Search for Neuroprotective Substances from Natural Resources

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Introduction

Excitatory amino acids are known to induce considerable neurotoxicity in central nervous system under abnormal conditions. It has been demonstrated that the glutamatergic system was involved in the development of neuronal cell death followed by a variety of traumas including ischemia, hypoglycemia and epilepsy[1,2] and certain neurodegenerative diseases such as Huntington's chorea, Parkinson's disease, Alzheimer's disease and AIDS neuropathology[3~6]. The glutamatergic system is classified as two main categories depending on the agonist preference of the postsynaptic receptor system. *N*-methyl-D-aspartate (NMDA) is one major ionotropic receptor, whereas the other comprises kainate and -amino-3-hydroxy-5-methyl-isoxasol-4-propionic acid (AMPA) receptors. Although the mechanisms leading to nerve cell death are largely unknown, there is an overwhelming amount of literature on the importance of calcium level in cells which is elevated by the activation of NMDA receptors. It has also been known that the AMPA/kainate receptor induces the Ca^{2+} influx directly through the Ca^{2+} permeable receptors and mediates part of the excitotoxicity in the central nervous system (CNS). Kainate is a powerful neurotoxin that produces selective neuronal damage in CNS and is employed as a tool to destroy postsynaptic elements in brain with preserving presynaptic structures[7]. Kainate preferentially destroys the CA3/CA4 hippocampal formation upon intraventricular administration to rat.

NMDA antagonists had been designed for treatment of acute onset neurodegeneration such as stroke and trauma and recently tried for clinical application[8]. Unfortunately, the hopes for application of NMDA antagonists in patients so far were limited by unacceptable side effects[9~12]. The next generation of glutamate antagonists, the kainate/AMPA antagonists,

turned out to be effective in reducing ischemic damage in rats subjected to global ischemia even when administered several hours after the ischemic insult[13,14].

During the screening for neuroprotective compounds against glutamate- and kainate-induced neurotoxicity using chick telencephalic and mouse cortical cell cultures, novel substances, designated as neuroprotectins A-C and curtisians A-D, have been isolated from natural resources. In this symposium, their chemical structures and biological activities will be presented.

Materials and Methods

General Experimental Procedures

Melting points were measured with a Yanaco MP-S3 micro melting point apparatus and were uncorrected. Specific optical rotations were determined with a Jasco DIP-371 digital polarimeter. UV-visible and IR spectra were recorded on Shimadzu UV-300 and Jasco A-102 spectrophotometers, respectively. Mass and high-resolution mass spectra were measured with a JEOL HX-110 spectrometer in the FAB mode, using glycerol matrix with polyethylene glycol as the internal standard. TLC plates (silica gel 60 F_{254}), ODS-TLC plates (RP-18 F_{254} s) and silica gel (Kieselgel 60, 70-230 mesh) were purchased from Merck Company. ODS-TLC was developed with 50 mM citrate buffer (pH 6.0) and acetonitrile (6:4). HPLC was carried out using a Cosmosil C18 column (ϕ4.6$\times$150 mm) eluted with 45% aq. acetonitrile/0.04% TFA. The flow rate was 0.8 ml per minute and the UV absorption of the eluate was monitored at 220 nm.

Culture of Chick Telencephalic Neuron and Neuroprotective Activity

Dissociated telencephalic neurons were prepared from brains of 5 day-old-chick embryos according to the method of Taguchi *et al*[15]. The telencephalic regions of chick embryos were dissected out in Ca^{2+} and Mg^{2+}-free phosphate buffer saline (pH 7.4) supplemented with 10 mM glucose. The removed tissues were treated with 0.0125% trypsin in the same medium for

30 minutes at 37°C. The tissues were then rinsed and saturated with 2-fold diluted Eagle's minimum essential medium supplemented with 10 mM glucose, 2 mM glutamine, 2 mM $CaCl_2$, 25 mM HEPES, 60 mM NaCl, 1 mM sodium pyruvate, 50 nM Na_2SeO_3, 0.1 mM choline-Cl, 0.1 mM inositol, 5 μg/ml insulin, 20 μg/ml ovotransferin, 20 μM progesterone, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The tissues were then dissociated in one ml of the same medium added with 0.2 mg DNase I. The dissociated neurons were adjusted at a density of 5×10^5 cells and plated in a volume of 200 μl per each well of 48-well microplates. These neurons were then cultured at 37°C in 5% CO_2 and in saturated humidity. After five days of culture, excitotoxicants and /or neuroprotectins were added into the culture medium. Neuroprotective activity was determined by the neuronal viability estimated by the MTT method at one day for kainate and two days for glutamate after initiation of exposure to glutamate/neuroprotectins or kainate/neuroprotectins. MTT tetrazolium salt (0.7 mg/ml) was added to neurons grown in 48-well microplates followed by incubation for 4 hours at 37°C in 5% CO_2 and in saturated humidity. The reaction medium was gently aspirated, washed with PBS buffer and then isopropanol containing 0.1 M HCl was added to solubilize the blue formazan product. Neuronal viability was estimated by quantifying soluble formazan using a Bio-Rad microplate reader at 540 nm. The activity was also confirmed by counting the number of survived neuronal cells.

Culture of mouse cortical Neuron and Neuroprotective Activity

Mouse cortical cell cultures were established from gestation day 15 ICR mouse embryos. The cortices were incubated in 0.8 mg trypsin/ml HBSS (Hanks' balanced salts solution) for 7 min and then rinsed three times in 10 ml of HBSS. Cells were then centrifuged at 1500 rpm (450 g) for 5 min. and after carefully remove media, resuspended the pellet by trituration through the narrowed bore of a fired-polished pasteur pipette in B27-supplemented neurobasal medium. The cortical cells were plated on 48-well plates (1×10^6 cells/dish) precoated with 100 ㎍/㎖ poly-D-lysine. The cortical cells were grown in neurobasal medium

containing B27- supplement, L-glutamine (final conc. 0.5 mM), 100 units/ml penicillin, and 100 ㎍/㎖ streptomycin at 37℃ in a humidified atmosphere of 5% CO2. Cultures were allowed to mature for 12 days before they were used for experiments. Neurobasal medium, which is derived from Dulbecco's Modified Eagle's Medium, was optimized for cell survival and the almost complete absence of glial cells.54) To assess neuroprotective activity, previously cortical cell cultures were replaced with neurobasal medium without supplement. Test compound was dissolved in methanol (final concentration, 1%). Cultures were pretreated with compounds for 1 h before exposed to 20 μM glutamate, 80 μM AMPA, 40 μM NMDA, and 200 μM kainate for excitotoxic injury and 50 μM H2O2 for oxidative injury, respectively. The cultures were then maintained for an additional 24 h in neurobasal medium. Neuronal viability was measured by the MTT assay which reflects mitochondrial enzyme function. MTT tetrazolium salt (1 mg/ml) was added to neurons grown in 48-well microplates followed by incubation for 1 h at 37℃ in 5% CO2 and in saturated humidity. The reaction medium was gently aspirated, and then DMSO was added to solubilize the blue formazan product. Neuronal viability was estimated by quantifying soluble formazan using a microplate reader at 540 nm. Cell viability was calculated as 100 × (OD of neurotoxins + compound-treated - OD of neurotoxins-treated) / (OD of control - OD of neurotoxins-treated).

Results and Discussion

Isolation of Neuroprotectins and Curtisians

Neuroprotectins A and B were isolated from the culture broth of *Streptomyces* sp. 60910. In brief, the cultured broth was centrifuged to give supernatant and mycelial cake. The supernatant was adjusted to pH 4.0 with 1N HCl and then partitioned between EtOAc and water. The EtOAc layer was concentrated *in vacuo* and the dried residue was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (10:1~1:4) to afford an active fraction. Active fraction was purified by ODS column chromatography in citrate buffer

(50 mM, pH 6.0) with increasing amounts of acetonitrile and by a column of MCI gel eluting with MeOH, followed by Sephadex LH-20 column chromatography to afford neuroprotectins A-B.

Curtisians A-D were isolated from the methanolic extracts of the fruiting body of *P. curtisii* (4.5 g, dry weight). The extract was concentrated *in vacuo*, and the concentrate was chromatographed on a column of silica gel eluting with $CHCl_3$ only and a mixture of $CHCl_3$-MeOH (10:1). The active fraction ($CHCl_3$-MeOH mixture) was concentrated *in vacuo* and then subjected to a column of Sephadex LH-20 eluted with $CHCl_3$-MeOH (2:1). The active eluate from the column was concentrated *in vacuo*, applied on preparative silica gel TLC plate and developed with a mixture of $CHCl_3$-MeOH (10:1). Two bands of crude curtisians with Rf values of about 0.5 and 0.35 were scraped out from a TLC plate. The compound from the band of Rf 0.5 was finally purified by reverse-phase HPLC (ODS column, 50% aq. CH_3CN) to give curtisians A (1.1 mg) and B (15 mg) with retention time of 19 and 35 min, respectively. The another band of Rf 0.35 was purified by reverse-phase HPLC (50% aq. CH_3CN) to give curtisians C (5.4 mg) and D (14.4 mg) showing the retention time of 18.5 and 26.5 min, respectively.

Physico-Chemical Properties of Neuroprotectins and Curtisians

Neuroprotectins were obtained as a yellowish brown solid. The molecular weights of neuroprotectins A and B were determined to be 1341 and 1357, respectively, by the FAB-mass measurements in the negative mode. Their molecular formulae were established as $C_{61}H_{45}N_7O_{16}Cl_6$ and $C_{61}H_{45}N_7O_{17}Cl_6$, respectively, by high-resolution FAB-mass analyses in combination with NMR spectral data. Bathochromic shifts in the UV spectra of these two compounds by the addition of alkali suggested the presence of phenolic hydroxyl functional groups in their molecules. IR absorptions at 3360 ~ 3380 and 1660 cm^{-1} implied the presence of hydroxyl and/or amide NH and amide carbonyl functions, respectively.

Curtisian A was obtained as a yellow solid while curtisians B, C, and D were obtained as a dark brown solid. Curtisians were readily soluble in dimethylsulfoxide (DMSO) and methanol, slightly soluble in $CHCl_3$ and insoluble in hexane and water. The UV absorptions of curtisians near 267 nm suggested the presence of aromatic functions in their structures. Their common IR absorption bands at 3450-3430, near 1775 and near 1740 cm^{-1} suggested the presence of hydroxyl group, carbonyl groups derived from phenyl alkanoate moiety and other carbonyl groups, respectively. The molecular formulae of curtisians A-D were determined to be $C_{31}H_{24}O_{10}$ (*m/z* 579.1254 $(M+Na)^+$ -1.3 mmu), $C_{33}H_{28}28O_{10}$ (*m/z* 584.1669 $(M+H)^+$ -1.3 mmu), $C_{36}H_{38}O_{15}$ (*m/z* 733.2112 $(M+Na)^+$ +0.4 mmu) and $C_{35}H_{32}O_{11}$ (*m/z* 651.1851 $(M+Na)^+$ +0.9 mmu), respectively, by high-resolution FAB mass spectroscopy in combination with ^1H and ^{13}C NMR spectra.

Structure Determination of Neuroprotectins and Curtisians

The structures of neuroprotectins A and B were determined to be aromatic peptides by various one- and two-dimensional NMR experiments including ^1H NMR, ^{13}C NMR, DEPT, DQF-COSY, HMQC and HMBC. In the ^1H NMR spectra measured in DMSO-d_6 or CD_3OD, nine exchangeable protons were observed in the down-field region due to hydroxyl or amide protons which were quenched by the addition of D_2O. Eighteen aromatic methine protons appeared in aromatic region in addition to *meta*-coupled aromatic protons. Furthermore, six methine protons assignable to α-methine protons of peptides were observed together with two methylene protons and *N*-methyl protons at 2.99 ppm. The ^{13}C NMR and DEPT spectra revealed the presence of a methyl, two methylenes, 7 sp^3 methines and 19 sp^2 methines, 16 sp^2 quaternary carbons, 7 oxygenated sp^2 quaternary carbons and 9 carbonyl carbons. The HMQC spectrum revealed the one-bond ^1H-^{13}C connectivities and the presence of five symmetrical aryl methines.

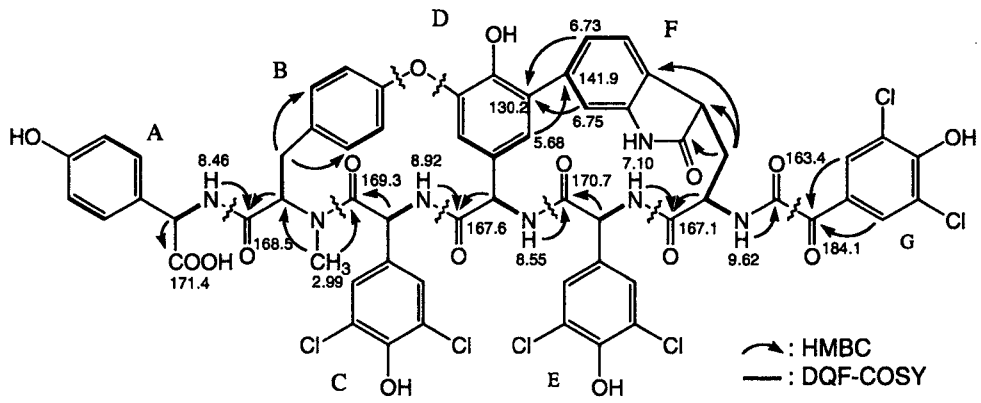
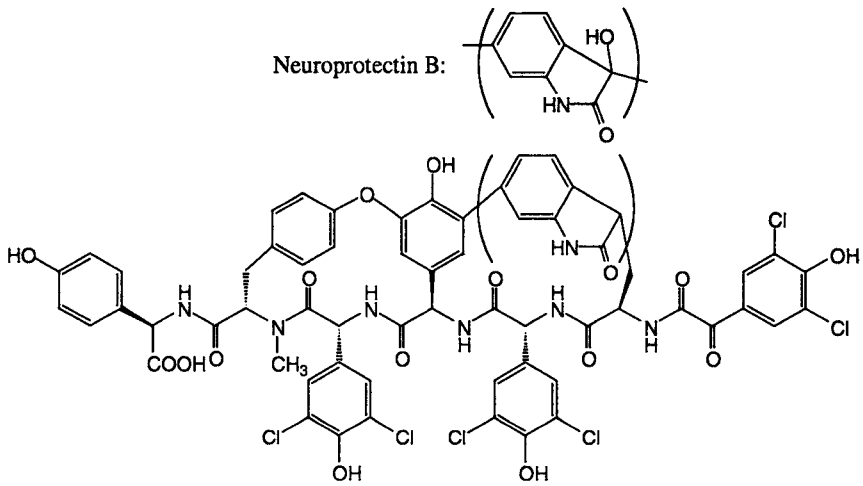


Fig. 1. Partial structures of neuroprotectin A elucidated by COSY and HMPC experiments



Neuroprotectin A

Fig. 2. Structures of neuroprotectins A and B.

The partial structures A to G consisting of amino acid residues were established by the DQF-COSY and HMBC experiments, as shown in Fig. 1. The 3,5-dichloro-4-hydroxyphenyl moieties assignable to partial structures C, E and G, and an oxindoline residue F were

confirmed by comparison of the ^{13}C chemical shifts with those of complestatin and 3-methyloxindoline. These chemical shift values for neuroprotectin A were in good agreement with those of the corresponding carbons of the 3,5-dichloro-4-hydroxyphenyl moiety and 3-methyloxindoline. These partial structures were connected by detailed analysis of the HMBC spectrum, which showed the correlations between the carbonyl carbon and amide proton of the neighboring amino acid. Non-equivalent proton and carbon chemical shifts of 1,4-disubstituted benzene for the partial unit B suggested that the partial units B and D should be bridged by an ethereal linkage as shown in the cases of complestatin and kistamicins. Finally, the remaining subunit G was deduced to be connected to the amide carbonyl carbon at 163.4 ppm. Thus, the structure of neuroprotectin A was determined as shown. The chemical structure of Neuroprotectin B was also assigned as shown in Fig. 2 by the same method as that of neuroprotectin A.

The ^{1}H NMR spectrum of curtisian A revealed the signals attributable to twenty two protons including thirteen aromatic protons originated from two 1,4-disubstituted benzenes and one phenyl, and three methyl protons of the acetyl group. The peaks observed in the ^{13}C NMR spectrum of curtisian A were seriously overlapped, suggesting that this compound has symmetrical structure. Direct ^{1}H-^{13}C connectivity was established by HMQC spectrum and further structural information was obtained by HMBC experiment. The *p*-terphenyl structure for curtisian A was suggested by the NOE experiment, which showed no NOE correlation between the protons of two *p*-hydroxyphenyl groups. It also should be noted that *p*-terphenyl moieties are ubiquitous among mushroom metabolites. Therefore, curtisian A was assigned as a *p*-terphenyl compound with the substituents of three acetyls and one benzoyl, as shown in Fig. 3. The structures of curtisians B, C, and D were also determined by the same method as that of curtisian A.

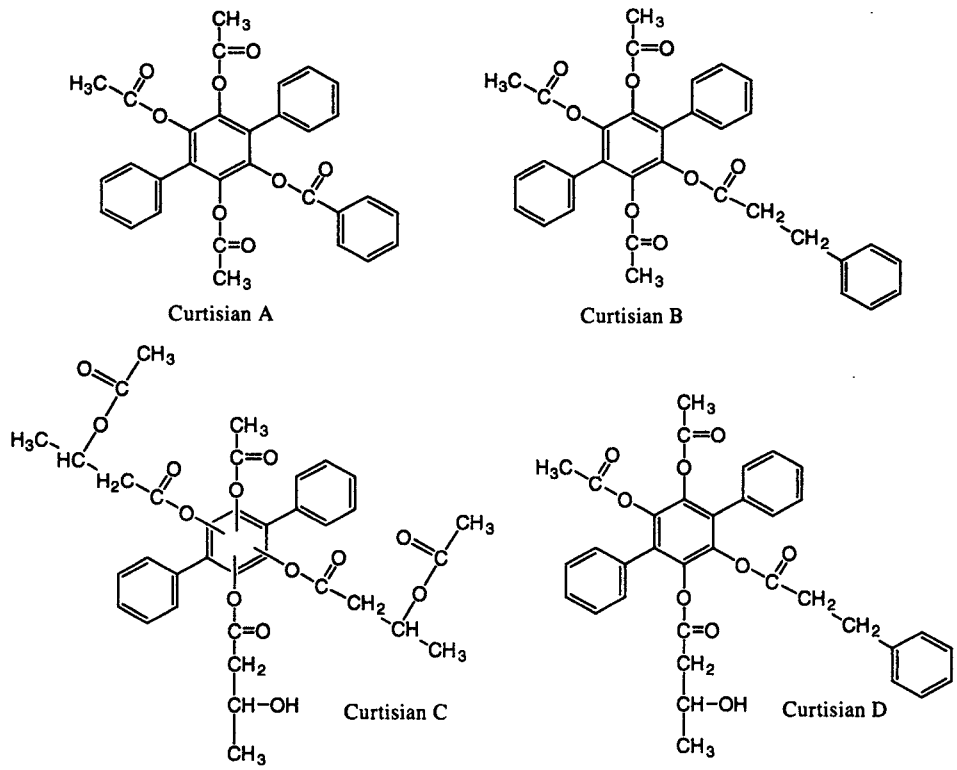


Fig. 3. Structures of curtisians A, B, C, and D.

Biological Activities of Neuroprotectins and Curtisians

The biological activity of neuroprotectins to protect neuronal cells from excitotoxicity was estimated by observing primary cultured chick telencephalic neurons upon treatment of excitatory neurotoxins. Exposure of 400 μM kainate in dissociated cell culture of chick telencephalic neurons resulted in neuronal cell death within 24 h. Kainate-induced neuronal degeneration, however, was completely blocked by the addition of 0.5 μM neuroprotectins in a dose-dependent fashion. The ED_{50} values of neuroprotectins A, B, complestatin and a non-NMDA antagonist DNQX against kainate-induced neurotoxicity was 0.21, 0.24, 0.14 and 2.27 μM, respectively, and neuroprotectins showed about ten-times higher activity than that of DNQX used as control. Neuroprotectins did not exhibit the antioxidative activity at concentrations effective to inhibit neuronal degeneration. This result implied that the neuroprotective activity of these compounds was not based on the antioxidant effect. In

addition, we investigated the receptor-binding affinity of neuroprotectin A with glutamate receptor subtypes. Although neuroprotectins completely blocked neuronal degeneration induced by glutamate, they did not show any significant affinity to known NMDA and non-NMDA binding sites. Detailed biological activities will be presented.

Curtisians protected mouse cortical neurons from glutamate-induced toxicity in a dose dependent manner. Of the glutamate receptor subtypes, curtisians were found to block NMDA receptor-mediated but not AMPA/kainate mediated cell death. In addition, curtisians exhibited potent antioxidative activity against hydroxyl radicals that were generated by the addition of H_2O_2, and lipid peroxidation in rat liver microsome, but no activity was detected in the superoxide, DPPH and ABTS radical scavenging systems, and in protection of N18-RE-105 cells subjected to glutamate-induced glutathione depletion. Curtisians only showed biological activity against oxidative damage caused by the iron-mediated Fenton reaction, which suggests that curtisians function as iron chelators. In iron chelation testing, based on DNA single strand breakage (SSB) induced by the addition of iron and H_2O_2, curtisians prohibited DNA SSB like iron chelator desferrioxamine. These results suggest that the neuroprotective action of *p*-terphenyl curtisians is dependent on their ability to chelate iron, and that in this context they may be useful as neuroprotective agents against neurological disorder, which result in neuronal cell death.

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