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Biochimica et Biophysica Acta 1283 (1996) 31–36

BBA Biochimica et Biophysica Acta

Role of proline residue in the channel-forming and catecholamine-releasing activities of the peptaibol, trichosporin-B-VIa

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Received 15 December 1995; revised 9 April 1996; accepted 9 April 1996

Abstract

Trichosporin-B-VIa (TS-B-VIa) has a Pro^{14}-kinked helical structure which is considered to be important for the formation of peptaibol-type ion-channels in lipid bilayer membranes. TS-B-VIa and its analog [Aib^{14}]TS-B-VIa with Pro → Aib substitution at position 14, resulting in a straight helical structure, were tested for ion-channel-forming activity in planar lipid bilayer membranes and for ability to induce catecholamine secretion from cultured bovine adrenal chromaffin cells. Voltage-dependent multi-channel conductance, which is characteristic of TS-B-VIa, was also observed for [Aib^{14}]TS-B-VIa. In single-channel measurements, current fluctuations induced by [Aib^{14}]TS-B-VIa had a shorter life-time and showed fewer substates than those induced by TS-B-VIa. Catecholamine secretion induced by these peptides at low concentrations is completely Ca^{2+}-dependent. At high concentrations, TS-B-VIa-induced secretion was partly independent of external Ca^{2+}, but this was not the case for the analog. The differences of behavior can be explained in terms of the differences of hydrophobicity, amphiphilicity, and magnitude of dipole moment due to the conformational changes around position 14 and the C-terminal domain caused by the Pro → Aib substitution.

Keywords: Trichosporin; *Trichoderma polysporum*; Peptaibol; Ion channel; Adrenal chromaffin cell; Catecholamine

1. Introduction

Trichosporin-Bs (TS-Bs) are icosapeptides isolated from the fungus *Trichoderma polysporum* (Link ex Pers.) Rifai (strain TMI 60146) [1,2]. TS-Bs are rich in an unusual amino acid, α-aminoisobutyric acid (Aib), and their N- and C-terminals are protected by an acetyl group (Ac) and phenylalaninol (Pheol), respectively. Because of these structural characteristics, TS-Bs are classified as members of the peptaibol family [3]. In previous papers, we reported that TS-Bs induce catecholamine secretion from bovine adrenal chromaffin cells [4] and uncouple oxidative phosphorylations in rat liver mitochondria [5]. These findings suggest that TS-Bs have ion-channel-forming and/or membrane-modifying activities, as do many peptaibols.

Comparing the sequences of ion-channel-forming peptaibols, such as alamethicins [6], suzukacillins [7], paracelsins [3], trichorzianines [8], hypelcins [9], and trichocellins [10], we noted that a Pro residue at the seventh position from the C-terminal is well conserved. TS-Bs also contain a Pro residue at this position. It is believed that the Pro causes a kink in the helical structure and provides conformational flexibility in the C-terminal half. The structure induced by Pro has been supposed to be important in voltage activation of peptaibol ion channels [11,12]. Pro residues are also found in other ion-channel-forming peptides [13–15] and in the transmembrane segments of some receptors and transporters [16]. The roles of these Pro residues are also of interest.

To investigate the function of the Pro residue in TS-Bs, we synthesized [Aib^{14}]TS-B-VIa, with a Pro → Aib substitution at position 14 of TS-B-VIa [17]. The sequences of

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PII S0005-2736(96)00070-3

TS-B-VIa and [Aib^{14}]TS-B-VIa are shown in Fig. 1. In our previous circular dichroism (CD) and NMR studies on the conformation of TS-B-VIa and [Aib^{14}]TS-B-VIa in methanol [18], we showed that TS-B-VIa has a fully helical structure with a kink that is stabilized by a $1 \leftarrow 4$ hydrogen-bond between the Leu^{12} CO and Val^{15} NH. The helical structure is composed of a 3_{10}-helix in the N-terminal first turn, α-helix in the middle region and 3_{10}-helix in the C-terminal moiety that follows Leu^{12}. In contrast, [Aib^{14}]TS-B-VIa predominantly has a straight α-helical structure except for a 3_{10}-helix in the N-terminal first turn. In this paper, we deal with the effects of the conformational differences caused by the Pro → Aib substitution on the ion-channel-forming properties and catecholamine-releasing activities.

2. Materials and methods

TS-B-VIa and [Aib^{14}]TS-B-VIa were synthesized as described previously [17].

Multi-channel and single-channel conductances were measured for planar lipid bilayer membranes doped with TS-B-VIa and [Aib^{14}]TS-B-VIa. In the measurements of multi-channel conductances, membranes were formed on a 1 mm hole in a Teflon chamber by the painting method [19]. The membrane forming solution was 1% egg-lecithin/cholesterol (4:1) in decane. An ethanolic solution of a peptide was added to one side (*cis* side) of the aqueous compartments, which contained unbuffered 1 M KCl solution. The other side (*trans* side) of the compartments was grounded. After allowing for partitioning equilibrium, a 0.01 Hz triangle-wave voltage (V) was applied to the membrane and the current–voltage curves were recorded. The relationship of multi-channel conductances (G) and V is empirically expressed by the following equation [11].

$$G = \Gamma C_p^n \exp(V/V_e) \quad (1)$$

where C_p is the peptide concentration, V_e the voltage that produces an e-fold change in conductance at a fixed concentration, n the number of peptides forming one ion channel, and Γ a parameter that is a function of various variables related to the experimental conditions. From the dipole realignment model [20,21], according to which dipoles of helical peptides are reoriented in the membrane along the direction of the electric field during the gating events, V_e is given by

$$V_e = kT/en\alpha \quad (2)$$

where k is Boltzman's constant, T the temperature, e the elemental charge and α the product of the charge on one peptide and its displacement during the gating event. If the helical peptides are parallel to the membrane surface in the absence of an electric field and are perpendicular to the membrane surface on the application of a sufficiently large voltage, the dipole moment μ of the peptide is

$$\mu = e\alpha d \quad (3)$$

where d is the thickness of the hydrophobic part of the membrane. In the calculation, we used $d = 3$ nm.

In single-channel conductance measurements, we used diphytanoylphosphatidylcholine (diphPC) membranes, which were formed on a 120 μm hole in a Teflon film sandwiched between two half cells by the method of Montal and Mueller [22]. A peptide was added to the *cis* side, and current fluctuations were measured under a constant *cis*-positive voltage. All experiments were conducted at room temperature.

Catecholamine secretion from cultured bovine chromaffin cells was measured as previously described [4]. Adrenal chromaffin cells were isolated from bovine adrenal glands by collagenase digestion. The cells in modified Eagle's minimum essential medium were maintained at 37°C in a CO_2 incubator and were used for experiments after 4 days of culturing.

3. Results

Fig. 2 shows multi-channel current–voltage (I–V) curves measured across egg-lecithin/cholesterol bilayers doped with TS-B-VIa and [Aib^{14}]TS-B-VIa. The peptides

TS-B-VIa:

1 14 20
Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Ile-Aib-Gly-Leu-Aib-**Pro**-Val-Aib-Aib-Gln-Gln-Pheol

[Aib^{14}]TS-B-VIa:

1 14 20
Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Ile-Aib-Gly-Leu-Aib-**Aib**-Val-Aib-Aib-Gln-Gln-Pheol

Fig. 1. Primary structures of TS-B-VIa and [Aib^{14}]TS-B-VIa.

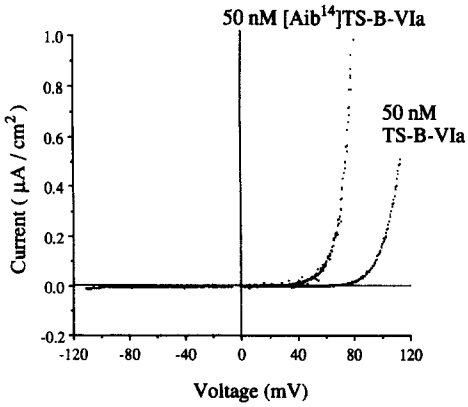

Fig. 2. Current–voltage curves of lipid bilayer membranes exposed to 50 nM TS-B-VIa and [Aib^{14}]TS-B-VIa in 1 M KCl. Membranes were formed from lecithin with cholesterol in unbuffered 1 M KCl. Peptides were added to the *cis* side of the membrane.

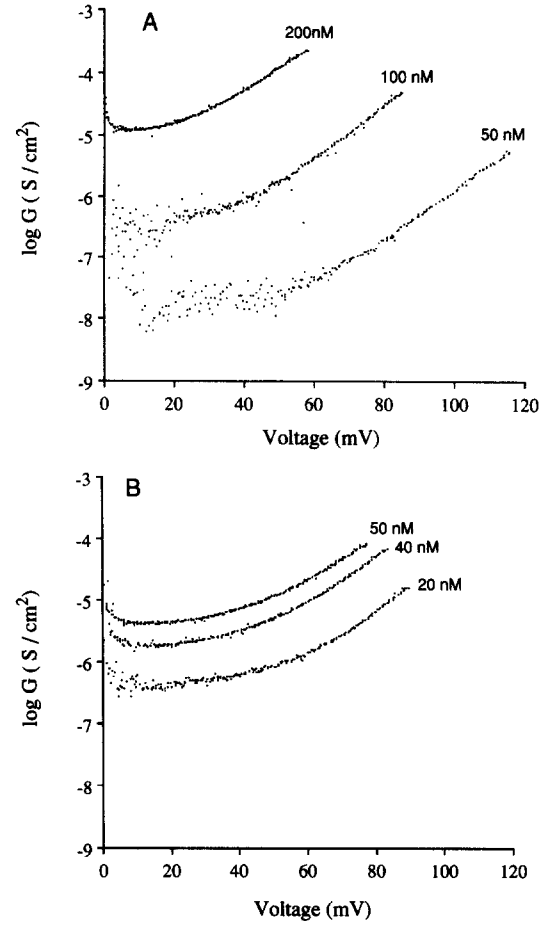

Fig. 3. Effect of peptide concentration on logarithmic conductance (log G)/voltage characteristics of TS-B-VIa (A) and [Aib^{14}]TS-B-VIa (B) doped membranes. Membranes were formed from lecithin with cholesterol in 1 M KCl containing TS-B-VIa (A) or [Aib^{14}]TS-B-VIa (B) at the concentrations indicated.

were added to only one side of the membranes (*cis* side). Both the peptides showed a similar I–V characteristic, which was highly asymmetric, i.e., a steep rise of the current was found only when the *cis*-side was positive. This suggests that the induction of characteristic I–V curves is not influenced by the substitution of Pro for Aib. However, the critical voltage above which the steep increase in current was observed was different between the two peptides at the same concentration. The critical voltage of [Aib^{14}]TS-B-VIa is lower than that of TS-B-VIa, indicating that [Aib^{14}]TS-B-VIa is more effective for channel formation than TS-B-VIa. Fig. 3 shows the membrane conductance (G) plotted logarithmically against the voltage. There are two components in the plots, as pointed out by Roy [23], i.e., a voltage-independent and a voltage-dependent conductance. In the log G vs. V plots, the voltage-dependent conductance showed a straight line, the slope of which was independent of the peptide concentration. The relationship of G and V is empirically expressed by Eq. (1). The value of V_e in Eq. (1) was determined from the slope of the log G/V plots, being 10.0 mV and 9.4 mV for TS-B-VIa and [Aib^{14}]TS-B-VIa, respectively (Table 1). As expected from Eq. (1), the conductance at a fixed voltage is proportional to the nth power of the peptide concentration. Hence, n is easily determined from the peptide concentration dependence of the conductance at a fixed voltage. When the concentration of TS-B-VIa was doubled, the conductance at a given voltage was increased by a factor of 116 times, whereas for [Aib^{14}]TS-B-VIa, the conductance was increased by a factor of 17 times (Fig. 3). Therefore, the relation between G and C_p can be expressed as $G \propto [C_p]^{6.8}$ for TS-B-VIa and $G \propto [C_p]^{4.2}$ for [Aib^{14}]TS-B-VIa, and thus the values of n are about 7 for TS-B-VIa and about 4 for [Aib^{14}]TS-B-VIa (Table 1).

Using Eq. (2), the values of a charge move on one monomer during the gatings (α) were calculated from the observed values of V_e as shown in Table 1. The dipole moments calculated from the values of α using Eq. (3) are also shown in Table 1. From this calculation, larger dipole moment was estimated for [Aib^{14}]TS-B-VIa.

In order to obtain well resolved single-channel currents, we used diphPC bilayer membranes formed on a smaller hole than that used for macroscopic measurements. Fig. 4 shows well resolved multilevel single-channel currents with six conductance substates induced by TS-B-VIa. The con-

Table 1
Multi-channel conductance parameters for TS-B-VIa and [Aib^{14}]TS-B-VIa.

	V_e (mV)	n	α	μ (D)
TS-B-VIa	10.0 ± 1.2	6.8 ± 0.4	0.37	53
[Aib^{14}]TS-B-VIa	9.4 ± 0.2	4.3 ± 0.3	0.62	92

V_e and n were estimated from log $G-V$ curves for 50, 100 and 200 nM TS-B-VIa and for 20, 40 and 50 nM [Aib^{14}]TS-B-VIa. Data are mean ± S.D. from four experiments at each concentration. α and μ were calculated from Eqs. (2) and (3), respectively.

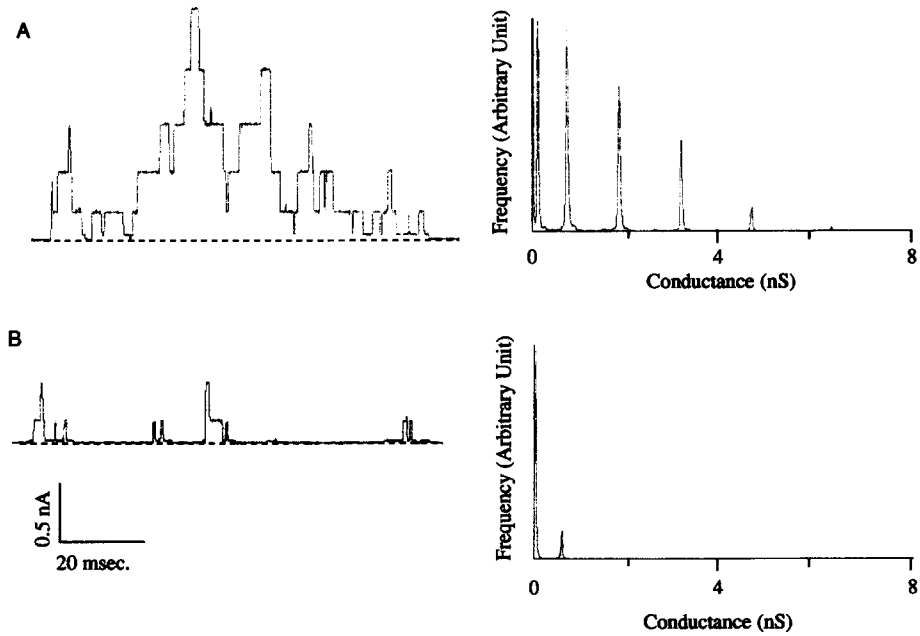

Fig. 4. Single-channel fluctuations of diphPC membranes doped with TS-B-VIa (A) and [Aib^{14}]TS-B-VIa (B) in 1 M KCl. TS-B-VIa and [Aib^{14}]TS-B-VIa were added to the *cis* compartment only, to make a final concentration of 4 nM. Applied voltage was 270 mV. Associated amplitude histograms are on the right of channel–current recordings.

ductance substates correspond to channels with different pore sizes, which are formed from different numbers of peptides, as described by Boheim [24]. The transitions between the substates, that are due to the changes in pore size, are caused by the uptake and release of peptide monomers into and from the channel aggregate. In contrast with TS-B-VIa, [Aib^{14}]TS-B-VIa showed only three conductance substates with shorter life-times than those of TS-B-VIa (Table 2). These results indicate that the Pro → Aib substitution reduces the stability of channel aggregates and decreases the number of monomers forming a channel. The fact that [Aib^{14}]TS-B-VIa forms smaller channel aggregates than TS-B-VIa is consistent with the estimated n values obtained from the multi-channel experiments. When the conductance value of the two peptides is compared for each substate, the [Aib^{14}]TS-B-VIa channel has a lower conductance than the TS-B-VIa channel, especially at the lowest level (Table 2).

Incubation of cultured bovine adrenal chromaffin cells with TS-B-VIa caused secretion of catecholamines from the cells. The increase in the catecholamine secretion was dependent on the concentration of TS-B-VIa (2–20 μM) (Fig. 5). The secretion at low TS-B-VIa concentrations (2–10 μM) was completely abolished by eliminating Ca^{2+} from the incubation medium, while at a higher concentration (20 μM), the secretion was independent of external Ca^{2+}. Therefore, TS-B-VIa-induced secretion occurs in both Ca^{2+}-dependent and -independent ways. [Aib^{14}]TS-

Table 2
Single-channel parameters of TS-B-VIa and [Aib^{14}]TS-B-VIa

	Substate	Conductance	Probability of opening [a]	Mean life-time (ms) [b]
TS-B-VIa	1	0.14	0.271	0.89
	2	0.75	0.306	1.11
	3	1.85	0.233	1.14
	4	3.17	0.146	1.42
	5	4.66	0.037	0.79
	6	6.33	0.007	0.61
[Aib^{14}]TS-B-VIa	1	0.05	0.848	0.69
	2	0.60	0.145	0.40
	3	1.64	0.007	0.29

[a] Open probability of each level within bursts; closed state was excluded.
[b] Mean life-time (τ) was obtained from the least-squares equation, $P_t = e^{-t/\tau}$, fitted to the plot of life-time probability (P_t) distribution for each substate.

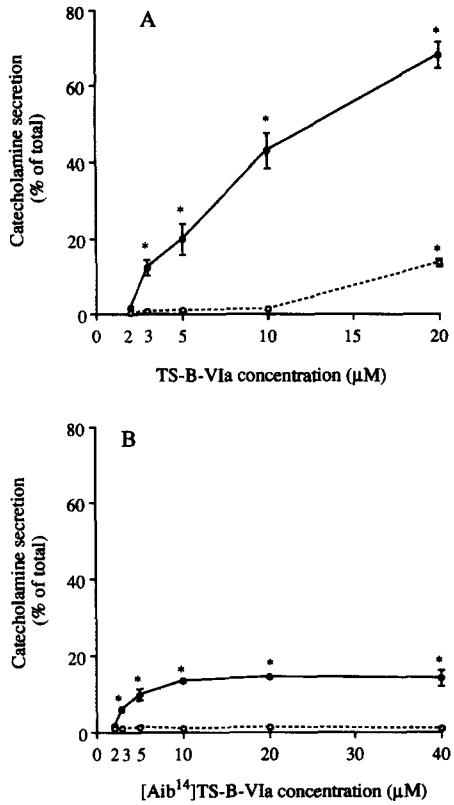

Fig. 5. Effect of TS-B-VIa (A) and [Aib^{14}]TS-B-VIa (B) on catecholamine secretion from cultured bovine adrenal chromaffin cells. The catecholamine secretion was measured after incubation of the cells for 10 min at 37°C with various concentrations of peptides, in 2.6 mM Ca^{2+}-containing (filled circles) or Ca^{2+}-free (plus 0.2 mM EGTA) medium (open circles). Catecholamine secretion is shown as a percentage of total catecholamine content. Data are means ± S.D. from three experiments. * $P < 0.001$, significantly different from control.

B-VIa-induced secretion increased concentration-dependently at low concentrations up to 10 μM, but at high concentrations, the secretion reached a plateau. Ca^{2+}-independent secretion was not observed even at high concentrations (20 and 40 μM).

4. Discussion

In this study, we have demonstrated that Pro^{14} → Aib^{14} substitution in TS-B-VIa does not alter the voltage activation of the ion channel, but affects the probability of the ion-channel formation, the life-time of the channel and the occurrence of multilevel conductances. The results obtained were similar to those reported for a Pro^{14} → Ala^{14} substituted alamethicin analog in which all Aibs were replaced with Leu [25] and for Pro^{14} → Ala^{14} substituted melittin [26], except that Pro^{14} → Ala^{14} substitution in the alamethicin analog had the effect that at a given concentration the I–V curve is shifted to higher V values, while in the case of Pro^{14} → Aib^{14} substitution in TS-B-VIa, it is shifted to lower V values. [Aib^{14}]TS-B-VIa increased the macroscopic conductance of planar lipid bilayer membranes more effectively than TS-B-VIa when compared at the same peptide concentration. This suggests that the Pro → Aib substitution increases the probability of ion-channel formation. The reason may be two-fold: the high hydrophobicity and the large dipole moment of [Aib^{14}]TS-B-VIa compared with native TS-B-VIa. The high hydrophobicity of [Aib^{14}]TS-B-VIa is deduced from the fact that [Aib^{14}]TS-B-VIa has a longer retention time than that of TS-B-VIa in reversed-phase HPLC [17]. This implies a high partition coefficient to the lipid membranes, thereby increasing the opportunity for ion-channel formation in the membrane. A similar relationship between the membrane conductance and the peptide hydrophobicity has been obtained for five TS-B analogs with various replacements of amino acid residues [27].

Since [Aib^{14}]TS-B-VIa has a higher helical content than TS-B-VIa and lacks a kink in the helical structure [18], the molecular dipole moment of [Aib^{14}]TS-B-VIa should be larger than that of TS-B-VIa. This prediction is consistent with the estimates of peptide dipole moment obtained from the macroscopic conductance measurements (Table 1). The large molecular dipole moment of [Aib^{14}]TS-B-VIa would also contribute to its high efficiency of ion-channel formation because it facilitates the orientation of the peptide in an electric field. Although the Pro → Aib substitution increases the probability of ion-channel formation, it decreases the stability of the ion channel, which is manifested as a decrease in the life time of the ion channel. The difference in the stability of the ion channel can be attributed to the structural differences between the two peptides in helix length, solvent-accessibility and steric hindrance. Our ^{1}H-NMR studies in methanol indicated that the conformational differences are mainly in the C-terminal domain from position 12 to 20, where a 3_{10}-helix is found for TS-B-VIa and an α-helix for [Aib^{14}]TS-B-VIa [18]. The length of TS-B-VIa including the 3_{10}-helix would be longer than that of [Aib^{14}]TS-B-VIa. In our recent study with several truncated TS-B-VIa synthetic analogs [28], it has been shown that the life-time and the stability of the ion channel are very sensitive to the length of peptaibols. Hence, the difference in helical length between [Aib^{14}]TS-B-VIa and TS-B-VIa would alter their single-channel behavior.

The peptaibol ion channels, which are assumed to consist of a bundle of helices, in membranes are stabilized by polar interactions between helices and between helices and water molecules in the pore region. [Aib^{14}]TS-B-VIa lacks solvent-accessible carbonyls, Aib^{10}CO and Gly^{11}CO, unlike native TS-B-VIa [18]. This may be another factor causing the low stability of [Aib^{14}]TS-B-VIa ion channels. Furthermore, a bundle of [Aib^{14}]TS-B-VIa helices would suffer steric hindrance owing to the bulky C-terminal residues because of the absence of a kink in the helix. This may be the third factor accounting for the low stability. TS-B-VIa forms a funnel-shaped pore, as proposed by Fox

and Richards [12], while straight [Aib^{14}]TS-B-VIa forms a cylindrical pore which would be narrower than that of TS-B-VIa. Therefore, the [Aib^{14}]TS-B-VIa channels always showed smaller unit conductance than the TS-B-VIa channels for all substates.

In bovine adrenal chromaffin cells, an influx of external Ca^{2+} into the cells is essential for triggering the secretion of catecholamines [29]. We found that incubation of chromaffin cells with TS-Bs causes Ca^{2+} influx into the cells and induces catecholamine secretion from the cells [4]. With TS-Bs at low concentrations, the secretion was completely dependent on external Ca^{2+}, while at high concentrations, the secretion was partly independent of external Ca^{2+}. We concluded that TS-Bs at higher concentrations impairs the cells and causes Ca^{2+}-independent secretion from the damaged cells [4]. The same result was obtained for TS-B-VIa-doped cells, whereas Ca^{2+}- independent secretion was not observed for [Aib^{14}]TS-B-VIa at higher concentrations (20 and 40 μM). This result suggests that the membrane-disordering effect of [Aib^{14}]TS-B-VIa at high concentrations is weaker than that of TS-B-VIa. The membrane-disordering effects may be enhanced by the Pro-kinked structure of TS-B-VIa.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research (07680629 and 07772095) from the Ministry of Education, Science and Culture of Japan.

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