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Structure-Function Relationship of Model Aib-Containing Peptides as Ion Transfer Intermembrane Templates[1]

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Peptaibols comprise a family of peptide antibiotics with high contents of 2-aminoisobutyric acid (Aib) residues and C-terminal amino alcohols. These peptides form α-helical structures leading to voltage-gated ion channels in lipid membranes. In the present study, amphiphilic helical Aib-containing peptides of various chain-lengths, Ac-(Aib-Lys-Aib-Ala)$_n$-NH$_2$ (n=1-5), were designed to investigate the mechanisms of the aggregation and transmembrane orientation of helical motifs in lipid bilayer membranes. Peptide synthesis was performed by the conventional stepwise Fmoc solid-phase method. The crude peptides were obtained in high yields (66-85%) with high purities (69-95%). Conformational analysis of the synthetic peptides was performed by CD spectroscopy. It was found that these peptides take on highly helical structures, and the helicity of the peptides increases with an increase in chain-length. The longest peptide, Ac-(Aib-Lys-Aib-Ala)$_5$-NH$_2$, self-aggregates and adopts a barrel-stave conformation in liposomes. Ac-(Aib-Lys-Aib-Ala)$_5$-NH$_2$ exhibited potent antimicrobial activity against Gram-positive bacteria. Patch-clamp measurements revealed that this peptide can form well-defined ion channels with a long lifetime at relatively low transbilayer potentials and peptide concentrations. For this peptide, the single-channel conductance of the most frequent event is 227 pS, which could be related to a single-state tetrameric pore.

Key words: Aib (2-aminoisobutyric acid)-peptide, antimicrobial activity, ion channel activity, peptide chain aggregation, solid phase peptide synthesis.

Ion channels are important in mediation of the transmembrane transduction of electrical and chemical signals between cells and cellular compartments. Recently, a number of physiologically important channel proteins were identified and sequenced, and analysis of their sequences suggested that they are composed of four or five domains, each of which contains a number of membrane-spanning helices (*1*). Natural ion channels are complex proteins that combine various electrophysiological functions—such as gating mechanism, ion selectivity, voltage dependence and conductance—in a single molecule. It is known that several antibiotic peptides (*e.g.* melittin, magainin and cecropin) interact with biological membranes, and with each other in the membranes to form ion channels (*2*). Alamethicin is a 20-residue peptide from the fungus, *Trichoderma viride*, which contains a high proportion of the unusual amino acid, 2-aminoisobutyric acid (Aib), in addition to two proline residues (*3*). The *N*-terminus of alamethicin is acetylated and the C-terminal residue is phenylalaninol. Its amino acid sequence is Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phe-*ol*. Alamethicin is capable of forming voltage-gated channels exhibiting very high conductance in lipid membranes. Peptaibols form helical structures in phospholipid membranes, and the presence of Aib, with its preference for certain dihedral angles most suitable for 3_{10}- or α-helical structures, gives a particular stability to their conformations in a variety of environments. In accordance with experimentally supported models, several molecules of these peptides associate in membranes to form a pore in the center of the assembly to allow ions to pass through. The use of synthetic peptides with protein-like ion channel functions is challenging. However, it has been shown that artificial functional

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Abbreviations: Aib (B), 2-aminoisobutyric acid; DCC, *N,N*-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DPhPC, diphytanoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; NMP, *N*-methylpyrrolidinone; Rink Amide resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin; RP-HPLC, reversed phase high performance liquid chromatography; TFA, 2,2,2-trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

proteins can be designed to control signals through lipid membranes, and to obtain an insight into the mechanisms of ion transfer (*4*). The design of artificial functional proteins has long been a top priority in several laboratories throughout the world. The major requisites for the successful design of pore-forming peptides are the abilities to form stable amphiphilic secondary and/or supersecondary structures inside or on the surface of lipid membranes, to allow moderate transmembrane conductance in the range of protein ion channels or pores, to possess ion selective properties to distinguish between different ions with different natures, and to exhibit other distinct electrophysiological functions such as voltage- or ligand-gating.

The relationship between various biological functions of Aib-containing peptides (for instance, ion transfer and lipid-peptide interaction) and their secondary and/or supersecondary structures has been widely investigated (*5-9*). However, detailed characterization of alamethicin-like pores is still needed for comparison with natural channel proteins. It would be interesting to elucidate the relationships between the conformations and functions of ion channel peptides, by extension of related integral membrane proteins, and development of methods for the prediction and modeling of membrane protein structures. In addition, Aib-containing peptides have been normally synthesized through tedious procedures that involve carefully chosen stepwise solution techniques and chemical segment condensations (*10, 11*).

In the present study, to gain an insight into the mechanisms by which amphiphilic α-helices can aggregate and conduct ions, we synthesized Aib-containing peptides of various chain lengths [Ac-(Aib-Lys-Aib-Ala)$_n$-NH_2 (n=1, 2, 3, 4, and 5: named BKBA-4, 8, 12, 16, and 20, respectively)] by the solid-phase method. Following the above-mentioned guidelines using a combination of CD spectroscopy and patch-clamp methodology, our efforts in this study are mostly directed towards the structural and functional characterization of stable transmembrane pore structures (with moderate conductance) formed by these synthetic peptides.

EXPERIMENTAL PROCEDURES

Materials—Peptide synthesis was carried out by the Fmoc strategy in disposable polypropylene vessels (Pierce, Rockford, IL). Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-OSu, HOBt, HOAt, HBTU, and HATU were obtained from Peptide Institute (Osaka) and Nova Biochem (Tokyo). Siliconized sample tubes were used to prevent any non-specific adhesion of the peptide to the tube walls. The standard amino acid mixture (type H), phenyl isothiocyanate (PITC), CH_3COONa, CH_3CN, and piperidine were from Wako Chemicals (Osaka). DMF and NMP were purchased from Kanto Chemicals (Tokyo). Rink Amide resin (0.37 mmol/g, 100-200 mesh) was from Nova Biochem. DPPC and DPPG were from Sigma (St. Lois, MO). Calcein was obtained from Dojindo Laboratories (Kumamoto). DPhPC was obtained from Avanti Polar Lipids (Alabaster, AL) as a 50 mg/ml chloroform solution. Alamethicin and gramicidin S were purchased from Sigma.

Fmoc-Aib-OH—To a solution of H-Aib-OH (1.03 g, 10 mmol) in an aqueous Na_2CO_3 solution (1.05 g/10 ml) was added Fmoc-OSu (4.03 g, 12 mmol) in acetone (20 ml) in portions over a period of 10 min at room temperature. The briskly stirred solution was maintained at pH 9-10 by the addition of a 1 M Na_2CO_3 solution. After stirring overnight the solution was evaporated and the residue was dissolved in water (10 ml). The aqueous solution was washed with ether (10 ml×3) and then acidified cautiously with 6 M HCl to pH 3. The white precipitate formed was extracted with EtOAc (30 ml×3). The combined EtOAc layers were washed with water (30 ml×3) and then dried over anhydrous Na_2SO_4. The filtrate was evaporated, and the residue was solidified by the addition of ether and petroleum ether. Yield, 3.1 g (80%). ^{1}H-NMR ($CDCl_3$) d: 1.55 (*bs*, 6H, CH_3×2); 4.19 (*t*, 1H, C*H*); 4.40 (*bs*, 2H, CH_2); 7.26 (*dd*, 4H, Ar-$H^{2,3,6, \text{and } 7}$); 7.78 (*dd*, 4H, Ar-$H^{1,4,5 \text{ and } 8}$). R_f ($CHCl_3$-MeOH-AcOH=95:5:1, by volume) 0.47.

Peptide Synthesis—Peptides were synthesized by the stepwise elongation of Fmoc-amino acid on Rink Amide resin (135.1 mg, 0.05 mmol). The coupling protocol was as follows: (i) preswelling of the resin with DCM (×3) and DMF (×3); (ii) deprotection of the Fmoc-group with 20% piperidine/DMF (1 min and 10 min); (iii) washing with DMF (2 ml×10); (iv) activation of the Fmoc-amino acid (0.25 mmol) with 0.45 M HOBt-HBTU in DMF (0.56 ml, 0.25 mmol) and 2 M DIEA/NMP (0.25 ml, 0.5 mmol) for 20 min; (v) coupling with the preactivated Fmoc-amino acid for 30 min; and (vi) washing with DMF (2 ml×10). These procedures (steps 2-6) were repeated until the desired compound was obtained. After removing the Fmoc-group, the revealed amino group was acetylated with AcOH (30 mg, 0.5 mmol), DCC (103 mg, 0.5 mmol), and HOBt (76.5 mg, 0.5 mmol). Final deprotection and cleavage of the peptide from the resin were achieved with 95% TFA/water for 90 min at room temperature. After removing the resin by filtration, the filtrate was concentrated with N_2 gas, and the crude product was precipitated and washed with ether. The crude peptides were purified by preparative HPLC on a Wakosil 5C4-200 column (10×250 mm i.d.) using the following solvent systems: (A) 95% water/5% CH_3CN/0.05% TFA; and (B) 5% water/95% CH_3CN/0.04% TFA. The purity of the peptides was confirmed by analytical HPLC on a Wakosil 5C4-200 column (4.6×250 mm i.d.) using the same solvent systems as described above. HPLC-purified peptides were characterized by electron spray ionization mass spectroscopy with a Finnigan MAT SSQ 7000 (Finnigan MAT, San Jose, CA). The results of mass spectrometry measurements were in agreement with theoretical data: BKBA-4, observed molecular weight 428.4 (calculated 428.5); BKBA-8, 797.9 (798.1); BKBA-12, 1,167.4 (1,167.5); BKBA-16, 1,536.7 (1,536.9); BKBA-20, 1,906.3 (1,906.4).

Circular Dichroism—The CD spectra were recorded with a JASCO J-720 spectropolarimeter with a cylindrical cell of 1 mm path length at room temperature. The instrument was calibrated with recrystallized camphorsulfonic acid-d_{10}. The CD cell 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 on the basis of the amino acid analysis data. Peptide solutions were prepared an hour before measurement. All spectra were the averages of 8 to 10 scans obtained by collecting data from 260 to 190 nm at 0.2 nm intervals, with a response time of 2 s for each point. The results were expressed as the

mean residue ellipticity. The predicted contents of secondary structures were calculated according to the Sreerama-Woody method (*12*).

Preparation of Liposomes—Small unilamellar vesicles were prepared by probe sonication of DPPC or DPPC/DPPG dispersions. Lipids were used as chloroform solutions. The chloroform was then evaporated off under a stream of nitrogen. The lipids were subjected to reduced pressure for 1 h and then resuspended in an appropriate buffer by vortexing. The resulting lipid dispersions were then sonicated for 10–20 min until clear solutions were obtained. The vesicle size was determined by the electro light scattering method (ELS-800, Photal, Otsuka Electronics). Examination of the grids demonstrated that the vesicles were unilamellar, with an average diameter of 60–75 nm.

Leakage Measurements—Unilamellar vesicles on trapping calcein were prepared as follows. A lipid film was hydrated with a 70 mM calcein solution (pH 7.4). The suspension was vortexed and sonicated. Untrapped calcein was removed by gel filtration [Sephadex G-25, 15×450 mm column, 10 mM Tris-HCl buffer (pH 7.4) as the eluent]. The lipid concentration was determined by phosphorus analysis (*13*). The vesicular suspension was mixed with the peptide in a 10 mM Tris-HCl buffer solution (pH 7.4) in a cuvette. The leakage of calcein out of the vesicles was monitored by measuring the fluorescence intensity at 520 nm (excitation at 490 nm) with a Shimadzu RF-510 spectrofluorimeter at 30°C. The fluorescence intensity corresponding to 100% leakage was determined by adding a Triton X-100 (10% in water) solution. The percentage leakage was determined using the equation: $100\times(F-F_0)/(F_t-F_0)$, where F is the fluorescence intensity achieved with the peptides, F_0 and F_t are the intensities observed without the peptide and after Triton X-100 treatment, respectively.

Antimicrobial Assays—The minimum amounts of BKBA peptides necessary for complete inhibition of the growth of *Bacillus subtilis* PCI 215, *Staphylococcus aureus* FDA 209P, and *Escherichia coli* B were determined by the dilution method in Bouillon agar medium. Plates were incubated at 37°C overnight. Alamethicin and gramicidin S were used as reference compounds.

Single-Channel Measurements—Patch-clamp experiments were performed as generally described, with minor modifications (*8*). The patch pipettes were of the hard-glass type, were prepared by the two-pull method to give an approximate diameter of 1 μm. The pipettes were used without being heat polished or siliconized. The electrolyte solutions comprised 0.5 M KCl solutions buffered with 5 mM HEPES at pH 7.4. The pipette tip resistance in electrolyte solutions was about 5 MΩ for a 0.5 M KCl solution. Peptide stock solutions were prepared as 10 or 100 μM buffer solutions. The final peptide concentration, when added to patch pipettes, was 100 nM. The chloroform solutions of DPhPC lipids were mildly evaporated, and the residual lipid was redissolved in hexane before being spread on an aqueous surface. Seals of 2 to 20 GΩ were formed at the tip of pipettes. Single-channel currents were measured using an Axopatch 1D patch-clamp amplifier (Axon Instruments) controlled with pClamp 6 (Axon Instrument) software. Data were filtered at 2 kHz frequency, stored directly on a disk, and analyzed with an Axograph (Axon Instruments).

RESULTS

Synthesis of Model Peptides—Peptide synthesis was carried out by the solid-phase method on Rink Amide resin using the Fmoc strategy. This is a clear-cut synthesis protocol for Aib-containing peptides (4–20 amino acid residues length) with conventional procedures. The crude peptides were obtained in high yields (66–85%). The purities of synthetic BKBA-4, 8, 12, and 16 were confirmed by RP-HPLC (95, 91, 85, and 76%, respectively). All of the side products were confirmed to have single- or multi-deletions of Aib, Lys, or Ala residues by ESI-MS analysis. These peptides could also be synthesized efficiently by the DCC-HOBt and HBTU methods. Purification of the synthesized peptides was carried out by preparative RP-HPLC. The homogeneities and structures of the purified peptides were verified by analytical RP-HPLC, amino acid analysis, and ESI-MS. Further elongation of the 16 mer peptide on the resin to synthesize BKBA-20 was carried out by the HATU-HOAt method. As shown in Fig. 1, the purity of the crude synthesized BKBA-20 was convincing (69% pure peptide). RP-HPLC and mass spectroscopic analysis of the final purified products indicated that all of the synthesized compounds were the target molecules of high purity.

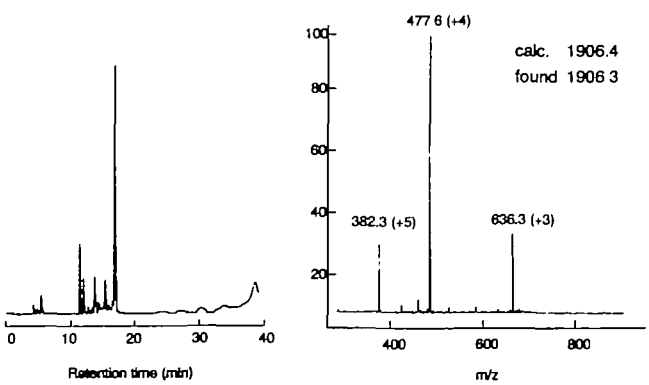

Fig. 1. **RP-HPLC profile and electrospray mass spectrum of BKBA-20 synthesized by the HBTU/HOBt and HATU/HOAt methods.**

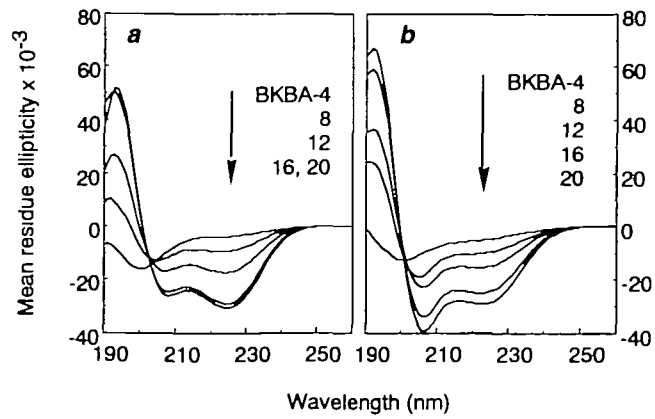

Fig. 2. **CD spectra of BKBA-4, 8, 12, 16, and 20.** The peptides (100 μM) were in (a) phosphate buffer (pH 7.4) and (b) TFE.

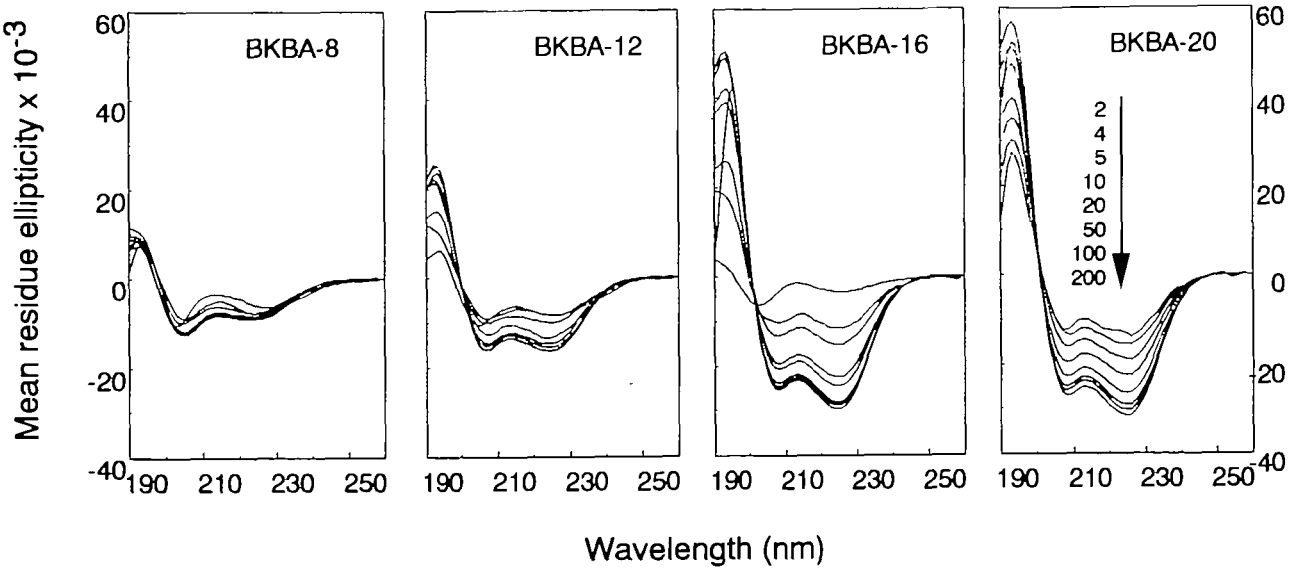

Fig. 3. **Concentration dependences of BKBA-8, 12, 16, and 20 in phosphate buffer (pH 7.4).** The peptide concentrations were 2, 4, 5, 10, 20, 50, 100, and 200 μM.

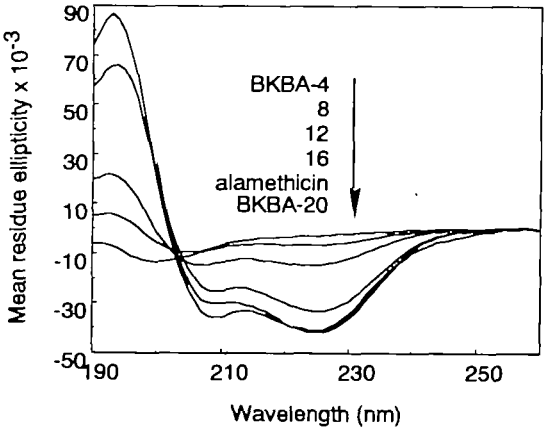

Fig. 4. **CD spectra of BKBA-4, 8, 12, 16, 20, and alamethicin.** The peptides (10 μM) were in the presence of DPPC liposomes (1 mM).

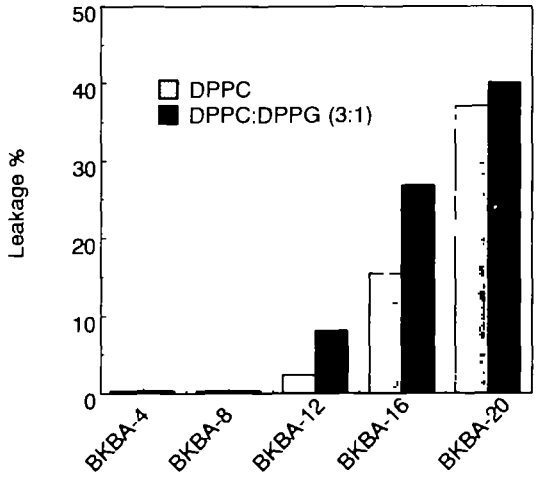

Fig. 5. **Profiles of calcein leakage from DPPC and DPPC:DPPG (3:1) liposomes induced by the BKBA peptides.** Peptide and liposome concentrations were 1 and 50 μM, respectively.

CD Conformational Analysis—Figure 2a shows the CD spectra of a series of BKBA peptides at 100 μM in phosphate buffer (pH 7.4). The CD spectrum of BKBA-4 exhibits a weak band at 200 nm, representative of a non-ordered structure. That of BKBA-8 shows signs of helical structure motifs. The CD spectra of peptides having longer peptide chains, *i.e.* BKBA-12, 16, and 20, under the same conditions, exhibit typical double minimum bands at 208 and 225 nm and a strong positive band at 196 nm, characteristic of the α-helix. The $[\theta]_{\pi-\pi^*}/[\theta]_{\pi-\pi^*\parallel}$ transition values of the spectra of BKBA-12, 16, and 20 were 1.03, 1.17, and 1.17, respectively. The helicities of the peptides increased as the peptide chain-length increased. In particular, BKBA-16 and 20 took on almost complete α-helical structures (77 and 85%, respectively). TFE can increase the helicity of single-stranded peptides and has been widely used as an α-helix-inducing cosolvent. In TFE, BKBA-12, 16, and 20 also exhibited significant α-helical contents (Fig. 2b). The values of $[\theta]_{\pi-\pi^*}/[\theta]_{\pi-\pi^*\parallel}$ see for BKBA-12, 16, and 20 were 0.68, 0.74, and 0.75, respectively. The molar ellipticity at 225 nm of peptides was not affected significantly in either TFE and the buffer. However, the 208 nm band in TFE was appreciably enhanced in negative ellipticity. In addition, the BKBA peptides exhibited more negative intensity with an increase in the peptide concentration (Fig. 3). For short peptides, an increase in the peptide concentration had little effect on the peptide ellipticity or the helix content. The most significant changes were observed for BKBA-16 and -20, which showed large increases in the molar ellipticity and helix content as the peptide concentration increased. BKBA-20 showed an α-helical structure even at 2 μM in the buffer ($[\theta]_{225} = -13{,}600$ deg·dm^2·mol^{-1}, helicity 40%). Moreover, the addition of DPPC liposomes induced an increase in the α-helical content of longer peptides, whereas BKBA-4 and 8 were identical with those in the buffer solution. Interest-

TABLE I. **Minimum inhibitory concentrations (mg/ml) of BKBA peptides against microorganisms.**

	S. aureus	*B. subtilis*	*E. coli*
BKBA-4	>100	>100	>100
BKBA-8	>100	>100	>100
BKBA-12	>100	>100	>100
BKBA-16	50	25	>100
BKBA-20	25	6.3	>100
Alamethicin	50	25	>100
Gramicidin S	25	12.5	>100

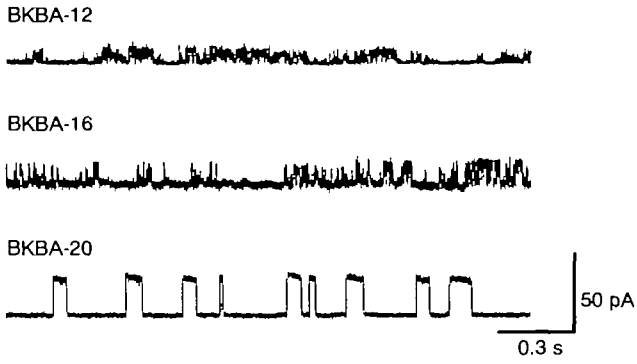

Fig. 6. **Conductance patterns of BKBA-12, 16, and 20 in DPhPC.** The electrolyte solution was 0.5 M KCl buffered with HEPES at pH 7.4. The electrolyte composition was symmetrical on both sides of the membrane, and peptide concentration was 100 nM. The applied membrane potential was +100 mV.

ingly, despite their clear differences in the buffer solution, the CD characteristics of BKBA-20 and alamethicin were very close in liposomes (Fig. 4). The CD spectra in DPhPC and acidic liposomes were similar to those in neutral liposomes (data not shown).

Dye Leakage Experiments—The effects of the peptides on the membrane structural perturbation were investigated on the basis of the release of calcein entrapped in phospholipid vesicles. The profiles of the dye leakage from DPPC and DPPC:DPPG (3:1) vesicles due to the actions of BKBA peptides are shown in Fig. 5. The dye leakage from liposomes was enhanced with an increase in the peptide chain-length. BKBA peptides exhibited slightly stronger leakage activities toward acidic DPPC:DPPG (3:1) vesicles compared with neutral DPPC vesicles.

Antimicrobial Activity—The minimum inhibitory concentrations (MICs) of the BKBA peptides, against *S. aureus, B. subtilis*, and *E. coli*, are listed in Table I. *S. aureus* and *B. subtilis* are Gram-positive, and *E. coli* is a Gram-negative bacteria. The MIC values for alamethicin and gramicidin S were used for comparison. Peptides containing 12 or fewer residues were completely inactive against the tested microorganisms. Elongation of the chain-length resulted in an increase in activity against Gram-positive bacteria. The activities of the 16-mer peptide and alamethicin were comparable. Further extension of the chain-length to 20 amino acids resulted in a higher activity profile than that of the cyclic peptide, gramicidin S, against Gram-positive bacteria. BKBA peptides, under our experimental conditions, are specific for Gram-positive and not Gram-negative bacteria, and this specificity can be attributed to differences in the membrane composition.

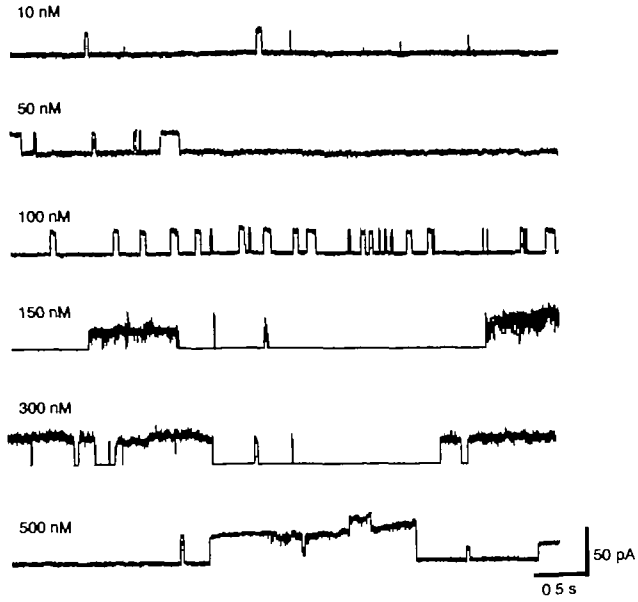

Fig. 7. **Concentration dependences of BKBA-20 conductance.** The electrolyte solution was 0.5 M KCl buffered with HEPES at pH 7.4. The electrolyte composition was symmetrical on both sides of the membrane. The applied membrane potential was +100 mV.

Ion Channel Formation—Single-channel currents of BKBA peptides were recorded at 100 mV in symmetric 0.5 M KCl using patch-clamp methodology. We used a DPhPC planar bilayer, which is neutral in net charge and known to form mechanically stable bilayer membranes. BKBA-12 and -16 showed channel-like activity at a low concentration (100 nM). However, their conductance patterns exhibited irregular stray fluctuations and an erratic increases in the membrane current (Fig. 6). BKBA-20 formed a stable channel readily, with a uniform and clear transition between the open and closed states of the channel. The single-channel conductance of the most frequent event for BKBA-20, calculated from Gaussian fits to current histograms, is 227 pS. BKBA-12 and -16 show comparably lower conductance (half to 2/3 of the BKBA-20 conductance). Most of the calculated values predicted that 3 or 4 helical subunits were involved in the channel structure of BKBA-20 (*14*). The relative probability for detecting conducting states was in the order of BKBA-20>-16>-12, the values being 1.0, 0.6, and 0.4 for BKBA-20, -16, and -12, respectively. This order clearly corresponds to the peptide chain-length. When BKBA-4 and -8 were incorporated into a lipid bilayar at a high concentration, only drifting of baseline currents was observed. The probability of the channel being in the open state (P_o) was dependent on the peptide concentration. The lifetimes of channels prolonged with an increase in the peptide concentration. The clear concentration-dependent behavior of BKBA-20 is shown in Fig. 7. Here, the probability of an open state increases with the peptide concentration. At 500 nM, the conductance changes from a single-state to a multi-state pattern.

DISCUSSION

Design of Model Peptides—The purpose of this study was to design a simple model peptide which takes on a trans-

membrane pore-forming structure in lipid bilayers. Studies involving numerous other peptides have indicated that those peptides which interact most with lipid bilayers have a strong helical propensity (*15*). We therefore chose only those amino acids, Aib, Ala, and Lys, which are strong helix formers. The Aib unit is particularly effective in suppressing the formation of β-sheet structures, as the torsion angles, ϕ and ψ, of the Aib residue are confined to the right- and left-handed helical regions of the conformational energy map (*16*). The amino acid sequence was designed to have alternating Aib and other amino acid residues to avoid adjacent Aib residues, which lead to synthetic difficulties (*17*). The residues were so positioned that a peptide with a helical conformation would have a hydrophobic face and a hydrophilic face with stacked Lys residues. The helical content of the peptide analogs increases progressively with the chain-length, resulting in concomitant enhancement of lipid interaction ability (*18*). In general, it has been estimated that a chain-length of at least 20 amino acid residues in an α-helical structure is required to span a typical lipid bilayer. Several Aib-containing model peptides have been reported to elucidate the structural 3_{10}-helix to α-helix transition (*19*). Such model peptides are usually short, less than 10 residues in length. Recently, Imanishi *et al.* showed that the amphiphilic peptides, poly (Lys-Aib-Ala-Aib) and (Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$, took on an α-helical conformation in aqueous solutions, and the amphiphilic helical structure was considered to be the reason why the peptides can induce the fusion of DPPC vesicles (*20, 21*). Moreover, the same group synthesized hydrophobic peptides, Boc-(Ala-Aib)$_n$-OMe (n=2, 4, 6, 8), as models of transmembrane structures (*5, 6*). It was found that these peptides exhibit a channel-like activity in lipid bilayer membranes. Jelokhani-Niaraki *et al.* have shown that a hydrophobic α-helical peptide composed of 16 amino acid residues assembles into an α-helix bundle with a particular transmembrane orientation in bilayer membranes (*7-9*). In the present study, to determine the relationship between the peptide chain-length and biological activity, we synthesized a series of peptides containing a repeat sequence of the tetrapeptide unit, Aib-Lys-Aib-Ala. BKBA-16 and -20 have chain-lengths capable of spanning a lipid bilayer to form pore structures. All peptides were blocked at their N- and C-termini with acetyl- and amide groups, respectively, to avoid the introduction of charges and possible unfavorable interactions with the α-helix macrodipole (*22, 23*).

Synthesis of Model Peptides—In spite of their interesting membrane-modifying properties, peptaibols, including alamethicin and its analogs, have been barely modified because of the synthetic difficulties. Due to the steric hindrance by the methyl groups on the Cα atom, the coupling efficiencies of Aib and the residue at the Aib+1 position in the stepwise assembly of peptide chains are usually poor (*17*). Consequently, these peptides have only been synthesized by means of tedious procedures involving carefully chosen stepwise solution techniques and chemical or enzymatic segment condensations (*10, 11*). The solid phase approach has been totally unsuccessful. To overcome this problem, several new carboxy-activating agents have been proposed (*e.g.* NCA, CIP, and PyBrop) (*24*). However, unusually long reaction times or increased reaction temperatures are necessary to perform these couplings. Recently, Fmoc amino acid fluorides were shown to be efficient, rapid-acting coupling reagents for both solution- and solid-phase synthesis, being particularly useful in the case of sterically hindered amino acids (*25, 26*). However, with this method, Fmoc amino acids are converted to the acid fluorides *via* tedious intermediation reactions. These derivatives should be isolated and purified by recrystallization prior to use. Moreover, of the 20-odd proteinogenic amino acids, His and Arg could not be converted to shelf-stable amino acid fluorides for use in peptide assembly (*27*). For these reasons, we chose the commonly used coupling reagents. Fmoc/HBTU chemistry has recently been applied to peptide synthesizers (*28*). In order to increase the yield of the coupling reaction, we improved the Fmoc-HBTU/HOBt activation procedure according to the Fast-Moc protocol (*29*). In our procedure, it is of great significance that Fmoc amino acids were well-preactivated with HBTU/HOBt. In addition, the final concentration in the coupling reaction was 0.3 M, which is 5- to 10-fold higher than in previous experiments. The outstanding effectiveness of the procedure was confirmed by the high purity of the crude products (Fig. 1). Such results were also obtained efficiently by the DCC-HOBt and HBTU (only) methods. Moreover, the use of Boc-amino acids also gave satisfactory results (data not shown). Thus, the synthesis of Aib-containing peptides was successfully accomplished with the commonly used coupling reagents such as HBTU-HOBt and DCC-HOBt, as well as HBTU.

Properties of BKBA Peptides—Several helical peptide models have been suggested to exhibit pore-forming ability by facilitating self-aggregation with a transmembrane orientation (*4-6*). These models were based on the ability of peptides to form alamethicin-like aggregates inside the hydrophobic area of membranes and to induce voltage-gated pores (*3*). The pore-forming mechanism of these aggregated helical peptides is basically different from these of other peptide channel-formers, such as linear gramicidin.

When BKBA peptides were loaded at comparatively high concentrations (10 mg/ml) for preparative RP-HPLC, two major broad peaks were observed (data not shown). However, the two materials were found to be homogeneous on analytical RP-HPLC and ESI-MS. Thus, the two peaks may be due to aggregated forms of the same peptide. The retention times of the purified synthetic peptides were delayed due to the increase in hydrophobicity as the chain-length increased. In size-exclusion chromatography experiments, BKBA-20 was eluted from agarose with a retention time corresponding to an apparent molar ratio (M_r) of 4,131 when compared with globular protein standards. This apparent value was 2.1 times the actual molecular weight (data not shown). This anomalous behavior on size-exclusion chromatography is characteristic of the rod-like shape of coiled-coil proteins. For example, the two stranded coiled-coil protein, tropomyosin, gave an apparent M_r that was 2.1 times the actual M_r of 66,000 (*30*). These results indicated that longer chain BKBA peptides possessed a strong tendency to aggregate, and that BKBA-20 associates *via* monomer to dimer equilibrium in the phosphate buffer.

CD spectra revealed that the peptides form highly helical structures, as observed previously for Aib-containing peptides (*5-7, 20, 21*). The helix contents increased with elongation of the chain-length (Fig. 2). On comparison of

the CD spectra of the peptides in the buffer and TFE, it was found that the helical contents of peptides (monitored at 225 nm) were not affected significantly in the two environments. However, the band at 208 nm increases dramatically in TFE. The reversal of the ellipticity ratio at 208 and 225 nm in TFE may reflect the transition from an aggregated state in the buffer to single-stranded helices in TFE (*31*). It seems that the $[\theta]_{\pi-\pi^*||}$ transition (208 nm CD band) is sensitive to single stranded helices *versus* interacting helices, as demonstrated here with highly helical structures. Aib-rich peptides are known to adopt a 3_{10}-helical structure with lengths shorter than 8 residues. Those with more than 8 residues prefer to form a mixed $3_{10}/\alpha$ or α-helix (*19*). The CD spectrum of a right-handed α-helix gives rise to two negative bands at 222 and 208 nm of almost equal intensity and a strong positive band at about 192 nm. In contrast, the 3_{10}-helix has a smaller ellipticity ratio $[\theta]_{n-\pi^*}/[\theta]_{\pi-\pi^*||}$ and a positive band near 195 nm with reduced intensity compared to that for the α-helix (*32*). The 3_{10}-helix becomes stable in nonpolar environments, leading to an effective increase in the intrapeptide hydrogen bond strength. Under aqueous conditions, the α-helix is stabilized by its inter-helical side-chain interaction. For these reasons, the helical structures of all BKBA peptides would be dominantly α-helical, with the possible exception of BKBA-8 in TFE. In a preliminary effort, $(Ala\text{-}Aib)_6$ was synthesized and confirmed to form a 3_{10}-helical structure by its IR spectrum (data not shown). On comparison of the CD spectra of the peptide and BKBA-12, it was shown that a lysine residue is important for induction of the α-helical conformation and peptide aggregation.

In an aqueous solution, the molar ellipticity of the longer peptides was generally concentration-dependent, and an aggregation-induced increase in helicity with an increase in peptide concentration was observed. The helicity of BKBA-16 and BKBA-20 reached a plateau at 20 μM peptide concentration with high negative $[\theta]_{225}$ values (Fig. 3). In contrast, the ellipticity of short peptides (12 residue or less) was very low, even at peptide concentrations as high as 200 μM, implying a low content of α-helix. A likely explanation for this concentration-dependent behavior with regard to the molar ellipticity is the formation of aggregates. When the concentration of a peptide is increased, the equilibrium between single-strand helices and the aggregate shifts toward the formation of the aggregate, which increases the α-helical content of the peptide. The results of our experiment clearly show that the helicity of peptides is enhanced by an increase in the peptide chain-length, which in turn promotes the peptide aggregation.

Activities of BKBA Peptides—The CD spectra of peptides in liposomes suggest a structure-function relationship for biologically active peptides (Fig. 4). Negatively charged DPPC:DPPG (3:1) liposomes were used to mimic bacterial exterior cell membranes, and neutral DPPC liposomes were used as a model membrane for mammalian cells. BKBA peptides formed α-helical structures with the same content in the presence of neutral and acidic liposomes. The longer peptides, BKBA-16 and -20, showed maximum helicity in the liposome preparation with a peptide/lipid ratio of at least 1/100. The membrane perturbation effect of peptides was evaluated by means of dye leakage experiments. These analogs caused greater leakage of the dye from acidic liposomes than from neutral liposomes, suggesting that electrostatic interactions contribute to their activities. Their perturbation abilities were correlated with the chain-length dependence on helicity in a phospholipid bilayer (Fig. 5).

The BKBA-16 and -20 peptides also exhibited potent antimicrobial activity against Gram-positive bacteria (Table I). In particular, BKBA-20 was more active than alamethicin, which is also 20 residues long. The differences between the two Aib-peptides may be a reflection of their amphiphilic properties. Many natural host defense peptides have the common features of being highly basic, due to the presence of multiple arginine and lysine residues, and of forming amphipathic structures (*33*). Their basic nature facilitates their interaction with the cell membrane, and their amphipathic character allows them to be incorporated into the membrane *via* a barrel-stave model or carpet-like model, ultimately leading to disruption of the structures (*2, 34, 35*). Peptaibol antibiotics such as alamethicin have a strong ability to form ion channels in planar lipid bilayers. However, these peptides are highly hydrophobic and are rich in hydrophobic amino acids. The results indicate that the amphiphilic property is the key factor for the antimicrobial activity. Furthermore, in the present study, BKBA-20 was also more active than gramicidin S. These results suggested that the potent activity of BKBA-20 could be due to its high binding affinity for cell membranes and that this affinity was further enhanced by the high pore-forming ability of the peptide. Under our experimental conditions, these peptides were largely ineffective against Gram-negative bacteria. The cell surface of Gram-negative bacteria is a complex structure composed of two membrane systems. The inner membrane contains the respiratory enzymes. The outer membrane, which contains negatively charged lipopolysaccharide molecules, acts as a barrier to hydrophobic molecules. However, other cationic antibiotics, such as magainins, can bind to the lipopolysaccharide molecules located on the exterior surface of the outer membrane, and perturb the structure of this membrane. Recently, Kondejewski *et al.* reported that gramicidin S is active against both Gram-positive and -negative bacteria, and that its activity is highly dependent on the methodology of assay used to measure activity (*36*).

Electrophysiological Studies—Current measurement of a planar DPhPC bilayer showed that BKBA-20 formed stable ion channels at the concentration of 100 nM. The currents flowing through single-channel units were clearly resolved as distinct unitary events that fluctuate between the open and closed states. In accordance with the rigid cylindrical model, it was found that BKBA-20 took on higher order structures ($N=4$ or more) as the simplest conducting structures in a planar bilayer. BKBA-12 and 16 also produced channel-like activity (Fig. 6). In contrast, irregular transient fluctuations and drifting of membrane conductance along a variable time course without a repeatable characteristic pattern were observed for shorter peptides. The frequency of observation of membrane conductance increased in the order of BKBA-20>-16>-12, suggesting that the increase in the peptide chain-length is correlated with the induction of ion permeability of phospholipid bilayers. These results indicated that a chain-length sufficient to span a lipid bilayer (*ca.* 30Å apart) of at least 20 residues is a requisite for the formation of a stable ion channel (*37*). BKBA-20 also induced concentration-de-

pendent channels which form single-state conductance patterns at low peptide concentrations (Fig. 7). Increasing peptide concentrations resulted in multi-state conductance patterns with elongation of the open lifetimes. The aqueous phase aggregation of channel-forming peptides at low concentrations suggests that preformed aggregates may be inserted into the membrane to constitute functional channels. It is worth noting that the rate of fluctuation of the open-close states is dependent on the formation of aqueous phase aggregates and their eventual insertion into the membrane.

In conclusion, it was found that there is a clear correlation between peptide chain-length, peptide aggregation and biological activity. Peptides adopting architectures similar to that of BKBA-20 are expected to be appropriate templates for intermembrane stable channel-forming structures. Thus, BKBA-based peptides and their modified versions can act as simple models for studying the structure-function properties of more elaborate ion channel proteins.

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REFERENCES

1. Jan, L.Y. and Jan, Y.N. (1992) Tracing the roots of ion channels. *Cell* **69**, 715-718
2. Bechinger, B. (1997) Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin. *J. Membr. Biol.* **156**, 197-211
3. Sansom, M.S.P. (1993) Structure and function of channel-forming peptaibols. *Q. Rev. Biophys.* **26**, 365-421
4. Lear, J.D., Wasserman, Z.R., and DeGrado, W.F. (1988) Synthetic amphiphilic peptide models for protein ion channels. *Science* **240**, 1177-1181
5. Otoda, K., Kimura, S., and Imanishi, Y. (1993) Orientation and aggregation of hydrophobic helical peptides in phospholipid bilayer membrane. *Biochim. Biophys. Acta* **1150**, 1-8
6. Otoda, K., Kimura, S., and Imanishi, Y. (1993) Effect of cation binding to hydrophobic helical peptides on orientation, aggregation, and ion-channel activity in phospholipid bilayer membranes. *J. Chem. Soc., Perkin Trans.* **1**, 3011-3016
7. Jelokhani-Niaraki, M., Yoshioka, K., Takahashi, H., Kato, F., and Kondo, M. (1992) Changes in conformation and antimicrobial properties caused by replacement of D-amino acids with α-aminoisobutyric acid in the gramicidin backbone: synthesis and circular dichroic studies. *J. Chem. Soc., Perkin Trans.* **2**, 1187-1193
8. Jelokhani-Niaraki, M., Kodama, H., Ehara, T., and Kondo, M. (1995) Conformational studies and pore-forming properties of α-aminoisobutyric acid analogue of gramicidin B. *J. Chem. Soc., Perkin Trans.* **2**, 801-808
9. Jelokhani-Niaraki, M., Nakashima, K., Kodama, H., and Kondo, M. (1998) Interaction and orientation of an α-aminoisobutyric acid- and tryptophan-containing short helical peptide pore-former in phospholipid vesicles, as revealed by fluorescence spectroscopy. *J. Biochem.* **123**, 790-797
10. Gisin, B.F., Davis, D.G., Borowska, Z.K., Hall, J.E., and Kobayashi, S. (1981) Synthesis of major component of alamethicin. *J. Am. Chem. Soc.* **103**, 6373-6377
11. Slomczynska, U., Zabrocki, J., Kaczmarek, K., Leplawy, M.T., Beusen, D.D., and Marshall, G.R. (1992) Facilitated synthesis of peptaibols: alamethicin via enzymatic segment condensation. *Biopolymers* **32**, 1461-1470
12. Sreerama, N. and Woody, R.W. (1993) A self-consistent method for the analysis of protein secondary structure from circular dichroism. *Anal. Biochem.* **209**, 32-44
13. Bartlett, G.R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**, 466-468
14. Sansom, M.S.P. (1993) Alamethicin and related peptaibols —model ion channels. *Eur. Biophys. J.* **22**, 105-124
15. Epand, R.M., Shai, Y., Sgrest, J.P., and Anantharamainh, G.M. (1995) Mechanisms for the modulation of membrane bilayer properties by amphiphatic helical peptides. *Biopolymers* **37**, 319-338
16. Burgess, A.W. and Leach, S.J. (1973) An obligatory α-helical amino acid residue. *Biopolymers* **12**, 2599-2605
17. Spencer, J.P., Antonenko, V.V., Delaet, N.G.J., and Goodman, M. (1992) Comparative study of methods to couple hindered peptides. *Int. J. Pept. Protein Res.* **40**, 282-293
18. Agawa, Y., Lee, S., Ono, S., Aoyagi, H., Ohno, M., Taniguchi, T., Anzai, K., and Kirino, Y. (1991) Interaction with phospholipid bilayers, ion channel formation, and antimicrobial activity of basic amphiphilic α-helical model peptides of various chain lengths. *J. Biol. Chem.* **266**, 20218-20222
19. Karle, I.L. (1996) Flexibility in peptide molecules and restraints imposed by hydrogen bonds, the aib residue, and core inserts. *Biopolymers* **40**, 157-180
20. Kono, K., Kimura, S., and Imanishi, Y. (1990) pH-dependent interaction of amphiphilic polypeptide poly(Lys-Aib-Leu-Aib) with lipid bilayer membrane. *Biochemistry* **29**, 3631-3637
21. Zhao, J., Kimura, S., and Imanishi, Y. (1995) Amphiphilic α-helical structure in water stabilized by dioctadecyl chain. *J. Chem. Soc., Perkin Trans.* **2**, 2243-2248
22. Shoemaker, K.R., Kim, P.S., York, E.J., Stewart, J.M., and Baldwin, R.L. (1987) Tests of the helix dipole model for stabilization of α-helices. *Nature* **326**, 563-567
23. Dasgupta, S. and Bell, J.A. (1993) Design of helix ends. Amino acid preferences, hydrogen bonding and electrostatic interactions. *Int. J. Pept. Protein Res.* **41**, 499-511
24. Alberico, F. and Carpino, L.A. (1997) Coupling reagents and activation. *Methods Enzymol.* **289**, 104-126
25. Wenschuh, H., Beyermann, M., Krause, E., Brudel, M., Winter, R., Schumann, M., Carpino, L.A., and Bienert, M. (1994) Fmoc amino acid fluorides: convenient reagents for the solid-phase assembly of peptides incorporating sterically hindered residues. *J. Org. Chem.* **59**, 3275-3280
26. Wenschuh, H., Beyermann, M., Haber, H., Seydel, J.K., Krause, E., and Bienert, M. (1995) Stepwise automated solid phase synthesis of naturally occurring peptaibols using Fmoc amino acid fluorides. *J. Org. Chem.* **60**, 405-410
27. Carpino, L.A., Beyermann, M., Wenschuh, H., and Bienert, M. (1996) Peptide synthesis via amino acid halides. *Acc. Chem. Res.* **29**, 268-274
28. Fields, C.G., Lloyd, D.H., Macdonald, R.L., Otteson, K.M., and Noble, R.L. (1991) HBTU activation for automated Fmoc solid-phase peptide synthesis. *Pept. Res.* **4**, 95-101
29. Schneider, J. and Kent, S.B. (1988) Enzymatic activity of a synthetic 99 residue protein corresponding to the putative HIV-1 protease. *Cell* **54**, 363-368
30. Hodges, R.S., Saund, A.K., Chong, P.C.S., St.-Pierre, S.A., and Reid, R.E. (1981) Synthetic model for two-stranded alpha-helical coiled-coils. Design, synthesis, and characterization of an 86-residue analog of tropomyosin. *J. Biol. Chem.* **256**, 1214-1224
31. Lau, S.Y.M., Taneja, A.K., and Hodges, R.S. (1984) Synthesis of a model protein of defined secondary and quaternary structure. *J. Biol. Chem.* **259**, 13253-13261
32. Toniolo, C., Polese, A., Formaggio, F., Crima, M., and Kamphuis, J. (1996) Circular dichroism spectrum of a peptide 3_{10}-helix. *J. Am. Chem. Soc.* **118**, 2744-2745
33. Saberwal, G. and Nagaraj, R. (1994) Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: facets of their conformational features, structure-function correlations and membrane-perturbing abilities. *Biochim. Biophys. Acta* **1197**, 109-131
34. Ojcius, D.M. and Young, J.D.E. (1991) Cytolytic pore-forming proteins and peptides: is there a common structural motif? *Trends Biochem. Sci.* **16**, 225-229
35. Shai, Y. (1995) Molecular recognition between membrane-spanning helices. *Trends Biochem. Sci.* **20**, 460-464
36. Kondejewski, L.H., Farmer, S.W., Wishart, D.S., Hancock, R.E.W., and Hodges, R.S. (1996) Gramicidin S is active against both gram-positive and gram-negative bacteria. *Int. J. Pept. Protein Res.* **47**, 460-466
37. Åkerfeldt, K.S., Lear, J.D., Wasserman, Z.R., Chung, L.A., and DeGrado, W.F. (1993) Synthetic peptides as models for ion channel proteins. *Acc. Chem. Res.* **26**, 191-197