

Alkylresorcinols in Barley (*Hordeum vulgare* L. *distichon*) Grains

Robert Żarnowski^{a,*}, Yoshikatsu Suzuki^b, Isamu Yamaguchi^b
and Stanisław J. Pietr^a

^a Department of Agricultural Microbiology, Agricultural University, Grunwaldzka 53, 50–375 Wrocław, Poland. Fax: +48-(0)71-3282868. E-mail: robert@ozi.ar.wroc.pl

^b RIKEN (The Institute of Physical and Chemical Research), Hirosawa 2–1, Wako-shi, Saitama 351–0198, Japan

* Author for correspondence and reprint requests

Z. Naturforsch. **57c**, 57–62 (2002); received August 8/September 7, 2001

Barley, Alkylresorcinols, Resorcinolic Lipids

This study was carried out to compare grains of barley (*Hordeum vulgare* L. *distichon*) regarding contents and compositions of 5-*n*-alkylresorcinols. Mixtures of resorcinol homologues were isolated from acetone extracts from five barley cultivars. These polyketide metabolites were identified by chromatographic and spectroscopic means. The content and homologue patterns among different varieties were similar. The predominant compounds were 1,3-dihydroxy-5-*n*-heneicosylbenzene (C_{21:0}), 1,3-dihydroxy-5-*n*-nonadecylbenzene (C_{19:0}) and 1,3-dihydroxy-5-*n*-pentacosylbenzene (C_{25:0}). The alkylresorcinol concentrations, in contrast to their compositions, depended on environmental and agricultural factors.

Introduction

5-*n*-alk(en)ylresorcinols, a group of naturally occurring polyketide-derived phenols, have been widely recognised since the 1930s as allergic constituents that in higher doses may cause contact dermatitis (Anderson *et al.*, 1931; Wasserman and Dawson, 1948). Over the years, a considerable amount of research has demonstrated that resorcinolic lipids can be found in various living organisms, such as lower and higher plants, fungi and bacteria (Kozubek and Tyman, 1999). Their occurrence in the *Gramineae* family has been ascertained including several utilitarian cereal species. Cereal alkylresorcinols were found to be mixtures of saturated, monoenoic and dienoic homologues with 13–29-carbon chains. In general, the amount of resorcinol derivatives in cereals is the highest in rye, lower in wheat, triticale, and other cereals.

Resorcinolic lipids are non-isoprenoid, long-chain, odd-numbered homologues of orcinol (1,3-dihydroxy-5-methylbenzene). These constituents are involved in multiple aspects of cellular biochemistry, membrane structures, and also physiology of organisms. Alkylresorcinols are also involved in a multitude of interactions with biological membranes, affecting their physicochemical properties. Due to their amphiphilic character, they are able to significantly modulate

activity of membrane-bound enzymes (Kieleczawa *et al.*, 1987; Sikorski *et al.*, 1987; Toyomizu *et al.*, 1993) and the fluidity of the membrane lipids (Kozubek and Demel, 1981; Hendrich and Kozubek, 1991). These lipids were found as an important part of the waxy epicuticular layer in cereal grains, stems and leaves. Due to their strong antibacterial and antifungal activity (Heinzen *et al.*, 1996; Żarnowski *et al.*, 1999), those compounds are biosynthesised specifically during the seedling stage to protect the plant against predators (Suzuki and Yamaguchi, 1998). These preformed antifungal compounds prevent the germination of fungal spores on the plant surface (Morrissey and Osbourn, 1999). At the same time, certain species of phytopathogenic fungi are able to biosynthesise resorcinolic lipids (Żarnowski *et al.*, 2000). These phenolic compounds in fungal cells protect them against fungicide action when the cultures are treated with exogenous alkylresorcinols (Żarnowski *et al.*, 1999; Żarnowski and Kozubek, 2001).

The importance of alkylresorcinols in the diet was demonstrated by a few reports (Pawlik *et al.*, 1976; Pawlik, 1979; Sedlet *et al.*, 1984). Rather negative effects of analysed compounds have been shown including serious growth inhibition and other pathological symptoms in several animal species. But those changes were observed only when considerably high doses of alkylresorcinols

were applied. Until now, however, there are no established toxicity levels of alkylresorcinols against mammalian organisms. On the contrary, *in vitro* studies on biological activities of alkylresorcinols indicated their strong antitumor action against certain cancer cell lines (Itokawa *et al.*, 1989; Matsumoto *et al.*, 1990). Moreover, alkylresorcinols exhibit the ability to protect cellular lipid components against oxidation processes (Kozubek and Tyman, 1999). Lack of toxic and carcinogenic effects of alkylresorcinols together with their antioxidant and antitumor properties suggests their possible participation in the protection of cells against cancer disorders.

Human population of today is concerned about having an adequate amount of fibres in the diet. It should be noted that various high fibre products contain up to a three-fold higher concentration of alkylresorcinols than the rye grains (Al-Ruqaie and Lorenz, 1992). Therefore, the consumption of these products might exert positive effects on human health.

The objective of this study was to determine alkylresorcinol content and homologue composition among investigated barley cultivars to estimate the usefulness of those cultivars from the nutritional point of view.

Experimental

Grain samples

Five qualified varieties of spring-crop barley (*Hordeum vulgare* L. *distichon*), cv. Rabel, cv. Rambo, cv. Rataj, cv. Rudzik, and cv. Scarlett, were studied. All varieties were cultivated on field plots at the Wrocław Agricultural University Plant Cultivation Experimental Station in Pawłowie, Poland. Complete cultivar vouchers are available from the Central Laboratory for Studies of Cultivable Plants (COBORU), Słupia Wielka, Poland. Plant material was harvested in 1998, except cv. Rudzik, which was collected in 1998 as well as in 1999. Grains were collected when the full maturity was achieved and then kept in moisture-proof containers until further laboratory analyses.

Isolation and purification of alkylresorcinols

The fraction of resorcinolic lipids was isolated from whole grains, except grains of cv. Rudzik har-

vested in 1998. Part of this sample (whole grains) was ground previously in a laboratory mill. From each grain sample, 30 g was soaked completely at room temperature with an equal amount of acetone. After 24 hrs, the acetone fraction was filtered through filter paper to remove any solid particles. The filtrate was saved and the plant material was soaked twice more with the same amount of acetone for 24 hrs each. All acetone filtrates were combined and the solvent was removed by vacuum evaporation on a rotavapor at 40 °C. The oily residue was redissolved in 0.2 ml of ethyl acetate and then applied on a 20 × 20 cm preparative TLC silica gel 60. Separation was carried out by chloroform/ethyl acetate (85:15, v/v). Afterwards, 1 cm wide strips of the gel on both sides of the plate were sprayed with aqueous 0.05% Fast Blue B × BF₄ (Chemapol, Prague, Czech Republic). Alkylresorcinols were identified by their characteristic reddish-violet colour and *R_f* value (Kozubek and Tyman, 1995). Parts of the gel containing compounds of interest were scraped off the plates and the material was extracted with ethyl acetate during occasional shaking for 2 hrs. After centrifugation (7500×g, 10 min), the supernatant was decanted and the remaining gel extracted once again. All supernatants were combined, concentrated *in vacuo* and then redissolved in 0.2 ml of ethyl acetate. The solution was applied on a similar preparative TLC plate and the chromatogram was developed by hexane/ethyl ether/formic acid (70:30:1, v/v). Next steps for resorcinols separation, gel staining and its extraction, centrifugation, and concentration, were repeated. The fraction of pure alkylresorcinols was redissolved in 0.2 ml of chloroform and used for further analysis. Each of the isolations was made at least in triplicate.

Determination of alkylresorcinols content

The microcolorimetric method (Tłuścik *et al.*, 1981) was used for quantitative determination of alkylresorcinols in analysed plant material. Briefly, the sample containing compounds of interest, dissolved in chloroform was put into a dry glass tube and the solvent evaporated with a stream of nitrogen gas. To the dry residue 4 ml of the reagent prepared by a 5-fold dilution with *n*-propanol of 0.05% (w/v) Fast Blue B × BF₄ in 5% acetic acid were added. The content was thoroughly mixed

using a Vortex mixer and left in the dark for an hour. The sample was read at 520 nm against the reagent blank. The content of alkylresorcinols was estimated using a calibration curve (1–10 µg) prepared by a suitably diluted stock solution of recrystallized pure 5-*n*-pentadecylresorcinol (Aldrich Chemical Co., Milwaukee, WI) as reference compound. Each determination was carried out in triplicate.

Alkylresorcinols homologue composition

The sample containing alkylresorcinols mixture was re-dissolved in 100 µl of ethyl acetate. 70 µl of the sample was added into a glass capillary-tube (ø ca. 2 mm, 5 cm). After removal of the solvent, 5 µl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was added and the tube was sealed and allowed to stand at 70 °C for 30 min. One µl of the derivatized sample was injected into HP 5890 Series II gas chromatograph connected to JEOL SX-102A mass spectrometer, at 70 eV with a gas flow rate of 1 ml/min of He. A DB-1 column (G & L Science, Tokyo, Japan; ø 0.25 mm × 15 m, 0.25 µm film thickness) was used and column oven temperature was programmed as follows: 130 °C for 1 min, 30 °C/min to 250, 15 °C/min up to 320 and 320 °C for 2 min. Sample injection was at 250 °C. Identification of each alkylresorcinol homologue was obtained from the molecular ion and common base peak ion at *m/z* 268 which is characteristic of these molecules. The retention time of each homologue was 9.3 min (M^+ 464, $C_{15:0}$), 10.4 min (M^+ 492, $C_{17:0}$), 11.6 min (M^+ 520, $C_{19:0}$), 12.7 min (M^+ 548, $C_{21:0}$), 13.8 min (M^+ 576, $C_{23:0}$) and 14.9 min (M^+ 604, $C_{25:0}$), respectively. The relative composition and total amounts of the homologue were estimated by the area of the base peak ion at *m/z* 268.

Chromatographic analyses

Additional identification of resorcinolic lipids was carried out using a set of chromatographic techniques. Normal-phase TLC separations were done on analytical and preparative layers on plastic and glass plates covered with silica gel Si60. After development of chromatograms and evaporation of solvents, the plates were sprayed with aqueous 0.05% Fast Blue B × BF_4 and alkylresorcinols were identified by their characteristic red-

dish-violet colour and R_f value. To determine composition of the homologues according to the length of the side chain, reversed-phase TLC on RP18 HPTLC plates (Kozubek, 1985) and normal-phase TLC on aluminium oxide (Tłuścik and Kozubek, 1984) were used. The presence and composition of homologues according to their unsaturation were determined by argentation chromatography on silica gel impregnated with 5% $AgNO_3$ (Kaczmarek and Tłuścik, 1984). All TLC plates were from Merck (Darmstadt, Germany). Solvents and reagents of highest available purity were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

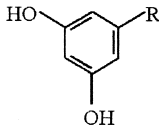
Results

Five different cultivars of barley were analysed for content and composition of resorcinolic lipids. Crude acetone extracts from dry mature grains were separated by TLC on silica gel developed with chloroform/ethyl acetate (85:15, v/v) mixture. Such purified alkylresorcinols' fractions were identified on TLC plates by their specific reddish-violet colour in reaction with diazonic salt Fast Blue B and their very characteristic mobility value (R_f), identical to authentic 5-*n*-pentadecylresorcinol.

The content of resorcinolic lipids was determined in purified fractions. Quantitation of alkylresorcinols in analyzed samples was done measuring the difference of absorbance of the colour complex between tested compounds and the diazonic salt. Quantitation in whole lipid extracts is an inadvisable method due to the presence of other non-resorcinolic substances cross-reacting with Fast Blue B. Extracts from each analysed sample should be first purified to remove contaminating components. Calculated values of alkylresorcinols content are summarised in Table I.

Eight resorcinol homologues diverse regarding their length of side-carbon chains as well as their (un)saturation, were found. The qualitative and quantitative patterns of homologues in different cultivars were rather similar. Regardless of the variety, the predominant compounds found were 1,3-dihydroxy-5-*n*-heneicosylbenzene ($C_{21:0}$ – ca. 40%), 1,3-dihydroxy-nonadecylbenzene ($C_{19:0}$ – ca. 29%), 1,3-dihydroxy-5-*n*-pentacosylbenzene ($C_{25:0}$ – ca. 19%), and 1,3-dihydroxy-tricosylbenzene ($C_{23:0}$ – ca. 11%). Only spurious amounts of

Table I. Alkylresorcinols in barley grains.

Cultivar	Year of harvest	Content ^d [mg/kg]	<div style="text-align: center;">  </div> <div style="text-align: center;">Homologue composition (% of total alkylresorcinol content)</div>							
			C _{15:0}	C _{17:0}	C _{19:0}	C _{19:1}	C _{21:0}	C _{21:1}	C _{23:0}	C _{25:0}
Rabel	1998	54.1	0.1	1.2	27.2	0.1	39.9	<i>t</i>	11.0	20.7
Rambo	1998	41.1	0.2	1.6	29.9	0.4	42.5	<i>t</i>	10.9	14.9
Rataj	1998	47.0	0.2	1.6	29.0	0.5	41.8	0.1	11.7	15.9
Scarlett	1998	44.1	1.2	2.0	31.3	0.6	42.8	<i>t</i>	10.1	12.5
Rudzik	1998	43.4	<i>nd</i>	<i>nd</i>	25.0	<i>nd</i>	37.6	<i>nd</i>	12.9	24.5
Rudzik ^g	1998	209.9	<i>nd</i>	<i>nd</i>	24.4	<i>nd</i>	38.7	<i>nd</i>	12.6	24.3
Rudzik	1999	73.9	<i>nd</i>	3.2	36.5	<i>t</i>	34.0	<i>nd</i>	8.2	18.2

^g – ground before laboratory processing; ^d – dry weight; *t* – trace; *nd* – not detectable; R: C₁₅–25 saturated or monounsaturated side chain.

1,3-dihydroxy-5-*n*-heptadecylbenzene (C_{17:0}) and of 1,3-dihydroxy-5-*n*-pentadecylbenzene (C_{15:0}), were found. Similarly, the content of monounsaturated homologues was very low, whereas diunsaturated resorcinol derivatives were not found.

The analysis of alkylresorcinols provided apparent evidence on their basic skeletal structure, regarding their alkyl chain length as well as the chain unsaturation degree. The unambiguous identification of those analyzed compounds was disclosed by the occurrence of characteristic base ionic peaks at *m/z* 267 and 268 and their mutual ratio 1:4 or 1:5 (Vincieri *et al.*, 1981). The same homologues were recognised using mass spectrometry as well as reversed- and normal-phase TLC techniques. Next, the application of the argentation chromatography allowed establishing homologue compositions diverse in saturation of the side-chain. It was found that all analysed barley varieties contained mostly saturated homologues and only trace amounts of monounsaturated homologues. Collected data are presented in Table I.

Discussion

In this report, we demonstrated the content and composition of resorcinolic lipids in grains of five barley cultivars. We found that cv. Rabel, cv. Rambo, cv. Rataj, cv. Scarlet and cv. Rudzik contain similar amounts of alk(en)ylresorcinols, up to 54 mg per kilogram (dry weight). Consequently, they may be classified as the group of low-resorcinol varieties.

The comparison of resorcinolic lipids' contents in milled and whole grains of cv. Rudzik showed some differences. We found that extraction from milled whole grains yielded nearly 4.8 times higher amount than from whole grains. Thereby, it suggests that the majority of these compounds is localised in the epicuticular wax zone (about 20%). This result is in good agreement with the prior report on localisation of alkylresorcinols in cereals (Verdeal and Lorenz, 1977; Thuścik, 1978; Salek, 1978), which showed bran to contain the highest alkylresorcinol level. Intermediate values were found in the shorts, whereas the flour fractions produced relative low values. This indicates that a gradient exists with the highest amount of the compounds in the pericarp, intermediate amounts in the aleurone layer, and relatively small but detectable amounts in the endosperm portion of cereal grain kernels. Our observation supports also the thesis of the protective role of these phenols in grain biology (Suzuki *et al.*, 1996). This assumption seems to be correct, the more so because it was earlier found that low-resorcinol cereal cultivars are more susceptible to fungal infections (García *et al.*, 1997; Żarnowski and Pietr, unpublished). There was also found that pathogenic microorganisms more often infect grains in damaged places. Additionally, the legitimacy of this thesis appears authentic due to antifungal activities of alk(en)ylresorcinols have been already reported in a few papers (García *et al.*, 1997; Żarnowski *et al.*, 1999).

In this paper, the fluctuation of alkylresorcinols content during consecutive followed vegetation periods has been also reported. We stated that amounts of alkylresorcinols in grains of cv. Rudzik were diverse in 1998 and 1999. Plants were cropped on the same field plots, so this observed variability undoubtedly is directly affected by environmental factors, such as climatic conditions, weather, and fertilisation. This finding is in a good agreement to the data of Wieringa (1987). Besides, Al-Ruqaie and Lorenz (1992) reported that cereal grains grown under the same agronomic and cli-

matic conditions during consecutive crop years showed only slight variations from year to year.

Acknowledgements

RŻ and YS contributed to this work equally. RŻ is deeply indebted to the Foundation for Polish Science for the National Scholarship for Young Scientists (Edition 2001). We would like to thank an anonymous reviewer as well as all those colleagues, who read this manuscript for their valuable comments.

- Al-Ruqaie I. and Lorenz K. (1992), Alkylresorcinols in extruded cereal brans. *Cereal Chem.* **69**, 472–475.
- Anderson H. H., David N. A. and Leake C. D. (1931), Oral toxicity of certain alkylresorcinols in guinea pigs and rabbits. *Proc. Soc. Exp. Biol. Med.* **28**, 609–612.
- García S., García C., Heinzen H. and Moyna P. (1997), Chemical basis of the resistance of barley seeds to pathogenic fungi. *Phytochemistry* **44**, 415–418.
- Heinzen H., Barra C., García C., García S., Larramendi S. and Moyna P. (1996), On the chemical ecology of epicuticular waxes from seeds. *Proceedings of the International Society for Chemical Ecology Meeting*, Prague. Abstract 42.
- Hendrich A. B. and Kozubek A. (1991), Calorimetric study on the interactions of 5-n-heptadec(en)ylresorcinols from cereal grains with zwitterionic phospholipid (DPPC). *Z. Naturforsch.* **46c**, 423–427.
- Itokawa H., Totsuka N., Nakahara K., Maezuru M., Takeya K., Kondo, M., Inamatsu M. and Morita H. (1989), A quantitative structure-activity relationship for antitumor activity of long-chain phenols from *Ginkgo biloba* L. *Chem. Pharm. Bull.* **37**, 1619–1621.
- Kaczmarek J. and Thućik 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.
- Kieleczawa J., Szalewicz A., Kozubek A. and Kulig E. (1987), Effect of resorcinols on electron transport in pea chloroplasts. *Progr. Photosynth. Res.* **2**, 585–587.
- 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 Demel R. A. (1981), The effect of 5-(n-alk(en)yl)resorcinols from rye on membrane structure. *Biochim. Biophys. Acta* **642**, 242–251.
- Kozubek A. and Tyman J. H. P. (1995), Cereal grain resorcinolic lipids: mono and dienoic homologues are present in rye grains. *Chem. Phys. 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.
- Matsumoto K., Fujimoto M., Ito K., Tanaka H. and Hirondo I. (1990), Comparison of the effects of bilobol and 12-O-tetradecanoylphorbol-13-acetate on skin and test of tumor-promoting potential of bilobol in CD-1 mice. *J. Toxicol. Sci.* **15**, 39–46.
- Morrissey J. P. and Osbourn A. E. (1999), Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.* **63**, 708–724.
- Pawlik J. (1979), Growth of chickens as influenced by alkylresorcinols of rye. *Rocz. Nauk Zootech.* **13**, 121–136.
- Pawlik J., Kudrewicz-Hubicka Z. and Wilusz T. (1976), Growth and dressing percentage of slaughter chicks, and behaviour of their serum proteins as influenced by rye and the alkylresorcinols contained in it. *Rocz. Nauk Zootech.* **5**, 109–125.
- Salek M. (1978), Determination of the 5-alkylresorcinol contents in rye grain and milling products. *Rocz. Państ. Zak. Hig.* **29**, 205–211.
- Sedlet K., Mathias M. and Lorenz K. (1984), Growth-depressing effects of 5-n-pentadecylresorcinol: A model for cereal alkylresorcinols. *Cereal Chem.* **61**, 239–241.

- Sikorski A. F., Michalak K., Bobrowska M. and Kozubek A. (1987), Interaction of spectrin with some amphipathic compounds. *Stud. Biophys.* **121**, 183–191.
- Suzuki Y., Esumi Y., Hyakutake H., Kono Y. and Sakurai A. (1996), Isolation of 5-(8'-heptadecenyl)-resorcinol from etiolated rice seedlings as an antifungal agent. *Phytochemistry* **41**, 1485–1489.
- Suzuki Y. and Yamaguchi I. (1998), Antimicrobial agents (phytoanticipins) in *Gramineae* crops produced specifically during seedling stage. *J. Pest. Sci.* **23**, 316–321.
- Tłuścik F. (1978), Localization of the alkylresorcinols in rye and wheat caryopses. *Acta Soc. Bot. Polon.* **47**, 211–218.
- 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.
- Toyomizu M., Sugiyama S., Jin R. L. and Nakatsu T. (1993), Alpha-glucosidase and aldose reductase inhibitors: constituents of cashew, *Anacardium occidentale*, nut shell liquids. *Phytother. Res.* **7**, 252–254.
- Verdeal K. and Lorenz K. (1977), Alkylresorcinols in wheat, rye and triticale. *Cereal Chem.* **54**, 475–483.
- Vincieri F. F., Vinzenzini M. T. and Vanni P. (1981), Extraction of active compounds from sarcotesta of *Ginkgo biloba* seeds: inhibition of some dehydrogenase activities. *Riv. Ital. E.P. P.O.S.* **63**, 79–82.
- Wasserman D. and Dawson R. (1948), Cashew nut-shell liquid. III. The cardol component of Indian cashew nut-shell liquid with reference to the liquid's vesicant activity. *J. Am. Chem. Soc.* **70**, 3675–3679.
- Wieringa G. W. (1987), On the occurrence of growth inhibiting substances in rye. PhD dissertation. Wageningen.
- Żarnowski R. and Kozubek A. (2001), Resorcinolic lipids as natural biofungicides. In the Proceedings of 13th International Reinhardtsbrunn Symposium on Modern Fungicides and Antifungal Compounds. 14–18 May 2001, Friedrichroda, Germany. Abstract p. 40.
- 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. Pol. Acad. Sci.: Biol. Sci.* **47**, 231–235.
- Żarnowski R., Lewicka T. and Pietr S. J. (2000), Production and secretion of 5-*n*-alkylresorcinols by *Fusarium culmorum*. *Z. Naturforsch.* **55c**, 846–848.