ELSEVIER

Biochimica et Biophysica Acta 1325 (1997) 209–214

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Role of the Gln/Glu residues of trichocellins A-II/B-II in ion-channel formation in lipid membranes and catecholamine secretion from chromaffin cells

Shun-ichi Wada [a], Akira Iida [a], Koji Asami [b], Eiichi Tachikawa [c], Tetsuro Fujita [d,*]

[a] *Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan*
[b] *Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan*
[c] *School of Medicine, Iwate Medical University, Morioka 020, Japan*
[d] *Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka 573-01, Japan*

Received 10 September 1996; revised 16 December 1996; accepted 16 December 1996

Abstract

Trichocellins (TC) A-II and B-II, 20-residue peptaibols isolated from conidia of the fungus *Trichoderma viride*, have the same sequence except for the residue at position 18. Both TCs were found to form voltage-dependent ion-channels in bilayer lipid membranes (BLM) and to induce catecholamine secretion from bovine adrenal chromaffin cells through Ca^{2+} influx. TC-A-II (Gln^{18}, neutral) was more effective than TC-B-II (Glu^{18}, charged) for macroscopic current induction in BLMs and for catecholamine secretion from chromaffin cells, suggesting that Glu^{18} is unfavorable for the ion-channel formation in BLMs and chromaffin cell membranes. Nevertheless, single-channel recordings indicated that TC-B-II forms larger pores with longer open lifetimes than those of TC-A-II. This indicates that the negatively charged carboxyl group of Glu at position 18 stabilizes larger pores. The effects of the negative charge of Glu^{18} on the activities were confirmed by the use of a TC-B-II analog containing the methyl ester of Glu^{18}.

Keywords: Trichocellin; Ion-channel; Single channel recording; Catecholamine secretion; Peptaibol

1. Introduction

Peptaibols [1], which are a family of antibiotic peptides containing α-aminoisobutyric acid (Aib) at a high ratio and having an amino alcohol at the C-terminus, show various biological activities, e.g., growth inhibition of various fungi and bacteria [2] hemolysis [3], uncoupling of oxidative phosphorylation in rat liver mitochondria [4–7], and induction of catecholamine secretion in bovine adrenal chromaffin cells [8,9]. These activities are related to their membrane-modifying and ion-channel-forming properties. Much attention has been devoted to the ion-channel formed by peptaibols, particularly alamethicin (Alm), because of their structural and functional similarities to biological ion-channels. The electrical properties of the ion channels have been extensively studied by electrophysiological methods in planar bilayer lipid membranes (BLM) [10–13], and have been interpreted in terms of the barrel-stave model [14,15] in which a bundle of parallel helices forms a hydrophilic cylindrical pore.

* Corresponding author. Fax: +81 720 663146.

PII S0005-2736(96)00260-X

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

TC-A-II: Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Iva-**Gln**-Gln-Pheol

TC-B-II: Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Iva-**Glu**-Gln-Pheol

Fig. 1. Sequences of TC-A-II and TC-B-II (Aib = α-aminoisobutyric acid, Iva = isovaline)

Recently, we have isolated trichocellin (TC), a mixture of 20-residue peptaibols, from conidia of the fungus *Trichoderma viride* [16,17], together with trichorovins [18] and trichodecenins [16], during a search for antibacterial substances. Ten kinds of TCs were purified and classified into two groups on the basis of whether the residue at position 18 is Gln or Glu: TCs-A with Gln^{18} and TCs-B with Glu^{18}. The common structural characteristics of TCs-A and -B are as follows: the N-terminal amino acid is protected by an acetyl group, the C-terminal amino alcohol is phenylalaninol (Pheol) and there is no proline at position 2, in contrast to Alm.

In this study, we have characterized the functional properties of the major components of TCs, TC-A-II (saturnisporin II [19]) and TC-B-II (for the sequences, see Fig. 1), by examining the catecholamine-releasing activity from bovine adrenal chromaffin cells and the ion channel-forming activity in planar BLMs.

Comparison between TC-A-II and TC-B-II allowed us to evaluate the effect of the negative charge at position 18 of TC-B-II on the activities. The results were confirmed by using TC-B-Me (the methyl ester of Glu^{18}), in which the negative charge of TC-B-II is neutralized.

2. Materials and methods

2.1. Peptides

The isolation and structural elucidation of TC-A-II and TC-B-II have been described elsewhere [15,16]. TC-B-Me was prepared by treatment of TC-B-II with diazomethane.

2.2. Measurements of catecholamine secretion

Bovine adrenal chromaffin cells were isolated by collagenase digestion as described previously [20]. The isolated cells were immediately suspended in Eagle's minimum essential medium containing 10% calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.3 μg/ml amphotericin B, and plated on 35-mm dishes, at a density of 2×10^6 cells/dish. The cells were maintained at 37°C in a CO_2 incubator (95% air/5% CO_2) and were used for experiments after 4 days culture.

The cultured chromaffin cells were washed twice with KRH [Krebs-Ringer-Hepes; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer solution (125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Hepes, 5.6 mM glucose and 0.5% bovine serum albumin) and then preincubated in KRH solution at 37°C for 10 min. The cells were washed again with the prewarmed KRH solution and incubated with or without a test peptide at 37°C for 10 min. The reaction was terminated by transferring the incubation medium to tubes placed in an ice-cold bath. The catecholamine secreted into the medium was extracted with 0.4 M perchloric acid and adsorbed on aluminum hydroxide. The amount of catecholamine (epinephrine + norepinephrine) was measured by the ethylenediamine condensation method [21]. A Ca^{2+}-free medium was prepared by omitting Ca^{2+} and adding 0.2 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid].

2.3. Electrophysiological measurements

Macroscopic current-voltage (I-V) curves of planar BLMs exposed to peptaibols were taken by imposing a triangular wave voltage (0.01 Hz) through a pair of Ag/AgCl electrodes. Planar BLMs of about 1 mm diameter were formed in 0.1 M $CaCl_2$ (unbuffered) by the painting method [22]. The lipid solution used contained 8.4 mg/ml egg phosphatidylcholine and 2.3 mg/ml cholesterol dissolved in n-decane. A small amount of an ethanolic peptide solution was added to one side (*cis*-side) or both sides of the membranes. V_c is defined as the critical voltage at which the current

curve crosses the straight line corresponding to a reference conductance of 0.3 $\mu S/cm^2$. The mean number (N_p) of peptide monomers that form one channel is given by $N_p = V_a/V_e$, where V_a is the slope of the V_c-log [peptide concentration] plots, and V_e is the voltage increment producing an e-fold conductance change [23].

In microscopic experiments, we used planar BLMs formed on a small hole (about 100 μm diameter) in a thin Teflon film (0.25 μm thickness) by the folding method [24] to obtain well-resolved single-channel recordings. The membrane-forming solution was 10 mg/ml diphytanoylphosphatidylcholine in hexane and the bathing solution was 1 M KCl or 0.5 M $CaCl_2$ (unbuffered). Peptides were added to only one side of the membranes (*cis*-side). Based on a model of a cylindrical pore filled with an electrolyte solution [10,12,13], predicted unitary conductances (G) are expressed by the relation $G = \pi a^2/[\rho(l + 0.5\pi a)]$, where a is the effective radius of the pore, ρ is the resistivity of the electrolyte solution, and l is the length of the pore. The radius, r, of the central pore surrounded by N_p cylinders of radius, R, is given by $r = R[1/\sin(\pi/N_p)\text{-}1]$. The effective pore radius, a, is given by $a = r\text{-}r_{ion}$, where r_{ion} is the radius of a permeant hydrated ion. The unitary conductances were calculated using $l = 3.5$ nm and $R = 0.65$ nm, which values were obtained for Alm by means of dielectric and ultracentrifugation measurements [25,26]. The resistivity (ρ) of the bathing solutions was measured with a 4192A LF Impedance Analyzer (Hewlett-Packard) at 100 kHz.

3. Results

3.1. Catecholamine secretion from chromaffin cells

When chromaffin cells were incubated with 10 μM TCs for 10 min in a KRH buffer solution containing Ca^{2+}, the secretion of catecholamine from the cells was 22.9% for TC-A-II, 1.8% for TC-B-II and 15.6% for TC-B-Me relative to the total catecholamine content in the cells (174 ± 13 nmol for 2×10^6 cells) (Fig. 2). The catecholamine secretion resulted from influx of Ca^{2+} into the cells, because there was no discernible secretion with a Ca^{2+}-free KRH solution. The catecholamine-releasing activities

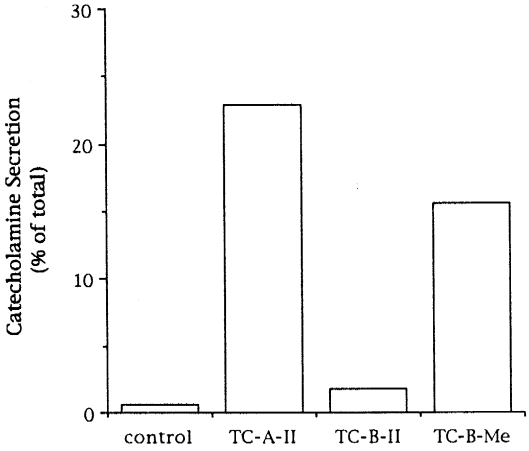

Fig. 2. Effect of 10 μM TC-A-II, TC-B-II and TC-B-Me on catecholamine secretion from cultured bovine adrenal medullary cells. The adrenal medullary cells were incubated in Ca^{2+}-containing medium for 10 min at 37°C.

of TC-A-II and TC-B-Me were about ten times that of TC-B-II and the order of potency was TC-A-II > TC-B-Me > TC-B-II. These results suggest that the catecholamine-releasing activity or the ability to induce Ca^{2+} influx depends strongly on the hydrophobicity or charge of the amino acid residue at position 18.

3.2. Ion channel formation in planar BLMs

3.2.1. Macroscopic current-voltage characteristics

When TC-A-II, TC-B-II and TC-B-Me were added to both sides of membranes, voltage-dependent symmetrical I-V curves were obtained (Fig. 3), while the addition of these peptides on one side provided asymmetrical I-V curves, with current increments at only *cis*-positive voltages, as with Alm [10–15] and trichosporins [27]. The current increased exponentially, as in the case of Alm and trichosporins, due to the simultaneous openings of large numbers of ion-channels depending on the applied voltage.

In order to compare the ability to form ion-channels among TCs, we used the parameter V_c. A lower value of V_c corresponds to a higher channel-forming activity. From the I-V curves shown in Fig. 3, the values of V_c were estimated to be 85 mV for TC-A-II, 96 mV for TC-B-II and 91 mV for TC-B-Me. The order of ion-channel-forming activity is therefore TC-A-II > TC-B-Me > TC-B-II. This order is identical to

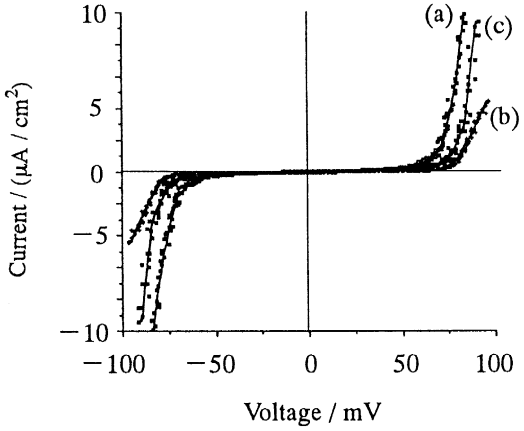

Fig. 3. Current–voltage curves of planar BLMs in a 0.1 M $CaCl_2$ solution containing 0.3 μM (a) TC-A-II, (b) TC-B-II or (c) TC-B-Me.

that found for the catecholamine secretion-inducing activity from chromaffin cells, indicating that the amino acid residue at position 18 plays an important role in both channel-forming and catecholamine-releasing activities.

The values of N_p were estimated to be 5.9 for TC-A-II and 6.2 for TC-B-II in the same manner as described for Alm [23], and the characteristic parameters for TC-A-II and TC-B-II derived from macroscopic I-V curves are listed in Table 1.

3.2.2. Microscopic single ion-channel behavior

Single ion-channel recordings for TC-A-II, TC-B-II and TC-B-Me were made in 1 M KCl and 0.5 M $CaCl_2$ solutions. The current fluctuations induced by the three kinds of TCs added to the *cis*-side were

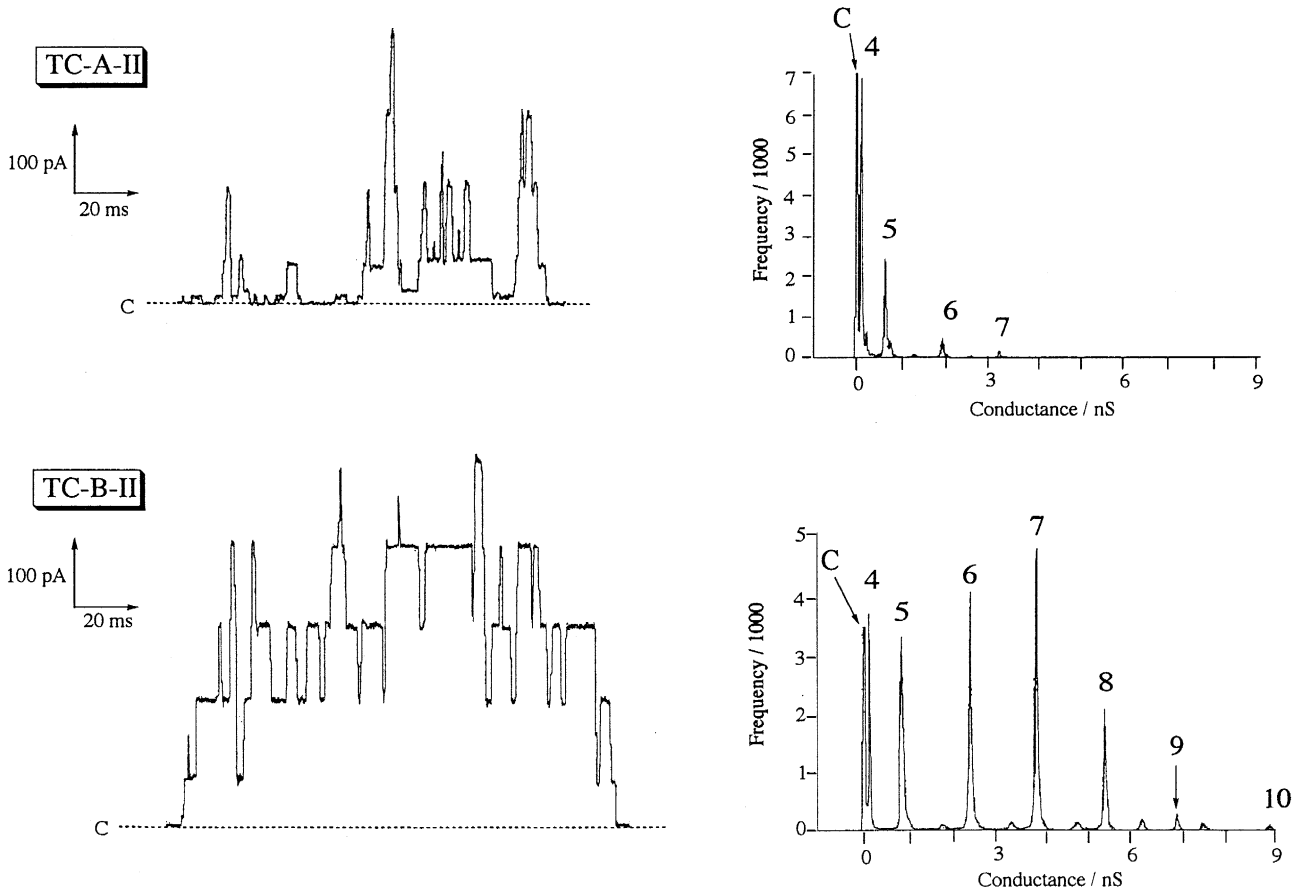

Fig. 4. Single ion-channel recordings (left) measured in 1 M KCl solution and the associated conductance histograms (right). C indicates the closed state. TC-A-II (top) or TC-B-II (bottom) was added to the *cis*-side at the concentration of 43 nM. The applied voltage of the *cis*-side relative to the *trans*-side was +120 mV. The numbers beside the peaks in the histograms are the estimated numbers of peptide monomers forming one pore.

Table 1
Characteristic parameters for TC-A-II and TC-B-II derived from macroscopic current–voltage curves

	V_c (mV)	V_a (mV)	V_e (mV)	N_p
	85.2 (0.3 μM)			
TC-A-II	64.4 (0.5 μM)	62.5	10.6	5.9
	96.3 (0.3 μM)			
TC-B-II	73.2 (0.5 μM)	64.5	10.4	6.2

observed only at *cis*-positive voltages (Fig. 4). The single ion-channel recording of TC-A-II differed from that of TC-B-II, but was similar to that of TC-B-Me (not shown). There were three (for TC-A-II and TC-B-Me) and six (for TC-B-II) discrete conductance levels (see the conductance histograms in Fig. 4), which were not integral multiples of each other (Table 2). According to the 'barrel-stave' model [14,15], the conductance levels correspond to pores made up of bundles of different numbers of peptides. The conductance values of TC-B-II were higher than those of TC-A-II and TC-B-Me at each conductance level. A similar tendency was reported for Alms Rf50 (Gln^{18}) and Rf30 (Glu^{18}) [14], as well as for 19-residue peptides, trichorzianins (Trz)-A IIIc (Gln^{18}) and B IIIc (Glu^{18}) [28,29].

According to a simple macroscopic approximation for the ion channel, i.e., a cylindrical pore filled with an electrolyte solution [10,12,13], the unitary conductance can be predicted. Thus, N_p can be estimated by comparing the observed unitary conductances with the predicted ones. Fig. 5 shows the relationships between the predicted and observed conductances for the data obtained with 1 M KCl. The observed conductance values are in good agreement with the predicted ones, and thus the conductance levels 1-6 correspond to the pores that consist of 4–9 monomers.

Table 2
Unitary conductance (G) of ion-channels induced by TC-A-II, TC-B-II and TC-B-Me (temperature = 25°C)

	G/nS in 1 M KC1			G/nS in 0.5 M $CaCl_2$		
Level	A-II	B-II	B-Me	A-II	B-II	B-Me
1	0.18	0.21	0.12	0.13	0.16	0.14
2	0.83	1.13	0.77	0.66	0.79	0.69
3	2.26	2.77	2.13	1.65	1.79	1.66
4	3.75	4.35	3.59	2.73	2.89	2.74
5	n.d.	6.05	n.d.	3.94	4.11	3.95
6	n.d.	7.14	n.d.	n.d.	5.45	n.d.

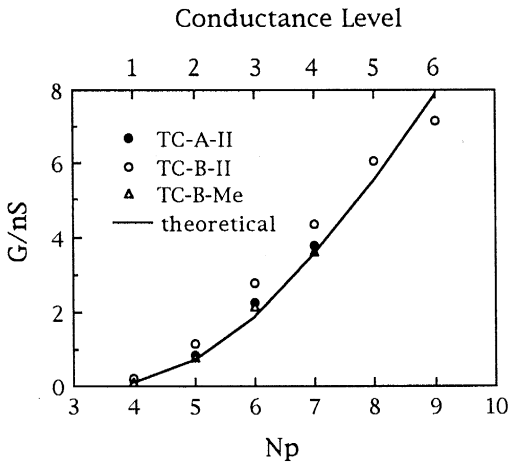

Fig. 5. Comparison of the unitary conductances observed in 1 M KCl with those calculated by use of a simple equivalent cylinder model with $R = 0.65$ nm, $l = 3.5$ nm [25,26], and $r_{ion} = 0.17$ nm [13].

The most frequently occurring pores for TC-A-II, TC-B-II and TC-B-Me were made up of 4, 7 and 4 monomers, respectively. Thus, TC-B-II has a tendency to form larger pores than TC-A-II and TC-B-Me.

Another difference among the TCs was found in the mean open life time (τ) at each conductance level, i.e., $\tau = 0.9$ ms for TC-A-II, $\tau = 1.8$ ms for TC-B-II and $\tau = 0.9$ ms for TC-B-Me for conductance level 3. This result implies that TC-B-II forms more stable pores than TC-A-II and TC-B-Me.

4. Discussion

Trichocellins have two forms that differ in whether the residue at position 18 is Gln or Glu, like Alm-Rf50 (Gln^{18}) and Alm-Rf30 (Glu^{18}) [30], and Trz-A IIIc (Gln^{18}) and Trz-B IIIc (Glu^{18}) [31–33]. The availability of these peptaibols allows us to investigate the role of the negatively charged carboxyl group at residue 18, close to the C-terminus, in the ion-channel formation and channel properties.

Our present study demonstrated that the negative charge of Glu^{18} is unfavorable for the activity to release catecholamine from chromaffin cells and for forming ion-channels in planar BLMs. A similar tendency has been found in experiments on leakage from carboxyfluorescein-entrapping liposomes and in

the inhibitory properties and cell lysis activity toward the amoeba *Dictyostelium* by Trz-A IIIc and Trz-B IIIc [34]. Vodyanoy et al. [35] found that Fraction 4 (Rf50) of Alm is more effective for forming ion-channels in planar BLMs than Fraction 6 (Rf30). Molle et al. [28] demonstrated that Trz-A IIIc shows greater efficiency for forming ion-channels than Trz-B IIIc. These results may be interpreted in terms of the hydrophobicity of peptaibols, which plays a key role in their partition between the membrane and aqueous phase, as reported previously [27]. High hydrophobicity would increase the concentration of peptaibols in the membrane, thereby facilitating ion-channel formation.

The stabilization of larger channels with longer life times was reported in the case of Trz-B IIIc [28]. In our single ion-channel experiments, it was found that TC-B-II formed long-lived, large pores compared with TC-A-II and TC-B-Me. The negative charge of Glu^{18} is thus important for stabilizing large pores in the planar BLMs. The electrostatic repulsion between the negative charges of Glu^{18} in a helix-bundle may prevent distortion or collapse of the central pore and hold it open. Further, as postulated for Alm [36], intermolecular hydrogen bonds between Glu^{18} and Gln^{19} could contribute to the stability of the channel.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research (05453180, 0633014, 06680558 and 07680629) from the Ministry of Education, Science and Culture of Japan.

References

[1] Brückner, H. and Przybylski, M. (1984) J. Chromatogr. 296, 263–275.
[2] Fujita, T., Takaishi, Y., Moritoki, H., Ogawa, T. and Tokimoto, K. (1984) Chem. Pharm Bull. 32, 1822–1828.
[3] Irmscher, G. and Jung, G. (1977) Eur. J. Biochem. 80, 165–174.
[4] Takaishi, Y., Terada, H. and Fujita, T. (1980) Experientia 36, 550–551.
[5] Mathew, M.K., Nagaraj, R. and Balaram, P. (1981) Biochem. Biophys. Res. Commun. 98, 548–555.
[6] Das, M.K., Raghothama, S. and Balaram, P. (1986) Biochemistry 25, 7110–7117.
[7] Okuda ,M., Iida, A., Uesato, S., Nagaoka, Y., Fujita, T., Takaishi, Y. and Terada, H. (1994) Biol. Pharm. Bull. 17, 482–485.
[8] Fonteriz, R.I., Lopez, M.G., Garcia-Sancho, J. and Garcia, A.G. (1991) FEBS Lett. 283, 89–92.
[9] Tachikawa, E., Takahashi, S., Furumachi, K., Kashimoto, T., Iida, A., Nagaoka, Y., Fujita, T. and Takaishi, Y. (1991) Mol. Pharmacol. 40, 790–797.
[10] Sansom, M.S.P. (1991) Prog. Biophys. Molec. Biol. 55, 139–235.
[11] Wooley, G.A. and Wallace, B.A. (1992) J. Membrane Biol. 129, 109–136.
[12] Sansom, M.S.P. (1993) Eur. Biophys. J. 22, 105–124.
[13] Sansom, M.S.P. (1993) Q. Rev. Biophys. 26, 365–421
[14] Boheimm G. (1974) J. Membr. Biol. 19, 277–303.
[15] Baumann, G. and Mueller, P. (1974) J. Supramol. Struct. 2, 538–557.
[16] Fujita, T., Wada, S., Iida, A., Nishimura, T., Kanai, M. and Toyama, N. (1994) Chem. Pharm. Bull. 42, 489–494.
[17] Wada, S., Nishimura, T., Iida, A., Toyama, N. and Fujita, T. (1994) Tetrahedron Lett. 35, 3095–3098.
[18] Wada, S., Iida, A., Akimoto, N., Kanai, M., Toyama, N. and Fujita, T. (1995) Chem. Pharm Bull. 43, 910–915.
[19] Rebuffat, S., Conraux, L., Massias, M., Auvin-Guette, C. and Bodo, B. (1993) Int. J. Peptide Protein Res. 41, 74–84.
[20] Tachikawa, E., Takahashi, S. and Kashimoto, T. (1989) J. Neurochem. 53, 19–26.
[21] Weil-Malherbe, H. and Bone, A.D. (1952) Biochem. J. 51, 311–318.
[22] Mueller, P., Rudin, D., Tien, H.T. and Wescott, W.C. (1962) Circulation 26, 1167–1171.
[23] Hall, J.E. and Vodyanoy, I. (1984) Biophys. J. 45, 233–247.
[24] Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3561–3566.
[25] Schwarz, G. and Savko, P. (1982) Bioelectromagnetics 3, 25–28
[26] Schwarz, G. and Savko, P. (1982) Biophys. J. 39, 211–219.
[27] Nagaoka, Y., Iida, A., Kambara, T., Tachikawa, E., Asami, K. and Fujita, T. (1995) Biol. Pharm. Bull. 18, 640–642.
[28] Molle, G., Duclohier, H. and Spach, G. (1987) FEBS Lett. 224 208–212.
[29] Duclohier, H., Molle, G. and Spach, G. (1989) Biochim. Biophys. Acta 987, 133–136.
[30] Pandey, P.C., Cook Jr., J.C. and Rinehart, K.L. (1977) J. Am. Chem. Soc. 99, 8469–8483.
[31] Bodo, B., Rebuffat, S., El Hajji, M. and Davoust, D. (1985) J. Am. Chem. Soc. 107, 6011–6017.
[32] El Hajji, M., Rebuffat, S., Lecommandeur, D. and Bodo, B. (1987) Int. J. Pepide Protein Res. 29, 207–215.
[33] Rebuffat, S., El Hajji, M., Hennig, P., Davoust, D. and Bodo, B. (1989) Int. J. Pepide Protein Res. 34, 200–210.
[34] El Hajji, M., Rebuffat, S., Le Doan, T., Klein, G., Satre, M. and Bodo, B. (1989) Biochim. Biophys. Acta 978, 97–104.
[35] Vodyanoy, I., Hall, J., Balasubramanian. T.M. and Marshall, G.R. (1982) Biochim. Biophys. Acta 684, 53–58.
[36] Fox, R.O. and Richards, F.M. (1982) Nature 300, 325–330.