Isolation and Structure Elucidation of Radical Scavengers from *Thymus vulgaris* Leaves

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2,2-Diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activity-guided fractionation of a leaf extract of *Thymus vulgaris* led to the isolation of the radical scavengers rosmarinic acid **1**, eriodictyol, taxifolin, luteolin 7-glucuronide, *p*-cymene 2,3-diol, *p*-cymene 2,3-diol 6−6′-dimer, carvacrol, thymol, and a new compound, **2**. The fractionation was considerably facilitated by using an on-line HPLC detector for radical scavenging activity. In this detector activity is monitored as the disappearance of the color of a postcolumn added stable radical after reacting with radical scavengers in a reaction coil. Compound **2**, which consists of rosmarinic and caffeic acid moieties linked via a C-3′−C-8″ ether bridge, was mainly elucidated by various NMR techniques and CD. Phenylpropanoid trimer **2** was a weaker and stronger radical scavenger than rosmarinic acid **1** in off-line TEAC and DPPH• assays, respectively.

Currently there is considerable interest in new natural antioxidants to replace synthetic ones such as BHT (butylated hydroxytoluene) for use in foods and cosmetics. Rosemary and sage extracts are already extracted on an industrial scale and used commercially. Recently we screened various extracts from several Lithuanian aromatic plants for their antioxidant activity.[1] In this screening thyme (*Thymus vulgaris* L. (Lamiaceae)) leaves appeared to be a promising source of natural antioxidants. In an effort to isolate and identify the active constituents, two different extracts of thyme leaves were fractionated on the basis of their activity in on-line HPLC and TLC radical scavenging assays.[2,3]

Results and Discussion

Thyme leaves were harvested from an experimental station in Lithuania and dried under mild conditions. To compare the influence of the initial extraction solvent, a methanolic and an acidic aqueous ethanolic extract were prepared and both extracts were fractionated by a combination of solvent partitioning, normal phase chromatography, size exclusion chromatography, and reversed-phase MPLC or HPLC. Between the two types of solvents no major differences in terms of radical scavengers present in the initial crude extract were observed. However the presence of 20% water and 1% acetic acid in one of the solvents significantly decreased the amount of chlorophyll present in the initial extract, thus facilitating further fractionation steps. For this reason the acidic aqueous solvent will be used for leaves in any future studies of this type. Whenever possible, fractions of similar composition were pooled after analysis by TLC or HPLC. The radical scavenging activity of each fraction was determined either by spraying the TLC plate with a solution of the blue-colored stable radical DPPH• or by HPLC analysis with on-line detection of radical scavenging activity.[2,3] When radical scavengers are present, the DPPH• radical is reduced to a colorless product. The reduction can be observed visually on TLC plates as a yellowish spot on a purple background or after an HPLC separation by a visible wavelength detector as a decrease in absorption at 517 nm.

Although radical scavenging activity should not be considered as being synonymous with antioxidant activity, it is a fact that all of the more powerful natural antioxidants such as rosmarinic acid, tocopherol, carnosol, and ascorbic acid are also strong scavengers of the DPPH• radical. Thus good activity in this test is a first indication of the presence of possible antioxidants. As an example of the application of on-line HPLC radical scavenging detection in Figure 1, the UV and DPPH• quenching profiles of a crude methanolic thyme extract are given. Two strongly active peaks in the middle of the chromatogram are clearly visible. Noteworthy is further the pronounced radical scavenging activity of two minor peaks in the UV profile corresponding to two apolar constituents which might otherwise have been overlooked. The on-line assay speeded up the fractionation process significantly. In the beginning of the chromatogram several weakly active polar constituents eluted. The fractionation led eventually to the isolation of seven active compounds.

They were identified by a combination of UV, MS, ^{1}H NMR, and ^{13}C NMR as the known compounds rosmarinic acid **1**, eriodictyol, taxifolin, luteolin 7-glucuronide, *p*-cymene 2,3-diol, and *p*-cymene 2,3-diol 6,6′-dimer. The identity of rosmarinic acid **1**, eriodictyol, and taxifolin was further confirmed by comparison of their R_f value on TLC and retention time on HPLC with those of reference compounds. Additionally the weakly active volatile compounds carvacrol and thymol were identified by GC−MS and by comparison of their GC and HPLC retention times and R_f value on TLC with those of standards. In Figure 1 thymol corresponds with the large UV-active peak at the end of the chromatogram. In the on-line DPPH• assay it showed despite its high concentration negligible activity. This is in contrast to their activity on TLC plates, indicating a slow reaction with DPPH•. All of these compounds except taxifolin and luteolin 7-glucuronide have been

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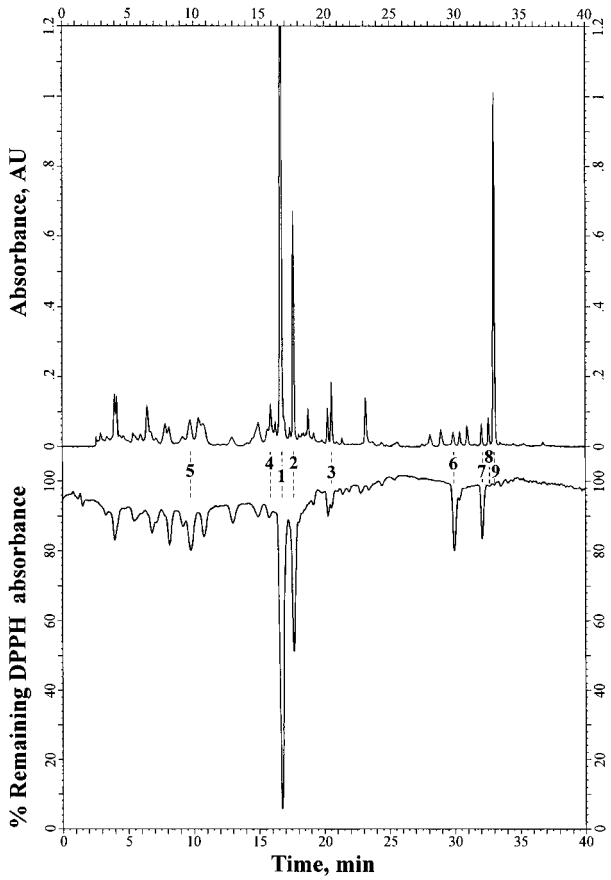


Figure 1. RP-HPLC profiles of a total methanolic extract of thyme leaves with UV 280 nm (upper trace) and DPPH• radical scavenging activity detection (lower trace). With the latter detector, negative peaks indicate activity. Numbers between the two traces are for peak assignments, 1: rosmarinic acid **1**, 2: 3′-*O*-(8″-*Z*-caffeoyl)rosmarinic acid **2**, 3: eriodictyol, 4: taxifolin, 5: luteolin 7-glucuronide, 6: *p*-cymene 2,3-diol, 7: *p*-cymene 2,3-diol 6,6′-dimer, 8: carvacrol, and 9: thymol. For chromatographic conditions see Experimental Section.

reported before in thyme,[4−10] and the presence of one or more phenolic groups explains their radical scavenging activity. The highly active compound **2** was new, and its structure elucidation is described below.

The chromatographic behavior of **2** on RP-HPLC was very similar to that of rosmarinic acid **1**, and only an acidified water−acetonitrile gradient gave two baseline separated peaks. Also the UV spectrum of **2** was reminiscent of that of rosmarinic acid with only a small shift of the high-wavelength maximum, thus suggesting a structure similar to that of **1**. FD mass spectrometry gave as molecular weight 538 with as most likely elemental composition $C_{27}H_{22}O_{12}$. This corresponded with the attachment of an additional caffeic acid moiety to rosmarinic acid **1**. A literature survey showed the existence of two independent reports on one and the same compound fitting the above description. Agata et al. reported melitric acid A **3** from *Melissa officinalis*,[11] while Zhang and Li isolated the same compound from *Salvia cavaleriei*.[12] They named it salvianolic acid I. When the spectral data of **2** were compared with those of **3**, it became apparent that they were almost but not quite identical. Next, all protons and carbons in **2** were assigned by comparison with literature and our own data for **2** and **3**, respectively, COSY, and direct and long-range $^1H-^{13}C$ correlated spectra (see Table 1). The correlations in the long-range COLOC spectrum are given in Figure 2. The assignment showed that there were significant differences in chemical shifts only for the middle caffeic acid unit in **2** and **3**. As both the $^1H-^1H$ coupling

Table 1. ^1H (400 MHz) and ^{13}C NMR (100 MHz) Data for **1**, **2**, and **3** (δ in ppm)

	$^1H^a$				$^{13}C^b$			
position	**1**	**2**	**2**	**3**[12]	**1**	**2**	**2**	**3**[12]
1					129.2	129.0	128.1	129.4
2	6.76	6.72	6.79	6.79	117.5	117.2	117.2	117.6
3					146.1	146.1	145.1	146.1
4					145.2	145.2	144.2	145.2
5	6.68	6.67	6.71	6.76	116.2	116.0	115.7	116.4
6	6.59	6.58	6.61	6.68	121.8	121.5	121.1	121.7
7a	2.97	2.96	2.98	3.04	37.8	36.9	36.9	37.9
7b	3.08	3.06	3.09	3.14				
8	5.13	5.13	5.18	5.22	74.5	73.9	73.3	74.9
9					173.5	172.6	170.7	173.7
1′					127.6	127.2	126.8	130.8
2′	7.02	6.94	7.14	7.18	114.3	114.5	116.7	116.7
3′					146.7	146.2	145.4	146.4
4′					149.7	151.0	150.1	148.3
5′	6.76	6.92	6.99	6.79	116.4	117.7	115.9	116.5
6′	6.93	7.17	7.27	7.01	123.2	125.3	124.4	122.2
7′	7.53	7.48	7.54	7.60	147.7	146.7	145.5	146.7
8′	6.25	6.17	6.26	6.39	115.1	114.8	114.6	116.4
9′					168.4	167.2	166.2	168.2
1″						125.5	125.1	125.8
2″		7.30	7.40	7.30		117.8	117.2	118.2
3″						146.4	145.3	146.4
4″						148.8	147.6	148.8
5″		6.75	6.83	6.80		116.1	115.1	115.7
6″		7.11	7.15	7.12		124.7	125.0	125.0
7″		7.34	7.34	7.37		129.1	128.6	129.2
8″						139.2	138.5	139.3
9″						166.0	164.4	167.0
solvent[c]	Me-d_4	Me-d_4	Ac-d_6	Ac-d_6	Me-d_4	Me-d_4	Ac-d_6	Ac-d_6

[a] Coupling constants: all 1,3,4-trisubstituted benzene rings showed similar coupling constants of 8 (*J*H5−H6) and 2 Hz (*J*H2−H6), *J*(H7′−H8′) 16 Hz, *J*(H7a−H7b) 14.3 Hz, *J*(H7a−H8) 4.3 Hz, *J*(H7b−H8) 8.6 Hz. [b] ^{13}C NMR assignments in methanol have been confirmed by 2D NMR techniques. [c] Me-d_4 = deuterated methanol, Ac-d_6 = deuterated acetone

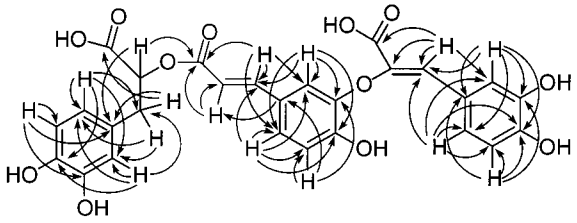
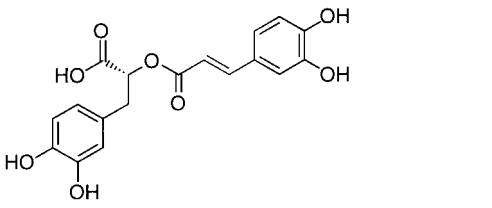


Figure 2. Observed COLOC correlations in 3′-*O*-(8″-*Z*-caffeoyl)-rosmarinic acid **2**.

constants and $^1H-^{13}C$ couplings unambiguously proved the presence of a caffeic acid unit, the only possible difference between **2** and **3** is the position of the attachment of the second caffeic acid unit. The only alternative attachment is to the other phenolic hydroxyl of the central caffeic acid moiety, i.e., position 3′ instead of 4′. Application of ^{13}C substituent parameter rules to the values reported by Agata et al. for **3** leads to significant upfield shifts for C-4′ and C-6′, moderate downfield shifts for C-1′, C-2′, and C-3′, and no or small changes for C-5′, C-7′, C-8′, C-8″, and C-9″. Except for C-3′ the expected changes were indeed observed, confirming the different mode of attachment. The shift of H-7″ indicated that the stereochemistry of the C7″−C8″ double bond is *Z*. If the stereochemistry would be *E*, which inter alia would correspond with the rare *Z*-caffeic acid as one of the building blocks, the H-7″ proton would be shifted more upfield.[11] The absolute configuration of **2** was determined by CD spectroscopy. As the chiral center and its immediate environment are identical to that of rosmarinic acid **1**, one would expect a similar CD spectrum if the configuration around C-8 is the same as in **1**. Indeed the CD spectra were only quantitatively different. Thus structure **2** is proposed for this new phenylpropanoid trimer.



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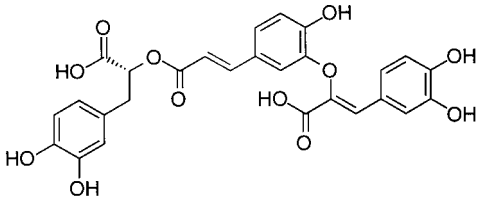

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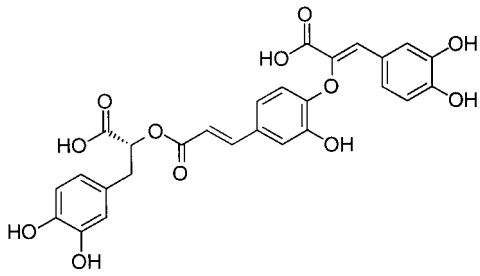

3

The activity-guided fractionation of Lithuanian thyme leaves led to the isolation of in total nine radical scavenging compounds. The simple phenolic monoterpenes thymol and carvacrol are well known and occur in hexane extracts and in the essential oil. They occur in rather high concentrations but are only weakly active in the DPPH• assay. In the on-line assay of the total extract these compounds hardly showed any activity (Figure 1). Two potent apolar radical scavengers are *p*-cymene 2,3-diol and *p*-cymene 2,3-diol 6,6′-dimer. Their concentration in a crude methanol extract is low, but they can be well observed with the HPLC on-line radical scavenging assay (Figure 1). Both their presence in thyme leaf extracts and their potent antioxidant activity have been reported earlier.[8−10] The two most important radical scavenging compounds were identified as rosmarinic acid **1** and 3′-*O*-(8″-*Z*-caffeoyl)rosmarinic acid **2**.

In off-line experiments the activity of these two compounds was determined by their reaction with the DPPH• radical and the $ABTS^{\bullet+}$ radical anion. At a molar ratio of test substance to DPPH• of 1:2, **1** and **2** showed 95% and 96% scavenging, respectively, after 15 min. For both compounds this was the end value. These values are not significantly different; however **2** showed much faster kinetics than **1**. The final value was reached for rosmarinic acid **1** after 15 min, while this result took only 3 min for **2**. After 1 min **1** and **2** showed 43% and 70% scavenging, respectively. The Trolox equivalent antioxidant capacity (TEAC) of the two compounds showed a different picture. In this assay rosmarinic acid **1** was considerably more active than the standard compound Trolox (water-soluble vitamin E), with a TEAC of 1.50 after 1 min, while **2** was significantly weaker than Trolox, with a value of 0.58. When these values were calculated after 6 min, they were 1.54 and 0.69 for **1** and **2**, respectively. Thus with the more reactive radical $ABTS^{\bullet+}$ **1** showed faster kinetics than **2**. There can be several reasons for the observed difference in antioxidant activity of these two compounds. First, the ability of compounds **1** and **2** to quench two different radicals (i.e., DPPH• and $ABTS^{\bullet+}$) can be different. Mantle et al. arrived at a similar conclusion when comparing the abilities of antioxidants to react with $ABTS^{\bullet+}$ and with radicals formed during the catalyzed oxidation of luminol.[13] Second, two basically different types of calculations were performed to obtain the TEAC and the $I_{DPPH\bullet}$ values. For the TEAC values, a graphical estimation of a slope of the line that represents inhibition as a function of concentration is carried out, while for the $I_{DPPH\bullet}$, a percentage of quenched DPPH• is arithmetically calculated. Finally, the speed of the radical quenching reaction for two analyzed compounds may also differ. As mentioned above, the TEAC value for rosmarinic acid **1** increased only 2.5% during the last 5 min of the reaction, while for 3′-*O*-(8″-*Z*-caffeoyl)-rosmarinic acid **2** this value during the same period increased 16%. This suggests that the reaction time of 6 min was not sufficient for a true estimation of compound's **2** reactivity with $ABTS^{\bullet+}$. It should be noted that the DPPH• reaction with antioxidants lasted almost 3 times longer. When analyzing the $ABTS^{\bullet+}$ quenching by various antioxidants, van de Berg et al. also concluded that most antioxidants, except Trolox, exhibit a slow reaction.[14] More studies employing different techniques including real food systems are needed to complete the evaluation of the antioxidant activity of 3′-*O*-(8″-*Z*-caffeoyl)rosmarinic acid **2**.

Experimental Section

General Experimental Procedures. All solvents used for extraction, partitioning, and fractionation purposes were redistilled, with the exception of MeOH and MeCN, which were HPLC grade. UV spectra were recorded on a Perkin-Elmer Lambda 18 UV/vis spectrometer. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter with a 10 cm cell with a sodium lamp in MeOH. Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter in MeOH. One-dimensional NMR spectra were recorded on a Bruker AC200 or Bruker AM-400 spectrometer using the residual solvent peaks as internal standard. Two-dimensional spectra (COSY, HETCOR, and COLOC) were all recorded on a Bruker AM-400. Field desorption (FD) and chemical ionization (CI, pos. and neg. mode, reaction gas CH_4) mass spectra were obtained on a MAT 95 mass spectrometer. Solvents were removed at 12−35 mmHg pressure at 45 °C with a Büchi RE 11 rotary evaporator combined with a Vacuubrand CVC2 vacuum pump and a Büchi 461 water bath. Freeze-drying was carried out in a Christ Alpha 1-2 freeze-dryer equipped with a Vacuubrand rotary vane vacuum pump. Column chromatography was performed using Fluka Si gel (Kieselgel 60, 230−400 mesh) and Pharmacia Sephadex LH-20. Medium-pressure liquid chromatography (MPLC) was carried out on a Jobin Yvon axial compression system equipped with a Gilson 303 pump and 802 C manometric module. The column was filled with Bakerbond 40 μm RP-18 stationary phase. Eluted fractions were collected with a LKB Bromma 2111 Multirac sample collector.

Semipreparative RP-HPLC was conducted with a Waters 600 E pump combined with a 250 × 10 mm Rainin Dynamax RP-HPLC column filled with Microsorb 5 μm C18 stationary phase, a Waters 990 photodiode array detector, and a Gilson 231 autosampling injector equipped with a 100 μL injection loop. The instrumental setup used for analytical HPLC was similar to that used for semipreparative purposes except for

the introduction of a Scientific Systems pulse damper and a 250 × 4.6 mm Alltima RP-HPLC analytical column filled with a C18 5 μm stationary phase. Isocratic elution with MeCN−H_2O (505:495) was used in semipreparative runs, while for analytical RP-HPLC a binary MeCN−H_2O gradient was used. Solvents A and B were MeCN−H_2O (25:75) acidified with 0.25% HOAc and 100% MeCN acidified with 0.25% HOAc, respectively. During the initial 3 min the eluent consisted of 100% A. Then, the percent of solvent A decreased to 65% in 11 min and remained at this percent during the next 7 min. During the following 7 min the percent of solvent A further decreased to 5%, where it remained during 12 min. In the next 3 min solvent A decreased to 0% and remained so during 2 min. Finally, solvent A returned to its initial percent in 5 min. HPLC on-line DPPH$^\bullet$ radical scavenging detection was carried out as described earlier.[2,3]

Thymol and carvacrol were purchased from Sigma-Aldrich, eriodictyol and (±)-taxifolin from Roth, and rosmarinic acid from Extrasynthèse. Luteolin 7-glucuronide unambiguously identified by 2D NMR techniques was available as a reference (Pukalskas, unpublished).

Plant Material. The thyme leaves (*Thymus vulgaris* L.) were collected in the period September 21−30, 1997, in the experimental garden of the Lithuanian Institute of Horticulture, Babtai, Lithuania. A voucher specimen has been deposited in the Lithuanian Institute of Horticulture, no. TV-97-20. The plant material was dried in a drying cabinet with forced ventilation at 40 °C for 2−3 days. The samples were packed in double-walled paper bags and stored at ambient temperature before use.

Extraction and Isolation. Procedure 1. Dried, ground thyme leaves (100 g) were extracted at 20 °C with 1 L of EtOH−H_2O−HOAc (80:19:1) under N_2 for 5 days. The obtained crude extract was filtered and successively extracted in a separatory funnel with petroleum ether (40−60 °C) and EtOAc. The petrol ether layer was not further processed. After removal of the EtOH with a rotary evaporator, the aqueous layer was extracted with EtOAc. The EtOAc layer was concentrated in vacuo at 45 °C, yielding 5.6 g of extract. This was fractionated on Sephadex LH-20 with EtOH and Me_2CO−H_2O (1:1), resulting in 35 fractions of 15 mL. Fractions were checked for their capacity to bleach DPPH$^\bullet$ on TLC. After evaporation active fractions were refractionated on Sephadex with the same eluents. Active fractions were pooled, concentrated, and successively fractionated on a Si gel column with hexane−EtOAc−HCO_2H (60:39:1) and hexane−EtOAc−HCO_2H (40:59:1). Three pure radical scavenging compounds were isolated and identified as eriodictyol **3** (15 mg), taxifolin **4** (63 mg), and rosmarinic acid **1** (390 mg).

The aqueous layer remaining after extraction with EtOAc was concentrated in vacuo at 65 °C. Any residual water was removed by freeze-drying for 48 h. This extract was partitioned between H_2O and n-BuOH. The aqueous extract was inactive and discarded. The BuOH layer, after drying and concentration, was separated by RP-MPLC on C-18 with THF−H_2O−HCO_2H (30:69:1) as eluent. Active fractions were pooled and separated by column chromatography on Sephadex LH-20 with EtOH−H_2O (1:1). Active fractions were pooled and concentrated (141 mg). This material was washed 4× with 50 mL of EtOAc−MeOH−H_2O−HCO_2H (87:10:2:1). The insoluble residue was centrifuged and dried in vacuo, resulting in a yellow powder (38.5 mg) identified as luteolin 7-glucuronide **5**.

Procedure 2. Dried, ground herb material (50 g) was extracted for 70 h under N_2 with 500 mL of MeOH. The extract was filtered and concentrated in vacuo at 40 °C, yielding 8.9 g of crude methanolic extract. The extract was partitioned between hexane and warm H_2O (45 °C). The hexane layer was dried over Na_2SO_4 and concentrated in vacuo, yielding 2.45 g of hexane extract. This extract was separated by CC on Si gel with toluene−Me_2CO−HCO_2H (184:15:1). Two active fractions, A and B, were obtained, which were both further purified by CC on Si gel with toluene−Me_2CO−HCO_2H (190:19:1) and on Sephadex with MeOH. Purified fraction A was further purified to homogeneity by crystallization from MeOH at −18 °C and semipreparative RP-HPLC on a C18 column with MeCN−H_2O (1:1). It was identified as *p*-cymene 2,3-diol **6** (11 mg). Purified fraction B was further purified to homogeneity by dissolving it in benzene, filtration, and evaporation of the benzene. It was identified as *p*-cymene 2,3-diol 6,6′-dimer **7** (11 mg).

The aqueous layer remaining after the hexane partitioning was successively extracted with t-BuMeO, EtOAc, and BuOH. The remaining aqueous layer was evaporated to dryness (3.1 g). This fraction was chromatographed on a Si gel column with toluene−Me_2CO−HCO_2H (133:66:1). In total 110 fractions were collected. Pooled fractions 69−100 contained 33 mg of 3′-*O*-(8″-*Z*-caffeoyl)rosmarinic acid **2**.

Assessment of Radical Scavenging Activity of Fractions. A fast screening method that involved spotting 8−10 μL of each collected fraction on a Merck Si gel 60 F_{254} 0.25 mm thickness TLC plate and spraying it with 0.1% DPPH$^\bullet$ solution in MeOH without chromatographic development was used to detect isolates with radical scavenging activity. Plates were examined after 15 min. Radical scavengers of active fractions bleached the purple background of DPPH$^\bullet$, giving a white-yellowish spot. Before pooling, active fractions were investigated by TLC followed by spraying with a 0.1% methanolic DPPH$^\bullet$ solution similar to a procedure described by Takao et al.[15] The TLC chromatography was carried out on Merck Si gel or RP-18 F_{254S} TLC plates with suitable eluents. Purity of combined fractions and radical scavenging activity of individual constituents were also evaluated with the on-line HPLC DPPH$^\bullet$ system.[2,3]

Off-line ABTS Radical Scavenging Assay. A 2.0 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) stock solution in phosphate-buffered saline (PBS buffer, 8.18 g of NaCl, 0.27 g of KH_2PO_4, 1.42 g of Na_2HPO_4, and 0.15 g of KCl dissolved in 1 L of ultrapure water) was prepared. If necessary, the pH was adjusted to 7.4 with NaOH solution. A 70 mM $K_2S_4O_8$ solution in ultrapure water was prepared. ABTS radical cation solution was produced by mixing 50 mL of ABTS stock solution with 200 mL of $K_2S_4O_8$ solution and leaving the mixture to stand in the dark at room temperature for 16−17 h before use. The radical was stable under these conditions for more than 2 days. For the radical scavenging assay the ABTS$^{\bullet+}$ solution was diluted with PBS buffer to an absorbance of 0.800 ± 0.030 at 734 nm. Stock solutions in MeOH of the compounds to be investigated were diluted with PBS so that after introduction of a 10 μL aliquot of each dilution into the assay each dilution produced a 10−80% reduction of the initial absorbance. After addition of 990 μL of diluted ABTS$^{\bullet+}$ solution to 10 μL of the test compounds or the Trolox standard (final concentration 0−2 μM) in EtOH or PBS, the absorbance was recorded exactly 1 and 6 min after the initial mixing. The testing was at ambient temperature (≈20 °C). Appropriate solvent blanks were run in each assay. All determinations were carried out in triplicate at each separate concentration of the standard and samples. Typically six concentrations were measured (e.g., 8−4−2−1−0.5−0.25 μM). The percentage inhibition of the absorbance at 734 nm was calculated and plotted as a function of the concentration of radical scavengers and of the Trolox standard. The concentration of test substance giving the same decrease of ABTS$^{\bullet+}$ absorbance at 734 nm as Trolox was calculated in terms of the Trolox equivalent antioxidant activity (TEAC) at two time points (1 and 6 min). The TEAC value is calculated by dividing the slope of the plot of the test substance by that of Trolox.

Off-line DPPH$^\bullet$ Radical Scavenging Assay. Radical scavenging activity against the stable radical DPPH$^\bullet$ was measured using the method of Von Gadow et al.,[16] which was modified as described below. Methanolic solutions (10^{-4} M) of DPPH$^\bullet$ and compounds to be tested and reference compounds (BHT and RA) were mixed in a 1 cm path length disposable plastic half-micro cuvette (Greiner Labortech, The Netherlands) in such way that the final molar ratios between the compound and DPPH$^\bullet$ were 1:10 or 1:2. The samples were kept 15 min in the dark at room temperature, and the decrease of absorbance at 515 nm was measured against methanol using a Specol 11 spectrophotometer (Carl Zeiss Jena). The absorbance of a blank sample containing the same amount of methanol and DPPH$^\bullet$ solution was prepared and measured

daily. DPPH• solution was freshly prepared daily and kept in the dark at 4 °C between the measurements. All determinations were performed in triplicate. The radical scavenging activity of the tested samples, expressed as percent inhibition, was calculated with the following formula:[2]

$$\% \text{ Inhibition} = [(AB - AA)/AB] \times 100\%$$

where AB = absorbance of the blank sample at $t = 0$ and AA = absorbance of tested antioxidant after 15 min.

Rosmarinic acid (1): $[\alpha]^{22}_D$ +81.6° (*c* 0.41, MeOH); CD (MeOH) $\Delta\epsilon$ (nm) +3.2 (332), +4.0 (297), −2.2 (233), +2.0 (218), 0.0 (215); UV (MeOH) λ_{max} 217, 231 (sh), 290, 329 nm; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz), see Table 1; EIMS *m*/*z* 201 (12), 200 (17), 199 (13), 126 (32), 125 (80), 124 (100), 123 (50), 113 (14), 78 (10), 77 (9); FABMS *m*/*z* 362 (19), 361 [M + H]$^+$ (27), 187 (26), 186 (67), 181 (36), 180 (34), 164 (25), 163 (83), 115 (19), 94 (100); R_f value on TLC and t_R with HPLC identical to those of an authentic reference; NMR data in agreement with those published.[17,18]

3′-*O*-(8″-*Z*-Caffeoyl)rosmarinic acid (2): $[\alpha]^{22}_D$ +40.3° (*c* 1.2, MeOH); CD (MeOH) $\Delta\epsilon$ (nm) +7.9 (327), +8.0 (297), +1.0 (275), +1.8 (252), −1.4 (233), +0.9 (221), 0.0 (217); UV (MeOH) λ_{max} 217 (sh), 231 (sh), 291, 322 nm; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz), see Table 1; COLOC spectrum, see Figure 2; FDMS *m*/*z* 538; pos. CIMS (CH_4) *m*/*z* 391 (80), 113 (24), 73 (22), 57 (100); neg. CIMS (CH_4) *m*/*z* 346 (88), 176 (100).

Eriodictyol: HREIMS *m*/*z* 288.0620 (calcd for $C_{15}H_{12}O_6$, 288.0634); R_f value on TLC and t_R with HPLC identical with those of an authentic reference; UV, EIMS, and ^{1}H NMR (400 MHz) data in agreement with those published.[19−21]

Taxifolin (synonym: dihydroquercetin): HREIMS *m*/*z* 304.0556 (calcd for $C_{15}H_{12}O_7$, 304.0583); R_f value on TLC and t_R with HPLC identical with those of an authentic reference; UV, EIMS, and ^{1}H NMR (400 MHz) data in agreement with those published.[19,22]

Luteolin 7-glucuronide: UV (MeOH) λ_{max} 255, 268 (sh), 348 nm; ^{13}C NMR (CD_3OD, 100 MHz) δ 183.0 (C4), 171.3 (C6″), 165.8 (C2), 163.2 (C7), 161.6 (C5), 157.7 (C9), 150.0 (C4′), 145.8 (C3′), 122.3 (C1′), 119.7 (C6′), 115.8 (C5′), 113.2 (C2′), 105.1 (C10), 103.1 (C3), 100.2 (C1″), 100.2 (C6), 95.0 (C8), 76.1 (C5″), 75.5 (C3″), 73.3 (C2″), 71.9 (C4″); EIMS *m*/*z* 286 (100); FABMS *m*/*z* 463 [M + H]$^+$ (25), 449 (15), 287 (35), 263 (20), 243 (30), 207 (80), 183 (40), 115 (100); FDMS *m*/*z* 463 [M + H]$^+$ (30), 449 (22), 447 (3), 288 (12), 287 (83), 286 (100); UV, EIMS, and ^{1}H NMR (400 MHz) data in agreement with those published.[19,23,24]

***p*-Cymene 2,3-diol:** GC-EIMS *m*/*z* 166 [M]$^+$ (30), 151 (100), 133 (10), 105 (10), 77 (5); ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in agreement with those published.[9]

***p*-Cymene 2,3-diol 6,6′-dimer (systematic name: 3,4,3′,4′-tetrahydroxy-5,5′-diisopropyl-2,2′-dimethylbiphenyl):** UV, EIMS, ^{1}H NMR (400 MHz), and ^{13}C NMR (100 MHz) in agreement with those published.[10,25]

Carvacrol: R_f value on TLC and t_R with HPLC and capillary GC identical to those of an authentic reference; UV and GC−MS in agreement with those published.[26,27]

Thymol: R_f value on TLC and t_R with HPLC and capillary GC identical to those of an authentic reference; UV and GC−MS in agreement with those published.[26,27]

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