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Comprehensive Characterisation of *n*-Alkylresorcinols and other Lipid Constituents of *Mercurialis tomentosa* L. from Alicate, Spain

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ABSTRACT

Mercurialis tomentosa L. has been used in Spanish ethnomedicine. In the present study the first phytochemical characterisation of a lipid fraction from *M. tomentosa* was performed. The $CHCl_3$ extraction of aerial parts from *M. tomentosa* and GC-MS investigations revealed the occurrence of cuticular lipid and wax constituents, like long chain *n*-alcohols and *n*-aldehydes (C_{22}-C_{30}), besides several aromatic constituents, *i.e.* phenylpropanoids and *n*-alkylresorcinols. The latter were further purified by CC and analysed by LC-MS^n. In contrast to other *Mercurialis* species, *i.e. M. annua*, *M. perennis*, which exclusively contain 5-*n*-alkylresorcinols (**1a-j**, C_n), mainly 5-*n*-alkyl-2-methylresorcinols (**2a-j**, C_n*) with side chain lengths of C_{15}-C_{25} were found in *M. tomentosa*, in addition to **1a-j**. Thus, the latter compounds may be utilised for analytical characterisation and authentication of *M. tomentosa* based on fingerprinting methods. For structure elucidation a novel facile total synthesis of one representative 5-*n*-alkyl-2-methylresorcinol homologue (**2d**, C_{19}*) was developed, starting with a *Grignard* reaction from a substituted benzoic acid chloride (**19**). The compound obtained by synthesis was identical to the natural product **2d** in terms of its

chromatographic and spectroscopic features. Futhermore, **2d** exhibited satisfactory DPPH free radical scavenging activity (IC_{50} = 37.8 µM) when compared to trolox (IC_{50} = 21.0 µM), corroborating the antioxidant features of these amphipathic molecules.

Introduction

Lipids are a large group of natural constituents found in higher plants and fulfill a multitude of biological functions. Besides carbohydrates, lipids in form of lipid bodies are the most important energy stores in all cells. Beyond, lipids are hydrophobic or amphipathic molecules that may biosynthetically originate from carbanion-based condensations of thioesters (acetyl-CoA to fatty acids, polyketides, *etc.*) and/or from carbocation-based condensations of isoprene units (prenols, sterols, *etc.*) [1]. Depending on their chemical structure, lipids may be divided into glycerol esters of fatty acids (acylglycerols), phospho- and sphingo-lipids (ceramides), glycolipids, phenolic lipids, isoprenoids (*e.g.* sterols, carotenoids) and waxes (*e.g.* long chain *n*-alkanes, ketones, fatty acids and *n*-aldehydes) [2]. The latter are found as cuticular waxes, constituting a natural barrier against environmental impacts, and providing a successful strategy ensuring plant survival in the dry gaseous environment [3][4].

In our exploration of medicinal plants as a source of novel bioactive lipid constituents we closer investigated the genus *Mercurialis* (dog's mercury), which comprises 8 to 10 species [5]. *Mercurialis* species are mainly distributed in the Eurasian area, whereas *M. leiocarpa* is found in Asia [6]. The plants are dioecious and belong to the *Euphorbiaceae* (spurge family). Various subspecies and hybrids have been described [7]. Since ancient times, *Mercurialis* species have been used in European ethnomedicine to treat several diseases such as purulent wounds, eczema, abscesses, but also as a laxative and to cure menstrual complaints [8].

Nowadays, particularly *M. perennis* is applied in medicine, especially for the treatment of slowly healing wounds, burns, conjunctivitis (inflamed eyes) and against hemorrhoids [9]. Comprehensive phytochemical investigations of *M. perennis* and *M. annua* have revealed a broad spectrum of nonpolar and polar secondary plant metabolites, such as low-molecular phenolics, alkaloids, sterols, tocopherols, depsides, flavonoids, and essential oil components [10-12]. In this course, the occurrence of 5-*n*-alkylresorcinol homologues in *M. perennis* and *M. annua*, as a first proof of this compound class in the *Euphorbiaceae* family was reported [13].

Based on these phytochemical analyses, we aimed to broaden our knowledge of the pharmacognosy of this plant family. Consequently, *Mercurialis tomentosa* L., a species widely spread on the Iberian Peninsula (Portugal, Spain and southern parts of France), came into the focus of our interest. *M. tomentosa* (Spanish names: 'Carra' or 'hierba de Santa Quiteria', *etc.*) has already been described in 1753 by *Linné* in the publication 'Species Plantarum' [14]. The plant is white-silverish haired, has a tap root and is growing in sunny and dry areas [15]. Compared to other *Mercurialis* species, which are herbaceous plants, *M. tomentosa* is a subshrub with lignified stalks.

Water decoctions of the entire plant have formerly been used in Spanish ethnomedicine for the treatment of external swellings, rheumatism, inflammations and internally as a purge or against intestinal problems [15-17]. Interestingly, pharmacological investigations could recently show *in vitro* anti-inflammatory effects by inhibition of PGE_2 and TNF-α in human monocytes, due to the application of a *M. tomentosa* EtOAc extract [18]. However, information on the natural constituents of *M. tomentosa*, let alone the active principles, are completely lacking in the literature. Thus, the current investigations focused on the analytical characterisation of lipid constituents from *M. tomentosa*.

Results and Discussion

For the investigation of lipid constituents, a $CHCl_3$ extract from fresh herbal parts of *M. tomentosa* was prepared and analysed by GC-MS. A complex peak profile was detected in the GC-MS chromatograms, covering a retention time range of t_R 40-60 min (*Fig. 1a*). By comparing the mass spectra of these peaks with the NIST database and with those of reference compounds, several long chain *n*-aldehydes and *n*-alcohols of carbon-chain lengths C_{22}-C_{30} were assigned, with the even-numbered homologues C_{24}, C_{26} and C_{28} predominating. Long chain *n*-aldehydes and *n*-alcohols are known to be typical constituents of cuticles, covering the aerial portions of land plants and building a protective layer against dehydration, UV irradiation, and pathogen attack [3][19]. Some cuticular wax components build crystalline like structures as platelets, tubules or threads [3]. No conspicuous crystalline structures were observed in the present study for *M. tomentosa*. SEM analysis revealed a continuous smooth cuticular wax film on trichomes and epidermis under the thick layer of trichomes (*Fig. 2a, b, c*). Thus, for *M. tomentosa* it becomes apparent that the trichomes have a function in UV protection or may serve as a protection against desiccation [20] and were developed, together with wax components, as a result of environmental adaptation. While the occurrence of *n*-aldehyde and *n*-alcohol homologues in quince wax (*Cydonia oblonga* L., Rosaceae) has been reported recently [21], these constituents have not been analysed before in the genus *Mercurialis*.

Furthermore, the $CHCl_3$ extract of *M. tomentosa* was scrutinised with regard to the occurrence of phenolic lipids, *i.e.* alkyl phenols. By selected ion monitoring (SIM) of base peak fragments at *m/z* 124 small amounts of some 5-*n*-alkylresorcinol homologues were detected in the GC-MS chromatogram of the $CHCl_3$ extract (data not shown). Furthermore, compounds with similar mass spectra were observed, exhibiting a 14 Da extension (CH_2) of the base peak fragment (*m/z* 138). However, the concentrations of these constituents were too

low to obtain significant mass spectra. Consequently, compound enrichment was achieved by loading a CH_2Cl_2 extract of *M. tomentosa* onto a polyamide column. After washing the column with CH_2Cl_2 to remove nonpolar constituents, *n*-alkylresorcinols were obtained as a highly concentrated fraction by elution with MeOH. Henceforth, GC-MS investigations of this fraction revealed a mixture of compounds derived from two structural types of *n*-alkylresorcinol homologues, as can be deduced from SIM of fragments at *m/z* 124 and 138, respectively (*Fig. 1b, c* and *Table 1*). By comparison of the mass spectra with literature data [13][22] the latter were identified as 5-*n*-alkylresorcinol- and 5-*n*-alkyl-2-methylresorcinol homologues (**1a-j** and **2a-j**, respectively), comprising side chain lengths of C_{15} – C_{25}. Structural assignment of the 5-*n*-alkylresorcinols (**1**, C_n) was further supported by comparison with a reference compound (**1d**, C_{19}).

While 5-*n*-alkylresorcinols (**1**, C_n) have previously been detected in *M. perennis* and *M. annua* [13], the occurrence of 5-*n*-alkyl-2-methylresorcinols (**2**, C_n*) has so far only been reported for two species of other plant families, *i.e. Polygonum maritimum* (Polygonaceae family) and *Dactylis glomerata* subsp. *hispanica* (Poaceae family) [22][23]. Accordingly, the newly detected homologous compounds (**2**, C_n*) may serve as specific chemotaxonomic markers for *M. tomentosa* (*Fig. 1b, c*), *e.g.* for comparative phytochemical studies considering further *Mercurialis* species.

The chain-length distribution of the *n*-alkylresorcinols was assessed based on the relative peak areas of the extracted ion chromatograms (*Fig. 3*). The odd-numbered C_{19} and C_{21} homologues of both *n*-alkylresorcinol types (**1d, f** and **2d, f**, respectively) were found to predominate. Noteworthy, also the piperidin-2,6-dione alkaloids hermidin (**5**) and hermidin quinone (**8**) were identified in the MeOH polyamide fraction of the CH_2Cl_2 extract as typical marker components of *Mercurialis* [10][12], based on their mass spectra and by comparison with synthetic reference standards (see GC-MS chromatogram, *Fig. 4*). In addition, several

low-molecular aromatic compounds, like *p*-hydroxybenzaldehyde (**3**), *p*-hydroxybenzonitrile (**4**), vanillin (**9**) and the phenylpropanoids coniferyl alcohol (**11**), *o*- and *p*-coumaric acid methyl and ethyl esters (**10**, **12** and **13**, respectively), and sinapyl alcohol (**16**), were detected (*Fig. 4*). Interestingly, phenylpropanoids, like *p*-coumaric acid esters, have been described as integral constituents of the cutin polyester matrix [24], while the cinnamoyl alcohols **11** and **16** are lignin monomers [25].

Moreover, the fraction eluted with MeOH from the polyamide column was also investigated by HPLC-APCI$^+$-MS^n to characterise the assigned *n*-alkylresorcinols more closely. All *n*-alkylresorcinols detected by GC-MS were corroborated by LC-MS (*Fig. 5*, *Table 1*). Expectably, differences in the fragmentation patterns of the 5-*n*-alkyl-2-methylresorcinols (**2**, C_n*) in the EI-MS and APCI$^+$-MS mode were observed (*Scheme 1*, *Table 1*). Thus, α-cleavage of the aliphatic side chain and subsequent *McLafferty* re-arrangement of the phenolic ring yielded a base peak fragment at *m/z* 138 and a minor fragment at *m/z* 137, due to the formation of a dihydroxytropylium cation in the EI-MS experiments [13][26]. In addition, fragments at *m/z* 151 and 180 were detected as a result of a β- and δ-cleavage, respectively (*Scheme 1*, *Fig. 6a*). In contrast, in the APCI$^+$ ionisation mode the preferential fragmentation is characterised by a release of the aliphatic side chain from the aromatic backbone, presumably by formation of a phenyloxonium cation (*m/z* 125) or phenoxy cation (*m/z* 123; see *Scheme 1*, *Fig. 6b* and *Table 1*), followed by the subsequent release of water to form a phenyl cation at *m/z* 107. The MS^n fragmentation patterns of the individual 5-*n*-alkyl-2-methylresorcinol- (**2**, C_n*) and 5-*n*-alkylresorcinol-homologues (**1**, C_n) in the APCI$^+$ mode were not consistent, which is probably due to differing concentrations of the analytes (*Table 1*). However, the LC-MS data supported the structure assignment of the target analytes **1** and **2**.

To further corroborate structure assignment and to obtain greater substance amounts, *e.g.* for pharmacological studies, an attempt to realise a total synthesis of one representative *n*-alkylresorcinol homologue, namely 5-*n*-nonadecyl-2-methylresorcinol (**2d**), was made. Several approaches for the synthesis of *n*-alkylresorcinols are known from literature [27-29]. However, we here report a synthesis of **2d** for the first time, starting from a *Grignard* reaction (*Scheme 2*): The substituted benzoic acid chloride **19**, obtained from **18** by chlorination with oxalyl chloride, was treated at -78^{o} with a THF solution of *n*-octadecylmagnesium chloride in the presence of bis-[2-(*N,N'*-dimethylamino)ethyl]ether [30], to yield ketone **20**. Subsequently, **20** was reduced via a *Wolff-Kishner* reaction at 200^{o} in the presence of hydrazine hydrate, KOH and triethylenglycol, to obtain **21**. Then, **2d** was yielded from the latter by removing the protective groups through de-methylation with BBr_3 in CH_2Cl_2 at 0^{o} (*Scheme 2*). With respect to its chromatographic and mass spectrometric features, **2d** matched with the respective natural constituent of *M. tomentosa*.

Various biological activities have been reported for *n*-alkylresorcinols in the literature. *n*-Alkylresorcinols are assumed to play an important role as phytoanticipins and allelochemicals [22][31]. *n*-Alkylresorcinols also exert cytotoxic, antimicrobial [32], anti-inflammatory (COX-1/2) [33] and antioxidant activity *in vitro* [34]. Exemplarily, we herein investigated the radical-scavenging activity of **2d** using the DPPH (1,1'-diphenyl-2-picryl-hydrazyl) assay. DPPH is an artificial, stable radical, frequently used for common antioxidant assays [35][36]. This compound dissolves in MeOH yielding a purple color. By adding antioxidants DPPH is reduced, thus forming a decolored reaction product (yellow color). This effect can be quantitated by spectrophotometric measurements. When **2d** was applied to DPPH a logarithmic concentration-absorption correlation was observed. From the resulting regression equation an IC_{50} of 37.86 µM was calculated for **2d** (*Table 3*). A five-fold higher IC_{50} as compared to **2d** has been reported in the literature for **1d** [34]. The activity of **2d** was

further compared to the common antioxidant trolox, which revealed a linear concentration-absorption correlation, from which an IC_{50} value of 21.03 µM was deduced, being in agreement with literature data [34].

Conclusions

The current analytical investigations into $CHCl_3$ extracts of *M. tomentosa* revealed the occurrence of several lipid components, which were detected for the first time in this plant species. Long-chain *n*-alcohols and *n*-aldehydes were identified as characteristic wax compounds, which may play a significant role in the regulation of the barrier properties of plant cuticles [3]. Furthermore, two homologous series of *n*-alkylresorcinols were analysed together with further phenolic components (*e.g.* phenylpropanoids) of similar polarity. The newly detected 5-*n*-alkyl-2-methylresorcinols (**2**, C_n*), which are quantitatively predominant among the phenolic lipids in *M. tomentosa*, were identified as specific markers. The latter may be utilised for chemotaxonomic differentiation of *Mercurialis* species and, thus, may help to align phytochemical data with phytogenetic studies already published for *Mercurialis* [6]. To support these findings a higher number of samples covering different geographic regions should be considered in future studies.

Moreover, by investigation of one representative 5-*n*-alkyl-2-methylresorcinol **2d** (C_{19}*) in the DPPH assay the antioxidant features of this compound class were assessed. Furthermore, since *n*-alkylresorcinols have recently been demonstrated to exert anti-inflammatory (COX-1/2) activities *in vitro* [33], it is assumed that the formerly described anti-inflammatory effects of a *M. tomentosa* extract [18] could be assigned to these phenolic lipid constituents. However, further investigations are required to ascertain this assumption.

Experimental Part

General

Source of reference compounds for GC analyses: 1-hexacosanol and 1-octacosanol (C_{26}/C_{28}-Alc, respectively), 4-hydroxybenzaldehyde (**3**), 4-hydroxybenzonitrile (**4**), ethyl 4-hydroxybenzoate (**7**), coniferyl alcohol (**11**), and *n*-hexadecanoic acid (**14**) were purchased from *Sigma-Aldrich* (Steinheim, Germany). The *n*-aldehydes (C_{26}/C_{28}/C_{30}-Ad), hermidin (**5**) and hermidin quinone (**8**) were synthesised according to previously published procedures [10] [21]. 5-*n*-Nonadecyl-resorcinol (**1d**, C_{19}) was obtained from *ReseaChem* (Burgdorf, Switzerland). 3,5-Dimethoxy-4-methylbenzoic acid (**18**) was purchased from *Alfa Aesar* (Karlsruhe, Germany), and Scopoletin (**15**) from *Phytolab* (Vestenbergsgreuth, Germany). Ethyl 4-(*E*)-hydroxycinnamate was obtained by synthesis from *p*-coumaric acid and EtOH, based on a literature protocol [37].

NMR Spectroscopy

NMR spectra were recorded in $CDCl_3$ using a *Varian Unity Inova* 500 MHz spectrometer. The measurement temperature was 298 K. Chemical shifts were expressed in δ [ppm] and referenced to residual (non-deuterated) solvent signals of $CDCl_3$ (^{1}H: δ(H) 7.27; ^{13}C: δ(C) 77.00). ^{13}C-NMR signal assignment of the novel compounds was based on 2D heteronuclear NMR experiments (gHMBC and gHSQC). ^{13}C-NMR chemical shifts of aromatic carbons were assigned by increments [38]. For evaluation of NMR spectra the program *SpinWorks 3.1.7.* (Copyright® 2010, K. Marat, University of Manitoba, USA) was used.

IR Spectroscopy

IR spectra of the synthesised compounds were recorded on a *Bruker Tensor* 27-FT-IR spectrometer at the *SGS Institute Fresenius* (Taunusstein, Germany).

Plant material

Aerial parts of *M. tomentosa* were collected at Alcocer de Planes, Alicante (Spain) in April 2015, air dried and stored at -80^{o} until investigation. A voucher specimen of *M. tomentosa* (voucher no. HOH-014615) was deposited in the herbarium of the Department of Botany at Hohenheim University (Germany). The plant material was identified by Prof. *O. Spring*.

Extraction and Chromatographic Purification of n-Alkylresorcinols

Dried herbal parts of *M. tomentosa* (25.0 g) were extracted with CH_2Cl_2 (250 ml). The mixture was flushed with nitrogen (N_2) for 10 min. and stored overnight in a refrigerator (+ 4^{o}). Afterwards, the plant material was filtered off over celite by vacuum suction, washed with CH_2Cl_2 (250 ml), and subsequently the extraction was repeated for a second time in the same manner. The solvent was removed from the combined extracts by vacuum rotary evaporation to yield 0.61 g of crude material. The latter was loaded onto a polyamide column (10.5 g, 20 cm x 2 cm, i.d.), preconditioned with CH_2Cl_2 (50 ml). Compound elution was performed with CH_2Cl_2 (150 ml, fraction discarded) and MeOH (150 ml). The solvent was removed *in vacuo* from the MeOH fraction to yield 0.04 g material, which was highly enriched in *n*-alkylresorcinols and analysed by GC-MS and LC-MS^{n}. At the same time, a

sample of fresh plant material (11.5 g) was extracted with $CHCl_3$ (250 ml) and stored at RT until investigation.

GC-MS Analysis

GC-MS analyses were performed with a *Clarus 500* (*PerkinElmer, Inc.*, Shelton, CT, USA) gas chromatograph with split injection (split ratio 30:1, injection volume 1.0 µl) coupled to a single quadrupole mass spectrometer. The column used was a *Zebron ZB-5ms* cap. column (60 m x 0.25 mm i. d. x 0.25 µm film thickness, 5% phenylpolysiloxane and 95% dimethylpolysiloxane coating; *Phenomenex*, Torrance, USA). Carrier gas: helium at a flow rate of 1 ml/min. The injector used was a PSS (programmed-temperature split/splitless injector; temperature: 250°). The temperature program for the column oven was 100 to 320° with a linear gradient of 4°/min and a final hold time of 30 min. The mass spectrometer was run in electron ionisation mode (70 eV).

RP-HPLC-APCI$^+$-MS^n Analyses

LC-MS analyses where carried out on an *Agilent 1200* HPLC system (*Agilent*, Waldbronn, Germany) equipped with a degasser, a binary pump, an autosampler, a thermostated column compartment and a diode array detector connected to a HCTultra ion trap MS detector equipped with an APCI ion source (*Bruker Daltonik GmbH*, Bremen, Germany). For chromatographic separation, a *Synergi 4u Hydro-RP 80A* column (150 x 2.0 mm i.d., 4 µm particle size, *Phenomenex*, Torrance, USA) was used at 25°C and a flow rate of 0.3 ml/min. The mobile phase consisted of H_2O (eluent A) and MeOH (eluent B). Elution started with 83% B, followed by a linear gradient to 100% B within 60 min, staying isocratic

for 5 min before re-equilibration to starting conditions within 5 min. The injection volume was 20 µl. Detection was performed by mass spectrometry with an APCI interface operating in the positive ionisation mode, applying the following parameters: HV capillary: -4000 V; dry gas N_2: 5.00 l/min with a dry gas temperature of 300°C; nebuliser: 40 psi; vaporiser temperature: 400°C. Full scan mass spectra (range from *m/z* 50 to 700) of the HPLC eluates were recorded during chromatographic separation. MS^n data were acquired in the auto MS/MS mode. The instruments were controlled by *Agilent ChemStation* (Rev. B.01.03 SR1) and *Bruker Daltonik EsquireControl* software (V6.1).

Synthesis of the Reference Compound 1,3-Dihydroxy-2-methyl-5-nonadecylbenzene (= *5-Nonadecyl-2-methyl-resorcinol,* **2d**). *3,5-Dimethoxy-4-methyl-benzoic acid chloride* (**19**)

A solution of 3,5-dimethoxy-4-methylbenzoic acid (**18**, 10.36 g, 52.80 mmol) in toluene (244 ml) was treated with 4 drops of DMF and cooled in an ice/NaCl-mixture. Subsequently, oxalyl chloride (9.5 ml, resp. 14.0 g, 110.30 mmol) was added dropwise under stirring, which was continued for 3 h 40 min, while the mixture was slowly warmed up to room temperature. Afterwards, toluene was removed by vacuum rotary evaporation to yield a crude product (11.3 g). For de-colorisation, the latter was dissolved in EtOAc (100 ml), 0.2 g of activated carbon was added to the solution, stirred for 5 min, and filtered over Celite by vacuum suction, which was washed with EtOAc (2 x 50 ml). The solvent was removed *in vacuo* from the combined filtrates to yield a light beige product (**19**, 11.0 g, yield: 97.0 % of the theory), which was used without further purification. GC-MS purity (70 eV): >99% (t_R min) *m/z* 216, 214 (6, 19, [M ($^{35}Cl/^{37}Cl]^+$), 179 (100,$[M-Cl]^+$), 151 (9), 136 (13), 121 (7), 106 (4), 91 (22), 77 (14), 65 (10), 51 (8).

1,3-Dimethoxy-2-methyl-5-(1-oxo-nonadecyl)benzene (*=5-(1-Oxononadecyl)-2-methylresorcinol,* **20**)

Bis-[2-(N,N-dimethylamino)ethyl] ether (9.5 ml, resp. 8.0 g, 50 mmol) was added dropwise under stirring to a solution of *n*-octadecylmagnesium chloride in THF (0.5 M, 100 ml) under N_2, which was cooled with a dry ice/Me_2CO mixture (-78°) [30]. Thereafter, a solution of 3,5-dimethoxy-4-methyl-benzoic acid chloride (**19**, 10.73 g, 50 mmol) in dry THF (50 ml) was added within 15 min, the reaction mixture was further stirred at -78° (20 min) and then for 5 h at room temperature. Thereupon, the reaction was quenched by adding a sat. ice-cooled NH_4Cl solution (150 g NH_4Cl/500 ml water). After extraction with Et_2O (3 x 200 ml), the combined extracts were dried (Na_2SO_4) and the solvent was distilled off by vacuum rotary evaporation. The crude solid product (22.7 g) obtained was treated with petroleum ether (250 ml, b.p. 40-60°) and the mixture stirred for 15 min. Then, the sediment was filtered off by vacuum suction, washed with petroleum ether (2 x 50 ml) and dried at room temperature to yield the crude product **20** (11.9 g). The latter was recrystallised from ethanol (96%, *v/v*, 500 ml) to give the pure **20** (10.8 g, yield: 50.0 % of the theory). White powder. M. p. 82° (in accordance with the literature [39]). R_f (SiO_2; *n*-hexane/EtOAc = 15:3 (*v/v*)) 0.65. UV(ACN): 216 (4.38), 274 (4.00). GC-MS purity (70 eV): >99.9% (t_R 58.0 min) *m/z* 432 (8, $[M]^+$), 404 (1, $[M\text{-}CO]^+$), 334 (0.6), 320 (0.7), 207 (6), 194 (100, $[C_{11}H_{14}O_3]^+$), 179 (54, $[C_{10}H_{11}O_3]^+$), 166 (8), 151 (7), 136 (5), 121 (3), 91 (9), 69 (6), 55 (13). APCI$^+$-MS *m/z* 433* $[M+H]^+$(MS^1); 281* $[M\text{-}C_9H_{11}O_2+H]^+$, 263 $[281\text{-}H_2O]^+$, 207, 193, 179, 165* $[C_{10}H_{13}O_2]^+$, 151, 137* $[C_8H_9O_2]^+$, 123 (MS^2); 95, 81, 68 (MS^3);*parent ions for the next fragmentation step. ^{1}H NMR ($CDCl_3$, 500 MHz): 7.15 (*s*, H-C(21/21')), 3.89 (*s*, H-(C25/25'), OCH_3), 2.94 (*t*, *J* = 7.4 Hz, H-C(2)), 2.14 (*s*, H-C(24)), 1.74 (*tt*, *J* = 7.4, 7.4 Hz, H-C(3)), 1.39 - 1.26 (*br s*, H-C(4)-C(18))**, 0.89 (*t*, *J* = 7.0 Hz, H-C(19)). ^{13}C NMR ($CDCl_3$, 125 MHz): 200.13 (C(1), C=O), 158.23 (C(22/22')), 135.58 (C(20)), 120.60 (C(23)), 103.32 (C(21/21')),

55.86 (C(25/25‘), OCH_3), 38.51 (C(2)), 31.92 (C(17)), 29.69, 29.67, 29.65, 29.63, 29.52, 29.51, 29.41, 29.35 (C(4)-C(16))**, 24.62 (C(3)), 22.68 (C(18)), 14.10 (C(19)), 8.68 (C(24)). ** 5 signals overlapped. IR (KBr, ν in cm^{-1}): 2998 (w, aromatic CH), 2949 (m, aromatic CH), 2916 (s, alkyl-CH), 2850 (s, alkyl-CH), 1668 (m, C=O), 1584 (m), 1473 (m), 1410 (m), 1324 (w), 1309 (m), 1237 (m), 1157 (m), 1141 (s, OCH_3), 861 (w), 822 (w), 715 (m).

1,3-Dimethoxy-2-methyl-5-nonadecylbenzene (=5-Nonadecyl-2-methylresorcinol dimethylether, **21***), via* Wolff-Kishner *Reduction*

A mixture of **20** (5.00 g, 11.56 mmol), triethylene glycol (63 ml), powdered KOH (2.59 g, 46.16 mmol) and aqueous NH_2OH solution (80%, *v/v*, 2.2 ml) was stirred under N_2 at 200° for 3 h. After cooling down, the reaction mixture was extracted with Et_2O (5 x 100 ml), the unified extracts were dried (Na_2SO_4) and the solvent was removed *in vacuo* to yield a yellow solid crude material. The latter was washed with Me_2CO (5 x 100 ml) and, after drying, the solid product recrystallised from EtOH (90 ml) to yield pure compound **21** (2.92 g, yield: 60.4% of the theory). M. p. 54.4° (in accordance with literature [39]). R_f (SiO_2; *n*-hexane/EtOAc = 19:1 (*v/v*)) 0.55. UV-VIS (ACN): 206 (4.60), *sh* 226. GC-MS purity (70 eV): >99.9% (t_R 54.4 min) *m/z* 418 (27, $[M]^+$), 376 (0.6), 334 (0.4), 250 (0.4), 208 (2), 179 (14, $[C_{11}H_{15}O_2]^+$), 166 (100, $[C_{10}H_{14}O_2]^+$), 151 (11), 135 (8), 121 (3), 105 (3), 91 (5), 79 (2), 69 (3), 57 (15). APCI$^+$-MS *m/z* 419*$[M+H]^+$ (MS^1); 153* $[M+C_{19}H_{38}+H]^+$ (MS^2); 138*[153-CH_3] (MS^3); 123 [138-CH_3], 109 [138-CH_2] (MS^4); * parent ions for the next fragmentation step. ^{1}H NMR ($CDCl_3$, 500 MHz): 6.38 (*s*, H-C(21/21‘)), 3.82 (s, H-C(25/C25’), OCH_3), 2.58 (*t*, *J* = 7.8 Hz, H-C(1)), 2.07 (*s*, H-C(24)), 1.62 (*tt*, *J* = 7.4, 7.4 Hz, H-C(2)), 1.33 - 1.27 (*br s*, H-C(3-C18))**, 0.89 (*t*, *J* = 6.9 Hz, H-C(19)). ^{13}C NMR ($CDCl_3$, 125 MHz): 158.13 (C(22/22‘)), 141.47 (C(20)), 111.60 (C(23)), 103.80 (C(21/21‘)), 55.69 (C(25/25’), OCH_3),

36.58 (C(1)), 31.92 (C(2)), 31.70 (C(3)), 29.70, 29.68, 29.65, 29.63, 29.56, 29.46, 29.36 (C(4-C17))**, 22.69 (C(18)), 14.11 (C(19)), 7.93 (C(24)).** 7 signals overlapped. IR (KBr, ν in cm^{-1}): 2995 (w, aromatic CH), 2955 (m, aromatic CH), 2916 (s, alkyl-CH), 2849 (s, alkyl-CH), 1606 (m), 1586 (s), 1471 (m), 1461 (m), 1414 (m), 1308 (w), 1239 (m), 1182 (w), 1140 (s, OCH_3), 818 m), 718 (m), 663 (w), 590 (w). Noteworthy, the solvent was removed *in vacuo* from the Me_2CO washings (see above), and the residual yellow solid was suspended in cooled Me_2CO (20 ml), filtered off and washed with cooled Me_2CO (20 ml) to yield *3-methoxy-2-methyl-5-nonadecyl-1,4-quinone* as a by-product (1.52 g, yield: 25.8% of the theory) after drying (M. p. 77-78°, M. $p._{Lit.}$ 68-74° [39]). R_f (SiO_2; *n*-hexane/AcOEt = 19:1 (*v/v*)) 0.22.

1,3-Dihydroxy-2-methyl-5-nonadecylbenzene (=*5-Nonadecyl-2-methylresorcinol,* **2d**)

A solution of **21** (1.00 g, 2.39 mmol) in CH_2Cl_2 (40 ml) was cooled with an ice/NaCl mixture under a N_2 atmosphere. BBr_3 (1.2 ml, resp. 3.16 g, 12.64 mmol) was added under stirring, and stirring was continued for 6 h 30 min, while the reaction mixture warmed up to room temperature. Then the reaction was quenched by adding a sat. aqueous $NaHCO_3$ solution (10%, w/v, 200 ml) and the mixture extracted with CH_2Cl_2 (3 x 100 ml). The combined extracts were dried (Na_2SO_4) and the solvent was removed *in vacuo*. Purification of the crude product (0.88 g) was achieved by vacuum liquid chromatography (VLC) on silica 60 G (100 g), preconditioned with *n*-hexane. Elution was performed with an *n*-hexane/AcOEt gradient (100/0 to 80/20, *v/v*). The corresponding fractions containing the target product (analysed by TLC) were combined and the solvent was distilled off *in vacuo* to afford the pure compound **2d** (0.65 g, yield: 69.8% of the theory). White solid, M. p. 107.4°. R_f (SiO_2; CH_2Cl_2/MeOH = 19:1 (*v/v*)) 0.55. UV/VIS (ACN): 204 (4.58), *sh* 226, 270 (2.98).

GC-MS purity (70 eV): > 98 % (t_R 57.6 min) *m/z* 390 (9), 348 (0.4), 306 (0.5), 236 (0.2), 222 (0.6), 207 (0.4), 180 (3), 165 (0.7), 151 (10), 138 (100), 137 (39), 123 (5), 109 (1), 91 (2), 83 (2), 69 (3), 57 (9). IR (KBr, ν in cm^{-1}): 3350 (brs, OH), 2954 (m, aromatic CH), 2917 (s, alkyl-CH), 2847 (s, alkyl-CH), 1632 (m), 1587 (m), 1523 (w), 1466 (m), 1426 (m), 1327 (m), 1169 (m), 1095 (m), 1072 (s), 924 (w), 843 (m), 721 (m), 661 (w). The MS and NMR data correspond to the literature (Sconamiglio et al. 2012). For $APCI^+$-MS data see Table 1.

DPPH Radical Scavenging Assay

The DPPH assay was performed according to a literature procedure [36]. In brief, different concentrations of the test compounds in MeOH (200 µl) were added each to 1800 µl of 1,1'-diphenyl-2-picryl-hydrazyl (DPPH, 100 µM) and incubated for 30 min at 37°. Then, the absorbance at λ_{max} 516 nm was measured using a Perkin-Elmer UV/VIS-spectrometer (Lambda 2). IC_{50} values were calculated from the plot of resulting absorbance/concentrations curves by regression analysis. Scavenging activities were expressed as IC_{50} values, which denote the concentrations of the test compound (µM) required to bring about a 50% reduction in absorbance relative to that of the control. The lower the IC_{50} value, the higher is the scavenging activity of the corresponding compound.

Scanning Electron Microscopy (SEM)

Samples of upper and lower surfaces of a fully developed leaf of a herbarium specimen (voucher no. HOH-014615) were prepared for scanning electron microscopy. The leaf samples, size 7-10 mm x 2-3 mm, were cut with a razor blade, and also a razor blade was used to partly remove the trichomes from the leaves, to reveal the cuticle of the epidermis

between the trichomes. The dry samples were mounted on adhesive carbon tabs on Al-stubs, sputter-coated with gold-palladium (*SCD 040*, Balzer Union, Wallruf, Germany) and investigated using a scanning electron microscope (*DSM 940*, Zeiss, Oberkochen, Germany) at 5 and 7 kV.

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Figures

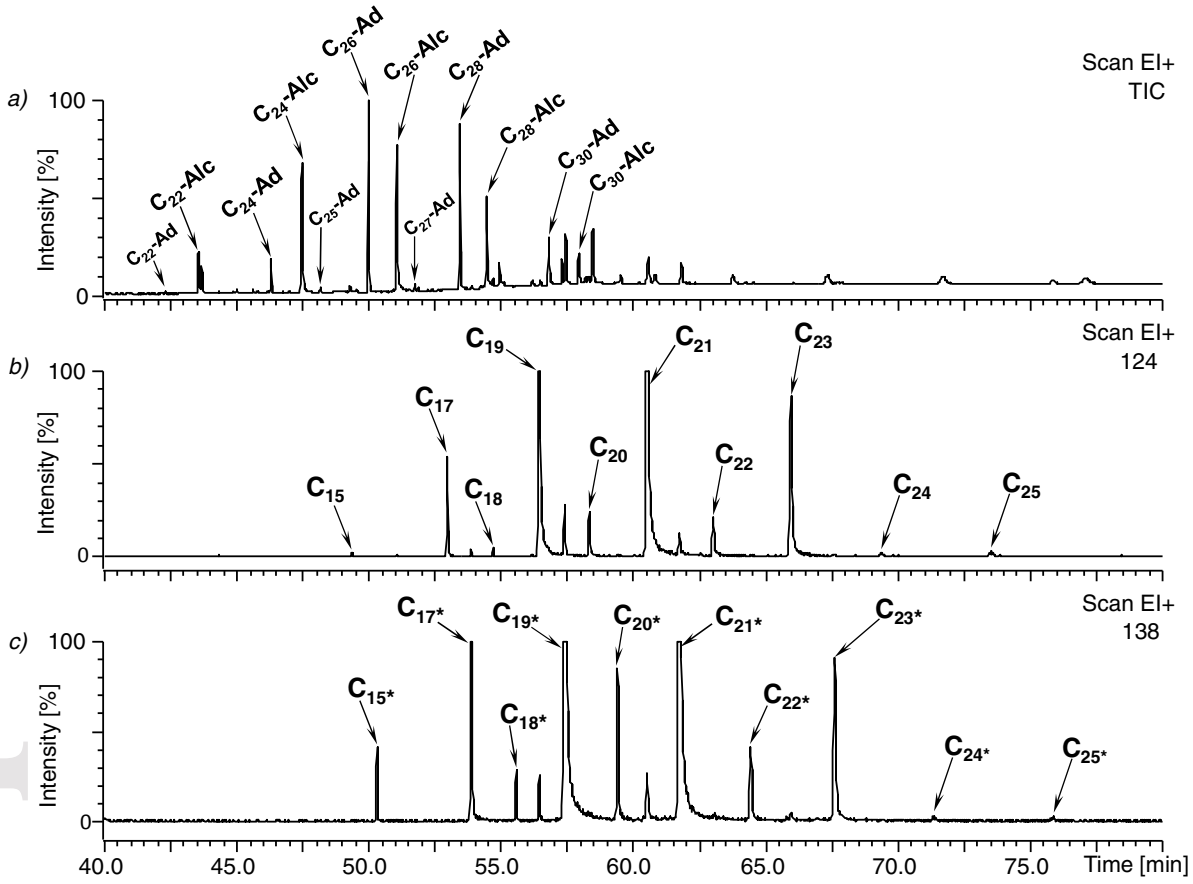


Fig. 1. Sections of a GC-MS profile showing lipid constituents of *M. tomentosa* L. *a*) Crude $CHCl_3$ extract, displays fatty *n*-alcohols (Alc) and *n*-aldehydes (Ad). *b*) MeOH fraction resulting from polyamide chromatography; Scan on *m/z* 124, exhibiting 5-*n*-alkylresorcinols (**1a-j**, C_n). *c*) Scan on *m/z* 138, exhibiting 5-*n*-alkyl-2-methylresorcinols (**2a-j**, C_{n*}).

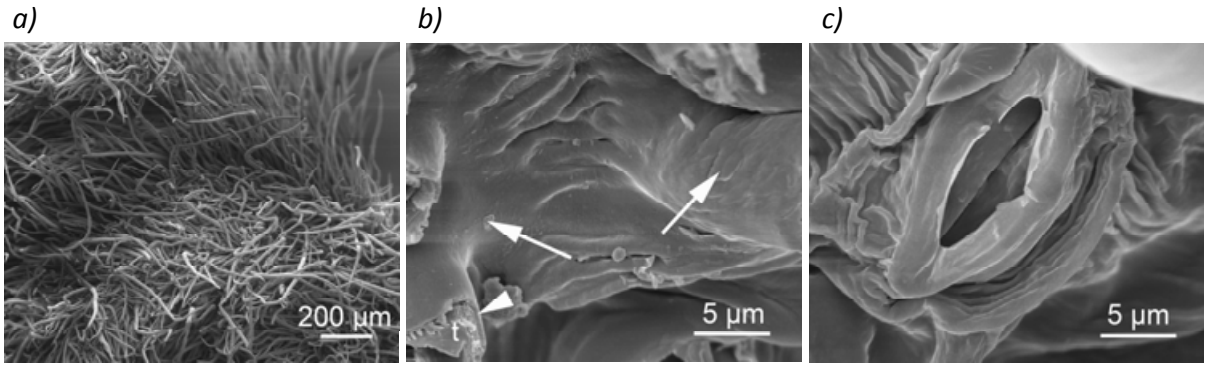


Fig. 2. Scanning electron micrographs of leaves of *M. tomentosa* L. *a*) Upper leaf surface densely covered with trichomes. *b*) Upper leaf surface after removing trichomes exhibiting a continuous smooth cuticle with only few wax particles (arrows); cut off trichome (t); cutting edge of the cuticle (arrow head). *c*) Undersurface showing the cuticular ledge of a stoma.

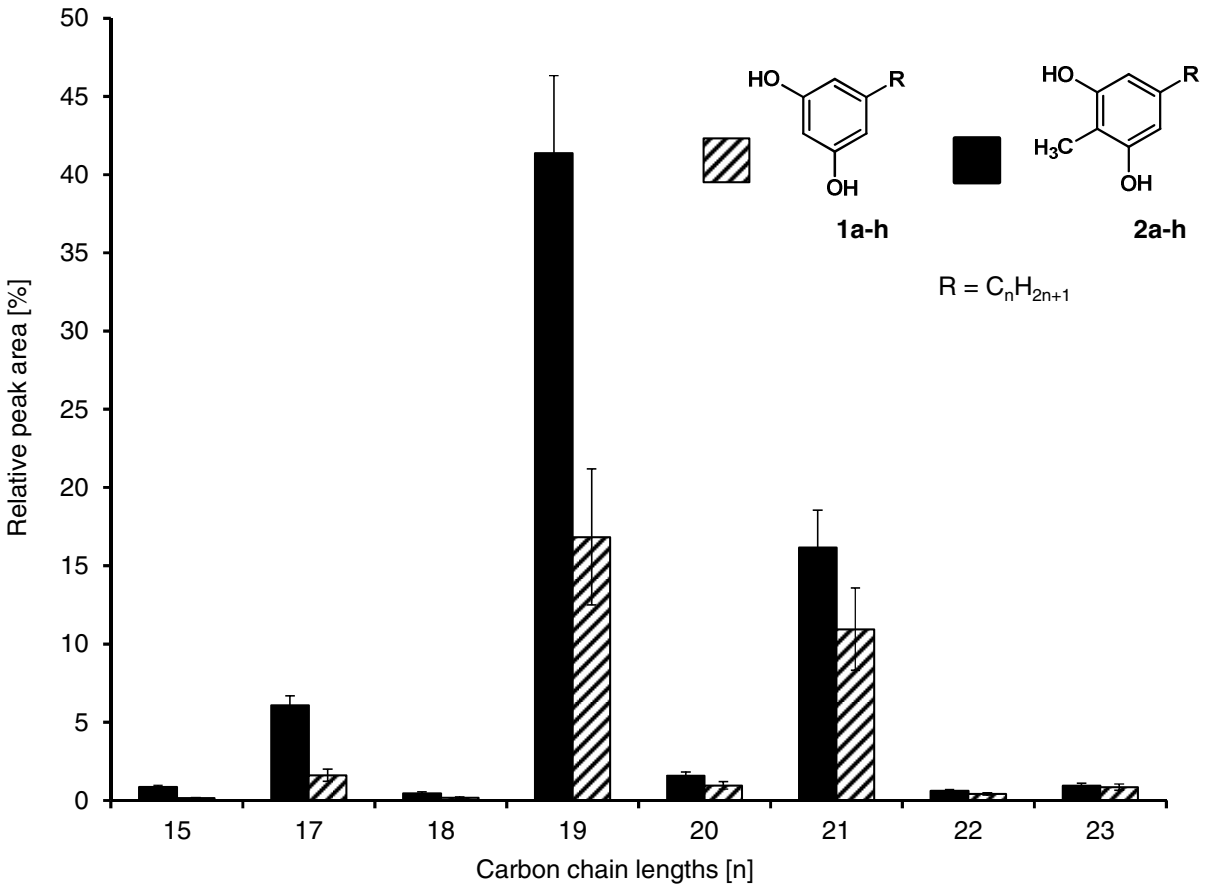


Fig. 3. Relative distribution of 5-*n*-alkylresorcinols (**1a-h**) and 5-*n*-alkyl-2-methylresorcinols (**2a-h**) in *M. tomentosa* (GC-MS area %, n = 3), based on chromatograms as shown in *Fig. 1*. The homologous **1i-j** (C_{24}-C_{25}) and **2i-j** (C_{24}*-C_{25}*) were not covered in this calculation due to their trace amounts.

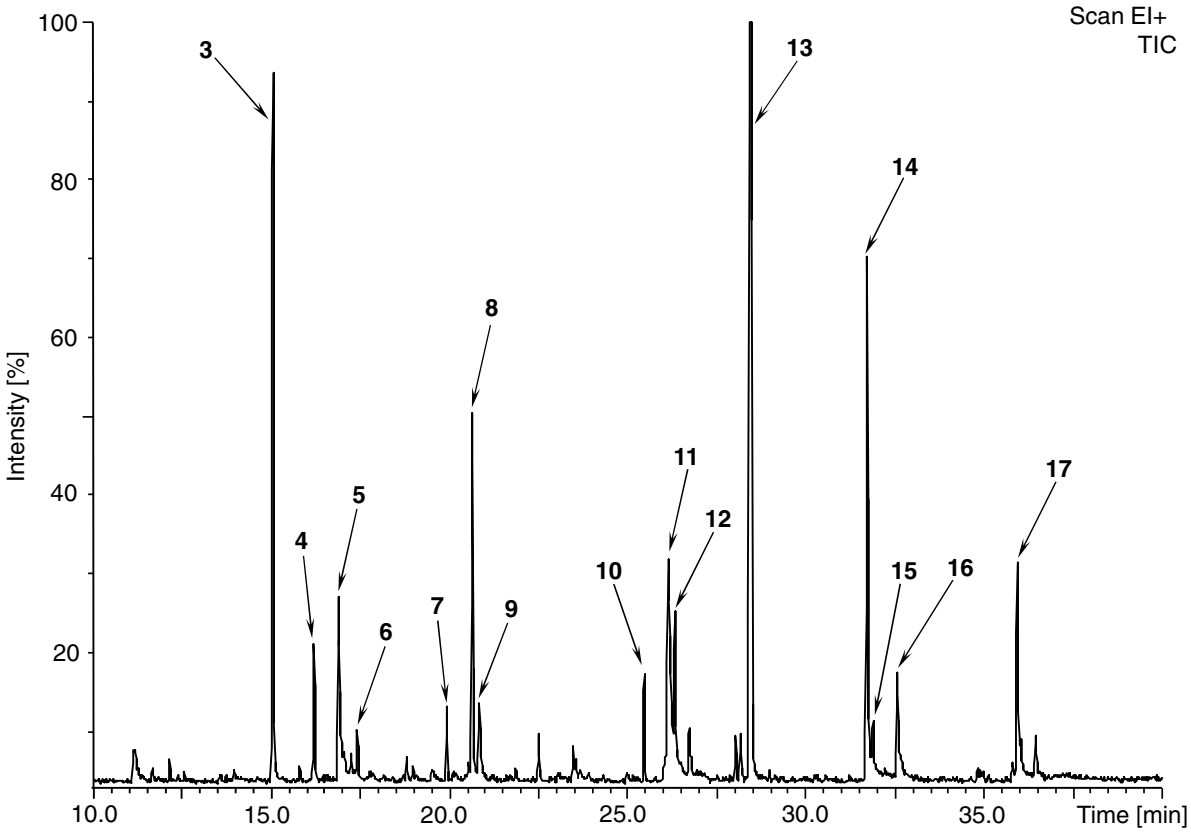


Fig. 4. Section of a GC-MS chromatogram of the MeOH fraction eluted from the polyamide column, exhibiting mostly aromatic constituents of *M. tomentosa*. For compound assignment see *Table 2*.

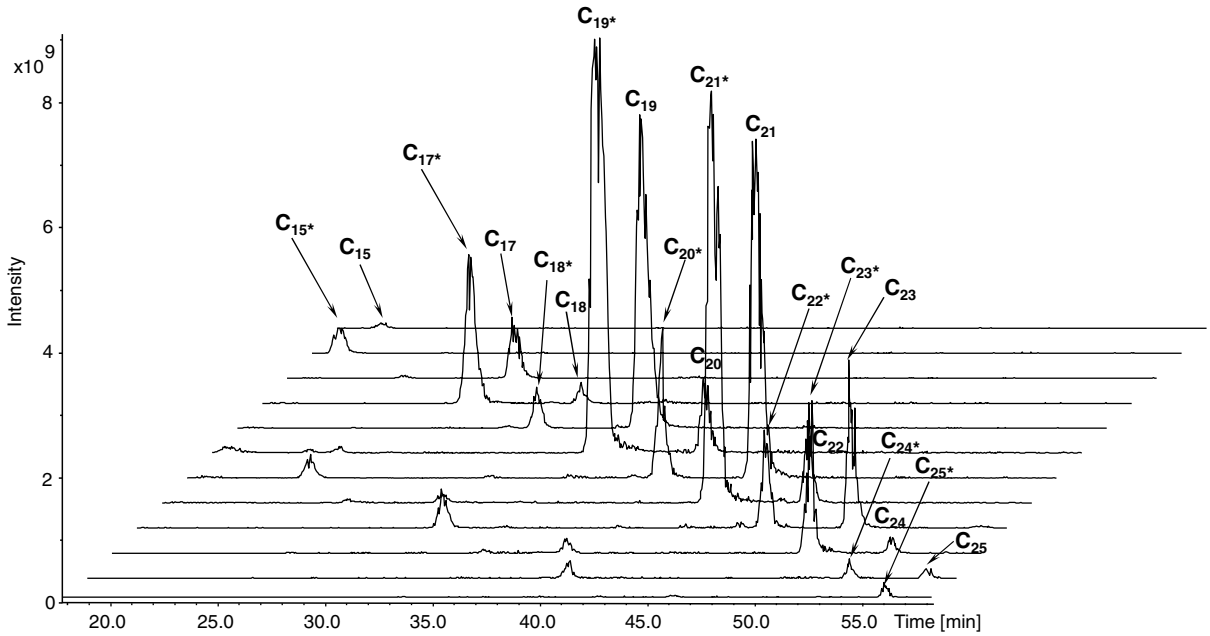


Fig. 5. Staggered illustration of LC-MS extracted ion traces $[M+H]^+$ of individual 5-*n*-alkyl- (**1a-j**, $\mathbf{C_n}$) and 5-*n*-alkyl-2-methylresorcinols (**2a-j**, $\mathbf{C_{n*}}$).

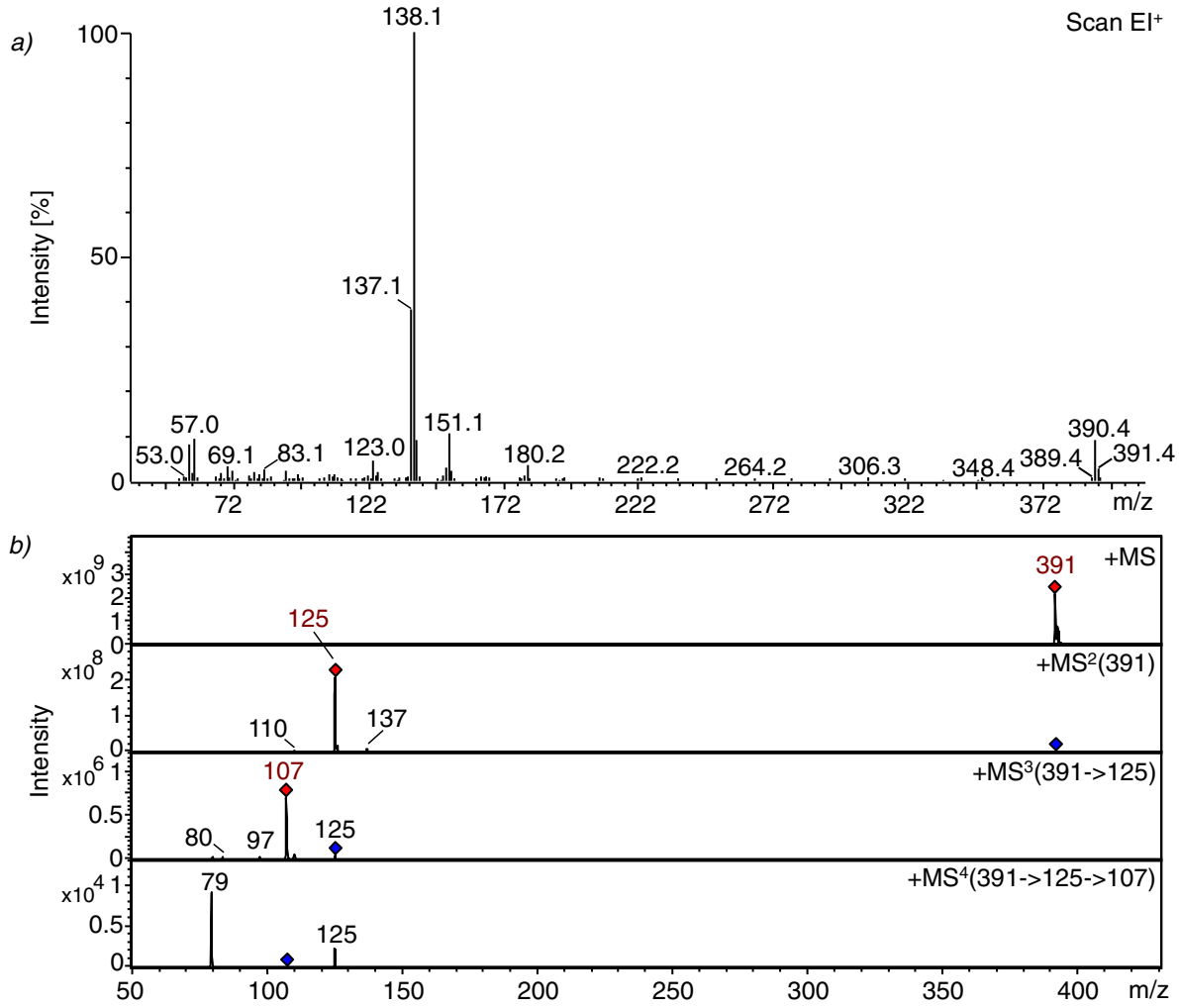
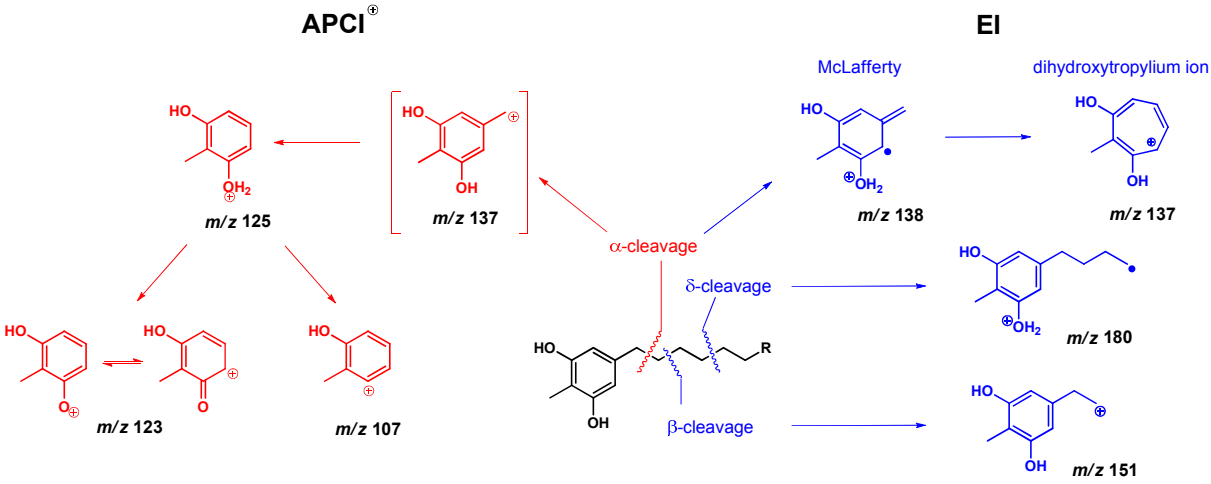


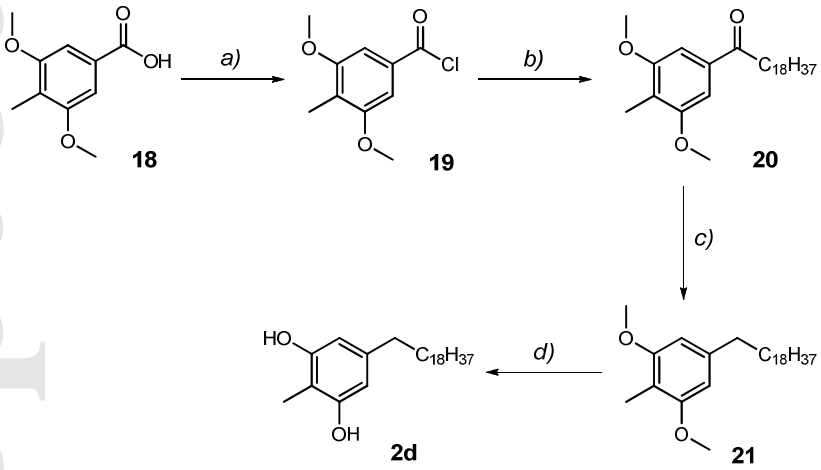
Fig. 6. EI- and APCI$^+$-MS^n mass spectra of compound **2d** ($C_{26}H_{46}O_2$; MW 390.64). *a)* EI spectrum (70 eV). *b)* APCI$^+$-MS^n data.

Schemes

Scheme 1. Proposed mass spectrometric fragmentation of 5-*n*-alkyl-2-methylresorcinols (**2**, C_n*) yielding their most intense daughter ions, observed in the $APCI^+$ and EI (70 eV) mass spectra.



Scheme 2. Synthesis of 2-methyl-5-nonadecyl-resorcinol (**2d**).



a) $(COCl)_2$, toluene, 0°. *b)* *n*-$C_{18}H_{37}MgCl$/THF, bis-[2-(*N*,*N*'-dimethylamino)ethyl]ether, -78°. *c)* NH_2OH, KOH, triethylenglycol, 200°, reflux. *d)* BBr_3/CH_2Cl_2, 0°.

Tables

Table 1. GC-MS and LC-MS data for 5-*n*-alkyl- (**1a-j,** C_n) and 5-*n*-alkyl-2-methylresorcinols (**2a-j**, C_n*), identified in the polyamide fraction (MeOH) of the $CHCl_3$ extract from *M. tomentosa*.

	GC-MS (EI)		LC-MS	Intrinsic ion peaks in the $APCI^+$ mode		
Compound side chain	t_R [min]	*m/z* [a] $[M]^+$	t_R [min]	$MS^1(M+H)^+$	MS^2	MS^3
1a, C_{15}	49.4[b]	n.d.	20.0	321	111	70
2a, C_{15}*	50.3	334	19.1	335	125	110, 107, 79
1b, C_{17}	53.0	348	28.3	349	111	93, 70
2b, C_{17}*	53.8	362	27.5	363	125	107, 78
1c, C_{18}	54.7	362	32.6	363	111	93
2c, C_{18}*	55.5	376	31.9	377	125	107
1d, C_{19}	56.4	376	36.5	377	111	70
2d, C_{19}*	57.4	390	35.6	391	125	107
1e, C_{20}	58.3	390	41.1	391	137, 125, 111	66
2e, C_{20}*	59.4	404	40.2	405	125	114, 107, 97
1f, C_{21}	60.5	404	44.1	405	123	95, 79
2f, C_{21}*	61.7	418	43.3	419	125	107
1g, C_{22}	63.0	418	47.7	419	123	-
2g, C_{22}*	64.4	432	47.0	433	125	107, 79
1h, C_{23}	65.9	432	50.8	433	123	-
2h, C_{23}*	67.6	446	50.2	447	125	107, 110
1i, C_{24}	69.4[b]	n.d.	53.9	447	n.d.	n.d.
2i, C_{24}*	71.4[b]	n.d.	53.2	461	n.d.	n.d.
1j, C_{25}	73.5[b]	n.d.	57.0	461	n.d.	n.d.
2j, C_{25}*	75.9[b]	n.d.	56.0	475	n.d.	n.d.

[a]) In addition also the fragments: *m/z* 166*, 137**, 124 (base peak)*** and 123**** were also detected for all 5-*n*-alkylresorcinols (C_n) and *m/z* 180*, 151**, 138 (base peak)*** and 137**** for the 5-*n*-alkyl-2-methylresorcinols (C_n^*), respectively (*δ-, **β-cleavage, ***McLafferty rearrangement, ****dihydroxytropylium ion).[b]) Detected only in traces. n.d. = not detected. The corresponding ions which were further fragmented in the MS^n mode are underlined.

Table 2. GC-MS data of constituents detected in the polyamide fraction (MeOH) of the *M. tomentosa* $CHCl_3$ extract.

No. [a])	Constituent	t_R [min]	calc. *M*r [Da]	Characteristic fragments, *m/z* (BPI [%])
3	4-Hydroxybenzaldehyde	15.0	122.12	122 (90), 121 (100), 93 (35), 65 (41)
4	4-Hydroxybenzonitrile	16.2	119.12	119 (100), 91 (23), 64 (17), 63 (13)
5	Hermidin	16.9	171.15	171 (99), 156 (36), 142 (100), 128 (11), 114 (26), 69 (24)
6	4-Hydroxacetophenone	17.4	136.15	136 (34), 121 (100), 93 (31), 65 (26)
7	Ethyl 4-hydroxybenzoate	19.9	166.17	166 (17), 138 (19), 121 (100), 93 (16), 65 (16)
8	Hermidin quinone	20.6	169.13	169 (38), 112 (36), 84 (18), 69 (100)
9	Vanillic acid	20.8	168.15	168 (100), 153 (75), 125 (21), 97 (40), 79 (10), 69 (19)
10	Ethyl 2-(*E*)-hydroxycinnamate	25.5	192.21	192 (46), 164 (11), 147 (100), 120 (38), 119 (29), 91 (28), 65 (27)
11	(*E*)-Coniferylalcohol	26.1	180.20	180 (75), 137 (100),131 (13), 124 (71), 119 (25), 109 (16), 103 (24), 91 (49), 77 (22), 65 (14)
12	Methyl 4-(*E*)-hydroxycinnamate	26.3	178.18	178 (66), 147 (100), 137 (22), 124 (13), 119 (37), 91 (39), 65 (27)
13	Ethyl 4-(*E*)-hydroxycinnamate	28.4	192.21	192 (49), 164 (12), 147 (100), 120 (36), 119 (29), 91 (29), 65 (24)
14	Palmitic acid	31.7	256.42	256 (31), 213 (18), 185 (10), 171 (10), 157 (9), 129 (33), 115 (13), 97 (29), 83 (37), 73 (98), 55 (100)
15	Scopoletin	31.9	192.17	192 (100), 177 (62), 164 (29), 149 (59), 121 (35), 79 (19), 69 (34)
16	Sinapylalcohol	32.5	210.23	210 (100), 182 (23), 167 (93), 154 (40), 149 (35), 135 (11), 121 (20), 106 (13), 91 (21), 77 (45)
17	α-Linolenic acid	35.9	278.43	278 (3), 249 (1), 222 (7), 149 (5), 135 (9), 121 (13), 108 (34), 93 (42), 79 (100), 67 (72)

[a]) For peak numbering see *Fig. 4.*

Table 3. IC_{50} (µM) of 5-nonadecyl-2-methyl-resorcinol (**2d**, **C_{19}***) and trolox according to the DPPH method.

Compound	Regression equation	R^2	IC_{50} (µM)
2d	y = 18.44 ln(x)-17.011	0.9975	37.86[a)]
trolox	y = 2.40 x-0.5825	0.9997	21.03[b)]

[a)]Means of triplicates (CV < 5%).[b)]Value in accordance with the literature [3