Novel Antioxidant Compounds from Tart Cherries (*Prunus cerasus*)

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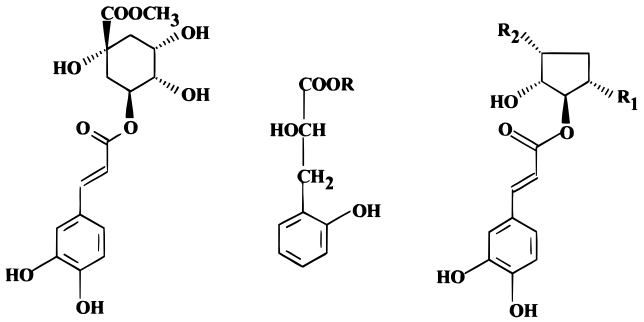
Received June 19, 1998

As indicated by an Fe(II)-induced liposome peroxidation bioassay, the EtOAc extract of tart cherries (*Prunus cerasus*) was found to have strong antioxidant activity. Purification of this extract afforded chlorogenic acid methyl ester (**1**) and three novel compounds, 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid (**2**); 1-(3′,4′-dihydroxycinnamoyl)-cyclopenta-2,5-diol (**3**), and 1-(3′,4′-dihydroxycinnamoyl)-cyclopenta-2,3-diol (**4**), as determined by their spectral data. At a 20-μM concentration, the antioxidant activities of compounds **3** and **4** were comparable to the antioxidant activities of caffeic acid, whereas compound **1** showed activity similar to chlorogenic acid. Also, these compounds showed antioxidant activities similar to the commercial antioxidants *tert*-butylhydroquinone and butylated hydroxytoluene. However, compound **2** was not active when tested at a 100-μM concentration.

Consumers are now including phytonutrients in their diet with the notion that antioxidant compounds may reduce the incidence of cancer and aging in humans. Free radicals are implicated in a number of pathological processes including aging, inflammation, reoxygenation of ischemic tissues, atherosclerosis, and cancer.[1] The harmful activities of free radicals such as hydroxyl radicals (OH·) initiate a chain reaction that causes lipid peroxidation, damage to enzymes and DNA, and cell death. Naturally occurring antioxidant components, such as flavonoids, can stabilize highly reactive and potentially harmful free radicals.[2] Many common foods contain nonnutritional components such as flavonoids and are considered to reduce the incidence of chronic diseases.[3,4] The antioxidant phenolic compounds are reported to remove free radicals and protect the structural integrity of cells and tissues.[5]

Preliminary antioxidant assays revealed good antioxidant activities in the MeOH and EtOAc extracts of Balaton tart cherries (*Prunus cerasus* L., Rosaceae), while hexane extracts showed little or no activity. Anecdotal reports indicate that consumption of tart cherries can alleviate the pain of gout and arthritis [6] and prompted us to investigate tart cherries for biologically active compounds. We have evaluated the antioxidant efficacy of *P. cerasus* constituents using an Fe(II)-induced peroxidation of liposome antioxidant bioassay.[7,8] In this paper, we report the isolation and identification of three novel antioxidant compounds **(2**−**4)** from the EtOAc extracts of tart cherries.



1 **2** R = H, **5** R = CH_3 **3** R_1 = OH, R_2 = H **4** R_1 = H, R_2 = OH

Results and Discussion

Ethyl acetate extracts of dried Balaton tart cherries were separated by medium-pressure liquid chromatography (MPLC), preparative TLC, and HPLC to yield compounds **1**−**4**. Both the ^{1}H and ^{13}C NMR spectral analysis of compound **1** revealed that the chemical shifts observed were identical to the published spectral data of the known compound, chlorogenic acid methyl ester.[9]

Compound **2** was obtained as a white solid. The molecular formula of this compound was determined as $C_9H_{10}O_4$ by FABMS. The ^{1}H NMR spectrum revealed two aromatic protons that appeared as doublets at δ 7.40 and 6.86, respectively. Another two aromatic protons in the molecule appeared as triplets at δ 7.22 and 7.00, respectively. This indicated that there is an ortho-substituted aromatic moiety in the molecule. The multiplets at δ 4.19 and 2.80 were assigned to oxygenated methine and methylene moieties, respectively. The ^{13}C NMR spectrum of **2** supported these assignments in addition to a carbonyl carbon at δ 178.6. The structure of this compound was further confirmed by the formation of product **5** from **2** by methylation. Methylation of **2** by CH_2N_2 yielded one unit each of $-OCH_3$ and $-COOCH_3$. These data confirmed the presence of a phenolic OH and a COOH in **2**. Therefore, the NMR confirmation of the identity of compound **2** as 2-hydroxy-3-(*o*-hydroxyphenyl)-propanoic acid is in agreement with the methylation data. Circular dichroism (CD) studies of **2** showed that it is a racemic mixture as evident from a straight line in the CD spectrum. To our knowledge, this is the first report of this compound as a natural product.

Compound **3** was obtained as a pale yellow gum. The ^{1}H NMR spectrum of **3** indicated two olefinic proton signals appearing as doublets at δ 7.45 and 6.19, respectively. A coupling constant of 15.9 Hz for these two protons suggested that they are trans oriented. The signals that appeared at δ 7.02, 6.97, and 6.75 were assigned to aromatic protons of a 3,4-dihydroxylcinnamoyl group, respectively, and were similar to the chemical shifts of chlorogenic acid. The peaks at *m*/*z* 180 and 163 in the EIMS of **3** confirmed that it contained a caffeic acid moiety. The signals at δ 5.17, 3.82, and 3.54 were assigned to three oxygenated protons, one (δ 5.17) esterified, as well as multiplets at δ 1.83, integrating for four protons of two methylene groups. These oxymethines appeared at δ 70.9 (× 2) and 67.5, respectively, in the ^{13}C NMR spectrum.

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10.1021/np980268s CCC: $18.00
Published on Web 11/25/1998

Compound **3** showed only one carbonyl carbon at 166.1 ppm. The fact that compound **3** has only one carbonyl carbon and no quaternary carbon around δ 70.9 suggested that the caffeic acid moiety was not connected to a quinic acid moiety but to a cyclopentane-2,5-diol moiety. From these spectral data, the structure of compound **3** was assigned as 1-(3′,4′-dihydroxylcinnamoyl)-cyclopenta-2,5-diol.

Compound **4** was obtained as a colorless oily product. The ^{1}H NMR spectrum of **4** revealed a 3,4-dihydroxylcinnamoyl moiety as in compound **3**. However, the two multiplets in **4**, appearing at δ 2.15 and 1.95, were assigned to two methylene groups, respectively. The DQFCOSY experiment showed that two CH_2 protons in **4** were correlated and adjacent to each other and also coupled to other hydrogens. The ^{13}C NMR spectrum of this compound revealed that there were only one carbonyl carbon, eight methine carbons, and two methylene carbons. Three of the methine carbons at δ 74.8, 73.0, and 68.3 were oxygenated and showed correlations to three methine protons at δ 3.64, 5.35, and 4.14, respectively, as evident from the HMQC spectrum. Also, five other methine carbons at δ 115.1, 116.5, 122.9, 146.8, and 115.8 showed correlations to three aromatic protons appearing at δ 7.04, 6.76, and 6.93 and two olefinic protons at 7.58 and 6.30 ppm, respectively. Therefore, compound **4** was assigned as 1-(3′,4′-dihydroxylcinnamoyl)-cyclopenta-2,3-diol.

CD measurements of compounds **3** and **4** did not show absorption maxima or minima. This seems to be because the cyclopentane moieties in **3** and **4** do not absorb in the UV region. However, both of these compounds gave observable peaks in their ORD spectra. Compounds **3** and **4** are novel.

The antioxidant activity of compounds **1**−**4** was determined by fluorescence spectroscopy,[10] and the activity was compared with caffeic acid; ferulic acid; chlorogenic acid; *p*-hydroxycinnamic acid; and two commercial antioxidants, *tert*-butylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT), each at a 20-μM concentration. In this assay, the lipid peroxidation was initiated by Fe^{2+}, and the rate of decrease of fluorescence intensity reflected the rate of lipid peroxidation. The inhibitory activities of Fe^{2+}-induced lipid peroxidation in the large unilamellar vesicles for compounds **3** and **4** were about 80% at 20 μM. Compound **1** showed about 50% inhibitory activity. However, **2** did not show antioxidant activity even when tested at a 100-μM concentration. The assay results showed that *p*-hydroxycinnamic acid is a weak antioxidant when compared to ferulic acid. However, the caffeic acid analogues, compounds **3** and **4**, showed the highest antioxidant activity in this assay. The percent inhibition of lipid peroxidation for TBHQ and BHT were > 90% at a 20-μM concentration (Figure 1).

The variation in antioxidant activity among caffeoyl esters is dependent on the hydroxyl substitution of the aryl ring. More than one hydroxyl substitution in the aryl ring enhanced the antioxidant activity. Introduction of a second hydroxyl group in the *ortho* position, as in caffeic acid, also enhanced the antioxidant activity. Methylation of the hydroxyl group in the *ortho* position of caffeic acid, as in ferulic acid, resulted in a decrease of antioxidant activity. This result is in agreement with published studies on the effects of hydroxycinnamates on the autoxidation of fats and lipids. [11,12] Our data suggest that caffeic acid is the best antioxidant, followed by compounds **4** and **3**, chlorogenic acid, and chlorogenic acid methyl ester (**1**). This trend in activity may be due to the difference in hydrophilicity or chelation properties of these compounds. It is interesting to note that the antioxidant activities of the novel caffeic acid analogues, **3** and **4**, are comparable to the commercial antioxidants BHT and TBHQ at the concentration tested.

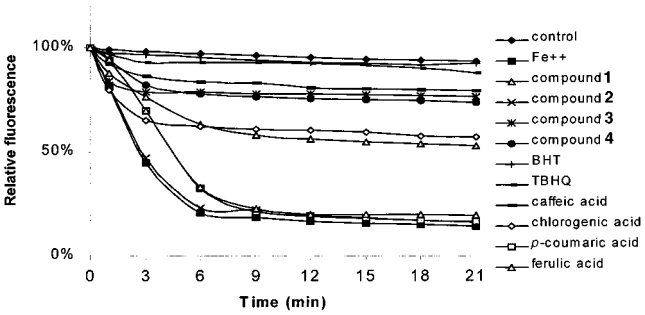

Figure 1. Antioxidant activities of compounds **1**, **3**, and **4** and some commercial antioxidants at 20 μM concentration. The antioxidant activity of compound **2** was measured at 100 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative intensity represents the fluorescence intensity at a given time divided by the initial intensity at the start of the assay. Values represent the means of duplicate measurements.

Experimental Section

General Experimental Procedures. Commercial antioxidants TBHQ and BHT were used as positive controls in the antioxidant assays. TBHQ was purchased from Eastman Chemical Products Inc., Kingsport, TN, BHT was purchased from National Biochemicals Corporation, Cleveland, OH. Si gel (60 mesh, 35−70 μm) used for MPLC was purchased from E. Merck, Darmstadt, Germany. TLC plates (GF Uniplate, Analtech, Inc., Newark, DE), after developing, were viewed under 254 and 366 nm. For preparative HPLC (LC-20, Japan Analytical Industry Co., Tokyo, Japan) purification, two Jaigel-ODS, A-343-10 (20 mm × 250 mm, 10 μm, Dychrom, Santa Clara, CA) columns were used in tandem. Peaks were detected using a model D-2500 Chromato-integrator connected with a UV detector. ^{1}H, ^{13}C, DQFCOSY, and HMQC NMR spectra were recorded on a Varian Unity 500 and an Inova 300 MHz spectrometer at 25 °C and referenced to the residual proton solvent resonance (CD_3OD at 3.30 and 49.0 ppm and DMSO-d_6 at 2.49 and 39.5 ppm, for ^{1}H and ^{13}C NMR, respectively). FABMS were obtained on a JEOL JMS−HX110 mass spectrometer using a glycerol matrix, and EIMS spectra were obtained on JEOL JMS-AX505 mass spectrometer. CD and optical rotatory dispersion (ORD) measurements were carried out using a JASCO J-710 CD-ORD spectropolarimeter (Japan Spectroscopic Co.). For CD/ORD measurements, test compounds were dissolved in MeOH (0.2 mg mL^{-1}), and CD/ORD were determined under the following conditions: scan mode (wavelength), band width (0.5 nm), sensitivity (50 m deg), response (1 s), wavelength range (200−400 nm for CD and 200−800 nm for ORD), step resolution (1 nm), scan speed (200 nm min^{-1}), and accumulation (1). Nitrogen (99.99%) was generated by a nitrogen generator model NG-150 at the rate of 15 L min^{-1}. UV spectra of compounds, in MeOH, were measured on a Shimadzu UV/vis spectrophotometer (Kyoto, Japan).

Plant Material. Pitted and individually quick frozen (IQF) Balaton cherries (*P. cerasus* L., Rosaceae), which were collected in July 1995, were obtained from commercial growers (Traverse City, MI) and supplied by the Cherry Marketing Institute, Inc. (Dewitt, MI).

Extraction and Isolation. IQF Balaton tart cherries (2 kg) were lyophilized at 10 °C and yielded 342 g of dried cherries. The Balaton dried cherries (340 g) were milled and extracted with hexane (500 mL × 3), EtOAc (500 mL × 3), and MeOH (500 mL × 3) to yield 0.71, 2.53, and 198.9 g of extracts, respectively.

The EtOAc extract of Balaton cherries (1.75 g) was fractionated by Si gel (100 g) MPLC using $CHCl_3$ and MeOH under gradient conditions, starting with 100% $CHCl_3$ and ending

with 100% MeOH. Fractions 1−4 (125 mL each, $CHCl_3$), 5−8 (100 mL each, $CHCl_3$−MeOH, 8:1), 9−12 (100 mL each, $CHCl_3$−MeOH, 4:1), and 12−16 (150 mL each, MeOH) were collected and combined after TLC analysis (Si gel plates developed with MeOH−$CHCl_3$, 16:1, for fractions 1−8 and MeOH−$CHCl_3$−HCOOH, 1:4:0.2, for fractions 9−16), to yield 85, 134, 330, 910, and 225 mg each of fractions A−E, respectively. Fractions A and B showed no antioxidant activity. Fractions C−E were further purified for antioxidant compounds.

Fraction C (250 mg) was purified by preparative silica TLC using MeOH−$CHCl_3$−HCOOH (4:1:0.2) as the mobile phase to yield compounds **1** (10.1 mg, R_f 0.50) and **2** (8.9 mg, R_f 0.67). The EIMS of **1** gave the molecular ion at *m/z* 368 (10) and fragment ions at *m/z* 353 (8), 311 (5), 180 (3), 163 (5), and 83 (10). Also, the FABMS of compound **1** gave two peaks at *m/z* 391 (25) $[M + Na]^+$ and 369 (3) $[M + H]^+$. ^1H and ^{13}C NMR spectra of compound **1** were identical to the published data of chlorogenic acid methyl ester.[8]

Compound 2: white solid; IR (film) ν_{max} 3316, 1728, 1590, 1406 cm^{-1}; UV λ_{max} (MeOH) 218 (3.04), 253 (3.42), 289 (3.66) nm; CD/ORD measurements gave straight lines indicating that compound **2** was obtained as a racemic mixture; ^1H NMR (CD_3-OD) δ 7.40 (1 H, d, J = 7.32 Hz, H-6′), 7.22 (1H, t, J = 7.57 Hz, 7,32 Hz, H-4′), 7.00 (1H, t, J = 7.57 Hz, 7.32 Hz, H-5′), 6.86 (1H, d, J = 7.57 Hz, H-3′), 4.19 (1H, m, H-2), 2.80 (2H, m, H-3); ^{13}C NMR (DMSO-d_6): δ 178.6 (C-1), 141.6 (C-2′), 133.4 (C-6′), 128.4 (C-4′), 123.8 (C-5′), 121.3 (C-1′), 109.2 (C-3′), 73.4 (C-2), 48.6 (C-3); FABMS *m/z* 183 (4) $[M + H]^+$.

Compound 3: fraction D (900 mg) purified using a preparative HPLC with the mobile phase being MeOH−H_2O (30:70) at a flow rate of 3 mL/min, to yield compound **3** (t_R 58 min, 9.4 mg); pale yellow oily compound; IR (film) ν_{max} 3351, 2926, 1669, 1599, 1379, 1267, 1076 cm^{-1}; UV λ_{max} (MeOH) 206 (3.99), 215 (4.00), 243 (3.83), 299 (3.86), 325 (3.89) nm; ORD (m deg) 336 (75), 316 (−44), 298 (−40), 260 (35), 240 (−22), and 216 (52) nm; ^1H NMR (DMSO-d_6) δ 7.45 (1H, d, J = 15.9 Hz, H-7′), 7.01 (1H, d, J = 1.8 Hz, H-2′), 6.96 (1H, dd, J = 8.1 Hz, 1.8 Hz, H-6′), 6.75 (1H, d, J = 8.1 Hz, H-5′), 6.19 (1 H, d, J = 15.9 Hz, H-8′), 5.17 (1H, m, H-1), 3.82 (1H, m, H-2), 3.54 (1H, m, H-5), 1.83 (4 H, m, H-3, H-4); ^{13}C NMR (DMSO-d_6) δ 166.1 (C-9′), 148.2 (C-3′), 145.5 (C-4′), 144.4 (C-7′), 125.7 (C-1′), 121.1 (C-6′), 115.8 (C-5′), 115.0 (C-8′), 114.6 (C-2′), 70.9 (C-2, C-5), 67.5 (C-1), 35.2 (C-3, C-4); FABMS *m/z* 281 (2) $[M + H]^+$; EIMS *m/z* 180 (93), 163 (100), 145 (20).

Compound 4: fraction E (225 mg) purified by preparative HPLC; The mobile phase was MeOH−H_2O (40:60) at a flow rate of 4 mL/min; Subfractions 1 (180 mg), 2 (10.8 mg), 3 (8.4 mg), 4 (8 mg), and 5 (10 mg) were collected. Subfraction 1 was not active and contained malic acid, as confirmed by its ^1H NMR spectrum. Fraction 2 (10.8 mg) was the most active and hence purified again by HPLC under the same conditions to yield compound **4** (t_R 34 min, 9.4 mg): oily compound; IR (film) ν_{max} 3372, 1692, 1603, 1277, 1184, 1074 cm^{-1}; UV λ_{max} (MeOH) 203 (3.95), 215 (3.94), 243 (3.76), 299 (3.81) and 327 (3.90) nm; ORD (m deg) 314 (−58), 288 (−61), and 234 (−61) nm; ^1H NMR (CD_3OD) δ 7.58 (1H, d, J = 15.9 Hz, H-7′), 7.04 (1H, d, J = 1.8 Hz, H-2′), 6.93 (1 H, dd, J = 8.2 Hz, 1.8 Hz, H-6′), 6.76 (1H, d, J = 8.2 Hz, H-5′), 6.30 (1H, d, J = 15.9 Hz, H-8′), 5.35 (1H, m, H-1), 4.14 (1H, m, H-3), 3.64 (1H, dd, J = 8.3 Hz, 3.1 Hz, H-2), 2.15 (2H, m, H-4), 2.15 (1H, m, H-5a), 1.95 (1 H, m, H-5b); ^{13}C NMR (CD_3OD) δ 169.0 (C-9′), 149.4 (C-3′), 146.8 (C-4′), 146.8 (C-7′), 128.0 (C-1′), 122.9 (C-6′), 116.5 (C-5′), 115.8 (C-8′), 115.1 (C-2′), 74.8 (C-2), 73.0 (C-1), 68.3 (C-2), 41.5 (C-5), 36.7 (C-4); FABMS *m/z* 281 (2) $[M + H]^+$; EIMS *m/z* 180 (34), 163 (100).

Methylation of Compound 2. *N*-Nitroso-*N*-methylurea (1.5 g) was slowly added to 100 mL of 25% KOH and 100 mL Et_2O mixture at 0 °C and reacted for about 1 h. The yellow ether layer containing CH_2N_2 was separated using a separatory funnel (500 mL) and washed with cold H_2O (100 mL) to remove excess KOH. Compound **2** (4 mg) was dissolved in MeOH and mixed with excess CH_2N_2 reagent (5 mL) in ether. The reaction mixture was kept at room temperature for 1 h. The solvent was then evaporated to afford compound **5** (4 mg): white solid; ^1H NMR (CD_3OD) δ 7.35 (1H, d, J = 7.32 Hz, H-6′), 7.25 (1H, dd, J = 7.57 Hz, 7.32 Hz, H-4′), 7.01 (1H, dd, J = 7.57 Hz, 7.32 Hz, H-5′), 6.87 (1H, d, J = 7.57 Hz, H-3′), 4.49 (1H, dd, J = 7.32 Hz, 4.88 Hz, H-2), 2.80 (1H, d, J = 12.45 Hz, 4.88 Hz, H-3a), 2.69 (1H, dd, J = 12.45 Hz, 7.32 Hz, H-3b), 3.69 (3H, s, OCH_3), 3.47 (3H, s, $COOCH_3$).

Antioxidant Assay. A mixture containing 5 μmol of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 0.015 μmol of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (Molecular Probe, Inc., Eugene, OR) was dried under vacuum using a rotary evaporator. The resulting lipid film was suspended in 500 μL of a solution containing NaCl (0.15 M), EDTA (0.1 mM), and MOPS (0.01 M) and subjected to 10 freeze−thaw cycles using a dry ice/EtOH bath. The buffer was previously treated with chelating resin, Chelex 100 (Sigma, St. Louis, MO) (5 g/100 mL buffer), to remove trace metal ion. The lipid−buffer suspension was then extruded 29 times through a Liposo-Fast extruder (Avestin, Inc., Ottawa, Canada) containing a polycarbonate membrane with a pore size of 100 nm to produce unilamellar liposomes. A 20-μL aliquot of this liposome suspension was diluted to 2 mL in Chelex 100-treated buffer containing 200 mM NaCl and 100 mM HEPES buffer at pH 7.0 and incubated for 5 min at room temperature, followed by incubation for another 5 min in the thermostatic cuvette holder (23 °C) of the spectrofluorometer. Peroxidation was then initiated by the addition of 20 μL of 0.5 mM stock $FeCl_2$ solution to achieve a final concentration of 0.5 μM of Fe^{2+} in the absence or presence of test compounds. The control sample did not contain either Fe^{2+} or any test compound. Fluorescence intensity of these liposome solutions at an excitation wavelength of 384 nm was recorded every 3 min on a fluorescence spectrofluorometer (SLM4800, SLM Instruments Inc., Urbana, IL) over a period of 21 min. The decrease in relative fluorescence intensity with time indicates the rate of preoxidation. The percent inhibition of the lipid oxidation was calculated using the equation: Percent Inhibition = $\{[(F_{rel})_{Pl} - (F_{rel})_{Fe}/[(F_{ref})_C - (F_{rel})_{Fe}]\} \times 100$, where: $(F_{rel})_{Pl}$ = relative fluorescence for the Fe(II) and test samples at the end of 21 min, $(F_{rel})_C$ = relative fluorescence for the control sample at 21 min, and $(F_{rel})_{Fe}$ = relative fluorescence for the Fe(II)-containing sample at the end of 21 min.[8]

Acknowledgment. This is a contribution from the Michigan State University Agricultural Experiment Station. Funding for this research was partially provided by The Cherry Marketing Institute, Michigan. The NMR data were obtained on instrumentation that was purchased in part with the funds from NIH grant no. 1-S10-RR04750, NSF grant no. CHE-88-00770, and NSF grant no. CHE-92-13241.

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NP980268S