 

# Chemical Conversion of Natural Polymyxin B and Colistin to Their *N*-Terminal Derivatives[1]

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The chemical conversions of natural polymyxin B and colistin, which are fatty-acylated cyclic decapeptides, to polymyxin (2–10) and colistin (2–10) derivatives were examined. The $N^{\alpha}$-free and side chain $N^{\gamma}$-protected nonapeptides, i.e., tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin B (2–10) and tetrakis($N^{\gamma}$-trifluoroacetyl)–colistin (2–10), were prepared by trifluoroacetylation of polymyxin B and colistin, followed by chemical cleavage with 50% methanesulfonic acid to remove $N^{\alpha}$-alkanoyl–$N^{\gamma}$-trifluoroacetyl–Dab–OH. The $N^{\gamma}$-protected nonapeptides were useful starting materials for the semi-synthesis of *N*-terminal derivatives by selective $N^{\alpha}$-acylation at Thr$^2$, followed by the removal of the $N^{\gamma}$-trifluoroacetyl protecting group with aqueous piperidine. Further, myristoyl–polymyxin B (2–10) and myristoyl–colistin (2–10) retained their antimicrobial activity with an MIC of 2–4 nmol mL$^{-1}$ against *Escherichia coli*, *Salmonella* Typhimurium, and *Pseudomonas aeruginosa*. They also retained their high lipopolysaccahride (LPS) binding activity. Acetyl–polymyxin B (2–10) and acetyl–colistin (2–10) exhibited very low biological activities, except for a high bactericidal activity specifically against *Pseudomonas aeruginosa* with an MIC of 2 nmol mL$^{-1}$. The distinct sensitivity of three Gram-negative bacteria tested toward acetyl-nonapeptides suggested that the *N*-terminal hydrophobic character of the fatty-acylated polymyxin peptides was necessary for the bactericidal activity against *Escherichia coli* and *Salmonella* Typhimurium, but not against *Pseudomonas aeruginosa*.

Polymyxin B is a cationic cyclic decapeptide antibiotic that contains six $\alpha,\gamma$-diaminobutyric acid (Dab) residues, among which the $\gamma$-amino group of Dab$^4$ is acylated by *C*-terminal Thr$^{10}$ to form a 23-member lactam ring. The $\alpha$-amino function of Dab$^1$ of polymyxin B is acylated by various fatty acids,[2–4] and the main component of polymyxin $B_1$ is acylated by (*S*)-6-methyloctanoic acid. The minor components of polymyxin $B_2$, $B_3$, $B_4$, $B_5$, and $B_6$, which have been structurally elucidated, differ only in their *N*-terminal alkanoyl moieties that comprise 6-methylheptanoyl, octanoyl, heptanoyl, nonanoyl, and 3-hydroxy-6-methyloctanoyl groups, respectively.[5] However, the skeletal structure of the peptide moiety is conserved, except for very small amounts of minor components that contain Ile instead of Leu$^6$ or Ser instead of Thr$^2$. Colistin A (polymyxin $E_1$) and colistin B (polymyxin $E_2$) have structures similar to those of polymyxin $B_1$ and polymyxin $B_2$, respectively. However, in both colistins, position 7 is occupied by D-Leu, while in polymyxin $B_1$ and $B_2$, the same positions are occupied by D-Phe.[4,8] Very small amounts of minor components of colistin have also been reported to contain Ile and Val instead of one of the Leu residues.[9]

The enzymatic digestion of polymyxin B or colistin with ficin or papain removes alkanoyl–Dab–OH to produce polymyxin B (2–10) or colistin (2–10), respectively.[10,11] This colistin nonapeptide has been used for the chemical synthesis of various alkanoyl–colistin nonapeptides and alkanoyl–aminoacyl–colistin nonapeptides by Chihara et al.[12–14] It is very important to utilize natural products for the synthesis of analogues, since the complete chemical construction of a compound with a complicated structure is laborious. Such a construction involves multiple synthetic steps, and considerable amounts of chemicals are used in the process, which may cause environmental problems.

The aim of this study is, first, to develop a semi-synthetic route to prepare polymyxin B (2–10) and colistin (2–10) derivatives from natural polymyxin B and colistin, respectively. The key step in the route for the preparation of $N^{\gamma}$-protected polymyxin B (2–10) and $N^{\gamma}$-protected colistin (2–10) with $N^{\alpha}$-free forms was the cleavage reaction of $N^{\gamma}$-protected polymyxin B or $N^{\gamma}$-protected colistin in aqueous methanesulfonic acid (MSA).[15–19] Second, we discuss herewith the role of the *N*-terminal fatty acyl groups of these peptides for the antibacterial and LPS binding activities by employing pure synthetic peptides.

## Results and Discussion

For the chemical conversion of natural polymyxin B and colistin to their nonapeptide derivatives, the key compounds, i.e., tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin B (2–10) and tetrakis($N^{\gamma}$-trifluoroacetyl)–colistin (2–10), were prepared according to the route shown in Fig. 1. Since commercially available polymyxin B is a mixture of numerous closely related peptides, the main peak in the HPLC chromatogram, which corresponds to polymyxin $B_1$, was isolated, and the pure polymyxin $B_1$ (**1**) was used. An excess of *S*-ethyl trifluorothioacetate was reacted with **1** in the presence of triethylamine to protect the

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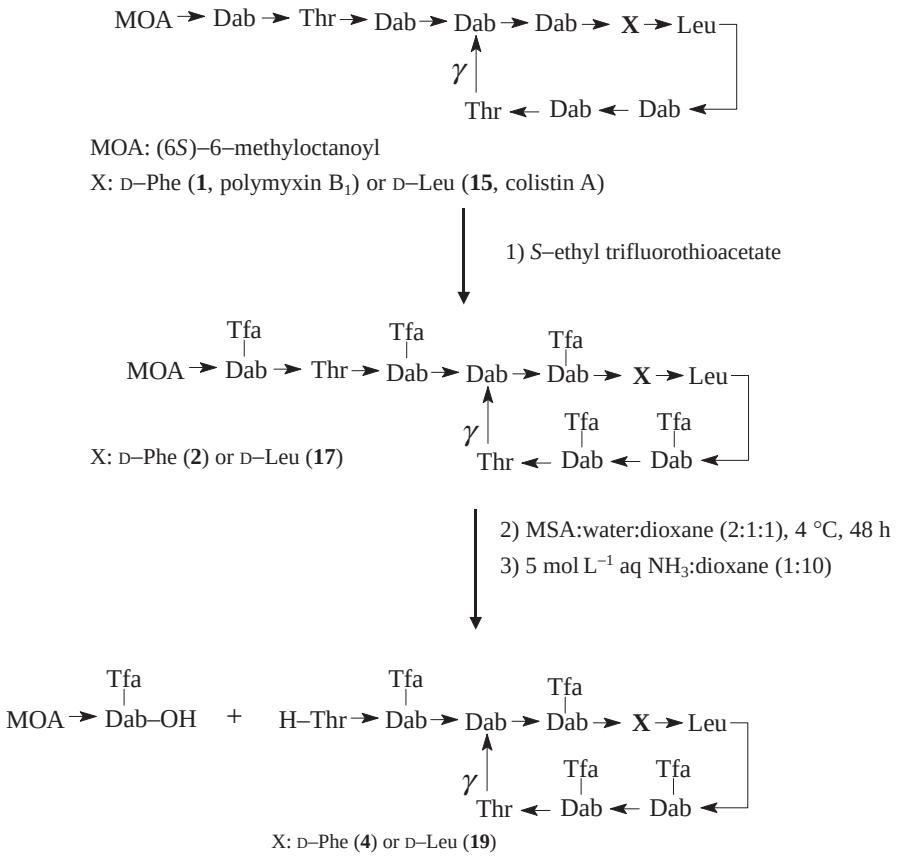



Fig. 1. Synthesis of tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin B (2–10) (**4**) from polymyxin $B_1$ (**1**) or tetrakis($N^{\gamma}$-trifluoroacetyl)–colistin (2–10) (**19**) from colistin A (**15**).

$\gamma$-amino functions of five Dab residues to yield pentakis-($N^{\gamma}$-trifluoroacetyl)–polymyxin $B_1$ (**2**). The cleavage reaction of **2** with MSA:dioxane:$H_2O$ (2:1:1) at a concentration of $5 \times 10^{-4}$ mol $L^{-1}$ was performed at 4 °C for 48 h to eliminate $N^{\alpha}$-[(*S*)-6-methyloctanoyl]–$N^{\gamma}$-trifluoroacetyl–Dab–OH. Excess MSA in the reaction mixture was eliminated by chromatography through an ODS column. After washing with water, the absorbed protected peptides were eluted with 75% dioxane. The cleavage products were treated with dilute ammonia solution, and tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin B (2–10) (**4**) was obtained in 7.7% yield after RP-HPLC purification.

The low yield of **4** was attributed to the nonspecific cleavage of the peptide bonds in **2** in the presence of a high concentration of methanesulfonic acid, as seen in the analytical HPLC chromatogram (Fig. 2A) of the hydrolysis reaction mixture. The time courses of **2** and the degradation products in peaks **a**–**e** are shown in Fig. 3. Since polymyxin B contains two Thr residues at positions 2 and 10, the migration of the Dab residues at positions 1 and/or 9 to the side chain hydroxy groups of $Thr^2$ and/or $Thr^{10}$ is thought to be involved. In fact, when the reverse N–O migration reaction was carried out using dilute ammonia,[20] the number of peaks in the HPLC chromatogram decreased, and the peak area of the desired product **4** as well as that of the starting material **2** increased (Fig. 2B). The degradation products, i.e., peaks **a**–**e** in the HPLC chromatogram in Fig. 2A, were isolated and analyzed by FAB-MS. The analysis data suggested that peaks **a**–**e** corresponded to the deduced structures **A**–**E** shown in Fig. 4. Furthermore,

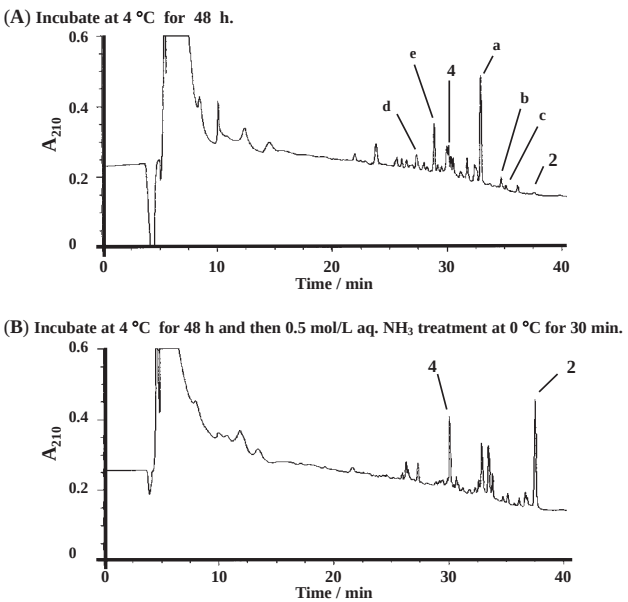



Fig. 2. HPLC profiles of the reaction mixture to yield tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin B (2–10) (**4**) from tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin $B_1$ (**2**) in MSA:water:dioxane (2:1:1). Column, Puresil C18 (4.6 × 250 mm); linear 30 min gradient elution from 4.8 to 76% $CH_3CN$ in 0.1% TFA; flow, 1 mL $min^{-1}$; detection, 210 nm.

the material corresponding to peak **e** was treated with dansyl (5-dimethylaminonaphthalene-1-sulfonyl) chloride, and the product had FAB-MS peak at $m/z$: 1813.6 ($[M + H]^+$), corresponding to di-dansylated **E**. Moreover, acid hydrolysis of di-dansylated **E** yielded $N^{\alpha}$-dansyl–Thr. Thus, it was revealed that the desired product **4** was produced partially from **E** through reverse N–O rearrangement.

Next, the preparation of the key compound **4** directly from commercially available polymyxin B sulfate complex was examined. Since most polymyxin B family peptides differ from each other only in terms of the fatty acid moiety with the same structure in the peptide region, they must produce homogeneous polymyxin B (2–10), when the *N*-terminal alkanoyl–Dab–OH is cleaved off. The HPLC chromatogram of the MSA-cleavage products of pentakis($N^{\gamma}$-trifluoroacetylated) non-purified polymyxin B (**3**) was more complicated than that of pentakis($N^{\gamma}$-trifluoroacetylated) purified polymyxin $B_1$ (**2**). However, compound **4** was obtained in the same yield (11.2%) and purity.

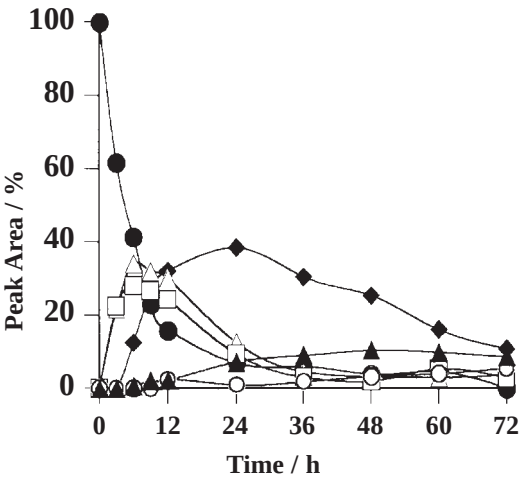
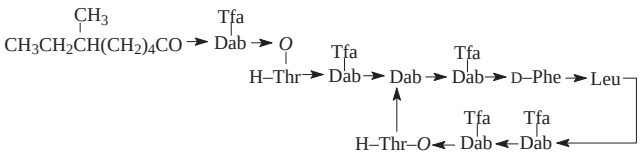
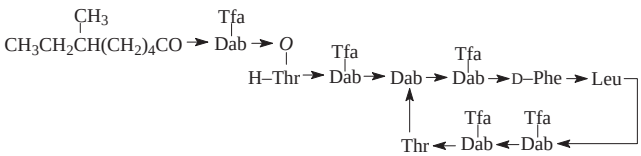



Fig. 3. Time courses of pentakis($N^{\gamma}$-trifluoroacetyl)–polymyxin $B_1$ (**2**) and its degradation products during incubation in MSA:water:dioxane (2:1:1) at 4 °C. Peaks of **2** (●), **a** (◆), **b** (△), **c** (□), **d** (○), and **e** (▲) on HPLC chromatogram (Fig. 2).

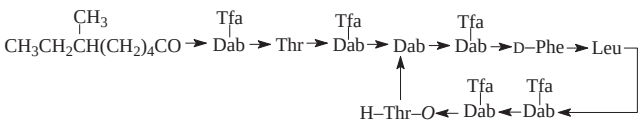
Starting from compound **4**, the preparation of the *N*-terminal analogs of polymyxin B was examined (Fig. 5). The treatment of **4** with aqueous piperidine to remove trifluoroacetyl groups on the $N^{\gamma}$-function of four Dab residues yielded **5**. The coupling of **4** with octanoic acid by using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) in the presence of triethylamine yielded $N^{\alpha}$-octanoyl–tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin B (2–10) (**6**), which was purified by gel-filtration using DMF–$H_2O$ (9:1) as the eluent. The trifluoroacetyl groups on **6** were removed by aqueous piperidine, and the resulting octanoyl–polymyxin B (2–10) (**7**) was purified by RP-HPLC. For the synthesis of polymyxin $B_3$, the coupling of **4** with Boc–Dab(Fmoc)–OH yielded Boc–Dab(Fmoc)–tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin B (2–10) (**8**), which was treated with TFA to remove Boc, after which octanoic acid was introduced. The simultaneous deprotection of Fmoc and the trifluoroacetyl groups of **8** with aqueous piperidine yielded polymyxin $B_3$ (**10**). Polymyxin B (2–10) (**5**), octanoyl–polymyxin B (2–10) (**7**), and polymyxin $B_3$ (**10**) obtained by the conversion procedure in this study were confirmed by FAB-MS analysis. Based on analytical RP-HPLC conversion products (**7** and **10**) were highly pure, and a single peak was observed in the co-elution chromatogram (Fig. 6) with authentic octanoyl–polymyxin B (2–10) and polymyxin $B_3$, which were chemically synthesized as previously



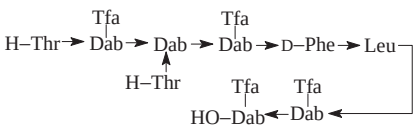

**A**: FAB–MS calcd $C_{66}H_{94}F_{15}N_{16}O_{18}$ $[M+H]^+$ 1683, found 1683.



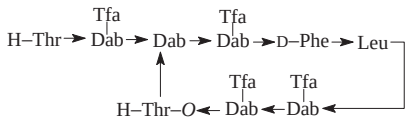

**B**: FAB–MS calcd $C_{66}H_{94}F_{15}N_{16}O_{18}$ $[M+H]^+$ 1683, found 1683.




**C**: FAB–MS calcd $C_{66}H_{94}F_{15}N_{16}O_{18}$ $[M+H]^+$ 1683, found 1683.

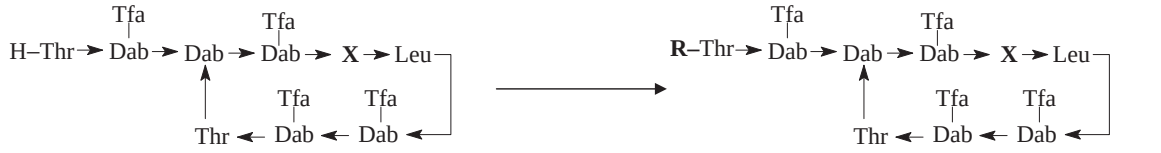



**D**: FAB–MS calcd $C_{51}H_{73}F_{12}N_{14}O_{16}$ $[M+H]^+$ 1365, found 1365.




**E**: FAB–MS calcd $C_{51}H_{71}F_{12}N_{14}O_{15}$ $[M+H]^+$ 1347, found 1347.

Fig. 4. Deduced structures of the products (**A**, **B**, **C**, **D**, and **E**) isolated from the peaks (**a**, **b**, **c**, **d**, and **e**) of HPLC chromatogram (Fig. 3A) and their FAB-MS data (found).




**4** or **19** → **5, 7, 10, 12, 14, 20, 22, 25, 27,** or **29**

| materials | | procedure | products | | |
|---|---|---|---|---|---|
| | **X** | | | **R** | **X** |
| **4** | D–Phe | **a**; DMF:$H_2O$:piperidine, at 4 °C for 48 h | **5** | H | D–Phe |
| | | **b**; 1) octanoic acid/HATU, 2) procedure **a** | **7** | octanoyl | |
| | | **c**; 1) Boc–Dab(Fmoc)–OH/HATU, 2) TFA, 3) procedure **b** | **10** | octanoyl–Dab | |
| | | **d**; 1) acetic anhydride/pyridine, 2) procedure **a** | **12** | acetyl | |
| | | **e**; 1) myristoic acid/HATU, 2) procedure **a** | **14** | myristoyl | |
| **19** | D–Leu | **a**; DMF:$H_2O$:piperidine, at 4 °C for 48 h | **20** | H | D–Leu |
| | | **b**; 1) octanoic acid/HATU, 2) procedure **a** | **22** | octanoyl | |
| | | **c**; 1) Boc–Dab(Fmoc)–OH/HATU, 2) TFA, 3) procedure **b** | **25** | octanoyl–Dab | |
| | | **d**; 1) acetic anhydride/pyridine, 2) procedure **a** | **27** | acetyl | |
| | | **e**; 1) myristoic acid/HATU, 2) procedure **a** | **29** | myristoyl | |

Fig. 5. Syntheses of the *N*-terminal analogs of polymyxin B and colistin.

reported.[21] Acetyl–polymyxin B (2–10) (**12**) and myristoyl–polymyxin B (2–10) (**14**) were prepared by the same route described above. This was done in order to examine the relationships between the *N*-terminal fatty acyl structure and the biological activity.

The chemical conversion of colistin to colistin (2–10) derivatives by the same route was also examined. Commercially available colistin sulfate complexes were composed of two major components, colistin A and colistin B, and various minor components. First, purified colistin A (**15**) was trifluoroacetylated, and the resulting pentakis($N^{\gamma}$-trifluoroacetyl)–colistin A (**17**) was cleaved by MSA-hydrolysis to yield tetrakis($N^{\gamma}$-trifluoroacetyl)–colistin (2–10) (**19**) (Fig. 1). Compound **19** was prepared in 6.2% yield after HPLC purification. When non-purified colistin was directly subjected to pentakis(trifluoroacetylation), followed by cleavage by MSA, **19** was obtained in 8.3% yield (calculated as pure colistin A for the starting complex materials). Starting from **19**, not only colistin (2–10) (**20**), octanoyl–colistin (2–10) (**22**), and octanoyl–colistin (**25**) but also acetyl–colistin (2–10) (**27**) and myristoyl–colistin (2–10) (**29**) were prepared by the same procedure as that described above for the polymyxin B derivatives (Fig. 5).

We previously reported a method for simple chemical deblocking with a high concentration of aqueous methanesulfonic acid (MSA) for *N*-terminal-blocked peptides such as pGlu–peptides[15–17] and myristoyl–glycyl–peptides (Myr–Gly–peptides).[18,19] Because Myr–Gly–peptides are hydrophobic, MSA:dioxane:$H_2O$ (2:1:1) was used to cleave off Myr–Gly–OH predominantly from Myr–Gly–peptides. In this study, the extremely hydrophobic derivatives of fully protected polymyxin B (**2** and **3**) and colistin (**17** and **18**) were subjected to the cleavage reaction by using a large amount of MSA: dioxane:$H_2O$ (2:1:1). However, the removal of $N^{\alpha}$-alkanoyl–$N^{\gamma}$-trifluoroacetyl–Dab–OH from the protected polymyxin B did not proceed as selectively as reported previously for the removal of Myr–Gly–OH from Myr–Gly–peptides. The trifluoroacetyl group was adopted as an acid-resistant protecting group of the side chain $N^{\gamma}$-amino functions of five Dab residues of polymyxin B. Fmoc was also examined as an acid-resistant protecting group; however, it was difficult to introduce it completely to five $N^{\gamma}$-amino functions when Fmoc–OSu was employed. FAB-MS analysis showed that the number of Fmoc groups introduced could not exceed four, probably because of steric hindrance, which was overcome by using trifluoroacetyl or 2,2,2-trichloroethoxycarbonyl (Troc) groups.

It was reported previously that various acyl–colistin (2–10) derivatives were prepared by the reaction of colistin (2–10) (colistin nonapeptide) with the corresponding acid chlorides, where the reaction was carried out at a pH of 5.0 in order to introduce an acyl group selectively into the $\alpha$-amino function of Thr.[12–14] However, in the preparation of octanoyl colistin nonapeptide, the product was contaminated with $N^{\gamma}$-octanoyl–nonapeptide(s) even after purification. In the synthesis of the $N^{\alpha}$-alkanoyl–monoaminoacyl derivatives of colistin nonapeptide, the efficiency of coupling with the $\alpha$-amino function of Thr was considered to be approximately 50–60%, which was estimated as DNP–Thr, and the remaining part contained in the preparation seemed to be occupied by acylated derivatives at one of the $\gamma$-amino groups of the nonapeptide.[14] Moreover, partial racemization at the $N^{\alpha}$-alkanoylamino acid might be unavoidable since non-urethane-type acylamino acids were directly activated for the introduction reaction. Therefore, as a semi-synthetic route for the *N*-terminal analog of polymyxin B and colistin, the $N^{\gamma}$-protected nonapeptide preparation with $N^{\alpha}$-free Thr described in this paper is advantageous

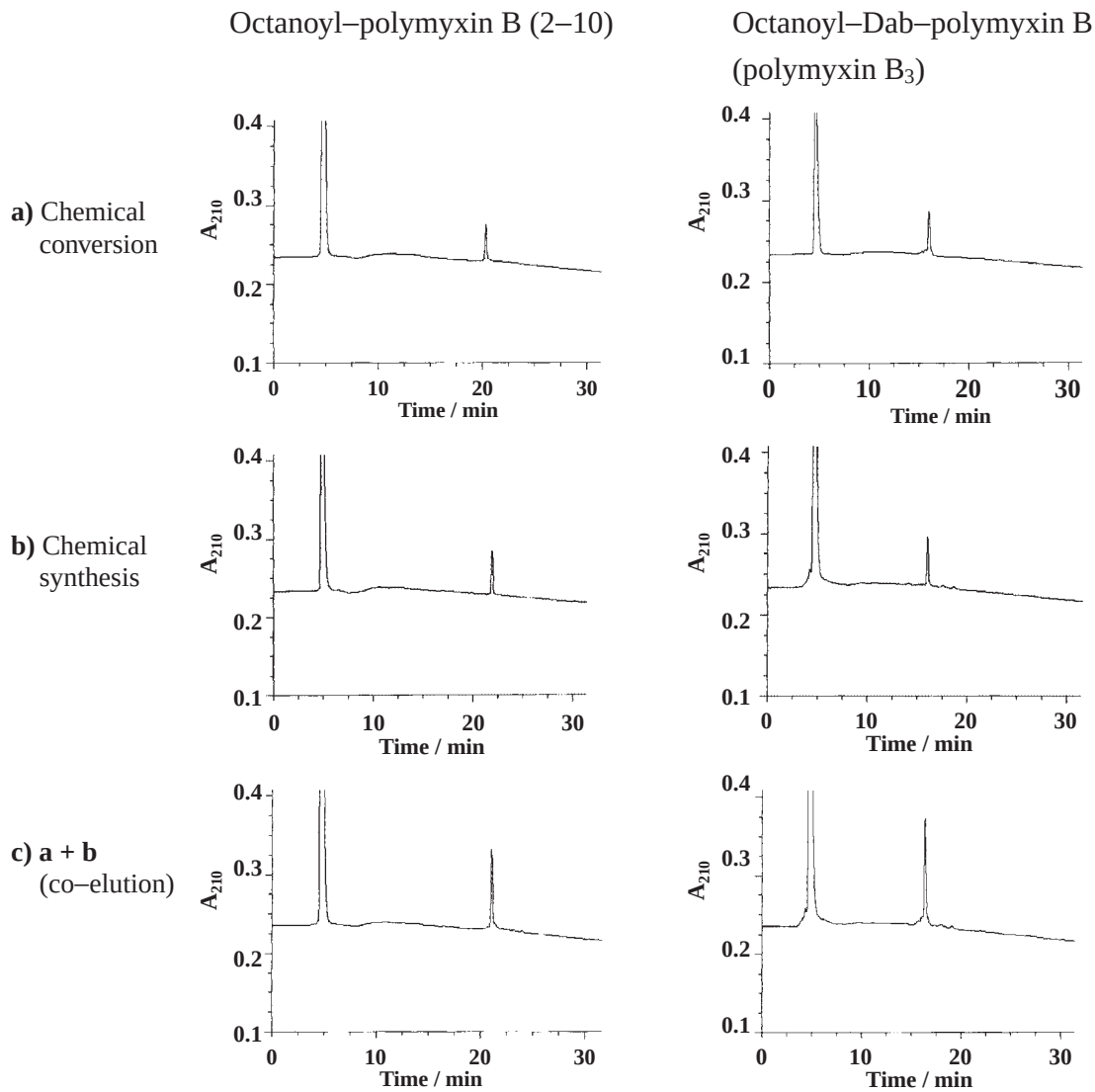



Fig. 6. HPLC profiles of octanoyl–polymyxin B (2–10) and octanoyl–Dab–polymyxin B (2–10) (polymyxin $B_3$) prepared by chemical conversion or chemical syntheses and their co-elution profiles. Column, Puresil $C_{18}$ (4.6 × 250 mm); linear 30 min gradient elution from 28.5 to 47.5% $CH_3CN$ in 0.1% TFA; flow, 1 mL $min^{-1}$; detection, 210 nm.

for obtaining a highly pure peptide to evaluate its biological activity. As described below, octanoyl–colistin (2–10) synthesized in this study showed potent bactericidal activity against *Escherichia coli*, while the preparation of this compound as reported 30 years ago was 10–20 times less active.[12] The difference in potency is thought to originate from the difference in the purities of the synthetic peptides, as described above. In other words, the *N*-terminal structure–activity relationship work should be re-examined with highly pure synthetic peptides.

The total solid-phase synthesis of polymyxin $B_1$ has been reported by Sharma et al. in 1999.[22] Subsequently, we have reported the synthesis of the polymyxin B derivatives.[23] Studies on the structure–activity relationship of the polymyxin peptides employing various synthetic peptides have been performed;[24–30] however, not many analogs have been reported to examine the role of the *N*-terminal fatty acyl group of these peptides with regard to their bactericidal activity. The introduction of $Ala_3$ and $Ala_6$,[27] and formyl–Met–Leu–Phe[30] into the *N*-terminal of polymyxin (2–10) has been reported to produce peptides without bactericidal activity, whereas the introduction of Fmoc[27] increased the activity. Previously, we have reported that the contribution of the $C_9$ fatty acyl groups of polymyxin B peptides to the LPS binding was slightly greater than that of the $C_7$ or $C_8$ fatty acyl groups by employing synthetic polymyxin B family peptides (polymyxin $B_1$, $B_2$, $B_3$, $B_4$, $B_5$, and $B_6$) and seven *N*-terminal fatty acid and/or amino acid deletion anologs.[21]

The assay results of the antimicrobial and LPS binding activities of acyl–nonapeptides prepared in this study are shown in Table 1 and Fig. 7. Myristoyl–polymyxin B (2–10) (**14**) and myristoyl–colistin (2–10) (**29**) exhibited considerable antimicrobial activity against the three Gram-negative bacteria tested. The degree of the activity was slightly lower than those of octanoyl–polymyxin B (2–10)[21] and octanoyl–colistin (2–10) (**22**). Acetyl–polymyxin B (2–10) (**12**) and acetyl–colistin (2–10) (**27**) exhibited very low antimicrobial activity against *Escherichia coli* and *Salmonella* Typhimurium. These results suggested the importance of the size of the fatty acyl groups

Table 1. Antimicrobial Activity of Synthetic Polymyxin Analogs

| Peptide | MIC/nmol mL$^{-1}$ | | |
|---|---|---|---|
| | *Escherichia coli* | *Salmonella* Typhimurium | *Pseudomonas aeruginosa* |
| Polymyxin B (Sigma) | 1.0 | 0.5 | 1.0 |
| Acetyl–polymyxin B (2–10) (**12**) | 32.0 | 128 | 2.0 |
| Myristoyl–polymyxin B (2–10) (**14**) | 4.0 | 4.0 | 4.0 |
| Octanoyl–colistin (2–10) (**22**) | 1.0 | 4.0 | 2.0 |
| Octanoyl–Dab–colistin (2–10) (polymyxin $E_3$) (**25**) | 1.0 | 1.0 | 1.0 |
| Acetyl–colistin (2–10) (**27**) | 128 | >512 | 2.0 |
| Myristoyl–colistin (2–10) (**29**) | 4.0 | 2.0 | 4.0 |

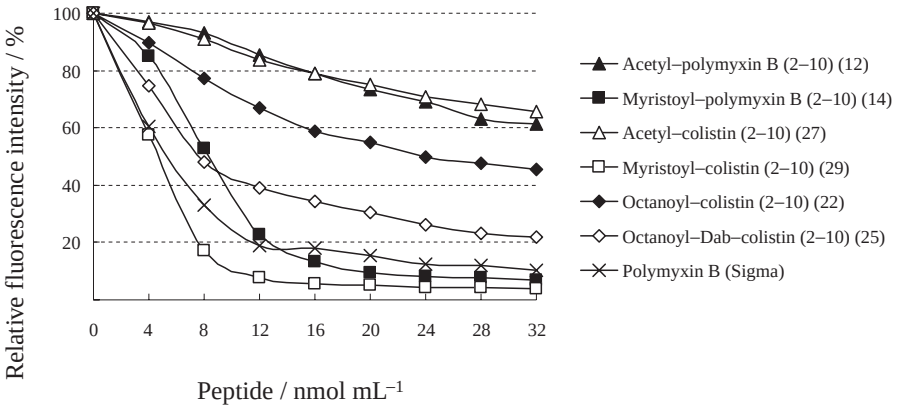



Fig. 7. Displacement of [Dab(Dansyl–Gly)$^1$]–polymyxin $B_3$ bound to LPS by synthetic polymyxin B and colistin analogs.

at the *N*-terminal of these peptides for the bactericidal activity and were consistent with a recent report, in which it is stated that the substitution of shorter acyl chains for (*S*)-6-methyloctanoyl of polymyxin $B_1$ yielded analogs with a decreased antimicrobial activity against *Escherichia coli*. In other words, the MIC values of hexanoyl, pentanoyl, and butanoyl analogs were 0.7, 11, and 23 μg mL$^{-1}$, respectively.[29] It is thought that the molecular mechanism of polymyxin peptides with the cell membrane of Gram-negative bacteria proceeds as follows: 1) the cyclic cationic domain of the molecule binds to the phosphates of the lipid A portion of LPS in the bacterial outer membrane, 2) the fatty acyl as well as the side chains of D-Phe (or D-Leu) and Leu at positions 6 and 7 interact with the hydrophobic parts of LPS, and 3) the binding to and the penetration into the cellular membrane disturb the membrane structure, leading to disruption and cell death. The present results showed that the medium size of the fatty acyl (octanoyl) appeared to be advantageous to bind to and disturb the membrane and to kill the bacteria. The acetyl group of the nonapeptides (**12** and **27**) appeared to be unable to participate in the biological function. However, it was interesting to note that, in this study, acetyl–nonapeptides **12** and **27** retained a higher bactericidal activity against *Pseudomonas aeruginosa* than myristoyl–nonapeptides **14** and **29**. The increased sensitivity of acetyl–nonapeptides **12** and **27** only against *Pseudomonas aeruginosa* is attributed to the difference in the cell membrane structure between *Pseudomonas aeruginosa* and *Escherichia coli* or *Salmonella* Typhimurium. The hydrophobic character of the *N*-terminal fatty acyl of polymyxin B peptides seemed to have a very limited contribution to the interaction with the cell membrane of *Pseudomonas aeruginosa*. In this regard, Tsubery et al. have pointed out recently that *Pseudomonas aeruginosa* is exceptionally susceptible toward polymyxin B nonapeptide.[25,27]

Myristoyl–nonapeptides **14** and **29** retained the LPS (derived from *Escherichia coli*) binding activity to the same degree as polymyxin $B_1$. On the other hand, acetyl–nonapeptides **12** and **27** exhibited a very low LPS binding activity. The binding assay results of **12** and **27** were compatible with their low antimicrobial activities against *Escherichia coli*. The low LPS binding assay results of **12** and **27** were in contrast to the high bactericidal activity against *Pseudomonas aeruginosa*, indicating that the in vitro binding assay results did not show a parallel disruptive effect on the living bacterial membrane of *Pseudomonas aeruginosa*.

## Conclusion

The $N^{\alpha}$-free and side chain $N^{\gamma}$-protected nonapeptides, i.e., tetrakis($N^{\gamma}$-trifluoroacetyl)–polymyxin B (2–10) (**4**) and tetrakis($N^{\gamma}$-trifluoroacetyl)–colistin (2–10) (**19**), were prepared from natural polymyxin B and colistin by trifluoroacetylation followed by chemical cleavage with 50% methanesulfonic acid. It was shown that they were useful starting materials for the semi-synthesis of the *N*-terminal derivatives of polymyxin B and colistin by selective $N^{\alpha}$-acylation at Thr$^2$. The myristoylation of **4** and **19**, followed by deprotection with aqueous piperidine, yielded myristoyl–polymyxin B (2–10) (**14**) and myristoyl–colistin (2–10) (**29**). Myristoyl–nonapeptides **14** and **29** retained their antimicrobial activity with an MIC of 2–4 nmol mL$^{-1}$ against *Escherichia coli*, *Salmonella* Typhimurium, and *Pseudomonas aeruginosa*, and they also retained a high LPS binding activity. Acetyl–polymyxin B

(2–10) (**12**) and acetyl–colistin (2–10) (**27**) exhibited very low antimicrobial activity against *Escherichia coli* and *Salmonella* Typhimurium, whereas they exhibited high antimicrobial activity specifically against *Pseudomonas aeruginosa*, thereby suggesting that there is a difference in the cell membrane structure of *Pseudomonas aeruginosa* from the other Gram-negative bacteria tested.

## Experimental

**General.** Fast-atom bombardment mass spectra (FAB-MS) were obtained on a JMS-DX300 mass spectrometer (JEOL Ltd., Japan). The optical rotations of the peptides were measured using a DIP-370 digital polarimeter (JASCO Co., Ltd., Japan). HPLC was performed on a system comprised of a PX-8010 gradient controller (Tosoh, Japan), two CCPD pumps (Tosoh), a Rheodyne 7125 injector (Rheodyne Inc., U.S.A.), or an AS-8020 automatic sample injector (Tosoh), a UV 8000 detector (Tosoh), and an 805 data station (Waters). HP-TLC was performed on precoated silica-gel plates (Kieselgel 60, E. Merck, Germany). $R_f{}^1$: BuOH:AcOH:AcOEt:$H_2O$ (1:1:1:1), $R_f{}^2$: BuOH:pyridine:AcOH:$H_2O$ (30:20:6:24).

**Polymyxin $B_1$ Pentahydrochloride (1).** Polymyxin B sulfate (Wako, 500 mg) was purified by RP-HPLC using a YMC Pack ODS-5ST column (20 × 150 mm) with 24% $CH_3CN$ in 0.1% TFA at a flow rate of 6 mL min$^{-1}$. The main peak ($t_R$: 25.6 min) was collected, and the combined eluate was lyophilized. The obtained powder was dissolved in water containing 1 mol L$^{-1}$ HCl (1 mL) and then re-lyophilized. Yield: 327 mg (68.4%). $[\alpha]_D^{25}$ −57.6° (*c* 0.5, 12% AcOH). FAB-MS calcd for $C_{56}H_{99}N_{16}O_{13}$ $[M + H]^+$ 1203, found 1203. HP-TLC: $R_f{}^1$ 0.13, $R_f{}^2$ 0.43.

**Pentakis($N^\gamma$-trifluoroacetyl)–Polymyxin $B_1$ (2).** Purified polymyxin $B_1$ pentahydrochloride (**1**) (166 mg, 0.11 mmol) was suspended in dimethyl sulfoxide (DMSO) (30 mL) containing triethylamine (TEA) (150 µL, 1.08 mmol) and *S*-ethyl trifluorothioacetate (341.6 mg, 2.16 mmol) was added under cooling. The mixture was allowed to react for 28 h at room temperature, while maintaining the pH at 7.8 with TEA. The product was precipitated by addition of cold $H_2O$ (60 mL) and collected by filtration and dried. It was then reprecipitated from DMSO (3 mL) and $H_2O$ (50 mL) followed by MeOH (3 mL), ether (15 mL), and petr. ether (15 mL). Yield: 163 mg (80.8%). $[\alpha]_D^{27}$ −38.6° (*c* 0.5, DMF), FAB-MS calcd for $C_{66}H_{94}F_{15}N_{16}O_{18}$ $[M + H]^+$ 1683.669, found 1683.7. HP-TLC: $R_f{}^1$ 0.90, $R_f{}^2$ 0.84.

**Pentakis($N^\gamma$-trifluoroacetyl)–Polymyxin B (3).** Polymyxin B sulfate (Wako, 1.5 g, 1.04 mmol, calculated as **1**) was suspended in a DMSO solution (300 mL) containing TEA (1.45 mL, 10.4 mmol) and *S*-ethyl trifluorothioacetate (3.29 g, 20.8 mmol) was added under cooling. The mixture was allowed to react for 28 h at room temperature while maintaining the pH at 7.8 with TEA. The product was obtained by solidification in the same manner as that described for **2**. Yield 1.69 g; 96.9%, (calculated as **2**). The crude product **3** was used for the next reaction without further purification. A small amount of the product **3** dissolved in DMSO was purified by RP-HPLC on a YMC Pack D-ODS-5ST column (150 × 20 mm) with linear gradient elution (30 min) from 51.3 to 55.9% $CH_3CN$ in 0.1% TFA as the eluent at a flow rate of 6 mL min$^{-1}$. The main peak material was lyophilized from dioxane and was characterized as **2** from the FAB-MS, HPLC, and HP-TLC analytical data, which were in complete agreement with those for **2**.

**HPLC Analysis of 2 and Its Degradation Products.** Solutions of 4 × 10$^{-4}$ mol L$^{-1}$ of **2** in MSA:dioxane:$H_2O$ (2:1:1) were prepared under ice cooling and divided into ten aliquots (100 µL each) in glass tubes with airtight caps. These were maintained at 4 °C, and each tube was removed at 0 h or an appropriate time point, and neutralized by adding 66.5 µL of water, 116.5 µL of dioxane, and 50 µL of 8 mol L$^{-1}$ NaOH. Subsequently, they were stored at −40 °C until analysis. To analyze the reaction mixture, the solution (125 µL) was examined by RP-HPLC using a Puresil $C_{18}$ column (250 × 4.6 mm) with gradient elution (30 min) from 4.8 to 76% MeCN in 0.1% TFA at a flow rate of 1 mL min$^{-1}$. The peak area of **2** at 0 h, measured at 210 nm, was taken as 100%, and those of the degradation products (peak **4**, peaks **a**–**e** in Fig. 2A) were measured and expressed as percentages (Fig. 3). To deduce the structures of the reaction products, each HPLC peak (Fig. 2A) was collected, and then the product was estimated by FAB-MS analysis. FAB-MS data and deduced structures (**A**–**E**) are shown in Fig. 4.

**Dansyl Derivatives of N–O Migration Products of 2 and *N*-Terminal Amino Acid Analysis.** Material **E**, isolated from peak **e**, (Fig. 2A) tested positive for ninhydrin. Lyophilized material **E** (205 µg) was dissolved in cold DMSO–DMF (1:1) (30 µL), and a solution of dansyl chloride (121 µg) in acetone (90 µL) and a 1% TEA solution in DMF (13 µL) were added. The mixture was allowed to react for 3 h in the dark at room temperature, and the solvent was evaporated. The main product (dansylated **E**) was isolated by HPLC on a YMC-$C_4$ column of (1 × 25 cm) with linear gradient elution (30 min) from 28.5 to 90% $CH_3CN$ at a flow rate of 2 mL min$^{-1}$. The main peak product ($t_R$: 23.6 min) was isolated and lyophilized. FAB-MS calcd for $C_{72}H_{93}F_{12}N_{16}O_{19}S_2$ $[M + H]^+$ 1813.6, found 1813.6, corresponding to di-dansylated **E**. The product (30 µg) was hydrolyzed with 6 mol L$^{-1}$ HCl vapor at 130 °C for 1 h, and then it was dissolved in EtOH (50 µL) was analyzed by HPLC on a Symmetry $C_{18}$ 3.5 µm column (4.6 × 150 mm) (Waters) employing linear gradient (40 min) elution from 8 to 64% $CH_3CN$ in 0.1% TFA at a flow rate of 0.8 mL min$^{-1}$, detection at 210 nm. The main peak appeared at the same retention time as that for authentic dansyl–Thr ($t_R$: 16.8 min). In the same manner as that described above for **E**, material **A** isolated from peak **a** (Fig. 2A), tested positive for ninhydrin. The reaction of **A** with dansyl chloride yielded a product eluting at 34.1 min by HPLC; this product was isolated and analyzed. FAB-MS calcd. for $C_{90}H_{116}F_{15}N_{18}O_{22}S_2$ $[M + H]^+$ 2149.8, found 2149.8, corresponding to di-dansylated **A**. Hydrolysis of di-dansylated **A** yielded dansyl-Thr on HPLC.

**Tetrakis($N^\gamma$-trifluoroacetyl)–Polymyxin B (2–10) Hydrochloride (4). Route A:** Compound **2** (150 mg, 90 µmol) was dissolved in a mixture of MSA (90 mL), $H_2O$ (45 mL), and dioxane (45 mL) and stirred at 4 °C for 48 h. The mixture was diluted with $H_2O$ (360 mL) and added to a Wakogel 50C18 column (1.8 × 4 cm), which was further washed with $H_2O$ (110 mL) to remove MSA. It was then eluted with 75% aqueous dioxane (100 mL). The eluate was lyophilized to yield a powder, which was dissolved in DMF (7 mL), and then, a dilute ammonia solution (5 mol L$^{-1}$, 0.7 mL) was added. The mixture was stirred for 30 min in an ice bath, and then the solvent was evaporated in vacuo. The crude product was dissolved in a 1:2 solution of DMSO:aqueous dioxane (75% v/v) (4 mL), and then purified by RP-HPLC on a YMC Pack D-ODS-5ST column (150 × 20 mm) with linear gradient elution (30 min) from 33.25 to 38% $CH_3CN$ in 0.1% TFA as the eluent at a flow rate of 5 mL min$^{-1}$. The eluate that contained purified **4** was evaporated and lyophilized from dioxane containing 1 mol L$^{-1}$ HCl (50 µL). The yield of **4** was

9.5 mg (7.7%). $[\alpha]_D^{25}$ −46.4° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{51}H_{71}F_{12}N_{14}O_{15}$ $[M+H]^+$ 1347.503, found 1347.5. HP-TLC: $R_f{}^1$ 0.75, $R_f{}^2$ 0.76.

**Route B:** Compound **3** (500 mg, 0.30 mmol, calculated as **2**) was dissolved in a mixture of MSA (300 mL), $H_2O$ (150 mL), and dioxane (150 mL) and stirred at 4 °C for 48 h. The mixture was treated in the same manner as described above in Route A. After HPLC purification, **4** was lyophilized from dioxane containing 1 mol $L^{-1}$ HCl (1 mL). The yield of **4** was 46.0 mg (11.2%). $[\alpha]_D^{25}$ −49.6° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{51}H_{71}F_{12}N_{14}O_{15}$ $[M+H]^+$ 1347.503, found 1347.5. HP-TLC: $R_f{}^1$ 0.75, $R_f{}^2$ 0.76.

**Polymyxin B (2–10) Pentahydrochloride (5).** Compound **4** (8.3 mg, 6 μmol) was dissolved in a mixture of DMF:$H_2O$:piperidine (5:6:2) (18 mL) and stirred at 4 °C for 48 h. The solvent was evaporated, and the residue was dissolved in distilled water (2.5 mL). The solution was then purified by RP-HPLC on a YMC Pack D-ODS-5ST column (150 × 20 mm) with linear gradient elution (30 min) from 4.75 to 23.75% $CH_3CN$ in 0.1% TFA as eluent at a flow rate of 5 mL $min^{-1}$. Purified **5** was lyophilized from distilled water containing 1 mol $L^{-1}$ HCl (50 μL). Yield: 2.6 mg (37.9%). $[\alpha]_D^{25}$ −43.2° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{43}H_{75}N_{14}O_{11}$ $[M+H]^+$ 963.574, found 963.6. HP-TLC: $R_f{}^1$ 0.04, $R_f{}^2$ 0.16.

**Octanoyl–Tetrakis($N^\gamma$-trifluoroacetyl)–Polymyxin B (2–10) (6).** To the solution of **4** (7.33 mg, 5.30 μmol) in DMF (250 μL), a mixture of octanoic acid (3.06 mg, 21.2 μmol) and HATU (8.05 mg, 21.2 μmol) in DMF (33.5 μL) was added. After addition of 4-methylmorpholine (NMM) (1.1 μL, 10.8 μmol) in DMF (10 μL) to the solution under ice cooling, the mixture was stirred at room temperature, and its pH was maintained at 7.8 with NMM overnight. The mixture was applied to a Toyopearl HW-40 column (100 × 2 cm), and eluted with DMF:water (9:1). The collected eluate containing the main product was combined and evaporated in vacuo. The residue was lyophilized from dioxane (30 mL). Yield: 6.02 mg (77.3%). $[\alpha]_D^{27}$ −33.8° (*c* 0.5, DMF), FAB-MS calcd for $C_{59}H_{85}F_{12}N_{14}O_{16}$ $[M+H]^+$ 1473.608, found 1473.6. HP-TLC: $R_f{}^1$ 0.89, $R_f{}^2$ 0.82.

**Octanoyl–Polymyxin B (2–10) Tetrahydrochloride (7).** Compound **6** (6.08 mg, 4.1 μmol) was treated with piperidine in the same manner as that described for **5**. The product was purified by RP-HPLC on a YMC Pack D-ODS-5ST column (20 × 150 mm) with linear gradient elution (30 min) from 38 to 66.5% $CH_3CN$ in 0.1% TFA as the eluent at a flow rate of 6 mL $min^{-1}$. Purified **7** was lyophilized from 75% dioxane containing 1 mol $L^{-1}$ HCl (50 μL). Yield: 4.18 mg (82.0%). $[\alpha]_D^{22}$ −50.0° (*c* 0.2, 12% AcOH), FAB-MS calcd for $C_{51}H_{89}N_{14}O_{12}$ $[M+H]^+$ 1089, found 1089. HP-TLC: $R_f{}^1$ 0.48, $R_f{}^2$ 0.46.

**Boc–Dab(Fmoc)–Tetrakis($N^\gamma$-trifluoroacetyl)–Polymyxin B (2–10) (8).** To a suspension of **4** (110.6 mg, 80 μmol) in DMF (1.1 mL), a mixture of Boc–Dab(Fmoc)–OH (140.96 mg, 320 μmol) and HATU (121.7 mg, 320 μmol) in DMF (100 μL) was added. After addition of NMM (16.3 μL, 160 μmol) in DMF (147 μL) to the solution under ice cooling, the mixture was stirred at room temperature, and the pH was maintained at 7.8 with NMM overnight. The product was purified on a Toyopearl HW-40 column in the same manner as that described for **6**. Yield: 107.6 mg (76.1%). $[\alpha]_D^{27}$ −30.6° (*c* 0.5, DMF), FAB-MS calcd for $C_{75}H_{97}F_{12}N_{16}O_{20}$ $[M+H]^+$ 1769.687, found 1769.69. HP-TLC: $R_f{}^1$ 0.90, $R_f{}^2$ 0.84.

**Octanoyl–Dab(Fmoc)–Tetrakis($N^\gamma$-trifluoroacetyl)–Polymyxin B (2–10) (9).** Compound **8** (28.3 mg, 16 μmol) was dissolved in 95% aqueous TFA (2 mL). The solution was stirred at room temperature for 1.5 h. After evaporation in vacuo to remove TFA, the product was lyophilized from dioxane containing 1 mol $L^{-1}$ HCl (0.1 mL). The resulting H–Dab(Fmoc)–tetrakis($N^\gamma$-trifluoroacetyl)–polymyxin $B_1$ (2–10) hydrochloride was dissolved in DMF (650 μL), and a mixture of octanoic acid (9.23 mg, 64.0 μmol) and HATU (24.3 mg, 64.0 μmol) in DMF (100 μL) was added under ice cooling. After the addition of NMM (3.3 μL, 32 μmol) in DMF (30 μL) to the ice-cooled solution, the mixture was stirred at room temperature, and its pH was maintained at 7.8 with NMM overnight. The product was purified on a Toyopearl HW-40 column in the same manner as that described for **6**. The product was lyophilized from dioxane. Yield: 24.6 mg (85.7%). $[\alpha]_D^{27}$ −27.4° (*c* 0.5, DMF), FAB-MS calcd for $C_{78}H_{103}F_{12}N_{16}O_{19}$ $[M+H]^+$ 1795.739, found 1795.74. HP-TLC: $R_f{}^1$ 0.92, $R_f{}^2$ 0.84.

**Octanoyl–Dab–Polymyxin B (2–10) Pentahydrochloride (Polymyxin $B_3$) (10).** Compound **9** (7.3 mg, 4.1 μmol) was treated with piperidine in the same manner as that described for **5** to remove Fmoc and trifluoroacetyl groups. The product was purified by RP-HPLC on a YMC Pack D-ODS-5ST column (20 × 150 mm) with linear gradient elution (30 min) from 19 to 28.5% $CH_3CN$ in 0.1% TFA as the eluent at a flow rate of 5 mL $min^{-1}$. Purified **10** was lyophilized from 75% dioxane containing 1 mol $L^{-1}$ HCl (50 μL). Yield: 4.2 mg (75.3%). $[\alpha]_D^{25}$ −56.5° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{55}H_{97}N_{16}O_{13}$ $[M+H]^+$ 1189, found 1189. HP-TLC: $R_f{}^1$ 0.10, $R_f{}^2$ 0.41.

**Acetyl–Tetrakis($N^\gamma$-trifluoroacetyl)–Polymyxin B (2–10) (11).** To a solution of **4** (27.65 mg, 20 μmol) in 90% DMF (500 μL), pyridine 2.25 μL (28 μmol) and acetic anhydride 2.65 μL (28 μmol) were added under ice cooling, and the mixture was then stirred at room temperature for 1 h. The product was purified on a Toyopearl HW-40 column in the same manner as that described for **6**. Yield: 24.87 mg (89.5%). $[\alpha]_D^{25}$ −52.0° (*c* 0.5, DMF), FAB-MS calcd for $C_{53}H_{73}F_{12}N_{14}O_{16}$ $[M+H]^+$ 1389, found 1389. HP-TLC: $R_f{}^1$ 0.80, $R_f{}^2$ 0.79.

**Acetyl–Polymyxin B (2–10) Tetrahydrochloride (12).** Compound **11** (20.84 mg, 15 μmol) was treated with piperidine in the same manner as that described for **5**. The product was purified by RP-HPLC on a YMC Pack D-ODS-5ST column (20 × 150 mm) with linear gradient elution (20 min) from 10 to 16% $CH_3CN$ in 0.1% TFA as the eluent at a flow rate of 5 mL $min^{-1}$. Purified **12** was lyophilized from 75% dioxane containing 1 mol $L^{-1}$ HCl (50 μL). Yield: 7.01 mg (36.0%). $[\alpha]_D^{25}$ −50.8° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{45}H_{77}N_{14}O_{12}$ $[M+H]^+$ 1005, found 1005. HP-TLC: $R_f{}^1$ 0.07, $R_f{}^2$ 0.33.

**Myristoyl–Tetrakis($N^\gamma$-trifluoroacetyl)–Polymyxin B (2–10) (13).** To the solution of **4** (41.48 mg, 30 μmol) in DMF (400 μL), a mixture of myristoic acid (27.4 mg, 120 μmol) and HATU (45.63 mg, 120 μmol) in DMF (150 μL) was added. After the addition of NMM (6.12 μL, 60 μmol) to the solution under ice cooling, the mixture was stirred at room temperature, and its pH was maintained at 7.8 with NMM for 4 h. The product was purified on a Toyopearl HW-40 column in the same manner as described for **6**. Yield: 41.94 mg (89.85%). $[\alpha]_D^{25}$ −36.7° (*c* 0.5, DMF), FAB-MS calcd for $C_{65}H_{97}F_{12}N_{14}O_{16}$ $[M+H]^+$ 1557, found 1557. HP-TLC: $R_f{}^1$ 0.91, $R_f{}^2$ 0.84.

**Myristoyl–Polymyxin B (2–10) Tetrahydrochloride (14).** Compound **13** (31.12 mg, 20 μmol) was treated with piperidine in the same manner as that described for **5**. The product was purified by RP-HPLC on a YMC Pack D-ODS-5ST column (20 × 150 mm) with linear gradient elution (40 min) from 38 to 44% $CH_3CN$ in 0.1% TFA as the eluent at a flow rate of 5 mL $min^{-1}$. Purified **14** was lyophilized from 75% dioxane containing

1 mol $L^{-1}$ HCl (50 μL). Yield: 9.51 mg (36.1%). $[\alpha]_D^{25}$ −46.9° (*c* 0.2, 12% AcOH), FAB-MS calcd for $C_{57}H_{101}N_{14}O_{12}$ $[M+H]^+$ 1173, found 1173. HP-TLC: $R_f{}^1$ 0.49, $R_f{}^2$ 0.49.

**Colistin A and B (15 and 16).** Colistin sulfate (Wako, 600 mg) was purified by RP-HPLC using YMC Pack D-ODS-5ST (20 × 150 mm) with 23% $CH_3CN$ in 0.1% TFA at a flow rate of 6 mL $min^{-1}$. The peak products corresponding to colistin A ($t_R$: 39.6 min) and colistin B ($t_R$: 21.7 min) were obtained and lyophilized. Colistin A (**15**), yield: 195.5 mg (33.6%). $[\alpha]_D^{25}$ −59.2 (*c* 0.5, 12% AcOH), FAB-MS: 1169 $[M+H]^+$, (calcd. for $C_{53}H_{101}N_{16}O_{13}$; 1169), HP-TLC: $R_f{}^1$ 0.11, $R_f{}^2$ 0.41. Colistin B (**16**), yield: 219.8 mg (38.2%). $[\alpha]_D^{25}$ −61.6° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{52}H_{99}N_{16}O_{13}$ $[M+H]^+$ 1155, found 1155. HP-TLC: $R_f{}^1$ 0.11, $R_f{}^2$ 0.41.

**Pentakis($N^\gamma$-trifluoroacetyl)–Colistin A (17).** Purified colistin A (**15**) (164 mg, 0.12 mmol) was trifluoroacetylated with *S*-ethyl trifluorothioacetate (380 mg, 2.4 mmol), and the product **17** was precipitated in the same manner as that described for **2**. Yield: 183 mg (95.6%). $[\alpha]_D^{27}$ −41.3° (*c* 0.5, DMF), FAB-MS calcd for $C_{63}H_{96}F_{15}N_{16}O_{18}$ $[M+H]^+$ 1649, found 1649. HP-TLC: $R_f{}^1$ 0.92, $R_f{}^2$ 0.84.

**Pentakis($N^\gamma$-trifluoroacetyl)–Colistin (18).** Colistin sulfate (Wako, 1.5 g, 1.1 mmol, calculated as **15**) was trifluoroacetylated with *S*-ethyl trifluorothioacetate (3.4 g, 22 mmol) and the product **18** was precipitated in the same manner as that described for **3**. Yield: 1.69 g (96.6%, calculated as **17**). A small amount of the product **18** dissolved in DMSO was subjected to RP-HPLC on a YMC Pack D-ODS-5ST column (150 × 20 mm) with linear gradient elution (30 min) from 51.3 to 55.1% $CH_3CN$ in 0.1% TFA as the eluent at a flow rate of 5 mL $min^{-1}$. Two main peaks were observed, and one of the peaks was characterized as **17** based on the FAB-MS, HPLC, and HP-TLC analytical data. The other peak corresponded to pentakis($N^\gamma$-trifluoroacetyl)–colistin B: $[\alpha]_D^{27}$ −41.1° (*c* 0.5, DMF), FAB-MS calcd for $C_{62}H_{94}F_{15}N_{16}O_{18}$ $[M+H]^+$ 1635, found 1635. HP-TLC: $R_f{}^1$ 0.92, $R_f{}^2$ 0.84.

**Tetrakis($N^\gamma$-trifluoroacetyl)–Colistin (2–10) Hydrochloride (19). Route A:** Purified **17** (150 mg, 91 μmol) was treated with MSA (90 mL), $H_2O$ (45 mL), and dioxane (45 mL), and the product was isolated in the same manner as that described for **4**. Yield: 7.6 mg (6.2%). $[\alpha]_D^{25}$ −42.4° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{48}H_{73}F_{12}N_{14}O_{15}$ $[M+H]^+$ 1313, found 1313. HP-TLC: $R_f{}^1$ 0.71, $R_f{}^2$ 0.73.

**Route B:** Pentakis($N^\gamma$-trifluoroacetyl)–colistin (**18**) (490 mg) was treated with MSA (300 mL), $H_2O$ (150 mL), and dioxane (150 mL), and the product was isolated in the same manner as that described for **4**. Yield: 34.1 mg (8.5%). $[\alpha]_D^{25}$ −40.4° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{48}H_{73}F_{12}N_{14}O_{15}$ $[M+H]^+$ 1313, found 1313. HP-TLC: $R_f{}^1$ 0.70, $R_f{}^2$ 0.73.

**Colistin (2–10) Pentahydrochloride (20).** Compound **19** (2.0 mg, 1.5 μmol) was treated with a mixture of DMF:$H_2O$:piperidine (5:6:2), and the product was isolated in the same manner as that described for **5**. Yield: 1.0 mg (60.7%). $[\alpha]_D^{25}$ −45.6° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{40}H_{77}N_{14}O_{11}$ $[M+H]^+$ 929, found 929. HP-TLC: $R_f{}^1$ 0.04, $R_f{}^2$ 0.16.

**Octanoyl–Tetrakis($N^\gamma$-trifluoroacetyl)–Colistin (2–10) (21).** Compound **19** (13.5 mg, 10 μmol) was coupled with octanoic acid (5.76 mg, 40 μmol) and HATU (15.2 mg, 40 μmol), and the product was isolated in the same manner as that described for **6**. Yield: 14.0 mg (97.3%). $[\alpha]_D^{27}$ −26.3° (*c* 0.5, DMF), FAB-MS calcd for $C_{56}H_{87}F_{12}N_{14}O_{16}$ $[M+H]^+$ 1439, found1439. HP-TLC: $R_f{}^1$ 0.89, $R_f{}^2$ 0.84.

**Octanoyl–Colistin (2–10) Tetrahydrochloride (22).** Compound **21** (2.3 mg, 1.6 μmol) was treated with DMF:$H_2O$:piperidine (5:6:2), and the product was purified by HPLC in the same manner as that described for **7**. Yield: 0.9 mg (46.9%). $[\alpha]_D^{23}$ −50.7° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{48}H_{91}N_{14}O_{12}$ $[M+H]^+$ 1055, found 1055. HP-TLC: $R_f{}^1$ 0.48, $R_f{}^2$ 0.46.

**Boc–Dab(Fmoc)–Tetrakis($N^\gamma$-trifluoroacetyl)–Colistin (2–10) (23).** Compound **19** (13.5 mg, 10 μmol) was coupled with Boc–Dab(Fmoc)–OH (17.6 mg, 40 μmol) and HATU (15.2 mg, 40 μmol), and the product was isolated in the same manner as that described for **8**. Yield: 17.3 mg (99.7%). $[\alpha]_D^{27}$ −26.1° (*c* 0.5, DMF), FAB-MS calcd for $C_{72}H_{99}F_{12}N_{16}O_{20}$ $[M+H]^+$ 1735, found 1735. HP-TLC: $R_f{}^1$ 0.92, $R_f{}^2$ 0.85.

**Octanoyl–Dab(Fmoc)–Tetrakis($N^\gamma$-trifluoroacetyl)–Colistin (2–10) (24).** Compound **23** (13.0 mg, 7.5 μmol) was treated with TFA, and the resulting H–Dab(Fmoc)–tetrakis($N^\gamma$-trifluoroacetyl)–colistin (2–10) was coupled with octanoic acid in the same manner as that described for **9**. The product was purified on a Toyopearl HW-40 column in the same manner as that described for **9**. Yield: 12.0 mg (90.9%). $[\alpha]_D^{27}$ −23.8° (*c* 0.5, DMF), FAB-MS calcd for $C_{75}H_{105}F_{12}N_{16}O_{19}$ $[M+H]^+$ 1761, found 1761. HP-TLC: $R_f{}^1$ 0.90, $R_f{}^2$ 0.85.

**Octanoyl–Dab–Colistin (2–10) Hydrochloride (Octanoyl–Colistin, 25).** Compound **24** (7.9 mg, 4.5 μmol) was treated with DMF:$H_2O$:piperidine (5:6:2) in the same manner as that described for **5**, and the product was purified by HPLC under conditions similar to those used for **10**. Yield: 2.5 mg (41.7%). $[\alpha]_D^{25}$ −51.5° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{52}H_{99}N_{16}O_{13}$ $[M+H]^+$ 1155, found 1155. HP-TLC: $R_f{}^1$ 0.10, $R_f{}^2$ 0.42.

**Acetyl–Tetrakis($N^\gamma$-trifluoroacetyl)–Colistin (2–10) (26).** Compound **19** (26.97 mg, 20 μmol) in 90% DMF (500 μL) was treated with pyridine 2.25 μL (28 μmol) and acetic anhydride 2.65 μL (28 μmol), and the product was isolated in the same manner as that described for **11**. Yield: 19.68 mg (70.7%). $[\alpha]_D^{25}$ −41.3° (*c* 0.5, DMF), FAB-MS calcd for $C_{50}H_{75}F_{12}N_{14}O_{16}$ $[M+H]^+$ 1355, found 1355. HP-TLC: $R_f{}^1$ 0.80, $R_f{}^2$ 0.79.

**Acetyl–Colistin (2–10) Tetrahydrochloride (27).** Compound **26** (27.08 mg, 20 μmol) was treated with DMF:$H_2O$:piperidine (5:6:2), and the product was purified by HPLC in the same manner as that described for **12**. Yield: 9.01 mg (40.36%). $[\alpha]_D^{25}$ −47.3° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{42}H_{79}N_{14}O_{12}$ $[M+H]^+$ 971, found 971. HP-TLC: $R_f{}^1$ 0.07, $R_f{}^2$ 0.33.

**Myristoyl–Tetrakis($N^\gamma$-trifluoroacetyl)–Colistin (2–10) (28).** Compound **19** (53.94 mg, 40 μmol) was coupled with myristoic acid (11.52 mg, 80 μmol) and HATU (30.4 mg, 80 μmol), and the product was isolated in the same manner as that described for **13**. Yield: 46.58 mg (76.5%). $[\alpha]_D^{25}$ −37.3° (*c* 0.5, DMF), FAB-MS calcd for $C_{62}H_{99}F_{12}N_{14}O_{16}$ $[M+H]^+$ 1523, found1523. HP-TLC: $R_f{}^1$ 0.91, $R_f{}^2$ 0.85.

**Myristoyl–Colistin (2–10) Tetrahydrochloride (29).** Compound **28** (30.44 mg, 20 μmol) was treated with DMF:$H_2O$:piperidine (5:6:2), and the product was purified by HPLC in the same manner as that described for **14**. Yield: 6.47 mg (25.2%). $[\alpha]_D^{25}$ −46.4° (*c* 0.5, 12% AcOH), FAB-MS calcd for $C_{54}H_{103}N_{14}O_{12}$ $[M+H]^+$ 1139.788, found: 1139.8. HP-TLC: $R_f{}^1$ 0.48, $R_f{}^2$ 0.48.

**Antimicrobial Activities of Synthetic Polymyxin B and Colistin Analogs.** The antimicrobial activities of synthetic peptides were estimated by the standard micro plate dilution method as reported previously.[21]

**LPS Binding Activity.** LPS binding activity was examined according to the method reported by Moore.[31] A solution of [Dab-(Dansyl–Gly)[1]]–polymyxin $B_3$ (4 nmol in 4 μL) and 30 μg of LPS (*E. coli*, serotype005:$B_5$, Sigma Chemical Co.) in 5 mM HEPES

buffer (pH 7.2, 1 mL) was incubated in quartz cuvette at 30 °C for 1 h, and then a peptide solution (1 μmol $mL^{-1}$) (4 μL each) was added in the same manner as reported previously.[21] The fluorescence spectra were measured using a fluorescence spectrophotometer F-4500 (Hitachi Instrument Co., Tokyo, Japan) at an excitation wavelength of 330 nm and an emission wavelength of 490 nm.

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