n-Alkylresorcinol Occurrence in *Mercurialis perennis* L. (Euphorbiaceae)

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Investigation of the dichloromethane extracts from herbal and root parts of *Mercurialis perennis* L. afforded a mixture of 11 homologous *n*-alkylresorcinols (ARs) with saturated odd-numbered alkyl side chains (C15:0–C27:0). In addition to three predominant ARs (C19:0, C21:0 and C23:0), a number of minor ARs were identified by use of LC-MS/MS and GC-MS techniques. Among the compounds detected, four uncommon ARs with even-numbered alkyl side chain lengths were also determined. The overall AR concentration in herbal parts was 7 to 9 times higher compared to that of the roots. The results presented may open a new view on the phytochemistry and pharmacognosy of *M. perennis* and other members of the Euphorbiaceae family.

Key words: Mercurialis, Euphorbiaceae, Acalypheae, *n*-Alkylresorcinols

Introduction

The herbal parts of dog's mercury (*Mercurialis perennis* L., Euphorbiaceae, subfamily Acalypheae) are used in remedies effective for topical treatment of inflammation, poorly healing wounds, sore or dry and inflamed eyes (Madaus, 1979). Applied orally the fresh herb or root are faintly poisonous and show a strong laxative and diurectic effect (Madaus, 1979; Blaschek *et al.*, 2008). In the European ethnomedicine *M. perennis* is administered for the treatment of various affections such as eczema, dropsy, and tumours (Blaschek *et al.*, 2008).

Previous phytochemical studies on *M. perennis* led to the identification of piperidine alkaloids (Swan, 1984, 1985; Lorenz *et al.*, 2009), flavonoid glycosides (Dumkow, 1969), simple phenolics, terpenes, triacylglycerols, tocopherols and sterols (Lorenz *et al.*, 2009). However, constituents from dog's mercury are still poorly known.

In continuation of our studies on the spectrum of bioactive metabolites from medicinal plants, dichloromethane (DCM) extracts of the herbal and root parts of *M. perennis* were investigated. As a previous study on the lipid constituents of *M. perennis* (Lorenz *et al.*, 2010) afforded a minor fraction that has not been characterized yet, the present work reports on the closer inspection of this fraction. Most unexpectedly, a complex mixture of *n*-alkylresorcinols (ARs) was found. Using LC-MS/MS and GC-MS techniques as well as chemical derivatization methods these compounds were identified as a homologous series of side chain-saturated ARs (C15:0–C27:0, see Fig. 1). ARs are a subclass of phenolic lipids which till now have been found in members from only a few plant families and display a multitude of biological activities (Kozubek and Tyman, 1999).

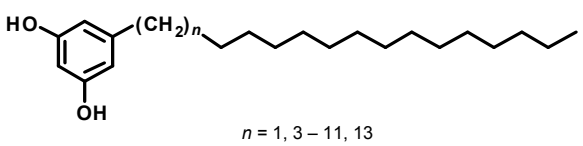

Fig. 1. Chemical structures of *n*-alkylresorcinols identified in *M. perennis*.

Materials and Methods

Extraction of the plant material

Herbal and root parts of *Mercurialis perennis* were collected in the mountain forest close to Bad Boll/Eckwälden (Baden-Württemberg, Germany) in April 2009 and identified by Prof. Otto Spring, Department of Botany, Hohenheim

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University, Stuttgart, Germany. Voucher specimens (HOH-006229-HOH-006232) are deposited at this department. The fresh plant material was kept at –80 ºC until investigation. Herbal parts of *M. perennis* (100.4 g) yielded 0.7 g crude DCM extract after repeated extraction (2 × 500 mL) at room temperature under nitrogen atmosphere. The crude DCM extract (0.7 g dissolved in 30 mL DCM) was loaded on a polyamide column (11 × 2 cm; particle size, 0.05–0.16 mm; Carl Roth, Karlsruhe, Germany) preconditioned with DCM (50 mL). Thereafter the column was washed with DCM (150 mL, fraction discharged) followed by methanol (200 mL). The yielded methanol fraction (70.0 mg) was chromatographed via vacuum liquid chromatography (VLC) on TLC grade silica gel (Merck) with a DCM/methanol mixture (49:1–48:2) yielding two AR-enriched fractions (23.0 + 3.0 mg) which were unified. For quantitative GC-MS analysis herbal or root parts (22.0–25.0 g) were immersed in DCM (250 mL). Subsequently, the plant material was minced by a Ultra-Turrax® (21,000 rpm; IKA-Werke, Staufen, Germany). A stream of nitrogen was bubbled through the extraction mixture for 5 min before and after Ultra-Turrax® treatment. The slurry was allowed to stand for 24 h. Afterwards the sediment was recovered by vacuum filtration over Celite and the filter cake re-extracted in the same manner again and finally washed with DCM (50 mL). Remaining water was removed from the combined filtrates by sodium sulfate and the DCM fraction evaporated to dryness under vacuum rotovaporation. For GC-MS measurements the residues were dissolved in chloroform (20 mL) containing the internal reference compound eicosane.

HPLC-DAD-MS/MS analyses

Chromatographic analyses were carried out with an Agilent 1200 HPLC system (Agilent Technologies Inc., Palo Alto, USA), equipped with a binary pump, a microvacuum degasser, an autosampler, a thermostatic column compartment and a UV-VIS diode array detector (DAD). The UV detection of the ARs was performed at 275 nm. A Synergy Hydro-RP column (4 μm, 2.0 × 150 mm i. d.; Phenomenex, Torrance, CA, USA) was used for chromatographic separation at 25 °C. The mobile phase consisted of water (mobile phase A) and methanol (mobile phase B) with a flow rate of 0.30 mL/min. Starting with 83% B at 0 min, a linear gradient was followed to 100% B at 60 min, kept for 5 min before re-equilibrating to starting conditions.

A purified AR fraction (23 mg, obtained via a procedure described above) was dissolved in methanol (10 mL). The resulting clear solution was filtered through a 0.45-μm GHP Acrodisc® membrane (PALL Life Sciences, Dreieich, Germany) before use. The injection volume of each sample was 20 μL. The HPLC system was coupled to a HCT ultraion trap (Bruker Daltonik, Bremen, Germany) fitted with an APCI source operating in the positive mode with the following parameters: HV voltage, –4000 V; dry gas, N_2; flow rate, 5.0 L/min; with a dry temperature set at 300 ºC; nebulizer, 2.72 atm; vaporizer temperature, 400 °C. Full scan mass spectra of the HPLC eluates were recorded during the chromatographic separation yielding $[M+H]^+$ ions. To obtain further structural information, these ions were trapped and fragmented to yield the precursor product patterns of the analytes. The mass range was recorded from *m/z* 50–1000 with a compound stability and trap-drive level of 100%. MS^n data were acquired in the auto-MS/MS mode. The instruments were controlled by an Agilent Chemstation and an EsquireControl Software.

Derivatization of the ARs for GC-MS analyses

To gain further structural information, the ARs were silylated or methoxylated and the so-obtained products were analyzed by GC-MS. The silylation of the analytes was implemented by treatment of the AR fraction (26 mg) with 0.5 mL chloroform and 0.2 mL silylating mixture Fluka I by Sweeley (45 min at 105 ºC) according to a previously reported procedure (Lorenz *et al.*, 2010).

The methoxylation was performed by treatment of the AR fraction [5.0 mg, dissolved in 10 mL methanol/water (9:1, v/v)] with a diazomethane/diethyl ether solution. The latter was prepared from *N*-nitrosomethyl urea (4.0 g in 48 mL diethyl ether) and an ice-cold aqueous potassium hydroxide solution (40%, 10 mL) by a literature procedure (Beckert *et al.*, 2009). After stirring (3 h) at room temperature the reaction mixture was purged with a vigorous stream of nitrogen to remove unreacted diazomethane and diethyl ether. Before GC-MS analyses the residual product was dissolved in methanol (1 mL).

GC-MS analyses

GC-MS was performed with a Perkin Elmer Clarus 500 gas chromatograph with split injection (split ratio, 30:1; injection volume, 1.0 μL) coupled to a mass detector. The column used was a Zebron ZB-5 ms capillary column (60 m × 0.25 mm i. d. × 0.25 μm film thickness, 5% phenylpolysiloxane and 95% dimethylpolysiloxane coating; Phenomenex). Helium was the carrier gas at a flow rate of 1 mL/min. The injector used was a PSS (programmed-temperature split/splitless injector; temperature, 250 ºC). The temperature program for the column oven was 100 to 320 ºC at 4 ºC/min with a final hold time of 30 min. The mass spectrometer was run in the electron ionization mode (70 eV). Synthetic AR reference standards (C19:0, C23:0, and C25:0) were purchased from ReseaChem (Burgdorf, Switzerland). The most abundant ARs were quantified in crude DCM extracts by GC-MS in the single ion mode (*m/z* 124) via external calibration using eicosane (*n*-C20) as internal standard. Three separate extractions were analyzed by GC-MS, each measured in triplicate (n = 3). Calibration curves were established for 1,3-dihydroxy-5-nonadecylbenzene (AR C19:0) in the range 0.003–0.113 mg/mL (r^2 = 0.997). The area percentages of the main ARs (C19:0, C21:0, and C23:0) were determined and calculated as C19:0 assuming equal peak sensitivities. AR concentrations were calculated on the dry matter basis of the plant material.

Results and Discussion

A purified AR fraction (see Materials and Methods) was analyzed by HPLC/DAD, using an APCI source in the MS/MS-mode according to Knödler *et al.* (2007, 2009). The HPLC chromatograms recorded at 275 nm showed several peaks whose UV spectra concordantly exhibited two specific maxima at 276 and 280 nm, characteristic for ARs (Knödler *et al.*, 2007). In the base peak chromatograms (BPC) the protonated molecular ions $[M+H]^+$ of the ARs were established. By fragmentation of these ions (MS^2) further intrinsic AR ions like *m/z* 123 and 111 were obtained. A graph of staggered extracted ion LC chromatograms (single ion mode, scanned on the molecular weight of the particular AR: $[M+H]^+$) is exemplarily shown in Fig. 2. However, sufficient structural assignment of the ARs was not possible by use

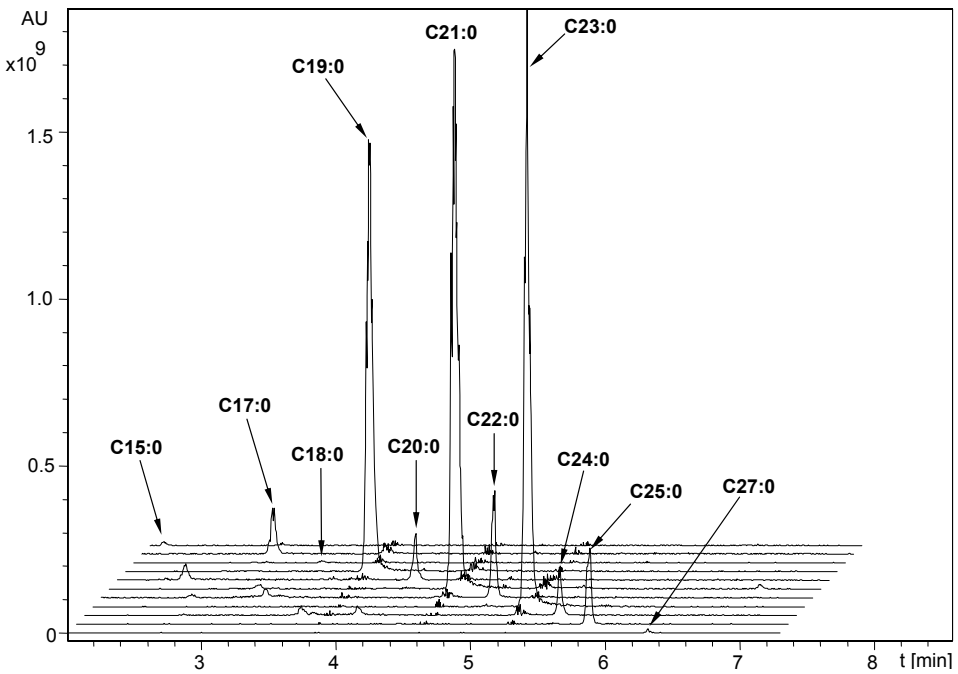

Fig. 2. Staggered illustration of LC-MS/MS extracted ion traces $[M+H]^+$ of individual *n*-alkylresorcinols.

of HPLC-MS. Therefore, a GC-MS method was established (see Materials and Methods). In the course of GC-MS investigations on the purified fraction 11 ARs could be detected. The derivatized ARs in which both phenolic hydroxy groups were silylated or methylated delivered additional chromatographic and mass spectrometric information and thus allowed a sound structural assignment (see Table I). Characteristic fragments at *m/z* 124, 268 and 152 were due to McLafferty rearrangement of the aromatic ring and observed as base peaks (100% BPI) in the EI-mass spectra of the non-derivatized, silylated and methylated ARs, respectively (Fig. 3, Table I). EI-MS fragmentation, *e.g.* formation of di-oxo-tropylium cations, *β*- and *γ*-cleavage at the side chain as well as a methyl cleavage from the TMSi derivatives (Fig. 3, Table I), support the structural characterization of the ARs and correspond with previous literature data (Ross *et al.*, 2004; Seitz, 1992). In addition to three predominant ARs (C19:0, C21:0, C23:0) four uncommon ARs with even-numbered side chain lengths (C18:0, C20:0, C22:0, C24:0) were detected in the extracts. While in most cases the aliphatic side chain of natural ARs is odd-numbered due to the specific biosynthetic assembly (Kozubek and Tyman, 1999; Suzuki *et al.*, 2003) the occurrence of even-numbered ARs in plants has only rarely been reported in the literature (Kato *et al.*, 1985).

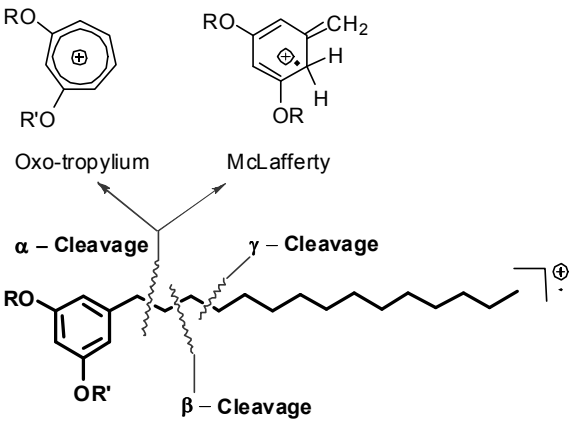

Fig. 3. Proposed mass spectroscopic fragmentation of *n*-alkylresorcinols and their oxo-substituted derivatives into their most abundant daughter ions, observed in the EI-mass spectrum (70 eV).

Finally, the structures of the ARs C19:0, C22:0, C23:0, and C25:0 were additionally proven by comparison of the chromatographic and mass spectrometric features with synthetic reference compounds.

Highest AR concentrations of C19:0, C21:0, and C23:0 were found by GC-MS in the herbal parts of *M. perennis* (134.4, 155.1, and 79.8 mg/kg, respectively). However, in root parts 7 to 9 times lower amounts of the ARs were determined

Table I. GC-MS data for the *n*-alkylresorcinols (ARs) and their trimethylsilyl and methyl derivatives.

n-Alkyl side chain	AR		1,3-Bis-TMSi-AR		1,3-Bis-methyl-AR	
	Rt [min]	*m/z*[a] $[M]^+$	Rt [min]	*m/z*[b] $[M]^+$, $[M–CH_3]^+$	Rt [min]	*m/z*[c] $[M]^+$
C15:0	48.7	320	46.7	464, 449	45.5	348
C17:0	52.3	348	50.1	493, 477	49.2	376
C18:0	54.0	362	51.7	507, 492	51.0	390
C19:0	55.7	376	53.4	521, 505	52.6	404
C20:0	57.6	390	54.9	534, 519	54.3	419
C21:0	59.6	404	56.6	549, 534	55.9	433
C22:0	62.0	418	58.3	563, 548	57.7	447
C23:0	64.8	433	60.4	577, 562	59.7	461
C24:0	68.2	447	62.8	591, 576	62.1	475
C25:0	71.9	461	65.4	605, 590	64.8	489
C27:0	[d]	–	[d]	–	71.8	517

[a] In addition to the fragments characteristic to the certain AR, fragments common for all homologous *n*-alkylresorcinols, *m/z* 166*, 137**, 124 (base peak)***, 123****, were also found.
[b] Fragments *m/z* 310*, 281**, 268 (base peak)***, 267**** for 1,3-bis-TMSi-ARs.
[c] Fragments *m/z* 194*, 165**, 152 (base peak)***, 151**** for 1,3-bis-methyl-ARs.
[d] Not detected.
(**γ*-Cleavage; ***β*-cleavage; ***McLafferty rearrangement; ****oxo-tropylium ion).

(17.7, 16.6 and 11.0 mg/kg, respectively). It may be assumed that the AR biosynthesis is accomplished in the herbal parts of the plant, the ARs afterwards being transported into the roots. The amphiphilic ARs (Fig. 1) are presumably located in the liposomal membranes of the plant cells where they mainly act as antioxidants (Kozubek and Nienartowicz, 1995). As pro-oxidative metabolic events are expected to be more dominant in the herbal parts, the quantitative distribution within the plant appears plausible.

ARs could also be detected in an aqueous fermented extract from *M. perennis* obtained via an official specification (HAB, 2008). This was quite unexpected because of the high log *P* values of ARs meaning that they are virtually insoluble in water (Ross *et al.*, 2004). Still, the ARs were in concentration ranges below the quantification limit even after pre-concentration steps.

While ARs have formerly been exclusively reported to be present in only a few plant families, *e.g.* Anacardiaceae (Knödler *et al.*, 2007, 2008; Skopp *et al.*, 1987), Araceae (Reffstrup and Boll, 1985), Gramineae (Seitz, 1992; Zarnowski *et al.*, 2004), Ginkgoceae (Fuzzati *et al.*, 2003), and Myristicaceae (Kato *et al.*, 1985) as well as in microorganisms (Kozubek and Tyman, 1999), their presence in the Euphorbiaceae family marks a novel finding. Since recent *in vitro* studies demonstrated that ARs exhibit a broad spectrum of beneficial biological activities, *e.g.* antioxidant (Kozubek and Nienartowicz, 1995), anti-inflammatory (COX-1 and COX-2) (Knödler *et al.*, 2008), antiparasitic (Valcic *et al.*, 2002), cytotoxic, and antimicrobial activity (Kozubek and Tyman, 1999), the present findings of ARs in *M. perennis* shed a new light on the bioactivity of extracts derived therefrom.

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