Alkylresorcinols in Fruit Pulp and Leaves of *Ginkgo biloba* L.

Ewa D. Żarnowska[§], Robert Żarnowski[§§] and Arkadiusz Kozubek*

Institute of Biochemistry and Molecular Biology, Department of Lipids and Liposomes, University of Wrocław, Przybyszewskiego 63/77, 51–148 Wrocław, Poland.
Fax: +48 (71) 3252930. E-mail: kozubek@angband.microb.uni.wroc.pl

* Author for correspondence and reprint requests

Z. Naturforsch. **55c,** 881–885 (2000); received May 15/July 12, 2000

5-*n*-Alkylresorcinols, 1,3-Dihydroxy-5-alk(en)ylbenzene, 1,3-Benzenediol, *Ginkgo biloba*

These studies were undertaken to characterise resorcinolic lipids (5-*n*-alk(en)ylresorcinols) composition and to determine their seasonal fluctuations in fruit pulp and leaves of *Ginkgo biloba* L. Resorcinolic lipid concentrations were consistently higher in fruit pulp than in leaves. In pulp, several mono- and di-unsaturated homologs of alkylresorcinols were the predominant group of analysed lipids. Contrary to the fruit pulp, only 5-*n*-pentadecylresorcinol was demonstrated in leaves. Initially, the alkylresorcinol's content both in pulp and leaves increased until June – July and decreased following seeds ripening. This trend continued until senescence of leaves in late September and October.

Introduction

Ginkgo biloba originates from Eastern China where it grows wild. This ancient species is at present widely cultivated as ornamentals either at botanical gardens or parks. *Ginkgo* tree is also planted as a valuable natural source of several groups of compounds, including essential oils, flavour and fragrance compounds, various lipid classes such carotenes and phenolic lipids, and alkaloids. These chemical components are important from pharmacological and medical point of view (Jarvis and Morgan, 1997). The green seed kernels are also edible (e.g., Kleijnen and Knipschild, 1992; Wada and Haga, 1997).

Uncommon properties of *Ginkgo* leaves are known in Japan since ancient times, where they are inserted between book pages as a preservative. An increased focus on studies of *Ginkgo* revealed a wide range of its important therapeutic and pharmacological effects on human health. Particularly, extracts from *Ginkgo* have a wide application for treating various cardiovascular and neurological diseases (Oyama *et al.*, 1994). Two groups of biologically active compounds – ginkgoflavonolglycosides and terpenoids – are used because of their favourable effect on central nervous system, insufficient blood supply and anoxia (Otta and Wójcik, 1998). Extracts of *Ginkgo* leaves, for similar reasons, have also been applied in cosmetology as a constituents of wide, multifunctional line of skin care products (Domagała, 1998). Besides beneficial effects, a negative influence of *Ginkgo* extracts, like e.g., dermatitis, developed in human and animal organisms, has been recognised. Those beneficial and negative effects are triggered off among others by phenolic compounds, which constitute an important group of the plant secondary metabolites. Among phenolic compounds, the presence of phenolic acids (protocatechoic, *p*-hydroxybenzoic, vanillic, caffeic, isovanillic, *p*-coumaric, ferulic, and sinapic) in *Ginkgo* leaves was described recently by Ellnain-Wojtaszek and Zgorka (1999). Furthermore, alkylphenols and their acidic derivatives – anacardic acids – are well known (Gellerman *et al.*, 1974). Historically, *Ginkgo biloba* is the first plant species, where another subclass of non-isoprenoid phenolic lipids – called 5-alkylresorcinols – has also been found (Morimoto *et al.*, 1968). The trivial name „bilobol" was used with reference to 5-*n*-pentadec-8(*Z*)-enyl-resorcinol (Fig. 1). According to the described biological activities (see e.g. (Kozubek and Tyman, 1999; Żarnowski *et al.*,

§ Present address: Wrocław University of Medicine, Department of Biophysics, Chałubińskiego 10, 50–368 Wrocław, Poland.
§§ Present address: Agricultural University, Department of Agricultural Microbiology, Grunwaldzka 53, 50–375 Wrocław, Poland.

0939–5075/2000/1100–0881 $ 06.00

1999), alkylresorcinols may exert a strong effect on living organisms and on the ecosystem.

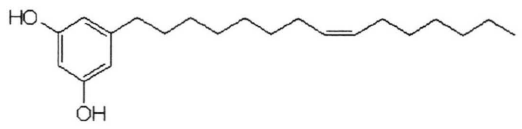

Fig. 1. Bilobol, 5-n-alkylresorcinol homologue predominant in *Ginkgo biloba*.

In this report, fluctuations in resorcinolic lipid content in *Ginkgo* fruit pulp and leaves during the growing season is demonstrated.

Materials and Methods

Plant material, harvesting procedures and sample preparation

Fruit pulp and leaves were collected in the University of Wrocław Botanical Garden periodically during the growth season in 1996. The *Ginkgo* specimen, with the thickness circumference of 270 cm, over 65 years of age consists of two trees: a male and a female that grow together. Pulp and leaves were harvested at intervals, beginning in April 1996 and ending in October 1996. The samples were cut with a cutter solely from female specimen. Seed kernel was removed from fruit pulp. Collected material was immediately frozen in liquid N_2 and kept in plastic bags at −70 °C until further laboratory analysis. Each of the collected samples were cut into small pieces and finely ground in a mortar.

Chemicals

TLC plates were from Merck, Poznań (Poland), silica gel Si60 for column chromatography from Baker, Łódź (Poland). Diazonium salt Fast Blue B × BF_4 was a kind gift from Lachema, Prague (Czech Republic). Standard alkylresorcinols and 5-n-pentadecylresorcinol were isolated chromatographically (Kozubek, 1985) from mature rye grains and from CNSL (Cashew Nut Shell Liquid, Cardolite Co., USA) and used as saturated congeners after hydrogenation of the enoic constituents. Other chemicals of the highest available purity were from Polskie Odczynniki Chemiczne (POCh), Gliwice (Poland).

Isolation and purification of resorcinolic lipids

Each of ground sample of plant material was extracted with acetone for 24 hr. The volume of the organic solvent was adjusted to soak the sample completely. The extracts were separated by filtration and plant material was re-extracted with a similar volume of acetone. Both extracts were combined and acetone was removed in rotary vacuum evaporator. The dry residue was redissolved in 1 ml of $CHCl_3$ and then applied onto a glass column (∅ 15 × 200 mm) filled with silica gel 60 suspended in $CHCl_3$. Flash column chromatography was carried out first with $CHCl_3$ and then with $CHCl_3$:AcOEt (85:15, v/v); 5 ml fractions were collected manually. Each fraction was tested for presence of alkylresorcinols by spotting (approximately 10 µl) on a small piece of silica gel covered TLC plate and wetting with aqueous Fast Blue B (0.1%, w/v). The appearance of characteristic red-violet colour indicated the presence of studied compounds (Kozubek and Tyman, 1995). Fractions containing alkylresorcinols were combined, concentrated under a stream of nitrogen gas and applied to preparative TLC plates (2 mm layer) covered with silica gel. Chromatograms were run in *n*-hexane:ethyl ether:formic acid (70:30:1, by v). After separation solvents were evaporated and plates masked with a piece of a cardboard precut to the size that left a 1 cm wide strip of the gel on the both sides of the plate and sprayed with aqueous Fast Blue B × BF_4. The band of alkylresorcinols has been identified both by its characteristic red-violet colour and the R_f value. Appropriate parts of the gel, corresponding with standard alkylresorcinols, were scrapped off, transfered into a dry tube and extracted with acetone for 2 hr. After centrifugation and removal of the solvent, the residue was dissolved in 0.5 ml of $CHCl_3$ and used for further analyses.

Identification of alkylresorcinols and analysis of homolog composition

Resorcinolic lipids were identified by chromatographic and spectroscopic means. Alkylresorcinols isolated from fruit pulp were further analysed in EI-MS (70eV) after their chemical modification into tetramethylsilyl ethers (TMS) derivatives. Mass spectral analyses were performed at The Instrumental Analyses Laboratory at the Faculty of

Chemistry, Adam Mickiewicz University, Poznan, Poland. Spectra were obtained by ESI MS (Finnigan Mat PSQ 700) and GC/MS on Hewlett Packard 5890 Series II gas chromatograph coupled with an EI mass spectrometer (AMD Intectra). Additionally, resorcinolic lipids isolated from leaves were analysed in ^{1}H-NMR (300 MHz, $CDCl_3$-CD_3OD), ^{13}C-NMR (75 MHz, $CDCl_3$-CD_3OD) and GC/MS (70 eV, DB-1 column, GC conditions: 80 °C for 1 min, 30°/min to 230, 10 °C/min to 320 and 320 °C for 2 min; injection at 250 °C). Data were compared with those reported elsewhere (Kozubek and Tyman, 1995; Kozubek and Tyman, 1999; Vincieri *et al.*, 1981). Besides MS analysis, composition of resorcinolic homologs according to the length of the side-chain was also determined using both the reversed-phase TLC plates (Merck 5914) (Kozubek, 1985) and the normal-phase TLC plates covered with aluminum oxide (Merck 5581) (Tłuścik and Kozubek, 1984). Composition of homologs according to their unsaturation of the side-chain was done in argentation chromatography on TLC silica gel plates impregnated with 5% silver nitrate in 50% aqueous methanol (Kaczmarek and Tłuścik, 1984).

Quantitative determination of alkylresorcinols

The microcolorimetric method (Tłuścik *et al.*, 1981) was used for quantitative determination of alkylresorcinols in plant material. Briefly, the sample containing alkylresorcinols, dissolved in chloroform was put into dry glass tube and the solvent was evaporated with the stream of nitrogen gas. To the dry residue 4 ml of the reagent prepared by 5-fold dilution with n-propyl alcohol of 0.05% (w/v) Fast Blue B × BF_4 in 5% acetic acid were added. The content was thoroughly mixed using a Vortex mixer and left in the dark for an hour. The colour developed was read at 520 nm against the reagent blank. The content of alkylresorcinols was estimated using a calibration curve (1–10 μg) prepared with 5-*n*-pentadecylresorcinol as a reference compound.

Statistical analysis

Each of samples was analysed in triplicate. The results of quantitative determinations were analysed statistically and standard error did not exceed 5%.

Results

Acetone extracts from *Ginkgo biloba* pulp and leaves were separated by CC and TLC on silica gel. Staining with Fast Blue B × BF_4 allowed the preliminary identification of alkylresorcinols, exhibiting characteristic red-violet colour and mobility value (R_f) that was identical to authentic 1,3-dihydroxy-5-alkylbenzenes (Kozubek and Tyman, 1995). The MS analysis of alkylresorcinols isolated from *Ginkgo* pulp and leaves, provided further evidence on their basic skeletal structure. This method enabled the determination of alkyl chain length as well as the chain unsaturation degree. Isolated material showed presence of characteristic for alkylresorcinols base ionic peaks. The unambiguous identification of alkylresorcinols was disclosed by the occurrence of peaks at *m/z* 123 and 124 and their mutual ratio 1:4 or 1:5. In samples from fruit pulp, the occurrence of several parent molecular ions with *m/z* masses from 320 to 432 confirmed presence of homologs from C15 to C23 with various degree of the side chain unsaturation. Additionally, the ^{1}H and ^{13}C NMR techniques were applied for analysis of alkylresorcinols isolated from leaves. In this case, only the presence of 5-*n*-pentadecylresorcinol was verified, only. Spectral data are summarized in Table I.

Quantitative determination of alkylresorcinols in analysed samples was made by a microcolorimetric method based on measurement of absorbances of the red-violet complex between alkylresorcinols and diazonic salt, Fast Blue B. Reversed- and normal-phase TLC analyses identified homolog compositions in plant samples. The same homologs were identified by electron impact mass spectrometry. Alkylresorcinol content is summarized in Table II.

Application of argentation chromatography allowed to establish enoic homologs compositions. It has been found that unripe pulp (sample P1) contains only saturated homologs. Fully mature pulp (sample P7) contained predominantly dienoic (about 83%) and monoenoic (13%) homologs. Only 4% of resorcinolic lipids with a saturated side chain were present in pulp tissues. In contrast, leaves contain only saturated alkylresorcinols during the whole growth season.

Table I. NMR and GC/MS data for 5-*n*-pentadecylresorcinol isolated from *Ginkgo biloba* leaves.

^{1}H NMR	0.88 (3H, t, *J*=6.6 Hz)
	1.25 (29H. broad s)
(300 MHz, in	1.56 (2H, broad m)
$CDCl_3$-CD_3OD)	2.46 (2H, t, *J*=7.7 Hz)
[δ ppm]	6.15 (1H, t, *J*=2.1 Hz)
	6.21 (2H, d, *J*=2.1 Hz)
^{13}C NMR	14.0 (t)
	22.6 (d)
(75 MHz, in	29.2 (d)
$CDCl_3$-CD_3OD)	29.3 (d)
[δ ppm]	29.4 (d)
	29.55 (d)
	29.58 (d)
	29.6 (d) × 5
	31.0 (d)
	31.8 (d)
	35.8 (d)
	99.8 (d)
	107.3 (d) × 2
	145.7 (s)
	157.1 (s) × 2
GC/MS	
	retention time 9 min 46 s
(70 eV, as the TMS derivative)	$[M^+]$=464

Table II. Alkylresorcinols in pulp (P) and leaves (L) of *Ginkgo biloba* L.

Sample*)	Date of harvest	Alkylresorcinol content [μg/g ± SE]
P1	29.05	34.31 ± 0.72
P2	04.06	71.86 ± 0.71
P3	24.06	454.38 ± 9.10
P4	04.07	315.41 ± 2.51
P5	11.07	302.71 ± 7.85
P6	22.07	282.75 ± 3.42
P7	08.10	222.98 ± 4.84
L1	29.05	26.56 ± 0.32
L2	04.06	37.10 ± 1.12
L3	24.06	42.33 ± 0.55
L4	04.07	87.09 ± 2.19
L5	11.07	59.25 ± 0.94
L6	08.10	31.40 ± 0.79

*) The sample codes are related to the date of sample collection (colum 2 of the Table).

Discussion

We have demonstrated that alkylresorcinols in *Ginkgo biloba* vary both in contents and composition depending on organ and time of vegetation. The level of resorcinolic lipid increased during seed development and was positively correlated with seed growth. While the intensity of seed growth slowed down, the content of alkylresorcinols was highest. Afterwards, the alkylresorcinol level decreased. This phenomenon may be explained either as a result of biochemical modification of resorcinolic lipids, such as glycosylation of hydroxyl groups, or as a consequence of alkylresorcinols deposition in other parts of the plant. Simultaneously, the level of side chain saturation has been changed from saturated into mono- and dienoic alkylresorcinols. These observations show resorcinolic lipids as first and foremost compounds abundant component of *Ginkgo* pulp. The majority of alkylresorcinol homologs in mature pulp were C15 and C17 congeners (over 98%) with most prominent C17:2 and C15:1 homologues (approx. 85%) – Table III.

Table III. Content of alkylresorcinol homologues in ripe fruit pulp of *Ginkgo biloba*.

Homologue	Average Percentage content*)	Content in fruit pulp [μg/g ± SE]
C 15:0	2.4	5.3 ± 0.7
C 15:1	32.1	71.6 ± 3.2
C 17:0	2.6	5.8 ± 1.2
C 17:1	8.3	18.5 ± 2.5
C 17:2	53.0	118.2 ± 6.3
other	1.6	3.6 ± 0.4

*) Of all alkylresorcinols present.

Our results indicate that *Ginkgo* tissues contain up to 400 mg of alkylresorcinols per kilogram. At the same time, plant tissues differ each with respect of phenolic lipid concentrations. Apparently this is important from the plant antipathogen-defence point of view. Biological activities of resorcinolic lipids vs. bacteria and fungi have already been reported, (e.g., Żarnowski *et al.*, 1999). The accumulation of alkylresorcinols in pulp may either limit the colonization of seeds with phytopathogenic microorganisms or change the plant attractivity to herbivores. The lower content in leaves probably is due to the fact that they contain also other bioactive compounds, either completely absent in pulp or at the very low concentration. Resorcinolic lipids may not only affect above mentioned microorganisms. Alkylresorcinols accumu-

lated in *Ginkgo* organs might also participate to some ecological functions, as important components in the soil when seeds are left in the field. Alkylresorcinols as plant phenols may persist in the soil and affect certain soil microorganisms. Thereby, they may actively participate both in the formation of microflora composition, and affect the process of decomposition of organic matter.

Acknowledgements

The authors kindly acknowledge Dr. Yoshikatsu Suzuki (Microbial Toxicology Lab, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama, Japan) for recording NMR spectra. This work was supported by a University of Wrocław research grant.

Domagała Z. (1998), Inter-fragrances products as an example of the maidenhair tree application in cosmetics. Ann. Dendrol. **46,** 155–158.

Ellnain-Wojtaszek M. and Zgorka G. (1999), High-performance liquid chromatography and thin-layer chromatography of phenolic acids from *Ginkgo biloba* L. leaves collected within vegetative period. J. Liquid Chrom. Rel. Technol. **22,** 1457–1471.

Gellerman J. L., Anderson W. H. and Schlenk H. (1974), Biosynthesis of anacardic acids from acetate in *Ginkgo biloba*. Lipids **9,** 722–725.

Jarvis A. P. and Morgan D. (1997), Isolation of plant products by supercritical-fluid extraction. Phytochem. Anal. **8,** 217–222.

Kaczmarek J. and Tłuścik F. (1984), Variability of alkylresorcinol content in rye (*Secale cereale* L.) grains. A comparative analysis with several species of the genus *Triticum*. Genet. Polon. **25,** 349–358.

Kleijnen J. and Knipschild P. (1992), Ginkgo biloba. Lancet **340**, 1136–1139.

Kozubek A. (1985), Isolation of 5-n-alkyl, 5-n-alkenyl- and 5-n-alkadienyl- resorcinol homologs from rye grains. Acta Aliment. Polon. **9,** 185–198.

Kozubek A. and Tyman J. H. P. (1995), Cereal grain resorcinolic lipids: mono and dienoic homologues are present in rye grains. Chemistry Physics Lipids **78,** 29–35.

Kozubek A. and Tyman J. H. P. (1999), Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. Chem. Rev. **99,** 1–26.

Morimoto H., Kawamatsu Y. and Sugihara H. (1968), Steric structure of the toxic substances from the fruit pulp of *Ginkgo biloba*. Chem. Pharmacol. Bull. **16,** 2282–2286.

Otta H. and Wójcik P. (1998), Maidenhair tree (*Ginkgo biloba* L.) in therapy. Ann. Dendrology **46,** 147–154.

Oyama Y., Fuchs P. A., Katayama N. and Noda K. (1994), Myricetin and quercetin, the flavonoid constituents of *Ginkgo biloba* extract, greatly reduce oxidative metabolism in both resting and Ca^{2+}-loaded brain neurons. Brain Res. **635,** 125–129.

Tłuścik F. and Kozubek A. (1984), Determination of cereal 5-n-alkylresorcinol homologs by thin layer chromatography on aluminium oxide. Chem. Anal.(Warsaw) **29,** 79–84.

Tłuścik F., Kozubek A. and Mejbaum-Katzenellenbogen W. (1981), Alkylresorcinols in rye (*Secale cereale* L.) grains. VI. Colorimetric micromethod for the determination of alkylresorcinols with the use of diazonium salt, Fast Blue B. Acta Soc. Bot. Polon. **50,** 645–651.

Vincieri F. F., Vinzenzini M. T. and Vanni P. (1981), Extraction of active compounds from sarcotesta of *Ginkgo biloba* seed: inhibition of some dehydrogenase activity. Rivista Italiana. E. P. P. O. S. **63,** 79–82.

Wada K. and Haga M. (1997), Food poisoning by *Ginkgo biloba* seeds. In *Ginkgo Biloba* – A Global Treasure. From Biology to Medicine, (Hori T., Ridge R. W., Tulecke W., Del Tredici P., Tremouillaux-Guiller J. and Tobe H., ed.) Springer, Heidelberg.

Żarnowski R., Kozubek A. and Pietr S. J. (1999), Effect of rye 5-n-alkylresorcinols on *in vitro* growth of phytopathogenic *Fusarium* and *Rhizoctonia* fungi. Bull. Polish Acad. Sci: Biol. Sci. **47,** 231–235.