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# Synthesis and paralytic activities of squaryl amino acid-containing polyamine toxins

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**Summary.** Eight analogs **4a-7a** and **4b-7b** of philanthotoxin (PhTX) from wasp venom and nephilatoxin-8 (NPTX-8) from spider venom whose tyrosine or asparagine linker is replaced by squaryl (sq) amino acid or 4-amino squaryl (4-asq) amino acid have been synthesized in an efficient manner via coupling of *N*-acyl squaryl amino acid intermediate **19** or **26** with the corresponding polyamine part. Preliminary bioassay using crickets revealed that the analogs substituted by glutamate-type squaryl amino acid-containing NPTX **7a** and **7b** showed more potent paralytic activities than that of NPTX-8.

**Keywords:** Philanthotoxin – Nephilatoxin-8 – Polyamine toxins – Paralytic activity -Glutamate receptors – Squaric acid – Squaryl amino acid – 4-Hydroxycyclobuten-1-one, 2,3-dioxocyclobutene – N-Acyl squaryl amino acid – Squaryl amino acid-containing polyamine toxins – Insecticidal activity

## Introduction

Squaric acid **(1)** has attracted much attention as an intriguing multi-functional organic molecule because of its characteristic physicochemical properties such as aromaticity, strong acidity, high reactivity to nucleophile, and strong chelating ability to metal ions (Fig. 1) (Seitz and Imming, 1992; Schmidt, 1980; West and Niu, 1969). Thus, squaric acid is often employed as a carboxylate isostere in medicinal chemistry (Butera et al., 2000; Shinada et al., 1999), as a functional dye in material science (Ashwell et al., 1995; Law, 1993), and as a building block in organic chemistry (Liebeskind, 1989; Moore and Yerxa, 1992; Ohno et al., 1998; Paquette, 1998). Recently, we have developed a new class of 4-hydroxy-2,3-dioxocyclobut-1-enyl (squaryl: sq) group-containing amino acids **2** and **3** whose $\beta$- or $\gamma$-carboxylic acid moiety of aspartic acid or glutamic acid is replaced with the sq group (Shinada et al., 1999). These amino acids can be viewed as novel entries of aspartic acid, glutamic acid, and tyrosin analogs which retain the aforementioned physicochemical properties of squaric acid. Indeed, the p*K*a value of the sq group was estimated to be 0 which is almost the same value as that of trifluoroacetic acid (TFA). Moreover, the glutamate-type analog **3** showed potent paralytic activity to crickets ($ED_{50}$: 0.98 $\mu$g/1 g of crickets) (Shinada et al., unpublished results) and neuro-excitatory activity in mammalian central nervous systems, which was classified as a selective agonist of ionotropic glutamate receptors (Shinada et al., 1999). These facts have proven that the sq group can act as an equivalent functional group with the $\gamma$-carboxylic acid of L-glutamate. The next step in our program was to extend the utility of these amino acids as an isostere of an amino acid possessing an aromatic moiety such as phenylalanine, tyrosine, or Dopa. Furthermore, it was of interest to examine whether the 4-amino-2,3-dioxocyclobut-1-enyl (4-asq) group, easily prepared from its 4-alkoxy derivative by treatment with ammonia, could mimic the amide group of asparagine or glutamine which are often found in biologically active polyamines (Fig. 2) (Kawai and Nakajima, 1993; Lachlan and Hodgson, 2002; McCormick and Meinwald, 1993; Schafer et al., 1994). In this paper, we wish to describe the synthesis of novel sq amino acid-containing polyamine toxins **4–7** whose tyrosine or asparagine residue is replaced by sq amino acid **2a** or **3a** or their 4-amino sq derivative **2b**

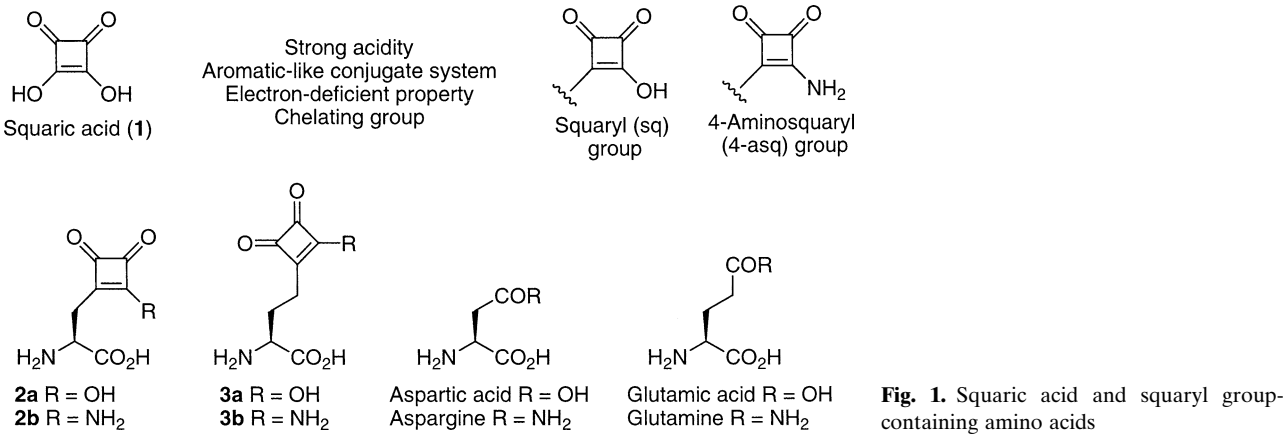



**Fig. 1.** Squaric acid and squaryl group-containing amino acids

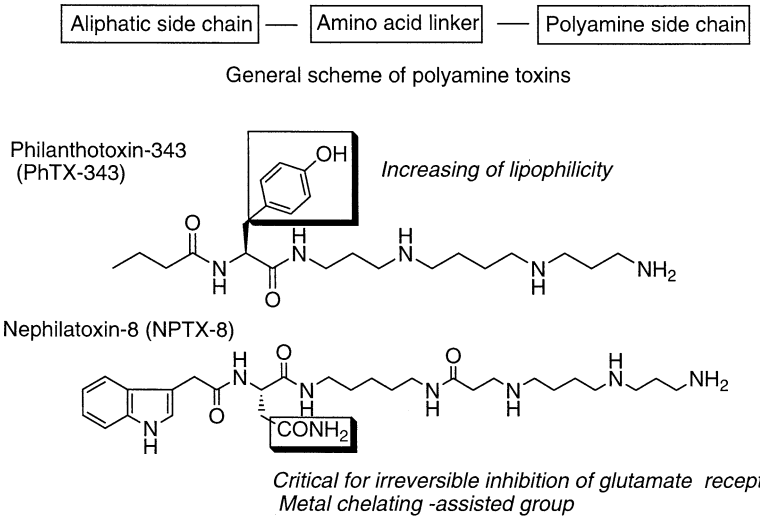



**Fig. 2.** Structures of philanthotoxin and nephilatoxin-8. Role of amino acid linkers

or **3b**, respectively. Paralytic activities of the synthetic compounds to crickets are briefly disclosed.

Several classes of polyamine toxins are isolated from wasp and spider venoms. Representative examples are philanthotoxin (PhTX) from *Philantus triangulum* (Eldefrawi et al., 1988) and nephilatoxins (NPTX) from *Nephila clavata* (Fig. 2) (Aramaki et al., 1986; Toki et al., 1988). It is well known that these polyamine toxins are used principally to inflict paralysis of insects by such arthropods (Kawai and Nakajima, 1993; McCormick and Meinwald, 1993; Schafer et al., 1994). Electrophysiological and biochemical studies have revealed that these toxins act as a potent antagonist of ionotropic glutamate receptors, specific to non-NMDA subtype, at many synapses in both vertebrates and invertebrates (Aramaki et al., 1986; Eldefrawi et al., 1988; Kawai and Nakajima, 1993; Lachlan and Hodgson, 2002; Schafer et al., 1994; Yoshioka et al., 1988)). Since glutamate receptors are implicated in the construction of memory and learning as well as in the pathogenesis of neuron damage to cause various neuronal diseases (Monaghan et al., 1989; Nakanishi and Masu, 1993; Shimamoto and Ohfune, 1996; Watkins et al., 1990), the polyamine toxins and their analogs are used as not only useful tools for the investigation of molecular mechanisms of glutamate receptors and their physiological functions but also as lead compounds to develop neuroprotecting drugs.

The structures of the toxins are comprised of an aliphatic part, an amino acid linker (tyrosine or asparagine), and a polyamine side chain. Several studies regarding the structure-activity relationship (SAR) of these toxins have been reported (Lachlan and Hodgson, 2002; Stromgaard et al., 2000; Wang et al., 2000), suggesting that the amino acid linker

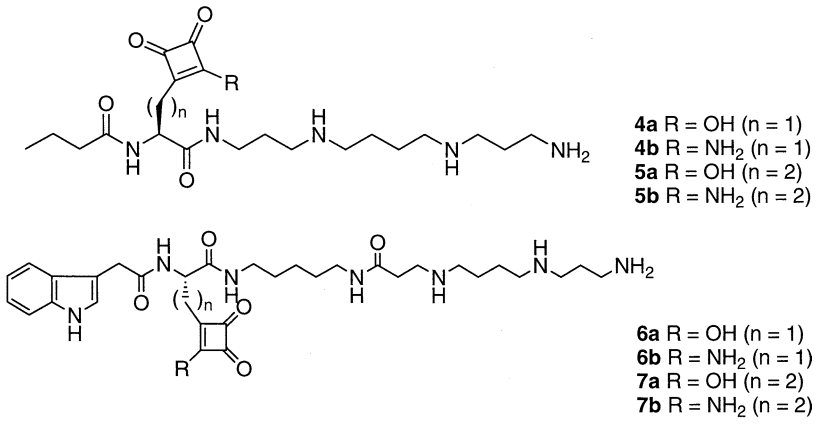
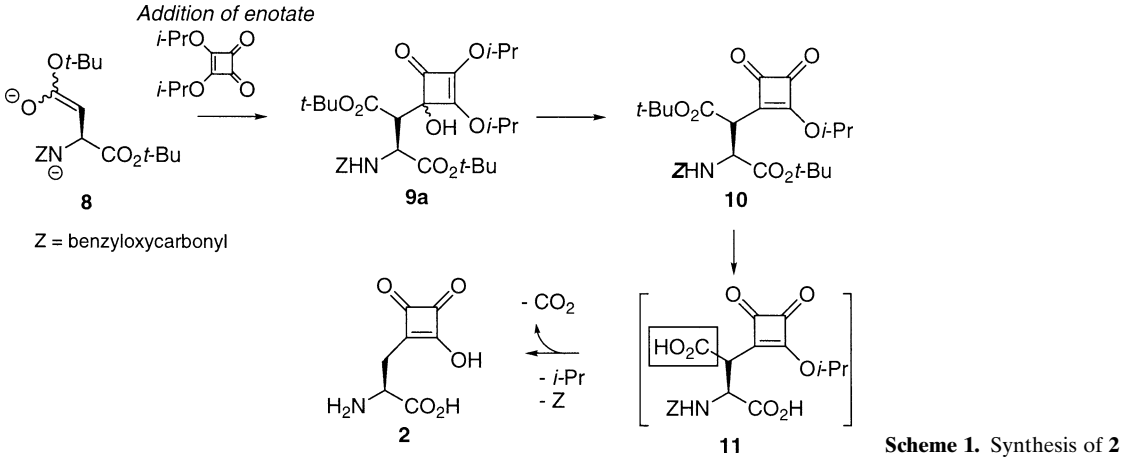



**Fig. 3.** Target compounds: Sq amino acid-containing PhTX-343 and NPTX-8




**Scheme 1.** Synthesis of **2**

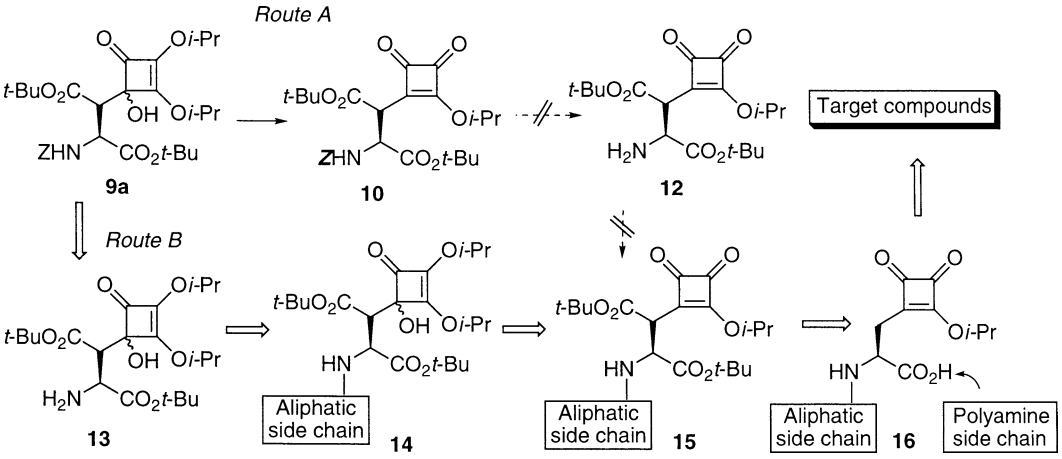
plays different roles for the biological activity: (i) the tyrosine part of PhTX is a lipophilic as well as a proton-donating functional group leading to an increase of binding affinity to the receptors, (ii) the asparagine moiety of NPTX considerably contributes to formation of a metal chelate complex in co-operation with the amino groups of the polyamine chain, and (iii) the presence of an asparagine moiety is crucial for the irreversible inhibition of glutamate receptors (Kawai and Nakajima, 1993; Lachlan and Hodgson, 2002; McCormick and Meinwald, 1993; Schafer et al., 1994). These hypotheses prompted us to incorporate the sq-containing amino acids **2a** and **3a**, and their 4-amino derivatives **2b** and **3b**, into the linker position of PhTX and NPTX-8, respectively. Since these amino acids and the corresponding 4-amino derivatives have unique proton-donating as well as metal chelating properties, it was expected that the sq amino acid-containing polyamine toxins **4–7** would provide new insights into the SAR studies of polyamine toxins (Fig. 3) (Hidai et al., 2000; Nihei et al., 2002; Stromgaard et al., 2000; Wang et al., 2000).

## Results and discussion

### *Synthetic plan of polyamine toxin analogs*

Synthesis of sq amino acid **2** has, previously, been performed by the following sequence of reactions: (i) addition of enolate **8** derived from aspartic acid to afford hydroxycyclobutenone **9**, (ii) acid-catalyzed dehydroxylation of **9** to cyclobutenedione **10**, (iii) removal of the protecting groups, and (iv) simultaneous decarboxylation from **11** to give **2** (Scheme 1) (Hayashi et al., 1997; Shinada et al., 1999).

Selective protection of the free amino acid **2** at the *N*-terminal or the 4-hydroxy group can not be performed due to its strong acidity and poor solubility in organic solvents. Therefore, introduction of a




**Scheme 2.** Synthetic plan of squaryl amino acid-containing polyamine toxins

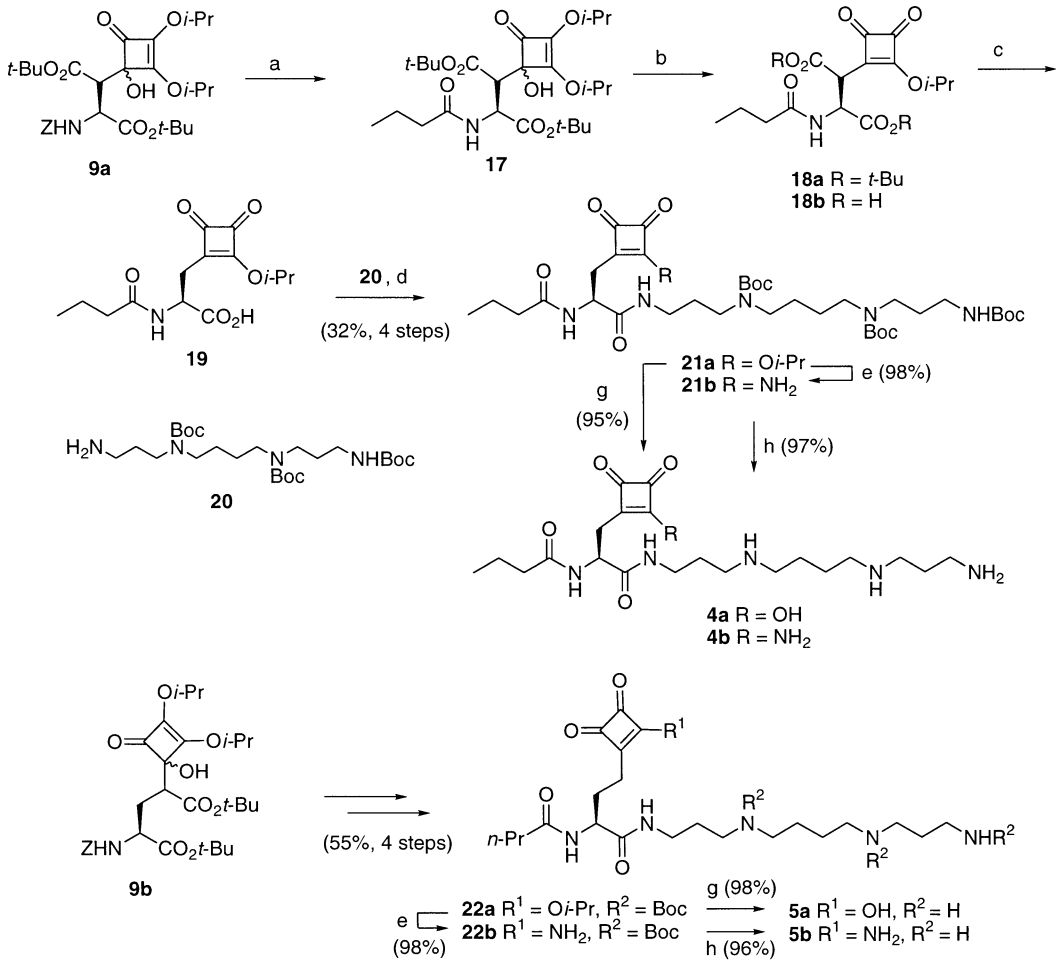
requisite aliphatic side chain into the *N*-terminal of the synthetic intermediate **9** or **10** was needed prior to removing the protecting groups. Although a protected cyclobutenone **10** was considered to be a plausible intermediate for the condensation with the acyl group (Route A), the Z group can not be removed under the catalytic hydrogenation conditions because of the preventative effects by the sq group as has been reported in our previous work (Shinada et al., 2000). Therefore, we chose the hydroxycyclobutenone **9a** as the starting material (Route B). We expected that the *N*-acylated intermediates **14** and its one-carbon homologue which corresponds to glutamate-type analog can be employed as a common intermediate for the synthesis of the target toxins **4–7**, respectively, as shown in Scheme 2.

### *Synthesis of PhTX analogs **4** and **5***

The removal of the Z group from **9a**, a key step to introduce the butyryl group to the *N*-terminal, proceeded smoothly under the hydrogenation conditions ($H_2$/Pd-C) to give free amine **13** (Scheme 2), which is in sharp contrast to the hydrogenation of **10**. To avoid the troublesome isolation procedure of the free amine, we considered that the butyryl group can be introduced simultaneously during the hydrogenation step. Thus, **9a** was subjected to the hydrogenation conditions in the presence of butyric acid anhydride. The reaction gave desired the *N*-butyryl **17** in one pot. The 4-hydroxycyclobuten-1-one moiety of **17** was transformed at this stage into the corresponding cyclobutenedione **18a** by treatment with a catalytic amount of hydrochloric acid in $CH_2Cl_2$. Selective removal of the *tert*-butyl groups was carried out using TFA to give **18b** where the isopropyl group remained unchanged. The removal of the $\beta$-carboxyl group was effected by the assistance of $Et_3N$ to give *C*-terminal free **19** (Shinada et al., 1999). Thus, *N*-acylated *C*-terminal free amino acid **19** (32% in 4 steps) was prepared from the hydroxycyclobutenone **9a**. Protected spermine **20** as the polyamine residue was prepared according to the reported procedure (Jasys et al., 1990). This was coupled with the *C*-terminal free amino acid **19** using 1-hydroxybenzotriazole (HOBt) and benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluoro-phosphate (BOP) to give **21a**. All the protecting groups on **21a** were removed simultaneously by treatment with 1 *N* HCl to give sq aspartic acid-containing PhTX **4a**. The 4-asq analog **4b** was prepared using protected **21a**. The reaction of **21a** with methanolic ammonia underwent substitution reaction at the *iso*-propyloxy group to give **21b**. The resulting 4-asq derivative **21b** was subjected to the deprotection using TFA to give **4b**. The synthesis of **5a** and **5b** (sq glutamic acid) which correspond to one-carbon elongated homologs of **4a** and **4b** started with hydroxycyclobutenone **9b**. This was converted into **5a** and **5b** in the same manner as those from **9a**. Thus, 4 types of sq- or 4-asq amino acid-containing PhTX were synthesized.

### *Synthesis of NPTX analogs **6** and **7***

The synthesis of NPTX-8 analogs **6** and **7** was performed according to the synthesis of the PhTX



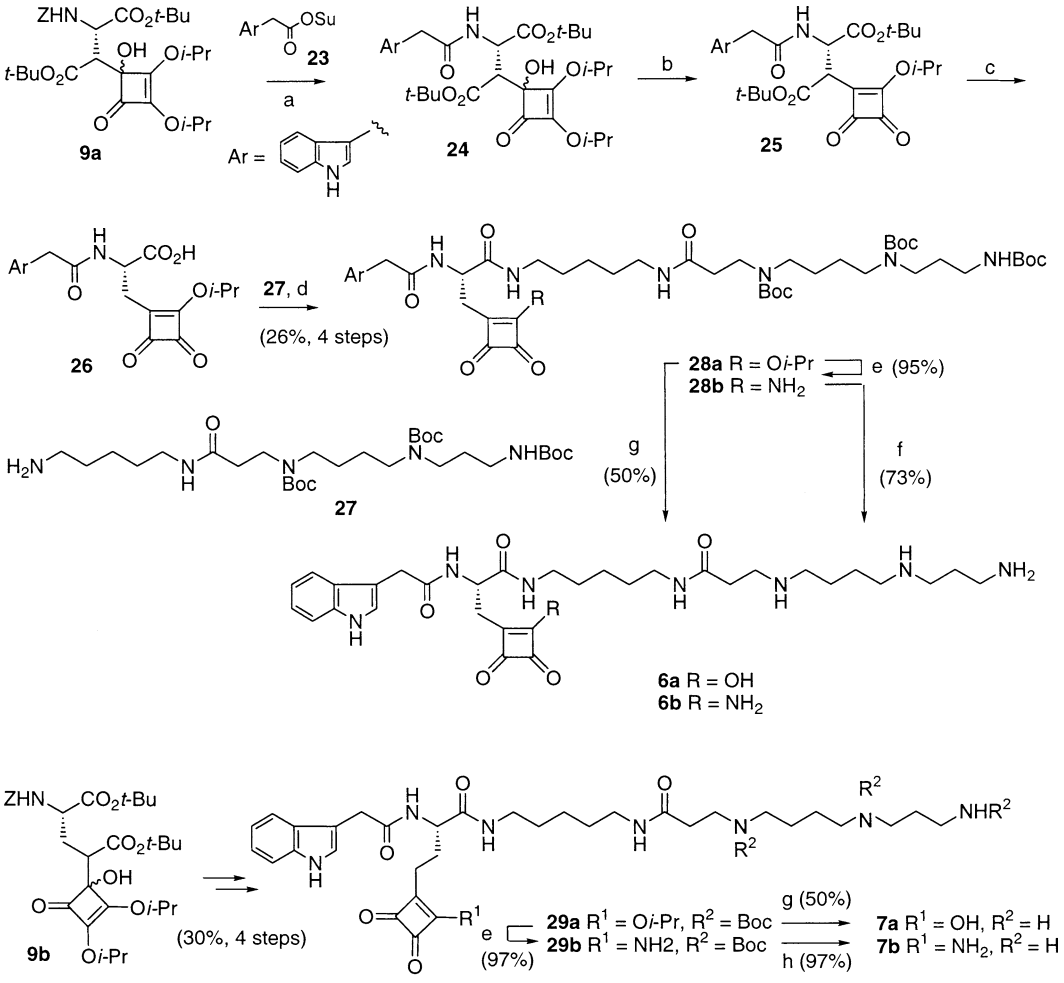

[a]Conditions (a) $H_2$, Pd-C, $(n\text{-}C_3H_7CO)_2O$, MeOH, rt, 1 h; (b) 12 *N* HCl, $CH_2Cl_2$, 0 °C, 1.5 h; (c) TFA, $CH_2Cl_2$, rt, 1.5 h, then $Et_3N$, rt, 5 min; (d) BOP, HOBt, *i*-$Pr_2NEt$, $CH_2Cl_2$, 0 °C to rt, 17 h; (e) $NH_3$, MeOH, rt, 1 min; (f) TFA, $CH_2Cl_2$, 0 °C to rt, 3 h; (g) 1 *N* HCl, acetone, rt, 2 h; (h)TFA, $CH_2Cl_2$, rt, 3 h.

**Scheme 3.** [a]Synthesis of PhTX analogs **4** and **5**

analogs (Scheme 4). Under the hydrogenation conditions, an active ester of indole acetic acid **23** was introduced to hydroxycyclobutenedione **9a** to give *N*-acylated derivative **24**. This was converted into cyclobutenedione **25**, which, upon deprotection and $Et_3N$-assisted decarboxylation, gave *C*-terminal free **26**. The overall yield from **9b** to **26** was 26% in 4 steps. The coupling of **26** with a protected polyamine residue **27**, prepared using known procedure (Jasys et al., 1990), was effected using HATU and HOAt, to afford fully protected sq amino acid-containing NPTX-8 **28a**. The protecting groups of **28a** were removed simultaneously with 1 *N* HCl to give **6a**. Treatment of **28a** with ammonia gave 4-asq derivative **28b**, which, upon exposure to TFA, gave **6b**. Sq glutamic acid-containing **7a** and **7b** were prepared from **9b** in the same manner as **6a** and **6b**. Thus, eight analogs of PhTX and NPTX-8 were synthesized.

### *Paralytic activities of sq AA-containing analogs for crickets*

Insecticidal activity of the synthetic analogs **4–7** was examined. Crickets (*Gillus bimaculata*) were used for measuring their paralytic activities. PhTX-343 and NPTX-8 were used as the standard samples for evaluation of the activities. The potency order of the PhTX analogs **4–5** was as follows: **5b** > **5a** > **4b** ≥ **4a**. These analogs were 10–100 fold less potent than PhTX-343. Analogs bearing sq glutamic acid **5a** and




[a]Conditions: (a) $H_2$, Pd-C, MeOH, rt, 5 h; (b)12 *N* HCl, $CH_2Cl_2$, 0 °C, 1 h; (c) TFA, $CH_2Cl_2$, rt, 3 h then $Et_3N$, rt, 5 min; (d) HATU, HOAt, *i*-$Pr_2NEt$, DMF, 0 °C to rt, 20 h; (e) $NH_3$, MeOH, rt, 1 min; (f) TFA, $CH_2Cl_2$, rt, 2.5 h; (g) 1 *N* HCl, acetone, rt, 12 h.

**Scheme 4.** [a]Synthesis of NPTX analogs **6** and **7**

4-asq glutamine **5b** were slightly more potent than those of the analogs bearing sq aspartic acid **4a** and 4-asq asparagine **4b**. Replacement of the phenol moiety of PhTX to a more hydrophilic sq and 4-asq group would lead to a decrease in the potency. Among the synthetic analogs, the most hydrophobic 4-asq analog **5b** exhibited the highest activity (Table 1). These results suggest that the hydrophobic phenol moiety of the tyrosin linker of PhTX plays an important role in the paralytic activities.

On the other hand, the paralytic activity of sq amino acid-containing NPTX-8, **6** and **7**, was much more potent than those of PhTX analogs **4** and **5**. Among them, the glutamine-type **7b** was found to be the most potent analog whose activity was more than ten times

**Table 1.** $ED_{50}$ of PhTX analogs

| Entry | $ED_{50}$ ($\mu$g/g) |
|---|---|
| **PhTX-343** | 0.980 |
| **4a** | 56.40 |
| **4b** | 54.36 |
| **5a** | 34.42 |
| **5b** | 26.42 |

that of natural NPTX-8 (Table 2). Since **7a** and **7b** possessing a glutamate- or glutamine-type sq linker showed more potent activity than asparatate-type **6a** and asparagine-type **6b**, hydrophobicity, i.e., the longer chain length of the linker would be a crucial factor for the activity, while the effects of the chelation

**Table 2.** $ED_{50}$ of NPTX analogs

| Entry | $ED_{50}$ ($\mu$g/g) |
|---|---|
| **NPTX-8** | 0.535 |
| **6a** | 3.841 |
| **6b** | 2.064 |
| **7a** | 0.132 |
| **7b** | 0.041 |

with metal ions such as $Mg^{2+}$ and $Zn^{2+}$ are not clear at this stage. This is the first example which demonstrates that the squaryl group-containing amino acid derivative enhanced the activity of the natural polyamine toxin. The neuropharmacological actions of the synthetic compounds in mammalian CNS are currently being investigated in our laboratories.

## Materials and methods

All reagents and solvents were purchased from either Aldrich Chemical Company, Inc., Merck & Co., Inc., Nacalai Tesque Company, Ltd., Peptide Institute, Tokyo Kasei Kogyo Co., Ltd., or Wako Pure Chemical Industries, Ltd., and used without further purification unless otherwise indicated. Dichloromethane ($CH_2Cl_2$) was distilled from phosphoric pentaoxide ($P_2O_5$). Methanol (MeOH) was distilled from magnesium turning and iodine. Optical rotations were taken on a JASCO P-1030 polarimeter with a sodium lamp (D line). FTIR spectra were measured on a JASCO FT/IR-420 infrared spectrophotometer. $^1$H NMR spectra were recorded on either JEOL JNM-LA 300 (300 MHz), or JEOL JNM-LA 400 (400 MHz). Chemical shifts of $^1$H NMR were reported in parts per million (ppm, $\delta$) relative to $CHCl_3$ (7.26 ppm) in $CDCl_3$, $CHD_2OD$ (3.3 ppm in $CD_3OD$), or HDO ($\delta$ = 4.80 ppm) in $D_2O$. High resolution mass spectra (HRMS) were obtained on JEOL JMS-AX500 for fast atom bombardment ionization (FAB). All reactions were monitored by thin-layer chromatography (TLC), which was performed with pre-coated plates (silica gel 60 F-254, 0.25 mm thickness, manufactured by Merck). TLC visualization was accompanied using UV lamp (245 nm) or a charring solution (ethanoic *p*-anisaldehyde, ethanoic phosphomolybdic acid, aqueous potassium permanganate and butanoic ninhydrin). Daisogel IR-60 1002W(40/63 mm) was used for flash column chromatography on silica gel. Reversed phase chromatography was performed on Cosmosil 140$C_{18}$-PREP.

### *Synthesis*

*General procedure for the preparation of coupling products **21a, 22a, 28a,** or **29a***

Hydroxycyclobutenone (**9a** or **9b**: a mixture of diastereomers, 0.2~0.5 mmol), acylating reagent (butyric acid anhydride or **23**, 0.4~0.6 mmol), and 10% palladium-carbon (20~40 mg) in methanol (4 mL) was stirred under $H_2$ for 1 h. The mixture was filtered to remove the catalyst and concentrated *in vacuo* to give curde *N*-acyl compound (**17**, **24**, or their one-carbon homologs) as a pale yellow oil. The crude mixture was used for the next step without further purification. To a solution of the crude mixture in dichloromethane (2~3 mL) was added 12 *N* HCl (10~15 $\mu$L) at 0°C. The mixture was warmed to room temperature, stirred for 1.5 h, and neutralized with saturated aqueous $NaHCO_3$. The mixture was poured into water and extracted with ethyl acetate (x 2). The combined organic layer was washed with brine and dried over anhydrous $MgSO_4$, and evaporated *in vacuo* to give a crude dicarboxylate as a pale yellow oil. To a solution of the crude mixture in $CH_2Cl_2$ (2 mL) was added trifluoroacetic acid (1 mL) at 0°C. The mixture was warmed to room temperature, stirred for 1.5 h, and evaporated *in vacuo*. To a solution of the residue in dichloromethane (2 mL) was added a few drops of triethylamine under vigorous stirring. The mixture was concentrated *in vacuo* to give a crude carboxylic acid. To a solution of the residue in $CH_2Cl_2$ (2 mL) was added, successively, 1-hydroxybenzotriazole (HOBt, 2 equiv), benzotriazole-1-yloxytris(dimethyl-amino)phosphonium hexafluorophosphate (BOP, 2 equiv), and diisopropylethylamine (3 equiv) at 0°C by 2 to 3 portions. The mixture was warmed to room temperature, stirred for 17~20 h, and poured into water. The mixture was extracted by ethyl acetate (x 2). The combined organic layer was washed with aqueous $NaHCO_3$, 1 *N* hydrochloric acid, and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by silica-gel column chromatography (elution with ethyl acetate/hexane = 1:1 to 1:0) to give **21a**, **22a**, **28a**, and **29a** as a pale yellow oil.

**21a**: 32% yield from **9a**. $[\alpha]D^{22}$ = +1.62 (c 2.7, $CHCl_3$); FTIR (neat) 3319, 2974, 2933, 2874, 1797, 1753, 1682, 1593, 1527, 1418, 1366, 1167 cm$^{-1}$; $^1$H NMR (400 MHz, $CDCl_3$)$\delta$ 1.42 (d, J = 6.2 Hz, 6 H), 3.03–3.08 (m, 2 H), 3.37 (br s, 1 H), 5.17–5.24 (m, 1 H), 5.37 (sept, *J* = 6.2 Hz, 1 H), 7.26–7.42 (m, 5 H); HRMS (FAB) *m/z* calcd for $C_{39}H_{68}N_5O_{11}$ $(M+H)^+$ 782.4914, found 782.4915.

**22a**: 55 % yield from **9b**. $[\alpha]D^{22}$ = +0.226 (c 5.3, $CHCl_3$); FTIR (neat) 3306, 2974, 2933, 2873, 1795, 1752, 1688, 1593, 1531, 1418, 1390, 1366, 1167 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$)$\delta$ 7.81 (br s, 1 H), 6.52 (br s, 1 H), 5.38 (sept, *J* = 6.4 Hz, 1 H), 5.33 (br s, 1 H), 3.50–3.00 (m, 13 H), 2.75–2.50 (m, 2 H), 2.30–2.10 (m, 6 H), 2.05–1.95 (m, 2 H), 1.55–1.65 (m, 6 H), 1.50–1.45 (m, 33 H), 0.92 (t, *J* = 7.2 Hz, 3 H); HRMS (FAB) *m/z* calcd for $C_{40}H_{70}N_5O_{11}$ $(M + H)^+$ 796.5072, found 796.5081.

**28a**: 26% yield from **9a**. $[\alpha]D^{22}$ = +3.62 (c 2.9, $CHCl_3$); FTIR (neat) 3317, 2975, 2933, 2865, 1790, 1752, 1672, 1590, 1536, 1419, 1366, 1166 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$)$\delta$ 7.52 (d, *J* = 7.9 Hz, 1 H), 7.38 (d, *J* = 7.9 Hz, 1 H), 7.21 (br s, 1 H), 7.16 (t, *J* = 7.9 Hz, 1 H), 7.07 (t, *J* = 7.9 Hz, 1 H), 5.80 (br s, 1 H), 5.30 (br s, 1 H), 5.32 (sept, *J* = 6.2 Hz, 1 H), 5.05 (br s, 1 H), 3.85 (d, *J* = 14.8 Hz, 1 H), 3.72 (d, *J* = 14.8 Hz, 1 H), 3.70–3.40 (m, 2 H), 3.40–3.00 (m, 18 H), 2.70–2.35 (m, 6 H), 1.64 (m, 2 H), 1.65–1.55 (m, 2 H), 1.55–0.75 (m, H); HRMS (FAB) *m/z* calcd for $C_{50}H_{78}N_7O_{12}$ $(M + H)^+$ 968.5708, found 968.5690.

**29a**: 30% yield from **9b**. $[\alpha]D^{22}$ = +1.25 (c 2.4, $CHCl_3$); FTIR (neat) 3307, 2975, 2932, 2866, 1794, 1750, 1673, 1590, 1539, 1418, 1390, 1366, 1249, 1165 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$)$\delta$ 9.60 (br s, 1 H), 7.52 (d, *J* = 7.9 Hz, 1 H), 7.33 (d, *J* = 7.9 Hz, 1 H), 7.14 (t, *J* = 7.9 Hz, 1 H), 7.12 (s, 1 H), 7.06 (t, *J* = 7.9 Hz, 1 H), 6.85–6.70 (br s, 2 H), 5.32 (sept, *J* = 6.2 Hz, 1 H), 4.46 (q, *J* = 5.9 Hz, 1 H), 3.70 (br s, 2 H), 3.45–3.35 (m, 2 H), 3.30–2.90 (m, 14 H), 2.65–2.05 (m, 5 H), 1.90 (m, 1 H), 1.65–1.55 (m, 2 H), 1.50–1.45 (m, 33 H), 1.45–1.15 (m, 8 H); HRMS (FAB) *m/z* calcd for $C_{51}H_{80}N_7O_{12}$ $(M + H)^+$ 982.5865, found 982.5867.

*General procedure for the deprotection of **21a, 22a, 28a,** or **29a**. Synthesis of sq amino acid-containing polyamine toxin **4a, 5a, 6a,** or **7a***

To a solution of a fully protected polyamine **21a**, **22a**, **28a**, or **29a** (5~10 mg) in acetone (1 mL) was added 1 *N* HCl (1 mL). The mixture was stirred for 2 h at room temperature and evaporated

*in vacuo*. The residue was purified on Cosmosil 140$C_{18}$-PREP (elution with 0.5% $H_2O$/TFA) to **4a**, **5a**, **6a**, or **7a** as a pale yellow oil.

**4a**: 95% yield from **21a**. $[\alpha]D^{22} = -0.469$ (c 3.2, MeOH); FTIR (neat) 3271, 3043, 2968, 1779, 1675, 1562, 1468, 1429, 1202, 1132, $cm^{-1}$; $^1H$ NMR (400 MHz, $CD_3OD$)$\delta$ 4.72 (t, $J$ = 6.1 Hz, 1 H), 3.18 (t, $J$ = 7.8 Hz, 2 H), 3.36–2.85 (m, 12 H), 2.27 (t, $J$ = 7.6 Hz, 2 H), 2.11 (quint, $J$ = 7.6 Hz, 2 H), 1.90–1.70 (m, 6 H), 1.67 (dd, $J$ = 14.9 and 7.3 Hz, 1 H), 1.63 (dd, $J$ = 14.9 and 7.3 Hz, 1 H), 0.94 (t, $J$ = 7.3 Hz, 3 H); HRMS (FAB) *m/z* calcd for $C_{21}H_{38}N_5O_5$ $(M + H)^+$ 440.2873, found 440.2873.

**5a**: 95% yield from **22a**. $[\alpha]D^{22} = -0.255$ (c 4.3, MeOH); FTIR (neat) 3264, 2963, 2813, 1776, 1699, 1674, 1646, 1559, 1202, 1133 $cm^{-1}$; $^1H$ NMR (400 MHz, $CD_3OD$) $\delta$ 4.24 (dd, $J$ = 8.3 and 5.8 Hz, 1 H), 3.38 (m, 2 H), 3.18 (t, $J$ = 7.6 Hz, 2 H), 3.12–3.30 (m, 8 H), 2.63 (dd, $J$ = 15.3 and 7.6 Hz, 1 H), 2.56 (dd, $J$ = 15.3 and 7.6 Hz, 1 H), 2.51 (t, $J$ = 6.6 Hz, 2 H), 2.20–2.08 (m, 3 H), 2.02–1.80 (m, 7 H), 1.64 (dd, $J$ = 14.9 and 7.3 Hz, 1 H), 1.60 (dd, $J$ = 14.9 and 7.3 Hz, 1 H), 0.94 (t, $J$ = 7.3 Hz, 3 H); HRMS (FAB) *m/z* calcd for $C_{22}H_{38}N_5O_5$ $(M\text{-}H)^-$ 452.2873, found 452.2860.

**6a**: 50% yield from **28a**. $[\alpha]D^{22} = +1.25$ (c 5.1, MeOH); FTIR (neat) 3285, 3050, 2938, 2858, 1779, 1675, 1565, 1458, 1436, 1202, 1134 $cm^{-1}$; $^1H$ NMR (300 MHz, $D_2O$) $\delta$ 7.48 (d, $J$ = 8.0 Hz, 2 H), 7.32 (s, 1 H), 7.21 (t, $J$ = 8.0 Hz, 1 H), 7.09 (t, $J$ = 8.0 Hz, 1 H), 4.69 (dd, $J$ = 6.6 and 5.1 Hz, 1 H), 3.82 (d, $J$ = 15.8 Hz, 1 H), 3.78 (d, $J$ = 15.8 Hz, 1 H), 3.23–2.80 (m, 14 H), 2.61 (t, $J$ = 6.8 Hz, 2 H), 2.15–2.00 (m, 2 H), 1.85–1.60 (m, 4 H), 1.50–1.05 (m, 8 H). HRMS (FAB) *m/z* calcd for $C_{32}H_{48}N_7O_6$ $(M + H)^+$ 626.3666, found 626.3661.

**7a**: 97% yield from **29a**. $[\alpha]D^{22} = -0.611$ (c 3.6, MeOH); FTIR (neat) 3285, 3044, 2943, 2860, 1777, 1675, 1559, 1458, 1434, 1202, 1133 $cm^{-1}$; $^1H$ NMR (400 MHz, $CD_3OD$) $\delta$ 7.55 (d, $J$ = 7.8 Hz, 1 H), 7.37 (d, $J$ = 7.8 Hz, 1 H), 7.21 (s, 1 H), 7.12 (t, $J$ = 7.8 Hz, 1 H), 7.04 (t, $J$ = 7.8 Hz, 1 H), 4.34 (dd, $J$ = 8.6 and 5.8 Hz, 1 H), 3.75 (s, 2 H), 3.20–2.80 (m, 16 H), 2.80–2.50 (m, 2 H), 2.45 (br s, 2 H), 2.20 (m, 1 H), 2.04 (m, 2 H), 1.95 (sept, $J$ = 6 Hz, 1 H), 1.62 (br s, 2 H), 1.48–1.40 (m, 4 H), 1.30–1.20 (m, 2 H). HRMS (FAB) *m/z* calcd for $C_{33}H_{50}N_7O_6$ $(M + H)^+$ 640.3823, found 640.3836.

*General procedure for the synthesis of 4-asq amino acid-containing polyamine toxin **4b, 5b, 6b, or 7b***

Ammonia gas was bubbled into a solution of **21a**, **22a**, **28a**, or **29a** (5~10 mg) in methanol (2 mL) for 1 min. The mixture was evaporated *in vacuo*. To a solution of the resulting 4-asq compound **21b**, **22b**, **28b**, or **29b** in acetone (1 mL) was added TFA (1 mL). The mixture was stirred for 2~3 h and evaporated *in vacuo*. The residue was purified on Cosmosil 140$C_{18}$-PREP (elution with $H_2O$) to give **4b**, **5b**, **6b**, or **7b** as a pale yellow oil.

**4b**: 95% yield from **21a**. $[\alpha]D^{22} = -0.085$ (c 3.5, MeOH); FTIR (neat) 3271, 3068, 2874, 1791, 1733, 1674, 1576, 1559, 1436, 1201, 1134 $cm^{-1}$; $^1H$ NMR (400 MHz, $CD_3OD$)$\delta$ 4.71 (t, $J$ = 6.8 Hz, 1 H), 3.31–3.29 (m, 2 H), 3.14 (t, $J$ = 7.6 Hz, 2 H), 3.10–2.95 (m, 10 H), 2.23 (t, $J$ = 7.4 Hz, 2 H), 2.09 (br quint, $J$ = 7.1 Hz, 2 H), 1.86 (br quint, $J$ = 7.1 Hz, 2 H), 1.85–1.72 (m, 4 H), 1.64 (dd, $J$ = 14.9 and 7.6 Hz, 1 H), 1.60 (dd, $J$ = 14.9 and 7.6 Hz, 1 H), 0.92 (t, $J$ = 7.3 Hz, 3 H); HRMS (FAB) *m/z* calcd for $C_{21}H_{38}N_6O_4$ $(M + H)^+$ 439.3033, found 439.3041.

**5b**: 97% yield from **22a**. $[\alpha]D^{22} = -0.882$ (c 1.7, MeOH); FTIR (neat) 3267, 2967, 2857, 1790, 1672, 1578, 1455, 1201, 1134 $cm^{-1}$; $^1H$ NMR (300 MHz, $CD_3OD$)$\delta$ 4.22 (dd, $J$ = 8.8 and 4.2 Hz, 1 H), 3.35–3.25 (m, 2 H), 3.20–2.96 (m, 10 H), 2.67 (t, $J$ = 7.4 Hz, 2 H), 2.24 (t, $J$ = 7.4 Hz, 2 H), 2.17 (m, 1 H), 2.07–1.98 (m, 3 H), 1.80–1.70 (m, 6 H), 1.64 (dd, $J$ = 14.8 and 7.3 Hz, 1 H), 1.59 (dd, $J$ = 14.8 and 7.3 Hz, 1 H), 0.94 (t, $J$ = 7.3 Hz, 3 H); HRMS (FAB) *m/z* calcd for $C_{22}H_{41}N_6O_4$ $(M + H)^+$ 453.3190, found 453.3198.

**6b**: 69% yield from **28a**. $[\alpha]D^{22} = +1.55$ (c 2.0, MeOH); FTIR (neat) 3296, 3068, 2930, 2855, 1789, 1672, 1577, 1432, 1202, 1134 $cm^{-1}$; $^1H$ NMR (400 MHz, $CD_3OD$) $\delta$ 7.47 (d, $J$ = 8.0 Hz, 1 H), 7.35 (d, $J$ = 8.0 Hz, 1 H), 7.20 (s, 1 H), 7.10 (t, $J$ = 8.0 Hz, 1 H), 6.98 (t, $J$ = 8.0 Hz, 1 H), 4.82 (dd, $J$ = 7.6 and 6.0 Hz, 1 H), 3.75 (d, $J$ = 14.8 Hz, 1 H), 3.71 (d, $J$ = 14.8 Hz, 1 H), 3.25–2.80 (m, 18 H), 2.56 (t, $J$ = 6.6 Hz 2 H), 2.07 (quint, $J$ = 7.8 Hz, 2 H), 1.66 (m, 4 H), 1.50–1.25 (m, 6 H), 1.19 (quint, $J$ = 7.8 Hz, 2 H); HRMS (FAB) *m/z* calcd for $C_{33}H_{50}N_7O_6$ $(M + H)^+$ 639.3982, found 639.3964.

**7b**: 94% yield from **29a**. $[\alpha]D^{22} = +0.067$ (c 5.9, MeOH); FTIR (neat) 3296, 3079, 2943, 2860, 1790, 1675, 1576, 1457, 1202, 1134 $cm^{-1}$; $^1H$ NMR (400 MHz, $CD_3OD$) $\delta$ 7.56 (d, $J$ = 7.8 Hz, 1 H), 7.36 (d, $J$ = 7.8 Hz, 1 H), 7.22 (s, 1 H), 7.12 (t, $J$ = 7.8 Hz, 1 H), 7.02 (t, $J$ = 7.8 Hz, 1 H), 4.32 (dd, $J$ = 8.8 and 5.4 Hz, 1 H), 3.75 (s, 2 H), 3.20–2.90 (m, 14 H), 2.91 (m, 1 H), 2.67–2.57 (m, 2 H), 2.51 (t, $J$ = 7.6 Hz, 2 H), 2.18 (m, 1 H), 2.10–1.95 (m, 2 H), 1.70 (br s, 4 H), 1.48–1.37 (m, 4 H), 1.32–1.18 (m, 2 H). HRMS (FAB) *m/z* calcd for $C_{33}H_{50}N_7O_6$ $(M + H)^+$ 639.3982, found 639.3964.

*Assays of paralysis activities*

Crickets (*Grillus bimaculatus*, 120~200 mg) were injected intrathoracically between the second and third pair of legs, with 1~5 $\mu$L of different doses of the polyamine toxins, using a 10 $\mu$L microsyringe. $ED_{50}$ values represent the effective dose to paralyze 50% of treated crickets at 10 minutes post-injection. The criterion for paralysis was the inability of crickets to upturn when they were placed on their back. The $ED_{50}$ values were obtained by probit analysis (Finney, 1962) of the data from four to six groups of 10 crickets each.

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