (4+) retains considerable activity. These findings clearly indicate that the free amino groups outside the cyclic peptide moiety of polymyxins are not crucial for the OM permeability-increasing action.

Materials and Methods

Preparation of Polymyxin B Heptapeptide (PBHP) Sulfate

PM B sulfate (200 mg, Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in 10 ml of 0.1 M phosphate buffer (pH 7.0) and 10 mg of Nagarse (from *Bacillus subtilis*, 6.3 DMC units/mg, Serva Feinbiochemica Co., Heidelberg, Germany) was added. The mixture was shaken at 37°C for 48 hours. Samples were withdrawn at intervals and analyzed for the presence of PBHP, Acyl-A_2bu-Thr-A_2bu, and other hydrolysis products by TLC and ninhydrin staining. The TLC solvent was the upper layer of *n*-BuOH - AcOH - H_2O (4 : 1 : 3, v/v) containing 1 : 20 pyridine (v/v). After the pH was lowered to 4 with 1 N H_2SO_4, the insoluble mass was removed by centrifugation (10,000 × *g*, 30 minutes). The supernatant was concentrated *in vacuo* to approx 1 ml, applied on a Sephadex G-25 column (60 × 0.9 cm, i.d., Pharmacia LKB, Uppsala, Sweden), and eluted with water. Fractions were analyzed by TLC as described above and the PBHP-containing fractions were concentrated.

Crude PBHP was further purified twice by reversed-phase HPLC using a YMC-Pack AM 324-10-ODS column (30 × 1.0 cm, i.d., Yamamura Chemical Institute, Kyoto, Japan), isocratic elution with 0.01 N H_2SO_4 in MeOH - H_2O (6 : 4), the flow rate of 1.0 ml/minute, and a variable-wavelength UV detector (875-UV, Japan Spectroscopic, Tokyo, Japan; set at 210 nm). The pH of the PBHP-containing fractions was raised to 4 with 0.1 M NaOH. Na_2SO_4 formed was removed by Micro-Acilyzer using a Aciplex AC-200-10 cartridge (Asahi Chemical Industry Co., Tokyo, Japan). The desalted solution was lyophilized, yielding 50 mg of PBHP sulfate as white powder.

Preparation of Polymyxin B Octapeptide (PBOP) Sulfate

PM B sulfate (200 mg, Sigma) was dissolved in 15 ml of 0.1 M phosphate buffer (pH 7.0) and 10 mg of bromelain (6 units/mg; Boehringer Mannheim, Germany) was added. The mixture was shaken at 37°C for 9 hours. The subsequent isolation of PBOP from the hydrolysate was carried out by using procedures identical to those employed in the purification of PBHP (see above). Seventy mg of PBOP sulfate as white powder was obtained.

Deacylpolymyxin B (DAPB) Sulfate and Polymyxin B Nonapeptide (PMBN) Sulfate

DAPB was prepared and its purity analyzed as described in our previous report[12]. PMBN (lot F7/1984) originated from Farmos Group Ltd. (Turku, Finland).

Purity Analysis of PBHP and PBOP by Using HPLC

Analytical reversed-phase HPLC was performed using a YMC-Pack ODS AM-312 column (150 × 6 mm, i.d.), isocratic elution with 0.01 N H_2SO_4 in MeOH - H_2O (35 : 65), the flow rate of 0.9 ml/minute, and the detection at 210 nm.

Amino Acid Composition Analysis

The amino acid composition analysis was performed with a Hitachi L-8500 automatic amino acid analyzer as described previously[12].

Amino Acid Sequence Analysis

The peptide sequences were determined with a pulse liquid-phase protein sequencer (Applied Biosystems model 473A, Foster City, CA, U.S.A.) from approximately 200 pmol of material.

FAB Mass Spectra

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

Bacterial Strains

Salmonella typhimurium LT2 strain SH5014 and *Escherichia coli* strain IH3080 are typical and well-characterized representatives of members of *Enterobacteriaceae* with normal OM permeability barrier function[7~10,16].

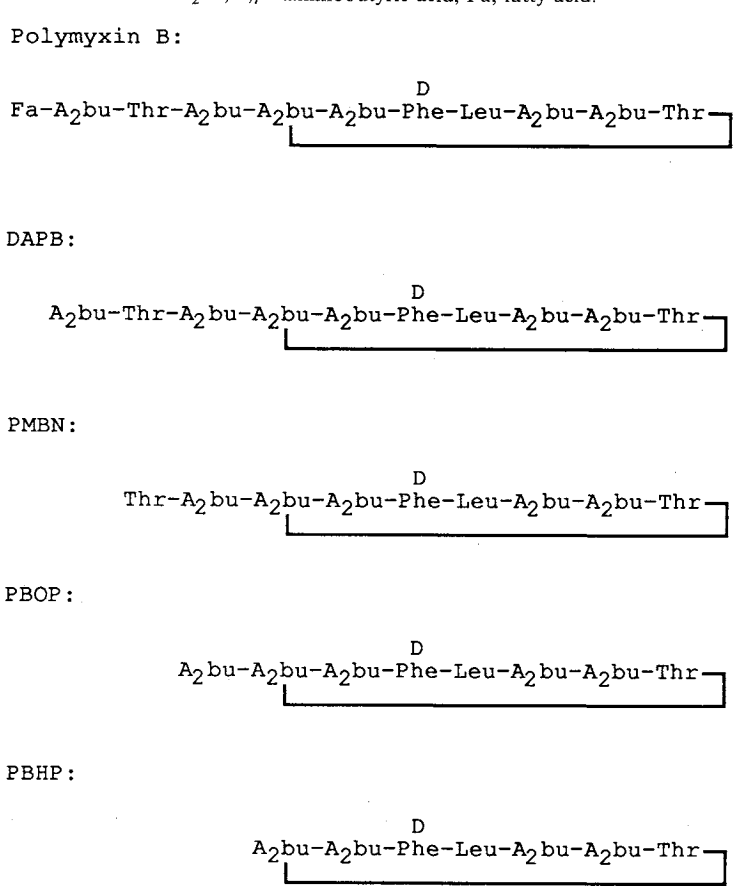
Assay for OM Permeability-increasing Activity

In the assay for OM permeability-increasing activity, antibiotic probes which permeate very poorly the intact OM but which traverse the damaged OM, were used. The assay was performed as a synergistic growth inhibition assay by using checkerboard dilutions and microtiter plates (Nunclon Delta, Nunc, Roskilde, Denmark) essentially as described earlier[10].

The assay medium was L broth and contained (per liter) 10 g of Tryptone (Difco Laboratories, Detroit, MI, U.S.A.), 5 g of yeast extract (Oxoid, Columbia, MD, U.S.A.), and 5 g of NaCl (pH 7.2). L broth containing increasing concentrations of the probe antibiotic (rifampicin, fusidic acid, erythromycin, novobiocin, source as in ref 12) was inoculated (10^4 cells/ml) with bacteria grown on L agar into the stationary growth phase. Aliquots (200 μl) of these inoculated, antibiotic-containing media were then pipetted into wells of a microtiter plate. Each well already contained increasing amounts of the polymyxin derivative to be tested in 20 μl of 0.9% NaCl (use of 0.9% NaCl and rapid addition of the assay medium minimized the adsorption of the polymyxin derivatives onto polystyrene). After an incubation of 18 hours at 37°C, the growth (A_{405}) was measured with a Titertek Multiscan spectrometer (Labsystems, Helsinki, Finland). Uninoculated drug-containing media were used to blank the spectrophotometer. The lowest concentration of an antibiotic which reduced the bacterial growth by $\geq$90% was interpreted as the MIC.

Fig. 1. Structure of PM B and its derivatives DAPB, PMBN, PBOP, and PBHP.

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



Results

Preparation of PBHP

PBHP (Fig. 1) was prepared using Nagarse, the alkaline serine protease of *B. subtilis*. This enzyme has been shown by SUZUKI *et al.*[17] to hydrolyze the A_2bu-A_2bu bond in the side chain of colistin (polymyxin E) and, accordingly, to yield cyclic colistin heptapeptide and the acyl tripeptide Acyl-A_2bu-Thr-A_2bu. Also a related protease, colistinase II from *B. polymyxin* var. *colistinus*, has been demonstrated to give those hydrolysis products of colistin[18].

The hydrolysis gave several peptide fragments as by-products, and therefore, the yield of pure PBHP was rather low. Amino acid composition of the purified PBHP preparation (A_2bu, Thr, Leu, and Phe in a molar ratio of 4.1 : 1.0 : 1.0 : 1.0) corresponded to that expected for PBHP. Edman degradation was applicable (because even the cyclic PBHP molecule has its α-amino terminus unblocked) and gave the expected sequence. The first cycle did not reveal any amino acid, as expected for cyclic PBHP (amide

Fig. 2. FAB-MS spectrum of PBHP (Panel A) and PBOP (Panel B).

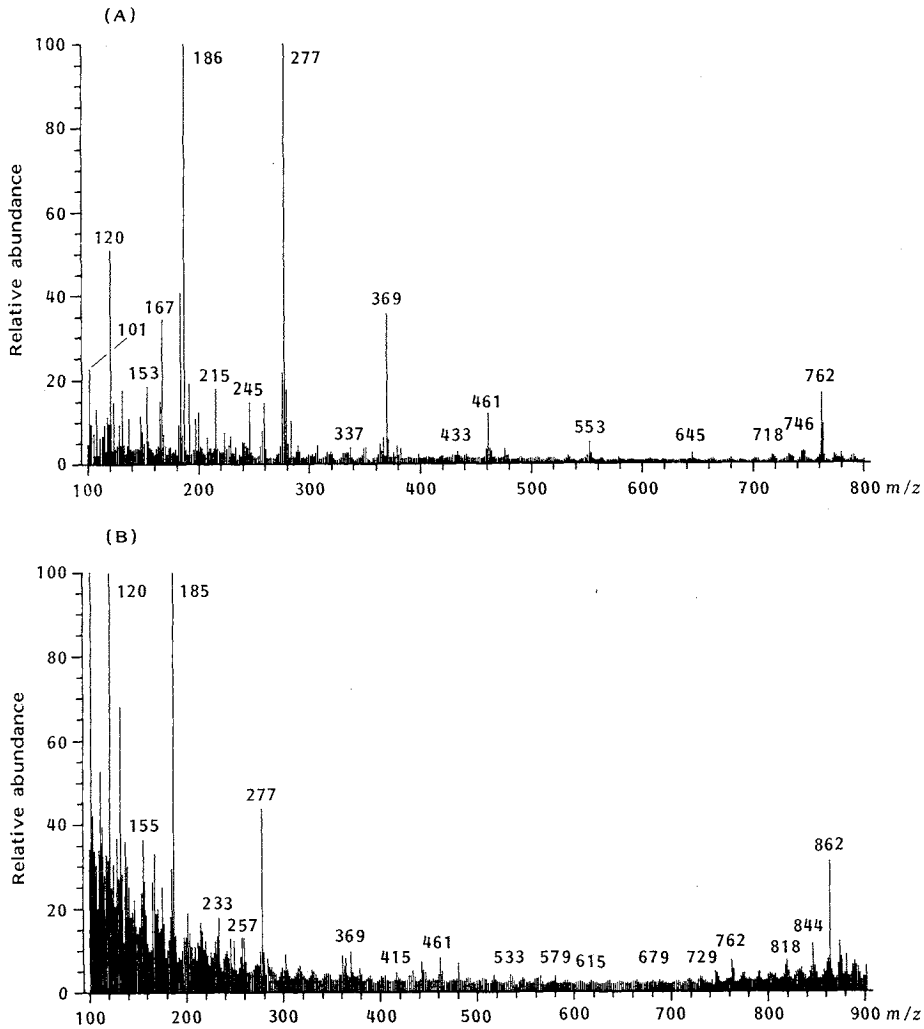
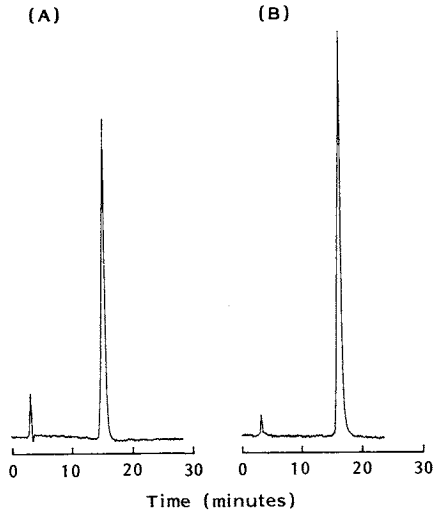


Fig. 3. HPLC of PBHP (Panel A) and PBOP (Panel B).



See Materials and Methods for analytical HPLC conditions. Sample size: 20 μg (for PBHP), 25 μg (for PBOP).

linkage between the γ-amino group of the N-terminal A_2bu residue and the carboxyl group of Thr). In MS (Fig. 2A), the protonated molecular ion $(M+H)^+$ had the *m/z* of 762. This also ascertained the cyclic structure.

In analytical HPLC (Fig. 3A), PBHP had the Rt of 15.1. Occasionally, a minor peak representing not more than 6% of the total was also found. No peak having the Rt of DAPB (12.0), PMBN (12.8), or PBOP (16.0) was observed.

Preparation of PBOP

To prepare PBOP, PM B was treated with bromelain, a plant proteinase which has previously been shown by KIMURA and co-workers to be useful in the preparation of colistin octapeptide[19]. As in the case of PBHP, several by-products were obtained during the hydrolysis and the yield remained rather low. Amino acid composition of the product (A_2bu, Thr, Leu, and Phe in a molar ratio of 5.2 : 1.0 : 1.0 : 1.0) and the amino acid sequence corresponded to that expected for PBOP. In sequencing, no amino acid was detected in the second cycle, indicating that the product had preserved its cyclic structure. In MS (Fig. 2B), the protonated molecular ion $(M+H)^+$ had the *m/z* of 862, as expected. The mass difference of 100 between this *m/z* and 762 (the *m/z* of PBHP, see above) corresponded to A_2bu. HPLC (Fig. 3B) indicated that the preparation was pure.

Intrinsic Antibacterial Activities of the Compounds

The intrinsic antibacterial activities of the compounds are shown in Table 1. No inhibition of the

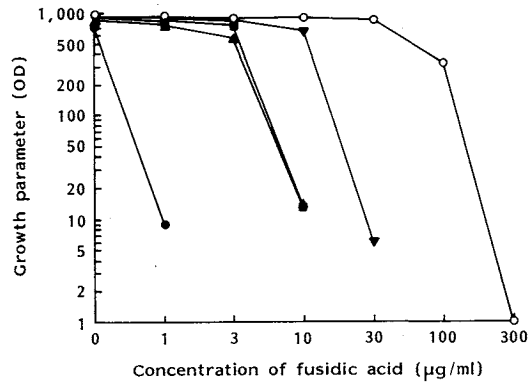
Table 1. Intrinsic antibacterial activity (MICs) of the polymyxin B derivatives studied[a]. The MIC of polymyxin B is also shown.

Polymyxin derivative	MIC (μg/ml) for	
	E. coli IH3080	*S. typhimurium* SH5014
Polymyxin B	0.3	0.3
DAPB	30	10
PMBN	300	>300
PBOP	≥300	≥300
PBHP	≥300	≥300

[a] As tested in L broth.

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

○ None, ● DAPB, ▲ PMBN, ■ PBOP, ▼ PBHP.



Escherichia coli IH3080 was grown in L broth containing increasing concentrations of fusidic acid as well as 3 μg/ml of a PM derivative (DAPB, PMBN, PBOP, or PBHP). After the incubation of 18 hours at 37°C, the growth was measured and related to the value 1,000 (representing the growth in the absence of any antibiotics and PM derivatives).

growth of *E. coli* or *S. typhimurium* was observed when PBHP or PBOP (in concentrations up to 100 μg/ml) were tested. As shown earlier[7,8,12], DAPB was weakly antibacterial (MICs 10~30 μg/ml) while PMBN practically lacked any direct antibacterial action.

Permeability-increasing Action

Both PBOP and PBHP increased the permeability of the OM, as tested in the antibiotic synergism assay (Fig. 4). As low a fusidate concentration as 10 μg/ml was able to inhibit by more than 98% the growth of *E. coli* in the presence of 3 μg of PBOP per ml, while in the absence of PBOP, a 30-fold higher concentration of fusidate was needed to cause the same inhibition. PBOP was as effective as the reference substance, PMBN. PBHP was slightly less active and reduced (at 3 μg/ml) the MIC of fusidate by a factor of 10. As described earlier[12], the other reference, DAPB was more potent than PMBN and reduced (at 3 μg/ml) the MIC of fusidate by a factor of 300. Accordingly, the positive charges present in the terminal A_2bu residue of DAPB are required for full permeabilizing activity but a notable activity remains when this A_2bu residue is removed.

Table 2 shows the results obtained by using increasing concentrations of the PM derivatives and several probe antibiotics. Identical amounts (1 μg/ml) of PMBN and PBOP were needed to sensitize *E. coli* to rifampicin by a factor of 100. Three μg/ml of PBOP was sufficient to sensitize the target to all the other tested antibiotics by a factor of ≥30, whereas in the case of two of these antibiotics, a somewhat larger amount of PMBN was required for a similar effect. Accordingly, it can be concluded that PBOP is a remarkably effective permeabilizer. Also PBHP was notably active, only slightly (maximally 3-fold)

Table 2. MICs of various hydrophobic probe antibiotics against *Escherichia coli* IH3080 in the absence and presence of the PM derivatives.

PM derivative	Probe antibiotic	MIC (μg/ml) of the probe antibiotic						
		PM derivative concentration (μg/ml)						
		0	0.3	1	3	10	30	100
DAPB	Rifampicin	10	3	0.1	0.1	0.1	0[a]	0
PMBN	Rifampicin		3	0.1	0.1	0.1	0.1	0
PBOP	Rifampicin		1	0.1	0.1	0.1	0.1	0.1
PBHP	Rifampicin		10	1	0.1	0.1	0.1	0.1
DAPB	Erythromycin	100	100	1	1	1	0	0
PMBN	Erythromycin		100	30	10	3	3	0
PBOP	Erythromycin		30	10	3	3	1	0.3
PBHP	Erythromycin		100	30	10	3	3	1
DAPB	Novobiocin	30	10	1	1	1	0	0
PMBN	Novobiocin		30	10	3	1	1	0
PBOP	Novobiocin		30	10	1	1	1	0.3
PBHP	Novobiocin		30	10	3	1	1	0.3
DAPB	Fusidic acid	300	100	1	1	1	0	0
PMBN	Fusidic acid		100	30	10	10	3	0
PBOP	Fusidic acid		100	30	10	3	1	1
PBHP	Fusidic acid		300	100	30	10	3	1
DAPB	Clindamycin	300	100	10	3	3	0	0
PMBN	Clindamycin		100	30	10	10	10	0
PBOP	Clindamycin		100	30	10	10	10	3
PBHP	Clindamycin		100	30	10	10	10	10

[a] 0, The PM derivative itself inhibited the growth.

less active than PBOP or PMBN (Table 2). Similar results were obtained when *S. typhimurium* was used as the test organism instead of *E. coli* (data not shown).

Discussion

In this paper, we have shown that PBOP is as effective a permeabilizer of the OM as PMBN. This is a new and remarkable finding and gives us evidence to suggest that, in the OM permeability-increasing action, the peptide side chain of the PM derivatives is less crucial than the cyclic portion. The preparation and purification of PBOP is at least as complicated as that of PMBN. However, our data can now act as a basis for the development of analogous, biologically active synthetic peptides.

The other finding in our study is even more significant. We were able to show that PBHP is as a permeabilizer almost as active as PMBN. This indicates that the suitable, compact array of the free amino groups of the heptapeptide ring gives polymyxin peptides their unique permeabilizing properties. Our PBHP preparation was only approx 3-fold less active than PMBN whereas ITO-KAGAWA and KOYAMA have previously shown that the analogous derivative of colistin, colistin heptapeptide (CHP) is approx 100-fold less active than colistin nonapeptide[11]. The difference between these results is remarkable and requires further study. The original chemical study[18] describing the preparation of CHP showed evidence that the preparation was cyclic (as is our PBHP). Therefore, it is probable that the CHP preparation used in the biological studies of ITO-KAGAWA and KOYAMA was also cyclic. No traces of DAPB, PMBN, or PBOP were found in our PBHP preparation, hence its activity was not due to any contamination by those biologically active PM derivatives. On the other hand, it is possible that acyl migration (N→O and/or $N^{\alpha}\rightarrow N^{\gamma}$) takes place during the enzymatic treatment and the purification of polymyxin heptapeptides[20]. The procedures employed in the present work and in that of ITO-KAGAWA and KOYAMA[18] remarkably differed from each other. In this light, our finding is especially important since it indicates that under certain conditions, a biologically active preparation can be achieved.

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