colorless crystalline compound. M.p. 110 °C. $[\alpha]_D^{18}$ +143.3° (c 1, EtOAc). Anal. Calcd for $C_6H_8N_2O_6$: C, 35.29; H, 3.92; N, 13.72. Found: C, 35.00; H, 4.04; N, 13.51. IR and ^{1}H NMR spectral data are in accordance with those in literature [10]. The melting point was determined in open capillaries on an Electrothermal apparatus and was uncorrected. Optical rotation was measured using a Perkin–Elmer 141 MC polarimeter.

3. Pharmacology

Rats of either sex weighing 280–360 g, were killed by a blow on the head and the superior mesenteric artery was isolated. The artery was immersed in Krebs–Ringer bicarbonate solution (mmol/l: NaCl, 118.3; KCl, 4,7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; CaEDTA, 0.026; glucose, 11,1). The rings (4 mm long) were mounted on pairs of stainless steel wire hooks, and connected to a force transducer (Hugo Sachs Elektronik), and suspended in an organ chamber filled with 60 ml of Krebs-Ringer bicarbonate solution (37 ºC; pH 7.4), which was bubbled with a gas mixture of 95% O_2/5% CO_2. Isometric tension was continuously recorded.

Each ring was gradually stretched to the optimal point (2 g) on its length-tension curve and allowed to equilibrate for 30 min [11]. The functional integrity of the endothelium was confirmed by the presence of an immediate relaxation induced by acetylcholine (10^{-6} mol/l) in rings contracted with phenylephrine (10^{-6} mol/l). All experiments were done on the precontracted preparations with phenylephrine [12].

References

1 Rang, H. P.; Dale, M. M.; Ritter, J. M.: Pharmacology, 4. Ed., p. 271, Churchill Livingstone, New York 1999
2 Laurence, D. R.; Benett, P. N.; Brown, M. J.: Clinical Pharmacology, 8. Ed., p. 426, Churchill Livingstone, Edinburgh, 1997
3 Ignaro, L. J.: Angew. Chem. Int. Ed. **38**, 1882 (1999)
4 Murad, F.: Angew. Chem. Int. Ed. **38**, 1856 (1999)
5 Holford, N.: Clinical Pharmacokinetics Drug Data Hand Book, 3. Ed., p. 12, Adis International, A Wolters Kluwer Company, Auckland, 1998
6 Stoss, P.: GPat 3124410, 1981; C.A. **98**, 161103 (1983)
7 Corey, E. J.; Schmidt, G.: Tetrahedron Lett. **5**, 399 (1979)
8 Čeković, Ž.; Tokić, Z.: J. Serb. Chem. Soc. **59**, 3 (1994)
9 Tokić Vujošević, Z.; Čeković, Ž.: Heterocycl. Commun. **7**, 43 (2001) and references cited therein
10 Tokić Vujošević, Z.; Čeković, Ž.: Synthesis, in press
11 Krstić, M. K.; Stepanović, R. M.; Krstić, S.; Katušić, Z.: Pharmacology **38**, 113 (1989)
12 Furchgott, R. F.; Zawadzki, J. V.: Nature **299**, 373 (1980)

Received November 21, 2001
Accepted February 6, 2002

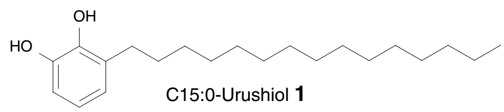
Radica Stepanović Petrović, Ph.D.
Assistant Professor
Department of Pharmacology
Faculty of Pharmacy
Vojvode Stepe 450, POB 146
11221 Belgrade
Yugoslavia

Dr. Willmar Schwabe Arzneimittel, Karlsruhe, Germany

Detection of allergenic urushiols in *Ginkgo biloba* leaves

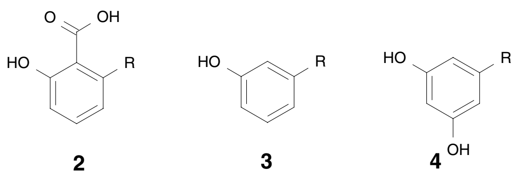
K. Schötz

Leaves of *Ginkgo biloba* are widely used to prepare extracts for the treatment of peripheral and cerebral circulatory disorders as well as dementia of different aetiology [1–3]. *Ginkgo*, like the members of the Anacardiaceae family [4], which include mango tree (*Mangifera indica*), cashew nut tree (*Anacardium occidentale* [5]), lacquer tree (*Rhus vernicifera* [6], *Melanorrhoea usitate* [7]), Indian marking nut tree (*Semecarpus anacardium* [8]), and the poison ivy, oak and sumac genera (*Toxicodendron*) [9, 10], is known to accumulate long chain alkylphenols. All of these plants are well known to induce allergic contact dermatitis [11]. Allergies towards Anacardiaceae are extremely frequent in the USA, where about 50–85% of the population is sensitive to members of this plant family [12]. A mixture of 3-n-pentadec(en)yl or heptadec(en)ylcatechols, commonly referred to as urushiols (e. g. derivative **1** with C15 : 0 side chain) has been found to be responsible for these reactions [13, 14].



C15:0-Urushiol **1**

In *Ginkgo* several long chain alkyl phenols such as ginkgolic acids **2,** cardanols **3** and cardols **4** have been observed to posses contact allergenic properties [15–18]. Since these alkylphenols have been show to cross react with poison ivy allergens, it has been suggested that **2** and **3** can be biotransformed to yield urushiols **1** [15]. These would provide a plausible explanation for the strong allergenic effects of *Ginkgo biloba*. However, it cannot be excluded that the biochemical machinery of *Ginkgo biloba* itself is able to process these long chain alkyl phenols to form urushiols **1**. Indeed, to our knowledge we here report for the first time that urushiols are natural constituents of *Ginkgo* leaves.



2 **3** **4**

Since long chain alkylphenols represent a substantial risk factor for adverse drug reactions, suitable techniques for elimination of these compounds from therapeutically used *Ginkgo* leaf extracts should be applied. As extremely low doses (5–50 ng) of urushiols are sufficient to elicit patch test reactions in humans [19], for the determination of such small quantities, especially in extracts, it is necessary to use high resolution separation techniques in combination with suitable detection sensitivity and selectivity. Method of choice for this application is derivatization gas chromatography mass spectroscopy (GC/MS [20], see ref. [21] for LC-ES-MS of acid derivatives). In addition, known and supposed constituents are required as reference substances. Compounds **2** and **3** (R = C13 : 0, C15 : 0,

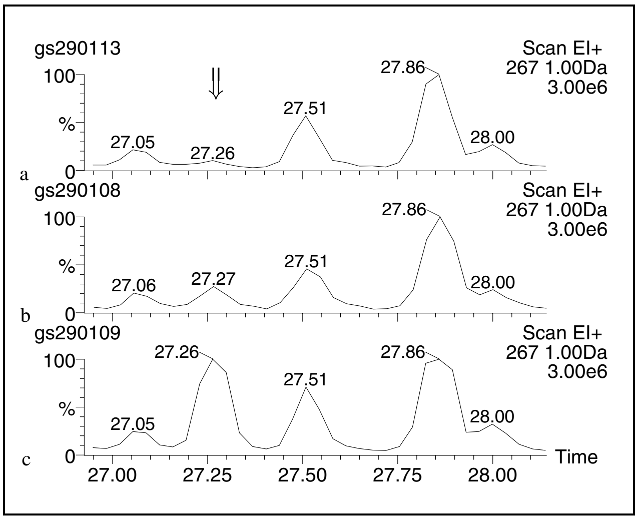


Fig. 1a–c: Comparison of GC/MS-chromatograms of a crude extract of *Ginkgo biloba* (displayed is the signal intensity at 267 amu). 1a: 1.5 µg crude *Ginkgo* leaf extract without addition of **1**, 1b: 1.5 µg crude *Ginkgo* leaf extract +0.3 ng urushiol **1**, 1c: 1.5 µg crude *Ginkgo* leaf extract +1.5 ng urushiol **1** (R_t: 27.26 min)

C15 : 1(8), C15 : 1(10), C17, C17 : 1(8), C17 : 1(10)) were synthesized according to modified published procedures [22–27] and used for the establishment of optimal GC conditions, allowing the separation of all fully silylated 14 compounds. For the verification of the detection system, unsaturated cardanols **3** and cardols **4** (C15 : 2, C15 : 3) from cashew nut shell liquid distillate (Palmer Inc.) were included in the analysis. As reference in the search for urushiols, two samples kindly provided from A. V. Del Grosso (FDA; synthetic C15 : 0(1), isolated C17 : 2 + C17 : 3) were used.

The results confirm that ginkgolic acids constitute the major group of long chain alkylphenols in *Ginkgo* leaves (approx. 20000 ppm). Cardanols (3-alkylphenols) were found at a concentration of about 1000 ppm [20], whereas no cardols [28] were detected. Surprisingly, it was now observed that *Ginkgo* also contains urushiols (about 100 ppm, whole group of catechol derivatives in dried leaves), which are by far the most important known contact allergenic compounds from plant sources. Representative GC/MS chromatograms of a lipophilic extract from *Ginkgo biloba* leaves are shown in Fig. 1a. Confirmation for the presence of 3-n-pentadecyl-catechol was obtained by addition of authentic **1** (Figs. 1b and 1c). The concentration of **1** (single compound C15 : 0, Fig. 1b) in the crude *Ginkgo* extract can be estimated to amount to about 30 ppm. In addition, the typical fragmentation pattern of the silylated catechol derivative of **1** was detected in other signals, arguing for a homologue series of compounds with different side chain length and saturation, known from the distribution of ginkgolic acids **2** and cardanols **3** (e.g.: C13 : 0, C15 : 1(8), C15 : 1(10), C17 : 1). Further work on these compounds will be published in due course.

Our results clearly demonstrate that urushiols **1**, like ginkgolic acids **2** and cardanols **3**, are natural constituents of *Ginkgo biloba* leaves. Since these compounds have not only been found to induce allergic contact dermatitis but also possess cytotoxic, mutagenic and tumorpromoting potential [29], these alkylphenols should be removed from commercial extracts as far as technically achievable. In the standardized *Ginkgo* extract EGb 761 the content of ginkgolic acids is restricted to a maximal concentration of 5 ppm by employing an appropriate manufacturing process. This limit value has also been included in the German Commission E monograph, the German Pharmacopoiea and the WHO monograph on *Ginkgo biloba*. During preliminary investigations using the above method with an estimated detection limit of about 0.05 ppm, no urushiols could be detected in EGb 761. This finding indicates that all alkylphenols are removed during production of EGb 761 in parallel. Thus, it is appropriate to use the predominant and easily detectable ginkgolic acids (for example by validated HPLC techniques) as marker substances for process control and assurance of pharmaceutical quality.

Experimental

1. Equipment

GC/MS: HP 5890 Series II, column: DB-5MS, 30 m, ID: 0.25 mm, (Agilent 122–5532); carrier gas: He, temperature program: 15 °C min^{-1} from 100 to 230 °C, 10 mins at 230 °C, gradient from 230 °C up to 300 °C with 3.5 °C min^{-1} and 5 mins final temperature 300 °C. Injection port: 230 °C, GC/MS-interface 250 °C. Injection volume 1 µl.

GC/MS: Trio2000 MicroMass (former Fisons). Software: MassLynx 3.2. Source: EI^+ (70eV), tungsten filament. Emission current: 200 µA. Measurement parameters: full scan for 2 s from 40 to 640 amu (continuum), inter scan time 0.1 s, runtime 45 min (solvent delay 5 mins), source temperature 180 °C, resolution HM/LM 12.5 arbitrary units, multiplier 500.

Silylation: Evaporated methanolic solutions of extracts (150 µg) were taken up in 100 µl of BSTFA (Pierce chemicals, Rockford, USA) and heated 30 mins at 100 °C. Aliquots of reference compounds were added at appropriate dilutions before the evaporation step.

2. Ginkgo leaf extract

Dried and milled *Gingko biloba* leaves 200 g were two times extracted at 60 °C with 2 l n-heptane and the combined filtrates evaporated to yield 16.72 g (8.4%) extract.

Acknowledgements: The author wish to thank E. Koch for critical advice in preparing the manuscript, K. Klessing for provision of synthetic ginkgolic acids and cardanols and H. Schneider for excellent technical assistance and measurement of GC/MS-spectra.

References

1 Liebgott, T.; Miollan, M.; Berchadsky, Y.; Drieu, K.; Culcasi, M.; Pietri, S.: Basic Res. Cardiol. **95**, 368 (2000)
2 Le Bars, P. L.; Kieser, M.; Itil, K. Z.: Dement. Geriatr. Cogn. Disord. **11**, 230 (2000)
3 Wesnes, K. A.; Ward, T.; McGinty, A.; Petrini, O. M.: Psychopharmacology **152**, 353 (2000)
4 Marks Jr., J. G.; DeMelfi, T.; McCarthy, M. A.; Witte, E. J.; Castagnoli, N.; Epstein, W. L.; Aber, R. C.: J. Am. Acad. Dermat. **10**, 627 (1984)
5 Paramashivappa, R.; Kumar, P. P.; Vithayathil, P. J.; Rao, A. S.: J. Agric. Food Chem. **49**, 2548 (2001)
6 Du, Y.; Oshima, R.; Kumanotani, J.: J. Chromatogr. **359**, 463 (1984)
7 Du, Y.; Oshima, R.; Yamauchi, Y.; Kumanotani, J.; Miyakoshi, T.: Phytochemistry **25**, 2211 (1986)
8 Gedam, P. H.; Sampathkumaran, P. S.; Sivasamban, M. A.: Phytochemistry **13**, 513 (1974)
9 Gross M.; Baer, H.; Fales, H. M.: Phytochemistry **14**, 2263 (1975)
10 ElSohley, M. A.; Adawadkar, P. D.; Benigni, D. A.; Watson, E. S.; Little Jr., T. L.: J. Med. Chem. **29**, 606 (1986)
11 Evans, F. J.; Schmidt, R. J.: Planta Med. **38**, 289 (1980)
12 Benezra, C. in Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular, and Medical Properties. p. 395, Alan R. Liss Inc., New York 1988
13 Majima, R.: Chem. Ber. **55**, 191 (1922)
14 Jefferson, A.; Wangchareontrakul, S.: J. Chromatogr. **367**, 145 (1986)
15 Lepoittevin, J.-P.; Benezra, C.; Asakawa, Y.: Arch. Dermatol. Res. **281**, 227 (1989)
16 Hausen, B. M.: Am. J. Contact. Dermatitis **9**, 146 (1998)
17 Koch, E.; Jaggy, H.; Chatterjee, S. S.: Int. J. Immunopharm. **22**, 229 (2000)
18 Stahl, E.; Keller, K.; Blinn, C.: Planta Med. **48**, 5 (1983)
19 Sasseville, D.; Nguyen, K. H.: Contact Dermatitis **32**, 182 (1995)
20 Cartayrade, A.; Bourgeois, G.; Balz, J.-P.; Carde, J.-P.: Trees **4**, 171 (1990)

21 Ndjoko, K.; Wolfender, J.-L.; Hostettmann, K.: J. Chrom. B **744**, 249 (2000)
22 Zehnter, R.; Gerlach, H.: Liebigs Ann. 2209 (1995)
23 Hamada, Y.; Hara, O.; Kawai, A.; Kohno, Y.; Shioiri, T.: Tetrahedron **47**, 8635 (1991)
24 Hauser, F. M.; Pogany, S. A.: Synthesis 814 (1980)
25 Kawamura, J.: Japan. J. Chem. **3**, 89 (1928)
26 Franke, K.; Masaoud, M.; Schmidt, J.: Planta Med. **67**, 477 (2001)
27 Verotta, L.; Peterlongo, F.: Phytochem. Anal. **4**, 178 (1993)
28 Zarnowska, E. D.; Zarnowski, R.; Kozubek, A.: Z. Naturforsch. **55c**, 881 (2000)
29 Jaggy, H.; Koch, E.: Pharmazie **52**, 735 (1997)

Received February 15, 2002
Accepted March 10, 2002

Dr. K. Schötz
Dr. Willmar Schwabe Arzneimittel
CF Naturstoffe
Willmar-Schwabe-Str. 4
Postfach 41 09 25
76209 Karlsruhe
karl.schoetz@schwabe.de

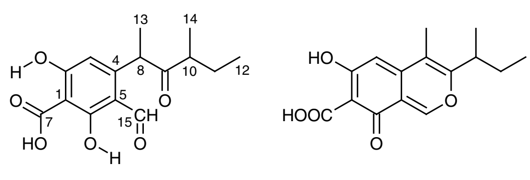
Department of Pharmaceutical Biology[1], Institute of Pharmacy, University of Greifswald, and Department of Chemistry of Natural Products[2], Society of Biotechnological Research, Braunschweig, Germany

Ascochital, a new metabolite from the marine ascomycete *Kirschsteiniothelia maritima*

C. Kusnick[1], R. Jansen[2], K. Liberra[1] and U. Lindequist[1]

The organic extracts of the culture filtrate of the marine ascomycete *Kirschsteiniothelia maritima* (Linder) D. Hawksw. (Pleomassariaceae) displayed antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Micrococcus flavus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Bioactivity guided separation of these extracts led to ascochital (**1**), a new aromatic aldehyde, besides the closely related ascochitine (**2**). Here we report on the isolation, structure elucidation and antibacterial activity of these compounds.

The fungal strain of *K. maritima* was taken from submerged wood and cultivated in a liquid shake culture on Hagem broth for 15 days. Chromatography of an EtOAc$-$Me$_2$CO extract of the culture broth on Sephadex LH-20 afforded two main products **1** and **2**. The major compound ascochitine (**2**), a cristalline yellow solid, was identified from its UV and NMR spectral data [1, 2]. Previously, **2** has been isolated as phytotoxic metabolite from cultures of *Ascochyta fabae* [3] and *A. pisi* [4]. The new metabolite, ascochital (**1**), was obtained as brown oil. Negative-mode DCI-MS and high-resolution DCI-MS of **1** established the elemental composition $C_{15}H_{18}O_6$ for the molecular ion at *m/z* 294. The ^{1}H- and ^{13}C NMR spectra of **1** in DMSO-d_6 were complicated by partial doubling of signals indicating the presence of two inseparable diastereomers in a nearly equivalent ratio (A and B in the Table). Because the doubled ^{13}C NMR signals appear as well separated pairs, all 25 observed signals could be assigned to the 15 carbon atoms. A doubled 2-butyl and $CH_3-CH<$ residue were identified in the ^{1}H,^{1}H-COSY spectrum. The analysis of the HMQC spectrum correlated all signals of carbon-bound protons with their respective ^{13}C NMR signals and further showed the presence of an aldehyde group ($\delta_{H/C} = 10.3/189$; C-15) and of an unsaturated methine group ($\delta_{H/C} = 5.8/107$; C-3). Thus, the signals of one keto group, a further five quarternary sp^2 carbons, and of two signals of H,D-exchangeable OH protons remained to be specified in the NMR spectra. A third exchangeable acidic proton, which was not visible in the ^{1}H NMR spectrum due to fast exchange with the residual water, was assigned to the carboxylic acid group ($\delta_C = 174$; C-7). The direct ^{13}C, ^{1}H NMR couplings ($^1J_{C,H}$) visible in the long-range ^{1}H, ^{13}C-correlation NMR spectrum (HMBC) revealed the aromatic character of methine C-3 by $^1J_{C,H} = 159$ Hz and confirmed the aldehyde group ($^1J_{C,H} = 174$ Hz). An obvious $^2J_{C,H}$ coupling of 21 Hz and a long-range correlation signal with the H-15 aldehyde proton identified C-5 as point of attachment for the aldehyde group. The HMBQ correlation between



1 **2**