ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2008, p. 3229–3236 Vol. 52, No. 9
0066-4804/08/$08.00+0 doi:10.1128/AAC.00405-08

Novel Polymyxin Derivatives Carrying Only Three Positive Charges Are Effective Antibacterial Agents▽

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Received 25 March 2008/Returned for modification 25 May 2008/Accepted 22 June 2008

The lack of novel antibiotics against gram-negative bacteria has reinstated polymyxins as the drugs of last resort to treat serious infections caused by extremely multiresistant gram-negative organisms. However, polymyxins are nephrotoxic, and this feature may complicate therapy or even require its discontinuation. Like that of aminoglycosides, the nephrotoxicity of polymyxins might be related to the highly cationic nature of the molecule. Colistin and polymyxin B carry five positive charges. Here we show that novel polymyxin derivatives carrying only three positive charges are effective antibacterial agents. NAB739 has a cyclic peptide portion identical to that of polymyxin B, but in the linear portion of the peptide, it carries the threonyl–D-serinyl residue (no cationic charges) instead of the diaminobutyryl-threonyl-diaminobutyryl residue (two cationic charges). The MICs of NAB739 for 17 strains of *Escherichia coli* were identical, or very close, to those of polymyxin B. Furthermore, NAB739 was effective against other polymyxin-susceptible strains of *Enterobacteriaceae* and against *Acinetobacter baumannii*. At subinhibitory concentrations, it dramatically sensitized *A. baumannii* to low concentrations of antibiotics such as rifampin, clarithromycin, vancomycin, fusidic acid, and meropenem. NAB739 methanesulfonate was a prodrug analogous to colistin methanesulfonate. NAB740 was the most active derivative against *Pseudomonas aeruginosa*. NAB7061 (linear portion of the peptide, threonyl-aminobutyryl) lacked direct antibacterial activity but sensitized the targets to hydrophobic antibiotics by factors up to 2,000. The affinities of the NAB compounds for isolated rat kidney brush border membrane were significantly lower than that of polymyxin B.

Whereas pharmaceutical companies have in recent years developed a considerable number of new drugs against multiresistant gram-positive bacteria, virtually no novel drugs, with the exception of tigecycline, are suitable for systemic therapy of infections caused by gram-negative bacteria (9, 14, 22, 27). Simultaneously, these bacteria are progressively becoming more and more multiresistant (extremely multiresistant [XMR]), or even panresistant, to the clinically available antibacterial agents (7, 14, 21, 27).

Today, major therapeutic problems are caused by carbapenemase-producing XMR strains of *Klebsiella pneumoniae*. The other notorious bacteria include the XMR strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. However, in the coming years, a far more serious threat will be the emergence of carbapenemase-producing XMR strains of *Escherichia coli*, a very common opportunistic pathogen. Accordingly, the need for novel agents against gram-negative bacteria is serious. The lack of such agents would result in a literal return to the pre-antibiotic era (23).

The current crisis has reinstated polymyxins as the drugs of last resort for treatment of serious infections caused by gram-negative XMR strains (4, 11, 12, 25, 35). Colistin (polymyxin E) and polymyxin B are cyclic lipodecapeptides, each carrying five free amino groups and, accordingly, five positive charges under physiological conditions (24, 26). Their first target in susceptible gram-negative bacteria is the outer membrane (OM), where these amino groups bind to the acidic lipopolysaccharide (LPS) molecules (18, 30). Polymyxins were discovered as early as 1947 but were largely abandoned owing to their nephrotoxicity and neurotoxicity and the discovery of better-tolerated drugs. Even though the toxicity of polymyxins may not be as high as that observed in the different settings of the past, it might still complicate the patient's therapy or even require its discontinuation (4, 5, 11, 12, 25, 35).

Numerous efforts have been made to render polymyxin less toxic. In colistin methanesulfonate (CMS), the free amino groups are blocked by sulfomethylation to yield an uncharged prodrug. CMS hydrolyzes in vivo as well as in aqueous solutions in vitro to liberate the compound with antibacterial activity (6, 10). In rats, an intravenous bolus of 3 mg of polymyxin B per kg of body weight (3) or 3 mg of colistin per kg (11) caused acute signs of toxicity (dyspnea, piloerection, and decreased movement), while no such signs were observed after an intravenous bolus of 15 mg of CMS per kg (11). CMS is currently used in systemic therapy. However, the nephrotoxicity of CMS does not appear to be lower than that of the unsulfomethylated form of polymyxin B currently used in systemic therapy (5). Furthermore, the degree of sulfomethylation differs between different CMS preparations (6, 10, 11).

Another approach to reducing toxicity has been to treat polymyxin with ficin or related enzymes that remove the lipid moiety (the 6-methyloctanoyl or 6-methylheptanoyl residue).

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▽ Published ahead of print on 30 June 2008.

The N-terminal amino acyl residue is lost as well. The resultant polymyxin nonapeptides (colistin nonapeptide and polymyxin B nonapeptide [PMBN]) have less acute toxicity than their parent polymyxins (3, 30) but are devoid of antibacterial activity. However, PMBN and colistin nonapeptide are very effective in synergistic combination with those antibacterial agents that are effectively excluded by the intact OM (8, 20, 30, 32, 33). Unfortunately, PMBN retains the nephrotoxicity of polymyxin B (30). It should be noted that PMBN and colistin nonapeptide carry five positive charges.

In this study, we evaluated the structure-function relationships and antibacterial properties of novel synthetic polymyxin derivatives that differ from all natural polymyxins by possessing no more than three positive charges under physiological conditions. Such derivatives can be expected to have toxicological and pharmacokinetic properties different from those of natural polymyxins.

MATERIALS AND METHODS

Peptide synthesis. Polymyxin derivatives were synthesized by conventional solid-phase chemistry using the standard Fmoc (fluorenylmethoxy carbonyl) protection strategy. The α-amino function was protected by Fmoc, and Fmoc was removed by 20% piperidine in dimethylformamide. The γ-amino group of the diaminobutyryl (Dab) residue involved in cyclization of the peptide was protected by *t*-butoxycarbonyl, an acid-labile group that was removed at the cleavage step. The functional group of asparagine was protected by tritylation. All the other amino acids with functional side chain groups were protected by benzyloxycarbonyl (Z), a group that is stable in the acid cleavage stage but can easily be removed after the cyclization reaction.

The synthesis employed *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HCTU) or *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) as an activator, and the steps were performed in a commercial automated synthesizer. The amino acids were purchased already protected from a standard supplier. Acylation was performed for 30 min by using a fourfold molar excess of each amino acid or the fatty acid, a fourfold molar excess of the activator HCTU or TBTU, and an eightfold molar excess of *N*-methyl morpholine.

The peptide was removed from the resin by reaction with a solution of 95% trifluoroacetic acid and 5% water for 2 h at room temperature, to yield the partially protected product. The resulting peptide was precipitated with diethyl ether.

The cyclization mixture comprised benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop), *N*-hydroxybenzotriazole, and *N*-methyl morpholine at molar excesses of 2, 2, and 4, respectively. The cyclization mixture was added to the peptide (dissolved in dimethylformamide) and allowed to react for 2 h. The cyclized, protected peptide was precipitated by the addition of cold diethyl ether. Any residual PyBop was removed by washing the peptide with water. The remaining side chain protection groups (Z) were removed by catalytic dehydrogenation (i.e., by subjecting the peptide, dissolved in acetic acid–methanol–water [5:4:1], to an atmosphere of hydrogen in the presence of a palladium charcoal catalyst).

The deprotected and cyclized peptides were purified by reversed-phase chromatography using conventional gradients of acetonitrile–water–trifluoroacetic acid. The product was dried by lyophilization. The purity, as estimated by reversed-phase high-performance liquid chromatography, was more than 95%.

Preparation of NAB739 methanesulfonate sodium salt. NAB739 methanesulfonate was synthesized using essentially the method described previously (34) for the synthesis of polymyxin B methanesulfonate. First a neutral formaldehyde solution (400 μl of 30% aqueous formaldehyde [brought to pH 7.2 with 1 N $NaHCO_3$]) and then a 1 N $NaHCO_3$ solution (2 ml) were added to NAB739 acetate (100 mg dissolved in 2 ml of water). The precipitated NAB739 formaldehyde derivative was filtered and washed with water. The moist solid was suspended in water (5 ml), and sodium metabisulfite (100 mg) was added. A clear solution was obtained after a few minutes and was freeze-dried. The flocculent white solid was extracted with warm acetone (7.5 ml) and dried in vacuo. The yield was 78 mg. Analysis of the product by electrospray ionization mass spectrometry revealed a predominant peak with a molecular mass of 1,075.3 Da, indicating that most of the derivative was sulfomethylated at each of the three Dab residues of the NAB739 compound. A minor peak representing NAB739 blocked randomly at two of the three Dab residues was also visible.

Microbial strains. *E. coli* IH3080 (K1:O18) is an encapsulated strain originally isolated from a neonate suffering from meningitis (33). Other *E. coli* strains included ATCC 25922; three extended-spectrum β-lactamase-producing strains (NCTC13351, NCTC13352, and NCTC13353) from the National Collection of Type Cultures, Colindale, United Kingdom; seven strains isolated from blood cultures in Sweden (from the Culture Collection, University of Gothenburg, Sweden [CCUG]); and five strains isolated from blood cultures at Helsinki University Hospital, Helsinki, Finland, in 2006 to 2007 (the F strains). Other microbial strains were from the ATCC (11 strains), the CCUG (3 strains), and Helsinki University Hospital (8 strains). *E. coli* SC 9252 and *E. coli* SC 9253 are well-characterized polymyxin-resistant *pmrA* mutants of *E. coli* K-12 (19, 26).

Antibacterial assays. To screen for both the direct antibacterial activities of the compounds and their abilities to sensitize a set of target bacteria to the hydrophobic model antibiotic (rifampin), the simple agar well diffusion method was used. LB agar plates (LB Agar Lennox; Difco, BD, Sparks, MD) and LB plates containing increasing concentrations (0.1 μg/ml, 0.3 μg/ml, and 1.0 μg/ml) of rifampin (Sigma-Aldrich, St. Louis, MO) were used. The plates were inoculated with bacterial suspensions in 0.9% NaCl (a 0.5 McFarland standard), according to CLSI standard M2-A9 (1) to produce even, confluent growth. Thereafter, small wells were cut (diameter, 2 mm; five wells per plate). Four quantities (0.4 μg, 1 μg, 4 μg, and 10 μg) of each compound dissolved in 0.9% NaCl were tested. Controls included 0.9% NaCl alone, as well as solutions of peptides with previously determined activities, such as polymyxin B sulfate (P0972; Sigma-Aldrich) and PMBN (Sigma-Aldrich). After an 18-h incubation at 37°C, the diameters of the growth inhibition zones were measured, and the corresponding surface areas (in square millimeters) were calculated.

MICs were determined using the agar dilution method according to CLSI protocol M7-A7 (2). Mueller-Hinton agar (Difco 225220; BD, Sparks, MD) plates containing increasing concentrations (0.25 to 32 μg/ml in twofold increments) of the test substances were used. The plates were inoculated (1 μl per spot, corresponding to approximately 10^4 CFU/spot) using a multipoint inoculator and were incubated for 16 to 20 h at 35°C.

The synergism of the compounds with other antibiotics was studied using E-strips (Biodisk Ltd., Solna, Sweden) on plates containing Mueller-Hinton agar (product no. LabO39; LabM Ltd., Bury, Lancashire, United Kingdom) with increasing concentrations of the compound under study (for NAB739, 0.125 μg/ml to 2 μg/ml in twofold increments; for NAB7061, 1 μg/ml to 16 μg/ml in twofold increments) as well as on Mueller-Hinton agar without the compounds. The inoculum was prepared according to the manufacturer's instructions and corresponded to that in CLSI standard M2-A9 (1). When the synergistic effects of NAB7061 on a large number of bacterial strains were studied, only one concentration of NAB7061 was used (4 μg/ml). Synergism with rifampin (Sigma-Aldrich) was also studied in microtiter plates using the checkerboard method, cation-adjusted Mueller-Hinton II broth (Difco 212322), and an inoculum size of 5×10^5 CFU/ml according to CLSI standard M7-A7 (2).

Affinities of the compounds to the BBM of the renal cortex. The binding of the compounds to the brush border membrane (BBM) of the renal cortex was measured indirectly by their abilities to inhibit the binding of radiolabeled gentamicin to the BBM. BBMs were isolated from the renal cortices of male albino rats by using the Mg^{2+}–EGTA precipitation technique as described previously (22). The binding of gentamicin was measured, according to the method described by Nagai et al. (16), by incubating BBM vesicles (20 μl) in 10 mM HEPES (pH 7.5) buffer with 300 mM mannitol in the presence of 20 μM [^{3}H]gentamicin (American Radiolabeled Chemicals, Inc., St. Louis, MO) with or without the compound to be tested or a positive control. After incubation for 60 min at 4°C, 1 ml of this buffer, ice-cold, was added, and the mixture was filtered through a Millipore filter (pore size, 0.45 μm; HAWP). The filter was washed with the buffer, and the radioactivity remaining in the filter was measured using a liquid scintillation counter. Fifty percent inhibitory concentrations (IC_{50}s) were determined as described previously (16) using the Hill equation.

Cytotoxicity assay on V79 cells. Cytotoxicity assays were performed using the Chinese hamster lung fibroblast cell line V79 (European Collection of Cell Cultures, Salisbury, United Kingdom). The cells were grown in Dulbecco's modified Eagle medium supplemented with 1% (vol/vol) antibiotic-antimycotic solution (10,000 U/ml penicillin, 10 μg/ml streptomycin, and 25 μg/ml amphotericin B), glutamine (2 mM), and heat-inactivated fetal bovine serum (10%), all from Sigma-Aldrich. The culture (2 to 3 days old; more than 50% confluent) was trypsinized, and a cell suspension in fresh medium was made and used to seed 60-mm-diameter petri dishes containing 5 ml of culture medium with approximately 200 cells each. Three replicate dishes were prepared for each treatment. After the dishes were incubated for 24 h at 37°C under a humidified atmosphere containing approximately 5% CO_2, the culture medium was replaced with fresh culture medium containing the appropriate concentrations (0.25 μg/ml to 128

TABLE 1. Antibacterial activities of the NAB compounds against *E coli* IH3080

Compound name	Structure[a]			No. of positive charges	Activity[b]	
	Fatty acyl moiety	Peptide sequence				
		Linear portion	Cyclic portion		Direct	With rifampin
Polymyxin B	MO(H)A	XTX	[XXfLXXT]	5	120 ± 11[c]	125 ± 17[d]
PMBN		+TX	[*******]	5	≤5 ± 0[c]	143 ± 34[c]
NAB7061	OA	TZ	[*******]	3	32 ± 22[c]	194 ± 27[c]
NAB718	OA	TZ	[**l****]	3	0	100
NAB719	OA	TZ	[******L]	3	50	180
NAB715	OA	TX	[*Z*****]	3	0	100
NAB716	OA	TX	[****Z**]	3	60	250
NAB717	OA	TX	[*****Z*]	3	0	170
NAB708	OA	TZ	[****Z**]	2	0	10
NAB726	OA	XTX	[*Z**Z**]	3	0	0
NAB725	OA	XTX	[*Z***Z*]	3	0	10
NAB739	OA	Ts	[*******]	3	171 ± 12[c]	214 ± 27[c]
NAB740	DA	Ts	[*******]	3	130	150
NAB743	OA	TS	[*******]	3	80	230
NAB746	OA	TN	[*******]	3	10	180
NAB733	OA	aa	[*******]	3	0	200
NAB734	OA	Ts	[***T***]	3	172 ± 25[d]	190
NAB737	OA	Tt	[***T***]	3	200	250
NAB738	OA	Ta	[***T***]	3	60	230
NAB748	OA	TTs	[*******]	3	160	190
NAB749	OA	ATs	[*******]	3	160	200

[a] One-letter codes for amino acyl residues are as follows: A, Ala; F, Phe, L, Leu; N, Asn; S, Ser; T, Thr; X, diaminobutyric acid; Z, aminobutyric acid. Lowercase underlined letters represent residues that are in the D configuration. MO(H)A is a mixture of methyl octanoic and methyl heptanoic acids; OA, octanoic acid; DA, decanoic acid. Asterisks in the cyclic-portion sequences indicate amino acyl residues identical to those in polymyxin B.

[b] Growth inhibition (in square millimeters) around a well that contains 10 μg of the peptide on an LB plate without rifampin (direct activity) or with rifampin (1 μg/ml).

[c] Values are means ± SD from at least three (three to eight) independent experiments.

[d] Values are means ± SD from two independent experiments.

μg/ml in twofold increments) of the test item or the positive control (phenol). After the 24-h treatment period under the growth conditions specified above, cultures were washed three times, covered with fresh medium, left to form colonies for 3 days, fixed with methanol, and stained with 5% Giemsa solution; then the colonies were counted.

RESULTS

Structure-function relationships of the polymyxin derivatives. To reduce the number of cationic charges, we removed both cationic Dab residues from the linear portion of the polymyxin molecule. NAB7061 lacks the N-terminal Dab of all polymyxins and carries the neutral aminobutyryl (Abu) residue instead of Dab at the C terminus of the linear portion of the peptide (Table 1). Furthermore, NAB7061 carries octanoyl residues as the fatty acyl residues, instead of a mixture of methyloctanoyl and methylheptanoyl residues (present in polymyxin B). Its cyclic portion is identical to that of polymyxin B.

As the test organism, we used the virulent encapsulated *E. coli* strain IH3080 (O18:K1), the same strain systematically used in our earlier studies. The direct antibacterial activity of NAB7061 was very weak, but the compound remarkably enhanced the activity of rifampin, indicating that it damages the bacterial OM permeability barrier in the same way as PMBN, the best-characterized OM permeability-increasing agent (30).

Essentially similar results were obtained with NAB718 and NAB719. The cyclic portions of these peptides are identical to those of colistin (polymyxin E) and polymyxin T, respectively.

Substituting a neutral amino acyl residue (Abu) for one of the cationic Dab residues in the cyclic portion of the peptide had little effect on the permeability-increasing activity when this change was compensated for by the introduction of one cationic residue in the linear portion of the peptide, as in compounds NAB715, NAB716, and NAB717. Without this compensation, the compound was virtually inert, as shown for NAB708. Substituting two Abu residues for the corresponding Dab residues in the cyclic peptide resulted in inactive compounds, even when the loss of positive charges in the cyclic portion of the peptide was compensated for by the introduction of two Dab residues in the linear portion of the peptide, as in NAB725 and NAB726.

In very sharp contrast to NAB7061 and all the other permeability-increasing polymyxin peptides that possess Abu at the C terminus of the linear portion of the peptide, NAB734, NAB737, and NAB739 all turned out to be very effective as direct antibacterial agents (Table 1). Their activities appeared to be superior even to that of polymyxin B. The linear peptide portions of NAB734 and NAB739 consist of Thr–D-Ser, while that of NAB737 consists of Thr–D-Thr. All these residues carry hydroxyl groups. The cyclic portion of NAB739 is identical to that of polymyxin B, while the cyclic portions of NAB734 and NAB737 are identical to that of polymyxin S.

Compared with NAB739, compounds NAB748 and NAB749, with linear portions Thr–Thr–D-Ser and Ala–Thr–D-Ser, respectively, were as active; NAB743 (with Thr-Ser) was somewhat less active; and compounds with Thr-Asn (NAB746), D-Ala–D-Ala (NAB733), or Thr–D-Ala (NAB738) were much less active as direct antibacterial agents.

TABLE 2. MICs of rifampin for *E. coli* ATCC 25922 in the presence of increasing concentrations of NAB739 or NAB7061[a]

Peptide concn (μg/ml)	Rifampin MIC (μg/ml)[b] in the presence of the indicated peptide by the following method:			
	Etest		Checkerboard	
	NAB739	NAB7061[c]	NAB739	NAB7061
No peptide	16	8–16	8	8
0.25	12	ND	8	ND
0.5	8	ND	8	ND
1	NA	0.19–0.38	8	8
2	NA	0.064–0.094	NA	0.5
4	NA	0.016–0.047	NA	0.125
8	NA	0.006	NA	0.031
16	NA	NA	NA	0.031

[a] Determined by Etest on Mueller-Hinton agar containing increasing concentrations of the peptide as well as by the checkerboard method in Mueller Hinton II broth. The FICI in the presence of NAB7061 were 0.071 to 0.078 by Etest and ≤0.128 by the checkerboard method (results of two independent determinations).

[b] ND, not done; NA, the peptide alone inhibited growth.

[c] Results of five (for no peptide and 4 μg/ml) or two (for all other concentrations) independent determinations.

Also a direct antibacterial agent, NAB740 differed from NAB739 by only two additional C atoms in its fatty acyl chain.

Similar findings were obtained in screening assays that employed *A. baumannii* ATCC 19606. Against this strain, NAB7061, NAB733, NAB738, and NAB746 had direct antibacterial activities (expressed as surface areas of growth inhibition) of 0 to 20 mm^2 only, whereas NAB739, polymyxin B, and NAB734 produced inhibition areas of 169 ± 26, 142 ± 26, and 87 ± 11 mm^2, respectively (averages ± standard deviations [SD] from two to five independent experiments). Furthermore, very similar differences in activities against *E. coli* IH3080 were found in screening assays that employed smaller amounts of the same compounds (0.4 μg, 1 μg, and 4 μg per well).

To show that the direct antibacterial activity of NAB739 is due to the presence of free amino groups in the cyclic portion of the peptide, we synthesized NAB739 methanesulfonate, a compound in which the free amino groups are sulfomethylated to yield a labile compound that slowly decomposes to free NAB739 (the nascent compound). In analogy to colistin methanesulfonate (see the introduction), a freshly made aqueous solution of NAB739 methanesulfonate was found to be only weakly bactericidal. When tested at a concentration of 2 μg/ml, it reduced the CFU of *E. coli* IH3080 only by approximately one-third, while a 2-day-old solution of NAB739 methanesulfonate or the control (free NAB739) reduced the CFU by 90% or 98%, respectively.

Comparison of the properties of NAB739 and NAB7061. NAB739 differs from NAB7061 only in possessing D-Ser instead of Abu in the C terminus of the linear portion of the peptide. The remarkable effect of this difference on antibacterial activity was verified by determining the MICs of these two compounds against *E. coli* ATCC 25922. Two methods were used: dilution in Mueller-Hinton agar and microdilution in Mueller-Hinton II broth (Table 2). In addition, to assess the observed synergism with rifampin in more detail, the fractional inhibitory concentration indices (FICI) of both compounds with rifampin were determined using two methods: the Etest on Mueller-Hinton agar containing increasing concentrations of the compound and the checkerboard method in Mueller-Hinton II broth. The MIC of NAB739 alone ranged from 1 μg/ml to 2 μg/ml. No measurable synergism with rifampin was found by either of the two tests. On the other hand, the MIC of NAB7061 ranged from 16 μg/ml to >16 μg/ml, and very strong synergism with rifampin was observed, with FICI as low as 0.071 to 0.078 by Etest and ≤0.128 by the checkerboard method. Accordingly, the alteration of a single amino acyl residue results in a very significant difference in biological activity.

MICs of NAB739, NAB740, and NAB7061. The MICs of NAB739 against *E. coli* were very close to those of polymyxin B (Table 3). For clinical isolates of *E. coli* (15 strains), both the MIC at which 50% of isolates were inhibited (MIC_{50}) and the MIC_{90} of NAB739 and polymyxin B were 1 μg/ml. For *Klebsiella* spp., *Enterobacter cloacae*, and *Citrobacter freundii* (11

TABLE 3. MICs (μg/ml) of selected NAB peptides and polymyxin B

Organism	MIC (μg/ml) of:			
	NAB739	NAB740	NAB7061	Polymyxin B
E. coli ATCC 25922	1	2	>32	1
E. coli IH3080	1	2	>32	0.5
E. coli (15 strains)[a]	0.5–1 (1, 1)	1–2 (2, 2)	16–>32 (>32, >32)	0.25–1 (1, 1)
K. pneumoniae CCUG45421	4	4	>32	1
K. pneumoniae (3 strains)	1–2	2–4	>32	0.5–1
K. oxytoca ATCC 13182	2	2	32	0.5
K. oxytoca CCUG51683	2	2	32	0.5
E. cloacae ATCC 23355	2	2	32	0.5
E. cloacae CCUG52947	2	2	>32	1
E. cloacae (3 strains)	2	2	32–>32	0.5
C. freundii ATCC 8090	2	2	16	1
A. baumannii ATCC 19606	8	4	>32	1
A. baumannii (2 strains)	4	2	>32	0.5–1
S. marcescens ATCC 8100	>32	>32	>32	>32
P. aeruginosa ATCC 27853	8	4	>32	1
E. coli SC 9252	32	8	>32	>32
E. coli SC 9253	>32	16	>32	>32
S. aureus ATCC 25923	>32	>32	>32	>32
C. albicans ATCC 28366	>32	>32	>32	>32

[a] Values in parentheses are the MIC_{50} and MIC_{90}, respectively.

TABLE 4. MICs of five antibiotics for *A. baumannii* and *P. aeruginosa* in the presence of NAB739[a]

Bacterial strain	Antibiotic	MIC (μg/ml)[b] at the following concn (μg/ml) of NAB739:						FICI
		0	0.125	0.25	0.5	1	2	
A. baumannii ATCC 19606	Clarithromycin	8	1.5	1	0.5	0.094	NA	0.250
	Rifampin	4	0.25	0.125	0.047	0.006	NA	0.126
	Vancomycin	>256	64	8	3	1	NA	≤0.156
	Fusidic acid	>256	32	12	4	1	NA	≤0.172
	Meropenem	1	1	0.5	0.25	0.125	NA	0.500
A. baumannii F264	Clarithromycin	6	2	1.5	0.5	0.125	NA	0.333
	Rifampin	12	0.5	0.38	0.094	0.016	NA	0.105
	Vancomycin	>256	256	16	3	1	NA	≤0.188
	Fusidic acid	>256	32	12	3	1	NA	≤0.172
	Meropenem	>32	32	6	1	0.38	NA	0.281
P. aeruginosa ATCC 27853	Clarithromycin	48	32	32	16	12	3	≤0.46
	Rifampin	32	32	32	32	16	4	
	Vancomycin	>256	>256	>256	>256	>256	>256	
	Fusidic acid	>256	>256	>256	>256	>256	192	
	Meropenem	0.25	0.25	0.25	0.25	0.25	0.25	

[a] Tested by using E-strips on Mueller-Hinton agar containing increasing concentrations of NAB739.
[b] NA, not applicable; NAB739 alone inhibited growth.

strains altogether), the MICs of NAB739 ranged from 1 μg/ml to 2 μg/ml, whereas for *K. pneumoniae* CCUG45421, the MIC was 4 μg/ml.

NAB740 was almost as active as NAB739 against *Enterobacteriaceae*, but for *P. aeruginosa* and *A. baumannii*, its MICs (4 μg/ml and 2 to 4 μg/ml, respectively) were slightly lower than those of NAB739. The MICs of NAB7061 were very high for all the strains studied. Bacteria that are known to be resistant to polymyxin B were resistant to NAB739 and NAB740, as well. These included *Serratia marcescens*, the polymyxin-resistant *pmrA* mutants of *E. coli* (19, 26), and microbes that do not belong to the gram-negative bacteria (*Staphylococcus aureus*, *Candida albicans*).

Effects of subinhibitory concentrations of NAB739 on the susceptibilities of *A. baumannii* and *P. aeruginosa* to antibacterial agents. The MICs of five antibacterial agents were determined for two strains of *A. baumannii* (ATCC 19606 and F264) and for *P. aeruginosa* ATCC 27853 by the Etest method on Mueller-Hinton agar containing increasing concentrations of NAB739 (Table 4).

Interestingly, NAB739, at a concentration as low as 0.5 μg/ml, could sensitize both *A. baumannii* strains to rifampin (sensitization factors, 85 to 128), clarithomycin (sensitization factors, 12 to 16), fusidic acid (sensitization factors, 64 to 85), and vancomycin (sensitization factor, 85). The MICs of vancomycin for both strains were as low as 3 μg/ml in the presence of NAB739 at 0.5 μg/ml. The FICI were very low. Strain F264 was resistant to meropenem, but in the presence of NAB739 (0.5 μg/ml) the MIC of meropenem was as low as 1 μg/ml. These findings were quite unexpected in light of the results obtained with *E. coli* strains under identical test conditions (Table 2; also data not shown). NAB739 had also some synergism (FICI, ≤0.46) with clarithromycin against *P. aeruginosa*.

Abilities of NAB7061 to sensitize *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* to antibacterial agents. MICs of a representative set of clinically used antimicrobial agents were determined for two strains of *E. coli* (ATCC 25922 and IH3080), as well as for *K. pneumoniae* ATCC 13883 and *E. cloacae* ATCC 23355, by using Etests on Mueller-Hinton agar containing increasing concentrations of NAB7061. Under the conditions used, no direct growth-inhibitory effect of NAB7061 on *E. coli* IH3080 or *K. pneumoniae* ATCC 13883 was detectable, even at 16 μg of NAB7061/ml, whereas 16 μg/ml and 8 μg/ml inhibited the growth of *E. coli* ATCC 25922 and *E. cloacae* ATCC 23355, respectively.

NAB7061 exerted strong synergism with rifampin, clarithromycin, azithromycin, erythromycin, and mupirocin against all four strains tested (Table 5). The FICI of the combination of NAB7061 and rifampin ranged from ≤0.073 to 0.260, and that of NAB7061 and clarithromycin ranged from 0.105 to 0.273. At a concentration of 4 μg/ml, NAB7061 sensitized the strains to rifampin by factors ranging from 170 to 1,500. Extremely high sensitization factors were also observed for clarithromycin (63 to 380), mupirocin (24 to 512), azithromycin (31 to 64), and erythromycin (21 to 48).

Low FICI, determined with *E. coli* ATCC 25922, were also observed for quinupristin-dalfopristin (≤0.250), fusidic acid (≤0.270), linezolid (≤0.344), clindamycin (0.417), and vancomycin (≤0.438), although the resulting MICs were reasonably low only in the case of quinupristin-dalfopristin (1.5 to 3 μg/ml). All these antibacterial agents are notably hydrophobic or large (vancomycin) molecules and are effectively excluded by the intact OMs of gram-negative bacteria.

No sensitization (sensitization factor, ≤2; determined using *E. coli* ATCC 25922 and NAB7061 at a concentration of 4 μg/ml) to piperacillin, ceftazidime, cefotaxime, levofloxacin, ciprofloxacin, meropenem, or tobramycin was found. All these agents are hydrophilic or relatively hydrophilic, and the intact OM is not an effective permeability barrier against them. Furthermore, no sensitization was found to the large lipoglycopeptide daptomycin or to tigecycline, a molecule that probably penetrates the OM rather easily, as do the tetracyclines.

Susceptibilities of other strains of gram-negative bacteria to NAB7061. The MICs of rifampin and clarithromycin for a

TABLE 5. MICs of rifampin, three macrolides, and mupirocin for four bacterial strains in the presence of increasing concentrations of NAB7061[a]

Antibiotic and strain	MIC (μg/ml) of the antibiotic at the following NAB7061 concn (μg/ml):				Sensitization factor[b] at 4 μg/ml NAB7061	FICI
	0	1	2	4		
Rifampin						
E. coli ATCC 25922	8–16	0.19–0.38	0.064	0.016–0.047	250–750	0.071–0.078
E. coli IH3080	8	0.25	0.19	0.023–0.047	170–350	≤0.055–0.093
K. pneumoniae ATCC 13883	16–>32	1.5–3	0.25–0.5	0.064–0.125	250–500	≤0.073–≤0.078
E. cloacae ATCC 23355	12	8–12	0.125	0.008–0.016	750–1,500	0.26
Clarithromycin						
E. coli ATCC 25922	16–48	0.5	0.19	0.094–0.125	170–380	0.137
E. coli IH3080	12	0.5	0.25	0.047–0.064	190–260	0.105
K. pneumoniae ATCC 13883	12	2	1	0.19	63	≤0.141
E. cloacae ATCC 23355	64–96	32	1.5	0.25–0.75	85–380	0.273
Azithromycin						
E. coli ATCC 25922	3–6	0.5	0.19	0.094–0.125	24–64	0.157
E. coli IH3080	2–4	0.75	0.19	0.064	31–63	0.173
K. pneumoniae ATCC 13883	2	0.19	0.19	0.047–0.064	31–43	≤0.126
E. cloacae ATCC 23355	6–8	4	1.5	0.19–0.25	32	0.438
Erythromycin						
E. coli ATCC 25922	32–48	6	2	0.75–1	32–48	0.188
E. coli IH3080	16	4	2	0.38–0.5	32–42	0.25
K. pneumoniae ATCC 13883	24	4	2	0.5–1	24–48	≤0.143
E. cloacae ATCC 23355	64	48	8	1.5–3	21–43	0.375
Mupirocin						
E. coli ATCC 25922	256–384	16	4	1.5–6	64–170	0.127
E. coli IH3080	128–192	16	12	1.5–3	64–85	0.146
K. pneumoniae ATCC 13883	64–96	24	12	2–4	24–32	≤0.167
E. cloacae ATCC 23355	48–128	24	8	0.19–0.25	250–512	0.293

[a] Tested by using E-strips on Mueller-Hinton agar containing increasing concentrations of NAB7061. For the growth-inhibitory concentrations of NAB7061 alone under these test conditions, see the text. For rifampin, data are results of five (0 and 4 μg/ml NAB7061) or two (1 and 2 μg/ml NAB7061; FICI) independent determinations. For all other antibiotics, data are results of two (0 and 4 μg/ml NAB7061) or one (1 μg/ml and 2 μg/ml NAB7061; FICI) independent determinations.
[b] Ratio of the MIC in the absence of NAB7061 to that in the presence of 4 μg of NAB7061/ml.

representative set of gram-negative bacterial strains, other than those listed in Table 5, were determined in the presence of 4 μg/ml NAB7061. For all strains of *E. coli*, *Klebsiella oxytoca*, *E. cloacae*, and *C. freundii* (15 strains altogether), the MICs of rifampin were as low as ≤0.125 μg/ml in the presence of NAB7061, and the sensitization factors ranged from 85 to 2,000 (Table 6). Similar results were obtained with clarithromycin; for 13 out of 15 strains, the MICs of clarithromycin were as low as ≤0.25 μg/ml, and for all 15 strains, the sensitization factors ranged from 90 to 1,000. Strains of *K. pneumoniae* remained somewhat more resistant to both antibiotics, and the sensitization factors ranged from 10 to 500.

For the three strains of *A. baumannii*, the sensitization factors ranged from 21 to 125, and the resulting MICs were quite low (rifampin MIC, ≤0.125 μg/ml; clarithromycin MIC, ≤0.5 μg/ml).

As expected, polymyxin-resistant species, i.e., *S. marcescens*, *Proteus mirabilis*, and *Proteus vulgaris*, were resistant to the sensitizing action of NAB7061. Furthermore, NAB7061 did not sensitize *P. aeruginosa*.

Affinities of NAB739, NAB740, NAB7061, and polymyxin B for the BBM of the renal cortex. The binding of the compounds to the isolated renal BBM was measured indirectly by determining their abilities to inhibit the binding of radiolabeled gentamicin to the BBM. The IC_{50}s for the compounds studied were as follows (averages ± SD for three parallel determinations, if not otherwise stated): 39.3 ± 5.5 μM (two independent experiments, each with three replicates) for polymyxin B, 90.2 ± 9.7 μM for unlabeled gentamicin, 187.3 ± 24.3 μM (two independent experiments, each with three replicates) for NAB7061, 198 ± 49 μM for NAB740, and 264 ± 91 μM for NAB739. The differences between the IC_{50} of polymyxin B and those of each of the NAB compounds were statistically significant ($P < 0.05$). Accordingly, it can be concluded that the affinity of NAB739 for the BBM is only approximately one-sixth or one-seventh that of polymyxin B and approximately one-third that of gentamicin. Similarly, the affinity of NAB7061 for the BBM is only one-fifth that of polymyxin B.

Cytotoxicity. NAB7061, NAB739, and NAB740 (all at 0.25 μg/ml to 128 μg/ml) showed no cytotoxicity against V79 Chinese hamster lung fibroblast cells (treatment time, 24 h).

DISCUSSION

The known difficulty in developing antibacterial agents against species of *Enterobacteriaceae*, *P. aeruginosa*, *A. baumannii*, and many other gram-negative bacteria is caused by the OMs of these bacteria. The OM is an effective permeability barrier to hydrophobic antibiotics as well as to large hydrophilic antibiotics that easily enter gram-positive bacteria (18).

TABLE 6. Susceptibilities of gram-negative bacteria to rifampin and clarithromycin in the presence of NAB7061[a]

Bacterial strain	Rifampin		Clarithromycin	
	MIC (μg/ml) in the presence of NAB7061[c]	Sensitization factor[b]	MIC (μg/ml) in the presence of NAB7061	Sensitization factor
E. coli (8 strains)	0.016–0.125	85–500	0.032–0.125	90–750
E. coli NCTC13353	0.064	125–250	1	>260
K. pneumoniae CCUG45421	2–3	10–20	24	10
K. pneumoniae (3 strains)	0.19–0.75	43–>170	0.25–2	24–170
K. oxytoca ATCC 13182	0.032–0.047	680–750	0.25	260
K. oxytoca CCUG51683	0.012–0.023	700–2,000	0.19	250
E. cloacae CCUG52947	0.032–0.047	500–1,000	0.38	350
E. cloacae (2 strains)	0.016–0.021	1,400–1,500	0.047–0.094	500–1,000
C. freundii ATCC 8090	0.023–0.032	500–1,000	0.125	250
A. baumannii ATCC 19606	0.094–0.125	24–32	0.5	50
A. baumannii (2 strains)	0.032–0.125	21–125	0.25–0.38	40–100
S. marcescens ATCC 8100	16	<2	96	<2
P. mirabilis ATCC 29906	1–6	<2	24	<2
P. vulgaris ATCC 13315	1–1.5	<2	24	<2
P. aeruginosa ATCC 27853	12–16	2	32	<2

[a] Tested by using E-strips on Mueller-Hinton agar. NAB7061 was used at 4 μg/ml.
[b] Ratio of the MIC in the absence of NAB7061 to that in the presence of 4 μg of NAB7061/ml.
[c] Results from two independent determinations.

In addition, effective efflux mechanisms that pump noxious substances out of the cell act synergistically with the OM permeability barrier (13). More than 95% of novel antibacterial agents isolated from nature or synthesized in the laboratory lack activity against *E. coli* but have activity against gram-positive bacteria, according to an analysis of agents reported from 1981 to 1992 (31).

However, the OM can also be regarded as the Achilles′ heel of gram-negative bacteria and an attractive target for antibiotic action. Polymyxins and many of their derivatives, as well as numerous other compounds that carry several positive charges, bind to the acidic LPS molecules on the outer surface of the OM, disorganize the highly ordered structure of the LPS layer, and destroy the permeability barrier function of the OM (30).

The action of polymyxins is rather specific. Typically, the MICs for gram-positive bacteria are high. The enantiomeric mirror analogue of PMBN (D-PMBN) completely lacks the antibacterial properties of PMBN, such as the ability to act synergistically with hydrophobic antibiotics (29). This further substantiates our original view that the antibacterial activity of polymyxins is determined not only by their cationic nature but also by proper conformation (30). Furthermore, all 11 synthetic analogues of PMBN, each having a rather subtle modification, were shown to be much less potent than PMBN (28).

In contrast to polymyxins, many other cationic peptides lack specificity. They are not only antibacterial but also active against eukaryotic microbes and mammalian cells. They are also easily inactivated by serum proteases.

In the present work, we found that several polymyxin derivatives are directly antibacterial even though they carry only three positive charges, all of them in the cyclic portion of the peptide. The MICs of NAB739 for *E. coli* strains are identical or almost identical to those of polymyxin B. NAB739 shows good activity against other polymyxin-susceptible strains of *Enterobacteriaceae*, as well. The presence of two hydroxyl groups in the linear portion of the peptide is very advantageous.

NAB739 alone was active against *A. baumannii*, but at low subinhibitory concentrations it also had remarkable synergism with several other antibiotics, including clarithromycin, rifampin, and vancomycin, against *A. baumannii*. A combination might be advantageous in preventing the development of resistance.

The methanesulfonate derivative of NAB739 might have some advantages over NAB739. For instance, in analogy to CMS, acute toxicity might be lower for the methanesulfonate derivative than for NAB739 itself.

NAB740 differs from NAB739 in carrying a decanoyl residue instead of an octanoyl residue as the fatty acyl moiety and by being somewhat more active against *P. aeruginosa*. NAB7061 lacks direct antibacterial activity but has strong synergism with several antibiotics against polymyxin-susceptible strains of *Enterobacteriaceae* and *A. baumannii*.

The cyclic portions of the NAB739, NAB740, and NAB7061 molecules are identical to that of polymyxin B. Hence, they could be made semisynthetically by using polymyxin B as the starting material. In addition, the cyclic portions of other naturally occurring polymyxins can be used, as shown in Table 1 for colistin, polymyxin S, and polymyxin T.

Polymyxins as well as other highly cationic agents release histamine from mast cells. This can complicate therapy for cystic fibrosis patients, who receive aerosolized polymyxin. Whether the histamine-releasing activity of the novel compounds is lower than that of polymyxins should be evaluated.

The nephrotoxicity of aminoglycosides, another group of antibiotics that carry several positive charges under physiological conditions, is manifested in the renal proximal tubules. Aminoglycosides are taken up by the epithelial cells of the proximal tubules and stay there for a long time (17). The binding site for aminoglycosides is believed to be acidic phospholipids in the BBM of the proximal tubular cells. In addition, megalin, a giant endocytic receptor abundantly present in the apical membrane of proximal tubules, plays an important role in the binding and endocytosis of aminoglycosides (17). Polymyxin B binds to megalin (15). Accordingly, the mechanism of

nephrotoxicity of polymyxins might be related to that of aminoglycosides. In this paper we showed that reducing the number of positive charges from five to three decreases the affinity of the compound for isolated rat kidney BBMs by a factor of 6 to 7.

Whether the NAB compounds with three positive charges are less nephrotoxic than polymyxin B and colistin remains to be seen. We are currently investigating the pharmacokinetic and toxicological properties of these compounds.

ACKNOWLEDGMENTS

J.F. thanks Sat Sandhu, who performed all of the milligram-level peptide synthesis chemistry. M.V., O.S., and J.A. thank Sini Virtanen for excellent technical assistance.

This study was funded by Northern Antibiotics Ltd.

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