

The Position of Aib Residues Defines the Antimicrobial Activity of Aib-Containing Peptides

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2-Aminoisobutyric acid (Aib,[1] 2-amino-2-methylpropionic acid) is an unusual amino acid, which is present in some antimicrobial peptides. To elucidate the impact of Aib residues on the conformation and biological activity of ion channel forming peptides, Aib residues in Ac-(Aib-Lys-Aib-Ala)$_5$-NH$_2$ were partially and fully replaced by Ala residues. A total of three analogs were thus synthesized. Two of these analogs, Ac-(Aib-Lys-Ala-Ala)$_5$-NH$_2$ (BKAA-20) and Ac-(Ala-Lys-Aib-Ala)$_5$-NH$_2$ (AKBA-20), had similar amino acid composition and were not hemolytic. BKAA-20 exhibited comparable antimicrobial activity to gramicidin S against Gram-positive bacteria (minimum inhibitory concentration: $\sim$3.13 μg ml^{-1}), whereas AKBA-20 was not active. Single-channel measurements showed that there was a clear correlation between the antimicrobial activity and the ion channel activity in these analogs. The third analog, Ac-(Ala-Lys-Ala-Ala)$_5$-NH$_2$, was biologically inactive. Circular dichroism spectroscopy revealed that the helix forming propensities of BKAA-20 and AKBA-20 were independent of the position of Aib residues in negatively charged phospholipid vesicles, as mimics of external cell membranes of bacteria. However, different positions of Aib residues in BKAA-20 and AKBA-20 affected their antimicrobial activity. It is indicated that the introduction of Aib residues into the peptide backbone not only promotes the generation of stable helical conformations, but also modulates the antimicrobial activity. The results of this study can implicate a deeper understanding of the role of Aib residues in the structure-activity relationships of helical pore forming peptides, which in turn promotes the rational design of effective antimicrobial peptides.

In the recent years, infection caused by microbes resistant to clinically used antibiotics has resulted in a serious threat to public health globally.[2–4] The structure-activity relationships of antimicrobial peptides have been widely investigated as part of a search for novel broad-spectrum antibiotics.[5–8] It is known that many of these peptides selectively interact with the negatively charged bacterial membranes and exhibit antimicrobial activity by permeabilizing the membrane via ion channel formation and/or disruption of its structure.[9] Naturally occurring antimicrobial peptides share some common features, despite the great diversity in their structures. The common cationic character of a majority of antimicrobial peptides is mostly due to the presence of lysine and/or arginine residues. Moreover, the presence of amphiphathic secondary structures, with polar side chains aligned along one side and hydrophobic residues along the opposite side, have also proved to be crucial for the interaction of these peptides with bacterial membranes.[10–12]

Peptaibols (peptide with Aib and C-terminal amino alcohol), such as alamethicin, were isolated from the fungi, and contain a high proportion of an unusual amino acid, 2-aminoisobutyric acid (Aib, 2-amino-2-methylpropionic acid). Peptaibols normally form α-and/or 3_{10}-helical conformations in lipid membranes, generate transmembrane ion channels, and exhibit strong antimicrobial and hemolytic activities. We have previously reported on the synthesis of an Aib-containing peptide, Ac-(Aib-Lys-Aib-Ala)$_5$-NH$_2$ (BKBA-20), which adopted a highly amphiphathic helical structure in 2,2,2-trifluoroethanol (TFE), phosphate buffer, and dipalmitoylphosphatidylcholine (DPPC) vesicles, exhibited well-defined ion channels in diphytanoylphosphatidylcholine (DPhPC) bilayers, and had potent activities against Gram-positive bacteria.[13] The impact of Aib residues and their position in the peptide backbone on the antimicrobial activity and ion channel formation ability of Aib-containing peptides can be of prime importance in understanding the structure-function relationships of the naturally occurring peptaibols and their synthetic analogs. So far, only a few reports have been dedicated to this subject.[14,15] In this study, to explore the role of Aib residues in the antimicrobial activity and ion channel formation of Aib-containing peptides, BKBA-20 was chosen as the template and Ala-substituted BKBA-20 analogs, (Ac-(Aib-Lys-Ala-Ala)$_5$-NH$_2$ (BKAA-20), Ac-(Ala-Lys-Aib-Ala)$_5$-NH$_2$ (AKBA-20), and Ac-(Ala-Lys-Ala-Ala)$_5$-NH$_2$ (AKAA-20)) were synthesized, and their conformation and biological activities were evaluated and compared to the original peptide. In these peptides, the total number of Lys residues, and therefore the overall positive charge of the peptides, is preserved. BKAA-20 and AKBA-20 share a similar number of Aib residues, while AKAA-20 lacks Aib residues. Consequently, it was found that all of the synthesized peptides interact with biological membranes and their helix-

forming propensities are comparable in negatively charged lipid vesicles. However, the positions of Aib residues in peptide sequences affected their interaction with membranes and antimicrobial activity.

Results and Discussion

Peptide Synthesis. Until lately, Aib-containing peptides had only been synthesized by stepwise solution techniques, since the solid-phase methods were mostly unsuccessful, or had very low yields. Recently, using Fmoc amino acid fluorides proved to be suitable, but not straightforward, for the solid-phase synthesis of Aib-containing peptides.[16,17] In a recent study, we successfully synthesized an Aib-containing peptide, BKBA-20, using conventional Fmoc-chemistry.[13] All four peptides of this study were synthesized in a similar manner. The Fmoc amino acid was pre-activated with HBTU-HOBt in the presence of DIEA, and the stepwise synthesis proceeded with reasonable yields. The crude peptides were obtained in high yields (81–96%). Purification of the synthetic peptides was carried out by preparative high-performance liquid chromatography (HPLC). The homogeneity and structures of the purified peptides were confirmed by analytical HPLC and MALDI-TOF mass spectrometry.

CD Spectroscopy. The secondary structures of peptides in various environments were studied by CD spectroscopy. The helical contents of peptides were calculated according to the Sreerama and Woody method,[18] and are summarized in Table 1. All four peptides exhibited helical structures with low helical contents in 50 mM (M = mol dm^{-3}) phosphate buffer (pH 7.4). Figure 1 shows the CD spectra of peptides in neutral phospholipid DPPC (Fig. 1a), and the negatively charged phospholipid DPPC/DPPG (dipalmitoylphosphatidylglycerol) (3:1) (Fig. 1b) as small unilamellar vesicles (SUVs). DPPC and DPPC/DPPG liposomes were used as models for mammalian erythrocyte and bacterial exterior cell membranes, respectively. In the presence of DPPC/DPPG SUVs, all four peptides exhibited comparable helical structures with typical double minimum bands at 208 and 225 nm and a strong positive band at 194 nm. Moreover, in 50% TFE/buffer, a membrane-mimicking environment,[19,20] the CD spectra of all four peptides also had comparable helical conformations (helical contents: 78–82%) (data not shown). Therefore, it is plausible for these peptides to have similar helix forming propensities in membrane-like environments, independent of the total number and the position of Aib residues.

However, the helical contents of peptides in DPPC SUVs differed from those in DPPC/DPPG SUVs. The spectrum of AKAA-20 in DPPC is quite similar to that of the peptide in buffer, suggesting that AKAA-20 may interact weakly with DPPC. The helical content of BKBA-20 in DPPC showed a similar value in DPPC/DPPG SUVs. Interestingly, AKBA-20 had a lower helical content than BKAA-20, indicating that the

Table 1. Helicities of Aib-Peptides in Neutral and Acidic Vesicles

	Helical contents / %[a]		
Peptides	Phosphate buffer	DPPC SUVs	DPPC/DPPG[b] SUVs
BKBA-20	39	80	80
BKAA-20	36	78	85
AKBA-20	43	59	88
AKAA-20	30	39	86

a) Helical contents were calculated according to the Sreerama and Woody method.[18] b) Molar ratio of DPPC:DPPG was 3:1. Concentrations of peptide and lipid were 10 μM and 2 mM, respectively.

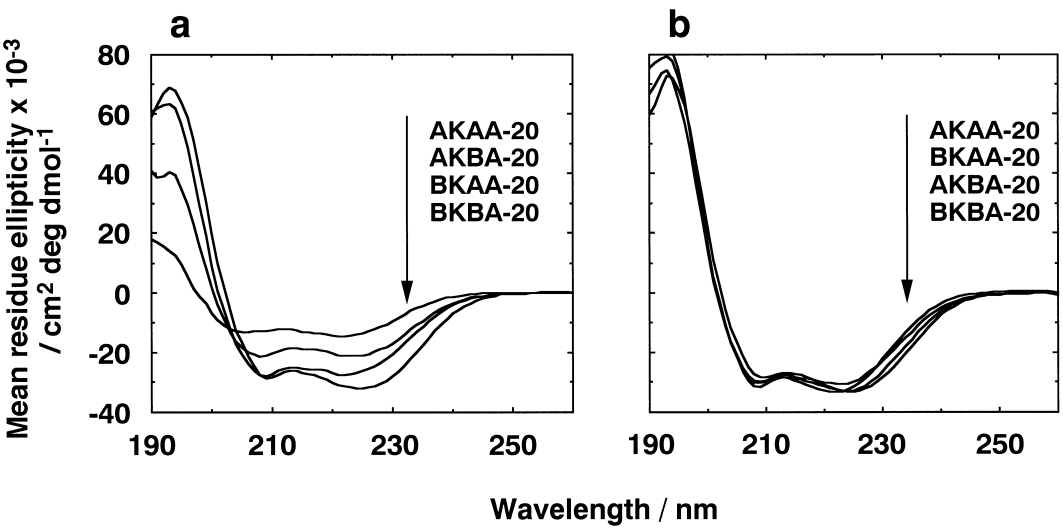

Fig. 1. CD spectra of Aib-containing peptides in (a) DPPC SUVs and (b) DPPC/DPPG (3/1) SUVs. Concentrations of peptide and lipid were 10 μM and 2 mM, respectively. Spectra were measured at 30 °C in 50 mM phosphate buffer (pH 7.4).

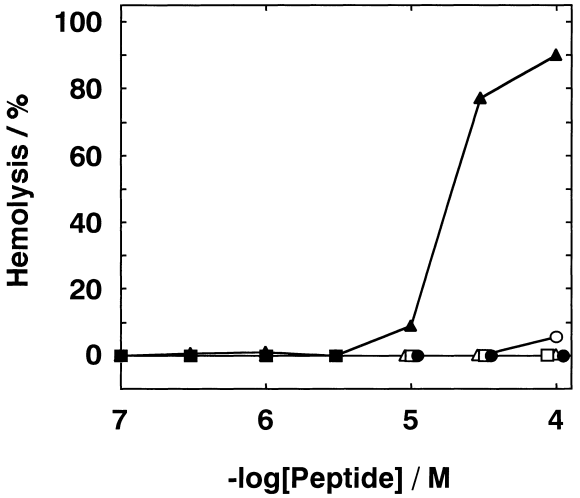

Fig. 2. Hemolytic activity of Aib-containing peptides for human erythrocyte (2%) in 10 mM phosphate buffer, 150 mM NaCl (pH 7.4). Peptides: BKBA-20 (open circle), BKAA-20 (open triangle), AKBA-20 (open square), AKAA-20 (closed circle), and alamethicin (closed triangle). The mixture of peptide and erythrocyte was incubated at 37 °C for 1 h. All data are mean values of three experiments.

conformations of the two peptides in DPPC SUVs were different in spite of the similarity in their amino acid composition and the intrinsic hydrophobicity (the total hydrophobicity of amino acid components of the peptide). In general, it seems that hydrophobicity is an important factor in the interaction of peptides with neutral lipid membranes.[21,22] The retention times on reversed-phase HPLC can be interpreted as measures of peptide hydrophobicity.[23] The retention times of BKBA-20, BKAA-20, AKBA-20, and AKAA-20 (determined on a C_{18} column) were 18.27, 17.42, 16.42, and 15.73 min, respectively. These results were well correlated with the helical contents in DPPC SUVs. The difference in the retention times of BKAA-20 and AKBA-20 can be due to a difference in their hydrophobicity, that is related to the ability of the peptide to form hydrophobic domains for interactions with the reversed-phase matrix. Similar observations on the relationship between peptide hydrophobicity and the retention time on HPLC have been reported both regarding α-helical[24,25] and β-sheet[32] peptides.

Hemolytic and Antimicrobial Activities. The biological activities of all four peptides were evaluated as hemolytic and antimicrobial activities. Figure 2 shows the hemolytic activity of peptides against human erythrocytes. Alamethicin, as a helical and highly hemolytic peptaibol, was used as a reference. The half-effective concentration of alamethicin was about 20 μM. The four peptides of this study exhibited very weak hemolytic activities. It has been reported that those peptides with higher hydrophobicity exhibited stronger hemolytic activities due to a hydrophobic interaction of the peptide with the erythrocyte membranes, which are mainly composed of neutral phospholipids.[21,24] In our HPLC analysis, the retention time of alamethicin was 26.36 min (more hydrophobic than the rest of the peptides of this study). Consequently, the results of hemolysis can be partly attributed to the hydrophobic character of the peptides. It seems that peptides with comparable structures and hydrophobicities lower than a certain threshold (as judged by the lower retention times compared to that of alamethicin) lose their hemolytic activities.

Table 2. Antimicrobial Activity of Aib-Containing Peptides

Peptides	MICs/μg mL^{-1} a)		
	S. aureus	*B. subtilis*	*E. coli*
Alamethicin	12.5	6.25	> 100
Gramicidin S	6.25	3.13	> 100
BKBA-20	6.25	0.78	> 100
BKAA-20	6.25	3.13	> 100
AKBA-20	> 100	> 100	> 100
AKAA-20	> 100	> 100	> 100

a) The MICs (minimum inhibitory concentrations) were determined by the dilution method in Bouillon agar medium. Plates were incubated at 30 °C at 14 h.

Table 2 shows the minimum inhibitory concentrations of the studied peptides against bacteria *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. None of the peptides were active against Gram-negative bacteria, *E. coli*. Antimicrobial positively charged helical peptides, such as magainins, which are active against *E. coli*, can interact with the surface of the bacterial outer membranes and permeabilize the inner membranes.[26,27] There is a possibility that due to their conformation and amphipathic pattern in an aqueous environment and/or lipid bilayer surfaces, despite the overall positive charge of the peptides (+5), none of the four peptides can effectively bind to (and therefore penetrate through) the surface of the bacterial outer membranes to approach the inner membranes of *E. coli*. Conversely, another possibility can be due to the strong interaction of the positive charges (Lys side-chains) with the negative charges on the bacterial membrane surface, which hinders any further penetration of peptides into the inner bacterial membranes. The CD spectra of peptides in negatively charged vesicles support the second assumption (strong interaction with the induction of highly helical structures in membranes, Fig. 1b). However, the first assumption, or a combination of both assumptions, cannot be completely ruled out due to the complexities of the bacterial membrane structures compared to simplified membrane models. The case is different for the Gram-positive bacteria. AKAA-20, which does not contain Aib residues, and AKBA-20 were not active against the examined strains of Gram-positive bacteria despite their amphiphathic cationic nature. In contrast, BKBA-20 showed potent activities against Gram-positive bacteria.[13] BKAA-20, having the same amino acid composition to AKBA-20, exhibited comparable activities to BKBA-20 and gramicidin S (a broad-spectrum potent cyclic antimicrobial peptide), and stronger activities than alamethicin against Gram-positive bacteria. These results indicated that the position of Aib residues in the peptide backbone affect their antimicrobial activity. In addition to electrostatic interactions, hydrophobic interactions play a major role in the partitioning of peptides into the lipid bilayer of biological membranes.[12] Juvvadi et al. reported that the antimicrobial activity of a cecropin A-melittin hybrid peptide could be modulated by the peptide hydrophobicity.[33] Likewise, the antimicrobial activity of magainin 2 analogs and

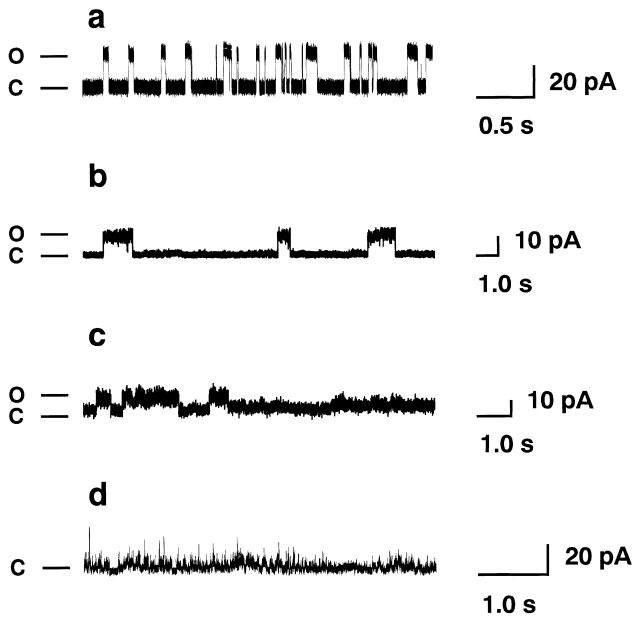

Fig. 3. Conductance patterns of (a) BKBA-20, (b) BKAA-20, (c) AKBA-20, and (d) AKAA-20 in DPhPC bilayer at 25 ± 2 °C. The electrolyte solution was 500 mM KCl buffered with 5 mM HEPES (pH 7.4). The electrolyte composition was symmetrical on both sides of the membrane, and peptide concentration was 100 nM. The applied membrane potential was 100 mV. The states of open and closed, determined from the current amplitude histogram, are represented as O and C, respectively.

the model peptides increases with enhanced peptide hydrophobicity.[22,24,28,29] From our results of HPLC analysis, the difference in the activity of BKAA-20 and AKBA-20 can be explained by relating it to the hydrophobicity of peptides. To evaluate the enzymatic stabilities of BKAA-20 and AKBA-20, a tryptic digestion assay was carried out. AKBA-20 had a 19-fold higher resistance to digestion than BKAA-20. This suggests that the difference in the antimicrobial activities of BKAA-20 and AKBA-20 can not be explained by the proteolytic effect of bacterial proteases on peptides.

Ion Channel Formation. Ion channel formation is one of the proposed mechanisms for the antimicrobial activity of peptides.[9] Figure 3 shows the conductance patterns of all four peptides, measured by the tip-dip patch-clamp technique. DPhPC lipid was selected for these experiments because of its ability to form mechanically stable membranes. BKBA-20 formed well-defined single-state channels at 100 mV membrane potentials[13] (Fig. 3a). BKAA-20 also formed ion channels, and its molecular assembly for channel formation was more homogeneous rather than BKBA-20 (Fig. 3b). Although AKBA-20 exhibited channel-like activities, its conductance patterns were irregular stray fluctuations with erratic increases in the membrane current (Fig. 3c). The conductances of BKAA-20 and AKBA-20 were 100 and 90 pS, respectively. However, the relative probability for detecting the conducting states was higher in BKAA-20 compared to AKBA-20. As shown in Fig. 3d, the non Aib-peptide, AKAA-20, did not form visible ion channels. Consequently, there was a clear correlation between the antimicrobial and ion channel activities in these analogs. Likewise, Béven et al. reported in their structure-activity relationships of alamethicin and related analogs that the antimicrobial activities of peptides were correlated with their ion channel activity.[34] The peptides that formed ion channels with defined conductance patterns, BKBA-20 and BKAA-20, were also more active against Gram-positive bacteria. Therefore, it is plausible that the ability to form ion channels is part of the mechanism of the antimicrobial activity of these peptides.

Conclusions

In the present study, the role of Aib in the biological activity of a series of model helical peptides was explored in some detail. Aib is commonly found in the naturally occurring antimicrobial peptides, peptaibols. Structure-function relationship studies in BKBA-20 (a helical and antimicrobial synthetic peptide) and its analogs suggested that the alternately arranged Aib residues are significant for not only inducing stable helical conformations, but also the biological activity of these peptides. The same alternative arrangement of Aib residues has also been observed in the biologically active peptaibols. It was found that the helix forming propensities of the synthesized peptides were independent of the position of the Aib residues in the negatively charged DPPC/DPPG vesicles, although the helical contents of peptides were sequence dependent and affected in DPPC vesicles. The peptide structures in membranes and their ion channel forming ability in lipid bilayers had a clear correlation with their antimicrobial activity against Gram-positive bacteria, but not with their hemolytic activity. Moreover, the positions of Aib residues in the peptide sequence affected the biological activity. An analog of BKBA-20, BKAA-20, had considerable antimicrobial activity, but was not hemolytic (a desirable therapeutic property). Another analog, AKBA-20, which was different from BKAA-20 only in the relative positions of Aib residues, was neither antimicrobial nor hemolytic. It is therefore shown that despite structural similarities in lipid membranes, the position of Aib residues and the overall change in the hydrophobicity of peptide molecules can significantly alter their biological activity. This is an interesting and important finding that can be utilized in designing of novel helical antimicrobial peptides possessing high therapeutic potencies without cytotoxicity. Presently, based on the results of this work and to optimize the therapeutic properties of Aib-containing helical templates, synthesis and structure–function studies of these peptides are being undertaken in our laboratories.

Experimental

General. Fmoc-Aib-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH, Rink Amide resins (0.56 mmol/g, 100–200 mesh), and HBTU were purchased from Calbiochem-Novabiochem (Tokyo, Japan). DCC, HOBt, and TFA were obtained from Peptide Institute (Osaka, Japan). The standard amino acid mixture (type H) and phenyl isothiocyanate were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). DPPC and DPPG were from Sigma (Missouri, USA). DPhPC was obtained from Avanti Polar Lipids (Alabaster, AL) as 50 mg ml^{-1} chloroform solutions. All other chemicals from Wako Pure Chemical Industries Ltd. were of special grade, and used without further purification.

RP-HPLC was conducted on a JASCO chromatography recorder (Tokyo, Japan) with a UV-970 Intelligent UV/Vis detector at 220 nm and 807 IT Intelligent Integrator. Eluents were solution A (95% H_2O/5% CH_3CN/0.05% TFA) and solution B (5% H_2O/95% CH_3CN/0.04% TFA). Mass spectra were measured on a MALDI-TOF MS (Voyager-DERP, PerSeptive Biosystems Inc., Framingham, MA) with α-cyano-4-hydroxycinnamic acid (Aldrich Chem. Co, Milwaukee, WI) as the matrix. Amino acid analysis was performed on a Pico Tag Workstation (Waters, Milford, MA) after hydrolysis in a constant-boiling hydrochloric acid at 110 °C for 24 h.

Peptide Synthesis. Peptide synthesis was carried out through a stepwise solid-phase peptide synthesis utilizing Fmoc-chemistry on as Rink Amide resins.[13] Ten-fold molar excess of the Fmoc amino acid was pre-activated with HBTU–HOBt in the presence of DIEA for 20 min. Coupling reaction was carried out for 50 min. Fmoc group was removed by 20% piperidine in DMF for 10 min. After acetylation by DCC/HOBt–AcOH, the resin was treated with TFA/H_2O (90/10, v/v) for 90 min at room temperature. The precipitated peptide by the addition of ether was purified by preparative HPLC on a Wakosil 5C4-200 column (10 × 250 mm). Gradient: 20–100% B in 60 min with a linear gradient at a flow rate of 2.0 mL min^{-1}. The homogeneity of the peptide was confirmed by analytical HPLC on a Wakosil 3C18 RS column (2.0 × 150 mm). Gradient: 0–100% B in 30 min with a linear gradient at a flow rate of 0.25 mL min^{-1}. Structures of peptides were verified by amino acid analysis and MALDI-TOF MS. De Filippis et al. reported that the reaction of phenyl isothiocyanate with Aib led to the formation of phenylthiocarbamyl (PTC)-Aib and cyclic phenylthiohydantoin (PTH)-Aib derivatives.[30] In our amino acid analysis, two peaks were observed, which corresponded to these derivatives. However, these peaks were not reproducible and, as a result, Aib residues were not estimated under our analysis conditions. The concentrations of peptide solutions were determined by estimations based on PTC-Ala and PTC-Lys derivatives. Synthesis of BKBA-20 has recently been reported in detail.[13]

BKAA-20. Yield of crude peptide, 90%. Amino acid ratios in acid hydrolysate: Ala 9.7, Lys 5.0. MS Found: *m/z* 1837.9. Calcd for $(M + H)^+$ 1837.3.

AKBA-20. Yield of crude peptide, 81%. Amino acid ratios in acid hydrolysate: Ala 10.1, Lys 5.0. MS Found: *m/z* 1837.2. Calcd for $(M + H)^+$ 1837.3.

AKAA-20. Yield of crude peptide, 96%. Amino acid ratios in acid hydrolysate: Ala 14.7, Lys 5.0. MS Found: *m/z* 1766.8. Calcd for $(M + H)^+$ 1767.1.

Preparation of Liposomes. Small unilamellar vesicles were prepared by probe sonication of DPPC or DPPC/DPPG (3:1 molar ratio) dispersions. Lipids were solved in chloroform and then dried in vacuum overnight. These lipid films were hydrated with 50 mM phosphate buffer solution (pH 7.4), voltexed at 50 °C for 30 min, and then sonicated for 20 min using Branson Sonifier 250 (Branson, Danbury, CT).

CD Spectra Measurements. CD spectra were measured on a Jasco J-720 spectropolarimeter (Tokyo, Japan) using a cylindrical cuvette of 1 mm path length. The instrument was calibrated with recrystallized camphorsulfonic acid-d_{10} (Katayama chemical, Osaka). The CD cuvette was washed with a concentrated NaOH aqueous solution between determinations to remove any peptide adhering to its inner surface. The peptide concentrations of stock solutions were determined based on a quantitative amino acid analysis. All staffs for preparing and transferring the solution were siliconized to prevent nonspecific adsorption of the hydrophobic peptides. Samples were incubated at room temperature for 30 min. All spectra were the average of 10 scans obtained by collecting data at 0.2 nm intervals from 260 to 190 nm at 30 °C. The helical contents were calculated according to the Sreerama and Woody method.[18]

Antimicrobial Assay. The minimum amounts of Aib-peptides necessary for complete inhibition of the growth of *Bacillus subtilis* PCI 215, *Staphylococcus aureus* FDA 209P, and *Escherichia coli* B were determined by dilution method in Bouillon agar medium. Plates were incubated at 30 °C for 14 h. Alamethicin and gramicidin S were used as reference compounds. The minimum inhibitory concentration (MIC) of each peptide is reported as the average of triplicate measurements.

Hemolytic Assay. The hemolytic activities of Aib-peptides were determined using human erythrocytes. The erythrocytes were collected from heparin-treated blood by centrifugation (at 2500 rpm at 4 °C for 5 min) and washed four times with phosphate-buffered saline (PBS: 10 mM phosphate buffer, pH 7.4/150 mM NaCl) to remove plasma and buffy coat. Suspensions of 2% erythrocytes in PBS with or without peptides were incubated at 37 °C for 1 h. Hemolysis was evaluated as the release of hemoglobin (absorbance at 570 nm) of the supernatant after centrifugation at 2500 rpm at 4 °C for 5 min. A 100% hemolysis was determined by hemoglobin release after the addition of 0.3% Triton X-100. Alamethicin was used as a reference compound. The hemolytic activity of each peptide is reported as the average of triplicate measurements.

Single-Channel Measurements. Patch-clamp experiments were performed using tip–dip technique at room temperature (25 ± 2 °C), as was generally described.[13,31] The patch pipettes were of the hard-glass type, and were prepared by the two-pull method by a pipettes-puller (Narishige, Tokyo) to give an approximate diameter of 1 μm. The electrolyte solutions were comprised of 500 mM KCl solutions buffered with 5 mM HEPES (pH 7.4). The electrolyte composition was symmetrical for both sides of the lipid bilayer. Peptide concentration was 100 nM, when added to patch pipettes. Chloroform solutions of DPhPC were mildly evaporated by N_2 flushing, and the residual lipids were redissolved in hexane (2 mg mL^{-1}) before being spread on an aqueous surface. DPhPC hexanic solution was added to the aqueous surface of electrolyte solutions (about 2 μL) in plastic dishes with 3.5 cm diameters. The single-channel current was measured using an Axopatch 1D patch-clamp amplifier (Axon Instruments) controlled with pClamp 6 (Axon Instruments) software. Data were filtered at 2 kHz frequency, stored directly on a hard disk, and analyzed with an Axograph (Axon Instruments).

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References

1 The abbreviations are according to biochemical nomenclature by IUPAC-IUB Joint Commission, *Eur. J. Biochem.*, **138**, 9 (1984), are used throughout. Unless otherwise specified, amino acids without Aib are L-stereoisomers. Additional abbreviations are as follows: Aib, 2-aminoisobutyric acid (2-methylalanine, 2-amino-2-methylpropionic acid); Boc, *t*-butyloxycarbonyl; CD, circular dichroism; DCC, dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DPhPC, diphytanoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; Fmoc, 9-fluore-

nylmethoxycarbonyl; HBTU, *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectroscopy; PBS, phosphate-buffered saline; Rink Amide resins, 4-(2,4-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resins; RP-HPLC, reversed phase high-performance liquid chromatography; SUVs, small unilamellar vesicles; TFA, 2,2,2-trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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