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In Vitro Activity-Guided Identification of Antioxidants in Aged Garlic Extract

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ABSTRACT: Activity-guided fractionation was applied on an aged garlic extract (AGE), reported to show strong antioxidant activity, in order to locate the key in vitro antioxidant ingredients by means of the hydrogen peroxide scavenging (HPS) assay as well as the ORAC assay. Besides the previously reported four tetrahydro-*β*-carbolines, (1*R*,3*S*)- and (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid and (1*R*,3*S*)- and (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid, LC-MS/MS, LC-TOF-MS, and 1D/2D-NMR experiments led to the identification of coniferyl alcohol and its dilignols (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol, (+)-(2*S*,3*R*)-dehydrodiconiferyl alcohol, *erythro*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether, and *threo*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether as the major antioxidants in AGE. The purified individual compounds showed high antioxidant activity, with EC_{50} values of 9.7−11.8 *μ*M (HPS assay) and 2.60−3.65 *μ*mol TE/*μ*mol (ORAC assay), respectively.

KEYWORDS: *aged garlic extract, antioxidants, dihydrodehydrodiconiferyl alcohol, dehydrodiconiferyl alcohol*

INTRODUCTION

Reactive oxygen species (ROS) are well known to be generated as byproducts of the normal cell aerobic respiration that is essential to life and play a crucial role in cell signaling and homeostasis.[1] However, overproduction of ROS causes undesirable oxidative stress, which is associated with chronic and degenerative diseases such as diabetes,[2,3] neurodegeneration,[4,5] cardiovascular disease,[6,7] and cancer[8,9] and is involved in the process of aging.[10,11]

Although not 100% effective, the human body has developed a very delicate system to eliminate free radicals from the body.[12,13] Vitamins C and E and antioxidant phytochemicals such as polyphenols have been thought to be responsible for most of the antioxidant activity in foods and were examined for their abilities to attenuate oxidative damage induced by ROS.[14,15]

Moreover, previous studies have provided strong evidence that aged garlic extract (AGE), manufactured by extracting fresh garlic with aqueous ethanol and maturing the extract for more than 10 months, exhibits strong antioxidant activity, e.g. by increasing the activity of superoxide dismutase, catalase, and glutathione peroxidase in vascular endothelial cells [16] as well as by scavenging H_2O_2 and free radicals.[17−19] AGE was also reported to exhibit cardioprotective,[20,21] liver-protective,[22,23] and cancer-preventing effects,[24,25] although the underlying chemical components have not yet been elucidated.

Besides some organosulfur compounds such as *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC), originating from garlic,[17] Maillard reaction products such as *Nα*-(1-deoxy-D-fructos-1-yl)-L-arginine (**1**; Figure 1) and the 1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acids **2**−**5** were identified in AGE as potent antioxidants.[26,27] Even though these previous studies gave a first insight into antioxidants in AGE, their antioxidant activities are assumed to be by far insufficient to show a major contribution to the overall antioxidative power of AGE.

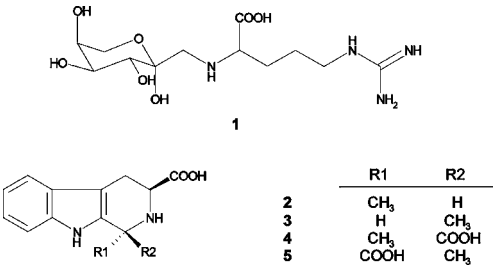

Figure 1. Chemical structures of antioxidants previously identified in aged garlic extract: *Nα*-(1-deoxy-D-fructos-1-yl)-L-arginine (**1**), (1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid (**2**), (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid (**3**), (1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**4**), and (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**5**).

The objective of the present study was, therefore, to locate the antioxidants in AGE by means of an activity-guided fractionation approach using the hydrogen peroxide scavenging (HPS) assay as well as the oxygen radical absorbance capacity (ORAC) assay and to identify their chemical structures by means of LC-MS and NMR experiments.

MATERIALS AND METHODS

Chemicals. 2,2′-Azo-bis(2-methylpropinamidine) (AAPH), fluorescein sodium salt (FL), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azinobis(3-ethylbenzothiazoline-6-

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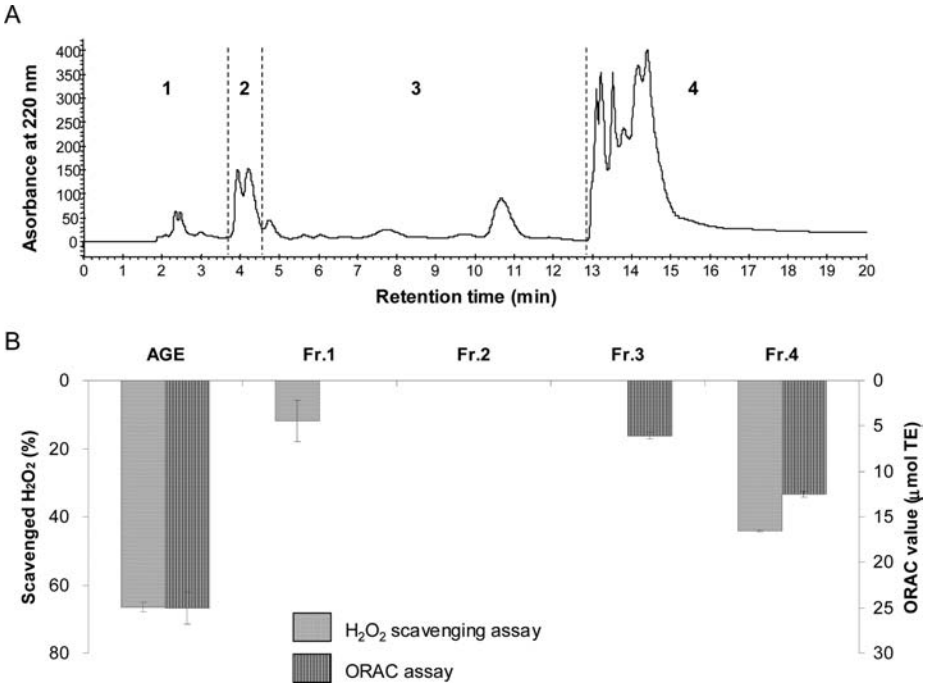

Figure 2. RP18-HPLC chromatogram of AGE separation (A) and antioxidant activity of AGE and HPLC fractions (B). Data given for HPS and ORAC activity are the means of three and four independent repetitions, respectively (error bars represent standard deviation, SD).

sulfonic acid) diammonium salt (ABTS), peroxidase from horseradish, quercetin, (−)-epicatechin, ascorbic acid, and coniferyl alcohol were purchased form Sigma-Aldrich (Steinheim, Germany), and hydrogen peroxide was from Merck (Hohenbrunn, Germany). Water for chromatographic separations was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany), and solvents used were of HPLC-grade (Merck, Darmstadt, Germany). Aged garlic extract was produced by Wakunaga Pharmaceutical Co. Ltd. by hydroalcoholic extraction of sliced, fresh garlic cloves in a stainless steel tank for 20 months at room temperature. The AGE samples used contained the well-known organosulfur compound *S*-allylcysteine in the concentration of 1.6−2.4 mg/g dry weight.[19] *Nα*-(1-Deoxy-D-fructos-1-yl)-L-arginine (**1**) was synthesized as reported in the literature.[26] Chemical synthesis of (1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid (**2**), (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid (**3**), (1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**4**), and (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**5**) was performed following a literature procedure.[27]

Hydrogen Peroxide Scavenging Assay. In accordance with a literature protocol,[26] sample solutions of AGE fractions and purified compounds were prepared in phosphate buffer (100 mM, pH 6.0) using the test materials in the concentration ratios naturally occurring in AGE. An aliquot (100 μL) of the sample solution or phosphate buffer (100 mM, pH 6.0) used for control was placed in each well of a 96-well clear microplate (VWR, Ismaning, Germany). Then, phosphate buffer (100 mM, pH 6.0; 30 μL) and aqueous hydrogen peroxide solution (500 μM in water; 10 μL) or phosphate buffer used for blank was added in each well. Afterward a solution of peroxidase (150 U/mL in water; 40 μL) and a solution of ABTS (0.1% in water; 40 μL) were added. After incubation for 15 min at 37 °C, the absorbance of each well was measured at 414 nm by means of a FLUOstar OPTIMA equipment (BMG LABTECH, Offenburg, Germany). Using the measured absorbance of the sample solution (AS), the sample-blank solution (ASB, without hydrogen peroxide), the control solution (AC, without sample solution), and control-blank solution (ACB, without sample solution and hydrogen peroxide), the scavenging effect (*E*) was calculated using the following formula, and EC_{50} was calculated by the probit method: $E = [(AC - ACB) - (AS - ASB)]/(AC - ACB) \times 100$.

Oxygen Radical Absorbance Capacity Assay. Following a literature protocol[28] with some modification, the peroxyl radical scavenging efficacy was measured using the ORAC assay. Sample solutions were prepared in the same way as in the hydrogen peroxide scavenging assay using phosphate buffer (10 mM, pH 7.4). A series of Trolox solutions (200, 100, 50, 25, 12.5 μM) was prepared by diluting an ethanolic solution of Trolox (2 mM) with phosphate buffer (10 mM, pH 7.4). Sample solution, Trolox dilution (25 μL), or phosphate buffer (10 mM, pH 7.4) used as blank was placed in wells of a 96-well black microplate (VWR). Then fluorescein sodium salt (150 μL in phosphate buffer, 10 nM) was added to each well, and the microplate was incubated at 37 °C for 30 min. Afterward, the decay of fluorescence was measured every 90 s at the excitation wavelength of 485 nm and the emission wavelength of 520 nm using a FLUOstar OPTIMA plate reader. The first three cycles were taken to determine the background signal. After 3 cycles, AAPH (25 μL in phosphate buffer, 240 mM) was added, and then the measurement was resumed and continued up to 90 min (60 cycles in total). The ORAC values were calculated according to the method of Cao et al.[29] Briefly, a standard curve was obtained from the area under the fluorescence versus time curve (AUC) for Trolox dilutions minus the area-under-the-curve (AUC) for blank. Then the AUC for the sample solution minus the AUC for the blank was calculated and compared to the standard curve. ORAC values were expressed as Trolox equivalents (μmol TE/μmol).

Fractionation of AGE. An aliquot of AGE (80 g, dry weight) was fractionated by using a preparative HPLC system (PrepStar, Varian, Darmstadt, Germany) consisting of two HPLC pumps (model SD-1), a two-wavelength UV detector (Prostar 325), and a fraction collector (model 701), equipped with a 50 × 200 mm, 8 μm, C-18 column (Microsorb, Varian, Darmstadt, Germany) packed by using a load-and-lock packing station (Varian, Darmstadt, Germany). Monitoring the effluent (100 mL/min) at 220 nm, chromatography was performed starting with aqueous formic acid (0.1% in water, pH 2.5) for 10 min, then increasing the methanol content up to 100% within 3 min, and finally held for 7 min at 100% methanol. Four fractions, namely, fractions 1 (28.3 g), 2 (0.3 g), 3 (6.7 g), and 4 (43.5 g), were collected (Figure 2), concentrated under reduced pressure at 40 °C, and then further separated on a semipreparative HPLC system (Jasco, Groß-Umstadt, Germany) equipped with a 250 × 21.2 mm, 5 μm, C-18

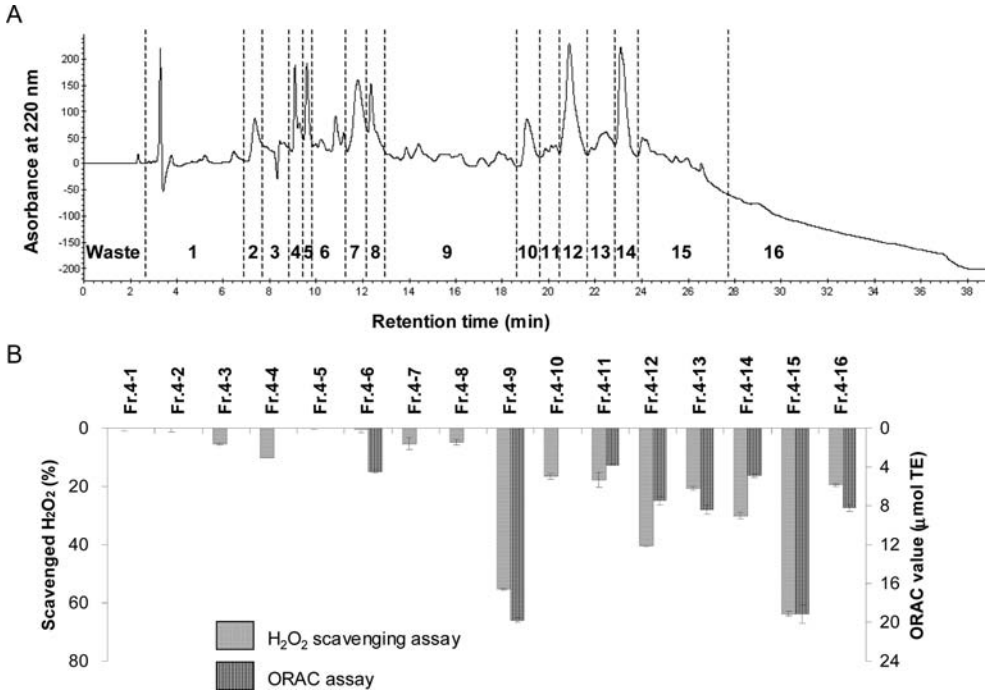

Figure 3. RP18-HPLC chromatogram of the separation of fraction 4 (A) and antioxidant activity of HPLC fractions (B). Data given for HPS and ORAC activity are the means of three and four independent repetitions, respectively (error bars represent SD).

column (Microsorb, Varian, Darmstadt, Germany) as the stationary phase consisting of two PU-2087 Plus pumps, a DG-2080-53 degasser, an LG-2080-02 gradient unit, and a 2010 Plus multiwavelength detector. Using a flow rate of 21.0 mL/min, an aliquot (10 g) of fraction 4 was fractionated using the following gradient of 0.1% aqueous formic acid (solvent A) and methanol (solvent B): 0−3 min (0% B), 3−33 min (0 → 90% B), 33−34 min (90 → 100% B), 34−39 min (100% B). Monitoring the effluent at 220 nm, a total of 16 subfractions were collected, separated from solvent under vacuum, and then freeze-dried to obtain fractions 4-1 (286 mg), 4-2 (47 mg), 4-3 (86 mg), 4-4 (455 mg), 4-5 (1542 mg), 4-6 (3129 mg), 4-7 (153 mg), 4-8 (46 mg), 4-9 (115 mg), 4-10 (9 mg), 4-11 (11 mg), 4-12 (25 mg), 4-13 (21 mg), 4-14 (6 mg), 4-15 (39 mg), and 4-16 (90 mg) with the yields given in parentheses (Figure 3). The corresponding fractions collected from four separations were combined. Fraction 4-14 was further separated by means of semipreparative HPLC (HPLC pump system PU 2087, high-pressure gradient unit, and PU-2075 UV detector, Jasco, Groß-Umstadt, Germany) equipped with a Luna PhenylHexyl 10 × 250 mm, 5 μm column (Phenomenex) as the stationary phase using the following gradient of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 4.2 mL/min: 0−35 min (22% B), 35−38 min (22 → 100% B), 38−42 min (100% B). Monitoring the HPLC effluent at 220 nm showed two main compounds, which were isolated, freed from solvent under vacuum, and freeze-dried to afford compounds **7** and **8** as white, amorphous powders in yields of 14.7 and 11.7 mg (Figure 4). LC-TOF-MS, 1D/2D-NMR, and circular dichroism (CD) spectroscopic experiments led to the identification of these compounds as (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol (**7**) and (+)-(2*S*,3*R*)-dehydrodiconiferyl alcohol (**8**), respectively.

(−)-(2*R*,3*S*)-Dihydrodehydrodiconiferyl alcohol, 7, Figure 4. UV/vis (MeOH/H_2O, 5:5, v/v): λ_{max} = 232, 281 nm. LC-TOF-MS (ESI−): *m/z* 359.1497 ($[M - H]^-$, measured; *m/z* 359.1495, calculated for $[C_{20}H_{24}O_6-H]^-$). CD (MeOH, 0.28 mmol/L): λ_{max} ($\Delta\varepsilon$) = 294 (−1.1), 242 (−2.8), 224 (+1.4). ^{1}H NMR (400 MHz, acetone-d_6, COSY): δ 7.04 [d, 1H, *J* = 1.9 Hz, H−C(2′)], 6.89 [dd, 1H, *J* = 1.9, 8.2 Hz, H−C(6′)], 6.81 [d, 1H, *J* = 8.2 Hz, H−C(5′)], 6.75 [d, 1H, *J* = 1.6 Hz, H−C(4)], 6.73 [d, 1H, *J* = 1.6 Hz, H−C(6)], 5.52 [d, 1H, *J* = 6.6 Hz, H−C(2)], 3.83 [s, 3H, H−C(7-OMe)], 3.82 [s, 3H, H−C(3′-OMe)], 3.76−3.92 [m, 2H, H−C(11)], 3.57 [t, 2H, *J* = 6.4 Hz, H−C(10)], 3.51 [m, 1H, *J* = 6.4, 6.6 Hz, H−C(3)], 2.62 [t, 2H, *J* = 7.7 Hz, H−C(8)], 1.79 [m, 2H, *J* = 6.4, 7.7 Hz, H−C(9)]. ^{13}C NMR (100 MHz, acetone-d_6, HSQC, HMBC): δ 148.35 [C(3′)], 147.35 [C(7a)], 147.19 [C(4′)], 144.89 [C(7)], 136.30 [C(5)], 134.70 [C(1′)], 130.00 [C(3a)], 119.54 [C(6′)], 117.57 [C(4)], 115.64 [C(5′)], 113.89 [C(6)], 110.45 [C(2′)], 88.18 [C(2)], 64.78 [C(11)], 61.84 [C(10)], 56.42 [C(7-OMe)], 56.27 [C(3′-OMe)], 55.10 [C(3)], 36.00 [C(9)], 32.70 [C(8)].

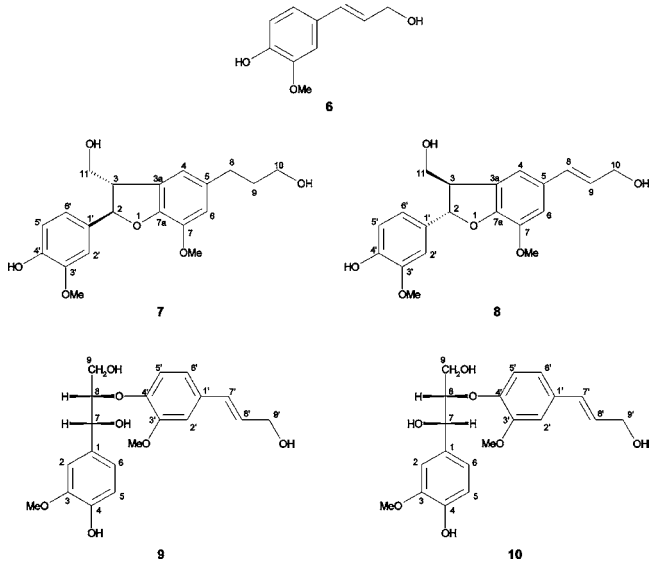

Figure 4. Chemical structures of coniferyl alcohol (**6**), (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol (**7**), (+)-(2*S*,3*R*)-dehydrodiconiferyl alcohol (**8**), *erythro*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether (**9**), and *threo*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether (**10**).

(+)-(2*S*,3*R*)-Dehydrodiconiferyl alcohol, 8, Figure 4. UV/vis (MeOH/H_2O, 5:5, v/v): λ_{max} = 225, 275 nm. LC-TOF-MS(ESI−): *m/z* 357.1350 ($[M - H]^-$, measured; *m/z* 357.1338, calculated for $[C_{20}H_{22}O_6-H]^-$); CD (MeOH, 0.28 mmol/L): $\lambda_{max}(\Delta\varepsilon)$ = 286 (+1.4), 231 (−1.2). ^{1}H NMR (400 MHz, acetone-d_6, COSY): δ 7.04

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[d, 1H, J = 1.9 Hz, H−C(2′)], 6.98 [d, 1H, J = 1.6 Hz, H−C(4)], 6.95 [d, 1H, J = 1.6 Hz, H−C(6)], 6.89 [dd, 1H, J = 1.9, 8.1 Hz, H−C(6′)], 6.81 [d, 1H, J = 8.2 Hz, H−C(5′)], 6.53 [d, 1H, J = 15.9 Hz, H−C(8)], 6.25 [dt, 1H, J = 5.3, 15.9 Hz, H−C(9)], 5.57 [d, 1H, J = 6.5 Hz, H−C(2)], 4.20 [d, 2H, J = 5.3 Hz, H−C(10)], 3.87 [s, 3H, H−C(7-OMe)], 3.82 [s, 3H, H−C(3′-OMe)], 3.93−3.79 [m, 2H, H−C(11)], 3.54 [dd, 1H, J = 6.3, 6.5 Hz, H−C(3)]. ^{13}C NMR (100 MHz, acetone-d_6, HSQC, HMBC): δ 151.77 [C(3′)], 149.27 [C(4′)], 148.84 [C(3)], 147.20 [C(4)], 133.79 [C(1)], 133.17 [C(1′)], 131.42 [C(7′)], 128.63 [C(8′)], 120.81 [C(6′)], 120.75 [C(6)], 118.87 [C(5′)], 115.85 [C(5)], 111.76 [C(2)], 111.30 [C(2′)], 87.14 [C(8)], 74.04 [C(7)], 63.75 [C(9′)], 61.92 [C(9)], 56.55 [C(3′-OMe)], 56.35 [C(3-OMe)].

Synthesis of *erythro*-Guaiacylglycerol-*β*-*O*-4′-coniferyl Ether (9) and *threo*-Guaiacylglycerol-*β*-*O*-4′-coniferyl Ether (10). Following a literature protocol[30] with some modifications, coniferyl alcohol (0.56 mmol) was dissolved in phosphate buffer (85 mL; 100 mM, pH 6.0), and a solution (5 mL) of horseradish peroxidase (3.4 μg/mL) in phosphate buffer and an aqueous solution (85 mL) of hydrogen peroxide (0.01% in water) were added dropwise within 30 min while stirring at room temperature. After 5 h of continued stirring, the reaction was stopped by the addition of hydrogen chloride (2 mL, 1 M), the reaction mixture was extracted with ethyl acetate (2 × 200 mL), the combined organic layers were separated from solvent under vacuum, the residue was taken up in methanol/water (75/25, v/v; 15 mL), and then the target compounds **9** and **10** were isolated by means of preparative HPLC (Jasco) equipped with a Luna PhenylHexyl 21.2 × 250 mm, 5 μm column (Phenomenex). Using a flow rate of 21 mL/min, chromatography was performed with the following gradient using 0.1% aqueous formic acid (solvent A) and methanol (solvent B): 0−12 min (10 → 20% B), 12−32 min (20 → 60% B), and 32−34 min (60 → 100% B). Monitoring the effluent at 220 nm, the effluent of the peaks detected at 16.2 and 16.8 min, respectively, were collected individually and freed from solvent under vacuum to give compounds **9** (4.0 μmol) and **10** (7.2 μmol) as white, amorphous powders after freeze-drying.

*erythro-Guaiacylglycerol-β-O-4′-coniferyl Ether, **9**, Figure 4.* UV/vis (MeOH/H_2O, 5:5, v/v): λ_{max} = 265 nm; LC-TOF-MS (ESI−): m/z 375.1446 ([M − H]$^-$, measured; m/z 375.1444, calculated for [$C_{20}H_{24}O_7$−H]$^-$). ^{1}H NMR (500 MHz, methanol-d_4, COSY): δ 7.02 [d, 1H, J = 1.9 Hz, H−C(2)], 7.00 [s, 1H, H−C(2′)], 6.87 [brs, 2H, H−C(5′, 6′)], 6.84 [dd, 1H, J = 1.8, 8.1 Hz, H−C(6)], 6.73 [d, 1H, J = 8.1 Hz, H−C(5)], 6.51 [dt, 1H, J = 1.4, 15.9 Hz, H−C(7′)], 6.24 [dt, 1H, J = 5.8, 15.9 Hz, H−C(8′)], 4.82 [overlapped, 1H, H−C(7)], 4.35 [m, 1H, H−C(8)], 4.19 [dd, 2H, J = 1.4, 5.8 Hz, H−C(9′)], 3.80 [m, 2H, H−C(9)], 3.80 [s, 3H, H−C(3′-OMe)], 3.80 [s, 3H, H−C(3-OMe)]. ^{13}C NMR (125 MHz, methanol-d_4, HSQC, HMBC): δ 151.93 [C(3′)], 148.96 [C(4′)], 148.72 [C(3)], 147.04 [C(4)], 134.10 [C(1)], 133.07 [C(1′)], 131.46 [C(7′)], 128.51 [C(8′)], 121.04 [C(6)], 120.66 [C(6′)], 118.92 [C(5′)], 115.66 [C(5)], 111.90 [C(2)], 111.40 [C(2′)], 86.22 [C(8)], 74.12 [C(7)], 63.76 [C(9′)], 62.23 [C(9)], 56.52 [C(3-OMe)], 56.34 [C(3′-OMe)].

*threo-Guaiacylglycerol-β-O-4′-coniferyl Ether, **10**, Figure 4.* UV/vis (MeOH/H_2O, 5/5, v/v): λ_{max} = 264 nm. LC-TOF-MS (ESI−): m/z 375.1447 ([M − H]$^-$, measured; m/z 375.1444, calculated for [$C_{20}H_{24}O_7$−H]$^-$). ^{1}H NMR (500 MHz, methanol-d_4, COSY): δ 7.09 [d, 1H, J = 1.9 Hz, H−C(2′)], 7.06 [d, 1H, J = 1.9 Hz, H−C(2)], 7.03 [d, 1H, J = 8.3 Hz, H−C(5′)], 6.95 [dd, 1H, J = 1.9, 8.3 Hz, H−C(6′)], 6.90 [dd, 1H, J = 1.9, 8.1 Hz, H−C(6)], 6.79 [d, 1H, J = 8.1 Hz, H−C(5)], 6.57 [d, 1H, J = 15.9 Hz, H−C(7′)], 6.30 [dt, 1H, J = 5.7, 15.9 Hz, H−C(8′)], 4.92 [d, 1H, J = 5.8 Hz, H−C(7)], 4.33 [m, 1H, H−C(8)], 4.24 [dd, 2H, J = 1.4, 5.7 Hz, H−C(9′)], 3.91 [s, 3H, H−C(3′-OMe)], 3.86 [s, 3H, H−C(3-OMe)], 3.77 [dd, 1H, J = 4.0, 11.9 Hz, H−C(9a)], 3.51 [dd, 2H, J = 5.3, 11.9 Hz, H−C(9b)]. ^{13}C NMR (125 MHz, methanol-d_4, HSQC, HMBC): δ 151.77 [C(3′)], 149.27 [C(4′)], 148.84 [C(3)], 147.20 [C(4)], 133.79 [C(1)], 133.17 [C(1′)], 131.42 [C(7′)], 128.63 [C(8′)], 120.81 [C(6′)], 120.75 [C(6)], 118.87 [C(5′)], 115.85 [C(5)], 111.76 [C(2)], 111.30 [C(2′)], 87.14 [C(8)], 74.04 [C(7)], 63.75 [C(9′)], 61.92 [C(9)], 56.55 [C(3′-OMe)], 56.35 [C(3-OMe)].

Ultra-Performance Liquid Chromatography/Mass Spectrometry (UPLC-MS/MS). Mass spectral analyses were performed on a Waters Xevo TQ-S mass spectrometer (Waters, Manchester, UK) coupled to an Acquity UPLC i-class core system (Waters, Milford, MA, USA) equipped with a 2 × 150 mm, 1.7 μm, BEH Phenyl column (Waters, Manchester, UK) in a column oven. Using the negative electrospray ionization mode (ESI−), capillary voltage (−2.0 kV), sampling cone voltage (50 V), source offset voltage (30 V), source temperature (150 °C), desolvation temperature (500 °C), cone gas (150 L/h), desolvation gas (1000 L/h), collision gas (0.25 mL/min), and nebulizer gas (6.5 bar) were set as given in parentheses. Calibration of the mass spectrometer in the range m/z 40−1963 was performed using a solution of phosphoric acid (0.1% in acetonitrile). The UPLC-MS/MS system was operated with MassLynx software (Waters, Manchester). Data processing and analysis were performed using TargetLynx 4.1 SCN 813 software (Waters, Manchester).

UPLC-MS/MS Detection of Compounds 7−10 in AGE. After 1:10 dilution with 20% methanol, aliquots (1 μL) of AGE were injected into the UPLC-MS/MS system equipped with a 2 × 150 mm, 1.7 μm, BEH Phenyl column (Waters, Manchester, UK). Operating with a flow rate of 0.4 mL/min and a temperature of 45 °C, chromatography was performed using the following gradient of 0.1% aqueous formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B): 0.0−9.0 min (12 → 40% B), 9.0−9.1 min (40 → 99% B), 9.1−9.6 min (99% B), 9.6−9.7 min (99 → 12% B), 9.7−10.2 min (12% B). ESI$^-$ mass and product ion spectra were acquired for compounds **7−10** with direct flow infusion using IntelliStart. The MS/MS parameters were tuned for each individual compound, detecting the fragmentation of the [M − H]$^-$ molecular ions into specific product ions after collision with argon. By means of the multiple reaction monitoring (MRM) mode, compounds **7** (m/z 359.3 → 341.1/329.1), **8** (m/z 357.3 → 339.3/327.2), **9** (m/z 375.2 → 327.2/149.2), and **10** (m/z 375.2 → 327.2/195.1) were analyzed using the mass transitions given in parentheses (20 ms duration). Comparison of retention times with the reference compounds, followed by cochromatography, led to the unequivocal identification of compounds **7−10** in AGE.

LC/Time-of-Flight Mass Spectrometry (LC-TOF-MS). Aliquots (1−5 μL) of the analytes dissolved in methanol/water (8:2, v/v; 1 mL) were injected into an Acquity UPLC core system (Waters, Milford, MA, USA) connected to a SYNAPT G2-S HDMS spectrometer (Waters, Manchester, UK) operating in the electrospray (ESI) modus with the following parameters: capillary voltage +2.5 or −3.0 kV, sampling cone 30, extraction cone 4.0, source temperature 150 °C, desolvation temperature 450 °C, cone gas 30 L/h, and desolvation gas 850 L/h. The instrument was calibrated over a mass range from m/z 50 to 1200 using a solution of sodium formate (0.5 mmol/L) in 2-propanol/water (9:1, v/v). All data were lock mass corrected using leucine enkephaline as the reference (m/z 556.2771 for [M + H]$^+$; m/z 554.2615 for [M − H]$^-$). Data acquisition and analysis was performed by using the MassLynx software (version 4.1; Waters).

Circular Dichroism Spectroscopy. CD spectra were acquired by means of a Jasco J810 spectropolarimeter (Jasco, Tokyo, Japan).

Nuclear Magnetic Resonance Spectroscopy. ^{1}H, ^{13}C, COSY, HSQC, and HMBC experiments were performed on an Avance III 400 MHz spectrometer with a BBO probe and an Avance-III-500 spectrometer, respectively, the latter of which was equipped with a Cryo-CTCI probe (Bruker, Rheinstetten, Germany). Methanol-d_4 and acetone-d_6 were used as solvents, and trimethylsilane (TMS) was used as the internal standard. Data processing was performed by using Topspin software (version 2.1; Bruker) as well as Mestre-C software (version 4.8.6; Mestrelab Research, Santiago de Compostella, Spain).

RESULTS AND DISCUSSION

Since no single antioxidant assay alone is able to give a comprehensive picture of the antioxidant capacity of the total aged garlic extract and fractions isolated thereof, the following study was performed with two different antioxidant assays as

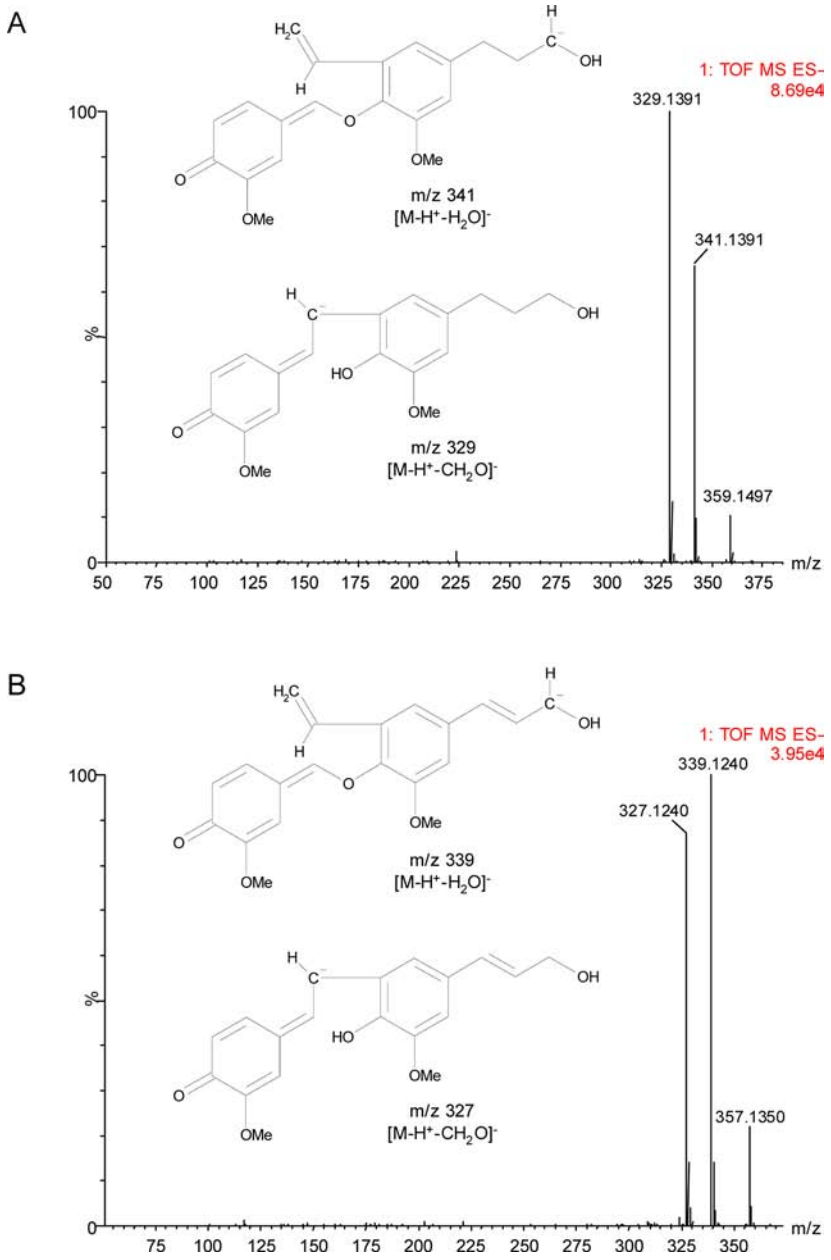

Figure 5. TOF-MS (ESI−) spectra of (A) (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol (**7**) and (B) (+)-(2*S*,3*R*)-dehydrodiconiferyl alcohol (**8**).

recommended in the literature.[31] The HPS assay, based on a single electron transfer mechanism, measures the radical reduction ability of an antioxidant by transferring one electron, whereas the ORAC assay, based on a hydrogen atom transfer mechanism, determines the radical-quenching ability of an antioxidant by hydrogen donation.

In Vitro Activity-Guided Fractionation of AGE. Aliquots of the aged garlic extract were separated by means of preparative RP18-HPLC to give four fractions (Figure 2A), which were used in their natural concentration ratios for the determination of their antioxidant activity using the HPS and the ORAC assay, respectively. Fraction 4 was identified with by far the highest antioxidant activity, accounting for 80% and 50% of the HPS and ORAC activity, respectively, found for the total AGE (Figure 2B). In comparison, fraction 1 showed only marginal activity in the HPS assay and did not exhibit any activity in the ORAC assay, whereas the opposite was observed for fraction 3. Fraction 2 did not show any activity in the assays used. UPLC-MS/MS screening of AGE for the known antioxidant *Nα*-(1-deoxy-D-fructos-1-yl)-L-arginine (**1**; Figure 1),[26] followed by cochromatography with the synthetic reference substance, revealed the Amadori compound **1** to be present in fraction 1 (data not shown). *Nα*-(1-deoxy-D-fructos-1-yl)-L-arginine was absent in fraction 4, and the following studies were focused on previously unknown antioxidants in fraction 4.

In order to identify the key antioxidants in AGE, the most active fraction (4) was further separated by means of RP-HPLC to give a total of 16 subfractions, namely, fraction 4-1 to 4-16, which were freed from solvent under vacuum and then used for the determination of their antioxidant activities using the HPS and the ORAC assay, respectively (Figure 3). The highest antioxidant activity in both assays was found for subfractions 4-

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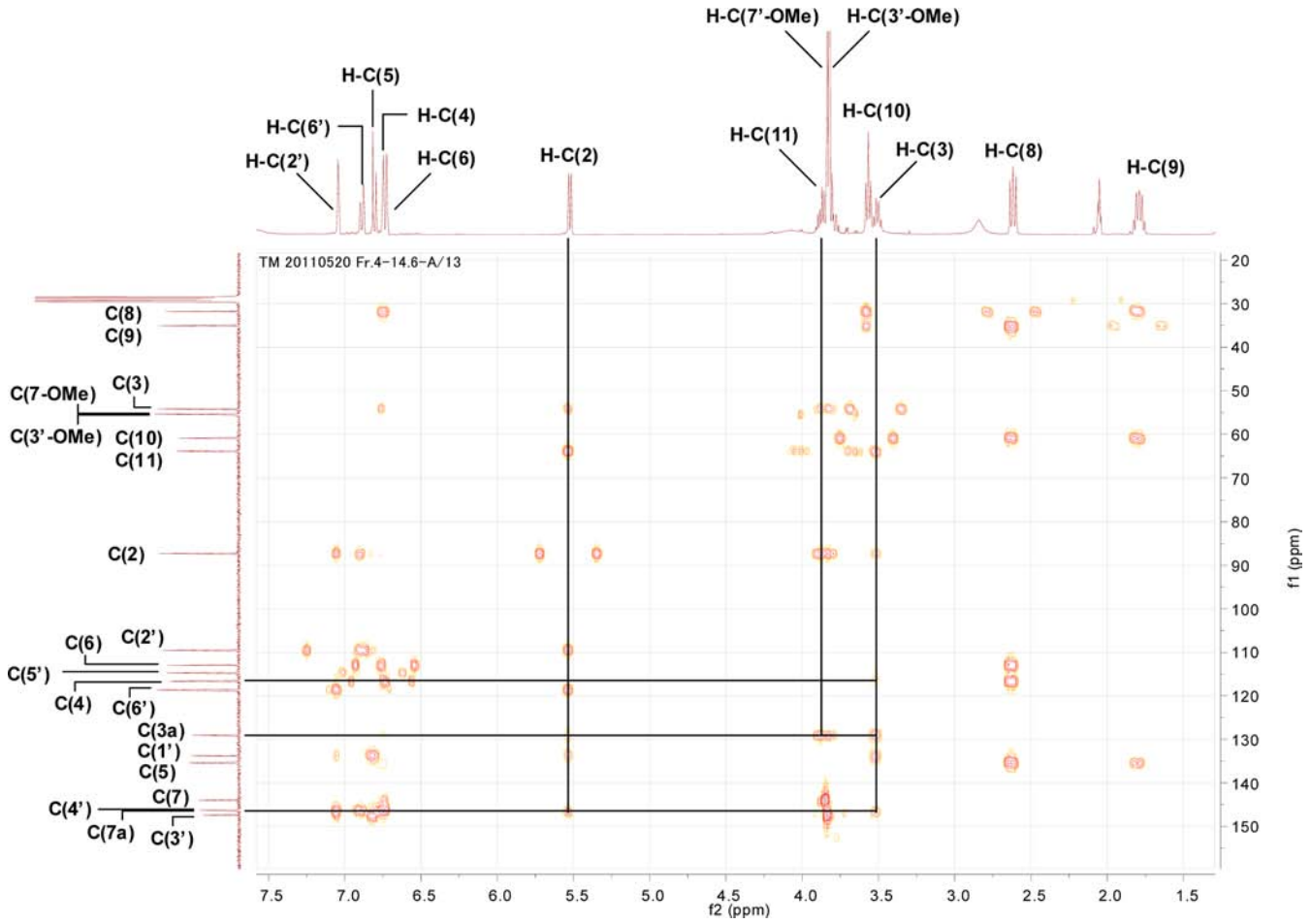

Figure 6. HMBC spectrum (400 MHz, acetone-d_6) of (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol (**7**).

9 and 4-15, followed by subfractions 4-12 to 4-14, and 4-16, respectively.

Rechromatography of fractions 4-9 and 4-15, followed by HPLC-MS/MS analysis, revealed the presence of the tetrahydro-*β*-carbolines **2**−**5**, reported recently as antioxidants in AGE,[27] besides a series of minor constituents, which could not be isolated in the purity and amounts needed for an unequivocal structure determination by NMR spectroscopy. HPLC-MS/MS analysis, followed by cochromatography with the corresponding reference compound, led to the identification of (1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**4**) and (1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid (**2**) as active antioxidant in the most potent fractions 4-9 and 4-15, respectively. In addition, fractions 4-11 and 4-12 were found to contain (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid (**3**) as the primary antioxidant, and (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**5**) was detected in fraction 4-11. Rechromatography of the active subfraction 4-14 indicated the presence of two unknown antioxidants (**7** and **8**), showing UV absorption maxima at 232 and 281 nm.

Structure Determination of Antioxidants 7 and 8. UPLC-TOF-MS analysis of compound **7** showed the three ions *m*/*z* 359.1497, 341.1391, and 329.1391 (Figure 5A), which matched the empirical formula of the pseudomolecular ion $[C_{20}H_{24}O_6-H]^-$ (calcd *m*/*z* 359.1495) and the fragment ions $[C_{20}H_{22}O_5-H]^-$ (calcd *m*/*z* 341.1389) and $[C_{19}H_{22}O_5-H]^-$ (calcd *m*/*z* 329.1389), most likely formed by cleavage of water (−18 Da) and formaldehyde (−30 Da), respectively. In comparison, the MS spectrum of **8** (Figure 5B) showed the pseudomolecular *m*/*z* 357.1350 and the fragment ions *m*/*z* 339.1240 and 327.1240, which are well in alignment with the cleavage of one molecule of water and formaldehyde, respectively. As literature studies reported on losses of 18 Da (water) and 30 Da (formaldehyde) in the MS spectra of dilignols,[32] compounds **7** and **8** were suggested to exhibit a dilignol structure.

Besides two methoxy groups, the ^{1}H NMR spectrum of **7** showed aromatic proton signals for H−C(2′), H−C(6′), and H−C(5′) resonating at 7.04, 6.89, and 6.81 ppm and indicated the presence of a 1,3,4-trisubstituted benzene ring. In addition, the *meta*-coupled aromatic protons H−C(4) and H−C(6), showing a coupling constant of *J* = 1.6 Hz, resonated at 6.75 and 6.73 ppm and indicated the presence of a 1,2,4,5-tetrasubstituted benzene ring. Moreover, three aliphatic protons were observed at 5.52 ppm [d, 1H, *J* = 6.6 Hz, H−C(2)], 3.51 ppm [dd, 1H, *J* = 6.4, 6.6 Hz, H−C(3)], and 3.76−3.92 ppm [m, 2H, H−C(11)], which coupled to each other, and another three aliphatic protons coupling with each other were observed at 3.57 ppm [t, 2H, *J* = 6.4 Hz, H−C(10)], 2.62 ppm [t, 2H, *J* = 7.7 Hz, H−C(8)], and 1.79 ppm [m, 2H, *J* = 6.4, 7.7 Hz, H−C(9)].

Well in agreement with the molecular formula $C_{20}H_{25}O_6$ proposed by TOF-MS, the ^{13}C NMR spectrum showed 20 carbon resonances. Comparison of ^{13}C NMR data with those obtained by means of a DEPT-135 experiment revealed nine

primary or tertiary carbons, four secondary carbons, and seven quaternary carbon atoms in compound **7**. Signal alignment by means of a heteronuclear multiple-bond correlation spectroscopy (HMBC) experiment revealed that compound **7** is composed of two phenylpropanoids. The HMBC experiment exhibits correlations of H−C(2) with C(3a) and C(7a), correlations of H−C(3) with C(4) as well as C(3a) and C(7a), and correlations of H−C(11) with only C(3a), thus implying that C(2) and C(3) are connected to an aromatic ring of another phenylpropanoid through an *O*-linkage and a *C*-linkage, respectively (Figure 6). Taking all spectroscopic data into consideration, compound **7** was identified as dihydrodehydrodiconiferyl alcohol (Figure 4). Although this compound was earlier reported in *Cleistopholis glauca* and *Lawsonia alba*,[33,34] this is the first report on the presence of **7** as an antioxidant in garlic.

The ^{1}H NMR signal pattern recorded for compound **8** showed major similarities to that observed for **7**, except for the aliphatic proton signals H−C(8) and H−C(9) at 6.53 and 6.25 ppm, respectively, showing a large coupling constant of J = 15.9 Hz and indicating a *trans*-configured double bond between C(8) and C(9) instead of a single bond. Careful signal assignment by means of heteronuclear correlation experiments (HSQC, HMBC) led to the unequivocal identification of compound **8** as dehydrodiconiferyl alcohol (Figure 4). Although published earlier as a phytochemical in *Rosa multiflora*,[35] this is the first report on compound **8** as an antioxidant in garlic.

Using the ^{1}H NMR signal and coupling constant of H−C(2) as diagnostic markers to differentiate between *cis*- and *trans*-diastereoisomers of dilignols,[36] the doublet signal of H−C(2) at 5.52 ppm (J = 6.6 Hz) of **7** and at 5.57 ppm (J = 6.5 Hz) of **8** clearly indicated a *trans*-configuration in both antioxidants. To confirm the absolute configuration of carbon atoms C(2) and C(3) in **7** and **8**, both compounds were analyzed by means of circular dichroism spectroscopy. As depicted in Figure 7, the

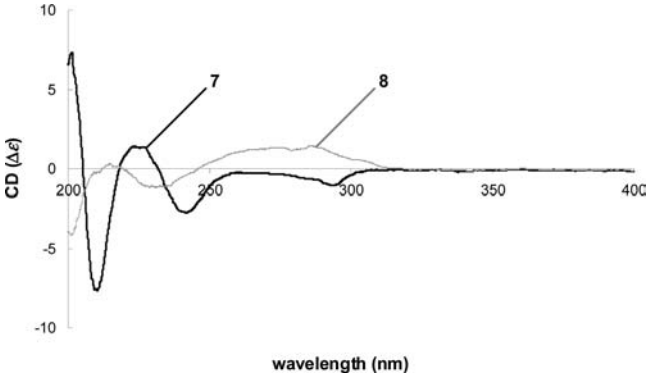

Figure 7. CD spectra of (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol (**7**) and (+)-(2*S*,3*R*)-dehydrodiconiferyl alcohol (**8**).

CD spectrum of **7** showed a positive Cotton effect at 224 nm and negative Cotton effects at 242 and 294 nm, being well in line with the data published for the (−)-(2*R*,3*S*)-configumer.[37] Therefore, the structure of antioxidant **7** could be deduced as (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol. On the other hand, comparison of the CD spectrum of **8**, exhibiting a negative Cotton effect at 231 nm and a positive Cotton effect at 286 nm (Figure 7), with literature data[38,39] enabled the identification of antioxidant **8** as (+)-(2*S*,3*R*)-dehydrodiconiferyl alcohol.

Preparation of *erythro*-Guaiacylglycerol-*β*-*O*-4′-coniferyl Ether (9) and *threo*-Guaiacylglycerol-*β*-*O*-4′-coniferyl Ether (10) and Identification in AGE. Dihydrodehydrodiconiferyl alcohol (**7**) and dehydrodiconiferyl alcohol (**8**) are reported as dimeric dehydrogenation products of coniferyl alcohol (**6**) formed by an enzymatic radical coupling of a *β*-radical of one coniferyl alcohol unit with a 5-radical of another coniferyl alcohol unit.[40] As coniferyl alcohol (**6**) is reported to dimerize preferentially to form *β*-*O*-4 intermolecular linkages,[41] the question arose as to whether dimers with a *β*-*O*-4 linkage do exist also in AGE. Therefore, reference material of the *β*-*O*-4 linked dilignols **9** and **10** (Figure 4) was synthesized by oxidative coupling of coniferyl alcohol with hydrogen peroxide in the presence of horseradish peroxidase. After chromatographic purification, the structures of **9** and **10** were confirmed by means of LC-MS, TOF-MS, and NMR spectroscopy and were in alignment with literature data.[42,43]

In order to investigate the presence of these dilignols in AGE, the MS/MS parameters were tuned for each individual compound, and then the AGE fractions were screened for **9** and **10** by means of UPLC-ESI-MS/MS. This MS screening, followed by cochromatography with the corresponding reference compounds, led to the unequivocal identification of *erythro*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether (**9**) and *threo*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether (**10**) in fractions 4-12 and 4-13, respectively. Moreover, their precursor molecule coniferyl alcohol (**6**) was identified in AGE fraction 4 by means of UPLC-MS/MS.

Antioxidant Activities of Purified Compounds from AGE. After checking the purity of each compound by means of HPLC-ELSD, UPLC-TOF-MS, and ^{1}H NMR spectroscopy, coniferyl alcohol (**6**), the dilignols **7**−**10**, and the compounds **1**−**5** (Figure 1) previously identified as antioxidants in AGE[26,27] were studied for their antioxidant activity using the HPS as well as the ORAC assay (Table 1). Analysis of the reference compounds ascorbic acid, quercetin, and (−)-epicatechin, well known for their high antioxidant activities, showed comparable activity of these compounds with that reported in the literature.[28,44,45]

As expected from the activity-guided fractionation, compounds **6**−**10** showed high antioxidant activities; for example, EC_{50} values of 9.7−11.8 *μ*M were found in the HPS assay and high activities of 2.60−3.65 *μ*mol TE/*μ*mol were found when using the ORAC assay. Compared to the low activities found for *Nα*-(1-deoxy-D-fructos-1-yl)-L-arginine (**1**) in the HPS (EC_{50}: 139.2 *μ*M) and the ORAC assay (0.01 *μ*mol TE/*μ*mol), the phenols **6**−**10** showed ∼13 and ∼330 times higher antioxidant activities, respectively. Coniferyl alcohol (**6**) and its dimers **7**−**10** showed comparable activities with the tetrahydro-*β*-carbolines **2**−**5**; for example, the HPS and the ORAC activities of (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol (**7**) and (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**5**) were in the same range. These data confirm previous studies reporting on the in vitro antioxidant activity of dilignols and their cellular activity against high glucose-stimulated ROS production.[46−49]

In conclusion, activity-guided fractionation using both the HPS and the ORAC assay enabled the identification of the tetrahydro-*β*-carbolines **2**−**5**, coniferyl alcohol (**6**), (−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol (**7**), (+)-(2*S*,3*R*)-dehydrodiconiferyl alcohol (**8**), *erythro*-guaiacyl-

Table 1. Antioxidant Activity of Compounds 1−10 and Reference Compounds

test compound	H_2O_2 assay[a,b] (EC_{50} in μM)		ORAC assay[c,d] (μmol TE/μmol)	
Nα-(1-deoxy-D-fructos-1-yl)-L-arginine (**1**)	139.2	(104.1−225.8)	0.01	(±0.00)
(1*R*,3*S*)-/(1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid (mixture of **2** and **3**)	168.2	(137.7−209.8)	1.48	(±0.06)
(1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**4**)	36.7	(29.0−49.7)	1.68	(±0.08)
(1*S*,3*S*)- 1-methyl-1,2,3,4-tetrahydro-*β*-carboline-1,3-dicarboxylic acid (**5**)	14.3	(12.2−16.9)	1.73	(±0.09)
coniferyl alcohol (**6**)	9.7	(8.6−11.1)	3.29	(±0.18)
(−)-(2*R*,3*S*)-dihydrodehydrodiconiferyl alcohol (**7**)	10.8	(9.2−12.8)	2.60	(±0.20)
(+)-(2*S*,3*R*)-dehydrodiconiferyl alcohol (**8**)	11.8	(10.2−13.9)	3.65	(±0.37)
erythro-guaiacylglycerol-*β*-*O*-4′-coniferyl ether (**9**)	10.3	(9.0−11.8)	3.27	(±0.14)
threo-guaiacylglycerol-*β*-*O*-4′-coniferyl ether (**10**)	11.0	(9.6−12.5)	3.53	(±0.09)
ascorbic acid	16.5	(15.0−18.3)	0.34	(±0.10)
quercetin	6.1	(5.3−7.1)	5.61	(±0.07)
(−)-epicatechin	4.1	(3.7−4.6)	9.65	(±0.53)

[a]The HPS assay was performed in three independent repetitions for each sample. [b]The range in parentheses represents 95% confidence interval. [c]The ORAC assay was performed in four independent repetitions for each sample. [d]The numerical value in parentheses represents the SD.

glycerol-*β*-*O*-4′-coniferyl ether (**9**), and *threo*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether (**10**) as potent in vitro antioxidants in aged garlic extract. Quantitative studies, followed by reconstitution and omission experiments, are currently in progress in order to answer the question as to what extent the individual compounds account for the in vitro antioxidant activity of the total AGE and to investigate potential "cocktail" effects between individual antioxidants. In addition, future studies need to focus on the bioavailability of each compound and its actual in vivo activity.

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Notes

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