An Approach to the Chemotaxonomic Differentiation of Two European Dog's Mercury Species: *Mercurialis annua* L. and *M. perennis* L.

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Mercurialis annua and *M. perennis* are medicinal plants used in complementary medicine. In the present work, analytical methods to allow a chemotaxonomic differentiation of *M. annua* and *M. perennis* by means of chemical marker compounds were established. In addition to previously published compounds, the exclusive presence of pyridine-3-carbonitrile and nicotinamide in CH_2Cl_2 extracts obtained from the herbal parts of *M. annua* was demonstrated by GC/MS. Notably, pyridine-3-carbonitrile was identified for the first time as a natural product. Further chromatographic separation of the CH_2Cl_2 extracts *via* polyamide yielded a MeOH fraction exhibiting a broad spectrum of side-chain saturated *n*-alkylresorcinols. While the *n*-alkylresorcinol pattern was similar for both plant species, some specific differences were observed for particular *n*-alkylresorcinol homologs. Finally, the investigation of H_2O extracts by LC/MS/MS revealed the presence of depside constituents. Whereas, in *M. perennis*, a mixture of mercurialis acid (=(2*R*)-[(*E*)-caffeoyl]-2-oxoglutarate) and phaselic acid (=(*E*)-caffeoyl-2-malate) could be detected, in *M. annua* solely phaselic acid was found. By comparison with synthesized enantiomerically pure (2*R*)- and (2*S*)-phaselic acids, the configuration of the depside could be determined as (2*S*) in *M. annua* and as (2*R*) in *M. perennis.*

Introduction. – The Euphorbiaceae comprise 300 genera with almost 7500 species [1][2]. The subfamily Acalyphoideae (457 species), which is represented by genera such as *Acalypha*, *Dalechampia*, *Mallotus*, *Mercurialis*, *Ricinus*, and *Trewia* [2][3], is attracting a distinct pharmaceutical interest recently. In the course of a natural-product screening program, the chemical constituents of a particular representative of this plant family, the perennial dog's mercury (*Mercurialis perennis* L.), was lately investigated in depth. A broad spectrum of lipophilic and hydrophilic constituents of *M. perennis* have been identified, *e.g.*, alkaloids, terpenes, sterols, low molecular weight phenolics [4], *n*-alkylresorcinols [5], depsides, as well as a great variety of flavonol glycosides [6].

Another widespread European species is annual dog's mercury (*M. annua* L.). Both *M. perennis* and *M. annua* have been used since ancient time in the European herbal medicine for the treatment of various medical indications like difficult healing wounds, sores and ulcers, burns and suppurations. Herbal parts of both plants are recommended as a cure in constipations, as well as for the treatment of anorexia and gynaecological disorders [7]. Although *Mercurialis* is no longer used in allopathy, the aqueous fermented or hydroalcoholic preparations obtained from the herbal parts continue to play a role in homeopathy and anthroposophic medicine [8]. However, a clear differentiation of both *Mercurialis* species, particularly with regard to phyto-

chemical and pharmacological properties, has not been carried out. Also chemotaxonomic differences of both *Mercurialis* species are not reported in the literature. Therefore, a close investigation on the phytochemical composition of *M. annua* was performed to compare its metabolic compound pattern with that of *M. perennis*.

While *M. perennis* is a typical forest plant, primarily found in shady beech woods on neutral or alkaline limestone soil (karst areas) [9][10], *M. annua* grows next to open agricultural fields or on wasteland rich in fertilizers with a high tolerance to nitrates and phosphates [7]. The flowering period of *M. perennis* is early spring (March–April), while late summer (August–September) is typical for *M. annua*. It was hypothesized that there are characteristic chemical-marker compounds in herbal parts for species differentiation. For that reason, plant material for phytochemical investigations was each collected during blossoming. Different types of extraction procedures were chosen to evaluate the complete spectrum of both lipophilic and hydrophilic natural constituents by using chromatographic and spectroscopic analyses.

Results and Discussion. – *Lipophilic Volatile Constituents.* Investigation of CH_2Cl_2 extracts from herbal parts of *M. annua* and *M. perennis* by GC/MS revealed several compounds such as alkaloids, monoterpenes, and aromatic alcohols. Assignments were performed according to the procedure described in [4]. In this manner, signals of seven known constituents, *i.e.*, benzyl alcohol (**2**), 2-phenylethyl alcohol (**3**), 6,6-dimethylbicyclo[3.1.1]heptan-2-one (**5**), *cis*-myrtanal (**6**), *cis*-myrtanol (**7**) hermidine (**9**), and hermidine quinone (**10**), were identified in extracts from both species (see *Fig. 1*, *a* and *b*). Three further signals, however, could not be readily assigned. Mass spectra at t_R 6.7, 9.1, and 16.0 min exhibited molecular-ion [M^+] peaks at *m*/*z* of 104, 121, and 122, respectively. By comparison of the mass spectra with the NIST database [11] and using commercially available reference compounds, these signals were identified as those corresponding to pyridine-3-carbonitrile (**1**), *N*-ethylaniline (**4**), and nicotinamide (**8**). Notably, all three constituents were detected for the first time in *Mercurialis*. While compound **4** was found in both species, **1** and **8** were exclusively identified in *M. annua* (*Fig. 1*, *a*). By GC/MS, the amounts of **1** and **8** were determined as 21.24 (±2.89) and 117.84 (±14.51) mg/kg, respectively, in the fresh plant material. To the best of our knowledge, **1** has not been previously reported as a natural compound. However, a closely related compound, 4-methoxypyridine-3-carbonitrile (**11**; see *Fig. 2*), the MeO derivative of **1**, has been earlier identified as a constituent of the tropical tree *Hernandia nyphaeifolia* (Hernandiaceae family) [12]. Findings of the pyridine alkaloids **1** and **8** in *M. annua* compelled us to more detailed chemotaxonomic and plant physiological considerations. Nitrile-containing piperidine alkaloids have been described from different tribes of the Euphorbiaceae family [3]. However, the Acalyphoideae family exhibits a certain type of cyanogenesis and forms biogenetically relevant 1-methyl-oxopyridine-3-carbonitriles, which in most cases are not cyanogenic [13]. For instance, ricinine (**12**), ricinidine (**13**), and nudiflorine (**14**; see *Fig. 2*) were found in seeds of the castor bean plant *Ricinus communis* and *Trewia nudiflora* [14][15]. Mallorepine (**15**) has been identified in *Mallotus repandus* [16]. From herbal parts of *Acalypha indica*, cyanopiperidone alkaloid glucosides such as acalyphine (**16**) have been isolated [17][18]. The presence of compound **1** in the same plant family is now established as another structural homolog. From the literature, it is known that nicotinic acid (**17**) and nicotin-

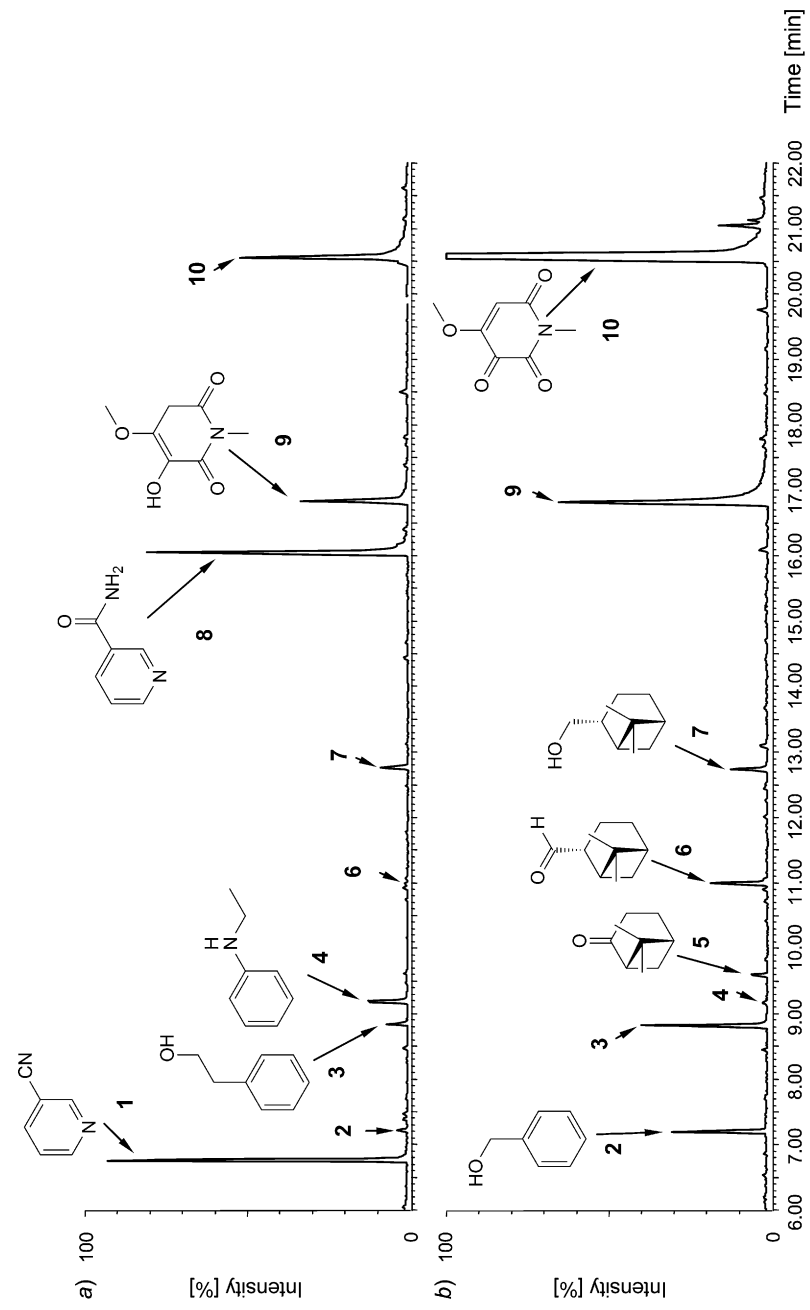


Fig. 1. *GC Fingerprints* (total ion chromatogram) *of* CH_2Cl_2 *extracts obtained from aerial parts of* M. annua (*a*) *and* M. perennis (*b*). Detected compounds: pyridine-3-carbonitrile (**1**), benzyl alkohol (**2**), 2-phenylethyl alcohol (**3**), *N*-ethylaniline (**4**), 6,6-dimethylbicyclo[3.1.1]heptan-2-one (**5**), *cis*-myrtanal (**6**), *cis*-myrtanol (**7**), nicotinamide (**8**), hermidine (**9**), and hermidine quinone (**10**). Peak assignments based on MS data (NIST) and comparison with reference compounds. Compounds **1** and **8** were exclusively detected in *M. annua*.

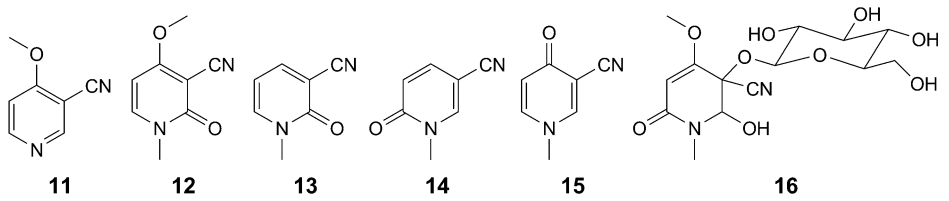
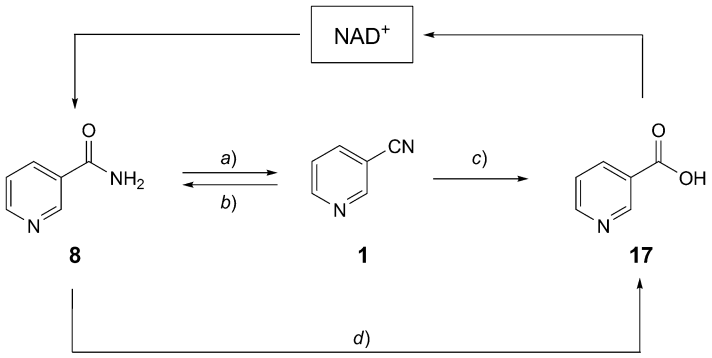


Fig. 2. *Known structurally relevant pyridine-3-carbonitrile and dihydro-oxo-pyridine-3-carbonitrile alkaloids, formerly identified in members of the Acalyphoideae family* (except **11** in the Hernandiaceae). 4-Methoxypyridine-3-carbonitrile (**11**), ricinine (=1,2-dihydro-4-methoxy-1-methyl-2-oxopyridine-3-carbonitrile; **12**), ricinidine (=1,2-dihydro-1-methyl-2-oxopyridine-3-carbonitrile; **13**), nudiflorine (=1,6-dihydro-1-methyl-2-oxopyridine-6-carbonitrile; **14**), mallorepine (=1,4-dihydro-1-methyl-4-oxopyridine-3-carbonitrile; **15**), acalyphine (=1,2-dihydro-2-hydroxy-4-methoxy-1-methyl-6-oxopyridine-3-carbonitrile 3-*O*-glucoside; **16**).

amide (**8**) are the precursors for the biosynthesis of piperidine-carbonitrile alkaloids (*e.g.*, ricinine (**12**)), indicating a close association of these compounds with the pyridine nucleotide (NAD) cycle [19][20]. Since the exact biosynthetic pathway is unkown, the simultaneous presence of the two structurally relevant alkaloids **1** and **8** in *M. annua* led to the assumption of a nicotinamide dehydratase/nitrile hydratase (nitrilase) enzyme system in the plant. The first enzyme converts carboxylic acid amides into the corresponding nitriles by dehydratation, and the second works in the opposite direction (see *Scheme 1*). Nitrile hydratase has previously only been detected in bacteria and fungi [21–23]. Its presence in higher plants would be a novelty. The exclusive presence of pyridine-3-carbonitrile (**1**) in *M. annua* and earlier reports on the mammalian toxicity after *Mercurialis* plant ingestion [24][25] open up the question if not hermidine (**9**) and its derivatives but rather **1** would be the toxic principle of the plant. Since *M. perennis* is typically found near shady woods, *M. annua* is omnipresent in many areas. From a chemo-ecological point of view, the occurence of **1** in *M. annua* is, therefore, plausible as a defense strategy against herbivores, while *M. perennis* assures its exis-

Scheme 1. *Presumed Pyridine Alkaloid Biosynthesis Cycle in* M. annua



a) $-H_2O$, Nicotinamide dehydratase. *b*) $+H_2O$, Nitrile hydratase. *c*) $+2\ H_2O$ ($-NH_3$), Nitrilase. *d*) $+H_2O$, ($-NH_3$), Amidase; NAD^+ = nicotinamide adenin dinucleotide.

tence by a perennial rhizome system. However, further studies are warranted to investigate the chemo-ecological role, biosynthetic pathways, and the toxicity of this alkaloid.

n-*Alkylresorcinol Fingerprint. n*-Alkylresorcinols were recently ascertained as novel lipophilic constituents of *M. perennis* and the Euphorbiaceae family [5]. In the present work, the chromatographic separation of the CH_2Cl_2 extracts of *M. annua* and *M. perennis via* polyamide yielded alkylresorcinol enriched fractions (MeOH), which were analyzed by GC/MS (mass scan on *m/z* 124): numerous side-chain saturated *n*-alkylresorcinols (side-chain lengths C15 : 0–C23 : 0) were detected, with C19 : 0 as the main constituent (see *Fig. 3*, *a* and *b*). The identification of these structures was based on published mass spectral data [5] and by use of a reference compound (3-nonadecylresorcinol, C19 : 0). While the *n*-alkylresorcinols were similar for both *Mercurialis* species, characteristic differences were found for their specific distribution (*Fig. 3*, *c*). Whereas, in *M. annua*, the shorter side-chain length homologs were dominant (C15 : 0, C17 : 0, C19 : 0, and C21 : 0), a tendency for longer-chain homologs (C19 : 0, C21 : 0, and C23 : 0) was observed for *M. perennis*. Thus, by analysis of the *n*-alkylresorcinol profile a clear assignment of the corresponding *Mercurialis* species appears to be feasible.

Hydrophilic Constituents. To profile hydrophilic constituents, H_2O extracts obtained from *M. annua* and *M. perennis* were investigated by LC(DAD)/MS/MS. Characteristic UV chromatograms are exemplarily shown in *Fig. 4*, *a* and *b*. While in *M. annua*, exclusively one main constituent at t_R 32.9 min was detectable, in the extract of *M. perennis* an additional signal at t_R 41.5 min was detected. The negative-ion mass spectra of these constituents exhibited peaks at *m/z* 591 and 619, representing $[2\,M-H]^-$ clusters, and their fragmentation into the pseudomolecular ions with peaks at *m/z* 295 and 309, respectively (see *Fig. 5*, *a* and *b*). In both mass spectra, the indicative fragment peak at *m/z* 179 [caffeic acid $-H]^-$ was observed. Furthermore, the compound at t_R 32.9 min showed a [malic acid $-H]^-$ fragmentation and the other at t_R 41.5 min a cleavage of a [2-hydroxyglutaric acid $-H]^-$ moiety. By close inspection of the MS data and UV absorption characteristics (see *Fig. 5*), the depside constituents phaselic acid (2-*O*-(*E*)-caffeoyl malic acid; **18**) and mercurialis acid ((−)-(2*R*)-2-[(*E*)-caffeoyloxy]-glutaric acid; **19**) were identified [6]. Whereas in *M. annua* only **18** was found, in *M. perennis* both compounds **18** and **19** were present. In the extract of *M. perennis,* the peak-area ratio of **18** to **19** was determined as 1.00 : 1.82 (see *Fig. 4*, *b*). The structure and configuration of the depside **19** was recently determined by NMR and chiral-resolution methods [6]. However, the absolute configuration of **18** in *M. annua* and *M. perennis* is still unresolved. Since the occurrence of both (2*R*)- and (2*S*)-**18** enantiomers has been reported from several higher plant species [26–29] as well as from lichens [30], it was interesting to elucidate the configuration of **18** in both *Mercurialis* species.

Configuration Determination of **18**. For configuration determination of **18**, constituents of the H_2O extracts of *M. annua* and *M. perennis* were hydrolyzed by treatment with aqueous 1N NaOH. The resulting malic acid (**23**) was extracted with AcOEt, followed by methylation with BF_3/MeOH (see *Scheme 2*, sequence *c*–*d*). The dimethylmalate enantiomers **21** thus obtained were separated on a chiral GC phase. In the hydrolysate of *M. annua*, an almost 1 : 1 mixture (2*R*)-**21**/(2*S*)-**21** was found, while in *M. perennis* mainly (2*R*)-**21** was present, beside small amounts of (2*S*)-**21** (see *Fig. 6*). The hydrolysis experiment indicated that in *M. perennis* the (2*R*)-**18** enantiomer is

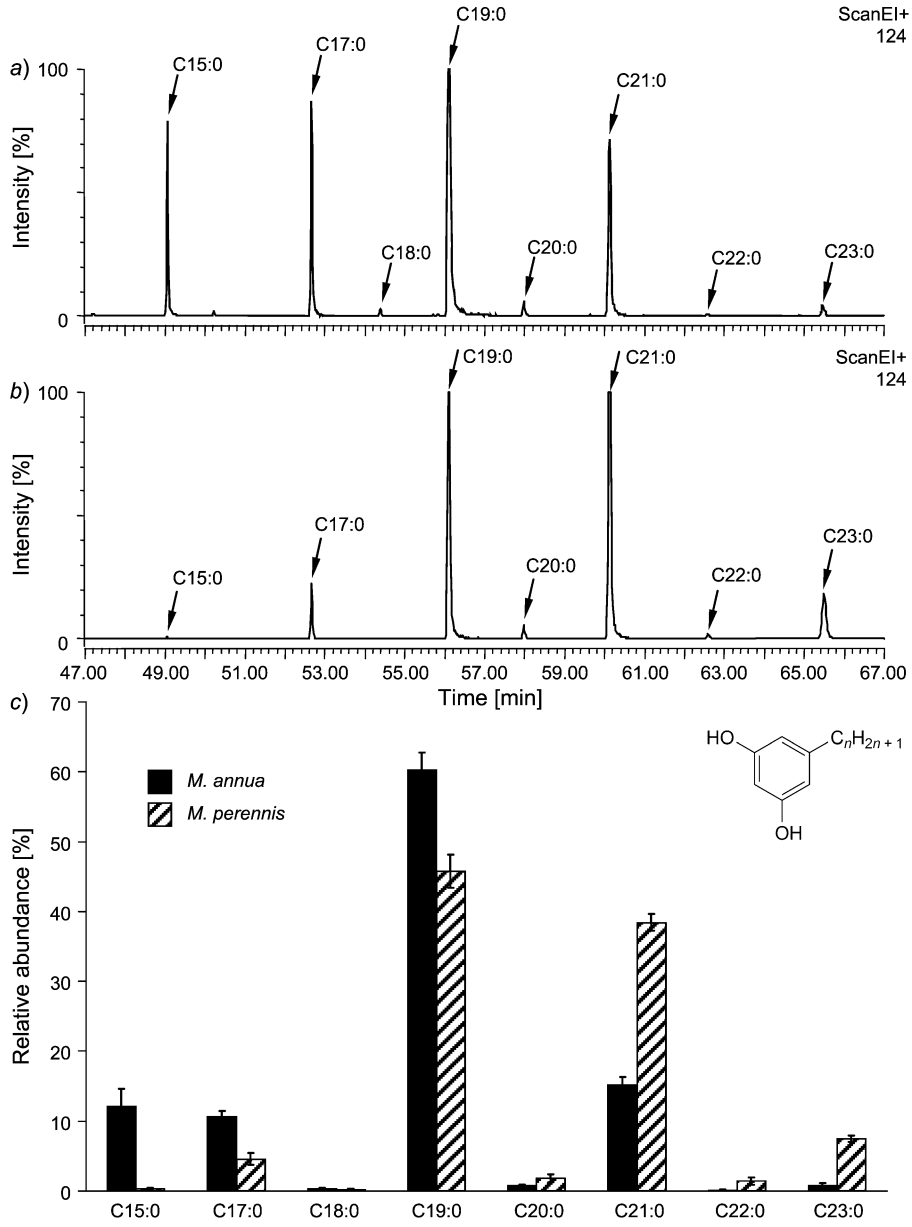


Fig. 3. *Ion chromatograms* (GC/MS, mass scan on *m*/*z* 124) *of* n-*alkylresorcinol-enriched fractions* (MeOH eluate from polyamide) *from* M. annua (*a*) *and* M. perennis (*b*). Peaks identified *via* previously published MS data [5]. c) *Side-chain length distribution of* n-*alkylresorcinol homologs* (C15 : 0–C23 : 0) *determined as relative abundance of peak areas* (three separate workups, each measured in triplicate). A generalized structural formula of the *n*-alkylresorcinol backbone is also given.

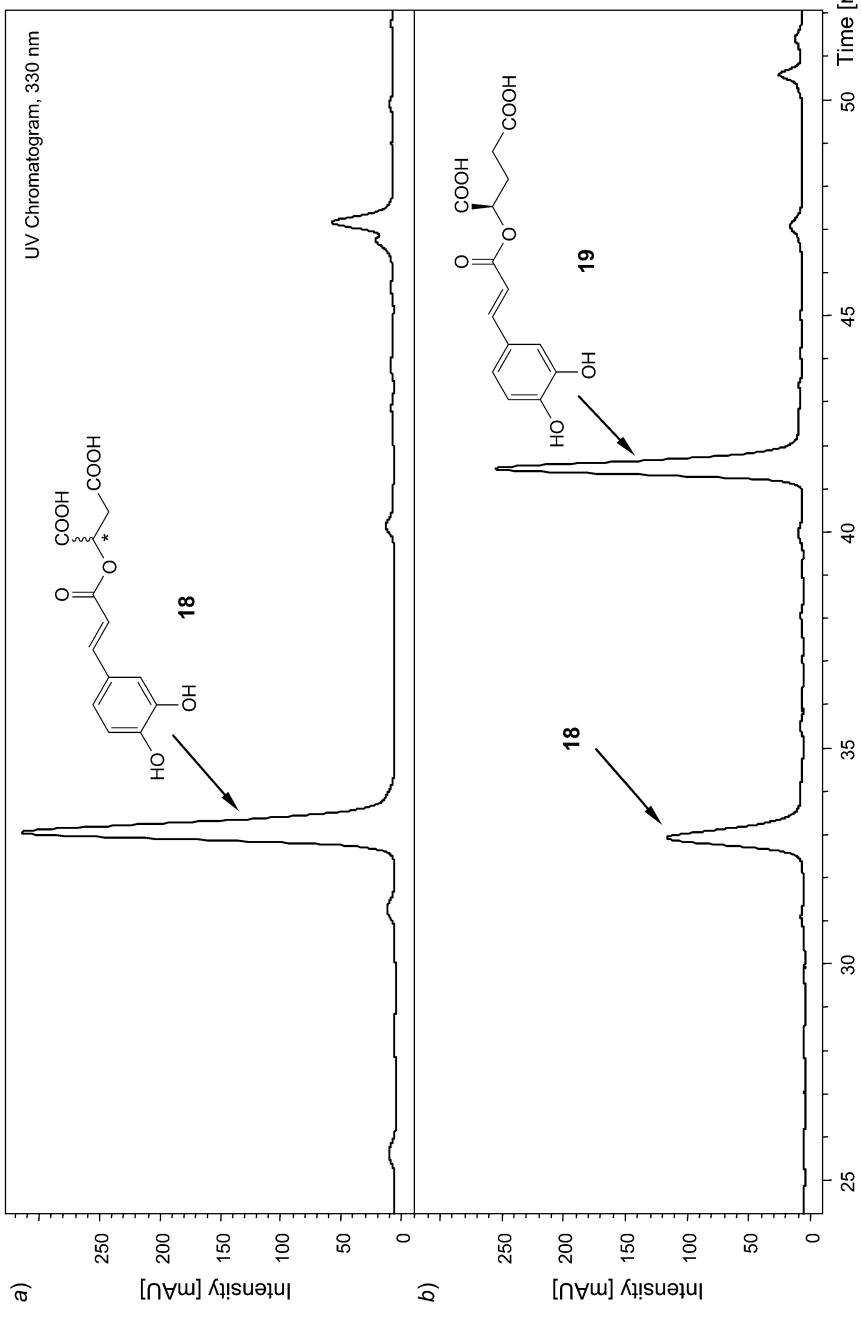


Fig. 4. *HPLC Chromatogram sections* (330 nm) *of H_2O extracts obtained from herbal parts of* M. annua (*a*) *and* M. perennis (*b*). The identification of **18** and **19** is based on LC/MS/MS data (see *Fig. 5*). The asterisk (*) in formula **18** indicates a variable configuration.

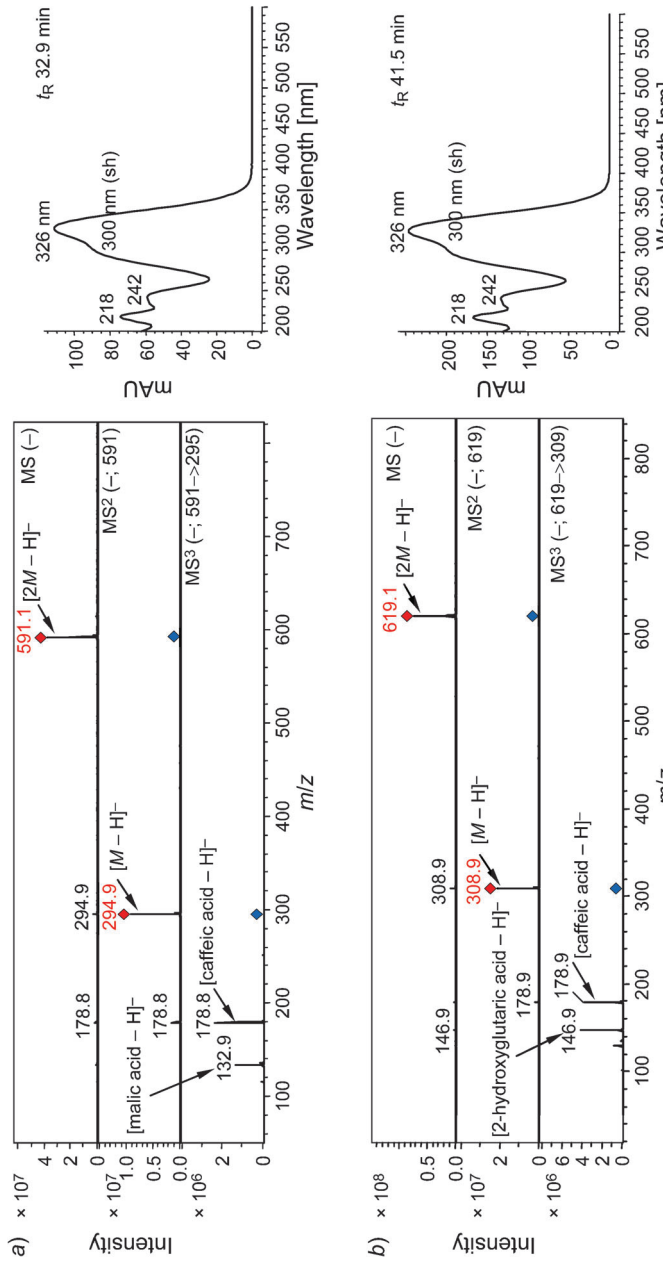
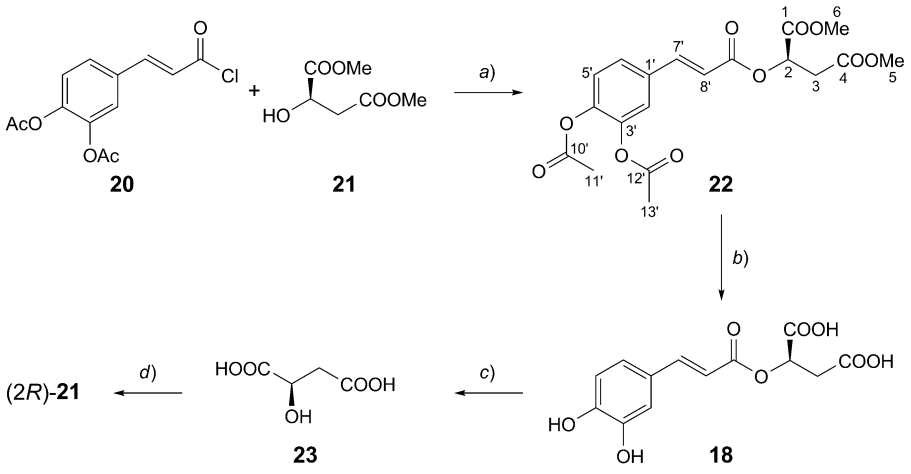


Fig. 5. a) *MS*$^{-}$, *MS*$^{2-}$, *and MS*$^{3-}$ *data of phaselic acid* (**18**; $C_{13}H_{12}O_8$, $M_r = 296.23$) *and* b) *mercurialis acid* (**19**; $C_{14}H_{14}O_8$, $M_r = 310.26$) *as obtained in the negative-ionization mode. Right:* UV Spectra of the corresponding compounds.

likely, but the absolute configuration of **18** in *M. annua* still remained unkown. An enzymatic analysis of the free (unesterified) malic acid (**23**) in *M. annua* and *M. perennis* revealed a predominance of the (2*S*)-**23** (L-malate) in comparison to the (2*R*)-**23** enantiomer (D-malate), however, without configurational information on the respective depsides (data not shown). Hence, an enantioselective synthesis of (2*R*)-**18** and (2*S*)-**18** was performed to obtain reference material for a chiral HPLC resolution experiment (see *Scheme 2*). For the new synthesis of enantiomerically pure (2*R*)-**18** and (2*S*)-**18**, an established protection-group concept was applied, formerly used by *Sefkow* for the synthesis of chlorogenic acids [31]. The reaction of (*E*)-3,4-bis(acetyloxy)cinnamoyl chloride (**20**) with the appropriate **21** enantiomer yielded the intermediate condensation product **22**. Subsequently, the protection groups were removed from **22** by treatment with a 1N HCl/THF mixture to yield the product (2*R*)-**18** or (2*S*)-**18**, respectively (*Scheme 2*). Finally, a separation of the synthesized enantiomer mixture (2*R*)-**18**/(2*S*)-**18** was achieved on a protein-based *Chiral-AGP*® HPLC column under reversed-phase conditions (see *Fig. 7, g*). By investigation of the *Mercurialis* H_2O extracts *via* chiral HPLC and spiking the samples with both enantiomerically pure stereoisomers of **18**, the presence of (2*S*)-**18** in *M. annua* and (2*R*)-**18** in *M. perennis* was established unequivocally (*Fig. 7, a–f*). The opposite configuration of **18** in both *Mercurialis* species leads to the question for their biosynthesis. *Sullivan* and *Zarnowski* have recently identified a gene in red clover (*Trifolium pratense*) encoding a hydroxycinnamoyl transferase (HCT-2), capable of transferring caffeoyl moieties from a coenzyme A (CoA)-thiolester to L-malic acid ((2*S*)-**23**) [29][32]. Even if the presence of such a hydroxycinnamoyl transferase in *Mercurialis* is likely, the different substrate specifity for (2*S*)-**23** or (2*R*)-**23** to form the depside enantiomers (2*R*)-**18** or (2*S*)-**18**, respectively, remains unclear and is an interesting task for further studies.

Scheme 2. *Synthesis of (2*R*)-Phaselic Acid* ((2*R*)-**18**). Analogously, the (2*S*)-**18** was synthesized. For stereochemical analysis, the hydrolysis of (2*R*)- or (2*S*)-**18** afforded (2*R*)- or (2*S*)-**21**, respectively, after methylation.



a) Pyridine, CH_2Cl_2, 4-(dimethylamino)pyridine (DMAP), reflux. The arbitrary atom numbering is shown for the intermediate **22**. *b*) 1N HCl, THF, reflux. *c*) 1. 1N NaOH; 2. 1N HCl. *d*) BF_3/MeOH.

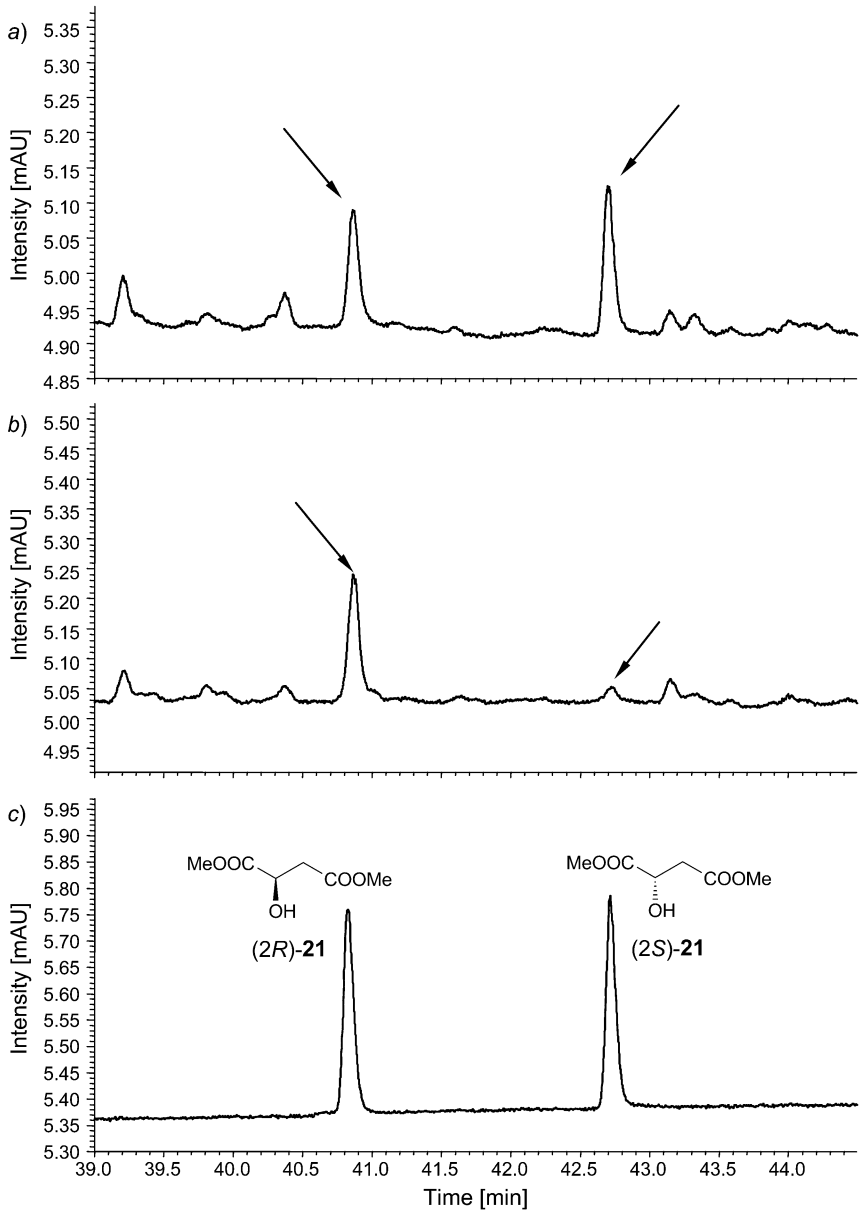


Fig. 6. *Chiral GC separation of hydrolyzed H_2O extracts from* M. annua (*a*) *and* M. perennis (*b*) *after treatment with BF_3/MeOH*. c) *Reference mixture of dimethyl (2*R*)- and (2*S*)-malate* ((2*R*)- and (2*S*)-**21**, resp., 1:1 (*w*/*w*)).

Conclusions. – By using different extraction procedures and analytical methods, lipophilic and hydrophilic metabolic profiles of *M. annua* and *M. perennis* were analyzed. Specific chemical markers such as the newly found pyridine alkaloids, the

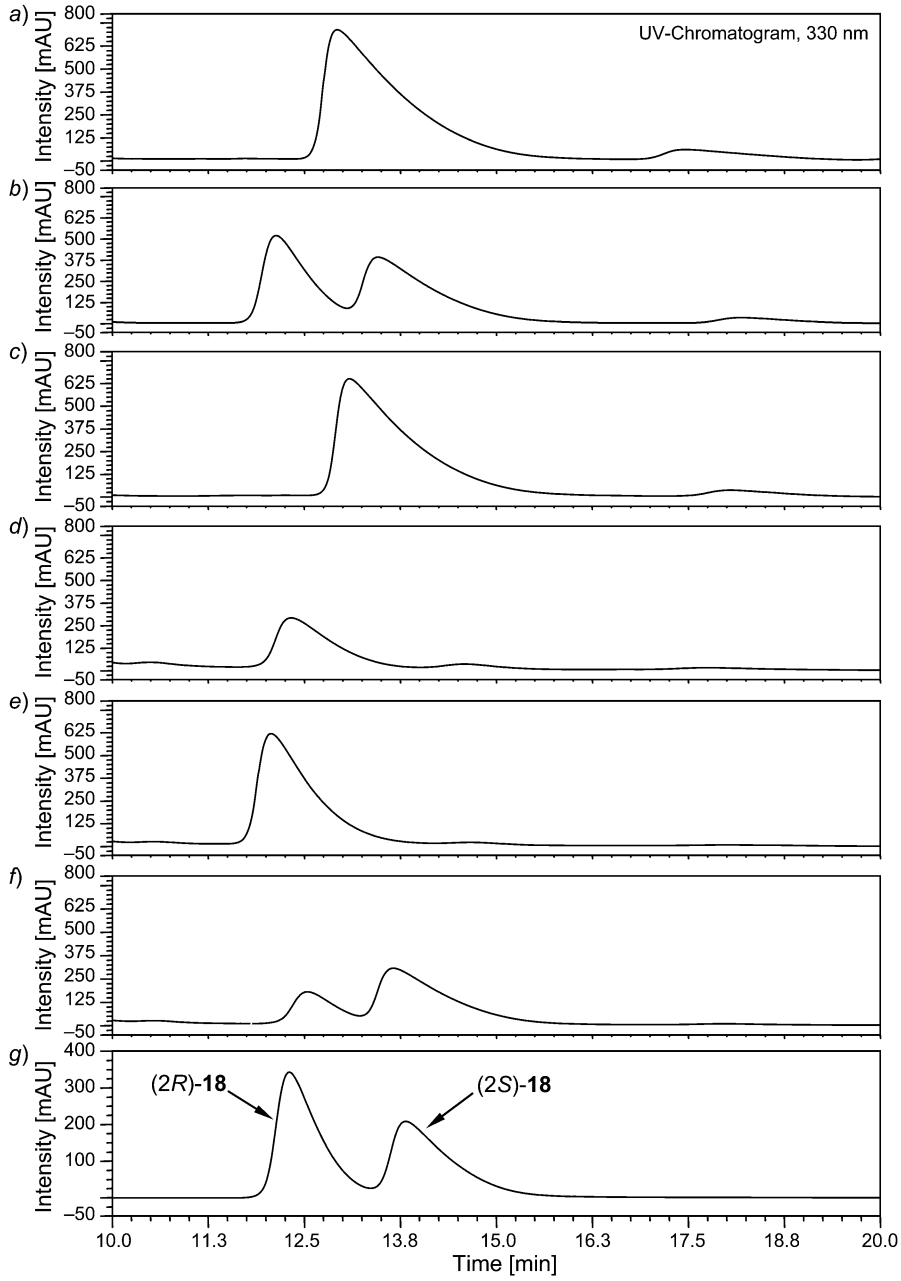


Fig. 7. *HPLC Chromatograms* (330 nm) *of* H_2O *extracts from* M. annua (*a*) *and* M. perennis (*d*) *on a* Chiral-AGP® *phase.* b) M. annua *spiked with (2*R*)-phaselic acid* ((2*R*)-**18**); c) M. annua *spiked with (2*S*)*-**18**; e) M. perennis *spiked with (2*R*)*-**18**; f) M. perennis *spiked with (2*S*)*-**18**; g) *reference mixture of (2*RS*)*-**18**.

distinguished *n*-alkylresorcinol fingerprints, as well as the differences in the depside composition and their respective configurations provide useful tools for a doubtless phytochemical differentiation of *M. annua* and *M. perennis*. Among others, these findings could be applied to the authentication of fresh plant material and herbal remedies obtained from both species, and provide a basis for further biochemical investigations. The phytochemical difference found may also trigger studies on the pharmacological potency of *M. perennis* and *M. annua* preparations.

Experimental Part

Reagents and Standards. Pyridine-3-carbonitrile, 4-(dimethylamino)pyridine (DMAP), dimethyl (+)-(*R*)- and (−)-(*S*)-malate were purchased from *Aldrich* (D-Steinheim). *n*-Octadecane, (−)-*cis*-myrtanol and C_{18}-reversed-phase (RP) silica gel *100* were obtained from *Fluka* (CH-Buchs). *N*-Ethylaniline and the boron trifluoride–methanol complex (20% BF_3 in MeOH (*w/w*)) were purchased from *Merck* (D-Darmstadt). Nicotinamide and *Amberlite® XAD-7HP* were from *Sigma–Aldrich* (D-Taufkirchen), 3-nonadecylresorcinol, C19:0 from *ReseaChem* (CH-Burgdorf), 2-phenylethyl alcohol and polyamide from *Carl Roth* (D-Karlsruhe). All other chemicals were obtained in anal. grade from *VWR* (D-Darmstadt). Hermidine and (−)-*cis*-myrtanal were prepared according to the procedure described in [4].

Plant Material. Herbal parts of *M. annua* were collected in September 2009 and 2010 on field borders at the northwest side of Magdeburg (Saxony-Anhalt, Germany). Herbal parts of *M. perennis* were collected in April 2009 and April 2010 in a limestone area of the mountain forest above Bad Boll/Eckwaelden (Baden-Wuerttemberg, Germany). The fresh plant material was mechanically cleaned and stored at −80° until investigation. Identification of the plant material was performed by Prof. *O. Spring*, and voucher specimens are deposited with the herbarium of the Department of Botany, Hohenheim University, Germany (*M. annua*, vouchers: HOH-012314, HOH-012315; *M. perennis*, vouchers: HOH-011281, HOH-011282, and HOH-011283).

CH_2Cl_2 Extraction. GC/MS Quantification of Pyridine-3-carbonitrile (**1**) *and Nicotinamide* (**8**). Frozen herbal parts of *M. annua* or *M. perennis* (50 g) were extracted with CH_2Cl_2 (500 ml) by *Ultrathurrax®* treatment (2 min at 15,200 rpm, *IKA-Werke*, D-Staufen). To prevent oxidative alterations, the slurry was bubbled with N_2 (10 min) before and after *Ultrathurrax®* treatment. After standing for 24 h at +4°, the plant material was filtered over *Celite* through a *Büchner* funnel. Then, the pulp was washed once with CH_2Cl_2 (50 ml) and extracted for a second time in the same manner. Subsequently, from the unified extracts the solvent was distilled off by rotary evaporation *in vacuo* (at 35°). For qual. GC/MS analysis of **1** and **8**, the residual spissum extract was dissolved in AcOEt (to 10 ml; containing the internal standard *n*-octadecane) and submitted to GC/MS. Amounts of **1** and **8** were analyzed by use of external calibration curves (calibration range: 0.16–6.44 mg/ml for compound **1**, $R^2 = 0.9997$; and 0.17–6.94 mg/ml for **8**, $R^2 = 0.9994$).

GC/MS Profile of n-*Alkylresorcinols.* The plant material (100 g) was extracted twice with CH_2Cl_2 according to the previous protocol. The *n*-alkylresorcinol containing extract was concentrated according to a modified literature procedure [5]. In brief, the spissum extract (dissolved in 30 ml CH_2Cl_2) was loaded on a polyamide column (11 × 2 cm; particle size, 0.05–0.16 mm) preconditioned with CH_2Cl_2 (50 ml). Thereafter, the column was washed with CH_2Cl_2 (100 ml, fraction disposed), followed by MeOH (100 ml). From the MeOH eluate, the solvent was removed *in vacuo*. To assess the *n*-alkylresorcinol profile, the residue was re-dissolved in MeOH (1.5 ml) and analyzed by GC/MS (mass scan on *m*/*z* 124).

H_2O Extraction. Herbal parts (50 g) were homogenized in distilled H_2O (63 ml) by *Ultrathurrax®* treatment (5 min at 15.000 rpm). Before and after homogenization, the material was flushed with N_2 to prevent air oxidation. After standing over night at +4°, the plant material was squeezed over a cloth strainer, and the obtained slurry was filtered over *Celite* through a *Büchner* funnel by vacuum suction. The resulting filtrates (*ca.* 70 ml) were stored at −80° until further investigation.

Anal. and Spectral Analyses. RP-HPLC/ESI-MS/MS Analyses were carried out on an *Agilent 1200 HPLC* system (*Agilent Technologies Inc.*, Palo Alto, USA), equipped with a binary pump, a micro-vacuum degasser, an autosampler, a thermostatic column compartment, and a UV/VIS diode array detector. A *ReproSil-Pur C18-AQ*® RP column (5-μm particle size, 250 × 4.0 mm i.d., *Dr. Maisch GmbH*, D-Ammerbuch-Entringen) was used for chromatographic separation at 23° and a flow rate of 1.0 ml/min. The UV detection of the depsides (see *Fig. 4*) was performed at 330 nm. The mobile phase consisted of $HCOOH/H_2O$ 0.2 : 99.8 (*v*/*v*); mobile phase *A*) and MeCN (100%; mobile phase *B*). Starting with 10% *B* for 25 min, a linear gradient was followed to 23% *B* at 60 min, remaining isocratically for 2 min, then increasing to 100% *B* at 65 min, keeping for 5 min, before re-equilibration to starting conditions. The H_2O extracts of *M. annua* or *M. perennis* were centrifuged at 19,064 g (5 min) before use. The injection volume of each sample was 20 μl. The LC system was coupled to an *HCTultra* ion trap (*Bruker Daltonik GmbH*, D-Bremen) with an ESI source operating in the negative-ion mode with the following parameters: HV voltage, 4000 V; dry gas, N_2, 12.00 l/min with a dry temp. set at 365°; nebulizer, 70 psi. 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 of recording was *m*/*z* 50–2000 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 (B.01.03) and EsquireControl Software (V6.1).

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-5ms* cap. column (60 m × 0.25 mm i. d. × 0.25 μm film thickness, 5% phenyl polysiloxane and 95% dimethyl polysiloxane coating; *Phenomenex*, Torrance, USA). He was the carrier gas at a flow rate of 1 ml/min. The injector used was a PSS (programmed-temp. split/splitless injector; temp., 250°). The temp. program for the column oven was 100 to 320° at 4°/min with a final hold time of 30 min. The mass spectrometer was run in the electron ionization (EI) mode (70 eV).

NMR Spectroscopy. The NMR spectra were recorded in $CDCl_3$ at 500 (^1H) and 125 MHz (^{13}C) with a *Varian Unity Inova* NMR spectrometer (D-Darmstadt). Chemical shifts are reported in δ [ppm] referenced to the residual solvent signal of $CHCl_3$ (^1H: δ 7.26; ^{13}C: δ 77.00 ppm). ^{13}C-NMR Signal assignment of the novel compound **22** was based on 2D-heteronuclear NMR experiments (gHMBC and gHSQC).

*Synthesis of the Reference Compounds (2*R*)- and (2*S*)-Phaselic Acid* ((2*R*)-**18** and (2*S*)-**18**, resp.). *4-[(1*E*)-3-Chloro-3-oxoprop-1-en-1-yl]benzene-1,2-diyl Diacetate* (**20**). Compound **20** was synthesized in 95% yield according to a literature procedure [31]. M.p. 87–88° ([33]: 80–83°). GC/MS Purity: GC/MS, (70 eV) > 99%; at t_R 34.2 min: 284 (1), 282 (4, M^+), 247 (3, $[M-Cl]^+$), 242 (9), 240 (23, $[M-COMe]^+$), 205 (14, $[M-COMeCl]^+$), 200 (17), 198 (50, $[M-2\,COMe]^+$), 163 (100, $[M-2\,COMeCl]^+$), 134 (15). The mass spectrum was essentially consistent with literature data [31].

*Dimethyl (2*R*)-2-({(2*E*)-3-[3,4-Bis(acetyloxy)phenyl]prop-2-enoyl}oxy)butanedioate* ((2*R*)-**22**). A mixture of **20** (1.74 g, 6.16 mmol), *dimethyl (+)-(*R*)-malate* ((2*R*)-**21**; 1.00 g, 6.17 mmol), DMAP (0.068 g, 0.556 mmol), pyridine (40 ml), and CH_2Cl_2 (136 ml) was heated under reflux for 7 h. Subsequently, the CH_2Cl_2/pyridine mixture was removed *in vacuo* by rotary evaporation, and the orange residue was applied to vacuum liquid chromatography (VLC). In brief, TLC-grade SiO_2 (75 g) was preconditioned with hexane (100 ml). Then, a soln. of the crude material (dissolved in 20 ml of AcOEt) was loaded on the column, which was again flushed with hexane (200 ml). By elution with a hexane/AcOEt linear gradient (8 : 2 to 3 : 7 (*v*/*v*), several fractions were obtained, monitored by TLC (SiO_2; mobile phase: 100% AcOEt). *Fractions 1* and *2* (hexane/AcOEt 4 : 6 (*v*/*v*) containing the target compound were combined, and the solvent was removed under reduced pressure. The residue obtained (1.82 g) was washed with hexane (2 × 50 ml) by ultrasound treatment. Thus, remaining starting material was removed with the supernatant. Further purification by repeated VLC afforded 1.36 g of pure (2*R*)-**22** (colorless sirup; 54% of the theory). TLC (SiO_2; 100% AcOEt): R_f 0.61. UV/VIS: 219 (4.21), 281 (4.36). ^1H-NMR ($CDCl_3$, 500 MHz): 7.66 (*d*, *J* = 16.0, H–C(7′)); 7.39 (*dd*, *J* = 2.0, 8.6, H–C(6′)); 7.35 (*d*, *J* = 1.9, H–C(2′)); 7.20 (*d*, *J* = 8.6, H–C(5′)); 6.42 (*d*, *J* = 15.7, H–C(8′)); 5.61–5.58 (*m*, H–C(2)); 3.77, 3.71 (2*s*, 2 MeO); 2.96–2.94 (*m*, CH_2(3)); 2.28, 2.27 (2*s*, 2 Me of Ac). ^{13}C-NMR ($CDCl_3$, 125 MHz): 169.46, 169.25

(C(1), C(4)); 167.89, 167.79 (C(10′), C(12′)); 165.21 (C(9′)); 144.29 (C(7′)); 143.67 (C(4′)); 142.37 (C(3′)); 132.83 (C(1′)); 126.44 (C(6′)); 123.87 (C(5′)); 122.83 (C(2′)); 117.82 (C(8′)); 68.31 (C(2)); 52.65, 52.11 (C(5), C(6)); 35.97 (C(3)); 20.53, 20.48 (C(11′), C(13′)). The ^{13}C-NMR assignments were based on gHSQC and gHMBC experiments. The arbitrary C-atom numbering is shown in *Scheme 2*. GC/MS at t_R 49.4 min; (70 eV): 408 (6, M^+), 377 (4), 366 (16, [M – COMe]$^+$), 324 (65, [M–2 COMe]$^+$), 247 (7), 205 (13), 162 (100), 145 (4), 134 (48), 113 (8). The enantiomer (2*S*)-**22** was prepared *via* a similar procedure. (2*R*)-**22**: $[\alpha]_D^{25}$ = –5.00 (c = 1.00, MeOH); (2*S*)-**22**: $[\alpha]_D^{25}$ = +4.93 (c = 1.01, MeOH).

*(2*R*)-Phaselic Acid* (= *(2*R*)-2-{[(2*E*)-3-(3,4-Dihydroxyphenyl)prop-2-enoyl]oxy}butanedioic Acid*; (2*R*)-**18**). In a mixture THF/1N HCl 1:3 (*v*/*v*, 75 ml), (2*R*)-**22** (1.15 g, 2.816 mmol) was dissolved under N_2. Then, the mixture was stirred and heated under reflux for 6 h. Afterwards, the pH was adjusted to 3.0 by dropwise addition of 1N NaOH, and the soln. was saturated with NaCl and extracted with AcOEt (3 × 100 ml). The combined AcOEt extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo* by rotary evaporation to yield 0.94 g of crude material. By VLC on a C_{18}-*RP*-SiO_2 column (100 g of TLC grade *100* C_{18}-*RP* SiO_2, preconditioned subsequently with MeOH and MeOH/H_2O, 10:90 (*v*/*v*)), the product was purified by elution with a linear MeOH/H_2O gradient from 10:90 to 30:70 (*v*/*v*). Fractions were analyzed by TLC showing that *Frs. 13–15* (MeOH/H_2O 30:70 (*v*/*v*)) contained the target product. From the combined fractions, the solvent was distilled off *in vacuo* to yield pure (2*R*)-**18** (0.41 g; 49% of the theory). Pale yellow resin. TLC (SiO_2; $CHCl_3$/AcOH/H_2O 10:8.4:1.6 (*v*/*v*/*v*)): R_f 0.32. HPLC Purity: > 99.9%, t_R 35.7 min. $[\alpha]_D^{24}$ = – 16.48 (c = 1.64, MeOH); $[\alpha]_D^{23}$ = – 32.9 (c = 2.06, H_2O); [30]: $[\alpha]_D$ = – 37.7 (c = 2.18, H_2O). UV/VIS: 219 (4.13), 245 (4.00), 331 (4.26)[1]). Compound (2*S*)-**18** was prepared in 51.5% yield in the same manner from the corresponding precursor. (2*S*)-**18**: $[\alpha]_D^{25}$ = – 15.96 (c = 1.75, MeOH); $[\alpha]_D^{23}$ = +29.6 (c = 1.75, H_2O); [26]: $[\alpha]_D$ = +31.5 (c = 1.36, H_2O)[1]).

*Configuration Analysis of Malic Acids, (2*R*)-***23*** and (2*S*)-***23****, by Hydrolysis of the H_2O Extract, and GC Separation of (2*R*)-***21*** and (2*S*)-***21*** after Derivatization.* The H_2O extracts of *M. annua* and *M. perennis* (35 ml each) were stirred for 24 h with 1N NaOH (10 ml). The resulting brown solns. were acidified with 1N HCl to pH 1.2 and diluted with H_2O (50 ml). The H_2O layers were saturated with NaCl and extracted with AcOEt (2 × 50 ml). Then, the combined AcOEt extracts were dried (Na_2SO_4), the solvent was removed by vacuum rotovaporation, and the residues obtained were treated with 20% BF_3 in MeOH (*w*/*v*; 1 ml) for 45 min at 105°. Subsequently, the mixtures were transferred into sat. NaCl soln. (5 ml) and extracted with hexane (1 × 5 ml). The hexane extracts were dried (Na_2SO_4) and evaporated to a small volume (1 ml) by a vigorous stream of N_2. From the soln. thus obtained, 1 µl was injected into a GC-FID (*Perkin–Elmer*, *Autosystem XL*), equipped with a chiral cap. column (*IVADEX-7*, 25 m × 0.25 mm i. d. × 0.25 µm film thickness, diethyl *tert*-butylsilyl β-cyclodextrine coating; *IVA Analysentechnik*, D-Meerbusch). He was used as carrier gas at a flow rate of 1.3 ml/min. The injector and detector temps. (FID) were set to 230°. The temp. program for the column oven was: 50–180° at 1.5°/min with a total run time of 87 min. For comparison dimethyl (+)-(*R*)- and (–)-(*S*)-malate ((2*R*)-**21** and (2*S*)-**21**, resp.) were used as reference.

*Analysis of Free (2*R*)-***23*** and (2*S*)-***23*** by Enzymatic Reaction.* To remove polyphenols and colorants, H_2O extracts of *M. annua* and *M. perennis* (4 ml each) were filtered twice by vacuum suction over an *Amberlite® XAD-7 HP* column (2-cm diameter, 2.5-cm length; washed before with 100 ml H_2O). The obtained filtrates were analyzed by use of commercially available D- and L-malic acid assays (*R-Biopharm AG*, D-Darmstadt). The amounts of D- or L-malic acids ((2*R*)- and (2*S*)-**23**, resp.) were determined *via* increase of NADH (by absorbance at 340 nm) which is formed by enzymatic conversion of malic acid, nicotinamide-adenine dinucteotide (NAD+), and D- or L-malate dehydrogenase, resp.: D-(L)-malic acid + NAD^+ → oxaloacetate + NADH + H^+ [34].

*Chiral HPLC Separation of (2*R*)-***18*** and (2*S*)-***18*** in the H_2O Extracts.* The configuration analysis of **18** was performed on a *Dionex Ultimate 3000* HPLC (*Dionex GmbH*, D-Idstein). A *Chiral-AGP®* column (*ChromTec-Ltd.*, 5-µm particle size, 150 × 2.0 mm-i. d., *Dr. Maisch GmbH*, D-Ammerbuch-Entringen) was used for chromatographic separation at 20°. The UV detection of the phaselic acid enantiomers was performed at 220 and 330 nm (*Fig. 7*). Using NaH_2PO_4 in H_2O (0.1M, pH 3.0, eluent *A*) and MeCN

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[1]) The anal. data were in accordance with those in the literature [26][30]. See also *Fig. 5*, *a*, for LC/MS/MS data.

(100%; eluent *B*), the following gradient at a constant flow rate of 0.21 ml/min was applied: 0–20 min, 0% *B*; 21–23 min, 0–10% *B*; 23–28 min, 10% *B*; 29–30 min, 10–0% *B*; 31–36 min, 0% *B*. The software used for HPLC system control and data processing was Chromeleon (V6.8; *Dionex GmbH*, D-Idstein). The H_2O extracts of *M. perennis* and *M. annua* (70 ml) were filtered by vacuum suction over SiO_2 (for TLC, layer: 1.5×3.5 cm), and the filtrate was centrifuged for 5 min at 14,000 rpm before use. The injection volume of each sample was 5 µl. Compounds (2*R*)-**18** and (2*S*)-**18** (2.0 mg/l and 1.8 mg/l resp.) were employed as reference.

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