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Formation of ion-selective channel using cyclic tetrapeptides

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ABSTRACT

It is important for ion channel peptides to have energetic stability and ion-selectivity for development of some medicines. In the present study, our objective was to achieve formation of energetically stable and ion-selective channels in the membrane using cyclic tetrapeptides. We succeeded in formation of energetically stable and ion-selective channels using two cyclic tetrapeptides cyclo(D-Ala-Dap)$_2$ (Dap; L-2,3-diaminopropionic acid) and cyclo(D-Ala-Glu)$_2$. The results of ion channel recording suggested that the cationic cyclo(D-Ala-Dap)$_2$ was resulted in Cl^- anion-selective and the anionic cyclo(D-Ala-Glu)$_2$ led to K^+ cation-selective ion channel formation, respectively. This ion selectivity may be attributed to the charge state of peptides. And a low-hydrophobic cyclic tetrapeptide; cyclo(D-Ala-Dap)$_2$ had a tendency to form stable ion channel compared to more high-hydrophobic ones; cyclo(D-Phe-Lys)$_2$, cyclo(D-Phe-Dap)$_2$ and cyclo(D-Ala-Lys)$_2$. Our findings will shed light on the field of ion channel peptide study, especially cyclic one.

1. Introduction

The use of ion channel peptides enables ions to pass through a lipid bilayer.[1–4] The mechanism of the ion channel is forming a pore structure in the lipid membrane, followed by passing ions into that pore over the opposite side of the membrane.[5] Ion concentration and its gradient at some compartments in bio-organism like cells are crucial for life-activity. If these important factors are disturbed and distracted, the life cannot sustain their activities. This concept applies the ion channel peptides to anti-microbial reagents.[6] It is important for ion channel peptides to have energetic stability and ion-selectivity for development of some medicines.

It has been numerously reported about linear ion channel peptides such as Alamethicin, Gramicidin and etc.[3,4] Recently, it has been reported that cyclic ion channel peptides by Ghadiri et al.[7] It has been observed that a high throughput rate of ion which demonstrates ion channel formation using the cyclic octapeptide cyclo(Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Gln-D-Leu). Their study has shown a self-assembled cylindrical β-sheet peptide structure 'nanotube' is formed by stacking the cyclic peptides in the membrane. As a result, ions pass through this hollow of nanotubes, and the ion channel formation is completed.

Our laboratory has reported the ion channel formation by cyclic octa-, hexa- and tetra- peptides.[8–10] According to the 'nanotube' ion channel, a diameter of cyclic tetrapeptide (about 0.2 nm) is too small to be an appropriate pore for passing the evaluated ions such as K^+ and Cl^- (dehydrated diameter of these ions are about 0.3 nm) even if the nanotube is formed. However, we previously reported that the possibility of ion channel formation using some cyclic tetetrapeptides.[9] That ion passing mechanism has been unknown.

In the present study, our objective was to achieve formation of energetically stable and ion-selective channels in the membrane using cyclic tetrapeptides (Fig. 1). We succeeded in formation of energetically stable and ion-selective channels using two cyclic tetrapeptides cyclo(D-Ala-Dap)$_2$ (Dap; L-2,3-diaminopropionic acid) (**1**) and cyclo(D-Ala-Glu)$_2$ (**2**). And a low-hydrophobic cyclic tetrapeptide; cyclo(D-Ala-Dap)$_2$ had a tendency to form stable ion channel compared to more high-hydrophobic ones; cyclo(D-Phe-Lys)$_2$ (**3**), cyclo(D-Phe-Dap)$_2$ (**4**) and cyclo(D-Ala-Lys)$_2$ (**5**).

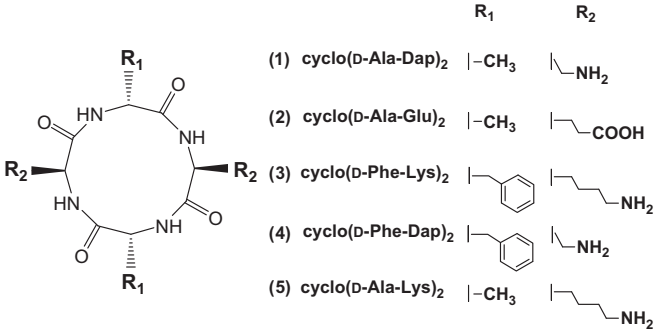

Figure 1. Chemical structures of cyclic tetrapeptides.

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2. Results

2.1. Preparation of cyclic tetrapeptides

The cyclic tetrapeptides were designed to contain alternating D- and L-amino acid residues can adopt a flat-ring conformation to stack cyclic peptides, followed by forming a contiguous hydrogen-bonded hollow structure 'nanotube'.[11] All peptides were synthesized by a combination of solid phase peptide synthesis and conventional solution-phase synthesis. After the linear peptide was elongated by Fmoc chemistry using solid phase synthesis, the cyclization of linear peptide was proceeded with HBTU. For avoid polymerization of the linear peptide, the cyclization was proceeded in high-diluted condition which peptide concentration was 0.5 mM in DMF. To facilitate purification with RP-HPLC, the low-hydrophobic cyclic tetrapeptides; cyclo(D-Ala-Dap)$_2$ (**1**), cyclo(D-Ala-Glu)$_2$ (**2**) and cyclo(D-Ala-Lys)$_2$ (**5**) were purified as protected states; cyclo(D-Ala-Dap(Boc))$_2$, cyclo(D-Ala-Glu(OtBu))$_2$ and cyclo(D-Ala-Lys(Boc))$_2$, respectively. Finally, these purified peptides were deprotected with TFA. The satisfactory analytical data about all peptides was obtained by analytical RP-HPLC and MALDI TOF-MS characterization.

2.2. Ion selectivity of cationic cyclic tetrapeptide cyclo(D-Ala-Dap)$_2$

The single-channel conductance recording was measured using the tip-dipping method to examine the difference of ion selectivity for cationic cyclic tetrapeptide cyclo(D-Ala-Dap)$_2$ (**1**) with peptide concentration 10 nM in 500 mM three kinds of electrolytes which were KCl, CsCl and KAsp (Potassium Aspartate), independently.[12] As shown in first and second rows of Figure 2, the conductance recording at KCl and CsCl measurements showed the stable and continuous current values in the range up to 100 pA which meant open–close pore transition states of ion channel formation. The frequency of recording for ion channel formation was two times out of five trials (2/5) about both KCl and CsCl measurements. However, it was not observed at KAsp measurement (frequency; 0/5) in this study.

2.3. Ion selectivity of anionic cyclic tetrapeptide cyclo(D-Ala-Glu)$_2$

The single-channel conductance recording was also measured for anionic cyclic tetrapeptide cyclo(D-Ala-Glu)$_2$ (**2**) with peptide concentration 10 nM in 500 mM three kinds of electrolytes which were KCl, CsCl and KAsp, independently. As shown in first and third rows of Figure 3, the conductance recording at KCl and KAsp measurements showed the stable and continuous current values in the range up to 100 pA which meant open–close pore transition states of ion channel formation. The frequency of recording for ion channel formation was 2/5 about both KCl and KAsp measurements. However, it was not observed current values that meant ion channel formation at CsCl measurement (frequency; 0/5) in this study.

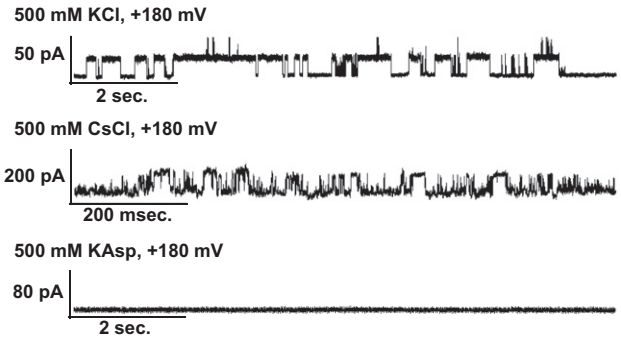

Figure 2. Ion channel recording of cyclo(D-Ala-Dap)$_2$ using three kinds of electrolytes under electrical potential.

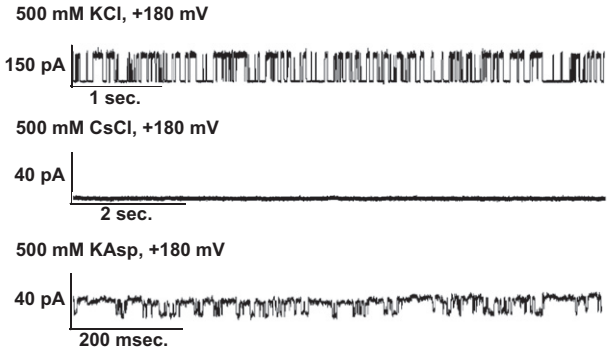

Figure 3. Ion channel recording of cyclo(D-Ala-Glu)$_2$ using three kinds of electrolytes under electrical potential.

2.4. Ion selectivity of mixing sample with cyclo(D-Ala-Dap)$_2$ and cyclo(D-Ala-Glu)$_2$

The single-channel conductance recording was also measured for mixing sample with cationic cyclic tetrapeptide cyclo(D-Ala-Dap)$_2$ (**1**) and anionic one cyclo(D-Ala-Glu)$_2$ (**2**) with molar ratio, 1:1. The mixing sample was prepared to add both 10 nM peptide solutions into a same tube with molar ratio, 1:1. It was also employed three kinds of electrolytes which were KCl, CsCl and KAsp, independently. As shown in first and third rows of Figure 4, the conductance recording at KCl and KAsp measurements showed the stable and continuous current values in the range up to 500 pA which meant open–close pore transition states of ion channel formation. The frequency of recording for ion channel formation was 1/5 about both KCl and KAsp measurements. However, it was not observed current values that meant ion channel formation at CsCl measurement (frequency; 0/5) in this study. The current values of at KCl and KAsp measurements using mixing sample (100–500 pA see in Fig. 4) were higher than that of at the measurements using each cyclic tetrapeptides separately (20–100 pA see in Figs. 2 and 3).

2.5. Evaluation of ion channel formation for cyclo(D-Phe-Lys)$_2$, cyclo(D-Phe-Dap)$_2$, cyclo(D-Ala-Lys)$_2$ and cyclo(D-Ala-Dap)$_2$

The single-channel conductance recording was also measured to evaluate the difference of molecular-hydrophobicity effects on ion channel formation for cyclo(D-Phe-Lys)$_2$ (**3**), cyclo(D-Phe-Dap)$_2$ (**4**),

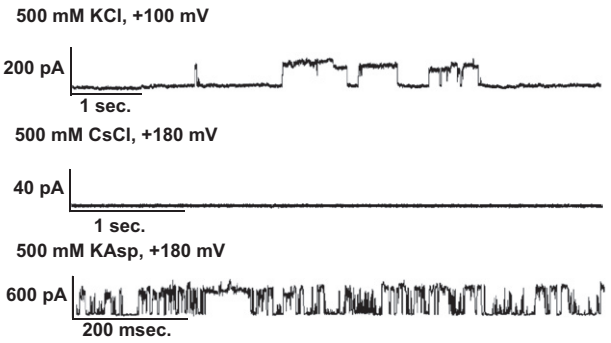

Figure 4. Ion channel recording of mixing sample of cyclo(D-Ala-Dap)$_2$ and cyclo(D-Ala-Glu)$_2$ with molar ratio 1:1 using three kinds of electrolytes under electrical potential.

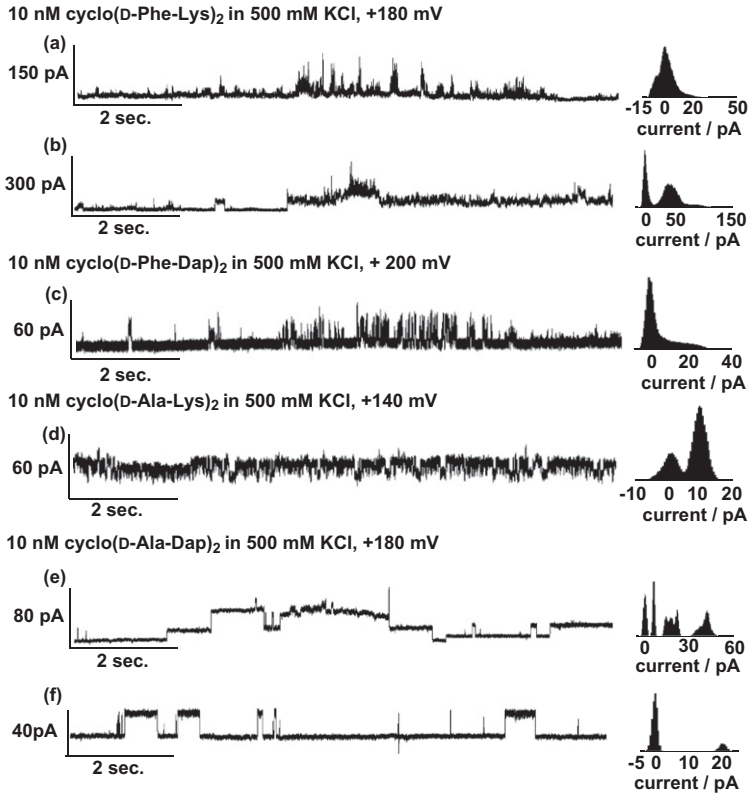

Figure 5. Ion channel recording of cyclo(D-Phe-Lys)$_2$, cyclo(D-Phe-Dap)$_2$, cyclo(D-Ala-Lys)$_2$ and cyclo(D-Ala-Dap)$_2$ using KCl solution under electrical potential. Left panel is a single ion channel conductance and right one is a histogram analysis for each conductance.

cyclo(D-Ala-Lys)$_2$ (**5**) and cyclo(D-Ala-Dap)$_2$ (**1**) with peptide concentration 10 nM in 500 mM KCl. As shown in Figure 5a–b (left panels), the recording for the peptide **3** showed fluctuated and discrete current values in the range up to 60 pA. To clarify the distribution of current value with its frequency for each single-conductance recordings, the recording data were converted to the histogram analysis data. From the histogram analysis of the peptide **3** shown in Figure 5a–b (right panels), some broad peaks were observed which meant the distribution of current values were dispersed. As shown in Figure 5c–d (left panels), the recording for the peptide **4**–**5** showed also fluctuated and discrete current values in the range up to 30 pA. From the histogram analysis of the peptide **4**–**5** shown in Figure 5c–d (right panels), some broad peaks were observed which meant the distribution of current values were dispersed. On the contrary, as shown in Figure 5e–f (left panels), the recording for the peptide **1** showed stable and continuous current values in the range up to 50 pA which meant open–close pore transition states of ion channel formation. From the histogram analysis of the peptide **1** shown in Figure 5e–f (right panels), some sharp peaks were observed which meant distribution of current values were constantly focused. In Figure 5e (left panel), the step-wise current changes were observed indicating multi-pore formation by the peptide **1**. From these results, it was preferable for energetically stable ion channel formation to use the low-hydrophobic cyclic tetrapeptide **1** compared to the high-hydrophobic cyclic tetrapeptide **3**–**5**.

3. Discussion

Many studies have been reported that the evaluation of ion channel formation using cyclic peptides.[7,10,13–18] For example, Ghadiri et al. have suggested that the cyclic ocatapeptide forming the self-assembled cylindrical β-sheet peptide structure called 'nanotube' by stacking the cyclic peptides in the membrane.[7] As a result, ions pass through this hollow of nanotubes, and the ion channel formation is achieved. According to the 'nanotube' ion channel model, it is necessary that a diameter of cyclic peptides should be larger than that of passing ions such as K^+ and Cl^- (dehydrated diameter of these ions are about 0.3 nm). However, a few reports about cyclic tetrapeptide such as tentoxin have been published though a diameter of cyclic tetrapeptide (about 0.2 nm) is smaller than that of K^+ and Cl^-.[14] The mechanism of ion channel formation by cyclic tetrapeptides is unclear for now. In the present study, it was also observed that ion channel formation using the cyclic tetrapeptides such as cyclo(D-Ala-Dap)$_2$ (**1**) and cyclo(D-Ala-Glu)$_2$ (**2**) (see in Figs. 2 and 3). This might indicate a nanotube-bundle model. It is a kind of self-organizing structure that nanotubes aggregate each other in the lipid membrane, followed by forming an intermolecular pore between nanotubes, which is similar to the bundle structure formed by linear ion channel peptide.[5] The possibility for nanotube-bundle model by cyclic peptides has been suggested before.[19] It is necessary to clarify the mechanism of the ion channel formation by cyclic tetrapeptide in the future.

From the results of ion selectivity evaluation, the ion channel formation was observed that the recording of the peptide **1** showed ion channel formation at KCl and CsCl, not at KAsp evaluating system (Figs. 2–4). On the other hand, that of the peptide **2** showed ion channel formation at KCl and KAsp, not at CsCl system. Similarly, that of the mixing sample showed ion channel formation at KCl and KAsp, not at CsCl system. Given the common ion species from these results, the use of the cationic peptide **1** was resulted in Cl^- anion-selective and the anionic peptide **2** led to K^+ cation-selective ion channel formation, respectively. This ion selectivity may be attributed to the charge state of peptides, which means that a cationic cyclic peptide attract an anion and anionic one attract a cation. It has been reported that ion selectivity has

changed cation-selective into anion-selective by altering the neutral amino acid residue; Gln to cationic one; Lys.[20] This might indicate that the charge state of residue function as an ion-filter. The ion selectivity of the mixing sample was similar to that of anionic cyclic peptide which showed K^+ cation-selective ion channels. Given electrostatic interaction between the cationic peptide **1** and the anionic peptide **2**, the bias of charge for each peptides may be canceled and approach to the neutral charge state. This ion-selectivity for mixing sample is interesting. But it is still uncertain about the detailed mechanism of ion-selectivity for the mixing sample. The current value in the recording of the mixing sample is lager than that of each cyclic peptide employed separately. It is often said that the current value is counterpart of the pore-size of ion channel.[21] The electrostatic interaction between cationic and anionic cyclic tetrapeptides in the mixing sample may lead to stability of pore-structure for formation of more large-size pore in the membrane.

From the results of single ion channel recording of the peptide **3**–**5** and the peptide **1**, the recording of the peptide **1** showed energetically stable ion channel formation compared to that of the peptide **3**–**5** (Fig. 5). The open-time of pore for the peptide **1** showed continuously about 10 s compared to that of the peptide **3**–**5** whose conductance recording showed the fluctuated and discrete current values change. The difference of stability for ion channel formation is dependent on the amino acid sequence. It may be preferable for cyclic peptides that contain less interactive factors such as low hydrophobic molecules whose have little bulky side-chain groups. Because the less interactive factor enable cyclic peptides to move and arrange the most appropriate conformation to achieve supramolecular-like structure such as 'nanotube-bundle' in the dynamic lipid membrane atmosphere. Therefore, it is possible that the use of the low-hydrophobic cyclic tetrapeptide **1** has the advantage for energetically stable and having constant pore-size ion channel formation compared to the high-hydrophobic cyclic tetrapeptide **3**–**5**.

4. Conclusion

In the present study, we achieved energetically stable and ion-selective ion channel formation in the membrane by substituting amino acid sequences in the cyclic tetrapeptides. With biasing electrostatic state of cyclic tetrapeptides turning into positive or negative charges, the ion-selective ion channel formations were observed. Furthermore, a low-hydrophobic cyclic tetrapeptide; cyclo(D-Ala-Dap)$_2$ had a tendency to form stable ion channel compared to more high-hydrophobic ones; cyclo(D-Phe-Lys)$_2$, cyclo(D-Phe-Dap)$_2$ and cyclo(D-Ala-Lys)$_2$. It requires further study that about the aggregating state, pore forming, and ion passing mechanism in the membrane by cyclic tetrapeptides. Our report indicates that the use of comparatively simple structure cyclic tetrapeptides can induce energetically stable and ion-selective ion channel formation in the membrane. These indication will become an important model at the field of ion channel peptide study, especially cyclic one.

5. Experimental

5.1. Materials and apparatus

9-Fluorenylmethoxycarbonyl (Fmoc) protected amino acids, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium (HBTU) and 2-chloritritylchloride resin were purchased from Novabiochem (Tokyo, Japan). *N*-hydroxybenzotriazole (HOBt) and 2,2,2-trifluoroacetic acid (TFA) were available from Peptide Institute (Osaka, Japan). Polypropylene vessels for solid phase peptide synthesis were purchased from Pierce (Rockford, IL, USA). Diphytanoylphosphatidylcholine (DPhPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA) as 50 mg/mL chloroform solution. Single-channel currents were amplified using an Axopatch 1D patch-clamp amplifier (Axon Instruments Inc., CA, USA) controlled by pClamp 6 software.

5.2. Peptide synthesis

Peptides were synthesized by Fmoc strategy. Peptides were elongated on 2-chlorotritylchloride resin. Amino acids were activated with 0.45 M HBTU and HOBt in DMF. Fmoc groups were removed with 20% piperidine in DMF. Linear tetrapeptides were cleaved from the resin using AcOH/TFE/DCM (2:2:6, v/v) solution. The detached peptides were cyclized with HBTU and HOBt in DMF. After evaporation of the solution, the residue was dissolved in ethyl acetate and washed with H_2O, 0.5 M $NaHCO_3$ and 5% $KHSO_4$. Organic phase was dried over Na_2SO_4. Solution was concentrated using evaporator and precipitated by diethyl ether. Side-chain protecting groups such as *tert*-butoxycarbonyl (Boc) or *tert*-butoxy ester (OtBu) was deprotected by 90% TFA solution. The crude peptides were purified by preparative RP-HPLC on a Wakosil ODS column (100% H_2O-0.1% TFA and 100% acetonitrile-0.08% TFA). Homogeneities and structures of all peptides synthesized in this study were confirmed by analytical RP-HPLC and MALDI TOF-MS analyses. MALDI TOF-MS for cyclo(D-Ala-Dap)$_2$: calcd *m/z* 315.17, found *m/z* 315.03 $[M+H]^+$, cyclo(D-Ala-Glu)$_2$: calcd *m/z* 401.16, found *m/z* 401.09 $[M+H]^+$, cyclo(D-Phe-Lys)$_2$: calcd *m/z* 551.33, found *m/z* 551.20 $[M+H]^+$, cyclo(D-Phe-Dap)$_2$: calcd *m/z* 467.24, found *m/z* 467.17 $[M+H]^+$, cyclo(D-Ala-Lys)$_2$: calcd *m/z* 399.27, found *m/z* 399.21 $[M+H]^+$.

5.3. Ion channel recording

Single ion channel recordings were carried out with the tip-dipping method at room temperature.[12] The pipettes were prepared from hematocrit hard glass capillaries. Settings in the pipette puller were adjusted to obtain a tip diameter of 1 μm. The pipettes were filled with solution of 0.5 M KCl buffered with 5 mM HEPES (pH 7.4) containing 10 nM peptides and Ag–AgCl electrode was inserted in the pipette. For ion-selectivity measurements, 0.5 M KCl was replaced either 0.5 M CsCl or KAsp ion species. Voltage was referenced to another Ag–AgCl electrode placed in the opposite side of the lipid bilayer. The pipette tip was immersed in a Petri-dish filled with the same buffer without peptides. After immersion of the pipette, DPhPC phospholipid monolayer was spread on the surface of the dish solution by carefully adding to the edge of the dish 0.5–1.0 μL of a 10 mg mL^{-1} solution of the lipid dissolved in hexane. After 10-minute waiting for the evaporation of the solvent from the surface of the solution, the tip was re-dipped in the solution to form a lipidbilayer on the tip. Amplified single-channel currents were filtered at 1 or 2 kHz and analyzed using Axograph 3.5 (Axon Instruments, Inc.).

Acknowledgments

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References and notes

1. Lear, J. D.; Wasserman, Z. R.; DeGrado, W. F. *Science* **1988**, *240*, 1177.
2. Spach, G.; Duclohier, H.; Molle, G.; Valleton, J.-M. *Biochimie* **1989**, *71*, 11.
3. Woolley, G. A.; Wallace, B. *J. Membr. Biol.* **1992**, *129*, 109.

4. Iwata, T.; Lee, S.; Oishi, O.; Aoyagi, H.; Ohno, M.; Anzai, K.; Kirino, Y.; Sugihara, G. *J. Biol. Chem.* **1994**, *269*, 4928.
5. Baumann, G.; Mueller, P. *J. Supramol. Struct.* **1974**, *2*, 538.
6. Kagan, B. L.; Selsted, M. E.; Ganz, T.; Lehrer, R. I. *PNAS* **1990**, *87*, 210.
7. Ghadiri, M. R.; Granja, J. R.; Buehler, L. K. *Nature* **1994**, *369*, 301.
8. Taira, J.; Osada, S.; Hayashi, R.; Yoshiki, M.; Tatebe, S.; Ueda, T.; Ehara, T.; Kodama, H. *Pept. Sci.* **2004**, *41*, 531.
9. Taira, J.; Osada, S.; Jelokhani-Niaraki, M.; Ehara, T.; Kodama, H. *Pept. Sci.* **2005**, *42*, 245.
10. Taira, J.; Osada, S.; Hayashi, R.; Ueda, T.; Jelokhani-Niaraki, M.; Aoyagi, H.; Kodama, H. *Bull. Chem. Soc. Jpn.* **2010**, *83*, 683.
11. Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. *Nature* **1993**, *366*, 324.
12. Taira, J.; Jelokhani-Niaraki, M.; Osada, S.; Kato, F.; Kodama, H. *Biochemistry* **2008**, *47*, 3705.
13. Ramesh, J.; Ghosh, J. K.; Swaminathan, C. P.; Ramasamy, P.; Surolia, A.; Sikdar, S. K.; Easwaran, K. R. K. *J. Peptide Res.* **2003**, *61*, 63.
14. Heitz, F.; Jacquier, R.; Kaddari, F.; Verducci, J. *Biophys. Chem.* **1986**, *23*, 245.
15. Clark, T. D.; Buehler, L. K.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1998**, *120*, 651.
16. Wang, D.; Guo, L.; Zhang, J.; Roeske, R. W.; Jones, L. R.; Chen, Z.; Pritchard, C. *J. Peptide Res.* **2001**, *57*, 301.
17. Ishida, H.; Qi, Z.; Sokabe, M.; Donowaki, K.; Inoue, Y. *J. Org. Chem.* **2001**, *66*, 2978.
18. Heitz, F.; Kaddari, F.; Mau, N. V.; Verducci, J.; Seheno, H. R.; Lazaro, R. *Biochimie* **1989**, *71*, 71.
19. Fernandez-Lopez, S.; Kim, H.-S.; Choi, E. C.; Delgado, M.; Granja, J. R.; Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxen, K. M.; Ghadiri, M. R. *Nature* **2001**, *412*, 452.
20. Starostin, A. V.; Butan, R.; Borisenko, V.; James, D. A.; Wenschuh, H.; Sansom, M. S. P.; Woolley, G. A. *Biochemistry* **1999**, *38*, 6144.
21. Sansom, M. S. P. *Eur. Biophys. J.* **1993**, *22*, 105.