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Re: Expediente No. 07.112.258

Solicitud de patente a nombre de **DR. WILLMAR SCHWABE GMBH & Co., KG.**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **DR. WILLMAR SCHWABE GMBH & Co., KG.**, domiciliada en Karlsruhe, Alemania, solicitante de la patente de la referencia, en respuesta al oficio No. 06017 notificado por fijación en lista el 18 de abril de 2012, nos permitimos informar lo siguiente:

Que atentamente presentamos ante ese Despacho copia informal del siguiente documento:

- ANSGAR ARENZ et al: "Occurrence of Neurotoxic 4'-O-methylpyridoxine in Ginkgo Biloba Leaves, Ginkgo Medications and Japanese Ginkgo Food", (1996), Planta Medica. Vol. 62, NR, 6 page(s) 548-551.

Con la presentación de estos documentos damos cabal cumplimiento al requerimiento realizado por esa oficina mediante el Artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina.

Señor Superintendente,

DILIA MARIA RODRIGUEZ D'ALEMAN

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Occurrence of Neurotoxic 4'-O-Methylpyridoxine in *Ginkgo biloba* Leaves, *Ginkgo* Medications and Japanese *Ginkgo* Food

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Abstract: 4'-O-Methylpyridoxine (ginkgotoxin) is a neurotoxic antivitamin B₆ which occurs in *Ginkgo biloba* L. seeds. Contrary to a previous report by Wada et al. (15), the toxin was also detected in *Ginkgo biloba* leaves. The leaves are a source of extracts (e.g. EGb761) employed in the preparation of *Ginkgo* medications. Consequently the toxin is also present in *Ginkgo* medications and is even detectable in homeopathic preparations. The toxin occurs also in boiled Japanese *Ginkgo* food. However, the amount of the toxin is likely to be too low to exert a detrimental effect after administration of the medication or ingestion of food.

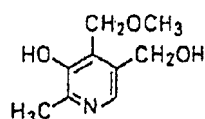
Key words: 3-Hydroxy-5-hydroxymethyl-4-methoxymethyl-2-methylpyridine, 5-hydroxy-4-(methoxymethyl)-6-methyl-pyridine-3-methanol, 4'-O-methylpyridoxine, ginkgotoxin, Gin-nan sitotoxism, *Ginkgo biloba* L., Ginkgoaceae.

Introduction

Ginkgo biloba L. (maidenhair tree, Ginkgoaceae) has attracted much attention for botanical, chemical, and pharmaceutical reasons (1, 2). The tree contains an array of different natural products (3) among which ginkgolides and bilobalide are unusual because in Nature they seem to be confined to *Ginkgo biloba* (4–7). Up to now no source other than *Ginkgo* is known for these natural products. The flavonoids occurring in *Ginkgo* plants have also been isolated (3, 8). As opposed to ginkgolides, however, they occur widespread in Nature.

The leaves of the *Ginkgo* plant are a source of extracts (EGb761) which exhibit a beneficial effect in the treatment of insufficient central and peripheral blood flow (2, 9). It is for this reason that *Ginkgo* medications are top-selling phytotherapeutic medications in Europe with sales of 800 Million Deutsche Mark annually (10).

Interestingly, the occurrence in *Ginkgo* seeds of a neurotoxic antivitamin B₆ (4'-O-methylpyridoxine) (Fig. 1) has also been reported (11). This toxic principle is likely the cause of "Gin-nan-sitotoxism" by interfering with the amino acid metabolism in patients with convulsions or even death as cardinal symptoms (11–14). A report in the literature on the absence of this toxin from leaves of the *Ginkgo* tree (15) seemed to indicate



Ginkgotoxin

4'-O-Methylpyridoxine

3-Hydroxy-5-hydroxymethyl-4-methoxymethyl-2-methylpyridine

Fig. 1 Structure and synonyms of ginkgotoxin.

that this toxin should also be absent from the extracts which are derived from leaves. Hence *Ginkgo* medications should also be devoid of 4'-O-methylpyridoxine. Analysis in our laboratory of samples from different *Ginkgo* trees showed, however, that this toxin also occurs in leaves. Hence, it is not surprising that the toxin is also present in *Ginkgo* medications including homeopathic preparations. The toxin is also detectable in boiled *Ginkgo* seeds used as a food in Japan.

Materials and Methods

Plant material

The following trees were used as a source of leaves and seeds. A female tree growing in the Hugo Haelschner-Straße in Bonn (height 8 to 9 m with 62 cm circumference at a height of 2 m above ground), a male tree growing in the Nussallee 6 in Bonn (height 12 m with 164 cm circumference at a height of 2 m above ground), a tree which had been generated by grafting a female twig onto a male plant in 1895 (height 5 m with 190 cm circumference at a height of 2 m above ground). This latter tree produces both male and female flowers, has male and female branches and carries a wealth of seeds every year. This tree grows in the Botanical Garden Bonn, Germany. Herbarium specimen collected by W. Lobin on June 27th (1996), Botanica Garden Bonn.

Quantitative analysis of 4'-O-methylpyridoxine

Leaves or seeds were frozen in liquid nitrogen and powdered in a mortar. The powder (300 to 500 mg) was transferred to test tubes and suspended in water (2 ml). The test tube was covered with a marble and heated (30 min, 100 °C) in a water bath. After cooling the suspension was centrifuged (30 min 10000 rpm) and the supernatant analyzed for 4'-O-methylpyridoxine (Fig. 2) using a Merck-Hitachi L-6200A HPLC

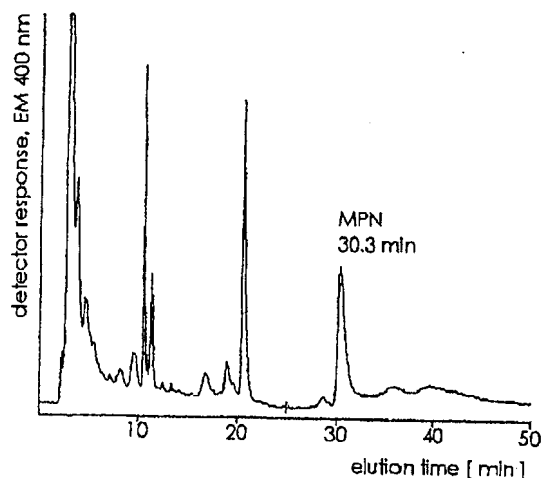


Fig. 2 HPLC trace of a homeopathic *Ginkgo* preparation ("Ginkgo loges Tropfen") (MPN = 4'-O-methylpyridoxine).

apparatus (Darmstadt, Germany), connected to a fluorescence detector (Shimadzu RF551 set to 330 nm (EX) and 400 nm (EM)). The extracts were separated on a Nucleosil 100 5 SA-column (250 × 4 mm ID) with a Nucleosil 100 5SA-guard column (30 × 4 mm ID) (Macherey-Nagel, Düren, Germany) with a flow rate of 1 ml · min⁻¹. As eluents solvents A (0.01 M hydrochloric acid) and B (potassium phosphate buffer, 0.5 M, pH 5) were used. Elution was started with solvent A (100%) increasing B from 0% to 10% over 6 min, followed by an increase to 20% B over 2 min. Subsequently B was increased to 100% over 7 min. After elution (100% B) for 13 min the column was reconditioned with 100% A for 15 min.

Preparative isolation of 4'-O-methylpyridoxine from leaves and from the medication "Tebonin-forte"

Freshly collected leaves (2 kg) were frozen in liquid nitrogen and powdered in a blender. The powder was suspended in water (4.5 l) and the suspension heated on a water bath (100 °C, 15 min). The extract was collected after centrifugation and the extraction repeated twice. The combined extracts were concentrated to 500 ml at 50 °C on a rotary evaporator. This extract or "Tebonin forte" were adjusted to pH 3 (conc. hydrochloric acid) and passed over a cation exchange column (Dowex 50 WX8 H⁺, 100–200 mesh, 100 g, diameter 3 cm). The column was washed (0.2 M hydrochloric acid) until the viscosity of the effluent of the column dropped significantly. 4'-O-Methylpyridoxine was eluted with a solution containing boric acid (0.2 M) and potassium chloride (0.2 M) adjusted to pH 6.8 with potassium hydroxide prior to elution. The eluate (500 ml) was taken to dryness in vacuum at 50 °C. The dried material was dissolved in hydrochloric acid (0.2 M; 250–300 ml) and again passed over an identical ion exchange column (vide supra). The column was washed (water) until the effluent was neutral and 4'-O-methylpyridoxine was eluted with ammonia solution (2%). The fraction containing 4'-O-methylpyridoxine (checked by HPLC) was concentrated to 1 to 2 ml and the toxin purified by paper chromatography (Whatman 3 MM, solvent: organic phase of a mixture of *n*-butanol, water, acetic acid = 4 : 5 : 1, v/v; *R_f* = 0.7). The toxin was eluted with water and rechromatographed on silica gel plates which had been washed previously with a mixture of chloroform and methanol = 1 : 1 (v/v). The chromatograms were developed in *tert*-butanol, ethyl methyl ketone, ammonia (5%), and water = 4 : 3 : 2 : 1. The toxin (*R_f* =

0.7) was eluted with methanol. Rechromatography was carried out on silica gel and the plates developed in toluene, ethanol, acetic acid = 5 : 4 : 1 (v/v). *R_f* = 0.45. Finally the toxin was eluted with methanol.

Identification of ginkgotoxin

This was done by ¹H-NMR and ¹³C-NMR (in deuteromethanol) on a 500 MHz NMR spectrometer AMX 500 (Bruker, Karlsruhe, Germany). The spectrum of the isolated material was indistinguishable from that of a synthetic sample (16).

Results

Two kg of leaves were harvested in August 1995 from a female tree and 2 kg from a male tree. Both lots were worked up separately. Preparative isolation of 4'-O-methylpyridoxine gave 4 or 6 mg, respectively. 4'-O-Methylpyridoxine was also isolated preparatively from 400 ml of the *Ginkgo* medication "Tebonin forte" with 2.9 mg overall yield. In each case the identity of the isolated product was verified by ¹H- and ¹³C-NMR spectroscopy as described under Materials and Methods. The data show that leaves (Fig. 3) and *Ginkgo* medications clearly contain 4'-O-methylpyridoxine.

Quantitative estimation of the amount of ginkgotoxin present in seeds and leaves was also carried out with the analytical method (fluorescence detection) described under Materials and Methods. The method is very sensitive (limit of detection < 0.5 µg) and lends itself to detect 4'-O-methylpyridoxine in homeopathic *Ginkgo* medications (Fig. 2). The time dependent formation in seeds and leaves is depicted in Fig. 3 a and 3 b. The data show that the highest amount of ginkgotoxin in seeds and leaves derived from the tree in the Botanical Garden occurs by the end of July and beginning of August (Fig. 3 a, b).

The same method was employed to analyze the amount of 4'-O-methylpyridoxine in different allopathic preparations (Table 1a). As expected, homeopathic preparations contain much less toxin (Table 1b).

4'-O-Methylpyridoxine was also isolated from Japanese *Ginkgo* food of two different brands purchased in a department store in Okayama, Japan, in May 1995 (Table 2). The *Ginkgo* food is sold after boiling which apparently removes 99% of the ginkgotoxin (Table 2) from the fresh seed. In both cases the sauce also contains ginkgotoxin, the amount of which does not exceed the ginkgotoxin in the boiled seeds significantly. Within a fresh seed the albumen is the tissue which contains most of the ginkgotoxin while the sarcotesta contains only a minor amount (Table 2). This low amount is also encountered in incompletely developed seeds lacking the albumen. These "seeds" consist of a sarcotesta and a sclerotesta only.

Discussion

Ginkgotoxin (4'-O-methylpyridoxine) is reported to cause convulsions in guinea pigs at an oral dose of 11 mg per kg body weight. Signs of intoxication are also paralysis of legs and auditory hyperalgesia. Following an oral dose of 50 mg per kg body weight death may result after ventricular fibrillation (14).

Gin-nan sitotoxism has sometimes occurred during food shortages in Japan (1930–1960, about 70 reports) and China

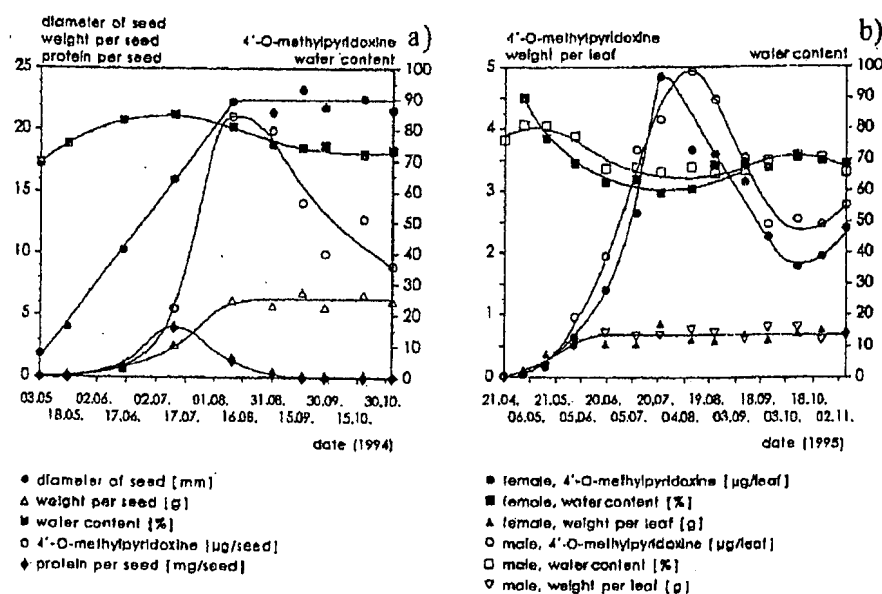


Fig. 3 Time dependent formation of ginkgotoxin in seeds (a) and leaves (b) of a grafted *Ginkgo biloba* tree in the Botanical Garden Bonn; "male or female" specifies determinations that were carried out with leaves from male or female branches. Weight of seeds or leaves are the mean of ten individual determinations. Individual determinations of 4'-O-methylpyridoxine were repeated two times. Data represent the mean of these determinations.

Table 1 4'-O-Methylpyridoxine in liquid allopathic (a) or homeopathic (b) medications. Standard deviation is less than $\pm 4.5\%$.

Medications ^c	Amount of 4'-O-methylpyridoxine ($\mu\text{g} \cdot \text{ml}^{-1}$)	Recommended daily intake (ml)	Maximum daily intake of 4'-O-methylpyridoxine (μg)
a) allopathic			
Tebonin forte	8.13	3 times 1 to 2 ml	48.78
Rökan	9.77	3 times 1 to 2 ml	58.62
Kaveri forte	3.80	3 times 1 ml	11.40
Gingium	7.18	3 times 1 to 2 ml	43.08
b) homeopathic			
<i>ba) orally applied medications</i>			
<i>Ginkgo biloba</i> Urtinktur Hanosan	0.301	1 to 3 times 0.25–0.5 ml	0.452
<i>Ginkgo biloba</i> Urtinktur DHU	0.589	3 times 0.25–0.5 ml	0.884
Ginkgo-loges Tropfen	1.995	3 to 6 times 1 ml	11.970
<i>Ginkgo biloba</i> Hevert comp. Tropfen	0.008	3 to 4 times 1 ml	0.032
Cereginkgo Tropfen	0.015	3 to 6 times 1 ml	0.090
<i>bb) medications applied by injection</i>			
Ginkgo-loges Injektionslösung	0.031	single dose, 2 ml	0.062
Hewe Ginkgo Injektionslösung	0.015	single dose, 2 ml	0.030
<i>Ginkgo biloba</i> comp. Hevert Injektionslösung	0.006	single dose, 2 ml	0.012
Cereginkgo Injektionslösung	not detectable	single dose, 2 ml	–

^c The medications listed in this Table are specified in the ABDA Databank, version 1.45/HOV.

with 27% lethality (11–14). A 21 months old patient with ginnan food poisoning was reported who had a $0.09 \mu\text{g}/\text{ml}$ serum level of ginkgotoxin 8.5 hours after ingestion of 50 *Ginkgo albumens* (12). 4'-O-Methylpyridoxine and its 5'-O-acetyl derivative were also reported to occur in pods of the Fabaceae plant *Albizzia tanganyicensis* (17). The seeds are responsible for neurological disorders and death in cattle and sheep in South Africa. Derivatives of pyridoxine were also isolated from *Albizzia julibrissin* Durazz (18) and *Albizzia versicolor* Welw. ex Oliver (19).

Ginkgotoxin may be an inhibitor in mouse brain of pyridoxal kinase (13) or of glutamate decarboxylase (14), a pyridoxal phosphate dependent enzyme. The detrimental effect of ginkgotoxin can be alleviated by pyridoxol (19). Our data show as opposed to a previous report (15) that ginkgotoxin is clearly

a constituent of *Ginkgo* leaves and occurs also in *Ginkgo* medications and Japanese *Ginkgo* food.

The leaf extract EGb761 employed in the preparation of some *Ginkgo* medications is standardized for a high level of flavonoids (24%) and terpene lactones (6%). The beneficial effect of the extract is attributed to both groups of natural products. The highest amount of ginkgolides is observed in late summer or early autumn and this coincides with a maximum amount of ginkgotoxin in the leaves and seeds (Fig. 3).

In spite of the presence of ginkgotoxin in the *Ginkgo* medications the extract of leaves was reported to be nontoxic (9). This may be due to the low concentration of ginkgotoxin which is much lower than the amount necessary to cause the detrimental effects mentioned above. A rather low concentra-

Table 2 4'-O-Methylpyridoxine in seed albumens and seed coats (sarcotesta) of different origin. Standard deviation is less than $\pm 4.5\%$.

Sample	4'-O-Methylpyridoxine	
	per fresh weight ($\mu\text{g} \cdot \text{g}^{-1}$) or ($\mu\text{g} \cdot \text{ml}^{-1}$)	per dry weight ($\mu\text{g} \cdot \text{g}^{-1}$)
albumen of seed harvested on Sept. 30th, 94	42.24	105.16
sarcotesta of seed harvested on Sept. 29th, 94	1.25	5.44
sarcotesta of "seed" lacking albumen harvested on Sept. 29th, 94	1.74	7.15
boiled seed (albumen) (japanese canned food)	0.46	1.32
sauce in can	0.65	—
boiled seed (albumen) (japanese food conserved in plastic foil)	0.27	0.75
sauce in plastic foil	0.38	—

tion of ginkgotoxin occurs also in boiled Japanese *Ginkgo* seeds which are used as a food. This and the fact that only a limited amount of boiled seeds are ingested during a meal in Japan may ensure that intoxications are normally not observed.

The fact that bilobalide present in *Ginkgo* plants decreases the severity of convulsions (20) may also be taken into account.

Acknowledgements

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