Studies on the Constitution of Edible and Medicinal Plants. I. Isolation and Identification of 4-O-Methylpyridoxine, Toxic Principle from the Seed of *Ginkgo biloba* L.

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A toxic substance, 4-O-methylpyridoxine, responsible for "gin-nan food poisoning" was isolated from seeds of *Ginkgo biloba* L. (Ginkgoaceae). It is proposed that the toxic principle causes food poisoning through not only antagonizing vitamin B_6 in the body but also inhibiting the formation of 4-aminobutyric acid from glutamate in the brain.

"Gin-nan" is the seed of *Ginkgo biloba* L. (maidenhair tree, Ginkgoaceae), and its albumen is used as a crude drug and food in China and Japan. In particular, it is used as an antitussive and expectorant in folk medicine. However, when this substance has been taken to excess during food shortages, "gin-nan food poisoning" has sometimes occurred in Japan (1930—1960, about 70 reports)[1] and in China.[2] The cardinal symptoms of this poisoning are mainly convulsions and loss of consciousness, and infants are particularly vulnerable. The sequela is not serious in survivors, but lethality is about 27% in Japan.

Although several investigations have been undertaken to elucidate the cause of this food poisoning,[1] it remained to be clarified.[3] This paper describes our finding that 4-O-methylpyridoxine isolated from *G. biloba* is the toxic substance that causes gin-nan food poisoning.[4]

The albumen of Ginkgo seeds was dried at 40 °C for 7 d and then ground. The powder was extracted with water at 4 °C for 2 d. The extract was treated as follows. An outline of the procedure is shown in Chart 1.

The aqueous extract was filtered and centrifuged. A large portion of this precipitate was starch. The toxicity of the supernatant was evaluated by oral acute toxicity tests using guinea pigs (about 300 g) of either sex. From later stages of the separation, test samples were dissolved or suspended in water at appropriate concentrations. Signs of toxicity were paralysis of legs, opisthotonus, clonic convulsions, and auditory hyperalgesia. All these signs were closely similar to those seen in humans, and occurred within 30—40 min after administration of the toxic fractions except for the first sample, which was fractionated at the first stage (2—3 h).

After removal of the precipitated proteinous substances, fractionations were carried out by partitioning between various organic solvents [petroleum ether, ethyl acetate (AcOEt), and n-butyl alcohol (BuOH)] and water. The obtained toxic fraction (BuOH layer) was further separated into two fractions by alumina column chromatography [eluent: $CHCl_3$–methyl alcohol (MeOH), 19:1, v/v]. When the first fraction was given to the guinea pigs, it induced

dried albumen (*Ginkgo biloba* L.)
| H_2O
filtrate
| centrifuged, 2000 rpm, 10 min
┌──────────────────────────┴──────────────────────────┐
precipitate [−]   supernatant [+: 38 ml/kg]
                                              | 1) 80°C, 1 h
                                              | 2) precipitated with EtOH
                              ┌───────────────┴───────────────┐
residue [−]   soluble portion [+: 4.3 g/kg]
                                              | 1) concentrated
                                              | 2) petroleum ether
                    ┌─────────────────────────┴─────────────────────────┐
p. ether layer [−]   aqueous layer [+: 2.9 g/kg]
                                              | AcOEt
              ┌───────────────────────────────┴───────────────────────────────┐
AcOEt layer [−]   aqueous layer [+: 1.1 g/kg]
                                              | BuOH
        ┌─────────────────────────────────────┴─────────────────────────────────────┐
aqueous layer [−]   BuOH layer [+: 190 mg/kg]
                                              | Alumina column chromatog.
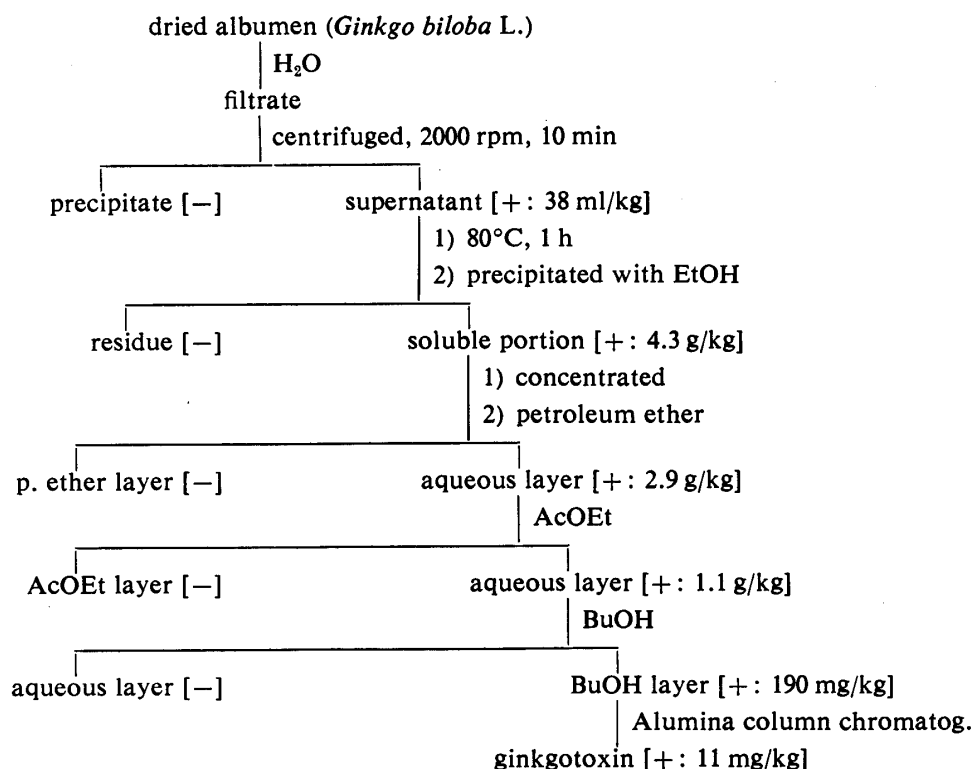   ginkgotoxin [+: 11 mg/kg]

Chart 1. Isolation of Ginkgotoxin from *G. biloba*

[+: ml, g, or mg/kg] indicates toxic fraction and dose. (Typical toxic symptoms occurred at this dose.)

[−] indicates nontoxic fraction. (In this fraction, no typical symptoms occurred when a dose greater than that of the corresponding toxic fraction was administered.)

the characteristic convulsions at an oral dose of only 11 mg/kg. The other fraction [149 mg/kg] did not show toxicity.

These isolation procedures afforded the "ginkgotoxin" (tentative name) in *ca.* 0.01% yield (dry weight). Ginkgotoxin had the molecular formula $C_9H_{13}NO_3$ [m/z 183.089 (M^+); $C_9H_{13}NO_3$ requires 183.089], and showed positive reactions with ferric chloride solution, and with Fast Blue B Salt (diazonium reagent) indicating that it is a hydroxypyridine.[5]

Its ^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra[6] showed the presence of an aromatic methyl group [2.42 (3H, s, C_2-C$\underline{H}_3$) and 18.4 (q, C_2-$\underline{C}H_3$)], a methoxy group [3.44 (3H, s, C_4-CH$_2$OC$\underline{H}_3$) and 58.8 (q, C_4-CH$_2$O$\underline{C}H_3$)], two methylene groups (–CH$_2$O–) [4.63 and 4.76 (each 2H, each s, C_4-C$\underline{H}_2$O– and C_5-C$\underline{H}_2$O–), 60.4 and 67.9 (each t, C_5-$\underline{C}H_2$O– and C_4-$\underline{C}H_2$O–)], an aromatic proton [7.90 (1H, s, C_5-H)], and a tetrasubstituted pyridine ring [138.8 (d, C_6), 131.3, 135.2, 147.6, and 152.4 (each s, C_5, C_4, C_2, and C_3)].

Two reasonable structures of ginkgotoxin, 4- or 5-*O*-methylpyridoxine, could be considered based on the above chemical reactions and spectroscopic data. As ginkgotoxin coupled with 2,6-dichloroquinonechloroimide (Gibbs reagent) in the presence of borate,[7] it was concluded to be 4′-substituted (*i.e.* 4-*O*-methyl-) pyridoxine (MPN). The mixed melting point with authentic 4-*O*-methylpyridoxine hydrochloride (mp 181 °C)[8] did not show depression. Ginkgotoxin was thus identified as 3-hydroxy-5-hydroxymethyl-4-methoxymethyl-2-methylpyridine (Chart 2).

Synthetic MPN is known to be a potent convulsive agent having antivitamin B_6 activities in man and in a variety of experimental animals such as mice, rats, cats, dogs, and monkeys.[9] It has already been proved that these convulsions caused by MPN can be prevented or stopped with pyridoxine (vitamin B_6).[10] This suggests that pyridoxine will prevent the

CH₂OR¹ structure with ginkgotoxin/pyridoxine labels
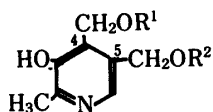
ginkgotoxin (MPN): $R^1 = CH_3$, $R^2 = H$
pyridoxine (vitamin B_6): $R^1 = R^2 = H$

Chart 2

symptoms of gin-nan food poisoning.

MPN is also known to inhibit the formation of 4-aminobutyric acid (GABA) from glutamate in the brain. MPN may compete with vitamin B_6 which serves as a coenzyme of glutamate decarboxylase.[11] As GABA is regarded as an inhibitory chemical transmitter, a deficiency of GABA in the brain may induce the seizures.[11] Therefore, it is reasonable to speculate that MPN in the seeds of *G. biloba* might induce convulsions through the same mechanism. The lack of vitamin B_6 during times of food shortage also may be one of the causes of "gin-nan food poisoning."

This is the first report of the isolation of MPN from a natural source.

Experimental

Melting points were taken on a Shimadzu MM-2 micro melting point apparatus and are uncorrected. The mass spectra (MS) were measured with a JEOL JMS-OISG-2 mass spectrometer. The ultraviolet (UV) spectrum was recorded with a Shimadzu UV-200 double beam spectrophotometer. The ^1H- and ^{13}C-NMR spectra was recorded with a JEOL FX-200 spectrometer in CD_3OD using tetramethylsilane as an internal standard. Alumina column chromatography was carried out on Aluminium oxid 90 (activity II—III, Merck).

Isolation of MPN from the Seeds of *Ginkgo biloba* L.——The albumen of Ginkgo seeds (4.5 kg) was dried at 40 °C for 7 d and then ground. The dried powder (2 kg) was extracted with distilled water (2.5 l) at 4 °C for 2 d. The precipitated starch was filtered off and centrifuged at 2000 rpm for 10 min. The supernatant (545 ml) was heated at 80 °C for 1 h on a water bath, and then EtOH (1650 ml) was added to the supernatant. The resulting precipitate was filtered off and the EtOH solution was evaporated to dryness on a water bath. The obtained residue (10.66 g) was dissolved in water (50 ml) and partitioned between water and petroleum ether (60 ml × 3), ethyl acetate (50 ml × 3), and butyl alcohol (BuOH, 50 ml × 3) successively. The BuOH layer was evaporated to dryness (320 mg). The resulting residue was chromatographed on alumina (30 g; solvent, $CHCl_3$–MeOH, 19 : 1, v/v), and two fractions were obtained. The first fraction (190 mg) contained the toxicity.

Ginkgotoxin (4-*O*-Methylpyridoxine, MPN)——Colorless oil. High-MS m/z: 183.089 (Calcd for $C_9H_{13}NO_3$: 183.089). MS m/z: 183 (M^+), 165 ($M^+ - H_2O$), 151 ($M^+ - CH_3OH$). FD-MS m/z: 184 ($M^+ + H$), 183 (M^+). UV $\lambda_{max}^{H_2O}$ nm (log ε): 250 (7.82), 329 (8.49). ^1H-NMR δ: 2.42 (3H, s), 3.44 (3H, s), 4.63 (2H, s), 4.76 (2H, s), 7.90 (1H, s). ^{13}C-NMR δ: 18.4 (q), 58.8 (q), 60.4 (t), 67.9 (t), 131.3 (s), 135.2 (s), 138.8 (d), 147.6 (s), 152.4 (s). Ginkgotoxin hydrochloride: colorless prisms from MeOH–acetone, mp 179—180 °C.

Animals——The studies were performed with Hartley guinea pigs (250—350 g) of either sex. Guinea pigs having the same body weight within 10% were utilized in the toxicity test at each isolation stage.

Samples for Toxicity Tests——Samples were dissolved or suspended in distilled water in appropriate concentrations at each isolation stage (see Chart 1).

Procedure of Toxicity Tests——The bioassay shown in Chart 1 was carried out as follows. For the studies with oral administration, guinea pigs were deprived of food for 12 h before toxicity tests. Water was available *ad libitum*. The administration volume of test sample was less than 6 ml/kg (except for the first isolation stage: 38 ml/kg). One test series for evaluation of acute toxicity extended over a period of 2—7 d, or sometimes longer.

Each evaluation consisted of 3 animals or more for each fraction. The items of observation were paralysis of legs, opisthotonus, clonic convulsions, and auditory hyperalgesia. These symptoms occurred within 30—40 min after administration of toxic fractions (except for the first extraction stage: 2—3 h).

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